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Polymer-lipid interactions: biomimetic self-assembly behaviour and surface properties of poly(styrene-alt-maleic acid) with diacylphosphatidylcholines

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ABSTRACT

Various lubricating body fluids at tissue interfaces are composed mainly of combinations of phospholipids and amphipathic apoproteins. The challenge in producing synthetic replacements for them is not replacing the phospholipid, which is readily available in synthetic form, but replacing the apoprotein component, more specifically, its unique biophysical properties rather than its chemistry. The potential of amphiphilic reactive hypercoiling behaviour of poly(styrene-alt-maleic acid) (PSMA) was studied in combination with two diacylphosphatidylcholines (PC) of different chain lengths in aqueous solution. The surface properties of the mixtures were characterized by conventional Langmuir–Wilhelmy balance (surface pressure under compression) and the du Noüy tensiometer (surface tension of the non-compressed mixtures). Surface tension values and ^{31}P-NMR demonstrated that self-assembly of polymer-phospholipid mixtures were pH and concentration-dependent. Finally, the particle size and zeta potential measurements of this self-assembly showed that it can form negatively charged nanosized structures that might find use as drug or lipids release systems on interfaces such as the tear film or lung interfacial layers. The structural reorganization was sensitive to the alkyl chain length of the PC.

Keywords: biomimetic, phosphorylcholine, surface properties, self-assembly, amphiphiles

1.- INTRODUCTION

There is an increasing interest in mimicking the molecular behaviour of natural systems. One important target is the spreading and lubrication behaviour of biological fluids such as tears, pulmonary surfactants and synovial fluid. These fluids exhibit some remarkable similarities in composition in that they all contain a phospholipid, dipalmitoyl phosphatidylcholine (DPPC), in combination with amphipathic apoproteins, such as SP-B and SP-C. The challenge in producing replacements for these fluids in treating lubricity deficiency diseases such as dry eye, respiratory distress syndrome (RDS) and arthritis is not replacing the phospholipid, which is readily available in synthetic form, but in replacing the apoprotein component. In this paper we investigate the self-assembly behaviour and surface properties of the poly(styrene-alt-maleic acid) – diacylphosphatidylcholine system that was initially identified in these laboratories as a potential reactive functional substitute [1,2].

The native lung surfactant consisting mainly of DPPC and the apoproteins SP-B and SP-C has been shown to greatly accelerate the kinetics of adsorption, decrease the surface tension upon compression to nearly zero, and induce good respreadability [3,4]. However, due to the relative scarcity, as well as hydrophobic and surface active nature of these proteins, it is difficult and costly to isolate them from natural sources in high purity [5]. For example, the complex structure of SP-B makes it challenging to synthesize chemically and then obtain a properly folded form. For that reason, analogues have been developed to mimic its biophysical properties instead of its chemistry. An effective lung surfactant must have at least three fundamental biophysical properties: (1) rapid adsorption to the air-water interface, (2) the ability to reach near-zero surface tension upon film compression, and (3) the ability to re-spread upon multiple compressions and expansions of surface area with minimal loss of surfactant into the subphase [6]. Non-natural analogues of SP-B such as amphiphilic peptides and peptoids have been previously studied [7,8]. However, since peptides are relatively expensive to synthesize and purify, there is great interest in developing synthetic analogs as protein mimics for therapeutic purposes [9,10].

A similar interfacial role has been identified in the tear film, although there the physiological role of the lipoidal component differs from that in the lung. The tear film has a coating of phospholipids, which are necessary for the formation of a stable tear film. In this regard, they must be clear and colourless, unlike conventional aqueous preparations of phospholipids that may be opaque.

Responsive hydrophobically associating polymers or hypercoiling polymers, can in many ways be considered to be analogous to apoproteins in their ability to form compact molecules with a defined secondary structure, and hence, functionality. These molecules are characterized by the presence of alternating charged and hydrophobic groups. The balance between charge repulsion and hydrophobic interactions is sensitive to environmental pH and therefore changes in pH above or below their pKa, produce controllable conformational changes and increased functionality. The change from a charged extended chain to a collapsed uncharged coil structure is sometimes referred to as "hypercoiling" behaviour and enables the polymer to act as a simple switch between an 'on' and 'off' state [2] in a reactive way. Copolymers of maleic acid and styrene have this behaviour. The potential of PSMA as a polymeric drug and its conjugation with several drugs has already been exploited with clinical success to treat several types of cancer [11,12], to inhibit human immunodeficiency virus type 1 (HIV-1) [13,14], and it has also been reported to be strong inhibitor of spermatozoa motility [15], among others. It represents a promising raw material due to its low cost and toxicity.

Phosphatidylcholines (PC) are considered as zwitterionic surfactant molecules due to the existence of two charged groups with different sizes [16]. Phosphatidylcholines can spontaneously organize into bilayers due to their cylindrical shapes [17]. Due to their amphiphilic nature, they are also called as membrane-forming lipids with the polar head group aligned towards the aqueous phase and the hydrophobic tail groups forming the hydrophobic core. The so-called hydrophobic effect is believed to be the main driving force in self-association [18]. They way in which they form the bilayers, their solubilities and their stability, depend strongly on their chain length and self-assembly properties [19,20].

PC-containing polymers have been widely used in various biomedical applications including contact lenses coatings, drug delivery systems, biochip-based diagnosis systems, blood-contacting medical devices, and tissue engineering devices [21-24]. When functional hypercoiling polymers are combined with film-forming lipids they associate to produce lipid–polymer nanostructures analogous to naturally occurring lipoprotein assemblies in the form of flattened disk-like molecular assemblies. The reactive pH-dependent complexation of anionic polyelectrolytes with phospholipid vesicles has been studied before [25]. Such behaviour was explained in terms of hydrogen bonding between the charged carboxylic acid pendant groups of the polymer and the phosphodiester head groups of the phospholipid.

Lowering of the pH caused a loss of charge and predominant hydrophobic interactions. Alternating copolymers of styrene and maleic acid (i.e. hydrolysed styrene/maleic anhydride polymers) have a pKa value in the region of 3.75-4.0, [26] the pKa for the individual acid functions being approximately 1.97 and 6.24. In this case, the amphipathic segments surround the phospholipid bilayer in a 'doughnut' arrangement [2,27]. As previously mentioned, PSMA-phospholipid complexes could be used in several applications related to medical conditions affecting mucosal surfaces. In addition, they could be used as biomimetic analogues for the tears, due to their transparency once they are formed. Preparation of clear solutions requires a lowering of the pH to 4-5. Upon decreasing the pH, the hydrophobic microdomains of PSMA disrupt the vesicle membranes by associating with the lipoidal core of the bilayer, resulting in a membrane reorganization and formation of small discoidal complexes. This results in the formation of optically clear, aqueous suspensions.

Although these complexes can be formed with DPPC it is necessary to maintain the mixture at an elevated temperature above the main phase transition temperature (melting point, Tm) of DPPC of around 42 ^{o}C. e.g. at about 50 ^{o}C, during the process. For the wider range of applications envisaged here, it is logical to examine systems that can be formed at room temperature which logically leads to the exploration of DMPC (1,2-dimyristoyl-sn-glycero-3-phosphocholine) and DLPC complexes (1,2-dilauroyl-sn-glycero-3-phosphocholine), with 14 and 12 carbon atoms (Tm 23 ^{o}C and −2 ^{o}C), respectively, so that they are in a disordered bilayer or liquid crystalline phase at room temperature.

The use of different analytical techniques to explore the association between anionic polyelectrolytes and lipid vesicles has long been investigated by several researchers using different approaches (e.g. fluorescence spectroscopy, differential scanning calorimetry, neutron scattering and FTIR [27,28]. In this study the Langmuir–Wilhelmy balance combined with the du Noüy tensiometer can analyse monolayers as simplified models since various parameters like density, packing, nature of lipids as well as the subphase composition, pH and temperature can be varied in a well-defined manner. For this reason, these techniques may provide information regarding conformational transitions that play a crucial role in the development of vesicular biomimetic systems [29-31]. Finally, the ability to form stable polymer-phospholipid complex nanoparticles, were analysed by means of sizes distribution, surface charges and morphologies.

2.- MATERIALS AND PHYSICO-CHEMICAL CHARACTERIZATION

2.1. Materials

Dilauroyl phosphorylcholine (DLPC, ≥99%, Mw 622 g/mol), was purchased from Sigma-Aldrich (Gillingham, UK), and dimyristoyl-phosphorylcholine (DMPC, ≥99%, Mw 678 g/mol) from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA). Styrene-maleic anhydride copolymer (50:50, 1600 MW), was purchased from MP-monomer polymer & Dajac Labs. All the solvents (HPLC grade) and the phthalate buffer tablets were from Fisher Scientific (Loughborough, UK). First, styrene-maleic acid copolymer was obtained as alternative copolymer of styrene and maleic anhydride by hydrolysis in water at basic pH adjusted by 1 M NaOH and was kept at 80 ^{o}C for 6 h. After hydrolysis, PSMA became soluble in weak acid.

2.2. Physico-chemical characterization

The Langmuir–Wilhelmy balance (NIMA Tech. Ltd., Coventry, UK) had a subphase area of 30x20cm and a maximum monolayer containment area of 535cm^2. It was equipped with an IU4 computer interface unit and operating software version 7.8. A stock solution of styrene maleic acid 1.9 mM (30 mg/ml) was prepared and stored at room temperature (20 ^{o}C). Stock solutions of the phospholipids were prepared in chloroform in the concentration of 2·mM (1.2-1.5mg/ml), and were sealed and stored at room temperature.

The Langmuir–Wilhelmy balance, which was kept inside a cabinet, was equipped with two barriers that moved simultaneously towards the centre of the trough, which allowed for symmetric compression of the film. It was fabricated in poly(tetrafluoroethylene) (PTFE), a material that avoids contamination of the surface. The surface pressure (π), varied as the area between the barriers was changed, and was measured by means of the Wilhelmy plate method where continuous surface pressure measurements are obtained by a transducer interfaced with the software. The performed experimental procedure consisted basically of the following steps: (1) Cleaning the trough and barriers with HPLC grade chloroform and HPLC ultra-pure water. (2) Addition of the subphase (HPLC ultra-pure water), the subphase temperature was kept constant and equal to 20 ^{o}C by means of an external temperature controller unit. (3) Attaching the Wilhelmy plate (strip of Whatman's Chromatography paper) connected to an electrobalance. (4) Spreading, drop by drop, of the phospholipid solution. (5) Evaporating the solvent for 10 min. (6) Compressing the film at 100 cm^2/min and recording

the π versus area data. PSMA studies followed an equivalent procedure but, the PSMA solution was spread over the water subphase. In the experiments examining the effect of PSMA on the molecular packing of phospholipids monolayers, the PSMA solution was injected beneath the pre-forming phospholipid monolayer. Spreading solutions were deposited onto the aqueous subphase with a Hamilton microsyringe. The pH-dependent self-association of the polymer and pH-dependent complexation of PSMA with phospholipids were studied with phthalate buffer of pH 4 as subphase.

To measure the surface tension of the polymer-lipid complexes the du Noüy tensiometer with Pt-Ir ring by the ring detachment technique was used. This method utilizes the interaction of a platinum ring with the liquid interface being tested. The ring is submerged below the interface and subsequently raised upwards. As the ring moves upwards it raises the meniscus of the liquid. This meniscus eventually tears from the ring and returns to its original position. Prior to this event, the volume of the meniscus, and thus, the force exerted, passes through a maximum value and begins to diminish prior to the actual tearing event. The calculation of the surface or interfacial tension by this technique is based on the measurement of this maximum force.

The ^{31}P-NMR spectra of the phospholipids and complexes were acquired at 121.5 MHz on Bruker Advance 300 Spectrometer. XWIN NMR Version 3.5 software was used to process the spectra. The spectrometer field was locked during acquisition by using D_2O. The proton-decoupled spectra were obtained using a relaxation delay of 4 s, spectral width of 18,248 Hz and acquisition time of 1.8 s. The number of acquisitions was 400 scans. All spectra were recorded at room temperature and the chemical shifts were measured relative to H_3PO_4 (85%) as an external reference. The water used throughout the experiment was deuterated water. The pH of solution was adjusted to the required value by using small amounts of NaOH or HCl solutions.

The particle size distribution, polydispersity and ζ potential of the polymer-phospholipid nanoparticles dispersions were measured by light scattering in a Zetaplus equipped with a He-Ne laser operating at a wavelength of 633nm. (BTC Brookhaven Instr. Corp.). Particle size was determined from the analysis of intensity fluctuations of scattered light from a suspension of particles undergoing brownian motion.

The morphology was assessed by cryo-SEM (JEOL 2100F equipped with a Gatan Orius camera, Gatan Tridiem Filter, and JEOL Digital STEM system). Briefly, samples were

deposited onto graphene oxide on lacey amorphous carbon grids (EM Resolutions). Samples were frozen using a Gatan CP3 Cryoplunge into liquid ethane and transferred under cryogenic conditions to a Gatan 914 Cryo tomography holder.

3.- RESULTS AND DISCUSSION

3.1. Surface pressure-Area isotherms

The advantage of using the Langmuir–Wilhelmy balance technique is that the measurement of the surface pressure as a function of surface area (π-A isotherms) can be conveniently carried out and analysed in various environmental conditions such as pH, type of subphase, deposition conditions, and temperature. Langmuir–Wilhelmy balance isotherms of the polymer-phospholipid mixtures are expected to exhibit a higher film collapse pressure (lower surface tension) that the phospholipids by themselves. Figure 1, shows the overlay of area-pressure isotherms (compression towards left-side, expansion towards right-side) on pH 4 buffer subphase for the monolayers of pure phospholipids (solid), pure PSMA (solid-dashed) and phospholipids in the presence of PSMA (dashed) performed in the Langmuir–Wilhelmy balance. The results showed that the surface pressure expansion-compression cycle of the phospholipids changed in the presence of PSMA, the maximum surface pressure increased, showing hysteresis in the form of change in the shape and in the maximum surface pressure reached.

(PLEASE INSERT FIGURE 1)

Polar head groups of pure phospholipids maintain their zero net charge at pH 4. When compressed, the surface pressure of the phospholipid monolayer increased, likely to be due to charge shielding effect, or lipid molecules strongly adsorbed to the phthalate, being desorbed from the interface. The collapse pressure increased as the chain length of the phospholipid increased (see in figures 1a and 1b, solid lines), which could reflect more pronounced repulsive interactions with increasing chain length. The absence of a phase transition between liquid-expanded (LE) and liquid-condensed (LC) states in DLPC and DMPC, confirmed that these phospholipids existed in the LE expanded state. This preference may be explained by the acyl chain length of DLPC (12 C) and DMPC (14 C), not being long enough in the measured concentration, to generate a strong hydrophobic interaction, as is required in the liquid-condensed state.

To analyse the monolayer behaviour of the PSMA, it is necessary to remember that it is an amphiphilic molecule. When spread on aqueous subphase, PSMA molecules arrange themselves with their apolar moieties (phenyl groups) oriented towards the air phase and their polar moieties (carboxyl groups) immersed in the subphase. The PSMA did not have a detectable surface pressure in water (data not shown), suggesting that it was not capable of forming the insoluble monolayer on the water subphase. This was perhaps related to the fact that it was mostly ionized in pure water and likely to dissolve in the bulk of the subphase than to spread on the air-water interface. A possible association of PSMA molecules in water was postulated by Garnier and coworkers [32], who proposed a zipper-like association mechanism. They further proposed equilibrium of the SMA polymer chains between the solution and the air-water interface. Interestingly, when the acidic buffer was used as subphase, the PSMA showed an isotherm (see figure 1 solid-dashed line). At this pH the ionization of the polymer was reduced due to the protonation of the carboxyl groups, which increased its hydrophobicity, thus leading the polymer to form an insoluble monolayer at the interface.

The incorporation of PSMA into the phospholipid monolayer was pronounced at pH 4 (see dashed lines in figure 1). This fact led the PSMA to complex with lipids through hydrogen bonding and hydrophobic interactions. In agreement with previous studies [2,33-36], the results obtained herein demonstrate a pH-dependent association of PSMA and phosphatidylcholines. The collapse pressure values were higher than those of the pure phospholipids, suggesting that the incorporation of PSMA raised the intermolecular interaction at the interface upon compression. The surface pressure values on the uncompressed state were also high (reduced surface tension, high surface activity), which means that the phospholipid monolayer at the interface may enhance the affinity of PSMA molecules to adsorb at the interface, possibly by associating the phenyl groups with their hydrophobic tails.

In the same way, previous studies in our group performed with the pulsatile bubble surfactometer showed surface tension changes in dynamic conditions upon compression-expansion cycles, which revealed unique surface activity of these systems [37]. The common basis for this behaviour of membrane proteins is found in amphipathic alpha helical structures. In the case of hypercoiling polymers it is a matter of balancing hydrophilic and hydrophobic groups, controlling their sequence distribution and molecular weight. An

optimal polymer conformation only occurred with a near-perfect alternation of monomer units, and this was the critical factor determining the ability of the copolymer to adopt a surface-active conformation, maximizing hydrogen and π-π bonding to form an amphipathic polymer coil analogous to that adopted by native solubilizing apoproteins and phospholipids [38]. Assemblies of alternating PSMA and phospholipids exhibited a low surface tension under conditions of dynamic surface compression, approaching those observed with commercially available artificial lung surfactants, the most surface-active natural products known, but at a fraction of the cost of such animal-derived products.

3.2. Surface Tension of PSMA in the presence of phospholipids

There have been numerous publications where surface tension techniques were used to investigate polymer-surfactant association. Taylor et al [39] proposed a plot of surface tension variation with the surfactant concentration, where a monotonous decrease with increasing surfactant concentration was observed, at the CMC (critical micelle concentration), the surface tension abruptly levels off and becomes essentially constant. On addition of polymer, on the other hand, a completely different behaviour is observed: At low surfactant concentration the surfactant starts to form micelles on the polymer. This is the critical aggregation concentration (CAC) for the system. After that, at high surfactant concentration, the CMC is reached.

In this work, the self-association in solution and adsorption behaviour at the air-water interface of PSMA with no compression was studied. The association behaviour of the polymer with the phospholipids was also investigated under acidic conditions.

(PLEASE INSERT FIGURE 2)

The effect of the pH on the surface tension profiles for the PSMA is compared in figure 2. It is worth mentioning that the pH affected the surface activity of the polymer, which increased as the pH decreased, giving a series of pH dependent surface activities: at pH 4 > at pH 6 > at pH 12. This means that non-ionized “neutral” chain segments are surface active, at previously shown for other polymers with carboxylic groups such as poly(acrylic acid) (PAA) [40]. However, PSMA shows a much bigger decrease in surface tension with the pH than PAA at low concentrations, showing a bigger sensitivity to pH due to its hypercoiling properties. The

surface tension decreases as the coiling of the polymer increases. The protonation of the free carboxylic groups of PSMA is known to trigger this transition from an expanded conformation at high pH to a relatively hydrophobic globular coil in acidic solution.

Overall, the decrease in surface tension observed at acid pH was maintained over the increase in concentration. At high concentrations the polymer is no longer properly spread and oriented on the water surface and it is being absorbed in the bulk, which means that the surface properties are no longer sensitive to changes in concentration. Led by these results, additional surface tension measurements were performed for low polymer concentrations as a mean to clarify the adsorption behaviour at pH 4. They were compared with the values obtained from the mixture of PSMA with increasing amounts of DMPC. It is known that the equilibrium surface tensions of phosphatidylcholines are independent of pH [41]. However, it is also known that protein-lipid interactions at air-liquid interfaces are dependent on electrostatic charges, cations, anions, distribution of protons, and surface potential, which are influenced by pH changes [42].

(PLEASE INSERT FIGURE 3)

Figure 3 shows the surface tension at different pH values of PSMA 0.2 wt.% and PSMA-DMPC at the same PSMA concentration and relative ratio polymer to phospholipid of 3 to 1 in weight. The hypercoiling behaviour of PSMA can be observed as a decrease in the surface tension, starting at neutral pH (near the first pKa value of maleic acid, see crossing lines) and moving down to acid values (near the actual pKa of the alternating copolymer). As the pH decreased, the acid groups of the maleic blocks that were exposed to the surface were influenced by the hydrophobic hypercoiling styrene domains of the copolymer, leading to a decrease in the overall surface tension. The influence of the DMPC in the overall surface tension of the polymer can be observed as an increase compared to the pure PSMA, due to a higher surface tension of the phospholipid, not as strongly influenced by the pH as the reactive PSMA. In addition, the presence of the lipid, decreases the total amount of polymer exposed to the surface, decreasing its influence in the surface tension measured there.

The surface tension of the DMPC and the polymer-DMPC mixture at pH 4 against logarithmic concentrations of the polymer are shown in Figure 4. It can be observed in the figure that the surface tension of PSMA began to decrease at polymer concentration of 10^{-3} wt.% and remained constant at concentrations above 0.2 wt.%. This finding demonstrates that the polymer started to adsorb and saturated the interface at concentrations of 10^{-3} wt.% and 0.2 wt.%, respectively, showing a great affinity to the surface rather than undergoing self-

association in the bulk of solution. Garnier et al [32] proposed that PSMA adsorbs at the interface as a 2-D random coil and that its surface coverage is inversely proportional to the aggregation size in solution. The saturation concentration at the interface is not always correlated to the CAC in solution, since the self-association of PSMA in solution is energetically favoured over adsorption at the interface. The surface behaviour of phospholipid DMPC solutions are shown in figure 4, as well (solid squares). From the figure the estimated CMC at pH 4 for DMPC was around 2 wt.%. It can be observed in the inset the influence of increasing amounts of DMPC on the surface tension of PSMA 0.2% (solid triangle with arrow is the starting point for the inset, with no DMPC). As the amount of DMPC increased the surface tension of the polymer increased, moving closer to the values of the pure phospholipid, the highest values observed for the PSMA-DMPC mixtures were observed at DMPC concentrations between 0.1 and 1 wt.%. It must be taken into account that these are uncompressed systems. Under compression, the surface tension would decrease dramatically (surface pressure dramatically increase, see figure 1). In the same way, when compressing phospholipid monolayers, they orientate on the surface showing an increase in the surface pressure up to the surface pressure of water, which translates in a decrease in the surface tension close to zero.

(PLEASE INSERT FIGURE 4)

The surface behaviour of phospholipids solutions at pH 4 as a function of their concentration is shown in figure 5 as well (solid circles and squares, respectively). From the figure the estimated CMC at pH 4 for DLPC was around 0.04 wt.%, and around 2 wt.% for DMPC. The effect of the addition of phospholipids on the surface tension behaviour of PSMA 0.2 wt.% may also be observed in the figure (white circles and squares, respectively).

(PLEASE INSERT FIGURE 5)

The initial surface tension plateau was observed at low lipid concentration region (10^{-5}-10^{-3} wt.% for DLCP and 10^{-5} to 10^{-1} for DMPC), suggesting that within this region the additional phospholipid molecules did not significantly adsorb at the interface. The surface tensions of the pure phospholipids were higher than those of their mixtures with PSMA at very low phospholipid concentration, which means that the main driver of the surface activity at this point was the polymer. At polymer-phospholipids mixtures concentrations of 0.01 wt.%, the surface tension of DLPC-PSMA began to drop, while the DMPC-PSMA began to rise. The

drop in surface tension of DLPC-PSMA was related to the beginning of the formation of surface-active polymer-lipid complexes at the interface. A further addition of lipid resulted in a formation of non-surface active polymer-micelle aggregates, were the lipids may adsorb in the form of micelles [43]. The final slight decrease and plateau in surface tension at high DLPC concentrations suggested that any further lipid added to the solution was not bound to the polymer and therefore lowered the surface tension down to ~25-30 mN/m, where the surface tension for the mixtures was almost similar to that for the pure lipid at the same lipid concentration. The small rise of surface tension of DMPC-PSMA mixtures has been shown in figure 4, as the amount of DMPC increased the surface tension of the polymer increased moving closer to the values of the pure phospholipid, however due to the presence of the PSMA in the mixture, the surface tension of the mixture never reached that of the pure DMPC. Once the surface tension of the pure DMPC and PSMA started decreasing the overall surface tension values of the mixture decreased as well, moving closer to pure PSMA values, rather than pure DMPC ones.

To sum up, at very low lipid concentrations, polymer was strongly adsorbed at the interface, displaying significant surface activity. At higher concentrations, the lipids were bound to the polymer chains, resulting in a surface-active polymer-lipid complex, which occurred at higher lipid concentration and it was formed by the micellization of the phospholipids onto the polymer chains. Finally, the polymer was saturated with the lipids. Above the saturation concentration, the lipids were not bound to the polymer and therefore lowered the surface tension and then, the coexistence of pure phospholipid micelles and mixed micelles of PSMA and phospholipids could be observed.

3.3. ^{31}P NMR

This technique helps to understand the conformational changes of the mixtures with the pH. The pH-dependent membrane disruptive activity of PSMA is believed to be associated with the conformational transition of the polymer. The protonation of the free carboxylic groups of PSMA is known to trigger this transition from an expanded conformation at high pH to a relatively hydrophobic globular coil in acidic solution. The collapsed polymer chain provides an increased number of hydrophobic sites which enhance polymer adsorption to the phospholipid and so the ability of PSMA to disrupt the lipid membrane.

As can be observed in Figure 6, at pH 10 (Fig.6a) the mixture of DLPC and PSMA showed two spectral peaks at chemical shifts around -10 and 0 ppm and the mixture of DMPC and PSMA showed one spectral peak at around 0 ppm (Fig.6d). This suggests that in the DLPC mixture there were two distinct populations of lipid experiencing different motional environment and in the DMPC mixture just one. The peak at -10 ppm in DLPC indicated a lamellar organization in the presence of PSMA. The peak at 0 ppm in both DLPC and DMPC suggested the presence of an isotropic phase (e.g., micellar). This is a phase where phosphate heads undergo rapid isotropic averaging motion, which produces a narrow symmetrical ^{31}P-NMR spectrum. Narrow signals at isotropic shift value have been observed in phospholipid systems by many authors and under various conditions [44-46]. The spectra indicated that under alkaline conditions, the polymer was capable of binding with the phospholipids. However, this cooperation might compete with other possible lipid self-associations such as bilayer. PSMA possibly modified the shape of the lipid assembly through hydrogen bonding, electrostatic and hydrophobic interactions, but one may assume that the binding strength of the polymer was not sufficient to induce all of the phospholipid to undergo the bilayer-nonbilayer transformation.

(PLEASE INSERT FIGURE 6)

As the pH of the mixtures of PSMA with the phospholipids decreased from 10 to 6, ^{31}P-NMR peaks appeared (Figs. 6b and 6e). In the case of DLPC (see Fig. 6b), the peak was broader and it was believed to be a result of the superposition of one peak corresponding to the isotropic phase and another corresponding to anisotropic lipid phase, however, this time, the anisotropic phase was other type instead of lamellar, evidenced by the disappearance of the lamellar peak at -10 ppm. It is important to note that apart from the micellar structure, other isotropic structures that give rise to a narrow isotropic peak include small vesicles, cubic, rhombic and inverted micellar structures [46-48]. The binding strength of the PSMA was strong enough to destabilize the lipid bilayer and induce the lipid to undergo bilayer-nonbilayer phase transformation. The binding of PSMA to phospholipid is believed to occur through a combination of hydrogen bonding and hydrophobic interactions, with electrostatic interactions being secondary. The PSMA molecules are believed to modify the shape of the phospholipids by surrounding the polar heads.

Further decreasing the pH of the polymer-lipid mixtures towards 4 (Figs. 6c and 6f), a sharper isotropic resonance with higher peak intensity was observed. This fact demonstrates that at these pH values, the phospholipid-polymer complexes favoured the formation of

micelles, small vesicles or other isotropic structures in which the motions were sufficiently fast to completely average the chemical shift anisotropy. The anisotropic phases may no longer exist under these conditions.

In addition, the ^{31}P-NMR spectra of the lipids and pure PSMA solution at pH 4 are presented. One may notice that the samples did not show any detectable NMR resonance. The obvious explanation for the PSMA sample is that it did not have any phosphorous nuclei to give rise to a ^{31}P-NMR spectrum. The case of the lipid samples may be explained by the molecular packing constraint of the lipids. As phospholipid molecules were dispersed in water, they tend to assemble into the lipid bilayers. This self-association is an example of the so-called hydrophobic effect, which has a large entropic contribution arising from the configurational entropy of the hydrogen bond networks within the water. Because of this effect, the lipid bilayers arrange themselves in such a way that they have no edges and thus form multilayer supramolecular structures, which constrain the molecular freedom of the phospholipid head groups. High resolution NMR spectra of phospholipids are not observed until the large aggregates are broken up by ultrasonication. This observation suggests that under these conditions, the lipids may self-assemble into multilayer supramolecular structures and that the phospholipid molecules within these structures may be strictly immobilized. This molecular packing constraint may explain the absence of ^{31}P-NMR signal shown in the figure.

3.4. Particle size and Zeta potential

At pH 4, the stable complexes made with both DLPC and DMPC showed nanometric monodisperse sizes, close to 50 nm in diameter. The polydispersity index was higher in the case of DLPC samples, and showed slightly more negative surface charge. This may be due to the shorter chain length, different solubility and different molecular structure of DLPC [49], which made its interaction with PSMA more difficult, leading to increased polydispersity and exposing more negative charge at the surface. It is widely accepted that most natural membranes are negatively charged because of the presence of variable quantities of negatively charged phospholipids. However, in this case, we have to take into account that even though phosphatidylcholine heads are zwitterionic (theoretically globally uncharged at neutral pH), it gives rise to a negative zeta potential in water. This has been interpreted in terms of hydration layers formed around the surface [50] and to the orientation of lipid head

groups [51]. Similar behaviour, with residual negative values but greater charge neutralization is observed at pH 4.

(PLEASE INSERT TABLE 1)

3.5. Morphology (cryo-EM)

Figure 7 shows cryo-EM micrographs of the complexes. These show rounded disc shapes of sizes smaller than 50 nm, which is consistent with the average diameters obtained from dynamic light scattering. Some aggregation of such small nanostructures into larger structures could be observed as interconnected white regions in figure 7a corresponding to PSMA-DLPC particles.

(PLEASE INSERT FIGURE 7)

4.- CONCLUSIONS

The aim of this work was to gain a better understanding of the biophysical properties of alternating low molecular weight alternating PSMA copolymer, as a potential synthetic analog with mimicking properties for the amphiphilic helical strands of apoproteins. For that purpose, pH-dependent complexation of this PSMA with phospholipid membranes at the air-water interface and the formation of nanostructures by self-assembly in aqueous solution at specific conditions were also studied. To achieve that, observation of several phospholipids monolayers were conducted in the absence and presence of PSMA, under compression by using the Langmuir Trough technique and with the du Noüy tensiometer under non-compressed conditions. The ability of the PSMA to interact with the phospholipid monolayer increased with decreasing pH. The structural reorganization of the phospholipid monolayer is also sensitive to molecular weight of the phosphatidylcholine. The surface tension curves of the mixtures showed that at very low lipid concentrations, the polymer was strongly adsorbed at the interface. At higher concentrations the micellization of the phospholipids over the polymer chains was observed. ^{31}P NMR showed that the strong interaction between polymer and lipid resulted in the disappearance of the bilayer structure of pure phospholipids and the formation of isotropic phases when they intertact with the polymer at medium-low pH. Finally, polymer-phospholipid complexes were formulated and the particle sizes and

morphology studies showed nanometric, monodisperse and negatively charged particles, that have potential use as nanocarriers at interfaces.

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FIGURE CAPTIONS

Figure 1. Overlay of surface-pressure –area isotherms on pH 4 phthalate buffer for pure phospholipids (solid), pure PSMA (solid-dashed) and their mixtures (dashed). a) DLPC, b) DMPC.

Figure 2. Effect of pH on uncompressed (du Noüy tensiometer) surface tension-concentration dependencies of PSMA: pH 4 (grey), pH 6 (dark grey), pH 12 (light grey).

Figure 3. Uncompressed (du Noüy tensiometer) surface tension at different pH values of PSMA 0.2 wt.% (●) and PSMA-DMPC mixtures (o) at the same PSMA concentration and relative ratio polymer to phospholipid of 3 to 1 in weight

Figure 4. Uncompressed (du Noüy tensiometer) surface tension at pH 4 against logarithmic concentrations of PSMA (Δ), DMPC (o), and their mixtures at 0.2 wt.% PSMA (inset).

Figure 5. Uncompressed (du Noüy tensiometer) surface tension at pH 4 against logarithmic concentrations of DLPC and DMPC. and surface tension of polymer-phospholipid mixtures upon addition of increased concentrations of phospholipid to a constant amount of 0.2wt.% PSMA: PSMA (Δ), DLPC (◊), DMPC (●), DLPC-PSMA (o), DMPC-PSMA (□).

Figure 6. Proton-decoupled ^{31}P-NMR spectra on aqueous mixtures of DLPC and PSMA at (a) pH10, (b) pH 6 and (c) pH 4, and spectra on aqueous mixtures of DMPC and PSMA at (d) pH10, (e) pH 6 and (f) pH 4. The spectra of pure phospholipids and pure PSMA at pH 4 are also shown below for comparison.

Table 1. Particle size distribution, polydispersity (PDI) and zeta potential (mV) of the two types of complexes, with their corresponding standard deviations.

Figure 7. Electron microscopy micrographs of (a) DLCP-PSMA complexes and (b) DMPC-PSMA complexes at pH 4

FIGURE 1

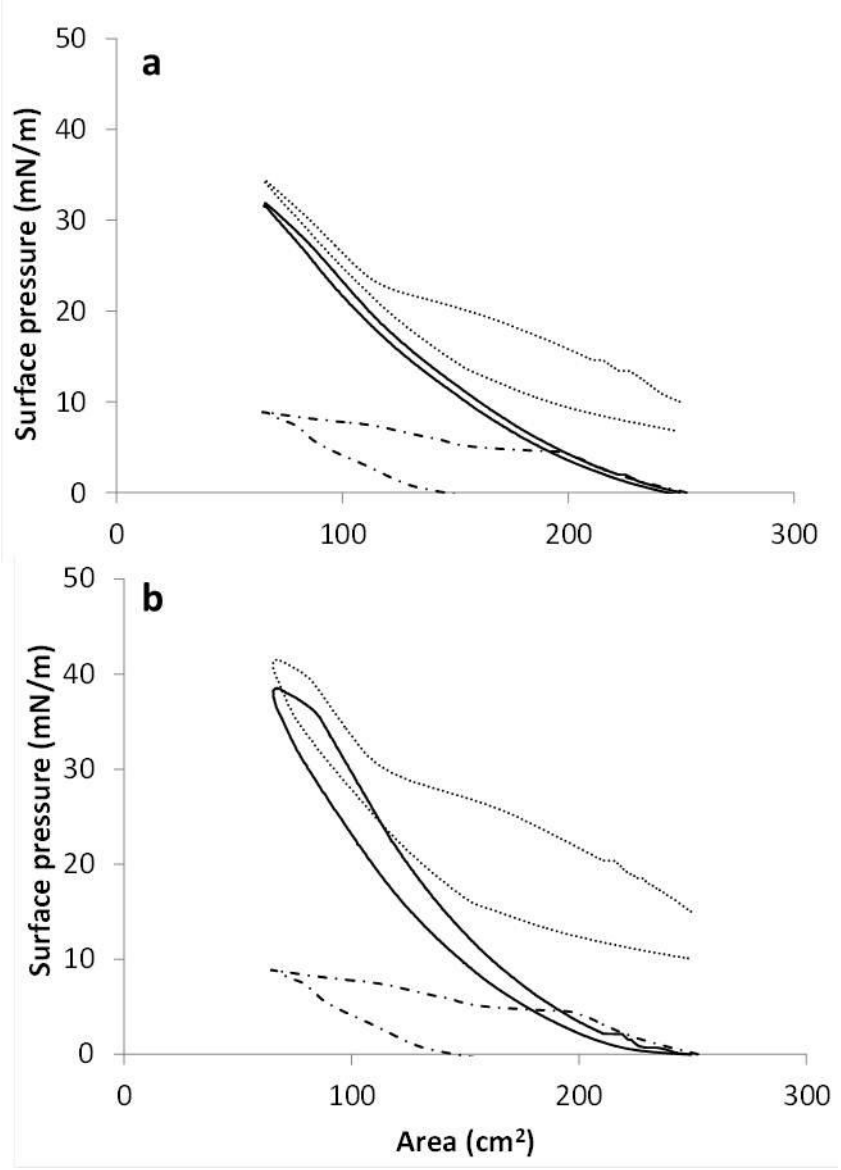
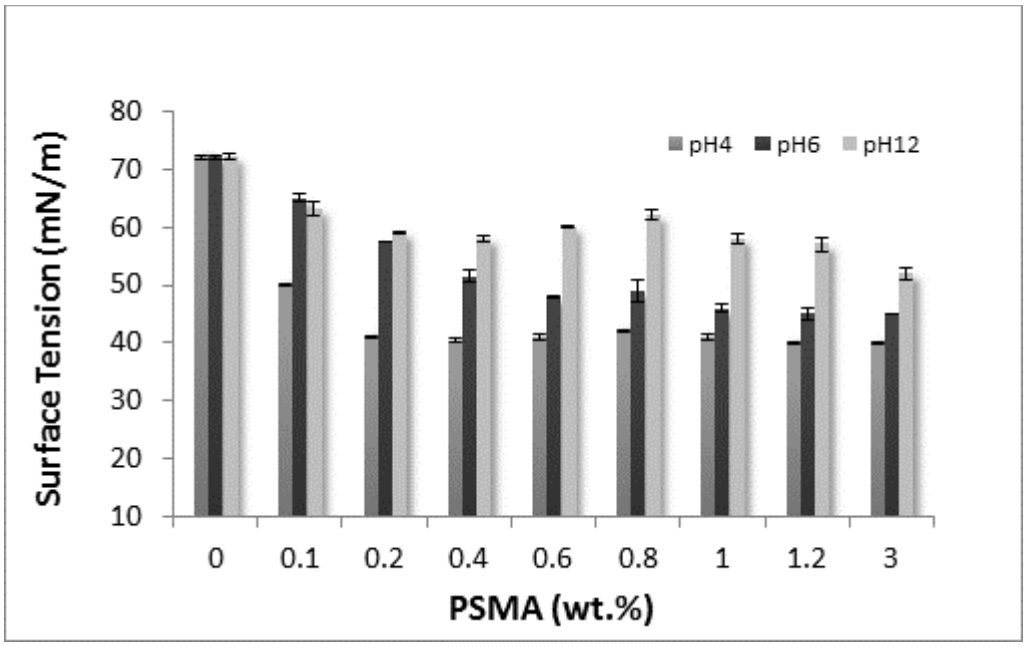

a
b
Surface pressure (mN/m)
Surface pressure (mN/m)
0
10
20
30
40
50
0
100
200
300
Area (cm²)

FIGURE 2



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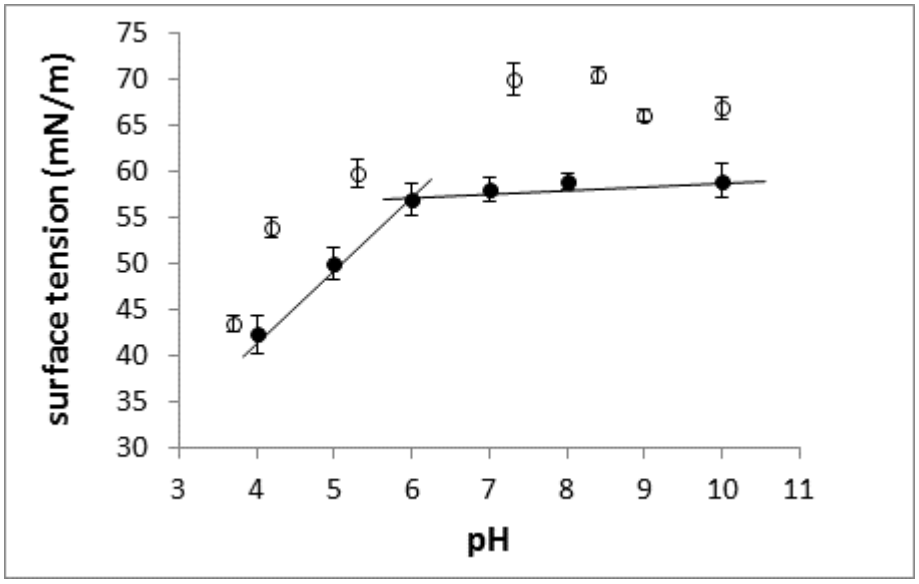

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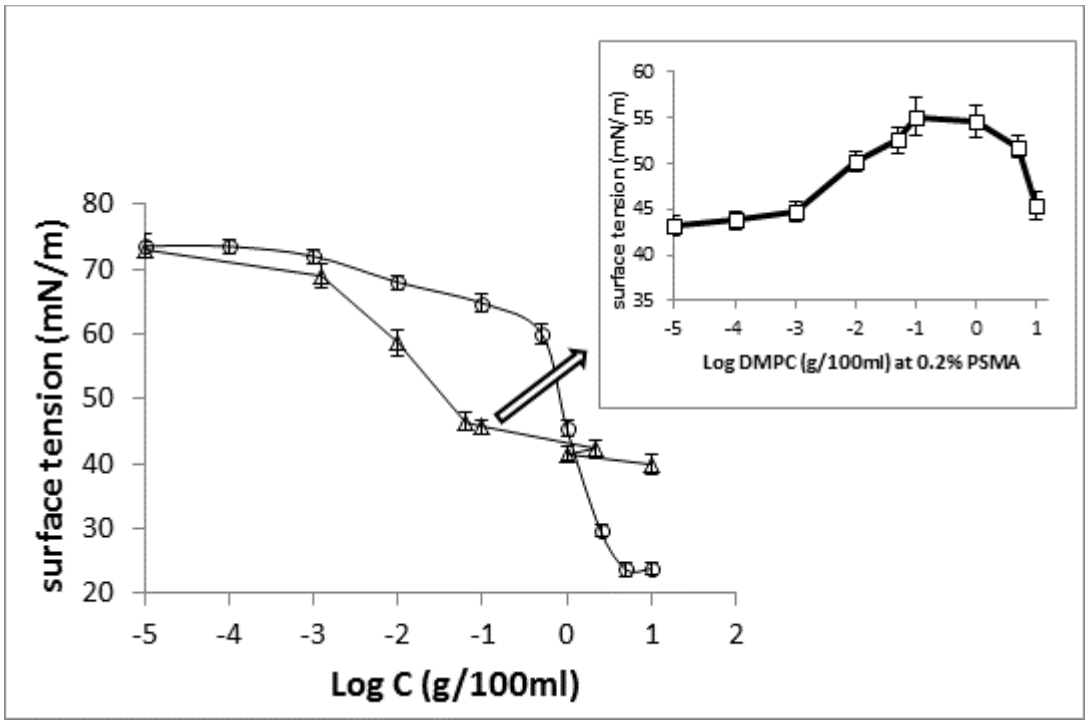

FIGURE 5

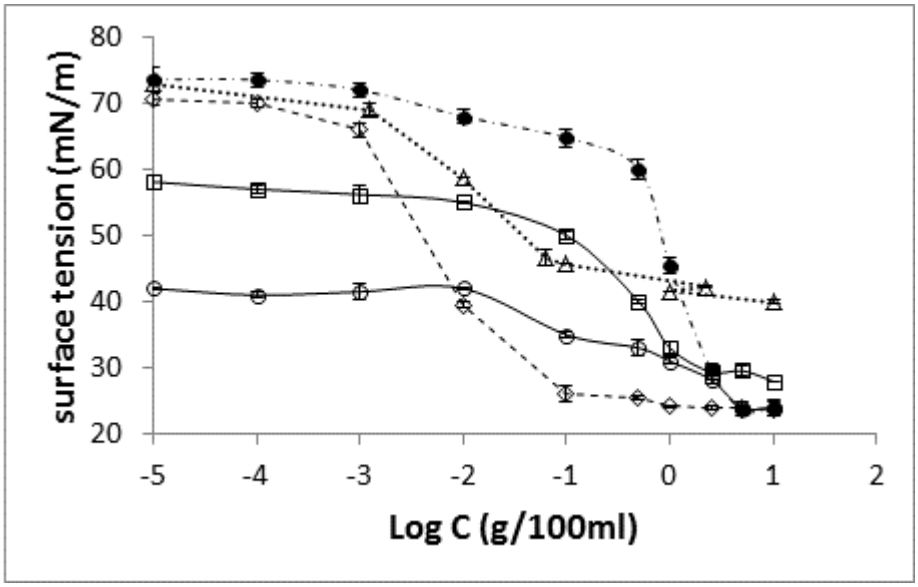

FIGURE 6

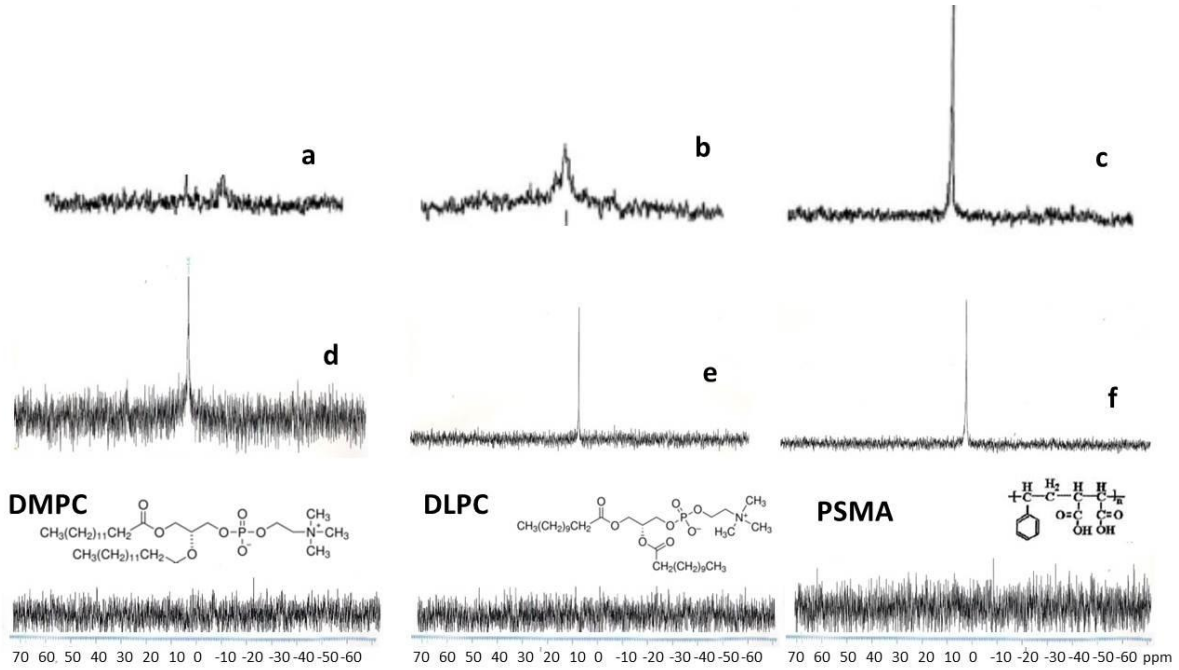

TABLE 1

SAMPLE	Size(nm)	PDI	Zeta Potential (mV)
PSMA-DLPC	53±1	0.438±0.016	-17.9±2.6
PSMA-DMPC	47±10	0.251±0.04	-14.6±7.5

FIGURE 7

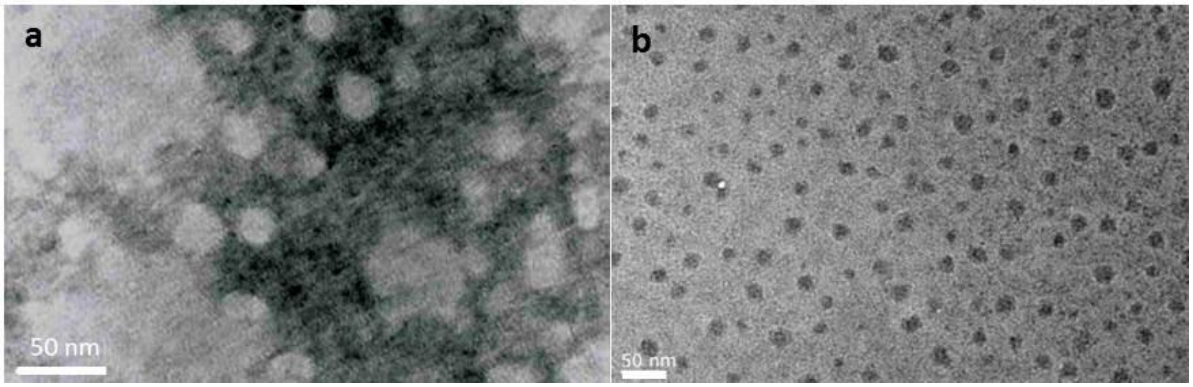

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Figure1

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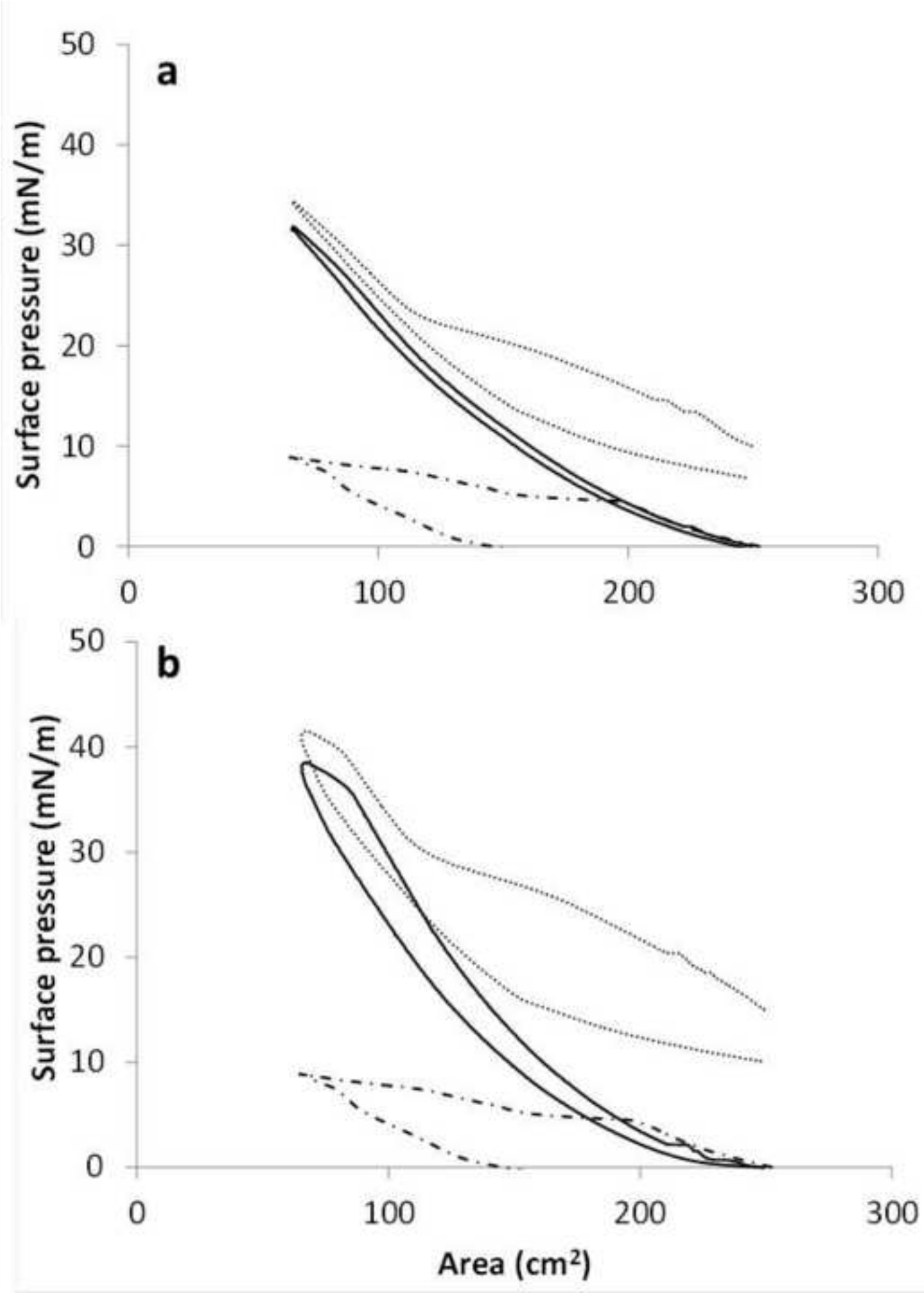

Figure2

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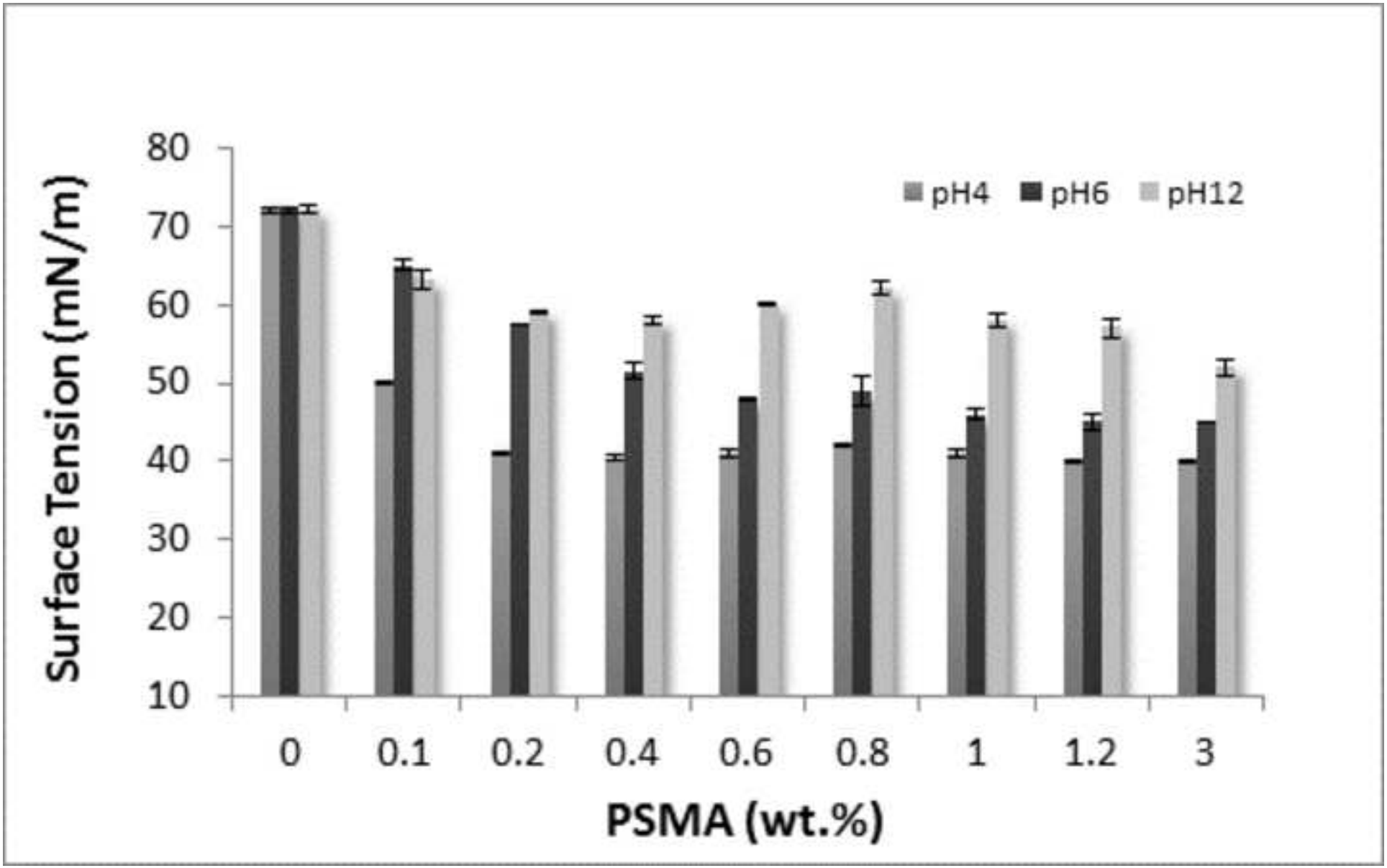

Figure3

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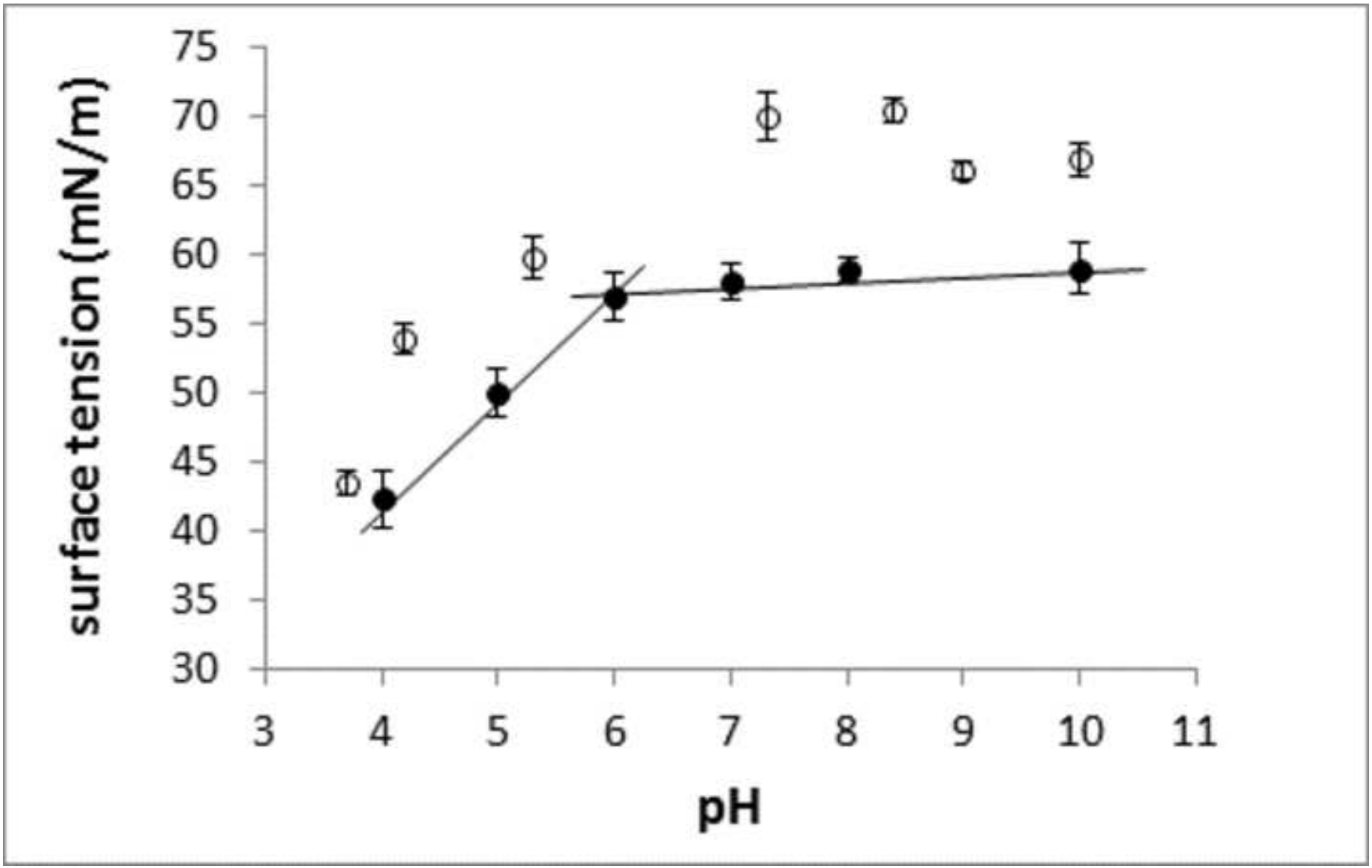

Figure4

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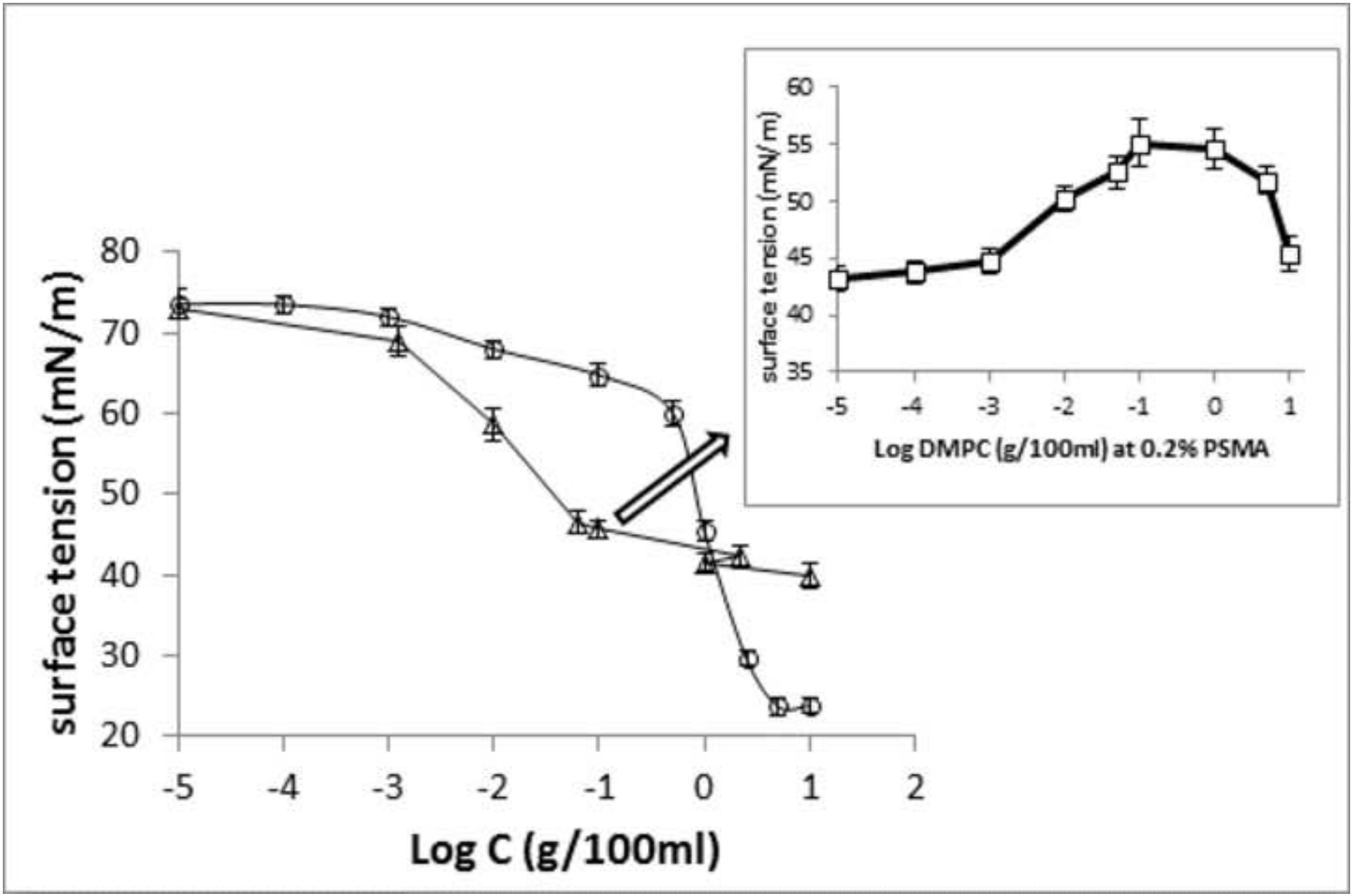

Figure5

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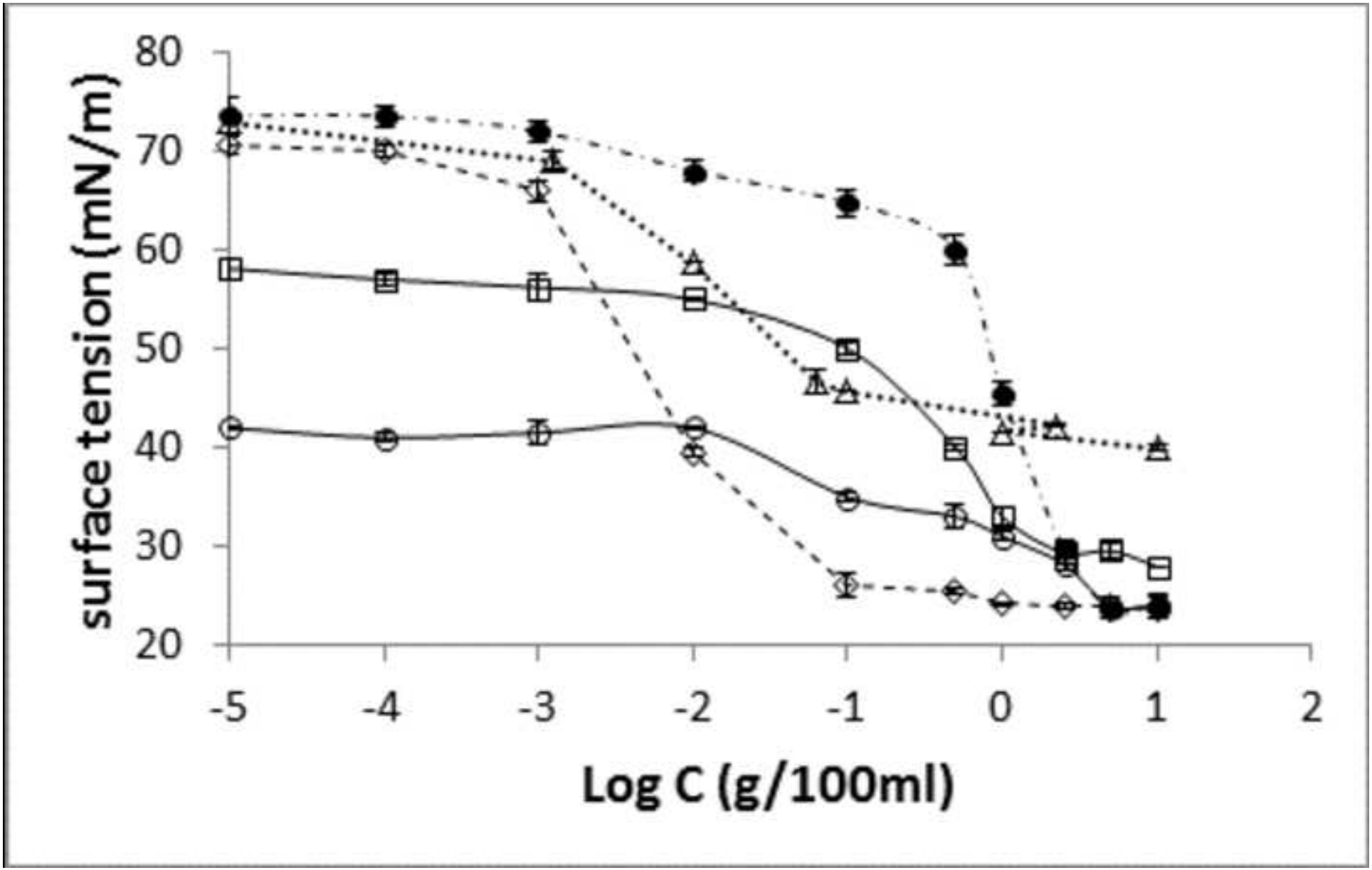

Figure6

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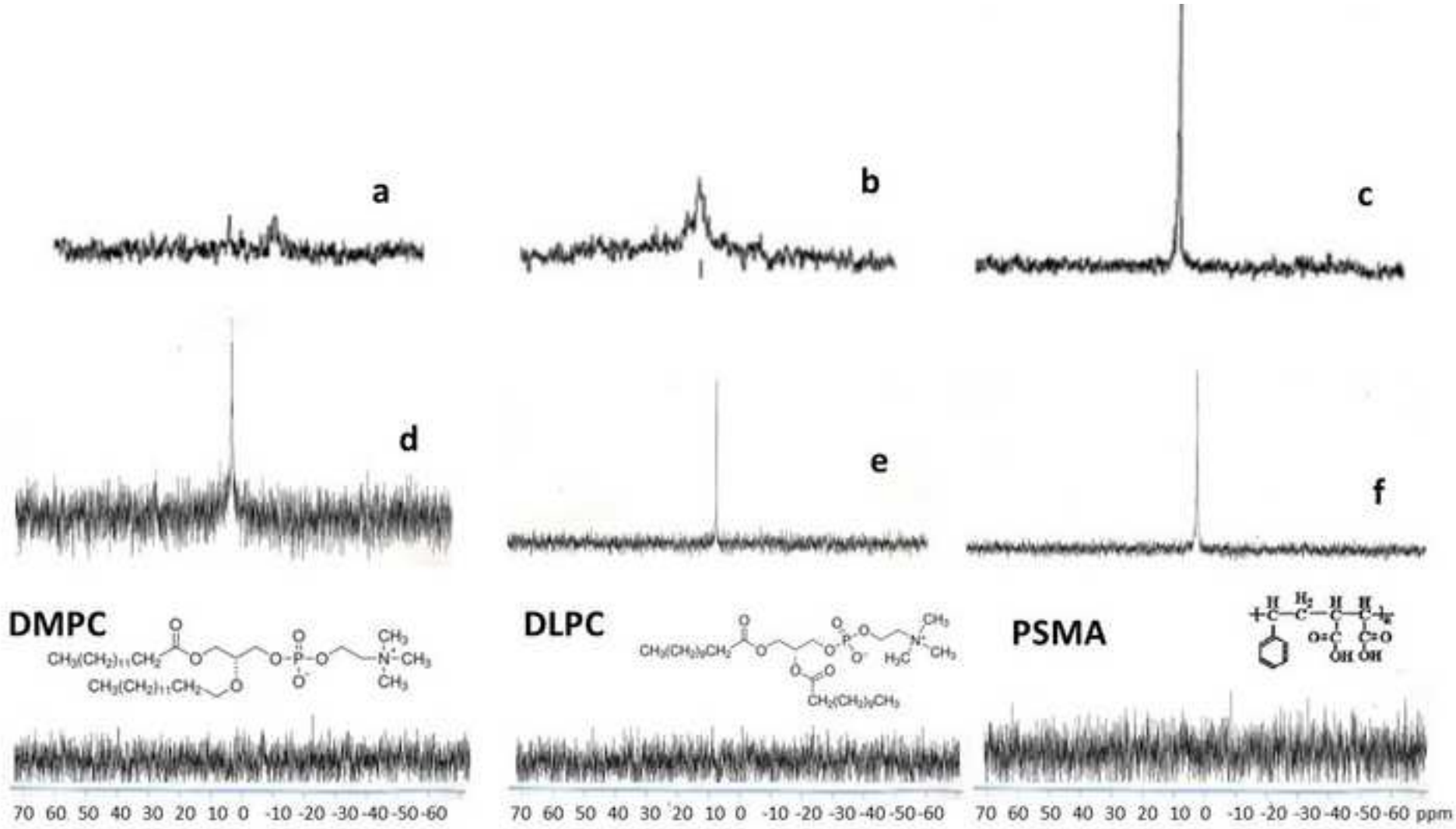

Figure7
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