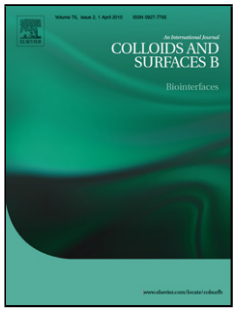

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PHENYLALANINE INTERACTION WITH LIPID MONOLAYERS AT DIFFERENT pHs.

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ABSTRACT

The influence of Phe on the surface pressure of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) monolayers at the air−water interface was studied at different initial surface pressures (26 and 40 mN/m) and two pHs (5.0 and 7.3) at constant temperature (20^{o}C). Changes produced by the aminoacid added to the subphase on the surface pressure and on the dipole potential of lipid monolayers were measured at a fixed area. Compressibility properties of the monolayers at different pHs were studied by (π–A) isotherms. The results suggest that Phe intercalates into a DPPC film at the air−water interface at pH 5 and forms a different arrangement at pH 7.3.

The possible relevance of these results of the effect of Phe in physiological conditions is discussed.

Abbreviations: DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine)-Phe (L-phenylalanine).

Keywords: Lipid monolayers - DPPC - Phenylalanine- surface pressure- dipole potential-pH.

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I. INTRODUCTION

Phe residues have been identified as a key component in the formation of amyloid structures under pathologically relevant concentrations which have similar biophysical and structural properties and are associated with a diverse group of diseases among them Alzheimer's disease, type II diabetes and prion disorders [1-3].

It has been also shown that Phe produces damage in thylakoid membranes at very low concentrations during freezing and that it induces leakage and membrane fusion in liposomes [4]. Apparently, at relatively low concentrations the damage is produced on membranes under stress conditions, i.e. partial dehydration. Therefore, the influence of Phe on the stability of membranes seems to be regulated by the water stress, probably, by positioning of the aromatic ring in the lipid-water interface, more precisely in the head group or glycerol backbone region [5]. In this regard, it is important to notice that after mechanical injuries, phenylalanine ammonium lyase (PAL) activation is followed by the synthesis of protective phenolic compounds to reduce the leakage of water [6].

The interaction of different amino acids with membranes used lipid monolayers as model systems [7-10]. In this type of system, previous studies with Phe were mostly carried out in conditions that differ from the

physiological ones, especially regarding to pH. This fact may be important to take into account when data are used to explain biological relevant processes.

In this context, the aim of this work is to evaluate the Phe interaction with lipid monolayers at pH 5.0 and at pH 7.3.

Monolayers spread at the air-water interphase allow obtaining information about the changes in the lipid packing and water accessibility measuring surface pressure at different areas and about the electrical changes produced in the membrane interface following the dipole potential.

II EXPERIMENTAL DETAILS

Materials.

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Chloroform, KCl and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were of analytical grade. Purity of lipids and buffers were checked by FTIR and UV spectroscopies (see supplementary material). To identify unknown or unexpected components in all chemicals used. selected spectra were compared with available libraries.

Fresh stock solutions of Phe were prepared immediately before each assay in order to avoid the fiber formation [11]. The pH of Phe solution in KCl was 5.0, indicating that the zwitterionic state of Phe would dominate in this condition. Water was of Milli-Q quality obtained in an Osmoion 10.2 equipment.

Surface pressure

Changes in the surface pressure of lipid monolayers upon addition of Phe to the subphase were measured at constant area at 20 ± 0.5 °C) in a Kibron Langmuir-Blodgett trough. A chloroform solution of lipids was spread on the aqueous interface (KCl or HEPES) to reach surface pressures of 26 ± 1 mN/m or 40 ± 1 mN/m. Phe solutions were injected in the subphase and the changes of surface pressure were recorded with time until a constant value was reached. No significant changes were observed in the values of surface pressure when Phe was injected in the absence of lipid or when HEPES was added to the solution.

Surface pressure-area (π-A) isotherms were obtained in the KSV trough (area: 240.00 cm^2) of a Wilhelmy balance provided by a Platinum probe of 39.24 mm^2. The Teflon trough and probes were washed and rinsed with water. The Platinum probe was flamed until glowed red-hot before each assay. The whole equipment was enclosed in an acrylic box to minimize solvent evaporation and to avoid contaminations from the environment during the study.

The trough was filled with the barriers fully open with the appropriate volume checking that the borders of the meniscus were even in the whole perimeter. Then the barriers were moved on the aqueous phase without lipids slowly to obtain a homogeneous surface, this procedure was made three times before adding the lipids

Before each experiment the water surface tension was checked with pure water to 72 mN m^{-1}.

Monolayers were allowed to stabilize during 30 min before measurements.

In these experimental conditions, in order to maintain the reproducibility of the isotherms the same amount of lipids was added to the surface of the subphase containing buffer, KCl or Phe solutions at pH 5.0 (in KCl) and 7.3 (in HEPES)

Compression rate for monolayers were in all the experiments 5 mm.min^{-1}, target pressure was set at 60 mN/m at a temperature of 20 ± 2°C.

The surface pressure (π) and the barriers were controlled by software purchased from NIMA (KSV-NIMA, Finland).

Control experiments in the absence of Phe were also carried out in the same conditions.

The number of determinations was increased in order to improve the reproducibility of the isotherms.

Reported data are the average of three different batches of lipid preparations. In each of them each sample was assayed at least by duplicate and averaged. Errors are reported as standard deviations.

Dipole potential

Dipole potential (Ψ_D) was determined in monolayers formed on an air–water interface by spreading chloroform solutions of lipids on an aqueous surface in the presence and the absence of Phe at both pHs. Different aliquots of lipids were added until a constant surface pressure was achieved [12,13].

The values of the interfacial potential were determined through a circuit of high impedance, connecting a vibrating electrode above the monolayer and a reference Ag/AgCl electrode in the aqueous subphase. The temperature was set at 20 ± 0.5 °C measured with a calibrated thermocouple immersed in the subphase. Therefore, the reference potential is constant at the temperature of measure [14].

Zero of the potential was reached with solutions of 1mM KCl and 10 mM HEPES for measurements at pHs 5.0 and 7.3 respectively.

III. RESULTS AND DISCUSSION

The interaction of Phe with DPPC was studied following the changes in surface pressure ($\Delta\Pi$) produced by Phe injected in the subphase underneath lipid monolayers stabilized on the air-water interface. Significant differences were found when the perturbation ($\Delta\Pi$) was analyzed at different initial surface pressure 26 - 40 mN/m and at different pHs 5.0 and 7.3 (Figure 1).

Data in Figure 1 A and B denote that at 26 mN/m subsequent additions of Phe do not cause significant changes in surface pressure at both pHs.

However, at 40 mN/m a higher increase in surface pressure than that at 26 mN/m at pH 5.0 is observed. Surprisingly, at pH 7.3 in the same degree of compression (40 mN/m) a consistent decrease in the pressure was observed.

The increase in surface pressure indicates a decrease of the excess surface tension of the monolayer after the lipids were spread. In contrast, a decrease in surface pressure denotes an increase in the surface tension, that is, towards values of pure water. This would suggest that in some extent lipids are being condensed in the surface exposing water regions.

In Figure 2, the kinetic profiles of the pressure changes at 40mN/m induced by 11 mM Phe at pH 5.0 or 7.3 is analyzed according to equation (1) [15]:

$$\Delta\Pi = -e^{-kt}\Delta\Pi_{max} + \Delta\Pi_{max} \quad (1)$$

where (k) is the adsorption rate constant. At pH 5.0 the rate of pressure change ($k = 5.4 \pm 0.06\ 10^{-4}\ s^{-1}$) is significantly lower than that observed at pH 7.3 ($k = 8.10\ \pm 0.1\ 10^{-1} s^{-1}$). This means that at pH 5 the insertion of Phe may have kinetic hindrances to reach stabilized positions, which do not exist (or exist at a much lesser extent) at pH 7.3.

The results of Figures 1 and 2 suggest that there is a different behavior of Phe in DPPC as a function of pH. It is well known that Langmuir isotherms allow to characterize phase behavior of a system under study [16,17]. In this context, compression curves were carried out in the presence and the absence of Phe at both pHs (Figure 3A). The comparison of the control curves of DPPC in the absence of Phe at pH 5 and pH 7.3 (full lines in part A) indicates that the buffer does not affect significantly the monolayer properties giving comparable extrapolated area per lipid values of 50.1 ± 1.0 $Å^2$ and 52.0 ± 1.0 $Å^2$, respectively [18]. At both pH conditions, Phe does not affect the initial surface tension of KCl or buffer without lipids.

In the presence of Phe (dotted lines), the profiles of the isotherms indicate that the coexistence of phases observed in DPPC at both pHs almost disappears. In addition, the estimated molecular area per lipid in the presence of 11 mM Phe at pH 5.0 (black line) (70.8 ± 3.7 $Å^2$) is greater than the changes observed at pH 7.3 (grey line) at the same concentration of Phe c.a. 60.6 $Å^2$ (see Table 1). Our results at pH 5 show the same trend as that reported in the literature by Petelska et al. although absolute values cannot be compared because the type of phosphatidylcholines is not reported [19].

The differences in the molecular area per lipid can be ascribed to a different insertion of the aminoacid in the interface. Since, according to the control assays in Figure 3A different pHs without Phe do not affect the surface pressure/area curves, this behavior can be ascribed to different conformation of the Phe molecule in the aqueous phase that modify its size and hydrophobicity according to its charge distribution. It has been reported that Phe conformation is sensible to the number of water molecules at which it may stabilize [20].

The increase in the molecular area per lipid (△Area = 19.9 $Å^2$ at pH 5.0**)** can be due to the insertion of the aromatic ring with a characteristic size and hydrophobicity and, therefore it is expected that this amino acid would influence the molecular packing. The insertion of aromatic rings has been found in other systems such as the analogue of tyrosine, arbutin [21]. Instead, the result at pH 7.3, in which significant lower area change is found, could indicate an interfacial interaction without a significant insertion of Phe. This is congruent with the observation that the kinetics to insert at pH 5.0 is much lower than that at pH 7.3, which would suggest that Phe does not penetrate the interface at pH 7.3.

In Figure 3B the values of the compressibility modulus and its variations as a function of the mean molecular packing areas were analyzed. The compressibility modulus gives a quantitative measure of the state of the monolayer. As it was reported, a defined minimum or abrupt variation of the slope of the curve of compressibility modulus versus area indicates with high sensitivity the occurrence of a change in the physical state of the monolayer, for example the coexistence of expanded-condensed phase transitions in the film [22].

The compressibility modulus was calculated by the following equation 2 [23]

$$C^{-1} = -A \cdot \left(\frac{d\Pi}{dA} \right)_T \qquad (2)$$

Compressibility modulus values in the presence of 11 mM Phe at pH 5.0 are lower than those obtained in its absence, indicating high elasticity in the system. It is possible that Phe inserts in open spaces in the monolayers giving a higher area per lipid and a lower compressibility. This insertion would account for the partial disappearance of the

coexistence in DPPC curves as shown in Figure 3A. At pH 7.3 a decrease on the compressibility modules (Figure 3B) is also observed while the area change is 8.6 $Å^2$. This also can be observed in panels A and B. At 26 mN/m, there are similar area changes at both pH induced by Phe. However, at 40 mN/m, the area change is only observed at pH 5.0 but not at pH 7.3.

The measurements of surface pressure, compressibility and calculated area per molecule suggest that Phe inserts differently at pH 5.0 than at pH 7.3 into DPPC monolayers at 40 mN/m. To support this suggestion the electrical properties were evaluated by measuring the dipole potential. In Figure 4, the change of the dipole potential as a function of the surface pressure is shown. At pH 5.0 the dipole potential of DPPC monolayers decreases in the presence of Phe from 442 ± 10 mV to nearly 286 ± 24 mV, i.e. $\triangle\psi$ = -156 mV (Table 1). This stabilization is reached at 15 mN/m This behavior is in agreement with the area increase observed in Figure 3 A. At pH 7.3, the dipole potential stabilizes at less than 5 mN/m in a higher value in comparison to that of monolayers without Phe opposite to that at pH 5.0. An explanation for this difference could be that Phe can fit in the membrane and oppose to the dipole at the interface more efficiently at pH 5.0 than at pH 7.3. At pH 7.3, the increase in dipole potential is compatible with dipoles organized at the external surface, i.e. without significant insertion (Figure 5).

In resume, at pH 5.0 we found a significant increase in area per lipid, concomitant with an increase on the surface pressure and a decrease on the dipole potential. A simple explanation of this result could be the formation of a complex between Phe and DPPC as previously proposed by Petelska et al. [20]. In this complex, the dipole of Phe opposes that of the lipid monolayer. As changes in the dipole potential may involve water dipoles reorganization it is likely that this complex may be formed at

expense of water removal [24,25]. At pH 7.3 no significant change of area were observed. In addition, the surface pressure decrease in condensed monolayer is accompanied by an increase of the dipole potential. How these results may be explained?. At pH 7.3, the dipole potential of DPPC is significantly lower than at pH 5, denoting that some dipoles are flat with respect to the plane or less water is polarized. Simon and McIntosh [26] have discussed about the contribution of the oriented dipoles in the head group region and other molecules to the dipole potential and also reported that the magnitude of the hydration pressure depends on the size of the dipole potential. This analysis allows us to explain our results with regard to the increase of the dipole potential and the decrease in surface pressure observed at pH 7.3. Besides the electric field produced by the dipoles could polarize the interface water yielding a different distribution of lipids around Phe. This means that the orientation of dipoles opposing normal to the surface favors the Phe interaction. In this condition the decrease in surface pressure induced by Phe could be explained by a direct interaction of the lipid head groups with amino acid, condensing some lipids in the surface, which would promote the formation of lipid free spaces. This would be congruent with the increase in surface tension at the air-water interface.

IV. CONCLUSIONS

Data in literature are reported at pH 5.0 and usually these results are extrapolated to physiological conditions. However, according to our results, the interactions of Phe at pH 7.3 seem to follow a completely different trend.

At pH 5.0, Phe would be inserted into the membrane increasing the distance between the lipid molecules, reducing the degree of order of the dipoles in the membrane and thereby creating a lower total dipole.

However, the opposite effect observed at pH 7.3, could be due to the interaction of Phe with phospholipids at the interface regions, generating a reconfiguration of the lipid arrangement with areas of higher lipid packing. This new arrangement in the monolayer causes the existence of a higher orientation of dipoles of lipid and water molecules contributing to a higher overall dipole moment.

Concluding remarks: At pH 5.0 Phe perturbs the structure of the monolayer forming a complex Phe-DPPC which is not found at pH 7.3. At pH 7.3 Phe may organize at the surface of the lipid arrangements suggesting a film adsorbed on the lipids rather than an insertion.

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TABLE I

Effect of Phe 11 mM on the dipole potential and areas of DPPC monolayers (40 mN/m) at different pHs

Lipid	Sub phase	pH	ψ (mV)	△ψ (mV)	Area/lipid (Å^2)	△Area (Å^2)	Δπ (mN/m)
DPPC	KCl	5	442 ± 10		50.1 ± 1.0		
DPPC	KCl/ Phe	5	286 ± 24	-156	70.8 ± 3.7	+19.9	4.3 ± 0.6
DPPC	HEPES 10 mM	7.3	152 ± 15		52.0 ± 1.0		
DPPC	HEPES 10 mM/ Phe	7.3	422 ± 13	+270	60.6 ± 0.6	+8.6	-11.8 ± 0.1

Errors are reported as standard deviations of three different batches of lipid preparations and averaged.

LEGENDS TO THE FIGURES.

FIGURE 1

A.- Perturbation of the surface pressure by the addition of increasing Phe concentration at the subphase at pH 5. Π^o = 40 mN/m (■), Π^o = 26 mN/m (●).

B.- Perturbation of the surface pressure by the addition of increasing Phe concentration at the subphase at pH 7.3. Π^o = 40 mN/m (■), Π^o = 26 mN/m (●). Temperature: 20 ^{o}C.

Errors are reported as standard deviations of three different batches of lipid preparations and averaged.

FIGURE 2

Variation of the surface pressure as a function of time after Phe injection to reach a final concentration of 1mM in DPPC monolayer spread on KCl pH 5.0 (A) o HEPEs 7.3 (B). Dashed lines (--) correspond to the fitting of data with the equation 1. The initial surface pressure was 40mN/m for both assays.

FIGURE 3

(A) Surface pressure /area per lipid isotherms of DPPC monolayers on KCl 1mM pH 5 (black full line) or HEPES 10 mM, pH 7.3 (grey lines grey) in the absence and at pH: 5 (dash black line) or pH: 7.3 (dash grey line) in the presence of Phe 11 mM.

B) Curves of inverse compressibility modulus/molecular area in KCl 1mM pH 5.0 with (dashed line) and without (full line) Phe.

C) Inverse compressibility of DPPC in HEPES 10 mM, pH 7.3 in the absence (full line) and the presence of Phe 11mM (dash line).

FIGURE 4.

Changes of dipole potential produced by 11mM Phe added to the subphase of KCl 1mM at pH 5.0 (•) and HEPES 10mM at pH 7.3 (◆) Errors are reported as standard deviation of three different batches of lipid preparations and averaged.

FIGURE 5

A) Scheme of the proposed location Phe in the lipid monolayer at the two different pHs studied.

B) Molecular area shift of the two Phe-DPPC arrangements.

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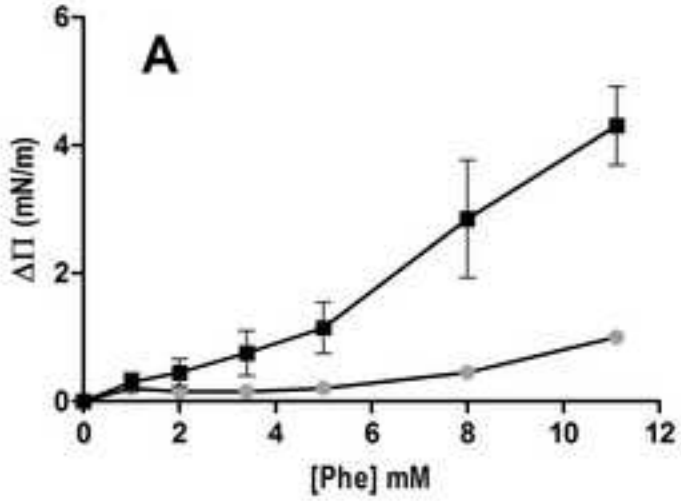
HIGHLIGHTS.

Phe inserts in DPPC monolayers at pH 5
Phe forms a complex with PC molecules.
Phe form fils on the monolayer surface at pH 7

Figure 1



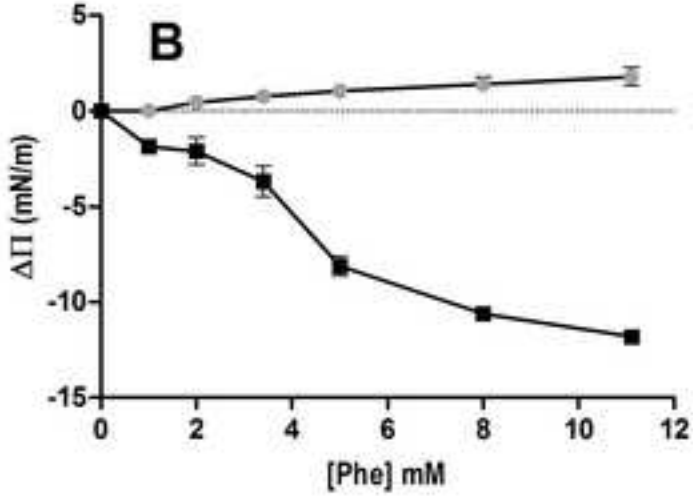


Figure 2

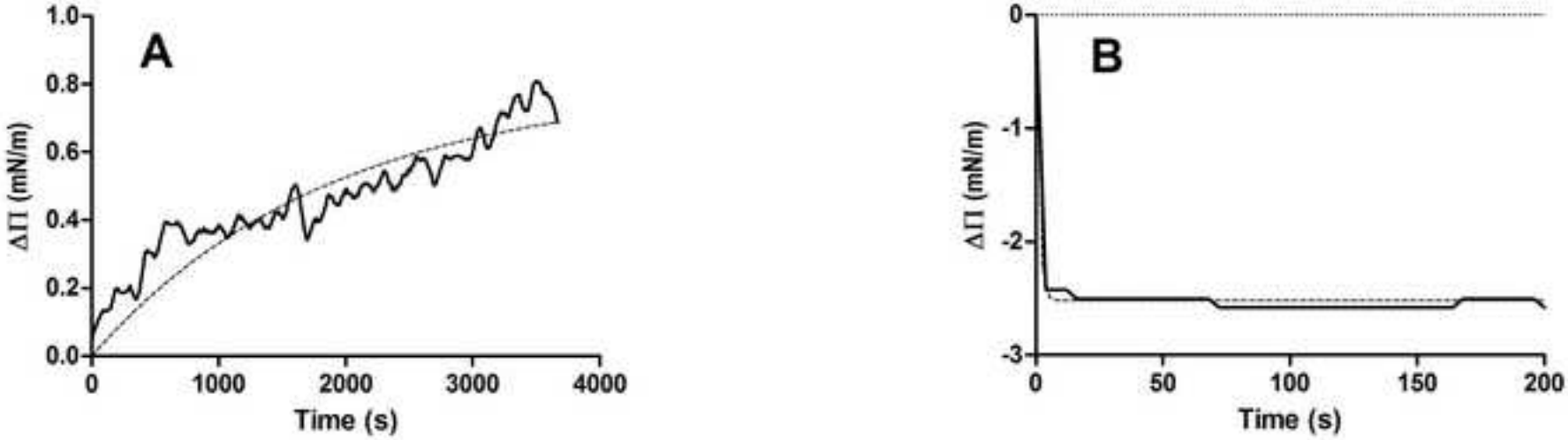


Figure 4

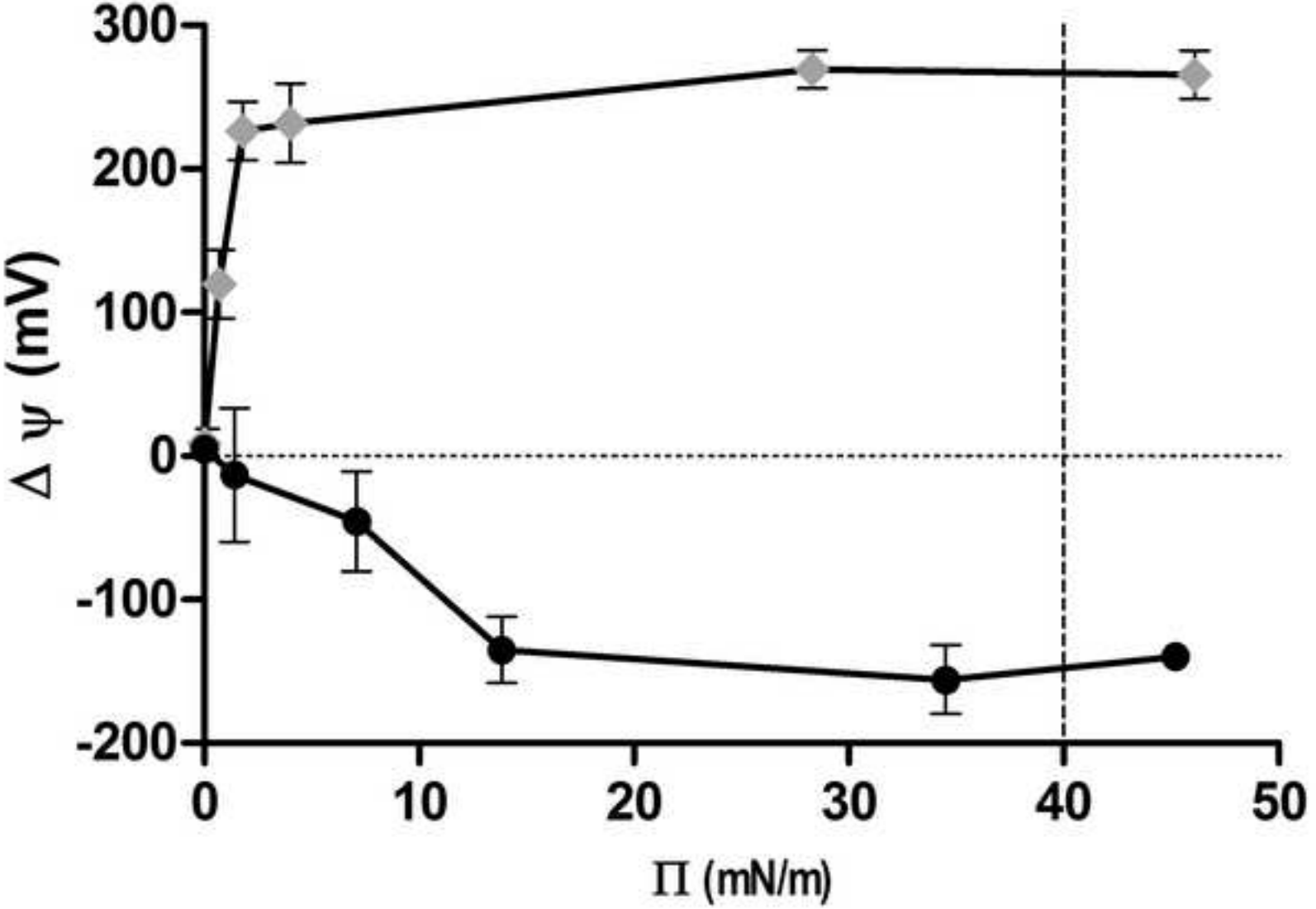


Figure 3A

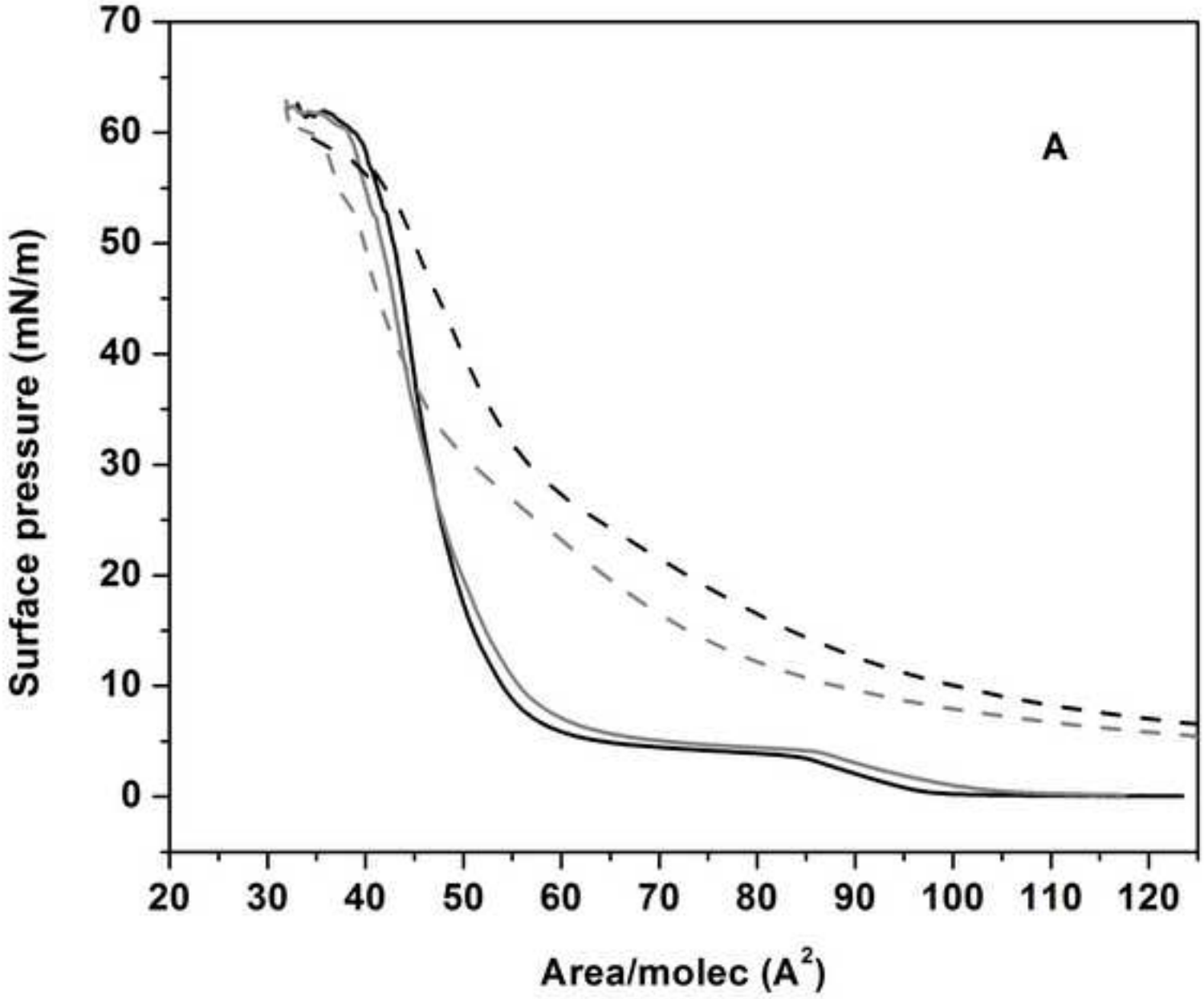

A
Surface pressure (mN/m)
Area/molec (A^2)
0
10
20
30
40
50
60
70
20
30
40
50
60
70
80
90
100
110
120

Figure 5

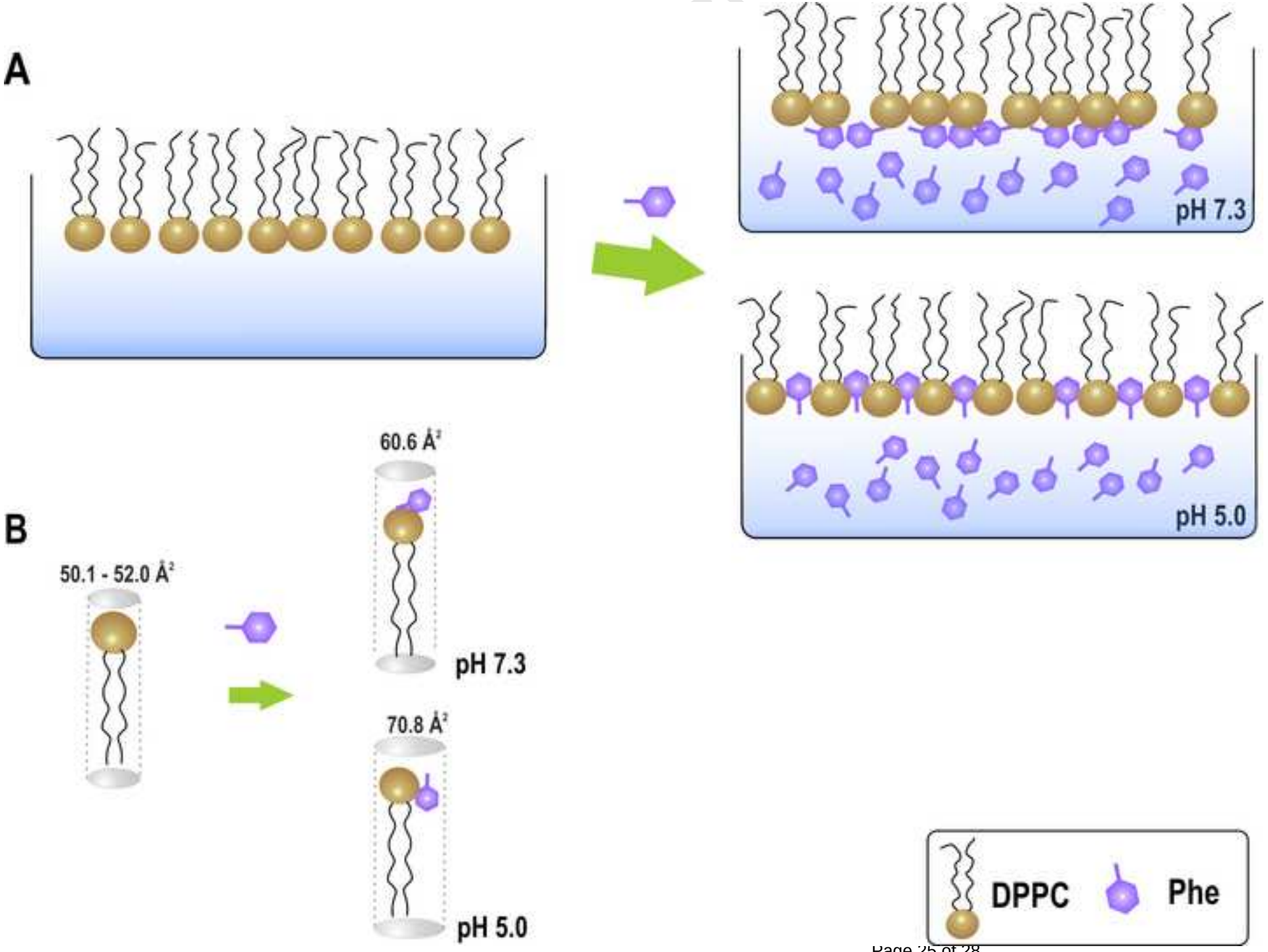


Figure 3B

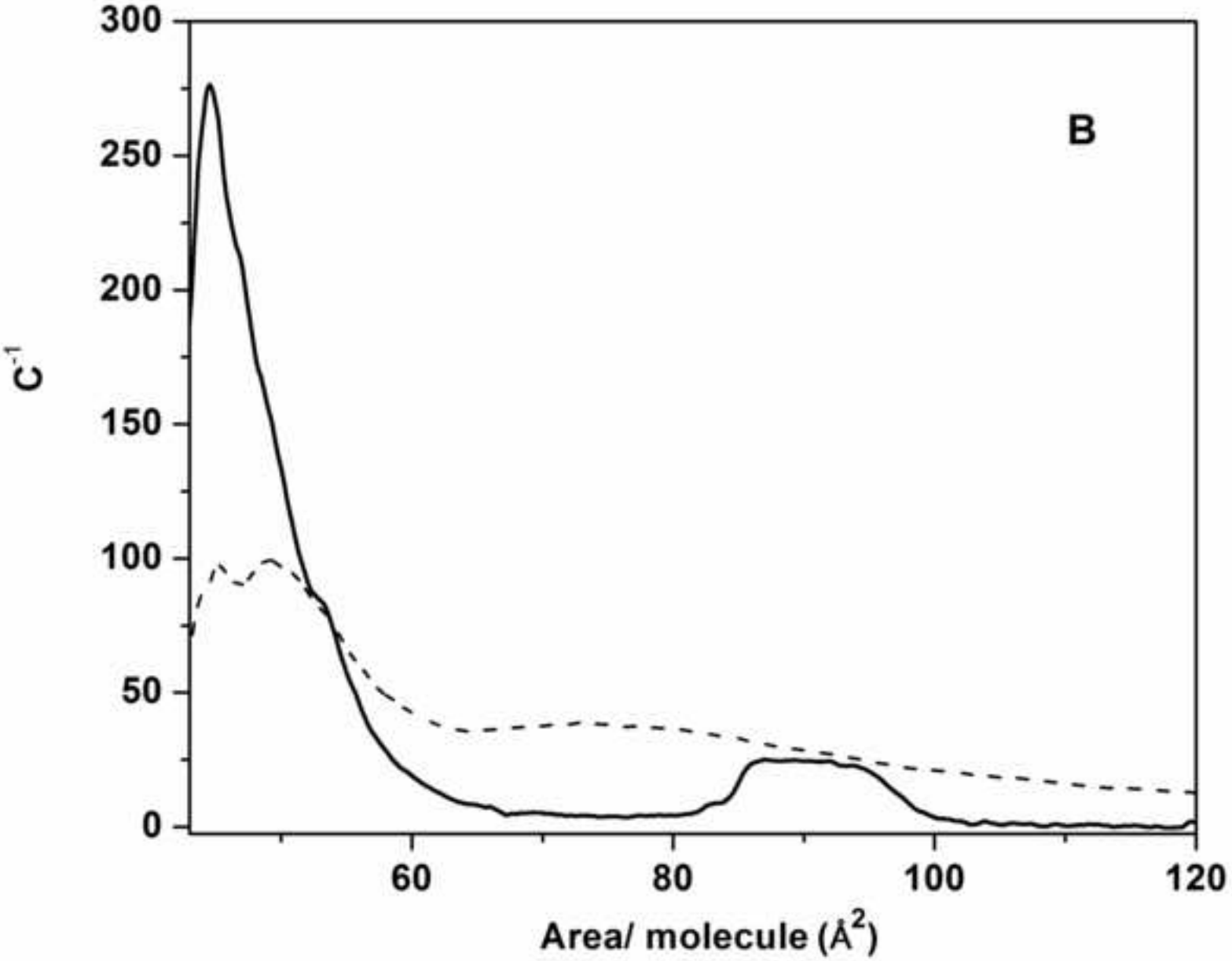
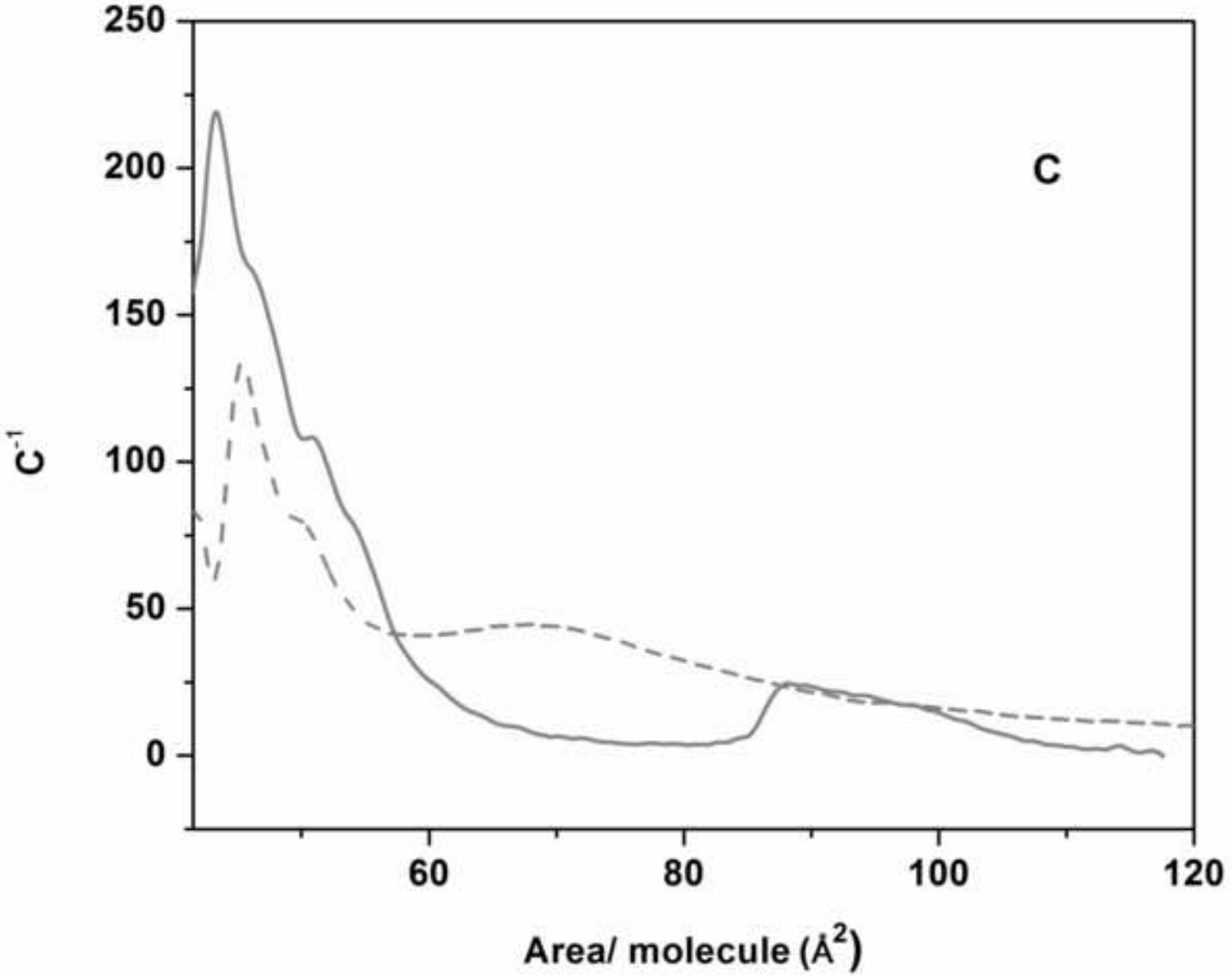


Figure 3C



Graphical Abstract (for review)