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Influence of Salt Concentration on the Assembly of DNA Multilayer Films

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DNA multilayer films are promising candidates for a plethora of applications, including sensing, diagnostics, and drug/gene delivery. Fabricated solely from DNA, the use of salt in forming DNA multilayers is crucial in promoting and maintaining hybridization of complementary base pairs by minimizing the repulsive forces between the oligonucleotides and preventing disassembly of the layers once formed. Herein, we examine the role of salt on the assembly of DNA films assembled from oligonucleotides composed of two homopolymeric diblocks (polyA_nG_n and polyT_nC_n) in salt concentrations ranging from 0.1 to 2 M. Using quartz crystal microgravimetry (QCM) and flow cytometry, we show that films assembled at high salt concentrations (2 M salt) exhibit a different morphology and are denser than those assembled from lower (1 M salt) salt solutions. Formation of the T·A*T triplex in solution and within the DNA film was also studied using circular dichroism (CD) and QCM, respectively. DNA films assembled using oligonucleotides of various lengths (20- to 60-mer) at high salt concentration (2 M salt) showed no significant influence on the film growth. This work shows that salt plays an important role in the assembly and final morphology of DNA multilayer films, hence enabling films with different properties to be tailored.

Introduction

DNA is a versatile building block for the construction of materials where control over the film properties such as structure, stability, and responsiveness in the physiological range is required. We have previously shown that films fabricated solely from synthetic DNA[1,2] can be engineered using a bottom-up approach known as the layer-by-layer (LbL) technique.[3,4] The use of different building blocks, altering the number of layers within the film, or varying the assembly conditions allows fine control over the properties of LbL thin films.[5−7] In contrast to electrostatically assembled polyelectrolyte multilayer films, the assembly of DNA multilayer films exploit the highly specific interaction between the complementary base pairs. The hybridization between complementary strands, which occurs in an antiparallel direction through the formation of two and three hydrogen bonds between T = A and C≡G base pairs,[8] enables greater control over the directionality of the strands within the film. This cannot be achieved in conventional (non-DNA) polyelectrolyte multilayer systems.

We have previously shown that the thickness and shrinkage or swellability of DNA multilayer films can be controlled through careful design of the strand sequence.[1,9] The stability of these films can also be manipulated by varying the length and terminating layer of the DNA building blocks[10] or by cross-linking the preformed films with oligonucleotides to attain greater film stability.[11] Another key parameter for the successful assembly of DNA multilayer films is minimization of the repulsive forces between the double-stranded oligonucleotides from which the films are assembled. This enables the oligonucleotides to come into close contact for hybridization by reducing repulsion between the layers, which can cause the film to disassemble. One way to overcome the repulsive forces between the DNA strands is through the use of salt in the deposition solution to shield the charges on the DNA phosphate backbones. Salt has been utilized in conventional polyelectrolyte LbL films to induce structural changes within the films.[12−14] The main effect of salt is to screen the electrostatic charges. Apart from reducing the repulsion between like-charged polyelectrolytes, salt can also change the polyelectrolyte conformation from an extended to a more coiled conformation.[15] As a result, thicker polyelectrolyte films are obtained due to the extrinsically compensated charges on the layers swollen from an increase in salt ions and water.[13] Salt has a similar effect on DNA, screening the negative charges on the phosphate backbone and thus minimizing repulsion between neighboring strands and increasing the probability of hybridization between complementary strands. Analogous to conventional polyelectrolytes,[16] DNA also "relaxes" in the presence of salt, and the persistence length of single-stranded DNA can vary from 3 nm under low salt (0.25 M NaCl) to 1.5 nm under high salt (2 M NaCl) conditions.[17] However, DNA has a tendency to form ordered structures in the presence of salt.

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It is well-known that while synthetic homooligonucleotides (e.g., polyG, polyA, and polyC) can exhibit a variety of conformations such as duplexes or quadruplexes under different solvent conditions, polyT does not exhibit any ordered conformation and adopts a single helix with its bases turned "out".[8] PolyG strands can self-assemble into highly stable tetraplexes, formed when stacks of four guanines associate via hydrogen bonding in a planar and cyclic arrangement. A tetraplex can be formed from (1) four individual guanine-rich strands,[18] (2) the intramolecular folding of a single guanine-rich strand,[19] or (3) the dimerization of two guanine-stabilized hairpin loops.[20] These structures are particularly stable in the presence of a cation such as potassium or sodium, which fills the void in the core of the four stranded structure lined with electronegative carbonyl oxygens.[21] Conversely, protonation of the adenine and cytidines below their respective pK_a value of 4 and 4.5 can lead to the formation of a double helix.[8] Thus, polyA adopts a single helix at neutral or alkaline pH but transforms into a double helix below pH 4. The pK_a value of the polyC strand, however, is different from that of the cytidines and can range between 5.7 and 7.[22,23] The electrostatic attraction between the positively charged bases and the negatively charged phosphates contributes to the stabilization of the double helices.[22] PolyA and polyC acidic helices are, however, destabilized at increasing ionic strengths.[22−24]

The presence of salt can also drive the assembly of secondary structures such as triplexes. There are two main classes of triplexes that can form: a YR*Y triplex consisting of one purine (R) and two pyrimidines (Y) and a YR*R triplex consisting of two purines and one pyrimidine.[25] The YR*Y ($C\cdot G^*C^+$ or $T\cdot A^*T$) triplexes form when the third strand is located in the major groove of the duplex by forming Hoogsteen hydrogen bonds with the purine strand of the duplex. Formation of the $C\cdot G^*C^+$ requires protonation of the third pyrimidine (C) strand and occurs under acidic conditions.[26] The presence of salt, however, decreases the pK_a of the cytosines within the triplex and hence has a destabilizing effect on the $C\cdot G^*C^+$ triplex.[27] In the case of the $T\cdot A^*T$ triplex, a higher countercation concentration provides better shielding of the electronegative charges on the phosphate backbones and thus stabilizes the $T\cdot A^*T$ triplex.[28] Conversely, the YR*R triplexes can only form in the presence of multivalent ions.[29] Hence, given the absence of multivalent ions and the slightly acidic pH in our experimental conditions (see later), we will only consider the formation of the $T\cdot A^*T$ triplexes in the DNA films. The use of salt at different concentrations can provide a means to control the formation of these ordered structures during fabrication of the DNA multilayer film. These ordered structures can be used to impart greater stability in the films that can improve the properties of the DNA films, which are known to be loosely bound and highly permeable.[1]

In this study, the growth and viscoelasticity of a 30-mer film assembled at different salt concentrations (0.1−2 M) were investigated on both planar and colloidal supports using quartz crystal microgravimetry (QCM) and fluorescence studies, respectively. The formation of the $T\cdot A^*T$ triplex was examined in both solution and for the first time, within the DNA multilayer film, using circular dichroism and QCM studies, respectively. CD provides a convenient means to assess conformational changes in the oligonucleotides as a function of ionic strength and other parameters. Oligonucleotides ranging from 10 to 60 bases long were also used to construct DNA films at 2 M salt to study the effect of oligonucleotide length on the assembly of the films at high salt concentration.

Experimental Section

Materials. Sodium chloride (NaCl), citric acid, sodium hydroxide, 3-aminopropyltrimethoxysilane (APS), absolute ethanol, and ammonia solution (28 wt %) were obtained from Sigma-Aldrich and used as received. High-purity water of resistivity greater than 18 MΩ cm was obtained from an inline Millipore RiOs/Origin system (Milli-Q water). A stock solution of saline sodium citrate (SSC) buffer was made up with 24.0 g of citric acid and 73.0 g of sodium chloride in 500 mL of water to give a final concentration of 2.5 M sodium chloride and 0.25 M sodium citrate (Na^+ concentration of 3.25 M). The pH of the buffer solution was measured with a Mettler-Toledo MP220 pH meter and adjusted to pH 6.5 using 1 M sodium hydroxide. SSC buffer solutions with salt concentrations of 0.1, 0.3, 0.5, 1, and 2 M were prepared by diluting the stock SSC buffer with water. All of the oligonucleotides used, including the 5′-end tetramethyl-6-carboxyrhodamine-labeled (TAMRA) poly$A_{15}G_{15}$, abbreviated as poly-$A_{15}G_{15}$-TAM, were custom synthesized by Geneworks (Adelaide, Australia). The synthesized oligonucleotides were rehydrated in Milli-Q water to give a stock concentration of 150 μM. The absorbance at 260 nm (A_{260}) of the oligonucleotide solutions were recorded using an Agilent UV−Vis 8453 spectrophotometer, and the concentration was calculated using the formula: oligonucleotide concentration = $A_{260}/\varepsilon_{260}$, where ε_{260} is the extinction coefficient of the single-stranded oligonucleotide. The stock oligonucleotide solution was diluted in SSC buffer to the concentrations listed in Table S1 (Supporting Information) as required for each experiment. Silica particles ($1.05 \pm 0.04\,\mu$m) were obtained from Microparticles GmbH (Umwelttechnologie-Zentrum, Germany) and amine-functionalized using the procedure described in the Methods section. Standard 5 MHz gold-coated AT-cut quartz crystals (Q-Sense AB, Västra Frölunda, Sweden) were cleaned with Piranha solution (70/30 v/v% sulfuric acid/hydrogen peroxide) and dried under a nitrogen stream. *Caution! Piranha solution is highly corrosive. Extreme care should be taken when handling piranha solution, and only small quantities should be prepared.* All gold crystals were treated for 10 min in a UV-ozone TipCleaner (Bioforce NanoSciences, Inc.) to remove any remaining contaminants prior to the experiments.

Methods. *Quartz Crystal Microgravimetry*. QCM measurements were carried out on a Q-Sense E4 instrument (Q-Sense AB, Västra Frölunda, Sweden) at a constant temperature of 23.8 °C throughout all experiments. All overtones measured (1st, 3rd, 7th, 11th, and 13th) showed the same trend; however, we report only the fifth overtone frequency and dissipation values in this paper. The large dissipation changes observed during the film assembly indicate a very soft film, which invalidate the assumptions behind the Sauerbrey equation.[30] Hence, the film buildup is qualitatively reported as a frequency change rather than as an absolute mass. The 30-mer film was assembled under the following SSC concentrations: 0.1, 0.3, 0.5, 1, and 2 M. DNA

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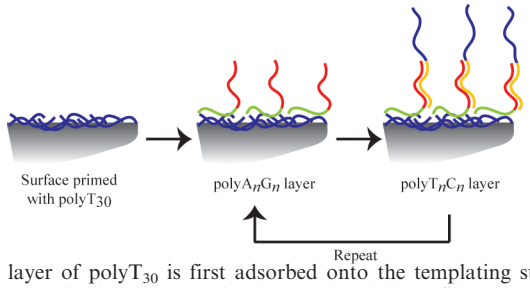
Scheme 1. DNA Multilayer Film Assembly[a]



[a] A layer of $polyT_{30}$ is first adsorbed onto the templating surface (planar or colloidal). To assemble the DNA multilayer film, alternating layers of $polyA_nG_n$ and $polyT_nC_n$ (where n refers to the number of A, G, C, or T bases) are successively deposited onto the surface. The scheme does not represent the actual orientations of the DNA strands.

films constructed from various lengths (10-, 20-, 30-, 40-, and 60-mer) were also assembled under 2 M salt concentrations. Typically, a DNA film was assembled by first adsorbing a priming layer of $polyT_{30}$ (225 μL of a 5 μM solution for 10 min) onto the gold-coated quartz crystal electrodes. To construct the multilayer film, alternating layers of $polyA_nG_n$ (225 μL of a 150 μM nucleotide solution (i.e., 5 and 15 μM for a 30-mer and 10-mer, respectively) for 20 min) and $polyT_nC_n$ (225 μL of a 150 μM nucleotide solution for 20 min) were successively deposited to a total of nine layers (including the $polyT_{30}$ layer), where n refers to the number of bases (Scheme 1). To ascertain whether T·A*T triplexes formed in the film, a layer of $polyA_{15}G_{15}$ was first deposited on a $polyT_{30}$ layer. This was followed by the deposition of either a $polyT_{15}$ or a $polyT_{15}C_{15}$ layer to form a T·A*T triplex. $PolyA_{15}$ was subsequently introduced over each film to examine whether the formed T·A*T triplex would be displaced. To verify whether T could associate with G, $polyC_{15}$ was first deposited on the $polyA_{15}G_{15}$ layer followed by a $polyT_{15}$ layer, which could then form a T·A*T triplex. Each film was rinsed in 1 mL of SSC buffer between each adsorption step to remove any loosely bound oligonucleotides. All solutions were flowed over the crystal sensor surface at a constant flow rate of 300 μL min^{-1}. The raw data were analyzed using the QTools 3.0.0.175 software.

Layer-by-Layer (LbL) Assembly on Silica Particles. Silica particles were amine-functionalized using the following procedure. 100 μL of a 5 wt % particle suspension was washed once in Milli-Q water. The particles were incubated in a mixture comprising 2 mL of ethanol, 400 μL of APS, and 100 μL of 28 wt % ammonia solution for 4 h. The silica particles were washed once in ethanol and three times in Milli-Q water to rinse off any unreacted chemicals before storing in 100 μL of Milli-Q water. The DNA multilayer films were fabricated using the general LbL method on silica particles were as follows. A priming layer of $polyT_{30}$ (280 μL of a 5 μM solution) was first deposited on the amine-functionalized silica particles (50 μL of 5 wt %). The films were constructed by coating the particles with alternating layers of $polyA_{15}G_{15}$-TAM and $polyT_{15}C_{15}$ (Scheme 1). For each layer deposition, the particles were incubated in 280 μL of a 5 μM DNA (150 μM nucleotide) solution for 20 min, followed by washing in SSC buffer to remove any excess DNA. A proportion of the particles were removed after deposition of each layer for analysis, and the amount of DNA used for subsequent layers was reduced proportionally to the remaining amount of particles. The average fluorescence intensity of the particles on the addition of every DNA layer was measured using a Partec FloMax space flow cytometer.[31] Analysis of all flow cytometry data was performed using the Partec FloMax software. The fluorescence intensity of the particles after the addition of each layer was taken at the different salt concentrations (0.1, 0.3, 0.5, 1, and 2 M SSC).

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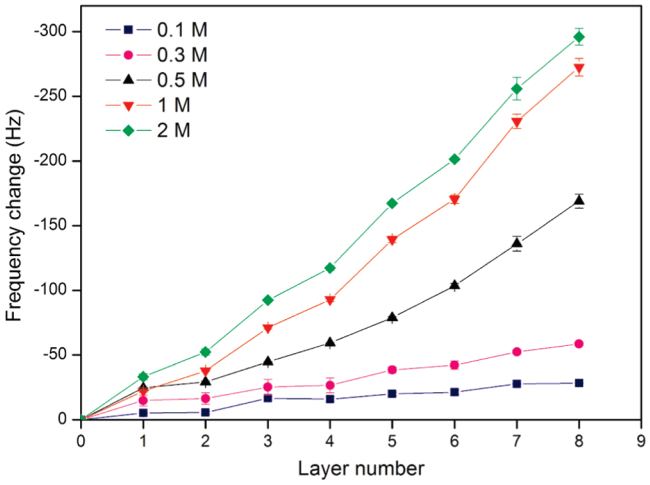

Figure 1. Influence of salt concentration on the growth of the DNA multilayer films. Layer 0 corresponds to the T_{30} precursor layer; odd and even layers correspond to the $A_{15}G_{15}$ and $T_{15}C_{15}$ layers, respectively.

Circular Dichroism Spectroscopy. The CD spectra were obtained using a Jasco J-815 circular dichroism spectropolarimeter with the temperature-controlled cell holder set at 24 °C. Each sample was prepared in 0.1, 0.3, 0.5, 1, and 2 M SSC buffer to DNA concentrations listed in Table S1 (Supporting Information). A 0.1 cm path cuvette cell was used, and the spectra contributions from the cuvette and buffer were subtracted from each collected sample. The CD spectra were averaged over four scans and collected from 310 to 220 at 0.1 nm intervals with a bandwidth of 1 nm. The raw spectra were smoothed by fast Fourier transform (FFT) where necessary.

Results and Discussion

Influence of Salt Concentration on Film Assembly. *Planar Supports*. Under certain assembly conditions in solution, $polyA_{15}G_{15}$ can form extremely stable complexes (known as frayed wires) where multiple strands of $polyA_{15}G_{15}$ can self-assemble into high molecular weight aggregates comprising of two structural domains; single-stranded adenine runs or "arms" and self-complexed guanines or "stems".[32,33] We have confirmed using CD that $polyA_{15}G_{15}$ does not form any frayed wires in solution under the conditions we used in our experiments, although our results suggest the formation of another complex, possibly a dimeric chair G quadruplex structure (see Supporting Information, Figure S1).[34]

Because of the high dissipation values observed during film assembly, the Sauerbrey equation[30] is not valid; hence, a quantitative evaluation of adsorbed mass is not possible. Thus, the layer buildup is qualitatively reported as a change in frequency. The growth of the 30-mer film under the various salt concentrations is observed by a decrease in frequency with deposition of each layer, as shown in Figure 1. This suggests that there is specific base-pairing, as earlier work has shown that hybridization between the layers only occurs between complementary base-pairs.[1] The efficiency and stoichiometry of association of the DNA chains are currently being examined by extensive modeling on the assembly of the DNA multilayer films. The QCM results clearly demonstrate that the growth of the film is dependent on the salt

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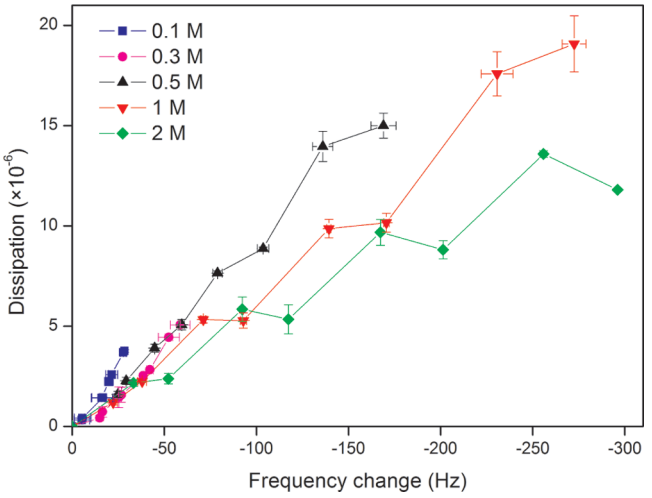

Figure 2. Plot of dissipation versus frequency change for DNA multilayer films assembled using polyA$_{15}$G$_{15}$ and polyT$_{15}$C$_{15}$ on a polyT$_{30}$ priming layer at various salt concentrations.

concentration in the assembly solution. A four-bilayer 30-mer film assembled under 0.1 M salt resulted in a decrease in frequency of only 28.4 Hz. The decrease in frequency for a film with the same number of layers but which was assembled under 0.3 M salt was 58.6 Hz. In contrast, a four-bilayer 30-mer film fabricated at 0.5 and 1 M salt showed frequency decreases of 169.0 and 272.5 Hz, respectively. A further increase in the salt concentration from 1 to 2 M resulted in a slight additional decrease in the film frequency to 296.0 Hz. These results suggest that although hybridization occurs at salt concentrations as low as 0.1 M, the use of high salt promotes the deposition of more DNA by increasing the packing of the DNA strands on the surface. Although the dependence of double-helix free energy on salt concentration has been explained by the counterion condensation theory,[35] at high NaCl concentration (1−2 M), the water structure and interaction with the DNA change significantly. This additional effect can play a role in increasing the stability of both duplex and triplex structures and resulting growth of the film.[36]

Figure 1 also shows a distinct stepwise growth at higher salt concentrations (1 and 2 M). A larger decrease in frequency was obtained with every A$_{15}$G$_{15}$ (T = A hybridization) layer compared to every T$_{15}$C$_{15}$ (C≡G) layer, which suggests that the difference in the hybridization properties of the TA and CG base pairs under various salt concentrations are amplified at higher salt concentrations.

The structure of a film can be better understood by studying the viscoelastic properties ("softness") of the film, which can be measured in terms of the dissipation factor of a film.[37] The dissipation, or damping (D), is a measure of the loss of energy from a freely oscillating quartz crystal as a result of a soft film adhered to the surface. It is defined as the energy lost (dissipated) during one oscillation cycle divided by the total energy stored in the oscillator.[37,38] Figure 2 shows the plot of the dissipation versus frequency change associated with each 30-mer layer deposited under the various salt concentrations. The results suggest the formation of two distinct film morphologies, which are defined by the salt concentrations under which the films were assembled. Films that were assembled under salt concentrations from 0.1 to 1 M showed increasing dissipation with the formation of each layer. As observed previously, the change in dissipation for the deposition of each AG layer (T = A hybridization) was greater than that for every TC layer (C≡G hybridization) at 0.5 M salt, a characteristic of diblock films.[1] This was attributed to the formation of three hydrogen bonds (C≡G) during deposition of the TC layer compared to the formation of two hydrogen bonds (T = A) during deposition of the AG layer.[1] The overall frequency change and dissipation of the film for the same number of layers were larger for the higher salt concentrations, indicating the deposition of more DNA. However, at 2 M salt concentration, a stepwise increase and decrease in the dissipation, corresponding to the deposition of every AG (T = A hybridization) and TC (C≡G hybridization) layer, respectively, was observed. The overall dissipation of the film after eight layers was lower than at the other salt concentrations, which indicates the formation of a denser film than the films assembled at lower salt concentrations. This suggests a film with a different morphology at 2 M, indicating a more complex growth mechanism. A possible explanation is the formation of T·A*T triplexes within the film. Such complexes are known to form at high salt concentrations of more than 200 mM Na^+.[28,39]

Using CD measurements (Figure 3a), the formation of the T·A*T triplex was first studied in solutions containing polyT$_{15}$ and polyA$_{15}$ in a 2 to 1 mole ratio at different salt concentrations (0−2 M). PolyT$_{15}$ and polyA$_{15}$ exist as single strands in solution in water and at the salt concentrations investigated (see Supporting Information, Figure S2). Our results showed that different spectra were obtained in water and in the presence of salt, indicating that there was no hybridization of the polyT$_{15}$ and polyA$_{15}$ strands in water. At increasing salt concentrations, a decrease in the ellipticity at 280 nm and a shift of the 260 nm peak to lower wavelengths with higher intensity was observed. There was also a decrease in the ellipticity below 220 nm at higher salt concentrations (data not shown). The negative peak between 210 and 220 nm is characteristic of a triplex T·A*T,[40] indicating the formation of a triplex T·A*T at high salt concentrations (> 0.5 M). Figure 3b shows the contribution of the triplex toward the CD signal at 2 M salt concentration, which shows a negative contribution below 240 nm. Although CD has been used to study the conformation of T = A within a PEI multilayer matrix,[41] given the complexity of our system due to the presence of different secondary structures (e.g., T·A*T triplexes, G quadruplexes) and T = A and C≡G, the CD profile (Figure S3, Supporting Information) is difficult to interpret and hence was not used to analyze the DNA multilayer films.

To demonstrate whether the formation of a T·A*T triplex occurred in the DNA multilayer film, a series of experiments were carried out at 0.5 and 2 M salt. In all of the experiments, polyA$_{15}$G$_{15}$ was deposited on a polyT$_{30}$ layer, leaving the G strands available for subsequent hybridization with C strands. However, when polyT$_{15}$ strands were introduced, decreases in film frequency of 19% and 21% at 0.5 and 2 M salt, respectively, were observed. This could be attributed to the hybridization between the polyT$_{15}$ and polyG$_{15}$ strands, as it is known that association between G and T is possible,[1] or the formation of a

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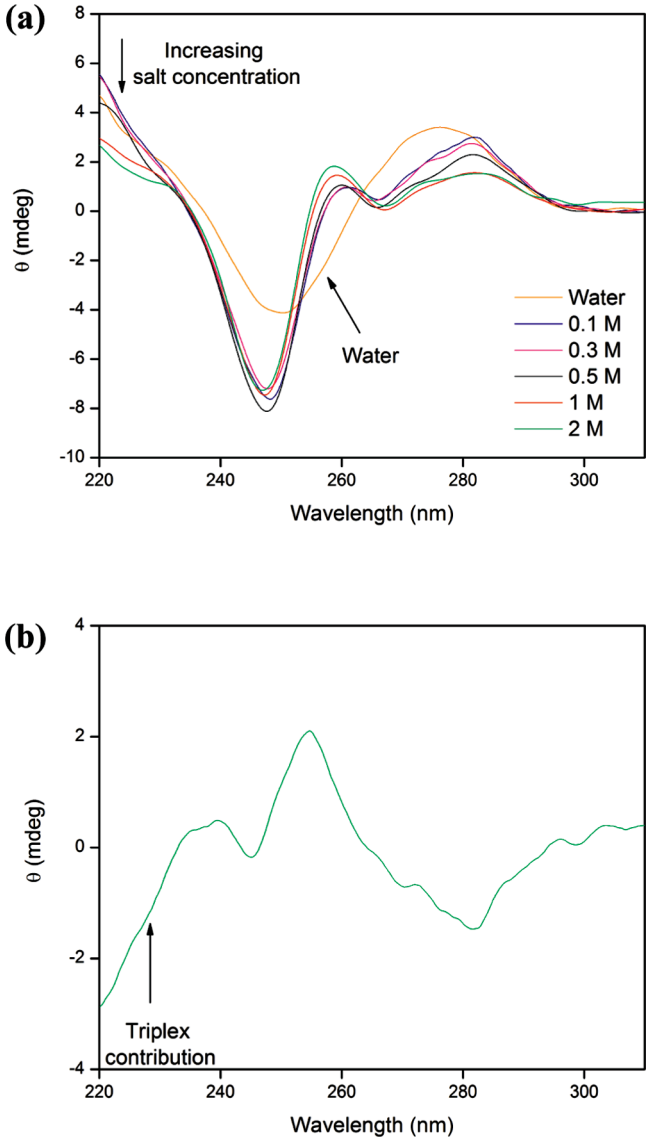

Figure 3. (a) CD spectra of $polyT_{15}$ and $polyA_{15}$ in a mole ratio of 2 to 1, respectively, at various salt concentrations (0−2 M). (b) Triplex contribution to the experimental CD spectra of a 2:1 mole ratio of $polyT_{15}$ and $polyA_{15}$ in 2 M salt. The spectrum is calculated by subtracting the 0.1 M spectra (assuming minimal triplex formation) from the 2 M salt spectra.

triplex through binding of the $polyT_{15}$ strands to the TA duplex. To confirm between these two possibilities, $polyC_{15}$ strands followed by $polyT_{15}$ strands were introduced over the $polyA_{15}G_{15}$ layer. The $polyC_{15}$ strands should bind to all of the available G strands on the surface, leaving no available G strands available to associate with the $polyT_{15}$ strands. However, the results showed that there was a 6% and 20% decrease in film frequency after $polyT_{15}$ was introduced (Figure S4, Supporting Information) at 0.5 and 2 M salt, respectively. This confirms the formation of T·A*T triplexes occurs more readily at higher salt concentrations.[28,39]

To determine whether the T·A*T triplexes could be displaced from the surface after formation, $polyA_{15}$ strands were introduced over a $polyT_{15}/polyA_{15}G_{15}$ film capped with either a $polyT_{15}$ or $polyT_{15}C_{15}$ layer assembled at 0.5 or 2 M salt. Figure S5 (Supporting Information) shows that the triplexes formed with the $polyT_{15}$ strands were almost completely removed, and only 3% and 2% of the triplexes remained after treatment with $polyA_{15}$ strands at 0.5 and 2 M salt, respectively. However, when $polyA_{15}$ was introduced to the triplexes formed using $polyT_{15}C_{15}$, an 8% increase in the film frequency (corresponding to a decrease in film mass) was obtained at 0.5 M salt. In this case, the $polyA_{15}$ strands approaching the surface can competitively hybridize with the $polyT_{15}$ diblock and displace the third $polyT_{15}C_{15}$ strand bound as a T·A*T triplex. A different behavior was observed at 2 M, where treatment of the triplexes formed using $polyT_{15}C_{15}$ resulted in a 7% decrease in the film frequency (corresponding to an increase in film mass). This suggests that the triplexes formed at higher salt are more stable and are not easily disrupted.

In light of the above findings, we propose an assembly mechanism for the DNA multilayer films at different salt concentrations (Scheme 2). The $polyA_{15}G_{15}$ strands are likely to adopt multistrand structures due to the formation of a dimeric chair G quadruplex structure (see Supporting Information, Figure S1), as the deposition of more than one strand of $polyA_{15}G_{15}$ onto the surface is associated with a larger decrease in the film frequency and an increase in film dissipation. When $polyT_{15}C_{15}$ is introduced, the $polyC_{15}$ strands are able to hybridize to the G_{15} region of the $polyA_{15}G_{15}$ strands, displacing the $polyA_{15}G_{15}$ strands involved in quadruplex formation. At the same time, the $polyT_{15}$ part of the strands are able to form triplexes with the T=A duplex. Hence, an overall decrease in frequency is observed. The DNA triplex is also known to be a more rigid structure[42] and thus increases the stiffness of the film, which was observed experimentally by a decrease in dissipation of the DNA film at 2 M salt concentration (Figure 2). When $polyA_{15}G_{15}$ is introduced again, while some of the triplex is removed to form duplexes in solution, hybridization also occurs with the free T strands on the surface. However, there is no displacement of the stable T·A*T triplexes at 2 M salt, and introduction of the $polyA_{15}G_{15}$ strands results in hybridization with the free T strands on the surface. The role of the triplexes in the DNA multilayer film growth is more clearly seen at 2 M, as the T·A*T triplexes are more stable at higher salt concentrations.[27]

To investigate whether high salt concentrations in the assembly solutions can be used to promote greater film growth from various oligonucleotide lengths, DNA films were also fabricated from a range of oligonucleotides (10, 20, 40, and 60 bases) in 2 M salt. We have previously reported that there is no growth of the 10-mer to form DNA multilayer films when assembled at 0.5 M salt.[10] Similarly, in the present study, we found that we could not obtain a film assembled using 10 base long oligonucleotides at 2 M salt (Figure 1). Although there was approximately twice the amount of $polyA_5G_5$ deposited at 2 M (−42.4 Hz) compared to 0.5 M (−21.4 Hz),[10] there was no subsequent DNA deposition to form a multilayer film. This is attributed to the low melting temperature of the hybridizing strands at room temperature.[43,44]

As seen in Figure 4, there was no significant difference in the overall frequencies of the films that were fabricated using oligonucleotides ranging from 20 to 60 bases at 2 M salt concentration. The effect of salt on oligonucleotides of different length is more prominent at 0.5 M than at 2 M.[10] For example, the 20-mer comprising eight layers assembled under 2 M (−291.7 Hz) salt showed a change in frequency of more than 7 times than when the

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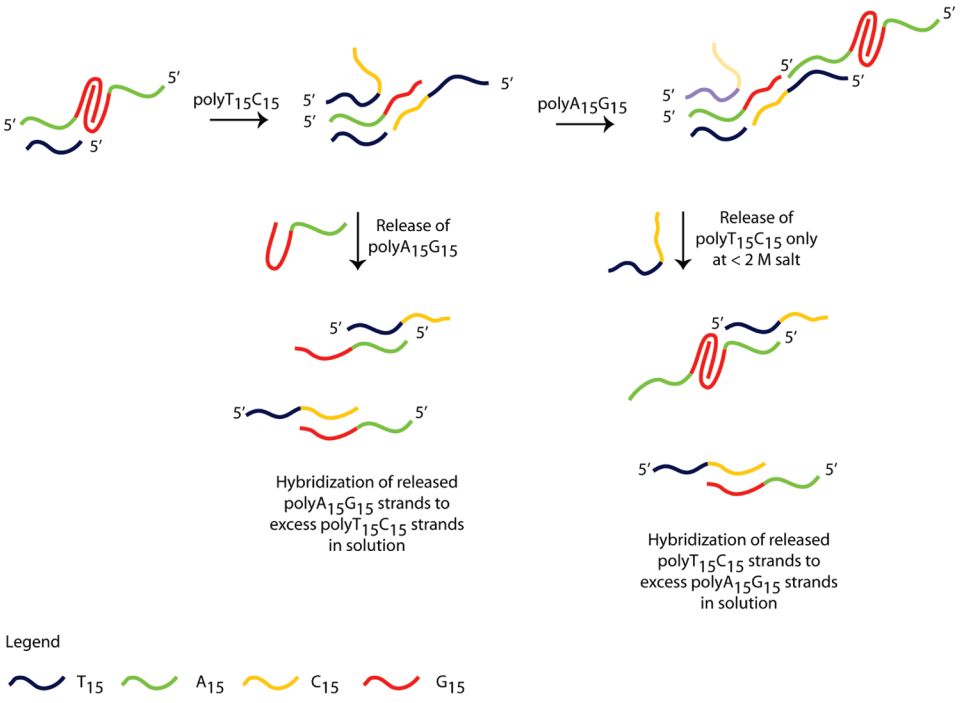
Scheme 2. Proposed Film Assembly of the DNA Multilayer Films at Salt Concentrations above 0.5 M[a]



[a] The T·A*T triplex is not displaced at high salt concentrations (2 M).

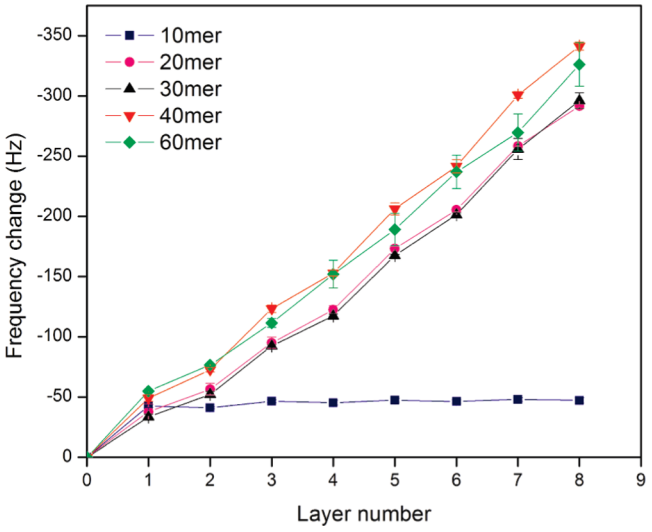


Figure 4. Growth of DNA multilayer films assembled using various oligonucleotide sizes (10, 20, 30, 40, and 60 bases) at 2 M salt concentration. Layer 0 corresponds to the polyT$_{30}$ layer; odd and even layers correspond to the A$_n$G$_n$ and T$_n$C$_n$ layers, respectively.

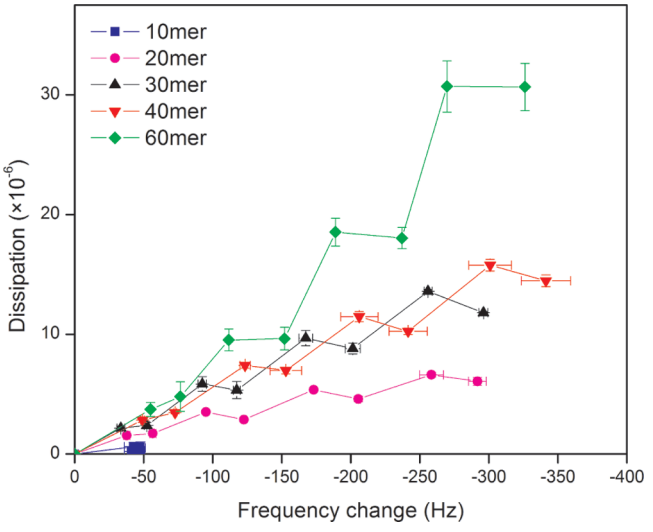


Figure 5. Plot of dissipation versus frequency change for DNA multilayer films assembled using various oligonucleotide sizes (10, 20, 30, 40, and 60 bases) at 2 M salt concentration.

film was deposited under 0.5 M salt (−39.8 Hz).[10] The eight-layer 30-, 40-, and 60-mer films (296.0, 341.3, and 326.1 Hz, respectively) assembled at 2 M showed approximately double the frequency decrease at 0.5 M (169.0, 182.4, and 152.0 Hz, respectively) salt. This suggests that the oligonucleotides are not as strongly bound when assembled at 0.5 M salt, compared to assembly at 2 M salt. It is also likely that the T·A*T triplexes are not as stable at lower salt solutions and do not contribute toward the layer growth.

The total frequency changes of films fabricated using 20-, 30-, and 40-mer oligonucleotides at 2 M were −291.7, −296.0, and −341.3 Hz, respectively. Stepwise growth was observed, where deposition of every A$_n$G$_n$ (T = A hybridization) layer resulted in more growth than the deposition of every T$_n$C$_n$ (C≡G hybridization) layer. When the films were fabricated using 60 base long oligonucleotides, although a larger decrease in frequency was obtained for the first bilayer (i.e., T$_{30}$, A$_{30}$G$_{30}$, and T$_{30}$C$_{30}$ layers) compared to the 20-, 30-, and 40-mer films, less DNA was subsequently deposited. The total change in frequency of the 60-mer film at 2 M was −326.1 Hz after eight layers.

As mentioned earlier, the dissipation can be used to better understand the viscoelasticity of the films. A decrease in the dissipation was associated with the deposition of every polyT$_{15}$C$_{15}$ layer when the 30-mer film was assembled under 2 M. This decrease was not noted at the lower salt concentrations, indicating the formation of a film with triplexes at 2 M assembly conditions. Our results in Figure 5 show a similar trend where a

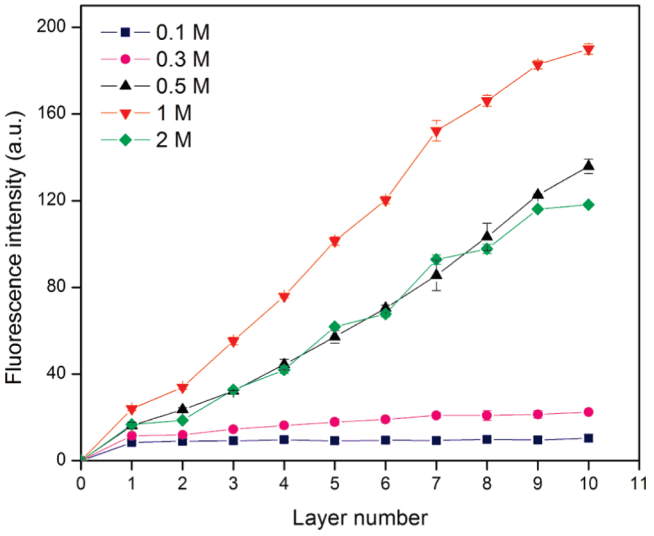


Figure 6. Growth of DNA multilayer films on colloidal supports, monitored using flow cytometry. Layer 0 corresponds to the T_{30} precursor layer; odd and even layers correspond to the $A_{15}G_{15}$-TAM and $T_{15}C_{15}$ layers, respectively.

corresponding decrease and increase in dissipation was observed with the deposition of every T_nC_n and A_nG_n layer, respectively. This was only observed when oligonucleotides ranging from 20 to 40 bases were used, suggesting that a film with a similar morphology is obtained. However, for the 60-mer films, there was no change in the dissipation when a polyT_nC_n layer was deposited. This film also had a lower frequency change (−326.1 Hz) and double the dissipation (30.7×10^{-6}) compared to the 40-mer film (−341.3 Hz, 14.5×10^{-6}), suggesting that the 60-mer film was highly swollen. It is possible that the longer and more flexible 60 base long oligonucleotides are entangled with the other strands on the surface, giving a water-swollen gel-like film structure with high film dissipation. These interactions increase the steric hindrance within the film, and less DNA is deposited with each subsequent layer. During poly$T_{15}C_{15}$ deposition, the formation of the T·A*T triplex should lead to a decrease in the film dissipation. However, the decrease is masked by the higher increase in dissipation due to the high water content of the film, and hence no overall change in the dissipation of the film is observed.

Colloidal Supports. By fluorescently labeling every alternate layer during the film buildup, a qualitative measure of the amount of DNA deposited at each layer on a colloidal template can be obtained.[31] The use of fluorescence is also useful in monitoring any conformational changes during film assembly as the fluorophore fluorescence is highly dependent on its surrounding environment. Figure 6 shows the average fluorescence intensity of silica particles coated with 30-mer films assembled at various salt concentrations. The results show negligible growth at 0.1 M salt (1.0 au per layer) and 0.3 M salt (2.2 au per layer), consistent with the findings on the planar surfaces (QCM studies) where the low growth was attributed to insufficient shielding of the DNA strands. A similar trend to the planar surface was obtained — the growth at 1 M (19.0 au per layer) was also higher than the growth at 0.5 M (13.6 au per layer). Interestingly, there was less fluorescence intensity for the film assembled at 2 M salt. However, the QCM results (Figure 1) demonstrate that a greater decrease in film frequency was obtained at higher salt concentrations. This suggests that the lower fluorescence intensity does not necessarily indicate the deposition of less mass on the colloidal particles, but rather the formation of a film with a different conformation at such a high salt concentration, which affects the fluorophore fluorescence. It is likely that there is some fluorescence quenching of the fluorophore within the dense film. TAMRA has been shown to self-quench at a Förster critical distance, R_0, of 44 Å due to exciton coupling.[45,46] In addition, even though the poly$T_{15}C_{15}$ strands were not labeled, there was an increase in the fluorescence intensity of the film after every deposition, which suggests that the film rearranges during layer formation. These results suggest that the morphology of the film assembled at 2 M salt is different to that of the films assembled at 0.1, 0.3, 0.5, and 1 M salt.

Conclusions

We have shown that the presence of salt is essential for the growth of DNA multilayer films. Although low salt concentrations (< 0.5 M) are sufficient for DNA hybridization and hence film formation, the DNA strands are likely to pack more densely on the surface at higher salt concentrations. The results showed that the frequency changes and dissipation increase with the formation of each layer assembled from 0.1 to 1 M salt. However, at 2 M salt, the dissipation observed for deposition of every $T_{15}C_{15}$ layer decreases, suggesting the formation of a denser film with a different morphology. We showed that the triplexes formed at 2 M using the poly$T_{15}C_{15}$ strands are not displaced when polyA_{15} is subsequently introduced. We also found that there was no significant difference in the overall frequency of the DNA films assembled using oligonucleotides of various lengths (20-, 30-, 40-, and 60-mer). The deposition of every T_nC_n layer led to a decrease in film dissipation in the 20-, 30-, and 40-mer films, but not in the 60-mer film. This was attributed to the possible masking of the decrease in dissipation associated with the formation of the T·A*T triplexes by the high water content of the gel-like film structure.

On the colloidal particles, a systematic increase in the fluorescence intensity of the film (indicating greater film growth) was obtained from 0.1 to 1 M salt. However, at 2 M salt, a decrease in the fluorescence intensity of the film was observed. From the QCM results, which show that more film is deposited at this salt concentration, the decrease in the fluorescence intensity of the film likely indicates the formation of a film with a different film morphology due to the presence of the T·A*T triplexes that are not stable in films obtained at lower salt concentrations.

Overall, the use of salt can be used to control the growth of DNA multilayer films and promote the formation of ordered structures such as triplexes, which are known to be more stable than duplexes, to tune the viscoelastic properties of the DNA films. This work is anticipated to be useful in engineering films for the encapsulation of drugs and vaccines for targeted drug delivery or for sensing applications.

Acknowledgment. We gratefully acknowledge the Australian Research Council for financial support (Discovery Project and Federation Fellowship schemes) and the Particulate Fluids Processing Centre for infrastructure support.

Supporting Information Available: CD spectra of poly-$A_{15}G_{15}$ and differential spectra of G_{15} at different salt concentrations (Figure S1); CD spectra of polyT_{15}, polyA_{15}, polyC_{15}, and polyG_{15} at different salt concentrations (Figure S2); CD spectrum of a five-bilayer 60-mer film in 0.5 M salt (Figure S3); percentage decrease in film frequency for the

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deposition of polyC_{15} followed by polyT_{15} on a poly$A_{15}G_{15}$ layer assembled at 0.5 and 2 M salt (Figure S4); percentage of film frequency remaining after introduction of polyA_{15} to a polyT_{15} surface assembled at 0.5 and 2 M salt and percentage increase or decrease in film frequency after introduction of polyA_{15} to a poly$T_{15}C_{15}$ surface assembled at 0.5 and 2 M salt (Figure S5). This material is available free of charge via the Internet at http://pubs.acs.org.