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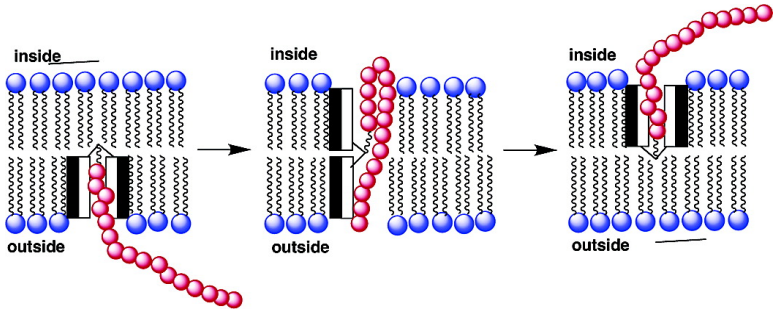

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Molecular Umbrella-Assisted Transport of an Oligonucleotide across Cholesterol-Rich Phospholipid Bilayers

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Abstract: A series of molecular umbrella conjugates, derived from cholic acid, deoxycholic acid, spermidine, lysine, and 5-mercapto-2-nitrobenzoic acid, have been synthesized and found capable of transporting an attached 16-mer oligonucleotide (S-dT_{16}) across liposomal membranes made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyldglycerol (POPG), and cholesterol [POPC/POPG/cholesterol (65/5/30; mol/mol/mol, v/v/v)] at 37 °C. Those molecular umbrellas containing four choloyl (or deoxycholoyl) groups resulted in significantly faster rates of transport as compared to those containing only two such moieties. A model that accounts for these membrane transport processes is proposed.

Introduction

Oligonucleotides have considerable promise as therapeutic agents for the treatment of a wide range of diseases.[1−15] Certain oligonucleotides, for example, which can act in an "antisense" manner (i.e., bind to a complementary segment of mRNA and block the transfer of genetic information from DNA to proteins), are currently in Phase 3 clinical trials for the treatment of nonsmall cell lung cancer, Crohn's disease, malignant melanoma, chronic lymphocytic leukemia, and multiple myeolma.[1] One antisense oligonucleotide (Vitravene) is now being used for the treatment of cytomegalovirus retinitis found in some AIDS patients.[1] Despite their broad potential as drugs, the delivery of oligonucleotides to mRNA in the cytoplasm of cells, in vivo, remains as a major challenge. In particular, it now appears that virtually all oligonucleotides, including those attached to molecular, macromolecular, and supramolecular carriers, first enter cells via endocytosis.[2,3] Subsequent release from endosomal-lysosomal compartments, by some unknown and inefficient mechanism(s), then delivers a portion of the oligonucleotide into the cytoplasm where it can reach its intended target. In principle, if one could deliver oligonucleotides directly into the cytoplasm by passive transport, this should lead to more effective drugs. Such a technique could also be considered for the gene therapy, as well as for the delivery of other large biologically active molecules, for example, proteins.

In previous work in our laboratories, we have shown that "molecular umbrellas" (i.e., molecules containing two or more facially amphiphilic units attached to a central scaffold) are capable of transporting small, covalently attached polar molecules such as glutathione and adenosine 5′-*O*-(3-thiotriphosphate) across phospholipid bilayers.[16,17] The fact that facial amphiphilicity is necessary for such transport has led us to propose a novel permeation mechanism, whereby the molecular umbrella moiety plays an active role in the translocation process. In other words, the enhanced transport is not simply the result of an altered hydrophobic/lipophilic balance of the attached agent.[17] A model for umbrella-assisted transport that we have previously proposed is illustrated in Scheme 1. Here, the shaded and unshaded rectangles represent hydrophobic and hydrophilic faces of the molecular umbrella moiety, respectively, and the lightly shaded oval represents an attached polar agent. In essence, the conjugate first approaches the bilayer in an exposed conformation (**A**), then flips into an adsorbed state (**B**), and finally enters the hydrocarbon interior in a shielded conformation (**C**). Subsequent diffusion across the membrane, 180° rotation, and reversal of steps **B** and **A** on the other side of the membrane

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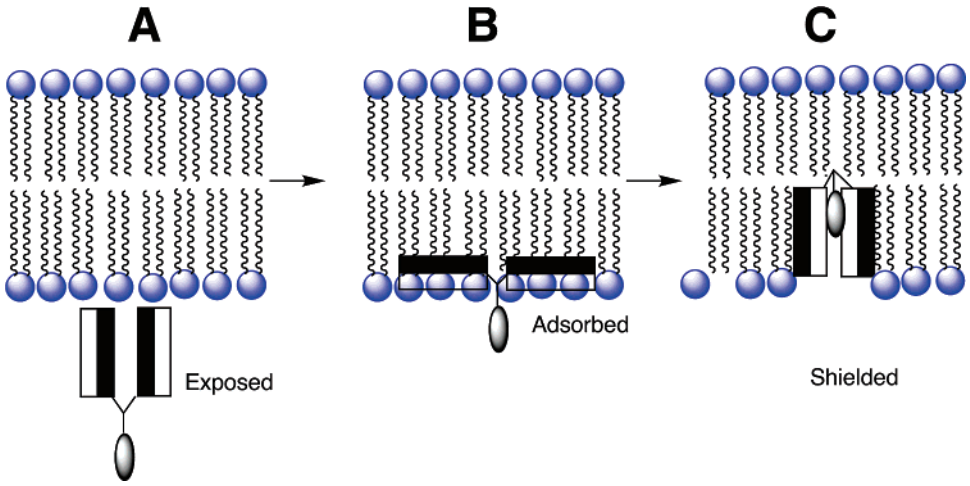
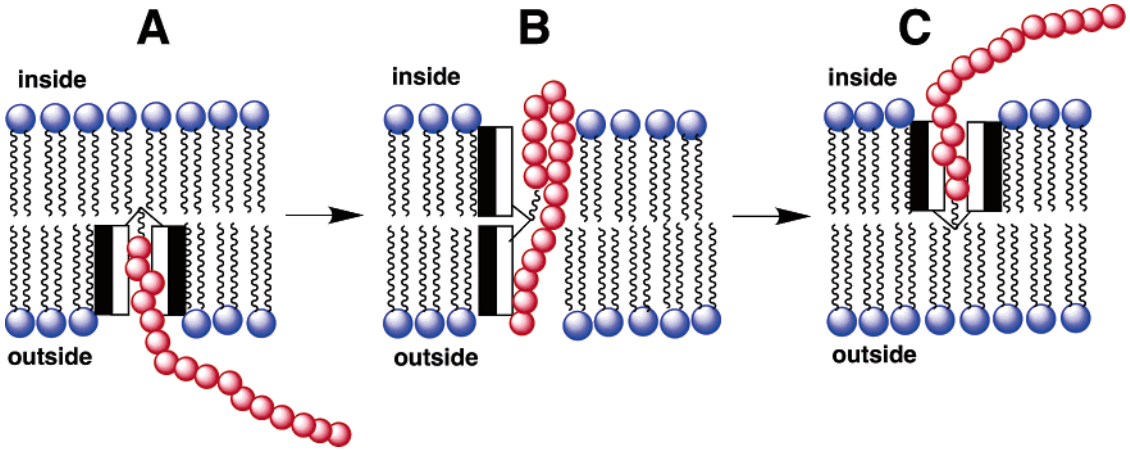
Scheme 1



Scheme 2



(not shown) then releases the conjugate into the adjoining aqueous phase. Thus, we postulated that the molecular umbrella moiety facilitates the transport of the polar agent by masking its hydrophilicity while in the hydrocarbon interior of the bilayer. The driving force for entry into the hydrocarbon interior is thought to derive from hydrophobic forces between the hydrophobic face of each facial amphiphile and the hydrocarbon chains of neighboring lipids.

Although our previous studies have focused, sharply, on the bilayer transport of small molecules, we have become keenly interested in the possibility that molecular umbrellas might also be used to facilitate the transport of oligonucleotides across lipid bilayers. An obvious issue of concern, however, is the relative size of an attached oligonucleotide. Specifically, modest-sized molecular umbrellas would not be able to shield the entire oligonucleotide. Nonetheless, it seemed to us that an attached molecular umbrella could "lead" the oligonucleotide into the lipid bilayer and still provide a pathway for translocation. In a sense, we envisioned the molecular umbrella acting like a leader sequence, which promotes the transport of an attached protein across the endoplasmic reticular membrane. How might this work? One possible scenario is illustrated in Scheme 2. Here, the conjugate first penetrates the membrane to produce a shielded conformation (**A**). Subsequent opening of the umbrella across the bilayer, and simultaneous folding of the oligonucleotide into a partial "U", could leave most of the membrane-internalized phosphate groups pointing toward the hydrophilic faces of the umbrella (**B**), allowing for translocation to **C**. Release into the adjoining aqueous phase would then involve simple desorption (not shown).

This translocation scenario raises several intriguing questions. First and foremost is whether this transport works at all, either by the process depicted in Scheme 2 or by any other mechanism. Second, because mammalian cells are rich in cholesterol, and because this sterol is known to have a strong condensing effect on fluid phospholipid bilayers, is transport possible across cholesterol-rich membranes?[18,19] Third, do these molecular umbrellas form fixed pores or defects in the membrane through which the oligonucleotides can pass?[20,21] Fourth, if translocation occurs, is its rate sensitive to the structure and composition of the umbrella moiety? In this paper, we describe experiments that address each of these questions.[22]

Experimental Section

Methods and Materials. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without

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further purification. *N*,*N*-Diisopropylethylamine (DIPEA) and 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) were obtained from Aldrich and used directly. All oligonucleotide conjugates were derived from $HO(CH_2)_6SS\text{-}dT_{16}$ (**5**), which was obtained from Oligos Etc. Inc. (Wilsonville, OR). House-deionized water was purified using a Milli-Q-filtering system (Millipore) containing one carbon and two ion-exchange stages, and used to prepare phosphate buffers [10 mM NaH_2PO_4, 100 mM NaCl, 1 mM EDTA, pH 7.2] and TRIS buffers (10 mM TRIS, 100 mM NaCl, 1 mM EDTA, pH 7.2). All UV measurements were made using a Cary 300 Bio UV−vis spectrophotometer. L-Lysine was used in the synthesis of **3**; DL-lysine was used for the synthesis of **4**. Lysine dicholamide, which was previously reported, was prepared using methods similar to those described herein for lysine dideoxycholamide.[23]

Lysine Dideoxycholamide. To a solution that was made from 2.0 g (5.09 mmol) of deoxycholic acid, 644 mg (5.6 mmol) of *N*-hydroxysuccinimide, and 40 mL of THF was added 1.16 g (5.6 mmol) of DCC. The mixture was stirred for 4 h at room temperature and filtered to remove the insoluble urea. To this filtrate was added a solution made from 292 mg (2.0 mmol) of DL-lysine, 2.09 mL of triethylamine, and 10 mL of water. After being stirred overnight, the mixture was poured into 50 mL of 1 M aqueous HCl. The resulting solid was collected by filtration and purified by column chromatography [silica gel, $CHCl_3/CH_3OH/H_2O$, (30/10/1, v/v/v)] to give 1.05 g (58%) of lysine dideoxycholamide having R_f 0.30, and ^{1}H NMR (CD_3OD, 500 MHz, 320 K) δ ppm: 0.69 (s, 6H), 0.92−1.9 (m, 66H), 2.00−2.30 (m, 4H), 3.15 (t, 2H), 3.52 (m, 2H), 3.94 (s, 2H), 4.29 (m, 1H).

Disulfide 6. To a solution that was made from 100 mg (0.112 mmol) of N_1,N_3-spermidine-dideoxy choleamide, 43 μL (0.306 mmol) of triethylamine, and 1 mL of DMF was added 35 mg (0.051 mmol) of bis{3-*O*-[*N*-1,2,3-benzotriazin-4(3*H*)-one]yl}-5,5′-dithiobis-2-nitrobenzoate.[24] After being stirred for 12 h at room temperature, the mixture was poured into 10 mL of a saturated sodium bicarbonate solution. The resulting precipitate was collected by filtration and purified by column chromatography [silica gel, $CHCl_3/CH_3OH$/30% NH_4OH (50/10/1, v/v/v)] to give 87 mg (79%) of **6** having R_f 0.47, and ^{1}H NMR (CD_3OD, 360 MHz, 320 K) δ ppm: 0.69 (s, 12H), 0.92−2.30 (m, 140H), 2.98 (br, 4H), 3.14 (br, 4H), 3.30 (m, 4H), 3.52 (m, 8H), 3.93 (s, 4H), 7.60 (d, 2H), 7.85 (m, 2H), 8.24 (m, 2H). MALDI-TOF MS *m*/*z* 2171 ($[M + Na]^+$).

Disulfide 7. To a solution that was made from 18.3 mg (0.046 mmol) of 5,5-dithiobis(2-nitrobenzoic acid) in 0.6 mL of anhydrous DMF and 0.017 mL (0.093 mmol) of DIPEA was added 32.2 mg (0.092 mmol) of *N*-*O*[1,2,3-benzotriazin-4(3*H*)-one]-yl tetramethylisothiuronium tetrafluoroborate. After the mixture was stirred in a sealed flask at room temperature for 3.5 h, the resulting suspension was added, in small portions, to a solution that was made from 183 mg (0.092 mmol) of N_1,N_3-spermidinebis(lysine-dicholeamide), 1.3 mL of anhydrous DMF, and 0.025 mL of DIPEA.[24] After being stirred at room temperature for 6.5 h, the mixture was poured into 30 mL of water that was saturated with $NaHCO_3$. The resulting precipitate was separated, washed with 2 × 25 mL of H_2O, dissolved in MeOH (1 mL), and purified by preparative TLC [(silica gel, $CHCl_3$/MeOH/H_2O, 70/30/3, (v/v/v)] to give 99.9 mg (50.2%) of **7** having R_f 0.67, and ^{1}H NMR (CD_3OD, 360 MHz, 320 K): 0.68 (s, 24H), 0.89−2.30 (m, 268H), 3.12 (m, 16H), 3.30 (m, 8H), 3.52 (m, 8H), 3.92 (m, 8H), 4.27 (m, 4H), 7.57 (d, 2H), 7.83 (m, 2H), 8.25 (m, 2H). MALDI-TOF MS *m*/*z* 4311 ($[M + Na]^+$).

Disulfide 8. Lysine dideoxycholamide (103 mg, 0.115 mmol) and *N*-hydroxysuccinimide (16 mg, 0.138 mmol) were dissolved in 1 mL of DMF, followed by the addition of DCC (30 mg, 0.145 mmol). After the solution was stirred for 5 h, spermidine (8 mg, 0.055 mmol) and DIPEA (60 μL, 0.345 mmol) were added. The mixture was stirred for an additional 12 h at room temperature and poured into 20 mL of saturated sodium bicarbonate. The resulting precipitate was collected by filtration and purified by preparative TLC using $CHCl_3/CH_3OH$/30% NH_4OH (40/10/1, v/v/v) to give 53 mg (51%) of the corresponding N_1,N_3-spermidinebis(lysine-dideoxycholamide) having R_f 0.69, and ^{1}H NMR (CD_3OD, 360 MHz, 320 K) δ ppm: 0.69 (s, 12H), 0.92−2.30 (m, 146H), 2.64 (m, 4H), 3.17 (m, 8H), 3.52 (m, 4H), 3.94 (s, 4H), 4.25 (m, 2H). MALDI-TOF MS *m*/*z* 4183 ($[M + Na]^+$).

To a solution that was made from 53 mg (0.028 mmol) of N_1,N_3-spermidinebis(lysine-dideoxycholamide), 13 μL (0.075 mmol) of DIPEA, and 1 mL of DMF was added 8.5 mg (0.0125 mmol) of bis{3-*O*-[*N*-1,2,3-benzotriazin-4(3H)-one]yl}-5,5′-dithiobis-2-nitrobenzoate.[24] After being stirred for 12 h at room temperature, the mixture was poured into 10 mL of a saturated sodium bicarbonate solution. The resulting precipitate was collected by filtration and purified by preparative TLC [silica gel, $CHCl_3/CH_3OH$/30% NH_4OH (50/10/1, v/v/v)] to give 40 mg (77%) of **8** having R_f 0.51, and ^{1}H NMR (CD_3OD, 360 MHz, 320 K) δ ppm: 0.66 (s, 24H), 0.89−2.30 (m, 292H), 3.12 (m, 16H), 3.30 (m, 8H), 3.50 (m, 8H), 3.92 (s, 8H), 4.30 (m, 4H), 7.52 (d, 2H), 7.74 (d, 2H), 8.20 (d, 2H).

Umbrella−Oligonucleotide Conjugates. The following procedure that was used for the synthesis of **3** was typical: A solution was prepared from 12.9 mg (2.8 μmol) of $HO(CH_2)_6SS\text{-}dT_{16}$ (**5**), 0.6 mL water, and 0.9 mL of TRIS buffer (10 mM TRIS, 100 mM NaCl, 1 mM EDTA, pH 7.2). To this solution was added 0.5 mL of 1.0 M dithiothreitol (DTT) in H_2O. After the mixture was stirred at room temperature for 17 h under an argon atmosphere, the solution was extracted with EtOAc (6 × 1.5 mL) to remove spent and unused DTT. A 10 μL-aliquot of the aqueous phase was analyzed by Ellman's reagent, which indicated a total thiol content of 3.89 μmol. The entire aqueous phase was then added to a solution that was made from 24.0 mg (5.6 μmol) of **7** plus 2.5 mL of methanol. The mixture was stirred under an argon atmosphere for 25 h, concentrated under reduced pressure (35 °C) to a volume of ca. 1 mL, and subjected to freeze-drying for 18 h. The residue was suspended in 0.5 mL of H_2O, and the solid was removed by filtration and washed with 4 × 250 μL H_2O. The combined aqueous phase was then dialyzed against 1000 mL of water using a dialysis membrane having MWCO 500. The contents of the dialysis bag were reduced in volume to ca. 500 μL by freeze-drying. Final purification (five 100-μL injections) was made by HPLC using a Breeze instrument (Waters), which was equipped with a Preparative Nova-Pak HR C18 reverse phase column (7.8 mm diameter, 300 mm length), using a flow rate of 3 mL/min and the following elution sequence: 0−3.5 min of solvent **A**, followed by a linear gradient from **A** to **B**, from 3.5 to 25 min, where **A** = 20% CH_3CN/80% 0.1 M aqueous triethylammonium acetate (v/v) and **B** = 80% CH_3CN/20% 0.1 M aqueous triethylammonium acetate (v/v). Detection was made by UV at 265 and 330 nm. The retention time of **3** under these conditions was 14.4 min. After collection, the volatile solvents were removed under reduced pressure followed by freeze-drying to give 7.5 mg (71%) of **3**. As a further test of purity, a portion of **3** was dissolved in TRIS buffer and added to a TRIS buffer that contained an excess of tris(2-carboxyethyl)phosphine hydrochloride (TCEP). The concentration of USH that was formed was found to be 69.3 μM (λ_{max} 425 nm). This same solution was also found to be 68.9 μM in oligonucleotide, based on a calibration curve made from **5** (λ_{max} 268 nm). Conjugate **3** showed the expected mass spectrum: MS(MALDI) $[M^{-16} + 15H^+]^-$ *m*/*z* 7122. Conjugates **2** (3.5 mg (39%), having a retention time of 13.5 min and $[M^{-16} + 15H^+]^-$ *m*/*z* 6048, and **4** (2.63 mg, 28%) having a retention time of 18.9 min and MS(MALDI) $[M^{-16} + 15H^+]^-$ *m*/*z* 7058 were prepared by similar procedures.

Efflux Experiments. A thin lipid film was formed on the inside walls of a glass test tube by evaporating a chloroform solution containing 44 mg of POPC/POPG (95/5, mol/mol) under a stream of argon. The film was dried under vacuum (0.5 Torr) for 18 h at room

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temperature, and hydrated with 2.2 mL of a 38 μM solution of **1** in TRIS buffer. The contents of the tube were then mixed via vortexing at room temperature for 30 min, and subjected to six freeze−thaw cycles (−196/40 °C). Subsequent extrusion through two 0.4 μm Nuclepore membrane (10 times), followed by extrusion through two 0.2 μm Nuclepore (15 times), afforded a dispersion, which was dialyzed against 1000 mL of TRIS buffer (two times) for 48 h, using dialysis tubing having MWCO 300 000. The quantity of **1** that remained associated with the liposomes was determined by withdrawing a 185 μL-aliquot, mixing it with 185 μL of a detergent solution [prepared by mixing 20% sodium dodecyl sulfate (SDS) in TRIS buffer with ethanol (1/2, vol/vol)], and analyzing the solution by UV (λ_{max} 268 nm). After the 48 h of dialysis, the dispersion was found to be 4.69 μM in **1**. The dispersion was further dialyzed against 1000 mL of TRIS buffer at room temperature and the decrease in concentration of **1** monitored by removing 185 μL aliquots and analyzing for residual conjugate. Similar procedures were used to monitor the efflux from cholesterol-containing liposomes, using a phosphate buffer. Efflux rates that were found for **1** using a TRIS buffer were similar to those found using a phosphate buffer.

Target Liposomes. A thin lipid film was formed on the inside walls of a glass test tube by evaporating a chloroform solution containing 44 mg of POPC/POPG/cholesterol (65/5/3), mol/mol/mol) under a stream of argon. The film was subsequently dried under vacuum (0.5 Torr) for 18 h at room temperature, and hydrated with 2.1 mL of phosphate buffer that was 2.0 mM (or 1.1 mM) in glutathione. The contents of the tube were then mixed via vortexing at room temperature for 30 min, and subjected to six freeze−thaw cycles (−196/40 °C). Subsequent extrusion through 0.4 μm Nuclepore membrane (10 times), followed by extrusion through 0.2 μm Nuclepore (15 times), afforded a dispersion that was dialyzed against 1000 mL of phosphate buffer (two times) for 48 h under an argon atmosphere, using dialysis tubing having a MWCO 300 000.

Analysis of Entrapped Glutathione. To determine the amount of entrapped glutathione, a 50 μL-aliquot of the dispersion was diluted with 150 μL of phosphate buffer, and mixed with 200 μL of a detergent solution [prepared by mixing a 20% sodium dodecyl sulfate (SDS) in TRIS buffer with ethanol (1/2, vol/vol)], plus 25 μL of 2.0 mM of 5,5′-dithiobis(2-nitrobenzoic acid) in phosphate buffer. The thiol content was then determined by UV (λ_{max} 425 nm) using a calibration curve (i.e., Ellman's method of analysis was used). This same method of analysis was used to determine the amount of entrapped GSH that was present in the vesicle dispersion at the end of the transport experiment.

Analysis of External GSH. Typically, a 500 μL-aliquot of a given dispersion was placed in the source side of a 2 mL equilibrium dialysis cell, and 500 μL of phosphate buffer was then added to the receiving side. A 100 nm Nuclepore membrane was used to separate both compartments. The receiving side was then analyzed for glutathione content after 132 min of dialysis under an argon atmosphere using Ellman's method with a calibration curve. Under the conditions used, the half-life for permeation of GSH across the Nuclepore membrane was 33 min.

Membrane Transport. In a typical transport experiment, 800 μL of a dispersion of target liposomes was heated to 37 °C and mixed with 70 μL of a 111 μM solution of **1** in phosphate buffer. Thus, the molar quantities of POPC, POPG, cholesterol, GSH, and **1** in 870 μL of this dispersion were 14.2 μmol, 1.1 μmol, 6.6 μmol, 86.2 nmol, and 7.7 nmol, respectively. This mixing was carried out in a heated cuvette (37 °C) of a UV spectrometer, which was equipped with a No-Air stopper and maintained under an argon atmosphere. After a 2-min incubation period, a baseline was recorded from 300 to 550 nm. The appearance of USH (λ_{max} 425 nm) was then used to monitor the thiolate−disulfide interchange reaction.

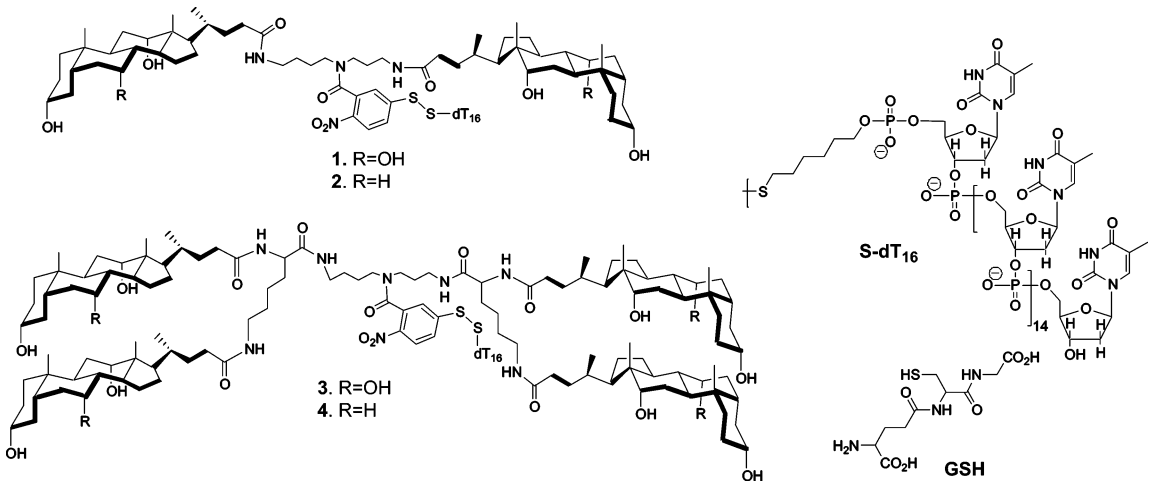
At the end of the kinetic run, an aliquot of the dispersion was destroyed with a sodium dodecyl sulfate solution to eliminate absorption contributions due to light scattering from the liposomes. This allows for a more accurate determination of the concentration of **1** that was initially present, and also the amount of **1** that remained at the end of the experiment. Thus, a 200 μL aliquot was withdrawn and diluted with 200 μL of a detergent solution [prepared by mixing 20% SDS in TRIS buffer with ethanol (1/2, vol/vol)]. The UV spectrum was recorded from 220 and 550 nm after the mixture was incubated for exactly 3 min at 25 °C, and the concentration of USH was determined. It should be noted that by disrupting the liposomes and diluting the solution, negligible reaction occurs between **1** and GSH within this 3-min period. Based on such analysis, the quantity of USH that was formed prior to destruction of the liposomes by SDS was in good agreement with the quantity that was determined by direct analysis of the dispersion, where light scattering effects were accounted for. Extending the incubation time of this SDS/ethanol solution to 40 min resulted in a maximum value of USH being formed, which provided an exact value for the initial concentration of **1** in the dispersion. This value was in good agreement with the oligonucleotide content, as determined by UV (λ_{max} 268 nm).

Analysis of Liposomal Dispersions after Partial Conversion. In a typical analysis, the concentration of unreacted **1** in the external aqueous phase (i.e., $USSO_{ext}$) was determined by the following procedure: When the transport experiment was stopped after 67 h (73% conversion), the entire dispersion was cooled to room temperature, placed in the source side of a 2-mL equilibrium dialysis cell, and dialyzed against an equal volume of phosphate buffer for 6.5 h. Analysis of a 400 μL-aliquot of the receiving side by UV (220−550 nm) yielded the concentration of $USSO_{ext}$ (λ_{max}330 nm). The total oligonucleotide content that was present in the receiving side (O_{ext}), which was also determined by UV analysis at λ_{max} 268 nm, represents the combination of $USSO_{ext}$ and the oligonucleotide that was cleaved by GSH and released into the external aqueous phase as $GSSO_{ext}$.

To estimate the percent of **1** that reacted with GSH and was captured by the aqueous compartment of the liposomes (i.e., $GSSO_{cap}$), the entire liposomal dispersion was first dialyzed against 1000 mL of phosphate buffer for 48 h at room temperature under an argon atmosphere. A 200 μL aliquot of the dispersion was then diluted with 200 μL of a detergent solution [prepared by mixing 20% SDS in TRIS buffer with ethanol (1/2, vol/vol)]. The UV spectrum of the resulting solution was then recorded between 220 and 550 nm to determine the total oligonucleotide content (λ_{max} 268 nm) that was associated with the liposomes (O_{lip}). An estimate of $GSSO_{cap}$ was made by subtracting the quantity of adsorbed molecular umbrella conjugate (i.e., $USSO_{ads}$) [calculated by subtracting the quantity of molecular umbrella−oligonucleotide in the external aqueous phase ($USSO_{ext}$) from the amount of unreacted **1**] from O_{lip}.

Partitioning Measurements. In a typical partitioning experiment, target liposomes were prepared from POPC/POPG/cholesterol (65/5/30, mol/mol/mol) using methods similar to those described above, except that glutathione was omitted. A phosphate buffer containing a given molecular umbrella−oligonucleotide conjugate was then added to the dispersion, such that the concentration of the conjugate was 10 μM and the total lipid content was 18 mg/mL. A portion of the dispersion (0.5 mL) was then placed in the source side of a 2 mL equilibrium dialysis cell (using a 100 nm Nuclepore membrane to separate both chambers), and the dispersion was allowed to equilibrate with 0.5 mL of phosphate buffer on the receiving side over a 48 h period. During this time, the cell was shaken in a 37 °C atmosphere. Analysis of the receiving side for the molecular umbrella−oligonucleotide conjugate was made, directly, by UV (λ_{max} 268 nm). The data were analyzed using a nonsaturable partitioning model, which yields the partition coefficient, K, from the equation $C_o/C = [(K)(L)/2(W)] + 1$, where C_o is the equilibrium concentration of the conjugate in solution that was measured in the absence of liposomes, C is the equilibrium concentration of the conjugate in solution that was measured in the presence of liposomes, L is the concentration of the lipids (a weighted

Chart 1



average molecular weight of 649 for the lipids was used), and W is the concentration of water, which was taken as 55.5 M.[25]

Results and Discussion

Experimental Design. The basic design that we have used for our molecular umbrella−oligonucleotide conjugates is similar to that used previously for conjugates formed from glutathione and adenosine 5′-*O*-(3-thiotriphosphate).[16,17] Thus, spermidine, cholic acid, deoxycholic acid, and 5,5′-dithiobis-(2-nitrobenzoic acid) were chosen as building blocks for the synthesis of four conjugates, **1**, **2**, **3**, and **4** (Chart 1). The use of the 5-mercapto(2-nitrobenzoyl) group as a linker for attaching the oligonucleotide to each molecular umbrella to give the corresponding conjugates (USSO) has three attractive features.[17] First, it allows for facile cleavage by liposome-entrapped glutathione via thiolate−disulfide interchange, the net result being the formation of the thiol form of the molecular umbrella (USH) and the corresponding glutathione conjugate of the oligonucleotide (GSSO) (eq 1). Second, the release of USH can be conveniently monitored by UV spectroscopy (λ_{max} 425 nm). Third, because GSH exists in the cytoplasm of cells at millimolar concentrations, such a linkage could be considered for drug delivery applications.

$$\text{USSO} + \text{GSH} \rightarrow \text{USH} + \text{GSSO} \qquad (1)$$

To place the shielding capacity of these molecular umbrellas into perspective, space-filling models of **1** and **3**, in exposed and shielded conformations, are shown in Figure 1. From these models, the umbrella moiety of **1** is estimated to cover ca. three nucleotide units. In contrast, the umbrella moiety of **3**, having lysine spacers at both ends of the spermidine backbone, covers ca. five nucleotide units. In addition, greater shielding of the nucleotide in **3** is possible due to the larger number of sterols that are present.

In terms of structure/activity considerations, comparison of **1** with **2** (a deoxycholoyl analog) allows one to judge the dependency of transport activity on having two versus three hydroxyls per sterol. A similar comparison can also be made with **3** and **4**. Comparison of **1** with **3**, and **2** and **4**, respectively, further allows one to probe the influence that the number of facial amphiphiles have on transport activity. For this investigation, a 16-mer oligonucleotide (S-dT_{16}) was chosen as a prototype because its length has relevance from a drug design standpoint. Liposomal membranes that were used as model membranes were made from 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidyldglycerol (POPG), and cholesterol [POPC/POPG/cholesterol (65/5/30, mol/mol/mol)] because they mimic the lipid framework of mammalian membranes.

Molecular Synthesis. The synthesis of **1** and **2** proved to be straightforward. Thus, reduction of $HO(CH_2)_6SS$-dT_{16} (**5**) with excess dithiothreitol, followed by reaction of the resulting HS-dT_{16} with 1 equiv of disulfide **6**, afforded **2** (Scheme 3). Compound **6** was prepared using the synthetic approach that is outlined in Scheme 4. Conjugate **1** was prepared by similar methods as previously described.[22]

The synthesis of **3** and **4** involved analogous coupling reactions using disulfides **7** and **8**, respectively. The latter were prepared by activation of cholic (or deoxycholic) acid, followed by condensation with lysine to give lysine dicholamide (or lysine dideoxycholamide). Subsequent activation of the carboxylic

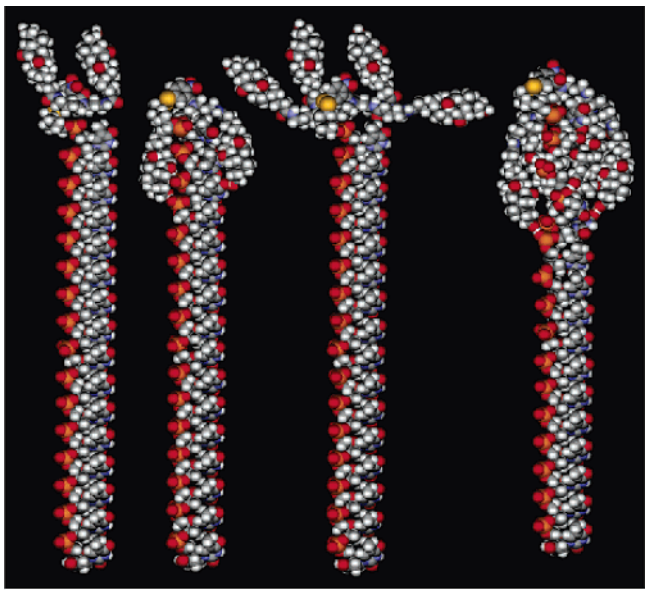
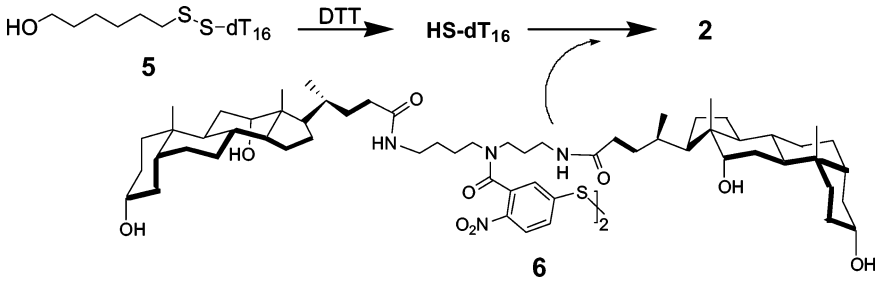


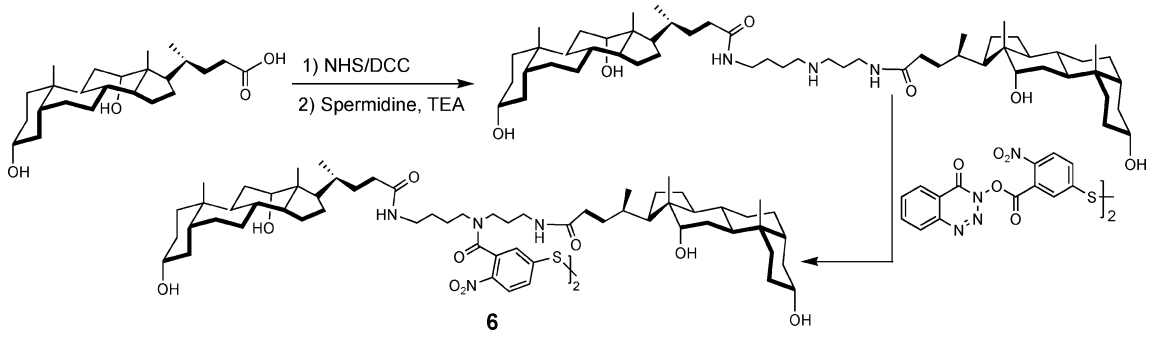
Figure 1. Space-filling models of **1** (left) and **3** (right) in an exposed and a shielded conformation.

(25) Boguslavsky, V.; Hruby, V. J.; O'Brien, D. F. *J. Pept. Res.* **2003**, *61*, 287−297.

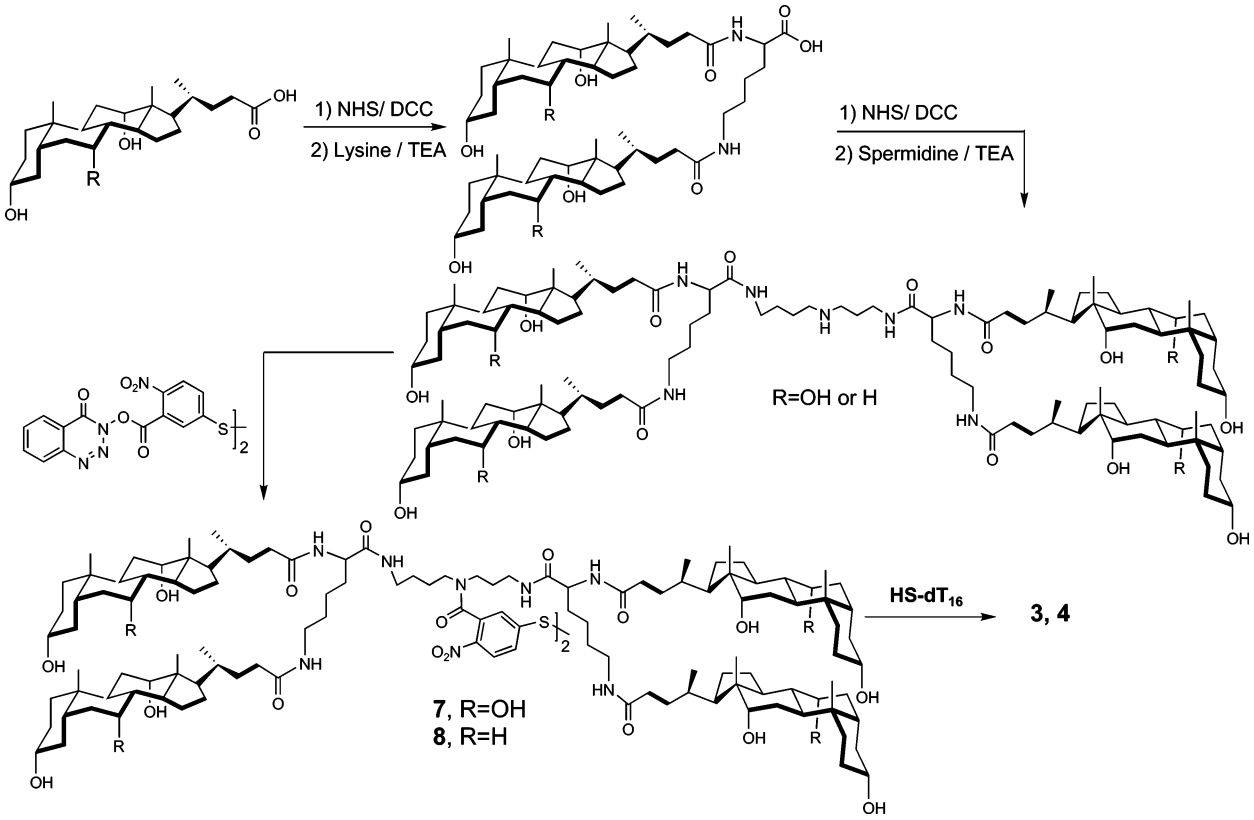
Scheme 3



Scheme 4



Scheme 5



group of the lysine moiety, and condensation with the primary amino groups of spermidine, followed by acylation with a doubly activated form of Ellman's reagent afforded **7** and **8** (Scheme 5).

Membrane Transport. Before comparing the transport properties of these molecular umbrella−oligonucleotide conjugates under cleavage conditions, we first examined the ability of **1** to cross phospholipid bilayers in its entirety, that is, in the absence of GSH. For this purpose, a liposomal dispersion was prepared from POPC/POPG/cholesterol (65/5/30, mol/mol/mol), using an aqueous solution containing **1**. Subsequent dialysis at room temperature for 48 h removed **1** that was present in the external aqueous phase. Under the experimental conditions used, the half-life for permeation of **1** across the dialysis membrane was 130 min. An extended dialysis was then performed to measure the efflux of the remaining conjugate (Figure 2). Because at least half of the liposome-bound conjugate must reside on the inside of the liposomes at the start of this extended

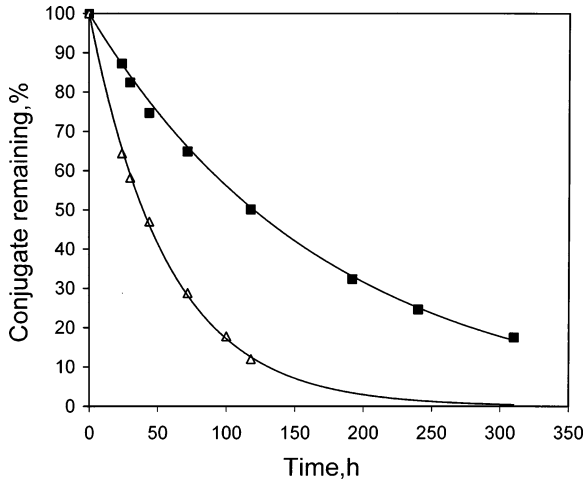


Figure 2. Efflux of **1** (■) and **3** (△) from liposomes made from POPC/POPG/cholesterol (65/5/30, mol/mol/mol) as a function of time at room temperature. Each solid line represents a first-order fit of the data.

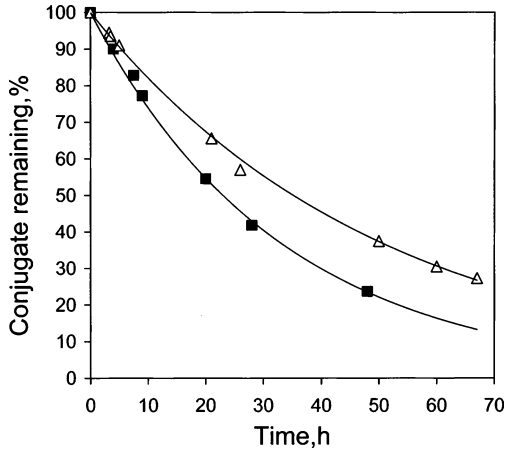


Figure 3. Plot of the percentage of **1** (△) and **2** (■) remaining as a function of time after exposure to liposomes made from POPC/POPG/cholesterol (65/5/30; mol/mol/mol, v/v/v) containing 2.0 mM of entrapped GSH. Each solid line represents a first-order fit of the data.

dialysis (i.e., solubilized in the aqueous interior and adsorbed on the inner surface), the observation of first-order release for more than one half-life establishes that **1** can cross the bilayer in its entirety. A similar experiment that was carried out in the absence of cholesterol gave the same efflux rate (not shown). With the larger, tetra-walled conjugate (**3**) as the permeant, a significantly faster efflux rate was observed (Figure 2).

In preliminary experiments, we found that **1** entered liposomes made from POPC/POPG (95/5, mol/mol) and reacted with entrapped GSH, obeying a first-order decrease in **1**.[22] The observed first-order rate constant (k_{obsd}) was 0.0197 ± 0.002 h^{-1} at 37 °C. To judge the effect that cholesterol has on this process, we carried out a similar experiment using liposomes made from POPC/POPG/cholesterol (65/5/30, mol/mol/mol). Within experimental error, the value of k_{obsd} was the same (i.e., 0.0195 ± 0.0013 h^{-1}, Figure 3). Analogous transport experiments that were carried out with **2** showed a modestly faster rate, where k_{obsd} was equal to 0.029 ± 0.002 h^{-1}. Similar experiments that were performed using the tetracholoyl and tetradeoxycholoyl analogues gave much faster rates, with values of k_{obsd} equaling 0.241 ± 0.016 and 0.105 ± 0.021 h^{-1} for **3** and **4**, respectively (Figure 4).

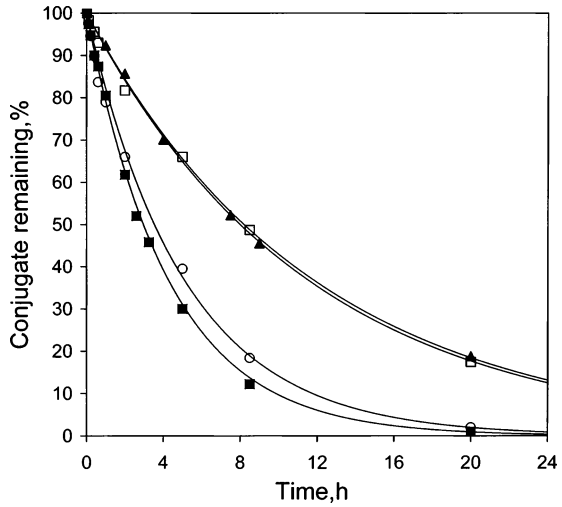


Figure 4. Plot of the percentage of **3** (■) and **4** (▲) remaining after exposure to liposomes made from POPC/POPG/cholesterol (65/5/30; mol/mol/mol, v/v/v) containing 2.0 mM of entrapped GSH at 37 °C; similar reactions were carried out using 1.1 mM entrapped GSH for **3** (○) and **4** (□).

Conjugates **3** and **4** were then examined for their activity dependence on the concentration of entrapped GSH. Within experimental error, no significant change in rate was observed when the GSH concentration was lowered from 2.0 to 1.1 mM; that is, at this lower concentration, the values of k_{obsd} for **3** and **4** were 0.227 ± 0.036 and 0.0838 ± 0.048 h^{-1}, respectively (Figure 4). These findings, together with the first-order dependency on the concentration of **3** and **4**, indicate that the rate of formation of USH is controlled by the permeation of individual conjugates across the membrane and not by chemical reaction.

To ensure that these thiolate−disulfide exchange reactions were occurring within the liposomes and not in the external aqueous phase, we analyzed for entrapped and external GSH at the end of each experiment. Specific procedures that were used are described in the Experimental section. In all cases, the percentage of entrapped GSH ranged from ca. 80−90% of its original value; less than 0.9% was present in the aqueous exterior (Table 1). These results indicate that leakage of entrapped GSH and oxidation due to adventitious oxygen were negligible under the experimental conditions used. At the same time, these results also demonstrate that the integrity of vesicles is maintained throughout these transport experiments. Dynamic light scattering measurements (Nicomp particle size analyses) also showed no significant change in particle size and size distribution of the target liposomes after exposure to the molecular umbrella−oligonucleotide conjugates and throughout the course of the transport experiments.

An additional series of analyses were performed to determine the quantity of cleaved molecular umbrella−oligonucleotide conjugate that had been captured by the liposomes (i.e., $GSSO_{cap}$), and also that which was released into the aqueous exterior ($GSSO_{ext}$). For those experiments in which the molecular umbrella−oligonucleotide conjugate (USSO) had fully reacted (i.e., those carried out with **3**), a determination of $GSSO_{cap}$ and $GSSO_{ext}$ was straightforward, because $GSSO_{ext}$ is readily removed from the dispersion by dialysis. For those cases in which USSO had undergone partial conversion, estimates of $GSSO_{cap}$ and $GSSO_{ext}$ can still be made. Thus, if we define O_{ext} as the total oligonucleotide that is present in the aqueous exterior, then $O_{ext} = GSSO_{ext} + USSO_{ext}$, where

Table 1. Composition of the Liposomal Dispersions after Transport[a]

USSO	time (h)	conversion (%)	GSH[b] (%)	O_{ext} (%)	O_{lip} (%)	$USSO_{ext}$ (%)	$USSO_{abs}$ (%)	$GSSO_{ext}$ (%)	$GSSO_{cap}$ (%)
1	67	73 ± 2.4	88 ± 1.1	42 ± 0.6	53 ± 0.6	14 ± 1.6	13 ± 2.9	28 ± 1.7	40 ± 2.9
2	42	71 ± 2.4	86 ± 1.1	45 ± 0.6	62 ± 0.5	17 ± 1.4	12 ± 2.8	28 ± 1.5	50 ± 2.2
3	20	99 ± 2.8	89 ± 1.1	2.8 ± 0.5	63 ± 0.5	0	1.0 ± 2.8	28 ± 2.1	62 ± 2.8
3[c]	20	98 ± 2.8	79 ± 1.1	2.8 ± 0.5	65 ± 0.5	0	2.0 ± 2.8	28 ± 2.1	66 ± 2.8
4	20	79 ± 2.5	82 ± 1.1	38 ± 0.4	61 ± 0.5	11 ± 1.3	10 ± 2.8	27 ± 1.4	51 ± 2.8
4[c]	20	83 ± 2.6	78 ± 1.1	31 ± 0.5	64 ± 0.6	12 ± 1.3	5 ± 2.9	18 ± 1.4	59 ± 3.0

[a] Transport experiments were carried out at 37 °C using liposomes made from 20 mg of total lipid/mL containing 2.0 mM entrapped GSH. At the start of each experiment, the concentrations of GSH and USSO in the dispersion were 100 and 10 μM, respectively. See text for definitions of O_{ext}, O_{lip}, $USSO_{ext}$, $USSO_{abs}$, $GSSO_{ext}$, and $GSSO_{cap}$. [b] Percent of GSH remaining at the end of the experiment. [c] 1.1 mM entrapped GSH.

Table 2. Rate Constants (k_{obsd}) and Partition Coefficients (K) for the Molecular Umbrella Conjugates

USSO[a]	k_{obsd} (h^{-1})[b]	K[c]
1	0.0195 ± 0.0013	2404 ± 17
2	0.029 ± 0.002	2444 ± 17
3	0.241 ± 0.016	3526 ± 24
4	0.105 ± 0.021	4248 ± 36

[a] Molecular umbrella−oligonucleotide conjugate. [b] For the conversion of USSO to GSSO. [c] Partitioning coefficient determined at 37 °C.

$USSO_{ext}$ is the unreacted conjugate in the aqueous exterior. Similarly, if we let O_{lip} represent the total oligonucleotide that is associated with the liposomes, this quantity represents the amount of **1** that is adsorbed to the membrane (i.e., $USSO_{ads}$) plus the amount of cleaved oligonucleotide that has been captured in the aqueous interior (i.e., $GSSO_{cap}$). In other words, $O_{lip} = GSSO_{cap} + USSO_{ads}$. Subtraction of experimentally measured value of $USSO_{ext}$ from the measured value of O_{ext} then gives $GSSO_{ext}$. Similarly, subtraction of $USSO_{ads}$ from the experimentally measured value of O_{lip} [where $USSO_{ads}$ is estimated by subtracting $USSO_{ext}$ from the percentage of **1** that remains at the end of the experiment] affords an estimate of $GSSO_{cap}$. Table 1 summarizes the results of such analyses. As is readily apparent, the majority of USSO was converted to $GSSO_{cap}$ in all cases, but a significant fraction of $GSSO_{ext}$ was also formed.

To obtain a more accurate assessment of the relative affinities of **1**, **2**, **3**, and **4** toward these cholesterol-rich liposomes, we measured their partitioning in the absence of GSH at 37 °C. Using the same concentrations of USSO and target liposomes that were used in the transport experiments, the partition coefficients, K, were found to be nearly identical for **1** and **2**, and very similar for **3** and **4**. In addition, conjugates **3** and **4** showed significantly greater affinity toward the liposomes as compared to **1** and **2** (Table 2).

Structure−Activity Relationships. In Table 2, we compare the partition coefficients for the molecular umbrella−oligonucleotide conjugates with the corresponding first-order rate constants that characterize these translocation events. From these data, it is clear that a molecular umbrella's ability to transport an oligonucleotide across a lipid membrane is strongly dependent on the number of facial amphiphiles present, but only modestly dependent on the change from three to two hydroxyl groups per sterol. The fact that the partition coefficient changes by only ca. 50% on going from **1** to **3**, but that k_{obsd} increases by more than 1 order of magnitude, indicates that the rate of the translocation processs, itself, is more important than membrane partitioning for overall membrane transport. The greater transport rates that are associated with the large molecular umbrellas, we believe, are a likely consequence of their greater capacity to shield the attached olignucleotide. The reason **2** is more active than **1**, but **3** is more active than **4**, is not presently clear. We suspect that these differences may also relate, in some way, to the shielding properties of these molecular umbrellas.

Mechanism of Transport. The precise mechanism by which these molecular umbrella−oligonucleotide conjugates cross liposomal membranes remains to be established. Our results, however, are certainly consistent with the process that is depicted in Scheme 2. Thus, the fact that a significant fraction of the cleaved oligonucleotide is detected in the external aqueous phase ($GSSO_{ext}$) can be accounted for by partial entry of GSH into the core of the membrane during stage **B**. Subsequent cleavage then releases the product into the aqueous interior as $GSSO_{cap}$ or into the external aqueous phase as $GSSO_{ext}$. The reduction in the quantity of $GSSO_{ext}$, which accompanies the lowering of the concentration of entrapped GSH (found with **4**), is consistent with this interpretation (Table 1). In particular, by lowering the entrapped GSH concentration, the rate of cleavage within the bilayer would be expected to be reduced, allowing more time for translocation and cleavage to occur on the inner surface and aqueous interior. Additionally, the fact that the very large oligonucleotide is transported across these lipid bilayers, but the much smaller glutathione molecule is not, rules out the possibility that the translocation takes place via fixed pores or defects.[26,27] Finally, the driving force for translocation under efflux conditions is relatively straightforward; it is simply the result of a concentration gradient (i.e., chemical potential) that exists across the membrane. In contrast, the situation under influx conditions is more complex due, in part, to the formation

(26) Although it is clear that these molecular umbrella−oligonucleotide conjugates, and also ones that have been cleaved by entrapped glutathione, do not produce pores that allow for the passage of glutathione, we cannot rule out the possibility that smaller permeants may cross these membranes.[20,21] The fact that a persulfated molecular umbrella, derived from eight choloyl groups, has been found to be nontoxic at concentrations as high as 1000 μg/mL does, however, offer significant encouragement that this molecular umbrella approach toward bilayer transport could be exploitable from a drug delivery standpoint. Detailed studies of the transport properties of these umbrellas are currently in progress.

(27) In our preliminary report, we described an additional control experiment in which the transport of a nonumbrella analogue of **1** was attempted, that is, a conjugate formed from S-dT_{16} and 5-mercapto-2-nitrobenzoic acid. Thus, incubation of this conjugate with liposomes [POPC/POPG (95/5, mol/mol)] containing entrapped GSH, under experimental conditions that were similar to those used for **1**, did not reveal a detectable USH (i.e., <2%) after 50 h. Evidence that this nonumbrella analogue was reactive toward GSH was also obtained by carrying out an additional experiment in which this conjugate (15 μM) was incubated with a vesicle dispersion that was 20 mg/mL in phospholipid and 71.4 μM in GSH. In this experiment, all of the GSH was present in the external aqueous phase; that is, the aqueous compartment of the vesicles contained only buffer. Analysis for USH showed a quantitative conversion after 2 h at 37 °C. The resulting dispersion was then subjected to the same dialysis conditions that were used in the transport experiment for **1**. In this case, dialysis led to the complete removal of the oligonucleotide from the vesicles.

of $GSSO_{ext}$ via the reaction of internalized GSH with membrane-bound USSO. Nonetheless, the facile cleavage of membrane-bound USSO, in general, by internalized GSH helps to maintain an excess of USSO in the exterior aqueous phase relative to the aqueous interior within the liposomes.

Conclusions

The results of this study show that molecular umbrella conjugates are capable of transporting a 16-mer oligonucleotide across cholesterol-rich phospholipid bilayers. The inability of a much smaller glutathione molecule to cross these membranes has ruled out the possibility of translocation occurring through fixed pores or defects. The observed kinetics further indicate that the overall rate of transport and cleavage is controlled by permeation and not by chemical reaction; partitioning measurements further indicate that permeation is dominated by translocation.

Taken together, the present findings indicate that the use of molecular umbrellas offers a promising new approach that may circumvent the long-standing problem of endocytosis, which has limited the development of oligonucleotides as therapeutic agents. Further studies aimed at probing the interactions of molecular umbrella−oligonucleotide conjugates with lipid bilayers in greater detail, and at defining structure/activity relationships in model as well as natural membranes, are currently in progress.

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