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Binding of α-lactalbumin to oleic acid monolayer and its relevance to formation of HAMLET-like complexes

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ABSTRACT

The α-lactalbumin from human milk forms a cytotoxic protein-fatty acid complex with oleic acid (OA) called HAMLET, which is probably formed in the stomach of a breastfed infant. However, the mechanism of this process is still unclear and in vivo synthesis of this tumoricidal complex has not yet been observed. We used a Langmuir monolayer approach to form an OA monolayer and study the interactions between this fatty acid and milk proteins. The results revealed irreversible adsorption of α-lactalbumin from bovine milk and human milk α-lactalbumin followed by the penetration of the OA monolayer. The process was found to be governed mainly by hydrophobic interactions between protein and the fatty acid. Binding of OA and α-lactalbumin led to the formation of a stable interfacial film that was recognisable as a HAMLET-like complex. These results give credence to the concept of HAMLET formation in a newborn's gastrointestinal system.

1. Introduction

The search for new, efficient methods of cancer treatment is an on-going research effort. Current procedures are based mostly on cytotoxic drugs, which have the complication of several side effects. Therefore, in recent years there have been reported studies on anticancer properties of several natural compounds. Among them, some components of human milk were identified as forming the tumoricidal complex HAMLET (human α-lactalbumin made lethal to tumour cells; Hakansson, Zhivotovsky, Orrenius, Sabharwal, & Svanborg, 1995). Its cytotoxicity was confirmed for skin papillomas, bladder cancer and glioblastoma (Jøhnke & Petersen, 2012).

The complex is formed from α-lactalbumin (α-LA) by removal of Ca^{2+} in the presence of a cofactor, C18:1 *cis* unsaturated fatty acid. The HAMLET is probably formed in a breastfed infant's stomach. Although the in vivo synthesis of HAMLET has not yet been observed, it is suggested that the complex is produced in the stomach because lingual and gastric lipases preferentially hydrolyse some triglycerides and release mainly oleic and palmitic acids (Kuwajima & Nakamura, 2016). Moreover, the HAMLET complex was obtained the first time by chance from human milk under conditions mimicking the infant's gastric environment (Hakansson et al., 1995). It is also notable that α-LA shows an extended lifetime in gastrointestinal tract in the presence of phosphatydylcholine. Moreover, due to the compact, globular structure of α-LA, it is relatively resistant to digestive proteases, such as pepsin and trypsin (Kamau, Cheison, Chen, Liu, & Lu, 2010).

α-LA can bind metal ions, including calcium, which is thought to play a role in the regeneration of native α-LA from the denatured form. On the other hand, native α-LA can be converted to the biologically active form by ion exchange chromatography in the presence of fatty acid. This major whey protein of mammalian milk consists of 123 amino acids and, apart from its metabolic role, it acts in the biosynthesis of lactose, enhances absorption of minerals and it is the source of amino acids and peptides with antibacterial properties. Binding of divalent ions by this protein may also indirectly influence the immune system of infants. Thus, α-LA is an example of a unique globular protein that has several independent physiological functions (Terazima, 2013).

Previous reports suggest that a lipid cofactor plays a key role in HAMLET formation, preventing the protein from folding to the native form that is not cytotoxic. Moreover, it was found that fatty acid takes part in activation steps leading to the killing of cancer cells. To address whether HAMLET complex formation is specific for oleic acid (OA), fatty acids differing in chain length, saturation and configuration of the double bond(s) were investigated. Oleic (C18:1:9 *cis*) and vaccenic (C18:1:11 *cis*) acids were identified as

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the most efficient cofactors in HAMLET complex formation. In addition, C16 or C20 *cis* unsaturated fatty acids and C18 *trans* fatty acids were unable to form biologically active complexes (Ho CS et al., 2012).

Despite numerous studies on the biomedical aspects of the complex, the mechanism of formation of the complex and its properties are not well described. In recent years there has been an ongoing discussion in the literature as to the contributions of OA and α-LA to the cytotoxicity of HAMLET and HAMLET-like complexes (Fontana, Spolaore, & Polverino De Laureto, 2013). Several issues were investigated in the past, such as the stoichiometry of the complex formation, the monomeric or oligomeric structure of α-LA, and the location of OA binding site in the protein as well as specific or nonspecific binding of OA to the protein. The HAMLET structure was recently the matter of scientific reports; some papers evidenced the stoichiometry to be 8.5 OA molecules bound to α-LA in HAMLET, but this value was different for milk of other mammals (Zhong et al., 2015). In other work up to 48 OA molecules were found to bind with protein (Wilhelm et al., 2009). Therefore, there are still new approaches required to investigate this issue.

The cytotoxicity was also confirmed for the bovine equivalent BAMLET (bovine α-lactalbumin made lethal to tumour cells) complex formed by OA and α-LA from bovine milk (Hoque et al., 2015; Rammer et al., 2010; Zhang et al., 2009). Similarly, anticancer properties were observed for other proteins from mammalian milk (Wilhelm et al., 2009), but the mechanisms of formation and activity seems to be different from that for HAMLET, BAMLET and other complexes. Therefore, intensive studies are needed to determine the influence of complexes containing polyunsaturated acids on normal and cancer cells, both in vivo and in vitro. The latter can be easily tested using insoluble monolayers composed of lipids that act as model biological membranes (Knobloc, Suhendro, Zieleniecki, Shapter, & Köper, 2015). On the other hand, this approach is also useful in investigating the interaction of water soluble, surface active compounds (including proteins) with lipid monolayers (Kiss, Dravetzky, Hill, Kutnyánszky, & Varga, 2008; Miano, Zhao, Lu, & Penfold, 2007).

Since OA is able to form a Langmuir monolayer at the air/water interface, the aim of this work was to find a procedure for HAMLET (or BAMLET) reconstruction using a Langmuir monolayer method and to investigate the molecular mechanism of the complex formation. It is worth noting that formation of HAMLET was proven possible the in aqueous phase (Knyazeva et al., 2008) and using many different approaches. Therefore, interactions of OA with α-LA or at the air/water solution interface can be studied. On the other hand, the in vivo synthesis of HAMLET or other HAMLET-like complexes has not been observed yet, therefore we expect our approach will mimic the natural environment of the infant's stomach and conditions of complex formation (in terms of pH, temperature and contact of free fatty acids with α-LA) and help to understand this intriguing phenomenon at the molecular level. To distinguish HAMLET-like complexes and other non-cytotoxic lipid-protein systems, we also used hen egg-white lysozyme as a reference to compare the binding properties of proteins.

The role of lipid-protein interaction is an important issue not only in cancer treatment or drug delivery, but also in the production of stable food emulsions (Barbana & Pérez, 2011; Chiralt, 2003; Dickinson, 2001). In this context, our study may also give insights into the molecular aspects of interaction between the components of infant formula. It would be extremely beneficial for babies who are not breastfed to consume the milk of the most similar composition to natural mothers' milk, especially the substances that have a large impact on human health.

2. Materials and methods

2.1. Materials

The fatty acid used in the experiments was *cis*-9-octadecenoic acid (i.e., oleic acid; 99%, Sigma Aldrich, St. Louis, MO, USA). Lysozyme from chicken egg (LYZ), α-LA from bovine milk type I (BLA I) and human milk α-LA (HLA) were also purchased from Sigma–Aldrich. For preparation of spreading solutions chloroform of high purity Uvasol (Merck) was used. The proteins were dissolved in ultrapure water (18 MΩ cm, 71.98 ± 0.01 mN m^{-1}). Hydrochloric acid (35–38%; Avantor Chemicals) was used to adjust the pH value of the water subphase.

2.2. Methods

2.2.1. Isotherm experiments

All isotherm experiments reported were performed using a Langmuir trough (KSV Nima, Helsinki, Finland) of surface area 273 cm^2 (364 × 75 mm). The subphase was ultrapure water (18 MΩ cm, 71.98 ± 0.01 mN m^{-1}) with HCl added to adjust the pH to 2. A platinum Wilhelmy plate connected to the balance measured the surface pressure π to a resolution of 4 μN m^{-1} as a function of the mean molecular area (A). The samples were spread on the subphase using a gas-tight Hamilton microlitre syringe and left for 5 min. After the evaporation of chloroform, the π-A isotherms were recorded upon compression of the area performed by symmetrical movable barriers. During the measurements the temperature was kept constant (36.6 ± 0.1 °C) with a Julabo circulator (Julabo). These experimental conditions were chosen to mimic the physiological environment of the human stomach. We used the lowest possible pH found in infant's stomach, which varies between 2 and 5 depending on health, age and feeding intervals. Before the experiment, the surface of the subphase was cleaned using a suction pump until the change in the surface pressure after maximum compression was below 0.2 mN m^{-1}.

2.2.2. Surface potential measurement

The surface potential (ΔV) was measured simultaneously with surface pressure using surface potential sensor. The non-destructive, non-contact vibrating capacitor method was applied (SPOT; KSV Nima). The instrument worked with two electrodes: the first immersed in the subphase and the vibrating electrode located just above the water surface. The surface potential was measured with the sensitivity of ±1 mV.

2.2.3. Brewster angle microscopy

Brewster angle microscopy (MicroBAM; KSV Nima) was used to visualise the monolayer morphology. The images were captured during the monolayer compression. A black glass plate was placed under the subphase to absorb the refracted beam. The resolution of image was approximately 6 microns $pixel^{-1}$ and the field of view was 3.6 × 4.0 mm. For microscopic studies, we kept the temperature constant at 21.0 ± 0.1 °C.

2.2.4. Relaxation/penetration experiments

Relaxation experiments were performed for pure OA monolayer and fatty acid/protein systems. The OA film was initially compressed to a desired surface pressure and then the surface area as well as barriers movement were recorded over time. To study the interactions between the components, the OA monolayer was first compressed to a desired surface pressure and then water solution of protein (100 μL) was injected underneath the film to the subphase using the microsyringe to obtain a concentration of protein equal to 0.01 g L^{-1}. The subphase was stirred using magnetic stirrer

placed under the trough. The observed changes in molecular area were presented as A/A_0, which is the ratio of actual molecular area in time t to the molecular area at time t_0 (the moment of injection) at constant surface pressure (Boisselier, Demers, Cantin, & Salesse, 2017).

3. Results and discussion

3.1. The surface pressure-area and surface potential-area isotherms

The π-A isotherms obtained for pure OA monolayer compressed at different rates indicated that the steepest curve was obtained for a compression rate equal to 10 mm min^{-1} (data not shown). Moreover, for this rate, the monolayer collapsed at π (π_{coll}) close to 30 mN m^{-1}, while for slower compression rates, π_{coll} was not achieved. This behaviour was reported earlier for other fatty acids (Heikkila, Kwong, & Cornwell, 1970). Therefore, all further compressions were performed at 10 mm min^{-1}.

As shown in Fig. 1A the collapse of OA monolayer was observed at $\pi = 30$ mN m^{-1}, which is only a little lower than the value reported by Fidalgo Rodríguez, Dynarowicz-Latka, and Miñones Conde (2017) for the same film spread at 10 °C on water. On the other hand, the limiting area is larger for OA spread on subphase at pH 2 and in 36.6 °C. Our results are in agreement with the earlier studies as the formation of stable monolayer by OA on water surface was earlier reported also by Gonçalves Da Silva and Romão (2005) and Gew and Misran (2017).

The compression of the OA monolayer accompanied by the surface potential measurement indicated a decrease of ΔV in the region of molecular areas below the lift-off area (i.e., 71 $Å^2$) followed by the continuous growth up to 0.12 V, which reflects ordering of the molecular dipoles at the interface and the arranging in a more perpendicular orientation to the interface by un-ionised OA molecules. The surface potential change is consistent with previous data, despite the different temperature applied (Marsden & Rideal, 1938); however, it is small in comparison with stearic acid that has a ΔV close to 0.390 V at the same pH as shown by Oliveira (1997), which can be explained by the ability of the saturated acid to have a less tilted orientation at the interface. At a surface pressure close to 30 mN m^{-1} the ΔV value became more stable indicating monolayer collapse. The shape of the ΔV-A curve is in agreement with the transitions observed in the π-A isotherm. The visible fluctuations in surface potential can be explained by the moisture-sensitive sensor that was affected by evaporation of the subphase at relatively high temperature.

It is known that stability, phase transition and collapse of fatty acid monolayers are related with the degree of unsaturation of the molecule, temperature, presence of cations or pH value (Kundu & Langevin, 2008). In our study we obtained the limiting area for OA monolayer $A_{lim} = 47.5$ $Å^2$, which is larger than the value reported earlier for 21 °C on water subphase (41 $Å^2$; Davies & Rideal, 1963). The flexibility of alkyl chains in fatty acid molecules was greater at higher temperatures, resulting in enhanced intermolecular collisions of the tails and the increased distance between the adjacent molecules at the interface. Obviously this intermolecular distance was also much higher than in the case of saturated fatty acids and it was reported to be as high as 6.40 $Å^2$ (Kanicky & Shah, 2002).

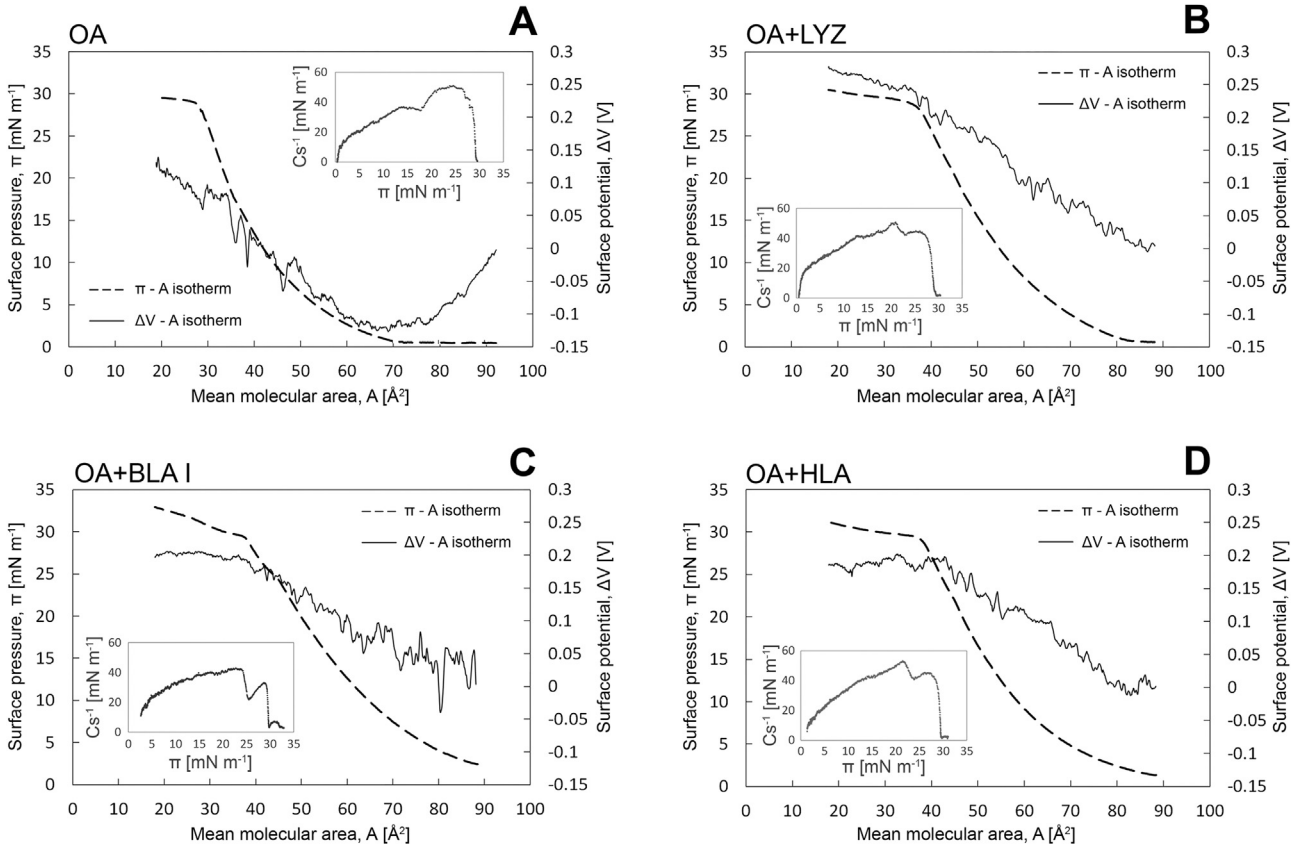

Fig. 1. Surface pressure-area (π-A) isotherms, surface potential-area (ΔV-A) curves and compression modulus (Cs^{-1}) as a function of surface pressure calculated from the isotherm data for (A) oleic acid, (B) oleic acid + lysozyme, (C) oleic acid + bovine α-lactalbumin and (D) oleic acid + human α-lactalbumin. All the monolayers were spread on water subphase at pH 2 and 36.6 °C; the protein concentration was 0.01 g L^{-1}.

Using the π-A isotherm, the static elasticity (compressional modulus, Cs^{-1}) was calculated for OA film according to the equation (Miller & Liggieri, 2009):

$$Cs^{-1} = -A\left(\frac{\partial \pi}{\partial A}\right)_t \quad (1)$$

The compressional modulus reflects variations in the physical state of the monolayers and molecular arrangement. The Cs^{-1} value ranges from 12.5 to 50 mN m^{-1} for liquid–expanded (LE) films, from 50 to 250 for liquid–condensed (LC) films and for Cs^{-1} >250 mN m^{-1} solid state (S) is observed (Davies & Rideal, 1963). The calculated values plotted in Fig. 1 as the function of surface pressure indicated formation of liquid-condensed film with the maximum molecular packing at 25.0 mN m^{-1}. This value corresponds with molecular area equal to 32.0 $Å^2$ and it results from nonlinear structure of unsaturated alkyl chains in OA which do not allow molecules to pack closely. Moreover, the compressibility of OA at pH 2 and 36.6 °C is significantly smaller than the value reported by Fidalgo Rodríguez, Dynarowicz-Latka, and Conde (2017) for 10 °C and water subphase.

The π-A isotherms and ΔV-A curves shown in Fig. 1B–D were recorded for an OA monolayer spread on a water subphase containing lysozyme, bovine α-lactalbumin or human α-lactalbumin. The molecular areas occupied by the protein-fatty acid systems were much larger than for pure OA, indicating adsorption of proteins at the interface; this was more effective for BLA I and HLA according to the limiting area being as high as 75 and 65 $Å^2$, respectively. Among all three proteins, only BLA I caused some fluidisation of the monolayer since the compression modulus decreased considerably below 50 mN m^{-1} in comparison with pure OA, while HLA and LYZ did not affect the packing density. On the other hand, all three proteins influenced the surface potential change, which in all cases reached ~0.20 V at collapse point. The shape of ΔV-A curves was consistent with the phase transition at the π-A isotherms for OA + HLA and OA + BLA I clearly indicating the collapse point. The growth of surface potential for OA + LYZ after reaching a surface area of 37 $Å^2$ suggested further orientational changes at the interface or a different mechanism of collapse than in the case of systems containing α-LA. Moreover, the apparent growth of initial surface pressure (i.e., before the compression began) in Fig. 1C,D indicated the interfacial activity of BLA I and HLA, which was confirmed in a separate experiment (see Fig. S1 in supplementary material). From comparison of all π-A isotherms and ΔV-A curves (see Fig. S1 in supplementary material) we concluded a significant influence of proteins on the organisation of the interfacial layer.

3.2. Hysteresis experiment and Brewster angle microscopy images

The stability and morphology of the film was investigated using Brewster angle microscopy during several compression-expansion cycles of pure OA film and OA spread on subphase containing 0.01 g L^{-1} of the selected protein, HLA. The hysteresis for OA (see Fig. 2A) obtained in the first cycle was quite large while that obtained in the next cycle was smaller. Starting with the 3rd cycle we observed decreasing lift-off area and collapse surface pressure accompanied by an identical shape of compression and expansion curves. This behaviour confirmed continuous degradation of the monolayer caused by dissolution of the material in the subphase. Higher temperature accelerated the monolayer degradation as shown in Fig. 2B. Simultaneously in the early stage of compression of pure OA we observed in BAM image typical gaseous state

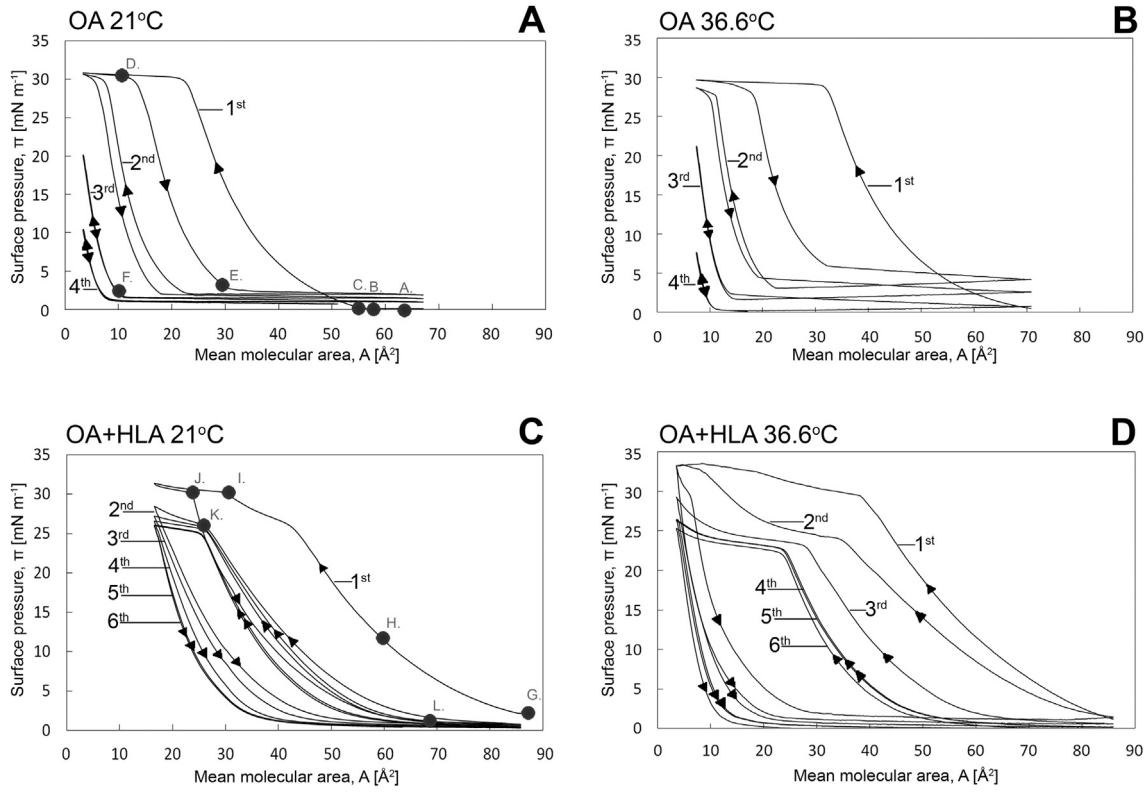

Fig. 2. The compression-expansion cycles obtained at subphase of pH 2 for (A) oleic acid at 21 °C, (B) oleic acid at 36.6 °C, (C) oleic acid + human α-lactalbumin (c_{HLA} = 0.01 g L^{-1}) at 21 °C, and (D) oleic acid + human α-lactalbumin (c_{HLA} = 0.01 g L^{-1}) at 36.6 °C. The compression rate was 10 mm min^{-1}. The letters in panels A and C correspond with the images given in Figs. 3 and 4, respectively.

represented by large areas coexisting with dark regions denoted partial covering the interfacial area by the film (Fig. 3A). Afterwards the foam-like morphology of the OA film was observed (Fig. 3B,C) and at $\pi = 30$ mN m^{-1} the collapse was confirmed (Fig. 3D). At the final stage of hysteresis experiment aggregates of OA appeared, as shown in Fig. 3F.

The hysteresis experiment for OA with HLA injected to the subphase revealed a significant increase of monolayer stability, as shown in Fig. 2C,D. The collapse surface pressure reached 30 mN m^{-1} in the first cycle and remained almost constant at 21 °C. The compression-expansion curves shifted to the lower molecular areas mostly in the first cycle. The BAM images captured simultaneously and shown in Fig. 4 indicated essential changes of the morphology of the OA monolayer in the presence of protein. It seems that stripe-like structure can be associated with the formation of mixed interfacial film formed by HLA molecules adsorbing between the fatty acid. This morphology can be related to different monolayer thickness affected by large HLA molecules distributed between small fatty acid molecules. Thus, the BAM images obtained confirmed the presence of the protein in the mixed film at the interface. The stability of such a mixed film may be the effect of the formation of the HAMLET-like complex between OA and HLA.

3.3. Relaxation/penetration studies

The data gathered in the relaxation experiment for pure OA indicated a significant decrease of A/A_0 ratio for all surface pressures (see Fig. 5A). Of several mechanisms of relaxation including desorption, collapse, evaporation, reaction at the interface, polar group hydration, etc., the simple dissolution mechanism can explain the results presented due to the linear character of the curves. The course of relaxation curves is somewhat different from the results reported for phospholipids where two-step processes were observed and the total material loss from monolayer was smaller and slower (Rodríguez Niño, Lucero, & Rodríguez Patino, 2008). On the other hand, in a manner similar to that for phospholipids, the gradual dissolution of the OA molecules into the subphase occurred at different rates and scale depending on surface pressure applied. Thus, the kinetics of the OA relaxation is strongly associated with molecular packing and the fastest dissolution was observed for higher values of π, demonstrating lower

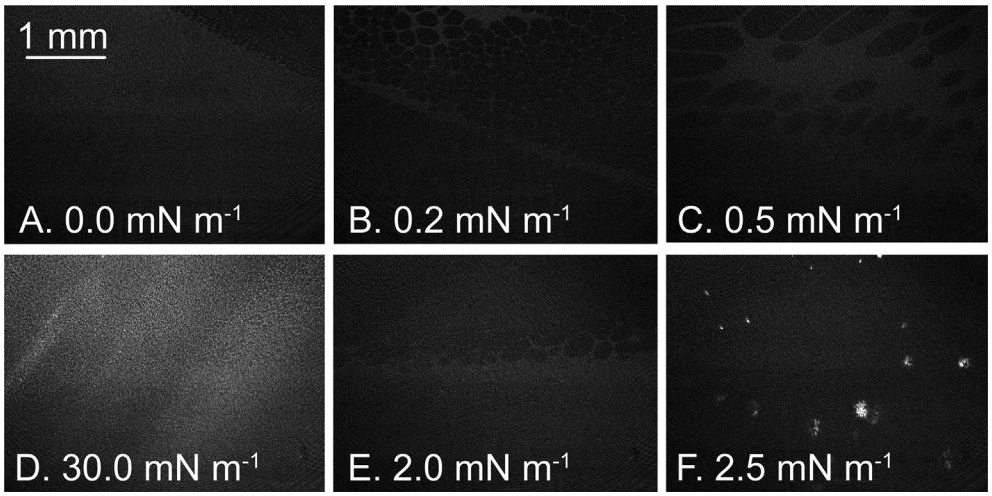

Fig. 3. Brewster angle microscope images captured for pure oleic acid during the hysteresis experiment at 21 °C. The letters on the images correspond with those indicated on the hysteresis loops in Fig. 2A.

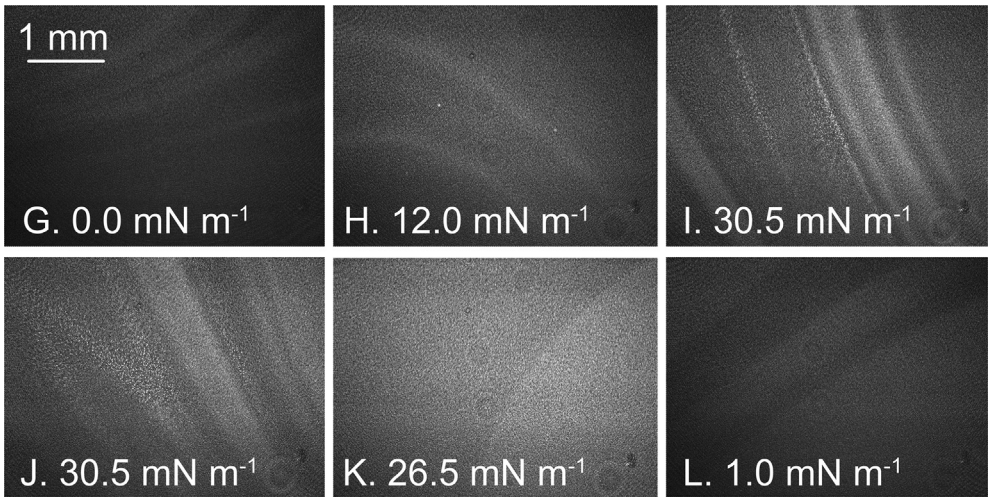

Fig. 4. Brewster angle microscope images captured for oleic acid + human α-lactalbumin during the hysteresis experiment at 21 °C. The letters on the images correspond with those indicated on the hysteresis loop shown in Fig. 2C.

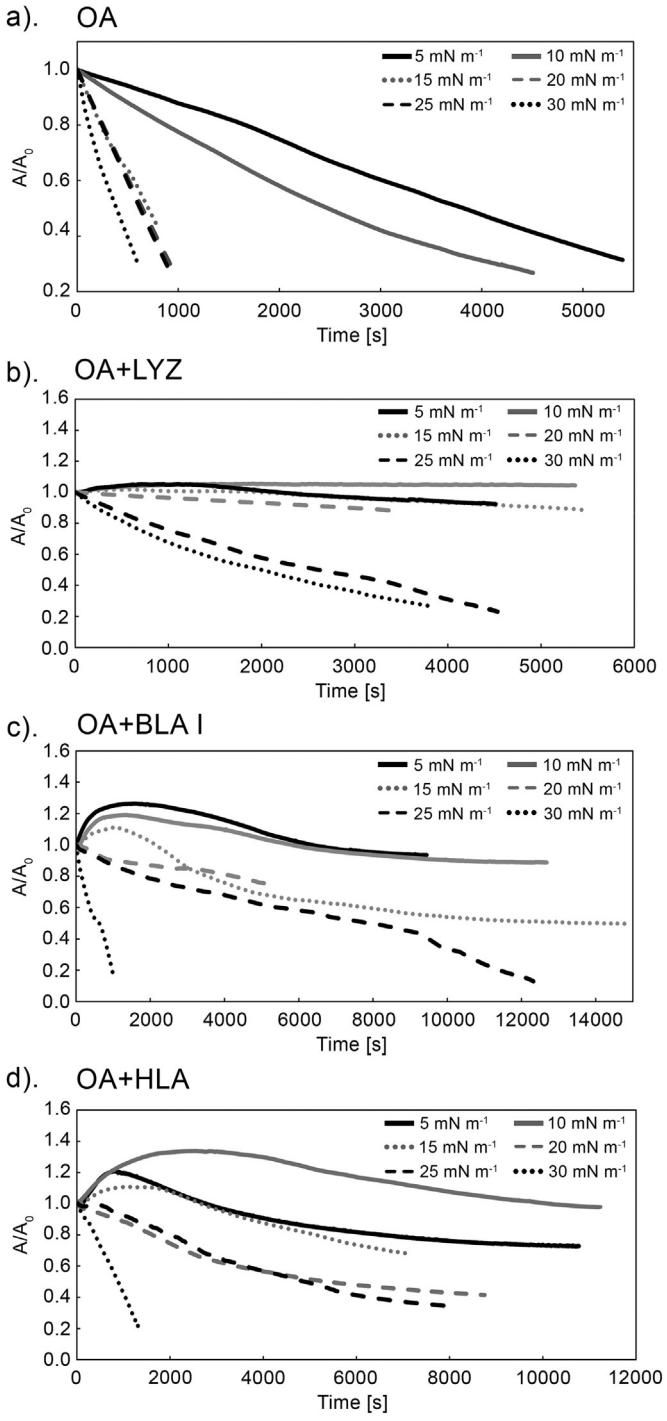

Fig. 5. Relaxation/penetration curves for (A) the oleic acid monolayer, and (B) lysozyme, (C) bovine α-lactalbumin and (D) human α-lactalbumin injected under the oleic acid monolayer after compression to desired surface pressures. In all experiments the subphase was at pH 2 and 36.6 °C; the protein concentration in the subphase was 0.01 g L^{-1}). The time t = 0 is related with the beginning of the relaxation at constant π or injection of the protein to the subphase at the given π.

stability of the condensed monolayer. The slower relaxation for the more expanded monolayer can be attributed to greater hydration of polar head groups in the film. Moreover, at pH 2 OA is far from its surface pKa, which is 9.85 according to Kanicky and Shah (2002). Under these conditions, OA forms an un-ionised film and there is a lack of ion–dipole interactions between carboxylic and carboxylate head groups that would stabilise the monolayer at pH ~ surface pKa. In other words, the weak cohesion between the molecules promotes the film relaxation. Moreover, the high temperature applied accelerates the monolayer dissolution.

The response of the OA monolayer to protein injection was monitored for BLA I and HLA since both proteins are able to form HAMLET-like complexes with OA. Moreover, the experiment with egg-hen white lysozyme was performed as reference. It was reported in the past that the interaction of water-soluble proteins with lipids film may appear both in the interfacial region (i.e., near the polar side of monolayer immersed in water subphase) and in the hydrophobic core (Hollmann, Delfederico, De Antoni, Semorile, & Disalvo, 2010). Generally, the interactions between proteins and lipids may result from hydrophobic interactions as well as electrostatic attraction between the charged molecules (Stă;nciuc, Aprodu, Râpeanu, & Bahrim, 2013). During the experiment performed at least two mechanisms play a role: a dissolution of protein in a lipid monolayer and a penetration of fatty acid film by the protein molecules. Moreover, on the basis of such an experiment, one can distinguish between these two mechanisms. It is also possible to observe competitive lipid-protein effect at the interface. From the curves presented in Fig. 5B it can be concluded that, at low surface pressures, lysozyme interacted with the OA monolayer. Instead of relaxation we observed a slight increase of relative area followed by stabilisation of the film for $\pi \leq 15$ mN m^{-1}, even for a long time period. The further slight decrease of A/A_0 by ~10% was observed only for 5 and 15 mN m^{-1}. From these results we drew conclusions about the complex mechanism of protein adsorption, penetration and incorporation in the monolayer at the air/water interface. In the expanded monolayer, the OA molecules are not densely packed and thus incorporation of protein into the film is facilitated. As the result, a mixed film consisting of OA and lysozyme is formed. In the case of monolayer compressed to 10 mN m^{-1}, the material loss was not observed at all; we noticed small increase of A/A_0 instead. It means that for this system, protein injection induced irreversible reorganisation at the interface. Lysozyme seems to act as a stabilising agent for the OA monolayer. The origin of this may be in: (i) reorganisation of water molecules in the expanded film, (ii) saturation of the interface by OA monolayer penetrated by the protein, (iii) lipid-assisted conformational changes in protein structure, (iv) intermolecular attractions between amino acid residues in lysozyme and OA. Lysozyme is known to be stable and biologically active in acidic pH and at this condition the polar groups at the surface of protein are able to interact with polar head groups of fatty acid molecules, while hydrophobic groups are localised in the interior of the molecule. All the above mechanisms seem to be possible; however, the processes observed are in most cases reversible.

In the case of the films compressed to $\pi \geq 20$ mN m^{-1}, lysozyme injected under the film of OA inhibited monolayer relaxation. It can be observed that loss of monolayer material is decelerated as compared with the case for pure OA. Moreover, the total A/A_0 decrease was considerably smaller than for pure OA. In this case the interaction of lysozyme with OA was exhibited mainly at the interfacial region without marked penetration of the film by protein. The gradual decrease of A/A_0 ratio for high surface pressures result from combined OA relaxation and a fast protein adsorption–desorption step.

In the next step, α-LA from bovine milk was injected under the OA monolayer preceded by a compression to different surface pressures and the response of the film was recorded, as shown in Fig. 5C. For 5 mN m^{-1} the observed increase of the relative molecular area reached maximum value equal to 1.25 and after almost 10000 s the A/A_0 ratio equilibrated at 1. Similar effect was observed

for 10 mN m^{-1}. This response of the OA monolayer on α-LA injection was much faster and stronger than in the case of lysozyme.

According to these results the adsorption of BLA I, saturation of the interface and irreversible binding of protein in the OA film appeared. The diffusion and adsorption of protein to fatty acid monolayer dominated in the first step, while the stabilising effect controlled by penetration of OA by the protein appeared in the later region. This mechanism of penetration was also demonstrated by bovine α-LA binding to phospholipid monolayers (Boisselier et al., 2017). A similar result was also shown for an oppositely charged monolayer of $C_{22}H_{45}SO_4Na$ and haemoglobin that strongly associated at the interface below the isoelectric point (Doty, Schulman, & Matalon, 1949). Here, the electrostatic forces can be neglected, since the OA at pH 2 is protonated. Thus, the nature of this interaction must be related mainly with hydrophobic interactions between OA chains and hydrophobic tryptophan residues from BLA I. This conclusion is in agreement with the results of Cawthern, Permyakov, and Berliner (1996) who observed such behaviour of OA and bovine α-LA at pH < 4, while at neutral pH the interaction was essentially electrostatic. Furthermore, protonated OA is able to form hydrogen bonds with amino acids, which may also stabilise the mixed monolayer at the interface. According to another study, α-LA was completely unable to permeate neutral liposomes composed of phospholipids (Boisselier et al., 2017).

Our results showed that electrostatic forces are not crucial for the penetration of fatty acid monolayer by BLA I and formation of a stable two-component film. Binding-mediated penetration was a mechanism proposed also for the other lipid-protein systems that exhibited a fast and strong response of the film on protein injection (Colacicco, 1970). It was suggested that protein adsorbed under the lipid film undergoes conformational changes that make it extremely surface active and able to penetrate the lipid film. In the case of α-LA and OA the same mechanism can explain the observed effects. Thus, the formation of BAMLET complex at the interface is very probable and it is in agreement with study of (Sullivan, Mok, and Brodkorb (2013) who proved the formation of BAMLET in pH 2.5.

Similar to the results obtained for lysozyme, the course of the curves for OA-BLA I system was dependent on the monolayer packing density. For $\pi = 15$ mN m^{-1}, the relative area increase was still observed, but shortly after that the monolayer relaxation proceeded and reached equilibrium at $A/A_0 \sim 0.6$. For higher surface pressures, OA relaxation was only slightly inhibited by BLA I injection as compared with pure OA.

In the case of HLA injected under the OA monolayer, the effects were similar to those obtained with BLA I, as seen in Fig. 5D. As expected, there was a distinct film expansion observed; however, the strongest response was recorded for 10 mN m^{-1} and for this monolayer state, the equilibrium was reached at the relative area higher than 1. Hence, binding-mediated penetration of OA by HLA molecules was suggested as the mechanism of the film stabilization, identical as for OA + BLA I system. Formation of stable lipid-protein complex after protein injection and adsorption in the OA monolayer can explain the results obtained for 10 mN m^{-1}. The OA relaxation was inhibited for the rest of surface pressures investigated, but this process was slightly faster for OA + HLA.

From comparison of the curves obtained for all systems, we found the variations in monolayer response with the protein type. The highest increase of molecular area at 5 mN m^{-1} was observed after injection of BLA I (see Supplementary material, Table S1). The magnitude of expansion reached 70%, indicating domination of binding-mediated mechanism of penetration. A little slighter effect was observed for HLA, while for lysozyme the interaction with OA was the weakest and mostly reversible. This difference may explain the ability of α-LA to form a cytotoxic complex, in contrast to egg-hen white lysozyme. Moreover, this finding is in agreement with reports that confirmed the highest potential of α-LA among other analogous proteins to bind to OA in a complex bioactive form (Mossberg, Hun Mok, Morozova-Roche, & Svanborg, 2010). This effect could be associated with probable calcium ion release from α-LA molecules in acidic pH that is essential for BAMLET or HAMLET formation. The other explanation may be related to the stoichiometry of binding, which is known to vary for different sources of α-LA; usually four to eight OAs are bound in the HAMLET (Brinkmann, Heegaard, Petersen, Jensenius, & Thiel, 2011; Wilhelm et al., 2009) Here, the same amounts of proteins were used in all the experiments, but the monolayer response was different for LYZ, BLA I and HLA. Binding of BLA I and HLA with OA occurred at various molecular packing, which may confirm the different stoichiometry of BAMLET and HAMLET complexes. From the results presented it can be concluded that OA is able to bind a larger number of BLA I molecules than it can bind HLA molecules. In this context, our approach seems to be also useful in the study of the binding parameters of other complexes forming at the interface.

To get the better insights into the dynamics and stoichiometry of binding, the time of maximum expansion and percentage of area expansion were compared for all the systems studied at various surface pressures on the basis of the penetration experiment (see Supplementary material, Fig. S2). For the liquid expanded film (LE) the rate of expansion was the greatest for HLA; however, the adsorption and penetration of all proteins through the OA monolayer was quite slow. The reason seems to be associated with conformational changes of the protein at the interface, which lost its tertiary structure and thus exposed the hydrophobic regions of the molecules. Only then the irreversible penetration and formation of the complex can take place. The final effect of these subsequent steps was stabilisation of the monolayer at the interface. Surprisingly the longest time needed to reach the maximum expansion was recorded at $\pi = 10$ mN m^{-1} for OA + HLA and OA + LYZ, and at 5 mN m^{-1} for OA + BLA I. This effect may be associated with some disturbances at the interfaces that occurred in the short-time region just after the injection (as indicated in Supplementary material, Figs. S3–5).

It is clear that the distance between the monolayer and diffusing molecules from the subphase was each time the largest for $\pi = 5$ mN m^{-1} and from this point of view, the time of maximal area expansion should change linearly with the increase of surface area. Hence, the nonlinearity of the curves in Fig. S2a in the LE region for OA + LYZ and OA + HLA must be related to a more complex behaviour. To explain this behaviour, the percentage of area expansion in the liquid-expanded region (as shown in Fig. S2b) indicates several effects. First, in the LE region the OA monolayer was affected the most by HLA at 10 mN m^{-1} and BLA I at 5 mN m^{-1}. This means that each injected component has its own kinetic pattern of complex formation. Secondly, the lowest percentage of expansion was obtained in the case of lysozyme, which does not form cytotoxic complex with OA. The reported data may be related to binding stoichiometry, which is different for both types of α-LA and other proteins, as mentioned earlier. Since all the injected proteins have similar molecular weight, it seems that OA interacts with them by binding different numbers of molecules, this being the largest for HLA and BLA I. According to the fact that the cytotoxicity of HAMLET-like complexes is related to the ability of protein to bind with a sufficient amount of lipids, the results shown in Fig. S2b confirmed higher potential of α-LA to form stable, tumoricidal complex with OA in comparison with lysozyme. Since OA was found to be a key factor in the anticancer activity of HAMLET and the protein was recognised as a carrier only, the more OA molecules that bind to α-LA, the larger the dose of antitumor agent available in the complex.

Moreover, by the monitoring the surface activity of water subphase containing injected protein without a fatty acid monolayer, it was found that interfacial activity of the proteins plays significant role in their binding with OA and consequently in cytotoxic activity. As shown in Supplementary material Fig. S6, the surface pressure of water increased with time up to several mN m^{-1} in the presence of α-LA and slightly in the presence of lysozyme. Different slope of the curves in the first 12 min indicated also that HLA and BLA I adsorbed and organised at the air/water interface more rapidly than LYZ. This suggested that HLA and BLA needed less time than lysozyme to diffuse, partially unfold and adopt thermodynamically favourable orientation at the interface.

Our results are consistent with the studies of Leman, Kinsella, and Kilara (1989), Suttiprasit, Krisdhasima, and McGuire (1992) and Alahverdjieva et al. (2008) who investigated the surface activity of globular proteins. The unfolding behaviour of α-LA at the interface was also reported in the literature (Cornec, Cho, & Narsimhan, 1999). Moreover, we compared our results from penetration experiment for α-LA with the data obtained in similar experiment using another milk protein, β-casein (β-CA). For the same concentrations of the proteins injected into the water subphase, the OA monolayer response was different (see Supplementary material Fig. S7). The continuous, intensive area expansion for OA + β-CA differs from the equilibrium reached by the system containing OA and α-LA. The observed effects can be explained by the unfolded structure of β-CA in contrast to α-LA being able to change its tertiary structure at the interface.

Finally, the results shown in Supplementary material Fig. S4a,b emphasised the role of monolayer packing in its response to protein injection. The LE-LC transition dramatically changed the dynamics of expansion produced by proteins. On the other hand, none of the substances caused area expansion in the condensed state due to lack of binding sites in the tightly packed film, thus in this region the relaxation was only inhibited.

4. Conclusions

Oleic acid spread at the air/water interface penetrated by milk proteins was successfully used as a model of infant's stomach where HAMLET complex may form. An approach to investigate the formation of biologically active fatty acid–protein complexes at the air/water interface at molecular scale and simulate in vivo processes was shown. Our study indicated the formation of a mixed OA-protein monolayer in an acidic environment and at physiological temperature, which may be relevant to HAMLET-like complex formation. In this study we found that α-LA can strongly interact with OA monolayer in several steps: (i) diffusion and adsorption at the interface, (ii) incorporation into the film, (iii) formation of protein-lipid complex between the molecules. In our approach, hydrophobic interactions were found to dominate over the electrostatic mechanism of interactions in a mixed film. We confirmed that protein penetration is sensitive to molecular packing of the OA monolayer; only an expanded film allows the protein to enter the monolayer by a binding-mediated mechanism. We suggest that formation of lipid-protein complexes is likely to occur at the interface. The use of different systems also gave us insights into the stoichiometry of binding of the proteins with OA at the interfaces, suggesting the largest number of HLA binding with OA.

We succeeded in the formation of stable protein-lipid complexes at acidic pH composed of OA and α-LA from bovine or human milk. These results are in agreement with the concept of HAMLET formation in a newborn's gastrointestinal system. Moreover, we investigated this phenomena on molecular scale with distinct kinetic steps. Thus, we showed that Langmuir monolayers serve not only as the source of model biological membranes that are frequently used to mimic the drug-lipid bilayer interaction, but also as a tool to study other physiologically relevant phenomena.

The next step in our research towards the understanding of HAMLET formation will be using interfacial rheology to study the viscoelastic properties of such a mixed film as well as determination of the cytotoxicity using the complex formed at the interface and transferred on a solid substrate.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2018.08.017.

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