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Structural modifications of lipid membranes exposed to statins – Langmuir monolayer and PM-IRRAS study

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ABSTRACT

The effects of selected statins on the structure and properties of lipid membranes composed of zwitterionic (1,2-dimyristoyl-sn-glycero-3-phosphocholine, DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine, DMPE) or anionic (1,2-dimyristoyl-sn-glycero-3-phospho-L-serine, DMPS) lipids were studied for the first time by Langmuir technique combined with polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS) and Brewster angle microscopy (BAM). The interactions of statins of different hydrophobicity: pravastatin, fluvastatin, and cerivastatin with the polar region of the lipids forming the membrane were monitored by PM-IRRAS and the changes of the overall monolayer structure and organization were described on the basis of surface pressure vs. area per molecule measurements and Brewster angle microscopy. Large differences in the action of each of the statins on the lipid monolayers were observed and explained by their different hydrophobicity combined with the different degree of hydration of the lipid polar headgroups in the monolayer. Monolayer fluidizing effect was connected with the interaction of statins in the headgroup region of the membrane affecting the original hydrogen bonding in the lipid layers. The most hydrophilic pravastatin interacted only with the polar head groups of the monolayer and affected the organization of the polar part of the lipid membrane by increasing the headgroups hydration. In the case of DMPS, the contribution of electrostatic interactions between the negatively charged headgroups and the drug was observed, and for this lipid especially strong dehydration effect of cerivastatin was revealed. It facilitated the incorporation of the hydrophobic part of the drug into the nonpolar region of the DMPS layer and in this case there was almost no fluidization of the layer. Strong dehydration effects may be dangerous for the lipid membranes and may also be one of the reasons to avoid cerivastatin in the therapies, despite its known efficacy especially in view of the large doses and prolonged application that are usually needed.

1. Introduction

Statins are the most common hypolipidemic drugs which act as competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-Coenzyme A (HMG-CoA) reductase (HMG-CoA reductase), the enzyme which catalyzes the rate-limiting step: the deacylation of HMG-CoA to CoA and mevalonate in the cholesterol biosynthetic pathway [1–4]. One of the mechanisms for controlling HMG-CoA reductase activity is the so-called negative feedback [5]. Reductase activity decreases as the concentration of mevalonate and thus cholesterol increases. Cholesterol is an important lipid in membranes of higher eukaryotes and accounts for ~30–50% of the total plasma lipid content. The unique structure of cholesterol and its ability to organize into distinct membrane domains contribute to the functional role of cholesterol in many processes e.g. signal transduction, trafficking, and pathogenesis [6]. Cholesterol is mainly produced in hepatocytes and from there it is transported throughout the body systems using lipoproteins, LDL and HDL [7]. Too high levels of LDL-cholesterol result in cardiovascular diseases and are nowadays the primary cause of disability and premature death [8,9]. Several approaches to treat hypercholesterolemia have been used worldwide since it represents a major risk for coronary heart disease.

Statins can be divided into those of natural and synthetic origin. Pravastatin used in our study belongs to the first group, while fluvastatin and cerivastatin are synthetic statins produced in biotechnological processes (Fig. 1) [10]. Statins have various structures, however, they all have the same pharmacophore group of the beta-hydroxy acid chain. They differ in the substituents that determine their lipophilicity. In their structure, statins are similar to the HMG-CoA molecule but show greater affinity towards HMG-CoA reductase [11]. It is important

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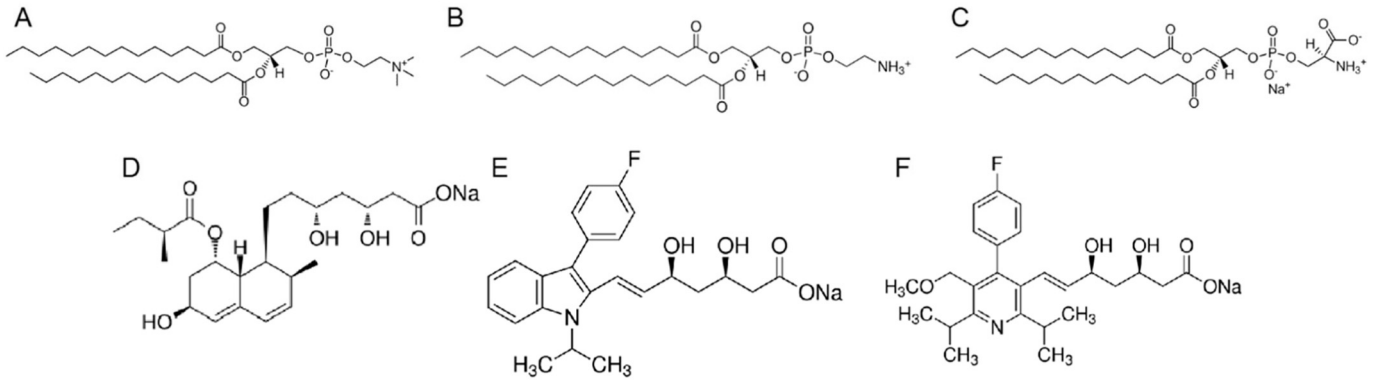

Fig. 1. Structural formulas of phospholipids: A) DMPC; B) DMPE; C) DMPS; and statins: D) pravastatin; E) fluvastatin; F) cerivastatin.

to take into consideration that they may not only interact with their therapeutic target protein but also they can modify the properties of the cell membranes, which would also cause alterations in the function of membrane proteins by changing e.g. packing of the lipid molecules around the proteins. An important direction of research on statins should be, therefore, to understand the changes that may occur in the lipid membrane under the influence of statins and factors that control drug penetration through these membranes.

The statins' effect on lipid membranes remains underexplored and the reports are scarce, since attention is focused rather on controlling cholesterol levels or statins' role in processes related to cancer. Larocque et al. discussed the interactions of fluvastatin with DMPC/DMPS bilayered micelles (bicelles) studied with infrared spectroscopy and NMR [12]. Fluvastatin was found to enter the DMPC/DMPS bilayer interface by interacting with the lipids' polar headgroups, while aromatic moieties partitioned into the nonpolar part of the bilayer. Lipid segregation also took place between the anionic and zwitterionic lipids in the membranes due to a preferential interaction of fluvastatin with DMPS. The interaction of a series of statin molecules: pravastatin, mevastatin, simvastatin, and fluvastatin with a phosphatidylcholine monolayer immobilized on porous silica particles has been studied using a biochromatographic approach (molecular chromatography) [13]. The immobilized artificial membrane provided a biophysical model system to study the binding of statin molecules to the lipid membrane. Mevastatin, atorvastatin, simvastatin and fluvastatin exhibited higher affinity towards the lipid monolayer surface than pravastatin. Thus, these statins were proposed to efficiently cross the cellular membrane without a transporter, simply by passive diffusion. Studies of mica supported lipid bilayers by atomic force microscopy have shown that statins affect nanomechanical stability of the supported bilayer and increase its heterogeneity [14]. The behavior of such membrane models depends, however, on the properties of the solid support, which is not present in the case of real membranes.

In this report we focus on showing how the contact with a solution containing statins affects the organization of simple one-component lipid layers (DMPC, DMPE or DMPS) treated as one leaflet of a lipid membrane, which is free from any rigid support (Fig. 1). The composition of model membranes was chosen based on the differences in the polar head group region properties. The lipids selected for the monolayer formation are the zwitterionic (DMPC, DMPE) and the negatively charged (DMPS) lipids. These lipids represent the groups of phospholipids, which are the main components of intestinal cell membranes, where the absorption of the selected drugs takes place [15].

Our approach is novel in that the lipid monolayer formation by the Langmuir technique at the air-solution interface allows the evaluation of statin induced changes on the molecular level - in the lipid molecules orientation and parts of the lipid interacting with the drug. Thus, the impact of those changes on the organization of the whole layer can be assessed. We selected 3 statins: pravastatin (PRA), fluvastatin (FLU) and cerivastatin (CER) differing in structure and lipophilicity to be added to the subphase (Fig. 1). The octanol–water partition coefficient logP, used to quantify the lipophilicity and to predict drug transport across biological membranes was reported to be 2.20 for pravastatin, which is the lowest value among the 3 drugs and indicates the weakest affinity for hydrophobic part of the monolayer [13]. Fluvastatin (logP = 4.50) and cerivastatin (logP = 3,40–4.15) contain aromatic rings in their structures, which increase their lipophilicity [13,16]. The above statins also differ in pK_a values. The pK_a value for pravastatin is 4.36, for fluvastatin 4.15 [13]. However, for cerivastatin two pK_a values are distinguished, pK_{a1} is 4.38 for the carboxylic acid form, while pK_{a2} for the pyridine form is 5.29 [17]. The investigated lipid monolayers consist of 3 common lipids. DMPC molecules are neutral with their polar head consisting of the uncharged choline. DMPE differs from DMPC in the structure of polar head - the hydrophilic part is less hydrated than the choline group and the NH_3^+ and PO_4^- groups interact forming hydrogen bonds. DMPS is a molecule with a negatively charged headgroup under conditions of our work [18,19]. Since the fatty acid residues are the same, the effect of polarity, charge and hydration of the lipid headgroups in the monolayer on the binding of statins can be compared. The molecular alterations of the lipid monolayer organization are elucidated using a combined approach involving measurements of surface pressure, Brewster angle microscopy and PM-IRRAS. Understanding of the effect of statins on the lipid component of the membrane is important for the appropriate treatment for hypercholesterolemia, choice of suitable statin and its dosing [20].

2. Experimental

2.1. Materials

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE), 1,2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS) were purchased from Avanti Polar Lipids (USA). High purity and anhydrous solvents: chloroform and methanol were used, both obtained from Sigma Aldrich (Poland). DMPC solution was prepared in chloroform, while DMPE and DMPS were dissolved in chloroform/methanol 4:1 v/v. Either pure water or phosphate-buffer saline (PBS) solution of pH 7.4 and concentration 0.01 M corresponding to the physiological conditions was used as the subphase. Pravastatin, fluvastatin and cerivastatin purchased from Sigma Aldrich were of high purity ≥98%. They were dissolved in water/PBS buffer subphase in order to obtain solutions at three concentrations (10^{-6}, $5 \cdot 10^{-6}$ and 10^{-5} M). The concentration values were chosen based on other studies on statin-lipid interactions as well as on in vitro studies on the possible statins' anticancer effect on human ovarian cancer cell

lines [21,22]. MilliQ water with resistivity 18.2 MΩ was used in the experiments.

2.2. Methods

2.2.1. Langmuir technique

The experiments were carried out using KSV-Nima Langmuir trough with Wilhelmy microbalance. The setup consists of two moveable, hydrophilic barriers and hydrophobic troughs of total areas of either 243 cm^2 or 587 cm^2. After careful cleaning of the trough with methanol and chloroform and rinsing with plenty of water, the phospholipid solutions in chloroform or chloroform:methanol 4:1 v/v were deposited on pre-cleaned surface of the subphase by a Hamilton syringe (50 μl or 100 μm). After that, organic solvent evaporation was allowed for 10 min. The measurement of surface pressure (±0.1 mN/m) was performed using filter paper as the Wilhelmy plate connected with the Wilhelmy balance. All experiments were carried out at room temperature (21 ± 1 °C).

2.2.2. Brewster angle microscopy (BAM)

Brewster angle microscopy images were recorded using UltraBAM with Nanofilm_ep3 setup (Accurion, Germany). BAM images were taken simultaneously with the measurement of surface pressure – area per molecule (π-A) isotherm. The actual size of the photographed area of monolayer is 800 μm × 430 μm.

2.2.3. Polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS)

PM-IRRAS experiments were carried out using trough of total area of 243 cm^2 and KSV-Nima software. A KSV-Nima PM-IRRAS (PMI 550) spectrometer was applied. The light beam (He-laser and IR) reached the air/water surface at the angle of 76°, the measurement range expressed in the wavenumber was between 700 cm^{-1} and 4000 cm^{-1} with the resolution of 8 cm^{-1}. On one arm of the goniometer there is an FTIR spectrometer with ZnSe polarization modulation unit (Hinds Instrument, USA), which was set to 1500 cm^{-1} to ensure its maximum efficiency in the polar head group region and on the other arm there is the MCT detector. The light was constantly modulated between the polarization p and s, so that the difference of the above-mentioned signals gives the signal for surface and their sum – the reference spectrum. If S is the final spectrum, it is defined as $S = \delta/\sigma$, where δ is difference of R_s and R_p (reflectivities of the s and p beam, respectively) and σ is the sum of the detected reflectivities of the p- and s- polarized light. Each measurement consisted of 3000 scans, thus the measurement time was 5 min. Before each measurement the background measurement for the pure subphase without phospholipid was performed. Each final spectrum was normalized, which was done by applying the following formula:

$$\Delta S = (S_\pi - S_0)/S_0, \quad (1)$$

where S_π is the spectrum of monolayer and S_0 is the background spectrum. Such background-corrected spectra are reported in this manuscript. Additionally, the PM-IRRAS spectra were also baseline-corrected. The measurements were made at the surface pressure of 30 mN/m, which was kept constant during the collection of the spectra.

3. Results and discussion

3.1. Langmuir monolayer studies of the interactions of statins with phospholipid membranes

In order to assess the effect of the three selected statins on the model phospholipid membranes the Langmuir experiments at the air-water interface were performed. As mentioned above, the model systems were two zwitterionic lipids (DMPC and DMPE) and one negatively charged lipid (DMPS). The drugs were dissolved in the subphase, on which the phospholipid monolayers were formed. The changes in the isotherm shapes and characteristic parameters due to the interactions with drugs were followed.

The surface pressure-area per molecule isotherms for the DMPC monolayer were recorded for the pure PBS buffer subphase or water and following the addition of statins: pravastatin, fluvastatin and cerivastatin (Figs. 2A and S1). The differences between isotherm parameters are collected in Table 1. DMPC is a neutral lipid with the uncharged choline and the film prepared on pure PBS buffer is in a liquid-expanded phase. The compressibility modulus allows one to describe the state of the monolayer at the air-solution interface [23]:

$$Cs^{-1} = -A\frac{d\pi}{dA} \quad (2)$$

The C_{smax}^{-1} value equal to 80 mN/m confirms the liquid-expanded form of the monolayer in agreement with the literature data (Fig. 2A and Table 1) [18].

The presence of each of the statins in the subphase caused a shift of the isotherms towards higher areas per molecule, as indicated by the value of the area, at which the "lift-off" point is seen. The largest changes are thus observed at small surface pressures, i.e. at early stages of monolayer formation. Although interactions of all statins with phosphocholine monolayers were visible by the changes of the isotherms in all cases, the largest increase in A_0 together with a significant increase in membrane fluidity are observed for fluvastatin (Fig. 2A and Table 1). Fluvastatin remains in the monolayer up to high surface pressures leading to the fluidization of the original monolayer. For cerivastatin a significant difference in the isothermal shape is observed compared to that of the DMPC monolayer formed on pure buffer subphase (Fig. 2A). Surface pressure begins to increase already at very large area per molecule values and the compression modulus decreases relative to that of the DMPC monolayer on pure subphase to the value of 52.0 mN/m indicating a highly expanded liquid phase (LE) [23]. In the liquid-expanded phase, the lipid molecules interact with each other mainly through polar heads and their hydrophobic tails are inclined at different angles to the interface [24]. It may be supposed that cerivastatin interacts with monolayer components mainly at the beginning of the compression at large areas per molecule and upon further compression it is expelled from the phospholipid monolayer, since the isotherm overlaps with the DMPC isotherm on pure buffer. The effect of pravastatin, which is the most hydrophilic drug, is almost negligible, as if it only slightly interacted with polar choline heads, not affecting the organization of the nonpolar part of the monolayer. Interestingly, there are no significant changes in the effect of the selected statins when the subphase is changed from PBS buffer to pure water (Fig. S1A and Table S1).

As described in the Introduction, DMPE differs from DMPC in the structure of polar head (Fig. 1), thus the hydrophilic part of this lipid is less hydrated than in the choline lipid [19]. In the case of DMPE monolayer formed on pure buffer, a well-developed phase transition is visible, which indicates the change of phase from LE to LC (Fig. 2B). The maximum value of compression modulus is 335 mN/m (Table 1), reflecting the solid nature of the film at larger surface pressures [25]. Comparison of the isotherms of DMPE monolayer in the presence of three statins in the PBS subphase suggests that each of the statins interacts with ethanolamine lipid, however, in a different way. Pravastatin shows the weakest effect upon the monolayer structure, although the shift of the isotherm towards higher areas per molecule without changing the isotherm shape reflects some interaction or incorporation of the drug into the layer (Fig. 2B). The increased lipophilicity of fluvastatin and cerivastatin compared to pravastatin as indicated by logP values [13,16] and also logD (distribution of the drug in octanol:water at physiological pH = 7.4) values [26] significantly facilitates interactions with hydrophobic chains of the fatty acid residues. As a result, the surface

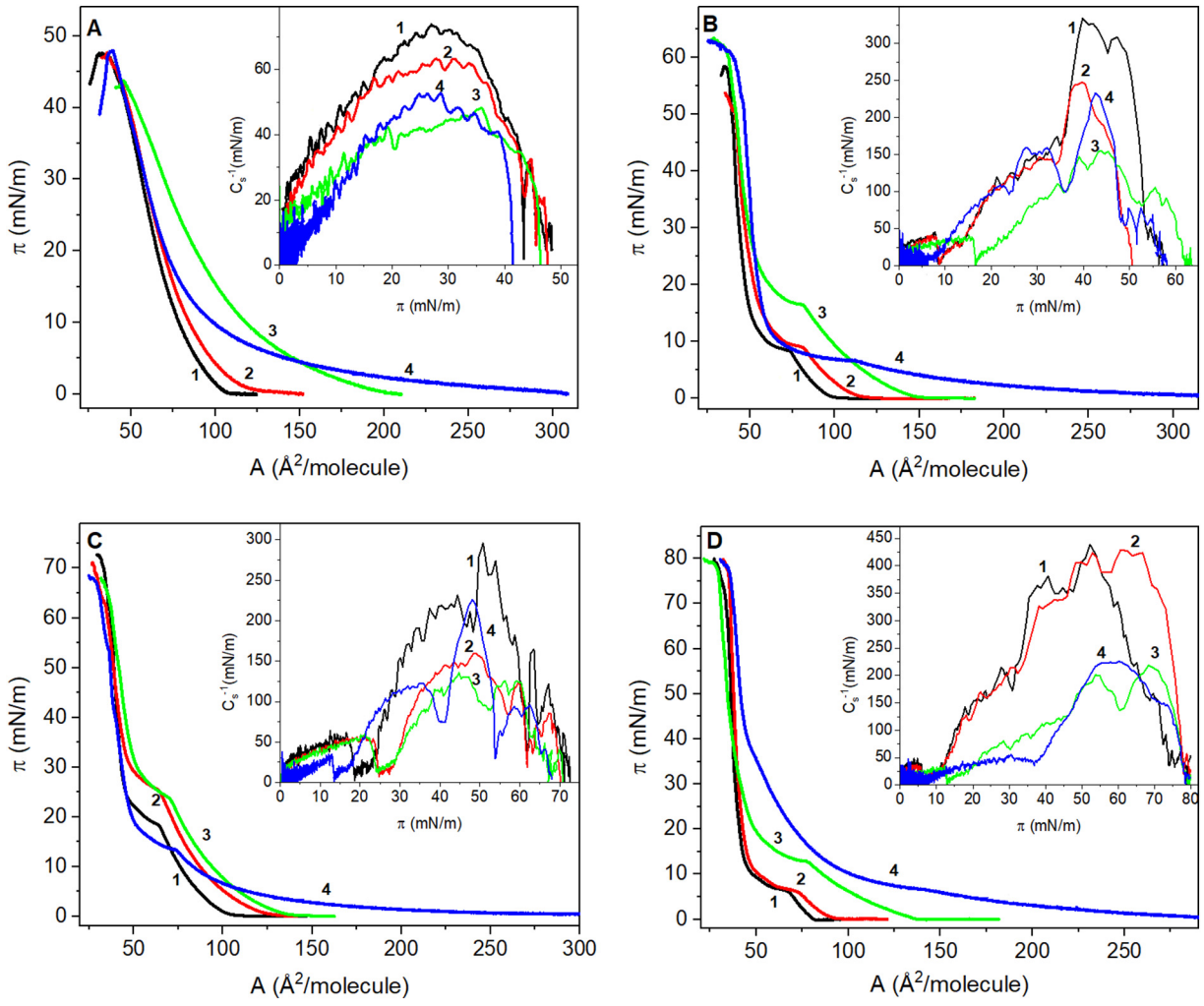

Fig. 2. Surface pressure – area per molecule (π-A) isotherms of A) DMPC; B) DMPE; C) DMPS monolayers formed on pure PBS buffer solution (black/1) and D) DMPS monolayers formed on pure water (black/1) and subphase containing 10^{-5} M pravastatin (red/2), 10^{-5} M fluvastatin (green/3) and 10^{-5} M cerivastatin (blue/4). Insets: compression modulus vs surface pressure plot. ($T = 21 \pm 1$ °C). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pressure, at which the phase transition occurs, is much higher (~17 mN/m) in the presence of fluvastatin, which suggests stronger interactions of this drug with model DMPE monolayer. As with DMPC, cerivastatin gets incorporated already at large areas per molecule and surface pressure at first slowly increases (Fig. 2B). However, cerivastatin causes relatively small changes of A_0 and C_s^{-1} (Table 1). Again, fluvastatin has the greater impact on lipid organization, as its addition causes the largest increase in A_0 value and largest decrease in the compression

Table 1
Characteristic parameters of DMPC, DMPE and DMPS Langmuir monolayers formed on subphases containing 10^{-5} M concentrations of pravastatin (PRA), fluvastatin (FLU) and cerivastatin (CER).

Subphase	A_0 (Å^2)	$A_{30\ mN/m}$ (Å^2)	A_{coll} (Å^2)	π_{coll} (mN/m)	Cs^{-1} (mN/m)
DMPC					
PBS pH = 7.4	78.5 ± 2.3	56.1 ± 0.7	38.5 ± 0.3	46.6 ± 0.6	80 ± 1
10^{-5} M PRA	90.7 ± 4.0	57.9 ± 1.2	39.5 ± 0.7	44.9 ± 1.7	65 ± 3
10^{-5} M FLU	112.8 ± 9.6	70.2 ± 4.8	44.3 ± 1.2	45.2 ± 1.1	55 ± 6
10^{-5} M CER	89.9 ± 0.8	57.0 ± 2.9	44.2 ± 5.9	46.2 ± 5.9	52 ± 12
DMPE					
PBS pH = 7.4	51.1 ± 0.3	45.2 ± 0.3	37.9 ± 0.8	56.1 ± 1.8	335 ± 9
10^{-5} M PRA	54.5 ± 0.9	46.6 ± 1.0	40.2 ± 1.2	53.1 ± 0.9	248 ± 1
10^{-5} M FLU	60.3 ± 0.0	49.7 ± 0.0	35.9 ± 0.4	59.7 ± 1.7	153 ± 7
10^{-5} M CER	57.4 ± 1.0	50.3 ± 0.9	37.4 ± 3.9	59.0 ± 0.8	211 ± 5
DMPS					
PBS pH = 7.4	50.9 ± 0.7	45.4 ± 0.0	32.4 ± 0.4	65.6 ± 1.1	305 ± 1
10^{-5} M PRA	52.2 ± 0.5	47.0 ± 2.1	32.1 ± 0.4	64.3 ± 0.2	152 ± 4
10^{-5} M FLU	58.9 ± 0.3	50.9 ± 1.4	34.6 ± 0.9	61.7 ± 3.5	136 ± 3
10^{-5} M CER	50.8 ± 0.7	41.2 ± 0.6	24.8 ± 0.5	65.0 ± 3.0	206 ± 5

modulus value, so that the layer formed on this subphase changes its phase to a less condensed (Table 1). According to the literature, fluvastatin may locate in the hydrophobic part of the membrane in an intermediate position in the upper region of the acyl chains, while cerivastatin may penetrate deeper the hydrophobic chains region [21]. The above-mentioned location of fluvastatin would result in the changes in the area per molecule as well as in the increase in the membrane fluidity as observed in Langmuir studies. The less pronounced effect of cerivastatin on the A_0 and C_s^{-1} may indicate that cerivastatin as the most hydrophobic drug easily penetrates the layers. Interestingly, the observed effect of statins on DMPE membranes does not depend on the subphase composition and was similar for monolayers formed on water (Fig. S1B and Table S1).

In order to get more insight into the effect of statins on the morphology of phospholipid monolayers Brewster angle microscopy has been also employed. The BAM images show clearly the different behavior of the DMPE monolayer compressed on subphases containing cerivastatin, fluvastatin and pravastatin (Fig. 3). The formation of typical, large flower-shaped domains can be observed for DMPE monolayers formed on pure buffer subphase. The domains appear at surface pressure of 10 mN/m, which corresponds to the LE-LC phase transition. The domain formation in the case of cerivastatin appears at similar surface pressures as for DMPE layer compressed on pure subphase, but their shape is altered and they become smaller. This effect becomes even more pronounced with further compression of the monolayer – the domains observed at 15 mN/m are small and round and finally coalesce to form more uniform monolayer. In the case of fluvastatin presence in the subphase, DMPE domains preserve their typical shape although are smaller and they start to appear at higher surface pressures compared to DMPE monolayer on pure buffer subphase. This also proves that fluvastatin fluidizes the monolayer so that the phase transition appears at much higher surface pressures. Weaker but similar effect is seen in the case of the hydrophilic pravastatin acting as a slightly fluidizing agent upon the DMPE monolayer: the domain formation and phase transition is only slightly affected. The BAM images are consistent with the result of the surface pressure – area per molecule isotherms.

The influence of different concentrations of statins on the properties of DMPE monolayers has been also investigated (Fig. S2). In the case of pravastatin, no significant differences in the isotherm characteristics were observed for different concentrations of this drug in the subphase.

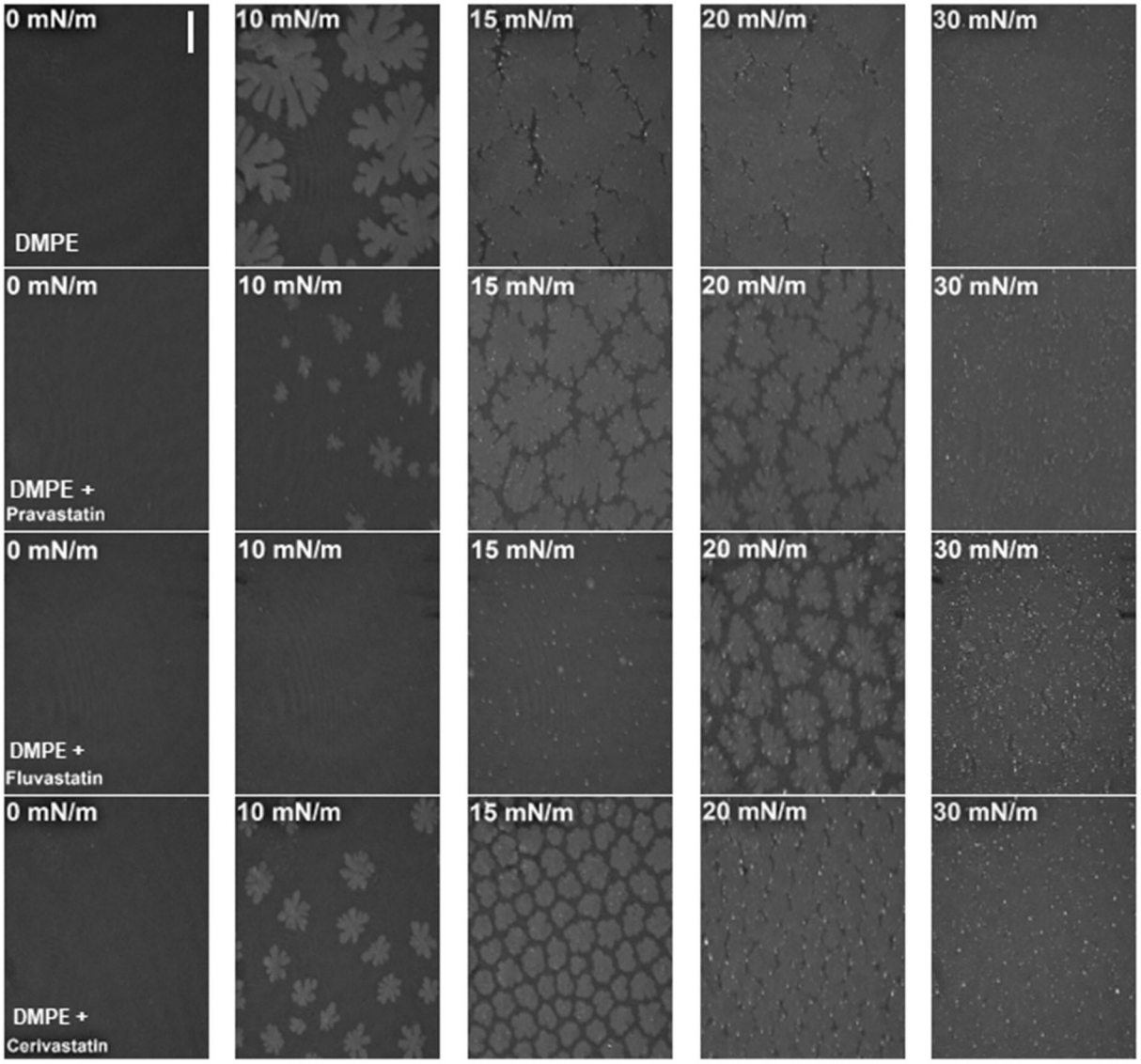

Fig. 3. BAM pictures obtained at selected surface pressures for DMPE monolayers formed on PBS buffer pH 7.4 and PBS pH 7.4 containing 10^{-5} M statins. ($T = 21 \pm 1$ °C). The scale bar is 100 μm.

The isotherms were only slightly shifted towards the larger areas per molecule, while preserving their shape. For fluvastatin the shape of the isotherm remains very similar with the increasing concentration, but the surface pressure of the phase transition increases with fluvastatin concentration. In the same time the maximum value of Cs^{-1} decreases (Table S2). However, these changes are not directly proportional to the drug concentration. The fluidization of the DMPE monolayer in the presence of cerivastatin is not concentration-dependent, since the maximum value of compression modulus is comparable irrespective of the cerivastatin concentration and implies only that the monolayer changes its phase from solid to liquid condensed (Table S2).

The third model system used to study interactions with statins employs DMPS, which is a phospholipid with a negative charge due to the presence of the serine in the polar head (Fig. 1). The DMPS monolayers are characterized by strong packing of lipid polar heads with acyl chains adopting orientation close to perpendicular to the air-water interface, which results in relatively low values of area per molecule in the well-organized monolayer [19,27]. Additionally, a plateau corresponding to LE-LC phase transition is clearly visible (Fig. 2C, D and Tables 1 and S1). DMPS monolayers also exhibit relatively high values of compression modulus corresponding to solid monolayer state [18]. However, it should be kept in mind that the negatively charged DMPS monolayer shows slightly different surface properties depending on the composition of the subphase, since the negatively charged polar heads of PS lipids interact electrostatically with the monovalent cations [28]. Therefore, the effect of the change of the subphase from water to buffer leads also to the differences in the effect of statins observed for the DMPS monolayer. The results obtained for the two types of subphases: water and buffer are compared (Fig. 2C, D).

When pure water is used as a subphase, the influence of pravastatin on DMPS monolayers is almost negligible (Fig. 2D). The presence of fluvastatin leads to the shift of the surface pressure of phase transition to the higher values and to the significant decrease in the maximum value of compression modulus, which proves the fluidizing effect of the drug. The most significant changes are observed for cerivastatin. The area per molecule increases significantly and the maximum value of compression modulus decreases (Table S1). Additionally, the shape of the isotherm changes, since the plateau region corresponding to the phase transition is no longer clearly visible (Fig. 2D). In the case of the application of PBS buffer as a subphase, the phase transition region is shifted to a higher surface pressure of almost the same value in the presence of both pravastatin and fluvastatin showing similar effect of these two drugs (Fig. 2C). Additionally, the shapes of the isotherms are also very similar. The biggest increase in the area per molecule (58.9 Å^2) and the largest decrease in compression modulus value (135.8 mN/m) is observed for fluvastatin (Table 1). This confirms the increase in fluidity. Cerivastatin behaves again in a different way: the isotherm shape changes significantly, the region of phase transition appears at lower surface pressures than on the pure subphase, and the maximum value of compression modulus is smaller suggesting that the layer changed its state from solid to liquid condensed (Table 1). However, this value is still significantly higher than the values obtained in the presence of pravastatin or fluvastatin.

The effect of different concentrations of the statins on DMPS monolayers formed on buffer subphase depends on the statin. The addition of pravastatin into the subphase does not change the shape of the isotherms significantly (Fig. S3). However, the presence of higher cerivastatin concentrations leads to unexpected changes in the structure of DMPS layers: the value of area per molecule at 30 mN/m becomes smaller than that for DMPS monolayer formed on pure buffer, and the maximum Cs^{-1} values are distinctly larger than in the presence of similar concentrations of the other two drugs (Fig. S3, Table S2). Thus, there is a clearly solidifying effect at higher concentrations of this statin, which resembles the influence of cholesterol upon the lipid layers. Such an ordering effect on the lipid membranes due to the presence of molecules possessing rigid, steroid-like moiety in the structure has been previously observed for example for a glyco-diosgenin, a synthetic surfactant [29]. Therefore, a similarity of cerivastatin to cholesterol should be taken into consideration in view of any applications of this statin as the cholesterol lowering drug, since substitution of cholesterol by cerivastatin may be another function of this drug apart from its role as the inhibitor of HMG-CoA reductase. Additionally, BAM images show the most homogenous layer at 30 mN/m in the case of cerivastatin in the subphase (Fig. S4).

3.2. PM-IRRAS studies of statins interactions with model membranes on water subphase

PM-IRRAS spectra provide useful information on the interactions of solution species with polar head groups of phospholipids. The spectra were collected in the presence of the three selected statins dissolved in the water subphase to obtain the final concentration of 10^{-5} M and compared with spectra collected for a phospholipid monolayer formed on pure water subphase. The water subphase was chosen due to the fact that for the two neutral lipids (DMPC and DMPE) no significant differences in the nature of the interactions of statins were observed when water subphase was exchanged to buffer. In the case of negatively charged DMPS monolayers the application of pure water subphase for PM-IRRAS studies allowed us to focus on the interactions of statins with polar head groups without interfering effect of cations present in the buffer solution, since it has been previously shown that the presence of salts may interfere with the interactions between statins and phospholipids [12]. Our intention was to investigate the effect of statins on lipid monolayers that mimic biological membranes. Therefore, prior to PM-IRRAS measurements, the phospholipid monolayers were compressed to 30 mN/m, at which lipid monolayers and bilayers are in corresponding states and the molecular organization and elastic compressibility modulus for monolayers and bilayers are comparable [30–32].

The spectra of the two regions corresponding to the polar head group (phosphate group) region between 1300 cm^{-1} and 1000 cm^{-1} and ester group region centered at ~1700 cm^{-1} were collected. Fig. 4 shows the spectra collected between ~1800 cm^{-1} and ~800 cm^{-1} for

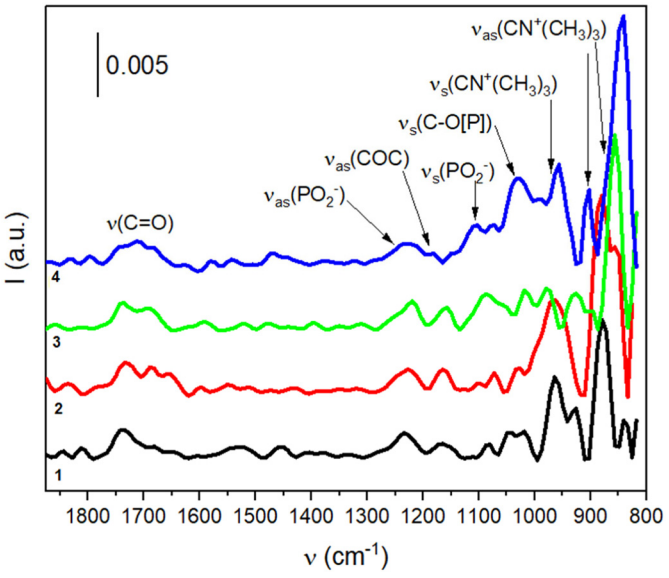

Fig. 4. PM-IRRAS spectra in the ~1800 cm^{-1} to ~800 cm^{-1} region of DMPC monolayers compressed to 30 mN/m on pure water subphase (black/1) and water subphase containing 10^{-5} M pravastatin (red/2), 10^{-5} M fluvastatin (green/3), 10^{-5} M cerivastatin (blue/4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

DMPC monolayers compressed to 30 mN/m on pure water and water containing three selected statins.

The first interesting spectral region centered at ~1700 cm^{-1} refers to the ester carbonyl group. The υ(C=O) band position is sensitive to the hydrogen bonding [33,34]. The maxima observed at 1740 cm^{-1} correspond to the non-hydrogen bonded ester groups, while shift of the band to the lower wavenumbers corresponds to the hydrogen-bonded groups. In the case of DMPC monolayers formed on pure water ester groups are mostly non-hydrated, since the band maximum at 1744 cm^{-1} is observed (Table 2). The band shift towards lower wavenumbers in the presence of pravastatin and fluvastatin is observed but it is within the spectral resolution of the equipment (8 cm^{-1}). It should be also noted that another band below 1700 cm^{-1} develops. Due to its position it may be attributed to the hydrogen-bonded component of the C=O band. Therefore, it may by postulated that the ester carbonyl group gets involved in the interaction with statins' OH group. Such interactions are favored for the most hydrophilic pravastatin and therefore this band is the most evident in the presence of this drug. In the case of cerivastatin the υ(C=O) band position is equal to 1712 cm^{-1}, which proves strong hydrogen bonding of the DMPC ester group due to the presence of this statin.

Another useful spectral region between 1300 cm^{-1} and 1000 cm^{-1} provides information on the phosphate group. The position of both symmetric and asymmetric bands and the broadening of the $\upsilon_{as}(PO_2^-)$ band located ~1230 cm^{-1} implies hydration of phosphate group. It is observed for both DMPC monolayer on pure water and on subphases containing statins. However, for fluvastatin and especially for cerivastatin a slight shift of the band position to lower wavenumbers is observed (Table 2). It suggests the increasing hydration of phosphate group compared to pure DMPC monolayers [33,34]. There is also another band observed in this spectral region located usually between ~1170 cm^{-1} and ~ 1180 cm^{-1}. This band corresponds to the asymmetric C-O-C stretch of the ester groups in the β and γ acyl chains of DMPC, υ_{as}(C-O-C) [34]. According to the literature, the position of this band at ~1180 cm^{-1} corresponds to the planar orientation of C-O-C group in γ acyl chain [35]. The shift of this band to lower wavenumbers ~1165 cm^{-1} signifies non-planar orientation of β acyl chain. Based on the data summarized in Table 2 it may be stated that the non-planar orientation prevails in the case of pravastatin and fluvastatin presence in the DMPC monolayer, while for cerivastatin probably the planar orientation of the chain dominates. Other bands can be also distinguished between 1050 cm^{-1} and 1030 cm^{-1} depending on the investigated system (Fig. 4). These bands may be attributed to the asymmetric single bond C—O stretch of the ester phosphate group υ_{as}(C-O[P]) at ~1050 cm^{-1} [36,37]. The exact position of these bands differs for DMPC in the absence and presence of statins. It is difficult to draw conclusion due to the uncertainty of the exact band position but undoubtedly this band becomes much better developed when cerivastatin is present in the monolayer, which also means that the drug interacts with polar head group region of DMPC molecules.

The analysis of the position of bands corresponding to the vibrations within choline group of DMPC molecule in the 1000–800 cm^{-1} spectral region, where C-N-C stretching bands are located, allows for obtaining the information on the conformation of the O-C-C-N frame of the choline group. This group can assume *trans* conformation, when bands corresponding to $\upsilon_s(CN^+(CH_3)_3)$ are observed at ~925 cm^{-1} and ~ 875 cm^{-1}. *Trans* conformation occurs for dry films. The positions of these bands at ~900 cm^{-1} and ~ 860 cm^{-1} correspond to the *gauche* conformation typical for hydrated films [38]. The other pair of bands observed at ~970 cm^{-1} and ~ 950 cm^{-1} are attributed to the $\upsilon_{as}(CN^+(CH_3)_3)$ vibrations [33]. Additionally, there might be also a band corresponding to the asymmetric PO single bond, which occurs at ~825 cm^{-1} [38]. However, band assignment in the low wavenumber region is difficult due to the low efficiency of PEM. In the case of DMPC monolayer formed on pure water subphase the band position observed for symmetric stretching $\upsilon_{as}(CN^+(CH_3)_3)$ clearly suggests the *trans* conformation of the choline frame (Table 2). Similar situation occurs for DMPC monolayers in the presence of pravastatin and fluvastatin, which show similar band positions to the pure DMPC monolayers, although not all bands are well developed for pravastatin and the band assignment is difficult for the lower wavenumber component for fluvastatin (Fig. 4). However, a different situation is observed for cerivastatin. In the presence of this drug in the DMPC monolayer compressed to 30 mN/m the asymmetric band is significantly shifted towards lower wavenumbers, ~902 cm^{-1} (Table 2). The second component of the symmetric stretching cannot be univocally assigned because the assignment of the intense band at ~840 cm^{-1} is difficult. However, it implies that the O-C-C-N frame of the choline group in this case is in *gauche* conformation. Therefore, it may be concluded that among the investigated statins only cerivastatin is able to interact strongly with choline group of DMPC, which results in the change in its conformation from *trans* to *gauche*.

Overall, it may be stated that among all the three investigated statins the most significant differences may be observed for the DMPC polar head and ester group region for DMPC monolayers in the presence of cerivastatin. This drug imposes changes in the conformation of choline group, increases hydration of phosphate and ester carbonyl group and influences the orientation of C-O-C group of acyl chains. It all proves the interactions of cerivastatin with DMPC and influences the conformation of the phospholipid molecules. Fluvastatin interacts mostly with phosphate group leading to its increased hydration, while the effect of pravastatin is not that significant.

As mentioned above, DMPC and DMPE molecules both have overall neutral charge (Fig. 1) but the three nitrogen-bound methyl groups of the DMPC polar head in DMPE are replaced by three hydrogen atoms [19]. Thus, the DMPE head group is less hydrated than that of DMPC. Fig. 5 shows the spectra recorded for DMPE monolayers formed on subphases containing the studied drugs.

In the spectral region around 1700 cm^{-1} there is a strong signal from the carbonyl group in the ester group at 1735 cm^{-1} [39]. The bands corresponding to υ(C=O) are well developed both for DMPE on a pure water subphase and with the addition of statins. Similar values have been obtained for the spectra (1736 cm^{-1}), except for the monolayer formed on the subphase with cerivastatin, since this value is slightly shifted towards higher wavenumbers compared to DMPE on pure subphase but the change remains within the resolution limit (Table 3). Therefore, it may be concluded that the ester group of DMPE remains non-hydrated in the presence of statins.

In the spectral region of the phosphate group vibration symmetric ($\upsilon_s(PO_2^-)$) and asymmetric ($\upsilon_{as}(PO_2^-)$) vibrations at 1090 cm^{-1} and 1230 cm^{-1} are observed, respectively [33]. The position of these bands suggests the hydration of phosphate group both in pure DMPE monolayers and monolayers in the presence of all statins. The wavenumber, at which symmetrical vibrations occur, have similar values (~1080 cm^{-1}), except for the monolayer formed on subphase containing pravastatin, when this band is observed at 1073 cm^{-1} (Table 3). It may suggest that pravastatin induces slightly increased hydration of phosphate group. Some shift of the ν_{as}(C-O[P]) band is also observed in the presence of pravastatin (Table 3).

Table 2
PM-IRRAS band position (in cm^{-1}) for DMPC monolayers formed on water and subphases containing 10^{-5} M concentrations of pravastatin (PRA), fluvastatin (FLU) and cerivastatin (CER).

Band	DMPC	DMPC + PRA	DMPC + FLU	DMPC + CER
υ(C=O)	1744	1736	1736	1712
$\upsilon_{as}(PO_2^-)$	1234	1226	1219	1219
υ_{as}(C-O-C)	1170	1165	1157	1180
$\upsilon_s(PO_2^-)$	1080	1072	1087	1072
$\upsilon_{as}(CN^+(CH_3)_3)$	964	964	979	956
$\upsilon_s(CN^+(CH_3)_3)$	925	–	925	902
	879	879	895	–

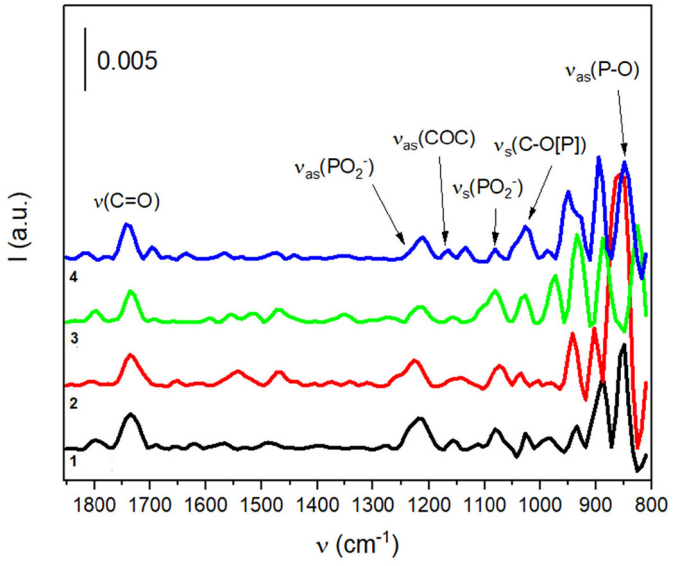

Fig. 5. PM-IRRAS spectra in the ~1800 cm^{-1} to ~800 cm^{-1} region of DMPE monolayers compressed to 30 mN/m on pure water subphase (black/1) and water subphase containing 10^{-5} M pravastatin (red/2), 10^{-5} M fluvastatin (green/3), 10^{-5} M cerivastatin (blue/4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

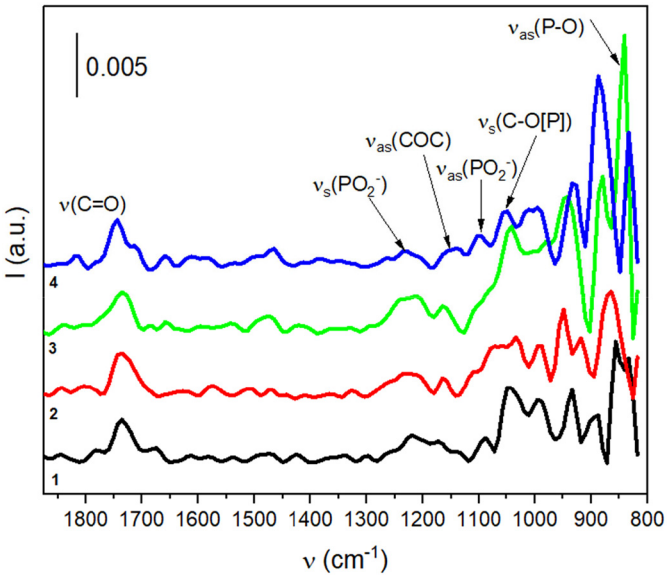

Fig. 6. PM-IRRAS spectra in the ~1800 cm^{-1} to ~800 cm^{-1} region of DMPS monolayers compressed to 30 mN/m on pure water subphase (black/1) and water subphase containing 10^{-5} M pravastatin (red/2), 10^{-5} M fluvastatin (green/3), 10^{-5} M cerivastatin (blue/4). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
PM-IRRAS band position (in cm^{-1}) for DMPE monolayers formed on water and subphases containing 10^{-5} M pravastatin (PRA), fluvastatin (FLU) and cerivastatin (CER).

Band	DMPE	DMPE + PRA	DMPE + FLU	DMPE + CER
υ(C=O)	1736	1736	1736	1740
$\upsilon_{as}(PO_2^-)$	1219	1226	1216	1214
υ_{as}(C-O-C)	1154	1162	1159	1167
$\upsilon_s(PO_2^-)$	1082	1073	1083	1078
υ_{as}(C-O[P])	1026	1036	1026	1023

Therefore, it may be concluded that hydrophilic drug such as pravastatin interacts with polar head group of DMPE, especially with phosphate group causing its increased hydration. Interestingly, for more hydrophobic drugs such as fluvastatin and cerivastatin no significant changes in the spectra in polar head group region are observed compared to DMPE monolayer on pure water. It suggests that those drugs do not impose any changes in the orientation of polar head group components. On the other hand, the changes in the DMPE isotherms were observed in the presence of these two drugs (Fig. S1B) suggesting some interactions. The PM-IRRAS results may support the explanation given based on literature data suggesting that fluvastatin may be located in the lower part of acyl chains [21]. Cerivastatin might be either penetrating the layers, not affecting heavily the orientation of polar heads at the air-water interface or might be partially removed from the layer underneath the polar heads of DMPE. Clearly, the effect of statins on DMPE monolayers is different to that on DMPC monolayers described above. It is again connected with the differences in the polar head group region of these lipids resulting in the different hydration and presence of hydrogen bonding between NH_3^+ and PO_4^- groups of DMPE molecules, which in turn prevents the statins from stronger interactions with DMPE polar heads.

In the case of DMPS monolayers on the water subphase similar bands as for DMPC and DMPE can be resolved both in the ester group region and phosphate group region (Fig. 6). In general, DMPS polar head groups are not involved in intramolecular interactions characteristic for DMPE (hydrogen bonding between NH_3^+ and PO_4^-), hence they are more hydrated than ethanolamine polar group [40]. Most significant changes occur in this case for solutions containing cerivastatin (Table 4).

In the presence of cerivastatin the υ(C=O) band is slightly shifted to higher wavenumbers compared to pure DMPS monolayer. It implies the decrease in the hydration of the ester group. It is also interesting to note that the lower-wavenumber component of C=O band located at 1712 cm^{-1} is well developed in this case. The same trend is observed for phosphate group bands located at ~1230 cm^{-1} and ~1090 cm^{-1}, which correspond to $\upsilon_{as}(PO_2^-)$ and $\upsilon_s(PO_2^-)$ band, respectively (Table 4). The position of these bands, especially the asymmetric one, for pure DMPS monolayer implies that phosphate groups are hydrated [33,34]. The presence of pravastatin and fluvastatin leads to the shift of the asymmetric band position towards slightly lower wavenumbers, which suggest even more hydration. However, cerivastatin induces opposite changes. The $\upsilon_{as}(PO_2^-)$ band is significantly shifted towards higher wavenumbers proving the dehydration of phosphate group. Consistently, the other band corresponding to phosphate group, $\upsilon_s(PO_2^-)$, is also shifted towards higher wavenumbers in the presence of cerivastatin (Table 4). These changes are similar to the effects observed for the interactions of PS lipids with divalent cations such as Ca^{2+} reported by Binder et al. [41] and Casal et al. [42]. The shifts of the bands were suggested to result from the interactions with calcium cations and dehydration of the phosphate group. The bands are shifted to higher wavenumbers and become narrower, which is associated with the reduction in motional freedom, leading to the immobilization of

Table 4
PM-IRRAS band position (in cm^{-1}) for DMPS monolayers formed on water and subphases containing 10^{-5} M concentrations of pravastatin (PRA), fluvastatin (FLU) and cerivastatin (CER).

Band	DMPS	DMPS + PRA	DMPS + FLU	DMPS + CER
υ(C=O)	1736	1736	1736	1743
$\upsilon_{as}(PO_2^-)$	1219	1211	1211	1234
υ_{as}(C-O-C)	1172	1165	1165	1140
$\upsilon_s(PO_2^-)$	1087	1080	–	1103
υ_s(C-O[P])	1049	1033	1041	1049
υ_{as}(P-O)	833	–	841	833

the entire phospholipid polar group [42]. Similar observations were made with respect to ester carbonyl group. The C═O bands became split giving rise to two components, similar to those observed in the case of cerivastatin (Fig. 6). Based on the above mentioned literature, there is another band, which is typical for PS groups and which might be also affected, namely the band due to the COO^- antisymmetric stretching mode located at 1620 cm^{-1} [42,43]. It has been previously reported that fluvastatin interacts through COO^- groups with mixed DMPC/DMPS bilayers causing a shift of this band to lower wavenumbers, which proves the formation of H-bonds between this group and polar moieties of the drug [12]. Unfortunately, in our case this band is not well developed. The band assignment and any comparison of the interactions of three statins with this part of DMPS molecule through hydrogen bonding is thus rather difficult (Fig. 6).

It may be concluded that the mechanism of cerivastatin interactions with DMPS polar head groups may be comparable to that of divalent cations such as Ca^{2+}, which consists in the removal of water of hydration from the phosphate and ester carbonyl groups. This mechanism is opposite to the one observed for DMPC monolayers, in the case of which increased hydration of the initially less hydrated choline and ester groups in the presence of cerivastatin was observed. These results stay in good agreement with the Langmuir monolayer studies. The reduction in motional freedom and the immobilization of the entire phospholipid polar group in the presence of cerivastatin results in the overall increase in the monolayer order, which is shown by the increase in the compression modulus values (Fig. 2D). On the other hand, the increased hydration of polar head group region of DMPS in the presence of pravastatin and fluvastatin leads to the increased overall fluidization of the DMPS monolayer and thus decrease in the Cs^{-1} value.

In this work we focus on the PM-IRRAS analysis of the polar headgroup part of lipid monolayers exposed to statins and therefore the experimental conditions were optimized for this IR region (Section 2.1). However, the analysis of the CH stretching region between 3000 and 2800 cm^{-1} may provide information on the changes in the orientation and conformation of the acyl chains (Fig. S5). The positions of the methylene symmetric and asymmetric bands provide information on the acyl chain conformation. The values lower than 2920 cm^{-1} and 2850 cm^{-1} for $\upsilon_{as}(CH_2)$ and $\upsilon_s(CH_2)$, respectively indicate that acyl chains are fully stretched and assume *all-trans* conformation [33,44]. Such a situation can be observed for both DMPE and DMPS monolayers formed on pure water subphase, since the methylene asymmetric and symmetric bands are located at ~2916 cm^{-1} and ~2847 cm^{-1} for both phospholipids (Fig. S5). These results are consistent with the PM-IRRAS data obtained for DMPS and DMPE bilayers supported on Au(111) electrodes [40]. However, when statins are present in the subphase, the general trend of the shift of the asymmetric band location towards higher wavenumbers (2924 cm^{-1}) can be noticed. Additionally, the tendency to increase the band width in the presence of statins can be observed. It all may indicate the increasing presence of *gauche* conformation and melting of the chains. Despite the fact that the above mentioned changes in the band location and width are close to the spectral resolution of the equipment and therefore can be only treated as general statements, these results are consistent with the conclusions drawn from the changes in the compression modulus (Figs. 2 and S1). The interactions of statins with both DMPE and DMPS monolayers lead to the decrease in the Cs^{-1} values, which points out to the fluidization of the monolayer. The only exemption is the effect of pravastatin on DMPS monolayers, when Cs^{-1} remains the same. It is also reflected by very similar PM-IRRAS spectra in the CH stretching region for DMPS on pure water and in the presence of pravastatin (Fig. S5C). In the case of DMPC monolayer, the band location obtained for DMPC on pure water is already shifted towards higher frequencies (2924 cm^{-1} and 2854 cm^{-1}, respectively), which is again consistent with PM-IRRAS literature data for supported DMPC bilayers [33]. It also stays in agreement with the values of Cs^{-1} showing that DMPC monolayers are in the liquid-expanded state. Pravastatin presence in the subphase leads to the slight increase in the Cs^{-1} value (Fig. S1 and Table S1) and thus to the shift of the band position towards lower frequencies (Fig. S5A), while fluvastatin and cerivastatin slightly decreases Cs^{-1} values and consequently moves the band positions again towards higher frequencies.

4. Conclusions

The surface pressure – area per molecule measurements allowed us to monitor the effect of three different statins on the structure of monolayers formed by the selected phospholipids. The results of those studies showed the degree of incorporation of the drugs into the layers and changes in the properties of the monolayers such as the liquid/solid phase transitions and stability upon compression in the presence of the drugs. On the other hand, the PM-IRRAS studies revealed the effect of the selected drugs on the polar head group region of the lipid layers and allowed us to explain the changes of the full monolayer properties observed using the Langmuir method. The BAM images confirm the results of the surface pressure – area per molecule isotherms proving that statins significantly alter the morphology of the layers leading to their fluidization. The isotherms show clearly that in the presence of statins the phase transitions present during the formation of the monolayers at the air-solution interface are shifted to higher surface pressures, which means that the less condensed form of the monolayer is favored when the layer is exposed to these statins.

Cerivastatin affects strongly the structure and properties of lipid monolayers and its behavior was found to depend on the degree of hydration of the lipid head groups and their charge. Cerivastatin imposed changes in the conformation of choline groups in the DMPC monolayer. The interactions with choline result in the change of the choline conformation from *trans* to *gauche* as confirmed by the shift of the band related to the –O-C-C-N frame of the choline group. This means that the head group becomes more hydrated compared to the same layer exposed to pure water subphase. PM-IRRAS studies revealed also the interaction with the ester group of DMPC. Cerivastatin present in the monolayer increased hydration of phosphate and ester carbonyl group and modified the orientation of C-O-C group of the acyl chains. As suggested based on the surface pressure measurements, the aromatic groups of this statin allow for a deeper penetration of the drug into the hydrophobic part of the membrane. This would make the drug more effective in lowering LDL cholesterol levels. However, it would also contribute to more negative side effects due to its deeper location between the lipid tails. It may be connected with higher risks of rhabdomyolysis as suggested by Galiullina et al. [21,45,46] and may be the reason for resigning from using cerivastatin in the treatment of hypercholesterolemia [47]. On the contrary, pravastatin and fluvastatin were found not to affect the choline group conformation of DMPC and the properties of the monolayers resembled those on pure aqueous subphase. Pravastatin had larger effect upon monolayers that consisted of lipids with less hydrated head groups and more involved in hydrogen bonding or other molecular interactions. A good example are monolayers of DMPE. In this case the interactions with most hydrophilic pravastatin are the most pronounced and the change of respective wavenumbers to lower values indicates higher hydration of the ester phosphate groups suggesting that the most hydrophilic drug is mostly active in the region of the head groups but does not affect significantly the organization of the lipid chains in the monolayer.

Significant changes occur in the case of DMPS monolayers especially upon contact with solutions containing cerivastatin. The results of PM-IRRAS studies are consistent with the Langmuir isotherm data. Interestingly, the observed changes are similar to the effects reported for PS lipids in the presence of divalent cations such as Ca^{2+} [41,42]. In these reports, the shifts of the bands were suggested to result from dehydration of the phosphate group and ester carbonyl groups due to interactions with calcium cations. Reduction in motional freedom, leading to the immobilization of the entire negatively charged phospholipid

polar group and to the overall increase in the monolayer order reflected by the increase in the compression modulus values may also explain the interactions with cerivastatin. This mechanism is opposite to that observed in the case of DMPC monolayers, where the increased hydration of the polar groups in the presence of cerivastatin occurred. It may be ascribed in the case of DMPS to the contribution of electrostatic interactions between the negatively charged headgroups and the drug. Interestingly, the other two statins, pravastatin and fluvastatin, do not cause such significant changes in the degree of hydration of polar heads of DMPS. Such strong dehydration effects may be dangerous for the lipid membranes and may also be one of the reasons to avoid this very effective statin especially when large doses and prolonged applications are needed.

CRediT authorship contribution statement

Michalina Zaborowska: Investigation, Validation, Visualization, Writing - original draft, Writing - review & editing. **Marcin Broniatowski:** Methodology, Writing - review & editing. **Paweł Wydro:** Methodology, Writing - review & editing. **Dorota Matyszewska:** Conceptualization, Investigation, Validation, Writing - original draft, Writing - review & editing, Supervision. **Renata Bilewicz:** Conceptualization, Writing - original draft, Writing - review & editing, Funding acquisition, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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