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Kappa-casein based electrochemical and surface plasmon resonance biosensors for the assessment of the clotting activity of rennet

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ABSTRACT

We report for the first time the development of *kappa*-casein (κ-CN)-based electrochemical and surface plasmon resonance (SPR) biosensors for the assessment of the clotting activity of rennet. Electrochemical biosensors were developed over gold electrodes modified with a self-assembled monolayer of dithiobis-*N*-succinimidyl propionate, while SPR measurements were performed on regenerated carboxymethylated dextran gold surfaces. In both types of biosensor, κ-CN molecules were immobilized onto modified gold surfaces through covalent bonding. In electrochemical biosensors, interactions between the immobilized κ-CN molecules and chymosin (the active component of rennet) were studied by performing cyclic voltammetry, differential pulsed voltammetry, and electrochemical impedance spectroscopy (EIS) measurements, using hexacyanoferrate(II)/(III) couple as a redox probe. κ-CN is cleaved by rennet at the Phe105–Met106 bond, producing a soluble glycomacropeptide, which is released to the electrolyte, and the positively charged insoluble *para*-κ-casein molecule, which remains attached to the surface of the electrode. This induced reduction of the net negative charge of the sensing surface, along with the partial degradation of the sensing layer, results in an increase of the flux of the redox probe, which exists in the solution, and consequently, to signal variations, which are associated with the increased electrocatalysis of the hexacyanoferrate(II)/(III) couple on the gold surface. SPR experiments were performed in the absence of the redox probe and the observed SPR angle alterations were solely attributed to the cleavage of the immobilized κ-CN molecules. Various experimental variables were investigated and under the selected conditions the proposed biosensors were successfully tried to real samples. The ratios of the clotting power units in various commercial solid or liquid samples, as they are calculated by the EIS-based data, were almost identical to those obtained with a reference method. In addition, EIS measurements showed an excellent reproducibility, lower than 5%.

1. Introduction

Rennet is a natural complex of enzymes (chymosin and pepsin) produced in any mammalian stomach, and it is widely used in cheese making industry as the major milk coagulant [1,2]. The milk-clotting activity of rennet relies on its ability to degrade casein micelles, which are composed of four casein fractions and among them, *kappa*-casein (κ-CN) at a percentage of 12% [3]. During the rennet coagulation of milk, chymosin cleaves κ-CN at the Phe105–Met106 bond producing the soluble glycomacropeptide (GMP, residue 106–169), which diffuses away from the micelle into the serum phase, and the positively charged (p*I* > 7) insoluble *para*-κ-casein molecule, which remains attached to the casein micelle [1,4,5]. This results in a reduction of the net negative charge of micelles, as well as, of the electrostatic repulsion among them. As a consequence, rennet-altered micelles become susceptible to aggregation [6].

The evaluation of the clotting power of rennet has always been a parameter of great importance from scientific, technological and commercial viewpoints. Since the first published method, based on visual observation of the formation of a clot [7], a number of methods, which also based on the visual observations of the coagulation point of milk, have been used to measure rennet activity [8–10]. Some of the drawbacks of these methods, which are associated with the subjective nature of the observation, have been adequately overcome by the IDF Standard 157A:1997 method [11].

Other methods based on blood clot timers [12], viscosity measurements [13–15], or measurements of near infra-red attenuation

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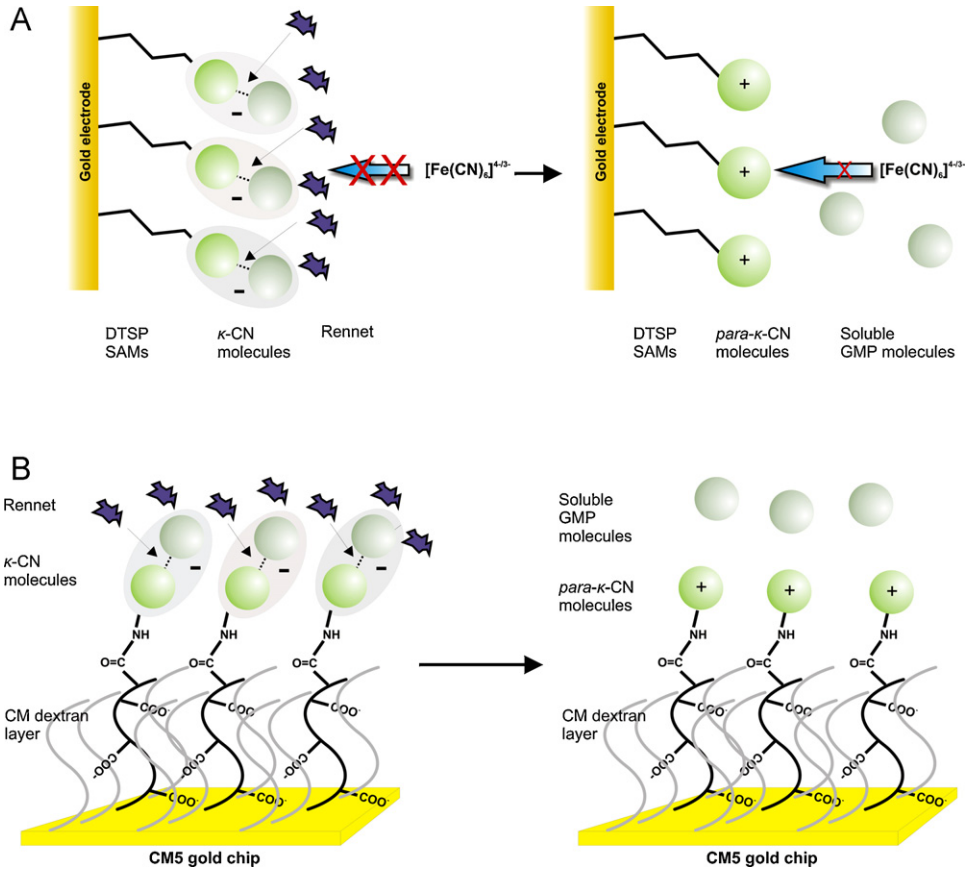

Fig. 1. Idealized view of (A) Au/DTSP/κ-CN electrodes and (B) CM5/κ-CN gold chips before and after their interaction with rennet.

through a milk sample during its coagulation [16] have also been proposed. In addition, spectrophotometric measurements [17], measurements of the electric conductivity [18] and a turbidity method [19] have also been proposed. Recently, a faradic impedimetric biosensor based on immobilized artificial casein micelles (ACM) was also proposed by our group [20].

Aiming to simplify the construction, and to improve the performance of the above biosensor, our endeavour was focused to redesign the sensing layer by immobilizing κ-CN, that is, the "active" component of milk micelles (Fig. 1). Using κ-CN as a sensing layer we succeeded to overcome problems associated with the short storage stability of the ACM stock suspension (one week) and the ACM size-dependent response of the resulting biosensors. Most importantly, a remarkable improvement of the reproducibility of the measurements from 9–16 down to <5% was achieved, thus meeting one of the most important criteria for a biosensor to pass from an academic concept to routine analysis. In this regard, lowering of the required incubation time from 15 down to 5 min is also considered as an important analytical feature of the newly proposed biosensors. In addition, the performance of the proposed biosensors at commercial solid or liquid samples was also investigated by employing different transduction techniques, including electrochemical impedance spectroscopy (EIS), differential pulse voltammetry (DPV), and surface plasmon resonance (SPR).

2. Experimental

2.1. Chemicals and solutions

Dithiobis-*N*-succinimidyl proprionate (DTSP), κ-casein (lyophilized powder from bovine milk, ≥70% κ-casein), Pronase E (Type XIV from *Streptomyces griseus*), and surfactant P20 were purchased from Sigma. Potassium ferrocyanide and potassium ferricyanide were from Merck. Sodium dihydrogen phosphate monohydrate, *N*-hydroxysuccinimide (NHS), bromoacetic acid, imidazole, Hepes, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), were purchased from Fluka. All other chemicals were from Merck and Sigma and double distilled water (DDW) was used throughout. Solutions of rennet were freshly prepared before use by dissolving the appropriate amounts of dry powder of calf and/or bovine rennet (Ipirotopoula, Greece; Chr. Hansen, Denmark) in a 20 mM solution of imidazole pH 5–6.5. Rennet samples in liquid form (Chr. Hansen; Danisco, Denmark) were mixed with a 1 + 3 volume of 26.7 mM imidazole and the pH of the mixture was adjusted to 5 with 0.1 M HCl.

2.2. Apparatus

EIS, DPV and cyclic voltammetry (CV) experiments were performed with the electrochemical analyzer PGSTAT12/FRA2 (Metrohm Autolab) in a one-compartment three electrode cell. Gold electrodes and a platinum wire were served as the working and auxiliary electrodes, respectively. The reference electrode was a Ag/AgCl/3 M KCl electrode and all potentials hereafter refer to the potential of this electrode. The impedance spectra were recorded over the frequency range 10^{-1}–10^{5} Hz, using a sinusoidal excitation signal, superimposed on a DC potential of +0.200 V. Excitation amplitude of 10 mV (rms) was used throughout. DPV grams were recorded over the potential range from 0.0 to +0.4 V, under the following parameters: modulation amplitude 50 mV, modulation time 60 ms, and interval time 150 ms. CV grams were recorded at a scan rate of 50 mV s^{-1}. All measurements were performed in a solution of 5 mM hexanocyanoferrate(II)/(III) (1 + 1 mixture) in a

50 mM imidazole buffer solution pH 6.5, containing 100 mM KCl, at room temperature.

SPR studies were conducted with a SPR Biacore 3000 (Biacore Life Sciences), equipped with a continuous flow system in which four channels are coupled in series.

2.3. Preparation of electrochemical sensors

Gold electrodes were constructed by using the commercial kit EasyCon (EasyCon Hellas, provided by Metrohm Autolab). Before use, gold electrodes of 2 mm active surface were polished with Al_2O_3 (0.01 μm grain size). Electrodes were electrochemically cleaned in 0.1 M H_2SO_4 by cycling from −0.6 to 1.6 V at a scan rate of 100 mV s^{-1}, until the CV becomes stable (approximately 25 cycles), and sonicated for 3 min in DDW. After this treatment, the electrodes were dipped into a solution of 1 + 1 + 5 (v/v) 25% NH_4OH + 30% H_2O_2 + H_2O for 10 min, washed with DDW, absolute ethanol and finally dried under argon. Gold electrodes were immersed in a solution of 2 mM DTSP in acetone for 2 h, rinsed thoroughly in fresh baths of acetone and dried under argon. Then, they were immersed in 300 μL of 0.5 mg mL^{-1} κ-CN in phosphate buffer pH 5.5 for at least 12 h. Finally, Au/DTSP/κ-CN electrodes were washed (3 × 10 min) to remove the non-chemically bound protein in phosphate buffer pH 5.5 under mild stirring.

2.4. Preparation of SPR gold chips

κ-CN molecules were immobilized onto CM5 (carboxymethylated dextran) Biacore chips, after the regeneration of their surface according to a previously reported method [21]. Used CM5 gold chips were immersed into a solution of Pronase E (1 mg mL^{-1} in 20 mM sodium phosphate, 150 mM NaCl pH 7.2) and allowed to react overnight at ambient temperature. Then, the chips were gently rinsed with DDW and immersed into a freshly prepared solution of 1 M bromoacetic acid dissolved in 2 M NaOH and allowed to incubate overnight at ambient temperature. Finally, they were rinsed with DDW and dried under argon.

Immobilization of κ-CN was accomplished by reaction of free primary amines on protein molecules with an *N*-hydroxysuccinimide ester activated surface. CM5 gold chips were equilibrated with HBS-EP buffer solution (10 mM Hepes, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20, pH 7.4), and then, using the following immobilization sequence routine, a number of solutions were mixed and injected over the surface through the automated robotics unit incorporated in the instrument: (i) equal volumes of 0.05 M NHS and 0.2 M EDC in HBS-EP for the activation of the carboxymethylated dextran, (ii) ligand solution (1 mg mL^{-1} κ-CN in a mixture 1 + 4 (v/v) 100 mM CH_3COONa pH 5.5 + 10 mM sodium acetate pH 4, which then was further diluted with a 10-fold volume of 10 mM sodium acetate pH 4 and filtered, (iii) 0.1 M ethanolamine in HBS-EP for the deactivation of residual NHS-esters, and (iv) HBS-EP solution to remove non-covalently bound ligand. A mobile phase of HBS-EP buffer solution was used throughout the experiments, while temperature was maintained constant at 25 °C, using a thermostat.

2.5. Procedure

Fully functionalized (Au/DTSP/κ-CN) electrodes were incubated in rennet solutions in 20 mM imidazole, pH 5–6.5 for a specified time interval at 25–37 °C, rinsed thoroughly with a 20 mM phosphate buffer solution pH 5.5 and transferred to the measuring cell. In SPR experiments, rennet samples were introduced in the carrier stream through the automated robotic unit.

2.6. Calculations

In EIS, the clotting power of the samples was represented with the relative change of the charge-transfer resistance (R_{ct}, which corresponds to the real impedance component at 0.1 Hz [20]) of the Au/DTSP/κ-CN electrodes, before and after interaction with rennet solution, $\Delta S(\%) = \{[R_{ct}$ (after the immersion to rennet)$\} - R_{ct}$ (before the immersion to rennet)$\}/R_{ct}$ (before the immersion to rennet)$\} \times 100$. In a similar manner, the relative change of the peak current (I_p) in DPV, $\Delta S(\%) = \{[I_p$ (after the immersion to rennet)$\} - I_p$ (before the immersion to rennet)$\}/I_p$ (before the immersion to rennet)$\} \times 100$ was used as a measure of the clotting power of the samples. In SPR, rennet milk-clotting activity was relied on the shift of the SPR angle (RU units) before and after the reaction with rennet.

3. Results and discussion

As illustrated in Fig. 1A, κ-CN is cleaved by rennet at the Phe105–Met106 bond, producing a soluble glycomacropeptide, which is released to the electrolyte, and the positively charged insoluble *para*-κ-casein molecule, which remain attached to the surface of the electrode. This induced reduction of the net negative charge of the sensing surface, along with the partial degradation of the sensing layer, results in an increase of the flux of the redox probe, which exists in the solution, and consequently, to signal variations, which are associated with the increased electrocatalysis of the hexacyanoferrate(II)/(III) couple on the gold surface. SPR experiments were performed in the absence of the redox probe (Fig. 1B) and the observed SPR angle alterations are solely attributed to the cleavage of the immobilized κ-CN molecules.

3.1. Electrochemical biosensors

3.1.1. Immobilization of κ-casein

Carbohydrate free κ-CN consists of 169 amino acids and has a molecular weight of 19 kD [22]. Preliminary experiments for the immobilization of κ-CN molecules were made from solutions of κ-CN in imidazole onto DTSP-modified gold electrodes. DTSP-based SAMs offer certain advantages, namely hydrophilicity and reactivity to primary amino-group containing biomolecules. Most importantly, there is no need to use cross-linkers, simplifying, in this way, the experimental procedure for the immobilization of the sensing layer [23]. Regarding the buffer of immobilization, even though imidazole was found to serve as the most suitable immobilization solution for casein micelles [20], it turned out to be not suitable for the immobilization of κ-CN. At the pH range 5–6, the solubility of κ-CN in imidazole is poor, whereas at pH 7 the resulting biosensors are not functional. On the other hand, the solubility of κ-CN in phosphate buffer was sufficient and the immobilization of κ-CN from such solutions led to the development of quite reproducible biosensors. This was particularly evident for the biosensors occurred at pH 5.5. The dependence of the performance of the biosensors on the pH of the immobilization solution is most likely to be attributed to structural reconformations of κ-CN at the tested pH values and, to the extent possible, its polymerization. In its native state, κ-CN is composed of monomeric polypetide chains and can range from a monomer to multimers, even larger than a decamer, due to the formation of disulfide-interchange linkage between Cys11–Cys11, Cys11–Cys88 and Cys88–Cys88 [24–26]. Trying to rationalize the performance of the biosensors at the two tested immobilization solutions over the pH range 5–7, it seems that both the pH and the composition of the immobilization solution control somehow the formation of these multimers, and consequently, determine the accessibility of chymosin to the bond Phe105–Met106 at the immobilized κ-CN molecules.

Protein loading was tested in order to define the κ-CN loading to obtain the maximum response. A saturation study was made using the Au/DTSP/κ-CN electrodes and four discrete κ-CN concentrations, that is, 0.125, 0.25, 0.5 and 1 mg mL^{-1} in phosphate buffer solution, pH 5.5. The sensitivity of the corresponding biosensors to a 0.2% (w/v) solution of rennet (Chr. Hansen, solid) in 20 mM imidazole pH 5 for 5 min at 37 °C was taken as the criterion for this study. The profile of the biosensors' sensitivity (data not shown) showed a maximum at a κ-CN concentration of 0.5 mg mL^{-1}, and therefore, further experiments were performed, applying the optimum κ-CN content onto the Au/DTSP/κ-CN electrodes. The decrease in the response observed at higher loadings is probably attributable to steric hindrance between chymosin and immobilized κ-CN active sites.

3.1.2. Buildup and performance of Au/DTSP/κ-CN electrodes

CV, EIS and DPV measurements during the various modification and interaction steps are illustrated in Fig. 2. Fig. 2A shows superimposed CVs of the redox couple using a scan rate of 50 mV s^{-1}. Formation of the DTSP SAMs results in a small decrease of both the oxidation and reduction peaks (scan b), indicating that under the specific experimental conditions (2 mM DTSP and 2 h incubation) the effect of the so formed SAMs on blocking interfacial electron transfer is rather low. On the other hand, the immobilization of κ-CN results in a substantial decrease of the redox peaks current (scan c). Besides the effect of κ-CN on blocking interfacial electron transfer, κ-CN generates a negatively charged interface that repels negatively charged $[Fe(CN)_6]^{4-/3-}$ anions, thus retarding the interfacial electron-transfer kinetics of the redox probe at the electrode surface. After interaction with rennet, the decrease of the net negative charge of the interface due to the formation of the positively charged *para*-κ-casein results in the increase of the flux of the redox probe at the electrode surface and, consequently, in the increase of the observed redox peaks current intensity (scan d), which lies between the values corresponding to Au/DTSP and Au/DTSP/κ-CN electrodes.

Similar to CV studies, the sequential construction sequence impedimetric spectra profiles (Fig. 2B) showed a progressive increase in R_{ct} values from 0.29 kΩ for bare gold electrodes (spectrum a) to 0.56 and 2.99 kΩ for Au/DTSP (spectrum b) and Au/DTSP/κ-CN (spectrum c) electrodes, respectively, followed by a decrease to 2.22 kΩ after the interaction of the fully functionalized biosensors (spectrum d) with rennet.

The stepwise construction of Au/DTSP/κ-CN electrodes, as well as their behavior after the interaction with rennet was also analyzed with DPV experiments (Fig. 2C). The intensity of the peak current I_p at +0.2 V, owed to the oxidation of the hexacyanoferrate(II) during the anodic scan from 0.0 to +0.4 V, was altered in a reverse manner compared with R_{ct} values at EIS experiments. Specifically, it was decreased from 83.4 μA for bare gold electrodes (curve a) to 57.4 and 8.5 μA for Au/DTSP (curve b) and Au/DTSP/κ-CN (curve c) electrodes, respectively, and then it was increased to 15.2 μA (curve d) due to the interaction of rennet with the immobilized κ-CN molecules.

3.1.3. Optimization of the working variables

The effect of the pH of the rennet solution, as well as of the incubation temperature on the performance of the impedimetric biosensors was also examined using a 0.2% (w/v) rennet solution in 20 mM imidazole and a constant incubation time of 5 min (Table 1). At pH 6, the response of the biosensors is almost nil, while at pH 6.5, the observed increase in the R_{ct} ($\Delta S > 0$) is not associated with interactions among chymosin and the immobilized molecules. It can most likely be attributed either to a rearrangement of the immobilized κ-CN or to (physical) adsorption of rennet's enzyme(s) over Au/DTSP/κ-CN electrodes.

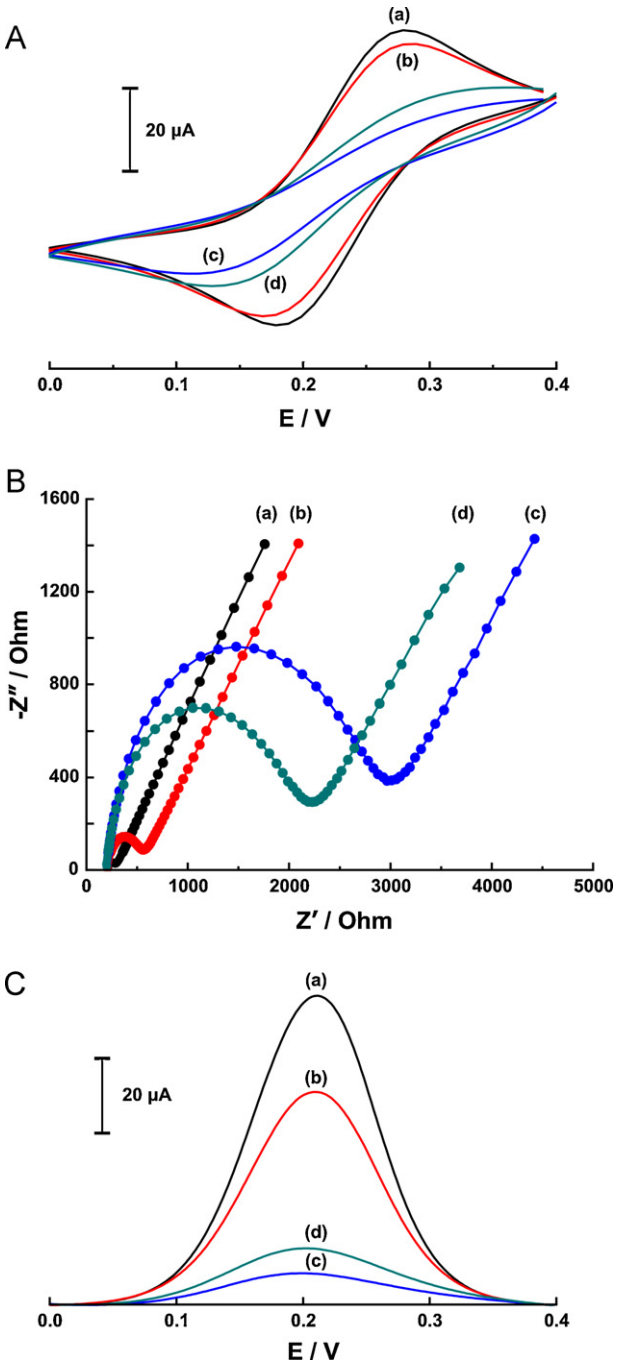

Fig. 2. (A) CV, (B) EIS and (C) DPV plots of Au/DTSP/κ-CN biosensors at various modification and interaction steps: (a) bare electrodes, (b) Au/DTSP electrodes, (c) Au/DTSP/κ-CN biosensors, and (d) the biosensors after incubation for 5 min at 37 °C in a solution of 1 + 3 (v/v) of rennet (Hansen, liquid) +26.7 mM imidazole pH 5.

The data tabulated in Table 1 also reveals that the interaction of the immobilized κ-CN with chymosin is favored at slightly lower pH values within the range 5–5.5. The highest responses were observed at pH 5 and 5.2. However, additional experiments performed with blank samples (pure imidazole solution) at these pH values showed that there is a significant drift of the signal, which, as is proven from the corresponding measurements at 30 °C and room temperature, is largely owing to heat treatment. In this regard, application to commercial products (see below) was performed at 30 °C and room temperature. Indeed, the observed signal changes at room temperature ($\Delta S = -14\%$) give promise that the proposed biosensors can be exploited for on-site applications.

Table 1
Signal changes, ΔS (%) of Au/DTSP/κ-CN biosensors after incubation in a solution of 0.2% (w/v) rennet (Hansen, solid) in 20 mM imidazole for 5 min at different pH values and incubation temperatures. A solution of 20 mM imidazole was used as blank.

pH	Temperature (°C)	After interaction with rennet[a]	After incubation to the buffer solution[a]
6.5	37	+20	
6	37	−3	
5.5	37	−28 ± 7	
5.2	37	−34 ± 3	−17 ± 5
5	37	−47 ± 3	−14 ± 1
5	30	−33 ± 2	−4 ± 1
5	RT	−17 ± 2	−3 ± 1

[a] Mean values ± standard deviation (n = 3). Replicates were conducted with three different electrodes.

The performance of the biosensors upon different concentrations of rennet ranging from 0% to 0.4% (w/v) was also tested. As can be seen in Fig. 3, Au/DTSP/κ-CN electrodes exhibit a concentration-dependent response, while the drop of the response at concentrated solutions of rennet (>0.2% (w/v)) might be attributed to (physical) adsorption of rennet's enzymes onto their surface, and thus, for applications at such concentrated protein solutions, post-blocking of the non-specific binding sites at the surface of the ready-to-use biosensors seems to be necessary. Moreover, the comparison of the performance of the biosensors at 30 and 37 °C, reveals that, essentially, the increase in the temperature does not offer any advantage regarding sensitivity. Over the concentration range from 0 to 0.2% (w/v) of rennet, the signal difference at the two tested incubation temperatures is almost constant and equal to that observed for the blank solution.

The measuring parameter increases proportional to the incubation time, up to a time interval of 15 min (data not shown). Longer incubation times, besides not being suitable for practical use, do not offer any benefits in terms of sensitivity. Here, it is important to note that compared with the related studies made with the artificial casein micelle-based biosensors [20], where the measuring parameter reached a maximum value after 60 min, Au/DTSP/κ-CN biosensors exhibit a considerably lower response time.

3.2. SPR studies

The SPR biosensor offers a wide range of unique benefits, including fast response, ability to measure several samples at the same time, and most importantly, it is a label-free, real-time analytical technology. However, the sensing surfaces of SPR detectors are expensive and account for the vast majority of the running costs for such instruments. For this reason, our study was entirely made by using regenerated CM5 gold chips, according to the method proposed by Groves et al. [22]. The different functionalization (see details in Section 2.4) and interaction steps on the SPR chips are illustrated in Fig. 4A and B, respectively. Since the measured response is directly proportional to the mass of the immobilized material, a drop in signal intensity is expected to occur with cleavage of immobilized casein. The signal difference occurring after point 5 (Fig. 4B) supports the effectiveness of the regeneration protocol and the suitability of the (regenerated) gold chips to re-serve as immobilization platforms.

After that point, the performance of plain CM5 (curve 4Ba, used as blank) and CM5/κ-CN (curve 4Bb) SPR chips were tested for two successive, 0.2% (point 6) and 0.1% (point 8) (w/v) rennet solutions in 20 mM imidazole pH 5. Plain CM5 chip gave no response to rennet

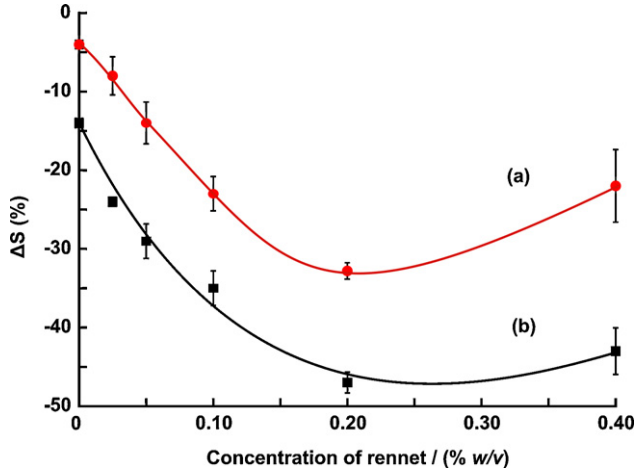

Fig. 3. Calibration curves at (a) 30 and (b) 37 °C. Experimental conditions: biosensors were incubated in rennet samples (Hansen solid) in 20 mM imidazole, pH 5 for 5 min. All measurements were performed in triplicates.

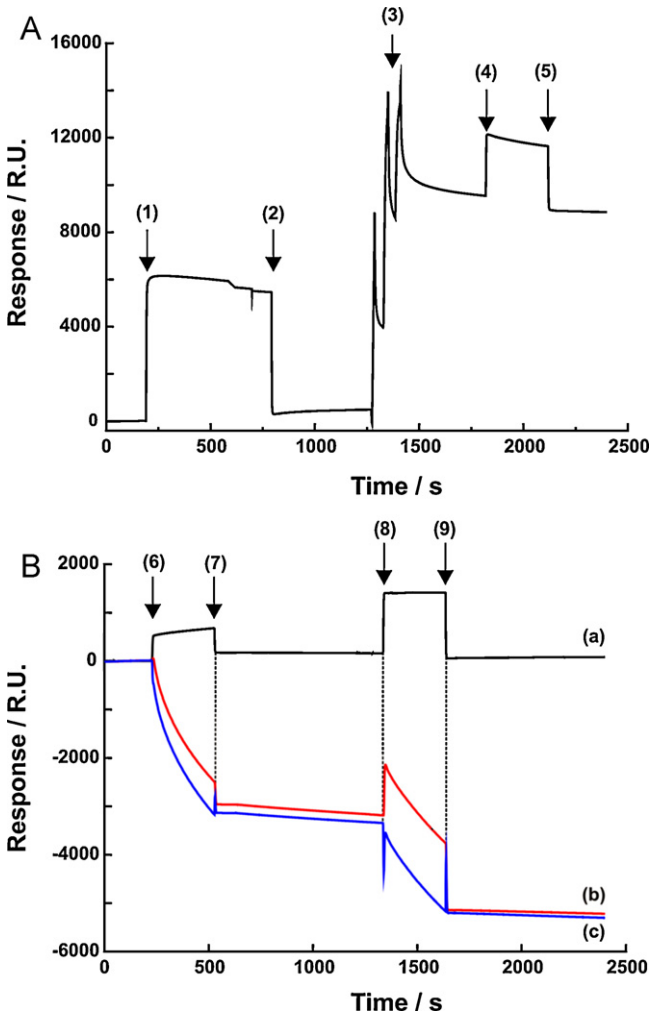

Fig. 4. (A) Stepwise construction of a CM5/κ-CN biosensor based on a regenerated CM5 gold chip: (1) activation of carboxymethylated dextran *via* ECS/NHS, (2) HBS-EP buffer solution, (3) sequence of addition of immobilization/HBS-EP buffer solutions, (4) deactivation of NHS-esters with ethanolamine, (5) HBS-EP buffer solution. (B) Performance of CM5 (curve a, used as blank) and CM5/κ-CN (curve b) SPR chips: (6) 0.2% (w/v) rennet (Ipirotopoula) in 20 mM imidazole, pH 5, (7) 20 mM imidazole, pH 5, (8) 0.1% (w/v) rennet (Ipirotopoula) in 20 mM imidazole, pH 5, (9) 20 mM imidazole, pH 5. Curve c corresponds to curve b, after subtraction of the baseline (curve a).

Table 2
Application of electrochemical and SPR biosensors to various rennet samples.

Samples	Clotting power, Berridge method	EIS at 30 °C (ΔS(%))[a]	EIS at RT (ΔS(%))[a]	DPV at 30 °C (ΔS(%))[a]	SPR at 25 °C (ΔRU)[a]
Danisco 1 + 3 v/v	61.900	−41 ± 5	−35 ± 5	103 ± 15	−6.37
Hansen 1 + 3 (v/v)	36.600	−23 ± 2	−14 ± 3	79 ± 12	−2.02
Hansen 0.1% (w/v)	130.000	−23 ± 2	−13 ± 2	54 ± 10	−6.23
Ipirotopoula 0.1% (w/v)	48.500	−9 ± 2	−6 ± 1	28 ± 10	−2.97

[a] Mean values ± standard deviation (n = 3). Replicates were conducted with three different electrodes or SPR chips.

solutions showing that (even after regeneration) carboxymethylated dextran layer preserves its excellent non-specific adsorption properties. On the other hand, the interaction of the functionalized CM5/κ-CN SPR chips with rennet samples gave rise to analytically useful, concentration-dependent signal changes. Curve c corresponds to curve b in Fig. 4B, after subtraction of the baseline (Fig. 4B curve a).

3.3. Application to commercial samples

The proposed electrochemical and SPR biosensors were further tested at various commercial solid and liquid samples. The performance of Au/DTSP/κ-CN biosensors was evaluated by both EIS and DPV measurements at 30 °C and room temperature (typically 23–24 °C), while the performance of SPR biosensors was evaluated at the fixed temperature of 25 °C established by the instrument. The observed signal changes were compared with the milk-clotting power, which was calculated by using the Berridge method [10]. As we can conclude from the data tabulated in Table 2, ΔS(%) values reflect the milk-clotting power of the tested samples, thus verifying the suitability of both the electrochemical and SPR biosensors to be used as a true alternative to the existing methods based on visual or optical observation of milk-clotting, incorporating the simplicity and advantages of biosensors.

It is important to note that impedimetric measurements at both 30 °C and room temperature, exhibit an excellent reproducibility (<5%). This feature along with other advantages associated with the use of electrochemical biosensors [27] can enable the use of the proposed biosensors for routine analysis. At DPV measurements on the other hand, the relative standard deviation was calculated 10–12%, which even though acceptable for routine analysis, is considerable higher than that calculated at EIS measurements. In addition, the reliability of each method can be justified by comparing the ratios of the clotting power units for the solid and liquid samples, as they occurred by the data presented in Table 2. According to the Berridge method, the ratios of the clotting power units are 61900/36600 = 1.69 and 130000/48500 = 2.68, respectively. Based on EIS data, the corresponding ratios are 1.78 and 2.55, almost identical to those calculated with the reference method. Based on DPV data, the corresponding values were calculated to be 1.30 and 1.93.Thus, overall, EIS was determined to be more reproducible and reliable than DPV.

The poor reproducibility of measurements has been recognized as one of the major obstacles associated with the use of electrochemical biosensors [27–29]. A general conclusion, however, regarding the reproducibility between EIS and dc based electrochemical biosensors was not possible to be drawn due to the lack of other comparative studies. In a previous comparative study between EIS and square wave voltammetry (an also dc based pulsed technique) [30], reproducibility was not included among the parameters taken into consideration.

4. Conclusions

This study employs single-use functional biosensors, based on thiol modified gold electrodes and κ-casein as sensing layer, as probes to evaluate the clotting power of rennet. The proposed biosensors were successfully tested at various commercial rennet samples in solid or liquid form.

The obtained results show that, overall, EIS is more reproducible and reliable than DPV at the electrochemical biosensors for the determination of the clotting power of rennet.

The comparison with a previous study made by our group based on artificial casein micelles, shows that κ-casein based biosensors, besides they need less effort to be constructed, are superior in terms of response time and reproducibility.

SPR biosensors based on regenerated CM5/κ-casein gold chips were successfully used for the assessment of the clotting activity of rennet at 25 °C. Preliminary results also showed that CM5/κ-casein gold chips can be used for more than one analytical run. Additional studies regarding the reusability of SPR chips are currently in progress in our lab.

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