# *trans*-2-Aminocyclohexanol as a pH-sensitive conformational switch in lipid amphiphiles†‡

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**Protonation-induced conformational change of lipid tails is reported as a novel strategy to render pH-sensitive lipid amphiphiles and lipid colloids.**

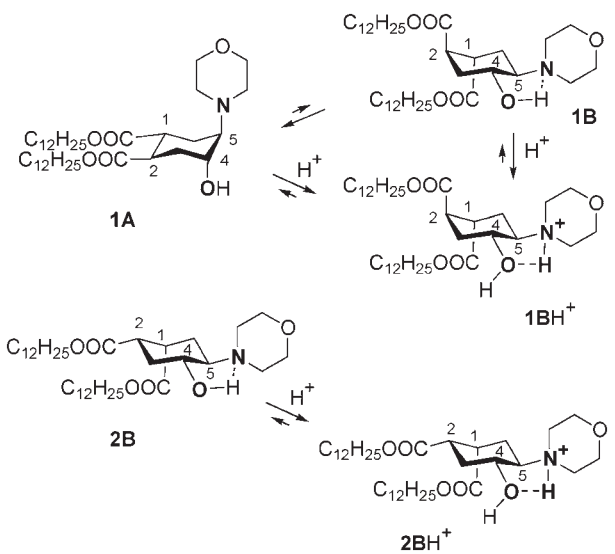
Conformationally controlled molecular switches are central devices of molecular machines[1] and provide a new and promising approach to materials with controllable properties. However, the concept of conformational switches has not yet been applied to the design of triggerable lipid amphiphiles. The potential advantage here is that the effect of the conformational switch in one molecule of lipid amphiphile can be amplified in its colloidal assemblies, such as liposomes.

Acid-sensitive lipid amphiphiles have drawn much interest as building blocks of colloidal drug and gene carriers that release therapeutic payloads specifically at low-pH target sites, such as solid tumors and inflamed tissues.[2] Although the reported acid-sensitive lipid amphiphiles have versatile chemical structures, their design falls into only two categories: (1) change of the hydrodynamic radius of the lipid headgroups by protonation with concomitant phase changes of the lipid bilayer,[3] and more recently, (2) hydrolysis of acid-labile lipids.[4]

Since the resistance of lipid bilayers against diffusion mostly relies on the stacking of the hydrophobic lipid tails,[5] a change of conformation of the lipid tails could also be exploited to trigger liposomes. Such consideration turned our attention to *trans*-2-aminocyclohexanols (TACHs), the protonation of which results in a strong hydrogen bond between the amino and the hydroxyl groups, forcing both these groups to adopt an equatorial conformation.[6,7] Further, this change can be mechanically transmitted by the cyclohexane ring to induce conformational changes of its remote substituents.[6–9] Previously, TACH-based pH-sensitive conformational switches were used to control the affinity of a crown ether and a podand to potassium ion.[7] Here we present TACH derivatives **1** and **2** (Scheme 1) as the first attempt to design conformationally switchable lipid amphiphiles and their pH-sensitive liposomes.

Based on our earlier studies on TACHs,[6,7] we anticipated that both *trans*-dodecyloxycarbonyl groups of **1** would adopt the equatorial conformation while the morpholine and the hydroxyl groups would be both in the axial conformation (**1A** in Scheme 1). Upon protonation, the axial-to-equatorial switch of the morpholine and the hydroxyl groups would force both ester groups into axial positions (**1B**$H^+$), thereby drastically increasing the separation of the lipid tails. In liposomes comprising **1**, this would loosen the packing of the lipid tails in the bilayer, leading to leakage and even to the collapse of the liposomes. To estimate the effect of such conformational change, the stereoisomer **2** (Scheme 1) with two dodecyloxycarbonyl groups in the *cis*-configuration was also studied for comparison. We expected this isomer to be always predominantly in a conformation with three substituents in the equatorial position (**2B**), and to show little or no conformational change upon protonation (**2B**$H^+$, Scheme 1).

Lipids **1** and **2** were prepared (Schemes S1, S2‡) using the approach developed previously.[7] The conformer populations ($n_A$, $n_B$) in the fast equilibrium [**1A**] ⇄ [**1B** + **1B**$H^+$] (Scheme 1) were estimated from an averaged signal width ($W = \Sigma J_{HH}$) measured as the distance between terminal peaks of a multiplet in $^1$H NMR: $W_{observed} = W_A n_A + W_B n_B$.[7] We used mainly the signal of H4, geminal to the hydroxyl group (Scheme 1), which was usually better resolved and located in a region apart from other signals. The parameter $W_B$ was assumed




**Scheme 1** pH-triggered conformational change of *trans*-2-aminocyclohexanol-derived lipids.

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to equal $W_{BH+} = W_{observed}$ in the presence of excess acid (25.3 Hz for **1**, 26.1 Hz for **2**), and $W_A$ was estimated as 9 Hz based on reported data for the related conformationally biased cyclohexanes.[7]

To characterize the pH-triggered conformational change, the lipids **1** and **2** were dissolved in $CD_3OD$, and the changes of their $^1H$ NMR spectra (300 MHz) were monitored during titration of the solution with trifluoroacetic acid (TFA).

As expected, lipid **2** was ~95% in conformation **2B** ($W_{H4}$ = 25.1 Hz), and this bias slightly increased in excess acid to ~100% of **2B**$H^+$ ($W_{H4}$ = 26.1 Hz) (Fig. 1). The H4 chemical shift changed from $\delta$ 3.53 at pH 8.3 (no acid added) to $\delta$ 3.82 below pH 2.5 (Fig. 2). The H5 signal of **2** moved downfield from $\delta$ 2.33 ($W_{H5}$ = 25.3 Hz) to $\delta$ 3.3 (unresolved), indicating deshielding caused by protonation/deuteration of the morpholine nitrogen.

The signal width of H4 in **1** increased from 13.2 Hz to 25.3 Hz (Fig. 1), indicating a sharp conformational switch from **1A** (~75% in original solution) to **1B**$H^+$ (~100% in excess acid). The H5 signal of **1** ($W$ = 13.7 Hz at pH 8.1; no acid added) became unresolved upon decrease of the apparent pH below 5, and migrated from $\delta$ 2.23 downfield to $\delta$ 3.3 below pH 3.5 (Fig. 2). In contrast, the multiplet H4 shifted upfield from $\delta$ 4.00 to $\delta$ 3.90 despite of the deshielding effect of the morpholinium group. Since axial protons typically give a signal at higher field than the otherwise equivalent equatorial protons, this observation supports an equatorial-to-axial change of H4, and thus the axial-to-equatorial change of the hydroxyl group. Further, the originally well-resolved signals of H1 ($\delta$ 2.98; $W$ = 22.3 Hz) and H2 ($\delta$ 3.09; $W$ = 23.1 Hz) in **1** became narrow and unresolved, and both shifted downfield to $\delta$ 3.4 upon the drop of pH (Fig. S1–S4‡). This attests for an axial-to-equatorial switch of H1 and H2, and thus for the equatorial-to-axial switch of the ester groups of the lipid tails.

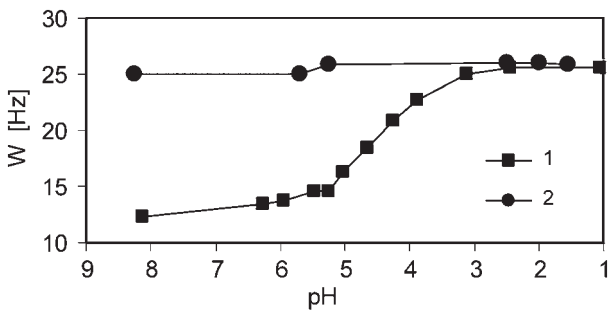


**Fig. 1** pH-dependent change of the H4 signal width ($W = \Sigma J_{HH}$) in the $^1H$ NMR spectra of **1** and **2** in $CD_3OD$.

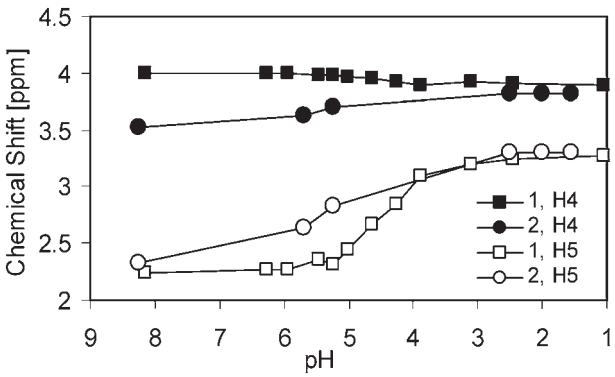


**Fig. 2** pH-dependent change of the chemical shift of H4 and H5 in $^1H$ NMR of **1** and **2** in $CD_3OD$.

Neither **1** nor **2** dissolves in water, which precluded their direct NMR characterizations in aqueous media. Nevertheless, **1** should undergo a similar pH-triggered conformational change at a water–bilayer interface as we found in highly polar, protic methanol.

As a first attempt to test the capacity of the lipid tail conformational change to trigger lipid colloids, we prepared liposome comprising 25 mol% **1**, and 75 mol% 1-palmitoyl-2-oleoyl-*syn*-glycero-3-phosphocholine (POPC), a common phospholipid that favors the formation of a lipid lamellar phase. As a monounsaturated lipid, POPC facilitates the mixing with both saturated and unsaturated lipids.[10] The liposomes contained fluorescent dyes, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX), so that liposome permeation could be monitored by dye release, which de-quenches their fluorescence.[10,11] In a pH 7.4 buffer, the **1**–POPC liposome preparation did not show substantial content leakage for more than 1 h at 37 °C. Upon exposure to mildly acidic media (pH 5.5), **1**–POPC liposome quickly released ~50% of the contents in 10 min.

Next we elected to test whether **1** can introduce pH-sensitivity to liposomes grafted with polyethylene glycol (PEG), a hydrophilic polymer commonly used to deter immune detection of liposomal drug/gene delivery systems by inhibiting their inter;actions with serum proteins.[12] We constructed a liposome comprising 5 mol% of a PEG–lipid conjugate, *N*-Palmitoyl-sphingosine-1-[succinyl-(methoxypolyethyleneglycol)2000] (mPEG2000-Ceramide), and 24 mol% **1** or **2**, together with 71 mol% POPC. The mPEG2000-Ceramide–**1**–POPC liposome preparation was stable in a pH 7.4 buffer for more than 8 months when stored at 4 °C and for more than 48 h at 37 °C (Table S1‡). Upon exposure to mildly acidic media (pH 5.5), mPEG2000-Ceramide–**1**–POPC liposome quickly released most of the contents (>80% in 20 min) (Fig. 3). In comparison, the analogous mPEG2000-Ceramide–**2**–POPC liposome released much less content (<30%) at pH 5.5 (Fig. 3), which probably occurred through the increase of the headgroup

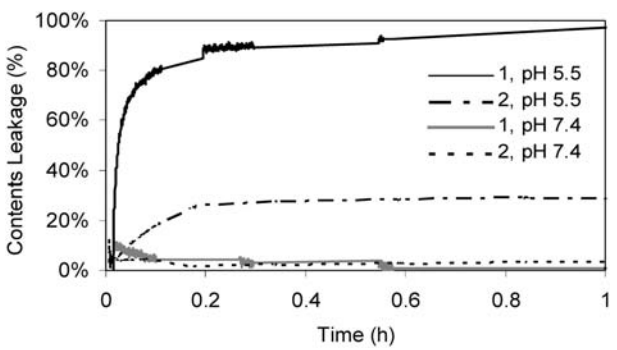


**Fig. 3** pH-dependent leakage of mPEG2000-Ceramide–**1**–POPC liposomes (1) and mPEG2000-Ceramide–**2**–POPC liposomes (2).

radius[3] and/or the change of interactions between the lipid headgroups[13] upon protonation of the morpholine group. Acid-induced lipid degradation can be excluded as a contributor to the observed leakages for the following reasons. First, lipids **1** and **2** were found to be stable overnight at a pH as low as 1 during our NMR analyses (Fig. 1 and 2). Secondly, our prior HPLC and liposome leakage studies[11,14] have shown that the ester groups in diacyl glyceride-based phospholipids and PEG–lipid conjugates do not undergo noticeable chemical degradation in mildly acidic media (pH 5.5) within the time span of the liposome leakage assays ($\sim$1 h, Fig. 3).

Fig. 2 shows that the morpholine group of **2** has virtually identical basicity compared with that of **1**. Lipid **2** differs from lipid **1** only in the configuration of one lipid tail substituent, and for this reason was unable to substantially change its conformation after protonation (Scheme 1, Fig. 1). The studies on the control liposome mPEG2000-Ceramide–**2**–POPC allowed the observation of all possible effects of the protonation of the morpholine group (change of headgroup radius, change of hydrogen bonding, *etc*.) on the liposome permeability, except for the effects caused by the change of conformation. Therefore, the much larger and faster leakage of mPEG2000-Ceramide–**1**–POPC liposome than mPEG2000-Ceramide–**2**–POPC liposome at pH 5.5 can be attributed to the pH-triggered conformational change of the lipid tails in **1**.

It is interesting that the leakage of mPEG2000-Ceramide–**2**–POPC liposome at pH 5.5 plateaued at about 30% (Fig. 3). One possible reason would be that the membrane destabilization by the protonation of **2** was so limited that the lipid bilayer managed to reorganize into a slightly different lamellar phase to retain most of the liposome content. In contrast, the drastic conformational change of the lipid tails of **1** would induce extensive reorganization of the membrane of mPEG2000-Ceramide–**1**–POPC liposome and thus a faster and almost complete liposome leakage.

When incubated in 75 vol% fetal bovine serum at 37 °C, the mPEG2000-Ceramide–**1**–POPC liposome released the encapsulated dyes by slow kinetics ($\sim$30% in 12 h, Fig. S7‡) that was virtually identical to that of the pH-insensitive control liposomes comprising 5 mol% mPEG2000-Ceramide and 95 mol% POPC. Since the clearance of liposomes by the immune system is initiated by liposome-serum protein interactions,[12] this observation suggests that pH-sensitive lipids based on lipid tail conformational change can be incorporated into PEG-coated liposomal drug/gene delivery systems without jeopardizing their ability to evade immune detection.

In summary, we have reported an unprecedented strategy to render triggerable lipid amphiphiles and lipid colloids: the protonation-induced conformational change of lipid tails using the *trans*-2-aminocyclohexanol group as a molecular lever. Lipid amphiphiles derived from *trans*-2-aminocyclohexanol can serve as versatile pH-sensitive molecular switches in drug and gene delivery systems (*e.g*. as helper lipids[15,16]). We are investigating the physicochemical mechanisms of the TACH-induced liposome leakage (possible involvement of raft formation or $L_\alpha \rightarrow H_{II}$ phase changes, *etc*.) and will report the findings in due course.

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