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RESEARCH PAPER

Assessment of goat milk adulteration with a label-free monolithically integrated optoelectronic biosensor

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Abstract The label-free detection of bovine milk in goat milk through a miniaturized optical biosensor is presented. The biosensor consists of ten planar silicon nitride waveguide Broad-Band Mach–Zehnder interferometers (BB-MZIs) monolithically integrated and self-aligned with their respective silicon LEDs on the same Si chip. The BB-MZIs were transformed to biosensing transducers by functionalizing their sensing arm with bovine k-casein. Measurements were performed by continuously recording the transmission spectra of each interferometer through an external spectrometer. The amount of bovine milk in goat milk was determined through a competitive immunoassay by passing over the sensor mixtures of anti-k-casein antibodies with the calibrators or the samples. The output spectra of each BB-MZI recorded during the reaction were subjected to Discrete Fourier Transform in order to convert the observed spectral shifts to phase shifts in the wavenumber domain. The method had a detection limit of 0.04 % (*v*/*v*) bovine milk in goat milk, dynamic range 0.1–1.0 % (*v*/*v*), recoveries 93–110 %, and intra- and inter-assay coefficients of variation less than 12 and 15 %, respectively. The proposed biosensor compared well in terms of analytical performance with a competitive ELISA developed using the same monoclonal antibodies. Nevertheless, the duration of the biosensor assay was 10 min whereas the ELISA required 2 h. Thus, the fast and sensitive determinations along with the small size of the sensor make it ideal for incorporation into portable devices for assessment of goat or ewe's milk adulteration with bovine milk at the point-of-need.

Keywords Monolithically integrated Mach–Zehnder interferometers · Label-free detection · Goat milk adulteration · Bovine k-casein determination

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Introduction

In recent years, the consumption of goat milk is increasing worldwide due to its higher nutritional value compared to bovine milk and to the fact that it causes fewer allergic reactions [1, 2]. Bovine milk contains more than 20 allergenic

proteins, while caseins and b-lactoglobulin are shown to cause the most allergenic effects [3]. Caseins represent four gene products named aS1-, aS2-, b- and k-casein, with the last one having a key role on the stability and coagulation of milk [4]. According to both EU (Directives 2000/13/EC and 2003/89/EC) [5, 6], and USA legislation [7], food industries are obliged to indicate the species origin of the milk used for food preparation to ensure its suitability for consumption by people prone to allergies. Especially, for cheese made from the milk of species other than bovine, the maximum content in bovine milk should be equal or lower than 1 % [8].

The higher price of goat milk with respect to bovine and its seasonal availability make it attractive target for possible adulteration [1, 8, 9]. To detect the fraudulent substitution of goat milk and its derivatives with bovine milk several analytical methods have been developed by employing a wide range of diverse principles of operation ranging from electrophoretic and chromatographic to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS), immunoassays, and DNA-based methods (PCR) [1, 10–20]. Among them, isoelectric focusing of γ-casein (C-terminal fragment of b-casein) is the reference method according to European Community for the detection of bovine milk in ewe, goat, and buffalo dairy products (EC regulation 273/2008) [21]. On the other hand, enzyme-linked immunosorbent assays (ELISAs) are the most attractive methods for the routine detection of bovine milk in non-bovine dairy products, since they are suitable for parallel analysis of a large number of samples in short time and could be automated [22–31]. However, these analytical techniques remain laborious, time consuming, require experienced personnel, and are not suitable for on-site analysis [32].

Lately, the development of label-free biosensors is gaining ground due to their potential for real-time and on-site analysis [32, 33]. To this direction, several biosensors have been developed to detect a wide range of substances, especially in milk and dairy products, using piezoelectric [34], electrochemical [35, 36] as well as optical transducers [32, 37–40]. Optical sensors present several advantages over their counterparts such as minimal interference from the sample, since the transducer is galvanically isolated from the excitation and detection electronics, and high potential for miniaturization and multi-analyte detection [32, 41]. Among them, Surface Plasmon Resonance (SPR) sensors are the most widely used and have been so far applied for the quantitative determination of caseins, whey proteins or bovine IgG in bovine colostrum, consumer milk, and hyperimmune milk powders as well as of plant protein adulterants in milk products [35–40, 42]. On the other hand, miniaturized label-free biosensors based on ring resonators [43], Young interferometry [44], and Mach–Zehnder interferometry [45, 46] have emerged as powerful tools for sensitive detection of multiple analytes in a single run.

The principle of operation of integrated optical biosensors is based on the use of planar waveguides where the waveguided modes interact with the biomolecular adlayers that grown as a result of coating or affinity reactions taking place on the waveguide surface. The waveguides are patterned on dielectric films covering lower refractive index planar substrates (bottom cladding layer) and are covered with a top cladding layer. Among the integrated optical biosensors, those based on Mach–Zehnder Interferometry (MZI) are between the most promising devices. In an integrated MZI, a single mode planar waveguide is split into two arms, the sensing arm (exposed to the sample) and the reference arm, by means of a Y-junction. The two arms recombine again at a second Y-junction, after a certain distance, and the two traveling waves interfere depending on their phase difference. In the conventional Single Wavelength MZI (SW-MZI), the output power of the interferometer is monitored. Biomolecular reactions that take place onto the waveguide surface over the sensing area change the effective waveguide index and higher order modes appear after the second Y-junction. Provided the waveguides are monomodal, the higher modes diffuse away. Thus, the fundamental mode photon flux detected at the end of the waveguide through an external photodetector provides a monitor of the adlayer growth on the sensing arm.

The majority of the SW-MZI devices that are employed in biosensing applications are fabricated on Si wafers with standard microelectronic processes and offer very high sensitivity that is defined mainly by the sensing arm length [46]. However, the light that is coupled to the MZI structure is provided by an external laser source, and thus size, cost, and ease of use issues of the measuring apparatus should be considered. In order to overcome the limitations of SW-MZI concept, we have recently demonstrated that MZI can also operate in Broad-Band scheme. Here, white light is fed at the input of the interferometer and on the output the spectral shifts are monitored. This particular implementation, Broad-Band Mach–Zehnder Interferometry (BB-MZI), provides significant advantages over SW-MZI since it solves the issues of phase ambiguity and signal fading [47]. Furthermore, in a recent publication, we have demonstrated that BB-MZI concept can be implemented on monolithically integrated transducers where the planar silicon nitride waveguides are self-aligned with silicon avalanche LEDs emitting in the visible and near IR spectral region [48].

In the present work, we evaluate the bioanalytical performance of the monolithically integrated silicon BB-MZI sensors through the development of a label-free biosensor for real-time detection of bovine milk in goat milk. The proposed biosensor consists of ten BB-MZIs, each one of them coupled with its own silicon LED, all integrated on the same chip of 34 mm^2 area (Fig. 1a). The bottom and the top claddings are silicon dioxide layers. The top cladding layer is removed over the 600-μm-long straight sensing arm (sensing area) to allow

for the analytical personalization of the chip through coating of recognition biomolecules on the exposed arms on each of the ten interferometers (Fig. 1b). An image of the chip with the fluidic module on top for the supply of reagents and the samples is presented in Fig. 1c. The transmitted light at the output of the BB-MZIs is monitored by an external spectrometer that is synchronized with the operation of the LEDs through an electrical- and computer-controlled multiplexer. This way, all ten sensors on the chip could be measured with the same spectrometer. The BB-MZI transducers were converted to biosensing elements by functionalizing the sensing arm with bovine k-casein and the determination of bovine milk content in goats' milk was achieved following a competitive immunoassay format by passing over the sensor mixtures of the samples with monoclonal anti-k-casein antibodies. The analytical characteristics of the sensor were compared to those of a competitive ELISA developed using the same antibodies.

Experimental

Reagents

Two mouse monoclonal antibodies against k-casein (clones 33-4G10 and 33-6A10) were provided by RIKILT (Wageningen, The Netherlands). k-Casein from bovine milk (MW approx. 19,000 Da), bovine serum albumin (BSA), anti-mouse IgG-HRP conjugate, 3-aminopropyltriethoxysilane (APTES) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-

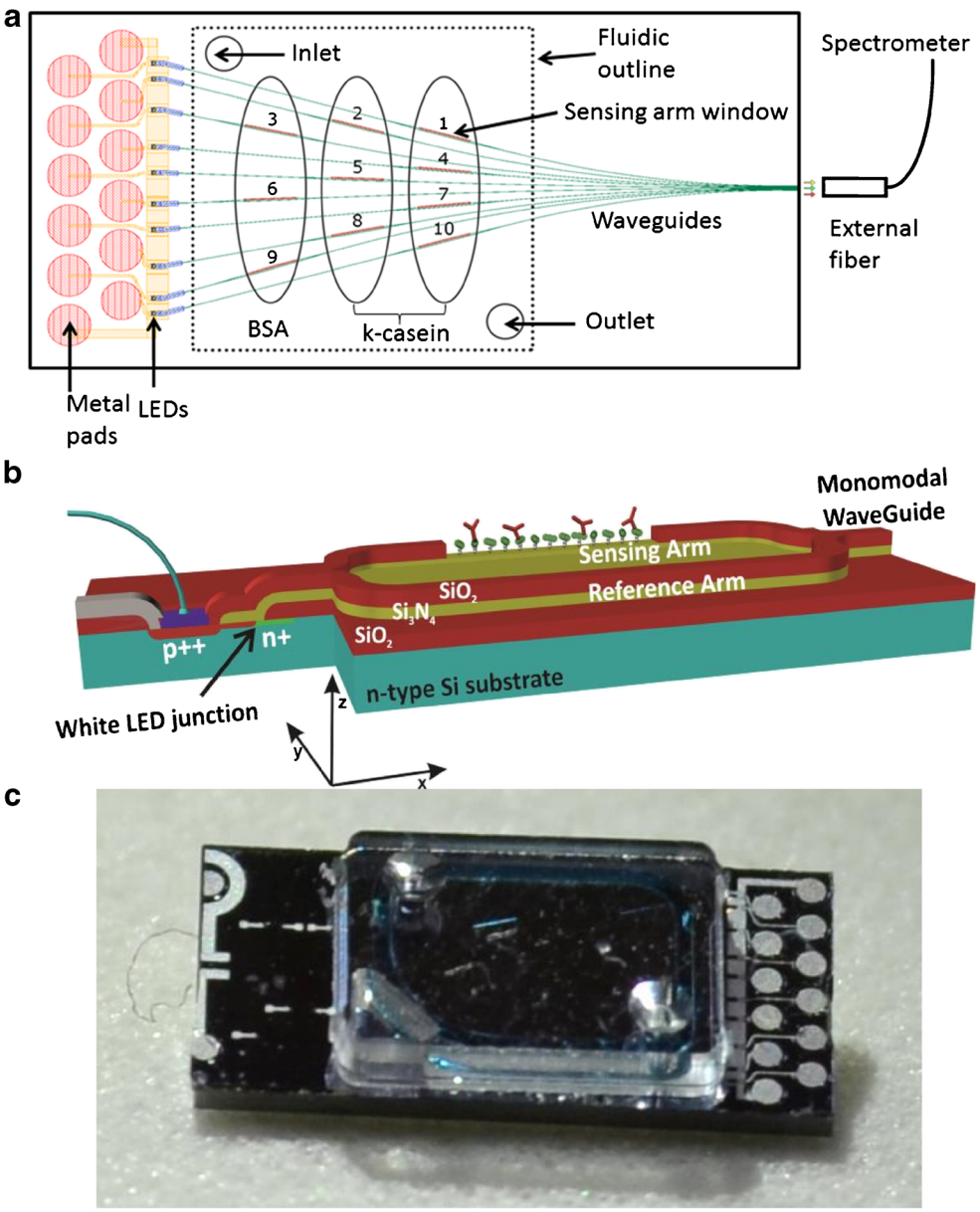

Fig. 1 (**a**) Schematic presentation of the silicon chip accommodating ten BB-MZI structures. Each BB-MZI structure is coupled to an LED (*at the left*). The output waveguides from all BB-MZIs converge in parallel near the emitting right edge. The spectra emitted from all BB-MZIs are measured by an external spectrometer through a pre-aligned fixed optical fiber. The fluidic module outline is also indicated (*dashed line*) along with the fluid inlet and outlet. The fluidic interfaces are outlined in ref. [48]. For the particular assay, seven out of the ten BB-MZIs are spotted with k-casein (specific signal) whereas the remaining three were coated with bovine serum albumin (non-specific binding). (**b**) 3-D representation of the BB-MZI device emphasizing the precise placement of the light-emitting junction right under the up-going segment of the silicon nitride waveguide (*yellow*). This placement is a result of fabrication processes that guarantee self-alignment of the emitting junction to the waveguide both in *X* and *Y* directions. The light emitter is a silicon avalanche diode that is known to emit light when reverse biased beyond its breakdown voltage. (**c**) Image of the chip with the fluidic on top

sulphonic acid) (ABTS) were purchased from Sigma-Aldrich (Darmstadt, Germany). Microtitration plates were from Greiner Diagnostic GmbH (Bahlingen, Germany). All other chemicals and reagents were obtained from Merck (Darmstadt, Germany). The water used throughout the study was doubly distilled.

Fabrication of the optoelectronic chips and output signal processing

The monolithic optoelectronic chip (Fig. 1c) is fabricated with mainstream silicon technology and integrates arrays of silicon avalanche diode LEDs along with optically coupled silicon nitride waveguides [41, 49]. The LEDs are silicon avalanche diodes that emit white light, when biased beyond their breakdown voltage. The light emission of the LED is confined in a very narrow region which makes possible high coupling efficiencies in such optocouplers in association with self-alignment techniques that place the emitting junction right under the waveguide front edge. The SiO_2 spacer next to the LED allows for the smooth waveguide bending from vertical to horizontal orientation and minimizes bending losses. The bottom and top silicon dioxide cladding layers have thickness of 3 and 2 μm, respectively. The top cladding area over the straight section of the sensing arm is etched away and is appropriately functionalized for the detection of the targeted analyte. The 150-nm-thick silicon nitride layer is patterned to a set of ten Mach–Zehnder interferometers each coupled to a silicon LED. The ten waveguides, after the second Y-junction, converge at the edge of the chip where an external fiber couples the emitted spectra to a miniaturized spectrometer (QE65000, Ocean Optics) (Fig. 1a). In Fig. 2, the output spectra from a MZI coated with 100 μg/mL k-casein received prior to (black line) and after reaction (red line) with the zero calibrators are presented. The output spectrum contains nearly sinusoidal oscillations characteristic of the two polarizations (TE and TM). The signal processing is done through Discrete Fourier Transform and in the wavenumber domain the spectra are transformed into two sharp peaks the phase of which provides the observable [49]. A 2π radian shift implies a spectral shift by a full period. All the output signal graphs presented in this work refer to spectral shifts in TE polarization.

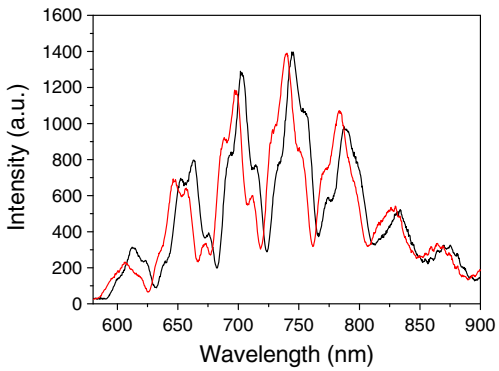

Fig. 2 Output spectra, as recorded by the spectrometer, from a BB-MZI coated with 100 μg/mL k-casein received prior to (*black line*) and after reaction (*red line*) with the zero calibrator. The adlayer growth causes a blue shift. There are two apparent oscillation frequencies: A higher for the TM polarization and a lower for the TE polarization [49]

Chemical and biological activation of the chips surface

Prior to activation, the silicon chips were cleaned and hydrophilized by O_2 plasma treatment (10 mTorr) for 30 s. Then, they were immersed for 2 min in a 0.5 % (*v*/*v*) APTES solution followed by gentle washing with water, drying under a nitrogen stream, and heating at 120 °C for 20 min [50]. For the biological activation of the chip, seven out of ten MZIs on each chip (Fig. 1a) were spotted with 100 μg/mL k-casein solution in 10 mM carbonate buffer, pH 9.2 (coating buffer), whereas the remaining three were spotted with 100 μg/mL BSA solution in the same buffer using the BioOdyssey Calligrapher Mini Arrayer (Bio-Rad Laboratories, Inc.). To ensure full coverage of the sensing areas with biomolecules, five lines of three spots each with a spot-to-spot distance of 120 μm were deposited onto each sensing arm using a solid pin with a 375-μm tip diameter. After spotting, the chips were incubated in a controlled humidity chamber (75 % humidity) overnight at 4 °C. Then, the chips were washed with 10 mM phosphate buffer, pH 7.4 (washing buffer), blocked with 100 times diluted raw goat milk in 10 mM phosphate buffer, pH 7.4, for 1 h, washed again as previously and dried under a nitrogen stream. Dried chips were used either immediately or kept at 4 °C in a desiccator until use.

Milk samples

Raw goat milk (n=12) and raw bovine milk (n=7) samples were collected from 14 farms located at different areas of South and Central Greece (Attika, Thessaly, Peloponnese). Raw ewe's milk was also obtained from three small farms located in the Attica region. Sodium azide (0.05 %, *w*/*v*) was added to the samples, which were transferred to the laboratory under cooled conditions, and stored in 10-ml portions at −20 °C for up to 6 months. Prior to analysis, the thawed samples were brought at room temperature (RT) and sonicated for 15 min. Pasteurized and highly pasteurized goat and bovine milk from different milk companies (DELTA FOOD S.A., EVOL The Union of Volos Agricultural Cooperatives, Olympos Larissa Milk Company S.A.) were purchased by local super markets.

Assay for the detection of bovine milk in goat milk with the MZI immunosensor

To perform the assay, the chips were covered with appropriate microfluidic modules to allow for the delivery of the samples to be analyzed (Fig. 2). The fluidic module is made of 800-μm-thick poly(methyl methacrylate) (PMMA) foil with milled fluid inlet and outlet ports (Electronic Supplementary Material (ESM), Fig. S1). The actual flow-cell is formed through a photopatterned 75-μm-thick dry photoresist film that is laminated onto the milled PMMA foil. This photoresist layer forms a pressure sensitive adhesive seal also acting as spacer between the chip and the PMMA. The fluidic module is attached onto the chip either manually or at wafer-level scale. The encapsulated chip was placed on a handling-frame and then inserted in the docking station of the benchtop measuring apparatus which provides both the electrical and fluidic connections (ESM, Fig S2). Prior to the assay, calibrators (0–1 % (*v*/*v*) bovine milk in goat milk) or samples, 50 times diluted in assay buffer (10 mM phosphate buffer, pH 7.4, containing 0.5 % (*w*/*v*) BSA), were mixed at 1:1 volume ratio with a solution containing the two anti-k-casein monoclonal antibodies (33-4G10 and 33-6A10) at 1 μg/mL each in assay buffer (final concentration of 0.5 μg/mL each), and preincubated for 15 min. After the equilibration of the chip with 100 times diluted goat milk in assay buffer, the mixtures of the calibrators or the samples with the antibodies were passed over the chip for 10 min with a flow rate of 40 μL/min (total sample volume 400 μL, corresponding to 4 μL of undiluted milk). The transmission spectra of all ten BB-MZIs are recorded in a period of 10 s with 1 s to be the integration time per BB-MZI. The signals are calculated after Discrete Fourier Transform of the output spectra as spectral shifts in TE polarization prior to and after reaction. For the preparation of the calibration curve, the percent ratios of the calibrators signal (S_x) to the zero calibrator signal (S_0) were plotted against the bovine milk content in goat milk.

ELISA procedure for the detection of bovine milk in goat milk

Microtitration wells were coated with 100 μL/well of a 1 μg/mL k-casein solution in coating buffer overnight at RT. Then, the wells were washed twice with 300 μL of washing buffer, blocked with 300 μL of 1 % (*w*/*v*) BSA solution in 0.1 M $NaHCO_3$, pH 8.5, for 1 h and washed twice with washing solution. Equal volumes of calibrators or samples 50 times diluted in assay buffer and antibodies solution containing the two anti-k-casein monoclonal antibodies, at 40 ng/mL each in assay buffer, were mixed and preincubated for 15 min. The assay was performed by adding 100 μL of the mixtures in each well and incubated under shaking for 1 h. Then, the wells were washed four times with washing solution and 100 μL of a 5 μg/mL anti-mouse HRP conjugate in 0.1 M Tris–HCl buffer, pH 8.25, containing 0.5 % (*w*/*v*) BSA, and 0.9 % (*w*/*v*) NaCl were added per well and incubated for 30 min at RT. After washing the wells four times with washing solution, 100 μL of H_2O_2/ABTS chromogenic substrate solution were added to each well and the optical density was measured at 405 nm after 30 min of incubation.

Results and discussion

Immunosensor development

Two different immunoassay configurations could be employed for the determination of analytes with the BB-MZI immunosensor; the direct one, involving anti-k-casein antibodies immobilization onto the BB-MZIs, and the competitive one where k-casein is immobilized on the sensing area and competes with the k-casein in the samples for a limited number of binding sites of the anti-k-casein antibodies in solution. The direct format is simpler and ideally could provide measurable signal by passing only the sample over the sensor. The magnitude of the signal in this case is proportional to the concentration of the analyte in the sample. In the competitive assay format, mixtures of antibody with the sample are run over the sensor and the maximum signal depends on the antibody concentration used. Both assay formats have been tested in the BB-MZI immunosensor. From preliminary experiments, it was found that chips modified with k-casein were more stable under dry storage conditions compared to chip modified with anti-k-casein antibodies and more robust with respect to chip regeneration. Since similar analytical sensitivities were obtained following the two immunoassay formats, the competitive one was selected for further investigation based on the immobilized k-casein stability and robustness.

To select the optimum k-casein concentration for immobilization onto the sensor surface, chips were spotted with solutions ranging from 5 to 200 μg/mL in coating buffer and assayed using a fixed antibody concentration (1 μg/mL of each monoclonal in the final assay mixture). As it is shown in Fig. 3, by increasing the k-casein concentration, the sensor response was increased and reached a maximum at concentrations equal to or higher than 100 μg/mL. Concerning the repeatability of the responses of the different BB-MZIs within the same chip (7 BB-MZIs functionalized with k-casein per chip), it was found that the coefficients of variation (CV) ranged from 35 to 15 % for k-casein concentrations ranging from 5 to 50 μg/mL, respectively. On the other hand, the within and between chips CVs of the responses received from chips spotted with 100 and 200 μg/mL k-casein concentration were in all cases less than 10 %. The quite lower dispersion of the output signals obtained using the higher coating concentrations could be ascribed to more homogeneous coverage of the sensing arm area with k-casein

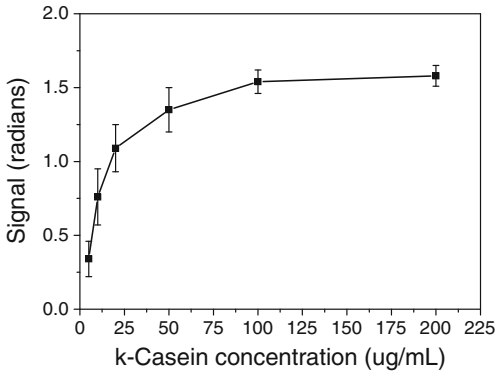

Fig. 3 Net mean signal values for zero calibrator versus the k-casein concentration used for coating. Each point is the mean value of seven BB-MZIs per chip; *Error bars* represent ± SD

molecules as compared to those obtained using lower coating concentrations. For this reason, a 100 μg/ml k-casein solution was used for further experiments.

Different blocking solutions including: (a) 1 % (w/v) BSA in 0.1 M $NaHCO_3$, pH 8.5, (b) 0.5 % (w/v) gelatin in 50 mM phosphate buffer, pH 7.4, or (c) 1 % (*v*/*v*) goat milk in 10 mM phosphate buffer, pH 7.4, have been tested with respect to zero calibrator signal and the elimination of non-specific binding as well. It was found that using gelatin as blocking agent there was a slight increase of the response of the 3 BB-MZIs spotted with BSA (non-specific signal) whereas the signal from the 7 BB-MZIs coated with k-casein was marginally decreased (~15 %) compared with the signals obtained from chips blocked using the other two blocking solutions. In addition, using these blocking agents very similar zero calibrator signals were obtained and no increase of the non-specific signal was observed. However, since equilibration of the chip was achieved quite faster (2–3 min) when 1 % (*v*/*v*) goat milk was used for blocking with respect to the blocking solution containing BSA (more than 10 min), blocking with 1 % (*v*/*v*) goat milk was selected for the final protocol.

Concerning the assay buffer, 10 mM phosphate, pH 7.4, containing 1 % (*v*/*v*) BSA was selected since it provided the highest zero calibrator signal compared to several other buffers tested.

Selection of the matrix for the preparation of calibrators

In order to select the most appropriate matrix for the calibrators preparation, several raw goat milk samples (n=12) from different farms as well as commercially available pasteurized (n=1) and highly pasteurized milk samples (n=2) were tested with respect to the signal they provided as zero calibrators. It was found that all milk samples tested provided very similar signals (n=15; CV=3.2). However, due to the seasonal availability of pasteurized goat milk in the market (September–March), raw goat milk which was kept frozen at −20 °C was selected as matrix for calibrators' preparation.

Effect of milk dilution

Milk is a complex biological fluid with high opacity due to the suspending particles of fat, proteins, and certain minerals that may affect signal response especially when measurements are based on evanescent wave interactions with the sample in the vicinity of the waveguide surface. In addition, its components could affect antibody-antigen reaction. For this reason, a range of goat milk dilutions (1/5–1/100) in assay buffer were tested. In Fig. 4, mean zero calibrator signals, the signals provided by a calibrator containing in all cases 0.005 % (*v*/*v*) bovine milk in goat milk after dilution, as well as the respective non-specific binding signals obtained from chips assayed with different goat milk dilutions are presented. Similar responses, without trend related to the milk dilution, were obtained in all cases both for the zero calibrator and the calibrator spiked with bovine milk. In addition, negligible non-specific binding signal was determined for all dilutions. Since the milk dilution does not affect the assay performance, for the analysis of real samples different dilutions could be employed to determine the bovine milk content. For the calibrators with bovine milk content ranging from 0 to 1 % (*v*/*v*), 50 times dilution was selected.

Optimization of the assay duration and antibodies concentration

The assay duration was optimized with respect to zero calibrator's signal. As it is shown in Fig. 5, using an antibodies solution at 1 μg/mL each, a sharp linear signal increase is observed for the first 15 min after the introduction of the zero

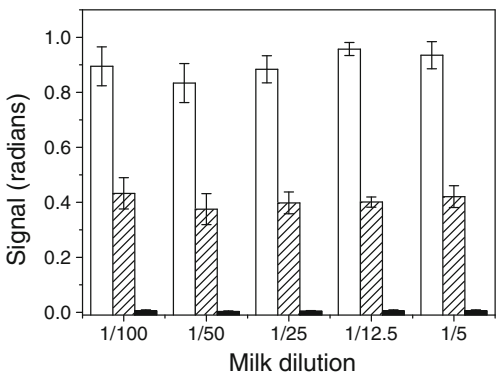

Fig. 4 Mean signal values for zero calibrator (*white bars*), a calibrator containing in all cases 0.005 % *v*/*v* bovine milk in goat milk after dilution (*hatched bars*), and the respective non-specific binding signals (*black bars*) obtained from chips assayed with different goat milk dilutions. *Bars* represent ± SD (n=7 for the specific signal, n=3 for the non-specific binding signal)

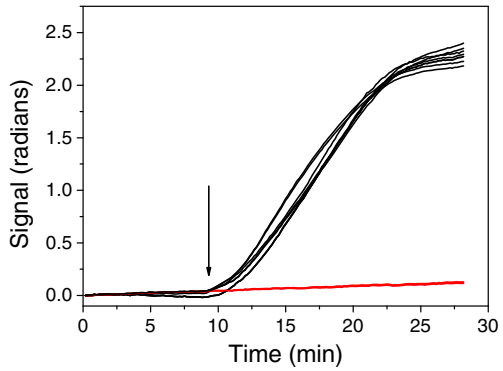

Fig. 5 Signal evolution obtained when running over the chip for 20 min a mixture of fifty times diluted goat milk with the two antibodies at 1 μg/ml final concentration each. The *arrow* indicates the introduction of the calibrator/antibodies mixture to the fluidic cell. *Red lines* represent responses from sensors coated with BSA (non-specific binding) while *black lines* responses from sensors coated with k-casein

calibrator/antibodies mixture. Marginal signal increase was obtained by further increasing the reaction time. However, after 10 min of reaction adequate signal was obtained (~70 % of the maximum), and thus, this reaction time was selected for the final application so as to shorten the duration of the analysis.

The effect of pre-incubation of calibrators with the antibodies solutions on the sensitivity and the dynamic range of the assay as well as on the run-to-run calibration curve repeatability was investigated. In Fig. 6, calibration curves obtained without pre-incubation or with 15 and 30 min pre-incubation of the mixtures of the calibrators with the antibodies, at 1 μg/mL each final dilution, are presented. The 15 and 30 min pre-incubation time resulted in superimposed calibration curves with significantly higher detection sensitivity compared to the calibration curve obtained without pre-incubation. Furthermore, the pre-incubation step improved considerably the run-to-run repeatability of the calibration curve. This improvement could be

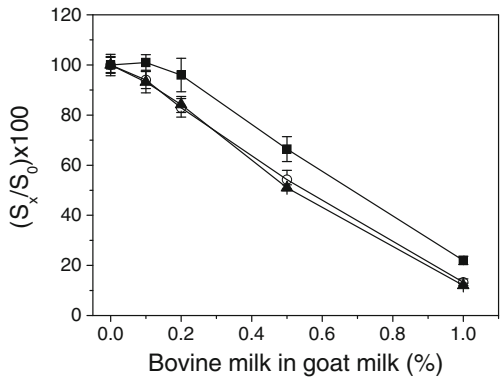

Fig. 6 Calibration curves obtained without (*filled square*) or with 15- (*filled upright triangle*) and 30-min (*empty circle*) pre-incubation of the calibrators with the antibodies solution. $(S_x/S_0)\times 100$ represents the percent ratio of each calibrator signal (S_x) to the zero calibrator signal (S_0). Each point is the mean value of 3 chips ± SD

ascribed to the elimination of time variations between the preparation of the different calibrators' mixtures with the antibodies and their introduction to the chip. Thus, a 15-min pre-incubation step of the calibrators or the samples with the antibodies was adopted in the final assay protocol.

Another important parameter optimized was the antibodies concentration. Several combinations of the two monoclonal antibodies concentrations have been tested so as to select the one providing adequate signal, high detection sensitivity, and the required dynamic range (up to 1 %). It was found that more sensitive calibration curves were obtained when the antibodies were used at a 1:1 concentrations ratio. In Fig. 7a and b, sensor responses provided by passing mixtures of different calibrators with the two antibodies at final concentrations of 1 and 0.5 μg/mL each, respectively, are presented. As it is shown, using the antibodies at final concentration of 0.5 μg/mL each, adequate signal (approximately 1 rad in 10 min) was obtained, although it was 40 % lower compared to that received using the antibodies at 1 μg/mL. However, as it is indicated in Fig. 7c, where the respective calibration curves are provided, the sensitivity of the assay obtained with the lower antibodies concentration was improved approximately 2.5 times compared to that obtained with the higher antibodies concentration without compromising the assay dynamic range. Therefore, for the assay the antibodies were used at a final concentration of 0.5 μg/mL each.

For the optimization of the assay conditions raw goat and bovine milk was used. Nevertheless, as it is mentioned above, raw, pasteurized, and highly pasteurized goat milk could be also used as matrix for calibrators' preparation. Since, the monoclonal antibodies employed in the present study are appropriate for the detection of raw and heat-treated bovine milk in milk of other species [51], raw, pasteurized, and highly pasteurized bovine milk are expected to perform similarly when used for the preparation of calibrators. Indeed, as it is shown in ESM Fig. S3, superimposed calibration curves were obtained using calibrators prepared by addition of raw, pasteurized, and highly pasteurized bovine milk in goat milk, either raw or pasteurized.

Moreover, the potential of the immunosensor to detect bovine milk in ewes' milk was investigated. Calibration curves obtained using as matrix for the calibrators sheep milk from three different farms spiked with raw bovine milk are provided in ESM, Fig. S4. As it is shown, the curves obtained using ewes' milk as matrix are very similar to those obtained when goat milk was used, indicating that the immunosensor developed could detect fraudulent addition of bovine milk to both ewes' and goats' milk.

Analytical characteristics of the immunosensor

The limit of detection (LOD) of the assay was determined as the concentration corresponding to signal equal to −3SD of

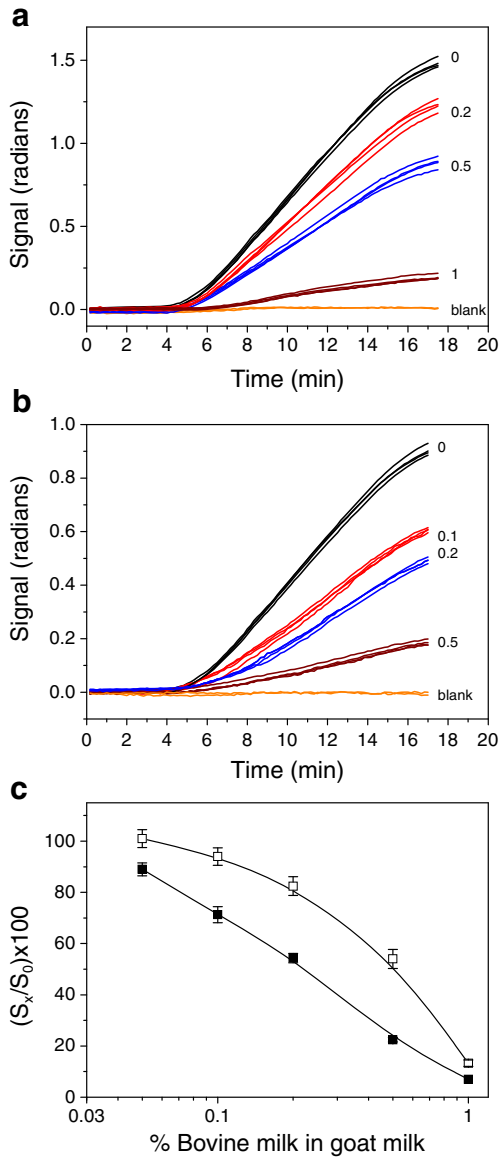

Fig. 7 Sensor responses when passing mixtures of different calibrators with the antibodies at 1 μg/mL (**a**) or 0.5 μg/mL (**b**) final concentration each, for 10 min. The signals of four different MZIs for each calibrator are illustrated (two MZIs for non-specific binding). (**c**) Calibration curves obtained with the antibodies at 1 μg/mL (*open symbols*) or 0.5 μg/mL (*closed symbols*). $(S_x/S_0)\times 100$ represents the percent ratio of each calibrator signal (S_x) to the zero calibrator signal (S_0). Each point is the mean value of seven waveguides per chip ± SD

the mean zero calibrator signals (21 replicate values from 3 chips; 7 MZIs per chip) and found to be 0.04 % (*v*/*v*). The working range of the assay was from 0.1 to 1 % (*v*/*v*) of bovine in goat milk, with an IC_{50} value of 0.22 % (*v*/*v*). Thus, the proposed immunosensor is appropriate for the detection of adulteration of goat milk since it can easily detect bovine milk at concentrations lower than 1 % (*v*/*v*), which according to EC Directives is the threshold for characterization of a cheese as originating from species other than bovine [5, 6].

Using the same antibodies, an ELISA method was also developed. This assay had an LOD of 0.004 % (*v*/*v*) of bovine milk in goat milk, and a dynamic range up to 0.5 % (*v*/*v*) (see ESM, Fig. S5) when the calibrators were used at the same dilution (50 times) as in the immunosensor. Thus, it was ten times more sensitive compared to the immunosensor; however, the duration of the ELISA was more than 2 h, whereas the time required for the assay in the immunosensor was 10 min (25 min including pre-incubation of the samples with the antibodies). Therefore, due to the short analysis time and the simplicity of the procedure (one step), the immunosensor developed is suitable for on-site determinations as opposed to ELISA (longer analysis time, three-step procedure).

Since the monoclonal antibodies used in this study have been raised against bovine k-casein [42], we evaluated also the analytical performance of the immunosensor developed in terms of k-casein determination. For this purpose, k-casein calibrators were prepared in 50 times diluted goat milk and assayed with the protocol applied for the determination of bovine milk. The calibration curve obtained is presented in Fig S6 (ESM). The LOD determined was 0.06 μg/mL, the IC_{50} was 0.45 μg/mL, and the dynamic range was up to 1 μg/mL.

Recovery experiments were performed by analyzing raw, pasteurized, and highly pasteurized goat milk samples (15 in total) spiked with known amounts of raw bovine milk (0.25, 0.75, 2, and 10 (*v*/*v*) %). In Table 1, the mean values (±SD) obtained from the 15 spiked samples along with the % recovery values are provided. The recovery values ranged between 93 and 110 % indicating high accuracy of the immunosensor developed.

The repeatability of the sensor was tested by running three control samples, i.e., raw goat milk spiked with 0.15, 0.3, and 0.75 % (*v*/*v*) bovine milk, which were aliquoted and kept frozen at −20 °C until use. The CVs of the concentrations determined from the different BB-MZIs of the same chip was less than 7 %, whereas the CVs of the mean concentrations determined using different chips run on the same day did

Table 1 Recovery of known amounts of bovine milks spiked in 12 different raw, 1 pasteurized and 2 highly pasteurized goat milk samples

Bovine milk added (%)	Bovine milk determined (%)	Recovery (%)
0	0.02±0.01[a]	
0.25	0.24±0.01	96.0±4.2
0.75	0.78±0.05	104±6.4
2.0	2.2±0.15[b]	110±6.8
10	9.3±0.85	93.0±9.1

[a] Mean values ± SD of all 15 samples

[b] Samples containing higher than 1 % (*v*/*v*) bovine milk were diluted appropriately so as the concentration to fall within the range of the calibrators

not exceed 12 %. Chips run on different days over a period of 1 month provided CVs up to 15 %.

Stability and regeneration of the immunosensor

Chips spotted and dried as described in "Materials and methods" were tested for a 1-month period and found to be stable. In particular, the mean signal value obtained from the different BB-MZIs of the chips ranged from 0.86 to 1.03 rad (see ESM, Fig. S7).

Chip regeneration is important for portable devices since it could reduce significantly the analysis cost. The potential of the chip to be regenerated was evaluated by performing repetitive assays with the zero calibrator over a period of 3 days. It was found that complete regeneration was achieved by running over the chip surface a 0.1 M glycine-HCl buffer, pH 2.5, for 5 min after immunoreaction. In Fig. 8, the mean values of the zero calibrator signals obtained from 7 BB-MZIs of chip regenerated several times in a 3 days period are presented. As it is shown, the chip could be regenerated for at least 12 times in a period of 2 days without any signal loss. In the third day, a 20 % signal reduction was observed compared with the signals obtained in the first 2 days; however, it should be noted that at the end of each day the sensor was washed with distilled water, dried, and kept at room temperature. Thus, the observed signal loss could be ascribed, besides to regeneration, to repetitive washing with water and drying.

Comparison with other immunosensors

In Table 2, the published LOD values and assay times are listed for immunosensors dealing with the determination of bovine milk and k-casein. The respective values of the immunosensor developed are also included in this table for comparison. In terms of k-casein determination, the proposed immunosensor with 10-min assay time is nine times more sensitive compared to the SPR immunosensor presented in ref. [37] with the same assay duration. The monoclonal antibodies employed in the present study are appropriate for the detection of raw and heat-treated cow and buffalo milk in milk of other species and sources [51]. In refs. [38] and [42], these antibodies have been employed for the determination of bovine milk in goats' and ewes' milk using a Spreeta-based biosensor and an SPR immunosensor using the Biacore 3000 instrument, respectively. Compared to the Spreeta-based biosensor with a run time of 7 min, the proposed immunosensor is four times more sensitive while it has comparable sensitivity with the SPR immunosensor which, however, is faster (5-min assay).

Table 2 Comparison of the proposed immunosensor with other literature immunosensors for determination of bovine milk and k-casein

Immunosensor/ device	Analyte	Analysis time	LOD	Ref. #
SPR immunosensor	Bovine k-casein in milk samples	10 min	0.45 μg/mL	[37]
Spreeta-based biosensor immunoassay	Bovine milk in ewes and goats milk	7 min	0.17 % (*v*/*v*)	[38]
SPR immunosensor	Bovine milk in ewes and goats milk	5 min	<0.1 % (*v*/*v*)	[42]
Immunosensor developed	Bovine milk in goats milk	10 min	0.04 % (*v*/*v*)	
	Bovine k-casein in goats milk		0.06 μg/mL	

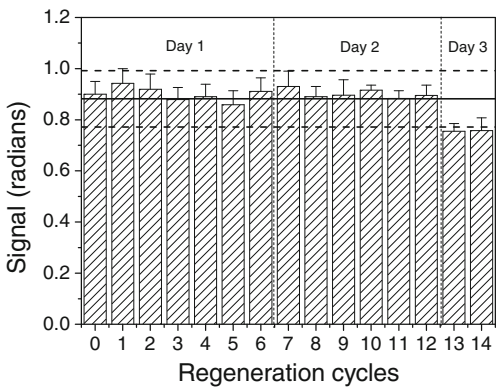

Fig. 8 Zero calibrator signals obtained from a single chip after repetitive regeneration steps over a period of 3 days. *Columns* represent mean values of 7 BB-MZIs; *error bars* represent ±SD. *Horizontal solid line* corresponds to the mean value of the 14 measurements; *dashed lines* refer to ±2SD

Conclusions

A miniaturized biosensor for the detection of bovine milk in goat milk was developed. The monolithic silicon interferometric chip provided label-free and real-time detection and quantification of bovine milk in goat milk using monoclonal anti-k-casein antibodies. The validation of the competitive immunoassay applied to the microchip indicated high sensitivity, accuracy, and reproducibility. The LOD which was achieved was low enough with respect to the required LOD according to the EC Directives for the detection of goat milk adulteration. In addition, the proposed immunosensor could be used for the determination of k-casein in bovine milk in order to evaluate the milk quality with respect to its nutritional value and cheese production as well. The silicon chip accommodates ten Broad-Band Mach–Zehnder Interferometric sensors and therefore could be employed for the simultaneous detection of multiple analytes after personalization of each sensing area with different recognition biomolecules. The high integration level and the small size of the sensor allows for the

potential realization of a portable measuring apparatus for fast on-site detection of fraudulent contamination of goat milk with bovine milk as well as in diverse applications regarding milk quality assessment.

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