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**Article:**

Sadeghpour, A, Rappolt, M, Ntountaniotis, D et al. (7 more authors) (2015) Comparative study of interactions of aliskiren and AT1 receptor antagonists with lipid bilayers. *Biochimica et Biophysica Acta - Biomembranes*, 1848 (4). 984 - 994. ISSN 0005-2736

<https://doi.org/10.1016/j.bbamem.2014.12.004>

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# Comparative Study of Interactions of Aliskiren and AT<sub>1</sub> Receptor Antagonists with Lipid Bilayers

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## ABSTRACT

The renin-angiotensin-aldosterone system (RAAS) plays a key role in the regulation of blood pressure. Renin is the rate limiting enzyme of the RAAS and aliskiren is a highly potent and selective inhibitor of the human renin. Renin is known to be active both in the circulating blood stream as well as locally, when bound to the (pro)-renin receptor ((P)RR). In this study we have investigated a possible mechanism of action of aliskiren, in which its accumulation in the plasma membrane is considered as an essential step for effective inhibition. Aliskiren's interactions with model membranes (cholesterol rich and poor) have been investigated by applying different complementary techniques: differential scanning calorimetry (DSC), Raman spectroscopy, magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectroscopy and small- and wide- angle X-ray scattering (SAXS and WAXS). In addition, *in silico* molecular dynamics (MD) calculations were applied for further confirmation of the experimental data. Aliskiren's thermal effects on the pre- and main transition of dipalmitoyl-phosphatidylcholine (DPPC) membranes as well as its topographical position in the bilayer show striking similarities to those of angiotensin II type 1 receptor (AT<sub>1</sub>R) antagonists. Moreover, at higher cholesterol concentrations aliskiren gets expelled from the membrane just as it has been recently demonstrated for the angiotensin receptor blocker (ARB) losartan. Thus, we propose that both the AT<sub>1</sub>R and the (P)RR-bound renin active site can be efficiently blocked by membrane-bound ARBs and aliskiren when cholesterol rich membrane rafts/caveolae form in the vicinity of the receptors.

**KEYWORDS:** Aliskiren, renin, PRR, DPPC bilayers.

## 1. Introduction

Hypertension is a risk factor associated with cardiovascular diseases, and the first leading cause of death in economically developed countries. For this reason, medicinal chemists aim to synthesize more effective and novel drugs, which can regulate the blood pressure with longer duration of action and fewer side effects. The most important system which interferes with the regulation of pressure is the Renin-Angiotensin-Aldosterone System (RAAS) [1, 2]. RAAS activation is stimulated by drop in blood pressure, loss of blood volume or reduction in plasma sodium concentration. These signals trigger the release of renin, a highly specific and selective aspartic protease, which cleaves angiotensinogen (Aog) to produce the inactive decapeptide angiotensin I (AngI). AngI is next converted by angiotensin-converting enzyme (ACE) to the active peptide angiotensin II (AngII), which causes vasoconstriction when it binds to the angiotensin subtype 1 receptor (AT<sub>1</sub>R) or stimulates the secretion of the hormone aldosterone. Since the rate-limiting step in this cascade is determined by renin to produce AngI, inhibition of this step is an effective therapeutic target against hypertension [3].

For many years, we are studying the interactions of AT<sub>1</sub>R antagonist molecules or angiotensin receptor blockers (ARB) that prevent AngII to exert its detrimental effects on AT<sub>1</sub> receptor, with the lipid bilayers and their receptor active site in order to comprehend their molecular basis of action. ARBs are hypothesized to act on the AT<sub>1</sub>R by a two-step process [4]. In the first step the drug is incorporated into the membrane, and in the second step it diffuses to the receptor site, where it binds to the active site of the AT<sub>1</sub>R [5-7].

For renin, the situation is to some extent different [8]. Renin circulates in the blood plasma, and thus a direct inhibition of renin in the systemic system by aliskiren is possible. However, renin binds with a high affinity to the (pro)-renin receptor ((P)RR) [9-11], and subsequent studies have shown that (P)RR-bound renin has a fourfold higher catalytic activity [12]. Thus a second, local mechanism of action of aliskiren is also possible (Fig. 1A). Due to its high lipophilicity ( $\log P$  octanol/water = 2.45 at pH 7.4 [13]) aliskiren is expected to accumulate as efficiently in lipid bilayers as the comprehensively studied ARBs [14-17]. In this study we therefore investigate the binding of aliskiren to different lipid bilayer models (both, cholesterol poor and rich) and put the results in context with the extensively studied AT<sub>1</sub>R antagonists for an improved understanding of the local interactions of aliskiren with (P)RR bound renin.

Cell plasma membranes have a complex architecture hosting various kinds of protein receptors which for the assembly of signalling molecules are believed to get organized in cholesterol rich micro-domains (rafts). The most abundant lipid species in the lipid matrix of the vascular smooth muscle cells [18] and sarcolemma cardiac membranes [19] are phosphatidylcholines (PCs). The most frequently found among them are PCs with oleic and linoleic chains, and further dipalmitoyl-phosphatidylcholine (DPPC). Hydrated DPPC bilayer models (poor and rich in cholesterol) have been therefore applied to investigate aliskiren-bilayer interactions (Fig. 1B and 1C) as it comprises very convenient mesomorphic states (gel, gel/fluid and fluid) and its thermal as well as dynamic properties have been studied thoroughly [20].

In this context, we have initiated a new research activity that aims to compare the membrane effects of aliskiren with those of AT<sub>1</sub>R antagonists, since both classes of molecules act in the same system and have been used synergistically to affect the biomembrane and increase their therapeutic index [21, 22].

To undertake this task, we have studied interactions between drug and lipid bilayers using differential scanning calorimetry, Raman spectroscopy, solid-state magic angle spinning (MAS) NMR spectroscopy and small- and wide angle X-ray scattering. In addition, we have complemented our studies using *in silico* MD simulations.

## **2. Materials and methods**

### ***2.1. Materials***

Dipalmitoyl-phosphatidylcholine and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL), and used without further purification. The salt form of aliskiren (aliskiren-hemifumarate) was kindly supplied by the Novartis (Basel).

### ***2.1. Differential scanning calorimetry***

For DSC experiments about 7 mg 50% (w/w) liposomal dispersions were used. The aliskiren concentrations were 5, 10, 15 and 20 mol% and the cholesterol concentration varied from 12-15 mol%. All samples were scanned from 25 to 50 °C at least three times until identical thermal scans were obtained using a scanning rate of 2.5 °C/min. Further details of

the sample preparation, set-up and data analysis can be found in our previous publications [14-17].

## **2.2. Raman spectroscopy**

The samples preparation for the Raman measurements was identical to that for DSC. Raman spectra were recorded with a Perkin-Elmer GX Fourier Transform spectrometer (Shelton, CT). Raman spectra of the examined samples were obtained in the frequency region of 3500–400  $\text{cm}^{-1}$  and in the temperature range 25 to 50 °C. Further experimental details can be found in our previous publications [14-17].

## **2.3. MAS NMR**

Sample preparation for solid-state NMR was identical to that for DSC. The samples (20  $\mu\text{L}$ ) were transferred to 3.2 mm zirconia rotors.  $^{13}\text{C}$  MAS and  $^{13}\text{C}$  CPMAS NMR spectra were obtained at 150.80 MHz with a 600 MHz Varian spectrometer (Palo Alto, CA). The spinning rate used was 5 kHz. The experimental temperatures were 25, 35, and 45 °C for CPMAS experiments and 45 °C for the MAS measurement. Chemical shifts were reported relative  $^{13}\text{C}$  resonance of tetramethylsilane. For both measurements number of scans was 400 and relaxation delay was 5 s. Polarization transfer in the CPMAS measurements was achieved with RAMP cross-polarization [23] (ramp on the proton channel) with a contact time of 5 ms. High-power continuous-wave hetero-nuclear proton decoupling was applied during acquisition.

## **2.4. X-ray scattering**

The samples for the X-ray scattering experiments were prepared in a similar way as for DSC measurements. Time resolved simultaneous small- and wide-angle X-ray scattering (SAXS and WAXS) experiments were carried out at the Austrian SAXS beamline at ELETTRA, Trieste [24, 25]. The experimental details of the set-up and sample environment are described in previous works [14-17]. The samples were heated from 20 to 60 °C and back to 20 °C with a scan rate of 1 °C/min taking every minute an exposure for 15 s. Static exposures were taken before and after each scan.

In the time resolved X-ray scattering experiments, the  $d$ -spacings of the gel and fluid phases were derived by standard procedures [26, 27]. In particular cases, the SAXS patterns were analysed globally applying the modified Caillé theory [28-30]. This global fitting

technique is described in details elsewhere [31-34]. The headgroup-to-headgroup thickness,  $d_{HH}$ , and the bending fluctuation (Caillé parameter),

$$\eta = \frac{\pi k_B T}{2d^2 \sqrt{(K_C B)}} \quad (1)$$

were directly obtained from the fits ( $K_C$ , denotes the membrane bending rigidity,  $B$  the compression bulk modulus,  $k_B$  the Boltzmann constant,  $T$  the temperature and  $d$  the lattice spacing).

## 2.5. Molecular dynamics

Aliskiren topology files were produced by PRODGR server. A bilayer of 128 dimyristoyl-phosphatidylcholine (DMPC) and another with DPPC molecules was simulated with a united atom representation and the topology files were downloaded from the Tieleman Web page [35-37], while 3655 water molecules were described by the Simple Point Charge (SPC) model. Three different concentrations of aliskiren were simulated i.e., 1 (equal to 0.8 mol%), 5 (4 mol%) and 11 (9 mol%) molecules, which are all placed initially in the aqueous phase. Each system was energy-minimized using the steepest descent method and next the molecular dynamics (MD) simulations were commenced for 250 ns. All simulations were performed with the MD package GROMACS 4.5.1. [36, 38-40]. Equations of motion were integrated with a 2 fs time step and all bonds were constrained to their equilibrium length with the LINCS algorithm. The temperature was kept constant at 325 K using the Berendsen thermostat with a 0.1 ps coupling time constant, while the Berendsen barostat was employed for the semi-isotropic pressure coupling of the bilayer at 1 bar. For the non-bonded interactions of the system, a cut-off radius of 10 Å was applied with pressure and energy correction terms due to the truncation of the potentials. The PME technique was used for the treatment of long range electrostatics.

The deuterium order parameter ( $S_{cd}$ ) of the alkyl tails is given by the following equation:

$$-S_{CD} = \frac{2}{3} S_{xx} + \frac{1}{3} S_{yy} \quad (2)$$

in which:

$$S_{ij} = \frac{1}{2} \langle 3 \cos \theta_i \cos \theta_j - \delta_{ij} \rangle \quad (3)$$

Details on how the components are computed can be found in other studies [36, 38].

### 3. Results

#### 3.1. Differential scanning calorimetry

The thermal changes of the DPPC and DPPC/cholesterol bilayers in the absence and presence of aliskiren have been analysed in DSC measurements (see Supporting Information, Fig. S1). In Table 1 the quantitative data of the diagnostic parameters ( $T_m$ ,  $\Delta T_m$ , and  $\Delta H$ ) are presented. In pure DPPC, both the pre-transition (lamellar gel phase,  $L_{\beta'}$  [41] to the ripple phase,  $P_{\beta'}$  [42]) as well as the main-transition (formation of the fluid lamellar phase,  $L_{\alpha}$  [43]) are observed. The recorded transition temperatures ( $T_{pre}=37.6\pm 0.3$ ,  $\Delta T_{pre}=1.0\pm 0.3$ ,  $T_m = 41.20\pm 0.1$ ,  $\Delta T_m = 0.9\pm 0.1$ ) and enthalpies ( $\Delta H_{pre}=1\pm 0.02$ ,  $\Delta H=7.33\pm 0.08$ ) for pure DPPC are in good agreement with literature values [20].

Addition of aliskiren at low concentration (5 mol%) broadens the main phase transition and abolishes the pre-transition without significant effect on the phase transition temperature,  $T_m$ , and enthalpies,  $\Delta H$ . The use of the higher concentrations of aliskiren (10-20 mol%), results in a further progressive decrease of  $T_m$  and an increase of  $\Delta H$ . Two concentrations of aliskiren (5 and 20 mol%) were used to examine its thermal effects on DPPC/cholesterol bilayers. In both cases the pre-transition gets suppressed, while the main phase transition broadens,  $T_m$  decreases slightly and  $\Delta H$  decreases (Table 1).

#### 3.2. Raman spectroscopy

Raman spectra of pure DPPC, DPPC/aliskiren (80/20), DPPC/cholesterol (85/15), and [DPPC/cholesterol (85/15)] / aliskiren (80/20) bilayers were obtained in a temperature range of 25-50 °C. Spectral bands in region from 1000-1150  $\text{cm}^{-1}$  are related to the hydrocarbon skeletal C-C stretching modes. In particular, the bands at  $\sim 1090 \text{ cm}^{-1}$  and  $\sim 1130 \text{ cm}^{-1}$  reflect the C-C stretching modes in *gauche* and *trans* conformations, respectively [16, 17, 44, 45]. The C-H stretching bands at  $\sim 2850 \text{ cm}^{-1}$  and  $\sim 2880 \text{ cm}^{-1}$  are due to symmetric and antisymmetric stretching modes in the methylene groups ( $\text{CH}_2$ ) of the alkyl chains, respectively, while the  $\sim 2935 \text{ cm}^{-1}$  band is correlated to the symmetric C-H stretching mode in the terminal methyl group [46-48]. The two intensity ratios  $I_{1090}/I_{1130}$  and  $I_{2850}/I_{2880}$  (Fig. 2) reflect the transition behaviour of the different bilayers studied and in particular describe the fluidity of the membranes. How aliskiren influences the order/disorder of the different model membranes is discussed in section 4.1.

### 3.3. MAS NMR

To obtain a detailed local information on the incorporation of aliskiren and cholesterol in the DPPC bilayers, we applied high resolution  $^{13}\text{C}$  MAS and CPMAS NMR spectroscopy [49] (Fig. 3). Observed chemical shifts for the carbons of DPPC are summarized Table 2 for DPPC, DPPC/cholesterol (85/15), DPPC/aliskiren (80/20), and [DPPC/cholesterol (85/15)] / aliskiren (80/20) bilayers (see also Supporting Information Tables S1 and S2). The spectra are presented according to the (i) headgroup, (ii) glycerol backbone, (iii) esterified carbonyl, and (iv) hydrophobic lipid chain regions (for carbon identifiers of DPPC refer to Fig. 1B).

#### 3.3.1. Head-group region

Minor changes are observed for the four preparations in the headgroup region ( $\text{N}(\text{CH}_3)_3$ , C-2'' and C-2'''). Head-group conformational changes from gel to liquid crystalline phase are less pronounced compared to that observed in the hydrophobic lipid chain region (cp. section 3.3.4). Further also the presence of aliskiren and cholesterol cause only minor chemical shift increases (maximum down field effect of 0.13 ppm). Indeed, chemical shifts of DPPC/aliskiren, DPPC/cholesterol and DPPC/cholesterol and DPPC/aliskiren preparations are alike, indicating that cholesterol and aliskiren are affecting the headgroup region similarly.

#### 3.3.2. Glycerol backbone region

A downfield shift was observed during the phase transition from the gel to liquid crystalline state for the four different membrane models ranging between 0.11-0.24 ppm indicating their conformational stability in this bilayer region (C-1, C-2, C-3). Cholesterol caused downfield changes up to 0.1 ppm, when comparing DPPC/cholesterol and DPPC bilayers at the same temperatures. When the DPPC/aliskiren and DPPC bilayers are compared, higher downfield shifts reaching 0.37 ppm are observed. DPPC bilayers containing both aliskiren and cholesterol show similar chemical shifts with those containing DPPC and aliskiren.

#### 3.3.3. Carbonyl region

The resolution for C-1' in the four preparations was not sufficient to follow the chemical shift changes during the phase transition. For C-2' a biphasic effect was observed, i.e., a downfield effect in the ripple phase and an upfield effect in the fluid lamellar phase. For C-3' a progressive upfield effect (decrease of chemical shift) was eminent as the temperature

increases reaching 0.7 ppm in the  $L_\alpha$  phase, indicating that this carbon in the carbonyl region behaves similarly to all other carbons at the hydrophobic region. Similar effects were observed for the DPPC/cholesterol sample. In DPPC/aliskiren bilayers a progressive upfield effect (<1 ppm) was observed for C-3' and C-2'. Similar upfield effect (<1 ppm) was also obtained in the case that both aliskiren and cholesterol are incorporated in the DPPC bilayers, i.e., DPPC/aliskiren and DPPC/cholesterol/aliskiren bilayers showed similar chemical shifts.

### 3.3.4. Hydrophobic lipid chain region

The chemical shifts decrease when DPPC bilayers undergo the transition from the lamellar gel phase  $L_\beta'$  (25 °C) towards the ripple phase  $P_\beta'$  (35 °C) and lamellar liquid crystalline phase  $L_\alpha$  (45 °C). This is due to the strong *trans-gauche* isomerization effects observed especially in the turnover to the  $L_\alpha$  phase. Upfield effect of the carbons which constitute the hydrophobic region was also observed in DPPC/cholesterol and DPPC/aliskiren bilayer samples. The most pronounced upfield effect was with  $(\text{CH}_2)_{10}'$  carbons reaching almost 3 ppm while with C-16', C-15' and C-14' the effect was ranging between 0.02-1.40 ppm.

## 3.4 X-ray scattering

In Fig. 4 an overview of the time-resolved SAXS/WAXS experiments is presented. In the contour plots high scattering intensities are colour-coded with red and orange, while lower scattering intensities are given in green and blue.

The structural changes in pure DPPC bilayers [50] are exemplified in Fig. 4A. At ambient temperatures the lamellar gel phase ( $L_\beta'$ ) is observed. The chains are packed in an orthogonal lattice [44,45] and tilted with respect to the bilayer plane about 32° [51]. Thereafter, the stable ripple phase ( $P_\beta'$ ) forms, followed by the lamellar fluid phase ( $L_\alpha$ ). Note, that the transition is not reversible, but in cooling direction two ripple phases form: the stable and the so-called metastable ripple phase ( $P_\beta'$  and  $P_{\beta' \text{ mtstbl}}$ ) [52]. The interaction of 10 mol% cholesterol with phosphatidylcholine bilayers is presented in Fig. 4B. Both in the gel and the fluid phase regime cholesterol induces in part a liquid ordered (lo) phase, where the lipids are free to laterally diffuse, but at the same time exhibit a certain chain order [53-55].

As can be seen in the SAXS patterns in Fig. 4C, aliskiren provokes in the gel phase the onset of an unbinding of the bilayers. The first order diffraction peak is centred at  $s = 0.007$

$\text{\AA}^{-1}$ , which corresponds to a  $d$ -spacing of about 140  $\text{\AA}$ . This means that the bilayers are about 100  $\text{\AA}$  apart. In other words the multilamellar vesicles (MLVs) are highly swollen with water. In the fluid phase regime the unbinding of bilayer membranes is complete, i.e., the bilayers are spatially uncorrelated.

In contrast to the effect of aliskiren alone, the incorporation of aliskiren together with cholesterol in the DPPC bilayers reduces the  $d$ -spacing drastically from 140 to 72  $\text{\AA}$  (Fig. 4D and Fig. 5B). The  $d$ -spacing of 72  $\text{\AA}$  in the lamellar gel-phase can actually be explained by a loss of chain tilt in the lamellar gel phase. This is supported by a determined bilayer thickness,  $d_{HH}$ , of 50  $\text{\AA}$  deduced from the electron density profile of the bilayer (Supporting Information Fig. S2-S3 and Table S3; note that a complete loss of chain tilt would result in a  $d$ -spacing of about 72  $\text{\AA}$  [49]). However, in the fluid phase a coexistence of highly swollen MLVs (maximum  $d$ -spacing is 102  $\text{\AA}$ , Fig. 5B) together with a high fraction of spatially uncorrelated bilayers is observed.

The temperature dependent lipid chain packing of the four different studied samples is presented in Fig. 6. The lipid chain packing gives a relatively good indication for  $T_m$ . The melting point for pure DPPC is determined with the WAXS recordings to be at 43  $^{\circ}\text{C}$ , while with decreasing lipid concentration the melting point is observed at 42, 40 and 38  $^{\circ}\text{C}$ , respectively. This is readily understood, since an increasing concentration of impurities in the form of cholesterol and/or aliskiren reduce the overall van der Waals energy. Another effect can be observed in the packing density of the lipid chains in the gel phase. For instance, at 37  $^{\circ}\text{C}$  the first order diffraction peak indicates an averaged nearest neighbour distance of the lipid chains of 4.27  $\text{\AA}$  for DPPC (area per chain 21.0  $\text{\AA}^2$ ). Aliskiren alone has a condensing effect ( $d = 4.20$   $\text{\AA}$  at 37  $^{\circ}\text{C}$ ), whereas 10 mol% cholesterol causes an overall looser chain packing in the gel phase: the  $d$ -spacing is about 4.31  $\text{\AA}$  in both the DPPC/cholesterol as well as the DPPC/aliskiren/cholesterol bilayer systems. This is understood, since cholesterol above a certain threshold (typically  $> 5$  mol%) induces in part the lo-phase, in which the apparent area per lipid is enhanced.

### 3.5. Molecular Dynamics

The effect of aliskiren molecules on DMPC and DPPC model membranes was examined with MD simulations at three different concentrations of the drug, i.e., for one, five and eleven molecules per 128 lipids (0.8, 4 and 9 mol%). The density profiles of all individual components in the system along the axis orthogonal to the bilayer ( $z$ -axis) are depicted in Fig.

7. Obviously aliskiren molecules diffuse into the interior of the bilayer even though their initial positions in the simulations were given in the aqueous phase.

Another important property of a lipid-bilayer system is the area per lipid which may be estimated by a variety of experimental techniques (e.g., NMR, X-ray diffraction). Most of the experimental values lie between 61-67 Å<sup>2</sup>/lipid indicating that the membrane is in the liquid crystalline phase. Similar results are obtained by our simulations as listed in Table S4. Moreover, there is an increase for the area per lipid as more aliskiren molecules are added. Focusing on the headgroup of lipids, the angle between the vector connecting P and N atoms and the z-axis is important. The presence of aliskiren does not affect the values of the angle as seen in Table 3. Furthermore, the radial distribution function (RDF) between water oxygen and phosphate oxygen was computed to evaluate the influence of the drug on the interface between DPPC and aqueous phase. No significant effect was observed by the aliskiren on the RDF (Fig. 7C).

The order parameters of DMPC bilayers are presented in Fig. 8A and are in agreement with reported data using 2H solid state NMR spectroscopy [56, 57]. They were averaged over the two alkyl tails of DMPC and clearly show upward shifting as the number of drug molecules increase. Fig. 8B instead represents the change of order parameter as the number of loaded aliskiren molecules varies from 1 to 11. Accordingly, the highest change in the order parameter occurs in carbon numbers of 6 to 9 (highlighted region in Fig. 10B). The results suggest that the aliskiren molecules mainly sit at the middle of the hydrophobic tails of the lipid. Note that both aliskiren simulation sets on DMPC and DPPC bilayers display very similar results, i.e., demonstrating that small chain length changes have no a great influence on the incorporation of aliskiren.

## 4. Discussion

### 4.1. Bilayer interactions with aliskiren

According to the differential scanning calorimetric data, aliskiren at 5 mol% broadens significantly the  $\Delta T_{1/2}$  showing that this low concentration acts as an “impurity”. At higher concentrations, aliskiren progressively lowers the phase transition temperature and increases  $\Delta H$ , while it affects less  $\Delta T_{1/2}$ . Similar increases in the  $\Delta H$ , at high concentrations (> 10

mol%) have also been observed for the AT<sub>1</sub>R antagonists losartan, irbesartan, valsartan and candesartan cilexetil, but not for candesartan. In addition, this progressive broadening of the phase transition and abolishment of the pretransition was more eminent to losartan, valsartan and candesartan cilexetil, but not for candesartan [16, 17, 58]. This increase of  $\Delta H$ , has been interpreted in our previous publications to be associated with partial interdigitation of the lipid chains in the gel phase. In fact the SAXS results of DPPC/aliskiren bilayers at 20 °C support this probable cause: the membrane thickness,  $d_{HH}$ , decreases from 44 Å for pure DPPC bilayers to about 31 Å in the presence of aliskiren (Fig. 9A).

Intramolecular *trans-gauche* conformational changes within the hydrocarbon chain region can be monitored directly by the intensity ratio  $I_{1090}/I_{1130}$  in Raman spectroscopy. The intensity ratio at this peak height allows the direct comparison of the bilayers disorder-order transitions between liposome preparations without or with drug incorporation. In Fig. 2A the changes in Raman ratio of  $I_{1090}/I_{1130}$  are presented. The transition temperatures are well in consistent with the results from the calorimetric measurements. DPPC bilayers show a strong  $\Delta I$ , the increase in Raman ratio across the gel to liquid crystalline phase ( $\Delta I=0.58$ ), which means rising up of about 80%. Incorporation of aliskiren into these bilayers induces higher disorder in gel phase and less disorder in fluid phase, when compared to the pure DPPC system. Note, the increase of this Raman ratio across the gel to liquid phase is only about 22% (from 0.88 to 1.1). The intensity ratio  $I_{2850}/I_{2880}$  (Fig. 2B) describes the main change occurring in the hydrocarbon-chain region of the lipids and corresponds to intermolecular interactions among aliphatic chains[59]. It is sensitive to subtle changes in conformational order from rotations, kinks, twists and bends of the lipid chains [60]. Alike to the ratio  $I_{1090}/I_{1130}$  the presence of aliskiren causes significant increase in the  $I_{2850}/I_{2880}$  meaning that drug loading causes disorder in the lipid bilayers in the gel phase. Thus, consistently the two ratios show that aliskiren causes fluidization of the lipid bilayers. However, the Raman spectroscopy results show distinct differences between the effects of aliskiren in DPPC bilayers as compared to AT<sub>1</sub>R antagonists. Aliskiren increases the *trans-gauche* ratio to a lesser extent than ARBs (e.g., olmesartan increases that ratio with significantly stronger extent, i.e. for 0.9 to 1.7 [16]).

<sup>13</sup>C MAS spectra were especially used to detect the aromatic region, while the <sup>13</sup>C CPMAS spectra of this region were not as eminent (Fig. 2). At 35 °C, the findings reveal a narrowing of the peaks in aliskiren loaded membranes as compared to the DPPC alone, indicating a change in fluidity of the lipid bilayers in the ripple phase regime (P<sub>β</sub>' phase).

When AT<sub>1</sub>R antagonists or aliskiren are present this phase gets suppressed, which is in agreement with DSC results. However, there is an exception to the rule, the ARB olmesartan does not cause an abolishment of the pre-transition and no narrowing of the peaks at 35 °C was observed indicating the complex motions of the lipids existing in the P<sub>β</sub> phase.

The interdigitation effect induced by aliskiren can be further corroborated by the X-ray diffraction data. As presented in the results section (Fig. 5A), the head-head distance ( $d_{HH}$ ) is about 31.4 Å in the presence of aliskiren. This value is far smaller compared to the  $d_{HH}$  value of 44.2 Å for pure DPPC bilayers [43]. Accordingly, one may conclude that the chain tilt loss is accompanied by the interdigitation of the hydrophobic layer by about 13Å (Fig. 9A). Consequently, one expects the area per lipid to increase, and this is exactly what the MD simulations data confirm as the aliskiren concentration increases (Table 3).

Another apparent effect of aliskiren is the induction of strong water swelling of the MLVs, i.e. the bilayers separation increases from 19 to 100 Å at 20 °C and from 28 to 130 Å at 60 °C (Fig. 9). Note also, that actually only a small fraction of the membranes remain spatially correlated in the fluid phase, while the biggest fraction of bilayers completely unbinds (cp. Fig. 5). In contrast in the gel phase the main fraction of the sample remains ordered. Here the swelling can be explained by electrostatic repulsion induced by the positively charged aliskiren molecules at pH 7 (aliskiren has only one pK<sub>a</sub> value of 15.9, and hence has strong basic properties [61]; membrane undulation usually play a minor role in the gel phase). In the fluid phase instead both electrostatic repulsion as well as the onset of membrane undulations will contribute to the increase in bilayer separation. This means that above the melting point the onset of Helfrich-undulations of the membranes delivers one part of the explanation. However, the electrostatic repulsion force is expected to diminish above  $T_m$ . As long as the partition coefficient of aliskiren can be assumed to be temperature independent, then this force diminishes in the fluid phase, simply because the surface charge density decreases (note, the area per lipid increases above  $T_m$ ). Thus, both increased membrane undulations and a higher partition coefficient of aliskiren in the lamellar fluid phase would be in accordance with the given observations.

#### **4.2 Location of aliskiren in the DPPC bilayers**

Molecular dynamic simulations and NMR spectroscopy data have been used to locate the topography of the aliskiren in the lipid bilayers. The NMR data show almost no change in chemical shifts within the headgroup and glycerol backbone and only minor changes in the

chemical shifts of carbonyl region, when aliskiren is incorporated in DPPC bilayers. However; drastic changes appeared in the hydrophobic lipid chain region with its maximum at the carbon number 10. Thus, aliskiren is mainly centred around this carbon, both in the gel and liquid phase. Further, the MD simulations approve this finding (see the density profiles in Fig. 7B). We note, that Pomes and co-workers [62] pointed out that water-defect and lipid surface reorganization processes for small molecule may take as long as several microseconds. These long term processes are not included in our MD simulations, however, aliskiren places itself quite deep in the bilayer, where interfacial water-restructuring mechanism play a minor role. Moreover, order parameter simulations demonstrate that aliskiren mainly accumulates at the centre of the hydrophobic lipid chain (Fig. 8B; corresponding to the carbon numbers 7 to 11 in DPPC given in Fig. 1B).

#### **4.3 The influence of cholesterol**

At 10 mol% cholesterol content, both in the gel and the fluid phase regime, cholesterol induces in part the liquid ordered (lo) phase causing a decrease of  $\Delta H$  by 30% (Table 1). However, progressive addition of aliskiren into the DPPC/cholesterol composites increasingly diminishes the effect of cholesterol. Both aliskiren and AT<sub>1</sub>R antagonist losartan act similarly against the lowering effect of  $\Delta H$  caused by cholesterol [63].

Furthermore, cholesterol itself induces the condensing effect in the fluid lamellar phase of DPPC bilayers. This is observed in the increase of the Raman ratio at  $I_{1090}/I_{1130}$  by only about 15%, however; addition of cholesterol together with aliskiren causes a further increase of Raman ratio during the main phase transition ( $\Delta I=1.55-0.96=0.59$ , Fig. 2A). Alike the presence of aliskiren/cholesterol causes significant increase also in the  $I_{2850}/I_{2880}$  ratio meaning that present of these two components together causes disorder in the lipid bilayers (Fig. 2B). Thus, it can be understood that aliskiren causes fluidization of the lipid bilayers even in presence of cholesterol. This is also confirmed by the bilayer thicknesses deduced from the SAXS data at 60 °C. The  $d_{HH}$  values for DPPC/aliskiren and DPPC/cholesterol/aliskiren do not differ significantly.

From the NMR data, it can be shown that cholesterol abolishes the pre-transition but, interestingly does not cause significant changes in the line-widths of the peaks (Fig. 3). This is an interesting observation and points out that, more complex motions are observed in DPPC/cholesterol bilayers as compared to DPPC/aliskiren or DPPC/cholesterol/aliskiren complexes.

While we have observed that aliskiren incorporation induces a strong increase in the bilayer separations due to electrostatic repulsion, this effect is reduced under the presence of 10 mol% cholesterol, i.e., we observe again a condensation of adjacent bilayers (see Fig. 9 bottom). A similar effect is apparent when losartan is interacting with DMPC/cholesterol membranes [15]. Initially unbound membranes start to realign in ordered stacks as the cholesterol content increases in the bilayer. Thus, it is tempting to assume that both losartan and also aliskiren get expelled from cholesterol rich membranes. This would at least deliver a straight forward explanation for the observed reduction bilayer separation distance.

In this respect, we briefly discuss a possible route of action of aliskiren. Due to its high lipophilicity [13] aliskiren is expected to accumulate as efficiently in lipid bilayers as the comprehensively studied ARBs [14-17] (Fig. 10, left hand side). However, when cholesterol rich domains (caveolae) would form in the direct vicinity of a (P)RR, then membrane-accumulated aliskiren molecules are likely to get expelled from the lipid bilayer due to the high concentration of cholesterol (cp. Fig. 4C and D), and a binding of the inhibitors to the active site becomes possible from the extracellular fluid (Fig. 10; right hand side). The same scenario could occur also for the mechanism of action of ARBs, i.e., also the AT<sub>1</sub>R could be efficiently blocked by for instance membrane-bound losartans, when a cholesterol rich membrane caveolae forms. This newly proposed mechanism is also interesting in the light of the increasingly investigated intracellular renin-angiotensin system [10, 64].

## 5. Conclusion

The interactions of aliskiren with cholesterol-rich and cholesterol-poor plasma membrane models have been investigated both experimentally (DSC, Raman spectroscopy, NMR, X-ray scattering) and by MD simulations. It turned out that aliskiren behaves in many respects in a similar fashion as the beforehand studied ARBs. Aliskiren incorporates in the bilayer with a high affinity, but positions itself a bit deeper in the membrane core as compared to most of the ARBs. Similarly, aliskiren induces a partial interdigitation of the lipid chain in the gel phase. As a consequence already at low concentrations the pretransition of DPPC gets suppressed. Moreover, the thermodynamic alterations (lowering of  $T_m$  and increasing of the main transition enthalpy) and fluidization effects on PC-model membranes have shown many parallels to the action of AT<sub>1</sub>R antagonists. Most interestingly, already the presence of 10 mol% cholesterol expels aliskiren at least in part from the membrane. This has led us to propose a refined mechanism of action of aliskiren for the case of its accumulation in the plasma membranes of e.g. vascular smooth muscle cells.

**Acknowledgements:** We kindly acknowledge Novartis for supplying us with aliskiren.

## Table Legends

**Table 1:** Representative diagnostic thermal effect values for DPPC and DPPC/cholesterol bilayers without or with aliskiren.

**Table 2:** Observed chemical shifts for the carbons of DPPC in  $^{13}\text{C}$  MAS and CPMAS spectra in a temperature range of 25-45 °C.

## Figure Legends

**Fig. 1.** Molecular structures of aliskiren (A) DPPC (B) and cholesterol (C).

**Fig. 2.** (A)  $I_{1090}/I_{1130}$  vs. temperature plots for pure DPPC ( $\blacklozenge$ ), DPPC containing 20 mol% of aliskiren ( $\blacktriangle$ ), DPPC containing 15 mol% cholesterol ( $\blacksquare$ ) and [DPPC/cholesterol (85/15)] / aliskiren (80/20) ( $\times$ ). The same symbolic is used for panel B representing  $I_{2850}/I_{2880}$  vs. temperature plots.

**Fig. 3.** Observed chemical shifts for the carbons of DPPC in carbon-13 CPMAS spectra at 25, 35 and 45 °C for (a) DPPC, (b) DPPC/cholesterol (85/15), (c) DPPC/aliskiren (80/20), and DPPC/cholesterol/aliskiren (68/12/20) bilayers.

**Fig. 4.** Temperature scan of multilamellar vesicles of (A) DPPC from 20 to 60 °C and back to 20 °C with 1 °C/min. Note at frame number 1 and 80 the sample temperature was 20 °C and the maximum temperature was reached at frame 40. For the phase assignments refer to the main text. (B) DPPC/cholesterol (90/10), (C) DPPC/aliskiren (83/17) and (D) [DPPC/cholesterol (90/10)] / aliskiren (83/17).

**Fig. 5.** The scattering profiles of A) DPPC/aliskiren and B) DPPC/cholesterol/aliskiren at two different temperatures. The profiles correspond to the dashed lines indicated in Fig. 4C and 4D. The solid lines are the global fits of the SAXS data (see section 2.4). Additionally the electron density map of the DPPC/cholesterol/aliskiren bilayer at 20 °C has been determined (see Supporting Information Fig. S2-S3 and Table S3).

**Fig. 6.** Lattice spacing of the first order diffraction peaks of DPPC (black), DPPC/cholesterol (90/10) (green), DPPC/aliskiren (83/17) (red), and of [DPPC/cholesterol (90/10)] / aliskiren (83/17) (blue). The WAXS analysis displays the nearest neighbour chain to chain distances derived from the first order diffraction peaks in the gel phase and rough distances determined from the position of the diffuse scattering maximum in the fluid phase.

**Fig. 7.** Molecular dynamics simulations of aliskiren DPPC membrane interactions. A) Snapshot of aliskiren in the DPPC bilayer (128 lipids, 3655 water molecules). B) Density profiles along z-axis of the DPPC bilayer for the system with 11 aliskiren molecules. C) Phosphate oxygen to water oxygen radial distribution function for the three different concentrations of aliskiren molecules.

**Fig. 8.** A) Deuterated order parameters for the alkyl tails of DMPC in presence of aliskiren molecules as obtained from the MD simulations. B) Difference in the order parameter between the alkyl chains of highest and lowest loaded aliskiren.

**Fig. 9.** Schematic representation of the pure DPPC, DPPC/cholesterol, DPPC/aliskiren and DPPC/cholesterol/aliskiren bilayers. For all four model membrane systems two adjacent bilayers are shown at 20 °C (A) and at 60 °C (B), respectively. Cholesterol molecules are colored in green and aliskiren in red. Above each scheme the polar thickness ( $d-d_{HH}$ ) and below the  $d$ -spacings are given. The data were derived from the SAXS data (see section 3.4).

**Fig. 10.** Schematic representation of cholesterol-induced release of aliskiren and losartan. Left hand side: aliskiren and losartan accumulate in the lipid bilayer, but not necessarily inhibit the (P)RR and AT<sub>1</sub>R. Angiotensinogen (Aog) gets cleaved by (P)RR-bound renin to angiotensin I (AngI), which by the angiotensin-converting enzyme (ACE) gets altered to the active peptide angiotensin II (AngII). Right hand side: As soon as a cholesterol rich caveolae forms, aliskiren and losartan get released from the bilayer and block the receptors.

## Tables

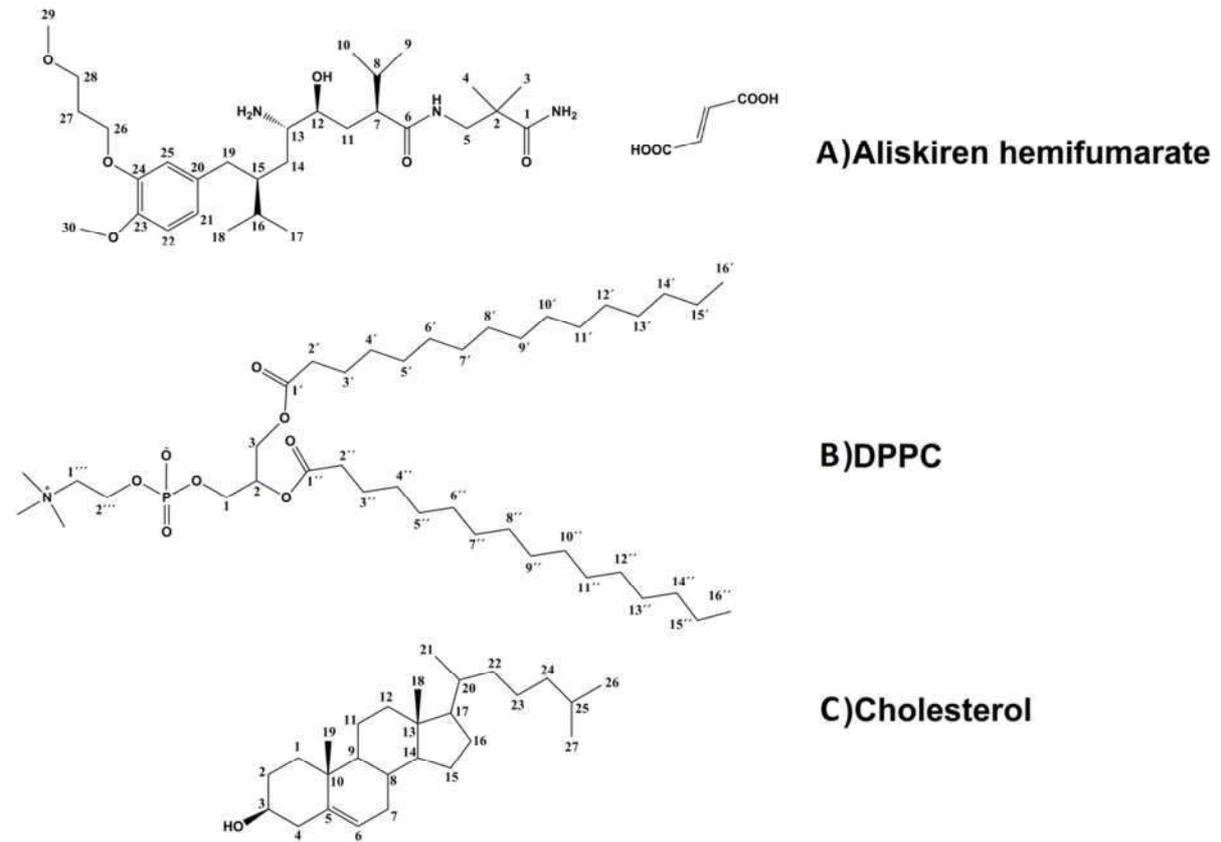
### Table 1

<b>Samples</b>	<b><math>T_m</math> (°C)</b>	<b><math>\Delta T_m</math> (°C)</b>	<b><math>\Delta H</math> (kcal/mol)</b>
<b>DPPC</b>	41.20±0.1	0.9±0.1	7.33±0.08
<b>DPPC/aliskiren 95:5</b>	40.93±0.1	1.6±0.1	7.86±0.08
<b>DPPC/aliskiren 90:10</b>	39.08±0.1	0.9±0.1	9.46±0.09
<b>DPPC/aliskiren 85:15</b>	38.13±0.1	1.1±0.1	10.04±0.10
<b>DPPC/aliskiren 80:20</b>	38.34±0.1	1.5±0.1	10.45±0.10
<b>DPPC/cholesterol 85:15</b>	40.60±0.1	1.0±0.1	5.12±0.05
<b>[DPPC/cholesterol 85:15]/aliskiren 95:5</b>	37.97±0.1	1.5±0.1	6.47±0.06
<b>[DPPC/cholesterol 85:15] /aliskiren 80:20</b>	36.80±0.1	1.9±0.1	7.33±0.07

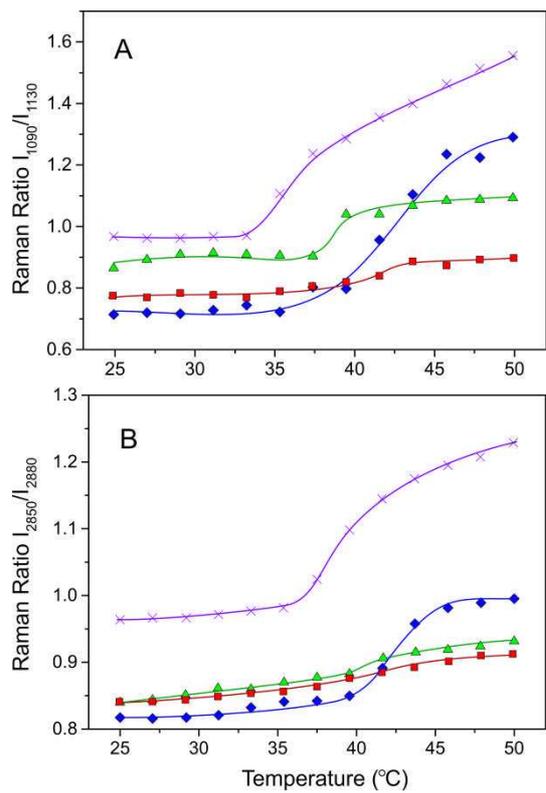
**Table 2**

<sup>13</sup> C-MAS														
T (°C)	Sample	Glycerol Carbons			Carbonyl Carbons			Hydrophobic Chain Region				Headgroup Region		
		C-1	C-2	C-3	C-1', C-1''	C-2', C-2''	C-3', C-3''	(CH <sub>2</sub> )' <sub>10</sub> , (CH <sub>2</sub> )'' <sub>10</sub>	C-14', C-14''	C-15', C-15''	C-16', C16''	N(CH <sub>3</sub> ) <sub>3</sub>	C-2'''	C-1'''
45	DPPC	63.80	71.41	64.41	174.02	34.81	25.86	31.10	32.83	23.41	14.49	54.89	66.82	60.21
45	DPPC/cholesterol (85/15)	63.88	71.45	64.53	174.19 +174.00	34.93	26.04	31.51	33.00	23.54	14.55	54.93	66.84	60.28
45	DPPC/aliskiren (80/20)	63.93	71.53	64.59	174.20	34.91	25.90 25.76	30.92 30.35	32.85	23.47	14.64	54.98	66.95	60.32
45	[DPPC/cholesterol (85/15)] / aliskiren (80/20)	63.92	71.48	64.59	174.20	34.91	25.90 25.75	30.96 30.36	32.85	23.47	14.64	54.95	66.93	60.30
<sup>13</sup> C-CPMAS														
T (°C)	Sample	C-1	C-2	C-3	C-1', C-1''	C-2', C-2''	C-3', C-3''	(CH <sub>2</sub> )' <sub>10</sub> , (CH <sub>2</sub> )'' <sub>10</sub>	C-14', C-14''	C-15', C-15''	C-16', C16''	N(CH <sub>3</sub> ) <sub>3</sub>	C-2'''	C-1'''
25	DPPC	-	71.22	64.38	172-174	34.98	26.72	33.25	34.22	24.44 24.17	14.65	54.76	66.63	60.22
25	DPPC/cholesterol (85/15)	-	71.22	64.48	172-174	35.15	26.82	33.26	34.26	24.48 24.15	14.70	54.79	66.66	60.26
25	DPPC/ aliskiren (80/20)	63.85	71.29	64.50	173-175	35.16	26.57	33.31 32.19	34.62	24.64 24.90	(15.06) 14.65	54.79	66.76	60.23
25	[DPPC/cholesterol (85/15)] / aliskiren (80/20)	63.83	71.33	64.52	173-175	35.22	26.63	33.32	34.65	24.68 24.91	(15.09) 14.61	54.81	66.74	60.25
35	DPPC	-	71.14	64.38	172-175	35.10	26.37	33.11	-	24.25	14.60	54.79	66.69	60.20
35	DPPC/ cholesterol (85/15)	-	71.21	64.47	172-175	35.19	26.46	33.10	-	24.29	14.65	54.92	66.81	60.33
35	DPPC/ aliskiren (80/20)	63.95	71.51	64.62	174.21	34.96	25.89	31.06 30.46	33.30 32.93	24.60 23.55	(15.12) 14.71	54.98	66.94	60.34
35	[DPPC/cholesterol (85/15)] / aliskiren (80/20)	63.91	71.44	64.59	174.17	34.91	25.90	31.00 30.42	32.89	23.53	14.66	54.93	66.87	60.31
45	DPPC	63.77	71.37	64.49	174.03	34.81	25.85	31.06	32.79	23.39	14.49	54.86	66.81	60.19
45	DPPC/ cholesterol (85/15)	63.86	71.43	64.55	174.17	34.93	26.02	31.43	32.97	23.53	14.55	54.93	66.85	60.27
45	DPPC/aliskiren (80/20)	63.93	71.53	64.56	174.16	34.93	25.87 25.76	30.89 30.32	32.82	23.48	14.63	55.00	66.97	60.32
45	[DPPC/cholesterol (85/15)] / aliskiren (80/20)	63.93	71.48	64.58	174.18	34.92	25.88 25.76	30.92 30.34	32.83	23.46	14.63	54.97	66.95	60.31

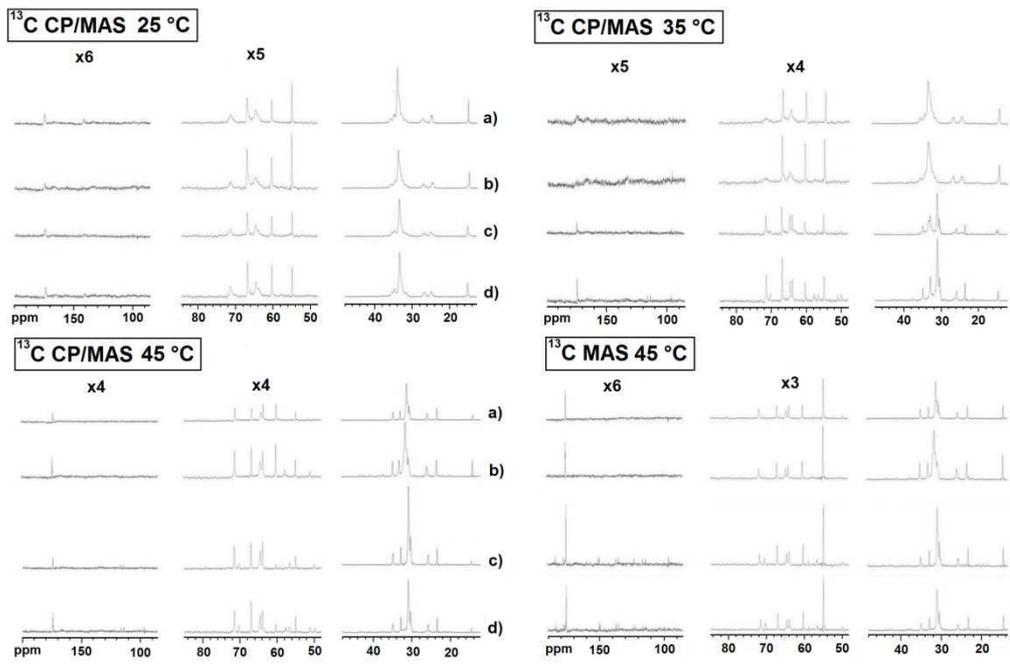
## Figures



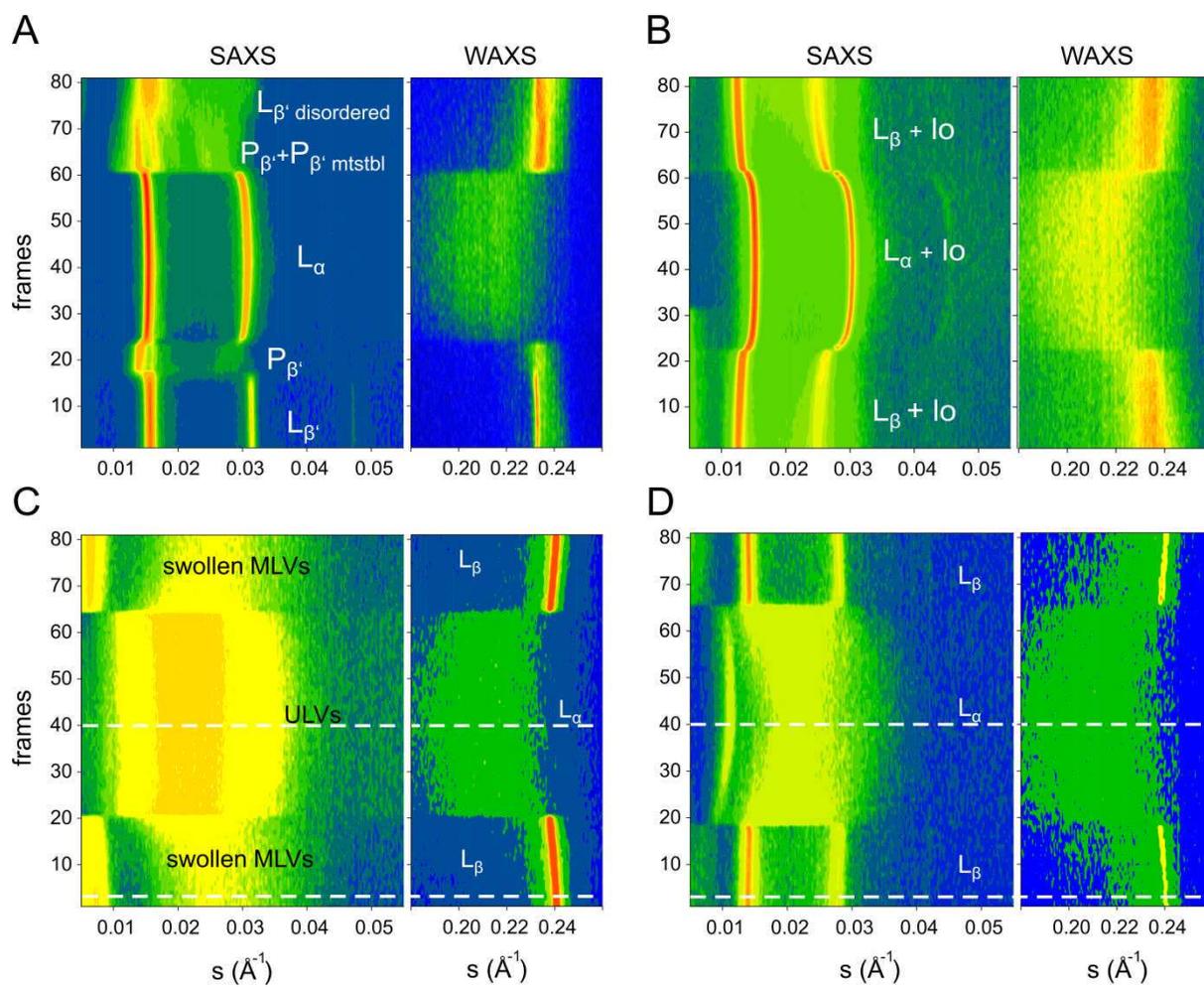
**Fig. 1**



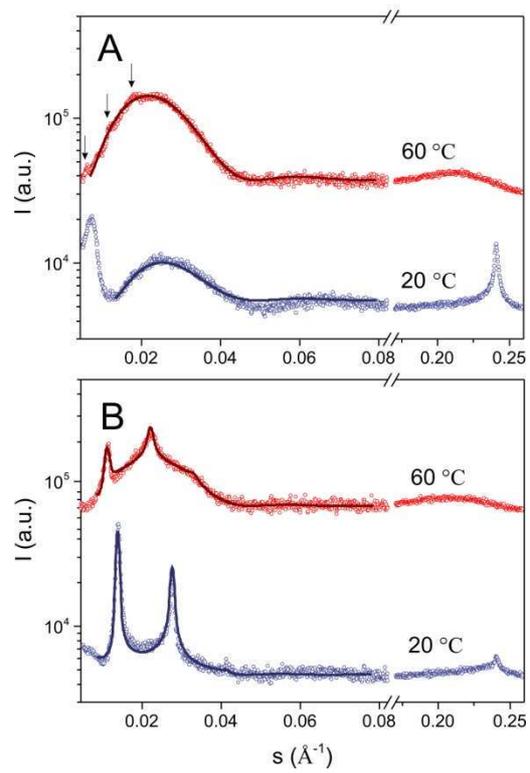
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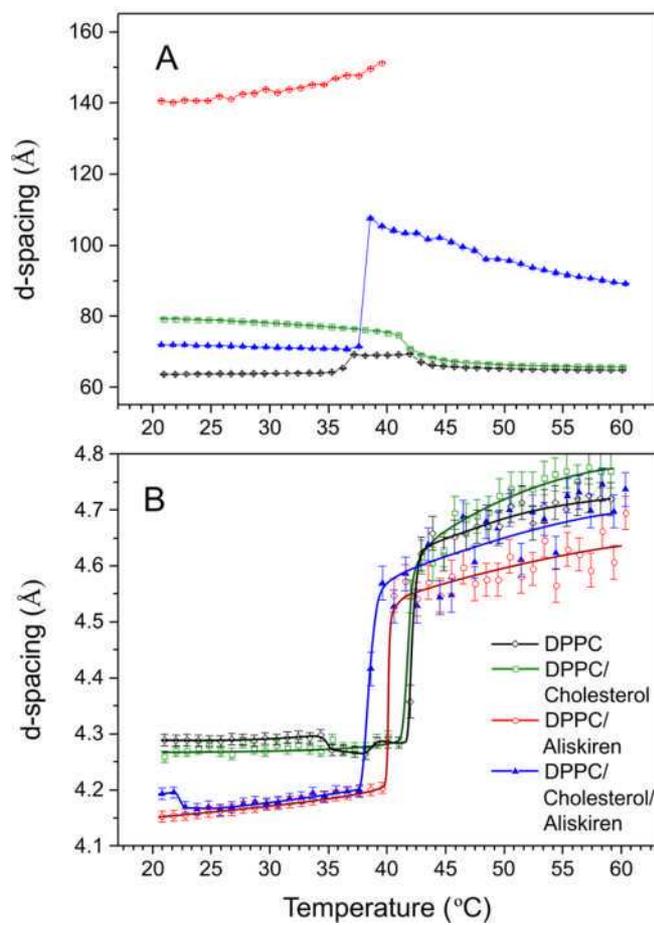
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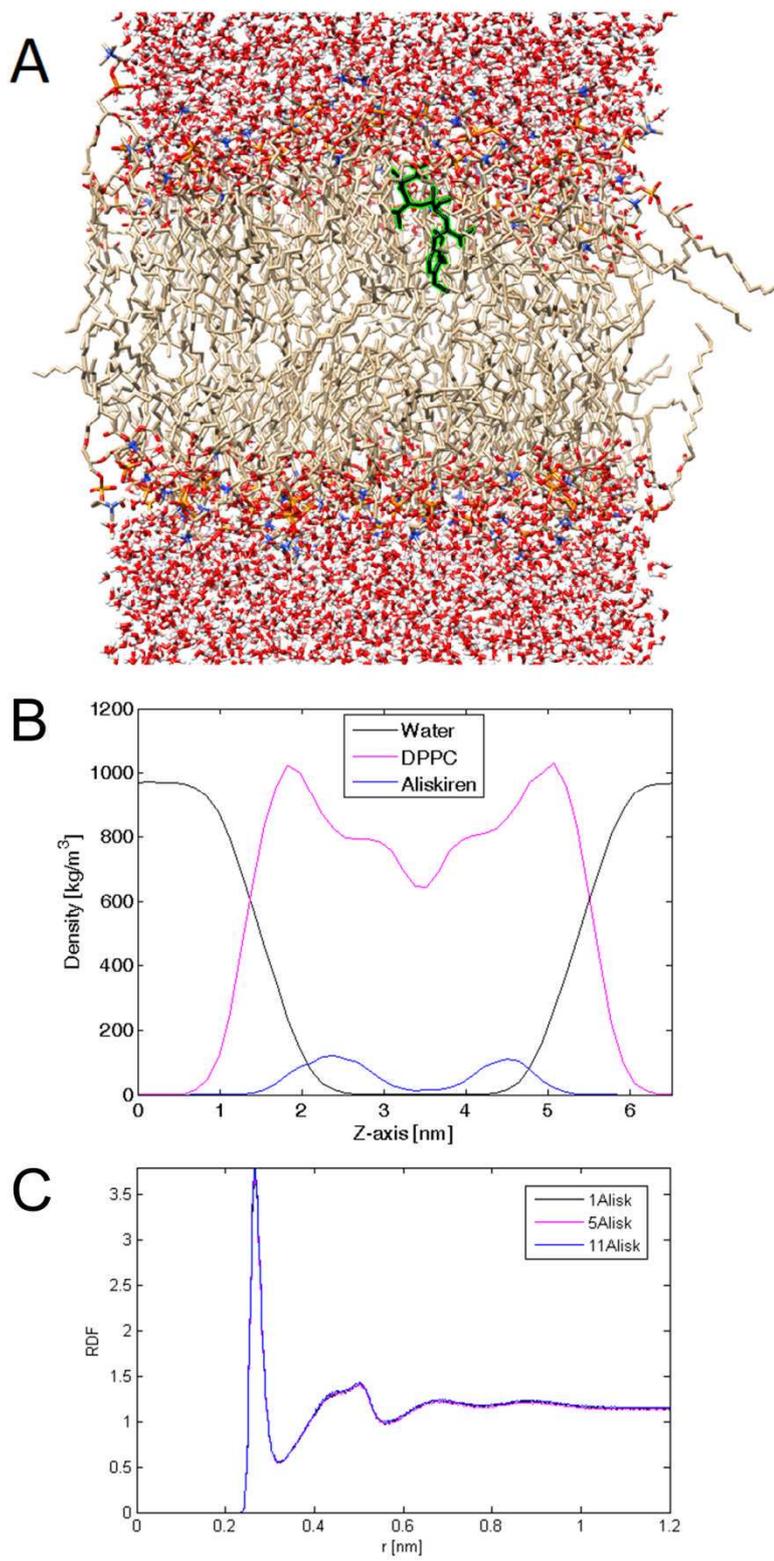
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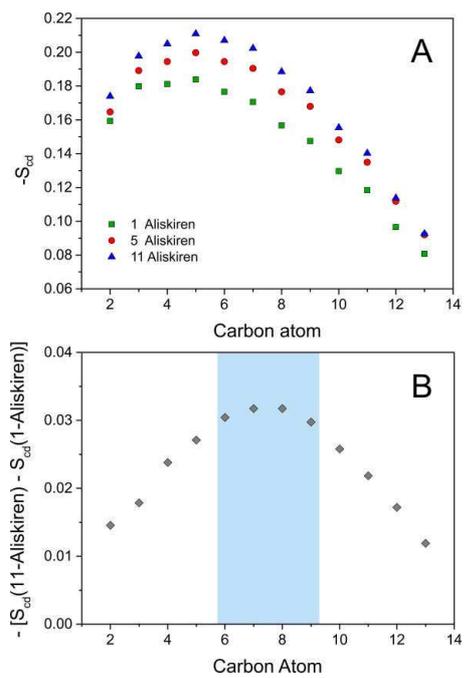
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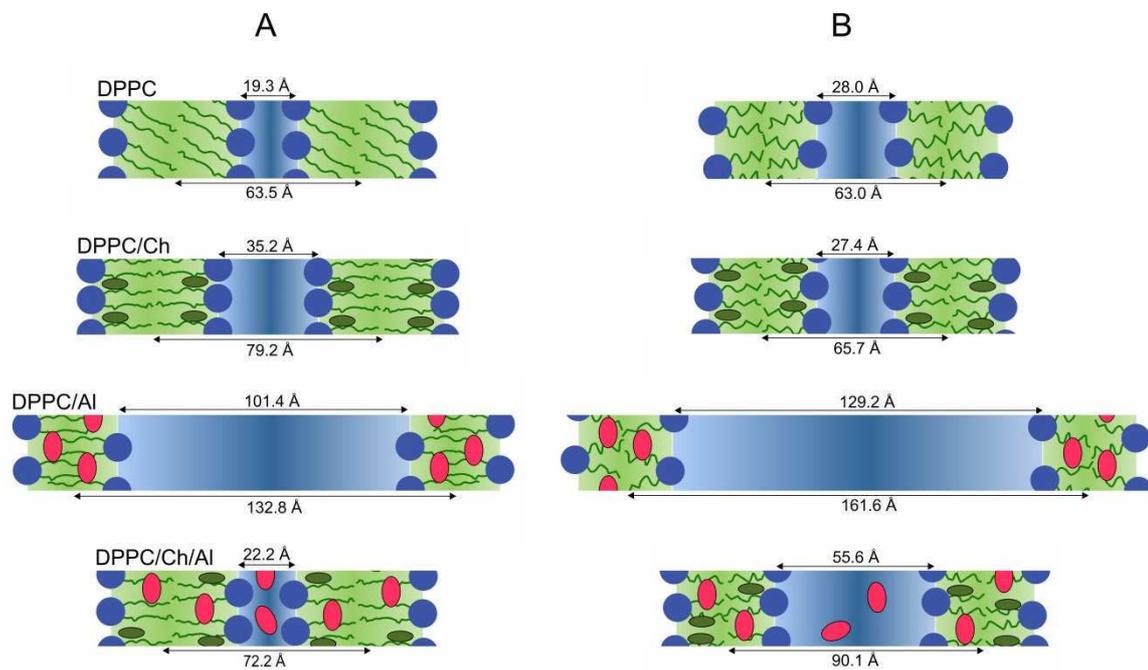
**Fig. 6**



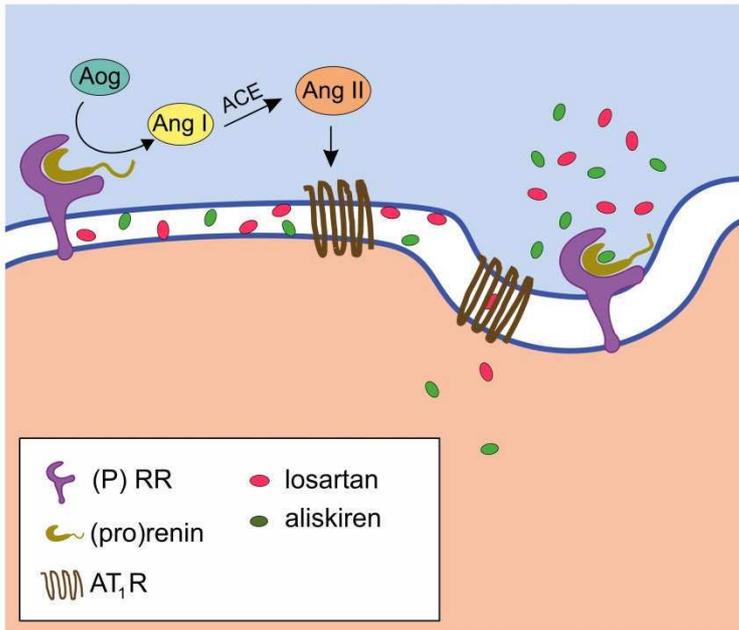
**Fig. 7**



**Fig. 8**



**Fig. 9**



**Fig. 10**

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