Communication

A Needle-and-Thread Approach to Bilayer Transport: Permeation of a Molecular Umbrella–Oligonucleotide Conjugate across a Phospholipid Membrane

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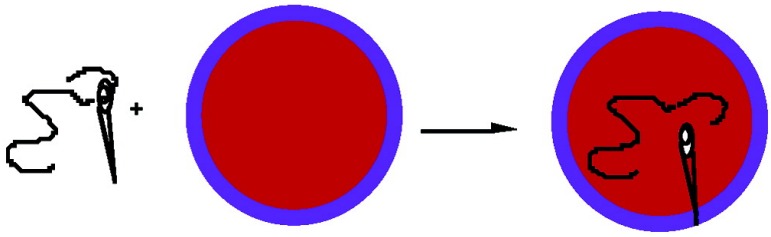

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A Needle-and-Thread Approach to Bilayer Transport: Permeation of a Molecular Umbrella−Oligonucleotide Conjugate across a Phospholipid Membrane

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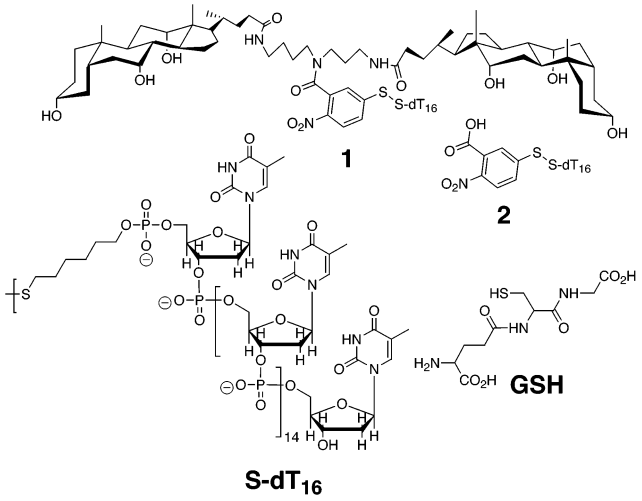
In this paper, we demonstrate the feasibility of using a molecular umbrella to transport a 16-mer oligonucleotide across a phospholipid bilayer. Specifically, we show that conjugate **1** is capable of delivering S-dT$_{16}$ into the aqueous compartment of large unilamellar liposomes containing entrapped glutathione (Chart 1). In a sense,

Chart 1



the molecular umbrella moiety functions like a "needle" in providing a pathway for the oligonucleotide (the "thread") to cross the membrane.

Previous studies from our laboratories have shown that molecular umbrellas (i.e., molecules bearing two or more facially amphiphilic units attached to a central scaffold) can transport small hydrophilic peptides such as glutathione (GSH) across phospholipid bilayers.[1−3] A long-standing question that we have sought to answer is whether molecular umbrellas can also transport much larger species, where only partial shielding is possible (Figure 1).

In the work reported herein, we have examined this question using a 16-mer oligonucleotide (S-dT$_{16}$) that has been covalently

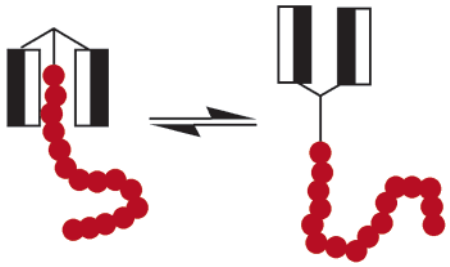
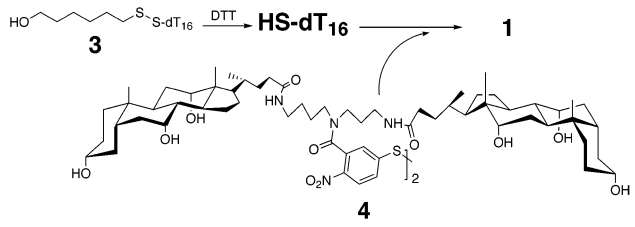


Figure 1. Stylized illustration of a molecular umbrella in a shielded conformation covering a small segment of an attached polar agent (left), and the same umbrella in a fully exposed conformation (right). Here, the shaded and unshaded rectangles represent hydrophobic and hydrophilic faces, respectively; the polar agent appears as a red beaded chain.

Scheme 1



attached to a di-walled molecular umbrella through a disulfide linkage (i.e., **1**). An oligonucleotide was specifically chosen for this investigation due to the broad potential of antisense oligonucleotides as therapeutic agents and because membrane transport has been identified as a major limiting factor.[4−8] Examination of space-filling models indicates that a maximum of about two nucleotides units of **1** can be shielded by the umbrella moiety, leaving a minimum of ca. 90% of the oligonucleotide exposed at all times. To judge the "needle-like" action of the umbrella moiety, an analogue (**2**) was also examined since it does not bear amphiphilic walls. Using an approach similar to that previously described, our evidence for vesicular entry rests on (i) the appearance of the umbrella-bound 5-mercapto(2-nitrobenzoyl) group (USH) that is generated from thiolate-disulfide interchange with entrapped GSH (eq 1), (ii) the absence of leakage of entrapped GSH, and (iii) a capture of the oligonucleotide by the vesicles that matches the extent of USH formation.[1−3]

$$\mathbf{1} + \mathrm{GSH} \rightarrow \mathrm{USH} + \mathrm{GSS\text{-}dT_{16}} \quad (1)$$

Conjugate **1** was synthesized by reducing HO(CH$_2$)$_6$SS-dT$_{16}$ (**3**) with excess dithiothreitol, followed by reaction of the resulting HS-dT$_{16}$ with 1 equiv of homodimer **4** to give **1** (Scheme 1).[2,9] Alternatively, reaction of HS-dT$_{16}$ with 5,5-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) afforded **2**.

Target vesicles were prepared from 1-palmitoyl-2-oleyol-*sn*-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidylglycerol (POPG) (95/5, mol/mol) (200 nm diameter, extrusion) using a TRIS buffer (10 mM TRIS, 100 mM NaCl, 1 mM EDTA, pH 7.2) that was 1.2 mM in GSH. We specifically chose POPC for these model studies because it closely resembles those phospholipids that are commonly found in mammalian cells. A small percentage of a negatively charged analogue, POPG, was also included in these membranes to increase the stability of the dispersion. Removal of non-entrapped GSH by dialysis afforded a vesicle dispersion that was 20 mg/mL in phospholipid and 0.210 mM in GSH.

In a typical transport experiment, 600 μL of the vesicle dispersion was mixed with 200 μL of a 28 μM solution of **1** at 37 °C under an argon atmosphere. The thiolate−disulfide interchange reaction was monitored by following the appearance of USH (λ_{max} 425 nm).

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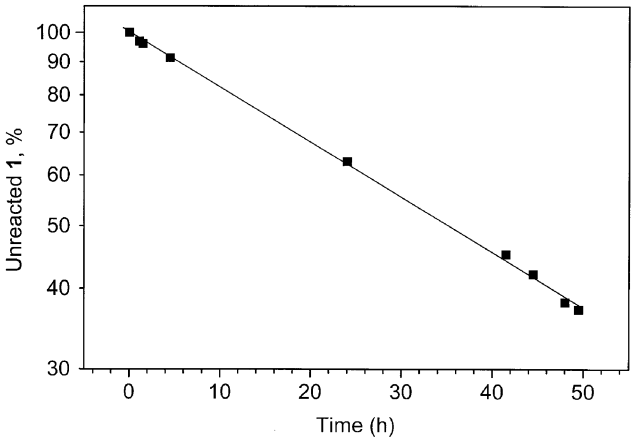

Figure 2. Semilogarithmic plot of the decrease of **1** during incubation with target vesicles containing GSH at 37 °C as a function of time.

A semilogarithmic plot of the percentage of **1** that remains in the dispersion as a function of time is shown in Figure 2. After a 50 h incubation period, when a 4.4 μM concentration of USH (63%) was produced, the dispersion was cooled to room temperature and placed in the "source" side of an equilibrium dialysis cell for 2 h. Analysis of the "receiving" side for GSH (Ellman's assay) showed no detectable thiol content ($<$0.1 mM). Analysis of the source side showed that the dispersion was 0.152 mM in GSH. Given the fact that the starting concentration of GSH in the reaction mixture was 0.158 mM, and that a 4.4 μM concentration of GSH was "spent" in forming USH, these results indicate that virtually all of the GSH remains entrapped within the vesicles and that the extent of oxidation due to adventitious oxygen was negligible.

To quantify the extent of capture of the oligonucleotide, the dispersion was further dialyzed against TRIS buffer (2 $\times$ 1000 mL for 24 h) to remove unreacted **1**. Analysis of the dispersion then showed an oligonucleotide concentration that was 4.2 μM (λ_{max} 268 nm), corresponding to a 62% capture. Thus, the extent of capture is in excellent agreement with the quantity of USH that was generated prior to dialysis.

An attempted transport of **2** was made by incubating this nonumbrella analogue with a similar dispersion of target vesicles at 37 °C. After 50 h, no USH (i.e., $<$2%) could be detected. To establish that **2** was reactive toward GSH, an additional experiment was performed in which **2** (15 μM) was incubated with a vesicle dispersion that was 20 mg/mL in phospholiplid 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.

Taken together, the present findings show that a molecular umbrella is capable of delivering S-dT_{16} to the aqueous interior of POPC/POPG vesicles containing entrapped GSH. If similar transport is possible across cellular membranes, this would have significant implications for the further development of antisense oligonucleotides as chemotherapeutic agents. Efforts currently underway are being aimed at (i) examining cellular uptake of antisense oligonucleotide-molecular umbrella conjugates, (ii) gaining insight into the mechanism by which **1** crosses phospholipid bilayers, and (iii) exploring the full scope of this needle-and-thread approach to bilayer transport. The results of these efforts will be reported in due course.

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Supporting Information Available: Procedures for preparing **1**, **2**, and target vesicles, analyzing GSH, and carrying out membrane transport experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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