



Milk Allergen Detection: Sensitive Label-Free Voltammetric Immunosensor Based on Electropolymerization

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Published online: 15 April 2020

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Abstract

We present in this work, first time in literature, a voltammetric label-free biosensor for detection of alpha lactalbumin (α -LB) in meal samples. Food allergy concern necessitates strict regulations in food labelling and innovative monitoring strategies for prevention of sepsis-related death. As major food allergens, dietary samples often contain whey proteins, including α -LB as one of the key allergenic. Available detection methods for α -LB are based on successive labelling steps and hence have long assay times with limited sensitivity. The new detection method presented here is based on capturing of α -LB via entrapped α -LB antibody (α -LB-Ab) through electropolymerization of polypyrrole (PPy) and then measuring the conductivity decrease by differential pulse voltammetry (DPV). This new label-free voltammetric biosensor for α -LB exhibits the best limit of detection (0.19 fg/mL) published so far in literature, with high selectivity and capability to quantify α -LB in real milk samples too.

Keywords Alpha lactalbumin · Milk allergens · Food allergy · Pyrrole · Electropolymerization · Immunosensor

1 Introduction

Food allergy is defined as an immune pathogenesis or a type I hypersensitivity immune response to some foods that contain certain food allergens [1]. Food allergies have increasingly become a public health issue and food safety concern since their prevalence is estimated to be between 4 and 8% in children and from 1 to 2% in adults [2, 3]. Hypersensitive immune allergenic response is known as anaphylaxis, and its management includes treatment of acute episodes and preventive measures by labelling products at high allergy risk [4].

As the most common food allergy in infants and young children, cow's milk allergy (CMA) affects between 2 and 3% of the general population [5, 6]. Cow's milk is known to contain about 30 to 35 g of proteins per litre that makes up to 3–3.5% protein. Those proteins can be sub-grouped into two main classes: caseins (80%) and whey proteins (20%). Among those proteins, only some types such as α -lactalbumin (α -LB), β -

lactoglobulin (β -LB), bovine serum albumin (BSA), immunoglobulins, lactoferrin and the four casein types are known to be allergenic [7]. Among milk allergens, a small (14,200 Da) protein that can bind to calcium, α -LB is reported to have an allergenic activity in 12% of the patients suffering from cow's milk allergy [8, 9]. As the second most abundant protein in bovine whey, α -LB is a globular protein made of 123 amino acids stabilized by four disulphide bonds [10]. The main function of α -LB is to catalyze lactose biosynthesis in the lactating mammary gland by being one of the two components of the lactose synthase enzyme [9]. α -LB is also known to be heat labile under its different forms from 78 to 94 °C and could be used as a marker for effective milk heat treatment [11]. Interestingly, the ratio whey to casein proteins is higher in human milk (60:40) than in cow's milk (20:80) [12]. Originally, infant milk formulas were based only on unmodified cow's milk proteins but whey proteins and in particular α -LB have been identified as beneficial for the infant's development [13]. Thus, current infant milk formulas are enriched in α -LB to mimic more closely the human breast-feeding milk [12].

Due to its important presence in bovine milk, its allergenic potential and its abundance as a supplement in infant milk formulas, the α -LB is an important target to detect in dietary samples. Among available food allergen detection methods, enzyme-linked immunosorbent assay (ELISA) is known to be the golden standard despite being time-consuming and requires

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labelling [14, 15]. On the other hand, biosensors offer great advantages for detection of biomarkers not only for food allergens [14] but also for drug monitoring [16] and disease biomarkers detection [17–20]. So far, several alternative biosensing methods have been proposed for food allergen detection based on visualized microarray immunoassays [21], surface plasmon resonance (SPR) [22], amperometric [23] or differential pulse voltammetry (DPV) [21, 24]. Concerning the α -LB detection, the current available methods are SPR [25], competitive fluorescence-linked immunosorbent assay (FLISA) [26], miniaturized optical biosensor [27] or biosensors based on amperometric measurements [28]. However, those methods either have high limit of detection [25] or require further materials during functionalization such as quantum dots [26], gold nanoparticles [29] or magnetic beads [28]. Therefore, there is a real need to develop new, more accurate, easy and cost-effective method for α -LB detection in dietary samples.

In this work, we propose for the first time to best of our knowledge the most sensitive label-free voltammetric biosensor for detection of α -LB from real milk samples based on electropolymerization based on polypyrrole (PPy), an organic conducting polymer that previously been shown to contribute increasing the electrode surface area and thus enhancing the loading capacity for efficient antibody [30] and nucleic acid immobilization [31]. The α -LB biosensor proposed here is very sensitive with limit of detection (LOD) of 0.192 fg/mL, which is ten times more sensitive compared with its commercial alternative, ELISA, and can be further optimized for multiplexed detection of food allergens thanks to addressable functionalization of electrodes with different biorecognition element.

2 Materials and Methods

2.1 Chemicals and Reagents Used

Recombinant Human α -LB protein (ab185402, 0.45 mg/mL) and α -LB antibody (α -LB-Ab) (ab182929, 1 mL) were purchased from Abcam. IgG antibody was bought from BioLegend. Albumin from human serum (HSA), pyrrole and phosphate buffer saline (PBS; $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$) were all purchased from Sigma. The measurements were performed with gold screen-printed electrodes (AuSPE) bought from DropSens. Blotto, non-fat dry milk was purchased from Santa Cruz Biotechnology. The dietary samples were all purchased from a local supermarket.

2.2 Methods

The experimental steps followed throughout the study are sketched in Scheme 1. The functionalization of the AuSPEs was done via immobilization of α -LB-Ab through electropolymerization of pyrrole. Electrochemical detection

was performed via DPV by measuring the oxidation peak current of $K_3[Fe(CN)_6]^{3-/4-}$. Experimental steps were detailed in the following sections.

2.2.1 Electrochemical Measurements

DPV measurements were performed using an electrochemical working station (Metrohm Autolab PGSTAT 302 N with the Nova 1.11 software) with a conventional three-electrode configuration. The potential was scanned from -0.2 to $+0.6$ V with a scan rate of 0.01 V/s. An equimolar solution of 5 mM- $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in 0.1 -M KCl was dropped on the electrodes for the measurement of peak current.

2.2.2 Biofunctionalization of AuSPEs for α -LB Capture

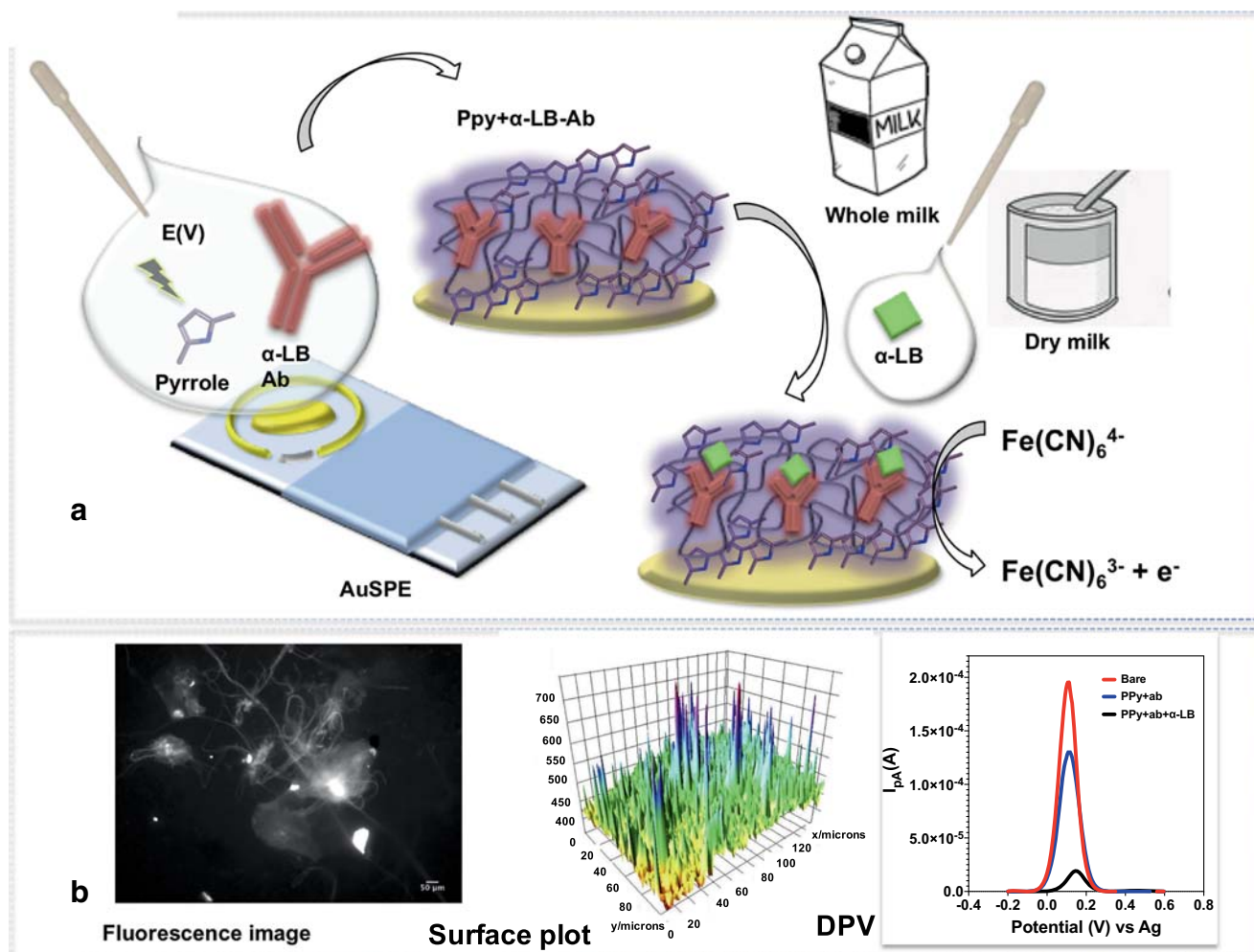
After α -LB-Ab was diluted in PBS to an optimized concentration, it was mixed ($1:1$, v/v) with a solution of 0.1 -M pyrrole (prepared in 0.7 -M KCl) and drop casted onto AuSPE surface. As determined in a previous work [31], a cyclic voltammetry (CV) procedure was applied for electropolymerization of pyrrole to PPy in order to encapsulate α -LB-Ab inside PPy. To do so, a potential scan from 0 to 0.850 V with a scan rate of 0.25 V/s was applied for 4 cycles. Afterwards, the electrodes were washed with 0.1 -M PBS, and then, DPV measurements of AuSPEs modified with PPy + α -LB-Ab were performed.

2.2.3 Electrochemical Detection of α -LB

PPy + α -LB-Ab-modified AuSPEs were incubated with diluted α -LB for 1 h at 4 °C. After incubation, electrodes were washed with PBS and DPV signal was measured. The optimum incubation time and temperature of α -LB were determined via several experiments with different incubation timings. Calibration curve experiments were thus performed using the recorded current signals in terms of α -LB concentration. The LOD and the limit of quantification (LOQ) were calculated from this calibration curve parameters as $3.3 \cdot s_{yx}/a$ and $10 \cdot s_{yx}/a$ respectively, with s_{yx} being the standard deviation of the residuals and a the slope of the calibration curve [32].

2.2.4 Selectivity Studies

Various selectivity checks were performed throughout this work. The first control study was done to assess if non-specific entrapment of α -LB occurs regardless of antibody capture. For this purpose, electropolymerization was performed without adding antibody solution to pyrrole but keeping all other parameters (see previous section) constant. After that, α -LB was incubated at optimal conditions (1 h at 4 °C). Another control study was to evaluate the selectivity of sensing surface as well as of the α -LB-Ab by comparing with a different antibody, IgG, which was similarly diluted in PBS



Scheme 1 Experimental steps followed throughout this study; biofunctionalization of Au SPE via α-lactalbumin antibody (α-LB Ab) through electropolymerization of pyrrole (Ppy) for capturing the α-LB in cow or dry milk samples (A). Characterization of the biosensor

surface via fluorescence microscopy imaging and detection of α-LB via DPV based on K₃[Fe(CN)₆]/K₄[Fe(CN)₆] oxidation signal before and after target capture (B)

(1/1000), and functionalization of AuSPEs was done similarly, and then the highest detectable α-LB concentration (0.2 nM) was incubated at the optimal conditions: 1 h at 4 °C. The last control test was performed to assess the selectivity α-LB-Ab by changing its target. For that, we functionalized the electrodes in the same manner and then incubated 0.2 nM of HSA at the optimal conditions used here as a model of a different non-specific target molecule.

2.2.5 Dietary Sample Analysis

Several dietary samples have been analyzed with the α-LB sensor: Ultra-high-temperature processing (UHT) cows' skimmed milk, non-fat dry milk and almond milk. Cows' milk samples were first diluted 1/1,000,000 in PBS and incubated

for 1 h at 4 °C on PPy + α-LB-Ab-modified AuSPEs. Measured DPV signals were used to calculate α-LB concentration via calibration curves performed for PBS. In addition to that, another calibration curve studies were performed by serial dilution of real milk samples to see the sensitivity changes over different measurement media. Almond milk was used as control real sample since it does not contain α-LB.

2.2.6 Fluorescence Microscopy Measurements

Axioplan2 upright fluorescence microscope (Carl Zeiss Microscopy GmbH, Germany) was used for fluorescence microscopy imaging. The image analysis was done by using ImageJ, a free software provided by the National Institute of Health (<http://imagej.nih.gov/ij/>).

2.2.7 Statistical Analysis

All data were obtained from three independent experiments and expressed by means $\pm$ SD. The means of two independent conditions were compared by using Student *t* tests. A *p* value < 0.05 was considered as significant.

3 Results and Discussion

The experimental steps conceptually shown in Fig. 1 were performed with the aim of label-free detection of α -LB based on oxidation peak current (IpA) measurement of $K_3[Fe(CN)_6]^{3-/4-}$ before and after α -LB incubation on PPy + α -LB-Ab-modified AuSPEs. In this work, on the contrary to other methods that employ electrochemically active intercalators such as Meldola's Blue [31] or enzymatic labels such as horseradish peroxidase [23], we use redox mediator $K_3[Fe(CN)_6]^{3-/4-}$ and provide a very simple biofunctionalization and detection method. The novelty of our work relies on biofunctionalization of AuSPEs.

More specifically, we have used electropolymerization-based immobilization strategy for the first time for label-free food allergen detection without any need for other surface modification strategy such as growing gold nanowires before polymerization-based doping of prostate specific antibody (PSA) and bovine serum albumin-blocking step after immobilization [30]. Previously, PPy polymerization was used also for miRNA detection but in that work, pencil graphite electrodes have been used and detection was label-based upon Meldola's Blue signal [33]. Therefore, our strategy is simple without need for additional surface treatment steps and applied first time for food allergen detection from real food samples. Antibody immobilization on AuSPEs was achieved via electropolymerization of pyrrole to PPy, thanks to electrostatic attraction between positively charged polypyrrole chains with the negative carboxyl groups on α -LB-Ab antibodies.

Optimization, selectivity and calibration studies were all performed by using DPV, while the characterization of the biosensor surface was done via fluorescence microscopy imaging.

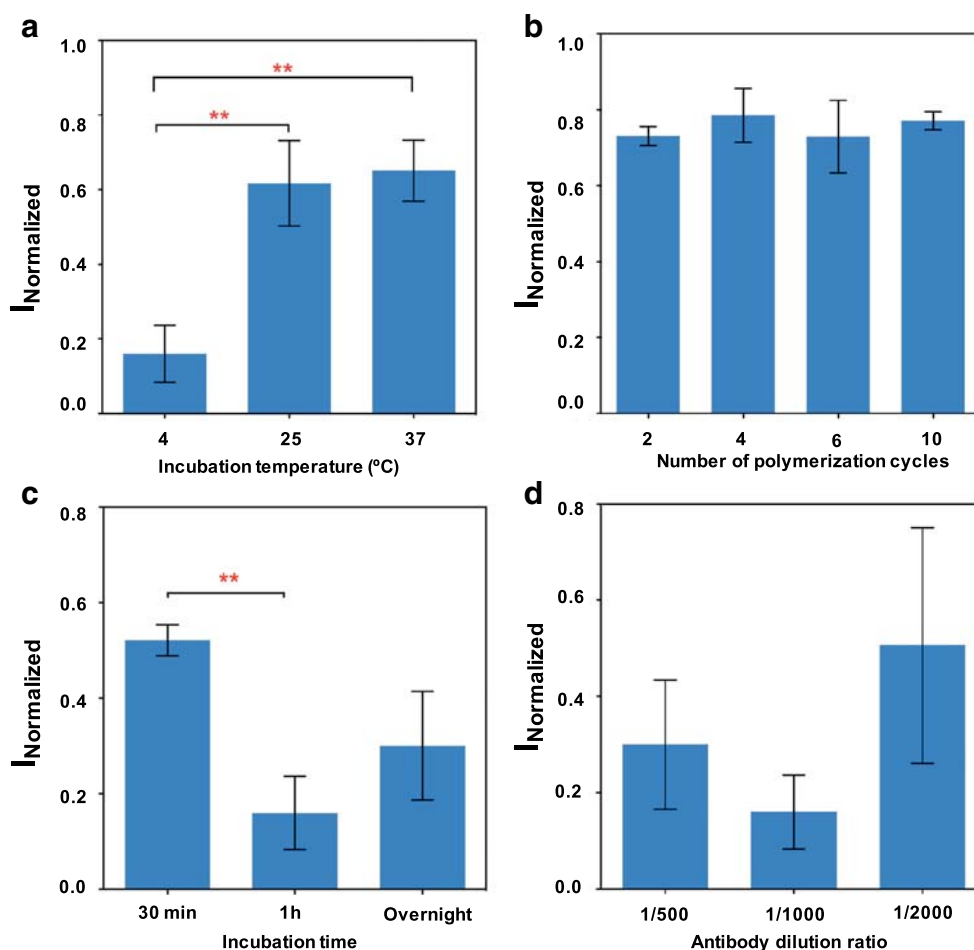


Figure 1 Experimental steps followed throughout this study; biofunction of Au SPE via (α -LB Ab) through electropolymerization of pyrrole (PPy) for capturing the α -LB in cow or dry milk samples (a). Characterization

of the biosensor surface via fluorescence microscopy imaging and detection of α -LB via DPV based on $Fe(CN)_6^{3-/4-}$ oxidation signal before and after target capture (b)

3.1 Optimization Studies for α -LB Biosensor

After proving that this method is suitable for α -LB detection, we moved to optimization studies for achieving the best biosensor performance. As it was clearly explained earlier [34], electropolymerization mechanism of pyrrole that is shown in Fig. 2A results in the inclusion of the counterion (C^-), which is usually the anion of the supporting electrolyte salt. Therefore, the chemical properties of the polymer can be controlled via certain parameters such as electrode substrate material, polymerization conditions such as number of cycles and applied potential (E_{app}), type of the monomer and type of counterion that can be provided by biological molecules such as antibodies. Counterion incorporation into PPy are reported to be primarily ionic, and in the case of large proteins, hydrophobic interactions, hydrogen bonding, dipolar interaction and London dispersion forces were reported to occur in the polymer backbone [34]. In order to determine the best conditions for parameters affecting the biosensor performance, we evaluated the normalized I_{pA} , $I_{Normalized}$, which is defined as ratio of I_{pA} before and after 0.2-nM α -LB incubation. As first optimization study, we have tested various incubation temperatures of +4, +25 and +37 °C and results can be seen in Fig. 2A. We concluded that the $I_{Normalized}$ is

significantly lower when α -LB is incubated at 4 °C rather than 25 °C (Student t test p value = 0.0045) or at 37 °C (p value = 0.0016). Therefore, we have chosen 4 °C as optimum incubation temperature for the antigen. Next, we have investigated the optimum number of cycles for CV procedure to grow antibody-doped PPy film. As it is clear in Fig. 2B, no significant differences were found between 2, 4, 6 or 10 cycles in terms of the values of $I_{Normalized}$. Thus, an intermediate cycle number of 4 was chosen as the best condition for pyrrole electropolymerization. After that, the effect of the antigen incubation time on the $I_{Normalized}$ was investigated. We have deduced from Fig. 2C that 30 min is not enough for the full interaction between α -LB-Ab and α -LB. Instead, after 1-h incubation of the antigen, significant decrease was observed (p value = 0.0017). However, incubation periods longer than 1 h did not result in a higher signal drop. Moreover, the difference between experiments with only 1 h and overnight is not statistically significant while standard deviation actually increases with overnight incubation. This last clearly shows that a more chaotic organization of the probes is obtained with overnight incubation and, therefore, the 30-min incubation is the one that allow working with a better sensing surface. In addition to that, since the biosensors have to be fast, the 1-h α -LB incubation was chosen as the optimal condition. The last

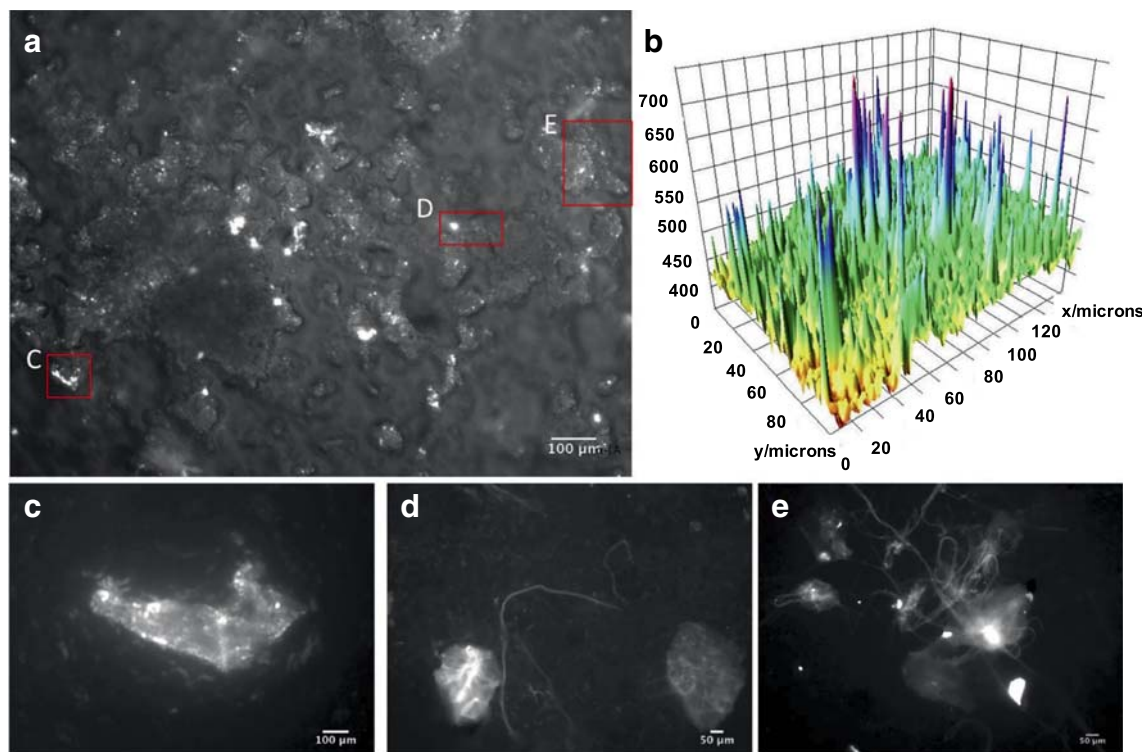


Figure 2 Optimization studies of the α -LB biosensor: bar graphs represent the normalized oxidation peak current value (the ratio of the peak currents after and before 0.2 nM α -LB Ab, $I_{Normalized}$) for different incubation temperature (A), number of polymerization cycles (B α -lactalbumin incubation time (C) and antibody dilution ratio (D). Data

are expressed as mean $\pm$ standard deviation of three different measurements. Experimental steps are detailed in methods section. T-test calculated p -values are 0.0045 (B, 25°C vs 4°C), 0.0016 (B, 37°C vs 4°C) and 0.0017 (C).

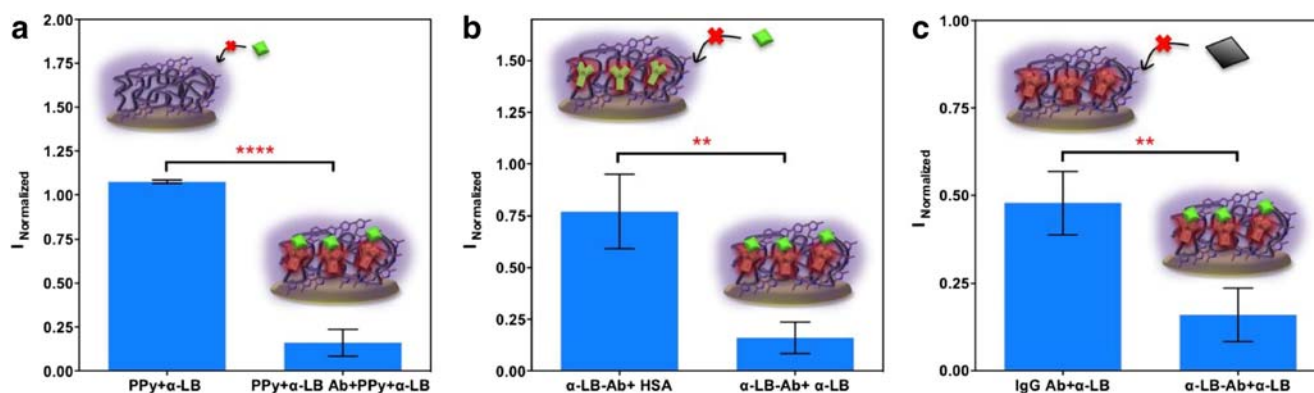


Figure 3 Fluorescence microscopy images of AuSPEs modified with AlexaFluor 647 tagged IgG protein with polypyrrole. ImageJ of the National Institute of Health (<http://imagej.nih.gov/ij/>) was used to analyze the images taken by Axioplan2 upright fluorescence

microscope at 20x objective. The plug-in Interactive 3D Surface Plot was used to generate a surface plot of the sensor surface in gray-scale and using the ‘spectrum’ LUT (from a different perspective) as in the image above.

condition to be optimized was the α -LB-Ab dilution factor. For this purpose, three different dilutions were tested (1/500, 1/1000 and 1/2000) and results are represented in Fig. 2D. No significant differences had been found between them, but the normalized oxidation peak current seems slightly lower for the 1/1000 dilution. Moreover, the error bars for the 1/2000 dilution are very big, probably due to over-diluted antibody, which cannot be homogeneously entrapped inside the PPy, and thus the reproducibility is low. On the other hand, the α -LB-Ab concentration for the 1/500 dilution seems to be more than enough since error bars are higher than the ones in 1/1000 dilution, which seems the optimum. As a result, the optimum conditions for the best α -LB performance were specified as 4 polymerization cycles with a 1/1000 dilution of the α -lactalbumin antibody and incubation of α -lactalbumin for 1 h at 4 °C.

3.2 Characterization of α -LB Biosensor Via Fluorescence Microscopy

One of the most important steps for immunosensors based on affinity sensing is to immobilize the antibody to the sensor surface in a most efficient manner, which means immobilization of maximum number of antibody in the right orientation. For this work, entrapment of antibody in electropolymerized PPy was used for sensor biofunctionalization. This method was previously used for microRNA detection [33] where Fourier transform infrared spectroscopy (FT-IR) was used for characterization of the sensor surface. In this work, we used for the first time fluorescence microscopy imaging of the sensor surface for characterization by using AlexaFluor 647-tagged IgG since the isotype of α -LB-Ab is also IgG

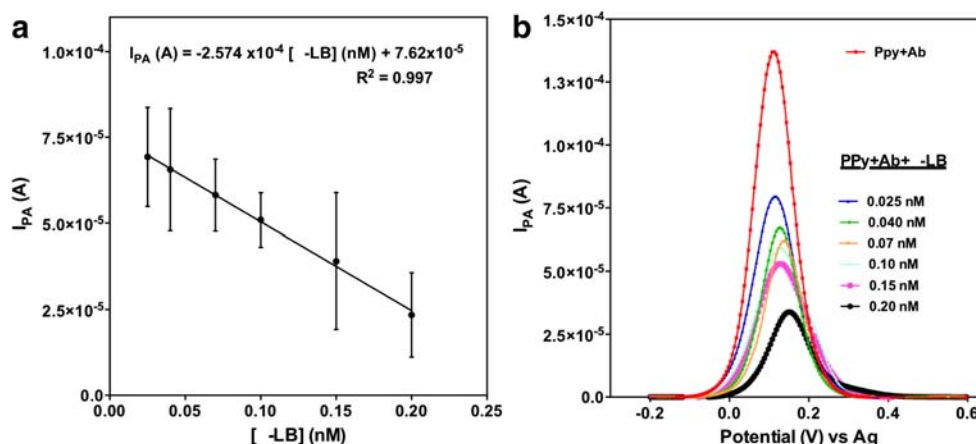


Figure 4 Control studies performed for the designed biosensor. Bar graphs represent the normalized oxidation peak current value, $I_{Normalized}$ to check the effect of α -LB Ab on α -LB and without α -LB (PPy+ α -LB) (A) or using IgG Ab (B) and selectivity control over HSA incubation (C).

Data are expressed as mean $\pm$ standard deviation of three different measurements. Experimental steps are detailed in methods section. T-test calculated p-values are < 0.0001 (A) 0.0094 (B) and 0.0055 (C).

and labelling proteins with fluorescence tags can also change their secondary structure. For this proof of concept in surface characterization, instead of α -LB-Ab, we have added AlexaFluor 647-tagged IgG protein into pyrrole solution for electropolymerization. As demonstrated in Fig. 3A, the sensor surface has high roughness due to PPy, and also, fluorescence signals spread through surface due to AlexaFluor 647-tagged IgG protein residues that can be seen in magnified images of the spots circled in Fig. 3A, C, D, and E. Fig. 3E clearly shows the entrapment of fluorescence-labelled proteins in PPy matrix that seems to have bundles and knots. Three-dimensional fluorescence intensity profile can be seen in Fig. 3B, where x -axis shows excitation and y -axis shows emission wavelength. The fluorescence intensity profile changes throughout the surface due to the non-organized and bundled PPy surface that AlexaFluor 647-tagged IgG protein is exposed to as expected. However, these images clearly show successful biofunctionalization of antibody after electropolymerization.

3.3 Selectivity Studies for the α -LB Biosensor

After the optimization and fluorescence microscopy-based characterization of the α -LB biosensor, we have performed control studies to assess the selectivity and the specificity of the designed biosensor. We have performed three sets of experiments for control study. All results of those studies were shown in Fig. 4. First of all, we checked the effect of α -LB-Ab on α -LB capture by functionalising AuSPEs only with PPy without any addition of α -LB-Ab. When the $I_{\text{normalized}}$ values

for PPy + α -LB and PPy + α -LB-Ab + α -LB were compared in Fig. 4A, it can easily be seen that $I_{\text{normalized}}$ resulted in a very significant decrease (p value < 0.0001) in presence of the α -LB-Ab. On the other hand, when there is no antibody present on the PPy film, no capture of α -LB was captured on the electrode surface, hence no signal reduction was observed. Thus, we conclude that the signal decrease after α -LB incubation is due to its interaction with its antibody α -LB-Ab but not just being non-specific binding to the polymer network. This is a very crucial parameter to check, since porous polymer structures can trap molecules and result in misleading information about biosensor readings. In the second control study, we have tested the selectivity of the biosensor in terms of different capturing antibody and different antigens. To do that, we have performed two different sets of experiments where we either changed the target antigen to HSA instead of α -LB (Fig. 4B) or changed the capturing antibody to IgG, instead of α -LB-Ab, for functionalization of AuSPE via electropolymerization (Fig. 4C). According to the HSA study, we deduced from Fig. 4B that even a very high concentration (0.2 nM) of HSA does not affect the $I_{\text{normalized}}$ value as much as α -LB does (p value = 0.0055). The results of capturing antibody change are shown in Fig. 4C. There, IgG antibody was similarly diluted over 1000 times. The resulting normalized peak oxidation current was compared with the original α -LB sensing scheme. A significant decrease (p value = 0.0094) is observed when α -LB antibody is used instead of IgG antibody, which means that the presented method is robustly based on antibody-antigen interaction rather than non-

Figure 5 Results of α -LB biosensor sensitivity studies: Calibration line drawn for DPV based detection of PBS in the concentration range of 0.025 nM - 0.20 nM (A) Differential pulse voltammograms recorded for various α -LB concentrations (B). Data are represented as mean $\pm$ standard deviation of three different experiments. Experimental steps are detailed in methods section.

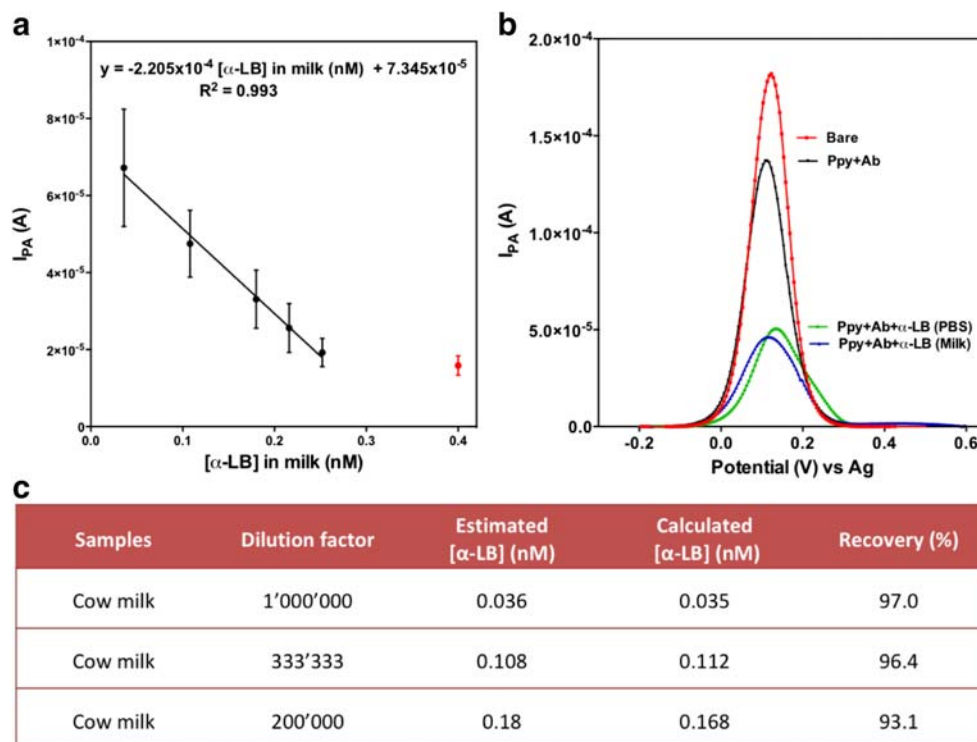


Table 1 List of reported milk antigens biosensor platforms

Analyte	Detection method	Detection range (pg/mL)	LOD (pg/mL)	Dietary sample	References
α -LB	Amperometric	37.0–5000	11.0	Milk	Montiel et al. (2016)
α -LB	Competitive fluorescence-linked immunosorbent assay (FLISA)	100–10 ⁶	100	Milk, yoghurt, peanut milk	Yang et al. (2014)
α -LB	SPR	10 ⁴ –10 ⁶	1.2 × 10 ⁸	Milk, colostrum, whey protein concentrates and infant formulae	Indyk (2009)
α -LB	Goldmag-based enzyme-linked immunosorbent assay	2.33 × 10 ³ –1.27 × 10 ⁵	1.72 × 10 ⁴	Bovine milk	Xu et al. (2017)
α -Lactalbumin, β -Lactoglobulin, lactoferrin	Visualized microarray immunoassay	4 × 10 ⁴ –5 × 10 ⁴	3 × 10 ³	UHT, skimmed and high-Ca milk	Li et al. (2017)
α -LB	Electropolymerization, label-free, DPV		355–2840	192.2	UHT, skimmed, dry, almond milk
					This work

specific binding of α -LB. All those results highlight the specificity of the biosensor towards α -LB detection, thanks to the use of α -LB-Ab and functionalization strategy.

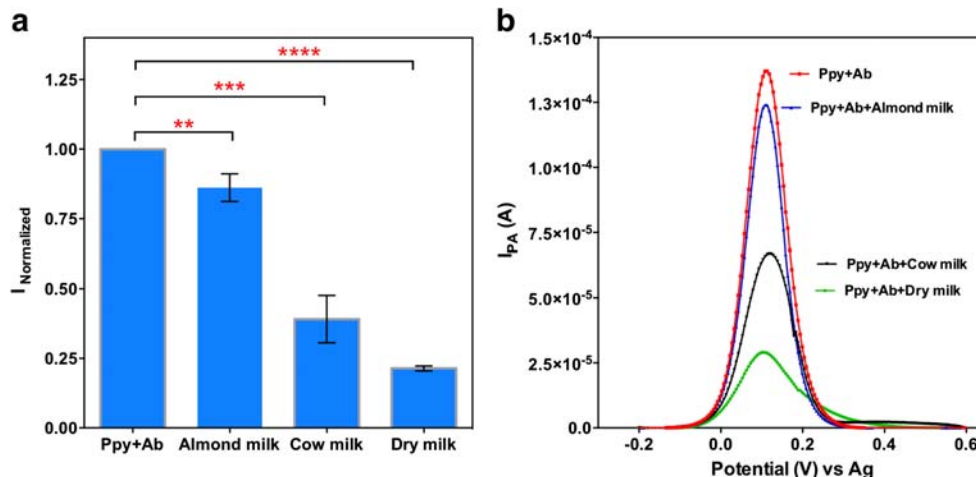
3.4 Sensitivity Studies

Having performed selectivity studies for the proposed system for α -LB detection, concentration studies have been conducted to assess the selectivity of the developed biosensor.

The calibration curve was drawn for concentration versus I_{pA} values, as can be seen in Fig. 5A, which is based on the raw data represented in Fig. 5B. To do so, different α -LB solutions with varying concentrations (0.025–0.2 nM) were prepared in PBS and were incubated on PPy + α -LB-Ab-modified AuSPEs at the optimum conditions determined earlier and I_{pA} was measured by DPV. From the equation of linear calibration curve ($R^2 = 0.9970$), LOD and LOQ values for the

biosensor were calculated as 0.0135 nM and 0.0410 nM respectively. Those values correspond to a detection range of 355–2840 pg/mL and a LOD of 192.2 pg/mL. Literature comparison of the LOD and linear detection range of the proposed biosensor are shown in Table 1. As can be seen from this table, available methods for α -LB detection is generally based on optical methods such as SPR [25], FLISA [26] and goldmag-based ELISA [35], or colorimetric methods like visualized microarray immunoassay [21], which had a higher LOD than the proposed method except FLISA. There is only one electrochemical method proposed for α -LB detection that is based on detection of α -LB via amperometric hydroquinone (HQ)/H₂O₂ system after capturing α -LB via magnetic beads. This method provides relatively lower LOD compared with our work. However, it requires extra steps such as magnetic bead modification and enzyme labelling and hence is more complex compared with our proposed label-free approach.

Figure 6 Real sample analysis via α -LB biosensor: Calibration drawn for DPV based detection of α -LB in cow milk in the concentration range of 0.025 nM - 0.20 nM (A) Differential pulse voltammograms recorded for the same α -LB concentrations in both cow milk and in PBS for comparison with bare or PPy+ α -LB AB modified electrodes (B), Comparison of the estimated (by detection) and calculated concentration values f α -LB for three different dilution factors of cow milk samples (C).



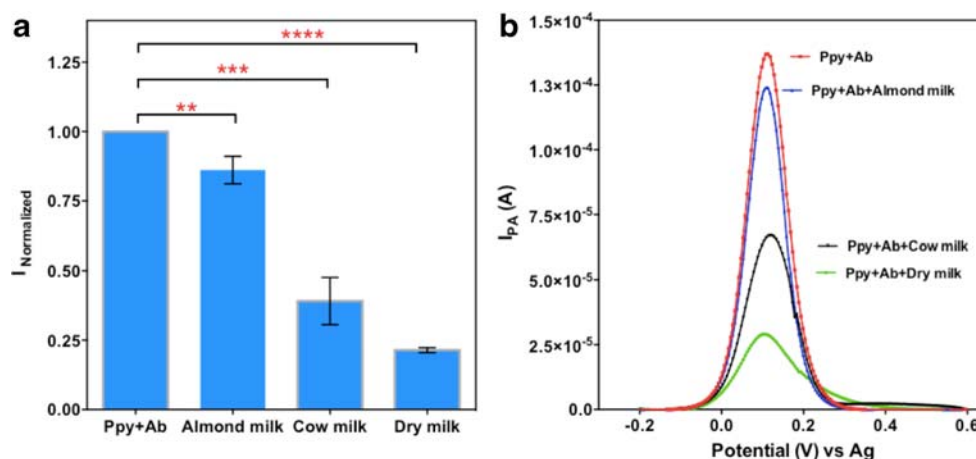


Figure 7 Control studies performed with different milk samples: Bar graphs with error bars representing the normalized oxidation peak current (the ratio of the peak currents after and before sample incubation with α -LB-Ab, $I_{\text{normalized}}$) for three different milk samples, cow, almond and dry milk, at same dilution ratio (1/1'000'000). T-test

calculated p-values are 0.0002 (***), 0.0012 (**) and < 0.0001 (****). (A) Differential pulse voltammograms recorded for almond milk, cow milk and dry milk at the same dilution ratio of 1/1'000'000 (B). Experimental steps are detailed in methods section

3.5 α -LB Detection in Real Dietary Samples

After determining the linear detection range and LOD, we tested our biosensor for detection inside real dietary samples. For this purpose, we used UHT whole milk, low-fat milk, dry milk and almond milk and performed calibration curve studies, as results were presented in the form of calibration curve Fig. 6A and raw differential pulse voltammograms Fig. 6B. The concentration of α -LB in milk was reported as 0.52 mg/mL as stated elsewhere [28]. In addition to that, Pingarrón et al. [28] used the dilution factor of 1000–2500,000 for magnetic bead-based immunosensor designed for α -LB detection compared with 2000–250,000 dilution factor of commercial ELISA kit. Therefore, we also have performed serial dilutions for milk samples in PBS to fall in the detection range, 355–2840 pg/mL, of the biosensor. Having prepared serial dilutions to prepare milk samples with different concentrations ranging from 0.036–0.25 nM, we have conducted concentration studies to get a calibration curve for real samples. The resulting calibration curve in real milk samples also presented linear tendency ($R^2 = 0.9934$). In order to compare the sensitivities in PBS and real samples, we have also calculated the recovery % of the α -LB considering three data points, I_{PA} values recorded for real milk measurements by recalculating the concentration by using calibration curve equation of Fig. 5A. The recovery percentage values are very high with very low relative error values (maximum 6.9%) as can be seen in Fig. 6C. Therefore, we conclude that the described sensing method is capable of detecting α -LB directly in real milk samples. Moreover, the calibration curve equations for PBS and real milk are very close, which in turn make us deduce that other milk components, such as fat or other proteins like caseins [36], do not have major interference effect for the biosensor, highlighting strong specificity of the

biosensor. Raw differential pulse voltammograms shown in Fig. 6B also show the similar signal at the same α -LB (0.10 nM) concentrations. The slight negative peak potential shift observed for milk measurement can be attributed to the pH difference of real milk and PBS, pH 6.4–6.8 and 7.4 respectively. In addition to previous selectivity studies we made, we also conducted control studies with dietary samples. For this purpose, we used almond milk as negative control since it is known to be very poor in protein and fat content that are less than 1% and 2% (w/w%) respectively [37]. In addition to that, it does not contain any α -LB and can serve as control sample for our work. All the parameters including dilution ratio was kept constant as other experiments performed with dry and UHT whole milk samples. For comparison, we have evaluated $I_{\text{normalized}}$ values for milk, dry milk and almond milk and results were represented as bar graphs in Fig. 7A. As it can be seen from this figure, the $I_{\text{normalized}}$ value for almond milk was above 0.85, whereas for cow's milk samples, this value was 0.36. The difference was statistically significant with a p value of 0.0012. Thus, we conclude that developed α -LB biosensor can discriminate between dietary samples that contain α -LB and the ones that do not. As another real sample control, we have chosen non-fat dry milk whose α -LB was assumed to be similar to whole UHT milk. Non-fat dry milk was dissolved in deionized water (5% w/v) and directly incubated on AuSPES modified with PPy + α -LB-Ab without any previous dilution. After DPV measurements, $I_{\text{normalized}}$ was calculated and compared with almond milk and cow's milk samples. The $I_{\text{normalized}}$ value for dry milk was 0.22, which is significantly lower than that for almond milk (p value < 0.0001). It was also lower than that for cow's milk. The main difference between those two milk samples are the fat content, since the tested cow's milk was 3.5%, whereas dry milk had no fat.

In addition to that, the dilution factor for dry milk was lower than the whole milk; therefore, we expect α -LB content to be higher in dry milk and hence result in lower $I_{\text{normalized}}$ value.

All in all, these results show that the designed biosensor can detect α -LB in dietary samples in a label-free way with a high sensitivity and selectivity.

4 Conclusions

For the first time in literature, we presented in this work the development of a sensitive label-free voltammetric biosensor for detection of α -LB in dietary samples based on electropolymerization of pyrrole. The parameters affecting the sensor performance such as number of polymerization cycle, optimum antibody dilution factor, antigen immobilization temperature and time were precisely optimized to get the best biosensor performance. The biosensor was tested over various milk samples to test the selectivity as well as sensitivity in real samples. Real sample studies proved the high selectivity of the biosensor, since the α -LB recovery rates were around 93.1–97.0% with a good sensitivity (LOD of 192.1 pg/mL). Our biosensor showed 10 – 10^5 times higher sensitivity compared with commercially available ELISA and other optical methods [21, 22, 35] presented in literature. In addition to the fact that this sensor is the first voltammetric sensor presented in literature, it also provides a simpler sensing scheme with only 1.5 h complete assay time compared with its only electrochemical equivalent, which is based on enzyme-based amperometric detection of α -LB via magnetic beads [28]. Thanks to its simplicity, high selectivity towards complex dietary samples and sensitivity, we believe that this biosensor could be used for food safety assessment tool by industry and consumers suffering from milk allergy.

Acknowledgements The authors would also like to thank Chêne Sophie Eglantine Tiffanie for preliminary experiments performed for this project.

Funding Information The authors would like to thank the Food and Nutrition Center at EPFL for funding of this work through the project titled: “Multi-Panel Lab-on-a-Chip System based on Electrochemical Sensors for Allergens Testing on Real Milk Samples in-the-field”.

Compliance with Ethical Standards

Does not apply/not applicable

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