Non-Leaky Vesicle Fusion and Enhanced Cell Transfection Using a Cationic Facial Amphiphile
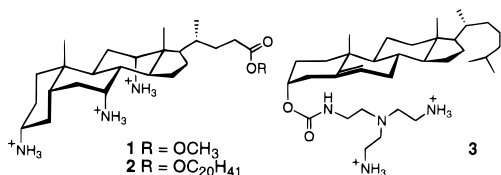
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Membrane fusion is an essential feature in many cellular processes such as endo- and exocytosis, fertilization, intracellular trafficking, and viral infection. Fusion is a complicated, multistep process and is known to be promoted by fusion proteins.[1] In many cases, the fusion activity has been localized to specific peptide sequences within these proteins, but there is conflicting evidence concerning the active secondary structure of these peptide sequences.[2] Often an amphiphilic α-helix is proposed, but in some cases there is evidence for a fusogenic β-sheet.[2,3] One rationalization of this observation considers overall structural amphiphilicity as the dominating factor rather than any specific structural element.[3] An alternative view is a peptide plasticity model which proposes that the fusion peptide can readily switch between a number of fusogenic structures.[4] A major reason for the mechanistic uncertainty is peptide flexibility, which makes it difficult to clearly define the structure of the kinetically active species. In an effort to help elucidate the factors that control membrane fusion, we are evaluating a range of structurally rigid architectures for their abilities to act as fusogens.[5] The advantage of this approach is that a rigid structure greatly simplifies the interpretation of results. In addition, there is the potential of discovering new types of fusion promoters that may be useful in drug delivery and gene therapy applications.

In this paper we describe the fusogenic ability of a facially amphiphilic steroid, a compound that can be viewed as a rigid mimic of a facially amphiphilic α-helix. Rigid, non-peptidyl facial amphiphiles have been reported before,[6,7] but to our knowledge they have not been evaluated as membrane fusogens. We compare the related facial amphiphiles **1** and **2** to the head−tail amphiphile **3**[8] and find that **2** is exceptionally good at promoting non-leaky vesicle fusion as well as human cell transfection.

1 R = OCH₃
2 R = OC₂₀H₄₁
3

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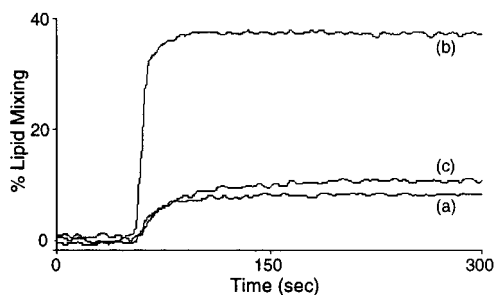
Initially, we evaluated the ability of externally added facial amphiphile **1** to promote the self-fusion of anionic vesicles at neutral pH. Addition of low concentrations of **1** (<15 μM) to 100 nm unilamellar vesicles composed of POPA:PE[9] (3:7) (50 μM total phospholipid) did not produce self-fusion, but at higher concentrations, amphiphile **1** did induce leaky self-fusion as judged by fluorescent contents mixing and leakage assays (e.g., 30 μM of **1** induced 12% contents mixing and 30% leakage).[10]

Recently, it was shown that the activity of a fusion peptide can be greatly enhanced if the peptide is "anchored" in the vesicle membrane.[11] With this work in mind we prepared analogue **2**, which contains a C-20 side chain, and conducted fusion studies using preformed cationic donor vesicles and anionic target vesicles. Two different vesicle compositions were evaluated. The first composition was PE:PC:X[9] where X is either **1**, **2**, or **3** for the cationic donor vesicles and X is POPA for anionic target vesicles. For each composition the concentration of X was adjusted so that the charge at the vesicle surface was a constant 15% (POPA is predominantly a monoanion at pH 7.4 and it was assumed that **1** and **2** are tricationic, whereas **3** is dicationic). Thus, the donor cationic vesicles were composed of PE:PC:X (14:5:1) for the facial amphiphiles **1** and **2**, and (28:9:3) for **3**. The target anionic vesicles were composed of PE:PC:POPA (14:3:3).

The donor vesicles were either unlabeled for lipid mixing and leakage assays or encapsulated the fluorescence quencher DPX for contents mixing assays.[9,10] The target vesicles were labeled with the resonance energy transfer probes NBD-PE and Rh-PE for lipid mixing assays, encapsulated the fluorophore ANTS for contents mixing assays, or encapsulated ANTS/DPX for leakage assays.[10] The fusion ability of each amphiphile was determined for 1:1 mixtures of donor and target vesicles with a final lipid concentration of 50 μM. As shown in Figure 1, donor vesicles containing **2** rapidly underwent 38% intermembrane lipid mixing, while donor vesicles containing **1** and **3** induced 10 and 11% lipid mixing, respectively. Donor vesicles containing **2** also induced significant internal contents mixing (20% after 10 min), whereas vesicles containing **1** or **3** were essentially ineffective (Figure 2). In the case of **2**, contents mixing occurs more slowly

(8) The synthesis of **1** has been reported before (Davis, A. P.; Pérez-Payán, M. N. *Synlett* **1999**, *S1*, 991−993). The syntheses of **2** and **3** are described in the Supporting Information.
(9) Abbreviations used in this report: ANTS, 1-aminonaphthalene-3,6,8-trisulfonate; DOPE, dioleoylphosphatidylethanolamine; DOTMA, *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride; DPX, *N,N′-p*-xylenebis-(pyridinium bromide); NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl); PC, egg phosphatidylcholine; PE, egg phosphatidylethanolamine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid; Rh-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl); TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.
(10) The modified literature methods (Düzgünes, N.; Wilschut, J. *Methods Enzymol.* **1993**, *220*, 3−14) used to measure lipid mixing, aqueous contents intermixing, and leakage are described in the Supporting Information. In short, lipid mixing was monitored by the probe dilution assay which uses the fluorescently labeled phospholipid, NBD-PE, and its resonance energy transfer quencher, Rh-PE. One vesicle population containing 0.3% of each the probes, NBD-PE and Rh-PE, is added to another population that is unlabeled. Lipid mixing is indicated by an increase in NBD-PE fluorescence intensity due to diminished quenching as the two probes are diluted. The contents mixing assay starts with two populations of vesicles, one encapsulating the fluorophore, ANTS, and the other containing the collisional quencher DPX. Fusion and mixing of aqueous contents results in a decrease in ANTS fluorescence intensity. The leakage assay starts with vesicles encapsulating a mixture of ANTS and DPX. Leakage of aqueous contents results in an increase in ANTS fluorescence intensity. All assays were reproduced independently at least once with an average uncertainty of less than 10%.
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Figure 1. Lipid mixing at 25 °C.[10] A population of unlabeled donor PE:PC:X (14:5:1) vesicles (25 μM) was added at $t = 50$ s to an equal population of target PE:PC:POPA (14:3:3) vesicles (containing 0.3% of the probes NBD-PE and Rh-PE) in 100 mM NaCl/5 mM TES buffer, pH 7.4. (a) X = **1**, (b) X = **2**, (c) X = **3**.
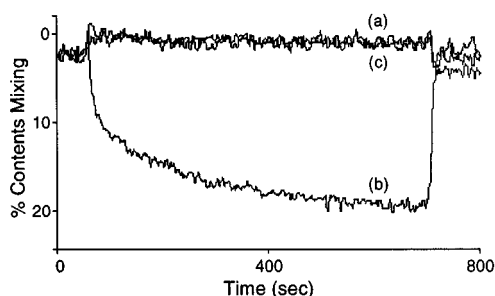

Figure 2. Contents mixing mixing at 25 °C.[10] A population of donor PE:PC:X (14:5:1) vesicles (25 μM) containing DPX was added at $t = 50$ s to an equal population of target PE:PC:POPA (14:3:3) vesicles containing ANTS in 100 mM NaCl/5 mM TES buffer, pH 7.4. (a) X = **1**, (b) X = **2**, (c) X = **3**. At $t = 700$ s, octaethylene glycol monododecyl ether was added to lyse the vesicles.

than lipid mixing, in agreement with a fusion mechanism that has hemifusion (joining and mixing of donor and target membranes) preceding pore formation and intermixing of aqueous vesicle contents.[1] Leakage studies revealed that fusion occurred without any leakage of aqueous contents (see Supporting Information).

A second, less-fusogenic vesicle composition, PC:cholesterol:X, was also studied.[11a] In this case the donor PC:cholesterol:X vesicles were 11:6:1 for **1** and **2** and 21:12:3 for **3**. The target vesicles were composed of PC:cholesterol:POPA (9:6:3). Very little contents mixing was observed with a 1:1 ratio of target-to-donor vesicles. However, at a 1:5 target-to-donor ratio, which increases the likelihood of the donor vesicles undergoing a detectable fusion event,[10] the donor vesicles containing **2** induced 32% contents mixing and 60% lipid mixing, while vesicles containing **1** induced 4% contents mixing and 13% lipid mixing, and vesicles containing **3** induced 8% contents mixing and 34% lipid mixing (see Supporting Information). As before, vesicle fusion occurred without any leakage.

The results clearly show that facial amphiphile **2** with a long hydrocarbon chain is a much better fusogen than analogue **1** or head–tail amphiphile **3**. It is also notable that fusion induced by **2** is non-leaky, which is very rare for a vesicle system.[11] Overall, our results correlate with the work of Pécheur and co-workers,[2] who showed that covalent attachment of an amphiphilic α-helical peptide to the donor vesicle surface greatly improves fusogenic activity. At this point we do not know exactly how amphiphiles

1–**3** are oriented in the vesicle membrane.[12] It is very likely that the head–tail amphiphile **3** lies in the donor membrane monolayer in a parallel orientation with the other phospholipids. The major role of its cationic headgroup is to induce aggregation and hemifusion with the anionic target membranes. In the case of **2** there is literature evidence that suggests its long ester chain may insert into the membrane and the facially amphiphilic steroid sits on the membrane surface.[13] The long ester chain may constrain the amphiphile's mobility, or it may produce a wedge-like molecular shape that induces negative or inverted curvature strain on the membrane outer monolayer, a phenomenon that is known to destabilize bilayers and stabilize fusion intermediates.[1–3,14] Thus, not only does cationic facial amphiphile **2** force the donor vesicles to aggregate with the anionic target vesicles, it also promotes the formation of a non-leaky fusion pore. Rigid, facial amphiphile **2** appears to be a good mimic of in vivo biological fusion peptide action and is likely to be a useful model system for mechanistic structure/activity studies.[2]

The ability of **2** to promote fusion suggests that it may be useful as a transfection agent since a likely critical step in transfection with cationic liposomes is fusion of the positively charged DNA/lipid particles with cell or endosomal membranes.[15,16] The transfection efficiency of a 1:1 mixture of **2** and DOPE[17] was compared to the widely used commercial formulation known as Lipofectin (1:1, DOTMA:DOPE).[9] Both vectors were tested for their ability to transfect cultured human embryonic kidney cells (HEK293) with a DNA plasmid that encodes for green fluorescent protein. The **2**:DOPE vector transfected five to eight times more cells than Lipofectin. There was no evidence of toxicity, as the cells remained attached to the plate and showed normal growth.

In summary, our results suggest that rigid, facial amphiphiles with lipophilic tails will be useful as functional mimics of amphiphilic fusion peptides. An attractive feature is the rigid structure which should simplify the interpretation of mechanistic studies. In the particular case of **2**, it should be synthetically straightforward to manipulate its structure and systematically probe the factors controlling membrane fusion. Facial amphiphiles such as **2** also have promise as novel cell transfection agents.

Supporting Information Available: Chemical structures, experimental procedures, fusion and transfection data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Control studies show that there is no loss of amphiphiles **2** and **3** from the vesicles after dialysis for 12 h, and only 15% loss of amphiphile **1** (see Supporting Information). Thus, the difference in fusogenic properties is probably not due to any difference in amphiphile/vesicle affinity, but more likely to a difference in amphiphile/membrane orientation.

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