Electrochemical immunosensor detection for lactoferrin in milk powder

Junyi Huang[1,2], Ziyu He[2,#] Jie Cao[2], Jiang Hong[2], Zhengjun Wu[1,*], Haiyan Gao[2], Xianyan Liao[2,*]

[1] State Key Laboratory of Dairy Biotechnology, Bright Dairy & Food Co. Ltd., Shanghai 200072, China;
[2] Key Laboratory of Food Nutrition and Function, School of Life Sciences, Shanghai University, Shanghai, 200444, China
[#]Co-first author: the contribution for this article is equal to Junyi Huang.
[*]E-mail: wuzhengjun@brightdairy.com, xyliao@shu.edu.cn

A lactoferrin (LF) immunosensor was fabricated by immobilizing an LF monoclonal antibody on a gold electrode, and the assembly process was tracked by cyclic voltammetric measurements and electrochemical impedance spectroscopy. A linear relationship between the immunosensor response current and the logarithm of the LF concentration was characterized to be in the range of 0.01-1000 ng mL^{-1} with a LF detection limit (LOD) of 4.9 pg mL^{-1}. The fabricated LF immunosensor was specific to LF with no response to interfering substances. The optimum response occurred at an incubation temperature of 37 °C and an incubation time of 60 min. In addition, the fabricated LF immunosensor exhibited a 4-week shelf life. Application of the fabricated LF immunosensor to real milk samples was demonstrated. In conclusion, we have developed a biosensor method for LF detection with high sensitivity and sufficient stability.

Keywords: Lactoferrin; Immunosensor; Bioelectrochemistry; Cyclic voltammetry

1. INTRODUCTION

LF is a natural glycoprotein and belongs to the milk transferrin family. LF is primarily found in mammalian milk and other secretions, such as saliva, tear, bile, pancreatic juice, and intestinal fluid [1-3]. The natural LF in breast milk promotes the development of the immune system in infants [4]. In addition, LF is not only involved in the transport of iron but also exhibits broad antibacterial, antioxidant, anticancer, and other biological functions [5-8]. Therefore, LF is an important additive in medicine, food, and cosmetics, such as health care products and infant formula [9]. LF also plays an

important role in the diagnosis and treatment of several clinical diseases [10-12]. Therefore, the development of a rapid and accurate LF detection technique is necessary.

The current LF detection methods, such as spectrophotometry, chromatography, and immunoassay techniques, have various drawbacks. The conventional immunological methods include radial immunodiffusion (RID) [13], radioimmunoassay (RIA) [14], and enzyme-linked immunosorbent assay (ELISA) [15]. RID and RIA are time consuming with low accuracy and radioactive risks. ELISA is accurate with a low detection limit. However, this method is operationally cumbersome, and the commercial assay kits are expensive. Among the normal physical and chemical analyses, HPLC is the most commonly used and the best choice for LF determination. However, these methods are too insensitive to quantitate low levels of LF in milk and have complicated sample pretreatment [16-18].

Due to the specific binding reaction between antigens and antibodies, immunoassay techniques are very useful. These methods include modern transducer-based biosensors (e.g., electrochemical biosensors [19]) and optical biosensors by utilizing surface plasmon resonance (SPR) optical detection [20]. Other techniques that exploit an automated latex assay have been reported for the determination of LF [21]. In the past 30 years, electrochemical immunosensors have attracted increasing attention in the field of analytical chemistry [22]. Based on the antigen and antibody recognition, the immune sensor has high sensitivity and specificity [23]. Moreover, electrochemical techniques have excellent sensitivity and ease of miniaturization and require low-cost and small samples. Therefore, the use of electrochemical biosensor method is expected to become the most popular LF detection method in the future. In our previous study, we used electrochemical techniques to study the bactericidal mechanism of LF [5]. In this manuscript, an electrochemical immunosensor for the detection of LF was developed by covalently attaching an LF antibody to a Au electrode. This immunosensor was fast and sensitive.

2. MATERIAL AND METHODS

2.1. Materials and reagents

Bovine LF, potassium ferricyanide ($K_3Fe(CN)_6$), bovine serum albumin (BSA), o-mercaptobenzoic acid (MBA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich (St. Louis, MO). The rabbit anti-bovine LF (anti-LF) was obtained from Abcam (England). All the other chemicals were of analytical grade. A diverse range of domestic commercial milk powders and infant formulas were purchased from Wal-Mart.

The phosphate buffered saline (PBS, pH 7.4) consisted of 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. The MBA solution (1%) was prepared with ethanol. All the other solutions were prepared in water purified using a Milli-Q purification system to greater than 18 MΩ followed by storage at 4 °C. The LF standard solutions were prepared by diluting the stock solution (1 mg mL^{-1}, by dissolving LF powder in PBS). The mouse anti-bovine antibody solution was diluted with PBS to 10 μg mL^{-1}. 1% BSA (W/V) was prepared with the same PBS.

2.2. Apparatus

For the cyclic voltammetry (CV) and electrochemical impendence spectroscopy (EIS) measurements, a CHI 660 Electrochemical system (CH Instrument, China) was used. This system has a three-electrode cell with a saturated calomel electrode as the reference electrode, a platinum sheet electrode as the counter electrode, and a modified gold disc electrode (0.025 cm^2) as the working electrode.

2.3 Pretreatment of the gold electrode

The gold electrode was pretreated prior to its further modification, as described below [24]. First, the gold electrode surface was polished to mirror smoothness using fine sand papers followed by applications of alumina slurry (with a particle diameter of 1.0, 0.3, and 0.05 μm sequentially) on a silk cloth. The smooth electrode was thoroughly ultrasonicated in ethanol followed by double distilled water for approximately 3 min to remove any adhesive particles. Next, the electrode was soaked in a freshly prepared piranha solution (volume ratio 1:3 for H_2O_2 and concentrated H_2SO_4) for 2 min and washed with pure water. Then, the electrode was electrochemically cleaned by CV scanning for 20 circles with a scan rate of 200 mV s^{-1} in 0.5 mol L^{-1} H_2SO_4. After thorough rinsing with pure water followed by drying with nitrogen, the clean bare electrode was ready for further modification.

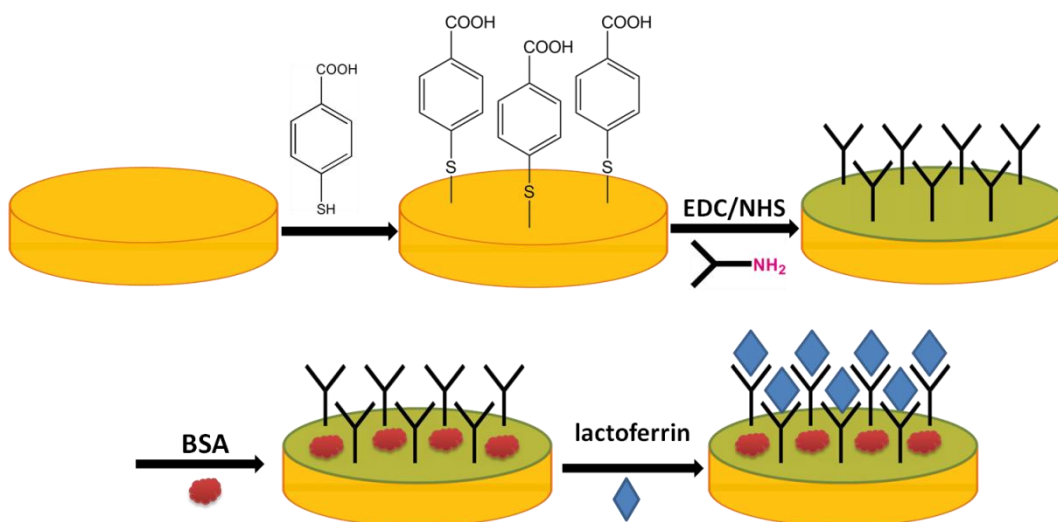*2.4 Fabrication of the immunosensor*

Figure 1. Schematic illustration of the stepwise immunosensor fabrication process.

Based on the method reported by Xiao [25], the procedure shown in Fig. 1 was employed to fabricate the immunosensor. First, a monolayer of MBA was formed on the electrode surface by

immersing the pretreated gold electrode in an ethanol solution with 1% MBA overnight. To provide high sensitivity and good reproducibility, suitable linker compounds must be identified to achieve a high antibody packing density on the electrode surface. The next step involves the activation of the self-assembled monolayer (SAM) by the formation of NHS esters in a 0.4 M EDC-0.1 M NHS solution. Then, the LF antibodies were immobilized on the electrode by forming amide bonds through their primary amines with the active NHS esters. The electrode was rinsed with pure water followed by drying with nitrogen. Finally, the electrode was immersed in a 1% BSA solution in PBS for 1 h to block any non-specific and unreacted sites.

2.5 Electrochemical measurements of milk samples

Milk powder samples were resuspended to 25 µg mL^{-1} in PBS solutions (pH 7.4) containing 5 mM Fe(CN)$_6$$^{3-}$/Fe(CN)$_6$$^{4-}$ (1:1). Next, the LF immunosensor was dipped into the PBS solution followed by 1 h of incubation at 37 °C. Then, the detection of LF was performed using CV at room temperature in a frequency range of -0.2 V to 0.6 V with a scan rate of 100 mV s^{-1}.

3. RESULTS AND DISCUSSION

3.1 Electrochemical characterization

CV and EIS are powerful tools for probing the surface modification features on electrodes and were employed in this study to characterize self-assembled monolayers (SAM). As shown in Fig. 2A, curve a reveals the reversible cyclic voltammogram of the Fe(CN)$_6$$^{3-}$/Fe(CN)$_6$$^{4-}$ redox probe at a bare Au electrode. In curve b, the faradic current is nearly blocked, resulting from a highly insulating surface due to the formation of the MBA-monolayer on the Au electrode after the pretreatment. Curve c was obtained after activation by the co-addition of NHS and EDC, which results in the formation of the NHS ester through the negatively charged terminal carboxylic group of MBA. An increased current response was observed in curve c due to the favoured transfer of the negative redox probe to the electrode surface in the presence of the positively/neutrally charged NHS ester. Curve d exhibited a decreased response current, suggesting that the penetration of the redox probe was reduced when antibody macromolecules (Ab) were immobilized on the Au-SAM. The further reduction in the response current in curve e resulted from BSA blocking of the unreacted terminal carboxylic group of MBA. Curve f exhibited a change after the introduction of LF (antigen, Ag), and the further decrease in the response current indicated binding of LF to the immobilized antibodies.

The corresponding Nyquist plots of the impedance spectra are shown in Fig. 2B. A bare gold electrode exhibits a small charge transfer resistance (Rct), which is manifested as a straight line (curve a). However, MBA that is conjugated on the electrode exhibited a large charge transfer resistance (curve b) due to the MBA-monolayer hindering electron transfer at the electrode surface. The resistance value became small again after the MBA-monolayer was activated by the EDC/NHS compound (curve c). When the rabbit anti-bovine LF antibody was introduced into the system (curve

d), the antibody bound to the MBA, and the gold electrode surface impedance value is further increased. After the electrode surface was blocked with BSA (curve e) and modified with the antigen (curve f), the impedance of the surface of the gold electrode continued to increase. These results are consistent with the CV measurements.
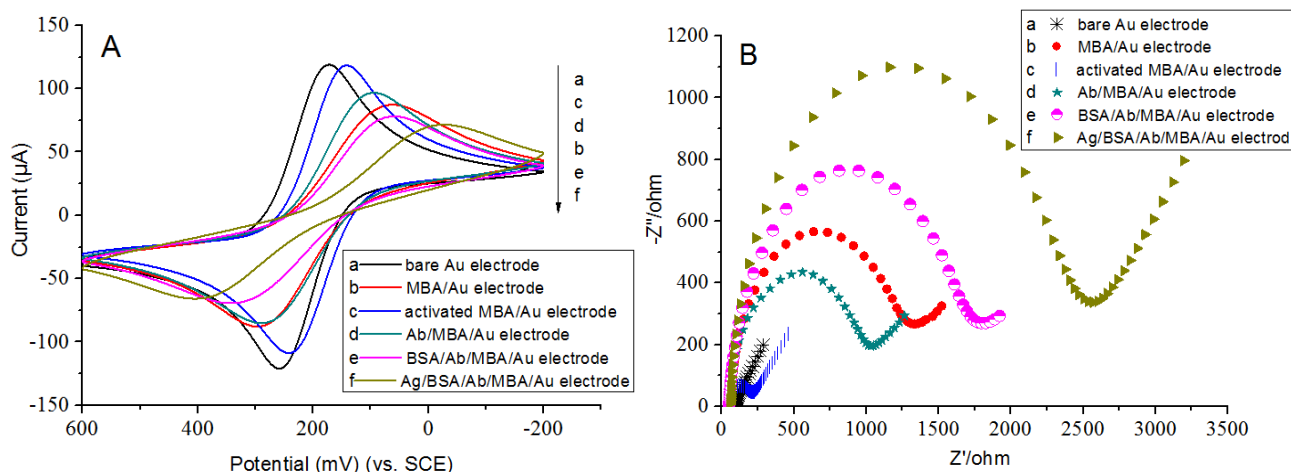

Figure 2. Cyclic voltammetry (A) and impedance complex plane plots (B) of the Au electrode in the presence of 5 mM $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$. (a) Bare Au electrode; (b) after modification with MBA; (c) after activation with EDC/NHS; (d) after antibody immobilization; (e) after BSA blocking; (f) after LF (1 ng mL^{-1}) binding. CV was carried out using a scan rate of 100 mV s^{-1}. EIS was carried out over a frequency range of 0.01 Hz-100 kHz at a signal amplitude of 10 mV.

3.2 Optimal conditions for the immunoreaction

To determine the optimal conditions for the immunoreaction, the v*arious* factors that affect the association between LF and its immunosensor were investigated (Fig. 3). The effect of the incubation temperature on the CV value for the antibody–antigen reaction was investigated in a temperature range of 20–60°C (Fig. 3A). The modified gold electrode was immersed in a 0.1 ng mL^{-1} LF solution at different temperatures for 60 min. The current decreased with the antibody-LF conjugate. The minimum current occurred at a reaction temperature of 37 °C. In addition, temperatures above or below 37 °C resulted in an increase in the reduction peak current. A high temperature may have resulted in denaturation of the corresponding proteins and temperatures lower than 37 °C may not favour antibody-antigen association. Therefore, 37 °C was selected as the working temperature for the fabricated LF immunosensor in this study.

The incubation time can also greatly affect the association of LF with LF antibodies. The prepared immunosensor gold electrode was immersed in a 0.1 ng mL^{-1} LF solution at 37 °C for different time periods. The effect of the reaction time on the response current is shown in Fig. 3B. The electrochemical response of the immunological reaction increased with the reaction time and reached a plateau at a reaction time of 60 min. As a result, 60 min was chosen as the optimal incubation time.
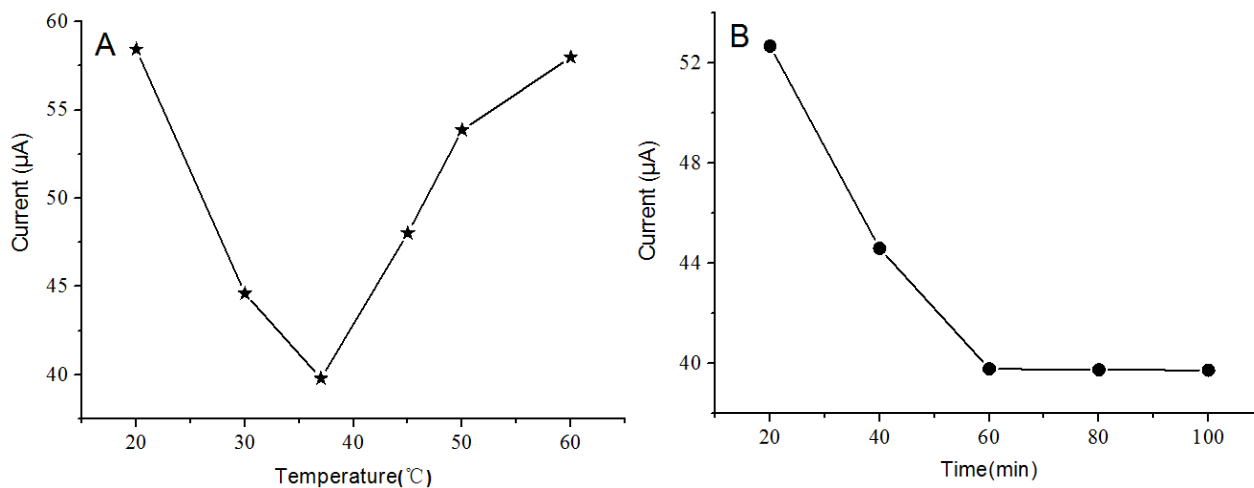
Figure 3. Effect of incubation temperature (A) and time (B) on the immunoreaction. The values are the average from 3 replicate measurements. The detection conditions are the same as those in Figure 2.

3.3 Calibration curve and limit of detection

To determine the response of the fabricated LF immunosensor to the LF concentration and the detection limit, the impendence spectra as a function of the LF concentration were recorded in PBS by CV under the optimized conditions (i.e., 37 °C working temperature and 60 min incubation time).
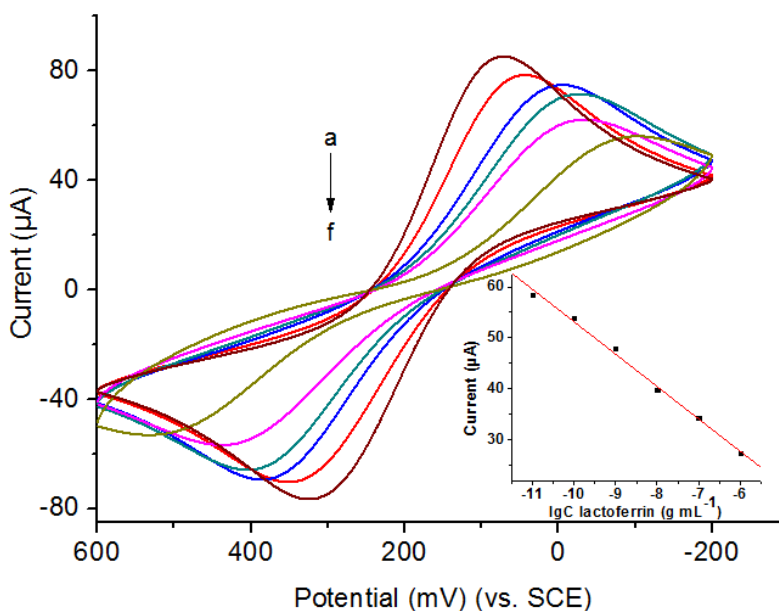

Figure 4. Cyclic voltammetry of the immunosensor with different concentrations of LF on their surface in the presence of $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ as a redox probe: (a) 10 pg mL^{-1}, (b) 100 pg mL^{-1}, (c) 1 ng mL^{-1}, (d) 10 ng mL^{-1}, (e) 100 ng mL^{-1}, and (f) 1 ug mL^{-1}.

As shown in Fig. 4, a linear relationship was observed between the response current and the LF concentration in logarithm in a range from 10 pg mL^{-1} to 1 µg mL^{-1} with a regression equation of I = - 10.9 - 6.4lg C (r^2 = 0.9946, C in g mL^{-1}, I in µA). The LOD was 4.9 pg mL^{-1} based on 3 S/N (signal/noise ratio). The limit of quantification was 26 pg mL^{-1} at 10 S/N. Optimization of the HPLC analysis was carried out using a standard solution of LF [26]. The calibration curve was extrapolated in a concentration range from 30 to 300 µg ml^{-1}. The LOD was determined to be 4.5 µg mL^{-1}. Liu et al. [17] applied the sandwich ELISA to the detection of LF, which linearly responded to LF standards in the 5–600 ng mL^{-1} range with a LOD of 3.23 ng mL^{-1}. Clearly, the LF immunosensor developed in this study has a broader detection concentration range and is more sensitive.

3.4 Specificity and stability of the immunosensor

The specificity of the LF immunosensor (i.e., the BSA/Anti-LF ab/MBA/Au electrode) was studied using CV measurements in the presence of different interfering substances. 200 ng mL^{-1} of various interfering substances (i.e., BSA, β-lactoglobulin, bovine haemoglobin, caseins, and vitamin C (Vc)) were incubated with 10 ng mL^{-1} LF. Fig. 5 shows the electrode reduction peak current in the absence or presence of different interfering substances. No obvious change in the response current in the absence or presence of different interferents was observed, and the reduction peak current of the electrode was stable at approximately 40 µA, indicating that the LF immunosensor is specific for the detection of LF and the presence of other substances does not affect its efficiency.

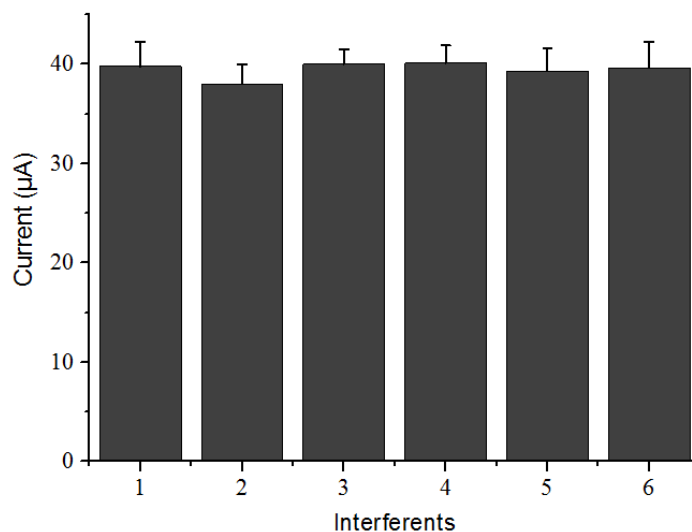**Figure 5.** Investigation of LF immunosensor specificity. The LF concentration was 10 ng mL^{-1}. (1) LF; (2) LF+200 ng mL^{-1} BSA; (3) LF+200 ng mL^{-1} caseins; (4) LF+200 ng mL^{-1} Vc; (5) LF+200 ng mL^{-1} β-lactoglobulin; (6) LF+200 ng mL^{-1} bovine haemoglobin.

The modified electrode was stored at 4 °C, and the current was measured weekly under the optimal conditions (Fig. 6). For the first 4 weeks, the BSA/Anti-LF ab/MBA/Au electrode exhibited good stability, and the electrode reduction peak current was stable at approximately 61 µA. After 4 weeks, the reduction peak current began to decline quickly from 61 µA in the fourth week to 58 µA in

the fifth week with a reduction of more than 10% reduction in the sixth week. Therefore, the shelf life of the modified electrode is at least 4 weeks.
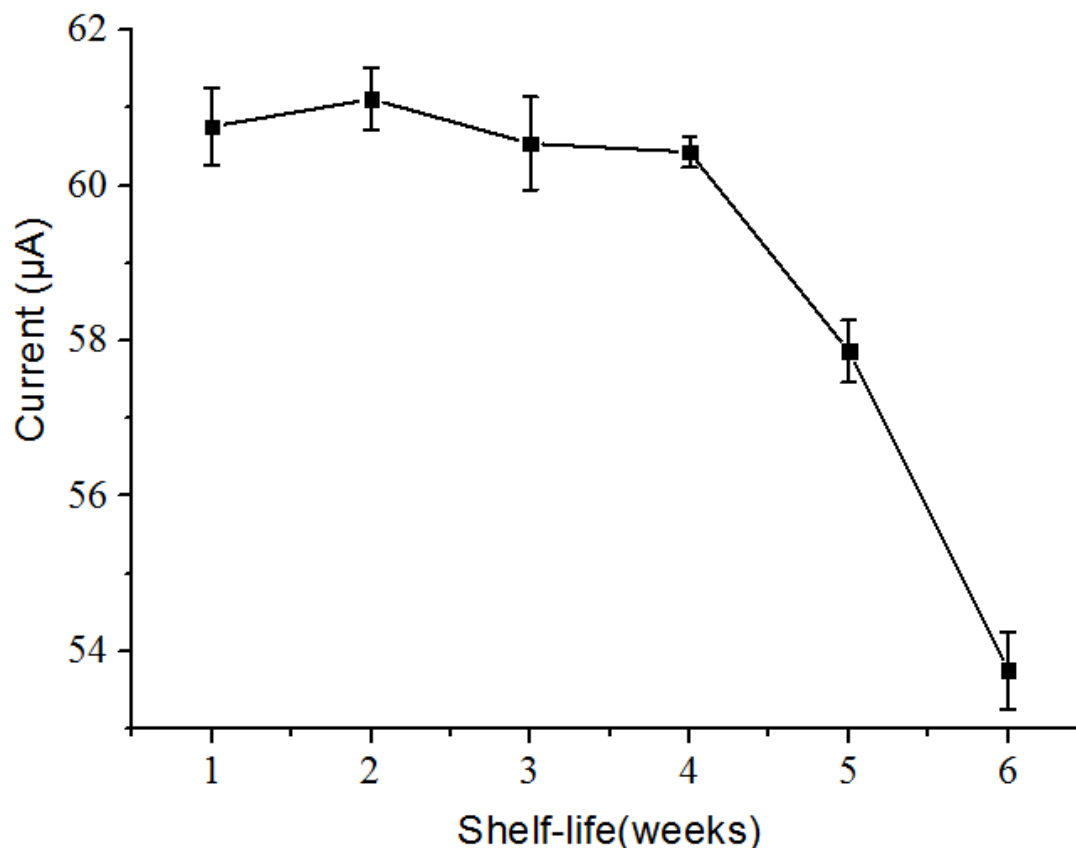

Figure 6. Relationship between the peak current and the storage time of the modified electrode at 4 °C.

3.5 LF assay in commercial milk powders

The LF isolated from cow's milk is typically added to commercial infant formulas. Therefore, the quality of infant formulas could be controlled through the determination of the LF content using the described biosensor immunoassay. Milk powder samples were selected and diluted to 25 μg mL^{-1} to prepare a LF content of approximately 10 ng mL^{-1} based on the labelled value. The LF content of each milk powder was calculated using the standard curve in Fig. 4. The results are shown in Table 1. The results indicated that the measured value was slightly lower than the labelled value for samples A to D, except sample B. The discrepancy may be due to different testing methods. Samples A to D could be added as fractionated bovine LF to supplementat infant formulas. The LF concentration range in infant formula ranges from 28 mg·100 g^{-1} to 100 mg·100 g^{-1}, which is in agreement with the Ministry of Public Health of China [27]. Although exogenous LF was not labelled for the two common milk powders, LF was detected in samples E (4 mg·100 g^{-1}) and F (16 mg·100 g^{-1}). Its LF content should be derived from raw cow's milk.

Table 1. LF content (mg 100 g^{-1}) of supplemented infant formulas based electrochemical immunoassay compared to label.

Sample	Label value	Measured value
A	100	94.7±2.6
B	68	74.5±3.2
C	35	28.2±1.6
D	38	31.1±1.8
E[a]	/	4.1±0.2
F[a]	/	16.9±1.2

Note: "/" means no label. Samples A to D are infant formula. [a] none lactoferrin-supplemented milk powder.

To test the accuracy of this method, the recoveries were evaluated by spiking 0.5, 1, 10, and 30 mg g^{-1} LF standards into sample E. The data are shown in Table 2. Using a background-subtraction measurement (measurement value minus the background value of 0.04 mg g^{-1}), the recovery rates were calculated and ranged from 94% to 107% with RSDs of 3.7-5.2%. These results indicated that the fabricated LF immunosensor method was sensitive for LF detection. In conclusion, a method for measuring the LF level in milk powder has been successfully established and can be applied to detect the LF level in other milk products.

Table 2. Results of spiked tests in skim milk powder (each fortified level was repeated six times).

Spiked (mg g^{-1})	Found (mg g^{-1})	Recovery[b] (%)	RSD (%)
0.5	0.51	94	5.2
1	1.06	95.7	4.9
10	10.44	104	4.5
30	32.14	107	3.7

[b] All recovery data were calculated by subtracting the background level of LF in milk powder (0.04 mg g^{-1}).

3.6 Comparison with other reported biosensors

Two biosensors for LF detection were reported by other researchers. One biosensor was developed for the detection of the LF content in commercial dairy products and employs SPR optical detection [20]. This technique is based on the specific recognition between antibody and antigen and has a similar detection range (0-1000 ng mL^{-1}) to the LF immunosensor reported in this manuscript but with a much smaller sensitivity (an instrumental LOD of 1.11 ng mL^{-1}). The second one was developed for the direct detection of the urinary tract infection biomarker LF [28]. This technique employs an electrochemical immunosensor that is based on a sandwich amperometric immunoassay. This method is less sensitive than the LF immunosensor reported in this study (with a detection limit of 145 pg mL^{-1}).

4. CONCLUSIONS

In this study, an electrochemical immunosensor for the detection of LF was developed by covalently conjugating the LF Mabs with MBA on a gold electrode. The LF was detected based on the increase in the impedance values upon LF binding with its antibody on the immunosensor. A linear relationship between the reduction peak current and the logarithm of the LF concentration was identified in the 0.01-1000 ng mL^{-1} range with a detection limit of 4.9 pg mL^{-1}. Therefore, the fabricated LF immunosensor is a feasible quantitative method with high sensitivity. The optimal working conditions (37 ℃ and 60 min) for the LF immunosensor and its shelf life (4 weeks) were also characterized. Furthermore, the fabricated LF immunosensor was highly specific for LF.

CONFLICT OF INTEREST
There is no conflict of interest.

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