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Micron-scale phase segregation in lipid monolayers induced by myelin basic protein in the presence of a cholesterol analog

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ABSTRACT

It was previously shown that myelin basic protein (MBP) can induce phase segregation in whole myelin monolayers and myelin lipid films, which leads to the accumulation of proteins into a separate phase, segregated from a cholesterol-enriched lipid phase. In this work we investigated some factors regulating the phase segregation induced by MBP using fluorescent microscopy of monolayers formed with binary and ternary lipid mixtures of dihydrocholesterol (a less-oxidable cholesterol analog) and phospholipids. The influence of the addition of salts to the subphase and of varying the lipid composition was analyzed. Our results show that MBP can induce a dihydrocholesterol-dependent segregation of phases that can be further regulated by the electrolyte concentration in the subphase and the composition (type and proportion) of non-sterol lipids. In this way, changes of the lipid composition of the film or the ionic strength in the aqueous media modify the local surface density of MBP and the properties (phase state and composition) of the protein environment.

1. Introduction

Biological membranes are highly dynamic organized interfaces, formed by a large diversity of lipids and proteins, that involve lateral clustering of their constituents [1]. Long standing, and yet not fully resolved questions about membranes are how the compositional heterogeneity regulates their organization and dynamic properties, and which are the physical factors involved in determining the lateral heterogeneity.

The surface organization of the monolayer formed with all the components of the myelin membrane at the air–water interface has been studied under controlled molecular packing conditions [2]. The components in the whole myelin monolayer are segregated in two phases over the full range of surface pressures until monolayer collapse (and beyond, see [3]). The surface pattern and its complex surface dynamics derives from the interaction of the major myelin proteins with the mixture of myelin lipids that, otherwise, self-organize as a tightly packed homogeneous membrane, with absence of segregated microdomains above a surface pressure of about 3 mN m^{-1} [4].

Ligand-labeling of some key components of myelin established that the surface pattern is brought about by the segregation of a phase containing the major myelin proteins together with some lipids (such as ganglioside GM1) that coexists with a phase enriched in other lipid components such as cholesterol, galactocerebroside and phosphatidylserine [5]. Although the distribution of components is maintained upon compression, the topography of the monolayer varies from a pattern of mostly rounded liquid domains to the coexistence of a liquid phase and a more viscous phase organized as a fractal pattern [6]. Mixed monolayers of the total myelin lipids with only one of the major myelin proteins, the Folch-Lees Proteolipid, indicated that the latter is required to stabilize the fractal phase. On the other hand, studies in monolayers of the total myelin lipid fraction containing only Myelin Basic Protein (MBP) showed that MBP induces the segregation of two phases, with MBP enriched in domains segregated from a lipid phase of low compressibility; MBP is squeezed out of the film at high surface pressures but remains associated to the interface [4]. Mixed monolayers of the total myelin lipids with both proteins reproduced, at defined proportions, the complex surface behavior of the whole myelin monolayer under compression [4].

MBP is the second most abundant protein of the myelin sheath and there are long standing evidences showing its importance for the compactness and integrity of this membrane in normal and neuropathological conditions [7,8]. MBP is a highly basic protein (PI>10), with a large percentage (24 mol%) of basic residues randomly distributed along its primary sequence. It has been postulated that MBP causes adhesion of the cytosolic surfaces of myelin by binding to negatively charged lipids in a surface charge-sensitive manner [8]. The native protein form exhibits about +19 net charges at pH 7 and a post-translationally modified less charged form (+13) is increased in Multiple Sclerosis [9]. The attraction and binding of MBP to bilayers of different lipid compositions is modulated by electrostatic interactions with the acidic lipid

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headgroups [10–12]. However, as measured with a variety of experimental techniques, hydrophobic interactions may also occur between parts of MBP embedded in the membrane and the hydrophobic chains of lipids [9,11–15]. Furthermore, the extent of insertion of MBP in the membrane and the degree of hydrophobic interaction may be regulated by several factors such as the lipid composition and phase state, the density of surface charges at the interface and the ionization state of the protein [12,16–19]. In this context, the presence of cholesterol was found to result in enhanced hydrophobic interactions of MBP with the membrane, more resistance to dissociation by salt concentration, and higher affinity binding than in liposomes lacking cholesterol [17,20–22]. In addition, several studies have also reported an enhanced ability of MBP to cause bilayer apposition, merging or aggregation, with liposomes of neutral and charged lipids in the presence of cholesterol [17,20–22].

In monolayers, MBP may also interact with pure zwiterionic membranes, although stronger interactions, and different surface arrangements, were described with anionic lipids films [23–26]. It was postulated that MBP interacts with acidic lipids in defined stoichiometries [8,15,26]. In experiments with vesicles of neutral and anionic phospholipid mixtures, MBP was found to segregate out the acidic lipid in a ratio of 27–34 lipid per protein molecules [27]. Studies with electron spin resonance also found a maximum of 36 acidic lipids directly interacting to an MBP molecule [12]. In addition, other studies in biphasic solvent systems showed that this ratio was dependent on the charge of the lipid, with the number of bound lipids varying in order to associate with 20–23 negative charges per MBP [28]. In systems containing acidic or neutral glycosphingolipids, calorimetric and monolayer studies indicated that MBP can lead to phase coexistence and sequester from 13 to 111 lipids, depending on the oligosaccharide chain complexity of the glycolipid and on the presence or absence of glycerophospholipid [15,26].

In summary, MBP is a flexible protein that may establish electrostatic and also hydrophobic interactions with lipid membranes, whose balance and strength can be regulated by several molecular factors that may lead to phase separation. In this work we investigated the possible origin and regulation of the domain segregation induced by MBP previously observed in monolayers prepared with the total lipid mixture extracted from myelin. The effect of electrostatics on the phase segregation was analyzed and, since a more fluid environment would probably stabilize MBP in the monolayer, the effect of the cholesterol content was also examined in view of its influence on the mechanical properties of membranes [29,30]. For these purposes, we studied by fluorescence microscopy the effect of changes in the proportion of MBP and dihydrocholesterol (dchol) on the surface pattern (segregated phases vs. homogeneous phase) of films of different composition of well defined synthetic phospholipids compressed at defined surface pressures. Dihydrocholesterol was mostly used instead of cholesterol because it shows an interfacial packing behavior and phase diagrams similar to those of cholesterol [31,32] and does not undergo detectable oxidation during experiments (some experiments with cholesterol were included as controls that showed similar behavior as the analogue). Considering a possible role of electrostatics, we also analyzed the effect of changing the proportion of charged lipids in the mixture and of variations of the ionic strength of the subphase. It is important to recall that the original asymmetry of components in the natural membrane is completely lost in these films (see [6,33]), and in some of the studied systems the composition was greatly simplified with respect to the natural mixture. We have no pretense of directly extrapolating the results obtained to the behavior of the natural myelin membrane, but to contribute to the understanding of some fundamental molecular factors by which MBP can induce surface pressure- and composition-dependent phase segregation of surface domains, modulated by the sterol and the phospholipid composition.

2. Materials and methods

2.1. Isolation procedures

An essentially protein-free myelin lipid fraction containing all the myelin lipids (except for the minor components represented by the more complex gangliosides [34]) was isolated from bovine spinal cord whole myelin following a procedure previously described [4]. The amount of protein remaining in the final extract, determined by standard procedures [35], was below 0.5 g/100 g of total lipids. The myelin basic protein was purified from bovine spinal cord as described previously by solubilization in solvent, followed by extraction in acid media and column chromatography [4].

2.2. Fluorescence microscopy

The surfaces of the films were observed by fluorescence microscopy while simultaneously registering the surface pressure vs. molecular area isotherms. The set up consisted of an automated Langmuir balance (KIBRON microtrough) with a Wilhelmy plate for surface pressure determination, mounted on the stage of a Zeiss Axiovert 200 (Carl Zeiss, Oberkochem, Germany) fluorescence microscope with a CCD video camera Zeiss commanded through the Axiovision software of the Zeiss microscope. Long distance 20× and 40× objectives were employed. Monolayers with variable mole fractions of probe were spread from chloroform/methanol solutions onto Milli-Q water or NaCl 0.10 M, at a molecular area larger than the lift off area. Before isometric compression of the film, the solvent was allowed to evaporate for 5 min. All experiments were performed at 24 ± 2 °C.

2.3. Lipids and fluorescent probes

The anionic fluorescent probes used were RhoeggPE (*N*-(lissamine rhodamine B-sulfonyl) diacyl-*sn*-glycero-3-phosphatidylethanolamine) with 55 % of unsaturated acyl chains and RhoC16PE (*N*-(lissamine rhodamine B-sulfonyl) dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine), both from Avanti Polar Lipids. We also used a positively charged probe, DiIC18 (1, 1′-dioctadecyl- 3, 3, 3′, 3′-tetramethylindocarbocyanine perchlorate) purchased from Molecular Probes. These probes exhibit a preferred partition into liquid-expanded phases. Dilauroyl phosphatidyl serine (dlps) and palmitoyl sphingomyelin (smC16) (*N*-Palmitoyl-D-*erythro*-Sphingosylphosphorylcholine) were from Avanti Polar lipids, Cholesterol and dihydrocholesterol (dchol) from Sigma. This cholesterol analog was used instead of cholesterol because it minimizes air oxidation during experiments and shows an interfacial behavior and phase diagrams as that of cholesterol [31,32]. All reagents were of the highest purity available and were used without further purification.

2.4. Phase diagram determination

The limits of the two phase coexistence region were taken as the surface pressure values at which the mixing/demixing of the phases occurs. For adequate experimental precision these points were determined during expansion of a previously compressed film; in these conditions those surface pressures correspond to the points where domains first emerge from an otherwise homogeneously fluorescent surface.

At least four different myelin lipid batches were used to reproduce the whole set of experiments. Each batch showed the same general tendency, with absolute values varying by less than 3 mN m^{-1} between batches. The diagrams for monolayers of myelin lipids correspond to representative results from a set of experiments performed with a single batch.

The phase diagrams were constructed by plotting the mole % of dchol and of MBP in the total mixture. The monolayers formed with four components (two phospholipids, dchol and the protein) were prepared with a fixed ratio of dlps/smC16 18.5:81.5, which corresponds approximately to the ratio of charged/neutral lipids in the non-cholesterol fraction of the total myelin lipid mixture [33].

3. Results

In a previous article [4], the surface behavior of monolayers prepared with the total myelin lipid mixture and MBP on ionic subphases was described. Fig. 1 shows that similar characteristics emerge when the monolayers are spread on pure water. Two immiscible liquid phases segregate at low lateral pressures and finally merge on compression. Both phases relax the line tension forming a pattern of circular segregated domains with enhanced boundary shape fluctuations as they approach the fusion point (Fig. 1B). The dark phase (low fluorescent probe solubility) was identified as a liquid cholesterol-enriched phase in whole myelin monolayers and in films of mixtures of MBP and total myelin lipids, and the bright phase (high fluorescent probe solubility) as an MBP-containing phase [2]. The overall behavior of these films is similar to the two-liquid immiscibility described for simple cholesterol–phospholipid mixtures at low cholesterol proportions, where cholesterol-rich and phospholipid-rich phases are formed. It is worth mentioning that in these simple mixtures, a second two-liquid phase coexistence can form at higher cholesterol concentrations, with distinctly different surface properties as smaller domain sizes and absence of a critical mixing point at room temperature. The limit between these two zones of two-phase coexistence (alfa and beta two phase zones in reference [32]) ranges from 25 to 43 mol% dchol (or cholesterol) depending on the phospholipid composition [36]. The MBP-induced segregation of phases was explored in simple phospholipid–dchol mixtures. The topographic features of these films are qualitatively similar to those of the MBP–myelin lipid monolayers (see inset in Fig. 2). The simple lipid mixture compositions contain sphingomyelin and phosphatidilserine, a representative sphingolipid and a charged phospholipid, respectively. We prepared a mixture of dlps/smC16/dchol, with the same proportions of negatively charged lipids to neutral non-sterol lipids (18.5:81.5) as in the total myelin lipid extract [33], and another with dlps as the only phospholipid component in order to analyze the effect of the non-sterol lipid composition. We used dlps as the charged phospholipid, as phosphatidylserines with longer saturated chains form solid–liquid two phase regions when mixed with dchol [37], and it was not our purpose to explore the effect of the solid phase state on the MBP induced segregation of phases.

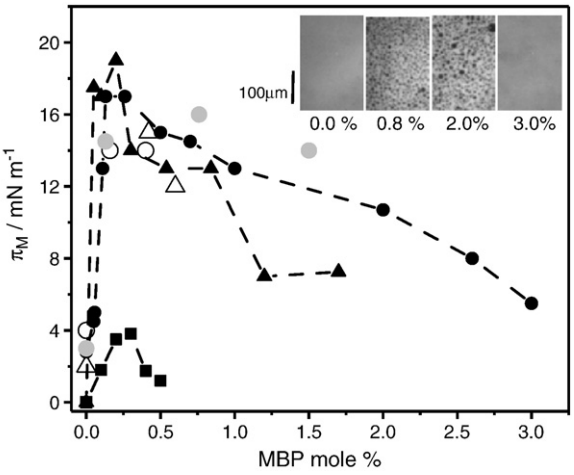

Fig. 2. Mixing/demixing lateral pressure as a function of the MBP content for monolayers of myelin lipid (squares), dlps/dchol 65:35 (black circles) and dlps/smC16/dchol 12:53:35 (black triangles) and their respective controls with cholesterol instead of dchol (open symbols). The gray symbols are for dlps/dchol in a proportion 58:42. The lateral pressures (π_M), determined by the Wilhelmy method, are those at which phase segregation is visualized by fluorescence microscopy using 0.8 mol% RhoC16 as the fluorescent probe (see the Materials and methods section). Subphase: Pure Water. The inset shows representative photos for the dlps/dchol 65:35 lipid mixture at 10 mN m^{-1} and at the indicated MBP proportions.

We inspected the behavior of the surface pattern upon compression for the different monolayer compositions. Section 3.1 describes the results found on pure water subphases and Section 3.2 analyzes the effect of adding salts to the subphase. Finally, Section 3.3 analyzes the influence of the monolayer composition on the phase segregation.

3.1. Monolayer behavior on pure water

Fig. 2 shows the mixing–demixing surface pressure vs. MBP mole % phase diagrams for films of MBP with purified myelin lipids from bovine spinal cord which contain about 40 mol% [33] of cholesterol (squares), dlps/dchol 65:35 (black circles), dlps/dchol 58:42 (gray circles) or dlps/smC16/dchol 12:53:35 (black triangles), on pure water. For the dlps/smC16/dchol, larger percent of dchol turns the mixture out of the alfa phase zone, therefore we only analyzed mixtures with 35% dchol or lower. The open circles and triangles in Fig. 2 correspond to the same mixtures described by the black circles

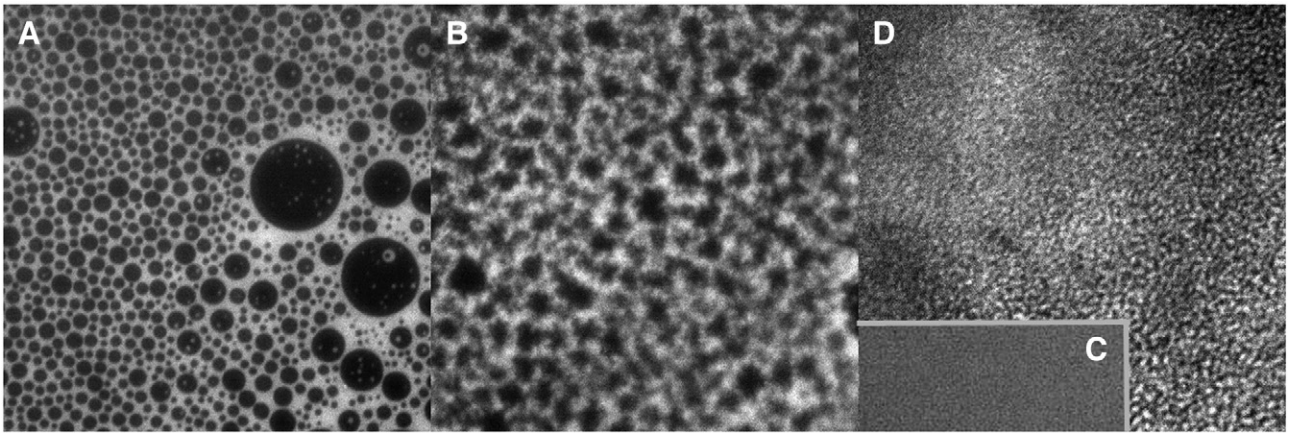

Fig. 1. Representative photos for monolayers on pure water formed by mixtures of myelin lipids with 0.2 mole % of MBP. (A) 2 mN m^{-1} during compression, (B) 3.7 mN m^{-1} during compression, (C) 5 mN m^{-1} during compression or expansion and (D) 3.6 mN m^{-1} during expansion. Notice that during compression, the domains fuse at 3.7 mN m^{-1} and the monolayer is homogeneous at higher pressures. During the expansion, the domains reappear at almost the same lateral pressure. Photos size: 200×200 μm (A, B and D) and 125×50 μm (C).

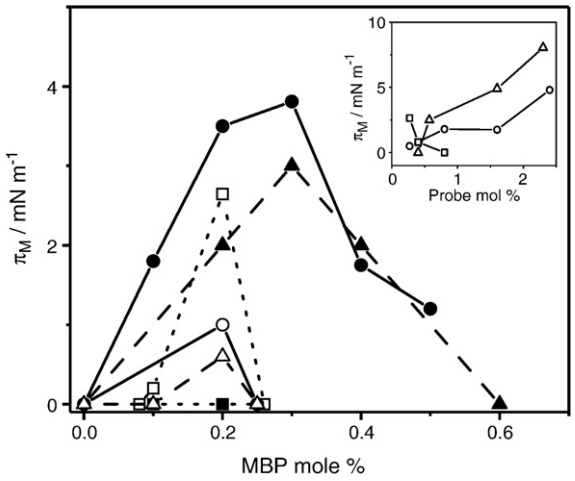

Fig. 3. Mixing/demixing lateral pressure as a function of the MBP content for monolayers of myelin lipids. The lateral pressures (π_M) were determined as in Fig. 2, in monolayers visualized by including the probes RhoeggPE (triangles), RhoC16 (circles) and DiIC18 (squares) as fluorescent probes at 0.8 mol% (filled symbols), 0.27 mol% (open squares, DiIC18) and 0.4 mol% (open triangles and circles, RhoC16 and RhoeggPE). Subphase: Pure Water. The inset shows the π_M values as a function of the proportion of probe for films with 0.2 mol% of MBP (symbols as in the main panel).

and triangles but with cholesterol instead of dchol. Each value of mixing/demixing lateral pressure (π_M) represents the surface pressure at which the domains reappear upon expanding a monolayer previously compressed until it becomes homogeneous, as revealed at the microscopic level (0.2 μm^2) by the probe distribution (see Materials and methods section). For each curve, the areas under the lines represent two phase regions and those above them correspond to a single homogeneously fluorescent phase. The inset shows photos for the dlps/dchol/MBP (65:35:MBP) mixture at 10 mN m^{-1} for the indicated MBP mole %.

The addition of relatively low proportions of MBP stabilized the two-phase state at lateral pressures higher than the mixing/demixing lateral pressures of the protein-free lipid mixtures (Fig. 2); notice that π_M is 3 mN m^{-1} for dlps/dchol while the myelin lipid mixture and the dlps/smC16/dchol mixture are already homogeneous near 0 mN m^{-1}. As the MBP proportion increases, the values of π_M first increase up to an optimal MBP concentration, and subsequently decrease for higher protein proportions. The mole fraction of MBP at which the segregated state becomes more stable is about 0.25 mol% for all lipid mixtures. The region of the phase diagram with two-phase coexistence, for mixtures of MBP and myelin lipid mixtures, is markedly reduced with respect to the phase diagram reported elsewhere on subphases with salts [4]. As shown below, the same difference is observed for simple mixtures (see Section 3.2).

The experiments were repeated using different probes to check for their possible effects on the surface pattern and phase behavior observed. We found an interesting effect of the type and proportion of the probe on the phase diagram of myelin lipid mixtures with MBP that was not observed in the simpler mixtures. In monolayers of the myelin lipid mixture with MBP, the presence of probes can displace to higher protein contents the surface pressure limit for the two-phase coexistence region and change the relative proportion of phases, while keeping their liquid phase state. In Fig. 3 we show the variation of π_M as a function of the proportion of MBP, in myelin lipid mixtures with different mole fractions of the fluorescent probes RhoC16 (circles), RhoeggPE (triangles), and DiIC18 (squares). When the concentration of RhoC16 or RhoeggPE increases from 0.4 mol% (open symbols) to 0.8 mol% (filled symbols), the values of π_M also increase. On the contrary, the increase of DiIC18 mole %, decreases the surface pressures of mixing. The formation of domains becomes stable only with low mole fractions of this probe (0.3 mol%, open squares). With 0.8 mol% no phase coexistence is observed (filled squares). The inset in Fig. 3 shows the variation of π_M with the proportion of the fluorescent probes in films with 0.2 mol% of MBP. The effects are rather noticeable even for slight variations of the amount of probes, all within the range of mole fractions usually employed to observe monolayers by fluorescence microscopy [2,31,32]. As stated before, in films of MBP mixed with dlps/dchol 65:35 or dlps/smC16/dchol 12:53:35, varying the proportion and type of probe made no change on the pattern or the π_M. Nonetheless, in these films, probes could still have an effect in the range of concentrations below those used for observations with fluorescence microscopy, as found by others on the miscibility transition temperatures of ternary vesicles [38].

Our results with the probes show that small changes in the proportion of a single component can modify the phase diagram of a mixture with all the lipid diversity of a whole natural membrane. Interestingly, the composition of the complex mixture is apparently

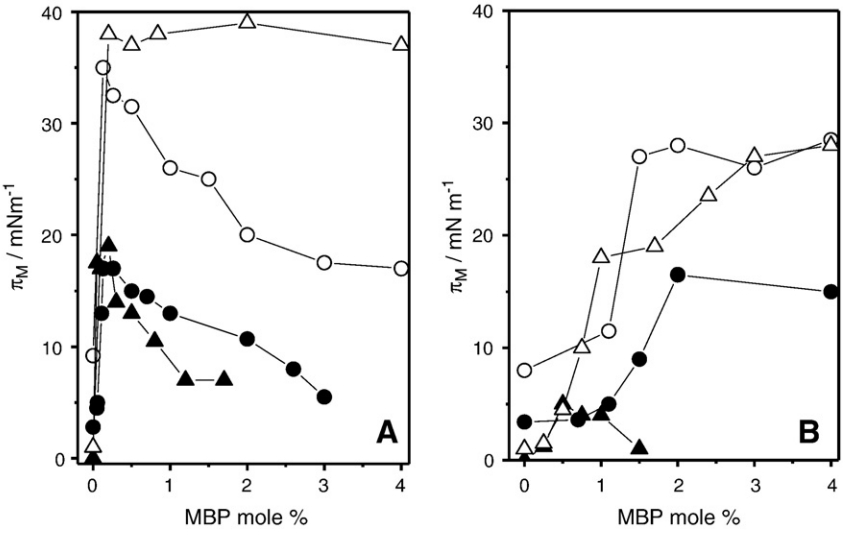

Fig. 4. Mixing/demixing lateral pressure (π_M) as a function of the MBP content for monolayers whose lipid fraction is (A) dlps/dchol 65:35 (circles) or dlps/smC16/dchol 12:53:35 (triangles). (B) dlps/dchol 80:20 (circles) or dlps/smC16/dchol 15:65:20 (triangles). Subphase: pure water (filled symbols) and NaCl 0.10 M (open symbols). Probe: 0.8 mol% RhoC16.

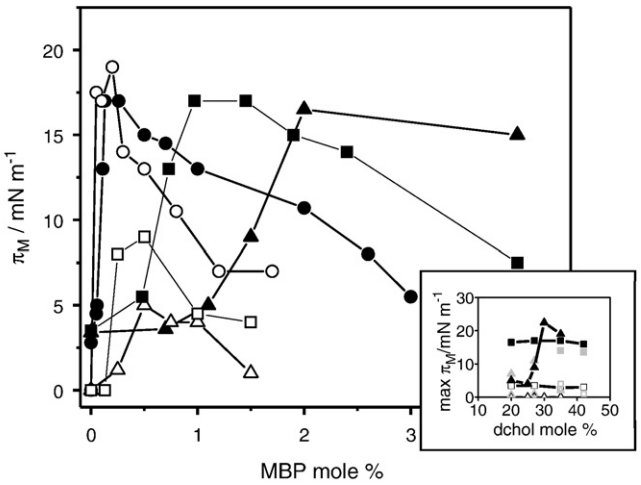

Fig. 5. Mixing/demixing lateral pressure (π_M) as a function of the MBP content for monolayers of dlps/dchol/MBP (filled symbols) or dlps/smC16/dchol/MBP (open symbols) at a dlps/smC16 18.5:81.5 with variable amounts of dchol. The percentages of dchol are 20 mol% (triangles), 27 mol% (squares) and 35 mol% (circles) in the lipid fraction. The inset shows the maximum π_M values as a function of dchol for dlps/dchol (squares) and dlps/smC16/dchol (triangles) films, with MBP (filled symbols) and without MBP (open symbols). Subphase: pure water. Probe: 0.8 mol% RhoC16. The gray symbols correspond to controls performed with cholesterol instead of dchol.

more sensitive to small perturbations of composition than the simpler mixtures. On the other hand, even when used in small fractions, probes are not just inert reporters since they can alter the monolayer membrane phase behavior, depending on their type, proportions and also on variations of the membrane composition. The opposing tendencies observed for the cationic DiIC18 and the anionic Rhodamine-derived probes on the surface pressure of mixing suggest that electrostatics might contribute to the effects. In whole myelin monolayers the segregation leads to an increased local concentration of MBP that could be favored, or antagonized, by molecules having the opposite or the same charge, respectively. In spite of the observed dependence of the two-phase limits with the probe type and concentration, the general mixing–demixing behavior of the myelin lipid mixture with MBP using different probes appears similar.

3.2. Effect of ions in the subphase

As mentioned in Section 3.1, when NaCl solution instead of pure water is used as subphase, the π_M values increase for all the systems studied. Fig. 4 shows the values of π_M as a function of the proportion of MBP in monolayers of dlps/dchol (circles) and dlps/smC16/dchol (triangles), on NaCl 0.1 M (open symbols), for films containing 35 mol % (Fig. 4A) and 20 mol% (Fig. 4B) of dchol. The π_M values on water are also shown for comparison (filled symbols). The addition of MBP to both lipid films with 35 mol% dchol produces a large increase in π_M, reaching surface pressures well above those of the protein-free lipid films on water. The π_M is maximum at about 0.1–0.2 mol% of MBP. For higher proportions, π_M decreases for dlps/dchol mixtures (Fig. 4A open circles) and remains the same for dlps/smc16/dchol (Fig. 4A open triangles), where the segregated state persists up to the monolayer collapse pressure. For mixtures with 20 mol% dchol, the presence of salts has the same stabilizing effect on the two-phase state at high lateral pressures (Fig. 4B).

In previous experiments with monolayers of the myelin lipid mixture on ionic subphases (10 mM Tris buffer, 100 mM NaCl and 20 mM $CaCl_2$) an increase of the π_M values was also observed for the same MBP proportion, similar to the dlps/smc16/dchol mixture, but at lower surface pressures [4]. The behavior on NaCl 0.10 M shows the trend reported in [4].

3.3. Effect of composition

3.3.1. The stability of the phase segregation depends on the dchol proportion

The effect of changing the proportion of dchol on the MBP induced lateral phase segregation is shown in Fig. 5 for monolayers spread on water subphase. In these curves, the π_M values as a function of the MBP proportion are plotted for films with 20 mol% (triangles), 27 mol% (squares) and 35 mol% (circles) of dchol in the lipid fraction. The non-sterol lipids are dlps (filled symbols) or a dlps/smC16 mixture (open symbols) in a proportion 18.5:81.5. The addition of relatively small amounts of MBP to mixtures with 35 mol% dchol increases the stability of the two phase state at high lateral pressures. In monolayers with lower mole % of dchol, addition of a higher proportion of MBP is required for inducing the separation of phases at pressures higher than the π_M value observed in protein-free monolayers. This effect is more marked in the absence of smC16. The same is also evident in the phase diagrams in Fig. 6, that show the composition zones with one phase (white areas) and two phases (dashed areas), at 9 mN m^{-1}, in films of dlps/dchol/MBP (Fig. 6A) and dlps/smC16/dchol/MBP (Fig. 6B). The open symbols represent

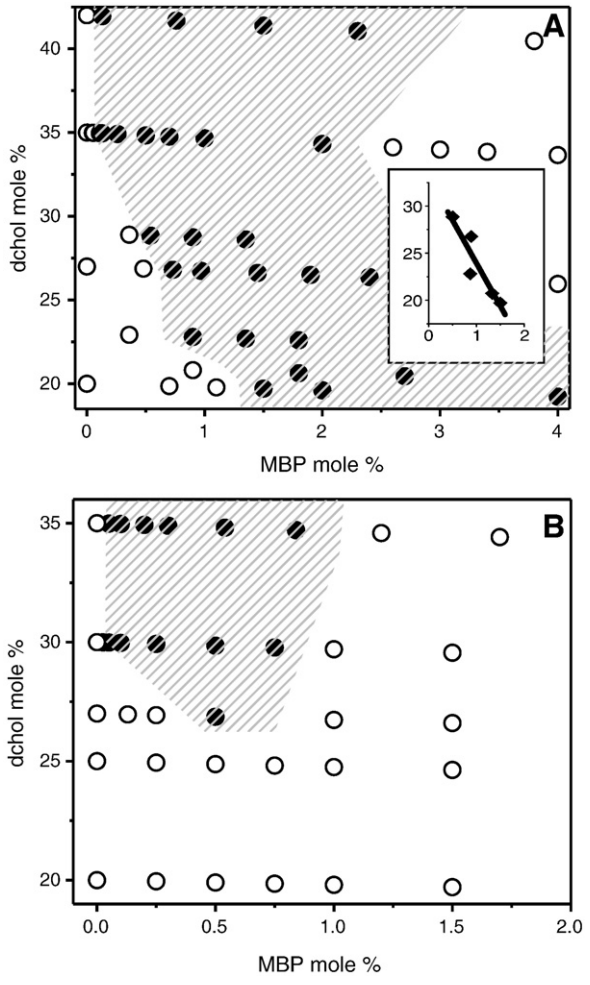

Fig. 6. Phase diagrams at 9 mN m^{-1} for mixtures of dlps/dchol/MBP (A) and dlps/smC16/dchol/MBP, where the dlps/smC16 ratio is 18.5:81.5 (B) with variable amounts of dchol. The dashed zone is a two phase region and the white zone is a one phase region. The filled and open symbols represent, respectively, the proportions of MBP at which two phases or one phase was observed. The inset in (A) shows the linearly interpolated composition values at 9 mN m^{-1} for the border between the one phase and the two phase region, for low MBP mole % (left border of the diagram). The line is a linear regression for those values, from which defined dlps/MBP and dlps/dchol ratios were estimated (see text). Subphase: pure water. Probe: 0.8 mol% RhoC16.

compositions were one phase is observed at this lateral pressure and the black symbols represent two phase zones. At least 35 mol% (Fig. 6A) or 30 mol% (Fig. 6B) of dchol is required to turn the homogeneous film into a heterogeneous one by the addition of a small proportion of the protein. For films spread on NaCl solutions the segregation induced by MBP shows a similar dependence with the proportion of dchol. The increase of the π_M values begins at about the same proportion of MBP as on water although, as above described, the region with two phases extends to higher surface pressures and increased mole % of MBP. This can be seen in Fig. 4 by comparing the curves on water (filled symbols) and on NaCl 0.10 M (open symbols) of the films with 35 mol% (Fig. 4A) and 20 mol% of dchol (Fig. 4B). On the other hand, lipid films of dlps or smC16/dlps without dchol do not result in macroscopic phase separation upon the addition of MBP in the range of 0.5–4 mol%, neither on NaCl nor on water (not shown). The results indicate that the phase segregation induced by MBP is finely regulated by the content of dchol, and furthermore strongly suggest that the sterol is required for the macroscopic phase segregation induced by MBP. It might be worth recalling that when phase segregation occurs in films of whole myelin and in mixtures with myelin lipids, MBP becomes excluded from the cholesterol enriched domains [2].

3.3.2. The phospholipid composition influences the dchol–MBP-induced phase segregation

The comparison of the curves in Fig. 5 reveals that the phospholipids composition of the mixture regulates the dependence of phase segregation on the proportion of dchol and of MBP. One clear difference is the mole % of MBP required to induce phase separation at a particular proportion of dchol, in mixtures with different non-sterol lipids. For example, in films with 20 mol% (triangles), or 27 mol% of dchol (squares), higher proportions of MBP are required to induce phase segregation when the non-sterol lipid is dlps (filled symbols) compared to a mixture of dlps and smC16 in a 18.5:81.5 ratio (open symbols). No difference is observed for films with 35 mol% dchol (circles), where even small proportions of MBP raise the surface pressure of mixing in both systems. The same occurs on NaCl subphases, showing different behavior for 20 mol% dchol (Fig. 4B), but not for 35 mol% dchol (Fig. 4A).

The inset in Fig. 5 shows the highest surface pressure at which MBP can induce segregation of phases for different proportions of dchol, in films with dlps (filled squares) or dlps/smC16 (filled triangles) as the non-sterol lipid. Each of these values corresponds to the maximum π_M of the curves shown in Fig. 5. The gray symbols in the inset correspond to control experiments performed with cholesterol instead of dchol and with the same MBP proportion. The values of π_M for pure lipid films are also shown in the same graph (open symbols) for comparison. The variation of the maximum pressures for demixing with the proportion of dchol clearly depends of the non-sterol lipid composition. In dlps/dchol films, the segregation induced by MBP occurs up to approximately the same surface pressure (about 17 mN m^{-1}) for all the analyzed proportions of dchol. On the other hand, in the films with smC16, there is a sharp increase of the maximum attainable π_M value at about 27 mol% of dchol.

The phase diagram of dlps/dchol/MBP films (Fig. 6A) shows that MBP and dchol coexist in one homogeneous phase (open symbols, white area) for low MBP and low dchol proportions (below 35 mol% of dchol). Moreover, the limit for the transition from the one-phase to the two-phase region occurs at increasingly higher proportions of MBP as the content of dchol decreases, meaning that the homogeneous phase state admits more MBP when less dchol is present in the film and vice-versa. A possible reason for this behavior is that MBP and dchol can remain within a same homogeneous phase up to limiting ratios of phospholipid/MBP and phospholipid/dchol are reached. The curves of π_M vs. MBP mole % indicate that the mixing–demixing pressures exhibit a steep rise on the low MBP mole % side (Figs. 4 and 5). This suggests that the limit of the one phase region may be set by defined stoichiometries since the transition to the two phase state occurs over a rather small range of proportions of MBP and dchol with respect to dlps. Assuming that on the one-phase to two-phase boundary all the dlps molecules are interacting with dchol (D) or MBP (M) in proportions D:1 and M:1, respectively, then the left phase segregation border of the phase diagram, should follow the equation:

$$\text{dchol mole \%} = \frac{100\%}{1+D} - \frac{1+M}{1+D}\ \text{MBP mole \%}.$$

With this model, we estimated the stoichiometry values at 7, 9 and 12 mN m^{-1} that are listed in Table 1. The inset in Fig. 6A shows the result at 9 mN m^{-1}. For the dlps/dchol interaction we found a proportion of 2:1, which coincides with a phospholipid/cholesterol (and phospholipid/dchol) ratio frequently associated with the formation of complexes [32]. Many mixtures of phospholipid–cholesterol show sharp changes in some physical properties at this ratio, usually interpreted as resulting from the formation of condensed complexes between the phospholipid and cholesterol. These complexes were predicted to exist in segregated and also in homogeneous phases [32]. On the other hand, the number of dlps molecules per MBP is in the range 24–26, which is within the values reported in the literature for the number of acidic lipids that interact with one MBP molecule [12,27,28].

For monolayers formed with dlps/smC16/dchol/MBP mixtures, at 35 and 30 mol% of dchol, small proportions of MBP raise the π_M value from 0 to about 17 mN m^{-1}, even when added in concentrations as low as 0.025 mol%. On the other hand, in films with 27 mol% of dchol, up to 0.15 mol% of MBP can mix in the lipid film without increasing π_M (Figs. 5 and 6B). This suggests that the limit composition for the segregation of MBP occurs between 27 and 30 mol% of dchol, near the 2:1 phospholipid/dchol ratio found for dlps/dchol mixtures. In dlps/smC16/dchol/MBP films, the dependence on the dchol proportion of the MBP mole % required for demixing occurs with a simultaneous decrease of the maximum π_M to low surface pressures (Fig. 5).

4. Discussion and conclusions

Our results show that MBP can induce a segregation of phases in a dchol dependent manner (the general behavior of cholesterol, used as a control in some experiments, is essentially the same, see Fig. 2). The absence of domains (at least larger than 0.2 μm^2), in a dchol free membrane with MBP, strongly suggests that dchol is indeed essential to induce the macroscopic phase separation created by the interaction of MBP with the lipids. The two-phase state becomes stable at higher lateral pressures over high ionic strength subphases, where some electrostatic-derived effects should be weakened. The dependence of domain formation on dchol and the effect of ions suggest that the main driving force for the segregation is not electrostatic. Moreover, in experiments with films of smC16 and dchol, where the sole negative lipid was the probe (less than 1 mol%), the interaction of MBP also induced two phase segregation [2]. The involvement of cholesterol, known to affect membrane mechanical properties [29], supports the existence of hydrophobic interactions between the protein and the lipids that require the insertion of parts of the protein inside the membrane. Several reports indicate that MBP can distort

Table 1
Number of dlps molecules interacting with MBP (M) and with dchol (D) in the left phase segregation border of the phase diagrams for mixtures of dlps/dchol/MBP (Fig. 6A), calculated as indicated in the text.

	7 mN m^{-1}	9 mN m^{-1}	12 mN m^{-1}
D	2.2 ± 0.3	2.0 ± 0.2	1.8 ± 0.1
M	26 ± 12	26 ± 8	24 ± 4

lipid membranes, in some cases favoring fluid patches, which should oppose to the ordering effect of cholesterol. For example, penetration/adsorption of MBP in bilayer vesicles can prevent crystallization of the lipid fatty acyl chains, and decrease the enthalpy of the main transition in various lipid systems [14,15], MBP can also induce liquid-expanded phases upon the interaction with condensed monolayers [18,25], and perturbs the lipid chain mobility in liposomes, as measured by electron paramagnetic resonance [12,13]. In monolayers of the myelin lipid mixture with MBP, we previously found that after the interaction of MBP with the rather incompressible myelin lipid film, the protein self-segregates in expanded phase domains, separated from a lipid phase with low lateral compressibility [4].

On the other hand, it has been observed that the increase of cholesterol content can reduce the binding of peptides to lipid membranes, and decrease the partition of transmembrane proteins to cholesterol-enriched phases [39]. As a counterpart, the exclusion of proteins or peptides from cholesterol enriched phases, can promote the formation of domains [30]. This mechanism of mutual exclusion may be relevant for the segregation of phases induced by MBP in the presence of cholesterol in the myelin lipid mixtures. So far, the ability of MBP to cause phase separation by sequestering acidic lipids from mixtures with neutral lipids has been well documented [10,14,15,23,27]. However, we observed that the interaction of MBP with acidic lipids, in monolayers with dlps and smC16, does not induce phase segregation in the absence of dchol. As mentioned before, in all the mixtures that we analyzed, dchol is required for MBP to promote domain formation. In this sense, the analysis of the phase diagram suggests that the transition to a two-phase state is observed when the molar proportions of phospholipid/MBP and phospholipid/dchol in the homogeneous phase, reach critical values. The role of dchol in the segregation process related to a particular stoichiometry is compatible with an effect on the packing order and elasticity of the membrane. In this regard, several lines of evidence have shown that membrane properties, especially those being influenced by the membrane free volume and tightness of lateral packing, are modulated by the proportion of cholesterol with sharp changes occurring at critical stoichiometries [32,40]. The stoichiometries reported here probably depend on the phospholipid–dchol and phospholipid–MBP interactions, possibly reflecting the distinct interaction that MBP and dchol establish with different types of phospholipids [15,23,25]. Interestingly, the dlps/MBP ratio, in films where the sole phospholipid is dlps, coincides with the number of anionic lipids reported to bind to MBP [12,13,28] and the dlps/dchol ratio coincides with a proportion where numerous mixtures of phospholipid and cholesterol show evidence of condensed complex formation [32].

In vesicles, cholesterol was shown to improve MBP-induced adhesion of liposomes, with a concomitant increase in the hydrophobic binding of MBP to the membrane [17,20,21]. Our present results may be in keeping with this concomitant effect of MBP and cholesterol since the transition from homogeneous to heterogeneous patterns modifies the local concentration of the protein as well as the mechanical properties and the composition of its local environment. In this sense, it was shown that the type of lipids and the phase state influence the extent of penetration of MBP in the membranes, with deeper penetration in liquid crystalline and liquid-expanded phases than in more condensed states [14,18,23,25]. This in turn would enhance the hydrophobic interactions that contribute to the binding/anchoring of MBP to the membrane.

Our results from MBP in simple lipid mixtures show that the phase state depends on multiple factors in a rather complex manner. The phase segregation is sensitive to variations of dchol and MBP proportions. Moreover, changing the composition of non-sterol lipids can modify the dependence of the two phase segregation on the proportion of dchol, regulating the concentration of MBP necessary to induce the formation of domains. In other words, modifying the composition of the phospholipids can change the susceptibility of the membrane to phase separate upon the interaction with MBP. The segregation is also regulated by changes of salt concentration in the subphase, with the domains becoming more stable on high ionic strength solutions, persisting up to higher pressures and greater proportions of MBP. In view of our results the synergic effect of MBP and dchol would permit a regulation of the lateral surface arrangement through changes of the salt concentration and of the composition of neutral or charged lipid species.

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