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Stable DNA Aggregation by Removal of Counterions

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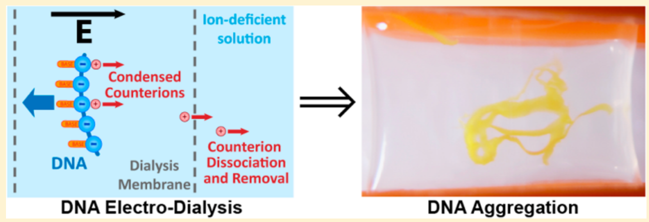
ABSTRACT: Negatively charged DNA can form extremely stable complexes with positively charged ions. These counterions are very difficult to remove from DNA; therefore, little is known about DNA behavior in their deficiency. We investigated whether removal of counterions from the strongly bound counterion layer would elicit any novel DNA properties or behaviors. In order to remove the tightly bound counterions, we used dialysis against deionized water in the presence of a strong (0.6 kV/cm) electric field. The electric field promoted the dissociation of the DNA−counterion complexes, while dialysis facilitated irreversible partitioning of counterions and DNA. Counterintuitively, when deprived of counterions, DNA precipitated from the solution into amorphous aggregates. The aggregates remained stable even when the electric field was turned off but readily redissolved when counterions were reintroduced. The phenomenon is likely explained by attraction of like-charged DNA polyions due to entropic-stabilization of condensed counterion layers.



DNA is an essential molecule of life. It is a biological polymer that bears a high density of negative charge due to ionization of phosphate groups in its backbone. In aqueous solutions, negatively charged DNA is always surrounded by an atmosphere of positively charged counterions. Their presence has an immense influence on properties and function of DNA. Counterions play an important role in the formation of secondary and tertiary structures of DNA, alter solubility and elasticity of DNA polymer chains, and modulate interactions of DNA with other molecules.[1−4] DNA counterions also facilitate the formation of biologically important DNA structures such as the four-way junction and the telomeric G-quadruplex.[5,6] In fact, the formation of a stable DNA double helix is only possible through neutralization of electrostatic repulsion between DNA strands by counterions.[7] Interaction of DNA with counterions can also cause structured DNA condensation and can influence DNA compaction by histones.[8,9] Counterion concentration and composition are commonly manipulated in order to fine-tune melting temperatures of DNA duplexes in PCR and hybridization assays.[10−12] Addition of counterions is a critical step in purification and concentration of DNA through ethanol precipitation.[13] Development of DNA-based nanostructures and nanocircuitry also heavily depends upon our understanding of counterion effects on DNA conformation and behavior.[14,15]

While the behavior of DNA in the context of excess counterions is well studied, very little is known about properties of DNA under severe counterion deficiency. Significant experimental and theoretical evidence suggests that, even when the solvent is deficient in counterions (e.g., deionized water), a stable layer of counterions (known as a condensed layer of counterions) remains bound to DNA.[16−19] The formation of the condensed layer is driven by the polyionic nature of DNA. It has been shown that the native charge density of DNA polyions is so high, that a counterion entering their vicinity would be unable to escape through thermal energy alone.[20,21] As a result, conventional deionization methods based on filtration, precipitation, or passive dialysis may not be able to remove condensed counterions. As such, condensed counterions are most likely carried over between samples along with DNA molecules. Furthermore, given the fact that DNA is always exposed to counterions during its synthesis, either in cells or in vitro, it can be argued that DNA−counterion complexes are present even in solutions of extremely low ionic strength. However, the theory suggests that an applied electric field can promote dissociation of this stable layer of ions from DNA.[22] Recently, we have shown that electric-field facilitated dissociation of condensed counterions occurs during DNA capillary electrophoresis, having a profound effect on electrophoretic mobility of DNA.[23] The goal of this work was to investigate whether the removal of counterions from the condensed layer would elicit any novel DNA properties or behaviors.

■ RESULTS AND DISCUSSION

We designed a simple procedure in which condensed counterions could be first dissociated from DNA by a high-strength electric field and, then, to prevent recondensation, could be permanently removed from solution by dialysis. An experimental setup is schematically shown in Figure 1A. A commercially synthesized and desalted DNA sample was dissolved in deionized water and placed into a semipermeable (transparent for small ions but nontransparent for DNA) dialysis bag (Figure 1B). The bag was, in turn, placed into an electroblotting chamber which

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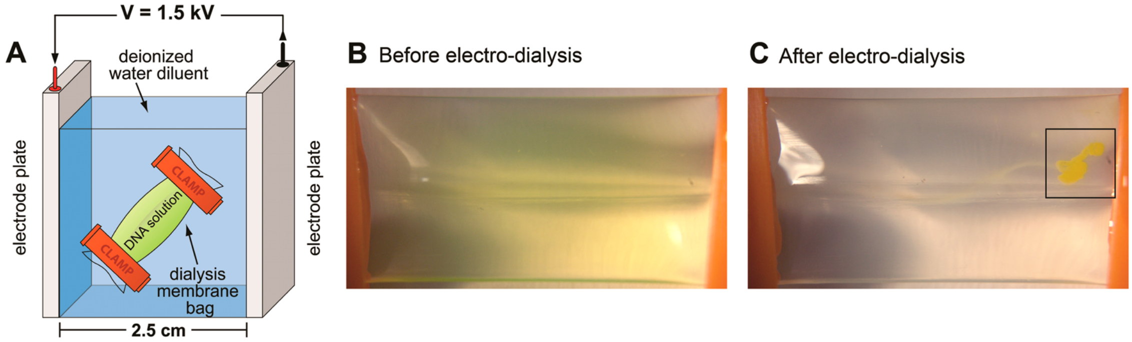

Figure 1. Precipitation of DNA by electrodialysis performed in a setup schematically depicted in panel A. The photographs at the right show 80-nt synthetic ssDNA in deionized water inside a semipermeable membrane bag before (B) and after (C) electrodialysis at 600 V/cm for a total of 5 min. The green color of the DNA solution and the yellow color of the DNA precipitate are due to the presence of fluorescein label on the DNA .The aggregate first forms at the inner side of the membrane closest to the positive electrode. The aggregate easily detaches from the membrane and tends to sink down in the dialysis bag by gravity.

incorporated two electrode plates capable of creating a uniform electric field across the chamber. The chamber was filled with deionized water to act as a dialysis diluent. The absence of salts in the sample and diluent allowed us to use a very high electric field without overheating the setup. Several 1 min long pulses of a constant electric field of 600 V/cm were then applied across the dialysis bag. The diluent was replaced with fresh deionized water after each pulse to ensure efficient removal of dissociated counterions. To our surprise, after just five 1 min pulses of the electric field we observed a previously unknown phenomenon: DNA precipitated out of solution (Figure 1C). The presence of the electric field was crucial: dialysis against deionized water at zero field strength did not produce any DNA precipitate even after several hours. Interestingly, electrodialysis of DNA samples has been previously performed by other groups; however, no DNA precipitation was observed in those experiments.[24,25] The reason, most likely, was the use of ion-containing dialysis diluents, which would have prevented the establishment of the required counterion deficiency.

With further examination, the obtained DNA precipitates displayed some remarkable properties. The precipitates were stable and did not redissolve after the electric field was turned off. Furthermore, the DNA precipitates remained insoluble after they were transferred into a fresh volume of deionized water, even after vigorous mixing and 24 h incubation. However, the precipitates did readily redissolve (within 1 min) when placed into a buffer solution or a salt-containing solution (Figure 2).

Using the developed electroprecipitation procedure, well-visible aggregates were formed after only 5 min in the electric field and 85% of the DNA precipitated within 20 min (Figure 3). The aggregates formed as an amorphous structure at the membrane wall closest to the positive electrode. The aggregates easily detached from the membrane and slowly sunk due to gravity. The pH values of the original solutions inside and outside of the dialysis bag were 3 and 6, respectively, and did not change significantly after the electrodialysis. No precipitation has occurred in control experiments in which the DNA solution was replaced with either deionized water or a solution of bovine serum albumin (BSA). Agarose gel electrophoresis of the buffer-dissolved precipitate showed a single band, with fluorescent properties and a migration pattern identical to those of the original DNA sample (Figure 3). This result suggests that electroprecipitation did not affect DNA integrity. Different types of DNA were successfully electroprecipitated, including several fluorescently labeled single-stranded DNA (ssDNA) of different lengths and nucleotide sequences, nonlabeled ssDNA, double-stranded DNA of various lengths from herring sperm extract, and purified plasmid DNA.

In the past, transient DNA aggregation under the influence of a strong electric field has been observed in elegant experiments by

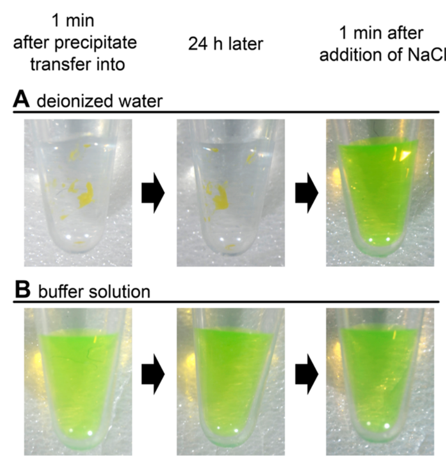

Figure 2. Ion-dependent solubility of electroprecipitated DNA. Photographs of two separate DNA precipitates after being transferred into deionized water (A) and 50 mM Tris-acetate buffer solution at pH 8.3 (B). The photographs were made 1 min and 24 h after the transfer and 1 min after subsequent addition of 1 mM NaCl. The samples were agitated by thoroughly vortexing them after the precipitate transfer and after the NaCl addition. The yellow color of the DNA precipitate and the green color of the DNA solution are due to a fluorescein label on the DNA.

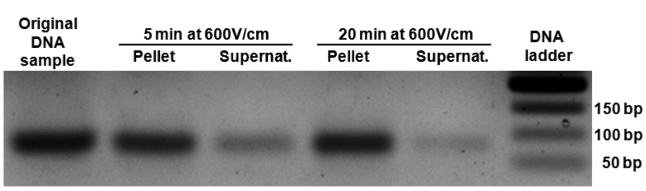

Figure 3. Agarose gel electrophoresis analysis of DNA integrity after electroprecipitation. From left to right the samples are: the original solution of 80-nt ssDNA, the precipitate (redissolved in 50 mM Tris-acetate buffer), and the supernatant after a 5 and 20 min electrodialysis against deionized water at 600 V/cm. The rightmost lane shows the migration of DNA molecular weight standards. DNA was imaged through fluorescence detection of fluorescein label on ssDNA and through ethidium bromide staining of the standards.

Maestre et al., and later by Viovy et al. and Doyle et al.[26−28] DNA aggregation occurred only in solutions with low ionic concentrations and at electric-field strengths above a certain threshold. In contrast to our precipitate, the aggregates observed by Maestre, Viovy, and Doyle were only stable in the presence of the electric field and spontaneously dissociated upon its removal. Most likely, the instability of the aggregates was due to reassociation of DNA with counterions which could not be permanently removed in the absence of dialysis. Viovy and Doyle proposed mechanisms for the formation of aggregates that involved redistribution of counterions along DNA molecules,[27−30] but these mechanisms do not explain the stability of aggregates observed by us.

If viewed purely through simple electrostatic consideration, our results are perplexing and counterintuitive. DNA counterions reduce electrostatic repulsion between DNA molecules by screening the negative charges in their backbones.[31] Therefore, their removal should increase repulsion between DNA molecules by intensifying the charge density of individual chains. Accordingly, aggregation of DNA is not intuitively expected under the condition of counterion deficiency. To explain the observed phenomenon, a more comprehensive consideration of counterion theory is required.

Counterion condensation theory was originally described by Oosawa and Manning several decades ago.[18,19] The theory describes the existence of two distinct subpopulations of DNA counterions: diffusely bound and condensed. Diffusely bound counterions behave as a gas-like cloud, separated from DNA by the entire Debye sphere. Condensed counterions, on the other hand, are much more closely associated with DNA. They occupy a rigid volume within the thickness of the first few shells of DNA-hydrating water molecules. The formation of these exceptionally stable counterion complexes is driven by the necessity to reduce the charge density of DNA below a certain threshold value. In solutions with severe counterion deficiency, when the diffusely bound counterion cloud is sparse and its charge-screening effects are low, the condensed counterion layers of two neighboring DNA molecules may exert an influence upon each other. Interestingly, calculations show that this mutual influence of condensed counterion layers may result in attraction between two DNA molecules.[32,33] The precise molecular-level mechanism of this short-range nonelectrostatic interaction is yet unknown, but it likely involves entropic stabilization of the DNA−counterion systems through increasing the volume available to condensed counterions. This theory has been proposed to explain the formation of fluid polyion clusters observed in low-salt solutions.[34] Our observations seem to be consistent with this theory. In our electrodialysis experiments, severe counterion deficiency was established to provide the required depletion of the diffusely bound cloud. Furthermore, the electrophoretic movement of DNA increases its local concentration at the dialysis membrane, making it more likely for DNA molecules to come sufficiently close for the short-range forces to take effect. Polarization and reorientation of DNA-condensed counterion complexes in the electric field may also play a role in facilitating the formation of the entropy-stabilized attractive forces. The remarkable stability of the aggregates in our experiments, however, suggests that additional physical phenomena may be involved. We have recently reported that the stably bound condensed counterions dissociate from DNA during capillary electrophoresis experiments, under the influence of an electric field of a comparable magnitude.[23] Combined with the currently reported strong dependence of aggregate solubility on the addition of external ions, it may be suggested that a partial depletion of the condensed counterion layer also takes place during electrodialysis. It is not clear what happens with DNA if its charge density grows beyond its threshold value, but perhaps, the observed aggregation is one of its manifestations. In either case, proving any of the available hypotheses requires a more detailed study of the reported phenomenon and a comprehensive analysis through molecular-dynamics simulations.

■ CONCLUSIONS

We describe a previously unreported phenomenon, in which DNA precipitated from the solution into amorphous aggregates as a result of electrodialysis against deionized water. The observed aggregates remained stable without the electric field but readily redissolved when counterions were reintroduced. The phenomenon is likely explained by attraction of like-charge DNA polyions due to interaction between condensed counterion layers. As such, our observations provide the first solid support for this theoretically predicted phenomenon. Further study of the phenomenon and its comprehensive analysis within the scope of the counterion theory is required to confirm or disprove the hypothesis.

■ MATERIALS AND METHODS

Reagents. All chemicals were purchased from Sigma-Aldrich (Oakville, ON) unless stated otherwise. The deionized water was freshly produced by a Millipore Milli-Q UV Plus instrument and had electrical resistance of ∼18 MΩ. Synthetic DNA was manufactured, desalted, and purified by Integrated DNA Technologies (Coralville, IA). All DNA was received as lyophilized pellets and resuspended in deionized water; no extra ions were added. A NanoDrop 1000 spectrometer (Thermo Scientific, Wilmington, DE) was used to verify DNA concentrations by measuring light absorbance at 260 nm. DNA concentrations were calculated on the basis of manufacturer-provided extinction coefficients. Unless stated otherwise, in all experiments, a fluorescein-labeled single-stranded (ss)DNA molecule was used, with nucleotide sequence of 5′-Fluorescein-CTT CTG CCC GCC TCC TTC CTG GTA AAG TCA TTA ATA GGT GTG GGG TGC CGG GCA TTT CGG AGA CGA GAT AGG CGG ACA CT-3′. In addition to this DNA molecule, electrodialysis was also performed with double-stranded (ds)DNA extract from herring sperm (Sigma-Aldrich), circular plasmid DNA purified from bacteria by QIAGEN Midiprep Kit, an AlexaFluor 488-labeled ssDNA molecule (5′-Alexa488-CTC CTC TGA CTG TAA CCA CGT GCC TAG CGT TTC ATT GTC CCT TCT TAT TAG GTG ATA ATA GCA TAG GTA GTC CAG AAG CC-3′), a nonlabeled ssDNA molecule (5′-GGT GGT GGT GGT GGT GGT GTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT GGT GGG TGG GTG GGT GG-3′), and a synthetic ssDNA library (5′-Fluorescein-CTT CTG CCC GCC TCC TTC CT-(N40)-AGA CGA GAT AGG CGG ACA CT-3′).

DNA Electrodialysis. For each dialysis experiment, 100 μL of a 50 μM DNA solution was prepared using deionized water. Three-cm-long portions of Spectra/Por 6 dialysis membrane bags (Spectrum Laboratories Inc., Rancho Dominguez, CA) with a molecular weight cutoff value of 25 kDa were used in all experiments. Prior to dialysis, the membrane bags were soaked in deionized water for 30 min and thoroughly rinsed by deionized water. The DNA solution was then transferred into the dialysis bag and clamped off at both ends, ensuring that no air bubbles were trapped inside the bag. For a passive dialysis experiment (with zero electric field strength), the membrane bag was placed into 500 mL of deionized water and incubated for 8 h. The diluent water was exchanged every hour, for a total of 8 times. After incubation, the solution was inspected for the presence of DNA precipitates. For electrodialysis experiments, the membrane bag with a DNA solution was placed into a Minive Blotter chamber (Amersham-GE Healthcare, Baie d'Urfe, QC) containing 300 mL of deionized water. A 1 min pulse of a 600 V/cm constant electric field was applied across the blotter chamber, after which the diluent water was exchanged. This procedure was repeated up to 20 times, with visible DNA precipitates usually appearing after 5−7 times. The precipitates were then picked up from the membrane bag using a micropipet tip and transferred into a test tube that contained 100 μL of either deionized water or 50 mM Tris-acetate buffer at pH 8.3. Control electrodialysis experiments were performed with deionized water and 1 mg/mL of BSA solution instead of the DNA solution. The pH of the solutions was determined by depositing small drops of sample onto Alkacid Test litmus paper ribbon (Fisher Scientific, Pittsburgh, PA, USA).

Solubility of DNA Aggregates. One hundred microliters of 50 μM fluorescein-labeled ssDNA solution was prepared and split equally between two dialysis bags to test the influence of counterions on the solubility of DNA precipitate (shown in Figure 2). The samples were concurrently subjected to electrodialysis in the same electroblotting chamber. Each of the formed precipitates was transferred into a separate vial containing 50 μL of either 50 mM Tris-acetate buffer solution at pH 8.3 or deionized water and thoroughly vortexed. The first set of photographs of the test tubes was taken 1 min after the transfer. The samples were further incubated for an additional 24 h at room temperature, and the second set of photographs was taken. Finally, 1 μL of 50 mM NaCl solution was added to each sample, to a final concentration of 1 mM of NaCl. Samples were thoroughly mixed by pipetting and photographed for the third time.

DNA Integrity. DNA integrity experiments were performed with two identical 100 μL aliquots of 50 μM DNA. Electrodialysis was concurrently performed with both DNA samples for five, 1 min pulses of a 600 V/cm electric field. At that point, one of the samples was removed from the electrodialysis chamber, and 15 additional 1 min cycles of electrodialysis were performed with the remaining sample. The precipitates from both samples were transferred into new vials, both containing 100 μL of 50 mM Tris-acetate buffer at pH 8.3. The supernatants that remained after electrodialysis were also collected. Samples of the original (nondialyzed) DNA solution, both redissolved precipitates, and their supernatants were diluted 100 times and loaded onto a 2.2% agarose gel. Electrophoresis was performed for 30 min at 100 V. DNA Molecular weight standards were visualized through ethidium bromide staining, while the ssDNA was visualized through fluorescein labeling. DNA solution, that was used for the 20 min electrodialysis experiment and redissolved in 50 mM Tris-acetate buffer at pH 8.3, was subjected to fluorescence measurements by the Nanodrop 3300 fluorometer (Thermo Scientific, Wilmington, DE) before and after the electrodialysis procedure to measure the efficiency of aggregate formation.

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Notes

The authors declare no competing financial interest.

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