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Effect of Molecular Crowding and Ionic Strength on the Isothermal Hybridization of Oligonucleotides

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Abstract

The isothermal hybridization of complimentary oligonucleotides, 15-mer, 25-mer, 35-mer, and a molecular beacon, was investigated under varying conditions of molecular crowding and ionic strength, using hypochromicity to follow strand pairing and polyethylene glycol as a crowding agent. Thermodynamic analysis of the results revealed the addition of counterions to the oligonucleotide backbones, $\Delta\psi$, to be dependent on the strand G-C content and the molecular crowding. A decrease in $\Delta\psi$ was observed with both increasing GC% and solution PEG content. In contrast, the number of bound water molecules depended on the activity of Na^+, where two regimes were observed. At $a_{Na^+} < 0.05$ and increasing molecular crowding, water molecules were released into the DNA solutions and oligonucleotide pairing was favored with both increasing hydrophobic forces, while at $a_{Na^+} \geq 0.05$, water molecules were bound to the strands and the extent of double strand formation decreased with increasing PEG wt%.

Keywords

polyethylene glycol; beacon; pairing; hypochromicity; sodium activity

INTRODUCTION

There is increasing attention directed to the understanding of biological processes both in vivo and in vitro under molecular crowding which is an important driving force in cellular and subcellular activities.1–8 A first example is the motility of spermatozoa prior to fertilization in the ovarian fluid which is a hypo-osmotic medium relative to the semen containing the spermatozoa. The authors of a recent study reported the change in motility and velocity of zebrafish sperm exposed to media at different concentrations of sucrose with decreasing performance at increasing osmotic pressures.9 Another example is the homeostasis of mitochondria, which is an osmotically controlled process.4, 10 In some pathological cases when the osmotic equilibrium is disrupted, water influx occurs into the mitochondria causing swelling subsequently the breakage of the organelles' outer membrane which leads to decreased mitochondrial activity hence cellular apoptosis.10

On another scale, interest is focused on the study of the packing of double stranded DNA into condensed phases in the nucleus of eukaryotes11–12 and in the capsid of viruses.13–18 Many factors control the packing of DNA; for example, multivalent cations, proteins,

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SUPPORTING INFORMATION AVAILABLE Additional experimental details, DNA melting curves, vapor pressure osmometry and analysis, circular dichroism and hypochromicity profiles, kinetics of pairing. This material is available free of charge via the Internet at http://pubs.acs.org.

liposomes, neutral crowding macromolecules.19 The osmotic pressure within eukaryotes can vary at different stages of the mitotic cellular division; whereas osmotic pressures in capsids can go up to 50 atm acting as a physical drive for the packing of the DNA and a biological trigger for its ejection.20¯21 Model viruses for the study are bacteriophages; the most common of them being bacteriophage λ. Phage λ's genome can be extended to 17000 nm while the inside diameter of its capsid is thousandfold smaller: approximately 25 nm.21 In order for the complete genome to fit in the capsid, the DNA strand has to be bent and closely packed which require forces that can overcome the repulsive forces between densely packed negatively charged strands. To understand the compacting mechanism into the close packed arrays, various groups studied the packing of DNA strands under osmotic stressing and in the presence of cationic species.22¯28 Rau et al. confined DNA strands into crystal structures against solutions of polyethylene glycol at different salt concentrations and studied the resulting structures by X-ray diffraction where the strands' interaxial distances decreased with increasing osmotic pressure.27

In addition to its compacting ability, the presence of the osmolytes has been shown to affect the thermodynamics of DNA stability. Spink and Chaires reported selective stabilization of DNA triple helices in the presence of polyethylene glycols while the stability of the double stranded DNA depended on the molecular weight of the osmotic stressing agent;29 enhanced stability was seen with high molecular weight stressors whereas the reverse was true with small osmolytes. DNA stability is not a recent interest. Numerous studies have explored the behavior and stability of DNA depending on pH, base sequence, end groups, cationic species, and osmolytes.30 Sucrose, ethylene glycol and small molecular weight PEGs have been shown to destabilize the duplexes, yet larger molecular weight PEGs which are excluded from the DNA strands change the enthalpies of duplex melting and stabilize the strands.31¯36

Studies of crowding and ionic effects on oligonucleotide pairing and stability have been accomplished via thermodynamic analysis of melting profiles of various double stranded DNA. *In vivo*, temperature change is not one of the physical parameters available for modifying DNA interactions. In this paper, we present a thorough investigation of different PEG-cation matrix contributions to the pairing of oligonucleotides under isothermal conditions. It should be noted, as explained by Parsegian et al., that the terms "osmotic stressing" and "macromolecular crowding" are equivalent.37 For our purposes, we will adopt the term molecular crowding to demonstrate its effect on the hybridization of DNA oligomers. The oligonucleotides studied were of different lengths; 15-mer, 25-mer and 35-mer and of varying G-C content. We report the crowding conditions, the chain length and GC content, and ionic strength as contributors to changing the equilibrium, the extent of counterion binding to the backbone of the oligonucleotides and the exchange of water molecules upon pairing of the oligonucleotides.

EXPERIMENTAL METHODS

DNA oligomers and "beacon" were obtained from The Midland Certified Reagent Company, Inc. and used without further purification (Table 1). Sodium phosphate monobasic, NaH_2PO_4; sodium phosphate, Na_2HPO_4; and sodium hydroxide, NaOH (1 N solution) were used as received from Fisher Scientific. Sodium chloride, NaCl; and sucrose, $C_{12}H_{22}O_{11}$ were from Sigma Aldrich. Polyethylene glycol, $(C_2H_4O)_n$ (PEG 4000; average M_w 3000), was obtained from TCI, Japan. For clarity, PEG 4000 will be denoted as PEG3.

The DNA samples were prepared by dissolving aliquots of complimentary single stranded oligonucleotides in cosolvent-phosphate buffer mixtures at 1.00 mM phosphate and pH 7.0 and different PEG3 content (1-30wt%). The final Na^+ concentration in the samples was

varied between 2 and 225 mM by the sequential addition of aliquots of 1.5 M NaCl to the sample solutions. The buffer capacity of all the solutions was tested by measuring the pH; an increase of 0.5 was observed over the range of NaCl and osmotic stressors used in this study.

Hypochromicity experiments at increasing ionic strengths were performed by collecting UV-visible spectra between 200 nm and 500 nm on a Varian Inc. CARY-100 spectrophotometer equipped with a Peltier 6 × 6 cell holder, a magnetic stirrer, and a temperature controller. Initially, the osmotic stressor/phosphate solutions were placed in the cell holder and kept at 20 °C for 5 minutes to establish temperature equilibrium. Aliquots of single strand oligomer were added to the solutions to yield 1 μM oligomer. Aliquots of the complimentary strand were then added to increase the total single strand concentration to 2 μM. Spectra were collected before and after each addition. Solutions were heated at 80 °C for 10 minutes followed by slow cooling at 1 °C/min to the desired temperature; 35 °C for the 15-mer, 55 °C for the 25-mer, and 67 °C for the 35-mer, where they were incubated for 1 h prior to the hypochromicity experiments. The working temperatures were determined through oligomer duplex melting experiments, described in Figure S1 in Supporting Information. Spectra were collected both after heating and cooling; a slight increase in absorbance was observed upon heating. After one hour of incubation at the desired temperature, aliquots of 1.5 M NaCl in the appropriate cosolvent-phosphate buffer, or solid PEG3, were added to the oligomer solutions and UV-vis scans were collected following 5 min temperature equilibration. Absorbance values at 260 nm were recorded and normalized using the equation: $\%=\frac{A-A_{\min}}{A_{\max}-A_{\min}}\times 100$. Hypochromicity of the beacon was performed in a similar fashion, with both increasing [NaCl] and PEG3 wt%, where the temperature of the beacon/phosphate buffer solution was kept constant at 75 °C. Scans were performed from 200 nm to 800 nm where three absorbance peaks were observed at 260 nm, 555 nm, and 590 nm the first corresponding to the DNA bases and the latter two to the Texas Red®, TR, and Black Hole Quencher-2™, BHQ-2, dye-quencher pair. The fluorescence of the beacon was analyzed on a Cary Eclipse equipped with a temperature controller. The solutions were irradiated at 590 nm and emission spectra were collected between 600 nm and 800 nm with an emission maximum at 615 nm.

Circular dichroism, CD, was performed for the conformational analysis of the oligomer strands. Spectra were collected with a CD spectrometer Model 410 by AVIV Biomedical, Inc. equipped with a temperature controller and a cell chamber purging system; a constant stream of N_2 was flushed through the chamber to purge it from ozone and eliminate water condensation on the sample cells at low temperatures. Solutions were prepared as described in the hypochromicity section. Three spectra were collected for each sample and averaged.

Kinetic studies were performed on the CARY-100 spectrophotometer to ascertain the extent of oligonucleotide hybridization at different PEG3-NaCl conditions. Solutions of complimentary strands at 1 mM phosphate buffer at pH 7.00 and varying PEG3 content were heated to the working temperature and absorbance at 260 nm was monitored against time before and after the addition of aliquots of NaCl. Once a constant signal was observed, the temperature of the samples was decreased to 20 °C and solutions were held at this temperature for 30 minutes. UV-vis spectra were collected to determine the absorbance at the latter temperature. Finally, the temperature was raised back to the working temperature and UV-vis spectra were collected after an equilibration period of 5 minutes.

Osmolalities of PEG3/NaCl/buffer and sucrose/buffer solutions were measured with a Vapro 5520 Vapor Pressure Osmometer (Wescor, Inc). Measurements were performed at 20 °C and at the same conditions as the hypochromicity experiments (see Figure S2 and Table S2).

RESULTS AND DISCUSSION

Hypochromism is a useful attribute of nucleic acids and remains the most studied phenomenon in DNA experiments.38 It relies on the decrease of absorbance of nucleotide bases between 250 nm and 270 nm. On forming a single strand the total absorbance of the nucleotides decreases by 20% due to the base-base interactions. Further decrease is observed, up to an additional 20%, when complimentary strands pair into a double helix where base stacking takes place.30 To understand the hybridization mechanism of complimentary strands under ionic and molecular crowding conditions, we took advantage of the latter feature.

The pairing of nucleotides depends on the complexity of the strands. The complimentary oligonucleotides used in this work, Table 1, were designed to minimize secondary interactions, consequently secondary structures, in order to approximate the experimental observations to a two state transition from complimentary single stranded DNA oligomers to double stranded pairs.

To mimic the cellular matrix, it is customary to use sugars and polyols as stressing agents. Figure 1 is a plot of the osmotic profiles of NaCl, sucrose, and PEG3 in 1 mM phosphate buffer at pH 7.0 determined by vapor pressure osmometry at 20 °C. Although our experiments are performed at higher temperatures, the data presented in Figure 1 is a good approximation of the osmotic behavior of the stressing agents under the conditions of the work herein as the activity of water is assumed to be minimally affected by temperature differences in the window of our investigations.35, 39

At high molecular weights, polyols are excluded from the DNA strands; therefore PEG3 does not directly interact with the strands but changes the activity of cosolutes around the oligonucleotide affecting the thermodynamics of DNA systems;34-35, 40 For example, Spink and Chaires reported a 5 °C increase of DNA duplex melting temperature in the presence of PEG 8000 at 19 wt% and 300 mM NaCl.29

In addition to changing the thermodynamics of DNA pairing, different molecular weight polyols can change the kinetics of the strand pairing reaction. The accepted pairing mechanism, in the presence of ions, describes the formation of a DNA double helix as a cooperative zip-up mechanism where the rate determining step is the nucleation step that depends on the solution ionic strength, followed by instantaneous zipping of the single strands.41-45 In a recent work, Gu et al.46 extended this mechanism to DNA pairing in molecular crowding conditions where they investigated the pairing of DNA oligomers in the presence of PEG 8000, PEG 600 and PEG 200 at 1 M NaCl. They also reported a decrease in the forward rate constants (pairing rate) with decreasing PEG molecular weight and attributed the insignificant changes in rate constants in high molecular weight PEG to the presence of pools of PEG-free solution where the DNA pairing proceeded without any change in the hybridization kinetics. Similarly, Schoen et al. observed insignificant changes in pairing kinetics of oligonucleotides in buffered dextran solutions at 20% wt/vol.47

Previous studies investigated the melting of double stranded DNAs to their complimentary single strands by spectroscopic or calorimetric analysis to establish the effect of ionic or molecular crowding species on the thermal stability of double stranded DNA. In this work, at first, we established the effect of sodium cations on the pairing equilibrium of the oligonucleotides described in Table 1 under isothermal conditions.

Figure 2 presents the hypochromicity of the 15-mer, 25-mer and 35-mer oligonucleotides with increasing ionic strength. The working temperature at which each solution was maintained was determined from melting experiments of corresponding duplexes in 2.5 mM

Na^+ in 1 mM phosphate buffer, see Figure S1. Kinetic measurements were conducted to ensure the points recorded represent the equilibrium values at each salt or PEG addition, see Supporting Information for details. The presence of cations in solution favors both the thermodynamics and the kinetics of DNA pairing.30 The concentration of Na^+ required for the pairing of complimentary oligonucleotides depends on the length of the strands, their composition, the end groups and the temperature of the system. It is well known that monovalent cations and some multivalent cations stabilize DNA double strands: first, through counterion condensation on the phosphate backbone which increases charge screening;48-52 second, by addition within the minor grooves of the double helix,53-56 where a spine of water and ions is formed.57-58 In addition to thermodynamic effects, the increasing ratio of cations to DNA increases the pairing rate of complimentary strands.43

From Figure 2, the range of NaCl concentration was determined for the three complimentary oligonucleotides. The onset of pairing of the 35-mer, at 67 °C, is observed at 25 mM Na^+, while the pairing reaches completion at 115 mM. Similarly, the 25-mer's pairing at 55 °C occurs between 35 mM and 222 mM, while the 15-mer at 35 °C pairs between 10 mM and 135 mM total Na^+ concentrations. The results are tabulated in Table S1.

To investigate the contribution of molecular crowding37 to the pairing of the oligonucleotides, the Na^+ concentration was maintained constant at 2 mM and the content of PEG was increased by sequential addition of solid PEG3 up to 30 wt%. The process was studied by spectroscopy and the absorbance profiles at 260 nm are shown in Figure 3.

The addition of PEG3 decreases the activity of water in solution (Figures S2-S4) causing a change in the thermodynamics of the system, which one might expect to favor strand pairing in order to decrease the hydrophobic effects present within bases of single stranded DNA. Although the content of PEG3 was increased to 30 wt%, the osmotic equivalent of approximately 0.5 M NaCl, no change in absorbance was observed with sequential addition of polyethylene glycol at 2 mM Na^+ until the ionic content was increased where the hypochromic effect was observed (open points in Figure 3).

To confirm this behavior, a molecular hairpin beacon was used.59 The beacon is a single stranded oligonucleotide where the complimentary ends are modified with a fluorophore molecule and a quencher molecule, respectively, Texas Red® at the 5′ end and Black Hole Quencher-2™ at the 3′ end. It has several advantages over separate complimentary strands; in addition to hypochromicity experiments, one can monitor, by fluorescence, the pairing of the complimentary bases which is a unimolecular process as opposed to bimolecular for the pairs of complimentary single strands. The energy transfer between the fluorophore and the quencher is controlled by FRET.60 When the beacon is in its open form the quencher molecule is far enough from the fluorophore to allow it to emit. On pairing, the quencher is in the vicinity of the fluorophore, energy transfer occurs, and the fluorescence is quenched. The technique offers higher sensitivity to signal change while studying the pairing of complimentary sequences.

Figure 4 shows the changes in fluorescence of the beacon-quencher pair at 75 °C with increasing Na^+ concentration in solution as well as PEG3 wt% at 2 mM NaCl. When NaCl is added to the solution of the beacon, the complementary ends of the beacon pair; the overall fluorescence signal of the solution gradually decreases as the pairing reaction is favored with increasing [NaCl]. Alternatively the fluorescence signal remains constant with increasing PEG3 wt% at low ionic strength; no signal quenching is observed as PEG3 is added to the beacon solution until the NaCl content is increased.

More evidence of the latter behavior is seen in the hypochromicity of the hairpin, see Figure S7. In addition, changes in the absorbance peaks of the dye-quencher pair are consistent with the above observations, Figure S8 and S9.

To see whether the oligonucleotides were binding to the PEG3 macromolecules, inhibiting the pairing of the strands, circular dichroism spectra of the oligomers were collected in PEG3 solutions. The CD signals at 275 nm and 245 nm increased as NaCl was gradually added to the solution of oligonucleotides while the addition of PEG3 at 2 mM Na^+ did not change the CD of the single stranded oligomers indicating no pairing of the strands and no direct interactions of the PEG3 macromolecules with the oligonucleotides, (see Figures S10 and S11). As severe molecular crowding is not sufficient, by itself, to induce strands to pair, we investigated the additive effect of PEG3 and Na^+ cations on the pairing of the oligonucleotides. Figure 5 presents the hypochromicity of the 35-mer with increasing NaCl concentrations in the presence of 0-30 wt% PEG3 in solution.

From Figure 5 it is clear that the pairing of the oligonucleotides requires a lower [NaCl] with increasing PEG3 concentration. In a recent study, Karimata et al.31 showed the increased stability of oligonucleotides under molecular crowding conditions at low salt concentrations. They investigated the latter phenomenon by performing melting experiments of oligomers and hairpins at varying PEG and NaCl compositions. In our experiments, for each oligonucleotide pair, we were able to quantify the NaCl concentrations needed to induce controlled pairing of the oligonucleotides at different PEG3 compositions and constant temperature, see Table S1.

In order to understand the cooperative effect of molecular crowding and sodium chloride on the hybridization, the thermodynamics of the different systems was investigated.61–63 The equilibrium hybridization of DNA is represented by:64

$$ssA.x_{Na}+.x'_{H_2O}+ssB.y_{Na}+.y'_{H_2O} \iff dsAB.Z_{Na^+}.z'_{H_2O}+\Delta n_w H_2O+\Delta n_s PEG+\Delta n_{Na^+} Na^+ \quad (1)$$

where *ssA, ssB, and dsAB* stand for the single stranded A and B and the double stranded AB oligonucleotides. *x, y* and *z* are, respectively, the number of sodium ions bound to the single strands and double strand. *x', y'* and *z'* are the number of water molecules at the single stranded and double stranded DNA. Δn_w, Δn_S, and Δn_{Na^+} are the changes in water molecules, PEG3 molecules and sodium ions in solution ($\Delta n_x = n_{x,f} - n_{x,i}$).

The equilibrium constant of the reaction is given by the following:31

$$K_{eq}=K_{obs}a_w^{\Delta n_w}a_s^{\Delta n_s}a_{Na^+}^{\Delta n_{Na^+}} \quad (2)$$

where K_{eq} is the true equilibrium constant of the pairing reaction, a_w, a_s, and a_{Na+} are the activities of water, PEG3 and sodium ions, respectively. K_{obs}, the observed equilibrium constant of the pairing reaction without the water and cosolute contributions, is obtained from spectroscopy by:30

$$K_{obs}=\frac{[AB]}{[A][B]}=\frac{2\alpha}{(1-\alpha)^2 C_T} \quad (3)$$

where [*A*] and [*B*] are the concentrations of the single strands, [*AB*] the concentration of the duplex, α, determined by spectroscopy, is the fraction of duplex to C_T and C_T is the total single strand concentration.

As observed in previous studies and the CD experiments of the oligomers in this present work, PEG3 does not interact with both the single strands and the double strands; therefore Δn_S is negligible and the activity contribution in Equation 2 is approximated by unity.[31, 35] At constant pressure and temperature conditions, the number of sodium ions added to the DNA strand during pairing can be evaluated from the slope of $\ln K_{obs}$ versus $\ln a_{Na^+}$[65]

$$\frac{d \ln K_{obs}}{d \ln a_{Na^+}} = -\left[\Delta n_w \left(\frac{d \ln a_w}{d \ln a_{Na^+}}\right) + \Delta n_{Na^+}\right] \quad (4)$$

where $\frac{d \ln a_w}{d \ln a_{Na^+}}$ is determined from osmotic pressure measurements of solutions of PEG3 with increasing NaCl content, see Figure S6.

Similarly, at constant sodium activity:

$$\frac{d \ln K_{obs}}{d \ln a_w} = -\Delta n_w \quad (5)$$

To determine the activity of sodium ions, the osmolality of the PEG3-NaCl/buffer solutions was measured by vapor pressure osmometry. Interaction of Na^+ ions with polyethylene glycol chains is only observed when the hydrophobicity of solutions is increased by the addition of organic solvents to the mixtures;[66-67] therefore the actual activities of the sodium ions can be calculated based on the osmometry measurements. Details are presented in the Supporting Information section (see Figures S2-S6 and Tables S2-S5). As a result, it is possible to look at the behavior of the single stranded oligomer pairing as a function of increasing Na^+ activity under different molecular crowding conditions. Figure 6 presents the calculated $\ln K_{abs}$ for the 35-mer versus $\ln a_{Na^+}$ at increasing PEG3 wt% from 0 to 30%.

The pairing equilibrium of DNA is highly dependent on both sodium cation concentration and molecular crowding. As seen in Figure 6 and in Figure S13, K_{obs} increases with increasing salt concentration but the extent to which it changes is dependent on the crowding condition of the solutions which is reflected in the $-\Delta n_{Na^+}$ values in Figure 7.

Counterion binding to polynucleotides is described by condensation theory, where one can calculate the number of counterions bound to the phosphate backbone assuming the structure and charge density on the rod-like DNA.[68-69] The theory predicts 0.88 sodium ion to be bound to a phosphate base in a B-form double stranded DNA and 0.71 sodium ion bound to a phosphate base in the single stranded form of the DNA in non crowded conditions.[70] Accordingly, upon pairing of complimentary single stranded DNAs, 0.17 sodium ion are added to each phosphate group, i.e. 0.34 per phosphate base pair.

When assuming DNA strands as ideal polyelectrolytes, one can use Equation 4 to determine directly the number of sodium ions added to the double strand as the duplex DNA is formed. Record et al.[63] introduced a non-ideality factor where the authors argue that the number of sodium cations bound, $\Delta\psi$, to the strands is twice the number calculated from Equation 4. Thus, $-\Delta n_{Na^+}$ values were multiplied by a factor of two in order to account for the non-ideal polyelectrolyte behavior of the oligonucleotides. Following this logic, for the 15-mer and

25-mer oligomers under “low” molecular crowding (0% - 5% PEG3), the numbers obtained are close to 0.34 ions per base pair as predicted by the counterion condensation theory, while the $\Delta\psi$ values of the 35-mer approach 0.17.

Although nonspecific counterion binding is considered to be independent of chain composition in a double stranded DNA, there is a decrease in $\Delta\psi$ values as the G-C content of the oligonucleotides is increased.70 Blake and Haydock reported the presence of 40% more Na^+ ions at the G and C residues than at the A and T bases in a single stranded DNA.71 The counterion binding per phosphate also decreases with increasing temperature, though not to a great extent.30 Finally, increasing chain length decreases the contribution of end group counterion binding to the averaged $\Delta\psi$ obtained for a base pair. We believe the additive effect of the latter three arguments is an explanation to why the $\Delta\psi$ values for the 35-mer are lower than those seen for the 15-mer and 25-mer.

The decrease in $\Delta\psi$ values with molecular crowding was observed by Spink and Chaires and was correlated to the decreasing water activity upon increasing concentration of ethylene glycol in the DNA solutions.35 To fully understand the decrease in $\Delta\psi$ values it is important to understand whether molecular crowding changes the counterion condensation on both the single stranded and double stranded DNAs.

The CD spectra of the double stranded oligonucleotides are consistent with the B-form helix, hence, according to the Watson-Crick Model of DNA,72-73 the double helical DNA is approximated to a cylinder of diameter 2 nm and a height increment per base pair of 0.34 nm. The change in structure for the 25-mer and the 35-mer oligonucleotides is quite evident and significant as the single stranded coils are transformed to respectively 8.5 nm and 11.9 nm cylindrical duplexes, while the 15-mer adopts a cylindrical height of 5.1 nm. In this case, it is safe to assume the counterions condensing on the double stranded DNA are predicted by condensation theory, since the duplexes are rod like cylinders with equally distributed charge densities on the outer surface. Therefore there are 0.88 sodium ions per phosphate group. On the other hand, the Na^+ condensation on the single stranded DNA depends on the molecular crowding of the solutions and their ionic strengths which alter the conformation of the strands. Nakano et al. studied the effect of molecular crowding on the conformation of double stranded, single stranded, hairpin DNA and single stranded RNA in 1 M NaCl solutions, where the double stranded DNA did not exhibit any change in structure whereas single stranded DNA oligomers exhibit some loss of helical character.74 The increase in molecular crowding of the single stranded DNA solutions forces the oligomers to adopt a more compact conformation relative to the macromolecule-free solutions which requires cations to condense on the phosphate backbone to eliminate charge repulsions. The decrease in $\Delta\psi$ values is therefore a result of the increase of sodium cations on the backbone of the oligonucleotides.

Further evidence of excluded volume effects75-76 is observed in the changes of the equilibrium constants at different ionic strengths and molecular crowding. At $a_{Na^+} \rangle 0.05$, the observed equilibrium constants of the 15-mer decrease, and to a greater extent those of the 25-mer and the 35-mer, with increasing PEG3 wt%. Zhou et al. recently reviewed the effect of molecular crowding on the association equilibrium of species represented as hard spheres with comparable diameter to the molecular crowding compound.75 Based on the available volume method, the authors demonstrated the decrease in the pairing equilibrium of hard spheres transforming to rod-like species with both increasing molecular crowding conditions and increasing length of the rod-like products.

At high salt concentrations and low molecular crowding (PEG3 < 5 wt%), the excluded volume change of pairing reaction of DNA single strands is relatively similar to the volume

change in the absence of molecular crowders, where the conformation of the single strands is not affected by the presence of PEG3. But with increasing molecular crowding the extent of pairing is diminished as more volume change is required to accommodate the double stranded DNA due to the coil-helix transition and the addition of water molecules to the double strands. On the other hand at $a_{Na^+} \langle 0.05$ an increase in K_{obs} is observed as the molecular crowding is increased. We think the favored pairing is a result of increasing hydrophobic forces within the single strands as the activity of water is decreased with the increasing PEG3 content. This latter is supported by the water molecules released from the strands upon pairing which will be described in the following section.

Finally, the water molecules exchanged during the pairing of DNA strands is studied at different sodium ion activities. The number of water molecules is determined using Equation 5 where the slopes of ln K_{obs} versus ln a_w, at constant Na^+ activities and at 5% ≤ $PEG3$ ≤ 30%, reveal an increase in the number of duplex-bound water molecules with increasing sodium ion activity up to 4.5 ± 0.5 molecules per base pair binding at a_{Na^+}=0.4. In contrast, water molecules are released from the DNA at $a_{Na^+} \langle 0.05$ and when extrapolated to salt-free conditions there are −4.0 ± 0.6 water molecules per base pair, independent of the oligomers' nature.

Previously, Spink and Chaires reported the release of four water molecules per base pair upon melting of the double stranded DNA strands,35 which were associated to the water molecules specifically bound to each nucleotide base pair.77 The increasing molecular crowding at low salt concentrations induces hydrophobic interactions within the single stranded DNA oligomers by releasing the water molecules bound to the hydrophobic sites of the nucleotide bases which results in the pairing of the complimentary strands. As discussed earlier at 2mM Na^+, 30 wt% PEG3 was not enough to push forward the equilibrium of pairing. From the plot of $\Delta\psi$ versus PEG wt%, Figure 7, the amount of PEG3 needed to induce the salt independent pairing of the oligonucleotides was estimated by extrapolation of the line fits to $\Delta\psi = 0$: 98%, 65% and 52% PEG3 wt% in the 15-mer, 25-mer and 35-mer DNA solutions respectively.

On the other hand at higher salt concentrations there is a gradual increase in the number of water molecules binding to the DNA strands which seems to level off at Na^+ activities greater than 0.4. In addition the number of bound water molecules, in the absence of A-tracts, appears to be independent of G-C content of the strands, Figure 7, leading us to believe that the addition of the water molecules is either at the phosphate backbone or the sugar bases and not the nucleotides.

CONCLUSIONS

We were able to determine the ionic and solvent contributions in the hybridization of oligonucleotides under molecular crowding conditions through spectroscopic studies of the hypochromic effect in oligonucleotides. Strands with different lengths and G-C content were analyzed to see the contribution of ionic activity in the pairing of the bases and the condensation of ions onto the phosphate backbone of the strands. The addition of ions on the double helical structure of the oligonucleotides decreased as the G-C content and length of the strands increased which is consistent with theoretical predication made through the counterion condensation theory. The effect of molecular crowding was observed through the decrease of ion binding onto the strands as the PEG3 content of the solutions was increased from 0% to 30% by weight which we believe is a result of cation condensation at the single strand due to compacting of the coil conformation with increasing crowding. Lastly, the hydration of the strands depended on the ionic conditions of the DNA solutions. Water

molecules were released into the DNA solutions at low salt concentrations whereas up to 4.5 water molecules were bound per base pair with increasing sodium ion activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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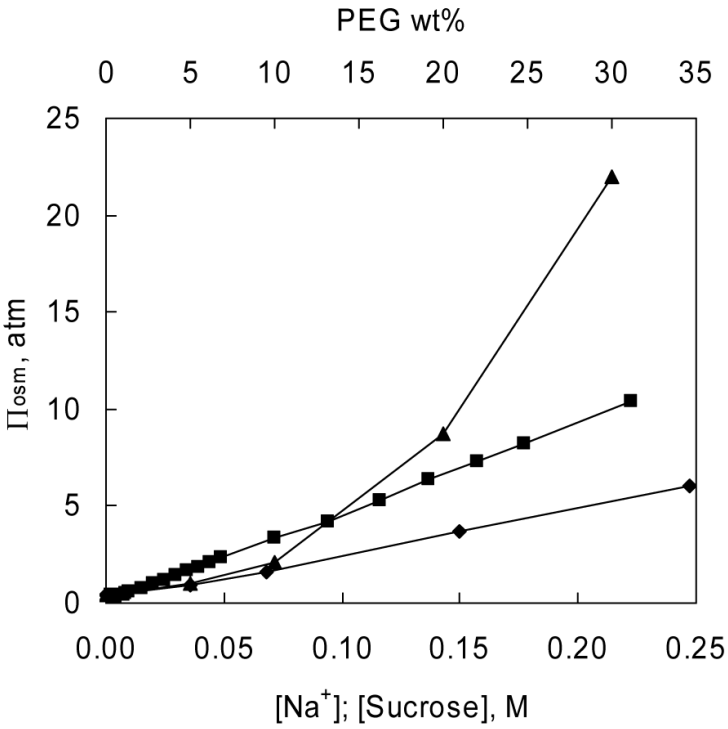

Figure 1.
Osmotic pressures of PEG3 (▲), sucrose (◆), and sodium chloride (■) solutions in 1 mM phosphate buffer @ pH 7.0 and 20 °C determined by vapor pressure osmometry. Sucrose and NaCl correspond to the primary x-axis. PEG data points are to the secondary x-axis.

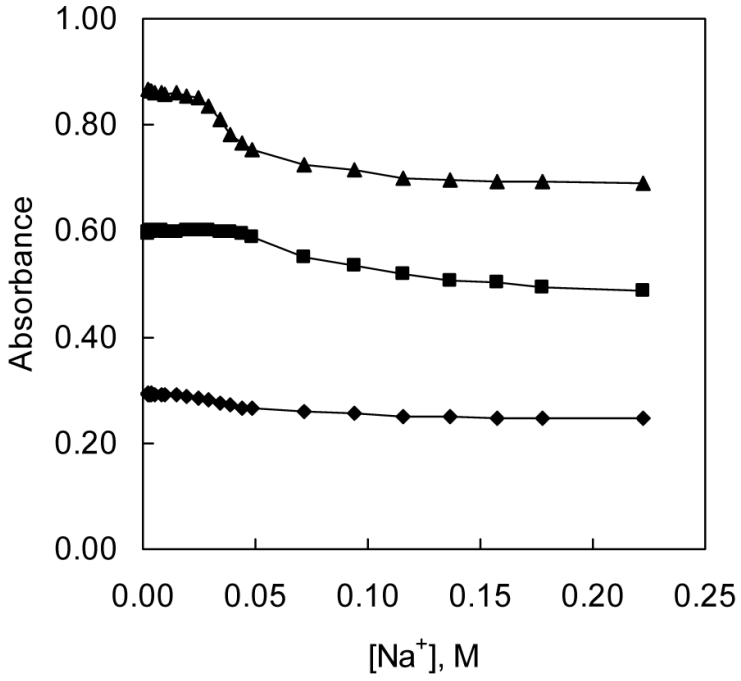

Figure 2.
Hypochromicity of DNA oligomers at 260 nm; 35-mer complimentary strands at 67 °C (▲), 25-mer complimentary strands at 55 °C (■), and 15-mer complimentary strands at 35 °C (◆) in 1 mM phosphate buffer at pH 7.0 with increasing concentration of Na^+ ions, no PEG3 added. Quantitative data on influence of NaCl and/or PEG on the 35-mer were extracted from expanded plots such as Figure 5.

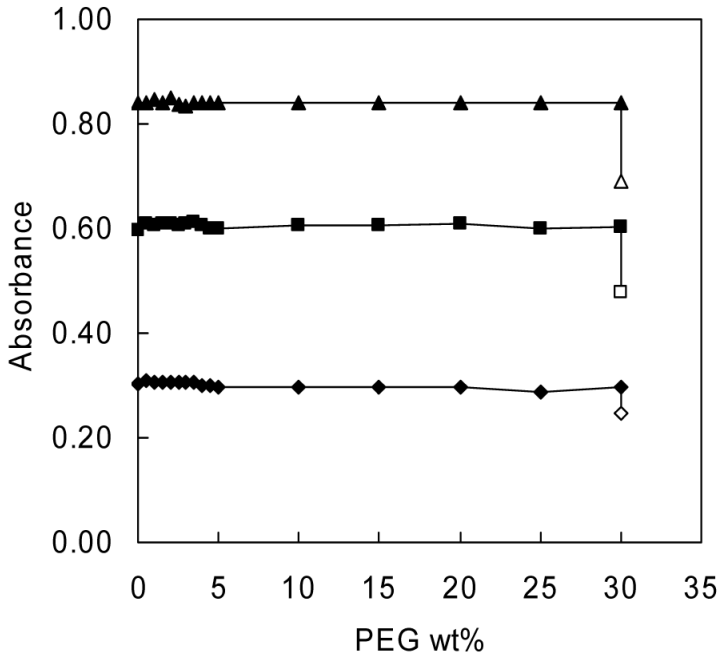

Figure 3.

Hypochromicity of DNA oligomers at 260 nm; 35-mer complimentary strands at 67 °C (▲), 25-mer complimentary strands at 55 °C (■), and 15-mer complimentary strands at 35 °C (◆) with increasing concentration of PEG3 in 1 mM phosphate buffer at pH 7.0 and 2 mM [Na^+]. Open points, △, □, ◇, correspond to the respective absorbance of oligomers at 260 nm after addition of Na^+ to the solution to a final concentration of 0.1 M.

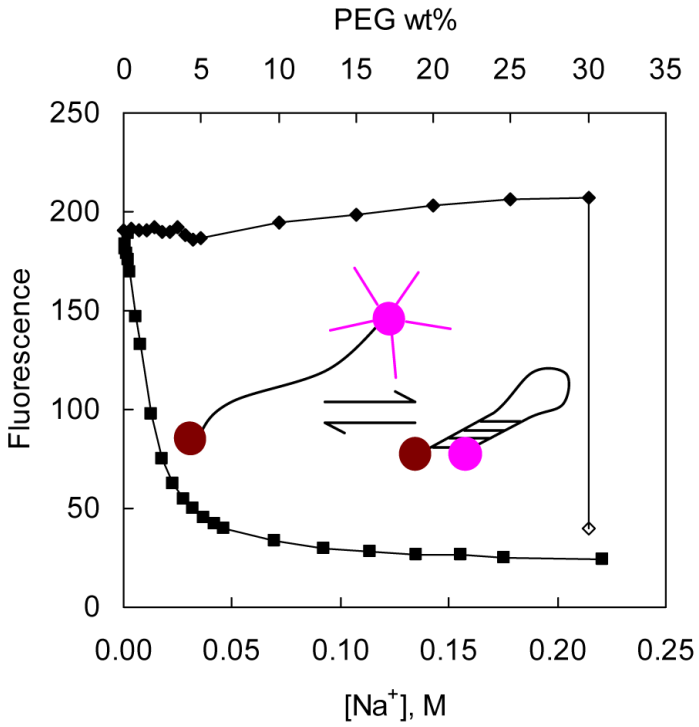

Figure 4.
Fluorescence signal decrease of beacon at 615 nm and at 75 °C under increasing ionic strength of solution with no added PEG3 (■ and primary x-axis); and under increasing osmotic stress at 2 mM Na^+ (◆ and secondary x-axis). The open point at 30 wt% PEG3 corresponds to the addition of NaCl to the solution to a final concentration of Na^+ of 0.125 M. The excitation wavelength was set at 592 nm. The inset is a cartoon depicting the fluorescence quenching observed upon the pairing of the complimentary ends of the hairpin.

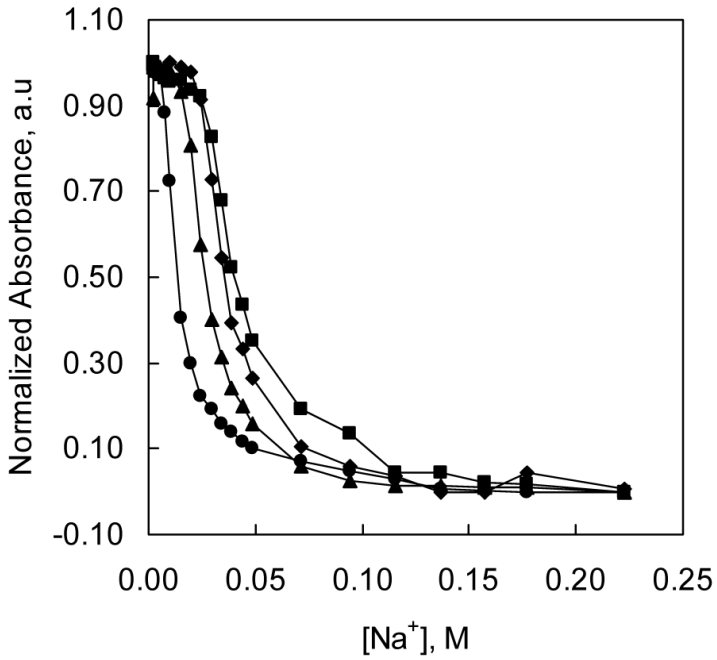

Figure 5.
Hypochromicity of complimentary DNA 35-mers at 260 nm and 67 °C in 1 mM phosphate buffer at pH 7.0 and increasing [NaCl] at: 0 wt% PEG3 (■); 1 wt% PEG3 (◆); 10 wt% PEG3 (▲); and 30 wt% PEG3 (●).

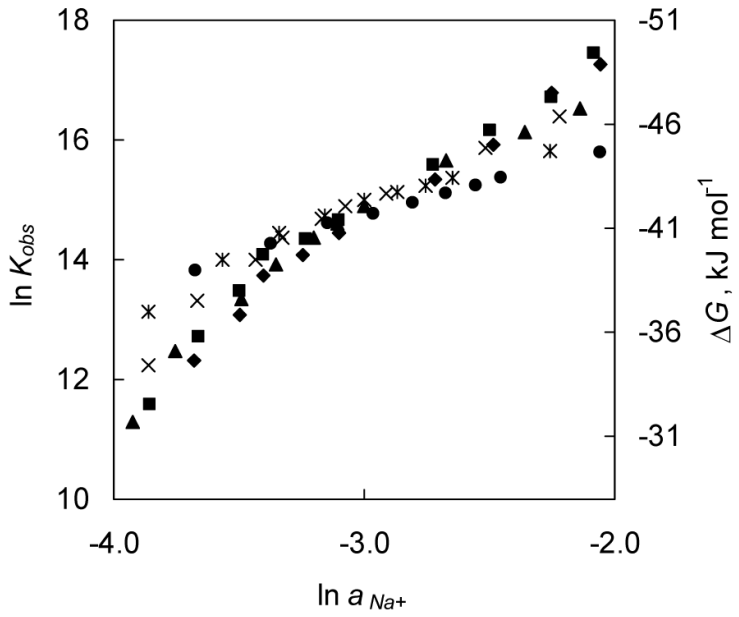

Figure 6.

Plots of ln K_{obs} versus ln a_{Na^+} of the 35-mer oligonucleotide in different molecular crowding conditions. Data points are at 0 wt% PEG3 (◆); 1 wt% PEG3 (■); 5 wt% PEG3 (▲); 10 wt % PEG3 (x); 20 wt% PEG3 (*); and 30 wt% PEG3 (●). The secondary y-axis is the ΔG values in kJ per mole of strand calculated at 67 °C.

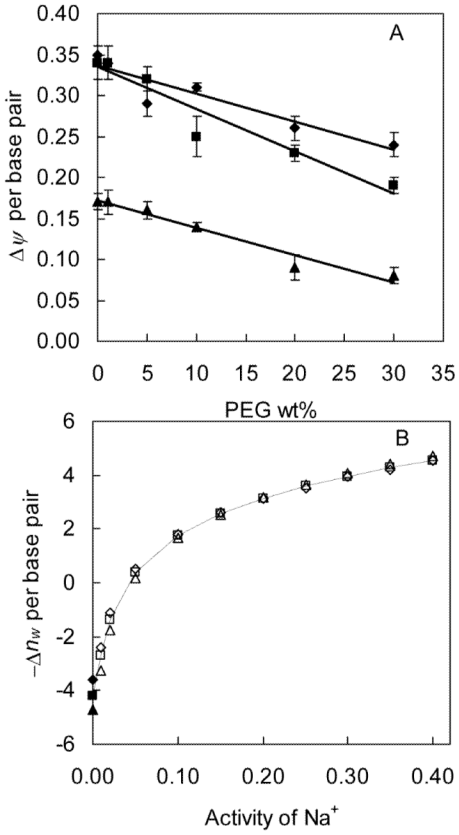

Figure 7.

Panel A: the number of sodium ions added, $\Delta\psi$, to the complimentary strands with increasing molecular crowding conditions. Lines are fits to the data. Panel B: The number of water molecules per base pair either released from, at $a_{Na^+} \langle 0.05$, or bound to, at $a_{Na^+} \rangle 0.05$. The full points at zero Na^+ activity indicate the extrapolated values for water molecules released from each strand. In both panels the points are respectively for, 35-mer @ 67 °C (△,▲); 25-mer @ 55 °C (□,■); 15-mer @ 35 °C (◇,◆)

Table 1

Complimentary DNA oligomers and molecular beacon used.

15-mer	5′-GTA TGA GAG ACT TTA-3′
	3′-CAT ACT CTC TGA AAT-5′
25-mer	5′-GGA TGA ATG ATT ATC TAG TAG TTC A-3′
	3′-CCT ACT TAC TAA TAG ATC ATC AAG T-5′
35-mer	5′-GCT ATG CCC ATT ACG TTC GTC ATG CTT GAC CCA GT-3′
	3′-CGA TAC GGG TAA TGC AAG CAG TAC GAA CTG GGT CA-5′
Beacon[a]	5′-/TR/**ACGGCGAGC**CACCAAATCTACTAA**GCTCGCCGT**/BHQ-2/-3′

[a]The complimentary parts in the beacon are depicted in bold letters.