



# Electrochemical magnetic beads-based immunosensing platform for the determination of $\alpha$ -lactalbumin in milk



Víctor Ruiz-Valdepeñas Montiel, Susana Campuzano\*, Rebeca M. Torrente-Rodríguez, A. Julio Reviejo, José M. Pingarrón\*

Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain

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## ABSTRACT

Alpha-lactalbumin ( $\alpha$ -LA) is one of the whey proteins in cows' milk that has been identified as allergenic. In this work, we present, for the first time, a very sensitive magnetic beads (MBs)-based immunosensor for the determination of  $\alpha$ -LA. A sandwich configuration involving selective capture and horseradish peroxidase-labeled detector antibodies was implemented on carboxylic acid-modified magnetic beads, captured magnetically under the surface of a disposable screen-printed carbon electrode for amperometric detection using the hydroquinone (HQ)/H<sub>2</sub>O<sub>2</sub> system. The  $\alpha$ -LA immunosensor exhibited a wide linear range (37.0–5000 pg/ml), a low limit of detection (LOD, 11.0 pg/ml) and noteworthy selectivity against other non-target proteins. The MBs-based immunosensing platform was applied successfully for the determination of  $\alpha$ -LA in several varieties of milk (raw and UHT cows' milk as well as human milk) and infant formulations. The results were corroborated with those obtained using a commercial ELISA method, thereby substantiating the analytical merits of this unique method.

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## 1. Introduction

Food allergy has become a major health concern of late. Currently, treatment for food allergy does not exist, and the only effective method to mitigate the potential health hazard is to avoid consumption of the allergen-containing food (Eissa, L'Hocine, Siaj, & Zourob, 2013). Among various food allergies, milk allergy is one of the most common instigators of an allergic response (Høst, 2002; Høst & Halken, 1990).

Cows' milk allergy (CMA) can be defined as any adverse reaction mediated by immunological mechanisms to cows' milk proteins. The estimated prevalence of CMA varies between 0.25% and 4.9% of the global population, being higher in children than adults (Fiocchi et al., 2010). Cows' milk contains more than 25 different proteins, but only whey proteins alpha-lactalbumin ( $\alpha$ -LA), beta-lactoglobulin ( $\beta$ -LG), bovine serum albumin (BSA), lactoferrin, and four caseins have been identified as allergens (Coscia et al., 2012). These milk proteins can be present in human breast milk and infant formulas as well as milk products, such as cheese and

yoghurt and “non-dairy” foods, which might contain milk-based contaminants or unlabeled additives. The most effective treatment of CMA is allergen avoidance. Although breast milk is the optimal nutritional choice for nursing infants, suitable milk substitutes for infants with CMA include soy hydrolyzed formulas, casein and whey hydrolyzed formulas, and amino acid formulas. However, due to clinically important residual allergenicity in some hypoallergenic formulas, controlled clinical testing is necessary before their use.

Lactalbumin is the albumin contained in milk and obtained from whey. There are alpha and beta lactalbumins, and both occur in milk.  $\alpha$ -Lactalbumin ( $\alpha$ -LA) is an acidic, single-chain Ca<sup>2+</sup>-binding protein (pI ~ 4.6) comprising 123 amino acids, no free thiols and four disulphide bonds, corresponding to a molecular mass of approximately 14 kDa, and is present in all mammalian milks. It is the second most abundant whey protein in cows' milk (~1 g/l) and the dominant whey protein in human milk.  $\alpha$ -LA binds calcium and zinc ions strongly, and might possess bactericidal or antitumor activity (Stănciuc & Răpeanu, 2010). A folding variant of human alpha-lactalbumin that can form in acidic environments, such as the stomach, called HAMLET, probably induces apoptosis in tumor and immature cells (Stănciuc & Răpeanu, 2010).  $\alpha$ -LA can also be considered as a potential marker of heat treatment because its denaturation in milk occurs on a large scale at temperatures between 70 and 96 °C (Jeanson, Dupont, Grattard, & Rolet-Répécaud, 1999).

\* Corresponding author.

E-mail addresses: [victor\\_lega90@hotmail.com](mailto:victor_lega90@hotmail.com) (V. Ruiz-Valdepeñas Montiel), [susanacr@quim.ucm.es](mailto:susanacr@quim.ucm.es) (S. Campuzano), [rebeca.magnolia@gmail.com](mailto:rebeca.magnolia@gmail.com) (R.M. Torrente-Rodríguez), [reviejo@quim.ucm.es](mailto:reviejo@quim.ucm.es) (A.J. Reviejo), [pingarro@quim.ucm.es](mailto:pingarro@quim.ucm.es) (J.M. Pingarrón).

$\alpha$ -LA forms the regulatory subunit of the lactose synthase heterodimer and  $\beta$ -1,4-galactosyltransferase ( $\beta$ 4Gal-T1) forms the catalytic component. Together, these proteins enable lactose synthase to produce lactose by transferring galactose moieties to glucose. This protein complex stimulates lactose production and is stimulated by the hormone prolactin. When an infant sucks on a nipple, this stimulates prolactin production, which causes the breast to fill with milk.  $\alpha$ -LA is of high nutritional value to the neonate, and is a particularly good source of the essential amino acids tryptophan and cysteine. Based on the accepted nutritional suitability of human milk, infant formulas derived from bovine milk are increasingly “humanised” with the addition of bovine whey protein fractions enriched with  $\alpha$ -LA to increase their nutritional value (Indyk, 2009; Kamau, Cheison, Chen, Liu, & Lu, 2010).

The significant contribution of  $\alpha$ -LA to the whey protein fraction of bovine milk, its allergenicity, an increasing trend towards its supplementation in infant formulas, and its potential as a marker of heat treatment have stimulated the need for reliable analytical methods for  $\alpha$ -LA determination at endogenous levels in milk, at supplemental levels in infant formulas and at pharmaceutical levels in milk protein isolates.

Although several methods have been reported for  $\alpha$ -LA detection including radial immunodiffusion (RID), nephelometric immunoassay (Cuillière et al., 1997), immunoelectrophoresis, enzyme-linked immunosorbent assays (ELISAs) (Šimíková, Lang, Semotán, & Kocent, 1987) and surface plasmon resonance (SPR) (Billakanti, Fee, Lane, Kash, & Fredericks, 2010), there remains an urgent need to develop alternative methods for quantification featuring reduced cost, improved sensitivity, selectivity and more rapid response. In this context, the development of new tools, minimizing limitations imposed by these methodologies and leveraging the high specificity of traditional immunochemical methods, is of great interest. In this sense, electrochemical immunosensors are envisaged as a valid alternative to classical methods for analysis of protein biomarkers, because they are amenable to direct electrical readout and well suited to rapid detection with high sensitivity and selectivity using low-cost instrumentation that is adaptable to portable, field-deployable embodiments, which is ideal for routine determination in the dairy industry. To the best of our knowledge, no electrochemical immunosensor has been described thus far for the determination of  $\alpha$ -LA.

Magnetic beads (MBs) have been utilized for the development of electrochemical immunosensors to improve their sensitivity, reduce the time for analysis and minimize matrix effects (Bange, Halsall, & Heineman, 2005; Conzuelo, Gamella, Campuzano, Reviejo, & Pingarrón, 2012; Esteban-Fernández de Ávila, Escamilla-Gómez, Campuzano, Pedrero, & Pingarrón, 2012; Nam, Thaxton, & Mirkin, 2003; Rosi & Mirkin, 2005; Willner, Baron, & Willner, 2007; Zhang, Cheng, Richter, & Greene, 2006). Electrochemical MBs-based immunosensors allow the analysis of complex samples without the need for sample preparation, including pre-enrichment or purification steps, due to the “extraction” of target analytes from the complex matrix (via magnetic attraction), and measurement of the electrochemical signal in a “clean” environment (Zacco, Pividori, Alegret, Galve, & Marco, 2006). These characteristics make functionalized MBs particularly relevant for immunosensors to be used in complex real sample matrices. In this paper, we describe the first electrochemical immunosensing platform for the determination of  $\alpha$ -LA using a sandwich configuration, screen-printed carbon electrodes (SPCEs) and HOOC-modified MBs (HOOC-MBs). The method developed involves the immobilization of the capture antibody on HOOC-MBs and an incubation step with a mixture of the analyte and a specific HRP-labeled detector antibody. The electrochemical detection of the enzyme reaction product was performed on disposable SPCEs using HQ as the electron transfer mediator and  $\text{H}_2\text{O}_2$  as the enzyme substrate.

The platform was applied successfully in the analysis of several varieties of milk samples and infant formulas, demonstrating its potential role in food safety.

## 2. Materials and methods

### 2.1. Apparatus and electrodes

Amperometric measurements were performed with a CH Instruments (Austin, TX) model 812B potentiostat controlled by software CHI812B. All measurements were performed at room temperature. The electrochemical transducers employed were screen-printed carbon electrodes (SPCEs) (DRP-110, DropSens, Spain) consisting of a 4-mm diameter carbon working electrode, a carbon counter electrode and an Ag pseudo-reference electrode, which were used in conjunction with a custom connector (DRP-CAC, Dropsens).

Homogenization of the solutions was facilitated by a Bunsen AGT-9 Vortex. A Thermomixer MT100 constant temperature incubator shaker (Universal Labortechnik) was also used. Magnetic separation steps for incubation/washing processes were performed using a Dynal MPC-S (product No. 120.20, Dynal Biotech ASA) magnetic particle concentrator. A neodymium magnet (AIMAN GZ) was used to control the attraction of the modified-MBs to the SPCE surface.

### 2.2. Reagents and solutions

Carboxylic acid-modified MBs (HOOC-MBs, 2.7  $\mu\text{m}$   $\varnothing$ , 10 mg/ml, Dynabeads<sup>®</sup> M-270 Carboxylic Acid, Cat. No: 14305D) were purchased from Invitrogen (San Diego, CA). NaCl (Cat. No: SO02241000), KCl (Cat. No: PO02001000), sodium di-hydrogen phosphate (Cat. No: SO03331000), di-sodium hydrogen phosphate (Cat. No: SO03371000), and Tris-HCl (Cat. No: TR04251000) were purchased from Scharlab. Tween<sup>®</sup>20 (Cat. No: 27,424-8), N-(3-di methylaminopropyl)-N'-ethylcarbodiimide (EDC, Cat. No: 171440100), ethanolamine, hydroquinone (HQ, Cat. No: 39,813-6), hydrogen peroxide (30%, w/v, Cat. No: H-9003), casein (Cat. No: C3400), alkaline phosphatase (from bovine intestinal mucosa, Cat. No: P5521), catalase (from bovine liver, Cat. No: C9322), lysozyme (from chicken egg white, Cat. No: L-6876) and bovine  $\beta$ -LG (Cat. No: L3908) were purchased from Sigma-Aldrich (St. Louis, MO). Ascorbic acid (Cat. No: 95209) was purchased from Fluka (St. Louis, MO). N-hydroxysulfosuccinimide (sulfo-NHS, Cat. No: 438650010) from Acros Organics and 2-(N-morpholino) ethanesulfonic acid (MES, Cat. No: 1080.25) and bovine serum albumin (BSA Type VH, Cat. No: 1066) were purchased from Gerbu. The goat anti-bovine  $\alpha$ -LA capture antibody (A10-128A) and the HRP-conjugated goat anti-bovine  $\alpha$ -LA detector antibody (A10-128P) employed in the investigations were components of the  $\alpha$ -LA ELISA Kit (Catalog No. E10-128), purchased from Bethyl Laboratories, Inc. This kit was also used for validation purposes.

The following solutions, prepared in deionized water, were employed: 0.05 mol/l phosphate buffer, pH 6.0; 0.1 mol/l phosphate buffer, pH 8.0; phosphate-buffered saline (PBS) consisting of 0.01 mol/l phosphate buffer solution containing 137 mmol/l NaCl and 2.7 mmol/l KCl, pH 7.5; 0.025 mol/l MES buffer, pH 5.0; 0.01 mol/l sodium phosphate buffer solution consisting of PBS with 0.05% Tween<sup>®</sup>20 (pH 7.5, PBST), and 0.1 mol/l Tris-HCl buffer, pH 7.2.

Activation of the HOOC-MBs was executed with an EDC/sulfo-NHS mixture solution (50 mg mL<sup>-1</sup> each in MES buffer, pH 5.0). The blocking step was accomplished with a 1 mol/l ethanolamine solution prepared in a 0.1 mol/l phosphate buffer solution, pH 8.0.

All chemicals used were of analytical-reagent grade, and deionized water was obtained from a Millipore Milli-Q purification system (18.2 MΩ cm).

### 2.3. Modification of MBs

A 3-μl aliquot of the HOOC-MBs commercial suspension was transferred into a 1.5 ml PCR reaction vial. Subsequently, the MBs were washed twice with 50 μl MES buffer solution for 10 min with continuous stirring (950 rpm, 25 °C). Between each step, the particles were concentrated using a magnet and, after 4 min, the supernatant was discarded. The MBs carboxylic groups were activated with a 35-min incubation in 25 μl of the EDC/sulfo-NHS solution (950 rpm, 25 °C). The activated MBs were washed twice with 50 μl of MES buffer and re-suspended in 25 μl of a 50 μg/ml anti-α-LA solution (in MES buffer). The anti-α-LA was captured on the activated beads over 15 min (at 25 °C) with continuous stirring (950 rpm). Subsequently, the anti-α-LA-modified MBs were washed twice with 50 μl of MES buffer solution. Thereafter, the unreacted activated groups on the MBs were blocked by adding 25 μl of the 1 mol/l ethanolamine solution (in 0.1 mol/l phosphate buffer, pH 8.0) and incubating the suspension, with continuous stirring (950 rpm), for 60 min at 25 °C. Following two washing steps with 50 μl of 0.1 mol/l Tris-HCl buffer (pH 7.2) and an additional washing iteration with 50 μl of PBST (pH 7.5), the anti-α-LA-coated MBs were re-suspended in 25 μl of a solution containing 1.0 μg/ml HRP-anti-α-LA and a variable concentration of the target antigen (in PBST, pH 7.5) before being incubated for 30 min (950 rpm at 25 °C). After two washing steps with PBST, the modified-MBs were re-suspended in 45 μl of 0.05 mol/l sodium phosphate buffer solution (pH 6.0).

### 2.4. Amperometric measurements

The re-suspended modified MBs were captured on the SPCE surface according to the procedure described earlier (Conzuelo et al., 2012). The magnet holding block with the SPCE, where the modified MBs were captured, was immersed into an electrochemical cell (see reference (Conzuelo et al., 2012) for details) containing 10 ml 0.05 mol/l phosphate buffer, pH 6.0 and 1.0 mmol/l HQ (prepared just prior to the electrochemical measurement). Amperometric measurements in stirred solutions were performed by applying a detection potential of −0.20 V vs. Ag pseudo-reference electrode on addition of 50 μl of a 0.1 mol/l H<sub>2</sub>O<sub>2</sub> solution until a steady-state current was obtained (approx. 1 min).

The amperometric signals provided throughout this report corresponded to the difference between the steady-state and background current. Unless otherwise indicated, each data point represents three independent measurements and the error bars indicate standard deviations inherent in the measurements.

### 2.5. Analysis of milk samples

The immunosensors developed were applied for the determination of α-LA in a variety of milk samples: raw and UHT cows' milk, human breast milk, and various infant formulas. The sample treatment consisted only of an appropriate dilution (factors between 1000 and 2,500,000, see Table 1) with the PBST (pH 7.5) buffer. Given the absence of matrix effects, after dilution to reduce the α-LA concentration in the samples to correspond with the linear portion of the calibration graph, determination of α-LA could be performed in a straightforward fashion – i.e. interpolating the amperometric signals obtained with the diluted samples with respect to the as-obtained calibration plot. The anti-α-LA-modified MBs were re-suspended in a 25 μl-aliquot of PBST-diluted milk supplemented with 1.0 μg/ml HRP-anti-α-LA and

**Table 1**

Determination of the α-LA (expressed in mg/ml) in different milk samples and infant formulas using the developed amperometric MBs-based immunosensor and comparison with the results obtained by a commercial ELISA spectrophotometric kit.

| Milk samples                | MBs-based immunosensor |                                                               | ELISA           |                                                             |
|-----------------------------|------------------------|---------------------------------------------------------------|-----------------|-------------------------------------------------------------|
|                             | Dilution factor        | [α-LA] <sub>found</sub>                                       | Dilution factor | [α-LA] <sub>found</sub>                                     |
| Raw cow milk                | 2,500,000              | (1.1 ± 0.1)<br>RSD <sub>n=3</sub> = 4.2%                      | 250,000         | (1.12 ± 0.03)<br>RSD <sub>n=3</sub> = 1.0%                  |
| UHT cow milk                | 1,000,000              | (0.52 ± 0.07)<br>RSD <sub>n=3</sub> = 7.0%                    | 100,000         | (0.56 ± 0.04)<br>RSD <sub>n=3</sub> = 3.9%                  |
| Human milk                  | 20,000                 | (0.55 ± 0.08) × 10 <sup>−3</sup><br>RSD <sub>n=3</sub> = 8.0% | 2000            | (0.6 ± 0.1) × 10 <sup>−3</sup><br>RSD <sub>n=3</sub> = 9.1% |
| Infant formulae 1           | 1,000,000              | (0.33 ± 0.04)<br>RSD <sub>n=3</sub> = 6.5%                    | 100,000         | (0.32 ± 0.02)<br>RSD <sub>n=3</sub> = 3.4%                  |
| Infant formulae 2           | 1,000,000              | (0.21 ± 0.01)<br>RSD <sub>n=3</sub> = 3.0%                    | 100,000         | (0.177 ± 0.007)<br>RSD <sub>n=3</sub> = 2.1%                |
| Hydrolyzed infant formula 3 | 1000                   | ND                                                            | 1000            | ND                                                          |

ND: non detectable.

incubated at 25 °C for 30 min under vigorous agitation (950 rpm). Thereafter, separation, washing and measurement protocols were similar to those described above for α-LA standard solutions.

For comparison purposes, the same milk samples were also analyzed using an ELISA and the same immunoreagents.

## 3. Results and discussion

The fundamental principles of the immunosensor configuration as well as of the electrochemical transduction are shown in Fig. 1. Briefly, the specific capture antibody (anti-α-LA) was immobilized covalently on to HOOC-MBs activated previously with an EDC/sulfo-NHS solution. Following a blocking protocol with ethanolamine, for the unreacted activated groups on the commercial MBs, anti-α-LA-MBs were incubated with the samples supplemented with the HRP-labelled detector antibody (HRP-anti-α-LA); the

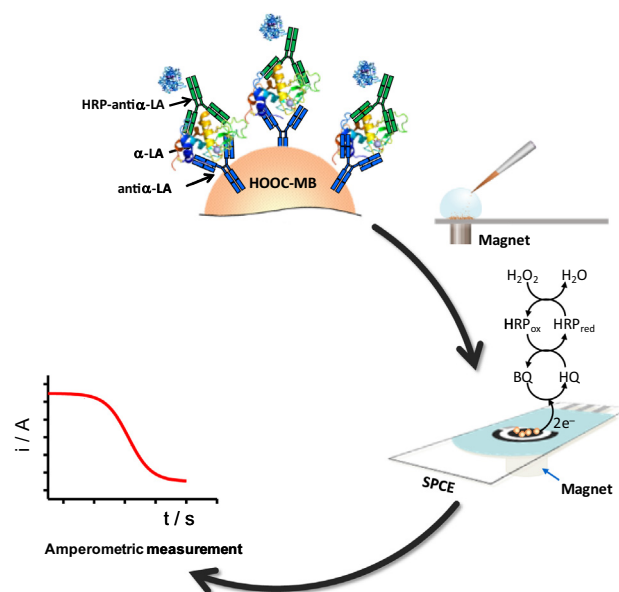


**Fig. 1.** Schematic illustration of the steps involved in the preparation of the α-LA sandwich MBs-based immunosensor (relative sizes of the components are not drawn to scale). The enzymatic and electrochemical reactions involved in the amperometric detection of the mediated reduction of H<sub>2</sub>O<sub>2</sub> with HQ at the SPCE are also shown.

target protein was sandwiched between the anti- $\alpha$ -LA immobilized on the MBs and the HRP-anti- $\alpha$ -LA.

The MBs bearing the sandwich immunocomplexes were captured magnetically on the electrode surface by placing SPCEs on a custom-fabricated magnetic holding block, and the biorecognition event was monitored by amperometric measurement of the reduction current generated with the HQ/H<sub>2</sub>O<sub>2</sub> system. Using this methodology, the SPCE acted only as the electrochemical transducer while all the immunoreactions occurred on the surface of the MBs.

### 3.1. Optimization of the working variables

The absence of non-specific adsorptions of  $\alpha$ -LA or HRP-anti- $\alpha$ -LA on anti- $\alpha$ -LA-free MBs was verified both in the absence and presence of 10 ng/ml  $\alpha$ -LA. Amperometric responses not significantly different from the blank were obtained, because there was an overlapping region in the error bars estimated for these signals as triple of the standard deviation ( $n=3$ ). Subsequently, all the experimental variables affecting the analytical performance of the immunosensors were optimized. The selection criterion applied for these variables was the largest current ratio obtained at  $-0.20$  V (vs. Ag pseudo-reference electrode) for 10 and 0 ng/ml  $\alpha$ -LA (signal-to-blank, S/B, ratio). This detection potential was optimized previously for the HQ/H<sub>2</sub>O<sub>2</sub> system (Gamella, Campuzano, Conzuelo, Reviejo, & Pingarrón, 2012). Table S1 (provided in the Supporting information section) summarizes the variables under investigation and the values selected for the preparation of the MBs-based immunosensor.

As examples, results obtained in the optimization of the anti- $\alpha$ -LA loading and incubation time with the mixture of  $\alpha$ -LA and HRP-anti- $\alpha$ -LA are provided in Fig. 2a and b), respectively. As expected, the current measured at  $-0.20$  V for 10 ng/ml  $\alpha$ -LA increased significantly with the anti- $\alpha$ -LA loading (Fig. 2a) up to 50  $\mu$ g/ml and then decreased – most likely due to the steric hindrance of the antigen when large amounts of the capture antibody are immobilized (Alves et al., 2015).

Regarding the incubation time with the  $\alpha$ -LA and HRP-anti- $\alpha$ -LA mixture, although there was no significant difference in the amperometric responses obtained in the presence of  $\alpha$ -LA, the non-specific adsorptions of the HRP-labelled antibody (in the absence of  $\alpha$ -LA) increased significantly for incubations longer than 30 min and, consequently, the resulting S/B ratio decreased above this value (Fig. 2b). The optimal discrimination obtained corresponded to an anti- $\alpha$ -LA concentration of 50  $\mu$ g mL<sup>-1</sup> and an incubation time of 30 min with a mixture of  $\alpha$ -LA and HRP-anti- $\alpha$ -LA; this protocol was selected for further studies.

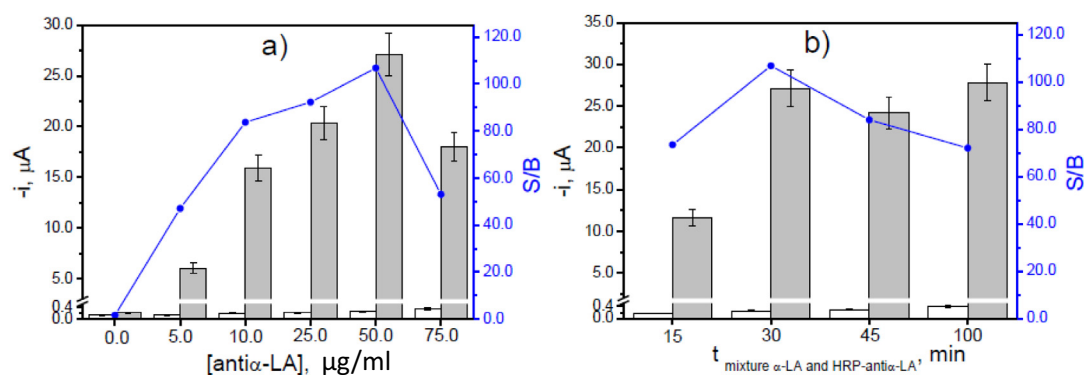


**Fig. 2.** Amperometric responses measured with the  $\alpha$ -LA sandwich MBs-based immunosensor for 0 (white bars) and 10.0 ng/ml  $\alpha$ -LA (grey bars) as well as the corresponding S/B ratios, as a function of the anti- $\alpha$ -LA loading (a) and incubation time with the mixture of  $\alpha$ -LA and HRP-anti- $\alpha$ -LA (b). Error bars provided indicate the standard deviation ( $n=3$ ).

### 3.2. Analytical characteristics

The reproducibility of the amperometric responses for 1 ng/ml  $\alpha$ -LA was evaluated with multiple identical immunosensors prepared on the same day and different days. Relative standard deviation (RSD) values for same-day and different-day preparations were 8.1 ( $n=10$ ) and 9.7% ( $n=7$ ), respectively, suggesting an acceptable reliability for the whole procedure including both the immunosensor fabrication protocol (MB modification and magnetic capture on the SPCE surface) and the amperometric transduction.

Working under optimized experimental conditions, a calibration plot for  $\alpha$ -LA was constructed in PBST (Fig. 3). A range of linearity ( $r=0.999$ ) between the measured current and the  $\alpha$ -LA concentration was found between 37 and 5000 pg/ml, with a slope value of  $2.97 \pm 0.04 \mu$ A  $\times$  ml/ng and an intercept of  $0.71 \pm 0.06 \mu$ A. The values for the limit of detection (LOD) and limit of quantification (LOQ) were calculated according using the  $3s_b/m$  and  $10s_b/m$  criteria, respectively, where  $m$  is the slope of the linear calibration plot, and  $s_b$  was estimated as the standard deviation of 10 amperometric signals measured without target  $\alpha$ -LA. Thus, the LOD and LOQ were 11.0 and 37.0 pg/ml, respectively. These low values make it possible to limit matrix effects associated with food

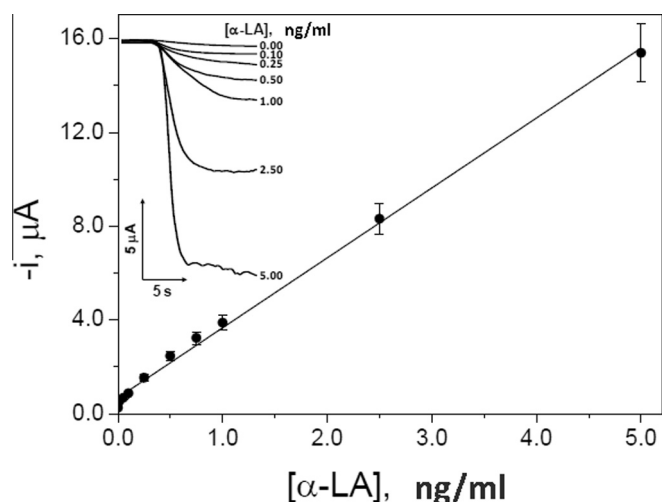


**Fig. 3.** Calibration plot constructed with  $\alpha$ -LA standards (prepared in PBST buffer, pH 7.5). Inset: Amperometric traces recorded with the immunosensor for the indicated  $\alpha$ -LA concentrations. Error bars provided indicate the standard deviation ( $n=3$ ) (b).

samples merely with an appropriate sample dilution without compromising the required sensitivity of the assay.

The methodology employed ensured the immunoreactions proceed only on the MBs and not on the electrode surface. To corroborate this, the storage stability of anti- $\alpha$ -LA-MBs conjugates was explored once the blocking step with ethanolamine was performed. Anti- $\alpha$ -LA-MBs were stored at 4 °C in PCR reaction vials containing 50  $\mu$ l of filtered PBS. Each working day, a few of the prepared conjugates were incubated with fresh solutions of HRP-anti- $\alpha$ -LA supplemented with 0 or 1 ng/ml  $\alpha$ -LA, according to the protocol described in the Experimental Section. No significant differences in the S/B ratio measured were apparent for a period of 12 days, indicating acceptable storage stability of the antibody-modified MB conjugates, which can be prepared and stored until required.

It is also important to note that an  $\alpha$ -LA immunosensor (electrochemical or otherwise) has not been described previously in the literature. The methodologies reported for the analysis of  $\alpha$ -LA provide LODs ranging from 1.5  $\mu$ g mL<sup>-1</sup> (turbidimetric immunoassays, (Cuillière et al., 1997)) to 0.12 mg/ml (SPR biosensor, (Indyk, 2009)), which are notably higher than those achieved with the immunosensor (11.0 pg/ml) as-developed. Although  $\alpha$ -LA spectrophotometric ELISA kits with slightly better LODs than achieved with the MBs-based immunosensor do exist, the inherent simplicity of the approach reported here can be claimed as an important practical advantage over the commercial ELISAs, which use tedious, time-consuming multistage processes and expensive detection instrumentation, making them difficult to implement as a tool for the development of user-friendly devices in on-site quality inspection of dairy products.

### 3.3. Selectivity of the immunosensor

The selectivity of the electrochemical MBs-based immunosensor towards  $\alpha$ -LA was evaluated against various non-target proteins (BSA, casein,  $\beta$ -LG, lysozyme, alkaline phosphatase, catalase and LPO), at concentrations found commonly in cows' milk, and the electroactive interferent ascorbic acid. The tests were performed by comparing values measured with the immunosensor for 0 and 1 ng/ml  $\alpha$ -LA, both in the absence and the presence of these compounds. Fig. 4 shows that no significant variation in

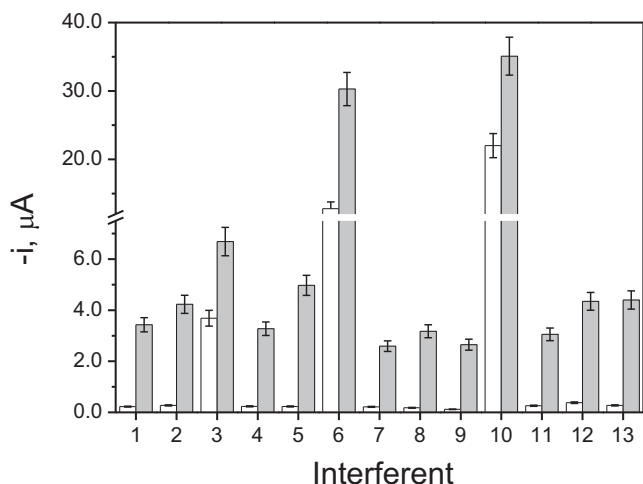


**Fig. 4.** Current values measured with the MBs-based immunosensor for 0.0 (white bars) and 1.0 ng/ml of  $\alpha$ -LA (grey bars) in the absence (1) and in the presence of 400  $\mu$ g/ml BSA (2), 28 mg/ml casein (3), 560 ng/ml casein (4), 0.5 U/l alkaline phosphatase (5), 3 mg/ml  $\beta$ -LG (6), 60 ng/ml  $\beta$ -LG (7), 5  $\mu$ g/ml catalase (8), 20  $\mu$ g/ml ascorbic acid (9), 2 mg/l LPO (10), 40 ng/l LPO (11), 2  $\mu$ g/ml lysozyme (12) and 40  $\mu$ g/ml lysozyme (13). Error bars provided indicate the standard deviation ( $n = 3$ ).

the S/N ratio was found in the presence of BSA, alkaline phosphatase, catalase or ascorbic acid. However, casein,  $\beta$ -LG, LPO and lysozyme interfered significantly in the  $\alpha$ -LA determination at levels typically found in milk (bars 3, 6, 10, and 12, respectively). While the high response observed for casein, lysozyme (structurally similar to  $\alpha$ -LA) and  $\beta$ -LG in the absence of  $\alpha$ -LA suggests cross-reactivity with the capture and detector antibodies. LPO has been reported to adsorb non-specifically on to carboxylated MBs, and catalyzes the oxidation of H<sub>2</sub>O<sub>2</sub> in a manner similar to HRP (Conzuelo et al., 2014; Resmini, Tripiciano, Rampilli, & Lodi, 1985). Nevertheless, as discussed below, the large sample dilution necessary to position the target analyte concentration in milk samples on the linear range of the  $\alpha$ -LA standard calibration graph minimized interference from these proteins, as presented in bars 4, 7, 11 and 13 of Fig. 3. For this reason, the immunosensor remains well positioned for use in the analysis of food samples.

### 3.4. Application to the analysis of milk samples

The MBs-based immunosensor was applied for the determination of  $\alpha$ -LA in several varieties of milk and infant formula. The possible impact of matrix effects was evaluated by constructing a calibration plot from the samples, which were diluted sufficiently with PBST and spiked with increasing amounts of a standard  $\alpha$ -LA solution, up to a maximum of 2.5 ng/ml. The slope obtained from the respective calibration plots in the different samples assayed were statistically similar to that obtained for buffered standard  $\alpha$ -LA solutions and, therefore, it was concluded that no significant matrix effects were apparent once the requisite dilution was performed. Accordingly,  $\alpha$ -LA quantification could be accomplished by simple interpolation of the current obtained for the diluted samples (Fig. 5) against the calibration plot constructed with  $\alpha$ -LA standards (Fig. 3). The respective dilution factors for each milk sample analyzed, which are required to position the target analyte concentration into the linear range of the calibration graph, are specified in Table 1.

The  $\alpha$ -LA content found in raw cows' milk (Table 1) was in agreement with the content described previously in mature bovine milk:  $\sim$ 1.2 g/l (Kamau et al., 2010). The lower content in UHT milk could be attributed to heat-denaturation of  $\alpha$ -LA during processing, which provokes unfolding of the molecule. These

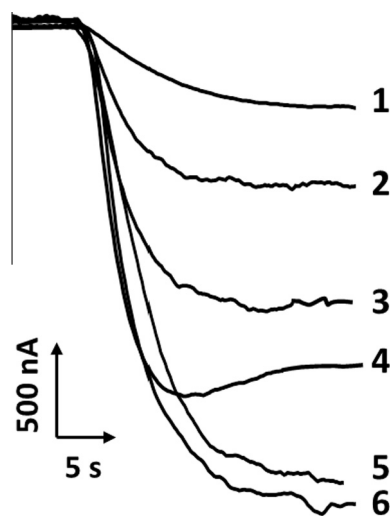


**Fig. 5.** Amperometric traces recorded with the MBs-based immunosensor in the absence of  $\alpha$ -LA (1) and in the presence of 1/20,000 PBST diluted human milk (2), 1/1,000,000 PBST diluted infant formula 2 (3), 1/1,000,000 PBST diluted infant formula 1 (4), 1/2,500,000 PBST diluted raw milk (5) and 1/1,000,000 PBST diluted UHT milk (6).

conformational modifications might induce the appearance of new epitopes at the surface of the molecule or the disappearance of those present in the native form of  $\alpha$ -LA (Jeanson et al., 1999). The results obtained in the analysis of raw and UHT commercial cows' milk were in close agreement with the percentages of the native  $\alpha$ -LA form present in these samples reported by others using two ELISA inhibition tests (Jeanson et al., 1999). This cross-validation demonstrates the potential of the proposed method in the identification of optimal heat treatment regimens at the industrial level; this could also find application in determination of the "thermal history" of milk powders that have usually undergone three or four consecutive heat treatments prior to sale.

The content of  $\alpha$ -LA in human breast milk was almost undetectable because the cohort of antibodies selected for the immunosensor were specific for bovine  $\alpha$ -LA, which has only 74% sequence homology with human  $\alpha$ -LA (with only a). As expected, no detectable  $\alpha$ -LA content was identified for hydrolyzed infant formula.

The results obtained in the analysis of these samples with the MBs-based immunosensor were compared with those provided by a commercial ELISA kit utilizing the same immunoreagents (see Table 1). By plotting the mean contents obtained with the immunosensor versus those provided with the ELISA kit, the resulting linear least-squares regression graph ( $r = 0.9983$ ) exhibits a slope of  $1.00 \pm 0.03$  and an intercept of  $0.01 \pm 0.02$ . Accordingly, a strong correlation was observed since the confidence interval (at a significance level of  $\alpha = 0.05$ ) for the slope and intercepts included unity and zero values, respectively. This comparison clearly indicates that the immunosensor exhibited no systematic errors and could be used successfully for the determination of  $\alpha$ -LA in milk samples.

Thorough comparison of the MBs-based immunosensor analytical performance against the commercial ELISA spectrophotometric kit allowed the following conclusions to be drawn: apart from the 279-fold lower LOD ( $0.28$  vs  $78$  pg  $\alpha$ -LA) achieved, the electrochemical immunosensor methodology was more rapid – the entire detection process required only 30 min (once the anti- $\alpha$ -LA-MBs are prepared), whereas the commercial ELISA kit often required 4 h from sample isolation to analyte quantification. Moreover, the spectrophotometric method was difficult to automate and hence difficult to employ *in situ*. Conversely, the use of the MBs-based immunosensor simplified the entire analytical procedure, requiring less reagents ( $25$  vs  $100$   $\mu$ l), and could be performed easily with an automated portable, low-cost, field-deployable instrument, which makes it a very attractive and user-friendly tool for routine determination of milk quality, infant formula quality assurance, and decentralization of agro-chemical analysis.

#### 4. Conclusions

In this work, a disposable amperometric MBs-based immunosensor for the rapid, specific and sensitive determination of  $\alpha$ -LA in milk samples and infant formulas was developed for the first time. The immunosensor possessed noteworthy analytical performance in terms of sensitivity, selectivity and reproducibility of the measurements in raw cows' milk without the need for complex or time-consuming pre-treatment steps, following dilution with an appropriate working buffer. The applicability of the MBs-based immunosensor as-developed was underlined by the implementation and analysis of different milk samples and infant formulas, and the results were validated successfully against a commercial ELISA spectrophotometric kit. The immunosensor performance is in accordance with such an ELISA kit in terms of rapidity, sensitivity, simplicity and cost and, therefore, it has potential as an alternative analytical tool to screen for the presence of  $\alpha$ -LA in

the dairy industry and pediatric foods. Moreover, the implementation of disposable mass-produced biosensors in conjunction with the simplicity, automation and miniaturization of the instrumentation constitute important advantages leading towards the integration of the method in portable (in-field), reliable and user-friendly analytical systems for milk and infant formula quality control.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.07.001>.

#### References

- Alves, R. C., Pimentel, F. B., Nouws, H. P. A., Marques, R. C. B., González-García, M. B., Oliveira, M. B. P. P., & Delerue-Matos, C. (2015). Detection of Ara h 1 (a major peanut allergen) in food using an electrochemical gold nanoparticle-coated screen-printed immunosensor. *Biosensors & Bioelectronics*, 64, 19–24.
- Bange, A., Halsall, H. B., & Heineman, W. R. (2005). Microfluidic immunosensor systems. *Biosensors & Bioelectronics*, 20, 2488–2503.
- Billakanti, J. M., Fee, C. J., Lane, F. R., Kash, A. S., & Fredericks, R. (2010). Simultaneous, quantitative detection of five whey proteins in multiple samples by surface plasmon resonance. *International Dairy Journal*, 20, 96–105.
- Conzuelo, F., Gamella, M., Campuzano, S., Reviejo, A. J., & Pingarrón, J. M. (2012). Disposable amperometric magneto-immunosensor for direct detection of tetracyclines antibiotics residues in milk. *Analytica Chimica Acta*, 737, 29–36.
- Conzuelo, F., Ruiz-Valdepeñas Montiel, V., Campuzano, S., Gamella, M., Torrente-Rodríguez, R. M., Reviejo, A. J., & Pingarrón, J. M. (2014). Rapid screening of multiple antibiotic residues in milk using disposable amperometric magnetosensors. *Analytica Chimica Acta*, 820, 32–38.
- Coscia, A., Orrù, S., Di Nicola, P., Giuliani, F., Rovelli, I., Peila, C., ... Bertino, E. (2012). Cow's milk proteins in human milk. *Journal of Biological Regulators and Homeostatic Agents*, 26, 39–42.
- Cuillière, M. L., Abbadi, M., Molé, C., Montagne, P., Béné, M. C., & Faure, G. (1997). Microparticle-enhanced nephelometric immunoassay of alpha-lactalbumin in human milk. *Journal of Immunoassay*, 18, 97–109.
- Eissa, S., L'Hocine, L., Siaj, M., & Zourob, M. (2013). A graphene-based label-free voltammetric immunosensor for sensitive detection of the egg allergen ovalbumin. *Analyst*, 138, 4378–4384.
- Esteban-Fernández de Ávila, B., Escamilla-Gómez, V., Campuzano, S., Pedrero, M., & Pingarrón, J. M. (2012). Disposable electrochemical magnetosensor for the determination of troponin T cardiac marker. *Electroanalysis*, 25, 51–58.
- Fiocchi, A., Brozek, J., Schunemann, H. J., Bahna, S. L., von Berg, A., Beyer, K., ... Vieths, S. (2010). World Allergy Organization (WAO) diagnosis and rationale for action against cow's milk allergy (DRACMA) guidelines. *World Allergy Organization Journal*, 3, 57–61.
- Gamella, M., Campuzano, S., Conzuelo, F., Reviejo, A. J., & Pingarrón, J. M. (2012). Amperometric magnetosensors for direct determination of D-dimer in human serum. *Electroanalysis*, 24, 2235–2243.
- Høst, A. (2002). Frequency of cow's milk allergy in childhood. *Annals of Allergy, Asthma & Immunology*, 89, 33–37.
- Høst, A., & Halken, S. (1990). A prospective study of cow milk allergy in Danish infants during the first 3 years of life. *Allergy*, 45, 587–596.
- Indyk, H. E. (2009). Development and application of an optical biosensor immunoassay for  $\alpha$ -lactalbumin in bovine milk. *International Dairy Journal*, 19, 36–42.
- Jeanson, S., Dupont, D., Grattard, N., & Rolet-Répécaud, O. (1999). Characterization of the heat treatment undergone by milk using two inhibition ELISAs for quantification of native and heat denatured  $\alpha$ -lactalbumin. *Journal of Agricultural & Food Chemistry*, 47, 2249–2254.
- Kamau, S. M., Cheison, S. C., Chen, W., Liu, X.-M., & Lu, R.-R. (2010). Alpha-lactalbumin: Its production technologies and bioactive peptides. *Comprehensive Reviews in Food Science and Food Safety*, 9, 197–212.
- Nam, J. M., Thaxton, C. S., & Mirkin, C. A. (2003). Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins. *Science*, 301, 1884–1886.

- Resmini, P., Tripiciano, C., Rampilli, M., & Lodi, R. (1985). Alcuni aspetti del controllo di qualità del latte al consumo. *Rivista della Società Italiana di Scienze dell'Alimentazione*, 14, 187–196.
- Rosi, N. L., & Mirkin, C. A. (2005). Nanostructures in biodiagnostics. *Chemical Reviews*, 105, 1547–1562.
- Simicková, M., Lang, B. A., Semotán, K., & Kocent, A. (1987). A non-competitive enzyme immunoassay of human  $\alpha$ -lactalbumin in biological fluids and tissue extracts. *Clinica Chimica Acta*, 163, 257–265.
- Stănciuc, N., & Râpeanu, G. (2010). An overview of bovine  $\alpha$ -lactalbumin structure and functionality. *The Annals of the University Dunarea de Jos of Galati Fascicle VI – Food Technology*, 34(2).
- Willner, I., Baron, R., & Willner, B. (2007). Integrated nanoparticle-biomolecule systems for biosensing and bioelectronics. *Biosensors & Bioelectronics*, 22, 1841–1852.
- Zacco, E., Pividori, M. I., Alegret, S., Galve, R., & Marco, M. P. (2006). Electrochemical magnetoimmunosensing strategy for the detection of pesticides residues. *Analytical Chemistry*, 78, 1780–1788.
- Zhang, H., Cheng, X., Richter, M., & Greene, M. I. (2006). A sensitive and high-throughput assay to detect low-abundance proteins in serum. *Nature Medicine*, 12, 473–477.

## SUPPORTING INFORMATION

### **Electrochemical magnetic beads-based immunosensing platform for the determination of $\alpha$ -lactalbumin in milk**

V. Ruiz-Valdepeñas Montiel, S. Campuzano\*, R.M. Torrente-Rodríguez, A.J. Reviejo, José

M. Pingarrón\*

<sup>1</sup>*Departamento de Química Analítica, Facultad de CC. Químicas, Universidad Complutense de Madrid, E-28040 Madrid, Spain.*

E-mails: victor\_lega90@hotmail.com, susanacr@quim.ucm.es, rebecca.magnolia@gmail.com, reviejo@quim.ucm.es, pingarro@quim.ucm.es. Tel. 34913944315, fax 34 913944329.

\* to whom correspondence is to be addressed

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**Table S1**

Optimization of the different experimental variables involved in the preparation of the electrochemical **MBs-based** immunosensor for  $\alpha$ -LA.

| Experimental variable                                                    | Tested ranges | Selected values |
|--------------------------------------------------------------------------|---------------|-----------------|
| $V_{\text{HOOC-MBs}}$ , $\mu\text{l}^*$                                  | 2–10          | 3               |
| [anti $\alpha$ -LA], $\mu\text{g/ml}$                                    | 5.0–75        | 50              |
| [HRP-anti $\alpha$ -LA], $\mu\text{g/ml}$                                | 0.5–5.0       | 1.0             |
| Incubation time with anti $\alpha$ -LA, min                              | 15–90         | 15              |
| Number of incubation steps                                               | 1 or 2        | 1               |
| Incubation time with $\alpha$ -LA and HRP-anti $\alpha$ -LA mixture, min | 15–60         | 30              |

\*Experimental variable optimized in a previous work (Ruiz-Valdepeñas Montiel et al., 2015).

## Reference

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