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# DNA duplex stabilization in crowded polyanion solutions

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**The melting temperature of duplex DNA is much higher in polyanions than that in non-ionic polymers with similar ionic strength, suggesting an additional electrostatic contribution on top of the excluded volume effect.**

Biological fluids and the cytoplasm contain concentrated biopolymers such as nucleic acids and proteins. They occupy ~20-40% of a live cell's volume, creating a crowded environment because of their mutual impenetrable property.[1] A thermodynamic consequence of macromolecular crowding is to favor reactions that produce reduced excluded volumes, such as DNA hybridization and protein oligomerization.[1,2] While most biochemical reactions have been studied only in simple buffers, the concept of macromolecular crowding has been increasingly appreciated in the past few decades.

Among the many biochemical reactions, DNA melting has received the most attention as a model system to understand the crowding effect.[3-6] Apart from its practical importance in DNA replication, biosensor development and therapeutics,[7,8] DNA melting can be conveniently monitored using many spectroscopic techniques. The affinity of DNA strands can be precisely tuned by varying DNA length, sequence, and buffer ionic strength. The melting temperature ($T_m$) of a DNA duplex is often increased by crowding agents since DNA (especially long DNA) melting is usually accompanied with an increase in the excluded volume. Non-ionic polymers such as polyethylene glycol (PEG) and dextran are among the most frequently used crowding agents.[4,9] Their specific chemical interaction with biopolymers such as DNA and proteins is relatively small (although still exist), so that their actions can be largely attributed to the excluded volume effect.[10,11]

We reason that using polyanions instead of non-ionic polymers might be a more accurate representation of cellular biopolymers since nucleic acids and most proteins are negatively charged.[12-14] One of the potential difficulties associated with using polyanions is the high salt concentration accompanying the polymer. For example, 10% (w/w) sodium polyacrylate (NaPAA) at neutral pH contains ~1.3 M $Na^+$. On the other hand, PEG can be prepared in the absence of any $Na^+$; the $Na^+$ concentration can be independently and precisely controlled by adding NaCl. A high $Na^+$ concentration makes it difficult to directly compare the crowding effect of NaPAA with PEG. Herein we mainly compared the trend of $T_m$ change and the highest $T_m$ that can be achieved, where a dramatic difference was observed among the tested polymers.

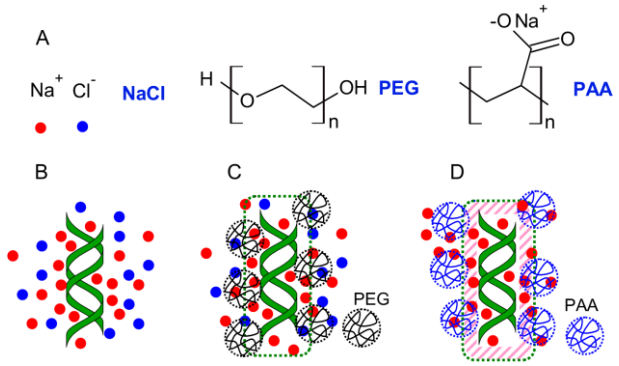


**Figure 1**. (A) The structures of the salt and polymers used in this study. Schematics of negatively charged duplex DNA dispersed in NaCl (B), in PEG and NaCl (C), or in polyanionic NaPAA (D). Electrostatic repulsion between PAA and DNA might not increase the excluded volume (green dotted lines) due to the extremely high salt concentration and short Debye length. Electrostatic force brought by the PAA chains (pink shaded lines) is likely to be the main reason for the additional stabilization.

We employed NaPAA as a model polyanion and three MWs were tested: 1200, 8000 and 15,000 (see Figure 1A for its structure). An AlexaFluor 488 labeled 12-mer DNA was hybridized to an Iowa Black labeled DNA to produce a DNA duplex. DNA melting was thus monitored by fluorescence enhancement.[15-19] The melting curves in the presence of increasing concentrations of NaPAA1200 are shown in Figure 2A, where typical DNA melting transitions are observed. The temperature corresponding to the maximal of the first derivative of a melting curve is $T_m$. In the absence of NaPAA, the DNA was dissolved only in 5 mM HEPES (e.g. ~2.5 mM $Na^+$) to give a $T_m$ of 38 °C. $T_m$ reached 50 °C with just 1% NaPAA, where the $Na^+$ concentration was ~120 mM from the polymer solution. As shown in Figure 2D (black dots), the $T_m$ value initially increased with NaPAA1200 concentration. After reaching the maximal $T_m$ of 68 °C in 20% NaPAA1200, further increase of the polymer concentration led to decreased stabilization. Therefore, NaPAA1200 has at least two types of actions on DNA stability, where the destabilizing factor exceeded the stabilizing factor at high polymer concentration.

For NaPAA8k (Figure 2B), normal DNA melting curves were obtained with up to 34% polymer concentration. The melting transition was very broad at 44% (e.g. spanning from 40 °C to 95 °C), which may suggest a different mechanism of melting. For this reason we do not include this data point for further discussion. The overall trend is quite different from that for NaPAA1200, since no dropping in $T_m$ is observed

with increasing of NaPAA8k concentration (Figure 2D, red dots). A very similar trend was obtained for NaPAA15k (Figure 2C). The highest tested NaPAA15k concentration was 34% because of its high viscosity.

The main reason for the drastic increase of $T_m$ by NaPAA is the $Na^+$ in the polymers. As shown in the top axis of Figure 2D, 10% (w/w) NaPAA contains ~1.3 M $Na^+$ and 30% gives 4 M $Na^+$, which is largely responsible for the increase of $T_m$ from 38 to ~71 °C. To calculate the $Na^+$ contribution, we next measured the melting of this DNA in various concentrations of NaCl in the absence of any polymer. Normal melting curves with a single melting transition were observed up to 3 M NaCl (Figure 3A). With 4 or 4.9 M NaCl, there appeared to be a secondary transition at ~30 °C. For these two samples the main transitions were taken as their $T_m$. The highest $T_m$ of 63 °C was observed with 1 M NaCl and further increase of NaCl led to decreased duplex stabilization. This trend is consistent with previous reports.[20] Since DNA is a highly negatively charged polymer, NaCl increases the $T_m$ of DNA by the charge screening effect of $Na^+$. This non-specific electrostatic screening is saturated at ~1 M $Na^+$. Further increase of the salt leads to other consequences such as its interaction with the surrounding water.[21,22] Anions (in this case $Cl^-$) have a greater effect on disrupting water structure compared to cations and they are responsible for the dropping of $T_m$.[20] To bring the $T_m$ from 63 °C to 71 °C, other factors in NaPAA must be considered besides $Na^+$. Similar observations were also observed with a FAM-labeled 12-mer or 24-mer DNA, indicating generality of our observation (Figure S2, ESI).

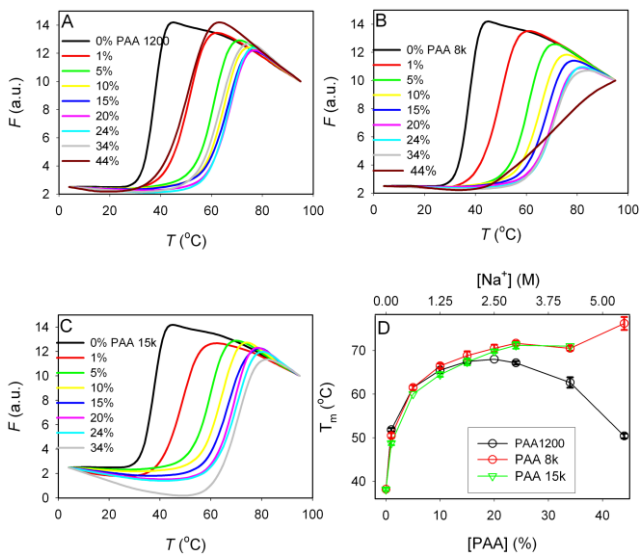


**Figure 2**. Normalized DNA melting curves in the presence of various concentrations of NaPAA1200 (A), NaPAA8k (B) and NaPAA15k (C). (D) $T_m$ as a function of NaPAA concentration (w/w) for these polymers.

We next consider the excluded volume effect. In order to also model a crowded environment, we further measured the $T_m$ in the presence of 10% PEG4k or PEG20k as a function of NaCl concentration (Figure 3, red and green dots). Note that the MW of each NaPAA repeating unit is about twice of that for PEG. At low NaCl concentrations, the $T_m$ was only slightly higher (e.g. < 1 °C) in the presence of PEG. With greater than 0.5 M NaCl, PEG even caused suppressed $T_m$.[10,23] Under all tested conditions, PEG induced stabilization never exceeded 1 °C and NaCl induced stabilization is maximally 24.6 °C. Since NaPAA8k and 15k can produce maximally ~33 °C increase in $T_m$, stabilization related the polymer charge effect is >7 °C.

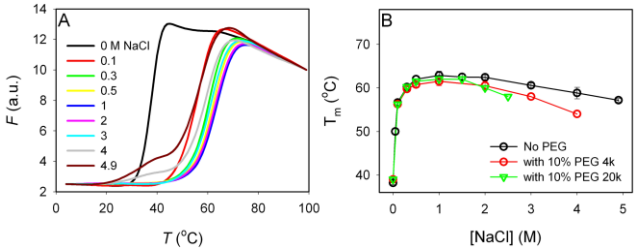


**Figure 3**. (A) Normalized DNA melting curves in the presence of various concentrations of NaCl. (B) $T_m$ as a function of NaCl concentration in the absence of PEG or with 10% PEG 4k or PEG 20k.

To understand the mechanism of DNA stabilization by NaPAA, we measured $T_m$ as a function of DNA concentration in 25% NaPAA8k or in 3 M NaCl (no polymer). By plotting $1/T_m$ as a function of DNA concentration $C$, thermodynamic parameters can be extracted from quation (1).

$$\frac{1}{T_m} = \frac{R}{\Delta H^\circ}\ln C + \frac{\Delta S^\circ - R\ln 4}{\Delta H^\circ} \quad (1)$$

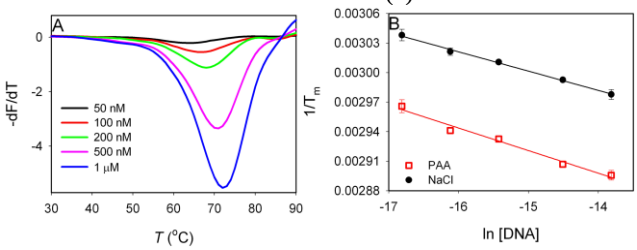


**Figure 4**. (A) The first derivatives of DNA melting curves as a function of DNA concentration in 25% NaPAA8k. (B) Thermodynamic analysis of DNA concentration dependent $T_m$.

The first derivatives of the DNA melting curves in 25% NaPAA8k are shown in Figure 4A, where the DNA concentrations were reflected by the area under each curve. The $T_m$ values shift to lower temperature as the DNA concentration is dropped, which is expected for duplex DNA melting. A plot was made in Figure 4B according to equation (1), where we obtained $\Delta H^\circ$ =101.4 kcal/mol in 3 M NaCl and 86.6 kcal/mol in 25% NaPAA8k. Therefore, enthalpy cannot explain the extra DNA stability brought by NaPAA since NaPAA requires less heat for the melting reaction. At the same time, $\Delta S^\circ$ =263 cal/K·mol in NaCl and 211.6 cal/K·mol in NaPAA. This means that the entropy increase after DNA melting is much smaller in NaPAA, which over compensates the enthalpy effect. In other words, the extra stability in NaPAA is an entropy effect. It is likely that the melted DNA strands are confined by the strong electrostatic repulsion of the surrounding PAA chains. It needs to be pointed out that the difference of free energy change $\Delta G^\circ$ is quite small in these two conditions. For example $\Delta G^\circ$ = 23.0 and 23.5 kcal/mol in NaCl and NaPAA, respectively, with a $\Delta\Delta G^\circ$ of just 0.5 kcal/mol.

Analyzing all the data together, we reason that duplex DNA stability in NaPAA is governed by the following factors: charge screening (e.g. effect of $Na^+$), anion effects on water, polymer chemical interactions with DNA, excluded volume effect, and electrostatic repulsion by PAA chains. With a high

polyanion or NaCl concentration, the effect of $Na^+$ is saturated for all the samples and is thus not considered here. A good starting point to compare the anion effect is the Hofmeister series (e.g. $SO_4^{2-}$ > $H_2PO_4^-$ > $CH_3COO^-$ > $Cl^-$ > $ClO_4^-$), which ranks anions in their ability to change water structure and it was initially generated by comparing protein solubility.[24] Similar studies have also been performed on DNA melting. For example, with 4 M salt, the destabilization of a DNA duplex follows this order $CF_3COO^-$ > $ClO_4^-$ > $CH_3COO^-$ > $Cl^-$.[22] By comparing the $T_m$ trend of NaCl and NaPAA1200, the latter has a larger destabilization effect since it induces a more drastic suppression of $T_m$ at high concentration than NaCl. This is consistent with that $CH_3COO^-$ is more destabilizing than $Cl^-$, and the PAA backbone is similar to $CH_3COO^-$.

PEG is known to interact with DNA bases via its methylene backbone to destabilize DNA;[11] PAA might also have such an interaction. If we assume that such chemical destabilizing effects and the disruption of water structure are independent of the MW of NaPAA, certain polymer length dependent effect must be playing an important role since NaPAA8k and 15k showed much higher $T_m$ than NaPAA1200 at high polymer concentrations. Next, we analyze the excluded volume effect. For our 12-mer DNA, PEG showed stabilization effect on DNA only when no NaCl was added. The stabilization effect of PEG disappeared even with just 100 mM NaCl (Figure 3B). Such salt concentration dependent PEG stabilization effect has been explained previously.[10] One reason for the lack of strong excluded volume effect is because the DNA we used was very short.[4] Equation (2) links the change of excluded volume $\Delta V_{ex}$ with $\Delta T_m$

$$\Delta T_m = \frac{RT_m^{0^2}}{\Delta H}\Delta V_{ex}C_p \qquad (2)$$

where $R$ is the gas constant, $\Delta H$ is the enthalpy of DNA melting, $T_m^0$ is the $T_m$ in the absence of the polymer, and $C_p$ is the molar concentration of the polymer.[9] Based on the fact that $\Delta T_m$ is almost zero (e.g. <0.8 °C), $\Delta V_{ex}$ should also be close to zero.

If we treat the effect of PAA to be purely excluded volume action by considering an extra volume contribution related to electrostatic repulsion, this additional volume change should be very moderate since the Debye length is so small in such high salt condition (e.g. <0.5 nm). Therefore, it is unlikely that the ~7 °C extra stabilization brought by high MW PAA can be completely attributed to an increased excluded volume change due to electrostatic repulsion. Instead, we propose that modulation of electrostatic repulsion between DNA chains by PAA should be an important reason. Such an electrostatic interaction cuased by concentrated negatively charged polymers has also been shown to condense long biological DNA,[14] to decrease double layer repulsion between negatively charged mica plates,[25] and to affect colloidal particle stability.[26]

In summary, we have measured the melting of a DNA duplex in polyanions and found that the ultimate stability of the DNA at high polymer concentration was significantly increased compared to any other conditions involving just NaCl or a mixture of NaCl with PEG. Since most of cellular biopolymers are polyanions, performing model reactions in negatively charged polymer solutions can offer further insights and better optimize sensors and devices inside cells.

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*Electronic Supplementary Information (ESI) available: materials and methods, original melting traces. See DOI...*

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