Compaction of negatively charged polyanions by polycations with different characteristics is investigated using Monte Carlo simulation in a coarse-grain model. Two different routes are tested and the results compared. In one, the polycation/polyanion charge ratio is varied by increasing the amount of polycations, keeping all the chain characteristics constant. In the other, the linear charge density of the polycations is altered but their number is kept constant. The set of systems in which the linear charge density changes is used as a model for a system comprising chains with different degrees of ionization under different pH conditions. In both cases, polycation/polyanion charge ratios ranging from 0.25 to 1.25 are addressed. The system with unitary charge ratio is common to both routes. It is seen that, although the overall trends followed by the two sets of systems are similar, marked differences can be discerned both for low charge ratios, and for the higher ones, where the systems are overcharged. Coexistence regimes are clearly detected in some of the systems. The results obtained computationally can be used to guide practical applications.

1. Introduction

The delivery of functional genes to a target cell is regarded by many as a potential revolution in medicine. The delivery of DNA is just the first step of the process and, once delivered to the cell, it needs to be expressed in order to produce a successful therapeutic effect. The human genome is coded in approximately 3 billion base pairs, which gives rise to an approximately 1.5 m long molecule. Therefore, DNA has first to be compacted into a complex of suitable dimensions to be able to pass the cell membranes and reach the nucleus.

Compaction of the genome is obtained via complexation of the DNA with small, highly positively charged proteins, the histones, into a large nucleoprotein complex that is referred as chromatin. DNA is a polyelectrolyte with a high linear charge density and strong electrostatic interactions are involved in all transitions of chromatin. It is therefore unsurprising that DNA condensation has attracted much interest from researchers. It has been mentioned in the literature that co-solutes with charge equal or larger than \( z = +3 \) have the ability to induce DNA compaction. Furthermore, highly charged entities, such as polycations, are very efficient in the compaction of DNA by forming DNA–polycation complexes (polyplexes), even in very dilute solutions. Thus, the expanding field of gene therapy has turned attention to polyplexes as promising novel systems for drug delivery. This has prompted the synthesis and characterization of new polycations, the study of their interaction with DNA and complex formation, as well as transfection efficiency determinations (see ref. 5–8 and references therein). Regarding polyplex formation, a large effort is being placed in the control of conformational aspects, compaction degree, size and polydispersity. The resulting complexes, apart from granting the condensation and protection of DNA, should also facilitate the uptake of the DNA into the cells. For an efficient transfection of the gene, the vector should be released from the endosomal compartment, migrate through the cytoplasm, be taken up by the nucleus and undergo at least partial decompaction for efficient transcription.

The endosomal escape is a critical factor for efficient gene delivery. Viruses, for example, escape from endosomes by exploiting the pH drop to trigger a cascade of events that ultimately allows their exit. This has naturally prompted a series of strategies to overcome this difficulty. Amongst these, we note the addition of agents into the transfection medium to help release the complexes from the endosomes, the incorporation of viral peptide sequences that are responsible for endosomal activity, or simply the use of polycations, such as polyethyleneimine, with intrinsic endosomal release activity due to their strong buffer capacity at physiological pH.

Due to the interest in these systems, a number of simulation studies have been performed concerning the compaction of polyelectrolytes in solution by the addition of oppositely charged polyelectrolytes. The majority of the studies were performed with polyelectrolytes with the same chain length and variation of the interaction strength, the size of the chains and the charge density of one of the chains. Asymmetric polyelectrolyte systems have also been considered. The effect of the length of the condensing agents and their number on the degree of compaction and the coil-to-globule transition of a single polyanion have been studied, as well as the influence of size, number and polydispersity of the polyanions on cluster formation. As a general rule, all the variations that have been described influence the formation and/or structure of the polyplexes.

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In this work, we use Monte Carlo simulations to look into the compaction of a large polyanion by pH-responsive polycations. For this, we inspect two sets of systems. In one, we keep the concentration of the polycation that acts as compacting agent constant, while increasing the respective charge density. This would correspond, experimentally, to an increased ionization, determined by pH variation. In the other, a constant linear charge density is imposed, but the concentration of the agent is increased. The aim is to investigate the conformational behavior of both the polyanion and shorter polycation chains, to compare the compacting action for similar polycation/polyanion charge ratios in the two sets of systems, and to identify the presence of coexistence and/or overcharging regimes.

2. Model and simulation details

Systems comprising one polyanion and smaller polycations at different mixing ratios have been studied using the primitive model, where all ions and chain monomers are considered as charged hard-spheres. The solvent is considered as a continuum with a dielectric permittivity, \( \varepsilon_s = 78.4 \), corresponding to that of water at the simulation temperature, \( T = 298.15 \) K. The model consists of four different types of charged particles: (i) connected, negatively charged spheres, representing a linear polyanion; (ii) connected, positively charged spheres, representing a linear polycation; (iii) positively charged spheres representing monovalent cations, counterions to the polyanion; (iv) negatively charged spheres representing monovalent anions, counterions to the polycations.

All interactions are assumed to be pairwise additive. The total potential energy, \( U \), is expressed as a sum of three contributions

\[
U = \sum U_{\text{elec}} + \sum U_{\text{ang}} + \sum U_{\text{bond}}.
\]

All particles interact via an electrostatic potential plus a hard-sphere repulsion, according to

\[
U_{\text{elec}}(r_{ij}) = \begin{cases} \infty, & r_{ij} < R_i + R_j \\ z_i z_j e^2 / 4 \pi \varepsilon_0 r_{ij}, & r_{ij} \geq R_i + R_j \end{cases}
\]

where \( z_i \) is the valence of particle \( i \), \( e \) denotes the elementary charge, \( R_{ij} \) is the distance between particle \( i \) and particle \( j \), and \( R \) is the corresponding hard-sphere radius of each particle (\( R = 2 \) Å for chain beads (monomers), monovalent anions and monovalent cations). Contiguous hard-spheres belonging to a chain also interact through a harmonic potential, \( U_{\text{bond}} \), given by

\[
U_{\text{bond}} = \frac{k_{\text{bond}}}{2} (r_{i,j+1} - r_0)^2
\]

where \( k_{\text{bond}} \) is the harmonic bond force constant (0.07 Nm\(^{-1}\)) and \( r_{i,j+1} \) is the distance between connected monomers with equilibrium separation \( r_0 = 5 \) Å imposed for the negative chain, whereas for the polycations a slightly larger value, \( r_0 = 5.6 \) Å, typical for polyamines, was used. The intrinsic, i.e., nonelectrostatic, chain stiffness is incorporated by defining the desired value for \( k_{\text{ang}} \) in the angular potential, \( U_{\text{ang}} \),

\[
U_{\text{ang}} = \frac{k_{\text{ang}}}{2} (\alpha_i - \alpha_0)^2
\]

where \( \alpha_i \) is the angle formed between the position vectors \( r_{i+1} - r_i \) and \( r_{i-1} - r_i \), \( \alpha_0 = 180^\circ \) is the reference angle, and \( k_{\text{ang}} = 0.002 \) J \( \text{r}^2 \) is the angular force constant.

In the present study, all particles are enclosed and free to move in a spherical cell of radius 400 Å.

The charge ratio, \( \beta \), between the polyanion (indicated by the subscript PA) and compacting agent (subscript PC) is defined as

\[
\beta = \frac{z_{\text{mon},\text{PC}} N_{\text{mon},\text{PC}} N_{\text{PC}}}{z_{\text{mon},\text{PA}} N_{\text{mon},\text{PA}}}
\]

where \( z_{\text{mon}} \) is the charge of the monomers, \( N_{\text{mon}} \) is the number of monomers in each chain, and \( N_{\text{PC}} \) is the number of polycation chains in the system.

In the present work, we modeled the titration of a polyanion composed of \( N_{\text{mon},\text{PA}} = 120 \) monomers with oppositely charged polyelectrolytes with a chain length \( N_{\text{mon},\text{PC}} = 30 \) monomers. Two sets of systems have been simulated for comparison. The distinctive characteristics of the different systems are summarized in Table 1. In the first set of systems, denoted I, we change the number of chains added to the polyelectrolyte solution with a constant unitary charge for each monomer. In the second set, II, the number of positive chains is kept constant while the linear charge density of the chains, calculated according to

\[
d_z = \frac{N_{\text{mon},\text{PC}} z_{\text{mon},\text{PC}}}{(N_{\text{mon},\text{PC}}-1) \langle R_{bb}^2 \rangle^{1/2}}
\]

and taking \( \langle R_{bb}^2 \rangle^{1/2} \) as the rms bead-bead separation for a single polycation chain calculated in the presence of its counterions, is varied by changing the charge of each monomer. All counterions are considered explicitly, and the overall charge neutrality of the systems is warranted. We have not considered increased salt concentration associated with pH buffering conditions.

For each system, Metropolis Monte Carlo simulations were performed in the canonical ensemble using the Molsim package. The efficiency of the simulations was improved by taking concerted moves in the chain particles. These included translation, end pivot rotation, and slithering. Equilibration was performed with no less than \( 5 \times 10^6 \) Monte Carlo steps and production runs typically comprised \( 5 \times 10^6 \) steps or more, each step corresponding to a trial move for every particle.

Table 1 Number of polycation chains, \( N_{\text{PC}} \), and linear charge density, \( d_z \), for each system under study

<table>
<thead>
<tr>
<th>Set</th>
<th>( \beta )</th>
<th>( N_{\text{PC}} )</th>
<th>( z_{\text{mon},\text{PC}}/e )</th>
<th>( d_z/e ) Å ( ^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.25</td>
<td>1</td>
<td>1.0</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>2</td>
<td>1.0</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>3</td>
<td>1.0</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4</td>
<td>1.0</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>5</td>
<td>1.0</td>
<td>0.17</td>
</tr>
<tr>
<td>II</td>
<td>0.25</td>
<td>4</td>
<td>0.25</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>4</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>4</td>
<td>0.75</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>4</td>
<td>1.0</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>4</td>
<td>1.25</td>
<td>0.14</td>
</tr>
</tbody>
</table>

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3. Results

3.1 Changing polycation concentration

Table 2 shows the values of the conformational indicators for the polyanion chains as the number of polycation chains, $N_{PC}$, is increased (set I). Also included are the corresponding values for the neutral chain, in which the charge of the monomers is zero and all other chain parameters are kept constant.

The radius of gyration, $\langle R_{gyr}^2 \rangle^{1/2}$, and the end-to-end distance, $\langle R_{ee}^2 \rangle^{1/2}$, have been calculated using

$$\langle R_{gyr}^2 \rangle^{1/2} = \left( \frac{1}{N_{mon}} \sum_{j=1}^{N_{mon}} |r_{N} - r_{CM}|^2 \right)^{1/2}$$

and

$$\langle R_{ee}^2 \rangle^{1/2} = \left( |r_{N_{mon}} - r_{1}|^2 \right)^{1/2}$$

where $r_i$ represents the position of particle $i$ and $r_{CM}$ is the position of the center of mass of the systems and $\langle \ldots \rangle$ is an ensemble average.

The persistence length, $l_p$, was calculated using the projection of angles between bond vectors according to

$$l_p = \frac{b \Sigma \cos \theta_i}{(\cos a)}$$

where $b$ is the bond length and $\theta_i$ the angle between bond vectors separated by $k$ bonds. Considering infinite chain and average quantities this leads to

$$l_p = \frac{\langle R_{inb} \rangle}{1 - (\cos a)}$$

The average values for the conformational indicators decrease monotonically from $\beta = 0$ to $\beta = 1$. In this latter case, they are also substantially smaller than those for the neutral chain. Definite compaction, however, is only visible for $\beta = 1$, as previously remarked. In fact, up to this point, systems have a monotonically from zero and all other chain parameters are kept constant.

Definite compaction, however, is only visible for $\beta = 1$, as previously remarked. In fact, up to this point, systems have a monotonically from zero and all other chain parameters are kept constant.

The average positioning of the polycation chains along the $y$-axis, $\langle R_{gyr} \rangle$, for the polyanions (Fig. 1) conveys the same type of information, but also reveals an interesting profile as $\beta$ exceeds 1. In fact, for increasing values of $\beta$ in the range $0 < \beta < 1$, we see a monotonic shift of the single maximum distribution towards very small dimensions. At $\beta = 1$, the distribution is very narrow, reflecting the lack of conformational freedom of very compact structures. Note the fact that the structures are more compacted than the corresponding neutral chain. However, for $\beta = 1.25$ we note the presence of a second maximum, corresponding to a situation of coexistence between compact and more extended polyanion conformations.

The compaction of the polyanion chain, as described in the previous paragraphs, has a counterpart in the evolution of the conformational properties of the polycation chains (Table 3). We note that for smaller values of $\beta$, the variation in the conformational indicators of the polycation chains is negligible. For $\beta = 1$, the dimensions of the longer polyanion and those of each polycation chain are comparable.

The analysis of the probability distributions of the radius of gyration, $P(R_{gyr})$, for the polyanions (Fig. 1) conveys the same type of information, but also reveals an interesting profile as $\beta$ increases. In fact, for increasing values of $\beta$ in the range $0 < \beta < 1$, we see a monotonic shift of the single maximum distribution towards very small dimensions. At $\beta = 1$, the distribution is very narrow, reflecting the lack of conformational freedom of very compact structures. Note the fact that the structures are more compacted than the corresponding neutral chain. However, for $\beta = 1.25$ we note the presence of a second maximum, corresponding to a situation of coexistence between compact and more extended polyanion conformations.

The compaction of the polyanion chain, as described in the previous paragraphs, has a counterpart in the evolution of the conformational properties of the polycation chains (Table 3). We note that for smaller values of $\beta$, the variation in the conformational indicators of the polycation chains is negligible. For $\beta = 1$, the dimensions of the longer polyanion and those of each polycation chain are comparable.

The analysis of the $P(R_{gyr})$ for the compacting agent (Fig. 2) allows a more accurate understanding of the conformational behavior of the polycation chains as $N_{PC}$ is increased. The distributions corresponding to $\beta = 0.25$ and $\beta = 0.5$ are very similar, both in the position of the maximum and dispersion. At $\beta = 0.75$ the probability distribution shows two clear maxima, in yet another instance of coexistence. For $\beta = 1.25$, the shape of the distribution is similar to that at $\beta = 1$, but wider and slightly displaced to higher values.

The average positioning of the polycation chains along the polyanion chain was assessed (Fig. 3). Particles are considered

![Fig 1 Probability distribution of the radius of gyration, $P(R_{gyr})$, for polyanions in the presence of an increasing number of polycation chains, $N_{PC}$, (set I). The values of the corresponding charge ratios ($\beta$) are indicated in the figure.](image-url)

**Table 2** Average properties of the polyanion chain in the presence of an increasing number of polycations, $N_{PC}$. Distances are given in Å.

<table>
<thead>
<tr>
<th>$\beta$</th>
<th>$N_{PC}$</th>
<th>$\langle R_{ee}^2 \rangle^{1/2}$</th>
<th>$\langle R_{gyr}^2 \rangle^{1/2}$</th>
<th>$\langle R_{inb}^2 \rangle^{1/2}$</th>
<th>$l_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>551 ± 5</td>
<td>195 ± 1</td>
<td>8.0 ± 0.1</td>
<td>41.99 ± 0.04</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>483 ± 3</td>
<td>161.0 ± 0.8</td>
<td>9.1 ± 0.1</td>
<td>33.88 ± 0.04</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>390 ± 4</td>
<td>132 ± 1</td>
<td>8.6 ± 0.1</td>
<td>28.15 ± 0.04</td>
</tr>
<tr>
<td>0.75</td>
<td>3</td>
<td>230 ± 5</td>
<td>84 ± 2</td>
<td>7.6 ± 0.1</td>
<td>25.38 ± 0.03</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>60.8 ± 0.5</td>
<td>31.6 ± 0.2</td>
<td>3.72 ± 0.1</td>
<td>23.95 ± 0.03</td>
</tr>
<tr>
<td>1.25</td>
<td>5</td>
<td>119 ± 7</td>
<td>48 ± 2</td>
<td>5.3 ± 0.2</td>
<td>24.74 ± 0.06</td>
</tr>
<tr>
<td>—</td>
<td>0</td>
<td>188 ± 2</td>
<td>76.0 ± 0.8</td>
<td>5.7 ± 0.1</td>
<td>25.59 ± 0.04</td>
</tr>
</tbody>
</table>

**Table 3** Average properties of the polycation chains for the systems where $N_{PC}$ was varied. Distances are given in Å. The first line presents the values for a single polycation chain, in the presence of the respective counterions. The last line refers to the corresponding neutral chain

<table>
<thead>
<tr>
<th>$\beta$</th>
<th>$N_{PC}$</th>
<th>$\langle R_{ee}^2 \rangle^{1/2}$</th>
<th>$\langle R_{gyr}^2 \rangle^{1/2}$</th>
<th>$\langle R_{inb}^2 \rangle^{1/2}$</th>
<th>$l_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>——</td>
<td>———</td>
<td>158.5 ± 0.1</td>
<td>56.85 ± 0.04</td>
<td>8.669 ± 0.005</td>
<td>39.69 ± 0.02</td>
</tr>
<tr>
<td>0.25</td>
<td>1</td>
<td>157.7 ± 0.9</td>
<td>52.1 ± 0.2</td>
<td>9.09 ± 0.04</td>
<td>33.6 ± 0.1</td>
</tr>
<tr>
<td>0.5</td>
<td>2</td>
<td>149.6 ± 0.9</td>
<td>50.3 ± 0.2</td>
<td>8.89 ± 0.04</td>
<td>32.76 ± 0.08</td>
</tr>
<tr>
<td>0.75</td>
<td>3</td>
<td>106 ± 1</td>
<td>39.2 ± 0.3</td>
<td>6.4 ± 0.1</td>
<td>27.72 ± 0.08</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>57.0 ± 0.4</td>
<td>26.9 ± 0.1</td>
<td>4.34 ± 0.03</td>
<td>24.98 ± 0.02</td>
</tr>
<tr>
<td>1.25</td>
<td>5</td>
<td>83.4 ± 0.6</td>
<td>32.9 ± 0.1</td>
<td>5.81 ± 0.03</td>
<td>26.34 ± 0.03</td>
</tr>
<tr>
<td>——</td>
<td>———</td>
<td>89.8 ± 0.4</td>
<td>34.6 ± 0.1</td>
<td>6.24 ± 0.03</td>
<td>25.49 ± 0.04</td>
</tr>
</tbody>
</table>
has been kept constant while the polycation/polyanion charge ratio is the conformational indicators of the polyanion (Table 4) is similar to the one described in the previous section. However, the values for $\langle R_{gyr}^2 \rangle^{1/2}$ are initially higher for the systems where $d_c$ has been varied. It is seen that, for $\beta = 0.75$, four chains with a lower $d_c$ (in set II) promote a slightly higher degree of compaction than three possessing a higher charge density (in set I). The $\beta = 1$ system is common to the two sets of systems, but towards the $\beta = 1.25$ overcharged complexes, we see that the expansion is more pronounced in the four chain, high $d_c$, system, than in the five chain counterpart, which displays a lower $d_c$ value in each chain.

Fig. 4 shows the probability distribution for $R_{gyr}$ of the polyanion chains for set II. As $d_c$ is increased, the nearly Gaussian-shaped distribution moves to smaller $R_{gyr}$ values. The dispersion of the distribution is not substantially altered as $\beta$ increases up to 0.75, but for $\beta = 1$, as previously mentioned, the distribution becomes very narrow. At $\beta = 1.25$, the distribution appears skewed to the left, ranging from the smallest values of $R_{gyr}$, corresponding to totally compact structures, to values reaching those of the most extended conformations for $\beta = 0.75$. In this case, and contrary to what was observed in the previous set of systems, coexistence is not observed for the overcharged complex.

The conformational behavior of the polycations of set I (Table 5) at low values of $\beta$ is clearly different from the one observed for the more highly charged compacting agents of the first set. For the lowest $\beta$, the polycation chains present a shape factor very close to that of a random coil (6.16 ± 0.01). This is to be expected due to the low charge present in each polycation chain. As $d_c$ is increased, the polycation chains firstly expand, go through a maximum at $\beta = 0.5$, then decrease towards $\beta = 1$ and increase again in the overcharged complex. It is interesting to note that the values of the persistence length $l_p$, are, in contrast to what happens in the case of an increasing number of chains, almost constant. This is a seemingly paradoxical observation: maintaining the properties of the polycation chain, we have a strong variation in $l_p$, but when the linear charge density of the chains is systematically increased, $l_p$ varies only slightly. The values of $l_p$ of the polycations in the absence of polyanion are depicted in Table 6 in conjunction with other parameters. By increasing $d_c$ on the chains, $l_p$ will naturally increase. The presence of the polyanion counteracts the potential increase and the values remain nearly constant.

The $P(R_{gyr})$ distributions of the polycations are presented in Fig. 5. They reflect what has been said above. Generally, less extended chain conformations, i.e., larger populations with

Table 4 Average properties of the polyanion in the presence of polycation chains with different effective charge. Distances are given in Å. For completeness, values for $\beta = 0, 1$, and the neutral chain are included, as in Table 2

<table>
<thead>
<tr>
<th>$\beta$</th>
<th>$\langle R_{cyl}^2 \rangle^{1/2}$</th>
<th>$\langle R_{gyr}^2 \rangle^{1/2}$</th>
<th>$\langle R_{cyl}^2 \rangle/\langle R_{gyr}^2 \rangle$</th>
<th>$l_p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>551 ± 5</td>
<td>195 ± 1</td>
<td>8.0 ± 0.1</td>
<td>41.99 ± 0.04</td>
</tr>
<tr>
<td>0.25</td>
<td>502 ± 5</td>
<td>173 ± 1</td>
<td>8.2 ± 0.1</td>
<td>37.85 ± 0.05</td>
</tr>
<tr>
<td>0.5</td>
<td>409 ± 5</td>
<td>144 ± 2</td>
<td>7.9 ± 0.1</td>
<td>32.25 ± 0.06</td>
</tr>
<tr>
<td>0.75</td>
<td>224 ± 5</td>
<td>83 ± 2</td>
<td>7.2 ± 0.2</td>
<td>27.00 ± 0.04</td>
</tr>
<tr>
<td>1.0</td>
<td>60.8 ± 0.5</td>
<td>31.6 ± 0.2</td>
<td>3.7 ± 0.1</td>
<td>24.95 ± 0.03</td>
</tr>
<tr>
<td>1.25</td>
<td>155 ± 6</td>
<td>58.3 ± 2.4</td>
<td>6.9 ± 0.2</td>
<td>24.62 ± 0.06</td>
</tr>
<tr>
<td>—</td>
<td>188 ± 2</td>
<td>76.0 ± 0.8</td>
<td>5.7 ± 0.1</td>
<td>25.59 ± 0.04</td>
</tr>
</tbody>
</table>
where the lowest conformational indicators. A decrease in the shape parameter for polyanions is observed when $N_{PC}$ or $d_z$ is increased, up to $\beta = 1$, followed by a shape parameter increase towards $\beta = 1.25$. The same is observed for the polycations in set I. In contrast, for systems where $d_z$ was varied, the polycation chains start with a value close to 6 (Gaussian chain), attain a maximum close to $\beta = 0.5$, and then follow the general behavior.

Some interesting aspects are additionally observed. The polyanions in set I display higher values of the shape parameter than those in set II for the same charge ratios, up to $\beta = 1$. Furthermore, the shape values for the polycations and polyanions of set I, for $\beta = 0.25$ and 0.5, are essentially the same, which stresses the correlation between the conformations of the compacting agent and compacted chain. For $\beta = 1$, the polycations are less compacted than the polyanions. Finally, for $\beta = 1.25$, the polyanions of set I show a lower shape value than the corresponding ones for set II, but the polycations display a very similar value, close to 6.

Table 5 Average properties of the polycations considering chains of different linear charge density, $d_z$, (set II). Distances are given in Å

<table>
<thead>
<tr>
<th>$\beta$</th>
<th>$(R_{ee}^2)^{1/2}$</th>
<th>$(R_{gyr}^2)^{1/2}$</th>
<th>$(R_{ee}^2)/(R_{gyr}^2)$</th>
<th>$l_P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>90.9 ± 0.2</td>
<td>34.89 ± 0.04</td>
<td>6.163 ± 0.008</td>
<td>25.86 ± 0.01</td>
</tr>
<tr>
<td>0.5</td>
<td>115.7 ± 0.6</td>
<td>41.3 ± 0.1</td>
<td>7.37 ± 0.03</td>
<td>26.71 ± 0.03</td>
</tr>
<tr>
<td>0.75</td>
<td>92.7 ± 0.9</td>
<td>35.6 ± 0.2</td>
<td>5.9 ± 0.1</td>
<td>26.08 ± 0.03</td>
</tr>
<tr>
<td>1.0</td>
<td>57.0 ± 0.4</td>
<td>26.9 ± 0.1</td>
<td>4.34 ± 0.03</td>
<td>25.98 ± 0.02</td>
</tr>
<tr>
<td>1.25</td>
<td>90 ± 1</td>
<td>34.8 ± 0.3</td>
<td>5.8 ± 0.1</td>
<td>26.74 ± 0.04</td>
</tr>
</tbody>
</table>
4. Discussion

In the previous section, we have presented the results obtained for all systems. In the following, we discuss the most interesting aspects, and analyze the differences in behavior of the two alternative compacting routes.

As $\beta$ was increased from 0 to 0.75, the conformational indicators for the large polyanion chain decrease in both sets of systems, showing the compacting action of the oppositely charged chains. The $R_{\text{gyr}}^2$ values were shown to be, in most cases, larger for the systems where $d_i$ was changed. It should be recalled that for $\beta < 1$, the linear charge density of the polycations in the systems of set II is smaller than the linear charge density of the polycations used in the set of systems for which polycation number (or concentration) is varied (set I). Although there are less polycation monomers in the systems of set I, the larger $d_i$ leads to a localized compaction capacity, at least up to values of $\beta$ close to 0.75. This also explains the larger values of the shape parameter. Additionally, the presence of 4 chains with low $d_i$ leads to uniform distribution of the neutralizing charge along the chain, and the effects of intrachain segregation are absent or negligible. The interaction between chitosan and DNA at different pH values has been studied, and it was observed that for the same mixing ratios (below 0.75), the systems with higher charge density presented a larger binding constant, which is in good agreement with the presence of 4 chains with low $d_i$.

We recall that, for the systems in set I with $\beta \leq 0.75$, the polycation chains preferentially occupy a part of the polyanion chain, although they slide along the chain. It has been suggested that the translational entropy of the polycations hinders the compaction of the polyanion. Experimentally, it was shown that for $\beta < 1.0$, the digestion of DNA inside the polyplexes by restriction enzymes proceeds more slowly than for free DNA but is not completely hindered. This indicates that the polycations bound to DNA do not hinder the specific recognition of DNA by enzymes, but slow down the process. It is suggested that the polycations are not rigidly bound to the DNA chain, but instead migrate from one region to another, opening sites for restriction.

It is interesting to note that for $\beta = 0.75$, four chains with lower $d_i$ promote an equal (or slightly higher) degree of compaction than three with a higher charge density. Electrostatics and entropy arguments effects would apparently suggest a more efficient action in the latter system. A plausible explanation is that (i) a larger charge density would promote a higher extension of the polycation chains, increasing the extension of the polyanion, complemented by the fact (ii) that four chains covering the polyanion cannot slide along the respective backbone, and this reduced entropy would result in a higher degree of compaction. In fact, systems at $\beta = 0.75$ possess some special characteristics, due to the substantial but incomplete degree of charge neutralization. The polyanion displays a broad $P(R_{\text{gyr}})$ distribution, and the respective polycation distribution is bimodal. These characteristics are visible in both sets of systems, and can be ascribed to some contribution from intrachain segregation. Fig. 8 illustrates some typical conformations for $\beta = 0.75$, in set I. Panel (a) shows a configuration in which the polyanion chain is partially folded, which means that some of the polycations are in a collapsed state, while the others are extended. A bridging analysis has shown three or four maxima in the probability of finding polycations associated with a certain number of polyanion monomers for systems in sets I and II, respectively (Fig. 9). The presence of such maxima seems to indicate some propensity of the system to collapse, partially, with full collapse of some of the polycation chains. In set I, the three polycation chains have a higher probability of being placed along the polyanion chain, as observed in the contact analysis of Fig. 3 and the representative snapshot in Fig. 8b. However, two other probability maxima could be discerned, one corresponding to polycation chains occupying nearly two thirds of the chain, which is consistent with the intrachain segregation mentioned above, and the other where the three polycations occupy almost the entire polyanion chain (Fig. 8c). The same applies to the corresponding system in set II, with the difference that four polycation chains are now present. In this case, structures range from more condensed ones, where all four polycations are associated to the majority of the polyanion chain (Fig. 10a), intermediate conformations with intrachain segregation induced by two or three polycations (Fig. 10b and c), and extended conformations where the four chains are placed more or less individually along the polyanion chain (Fig. 10d). Consequently, the polyanion of system $\beta = 0.75$ of set II presents a broader $P(R_{\text{gyr}})$ distribution when compared with $\beta = 0.75$ of set I (Fig. 1 vs. Fig. 4). We recall that intrachain segregation has been observed in large T4DNA molecules using fluorescence microscopy. The same analysis (data not shown), conducted for pure conformational states, reveals that extended structures present only one maximum ($\beta = 0.25$ and 0.5), and the same happens for compact structures ($\beta = 1.0$). In the former case, the maxima are dictated by the superposition of an extended polycation over an extended polyanion, and is an almost direct measure of how many polyanion segments are encompassed between the ends of the polycation. In the latter, each polycation covers most of the polyanion.

The most compact structures were obtained for $\beta = 1.0$, the common system (Fig. 11). Also, the size distribution is narrower and the number of polycation monomers in contact with each polyanion monomer is uniform throughout the polyanion chain, with the exception of the end monomers.
Whereas in the range $0 < \beta < 1$ the polyanions in the systems of set I always display a more extended conformation than the equivalent systems with variations in $d_z$, for a mixing ratio corresponding to $\beta = 1.25$ we found the opposite behavior. It seems that it is easier to accommodate five chains with a relatively low charge density than four more highly charged. A closer look at the contact analysis shows that there is some segregation between the four polycation chains of set II, whereas set I presents a more uniform distribution of the polycation chains along the polyanion. Also, the snapshots (Fig. 12a and b) indicate some probability of finding at least one isolated polycation chain along the polyanion chain in set II, which reduces the probability of finding very compact structures. The bimodal distribution of the polycation sizes of set II is in clear agreement with these observations, more extended polycation conformations coexist with more compacted ones. Furthermore, more compact polyanion structures are generally found when no isolated polycations are present (Fig. 12c).

For set I, on the other hand, the excess of positive charge in the complex can be more easily dealt with by having parts of the polycations (tails) sticking out of the complex (Fig. 13a) with the polyanion keeping a relatively compacted form. This type of complex would presumably be ideal for the delivery of genes to cells. The overall positive charge acts to electrostatically stabilize the complex, avoiding aggregation and precipitation, and it facilitates the approach to the cell membranes. The size would be sufficiently small to overcome
the cell membrane and yet sufficiently expanded to allow the access of the DNA to the cell machinery. There is also some probability of finding more expanded complexes, in coexistence with the above ones, as shown by the $P(R_{gyr})$ distributions of the polyanion (Fig. 1 and the illustration in Fig. 13b). Note here, again, that the presence of one or more extended polycation chains along the polyanion chain (intrachain segregation) substantially reduces the degree of compaction of the polyanion. In close resemblance to the systems at $\beta = 0.75,$ the radius of gyration presents a larger distribution in the case where the charge density of the polycations is varied.

In Fig. 14, we collect the distributions for $P(R_{gyr})$ of the polycations in gradient plots, for sets I and II (panels (a) and (b), respectively). These plots highlight the conformational differences undergone in each set as the charge ratio is increased, and also the differences between the two sets. In both we observe two clearly separated regions: the low $\beta$ extended chain region and the high $\beta$ compact structures. Also, the absence of extended conformations can only be observed in the vicinity of $\beta = 1.$ Clear coexistence is found for $\beta = 0.75$ in both systems. For $\beta = 1.25$ coexistence is not detected, except for a “shoulder” in the distribution of the $P(R_{gyr})$ in set II, for larger values of this parameter. We note also that, in general, set I displays extended conformations that differ more from the compact structures than those found in set II. In the latter, the extended structures vary in size as $\beta$ is increased, with a maximum close to $\beta = 0.5$ as previously indicated.

In summary, the observations indicate extended chains, clear coexistence regimes, and compact structures depending on the value of $\beta.$ A possible explanation for these different regimes lies in the enthalpic and entropic components of the systems. For low $\beta$, polycation chains are free to move along, or close to, the longer polyanion chain. Partial collapse would hinder this translational freedom, and the enthalpic gain does not compensate the entropic loss ($\beta = 0.25$ and 0.5 in our systems). When more polycation chains are present ($\beta = 0.75$), the translational freedom is reduced. Some or all of the shorter chains (and part of the longer one) may collapse. The enthalpic gain is now closer to the entropic loss. It seems some emphasis is put on situations in which the polycation chains that have collapsed are totally, rather than partially, collapsed. In this case, a partial collapse would correspond to a full loss of translational entropy, compensated only by a partial enthalpic gain, which makes it less favorable. Naturally, these remarks essentially refer to set I, in which the translational entropy is larger for the smaller values of $\beta,$ but they also...
apply to set II. For compact structures, the enthalpic gain dominates ($\beta = 1.0$). For even higher values of $\beta$, the pattern is more complex, and clearly dependent on the characteristics of the polycations, as inferred from the differences between sets I and II.

Finally, it is worthwhile remarking that it has been observed experimentally that polyanion-rich solutions (e.g. $\beta = 0.75$) are always more turbid than polycation-rich ones (e.g. $\beta = 1.25$).\cite{31} This is due to the presence of complexes with larger dimensions and/or a larger number of complexes in solution. This is in good agreement with the results presented here; Fig. 1 shows that undercharged polyanions present a much wider distribution of sizes, and higher probabilities of finding larger complexes than the overcharged ($\beta = 1.25$) counterpart.

5. Conclusions

We have studied the compaction of negatively charged polynions by polycations of different characteristics. In set I we have varied the number of polycations, keeping their charge density constant, and in set II we have kept the number of polycations constant and varied their charge density. Polycation/polyanion charge ratios ranging from 0.25 to 1.25 were addressed, with the system with $\beta = 1$ being a system common to both sets.

The two routes of compaction display similar trends, as far as average conformational indicators are considered. However, the behavior of the two sets of systems differs in a number of details. Set I generally produces more compact structures, irrespective of the charge ratio. These are accompanied by narrower distributions.

Situations in which coexistence is visible arise, in both sets, close to a unitary charge ratio ($\beta = 0.75$ and $\beta = 1.25$). These seem to be related with intrachain segregation, involving a discrete number of polycation chains. Additionally, systems that favor intrachain segregation show a larger dispersion in sizes and conformations and, concomitantly, a lower degree of compaction.

We note that most experimental applications benefit from a monodisperse population of complexes. Also, the absolute charge is of paramount importance. If undercharged complexes are more desirable for the application in question, then using fewer polycations with higher density, i.e. lower pH values, leads to slightly less polydisperse complexes and more compact structures. For applications where overcharged complexes are required, there are two possibilities, according to what is required. Polynplexes prepared at lower pH values are more expanded and polydisperse. At higher pH values, the polyanion is generally more compact and the excess charge is accommodated in the form of tails protruding from the complex. We speculate that these complexes are the most appropriate for gene delivery since the “positive tails” would act to electrostatically stabilize the complex, avoiding aggregation and precipitation, and facilitating the approach to the cell membranes. The size would be sufficiently small to overcome the cell membrane and yet sufficiently expanded to allow the access of DNA to the cell machinery, in what is presumably a very favorable situation for gene delivery into cells.

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References