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Research Article

Microfluidic tool coupled with electrochemical assay for detection of lactoferrin isolated by antibody-modified paramagnetic beads

Lactoferrin (LF) is approximately 80 kDa iron-binding protein, which is important part of saliva and other body fluids. Due to its ability to bind metal ions, it has many biologically important functions. In this study, a method for the isolation of LF from a biological sample using robotically prepared antibody-modified paramagnetic particles was developed using robotic pipetting station. The method consisted of the following optimised steps. Protein G was bound on the paramagnetic particles, on which goat antibody (10 μg) was linked. LF was subsequently added to microtitration plate, which had affinity to goat antibody and the interaction lasted for 30 min. We found that the highest signals were obtained using the combination of goat antibody 1:3000, murine antibody 1:5000 and conjugate 1:1500. Horseradish peroxidase reducing 3,3′,5,5′-tetramethylbenzidine (TMB) was linked to the merged complex. The resulted product of this reaction was subsequently analysed spectrometrically with detection limit (3 S/N) as 5 ng/mL. In addition, we also determined TMB by stopped flow injection analysis with electrochemical detection. The limit of detection (3 S/N) was estimated as 0.1 μg/mL. To compare spectrometric and electrochemical approach for detection of TMB, calibration range of bead-LF-antibodies complex was prepared and was determined using a least-squares correlation with coefficient R^2 higher than 0.95, indicating a very good agreement of the results obtained.

Keywords:
Electrochemistry / ELISA / Lactoferrin / Magnetic particles / Stopped flow injection analysis
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1 Introduction

Saliva is the product of the salivary gland secretory cells containing glycoproteins, proteins, enzymes, hormones and minerals [1]. Saliva composition varies depending on the current physiological state of an organism and processes controlled by autonomic nervous system on the basis of the conditioned and unconditioned reflexes [1]. Saliva involves in the transfer of flavour to the taste buds, moisturizes the mouth, facilitates dilution and swallowing food, breaks down carbohydrates and fats into simpler compounds and exhibits antimicrobial, antiseptic and protective effects [2]. Lactoferrin (LF), protein contained in saliva, is one of the components of innate immunity due to its antimicrobial, anti-inflammatory effects that result from its structure. It consists from two domains, which have the ability to bind metal ions, mostly Fe^{2+} or Fe^{3+}, but also Cu^{2+}, Zn^{2+} and Mn^{2+} [3, 4], which are required for bacteria growth [5]. The occurrence of this glycoprotein was observed in several mucosal secretions as breast milk, tears, blood, saliva, sweat, semen or vaginal discharge [3]. In addition, the enhanced level of LF in the blood is associated with inflammatory processes in the body [6].

ELISA [7–9], RIA [10, 11] or luminescence-based immunoanalysis [12] are commonly used for determination of LF. This protein can be also determined using other methods like CE [13] and/or flow injection analysis with electrochemical detection [14]. Biosensors with detection limits down to hundreds of pg/mL are suitable for determination of LF in urine samples, where LF is present in very small concentrations [15]. In addition to determination assays, affinity CE was used to investigate the binding of heparin to LF [16].

Nano- and microparticles are increasingly used in immunoassays both for molecules labelling as gold nanoparticles used for CA15–3 antigen determination [17] and for immobilization of target compounds including antibodies [18]. Besides particles without magnetic properties, advantages of paramagnetic particles using are simple separation

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Abbreviations: CV, cyclic voltammetry; **FPLC**, fast protein LC; **HRP**, horseradish peroxidase; **LF**, lactoferrin; **SFIA**, stopped flow injection analysis; **TMB**, 3,3′,5,5′-tetramethylbenzidine

Colour Online: See the article online to view Figs. 1–4 in colour.

of immunocomplexes and reactants, relatively large surface area for molecules immobilization, implementation to microdetection systems and therefore possibility to use smaller amounts of sample, to reduce reaction times and to enhance selectivity and reproducibility [19]. Combination of electrokinetic pumping and manipulation in a microfluidic device employing magnetic particles as a solid support was described for detection of rabbit IgG as model protein [20].

In this study, a method for the isolation of LF from a biological sample using antibody-modified paramagnetic particles was developed. The whole process included several steps, which have been optimised. The effectiveness of each step was monitored using spectrophotometric and electrochemical techniques. We also suggested a procedure for the automated preparation of the initial magnetic particle conjugates with antibody. After the isolation of LF by the optimised procedure, concentration of the protein of interest was determined using previously published flow injection analysis with electrochemical detection [21].

2 Materials and methods

2.1 Chemicals and pH measurement

3,3′,5,5′-Tetramethylbenzidine (TMB), Na_2CO_3, $NaHCO_3$, BSA, human IgG, NaCl, Na_2PO_4 and $NaHPO_4$ were purchased from Sigma Aldrich (St. Louis, USA). HPLC-grade methanol (>99.9%; v/v) was from Merck (Dortmund, Germany). Other chemicals were purchased from Sigma Aldrich in ACS purity unless noted otherwise. Stock standard solutions of LF (1 mg/mL) were prepared with ACS water (Sigma-Aldrich) and stored in dark at −20°C. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by software MultiLab Pilot (Weilheim). The pH electrode (SenTix H, pH 0–14/0–100°C/3 mol/L KCl) was regularly calibrated by set of WTW buffers (Weilheim). Polyclonal goat anti-LF, monoclonal murine anti-LF antibodies and chicken-HRP conjugate were purchased from SantaCruz Biotechnology (USA). Polyclonal rabbit anti-mouse conjugate with alkaline phosphatase (AP-conjugated rabbit anti-mouse IgG) was purchased from Dako (Denmark). Magnetic microparticles Dynabeads Protein G were from Invitrogen (Norway). Plastic (tips, DWP plates) used was low retention and low protein binding and was purchased from Eppendorf (Germany).

2.2 Isolation of LF by fast protein LC

LF from human saliva was isolated using fast protein LC (FPLC) protocol by Zitka et al. [22]. The saliva was obtained from healthy man (age 26 years) using Salivette tubes (Sarstedt, Germany). The obtained fractions of LF were dialyzed on cellulose ester membranes 0.1–1 kDa (Float-A-Lyzer G2, Spectra Pro, USA) 24 h, 4°C and lyophilised (Christ Alpha 1–2) 24 h under 1^{-10} mBar and −50°C. Lyophilised LF was dissolved in ACS water to concentration 1 mg/mL and filtered using cut-off filter (Amicon Ultra-2, Ultracel-30 Membrane, 30 kDa, Millipore, Ireland). The concentration of LF standard was measured spectrometrically.

2.3 ELISA

Dilution of the coating, primary and secondary antibodies for LF immunodetection was tested by ELISA. Microtitration plate was coated with 100 μL per well of polyclonal goat anti-LF antibody (SantaCruz Biotechnology) diluted 1:5000 or 1:3000 in 0.05 M carbonate buffer (0.032 M Na_2CO_3 and 0.068 M $NaHCO_3$, pH 9.6) at 4°C for 16 h. After coating the free surface of the wells was blocked with 150 μL per well of 1% BSA w/v in PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, and 4.3 mM Na_2HPO_4, pH 7.4) for 30 min at 37°C, then the wells were washed 5× with 350 μL of 0.05% v/v PBS-T (Hydroflex, TECAN, USA). Then, 100 μL of the sample of LF standard was added and the microplate was incubated at 37°C for 1 h. After washing with PBS-T, 100 μL of monoclonal murine anti-LF antibody (SantaCruz Biotechnology) in dilution 1:5000 or 1:10 000 in PBS was added and the plate was incubated for 60 min at 37°C. After washing with PBS-T, 100 μL of chicken anti-mouse-HRP conjugate (SantaCruz Biotechnology) in dilution of 1:1500 or 1:2000 was added and the plate was incubated for 60 min at 37°C. After incubation and washing 100 μL of 0.001% w/v TMB in 0.2 M sodium acetate adjusted to pH 5.8 with citric acid with 0.037% v/v of H_2O_2 was added. After 30 min, the reaction was stopped with 50 μL of H_2SO_4 and after additional 5 min the absorbance was read at 450 nm (Infinite M200 Pro, Tecan, USA).

2.4 Immobilization of antibodies to the paramagnetic beads

The procedure of antibodies preparation and immobilization to the beads was adopted from suppliers manual (Invitrogen). The magnetic beads with protein G (DB-G) (25 μL) were washed twice in the 100 μL of PBS buffer. Goat antibody against LF (10 μg in 100 μL of PBS) was added to DB-G and the Ab-DB-G complex was incubated for 30 min at room temperature in a multi-spin MSC-3000 centrifuge (Biosan, Latvia) to avoid beads sedimentation. During this incubation the antibody was bound to the Dynabeads via its Fc region. After that, tubes were placed on a Dynal Magnetic Particle Concentrator (Invitrogen), thus, the beads migrated to the side of the tube facing the magnet and allowed for easy removal of the supernatant. Unbound antibody was removed and the samples were washed with 100 μL of PBS and the beads were blocked with 0.1 mg/mL of nonspecific human IgG for 30 min in a multi-spin centrifuge. After the washing

the beads were resuspended in 100 μL of PBS with 0.01% Tween-20 and stored for further usage at 4°C.

2.5 Dot-immunobinding assay and SDS-PAGE

Dot-immunobinding assay was used to verify the antibodies binding to LF standards. Two microlitre of the sample was pipetted on a PVDF membrane (Bio-Rad, USA) and let to dry. Then the membrane was blocked with 1% milk and incubated with primary antibodies in dilution of 1:200 in PBS overnight with rotation. Then, the membrane was three times washed with PBS buffer containing 0.05% v/v Tween-20 (PBS-T) and incubated with secondary antibodies in dilution of 1:500 in PBS for 1 h. After the three times washing with PBS-T the membrane was incubated with a chromogenic substrate for HRP (0.4 mg/mL 3-aminoethyl-9-carbazole in 0.5 M acetate buffer with 0.1% H_2O_2, pH 5.5), after the adequate development the reaction was stopped by rinsing with water, dried and scanned.

SDS-PAGE was performed using a Mini Protean Tetra apparatus with gel dimension of 8.3 × 7.3 cm (Bio-Rad). First 12.5% w/v running, then 5% w/v stacking gel was poured. The gels were prepared from 30% w/v acrylamide stock solution with 1% w/v bisacrylamide. The polymerization of the running or stacking gels was carried out at room temperature for 45 or 30 min, respectively. Prior to analysis the samples were mixed with non-reduction sample buffer in a 2:1 ratio. The samples were incubated at 93°C for 3 min, and the sample was loaded onto a gel. For determination of the molecular mass, the protein ladder 'Precision plus protein standards' from Bio-Rad was used. The electrophoresis was run at 150 V for 1 h at laboratory temperature (23°C) (Power Basic, Bio-Rad, USA) in Tris-glycine buffer (0.025 M Trizma-base, 0.19 M glycine and 3.5 mM SDS, pH 8.3). Then the gels were stained with silver according to Krizkova et al. [23].

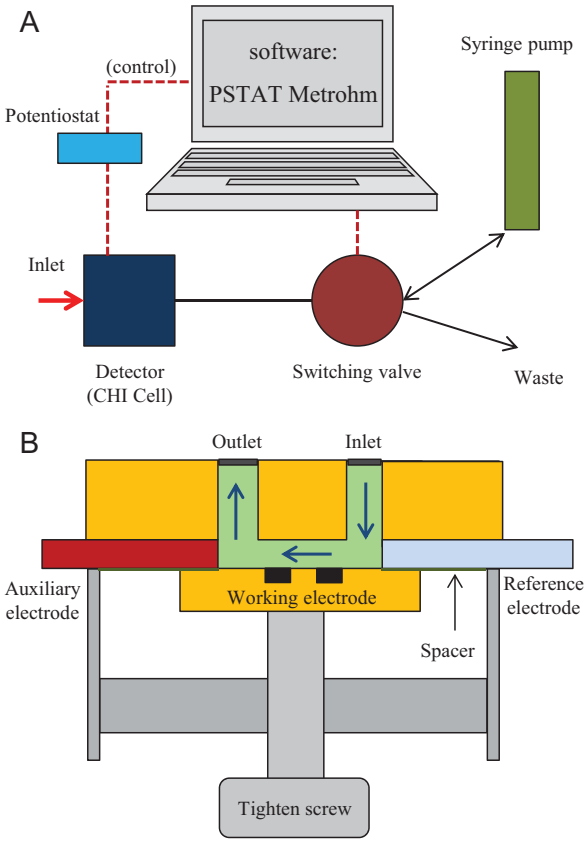

Figure 1. (A) Scheme of SFIA system. (B) Scheme of the electrochemical flow cell (CHI cell).

2.6 Robotic pipetting station

For automated samples handling prior to their electrochemical analysis, an automated pipetting station Ep-Motion 5075 (Eppendorf) with computer controlling was used. Positions C1 and C4 were thermostated (Epthermoadapter PCR96). The samples can be placed in position B3 Ep 0.5/1.5/2 mL adaptor. In B1 position Module Reservoir for washing solutions and waste were placed. Tips were placed in positions A4 (ePtips 50), A3 (ePtips 300) and A2 (ePtips 1000). Transfer was ensured by a robotic arm with pipetting adaptors (TS50, TS300, TS1000 – numeric labelling refers to maximal pipetting volume in microlitre) and a gripper for platforms transport (TG-T). The program sequence was edited and the station was controlled in pEditor 4.0. For samples preparation two platforms were used: Thermorack for 24× 1.5–2 mL microtubes (Position C3), which was used for storage of working solutions, 96-well DPW plate with well volume of 1000 μL (Position C1), which was thermostated. After the immunoseparation and enzymatic reaction, the magnetic particles were forced using Promega magnetic pad at position B4 (Promega, USA) and the solutions were transferred to a new DPW plate, in which HRP determination was performed.

2.7 Stopped flow injection analysis (SFIA)

For electrochemical detection of TMB, miniaturized microfluidic system for low volume coupled with automated electrochemical detection was used [21]. The system is composed from programmed syringe pump (Model eVol, SGE Analytical Science Pty, Australia), three-way 2-position selector valve (made from six-way valve) (Valco Instruments, USA), dosing capillary, which is entered to the electrochemical flow cell (CH Instruments, USA) and a prototype of miniaturized micropotentiostat (910 PSTAT mini (Metrohm, Switzerland)). The scheme of the instrument is shown in Fig. 1A with detailed electrochemical flow cell (Fig. 1B). Programmed syringe pump enables precise sample injections (units of microlitre with error lower than 5%). To prepare a fully automated system, switching valve enabling switching between the off waste and sample flow was placed into the system. Flow cell in volume of 500–1000 nL with electrochemical detection (working electrode: glassy carbon

Table 1. Comparison of immunoreactivity of available LF standards[a)]

Standard	Equation of regression 1:1500	R^2	Equation of regression 1:2000	R^2
1	$y = 0.09 \cdot 10^{-3}x + 7.54 \cdot 10^{-3}$	0.70	$y = 1.14 \cdot 10^{-3}x + 17.70 \cdot 10^{-3}$	0.73
2	ND	ND	$y = 0.1 \cdot 10^{-3}x + 0.86 \cdot 10^{-3}$	0.56
3	$y = 0.21 \cdot 10^{-3}x + 1.62 \cdot 10^{-3}$	0.88	$y = 0.31 \cdot 10^{-3}x + 2.48 \cdot 10^{-3}$	0.90

a) Goat antibodies 1:1500.
ND: not detected.

Table 2. Comparison of immunoreactivity of available LF standards[a)]

Standard	Equation of regression 1:1500	R^2	Equation of regression 1:2000	R^2
1	$y = 0.33 \cdot 10^{-3}x + 0.98 \cdot 10^{-3}$	0.94	$y = 0.27 \cdot 10^{-3}x - 3.08 \cdot 10^{-3}$	0.79
2	ND	ND	$y = 0.23 \cdot 10^{-3}x - 4.01 \cdot 10^{-3}$	0.43
3	$y = 2.48 \cdot 10^{-3}x - 2.70 \cdot 10^{-3}$	0.99	$y = 1.09 \cdot 10^{-3}x + 4.98 \cdot 10^{-3}$	0.96

a) Goat antibodies 1:3000.
ND: not detected.

electrode, auxiliary electrode: platinum, reference electrode: Ag/AgCl 3 M KCl) was used for a measurement.

The sample (10 μL) was injected by automated syringe (SGE Analytical Science, Australia) through flow cell in speed of 1.66 μL/s. The flow cell was cleaned by rinsing with 200 μL ethanol in water (75% v/v), then with 200 μL of 100% methanol and stabilized with 200 μL of the supporting electrolyte. Cleaning was applied after 50 measurements. The data obtained were processed by PSTAT software 1.0 (Metrohm). The experiments were carried out at 20°C.

2.8 Detection of TMB products

Supporting electrolyte for electrochemical detection of TMB was 0.05 M carbonate buffer pH 9.6. Detection parameters of cyclic voltammetry (CV) method were as follows: cyclic scan from 0 to +1000 mV and back to 0 mV, scan rate 20 mV/s. Analysis of calibration curve of TMB was carried out using method of differential pulse voltammetry where parameters were as follows: initial potential E 0.8 V, final potential –0.6 V, amplitude (V) = 0.05, pulse width (s) = 0.0167, pulse period (s) = 0.2, deposition potential (V) = 0.2, deposition time (s) = 30, sensitivity (A/V) 2.10^{-5}.

2.9 Descriptive statistics

Data were processed using Microsoft EXCEL® (USA) and STATISTICA.CZ Version 8.0 (Czech Republic). Results are expressed as mean ± SD unless noted otherwise (EXCEL®). The detection limits (3 S/N) were calculated according to Long and Winefordner [24], whereas N was expressed as standard deviation of noise determined in the signal domain unless stated otherwise.

3 Results and discussion

3.1 ELISA – Testing of LF standards

Immunoreactivity of LF standards with murine and goat anti-LF antibodies was tested by ELISA. Three LF standards were used: LF isolated from human saliva using FPLC according to protocol published by Adam et al. [25], commercially available standards of LF from Biopole and from Sigma-Aldrich, hereafter referred to as standard 1 (human), 2 (Biopole) and 3 (Sigma). LF concentration ranging from 2.5 to 40 ng/mL was prepared. Two dilutions of goat antibodies (1:1500 and 1:3000), three dilutions of murine antibodies (1:5000, 1:10 000 and 1:15 000) and two dilutions of labelled antibodies (1:1500 and 1:2000) were tested. The obtained equations and regression coefficients are shown in Tables 1 and 2. It is apparent that the linearity of the measured lines was strongly dependent on the amount of antibody used for the covering of plate, on the amount of labelled antibody and on the amount standard used. The best linearity ($R^2 = 0.99$) was obtained using standard no. 3 using 1:3000 goat antibody, 1:5000 murine antibody and 1:1500 labelled antibody. In contrast, the addition of 1:1500 goat antibodies did not show good linearity of the calibration curve with $R^2 = 0.70$ using 1:500 labelled antibody, and $R^2 = 0.73$ using 1:2000 labelled antibody. The calibration curve obtained from the analysis of standard no. 2 did not show good linearity and, using 1:1500 labelled antibody, calibration curve showed no trend. It is therefore obvious that the standard no. 2 does not bind to antibodies and therefore cannot be used for further analysis. Standard no. 3 shows good linearity using 1:500 goat antibody and 1:2000 labelled antibody with $R^2 = 0.9$. When applying 1:3000 goat antibody and 1:15 000 labelled antibody, the coefficient of determination of measured dependence was $R^2 = 0.94$. The worse regression coefficient in comparison with standard no. 1 can be associated with

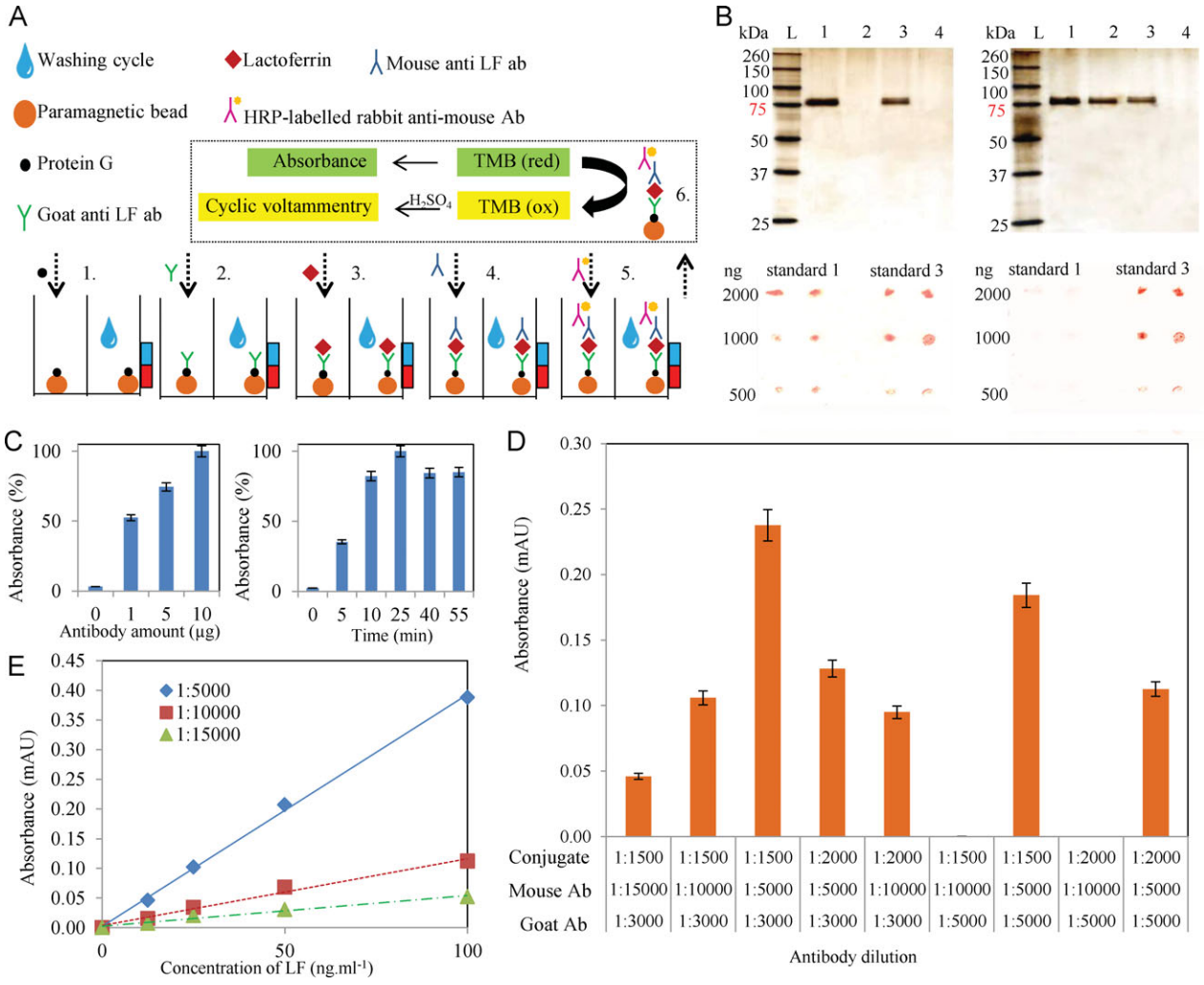

Figure 2. (A) Scheme of bead-LF-antibodies complex. Polyclonal goat antibodies against LF were immobilized onto paramagnetic particles coated with protein G. After binding with LF, monoclonal murine antibody with rabbit conjugate containing HRP against LF was used for detection of this protein. (B) Verifying the functionality of the antibodies and comparison of their immunoreactivity with the standards of LF (standard 1 – Human, standard 3 – Sigma). Left: goat antibodies, right: murine antibodies. SDS-PAGE sample standard after immunoextraction of LF. Lane 1: 500 ng of LF (standard no. 1) before immunoextraction. Lane 2: 500 ng of LF (standard no. 1) after immunoextraction. Lane 3: 500 ng of LF (standard no. 3) before immunoextraction. Lane 4: 500 ng of LF 2 (standard no. 1) after immunoextraction (top) and dot blot of LF standard (bottom). (C) Dependence of signal intensity on amount of coating goat antibodies; 10 µg corresponds to 1:3000 dilution (left), dependence of signal intensity on time of interaction (right). (D) LF signal (80 ng/mL, standard no. 3) measured by using of various combinations of the antibodies and conjugate (goat antibody 1:3000, and conjugate 1:1500). (E) Calibration curve of LF (standard no. 3) measured using various concentration of murine antibody 1:5000, 1:10 000 and 1:15 000.

the fact that standard no. 3 exhibits poor immunoreactivity with used antibodies, probably due to preparation protocol or impurities, which may interfere with the immunoreaction. Based on the results obtained, LF standards nos. 1 and 3 were used in the following experiments.

3.2 Preparation of bead-LF-antibodies complex

In the following part of our study, the immunoseparation of LF was based on magnetic beads modified by sandwich ELISA. Scheme of bead-LF antibodies complex is shown in Fig. 2A. Goat antibody was linked to paramagnetic particles via protein G, subsequently LF was added and murine antibody was bound in the following step. Formation of the immunocomplex was determined with anti-mouse Igs HRP conjugate and TMB. The absorbance of the reaction product was measured at 450 nm.

Before optimising of formation of bead-LF-antibodies complex, the applicability of antibodies for immunoextraction of LF was verified by SDS-PAGE and dot-immunobinding assay. Immunoextraction of LF was designed that 10 µL of beads modified by goat or murine antibody according to the Chapter 2.3 was pipetted to 100 µL of LF (125 µg/mL). Further, samples were shaken for 60 min, then, the liquid was pipetted away and used for SDS-PAGE analysis. For analysis by SDS-PAGE, 500 ng of LF standards before and after

immunoextraction was applied into the wells of a gel. In both standards before immunoextraction, we detected a band with an approximate molecular weight of 80 kDa, which corresponds to LF. After immunoextraction with goat antibodies in standard samples nos. 1 and 3, we did not detect any band after immunoextraction, which indicates that both standards of LF were bound to the antibodies immobilized onto the paramagnetic beads (Fig. 2B, left). When using murine antibodies, we detected weak band in standard no. 1 (Fig. 2B, right). This means that the standard no. 1 bound to murine antibodies on the paramagnetic particles only slightly and remained in solution (Fig. 2B).

Using the dot-blot analysis, it was confirmed that both types of antibodies were able to detect less than 200 ng LF of standard no. 3. In standard no. 1, immunoreactivity was demonstrated only with goat antibodies. Therefore, it can be concluded that this standard was unusable for sandwich type of analysis, which is consistent with results obtained by ELISA (Table 1). It clearly follows from the results obtained that standard no. 3 was used for the following experiments.

Subsequently, the concentration of goat antibodies coupled to paramagnetic particles (1, 5 and 10 μg) was optimised. Detection of antibodies was carried out spectrophotometrically using chicken anti-goat Igs conjugated with HRP. It clearly follows from the results shown in Fig. 2C that the highest absorbance was detected using 10 μg. When optimising interaction time, the following ones were tested as 15, 30, 45 and 60 min. The most suitable time for an interaction of antibodies with LF was 30 min. At higher interaction time, the absorbance slightly decreased (Fig. 2C).

Considering the fact that we planned linking of other antibodies, which also binds to the G protein, we had to block free surface with nonspecific Igs. Blocking increased the detected signal by 3%, which is negligible for our experimental purposes (not shown). The further step was to optimise amount antibodies used for construction bead-LF-antibodies complex. Comparison of signal of LF (80 ng/mL) determined by various concentrations of antibodies, which were selected based on the results obtained by ELISA (combination of goat antibody 1:3000 or 1:5000; murine antibody 1:5000, 1:10 000 or 1:15 000; and conjugate 1:2000 or 1:1500), is shown Fig. 2D. It clearly follows from the results obtained that the highest signals were obtained using the combination of goat antibody 1:3000, murine antibody 1:5000 and conjugate 1:1500. It is also evident that the concentration of murine antibody (the first three columns) was the most critical for obtaining the highest signal heights. Therefore, we aimed our attention at the influence of murine antibodies dilution on LF signal. For this purpose, goat antibody 1:3000 and conjugate 1:1500 was used. There were tested again the following dilution 1:5000, 1:10 000 and 1:15 000. The measured dependence of the signal height on LF concentration within the range from 10 to 100 ng/mL is shown in Fig. 2E. Regression coefficients were greater than 0.97 using all dilutions. Using 1:15 000, the signal was, however, very low. This suggests that the concentration of murine antibody required for further signal amplification was not reached. Using dilution of 1:10 000 increased regression coefficient to 0.99. Using 1:5000, we detected the highest signal and the regression coefficient was greater than 0.99. Sensitivity expressed as slope of the curve enhanced by 22× and/or 78× in the case of using of 1:10 000 and/or 1:5000 dilution, respectively. We confirmed our results that the highest signals were obtained using the combination of goat antibody 1:3000, murine antibody 1:5000 and conjugate 1:1500. Detection limit (3 S/N) was estimated as 5 ng/mL.

Table 3. Analytical parameters of other related methods for determination of LF

Method	Linearity	LOD	Recovery (%)	RSD (%)	Reference
ELISA[a)] (ng/mL)	0.625–40	0.6	98	7.8	[8]
Immunosensor–amperometric detection (μg/mL)	56–8000	24	100	5.4	[26]
CE (μg/mL)	10–400	3	91	2.4	[29]
SFIA (μg/mL)	0.78–100	0.4	93	3.1	–

a) Commercial kit (Abcam, Cambridge, United Kingdom).

3.3 SFIA analysis of TMB

Campanella et al. determined LF using amperometric immunosensor, which consisted of hydrogen peroxide electrode coated with the immobilized antibodies against LF. This method is less time consuming (time accumulation 1 h), but the detection limit was estimated to 3 μg/mL [13, 15, 26, 27]. Amperometric immunosensor was tested for diagnosis of urinary tract infection by determination of LF level with detection limit 145 pg/mL [28]. Short overview of the mostly used methods for LF determination [8, 26, 29] and their comparison with the suggested method is shown in Table 3. To our knowledge there have not been described microfluidic bead-based immunosensor for LF determination. Therefore, we attempted to apply the above-mentioned results for suggestion of microfluidic bead-based immunosensor.

TMB is used as a substrate to generate detectable signal in ELISA. The reaction between the TMB substrate and HRP produces a measurable blue colour change that correlates with analyte level. After adding stop solution (acid), yellow complex is formed (Fig. 3A). TMB also exhibits electrochemical activity and therefore can be measured by using electrochemistry, which is especially useful for miniaturization and sensors [25, 30]. For electrochemical analysis of TMB, SFIA as described by Zitka et al. [21] was used. For observing of redox change of TMB we added 185 μL of stock solution of TMB into the rest of reagents, which were as follows: 1.9 μL of H_2O_2, 92.5 μL of HRP with antibody (diluted 1:10) and finally 1720 μL of substrate buffer. Characterization of conversion of 1 μM TMB within time interval from 5 to 60 min was carried out by CV. When interlaying the obtained cyclic voltammograms showed in Fig. 3B, reduction peaks are detected at approximately 0.25 and 0.35 V. Their height gradually decreased from the beginning of the measurements. The

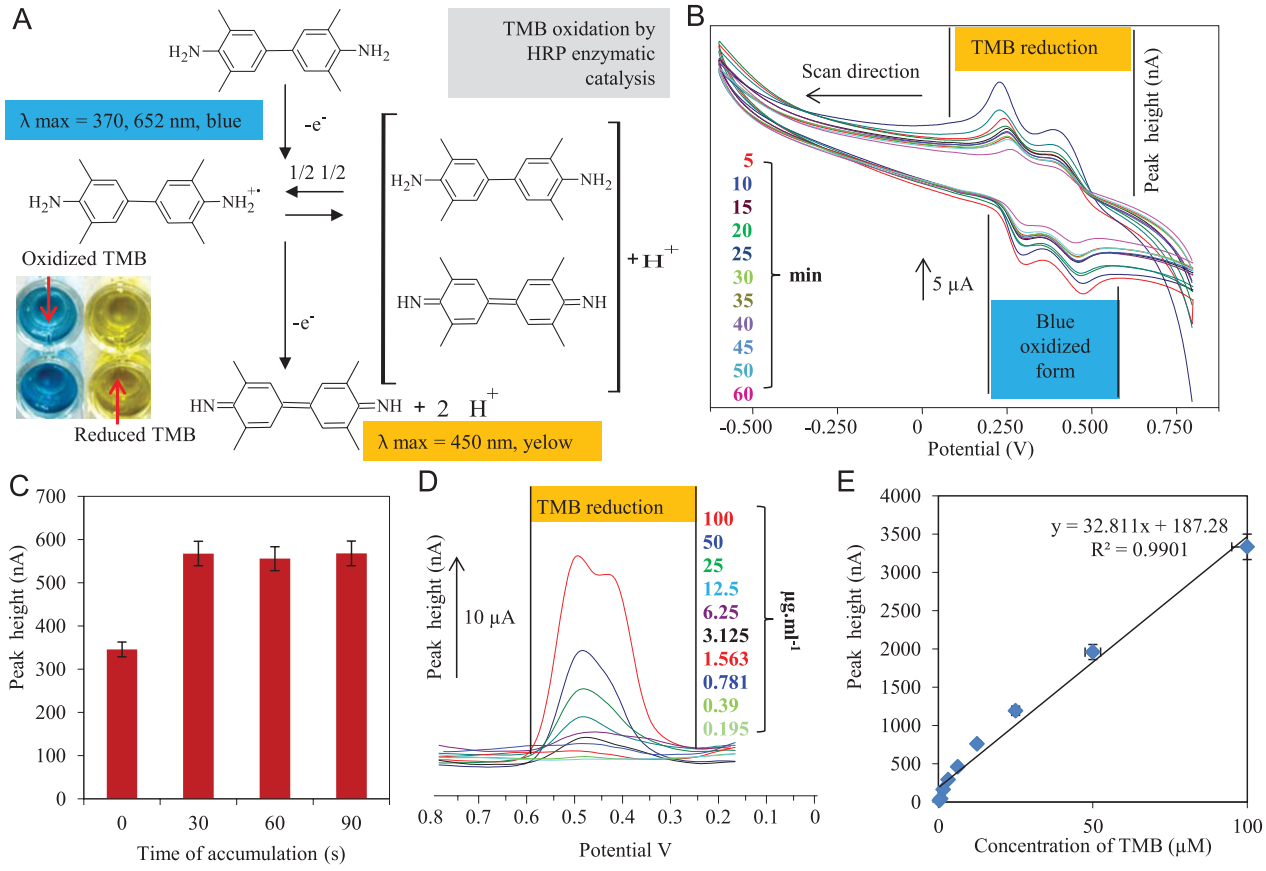

Figure 3. (A) Reaction scheme of the TMB conversion from reduced to oxidized form by HRP. (B) Time dependence of TMB (1 µM) conversion in the presence of 30% H_2O_2 catalysed by HRP as overlay of cyclic voltammetric scans. (C) The influence of time of accumulation on peak height of TMB (1 µM) measured by differential pulse voltammetry (DPV). (D) Differential pulse voltammograms of various concentration of TMB. (E) Calibration curve (0.195–100 µg/mL) of TMB (reduction signals) measured by DPV.

height of the oxidation peaks at 0.260 and 0.280 V slightly increased with a longer interaction time up to 20 min, then, the peaks decreased. Based on the potentials of reduction and oxidation peaks, it is evident that the reaction is reversible.

After that we characterized the basic electrochemical behaviour of TMB, we used differential pulse voltammetry for detection of TMB due to better sensitivity of the measurement compared to CV. Primarily, we optimised accumulation time of TMB (1 µM) at the surface of working electrode (Fig. 3C). It clearly follows from the results obtained that 30 s long interaction time caused marked enhancement of the signal. Under longer accumulation time (60 and 90 s), the signal did no change. Our effort was to develop a sensitive electrochemical detection of TMB, which would be also less time consuming. Therefore, we selected accumulation time of 30 s. Using this accumulation time, calibration curve for TMB within the range from 0.195 to 100 µg/mL was measured (Fig. 3D), which showed a linear trend with the following equation $y = 32.811x + 187.28$, $R^2 = 0.9901$, RSD = 6.7% and $n = 5$ (Fig. 3E). The limit of detection (3 S/N) for TMB was estimated as 100 ng/mL.

3.4 Correlation between spectrophotometric and electrochemical determination

To compare spectrometric and electrochemical approach for detection of TMB, calibration range of bead-LF-antibodies complex was prepared within the range from 2.5 to 80 ng/mL. Standards nos. 1 and 3 were tested and standard 2 was not used, because there was not detected any interaction with antibodies. A linear dependence with $R^2 = 0.99$ was measured using standard no. 3 (Sigma-Aldrich, Fig. 4A). On the other hand, the calibration standard no. 1 showed increasing trend only with $R^2 = 0.91$, which is probably caused by impurities occurring in the sample prepared according to Section 2.2, because the isolated LF was not further purified. Calibration of bead-LF-antibodies complex detected electrochemically was based on reduction signals of TMB (Fig. 4B). Concentration dependence shows a downward trend due to the fact that the decrease of TMB peak is measured. The regression coefficient of standard no. 1 was $R^2 = 0.92$ and of standard no. 3 was $R^2 = 0.98$, which is in good agreement with the spectrophotometric assay.

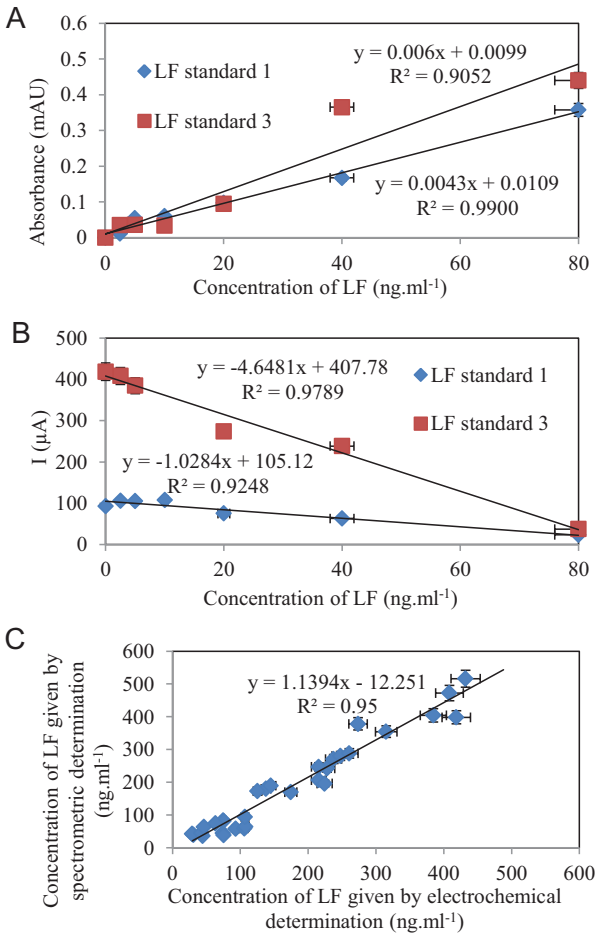

Figure 4. Comparison of the results of electrochemical and spectrometric detection. (A) Calibration curve of LF standards nos. 1 and 3 obtained by spectrometry. (B) Calibration curve of LF standard s nos. 1 and 3 obtained by electrochemistry. (C) Correlation between the concentrations of LF detected by spectrometry and electrochemistry.

The correlation between the concentrations of LF in Sigma-Aldrich standard and in human saliva isolated according to Section 2.2, detected using spectrophotometric and electrochemical assay was determined using a least-squares correlation with coefficient R^2 higher than 0.95, indicating a very good agreement of the results obtained (Fig. 4C).

4 Concluding remarks

In this study, we developed a bead-based immunosensor of LF coupled with electrochemical detection using microfluidic SFIA system with amperometric detection of TMB. Liquid handling during beads preparation was processed by fully automated pipetting system. This immunosensor was further tested for determination of LF obtained by FPLC separation and compared it with determination of commercially available LF standard. It follows from the results obtained that SFIA coupling with bead-based immunoassay has a good potential to be useful for analysis of samples obtaining LF including blood and urine. In comparison with other electrochemical approaches, the suggested tool is more than twofold more sensitive compared to other electrochemical tools. Moreover, dynamic range of the suggested method is better than ELISA.

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