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Many Facets of the Polyelectrolyte and Oppositely Charged Colloidal Particle Complexation: Counterion Release and Electrical Conductivity Behavior

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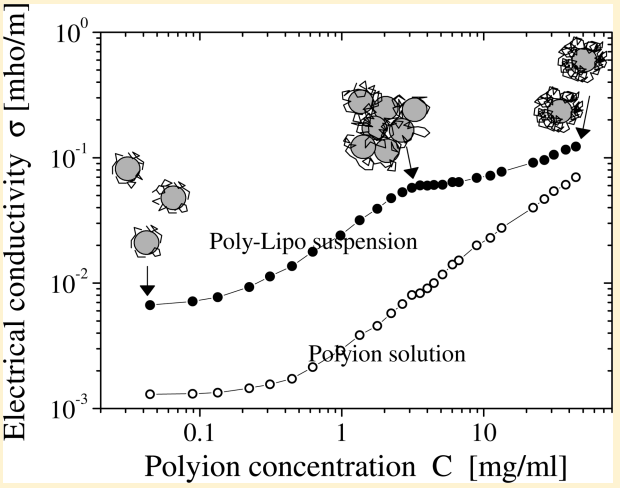
ABSTRACT: The *lateral* correlated adsorption of polyions onto oppositely charged vesicles, leading to the formation of stable equilibrium clusters of mesoscopic size, is associated to the release of a fraction of counterions, initially condensed on the polyion chains. This ulterior release of counterions provokes an increase of the number of *free* ions, besides the ones due to the partial ionization of both charged particles and polyions, that can be appropriately monitored by means of electrical conductivity measurements of the whole system. We have investigated this behavior in a suspension of cationic vesicles made up by dioleoyl trimethyl ammonium propane (DOTAP) liposomial vesicles interacting with an anionic polyelectrolyte composed by polyacrylate sodium salt. This system has been in the past extensively studied by us by means of different experimental techniques, and its behavior has been sufficiently characterized, as far as hydrodynamic and electrical properties are concerned. In this note, we report on the dc electrical conductivity behavior during the whole aggregation process, from the single polyion-coated liposomal particles, to polyion-induced liposome clusters, to finally polyion-fully covered liposomes, in polyion excess conditions. We have evaluated the excess of released counterions on the basis of the standard theory of the electrical properties of aqueous charged solutions and compared this quantity with the one predicted by the *lateral* correlation adsorption model. The agreement is quite good, offering strong experimental evidence of the role played by the release of counterions in the aggregation process. Finally, we have considered a similar liposomial system, where the *lateral* correlation adsorption was inhibited by structural reasons, having replaced the polyion by a simple electrolyte, whose dissociated ions will adsorb randomly at the particle surface, rather than in a correlated manner. In this case, no counterion release upon complexation occurs, and the electrical conductivity of the suspension approaches the one theoretically expected.



1. INTRODUCTION

The aggregation of charged colloidal particles induced by interactions with oppositely charged polyions has attracted a great deal of interest in the past few years because of its relevance both in soft-matter physics and for the implications in biomedical applications, for example, in nonviral gene therapy and, more generally, in biotechnology.[1−4]

The self-assembling polyion-induced complexes, controlled by an interparticle potential with a short-range attraction and a screened electrostatic repulsion, have witnessed an increasing large interest because of the extremely varied phenomenology they present,[5,6] depending, besides the chemistry of the components, on various environmental physicochemical parameters and, moreover, on sample preparation and mixing procedure.

A common way to realize these aggregates is obtained by the addition of *nonadsorbing* polyions, which produce an unbalanced osmotic pressure in the depletion region, leading to an attractive effect by depletion interaction.[7,8] In our systems, on the contrary, we will promote the aggregation of the charged particles by the addition of oppositely charged *adsorbing* polyions which form a strongly correlated liquid-like structure onto the particle surface. In this case, the short-range attraction, and hence the aggregation effect, is driven by the correlation energy gain at the place of contact when two approaching particles touch each other. This mechanism differs from the ones known up to now and produces, in a certain way, an unexpected and intriguing phenomenology,

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in particular, a correlation between the overall size of the complexes and their overall electrical charge.

The central idea is that, due to the strong interactions with a charged surface, polyions do not position themselves randomly (like as in a three-dimensional space) but form a strong correlated liquid on the surface, this liquid being reminiscent of a Wigner crystal.[9] This correlated adsorption results in a short-range attractive potential between the particles.

Among the systems subjected to this phenomenology, we have extensively investigated liposomes composed by cationic lipids (unilamellar lipidic vesicles encompassing an aqueous core) and linear anionic polyions, which react spontaneously by electrostatic interactions. In this context, polyions can be considered as an "*electrostatic glue*" for charged particles. They allow us to build up a particle cluster of appropriate size, stable over a long period of time and, moreover, with size that can be controlled (and modified, if we want) continuously by the ionic strength of the suspending aqueous medium.

From a theoretical point of view, the aggregation of charged mesoscopic particles induced by oppositely charged polyions has been extensively investigated by Nguyen, Shklovskii, Grosberg, and others[10−13,37] on the basis of what is known as *lateral* correlated adsorption. When each particle adsorbs many polyions, they strongly repel each other at the particle surface and form a strongly correlated two-dimensional liquid.[9]

These aspects, as far as liposomes built up by DOTAP lipids and anionic polyelectrolytes (polyacrylate sodium salt) are concerned, have been extensively investigated in the past few years, from both experiments and simulations, and a well-consolidated picture of the aggregation process has been reached.[14−19]

However, an aspect remains not completely clarified, i.e., the role played by counterions in the aggregate formation or, in other words, whether or not there is a further release of conterions, besides the ones deriving from the initial (partial) ionization of liposomes and polyions, as a direct consequence of the correlated adsorption of polyions on the particle surface.

Evidence of the role played by counterions in the spontaneous formation of lipoplexes (restructured aggregates of liposome particles in the presence of DNA) has been previously discussed by Wagner et al.,[20] who evidenced that, in the conditions under investigation, the aggregation is entropy driven, the driving force being the release of counterions initially condensed on the polyion upon adsorption. More recently, however, Radeva et al.,[21] in the case of *highly* charged polyions and *weakly* oppositely charged particles, have shown that complexation does not lead to counterion release from the polyions, the role of condensed counterions being confined to the charge inversion effect.

Electrical conductivity, depending, at least in principle, on the totality of the charged entities present in the system, both at mesoscopic and microscopic level, should be able to clarify this important point.

Within this framework, from an experimental point of view, the overall phenomenology observed in this kind of systems (here we are dealing, in particular, with cationic DOTAP liposomes and polyacrylate polyions) is summarized in Figure 1, where we show the electrical conductivity σ of the liposome−polyion system during the whole process of complexation (as a function of the polyion concentration), together with the two prints of the correlated polyion adsorption, i.e., the *reentrant condensation* (inset A) and the *charge inversion* (inset B).

Reentrant condensation clearly evidences the progressive formation of aggregates (in this case, from 200 nm to 1 μm in size) up

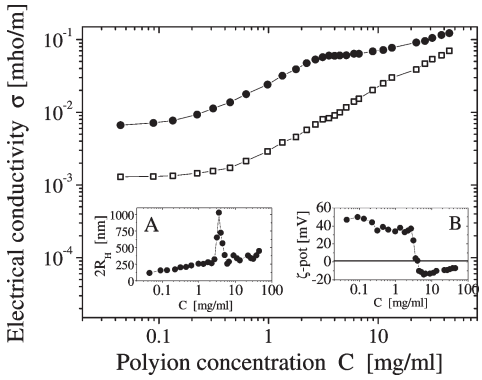

Figure 1. Summary of the phenomenology of the polyion-induced liposome cluster aggregation as far as the electrical conductivity behavior is concerned. (●) the dc electrical conductivity of DOTAP liposome suspension in the presence of a different amount of polyacrylate [PAA] polyions; (□) the dc electrical conductivity of polyacrylate aqueous solution as a function of the polyion concentration. Inset A: the average size of the polyion-induced aggregates measured by dynamic light scattering technique as a function of the polyion concentration (reentrant condensation). Panel B: ζ-potential of polyion-induced aggregates as a function of the polyion concentration (charge inversion crossing the isoelectric point).

to the isoelectric point, followed by a progressive reduction of the aggregate size toward the one of the initial particles. Charge inversion occurs when, at the surface of the mesoscopic charged particle, more counterions than necessary to neutralize it collapse. The resulting complexes can therefore display an overall charge of opposite sign to the one the particles originally bear. These two effects are strongly correlated, the presence of the former being a consequence of the latter and vice versa.

This paper deals with the dc (low-frequency) electrical conductivity of positively charged lipidic vesicle (liposome) aqueous suspensions in the presence of an increasing amount of negatively charged polyelectrolytes, in the whole concentration range, from well below to well above the isoelectric point. We will show that, in this system, there is a continuous release of counterions in the bulk solution up to the isoelectric point, when the aggregates reach their maximum size and their overall charge approaches zero. The amount of this counterion release is exactly the one predicted by the Nguyen et al. model[10−13,22,23] on the basis of the *lateral* correlated polyion adsorption. This finding can be considered an experimental confirmation that the so-called *charge patch* attraction produces stable aggregates of mesoscopic size, resulting from the complexation of individual particles (the single liposomes) which maintain their initial integrity.

2. EXPERIMENTAL SECTION

Dioleoyl trimethyl ammonium propane [DOTAP] and dipalmitoyl glycero phosphate [DPPA] were purchased from Avanti Polar Lipids [Alabaster, Alabama] and used without further purification. The anionic polyelectrolyte employed was polyacrylate sodium salt [Na−PAA], a highly charged, flexible polyion purchased from Polysciences, Inc., Warrington, as 0.25 wt/wt solution in water, with a nominal molecular weight of 60 kDa. The lipids were dissolved in chloroform−methanol (1:1 vol/vol) at a concentration of 10 mg/mL. After solvent evaporation, dried lipid films were hydrated with deionized water (electrical conductivity less than 10^{-6} Ω^{-1}/cm at room

temperature). To form unilamellar vesicles, the lipid solution was sonicated at a temperature of 25 °C for 1 h in pulsed-power mode until the solution appeared optically transparent in white light. The solution was later filtered through Millipore polycarbonate filters 0.45 μm in size.

The liposome and polyion-coated liposome size (average radius R) and size distribution (polydispersity) were obtained from dynamic light scattering measurements (Brookhaven Digital Autocorrelator mod. BI-9000AT). The ζ-potential was measured by means of a laser Doppler electrophoresis technique, using a MALVERN Zetamaster apparatus equipped with a 5mW HeNe laser.

The polyion adsorption at the liposome surface induces a progressive increase of the cluster size up to the isoelectric point, where the DOTAP to PAA concentration ratio is about 6.2 g/g. Above the isoelectric point, in polyion excess conditions, each liposome is heavily coated by adsorbed polymers, and its size reaches values of about 250−260 nm.

The electrical conductivity σ of the liposome suspensions and polyion-induced liposome aggregates was measured in the frequency range from 1 kHz to 10 MHz, at the temperature of 25.0 °C, by means of a Radiofrequency Impedance Analyzer Hewlett-Packard model 4291A. Details of the experimental procedure have been reported elsewhere.[24,25] Although the measurements extend over a wider frequency range, here we consider exclusively the low-frequency value, neglecting the effects associated with the heterogeneity of the system and the effects associated with the surface ionic polarizations. A brief account of these measurements with some preliminary results, in light of the lateral correlation model, has been previously reported elsewhere.[26]

3. ELECTRICAL CONDUCTIVITY: AN OVERVIEW

The electrical conductivity of an ensemble of charged particles moving under the influence of an external electric field is due to the contribution of three independent terms, i.e., the electric charge (ze) that each particle bears, the numerical concentration c, and the mobility u of each carrier, defined as the average velocity normalized to the electric field of unit strength, i.e.

$$\sigma = \sum_i (|z_i|e)c_i u_i \qquad (1)$$

Equation 1 is a rather general expression, and it must be specified for the different systems we are dealing with.

3.1. Electrical Conductivity in the Absence of Complexation. Let us consider, right away, the case of an absence of complexation. We are dealing with a solution containing polyions at a numerical concentration N_P with degree of polymerization N, monomer size b, and contour length $L = Nb$ and structural charge density $\eta = e/b$. Because of the counterion condensation,[27−29] each polyion bears an electric charge $Q_P = Nze/\xi$ and releases in the aqueous phase a number of counterions $N(1 - f)$ and hence a charge $Q_P = Nze(1 - f)$. Here, f is the fraction of counterion condensation, and consequently $(1 - f)$ is the fraction of free counterions in the bulk solution.

Within the Onsager−Manning condensation model,[27−29] when the charge parameter $\xi = l_B/b$ is larger than unity (for monovalent ions), the polymer chain has an effective renormalized charge density $\eta_c = e/l_B$ (smaller than the nominal charge density $\eta = e/b$), and the fraction $(1 - f)$ of free counterions is given by $(1 - f) = 1/\xi = b/l_B$. Here, $l_B = e^2/(\varepsilon K_B T)$ is the Bjerrum length, with $K_B T$ being the thermal energy.

Also in the case of spherical charged liposomal vesicles, a charge renormalization occurs,[30−32] each particle bearing an apparent charge $Q_L = Z_{bare}e(1 - g)$ much smaller than the bare charge $Q = Z_{bare}e$. Here, g is the fraction of counterion condensation and plays the same role of f, already introduced in the case of the polyion chains. Consequently, the concentration of free counterions originated from partial ionization of each vesicle is $Z_{bare}(1 - g)$, and the charge released in the solution for each liposome is $q_L = Z_{bare}e(1 - g)$.

In the case of DOTAP liposomes (counterion Cl^-) and PAA polyions (counterions Na^+), at the numerical concentration of N_L and N_P, respectively, the electrical conductivity, following the additivity rule of eq 1, can be written as

$$\sigma = Q_L N_L u_L + Q_P N_P u_P + q_L(1-g)N_L u_{Cl^-} + q_P N_P u_{Na^+} \qquad (2)$$

where u_L, u_P, u_{Cl^-}, and u_{Na^+} are the mobilities of liposomes, polyions, and Cl^- and Na^+ ions, respectively. In the usual notations, the last two terms in the right-hand side of eq 2 can be rewritten as

$$\sigma = Q_L N_L u_L + Q_P N_P u_P + \frac{1}{2}(1-g)C_{Lip}\lambda_{Cl^-} + N(1-f)C_P\lambda_{Na^+} \qquad (3)$$

where C_{Lip} and C_P are the concentration of the lipid molecules and the polyions, expressed in mol/cm^3, respectively; and λ_{Cl^-} and λ_{Na^+} are the equivalent limit conductances, expressed in mho·cm^2·equiv^{-1}, of Cl^- and Na^+ ions, respectively. We have expressed the concentration C_L of liposomes through the concentration C_{Lip} of the lipid molecules which form them by means of the relation $C_{Lip} = 2C_L(4\pi R^2/s_0)$, where R is the external radius of the liposome and s_0 is the area of the polar head of each lipid molecule. The factor 2 takes into account the fact that the release of counterions into the aqueous solution derives only from the external surface of the liposome.

The first two contributions of eq 3, due to the single liposomes and to the polyion chains, are generally negligible because of their small mobilities, and consequently, the overall electrical conductivity is, to a first approximation, due to the contribution of Cl^- and Na^+ ions, deriving from the partial ionization of the liposome particles and of the polyions.

3.2. Electrical Conductivity in the Presence of Complexation. In the presence of polyion adsorption, and hence in the presence of liposome complexation, the above picture must be modified since a further release of counterions, deriving from each polyion, occurs. Consequently, in the electrical conductivity of the suspension, eq 3, a further contribution must be added. According to the model of the lateral correlated adsorption,[10−13] the *effective* polyion charge density upon adsorption varies from η_c to η^* so that the change in the polyion charge induced by its adsorption at the particle surface is given by

$$\Delta Q_P = Nb(\eta^* - \eta_c) \qquad (4)$$

In this case, the whole conductivity, neglecting, as above, the contribution of the liposomes and polyions and maintaining only the contributions of Cl^- and Na^+ ions, becomes

$$\sigma = \frac{1}{2}(1-g)C_{Lip}\lambda_{Cl^-} + N(1-f)C_P\left[\frac{\eta^*}{\eta_c}\right]\lambda_{Na^+} \qquad (5)$$

The key role of the process is assumed by the parameter η^*. The merit of the lateral correlated adsorption model is that an explicit expression for η^* is available.

According to Nguyen et al.,[10−13,22,23] in the presence of linear polyions of radius a and effective charge density η_c (negative to make the sign consistent with the case of Na−PAA) and a surface with positive charge density ϱ_0, the counterion release, upon the aggregate formation, is governed by two characteristic lengths, i.e., the screening length r_s, due to monovalent counterions in the bulk solution, and the spacing $A_0 = \eta_c/\varrho_0$ between the adsorbed polyions, when they neutralize the charged surface.

If $r_s \ll A_0$, counterions from the polyions are not released, and the polyions maintain their charge density η_c. On the contrary, if $r_s \gg A_0$, the charge on the surface forces the polyion to release some of its "condensed" counterions so that its effective charge density η^* becomes larger than η_c. By imposing the appropriate conditions of equilibrium for the chemical potential of ions in the system, Nguyen et al.[10−13,22,23] derived the following expression for η^*

$$\eta^* = \eta_c \sqrt{\ln(r_s/a)/\ln(A_0/2\pi a)} \qquad (6)$$

Equation 5 furnishes the expression of the electrical conductivity during the whole process of liposome cluster formation, until the maximum size (at the isoelectric point) is reached.

Once the liposome aggregation is completed, the further adding of polyions produces the beginning of the reentrant aggregation process which ends when each liposome is overcoated and inverts its electric charge.

In these conditions, during the reentrant process, the electrical conductivity of the polyion−liposome suspension can be written as

$$\sigma = \sigma_c + (C_P - C_{Pc})N(1-f)\lambda_{Na^+} \qquad (7)$$

where σ_c and C_{Pc} are the electrical conductivity and the polyion concentration at the isoelectric point, respectively, when the maximum aggregate sizes are reached.

4. RESULTS AND DISCUSSION

Equations 5 and 7 describe the electrical conductivity in the whole liposome aggregation process. However, they depend on two parameters, f and g, which represent a kind of charge renormalization parameter. Their values can be evaluated, at least to a first approximation, from electrical conductivity measurements of the liposome suspensions at the different concentrations investigated and of the polyion solution at the different concentrations in the absence of liposomes, respectively. In these two cases, the electrical conductivity is given by

$$\sigma = \frac{1}{2} C_{lipid}(1-g)(\lambda_{lipid} + \lambda_{Cl^-}) \qquad (8)$$

$$\sigma = C_P(1-f)(\lambda_p + \lambda_{Na^+}) \qquad (9)$$

Also in this case, we can neglect the contributions of liposomes and polyions and consider only the contributions of small ions in the solution. The results are shown in Figure 2, where we report the values of f and g as a function of the polyion and lipid concentration. On the basis of the Manning counterion condensation model,[27−29] in linear polyions, the fraction f of condensed counterions is given by $f = 1 - 1/\xi = 1 - b/l_B$. In the present case, $b = 2.52 \times 10^{-8}$ cm, and consequently, we expect a fraction $f = 0.65$. This value does not differ too much from the one evaluated experimentally, at least in a wide range of intermediate concentrations. Deviations occur at very low polyion concentrations and at very high polyion concentrations. These deviations

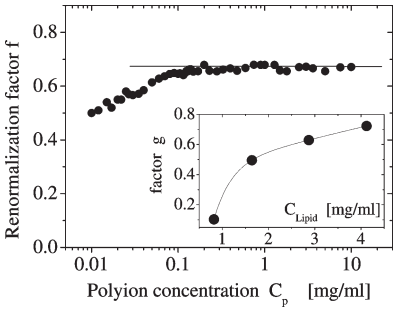

Figure 2. Charge renormalization factor f as a function of the polyion concentration C_P derived from the measured value of the electrical conductivity σ of the polyion solution, on the basis of eq 9. The inset shows the charge renormalization factor g as a function of the lipid concentration C_{lipid} derived from the measured value of the electrical conductivity σ of the liposome solution, on the basis of eq 8.

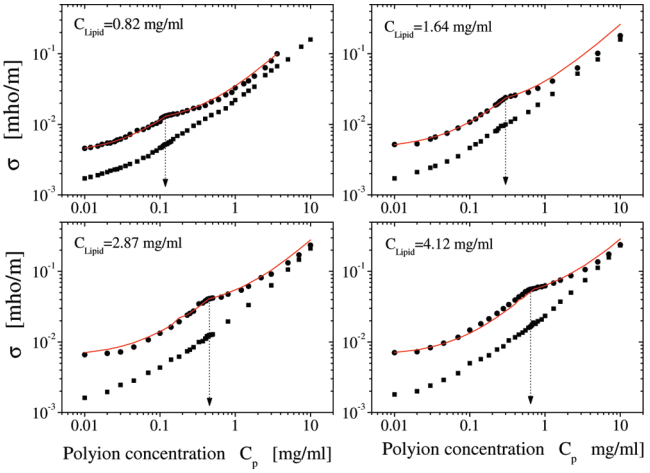

Figure 3. Electrical conductivity increment σ of polyion-induced liposome aggregates as a function of the polyion concentration in the aqueous phase, for four different liposome concentrations C_{lipid} (from 0.5 to 4.5 mg/mL of DOTAP lipids). The conductivity σ of the polyion solution as a function of the concentration C_P, in the absence of the liposome component, is shown for comparison. The full lines represent the calculated values on the basis of eq 5, below the isoelectric condition, and eq 7, above the isoelectric condition. The arrows mark the polyion concentration at the isoelectric condition, when the overall charge of the aggregates approaches neutrality.

are expected and have been observed in various polyelectrolyte solutions.[33] As far as the value of the parameter g is concerned, we observe a clear dependence on the lipid (and hence liposome) concentration (see inset of Figure 2). In this case, that is for spherical particles, the counterion condensation effect is much less investigated, and the values of the charge renormalization parameter g is less defined.[30,32] However, in the evaluation of the electrical conductivity, we will use for g the values experimentally found for each liposome concentration.

The results of our analysis are summarized in Figure 3, where we show, for each of the four liposome concentrations investigated, the measured electrical conductivity in the polyion-induced liposome aggregation process together with the electrical conductivity of the polyion solution. As can be seen, before and after the isoelectric point, the slope of the electrical conductivity, as a function of the polyion concentration, changes

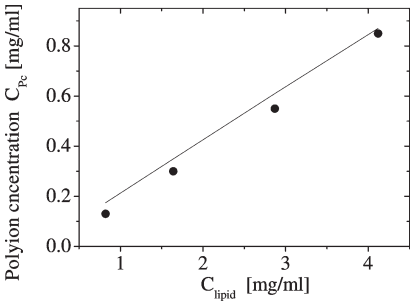

Figure 4. Polyion concentration C_{Pc} at the isoelectric point (maximum aggregate size) as a function of the liposome concentration expressed through the concentration C_{lipid} of the lipid molecules of DOTAP. The full line represents the value calculated on the basis of eq 11, with the same set of parameters employed in the evaluation of the electrical conductivity.

markedly, this change being due to the further amount of the release of counterions upon polyion adsorption. The calculated values of the electrical conductivity on the basis of eq 5 (before the isoelectric point) and of eq 7 (after the isoelectric point) agree in a very satisfactory way with the values experimentally measured.

This finding is the first strong evidence of the role played by counterions in the complexation processes and, moreover, represents a quantitative corroboration for the lateral correlated adsorption on the basis of electrical conductivity measurements.

A further comment is in order. According to the above stated model, the competition between attraction of polyions to the particle surface and the repulsion of neighboring polyions produces a change in the surface particle charge density that goes from the value ϱ_c to the value ϱ^*, that, for $r_s \gg A_0$, reads

$$\varrho^* = (\eta_c/2\pi a)\exp(-\sqrt{\ln(r_s/a)\ln(A_0/(2\pi a))}) \qquad (10)$$

This change allows us to predict the concentration C_{Pc} of polyions at which the isoelectric point is attained, in correspondence to the maximum size of the liposome cluster aggregates. At this concentration, the balance between liposome and polyion charges implies that

$$C_{Pc} = \left(1 - \frac{\varrho^*}{\varrho_c}\right)\frac{C_{lipid}}{fN} \qquad (11)$$

Figure 4 shows a comparison between the values of the polyion concentration C_{Pc} in correspondence of the isoelectric point (maximum aggregate size) as a function of the lipid concentration and the values calculated according to eq 11. As can be seen, considering that we employed the same set of parameters used in the evaluation of the electrical conductivity, the agreement is quite good.

Going back to the main problem we are dealing with, we stress that, in our system, liposome aggregation is attained thanks to the lateral correlation in the polyion adsorption onto the particle surface. This means that the particle surface charge heterogeneity should play a crucial role. These heterogeneities can be easily taken into account by considering the variance S_V^2 of the particle surface potential V_0, using the following simple toy model, which describes a heterogeneous flat surface.

In general, for any scalar observable $F(\vec{s})$ defined on a square region of area L^2, which assumes the value $-A$ in the whole

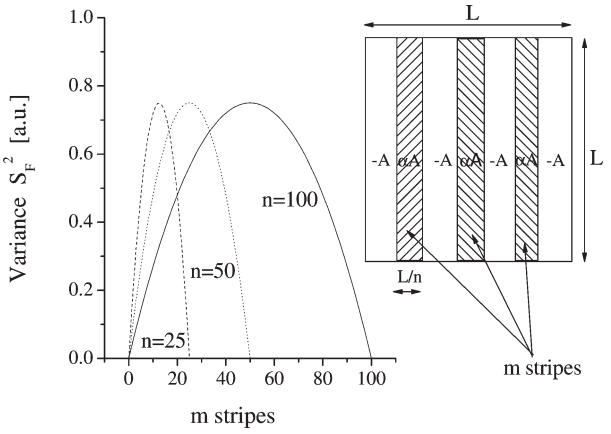

Figure 5. Simple representation of a stripe-decorated square region, resembling charged polyions adsorbed at the surface which tend to be parallel to each other due to their strong lateral repulsion. An observable $F(s)$ is defined in the region of area L^2, assuming the value $-A$ and αA outside and inside the stripes (dashed region), respectively. On the left, the variance S_F^2 of $F(s)$ as a function of the number m of stripes. Curves are calculated from eq 14 for $A = 1$, $\alpha = 2$, and $n = 25$, 50, 100, respectively.

square apart from m small stripes of thickness L/n ($n \geq m$) where it is equal to αA ($\alpha > 0$), its mean value $F(\vec{s})$ can be calculated as

$$\langle F(\vec{s})\rangle = \frac{1}{L^2}\int_{L^2} F(\vec{s})\,\mathrm{d}\vec{s} \qquad (12)$$

Hence, the mean value of $F(\vec{s})$ and its variance on the stripe-decorated square region, as a function of the number m of stripes, are given by

$$\langle F(\vec{s})\rangle = A\left[\frac{m}{n}(1+\alpha) - 1\right] \qquad (13)$$

$$S_F^2(m) = (\langle F(\vec{s})^2\rangle - \langle F(\vec{s})\rangle^2)$$

$$= A^2(\alpha+1)^2\left[\frac{m}{n} - \frac{m^2}{n^2}\right] \qquad (14)$$

As shown in Figure 5, $S_F^2(m)$ is a nonmonotonous function of m, assuming its maximum value for $m = n/2$. It is worth noting that, when $m = n/2$, the mean value of the observable can be written as

$$\langle F(\vec{s})\rangle = \frac{A}{2}[\alpha - 1] \qquad (15)$$

showing that, in correspondence of the maximum of the variance, the mean value of the observable $F(\vec{s})$ is zero if, and only if, $\alpha = 1$.

Expressions 13 and 14 can be adapted to the case of polyion-coated spherical particles when the parameters m and n and A are expressed in terms of the charge ratio ξ_s (defined as the polyion to liposome charge ratio), the size of particle R, the size of the chains, and the surface potential $V(\xi = 0) \equiv V_0$ of the colloidal particle. In particular, n can be written as the ratio between the area s_P covered by a single patch and the whole available area $4\pi R^2$. If we approximate the area of the *polyelectrolyte patches* with squares of side $R_{ee}/(2)^{1/2}$, we can finally write

$$n = \frac{4\pi R^2}{s_P} = \frac{8\pi R^2}{R_{ee}^2} \qquad (16)$$

$$m = \frac{Z_{bare}(1-g)\xi_s}{N} \qquad (17)$$

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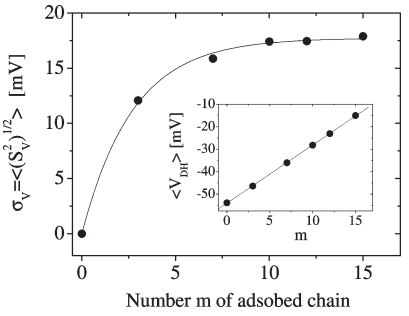

Figure 6. Standard deviation $\sigma_V = (S_V^2)^{1/2}$ at the particle surface potential derived from simulations (black circles) and values calculated on the basis of eq 19. The inset shows the mean value of the surface potential in the Debye−Hückel approximation derived from simulations (black circles) and values calculated on the basis of eq 18.

and, hence

$$< V(\xi_s) > = M\xi_s - V_0 \tag{18}$$

$$S_V^2 = M^2\xi_s^2\left[\frac{1}{\xi_s}\left(\frac{8\pi NR^2}{Z_{bare}(1-g)R_{ee}^2}\right) - 1\right] \tag{19}$$

where M is given by

$$M = \frac{V_0 Z_{bare}(1-g)R_{ee}^2}{8\pi NR^2}(\alpha + 1) \tag{20}$$

To further investigate the electrostatic heterogeneity of the single aggregate for different values of ξ_s, we calculated the mean value of the Debye−Hückel potential $<V_{DH}>$ and its variance S_V^2 at a fixed distance from a polyion−liposome complex, according to the expressions

$$\langle V_{DH}\rangle = \frac{\sum_{j=1}^{N_s}\sum_{i=1}^{N_c} V_{DH}(z_i, \vec{r}_j)}{N_s} \tag{21}$$

$$S_V^2 = \left[\frac{\sum_{j=1}^{N_s}(\sum_{i=1}^{N_c} V_{DH}(z_i, \vec{r}_j) - \langle V_{DH}\rangle)^2}{N_s}\right] \tag{22}$$

where $N_s = 10^4$ is the number of points on a spherical grid surrounding the entire polyion-coated liposome complex; N_c is the total number of charged points; z_i is the valence of the ith charge; and r_j represents a vector indicating the jth grid point.

A typical result showing the average potential $<V_{DH}>$ and the average standard deviation $\sigma_V = (S_V^2)^{1/2}$ obtained by simulations (for details of Monte Carlo simulation in the Debye−Hückel approximation, see Truzzolillo et al.[34]) is reported in Figure 6, together with the corresponding values of the potential (eq 18) and its variance (eq 19) as a function of the charge ratio ξ_s (below the isoelectric point), calculated on the basis of the model sketched in Figure 5. As can be seen, the agreement, even if the model is rather crude, is quite good.

4.1. Liposome-Simple Electrolyte Complexation (Absence of Lateral Correlation Adsorption). Interactions of liposomes with simple electrolytes, i.e., in the absence of a correlated adsorption due to the lack of an association of co-ions along the polymer chain, yield to a completely different phenomenology, marked by

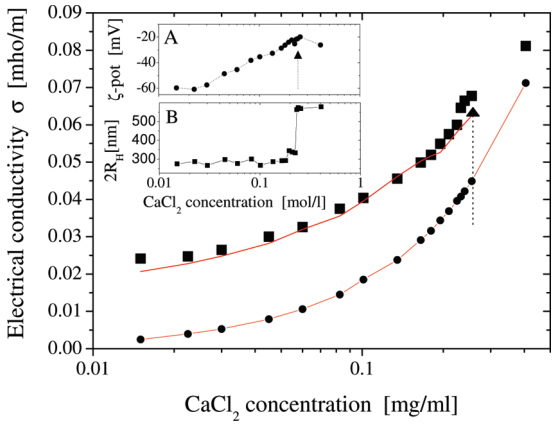

Figure 7. Electrical conductivity σ of a DPPA liposome suspension in the presence of different amounts of $CaCl_2$ electrolyte solution. The continuous line represents the calculated values according to eq 26. The electrical conductivity of the simple electrolyte solution (black circles) is also shown for comparison. The arrow marks the electrolyte concentration at which aggregation (coagulation) occurs. Inset A shows the values of the ζ-potential and inset B the size (the hydrodynamic diameter $2R_H$) of the liposomes as a function of the electrolyte concentration.

the absence of the two prints we have associated with the formation of a cluster phase, i.e., the reentrant condensation and the charge inversion. This different phenomenology is summarized in Figure 7, where we show the electrical conductivity of a DPPA liposome suspension (lipid concentration C_{lipid} = 1 mg/mL) in the presence of a different amount of $CaCl_2$ electrolyte solution, together with the corresponding values of the ζ-potential and the average size R of the resulting aggregates. In this case, the partial ionization of liposomes imparts to the particle surface a negative charge and the concurrent presence in the solution of positive counterions (Na^+ ions). Because of the lack of a short-range correlation, differently from the case of a polyion chain, counterions adsorb onto the particle surface randomly, reducing the overall charge toward zero, without the presence of a charge inversion effect. Moreover, the random adsorption prevents the reentrant condensation effect. The absence of both these effects is well documented in the results presented in Figure 7 (panels A and B).

The process is regulated by the association constant γ between cations and charged groups at the liposome surface. In this case, the electrical conductivity σ of the liposome suspension in the presence of $CaCl_2$ electrolyte solution is given by

$$\sigma = N_L Q_L(ze)(u_L + u_{Na^+}) + 2N_s(ze)(u_{Ca^{++}} + u_{Cl^-}) \tag{23}$$

where the number of charges Q_L at the liposome outer surface depends on the association constant γ of DPPA for Ca^{2+}. The decrease of the surface charge Q_L, relative to the value $Q_{bare}(1 - g)$ in the absence of the electrolyte solution, is described by the relation[35,36]

$$Q_L = (1-\gamma)Q_{bare}(1-g) = \frac{1}{1 - K[Ca^{++}]}Q_0(1-g) \tag{24}$$

with $[Ca^{+2}]$ given by

$$[Ca^{++}] = C_s \exp(-e\varphi_0/K_B T) \tag{25}$$

Here, N_L and N_s are the numerical concentrations of liposomes and electrolyte, respectively. In the numerical evaluation of eq 24,

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the potential φ_0 is supposed to be the same as the measured ζ-potential. Equation 23, written with the usual notations, becomes

$$\sigma = \frac{1}{2} C_{\text{lipid}}(1-g)\frac{1}{1+KC_s \exp(-e\varphi_0/K_B T)}(\lambda_{\text{lipid}}+\lambda_{Na^+})+ \quad (26)$$

$$2C_s(1-\exp(-e\varphi_0/K_B T))\lambda_{Ca^{++}} + 2C_s\lambda_{Cl^-} \quad (27)$$

where C_{lipid} and C_s are the concentrations in moles per unit volume of the lipid molecules and the salt molecules, respectively, and λ_{lipid}, λ_{Na^+}, $\lambda_{Ca^{++}}$, and λ_{Cl^-} are the equivalent conductances of DPPA molecules and of Na^+, Ca^{+2}, and Cl^- ions, respectively. The constant K is the intrinsic binding constant of DPPA for Ca^{+2} ions, and the factor g is the charge renormalization factor, already introduced, to take into account the partial ionization of the DPPA liposomes. The only parameter that was allowed to be changed to get a better fit was the binding constant K, where a value of K = 0.01 L/mol was used.

The predicted values are shown and compared with the experimental results in Figure 7. As can be seen, the agreement with the experimental values is rather good over the whole salt concentration, below the coagulation concentration. In this case, the correlated adsorption does not play any role, and the experimental values are well accounted for by a simple additivity relationship such as eq 23, taking into account the association of cations with the particle surface charge groups.

5. CONCLUSIONS

We are providing experimental evidence that, in the process of polyion-induced liposome aggregation, giving rise to stable, equilibrium clusters of mesoscopic size, the further release of counterions, over the amount predicted by the counterion condensation, is the key parameter of the whole process. This counterion release has been predicted by the *lateral* correlation in the polyion adsorption onto an oppositely charged surface, depending on a delicate balance between a short-range attraction and a screened electrostatic repulsion component in the interparticle potential. The release of counterions in the bulk solution due to the polyion adsorption produces an increment of the electrical conductivity of the whole solution, over the value expected for a solution of liposomes and polyions, having already taken into account the renormalization of their charges. By measuring the dc electrical conductivity of a liposome suspension in the presence of different (and increasing) amounts of oppositely charged counterions, the excess of counterions released in the adsorption process can be easily evaluated. We find that this counterion amount is, within the experimental uncertainties, exactly the one predicted by Nguyen et al.,[12] and by Grosberg et al.,[37] who argued that the entropy gain of the counterions released by adsorbing polyions drives both the two characteristic effects of charge inversion and reentrant condensation. This finding represents a first strong support to the influence of the *lateral* correlation in the formation of stable and equilibrium clusters of charged vesicles (liposomes in this case), stuck together by oppositely charged polyelectrolytes. Counterion release in the cationic liposome−DNA complexation process has been also reported by Wagner et al.[20] some years ago, but in that case, a complete restructuring of the complexes occurs, the aggregates resulting in an array of stacked lipid bilayers with DNA chains sandwiched between them. This restructuring implies the rupture of the lipidic vesicles and, in this case, the effects of the lateral correlation in light of the Nguyen et al. model are less clear and evident. Therefore, to our knowledge, the present study offers the first experimental evidence of counterion release in clusters where the single vesicles maintain their integrity, the role of the polyions being the one to provide the *electrostatic glue* to stick together different vesicles. The advantages in dealing with systems organized as the ones considered here, as far as the possibilities in biotechnological and biomedical applications are concerned, are absolutely conspicuous, suffice it to mention the possibility of the encapsulation of different drugs in different vesicles of the same cluster in multidrug delivery techniques.

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