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

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Received (in Cambridge, UK) 7th May 2008, Accepted 18th June 2008
First published as an Advance Article on the web 5th August 2008
DOI: 10.1039/b807704e

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 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. 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, and more recently, (2) hydrolysis of acid-labile lipids.

Since the resistance of lipid bilayers against diffusion mostly relies on the stacking of the hydrophobic lipid tails, 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. Further, this change can be mechanically transmitted by the cyclohexane ring to induce conformational changes of its remote substituents. Previously, TACH-based pH-sensitive conformational switches were used to control the affinity of a crown ether and a podand to potassium ion. 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, 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 (1BH⁺), 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 (2BH⁺, Scheme 1).

Lipids 1 and 2 were prepared (Schemes S1, S2) using the approach developed previously. The conformer populations (nA, nB) in the fast equilibrium [1A] ↔ [1B + 1BH⁺] (Scheme 1) were estimated from an averaged signal width (W = Σ IHH) measured as the distance between terminal peaks of a multiplet in 1H NMR: Wobserved = WAH_A + W_AH_B. 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...
to equal $W_{BH^+} = W_{\text{observed}}$ 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\(_3\)OD, and the changes of their \(^1\text{H} \) NMR spectra (300 MHz) were monitored during titration of the solution with trifluoroacetic acid (TFA).

As expected, lipid 2 was ~95% in conformation \(2B \) (\(W_{1H^+} = 25.1 \) Hz), and this bias slightly increased in excess acid to ~100% of \(2BH^+ \) (\(W_{1H^+} = 26.1 \) 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 (\(W_{1H^+} = 25.3 \) 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 1BH\(^+\) (~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.

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 interactions with serum proteins.\(^12\) We constructed a liposome comprising 5 mol% of a PEG–lipid conjugate, N-Palmitoyl-sphingosine-1-[succinyl-(methoxy(polyethylene)glycol)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
the pH-triggered conformational change of the lipid tails in 2

ion by the protonation of

One possible reason would be that the membrane destabilization of the morpholine group (change of headgroup radius, drastic conformational change of the lipid tails of 1 phase to retain most of the liposome content. In contrast, the bilayer managed to reorganize into a slightly different lamellar phase.

Therefore, the much larger and faster leakage of mPEG2000-Ceramide–POPC liposome than mPEG2000-Ceramide–POPC liposome at pH 5.5 can be attributed to the pH-triggered conformational change of the lipid tails of 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 (∼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 lipids15,16). We are investigating the physicochemical mechanisms of the TACH-induced liposome leakage (possible involvement of raft formation or Lα → HII phase changes, etc.) and will report the findings in due course.

This work was supported by SAAAGrants (X.G., V.V.S.) and Eberhardt Research Fellowship (V.V.S.) of University of the Pacific. We thank Dr Roshanak Rahimian for the use of the fluorometer. We thank the reviewers for insightful comments.

Notes and references