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Zeta potential and surface free energy changes of solid-supported phospholipid (DPPC) layers caused by the enzyme phospholipase A_2 (PLA_2)

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Abstract Stability and wetting properties changes of systems formed of phospholipid DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) layers covering silica particles or glass slides due to the phospholipase A_2 (PLA_2) action were determined by zeta potential measurements and the surface free energy evaluation, respectively. The comparison of the zeta potential and surface free energy, which was evaluated from advancing and receding contact angles via applying models of interfacial interactions, i.e. van Oss et al. (LWAB) and contact angle hysteresis (CAH), was found to be helpful for better understanding the mechanism of PLA_2 action on the lipid layers, what is discussed in the paper.

Keywords Phospholipase A_2 · DPPC · Zeta potential · Surface free energy

1 Introduction

The application of zeta potential and contact angle measurements to investigate biological materials and lipid membranes is still relatively new. Supported phospholipid layers can be good models for the biophysical studies of drug delivery systems because of their low toxicity and in addition they have physicochemical properties corresponding to the physiological lipids that build biological membranes (Heurtault et al. 2003; Troutier and Ladavière 2007). Furthermore, enzymes can play an essential role in the release of some drugs in cells; this is the case of phospholipases that catalyze hydrolysis of different ester bonds in phospholipids (Jørgensen et al. 2002). Therefore, such enzymes can be used to control the hydrolysis process involving gradual membrane degradation that finally may lead to release of the content, as well as to carry out reactions taking place in organisms with the use of phospholipases as catalysts, sensors and mediators.

Phospholipids dispersed in water tend to aggregate to form 'micelles' (spherical and bilayer) because of their amphiphilic molecular structure. As it is well known, it is the bilayer structure that is the most important in determining the cell structure. Although lipids are insoluble in water they are soluble in organic solvents, and this makes it possible to deposit them on a solid support. This can be done directly, or alternatively, they can be transferred to the support after spreading them at the air/water interface, by applying the Langmuir-Blodgett (Blodgett and Langmuir 1937) or Langmuir-Schaefer techniques (Langmuir and Schaefer 1938). These procedures allow formation of organized lamellar structures on the solid support that greatly mimic biological membranes (biomimetic systems).

As mentioned, the enzymes known as phospholipases can catalyze the hydrolysis of ester bonds in phospholipids forming a membrane. For example, phospholipase A_2 (PLA_2) is an interfacially activated enzyme that catalyzes the hydrolysis of the *sn*-2 acyl ester linkage of phospholipids. As a consequence of such chemical hydrolysis, lysophosphatidylcholine and fatty acids are formed resulting in the transformation of the lipid assembly from a lamellar to a micellar system. PLA_2 exhibits an increased activity on ordered lamellar lipid structures in comparison to monomolecularly dispersed lipids (Dennis 1983; Grandbois et al. 1998; Mouritsen et al. 2006; Nielsen et al. 1999; Wacklin et al. 2007). The reorganization influences the surface properties,

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whose changes can probe the reaction, together with the occurrence of the hydrolysis products, their aggregation at the interface and/or dissolution in the bulk phase.

Zeta potential and surface free energy are important and useful surface characterizing parameters, which can be used to predict wettability and to control stability of colloidal suspensions or emulsions, and also to understand aggregation processes (Chibowski 2003; Chibowski et al. 2008; Heurtault et al. 2003; Troutier and Ladavière 2007; van Oss et al. 1988).

The purpose of this paper is the investigation of the hydrolysis process taking place in DPPC layers deposited on silica particles or glass slides, due to the action of the enzyme phospholipase A_2. This is done via zeta potential and surface free energy determinations as a function of time during 2 h after the beginning of the experiments. Because activity of enzyme is also depended on the structure of the layers whose properties are influenced by the solid support, changes of the above two parameters determined as a function of time should deliver useful information about the hydrolysis reaction taking place in the DPPC layers. This is because the changes result from the appearance of polar and ionic products of the reaction. Therefore, in our opinion such investigation accomplishes data obtained with different techniques.

2 Experimental

2.1 Materials

DPPC (1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine) (semi-synthetic, 99%) was purchased from Sigma and used without further purification. **Phospholipase A_2** from hog pancreas (Fluka, 200 U/mg, one U is the enzyme amount that hydrolyzes 1 μmol of 3-*sn*-phosphatidylcholine per one minute at pH = 8 at 37 °C) was used as received after dissolving it in **buffer TRIS** containing 10 mM Trihydroxymethylaminomethane (POCH Gliwice), 5 mM $CaCl_2$ (POCH Gliwice), 100 mM NaCl (POCH Gliwice) adjusted to pH 8.9, to catalyze the hydrolysis of the supported phospholipid films. **Water** from Milli-Q system (resistivity 18.2 MΩ cm) was employed for dissolving the reagents. **Silicic acid** ($SiO_2 \cdot xH_2O$, from Riedel-de Haën, Germany) purified according the procedure described by Preočanin and Kallay (2006) and **glass slides** ($38 \times 26 \times 1$ mm^3, Comex, Wroclaw) treated with piranha solution (H_2O_2:H_2SO_4, 1:1) were applied as a solid support for the phospholipid layers. The treatment was applied to produce more hydrophilic glass surface because of –OH group formation. In consequence the phospholipid can be stronger adsorbed to the surface.

2.2 Methods

2.2.1 Zeta potential measurements

In this study the powdered samples of SiO_2 of known specific surface were first precovered with calculated mono- or bi-DPPC layer (or less than the monolayer and more than bilayer) by contacting the samples with the DPPC dissolved in chloroform and allowing the solvent to evaporate. Then the samples were dispersed in 0.1 M NaCl (natural pH = 5.9) or buffer (TRIS, pH = 8.9) solutions with the help of ultrasounds (45 W for 3 min). In the first series the zeta potentials (from Helmholtz-Smoluchowski equation) were determined for the samples during 2 h using a Brookhaven ZetaMeter, with the help of which also the diameters could be measured. In the second series of experiments, after suspension sonication, a defined amount of the enzyme was added and immediately the zeta potential and mean diameters were determined as function of time, also up to 2 h. In the studied systems the κa value (where κ is the Debye-Hückel parameter and a is the particle radius) was always greater than 100. Therefore the Helmholtz-Smoluchowski equation can be applied. For comparison, the parameters for bare SiO_2 surface were determined too. The mean zeta and diameter values were based on 10 measurements.

2.2.2 Surface free energy determination

For the energy study monolayers were deposited on glass surfaces by the Langmuir-Blodgett method and bilayers by Langmuir-Schaefer technique, keeping a constant 35 mN/m surface pressure of the DPPC monolayer spread on the water, using an automatically controlled Langmuir-Blodgett trough KSV 2000 (Finland). The effect of the enzyme on the wettability of the phospholipid membranes was examined at room temperature by incubation of the prepared samples in the buffer solution containing phospholipase A_2 at different contents, i.e. 2 U/mL; 0.2 U/mL and 0.02 U/mL, and for chosen periods of time: after 5, 15, 30, 60 and 120 min after the enzyme injection. Then the samples were dipped three times in Milli-Q water and dried in a vacuum desiccator for at least 12 h before contact angle measurements.

The surface free energy and its components (changes in the hydrophobic/hydrophilic character of the solid supported lipid membranes) for untreated and PLA_2 treated DPPC mono- and bi-layer was evaluated from the advancing and receding contact angles of water, formamide and diiodomethane, using two different models of interfacial interactions, namely, van Oss et al. (LWAB) (van Oss et al. 1988) and the contact angle hysteresis (CAH) (Chibowski 2003). The contact angles were measured using GBX Contact Angle Meter (France) equipped with camera and computer software for the contact angle calculation from the shape of the settled droplet.

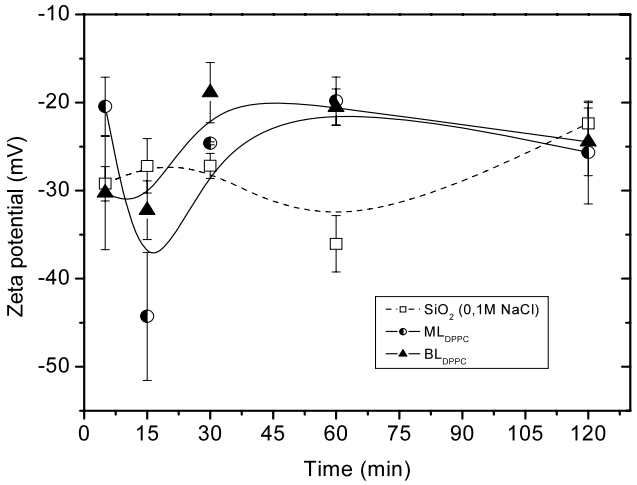

Fig. 1 Zeta potential changes of bare SiO_2 and covered with mono- and bi-layer of DPPC in 0.1 M NaCl

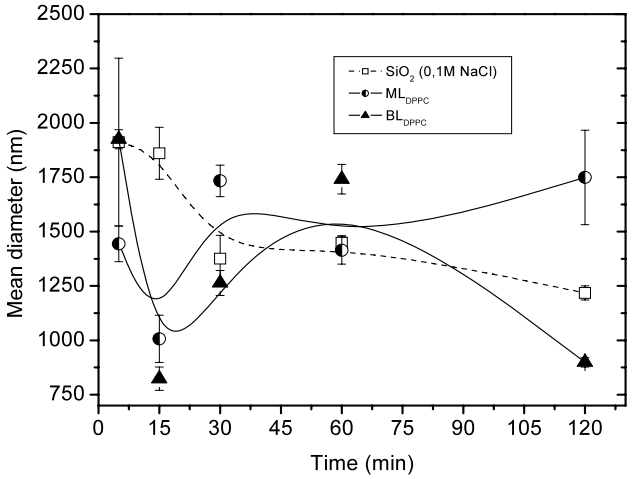

Fig. 2 Mean diameter changes of bare SiO_2 and covered with mono- and bi-layer of DPPC in 0.1 M NaCl

3 Results and discussion

3.1 Time evolution of zeta potential and mean particle diameter

In Fig. 1 the zeta potentials of SiO_2 both bare and covered with a statistical mono- or bi-layer of DPPC in NaCl are shown. After 2 h the zeta potential is practically the same as that of bare SiO_2 and the changes for mono- and bi-layer run in a similar way. The DPPC molecule is neutral in a broad range of pH, although it possesses positive $–N^{(+)}(CH_3)_3$ and negative $–OPO_3^-–$ groups. In the presence of sodium chloride, after the first 15 min, a decrease in negative zeta potential occurs. The $–N^{(+)}(CH_3)_3$ groups of phosphatidylcholine molecules interact electrostatically with negatively charged silica surface. However, in real systems it is hardly possible to obtain uniform monolayer coverage of the silica surface, especially in aqueous solutions where the hydrophobic tails of the DPPC molecules should tend to interact each other, what is energetically favorable. Hence, it is more probable that the molecules will rather form the bilayer patches, nonuniformly covering the particle surface. Such process may occur during the first 15 min and lead to more negative zeta potential increase than that of bare silica (Fig. 1). In the case of the bilayer, $–N^{(+)}(CH_3)_3$ groups of the outer DPPC layer can interact with OH^- ions, whereas $–OPO_3^-$ groups with Na^+. The changes in the mean diameters (Fig. 2) indicate that in DPPC-free suspension the sedimentation of large SiO_2 particles occurs during 2 h (the monotonically decreasing diameters). If DPPC is adsorbed on the particles first rapid sedimentation takes place (first 15 min) and then aggregation of the particles, and surprisingly, more stable is the suspension with statistical monolayer of DPPC coverage. Therefore, one can consider a steric stabilization process in this case.

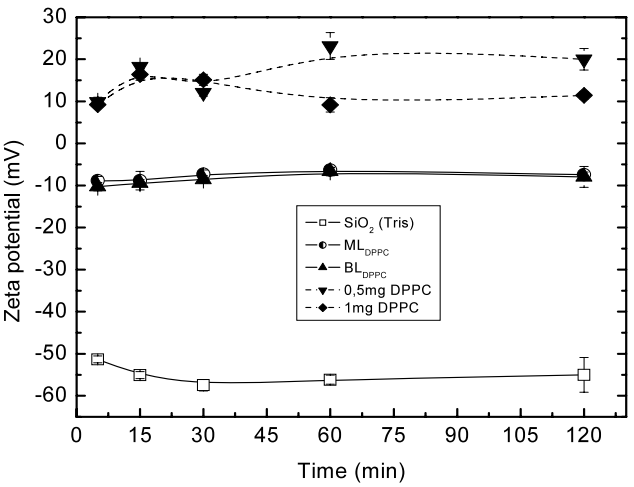

Fig. 3 Zeta potential changes of bare SiO_2 and covered with mono- and bi-layer of DPPC in TRIS buffer

In the second series of experiments the changes in zeta potential and mean diameter of the studied system ($DPPC/SiO_2$), were evaluated in TRIS buffer (Figs. 3 and 4). In this case also more than a calculated DPPC bilayer was deposited on the SiO_2 surface. Stable and more negative zeta potentials of bare SiO_2 (ca. −55 mV) were measured in the buffer than in NaCl solution (Fig. 3). The reason can be found in the favorable adsorption of Tris cations on the silica surface where also Na^+ and Ca^{2+} cations are present. Because the pKa of Tris molecule is 8.1, hence protonated and deprotonated form can occur at pH higher than pKa. Therefore at pH 8.9 both forms of the molecule can exist. In this system the Tris cations interact by attractive electrostatic forces with the negatively charged silica surface. Moreover, the Tris cation possesses three –OH alcohol groups and is much larger than Na^+ and Ca^{2+}. These –OH groups can

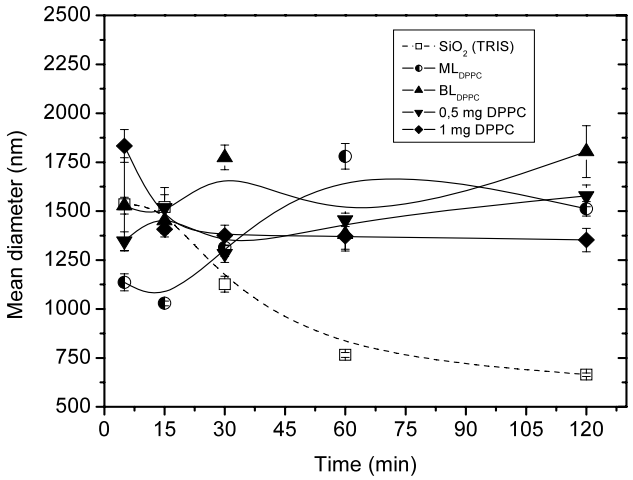

Fig. 4 Mean diameter changes of bare SiO_2 and covered with mono- and bi-layer of DPPC in TRIS buffer

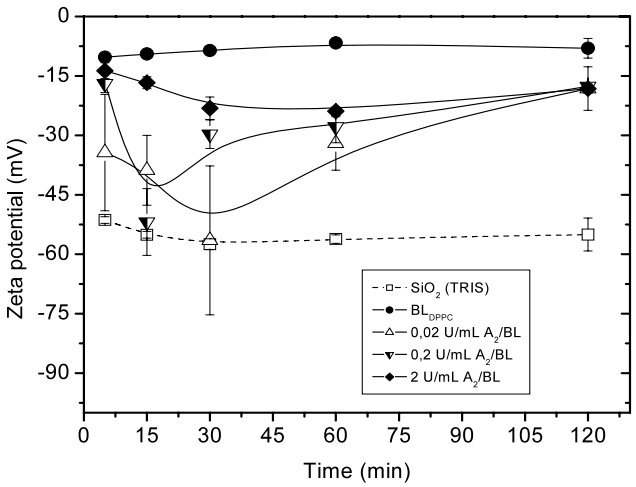

Fig. 5 Zeta potential changes of SiO_2/DPPC bilayer in TRIS buffer due to enzyme PLA_2 action

prevent SiO_2 particles from the aggregation process. But in Fig. 4 the mean diameter of bare SiO_2 particles decreases during 2 h, which means that sedimentation of larger aggregates (particles) occurs. Moreover, −55 mV zeta potential is not sufficient to stabilize the particles. This is not the case if DPPC is present (Fig. 3).

In the DPPC presence the zeta potentials are lower and also stable. Moreover the values are the same for mono- and bi-layer (−10 mV) and they are about 40 mV smaller than those of bare SiO_2 surface. However, while the mean diameter of bare SiO_2 decreases from ca. 1500 nm to 600 nm during 2 h so if DPPC is present the diameters generally change much less (Fig. 4). This indicates a steric stabilization in the presence of DPPC and hence a shift of the slip plane at the particles (Heurtault et al. 2003). Because the slip plane is situated in the electrolyte phase, the presence of ordering of DPPC hydrocarbon tails will shift outward the plane in comparison to bare silica surface. At the larger surface coverage than a statistical DPPC bilayer, at pH = 8.9 the zeta potential sign reversal takes place, which is positive (10–20 mV) and also stable. The reason can be found considering $–N^{(+)}(CH_3)_3$ groups oriented outward if sufficient amount of the phospholipid molecules is present on the SiO_2 surface, which results in positive zeta potential. Evidently, in the buffer at pH 8.9 the electrokinetic potential settles fast and in the presence of DPPC the mean diameters are more stable, fluctuating between 1110–1800 nm for the all tested surface coverages.

Neutral or zwitterionic lipids exhibit poor affinity to SiO_2 particles probably because of insufficient number of hydrogen bonds that can form between lipids and silanol groups (Rapuano and Carmona-Ribeiro 1997). Moura and Carmona-Ribeiro (2005) evidenced that an increase in ionic strength of the solution improved neutral lipid adsorption. Although Tris cations, being positive, can bound to negative

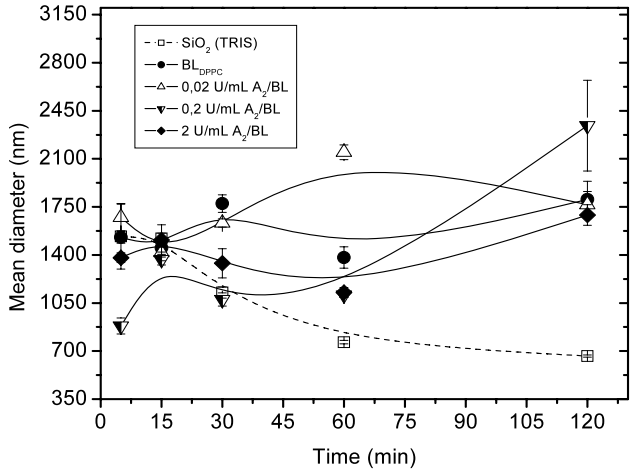

Fig. 6 Mean diameter changes of SiO_2/DPPC bilayer in TRIS buffer due to enzyme PLA_2 action

phosphate groups of lipids, they are larger than Na^+ ions so it is hardly possible that they penetrate into the binding sites of the phosphate groups present between DPPC zwitterionic molecules in the deposited layer. Therefore Tris cations increase only the ionic strength of the medium (Satoh 1995).

It should be also taken into account that in the buffer solution there are also present calcium ions, which are able to bind, orient and polarize the carbonyl group of the phospholipid, resulting in a more positive charge on this carbonyl carbon atom. This is in favor of the nucleophilic attack by the water molecule during hydrolysis (Verheij et al. 1980). The final effect of the influence of the cations is the decrease in the negative zeta potential of the studied systems.

The influence of different amounts of PLA_2 enzyme present in the buffer on DPPC monolayer and bilayer appeared to be very similar. Therefore only the results for bilayer are discussed below. Figures 5 and 6 show the zeta

potential and mean diameter changes for DPPC bilayer deposited on silica. The zeta potentials in the presence of PLA_2 enzyme are in within those of bare and the mono- or bilayer covered surfaces, and they change during 2 h. Focusing on the bilayer one can see that the greatest changes and the lowest reproducible values were obtained at the lowest the enzyme amount. The most characteristic is that after 2 h irrespective on the enzyme amount added the zeta potential value is the same, ca. −18 mV. The corresponding changes in the mean diameter are more complicated. After PLA_2 addition the diameter fluctuates during 2 h depending on the enzyme content, but finally it is comparable to that with DPPC bilayer. The greatest changes are observed at 0.2 U/mL content.

The enzyme causes DPPC hydrolysis, whose products are lysophospholipid and palmitic acid, which is negatively charged. In result of the hydrolysis the SiO_2/DPPC surface becomes more negatively charged. The affinity of the hydrolysis products to non-hydrolyzed part of the bilayer may prevent their dissolution into the bulk phase until a certain products concentration is reached (Nielsen et al. 1999). It means that the hydrolysis products, being amphiphiles, accumulate in the lipid bilayer forming non-lamellar phases (domains), the presence of which strongly influences the bilayer properties (Mouritsen et al. 2006). Generally, it results in increased negative zeta potential (Fig. 5). These changes may increase activation of the enzyme, leading finally to break-down of the bilayer structure (Mouritsen et al. 2006). It is likely that after dissolution of the reaction products in the bulk phase the negative zeta potential decreases and after 2 h the zeta potential of the particles is the same (−20 mV) irrespectively on the PLA_2 amount used, but it is much smaller than that of bare SiO_2 surface (−55 mV), and only 10 mV higher than that of SiO_2/DPPC before the enzyme treatment (Fig. 5). This can be the result of concurrent re-adsorption on the silica particles of different ions present in the bulk phase.

3.2 Surface free energy changes

The changes in wettability of solid-supported phospholipid layers by evaluation of the surface free energy were also studied. The magnitude of the surface free energy results from the kind and strength of intermolecular interactions. The energy determination was based on the contact angle measurements of three probe liquids, namely, water, formamide and diiodomethane, using two theoretical approaches: the Lifshitz-van der Waals/Acid-Base (LWAB) (van Oss et al. 1988) and the Contact Angle Hysteresis (CAH) (Chibowski 2003, 2005, 2007). In the former model van Oss et al. expressed surface free energy as a sum of the apolar interactions, called Lifshitz-van der Waals γ_S^{LW}, and the polar Lewis acid-base interactions γ_S^{AB} (LWAB approach).

$$\gamma_S^{tot} = \gamma_S^{LW} + \gamma_S^{AB} = \gamma_S^{LW} + 2(\gamma_S^+ \gamma_S^-)^{1/2} \tag{1}$$

where γ_S^- is the electron-donor and γ_S^+ is the electron-acceptor component of the energy. Then using this approach the work of adhesion (W_A) of solid/liquid can be calculated:

$$W_A = \gamma_L(1+\cos\theta) = 2(\gamma_S^{LW}\gamma_L^{LW})^{1/2} + 2(\gamma_S^+\gamma_L^-)^{1/2} + 2(\gamma_S^-\gamma_L^+)^{1/2} \tag{2}$$

where subscripts 'S' and 'L' mean solid and liquid, respectively.

In the contact angle hysteresis approach (CAH) total surface free energy of a solid γ_S^{tot} can be related to surface tension of the probe liquid γ_L and hysteresis of the contact angle of this liquid, which is defined as the difference between advancing θ_a and receding θ_r contact angles.

$$\gamma_S^{tot} = \frac{\gamma_L(1+\cos\theta_a)^2}{(2+\cos\theta_r+\cos\theta_a)} \tag{3}$$

It should be stressed that the measured zeta potentials deal to the SiO_2/DPPC dispersion and the surface free energy deals with DPPC layers supported on planar glass plates. However, in both systems the surfaces should possess analogical properties.

The surface free energy changes of DPPC mono- and bilayers due to PLA_2 treatment are shown in Figs. 7 and 8, respectively. It was found that both mono- and bi-layer of DPPC deposited on glass surface caused significant decrease in glass surface free energy, from about 57 mJ/m^2 for bare glass surface to ca. 35–29 mJ/m^2 for the surface with mono- or bi-layer of DPPC if calculated from van Oss model (1 and 2). It can be speculated that the lower total surface free energy of the bilayer than that of monolayer is probably caused by reorientation of the upper DPPC layer molecules. Hence, the energy decrease is mainly due to decrease in the surface hydrophilicity.

From the contact angles it was found that in case of DPPC mono- and bi-layer deposited on glass the enzyme phospholipase A_2 (PLA_2) in buffer (TRIS, pH = 8.9) caused the hydrolysis of the layers at a rate dependent on the enzyme concentration (Figs. 7 and 8). This can be justified by the decrease in the layer surface hydrophobicity due to liberation of the palmitic acid and lysophosphatidylcholine by breaking down ester bond in *sn*-2 position of the DPPC molecule. However, the hydrolysis products are considered to concentrate first in the phospholipid layer because of their affinity to the lipid layer and then dissolve in the solution (Nielsen et al. 1999). At the enzyme concentration 2 U/mL, already after 5 min the increase in the surface free energy shows that the process has occurred fast. At the lowest concentration

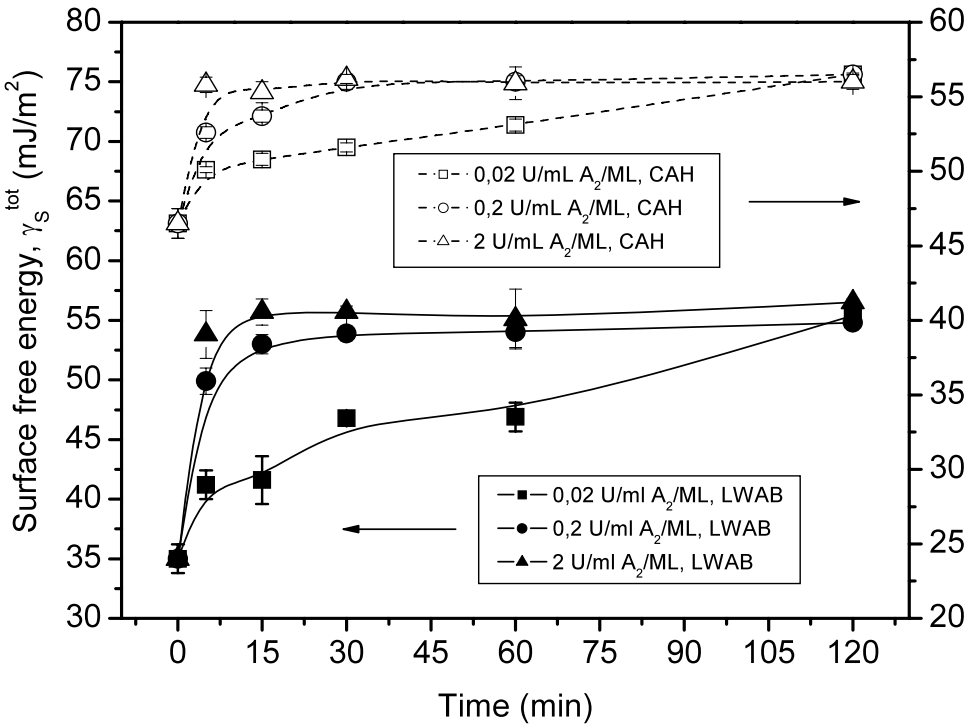

Fig. 7 Surface free energy changes (in mJ/m^2) of the DPPC monolayer deposited on glass, caused by phospholipase A_2 action, calculated from van Oss (LWAB) and Contact Angle Hysteresis (CAH) approaches

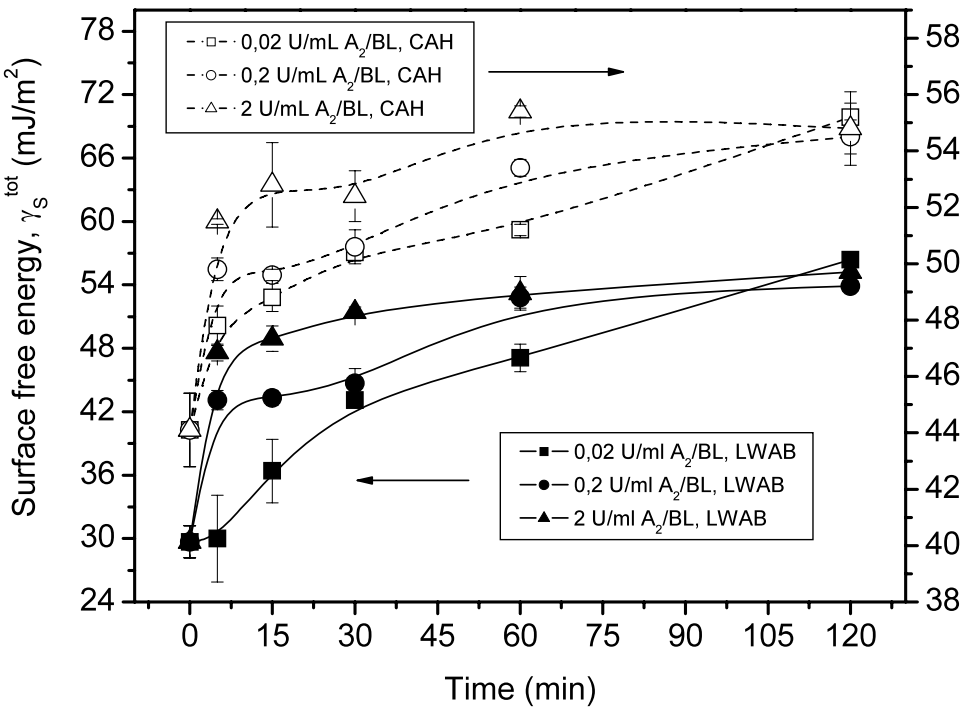

Fig. 8 Surface free energy changes (in mJ/m^2) of the DPPC bilayer deposited on glass, caused by phospholipase A_2 action, calculated from van Oss (LWAB) and Contact Angle Hysteresis (CAH) approaches

(0.02 U/mL) a gradual increase in the total surface free energy as a function of time can be seen. However, after 2 h of the samples contact with the enzyme the values of free energy overlap, regardless of the enzyme concentration, and equal to ca. 55 mJ/m^2 for mono- and bi-layer if determined from van Oss approach (Figs. 7 and 8). This indicates accomplishment of the hydrolysis reaction in both cases. The trend of the changes is similar, but in case of the bilayer the surface free energy values are lower than for the monolayer. This is because of double amount of the phospholipid material present and the prolongation of the hydrolysis time. The energy plateau for the concentrations 0.2 and 2 U/mL is

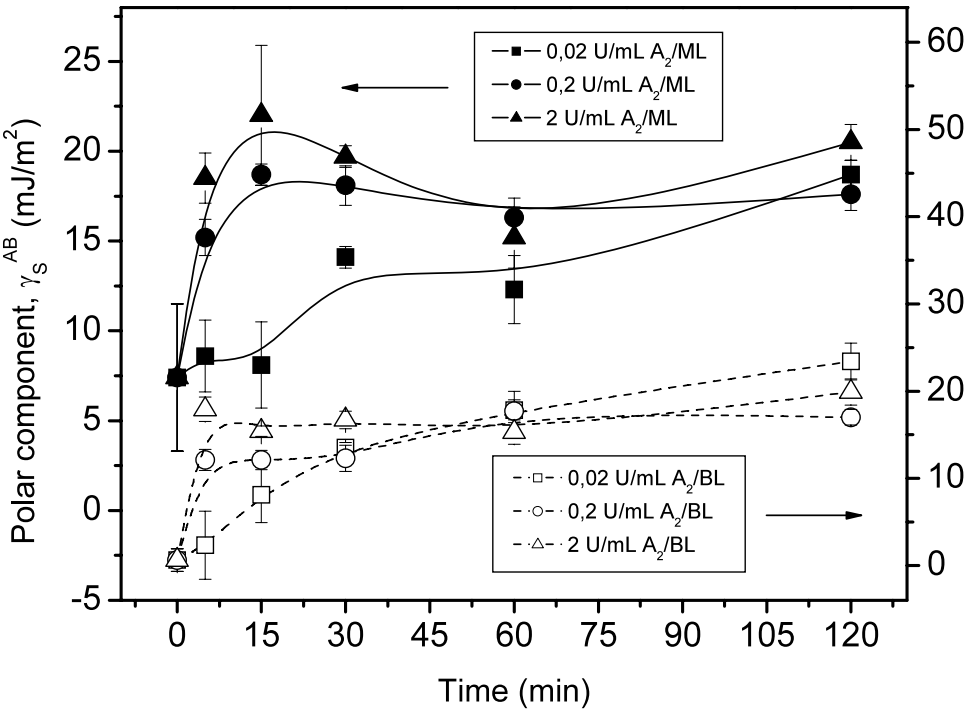

Fig. 9 Polar interaction changes (in mJ/m^2) of the DPPC mono- and bi-layer deposited on glass, due to phospholipase A_2 action, calculated from van Oss (LWAB) approach

observed after 60 min and again 2 h treatment results in the convergent values of energy (Figs. 7 and 8).

In the figures there are also plotted changes in the surface free energy calculated from contact angle hysteresis of water, formamide and diiodomethane using (3), which are arithmetic means. As can be seen, the changes are similar to those evaluated from van Oss approach, which confirm reliability of both methods for the surface free energy changes tracking, despite that the values calculated from CAH approach are higher, which results from applying the advancing and receding contact angles for calculation (Chibowski 2003), and hence they reflect the interfacial interactions at a closer intermolecular distance. The detailed discussion of this problem has been published elsewhere (Chibowski 2003, 2005, 2007).

The PLA_2 action results in an increase in acid-base interactions (Fig. 9), which probably is due to the presence of polar products of the hydrolysis (palmitic acid and lysophosphatidylcholine) at the interface. The polar component of the energy was determined from van Oss approach as the geometric mean of electron-donor and electron-acceptor parameter: $\gamma_S^{AB} = 2(\gamma_S^- \gamma_S^+)^{1/2}$ (1). Moreover, during the layer degradation more and more patches of bare glass surface are accessible for the probe liquids during the contact angle measurements, hence, the surface free energy is finally comparable to that of bare surface of the glass support.

The changes in hydrophilic/hydrophobic character of the surface during hydrolysis, can be better depicted by plotting the work of spreading, W_S, for water on the hydrolyzed layers, which is the difference between the work of adhesion $W_a = \gamma_L(1 + \cos\theta_a)$ and the work of cohesion $W_C = 2\gamma_L$. The work of spreading values are shown in Fig. 10. It is clearly seen that the work of spreading is lower for bilayer than that for monolayer. Hence, during the hydrolysis the bilayer is more hydrophobic than monolayer, which is understandable considering DPPC molecules orientation in the bilayer deposited on polar glass surface.

4 Conclusions

The changes in the zeta potential and the surface free energy of solid supported phospholipid DPPC membranes treated with phospholipase A_2 at its different concentrations were investigated. The obtained changes of these two parameters indicate that the phospholipid hydrolysis takes place to an extent dependent on the enzyme content. It is found that the zeta potential of bare silica particles is more stable in TRIS buffer than in NaCl, probably because of the preferred Tris cations adsorption on the silica surface preventing particle aggregation. The addition of DPPC to the system in amounts equivalent to mono- or bi-layer generally decreases the zeta potentials, however the dispersion is stable during the experiment time, probably because of steric stabilization. During PLA_2 enzyme action an increase in negative zeta potential is observed caused by the hydrolysis products, especially negatively charged palmitic acid molecules, which first can form some domains inside the bilayer and than after reaching the appropriate domain size dissolve in the solution, thus decreasing the negative zeta potential value.

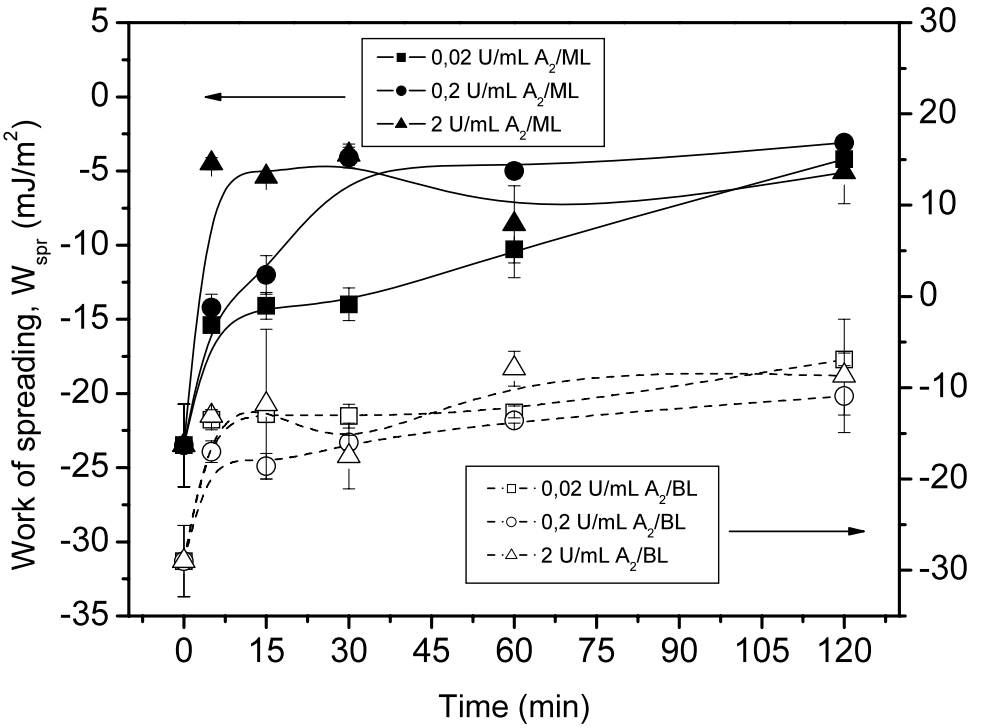

Fig. 10 Work of spreading (in mJ/m^2) of the DPPC mono- and bi-layer deposited on glass, due to phospholipase A_2 action

In the presence of PLA_2 the increase in the hydrophilicity (the surface free energy) of the layers deposited on glass lasted for up to 2 h, although at the enzyme concentration 2 U/mL about 80–90% of the changes took place within first 5 min and the surface became hydrophilic, practically to the same extent as the bare glass support surface. However, at 0.02 U/mL the gradual increase in the surface free energy is observed. This suggests that the methodology described allows to control hydrolysis process in order to obtain surfaces of defined hydrophobic/hydrophilic properties. It seems to us that, these results allow for better insight into the relation between the lipid layers properties and processes taking place at the modified surfaces.

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