Impedimetric Biosensor for the Assessment of the Clotting Activity of Rennet

Maria A. Panagopoulou,† Dimitrios V. Stergiou,† Ioannis G. Roussis,‡ and Mamas I. Prodromidis*†

Laboratories of Analytical Chemistry and Food Chemistry, Department of Chemistry, University of Ioannina, 451 10 Ioannina, Greece

Cheese production is relied upon the action of rennet (a mixture of chymosin and pepsin) onto casein micelles of milk. For the first time, the monitoring of this interaction with electrochemical impedance spectroscopy (EIS) was used to develop a faradic impedimetric biosensor for the assessment of the clotting activity of rennet, using hexacyanoferate(II)/(III) couple as a redox probe. Gold electrodes were modified with self-assembled monolayers of different thiols (thiolic acid, dithiobis-N-succinimidyl propionate, and cysteamine), and (artificial) casein micelles were immobilized on the modified gold surfaces. The proposed method is based on the measurement of charge-transfer resistance (Rct) changes attributed to the degradation of the negatively charged immobilized casein micelles by rennet to neutral biostructures. This action results in the increase of the flux of the redox probe, which exists in the bulk solution, to the surface. The evaluation of the clotting power of rennet has always been a parameter of great importance from scientific, technological, and commercial viewpoints. The importance of this parameter has raised the scientific interest since the end of the 19th century when the first method, based on visual observation of the formation of a clot, was published. Since then, numerous methods, which also depend upon visual observations of the coagulation point of the milk, have been used to measure rennet activity. Even though they are still in routine use, they suffer from the subjective nature of the observation, especially when

The proposed biosensors were successfully tried for various commercial samples. The casein content of milk represents about 80% of milk proteins. The principle casein fractions are αs1-, αs2-, β-, and κ-casein (κ-CN), and their distribution in cow’s milk is 38, 10, 40 and 12%, respectively. In combination with appreciable quantities of colloidal calcium phosphate (CCP) nanoclusters, they appear in the form of not-quite spherical colloids, 50–500 nm in diameter, called casein micelles. The distinguishing property of all caseins is their low solubility at pH 4.6. Rennet is a natural complex of enzymes produced in any mammalian stomach, and it is widely used in the cheese making industry as the major milk coagulant. The milk-clotting activity of rennet relies on its ability to degrade casein micelles, and this action depends on the chymosin (pI ≈ 4.6) content of the complex. Chymosin proportion may differ by source (cow, lamb, goat, chicken, camel, or nonanimal sources) and age, accompanied by a concomitant alteration of pepsin content. The proportions normally present in commercial rennets are 70% chymosin and 30% pepsin.

Rennet coagulation of milk may be divided into primary (enzymic hydrolysis) and secondary (aggregation) stages, although these stages normally overlap to some extent during cheese making. During the primary stage, chymosin hydrolyses the outer “hairy layer” of κ-CN (Figure 1A). More specifically, κ-CN is cleaved by rennet 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 (pI > 7) insoluble para-κ-casein molecules, which remain attached to the casein micelle. 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 (Figure 1B). The nature of the attractive forces during the aggregation of casein micelles is still not completely clear, although calcium bridges, van der Waals forces, and hydrophobic interactions appear to be involved.

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(9) Sohrab, F. Milch-Zeitung 1877, 6, 497–501.
different persons in different laboratories are involved. Some of these drawbacks have been adequately overcome by the joint IDF/ISO Standard - ISO 11815/IDF 157:2007 method, which uses reference rennet powder with an overall milk-clotting activity of 1000 IMCU g\(^{-1}\) (IMCU = international milk-clotting units).\(^{13}\)

Other methods based on blood clot timers\(^{14}\) or on viscosity measurements of renneted milk\(^{15-17}\) have also been proposed. In addition, spectrophotometric measurements of milk-rennet reaction products at 290 nm,\(^{18}\) measurements of the electric conductivity,\(^{19}\) and a turbidity method\(^{20}\) have also been proposed. The first instrument proposed for measuring milk clotting, the Formagraph, was developed in 1980.\(^{21,22}\) Its operation is based on the movement of a small pendulum immersed in oscillating samples of rennetted milk and a light flash for recording the position of the pendulum, as it alters due to the milk clotting, on a photographic paper. Formagraph was then replaced by the Optigraph (Ysebaert, France),\(^{23}\) an also bench-type instrument, which employs measurements of near infra red attenuation through a milk sample during its coagulation. According to a recently published comparative study, results received by both instruments correlate well.\(^{24}\)

Aiming to develop an inexpensive and easy-to-perform method, compatible with the modern sensor technology, artificial casein micelles (ACM), made by commercially available sodium caseinate, were immobilized onto self-assembled monolayers (SAMs) of thiol-modified gold electrodes (Au/SAMs). Employing faradic impedimetric measurements before and after the immersion of the electrodes (Au/SAMs/ACM) in rennet samples, clotting power is expressed by the relative decrease of the charge-transfer resistance, R\(_{ct}\).

**EXPERIMENTAL SECTION**

**Chemicals and Solutions.** Sodium caseinate, thioic acid (TA), dithiobis-N-succinimidyl propionate (DTSP), cysteamine (CYS), lysine, and glutaraldehyde (GA; ∼25% in water; kept in sealed vials under argon at +4 °C) were purchased from Sigma. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), imidazole, D-(+)-gluconic acid \(\delta\)-lactone, absolute ethanol, potassium ferrocyanide, and potassium ferricyanide were from Merck. All other chemicals were from Merck and Sigma and double distilled water (DDW) was used through-

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\(^{14}\) de Man, J. M.; Batra, S. C. Dairy Ind. 1964, 29, 32–33.


\(^{19}\) Green, M. L. J. Dairy Res. 1977, 44, 159–188.


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out. Rennet solutions were prepared before use by dissolving 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 an equal volume of 40 mM imidazole, and the pH of the mixture was adjusted to 5 with 0.1 N HCl. A 20 mM imidazole solution, pH 6.5, was used in various washing steps throughout the build-up and storage of the biosensors. A 50 mM phosphate buffer (PB) solution, pH 7, was used for the activation of amine groups at Au/CYS electrodes with glutaraldehyde.

**Preparation of Artificial Casein Micelles.** Suspensions of artificial casein micelles (ACM) were weekly prepared by a modified Patent method.25 Powdered sodium caseinate (6.3 g) was carefully added into 50 mL of hot DDW (∼55 °C) and mixed under stirring for about 6 to 7 h to achieve a homogeneous opaque solution. The solution was left to reach a temperature of 37 °C, and then, 4.56 mL of an aqueous solution of 0.87 mM CaCl₂ was added dropwise into the caseinate solution with a constant mixing over a 20 min period of time under stirring. Then, 2.98 mL of an aqueous mixture of 17.1% (w/v) K₂HPO₄ and 1.8% (w/v) NaOH was introduced dropwise into the stirred reaction medium, with a constant mixing over a period of 6 min. Afterward, the mixture was kept under stirring for 3 h at room temperature and remained for at least 12 h at 4 °C before use. The size of ACM depends on the addition order of the reagents in the mixture and the mixing rate.26 The as-produced ACMs have a mean diameter of 250 nm.25

**Apparatus.** Electrochemical impedance spectroscopy (EIS) 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 (IJ Cambria) electrode, and all potentials reported hereafter refer to the potential of this electrode. The impedance spectra were recorded over the frequency range of 10⁻¹⁻¹⁰⁻² Hz, using a sinusoidal excitation signal, superimposed on a DC potential of +0.200 V. Excitation amplitude of 10 mV (rms) was used throughout. All measurements were performed in a solution of 5 mM hexacyanoferrate (II)/(III) (1 + 1 mixture) in a 50 mM imidazole buffer solution, pH 6.5, containing 100 mM KCl, at room temperature.

FT-IR studies were conducted with an FT-IR optical spectrometer (Thermo-Electron) over gold surfaces, which had been functionalized according to the procedures described below. The surface topography of ACM-modified gold surfaces, before and after their immersion in rennet solutions, was studied by atomic force microscopy (AFM), at tapping mode, using a Nanoscope IIIA instrument (Digital Instruments).

**Formation of SAMs and Immobilization of ACM.** Gold electrodes were constructed using the commercial kit EasyCon (EasyCon Hellas, provided by Metrohm Autolab). Before use, gold electrodes of 2 mm active surface were polished with Al₂O₃ (0.01 µm grain size) and sonicated for 3 min in DDW. After polishing, gold surfaces were cleaned by dipping into a solution of 1 + 1 w/v mixture of 17.1% (w/v) K₂HPO₄ and 1.8% (w/v) NaOH. A 20 mM imidazole solution, pH 6.5, was used in various washing steps throughout the build-up and storage of the biosensors. A 50 mM phosphate buffer (PB) solution, pH 7, was used for the activation of amine groups at Au/CYS electrodes with glutaraldehyde.

**Au/TA Electrodes.** Gold electrodes were immersed in a solution of 0.2 M TA in ethanol for 20 h, rinsed thoroughly in fresh baths of absolute ethanol, and dried under argon. Activation of the terminal carboxyl groups was done in a freshly prepared mixture of 0.2 M EDC and 0.05 M NHS in DDW for 30 min under stirring. Activated surfaces were thoroughly washed with the same solvent, and finally, 1 mL of the ACM suspension [undiluted, 1 + 1, 2 + 1, 4 + 8 (v/v) mixtures of it with 0.2 M NaCl, all at pH 6.5, and 1 + 4 (v/v) mixture with 0.2 M NaCl at pH 5.6] was placed, with the aid of a plastic cup, over the electrode surface for at least 12 h in a humidified glass chamber. Adjustment to pH 5.6 was made by adding appropriate amounts of a 0.2 M solution of gluconic acid δ-lactone in the ACM/NaCl mixture.

**Au/DTSP Electrodes.** Gold electrodes were immersed in a solution of 2 or 5 mM DTSP in acetone for 4 h, rinsed thoroughly in fresh baths of acetone, and dried under argon. Then, 1 mL of the ACM suspension [undiluted and as an 1 + 4 (v/v) mixture with 0.2 M NaCl, pH 6.5] was placed, with the aid of a plastic cup, over the electrode surface for at least 12 h in a humidified glass chamber.

**Au/thiol/ACM Electrodes.** Fully functionalized (Au/thiol/ACM) electrodes were immersed in a 0.1 M solution of lysine to deactivate the remaining amine-active groups, washed thoroughly with 20 mM imidazole solution, pH 6.5, and stored in the same buffer until use.

Fixation of immobilized ACM (if it is stated) was made by incubating the fully functionalized (Au/TA/ACM) electrodes in a solution of 2.5% glutaraldehyde in PB solution for 1 h under mild stirring. After thoroughly rinsing with the same buffer solution, to remove the physically absorbed glutaraldehyde, 1 mL of the ACM suspension [undiluted and as an 1 + 4 (v/v) mixture with 0.2 M NaCl, pH 6.5] was placed, with the aid of a plastic cup, over the electrode surface for at least 12 h in a humidified glass chamber.

**Procedure.** Fully functionalized (Au/thiol/ACM) electrodes were incubated in rennet solutions in 20 mM imidazole, pH 5–6.5, for a specified time interval at 37 °C, rinsed thoroughly with a 20 mM imidazole solution, pH 6.5, and transferred to the measuring cell. All the optimization studies were made with the product “Ipirotopoula”.

**Calculations.** Throughout this study, the relative change of the signal of the Au/thiol/ACM electrodes, before and after their immersion to the rennet solution, ΔS (%), which is expressed as

\[
\Delta S(\%) = \left(1 - \frac{R_{ct}(\text{after the immersion to rennet})}{R_{ct}(\text{before the immersion to rennet})}\right) \times 100, \nonumber
\]

was taken as a measure of the clotting power of the tested samples. Relative signal changes (R_{ct}) were calculated using the equation

\[
R_{ct} = \frac{I_2-I_1}{I_1}, \nonumber
\]

where I₁ is the current before the immersion of the electrode to rennet, and I₂ is the current after the immersion of the electrode to rennet.
**RESULTS AND DISCUSSION**

Rennet action results in the degradation of the negatively charged immobilized ACM and the formation of denuded, neutral biostructures (Figure 1A). This action has a dual effect, namely, (i) elimination of the negative charge, which repulses the negatively charged redox probe and (ii) partial aggregation of ACM (Figure 1B), which increases the part of nonblocked electrode surface. Both effects result in the increase of the flux of the redox probe, which exists in the bulk solution, to the surface of the electrode and, consequently, in the decrease of $R_t$. Referring to immobilized ACM, the stage of aggregation is expected only among adjacent casein micelles. On the basis of the atomic force micrographs of the ACM-modified gold surfaces taken before (Figure 1C) and after (Figure 1D) their incubation in rennet solutions, an idealized view of this process is illustrated in Figure 1A.B.

A determining factor in the performance of the proposed biosensors is the design of the immobilization platform in order to achieve functional surfaces. For this reason, our study includes modification of the gold surfaces with SAMs bearing negatively (TA), neutrally (DTSP), or positively (CYS) charged terminal groups. A detailed description of the performance of the corresponding biosensors is given below.

**Au/TA/ACM Biosensors. Immobilization of ACM.** Protein immobilization via carbodiimide chemistry is favored when the pH of immobilization lies between the isoelectric point of the protein and the $pK_a$ of the surface. Activation with EDC/NHS roughly results in a 30–40% transformation of the carboxyl groups to amine-active succinimide esters, and thus, the immobilization platform carries negative charge. At pH values lower than the isoelectric point, proteins carry a net positive charge and, due to electrostatic interactions, are effectively preconcentrated on the immobilization carrier. Thus, the pH of the immobilization solution is a crucial parameter to receive high yields of immobilization, and actually, this is a conflicting point between previous published works referring to the immobilization of casein micelles onto gold surfaces modified with SAMs of 11-mercaptoundecanoic acid.28,29 However, the low solubility of caseins at pH values near their isoelectric point does not allow them to efficiently preconcentrate near the surface of the electrode. To overcome this problem, immobilization experiments were performed with solutions of high ionic strength.

| $R_{ct}$ values were calculated by fitting the impedance data to a Randles equivalent circuit
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In a series of comparative experiments, immobilization of ACM was tested from the original suspension and 1 + 1, 1 + 2, and 1 + 4 (v/v) mixtures of it with a solution of 0.2 M NaCl or DDW. From the results tabulated in Table 1, we can assume that diluted mixtures of ACM, especially those containing NaCl, serve as the most suitable immobilization solutions. Sodium chloride at a dilution ratio of 1 + 4 seems to provide an effective shield to the negative charge of ACM and an increased concentration of ACMs close to the immobilization surface, resulting in higher coupling yields. Dilution ratios and the concentration of NaCl were selected to preserve the 3D-structure of ACMs. Higher dilution ratios (1 + 8) or higher concentrations of NaCl result in unstable mixtures (precipitation of ACMs over time) due to an ion exchange process with the calcium cations present in the micelles.30,31 On the other hand, mixtures up to 1 + 4 (v/v) with 0.2 M NaCl are quite stable over time (see IR studies below).

Moreover, in both 1 + 2 and 1 + 4 mixtures, lowering of the pH of the immobilization solution from 6.5 to 5.6 leads to higher coupling yields, as at more acidic regimes the repulsing electrostatic forces between ACMs and the immobilization surface become weaker.28,29

**CV and EIS Studies.** CV and EIS spectra during the various modification and interaction steps are illustrated in Figure 2A.B, respectively. Figure 2A shows superimposed CVs of the redox couple using a scan rate of 100 mVs$^{-1}$ at (a) bare, (b) Au/TA, and (c) Au/TA/ACM electrodes before and after (d) their immersion to a 0.2% solution of rennet in 20 mM imidazole solution, pH 5, at 37 °C for 2 h. Formation of the SAM of thiotic acid inhibits the electrochemical reaction, thus both oxidation and reduction peaks disappear. Immobilization of bulky ACM results in further decrease of the current due to the effect of the ACM on blocking interfacial electron transfer. It is, also, important to note that both layers (SAMs of TA and ACM) generate a negatively charged interface that repels negatively charged [Fe(CN)$_6$]$_{4^-}$/3$^+$ anions. This repulsion is anticipated to retard the interfacial electron-transfer kinetics of the redox

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with rennet enhances the interfacial electron-transfer kinetics of the redox probe at the electrode surface, thereby decreasing $R_\text{ct}$ (spectrum in Figure 2Bd), that is $R_\text{ct}(b) < R_\text{ct}(d) < R_\text{ct}(c)$. The spectrum in Figure 2Be corresponds to Au/TA/ACM biosensors after their incubation in a solution of 20 mM imidazole, pH 5 (blank), at 37 °C for 2 h.

**Performance of Au/TA/ACM Biosensors.** On the basis of the results tabulated in Table 1, the action of rennet onto immobilized ACM was investigated at Au/TA/ACM electrodes made from a $1 + 4 \, \text{v/v} \, \text{ACM} + 0.2 \, \text{M NaCl}$ mixture at pH 6.5. Electrodes made from the same mixture at pH 5.6 showed a similar performance, and thus, results are not included. According to the literature, the formation of casein micelle aggregates is favored in low pH values and in the presence of calcium cations. An analogous behavior is observed at EIS studies. The bare gold electrode shows a very small semicircle domain implying a very fast electron-transfer process with a diffusional limiting step ($R_\text{ct}$ values (spectra in Figure 2Bb,c, respectively). The drop of the response at concentrated ($>0.2\, \text{w/v}$) solutions of rennet might be attributed to (physical) adsorption of rennet’s enzymes over Au/TA/ACM electrodes, and thus, for applications at such concentrated protein solutions, postblocking of the nonspecific binding sites seems to be necessary. On the other hand, the performance of Au/TA/ACM biosensors, at different incubation times, indicates that the kinetics of the reaction is rather slow. The measuring parameter reaches a maximum value after, at least, 120 min, while at shorter incubation times (30 min), where the applicability of the proposed biosensors is more attractive, the observed signal changes of $-22$ and $-34\%$, which were obtained at pH 5.2 and 5, respectively, indicate the suitability of the proposed biosensors for the evaluation of the clotting power of rennet.

The impedimetric behavior of the Au/TA/ACM electrodes at different concentrations of rennet (0.1, 0.2, and 0.4%), incubation times (30, 60, and 120 min), buffer solutions (imidazole, 3-(N-morpholino)propanesulfonic acid (MOPS), and N2-hydroxyethylpiperazine-N2-ethanesulfonic acid (HEPES)), and after fixation of the immobilized ACMs with glutaraldehyde was also studied, and the results are collectively presented in Figure 3. The drop of the response at concentrated ($>0.2\, \text{w/v}$) solutions of rennet might be attributed to (physical) adsorption of rennet’s enzymes over Au/TA/ACM electrodes, and thus, for applications at such concentrated protein solutions, postblocking of the nonspecific binding sites seems to be necessary. On the other hand, the performance of Au/TA/ACM biosensors, at different incubation times, indicates that the kinetics of the reaction is rather slow. The measuring parameter reaches a maximum value after, at least, 120 min, while at shorter incubation times (30 min), where the applicability of the proposed biosensors is more attractive, the observed signal change is $-15\%$. Finally, in accordance with previous works, imidazole buffer solution seems to be the optimum choice for the stabilization of immobilized casein micelles. Substantially lower signal changes were observed when experiments were performed in MOPS or HEPES buffer solutions.


and (h) fixation of the immobilized ACM with glutaraldehyde.

20 mM imidazole, 20 mM HEPES, and 20 mM MOPS, respectively; incubation for 2 h at 37 °C in 20 mM imidazole, pH 5.0, for 2 h at 37 °C in 20 mM imidazole, pH 5, in 20 mM imidazole, 20 mM HEPES, and 20 mM MOPS, respectively; and (h) fixation of the immobilized ACM with glutaraldehyde.

A control experiment, which was designed to support our assumptions regarding the nature of ACM—rennet interaction and, consequently, the mechanism of the signal production was performed after fixation of immobilized casein micelles with glutaraldehyde. This treatment stabilizes the immobilized ACM and leads to the formation of a compact layer due to the interconnection of immobilized ACM through Schiff bonds between the aldehyde groups of glutaraldehyde and the primary amines in casein micelles. The remarkable increase of $R_\text{ct}$ at glutaraldehyde-fixed Au/TA/ACM electrodes (1.2 MOhm (data not shown) compared with that of 325 kOhm (Figure 2B)), as well as the nil effect of rennet on them (Figure 3, is probably attributable to an extended blocking effect to redox species in solution and to a limited access of chymosin to κ-CN fractions, respectively, due to the formation of the aforementioned compact layer of immobilized ACMs.

Performance of Au/CYS/ACM Biosensors. Au/CYS/ACM biosensors, based on SAMs developed from 10 or 20 mM cysteamine, were also examined in their efficiency to probe the action of rennet toward immobilized ACM. Both biosensors, after their immersion in a 0.2% w/v solution of rennet in 20 mM imidazole, pH 5, for 2 h at 37 °C, gave almost the same response $\Delta S (%) = -45\%$ (data not shown), which is higher compared with that observed using Au/TA/ACM biosensors $\Delta S (%) = -35\%$. In contrast with the TA-modified based electrodes, Nyquist plots of CYS-modified based electrodes (Figure 4) include a semicircle (charge-transfer controlled behavior at high frequency region) followed by a straight line (diffusion-controlled behavior at low frequency region), thus allowing $R_{\text{ct}}$ values to be calculated by fitting the impedance data to the Randles equivalent circuit $R_s(Q_{dl}(R_{\text{ct}}, W))$ illustrated in side panel of Figure 4. Observed alterations of the $R_{\text{ct}}$ values in various modification and interaction steps are attributable to similar phenomena, which are described above for Au/TA/ACM electrodes. The most noticeable difference is the magnitude of recorded $R_{\text{ct}}$ values (in the range of a few hundred Ohm) due to the electrostatic attraction between the oppositely charged redox anions and the CYS-modified gold surface.

Figure 3. Signal changes, $\Delta S (%)$, of Au/TA/ACM biosensors after (a), (b), and (c) incubation in a 0.2% w/v solution of rennet in 20 mM imidazole, pH 5.0, at 37 °C for 30, 60, and 120 min, respectively; (d), (e), and (f) incubation in 0.2, 0.1, and 0.4% w/v solutions of rennet in 20 mM imidazole, pH 5, for 2 h at 37 °C, respectively; (g) incubation for 2 h at 37 °C in 0.2% w/v solutions of rennet, pH 5, in 20 mM imidazole, 20 mM HEPES, and 20 mM MOPS, respectively; and (h) fixation of the immobilized ACM with glutaraldehyde.

Figure 4. Nyquist plots of Au/(10 mM)CYS/ACM biosensors at various modification and interaction steps: (a) bare gold electrodes, (b) Au/CYS electrodes, (c) Au/CYS/ACM biosensors, and (d) the biosensors after incubation for 2 h at 37 °C in a 0.2% w/v solution of rennet in 20 mM imidazole, pH 5, and (e) in a solution of 20 mM imidazole, pH 5, which was used as a blank. Inset panel, Randles equivalent circuit $R_s(Q_{dl}(R_{\text{ct}}, W))$.

Performance of Au/DTSP/ACM Biosensors. The use of DTSP-SAMs as immobilization platforms offers certain advantages, as such monolayers provide interfaces with specific hydrophilicity and desired chemical reactivity to amino-group containing biomolecules. In addition, there is no need to use coupling agents or activators, simplifying, in this way, the experimental procedure for the construction of the biosensors. Preliminary experiments were conducted with gold electrodes modified with 2 or 5 mM DTSP, while the immobilization of casein micelles was tried using the undiluted suspension of ACM or an 1 + 4 v/v mixture of ACM with 0.2 M NaCl. The impedimetric profile of Au/(2 mM)DTSP/ACM electrodes, made from the original suspension of ACM, at the different modification and interaction steps, is illustrated in Figure 5A. These electrodes showed the best performance (comparative data not shown), and thus, they were further used to investigate the behavior of the biosensors to different concentrations of rennet and incubations times. The data shown in Figure 5B indicates that the measuring parameter increases proportional to the concentration and the incubation time, up to a time interval of 60 min. Longer incubation times, besides that they are not indicated for practical use, do not offer any benefits in terms of sensitivity. Indeed, in two out of three tested concentrations, the observed $\Delta S (%)$ values are slightly lower. This behavior can, also, be attributed to physical absorption of rennet’s enzymes onto the surface of the electrodes over prolonged incubation interval times. Data, also, reveals that, compared with the Au/TA/ACM biosensors, the kinetics of the reaction is considerably faster. For an incubation time of 30 min, the absolute signal changes increase from −15% (Au/TA/ACM biosensors) to −55%, while at incubation time intervals of 15 min, analytical useful signal changes of −32% can be achieved.

The proposed biosensors were further tested in various commercial samples in both liquid and solid form, and the observed signal changes were compared with the milk-clotting power, as it was determined in our laboratory, using the Berridge method. As can be seen in Table 2, $\Delta S (%)$ values reflect the milk-clotting power of the tested samples, indicating the suitability


of the proposed biosensors for the evaluation of the clotting power of rennet in both liquid and solid samples. It is important to note that analytical useful $\Delta S$ (%) values can also be obtained within 5 min of incubation, thus making the proposed biosensors especially attractive for routine use. Further evaluation of the observed signal changes is beyond the scope of this work. The response of the proposed biosensors should be correlated with units of milk-clotting power under specific measuring conditions.

**FT-IR Studies.** The suitability of the $1 + 4 \, v/v$ ACM + 0.2 M NaCl mixture at pH 6.5, as immobilization solution for the modification of Au/TA electrodes, was confirmed by IR studies. As shown in Figure 6A, the absorption bands of amide I and amide II, which are closely connected with the structure of ACMs, are identical to those recorded for the undiluted suspension of the ACM. Immobilization of ACMs onto the TA- and DTSP-modified gold surfaces as well as the interaction of them with rennet were also probed with IR spectroscopy. In particular, we recorded the IR spectra of the fully functionalized surfaces before and after their incubation in the working buffer solution (blank) and in a 0.2% w/v solution of rennet in 20 mM imidazole, pH 5.0, used as a blank (blue line). Spectra were recorded over the range 400–4000 cm$^{-1}$; however, for better observation of amide I and amide II bands, a selected area over 1200–1800 cm$^{-1}$ is only presented. The spectra of immobilized ACM (Figure 6B,C) show a high degree of similarity to that of the suspension of ACM (Figure 6A), indicating that covalent binding to thiol SAMs does not significantly impair the structure of the micelles. An average shift of 27 cm$^{-1}$ for amide I and amide II bands is observed. 

**Table 2. Application of Au/DTSP/ACM Biosensors to Various Rennet Samples**

<table>
<thead>
<tr>
<th>sample</th>
<th>incubation time, min</th>
<th>form</th>
<th>clotting power (Berridge method)</th>
<th>proposed biosensors $\Delta S$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hansen</td>
<td>15</td>
<td>liquid</td>
<td>1:36 600</td>
<td>-24</td>
</tr>
<tr>
<td>Danisco</td>
<td>15</td>
<td>liquid</td>
<td>1:61 900</td>
<td>-51</td>
</tr>
<tr>
<td>Hansen</td>
<td>15</td>
<td>solid</td>
<td>0.05% w/v</td>
<td>1:130 000</td>
</tr>
<tr>
<td>Hansen</td>
<td>5</td>
<td>solid</td>
<td>0.2% w/v</td>
<td>1:48 500</td>
</tr>
<tr>
<td>Hansen</td>
<td>5</td>
<td>solid</td>
<td>0.2% w/v</td>
<td>1:130 000</td>
</tr>
<tr>
<td>Ipirotopoula</td>
<td>5</td>
<td>solid</td>
<td>0.2% w/v</td>
<td>1:48 500</td>
</tr>
</tbody>
</table>

$^a$ At electrochemical measurements, the standard deviation of the mean ranges from 9 to 16%, $n = 3$.  

of the proposed biosensors for the evaluation of the clotting power of rennet in both liquid and solid samples. It is important to note that analytical useful $\Delta S$ (%) values can also be obtained within 5 min of incubation, thus making the proposed biosensors especially attractive for routine use. Further evaluation of the observed signal changes is beyond the scope of this work. The response of the proposed biosensors should be correlated with units of milk-clotting power under specific measuring conditions.

**FT-IR Studies.** The suitability of the $1 + 4 \, v/v$ ACM + 0.2 M NaCl mixture at pH 6.5, as immobilization solution for the modification of Au/TA electrodes, was confirmed by IR studies. As shown in Figure 6A, the absorption bands of amide I and amide II, which are closely connected with the structure of ACMs, are identical to those recorded for the undiluted suspension of the ACM. Immobilization of ACMs onto the TA- and DTSP-modified gold surfaces as well as the interaction of them with rennet were also probed with IR spectroscopy. In particular, we recorded the IR spectra of the fully functionalized surfaces before and after their incubation in the working buffer solution (blank) and in a 0.2% w/v solution of rennet in 20 mM imidazole, pH 5.0, used as a blank (blue line). Spectra were recorded over the range 400–4000 cm$^{-1}$; however, for better observation of amide I and amide II bands, a selected area over 1200–1800 cm$^{-1}$ is only presented. The spectra of immobilized ACM (Figure 6B,C) show a high degree of similarity to that of the suspension of ACM (Figure 6A), indicating that covalent binding to thiol SAMs does not significantly impair the structure of the micelles. An average shift of 27 cm$^{-1}$ for amide I and amide II bands is observed. 

Specifically, the IR spectra of immobilized ACMs over SAMs of TA and DTSP exhibit the characteristic amide I band, which is primarily attributed to an out-of-phase combination of C=O and C−N stretchings of amide groups, at 1660 and 1655 cm\(^{-1}\), respectively. Also, the amide II band, which is attributed to an out-of-phase combination of in-plane C−N stretching and N−H bending of amide groups, is observed at 1549 and 1544 cm\(^{-1}\), respectively.

By comparing the spectra corresponding to Au/TA/ACM surfaces before and after their immersion in the solution of rennet, the observed decrease of the intensity of amide I band suggests a partial loose of \(\alpha\)-helix content,\(^{37}\) due to the aggregation of casein micelles. This decrease is accompanied by the appearance of two shoulders at 1620 and 1680 cm\(^{-1}\), attributable to the intermolecular \(\beta\)-sheet aggregates, even though the aggregation process is more complex than a simple \(\alpha\)-helix to aggregated \(\beta\)-sheet transition.\(^{38}\) The spectral contributions of \(\beta\)-sheet aggregates are not clearly detectable, due to the low concentration of immobilized casein micelles over the examined surfaces; however, they appear with low intensities (the overall amide I band is appearing asymmetric around 1660 cm\(^{-1}\)). Other spectral observations based on amide II or/and amide II′ are not further discussed, as the low signal-to-noise ratio at those bands cannot ensure the extraction of safe conclusions. Finally, IR spectra corresponding to Au/TA/ACMs surfaces, before and after incubation in blank solution, are almost identical, thus suggesting that, in the absence of rennet, the secondary structure of casein micelles remains unaffected.

**CONCLUSIONS**

This study employs single-use functional biosensors, based on gold electrodes modified with negatively, neutrally, and positively charged thiol-based SAMs, as probes to evaluate the clotting activity of rennet for the first time. Biosensors that developed on dithiobis-N-succinimidyl propionate SAM were found to exhibit better sensitivity and faster kinetics compared with those developed on thiocic acid or cysteamine SAMs.

The proposed biosensors were successfully tested at various commercial samples in powder or liquid form. Relative changes of the charge-transfer resistance reflect the milk-clotting activity, as it is calculated with a reference method. Interestingly, reliable signal changes were also obtained within 5 min of incubation, indicating the suitability of the proposed biosensors for use in routine analysis. In our opinion, the present work offers a true alternative to the existing instrumental methods or methods based on visual observation, incorporating the simplicity and advantages of biosensors.

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