Molecular Umbrella-Assisted Transport of a Hydrophilic Peptide Across a Phospholipid Membrane

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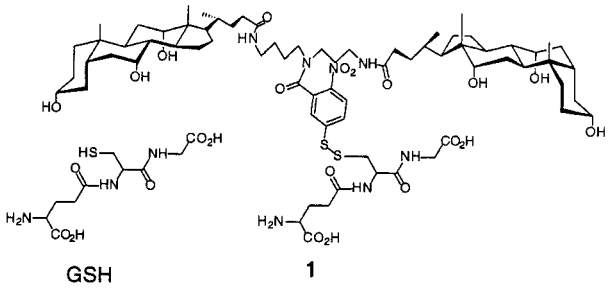
In this paper, we demonstrate the feasibility of using a molecular umbrella as a vehicle for transporting a hydrophilic peptide across a phospholipid bilayer. Specifically, we show that the covalent attachment of glutathione (γ-Glu-Cys-Gly, GSH) to a di-walled molecular umbrella, derived from cholic acid and spermidine, yields a peptide−umbrella conjugate (**1**, USSG) that can readily permeate bilayers composed of 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC).



One of the most significant challenges presently facing medicinal chemists is to find ways of promoting the passive transport of polar, biologically active agents (e.g., peptides, antisense oligonucleotides, and DNA) across lipid membranes.[1,2] In an effort to create a general solution to this problem, we have been developing a novel class of amphiphilic molecules termed, "molecular umbrellas".[3] In essence, a molecular umbrella consists of two (di-walled) or more amphiphilic walls (rigid hydrocarbon units that maintain a hydrophobic and a hydrophilic face) that are coupled to a central scaffold. Our working hypothesis has been that such a molecule should facilitate the transport of an attached agent by adopting a shielded conformation, thereby hiding its hydrophilicity from the hydrophobic core of a lipid bilayer. As a first step toward this goal, we have shown that molecular umbrellas display "molecular amphomorphism", that is, the ability to form a shielded or exposed conformation in hydrophobic and hydrophilic environments, respectively. In this paper, we report the first example of umbrella-assisted permeation through a lipid bilayer.

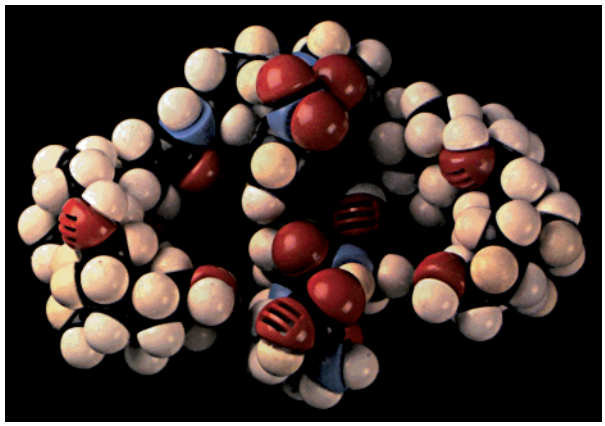
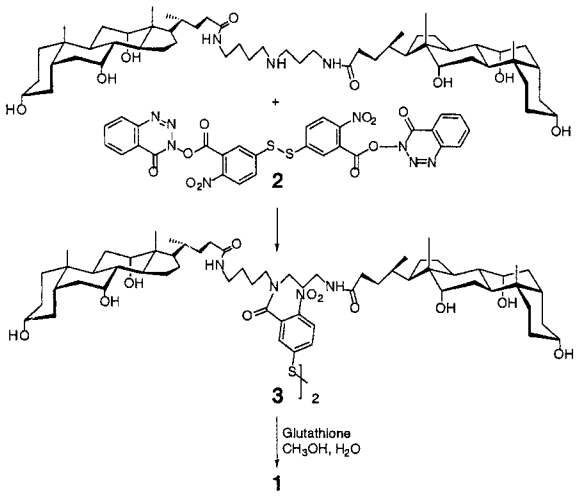


Figure 1. CPK molecular model of **1** arranged in a shielded conformation.

Scheme 1



Our design of **1** was based on five considerations. First, we viewed GSH as a reasonable drug model based on its strong hydrophilicity. In particular, GSH would not be expected to cross a lipid bilayer, in the absence of a carrier. Second, GSH can be covalently attached to, as well as released from, a molecular umbrella by taking advantage of thiolate−disulfide interchange chemistry. Third, the 5-thiol(2-nitrobenzoyl) "handle" would allow one to monitor the release of GSH within the interior of the vesicle via its strong UV absorption. Fourth, previous studies from our laboratory have shown that di-walled molecular umbrellas derived from cholic acid and spermidine exhibit molecular amphomorphism, which is a likely prerequisite for membrane transport.[3] Fifth, examination of **1** by CPK molecular models indicate that such an umbrella can provide significant coverage for an attached GSH molecule (Figure 1).

The synthetic approach that was used to prepare **1** is summarized in Scheme 1. In brief, reaction of N^1,N^3-spermidinebis-[cholic acid amide] with 5,5′dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), which had been doubly activated with 3-hydroxy-1,2,3-benzotriazin-4(3H)-one (i.e., **2**) afforded an umbrella dimer (**3**).[4] Subsequent reaction with GSH yielded the desired umbrella, **1**, having λ_{max} 334 nm (ϵ 7.142 × 10^3 M^{-1}

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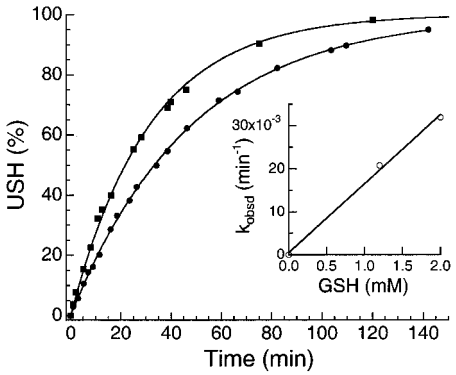
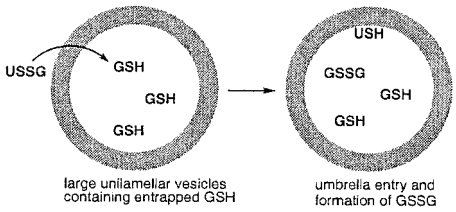


Figure 2. Pseudo-first-order appearance of USH for reaction of 0.2-μm POPC vesicles containing 1.2 mM GSH (●) and 2.0 mM GSH (■) with **1** at 23 °C; inset shows the k_{obsd} versus the internal concentration of GSH.

Scheme 2



cm^{-1}, borate buffer, pH 7.0).[5] The corresponding thiol, generated via the reduction of **1** with tris(2-carboxyethyl)phosphine) (TCEP), exhibited a λ_{max} 427 nm (ϵ 7.007 $\times$ 10^3 M^{-1} cm^{-1}, borate buffer).[6]

To test for membrane transport, we examined the ability of **1** to enter a phospholipid vesicle containing entrapped GSH. Evidence for such entry would then rest upon (i) the formation of oxidized glutathione (GSSG) *within the interior of the vesicle*, (ii) the appearance of the thiol form of the umbrella (USH), and (iii) the absence of release of GSH into the external aqueous phase, that is, chemical reaction taking place within the vesicle interior (Scheme 2).

With this experimental design in mind, large unilamellar vesicles were prepared from POPC (200 nm diameter, extrusion) in borate buffer (pH 7.0) containing 1.2 mM GSH. Removal of nonentrapped GSH via gel filtration and dialysis afforded a phospholipid dispersion (20 mg/mL, 26 mM). In a typical experiment, 200 μL of this dispersion were mixed with 300 μL of a 20 μM solution of **1** in borate buffer. At such a concentration, the umbrella molecule exists in solution as a free monomer (the critical micelle concentration of **1** is 60 μM, surface tension method). Mixing was carried out in the "source" side of a 1.5-mL equilibrium dialysis cell (containing an equal volume of borate buffer in the "receiving" side) using a wrist-action shaker. The extent of thiolate-disulfide interchange was then monitored in the source side by the appearance of USH (Figure 2). A similar experiment that was carried out, in which the entrapped concentration of GSH was increased to 2.0 mM, resulted in a proportional increase in the observed pseudo-first-order rate constant (Figure 2). These results indicate that the rate of formation of USH is controlled by *chemical reaction*, and not by permeation across the lipid bilayer (eq 1). Binding studies indicate that both USSG and USH have a high affinity toward POPC membranes; that is, centrifugation of multilamellar dispersons of POPC containing USSG or USH resulted in more than 94 and 90% removal of each from solution, respectively.

$$\text{USSG} + \text{GSH} \rightarrow \text{USH} + \text{GSSG} \qquad (1)$$

To determine whether GSH was fully retained by the vesicles, the receiving side of the cell was analyzed for GSH by reaction with Ellman's reagent. In essence, no GSH could be detected ($<$0.1 μM) after 24 h. It should be noted that complete release of GSH would correspond to a thiol concentration of $\sim$28.5 μM in both the source and receiving side of the cell, since the half-life for permeation of GSH across the dialysis membrane is 200 min. Analysis of the source side for residual thiol (Ellman's reagent), after destroying the vesicles with sodium dodecyl sulfate, confirmed the presence of 22.6 nmol of GSH. This provides an excellent mass balance, since complete reaction of GSH with **1** would, theoretically, leave 23.5 nmol of the tripeptide within the vesicles.

To establish that GSSG was formed *within* the vesicles, we analyzed for GSSG in both the source and receiving side after a 28 h period. It should be noted, that GSSG crosses the dialysis membrane with a half-life of $\sim$8 h. Analysis of GSSG in the source side was made by a combination of disulfide reduction with excess of TCEP, and back-titration with 4,4′-dipyridyl disulfide (Aldrithiol-4)).[7,8] To prevent GSH from reacting with Aldrithiol-4, strongly acidic conditions were used in this analysis.[8] Thus, when 12 μM (6 nmol) of **1** and 57 μM (28.5 nmol) of vesicle-entrapped GSH were introduced into the source side, and the vesicles allowed to react/dialyze for 28 h, 10.9 μM (5.45 nmol) of GSSG and 45.2 μM (22.6 nmol) of GSH were detected in the source side. Analysis of the receiving side for GSSG, using a more sensitive fluorescamine assay, indicated the presence of less than 0.24 μM (0.12 nmol) of the peptide.[9,10] As a further control, GSSG was added, *externally*, to the vesicle dispersion (corresponding to 10 μM of external GSSG in the source side), and the receiving side then analyzed for GSSG. After 8 h of dialysis, the concentration of GSSG that appeared in the receiving side was 2.2 μM. Thus, adsorption of GSSG to the lipid membrane is negligible.

Taken together, the present findings provide compelling evidence for umbrella-assisted transport of GSH across bilayers of POPC. The fact that the rate of formation of USH is controlled by chemical reaction further indicates that the half-life for membrane transport must be $\ll$22 min. Finally, it should be noted that the presence of millimolar concentrations of GSH within the cytoplasm of mammalian cells suggests that the chemistry described herein can be translatable into practical prodrug delivery devices. Efforts aimed at extending this methodology to peptides of therapeutic interest (e.g., enkephalins), as well as to antisense oligonucleotides are currently in progress.

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Supporting Information Available: Procedures for the synthesis of **1**, kinetic experiments, and analyses of GSH and GSSG (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(5) The borate buffer that was used in all of the experiments described herein was composed of 0.1 M H_3BO_3 that contained 2 mM EDTA, and was adjusted to pH 7.0 by titration with 1 M NaOH.

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(8) Control experiments established that under the conditions used, reaction of TCEP with Aldrithiol-4 is complete, while reaction of GSH with Aldrithiol-4 is negligible.

(9) The fluorescamine assay could not be used on the source side due to interference with the lipids and with GSH.

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