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Complexation of phosphocholine liposomes with polylysine. Stabilization by surface coverage versus aggregation

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Abstract

Complexation between linear poly-L-lysine (PLL) and negatively charged phosphocholine unilamellar liposomes has been investigated by means of dynamic light scattering, microelectrophoresis, and differential scanning calorimetry. It is found that complexation results in charge inversion (vesicle coating/stabilization) or vesicle aggregation depending on various experimental conditions. Complexation in dependence on PLL concentration and molecular mass, lipid phase state, rate and order of liposome and PLL mixing and time evolution of complexes are investigated and discussed. Aggregation profiles are determined and size distribution of the aggregates formed is studied, leading to the possibility of aggregation control. The time evolution of vesicle aggregation shows particle enlargement consisting in particle growth up to the irreversible formation of thermodynamically stable aggregates of about 2 μm in diameter. The formation of stable aggregates is in agreement with theoretical predictions of colloid particles aggregation by an interplay of long range electrostatic repulsion and short range attraction. Differential scanning calorimetry reveals that physical adsorption occurs exclusively on the vesicle surface and the lipidic organization is not significantly disturbed. The present study describes multivariable aspects of the complexation process between liposomes and polyions which results in the formation of a new class of still poorly defined colloids. These results allow establishing and optimization of a procedure for fabrication of polycation-stabilized vesicles to be used for various applications such as drug delivery.

Keywords: Polylysine; Liposomes; DPPC; Adsorption; Coating; Stabilization; Aggregation

1. Introduction

Interaction of liposomes to each other and with macromolecules attracts great attention because of the interest to simulate intercellular, polymer–cell and liposome–cell interactions, [1–3] as well as the interest in soft-matter physics [4,5].

Liposome complexation with polyelectrolytes has been thoroughly investigated in recent years by many authors. Together with lipoplexes (complexes of DNA with positively charged vesicles) [6–14], complexation of negatively charged vesicles with polycations was intensively studied [15–24]. The latest attracts attention due to similar processes happening in living organisms when negatively charged cells interact with positively charged macromolecules. Moreover, various polycations were found to increase the permeability of cell membranes [25,26].

Besides simulation of processes in living systems and fundamental interest in liposome–polymer interactions, studies of covering of vesicles by physical polymer adsorption as an alternative to stealth liposomes [27] is important in order to stabilize such fragile colloidal carriers. Recently, we have shown that PLL coating of liposomes stabilizes them against disruption upon adsorption of vesicles on charged surfaces [28,29]. This is of high interest to prepare stable surface-modified liposomes to be used for medical and non-medical applications [30–33].

The phenomenology of complexation between a polyelectrolyte and a charged colloidal particle of opposite sign has

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attracted much attention but is rather complicated and depends on a variety of parameters including the charge density of the polyion and of the particle surface, the ionic strength, and the flexibility of the polyelectrolyte backbone. We have chosen a positively charged, linear, and flexible polyelectrolyte, PLL, to be complexed in the presence of low salt content (15 mM NaCl) with charged liposomes having about 10% of charged surface groups. We chose to work at such a small value of the ionic strength in order to reduce the probability of vesicle aggregation. In addition a pretty small fraction of charged lipids was selected because at high charge density (mole fraction of charged groups exceeding 30%) a complete vesicle disruption upon complexation with the synthetic polycation poly(*N*-ethyl-4-vinylpyridinium bromide) was reported [17].

Complexation of anionic liposomes with polycations was intensively studied for synthetic [15–18,34,35] and natural ones [20–24] showing a strong tendency to vesicle aggregation. This research has revealed a variety of interesting observations like transmembrane migration, reversible adsorption of the adsorbed polyelectrolyte molecules, etc. However, no study was done concerning the mechanism, the structure and time evolution of the formed aggregates as well as complex preparation conditions giving the possibility to efficiently control the aggregation/stabilization. The present work addresses these questions giving additional knowledge on the complexation mechanism of vesicles with charged macromolecules.

In this study the interaction of linear PLL with unilamellar phosphocholine liposomes has been investigated by combining dynamic light scattering, microelectrophoresis techniques, and differential scanning calorimetry. The effect of PLL/liposome charge ratio, PLL concentration and molecular mass, lipid phase state as well as mixing rate of PLL and liposome solutions on the complexation process are revealed and discussed. Aggregation of liposome–PLL complexes and time evolution of aggregation are under consideration.

2. Materials and methods

The used chemicals are the following: 1,2-Dipalmitoyl-*sn*-Glycero-3-Phosphocholine (DPPC), 1,2-Dipalmitoyl-*sn*-Glycero-3-(Phospho-*rac*-(1-glycerol)) (Sodium Salt) (DPPG) were purchased from Avanti Polar Lipids. Cholesterol (CL, C-8667, Sigma, USA), tris(hydroxymethyl)aminomethane (TRIS, Sigma, T1503, USA), NaCl (Prolabo, France), Poly-L-lysine hydrobromide (PLL) with viscosimetric molecular masses of 2, 28, and 280 kDa (Sigma, ref. P8954, P7890, and P1399, respectively), Triton X-100 (Aldrich, 23,472–9) and 5(6)-carboxyfluorescein (CF, Fluka, 21877). Throughout the study, 10 mM TRIS-buffer containing 15 mM NaCl, pH 7.4 was used and mentioned in the text as TRIS-buffer. All materials were used without further purification. The water used in all experiments was prepared in a three stage Millipore Milli-Q Plus 185 purification system and had a resistivity higher than 18.2 MΩ·cm.

2.1. Vesicle preparation

Unilamellar liposomes composed of a DPPC/DPPG/CL mixture (80/10/10 w/w) and loaded with CF were prepared by mechanical extrusion. Lipids and cholesterol were dissolved in chloroform (DPPG was dissolved in chloroform: methanol mixture, 1:1 v/v) and mixed in 100-ml round bottom flask. The solvent was removed by rotary evaporation at 35 °C and intensive rotation under a pressure of 300 mbar during 20 min, then overnight at 30 mbar (room temperature). The thin lipid film (total mass of 10 mg) was hydrated at 55–60 °C with 2 ml of a 0.2 mg/ml solution of CF in TRIS-buffer. The obtained turbid suspension was then subjected to 11 freeze–thaw cycles (quenching in liquid nitrogen for 8–10 min followed by heating in water at 55–60 °C for 15 min). One milliliter of the suspension was then extruded 13 times through track-etch polycarbonate membranes with a stainless steel Avanti Mini Extruder operated at 55 °C. The extrusion was done firstly with a 400-nm polycarbonate membrane followed by extrusion with a 100-nm membrane at the same temperature conditions. Un-entrapped CF was removed by gel-permeation chromatography at a pressure of 1 atm. To this aim a 2×20 cm^2 glass column filled with Sephadex G-50 (gel volume 35 ml) equilibrated with TRIS-buffer was used. The fraction of the liposomes (elution volume 12–16 ml) was collected and diluted to a total volume 20 ml. The liposome suspension was finally stored at 4 °C and used within 2 weeks.

2.2. Determination of lipid concentration

The efficiency of liposome fabrication was determined as the ratio between lipid content in liposome solution to the amount of lipids used to prepare the vesicles. To this aim, the lipid concentration in the liposome solution was determined by the Böttcher method [36]. Content of DPPG was calculated using the assumption that the DPPC:DPPG:CL ratio in the final liposome preparation is equal to the ratio used to prepare the liposomes.

2.3. Interaction of PLL with vesicles

Half a milliliter of PLL solution in 2 ml Eppendorf tubes was placed in an Eppendorf Thermomixer Compact (Sigma, USA) at temperature of 25 °C or 54 °C under agitation (950 or 300 rpm). Liposome solution (0.35 mg/ml lipid concentration) was heated to 25 °C or 54 °C and dropped with a constant rate during one min into the agitated PLL solution (10^{-4} to 10^{-1} mg/ml) maintained at the same temperature. Mixing of the liposome and PLL solutions at 54 °C has been done inside an oven in order to keep a nearly constant temperature. Then agitation was stopped. Dynamic light scattering measurements were performed 5 or 30 min after liposome and PLL solution mixing and each measurement (3 accumulations of 30 s) was repeated 2–3 times for reproducibility.

2.4. Dynamic light scattering

Dynamic light scattering measurements were performed using an HPPS 500 apparatus (Malvern Instruments, UK). Light scattered by the sample was detected at an angle of 173° with laser source operating at a wavelength of 632.8 nm. The sample cuvette was placed in a thermostated cell maintained at varying temperatures between 10 and 55 °C. The analysis of DLS autocorrelation functions of the scattered light intensity has been carried out with the ALV-correlator v3.0 software, which allowed to determine the distribution of the scattered intensity versus the hydrodynamic radius.

2.5. Electrophoretic mobility

Electrophoretic mobility of liposomes and liposome–PLL complexes was measured by laser microelectrophoresis in a thermostated cell using a Zetasizer Nano ZS (Malvern, UK). The zeta-potential was calculated from the measured electrophoretic mobility using the Schmolukovski equation.

2.6. Differential scanning calorimetry (DSC)

DSC measurements were performed using the Micro-Calorimetry System (MCS DSC, MicroCal Inc., Northampton, MA, USA) at a temperature ranging from 15 to 60 °C with a scan rate of 0.5 °C/min. Before use, the samples with a lipid content of 0.7 mg/ml were degassed. TRIS-buffer was used as reference solution. The scanning was done 3–4 times after cooling down to the initial temperature in order to check for the reproducibility and the reversibility of the phase transition.

3. Results and discussion

3.1. Liposome complexation with PLL

It is found that upon complexation two different situations are encountered: the liposomes can be covered well with PLL molecules and stay in a non-aggregated state or they can aggregate to each other (Fig. 1, A→B and A→C, respectively). PLL–liposome complex formation induced by mixing of liposome and PLL solutions has been studied by measuring the average hydrodynamic diameter and electrophoretic mobility of the diffusing complexes in the solution using the dynamic light scattering technique. PLL with molecular masses of 2, 28, and 280 kDa (for short PLL2, PLL28, and PLL280, respectively), mixing rate and mixing order, as well as lipid phase state (solid and liquid) and duration of storage between the preparation and the measurement were considered to have a possible effect on complexation. PLL concentration was varied from 10^{-4} to 10^{-1} mg/ml corresponding to the dilute regime of polyelectrolyte solution. Indeed, in this PLL concentration range, the volume fraction of PLL varied between 7×10^{-8} and 7×10^{-5} assuming a partial specific volume of 0.7 $cm^3\ g^{-1}$ as is usually done for polypeptides [37]. It should be mentioned that a liposome solution of constant vesicle concentration was added drop-wise to a PLL solution of various concentrations at a constant dropping rate (if it is not specified) in order to standardize the mixing procedure and to have PLL excess during the interaction in such a way to minimize possible aggregation.

DPPG is a negatively charged lipid, while DPPC is a neutral lipid, and CL is uncharged. Thus, PLL interaction with vesicles is determined by polycation binding to phosphate groups of DPPG. A plot of the normalized hydrodynamic diameter of the particles formed (d/d_o, were d is an average diameter of the formed particles, $d_o=(129\pm2)$ nm is the diameter of native vesicles) versus charge molar ratio of the mixture between charged lipid and the total amount of available PLL, (Z=PLL/DPPG) gives a complexation profile. However, the average hydrodynamic diameter used to construct such complexation profiles does not give a real idea of the diameter distribution of the formed particles in the system. However, any changes in the shape or location of this profile indicate changes in the complexation/aggregation process and provide some useful information.

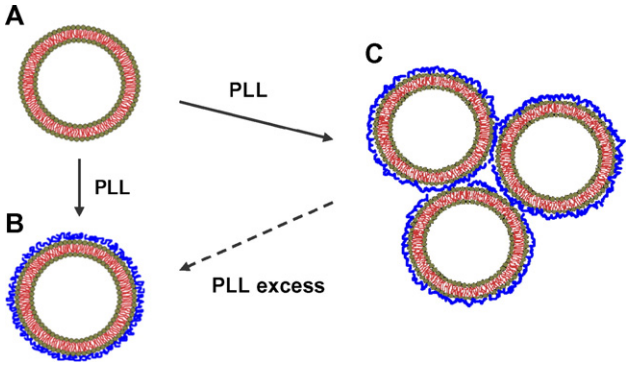

Fig. 1. Scheme of complexation between liposomes (A) and PLL leading to formation of stable PLL-covered single liposomes (A→B) or liposome aggregates (A→C). Disaggregation induced by PLL addition is depicted with a dashed arrow (C→B).

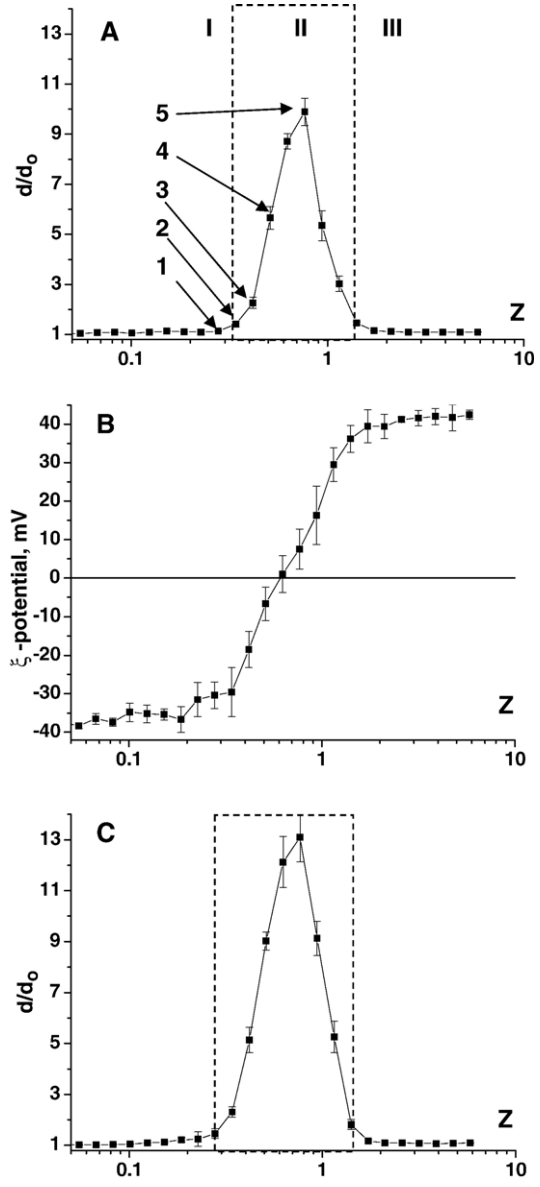

Fig. 2. Normalized particle diameter (d/d_o) versus PLL/DPPG charge ratio Z of the liposome–PLL280 complex. Measurements were done 5 min (A) or 30 min (C) after complex preparation by mixing of PLL and liposome solutions at 950 rpm. (B) Zeta-potential of particles studied in panel A. Aggregation range is depicted with dashed lines. d and d_o—average hydrodynamic diameters of complexated and native vesicles, respectively (d_o=129 nm). All experiments were carried out at 25 °C.

A typical complexation profile for PLL280 is presented in Fig. 2A. With an increase in the PLL concentration, and hence an increase in Z, the average size of the particles present in the system, starting from a value close to that for native vesicles ($d/d_o=1$), increased to values exceeding 1 μm due to formation of aggregates, and then decreases again to a final value of about 1.1 corresponding to well-covered PLL-stabilized liposomes.

The complexation profile might be divided into three regions—I, II, and III as could be seen from Fig. 2A. Region I

corresponds to the beginning of liposome coating with PLL until to a state, $Z \sim 0.3$ when aggregation between liposomes is taking place. Region II, for Z values between about 0.3 and 1.1 corresponds to what we will call the "aggregation range". We define this "aggregation range" as appearance of intensity-weighted peak corresponding to particles containing at least dimers—triplets or higher order aggregates. However, since the aggregates may be anisotropic and we calculate only the hydrodynamic radius of their equivalent sphere, we will not heavily emphasize upon their aggregation number. Note that the precise width of the "aggregation range" is defined by the sensitivity of the used light scattering apparatus. This is due to the fact that aggregates scatter light considerably stronger than non-aggregated vesicles. In region III of the aggregation profile, it seems that single PLL-coated liposomes are formed again upon mixing of vesicles and PLL solutions. This is apparent from the constant value of the particle diameter. However, the obtained value a little exceeded the diameter of the uncoated vesicles because of adsorbed PLL molecules which necessarily will increase the hydration diameter of the vesicle. For clarity, the "aggregation range", or region II, is depicted as a dashed frame in all graphs presenting a complexation profile.

As already mentioned, the average hydrodynamic diameter used to construct a complexation profile cannot provide one with a precise diameter distribution of the particles present in solution. Therefore, particle size distributions were examined for several experimental points in Fig. 2A. Fig. 3A represents

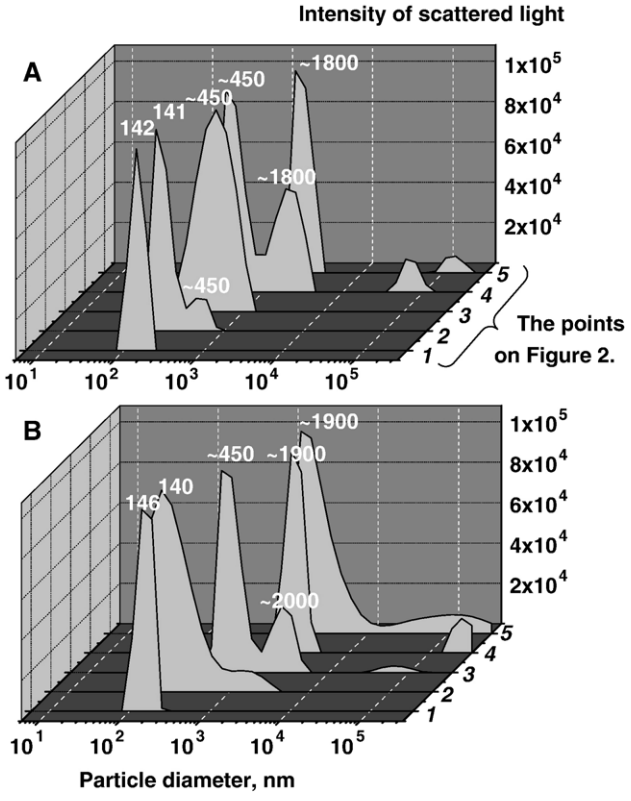

Fig. 3. Intensity-weighted size distributions of particles formed by PLL–liposome complexation corresponding to samples depicted as points 1–5 in Fig. 2A. An average particle diameter is presented above the defined peak. Measurements are done 5 (A) and 30 (B) min after complexation at 25 °C.

intensity-weighted size distributions of particles formed for the points 1–5 in Fig. 2A. Lane 1 of Fig. 3A corresponds to the last point of region I in the aggregation profile displayed in Fig. 2A. One well-defined peak for this point indicates the presence of single vesicles of about 140 nm in diameter (normalized diameter closed to 1.1). The fact that the vesicle diameter exceeded slightly the diameter of native vesicles (129 nm), is certainly due to the effect of surface coverage with PLL molecules. Lane 2 corresponds to the first point of region II ("range of aggregation" in Fig. 2A). A second peak, corresponding to diameters of about 400–500 nm together with a peak of single vesicles is observed. This new peak corresponds clearly to the appearance of liposome aggregates. Thus, the average calculated diameter for the distribution of lane 2 is equal to 183 nm (normalized diameter of 1.4) because of the two detected populations of particles. Lane 3, corresponding to Z of about 0.4, shows the exclusive presence of small aggregates while the peak corresponding to single particles has totally disappeared. The corresponding average normalized diameter of about 2.5 confirms the presence of only small aggregates. Together with small aggregates, a population of larger particles of about 2 μm in diameter is apparent in lane 4, while lane 5 corresponds to the presence of a unimodal population of particles of about 2 μm in diameter. It should be noted that for lanes 4 and 5, very large aggregates of about 100 μm in diameter, were also detected.

The cupola-like shape of the aggregation profile (Fig. 2A) is explained by an increase of the population of particles with larger size. Big micron-sized particles (see Fig. 3A, lanes 4 and 5) scatter a lot of light but are present in a very small concentration in the mixture. Hence they are not found at all in a number-weighted size distribution analysis. Crossing the threshold of the aggregation profile (Fig. 2A), the situation is similar (profile is symmetric). A precise understanding of particle enlargement will now be considered with respect to both the mechanism of aggregates formation and the time evolution of the aggregation process.

3.2. Mechanism of aggregation

Aggregation of colloids interacting with oppositely charged molecules can occur for a variety of particle sizes in different time scales, strongly depending on the interaction strength between colloidal particle and aggregation inducers—macromolecules [4,38,39], surfactants [40], salts [41]. The reversible or irreversible character of the aggregation mechanism leads to uni- or multi-modal distribution of growing aggregates [39–41].

When the charge provided by the adsorbed PLL coating is sufficient to neutralize the liposome surface charge, the PLL-coated particles have strong tendency to form aggregates. This is clearly indicated in Fig. 2A by means of the particle size increase. The reason behind liposome aggregation may be a non-homogeneous overcompensation of surface charge of liposomes by the adsorbing PLL molecules. The aggregates could be formed when one liposome with a polycation-covered domain interacts with another liposome by binding to its non-covered oppositely charged domain. The

closer to charge neutralization, the bigger is average size of aggregates.

However, the maximum of the aggregation profile corresponds to Z close to 0.7, but not to the expected isoelectric point of $Z=1$. This is not surprising if one considers the structure of the lipidic bilayer. Taking into account a homogeneous distribution of DPPG molecules in the inner and the outer leaflet of the lipidic bilayer, the number of DPPG molecules exposed to direct contact with PLL in solution is half the number of initially provided DPPG molecules. Assuming that the number of lipid molecules is the same on both leaflets of the vesicular membrane is a very good approximation for vesicles larger than 100 nm in diameter since the effect of the membrane thickness (about 5–6 nm) and curvature can be neglected with respect to the average vesicle diameter. However, it holds only if any lipid migration between both leaflets is restricted. The Z value of about 0.7 at the maximum of the aggregation profile in Fig. 2A occurs at a larger Z value than the expected one (0.5) on the basis of the occurrence of electrostatic interactions. The difference between the observed maximum positions in the aggregation profile with respect to the expected position could be attributed to the adsorption of a PLL amount larger than necessary to just neutralize the vesicle surface. This phenomenon is known as charge inversion which is due to lateral correlation between adsorbing molecules [4,38]. Hartmann and co-authors reported that just a half of PLL groups are bound to the charged lipid membrane [22].

The shape of the electrophoretic titration curve, constructed by measuring the electrophoretic mobility for each sample at a different Z value is presented in Fig. 2B. It confirms the previously suggested charge inversion of the liposome–PLL complexes above Z values of 0.7. A negative value of the ξ-potential is changed to a positive one within the "aggregation range". The biggest aggregates have an average charge close to zero, indicating charge neutralization of the covered vesicles. When the surface charge is close to zero, electrostatic repulsion between complexes is absent on average and the presence of an attractive potential, attributed to Van der Waals forces, leads to strong particle aggregation. When the surface charge of the liposomes is reversed, here for $Z>0.7$, an electrostatic repulsion force prevents aggregation and leads to the formation of single PLL-coated vesicles. Single vesicles are detected at $Z<0.3$, in region I of the complexation profile, when the PLL concentration is not high enough to change the vesicle surface charge by a significant amount.

In addition the vesicles used in this study were filled with CF. Absence of dye leakage from the internal compartment of the vesicles allows one to test liposome integrity upon complexation. No fluorescence was found after the big aggregates (point 5 in Fig. 2A) were separated from solution by filtration through ultrafree microcentrifuge filters (Sigma, MWCO 100 kDa). This indicates that integrity of vesicles just after aggregation is saved (the aggregates were filtrated just after preparation). This observation is in agreement with the proposed scheme depicting the aggregate structure (Fig. 1). However, it is not unexpected that some structural transformations occur upon vesicle aggregation which would lead to membrane rupture or fusion [42]. Therefore, a detailed study of liposome stability and of the structure of vesicles coated with a PLL layer is the topic of a separate research (D.V. Volodkin, H. Mohwald, J.-C. Voegel and V. Ball, unpublished data).

3.3. Time evolution of the aggregation

Time evolution has also to be considered in order to investigate the aggregation mechanism. The visual observation of the aggregate containing suspension shows the following: the complexes formed in region II do not sediment in a time interval of about a few hours. But after 5–10 h a white precipitate is formed. This precipitate consists of huge aggregates. In addition, it is observed that the closer to the top of aggregation profile, the shorter is the time needed to obtain a precipitate. This is related to the previous observation that the average particle charge is close to zero in the region of the maximum in the aggregation profile. At the beginning of the aggregation profile, for $Z \sim 0.3$–0.4 (for instance the experimental point 2, Fig. 2A), aggregation is negligible and no sedimentation is observed at all even after a few days indicating that the solution consists of a stable colloidal suspension. It has to be noticed that in this region of the aggregation profile the particles have a sufficient zeta potential (−30 mV) to prevent formation of large clusters, (Fig. 2B).

Fig. 2A and C shows the complexation profiles obtained 5 and 30 min after complex formation, respectively. One can see that the "aggregation range" is practically not changed after prolonged incubation of the formed complexes, but the height of the profile is significantly increased. This strongly suggests that aggregates are growing with time. Fig. 3A and B presents the intensity-weighted size distribution of particles formed at experimental points 1–5 (Fig. 2A), 5 and 30 min after mixing of the vesicle and PLL solutions, respectively. With time, peaks corresponding to larger aggregates are appearing (lane 3) or growing (lane 4). Careful analysis of the graphs A and B (Fig. 3) revealed the existence of a three-modal population of aggregates—(i) small aggregates corresponding mainly to single vesicles or very small aggregates, (ii) aggregates of about 2 μm in diameter, and (iii) huge aggregates of about 100 μm in diameter. It is observed that vesicles from population (i) aggregate progressively to be transited to belong to population (ii): 30 min after complexation the small aggregates are partially or completely enlarged to 2-μm-sized aggregates (see lanes 2–4 in parts A and B of Fig. 3). However, as vesicles reach this size of around 2 μm in diameter, further growing was not observed at least in the time interval between 5 and 30 min. For Z around 0.7 huge micron-sized aggregates are present in very small amounts even after 30 min of storage.

Taking into account all these observations, one can try to draw some conclusions about the investigated aggregation mechanism. The presence of well-defined populations of aggregates (Fig. 3) indicates that the aggregation process does not involve simple particle enlargement, but accumulation of aggregates of a finite size. Upon aggregation large aggregates coexist in equilibrium with smaller ones. Hence, one can conclude that the interaction between the vesicles and PLL leads

to the formation of thermodynamically stable or metastable aggregates with a diameter around 2 μm - population (ii). These aggregates always appear when aggregation takes place and their relative population is increasing with time.

These stable 2-μm-sized aggregates may contain thousands of liposomes sticking to each other. Formation of such aggregates can be explained by a "stabilization mechanism" which has been previously reported in the literature by Groenewold and Kegel [39]. These authors have shown that a small charge on a single colloidal particle is sufficient to stabilize large particle aggregates with a high aggregation number. This leads to formation of big aggregates on the order of some micrometers. This mechanism has been used to describe [9] the formation of lipoplexes of well defined size. This stabilization arises in a system characterized by the presence of long-range electrostatic repulsions and short-range attractive forces. The basic assumption of this mechanism concerns the presence of a small amount of charge on each complex colloidal particle. Up to a critical aggregate size (in our case 2-μm-sized particles), repulsion forces between particles are not sufficient to counterbalance the short-range attraction, which finally leads to efficient particle sticking. Upon additional growth of aggregates a sufficient charge should be accumulated and the aggregate stops to grow. Close to zero charge provokes clustering of the stable aggregates leading to a huge precipitate—which in the present system leads to the third (iii), growing up with time, aggregate population. This clustering proceeds over a long time scale, typically hours or even days.

Sennato et al. [11] reported the formation of polyacrylic acid-coated cationic liposome aggregates. Coexistence of two components—stable aggregates of 100–500 nm in diameter and growing unstable large aggregates of micrometer size were also observed. The coexistence of these different aggregate populations is in agreement with our findings. Formation of such stable aggregated particles is pretty peculiar for colloidal particles, and the observation of similar behaviours in the case of cationic and anionic vesicles gives evidence that electrostatic forces are responsible for complexation/aggregation.

3.4. Reversibility of aggregation

It is now of interest to assess if the observed aggregation process is of reversible or irreversible nature. It has to be noted that at low Z values (region I of the complexation profile) no large aggregates are found, as for the experimental point 1 in Fig. 2A. However, to get a complex at such a Z value a liposome solution was dropped into a PLL solution during 1 min. Upon this time, Z is shifting from high values of about 9 to the final one of about 0.3 (calculated from the variation of the number of added drops of the liposome solution: this number is equal to 1 after the first addition and the final value is of about 30). Upon vesicle titration, Z passes through all the values corresponding to the "aggregation range", region II (Z=0.35–1.1). Hence two situations are possible: either there are some aggregates that are formed and are subsequently dissolved (see Fig. 1C→B) or the amount of time available for aggregation, about 1 min, is not sufficient. At this stage we cannot conclude about the reversible–irreversible character of the aggregation process.

In order to check the reversible character of the described aggregation process, an excess of PLL up to a final ratio exceeding Z=5 was added to the samples prepared at experimental conditions corresponding to the points 2 and 4 (Fig. 3A). The size distribution of the new particles in the final mixture was then examined 10 min after PLL addition. The peak at about 450 nm in diameter (lane 2 in Fig. 3A) disappeared, while no changes were found when particles corresponding to the lane 4 in the same figure were also subjected to the same PLL addition. This confirms our prediction that the particle population of about 2 μm corresponds to highly stable aggregates. Formation of such thermodynamically stable aggregates takes some time. If these aggregates are not formed, smaller aggregates, which are less stable, could be dissolved by addition of excess of PLL or liposomes entering regions III or I, respectively. This is happening upon dropping (see above). However, when stable aggregates are formed, the aggregation is not reversible anymore.

To follow the formation of stable 2 μm sized aggregates, a liposome solution was mixed with a PLL solution to give a Z value of 0.7 (corresponding to the maximum of the of aggregation profile). The change of the solution turbidity was then followed as a function of time. A quasi steady turbidity

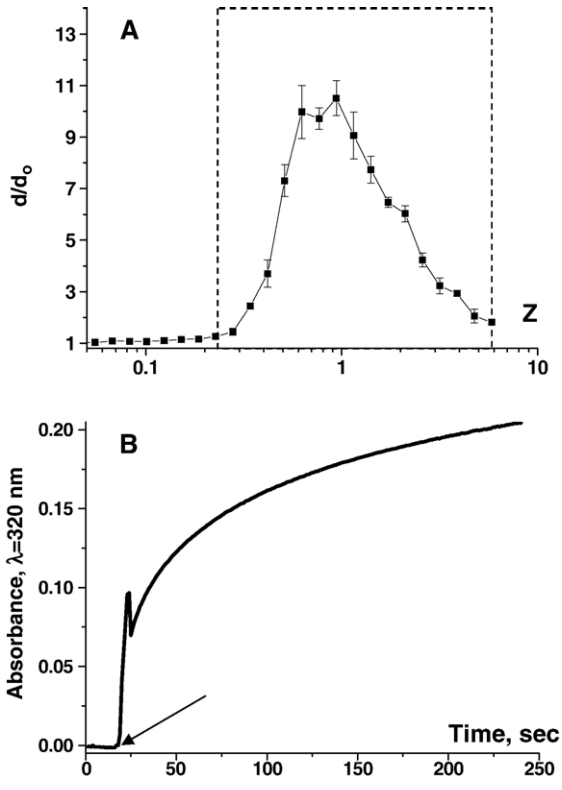

Fig. 4. (A) Normalized particle diameter (d/d_o) versus PLL/DPPG charge ratio of the liposome–PLL280 complex. Measurements were performed 5 min after complex preparation by mixing of PLL and liposome solutions at 300 rpm. (B) Increase of optical density (at 320 nm) of a solution obtained by mixing a liposome solution and PLL280 solution, at a time indicated by the arrow, at molar charge ratio Z=0.7. All experiments were carried out at 25 °C.

level was achieved in about 4 min (Fig. 4B). The rise of optical density just after mixing could be due to formation of various small aggregates followed by a continuous growth of irreversible stable aggregates. Thus, one can conclude that upon dropping of liposomes into a PLL solution, which is achieved during 1 min, time is not sufficient to lead to the formation of irreversible aggregates, but the formed small aggregates are dissolved towards single particles. Therefore, the samples prepared at $Z<0.3$ contain no aggregates (see the beginning of the paragraph 3.4).

3.5. Order and mixing rate of liposome and PLL solutions

It should be stressed that the "aggregation range" (Fig. 2A) is rather wide since it extends from $Z=0.3$ to $Z=1.1$. Since we have found above that the formation of small aggregates is due to a kinetic effect it is worth to study the evolution of the aggregation profile shape in dependence of the mixing rate of liposome and PLL solutions. Indeed by changing this parameter one changes the transport rate of interacting species and this could well be the rate limiting step of the whole aggregation process.

Only small attention has been given to study the influence of the component mixing order and of the mixing rate of liposomes and polyions upon preparation of their complexes. Kennedy et al. [43] have studied the influence of the order of component addition on the structure of lipoplexes obtained upon mixing DNA and anionic vesicles made from 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine. They have reported different structures when either the vesicles were injected in the DNA solution or when the DNA solution was dropped into the vesicular solution. However, the formed complexes were not coated vesicles but lipid–DNA particles obtained after vesicle rupture. The vesicle rupture could have been caused by strong DNA–lipid interaction when vesicles are composed of charged lipids. Such vesicle ruptures have indeed been observed when the fraction of the vesicle headgroups carrying a charge exceeded 30% [17].

We demonstrate here that the mixing rate has a strong effect on complexation. Figs. 2A and 4A represent the normalized diameter of liposome–PLL complexes formed upon mixing liposomes and PLL solutions at an agitation speed of 950 and 300 rpm, respectively. In these experiments, the time needed to drop the vesicles in the PLL solution as well as all the other experimental conditions were not changed. It is evident from the graphs that upon a decrease of the agitation speed, the "aggregation range" is significantly wider. Shaking of liposomes at 950 rpm does not induce any change in the liposome size indicating no effect of agitation. Formation of liposome–PLL complexes depends on component intermixing and, hence, on transport processes of PLL to the surface of the negatively charged vesicles. This means that vesicle surface overcharging upon PLL adsorption is very fast—it takes parts of seconds. Upon decrease of the agitation speed, a complete intermixing of PLL and liposomes takes longer time and the rate at which PLL is provided in the non-agitated layer around each vesicle is too small to allow for homogeneous charge overcompensation. This results in widening of the aggregation profile. In addition, the complexation profile becomes asymmetric as seen in Fig. 4A.

The complexation profile obtained at an agitation speed of 950 rpm becomes considerably wider if the order of mixing of the PLL and liposome solutions is reversed - PLL solution is added to liposome solution (data not shown). This observation again points to a very fast PLL surface overcharging taking less time than complete intermixing. The width of the aggregation profiles obtained at different agitation speeds and mixing order were practically not changed when the aggregates were characterized by light scattering after prolonged time of storage. The time needed to build up small and also stable large irreversible aggregates also strongly depends on the liposome concentration. Indeed at slow agitation (300 rpm instead of 950 rpm) seconds are needed for complete intermixing, and high local concentration of liposomes leads to faster formation of rather large amount of stable aggregates. As a result, the aggregation profile is certainly wider due to the presence of irreversibly grown particles (see Figs. 2A and 4A).

3.6. Effect of PLL molecular mass on the complexation profile

It is also worth to consider the effect of PLL molecular mass on the shape of the aggregation profile since this parameter is expected to influence the charge overcompensation which was shown to be associated with the stabilization of non-aggregated vesicles (Fig. 2B). To this aim, we investigated the complexation between liposomes and PLL molecules with a viscosimetric molecular mass of 2, 28, and 280 kDa. Fig. 5A–C presents the aggregation profiles for PLL of various molecular masses (all the other parameters are held constant). Upon a decrease of the molecular mass of the PLL chains the "aggregation range" becomes wider and at the extreme case, for PLL2, the aggregation profile could not be defined at all (it was often not possible to calculate the size distribution from the obtained autocorrelation function due to the presence of very big aggregates whose size certainly exceeds the size obtained for other PLL samples). At first glance, this result is unexpected because of the following reason. It is more difficult for high-molecular-weight polymer to realize multiple ionic contacts with liposome membrane due to slower diffusion to liposome surface and higher number of the ionic contacts per one molecule. This should result in slower surface coating and hence charge inversion for PLL280 than for PLL28 and PLL2 and as a consequence stronger aggregation in the case of high-molecular-weight polymer. Moreover, a "bridge aggregation" could be more pronounced in the case of longer chains. However, the experimental results are opposed to the expected ones. The actual experimental finding was that longer chains are more efficient in reducing the width of the aggregation profile and this observation was explained by a careful analysis of the zeta-potential of the particles used for construction of the aggregation profile for PLL of different molecular masses. These experiments were all done at 25 °C, hence when the lipids are in solid state. A typical zeta-potential curve for PLL280 is presented in Fig. 2B. At high Z, the ξ-potential reaches a plateau value indicating surface overcharging. However, these

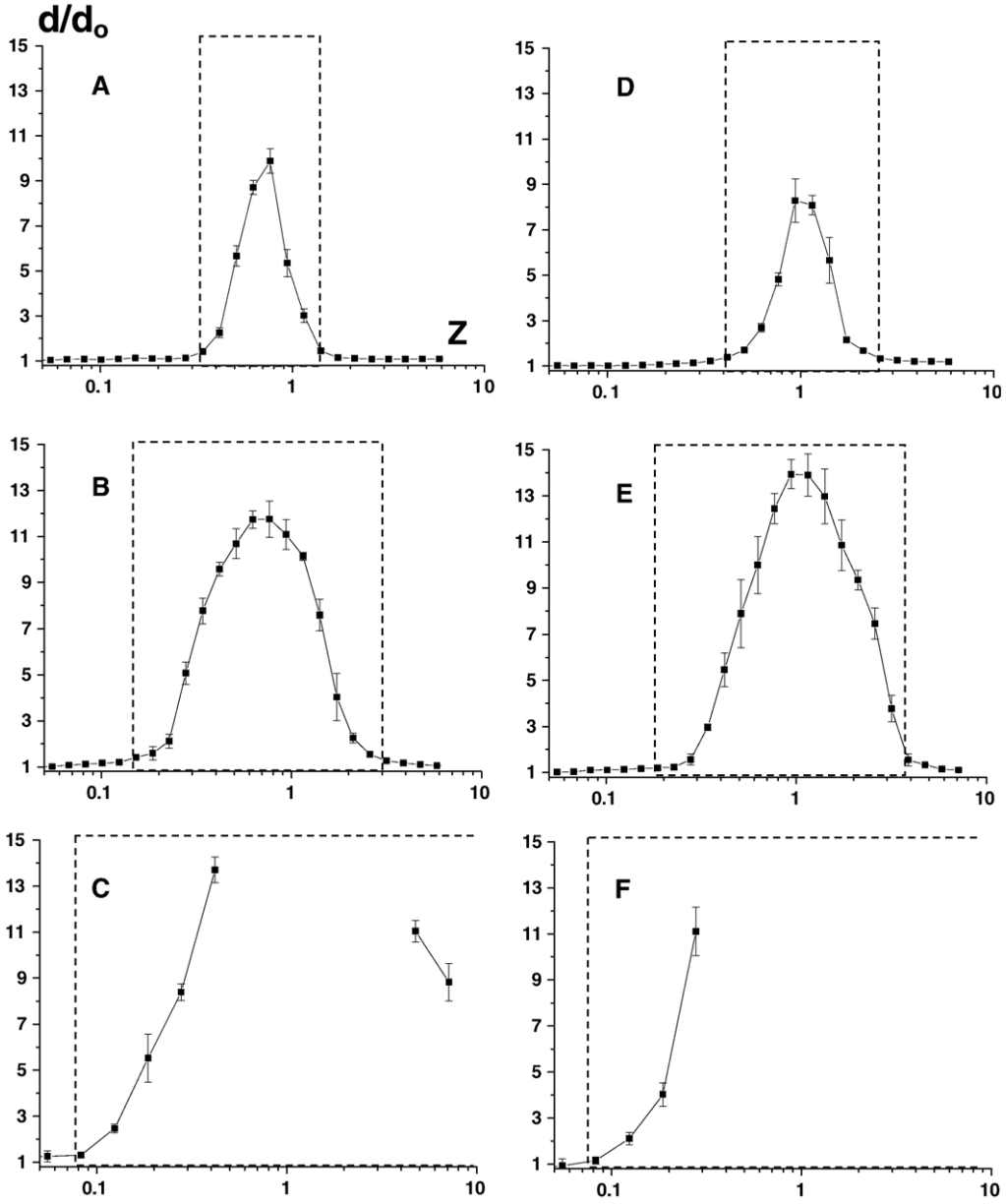

Fig. 5. Complexation profiles (normalized particle diameter d/d_o as a function of molar charge ratio (Z)) obtained at 25 °C (54 °C) for PLL280—A(D), PLL28—B(E), PLL2—C(F). The aggregation range is depicted with dashed lines. The diameter of uncomplexated liposomes at 25 and 54 °C are 129 nm and 74 nm, respectively.

plateau values are molecular mass dependent and were found to be close to 36, 38, and 8 mV for PLL280, PLL28, and PLL2, respectively. It is known that charged PLL of small molecular mass (3 kDa) adsorbs in a relatively flat conformation on a negatively charged surface [44]. At pH 7.4 PLL molecules are positively charged (pKa of the PLL is close to 10 [20,44]) and one can expect that liposomes will be coated with a rigid layer of PLL2 molecules due to electrostatic repulsion between neighboring charged side chains. Upon an increase of the PLL chain length, the number of tails and loops of PLL molecules exposed to solution is increased. This must impart larger overcharging of the vesicles and, as a result, better stabilization of PLL-coated vesicles against aggregation.

Besides a difference in surface overcharging, hydrodynamics can also play a role. High-molecular-weight PLL280 needs longer time to diffuse in close contact with the vesicle membrane than chains with a higher diffusion coefficient.

In the literature some studies of the influence of polymer molecular mass on complexation of charged vesicles with oppositely charged polyelectrolytes have been performed. For example, lipoplex formation with flexible poly(acrylic acid) of 5 and 225 kDa did not display a significant difference in the shape of the aggregation profile [7,9]. In the present study, complexation between PLL and anionic vesicles depends strongly on the PLL molecular mass. Probably, adsorption of poly(acrylic acid) leads to better surface stabilization against aggregation (coating is more homogenous) due to a more flexible conformation of the adsorbed poly(acrylic acid) molecules. Indeed it is known that PLL can form β-sheets upon interaction with lipid bilayers [45] and such β-sheets should increase the local rigidity in their

environment. All our observations and those described in the literature highlight a high sensitivity of colloid-polyion aggregation on the nature of the used polyion.

The thickness of the PLL layer on the vesicle surface calculated as the difference between the diameter of PLL-covered vesicle and the uncovered one at excess of PLL ($Z>5$) was evaluated for PLL of different molecular masses. For PLL280 and PLL28 the hydrodynamic thickness of the adsorbed layer was found to be 11 and 7 nm, respectively. It is not possible to determine the thickness for a PLL2 layer because of strong aggregation, but one can expect values of about few nanometres. Such small thickness can be expected when the molecules are adsorbing in a flat conformation. The tails and loops of PLL with higher masses providing larger thicknesses of the adsorbed layer.

It should be noted that for both PLL280 and PLL28 a size of stable aggregates of about 2 μm in diameter was observed. The small difference in size indicates that "bridge aggregation" does not appear for the employed PLL molecular masses when the vesicle diameter is considerably larger than the PLL molecule.

3.7. Effect of lipid phase state on the aggregation

Fig. 5D–F presents complexation profiles for PLL280, PLL28, and PLL2 when vesicle coating was performed at 54 °C. At this temperature the vesicles are in a fluid state as will be shown by means of differential scanning microcalorimetry. The shape of the aggregation profiles (which is again not defined for PLL2) are similar to those obtained at 25 °C, a temperature at which the vesicles are in the gel state (Fig. 5A–C), but the maxima of the profiles are shifted closer to 1 at the higher temperature (for the vesicles in the gel state, the Z value at the maximum of the profile is close to 0.7). This shift can be easily explained by increasing the number of ionic contacts between PLL and DPPG. Above the phase transition temperature of the lipid mixture, the vesicular membrane is in a fluid state. Lipid molecules in such a membrane not only acquire lateral mobility but might also migrate between the membrane leaflets (by the so called *flip-flop* mechanism). This may lead to an increase in the number of DPPG molecules in the outer leaflet of the vesicle membrane upon complexation with PLL. If all available PLL molecules could come into contact with DPPG molecules, the maximal number of DPPG molecules in the outer leaflet could be doubled (all DPPG molecules from the inner leaflet would have then migrated to the outer one).

Thus, a larger amount of PLL can be adsorbed when complexation is done at 54 °C. Measurement has been carried out at 25 °C. Therefore the ξ-potential of particles prepared in excess of PLL ($Z=7$) is higher when complexation is carried out at 54 °C. The values of 36, 38, and 8 mV (complex prepared at 25 °C) and 43, 41, and 17 mV (complex prepared at 54 °C) obtained for PLL280, PLL28, and PLL2, respectively can thus be explained.

3.8. Effect of PLL coating on liposome phase transition

The phase transition from the gel state to the liquid state of native liposomes and liposomes covered with PLL-280 was registered by differential scanning microcalorimetry. PLL 280 was chosen for this study because it was shown to produce a narrow "aggregation range" and very easy production of monodispersed coated vesicles. The PLL concentration used in these experiments, $Z=2$; exceeded the critical value needed to neutralize the liposome charge. Fig. 6 shows typical calorimetric traces for native liposomes (A) and PLL-covered ones (B). Calorimetric titration was repeated several times and no pronounced difference between the different curves was observed, indicating the reversible character of the phase transition.

DSC thermograms of native liposomes are characterized by a sharp peak with a well-defined maximum at (40.4±0.2) °C, reflecting homogeneous distribution of DPPG and CL molecules into the liposome membrane. This value coincides with the expected transition temperature of DPPC and DPPG (40.5 °C in 50 mM TRIS, pH 7.4 [46]) as main components of the liposome membrane. 10% w/w of CL does not have any observable effect on the phase transition. PLL-coated vesicles have very similar calorimetric curves as native ones, with a maximum at (40.6±0.3) °C.

Generally one can expect competing effects if a polycation binds to a charged membrane. The Coulombic repulsion between headgroups is reduced leading to an increase of the transition temperature, but the binding also introduces disorder favouring the fluid state. Yaroslavov et al. [20] have observed a strong increase of the transition temperature, up to several degrees, when polylysine interacted with charged vesicles. This was due to segregation of charged lipids migrating to the outer

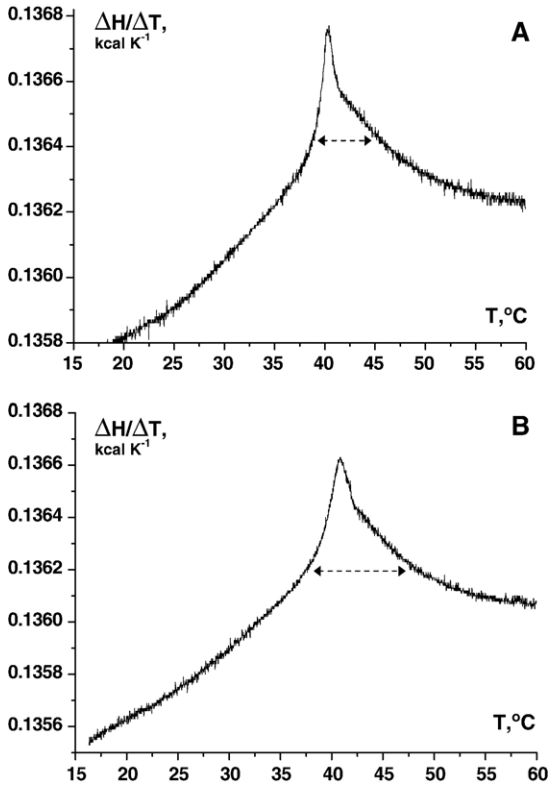

Fig. 6. DSC heating curves of uncoated (A) and PLL-coated liposomes (B). Lipid concentration is 0.7 mg/ml for both. Maximum for the curves is 40.4±0.2 °C and 40.6±0.3 °C for panels A and B, respectively.

leaflet of the vesicle membrane. In our case transition temperatures of DPPG and DPPC are the same, and one does not expect any changes in the maximum of calorimetric peak upon such a "flip-flop".

A little widening of the transition peak (dashed arrow in Fig. 6 helps to see the difference) is observed for PLL-covered vesicles suggesting less homogeneous distribution of DPPG in the bilayer membrane due to DPPG migration to the outer leaflet and formation of lipid domains as described in the literature [20] (Fig. 6). While PLL adsorption on DPPG membrane induced a change in transition temperature up to a few degrees, slight difference in the transition peaks may be due to the low fraction (about 10%) of charged lipid causing small structural changes upon complexation with PLL. Multiple repeating of calorimetric titration (0.5 °C/min) shows the same calorimetric curves, thus the time allowed for DPPG migration to the outer leaflet is enough. Moreover, an increase in zeta-potential for the covered particles at high temperature clearly indicates that this "flip-flop" process is already achieved in less than 5 min.

Changes in membrane permeability as a result of interaction with PLL is of high interest for studying the polycation-stabilized liposomes to be used for drug delivery applications. This topic is under consideration in our separate research [D.V. Volodkin, H. Mohwald, J.-C. Voegel and V. Ball, unpublished data].

4. Conclusions

This work is devoted to investigate of the complexation between unilamellar liposomes and oppositely charged polypeptide PLL. The structure of the resulting complexes ranges from isolated polyion-coated (stabilized) liposomes to the formation of vesicle aggregates. The complexation is examined to be affected by PLL/lipid ratio, order and mixing rate of liposome and polycation solutions, molecular weight of PLL, and lipid phase.

Vesicle aggregation observed at charge neutralization is caused by non-homogeneous liposome charge overcompensation with PLL. Characteristic polycation concentrations have been evidenced, beyond which the aggregates start to grow resulting in formation of three aggregate populations—small aggregates, which are growing to a second population of irreversible aggregates whose size (about 2 μm) is not changed with time, and large clusters of hundred microns in size. The time evolution of aggregation revealed a tendency for accumulation of irreversible aggregates of about 2 μm that is explained by a high thermodynamic stability of such structures having zero charge and formed by a "stabilization mechanism" when the long-range repulsion forces between particles counterbalance the short-range attraction. Manipulation of the nature of the polyion, rate/order and ratio of liposome and polyion mixing allows one to tailor properties of the resulting liposome–polyelectrolyte complexes.

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