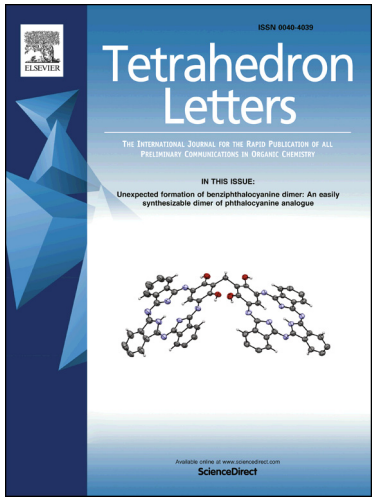

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A new type of pH-sensitive phospholipid

Li Zhan[a], Shuyuan Chang[a], Ting Liu[a], Shanbao Yu[b], Hui Li[b], Minjie Lu[c], Yong Zhu[c], Yu Luo[a,*], Jiahui Yu[a], Fan Yang[a] and Jie Tang[a]

[a] School of Chemistry and Molecular Engineering, East China Normal University, 500 Dongchuan Road, Shanghai 200241, China
[b] PharmaBlock Sciences (Nanjing) Inc., 10 Xuefu Road, Nanjing 210032, China
[c] Suzhou Highfine Biotech. Co., Ltd., 32 Hongxi Road, Suzhou 215129, China

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ABSTRACT

We describe the synthesis and characterization of a type of pH-sensitive pentaerythritol phospholipids, using a trialkoxybenzylidene acetal as the acid-labile moiety. This lipid was prepared by an eight-step synthesis via a latentiation strategy. Liposomes were prepared via the thin film extrusion method. The changes of liposomal sizes were measured by dynamic light scattering. Content release rates of the liposomes as a function of pH were monitored by using a calcein fluorescence dequenching assay. These results indicated that this new liposomal system was capable of releasing its contents under mildly acidic conditions. At last, in vitro cytotoxicity was assayed against three cell lines, suggesting this type of phospholipids was low toxic.

Since the discovery of liposomes by British haematologist Bangham in 1961,[1-3] liposomes have undergone intensive investigation as drug delivery vehicles for over 50 years, resulting in a number of successful applications for cancer chemotherapy, fungal infections, and infection imaging. So far, the liposomal drugs like Doxil and Ambisomes have reached the market. Liposomes are most often composed of phospholipids, especially phosphatidylcholine (PC), but may also include phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cholesterol (Figure 1).

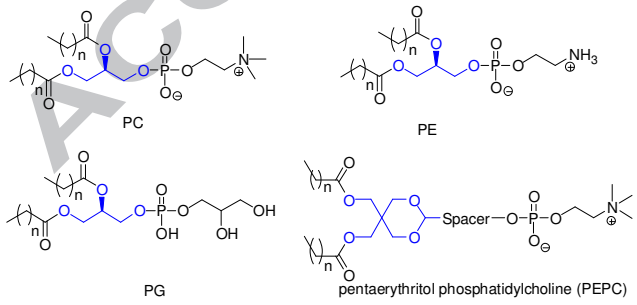


Figure 1. Conventional phospholipids and pentaerythritol phospholipids

The most significant obstacles of conventional liposomes are their rapid blood clearance, reticuloendothelial system localization and lack of site-specific targeting. So far, the development and application of novel targeting and/or release triggering liposomes to improve the therapeutic index of encapsulated drugs are widely reported in the literature. Among these "smart" liposomes, low pH-sensitive liposomes have attracted much interest.[4-6] Ideally, these liposomes should be relatively stable under the physiological pH but should be triggered to release their contents upon exposure to decreased pH at the targets. The rationale of pH-sensitive liposome is that the decrease of pH occurs in many physiological and pathological progressions such as endosome processing, tumor growth, inflammation and myocardial ischemia. It has been found that molecules internalized within cells via the endocytic pathway encounter a significant pH drop from neutral to pH 5.9–6.0. The pH could further reduce from 6.0 to 4.0 during progression from late endosomes to lysosomes.[7,8]

Principally, pH-sensitive liposome system can be classified into two different approaches: (1) formulations containing polymorphic lipids or fusion proteins; (2) lipids containing an acid-cleavable component. To date, a variety of pH-sensitive linkers, such as hydrazones, vinyl ethers, and ortho esters have been applied to pH-sensitive liposomes.[9-14] Although acetals as pH-sensitive moieties have been widely investigated in other kinds of drug delivery systems,[15-19] they were seldom used in pH-sensitive liposomes. In addition, most phospholipids commonly use glycerols as their skeletons. Herein, we describe a type of pentaerythritol phosphatidylcholine (PEPC), using pentaerythritols as the skeletons and acetals as pH-sensitive moieties (Figure 1).

Since a pentaerythritol has four hydroxyls, two of them can be acylated with long chain fatty acids. The other two can condense with an appropriate aldehyde to construct an acetal. It was well

known that the structure of the aldehyde had a great effect on the rate of hydrolysis.[20] We first prepared three pentaerythritol acetals (**1**, **2** and **3**) according to reported methods, and examined their hydrolysis rate by ^{1}H NMR at pH 5.0.[21] As shown in Figure 2, the compounds **1** and **2** were relatively stable at pH 5.0, but the compound **3** exhibited an obvious degradation at this pH. Besides, phenyl rings with more alkoxy groups could hydrolyze faster, suggesting electron-donating groups could accelerate the hydrolysis rate. These data were in accordance with others' results that trimethoxybenzylidene acetals are more acid-sensitive.[15-19]

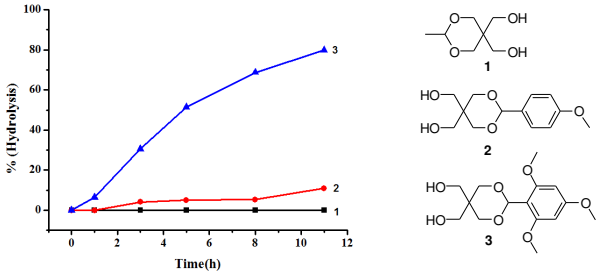
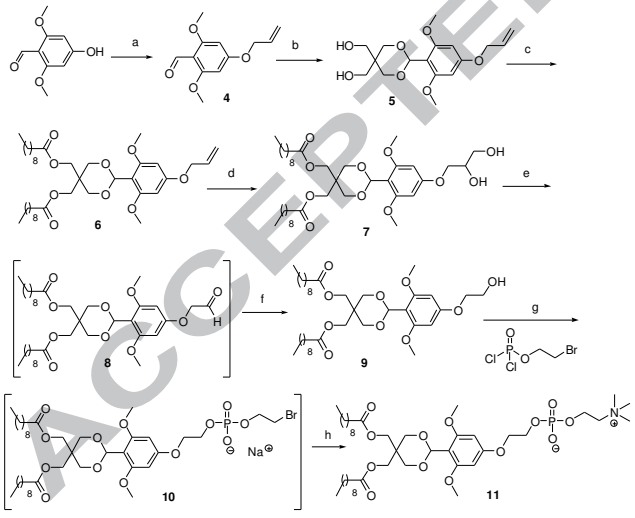


Figure 2. The hydrolysis rate of different pentaerythritol acetals at pH 5.0

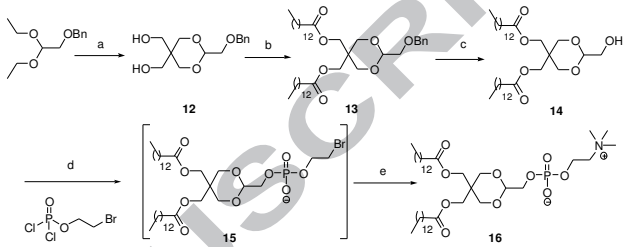
Thus, 1,3,5-trialkoxybenzene could serve as the best acid-sensitive "spacer" in the pentaerythritol phospholipid. However, the synthetic work of this type of acid-sensitive lipid did not proceed smoothly at the beginning, because of the involvement of a variety of functional groups. The acetal was acid-sensitive, while the ester was base-sensitive. In addition, this benzylidene acetal was also vulnerable to palladium-catalyzed hydrogenation. Several synthetic routes were attempted, but failed to produce the key intermediate **9** (Scheme 1). At last, a latentiation strategy was employed. A terminal double bond was first introduced and then transformed into a primary alcohol. The successful synthetic route was outlined in Scheme 1.



Scheme 1. Reagents and conditions: (a) allyl bromide, K_2CO_3, DMF, 85%; (b) pentaerythritol, TsOH, DMF, 92%; (c) capric acid, DCC, DMAP, CH_2Cl_2, 94%; (d) NMO, OsO_4, acetone, 87%; (e) $Pb(OAC)_4$, CH_2Cl_2; (f) $NaBH_4$, THF, MeOH, two steps 70%; (g) (i) Py, $CHCl_3$; (ii) $NaHCO_3$, H_2O; (h) $N(CH_3)_3$, $CHCl_3$, three steps 72%.

Thus, 2,6-dimethoxy-4-hydroxybenzaldehyde was reacted with allyl bromide to give the compound **4** in a 85% yield, which was condensed with pentaerythritol under acidic conditions to yield the acetal **5** in an excellent yield. The acetal **5** was subjected to acylation with capric acid to afford the ester **6**. However, the ozonolysis of the terminal double bond of **6** failed to give the aldehyde **8**. Alternatively, **6** was subjected to the dihydroxylation with osmium tetraoxide and smoothly produced the diol **7** in a 87% yield. The oxidative cleavage of the diol **7** with sodium periodate solution resulted in low yields, with a large amount of starting materials remained. Since **7** was very hydrophobic and had a low solubility in the aqueous media, lead tetraacetate was a good alternative reagent. Thus, the oxidative cleavage of the diol **7** with lead tetraacetate in dichloromethane gave the desired aldehyde **8**, which was reduced to afford the key intermediate **9**. The primary alcohol was then coupled with 2-bromoethyl dichlorophosphate and hydrolyzed to yield the bromide **10**, which was further reacted with trimethylamine to afford the target molecular **11** in good yields.

To further verify the pH-sensitivity of this spacer, another pentaerythritol lipid **16**, using an alkane as the spacer, was also prepared. This lipid was assumed to be less acid-sensitive and could serve as a control. The synthesis of this lipid was outlined in Scheme 2.



Scheme 2. Reagents and conditions: (a) pentaerythritol, TsOH, DMF, 50%; (b) myristic acid, DCC, DMAP, CH_2Cl_2, 90%; (c) Pd/C, THF, H_2, 80%; (d) (i) Py, CH_2Cl_2; (ii) $NaHCO_3$, H_2O; (e) $N(CH_3)_3$, $CHCl_3$, three steps 65%.

2-Benzyloxy-1,1-dimethoxyethane was condensed with pentaerythritol under acidic conditions to yield the acetal **12** in an 50% yield. Subsequently, **12** was coupled with myristic acid to give the ester **13**, which was then hydrogenated to afford the primary alcohol **14** in good yields. At last, the compound **14** was phosphorylated and hydrolyzed to yield the bromide **15**, and then subjected to nucleophilic substitution to produce the desired compound **16** in moderate yields.

Since PEPC was designed for the acid-triggered drug delivery, we were most interested in the following questions. (1) Could PEPC form liposomes through self-assembly? (2) If PEPC formed liposome, could acid trigger the content release?

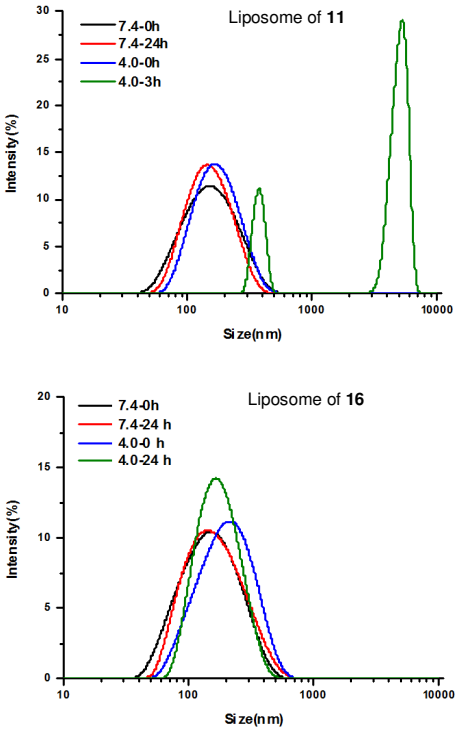


Figure 3. Changes in the size of liposomes in response to different pH

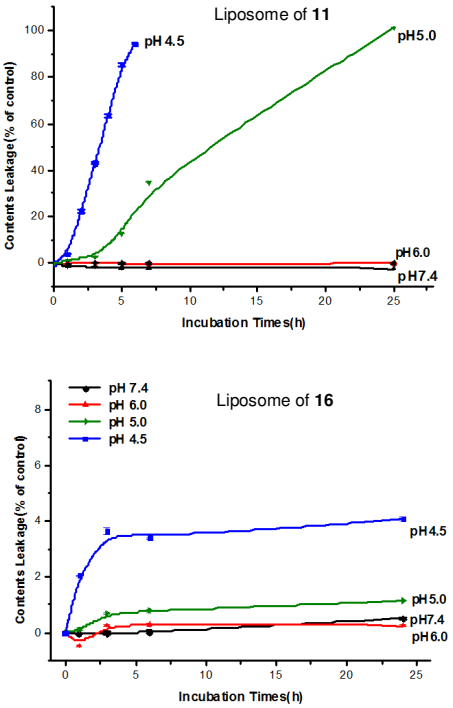


Figure 4. The calcein release from PEPC liposomes at different pH

To answer these questions, we first prepared empty liposomes of **11** and **16**, by conventional liposomal preparative methods.[22] The average sizes of the vesicles were determined by dynamic light scattering (DLS). The liposome of **11** had an average diameter of 134 nm (PDI = 0.119), while the liposome of **16** had an average diameter of 184 nm (PDI = 0.164). To study the acid-sensitive process, the size destabilization of PEPC liposomes in response to different pH values at different intervals was determined using DLS measurements (Figure 3). Under acidic conditions or neutral conditions, the liposomal size of **16** remained basically stable. However, the liposomal size of **11** was stable at pH 7.4 but increased quickly with decreasing pH, suggesting **11** was much more acid-sensitive than **16**. This phenomenon could be ascribed to the cleavage of the cyclic acetal moiety. Namely, the acetal could be cleaved under mildly acidic conditions and produce a hydrophobic unit, which was not soluble in water. In addition, we also examined the hydrolysis of compound **11** by ^{1}H NMR at pH 4.5 (see the supporting information). The results indicated that this compound was acid-labile, indeed.

Content release rates of PEPC liposomes as a function of pH were monitored by using a calcein fluorescence dequenching assay.[14] As calcein encapsulated at self-quenching concentration leaks into the extraliposomal solution, an increase in calcein fluorescence occurs. Liposomes containing calcein (50 mM) were incubated in buffer solutions of pH 4.5, 5.0, 6.0 or 7.4 at 37 °C and the time-dependent change in calcein fluorescence monitored at 527 nm. The calcein release percentage at each time point was calculated as a ratio versus 100% release by addition of Triton X-100 to the liposome sample. In this study, liposomes were modified with cholesterol to increase the stability. In the new formulation, the ratio of PEPC to cholesterol was 2 to 1. The calcein release from the liposome of **11** was obviously pH-dependent (Figure 4). After 5 hours, an over 80% release was observed at pH 4.5. However, no release occurred over 24 h at pH 7.4. As for the compound **16**, calcein release almost did not occur over 24 h even at pH 4.5. These data show that acetal of 1,3,5-trialkoxybenzaldehyde is more acid-sensitive and prone to hydrolysis than that of aliphatic aldehyde.

At last, in vitro cytotoxicity of compound **11** was tested by MTT assays in A549, HepG2 and MGC803 cells. The cells cultured after 72 h in the presence of **11** retained high cell viability even at high concentrations (50 ug/mL) (Figure 5). This result suggests that this amphiphilic lipid may be a safe drug carrier in vivo.

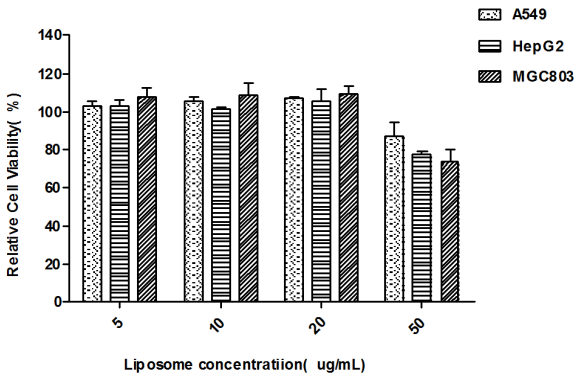


Figure 5. In vitro cell viabilities measured by the MTT assay after culture of the cells with **11** as functions of different concentrations

In conclusion, we have successfully developed pH-responsive liposomes comprising of a novel acid-labile cyclic acetal as the pH-sensitive moiety and pentaerythritol as the skeleton. This lipid could undergo self-association in aqueous media to form its liposomes. The sizes of the liposomes increased with prolonged time in acidic aqueous solution due to the hydrolysis of the acid-labile acetal. Calcein release rates further confirmed the pH sensitivity of this novel type of liposome. In addition, in vitro cytotoxicity assay showed that this lipid had low cytotoxicity. Thus, the present pH-sensitive liposomes can be used as a potential smart drug carrier.

Acknowledgments

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Research highlights

(1) A new type of pH-sensitive phospholipids with an acetal as the acid-labile moiety.
(2) This lipid was prepared by an eight-step synthesis via a latentiation strategy.
(3) This liposome could release its contents under mildly acidic conditions.
(4) This type of phospholipids was low toxic.

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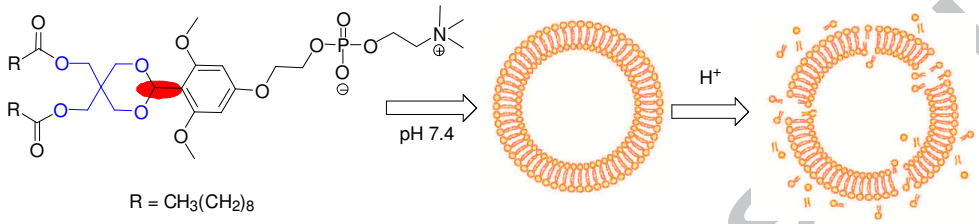

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