Interactions of antisense DNA oligonucleotide analogs with phospholipid membranes (liposomes)

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ABSTRACT

Antisense oligonucleotides have the ability to inhibit individual gene expression in the potential treatment of cancer and viral diseases. However, the mechanism by which many oligonucleotide analogs enter cells to exert the desired effects is unknown. In this study, we have used phospholipid model membranes (liposomes) to examine further the mechanisms by which oligonucleotide analogs cross biological membranes. Permeation characteristics of ³²P or fluorescent labelled methylphosphonate (MP-oligo), phosphorothioate (S-oligo), alternating methylphosphonate-phosphodiester (Alt-MP) and unmodified phosphodiester (D-oligo) oligodeoxynucleotides were studied using liposomal membranes. Efflux rates ($t_{1/2}$ values) at 37°C for oligonucleotides entrapped within liposomes ranged from 7–10 days for D-, S- and Alt-MP-oligos to about 4 days for MP-oligos. This suggests that cellular uptake of oligonucleotides by passive diffusion may be an unlikely mechanism, even for the more hydrophobic MP-oligos, as biological effects are observed over much shorter time periods. We also present data that suggest oligonucleotides are unlikely to traverse phospholipid bilayers by membrane destabilization. We show further that MP-oligos exhibit saturable binding (adsorption) to liposomal membranes with a dissociation constant (K_d) of around 20nM. Binding appears to be a simple interaction in which one molecule of oligonucleotide attaches to a single lipid site. In addition, we present water-octanol partition coefficient data which shows that uncharged 12–15 mer MP-oligos are 20–40 times more soluble in water than octanol; the low organic solubility is consistent with the slow permeation of MP-oligos across liposome membranes. These results are thought to have important implications for both the cellular transport and liposomal delivery of modified oligonucleotides.

INTRODUCTION

Antisense oligodeoxynucleotides have been used to selectively inhibit gene expression in a number of cell culture systems and have potential as therapeutic agents [for review, see 1–4]. Antisense oligodeoxynucleotides can be targeted either to specific sequences in double stranded DNA, thereby forming a local triple helix which interferes directly with transcription [5–6] or alternatively, they can be targeted to specific sequences within RNA (pre-mRNA or mRNA) molecules to inhibit gene expression by affecting RNA processing or ribosomal translation [1–3]. Using such a strategy, oligonucleotides have successfully been used in cell culture to inhibit the expression of specific oncogenes such as c-myc [7–8], and c-myb [9] and also viral genes such as those of HIV [10–11], influenza virus [12] and herpes simplex virus [13]. In addition, one example of efficacy of methylphosphonate oligonucleotides against c-myc in transgenic mice has been reported recently [14].

Early work in the antisense field was carried out using conventional phosphodiester DNA oligodeoxynucleotides (D-oligos). Although these compounds have been successfully used as sequence-specific antisense agents in some cell culture experiments [1–3], the therapeutic potential of D-oligos is limited by their poor biological stability [15–16]. As a result, structural analogs such as phosphorothioate (S-oligo) and methylphosphonate (MP-oligo) oligonucleotides with enhanced nuclease stability have been developed [1–4]. We have previously shown that MP-oligos are more resistant to nucleases present in nuclear and cytoplasmic extracts compared to S-oligos, while both are considerably more stable than D-oligos [16]. These chemically modified analogs are also thought to have improved cellular transport characteristics.

D-oligos, which are polyanions, have been reported to enter cells by receptor mediated endocytosis [17–18]. Using acridine as a fluorescent marker at the 5' end of D-oligo homopolymers, Loke et al [17] showed that uptake of these D-oligos into HL60 cells was saturable, dependent on temperature and could be competed with polynucleotides of any length, even

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mononucleotides such as ATP, provided they possessed a 5'-phosphate moiety. The authors suggested that the uptake of D-oligo was specific and were able to identify a 80 kDa putative receptor protein using oligo-(dT)-cellulose affinity chromatography. Binding of D-oligo to this protein could be competed with excess phosphorothioates, but not methylphosphonates, suggesting that the latter may utilize an alternative receptor protein or an entirely different mechanism of cellular uptake.

Early reports [17,19] on the kinetics of S-oligo uptake into cultured cells and the fact that S-oligos compete for cellular uptake with D-oligos suggests that these analogs may also enter living cells by endocytosis, presumably mediated by the same receptor protein. However, little is known about the mechanism by which MP-oligos traverse cell membranes. Since MP-oligos are neutral and relatively hydrophobic, these molecules have been previously thought to enter cells by passive diffusion [20]. In this and a companion paper [21], we present data indicating that simple diffusion is unlikely to be the predominant mode of cellular uptake for methylphosphonate oligonucleotides. Furthermore, based on our model membrane studies, we suggest that transport of oligonucleotides across endosomal membranes subsequent to endocytosis must be mediated by a mechanism other than passive diffusion.

Although living cells are the most appropriate model for studying membrane transport properties (as used in our companion paper [21]), information derived from such studies does not always lend itself to clear interpretation. An alternative approach to understanding the mechanism of cellular transport of oligonucleotide analogs is to use phospholipid vesicles (liposomes) as model membranes. Liposomes have extensively been used to study the membrane permeation characteristics of conventional drugs and small ions [22–28]. By careful selection of the lipid composition and morphology, liposomes may also provide a useful system for studying the transport of materials across intracellular membranous organelles such as endosomes. In addition liposomes have been suggested as a potential drug carrier system for antisense oligonucleotides [29–30], but little is known about the interactions of oligonucleotides with liposomal membranes.

In this paper, we report for the first time data on the interaction of oligonucleotides with phospholipid membranes and the permeation characteristics of oligonucleotide analogs across such membranes. We show that methylphosphonates and to a lesser extent the phosphorothioate analogs bind to model lipid membranes. The binding characteristics of MP-oligos suggest that a simple molecular interaction is involved in which one molecule of the oligonucleotide interacts with one binding site on the lipid membrane. Partition coefficient measurements suggest that fully modified MP-oligos, although not charged, still prefer to accumulate into the aqueous phase. The permeation of all oligonucleotide analogs across liposome model membranes was relatively slow with efflux half lives of greater than 4 days. This suggests that simple diffusion of oligonucleotides, including the MP-analogs, is unlikely to be the mechanism of cellular uptake, as biological effects in inhibiting individual gene expression are typically seen over much shorter periods of time. Indeed in the companion paper [21], we show that uptake of 15 mer methylphosphonate oligonucleotides into CHO cells (using either a ^{32}P or fluorescent label at the 5' end) occurs relatively rapidly and plateaus within 10–12 hours at 37°C.

METHODS

Oligonucleotide Synthesis

Antisense DNA oligodeoxynucleotides and their structural analogs (see Table 1 for description and sequences of oligonucleotides) were synthesized on an automated DNA synthesizer (Model 380B, Applied Biosystems, Foster City, CA) at the University of North Carolina School of Medicine. D-oligos, AM-oligo (alternating methylphosphonate and conventional phosphodiester linkages) and MP-oligos were synthesized using standard β -cyanoethyl cycles [31]. S-oligos were synthesized, and purified using HPLC, at ISIS Pharmaceuticals (Carlsbad, CA) and were a gift from Dr. C. K. Mirabelli. The terminal nucleotide of S, MP and AM-oligos contained an unmodified phosphodiester bond to facilitate 5' end radiolabelling. Di(deoxyinosine) methylphosphonate, as used in the partition coefficient studies, was prepared according to the pentavalent route of Miller, et al. [32], and the fully modified methylphosphonates (a dodecamer 5'-dATGCCCCCTCAAC-3', and a pentadecamer 5'-dCTGAAGTGGCATGAG-3') were prepared according to the trivalent route of Agrawal and Goodchild [33]. These methylphosphonate oligonucleotides were fully substituted with a methyl group at every internucleotide linkage including the terminal linkages and were purified by normal or reversed phase liquid chromatography.

5' end ^{32}P radiolabelling of oligodeoxynucleotides

Oligonucleotides were 5' end labelled with γ -[^{32}P]-labelled ATP using T4 polynucleotide kinase (Promega) in 100 mM Tris pH 7.5, 20 mM MgCl_2 , 10 mM DTT, 0.2 mM spermidine and 0.2 mM EDTA at 37°C for 30 min as recommended by the supplier. The specific activity was usually 10^8 – 10^9 cpm/ μg . Radiolabelled oligodeoxynucleotides were purified by PAGE (15% polyacrylamide containing 7M urea). Excised bands were eluted in either water (D- and S-oligos) or 20% ethanol/water (AM and MP-oligos), desalted by ethanol precipitation [34], and resuspended in water prior to use.

Preparation of fluorescent dye-conjugated methylphosphonate oligonucleotides

Methylphosphonate oligonucleotide (15 mer) with a sequence antisense to the site overlapping the AUG initiation codon of the human MDR-1 gene (see Table 1) was synthesized using an automated DNA synthesizer as described above. The oligonucleotide was detritylated, deprotected and purified according to a standard protocol [35]. In the last coupling cycle, an amino hexyl phosphate linker (AminoLink 2; Applied Biosystems, Foster City, CA) was attached to the 5' end of the oligonucleotide. The 5' aminolinked-oligonucleotide (50 μg) and tetramethylrhodamine isothiocyanate (100 μg) (10 mg/ml solution in dimethylformamide; Research Organics, Cleveland, Ohio) were conjugated in 200 mM sodium carbonate/sodium hydrogen carbonate buffer (pH 9) and the reaction mixture was left for 12–16 hours in the dark at room temperature. The fluorescently labelled oligonucleotide was separated from unreacted dye by passing the reaction mixture through a 3ml sephadex G25 column. The purity of the eluted material was checked by 15% PAGE which was carried out as described above. The purified fluorescently conjugated oligonucleotide was typically lyophilized (to reduce volume to $\sim 100\mu\text{l}$) prior to use.

Partition Coefficients

Aliquots of methylphosphonate oligomers were dried down in a vacuum evaporator. Then 0.5 ml each of octanol and water were added, and each vial was vortexed for 30 min. The phases were then allowed to separate for about 3 hrs. Equal aliquots were taken separately from the octanol and water phases and dried down as before. The dried products were then dissolved in solvent M (25% acetonitrile, 25% ethanol, 50% water), and their absorbance spectra were measured from 220 to 320 nm in a Shimadzu UV160 spectrophotometer. The peak absorbances at 250 nm, for di(deoxyinosine), or at 260 nm, for the longer methylphosphonate oligomers, were corrected for background optical density and used to calculate the partition coefficients. For the phosphorothioates we employed analysis by reversed phase liquid chromatography [36].

Preparation of Liposomes

The liposomes used in this study were composed of either: a) Egg Phosphatidylcholine (PC) 60%, Dimyristoylphosphatidylglycerol (DMPG) 20% and Cholesterol (CH) 20% w/w, b) Dipalmitoylphosphatidylcholine (DPPC) 70%, DMPG 20% and CH 20%, or c) PC 70% and DMPG 30% w/w. Phospholipids used were purchased from Avanti Polar Lipids (Birmingham, AL, USA) while cholesterol was purchased from Sigma Chemical Co. (St Louis, Mo, USA). Lipid mixtures (5–20 mg) were evaporated to dryness from chloroform solutions in a round-bottomed flask (100 ml volume). Liposomes were prepared by rehydrating dried lipid films with 1 ml phosphate buffered saline (PBS), pH 7.4, containing fluorescent or radiolabelled antisense oligonucleotides (~10 nM) at 50–55°C. The resultant multilamellar vesicles were cooled to room temperature and then either extruded through a 0.6 µm polycarbonate filter (this preparation termed MLV-liposomes) or subjected to five Freeze (in methanol/solid CO₂) And Thaw (37°C) cycles prior to extrusion through a 0.6 µm polycarbonate filter (this termed as FATMLV-liposomes) [23–24]. Particle size analysis of such FATMLV preparations, by a quasielectric light scattering method described elsewhere [37–38], indicated that vesicle diameters were typically around 460 ± 200 nm (Mean ± S.D.).

Binding (adsorption) of oligonucleotides to lipid membranes

For the adsorption studies, known amounts of 'empty' FATMLV-liposomes (prepared as described above but without entrapped oligonucleotide) were resuspended in 1 ml PBS containing 5' end ³²P labelled oligonucleotides (~10 nM) and incubated at 37°C for timed intervals. At the required time period, unbound oligonucleotide was removed from the liposomes by subjecting the preparation to 4 × 1 ml PBS vortex washes (preliminary studies showed that 4 washes were sufficient for this purpose (data not shown)). In between washes, the liposomes were microfuged at 1100 rpm for 5 min (Biofuge B, American Scientific Products). After washing, the lipid pellet was resuspended in 1 ml PBS and the amount of radiolabelled oligonucleotide strongly adsorbed onto the liposomes was determined by liquid scintillation counting.

Efflux of oligonucleotides from FATMLV-liposomes

FATMLV-liposomes (20 mg lipid) with entrapped fluorescent or radiolabelled oligonucleotides (~10 nM) to be used in efflux studies were also subjected to the above washing procedure to remove unincorporated oligonucleotide. The efflux of entrapped antisense oligonucleotides was carried out in 1 ml PBS (containing 0.01% w/v sodium azide as an antimicrobial) at 37°C. Aliquots (75 µl) of the lipid-PBS suspension were removed periodically over a period of 5 days, diluted to 1 ml with PBS and rapidly microfuged (1100 rpm; 5 min) to separate free or effluxed oligonucleotide (present in supernatant). The amount of radiolabelled oligonucleotide retained within the liposomes (pellet) was determined by liquid scintillation counting. In the case of fluorescently (TMRITC) labelled oligonucleotides, liposomes were dissolved in ethanol (1 ml) and the amount of oligonucleotide retained within liposomes was determined by visible spectroscopy (λ_{max} = 540 nm (in ethanol)). Efflux rates of tritiated sucrose ([6,6'(n)-³H] sucrose) (2 µCi per 20 mg lipid; specific activity of 5.89 Ci/mmol; Amersham, USA) and tritiated glucose (D-[1-³H] glucose) (2 µCi per 20 mg lipid; specific activity of 2.3 Ci/mmol; Amersham, USA) whose entrapment and efflux properties are well characterized [22,25–26] were used as controls.

Table 1. Molecular characteristics of oligonucleotides used.

Description of Oligonucleotide	Symbol	Length (nt)	Sequence	Target Site
Unmodified phosphodiester	D-oligo	14 mer	5' TAC CAA CCT GCC CA 3'	5' Splice site in human β -globin gene
		7mer	5' TAC CAA C 3'	
		4mer	5' TAC C 3'	
Phosphorothioate	S-oligo	14mer	5' TAC CAA CCT GCC CA 3'	AUG initiation codon of human multiple drug resistance gene (MDR-1)
Alternating phosphodiester and methylphosphonate	AM-oligo	14mer	5' TAC CAA CCT GCC CA 3'	
Methylphosphonate	MP-5'ss-oligo	14mer	5' TAC CAA CCT GCC CA 3'	
	MP-mdr-oligo	15mer	5' CTC CAT CAC CAC CTC 3'	

Stability of oligonucleotides in liposomes

The biological stability of oligonucleotides both within liposomes and in the efflux medium (PBS containing 0.01% w/v sodium azide) during the course of the experiment was assessed by PAGE (15% polyacrylamide containing 7M urea). In the case of entrapped oligonucleotides, FATMLV-liposomes were permeabilized with Triton X100 (0.5% v/v) to free entrapped oligonucleotide prior to analysis by PAGE.

RESULTS

Entrapment efficiency of antisense oligonucleotides in MLV and FATMLV-liposomes

One of the prime concerns in using liposomes as model membranes for determining efflux (transport) of oligonucleotides was whether sufficient amounts of oligonucleotide could be entrapped within lipid vesicles. We compared the entrapment efficiencies of two fabrication techniques commonly used to prepare liposomes.

Table 2 shows the relative efficiencies with which MLV and FATMLV-liposomes were able to entrap 5' end ³²P-labelled oligonucleotides. MLV-liposomes were only able to entrap a small percentage of the total oligonucleotide added (less than 2%) whereas by preparing liposomes by the freeze and thaw technique (FATMLV-liposomes; see Methods) significantly higher entrapment of oligonucleotides (up to 30% for MP-oligos) could be achieved. This finding was consistent with those reported previously for more conventional therapeutic agents encapsulated in liposomes [23–24] and led us to use FATMLV liposomes for the majority of our subsequent studies.

Sucrose was used as a marker for the aqueous compartment of liposomes. If oligonucleotides were entirely entrapped within the aqueous compartment of liposomes then their corresponding entrapment values should concur with those obtained with this marker compound. The fact that significantly greater values for the entrapment efficiencies of S-oligo and in particular, MP-oligo were observed in both MLV and FATMLV liposomes when compared to sucrose, suggested that these analogs were either strongly adsorbing onto the surface of lipid bilayers or significantly partitioning into the lipid membrane. This led us to examine the adsorption characteristics of MP-oligos to liposomes. In order to compare the relative extents of binding for the different oligonucleotide analogs, we incubated fixed concentrations of ³²P-labelled analogs with known amounts of 'empty' liposomes for a 2 hour period (preliminary studies showed that binding of oligonucleotides was complete within 30 min; data not shown) at 37°C and determined the amount of label associated with liposomes after suitable washing (see methods section). The results are included in Table 2. Indeed, a significant portion of the MP-oligo and, to a lesser extent S-oligo, was bound to the outside of liposomes. In contrast, there was minimal adsorption of D-oligo or ALT-MP-oligo to liposomes. Thus for these derivatives, the oligonucleotide incorporated into liposomes is likely to be in free solution within the internal aqueous compartment of these lipid vesicles (see also partition coefficient data below).

Binding characteristics of methylphosphonate oligonucleotides with model lipid membranes

To further study the binding characteristics of methylphosphonates we used a 15 mer oligonucleotide complementary to

Table 2. Entrapment efficiencies and adsorption characteristics of liposomal antisense oligonucleotides.

Compound	Lipid Mass (mg)	Incorporation Efficiency (%)		Adsorption (%)
		MLV	FATMLV	
Sucrose	10	0.47	7.50	0.16
D-oligo	10	0.48	7.56	0.17
Alt-MP-oligo	10	0.90	8.10	0.90
S-oligo	10	1.03	10.01	3.60
MP-oligo	5			10.15
	10	1.45	25-30	21.60
	20			30.71

Entrapment (incorporation) efficiencies are given for both multilamellar liposome preparation (MLV) and freeze and thaw multilamellar liposome preparation (FATMLV). The adsorption data given is for FATMLV-liposomes. Mean of at least three experiments is given. Data are expressed as % of total cpm either adsorbed onto or incorporated into the liposomes. Under the conditions used, incorporation would include both entrapment into the internal aqueous compartments and adsorption onto liposomal membranes.

the human MDR-1 gene (MP-mdr-oligo) (see Table 1). Figure 1a shows the binding of MP-mdr-oligo to FATMLV liposomal membranes as a function of concentration suggesting that binding was saturable. The binding data are also represented as a Scatchard plot in Figure 1b. The linear fit suggests that a simple interaction occurs during binding in which one oligonucleotide molecule interacts with one binding site present on the lipid membrane. Binding parameters derived from this plot such as the dissociation constant (K_d), and binding site density are summarized in the legend to the figure.

Partition Coefficients

To investigate whether MP-oligos might exhibit significant partitioning into the lipid phase, water-octanol (w/o) partition coefficients were measured. Although w/o coefficients probably do not quantitatively reflect partitioning of large molecules such as oligonucleotides into lipid bilayers, they do offer a convenient estimate of relative lipophilicity. Partition coefficients for oligodeoxynucleoside methylphosphonates from octanol into water at room temperature are shown in Table 3. For comparison, the reported w/o partition coefficient for (dimethyl) methylphosphonate was 4.5 ± 1.2 (Mean $\pm$ S.D) [36] and that of a 16 mer D-oligo, 5' dCTGACAACATGACTGC 3', has been reported to be 5.7×10^4 [39]. The w/o partition coefficients of the methylphosphonate oligomers were all much greater than that of the model compound (dimethyl) methylphosphonate, implying that deoxynucleoside moieties increase the polarity of the compound. Significantly, all of these coefficients are greater than one, indicating that the methylphosphonates are more soluble in water than in octanol. The coefficients of the two diastereoisomers of di(deoxyinosine) differed, but the difference was not statistically significant ($P < 0.23$).

We also attempted to determine the w/o partition coefficient of a 14 mer S-oligo antisense to the 5' splice site of β -globin gene (see Table 1 for sequence). In the case of this phosphorothioate, no detectable partition of the oligomer into octanol could be detected by simple absorbance measurements, so measurements were attempted by liquid chromatography [36]. Despite repeated trials with large samples, no phosphorothioate

Figure 1a. Binding of MP-mdr-oligo to FATMLV liposomal membranes as a function of oligonucleotide concentration. A total lipid mass of 2 mg was used in each binding experiment. The required amount of 5' end fully substituted methylphosphonate labelled MP-mdr-oligo (15mer) was incubated with the lipid mass (liposomes) in 1 ml PBS (pH 7.4) as described in the Methods section. Each data point represents the mean of triplicate experiments. **b.** Scatchard plot of MP-mdr-oligo binding to FATMLV liposomal membranes. Calculations based on the plot indicate a dissociation constant (K_d) of 19.8 nM and the amount of binding to be equivalent to 0.97 ng oligonucleotide/mg of lipid.

peaks were found from octanol fractions. Given the sensitivity of the chromatography and detection system, one may estimate that the partition coefficient of the phosphorothioate from octanol into water is greater than 8,000. This estimated lower limit coefficient of the S-oligo may be compared with the reported coefficient of 57,000 for a conventional phosphodiester hexadecamer [39]. In the latter work, the observed partition coefficients fell smoothly to 220 as up to 13 of the 15 normal internucleotide linkages were replaced by 2-methoxyethyl phosphoramidates [39]. Hence, among phosphodiester, phosphorothioate, phosphoramidate, and methylphosphonate linkages, there is a reasonable correlation between partition coefficients and linkage polarities.

In terms of oligonucleotide entrapment within liposomes, these results suggest that the major portion of any given oligonucleotide analog exists in free solution within the aqueous compartments of the liposomes rather than being dissolved in the membrane lipid. This includes the MP-oligos, which also show, however, substantial surface binding to the outside of liposomes (see above).

Efflux kinetics of oligonucleotide analogs across model lipid membranes (liposomes)

In Figure 2A a typical efflux profile of 5' end 32 P labelled oligonucleotides (14 mer) from FATMLV liposomes is compared with that of tritiated D-glucose, a marker compound known to diffuse across bilayers [22,25–26]. The rate of permeation of D-oligo (charged molecule) across these model membranes was very slow (efflux $t_{1/2}$ of about 9 days) compared to that of glucose (efflux $t_{1/2}$ of about 1 day). Likewise permeation of other oligonucleotide analogs including MP-oligos was also relatively slow (efflux $t_{1/2} > 4.4$ days) (see Table 4). For a given lipid composition (PC: DMPG: CH; 60:20:20), the time taken for 50% of the entrapped oligonucleotide to efflux from liposomes (Efflux $t_{1/2}$) ranged from about 7–10 days for D-, S-, and AM-oligos to about 4 days for one of the MP-oligos (32 P labelled MP-mdr-oligo).

The fact that glucose, a marker with relatively rapid efflux across membranes ($t_{1/2} \sim 24$ hr) and sucrose, a marker molecule with slow efflux ($t_{1/2} \sim 10$ days), behaved as expected suggests that

Table 3. Water:Octanol partition coefficients of oligodeoxynucleoside methylphosphonates.

Compound	Sequence	Partition Coefficients (a)
Probable R isomer of Di(deoxyinosine) methylphosphonate	II (fast)*	113 $\pm$ 14
Probable S isomer of Di(deoxyinosine) methylphosphonate	II (slow)*	139 $\pm$ 15
Methylphosphonate dodecamer	5' ATGCCCTCAAC 3'	24 $\pm$ 2
Methylphosphonate pentadecamer	5' CTGAAGTGGCATGAG 3'	40 $\pm$ 5

^a Five separate aliquots were measured from each phase. The corrected absorbances from octanol phases were divided into the absorbances from aqueous phases to get water:octanol partition coefficients. Results shown are $\pm$ one standard deviation.

* Fast and Slow indicate the elution times of the two stereoisomers from reversed phase liquid chromatography. The methylphosphonates used are fully modified with a methyl substituent at every internucleoside linkage.

the fluxes determined for oligonucleotides were valid (see Table 4). The rate of permeation of D-oligos across lipid bilayers appeared to be only slightly affected by chain length (see Table 4) suggesting that the degree of polarity may be a greater determinant of transport across membranes for polyanionic oligonucleotides. In order to determine that the relatively slow efflux of methylphosphonate oligonucleotides was not influenced by the 32 P label, studies were performed with fluorescent TMRITIC conjugated MP-mdr-oligo. Essentially similar transport (i.e. slow) kinetics across lipid bilayers were observed for TMRIT-

Figure 2. A comparison of efflux profiles of 5' end ^{32}P labelled modified oligonucleotides and tritiated glucose from FATMLV liposomes. The % of oligonucleotide retained in FATMLV liposomes (plotted on log scale) is shown as a function of time in days. Conventional phosphodiester oligonucleotides (D-oligos) and modified oligonucleotides (ALT-MP, S- and MP-oligos) used were all 14 mer with a sequence antisense to the 5' splice site of the human β -globin gene (see Table 1 for sequence and description of modified oligonucleotides).

C conjugated MP-mdr-oligo (efflux $t_{1/2}$ of 6.2 days) compared to 5' end ^{32}P labelled MP-mdr-oligo (efflux $t_{1/2}$ of about 4.4 days). In an attempt to assess whether the extent of MP-oligo binding to the outside of the liposome surface would influence efflux kinetics, we determined the rate of ^{32}P labelled MP-oligo desorption and found that desorption rate was even slower than efflux (data not shown); this suggests that the bound fraction was not playing a significant role during the efflux measurements. Changes in lipid composition, including the presence or absence of cholesterol or use of saturated or unsaturated phosphatidylcholine, also had little influence on the efflux kinetics of D-oligos across lipid bilayers suggesting, once again, that polarity of the oligonucleotides may be a greater determinant of transport kinetics than membrane composition (see Table 4). It seems that so long as stable liposomes are formed, the permeation rates of oligonucleotides are quite slow. The difference in efflux kinetics between 5' end ^{32}P -labelled MP-5'ss and MP-mdr-oligos suggests that transport may be somewhat influenced by oligonucleotide sequence; however, it should be noted that both oligonucleotides had similar G:C ratios (see Table 1).

Since transport of methylphosphonate oligonucleotides by simple diffusion across these model lipid membranes was very slow and since biological effects in cultured cells were seen over much shorter time intervals [40–41] we question the possibility of these molecules entering cells by passive diffusion. On the other hand, it is conceivable that the tiny amounts of oligonucleotide entering the cell at early times may be sufficient to cause the observed biological effects.

Effect of MP-oligos on bilayer stability

Our studies seem to rule out passive diffusion as a means of oligonucleotide entry into cells. However, since MP-oligos bind to lipid membranes and are partially hydrophobic in nature, they might conceivably have detergent-like properties. Thus, at high concentrations they might destabilize membranes transiently and potentiate their own entry into cells. Hence, we tested the possibility that MP-oligos might destabilize membranes. To study this we entrapped tritiated sucrose into FATMLV liposomes and

Table 4. Summary of oligonucleotide efflux kinetics across model lipid membranes (FATMLV-liposomes).

Compound	Length (nt)	Label used	Efflux $t_{1/2}$ (days)
a) Lipid composition PC:DMPG:CH (60:20:20)			
Sucrose		^3H	10.1
Glucose		^3H	1.1
D-oligo	14 mer	^{32}P	7.2
D-oligo	7 mer	^{32}P	9.1
D-oligo	4 mer	^{32}P	8.7
S-oligo	14 mer	^{32}P	9.3
AM-oligo	14 mer	^{32}P	10.3
MP-5'ss-oligo	14 mer	^{32}P	7.2
MP-mdr-oligo	15 mer	^{32}P	4.4
MP-mdr-oligo	15 mer	TMRITC	6.2
b) Lipid composition DPPC:DMPG: CH (60:20:20)			
D-oligo	14 mer	^{32}P	7.1
c) Lipid composition PC : DMPG (70:30)			
D-oligo	14 mer	^{32}P	9.7

added 50 μM concentration of cold MP-oligo to the efflux medium (1 ml PBS). Membrane destabilization by cold oligonucleotide would be expected to accelerate the rate of sucrose efflux. The results in Table 5 show that when using cholesterol containing liposomes at physiological pH conditions (pH 7.4), efflux of sucrose was not significantly affected by cold MP-oligo. Thereby suggesting that membrane destabilization is an unlikely mechanism for cellular entry of MP-oligos.

On the assumption that MP-oligos, in the same manner as D- and S-oligos, may enter cells by endocytosis and thus be exposed to a low pH environment [22,42], we investigated the possibility that MP-oligos might destabilize membranes in an acidic environment (see Table 5). In this case, we chose a model lipid membrane composition which contained no cholesterol since endosomal membranes are thought to have little or no cholesterol [22]. Again, since efflux of sucrose was not affected, it appears unlikely that oligonucleotides by themselves, even at acidic pH, are likely to mediate their transport across lipid bilayers by membrane destabilization.

Stability of liposomal oligonucleotides

Since efflux of oligonucleotides from liposome membranes was monitored over a period of several days, we determined whether the entrapped oligonucleotides remained stable over the time

Table 5. Efflux kinetics of sucrose from model lipid membranes in the presence of excess cold MP-mdr-oligo under normal physiological and acidic pH environments.

Lipid composition % w/w	pH of experiment	Excess MP-oligo concentration	Efflux $t_{1/2}$ of sucrose
PC 60 DMPG 20 CH 20	7.4	0	10.1 days
	7.4	50 μ M	11.5 days
PC 70 DMPG 30	4.5	0	13.1 days
	4.5	50 μ M	13.5 days

period of the experiment. To do this, we looked at the stability of the unmodified oligonucleotide (D-oligo) as we have previously found this oligonucleotide to be the least stable of all the oligonucleotide types used in this study [15–16]. Figure 3 shows that liposomal D-oligo remained stable and was not degraded during the course of the experiment, indicating that efflux of the intact oligonucleotide was being monitored and not that of degraded products. The D-oligo that had effluxed out of the liposomes and was present in the efflux medium (PBS, pH 7.4) was also intact as no degradation products were observed over an 8 day period (Figure 3, lane C).

DISCUSSION

Few published reports exist on the cellular uptake mechanism(s) of antisense oligonucleotides, especially modified oligonucleotides such as the methylphosphonates (MP-oligos). In this study we have used model lipid membranes to further understand possible cellular transport characteristics of modified oligonucleotides.

In this article we have shown that significant amounts of oligonucleotides can be encapsulated into FATMLV-liposomes and that these had superior entrapment efficiencies compared to MLV preparations (see Table 2). This fact was not only useful in employing such liposomes as model membranes for the accurate determination of efflux kinetics but will also be important if liposomes are to be used as drug delivery carriers for antisense oligonucleotides [29–30]. We have also shown that methylphosphonate oligonucleotides and to a lesser extent, phosphorothioates bind to lipid membranes (see Table 2 and Figure 1). This finding suggests that these molecules are likely to bind to cell membranes as well, and possibly to the membranes of cell organelles. Thus, lipid binding may influence cellular transport and cellular distribution of these antisense agents.

On the basis that methylphosphonate oligonucleotides lack a charge, they were thought to be very hydrophobic with poor aqueous solubility. Our results on the water-octanol partition coefficients (see Table 3) suggest that, although relatively more hydrophobic than the polyanionic D- and S-oligos, fully

Figure 3. Stability of 5' end 32 P labelled D-oligo in FATMLV liposomes and efflux medium after 8 days. Stability was analysed by PAGE (15% polyacrylamide; 7 M urea). The absence of degradation products migrating below the control 14 mer D-oligo (lane A) on the denaturing gel shows that the oligonucleotide remained stable throughout the period of the efflux experiments both when entrapped within FATMLV-liposomes (lane B) or free in the efflux medium (lane C).

substituted MP-oligos still prefer to reside in the aqueous phase, exhibiting much greater solubility in water compared to octanol. Furthermore, because of their apolar nature, it had previously been thought that methylphosphonate oligonucleotides entered cells by passive diffusion [20]. Our results on efflux kinetics of MP-oligos across liposomal membranes suggest that passive diffusion would be a very slow process requiring days for even a small fraction of the oligonucleotide to gain access into cells (efflux $t_{1/2}$ >4 days observed with FATMLV liposomes). Hence, passive diffusion may not be the predominant mechanism by which these molecules enter living cells as antisense effects have been shown over much shorter periods of time [40–41]. Indeed in a companion paper we show that uptake of methylphosphonate oligonucleotides into CHO cells occurs relatively rapidly, plateaus within 10–12 hours, and seems to involve active endocytosis [21].

Relatively slow efflux kinetics across liposomal membranes were also observed for unmodified oligonucleotides (D-oligos), phosphorothioates (S-oligos) and those containing alternating methylphosphonate-phosphodiester linkages (AM-oligos) suggesting these oligonucleotides are also unlikely to enter cells by passive diffusion. These results are in agreement with the finding that D-oligos and S-oligos enter cells by receptor mediated endocytosis [17–18]. Although at this point it is not clear how AM-oligos enter cells, it is likely that since they are negatively charged macromolecules, they may also enter cells by endocytosis. However, whether this would involve a receptor protein and whether this would be the same as that mediating the transport of unmodified nucleotides remains to be ascertained.

The fact that MP-oligos exhibited binding to model lipid membranes led us to examine the hypothesis that at the relatively high concentrations often used for manipulation of gene expression (>50 μ M) these molecules might enter cells by destabilizing the plasma membrane. The fact that MP-oligos were not able to significantly influence the efflux of sucrose across liposomal membranes suggests that their cellular uptake is not likely to involve membrane destabilization.

On the assumption that MP-oligos, in the same fashion as D-oligos and S-oligos, may enter cells by endocytosis, we examined membrane transport of these molecules across model lipid membranes whose composition was chosen to resemble that of endosomes. Endosomes are acidic compartments in which macromolecules become entrapped following uptake by endocytosis. Hence, a necessary prerequisite to oligonucleotides exerting intracellular biological effects in the cytoplasm and/or nucleus is their efflux from endosomes and/or lysosomes, the first compartments encountered by oligonucleotides upon cellular entry by endocytosis. Clearly both D-oligos and S-oligos are able to escape from the endosomal compartments as they can exert sequence specific antisense effects [1–4]. The question is how do oligonucleotides get transported across endosomal membranes? Our results suggest that passive diffusion is again an unlikely mechanism (see Table 5). Furthermore, Table 5 shows that oligonucleotides by themselves are unlikely to perturb the endosomal membrane to cause efflux from these acidic endosomal compartments. The fact that release of viruses [42] and bacterial toxins [43] from endosomes is facilitated by proteins which undergo conformational changes at low pH to transiently destabilize the membrane leads us to speculate that oligonucleotide efflux from endosomal (and lysosomal) compartments may also be mediated by one or more proteins present in the bilayer. However, since endosomes are regularly fusing with other vesicles (e.g. lysosomes) [22,27] simple leakage of oligonucleotide during rupture or membrane fusion processes cannot be discounted.

It is only after the oligonucleotide is able to escape the endosomal compartment that it will be able to reach its intended biological target. Recent reports suggest that once oligonucleotides are introduced into the cytoplasm of cells, they rapidly diffuse to and localize within the nucleus. The intranuclear distribution observed is thought to depend on the oligonucleotide chemistry [44]. Chin et al [44] found that D-oligos and to a lesser extent, S-oligos colocalized with concentrated regions of small nuclear ribonucleoproteins (snRNPs), whereas MP-oligos colocalized with concentrated regions of genomic DNA. Quite independently, Leonetti et al [45] have reported similar observations. These authors found that translocation and localization of oligonucleotides occurred independent of oligonucleotide length and sequence and also independent of cell type used. By incubating photosensitive ³²P-labelled oligomer derivatives and photo-crosslinking to proteins of intact nuclei, they were able to show that binding of these oligomers occurred predominantly on a set of nuclear proteins ranging from 36–50 kDa [45]. Clearly the extent of oligonucleotide binding to proteins is an important determinant of cellular distribution. Furthermore, since hydrophobic oligonucleotide analogs, such as MP-oligos, also bind extensively to lipids (see Table 2) it is conceivable that binding to the membranes of cell organelles may also influence cellular distribution. Thus, the ratio of lipid to protein binding for a given oligonucleotide chemistry may prove to be an important determinant of intracellular distribution.

In contrast to the finding that oligonucleotides exhibit nuclear localization, reports in the literature exist in which antisense activity has been observed with oligomers targeted to vesicular stomatitis virus, a virus whose replication cycle is restricted entirely to the cytoplasm [46–47]. The exact relationship between nuclear distribution and the antisense activity of oligonucleotides remains to be ascertained. This information will be useful in the design and delivery of more efficient antisense compounds.

The above discussion suggests that delivery systems which may enhance cellular uptake and/or enhance efflux from endosomal compartments will be useful in the delivery of antisense agents. Liposomes, as carriers for the delivery of antisense compounds, may provide a system which allows both goals to be achieved. They offer a natural delivery system which is not only biocompatible and biodegradable but can potentially enhance cellular uptake of oligonucleotides. In addition to protecting oligonucleotides from enzymatic degradation, liposomes also offer the potential of producing controlled and sustained release formulations. The fact that efflux of oligonucleotides from liposomes was shown to be slow and sustainable over a period of days (see Figure 2 and Table 4), suggests that they may be useful in delivering oligonucleotides against genetic targets that have a slow turnover, such as the MDR-1 gene product p-glycoprotein [40,48]. Furthermore, the use of pH-sensitive liposomes [49] may promote efflux of oligonucleotides from endosomal compartments. Such liposomes destabilize membranes or become fusogenic when exposed to acidic conditions such as those found in endosomal compartments. Targeting liposomes to cells using surface antibodies may also be possible [30].

In conclusion, liposomes provide a useful model membrane system for further understanding the mechanisms of cellular transport of modified antisense oligonucleotides. The data presented here suggests that passive diffusion is an unlikely mechanism of cellular uptake of modified oligonucleotides including the MP-oligos. However, the relatively slow efflux of oligonucleotides from liposomes may be useful in the sustained delivery of these molecules especially to targets with a slow turnover.

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