



Effect of SAM chain length and binding functions on protein adsorption: β -Lactoglobulin and apo-transferrin on gold



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ABSTRACT

Controlled immobilization of proteins is crucial in many applications, including biosensors. Allergen biosensing, for example, requires molecular recognition of suitably immobilized proteins by specific antibodies and sensitive measurement of this interaction.

Self-assembled monolayers (SAMs), terminated by active functions, and are of great interest for the immobilization of biomolecules. The efficiency of further biorecognition involving molecules immobilized on these surfaces demonstrates an interesting dependence on the chain length and terminal function of the SAM. This motivated us to investigate adsorption of two proteins both known as milk allergens— β -lactoglobulin and apo-transferrin—on amine-terminated SAMs. We varied the chain length by using either short or long chain amine-terminated thiols (cysteamine, CEA, and 11-mercaptopundecylamine, MUAM). We also investigated the influence of the addition of a rigid cross-linker, *p*-phenylene diisothiocyanate (PDITC), to these amine layers prior to protein adsorption. Protein binding was studied using polarization modulation-infrared reflection absorption spectroscopy (PM-IRRAS) and atomic force microscopy (AFM) to characterize their amount and dispersion. We found that protein immobilization varies with SAM chain length and is also influenced by the presence of a cross-linker. The presence of a rigid cross-linker favours the binding of proteins on long chain SAMs, while the effect is almost nonexistent on shorter chains. In addition, the presence of the cross-linker induces a better dispersion of the proteins on the surfaces, regardless of the length of the thiols forming the SAMs. The effects of chain length and chemistry of protein binding are discussed.

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1. Introduction

Sensitive biosensors require the sensing surface to be optimized for the binding of the bioreceptor. Alkanethiols and their derivatives form self-assembled monolayers (SAMs) on a number of metals and are well-suited for this purpose due to their ease of formation, their versatile surface properties through functionalization and patterning, and their compatibility with a number of characterization techniques (including PM-IRRAS, QCM, SPR, XPS and AFM) [1–4]. Thiols on gold have been particularly valuable systems as they are simple to use, compatible with biomolecules and often already well characterized. The structure and properties

of a SAM are dependent on the type (or types) of thiolate molecules used in its formation. Researchers have developed techniques to alter the functionalization of thiolate SAMs and to permit various types of interactions with biomolecules, including both resistance to and optimized control over protein adsorption [2,5–7], the latter being the subject of this paper.

In addition to their tail functionality, thiolate chain length is an important factor in determining the properties of the SAM. When *n*-alkanethiols or alkanethiols terminated with small functional groups (i.e. —NH_2 , —COOH , —CN) are used to form SAMs, those with longer alkyl chains consistently form more ordered layers than those formed from shorter chains [8]. Long chains tend to align and form dense, crystalline-like arrangements, thanks to interactions between alkyl chains.

Recently, it has been shown that SAMs of thiols containing aromatic rings permits high intermolecular interactions, along with higher rigidity of the SAMs compared to alkyl-based SAMs [9]. Though aliphatic thiols gave rise to the majority of the studies of

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SAM structure, more recent investigations demonstrated that aromatic chains within the ring may both increase the ordering and stability of the layer—via π – π interactions and cross-linking—and change the electron properties and wettability of the whole layer [10–13].

The amount and orientation of proteins bound to a SAM depends strongly on the nature of the SAM [14,15]. The orientation of proteins on a surface determines which of their sites are accessible for binding. Control over orientation and thus subsequent accessibility is a key factor in understanding biomolecular recognition and in improving biosensor sensitivity and selectivity. This principle is most commonly employed when immobilizing antibodies, for which the F(ab')₂ fragment must be accessible for binding, leaving the Fc fragment to ideally be bound to the surface [16,17]. The amount of proteins adsorbed can also influence the subsequent biorecognition reactions. Specifically, high protein binding reduces the efficiency of biorecognition reactions likely due to steric hindrance—referred to here as “crowding effects” [14,16–19].

A better understanding of these parameters when using alkylthiol SAMs to immobilize proteins, is the goal of the present work, where two proteins of different sizes, β -lactoglobulin and apo-transferrin, were chosen to evaluate the influence of the SAM structure upon the recognition efficiency. β -Lactoglobulin is a globular protein found in milk that has been identified as an allergen. It has a mass of 18 kDa but at physiological pH levels it is typically found in its dimerized form which measures $3.5 \text{ nm} \times 1.8 \text{ nm}$. β -Lactoglobulin is an acid protein with an isoelectric point of 5.1 [20]. Apo-transferrin is a glycoprotein found in blood plasma that binds to iron and controls iron levels in the body; it has also been identified as a potential allergen in milk. It is an ellipsoid protein weighing approximately 80 kDa with a size of $6.2 \text{ nm} \times 2.5 \text{ nm}$ and having an isoelectric point of 5.9 [21]. Besides the interest of immobilizing two proteins of different sizes, studying the recognition of their corresponding IgEs is the first step towards the development of biosensors that can be used to identify a potential milk allergy.

In this work, we immobilized β -lactoglobulin and apo-transferrin on organic thiol SAMs of different alkyl chain lengths using two different immobilization methods, Cf. Fig. 1. SAMs of cysteamine (CEA) or 11-mercaptoundecylamine (MUAM) were formed on gold surfaces. To these surfaces, we covalently bound proteins to the terminal amine groups either directly, through activation of the acid groups on the proteins, or by attaching a rigid cross-linking group to the thiol chains that can bind the amine groups of the proteins. Protein binding was studied using polarization modulation-infrared reflection absorption spectroscopy (PM-IRRAS) and atomic force microscopy (AFM) to characterize their amount and dispersion. We then examined protein accessibility through biorecognition by a corresponding IgG. Finally, we correlated the initial surface characteristics with protein binding and biorecognition results to better understand how SAMs of different chain lengths affect protein adsorption.

2. Experimental

2.1. Materials

Cysteamine (CEA), 11-mercaptoundecylamine (MUAM), *p*-phenylene diisothiocyanate (PDITC), *N*-hydroxysuccinimide (NHS), *N*-1-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), Tween 20, β -lactoglobulin from bovine milk, bovine apo-transferrin, anti-transferrin antibody produced in chicken, phosphate buffered saline (PBS) and bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). Rabbit serum containing specific IgG against β -lactoglobulin (633 mg/L), was provided by the

allergy research team at ESPCI/ParisTech LECA, UMR 7195. All solvents were reagent-grade. Reagents were used without any further purification. Experiments were carried out under ambient conditions.

All experiments were performed on borosilicate glass substrates ($11 \text{ mm} \times 11 \text{ mm}$) coated first with a $2.5 \pm 1.5 \text{ nm}$ thick layer of chromium then a $250 \pm 50 \text{ nm}$ thick layer of gold (Arrandee, Werther, Germany). They were annealed in a butane flame to ensure good crystallinity of the topmost layers, then cleaned with UV-ozone and rinsed in a bath of absolute ethanol (5 mL/chip, 5 min).

2.2. Methods

2.2.1. SAMs formation and activation

Gold-coated sensor chips were immersed in solutions of CEA or MUAM in ethanol (1 mM, 5 mL/chip) and left covered overnight with gentle agitation. The chips were then washed once with ethanol, ultrasonicated for 30 s in order to remove all thiol molecules not covalently bounded to the gold surfaces, then washed once again with ethanol and once with water.

For sensor chips incorporating a PDITC cross-linking group, the chips were immersed in a solution of PDITC in a 9:1 mixture of dimethylformamide (DMF) and pyridine (0.2% w/v). After 2 h, the chips were removed and washed once in DMF/pyridine 9:1, twice in ethanol, and once in water with gentle agitation.

2.2.2. Protein immobilization

β -Lactoglobulin and apo-transferrin solutions of 50 mg/L ($2.78 \mu\text{M}$ and $0.625 \mu\text{M}$ respectively) were prepared in PBS. Two different immobilization methods were used, which are shown in Fig. 1.

2.2.2.1. Amine terminated surfaces: Immobilization by acid groups. Either β -lactoglobulin or apo-transferrin was directly immobilized on the SAMs modified surface following activation of the protein carboxylic acid groups through treatment with a solution of NHS and EDC (each at 1.2 equivalents by mass) for 2.5 h. The proteins were then immobilized by placing a 150 μL drop on the chip surfaces and left for 1 h. The chips were then washed twice in PBS with 0.1% Tween 20 and once in water (5 mL/chip, 5 min) with gentle agitation.

2.2.2.2. Cross-linker terminated surfaces: Immobilization by amine groups. For samples using surfaces incorporating PDITC, a 150 μL drop was placed on each sensor chip and left for 1 h. The chips were then washed twice in PBS with 0.1% Tween 20 and once in water (5 mL/chip, 5 min) with gentle agitation.

2.2.3. Bioaffinity recognition by antibodies

Antibody solutions were prepared at 1/50 dilutions in PBS, resulting in a concentration of 12.7 mg/L (133 nM) and 20 mg/L (84 nM) for β -lactoglobulin and apo-transferrin antibodies respectively. A 150 μL drop was placed on each sensor chip and left for 1 h. The chips were then washed twice in PBS with 0.1% Tween 20 and once in water (5 mL/chip, 5 min) with gentle agitation.

2.3. Characterization techniques

2.3.1. PM-IRRAS

Polarization modulation-infrared reflection absorption spectroscopy (PM-IRRAS) spectra were recorded using a commercial THERMO Nexus 5700 spectrometer. The external beam was focused on the sample with a mirror at an optimal incident angle of 85° . A ZnSe grid polarizer and a ZnSe photoelastic modulator, modulating

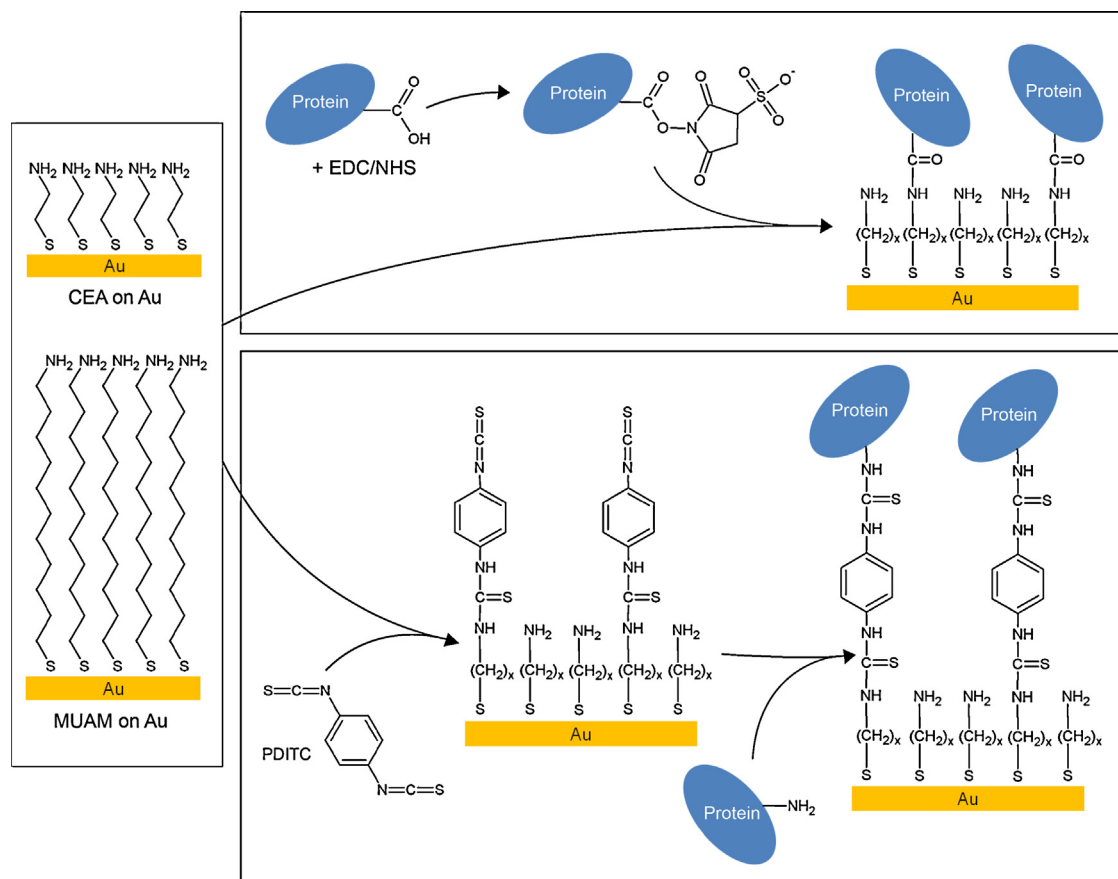


Fig. 1. Schematic drawing of protein immobilization methods on SAMs made from amine-terminated short-chain thiols (cysteamine, CEA) and long-chain thiols (11-mercaptopundecylamine, MUAM). (Top) Immobilization on surface amine groups following EDC/NHS activation of acid groups on proteins. (Bottom) Cross-linker binding and protein immobilization by reaction with amine groups on proteins.

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 MCT detector. The presented spectra result from the sum of 256 scans recorded with 8 cm^{-1} resolution. The PM-IRRAS signal is given by the differential reflectivity,

$$\frac{\Delta R}{R} = \frac{R_p - R_s}{R_p + R_s}$$

Results given represent an average of two to four measurements and error values are given as the standard deviation to give an idea of variability between measurements.

2.3.2. AFM analysis

AFM images were recorded using a commercial AFM (NanoScope 8 MultiMode AFM, Bruker Nano Inc., Nano Surfaces Division, Santa Barbara, CA) equipped with a $150 \times 150 \times 5\text{ }\mu\text{m}$ scanner (J-scanner). The substrates were fixed on a steel sample puck using a small piece of adhesive tape. Images were recorded in peak force tapping mode in air at room temperature ($22\text{--}24^\circ\text{C}$) using oxide-sharpened microfabricated Si_3N_4 cantilevers (Bruker Nano Inc., Nano Surfaces Division, Santa Barbara, CA). The spring constants of the cantilevers were measured using the thermal noise method, yielding values ranging from 0.4 to 0.5 N/m. The curvature radius of silicon nitride tips was about 10 nm (manufacturer specifications). All images shown in this paper are flattened data using a third-order polynomial built-in the Nanoscope Analyse Software v. 1.30 also provided by Bruker Nano Inc.

3. Results

3.1. SAM formation and cross-linker binding

Self-assembled monolayers (SAMs) based on two types of amine-terminated alkanethiols with different chain lengths, cysteamine (CEA) and 11-mercaptopundecylamine (MUAM), were formed on gold surfaces. For samples on which proteins were to be immobilized by their amine groups, a cross-linking group was bound to the amine-terminated thiols (Fig. 1). The surfaces were characterized by PM-IRRAS

3.1.1. PM-IRRAS characterization

The PM-IRRAS spectra of layers of CEA or MUAM formed on gold surfaces are given in Fig. 2. The spectra show several bands in the $2928\text{--}2854\text{ cm}^{-1}$ region, assigned to the asymmetric and symmetric $\nu\text{C-H}$ modes of the alkyl chains [22,23]. The peak intensity for the MUAM samples is greater than that of CEA, which correlates with the longer chain length. Others have shown that the position of the symmetric and asymmetric CH_2 stretching bands is indicative of the order and therefore crystallinity of a SAM [8,13,22], with lower wavenumbers corresponding to a higher degree of order. As expected, MUAM SAMs show peaks at slightly lower wavenumbers (2924 cm^{-1} and 2853 cm^{-1}) than CEA SAMs (2928 cm^{-1} and 2854 cm^{-1}) confirming that the long chain layers are better ordered than the short chain layers.

Upon PDITC binding, a slight shift of the symmetric and asymmetric CH_2 stretching bands to lower wavenumbers appears on both long and short chain surfaces indicating an increase in chain

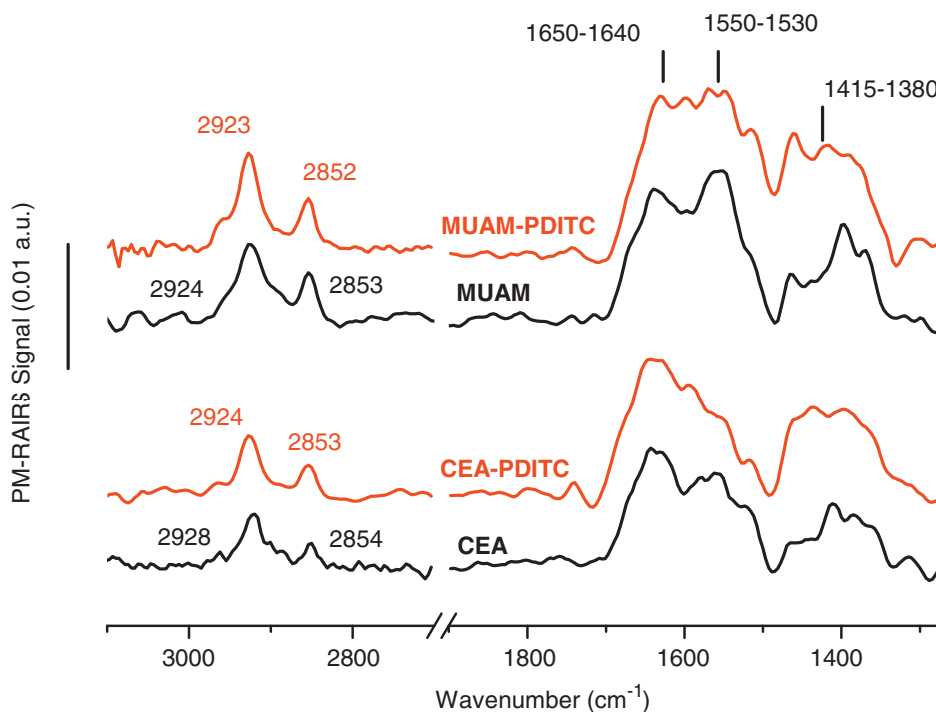


Fig. 2. PM-IRRAS spectra of Au-CEA, Au-CEA after PDITC binding, Au-MUAM and Au-MUAM after PDITC binding.

order. This effect is more pronounced for the CEA layer where, after PDITC binding, the C–H asymmetric stretch band shifts from 2928 cm^{-1} to 2924 cm^{-1} . The orientation of PDITC on the surface may allow intermolecular interaction via π orbitals and/or potentially add rigidity to the structures leading to improved ordering [13,24,25].

On CEA and MUAM spectra, bands in the lower wavenumber region at $1650\text{--}1640\text{ cm}^{-1}$ can be attributed to the N–H deformation vibration, thus confirming the presence of primary amine groups. The peak around 1550 cm^{-1} may be due to the deformation vibration of protonated amine groups. A peak at about 1460 cm^{-1} , more present on the MUAM spectra than on the CEA spectra, can be assigned to the CH_2 scissor vibrations [23].

Following PDITC addition, the peak intensities in this region slightly increase; in particular, the band at 1650 cm^{-1} increases, which may be due to the imine $\text{C}=\text{N}$ stretch of the unreacted isothiocyanate of PDITC, or to remaining primary amine groups due to incomplete cross-linker binding. The intensity increase in the $1380\text{--}1450\text{ cm}^{-1}$ region may be due to $\text{--N}=\text{C}=\text{S}$ groups of PDITC [23,26].

3.2. Protein immobilization

Apo-transferrin and β -lactoglobulin binding was then studied by PM-IRRAS and AFM. For covalent binding on amine-terminated surfaces (CEA or MUAM without cross-linker), the acid groups on the proteins were activated in an EDC/NHS solution, then allowed to bind to the surfaces, while on surfaces bearing the PDITC cross-linker, proteins in a buffered solution were dropped on the surfaces for reaction by their amine groups (Fig. 1). After protein binding and characterization, biorecognition of the corresponding rIgG antibodies was monitored by PM-IRRAS.

3.2.1. PM-IRRAS characterization of the protein layer

Protein binding was evidenced by the presence of intense bands at 1660 cm^{-1} and 1550 cm^{-1} , attributed to amide I and amide II

vibrations respectively; see all spectra labeled (a) on Fig. 3 for β -lactoglobulin binding (spectra following apo-transferrin binding are in the supplementary material section Fig. S1). Comparing band areas provides semi-quantitative information on the amount of bound proteins on the different surfaces [17]. The changes in peak areas are given in Table 1.

The amount of bound β -lactoglobulin varied with chain length. Binding was 60% less on MUAM compared with CEA. However, when a cross-linker was added, binding on MUA increased leading to an amide band area about 60% larger on long than on short chains. On CEA, adding a cross-linker led to a slight decrease in the amide band area, while on MUAM, the same treatment resulted in a signal about two times greater.

Similar trends occurred upon transferrin binding, see Fig. S1 and Table 1. The amide band area following transferrin immobilization was 40% lower on MUAM compared to CEA without cross-linker, but adding a cross-linker led to an increase in protein binding on long chains, the amide band area being multiplied by a factor of three, while the signal hardly changed on short chains (only a 7% decrease) following cross-linker addition.

3.2.2. AFM characterization of the protein layer

The dispersion of proteins on the different surfaces was investigated by AFM.

Fig. 4 shows β -lactoglobulin immobilized on the four different surfaces, each at two different length scales ($10\text{ }\mu\text{m} \times 10\text{ }\mu\text{m}$ and $1\text{ }\mu\text{m} \times 1\text{ }\mu\text{m}$). On both short and long chain amine-terminated thiol surfaces, β -lactoglobulin was found to preferentially bind to the edges of terraces forming thick lines. The smaller scale images (lower inserts of Fig. 4) show that some binding occurs on the terrace surfaces, but without forming a homogeneous layer. On the cross-linker terminated surfaces, for both CEA and MUAM layers, the dispersion was improved and protein binding preferentially occurred on the terrace surfaces. The dispersion on cross-linker terminated long chains appeared more homogeneous than on cross-linker terminated short chains. At the larger scale, agglomerates on the order of several hundred nanometers can be

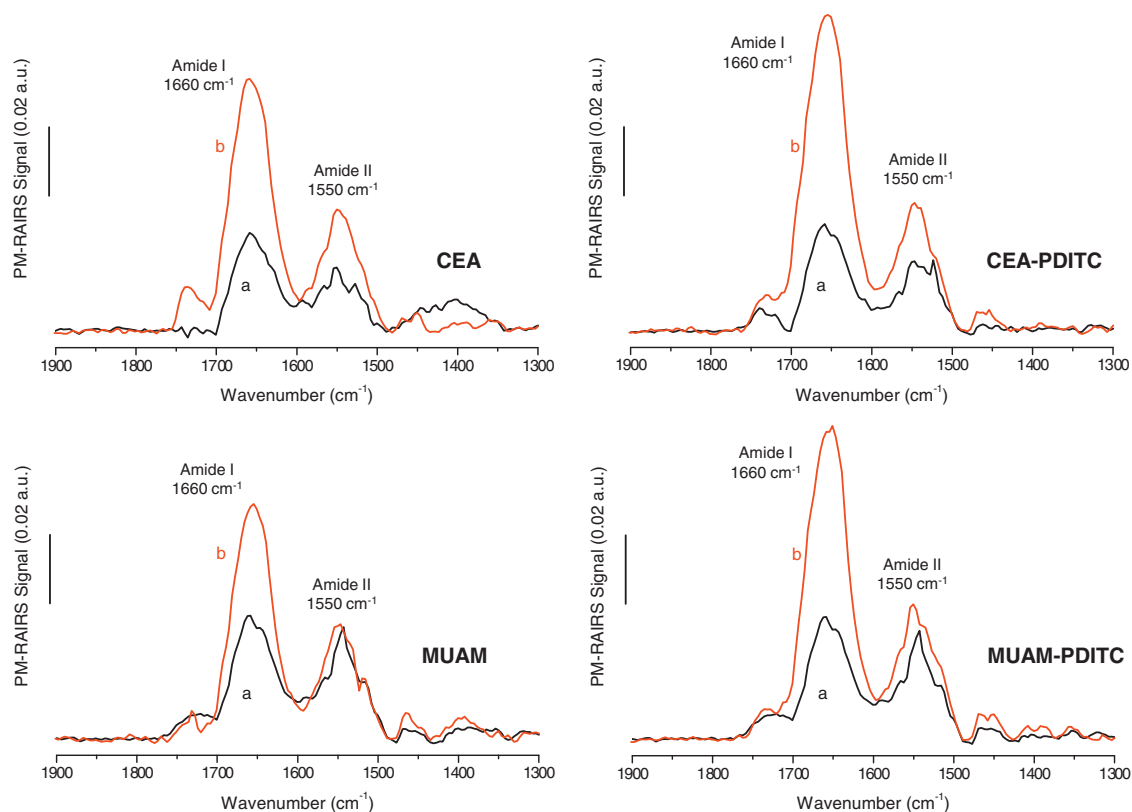


Fig. 3. PM-IRRAS spectra after β -lactoglobulin (a) and antibody recognition (b) on CEA, MUAM, CEA-PDITC and MUAM-PDITC.

observed on the CEA-coated surfaces with or without using a cross-linker.

AFM data of apo-transferrin on the various surfaces (shown in the supplementary material section, Fig. S2) present the same tendencies as β -lactoglobulin. On amine-terminated surfaces, apo-transferrin preferentially binds at the edges of the gold terraces, particularly on long chain-coated surfaces where clear protein lines appear along terrace edges. When using a cross-linker to bind apo-transferrin, binding preferentially occurs on the terraces, thus leading to a more homogeneous protein layer. When comparing the CEA-PDITC and MUAM-PDITC layers, one observes some agglomerates on the short chain layer while very few appear on the long chain layer.

Irrelevant of protein type, a better dispersed layer results on SAMs incorporating a PDITC cross-linker than on amine-terminated SAMs.

3.2.3. Biorecognition by specific antibodies

Antibody recognition of the proteins was monitored by measuring the changes in the amide I and II bands. Results are shown

in Table 1. A useful metric to compare biorecognition on different surfaces is the ratio between IR areas after antibody and protein binding, adjusted for the appropriate molecular masses (see last line of Table 1).

The amide band areas suggest that anti- β -lactoglobulin and anti-transferrin binding was higher on MUAM than on CEA, without cross-linker. The values observed on MUAM without cross-linker are very high, but likely due to non-specific physisorption of antibodies because of abnormally low protein coverage. In all other cases, the Ab/ β -lactoglobulin ratio appears to be almost constant, ≈ 0.2 , no matter the length and terminal function of the SAM; a slight enhancement is observed on CEA when adding the cross-linker.

Upon anti-transferrin binding on amine-terminated surfaces (without cross-linker), the amide band area was higher on long than on short chains and also higher in the presence of a cross-linker. The difference due to chain length was lower when a cross-linker was added. Note again that the difference in binding efficiency with a cross-linker was small, only 20% lower on long chains compared with short chains.

Table 1

Change in area of amide I and II ($1660\text{ cm}^{-1} + 1550\text{ cm}^{-1}$) peaks from PM-IRRAS following binding and ratio of bound antibodies to corresponding proteins adjusted for mass (errors correspond to standard deviation from measurements of two to four separate chips).

	Surfaces without cross-linker		Cross-linker terminated surfaces	
	CEA	MUAM	CEA	MUAM
β -Lactoglobulin binding	2.5 ± 0.1	0.9 ± 0.2	2.3 ± 0.2	3.5 ± 0.3
Anti- β -lactoglobulin binding	4.6 ± 0.2	5.2 ± 0.2	4.9 ± 0.2	7.5 ± 0.3
Ab/Pr (adjusted for mass)	0.22	0.70	0.26	0.26
Apo-transferrin binding	2.8 ± 0.2	1.7 ± 0.2	2.7 ± 0.2	4.7 ± 0.4
Anti-transferrin binding	0.7 ± 0.1	1.4 ± 0.1	1.1 ± 0.1	1.9 ± 0.1
Ab/Pr (adjusted for mass)	0.12	0.50	0.20	0.22

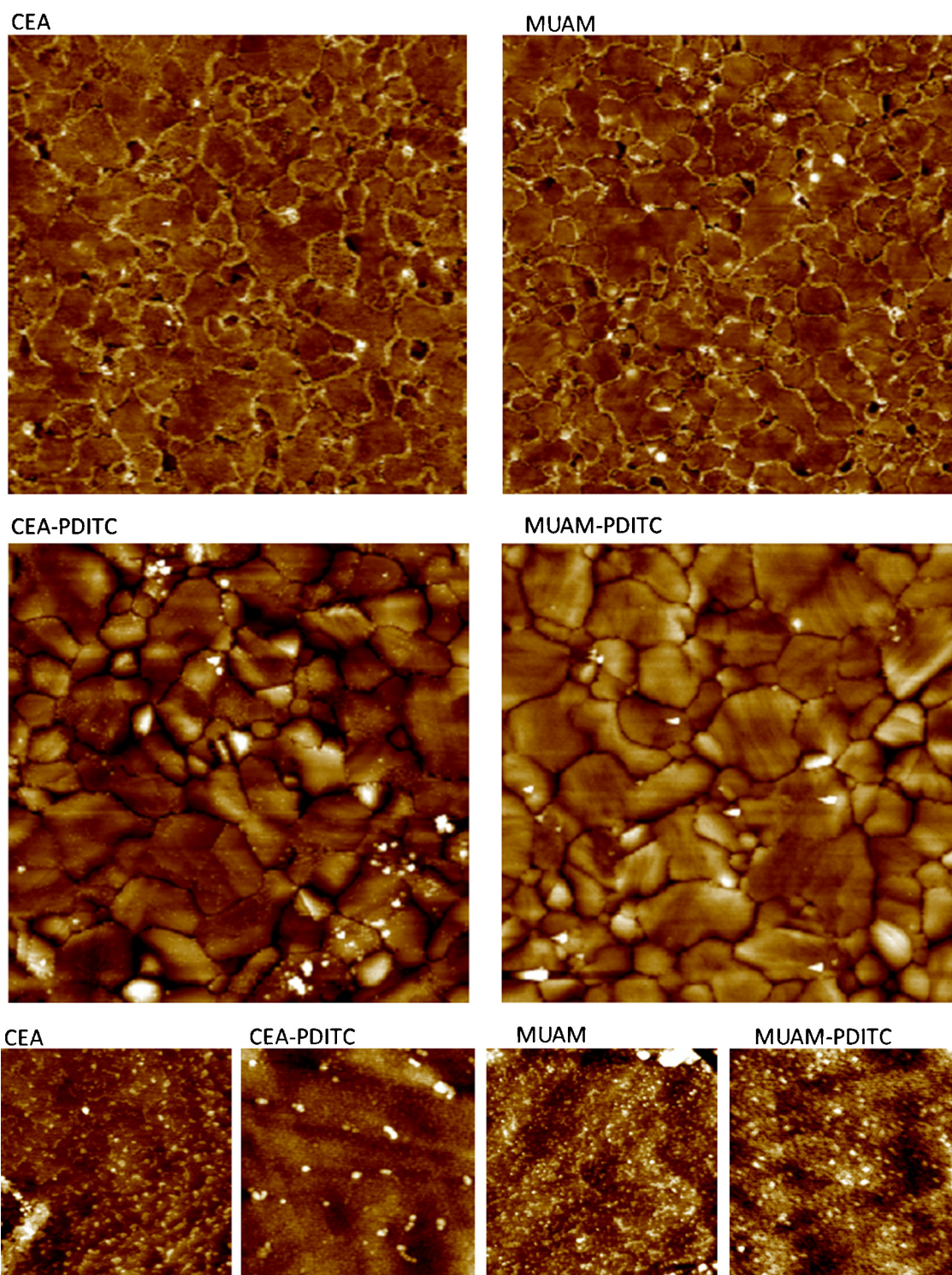


Fig. 4. AFM images of gold-coated substrates following SAM formation and β -lactoglobulin binding. Upper images: scan area: $10\ \mu\text{m} \times 10\ \mu\text{m}$, height scale: 40 nm. Lower images: scan area: $1\ \mu\text{m} \times 1\ \mu\text{m}$, height scale: 10 nm.

To summarize, recognition efficiency between anti-transferrin and transferrin, or anti- β -lactoglobulin and β -lactoglobulin, followed similar trends. On long chains, binding efficiency values were three times greater than on short chains without cross-linker, but this is likely due to non-specific physisorption; once a cross-linker was added, we observed almost no change (about 5%). Again, it is remarkable to observe an almost constant protein-antibody ratio on most SAM layers and for the two proteins.

4. Discussion

In this study, we investigated the binding of two proteins, β -lactoglobulin and apo-transferrin, mainly differing by their sizes, on two amine-terminated SAMs built from short chain cysteamine (CEA) and long chain 11-mercaptoundecylamine (MUAM) amine-terminated thiols. Two immobilization methods were used to attach proteins to the surfaces: a direct binding to the terminal

amine functions, after activating the acid groups of protein in solution, and a binding through the addition of a rigid cross-linker (PDITC), that reacts with the amine groups of proteins. Although the studied proteins were very different in size, the same trends were observed. The influence of the chain length on the amount of adsorbed proteins was very dependent on the method of immobilization.

When proteins were bound via their acid groups directly to the SAMs, through activation in solution of their acid groups, the adsorption on CEA short chains was favoured for both β -lactoglobulin and apo-transferrin. Interestingly, AFM images showed that a non-negligible part of adsorbed proteins were located in the defects on the gold grain boundaries. We previously observed preferential adsorption of β -lactoglobulin in these cracks suggesting that the presence of multiple amine groups around the protein favours its grafting through acid functions [27]. Indeed, Love et al. showed that these defects display many functional groups which can constitute a kind of “cage” (Fig. 7 in Ref. [1]). These groups may thus offer the protein multipoint binding to the surfaces explaining the high density in proteins around these defects. Moreover, as the long chains are more ordered than the short ones (see IR data), proteins are preferentially adsorbed on planar gold terraces rather than in the defects on the gold grain boundaries. Thus, fewer multipoint binding sites would be available for protein grafting. This difference may explain the lowest amount of protein adsorbed on long chain MUAM compared to the short CEA.

When proteins were bound via their amine groups through the addition of a rigid cross-linker, PDITC, adsorption on long MUAM SAMs was favoured for both proteins. In this case, the terminal groups of proteins involved in the binding are amine rather than the acid ones reacting in the previous case. The AFM investigation of protein adsorption showed another interesting difference, the absence of proteins in the defects on the gold grains boundaries. Similar trends were observed previously upon studying β -lactoglobulin grafting via its amine groups on mercaptoundecanoic acid SAM (see Fig. 2 in Ref. [27]). We may thus assume that proteins linked through their amine functions do not react with these cage-shaped binding sites, but more likely with sites consisting of planar terminal reactive functions. Furthermore, this planar geometry for protein amine groups' binding sites is consistent with the higher adsorption on long and better-ordered MUAM SAMs.

Studying the molecular recognition of immobilized proteins by specific antibodies was not very informative when proteins were bound via their acid groups on amine terminated SAMs; the results were affected by the low protein coverage. When the proteins were immobilized on the cross-linker-terminated surfaces, the protein-antibody ratio for β -lactoglobulin was remarkably close for both proteins, and almost constant on the two chain length thiols. This low variability suggests similar orientations of proteins on short CEA and long MUAM chains. In conclusion, the SAM length seems to affect the amount of adsorbed proteins, offering a higher density of binding sites on short chains when proteins are immobilized through their acid groups, while a higher density is observed on long chains when protein immobilization is carried out through their amine groups. Regarding the orientation and accessibility of protein layers, no impact of SAM length was observed; the nature of the chemical groups involved in the interaction rather governs the recognition. One interesting perspective to this work would be the investigation of grafted protein orientation using ToF-SIMS. This technique has already been used to determine the orientation of physically adsorbed β -lactoglobulin on amine and alkyl terminated SAMs [15]. This work suggests that, in the case of β -lactoglobulin adsorption on amine-terminated surfaces, binding may occur between the surface amine groups and activated

glutamic acid groups found in the Glu–Asn–Gly–Glu sequences of the protein. Future work would involve further analysis of the orientation of grafted proteins and correlation to molecular recognition by specific antibodies.

5. Conclusion

We investigated the adsorption of two proteins both known as milk allergens, β -lactoglobulin and apo-transferrin, on amine-terminated SAMs. Two different SAMs were built by using either short or long chain amine-terminated thiols (cysteamine, CEA, and 11-mercaptoundecylamine, MUAM). Two binding modes were explored for each of these SAMs: amide binding through the activation of protein acid groups, and reaction with the amine groups of protein upon addition of a cross-linker, *p*-phenylene diisothiocyanate (PDITC). The same tendencies were observed for both proteins: protein immobilization varied with SAM chain length and with the presence or absence of a cross-linker. When proteins were bound via their acid groups, adsorption was favoured on CEA short chains in cage-like binding sites. Conversely, when proteins were bound via their amine groups, adsorption was favoured on long MUAM SAMs and the binding sites seemed to consist of planar terminal reactive functions. We assessed the molecular recognition of immobilized proteins by specific antibodies and observed no influence of SAM length on the orientation and accessibility of proteins; rather, the nature of the chemical groups involved in the interaction played a larger role in their recognition. Future investigations into protein orientation using ToF-SIMS are in progress and should enable a better understanding, and thus control, of protein orientation on these SAMs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.colsurfb.2014.01.031>.

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