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In vitro selection of DNA aptamers targeting β-lactoglobulin and their integration in graphene-based biosensor for the detection of milk allergen

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ABSTRACT

Food allergy has increased rapidly in recent years affecting millions of people worldwide. With the increased consumption of packed food nowadays, a sensitive, accurate and rapid screening method for potential food allergens has become an urgent need in order to protect the sensitive consumers from life-threatening reactions. The current detection methods for food allergens are mostly based on immunoassays which are costly and times-consuming. In this work, we developed an aptamer/graphene-based electrochemical biosensor for on-step, sensitive and low cost detection of β-lactoglobulin (β-LG) milk protein, one of the most common food allergens specially in infants. The selection of DNA aptamers against the two β-LG variants A and B was successfully realised using systemic evolution of ligands by exponential enrichment (SELEX) method. Among the selected aptamers, BLG14 aptamer sequence has shown high affinity and specificity to both β-LG A and B with dissociation constants (K_ds) of 82 and 80 nM, respectively as calculated using fluorescence binding assays. The aptamer was then integrated in a voltammetric biosensor utilizing graphene-modified screen printed carbon electrodes. The binding is monitored by following the change in the square wave voltammetry (SWV) reduction peak signal of ferrocyanide/ferricyanide redox couple due to the removal of the negatively charged aptamers from the surface upon protein binding. This one-step "signal on" biosensor takes 20 min for the sensitive and selective detection of β-LG without using any labelling or sophisticated designs. The method was also tested in spiked food sample extract achieving good recovery percentage. The integration of the novel aptamer in the graphene biosensor allows a promising way for cost-effective, rapid and sensitive on-site detection of milk allergen in food stuff.

1. Introduction

Food allergy is an immune system reaction to a particular food which results in a symptoms such as swelling, rash, difficulty breathing, itchiness and in some cases can cause severe life threatening reaction (Cianferoni and Spergel1, 2009; Sicherer and Sampson, 2009). Milk is the most common food allergy particularly in infants (Lifschitz and Szajewska, 2015). Among milk proteins, β-LG is found to be the most important cause of the cow's milk allergy (Høst and Halken, 1990). The prevalence of food allergies has increased over the years leading to major global public health problem. Food allergy cannot be treated and if a person has a severe allergy, he must avoid eating the specific allergen -containing food. Food labelling rules in most of the countries state that the eight most common allergens (eggs, fish, milk, tree nuts, peanuts, shellfish, soya and wheat) must be declared in the ingredients list on the labels of pre-packed foods (Allen et al., 2014). However, traces of allergens can get into the food by accident during manufacturing or packaging. Therefore, an efficient detection tools for small amounts of food allergies in food products are highly demanded to protect the public health. These detection tools can be used by both food companies and public health authorities.

Many analytical methodologies have been developed for the quantification of β-LG. Chromatographic methods such as HPLC (Rena et al., 2010; Xiao-yu et al., 2012), capillary electrophoresis with laser-induced fluorescence detection (Pelaez-Lorenzoa et al., 2009), size-exclusion chromatography (Dijk and Smit, 2000), and enzyme linked immunoaffinity chromatography (Puerta et al., 2005) have been reported. Immunoassays such as enzyme-linked immunosorbent assays (ELISA) (Luis et al., 2009) have been also widely used for the detection and quantification of β-LG allergen in food samples. However, chromatography requires extensive sample processing to yield a concentrated and “clean” sample for reliability. Moreover, chromatography needs large and expensive instruments, therefore it is not suitable for on-site monitoring. ELISA kits for β-LG are

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commercially available and relatively sensitive. However, it is difficult to be applied for screening of large number of samples due to the high cost and long analysis time. Therefore, few biosensing platforms for β-LG have been appeared as alternatives to the conventional detection methods. Optical biosensors based on resonance enhanced absorption (Hohensinner et al., 2007) or surface plasmon resonance (Billakanti et al., 2010; Wu et al., 2016) have been demonstrated. Despite of the sensitivity of these sensors, they are also costly and require bulky equipments. Electrochemical biosensors are more favourable due to their low cost and capability of multiplexed analysis and miniaturization. Thus, we have recently reported (Eissa et al., 2012) a label free voltammetric immunosensor for β-LG using diazonium functionalized graphene electrodes showing high sensitivity and selectivity. Ruiz-Valdepeñas Montiel et al. (2015) have also reported an electrochemical magnetoimmunosensor for detection of β-LG using a sandwich configuration. Although that these immunosensors have offered significant advantages in detection, the instability and the high cost of the used antibodies remain major challenges. These issues limit the application of these immunosensors and motivated us to develop novel recognition receptor for β-LG that can avoid such limitations.

Aptamers have recently appeared as excellent alternatives to antibodies because of their stability, low cost and easy of synthesis. Aptamers are produced using in vitro selection procedure known as systematic evolution of ligands by exponential enrichment (Sampson, 2003). Many aptamers have been identified in the past two decades against various targets and some of them has been successfully integrated in different biosensing platforms (Radi, 2011). To the best of our knowledge, until now only few aptamers targeting allergens have been selected. Aptamers against lysozyme have been identified and successfully applied for egg allergen detection using electrochemical biosensors showing a detection limit of 36 nM (Huang et al., 2009; Rodríguez and Rivas, 2009). Similarly, DNA aptamers against Lup an 1 have been selected using SELEX by Nadal et al. (2012) with an affinity in the nanomolar range and applied for Lup an 1 allergen detection using FRET biosensor. Tran et al. (2013) have also reported the selection of aptamers against Ara h 1 peanut allergen and their application in SPR biosensor. Weng et al. have also shown the functionalization of Ara h1 aptamer with quantum dots and its integration in microfluidic graphene oxide biosensor (Weng and Neethirajan, 2016).

Graphene as a two dimensional carbon nanomaterial offers large surface area for immobilization of biomolecules and fast electron transfer rate which shown to be beneficial for electrochemical biosensing applications (Eissa et al., 2015a). Aptamers have been successfully integrated with graphene materials to develop low cost, sensitive and selective biosensors for different target analytes such as thrombin (Chang et al., 2010), ochratoxin A (Sheng et al., 2011), bisphenol A (Zhu et al., 2015) and microcystin (Eissa et al., 2014). The strong adsorption of the DNA on the graphene surface enables the simple fabrication of the aptasensor and offers a capability of applying a label-free detection scheme based on the dissociation of the aptamer from graphene surface upon protein/ aptamer complex formation.

In the work, we report for the first time, the selection of DNA aptamers against β-LG and the integration of one of the selected aptamers in a simple label-free voltammetric biosensor using graphene electrodes. This novel aptasensor offers a promising, simple, low cost and highly stable detection strategy that can be used by non specialized personnel replacing the current sophisticated immunoassays.

2. Experimental

2.1. Materials and reagents

The DNA library (ATACCAGCTTATTCAATT - N_{60}-AGATAGTAAGTGCAATCT-3), primers for polymerase chain reaction (PCR), and the aptamer sequences were custom-synthesized by Integrated DNA Technologies Inc. (Coralville, USA). Ethylenediaminetetraacetic acid (EDTA) disodium dehydrate, sodium carbonate anhydrous, acrylamide/bis-acrylamide (40% solution), sodium bicarbonate, sodium azide, boric acid, tris-base, urea and Taq plus DNA polymerase were obtained from Bioshop Inc. (Ontario, Canada). HRP-labelled IgG antibody, 3,3′,5,5′-tetramethylbenzidine (TMB) stabilized chromogen and TOPO TA Cloning Kit with One Shot MAX Efficiency DH5α-T1 were purchased from Invitrogen (NY, USA). β-Lactoglobulin (mixture of A and B), β-Lactoglobulin A and B from bovine milk, anti- β-lactoglobulin antibody produced in rabbit, N-hydroxysuccinimide (NHS)-activated Sepharose™ beads, potassium ferricyanide ($K_3Fe(CN)_6$), potassium ferrocyanide ($K_4Fe(CN)_6$), sodium acetate, acetic acid, bovine serum albumin (BSA), sulphuric acid, magnesium chloride, sodium chloride, Tween 20, potassium dihydrogen orthophosphate and dipotassium hydrogen orthophosphate were purchased from Sigma (Ontario, Canada). The food sample is a meal replacement mixture, formula 1 was purchased from Herbalife (Los Angeles, USA). Amicon Ultra-0.5 ml centrifugal desalting filters with a 3 kDa molecular cut-off were purchased from EMD Millipore (Alberta, Canada). Centrifuge tube filters with a cellulose acetate membranes with pore size of 0.45 μm were obtained from Corning life sciences (Tewksbury MA, USA). The binding buffer that was used for the selection consists of 50 mM Tris, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$. Elution buffer is 7 M urea in binding buffer. 0.1 M $NaHCO_3$ containing 0.5 M NaCl, pH 8.3 was used for the coupling of the β-LG A and B to the NHS activated sepharose beads. Tris-EDTA buffer (TE) is 10 mM Tris, pH 7.4, 1 mM EDTA. A 10 mM phosphate buffered saline (PBS) solution (pH 7.4) was used for the ELISA experiments and to prepare the $[Fe(CN)_6]^{4-/3-}$ redox solution for measurement. All solutions were prepared using Milli-Q grade water.

2.2. Instruments

Electrochemical experiments were performed using Autolab PGSTAT302N (Eco Chemie, The Netherlands) potentiostat/galvanostat, controlled by Nova 1.11 software. The electrochemical aptasensor experiment were done using disposable graphene-modified screen printed carbon electrodes (GSPE) were purchased from Dropsens, Inc. (Spain, ref. 110GPH). The electrodes consist of three-electrode configuration, which comprises a carbon working electrode modified with graphene, carbon counter and silver reference electrode. The electrodes were connected to the poteniostat using an electrode connector. The graphene electrode surface has been previously characterized using X-ray photoelectron spectroscopy (XPS) and Scanning electron microscopy (SEM).(Eissa and Zourob, 2012).

2.3. Aptamer selection protocol

The aptamer selection using SELEX and characterization methods are described in the Supporting information.

2.4. β-lactoglobulin aptasensor fabrication on graphene electrodes

The unlabeled aptamer BLG14 sequences were synthesized after eliminating the primers sequences (Table 1) and immobilized on the GSPE surface by physical absorption. In order to optimise the aptamer concentration, fifty micro litres of different concentrations of the aptamer (0.5–20 μM) in binding buffer were incubated on the graphene surface for 2 h. The aptasensors which used for the final detection were prepared by incubating 50 μl of 10 μM aptamer for 2 h on the GSPEs. After that, the GSPEs were extensively washed with binding buffer in order to remove excess unbound aptamers to the graphene surface. For the sensing and selectivity experiments, 50 μl of the desired concentrations of β-LG or BSA solutions in binding buffer were incubated on the GSPEs at room temperature for the optimized duration (20 min). Then, the GSPEs were washed with 50 mM Tris-HCl

Table 1
Sequences and dissociation constants (K_d) of the selected aptamers and β-lactoglobulin A and B.

aptamer number	**Aptamer sequence**	**K_d (nM)**	
		B-LG A	**B-LG B**
BLG1	GGCGATCGGACCACAGTACCCGCCCACCAGCCCCAGCATCATGCCCTCGTTGTGGTGTG	1048 ± 120	354 ± 30
BLG3	CACCAACGGACATCAGTACCCACCCGCCAGCCCCAACATCATGCCCATTCTGCGTGTCG	4830 ± 200	102 ± 50
BLG7	GGCGACACAGCAGCACACCCTCCCACCAGCCCCATGCAACGAAGCCCACG TCCGCCGCG	160 ± 30	–
BLG10	GGACACGGCAAAGGGGTATAGCCTACCGGACCGTGAACATGGAATGATGTGCTGCGTGG	–	–
BLG12	CGACACGCATGCAGCATTACCCACCCGCCAGCCCCGACGTCAACCGATCCGTGTACGTG	324 ± 33	254 ± 25
BLG14	CGACGATCGGACCGCAGTACCCACCCACCAGCCCCAACATCATGCCCATCCGTGTGTG	82 ± 30	80 ± 26

buffer, pH 7.4 containing 0.05% Tween 20 and the SWV measurements were recorded.

2.5. Electrochemical measurements

The SWV measurments were conducted in 10 mM $[Fe(CN)_6]^{3-/4-}$ redox couple dissolved in a solution of 0.1 M PBS buffer, pH 7.4. The parameters used for the SWV measurements: amplitude 20 mV; step potential −5 mV; interval time 0.04 s; scan rate 125 mV s^{-1}, and frequency 25 Hz.

2.6. Application of the β-lactoglobulin aptasensors in real food sample extract

The food sample extract was prepared following the method that we reported previously (Eissa et al., 2012). 0.1*g* of β-lactoglobulin free food sample powder was mixed with 1 ml of binding buffer and mixed at 60 °C for 1 h. The mixture was then centrifuged at 20,000*g* for 15 min and the clear supernatant was collected. The supernatant was diluted 1: 10 in binding buffer and spiked with different concentrations of β-LG (0.1, 1.0, 10 ng/ml). Finally, the spiked extracts were incubated for 20 min with the β-LG aptasensor at room temperature and the voltammetric measurements were recorded as described in the previous section.

3. Results and discussion

3.1. Attachment of β-lactoglobulin proteins to the NHS-activated sepharose beads

A mixture of β-LG A and B, the most common cow's milk β-LG variants, was used as a target for SELEX in order to ensure the selection of aptamers that bind to both variants. The proteins were attached to sepharose beads as a solid support to enable the partitioning of the bound from the unbound aptamers throughout the selection procedure. The coupling of the β-LG to the sepharose beads was done via the reaction of the amine groups of the proteins with the activated NHS terminal of the beads to form stable amide linkage (Fig. S1). The accomplishment of the conjugation reaction was then investigated by performing direct ELISA on the β-LG-conjugated beads. The development of the blue color with the β-LG-beads confirmed the successful realization of the covalent attachment.

3.2. In Vitro selection of DNA aptamers against β-lactoglobulin

A SELEX procedure was carried out for the identification of specific aptamers for β-LG following the protocol that we reported previously (Eissa et al., 2013, 2015b; Ng et al., 2012). First, the β-LG beads were incubated with the DNA library which consists of 1.8×10^{15} random oligonucleotide sequences. Then, a partitioning step was performed in order to separate the beads-bound DNA from the unbound DNA by washing the beads with binding buffer in a centrifugal tube. This was followed by elution of the beads-bounds DNA via denaturation process by heating at 90 °C in elution buffer containing urea. After desalting the eluted DNA, a PCR amplification was done using a set of primer sequences that we successfully applied in other SELEX screenings (Eissa et al., 2013, 2015b; Ng et al., 2012). A fluorescent labelled forward primer was used to facilitate the quantification of the eluted DNA in each round in order to monitor the enrichment of the specific aptamers by calculating the DNA recovery percentage. Moreover, the fluorescent label enables the separation of the fluorescent ssDNA aptamer from the double-stranded PCR product using denaturing PAGE. This round of incubation, separation, PCR amplification and purification of ssDNA aptamers was repeated few times until the highest affinity aptamers against β-LG were obtained.

After eluting the bound DNA to the positive β-LG beads in each round, the fluorescence intensity of the eluted DNA solution was measured and the DNA recovery percentage was calculated. Fig. 1A shows the plot of the percentage of DNA recovery versus the number of the SELEX cycle. This recovery profile (Fig. 1A) is used to monitor the enrichment of the specific DNA to β-LG throughout the SELEX process. A small increase in the DNA recovery was observed in the first four rounds where the maximum amount of beads were used (100 µl). However, a significant increase in the recovery was obtained

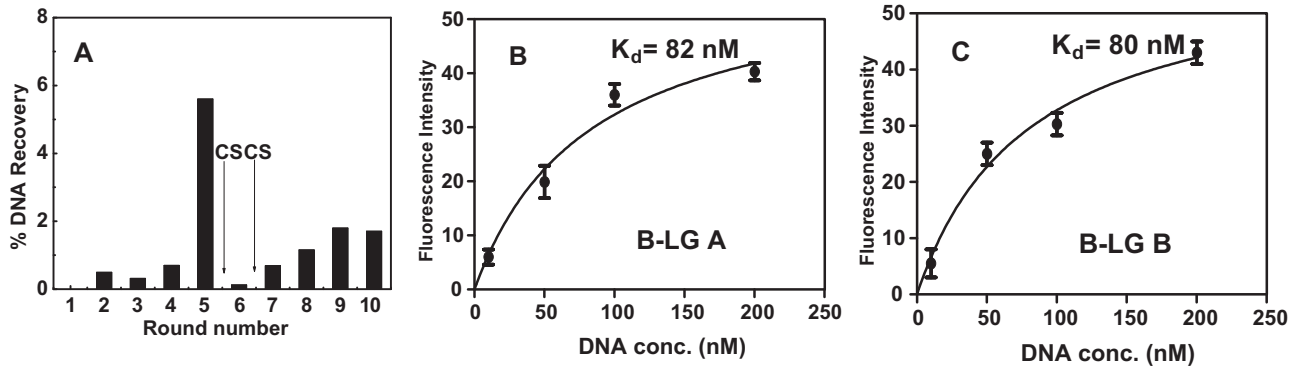


Fig. 1. (A) The recovery profile of the eluted DNA during the selection cycles. (B) Binding affinity curve obtained using fluorescence assays of the aptamer BLG14 and β-lactoglobulin A. (C) Binding affinity curve of the aptamer BLG14 and β-lactoglobulin B. The error bars represent the standard deviations of the measurements.

at round five.

It is well known that some of the DNA sequences in the library can also bind to the beads matrix. For that, it was necessary to carry out a counter selection round nearly midway on the SELEX screening. The counter selection was performed using negative blocked NHS activated beads in order to exclude the DNA which binds to the beads and to allow the selection of the specific β-LG aptamers. The counter selection was done after the fifth selection round where high recovery percentage was observed. The selected DNA pool from the fifth round was incubated with the negative beads, followed by washing the beads and incubating the DNA collected from the washes with the positive β-LG beads. After the counter selection, a dramatic decrease in the DNA recovery was obtained, likely due to the elimination of the DNA sequences that bind to the sepharose beads matrix. Then, another counter selection cycle was performed after the six cycle in order to ensure the removal of most of the nonspecific sequences. More stringent conditions were then applied for selection in the subsequent rounds as shown in Table S1 by decreasing the amount of beads and the incubation time. The decrease in the amount of the used beads/concentration of the protein and the binding time allows for competition between the sequences which should lead ultimately to select the tighter binding aptamers (McKeague et al., 2010). A gradual increase in the DNA recovery was observed until the recovery reached plateau which suggests the enrichment of β-LG-specific DNA. Therefore, the selection process was stopped at round 10 and the DNA was cloned into E coli competent cells.

Then, we randomly picked some clones for sequencing. The identified sequences were analysed using PRALINE software by multiple sequence alignment (Fig. S2). Many sequences were similar and significant sequence convergence was seen indicating the success of the enrichment of the specific DNA at round 10. The sequences were grouped based on their sequence similarity and the binding affinity of six aptamers to both β-LG A and B was tested individually.

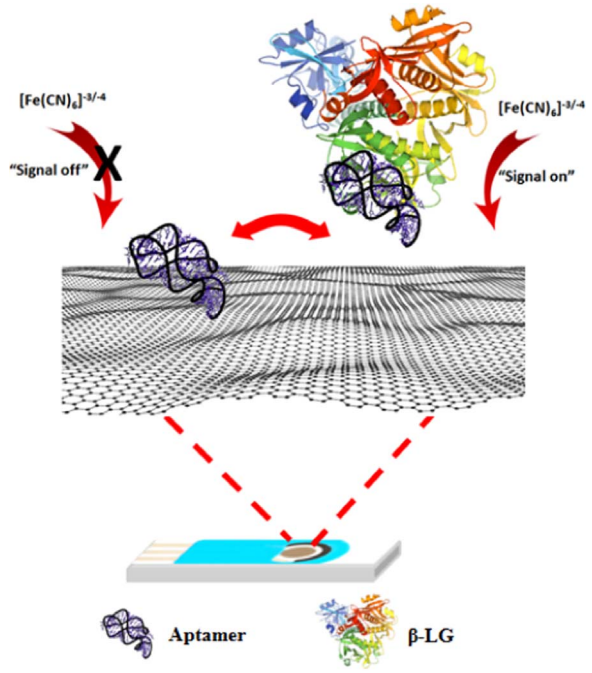
3.3. Binding affinity studies and determination of dissociation constants of the aptamers to β-lactoglobulin A and B

A fluorescence based assay was used to study the binding affinity of the selected aptamers to both β-LG A and B proteins. For that, the two proteins were individually immobilized on the NHS-activated beads. The binding assays experiments were conducted using constant amount of β-LG beads and different concentrations of fluorescein-labelled aptamers (0–200 nM) using the same binding protocol that was used in the selection experiments. After binding, β-LG beads were washed and the bound DNA were eluted. The binding curve was obtained by measuring the fluorescence of the eluted DNA after binding with different aptamer concentration (Fig. 1B and C). The binding curves of the selected aptamers were fitted using a non linear regression model that was used to calculate the dissociation constants (K_d).

As shown in Table 1, the six tested aptamer sequences showed different binding affinities to β-LG A and B. Only one sequence did not show any binding affinity to both proteins which we speculate that it binds to the beads matrix. Aptamers that bind to both protein variants (BLG12 and BLG14) as well as specific aptamers to β-LG A (BLG7) and β-LG B (BLG3) were obtained. Interestingly, it was observed that the aptamer BLG14 exhibited the highest binding affinity to both β-LG A and B with almost similar K_d of 82 and 80 nM, respectively. Therefore, The aptamer BLG14 was chosen to be applied for β-LG detection.

3.4. Voltammetric aptasensor for β-LG detection

The aptamer BLG14 was used to fabricate a label-free aptasensor on a graphene transducer utilizing SWV detection. Scheme 1, shows the biosensing mechanism of the developed aptasensor based on the voltammetric signal change of a redox probe due to the adsorption/release of the aptamer from the graphene electrode surface. The aptamers were first immobilized on a graphene-modified screen printed carbon electrodes (GSPE) by physical adsorption which causes a decrease in the voltammetric signal of a ferrocyanide redox probe (signal off). The stability of this adsorption is induced by the π- π stacking interactions between the nucleobases of the DNA aptamer and the graphene hexagonal lattice. However, upon binding of the β-LG protein to the aptamer, an increase in the voltammetric signal is observed (signal on) likely due to the partial release of the DNA from the surface as previously reported elsewhere (Chang et al., 2010; Sheng et al., 2011).



Scheme 1. β-lactoglobulin detection scheme based on aptamer-functionalized graphene screen printed electrodes.

Fig. 2A shows the $[Fe(CN)_6]^{3-/4-}$ reduction peak in SWV at the GSPE before and after the aptamer immobilization and protein binding. The bare graphene electrode exhibit a sharp well-defined peak in the cathodic scan. This peak decreased dramatically after the physical adsorption of the aptamers on the graphene surface due to the shielding of the surface with the negatively charged DNA that causes a repulsion with the redox probe (Chang et al., 2010). However, after β-LG binding, the intensity of the cathodic peak increased, suggesting the enhancement in the electron transfer efficiency. This increase in the voltammetric peak current after β-LG binding was exploited for the detection. We attribute this enhancement in the current to the release of the aptamer from the graphene surface upon β-LG binding due to the change in the conformation of the aptamer. This release leads to a decrease of the overall negative charge on the surface causing better access of the redox molecules to the graphene (Chang et al., 2010; Sheng et al., 2011).

3.5. Optimization of the aptasensor

In order to achieve the maximum response for the aptasensor, the aptamer concentration on the graphene electrodes were optimized. Fig. 2B, shows the gradual decrease in the SWV peak after incubation with different concentrations of the aptamer. As shown in the inset of Fig. 2B, the maximum change in the peak current (i-i^o) was observed when the GSPEs were incubated with 10 μM solution of the aptamer. This concentration ensures maximum coverage of the graphene surface with the immobilized aptamers which minimizes the nonspecific

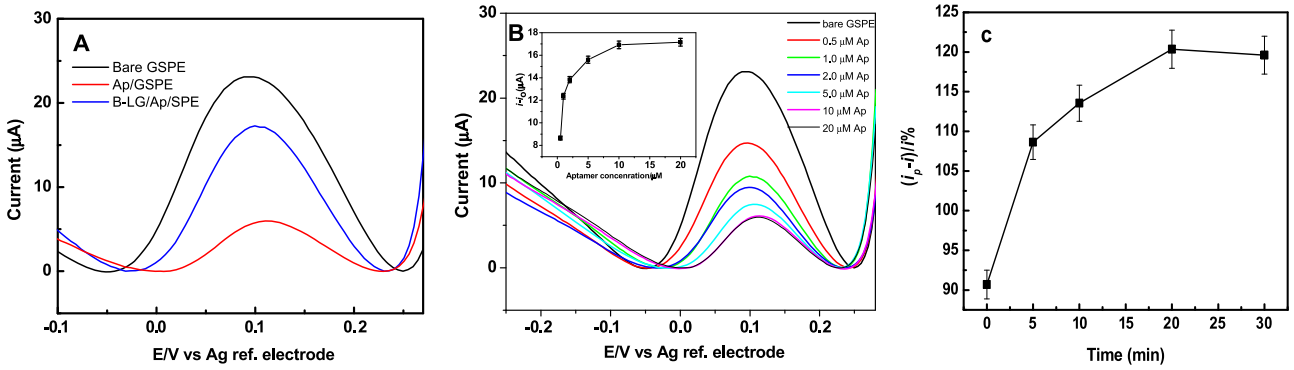


Fig. 2. (A) Square wave voltammograms of 10 mM $[Fe(CN)_6]^{4-/3-}$ redox solution in PBS, pH 7.4 for bare GSPE (black), BLG14 aptamer-functionalized GSPE (red), and after bind with β-LG (blue). (B) SWVs of $[Fe(CN)_6]^{4-/3-}$ for GSPE after 1 h incubation with different concentrations of BLG14, the inset is the $[Fe(CN)_6]^{4-/3-}$ peak current change versus the aptamer concentration. (C) Effect of incubation time on the aptasensor response signal ($(i_p\text{-}i)/i$ %), concentration of β-LG=10 ng/ml. The error bars represent the standard deviations of the measurements. (For interpretation of the references to color in this figure legend,the reader is referred to the web version of this article)

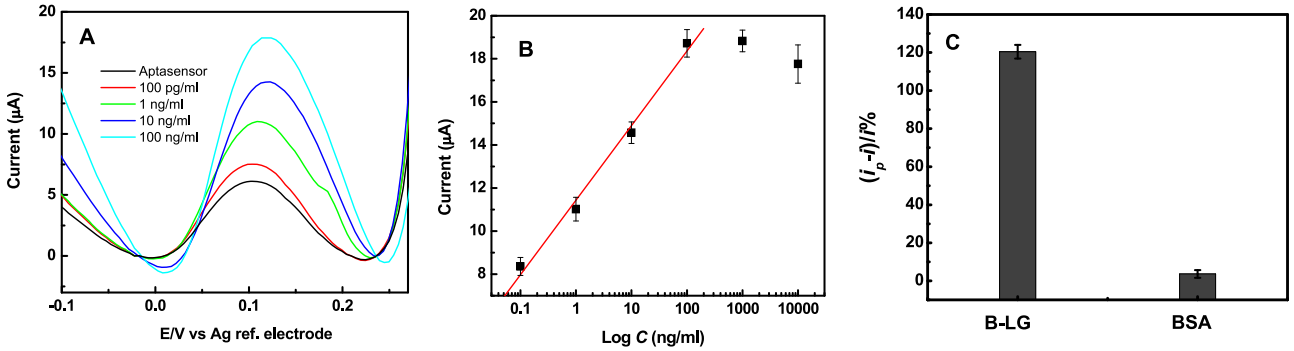


Fig. 3. (A) Square wave voltammetric response of the aptasensor after incubation with different concentrations (100 pg/ml- 10 μg/ml) of β-LG. (B) The calibration curve of the aptasensor (a plot of the peak current versus the logarithm of the β-LG concentrations). (C) Aptasensor response to 10 ng/ml of β-LG and BSA. The error bars represent duplicate measurements.

Table 2
Determination of β-lactoglobulin in spiked food extract.

Sample	Standard concentration (ng/ml)	Amount found (ng/ml)	Recovery %	RSD%
1	0. 1	0.090	90.0	4.9
2	1	0.910	91.0	5.3
3	10	0.95	95.0	5.5

adsorption. The binding time of the β-LG to the aptasensor was also optimized to achieve the largest signal change. As shown in Fig. 2C, the aptasensor response signal (expressed as $(i_p\text{-}i)/i$ %) increased with increasing the incubation time of the proteins on the aptasensor surface from 5 to 20 min after which no more change on the response was observed. Therefore, the protein was incubated for 20 min in all the subsequent experiments.

3.6. β-lactoglobulin aptasensor dose response, selectivity and stability

The aptasensor response was expressed as the SWV reduction peak current of the $[Fe(CN)_6]^{3-/4-}$ upon binding with the β-LG protein. In order to assess the aptasensor detection range, the SWV was measured after incubation with different concentrations (100 pg/ml to 10 μg/ml) of β-LG in binding buffer. As shown in Fig. 3A, the voltammetric peak current increased with increasing the concentration of the β-LG protein from 100 pg/ml to 100 ng/ml after which the higher protein concentration did not result in any more signal change. The calibration curve was constructed by plotting the aptasensor response versus the logarithm of the β-LG concentrations as shown in Fig. 3B. The calibration curve showed linear relationship up to 100 ng/ml which can be fitted with regression equation: (i_p =11. 4+3.36×log$C$ [ng/ml], showing a correlation coefficient of 0.995 (N=4). The detection limit of the aptasensor was calculated as 3σ/b, where σ is the standard deviation of the blank samples and b is the slope of the straight line to be 20 pg/ml. This detection limit is lower than the commercial ELISA, HPLC (Rena et al., 2010), capillary electrophoresis (Pelaez-Lorenzoa et al., 2009) as well as other reported optical (Hohensinner et al., 2007) (Wu et al., 2016) and electrochemical (Ruiz-Valdepeñas Montiel et al., 2015) immunosensors (Table S2).

Fig. 3C shows the selectivity of the aptasensor for β-LG against BSA as a non specific control protein. The aptasensors were incubated with 10 ng/ml solution of either β-LG or BSA and the SWV signal was recorded. The results showed no significant response of the aptasensors incubated in BSA against high signal with the β-LG protein indicating the high selectivity of the selected aptamers.

The stability of the developed aptasensor was also studied after storage for a week and no loss of activity was observed (data not shown). Moreover, we have previously studied the response of a graphene aptasensor for microcystin-LR prepared by physical adsorption of the results confirmed the stability of the aptasensor over one month (Eissa et al., 2014).

3.7. Application of β-lactoglobulin aptasensor in real food sample extract

The developed aptasensor was tested in spiked food extract in order to investigate the effect of the food matrix on the sensor response. Diluted β-LG-free food sample extracts were spiked with three different concentrations of β-LG and measured using the developed aptasensor. Table 2 shows the recovery percentage obtained using the aptasensor ranging from 90 to 95%) indicating non significant interference from

the food matrix. These results confirms that the developed aptamer/graphene biosensor can be successfully applied for the detection of milk allergen in food samples.

4. Conclusion

We have selected for the first time high affinity and specificity aptamers that bind to the milk allergen β-LG. The aptamer BLG14 which exhibited the highest affinity to both β-LG A and B protein variants with k_d of 82 and 80 nM, respectively, has been integrated into a graphene-based biosensor for quantitative detection of β-LG. The biosensor exploits the conformational change of the aptamer sequence upon binding with the β-LG protein which results in the release of the aptamer from the graphene surface. The novel developed electrochemical graphene-based aptasensor combines the low cost, stability and specificity of the aptamers with the ease of synthesis and fast electron transfer efficiency of graphene leading to a good sensitivity and selectivity. The detection limit was calculated to be 20 pg/ml that is much lower than other reported approaches with complicated fabrication and/or detection strategy. The aptasensor has showed good recovery percentage when applied in spiked food sample extract.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2016.12.020.

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Supporting Information

In vitro selection of DNA aptamers targeting β-lactoglobulin and their integration in graphene-based biosensor for the detection of milk allergen

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Coupling of β-LG A and B to the NHS activated beads

The coupling reaction is shown in the schematic diagram (Figure S1). Stock solutions of β-LG A and B (1.0 mg/ml) in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) were prepared. Two millilitres of the NHS activated sepharose beads were washed for 15 min with 1 mM HCL to remove the preservative solution and activate the terminal groups. The beads were then incubated with the stock solution of β-LG (1:1 volume ratio) in polypropylene tubes with mixing end-over-end for 4 hours at 4 ^{o}C. After the incubation is done, the beads were rinsed five times with coupling buffer in order to remove the excess unreacted β-LG. The free NHS active groups on the beads were blocked by incubating the beads with 0.1 M Tris-HCL buffer pH 8 with mixing for 1 hour. then, the β-LG beads were washed extensively with three cycles of alternating pH consisting of a wash with 0.1 M acetic acid/sodium acetate, pH 4 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCL buffer pH 8 containing 0.5 M NaCl. The negative NHS activated beads were treated the same way by blocked them using 0.1 M Tris-HCL buffer pH 8

for the counter selection cycles. The β-LG beads and the negative beads were kept in 50 mM Tris-HCl buffer pH 7.5 at 4^{o}C until further use. The Immobilization of β-LG on the beads was verified by performing direct ELISA for the coupled beads and the negative beads as control. To perform the ELISA experiments, 30 µl of each beads were washed with PBS buffer pH 7.4 and blocked with 2% BSA in PBS buffer for 2 hours. Then the beads were incubated with diluted anti- β-LG antibody (1:100) in PBS buffer, pH 7.4. After washing, the beads were incubated with HRP-labelled secondary antibody (diluted 1:1000) for 1 h. Then the beads were washed three times with PBS buffer and the stabilized chromogen (TMB) solution was added. A blue color was produced in the β-LG -coated beads, while no color was seen in the negative beads confirming the success of the conjugation reaction. After storing the beads for few weeks during the selection process, the stability of the β-LG -beads was again tested using ELISA

In vitro selection of the DNA Aptamer against β-LG

A random ssDNA library (3 nmol or 1.8×10^{15} sequences) was used for the SELEX experiments Each sequence consists of central random region of 60 nucleotides with two fixed regions of 18 nucleotides-sequences at the 3' and 5' ends. These regions are the primer binding sites for the PCR amplification. 100 µl of β-LG -beads were rinsed few times with binding buffer. (3 nmol at the first selection round, and 150 pmol in the subsequent rounds) of ssDNA pool were heated to 90°C for 5 minutes, cooled at 4°C for 10 minutes, kept at 25° C for 5 minutes and then added to the washed β-LG -beads in 300 µL binding buffer in a centrifuge filter tube. The mixture was incubated at room temperature with end-over-end rotation for 2 hours. The beads were then washed few times with binding buffer until very low fluorescence is detected in the last wash. The bound DNA to β-LG -beads was then eluted with 400 µL aliquots of elution buffer for 6 times with heating at 90°C for 10 minutes until no DNA is detected in the fluorescence

measurements. The eluted DNA was concentrated and desalted by ultrafiltration device. In the counter selection cycles after round 5 and 6, the DNA pool was first incubated with the negative blocked NHS beads, washed DNA was collected, subjected to the same heating and cooling treatment as previously described and subsequently incubated with β-LG-beads. In the last rounds the SELEX was applied using more stringent conditions as shown in table S1

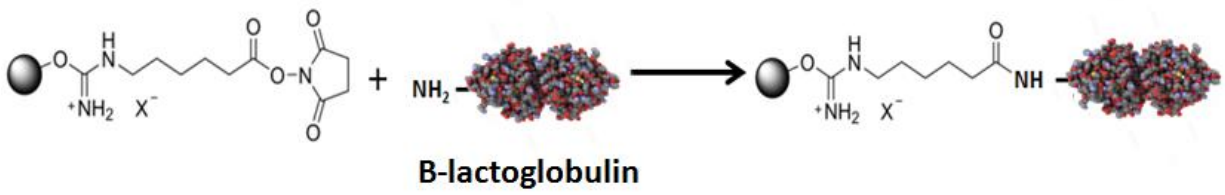

Fig. S1. Attachment of B-LG A and B to the NHS-activated sepharose beads

Table S1 Summary of the condition of the selection cycles

Selection round	Counter selection	Amount of β-LG beads (µl)	Incubation time with CBZ beads (min)
1-5	0	100	120
6	100 µl negative beads	100	120
7	0	100	120

8	0	70	60
9	0	70	50
10	0	60	30

PCR amplification and PAGE extraction

The selected DNA pool after each round was amplified by PCR in 15 parallel 75µL reactions each containing 2 units of Taq Plus and polymerase buffer, 2 mM $MgCl_2$, 200 µM dNTP, 0.2 µM of forward and reverse primers. The primers were modified with fluorescein and a PEG linker followed by a poly-A tail as reported previously (Eissa et al. 2013; Eissa et al. 2015; Ng et al. 2012). Forward primer: 5′-fluorescein-ATACCAGCTTATTCAATT-3′; reverse primer: 5′-poly(dA20)-PEG6-AGATTGCACTTACTATCT-3′. PCR conditions: 94°C for 10 minutes, followed by 25 cycles of 94°C for 1 minute, 47°C for 1 minute, 72°C for 1 minute, and a final extension step of 10 minutes at 72°C. PCR products were dried by SpeedVac, resuspended in water and formamide (50:50 v/v) and heated to 55°C for 5 minutes. The relevant ssDNA (labelled with fluorescein) was separated from the double stranded PCR product in 12% denaturing PAGE and eluted from the gel band by freeze-thaw cycle. Eluted ssDNA in TE buffer

was concentrated, desalted by ultrafiltration, quantified by UV and used for the next selection cycle.

Cloning and sequencing of selected DNA

After ten selection rounds when the DNA recoveries began to plateau as monitored by measuring the fluorescence intensity of the eluted DNA, the selected ssDNA were amplified with the non-modified primer set and cloned into pCR2.1-TOPO vector using the TOPO TA Cloning Kit. Colonies were grown on LB-agar medium supplemented with ampicillin, X-Gal and IPTG. Positive colonies were picked and grown in liquid media. ssDNA inserts were PCR amplified using the M13 forward and reverse primer sites within the vector and sequenced. Sequences of the selected ssDNAs were analyzed and aligned using PRALINE (Simossis et al. 2005) (Figure S2).

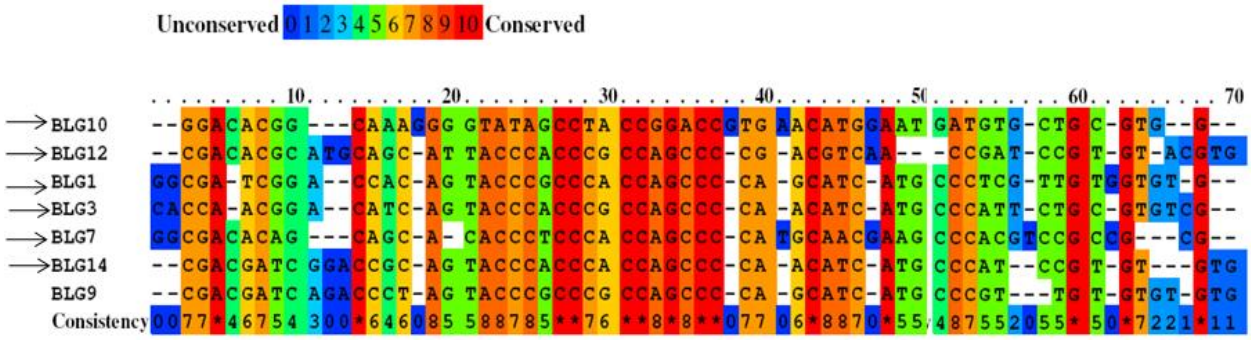

Fig. S2. Multiple sequence alignment of the selected aptamers

Dissociation constants determination by fluorescence assay

The dissociation constants of the selected aptamers for β-LG A and B were determined by performing binding assays as described above using various concentrations of the aptamer (0

to 200 nM). Different concentrations of each sequence in binding buffer were incubated with 20 µL of β-LG A and B beads in a centrifuge tube after being subjected to the heating and cooling treatment as described above in the selection step. The fluorescence of the eluted DNA from each sample was measured, and the saturation curve was obtained. The dissociation constant (K_d) for each sequence was calculated using non linear fitting of the curve.

Comparison of sensitivity of the different reported detection methods of β-lactoglobulin

Table S2. Comparison of sensitivity of the different reported detection methods of β-lactoglobulin

Method	**Limit of detection**	**Analytical range**	**Reference**
Voltametric aptasensor	20 pg mL^{-1}	100 pg mL^{-1}-100 ng mL^{-1}	This work
Capillary electrophoresis	9.2 ng mL^{-1}	-	(Pelaez-Lorenzoa et al. 2009)
Commercial ELISA	-	0.1- 1.0 µg ml^{-1}	(ELISA Systems, Australia)
HPLC	0.06 µg mL^{-1}	0.4-60 µg mL^{-1}	(Rena et al. 2010)

Electrochemical magneto immunosensing	0.8 ng mL^{-1}	2.8–100 ng mL^{-1}	(Ruiz-Valdepeñas Montiel et al. 2015)
Voltammetric immunosensor	0.85 pg mL^{-1}	1 pg mL^{-1}- 100 ng mL^{-1}	(Eissa et al. 2012)
Optical near field biosensor	-	100 ng mL^{-1}-100 µg mL^{-1}	(Hohensinner et al. 2007)
Surface plasmon resonance	5.54 ng mL^{-1}	5.0 ng mL^{-1}-4000 ng mL^{-1}	(Wu et al. 2016)

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