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Lactoferrin determination using flow or batch immunosensor surface plasmon resonance: Comparison with amperometric and screen-printed immunosensor methods

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ABSTRACT

In mammalian secretions, such as milk, lactoferrin is an important iron-binding glycoprotein, present in large quantities; it is also contained in powdered milks for babies sold in drugstores. Our team tested the feasibility of constructing a new immunosensor for lactoferrin analysis based on surface plasmon resonance (SPR) using two different devices and operating in batch or flow mode. The new method proposed is not competitive but "direct" and has the advantage of halving the measurement time which was deemed to be too long as is often the case in previous research when competitive immunological methods were used. Lastly, detailed comparison was made of the analytical features of new devices with those of classical or screen-printed immunosensor methods and the advantages and disadvantages of the new SPR method investigated. Lastly several applications and comparisons were carried out on cow and goat milk and on different types of dried milk for babies.

1. Introduction

Lactoferrin is a protective protein that plays an important role in the transfer of passive immunity from the mother to the neonate [1–4]. This protein is actually an iron-binding glycoprotein of the transferrin family, first isolated in cow's milk [5,6] and subsequently in human milk. Lactoferrin is considered a multifunctional or multi-tasking protein and plays several biological roles [7–11]. It appears to have antibacterial, antiviral, antifungal, anti-inflammatory, antioxidant and immunomodulatory properties. 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 human and animal milk and in dairy milk products for unweaned babies. One year ago we fabricated new immunosensors for the analysis of lactoferrin protein in human and bovine milk.

To this end we tested Clark transducers, while in all cases peroxidase was used as marker [12]. In the earlier research [12] the measurement method used, of the ELISA type or similar [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]. Further pursuing this type of research, we actually developed also classical or screen-printed amperometric immunosensors using an amperometric H_2O_2 electrode as transducer and the peroxidase enzyme as marker to determine lactoferrin. Lastly our team tested the feasibility of constructing immunosensors for lactoferrin determination based on surface plasmon resonance (SPR) transduction. The analytical features of this device are compared in the present paper with those of classical or screen-printed amperometric immunosensors above cited. Peroxidase as enzymatic marker and the "competition" procedure were used in the latter two cases. Conversely, the SPR transduction technique, used in the present research allowed a "direct" measurement procedure to be used [16,17]. In addition, in the case of the SPR device two different measurement procedures were performed by operating in batch or in flow mode.

2. Experimental

2.1. Apparatus

The surface plasmon resonance (SPR) experiments were performed in batch mode using a SPRINGLE Instrument (EcoChemie B.V., Utrecht (The Netherlands)), the assembly of which was based on the Kretschmann configuration: it works with a laser diode set at a wavelength of 670 nm, using a vibrating mirror to

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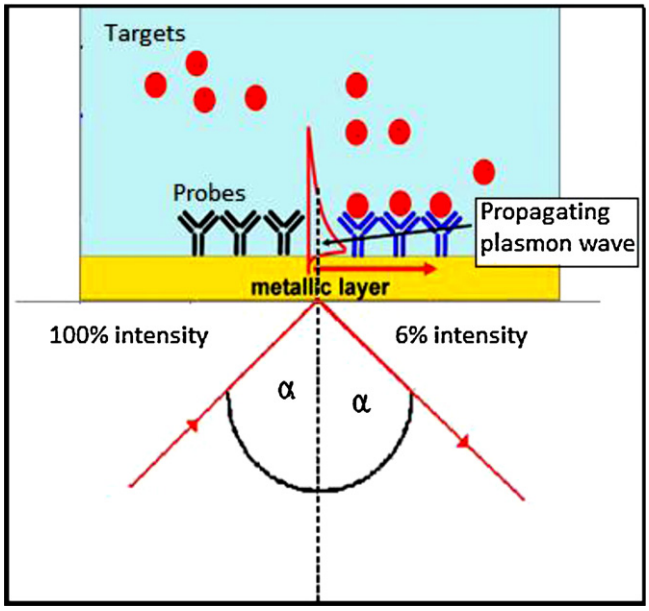

Fig. 1. Measurement scheme used with surface plasmon resonance operating in batch mode.

modulate the angle of incidence of the polarized light beam on the SPR substrate. The latter is constituted (Fig. 1) by a gold sensor disk (25 mm in diameter) mounted on a semicylindrical lens (with refractive index-matching oil) to form the base of a Teflon cuvette whose temperature is maintained at 25 ± 1 °C. The intensity of the reflected light is a minimum at the angle of resonance; this angle can be measured over a range of 4° with a resolution of 1 m using a diode detector. The instrument is equipped with an autosampler driving a controllable aspirating–dispensing–mixing pipette used to add samples to the cuvette and ensure constant mixing by aspiration and dispensing during measurements: this kind of experimental arrangement ensures a homogeneous solution and reproducible hydrodynamic conditions. Instrumental control and data acquisition are obtained through connection to a laptop computer running SPR software from Eco Chemie, version 4.1.2. For the measurement performed in flow mode (Fig. 2) a Biosuplar 400 T, Analytical μ-Systems Department of Mivitec GmbH (Sinzing, Germany) was used. This device is assembled using Kretschmann geometry. The main element of the device is the retroreflecting measurement prism, which is installed on a rotating table (see Fig. 2). A semiconductor laser is used as a light source in the device. Polarized light from the laser is split into two beams by a beam splitting device. These two beams allow two-channel device operation mode. The accepted optical arrangement provides design compactness and weak dependence of positions of light spot on the chip and on the registration system photodiode which is important in ensuring the accuracy of the SPR curve measurement. For lactoferrin analysis using a classical immunosensor a mod. 551 VA-Detector Amel potentiostat was connected to an amperometric hydrogen peroxide electrode (see Fig. 3(a)) from Universal Sensor Inc., New Orleans (USA), Mod. 4006a and to a mod. d5126-2 Omniscribe analog recorder. The test solution was contained in a thermostated cell at 25 °C and kept under constant magnetic stirring (Amel Instruments: mod. 291/lf). For screen-printed measurements an amperometric screen-printed transducer, fabricated as shown in Fig. 3(b) and with Pt working electrode modified by electrodeposited Prussian Blue polarized at 0 mV vs pseudo reference electrode Ag/AgCl, was purchased from BVT Technologies (Czech Republic), and connected for the measurements to a PalmSens Electrochemical Interface, Palm Instruments B.V. (The Netherlands).

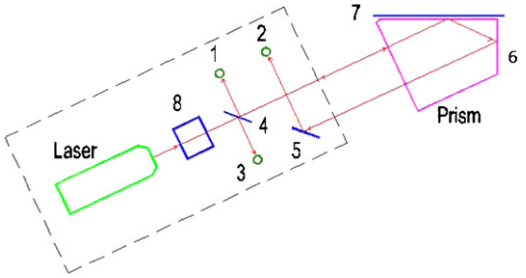

Fig. 2. A semiconductor laser is used as a light source in the device. Polarized light of the laser is split into two beams by beam splitting means 8. These two beams provide two-channel device operation mode. Beam in the first channel falls on the measurement prism 6 and further on the sensor chip 7, passing the transparent plate 4 that serves for take-off of the part of the beam energy on the photodiode 3 and part on photodiode 1 for laser power control. The point of incidence of beam on the chip coincides with the table rotation axis. The beam reflected from the chip falls on the photodiode 2 of the registration system after turning by 90° prism angle and turn mirror 5. The accepted optical arrangement provides design compactness and weak dependence of positions of light spot on the chip and on the registration system photodiode what is important for ensuring of accuracy of the SPR curve measurement.

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), β-casein from bovine milk (catalogue number C-6905-1g), β-lactoglobulin from bovine 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 G (catalogue number I-5256), immunoglobulin A (catalogue number I-5256) and the biotinylation kit, supplied by Sigma Immunochemicals, composed of biotinylation Reagent (BACSulfoNHS, 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 liter 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^{-1}, 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. The 11-mercaptoundecanoic acid (MUA) (95%), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) (commercial grade), N-hydroxysuccinimide (NHS) (98%), were purchased from Sigma–Aldrich (St. Louis, MO, USA); all other reagents were of analytical grade. All solutions were prepared using ultrapure deionized water (resistance: 18.2 mΩ × cm at 25 °C; TOC < 10 g mL^{-1}) obtained using a Direct-Q UV3 Millipore Instrument (France). The gold disks SensorDisc Au bare gold for SPR analysis was purchased from Xantec Byoanalitics (Duesseldorf, Germany).

2.3. Samples

Different samples (A, B, C and D*) of infants' powdered milk, produced by different pharmaceutical firms, were analyzed.