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Further development of lactoferrin immunosensor (part III)

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ABSTRACT

The last year we fabricated some new immunosensors for the analysis of lactoferrin protein. In the present research the immunological and analytical characteristics of the immunosensor method have been extensively investigated. The study was therefore extended to cover the ability of the analyte and the corresponding antibody to produce the immunocomplex. A rough estimation of the K_{aff} value was obtained at the midpoint of the Langmuir curve, where $K_{aff} = 1/IC_{50}$. The K_{aff} value was found to be of the order of $10^6\ M^{-1}$. In addition we attempted in the present study to reduce the excessively long time required for each measurement, due to the fact that, in the previous researches the measurement procedure used was the classic "competition" ELISA type, employing an ad hoc enzymatic marker. One possible way of reducing measurement time was found to be to perform a kind of completely innovative "direct" measurement developed by us, which still uses an enzymatic marker and an amperometric transducer, as in the previous competition method.

1. Introduction

Lactoferrin, an iron-binding glycoprotein of the transferrin family, was first isolated from cow's milk [1,2] and subsequently from human milk. However, it is also contained in human saliva and tears [3–5]. Lactoferrin is considered a multifunctional or multi-tasking protein and plays several biological roles [6–8]. It appears to have antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and immunomodulatory properties.

Lactoferrin is a protective protein that plays an important role in the transfer of passive immunity from the mother to the neonate [9–11]. The increasing commercial interest in exploiting the therapeutic value of lactoferrin has stimulated interest in developing reliable assays for its determination at the endogenous level in milk, in dairy milk products for unweaned babies and in saliva. One year ago we fabricated new immunosensors for the analysis of lactoferrin protein in human and bovine milk. To this end we tested three different transducers, while in all cases peroxidase was used as marker [12]. In the earlier research [12] the measurement method, of the ELISA type [13,14], was always competitive and separative. This immunosensor method has already been successfully applied in the analysis of different types of human and animal milk [12,15]. In the present work we have made a more in-depth study of several important aspects of the method, such as its ability to form the antibody complex by measuring the affinity constants. Lastly, a new measurement method, no longer competitive but "direct" is proposed which has the advantage of halving the measurement time which was deemed to be too long [12] as is often the case when competitive immunological methods are used [14,12].

2. Experimental

2.1. Apparatus

The amperometric measurements were carried out in a 10 mL thermostated glass cell kept under constant stirring. The amperometric measures for the oxygen were performed using an oximeter (Amel model. 360), connected to a recorder (AMEL mod. 868) and a Clark electrode type amperometric detector, supplied by Amel (mod. 332). For the amperometric H_2O_2 measures an amperometric biosensor detector was used, coupled to an amperometric hydrogen peroxide electrode Mod. 4006, both from Universal Sensor Inc., New Orleans (USA), and connected to an Amel mod. 868 analog recorder. The potentiometric measurements were carried out using a potentiometer (Orion model SA 720) connected to a recorder (AMEL mod. 868) and with an iodide electrode from Orion Research Inc., Boston (USA), mod. 94–53.

2.2. Materials

Ny+ Immobilon affinity membrane (positively charged nylon membrane with 0.45 μm porosity) was from Millipore Corporation (NY). Anti-lactoferrin (catalogue number L-3262), lactoferrin from bovine milk (catalogue number L-9507), lactoferrin from human milk (catalogue number L-61326), β-casein from bovine milk (catalogue number C-6905-1g), β-lactoglobulin from bovine

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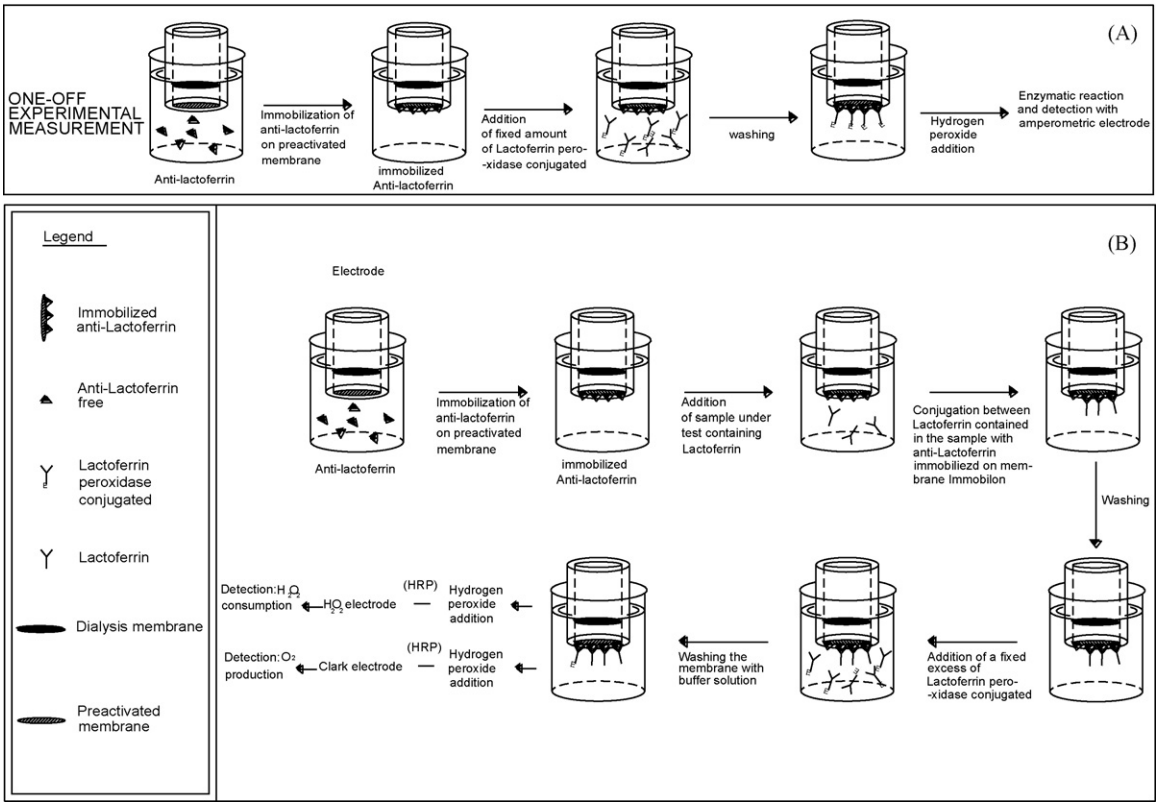

Fig. 1. (A) Scheme of one-off experimental measurement performed to check the concentration of the labelled antigen required to form the complex with the respective full antibody, which was immobilized on an ad hoc Immobilon membrane. (B) Scheme of the test for lactoferrin determination by immunosensor using the "direct" method.

milk (catalogue number L-0130), α-lactalbumin from bovine milk (type I, catalogue number L-5385), α-lactalbumin from human milk (catalogue number L-7269), Immunoglobulin A (IgA) from human colostrum (catalogue number I-2636), Immunoglobulin G (IgG) from bovine serum (catalogue number I-5256) and the biotinylation kit, supplied by Sigma Immunochemicals, composed of: biotinylation Reagent (BAC-SulfoNHS, i.e. biotinamido hexanoic acid 3-sulfo-N-hydroxysuccinimide ester), 5 M sodium chloride solution, micro-spin Column (2 mL) (practically consisting of a small empty cylindrical vessel pre-packaged with Sephadex G-50), 0.1 M sodium phosphate buffer pH 7.2, 0.01 M phosphate buffer saline (PBS) pH 7.4 (reconstituted with 1 L of deionized water to give 0.01 M phosphate buffer, 0.138 M NaCl, 2.7 mM KCl, pH 7.4); ExtrAvidin® peroxidase (containing 0.2 mL of ExtrAvidin peroxidase conjugate at 2.0 mg/mL, supplied with 0.01% thimerosal), dialysis membrane (art. D-9777), albumin (from bovine serum) (BSA) and TRIS (hydroxymethyl-aminomethane), Tween®-20 from Sigma–Aldrich; monobasic potassium phosphate, bibasic potassium phosphate and all other solvents or reagents of the highest purity were from Carlo Erba, Milan, Italy.

2.3. Samples analyzed

Three analyzed samples of human saliva were donated by the authors of this paper. Two different samples (named A and B) of powdered milk for newborns, produced by different pharmaceutical firms, were also analyzed, in one of which lactoferrin had also been added by the producer. Both samples were purchased from a local drugstore.

3. Methods

3.1. Lactoferrin biotinylation and extravidin-peroxidase conjugation

The avidin–biotin peroxidase conjugation technique is based on the use of a biotinylated antigen and an avidin horseradish peroxidase conjugate as part of the labelling system. The technique exploits the high affinity binding of biotin to avidin. The BiotioTag kit is specially designed for the small scale labelling of antibodies using biotinamido hexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester (BAC-SulfoNHS) as the labelling reagent. The entire procedure was illustrated in previous paper [12].

3.2. Anti-lactoferrin, or lactoferrin immobilization on Immobilon membrane

A commercial Immobilon membrane was used for antibody immobilization. It consisted of a positively charged nylon membrane with polyester reinforcement optimized for reliable and reproducible transfer, immobilization, hybridization, and subsequent reprobing. The Immobilon Ny+ Membrane was cut into 1 cm^2 surface area disks and 50 μL of a 2.0 mg mL^{-1} anti-lactoferrin solution was directly deposited on the membrane surface. The membrane was then dried at room temperature for about 24 h and stored at 4 °C.

When the competition method was used and the second measurement procedure was employed [12,15], the immobilization of lactoferrin in the Immobilon membrane was also necessary. This was obtained using the same procedure as for the anti-lactoferrin described above.

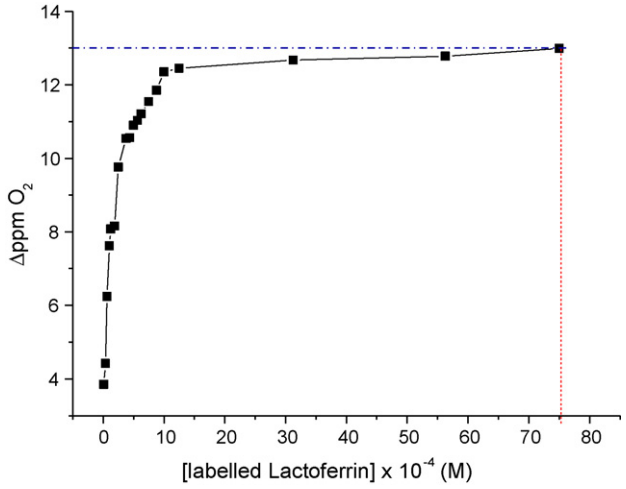

Fig. 2. Example of the experimental curve constructed to check the true concentration value used as a one-off experimental measurement and the corresponding signal. Clark electrode used as transducer.

3.3. Immunosensor assembly

Three different types of electrochemical transducers were used alternately in the competition method [12]: an amperometric electrode for H_2O_2 determination, a gaseous diffusion amperometric electrode for O_2 determination (Clark type electrode) and an ion selective electrode for iodide (see the assembly illustration reported in detail in previous papers [12,15]).

3.4. Determination of lactoferrin by immunosensor using competition method

(A) First competition procedure: the competition between a fixed amount of lactoferrin biotin–avidin–peroxidase conjugated and the non-conjugated lactoferrin (i.e. the lactoferrin sample to be measured), both free in solution, for anti-lactoferrin immobilized in membrane, formed the basis of the method.
(B) Second competition procedure: the competition between lactoferrin immobilized in the membrane and lactoferrin free in solution (to be measured) for a fixed amount of anti-lactoferrin biotin–avidin–peroxidase conjugated free in solution, formed the basis of the method.

Both these competition methods were described and illustrated in detail in a previous paper [12].

3.5. Determination of lactoferrin by immunosensor using the direct method

In order to reduce analysis time we developed a new procedure for performing the measurement "directly", that is, without introducing the competition step.

For the purpose of direct measurement of a real sample a completely innovative procedure was developed: first of all, a one-off experimental measure was performed (Fig. 1(A)) to determine the concentration of the labelled antigen required to form the complex with the full respective antibody, which was immobilized on the Immobilon membrane.

Rather than performing a single one-off experimental measure to check the concentration of the labelled antigen required to complex the full antibody, which was immobilized on the Immobilon membrane, what was actually done was to construct the ad hoc saturation curve, reported in Fig. 2. In practice, to this end, the immunosensor with the Immobilon membrane (on which the anti-lactoferrin was immobilized), secured to the head of the amperometric electrode for hydrogen peroxide, was dipped into the thermostated measurement cell containing 5 mL of phosphate buffer (pH 8,0; 0.1 M), to which a fixed volume (of the order of 100 μL) of a 1 mg mL^{-1} solution of lactoferrin peroxidase conjugated was added. The subsequent enzymatic measure was performed in the same cell, which contained the customary 5 mL of phosphate buffer, to which 20 μL of H_2O_2 solution 1% (v/v) had been added, using an amperometric transducer such as a Clark electrode or an H_2O_2 electrode.

This procedure was repeated for increasing concentrations of lactoferrin peroxidase conjugated so as to ensure that the concentration of the antigen conjugated in the measuring cell was maintained between 10^{-9} and 10^{-4} M. The curve obtained by plotting the current values (nA) recorded against the increasing concentrations of conjugated lactoferrin (see Fig. 2) made possible to determine the theoretical value of the labelled antigen concentration which was necessary to complex the full immobilized antibody value, namely 7.5×10^{-5} M. Lastly the signal in nA corresponding to this concentration was read off on the ordinate of the curve in Fig. 2.

Each "direct" test was then performed (Fig. 1(B)) by complexing a part of the molecules of the antibody immobilized in the Immobilon membrane with the antigen to be measured, which was contained in the real sample. Lastly, after washing, the complexation of the remaining immobilized antibody was "completed" using a fixed labelled antigen excess solution having the same concentration as that found by the one-off experimental measure. The customary enzymatic measurement was then performed, after washing, by adding the specific substrate of the enzyme to the renewed buffer solution in which the immunosensor was again immersed (see Fig. 1(B)).

This yielded the signal which would be used to enter in the calibration curve by taking the difference between the signal recorded during the one-off measurement and the one obtained by the test described above.

In practice, to perform each direct test, first the immunosensor with the immobilized antibody was treated with buffer solution containing BSA (5 mL of Tris–HCl buffer solution (pH 8.0; 0.1 M), 0.05% by weight of Tween®-20 and 2.5% by weight of BSA (bovine serum albumin), which was used to minimize non-specific absorption on the membrane). The buffer solution in the measuring cells was then renewed with 5.0 mL of the 0.1 M phosphate buffer (pH 8.0), into which the immunosensor was dipped; the unknown sample containing the antigen to be measured was then added and the whole antigen was complexed with the antibody immobilized in the membrane; then, the complexation of the immobilized antibody was "completed" by dipping the immunosensor for the second time in the same renewed buffer solution containing 7.5×10^{-5} M of peroxidase labelled antigen (i.e. the concentration found as described in the one-off experimental measurement). The enzymatic measurement was then performed, after washing, by adding 20 μL of H_2O_2 solution 1% (v/v). Lastly, the difference between the signal recorded during the one-off measurement and that obtained during the measurement described above was calculated and used to determine the lactoferrin content of the sample, utilizing the relative calibration curve. This measured signal (in nA) actually correlated directly with the lactoferrin concentration to be measured. In this case, the higher the concentration of not labelled lactoferrin free in solution, the stronger the signal produced by adding the hydrogen peroxide. Indeed, the lower the conjugated lactoferrin bound to the antibody immobilized on the Immobilon membrane, the lower the H_2O_2 consumed in the enzymatic reaction, and therefore the higher the signal of the H_2O_2 oxidized at the amperometric electrode.

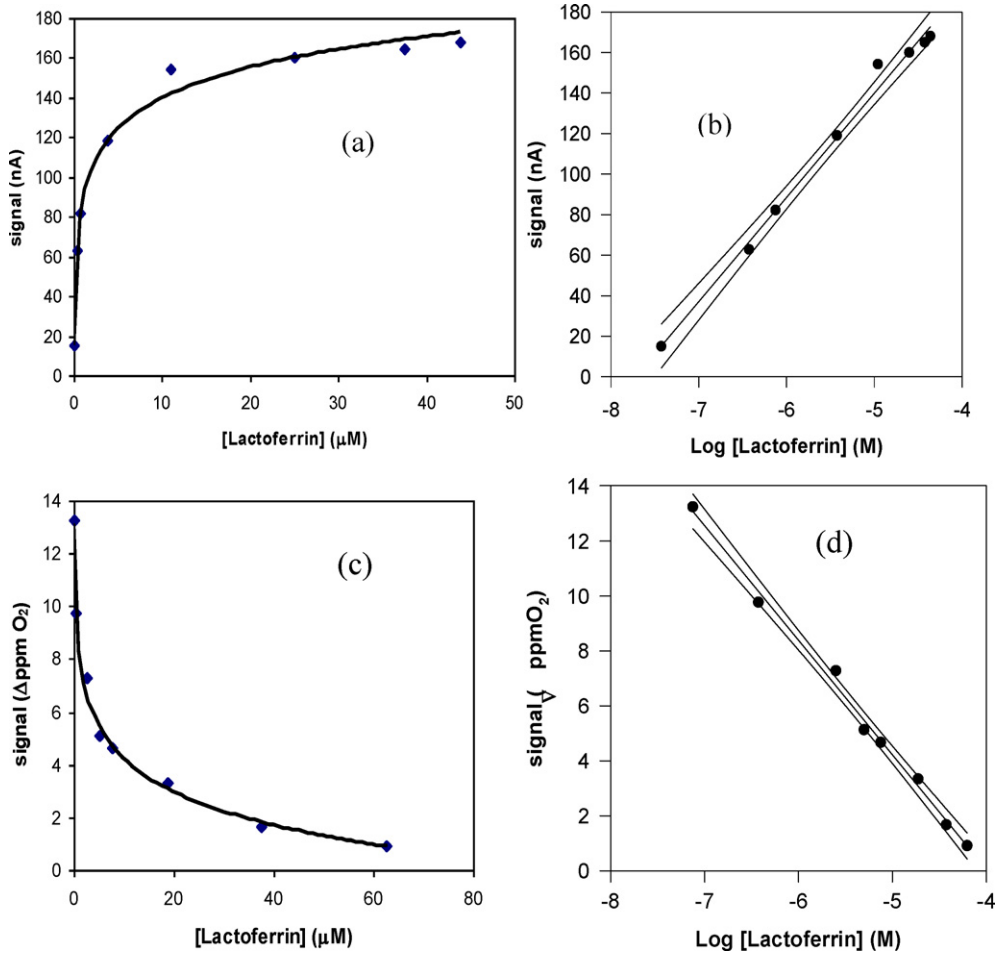

Fig. 3. "Direct" method: (a) behaviour of the immunosensor response as a function of increasing lactoferrin concentration using Immobilon membrane and an amperometric electrode for H_2O_2 as transducer and (c) using a Clark electrode as transducer; (b) and (d) corresponding respective calibration curves and confidence intervals for the lactoferrin determination, obtained using a semilogarithmic scale and H_2O_2 electrode or a Clark electrode, respectively as transducer.

The calibration curve was constructed analogously by each time adding increasing volumes (20–200 μL) of 10^{-6} (or 10^{-7}) M of lactoferrin standard solution to 5.0 mL of the buffer solution contained in the measurement cell, then following the same procedure as described above for the determination of an unknown sample, each time recording the difference between the signal recorded using the one-off measurement and that obtained at the end of each test; a response curve shown in Fig. 3(a) was thus recorded. Lastly the calibration curve was obtained by plotting this difference in current signal versus the increasing logarithm of the final lactoferrin concentration (Fig. 3(b)). This calibration curve was then used to determine the concentration of the unknown lactoferrin in any real sample. The same schematic sequence was used to construct a calibration curve to determine the lactoferrin when the amperometric gas diffusion electrode for O_2 was utilized as transducer instead of the H_2O_2 amperometric electrode. In this case, however, since the electroactive species (i.e. the oxygen in solution) is produced (unlike the H_2O_2, which is instead consumed) during the enzymatic reaction, an inverse correlation occurs (Fig. 3(c)) and so the amperometric signal decreases with increasing concentration of the lactoferrin measured (Fig. 3(d)).

3.6. Determination of K_{aff} for lactoferrin with direct or competition procedure

Since immunoreagent quality and characteristics are of the greatest importance in any immunological method, tests were also performed to check the ability of the test analyte to produce the immunocomplex with the antibody used.

A typical dose–response Langmuir curve of sigmoidal shape was obtained when the bound fraction (B/B_0) was plotted versus the logarithm of increasing final concentrations of the added standard solution. The IC_{50} is defined as the concentration of the analyte that inhibits 50% of the original observed signal (in nA) [16,17]. In our case IC_{50} is the current at which 50% of the antibody complex was reached. A rough estimation of K_{aff} of the analyte under test was obtained at the midpoint of the Langmuir curve, where $K_{aff} = 1/IC_{50}$.

The affinity constant values were thus calculated, using two different data sets, obtained by applying both the competition and the direct methods and using as transducer the electrode for H_2O_2 or else the one for oxygen. Moreover, in the case of the competition method also the electrode for iodide was used, and both the first and the second operating procedures were used.

3.7. Lactoferrin measurements and "recovery" test in real samples of powdered milk

The optimized immunosensor was used to determine the lactoferrin concentration in two different samples of powdered baby's milk, to one of which lactoferrin had been added by the manufacturer. The hydrogen peroxide transducer was in all cases employed using both the direct and the competition methods (first pro-

Table 1
Analytical characterization of immunosensors for lactoferrin determination, using the "direct" method and two different transducers, respectively.

Methods	Determination of lactoferrin by means of immunosensors, using "direct" method: equations of calibration curves and main analytical data	
Employed transducer	H_2O_2 electrode	Clark electrode
Regression equation (Y = a.u., X = M)	$Y = +51.4(\pm5.24)\log X + 396.1(\pm38.2)$	$Y = -4.22(\pm0.98)\log X - 16.9\ (\pm12.2)$
confidence level $(1-\alpha) = 0.95$	$(n-v) = 6$; $(t = 2.45)$	$(n-v) = 6$; $(t = 2.35)$
Linear range (M)	4.0×10^{-5} to 3.5×10^{-8}	4.0×10^{-5} to 7.0×10^{-8}
Correlation coefficient	0.9910	0.9976
Repeatability of the measurement (as pooled SD%)	6.0	6.1
Low detection limit (LOD) (M)	1.8×10^{-8}	3.8×10^{-8}
Instrumental response time (min)	5	5

a.u. = nA when using H_2O_2 as transducer; a.u. = Δppm O_2 when using Clark electrode as transducer. Operating conditions—buffer solution: Tris (pH 8.0, 0.1 M); conjugation temperature: 25 °C; conjugation time: ≤15 min. Total measurement time: ≤30 min in all cases.

cedure); this was because, by using two measurement methods (competition and direct), we actually found in both cases a wide linear range and a low detection limit, but, above all, a high sensitivity; both methods were therefore applied for the purpose of comparison.

To measure the lactoferrin protein in two analyzed infant powdered milk formulas, a fixed measure (5 g) of each product was dissolved in 30 mL of phosphate buffer solution (pH 8.0; 0.1 M), following the instructions for use printed on the packages. On the package containing sample (B) of powdered milk for infants, to which the manufacturer had added lactoferrin, it was stated that the ration prepared as described above made available to the infants about 140 mg L^{-1} of lactoferrin.

4.0 mL of this solution was added to the measuring cell containing 1.0 mL of phosphate buffer. The measure was then carried out using both the competition and the direct methods as described above.

Recovery tests were also performed on the two diluted powdered milk samples. For the recovery tests the two powdered baby's milk samples were spiked with known volumes of standard lactoferrin solution so as to obtain a final concentration in the measuring cell of about 10^{-7} M in lactoferrin in the phosphate buffer solution (0.1 M, pH 8.0). To this end a solution of lactoferrin was prepared by dissolving 1.0 mg of lactoferrin in 1.0 mL of phosphate buffer and 50 μL of this solution was added to the buffer solution containing 150 μL of powdered milk samples. The lactoferrin concentration in the samples was determined before and after the addition using both the direct and the competition method.

3.8. Lactoferrin measurements in real samples of human saliva

To measure the lactoferrin protein in two samples of human saliva, donors were asked to provide samples of about 4.5 mL of saliva which were then diluted with 0.5 mL of phosphate buffer (pH 8.0; 0.1 M). 5 mL of the solution thus obtained were transferred to the measuring cell. Determination was performed using both procedures (competition and direct) as described in Sections 3.5 and 3.6 above. Further tests were run to determine lactoferrin in another sample of saliva, but performing the measure using Gran's Plot method applied to the solution of a third saliva sample prepared as described above. To this sample aliquots of known and increasing concentration of standard lactoferrin solution (2 mg mL^{-1}) were added in order obtain a final concentration of lactoferrin added to the measuring cell of about 0.5, 2.5 and 5.0×10^{-7} M in phosphate buffer solution 0.1 M, pH 8.0. The lactoferrin concentration of the saliva sample was determined by checking the abscissa value at the intersection between the *Y*-axis and the straight line obtained by plotting the signal obtained (in nA) against the final lactoferrin concentration after each addition of the standard lactoferrin solution to the buffer solution containing saliva placed in the measuring cell. Determination was performed using both the direct and the competition method.

4. Results and discussion

In the immunosensors for direct measurement previously described in the literature [18,19], that is, which do not involve any "competition" procedure, the signal is often obtained directly

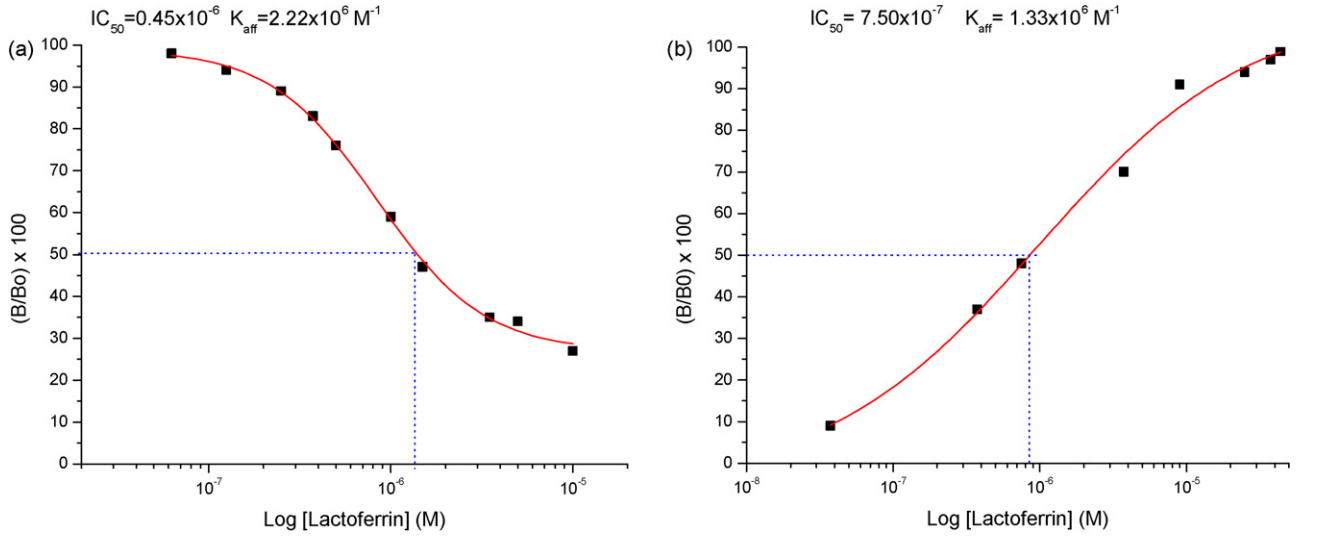

Fig. 4. Typical examples of IC_{50} and K_{aff} determination by Langmuir curves using: (a) first competition procedure and Clark electrode as transducer; (b) "direct" method and H_2O_2 electrode as transducer.

Table 2
K_{aff} values obtained using the immunosensor and the first, or the second competition method and alternately one of the three different transducers; in addition values using the "direct" method and an H_2O_2, or Clark electrode as transducer.

Method	Transducer used	IC_{50} ($n = 5$; RSD% ≤ 5)	K_{aff} (M^{-1}) ($n = 5$; RSD% ≤ 5)
1° Competition method	H_2O_2 electrode	6.75×10^{-7}	1.48×10^{6}
	Clark electrode	0.45×10^{-7}	2.22×10^{6}
	Iodide electrode	3.24×10^{-7}	3.10×10^{6}
2° Competition method	H_2O_2 electrode	7.00×10^{-7}	1.42×10^{6}
	Clark electrode	0.85×10^{-7}	1.16×10^{6}
	Iodide electrode	5.35×10^{-7}	1.87×10^{6}
Direct method	H_2O_2 electrode	7.50×10^{-7}	1.33×10^{6}
	Clark electrode	3.55×10^{-6}	0.28×10^{6}

Table 3
Determination by immunosensor of lactoferrin in two pharmaceutical powdered milks recommended for an unweaned infant diet. Values expressed both in (mg L^{-1}) and in μM.

Matrix	Direct method Found lactoferrin concentration (mg L^{-1}) $n = 5$; RSD% ≤ 5	Direct method Found lactoferrin concentration (μM) $n = 5$; RSD% ≤ 5	Competition method Found lactoferrin concentration (mg L^{-1}) $n = 5$; RSD% ≤ 5	Competition method Found lactoferrin concentration (μM) $n = 5$; RSD% ≤ 5
Powdered milk (A)	7.68	0.96×10^{-1}	7.92	0.99×10^{-1}
Powdered milk (B)[a]	80.8	1.01	87.2	1.09

(A and B) are two commercial powdered milks for unweaned diet produced by different pharmaceutical firm.
[a] Product with lactoferrin added by the producer.

Table 4
Recovery tests of added lactoferrin in pharmaceutical powdered milks recommended for unweaned infants.

Matrix	Found lactoferrin concentration (μM) (n = 5); RSD% ≤ 5	Added lactoferrin concentration (μM)	Experimental lactoferrin concentration (μM) (n = 5); RSD% ≤ 5	Recovery % lactoferrin concentration in biological matrix
Competition				
Powdered milk (A) (diluted 1:2)	5.22×10^{-2}	1.00×10^{-1}	15.02×10^{-2}	98.7
Powdered milk (B)[a] (diluted 1:3)	3.68×10^{-1}	1.00×10^{-1}	4.87×10^{-1}	104.0
Direct				
Powdered milk (A) (diluted 1:2)	4.76×10^{-2}	1.00×10^{-1}	14.96×10^{-2}	101.4
Powdered milk (B)[a] (diluted 1:3)	3.16×10^{-1}	1.00×10^{-1}	4.38×10^{-1}	105.2

(A and B) are two commercial powdered milks for unweaned diet produced by different pharmaceutical firm.
[a] Product with lactoferrin added by the producer.

as a result of immunocomplex formation (which is the cause of the membrane potential variation) and they usually do not make use of a marker. This kind of immunosensor has usually not been very successful for various reasons (weak signal, high noise, poor repeatability) even though they afford a considerable reduction in analysis time. Nevertheless we actually resumed studies on this type of immunosensor. Also in this case, however, that is for "direct" measurement, unlike what is usually reported in the literature for "direct" immunosensors, an enzymatic marker was again used to perform the electroenzymatic measurement, while the transducer used was again of the amperometric type, as described in Section 3.5.

The behaviour of the immunosensor response for the lactoferrin determination, obtained using the direct method and a hydrogen peroxide transducer, is shown in Fig. 3(a). An analogous response for the immunosensor, equipped by Clark electrode, is shown in Fig. 3(c), while the respective calibration straight lines, obtained from the same data, but using the semilogarithmic scale, are shown respectively in Fig. 3(b) and (d). The comparison of results (summarized in Table 1) referring to the analytical characterization of the direct method using two different transducers and the respective equations of calibration straight lines reported in Fig. 3(b) and (d), with the data of calibration straight line found using the competition procedure reported in the previous papers [12,15], shows that the low detection limit (LOD) for lactoferrin is of the order of about 10^{-8} M, in all cases; however, in both cases the sensitivity, expressed as the slope of the calibration straight line, is found to be higher if the hydrogen peroxide electrode is employed as transducer, than when the measurements are carried out using the Clark electrode; lastly the linear range is about three decades in all cases.

Because the immunoaffinity characteristic of the antigen for the immunoreagent is of the utmost importance in any immunological

Table 5
Determination of lactoferrin in two human saliva samples (A and B) obtained by immunosensor and respective calibration curves, using "direct" or competition method; (C) "direct" or competition measurement of lactoferrin concentration in a third saliva sample using Gran's Plot method.

Range values of found in literature [4] (mg L^{-1})	Sample	Lactoferrin value found in saliva using "direct" method (mg L^{-1}) $n = 5$; RSD% ≤ 5	Lactoferrin value found in saliva using competition method (mg L^{-1}) $n = 5$; RSD% ≤ 5
1.5–12.5	A	4.62	5.92
1.5–12.5	B	6.51	6.60
1.5–12.5	C	9.44	10.9

method, as stated previously, the present study was also extended to the ability of the lactoferrin to produce the immunocomplex with the corresponding antibody. A rough estimation of the K_{aff} value was obtained at the midpoint of the Langmuir curve, where $K_{aff} = 1/IC_{50}$.

In our case IC_{50} represents the concentration of the analyte that binds 50% of the antibody to the sensor surface, as B/B_0 is the bound fraction [20].

Fig. 4 shows examples of two typical Langmuir curves obtained by expressing the bonded fraction $(B/B_0) \times 100$ as a function of the logarithm of increasing lactoferrin concentration and using first competition or direct method, respectively. Analogous curves were also constructed using the values obtained by applying the first, or second competition procedure and using as transducers the electrode for H_2O_2 or alternatively the Clark electrode, or lastly the iodide electrode. These curves were used to obtain the graphic values of K_{aff} shown in Table 2. Table 2 also shows the values of K_{aff} obtained using the data produced by applying the direct method. It should be noted that all the K_{aff} values found, in Table 2, are very similar, and nearly all of the order of $10^6\ M^{-1}$. On the other hand these values were found to be in good agreement with the scanty data available on the topic in the literature [21–23].

Lastly the direct method was used to determine lactoferrin in twos powdered milk samples used as infants' food. Table 3 shows the values obtained, expressed both in $mg\ L^{-1}$ and μM. For the sake of comparison, the same table also shows the lactoferrin values obtained applying the competition method on the same samples. As can be seen the latter are in excellent agreement with the values obtained applying the direct method. It should also be noted how in sample (B), lactoferrin content is about 10 times greater than in sample (A). This is due to the fact that, in sample (B), the manufacturer added lactoferrin to the product in order to bring the content of this protein in the sample closer to the values of lactoferrin contained in fresh human milk about 8 months after childbirth [12,24]. This provides a significant indicator of the importance that pharmaceutical firms operating in the infant food sector are currently attributing to this polyfunctional protein. Table 4 on the other hand shows the values of the 'recoveries' obtained using the standard addition method applied to the two powdered milk samples tested. As can be seen, whether performing the measures with the direct method or the competition method the recovery values are always close to 100%. Even if this is not sufficient in itself to demonstrate the accuracy of these immunosensor methods, it may in any case be considered a necessary condition for validating the method's accuracy. Moreover, the precision may be considered practically the same for both methods (direct or competition) and in any case always acceptable (RSD ≤ 5). Furthermore, these immunosensor methods have proved to be relatively robust as, when operating in a buffered environment, for example, they are not subject to any variation in pH which might be induced by the addition of the test sample. Lastly, it has been shown that even small variations in the temperature of the measuring cell, which is thermostated to ambient temperature, or of the time of incubation or competition, have proved not to be critical factors.

In addition to the powdered milk applications, lactoferrin measures were also performed on human saliva samples. Table 5 shows the results obtained for two samples of this type using both methods (direct and competition) and using the respective calibration curves to obtain the concentration of lactoferrin contained in two respective saliva samples. Table 5 also shows a comparison of the values obtained for a third saliva sample, although this time produced by applying the Gran's Plot method. It should be noted how, in the latter sample, the lactoferrin concentration was found to be higher than in the previous two samples but also how, in all three saliva samples tested, the concentrations found always lay in the range of lactoferrin concentration values reported in the literature.

Table 6
Percent cross-selectivity values for lactoferrin immunosensors versus several common proteins contained in milk and saliva.

Protein	Order of magnitude of protein molecular weight (kDa)	Percent cross-selectivity values for lactoferrin immunosensor $n = 5$; RSD% ≤ 5
Human lactoferrin	80.0	100.0
Bovine lactoferrin	80.0	92.5
Casein	24.0	11.3
Lactoglobulin	18.4	30.4
Bovine lactalbumin	14.2	23.3
Human lactalbumin	14.2	25.2
IgA	150.0	4.4
IgG	150.0	3.5

Also the latter tables indicate the perfect agreement between the two immunosensor methods (direct and competition).

Several experimental results also shown that the response of the immunosensor equipped with the antibody reported in Section 2.2 was found not to be appreciably different and to be good enough both for human and bovine lactoferrin (slightly lower in the latter case, but only by about 7%) (see Table 6). Moreover, this was to be expected, as the proteins of the two species present homologous sequences, as reported in the literature [25,26].

Lastly, we also ascertained that the immunosensor method, as for the majority of immunological tests involving proteins, is not subject to any interference from the other small electrolytes present in the solution [27].

Tests were also run to assess selectivity towards other proteins (see cross-selectivity values reported in Table 6). It emerged that the immuglobulins A and G display practically no interference in lactoferrin measures, and even casein interferes only to a minor extent. Moreover, casein can easily be separated out by precipitation simply by lowering the sample pH to about 4.5 [28]. In practice, only the lactoglobulins and lactoalbumins can act as possible non-negligible interferents. Also in this case, however, if it proved necessary to eliminate them, this could possibly be done by means of ultrafiltration or centrifugation, exploiting the fact that their molecular weight (≈18,000 Da) is much lower than that of lactoferrin (≈80,000 Da) [29].

5. Conclusions

In conclusion, the two different methods developed, i.e. competition and "direct", involve above all very different measurement times. Indeed, in the competition method, the competition step lasts about 1 h, which considerably prolongs the total analysis time, while in the direct method each of two conjugation steps leading to the formation of the immunocomplex lasts about 15 min, for a maximum total time of about half an hour. However, the main analytical data for the two methods are comparable; also the measured affinity constant is found to be of the same order using the two methods. Furthermore, the results, obtained in real samples were always satisfactory from the analytical point of view whether the direct or the competition method was used.

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