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Counterion release in overcharging of polyion-liposome complexes

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We present a set of electrical conductivity measurements of a mesoscopic equilibrium cluster phase in the aggregation process of charged particles induced by oppositely charged polyions. These measurements supply strong experimental evidence that correlated adsorption of polyions is driven by the counterion release. This phenomenon, known to occur in DNA-liposome mixtures in lamellar phase, i.e., when liposomes fuse together to form a sandwichlike structure encompassing DNA chains, was not previously observed in aqueous suspension of clusters of intact liposomes stuck together by polyions to form reversible aggregates. A simple statistical model of the lateral correlation of polyions at the particle surface justifies quantitatively the observed behavior of the counterion release, as shown by electrical conductivity measurements.

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The lateral correlation in the adsorption of polyelectrolytes onto oppositely charged spherical surfaces, yielding the formation of large equilibrium complexes, induces two different intriguing phenomena. The first one, known as "charge inversion," occurs when a strongly charged object binds so many oppositely charged counterions that its effective charge inverts the sign [1]. Concomitant to the "charge inversion," there is a "reentrant" condensation effect [2], consisting in aggregates whose average size increases until reaching a maximum at the point of charge inversion, decreasing, afterwards, to the initial values. These two phenomena, which have been observed in experiments [3,4] and simulations [5,6], are of special interest in biological systems such as DNA-liposome complexes [7], polyelectrolyte-micelle [8], and DNA-dendrimer complexes [9], and have recently attracted significative attention.

Both of these two effects characterize a class of colloids where the balance between short-range attractive (charge-patch attraction) and long-range repulsive interaction gives rise to an equilibrium cluster phase, where particles form reversible, relatively large, aggregates. In these colloidal systems, the peculiar mechanism that justifies the short-range attraction is due to the adsorption of polyions on the oppositely charged surface, forming a two-dimensional strongly correlated short-range-order structure [10].

Only recently has the role of the overall charge density of these aggregates become more evident [11–13], while the effect of counterions on the complex formation is still not completely clear. The importance of the counterion release in the complex formation was pointed out by Rädler *et al.* [7] and by Wagner *et al.* [14], who identify this phenomenon as the driving force for the DNA-liposome aggregation, when DNA chains and cationic liposomes fuse together.

In this paper, we are presenting a set of low-frequency electrical conductivity measurements that provides evidence for the role of the counterion release in the polyion-induced liposome aggregation. We show that, when polyions adsorb onto the particle surface, forming a strongly lateral correlated adsorption [10,15], the release of counterions from the polyion and, partially, from the liposome particle surface is the key parameter in the organization of the resulting aggregates. The release stops at the isoelectric condition, when the polyion-coated liposome aggregates are close to neutral.

We used cationic liposomes built up by dioleoyltrimethylammonium propane (DOTAP) (chloride salt) prepared according to the standard procedure, resulting in unilamellar vesicles of an average diameter of about 60 nm and a polydispersity of the order of $p=0.2$. The liposome complexation was induced by poly(acrylate) polyion, a simple anionic highly charged polyelectrolyte of 60 kD molecular weight. We used poly(acrylate) both in salty form (Na-PAA) and in acidic form (H-PAA), added in an appropriate amount to liposome aqueous suspensions, containing a varying volume fraction from $\Phi=2.8\times10^{-3}$ to 1.4×10^{-2}. The polyion concentration was changed from 0.008 to 10 mg/mL in order to cover the whole concentration range where the reversible aggregate phase forms [16]. (The pH of the suspensions varied from 6.0 to 7.0.)

We have investigated the polyion-induced liposome aggregation by characterizing the size of the resulting complexes ("reentrant" condensation effect) through dynamic light-scattering measurements and their electrical behavior ("charge inversion" effect) through electrophoretic mobility and low-frequency electrical conductivity measurements. This latter characterization allows us to evaluate the counterion release during the complexation process.

The size and size distribution of the liposomes and the polyion-induced liposome aggregates were determined from the scattered light-intensity–intensity-correlation functions collected employing a Brookhaven BI9000AT logarithmic correlator and analyzed using the standard data analysis algorithm CONTIN [17] that furnishes the average translational coefficient $\langle D\rangle$, resulting in an average hydrodynamic radius R given by the Stokes-Einstein relationship. The effect of the charge inversion in the polyion-liposome complexes during the overall aggregation was investigated by means of electrophoretic mobility measurements carried out by means of the laser Doppler electrophoresis technique using a MALVERN Zetamaster apparatus equipped with a 5 mW HeNe laser. The electrical conductivity σ of the liposome suspension was measured at a temperature of $(2.5\pm0.1)\,^{\circ}$C by means of radio-frequency impedance analyzers HP mods 4291A and 4294A in the frequency range from 1 kHz to 2 GHz. In this

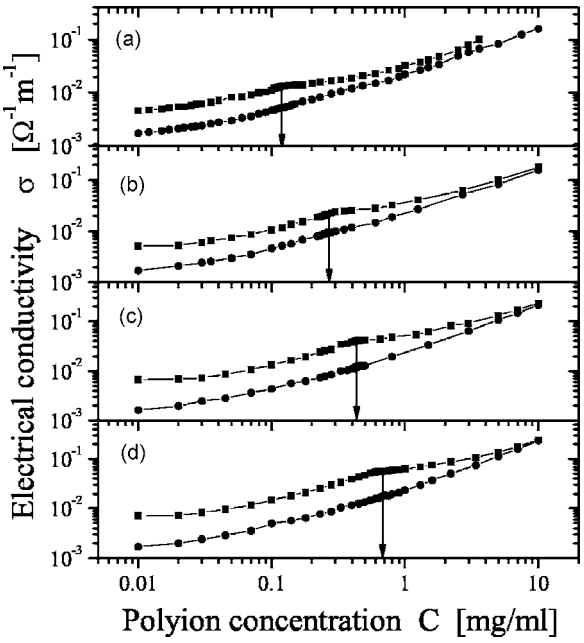

FIG. 1. The low-frequency electrical conductivity σ of polyion-induced liposome aggregates as a function of the Na-PAA polyion concentration. Polyion in saline form is employed, so that the counterion is Na^+. Experiment was carried out at four different liposome concentrations: (a) 0.82 mg/mL; (b) 1.64 mg/mL; (c) 2.87 mg/mL; (d) 4.12 mg/mL. The conductivities of the polyion solution in the absence of liposomes (●) are also shown for comparison. The arrows mark the isoelectric condition.

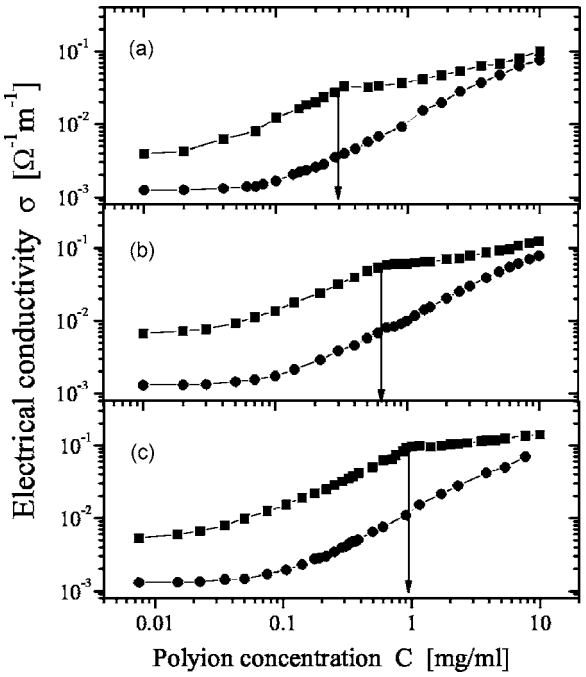

FIG. 2. The low-frequency electrical conductivity σ of polyion-induced liposome aggregates as a function of the H-PAA polyion concentration. Polyion in acidic form is employed, so that the counterion is H^+. Experiment was carried out at three different liposome concentrations: (a) 0.838 mg/mL; (b) 1.67 mg/mL; (c) 2.86 mg/mL. The conductivities of the polyion solution in the absence of liposomes (●) are also shown for comparison. The arrows mark the isoelectric condition.

paper, we will deal exclusively with the low-frequency limit of the measured electrical conductivity. The main results are summarized in Figs. 1 and 2, where σ is shown as a function of the amount of the polyion added, for different values of the liposome concentrations. Figure 1 refers to polyions in salty form and Fig. 2 to polyions in acid form. As can be seen, at a given polyion concentration, the increase of the electrical conductivity changes markedly its slope, in correspondence of the isoelectric point, where the overall charge of the liposome is compensated by the adsorbed polyions and the effective charge of the complex is close to zero. This scenario is clearly confirmed by results shown in Fig. 3, where the change in the slope at the charge neutralization condition (panel A) is accompanied by an inversion of the electrophoretic mobility u ("charge inversion," panel B) and by the maximum in size of the aggregates ("reentrant" condensation, panel C). Analogous behavior is shown by aggregates induced by polyions in salty form (data not shown). The increase of the conductivity approaching the isoelectric point is the print of the counterion release induced by correlated polyion adsorption.

We consider a solution containing polyions at a numerical concentration N_p with degree of polymerization N, "structural" monomer size b, and contour length $L=Nb$. Polyions behave as a charged line with an associated logarithmic electrostatic potential strong enough to bind oppositely charged counterions. Consequently, each polyion bears an electric charge $Q_{\mathrm{eff}}=fNe$ and releases in the aqueous phase a number of counterions Nf, each of them with an elementary charge e. Here, f is the fraction of the effective ionized charged groups on the polyion chain and consequently the fraction of free counterions in the bulk solution. Within the Onsager-Manning condensation model [18], when the parameter $\xi=l_B/b$ is larger than unity (for monovalent ions), the polymer chain has an effective charge density η_{eff} (smaller than the nominal charge density $\eta=e/b$) that is renormalized to a constant value $\eta_{\mathrm{eff}}=e/l_B$, and the fraction f of free counterions is given by $f=1/\xi=b/l_B$. Here, $l_B=e^2/(\epsilon K_B T)$ is the Bjerrum length. Thanks to the polyion renormalized charge density, the concentration of free counterions in the bulk solution is $C_p=N_p(N/\xi)=N_pNb\eta_{\mathrm{eff}}/e=N_pNf$. Complementary, analytical, and numerical solutions of the Poisson-Boltzmann equation [19,20] suggest that, also in the case of spherical charged liposomal vesicles, a charge renormalization occurs, with an apparent charge $Q=Z_{\mathrm{eff}}e$ much smaller than the bare charge $Z_{\mathrm{bare}}e$. Consequently, the concentration C_L of free counterions originating from ionization of the exterior leaflet of the liposome bilayer is $C_L=(1/2)gN_L$, where N_L is the concentration of lipids and g is the charge renormalization factor.

According to Nguyen *et al.* [15,21], in the presence of linear polyions of radius a and effective charge density η_{eff} (negative to make the sign consistent with the case of Na-PAA or H-PAA) and a surface with positive bare charge density ϱ_0, the counterion release, upon the aggregate formation, is governed by two characteristic lengths: the screening length r_s, due to monovalent counterions in the bulk solution,

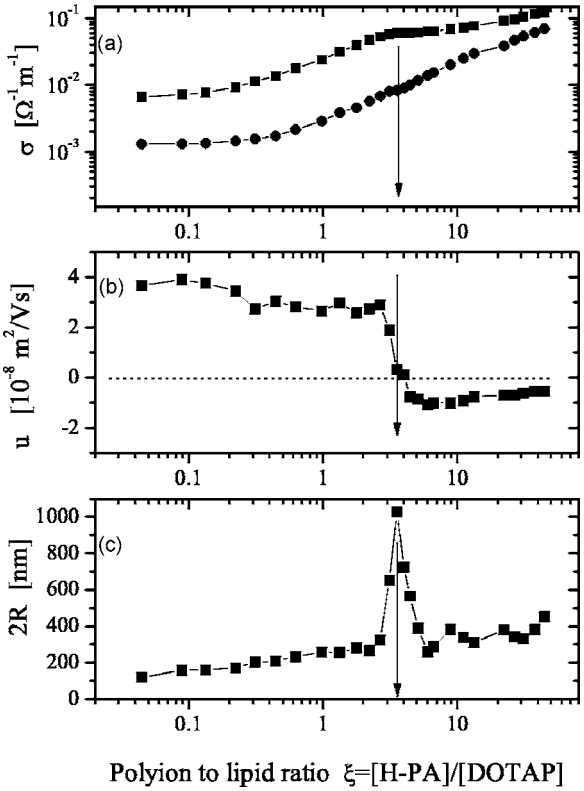

FIG. 3. (a) The electrical conductivity σ (■) compared with the values of the H-PAA polyion solution (●); (b) the electrophoretic mobility u; (c) the average hydrodynamic diameter $2R$ of the polyion-induced liposome aggregates as a function of the polyion to lipid molar ratio ξ=[H-PA]/[DOTAP]. The liposome concentration is 1.67 mg/mL and the polyion is in acidic form. The arrows mark the isoelectric condition.

and the spacing $A_0=\eta_{\text{eff}}/\varrho_0$ between the adsorbed polyions, when they neutralize the charged surface. If $r_s \ll A_0$, counterions from the polyions are not released and they maintain their charge density η_{eff}. 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 charge density η becomes larger than η_{eff}. By imposing the appropriate conditions of equilibrium for the chemical potential of ions in the system, Nguyen *et al.* [15,21] derived the following expression for η: $\eta=\eta_{\text{eff}}\sqrt{\ln(r_s/a)/\ln(A_0/2\pi a)}$. Analogously, the surface charge density ϱ_0 of the particles varies, assuming the value $\varrho=(\eta_{\text{eff}}/\pi a)\exp\{-\sqrt{\ln(r_s/a)\ln[A_0/(2\pi a)]}\}$ that, for $r_s \gg A_0$, remains smaller than ϱ_0.

In this context, it is easy to calculate the electrical conductivity σ of the liposome aggregate suspension during the whole polyion-induced complexation [25]. Assuming additivity and considering the contribution of the counterions alone, the conductivity σ can be written as $\sigma=C_L\lambda^- + C_p(1+\eta/\eta_{\text{eff}})\lambda^+$, where the first term represents the contribution of ions (Cl^- with an equivalent conductance λ^-) derived from the partial dissociation of the lipid polar head groups and the second one the contribution of counterions (Na^+ or H^+, with an equivalent conductance λ^+, according to the chemical state of the polyion, Na-PAA or H-PAA) due to the partial ionization and to the further release upon polyion adsorption.

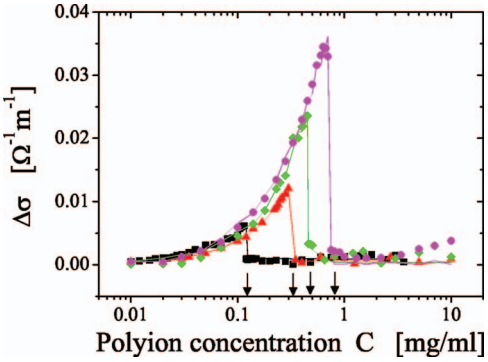

FIG. 4. (Color) The increment in the electrical conductivity $\Delta\sigma$ associated with the counterion release during the polyion-induced liposome complexation, for different lipid concentrations: (■) 0.82 mg/mL; (▲) 1.64 mg/mL; (♦) 2.87 mg/mL; (●) 4.12 mg/mL. The full lines are the values calculated according to the correlated adsorption model. The arrows mark the concentrations at which isoelectric condition occurs.

In order to make a comparison with the experimental results (Figs. 1 and 2), we have determined the renormalizaton factors g and f from measurements of single liposome suspensions and simple polyion solutions. The data have been analyzed within the polyelectrolyte conductivity theory, and values of f and g, in agreement with previous determinations, have been obtained [22–24]. The final results are shown in Fig. 4, where we present the experimental conductivity increment $\Delta\sigma=C_p\lambda^+\eta/\eta_{\text{eff}}$ associated with the counterion release, compared with the values derived from the correlated adsorption model. The data clearly indicate that the correlated adsorption is driven by the counterion release and clearly the phenomenon ends at the isoelectric point.

Moreover, the above-stated model is able to predict, through the dependence of ϱ, the polyion concentration C_0 at which charge neutralization occurs. The balance between the liposome and the polyion charges implies $(\varrho_0-\varrho)N_L S_0=2N_p fNe$, resulting in a concentration $C_0=(1-\varrho/\varrho_0)N_L/(fN)$. For example, in the case shown in Fig. 1, we obtain values of $C_0 \simeq 0.17$, 0.35, 0.60, and

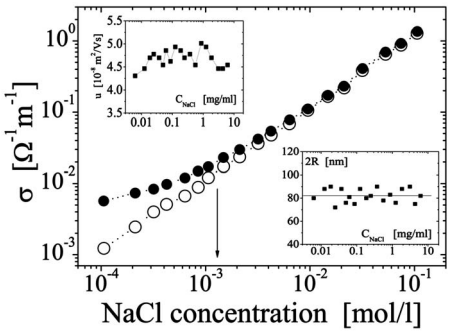

FIG. 5. The electrical conductivity σ of liposome suspension as a function of the simple salt (NaCl) concentration. The lipid concentration is 0.8 mg/mL. The arrow marks the isoelectric point. The upper and lower panels show the constancy of the aggregate size (absence of "reentrant" condensation) and the constancy of the electrophoretic mobility u (absence of "charge inversion").

0.87 mg/mL that are in very good agreement with those experimentally observed (indicated by the arrows in Fig. 1). The same happens for H-PAA polyions.

A final comment is in order. Figure 5 shows the electrical conductivity σ of a DOTAP liposome suspension in the presence of small-size univalent counterions (derived from the dissociation of NaCl salt). As can be seen, the electrical conductivity behavior is thoroughly different. Although the same number of charges effectively added in the aqueous solution has been employed, the lack of lateral correlation in the ion adsorption leads, at the isoelectric point, to the absence of any change of slope in the electrical conductivity, accompanied by the absence of the "reentrant" condensation (Fig. 5, lower panel) or "charge inversion" (Fig. 5, upper panel). This finding gives further evidence that charge inversion and counterion release are determined by polyion correlated adsorption.

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[25] The contribution of each liposomal particle (in spite of its charge) to the overall electrical conductivity is negligible because of the very low volume fraction Φ. At a lipid concentration of 2 mg/mL, for liposomes $R=50$ nm in radius, Φ is of the order of $\Phi \sim 10^{-3}$. Consequently, we expect a change in the conductivity of the disperding medium of the order of $2(1-\Phi)/(2+\Phi)$, within the experimental uncertainties.