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Effect of phospholipid and (phospho)lipase modification on interfacial properties of oil/water emulsion

Agnieszka Ewa Wiącek

Department of Interfacial Phenomena, Faculty of Chemistry,
Maria Curie-Skłodowska University,
Maria Curie-Skłodowska Sq. 3, 20-031 Lublin, Poland,
email: a.wiacek@poczta.umcs.lublin.pl

Dedicated to Professor Bronisław Jańczuk
on the occasion of his 70th birthday

This review paper focused on the effect of typical phosphorlipid (or lecithin) and enzyme modification on electrokinetic parameters of oil/water emulsion. Physicochemical properties of the systems were investigated taking into account the effective diameter of the droplets as well as the zeta potentials using the dynamic light scattering technique. The effect of phospholipid and phospholipase modification on interfacial properties of o/w emulsion was examined as a function of temperature, pH and ionic strength (effect of Na^+ or Ca^{2+} ions which occur in the physiological fluids). The particular role of Ca^{2+} ions in the dispersions with zwitterionic phospholipids (the main components of biological membrane) was confirmed.

The phospholipids dipalmitoylphosphatidylcholine, DPPC or dioleoylphosphatidylcholine, DOPC having the same headgroup bound to the apolar tail composed of two saturated or unsaturated chains were used as stabilizing agents. The effective diameters do not always correlate with the zeta potentials. A possible reason for such behaviour might a mechanism different from the electrostatic stabilization. Phospholipids and their mixtures (e.g. lecithin) may undergo spontaneous aggregation in aqueous solution and self-

organize into liposomes, which possess different sizes and surface affinities. These unique behaviours of phospholipid dispersion can be controlled using the investigated parameters. These findings are expected to increase in importance as phospholipid systems see more use in self-assembly applications.

The other aim of the paper was the comparison of the enzyme phospholipase influence on lipid hydrolysis in the o/w emulsion environment. The work is the study which presents the twofold effect of ethanol dipoles on phosholipid hydrolysis. It is believed that the enzyme effect on the phospholipid aggregation behaviour at the oil-water interface will be helpful for understanding different biological phenomena.

Keywords: dynamic light scattering**,** phospholipid, phospholipase, zeta potential, effective diameter.

1. INTRODUCTION

With biotechnological and pharmaceutical applications in mind the electrokinetic phenomena and interfacial properties of the dispersed systems in the presence of lipids or phospholipids, as well as effects of phospholipases are important for the occurring processes. Phospholipids appear very appropriate because they are principal components of cell membranes. Due to amphiphilic molecules phospholipids are applied as efficient, lipophilic emulsifiers and stabilizers. On the other hand, the phospholipid liposomes are used as intercellular transport tools for vitamins, drugs, proteins, controllers of hydrophobic-hydrophilic balance, catalysts, sensors and wetting enhancers [1-6]. Generally in the phospholipid dispersed systems zeta potential (surface charge), particle size, temperature, pH and ionic strength are important parameters for their characteristics and stability. The pH and ionic strength changes induce those in the electrical charge of phospholipid layer or liposome surface. This is due to the fact that the phospholipid molecule contains $–PO^-$ and $–N(CH_3)_3$ groups, which are in equilibrium with H^+ and OH^-, as well as other ions present in the solution. In the phospholipid dispersion vesicles could be also formed owing to the fact that most phospholipids undergo spontaneous aggregation in water or in aqueous electrolyte solution if shaken. Effect of phospholipid on interfacial properties of dispersed system can be modified by one or a few parameters to obtain interesting results with emphasis on the unique phospholipid phenomena.

This review describes dynamic light scattering, electrophoresis and turbidity measurements of a series of system with phospholipids, which is closest to the concentrations and components of biological relevance. The observation of the changes in droplet size and zeta potentials is a necessary requisite to get information about the complex emulsions stability and possibility of application. The changes are explicable in terms of expected physical principles useful for comparison with the results of other techniques and past experimentation. Physico-chemical properties are important in such systems because are they connected with their biocompatibility. It seems interesting to consider the possibility of functionalizing the model dispersions with the membrane-active biomolecules as the systems of biological significance with the desired biocompatibility. Such investigation of multicomponent lipid-base dispersions is still lacking, because the electrokinetic phenomena on different interfaces are still not well recognized.

It is well-known that dispersion systems with (81hosphol)lipids or (81hosphol)lipids aggregates are very useful as delivery vehicles for drugs and enzymes. On the other hand, enzymes are very sensitive to environmental parameters and chemical characterization of different phospholipid aggregates is necessary to understand their biological efficiency fully [7-12]. The investigated systems are a satisfactory base for biotechnological research. Following this we continued work by investigating the lipid dispersion system with phospholipases. Studies on phospholipases shed light on some of their molecular structure properties, conformational changes occurring in the presence of lipids and phospholipids, 81hosphol of the enzymes existing at interfaces and characteristics of dispersion systems at all. The important aspect of enzyme investigations in dispersion systems is interfacial state of the substrate, i.e. a different form of aggregates: micelles, vesicles or liposomes. Various mechanisms of enzymes adsorption at the surface of aggregated substrate particles (oil droplets, lipid bilayers or monomolecular lipid films) can result from the substrate specificity [13-17]. After adding lipase, electrostatic repulsion may occur between the negatively-charged active site of the enzyme and the ionized fatty acids originating from hydrolysis. The phospholipases are activated at the interface between the water and phospholipid layer surfaces. Much higher phospholipases enzyme activity is observed at such interfaces as vesicles, monolayers or bilayers than in isotropically dispersed phospholipids [16, 17].

Phospholipid monolayers are used as model systems to study in general stereospecific reactions, which enable structural control at the living cells interfaces. The results observed for the hydrolysis of typical phospholipids (DPPC or DOPC) proved that the systems with PLA_2 are very sensitive to the conditions and experiment environment because of its stereoselectively. For the reason that enzymes are active especially at the interfaces their action should also reflect in the electric potential changes. The enzyme activity can be evaluated by changing the dispersion composition [18-23].

The experimental findings were supported by calculation of the electric charge in the shear plane of electrical double layer. Having determined zeta potentials and droplet diameters, the electric charge can be calculated in the presence and absence of the enzyme. As a result it is possible to evaluate the effect of the enzyme on kinetic dispersion characterization. Specific 82hospholipid systems have been studied rarely, although they are often found in natural and synthetic products. By employing a few methods a number of lipid dispersed systems of potential biological application have been characterized. In this way, we hope to emphasize and better understand the processes occurring in biological cells [24-36].

2. MATERIALS AND METHODS

The main materials used in this research were phospholipids 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine, DPPC and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, DOPC (both 99% pure). The phospholipids were purchased from Sigma-Aldrich Chemical Co. (USA). *n*-tetradecane (>99%) was from Fluka (Germany). The substances were used as obtained without purification. The used concentration of DPPC and DOPC gave the same monolayer or bilayer coverage of oil droplets regarding 50 $Å^2$ for the cross-section surface of DPPC molecule and 87.9 $Å^2$ for the DOPC molecule. To compare in measurements the mixture of phospholipids was also used, e.g. cosmetic lecithin. It was purchased from ICHEM Poland. Purity of cosmetic lecithin was investigated by HPLC and Mass Spectroscopy techniques. The main component was phosphatidylcholine (75%), triglycerides (14 %), aliphatic acids (2%), amount of other phospholipids (e.g. cholesterol) was about 9%.

The enzyme phospholipase A_2 (PLA_2) (from *hog pancreas*) was obtained from Fluka and also used as received. The activity of used

enzyme PLA_2 at pH 8 and 37°C was 200 U/mg. That means that enzyme hydrolyzes 200 μmol of 3-*sn*-phosphatidylcholine per one minute (in the presence of Ca^{2+} millimolar amount for phospholipase). In the experiments the phospholipids amount was much lower and hence one may expect that the hydrolysis process should be fast. The appropriate amount of enzyme was added 1 min before the end of the emulsion homogenization (which lasted 10 min at 10,000 rpm) to obtain its final concentration 2 U/cm^3. The incubation time of enzyme (1 min) was chosen in order to assure the enzyme binding to the phospholipid molecules. The pH 8 was adjusted by the addition of NaOH (POCh S.A., Poland) to study the effect of enzyme in the optimal environment. Also, the effect of ions 1 mM NaCl and 1mM $CaCl_2$ was investigated. NaCl and $CaCl_2$ were analytical grade reagents (POCh S.A., Poland).

2.1. Effective diameter and zeta potential measurements

The definition of dispersion systems (e.g. emulsions) is based on the size characteristic of the structure. Well-formulated emulsions should display a narrow size distribution in the 1-1000 nm range. Larger droplets and the increase of their number in time can be the indicator of physical instability. Generally, the size is considered a major issue for biotechnological, medical and pharmaceutical applications since it greatly influences the *in vitro* and *in vivo* studies.

The particle size and multimodal size distribution of the suspensions were measured by the dynamic light scattering (*DLS*) method (also known as photon correlation spectroscopy, *PCS* or quasi-elastic light scattering, *QELS*) using a Zeta Plus Zetameter (Brookhaven Instruments Corporation, USA) at times 5, 15, 30, 60, 120 min and 24 h after emulsion preparation. Dynamic light scattering *DLS* is the powerful method for measurements of nanoparticle system size. *DLS* measures the fluctuation of the scattered light intensity caused by a random movement of the particles or droplets considered as spherical systems. As it is well known, the method provides information on the size distribution of the dispersed particles by analysis of the autocorrelation function of the laser light scattered by the particles undergoing Brownian motion. The method can also be used for the investigation of the state of charge at the liquid-liquid or solid-liquid interface. From the oscillations of the autocorrelation function the electrophoretic mobility distribution can be also obtained. All experiments (besides effect of temperature) were performed at 20 ± 1°C, and the average results of minimum ten repeated runs were

calculated. The zeta potential was evaluated from the electrophoretic mobility data [26, 32].

2.2. Preparation of emulsions

The emulsions were prepared as follows: *n*-tetradecane was dispersed in DPPC, DOPC or cosmetic lecithin/electrolyte solution (or ethanol solution) by mechanical stirring at 10 000 rpm for 10 min (Heidolph Homogenizer) at natural pH or pH 8 at 20 and 37°C ±0.5°C. The pH value of emulsions was regulated by adding a suitable amount of 0.1 M NaOH and was measured using a pH-meter (Mettler Toledo, Switzerland). The effective diameters were determined by means of Brookhaven Zetameter. If the system is polydisperse, the effective diameter weighed by intensity is a value calculated from the averaged intensity of the scattered light by each particle [26]. In the series of experiments with the enzyme, the enzyme was added after 9 min of the emulsion homogenization. After next 1 min of the emulsion homogenization the effective diameters were determined as a function of time up to 2h. It appeared that in most systems this time was sufficient to achieve the 'electrokinetic equilibrium'. For most emulsions two measurement runs of the electrophoretic mobility were taken with five cycles in each run. The zeta potential was calculated from the mobility data [26] and the reproducibility of the results was better than 5%.

3. RESULTS

3.1. Effect of phospholipid

Dipalmitoylphosphocholine, DPPC (area per molecule, S = 50Å^2) and dioleoylphosphocholine, DOPC (area per molecule, S = 87.9 Å^2) are the representatives of very important biologically phospholipids, saturated and unsaturated respectively. Their structures are presented in Figs. 1 and 2.

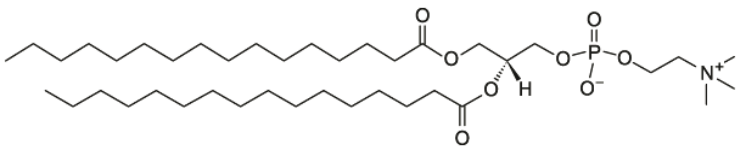

Fig. 1. Molecular structure of DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine).

The phospholipids differ from each other in the presence in the dioleoylphosphocholine, DOPC molecule one double –CH=CH– bond in each of the fatty acid acyl chains. In addition to the variety of lyotropic phases, phosphatidylcholines exhibit rich thermotropic phase behaviour.

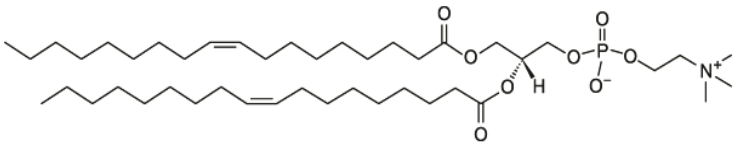

Fig. 2. Molecular structure of DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine).

The presence of cis double bonds in acyl chains causes the greatest decrease of the liquid crystalline-to-gel transition temperature, in this case from about 41°C to –20°C. Due to the low T_m dioleoylphosphocholine aqueous solutions exhibit a fluid phase throughout the temperature range studied. Depending on the solution pH a variety of phospholipid configurations can be envisaged to determine in what way the apolar tails are arranged (horizontally or vertically) with the polar heads directed either towards or away from the oil droplets [37]. Considering the effect of phospholipid modification on the electrokinetic potential of oil/water emulsions, there should be noticed that their molecules are zwitterions in the aqueous medium [38, 39]. Even though in the wide pH range both phospholipids have a zero net electric charge, their charged groups or electron–donor atoms can interact with charged substrates or with water dipoles [40, 41]. On the other hand, cis double bonds make DOPC molecules less rigid and explain its larger area, compared to the DPPC area per molecule [42, 43]. The authors [44], investigated the phase transitions of phospholipids (e.g. DPPC and DOPC) bilayers in a range of temperatures from 20°C to 60°C by AFM measurements. The layer fluidity increase is possible by regulating the lipid composition through changes in the degree of saturation and chain length. It is very useful because increase in the membrane fluidity is necessary for the optimal physiological function [44, 45].

The properties of the systems with phospholipid are sensitive to the conditions and environment in which the experiments are conducted. Electrolyte and pH affect specially the dispersion zeta potential. They are also characteristic of a physiological medium. The phospholipid molecules are zwitterionic and in quite a large pH range they do not possess net charge [40]. Though, the changes of electrokinetic parameters

may also result from ionic strength dependent on changes in orientation of the polar head of phospholipid molecules. Depending on the kind of electrolyte and its concentration behaviour of phospholipid molecules is different and could create various DPPC aggregates in the system.

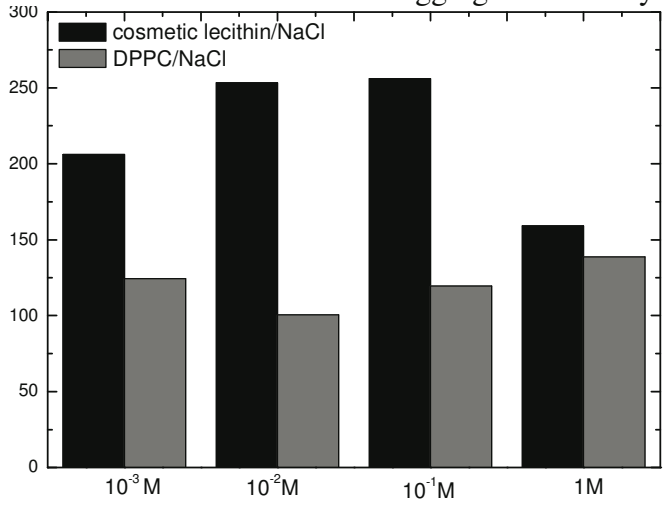

Fig. 3. Effective diameter of DPPC (or cosmetic lecithin) aggregates as a function of concentration of NaCl solution at 20°C.

In Figs. 3 and 4 effective diameters and zeta potentials of DPPC (or cosmetic lecithin) vesicles in the NaCl solution are presented. Na^+ ions can be bounded to the phosphate groups of phospholipid and slightly reduce the zeta potential of the phospholipid vesicles. This effect increases with the electrolyte concentration increase in both cases, for one phospholipid, DPPC and also for the mixture of phospholipids. This is a significant difference in the properties of the systems with DPPC or cosmetic lecithin presence in the NaCl electrolyte solution. The size of cosmetic lecithin vesicles increases with the electrolyte concentration in the range $10^{-3} - 10^{-1}$ M of NaCl and are twice higher than the size of DPPC vesicles. For 1M NaCl the size of both kinds of vesicles are comparable. In Fig. 3 it can be also seen that the most stable vesicles in the NaCl solution were obtained for zwitterionic DPPC although low values of zeta potential (see Fig. 4). On the basis on these results, it may be concluded that the phospholipid vesicles dispersed in the solution stabilize the system mainly as a consequence of steric stabilization.

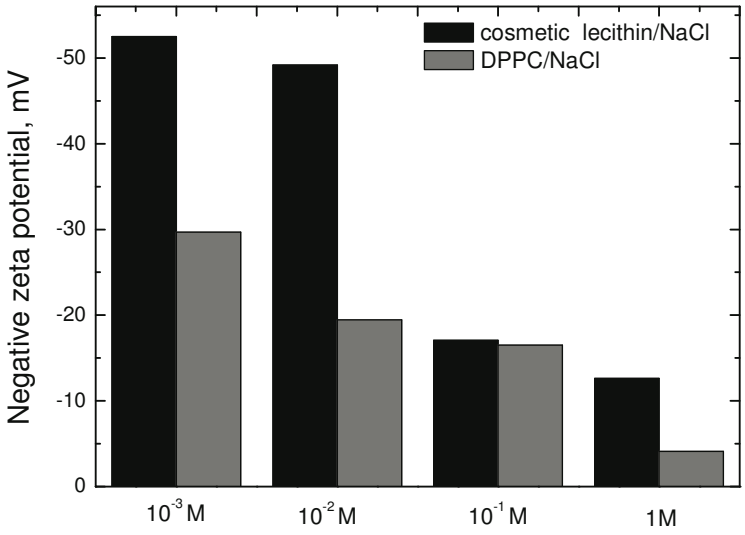

Fig. 4. Zeta potential of DPPC (or cosmetic lecithin) aggregates as a function of concentration of NaCl solution at 20°C.

On the other hand, monovalent cations adsorbing at the hydrophilic part of phospholipid vesicles decrease the negative zeta potential as a consequence, less negative residual charge is present on the phosphorlipid layer surface. The zeta potential decreases with the increasing electrolyte concentration and compression of the double layer thickness takes place. This effect is clearly visible for the cosmetic lecithin vesicles in the NaCl solution. For DPPC dispersions the effect is the same, but to a lesser extent. Surprisingly, similar values of zeta potential of both kinds of phospholipid vesicles were obtained for the 0.1 M NaCl solution.

Finally, the role of mono-, bi- and trivalent cations on the turbidity and transmittance of the investigated systems with the cosmetic lecithin aggregates was investigated. It follows from the experimental studies that the liposomes of each phospholipid had negative zeta potentials and generally, the addition of electrolyte reduces the zeta potential. The similar situation is observable for turbidity measurements. The presence of bivalent cations decrease the value of turbidity from 90 % to 61.8 %, and the presence of La^{3+} cations causes the highest decrease from 90 % to 57 %. The effect of increasing ionic strength on transmittance is of course opposite to the above described effect. It should be also stressed that the effect of ionic strength is very important for lipid dispersion from a biotechnological point of view. Physiological ions (e.g Na^+ or Ca^{2+} ions) can interact with a negative part of the polar head group. It follows

from the molecular simulation [46] and also from the experimental studies [47] that roughly two phospholipid molecules are bound to one Na^+ cation which loses many of its coordinating water molecules. Figaszewski and co-workers suggested that Na^+ can be bounded to the phosphate groups of phosphatidylcholine, thus weakening the internal salt linkage between the phosphate and trimethylammonium groups [48].

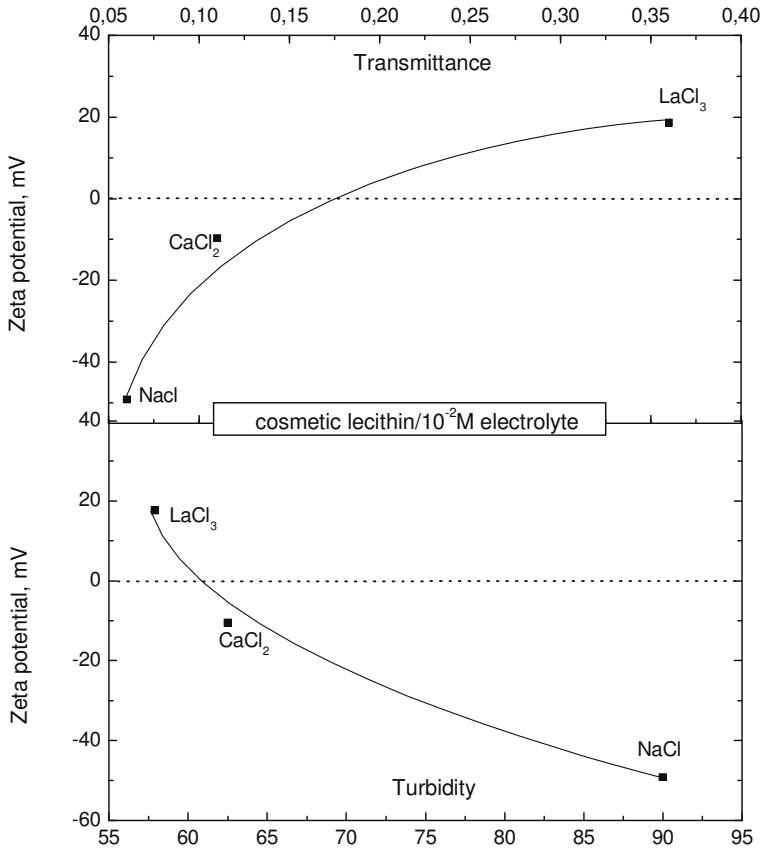

Fig. 5. Relationship of zeta potential vs. transmittance (or turbidity) for the cosmetic lecithin aggregates in 10^{-2} M electrolyte.

On the other hand, the binding of ions to phospholipid vesicles is interesting considering characteristics of phospholipid layer. Bartucci and co-workers [49] using the electron spin resonance spectroscopy studied the effect of high electrolyte concentration (up to 3M) on the unilamellar phospholipid vesicles. They found that the main transition of phosphatidylcholine was not affected. However, in 1 M electrolyte at pH = 3 the pretransition disappeared and at pH = 5 it was shifted from

25°C to 31°C. Moreover, Makino and co-workers [50] suggested that the polar head of phospholipid can reorient depending on the temperature and ionic strength which appeared in the zeta potential changes. Summing up, it may be concluded that the behaviour of the discussed phospholipid systems closely depends on the kind of environment (electrolyte concentrations and ions valence) and is strictly connected with behavior of emulsion systems with phospholipid.

Depending on the kind of electrolyte and its concentration phospholipid molecules behaviour is different and the formation of various phospholipid aggregates in the system is possible. In the *n*-tetradecane/electrolyte emulsions hydrophobic tails of the phospholipid molecule are immersed in the oil droplets. It is obvious, that for the concentrations of phospholipid greater than the appropriate c.m.c., adsorption of fragmentary bilayer, bilayer or vesicles, not individual molecule on oil droplet is more possible. A probable scheme of phospholipid/oil interface is presented in Fig. 6.

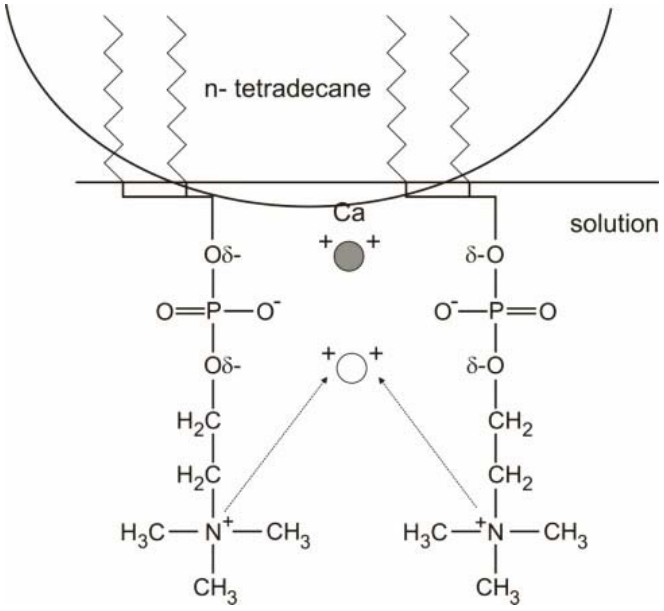

Fig. 6. Scheme of probable *n*-tetradecane/phospholipid interface.

The phospholipid molecules presence usually increases the emulsion droplet size and decreases the zeta potential. On the basis of literature and the experimental results, it is concluded that the lower negative zeta potentials result from the positive $-N^+(CH_3)_3$ groups of phospholipids

present on the oil droplet surface and possibility of ordering of water dipoles. In the emulsion containing phospholipids at the oil droplets/water interface the lipids are aligned with their hydrophilic parts facing the water phase [28, 29, 38]. Since the concentration of phospholipid in this investigation was greater than the appropriate c.m.c., adsorption of its individual molecules rarely occurred.

Applied phospholipids prefer bilayer or vesicle structure, so it is possible that the fragmentary bilayer is present on the *n*-tetradecane droplets. Due to the random nature of the bilayer folding, the formation of vesicles is non-uniform in both shape and size.

It can be assumed that the measured zeta potential is only that of *n*-tetradecane/phospholipid dispersion or phospholipid vesicles themselves, but in both systems the phospholipid aggregates should possess analogous properties and influence on the zeta potential in the same way.

3.2. Effect of temperature

With the presented aim the effect of temperature for the phospholipid modified emulsion was investigated. Regarding temperature of phase transition of phospholipids and to simplify the results description of the effect of temperature was investigated only for the emulsions with DPPC and DOPC, excluding those with cosmetic lecithin. During temperature increase Leonenko et al. [44] observed several DPPC phase transitions with a broad main transition. For DOPC the AFM topography images showed at room temperature the formation of bilayer patches and in the range 37–40°C the expansion of fluid-phase bilayer. On the basis of the obtained results authors [44] stated that the electrostatic forces on DOPC were the same order of magnitude as for DPPC, hence the greatest difference between phospholipid behavior comes from the short-range forces. The major contribution to the layer structure changes is provided by those in steric forces [45]. Additionally, at room temperature the thickness of layer of both phospholipids is very similar. But with the temperature increase, the DOPC thickness was reduced as the effect of the increase of the phospholipid tails lateral mobility and significant decrease of repulsive forces.

The zeta potential from the electrophoretic mobility and effective diameters of *n*-tetradecane droplets with and without dispersed DPPC or DOPC (in electrolyte solution) were measured as a function of time at two different temperatures, 20 and 37°C. The addition of phospholipid decreases the initially negative zeta potential of the *n*-tetradecane

emulsion, and this effect is more evident in the case of DPPC at a physiological temperature near its main temperature transition.

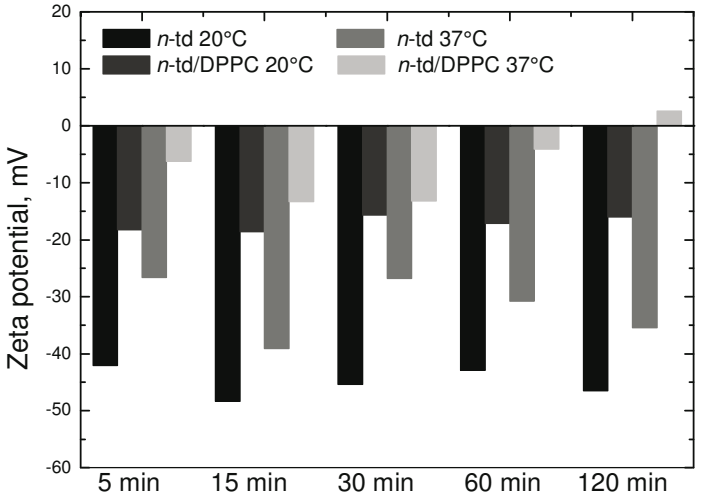

Fig. 7. Zeta potential of *n*-tetradecane droplets or *n*-tetradecane droplets with DPPC at 20 and 37°C as a function of time.

With the increased thermal energy the effect of phospholipid on the zeta potential at 37°C is diminished to about 5-15 mV only. After 2h the value close to 0 mV was obtained (Fig. 7). Makino et al. [50] studying DPPC liposomes suggested that the zero zeta potential occurred if the DPPC polar heads were orientated parallel to the liposome surface. As expected after the phospholipid addition changes of zeta potential are more visible for the DPPC molecules because at 37°C (near its main temperature transition, 41°C) the fluid phase appears. At 20°C the DPPC molecules are in the gel phase. At room temperature the presence of DPPC does not significantly influence the size of the emulsion droplets, which are a little smaller (about 300 nm) than those in the DPPC-free emulsion (Fig. 8). During the first minutes a small decrease in the diameters is observed, which can mean that some amount of larger droplets coalesced and floated up. Then the diameters are quite stable for 2 h, although a value of zeta potential was about –15 mV. It is suggested that the electrostatic repulsion and steric stabilization are present.

The changes in the phase structure to the fluid-like layer of DPPC occurring at physiological temperature, might be responsible for oil coalescence. This effect is not observed for the DOPC emulsions (Figs. 8 and 10). DOPC at the two temperatures is after its main temperature

transition, which is known as much below 0°C. Over a wide range of temperatures, DOPC mixes with water forms a fluid lamellar structure. The double bond in the hydrocarbon chain weakens molecular interactions and packing of the hydrocarbon chains. To some extent these changes in the phospholipid molecule structure are reflected in the effective diameters of the *n*-tetradecane droplets, which are shown in Fig. 10.

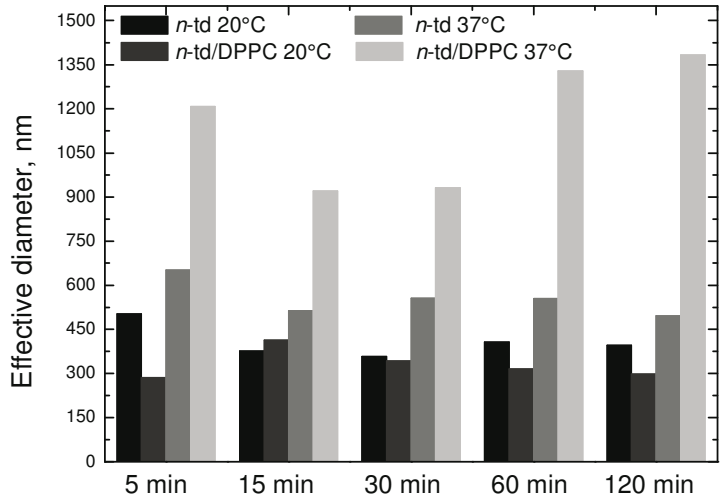

Fig. 8. Effective diameter of *n*-tetradecane droplets or *n*-tetradecane droplets with DPPC at 20 and 37°C as a function of time.

In contrast t the polymorphism of DPPC molecules, the DOPC molecules form a liquid-expanded structure under both investigated temperatures and aqueous phase pH. On the other hand, the changes of zeta potential by DOPC are visible at both temperatures (Fig. 9) probably as an effect of a loose packing of this phospholipid on *n*-tetradecane droplets, because of the presence of double bonds in its molecule. External parameters such as temperature appear to be of primary importance for the system stability**.**

The effect of increasing temperature on the effective diameter of *n*-tetradecane/DOPC emulsion and DOPC aggregates changes is presented in Fig. 10. The largest droplet size of emulsion was obtained at 37°C about 840 nm for 5 min-old emulsion. For the DOPC aggregates the role of steric stabilization is dominant because the effective diameters are stable during 2h measurements although very low values of zeta potential from –5 mV to 1 mV were obtained (Fig. 9).

DOPC is after its main temperature transition, therefore as expected the effect of temperature on the DOPC aggregates is minor comparing this effect on the oil/DOPC emulsion.

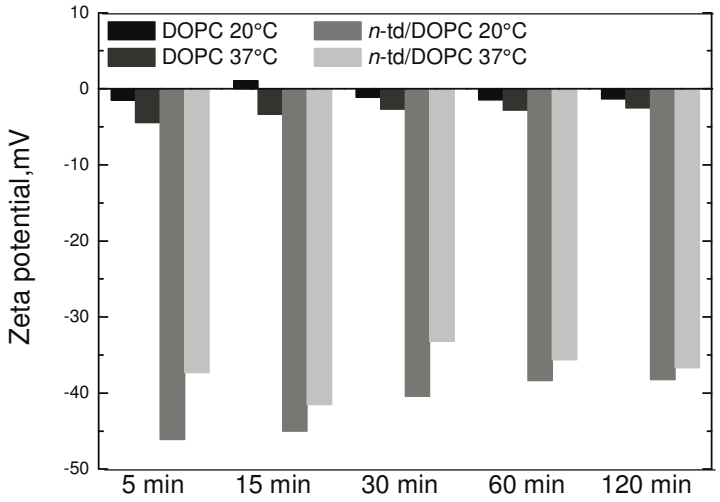

Fig. 9. Zeta potential of DOPC aggregates or *n*-tetradecane droplets with DOPC at 20 and 37°C as a function of time.

At 37°C the structure of DOPC layer can be slightly looser, which can also affect the zeta potential and size of droplets by reorienting the phospholipid polar heads. Additionally, the lamellar gel phase of lipid molecules containing unsaturated acyl chains is more disordered than that of the saturated lipid.

The effect of temperature on *n*-tetradecane emulsion properties is also visible. After the increase from room to physiological temperature the negative zeta potential decreases in the range about 10-15 mV (Fig. 9). The change of zeta potential could be an effect of another orientation of DOPC polar heads on *n*-tetradecane droplets after an increased thermal energy, but not the phase transition, because as was mentioned DOPC at the investigated temperatures is already after its main temperature transition. On the basis of the obtained results it is confirmed that the electrostatic and steric interactions in the emulsion with the adsorbed phospholipid layer are strictly dependent on the composition of the layer and solution pH.

The largest changes of the emulsion zeta potential occur during the first hour after emulsion homogenization. After 2 h the values of emulsion zeta potential at both investigated temperatures, are similar about –38 mV (Fig. 9), but the dependences of effective diameter as

a function of time are different (Fig. 10). Due to the change of DOPC layer structure adsorbed on *n*-tetradecane the largest droplets are observable at physiological temperature although high negative values of zeta potential about –40 mV were measured (Figs. 9 and 10).

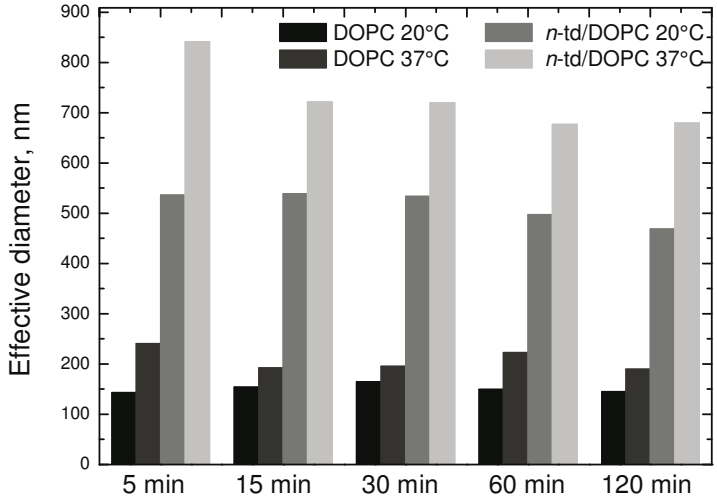

Fig. 10. Effective diameter of DOPC aggregates or *n*-tetradecane droplets with DOPC at 20 and 37°C as a function of time.

3.3. Effect of pH

Phospholipid aggregates formed are stable and additionally could stabilize *n*-tetradecane droplets in the emulsion. As was mentioned early, monovalent cations Na^+ can be bounded to the phosphate groups of phosphatidylcholine and reduce the zeta potential of the emulsion droplets. Multivalent cations, e.g. Ca^{2+} (or La^{3+}) adsorb on the hydrophilic part of phospholipids molecules or phospholipids vesicles and considerably reduce the zeta potential reversing the sign. Alternatively, only divalent cations have ability to promote DPPC vesicles formation and its adsorption on oil droplets. Hence, the effect of Ca^{2+} ions on the emulsion behaviour was also investigated and is described below.

In the investigated emulsions the DPPC vesicles, remaining in the bulk solution, contribute to the observed changes in the electrokinetic potential and diameters. This is a significant difference in the properties of the systems with the DPPC presence in the NaCl or $CaCl_2$ electrolyte solution (Figs. 11-14).

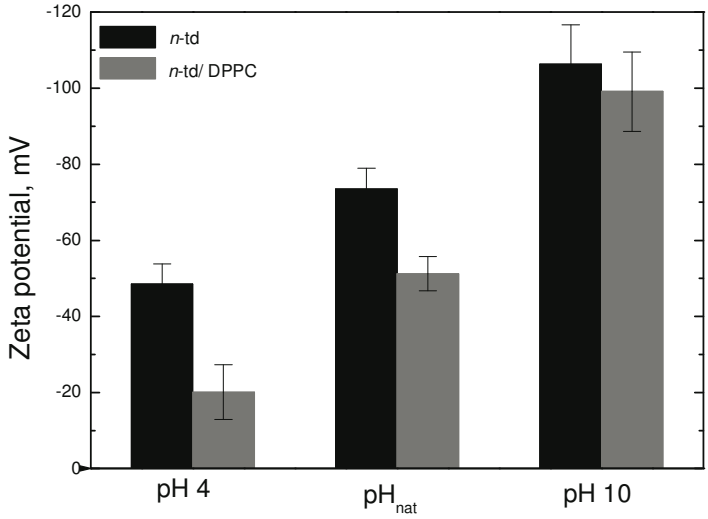

Fig. 11. Zeta potential of *n*-tetradecane droplets or *n*-tetradecane droplets with DPPC in 10^{-3} M NaCl solution at 20°C at three different pH values.

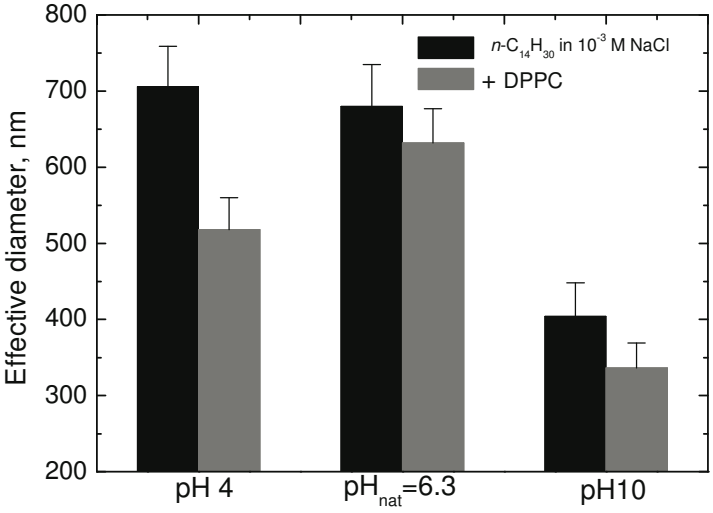

Fig. 12. Effective diameter of *n*-tetradecane droplets or *n*-tetradecane droplets with DPPC in 10^{-3} M NaCl solution at 20°C at three different Ph values.

Divalent cations have ability even at low concentration to promote DPPC vesicles adsorption and bilayer formation. Moreover, these cations are known to bind strongly to the DPPC head group, thereby screening repulsion, and perhaps promoting deposition of the additional bilayer. In the case of emulsion at acidic and natural pH the reversal of the ζ

potential is observed (Fig. 13), which is probably due to specific adsorption of Ca^{2+} at the outer surface of DPPC vesicles. The same conclusion was drawn on the basis of measurements of electrophoretic mobilities of phosphatidylcholine (from egg yolk) vesicles under different concentrations of electrolytes. The magnitude of the electrokinetic potential was influenced strongly by composition of the aqueous phase. The zeta potential becomes smaller with the increasing electrolyte concentration as a result of shrinkage of the electrical double layer at high ionic strength. It means that the specific adsorption of Mg^{2+} ions should be taken into account.

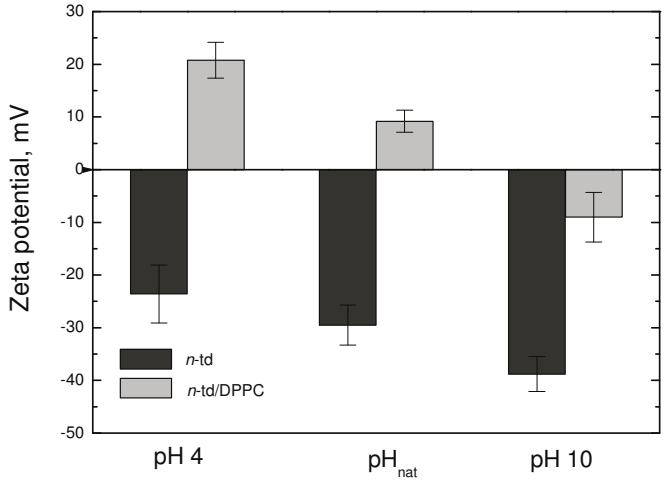

Fig. 13. Zeta potential of *n*-tetradecane droplets or *n*-tetradecane droplets with DPPC in 10^{-3} M $CaCl_2$ solution at 20°C at three different pH values.

Another explanation of calcium effect on the DPPC molecules in the aqueous system is the fact that calcium ions are able to bind, orient and polarize the carbonyl carbon atom in the phospholipid molecule. This is in favour of the nuclophilic attack by the water molecule during hydrolysis. As a result, less negative residual charge is present on this carbonyl carbon atom, which appears in the decrease of the negative zeta potential. It should be also stressed that an increase in ionic strength of the solution improved neutral lipid adsorption on oil droplets and system stability. Ca^{2+} ions in physiological concentrations may stabilize membranes. The importance of calcium ions is implicated in both structural and functional activities including besides membrane stability, enzyme activation and ion conductance. The effect of Ca^{2+} ions is very important in drug (or even nucleic acid) delivery systems.

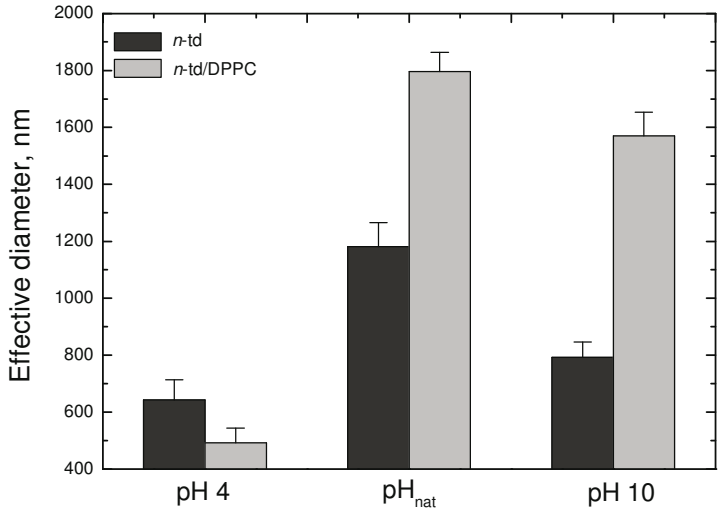

Fig. 14. Effective diameter of *n*-tetradecane droplets or *n*-tetradecane droplets with DPPC in 10^{-3} M $CaCl_2$ solution at 20°C at three different Ph values.

3.4. Electrokinetic charge

According to the DLVO theory, interactions between the *n*-tetradecane droplets with the adsorbed phospholipid and between its vesicles are controlled by the van der Waals and electrostatic forces. From a theoretical point of view, vesicles and cells are usually regarded as colloidal particles. The application of the DLVO theory to study interactions between cells has been reported before [19]. In the studies, variation in composition, separation distances between layers and layers thickness were considered in the calculation of the interactions between phospholipid vesicles for both planar and spherical systems. The magnitude of the van der Waals forces varies depending on the kind of electrolyte and could be scaled with the Hamaker constant for two droplets or two vesicles interacting in the medium. Moreover, non-DLVO forces such as steric forces and hydration effect on the DPPC molecules adsorbed on the *n*-tetradecane surface should be taken into account [19, 30-36]. Using the highly sensitive dynamic AFM technique, Higgins et al. studied the hydration forces between the oil and DPPC aggregates and found four distinct regions of water on the DPPC membrane surface [51]. The simulated and experimental results show the presence of clathrate-like structures of DPPC solvating $-N^+(CH_3)_3$ positively charged

groups too. Therefore, it can be concluded that the water molecules are located between the polar heads of DPPC molecules, which are adsorbed at the droplet surface [51, 52].

In the *n*-tetradecane/electrolyte emulsion stabilized by the phospholipid vesicles the zeta potential may be related to acid–base interactions. During experiments for the systems with the presence of phospholipids the negative zeta potential decreases, which could indicate that the DPPC molecules replace water immobilized dipoles near the *n*-tetradecane droplets and form hydrogen bonds with the dipoles. Additionally, steric forces related to membrane undulation or bulging are proposed to arise from the short-range intermolecular forces among phospholipid molecules, but act repulsively on a longer lateral scale [30-36]. On increasing the diameters the van der Waals attraction probably becomes dominant over the repulsive force by hydration. From the analysis of a similar system using electrostatic interaction, it was concluded that the interaction of phosphatidylcholine vesicles with the octane/10^{-3} M $MgCl_2$ interface rather occurs in the constant surface charge condition, not with the constant surface potential. In the case of the oil/water systems, potential energy gives negative values indicating the existence of an attractive force. They also concluded that the adsorption of the vesicles at the oil/water interface is determined by the DLVO force in the initial stage, but is mainly determined by the short range interaction in the final stage.

Zeta potential is an useful indicator of particle (emulsion droplets) surface charge to predict the stability of systems during the storage and in the biological medium. At this time it is stated that the zeta potentials above 25 mV are required for total electrostatic stabilization [24-26]. This rule cannot be precisely applied for the systems which contain steric stabilizers, e.g. polymers, phospholipids or protein, because their presence usually decreases the zeta potential due to the shift in the shear plane and steric stabilization should be taken into account. The obtained zeta potentials of *n*-tetradecane or *n*-tetradecane/phospholipid emulsions are negative and relatively stable, especially for the emulsion with DOPC (Figs. 7, 9). Most of the oil-in-water emulsions show negative zeta potentials because as we suggested in previous papers [24, 25, 28, 29] the water (ethanol) dipoles might play a role in the zeta potential creation. H^+ and OH^- ions were found to be potential-determining for the *n*-alkane/electrolyte, *n*-alkane/alcohol and *n*-alkane/protein emulsions. After the phospholipid addition the negative zeta potentials decrease, which can indicate that phospholipids adsorb on the *n*-tetradecane

surface. Differences between the effect of phospholipid on emulsion electrokinetic properties are dependent on the phospholipid structure presented in Figs. 1, 2.

At natural pH some DOPC and DPPC molecules will be positively charged because their pK_1 is in the range 3.8-4.0. It can be assumed that phospholipid molecules adsorb on the *n*-alkane surface with their hydrophilic heads directed toward the aqueous phase. The lipid molecule possesses a long hydrophobic tail, so its molecules may be 'immersed' in the *n*-alkane droplet, and then the polar head groups should be oriented toward the ethanol solution**.** This conclusion may be supported by measurements of others. Lately authors [53] have carried out the experiments of phospholipid coating on the glass plates hydrophobized by contact with *n*-tetradecane. They suggested that the DPPC molecules may dissolve to some extent in the thick *n*-alkane film and then expose their polar heads over the film surface thus producing large polar electron–donor interactions. Moreover, using contact angle and electrophoretic mobility measurements they stated that on the hydrophobized glass surface the saturated phospholipid layers are highly ordered and polar heads are accessible for water molecules to interact with by hydrogen bonds. The average thickness of formed *n*-tetradecane layer should have properties comparable to those of bulk *n*-tetradecane [53].

3.5. Effect of ethanol

In the technological systems the role of alcohol on the arrangement of lipids and hydrogen bonding creation should be taken into account [54]. The layer created by the phospholipid molecules in the ethanol solution is more flexible than in the pure water. Binding of ethanol molecules decreases the dipole moment of phospholipid molecule [55]. The amphiphilic nature of ethanol favours an interfacial location, and interactions are driven by both the opportunity for hydrogen bonding and hydrophobic interactions. Its presence has an effect on the main transition temperature causing distinct changes in the mechanism of phosphor-lipid/water (ethanol) phase structuring. Moreover, transition reversibility at low ethanol concentrations but irreversibility at its high concentrations was found.

Ethanol partitioning is more favourable in the unsaturated bilayers, which are characterized by their more disordered nature compared to the saturated layers. Regarding this, the effects of ethanol on oil emulsions with saturated or unsaturated phospholipid were investigated (DPPC and DOPC). In the case of *n*-tetradecane/phosphatidylcholine-water (ethanol)

emulsion, it can be concluded that ethanol molecules are located between the polar heads of DPPC molecules which stretch the droplet surface. During molecular simulation and spectroscopic methods authors [54-56] found that ethanol can form hydrogen bonds with the phosphate acceptor sites of phospholipid molecules and its molecules were located between the phosphate and the carbonyl groups.

For unsaturated lipids, a considerable amount of disorder exists not only in the liquid phase, but also in the gel one, which contrasts the behaviour observed for the saturated phospholipids. At room temperature the DPPC molecules are in the gel phase. At 37°C the fluid to gel ratio may be close to 1. Also occurrence of ethanol in the *n*-tetradecane/phospholipid emulsions could cause the earlier phase transition of phospholipids. Ethanol and water molecules form hydrogen bonds with the phospholipid molecules which are of comparable strength [25]. Phospholipid molecules can replace water and ethanol immobilized dipoles at the droplet surface and form hydrogen bonds with them, which appears in the change of the system zeta potential [24, 25, 28, 29]. Furthermore, the phospholipase activity might be greater in the ethanol-treated systems, but the literature data on this effect are ambiguous [57, 58]. The role of alcohol dipoles was successfully demonstrated in the zeta potential formation of *n*-tetradecane droplets. Those investigations provide insight to the properties of the PLA_2 hydrolysis process enhanced by added ethanol, which will be discussed in the next section.

3.6. Effect of enzyme

Investigations of the phospholipid lipolysis by PLA_2 and the efficiency of the enzyme action in the emulsion environment are deficient. Phospholipases A_2 are a family of small, water soluble lipolytic enzymes. These enzymes can be found both inside and outside the cells showing high affinity for the aggregates of phospholipid (vesicles, micelles and bilayers). In the case of PLA_2 enzyme, one ester bond at *sn*-2 position in the phospholipid molecule is hydrolyzed producing one molecule of fatty acid and a water soluble lysophospholipid molecule. Wacklin et al. [59] suggest, that accumulation of fatty acid molecules at the interface is accompanied by the increased adsorption of PLA_2, which enhances the hydrolysis. Since membranes under physiological conditions are in contact with electrolyte solution, their specific interactions with ions are a matter of substantial interest. Ions play an essential role not only in the structure, dynamics, and stability of membranes, but also for binding of proteins and membrane transport [60-63]. The present study

explores the effects of both electrolyte ions and ethanol molecules on phospholipid hydrolysis by phospholipase. The same is important in the emulsion with phospholipids. It seemed interesting to investigate the effect of ions on the properties of *n*-tetradecane/phospholipid emulsions. The obtained changes of zeta potential and effective diameter of *n*-$C_{14}H_{30}$ /DOPC emulsions in ethanol solutions at 37°C (at pH = 8) with and without PLA_2 enzyme is presented in Figs. 15-16.

The obvious role of ions for the systems with charged lipids is to compensate the lipid charge. However, several studies have confirmed that ions interact in an analogous way in the zwitterionic lipid bilayers [64]. This effect is clearly visible because after introducing ions to the solution, the evident decrease in negative zeta potential value is observed. Both Na^+ and Ca^{2+} ions bind to the head-groups of phospholipid and alter e.g. the lipid head dipole orientation. Ion pairing drives alkali cations to the negatively charged phosphate and carbonyl groups. Alternatively, not bound chloride anions are uniformly distributed in the water phase near the membrane surface or are co-adsorbed at the choline region in the outer part of the membrane [61, 63]. Distribution of ions close to the phospholipids molecules is confirmed by change of zeta potential in the direction to positive values. In the *n*-tetradecane/DOPC emulsions in 1 M ethanol the effect of calcium chlorides is not compensated by ethanol because modification of zeta potential after ions introduction is large about 20 mV (see Fig. 15).

On the basis of molecular dynamic simulations in 1 M NaCl the number of bound ions was calculated to be about one cation per three lipid molecules [65, 66]. Lee and co-workers [67] for phospatidylcholine observed complexes including three, four and five lipid molecules and found that four-coordinate Na^+ was the most common [67]. The presence of sodium ions rigidifies the headgroup region and ions also act to increase attractions between the neighbouring lipids. This effect is more significant for bivalent ions because the degree of chloride penetration to the phospholipid layer is higher for Ca^{2+} than for Na^+.

Indeed, we obtained very stable values of zeta potential and effective diameter for the emulsions with calcium ions, which confirms a stabilizing effect of Ca^{2+} on the adsorbed lipid layer in the *n*-tetradecane/DOPC emulsions. In the *n*-tetradecane/DOPC emulsions the electrolyte ion distribution near oil droplets generates a dipole moment that opposes that of the lipid headgroups modulated by the polarized ethanol molecules. Furthermore, the water polarization will change sign and be reduced considerably compared to that in the salt-free

system, resulting in a net potential change. The influence of phospholipase enzyme on the DOPC hydrolysis in the emulsion environment was investigated. The obtained relationships for the *n*-tetradecane/DOPC emulsions in the $CaCl_2$ solution at 37°C are presented in Figs. 15 and 16.

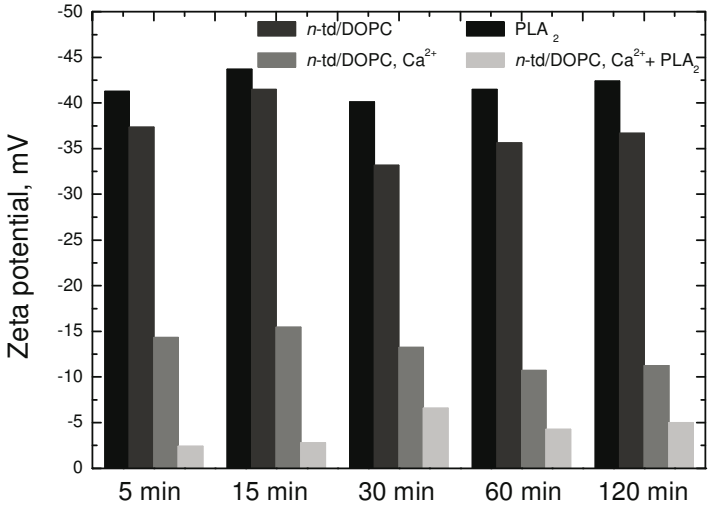

Fig. 15. Zeta potential of *n*-tetradecane droplets with DOPC (and Ca^{2+}) or in the presence of PLA_2 at 37°C as a function of time at pH 8.

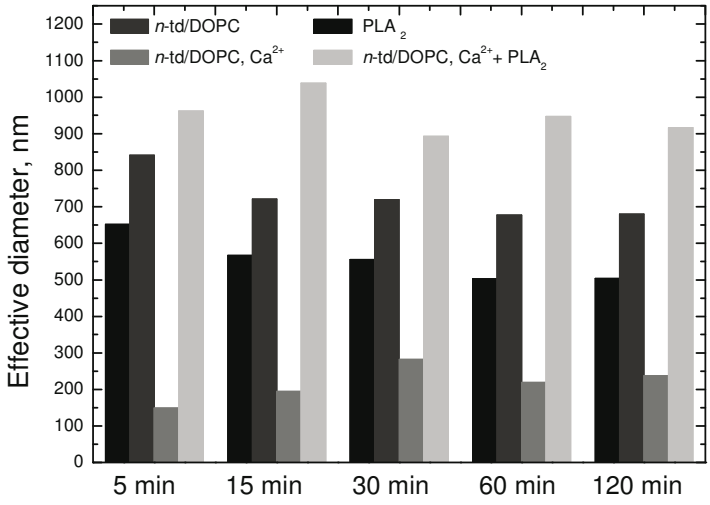

Fig. 16. Effective diameter of *n*-tetradecane droplets with DOPC (and Ca^{2+}) or in the presence of PLA_2 at 37 °C as a function of time at pH 8.

Evidently, the PLA_2 enzyme action appears in the zeta potential and the effective diameter changes, which depends also on kind of electrolyte and valence of ions. Besides the fact that there are many different mechanisms of phospholipase action on the phospholipid layer and rates of hydrolysis reaction, it is well known that PLA_2 enzyme hydrolyzing the ester bond at the second position in the DOPC molecules causes formation of bioactive products (oleic acid and lysophospholipid) [68].

The enzymatic lipolytic reaction is an important case of heterogeneous catalysis, where the water–soluble enzyme acts at the interface of insoluble lipid substrates. The amount of phospholipid used in our experiments, which can be hydrolyzed with respect to the activity of used phospholipase is much lower than its value. Consequently one may expect that the enzyme should hydrolyze all phospholipid molecules and the process should be fast. Indeed, the results of zeta potential and effective diameter of emulsion droplets at 37°C suggest that generally the hydrolysis reactions run fast because in most systems the potentials are changed soon after the enzyme addition. The largest changes of zeta potential are during the first 30 minutes of measurement. The previous studies showed that phospatidylcholine molecules deposited on the solid support underwent hydrolysis in less than 1 h. In the dispersion system with a large area (volume) practically all enzyme molecules initially present in water are fixed at the interface. Similarly to our previous paper describing DPPC emulsions [24, 25, 28, 29] we stated that the emulsion droplets with the adsorbed DOPC have a large total surface and this is probably the additional reason for speed enzyme action.

Kinetics of enzyme hydrolysis at different interfaces depends on the substrate organization and molecular environment of the catalytic reactions. Mircheva et al. [69] studied the hydrolysis of dioleoylphosha-tidylcholine by PLA_2. The measurements of surface area and surface pressure at the air-water interface showed that the enzymatic catalytic activity occurring in the monolayer is more efficient than at the vesicles or nanocapsule interfaces. Under the experimental conditions, at 37^oC the lipid bilayer of DOPC is in the liquid-crystalline state. PLA_2 penetration and activity in the case of such structured DOPC is similar to the native PC hydrolysis [68]. On the other hand, the cyclic voltametric techniques [8] have shown that the DOPC hydrolysis process by PLA_2 is fitted well by the first-order kinetic equation. The authors found that under the two pH conditions studied (7.5 and 8.6), the highest activity of enzyme was at about 6 mM Ca^{2+} concentration. However, even when calcium ions were

not added, there was still some hydrolytic activity probably as the effect of residual Ca^{2+} present during the enzyme preparation.

The above quoted data shed light on our results. All investigated systems with Ca^{2+} ions presence are very stable when equilibrium is achieved. The slight decrease in the negative zeta potential of emulsion caused by phospholipase enzyme at 37°C (Fig. 15) from about –12 mV to –4 mV could be explained by readsorption of the hydrolysis products, first oleic acid and also calcium ions, on the droplet surface. During the DOPC hydrolysis occurring lysophospholipid might form the micellar aggregates. Oleic acid molecules remain on the droplet surface or may form an inverse hexagonal phase [69]. On the basis of the obtained relationship of zeta potential and effective diameter we stated that hydrolysis starts during homogenization and ends within 30 min. The changes of zeta potential and size of emulsion droplets are visible immediately after the first 5 min. After 30 minutes the hydrolysis slows down or even ends, because after that time no change of measured parameters is can be practically seen. Wacklin et al. [59] suggest that accumulation of fatty acid molecules at the interface is accompanied by the increased adsorption of PLA_2, which increases hydrolysis. As mentioned earlier, the rate of DOPC hydrolysis in the investigated oil/water emulsions may be also advanced by ethanol molecules. Kisiel et al. [60] studied the influence of ethanol on the activity of pancreatic phospholipase A_2. Based on the obtained results they stated that in the presence of ethanol (3-9%) the rate of DOPC hydrolysis in the binary liposomes increases by a factor of 2-3.5.

Moreover, Leonenko et al. [44] found that at room temperature the thickness of DPPC and DOPC layers is very similar, but with the temperature increase, the DOPC thickness is decreased considerably. It is known that lipid compression is much easier in the fluid phase due to the higher disorder of tails, mobility of lipids and lower tail density. Such conclusion proved the changes of the emulsion effective diameter as a function of time presented in Fig. 10. The smallest size of droplets (c.a. 220 nm) which are very stable was obtained for *n*-$C_{14}H_{30}$ /DOPC, Ca^{2+} emulsion in comparison to *n*-tetradecane/DOPC emulsion (c.a. 700 nm), which suggests the stabilizing effect of bivalent ions. Besides, the cofactor function in phospholipid hydrolysis by phospholipase, Ca^{2+} ions can stabilize the colloidal system by electrostatic forces. After the PLA_2 enzyme addition significant increase of effective diameter to about 960 nm is observed (Fig. 10). This results from reduction of electrostatic forces decreased and that after DOPC hydrolysis

the role of steric stabilization is diminished too. The products of hydrolysis not soluble in water probably remain on the oil droplet surface. Consequently, the effective diameters may be larger than those of *n*-tetradecane/DOPC droplets. Moreover, the accumulation of DOPC hydrolysis products can modify the enzyme activity and the kinetic constant enhances enzyme autoactivation [69].

4. CONCLUSIONS

Studies of phospholipid system properties are of importance to many areas of research and technology [1-12]. The aim of this paper was the investigation of electrokinetic properties of phospholipids aggregates (or cosmetic lecithin aggregates) and analogous system in the emulsion environment. The main used technique was the dynamic light scattering. The behaviour of *n*-tetradecane/zwitterionic phospholipid systems was a direct consequence of different molecule structures and main temperature transition of phospholipid. In general, the steric barrier brought by liquid-crystalline structures is assumed to be the main stability mechanism for the oil/phospholipid emulsions. The effect of ethanol on both investigated emulsions was comparable probably because chain unsaturation has only a minor effect on the ethanol distribution function. It was also successfully demonstrated that the hydrolysis reactions run fast advanced by ethanol molecules. In most systems the change of the zeta potential and the size of emulsion droplets was visible immediately after the enzyme addition.

The other aspect of investigation was the characterization of oil/DOPC in the electrolyte emulsions with PLA_2 and the effect of physiological ions. Both Na^+ and Ca^{2+} ions bound to the head-groups of phospholipid alter their structural layer properties. Generally physiological ions act to increase attractions between the neighbouring lipids. In the investigated emulsion it was manifested as a decrease of the effective diameter**.** The obtained results confirmed the particular role of calcium ions in the colloidal systems. Besides the cofactor function of Ca^{2+} ions in the phospholipid hydrolysis, their stabilization function due to electrostatic forces was concluded. The ionic strength effects on phospholipid behaviour are of considerable importance and our findings provide further insight into the effects of salt on cell membranes. Knowledge of these relations with the corresponding interfacial phenomena can be extended towards a multicomponent lipid-based

emulsion prepared under controlled conditions. It seems that the investigated emulsions are a model system for biotechnological research and for understanding of many biological phenomena.

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CURRICULUM VITAE

Agnieszka Ewa Wiącek *studied chemistry at Maria Curie-Skłodowska University and in 1994 was employed as a research assistant at the Department of Physical Chemistry. She received Ph.D. degree in chemistry in September 2000 on the base of PhD. thesis including the effect of natural stabilizers on oil/water emulsion stability. In 1997-2004 she was an examiner of Recruit-ment Commission of Chemistry Faculty in Lublin and outer cities. Her research interests is gene-rally concentrated on physico-chemical properties of dispersed systems (mainly emulsion) and their stability; the structure and wettability of the polymer and biopolymer layers; interfacial phenomena and the electrokinetic properties of solid-liquid interface. In 2013 she received Dr. habilitation degree in physical chemistry writing dissertation titled "Effect of the selected biologically-active substances, mainly phospholipids and (phospho)lipases on the interfacial properties of dispersed systems". She published over 35 scientific papers in professional journal (including 12 monoauthor's paper and chapter in monograph of Marcel Dekker). Additionally she is an author of 40 conference' articles.*