Large equilibrium clusters in low-density aqueous suspensions of polyelectrolyte-liposome complexes: A phenomenological model

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In this paper, we revisit some of our previous results together with further experimental evidences for the existence of large equilibrium clusters in low-density aqueous colloidal suspensions, resulting from the screening of charged spherical macroions by oppositely charged linear polyelectrolytes. The aggregation process is described by a simple phenomenological model where aggregates interact via a long-range repulsion Yukawa potential and some supports to the equilibrium properties of the resulting aggregates, on the basis of dynamic light scattering, ζ potential, and transmission electron microscopy techniques, have been provided.

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Systems of interacting colloidal particles and charged polymers have recently attracted great interest, due to their relevance in a number of biological and technological processes [1]. In this Rapid Communication, we investigate the formation of polyelectrolyte-lipid complexes (lipoplexes) in aqueous solutions, consisting of linear, highly charged, anionic polyelectrolytes, and oppositely charged (cationic) liposomes. This system is characterized by a pronounced "charge inversion" that is responsible for the formation of large equilibrium clusters. Under certain conditions, this cluster phase seems to undergo a gelation process, exhibiting an aging behavior. Charge inversion occurs when, at the surface of a mesoscopic charged particle (macroion), more counterions than necessary to neutralize it collapse. As a consequence, the resulting complex displays an overall charge, whose sign is opposite to the one the particle originally bears [2]. This phenomenon, originating by the strong lateral correlation between the adsorbed polyions, depends on ion valence and size. When oppositely charged macroions of comparable size and valence interact, as is the case of anionic polyelectrolytes and cationic liposomes, "giant" charge inversion appears [3]. From the strongly correlated polyelectrolyte adsorption at the liposome surface, a short-range, attractive potential stems, that, close to the stoichiometric isoelectric point, causes the aggregation of the lipoplexes into larger clusters. Avoiding each other and residing as far away as possible to minimize their electrostatic interactions, adsorbed polyions leave the particle surface partially uncovered: lateral correlation causes a more or less ordered distribution of domains with excess negative charge (polyelectrolyte domains) and excess positive charge (polyelectrolyte-free domains) [4]. From such a nonhomogeneous surface charge distribution, a short range "dipolar" attractive potential between lipoplexes ("charge patch" attraction) can arise [5]. Close enough to the isoelectric point, due to a balance between electrostatic repulsion and charge patch attraction, lipoplexes could aggregate, forming large equilibrium clusters.

By combining dynamic light scattering (DLS), ζ potential, and transmission electron microscopy (TEM) techniques, we observed lipoplex complexation by mixing in an aqueous solution a highly negatively charged, flexible polyelectrolyte with positively charged liposomes. We employed a sodium salt of poly(acrylic acid), [-$CH_2CH(CO_2Na)$-]$_n$ (NaPAA) (Polysciences Inc. Warrington, PA) of three different molecular weights (5, 60, and 225 kD) and a cationic lipid, 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) (Avanti Polar Lipids, Alabaster, AL). For all DLS measurements, a fixed angle (137.5) optical fiber probe [Brookhaven fiber optic quasielastic light scattering (FOQELS)] has been employed. Figure 1 shows the reentrant condensation of lipoplexes, at different polymer/lipid molar ratios ξ (monomoles of polyelectrolyte/moles of lipid), the maximum size of aggregates corresponding to the isoelectric point. By changing the liposome surface charge density (adding, for example, a neutral phospholipid, Dioleoyl-Ethanolamine, to the liposome composition) the peak is attained at different polymer contents, but invariably occurs at the isoelectric point. For each measurement, a different sample was pre-

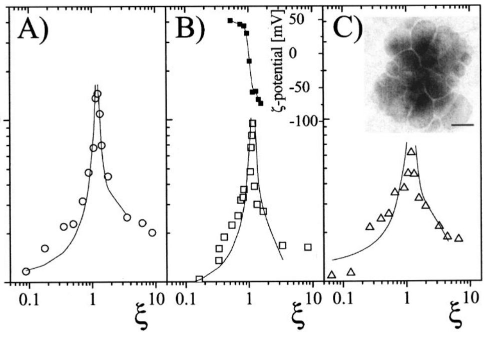

FIG. 1. Average radius $\langle R\rangle/\langle R_0\rangle$ (from DLS measurements) of aggregates at different polyion/lipid molar ratios ξ. $\langle R_0\rangle$ ($\simeq$40 nm) is measured in the absence of polyion. Three different molecular weight polyelectrolytes are employed: (a) (○): Mw $=225$ kD; (b) (□): Mw$=60$ kD; (c) (△): Mw$=5$ kD. The lines are calculated on the basis of Eq. (2). In panel (b) the typical behavior of the ζ potential is also shown (■), the maximum size of aggregates being attained at the isoelectric point. In panel (c), the inset shows a TEM image of a typical aggregate; the bar is 50 nm.

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pared, adding the appropriate amount of polymer to an aliquot of the liposome suspension in one single step. TEM images [6] show that aggregates of increasing size are formed by the clustering of individual liposomes, whose approximately spherical shape, although partially deformed, is still clearly recognizable (as also observed by Huebner *et al.* by cryo-TEM [7] in similar conditions). The smaller clusters have an approximately spherical shape, but close to the peak, larger aggregates assume a more elongated appearance. Radii obtained from DLS agree with those estimated from a statistical analysis of several TEM images on different samples. At sufficiently low polymer concentration, up to $\xi \leqslant 10$, the polyion is completely adsorbed, ruling out depletion interaction as a possible origin of lipoplex aggregation. The "bridging" effect can also be excluded since, for all the molecular weights employed, the end-to-end polymer length is shorter than the liposome size. For NaPAA interacting with colloidal particles of comparable size, significant bridging effects have been observed only at molecular weights larger than 250 kD [8]. The strength of charge patch attraction depends on the heterogeneity of the charge distribution at lipoplex surface: a more homogeneous coating results in a more homogeneous charge distribution and in a weaker attraction. Actually, shorter polyions provide more uniform charge distributions and, consequently, aggregate size shows less pronounced peaks, as observed in Fig. 1. This phenomenology differs from the one observed at higher polymer concentration ($\xi > 10$), where a Derjaquin-Landau-Verwey-Overbeck (DLVO) growth seems to apply [6,9].

A semiquantitative description of the aggregation behavior, close to the isoelectric point, can be derived on the assumption that lipoplex clusters are equilibrium aggregates. Their average size $\langle R \rangle$ results from a balance between the (screened) electrostatic repulsion, due to their residual overall charge, and the short-range attractive potential, due to the nonhomogeneous distribution of the polyion-coated domains. Once the balance is attained, the last lipoplex of radius R_0 sticks to a cluster of radius R already containing $N \sim (R/R_0)^3$ identical lipoplexes. From now on, repulsion due to the charge built up on the cluster exceeds local (i.e., independent of aggregation number N) attractive interaction and no other lipoplex can stick. The pair-interaction screened potential between two spherical macroions at distance r is well described [10] by the Yukawa potential

$$V_{12}(r)/K_BT = Z_1Z_2l_B\left(\frac{\exp(kd/2)}{1+kd/2}\right)^2\frac{\exp(-kr)}{r}, \qquad (1)$$

where Z_1, Z_2 are their effective valences, d the distance between their centers when the two particles are touching, l_B is the Bjerrum length, and k^{-1} the Debye screening length. In our case, counterions released both by liposomes and polyelectrolytes contribute to k, hence k and Z_i are not independent quantities [11]. The balance condition, resulting in an aggregate of $(N+1)$ lipoplexes, is reached when charge patch attraction equals electrostatic repulsion at $r=R+R_0$

$$Z_L^2\left(\frac{R}{R_0}\right)^3\frac{l_B}{[1+k(R+R_0)/2]^2}\frac{1}{(R+R_0)} = \mathcal{E}, \qquad (2)$$

where Z_Le and $Z_L(R/R_0)^3e$ are the charges of the sticking single lipoplex and of the N-lipoplex aggregate, respectively. $Z_Le = Z(1-2f\xi)e$ is the charge of a single lipoplex, considering the polymer adsorption, with Z the number of effective charges at the liposome surface (without polyion adsorption) and f a factor that takes into account the effective ionization of the polyelectrolyte. The charge patch attractive energy $\mathcal{E}$ is assumed independent of the aggregation number N. Equation (2) is an oversimplification, since this equation would be valid for two identical spheres. Even though, as it has been recently demonstrated [12], the repulsive part of the resulting cluster-cluster potential is again of the Yukawa type, the use of Eq. (1) for describing cluster-monomer interactions should be regarded as heuristic. Moreover, larger clusters may be not spherical in shape, but elongated (as it is expected, to minimize long-range repulsion). Nevertheless, this simple model provides a reasonable agreement with observations. Curves in Fig. 1 are calculated with three adjustable parameters: the energy $\mathcal{E}$, the liposome valence Z, and the effective polyion ionization f. For the three molecular weights employed, experimental data can be fitted using only one value for $f(\approx 0.4)$ and $Z(\approx 3.3\times 10^3)$, and with $\mathcal{E}$ that varies between $\approx 1.4\times 10^{-17}$ and $\approx 3.5\times 10^{-17}$ J [13]. At a molar ratio $\xi \approx 1$, aggregates are approximately neutral. However, if vesicles in the aggregates keep their shape, as TEM images demonstrate, only charges on the external leaflet of the lipid double layer are involved and only approximately one half of lipid charges have to be neutralized by the polyion, with an expected isoelectric point at $\xi \approx 0.5$. The apparent inconsistency can be overcome considering the counterion condensation, as Aubouy *et al.* [14] have recently pointed out. Due to the charge confinement effect, counterions are not (completely) released when a polyion approaches an oppositely charged surface. On this basis, it is not surprising that the isoelectric condition is experimentally found at about $\xi = 1$, since condensed counterions ($f \approx 0.4$) take part in the overall charge balance. The fitted value of Z is in reasonable agreement with the liposome charge obtained from the ζ potential ($Z \approx 2.3 \pm 0.3 \times 10^3$) [15].

Figure 2 illustrates the effect of the addition of a simple electrolyte (NaCl) to the lipoplex suspension. In the inset, the curves calculated on the basis of Eq. (2) show a good agreement with experimental data. In this case, a larger value of $Z \approx 6.9\times 10^3$ is obtained, the charge fraction f is approximately constant (≈ 0.45), while the energy $\mathcal{E}$ decreases from $\approx 3\times 10^{-18}$ J to $\approx 5\times 10^{-19}$ J when NaCl increases from 5×10^{-3} to 0.4 M. This decrease is consistent with the electrostatic nature of charge patch attraction that is screened at increasing salt content, even though, in our model, the dependence of $\mathcal{E}$ on k^{-1} is not considered explicitly [16]. In Fig. 3, the aggregation behavior of lipoplexes at three different polyion/liposome ratios as a function of the NaCl concentration is shown. Again, the agreement with Eq. (2) is surprisingly good. The assumption that the observed clusters are equilibrium aggregates has been tested by mixing, at different proportions, two samples prepared at the same polymer/lipid ratio ($\xi = 0.5$), but with a different amount of the simple salt added. Figure 4 shows the particle size distribution for the unmixed samples: Fig. 4(a) 0.004 M NaCl; Fig. 4(b): 0.35 M NaCl, and for their 1:1 vol/vol mixture [Fig. 4(b), empty bars]. For comparison, the size distribution of a

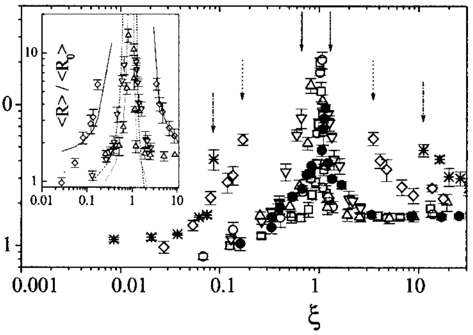

FIG. 2. Average radius, $\langle R\rangle/\langle R_0\rangle$, of lipoplex clusters as a function of the polyion/lipid molar ratio, ξ, in the presence of different amounts of simple electrolyte (NaCl): (∗) 0.5 M; (◇) 0.4 M; (▽) 0.3 M; (△) 0.1 M; (○) 0.05 M; (□) 0.005 M. In all samples, the molecular weight of the polyion was 60 kD. For comparison, results obtained in the absence of added salt (●) are also shown. Arrows mark the boundaries of polyion concentration ranges where the DLS correlation functions display two characteristic decay times (2τ region). These regions widen with the increase of the salt concentration: solid arrows, 0.3 M; dotted arrows, 0.4 M; dash-dotted arrows, 0.5 M. In the inset: the curves calculated on the basis of a fitting of Eq. (2) to experimental values, at selected concentrations of added NaCl: (△) 0.1 M; (▽) 0.3 M; (◇) 0.4 M.

sample obtained by adding NaCl to a final concentration of 0.17 M is also shown [Fig. 4(c), full bars]. The quite good coincidence of the two distributions is a strong evidence of the reversible character of lipoplex aggregation and qualifies the clusters as equilibrium aggregates. The possibility of large equilibrium aggregates has been recently addressed by Groenewold and Kegel [17], although in a different condition (nonaqueous solvents) in systems characterized by long-range repulsive and short-range attractive interactions. Using numerical simulations, Sciortino *et al.* [12] have recently shown an equilibrium cluster phase in such systems, where an arrested disordered state, at small volume fraction, can be generated. Actually, referring again to Fig. 2, in a region around the neutralization condition (marked by arrows), a different dynamics is observed. Within this region, DLS correlation functions become markedly nonexponential, with characteristic times that increase as a function of the waiting time, t_w, showing typical aging behavior. An analysis of the correlation functions results in two correlation times (Fig. 5). While the longer time, τ_s, increases with t_w, the shorter one, τ_f, remains approximatively constant. To clarify this aspect, we have measured the absolute scattered light intensity as a function of the aggregate concentration, c_a, for three different samples (salt concentration $c_s=0.3M$, $\xi=0.6$ and $\xi=0.9$; $c_s=0$, $\xi=0.9$), within and outside the region where the two relaxation times (2τ region) appear. For all the three samples, the quantity Kc_a/R_θ, where R_θ is the Rayleigh ratio and K an optical constant, varies linearly on c_a. However, while for the two samples outside the 2τ region, Kc_a/R_θ remains constant

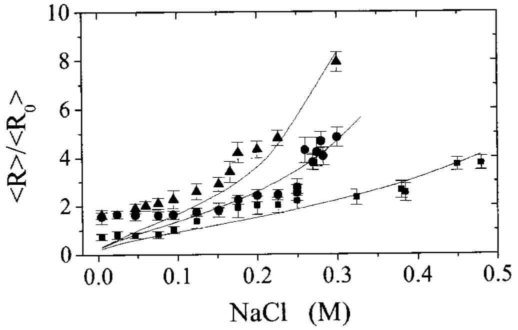

FIG. 3. Average radius of lipoplex clusters as a function of the added simple salt (NaCl) concentration, at three polyion/lipid molar ratios: (▲) $\xi=0.64$; (●) $\xi=0.53$; (■) $\xi=0.26$. The molecular weight of the polyion employed is 60 kD. The full lines are the model fitting, as discussed in the text.

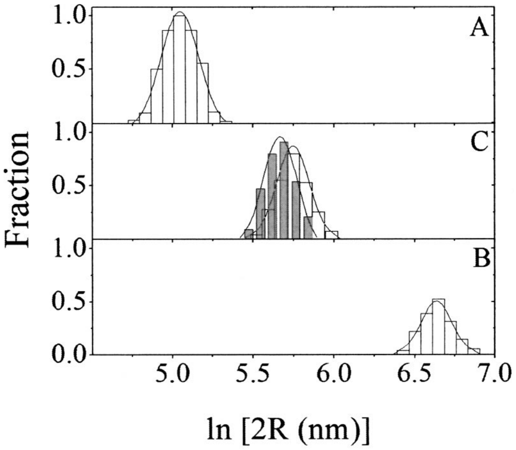

FIG. 4. Particle size distribution at different NaCl electrolyte concentrations: (a) 0.004 mol/l; (b) 0.35 mol/l; (c) (empty bars) 1:1 vol/vol mixture of (a) and (b), resulting in a final concentration of 0.17 mol/l; (c) (full bars) the sample obtained adding directly NaCl to the lipoplex suspension to a final concentration of 0.17 mol/l. The polyion/lipid ratio is $\xi=0.5$. The autocorrelation functions of the intensity of the scattered light have been analyzed by using a non-negatively linear sampling (NNLS) algorithm. The distributions are normalized to the intensity of the scattered light measured for sample (a).

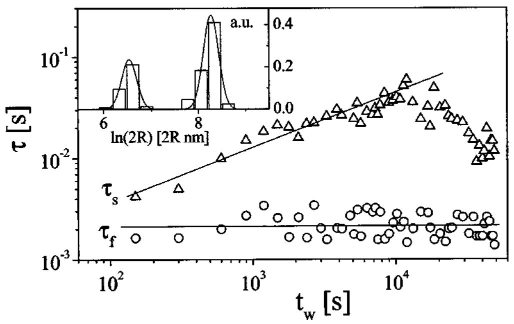

FIG. 5. The analysis of DLS correlation functions at different sample ages, in terms of time distribution (obtained by the inverse Laplace transformation algorithm CONTIN), reveals the presence of two peaks. A first one, centered at 2×10^{-3} s, is independent of the sample age t_w. The second one increases with t_w up to $\approx10^4$ s and then decreases. The inset shows a typical distribution of correlation times obtained from a measured correlation function. The age dependence of τ_s shows a logarithmic regime with an exponent 0.55.

in the whole dilution range, for the sample inside the 2τ region, the slope of Kc_a/R_θ vs c_a is large and positive, indicating a repulsive interaction among the particles. This result strongly supports the interpretation of the anomalous relaxation observed close to the isoelectric point, at sufficiently high salt content, as due to the strong interaction between particles. Moreover, TEM images, in these experimental conditions, do not show any evidence for the presence of the huge aggregates that should correspond to the longer correlation times τ_s, resulting in an apparent small diffusion coefficient. The presence of two relaxations is typical of arrested systems [18], where each particle is described as being trapped in a transient cage formed by its surrounding neighbors. In this picture, the faster relaxation is due to the fast diffusive displacement of the particle within its cage, while the slower one is due to the process of escaping from the cage. These findings suggest that, in a region around the isoelectric point, an arrested phase might exist when, due to the increase in the overall charge of aggregates, interclusters repulsion leads to a restricted mobility.

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[15] From the area that a DOTAP molecule occupies in a monolayer (≈ 65 Å^2), a stoichiometric value of $Z = 3.5 \times 10^4$ should result. The observed difference indicates a large counterion condensation, which reduces the effective charge on the liposome surface.

[16] It is worth noting that the Debye length k^{-1} decreases from ≈ 20 to ≈ 3 nm, as the salt concentration is increased. Intuitively, this small length compared with liposome size should rule out repulsions over distances of the order of lipoplex diameter. However, this effect is compensated by the large increase of the repulsion strength, due to the overall charge that builds up on the clusters.

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