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ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · APRIL 2008

Impact Factor: 3.3 · DOI: 10.1021/jp0775449 · Source: PubMed

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Hybrid Niosome Complexation in the Presence of Oppositely Charged Polyions

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Received: September 19, 2007; In Final Form: December 27, 2007

We have investigated the formation of complexes between negatively charged niosomal vesicles (hybrid niosomes), built up by dicethylphosphate [DCP], Tween 20 and Cholesterol, and three linear differently charged cationic polyions, such as α-polylysine, ϵ-polylysine, and polyethylvinylpyridinium bromide [PEVP], with two different substitution degrees. Our aim is to investigate the interaction mechanism between anionic−nonionic vesicles (hybrid niosomes) and linear polycations, characterizing the resulting aggregates in view of possible applications of these composite colloidal particles as vectors for multidrug delivery. In order to explore the aggregation behavior of the complexes and to gain information on the stability of the single niosomal vesicles within the aggregates, we employed dynamic light scattering (DLS), laser Doppler electrophoretic measurements, and fluorescence measurement techniques. The overall phenomenology is well described in terms of the re-entrant condensation and charge inversion behavior, observed in different colloidal systems. The aggregate size and overall charge depend on the charge ratio between vesicles and polyions, and the aggregates reach their maximum size at the point of charge inversion (re-entrant condensation). While the overall phenomenology is similar for all three polycations investigated, the stability and the integrity of the hybrid niosomal vesicles forming the aggregates strongly depend on the chemical structure of the polycations. The role of the polycations in the aggregation process is discussed by identifying specific interactions with the niosomal membrane, pointing out their importance for possible applications as drug delivery vectors.

1. Introduction

In the past few years, interactions of liposomal vesicles with oppositely charged polyelectrolytes have been extensively investigated.[1−5] These systems attracted much interest in view of the possibility of mimicking interactions of biological membranes with proteins, polymers, and genes,[6] as well as for the relevance of these systems to soft-matter physics, both from a theoretical[7,8] and from a practical point of view, in different fields of nanotechnology and biotechnology.[9] In particular, because of their capability to host both apolar and polar compounds (via interactions with the lipid bilayer or dissolution in the aqueous core, respectively), liposomes have been largely investigated as vectors for different drugs, genes, and DNA[6,10] and for the intracellular delivery of genetic material in gene therapy.[6] Their use as a pharmaceutical delivery system has been well established, and at present, investigations proceed exploring the potential of new formulations, with the aim of developing new drug-delivery technologies.[11] As an example, complexation of cationic liposomes with DNA has been thoroughly investigated in recent years by many authors,[12] on view of the possible use of the resulting structures, known as lipoplexes, as DNA-delivery vectors.

As we have recently shown,[1,13−16] by adding a proper amount of a polyelectrolyte to a suspension of oppositely charged liposomes, polyelectrolyte chains rapidly adsorb onto the liposome surface, and the resulting "polyelectrolyte-decorated" liposomes (pd-liposomes) form aggregates whose size and net charge are controlled by physicochemical parameters (such as the polyelectrolyte−liposome charge ratio, the length and the linear charge density of the polymer, the ionic strength of the solvent, and the charge density at the liposome surface) but are largely independent of the details of the chemical structure of the components.

Different combinations of linear polyelectrolytes (cationic,

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10.1021/jp0775449 CCC: $40.75
Published on Web 02/29/2008

as for example, double or single stranded DNA or sodium polyacrylate or anionic, as for example, polylysine) and oppositely charged liposomes, built up by natural or synthetic lipids, show a similar phenomenology, characterized by the presence of a significant overcharging, accompanied by a reentrant aggregation.[13,17,18] Overcharging occurs when more polyelectrolyte adsorbs at the liposome surface than is needed to neutralize the vesicle's own charge, so that, by increasing the polyelectrolyte−liposome molar ratio, the net charge of the pd-liposome inverts its sign. The second effect, concomitant to the overcharging (or charge inversion), consists of the formation of particle aggregates whose average size increases upon increasing the polyion concentration until it reaches a maximum (at the point of charge inversion), decreasing afterward to the initial value. Due to some balance between electrostatic repulsion and attraction resulting from the nonuniform (correlated) adsorption of the polyions at the liposome surface,[19] the pd-liposomes aggregate in clusters, whose size reaches a maximum in correspondence to the point of charge inversion.[16] Within these aggregates, liposomes maintain their individuality,[2] and the content of their aqueous core is generally not released in the surrounding medium. These characteristics and the possibility of assembling together, within the same aggregate, liposomes loaded with different substances[2,20] make this system suitable, in principle, for the formulation of innovative vectors for multidrug delivery.

In our previous studies on pd-liposomes,[1,13−16] we employed a cationic lipid, dioleoyltrimethylammonium propane (DOTAP). This choice was simply a consequence of its popularity as a vector for gene therapy. However, the toxicity of cationic lipids is well established.[6] In DNA gene therapy, with the polyelectrolyte itself being the substance to be transferred and DNA being a polyanion, the choice of cationic liposomes to form the complexes is forced.

In this context, with the perspective of using pd-liposome clusters as general-purpose multicompartment vectors for drug delivery, the polyelectrolyte has simply the role of an "electrostatic" glue, so that any suitable biocompatible couple of oppositely charged polyelectrolytes and liposomes can be, in principle, employed. In particular, negatively charged lipids can be used, which do not have the toxic effect shown by the cationic ones, combined with fully biocompatible cationic polyelectrolytes.

Complexation of negatively charged vesicles with polycations has been very intensively studied. These systems have attracted great attention also due to the similarity with the interaction processes occurring in living organisms when charged macromolecules interact with cell membranes that have a negative net charge. These investigations were mainly aimed at gaining some information, at a physical level, on the effects of the adsorption on the structure and the permeability of a lipid bilayer.[21,22]

Until now, these investigations have concerned several formulations of negatively charged phospholipidic liposomes and several natural and synthetic polycations, such as DPPG−DPPC and DPPS vesicles with polycationic amino acids such as polylysine and polyhistidine,[5,23−25] or DPPC−DPPG−cardiolipin with synthetic polycations, such as the poly ethylvinylpyridinium bromide.[5,22]

In the past few years, in addition to liposomal vesicles, new formulations of vesicles consisting of one or more nonionic surfactant bilayers (*N*on-*IO*nic lipo*SOMES*, or niosomes)[26,27] have been investigated as potential drug-delivery systems for different routes of administration, that is, intravenous, intraperitoneal, oral, or transdermal (due to their ability to cross the dermal horny layer). Niosomal vesicles need a formulation procedure similar to the one of phospholipidic liposomes, with the advantage of a lower cost, higher chemical stability, and lower toxicity. A net surface charge can be easily conferred to these vesicles by adding, during the formulation procedure, small amounts of ionic surfactants, obtaining hybrid or ionic−nonionic liposomes (hybrid niosomes).

Interactions of these new vesicles with polyelectrolytes have not been examined so far, in particular with regard to the problem of the stability of the vesicles within the aggregates. Because of the great potentiality of the niosomal vesicles and the relevant interest of vesicle−polyelectrolyte aggregates in view of their use as vectors for multidrug delivery, we consider these investigations on hybrid niosomes in the presence of charged polycations useful to extend the scenario of the interaction of complex charged macromolecules with lipid bilayers.

To this aim, we have investigated the complexation between negatively charged niosomal vesicles and three different polycations. In this work, we employed a basic formulation of unilamellar niosome vesicles made by a mixture of polysorbate 20 (Tween 20) and cholesterol, whose preparation and chemical−physical stability have been assessed in previous studies.[28] Tween 20 is a nonionic surfactant, the use of which was well established in biological and pharmaceutical studies because of its stability and relative nontoxicity. Cholesterol, due to its shape properties, is employed to allow the formation of unilamellar vesicles with Tween 20[27] since it was found to increase the packing density of vesicles and to reduce the bilayer permeability. A net negative charge is imparted to the vesicles by adding to the formulation a ionic surfactant, dicethylphosphate (DCP), at an appropriate concentration.

In order to induce aggregation, we used two kinds of polycations, polylysine, both in the form of the α- and ϵ-homopolymer,[29] and polyethylvinylpyridinium bromide (PEVP), synthesized from polyvinylpyridine, at two different charge fractions. Polylysine is widely used in membrane research, and its interaction with the natural phospholipid bilayer is well understood. However, its interaction with niosomes was not previously characterized. PEVP is one of the main polycations used for the preparation of polyplexes (i.e., complexes forming by mixing DNA and synthetic polycations), whose interaction with cells does not show any significant toxicity.[30]

Previous studies[31,32] on anionic liposomes evidenced that adsorption of oppositely charged polyelectrolytes to the bilayer surface can strongly modify the properties of the liposomes themselves, with possible vesicle disruption upon complexation, depending on the liposome composition and surface charge. Since several works showed that the modalities of interaction depend on the polycation−charged lipid molar ratio,[33,34] we investigated the hybrid niosomes−polycation complexation at a wide range of polycation concentrations, at a fixed niosomal concentration and composition.

The present work represents a further extension of the study of the aggregation mechanism of charged vesicles induced by oppositely charged macromolecules in terms of physical properties of the complexes such as size stability, surface charge, and membrane permeability in a novel system of hybrid niosomal vesicles, in view of possible biotechnological applications.

In this study, the interaction of different polycations (α- and ϵ-polylysine and PEVP) with unilamellar hybrid niosomes has been investigated by combining dynamic light scattering, laser Doppler electrophoresis techniques, and fluorescence measure-

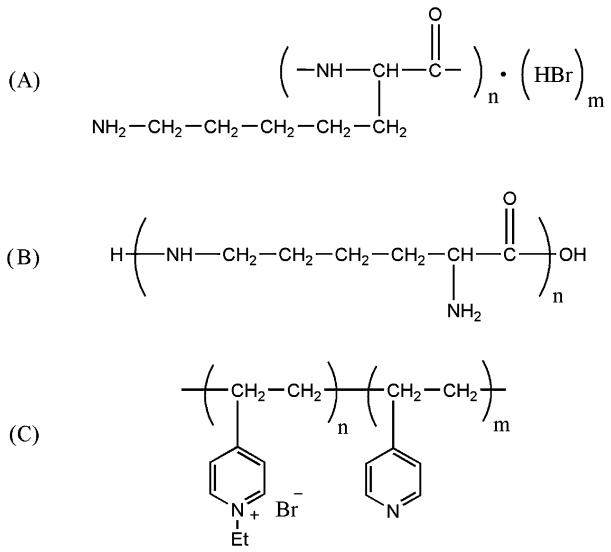

Figure 1. The structure formula of the polycations employed in this study; (A) α-polylysine hydrobromide, (B) ϵ-polylysine, and (C) PEVP.

ments. The effect of the polycation structure and polycation/DCP charge ratio is investigated and discussed within the framework of the re-entrant condensation and charge inversion behavior found in the aggregation of cationic liposomes and polyelectrolytes and widely discussed in a recent series of papers.[1,13−15,35,36]

2. Materials and Methods

2.1. Materials. Dicetylphosphate (DCP) was obtained from Sigma (St. Louis, MO), the nonionic surfactant polysorbate 20 (Tween 20) ($C_{58}H_{114}O_{26}$) was obtained from Merck (Darmstadt), and Cholesterol (Chol) was from Carlo Erba (Italy). Sephadex and HEPES salt (*N*-(2-idroxyethyl)piperazine-*N*0-(2-ethanesulfonic acid)) were Sigma-Aldrich products. All other products and reagents were of analytical grade. Solutions were prepared by using bidistillate water further purified through a Milli-Q water system (Millipore, Billerica, FL).

α-Polylysine hydrobromide with a degree of polymerization (DP) in the range of 130−300 was purchased from Sigma. ϵ-Polylysine with a DP of 30 was a gift from Chisso Corporation (Yokohama, Japan). This polymer is the natural product of the aerobic fermentation of *Steptomyces albulus*. ϵ-Polylysine was in the basic form and was converted to Cl salt by titration with HCl followed by extensive dialysis to eliminate the H^+ excess.

The polyvinylpyridine polymer was obtained from Polyscience. Poly(*N*-ethyl-4-vinylpyridinium bromide) (PEVP) was prepared by quaternization of polyvinylpyridine according to the procedure described in the literature.[37] Two samples containing about 5 and 35% of residual 4-vinylpyridine units, whose copolymer composition was determined by NMR measurements, were employed.

The chemical structures of the three polycations investigated, α-polylysine, ϵ-polylysine, and PEVP, are shown in Figure 1.

2.2. Vesicle Preparation and Purification. Unilamellar hybrid niosomal vesicles were obtained from a Tween 20−Chol−DCP dispersion according to the following procedure. Appropriate amounts of Tween 20 (45.7 mg), cholesterol (29.0 mg), and DCP (20.5 mg) were dissolved in a 25 mL chloroform−methanol (3:1) mixture in a round-bottomed glass vessel. After overnight solvent evaporation, the film was hydrated by addition of 25 mL of Hepes pH 7.4 buffer solution (10^{-2} M). The dispersion was vortexed for about 5 min and then sonicated for 5 min at 60 °C using a microprobe operating at 23 kHz and at an amplitude of 6 mm (Vibracell-VCX 400-Sonics).

Vesicle dispersion was purified by gel filtration on Sephadex G75 (glass column of 50 × 1.8 cm) using Hepes buffer as the eluent. The purified vesicle suspension was then extruded using cellulose filters with a pore diameter of 200 nm.

According to a quantitative evaluation[38] of the Tween 20 concentration carried out on a purified preparation, the percentage of this surfactant actually structured to form vesicles was determined to be 40.7% of the initial amount. The DCP structured amount was evaluated according to the spectrophotometric method proposed by IRSA-CNR and reported in detail in ref 39, giving a value of 20%. The cholesterol amount structured in the vesicles was 11.7%.

We checked the niosome vesicle integrity during the polycation-induced complexation by means of fluorescence measurements. For the preparation of fluorescent dye-loaded vesicles, the dye (calcein) was dissolved in the aqueous phase employed for the rehydration of the surfactant/Chol/DCP dried film. Excess dye present in the dispersant medium was eliminated by extensive dialysis (cellulose dialysis bag membrane 8000 MWCO-Spectrum, The Netherlands). The calcein concentration inside of the vesicles was approximately 10^{-2} M. At this concentration, the dye is self-quenched and does not produce any fluorescence if the vesicles maintain their integrity.

2.3. Preparation of Polycation−Hybrid Niosome Complexes. The polycation−hybrid niosome complex formation was promoted by adding 350 μl of polycation solution (prepared at an appropriate concentration) to an equal volume of hybrid niosome suspension at a concentration of 0.5 mg/mL in a single mixing step and gently shaking by hand, with a final niosomal concentration of 5×10^{15} particle/mL. This procedure has been followed in all of the experiments. In the case of polylysine polycations, in order to avoid sample annealing, samples were stored at 4 °C until used.

2.4. Dynamic Light Scattering Measurements. The size and size distribution of hybrid niosomes and polyion−hybrid niosome aggregates were measured at the temperature of 25 °C by means of the dynamic light scattering (DLS) technique, using a Malvern NanoZetaSizer spectrometer, equipped with a 5 mW HeNe laser (wavelength $\lambda = 632.8$ nm) and a digital logarithmic correlator. The normalized intensity autocorrelation functions were detected at a 90° angle and analyzed by using the Contin algorithm[40] in order to obtain the decay time of the electric field autocorrelation functions. The decay time is used to obtain the distribution of the diffusion coefficient D of the particles, which can be converted into an effective hydrodynamic radius R_H using the Stokes−Einstein relationship $R_H = K_BT/6\pi\eta D$, where K_BT is the thermal energy and η the solvent viscosity. Hybrid niosomes in the suspension show a distribution of size that, although rather narrow, is not fully negligible. The values of the radii reported here correspond to the intensity-weighted average.[41]

2.5. ζ-Potential Measurements. The electrophoretic mobility measurements were carried out by means of the laser Doppler electrophoresis technique using the Malvern NanoZetaSizer apparatus equipped with a 5 mW HeNe laser. The mobility u was converted into the ζ-potential using the Smoluchowski relation $\zeta = u\eta/\epsilon$, where η and ϵ are the viscosity and the permittivity of the solvent phase, respectively.

3. Results

Hybrid niosomes have an average diameter of about 160 nm, as obtained by dynamic light scattering measurements, and have

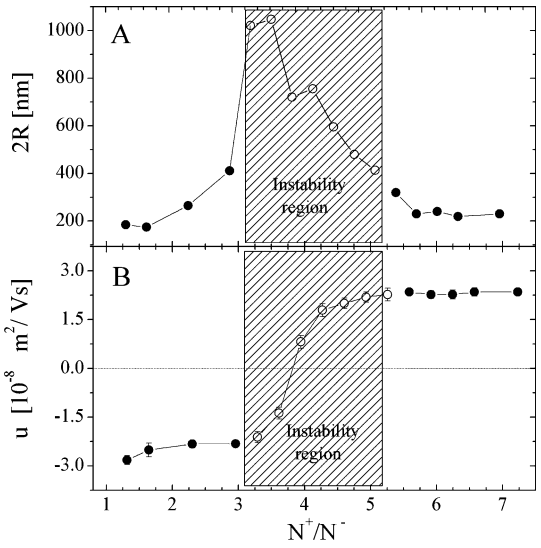

Figure 2. The average hydrodynamic diameter 2*R* (panel A) and the average electrophoretic mobility *u* (panel B) of α-polylysine−niosome structures as a function of the charge ratio N^+/N^-. The shadow region marks where instability of the aggregates is observed. Within this nonequilibrium region, values of the hydrodynamic diameter and electrophoretic mobility reported represent only the values measured during the initial 3 min of the aggregation.

a large negative electrophoretic mobility of 3.9×10^{-8} m^2/V s, which corresponds to a ζ-potential of about −50 mV, due to the presence of DCP head groups that carry a negative charge at the pH of the experiment, pH = 7.4 (the molar fraction of DPC molecules in the niosomal vesicles is 0.2). By adding appropriate amounts of polycations to the niosome suspension, large complexes rapidly form, whose size depends in a characteristic way on the concentration ratio of the polyelectrolytes and the niosomes.

We have investigated the aggregation behavior of negatively charged niosomal vesicles in the presence of three different cationic polyions, α-polylysine, ϵ-polylysine, and PEVP, with two different (nominal) charge fractions, 0.95 and 0.65.

In order to present our results in a unified way, in all of the experiments, we define a "stoichiometric" charge ratio, N^+/N^-, as the ratio between the total number N^+ of the positive charges on the polyion and the total number N^- of the negative charges of DCP molecules in the whole suspension

$$\xi = \frac{N^+}{N^-} = \frac{C_M}{M_{wM}} \frac{M_{wD}}{C_D} \quad (1)$$

where C_M and C_D are the (weight) concentrations of the polyion and DCP into the sample solution, respectively, and M_{wM} and M_{wD} are the molecular weight of the repeating unit of the polyion and the molecular weight of DCP. In all of the experiments, the concentration of DCP was fixed to the value of 0.15 mg/mL, and the molar charge fraction in the vesicle was 0.2. In the presence of PEVP, the ratio N^+/N^- was calculated taking into account the two charge fractions in the polymer chain, 0.95 and 0.65.

Figure 2 shows the behavior of the hydrodynamic diameter 2*R* (panel A) and the corresponding electrophoretic mobility *u* values (panel B) of hybrid niosomes in the presence of different concentrations of α-polylysine in the solution. The addition of α-polylysine promotes the progressive increase of the diameter of the complexes, starting from a value close to the diameter of pure hybrid niosomes when only a few polycations adsorb on the vesicles, and growing to larger and larger sizes due to the subsequent aggregation of polyion-decorated vesicles. Correspondingly, the value of the electrophoretic mobility *u* of the polylysine−niosome complexes increases, approaching less negative values until the point of charge inversion (isoelectric point) is reached. This behavior is connected to the progressive charge neutralization of the complexes, promoted by the adsorption of positive charges of polylysine units onto the negative charges at the vesicle surface.

Further addition of polylysine promotes the reversal of electrophoretic mobility, at a value of about $N^+/N^- \approx 3.8$, toward positive values and the decrease of the diameter of the complexes toward lower values. At the higher values of the charge ratio ξ, the aggregates reach a size similar to the one measured at low polylysine concentrations, at a ξ value between 1 and 2. This peculiar behavior is known in the colloid literature[42] as re-entrant condensation and is associated to the charge inversion of the polyion-decorated vesicles.[43] This behavior was observed in other systems, such as co-suspensions of dendrimers and DNA,[44] polyelectrolytes and micelles,[45] and mixtures of cationic liposomes and anionic polyelectrolytes.[46] The aggregation mechanism and the formation of stable structures were justified in light of a balance between electrostatic repulsion and short-range effective attraction interactions due to the nonuniform charge distribution at the particle surface (charge patch attraction).[47]

Although the overall behavior observed in this case is similar to the one of other similar systems,[48] α-polylysine-induced aggregates show a marked instability in a range of polymer concentrations close to the stoichiometric isoelectric point. In Figure 2, the shaded region marks this range. Here, a time-dependent change of the size of the aggregates, accompanied by a corresponding change in their electrophoretic mobility, is observed. While in panels A and B of Figure 2, solid symbols represent the values of the hydrodynamic diameter and electrophoretic mobility that remain constant in time, open symbols represent only the values measured in the initial stage of the aggregation within the first 3 min from the polyelectrolyte−niosome mixing since, in this range of values of ξ, all of the samples evolve in time.

The time evolution of the hydrodynamic radius, normalized to the radius of hybrid niosomal vesicles (≃160 nm in diameter), is shown in Figure 3 for the different polyion−lipid charge ratios investigated. Aggregates close to the point of charge inversion, where a complete charge neutralization is almost reached, show a more rapid growth with respect to the ones prepared far from the charge neutralization condition. Moreover, these rapid growing aggregates reach sizes larger than 8−10 μm in a relatively small time interval, and then, they precipitate in macroscopic flocs.

Actually, a complete description of the behavior of these unstable aggregates is even more complicate since, for all of these samples, at the different molar fractions investigated within the instability region, two different populations are observed. As it is shown in the inset of Figure 3, for the samples at molar ratios N^+/N^- of 3.3 and 4.6, all of the unstable samples show a large component (open symbols) that precipitates above a given size and a small one (closed symbols) that evolves in time.

The other two polyions investigated, ϵ-polylysine and PEVP, behave differently from α-polylysine since the marked instability close to the point of charge inversion does not appear.

The hydrodynamic diameter and the electrophoretic mobility of the complexes is shown in Figure 4 for ϵ-polylysine and in Figures 5 and 6 for PEVP at two different substitution degrees (95 and 65%, respectively). These polycations promote the formation of stable aggregates in the whole concentration range

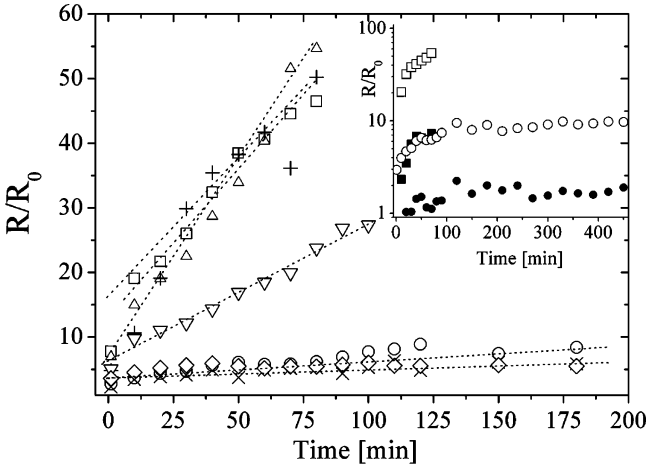

Figure 3. The normalized hydrodynamic radius R/R_0 of the largest α-polylysine−niosome structures formed within the aggregation region as a function of time, for the different polycation−lipid molar ratios N^+/N^-: (□) 3.3; (△) 3.6; (+) 3.9; (▽) 4.3; (○) 4.6; (×) 4.9; and (◇) 5.3 Dashed lines superimposed on the experimental data are only to guide the eyes. In the inset, for the samples with $N^+/N^- = 3.3$ (open symbols) and 4.6 (closed symbols), the average radius of the two particle populations present in the instability region is shown. The fast-growing population (squares) rapidly flocculates and disappears from the suspension. The small-size particles (circles) grow faster and reach a larger limiting size for a higher α-polylysine content.

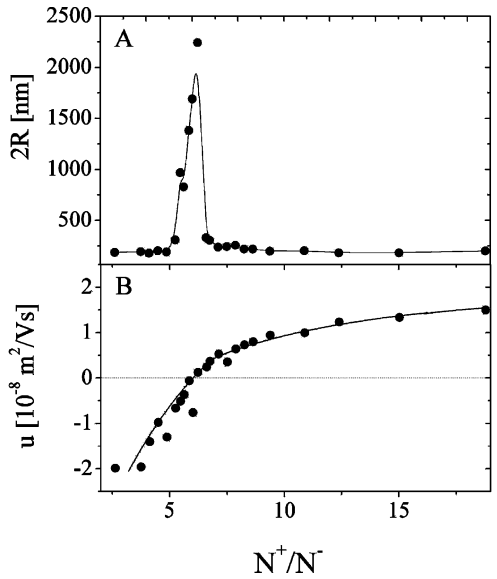

Figure 4. The average hydrodynamic diameter 2*R* (panel A) and the average electrophoretic mobility *u* (panel B) of ϵ-polylysine−hybrid niosome aggregates as a function of the polylysine−DCP charge ratio $\xi = N^+/N^-$.

investigated, and no time dependence of the hydrodynamic radius is observed.

In order to check the integrity of the hybrid niosomes during the whole aggregation process, we have monitored the leakage of a dye (calcein) entrapped in the interior of the hybrid niosomal vesicles. The possible release of calcein induced by the complexation with the polyions was studied as a function of the polyion−lipid charge ratio at a temperature of 25 °C. The percent I_F of dye leakage from the aggregates was calculated as

$$I_F = \frac{I - I_N}{I_E - I_N} \qquad (2)$$

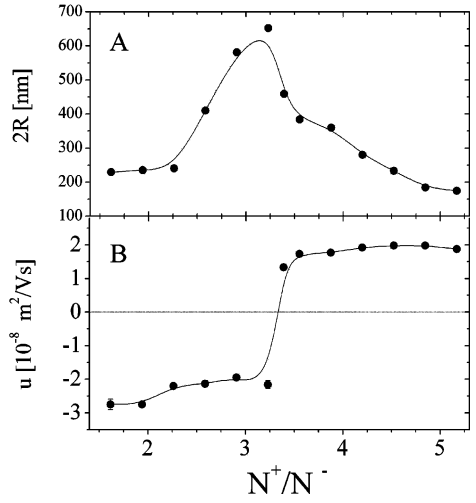

Figure 5. The average hydrodynamic diameter 2*R* (panel A) and the average electrophoretic mobility *u* (panel B) of PEVP 95%−niosome structures as a function of the PEVP−DCP charge ratio $\xi = N^+/N^-$.

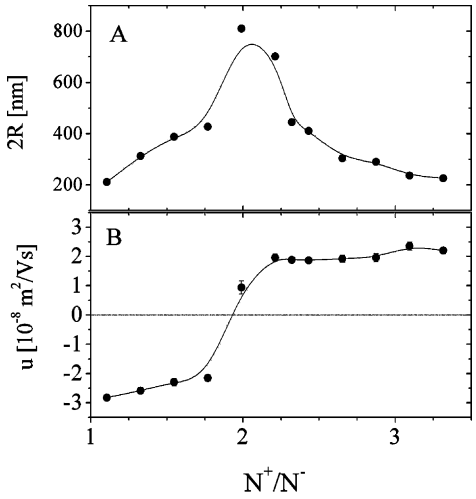

Figure 6. The average hydrodynamic diameter 2*R* (panel A) and the average electrophoretic mobility *u* (panel B) of PEVP 65%−niosome structures as a function of the PEVP−DCP molar ratio $\xi = N^+/N^-$.

where *I* is the fluorescence intensity of the polyion−hybrid niosome sample, I_N the one of the pure hybrid niosomes, and I_E the value corresponding to the complete rupture of the vesicles caused by adding, for example, ethanol to the suspension.

Hybrid niosomes were loaded with calcein at a self-quenched concentration inside of the aqueous core. A residual fluorescence was observed even after the exhaustive dialysis needed to eliminate the dye from the dispersant. This fluorescence is probably due to some amount of calcein that remained entrapped in the external hydrophilic layer of polyethylene residues at the surface of the hybrid niosomes. However, after one week of incubation at 25 °C, no increase of this residual fluorescence was observed, indicating that the membrane of DCP−Chol−Tween20 hybrid niosomes is impermeable at the conditions of the experiment (25 °C, pH = 7.4).

For each α-polylysine−niosome sample prepared at different charge ratios, the fluorescence intensity was studied after 30 min from preparation (Figure 7). When the hybrid niosomal vesicle were mixed with α-polylysine polyions, the fluorescence intensity increased significantly with respect to the value of pure hybrid niosomal vesicles. No time evolution of the fluorescence intensity was observed. At increasing polylysine concentration, the fluorescence intensity increased until a saturation region was reached through the "size instability" region (marked by the

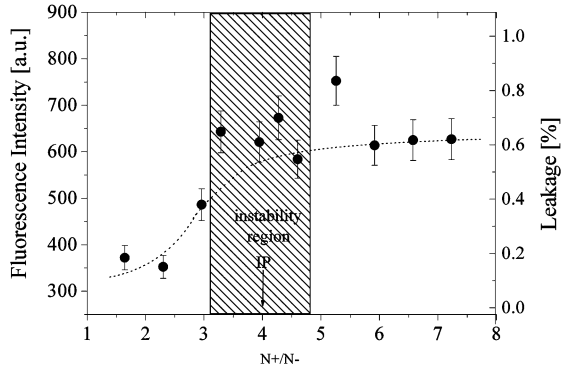

Figure 7. Fluorescence intensity (arbitrary units) and the corresponding leakage percentage of α-polylysine−niosome aggregates as a function of the charge ratio $\xi = N^+/N^-$. Uncertainties have been estimated on the basis of measurement reproducibility. The vertical arrow marks the concentration where the isoelectric point (IP) occurs. The dashed line is to guide the eyes only.

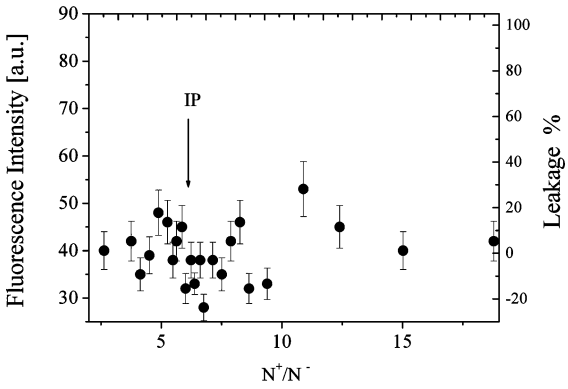

Figure 8. Fluorescence intensity (arbitrary units) of ϵ-polylysine−niosome aggregates as a function of the charge ratio $\xi = N^+/N^-$. Uncertainties have been estimated on the basis of measurement reproducibility. The vertical arrow marks the concentration where the isoelectric point (IP) occurs. The dashed line is to guide the eyes only.

shaded region in Figure 7, where larger aggregates precipitate). The observed behavior appears to be correlated with the stability property of the aggregates since the dye release reaches its plateau at the charge ratio where the instability region begins. Polylysine induces a maximum dye leakage which is around 60−70% of the complete release caused by the vesicle rupture induced by ethanol.

A completely different behavior was found in the presence of ϵ-polylysine and PEVP 95% polyion (Figures 8 and 9, respectively). No significant difference between the fluorescence of aggregates and that of pure hybrid niosomal vesicles was observed in this case, in the whole range of charge ratios investigated.

4. Discussion

The phenomenology of the complexation process observed with negatively charged hybrid niosomal vesicles in the presence of different oppositely charged polycations, that is, α-polylysine, ϵ-polylysine, and PEVP, is qualitatively the same for all of the polyions employed, showing the usual re-entrant condensation and charge inversion, with the exception of α-polylysine, where a marked instability of the aggregates was observed close to the point of charge inversion, with a substantial release of the aqueous core content.

The aggregation mechanism leading to the formation of large stable clusters of polyion-covered particles is rather general since it is independent of the details of the chemical structure of the

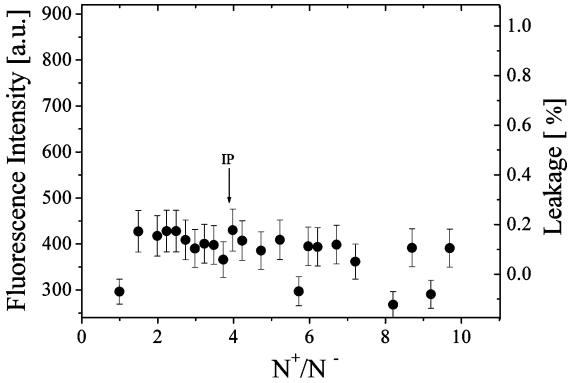

Figure 9. Fluorescence intensity (arbitrary units) of PEVP 95%−niosome aggregates as a function of the charge ratio $\xi = N^+/N^-$. Uncertainties have been estimated on the basis of measurement reproducibility. The vertical arrow marks the concentration where the isoelectric point (IP) occurs. The dashed line is to guide the eyes only.

components. This is understood since the aggregation mechanism is essentially of electrostatic origin and polyions act as an "electrostatic glue" to stick together like-charged vesicles. The formation of aggregates of finite size is determined by the balance between electrostatic repulsion due to the small effective charge of the polyion-coated vesicles and short-range attractive interactions. This attractive potential depends on the nonuniform distribution of the polyions adsorbed at the liposome surface ("charge patch" attraction[49]). The maximum size of aggregates is reached at the isoelectric point, where the charges on the vesicles are neutralized by the absorbed polyions and the repulsion between polyion-decorated vesicles is negligible. At higher polyelectrolyte content, since more polyions than necessary to neutralize the vesicle charge can adsorb (overcharging), a charge inversion phenomenon occurs, and the size of the clusters begins to decrease until it reaches values similar to the ones obtained at low polyelectrolyte concentration, at the beginning of the aggregation process. The shape of the electrophoretic mobility curves as a function of the N^+/N^- molar ratios (see Figures 2, 4, and 5, panel B) confirms the above-stated aggregation mechanism. Within this description, the maximum size (and the vanishing electrophoretic mobility) is reached close to the point of charge inversion.

PEVP− and ϵ-polylysine−hybrid niosome aggregates are stable with time for all of the molar ratios investigated, independent of the different linear charge density on the polymer chain and of the different chain flexibility. On the contrary, α-polylysine−niosome aggregates show a marked size instability in the region of charge neutralization, the aggregate size increasing with time with a complicated rate, depending on the polycation−lipid molar ratio. Immediately after mixing, large aggregates form, whose initial diameter shows, as a function of the polymer−lipid charge ratio ξ, a behavior that is compatible with the usual overcharging−re-entrant condensation scenario. However, the size of these aggregates keeps growing with time, at a rate that depends on the polyion−lipid charge ratio, with the more rapid growth being observed close to the charge neutralization point.

This time-dependent behavior within the above-stated phenomenology, considering only the effect of nonuniform charge distribution based on the electrostatic interactions, remains unexplained. The observed instability and the partial rupture of the membrane induced by α-polylysine, as evidenced by the fluorescence measurements, suggests that other nonelectrostatic interactions play a role in the aggregation mechanism induced by α-polylysine.

A similar behavior, with formation of large unstable aggregates, was observed in other systems built up by α-polylysine and negatively charged liposomes. According to Walter and coworkers,[24] in liposomes composed of phosphatidylserine (PS) and phosphatidylcholine (PC), α-polylysine induced large aggregation and leakage of the liposome content, accompanied by vesicles fusion, the extent of which depends on the fraction of negatively charged lipids in the vesicles, the relative amount of polycations, and the pH. In the presence of polylysine and PS liposomes, Hammes et al.[50] observed, by means of the electron microscopy technique, a dramatic rearrangement of the liposomes into large aggregates.

In our systems, fluorescence measurements show that there is at least the rupture of the niosome bilayer, probably accompanied by a partial fusion and/or rearrangement of the bilayer itself. This hypothesis could justify both the instability of the aggregates that continue to grow in time and, moreover, the anomalous growing laws, which differ from both an exponential growth and a power law growth that usually characterize the time behavior of aggregating colloidal systems.[51]

It is known that the α-polylysine adsorption is associated with leakages of the vesicle content, attributed to the insertion of the polycations into the double layer[52] favored by hydrophobic interactions with the aliphatic core of the bilayer. In fact, due to the negative charge density of the vesicle surface, the adsorbed polylysine tends to be completely neutralized. As a consequence, α-polylysine undergoes a conformational change from a random coil to an α-helix transition. Raman studies[25] and circular dichroism data[50] on the effect of α-polylysine on DPPG bilayers evidence a transition from a disordered to an ordered conformation of the polyion, accompanied by structural effects on the bilayer. It has been reported that α-polylysine bound to a double layer has a hydrophobic component as well an electrostatic interaction.[53] The presence of these two components promotes a more complex mechanism of binding, involving hydrophobic forces, which would lead to a penetration of the α-helix tracts into the bilayer.

In the presence of PEVP, the interaction of polycations with hybrid niosomal vesicles of DCP−Tween 20−Chol gives rise to the formation of stable aggregates at all of the charge ratios investigated. Moreover, fluorescence measurements indicate that PEVP adsorption on the vesicle surface occurs without any leakage of the vesicular aqueous content. It must be noted that, in the case of liposomes of phosphatidylcholine and cardiolipin, PEVP promotes the formation of aggregates, whose structure and stability critically depends on the concentration of the negatively charged lipid in the bilayer.[5,22] Kabanov and coworkers[5,22] found that, with vesicles of PC and cardiolipin, for mole fractions of charged lipids below 0.3, the integrity of the vesicle remains unaffected after PEVP adsorption. On the contrary, at higher contents of the charged lipids, PEVP completely destroyed liposomes, promoting the formation of strong nonvesicular complexes.

In our investigations, the molar fraction of the charged lipid (DCP) employed was on the order of 0.25, and our results indicate that, in these conditions, PEVP adsorption occurs without damaging the structure of polycation−niosomes aggregates.

5. Conclusions

In this work, we describe the complexation process between negatively charged vesicles (hybrid niosomes) built up by DCP−Chol−Tween 20 and different polycations, that is, α-polylysine, ϵ-polylysine, and PEVP at two different degrees of substitution.

At all of the experimental conditions investigated, we observe aggregation of hybrid niosomal vesicles induced by the presence of oppositely charged polycations. The size and the charge of the resulting structures strongly depend on the charge ratio between the moles of charged lipids and polyions. The aggregation proceeds from isolated polyion-decorated niosomes, with an overall positive charge at low polyion content, to larger, almost neutral, aggregates of polyion-decorated niosomes, close to the charge neutralization point. Increasing further the polyelectrolyte content in the overcharging region, the negative polyion-decorated niosomes form smaller and smaller aggregates until, at high polyelectrolyte content, the heavily polyion-coated vesicles are not able to aggregate any longer. This complex phenomenology has been described for a variety of colloidal systems within the framework of the re-entrant condensation and the charge inversion effects.

However, within this scenario, some significant differences appear, depending on the chemical structure of the polycation employed. The details of the polycation determine the stability properties of aggregates, in terms of the size and membrane permeability, because of the possible occurrence of nonelectrostatic interactions with the bilayer, causing the destabilization of the structure of the double layer itself.

α-Polylysine destabilizes aggregate structures formed by DCP−Tween 20−Chol niosomal vesicles, promoting the formation of unstable clusters at polycation−lipid molar ratios close to the neutrality condition. These aggregates leak their aqueous content and probably undergo vesicle fusion. This behavior is attributed to the penetration of polylysine into the double layer, occurring as a consequence of the conformational transition from a random coil to an α-helix structure.

Our measurements support evidence that the stability properties of the aggregates depend on the specific structure of the polycation employed. When α-polylysine is replaced by ϵ-polylysine, a homopolymer of lysine where the formation of the α-helix is inhibited, polycation−hybrid niosomal vesicles aggregate according to the re-entrant condensation behavior with the formation of stable clusters, where the integrity of the individual vesicles is preserved. Also, when synthetic PEVP polycations are employed, hybrid niosomal vesicles show the usual aggregation behavior also observed in the presence of ϵ-polylysine, thus indicating that PEVP polycations are effective complexing agents of oppositely charged niosomes.

The understanding of changes in the aggregation behavior and in the membrane permeability of the hybrid niosomes as a result of the interaction with a polyelectrolyte is of interest in the investigation of polyion−vesicle aggregates to employ as drug-delivery vectors. Hybrid niosomal vesicles are largely employed as delivery vectors, and the investigation of their interactions with polycations could open new perspectives in the rapid developing field of drug delivery.

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