On the effect of temperature on the reentrant condensation in polyelectrolyte-liposome complexation

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Abstract

Interactions of oppositely charged macroions in aqueous solution give rise to intriguing aggregation phenomena, resulting in finite-size, long-lived clusters, characterized by a quite narrow size distribution. Particularly, the adsorption of highly charged linear polyelectrolytes on oppositely charged colloidal particles is strongly correlated and some short-range order arises from competing electrostatic interactions between like-charged polymer chains (repulsion) and between polymer chains and particle surface (attraction). The charge inversion observed for the polyelectrolyte-decorated primary particles has been recently explained in terms of correlated adsorption. In these systems, long-lived clusters of polyelectrolyte-decorated particles form in an interval of concentrations around the inversion point. However, the mechanisms that drive the aggregation and stabilize, at the different polymer/particle ratios, a well defined size of the aggregates are not completely understood. Nor is clear the role that the correlated adsorption plays in the aggregation, although the importance of ’patchy interactions’ has been stressed as the possible source of attractive interactions between colloidal particles. Different models have been introduced to explain the formation of the observed finite-size cluster phase. However a central question still remains unanswered, i.e., whether the clusters are true equilibrium or metastable aggregates. To elucidate this point, in this work, we have investigated the effect of the temperature on the formation of the clusters. We employed liposomes built up by DOTAP lipid interacting with a simple anionic polyion, sodium polyacrylate, over an extended concentration range below and over the isoelectric condition. Our results show that the aggregation process can be described by a thermally-activated mechanism.
1 Introduction

Charged colloidal particles in the presence of highly charged linear polyelectrolytes self assemble in long-lived, finite-size mesoscopic aggregates. Despite the increasing body of experimental and theoretical work \[1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11\], the mechanisms that drive the aggregation and stabilize the aggregate size are not completely understood.

The strong and relatively long-ranged electrostatic interactions, the screening due to the diffuse ionic layers surrounding the particles, and the simultaneous presence of macroions different in geometry and in charge density (the linear polyelectrolytes and the bulky particles), make these systems inherently complex. The formation of the clusters is hence the result of a delicate balance between attractions and repulsions characterized by different ranges and also differing in their nature (mainly electrostatic \[8\] but also hydrophobic \[12, 5\] interactions).

The adsorption of linear polyelectrolytes on the surface of the oppositely charged particles occurs in a highly correlated manner. The competing interactions between polymers and surface (attraction) and between the polymers (repulsion) results in a locally ordered, correlated adsorption of the chains \[13, 14, 15, 16, 17\]. This non-uniform distribution gives rise to a non-uniform distribution of the surface electric charge, with alternate patches where the charge of the polymer or that of the bare particle surface are locally in excess. This correlated adsorption is the cause of the counterintuitive phenomenon of the ‘overcharging’, or charge inversion, which occurs when more polyelectrolyte adsorbs than is needed to neutralize the original charge of the surface, so that the net charge of the polymer-decorated surface inverts its sign.

From the first observations of the destabilizing effect of polyelectrolytes on colloidal suspensions, attractive ‘bridging’ forces have been proposed as the cause of aggregation \[18\]. However, when a polyelectrolyte solution and a suspension of oppositely charged colloidal particles are mixed together, the adsorption of the polymer on the particles and the aggregation occur on two different timescales. Being favored by the huge surface/volume ratio of the colloid, with the surface ‘finely distributed’ within the whole volume of the host phase, the adsorption should be completed in a few seconds from the mixing. Conversely, the aggregation process, due to the lower diffusivity of the bulkier colloidal particles should occur on a longer timescale. To this reasonable picture has been recently given a solid experimental basis \[8\].

As a consequence of the different timescales, the ‘primary particles’ that are involved in the aggregation process are the polyelectrolyte-decorated particles. This observation is not to be overlooked, since it suggests a useful shift of the perspective to guide the investigation of this phenomenology. Rather than hypothesizing complex scenarios of ‘polyelectrolyte-mediated’ interactions, where the polyelectrolytes, free in the solution among the particles, drive the aggregation of the colloid by a mix of osmotic, electrostatic screening and bridging effects, one has to deal with a much simpler system of (non-uniformly) polymer-coated particles.
There is much experimental evidence \cite{19, 20} as well as theoretical arguments \cite{15, 21, 22} in favor of a configuration of the adsorbed polyelectrolyte chains that, although with several loops and possibly dangling ends, lie quite flat on the particle surface, particularly if the the charge densities on the surface and/or on the chains are high \cite{23}. Also these considerations suggest that bridging mechanism should be excluded as the cause of aggregation, except perhaps for very long polyelectrolytes and very small particles.

The aggregation of charged colloids induced by oppositely charged polyelectrolytes, and the stability of the resulting clusters, appears mainly governed by electrostatic forces \cite{24, 10, 11}. While repulsions derive the electrostatic diffuse layer overlap, as described in the classical Derjaguin-Landau-Verwey-Overbeek theory (DLVO), attractions, besides the ubiquitous van der Waals dispersion forces, include an additional non-DLVO attractive force, originating from 'patch-charge' heterogeneities \cite{25, 26, 27}. In a broad sense, this interaction can still be viewed as a sort of bridging, since chain configurations span the inter-particle region to gain substantial entropy \cite{28}.

On the basis of the classical DLVO theory, one expects a stable suspension far away from the isoelectric point (IP), and a destabilization, with the formation of larger and larger aggregates that eventually precipitate, close to the IP. In fact, associated to the progressive reduction of the net charge of the polyelectrolyte-decorated particles, an increase of the cluster size is observed. The largest size of the aggregates is observed close to the isoelectric point, while any further addition of polyelectrolytes promotes a progressive decrease of their size. Eventually, when the particle surface is completely saturated by the adsorbed polyelectrolyte and the overcharging has reached its plateau value, the size of the particles in the suspension equals again the size of the primary particles (‘reentrant condensation’) plus a thin layer of adsorbed polymer \cite{3, 8, 4}. This reentrant condensation has been observed in a variety of colloidal systems, ranging from polyelectrolyte-micelle complexes \cite{2}, latex particles \cite{29}, dendrimers \cite{30}, phospholipid vesicles (liposomes) \cite{31, 3, 8}, to ‘hybrid niosomal’ vesicles \cite{12}. Although the adsorption patterns are further complicated by the presence of short-range interactions specific to the different systems, the similarities in the observed behavior for such different systems strongly indicate that the overall phenomenology is mainly governed by electrostatic interactions, arising from double layer overlap (repulsion) and surface charge non-uniformity (attraction).

DLVO theory does not account for these long-lived finite-size aggregates. By itself, the theory only provides a description of the stabilization/destabilization behavior. Once the colloid had been destabilized, the size of the clusters evolve along dynamics that depends on both particle diffusivity and sticking characteristics (diffusion limited or reaction limited cluster aggregation, for example) but, in principle, the aggregation eventually results in a phase separation. The formation of metastable finite-size clusters is not contemplated by the theory, but is the result of some different mechanisms. Usually, a metastable cluster phase is considered the consequence of a ‘frustrated aggregation’ due to the decreasing diffusivity that progressively reduces the chance of an encounter for the larger
clusters. Then the metastable cluster phase is 'kinetically stabilized'. However, within this theoretical framework, it is difficult to account for the dependence of the aggregate size on their net residual charge.

A different mechanism of stabilization of a cluster phase has been described in the case of colloids characterized by the presence of short-range attractive and long-range repulsive interactions [32, 33, 34, 35, 36]. Different authors have hypothesized that stable clusters can result from the equilibrium of long-ranged screened electrostatic repulsions and short-range attractions, where the like-charged primary particles within the same clusters repel each other even though they are far apart and not in a direct contact. However, due to the very small screening length in aqueous systems, when compared to the size of lipid vesicles, these model does not seem applicable to polyelectrolyte-liposome suspensions.

We have recently shown that the formation of a cluster phase and also the observed increase, with the aggregate size, of the apparent cluster-cluster repulsive interactions as measured by the second virial coefficient [10], can be effectively taken into account the aggregation of the polyelectrolyte-decorated particles as a thermally activated process.

Velegol and Twar [27] have recently developed an analytical model for the potential of mean force between non-uniformly charged particles. They have shown that a non-uniform charge distribution at the surface of colloidal particles gives place to an inter-particle potential that, even in the case of same-sign charged particles, has an attractive component. The model is based on the Derjaguin approximation and on an extension of the Hogg-Healy-Fuerstenau [HHF] model [37].

The resulting potential depends on the values of the average electrostatic surface potential, Φ, and on its standard deviation, σ. This inter-particle potential, for some combinations of the values of Φ and σ, shows a maximum, close to the particles surface, representing an energy barrier that particles have to pass for sticking together. The height H of this barrier increases with the radius of curvature, R, of the surface of the two approaching particles, i.e., for deformable particles (as is the case of polyelectrolyte decorated lipid vesicles), with the aggregate size, and depends on the surface potential Φ, going approximately as $H \propto R^2$. By adding to this potential a contribution from van der Waals attraction, operating at smaller distance, this picture does not result substantially modified.

As a further test of this picture, in this paper, we investigate the effect of the temperature on the aggregation behavior of colloidal charged particles induced by addition of oppositely charged polyanions. We will show that, as is expected for a thermally activated process, the size of the aggregates increases when they are formed at higher temperatures. Here, we deal with the complexation between an anionic linear polyelectrolyte, sodium polyacrylate [NaPA], and liposomes built up with the cationic lipid di-oleoyl-trimethyl-ammonium-propane [DOTAP] carried on at different temperatures, from 5 to 80 °C. Although the range of temperature variation is intrinsically limited by the liposome stability and by the aqueous nature of the dispersing medium, a significant effect is observed on cluster
properties, particularly on their size that, close to the isoelectric point, increases appreciably when the temperature is increased from 5 to 80 °C.

2 Experimental

2.1 Materials

Positively charged liposomes were prepared by employing the cationic lipid dioleoyl-trimethyl-ammonium-propane [DOTAP], purchased from Avanti Polar Lipids (Alabaster, AL) and used without further purification. The negatively charged polyelectrolyte sodium polyacrylate, $[-CH_2CH(CO_2Na)\cdots]_n$ [NaPAA], with nominal molecular weight 60 kD, was purchased from Polysciences Inc. (Warrington, PA) as 25 % aqueous solution. All liposomal samples and polyelectrolyte solutions were prepared in Milli-Q water, with electrical conductivity less than $1 \cdot 10^{-6}$ mho/cm.

2.2 Preparation of cationic lipid-polyion complexes

Liposomes were prepared by dissolution of an appropriate amount of DOTAP in methanol-chloroform solution (1:1 vol/vol). After overnight vacuum rotovaporation of the solvent, the dried lipid film was re-hydrated with Milli-Q quality water. The re-hydration process was carried out for 1 hour at a temperature of 40°C, well above the main phase transition temperature of this lipid ($T_f \approx 0°C$). In order to form small uni-lamellar vesicles, the lipid solution was sonicated for 1 hour at a pulsed power mode, until the solution appeared optically transparent in white light; the solution was then filtered by means of a Millipore 0.4 µm polycarbonate filter. For all the experiments, liposomes were prepared at a concentration of 1.5 mg/ml, corresponding to approximately $1.5 \cdot 10^{13}$ particle/ml (average radius 40 nm) and the solutions were stored at 4°C. For all the preparations, the size distribution of the DOTAP liposomes was log-normal with a mean hydrodynamic radius $40 \pm 5$ nm and with a polydispersity of the order of 0.2, as expected for a rather homogeneous particle suspension.

Measurements on liposomes-polyelectrolyte complexes were performed at various temperatures, from 5 to 80 °C. Lipoplexes were formed immediately before each measurement by mixing equal volumes of the liposome suspension and polyelectrolyte solutions at the appropriate concentration, as elsewhere described [4, 35]. Before mixing, liposomes and polyelectrolyte solutions were thermostatted at the desired temperature. The mixed sample was placed in a thermostatted cell for the measurement of both electrophoretic mobility and size and size distribution. Electrophoretic mobility measurements were performed 5 minutes after the mixing, immediately followed by size determination. The temperature was controlled within ±0.1°C.
2.3 Electrophoretic mobility and Dynamic light scattering measurements

Both the electrophoretic mobility and the size and size distribution of the suspended particles were measured by means of an integrated apparatus, NanoZetaSizer (Malvern Instruments LTD, UK) equipped with a 5 mW He-Ne laser. In this way the electrophoretic parameters and the size of the particles can be measured on the same sample and almost simultaneously, reducing the experimental uncertainties related to sample preparation, thermal gradients and convective movement that, in the presence of large aggregates within the suspension, can be considerable.

The electrophoretic mobility measurements were carried out by means of the laser Doppler electrophoresis technique. The mobility \( u \) was converted into the \( \zeta \)-potential using the Smoluchowski relation \( \zeta = u\eta/\epsilon \), where \( \eta \) and \( \epsilon \) are the viscosity and the permittivity of the solvent phase, respectively.

Size and size distribution of liposomes and polyions-liposome aggregates were measured by means of dynamic light scattering (DLS) technique, collecting the normalized intensity autocorrelation functions at an angle of 173° and analyzing the collected data by using the CONTIN algorithm [39], in order to obtain the decay time distribution of the electric field autocorrelation functions. Decay times are used to determine the distribution of the diffusion coefficients \( D \) of the particles, which in turn can be converted in a distribution of apparent hydrodynamic radii \( R_H \) using the Stokes-Einstein relationship \( R_H = K_B T / 6\pi\eta D \), where \( K_B T \) is the thermal energy and \( \eta \) the solvent viscosity. The values of the radii shown here correspond to the average values on several measurements and are obtained from intensity weighted distributions.

2.4 Simulations

We simulated the aggregation behavior of a system composed by \( N_p = 10000 \) particles of initial diameter \( 2R = 80 \) nm in a cubic box of volume \( V \) with packing fraction \( \phi = 4\pi\rho R^3/3 = 0.01 \), where \( \rho = N_p/V \) is the number density. We carried out MC simulation using local metropolis algorithm at \( T = 298 \) K. Particles interact via a short-range potential defined by eq. [30]. The parameters of the simulations have been detailed elsewhere [40]. We only recall here that to incorporate a Brownian dynamics in the MC algorithm, the \( i \)th particle is selected with a probability proportional to \( R_0/R_i \), where \( R_0 \) is the initial radius and \( R_i \) is the radius of the \( i \)th aggregate. Each selected aggregate is then moved in each direction by a random quantity (uniformly distributed between 0.2 nm). Since the aggregation process progressively slows down, simulations were interrupted when a plateau in the time dependence of the aggregate average radius was reached.
3 Results and discussion

We have investigated by means of electrophoretic and dynamic light scattering measurements the complexation of cationic DOTAP liposomes in the presence of anionic NaPA polyelectrolytes, as a function of the temperature in the interval from 5 to 80 °C.

Preliminarily, the thermal behavior of the liposome dispersion in the absence of the polyelectrolyte was investigated. The temperature of the liposome dispersion was varied by one step, by immersing the samples, at the initial temperature of T=25 °C, in a thermostatted bath at the desired final temperature and allowing to thermalize for 30 minutes before measuring the size. Following this procedure, no significant variation of liposome size is observed due to temperature change. It was found that different thermal protocols induce large variations of liposome size, as it was observed for other liposome systems [41]. For example, by varying the temperature very slowly, in steps of 1 ÷ 2 °C, and allowing to thermalize for several minutes after each step, we have observed a decrease in the vesicles radius as large as 50% in passing from T=5 to T=80 °C (data not shown). A small, but significant, increase of the ζ-potential was observed at increasing temperature, independently of the procedure employed to vary the temperature (data not shown). Taking the advantage of these preliminary investigations, only the the procedure of ‘rapid immersion’ was employed.

The stability of DOTAP liposome maintained at the desired temperature within the interval 5±80 °C was checked by repeated measurements of size and ζ-potential. Over a period of 24 hours nor the size nor the ζ-potential show appreciable variations. This high stability of DOTAP liposomes was largely expected, since in the whole interval of temperatures investigated the double layers of this lipid are in the liquid phase (the main transition temperature of this lipid is close to 0 °C) and do not undergo any phase transition that could facilitate a lipid restructuring [42].

Analogously, also the complexes that form on mixing liposomes with the polyelectrolyte remain stable in time over more than 12 hours, exhibiting a mono-modal log-normal distribution when analyzed by CONTIN. Only very close to the isoelectric point, large clusters appear, that rapidly precipitate, this phenomenon being more pronounced at the higher temperatures investigated.

As usual [38, 43], the behavior of the ζ-potential and hydrodynamic radius of complexes is shown as a function of a ‘stoichiometric’ charge ratio ξ, defined as the ratio between the total number $N^-\,$ of the negative charges on the polyion chains and the total number $N^+$ of the positive charges on DOTAP molecules in the whole suspension

$$\xi = \frac{N^-}{N^+} = \frac{C_M}{M_{w_M}} \frac{M_{WD}}{C_D}$$

where $C_M$ and $C_D$ are the (weight) concentrations of the polyion and DOTAP, respectively, and $M_{w_M}$ and $M_{WD}$ are the molecular weights of the repeating unit of the polyion and of DOTAP. This definition of $N^-/N^+$ considers all the
Fig. 1 shows the hydrodynamic diameter $2R$ and the corresponding $\zeta$-potential as a function of $\xi$ for the liposome-polyelectrolyte complexes, at some selected temperatures in the range from 5 to 80 $^\circ$C. At all the temperatures investigated, liposome-polyelectrolyte complexes show a 'reentrant condensation' accompanied by a $\zeta$-potential inversion, i.e. the inversion of the sign of their net electric charge. Close to the inversion point (isoelectric point), the complexes reach their maximum size. However, at any given polyelectrolyte-liposome ratio, the size of the aggregates depends on the temperature, the largest clusters being observed at the higher temperatures. This result indicates that aggregation is favored by higher temperatures, as it should be in a thermally activated process.

We have recently shown that the observed reentrant condensation and the accompanying charge inversion can be both explained in terms of the correlated adsorption of polyelectrolytes on the oppositely charged liposomes [38, 4]. The resulting attraction between the polyelectrolyte-decorated particles is due to the non-uniform distribution of the net electrostatic charge at their surface [10, 40].

The adsorption of the linear polyelectrolytes on the surface of the oppositely charged particles occurs in a highly correlated manner. In fact, the competing interactions between polymer and surface (attraction) and between polymers (repulsion) result in a locally ordered, correlated adsorption of the polymer [13, 14, 15, 16, 17].

The non-uniform distribution of the adsorbed chains gives also rise to a non-uniform distribution of the surface electric charge, with alternate patches where the charge of the polymer or the one of the particle surface are locally in excess.

Velegol and Twar [27] have recently developed an analytical model for the potential of mean force between non-uniformly charged particles. They have shown that a non-uniform charge distribution at the particle surface results in an inter-particle potential that, even in the case of particles that bear a net charge of the same sign, has an attractive component. The model is based on an extension of the Hogg-Healy-Fuerstenau [HHF] model [37] and on the Derjaguin approximation. This approximation holds when the characteristic radius of the smaller sphere is much larger than the characteristic length scale for the interaction [41], i.e. the Debye screening length.

By using the Derjaguin approximation, the generic force $F(h)$ between the surfaces of two spheres of radii $R_1$ and $R_2$, at a distance $h$ in terms of the potential $G(h)$ that would be observed the two surfaces were infinite planes at the same distance $h$, can be written as [44]

$$F(h) \propto \frac{R_1 R_2}{R_1 + R_2} G(h)$$  \hspace{1cm} (2)

This expression clearly shows that, as a general rule and independently of the nature of the inter-particle potential, whenever the Derjaguin approximation holds, when their radius increases, the force between two spherical particles
also increases, toward the limiting force observed for two planes facing each other.

According to Veleogol and Twar, the mean force pair interaction potential between two spherical particles \(A\) and \(B\) bearing a non-uniformly distributed electric charge on their surface can be written, in units of the thermal energy \(k_B T\), as

\[
\langle \Phi \rangle = \frac{\epsilon \pi R_A R_B}{R_A + R_B} \left[ (\zeta_A^2 + \zeta_B^2 + \sigma_A^2 + \sigma_B^2) \ln(1 - e^{-2\kappa H}) + 2\zeta_A \zeta_B \ln \left( \coth \frac{\kappa H}{2} \right) \right]
\]  

(3)

were \(H\) is the distance between the surfaces of the two approaching particles, \(\epsilon\) the permittivity of the medium and \(\kappa^{-1}\) the Debye screening length. This potential depends on the values of the average electrostatic surface potentials, \(\zeta_i\)-potential, and on their standard deviations \(\sigma_i\), combining a net charge-dependent monopole term (for \(\zeta_i \neq 0\)), which is repulsive for like-charged particles, and an always attractive multipole term \((\sigma_i \neq 0)\) arising from the charge heterogeneity. These two components lead up to a global maximum and to a potential barrier. The height of the potential barrier that two approaching particles must overcome in order to stick together and the separation \(H_{\text{max}}\) between the particle surface at which this maximum occurs can be evaluated from eq. \(3\). For two identical particles \((R_A = R_B = R)\) we obtain

\[
\Phi_{\text{max}} = \frac{\pi \epsilon R \left( \zeta^2 + \sigma^2 \right) \ln \left[ 1 - \left( \frac{\zeta^2}{\zeta^2 + \sigma^2} \right)^2 \right] + \zeta^2 \ln \left[ \frac{2\zeta^2 + \sigma^2}{\sigma^2} \right] }{\kappa}
\]

(4)

and

\[
H_{\text{max}} = \frac{1}{\kappa} \ln \left( \frac{\zeta^2 + \sigma^2}{\zeta^2} \right)
\]

(5)

respectively. The height of this barrier increases with the radius of curvature, \(R\), of the surface of the two approaching particles, i.e., for deformable particles (as is the case of polyelectrolyte decorated lipid vesicles \[10\]) with the aggregate size.

At each temperature, the increase of the height of the potential barrier favors the formation of finite size aggregates. Within this framework, the observed increase of the size of the aggregates at the higher temperatures can be easily explained. In fact, as the temperature is increased, the fraction of particles that have a sufficient thermal energy to overcome the potential barrier increases. Since the barrier height itself increases with the size of the aggregates, larger and larger aggregates are stabilized at higher temperatures.

The aggregation behavior of a system of non-uniformly charged particles was recently investigated by the present authors by means of Monte Carlo simulations \[40\] in order to derive the limiting size of the aggregates. Simulations qualitatively reproduce the observed trends in different colloidal systems. As an example, in fig. \[2\] it is shown the typical time evolution of the normalized
mean radius of the clusters that are formed by non-uniformly charged particles interacting through the potential described by equation\textsuperscript{3}. Fig. 2 shows the effect on this evolution, for two different average surface electrostatic potentials $\zeta = 15$ mV and $\zeta = 11$ mV, for a fixed value of the non-uniformity parameter $\sigma = 15$ mV. After an initial transient regime the radius of the aggregates increases in a similar way for the two considered $\zeta$-potential values. The growth process slows down at long times and the aggregate size reaches a long-time limit value that depends on the electrical surface parameters $\zeta$ and $\sigma$. For the same value of surface inhomogeneity $\sigma$, the kinetic arrest is reached at higher values of mean cluster size when surface potential $\zeta$ is lower (see inset of Fig. 2).

In all our simulations the plateau is reached when the height of the potential barrier between the particles is $\approx 10 K_B T$. This observation provides a strong support to the hypothesis that the dynamics slows down and the arrest of the growth observed on the timescale sampled by our simulation is controlled by a mechanism of 'thermal stabilization', i.e., the increase of the potential barrier with the radius stabilizes a 'limiting size' of the aggregates. Fig. 3 shows the comparison between the mean cluster size reached at the kinetic arrest obtained by MC simulations (filled circles) and the ones calculated according to eq. 4 within the framework of Velegol and Twar model, with $\Phi_{max} = 10 K_B T$, as a function of $\zeta$ and as a function of $\sigma$ (inset, Fig. 3). The observed agreement supports the evidence for a kinetic arrest at a well defined value of repulsive interactions between particles.

Here, we only report on the effect of the temperature on the size of the aggregates. Fig. 4 shows the limiting radius of the aggregates, as a function of $\zeta$-potential, according to the eq. 4 at the different temperatures. In the inset, the effect of the charge inhomogeneity parameter $\sigma$ on the mean force potential is shown as a function of the minimal distance $H$ between the surfaces of the two approaching particles. These behaviors justifies the increase of the size of the clusters as the temperature is raised for all the values of the surface potential.

The experimental results shown in Fig. 1 can be compared in a semi-quantitative way with the predictions of the model. Expressing the $\zeta$-potential as a function of the polyelectrolyte-liposome charge ratio $\xi$ (Fig. 1 bottom panel), the predicted average radius of the aggregates can be calculated as a function of $\xi$ from eq. 4.

Charge inhomogeneity is more difficult to evaluate. Because of the irregular shape and of the high deformability of our aggregates, a direct measurement of the $\sigma$ values by electrophoretic rotation appears difficult. However, a dependence of $\sigma$ on $\xi$ can be hypothesized on the basis of simple considerations. $\sigma$ is expected to be close to zero at the beginning of the aggregation process, where the aggregates show a $\zeta$-potential very similar to the one of bare primary particles. At these values of the charge ratio, only a few polion chains adsorb individually on the liposome surface and these isolated spots produce negligible inhomogeneity on the average. The inhomogeneity of the adsorption layer increases with the increase of the polion content, and $\sigma$ is expected to reach its maximum value at the isoelectric point, and then it is expected to decrease again as the adsorbed polion layer becomes more and more compact. This
behavior can be modeled assuming a Gaussian dependence of $\sigma$ on $\xi$, whose width is calculated as the half-height width of the condensation region at the different temperatures (Fig. 1 upper panels), while the amplitude is a free parameter to be determined by the fitting procedure. Finally, the value of the potential barrier height $\Phi_{\text{max}}$ in eq. (4) is somewhat arbitrarily fixed at $10 K_B T$. This choice appears justified by MC simulations, showing that for this height a dynamical arrest of the aggregation occurs (see, for example, Fig. 2, 3 and ref. [40]). With all these assumptions, the eq. (4) can be inverted and the expected limiting radius calculated in the whole range of the charge ratio $\xi$ investigated.

Fig. 5 shows a typical result of this analysis. The diameters measured at $20 \degree C$ (symbols) are compared with the $R(\zeta, \sigma)$ curve calculated on the basis of the eq. (4) (continuous line). An analogous reasonable agreement is found for all the temperatures investigated (data not shown).

Interestingly, the amplitude of the gaussian curve that represents the dependence of $\sigma$ on $\xi$, i.e. the maximum value assumed by $\sigma$, does not vary significantly with the temperature (the values being comprised within the interval $50 \pm 5 \text{ mV}$), indicating that inhomogeneity of the surface polion coverage at the isoelectric point is largely independent of temperature.

It must be noted that the final cluster size has an explicit dependence on temperature through the expression of the potential barrier height (eq. (4)). It could be interesting to determine if temperature changes affect the cluster size according to what is predicted by the explicit dependence on the temperature $T$, or if other effects have to be considered. To this aim, we have plotted together all the data at the different temperatures in Fig. 6, shifting the position of the aggregation peaks respect to the one at $80 \degree C$. Fig. 6 shows that all the experimental data, in the whole range $5 \div 80 \degree C$, collapse on a master curve when re-scaled, suggesting that the aggregation kinetics of polion-decorated liposome in the temperature range investigated is the same, independently of temperature, and it is typical of a thermal activated process.

This result is expected within the framework of Velebol and Twar model [45]. Considering the dependence of $R$ on $\zeta$-potential and $\sigma$, (eq. (4)), once that the dependence $\zeta(\xi)$ and $\sigma(\xi)$ are evaluated at each given temperature with the aid of experimental data, only the linear dependence on temperature, in the potential barrier height, remains.

At the isoelectric point, where large flocs are observed, higher temperatures seem to promote a more rapid gross aggregation. The universality of the aggregation appears to be valid within the condensation region but not at the isoelectric point. In fact, data of clusters with almost zero $\zeta$-potential fail to collapse on the master curve, due to large fluctuations of the measured size at the isoelectric point, where $\zeta$-potential has vanishing values.

Notably, at the lower temperature investigated, 5 $\degree C$, on the same experimental time scale employed in the other measurements, flocculation at the isoelectric point is not observed and almost-neutral clusters remain stable.

The charge ratio $\xi$ where the maximum size of the aggregates is observed does not vary significantly at the different temperatures (upper panels, fig. 1), as well as the position of $\zeta$-potential sign reversal, corresponding to charge
The increase of temperatures (bottom panels, Fig. 1) promotes a slight increase of the \( \zeta \)-potential of the complexes at charge ratios below the isoelectric point. This effect is better observed in Fig. 7, where the data are properly shifted to make the different positions of the isoelectric point coincident. In the regions below and above the isoelectric point, \( \zeta \)-potential increases with temperature. This small increase can be explained as the result of two combined effects.

First, the effect of counterion condensation on both polyelectrolytes and liposomes must be taken into account. Different theoretical models hypothesize that the counterions, on approaching to oppositely charged surface, 'condensed' around a highly charged linear polyelectrolyte would not necessarily be released because of entropic effects [46, 15, 47]. Recently, these hypotheses have also been substantiated by experimental evidence [48, 49, 7]. At the higher temperature, the increasing entropy of counterions reduces the effects of 'condensation' [50] so that the effective charge of the bare surface increases. The second effect is connected to the conformational entropy of the adsorbed polyions, that with the increase of the polyion content, and hence of the surface coverage, becomes relevant. In fact, at low temperatures, polyions tends to assume a more flat conformation on liposome surface, thus contributing a stronger screening effect, which reflects in the lower values of the \( \zeta \)-potential observed. At higher temperatures, polyions assume a more disordered conformation with more loops. A similar mechanism can be invoked to interpret the behavior observed above the isoelectric point. At charge ratios close to 1.5 above the isoelectric condition, a sensible increase of the \( \zeta \)-potential (in absolute values) with the temperature is observed. At \( T=80 \, ^\circ \text{C} \) above \( \xi \approx 1.5 \), \( \zeta \)-potential values begin to stabilize, as if a maximum polyion adsorption were reached. Again, this behavior could be connected with the temperature-depending adsorption properties of polyions, when, with the increase of the thermal energy, polyion chains are less strongly bound to the oppositely charged surface, until a desorption limit is reached [51].

4 Conclusion

We have investigated the effect of temperature on the reentrant condensation of polyelectrolyte-liposome complexes, in a temperature range from 5 to 80 °C. At all the temperatures investigated, the observed phenomenology is similar, with the formation of polyion-liposome complexes which aggregate in large clusters close to the isoelectric point. Temperature does not modify the overall aspect of the phenomena. However, significant changes of size and \( \zeta \)-potential values of the polyion-decorated liposome aggregates are observed, connected to the increase of temperature.

The observed effects can be interpreted in terms of a thermally activated process, where the potential barrier that controls the aggregation arises from the combined effect of the electrostatic repulsions, due to the residual net charge on the primary particles (the polyelectrolyte-decorated liposomes), and of an
attractive term due to the non-uniformity of the adsorbed chains and hence of the surface electric potential.

The observed behavior is interpreted within the framework of Velegol and Twar model [27], that takes into account the effect of charge anisotropy on aggregating particles. A good agreement is found between calculated and experimental values of cluster size, for all the temperatures investigated. More interestingly, the analysis according to the Velegol and Twar model evidences that, once the dependence of radius on temperature is taken into account, interactions of oppositely charged polyelectrolytes and liposomes show universality properties. In fact, cluster sizes within the reentrant condensation region collapse on a single master curve, thus excluding the presence of non-electrostatic interactions or effects connected with cluster growth rate on the observed aggregation.

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Fig. 1 - Reentrant condensation (aggregate diameter $2R$, upper panels) and charge inversion ($\zeta$-potential, bottom panels) of cationic DOTAP liposomes in the presence of anionic NaPAA polyelectrolyte. Data are shown as a function of the polyanion-lipid charge ratio parameter $\xi$ for the different temperatures investigated. Lines connecting experimental data point guide eyes only. It has to be noted that, at the isoelectric point, due to the intrinsic instability of the system, the data represent the initial size or $\zeta$-potential values of flocculating aggregates.

Fig. 2 - Some typical MC-step evolution of normalized mean cluster radius $<R>/R_0$ -1. Simulations have been carried out for different values of $\xi$ with a constant value of the standard deviation $\sigma=15$ mV, $\oslash: \zeta=15$ mV, $\odot: \zeta=11$ mV. The inset shows a magnification of the evolution at longer times of the mean size, in the region where kinetical arrest is reached and mean cluster size is stable.

Fig. 3 - Comparison between mean cluster size at the kinetic arrest calculated according to the eq. 4 (continuous line) with $\Phi_{\text{max}}=10$ $K_B T$ and obtained by MC simulations (filled circles), as a function of the electrostatic parameter $\zeta$ with fixed $\sigma=15$ mV. Inset shows mean cluster size according to eq. 4 (continuous line) and MC simulations (filled circles), at fixed $\zeta=15$ mV, as a function of $\sigma$. Each value of cluster size determined by MC simulations is calculated as the mean value of the last five points obtained in the MC simulations.

Fig. 4 - Mean equilibrium radius of clusters at the different investigated temperatures as a function of the $\zeta$-potential, obtained by means of MC simulations according to eq. 4 with $\Phi_{\text{max}}=10$ $K_B T$ and $\sigma=15$ mV (solid line: 5 °C, dashed line: 20 °C, dotted line: 40 °C, dash-dot line: 60 °C, dash-dot-dot line: 80 °C). Inset shows potential curves as a function of the minimal distance between particle surfaces $H$, at fixed $\zeta$-potential (15 mV), calculated for increasing values of $\sigma$ (solid line: 35 mV, dashed line: 30 mV, dotted line: 25 mV, dash-dot line: 20 mV, dash-dot-dot line: 15 mV).

Fig. 5 - Average hydrodynamic diameter of lipoplex clusters at 20 °C as a function of the polyanion-lipid charge ratio $\xi$. Line is calculated on the basis of eq. 4 within the Veleigol and Twar model for inhomogeneously charged interacting colloidal particles.

Fig. 6 - Average hydrodynamic diameter of lipoplex clusters normalized with respect to the temperature at which measurement were performed, for the various temperatures investigated (□: 5 °C, $\odot$: 20 °C, $\triangle$: 40 °C, $\triangledown$: 60 °C, $\oslash$: 80 °C). Cluster size is rescaled with respect to the liposome size and peak position is shifted with respect to the position at 80 °C. Errors have been evaluated of the order of 10 % of data. Line is to guide eyes only.
Fig. 7- \( \zeta \)-potential curves as a function of the charge ratio \( \xi \), for the different investigated temperatures (\( \Box: 5 \, ^\circ\text{C}, \, \circ: 20 \, ^\circ\text{C}, \, \Delta: 40 \, ^\circ\text{C}, \, \triangledown: 60 \, ^\circ\text{C}, \, \diamond: 80 \, ^\circ\text{C} \)). Lines represent linear fit on data, in regions below and above the isoelectric condition.
Figure 1:
Figure 2:
Figure 3:
Figure 4:
Figure 6:
Figure 7:
