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ORIGINAL CONTRIBUTION

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Mixed Langmuir monolayers of cholesterol and 'essential' fatty acids

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Abstract Langmuir monolayers of cholesterol and various fatty acids, such as stearic, oleic, linoleic, α-linolenic, and arachidonic acids, spread at the air/water interface are investigated. The system of cholesterol and stearic acid is found to be immiscible, with only one collapse, occurring at the same surface pressure for all composition range. However, surface pressure (π) – area (A) isotherms of cholesterol/unsaturated fatty acids show a characteristic course with two collapse states. The pressure of the first collapse varies with the proportion of the components in the mixture, while the second collapse, occurring at the surface pressure characteristic of cholesterol alone, is independent of mole fraction of the investigated fatty acid. The application of the surface phase rule indicates that the unsaturated fatty acids/cholesterol mixtures are miscible up to the surface pressure corresponding to the first collapse. Negative values of the excess free energy of mixing in all composition ranges prove that the mixtures are stable. The interactions existing in mixtures of cholesterol and unsaturated fatty acids possessing even numbers of double bonds are strongest in the lower region of fatty acid proportion, and the results are consistent with the minimum values of the excess free energy of mixing, indicating the most stable mixtures. For cholesterol and unsaturated fatty acids with odd numbers of double bonds the behavior is different, and the strongest interactions occur in both low and high regions of mole fraction of an acid.

Key words Mixed monolayers · Cholesterol · Unsaturated fatty acids · Interaction · Air/water interface

Introduction

Many phenomena that occur in nature take place at interfaces and involve only surface regions while the bulk almost does not participate in the overall process. The air/water interface is of particular relevance, due to its abundance; however, it remains one of the most difficult to investigate. In the past two decades a number of sophisticated, surface-sensitive physicochemical techniques have been developed for investigating the free water surface, including spectroscopic and optical techniques [1–3]. Traditional methods, which have been known and applied since the beginning of this century when Irvin Langmuir introduced the experimental and theoretical concepts on insoluble monolayers are, nevertheless, still very popular. In particular, surface pressure (π) measurements are widely used in order to determine a number of physicochemical parameters at the interface (such as the equilibrium constant, pH, energy relaxation, rate of chemical reaction), the knowledge of which are of utmost importance as they affect biological activity [4]. A number of publications have proved that the monolayer technique is entirely suitable for researching properties of physiological active materials [5, 6].

Since the analysis of surface pressure (π) – area (A) isotherms has been found to be successful in providing valuable information on the interaction in mixed biological systems (see [6] for a review), in this work we have applied the monolayer technique to examine the interactions in mixed systems of cholesterol with a series of physiologically important, unsaturated fatty acids (UFAs). As has long been known, *cis*-unsaturated fatty acids play a very important role in human nutrition and metabolism [7]. The following compounds are refered to as "essential" fatty acids (EFA): monounsaturated *oleic acid*, which belongs to the ω9 family, and polyunsaturated *α-linolenic* (ω3 family) and *linoleic acids* (ω6 family). The latter polyunsaturated fatty acids, provided in vegetable oils, are desaturated and elongated in the organism to produce arachidonic acid (from linoleic acid) and eicosapentaenoic and docosahexaenoic acids (from α-linolenic acid) [8]. They act as precursors of prostaglandins and related substances, generally refered to as eicosanoids [9]. Another positive role of EFAs is to reduce hypercholesterolemia – and thus decrease one of the main factors involved in heart disease [10] – by either lowering the level of LDL-bound cholesterol in blood (oleic acid) or diverting cholesterol to other body compartments (linoleic acid) [11].

Since both cholesterol [12] and unsaturated fatty acids [13] are capable of Langmuir monolayer formation at the air/water interface, the monolayer technique seems to be an appropriate tool for examining in vitro hypocholesterolemic effect of EFAs. The nature and strength of interaction is discussed applying the methodology described below. The interactions existing in cholesterol/EFAs are compared with those occurring in the mixtures of cholesterol with stearic acid. This classical film-forming compound serves as a model lipid-like molecule in many monolayer studies.

Interactions in Langmuir films

Interactions in mixed insoluble monolayers can be studied from the point of view of miscibility between their components. In this approach, the mixed monolayer is treated as a 2D solution and the interpretation of the existence of interactions is based on simple additivity relations of component mean molecular areas (ω) at various surface pressures, or surface pressures (π) at constant area as a function of their composition [14]. The linear dependence can indicate ideal mixtures of non-interacting molecules, or complete immiscibility of the components. On the other hand, deviations from these conditions indicate miscibility and non-ideality.

Quantitatively, molecular interaction, or rather "excess" interactions [14], are usually expressed as an excess of thermodynamic functions. The excess free energy of mixing (ΔG_{exc}) can be evaluated directly from the π/A isotherms using the following equation [15, 16]:

$$\Delta G^{exc} = \int_0^{\pi} \omega^{exc} d\pi \tag{1}$$

If the temperature dependence of the investigated mixed system is known, one can also calculate the excess entropy (ΔS_{exc}) using the Gibbs-Helmholtz equation for 2D systems [15], and then the excess enthalpy (ΔH_{exc}) of mixing.

From ΔG^{exc} values, the interaction parameter (α) and the interaction energy (Δh) can be calculated as follows [17, 18]:

$$\alpha = \frac{\Delta G^{exc}}{RT(X_1 X_2^2 + X_2 X_1^2)} \tag{2}$$

$$\Delta h = \frac{RT\alpha}{Z} \tag{3}$$

where Z is the coordination number, which can be taken as 6, according to the Quickendem and Tan model [19].

Experimental

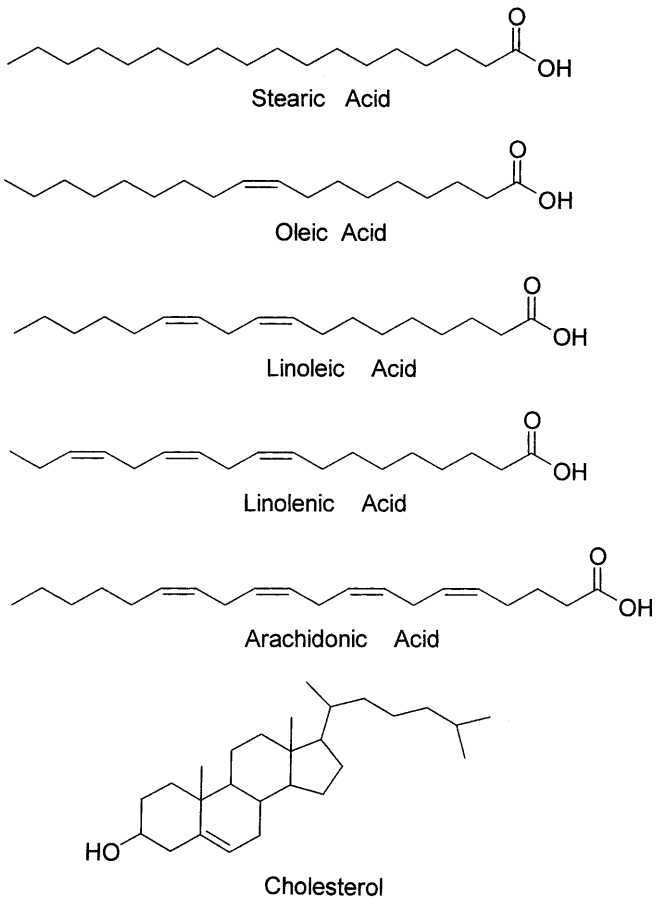
Cholesterol (98%), stearic acid (STA, octadecanoic acid, 99%), oleic acid (OA, *cis*-octadecenoic acid, 99%) linoleic acid (LA, *cis*-9-*cis*-12-octadecadienoic acid, 99%), α-linolenic acid (α-LA, *cis*-9-*cis*-12-*cis*-15-octadecatrienoic acid, 99%) and arachidonic acid (ARA, *cis*-5-*cis*-8-*cis*-11-*cis*-14-eicosatetraenoic acid, 99%) were purchased from Sigma and used without further purification.

The spreading solutions for the Langmuir experiments were prepared by dissolving the compound in spectroscopic grade chloroform/ethanol (4:1 v/v) mixture. Ultrapure water produced by a Milli-Q water purification system (resistivity = 18.2 M cm) was used as subphase. A fixed total number of molecules of the two components in mixture (2.3×10^{16}) was always deposited on the subphase, using a Microman-Gilson microsyringe precise to ± 0.2 μl. After spreading, the monolayers were left for 5 min for the solvent to evaporate, and then compression was initiated. Routine monolayer studies were carried out with a Lauda FW-1 balance furnished with a Teflon trough (total area = 562 cm^2), placed on an anti-vibration table. Monolayers were compressed with a barrier speed of 99 cm^2/min. This rate ensured reproducible results, especially for unsaturated fatty acids which are known to have the tendency to dissolve in the aqueous subphase.

The surface pressure of the floating monolayer was measured to an accuracy of ± 0.1 mN/m. Experiments were performed at various temperatures ranging from 5 °C to 30 °C. The subphase temperature was controlled thermostatically to within 0.1 °C by a circulating water system.

Results and discussion

The schematic representation of the investigated compounds is shown in Scheme 1. Surface pressure/area isotherms for the investigated fatty acids alone and their mixtures with cholesterol are presented in Fig. 1a–e. Although the experiments were performed at various subphase temperatures, ranging from 5 °C to 30 °C, the



Scheme 1 Chemical formulas of the investigated compounds

overall behavior is essentially the same for all temperatures studied. Monolayers of cholesterol and stearic acid are almost not influenced by temperature; however, the isotherms of unsaturated fatty acids tend to shift gradually towards lower molecular areas with increasing temperature. This effect has already been observed for unsaturated fatty acids, and is due to the enhanced dissolution of monolayer molecules into the subphase, caused by the presence of double bond(s) in the apolar chain [13]. To minimize the effect caused by the loss of molecules from the surface, the analysis of the results is restricted to low subphase temperature and the results in Fig. 1 are represented only by the isotherms recorded at 5 °C.

Surface pressure/area isotherms

STA/Cholesterol

The monolayer of cholesterol is a condensed-type of low compressibility (2.6×10^{-3} m/mN; compressional modulus, $c_s^{-1} = 385$ mN/m) with the limiting molecular area, ω_0 (obtained by extrapolation of the steep linear part of the π/A isotherm to $\pi = 0$) ca. 39 Å^2/molecule. This is in agreement with values already reported for Langmuir monolayers of cholesterol [12]. The film collapses at ca. 53 mN/m at an area of 35 Å^2/molecule. As has already been observed for other sterols [20], the isotherms recorded at temperatures of 5–30 °C nearly coincide.

Stearic acid, a model film-forming molecule, is certainly one of the most frequently investigated substances for monolayer studies. The well-known isotherm of stearic acid shows – characteristic of this compound – phase transition from LE to LC state at $\pi \sim 24$ mN/m and area of 17 Å^2/molecule, with the limiting molecular area $\omega_0 = 20$ Å^2/molecule. Upon dynamic compression conditions applied in this work, the monolayer collapses at ca. 60 mN/m.

All mixed STA/cholesterol isotherms lie between those for pure components. Apart from the isotherm for $X_{STA} = 0.9$, which resembles that for pure stearic acid in the sense of the presence of typical phase transition, all the remaining mixtures show the characteristics of cholesterol-type monolayer and collapse at the same pressure as that for pure cholesterol. Our previous detailed investigation on STA/cholesterol mixtures [21] revealed that, upon further compression after the collapse, the surface pressure of mixed monolayers rises again and a second collapse, at roughly the same surface pressure as it occurs for pure stearic acid monolayer, appears in the course of the isotherm. Such behavior of a mixed monolayer, with two clear collapse states corresponding to pure components, indicates that the system is immiscible [2, 22].

Unsaturated fatty acids/cholesterol

Figure 1b–e shows the pressure/area isotherms at 5 °C for mixtures of cholesterol with OA, LA, α-LA, and ARA. The expanded character of the isotherms of unsaturated fatty acids is typical of "liquid" surfactants [13]. The lift-off areas (70–75 Å^2/molecule) are much higher than that for stearic acid (25 Å^2/molecule), and the limiting molecular areas are 53.5 Å^2/molecule for OA and 44.5 Å^2/molecule for LA and α-LA acids, and 63 Å^2/molecule for ARA. The collapse pressure is close to 30 mN/m and is gradual or liquid-like, contrary to the sharply defined or solid-like collapse observed for "solid" surfactants, as stearic acid or other saturated long-chain fatty acids. The compressibility is in order of ca. 0.02 m/mN which is much greater than for stearic acid or cholesterol.

The general behavior of mixed systems of cholesterol and the investigated UFAs is similar; first it is evident that cholesterol increases the collapse pressure in its mixtures with unsaturated fatty acids. As a result, the

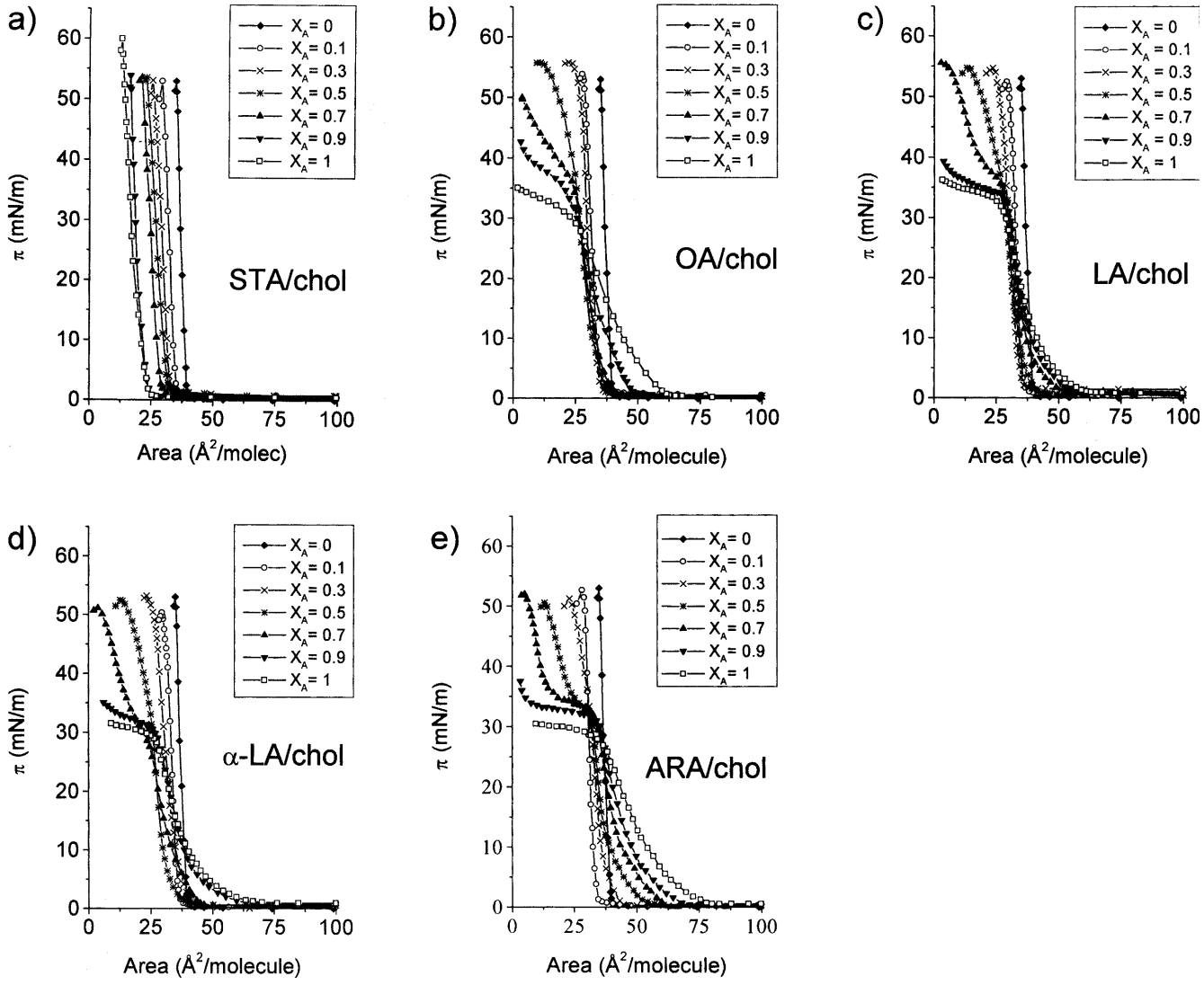


Fig. 1a–e Surface pressure/area (π/A) isotherms of cholesterol in mixtures with: **a** stearic acid; **b** oleic acid; **c** linoleic acid; **d** α-linolenic acid; **e** arachidonic acid spread on water subphase at 5 °C

mixed film can be compressed to a condensed state provided that cholesterol is present in the monolayer in excess. The lift-off areas for mixtures containing fatty acids in the excess ($X_A = 0.9$ for OA/cholesterol; $X_A = 0.7$–0.9 for LA and α-LA/cholesterol systems, and $X_A = 0.5$–0.9 for ARA/cholesterol) lie between those for pure components. For the remaining mixtures, the isotherms are shifted towards lower molecular areas, indicating the presence of interaction between film components. Pure monolayers of cholesterol and unsaturated fatty acids collapse at significantly different surface pressures. In contrast to STA/cholesterol systems, in which the mixed monolayers collapse at the same surface pressure, the behavior of mixtures with UFAs is quite different. Detailed analysis of the isotherms indicates that for all mixtures studied, except that of $X_A = 0.1$, two collapse states appear in the course of the isotherm. As can be seen in Fig. 2, the surface pressure of the first collapse varies with the monolayer composition, while the second collapse, which occurs at higher pressures, does not depend on the proportion of particular components (see Fig. 1), and actually reflects the collapse of pure cholesterol.

The application of the surface phase rule [23, 24] can be helpful for the interpretation of such unusual behavior. At constant temperature and external pressure, the degrees of freedom f of the monolayer equals $c–p + 1$, where c is the number of components at the interface (two for the systems investigated herein), and p is the number of phases in equilibrium (the bulk water and air are excluded). In the case of a miscible system, there are two phases ($p = 2$) at the collapse, i.e., collapsed film and the coexisting monolayer, and thus $f = 1$. Thus, the collapse pressure varies with the

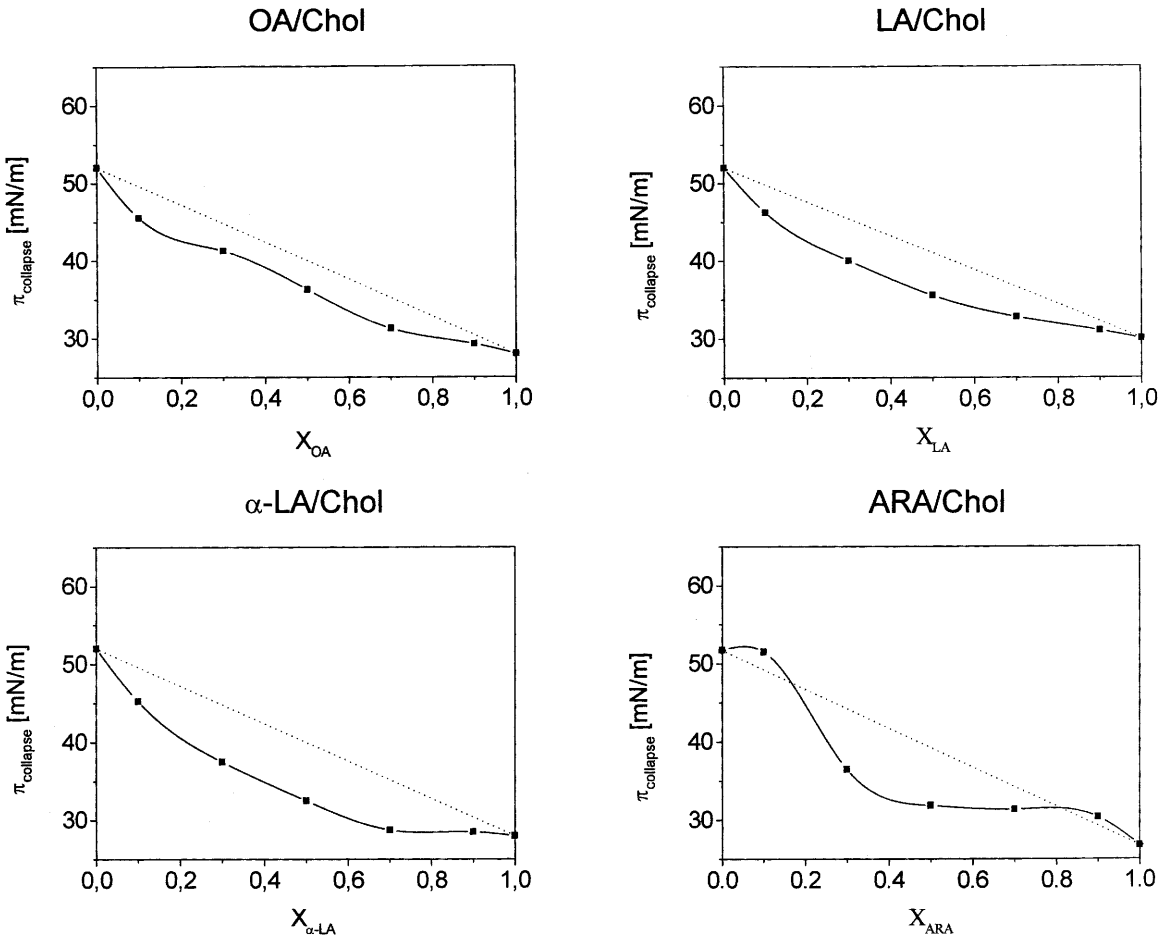


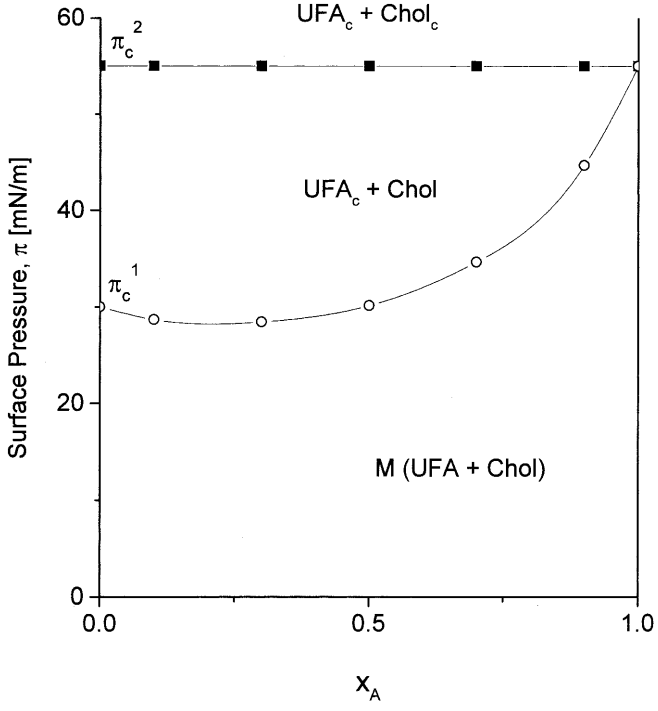
Fig. 2 Surface pressure of the first collapse as a function of composition for mixed monolayers of cholesterol and unsaturated fatty acids

monolayer composition. On the other hand, if the system is immiscible, there are three coexisting phases at the collapse, f = 0, and the collapse pressure is thus independent of the composition.

Analysis of the investigated systems indicates that the monolayers are miscible only in the surface pressure region below the first collapse (up to ca. 35 mN/m). At this pressure (π_c^1), the fatty acid is expelled from the mixed film (UFA_c). As can be seen in Fig. 2, values of π_c^1 differ from that for a fatty acid alone. Thus, it may be assumed that, before the first collapse, the mixed film is formed which deviates from ideal behavior. The interaction between the components causes a fatty acid to be squeezed out of the film at higher surface pressures than that in a monolayer of a fatty acid alone. Upon further compression the surface pressure continues to rise, due to the presence of cholesterol in the monolayer (Chol), which is finally expelled from the surface at a pressure of ca. 55 mN/m, corresponding to the second collapse ($Chol_c$). The diagram in Scheme 2 illustrates the phases existing in mixed investigated monolayers at different surface pressures. The region M denotes mixed monolayer with miscible components, i.e., cholesterol and a fatty acid in a monolayer form ($f = 2$ and $p = 1$). The curved line with open circles, representing π_c^1, indicates the equilibrium between collapsed fatty acid (UFA_c) and cholesterol in a monolayer form (Chol) (f = 1, p = 2), while the straight line with solid squares, connecting surface pressures of the second collapse (π_c^2), indicates the presence of collapsed cholesterol ($Chol_c$), collapsed unsaturated fatty acid (UFA_c), and cholesterol in a monolayer state (Chol) (f = 0, p = 3).

Analysis of the interaction in the mixed monolayer of fatty acids/cholesterol

Since UFAs and cholesterol were found to be miscible up to ca. 35 mN/m, the analysis of the interaction is restricted to the region below this value of surface pressure. Figure 3 illustrates the dependence of mean molecular area (ω) in the mixed monolayer as a function of its composition, expressed by the mole fraction of individual fatty acids (X_A), at constant surface pressure



Scheme 2 Phase diagram of mixed system of cholesterol and an unsaturated fatty acid

of 5 mN/m, 10 mN/m, and 20 mN/m. The dashed lines correspond to the values of ω calculated on the basis of the additivity rule. Except for the immiscible stearic acid/cholesterol system (small deviations are due to experimental errors), all other mixtures show behavior characteristic of miscible, non-ideal systems, with marked deviations from linearity. This was actually expected upon analyzing the values of the first collapse of the investigated monolayers. These negative deviations, proving film contraction, seem to be strongest for equimolar mixtures, except for ARA/cholesterol for which the minimum occurs for the composition of X_A~0.1. A more quantitative analysis is based on calculation of the excess free energy of mixing (ΔG_{exc}) (Fig. 4). Negative values of ΔG_{exc} for all the investigated mixtures at all composition ranges confirm that the mixed films are stable. The minimum value, indicating the highest monolayer stability, occurs for the mixtures of $X_A = 0.5$ for α-LA/cholesterol while for ARA and LA/cholesterol X_A is in the region of 0.1–0.3. For the mixtures with OA there is a broad region of low ΔG_{exc} values, where X_a is 0.2–0.8, and no sharp minimum can actually be distinguished. In fact oleic acid, with only one double bond in the apolar chain, is the most flexible of all those unsaturated fatty acids investigated here, and the mixed monolayers prove to be of the same,

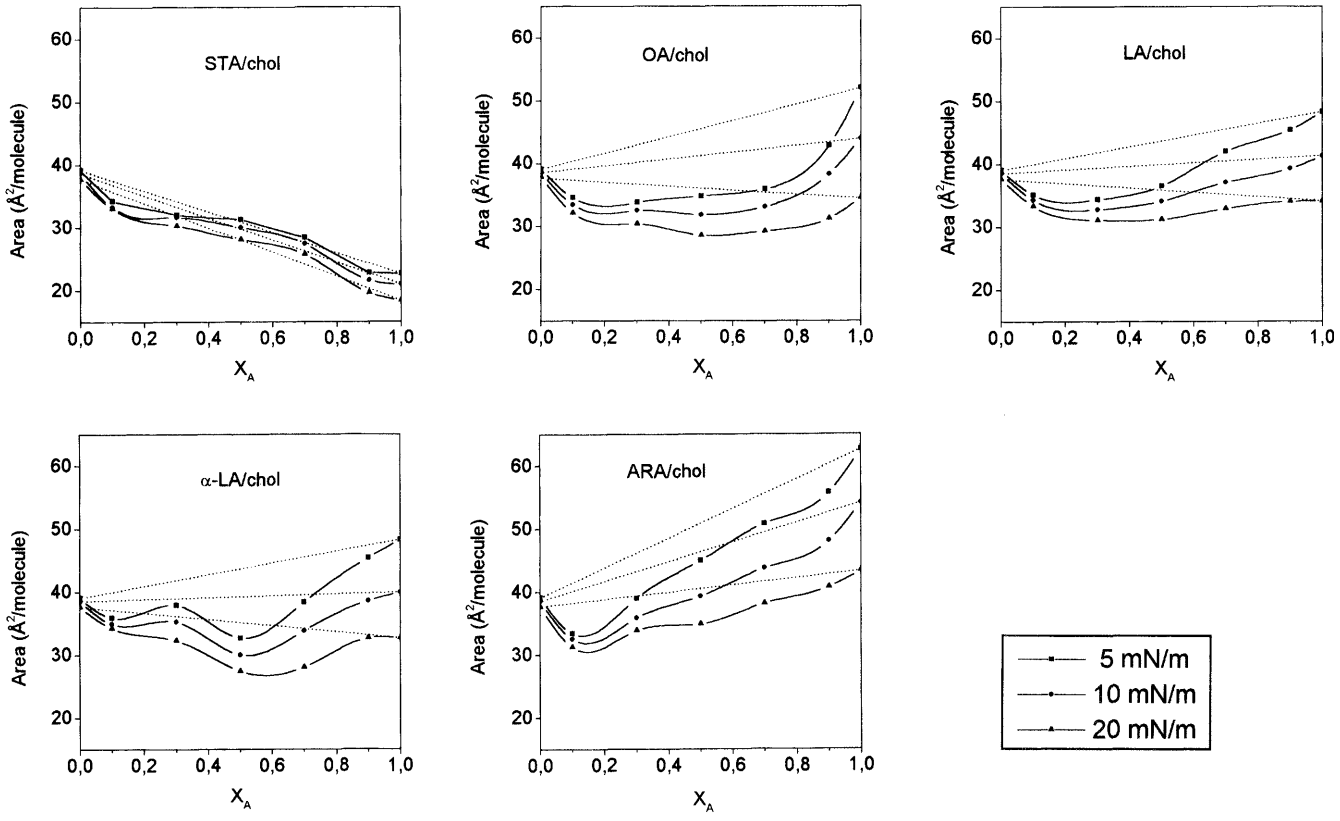


Fig. 3 Dependence of mean molecular area on the molar fraction of particular fatty acid in investigated mixed systems

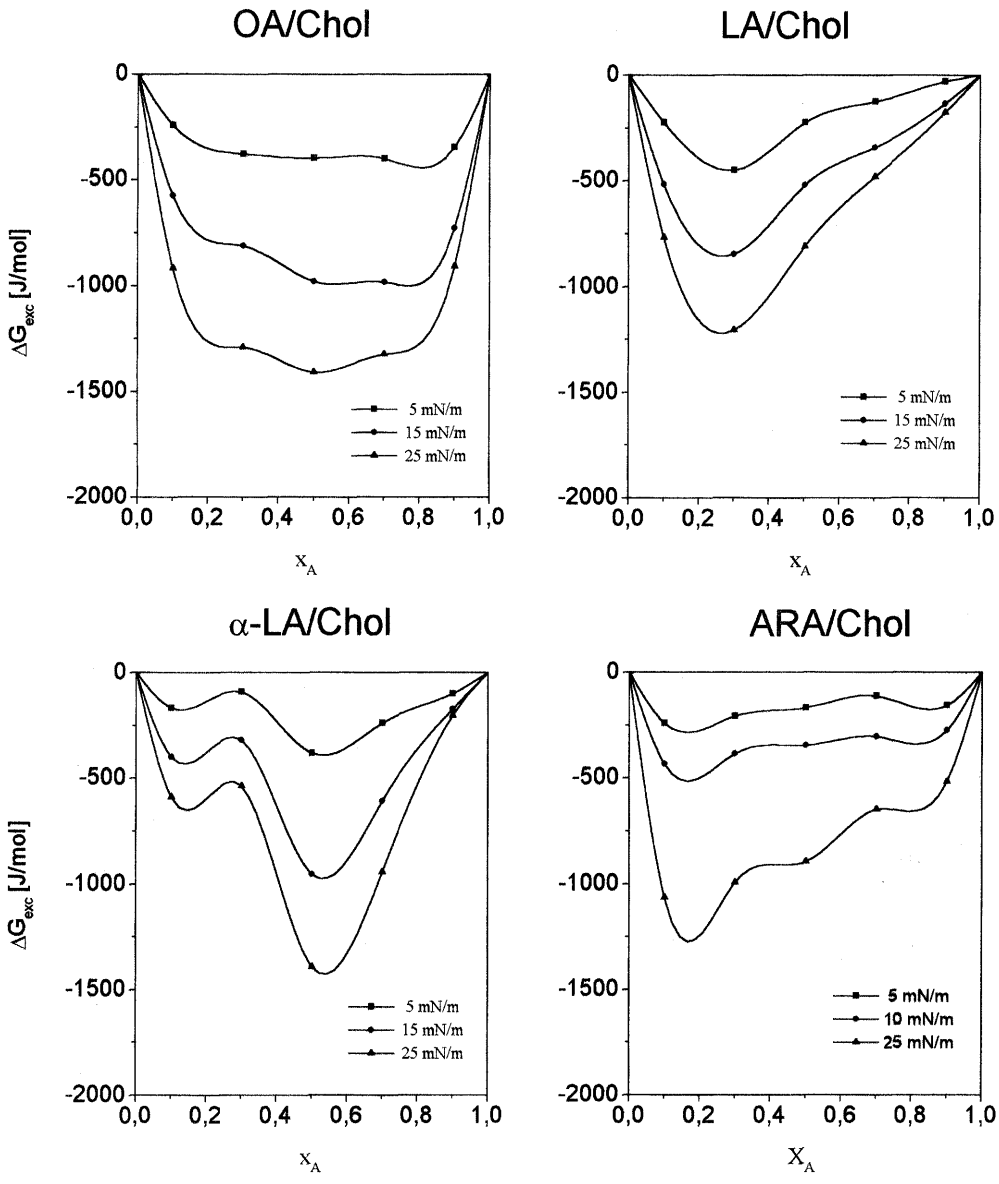


Fig. 4 The excess free energy of mixing (ΔG_{exc}) as a function of mole fraction of particular fatty acid (X_A) in the mixtures with cholesterol

high stability throughout almost all the composition range. It is also worth pointing out that fatty acids with even numbers of double bonds behave similarly, i.e., LA and ARA (two and four double bonds, respectively), but differently to those with odd numbers of unsaturated bonds (OA and α-LA).

For further insight into the strength of interaction, the interaction parameter, α, was calculated according to Eq. (2). Knowing α, the values of the interaction energy, Δh, can easily be calculated from Eq. (3). Since both dependencies, i.e., $\alpha = f(X_A)$ and $\Delta h = f(X_A)$, show similar trends, only one of them is shown in the paper ($\alpha = f(X_A)$) (Fig. 5). The minimum values, proving the strongest interaction, occur for the mixtures of $X_a \approx 0.2$ for LA and ARA/chol, and are consistent with ΔG_{exc} calculations, indicating the mixtures of maximum stability. However, the results obtained for mixtures of cholesterol with OA and α-LA are quite different. Here, the strongest interactions seem to appear for mixtures in both low (0.2) and high (0.8) regions of a fatty acid proportion.

The above discussion is based on the enthalpic origin for the influence of cholesterol on the phase behavior. However, there is also another effect which has to be considered here. Cholesterol has more hydrophobic character compared to fatty aids, and this may lead to entropic contribution which has been proved to lower the interfacial energy between coexisting fatty acid phases [25]. However, as discussed above, the loss of monolayer material from the surface by dissolution at higher temperatures does not allow us to calculate the entropic contribution in a reliable way.

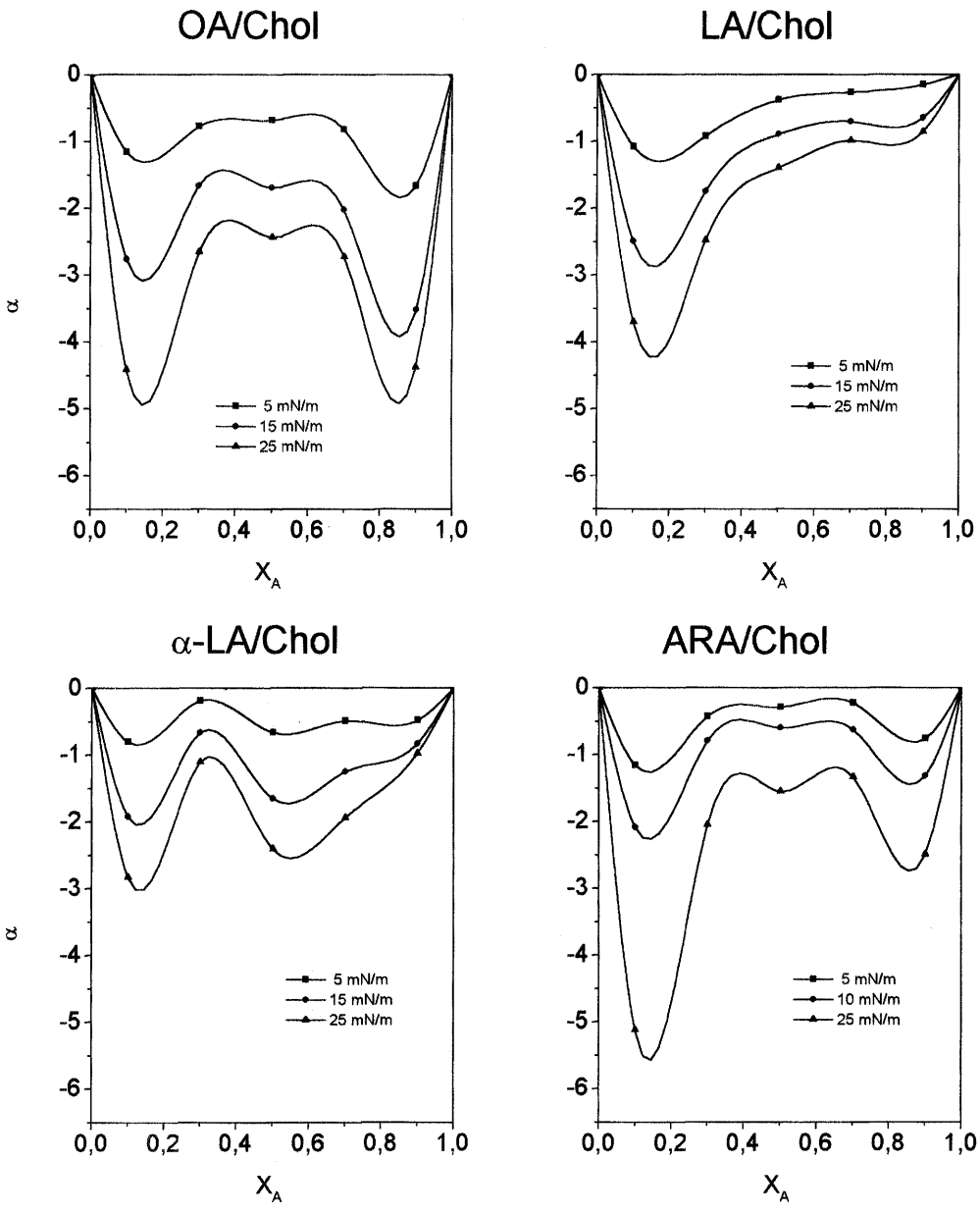


Fig. 5 The interaction parameter, α, as a function of mole fraction of particular fatty acid (X_A) in the mixtures with cholesterol

Although the behavior of cholesterol in mixtures with unsaturated fatty acids was found to be different and dependent on the number of double bonds in the apolar chain of a fatty acid molecule, the results obtained clearly show that strong interactions exist between particular components in the investigated mixed systems.

Conclusions

A monolayer study of cholesterol in its mixtures with unsaturated fatty acids, such as oleic, linoleic, α-linolenic, and arachidonic, reveals that, in contrast to immiscible cholesterol/stearic acid, these systems are miscible and interacting, as evidenced by the excess functions and parameters of interaction. The behavior of mixed films of cholesterol with unsaturated fatty acids was found to be dependent on the number (odd or even) of double bonds present in the apolar part of a fatty acid molecule. Although the relevance of the monolayer studies to the living systems has to be considered with care, our result may suggest that the hypocholestrolemic effect of unsaturated fatty acids in vivo depend on their interaction with cholesterol.

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