Drug Delivery

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

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Abstract: *We report the use of switchable lipids to improve the 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 spectroscopic studies. When incorporated in a liposome formulation, the switchable lipids triggered bilayer destabilization through fusion even in the presence of poly(ethylene glycol). We observed 88% release of sulforhodamine B in 15 min at pH 5, and the liposome formulations demonstrated high stability at pH 7.4 for several months. By using sulforhodamine B as a model of a highly polar drug, we demonstrated fast cytosolic delivery mediated by endosomal escape in HeLa cells, and no toxicity.*

Precise intracellular delivery is essential for the bioactivity of many classes of bioactive macromolecules, for example, 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 the physiological environment to provide stimuli-responsive properties and targeted drug delivery.[2] In particular, pH-sensitive liposomes improve the cytosolic delivery of drugs by supporting endosomal escape after endocytosis,[3] which to date remains an obstacle to DNA delivery.[4]

It is possible to introduce pH sensitivity by including hydrolyzable 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 1 h.[3c,6] Over the past decade, this issue has fostered the development of fast-response escape strategies, including the addition of fusogenic peptides,[7] titratable polyanions,[8] or charge-switching lipids[9] to liposomes. Unfortunately, such strategies often fail in the 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] and thus show much promise for translation to the clinic.[12]

The switchable lipids reported herein function on the basis of a molecular switch. Molecular switches are dynamic devices designed to change conformation in response to stimuli, such as certain pH values, light, or ions.[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 lipidlike 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 study was to optimize a PEGylated liposomal preparation to have fast-responding (< 30 min) lipidic-bilayer-destabilization properties and endosomal-escape capabilities at acidic pH values (5–5.5)[16] while remaining stable at the blood pH value of 7.4.

We introduced two alkyl chains on a di(methoxyphenyl)-pyridine pH-switchable unit and added a polar headgroup at the *para* position to the pyridine N atom to obtain lipidlike switches (see Figures S1–S5 in the Supporting Information for structures and synthetic details). In silico predictions indicated that the p*K*a value of the pyridine ring was strongly dependent on the nature of the headgroup (Figure 1A). Three headgroups were selected to cover a wide range of pKa_{pyr} values.

The conformational change of lipid **2** was examined by ^{1}H NMR titration (Figure 2). Lipid **4**, a non-methoxylated derivative of lipid **2** that is unable to switch and lock its conformation at acidic pH values, was also subjected to ^{1}H NMR titration. In both cases, protonation of the pyridine ring affected the H_{3py} atom, which was drastically deshielded when the pH value decreased. Similar behavior was also observed for switchable lipids **3** and its analogue with a COOH headgroup and $C_{10}H_{21}$ alkyl groups (see Figure S7), and had been observed for the previously reported molecular tweezer.[15]

More notable is the behavior of the H_o atom. For the non-methoxylated lipid **4**, the H_o atom facing the pyridine nitrogen atom became shielded upon protonation of this nitrogen atom (Figure 2B). Interestingly, this effect was not observed for the switchable lipid **2** (Figure 2A), thus suggesting that rotation about the $C_{pyridine}$–C_{phenyl} bond occurred to move the H_o atom away from the NH^+ group and counterbalance the shielding effect. The conformational change of switchable lipid **2** was further confirmed by nuclear Overhauser cross-relaxation spectroscopy (see Figure S8).

The switchable lipids **1**–**4** and two others with the headgroup $N(CH_3)_2$ and either $C_{10}H_{21}$ or $C_{14}H_{29}$ chains were

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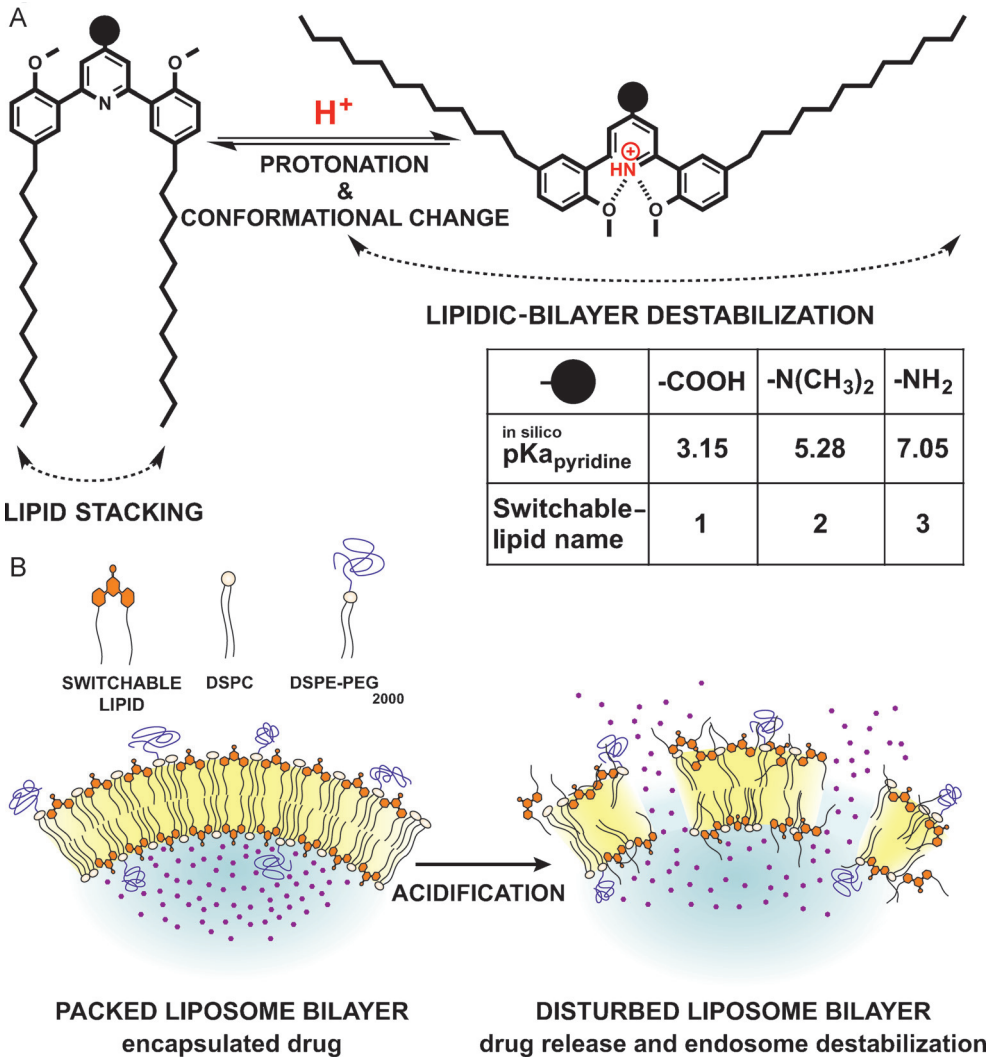

Figure 1. Graphical representation of the liposomal pH-sensitive delivery system based on a conformational switch. A) Protonation-induced conformational change of the pH-sensitive switchable lipids. B) Disruption of the lipidic bilayer of the liposome upon acidification, leading to drug release and endosome destabilization. DSPC = 1,2-dioctadecanoyl-*sn*-glycero-3-phosphocholine, DSPE-PEG_{2000} = *N*-(carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine.

incorporated in liposomal preparations at 25, 50, or 75 mol% along with the colipids DSPC and DSPE–PEG_{2000} (5 mol%). All liposome formulations exhibited a hydrodynamic diameter below 200 nm. HPLC analysis of purified liposomes confirmed the integration of 75–95% of the switchable lipids into the liposome membrane (see Table S9). With the exception of lipid **3**, whose pKa_{pyr} value is close to pH 7.4, all preparations were stable for over 3 months when stored at 4 °C.

The disturbance properties of the switchable lipids were studied by monitoring the pH-triggered release of the encapsulated sulforhodamine B dye. Total release was observed within 5 min at pH 4.5 with 75 mol% of **2** (Figure 3A). The rate of release increased as the pH value decreased and as the content of the switchable lipid in the formulation increased (Figure 3A,B; see also Figure 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 those observed for hydrolyzable linkages, which require hours to release their content.[6c,18] The contribution of the conformational change to the destabilization of the liposomal bilayer was further confirmed by the removal of **2** from the preparation or its replacement with **4**, which abolished the release of sulforhodamine B (Figure 3B).

Responsive release from liposomes incorporating **2** took place from pH 6.5 and was observable over about 2 pH units (Figure 3C). Sigmoidal fitting of the pH-dependent leakage profile allowed the estimation of a pKa_{pyr} value of 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 (see Figure S11), whereas liposomes with lipid **3** (NH_2 headgroup) were unstable at pH 7.4 (see Table S9). Furthermore, no impact of the alkyl chain was evidenced, since all preparations incorporating *N*,*N*-dimethyl-based switchable lipids ($C_{10}H_{21}$ alkyl groups; $C_{12}H_{25}$ alkyl groups (lipid **2**); $C_{14}H_{29}$ alkyl groups) exhibited similarly efficient and quick pH-triggered release (see Figure S12). These experiments confirm the in silico predictions and suggest that the p*K*a value of the system can be tuned by the nature of the headgroup, but not by the chain length.

The fusogenic properties of liposomes are usually restricted by the presence of PEG.[10a,b,19] In the present study, pH-triggered release occurred in the presence of DSPE–PEG_{2000} (5 mol%). To better understand the release mechanism at play, we conducted lipid-mixing assays with 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 the presence of lipid **2**. In contrast,

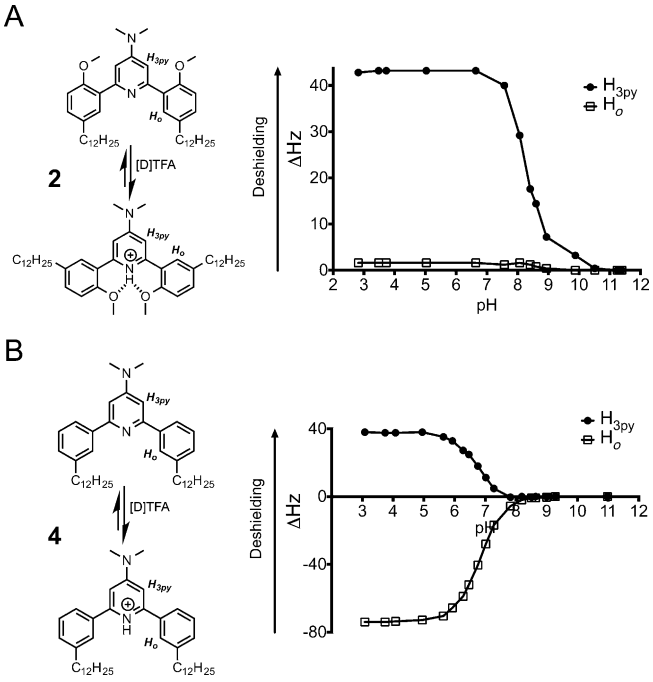

Figure 2. NMR titration experiments investigating the pH-dependent conformational change of A) the switchable lipid **2** and B) its non-methoxylated counterpart **4**.

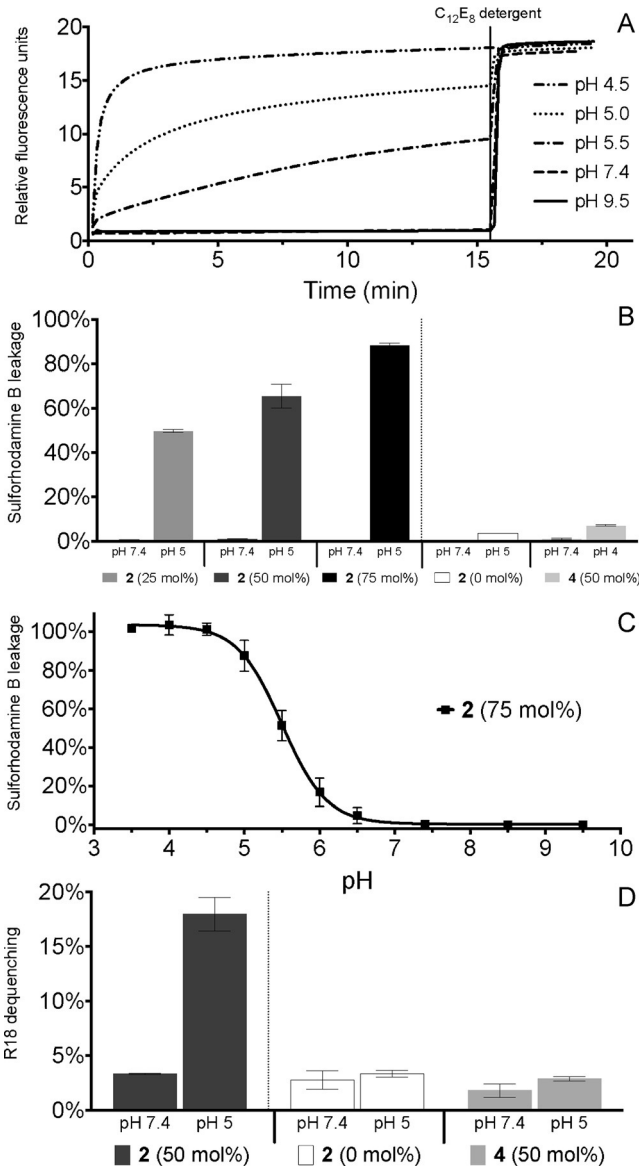

Figure 3. A) Kinetic profiles of sulforhodamine B release for the formulation incorporating the switchable lipid **2** (75 mol%). B) Sulforhodamine B leakage from liposome preparations incorporating the switchable lipid **2** (25–75 mol%), no switchable lipid, or the non-methoxylated switchable lipid **4** (50 mol%) after incubation for 15 min under neutral or acidic conditions. C) Leakage from the liposomal formulation incorporating the switchable lipid **2** (75 mol%) after incubation for 15 min at various pH values. D) R18 lipid-mixing experiments with liposomal formulations incorporating the switchable lipid **2** (50 mol%), no switchable lipid, or the non-methoxylated switchable lipid **4** (50 mol%) after incubation for 15 min under neutral or acidic conditions.

only limited dequenching was observed at pH 7.4 or when lipid **2** was removed or replaced with lipid **4**. An increase in the ratio of unlabeled liposomes favored interliposomal fusion and resulted in increased lipid mixing (see Figure S13B). These results confirm the fusogenic properties of switchable lipids and their compatibility with PEG shielding, thus highlighting their high in vivo potential.

To verify our hypothesis of endosomal escape and cytosolic delivery, we used fluorescence microscopy to investigate the intracellular trafficking observed with the pH-sensitive delivery system. Sulforhodamine B was chosen as a hydrophilic model drug because it retains its charges at endosomal pH values and is therefore unable to cross the cytoplasmic and endosomal membranes on its own.[21] HeLa cells were incubated for 1, 2, and 4 h with liposomes incorporating the switchable lipid **2** (50 mol%) and loaded with sulforhodamine B (Figure 4). After 1 h, a strong punctate colocalization of sulforhodamine B and LysoTracker showed that the liposomes were internalized through the endosomal pathway. A Manders colocalization coefficient (MCC) 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 the MCC value (to 0.28 and 0.29, respectively). As a control, liposomes without **2** were unable to escape the endosomal pathway, as the MCC value remained unchanged at the end of the 4 h incubation period (see Figure S14B). Unsurprisingly, the incubation of cells with free sulforhodamine B for 4 h resulted in the absence of red fluorescence (see Figure S14C): a reminder of the critical need for 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.

In parallel, we examined the cytotoxicity of the pH-sensitive liposome formulation on HeLa cells and its hemolytic activity on human red blood cells (see Figure S15). No toxicity or hemolytic activity were observed up to a concentration of 500 μM, thus hinting at the safety of this system for systemic injection.

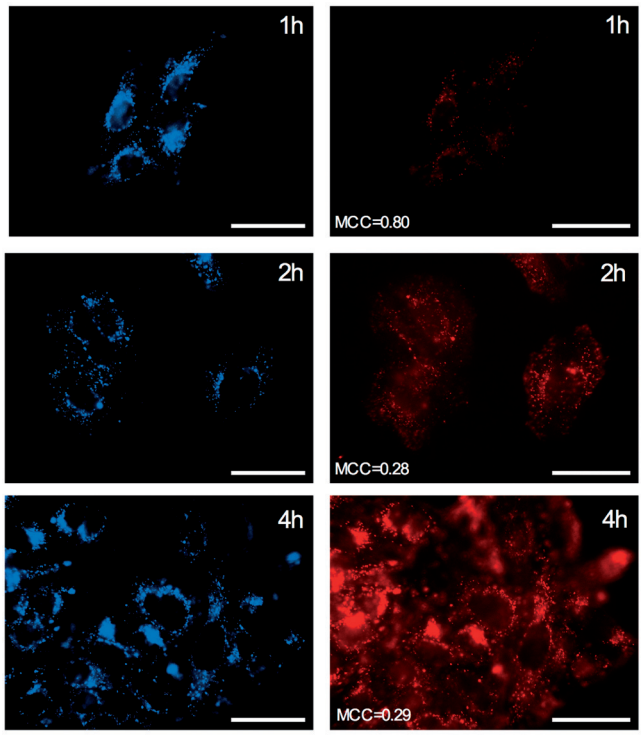

Figure 4. Fluorescence microscopy images of HeLa cells after incubation for 1, 2, and 4 h with the pH-sensitive formulation (switchable lipid **2**, 50 mol%) loaded with sulforhodamine B. Scale bar: 20 μm.

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

Keywords: conformational change · drug delivery · endosomal escape · liposomes · pH responsiveness

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