Review

Vyacheslav V. Samoshin*

Fliposomes: stimuli-triggered conformational flip of novel amphiphiles causes an instant cargo release from liposomes

Abstract: This review presents a new strategy for the design of stimuli-responsive liposomes for targeted delivery – the construction of a liposome membrane (lipid bilayer) using amphiphiles able to perform a stimuli-triggered conformational flip ('flipids'). When done simultaneously by a major or significant part of the bilayer molecules, this massive flip disrupts the liposome membrane and induces a rapid release of the liposome load specifically in response to the initial stimulus. The conformational switches incorporated into the amphiphilic molecules could potentially be controlled by various internal or external factors (pH, metal complexation, light, electric field, etc.). Using this concept, we designed a series of pH-triggerable flipids, and prepared and tested 'fliposomes' with extraordinary characteristics: high stability in storage and in serum combined with an instant release of their cargo in response to a weakly acidic medium.

Keywords: acid-triggered leakage; conformational switch; controlled drug release; peacock effect; pH-sensitive liposomes.

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Introduction

Liposomes are particulate vesicles made of one or several lipid bilayers enclosing an aqueous core and able to incorporate a variety of hydrophilic or lipophilic substances within the core or within the lipid membrane, respectively. Liposomes are mainly used as a potent drug delivery system (1–16), as they prolong the life of pharmaceuticals in the bloodstream. This allows the drugs to reach a destination without being decomposed. Therefore, lower doses could be used. For this and other reasons, toxic drugs often show a relevant improvement in their therapeutic index when they are administered as liposomal formulations. Currently, several liposome-based therapeutics are clinically approved (1–6). To provide a more efficient and targeted drug delivery, the liposomes should release their content exactly at the designated place in response to certain factors characteristic for a disease (pH, enzymatic activity, redox potential, temperature) or to a stimulus applied by external devices (temperature, light, radiation, ultrasound, and electric and magnetic field) (1–9, 15–20). These internal or external stimuli initiate a perturbation or even destruction of the liposome membrane (lipid bilayer), which triggers a liposome discharge. Thus, the leakage of the most studied thermosensitive liposomes is caused by the formation of local defects and phase transition in the lipid bilayer (1–4, 8, 12). Another classic approach is chemical or enzymatic cleavage of molecules constituting the liposome membrane (2–5, 9, 10, 16). Liposomes are often rendered stimuli sensitive by incorporation of additional stimuli-responsive components (peptides, proteins, polymers, metal nanoparticles, etc.) in relatively small amounts (1–7, 9–11, 15–24).

A novel promising strategy described here is based on a stimuli-triggered conformational flip performed simultaneously by a significant part of amphiphilic molecules composing the liposome membrane. This transformation does not require chemical cleavage of molecules or their relocation, and it is very fast. The amphiphiles have to be equipped with the stimuli-responsive conformational switches. Mechanical or conformational molecular switches are molecular systems that reversibly change the relative orientation of their parts under external influence. They play a central role in the design of molecular machinery, controllable compounds, and intelligent materials for possible use in many applications, including drug release, new sensor techniques, or information storage and transmission (25–31). Cyclohexane-based molecular devices have been designed as a new type of such switches (29–31).

pH-responsive systems

Acid-induced release is a promising approach to the design of liposomes as drug/gene delivery systems because increased acidity is characteristic for numerous physiological and pathological conditions, including endosome processing, inflammation, ischemia, and solid tumor growth (2–7, 10, 13–15). During the last three decades, this idea developed into many strategies for construction of pH-sensitive liposomes. One of the major approaches employs an acid-triggered change of interactions between molecules in a mixture of non-polar and anionic lipids that results in phase transition (5) and/or formation of domains with 'leaky' interfaces (32, 33). Variation of the pKa of anionic lipids and their molar ratio can slightly modify the pH sensitivity. Another popular concept is based on the acidic hydrolysis of ortho-esters, acetals, hydrazones, vinyl ethers, or other acid-labile linkers in lipidic amphiphiles transforming the latter into destabilizing detergents or conical lipids (2, 3, 5, 9, 10, 16, 34, 35). These reactions often require several hours to proceed, which may be a problem for timely drug release. Liposomes can also be made acid sensitive by incorporation into the lipid bilayer of additional minor components: peptides or polymers designed to have a pH-dependent conformational change and change of solubility, thus causing membrane perturbation, pore formation, phase separation, and fusion (2, 4, 10, 19–24). The relatively complicated syntheses of such components and their limited variability may be a disadvantage of this approach.

We recently suggested a novel strategy to render liposomes pH sensitive: a protonation-induced conformational switch of hydrocarbon chains in latent amphiphiles composing the liposome membrane (30, 36–42). Our liposome design is based on a drastic conformational flip performed simultaneously by a significant part of these structurally simple and synthetically accessible pH-sensitive molecules upon protonation. In our studies, the pH-responsive amphiphiles constituted from 25% to 90% (in most experiments, 50 mol%) of the whole lipid composition (36–42). This massive uprising disturbs the liposome membrane instantaneously and induces a rapid release of the liposome payload specifically in response to increased acidity of the medium.

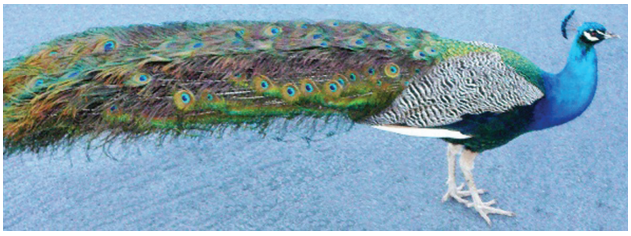
To highlight the key role of the conformational flip in pH-triggered liposome leakage, we introduced the terms 'flipids' for amphiphiles equipped with a pH-sensitive conformational switch and 'fliposomes' for liposomes composed of this material (30, 36–42). This should not be confused with a lipid translocation (flip-flop) that was involved in many reported lipid phase changes (43–46). The lipid motion from one leaflet of the bilayer to the other is unlikely to significantly contribute to the pH-triggered release from the fliposomes because of its much slower kinetics: hours for flip-flop (44) versus seconds for fliposome leakage.

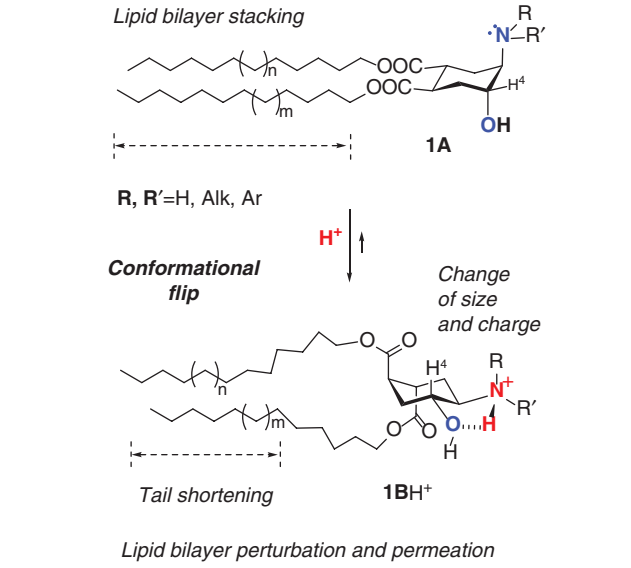
The promising preliminary results (36) encouraged us to study the physicochemical and biological properties of this novel type of liposomes in more detail. The compatibility of the pH-sensitive conformational trigger with commonly used lipids, ability to control the rate and extent of liposome content release, mechanism of lipid membrane destabilization, and viability of fliposomes for drug delivery have been studied (36–42). Of special interest was the ability of flipids to trigger the PEGylated liposomes due to their prolonged life in blood circulation and successful applications in drug/gene delivery (2, 4, 5, 10, 14, 34).

To address these issues, we designed, prepared, and characterized a series of PEGylated fliposomes (36–42). The pH-driven conformational interconversion of flipids was studied by ^{1}H nuclear magnetic resonance (NMR) titration. pH-triggered release from the fliposomes was measured using the 8-aminonaphthalene-1,3,6 trisulfonic acid/*p*-xylene-bis-pyridinium bromide (ANTS/DPX) fluorescent assay. Freeze-fracture electron microscopy (FFEM) was used for probing the mechanism of the acid-triggered lipid membrane destabilization. Selected lipid compositions were used to construct liposomes encapsulating a widely used anticancer drug methotrexate (MTX), followed by the characterization of their pH-triggered drug release by equilibrium microdialysis and anticancer activity in HeLa cells (human cervical cancer). By incorporation of 50 mol% flipids into liposome membranes containing also phospholipids and $mPEG_{2000}$-ceramide, we constructed pH-triggerable fliposomes with extraordinary characteristics: high stability in storage and in serum combined with an instant release of their cargo in response to a weakly acidic medium (36–42). The MTX-loaded liposomes demonstrated much higher cytotoxicity in HeLa cells than the free drug, indicating that they can serve as viable drug delivery systems (37).

Trans-2-aminocyclohexanol-based flipids

The first amphiphiles with the pH-triggerable conformational switch were derivatives of *trans*-2-aminocyclohexanol **1** that performed an acid-induced ring flip (36–41), spreading their lipophilic tails like peacocks (this conformational change can be dubbed a 'peacock effect'; Figure 1). Previously, we used the *trans*-2-aminocyclohexanol moiety





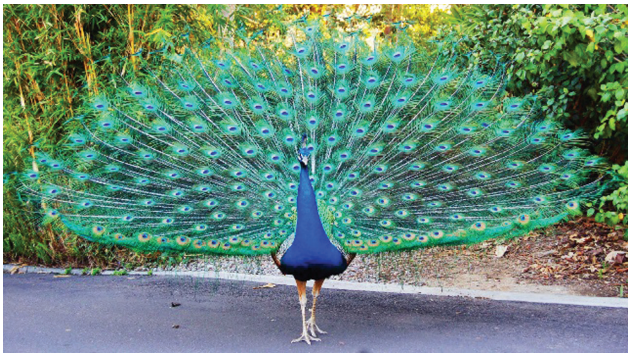


Figure 1 Protonation-induced conformational flip causes spreading and efficient shortening of the lipophilic tails (a 'peacock effect') along with a change of the charge, effective size, and shape of the polar head resulting in a quick perturbation of the lipid bilayer, lipid phase separation, and fast content leakage from the fliposomes (30, 36–41).

to construct the conformationally controlled crown ethers and podands (29, 30, 47, 48).

The driving force of this dramatic acid-triggered transition is a strong, protonation-generated intramolecular hydrogen bond of HO···H-N$^+$ type and an electrostatic/dipole-dipole attraction stabilizing a conformer with the *gauche* form of O-C-C-N fragment (**1B**H$^+$ in Figure 1). This impulse results in a conformational flip of the cycle that moves the ester groups COOR at the other end of the molecule away from each other into axial positions. The relocation of substituents changes their intra- and intermolecular interactions, for example, their ability to form complexes with cations or to pack into lipid bilayers (depending on the nature of substituents).

We examined the chair-chair flip of the cyclohexane ring in flipids **1** (Figure 1) in solution by the proton NMR, ^{1}H NMR (36–41). The vicinal coupling constants $^3J_{HH}$ between several protons attached to the cyclohexane moiety and their chemical shifts are strongly conformation dependent, which allows an assignment of a predominant conformation and an estimation of the position of equilibrium. To characterize the acid-induced swing of the conformational equilibrium, the changes of ^{1}H NMR spectra were monitored during titration of the diluted d_4-methanolic solutions of **1** with *d*-trifluoroacetic acid (Figure 2). The observation of a single set of well-resolved multiplets in the course of acidification attested to high rates of both the acid-base and the conformational equilibria on the NMR time scale. During the incremental addition of acid, the signal parameters changed significantly, indicating a strong protonation-induced shift of the conformational equilibrium from **A** to **B**D$^+$ (~100% in excess acid). This shift did not occur gradually over the whole course of titration, but happened only within a narrow pH range, the value of which depended on the basicity of the amino group. Using the change of $\Delta G_{B\text{-}A}$ values for the conformational equilibrium in methanol solution upon addition of acid, we estimated the power of this conformational pH trigger to be ≥11–12 kJ/mol. Remarkably, the

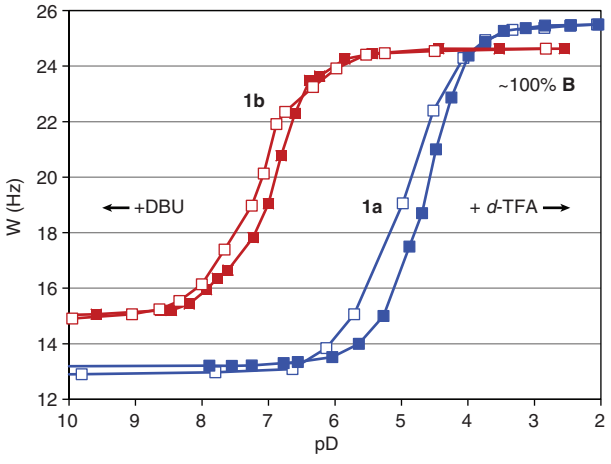


Figure 2 Change of the signal width (W=ΣJ_{HH}) for the proton H4 in ^{1}H NMR due to the conformational switch of the flipids **1a** (NRR′=morpholinyl, n=m=1) and **1b** (NRR′=$CH_3OCH_2CH_2NH$, n=m=1) in CD_3OD solution caused by titration with *d*-TFA (■) and then by backward titration of the resulting acidic solution with DBU (□) (39).

intramolecular hydrogen bond HO···H-N$^+$ wins a competition with hydrogen bonding to solvent molecules. Similar results were obtained for aqueous solutions of the model *trans*-2-aminocyclohexanols equipped with ethyl groups instead of long hydrocarbon chains and therefore slightly soluble in D_2O (41).

PEGylated liposomes that encapsulated a fluorescent dye (ANTS) and a quencher (DPX) were prepared by the freeze-thawing method. Starting with any particular lipid composition, this method yielded liposomes of reproducible colloidal properties and thus allowed studies on the relationship between the lipid composition of the liposomes and their acid-triggered release of contents (36–41). Noteworthy, after storage at 4°C for >6 months, the optimized liposome formulations (**1**/POPC/PEG-ceramide, 50:45:5 mol%) did not show noticeable changes in hydrodynamic diameter or polydispersity index, and kept a near-zero ζ-potential. We measured the leakage of ANTS/DPX after injecting a small aliquot of the fliposome preparation into buffer solutions with pH varying from 9.5 to 2.8 as exemplified for the case of two flipids in Figure 3.

As the initial horizontal part of the titration curves shows, fliposome leakage does not occur in basic (for **1a** and **1b**) or neutral (for **1a**) medium. However, the increase of acidity brings about a dramatic jump of leakage between pH 8.5 and 6.5 in the case of fliposomes containing 50 mol% **1b**, and between pH 6.0 and 4.0 in the case of fliposomes with 50 mol% **1a**. The relative position of these transitional areas meets the expectation of a higher basicity for the secondary amine **1b** as compared with the tertiary amine **1a** (in protic solvents). The resulting diagrams are very similar to the NMR titration curves for the conformational flip of amphiphiles **1a** and **1b** (Figure 2). We consider this similarity as an evidence for the intrinsic dependence of the pH-induced fliposome leakage on the pH-triggered conformational flip of the amphiphiles **1**.

Further evidence for the predominant role of conformational flip in the liposome leakage was obtained from the comparison of diastereomeric amphiphiles **1a** and **2** (Scheme 1) (36, 37). Compound **2** differs from flipid **1a** only in the configuration of one lipid tail substituent; however, for this reason, it is unable to noticeably change its conformation after protonation. Thus, the results for the control **2**/POPC/PEG-ceramide liposomes allowed observation of all possible effects of the protonation of morpholine group on the liposome permeability (change of headgroup charge and radius, change of hydrogen bondings, etc.), except for the effects caused by the change of conformation. Therefore, the much larger and faster acid-induced leakage of **1**/POPC/PEG-ceramide liposomes than that of **2**/POPC/PEG-ceramide liposomes could be attributed to loosening of the liposome membrane caused by the pH-driven conformational change of the lipid tails in **1a**. As expected, control liposomes containing neither **1a** nor **2** were not responsive to lowered pH (36, 37).

FFEM allowed us to gain more insight into the mechanism of triggered release from fliposomes. This powerful technique not only can image structures of lipid colloids at nanometer resolution but also takes snapshots of lipid phase transformation by rapid freezing. The unloaded **1**/POPC/PEG-ceramide (50:45:5) fliposome formulation was studied both at pH 7.4 and after exposure to pH 5.5 (Figure 4) (37). At both pH values, the samples contained vesicles, although a large part of them had dissipated in the more acidic medium. The diameters of the fliposomes measured on the electron micrographs ranged from 20 to

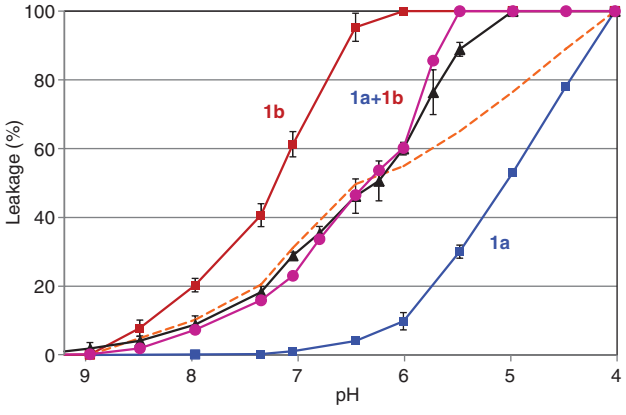
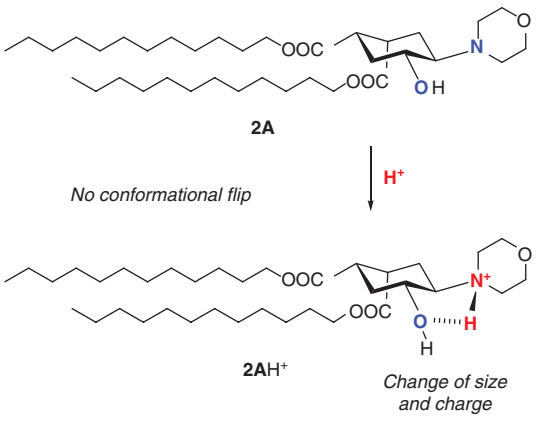


Figure 3 pH dependence of the ANTS/DPX leakage from **1**/POPC/PEG-ceramide fliposomes made with 50 mol% of flipid **1a**, or **1b**, or their 1:1 blend (**1a+1b**).
The black curve (▲) was obtained for the 1:1 mixture of fliposomes **1a** and **1b**. The dashed line is an average of the curves **1a** and **1b** (39).



Scheme 1 Absence of protonation-induced conformational flip in amphiphile **2** (36, 37).

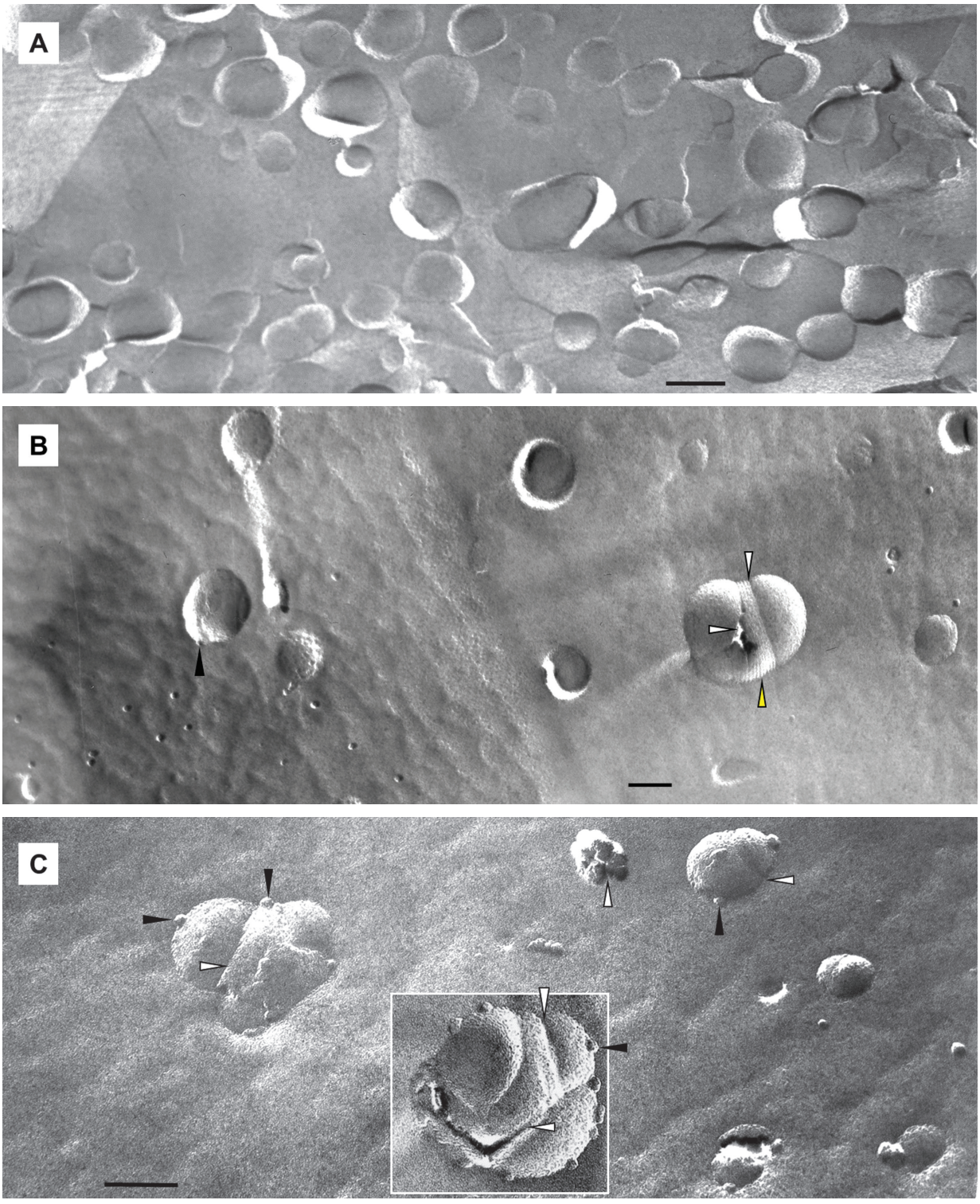


Figure 4 FFEM of the **1**/POPC/PEG-ceramide fliposome formulation at pH 7.4 (A) and 5 min after adjusting the pH to 5.5 with diluted acetic acid (B, C).
Examples of division, buds, and stripes are shown with white, black, and yellow arrows respectively. The bars represent 100 nm (37).

230 nm, with an average diameter of about 100 nm. At pH 7.4, the fliposome formulation appeared to be composed mostly of unilamellar vesicles (Figure 4A). Upon decrease of pH from 7.4 to 5.5, the remaining fliposomes underwent drastic morphological transformations (Figure 4B,C), including division, fusion, and budding.

It appears that a large number of buds pinched off the fliposomes as freely suspended small spherical particles. Defects of lipid packing that resembled long and deep cracks were also observed on some of the dividing fliposome membranes.

On the basis of ^{1}H NMR studies of the pH-induced conformational flip of compounds **1**, the fluorometric and ultraviolet studies of the pH-induced leakage of fliposomes, and the FFEM images of the morphological changes of the **1**/POPC/PEG-ceramide fliposomes, we proposed a novel mechanism of the pH-triggered content release from fliposomes (37). The protonation generates a strong intramolecular hydrogen bond between the amine and the neighboring hydroxy group, which flips the chair conformation of the cyclohexane ring and mechanically switches its two remote ester groups from equatorial to axial positions (Figure 1). Thereby, the spatial separation of the two lipid tails of **1** is increased, especially at their proximal end (peacock effect). At the distal end, the conformational freedom and the hydrophobic interactions may allow the chains to partially remain packed or to re-pack in the lipid bilayer, although with a decreased packable length of the hydrophobic moiety. Besides this conformational shortening and widening, as flipid **1** is protonated, its head group assumes a positive charge and increases its hydrodynamic size as a result of higher hydration. This leads to additional electrostatic and steric repulsion. All these changes are very fast, and they directly perturb the lipid bilayer and could induce phase separation of the fliposome membrane into thinner and thicker domains that are rich in **1** or in longer POPC molecules, respectively. The encapsulated contents of the fliposomes would then quickly leak through defects between the domains. Some domains of monolayers would subsequently bud from the fliposome membranes as small micelles, as observed in FFEM (Figure 4) (37).

Peacock effect: heads or tails?

A simple synthetic scheme used for preparation of flipids **1** allows substantial flexibility in the design of these molecular devices (30, 36–41). Their parameters can be conveniently modified by structural variation of all substituents, especially the hydrocarbon tails and the amino group in the relatively polar heads of the molecules.

Because the basicity of amines is determined by the nature of groups attached to the nitrogen atom, the flipid pH sensitivity can be tuned by appropriate modification of the amino group structure. This may allow tailoring of fliposomes for the triggered release at certain pH values. Moreover, by mixing such liposome preparations, each with different pH sensitivity, one could potentially obtain a system for simultaneous administration, but independent delivery, of different drugs that would be released each at a specific pH value. Furthermore, the reversibility of conformational flip upon addition of a base (Figure 2) suggests that fliposomes prepared in acidic medium could be used for targeted delivery to the more basic places, for instance, to the small intestine (49). We explored some of these possibilities (39–41).

We synthesized a series of flipids **1** with a variety of 'heads' and 'tails', and studied the pH dependence of the ANTS/DPX leakage from the corresponding **1**/POPC/PEG-ceramide fliposomes (36–41). The measured pH ranges for the leakage varied from pH 9 to pH 4 depending on the nature of the amino group (as shown in Figure 3 for flipids **1a** and **1b** with different basicity). Thus, the pH sensitivity of a fliposome preparation can be indeed custom-tailored. Very conveniently, the leakage range for each flipid can be accurately predicted from the results of ^{1}H NMR titration (Figure 2).

Intriguing results were obtained when we used a 1:1 blend of flipids **1a** and **1b** for preparation of (**1**+**2**)/POPC/PEG-ceramide fliposomes (39, 40). Because the pH ranges for leakage of the fliposomes based on just one of these triggers practically do not overlap (Figure 3), one could expect the mixture of both flipids to produce an additive effect. That would extend the leakage over a pH range from 8.5 through 4.0 (dashed line in Figure 3 representing an average of the curves for **1a** and **1b**). Instead, in our experiments, the release was complete by pH 5.5. The first (more basic) part of the experimental curve, which can be credited to the conformational flip of **1b**, practically coincides with the expectation (the dashed line). However, the second part of the curve, which was supposed to depend on switching of **1a**, goes up and achieves the complete leakage much 'earlier' on the acidity scale than expected.

Very similar results have been obtained when we mixed two separate fliposome formulations, one based on **1a** (50 mol% of lipid composition) and the second based on **1b** (also 50 mol%). We supposed that fliposomes in this mixture of formulations were to respond to the change of pH independently – first those containing the more basic **1b** and then those containing **1a**. However, in this case, the second kind of fliposomes released its content also at less acidic conditions than expected, and the content release was complete by pH 5.0 (Figure 3).

Thus, when the flipids are blended before the preparation of fliposomes, or when the ready fliposome formulations are mixed, those flipids do not respond to

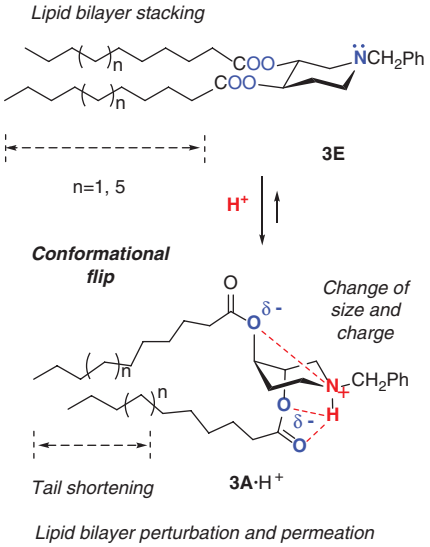
acidification independently. There appears to be some complicated interaction between them. A plausible explanation of the observed effects may be as follows. When the more basic molecules **1b** become protonated, the fliposomes that contain them are partially or completely destroyed. The ions **1b**H^+ thus released into the bulk aqueous media may play a role of a detergent, which permeates other fliposomes and releases their content. In other words, the acid-induced release of fliposome cargo may sometimes have an autocatalytic character. To verify this hypothesis, we performed the standard experiment by injection of the ANTS/DPX fliposome preparation based on **1a** into buffer solutions with pH 7.5 and 5.5. Then, we added the dispersion of **1b** in identical buffer. The molar amounts of **1a** and **1b** were equal. The addition of **1b** produced no change when the medium had pH 7.5. However, the fluorescence released by the fliposome preparation of **1a** in the pH 5.5 buffer increased upon injection of **1b**. This observation seems to confirm the ability of protonated compound **1b** to act as a detergent.

As the structure of lipid tails is critically important for the stability of lipid bilayers, the ability to release the liposome content could be tuned by appropriate design of these hydrophobic parts. We prepared and characterized the fliposomes containing new *trans*-2-morpholinocyclohexanol-based flipids equipped with either longer hydrocarbon chains, bent or branched hydrocarbon chains, and compared them with fliposomes based on flipid **1a** (38, 40, 41). The pH-driven conformational flip was studied by ^{1}H NMR titration. The ^{1}H NMR and conformational parameters were practically identical between these flipids in all the studied conditions. Thus, the elongation, bending, and branching of the hydrocarbon chains did not produce any noticeable conformational effect in the polar heads of these structures and did not alter their pH sensitivity. (This observation suggests *inter alia* that in future studies, simpler model compounds with short alkyl tails can be used for a preliminary estimation of conformational properties of the designed flipids.) The pH dependence of the ANTS/DPX leakage from the fliposomes made of these flipids was similar to the NMR titration curve, which confirmed the intrinsic dependence of the pH-induced liposome leakage on the pH-triggered conformational flip.

However, the elongation and branching of the hydrocarbon chains reduced the content release from fliposomes (38, 40, 41). This observation may be considered as an additional confirmation to the proposed mechanism of pH sensitivity, wherein bilayer destabilization starts with an acid-triggered conformational flip of the *trans*-2-aminocyclohexanol moiety (the polar head group) that increases the spatial separation of the two lipid tails, which results in a set of membrane perturbations that cause the leakage (Figure 1). The tails should be separated the most in the vicinity of the polar head and may partially fold back at the distal end to re-pack in the lipid bilayer. However, the packable length of the tails would decrease (Figure 1), thus inducing the phase separation of the lipid bilayer and liposome leakage. Therefore, the effect of the conformational flip in the polar head on the packing of the tails should relatively decrease as the flipids carry longer tails. On the other hand, membrane perturbation by the conformational flip is generally possible because of a certain degree of order in the hydrophobic part of the lipid bilayer. When some additional disorder (fluidity) is introduced from the very beginning by the asymmetrically branched groups, the effect of the conformational flip on the membrane integrity becomes less significant. These results suggest that a better performance of the flipids may be achieved by making their hydrocarbon chains shorter and/or by introducing additional rigid fragments into their lipid tails. These hypotheses are currently under exploration.

Piperidinol-based flipids

We recently suggested the *trans*-3,4-bis(acyloxy)-piperidine structure **3** (Scheme 2) as a new platform for the pH-triggered conformational switches (42, 50, 51). The driving



Scheme 2 Protonation-induced conformational flip, shortening of the lipid tails, and change of the charge, effective size, and shape of the polar head cause a quick perturbation of the lipid bilayer, lipid phase separation, and fast content leakage from the fliposomes (42).

force in these devices is the same as in flipids **1**: a strong, protonation-generated intramolecular hydrogen bond of O···H-N^+ type and an electrostatic/dipole-dipole attraction stabilizing a conformer with the *gauche* form of the O-C-C-N fragment (**3A**H^+ in Scheme 2).

Applying the approaches and methods developed for the cyclohexane derivatives **1**, we studied the conformational behavior of the piperidine-based flipids **3** and their properties as the lipidic components of fliposomes. The ^{1}H NMR data showed that flipids **3** changed conformation when acidity increased, and this flip occurred between apparent pD 5.5 and 3.5 in d_4-methanol solution. The fliposomes comprising flipid **3**, POPC and PEG-ceramide, were tested using the ANTS/DPX fluorescent assay and demonstrated a leakage that started at pH 5.6 and became substantial at pH 4.0. The release results achieved thus far with the flipids **3** are below the efficiency of the fliposomes containing *trans*-2-aminocyclohexanol-based flipids **1**. Perhaps, the perturbation of the lipid bilayer is stronger in the case of flipids **1** because the latter have more substituents, which drastically changed their position during the acid-triggered conformational flip (Figure 1).

Targeted drug and gene delivery

To confirm that fliposomes are capable of releasing encapsulated drugs in response to lowered pH, we constructed the **1a**/POPC/PEG-ceramide (50:45:5) and the **1a**/POPC/PEG-DPPE fliposomes (50:45:5) containing the anionic anticancer drug MTX (37). Both fliposome formulations were subjected to equilibrium microdialysis as a more versatile method to quantify drug release regardless of its fluorescence. The percentage of MTX release was determined by measuring the UV absorbance (λ=306 nm) of the free drug in the solution outside the dialysis bag after the microdialysis had reached equilibrium. None of the fliposome formulations released a significant amount of MTX after dialysis at pH 7.4 for 4 h. Upon dialysis at pH 5.5, both fliposome formulations containing flipid **1a** released most of the encapsulated MTX. The concentration of MTX generated in the buffer chamber by far exceeded the typical levels of MTX in patients' plasma and serum. For comparison, liposomes containing the diastereomeric amphiphile **2** released much less MTX after microdialysis at pH 5.5 (37). Such observations confirm that pH-triggered release of contents from fliposomes (ANTS, MTX, etc.) results mainly from the conformational change of the lipid tails, which takes place in **1a** but not in its diastereomer **2**.

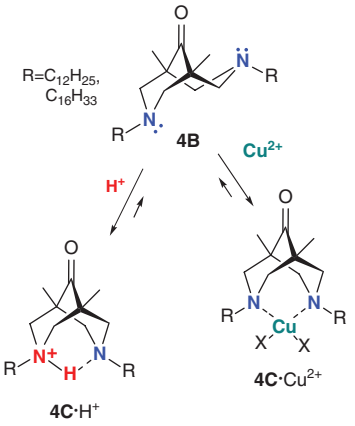
Finally, to test whether fliposomes can serve as viable drug delivery systems, selected liposome formulations encapsulating the anticancer drug MTX were applied to HeLa cells followed by an assessment of the cell viability using the MTS assay (37). HeLa cells treated with 1 μM (final concentration) of MTX solution retained 65.0±4.3% viability, consistent with prior reports on the anticancer activity of the free drug. In comparison, HeLa cells treated with MTX-loaded fliposomes built of **1a**/POPC/PEG-ceramide (50:45:5) showed a significantly lower viability (39.4±4.3%), indicating the superior anticancer cytotoxicity of such liposomal formulations of MTX compared with the free drug at the same dosage. The HeLa cells treated with MTX-loaded liposomes containing the diastereomeric analogue **2** instead of **1a** retained most of the viability, thus highlighting the importance of the conformational flip in enhancing the cytotoxicity of the payload MTX. The cell culture media were buffered at ~pH 7.4; therefore, discharge of the **1a**/POPC/PEG-ceramide/MTX fliposomes most likely took place in the acidic endosomal compartment of the HeLa cells after their cellular uptake. Because MTX is too hydrophilic to passively diffuse across biomembranes, it is likely that the fliposomes not only released MTX in response to the lowered pH in the endosome but also facilitated the diffusion of the released MTX from the endosome to the cytosol. One possible mechanism of such facilitated diffusion could be a destabilization of the endosomal membranes by the micelles generated from the destruction of fliposomes.

It is known that pH sensitivity improves the efficiency of a number of gene delivery systems, including viral vectors, polyplexes, and lipoplexes (10, 52). Inspired by the drug delivery experiments, we started exploring the possibility to use flipids **1** as helper lipids in cationic lipoplexes for gene transfection. The preliminary tests using luciferase assay produced very promising results (41, 53), and this project is currently in progress.

Metal-responsive systems

Another group of researchers suggested 3,7-diazabicyclo[3.3.1]nonan-9-one **4** (Scheme 3) as a conformational switch for the lipid-like amphiphiles (54, 55). In neutral and weakly basic media, these compounds adopt a chair-boat conformation (**4B**) that flips into a chair-chair form (**4C**) in the presence of acid or upon complexation with metal cations.

The liposomes with compounds **4** incorporated into membrane released their fluorescent cargo in response



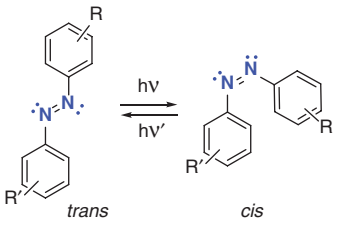
Scheme 3 Protonation- or complexation-induced conformational flip causes a perturbation of the lipid bilayer and the content leakage from fliposomes (54, 55).

to the addition of aqueous $CuSO_4$. This effect was attributed to the complexation-induced change of conformation, which affected the package of lipids in the bilayer and sharply increased the permeability of the liposomal membrane. The liposomal containers with such flipids can be used for the encapsulation and following release of drugs that control the copper level in patients with various pathologies, e.g., hepato-cerebral dystrophy (Wilson disease) (54, 55). Interestingly, the acid- or metal-triggered flip brings the hydrocarbon tails in **4** closer to each other – the change in the direction opposite to the peacock effect observed for flipids **1** (see above).

Phototriggerable flipids

In terms of the dramatic change of molecular shape and size, the conformational switch of flipids is similar to the *cis-trans* photoisomerization of azobenzene derivatives (56). Mechanistically, the isomerization of the latter can be described as a conformational flip proceeding through inversion at the nitrogen atom (Scheme 4) (56). Therefore, the lipidic amphiphiles equipped with the azobenzene photo-switch (1, 57, 58) match the definition of flipids presented above.

The artificial lipids containing the azobenzene moiety were synthesized and demonstrated the ability to cause a disruption of the bilayer packing and the release of entrapped solutes from liposomes upon light irradiation either *in vitro* or in cell cultures (1, 57, 58). The phototriggerable lipids synthesized thus far are triggerable by light in the UV or visible range, which has a limited ability to penetrate into biological tissues and deliver sufficient photon



Scheme 4 Photoisomerization of the lipidic azobenzene derivatives causes a perturbation of the lipid bilayer and the content leakage from liposomes (1, 57, 58).

energy. This poses certain limitations on the potential *in vivo* application of such delivery systems (1). However, a new approach that is currently gaining momentum may help overcome this problem: the use in photochemotherapy of compounds and materials designed to upconvert near-infrared light into higher-energy visible photons. It has been applied to activation of ruthenium complexes suggested as prodrugs for photoactivatable anticancer therapy (59, 60). Perhaps, the same or similar compounds could help trigger the azobenzene photoisomerization in lipid amphiphiles by infrared light.

Outlook

The first attempts to design stimuli-triggerable, especially pH-triggerable, amphiphiles equipped with conformational switches (flipids) and to use them as construction material for stimuli-responsive liposomes (fliposomes) produced encouraging results. Considering a broad variety of known and potential conformational switches, this strategy promises to be the subject of intense investigation in the following years.

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