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Binding of phosphorothioate oligonucleotides to zwitterionic liposomes

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Abstract

Although double-stranded DNA (dsDNA) has been shown to bind to zwitterionic lipids, it has been reported that this association is stronger for disordered (L_α) phase lipids than for well-ordered (L_β) lipids. In this work, the interaction of single-strand phosphorothioate oligonucleotides (ONs) with unilamellar liposomes of saturated and unsaturated zwitterionic phosphocholines (PCs) and phosphoroethylamine (PE) was investigated. It is shown that the association of phosphorothioate ONs to diacyl glycerophosphocholines is strong, but only for L_β phase or otherwise ordered bilayers. There is no measurable affinity for PE lipids. The apparent affinity of three different phosphorothioate ONs for L_β phase 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) has been measured and the dissociation constants were on the order of 10^{-7} M. Purine-rich ON sequences had stronger binding to DPPC liposomes than did pyrimidine-rich sequences, but there were other sequence-dependent factors. This exceptionally high affinity could be an important consideration in ON uptake, delivery, and biodistribution.

Keywords: Antisense oligonucleotide; DNA; DPPC; Lipid; Phosphorothioate

1. Introduction

Successful development of oligonucleotide (ON) therapeutics requires that these large, highly charged molecules penetrate the cytoplasmic membrane and enter the cell. Depending on the route of administration, additional membrane crossings may also be required. Thus, it is important to understand the details of ON interactions with membranes and their components.

Many endogenous, cationic membrane components are capable of binding DNA. Sphingolipids and basic nuclear proteins bind to double-stranded DNA (dsDNA). Synthetic cationic lipids such as DOTAP are widely used for transfection [3–5], and the structure of dsDNA-cationic lipid bilayers has been determined by X-ray diffraction [6,7].

Anionic phosphorothioate ONs have been shown to interact strongly with anionic lipids either in monolayers [8] or bilayers [9] through divalent cation mediators such as calcium. The structure of the lipid assembly is altered by the binding of the divalent cation and altered differently by the binding of divalent ion and single-strand DNA (ssDNA) (Rhodes et al., unpublished data). It appears that the effect of the divalent ion is to increase order in the lipid assembly and the effect of the ssDNA is to disorder the lipid assembly, whether the lipids are in the form of a monolayer or bilayer. More recently, it was shown that the conformations of ONs are altered by ion-mediated binding to anionic lipids [9].

Since successful delivery of DNA to cells implies close interaction of the DNA with cellular membranes that contain large amounts of zwitterionic lipids, it is important to understand how double-strand DNA (dsDNA) or ONs interact with zwitterionic lipid bilayers. Polynucleotides or RNA can bind to phosphocholine (PC) or PC–cholesterol mixtures in the presence of divalent cations (Mg^{2+}, Ca^{2+}, etc.) [10–12]. It is less well known that dsDNA or ONs can associate with bilayers of zwitterionic lipids such as PCs without divalent ion mediators. Bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) supported on flat surfaces were shown by scanning microscopy to bind dsDNA [13]. Unsaturated lipids such as 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were shown to bind dsDNA even more effectively, and a theoretical model was developed which explained the binding in terms of greater flexibility of DOPC [1,2]. Whether this is a general phenomenon which applies to zwitterionic liposomes was not known. This report describes qualitative and quantitative measurement of ON interactions with zwitterionic lipid bilayers.

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2. Materials and methods

2.1. Materials

All lipids [DPPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), DOPC, 1,2-ditricosanoyl-*sn*-glycero-3-phosphocholine (DTPC), 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)] sodium (DPPG), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoroethylamine (DPPE), 1-palmitoyl-2-oleoyl-*sn*-glycer-3-phosphoroethylamine (POPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoroethylamine (DOPE)] were purchased from Avanti Polar Lipids (Alabaster, AL), and were used without further purification. Phosphorothioate ONs were generously donated by ISIS Pharmaceuticals, Inc. (Carlsbad, CA), and were used without further purification. ON sequences used in this work are shown in Table 1. *R*, the ratio of the number of purines to the number of pyrimidines, ranged from 0 to 2.33.

Oligreen ssDNA label was purchased from Molecular Probes (Eugene, OR). All other reagents were purchased from Sigma (St. Louis, MO). Distilled water was deionized to >18 MΩ cm using a Barnstead Nanopure water filtration system. HEPES buffer (10 mM, pH 7.4) was used for all experiments.

2.2. Liposome preparation

Liposomes were prepared by extrusion, as described previously [9]. Briefly, lipid stock solution in chloroform (40 mg/ml) was shell-dried in test tubes under dry nitrogen, and then further dried under vacuum overnight. After adding buffer to the dried liposomes to yield a 10 mg/ml suspension, the capped tube was heated to a temperature greater than the main phase transition (melting) temperature (T_m) for 15 min, with repeated vortexing for 2 min. This procedure yielded a multilamellar vesicle (MLV) suspension. The MLVs were extruded 21 times at $T>T_m$ with an Avanti extruder using a 200-nm microporous filter.

2.3. Gel exclusion

ONs were added to the liposomes following extrusion. To a 10 mg/ml suspension of 200 nm liposomes, ON was added to yield the desired concentration, typically 600 ng/ml. Following 60 min of equilibration at a specific temperature, the samples were fractionated by size exclusion using a low pressure Sephadex G-75 column (typically 15 × 250 mm). Samples were equilibrated and columns run at 4, 10, 22 and 28 °C using temperature-controlled rooms. Liposomes emerged in the void volume and ONs were partially retained as a baseline separated peak (Fig. 1A).

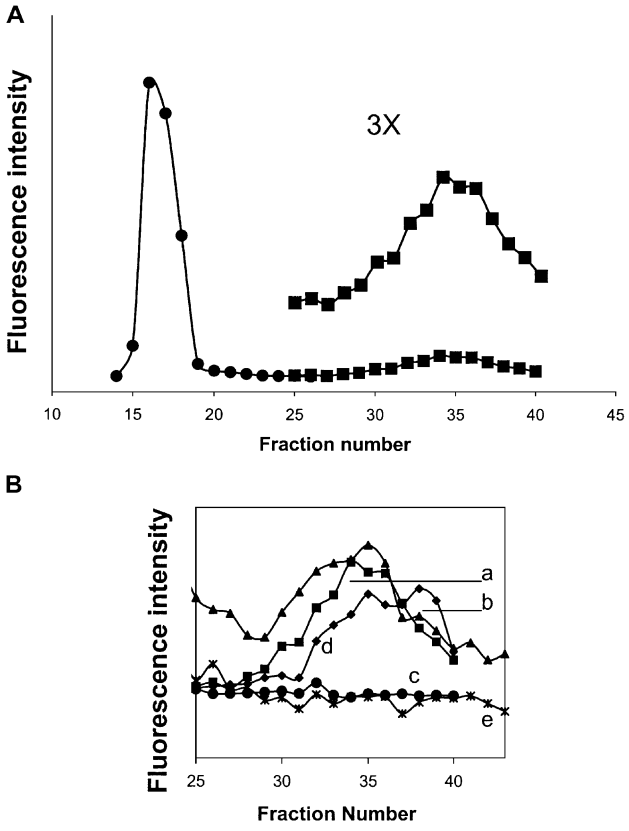

Fig. 1. Typical elution profiles for liposomes and oligonucleotides run individually on G-75 Sephadex. (A) Liposome alone (●) and oligonucleotides alone (■). (B) Elution profiles for oligonucleotides alone and in mixtures with different liposomes; three-fold of original intensity (a) Oligonucleotide alone (■), (b) DPPG + ODN (▲), (c) DPPC + ODN (●), (d) DOPC + ODN (♦), (e) DTPC + ODN (∗).

2.4. Fluorescence assays

Samples were assayed using a fluorescence spectrophotometer (Spex 332). Liposome containing fractions could be detected visually as a result of light scattering, and for most experiments, were assayed in the spectrophotometer using

Table 1
ON sequences used in this work

Compound	ISIS no.	Sequence	*R*
1	T27	5′-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-T-3′	0.00
2	1939	5′-C-C-C-C-C-A-C-C-A-C-T-T-C-C-C-C-T-C-T-C-T-C-3′	0.11
3	6547	5′-G-T-G-C-T-C-A-T-G-G-T-G-C-A-C-G-G-T-C-T-3′	0.55
4	5652	5′-T-T-G-G-G-G-T-T-G-G-G-G-T-T-G-G-G-G-T-T-3′	1.50
5	4274	5′-C-A-T-G-G-C-G-G-G-A-C-T-A-C-G-G-G-G-G-C-C-3′	1.63
6	2855	5′-G-T-G-G-G-C-C-A-T-G-A-T-G-A-T-G-G-A-A-G-G-3′	2.33

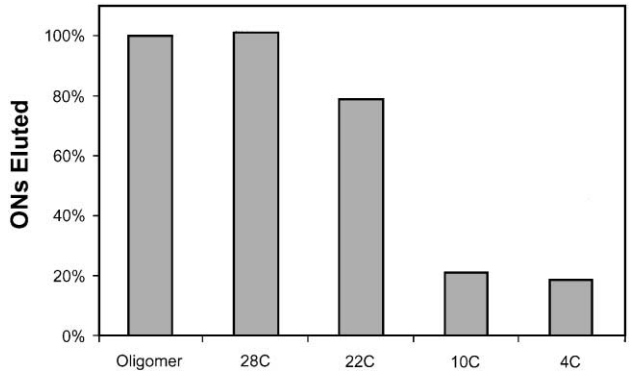

Fig. 2. Elution of ON **3** in the presence of DMPC.

the light scattering signal. ONs were assayed using a modified Oligreen assay. Oligreen concentrated reagent was diluted in near-darkness 1:800 in 10 mM Hepes buffer, pH 7.4. In the dark, 100 μl of the fraction to be assayed, 500 μl of Oligreen working stock, and 400 μl of Hepes buffer were added to a disposable polystyrene cuvette. The samples were incubated for 10 min and fluorescence measured using $\lambda_{ex}=493$ nm and $\lambda_{em}=524$ nm. To protect samples from photobleaching by ambient light, all sample handling and readings were performed in a darkened room.

2.5. Binding affinity

ONs were added to 400 μl of 4 mg/ml extruded liposome suspension in polypropylene microcentrifuge tubes to yield final ON concentrations of 60–1800 nM. Buffer was added to yield a final volume of 0.5 ml. Control samples contained ON only (oligomer control) or liposomes only (liposome control). All samples were allowed to incubate for 60 min at 20 °C and then centrifuged at 28,000 × *g* for 30 min at 20 °C. From each sample, 20 μl of supernatant was removed and assayed for ON using the Oligreen assay. The fluorescence intensities (*F*) of the samples were corrected using $F_{\text{liposome control}}$ to account for light scattering from liposomes that may not have pelleted. For the total ON concentration, $F_{\text{oligomer control}}$ (corrected for instrument blank) was used to allow for possible loss of ON from the supernatant. The fraction of ON bound was calculated by:

$$\text{Fraction bound} = \frac{(F_{\text{oligomer control}} - F_{\text{blank}}) - (F_{\text{sample}} - F_{\text{liposome control}})}{(F_{\text{oligomer control}} - F_{\text{blank}})}.$$

These results were interpreted as binding data using the lipid concentration (4.29 mM), and an apparent dissociation constant was calculated using a Scatchard model. Although the lipid concentration was an overestimate (since the inner bilayer leaflet was not available to the ON) and it is likely that more than one lipid is involved per ON, this calculation was intended to provide an "apparent affinity" and yields a conservative estimate.

3. Results

Based on the repulsive interaction due to like molecular charges and previous results using Langmuir films, ONs were not expected to bind to anionic liposomes in the absence of divalent cation [8]. Fractionation of mixtures of ON **3** and DPPG liposomes showed that the liposomes and ON **3** eluted at the positions expected based on control experiments in which the individual components were run (Fig. 1B, trace b). The amount of ON **3** eluting in the second peak is approximately equal to the total amount of ON **3** applied to the column. Therefore, ON **3** appeared not to have significant affinity to DPPG liposomes.

When ON **3** was mixed with DPPC liposomes and then subjected to the same fractionation procedure, the elution pattern was quite different; no free ON could be detected (Fig. 1B, trace c). This assay is sensitive to 5 ng/ml, less than 1% of the starting concentration. To further characterize the strong association of this ON **3** with DPPC, similar experiments were conducted using liposomes made with other lipids.

Since work by others with supported lipid bilayers had shown that the affinity of DNA for PC lipids was affected by phase [1,2,13], additional experiments were performed using DOPC, which has a main thermal phase transition ($L_\beta \rightarrow L_\alpha$) at −18.5 °C [14], and DTPC, which has a main thermal phase transition ($P_\beta' \rightarrow L_\alpha$) at 78.9 °C [15]. Thus, at 22 °C, the bilayer phases for DOPC and DTPC were L_α and L_β, respectively. Fig. 1B (traces d and e) showed that ON **3** had negligible association with DOPC (L_α) liposomes and essentially complete association with DTPC (L_β) liposomes. The trends of these observations were opposite to those of Fang and Yang [2] and the theoretical explanation of Dan [1]. In order to confirm that our result was a general phenomenon based in lipid ordering, further investigation

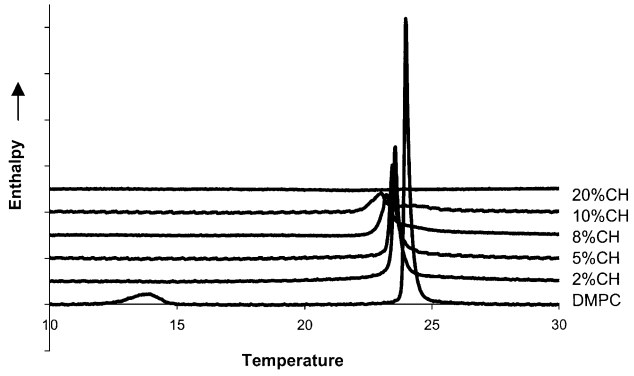

Fig. 3. DSC thermograms from DMPC and DMPC–cholesterol mixtures. Scans are offset in the vertical direction for clarity, but are not offset along the *T* axis.

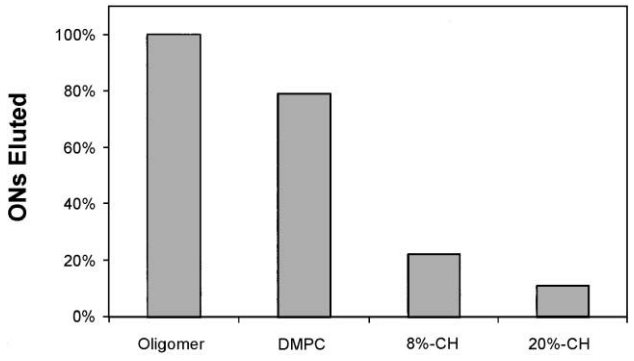

Fig. 4. Unbound oligonucleotide in oligonucleotide–liposome mixtures at 22 °C. Increased cholesterol content in the liposomes decreased the level of unbound oligonucleotide.

of the lipid phase dependence of ON association was undertaken.

Bilayers of DMPC exhibit a phase transition ($\ell_o \rightarrow \ell_d$) at 23.5 °C [16] that allows for facile manipulation of these lamellar phases under moderate temperature conditions. Using Hepes buffer, which has a low temperature coefficient, ON **3**-liposome association measurements were carried out at 4, 10, 22 and 28 °C. Fig. 2 shows that ON **3** bound to DMPC liposomes only at temperatures corresponding to ℓ_o phase. For liposomes at temperatures above T_m (ℓ_d phase) or at temperatures between the pretransition temperature, T_p (13.5 °C) and T_m, there was no measurable ON associated with the liposomes.

Addition of cholesterol increases ordering of the hydrophobic chains in DMPC bilayers. Depending on the conditions, ℓ_o or ℓ_d phases could coexist below T_m [17–19] and the relative proportion of each shifted by addition of cholesterol. To demonstrate that ON association with PC lipid bilayers is related to lipid order, various amounts of cholesterol were added to DMPC preparations. DSC results (Fig. 3) confirm that the phase composition is gradually

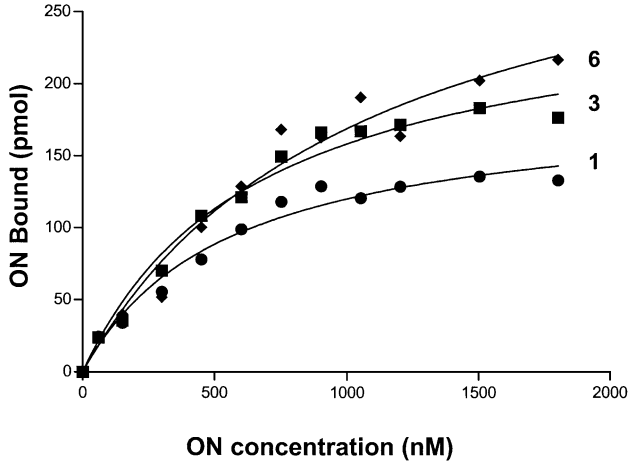

Fig. 5. Binding of ON **1** (●), **3** (■) and **6** (♦) to DPPC liposomes. The DPPC concentration was 4 mg/ml (4.29 mM) and the oligonucleotide concentrations ranged from 60 to 1800 nM.

Table 2
Scatchard parameters for ON **1**, **3** and **6** binding to DPPC liposomes

	1	**3**	**6**
B_{max} (nM)	375	526	659
K_D (nM)	559	658	739
ΔG (kCal)	−4.32	−4.43	−4.51

shifted by addition of cholesterol, consistent with previous data [19–21] which demonstrated increased lipid order, in spite of lower T_m and decreased ΔH. Fig. 4 shows that at 22 °C, near the phase transition temperature, addition of 8% or 20% cholesterol significantly increased the degree of ON **3** binding to liposomes. Compared to DMPC alone, addition of 20% cholesterol increased ON **3** binding by ~78%. These results provided convincing evidence that ON **3** bound preferentially to ordered phases.

A centrifugation assay was used to quantitatively measure the affinity of ON **3** binding to liposomes. Typical binding data are shown in Fig. 5. The binding isotherm appears to reflect saturable binding even though the estimated coverage of the liposome (based on typical molecular dimensions) was less than 2% at the highest ON concentrations. Although the binding of a molecule of ON **3** to the liposome is likely to involve a site with more than one lipid molecule, we do not yet have enough information to estimate the number of lipids involved per ON **3** molecule. Therefore, dissociation constants were calculated with Scatchard analysis, using a molar concentration for both lipids and ON **3**. In other words, the "binding site" for the ON was assumed to be a single lipid molecule. Using this conservative assumption, $\boldsymbol{K}_D = 658$ nM for ON **3** and DPPC.

Two additional ONs were tested to demonstrate that this high affinity association is a general property, not unique to a specific ON **3** sequence. Using the binding assay described above, dissociation constants were also measured for ON **1** and ON **6**. Table 2 summarizes the results of these binding measurements. All three ONs exhibited high-affinity, saturable binding to ordered DPPC liposomes. The interaction between liposome and ONs is favorable from the free energy point of view.

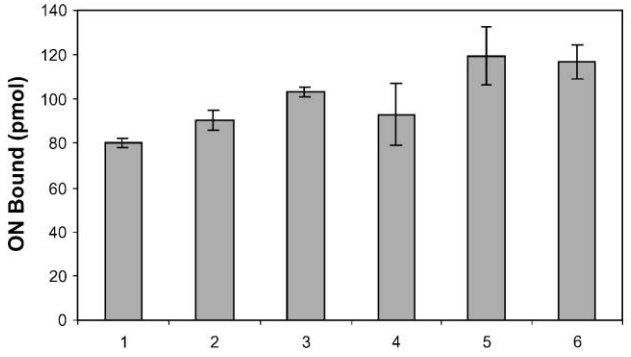

Fig. 6. Binding of ONs to DPPC liposomes. The DPPC concentration was 4 mg/ml (4.29 mM) and the oligonucleotide concentration was fixed at 902 nM. *R* is ratio of number of purines to pyrimidines.

Some reports have shown that ON uptake is sequence dependent. Iverson et al. [22] demonstrated that purine-rich ONs have higher cellular uptake than do those with pyrimidine-rich sequences. To assess base composition dependence, binding measurements were performed using six different ON sequences with varying purine/pyrimidine ratios (R). Identical concentrations of DPPC liposomes (4 mg/ml) and ONs (600 ng/ml) were used in each assay. Fig. 6 shows that for ON **1** ($R=0$), 21% of the ON was bound under these conditions, but for ON **6** ($R=2.33$), 31% was bound. In general, a trend was observed in which binding increased with R, increasing 46% as R increased from 0 to 2.33. The point at $R=1.5$ appears to deviate from this trend (see Discussion).

Phosphoroethylamine (PE) is a major zwitterionic component in cell membrane lipids. PE and PC together can comprise 50–60% of total lipid in some cells. The only structural difference between PE and PC is the amine in the headgroup. To determine whether the observed affinity of ON for PC was a general property of zwitterionic lipids, we repeated the centrifugation binding assay using DPPE and POPE. DSC with POPE and DPPE indicated that at $T \leq 22$ °C, DPPE and POPE were in an ordered phase. The fraction of ON **3** bound to DPPE or POPE at $T=20$ °C or $T=4$ °C was less than 2% (Fig. 7). The assay could not be performed with DOPE since these lipids did not form good pellets.

Binding affinity of ON **3** to mixtures of DPPC and DPPE exhibited a lipid composition-dependence (Fig. 8). For lipid compositions with $\leq 50\%$ DPPC, a floating aggregated phase appeared following extrusion, so neither the centrifugation assay nor the chromatographic assay could be performed. For the lowest measurable DPPC content (52% DPPC), the affinity was quite low, but at higher proportions of DPPC the affinity increased to a maximum at $\sim 80\%$ DPPC. The affinity appeared to remain at a high level from 80% DPPC to 100% DPPC.

Aggregation was not observed under any of the conditions described in this study. Aggregation and/or precipitation has been observed under other conditions (e.g. very high salt concentrations, some cationic lipid formulations) and corresponding changes in the spectroscopic characteristics of the ONs have been documented. However, none of these indicators suggest that aggregation had occurred in this study.

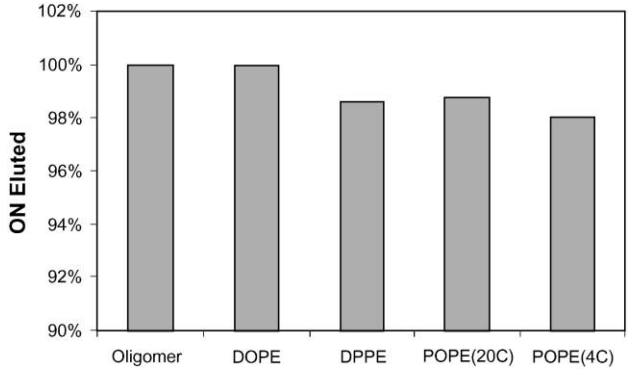

Fig. 7. Binding of ON **3** to PE liposomes. The PE concentration was 4 mg/ml and the ON concentration was 902 nM.

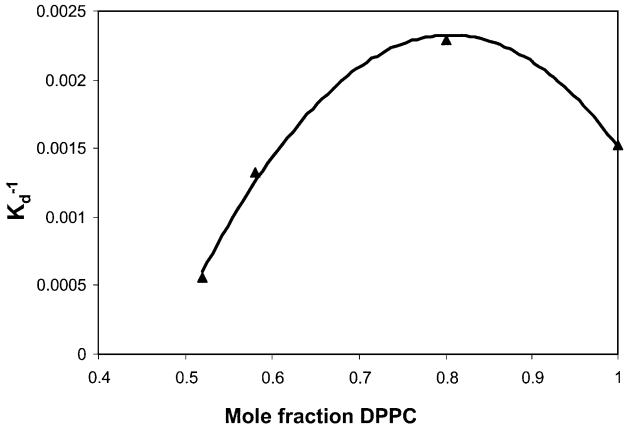

Fig. 8. Binding affinity of DPPC–DPPE mixtures.

4. Discussion

In the centrifugation assay, it was expected that all or nearly all of the liposome and/or liposome–ON complex would be pelleted. We carried out a liposome control experiment to confirm this assumption using light scattering. In regions of the UV–Vis spectrum where no absorption bands exist, any optical density (OD) should be due to light scattering. The functional form of the absorption spectrum of extruded DPPC liposomes confirms this, and standard curves based on OD at selected wavelengths with various liposome concentrations showed that this approach could be used to assess liposome content. Such measurements of DPPC supernatant after 30 min of centrifugation showed that 3.7% liposome vesicles remained in supernatant. Although this incomplete fractionation contributes to the error of determining the apparent affinity, the overall conclusion and relative magnitude of the data are not affected.

Binding of anionic ONs to cationic liposomes is clearly driven by ionic interactions. Previous results with ON binding to anionic lipids through divalent cation mediators are probably also driven primarily by ionic interactions [8]. The driving force behind DNA binding to PC (zwitterionic) bilayers is less obvious. Shepherd and Buldt [23] reported that an electrostatic (ion–dipole) interaction between DNA anions and the large dipole moment of the DPPC headgroup is a significant factor in dsDNA binding to DPPC. Theoretical calculations indicate that the binding strength between dsDNA and DPPC is about one-quarter that between DNA and cationic lipids [13,24]. Data from Malghani and Yang [13] clearly demonstrate DPPC/DNA affinity and calculations from Malghani and Yang [13] and Dan [1] provide feasible explanations.

According to Fang and Yang's [2] data and Dan's [1] model, the binding of a uniformly charged DNA rod causes lipids around the rod in a fluid-phase, disordered fluid bilayer to creep up at the sides of the rod, thus increasing the area of interaction. In this model, binding would be more effective for fluid-phase lipids since they would have more mobility. However, the physical properties of the immobilized lipid bilayers may not be representative of biological membranes in terms of dynamics and mechanical properties. Liposomes may thus represent a better model of a cellular membrane.

We have shown that ONs bind strongly to PC liposomes, and that the binding is phase dependent. Data from DOPC, DPPC and DTPC provide a basis for comparison of L_α and L_β phases, and it appears that ONs bind only to the L_β phase. Data from DMPC at 28 °C and at lower temperatures (4 and 10 °C) provide comparison of ℓ_o and ℓ_d phases. Data from DMPC–cholesterol mixtures showed increased binding with added cholesterol. We concluded more generally that ONs bind tightly to ordered PC headgroups. Furthermore, the binding of ONs to PC bilayers was composition-dependent, with purine-rich ONs exhibiting stronger binding. The binding of ON **4** was lower than would be expected from this trend. This ON is a series of three G_4 sequences and strongly favors formation of G-tetraplex [25]. This may be explained if the monomer-tetraplex equilibrium decreases the ON monomer available for binding and tetraplex binding is weaker than monomer binding.[1]

Based on spectroscopic data [26,27] and HPLC data from this laboratory (unpublished observations), ON binding to PC bilayers may be related to a specific interaction of bases with the ordered interior of lipid bilayers. Maltseva et al. [26] found that for poly (A) in the presence of liposomes, the IR bands from adenenine shift greatly. Acid treatment of poly (A) led to 70% loss of bases and elimination of poly (A) binding. DSC experiments (data not shown) carried out to determine whether the bases were buried into the bilayer core were inconclusive. Results of DSC scans with DPPC liposomes indicated negligible increases (<0.1 °C) in T_m values when ONs were added to preformed liposomes, and small ($\sim$ 10%) increases in ΔH. Present results do not allow a definite conclusion as to the location of the liposome-associated ONs. However, based on data of others [26,27] and preliminary HPLC results with immobilized artificial membrane (Regio IAM) columns, involvement of bases with the hydrophobic core is possible (unpublished data).

In our binding assays with DPPC vesicles, the weakest binding affinity was $\sim$ 700 nM (for ON **6**). The binding was saturable for all ONs tested, and K_D values were all submicromolar. The observed binding is consistent with the reported research of Juliano et al. [28,29] in which adsorption of phosphorothioate ONs to mixed component liposome (containing egg PC or DPPC). Considering the size of $\sim$ 20-base ONs and the area of PC lipids, the "binding site" probably consists of approximately 10 lipid molecules. If this estimate is correct, the effective K_D may be closer to 10^{-8} M.

Why did the ONs prefer the gel-like ordered phase rather than liquid crystal fluid phase? At room temperature, the mean area of a L_β phase DPPC headgroup is approximately 50 Å^2 [30] and the diameter is 7.9 Å. The distance between two base pairs in B-DNA is 3.6 Å. Thus, two anionic charges from the ON could be associated with each lipid headgroup. For DOPC at room temperature, however, the mean molecular area per lipid is $\sim$ 82 Å^2 [30]. The physical dimension of the headgroup has not changed, but the inter-headgroup spacing and the headgroup conformation are different due to the increased area of the fluid acyl chains. Thus, one could envision "gaps" between neighboring DOPC headgroups, such that some of the ON anions would be unable to interact with the headgroup, and the interaction between ON and lipid would be weakened.

An alternative explanation would involve affinity decreased by increased fluctuations in the disordered bilayers. The dynamic behavior of fluid bilayers is quite different from that of ordered bilayers, so the entropic contribution to the overall affinity could be compromised.

Interaction of ON **3** with PE lipids was quite different from that with PC lipids. The same assays were used to assess the binding interaction, but we found that for DPPE, which was in L_β phase at room temperature, there was only $\sim$ 2% ON **3** binding. For POPE at 20 and 4 °C the bound fractions were 1.26% and 1.99%, respectively. Thus, even for ordered phases of PE lipids, there is no significant interaction with ON **3**. The likely explanation for the difference between PC and PE is the headgroup chemistry. The nitrogen in the PC headgroup is in a trimethylamino group, but the nitrogen in PE is in a primary amine, which easily forms hydrogen bonds with water or neighboring lipid headgroups. In our assay, PE–PE interaction through hydrogen bonding could be preferred over PE–ON interaction. Alternatively, the tendency of PE to form inverted hexagonal (H_{II}) phase could affect the lipid–lipid interaction and/or the local surface structure on the membrane.

For DPPC–DPPE mixtures, ON **3** binding increases with increasing DPPC content, but ON **3** does not bind until DPPC content is over 52% and reaches maximal binding at 80% DPPC. We do not have sufficient information yet for a definitive model to explain these results, but the immiscibility of DPPC and DOPE [15] suggests a possible explanation. If the association of ON with PC requires an ordered domain and the order in a PC domain in a mixed-composition system diminishes near the edge of the domain, it stands to reason that the area of ordered PC might not reach adequate size until a critical composition. Similarly, once an

[1] That the $R=1.5$ data point was significantly lower was supported by fitting the data to a second order polynomial with and without the $R=1.5$ data point. The calculated R^2 values were 0.36 and 0.91, respectively. Other methods of analysis also demonstrated that the $R=1.5$ data point was significantly lower than expected based on the other results.

adequate ordered PC area has been achieved such that the equilibrium distribution of bound ON has been reached, additional surface area will not affect the proportion of bound PC. This is entirely speculative; a definitive model will require additional data.

The binding of ON to lipid membranes is a key component of uptake. The observed high affinity binding of ON to PC lipid bilayers may have important implications for cellular uptake and subcellular distribution of these macromolecules. The accepted mechanism for cellular uptake of ONs is by receptor-mediated endocytosis [31], but there is clear evidence of receptor-independent uptake as well [32]. The binding of anionic molecules like ONs to cationic membrane components like sphingolipids suggests one possible mechanism of membrane association, but the binding of ONs to the more ubiquitous PCs would imply a potentially more widespread uptake mechanism. Additional work in our laboratory is exploring the possibility that other well-ordered domains including PC and other lipids may be involved in ON interaction with cell membranes.

5. Conclusions

The association of ONs with PC liposomes is surprisingly strong and dependent on the lipid phase. In contrast to predicted binding behavior based on dsDNA adsorption to supported bilayers, the association of ONs with liposomes requires L_β-phase bilayers. Theoretical analysis of dsDNA-lipid association suggested that the enhanced binding of dsDNA to L_α bilayers was due to flexibility in the bilayer structure arising from the fluid acyl chain. We suggest that these results differ from ours because liposomes are capable of deformation in the bilayer normal direction, whereas the supported bilayers are rigidly constrained.

Note added in proof

The importance of lipid order in ON association with lipids is emphasized by our recent results with sphingolipids, naturally occurring cationic lipids. We (A. Schenkel) showed that several ONs exhibit high affinity for certain mixed-composition, sphingolipid-containing liposomes. This is not surprising, considering the likely charge-charge interaction. Under the conditions where high affinity ON binding was observed, the sphingolipid-containing liposomes appeared to be well ordered, based on DSC experiments. We were very interested to note that when the well ordered sphingolipid domains were disrupted by doing the binding experiments at $T>T_m$, ON binding became insignifi cant. This did not appear to be due to conformational changes of the ON because the circular dichroism spectrum of the ON did not decrease significantly until much higher temperatures. Thus, even in certain cationic lipid systems, lipid order is a prerequisite for ON binding.

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