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Mixtures of lecithin with polymerizable derivatives of cholesterol

A monolayer film balance study

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Abstract. One of the best investigated binary lipid mixtures is the lecithin-cholesterol system. We show here that it is possible to modify the cholesterol in such a way that it can be polymerized without changing its behaviour in mixtures with lecithin. The polymerizable derivatives exhibit a very similar phase diagram in the mixture with dipalmitoylphosphatidylcholine as the cholesterol itself. This is demonstrated by filmbalance measurements.

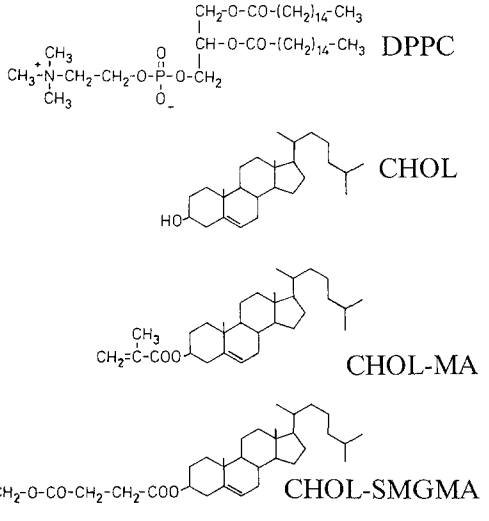
Key words: Monolayer, polymerizable lipid, cholesterol, mixture

Introduction

Since the 'condensing effect' of cholesterol upon lecithin monolayers was first detected (Leathes 1925), this system has been measured many times (e.g. Philips et al. 1970; Cadenhead et al. 1976; Pagano and Gershfeld 1973; Mueller-Landau and Cadenhead 1979a, b; Cadenhead and Mueller-Landau 1979). Some years ago the monolayer phase diagram of the dipalmitoylphosphatidylcholine cholesterol (DPPC-CHOL) system was established (Albrecht et al. 1981), but the nature of the phases is yet unknown. Our idea was to modify one of the components in such a way, that they can be polymerized, either in monolayers or after transferring them onto a solid support, using the Langmuir Blodgett technique (LB-mono- and multilayers, Langmuir 1920; Blodgett and Langmuir 1931), or one of the more recent modifications of it (Langmuir and Schaefer 1938). This should open several possibilities to discern homogeneous phases and heterogeneous areas of the phase diagram. To gain information about a specific mixture, however, the modified substances have to show the same phase diagram as the original materials. Preliminary experiments with polymerizable lecithin showed that the phase diagram was changed completely (Büschl et al. 1982). The results presented here show, that the modification of the cholesterol seems to be the better way. This is just following the example of nature, where the cholesterol is found in many modified versions and the modified molecules show basically the same effect upon membranes.

Polymerizable lipids are also of interest in other respects, because they can be used to stabilize synthetic membranes. It is possible to produce synthetic vesicles which exhibit increased stability when compared to that of natural membranes (Bader et al. 1985), or bilayer lipid membranes (BLMs) of increased long term stability (Benz et al. 1982) or LB-multilayers which are stable to solvents and aggressive media (Albrecht et al. 1984).

The following materials have been used:



We compare the monolayer phase diagrams of DPPC-CHOL with DPPC-CHOL-MA and DPPC-CHOL-SMGMA.

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Experimental

Materials

The water used for all filmbalance curves was purified with a Millipore water purification system (Milli-Q, 4 bowl). This system was fed with distilled water.

The DPPC was purchased from Fluka (purissimum grade) and used without further purification. Isotherms and isobars (Fig. 1) of that material indicate adequate purity. The isotherms were reproducible within the linewidth of the recorder and the isobars coincide within the resolution of the recorder. Furthermore, hysteresis curves of the material (Fig. 2) show a very good reproducibility. The curve in Fig. 2 has been compressed first to 40 mN/m and then expanded to an area much larger than the offset point after waiting for 1 h. After waiting for another hour, the film has been compressed again beyond the breakdown point. We found, that the measurement of such curves is a more precise proof of the purity of a lipid than most other methods, like TLC or elemental analysis; it is at least as good a test, as DSC curves of a preparation in water (Mabrey et al. 1978).

The cholesterol used (Fluka, purissimum) was tested in the same way (Isotherms, hysteresis-isotherms) with similar results. In that case, however, the method is not such a thorough test, because the cholesterol monolayer isotherm is a straight line only (Fig. 3, leftmost isotherm). The cholesterol and the cholesterol derivatives were recrystallized immediately before use. The derivatives showed the same good reproducibility of filmbalance curves as the other materials.

For spreading, a 9 to 1 mixture (vol-vol) of hexane (Merck, Uvasol) and ethanol (Merck, p.a. grade) was used. This spreading solvent has proven to be of a very reliable purity and it is routinely checked by measuring curves of a well known substance (Palmitic acid, reference for GC). This is at the same time a check of the purity of the water and the cleanliness of the filmbalance (Albrecht 1983).

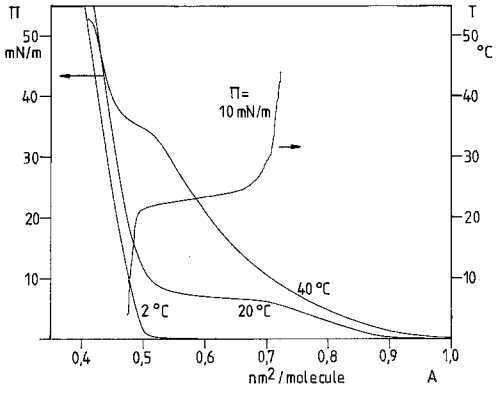

Fig. 1. Isotherms and isobar of DPPC

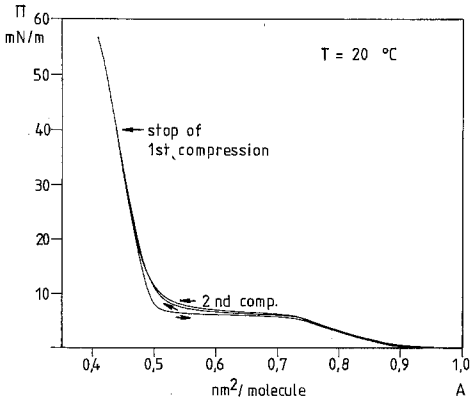

Fig. 2. Hysteresis isotherm of DPPC. Between compression and expansion and recompression the barrier has been halted for 1 h

Synthesis

CHOL-MA (Shibaev et al. 1979). 2 g (5.2 mmol) cholesterol (Fluka, purissimum) were dissolved in 40 ml of absolute chloroform. To this solution 2 ml of freshly distilled triethylamine was added. The mixture was cooled down to 0 °C and a solution of 0.5 ml (5.2 mmol) of methacrylic acid chloride (Fluka, p.a.) in 10 ml of absolute chloroform containing 10 mg hydroquinone (as inhibitor) was added dropwise. The reaction mixture was stirred for 30 min at 0 °C and then 5 ml of methanol were added. The solvent was evaporated under reduced pressure and the crude reaction product purified by silica gel column chromatography using hexane-ethylacetate 20/1 (vol/vol) as eluent. Recrystallization from methanol completed the purification of the product (m.p. 112 °C, yield: 34%).

CHOL-SMGMA. 0.5 g (1mmol) cholesteryl-hydrosuccinate (Serva, p.a.), 0.14 g glycolmethacrylate (Fluka, p.a.), 10 mg dimethylaminopyridine and 5 mg hydroquinone were dissolved in 10 ml of absolute chloroform. The solution was cooled down to 0 °C with an ice-bath. Then a solution of 0.26 g (1.24 mmol) of dicyclohexylcarbodiimide in 5 ml of absolute chloroform was added dropwise and the reaction mixture was stirred for 3 h at 0–5 °C. After filtration of the urea the solvent was evaporated under reduced pressure. Purification of the crude reaction product was done by silica gel column chromatography using hexane-ethylacetate 5/1 (vol/vol) as eluent followed by recrystallisation from methanol (m.p. 72.5–73 °C, yield: 69%).

Methods

The isotherms have been measured with a computer controlled filmbalance, as described earlier (Albrecht 1983), but the programs have been improved since. Most of the curves were measured in the conventional way, that is, the compression rate of the moving barrier of the filmbalance is constant (we chose 10 min/sweep or typically about 12 Å/molecule/min), but some of the curves have been measured using the "thermodynamic" method. In that mode the barrier does not advance the next step until the pressure is stable again (within the resolution of the A/D converter of the pressure pickup system of 0.03 mN/m). The aim of this measuring mode is to come closer to the thermodynamic equilibrium. However real thermodynamic equilibrium cannot be reached. The results obtained in this way are essentially the same as in the standard mode at a very slow compression rate (> 1 h/sweep).

For each curve the substrate (pure water) has been changed and the computer does not begin a measurement before the temperature is stable within 0.1 Centigrade. The temperature for all experiments was 25 °C.

For spreading a gas-tight glass syringe with a Teflon plunger and an adjustable stopface (Kloehn Co., Whittier, Calif.) has been used to make sure that the amount spread (80–150 µl) is always precisely the same for each set of curves. The mixtures have been prepared from equimolar stock solutions by mixing appropriate amounts.

For the experiments where the monolayer has been irradiated, a commercial filmbalance (MGW Lauda, West-Germany) has been used after several modifications. A low pressure mercury UV-lamp has been installed in the lid of the device. Before and during irradiation the balance was flushed with nitrogen. The compression barrier has been slightly modified to overcome problems with tiny leaks and the pressure pickup barrier has been changed to a design without any bonding tape. The original design caused problems because some of the glue of the Teflon bonding tape used was found in the films.

For polymerization the filmbalance was flushed with nitrogen and the film was kept under a constant pressure of 10 mN/m while the UV-lamp was on. The intensity of the low pressure mercury lamp was 7 mW/cm² and was homogeneous in the plane of the monolayer. Most of the radiation power is concentrated in the 254 nm line. The time for irradiation was 6–8 h. The long time required causes problems with the precision of the measurements. During 6 h traces of impurities (from the trough or the nitrogen) can accumulate and some substrate evaporates. Furthermore the pressure pickup system changes slightly with temperature under the influence of the UV-lamp. Thus the pressure varied between 10 and 15 mN/m.

Results and discussion

Figure 3 shows isotherms of DPPC-CHOL mixed monolayers at different compositions and three typical area composition curves. These curves are in accord with the results published a few years ago (Albrecht et al. 1981) and are given here for comparison with the following data. Figure 4 shows isotherms for the DPPC-CHOL-MA mixture. One striking difference is that the curve of the pure CHOL-MA is more expanded and that the breakdown point is much lower compared to the curve of cholesterol itself. This is obviously the influence of

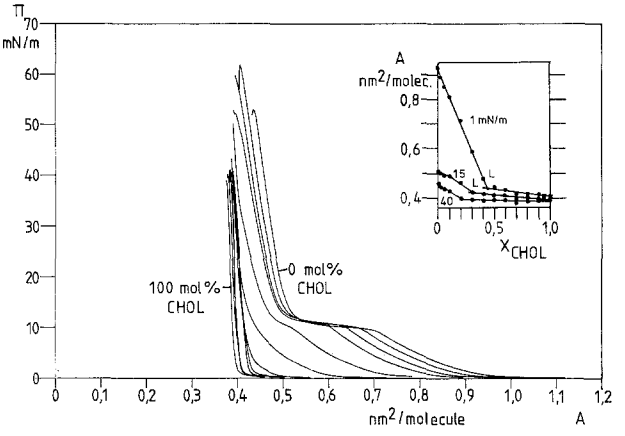

Fig. 3. Isotherms of the DPPC-CHOL mixtures at 25 °C and a content of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mol% cholesterol. The *inset* shows 3 representative area-composition curves at 1, 15 and 40 mN/m

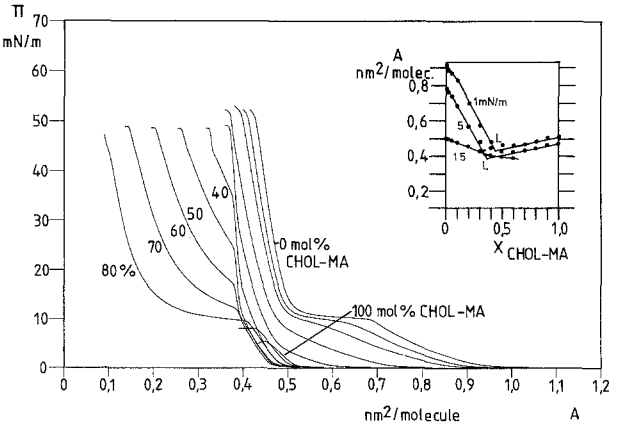

Fig. 4. Isotherms of the DPPC-CHOL-MA mixtures at 25 °C and a concentration of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mol% CHOL-MA. The *inset* shows 3 typical area-composition curves at 1, 5 and 15 mN/m

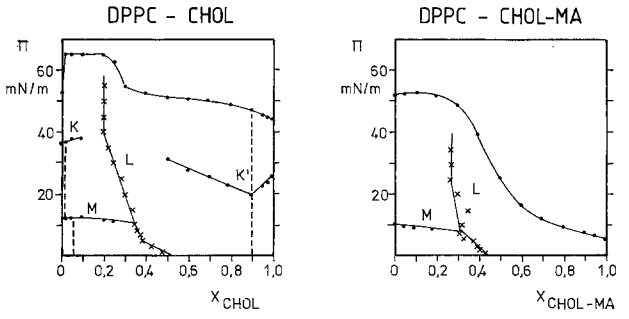

Fig. 5. Comparison of the phase diagrams of DPPC-CHOL and DPPC-CHOL-MA. The topmost line is the pressure, where the film breakdown occurs. The M-line indicates the main transition, the L-line is the border between lecithin line and cholesterol like behaviour of the mixed films. C and S lines denote the borders of ideal miscibility. K and K' denote a solid-solid transition, found in CHOL and DPPC

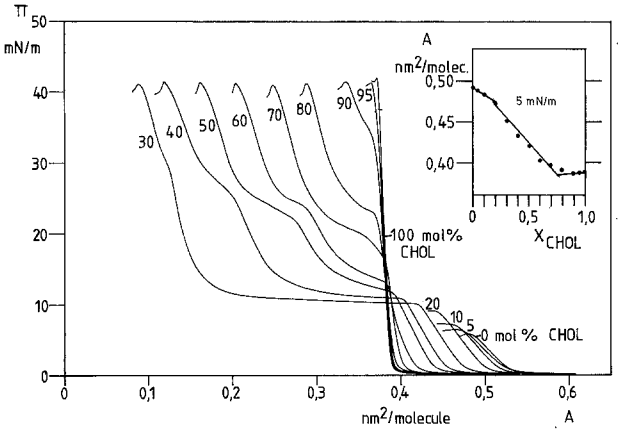

Fig. 6. Isotherms of the CHOL-MA-CHOL mixtures at 25 °C and a content of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 95 and 100 mol% cholesterol. The inset shows a representative area-composition curve at 5 mN/m

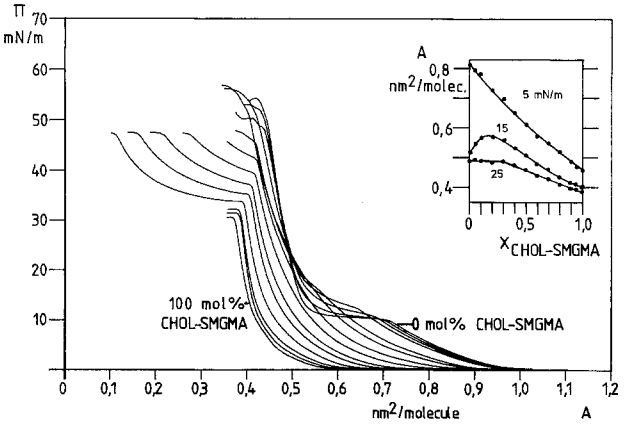

Fig. 7. Isotherms of the DPPC-CHOL-SMGMA mixtures at 25 °C and a concentration of 0, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mol% CHOL-SMGMA. The inset shows three typical area-concentration curves at 5, 15 and 25 mN/m

the methacrylate moiety, linked to the headgroup of the cholesterol. Nevertheless, except for that differences, the behaviour of the mixture is almost identical to the DPPC-CHOL system. Figure 5 shows the phase diagrams of both mixtures. The only major difference is the topmost line, that gives the breakdown pressure. According to the nomenclature used in a previous paper (Albrecht et al. 1981) the other lines are: M main transition, L the break in the area-composition curves and in some of the isotherms, where the curves change the character from (soft) lecithin like to (hard, less compressible) cholesterol like. The vertical lines on the left and the right hand side give the end of the ideal miscibility of either compound in the other and K denotes a solid-solid transition. The same symbols are used to mark the corresponding points in Figs. 3 and 4.

Some of the curves in Fig. 4 are drawn beyond the breakdown point, because there is an interesting detail to be seen. There is a second breakdown point, indicating that there are two immiscible phases present in the area right of the L-line. The first breakdown depends upon concentration and is due to a phase with variable content of DPPC in cholesterol. The second breakdown is due to a mixture with fixed composition (at a fixed pressure and temperature), corresponding to a point on the L-line. The area where this breakdown occurs corresponds to the expected percentage of that phase according to the phase diagram. This is clear and direct evidence for a two phase region to the right of the L-line, as suggested already for the DPPC-CHOL mixture (Albrecht et al. 1981). When measuring these curves in the "thermodynamic" mode, the second breakdown is not reached because after the first breakdown the monolayer is in a metastable state.

We did not try to polymerize pure CHOL-MA monolayers by UV-irradiation because of the low breakdown pressure and the difficulties in keeping the pressure constant below that breakdown pressure, which is reached at 5 mN/m. However, CHOL-MA can be polymerized by UV-irradiation in mixtures with DPPC. Under the conditions described above the reaction takes about 6 h. Because of the difficulties described above we did the polymerization only with the mixture containing 80 mol% of CHOL-MA and did not try to polymerize any of the other mixtures of that material with DPPC.

Figure 6 shows the curves of the mixture of CHOL with CHOL-MA. Because the mixtures of both with DPPC exhibit a similar phase diagram, we expected ideal miscibility, but the measurement shows a more complex behaviour. Some of the curves are again given past the breakdown point. The area-composition curve shows two breaks at

about 20% and 80%. The most likely explanation for these curves is the existence of miscibility of the pure compounds up to about 20% of either material and in between a phase separation. The isotherms between 20 and 80 mol% again show, as in the previous mixture, a double breakdown point.

The monolayer isotherm of CHOL-SMGMA (leftmost in Fig. 7) is intermediate between the curves of CHOL and CHOL-MA. Therefore we had expected to find a similar mixing behaviour for the mixtures with DPPC as for the DPPC-CHOL system. At first glance the isotherms of the DPPC-CHOL-SMGMA mixtures given in Fig. 7 show that this seems not to be the case. The area-composition curves are more or less straight lines, except around the main transition. The breakdown is shifted continuously from the value of pure DPPC to the value of CHOL-SMGMA. This is characteristic for ideal mixtures. Complete immiscibility would show the same area-composition curves, but the breakdown values would not be shifted and both breakdown should be visible in the curves, when measuring past the first, lower breakdown. In addition the main transition of DPPC should be visible up to low DPPC concentrations and not disappear between 10 and 20 mol% CHOL-SMGMA.

CHOL-SMGMA can be polymerized at the water surface. Unfortunately these experiments are not of high precision, as mentioned in the experimental section. Figure 8 shows the curve of CHOL-SMGMA before and after polymerization. The polymer curve is more expanded and shows a higher breakdown pressure. For determining the time of polymerization the area is plotted versus time. These curves show a stable area up to switch on of the UV-lamp and then an increase in area that reaches saturation after about 6 h. We also measured this for the 20, 40, 60 and 80 mol% mixtures and found that the polymerization kinetics and the time required are the same for all samples. The total increase in area is, within the poor precision, proportional to the amount of polymerizable lipid. This is in contradiction to the assumption of a homogeneously mixed film, because then the polymerization should be hindered compared to the pure CHOL-SMGMA film.

Because of this discrepancy we had a closer look at the mixed isotherms of the DPPC-CHOL-SMGMA system and found, that this material might also have a phase diagram similar to the DPPC-CHOL system, but the L-line might be shifted more to the left (lower concentrations) and therefore the 'condensing effect' is not visible in the area-concentration curves. It might be much smaller anyway. If one assumes that this is the case, then it is also found that the breakdown pressure of the curves

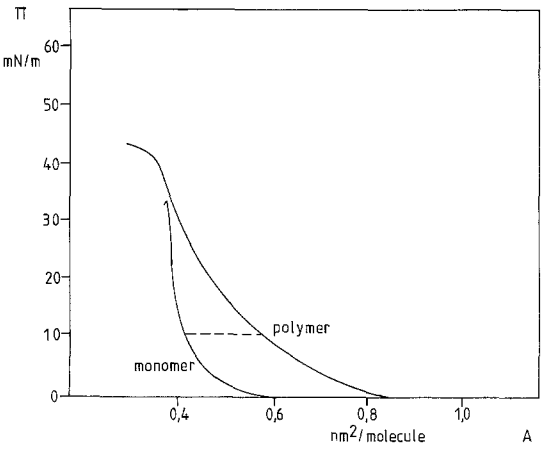

Fig. 8. Isotherm of CHOL-SMGMA before *(left)* and after *(right)* polymerization

does not vary much for the 0%, 2% and 5% curves and decreases afterwards continuously to the value of CHOL-SMGMA. Again a 'second breakdown' is found, when the curves are measured beyond the breakdown, indicating the same behaviour as for the first two systems. Two discrepancies are to be explained in that case. The first is why the main transition moves to substantially higher pressures, before it disappears and the second is why the breakdown pressure of the second phase (CHOL-SMGMA rich) is not constant. The first point can be answered easily. It might be the influence of traces of impurities, which are still present in the material despite the thorough checking. Some previous measurements of the DPPC-CHOL system (Cadenhead et al. 1976) showed the same increase in the main transition pressure. The second point is more crucial. It might be, that the breakdown pressure varies with the size of the clusters of CHOL-SMGMA. If one assumes, that after spreading the homogeneous mixture the same number of patches is formed, their size depends upon the concentration of the patches forming phase. Small patches have a lower ring tension (like small droplets of water have a smaller surface tension), thus being stable to a higher external pressure.

Conclusion

We could show, that it is possible to modify quite drastically the chemical structure of one of the components of a binary lipid system without changing the mixing behaviour very much. The modified molecules (Cholesterol) can be polymerized, as demonstrated in monolayers at the water surface.

This opens a variety of possibilities to stabilize membranes without changing other properties. So

for example cholesterol containing vesicles should exhibit a viscoelasticity of their membrane which allows them to pass through even the smallest blood vessels in the body similar to the findings obtained by modifying the Cholesterol content of red blood cells (Chabanel et al. 1983). If vesicles containing polymerized cholesterol derivatives have a similar viscoelasticity, this would be an extremely stable model system to study the action of liposomes in the body.

Further experiments with monolayers and bilayers along that line will have a good chance of producing a better understanding of the model membrane systems.

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