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# Chemistry specificity of DNA-polycation complex salt response: A simulation study of DNA, polylysine and polyethyleneimine<sup>†</sup>

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In this work, the chemistry specific stability determining factors of DNA-polycation complexes are examined by all-atom molecular dynamics simulations. To this end, we conduct a systematic variation of polycation line charge through polyethyleneimine (PEI) protonation and polycation chemistry via comparison with poly-L-lysine (PLL). Our simulations show that increasing line charge of the polycation alone does not lead to more salt tolerant complexes. Instead, the effective charge compensation by the polycation correlates with increased stability of the complex against additional salt. The salt stability of PEI-DNA complexes also links to the proton sponge property of weak polycations, commonly assumed to be behind the effectivity of PEI as a gene delivery vector. Examination of the complexes reveals the mechanism behind this behaviour; more  $\text{Cl}^-$  ions are attracted by the protonated complexes but, in contrast to the common depiction of the proton sponge behaviour, the ion influx does not lead to swelling of the complex structure itself. However, PEI protonation leads to release of PEI while DNA remains tightly bound to the complex. Jointly, these findings shed light on the stability determining factors of DNA-polycation complexes, raise charge distribution as an important stability determining contributor, and indicate the effectivity of PEI in gene delivery is likely to result from the freed PEI facilitating gene transfection.

## 1 Introduction

DNA is a charged polymer, a polyelectrolyte (PE), with a relatively high negative charge. In aqueous solution, DNA readily complexes with cationic polyelectrolytes and this DNA-polycation complexation has been demonstrated to be an effective means of transfecting genetic material in gene therapy<sup>1,2</sup>. As opposed to viral vectors, polyelectrolyte complexes, polyplexes, offer a infection free gene delivery. On the other hand, gene transfection is a multi-stage process where the design of an optimal carrier is a trade-off of several properties, such as, complex charge, complex stability, and complex size<sup>3–5</sup> as the delivery vector has to provide both adequate protection, and efficient and timely release of the gene<sup>6</sup>.

In general, PE complexes are known to respond to presence of electrolytes (e.g. salt). Excess salt can affect the solubility<sup>7</sup>, kinetics<sup>8</sup>, composition<sup>9,10</sup>, or even lead to complete dissociation<sup>11</sup> of the complex. Against this background, it is not surprising that additional salt also affects the gene delivery by PE complexes: Zelikin *et al.* have correlated the transfection

efficiency of a DNA-PE complex with the tolerance of the complex to the addition of salt<sup>12</sup>. Furthermore,  $\text{Ca}^{2+}$  ions are known to promote transfection, possibly by regulating the size or aggregation of the complexes, or by aiding in the release of the genetic material from the complex<sup>13–15</sup>. Indeed, we recently demonstrated the sensitivity of poly-L-lysine-DNA complexes to  $\text{Ca}^{2+}$  ions and provided an atomistically detailed description of the dissociation mechanism via molecular simulations<sup>16</sup>. Therefore, the response of polycation-DNA complexes to small ions is a significant topic with direct implications to the design of efficient gene carriers. Here, we address this by studying the complexation of two polycations, poly-L-lysine (PLL) and polyethyleneimine (PEI), with DNA, and the reaction of these complexes to excess ions via molecular dynamics (MD) simulations.

Both PEI and PLL have been widely studied as DNA delivery vectors with interest in efficiency of target reaching, release dynamics and mechanics. Out of the two, PEI is often considered more promising due to its ability to change its protonation state under physiological pH range. A host of both experimental<sup>4,17–20</sup> and computational work<sup>21–27</sup> on these complexes exists. That said, response of the DNA-polycation complexes to increasing concentrations of monovalent and divalent ions has not been compared earlier via simulations. A comparison of these chemically quite different polycations allows us to systematically address the influence of, e.g., protonation level and polycation chemistry on

<sup>†</sup> Electronic Supplementary Information (ESI) available: Summary of simulated systems, representative figure of open complex for 2xPEI25-DNA in 1.04 M NaCl, and time development of the minimum contact distances between polycations and DNA showing more in detail the fluctuations and precise detachments. See DOI: 10.1039/b000000x/

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polycation-DNA complex behaviour in salt.

The salt response of PEI-DNA complexes with different PEI protonation states is particularly interesting from the point of view of the so called proton sponge hypothesis<sup>28,29</sup>. For successful transfection, the PEI-DNA polyplexes have to be transported from endosomes to the nucleus. The proton sponge hypothesis postulates that the efficient buffering capacity of PEI in the low pH of endosomes is behind the efficiency of PEI as a transfection vector: PEI is able to absorb protons, which leads to more protons being pumped into the endosome and increases the influx of  $\text{Cl}^-$  ions as charge neutrality has to be maintained. Increased ionic strength then produces osmotic swelling, and the combination of the osmotic swelling and swelling of the PEI-DNA complex due to repulsion between protonated amine groups causes endosome rupture with subsequent release of its contents. The proton sponge hypothesis is widely used, but data both supporting<sup>30–32</sup> and seemingly in disagreement<sup>4,33,34</sup> with this theory exist. Molecular simulations allow us to evaluate the changes in the complexation in detail. Previously the effect of PEI protonation state on DNA binding<sup>21,22</sup> and behaviour in solution<sup>26</sup> have been studied via simulations, but the results therein have not been explicitly related to the mechanism of proton sponge behaviour, nor is the effect of additional  $\text{Cl}^-$  ions addressed.

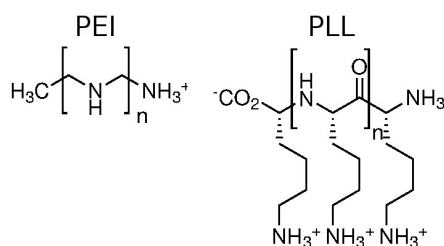
In this work, we examine 1) the dependence of amount of PEI complexed to DNA on PEI protonation state and 2) the salt response of the complexes in terms of polymer charge and polycation chemistry (PEI vs. PLL), as well as, in terms of complex structure and amount of polymer adsorbed. Finally, we 3) study the inner workings of the proton sponge hypothesis by connecting the contributions from PEI protonation, complex structure, and the presence of excess  $\text{Cl}^-$  ions.

## 2 Methods and simulated systems

The complexation and decomplexation of DNA with three different polycations, PLL, PEI50 and PEI25, is studied in this work. PLL, as a strongly charged polycation, can be expected to be completely protonated under biological conditions; the PLL used in the simulations is a fully protonated linear polypeptide. In contrast, the protonation degree of PEI as function of pH is unknown and estimates range between 10% and 90% at pH 7, see Refs.<sup>35,36</sup>. Furthermore, the structure of the polymer<sup>37</sup>, polyanion binding, and the ionic strength of the solution also affect the pKa of the PEI protonation sites<sup>24</sup>. Therefore, we choose to employ linear polyethyleneimine molecules with every second (50%) or every fourth (25%) backbone nitrogen (N) protonated. These are referred PEI50 and PEI25, respectively. The choice of setting the protonated groups at regular intervals and thus maximizing their separation in the simulated PEI molecules is a reason-

able approximation, as it minimises the interaction between the protonated groups and produces a uniform charge distribution. Monte Carlo simulations of PEI protonation confirm this configuration<sup>24</sup>, and similar choices for PEI protonation degree and configuration have previously been used for MD simulations of PEI<sup>21</sup>.

The molecular structures of the polycations are shown in Fig. 1. The protonated groups of both PEI ( $\text{NH}_2^+$ ) and PLL ( $\text{NH}_3^+$ ) are from now onward referred as  $\text{N}^+$  for simplicity of notation. The polycations in our simulations have a length of 20 monomers resulting in total charges of 20e per PLL, 10e per PEI50, and 5e per PEI25 strand. The DNA molecule used in the simulations is the Drew-Dickerson dodecamer d(CGCGAATTCGCG), which carries a net charge of  $-22e$ .



**Fig. 1** Molecular structures of unprotonated PEI and fully protonated PLL with the repeating units and simulation termini visible. For PEI25, every second N and for PEI50, every fourth N in the repeat units is protonated in our simulations. The PEI protonated groups are  $\text{NH}_2^+$  and for PLL protonation results in  $\text{NH}_3^+$ .

First, the DNA strand was placed in a simulation box of  $10 \times 10 \times 10 \text{ nm}^3$  together with two polycation molecules (two PLLs, PEI50s or PEI25s) and solvated with water. In the absence of salt, polycations spontaneously complex with DNA. These complexes were then simulated over a period sufficient to structurally stabilize them (tens of nanoseconds) after which they were exposed to different NaCl and CaCl<sub>2</sub> concentrations. The simulations were run for 200 ns. The NaCl concentrations were counterions (no excess salt), 0.27 M, 0.52 M, and 1.04 M NaCl, and CaCl<sub>2</sub> concentrations 0.13 M, 0.27 M, 0.39 M, 0.52 M, and 1.04 M.

For PLL, the two chains provide a strongly overcharged complex to which additional PLL chains are unlikely to adsorb but for PEI, the two polycation strands are insufficient to compensate the DNA charge. Therefore, the saturation of the PEI-DNA complex was studied by introducing additional PEI50 or PEI25 molecules one-by-one into the solution until the added strands would no longer adsorb into the complex. The saturated complexes contained 4 PEI50 molecules and 5 PEI25 molecules. The saturated complexes were simulated without excess salt and in the presence of 0.27 M NaCl for 160 ns. A table summarizing the all the simulated systems is presented in Supplementary Information<sup>†</sup> (SI).

Simulations were performed with the GROMACS 4.5.5<sup>38</sup> suite using the Amber99bsc0<sup>39,40</sup> force field for PLL and DNA. For PEI molecules, an Amber99ff<sup>39</sup> compatible parametrization constructed by Ziebarth and Wang was employed<sup>22</sup>. The usage of Amber99bsc0 force field ensures the stability of the DNA molecule for the whole duration of the simulations. Consistent with the Amber force-fields, the water in all simulations is modelled by explicit TIP4P-Ew water model<sup>41</sup>.

The Joung and Cheatham ion model<sup>42</sup> was used for the monovalent  $\text{Na}^+$  and  $\text{Cl}^-$  ions. As equivalent  $\text{Ca}^{2+}$  parametrization is not available, the standard Amber  $\text{Ca}^{2+}$  ions were utilized<sup>43</sup>. The Joung-Cheatham model for the monovalent ions was employed to prevent the unrealistic crystallization observed with standard Amber99 ions in high salt concentrations<sup>44,45</sup>. Accordingly, we do not observe salt crystallization in our simulations. That said, the Joung-Cheatham ions have been reported to bind strongly to phosphate which could lead to an over-neutralization of the DNA in this work with respect to other ion models<sup>46</sup>. Indeed, different ion parametrizations have been observed to differ in their binding to DNA<sup>46,47</sup>, and reliable modelling of interactions with divalent ions is particularly challenging as most classical force fields are not able to properly capture the polarizability and charge transfer effects that are involved<sup>48,49</sup>.

Electrostatics were treated with the full PME method<sup>50</sup> and the temperature was controlled by the stochastic rescaling thermostat of Bussi et al.<sup>51</sup> with reference temperature at 300 K and time constant of 0.1 ps. Pressure was maintained at 1 bar using Parinello-Rahman barostat<sup>52</sup> with time constant of 2 ps. All presented simulation snapshots were generated using VMD<sup>53</sup>.

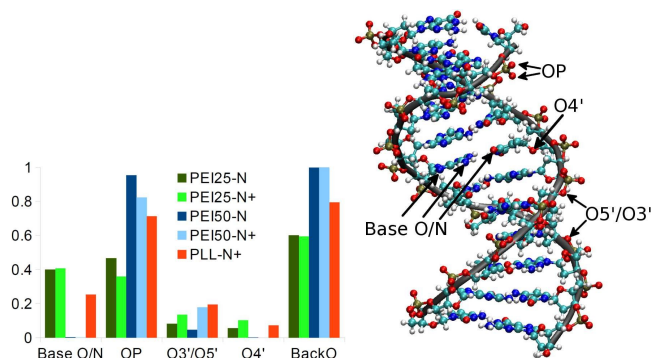
### 3 Results and discussion

#### 3.1 Structure of the complexes in the absence of added salt

In the absence of added salt, DNA rapidly forms a complex with two PLLs, PEI25s or PEI50s initially introduced into the solution. For PLL, a significant overcompensation of the DNA charge ( $-22e$ ) occurs already by the adsorption of the two PLL chains (charge  $40e$ ). However, the adsorption of two PEI50s (charge  $20e$ ) or two PEI25s (charge  $10e$ ) molecules is insufficient to compensate for the DNA charge. Therefore, we determined the maximum amount of PEI molecules adsorbing spontaneously to the PEI-DNA complexes by addition of PEI molecules one-by-one into the solution. In these simulations, we observe the DNA strand can complex with 4 PEI50 molecules ( $N/P=3.6$ ) and 5 PEI25s ( $N/P=4.5$ ).  $N/P$  ratios (the ratios of amine groups of cationic polymers to those of the DNA phosphate groups) have been connected with trans-

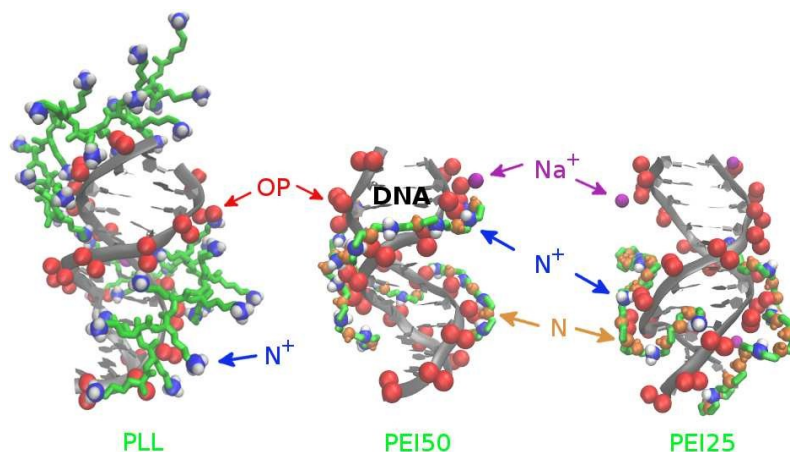
fection efficiency<sup>54,55</sup> and are therefore reported here. Both saturated complexes result in overcharging of the complex. The resulting total charge is  $18e$  by complexation of  $4 \times \text{PEI50}$  chains and  $3e$  by complexation of  $5 \times \text{PEI25}$  chains. For the  $2 \times \text{PLL}$ -DNA complex, the overcharging is  $18e$ . The absolute numbers here are dictated by the DNA and polymer chain lengths employed in the simulations. Furthermore, the finite length of the molecules in the simulations and local adsorption barriers might have an effect on the extent of complexation.

We first quantify and compare the binding of the two initially adsorbed polycations and the saturated complexes. The initially adsorbed polycation chains represent the most efficient polymer binding in the complex (relevant for addressing full dissociation), but the saturated complexes better reflect the complex under equilibrium conditions and in the presence of excess PEI. We find that the conformational characteristics and binding patterns of the  $2 \times \text{PEI}$ -DNA complexes and the saturated complexes of  $5 \times \text{PEI25}$ -DNA and  $4 \times \text{PEI50}$ -DNA are analogous. The less charged PEI25s have more convoluted conformations in the complex and are more able to match their neutral (and therefore more hydrophobic) sections with the DNA grooves. PEI50s align neatly on top of the phosphate backbone and occasionally form bridges over the grooves to reach other parts of the phosphate backbone without actually residing in the grooves.



**Fig. 2** The distribution of DNA binding sites of charged ( $\text{N}^+$ ) and uncharged ( $\text{N}$ ) polycation nitrogen groups in the  $2 \times \text{polycation}$ -DNA complexes (at left) and the DNA binding sites and their labels (at right). The binding is presented as a ratio to all bound nitrogens of each type. The group BackO includes DNA OP, O4' and O3'/O5' sites. Figure 3 labels the charged and uncharged polycation nitrogen groups.

PEI50 molecules bind efficiently to DNA: for the  $2 \times \text{PEI50}$ -DNA complex, 69% of all the PEI50 nitrogens are in contact with DNA. In contrast, the same is true for only 40% of PEI25 nitrogens in the  $2 \times \text{PEI25}$ -DNA complex. The protonated nitrogens prefer binding to DNA with 69% of the charged  $\text{N}^+$ s connected with DNA in the  $2 \times \text{PEI25}$ -DNA complex and mere 30% of the non-protonated Ns near the DNA.



**Fig. 3** Representative simulation snapshots of PLL, PEI50, and PEI25 complexes with DNA. The  $N^+$  groups of both polycations, DNA OP sites, and the counterions are labeled. Water, and counterions beyond 1 nm of DNA are omitted for clarity.

For the 2xPEI50-DNA complex, 85% of the charged  $N^+$ s and 52% of the non-protonated Ns are connected with DNA. The percentages of bound nitrogens change  $\leq 10\%$  between the 2xPEI25-DNA and the 5xPEI25-DNA complexes. A more significant drop occurs between the 2xPEI50-DNA and the 4xPEI50-DNA complex where the percentage of bound nitrogens is reduced from 69% to 49% upon saturation. This is likely an effect of crowding in the preferred binding sites. Nevertheless, the overall DNA-binding pattern remains similar: the charged nitrogens attach dominantly to the DNA phosphate oxygens. Particularly, PEI50 seems to orient almost perfectly on the phosphate backbone of the DNA. Fig. 2 indeed reveals both the protonated and non-protonated backbone nitrogens in PEI50 prefer strongly binding to the DNA-backbone, especially to the phosphate oxygens (OP).

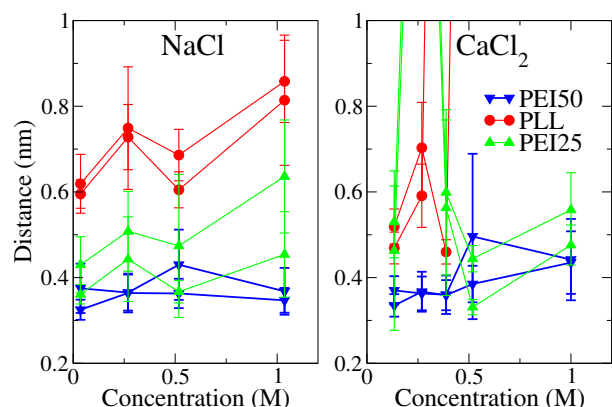
The less charged PEI25 shows a different binding pattern: both the protonated and non-protonated nitrogens of PEI25 bind also to the more deeply buried basic O and N sites of the DNA. While the protonated  $N^+$ s of PEI25 prefer connections with the DNA backbone analogous to PEI50, PEI25 loops and twists more easily at non-protonated sections. This provides flexibility. The loops and twists can make parts of the PEI25 molecules stick out of the polymer complex or enable it to reside also in the minor groove, see Fig. 3. The difference in conformations of PEI50 and PEI25 reflects also on the size of the complex. As Fig. 4 shows, on average PEI25 resides slightly further from the DNA surface than PEI50. For both PEI50 and PEI25, the saturated complexes have larger average nitrogen-DNA distance ( $\sim 0.5$  nm) than the 2xPEI complexes with 4xPEI50 being slightly more compact than 5xPEI25. The increase in size compared to 2xPEI complexes reflects both the larger amount of polymer in the complex and the looser binding of additional PEI compared to the first two PEI chains.

PLL has a higher charge per length than either of the PEIs studied in this work. However, the specific chemistry of PLL weakens the binding to DNA: only 30% of the  $N^+$ s of the 2xPLL system bind directly to DNA in the simulations. Analogous to PEI, the charged  $N^+$  tips of PLL prefer contacts with the DNA backbone sites, but the brush-like structure of the PLL does not allow PLL to utilize all its charged nitrogens in binding: some of the  $N^+$ -tips point away from the DNA. As with PEI25, PLL also has contacts to the basic ON sites in the DNA grooves. PLL has three dominant conformations in binding to DNA: one of the PLLs typically resides in the DNA major groove and the other either loosely oriented on top of the minor groove or attached to the DNA with the PLL backbone parallel to the axis of the DNA, see Fig. 3. As indicated by the larger backbone nitrogen-DNA distance in Fig. 4, the brush like chemistry of PLL also increases the size of the complex compared to PEI-DNA complexes.

### 3.2 Complexes and excess salt

Next, we exposed the complexes to excess NaCl or CaCl<sub>2</sub>. We focus on the salt tolerance of the complexes containing two polycation molecules as the characteristics of these complexes are similar to the saturated ones and the two initially adsorbed polycation chains are likely to represent the last stage of complete complex dissociation. The behaviour of the saturated complexes in excess salt is discussed with respect to the proton sponge hypothesis below.

In agreement with our earlier work<sup>16</sup>, monovalent salt is unable to induce full decomplexation in any of the studied 2xpolycation-DNA systems within the 200 ns time-scale of the simulations, see Fig. 6. However, both the 2xPEI25-DNA and the 2xPLL-DNA complex strongly fluctuate at the highest concentration of monovalent salt examined here, 1.04 M

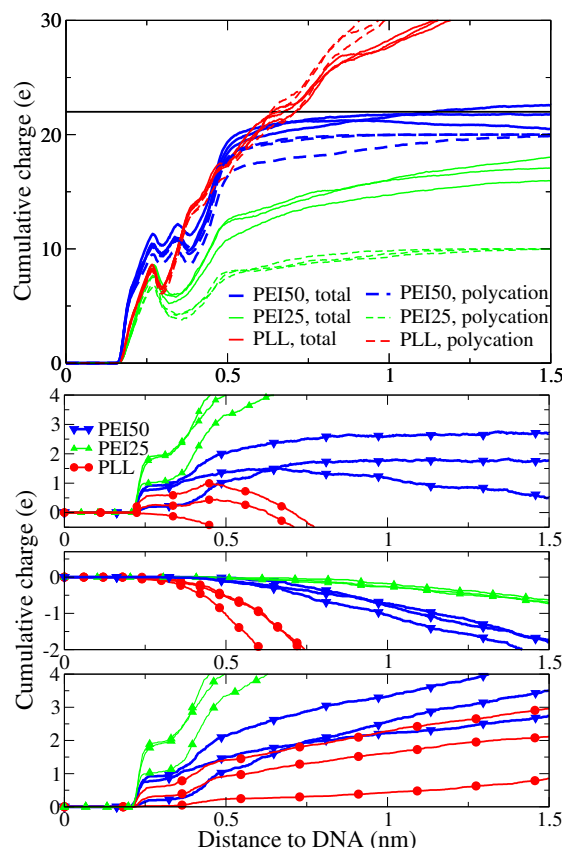


**Fig. 4** Average distance of PLL and PEI backbone nitrogen atoms to closest DNA atom at varying salt concentrations during the last 100 ns of the simulations. The two data sets represent each of the polycations in the 2xpolycation-DNA complexes. Error bars show fluctuations (one standard deviation from the mean).

NaCl. In CaCl<sub>2</sub>, full detachment of both PEI25s at 0.27 M is observed. For PLL, complete dissociation occurs at 0.52 M CaCl<sub>2</sub>, and one of the two PLL chains detaches at 0.39 M CaCl<sub>2</sub><sup>16</sup>. PEI50 is more tolerant against the increase of ionic strength in the solution and does not detach or in any of the studied salt concentrations in our simulations.

Both PEI25 and PLL detach via similar, zipper-like mechanism<sup>16</sup> in which the polycation-DNA charge-charge connections break one-by-one as the small cations absorb from the solution to the DNA surface replacing the DNA-polycation contacts. However, PLL responds to addition of salt significantly faster than PEI in the simulations: the PLL decomplexations upon addition of CaCl<sub>2</sub> occur within 60 ns whereas PEI reacts slower to added salt with onset of both the decomplexation and fluctuations mostly only after 60 ns. Additionally, the 2xPEI25-DNA complex at 1.04 M NaCl exhibits a particularly long-lasting (> 100 ns) sequence of small detachments where the polymer is barely connected with the DNA. Such data indicates a PEI25 strand is attached to DNA by one or two weak contacts with most of the polymer pointing outwards from the complex. A representative simulation snapshot is given in SI<sup>†</sup> along with the 2xpolycation-DNA minimum distances as function of time.

Therefore, PEI50 complexation with DNA is the least sensitive to addition of salt whereas PEI25 and PLL decomplex more easily in our simulations. As the decomplexation is induced by Ca<sup>2+</sup> ion binding to the OP sites of DNA and locally reversing charge<sup>16</sup>, the difference in the sensitivity to added salt can be clarified by comparing the binding patterns of the polycations, see Fig. 2. In particular, the brush-like structure of PLL leaves more DNA OP sites open. In contrast, PEI50 wraps on the DNA backbone with almost all of the charged



**Fig. 5** The cumulative charge distributions of the 2xpolycation-DNA complexes with counterions. The graphs present the total charge, the polycation charge contribution, and the ionic charge contributions. Distributions are time averages over 5 ns with three lines for each distribution demonstrating the magnitude of variation in data. Distance is measured as the distance to the closest DNA atom and the horizontal line shows the level where overcharging takes place.

N<sup>+</sup>s shielding DNA OP sites and the worm-like chemistry of PEI enables also the non-charged Ns to prevent ion condensation to DNA. This makes the PEI50 efficient in charge compensation, and the PEI50-DNA complex more tolerant against the addition of divalent salt.

### 3.3 Charge distribution without and with excess salt

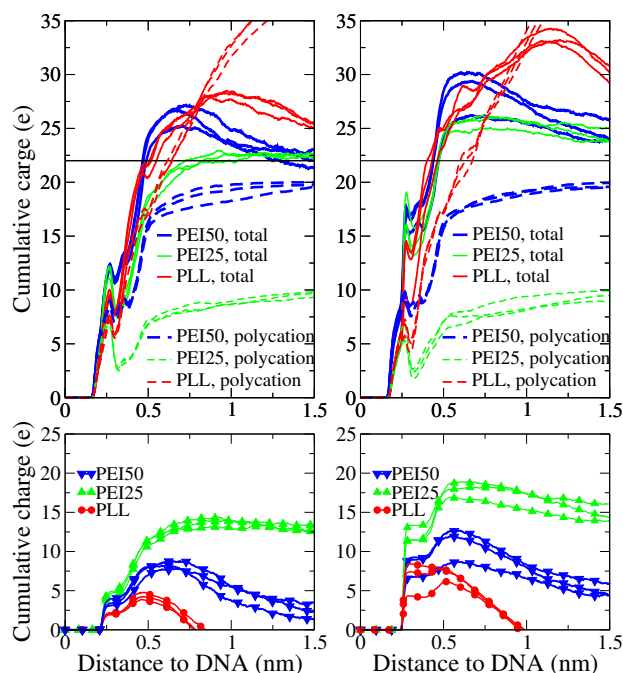
To further elucidate the role of polycation charge content and charge distribution on the salt tolerance of the polycation-DNA complexes, the intake of ions into the complexes and the charge neutralization characteristics of the different polycations were quantified via cumulative charge distributions around the DNA strand without and with excess salt. Cumulative charge distributions depict the amount of charge contained within a distance from the DNA surface. Comparison of the

cumulative charge distributions of the different 2xpolycation-DNA complexes without additional salt in Fig. 5 reveal quite expectedly, that PEI50 is more efficient than PEI25 in neutralizing DNA charge. Furthermore, PEI50 is also more effective close to DNA than PLL even though PLL has a higher charge per length. Complexation with PLL results in a more spread out overcharging peak. This is a consequence of both the larger charge content and the brush-like structure of the PLL.

As both the two PEI25 and PEI50 molecules contain less charge than the DNA strand they complex with, the 2xPEI-DNA complexes in our simulations remain undercharged. Therefore, even in the absence of excess salt, cations play a role in neutralizing the DNA charge in solution. As expected, more  $\text{Na}^+$  ions reside near the DNA in the 2xPEI25-DNA complex than in the 2xPEI50-DNA complex. In contrast, the 2xPEI50-DNA attracts more  $\text{Cl}^-$  ions than the 2xPEI25-DNA. With PLL, the less effective DNA charge compensation by the PLL than by PEI50 is also reflected in the amount of  $\text{Na}^+$  ions within the complex. Despite PLL-DNA complex being overcharged, similar amounts of  $\text{Na}^+$  ions are observed within the PLL-DNA and the PEI50-DNA complex in the simulations. The overcharging of PLL-DNA also attracts a considerable amount of  $\text{Cl}^-$  ions, which enable the gradual charge neutralization of the complex charge in the solution.

An intake of cations into the complex takes place when additional salt is introduced into the system. In Fig. 6, the cumulative charge distributions of the 2xpolycation-DNA complexes in 0.52 M NaCl and in 0.13 M  $\text{CaCl}_2$  are compared. Notably,  $\text{Ca}^{2+}$  is considerably more effective in penetrating the polycation-DNA complex than  $\text{Na}^+$ : Despite the lower ionic strength of 0.13 M  $\text{CaCl}_2$  compared to 0.52 M NaCl, 2-3 times more ionic charge resides near the DNA when  $\text{Ca}^{2+}$  is present. The stronger ability of  $\text{Ca}^{2+}$  to penetrate the complex seems to be independent of the polycation chemistry, and relates to the nature of divalent vs monovalent ions<sup>56-58</sup>, and in particular, to the presence of correlation effects. This strong tendency of  $\text{Ca}^{2+}$  to reside near the DNA surface, and bind to the OP sites, makes it more effective in inducing decomplexation compared to  $\text{Na}^+$ , as indicated by our earlier work<sup>16</sup>.

On average, a stronger charge of the polycation leads to less positive ions in the vicinity of the DNA. This trend is more clear when comparing PEI25 and PEI50, whereas the PEI50 and PLL allow very similar amount of cation influx into the complex, despite the large difference in charge content. On the other hand, the build-up of  $\text{Cl}^-$  ions differs significantly between PEI-DNA and PLL-DNA complexes: for the 2xPLL-DNA complex, a considerable decrease in the cumulative ionic distribution occurs in the same region where the outer parts of the PLL are. This is in contrast to PEI50 where much more moderate decrease in ionic charge is observed after the polycation charge has virtually reached its maximum.



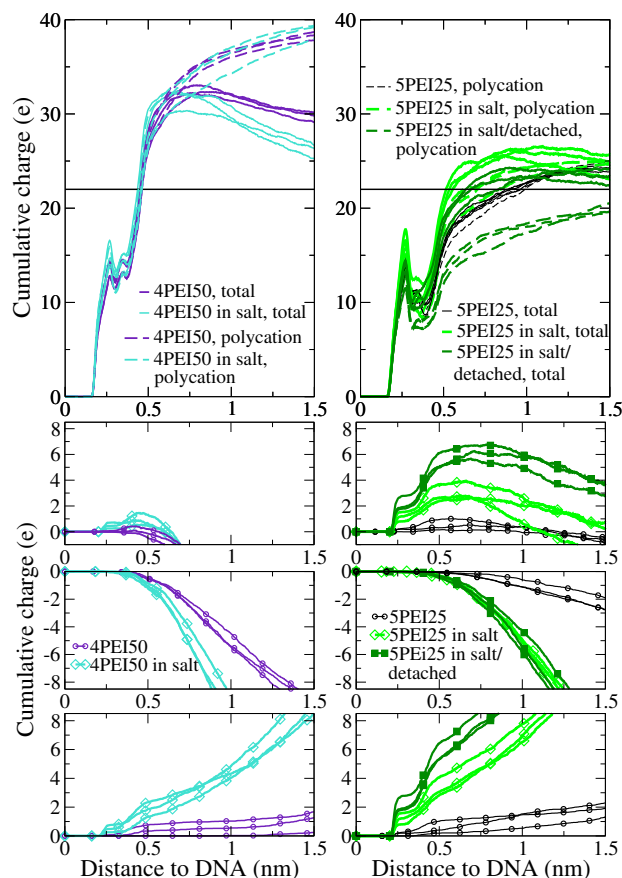
**Fig. 6** The cumulative charge distributions of the 2xpolycation-DNA complexes in 0.52 M NaCl (at left) and in 0.13 M  $\text{CaCl}_2$  (at right). The graphs present the total charge, the polycation charge contribution, and the ionic charge contributions. Distributions are time averages over 5 ns with three lines for each distribution demonstrating the variations in data. Distance to DNA is measured as the distance to the closest DNA atom.

For PEI25, the  $\text{Cl}^-$  ions are situated even further away.

We have demonstrated that influx of positive ions into the PEI50 complex is similar to the PLL complex. Out of the two, PLL has fewer  $\text{N}^+$ s bound to DNA, which shows up as smaller amount of polycation charge near DNA surface compared to PEI50. Therefore, less ions are needed to break the  $\text{N}^+$  contacts and similar amounts of cations in the PEI50-DNA and PLL-DNA complex can translate into more efficient loosening of the PLL-DNA complex compared to the PEI50-DNA complex.

To complement the analysis above, the charge distributions of the saturated 4xPEI50-DNA and 5xPEI25-DNA complexes without and with excess salt (0.27 M NaCl) are next discussed. These charge distributions are presented in Fig. 7. As already indicated by our comparison of the 2xPEI-DNA and the 2xPLL-DNA complexes, less  $\text{Na}^+$  ions (positive ionic charge) penetrate the saturated 4xPEI50-DNA and 5xPEI25-DNA complexes than the undercharged 2xPEI-DNA complexes. In contrast, the overcharged complexes attract more negative  $\text{Cl}^-$  ions. This is true also for the more charged 4xPEI50-DNA complex in comparison to the 5xPEI25-DNA complex. As the average distance of PEI backbone nitrogens

to the DNA surface is  $\sim 0.5$  nm in the saturated complexes, the  $\text{Cl}^-$ s dominantly remain outside the complexes.



**Fig. 7** The cumulative charge distributions of the saturated 4PEI50-DNA (at left) and 5PEI25-DNA (at right) complexes with counterions and excess 0.27 M NaCl. For the 5PEI25-DNA complex, data for both before and after detachment of one of the 5 polycations in salt is presented. The graphs present the total charge, the polycation charge contribution, and the ionic charge contributions. Distributions are time averages over 5 ns with three lines for each distribution demonstrating the variations in data. Distance to DNA is measured as the distance to the closest DNA atom.

Upon introduction of excess salt into the 4xPEI50-DNA system, the amount of  $\text{Cl}^-$  in the vicinity of the complex increases. Still, few of these additional  $\text{Cl}^-$ s penetrate the complex and no evident displacement of the PEI50 charge further away from the DNA surface is detected. However, PEI25 responds differently: the PEI25 molecules are more loosely bound, and exposure to 0.27 M NaCl even releases one of the five complexed PEI25s from the saturated complex. Indeed, a comparison of the charge distributions in Fig. 7 reveals the excess NaCl has very little effect on the 4xPEI50-DNA complex, while the 5xPEI25-DNA system shows an influx of pos-

itive ions even before one of the polymers detaches. The distribution of  $\text{Cl}^-$  ions does not change upon detachment which indicates that in this system  $\text{Na}^+$  dominates in inducing the detachment.

### 3.4 Discussion

Here, we have conducted an atomistically detailed computational study on the salt stability of three different types of polycation-DNA complexes. The study connects polycation charge content, specific chemistry, and especially charge compensation effectivity to the tolerance of polycation-DNA complex against additional salt. The comparison between simulation results and experimental data on DNA-polycation complexation and on the response of the complex to salt is hindered by the uncertainty in protonation states and specific chemistry of the polymers (e.g., branching) utilized in the experiments. In particular, the protonation ratio of PEI as a function of pH is not known, and within a force-field based MD simulation we cannot dynamically account for changes in protonation due to e.g. polyanion binding – the employed models are average representations of smaller and larger PEI protonation ratios and the results should be considered as indications of what happens with an increasing protonation ratio of PEI. Therefore, we analyze our results more on qualitative than quantitative level.

In our simulations, PEI50 binds dominantly to DNA OP sites following the DNA phosphate backbone rather strictly while PEI25 adopts much more convoluted, loopy conformations with the non-charged polymer sections going for the grooves. The differences in PEI25 and PEI50 binding to DNA reported here agree with previous simulational studies<sup>21,22</sup>, and experiments<sup>59</sup> where deprotonated PEI is was more prone to bind to DNA grooves, and it was suggested that this binding is driven by the release of water. In contrast, protonated PEI preferred DNA backbone. Our data indicates a similar effect in microscopical level with charged PEI sites preferring contacts with the DNA phosphate oxygens and longer stretches of the neutral polymer residing in the grooves. The PLL also prefers attaching to OP sites, as observed already in the previous simulations<sup>22,23</sup>, but due to its brush-like structure, PLL is not able to utilize all the charged  $\text{N}^+$  tips in binding. The structure also leads to bulkier complexes compared to PEI, which has been indicated experimentally<sup>11,19</sup> as well.

We found that exposing the complexes to monovalent salt caused fluctuations at 1.04 M concentration for the 2xPEI25-DNA and 2xPLL-DNA complexes, and detached one of the five complexed PEI25s from the saturated 5xPEI25-DNA complex at 0.27 M NaCl. Divalent cations were much more efficient in dissociating the complex and  $\text{CaCl}_2$  was able to induce a full detachment of one (at 0.39 M) or both (at 0.52 M) of the polycations from the 2xPLL-DNA complex. Additionally, both



PEI25s were detached when 2xPEI25-DNA complex was introduced to 0.27 M  $\text{CaCl}_2$ . In contrast, PEI50 complexes were extremely stable both in monovalent and in divalent salt, and PEI50-DNA complexes did not dissociate or strongly fluctuate over the duration of the simulations.

Interestingly, the PEI25-DNA zipper opens more gradually than the PLL-DNA. A particularly long lasting "open" state of 2xPEI25-DNA complex was detected in 1.04 M NaCl. Similar observations have been made experimentally in the fluorescence spectroscopy studies by Vuorimaa *et al.* where PEI-DNA complexes present a range of intermediate states between bound and freed PEI, whereas the distinction between free and bound states is pronounced for PLL<sup>60</sup>. The PEI Vuorimaa *et al.* studied was branched compared to our linear molecule which hinders us from making definite observations on specific conformations of these intermediate binding states between DNA and PEI.

The experimental melting and dissociation concentrations are in line with our observations. Bertschinger *et al.* observed significant release of DNA from PEI-DNA complexes only in NaCl concentrations above 1 M<sup>4</sup>. For PLL-DNA complexes, 0.8 M NaCl has been reported to induce a melting transition<sup>11</sup>, and specifically, Ref. 61 reports that a half of the DNA-PLL complexes dissociate at 0.86 M whereas no complexes are present at 1.3 M NaCl. For divalent  $\text{MgCl}_2$ , the corresponding concentrations are 0.2 M and 0.5 M.

Some concern has been raised<sup>46,47</sup> that the ion model used here may overestimate the interactions with the DNA phosphate sites, which are in key role in polycation binding to DNA. Consequently, we note that even though our choice of ion model is well founded, and the decomplexation concentrations reported here are in line with experimental reports, the values reported in this work should be regarded qualitatively; this is accounted for in evaluating the results. Furthermore, we verified the ion response by running the simulations also with the original Amber ions<sup>39,43</sup> with similar results (data not shown) providing confidence on the behavior.

Our data suggests that PLL, which has the highest charge per length, is more prone to detach from the complex than the less charged PEI50. In contrast, for the chemically equivalent PEI50 and PEI25, decreasing the protonation degree from 50% (PEI50) to 25% (PEI25) produces complexes that are less stable against addition of salt. Therefore, charge per length alone is an insufficient means to predict complexation strength, or salt solution stability of these complexes. Specific chemical detail of the molecules can help in explaining these findings. PLL binds to DNA inefficiently due to its molecular structure which does not allow all of its cationic groups to position themselves optimally. This results in complexes in which DNA charge remains more accessible to ions, and thus decomplexation can occur more easily than with, e.g., PEI50 whose worm-like molecular structure allows the poly-

mer to cover the DNA OP sites almost perfectly. For PEI25, the lower protonation degree again leads to less charge-bound  $\text{N}^+$ s to be replaced by ions, and more open DNA OP sites for the ions to condense to. This causes larger mean polycation distance to DNA, increased fluctuations of the complex, and consequently, to lower tolerance to added salt detected in our simulations. Our observations that decreasing PEI protonation state leads to less stable complexes is in full agreement with both previous simulational work<sup>21</sup> and experimental work, where DNA was released from PEI-DNA complex by alkaline pH<sup>62</sup>, as well as, observations of looser binding of DNA-PEI complexes in neutral pH versus low pH<sup>59</sup>.

Instead of charge per length, the overall charge density of the polycation seems to determine the effectivity of charge compensation. We modified this by altering two properties of the polymers. First, the charge per length of the polycation was controlled by decreasing the protonation ratio in PEI from 50% to 25%. Second, the structure of the polycation was altered: the brush like chemistry of PLL pushes the charged sites further apart from each other and the PLL backbone, whereas in PEI the  $\text{N}^+$ s in PEI are part of the backbone. This can be seen as increase in the polymer radius and consequently as a decrease in the charge density and charge compensation efficiency provided by the polymer. As a result, PLL charge around the DNA is more spread out, and more ions can penetrate into the complex and participate to the compensation of the DNA charge near the surface of DNA.

Indeed, our analysis shows that the most salt tolerant PEI50 has highest charge density near the DNA surface, and results in most effective charge compensation. The less stable PLL and PEI25 are also less effective in compensating the DNA charge close to the DNA surface. 2xPEI25-DNA complexes have the largest amount of cations in the complex and the more protonated 2xPEI50-DNA complexes attract similar amounts of cations near the DNA surface as the overcharged 2xPLL-DNA complexes. Interestingly, the  $\text{Cl}^-$  ions are able to reside in the PLL-DNA complex, which could provide an additional driving force for decomplexation by neutralizing the PLL charge inside the complex.  $\text{Cl}^-$ s may decrease the Coulombic energy gained by formation of PLL-DNA contacts and thus increase the salt sensitivity of PLL-DNA complexes. Indeed, for strongly charged polymers, favourable counterion-polyelectrolyte contacts can even lead to positive Coulombic energy of complexation<sup>63</sup>.

The correlation between the effectivity of charge compensation and the salt tolerance we suggest is supported by experiments: firstly, a higher polycation charge per length has been observed to lead to higher tolerance of the polycation-DNA complex to the addition of salt by several studies<sup>12,64,65</sup>. Gabrielson and Pack studied the effect of degree of acetylation of PEI to DNA-PEI complexes and observed that increased acetylation leads to both looser complexes and increased re-

lease of DNA from the complex by heparan sulphate<sup>65</sup>. Accordingly, Gabrielson and Pack proposed that reduced positive charge of acetylated complexes leads to weakened electrostatic interaction between DNA and PEI. Additionally, Akinc *et al.* observed that N-quaternizing the PEI charged nitrogens with side methyl and ethyl side chains made PEI-DNA complexes less tolerant against salt than unmodified PEI. The PEI with longer ethyl chains, translating to decreased charge density, was most prone to decomplex<sup>30</sup>.

In simulations, the more effective charge compensation by linear PEI compared to PLL has been noted previously by Ziebarth and Wang<sup>22</sup>. Sun *et al.*<sup>21</sup> indicated that an increase in branching has a similar effect in PEI binding to DNA as a decrease in charge per length. For linear vs. grafted PLLs, Elder *et al.*<sup>27</sup> detected a decrease in electrostatic binding energy between DNA and PLL as a function of increasing grafting length. All these priorly observed trends in polycation-DNA complex binding strength are linked to the charge compensation efficiency and to the salt tolerance of DNA-polycation complexes by our findings.

A wide debate in the polyelectrolyte community persists whether the complexation of two polyelectrolytes is driven by the ionic interactions between the two polyelectrolytes, by the entropic gain from the release of counterions upon complexation, or by water<sup>66</sup>. In addition to screening the ionic contacts between the polymers, salt affects complexation by suppressing the entropy gained by the release of ions. Ou and Muthukumar<sup>63</sup> concluded that additional salt always leads to weaker complexes but salt affects the interplay of Coulombic interactions and counterion release entropy differently at low and high polymer line charges: for weakly charged polymer, the salt weakens the complex by decreasing both the Coulombic binding energy gain and the free energy gain by release of counterions upon complexation. For strongly charged polymer, salt can make the Coulombic binding between complexes more favorable to complexation via screening but this effect is countered by even stronger suppression of the ion release entropy. Therefore, if counterion release entropy is assumed to fully dominate and chemical detail is omitted, the polymer with higher line charge (more counter ions), would be more prone to decomplex upon addition of salt. This means that, in our systems, PLL would be most affected by the suppression of the release entropy, and indeed, the PLL complexes are sensitive to salt in our simulations. That said, complexation of PEI25 should produce lowest ion release entropy and yet, also the PEI25-DNA complexation is sensitive to salt. The intermediate species PEI50, however, is very stable.

At a more complex level, the extent of ion release entropy is also dependent on the ability of the polycation to exclude ions from the complex which is, according to our results, related to the effectivity of charge compensation provided by the polycation. Interestingly, many of the common expressions<sup>63,67,68</sup>

for calculating the ion release entropy in complexation assume that complexation excludes almost all the ions from the complex, and the increase of salt concentration does not significantly alter the number of ions bound to the polymers. As the charge distributions presented in Figs. 5-7 show, these assumptions approximately hold for monovalent, but less so for divalent ions, and also the chemical structure of the polymer affects these quantities. Even the overcharged complexes, like the 2xPLL-DNA complex, can allow some cationic flux into the complex. If ion release entropy strongly dominates upon complexation, it is peculiar a high concentration of 1.04 M NaCl is not sufficient for inducing decomplexation. That said, we cannot conclude beyond discussion the entropic contributions due to ion release and binding.

Another commonly mentioned contributor to complex formation and stability is the flexibility of the polymer backbone<sup>27,69,70</sup>. In the complex, the polymer degrees of freedom are constrained compared to being free in a solution. In principle, a more flexible polymer gains more entropy when released from the complex. Out of the polycations studied here, PEI25 is by far the most flexible, and flexibility may provide an additional driving force favouring PEI25 decomplexation. That said, high ionic strength has been suggested to alleviate the effect of flexibility and other structural properties on the conformations adopted by the free polycation in solution<sup>58</sup>. Therefore additional salt potentially diminishes the role of flexibility in determining complex stability of chemically different polyelectrolytes.

In summary, the effectivity of charge compensation by the polycation seems to correlate with the sensitivity of the complex against the addition of salt. The efficient charge compensation leads to both efficient ionic binding between the polyelectrolyte and strong exclusion of cations from the complex in the systems studied here. The suppression of ion release entropy gained upon complexation may facilitate the salt induced decomplexation of PLL compared to PEI. That said, we observed the divalent ion being more efficient in inducing decomplexation, and at the same time our data shows that the behaviour of the divalent ion in the complexes, in the concentrations studied here, violates the assumptions behind the theories underlining the importance of ion release entropy upon complexation.

We have argued above that increasing in the line charge of PEI makes the PEI-DNA bond stronger and the complex more stable in salt solution. On first glance, this seems to be incompatible with the proton sponge hypothesis where the protonation of PEI enhances the transfection of DNA. However, according to the proton sponge mechanism, protonation aides transfection via inducing influx of  $\text{Cl}^-$  ions and consequent osmotic swelling of the endosome<sup>28,29</sup> which will lead to DNA release. In the simulations, we did see  $\text{Cl}^-$  ions aggregating strongly within the overcharged PLL-DNA complex.

As both of the 2xPEI-DNA complexes remain undercharged, a meaningful discussion the proton sponge property of PEI-DNA complexes therefore involves the saturated 4xPEI50-DNA and 5xPEI25-DNA complexes and their reaction to additional ions.

Firstly, 20% less of the more protonated PEI50 in molecular weight adsorbs spontaneously on the DNA compared to PEI25 in our simulations. This is reasonable since the more protonated PEI is more effective in compensating the DNA charge and therefore, less PEI is needed. The increased protonation also leads to crowding in the preferred DNA OP binding sites. Interestingly, the difference in DNA complexation ability of PEI50 and PEI25 indicates that the protonation of PEI occurring in low pH might release PEI from the PEI-DNA complex and increase the amount of free PEI in the solution with DNA still tightly protected by bound PEI. More so, theoretical considerations suggest that free PEI molecules are essential for inducing sufficient osmotic pressure increase to cause endosome rupture<sup>71</sup>. Experimentally, free PEI has been reported to aid gene transfection<sup>72</sup> and  $\text{Cl}^-$  accumulation<sup>31</sup> at endosomal level. In addition, there is evidence of free PEI facilitating the transfection also in other stages of the process<sup>72</sup>, such as by blocking the cell-surface glycosaminoglycans known to inhibit PEI-mediated transfection<sup>18</sup>.

In our simulations, no significant loosening of the complex structure was observed for 4xPEI50-DNA when exposing the complex to additional monovalent salt. Quite expectedly, the more overcharged 4xPEI50-DNA complex attracts more  $\text{Cl}^-$  ions than the 5xPEI25-DNA complex. That said, the  $\text{Cl}^-$ s do not notably penetrate between the DNA and PEI molecules, even when the complex is exposed to additional salt. Only little  $\text{Cl}^-$  penetration is observed despite the fact that the  $\text{Cl}^-$  concentration used here, 0.27 M, is considerably higher than the experimentally estimated maximum  $\sim 0.1 \text{ M}$ <sup>31</sup>. In contrast, the full detachment of one of the less protonated PEI25 molecules from the 5xPEI25-DNA complex occurred in our simulations upon addition of salt. However, the detachment is propelled by the positive ion ( $\text{Na}^+$ ) and not the negative ion ( $\text{Cl}^-$ ) entering the complex. Accordingly, partial release of DNA from the PEI-DNA complexes at low pH has been observed<sup>4</sup> *in vitro*, but the release was suggested to result from the neutralization of DNA phosphate oxygens with  $\text{H}^+$  ions instead of resulting from an influx of  $\text{Cl}^-$  ions into the complex.

In summary, our results shed light on the detailed mechanism of proton sponge behaviour. We suggest that protonation of the PEI strand can facilitate the endosome rupture via two routes. Firstly, the more protonated PEI produces more overcharged complexes which can attract more  $\text{Cl}^-$  ions and cause an increase in the osmotic pressure. Secondly, increasing protonation can release PEI molecules. The free PEI can both aid in bursting the endosome and facilitate other

stages of transfection. Therefore, it is reasonable that the proton sponge effect can cause endosomal rupture. However, in contrast to a common depiction of the proton sponge mechanism<sup>29,30,73</sup>, protonation and enhanced repulsion between protonated groups does not lead to swelling or loosening of the complex structure itself. Instead, our simulations show increased protonation leads to more stable and compact complexes. This would hinder the transcription of the genetic material residing inside the carrier complex. A successful delivery of genetic material may therefore require an additional step after the endosomal rupture where the complex is destabilized again by, for example, deprotonation and presence of multivalent ions.

## 4 Conclusion

To our knowledge, we present here the first molecular simulation characterization of polycation-DNA complex stability against added salt which probes systematically the effect of polymer charge per length and specific chemical structure. We have established that higher total charge or higher charge per length of polycation does not directly translate into higher stability of DNA-polycation complex against increasing salt concentration. Instead, the effectivity of charge compensation by the polycation, related to the effective charge density of the polymer, seems to correlate with the sensitivity of the complex against the additional salt with efficient charge compensation leading in stable complexes. Via extensive comparison with preceding experimental data, simulations and theoretical work, our study provides an atomistically detailed view on the mechanism through which these polymer properties can affect complex stability in salt solutions.

Furthermore, we also provided a detailed description of the proton sponge behaviour of PEI-DNA complexes: protonated PEI complex does indeed attract more  $\text{Cl}^-$  ions and protonation can facilitate transfection by release of PEI from the complex without decomplexation of the polyplex. Contradictory to a common description of the proton sponge mechanism, protonation does not lead to swelling of the complex structure. Instead, we find the complexes of more protonated PEI to be more compact.

The findings mean that tailoring the PE charge distribution could be a useful tool in designing ion resistant PE complexes. Joint together with the more detailed understanding of the proton sponge behavior presented here, these results bear a particular significance in developing more efficient polycation carriers for DNA delivery. Altogether, the results show that all-atom molecular dynamics simulations are a powerful tool both in elucidating existing experimental data and generating new fundamental understanding in such a intricate topic as polyelectrolyte complexation.

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