

Effect of electrostatic interactions on binding and retention of DNA oligomers to PNA liposomes assessed by FRET measurements

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Abstract

A FRET-based method is used to observe the desorption of di-alkyl peptide nucleic acid amphiphiles (PNAA) from liposomes occurring on binding of complementary DNA oligomers. PNA liposomes were prepared containing fluorescein-labeled PNAA and rhodamine-labeled dipalmitoylphosphoethanolamine (DPPE). These liposomes showed efficient energy transfer from the fluorescein to rhodamine, with an average donor-to-acceptor distance of 5.91 nm. In low-ionic-strength buffer (50 mM Tris–HCl, pH 8.0), the FRET signal was maintained in the presence of a stoichiometric amount of 10- and 20-mers DNA complements, but the signal attenuated for 40-mer complements, indicating that DNA first binds the PNAA before the PNAA/DNA duplex desorbs from the lipid bilayer. The FRET signal was maintained in the presence of 10-, 20-, 40-, and 60-mer DNA in high ionic-strength buffer, showing that the driving force for the desorption is electrostatic repulsion between the bound DNA oligomer and the liposome surface. This conclusion is corroborated by comparison of the PNA/DNA binding energy, the energy of adsorption of the di-alkyl PNAA to the lipid bilayer, and a calculation of the DNA/lipid bilayer electrostatic repulsion using the linearized Poisson–Boltzmann equation.

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1. Introduction

Liposomes functionalized with DNA oligomers have been developed with an eye toward highly sensitive DNA sensing [1,2] for use in anti-sense gene delivery [3] and as a platform for biophysical measurements [4]. These biomimetic materials can be used to encapsulate a high concentration of fluorescent or bioactive material and selectively release the contents in the presence of external stimuli. In most cases, liposomes are composed primarily of naturally occurring phospholipids that are biodegradable and, with suitable protection by surface-attached polymers, are non-immunogenic. As negatively charged polyelectrolytes, DNA oligomers attached to the liposome surface confer a negative charge upon it that electrostatically repels target DNA from solution, and can be expected to limit the extent of DNA binding to these surfaces.

An alternative is the use of the uncharged DNA analog “peptide nucleic acid” (PNA) which has the phosphodiester backbone of DNA replaced with an uncharged polyamide backbone [5,6]. PNAs form highly stable duplexes with complementary DNAs owing to the limited electrostatic repulsion between the two strands of a PNA/DNA duplex [7]. In a previous report [8] we described a self-assembling “PNA amphiphile” (PNAA) consisting of a 10-mer PNA peptide linked to a synthetic di-alkyl tail (Fig. 1). These molecules can be co-extruded with conventional phospholipids to form liposomes with specific DNA binding activity. Careful design of the PNAA was required for proper liposome extrusion and DNA binding function, most notably the inclusion of a water-soluble spacer group to encourage DNA binding along with four glutamic acid residues (negatively charged at pH 7–8) to provide for proper hydration.

An unexpected observation was made when using DNA oligomers longer than the PNAA sequence attached to PNA liposomes to assess binding in capillary electrophoresis. Unbound stretches of DNA extending away from the liposomes had a destabilizing effect on the PNA/DNA duplex, while those extending toward the liposome had a stabilizing effect. This was

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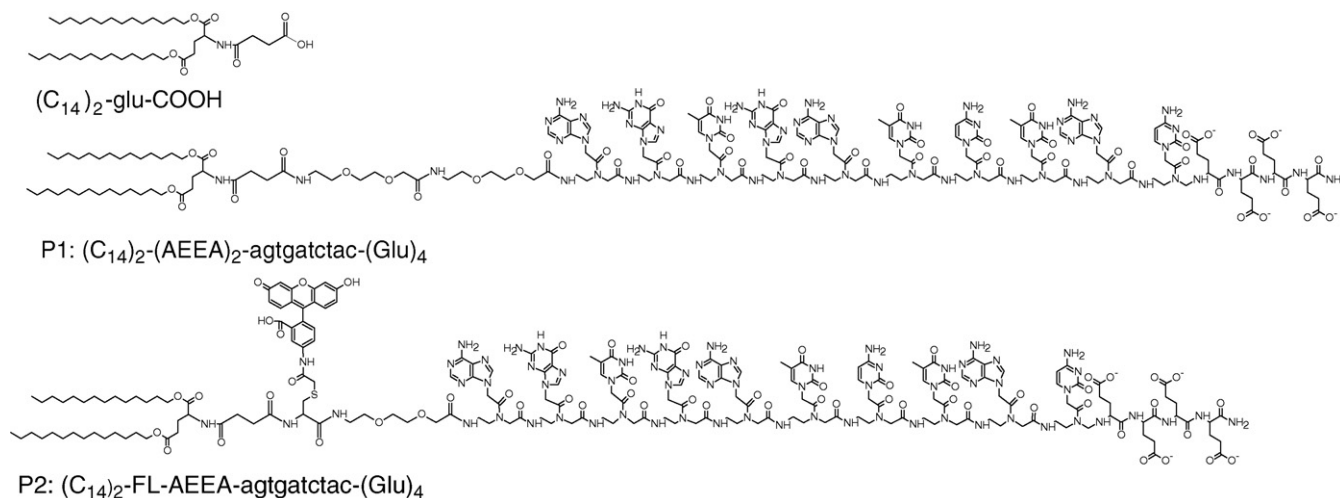


Fig. 1. Chemical structures of 10-base PNA (P1) and its fluorescein-labeled analog (P2).

explained by the existence of repulsive, electrostatic interactions between the glutamic acid residues of the PNA and the negatively charged phosphodiester backbone of the overhanging DNA bases. Favorable interactions between overhanging DNA stretches on the liposome side were ascribed to hydrophobic contacts between the unbound DNA bases and the interior of the lipid bilayer. The relative importance of electrostatic repulsion is typically addressed by performing binding experiments under conditions of high salt, where repulsions are screened.

Since capillary electrophoresis is not suitable for high ionic strength measurements, we have synthesized fluorescein-labeled PNA to carry out quiescent fluorescence resonance energy transfer (FRET) measurements on the surface of rhodamine-labeled liposomes. We employ FRET to elucidate the hydrophobic interactions between DNA and PNA liposomes by measuring the retention of PNA/DNA duplexes in lipid bilayers. The retention of PNA/DNA duplexes within the lipid structure is desirable for these liposomes to be used as signal-amplifying tags in biosensing applications.

2. Materials and methods

All solvents used here were HPLC grade (Fisher Scientific, Pittsburgh, PA) and used as received unless otherwise noted. Deionized water was purified to a final resistivity of 18.2 MΩ cm (Barnstead Nanopure, Barnstead International, Dubuque, IA). Glassware was cleaned with a chromate cleaning solution (Fisher) and rinsed copiously with water prior to use.

2.1. Synthesis of di-alkyl base amphiphile

(C₁₄)₂-glu-C₂-COOH (Fig. 1) was synthesized by a previously reported method [8–10]. Briefly, 7.36 g of a 1:2:1.5 molar mixture of L-glutamic acid, tetradecanol, and *p*-toluenesulfonic acid was dissolved in ~500 mL of toluene and heated under reflux until a stoichiometric amount of water was recovered in a Dean-Stark trap (~6 h). 7.27 g of the above product, 1.25 equiv. of diisopropylethylamine (DIPEA), and 1.15 equiv. of succinic

anhydride were then dissolved in 200 mL of THF/chloroform (1:1) and the resulting reaction mixture stirred for 2 h at 40 °C. The product (C₁₄)₂-glu-C₂-COOH, was concentrated by rotary evaporation and recrystallized three times from ethanol. The purity of both reactions was >98% as measured by electrospray ionization mass spectrometry (ThermoFinnegan LCQ, Woburn, MA).

2.2. Synthesis of PNA amphiphiles (PNA)

Unlabeled PNAs (P1, Fig. 1) were synthesized using Fmoc/Bhoc-protected monomers and an Fmoc-protected PAL-PEG-PS resin (Applied Biosystems, Foster City, CA) using a previously reported solid-phase peptide synthesis protocol [8,11]. Fmoc-protected PNA monomers and an Fmoc-protected ethylene glycol spacer (8-amino-3,6-dioxaoctanoic acid, AEEA) were obtained from Applied Biosystems. Fmoc-protected glutamic acid was obtained from Peptides International (Louisville, KY). A stock solution of each monomer in 1-methyl-2-pyrrolidinone (NMP) (“monomer solution”) was prepared at a concentration of 0.215 M and stored in a refrigerator for no more than 1 month. Each of these monomers was coupled to the peptide following the same procedure.

Prior to synthesis, 100 mg of resin was gently shaken in dichloromethane (DCM) for several hours. The resin was then deprotected by incubation with 20 vol.% piperidine in *N,N*-dimethylformamide (DMF) for 8–12 min. For monomer coupling, a solution of ~0.5 mL of monomer solution (adjusted to provide a five-fold excess of monomer to reported active sites), 0.5 mL of PNA base solution (Applied Biosystems), and 0.5 mL of PNA activator (Applied Biosystems) was allowed to stand for 2 min, then was shaken with the resin for 1 h. After coupling, the resin was washed with DCM and DMF, and capped by a 10 min incubation of the resin with the acetic anhydride. The resin was washed again with DCM and DMF and the process was repeated with 20 min coupling steps until the desired PNA peptide was synthesized. The di-alkyl amphiphile (C₁₄)₂-glu-C₂-COOH was attached to the N-terminus of the chain as the final step in the syn-

thesis, following the same coupling procedure as the protected monomers. The resin was soaked in a mixture of 4:1 v/v trifluoroacetic acid (TFA): *m*-cresol (total volume = 2 mL) for 2 h to cleave the PNAA from the resin and remove Bhoc side protecting groups. The product was purified by HPLC (Delta 600, Waters, Wilford, MA) using a Symmetry300 C₄ column (Waters) with a particle size of 5 μm. Elution of the PNAA product was achieved using a 30 min linear gradient (0.1% TFA in acetonitrile to 0.1% TFA in water) and a 1 mL/min flowrate. MALDI-TOF mass spectroscopy was performed on the PNAA product and showed good agreement between expected and observed PNAA molecular weights. A matrix of α-cyano-4-hydroxycinnamic acid was used and the instrument (Voyager STR, PerSeptive Biosystems, Framingham, MA) operated in positive polarity, reflector mode (accelerating voltage = 20 kV).

2.3. Synthesis of fluorescein-labeled PNAA

Fluorescein-labeled PNAA (P2, Fig. 1) were synthesized by pre-cleavage linking of a fluorescein derivative (5-iodoacetamidofluorescein, Molecular Probes, Eugene, OR) to a modified cysteine residue (*N*-α-Fmoc-*S*-*t*-butylthio-L-cysteine, Novabiochem, San Diego, CA) in the PNAA peptide [12–14]. The modified cysteine was coupled to the chain as other monomers described above, added between the AEEA spacer and the PNA monomer nearest the di-alkyl tail (Fig. 1). Coupling of the fluorescein derivative was accomplished on resin, prior to cleavage and deprotection of the PNAA. A stock solution of the fluorescein derivative (“fluorescein solution”) in NMP was prepared at a concentration of 0.215 M and stored in a refrigerator for not more than 1 month. Stock solutions of dithiothreitol (DTT) (0.1 M in DMF) and NH₄HCO₃ (0.2 M in water) were also prepared and used the same day.

After synthesis of the PNA peptide and conjugation of the lipid tail, the modified cysteine residue was deprotected by addition of 1 mL of DTT solution and 0.5 mL of NH₄HCO₃ solution. The solutions and resin were mixed for 4 h. Activated linkage of fluorescein to the deprotected cysteine was accomplished by addition of ~0.25 mL of fluorescein solution (adjusted to give 2.5-fold excess over reported resin active sites) and ~0.06 mmol of *N,N*-diisopropylethylamine (adjusted to give three-fold excess) in 1.5 mL of DMF. The solutions and resin were mixed for 2.5 h. Cleavage and purification of the P2 product were carried out as above. Again, MALDI-TOF mass spectroscopy was performed on the PNAA product and showed good agreement between expected and observed PNAA molecular weights. Yields for syntheses of fluorescein-labeled PNAA (P2, Fig. 1) were somewhat lower than those of P1, possibly due to the cleavage of some peptides from the resin during the thiol reaction.

2.4. Liposome extrusion

A 1:74:20 solution of rhodamine-labeled dipalmitoylphosphoethanolamine (DPPE-rhod), distearylphosphatidylcholine (DSPC), and cholesterol (Avanti Polar Lipids Inc., Alabaster, AL) in chloroform was prepared along with 1 mg/mL solutions

of P1 and P2 in 1:1 chloroform/methanol. Prior to lyophilization, appropriate amounts of each solution were mixed to yield the desired molar ratio of each constituent (about 3 μmol total lipid). The mixture was vortexed and lyophilized in small glass vials to yield a dry lipid film. The film was hydrated in 50 mM Tris buffer (1.5 mL, pH 8) to a final lipid concentration of 2 mM. The resulting lipid suspension was subjected to 5–10 freeze–thaw cycles before pressure filtration (nitrogen) through two 0.1 μm Nucleopore filters (Whatman, Clifton, NJ) in a stainless steel extruder (Northern Lipids, Vancouver, Canada). Ten extrusion cycles were performed at 60 °C and 350 psi to yield small unilamellar vesicles (SUV) with a narrow size distribution. This temperature was set above the chain-melting transition temperature of (C₁₄)₂-glu-C₂-COOH under these conditions ($T_c = 32.3$ °C) [10]. All SUV samples were checked for proper size distributions by dynamic light scattering (DLS, Malvern Zetasizer 3000HS, Malvern, UK), and tight distributions centered on 100–110 nm were obtained in all cases.

2.5. Fluorescence measurements

Prior to fluorescence measurements, the concentration of P1 and P2 in the extruded PNAA liposomes was measured by UV absorbance. A wavelength scan (between 230 and 700 nm) was collected on a 100-fold dilution of the extrudate (50 mM Tris–HCl, pH 8.0). Scattering effects were removed from the scan by subtracting a power-law fit of the non-absorbing portions of the scan (exponent = –4) [15]. The extinction coefficient for P1 (1.035×10^5 L mol^{–1} cm^{–1}) was used to calculate the total amount of P1 and P2 in the sample (neglecting the absorbance of fluorescein in P2), yielding a total concentration of PNAA in the extrudate of 42 μM. DNA oligomers were obtained from Integrated DNA Technologies (Coralville, IA) and used as received. Stock solutions of ~1 mM DNA oligomers were prepared in 50 mM Tris buffer (pH 8.0) using UV measurements to set the required dilution. For the fluorescence measurements, appropriate aliquots of the PNAA liposome solution and the DNA stock solution were diluted with 50 mM Tris buffer to a total volume of 0.2 mL with 2 μM PNAA and 2 μM DNA. For liposomes with a composition of P2:P1:DPPE-rhod: DSPC:chol = 1:4:1:74:20, this corresponds to a concentration of 0.4 mM for the rhodamine in DPPE-rhod and 0.4 mM for the fluorescein in P2.

Samples were loaded into wells of a 96-well, black-side, clear-bottom polystyrene microtiter plate (Costar, Corning, NY) for fluorescence resonance energy transfer (FRET) measurements. FRET measurements were made with a dual-mode, dual-monochromator microplate reader with 9-nm bandwidth (SpectraMax M2, Molecular Devices, Sunnyvale, CA). For FRET measurements, the excitation wavelength was 493 nm, and the emission was scanned from 493 to 750 nm in 1 nm increments. A 515 nm high-pass filter was used to minimize the amount of scattered excitation light that reached the emission detector.

The critical Förster distances [16–18] as well as the distances between donor–acceptor fluorescent groups, were calculated by a method outlined by Ratilainen et al. [19] using PhotochemCAD software (<http://www.photochemcad.com>). In short, the

rate of energy transfer from a donor to an acceptor is given by:

$$k_T = \frac{1}{\tau_D} \left(\frac{R_0}{R} \right)^6 \quad (1)$$

where τ_D is the lifetime of the donor in absence of the acceptor, R_0 the critical Förster distance, and R is the actual distance between donor–acceptor. R_0 is the distance at which the energy transfer accounts for half of the deactivation processes of the donor:

$$(R_0 \text{ (cm)})^6 = (8.79 \times 10^{-25}) \kappa^2 n^{-4} \phi_D J_{DA} \quad (2)$$

where κ^2 is the orientation factor, n the refractive index of the medium between the donor–acceptor, ϕ_D the quantum yield of the donor, and J_{DA} is the overlap integral (Eq. (3)). κ^2 was set to 2/3, which is the accepted value for randomized orientation of the donor and acceptor transition dipoles [20,21]. ϕ_D was set to 0.93, the quantum yield of fluorescein [22]. The overlap integral describes the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor:

$$J_{DA} = \frac{\int f_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda}{\int f_D(\lambda) d\lambda} \quad (3)$$

where $f_D(\lambda)$ is the corrected emission spectrum of the donor and $\varepsilon_A(\lambda)$ is the absorption spectrum of the acceptor. The energy transfer efficiency, E , is calculated by comparing the fluorescence intensity, I_{DA} , of the donor in the donor–acceptor duplex with the intensity, I_D , of the donor by itself:

$$E = 1 - \frac{I_{DA}}{I_D} \quad (4)$$

The distance between the donor–acceptor pair (R) is given by:

$$R = R_0 \left(\frac{1}{E} - 1 \right)^{1/6} \quad (5)$$

3. Results and discussion

The main goal of this work is to better quantify the extent of DNA hybridization to liposomes containing di-alkyl PNA amphiphiles (PNAA) using fluorescence methods. In a previous report [8] we used shifts in electrophoretic mobility assessed using capillary electrophoresis (CE) to identify specific binding of DNA oligomers to the PNA liposomes. We found significant mobility shifts on addition of complementary DNA of equal size to the PNA peptide or complementary DNA with short, unbound regions on the proximal side of the duplex. Complementary DNA with overhangs on the *distal* side of the duplex (farthest from the lipid bilayer) showed no shift in mobility, despite the fact that similar PNA amphiphiles in solution avidly bind complementary DNA oligomers with overhangs on either side [11]. Since interpretation of electropherograms only reveals changes in the electrophoretic mobility of PNAA liposomes, it is not clear whether the distal-overhang DNA failed to bind to the PNAA anchored in liposomes, or if the bound PNAA/DNA duplex desorbed from the lipid bilayer without a significant change in liposome mobility. Additionally, the CE method places

constraints on the ionic strength of solutions used, so high salt conditions (where electrostatic repulsions are screened) could not be interrogated.

Fluorescence resonance energy transfer (FRET) methods are a powerful means of assessing biomolecular binding events by labeling receptors and ligands with fluorophores with spectral overlap. Toward that end, we synthesized a PNA amphiphile (P2, Fig. 1) that contains a fluorescein group near the lipid tails. This was accomplished by incorporation of a modified cysteine residue in the solid-phase synthesis of the PNAA, and linking a derivatized fluorescein probe to it prior to cleavage of the PNAA to the support [12]. Given this labeled PNAA, binding of DNA to PNA in liposomes could be accomplished in a quiescent solution through the use of labeled DNA oligomers. We did attempt this, but found that the presence of fluorophores on the DNA oligomers either interfered with PNAA/DNA hybridization, or located the fluorophore too far from its FRET partner on the PNA strand to effect energy transfer.

Instead, we have added some rhodamine-labeled lipids to the liposome formulation to serve as a FRET acceptor for the fluorescein on the PNAA strand. A significant FRET signal should be expected when the PNAA are retained in the lipid bilayer, but the signal should be relieved when the PNAA is removed, for example, by hybridization to DNA with attendant electrostatically-induced desorption of the PNAA/DNA duplex. A shortcoming of this approach is that binding of the unlabeled DNA to the liposome surface cannot be directly measured, only inferred by a PNAA desorption event.

First, we ran a control experiment to ensure that 1 mol% fluorescein-labeled PNAA (P2) and 1 mol% rhodamine-labeled DPPE (DPPE-rhod) on the surface would produce a detectable amount of energy transfer (Fig. 2). The energy transfer from fluorescein (donor) to rhodamine (acceptor) indicates that the two groups are closer than the Förster critical distance (R_0). In Fig. 2, we see a significant reduction in the fluorescein peak accompa-

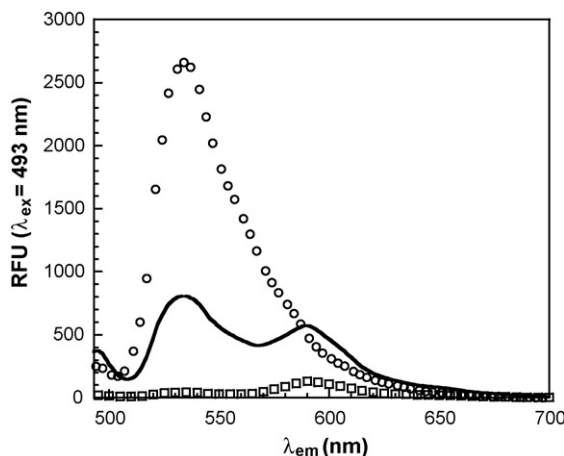


Fig. 2. Resonance energy transfer between rhodamine-labeled bilayer lipids and fluorescein-labeled PNAA (P2) in 50 mM Tris-HCl (pH 8.0). (○) Fluorescein-labeled liposomes (P2:P1:DSPC:chol 1:4:75:20); (□) rhodamine-labeled liposomes (P1:DPPE-rhod:DSPC:chol 5:1:74:20). Solid curve corresponds to “FRET-labeled” liposomes (P2:P1:DPPE-rhod:DSPC:chol 1:4:1:74:20). [PNAA] = 2 μ M; [P2] = [DPPE-rhod] = 0.4 μ M.

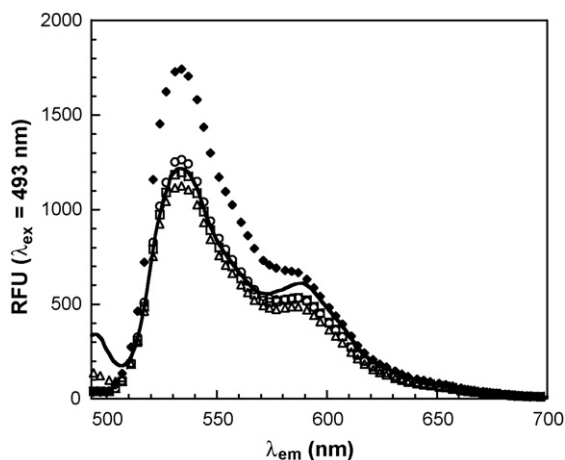


Fig. 3. Effect of complementary DNA oligomers on energy transfer between labeled PNA and labeled bilayer lipid under low salt conditions. PNA liposomes have the composition (P2:P1:DPPE-rhod:DSPC:chol 1:4:1:74:20), with [PNA] = [DNA] = 2 μ M in 50 mM Tris–HCl (pH 8.0). The DNA sequence is 5'-T_X-GTAGATCACT-T_X-3'. (○) 10-base DNA (X = 0); (□) 20-base DNA (X = 5); (◆) 40-base DNA (X = 15); (△) 60-base DNA (X = 25). Solid curve corresponds to PNA liposomes in the absence of DNA. [P2] = [DPPE-rhod] = 0.4 μ M.

nied by an increase in the rhodamine peak when both groups were present in the lipid formulation. We calculated the average donor–acceptor distance (R) to be 5.91 nm and $R_0 = 6.78$ nm from Eqs. (1)–(5) (see Section 2), a reasonable value given the low coverage of P2 and DPPE-rhod on the liposome surface.

The fluorescence spectra for the labeled PNA liposomes (containing 1:4:1:74:20 of P2:P1:DPPE-rhod:DSPC:cholesterol) in 50 mM Tris–HCl buffer (pH 8.0) showed no changes from the control experiment (solid line) on addition of an equimolar amount of complementary DNA oligomers 10–20 bases in length (Fig. 3). When we repeated the experiment with 40-base DNA, we noticed an increase in the fluorescein peak, indicating reduction of energy transfer, most likely due to extraction of PNA from the lipid matrix. Interestingly, when we carried out an experiment with 60-base DNA, the FRET signal once again matched the control experiment. We believe that electrostatic repulsion between the liposome surface and DNA in solution may prevent PNA/DNA hybridization with 60-base DNA. Fig. 4 shows results of the same experiment under high salt conditions (1 M Tris buffer, pH 8.0). In this case, we observed that FRET signal did not change from the control for all oligomer lengths tested (10, 20, 40 and 60 bases). Taken together, we conclude that desorption of the PNA from the liposome surface only occurs when an equimolar amount of 40-base DNA is added.

The low salt data of Fig. 3 were carried out in 50 mM Tris–HCl buffer, the same conditions used in our previous CE work, where hybridization and retention of 10- and 20-mers DNA to the PNA liposomes was conclusively demonstrated. Apparently, these shorter DNA oligomers do not provide sufficient electrostatic repulsion to drive desorption of the PNA/DNA duplex from the lipid bilayer. In the case of 40-base DNA, the attenuation of FRET signal can only be explained by a hybridization-induced desorption of the PNA from the liposome. This is likely caused by the added repulsion between the

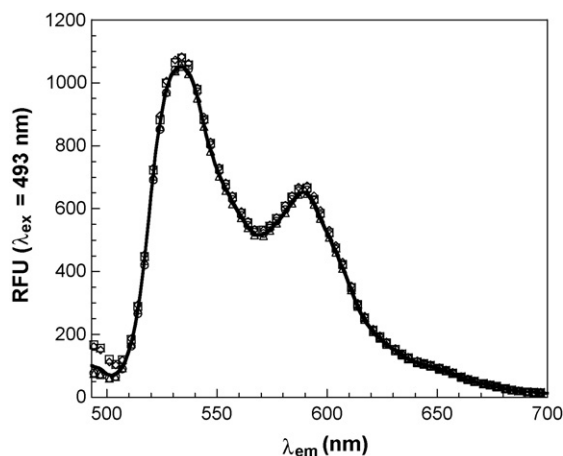


Fig. 4. Effect of complementary DNA oligomers on energy transfer between labeled PNA and labeled bilayer lipid under high salt conditions. PNA liposomes have the composition (P2:P1:DPPE-rhod:DSPC:chol 1:4:1:74:20), with [PNA] = [DNA] = 2 μ M in 1 M Tris–HCl (pH 8.0). The DNA sequence is 5'-T_X-GTAGATCACT-T_X-3'. (○) 10-base DNA (X = 0); (□) 20-base DNA (X = 5); (◆) 40-base DNA (X = 15); (△) 60-base DNA (X = 25). Solid curve corresponds to PNA liposomes in the absence of DNA. [P2] = [DPPE-rhod] = 0.4 μ M.

longer bound DNA and the negatively charged liposome. In the case of 60-base DNA, one would expect PNA desorption to occur as well, but the FRET signal is retained, and we conclude that PNA/DNA hybridization is now kinetically limited by the larger electrostatic barrier between the liposome and the 60-base DNA.

No PNA extraction was observed under high salt conditions (1 M Tris buffer, pH 8.0) for any of the DNA oligomers. Studies on PNA peptides [7] and PNA amphiphiles [11] have shown a weak stabilization of the PNA/DNA duplex on addition of salt. Additionally, the liposomes carry a slight negative charge and one would expect added salt to screen repulsions between vicinal DNA and the PNA liposomes. As such, we expect that the complementary DNA oligomers bind PNA under high salt conditions (1 M Tris–HCl) without desorption from the liposome surface for all oligomer lengths tested. It should be noted that we cannot completely rule out a lack of PNA/DNA binding under high salt conditions as this could not be measured using CE or labeled DNA oligomers for reasons described above.

These conclusions are supported by calculations of the electrostatic repulsion between the liposome surface and DNA oligomers in solution using the linearized Poisson–Boltzmann equation. The charge on the DNA oligomer (Q) was estimated by multiplying the number of phosphate groups by the fraction of charges not compensated for by Manning condensation (0.56) [23]. The surface potential of the PNA liposomes was taken from CE-derived electrophoretic mobility data. For the two salt conditions studied, the Debye screening length (κ^{-1}) was 1.36 and 0.31 nm, and given the radius of the liposomes (~ 100 nm) the Helmholtz–Smoluchowski expression gives a reasonable estimate of zeta potential (ζ) for the PNA liposomes [24]:

$$\zeta = \frac{u\mu}{\epsilon_r \epsilon_0} \quad (6)$$

Here u is the liposome mobility, μ the viscosity of the solution (0.0089 poise), ε_r the dielectric constant of water (78.5), and ε_0 is the dielectric permittivity ($8.85 \times 10^{-12} \text{ C V}^{-1} \text{ m}^{-1}$). Previous CE experiments showed that the PNAA liposomes had $u = -3.5 \times 10^{-4} \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ [8], and this value changed only slightly with the addition of complementary DNA. Eq. (6) gives $\zeta = -44.8 \text{ mV}$ for the PNAA liposomes, which is a good approximation of the surface potential (ϕ_0).

An expression for the electrostatic interaction energy (W) between a linear string of charge with uniform charge density and a charged surface a distance R away was given by Tsao [25]. In the absence of fixed dipole interactions, the expression is as follows:

$$W(R) = Q\phi_0 \left[2 \exp(-\kappa R) \mp \frac{1}{8} \frac{\kappa Q}{4\pi\varepsilon_r\varepsilon_0\phi_0} \int_{-1}^1 \int_{-1}^1 \left[\frac{\exp[-\kappa |2\vec{R} + (L/2\eta)\vec{p} - (L/2\eta')\vec{p}'|]}{|2\vec{R} + (L/2\eta)\vec{p} - (L/2\eta')\vec{p}'|} \right] d\eta d\eta' \right] \quad (7)$$

where L is the length of the linear string of charge, \vec{R} is a vector directed normal to the surface and terminating at the center of the string, \vec{p} a unit vector in the direction of the string orientation, and \vec{p}' is a unit vector in the direction of string's charge image. The sign of the RHS of the expression gives either the constant charge or constant potential solution.

To apply Eq. (7) to our situation, some simplifying assumptions were made. First, the DNA oligomer was treated as an extended linear string of charge, with length equal to the contour length of DNA oligomers (0.43 nm/base) [26]. The chain was oriented parallel to the liposome surface. The Tris–HCl buffer was treated as a 1:1 electrolyte so κ was calculated by:

$$\kappa = 3.30 \times 10^9 \sqrt{C_0} \quad (8)$$

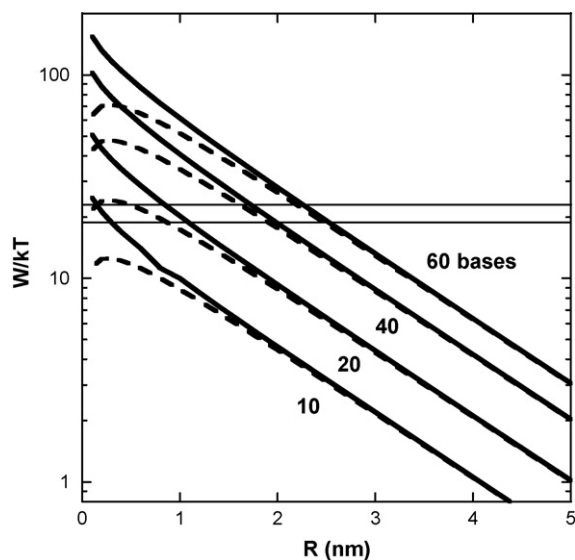


Fig. 5. Electrostatic repulsion (W) between the liposome surface and a fully extended DNA oligomer of varying length (10–60 bases) oriented parallel to the liposome surface under low salt conditions (50 mM 1:1 electrolyte). Calculations were made by Eqs. (7) and (8) using parameters described in the text (both constant potential and constant charge solutions are shown). Horizontal, solid lines correspond to the PNA/DNA binding energy (19.3 kT) and energy of extraction for a 14-carbon di-alkyl lipid (22.8 kT).

where C_0 has units of [M] and κ has units of [nm^{-1}]. Results of this calculation are given in Fig. 5 (50 mM Tris–HCl) and Fig. 6 (1 M Tris–HCl) for each of the DNA oligomers studied here. We have also included the free energy of hybridization for the PNAA/DNA duplex, taken from a van't Hoff analysis of UV-melting data (-47.8 kJ/mol ; 19.3 kT) [11] and the energetic penalty for extraction of a 14-carbon di-alkyl lipid from a bilayer matrix (22.8 kT) [27].

For a fair comparison, we need to specify the location of the 10-mer binding site of the PNAA near the lipid bilayer. While this cannot be conclusively specified, the contour length of the ethylene glycol spacer of P1 (4 EO monomers) and P2 (2 EO

monomers plus the fluorophore) is about 1.5–2.0 nm [28]. During the design of the PNAA systems, we found that the use of the spacer was required to provide for DNA hybridization, even though the PNA binding sequence is about eight times longer than the phospholipid headgroups. This indicates that without a spacer, the PNA peptide lies flat on the liposome surface, shielding it from interactions with oligomers in solution. The addition of the spacer may lift the PNA peptide from the surface, maintaining its flat orientation.

The observed FRET results can be explained by a simple energetic comparison of electrostatic repulsion and PNAA/DNA binding energy. At low salt conditions, the electrostatic repul-

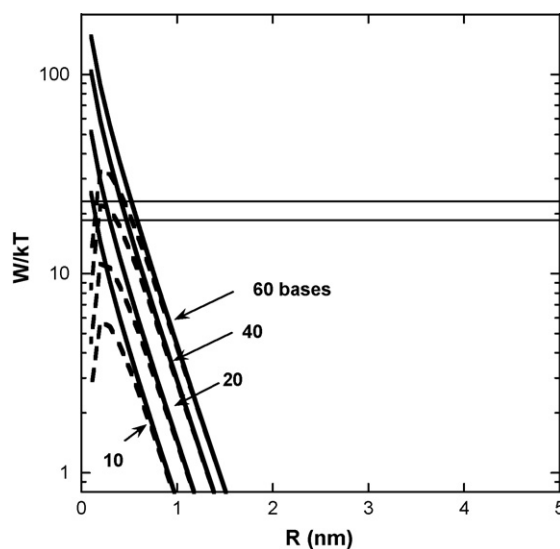


Fig. 6. Electrostatic repulsion (W) between the liposome surface and a fully extended DNA oligomer of varying length (10–60 bases) oriented parallel to the liposome surface under high salt conditions (1 M 1:1 electrolyte). Calculations were made by Eqs. (7) and (8) using parameters described in the text (both constant potential and constant charge solutions are shown). Horizontal, solid lines correspond to the PNA/DNA binding energy (19.3 kT) and energy of extraction for a 14-carbon di-alkyl lipid (22.8 kT).

sion at $R = 1.5$ nm is lower than the PNAA/DNA binding energy for 10- and 20-mers, but the electrostatic repulsion is larger for the 60-mer. Binding and retention of DNA is favorable for the 10- and 20-mers, so PNAA desorption does not occur. The 60-mer DNAs do not bind the PNAA, so no desorption can occur. About 40-mer DNA have a repulsion comparable to both the binding energy and the lipid extraction energy. Here, we expect that the repulsive barrier can be surmounted by Brownian motion over a reasonable time scale, allowing binding to occur. At that point, the kinetics of dissociation of the duplex prevent the immediate separation of the strands and the PNAA/DNA duplex desorbs instead. A similar hybridization-induced desorption of PNAA from surfactant micelles has been observed by sequence-dependent shifts in the critical micelle concentration [29]. In 1 M Tris–HCl, the electrostatic repulsion is comparably small at $R = 1.5–2.0$ nm and hybridization can occur without electrostatically-induced desorption.

4. Conclusions

Fluorescein-labeled PNA amphiphiles were synthesized and co-extruded with rhodamine-labeled phospholipids to yield PNA liposomes exhibiting fluorescence resonance energy transfer (FRET) between the two fluorophores. In the presence of a 40-mer DNA complement in 50 mM Tris–HCl, a hybridization-induced desorption of the PNAA from the lipid bilayer was observed by an attenuation of FRET signal. FRET signal was maintained after introducing shorter oligomers, or oligomers between 10 and 60 bases in high salt (1 M Tris–HCl). In low salt (50 mM Tris–HCl), FRET signal was also maintained in the presence of 60-mer complements, which evidently fail to bind to PNAA due to longer-ranged electrostatic repulsion. While this observation places some limits on the range of ionic strength and target DNA lengths that will be retained in liposomes, we have also shown that hybridization-induced desorption of PNAA is mitigated under high salt conditions where electrostatic repulsion between the oligomer and the liposome surface is screened.

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