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Keywords: MPP1; Protein-lipid interactions; Flotation assay; Langmuir-Blodgett monolayer

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MPP1 interacts with DOPC/SM/Cholesterol in an artificial membrane system using Langmuir-Blodgett monolayer

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Abstract: The interaction between Membrane Palmitoylated Protein -1 (MPP1) with lipid bi- and mono-layers composed of a DOPC/SM/Chol mixture was investigated. MPP1 co-migrates with liposomes to the top of the liposome flotation gradient, indicating binding of MPP1 with liposomes. The injection of MPP1 into the subphase of an LB monolayer of the above lipid composition induced an increase in surface pressure, indicating that MPP1 molecules were incorporated into the lipid monolayer. The compressibility modulus isotherms of MPP1, lipids and lipid-MPP1 films have essentially different shapes from one another. Pure MPP1 isotherms were characterized by a peak in surface pressure of 25-35 mNm^{-1}. This transition disappears in isotherms obtained with lipid monolayers in the presence of MPP1, which suggests an interaction between the protein and the lipid monolayers. In addition, this interaction is sensitive to the presence of cholesterol in the lipid monolayer, as adding of MPP1 into the subphase of lipid monolayers containing cholesterol resulted in a much larger increase in surface area than when MPP1 is injected into the subphase of a lipid monolayer devoid of cholesterol. In conclusion, the

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Introduction

Membrane Palmitoylated Protein 1 (MPP1), is a member of a family of membrane associated guanylate kinase homologue proteins (MAGUKs) and was originally identified as a membrane skeleton protein in erythrocytes (Podkalicka et al., 2015; Dimitratos et al., 1999; Quinn et al., 2009). Previous studies have confirmed that MPP1 anchors to the lipid bilayer in the erythrocyte membrane by constituting a ternary-complex with glycophorin C and protein 4.1 (Nunomura et al., 2000; Alloisio et al., 1993; Mburu et al., 2006). Human erythrocyte MPP1 consists of a single PDZ domain, a central SH3 domain, a C-terminal GUK domain and a D5 motif located between the SH3 and GUK domains (Ruff et al., 1991; Fanning and Anderson, 1996; Seo et al., 2009). Recently, MPP1 was shown to play a crucial role in lateral membrane organization that may be involved in the molecular mechanism of a yet-unexplored haemolytic anaemia (Łach et al., 2012). However, the interaction of MPP1 with membrane proteins leading to resting-state raft-stabilization does not involve membrane skeleton proteins such as actin, since extraction and/or depolymerisation does not affect the amount of DRM and membrane-fluidity, as measured by FLIM of the di-4 probe (Łach et al., 2012; Biernatowska et al., 2013). On the other hand, our team have recently proposed that MPP1 interacts with flotillins in the native membrane and in DRMs (Biernatowska et al. submitted).

The current view of the biological membrane is that lipids and proteins mutually interact in a dynamic but transient way to accomplish membrane functions. It has become clear now that lateral heterogeneity strongly influences our concepts of the structure of the lipid bilayer and that

lipid and protein sorting is highly dependent on the lateral organization of the membrane (Engelman, 2005). The enrichment of sphingomyelin and cholesterol in the membrane, in general, and in membrane raft domains, in particular, has been considered by several studies reporting on the roles of these lipids within the membrane, including the interactions between these two principal components (Collado et al., 2005; Frazier et al., 2007; Coste et al., 2006; Devanathan et al., 2006). Packing defects and lateral heterogeneity may facilitate a number of biological functions of the membrane. Therefore, it is relevant to understand the forces controlling the lateral ordering and diffusion of lipids and their basic physical chemistry. In brief, laterally separated phases may be induced by an enzymatic cleavage of lipids (Holopainen et al., 1998), temperature (Mouritsen, 1991), surface electrostatic associations (Rytömaa and Kinnunen, 1996), and lipid-lipid (Söderlund et al., 1999; Lehtonen et al., 1996) or lipid-protein (Mouritsen and Bloom, 1984) interactions. The ternary mixture consisting of dioleoylphosphatidylcholine (DOPC), sphingomyelin (SM) and cholesterol (Chol) have been used as a membrane-mimicking model (Nyholm et al., 2011; Yuan et al., 2002; Kulma et al., 2010; Tsukamoto et al., 2014). It has been shown that this lipid mixture can imitate the phase-separation of cell membranes (Bezlyepkina et al., 2013), which provides an experimental alternative to native plasma membranes containing major membrane components, including phospholipids, sphingolipids and cholesterol.

A growing number of studies describe membrane-mimicking models, including liposomes and Langmuir-Blodgett (LB) monolayers. In this study, we ask the question as to whether MPP1, as single protein component, has the capability to interact with lipid bi- or mono-layers which exhibit a complex lateral heterogeneity (DOPC/SM/Chol, 1:1:1 molar ratio) in two separate model systems, namely a liposome-flotation assay using liposomes, or with a lipid monolayer prepared from the lipid mixtures at the air-water surface using the Langmuir Blodgett (LB)

monolayer film technique. LB monolayer is a powerful technique that allows the formation of monolayer lipid films, together with introducing protein into the subphase buffer at a range of concentrations and under the desired variable physiologically, compatible conditions including subphase buffer, pH and temperature. The results of this study provided direct evidence of such an interaction and suggests the possible participation of this binding mechanism in lateral organization of the membrane.

Material and methods

Overexpression and purification of MPP1 in a bacterial system

The MPP1 protein construct was obtained by subcloning the MPP1 gene sequence (Sequence ID: NP_002427.1) into the pRSET A plasmid (Invitrogen) using BamHI and XhoI restriction enzymes (Promega). In order to express the MPP1, *Escherichia coli BL21* were transfected with purified plasmid constructs. After expression of MPP1 in *Escherichia coli BL21* cells, using IPTG as an inducer for 16 hours at 18°C, the recombinant protein with a His6-tag at the amino terminus was extracted with 8 M urea in 20 mM Tris-HCl, 150 mM NaCl and pH 8 and affinity purified on immobilized Ni-NTA-affinity resin (Qiagen) on a Econo-Pac® 10 DG chromatography column (Bio-Rad Laboratories). Lowering the temperature to 18ºC after bacterial induction was chosen to prevent the formation of inclusion bodies and to improve protein solubility.

The purified protein was then analysed using SDS polyacrylamide gel electrophoresis with a Coomassie blue stain. The concentration of MPP1 was calculated using an absorbancy coefficient at 280 nm calculated using ExPASy ProtParam program (Wilkins et al., 1999). MPP1 was centrifuged at 10,000 rpm to remove any precipitated material and to ensure the homogeneity of the sample before use. Circular Dichroism measurements of the proteins was performed after

dialysis on a JASCO J-815 (Spectroscopic Co. Ltd, Japan). The spectra were measured from 20-70°C at 0.2 nm resolution from 190 to 240 nm in Tris-HCl buffer containing 5 mM Tris-HCl, 50 mM NaCl and pH 7.4.

Lipids

Sphingomyelin (egg), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) were purchased from Avanti Polar Lipids and Cholesterol (Chol) was obtained from Northern Lipids. Lipid concentrations were quantified by phosphate analysis (Rouser et al., 1966). Cholesterol concentration was quantified using the Cholesterol Kit (BioSystems).

Flotation assay

Liposome preparation was produced by the technique of hydration of a dry lipid film (Bangham et al., 1965; Morton et al., 2012). Briefly, chloroform solutions of the individual lipids DOPC, SM and Cholesterol were mixed at 1:1:1 molar ratio in a round-bottom flask and chloroform was then evaporated in a nitrogen stream to obtain thin film. The film was then further dried in a vacuum desiccator for at least 2 hours or overnight. The lipids were resuspended in HBS buffer (20 mM HEPES, 150 mM NaCl, pH 7.4). The hydrated liposomal suspension was subsequently extruded at a temperature of 64ºC under high pressure of gaseous nitrogen through a 0.4 µm polycarbonate membrane filter. The filter was then replaced by 0.2 µm and 0.1 µm-pore membranes, and the extrusion cycles were repeated 10 times independently for each filter. The size of the liposomes was determined using a ZetaSizer (Malvern). Liposomes were stored at 4°C until use.

MPP1 at concentrations of 50 and 150 nM, and 0.4 mg/ml lipid-liposomes were prepared in HBS buffer to a final volume of 250 µl and incubated at room temperature for 30 minutes. Control

samples contained MPP1 at the same concentrations without liposomes. After incubation, samples were transferred to ultracentrifuge tubes and mixed with 250 µl of 60% sucrose by pipetting up and down few times. The samples were overlaid with 0.8 ml of 15% sucrose, 1.8 ml of 10% sucrose in HBS buffer, and finally with 1 ml of HBS buffer without mixing. The samples were then centrifuged at ~200 000x g (45 000 rpm, 60Ti rotor) for 2 hours at 4°C. After centrifugation, 6 fractions were taken, starting from the top of gradient, and after addition of SDS to a final concentration of 1%, fractions were analyzed via dot-blot assay. After equal volumes of samples were loaded into the wells of a dot-blotter (Hoefer Scientific Instruments), the membrane was blocked for 1 hour at room temperature after entire samples were filtered through the membrane. The samples were incubated with primary mouse monoclonal anti-MPP1 antibodies (Abnova, 1:1000) for 3 hours at room temperature or, alternatively, overnight at 4°C. Membranes were washed three times for 5 min in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween, pH 7.4). Secondary goat anti-rabbit antibodies (Santa Cruz 1:10000) were then added and incubated for 1 hour at room temperature. ECL was used for detection and viewed using UVP Bio Spectrum Imaging System (United Kingdom).

Langmuir-Blodgett monolayer

Monolayer experiments were carried out as described previously by Grzybek *et al.* (2009) The measurements were performed using a 70 cm^2 teflon Langmuir trough connected with motorized barriers (Nima Technology) equipped with a Nima tensiometer ST 9000 (Nima Technology) along with a filter-paper Wilhelmy-plate (KSV Nima, Biolin Scientific) to measure surface pressure. The trough was placed in an enclosed chamber, facilitating flushing with a nitrogen stream and temperature was controlled by a water jacket (Julabo F12, Germany) to 22°C. A subphase buffer (60 ml), containing 5 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 0.5 mM

DTT and pH 7.4 was used. Monolayers were formed by injecting a chloroform solution of a lipid-mixture with a Hamilton syringe on the surface of the subphase buffer. After spreading, the solution was left for 10 min to allow for solvent evaporation and a plot of the surface pressure (Π) versus molecular area (A) was plotted. For each experimental condition, at least 3 independent monolayers were prepared and, for each lipid mixture monolayer, 4-5 isotherms were recorded without reaching the collapse pressure, prior to adding any protein. When similar lipid isotherms are obtained, the protein is then injected. Aliquots, 600 µl of MPP1 dialyzed against the subphase buffer were injected into the subphase. The surface pressure against the area were recorded after stirring the subphase for 4 minutes using a small stirrer bar, followed by another 4 minutes of stabilization. For analysis, the most consistent isotherms from each monolayer were chosen. Independent and dependent variables collected by the instrument were imported into the Excel file and surface compressibility modulus (Cs^{-1}) of the monolayer was calculated from the first derivative of the monolayer surface pressure and area per molecule data using a formula:

$Cs^{-1} = -A \times (\Delta\Pi/\Pi A)$ (Gicquaud et al., 2003),

where A is the area per molecule at indicated surface pressure and Π is the corresponding surface pressure.

GraphPad PRISM® 6 Software was used in Scatchard analysing to perform nonlinear regression curve fit of one site binding. The results calculated by GraphPad displays the best-fit values for the binding parameter, i.e. equilibrium dissociation constant (K_D). Standard deviation and Student's t test was used to assess the variability of obtained data applying MS Excel procedures.

Results

Liposome flotation assays

Coomassie blue stained SDS-10% PAGE electropherogram of purified recombinant MPP1is shown in Figure 1*A*. Binding of MPP1 to liposome membrane was assessed using liposomes composed of DOPC, SM and Cholesterol (1:1:1 molar ratio) mixture. MPP1 was incubated with liposomes at room temperature for 30 minutes. Next, an equal volume of dense sucrose solution was added and the final mixture was placed at the bottom of a sucrose gradient and overlayered with a series of lower density solutions and then ultra-centrifuged (see Materials and Methods). If MPP1 binds to liposomes it will float towards the top of the centrifugation tube. An example of the dot-blot assay of gradient fractions of liposome mixtures containing 50 and 150 nM MPP1 is shown in Figure 1*B*, lanes a and b. Lane c in the same Figure shows a result of a dot assay of a 150 nM MPP1 sample in which liposome suspension was omitted. When the incubation mixture contained both liposomes and MPP1 (Fig.1*B*ab) a reasonable fraction of MPP1 is found in the top of the density gradient, while in the absence of liposomes it can be found only in bottom fractions. These results may indicate that MPP1 interacts with liposomes.

Langmuir-Blodgett monolayer study

Surface Pressure−Area Isotherms.

To gain a more detailed insight into possible MPP1-lipid interactions, we investigated the effect of MPP1 on LB lipid monolayers composed of 1:1:1 molar ratios of a DOPC/SM/Chol mixture. In a series of experiments, a lipid monolayer was formed and MPP1 was then injected into the subphase buffer under the lipid monolayer. Figure 2*A* shows the surface pressure-area isotherms of the lipids alone (dotted curve) or with MPP1 present in the subphase (solid curve), and the

isotherms of pure MPP1 without lipids as a control (dashed curve). The surface pressure of the lipid isotherms alone increases to a maximum value at ~41 mNm^{-1} as the surface area is reduced during compression. The addition of MPP1 in the subphase modifies the behavior of the isotherms. The isotherms show that, when MPP1 is present in the subphase of a lipid monolayer, the recorded initial surface pressure is higher than that of the lipid monolayer alone and protein alone. Moreover, at any given pressure, the addition of MPP1 significantly increased the pressure of the lipid monolayer. The complex features of the surface pressure-area isotherms of the obtained isotherms can be better shown by plotting the compressibility modulus (Cs^{-1}). The pure MPP1 isotherms are characterized by a peak that implies an apparent change in the protein conformation at the air-liquid interface at a surface pressure of 25 - 35 mNm^{-1}. It is interesting that this transition at 25 - 35 mN m^{-1} disappears in the isotherms obtained in the presence of a lipid monolayer, suggesting that MPP1 incorporation into lipid monolayer results in a restructuring of the lipid/protein packing at the surface (Fig. 2*B*). The inset in Figure 2*A* shows the area increment, ΔA, resulting from MPP1 incorporation into the lipid monolayer from the subphase. By progressively restricting the monolayer area and increasing the surface pressure, this effect (ΔA) decreased but did not totally disappear. This means that the area of the lipid-MPP1 monolayer in the presence of the protein in the range of the physiological pressures, >30 mNm^{-1}, is larger than the area of lipid monolayers in absence of protein (inset Fig. 2*A*).

It was interesting to compare the above described interaction with the interaction of MPP1 with a lipid monolayer containing charged lipid, i.e. one in which DOPC was substituted by DOPS. The Π-A and Cs^{-1}-Π isotherms of DOPS/SM/Chol lipid mixture show that, when MPP1 is injected into the subphase, the compression isotherm is characterized by a plateau (Π-A) or peak in the Cs^{-1} isotherm at the area corresponding to the surface pressure of 23-35 mNm^{-1} (Fig. 2*C* and 2*D*). In the case of this monolayer, the peak (Cs^{-1}-Π) or plateau (Π-A) does not disappear in the

presence of MPP1. It is interesting that this value is similar to that obtained for a pure MPP1 (dashed lines) and the penetration of the protein into the air-water interface has dramatically decreased at this value (inset Fig. 2*C*). These results indicate that the behavior of the protein at the air-liquid interface is also dependent upon the composition of the lipid monolayer.

Effect of the initial MPP1 concentration in the subphase on monolayer surface pressure

The increase of surface pressure ($\Delta\Pi$) of the DOPC/SM/Chol (1:1:1) monolayer after injection of MPP1 into the subphase was monitored and plotted as a function of concentration and a hyperbolic curve was obtained (Fig. 3). To estimate the apparent equilibrium dissociation constant (K_D) for the MPP1-lipid interaction, this data was fitted to a nonlinear regression fit using GraphPad PRISM 6 software (Fig. 3). The determined in such a way value of an apparent equilibrium dissociation constant (K_D) was 34.87 ± 6.6 nM and B_{max} which is maximal change in the surface pressure of 26.8 ± 2.0 mNm^{-1}. The data from the Scatchard plot showed a straight line, which implies a one-site binding interaction (inset Fig. 3).

Characteristics of surface pressure–area isotherms of DOPC/SM/Chol with different MPP1 concentrations in the subphase.

Figure 4*A* shows the Π–A isotherms of MPP1-lipid monolayers consisting of DOPC/SM/Chol with various concentrations of MPP1 in the subphase varied in the range of 5 to 40 nM. The initial surface pressure of lipid monolayer increases with increasing MPP1 concentration in the subphase. This indicates that MPP1 molecules are incorporated into the lipid monolayer from the subphase. Above the mentioned above transition at surface pressure of 25-35 mNm^{-1}, the isotherms of DOPC/SM/Chol monolayer when the subphase contains ≥20 nM MPP1 approach and overlap each other. The Cs^{-1}-Π curves (Fig. 4*C*) show that when the MPP1 concentration in

the DOPC/SM/Chol monolayer subphase exceeds 20 nM the transition at 25-35 mNm^{-1} (Fig. 2) starts to be more pronounced than this observed for the MPP1 concentrations ≤20 nM MPP1(Fig. 4*C*).

To further characterize the MPP1-Lipid monolayers, the change in surface area of lipid-MPP1 monolayers at a surface pressure of 20, 25, 30, 35 and 40 mNm^{-1} were plotted against MPP1 concentrations. At a pressure <35 mNm^{-1}, the change in the area corresponding to the protein incorporation increases with increasing protein concentration. On the other hand, at a surface pressure of 35 and 40 mNm^{-1} the change increasing up to protein concentration of 20 nM, whereas the change in the area in the lipid-MPP1 monolayers containing ≥20nM MPP1 was concentrations independent. When the points were fitted into a hyperbola, the obtained values of K_D were in the range of 2.6 nM for 40 mNm^{-1} to 20.5 nM for 20 mNm^{-1}, maintaining relatively stable values for various surface pressure. Moreover, as Cs^{-1}- Π dependence indicates, monolayer in this MPP1 concentration (30-40 nM) and Π (25-30 mNm^{-1}) range resembles characteristics of pure MPP1 at air-water interface (compare Fig. 2*B* and 4*C*). Possible explanation of this effect is given under "Discussion".

Effect of cholesterol on the interaction of MPP1 with lipid membranes.

To test whether presence of cholesterol has an effect on interaction of MPP1 with lipid monolayers, we measured the changes in the surface area (ΔA) of the DOPC/SM/Chol 1:1:1 monolayer and DOPC/SM 2:1 monolayer after the addition of 20 nM MPP1 into the subphase. The presence of cholesterol in the DOPC/SM monolayer facilitates the penetration of the monolayer by MPP1. Namely, the injection of 20 nM MPP1 into the subphase resulted in a 2-fold increase (at 20 mNm^{-1}) in ΔA values compared to those observed in the case of the absence of cholesterol. Moreover, at the higher surface pressure the higher this difference was, reaching at

least a four-fold increase in the ΔA values presence of cholesterol in the monolayer (Fig. 5). When Student's t test was applied for comparison of data obtained for DOPC/SM/Chol vs DOPC/SM monolayers at all surface pressure values highly significant differences were observed ($0.0001<p<0.002$). For The data presented above indicates rather a strong dependence of the MPP1-lipid monolayer interaction on cholesterol.

Discussion

Previous studies involving MPP1 have mainly focused on protein-protein interactions, rather than the binding of MPP1 to the membrane lipid bilayer. Here, we investigated the interaction between MPP1 and monolayers composed of two lipid mixtures, namely, a lipid mixture composed of composed of DOPC, SM and cholesterol at a molar ratio of 1:1:1. The qualitative results obtained from the flotation assay showed that a fraction of MPP1 molecules was associated with the liposome fraction from the top of the gradient, indicating that MPP1 binds to the liposomes. While MPP1 was also found in the bottom fraction of the gradient (Fig. 1*B*), which is highly dependent on the K_D values of the interaction between the molecules. In addition, the *in silico* binding analysis by molecular modeling have predicted the ability of the MPP1 homodimer formation which may result from the known fact that domains on the MPP1 can bind to each other, such as the SH3+HOOK domain and an end fragment of MPP1 that contains the GUK domain (Gosens et al., 2007). Formation of the homodimer might be responsible for preventing MPP1-lipid interaction.

More quantitative evidence of the interaction of MPP1 with lipids was also demonstrated via the Langmuir-Blodgett method with lipid monolayer films. The LB monolayer method provides an insight into whether the interaction between the protein and lipids affects the properties of the lipid monolayer. The increase in the surface pressure of the lipid monolayer composed of the -

DOPC/SM/Chol mixture after injection of MPP1 into the subphase indicates that MPP1 molecules penetrate into the lipid monolayer (Fig. 2). This increase in the initial pressure is similar to that obtained by Cytochrome C interaction with a cardiolipin phospholipid monolayer (Marchenkova et al., 2015), as well as to that observed for the interaction of a synthetic antimicrobial peptide, called V4, with both POPG and POPC monolayers (Yu et al., 2009). The observed effect was dependent upon the concentration of the protein in the subphase (Figs 3 and 4). However, when DOPC in the lipid monolayer film is substituted with DOPS, a negatively charged phospholipid, the properties of the monolayer seem less affected by MPP1, as the compressibility isotherm displays a peak at Π range 25-30 mNm^{-1} at an MPP1 concentration of 10 nM (Fig. 2). This may indicate a rather smaller fraction of MPP1 bound or forming a common phase with DOPS/SM/Chol monolayer. Therefore, the nature of the polar lipid head- groups may also play a role in the lipid-protein interaction.

The presence of lipid monolayers at the surface enhanced the attraction of MPP1 to the interface, since such monolayers were immediately modified in the presence of 5 nM MPP1, as shown by the immediate ΔΠ increase (Figs. 3 and 4). The DOPC/SM/Chol isotherms containing ≤30 nM MPP1 are also characterized by a transition at 25-35 mNm^{-1} (Fig. 4). Moreover, as Cs^{-1}-Π dependence indicates, monolayer in this MPP1 concentration and Π range resembles the characteristics of pure MPP1 protein at the air-liquid interface (compare Fig. 2*B* and 4*C*). This effect could be a result of a partial, transitory separation of the "protein domain" (phase) from the lipid-protein domain which is followed by squeezing out the protein from the monolayer at a surface pressure of around 35-40 mNm^{-1} (compare Fig. 4*A* and 4*C*). The saturation in the increase in area in the higher Π range could be due to the exchange of lipid molecules for MPP1 between the monolayer and the interphase and the binding of these lipid molecules by "interphase" MPP1. It should be noted that a certain amount of the protein remains tightly bound to the monolayer as

297 above squeezing out of protein at high surface pressure (>35 mNm^{-1}) led to an overlapping of the
298 isotherms on each other, but not an overlap with the isotherm of the pure lipid monolayer. A
299 similar mode of action has been described elsewhere, when the interaction and the incorporation
300 of human serum albumin within an octadecylamine monolayer was studied by Fan et al. (2005)
301 and was interpreted as the insertion of human serum albumin molecules into octadecylamine
302 monolayer.
303 As reported for other protein-lipid monolayer systems (Krol et al., 2000; Girard-Egrot et al.,
304 2004), the stability of mixed lipid–protein monolayers at high surface pressure without significant
305 loss of protein from the monolayer indicates a strong interaction between the protein and the
306 lipid. Moreover, the symmetric peaks from the Cs^{-1} curves, and the linear fits provided from
307 Scatchard plots, indicate that a one-step transition process is involved in the MPP1-lipid
308 monolayer interaction (Zhi-Wu Yu et al., 2002; Dziri et al., 1997; Ahluwalia et al., 1991).
309 The addition of MPP1 to the sub-phase underneath a monolayer of a lipid mixture without
310 cholesterol induced a smaller change in the area at constant surface pressure compared with that
311 obtained for the same lipid mixture containing cholesterol. This decrease in area may indicate a
312 structural modification or molecular rearrangement within the lipid monolayer that is specific to
313 the type of monolayer composition, that is, either the MPP1 can bind directly to cholesterol,
314 which possibility was shown by Listowski et al. (2015) or that cholesterol modulates the
315 arrangement of the lipid monolayer, i. e. inducing a phase separation (e.g. Grzybek et al., 2009),
316 which enhances the binding of MPP1. Further studies should bring explanation of this
317 mechanism.
318 Our previous simple modelling study on MPP1 (Listowski et al., 2015) showed that this protein
319 may contain two hydrophobic/amphipathic stretches of ~12-18 amino acid residues which could
320 be responsible for binding/penetration of lipid bi- and mono-layers via hydrophobic of these

interactions. The effect of ionic strength and pH would shed some light on this issue. However, using low and high ionic-strength or pH buffers strongly affects the stability of the protein. Therefore, studies in this area were constrained to conditions within which the protein would not be affected, and we were only able to use rather a low range of ionic strength and pH values. Using 50, 150 and 250 mM NaCl did not affect strongly this interaction, but a small minimum of Cs^{-1}_{max} was observed, suggesting the optimum conditions for the interaction. This was not observed in the case of the lipid monolayer mixture containing DOPS (Fig. 6). Studied range of pH indicated a small decrease in interaction at pH 8.2 of MPP1 with studied monolayers (Fig. 7). Overall, it seems that observed binding/penetration results from mixed hydrophobic-hydrophilic protein-lipid interactions which should be a subject of further studies by using other approaches. Our data using the above techniques demonstrate that MPP1 binds lipid bi- and mono-layers composed of DOPC/SM/Cholesterol. When the interaction of MPP1 with lipid monolayer mixtures was characterized, the results indicate that the extent of MPP1 binding was concentration-dependent, suggesting for the first time that MPP1-membrane binding may involve a non-proteinaceous component. It should be noted that this interaction may, at least in part, explain the participation of MPP1 in resting-state raft organization in erythroid cells

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

1. Interaction of MPP1 with liposomes ***A***, MPP1 was analysed by SDS-10% PAGE, Coomassie staining and size-calibrated using standard molecular weight markers. ***B,*** Interaction of MPP1 with liposomes composed of DOPC/SM/Chol at molar ratio1:1:1. Flotation assay of recombinant MPP1 added to lipid liposomes (0.4 mg/mL and 100-nm average diameter). Fractions were collected from top to bottom of the centrifuge tube and MPP1 in each fraction was analysed by Dot-blot assay with mouse monoclonal anti-MPP1 antibodies. Flotation of MPP1 depends on the presence of liposomes. (Lanes **a** and **b**) 50 nM and 150 nM MPP1 respectively with liposomes; MPP1 co-migrate with liposomes to the top of the gradient. (Lane **c**) 150 nM MPP1 alone in the bottom of the gradient.

2. Surface Pressure−Area Isotherms of MPP1 with lipid monolayers. ***A*** and ***C,*** The compression isotherms for the lipid monolayers alone (dotted curve), MPP1 alone (dashed curve), and lipid monolayers in the presence of 10 nM MPP1 in the subphase (solid curve). ***B*** and ***D,*** The compressibility modulus, $Cs^{-1}-\Pi$ isotherms, as a function of the surface pressure of the films was calculated from the corresponding Π -A isotherms. ***A,B,*** DOPC/SM/Chol, ***C,D,*** DOPS/SM/Chol both (1:1:1) molar ratio. Insets: The dependence of ΔA on Π_0 is presenting the MPP1 contribution in the monolayer calculated as the difference between the MPP1-lipid monolayer isotherms (curve 1) and lipid monolayers alone (curve 2) at the same surface pressure. Π_0, surface pressure; ΔA, an increment of the surface area from the lipid monolayer without MPP1 to the same lipid monolayer after MPP1 injection at the same surface pressure. The surface area is a relative value because the area per molecule of MPP1 cannot be calculated, as the size of MPP1 is undetermined.

493

494 **3. The effect of the initial protein concentration in the subphase as a function of MPP1**
495 **concentration.** Inset table presents a summary of results obtained from the nonlinear regression
496 fit. Scatchard Plot depicting the analysis of the curve data. MPP1 was injected into the subphase
497 beneath a monolayer of DOPC/SM/Chol (1:1:1) at 22°C. One-site binding was used to fit the
498 adsorption of MPP1 into the surface monolayer. Bmax = maximal change in surface pressure, K_D
499 = equilibrium dissociation constant. Error values = standard error of fit.

500

501 **4. MPP1-lipid isotherms using a DOPC/SM/Chol lipid mix with different concentrations of**
502 **MPP1 in the subphase.** ***A,*** The Π–A isotherms of MPP1- lipid monolayers in the presence of
503 different MPP1 concentrations in the subphase. ***B,*** The change in area of MPP1- lipid monolayers
504 versus MPP1 concentration. ***C,*** Cs^{-1}-Π isotherms calculated from Π–A isotherms in ***A***.
505 DOPC/SM/Chol isotherms at different MPP1: brown line, 5 nM, blue line, 10 nM, green line, 20
506 nM, yellow line, 30 nM, black line, 40 nM, and dotted black line, the lipid monolayer without
507 MPP1.

508

509 **5. Effect of cholesterol on the interaction of MPP1 with lipid monolayers.** The change in the
510 area, ΔA, induced by MPP1 at different surface pressure values in lipid monolayers with and
511 without cholesterol. The difference between the surface-area recorded for a lipid film in the
512 presence of MPP1 in the subphase and for the corresponding film of lipid recorded in the absence
513 of MPP1 as a function of the surface area available on the trough. The concentration of MPP1 in
514 the subphase is 20 nM. DOPC/SM/Chol (black column) and DOPC/SM (white dotted bars). Error
515 bars = ±S.D. For all pairs (DOPC/SM/Chol vs DOPC/SM) obtained p values for Student's t test
516 analyses were smaller than 0.002 for n=4.

6. The effect of NaCl on the phase behavior of MPP1-lipid monolayers. ***A*** and ***C,*** The compression isotherms for lipids in the presence of 50 mM (dotted curve), 150mM (dashed curve), and 250 mM NaCl in the subphase (solid curve). ***B*** and ***D,*** The compressibility modulus, Cs^{-1}–Π isotherms, as a function of the surface pressure of the films, was calculated from the corresponding Π-A isotherms. ***E,*** Maximum compressibility modulus, $Cs^{-1}{}_{max}$, values as a function of salt concentration for the MPP1- lipid mixtures. The surface area is a relative value for the reason mentioned in Figure 2 legend. Error bars = ±S.D, n=3.

7. The effect of pH on the phase behavior of MPP1-lipid monolayer interactions. ***A*** and ***C,*** The compression isotherms for lipids in the presence of subphase buffer at pH 6.0 (dotted curve), pH 7.4 (dashed curve), and pH 8.2 (solid curve). ***B*** and ***D,*** The compressibility modulus, Cs^{-1}–Π isotherms, as a function of the surface pressure of the films, was calculated from the corresponding Π-A isotherms. ***E,*** Maximum compressibility modulus, $Cs^{-1}{}_{max}$, values, expressed as a function of pH on MPP1-lipid mixtures. The surface area is a relative value for the reason mentioned in Figure 2 legend. Error bars = ±S.D, n=3.

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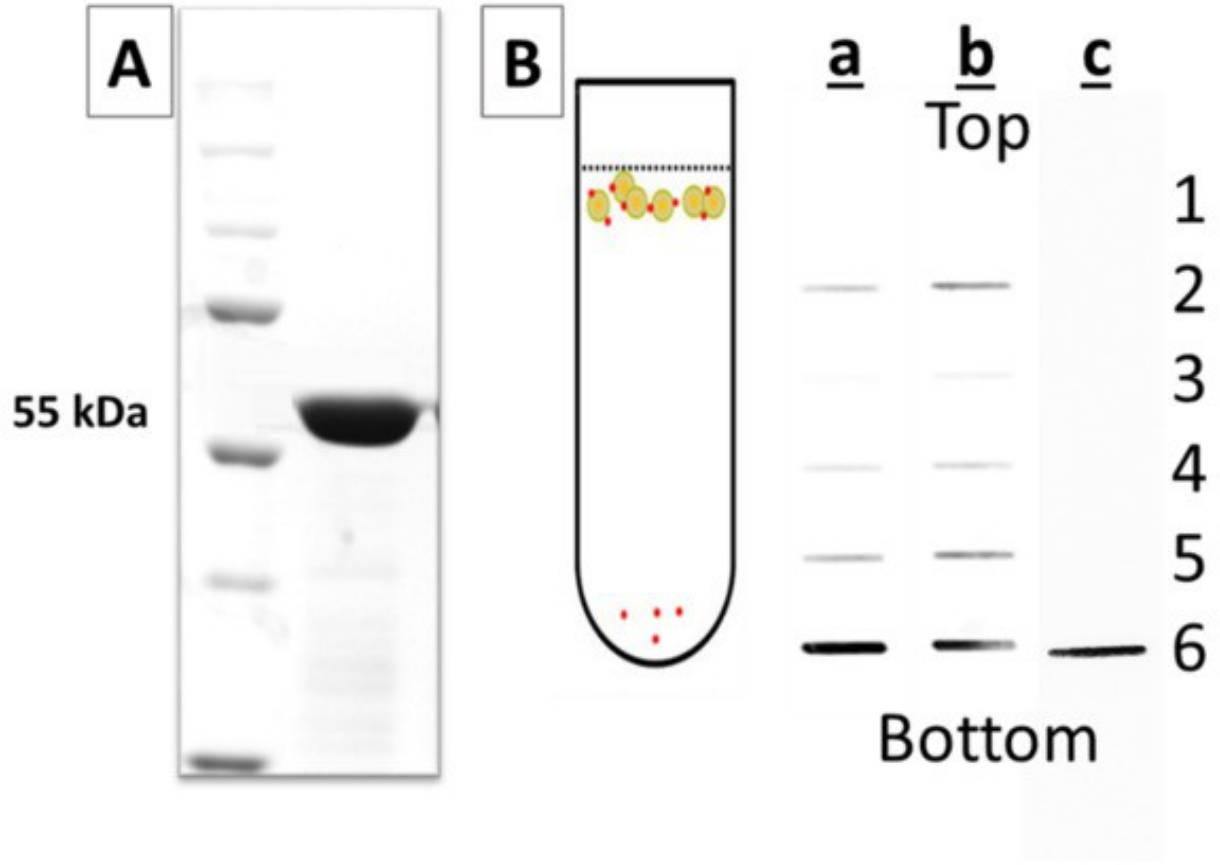

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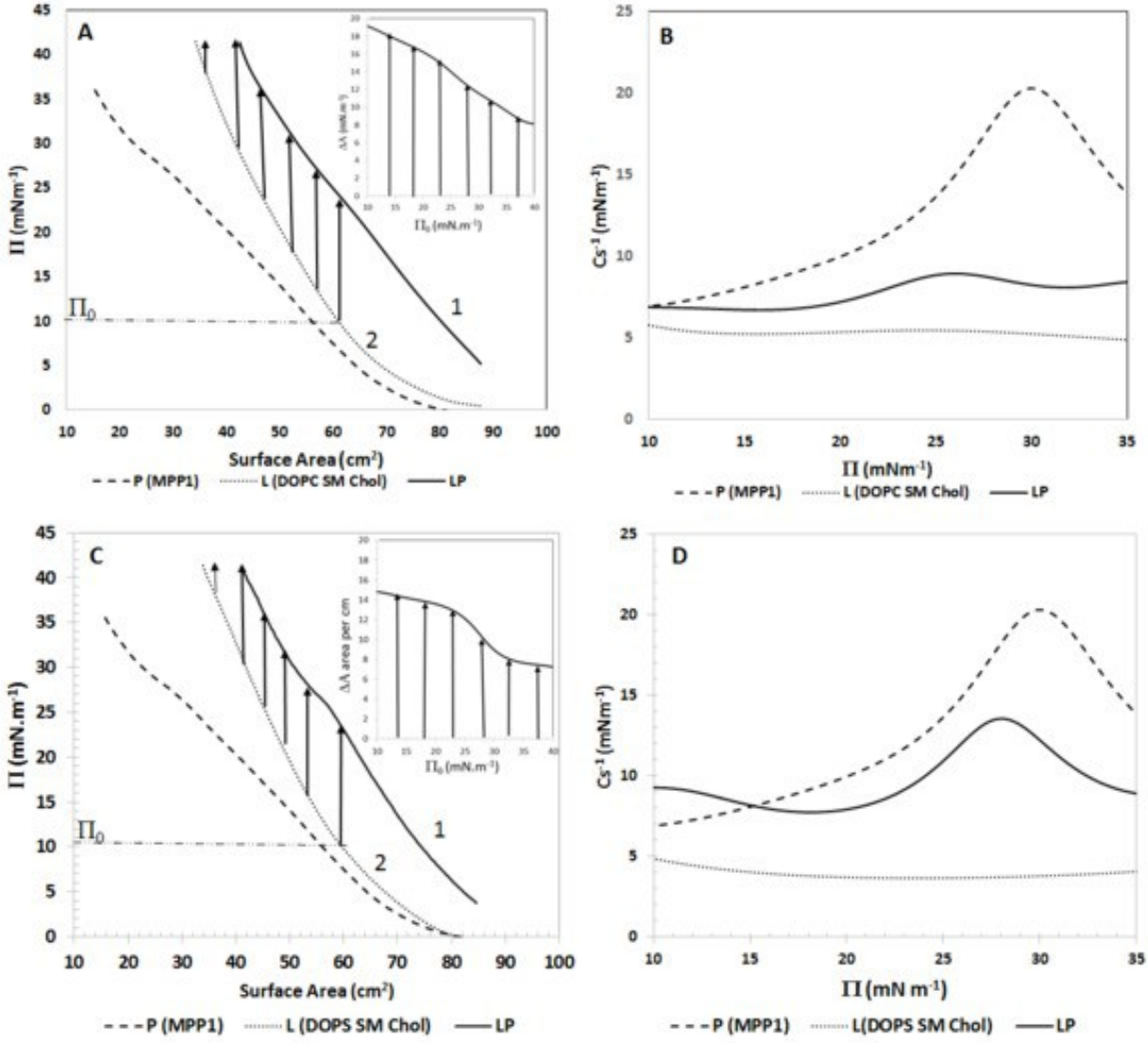

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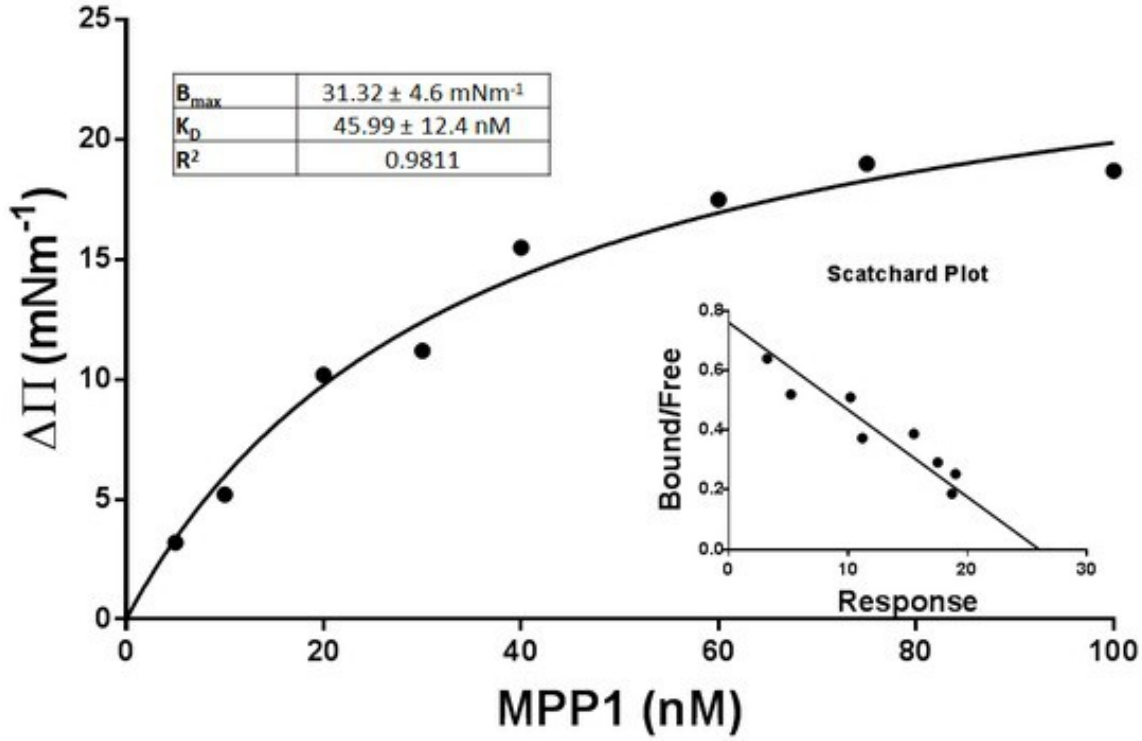

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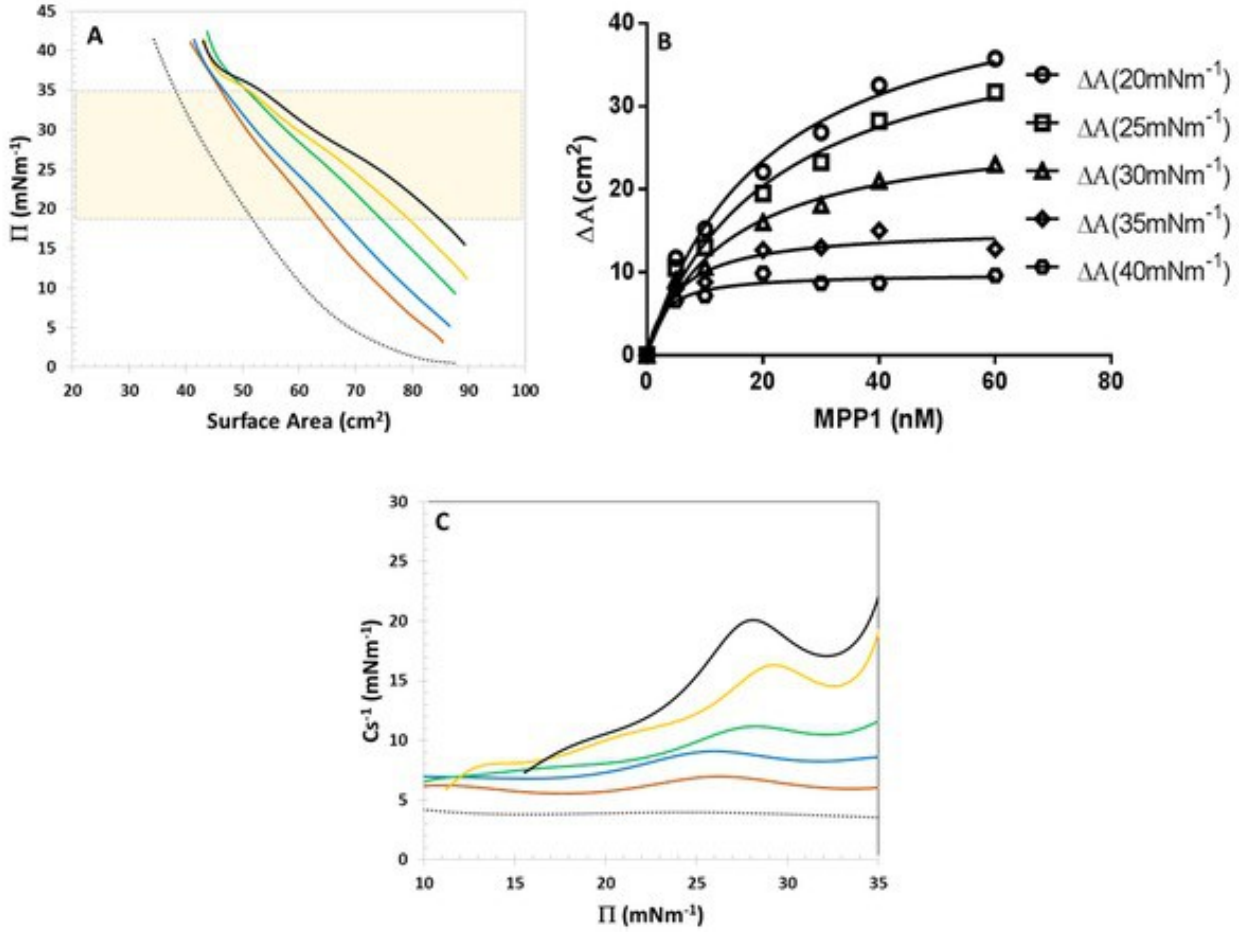

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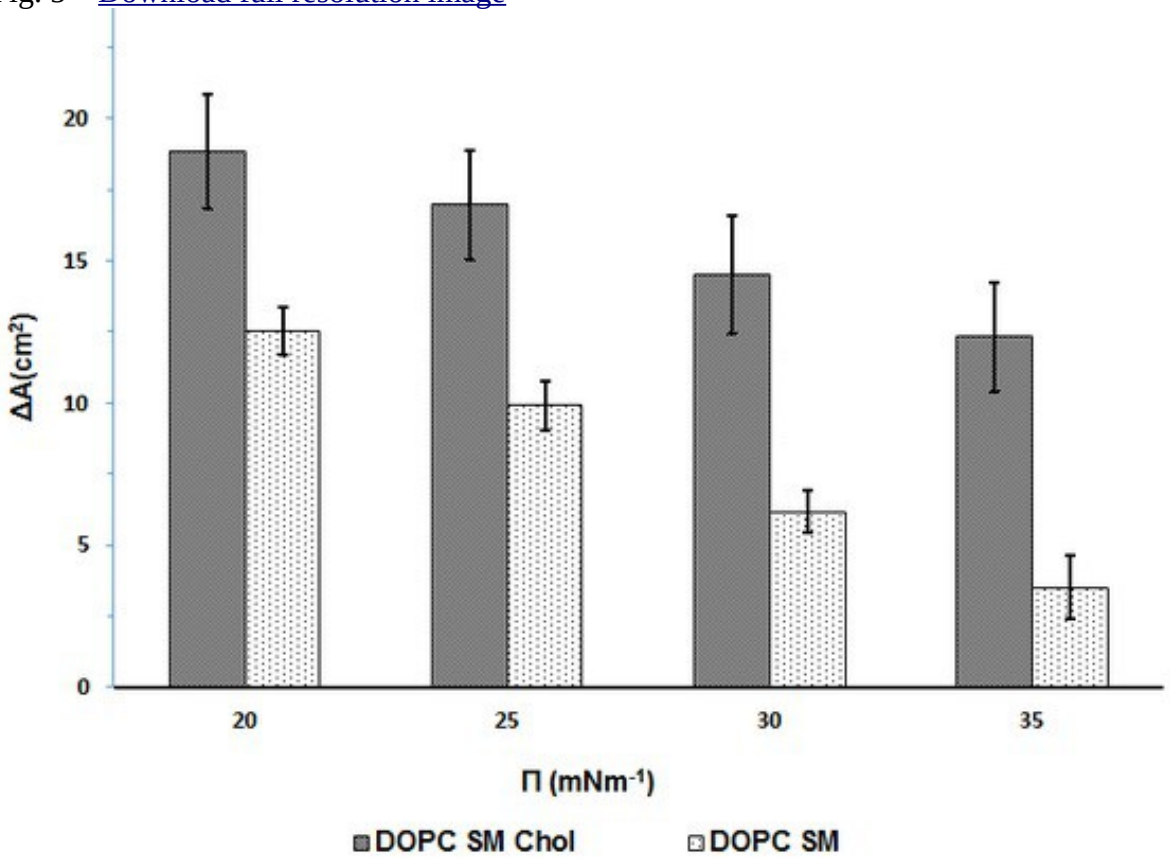

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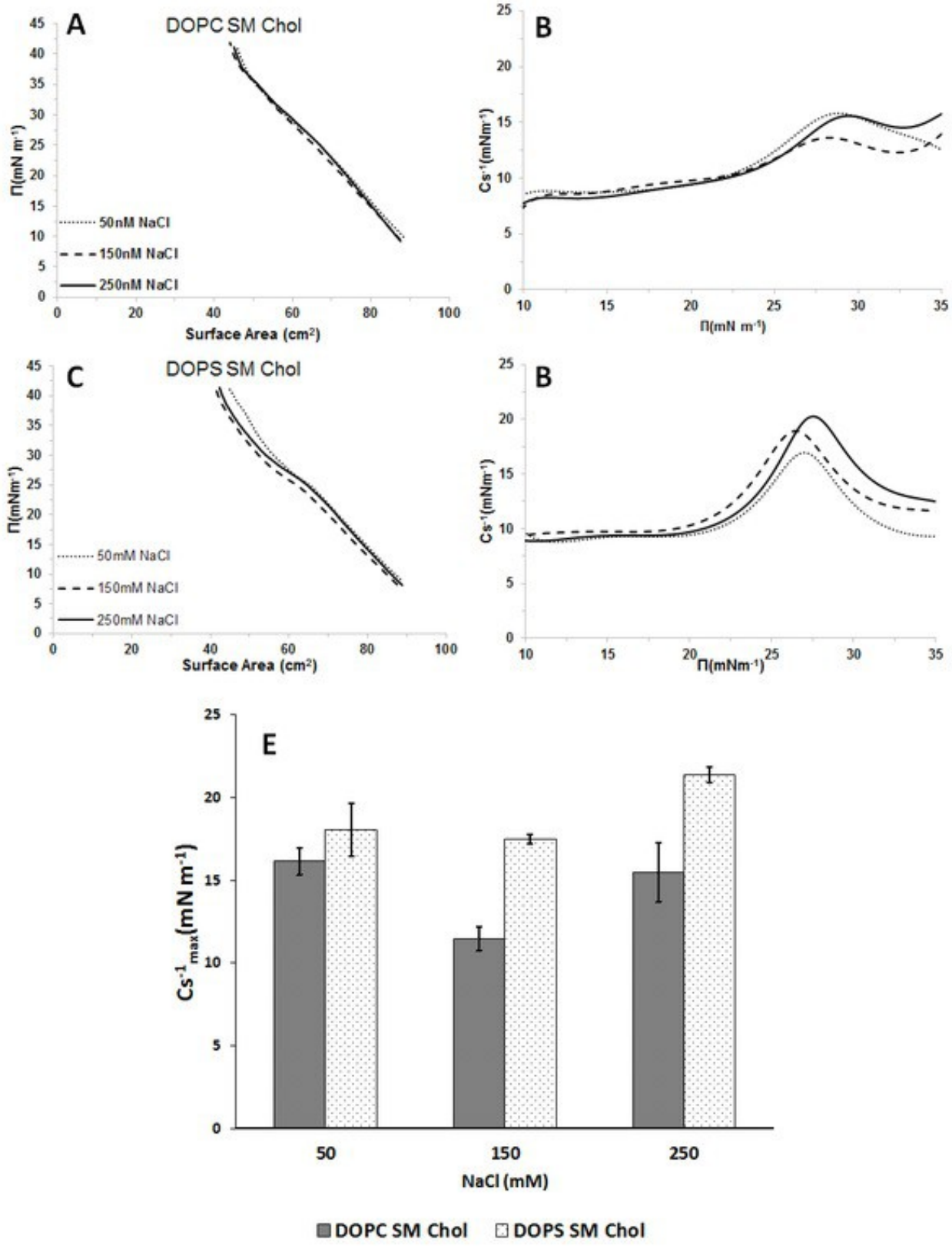

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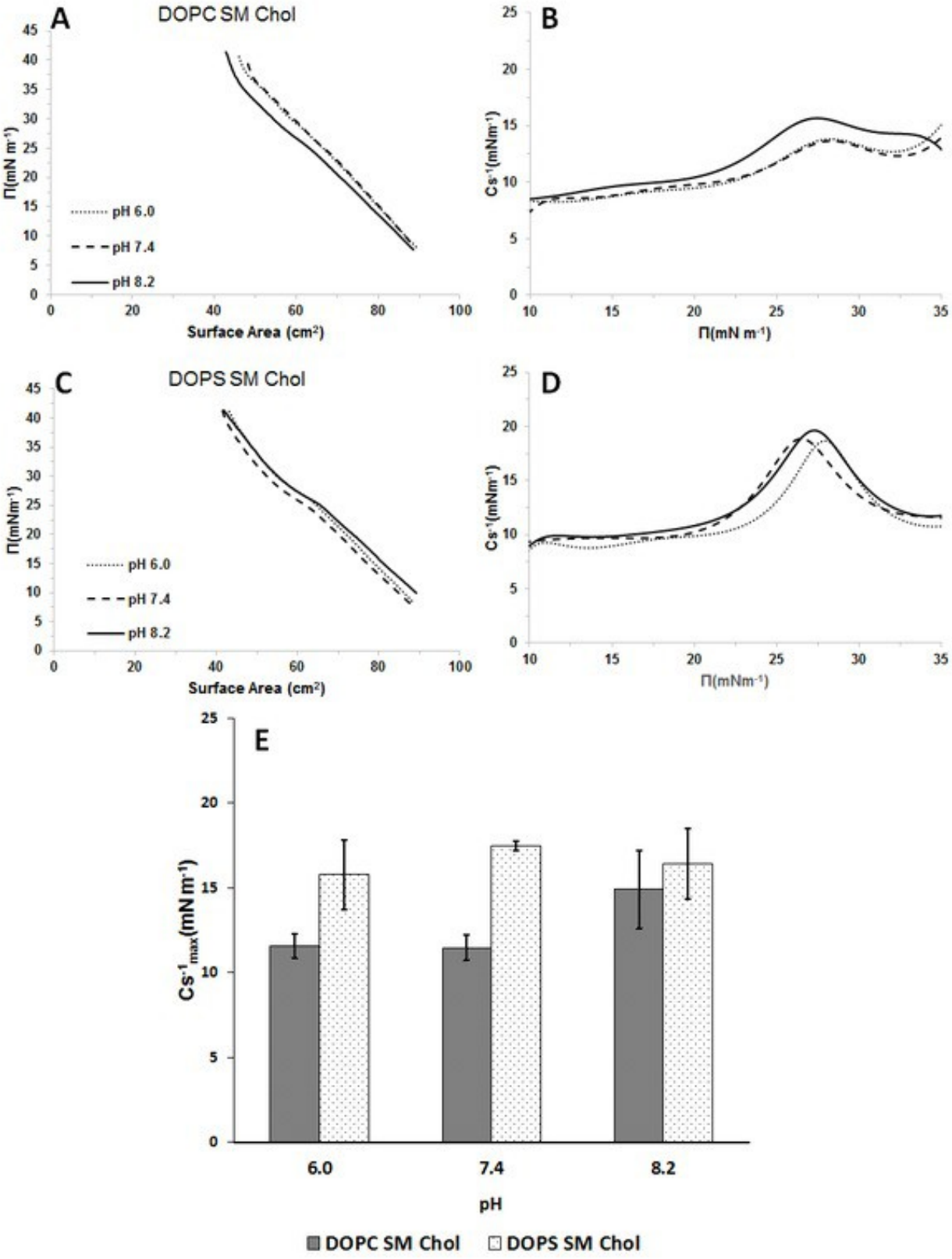

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