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# Investigation of an Allergen Adsorption on Amine- and Acid-Terminated Thiol Layers: Influence on Their Affinity to Specific Antibodies

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This work describes the controlled immobilization of a recognized allergen, beta-lactoglobulin, onto gold transducers with the aim of optimizing the elaboration of a biosensor directed against allergen-produced antibodies. This protein was immobilized on both amine- and acid-terminated thiol self-assembled monolayers, and the influence on its affinity to a specific IgG was investigated. For amine-terminated layers, the  $\beta$ -lactoglobulin was immobilized via its surface acid functions implying an activation step with 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride/ester of *N*-hydroxysuccinimide (EDC-NHS). Conversely, the grafting on acid-terminated layer takes advantage of the accessible amine groups that react with the activated acidalkylthiols. The resulting layers of  $\beta$ -lactoglobulin were then submitted to various concentrations of rabbit serum containing  $\beta$ -lactoglobulin specific rabbit immunoglobulin (rIgG), and the antigen/antibody affinity was evaluated using modulated polarization-infrared absorption spectroscopy (PM-IRRAS) and Fourier transform surface plasmon resonance (FT-SPR). Even though for similar concentration, the amount of adsorbed  $\beta$ -lactoglobulin was identical on both surfaces, atomic force microscopy (AFM) images showed a better dispersion for amine-terminated layers. Moreover, the affinity to specific IgG, estimated under static conditions by PM-IRRAS and under dynamic conditions by SPR, was different. Grafting  $\beta$ -lactoglobulin via its acid groups gave an affinity constant 3 times higher than its immobilization via its amine groups despite the fact that the amount of accessible recognition sites appeared to be similar for both systems. This work underlines the importance of the involved chemical groups upon protein immobilization on their biological activity and will be essential for the construction of nondirect biosensors for detecting specific immunoglobulin E (IgE) of allergens.

## 1. Introduction

Allergy to milk and its derivatives is a crucial issue mainly for young children.<sup>1–3</sup> Actually, introducing milk and its derivatives in an allergic organism is carried out in the hospital in the presence of reanimation staff to avoid vital deficiency.<sup>4</sup> Because of the prevalence of IgE-mediated allergy,<sup>5</sup> detecting the presence of these antibodies in patients' blood is of deep importance. Unfortunately, IgE are present at a very low amount in blood ( $IgE/IgG = 10^{-3}$ ) making their detection with a high sensitivity still a great challenge. During the past decade, biosensors have raised increasing interest for the detection of several types of targets such as toxins, bacteria, or pollutants<sup>6–8</sup> and also allergens.<sup>9</sup> Indeed, biosensors ideally combine speed of analysis (hours instead of days), cost effectiveness, and reasonably good sensitivity.<sup>10–12</sup> When designing any biosensing device, the critical step lies in the construction of the so-called sensing layer that includes the element of biorecognition. When the target is IgE, the biosensing layer is often constructed by immobilizing a specific allergen on which the IgE will be recognized.<sup>13</sup> A revealing step implying a secondary anti-IgE

antibody is then applied. The immobilization of the allergens on the transducer surface is crucial because it must guarantee an efficient and specific recognition. Biosensors usually do not include a revealing step. Therefore, the importance of functionalization is absolutely critical because it is the only step providing specificity. In this work, we investigate and optimize this step for a well-identified milk allergen,  $\beta$ -lactoglobulin.

$\beta$ -Lactoglobulin is the major protein in the whey of ruminant milk with a 2–3 g/L concentration.<sup>14,15</sup> The monomeric form of  $\beta$ -lactoglobulin consists of polypeptidic chains with 162 amino acid residues.<sup>16–18</sup> However, this monomeric form is only predominant at low pH; at pH > 3.5, the intermolecular repulsions between monomeric forms decrease and the protein is found predominately as dimers in physiological conditions.<sup>19,20</sup> The equilibrium between monomeric and dimeric forms was widely studied; it depends on a complex interplay of pH and ionic strength.<sup>20–22</sup>  $\beta$ -Lactoglobulin is an acid protein; its isoelectric point is 5.1<sup>23</sup> because of the high number of acid functions on its surface as shown on its 3D structure.<sup>16,17</sup>

In the case of gold-coated transducers, protein platforms are conveniently constructed from self-assembled monolayers of functional thiols by direct covalent attachment.<sup>24,25</sup> Tuning the terminal function of thiols allows grafting  $\beta$ -lactoglobulin either via its acid terminal groups or via the amine ones. In this work, we investigated both possibilities to check the influence on the amount of immobilized proteins and also the consequence on

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the molecular recognition of specific rabbit IgG (rIgG). Two different self-assembled monolayers (SAMs) were constructed using cystamine or diluted mercaptoundecanoic acid. The first molecule led to an amine-terminated layer enabling the grafting of  $\beta$ -lactoglobulin via its acid groups, whereas the second acidic thiol enabled the grafting via the amine functions of  $\beta$ -lactoglobulin. The adsorption of  $\beta$ -lactoglobulin was investigated ex situ using modulated polarization-infrared absorption spectroscopy (PM-IRRAS)<sup>26–29</sup> and in situ using Fourier transform surface plasmon resonance (FT-SPR).<sup>30,31</sup> PM-IRRAS takes advantage of the peptidic signals of the proteins, that is, amide I and II absorption bands at 1650 and 1550  $\text{cm}^{-1}$ , respectively. Binding of proteins leads to an increase of the intensity of these bands proportional to the amount of surface-bound proteic material. FT-SPR measures the reflectivity of a near IR light on a gold surface; the wavelength of the IR light can be varied with high precision thanks to an FT spectrometer.<sup>31</sup> The binding of successive molecular layers on a gold surface can be monitored thanks to the shift of the wavenumber corresponding to the minimum of reflectivity. Eventually, the dispersion of  $\beta$ -lactoglobulin was investigated by atomic force microscopy (AFM). The influence of the grafting groups on the molecular recognition of a specific rIgG was also studied by FT-SPR and PM-IRRAS to determine the affinity constant in each case and to compare the layer efficiencies.

## 2. Experimental Section

**2.1. Materials.** (a) **Chemicals.** 11-Mercaptoundecanoic acid (MUA), 6-mercaptophexanol (MOH), cystamine dihydrochloride (CA), *N*-hydroxysuccinimide (NHS), 1-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), polyethylene glycol sorbitan monolaurate (Tween 20), and  $\beta$ -lactoglobulin (A + B) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Rabbit serum containing specific IgG against  $\beta$ -lactoglobulin ( $\sim$ 650 mg L<sup>-1</sup>) was provided by the team “allergy and environment”<sup>32</sup> (ESPCI/ParisTechLECA, UMR 7195). All solvents were reagent-grade. Reagents were used without any further purification. Experiments were carried out at room temperature if not specified otherwise.

For PM-IRRAS analyses and AFM images, the surfaces, constituted of glass substrates (11 mm × 11 mm) coated successively with a 50 Å thick layer of chromium and a 200 nm thick layer of gold, were purchased from Arrandee (Werther, Germany). The gold-coated substrates were annealed in a butane flame to ensure a good crystallinity of the topmost layers and were rinsed in a bath of absolute ethanol for 15 min before adsorption.

FT-SPR sensors chips are constituted of optical glass substrates (18 mm × 18 mm) coated successively with a 15 Å thick layer of chromium and a 50 nm thick layer of gold (GWC technologies, United States). Prior to use, they were cleaned in an ozone cleaner (Bioforce Nanoscience) rinsed by ethanol and were dried under nitrogen flow. They were not annealed because of the rather thin layer of gold and the fragility of optical glass. They were then functionalized following the same procedure as for the IR sensors.

(b) **Self-Assembled Monolayer Formation and Grafting of  $\beta$ -Lactoglobulin.** Acid-Terminated Layers. Gold substrates were immersed in a binary mixture at 0.01 M (25/75) of MUA (2.5 mM) and MOH (7.5 mM) in 10 mL of absolute ethanol for 3 h and were thoroughly rinsed in ethanol (2 × 5 min) and Milli-Q water (1 × 5 min) and were dried under a flow of dry nitrogen.

The substrates were treated with a solution of NHS (60 mM) and EDC (30 mM) in ultrapure water for 90 min, were rinsed

in Milli-Q water (2 × 5 min), and were dried under a flow of dry nitrogen.

Immobilization of  $\beta$ -lactoglobulin (50 mg/L in phosphate buffer saline pH 7.5: PBS) on gold surfaces was carried out by depositing a 150  $\mu\text{L}$  drop of  $\beta$ -lactoglobulin solution on the Au modified substrates at room temperature for 2 h and by covering with a glass lid to minimize evaporation. After the immobilization step, the modified gold wafers were vigorously rinsed in PBS with 0.1% of Tween 20 (2 × 5 min) and Milli-Q water (1 × 5 min) with agitation and were dried under a flow of dry nitrogen.

**Amine-Terminated Layers.** The substrates were immersed in 10 mL of a 30 mM solution of cystamine (CA) in water for 12 h, were rinsed in Milli-Q water (2 × 5 min), and were dried under a flow of dry nitrogen.

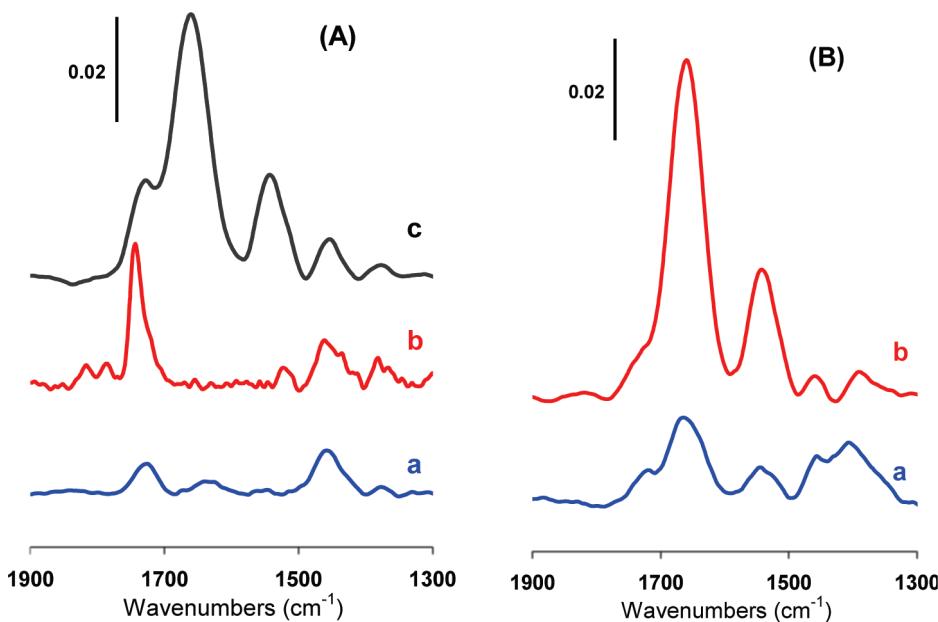
Before the immobilization step, carboxylic acid functions of  $\beta$ -lactoglobulin (50 mg/L in PBS) were activated with a solution of NHS (1.2 equiv) and EDC (1.2 equiv) in ultrapure water for 4 h. Then, immobilization was carried out by depositing a 150  $\mu\text{L}$  drop of  $\beta$ -lactoglobulin activated solution on the Au modified substrates at room temperature for 2 h and by covering with a glass lid. After the immobilization step, the modified gold wafers were vigorously rinsed in PBS with 0.1% of Tween 20 (2 × 5 min) and Milli-Q water (1 × 5 min) with agitation and were dried under a flow of dry nitrogen.

(c) **Bioaffinity Adsorption of Specific  $\beta$ -Lactoglobulin IgG.**  $\beta$ -Lactoglobulin coated sensors were submitted to dilute rabbit serum solutions by depositing a 150  $\mu\text{L}$  drop at room temperature for 1 h. Dilutions were 1/10, 1/50, 1/250, 1/1000, and 1/3000 in PBS that correspond to a  $\beta$ -lactoglobulin specific rIgG concentration of 63.3, 12.7, 2.5, 0.6, and 0.2  $\mu\text{g}/\text{mL}$ , respectively. After the immobilization step, the modified gold wafers were vigorously rinsed in PBS with 0.1% of Tween 20 (2 × 5 min) and Milli-Q water (1 × 5 min) with agitation and were dried under a flow of dry nitrogen.

**2.2. Techniques. PM-IRRAS.** PM-IRRAS spectra were recorded on a commercial Thermo-scientific (France) Nexus spectrometer. The external beam was focused on the sample with a mirror at an optimal incident angle of 80°. A ZnSe grid polarizer and a ZnSe photoelastic modulator, modulating the incident beam between p- and s-polarizations (HINDS Instruments, PEM 90, modulation frequency = 37 kHz), were placed prior to the sample. The light reflected at the sample was then focused onto a nitrogen-cooled mercury cadmium telluride (MCT) detector. The presented spectra result from the sum of 128 scans recorded with 8  $\text{cm}^{-1}$  resolution. The PM-IRRAS signal is given by the differential reflectivity  $\Delta R/R = (R_p - R_s)/(R_p + R_s)$ .<sup>33,34</sup>

**FT-SPR.** Fourier transform surface plasmon resonance (FT-SPR) systems perform wavelength scanning SPR detection in the near-infrared at a fixed angle of incidence.<sup>30,31</sup> Measurements were performed at room temperature with an SPR 100 module from ThermoFisher Scientific (France) equipped with a flow cell mounted on a goniometer. It was inserted in a Thermo Nexus FT-IR spectrometer using a white tungsten–halogen light source. The incidence angle was adjusted to have a minimal reflectivity located at 9000  $\text{cm}^{-1}$  at the beginning of each experiment to be in the best sensitivity region of the InGaAs detector. The  $\beta$ -lactoglobulin solutions were injected using a peristaltic pump Masterflex (Cole Palmer) at 100  $\mu\text{L}/\text{min}$  flow.

**AFM.** Atomic force microscopy images were recorded with a commercial diCaliber AFM microscope from VEECO Instruments Inc. The images were recorded only for PM-IRRAS substrates; FT-SPR sensors were not used because they were



**Figure 1.** PM-IRRAS spectra. (A) After mercaptoundecanoic acid grafting (a), after activation (b), and after  $\beta$ -lactoglobulin immobilization. (B) Upon cystamine grafting (a) and after  $\beta$ -lactoglobulin immobilization (b).

not annealed in a butane flame and the gold surface was not flat enough. Topographic images were taken in noncontact dynamic mode also known as tapping mode. Silicon nitride tips (resonance frequency of  $\sim 280\text{--}400$  kHz, spring constant of  $20\text{--}80$  N/m) have been used. Images were obtained at a constant speed of 2 Hz with a resolution of 512 lines of 512 pixels each. The raw data were processed using the imaging processing software di SpmLabAnalysis from Veeco Instruments Inc.

### 3. Results and Discussion

**SAM Formation and  $\beta$ -Lactoglobulin Immobilization.** The PM-IRRAS spectra corresponding to the successive steps enabling the adsorption of  $\beta$ -lactoglobulin on amine- and acid-terminated layers are shown in Figure 1. For acid-terminated layers, the SAM was constructed using a mercaptoundecanoic acid/mercaptohexanol (25/75) mixture to avoid nonspecific adsorption. Indeed, previous works evidenced that working with mixed SAM reduces considerably the nonspecific adsorption compared to pure MUA SAMs.<sup>28</sup> We experimented with the grafting of  $\beta$ -lactoglobulin on the pure SAM of mercaptoundecanoic acid, but the results (not shown) are not as good (lower amount of fixed proteins, decrease of rIgG recognition). Thus, we focused on mixed SAMs. The formation of these SAMs is evidenced on gold surfaces by the appearance of IR absorption bands of the symmetric  $\nu\text{COO}^-$  and the  $\delta\text{CH}_2$  around  $1450\text{ cm}^{-1}$  and also the  $\nu\text{COOH}$  at  $1723\text{ cm}^{-1}$  (Figure 1A-a). The symmetric and asymmetric  $\nu\text{CH}$ 's in the  $\text{CH}_2$  groups of the chain (not shown) were also observed at  $2927$  and  $2860\text{ cm}^{-1}$ , respectively.

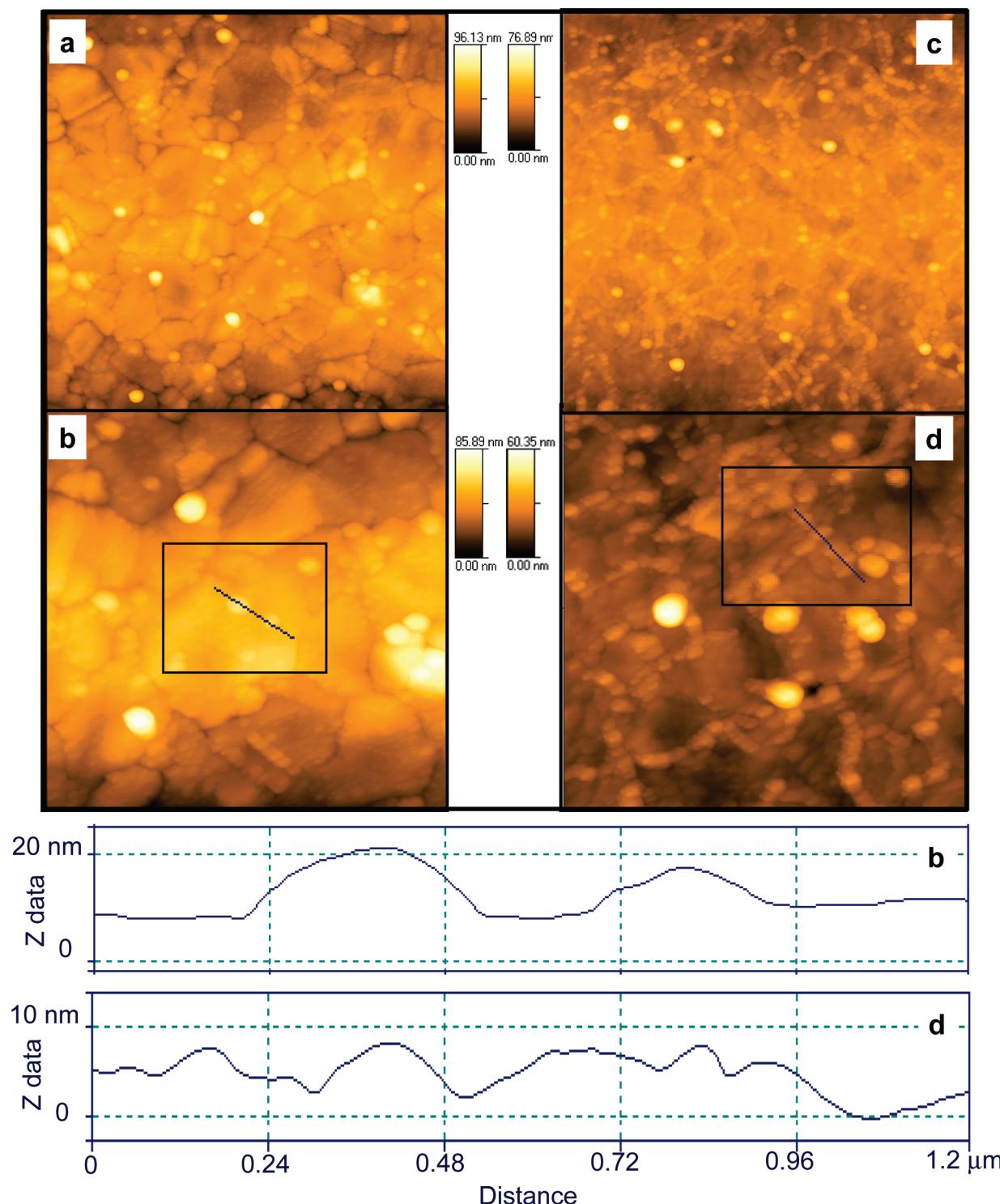
Before grafting  $\beta$ -lactoglobulin, the acid functions are activated using NHS-EDC, and the most intense band characteristic of ester functions appears at  $1735\text{ cm}^{-1}$  (Figure 1A-b). Upon grafting  $\beta$ -lactoglobulin, the ester band intensity decreases and the amide bands I and II appear at  $1650$  and  $1550\text{ cm}^{-1}$ , respectively (Figure 1A-c).

The PM-IRRAS spectrum of a gold surface modified by CA is displayed in Figure 1B-a, and two main vibration bands are observed. One positioned at  $1575\text{ cm}^{-1}$  could be attributed to the deformation symmetric vibration  $\delta_{\text{NH}}$  of primary ammonium functions, and a second one positioned at  $1660\text{--}1630\text{ cm}^{-1}$

likely includes the stretching vibration  $\delta_{\text{NH}}$  of primary amine functions and the deformation asymmetric vibration  $\delta_{\text{NH}}$  of primary ammonium functions.<sup>35</sup> The  $\text{CH}_2$  vibration bands at  $2927$  and  $2860\text{ cm}^{-1}$  were also observed (not shown). The same activation procedure as for MUA/MOH layers was chosen but was applied in solution onto  $\beta$ -lactoglobulin molecules instead of activating the functionalized surfaces. In this case also, the grafting of  $\beta$ -lactoglobulin was efficient and was evidenced on PM-IRRAS spectra by the amide bands (Figure 1B-b). The areas of these bands,  $2.6$  and  $2.4$  au on CA and MUA/MOH layers, respectively, allow a semiquantitative estimation of the amount of grafted proteins.<sup>26</sup> These values indicate that for both systems, the amounts of adsorbed proteins are comparable. This result might be surprising since the  $\beta$ -lactoglobulin outmost layer displays much more acid than amine functions, which leads to the expectation of an easier, or more likely, binding of proteins, whatever their orientation, when interacting with a CA SAM.

These similar amounts may be explained if we consider that steric hindrance on the surface is the limiting factor of the total number of adsorbed proteins rather than the number of accessible reactive groups at this concentration ( $50\text{ }\mu\text{g/mL}$ ). The saturation coverage was likely reached in both cases. To check that, we varied the concentration of  $\beta$ -lactoglobulin, in the  $0\text{--}100\text{ }\mu\text{g/mL}$  range, interacting with the MUA/MOH SAM (see Supporting Information). The results evidenced that beyond a concentration of  $20\text{ }\mu\text{g/mL}$ , the amide band area does not vary indicating a stable amount of adsorbed  $\beta$ -lactoglobulin and corroborating the saturation coverage at a concentration of  $50\text{ }\mu\text{g/mL}$ . All these IR data indicate that saturation coverage is reached at this concentration.

The  $\beta$ -lactoglobulin layers were studied by AFM to compare the protein adsorption modes on the two systems. AFM images of bare gold surfaces are shown in the Supporting Information. On AFM images, shown in Figure 2, one can observe that, though the surfaces were both saturated (from IR data), protein adsorption sites and dispersion show some slight differences. On mixed MUA/MOH layers, upon grafting through the amine functions of  $\beta$ -lactoglobulin, the macromolecules are mainly located on gold terraces with a protein height comprised between  $5$  and  $10\text{ nm}$ , which is consistent with the  $\beta$ -lactoglobulin

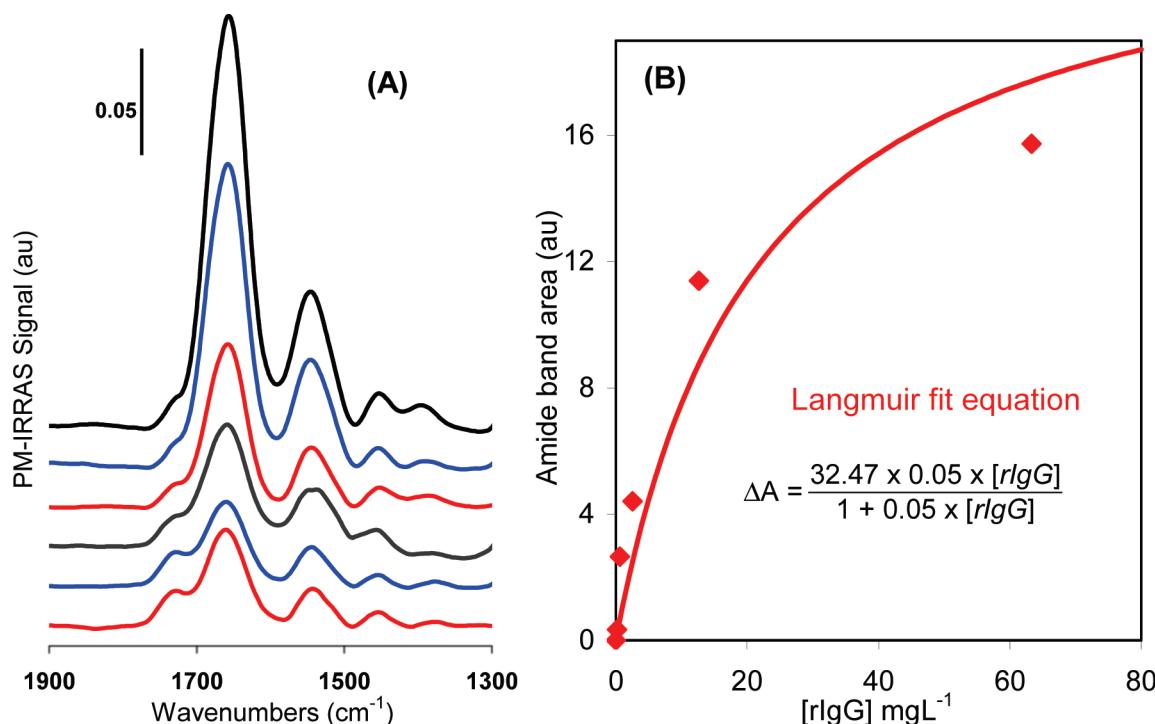


**Figure 2.** AFM images of  $\beta$ -lactoglobulin layers on mixed MUA/MOH (a,  $10 \times 10 \mu\text{m}$ ; b,  $4.7 \times 4.7 \mu\text{m}$ ) and CA SAM (c,  $10 \times 10 \mu\text{m}$ ; d,  $4.7 \times 4.7 \mu\text{m}$ ).

size.<sup>16,17</sup> In addition, one can observe a significant amount of aggregates with a height comprised between 40 and 50 nm. Conversely, on cystamine layers, the adsorption takes place all over the surface with preferential adsorption sites located at the frontier between gold terraces. In this case, the average height of protein layer is consistent with a well-dispersed monolayer of  $\beta$ -lactoglobulin (5–10 nm); isolated proteins can be well distinguished. Few aggregates are observed, but they are smaller than on the acid-terminated layer (height  $\sim 25$  nm). In agreement with IR data, AFM images showed an equivalent amount of adsorbed  $\beta$ -lactoglobulins using either thiol layer that

were better dispersed on CA SAMs. This might be correlated to the structure of the SAM layers themselves; the mixture of an acid/alcohol-terminated thiol does not lead to a homogeneous layer of the two thiols but likely adsorbs forming acid- or alcohol-terminated rich domains,<sup>36</sup> while the pure CA layer is obviously more homogeneous.

**Affinity Constant Estimation: Specific rIgG Adsorption.** Before studying the recognition of specific rIgG, the  $\beta$ -lactoglobulin surfaces were submitted to a goat serum free of specific rIgG. The use of mixed SAM (MUA/MOH) lowers nonspecific adsorption strongly. Contact of a  $\beta$ -lactoglobulin on MUA/MOH



**Figure 3.** (A) PM-IRRAS spectra obtained on acid-terminated layers upon interaction with rabbit serum with increasing concentrations of rIgG from bottom to top: 0, 0.2, 0.6, 2.5, 12.7, and 63.3  $\mu\text{g/mL}$ . (B) Amide band area as function of rIgG concentration; also shown is the Langmuir linear transform equation determined by the fit to the experimental data.

layer with the goat serum induces a small increase of amide bands (lower than 5% of total amide band area) likely because of the adsorption of a few nonspecific proteins. Since the usual blocking proteins, BSA or ovalbumin, are also considered as potential allergens, we could not apply such a blocking step. Observing a small amount of nonspecifically adsorbed proteins is thus not surprising; we took it into account to avoid any overestimate of specific rIgG adsorption. We are now working on new systems to definitively suppress nonspecific adsorption.

Specific rIgG recognition of  $\beta$ -lactoglobulin was studied by two techniques: PM-IRRAS and FT-SPR. The experimental conditions were different since for the latter technique protein adsorption was carried out *ex situ* in static conditions with rinsing and drying at each step, whereas for FT-SPR measurements, the whole experience took place *in situ* in dynamic conditions using a peristaltic pump to inject and let circulate protein solutions. The protein concentrations were kept constant for the two techniques. We previously compared the efficiency of these two techniques and observed a good agreement concerning the amount of specific adsorption sites at saturation.<sup>30</sup> However, this saturation was reached for a lower concentration when working in dynamic mode and was explained by diffusion that occurs under flux and that enhances the reactivity of surface groups specially when working with small planar surfaces. Thus, we expected higher affinity constants when estimating in dynamic conditions.

The PM-IRRAS spectra obtained upon interaction with rabbit serum, when decreasing dilution, are shown in Figure 3A and Figure 4A for MUA/MOH and CA attachment layers, respectively. In both cases, one observes an increase in amide band area upon successive injections of more and more concentrated rabbit serum. These integrated areas were reported as a function of the specific rIgG concentration, and the curve shape indicated a Langmuir type adsorption (see Figure 3B and Figure 4B for MUA/MOH and CA layers, respectively).

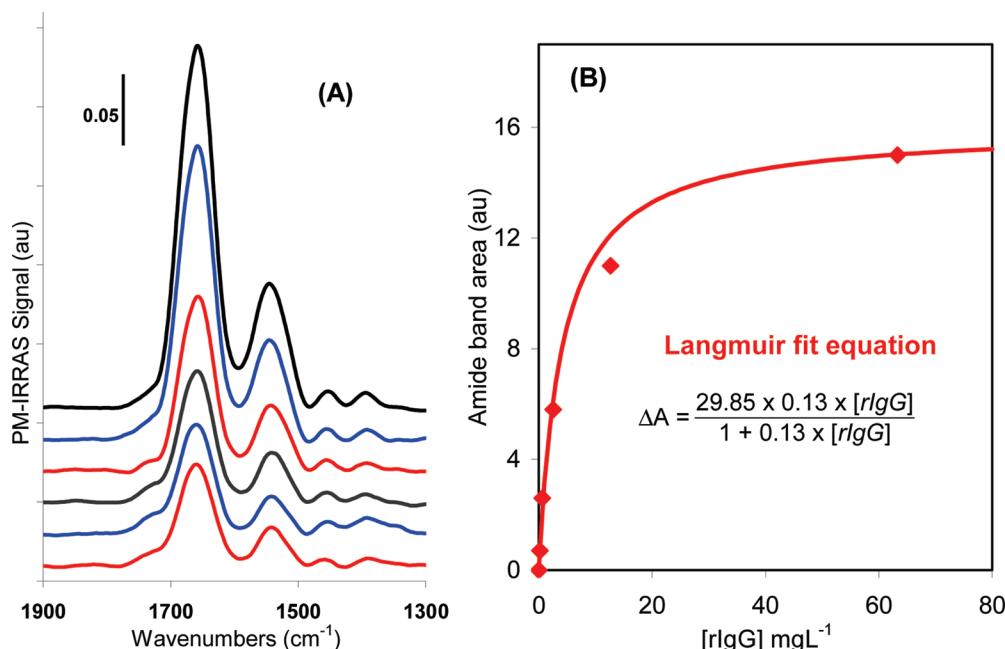
The studied system is not in complete agreement with the Langmuir model. Indeed, the Langmuir model is very simplified, and among its several assumptions, this model implies that all the adsorption sites have the same adsorption energy and that the adsorption of a molecule at one site does not affect either the availability of the other sites or their energy of adsorption.<sup>37,38</sup> We are aware that these criteria cannot be fulfilled especially at high rIgG concentration. Obviously, the protein has several adsorption sites whose nature and strength depend on  $\beta$ -lactoglobulin orientation on the surface. However, we assume that the model can be used in this case to estimate an average affinity value and a site density.

The data points at low concentration were fitted with a nonlinear curve fitting procedure according to the following Langmuir equation:

$$\Delta A = \frac{\Delta A_{\max} \times K \times [\text{rIgG}]}{1 + K \times [\text{rIgG}]} \quad (1)$$

where  $\Delta A$  is the variation of the area of the amide I and II bands and  $K$  is the affinity constant.

The fit results are also shown in Figure 4B and Figure 3B for MUA/MOH and CA layers, respectively. The  $\Delta A$  is similar using CA or MUA/MOH ( $\sim 30$  au in both cases) which indicates that the theoretical saturation coverage does not depend on the functions involved in  $\beta$ -lactoglobulin grafting. This is reasonable since (1) the used antibodies are polyclonal and can thus recognize various epitopes on  $\beta$ -lactoglobulin and (2) the limiting factor is often due to accessibility, and for both cases, we observed comparable amounts of  $\beta$ -lactoglobulin on gold surfaces. A very interesting difference is observed in the affinity constants obtained when immobilizing  $\beta$ -lactoglobulin via its amine functions on MUA/MOH layers or via its acid functions on CA layers. Indeed, these values, estimated from the above-



**Figure 4.** (A) PM-IRRAS spectra obtained on amine-terminated layers upon interaction with rabbit serum with increasing concentrations of rIgG from bottom to top: 0, 0.2, 0.6, 2.5, 12.7, and 63.3 µg/mL. (B) Amide band area as function of rIgG concentration; also shown is the Langmuir linear transform equation determined by the fit to the experimental data.

**TABLE 1: rIgG/β-Lactoglobulin Ratio Based on PM-IRRAS Data for Various Concentrations of rIgG in Rabbit Serum**

[rIgG] µg/mL	0.2	0.6	2.5	12.7	63.3	saturation (from Langmuir fit)
MUA/MOH	0.01	0.07	0.18	0.52	0.62	1.22
CA	0.02	0.09	0.20	0.47	0.57	1.10

discussed model, were equal to 0.05 and 0.13 L mg<sup>-1</sup> on the MUA/MOH and the CA layer, respectively, that is, 2.5 times higher in the latter case. The main difference between these two layers is the involved functions in the immobilization of β-lactoglobulin and its further dispersion on the surface. The higher affinity constant recorded when β-lactoglobulin is immobilized via its acid functions on a CA layers suggests a better recognition of specific rIgG than when the protein is bound via its amine functions; one may deduce that the epitopes involved in the molecular recognition are stronger in the vicinity of its amine functions or that binding the protein via its acidic functions induces less modifications of protein conformation. At high rIgG concentrations, the experimental amide band areas are closer to the Langmuir fit data for CA than for MUA/MOH underlayers.

Taking the molecular weight of the adsorbed proteins into account, the amide band area allows to compare the amounts of adsorbed molecules.<sup>26</sup> We applied this to estimate the rIgG/β-lactoglobulin ratio. The resulting data are shown in Table 1 together with the data at saturation coverage determined from the Langmuir fits. One observes an increase of this ratio with the rIgG concentration, more rapid in the case of the CA layer, with a similar maximum rIgG/β-lactoglobulin ratio close to 1. Thus, even though each rIgG is able to recognize two β-lactoglobulin molecules, the maximum saturation coverage appears to be reached with a ratio close to unity; going beyond 1/2 is nothing but an indication of the high dispersion of β-lactoglobulin on the surface.

These two systems were also studied by FT-SPR in dynamic conditions. The SPR spectra obtained upon admission of rabbit

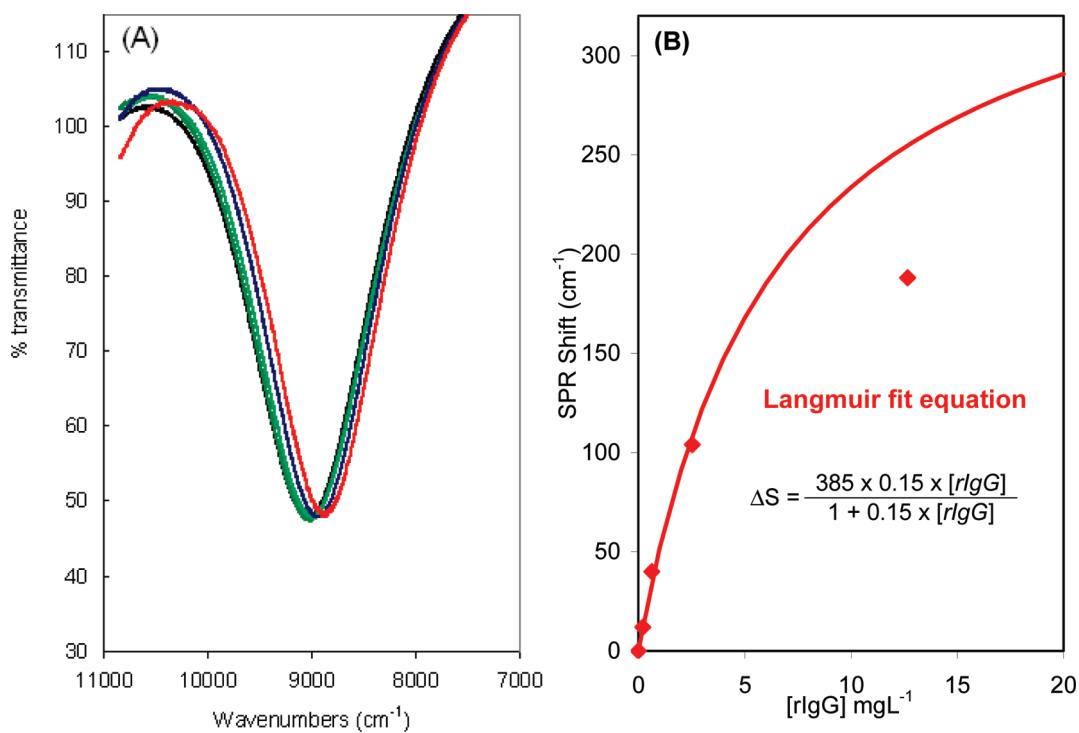
serum solutions of decreasing dilution are shown in Figure 5A and Figure 6A for MUA/MOH and CA layers, respectively. For both cases, one sees successive drops of the wavenumbers clearly indicating increasing amounts of proteins that bond to the surface when increasing the rIgG concentration in the buffer. These wavenumber shifts were reported as a function of the specific rIgG concentration, and the curve shape indicated a Langmuir type adsorption (Figure 5B and Figure 6B for MUA/MOH and CA layers, respectively). The data points were also fitted with a nonlinear curve fitting procedure according to the following Langmuir equation:

$$\Delta S = \frac{\Delta S_{\max} \times K \times [rIgG]}{1 + K \times [rIgG]} \quad (2)$$

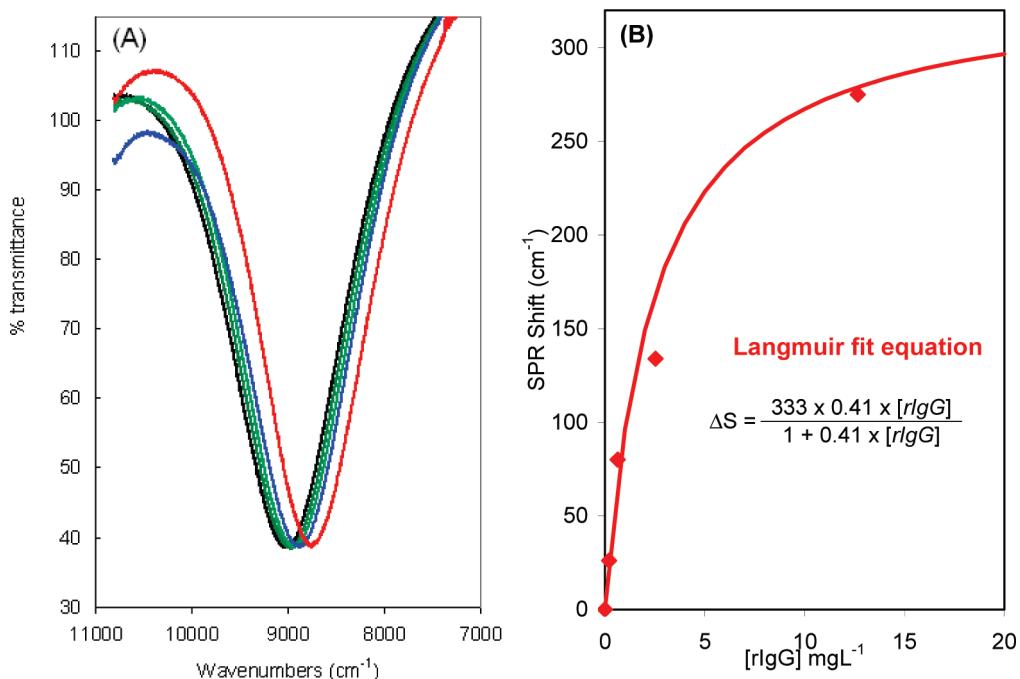
where  $\Delta S$  is the SPR wavenumber shift and  $K$  is the affinity constant.

Here again, the fit results, shown in Figure 5B and Figure 6B for MUA/MOH and CA layers, respectively, are consistent with the Langmuir model. As for PM-IRRAS, the measured SPR shift, at high rIgG concentration, is below the one expected from a Langmuir behavior for the MUA/MOH underlayers. This is obviously related to the poorer dispersion of the proteins on such a layer with the Langmuir model being restricted to noninteracting adsorption sites. This result evidences the dramatic influence of the β-lactoglobulin grafting procedure on the further molecular recognition.

Intercept points may, however, be calculated taking into account the data at low rIgG concentration; they are the same using CA or MUA/MOH layers suggesting identical amounts of specific adsorption sites. The calculated affinity constants were 0.15 and 0.41 L mg<sup>-1</sup> for the MUA/MOH and the CA layer, respectively. These values are higher than those obtained by PM-IRRAS; the difference, directly related to the kinetics of adsorption, may be explained by the dynamic mode used in FT-SPR, whereas the PM-IRRAS experiments were carried out



**Figure 5.** (A) SPR curves obtained on acid-terminated layers upon interaction with rabbit serum with increasing concentrations of rIgG from left to right: 0, 0.2, 0.6, 2.5, and 12.7  $\mu\text{g}/\text{mL}$ . (B) SPR shift as function of rIgG concentration; also shown is the Langmuir linear transform equation determined by the fit to the experimental data.



**Figure 6.** (A) SPR curves obtained on amine-terminated layers upon interaction with rabbit serum with increasing concentrations of rIgG from left to right: 0, 0.2, 0.6, 2.5, and 12.7  $\mu\text{g}/\text{mL}$ . (B) SPR shift as function of rIgG concentration; also shown is the Langmuir linear transform equation determined by the fit to the experimental data.

ex situ in static conditions.<sup>30</sup> Like PM-IRRAS, the FT-SPR technique indicates different affinity constants on the MUA/MOH and the CA layers, and the calculated  $K$  value is 2.5 times higher for the latter than for the former. In other words, both PM-IRRAS and FT-SPR measurements point out a better recognition between rIgG and  $\beta$ -lactoglobulin when the protein is grafted via its acid groups on gold surface.

Thus, the nature of surface functions involved upon the  $\beta$ -lactoglobulin grafting on gold surfaces affects its grafting mode, that is, its conformation and its dispersion, onto gold transducers, and these two factors may favor the exposure of the protein epitopes and may be determining for the molecular recognition phenomena.  $\beta$ -Lactoglobulins are better dispersed on the CA layer and thus are likely more accessible to the rIgG

than on the diluted MUA/MOH layer. As a result, grafting this protein via its acid groups on a CA layer led to an affinity constant 2.5 higher than upon using the external amine functions of  $\beta$ -lactoglobulin for the immobilization.

## Conclusion

The grafting of an allergen,  $\beta$ -lactoglobulin, on gold transducers to build up an allergenic antibody biosensor was investigated. Two different self-assembled monolayers (SAM) were explored: an amine-terminated SAM and an acid-terminated one.  $\beta$ -Lactoglobulin was grafted via its acid or amine groups on the amine- or acid-terminated layers, respectively. Even though both acid and amine grafting led to a similar amount of  $\beta$ -lactoglobulin, we observed some difference in the protein dispersion and an interesting influence of the immobilization technique on the molecular recognition of specific rIgGs. Immobilizing the  $\beta$ -lactoglobulin protein via its acid functions led to a better affinity, 2.5 times higher, than when immobilized via its amine groups toward rIgGs; this is probably due to a better accessibility of the epitopes. This recognition was studied using PM-IRRAS and FT-SPR, and these two techniques led to consistent results regarding the amount of specific adsorption sites and  $\beta$ -lactoglobulin/rIgG affinity values.

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**Supporting Information Available:** (1) Investigation of the saturation coverage of  $\beta$ -lactoglobulin on gold transducers using PM-IRRAS. (2) AFM images of bare gold surfaces. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## References and Notes

- (1) Rona Roberto, J.; Keil, T.; Summers, C.; Gislason, D.; Zuidmeer, L.; Sodergren, E.; Sigurdardottir Sigurveig, T.; Lindner, T.; Goldhahn, K.; Dahlstrom, J.; McBride, D.; Madsen, C. *J. Allergy Clin. Immunol.* **2007**, *120*, 638.
- (2) Moneret-Vautrin, D. A.; Kanny, G.; Morisset, M.; Flabbee, J.; Guenard, L.; Beaudouin, E.; Parisot, L. *Allergy* **2001**, *56*, 1071.
- (3) Kanny, G.; Moneret-Vautrin, D. A.; Flabbee, J.; Beaudouin, E.; Morisset, M.; Thevenin, F. *J. Allergy Clin. Immunol.* **2001**, *108*, 133.
- (4) Vlieg-Boerstra, B. J.; Dubois, A. E. J.; van der Heide, S.; Bijleveld, C. M. A.; Wolt-Plomp, S. A. A.; Oude Elberink, J. N. G.; Kukler, J.; Jansen, D. F.; Venter, C.; Duiverman, E. *J. Allergy* **2008**, *63*, 903.
- (5) Kanny, G. *Allerg. Immunol.* **2001**, *33*, 351.
- (6) Delibato, E.; Bancone, M.; Volpe, G.; De Medici, D.; Moscone, D.; Palleschi, G. *Anal. Lett.* **2005**, *38*, 1569.
- (7) Campas, M.; Prieto-Simon, B.; Marty, J.-L. *Talanta* **2007**, *72*, 884.
- (8) Boujday, S.; Gu, C.; Girardot, M.; Salmain, M.; Pradier, C.-M. *Talanta* **2009**, *78*, 165.
- (9) Yman, I. M.; Eriksson, A.; Johansson, M. A.; Hellenes, K.-E. *J. AOAC Int.* **2006**, *89*, 856.
- (10) Hall, R. H. *Microbes Infect.* **2002**, *4*, 425.
- (11) Patel, P. D. *J. AOAC Int.* **2006**, *89*, 805.
- (12) Lazcka, O.; Del Campo, F. J.; Munoz, F. X. *Biosens. Bioelectron.* **2007**, *22*, 1205.
- (13) Abassi, Y. A.; Jackson, J. A.; Zhu, J.; O’Connell, J.; Wang, X.; Xu, X. *J. Immunol. Methods* **2004**, *292*, 195.
- (14) Walstra, P.; Jenness, R. *Dairy Chemistry and Physics*; Wiley: New York, 1984; p 467.
- (15) Rizzuti, B.; De Santo, M. P.; Guzzi, R. *Langmuir* **2010**, *26*, 1090.
- (16) Monaco, H. L.; Zanotti, G.; Spadon, P.; Bolognesi, M.; Sawyer, L.; Eliopoulos, E. E. *J. Mol. Biol.* **1987**, *197*, 695.
- (17) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. *Nature* **1986**, *324*, 383.
- (18) Taheri-Kafrani, A.; Asgari-Mobarakeh, E.; Bordbara, A.-K.; Haertlé, T. *Colloids Surf., B* **2010**, *75*, 268.
- (19) Townend, R.; Winterbottom, R. J.; Timashoff, S. N. *J. Am. Chem. Soc.* **1960**, *82*, 3161.
- (20) Graziano, G. *Biopolymers* **2009**, *91*, 1182.
- (21) Kontopidis, G.; Holt, C.; Sawyer, L. *J. Mol. Biol.* **2002**, *318*, 1043.
- (22) Sawyer, L.; Kontopidis, G. *Biochim. Biophys. Acta* **2000**, *1482*, 136.
- (23) Wong, D. W. S.; Camirand, W. M.; Pavlath, A. E. *Crit. Rev. Food Sci. Nutr.* **1996**, *36*, 807.
- (24) Briand, E.; Salmain, M.; Herry, J.-M.; Perrot, H.; Compere, C.; Pradier, C.-M. *Biosens. Bioelectron.* **2006**, *22*, 440.
- (25) Chaki, N. K.; Vijayamohan, K. *Biosens. Bioelectron.* **2002**, *17*, 1.
- (26) Boujday, S.; Bantegnie, A.; Briand, E.; Marnet, P.-G.; Salmain, M.; Pradier, C.-M. *J. Phys. Chem. B* **2008**, *112*, 6708.
- (27) Boujday, S.; Briandet, R.; Salmain, M.; Herry, J.-M.; Marnet, P.-G.; Gautier, M.; Pradier, C.-M. *Microchim. Acta* **2008**, *163*, 203.
- (28) Briand, E.; Gu, C.; Boujday, S.; Salmain, M.; Herry, J. M.; Pradier, C. M. *Surf. Sci.* **2007**, *601*, 3850.
- (29) Briand, E.; Salmain, M.; Compere, C.; Pradier, C.-M. *Biosens. Bioelectron.* **2007**, *22*, 2884.
- (30) Boujday, S.; Methivier, C.; Beccard, B.; Pradier, C.-M. *Anal. Biochem.* **2009**, *387*, 194.
- (31) Frutos, A. G.; Weibel, S. C.; Corn, R. M. *Anal. Chem.* **1999**, *71*, 3935.
- (32) Godfrin, D.; Senechal, H.; Sutra, J.-P.; Busnel, J.-M.; Desvaux, F.-X.; Peltre, G. *J. Immunol. Methods* **2007**, *326*, 83.
- (33) Barber, B. J.; Green, M. J.; Saez, E. I.; Corn, R. M. *Anal. Chem.* **1991**, *63*, 55.
- (34) Buffeteau, T.; Desbat, B.; Turlet, J. M. *Appl. Spectrosc.* **1991**, *45*, 380.
- (35) Socrates, G. *Infrared Characteristic Group Frequencies: Tables and Charts*, 2nd ed.; J. Wiley & Sons: New York, 1994.
- (36) Tielens, F.; Costa, D.; Humboldt, V.; Pradier, C.-M. *J. Phys. Chem. C* **2008**, *112*, 182.
- (37) AltIn, O.; Özbelge, H. Ö.; Dogu, T. *J. Colloid Interface Sci.* **1998**, *198*, 130.
- (38) Bolstera, C. H.; Hornberger, G. M. *Soil Sci. Soc. Am. J.* **2007**, *71*, 1796.

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