# Switchable Lipids:Conformational Change for Fast pH-Triggered Cytoplasmic Delivery

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# Switchable Lipids: from Conformational Change to Fast pH-Triggered Cytoplasmic Delivery

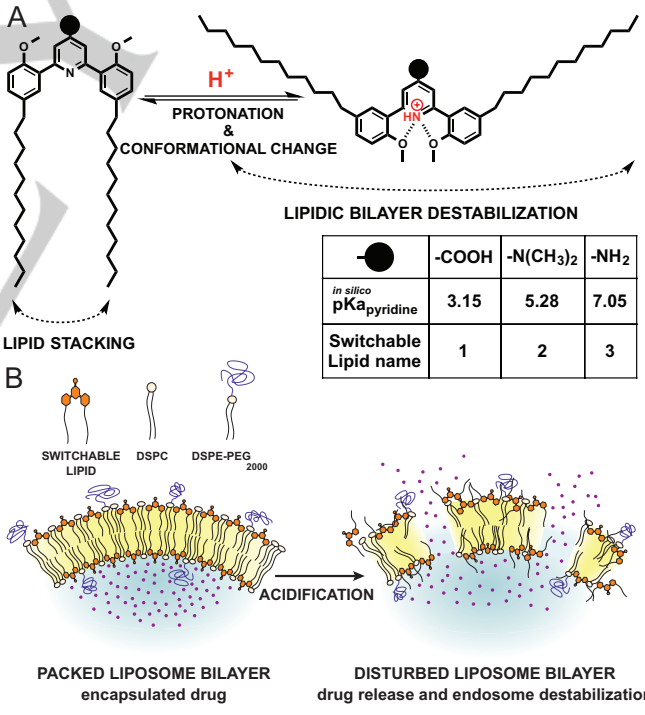
Warren Viricel[a], Amira Mbarek[a] and Jeanne Leblond*[b]

**Abstract:** We report the use of switchable lipids to improve endosomal escape and cytosolic delivery of cell-impermeable compounds. The system is based on a conformational reorganization of the lipid structure upon acidification, as demonstrated by NMR studies. When incorporated in a liposome formulation, the switchable lipids triggered bilayer destabilization through fusion even in the presence of poly(ethylene glycol). Eighty eight percent sulforhodamine B release was achieved in 15 minutes at pH 5 while high stability was demonstrated at pH 7.4 for several months. Using sulforhodamine B as a model of highly polar drug, we demonstrated fast cytosolic delivery mediated by endosomal escape in HeLa cells, and no toxicity.

Precise intracellular delivery is essential for bioactivity of many classes of bioactive macromolecules, e.g. anticancer drugs that must overcome cancer resistance mechanisms or siRNA that must reach the cytosol of target cells.[1] Smart delivery systems capitalize on local changes in physiological environment to provide stimuli-responsive properties and targeted drug delivery.[2] In particular, pH-sensitive liposomes improve cytosolic delivery of drugs by supporting endosomal escape after endocytosis[3], which to date remains an obstacle to DNA delivery.[4]

pH-sensitivity can be obtained by including hydrolysable linkages within the lipid structure.[5] The main difficulty with this strategy is to achieve degradation within the time scale of endosomal maturation, which is under an hour.[3c,6] Over the past decade, this issue has fostered the development of fast-responding escaping strategies including the addition of fusogenic peptides[7], titratable polyanions[8], or charge-switching lipids[9] to liposomes. Unfortunately, such strategies often fail in presence of poly(ethylene glycol) (PEG), which is necessary to improve circulation times.[2b,10] Limited approaches consolidate fast-responding pH-sensitivity and PEGylation[8c,11], making them bear much promise for translation to the clinic.[12]

The switchable lipids reported herein function based on a molecular switch. Molecular switches are dynamic devices designed to change conformation in response to stimuli such as pH, light or ion.[13] Such systems have been largely explored in sensing, but have only recently been considered for biological applications.[14] We previously reported a pH-responsive molecular tweezer able to bind and release a substrate in a pH-dependent fashion.[15] In this study, we constructed lipid-like switches that can integrate into the structure of liposomes (Figure 1A). It was hypothesized that, upon protonation, hydrogen bonding opportunities would favor a change in the relative orientation of the hydrocarbon chains of the switchable lipids, which would disturb the lipid packing of the liposomes, provoke the release of their cargo and confer endosomal escape properties (Figure 1B). The aim of this work was to optimize a PEGylated liposomal preparation presenting fast-responding (< 30 minutes) lipidic bilayer destabilization properties and endosomal escape capabilities at acidic pH values (5-5.5)[16] while remaining stable at blood pH 7.4.




| | -COOH | -N(CH$_3$)$_2$ | -NH$_2$ |
|---|---|---|---|
| *in silico* pKa$_{pyridine}$ | 3.15 | 5.28 | 7.05 |
| Switchable Lipid name | 1 | 2 | 3 |

**Figure 1.** Graphical representation of the liposomal pH-sensitive delivery system based on conformational switch. **(A)** Protonation induced conformational change of the pH-sensitive switchable lipids. **(B)** Disruption of the lipidic bilayer of the liposome upon acidification, leading to drug release and endosome destabilization.

Two alkyl chains were flanked on the di-(methoxyphenyl)pyridine pH-switching unit and a polar headgroup was added in *para* position in order to obtain lipid-like

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switches. *In silico* predictions indicated pKa of the pyridine ring to be strongly dependent on the nature of the headgroup (Figure 1A). Three headgroups were selected to cover a wide range of $pKa_{pyr}$ values. Structures and synthetic details are described in Figures S1-S5.

The conformational change of lipid **2** was examined by $^1$H-NMR titration (Figure 2). $^1$H-NMR titration was also performed on lipid **4**, a non-methoxylated derivative of lipid **2**, which is unable to switch-and-lock its conformation at acidic pH. In both cases, protonation of pyridine affected the $H_{3py}$ proton, which drastically deshielded when pH decreased. A similar behavior also occurred for the other switchable lipids **3** and **7** (Figure S7) and had been observed for the previously reported molecular tweezer.[15]

More noteworthy is the behavior of the $H_o$ proton. For the non-methoxylated lipid **4**, the $H_o$ proton facing the nitrogen atom became shielded upon protonation of the $NH^+$ group located in its proximity (Figure 2B). Interestingly, this was not observed for the switchable lipid **2** (Figure 2A), suggesting that rotation along the $C_{pyridine} - C_{phenyl}$ bond occurred, moving $H_o$ away from the $NH^+$ group and thus counterbalancing the shielding effect. The conformational change of switchable lipid **2** was further confirmed by nuclear overhauser cross relaxation spectroscopy (Figure S8).

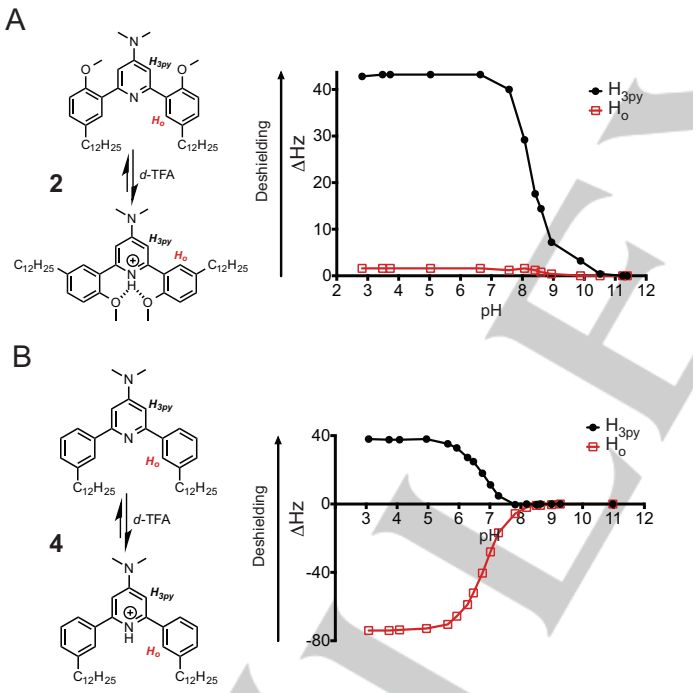


**Figure 2.** NMR titration experiments of the pH-dependent conformational change of **(A)** the switchable lipid **2** and **(B)** its non-methoxylated counterpart **4**.

The switchable lipids **1**-**6** were incorporated in liposomal preparations at 25, 50 or 75 mol% along with the co-lipids DSPC and $DSPE\text{-}PEG_{2000}$ (5 mol%). All liposome formulations exhibited a hydrodynamic diameter below 200 nm. HPLC analyses of purified liposomes confirmed integration of 75-95% of the switchable lipids into the liposome membrane (Table S9). With the exception of lipid **3**, whose $pKa_{pyr}$ is close to pH 7.4, all preparations were stable over 3 months of storage at 4°C.

Disturbance properties of the switchable lipids were studied by monitoring the pH-triggered release of the encapsulated sulforhodamine B dye. Total release was achieved in 5 minutes at pH 4.5 using 75 mol% of **2** (Figure 3A). The rate of release increased with decreasing pH and with increasing content of the switchable lipid in the formulation (Figures 3A, 3B, and S10). These fast kinetic results are common to other systems based on a conformational change such as carboxylated polymers and pH-sensitive peptides,[8c,17] but contrast with hydrolysable linkages that require hours to release their content.[6c,18] Contribution of the conformational change to destabilization of the liposomal bilayer was further confirmed after removing **2** from the preparation or replacing it with **4**, which abolished the release (Figure 3B).

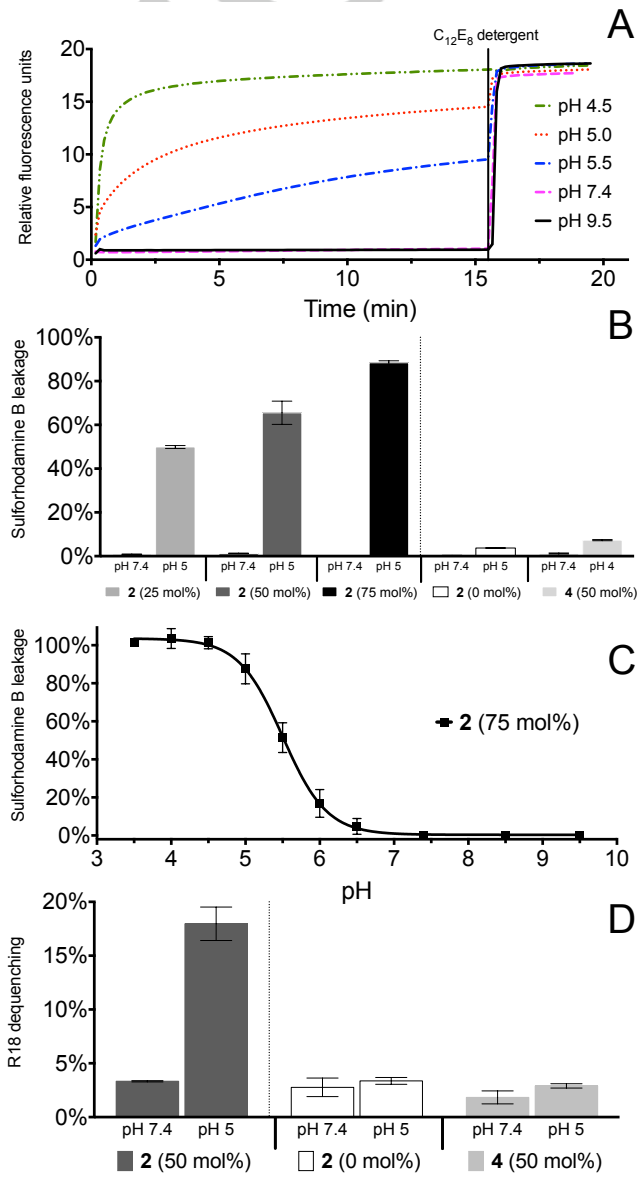


**Figure 3. (A)** Kinetic profiles of sulforhodamine B release for the formulation incorporating the switchable lipid **2** (75 mol%). **(B)** Sulforhodamine B leakage from liposome preparations incorporating the switchable lipid **2** (25-75 mol%), no switchable lipid or the non-methoxylated switchable lipid **4** (50 mol%) after

15 min incubation at neutral or acidic pH. **(C)** Leakage from the liposomal formulation incorporating the switchable lipid **2** (75 mol%) after 15 min incubation at various pH. **(D)** R18 lipid-mixing experiments of the liposomal formulation incorporating the switchable lipid **2** (50 mol%), no switchable lipid or the non-methoxylated switchable lipid **4** (50 mol%) after 15 min incubation at neutral or acidic pH.

Responsive release from liposomes incorporating **2** took place starting from pH 6.5 and was observable over ~2 units of pH (Figure 3C). Sigmoidal fitting of the pH-dependent leakage profile allowed estimating a $pKa_{pyr}$ at 5.50, in agreement with the *in silico* prediction of 5.28.

Structure-activity relationships revealed that other headgroups were less suitable for endosomal escape: liposomes with lipid **1** (COOH headgroup) were unable to destabilize the liposome bilayer even at pH 3.5 (Figure S11) while liposomes with lipid **3** ($NH_2$ headgroup) were unstable at pH 7.4 (Table S9). Furthermore, no impact of the alkyl chain could be evidenced, since all preparations incorporating *N*,*N*-dimethyl-based switchable lipids (**2**, **5** and **6**) exhibited similarly efficient and quick pH-triggered release (Figure S12). These experiments confirm the *in silico* predictions suggesting that the pKa of the system can be tuned by the nature of the headgroup, but not by the chain length.

Fusogenic properties of liposomes are usually restricted by the presence of PEG.[10a-b,19] In the present study, pH-triggered release occurred in presence of 5 mol% DSPE-$PEG_{2000}$. In order to better understand the release mechanism at play, lipid mixing assays were conducted using pH-sensitive liposomes containing the switchable lipid **2** labeled with octadecyl rhodamine B (R18) and model unlabeled phospholipid vesicles (Figure 3D).[17,20] Dequenching, which is indicative of lipid mixing, occurred within minutes under acidic conditions in presence of lipid **2** In contrast, only limited dequenching was observed at pH 7.4 or when lipid **2** was removed or replaced with lipid **4**. Increasing the ratio of unlabeled liposomes favored inter-liposomal fusion and resulted in increased lipid mixing (Figure S13B). These results confirm the fusogenic properties of switchable lipids and their compatibility with PEG shielding, highlighting their high *in vivo* potential.

To verify our hypothesis of endosomal escape and cytosolic delivery, the intracellular trafficking achieved with the pH-sensitive delivery system was investigated using fluorescence microscopy. Sulforhodamine B was chosen as a hydrophilic model drug because it retains its charges at endosomal pH and is therefore unable to cross the cytoplasmic and endosomal membranes on its own.[21] HeLa cells were incubated with sulforhodamine B-loaded liposomes incorporating 50 mol% switchable lipid **2** for 1, 2, and 4 h (Figure 4).

After 1 h, a strong punctate colocalization of sulforhodamine B and LysoTracker® was observed, showing that the liposomes were internalized *via* the endosomal pathway. A Manders' colocalization coefficient (MCC) value of 0.80 confirmed this observation. After 2 and 4 h, cells showed both punctate and diffuse red fluorescence throughout their cytoplasm, as evidenced by the significant drop in MCC values (respectively 0.28 and 0.29). As a control, liposomes without **2** were unable to escape the endosomal pathway, as MCC remained unchanged at the end of the 4-h incubation period (Figure S14B). Unsurprisingly, incubating cells with free sulforhodamine B for 4 h resulted in the absence of red fluorescence (Figure S14C), reminding the critical need of active delivery for hydrophilic drugs. Altogether, these results confirm that our delivery system based on a pH-triggered conformational change enables fast and efficient endosomal escape.

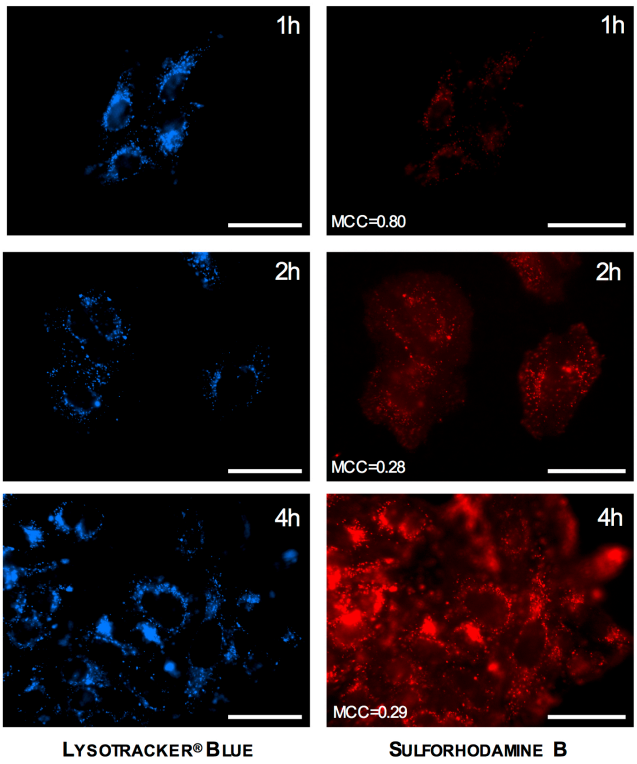


**Figure 4.** Fluorescence microscopy images of HeLa cells after 1, 2 and 4 hour incubation with the sulforhodamine B-loaded pH-sensitive formulation (switchable lipid **2;** 50 mol%). MCC = Manders' colocalization coefficient. Bar = 20 µm.

In parallel, we examined the cytotoxicity of the pH-sensitive liposome formulation on HeLa cells and their hemolytic activity on human red blood cells (Figure S15). No toxicity as well as no hemolytic activity could be seen up to 500 µM, hinting towards the safety of this system for systemic injection.

In summary, a new pH-sensitive liposomal delivery system was developed using switchable lipids that change conformation upon endosomal acidification. The liposomes quickly delivered a highly polar compound to the cytosol *via* efficient endosomal escape and remained effective despite the presence of a PEG corona at their surface. Such lipids can easily be included in existing liposomal formulations to enhance the intracytosolic bioavailability of hydrophilic drugs and nucleic acids.

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**Keywords:** conformational change • drug delivery • endosomal escape • liposomes • pH-responsiveness

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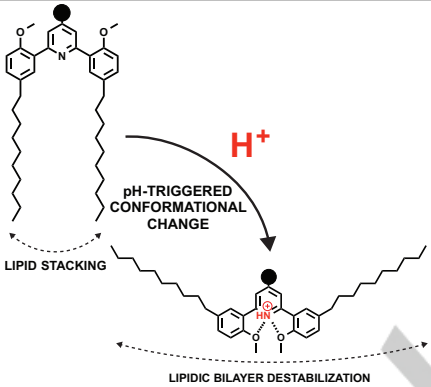
# COMMUNICATION

**Switch it on!** Integration of new pH-sensitive switchable lipids into poly(ethylene glycol)-coated liposome formulations enables fast and efficient cytoplasmic delivery of polar compounds *via* an endosomal escape mechanism. The formulations thus formed are stable at pH 7.4 and upon storage but instantly destabilize at endosomal pH values (5-5.5).

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