Chemical Evidence for Transbilayer Movement of Molecular Umbrellas

Sarinya Shawaphun, Vaclav Janout, and Steven L. Regen*

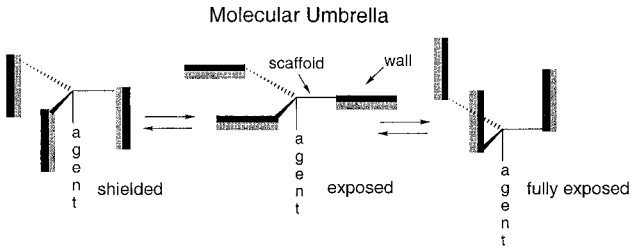
Contribution from the Department of Chemistry and Zettlemoyer Center for Surface Studies, Lehigh University, Bethlehem, Pennsylvania 18015

Received February 12, 1999

Abstract: Chemical evidence has been obtained for transbilayer movement of a di-walled and a tetra-walled molecular umbrella in large unilamellar vesicles (200 nm) derived from 1-palmitoyl-2-oleyol-*sn*-glycero-3-phoshatidylglycerol (POPG). A di-walled molecular umbrella (**1**), bearing 2-mercaptopyridine (2-MP) as a "reactive tag", was synthesized by reaction of N^1,N^3-spermidinebis[cholic acid amide] (**3**) with [*N*-1,2,3-benzotriazin-4(3H)one-yl]-3-(2-pyridyldithio)propionate [BPDP]. An analogous tetra-walled umbrella (**2**) was also prepared by condensing Fmoc-protected iminodiacetic acid with two molecules of **3**, deprotecting the secondary amino group, and coupling the resulting intermediate (**4**) with BPDP. Reaction of vesicle-bound **1** with external glutathione (GSH) resulted in a rapid and quantitative release of 2-MP. A similar thiolate−disulfide interchange reaction that was carried out between membrane-bound **1** and GSH, which was captured within the aqueous interior of the vesicles, also resulted in rapid and complete release of 2-MP. These results, together with the fact that GSH does not permeate across the POPG vesicle membranes, provides compelling evidence for rapid transbilayer movement. Reaction of membrane-bound **2** with external GSH also resulted in the rapid and quantitative release of 2-MP. The significance of these findings, with regard to the current view of molecular size restrictions on membrane permeability, is briefly discussed.

Introduction

We have recently introduced a new class of surfactants termed "molecular umbrellas" that has been designed to enhance the permeability of polar molecules across lipid bilayers.[1] Our ultimate goal is to exploit such compounds as drug carriers for the delivery of hydrophilic agents into cells.[2] In essence, a molecular umbrella consists of two or more facial amphiphiles (i.e., rigid units having a hydrophobic and a hydrophilic face) that are coupled to a central scaffold.[3,4] Our working hypothesis has been that an umbrella should facilitate the transport of an attached agent by shielding its hydrophilicity from the hydrophobic core of a membrane. Studies that have been carried out to date have shown that molecular umbrellas exhibit "molecular amphomorphism", i.e., the ability to adopt a shielded and an exposed (or a fully exposed) conformation when immersed in hydrophobic and hydrophilic environments, respectively.



One central question that has been of considerable concern to us, however, is whether molecular umbrellas are capable of crossing lipid bilayers based on their *size*. In particular, the current view that molecules larger than phospholipids (ca. 750 Da) diffuse across lipid bilayers at extremely low rates casts doubt on the viability of this approach.[5] Expressing this concern in terms of the permeant's permeability coefficient (*P*), one would expect that even if attachment of an agent to a molecular umbrella were to significantly increase its membrane/water partition coefficient (*K*), a larger reduction in its diffusion coefficient (*D*) would lead to a net lowering in overall membrane permeability; i.e., eq 1, where *L* is the thickness of the bilayer.

$$P = \frac{DK}{L} \tag{1}$$

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10.1021/ja9904641 CCC: $18.00
Published on Web 06/06/1999

In this paper, we present chemical evidence that a tetra-walled molecular umbrella having a molecular weight of 2146 Da can move across phospholipid bilayers with a half-life that is <20 min. Such a rate far exceeds that associated with the transbilayer movement of phospholipids themselves, which have half-lives that are on the order of days to weeks.[6] In a broader context, our results reported herein cast serious doubt on the notion of a ca. 750 Da molecular weight cutoff for rapid transbilayer movement—a notion that has, until now, discouraged the exploration of large molecules as carriers of drugs for cellular delivery via passive transport.

Experimental Section

General Methods. Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. All ^{1}H NMR spectra were recorded on a Bruker 360 MHz instrument; chemical shifts are reported in ppm and were referenced to residual solvents. The preparation of N^1,N^3-spermidinebis-[cholic acid amide], which was required for the synthesis of di- and tetra-walled umbrellas, has previously been described.[1b]

***N*-[*O*-1,2,3-Benzotriazin-4(3H)one-yl]-3-(2-pyridyldithio)propionate [BPDP].** Procedures that were used for the synthesis of BPDP were similar to those that have previously been described for the preparation of *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP).[7] To a solution of 2-carboxyethyl-2-pyridyl disulfide (0.50 g, 2.3 mmol) in 10 mL of CH_2Cl_2 was added *N*-hydroxybenzotriazinone (0.40 g, 2.5 mmol) as a powder. After addition of dicyclohexylcarbodiimide (0.53 g, 2.5 mmol), the resulting solution was stirred for 3 h at room temperature. Removal of the resulting urea by filtration, followed by concentration under reduced pressure and recrystallization (two times) from ethanol afforded 0.3 g (61%) of BPDP having mp 59−61 °C and ^{1}H NMR ($CDCl_3$) δ 3.2 (m, 4 H), 7.1−8.5 (m, 8 H). Anal. Calcd for $C_{15}H_{12}N_4O_3S_2$: C, 49.98; H, 3.36; N, 15.56. Found: C, 49.83; H, 3.45; N, 15.54.

Di-walled Umbrella (1). To a heterogeneous mixture that was composed of N^1,N^3-spermidinebis[cholic acid amide] (0.08 g, 0.086 mmol), 2 mL of CH_2Cl_2, and diisopropylethylamine (0.044 g, 0.34 mmol) was added BPDP (0.032 g, 0.089 mmol). The mixture became homogeneous within 1 min. After the mixture was stirred for 1 h at room temperature, the solvent was removed under reduced pressure and the residue then dissolved in 2 mL of CH_3OH. The organic product was then precipitated by addition to an aqeuous solution that was made from 20 mL of saturated $NaHCO_3$ and 1 mL of 10% Na_2CO_3. The solid was then triturated with 2 × 5 mL of water and purified by preparative thin-layer chromatography [silica, $CHCl_3$/CH_3OH/H_2O, 103/27/3 (v/v/v)] to give 71 mg (73%) of the corresponding di-walled umbrella bearing a reactive "tag" (i.e.,**1**), which was characterized by R_f 0.68 and ^{1}H NMR [$CDCl_3$/CD_3OD, 2/1 (v/v)] δ 0.66 (s, 6 H), 0.80−2.30 (m, 60 H), 2.36 (t, 2 H), 2.80 (t, 2 H), 2.96−3.30 (m, 8 H), 3.35 (s, 2 H), 3.78 (bs, 2 H), 3.92 (bs, 2 H), 7.18 (m, 1 H), 7.75 (m, 2 H), 8.38 (d, 1 H). HRMS for $(C_{63}H_{103}N_4O_9S_2)^+$: calcd, 1123.7167; found, 1123.7165.

Di-walled Umbrella−Glutathione Conjugate. The di-walled umbrella **1** (0.071 g, 0.063 mmol) was dissolved in 2.0 mL of CH_3OH and then added to a solution of glutathione (0.0193 g, 0.063 mmol) in 0.4 mL of water. After the mixture was stirred at room temperature for 24 h under a nitrogen atmosphere, the solvent was then removed under reduced pressure, and the desired conjugate purified by column chromatography [silica, $CHCl_3$/CH_3OH/H_2O, 65/40/10 (v/v/v)] to give 52 mg (63%) of the double-walled umbrella−glutathione conjugate having R_f 0.48 and ^{1}H NMR (CD_3OD) δ 0.67 (s, 6 H), 0.87−2.24 (m, 62 H), 2.73 (m, 2 H), 2.96 (m, 2 H), 3.20 (m, 8 H), 3.30−3.60 (m, 7 H), 3.78 (s, 2 H), 3.93 (s, 2 H), 4.57 (m, 1 H). HRMS for $(C_{68}H_{115}N_6O_{15}S_2)^+$: calcd, 1319.7862; found, 1319.7876.

Fmoc Carbamate of Iminodiacetic Acid. To a solution of iminodiacetic acid (0.49 g, 3.68 mmol) in a mixture of 4 mL of saturated aqeuous $NaHCO_3$ and 5 mL of dioxane was added 9-fluorenylmethylcarbonyl chloride (Fmoc-Cl) (0.90 g, 3.67 mmol) portionwise at room temperature. The reaction mixture was stirred in a closed flask at room temperature for 20 min, and the solvent then removed under reduced pressure (40 °C). The resulting oil was acidified via addition of 20 mL of 1 M HCl. The colorless solid product was then dissolved in 20 mL of $CHCl_3$ and washed, sequentially, with 10 mL 1 M HCl (twice) and 10 mL of water (three times). Removal of $CHCl_3$ under reduced pressure and recrystallization (twice) from 30 mL of CH_3OH afforded 0.75 g (58%) of the Fmoc carbamate of iminodiacetic acid, having ^{1}H NMR [CD_3OD/$CDCl_3$, 2/1 (v/v)] δ 4.10 (d, 4 H). 4.20 (t, 1 H), 4.34 (d, 2 H), 7.27 (t, 2 H), 7.31 (t, 2 H), 7.58 (d, 2 H), 7.74 (d, 2 H).

Fmoc-Protected Tetra-walled Umbrella. To a solution that was composed of Fmoc$N(CH_2CO_2H)_2$ (0.0192 g, 0.054 mmol), diisopropylethylamine (0.0156 g, 0.12 mmol), and 0.3 mL of anhydrous DMF was added the tetrafluoroborate salt of tetramethyluronium succinimide (TSU, 0.036 g, 0.19 mmol). The reaction mixture was stirred at room temperature for 75 min and then diluted with 0.2 mL of DMF and 0.1 mL of diisopropylethylamine, followed by direct addition of N^1,N^3-spermidinebis[cholic acid amide] (98.5 g, 0.106 mmol). After the mixture was stirred for 24 h, the solvents were removed under reduced pressure (23 °C, 0.5 mmHg). The crude product was dissolved in 1 mL of CH_3OH and then precipitated by pouring into 20 mL of saturated $NaHCO_3$. The precipitate was washed with 10 mL of water (twice) and purified by preparative thin-layer chromatography [1 mm EM Science silica, $CHCl_3$/CH_3OH/H_2O, 65/25/3 (v/v/v)] to give 0.072 g (69%) of the Fmoc-protected tetra-walled umbrella having R_f 0.75 (Note: the R_f is extremely sensitive to solvent polarity) and ^{1}H NMR (CD_3OD) δ 0.60 (d, 12 H), 0.63−2.19 (m, 132 H), 2.95−3.25 (m, 16 H), 3.35 (m, 4 H), 3.73 (s, 4 H), 3.86 (s, 4 H), 4.00 (bs, 4 H), 4.14 (d, 2 H), 4.35 (t, 1 H), 7.22−7.71 (m, 8 H).

Tetra-walled Umbrella 2. A solution of the Fmoc-protected tetra-walled umbrella (150 mg, 0.07 mmol) in 2 mL of CH_3OH plus 0.10 mL of piperidine was stirred at 40 °C for 5 h. Removal of solvent under reduced pressure and purification by column chromatography [silica, $CHCl_3$/CH_3OH/H_2O, 103/27/3 (v/v/v)] afforded the corresponding deprotected umbrella (**4**) having R_f 0.38 (the protected umbrella has an R_f of 0.70 under undentical TLC conditions) and ^{1}H NMR (CD_3OD) δ 0.70 (s, 12 H), 0.91−2.30 (m, 132 H), 3.17 (m, 8 H), 3.35 (m, 12 H), 3.54 (bd, 4 H), 3.78 (bs, 4 H), 3.94 (bs, 4 H).

To a heterogeneous mixture of **4** (0.082 g, 0.042 mmol) in 1 mL of CH_2Cl_2 and 0.044 g (0.34 mmol) of *N*,*N*-diisopropylethylamine was added a solution of BPDP (0.0152 g, 0.042 mmol) in 0.3 mL of CH_2Cl_2. After addition of 0.15 mL of CH_3OH, the reaction mixture became homogeneous and was stirred for 24 h at room temperature. The solvent was then removed under reduced pressure, and the residue dissolved in 2 mL of CH_3OH. Subsequent precipitation by addition of 20 mL of water plus 1 mL of saturated $NaHCO_3$, followed by washing with 5 mL of water (twice) and purification by preparative TLC [silica, $CHCl_3$/CH_3OH/H_2O, 65/25/3 (v/v/v)] afforded 62 mg (69%) of the tetrawalled umbrella bearing a reactive "tag" (i.e., **2**), having R_f 0.71 and ^{1}H NMR (CD_3OD) δ 0.69 (s, 12 H), 0.90−2.29 (m, 132 H), 2.70 (t, 2 H), 3.04 (t, 2 H), 3.10−3.30 (m, 20 H), 3.78 (bs, 4 H), 3.94 (s, 4 H), 4.22−4.36 (m, 4 H), 7.21 (m, 1 H), 7.81 (m, 2 H), 8.39 (d, 1 H).

Tetra-walled Umbrella−Glutathione Conjugate. A solution of **2** (0.052 g, 0.024 mmol) in 1 mL of CH_3OH was added to a solution of glutathione (0.0075 g, 0.024 mmol) in 0.1 mL of water, and the mixture allowed to stir at room temperature for 24 h under a nitrogen atmosphere. An additional 0.005 g of glutathione was then added, and the mixture stirred for an additional 48 h. Removal of solvent under reduced pressure, followed by purification via column chromatography [silica, $CHCl_3$/CH_3OH/H_2O, 65/25/3 (v/v/v)] afforded 11 mg (20%) of the tetra-walled umbrella−glutathione conjugate having R_f 0.32 and ^{1}H NMR (CD_3OD) δ 0.70 (s, 12 H), 0.90−2.24 (m, 120 H), 2.58 (m, 2 H), 2.96 (m, 2 H), 3.18 (m, 8 H), 3.30−3.60 (m, 17 H), 3.78 (s, 4 H), 3.92 (s, 4 H), 4.17 (d, 4 H), 4.69 (m, 1 H). HRMS for $(C_{127}H_{211}N_{10}O_{25}S_2Na_2)^+$: calcd, 2386.4784; found, 2386.4879.

Thioether Analogue of 2. To a stirred solution of **4** (80 mg, 0.041 mmol) in 0.5 mL of 2-methyl-1-pyrrolidone was added 3.7 mg of acryloyl chloride (0.041 mmol). The mixture was stirred at room

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temperature for 20 h and then poured into 10 mL of an aqueous solution that was saturated with $NaHCO_3$. The precipitate that was formed was washed with 2 × 10 mL of water, dissolved in 2 mL of CH_3OH, and purified by preparative TLC [silica, $CHCl_3/CH_3OH/H_2O$, 105/27/3 (v/v/v)] to give 43.5 mg of the corresponding acrylamide adduct having R_f 0.55 and ^{1}H NMR (CD_3OD) δ 0.69 (s, 12 H), 0.90−2.29 (m, 120 H), 3.17 (m, 8 H), 3.30 (m, 12 H), 3.79 (s, 1 H), 3.91 (s, 1 H), 4.28 (s, 2 H), 4.49 (bs, 2 H), 5.70 (d, 1 H), 6.17 (d, 1 H), 6.46 (m, 1 H).

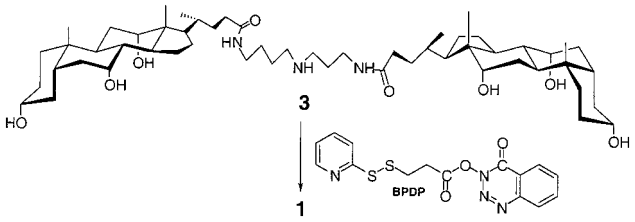
A solution of the acrylamide adduct of **4** (22.5 mg, 0.0205 mmol) and 2-mercaptopyridine (110 mg, 1 mmol) in 0.6 mL of pyridine was stirred at 45 °C for 48 h. The solvent was then removed under reduced pressure, and the resulting solid washed with water (5 × 10 mL) and purified by preparative TLC [silica, $CHCl_3/CH_3OH/H_2O$, 105/27/3 (v/v/v)] to give 17.7 mg (40%) of a thioether analogue of **2** having R_f 0.65 and ^{1}H NMR (CD_3OD) δ 0.69 (s, 12 H), 0.90−2.29 (m, 120 H), 1.77 (m, 2 H), 3.15 (m, 8 H), 3.29−3.40 (m, 16 H), 3.77 (s, 4 H), 3.92 (s, 4 H), 4.23−4.35 (m, 4 H), 7.04 (m, 1 H), 7.23 (d, 1 H), 7.56 (t, 1 H), 8.37 (d, 1 H). HRMS for $(C_{122}H_{201}N_8O_{19}S)^+$: calcd, 2114.4729; found, 2114.4673.

Vesicle Preparation. Typically, a thin lipid film was deposited onto the inner surface of a 13 × 100 mm test tube by evaporating a solution of POPG (20 mg, 26 μmol) plus 0.26 μmol of **1** (or **2**) in 2 mL of chloroform under a stream of argon. The film was then dried under reduced pressure (23 °C, 24 h, 150 mmHg), redispersed in 2 mL of borate buffer (0.1 M H_3BO_3, 2 mM EDTA, pH 8) via vortex mixing at room temperature for 30 s, incubated for 5 min, followed by additional vortex mixing for 30 s, and finally incubated for 20 min. The test tube was subsequently plunged into liquid nitrogen for ca. 30 s, and then plunged into a water bath (room temperature) for ca. 2 min. This freeze−thaw cycle was repeated five times. The resulting lipid dispersion was sequentially extruded through 0.4 μm Nuclepore membranes (10 extrusions), followed by extrusion through 0.2 μm Nuclepore membranes (10 extrusions). The vesicles were then purified by gel filtration chromatography with use of procedures similar to those previously described.[9]

Umbrella and Phospholipid Analysis. Aliquots (200 μL) of vesicles were taken before and after gel filtration and analyzed for umbrella content. Typically, a solution of sodium dodecyl sulfate (SDS, 560 μL of a 0.17 M solution in borate buffer, pH 8) was first added to each aliquot to produce a clear solution. Then, 40 μL of a 200 mM solultion of tris(2-carboxyethyl)phosphine hydrochloride (TCEP) in borate buffer was added, and the mixture allowed to incubate for 5 min at room temperature. The quantity of 2-mercaptopyridine, which was liberated, was then directly analyzed by UV (345 nm) with use of calibration curves derived from known mixtures containing 10 mM TCEP and 0.12 mM SDS in borate buffer, pH 8. Analysis of the phospholipid content before and after gel filtration was made by standard phosphorus analysis.

Kinetics of Release of 2-Mercaptopyridine. The kinetics of release of 2-MP was studied by using a 1-mL equilibrium dialysis cell. This cell consisted of two 1-mL chambers that were separated by a Spectrapor 7 dialysis membrane (50 000 MW cutoff) that had been cut from a 23 mm diameter dialysis tube. Prior to use, this tubing was soaked in 150 mL of water for 24 h at 10 °C. Fresh water was exchanged four times during this period. After the fourth exchange, the membrane was placed in borate buffer (0.1 M H_3BO_3, 2 mM EDTA, pH 8) for at least 12 h, and the dialysis cell was then assembled by placing the dialysis membrane between the two 1-mL chambers. A vesicle dispersion (1 mL) was added to one of the chambers immediately after gel filtration and 1 mL of borate buffer was added to the remaining chamber. A 20-μL aliquot of 65 mM glutathione in borate buffer (0.1 M H_3BO_3, 2 mM EDTA, pH 8) was then added to both chambers, and the cell immediately attached to a Burrell wrist action shaker and agitated at a maximum rate at ambient temperature (23 °C). Note: To ensure efficient agitation, small air bubbles should be visible in each chamber. An aliquot (ca. 0.7 mL) was taken from the chamber that was devoid of vesicles (receiving side) and analyzed for 2-mercaptopyridine by UV (345 nm). This same solution was then returned to the chamber, and the amount of 2-mercaptopyridine that was released was measured as a function of time.

Scheme 1

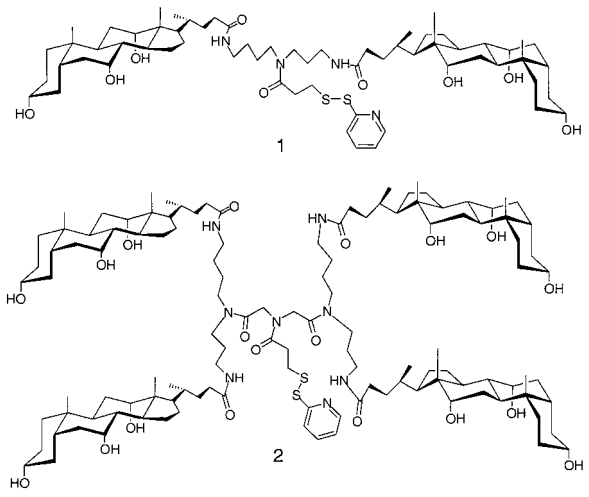


Reaction of Vesicle-Bound 1 with Captured Glutathione. Procedures that were used for vesicle formation were similar to those described above, except that the buffer that was used contained 3 mM GSH, and the hydration, extrusion, and gel filtration (two times) steps were carried out at ca. 0 °C. In these experiments, the molar ratio of entrapped GSH/molecular umbrella was ca. 3/1.

Membrane Impermeability of Glutathione. POPG-based vesicles were formed with use of procedures similar to those described above. In this case, however, 1 mol % of a thioether analogue of **2** was incorporated into the membrane, and 1.3 mM of glutathione was included in the borate buffer. Aliquots (80 μL) of the vesicles were taken before and after gel filtration and were analyzed for glutathione content by use of a fluorescamine assay and appropriate calibration curves.[9] Prior to analysis, Triton X-100 [200 μL of a 10% borate solution (w/w)] was mixed with the aliquot to produce a clear solution. Then, 20 μL of 20 mM fluorescamine in acetone was added, and the solution allowed to incubate for ca. 20 min. The percent leakage of glutathione was then analyzed as a function of time by taking aliquots (80 μL) from the receiving side of the dialysis cell and analyzing them by use of the fluorescamine assay.

Results

Umbrella Design and Synthesis. Molecular umbrellas **1** (1124 Da) and **2** (2146 Da) were specifically designed for this study to probe transbilayer movement. Here, 2-mercaptopyridine (2-MP) was chosen as a "reactive tag" based on its ability to bind to (and to be cleaved from) an umbrella via a disulfide linkage and its strong UV absorption, which permits direct analysis. Umbrella **1** was prepared by reaction of N^1,N^3-spermidinebis[cholic acid amide] (**3**) with [*N*-1,2,3-benzotriazin-4(3H)one-yl]-3-(2-pyridyldithio)propionate [BPDP] (Scheme 1).[7] The tetra-walled umbrella, **2**, was synthesized by condensing Fmoc-protected iminodiacetic acid with two molecules of **3**, deprotecting the secondary amino group, and coupling the resulting intermediate (**4**) with BPDP (Scheme 2).



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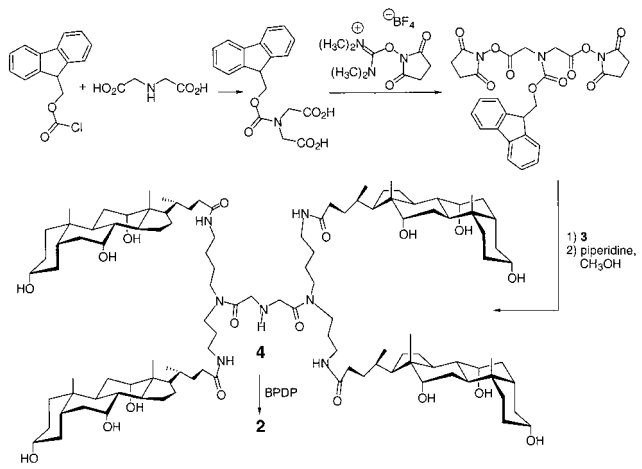
Scheme 2



Affinity of Molecular Umbrellas toward Phospholipid Vesicles. Thin films composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) plus **1** or **2** (deposited from a chloroform solution) were dispersed in borate buffer (pH 8) and subjected to freeze−thawing and extrusion, affording large unilamellar vesicles (200 nm diameter).[8] Under these conditions, the molecular umbrellas are expected to be incorporated into both halves of the bilayer and/or the aqueous phase; i.e., such a method has been termed a "double-sided" addition.[9] Analysis of the vesicles for phospholipid and umbrella content, before and after gel filtration, indicated that both umbrellas were strongly bound to the membrane. Thus, gel filtration of a 13 mM phospholipid dispersion resulted in a reduction of **1** from 1.3 to 1.0 mol % (based on POPG); with a similar loading of **2**, the umbrella content remained unchanged after gel filtration.

Reaction with External Glutathione. Since **1** and **2** have high affinity to POPG membranes, we tested for transbilayer movement by measuring the release of 2-MP from vesicles (formed via double-sided addition) by reaction with an externally added, membrane-impermeable thiol, i.e., glutathione (Glu-Cys-Gly, GSH).[10,11] In principle, if thiolate−disulfide interchange (liberating 2-MP and forming a umbrella−glutathione conjugate) were much faster than transbilayer movement, then the release of 2-MP should exhibit biphasic kinetics.[12] If chemical reaction were slower than transbilayer movement, however, then the appearance of 2-MP would follow pseudo-first-order kinetics.

Reaction of a 1.3 mM solution of GSH with 13 mM POPG vesicles containing 1 mol % of **2** (placed in the source side of a dialysis cell, 23 °C, pH 8) resulted in a *quantitative* release of 2-MP (analyzed from the receiving side of the cell). This release followed pseudo-first-order kinetics and was characterized by a half-life of ca. 4 h; increasing the concentration of GSH to 5.0 mM resulted in a proportional increase in rate (Figure 1). Analysis of the dispersion before and after reaction by dynamic light scattering showed no change in particle size. Similar experiments that were carried out with identical concentrations of **1** and POPG vesicles, in the presence of 5 mM of GSH, afforded a half-life for release of 5 h; when the initial concentration of GSH was 10 mM, the rate increased proportionally (Figure 2). Analysis of each dispersion by TLC confirmed the presence of the expected tetra-walled and di-walled glutathione conjugates, based on comparison with authentic samples. In a separate experiment, the time that was required for 50% of pure 2-MP (nonconjugated form) to cross the dialysis membrane was ca. 15 min. Thus, permeation of 2-MP across the dialysis membrane is not rate-limiting under these conditions.[13] The faster rate of release of 2-MP from **2** is,

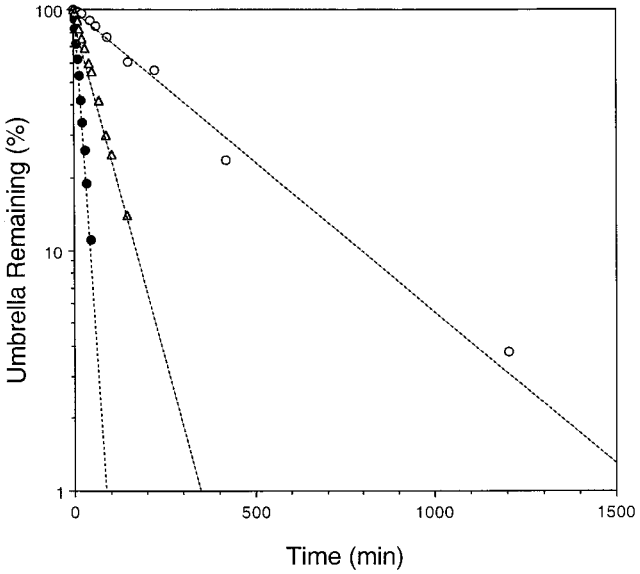


Figure 1. The percentage of vesicular **2** remaining as a function of time for the reaction of 13 mM POPG containing 1 mol % of **2** with (○) 1.3 mM GSH, (△) 5.0 mM, and (●) 25 mM GSH in borate buffer (pH 8.0).

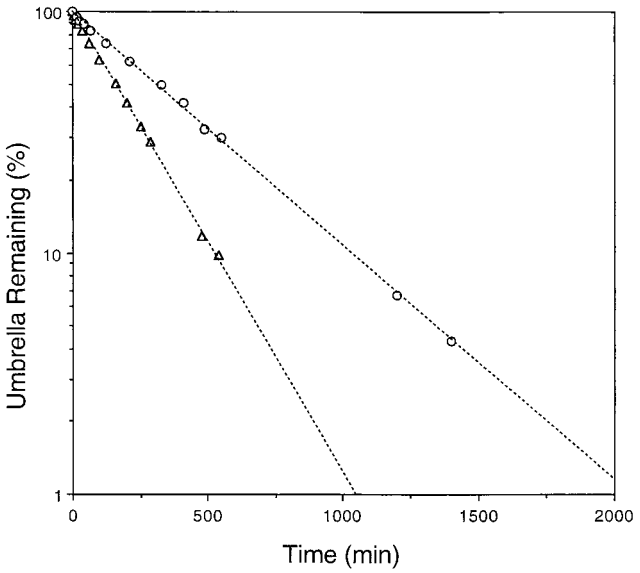


Figure 2. The percentage of vesicular **1** remaining as a function of time for the reaction of 13 mM POPG containing 1 mol % of **1** with (○) 5.0 mM GSH and (△) 10.0 mM GSH in borate buffer (pH 8.0).

(10) Vesicles derived from POPG, which contained 1.3 mM GSH within their aqueous interior, showed negligible peptide release after 24 h. The half-life for permeation of GSH across the dialysis membrane is ca. 2 h, in the absence and in the presence of vesicles containing a thioether analogue of **2**.

(11) Very poor water solubility of **1** and **2** prevented their external addition to vesicles containing entrapped GSH.

(12) Examination of **1** and **2** by CPK models indicates a maximum depth of penetration of the disulfide moiety in an exposed conformation (sterols lying flat at the water/membrane surface) of ca. 16 Å. For a fully exposed conformation (where the hydrophobic side of each sterol faces the alkyl chains of a neighboring phospholipid), the maximum depth of penetration is ca. 30 Å. Thus, incorporation of these umbrellas into a unilamellar vesicle having a bilayer thickness that exceeds 30 Å should limit their accessibility toward an externally added, membrane-impermeable reagent to only those disulfide moieties that are located in the outer monolayer leaflet. Since POPG membranes have a bilayer thickness of ca. 37 Å (Lehtonen, J. Y. A.; Kinnunen, P. K. J. *Biophys. J.* **1997**, *72*, 1247), the observation of total release of 2-MP provides compelling evidence for transbilayer movement.

we believe, a likely consequence of greater reactivity due to a more exposed conformation at the membrane surface. When POPG-bound **2** was exposed to much higher concentrations of GSH (25 mM), the rate of release of 2-MP, as expected, corresponded to the rate of permeation of free 2-MP across the dialysis membrane (Figure 1).

Reaction with Internal Glutathione. To ensure that all of the umbrella molecules are accessible to the inner surface of the membrane, as well as the external surface, we determined the extent of thiolate−disulfide interchange between membrane-bound **1** (prepared by double-sided addition) and GSH that was captured within the aqueous interior of the vesicles. For these experiments, chemical reaction during vesicle formation and gel filtration was minimized by carrying out these steps at ca. 0 °C. The total amount of time that was required to prepare and purify the vesicles (prior to adding them to the source side of the dialysis cell) was 120 min. Under the conditions that were used, where 3.0 mM GSH was entrapped in the vesicles (corresponding to 3 equiv relative to the membrane-bound umbrella), less than 5% of the umbrella-bound 2-MP was released after 1.2 h (analysis of the receiving side of the dialysis cell); extending the reaction time to 54 h resulted in a quantitative release of 2-MP. Subsequent analysis of the receiving side for GSH after the reaction was complete indicated negligible release of the peptide from the vesicles.

Discussion

Taken together, the results presented herein provide a compelling case for rapid transbilayer movement of **1** and **2** in bilayers of POPG. Since these thiolate−disulfide interchange reactions were found to be pseudo first order, when GSH was introduced externally, and since the bilayer is impermeable to GSH, this must mean that (i) transbilayer movement is much faster than chemical reaction or that (ii) all of the umbrella molecules are localized exclusively on the external surface of the vesicles. The fact that a 3-fold excess of internalized GSH resulted in a quantitative release of 2-MP from **1**, together with the fact that the release of GSH to the external bulk phase was negligible, provides compelling evidence that the umbrella is not exclusively localized on the vesicle exterior. Morever, if chemical reaction were taking place in the center of the membrane, then the GSH that reaches the center should be able to diffuse to the other side; i.e., GSH should be able to permeate the membrane. The fact that the bilayer is impermeable to GSH, however, rules out such a possibility. On the basis of these findings, we infer that the transbilayer diffusion rate **1** is very fast relative to the time scale of chemical reaction. Although we were not able to investigate the reaction of membrane-bound **2** with internalized GSH, because of its greater reactivity, the absence of literature precedent for exclusive placement of a membrane component on the outer surface of vesicles (prepared under double-sided conditions), together with the results obtained with **1**, makes it highly probable that **2** is also distributed on both sides of the membrane. On the basis of the results that are presented in Figure 1, an upper limit for the half-life for transbilayer movement of the tetra-walled molecular umbrella (**2**) can be estimated to be ca. 15 min in POPG membranes; from the results shown in Figure 2, an upper limit for the half-life for transbilayer movement of **1** is ca. 2.5 h. Thus, both umbrellas diffuse across POPG membranes at rates that far exceed those associated with the transbilayer movement of phospholipids, themselves.

Although the present findings yield insight into the diffusional properties of molecular umbrellas **1** and **2**, they do not provide information concerning their thermodynamically favored orientation within and/or on the surface of these membranes. It is noteworthy, in this regard, that this same question of membrane orientation of bile acids remains largely unresolved.[14] Results from recent monolayer studies involving undissociated bile acids tend to suggest that penetration of bile acid-based molecular umbrellas within phospholipid bilayers is thermodynamically unfavorable, i.e., that the sterol prefers to lie parallel to the membrane−water surface with the hydrophilic face pointing toward the aqueous phase.[14]

Conclusions

In this study, we have presented compelling chemical evidence in support of rapid transbilayer movement of a di-walled and a tetra-walled molecular umbrella molecule in POPG membranes. Our findings provide further incentive for exploring the permeation behavior of umbrella−drug conjugates in detail. At the same time, these results draw attention to the fact that molecular structure and composition can play a greater role in defining transbilayer movement than molecular size—a fact that should stimulate other approaches to the design of novel drug delivery vehicles.

Acknowledgment. We are grateful to the National Institutes of Health (PHS Grant GM51814) for support of this research.

JA9904641

(13) Vesicles that were formed in the presence of 1 mol % of a thioether analogue of **2** (prepared by condensing **4** with acryloyl chloride, followed by conjugate addition of 2-mercaptopyridine) plus 1.3 mM GSH, followed by gel filtration, resulted in entrapment of the peptide without signficant efflux after 24 h. These results indicate that such tetra-walled umbrellas do not disrupt the integrity of POPG bilayers.

(14) Fahey, D. A.; Carey, M. C.; Donovan, J. M. *Biochemistry* **1995**, *34*, 10886.