Enzymatic Release of Antitumor Ether Lipids by Specific Phospholipase A_2 Activation of Liposome-Forming Prodrugs

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An enzymatically activated liposome-based drug-delivery concept involving masked antitumor ether lipids (AELs) has been investigated. This concept takes advantage of the cytotoxic properties of AEL drugs as well as the membrane permeability enhancing properties of these molecules, which can lead to enhanced drug diffusion into cells. Three prodrugs of AELs (proAELs) have been synthesized and four liposome systems, consisting of these proAELs, were investigated for enzymatic degradation by secretory phospholipase A_2 (sPLA$_2$), resulting in the release of AELs. The three synthesized proAELs were (*R*)-1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (1-O-DPPC), (*R*)-1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphoethanolamine poly(ethylene glycol)$_{350}$ (1-O-DPPE-PEG$_{350}$), and 1-O-DPPE-PEG$_{2000}$ of which 1-O-DPPC was the main liposome component. All three phopholipids were synthesized from the versatile starting material (*R*)-*O*-benzyl glycidol. A phosphorylation method, employing methyl dichlorophosphate, was developed and applied in the synthesis of two analogues of (*R*)-1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphoethanolamine poly(ethylene glycol). Differential scanning calorimetry has been used to investigate the phase behavior of the lipid bilayers. A release study, employing calcein encapsulated in non-hydrolyzable 1,2-bis-*O*-octadecyl-*sn*-glycero-3-phosphocholine (D-O-SPC) liposomes, showed that proAELs, activated by sPLA$_2$, perturb membranes because of the detergent-like properties of the released hydrolysis products. A hemolysis investigation was conducted on human red blood cells, and the results demonstrate that proAEL liposomes display a very low hemotoxicity, which has been a major obstacle for using AELs in cancer therapy. The results suggest a possible way of combining a drug-delivery and prodrug concept in a single liposome system. Our investigation of the permeability-enhancing properties of the AEL molecules imply that by encapsulating conventional chemotherapeutic drugs, such as doxorubicin, in liposomes consisting of proAELs, an increased effect of the encapsulated drug might be achievable due to an enhanced transmembrane drug diffusion.

Introduction

Conventional cancer treatments, such as surgery, radiotherapy, and chemotherapy, require early localization of the tumor in order to be effective. Upon late localization, surgery and radiotherapy in particular become less effective and extensive chemotherapeutic treatments are required. In recent years, an increased understanding of human cancer has given rise to new and improved chemotherapeutic agents. However, the systemic use of these chemotherapeutics is severely limited by the fact that anticancer agents in general are extremely toxic to healthy as well as malign cells. The search for tumor-targeting drugs and drug-delivery systems to circumvent these complications has been intense. The ultimate goal is to find a system that targets pathological tissue and avoids healthy organs and tissues and in this way circumvent the general problem with a very small difference between therapeutic and toxic doses.[1] Of all drug-delivery systems, liposomes are among the most extensively studied. Being composed of naturally occurring substances, they are biocompatible, biodegradable, and nontoxic. In general, liposomes have a low degree of accumulation in the heart, kidneys, and tissue of the nervous system, and the toxicity of many drugs is therefore significantly reduced when encapsulated into liposomal carriers.[1] It has been found that tumors in general have a vasculature that is leaky toward small particles.[2] This opens up a possibility for small liposomes (<200 nm) to extravasate and accumulate in tumors.[3] To obtain a passive diffusion of liposomes into the porous cancer tissue, it is necessary to "coat" the liposome surface to increase the vascular circulation time. Incorporation of lipids with covalently attached poly(ethylene glycol) (PEG) has been shown to increase the liposome circulation time[4] without causing considerable leakage of the encapsulated material.[5] After passive accumulation in the porous cancer tissue, the encapsulated drugs will eventually diffuse out of the liposomes, possibly by

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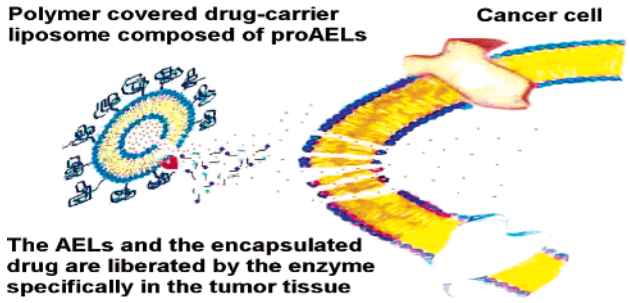


Figure 1. Drug delivery principle. Secretory phospholipase A_2 ($sPLA_2$) hydrolyzes the liposome membrane, thereby releasing both activated antitumor ether lipids (AELs) and the encapsulated drug. The AELs are cytotoxic to cancer cells. Furthermore, synergistically with the generated fatty acids, they function as permeability enhancers that promote drug uptake by the cancer cells.

nonspecific degradation.[6] However, it has also become evident that today's marketed PEG liposomes, containing high amounts of cholesterol, are very stable both in the blood stream and in the cancer tissue, resulting in a low and slow drug release in the tumor.[7,8] It would, therefore, be a substantial improvement if one could trigger the release of drugs specifically at diseased sites. It has been suggested that site-specific triggered release can be achieved by the design of liposomes that are sensitive to local hyperthermia,[9,10] by light- and pH-sensitive liposomes[11−15] and by liposomes that are destabilized by enzymes overexpressed in diseased tissue.[16]

Jørgensen et al.[17] have shown that polymer-covered liposomes are susceptible to degradation by $sPLA_2$, which occurs at elevated levels in the evading zone of tumor tissue,[18] possibly as an important part of the host's defense mechanism.[19−21] $sPLA_2$ is furthermore known to be up-regulated during infectious and inflammatory diseases.[22−25] An interesting feature of the enzyme is a much higher activity toward aggregated lipid substrates, such as liposomes, compared to lipid monomers. In addition, the enzyme activity is, to a large extent, influenced by the physical properties of the aggregated substrates, e.g., liposome size, charge, microstructure, and heterogeneity.[26−28] This finding has led us to develop a novel drug-delivery concept employing $sPLA_2$ as a target-specific trigger.[29] By formulating liposomes that are $sPLA_2$-degradable, it may be possible to obtain a site-specific drug release and further profit from the lysolipid's detergent-like properties (Figure 1). The fatty acid and lysolipid hydrolysis products, generated by $sPLA_2$, have furthermore been shown to display a synergistic effect as permeability enhancers.[30]

The aim of the present work has been to synthesize and characterize phospholipids with a non-hydrolyzable ether bond in the 1-position (1-*O*-phospholipids). These lipids will not only be capable of forming liposomes and carrying water-soluble drugs but will also function as prodrugs using $sPLA_2$ as the activating enzyme. When $sPLA_2$ hydrolyzes the 1-*O*-phospholipid-based liposomes, highly cytotoxic lysolipids (antitumor ether lipids, AELs) are formed. A large variety of AELs have been synthesized and tested in a variety of cell and animal tumor assays.[31] Many are very potent, but a general problem has been the toxicity toward red blood cells. The $sPLA_2$ activation of proAELs reported herein circumvents the

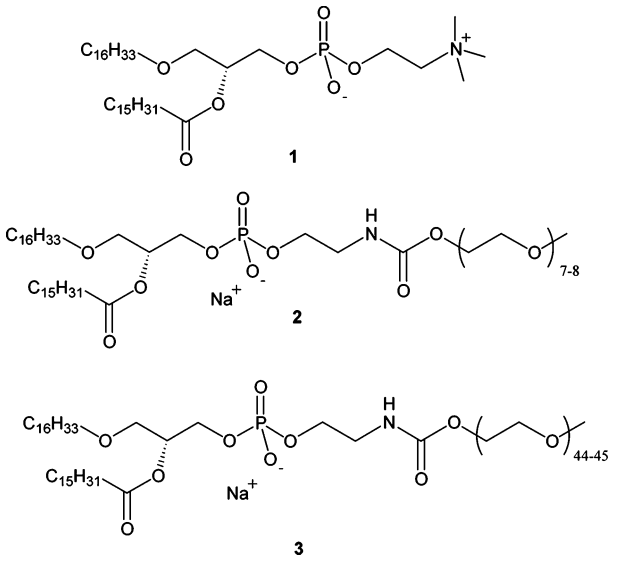
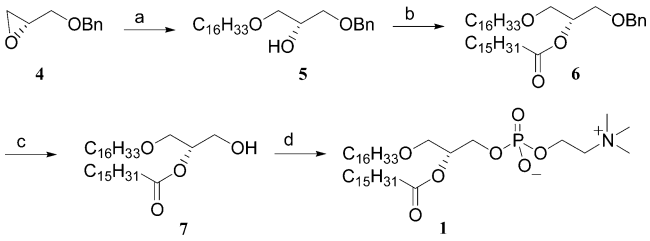


Figure 2. Chemical structures of the three different proantitumor ether lipids (proAELs) that have been synthesized and investigated with respect to their physical properties and ability to constitute a novel liposome-based drug-delivery system: (**1**) (*R*)-1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (1-O-DPPC); (**2**) (*R*)-1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphoethanolamine poly(ethylene glycol)$_{350}$ (1-O-DPPE-PEG$_{350}$); (**3**) 1-O-DPPE-PEG$_{2000}$.

Scheme 1. Synthesis of 1-O-DPPC[a]



[a] (a) $C_{16}H_{33}OH$, NaH, THF, DMF, 16h, 80 °C; (b) palmitoyl chloride, pyridine, petroleum ether, 16 h, room temp; (c) H_2, Pd−C, EtOAc, 1.5 h, room temp; (d) (i) $POCl_3$, Et_3N, CH_2Cl_2, 30 min, room temp, (ii) pyridine, choline tosylate, 16 h, room temp.

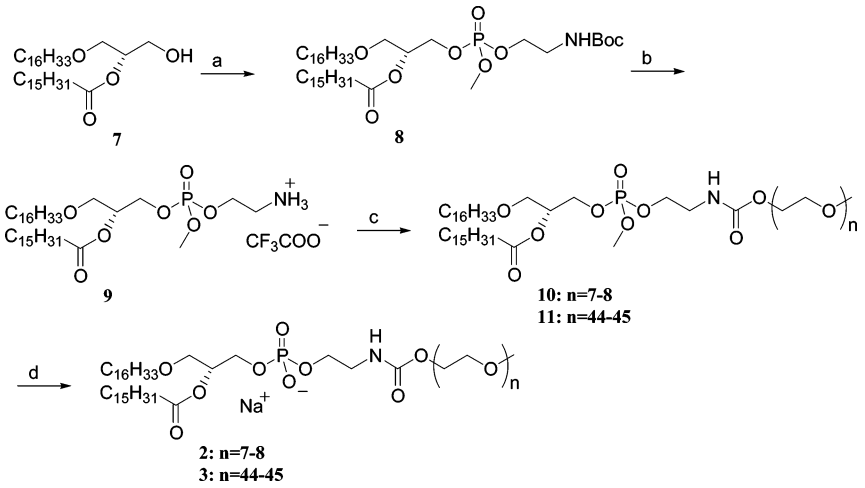
hemolytic limitations of using AELs as drugs in cancer treatment and furthermore offers a site-specific release of a liposome-carried drug, such as doxorubicin.

We report the synthesis of (*R*)-1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (1-O-DPPC) and two analogues of (*R*)-1-*O*-hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphoethanolamine poly(ethylene glycol) (1-O-DPPE-PEG) (Figure 2). We have investigated the physical properties of liposomes composed of these lipids and studied the $sPLA_2$ activity toward different compositions. In addition, the synergistic membrane permeability enhancing properties of the hydrolysis products have been studied. The results substantiate the idea of using $sPLA_2$ as a prodrug/drug-delivery trigger that can specifically activate and release drugs in pathological tissue. The work has been focused on clarifying fundamental questions involved in a rational development of a novel anticancer liposome technology based on 1-O-DPPC and various analogues as lipid prodrugs.

Results and Discussion

Synthesis of proAEL 1, 2, and 3 (Figure 2). The synthetic approach to all three phospholipids involves the synthesis of **7** (Scheme 1), from which either

Scheme 2. Synthesis of 1-O-DPPE-PEG_{350} and 1-O-DPPE-PEG_{2000}[a]



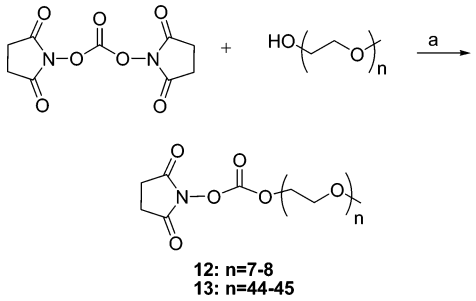
[a] (a) (i) $MeOPOCl_2$, TMP, toluene, 16 h, room temp, (ii) *N*-BOC-ethanolamine, TMP, 24 h, room temp; (b) TFA, MeOH, CH_2Cl_2, 0.5 h, 0 °C; (c) **12** or **13**, Et_3N, $CHCl_3$, 2 h, 40 °C; (d) NaI, 2-butanone, 2 h, 75 °C.

phosphatidylethanolamine or phosphatidylcholine is formed. The ring opening of epoxide **4** with sodium cetyl alcoholate was considerably improved compared to the reported procedure by Hirth et al.[32] by changing reaction conditions. The original conditions, where DMF was used as solvent, resulted in substantial dimerization, which gave yields between 30% and 40%. We found that THF/DMF 1:1 was a superior solvent system. **5** was used without purification, and acylation using palmitoyl chloride gave **6** in 70% yield over the two steps. We have found that using palmitic acid with standard DCC acylating conditions works equally well. Deprotection of **6** using H_2/Pd−C gave **7** in quantitative yield. Compound **7** is not stable under acidic conditions because it is prone to acyl migration. The rearrangement is not detected under basic or neutral conditions within 2 days and is therefore sufficiently stable to be employed in the phosporylation reactions.

The coupling of the choline headgroup to form **1** was performed by the use of phosphorus oxychloride and choline tosylate, applying standard conditions.[32;33] We used a MB-3 ion-exchange column prior to column chromatography to remove salts because washing is not possible because of the amphiphilic properties of the molecule. This reaction has suffered from inconsistent yields,[34] which we also experienced initially when using $CHCl_3$ as solvent (0−80% yields were obtained). Using dichloromethane instead gave consistently high yields around 77%. The overall yield in the synthesis of **1** from **4** was a very competitive 54%.

Preparation of the protected phosphatidylethanolamine **8** (Scheme 2) toward the synthesis of the polymer-covered lipids was initially attempted with $POCl_3$ as the phosphorylation reagent.[35−37] We encountered several drawbacks with this reagent in the synthesis of **8**. This led us to develop a new phosporylation method using commercially available methyl dichlorophosphate. The first attempts were performed in chloroform and with triethylamine as base. This resulted in complex mixtures, which were impossible to purify. A new attempt was made using triethylamine and pyridine. The idea was that pyridine would function as an activating agent and triethylamine as a proton scavenger, keeping pH high and preventing acyl migration; however, this also resulted in complex mixtures. A more sterically hindered base, tetramethyl piperidine (TMP), was tried, and this resulted in the desired compound, easily separated from the byproducts. Toluene was used instead of chloroform, which resulted in an improved yield. Furthermore, the use of toluene has the advantage that salts precipitate and can be filtered off prior to column chromatography. We have found this new phosphorylation method superior when using cheap headgroups that can be used in excess. The method gave **8** in 70% yield. The deprotection of **8** was performed using TFA,[38] giving **9** in quantitative yield, which without purification was coupled to the activated poly(ethylene glycol) **12** and **13** (Scheme 3).[39,40] **10** was purified by successively extracting toluene with water to remove unreacted polymer, followed by column chromatography, resulting in a 74% yield**. 11** could not be purified the same way because of the water solubility of the PEG_{2000}. Unreacted PEG_{2000} could not be separated from the desired product but was removed in the final step. The deprotection of **10** and **11** was performed with NaI,[41,42] resulting in a 95% yield of **2** and a 70% yield of **3** over two steps. Because of the size and polarity of **3**, it does not have simple properties in relation to column chromatography. Even though TLC showed an R_f of 0.3 in 12% methanol/dichloromethane, it ran extremely slowly on a silica column. The polarity of the solvent was raised to 50% MeOH/DCM before **3** came through. This was ascribed to the size of the PEG_{2000} making **3** a macromolecule.

Scheme 3. Synthesis of Activated PEG[a]



[a] (a) Et_3N, ACN, 4 h, room temp.

Table 1. Differential Scanning Calorimetry Data Showing the Melting Enthalpy, ΔH_m, and Peak Position T_m of the Main Phase Transition

	1-O-DPPC	95% 1-O-DPPC 5% 1-O-DPPE-PEG$_{350}$	90% 1-O-DPPC 10% 1-O-DPPE-PEG$_{350}$	95% 1-O-DPPC 5% 1-O-DPPE-PEG$_{2000}$
ΔH_m (kcal/mol)	9.45	8.26	7.99	7.5
T_m (°C)	43.01	42.64	42.43	42.86

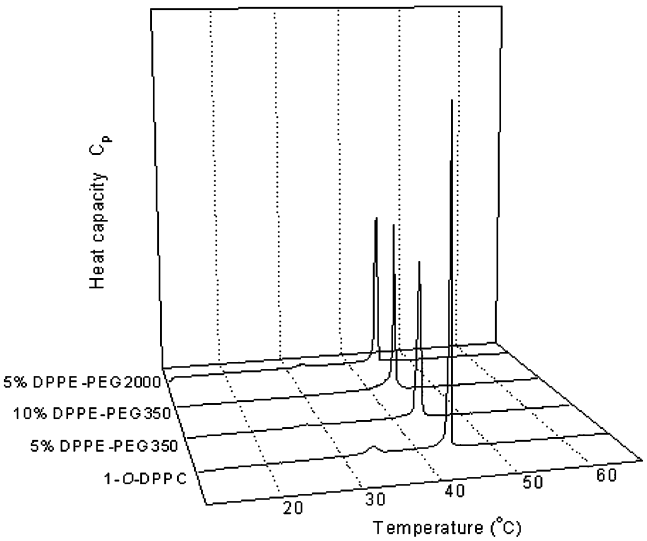


Figure 3. Differential scanning heat-capacity curves obtained at a scan rate of 10 °C/h for 1-O-DPPC liposomes containing 0% 1-O-DPPE-PEG, 5% 1-O-DPPE-PEG$_{350}$, 10% 1-O-DPPE-PEG$_{350}$, and 5% 1-O-DPPE-PEG$_{2000}$.

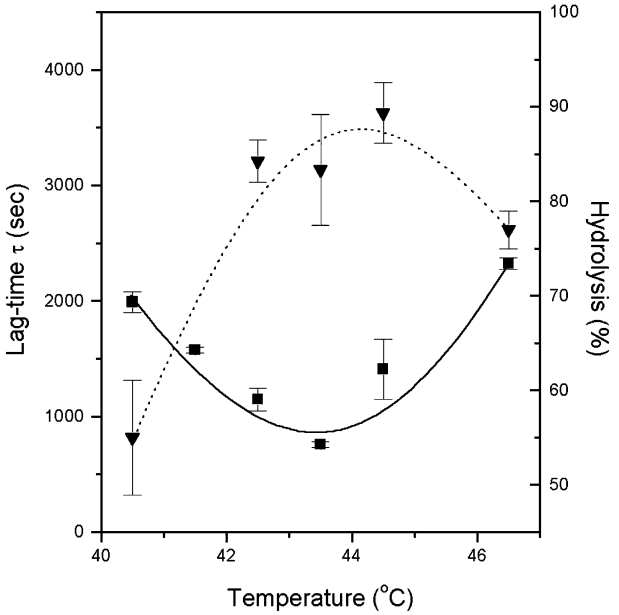


Figure 4. sPLA$_2$ hydrolysis of 1-O-DPPC liposomes. Lag-time measurements (solid line) show a minimum just above the main phase transition. The degree of hydrolysis 1000 s after the burst measured by HPLC is also illustrated (dashed line).

Thermal Analysis. Figure 3 shows differential scanning calorimetry results obtained for four different multilamellar liposome systems. We have studied pure 1-O-DPPC liposomes together with mixtures of 1-O-DPPC and 1-O-DPPE-PEG. The thermograms reveal that all formulations form liposomes. The DSC scan of 1-O-DPPC shows the main phase transition T_m at 43 °C and a pretransition where the lipid membrane goes from the gel to the rippled phase. This is the usual phase behavior of diacylphosphatidylcholines,[43] thereby showing that the C-1 ether linkage does not change the phase behavior drastically. The main phase-transition temperature is situated slightly above T_m of DPPC, indicating that there are more hydrophobic interactions between the aliphatic chains. However, the width of the transition, $T_{1/2}$, of multilamellar vesicles (MLVs) is larger for 1-O-DPPC ($T_{1/2}$ = 0.24 °C) than for DPPC ($T_{1/2}$ = 0.17 °C), indicating a less abrupt change in the membrane structure. The increase in the enthalpy of melting, compared to the enthalpy of melting of DPPC (ΔH_m = 8.39 kcal/mol [44]), suggests that 1-O-DPPC (ΔH_m = 9.45 kcal/mol) forms more stable liposomal membranes because of a tighter packing of the lipids. T_m and ΔH_m values for the multilamellar liposomes of 1-O-DPPC with 5% and 10% 1-O-DPPE-PEG$_{350}$ and 5% 1-O-DPPE-PEG$_{2000}$ are given in Table 1. It can be seen that incorporation of polymer-coated lipids lowers ΔH_m, indicating weaker interactions in the bilayer. In particular, the decrease in ΔH_m for 1-O-DPPE-PEG$_{2000}$ suggests that a large headgroup has an impact on the packing properties of the aliphatic chains. ΔH_m remains nearly the same for both concentrations of PE-PEG$_{350}$ in accordance with results presented by Kenworthy et al.[45]

sPLA$_2$ Lag-Time Measurements on 1-O-DPPC Liposomes. Figure 4 shows the observed sPLA$_2$ lag times in the temperature region of the main phase transition (38.5−46.5 °C), together with the degree of hydrolysis measured by HPLC 1000 s after the burst. The lag time, τ, reflects the elapsed time prior to accelerated hydrolysis (burst), which can be monitored by intrinsic fluorescence from the enzyme (see Figure 5a). Simultaneously with the sudden increase in the measured fluorescence, a decrease in the 90° static light scattering is observed. This is due to a change in the lipid morphology as nonbilayer-forming lysophospholipids and fatty acids are formed. Comparison of the DSC scan and the lag-time measurements shows that the lag-time minimum correlates with the main phase transition. This is in accordance with results found by Hønger et al.[26] Interestingly, the minimum in the lag time furthermore corresponds to the maximum in the degree of hydrolysis (Figure 4).

Calcein Release by Lysophospholipid Perturbation. Calcein release from D-O-SPC liposomes can be used as a model system for analyzing and substantiating the idea of using a liposomal carrier system as a combined prodrug and drug-delivery system that enhances the membrane permeability, resulting in an increased diffusion of the released drugs over the cell membrane.[46] The drug carriers are unilamellar liposomes of 1-O-DPPC lipids with different concentrations of 1-O-DPPE-PEG lipids. These liposomes are stable and long-circulating in the bloodstream because of the steric repulsion of the polymers but are succeptible to sPLA$_2$ attack, resulting in liposome destabilization followed by

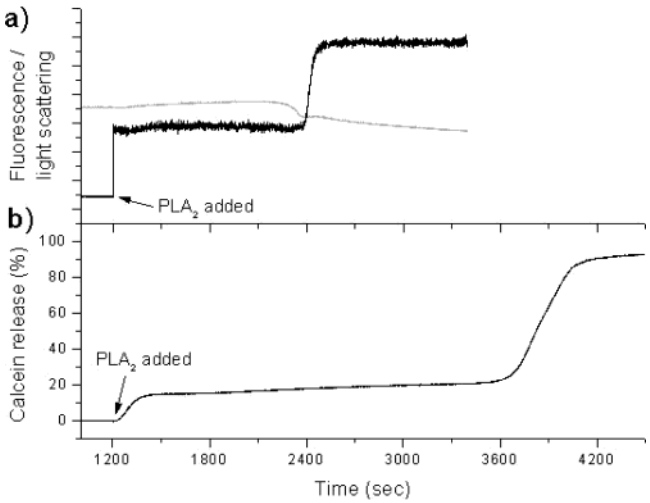


Figure 5. (a) Charateristic reaction time profile at 40.5 °C for $sPLA_2$ (*A. piscivorus piscivorus*) acting on 1-O-DPPC liposomes, measured by intrinsic fluorescence from the enzyme (black line) and at 90° static light scattering (gray line) from the lipid suspension. The scale is arbitrary. (b) Released calcein from D-O-SPC target liposomes at 40.5 °C. The measurements show that calcein release succeeds hydrolysis of the drug carrier liposomes, as should be expected.

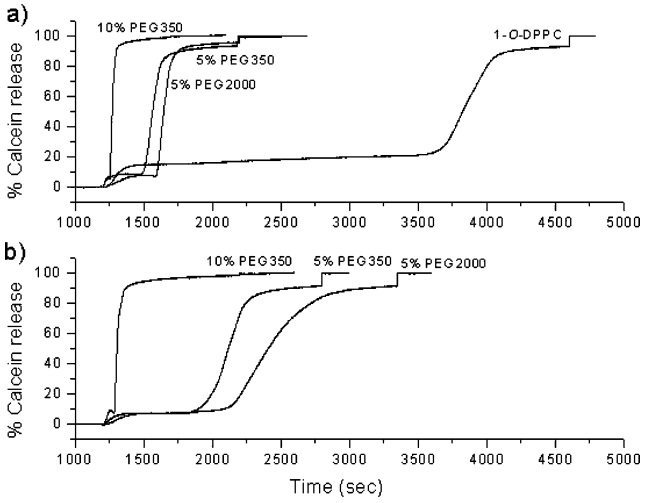


Figure 6. Release of the fluorescent calcein from the target D-O-SPC liposomes as a function of time. $sPLA_2$ was added after 20 min to a liposome suspension of D-O-SPC and 1-O-DPPC incorporated with 1-O-DPPE-PEG lipids: (a) temperature was 40.5 °C; (b) temperature was 38.5 °C. The experiments were stopped, after the burst had occurred, by addition of Triton X-100, which leads to complete release (100%) of calcein.

lysolipid (AEL) and fatty acid release. The D-O-SPC liposomes with encapsulated calcein are inert to $sPLA_2$ degradation and serve as a model of the membrane of the tumor cells. This makes it possible to measure the permeability-enhancing properties of the released AEL molecules from $sPLA_2$ hydrolysis of the carrier liposomes simply by measuring the amount of calcein released from the target liposomes.

The results obtained by the lag-time measurements show a strong time-dependent correlation with the calcein release. Noticeably, the burst indicated by intrinsic fluorescence from $sPLA_2$ happens prior to calcein release. Figure 5 shows that we can use the calcein release measurements as a consistent and direct indication of the $sPLA_2$ lag-time (burst) behavior.[47] Intrinsic fluorescence and HPLC measurements of the mixed liposome systems are very similar to the pure 1-O-DPPC system (data not shown). The lag time is, however, shorter because of the negative charge introduced by the 1-O-DPPE-PEG lipids. Figure 6a shows the degree of calcein release from D-O-SPC liposomes at 40.5 °C as a function of time after the addition of $sPLA_2$ to the different liposome suspensions. A small release of calcein is seen shortly after addition of $sPLA_2$. This release is difficult to explain; however, early $sPLA_2$ studies have shown that a short initial burst occurs followed by a lag phase in $sPLA_2$ hydrolysis of zwitterionic phospholipid vesicles.[48] The time-release profiles for the four liposome systems investigated show that liposomes containing 1-O-DPPE-PEG lead to a fast calcein release in accordance with lag-time experiments on polymer-covered liposomes.[28] It is seen that there is very little difference in the calcein release for 1-O-DPPE-PEG_{2000} and 1-O-DPPE-PEG_{350} when incorporated in the same concentration, which indicates a similar susceptibility of these membrane systems for $sPLA_2$ hydrolysis. However, at 38.5 °C (Figure 6b) the difference is more substantial because the steric barrier of the PEG_{2000} polymer becomes more dominating when the enzyme activity is lower. This does not reveal anything about the perturbing ability of the two lipids but reflects a lower enzyme affinity and/or accessibility to the membrane surface at this temperature.[47] 1-O-DPPE-PEG_{350} is expected to be less perturbing than 1-O-DPPE-PEG_{2000} based on molecular-shape considerations. The large headgroup of 1-O-DPPE-PEG_{2000} is expected to induce a more pronounced curvature stress in the membrane. However, to answer the question of which hydrolyzed 1-O-DPPE-PEG (1-O-2-lyso-PPE-PEG) possesses the highest ability to perturb the D-O-SPC membrane and thereby enhances drug uptake, one needs to account for the effective concentration of the 1-O-2-lyso-PPE-PEG in the target membrane, which is a result of the water-solubility difference of the monomers. It is notable, however, that 1-O-DPPC with 10% 1-O-DPPE-PEG_{350} shows nearly complete calcein release. From HPLC measurements (data not shown), we know that this formulation gives a very high degree of hydrolysis at 38.5 °C, which could be the reason for the more complete calcein release. Another explanation could be that 1-O-2-lyso-PPE-PEG_{350} perturbs the membrane more than 1-O-2-lyso-PPC does because of the difference in the overall molecular shape.

There is evidence that the negative charge on 1-O-DPPE-PEG lipids is the main contributor to the enhanced $sPLA_2$ activity. The length of the PEG_{2000} should give rise to a much higher steric repulsion of the enzyme than PE-PEG_{350} does. However, the apparent steric repulsion observed at 40.5 °C is overshadowed by the electrostatic attraction, resulting in a short lag time and fast hydrolysis of the liposomes. At lower temperatures (38.5 °C), the steric repulsion of PE-PEG_{2000} compared to PE-PEG_{350} becomes more pronounced and results in an increased lag time. These results substantiate the results presented in early articles,[49−52] describing reduced enzyme interaction, and the finding that $sPLA_2$

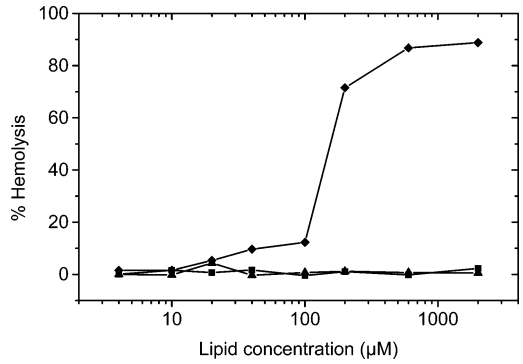


Figure 7. Percent hemolysis of human red blood cells as a function of lipid concentration. The liposomes were composed of 100% 1-O-DPPC (squares) and 95% 1-O-DPPC with 5% 1-O-DPPE-PEG_{2000} (triangles) and compared with ET-18-OCH_3 (diamonds) (forms micelles).

has increased activity toward PE-PEG doped liposomes[47] because of the introduction of a net negative charge.[28]

In Vitro Study of Red Blood Cell Hemolysis by proAEL Liposomes. The thoroughly investigated AEL compound ET-18-OCH_3 (Edelfosine) is known to be very cytotoxic toward tumor cells. However, the therapeutic use of ET-18-OCH_3 as an anticancer drug has been restricted by its strong hemolytic properties.[53] The disruptive nature of ET-18-OCH_3 on the membrane of red blood cells is closely associated with its detergent-like properties. It has been shown that the association of ET-18-OCH_3 with stable liposomes greatly reduces the hemolytic effects in vitro and in vivo.[54,55] However, the toxic dose is still very low. Two of the liposome systems studied in the present paper have been compared with ET-18-OCH_3. The synthesized proAELs are close proanalogues of ET-18-O-CH_3 and become cytotoxic once activated by $sPLA_2$. These proAEL analogues are consequently very interesting as chemotherapeutic agents if the severe side effects, such as hemolysis, can be removed by localized activation by $sPLA_2$ specifically in tumor tissue. Figure 7 shows the comparison of the hemolytic properties of two liposome proAEL systems with ET-18-O-CH_3. It is clear that the masked proAELs are significantly less hemolytic and nontoxic against human red blood cells. These results suggest that the proAEL liposome systems may be worthy candidates for the design of novel tumor-targeting drug-delivery formulations that can be administered intravenously.

Conclusion

Results have been presented that support and elaborate earlier results by Vermehren et al.[47,56] and Jørgensen et al.[28] It is shown that there is an increased $sPLA_2$ activity toward liposomes composed of zwitterionic phospholipids, such as 1-O-DPPC, when incorporated with negatively charged 1-O-DPPE-PEG. Through the synthesis of prodrug lipids of 1-O-DPPC and 1-O-DPPE-PEG, it has been possible to substantiate that $sPLA_2$ does not express a significant difference in activity toward liposomes composed of 1-O-phospholipids compared to liposomes composed of diacylphospholipids.[47] By employment of D-O-SPC liposomes with encapsulated calcein as a simplified model of a target membrane, it has furthermore been possible to show that the generated AELs and fatty acid hydrolysis products of $sPLA_2$ possess a membrane-perturbing effect and cause a substantial calcein leakage from the target liposomal membranes. This indicates, in relation to medical applications in cancer treatment, that there will be an increased diffusion of the released drugs into the cancer cells. This could potentially lead to an enhanced efficacy of the carried antitumor drugs. Hemolytic experiments on red blood cells have demonstrated that proAEL liposomes composed of pure 1-O-DPPC and 1-O-DPPC doped with 5% 1-O-DPPE-PEG_{2000} have a very low hemolytic toxicity.

The results presented are promising in relation to the development of a new liposome-based drug and drug-delivery system that target cancer tissue with elevated levels of $sPLA_2$. The proAEL liposomes offer a cancer-targeted release of a carried drug, such as doxorubicin, through their susceptibility to $sPLA_2$ degradation. In addition, the idea combines the use of liposomes as drug carriers with a unique and novel lipid-based prodrug that is activated by $sPLA_2$ specifically in tumor tissue. The resulting antitumor ether lipids (AELs) are known to display promising anticancer activity but have so far been limited in use because of their hemolytic properties. The proAEL concept is designed to circumvent these limitations and might even enhance the efficacy of the carried drug through the increased permeability of the target cell membrane. The results presented herein constitute a model study that is necessary for clarifying the suitability and potential of using $sPLA_2$ as a site-specific trigger for the release of antitumor ether lipids specifically in tumor tissue. We have provided the fundamental building blocks of the concept, which are required in order to take the next rational steps into biological studies.

Experimental Section

Materials and Liposome Preparation. Edelfosine (ET-18-OCH_3), which was used as reference in the blood hemolysis study, was purchased from Avanti Polar Lipids, Birmingham, AL. All other lipids were synthesized in our laboratories as described in Results and Discussion. 1-O-DPPC multilamellar liposomes were prepared by hydrating the lipids in a Hepes buffer solution for 1 h at 55 °C. The lipid suspension was vortexed every 15 min. Mixed liposomes of 1-O-DPPC and 1-O-DPPE-PEG were prepared by dissolving the weighed amounts in chloroform. The solvent was removed using a gentle stream of nitrogen, after which the lipid film was dried overnight under reduced pressure. The lipid film was hydrated in Hepes buffer using the same conditions as for 1-O-DPPC. Unilamellar liposomes of narrow size distribution were made by extrusion of the multilamellar liposome suspension 10 times through two stacked 100 nm polycarbonate filters.[57] The Hepes buffer used was prepared from 0.15 M KCl, 1 mM NaN_3, 0.03 mM $CaCl_2$, 0.01 mM EDTA, 0.01 M Hepes (pH 7.5). The employed $sPLA_2$ (*Agkistrodon piscivoros piscivoros*) was isolated and purified from snake venom.[58] This enzyme is structurally similar to mammalian $sPLA_2$, indicating a similar catalytic hydrolysis mechanism for phospholipid substrates.[59]

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) of 5 mM multilamellar liposomes was performed using a Microcal MC-2 (Northhampton, MA) ultrasensitive power-compensating scanning calorimeter equipped with a nanovoltmeter. The scans were performed in the upscan mode at a scan rate of 10 °C/h. An appropriate baseline has been subtracted from the calorimetric curves.

Phospholipase A_2 Lag-Time Measurements. Assay conditions for the $sPLA_2$ lag-time measurements were 0.15 mM unilamellar liposomes, 150 nM $sPLA_2$, 0.15 M KCl, 1 mM NaN_3, 0.03 mM $CaCl_2$, 0.01 mM EDTA, and 0.01 M Hepes (pH

7.5). The catalytic reaction was initiated by adding 8.9 μL of a 42 μM $sPLA_2$ stock solution to 2.5 mL of a thermostated liposome suspension equilibrated 20 min prior to addition of the enzyme. The time elapsed between addition of $sPLA_2$ and a sudden increase in the intrinsic fluorescence emission from $sPLA_2$ (tryptophan), due to a burst in the $sPLA_2$ activity, is defined as the characteristic lag time of the enzyme.[26] The emission takes place at 340 nm after excitation at 285 nm. The total amount of hydrolyzed lipid 1000 s after the $sPLA_2$ burst was determined by HPLC using a 5 μL diol column, a mobile phase composed of $CHCl_3$/MeOH/H_2O (730:230:25, v/v), and an evaporative light scattering detector.

Phospholipase A_2 Calcein Release Measurements. Assay conditions for the $sPLA_2$ calcein release measurements performed in order to investigate the permeability-enhancing effect of the generated AELs and fatty acid hydrolysis products were 25 μM unilamellar proAEL liposomes, 25 μM 1,2-bis-*O*-octadecyl-*sn*-glycero-3-phosphocholine (D-O-SPC) liposomes with encapsulated calcein, 25 nM $sPLA_2$, 0.15 M KCL, 1 mM NaN_3, 0.03 mM $CaCl_2$, 0.01 mM EDTA, and 0.01 M Hepes (pH 7.5). The catalytic reaction was initiated by adding 1.5 μL of a 42 μM $sPLA_2$ stock solution to 2.5 mL of a thermostated liposome suspension equilibrated 20 min prior to addition of the enzyme. The calcein release from the D-O-SPC liposomes was measured by the fluorescent intensity at 520 nm after a 492 nm excitation. The D-O-SPC liposomes were prepared by hydrating a D-O-SPC lipid film with a Hepes buffer (pH 7.5) containing 20 mM calcein for 1 h at 65 °C. The lipid suspension was vortexed every 15 min. The multilamellar liposomes were extruded as described above, and the resulting unilamellar liposomes were separated from free calcein using a chromatographic column packed with sephadex G-50. The measurements were performed at temperatures where both the carrier and the target liposomes are in the gel state. The amount of calcein released is calculated as

$$\% \text{ release} = 100 \times \frac{I_{F(t)} - I_B}{I_T - I_B} \qquad (1)$$

$I_{F(t)}$ is the measured fluorescence at time t, I_B is the background fluorescence, and I_T is the fluorescence measured after complete calcein release upon addition of Triton X-100. Calcein is entrapped in a self-quenching concentration at 20 mM, which is the reason for increased fluorescence upon release from D-O-SPC liposomes. A linear relation between the fluorescence measured and calcein released exists with the D-O-SPC liposome/calcein concentration used in the experiment.

Hemolysis Assay. The hemolysis assays were performed as described by Perkins et al.[60] 0.5 mL of the lipid suspensions was mixed with 0.5 mL of a washed human red blood cell (RBC) suspension [4% in PBS (v/v)] and incubated with constant agitation at 37 °C for 20 h. A negative reference (0% hemolysis) was prepared by mixing 0.5 mL of the RBC suspension with 0.5 mL of PBS. A positive reference (100% hemolysis) was prepared by adding 0.5 mL of 10% Triton X-100 to 0.5 mL of RBC suspension. The incubated samples were centrifuged (2000 rpm) for 10 min at 20 °C. An amount of 400 μL of the supernatant was diluted with 2 mL of buffer solution (PBS). Percent hemolysis was measured as absorbance at 550 nm using a Perkin-Elmer 320 spectrophotometer.

Synthesis. Reactions involving air-sensitive reagents were carried out under N_2 using the syringe−septum technique. THF was freshly distilled over sodium/benzophenone ketyl. DMF, CH_2Cl_2, $CHCl_3$, toluene, pyridine, TMP, and Et_3N were dried over 3 Å molecular sieves. Ether refers to diethyl ether. Reagents were purchased from Sigma-Aldrich Chemical Co. and used without further purification. Column chromatography was performed using silica gel (35−70 μm, 230−400 mesh). HPLC analysis was performed using a 5 μL diol column, a mobile phase consisting of $CHCl_3$/MeOH/H_2O (730:230:25, v/v), and an evaporative light scattering detector. ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 and 75 MHz, respectively.

1-*O*-Hexadecyl-3-*O*-benzyl-*sn*-glycerol (5). To a flame-dried flask (100 mL) containing washed NaH (0.153 g, 6.40 mmol) under N_2 at 0 °C was added cetyl alcohol (1.48 g, 6.10 mmol) in dry THF (25 mL). The NaH was washed by a continuous extraction of a NaH dispersion (60% mineral oil) with dry petroleum ether. The reaction mixture was heated to 80 °C for 1 h. (*R*)-*O*-Benzyl glycidol (**4**) (0.5 g, 3.05 mmol) was added and then DMF (25 mL) dropwise over 5 min. The reaction mixture was stirred overnight at 80 °C. The reaction mixture was cooled to room temperature and stirred for 20 min upon addition of water (2.5 mL). Removal of solvent in vacuo gave a brown residue, which was redissolved in ether (40 mL), washed with brine (3 × 30 mL), and dried over Na_2SO_4. The resulting yellow solid was purified on a short column (5 cm) using 20% ether/CH_2Cl_2 to give 1-*O*-hexadecyl-3-*O*-benzyl-*sn*-glycerol (**5**), crude, R_f = 0.44 (ether/CH_2Cl_2 1:4), which was used without further purification.

1-*O*-Hexadecyl-2-palmitoyl-3-*O*-benzyl-*sn*-glycerol (6). Crude **5** was dissolved in petroleum ether (60 mL). Pyridine (1.46 mL, 18.3 mmol) and palmitoyl chloride (4.5 mL, 14,7 mmol) dissolved in petroleum ether (80 mL) were added, and the mixture was stirred for 16 h at 80 °C, after which TLC (petroleum ether/CH_2Cl_2/ether 20:12:3) indicated that the reaction was complete. The reaction was quenched with water (15 mL) and the mixture was stirred 30 min, after which petroleum ether (100 mL) was added. The solution was washed with 0.8 mL of HCl (2 × 50 mL), H_2O (2 × 50 mL), $NaHCO_3$ (4 × 25 mL), and H_2O (2 × 50 mL), dried over $MgSO_4$, and concentrated. Purification by flash chromatography (petroleum ether/CH_2Cl_2/ether 60:12:3) gave 2,39 g (70%) of **6**. R_f = 0.51 (petroleum ether/CH_2Cl_2/ether 20:12:3). Anal. Calcd: C, 78.21; H, 11.78. Found: C, 78,24; H, 11.59. ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.32 (m, 5H, Ph), 5.18 (quintet, 1H, J = 5.1 Hz, CH), 4.48−4.60 (AB, 2H, J = 12.2 Hz, CH_2Ph), 3.62 (d, 2H, J = 5.4 Hz, CH_2CHCH_2), 3.57 (d, 2H, J = 5.2 Hz, CH_2CHCH_2), 3.42 (m, 2H, $C_{15}H_{31}CH_2O$), 2.33 (t, 2H, J = 7.4 Hz, CH_2COO), 1.62 (quintet, 2H, β-CH_2), 1.52 (quintet, 2H, β-CH_2), 1.26 (br s, 50H, 25 × CH_2), 0.88 (t, 6H, 2 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.5 (COO), 138.1, 128.4, 127.7 127.6 (Ph), 73.2, 71.5, 69.1, 68.7 (4 × CH_2O), 71.2 (OCH), 34.4 (CH_2COO), 31.8, 29.7, 29.6, 29.5, 29.4, 29.3, 29.2, 29.0, 25.9, 24.9, 22.6 (CH_2 aliphatic), 14.0 (2 × CH_3).

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycerol (7). To a flame-dried flask (50 mL) with Pd−C 10% (22 mg) under N_2 was added ethyl acetate (5 mL), and then **6** (195 mg, 0.30 mmol) was dissolved in ethyl acetate (10 mL). The flask was fitted with a balloon containing H_2, and the mixture was stirred for 90 min. The solution was filtered through a glass filter, which was washed successively with CH_2Cl_2. Concentration in vacuo gave 166 mg (99%) of **7** as a white solid, which was dried at 0.1 mmHg for 1 h and used in the next step. ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.99 (quintet, 1H, J = 5.1 Hz, CH), 3.82 (d, 2H, J = 4.8 Hz, CH_2CHCH_2), 3.62 (ABX, 2H, J = 5.0 Hz, CH_2-$CHCH_2$), 3.45 (m, 2H, $C_{15}H_{31}CH_2O$), 2.29 (t, 2H, J = 7.2 Hz, CH_2COO), 1.58 (quintet, 4H, 2 × β-CH_2), 1.26 (br s, 50H. 25 × CH_2), 0.88 (t, 6H, 2 × CH_3).

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphocholine (1). To a solution of $POCl_3$ (35.1 μL, 0.388 mmol) in dry CH_2Cl_2 (1.5 mL) at 0 °C was added a solution of **7** (160 mg, 0.290 mmol) and Et_3N (54 μL, 0.388 mmol) in CH_2Cl_2 (3.5 mL) dropwise over 20 min. The reaction mixture was stirred for 30 min under N_2 at room temperature, after which pyridine (200 μL, 2.49 mmol) and choline tosylate (128 mg, 0.465 mmol) were added. The reaction was stirred overnight at room temperature, resulting in a yellow solution. Water (0.2 mL) was added, and the reaction mixture was stirred for 40 min and concentrated to a white foam by azeotropic distillation with ethanol. The residue was dissolved in THF/H_2O 9:1 and slowly passed through an MB-3 column (5 cm), and the solvent was removed by azeotropic distillation with ethanol in vacuo. The crude product was purified by column chromatography (CH_2Cl_2/MeOH/H_2O 65:25:4), giving **1** in 159 mg (77%) as a white solid. R_f = 0.37 (CH_2Cl_2/MeOH/H_2O 65:25:4), >99% pure by HPLC, $[\alpha]^{25}_D$ −3.37° (*c* 0.5, $CHCl_3$/MeOH 1:1) (lit.,[61] $[\alpha]^{25}_D$

−3.38°, *c* 0.53 $CHCl_3$/MeOH 1:1). Phospholipase A_2 showed >95% conversion. Anal. Calcd for $C_{40}H_{82}NO_7P \cdot H_2O$: C, 65.09; H, 11.47; N, 1.90. Found: C, 64.94; H, 11.42; N, 1.90. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.13 (quintet, 1H, CH), 4.28 (m, 2H, PO$CH_2$$CH_2N^+$), 3.92 (m, 2H, CH_2CHCH_2), 3.79 (m, 2H, POCH$_2$$CH_2N^+$), 3.55 (m, 2H, CH_2CHCH_2), 3.40 (m, 2H, $C_{15}H_{31}$-CH_2O), 3.33 (s, 9H, $(CH_3)_3N^+$), 2.30 (t, 2H, J = 7.3 Hz, CH_2-COO), 1.59 (quintet, 2H, β-CH_2), 1.51 (quintet, 2H, β-CH_2), 1.26 (br s, 50H. 25 × CH_2), 0.88 (t, 6H, 2 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.6 (COO), 72.0 (CH_2CHCH_2, J = 7.9 Hz), 71.6 ($C_{15}H_{31}CH_2O$), 69.3 (CH_2CHCH_2), 66.1 (POCH$_2$$CH_2N^+$, J = 5.7 Hz), 63.8 (CH_2CHCH_2, J = 5.4 Hz), 59.3 (PO$CH_2CH_2N^+$, J = 4.8 Hz), 54.2 ($(CH_3)_3N^+$), 34.4 (CH_2COO), 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.0, 24.9, 22.5 (CH_2 aliphatic), 13.9 (2 × CH_3).

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-*O*-methyl-*N*-BOC-ethanolamine (8). To a solution of MeO-$POCl_2$ (136 μL, 1.36 mmol) and TMP (240 μL, 1.42 mmol) in dry toluene (2 mL) under N_2 at −25 °C was added **7** (343 mg, 0.62 mmol) in toluene (10 mL) by dropwise addition. The reaction mixture was stirred overnight at room temperature, after which only traces of starting material were indicated by TLC. TMP (366 μL, 2.17 mmol) and *N*-BOC-ethanolamine (575 μL, 3.71 mmol) were added, and the reaction mixture was stirred overnight at room temperature. TLC indicated traces of monochlorophosphate, R_f = 0.81 (CH_2Cl_2/EtOAc 7:3). The formed TMP-Cl salt was removed by filtration, and the solvent was removed under reduced pressure. The product was purified by column chromatography (CH_2Cl_2/EtOAc 7:3) to give 343 mg (70%) of **8** as a white solid. R_f = 0.32 (CH_2Cl_2/EtOAc 7:3). Anal. Calcd for $C_{43}H_{86}NO_9P$: C, 65.20; H, 10.94; N, 1.77. Found: C, 65.32; H, 11.01; N, 1.83. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.17 (m, 1H, J = 5.2 Hz, CH), 4.21, 4.10 (m, 4H, POCH_2CH_2N CH_2CHCH_2), 3.78 (dd, 3H, J = 11.2 Hz, 3.6 Hz, CH_3OP), 3.56 (d, 2H, J = 5.3 Hz, CH_2CHCH_2), 3.43 (m, 4H, POCH$_2$$CH_2N$ $C_{15}H_{31}CH_2O$), 2.34 (t, 2H, J = 7.4 Hz, CH_2COO), 1.62 (quintet, 2H, β-CH_2), 1.54 (quintet, 2H, β-CH_2), 1.45 (s, 9H, $(CH_3)_3$), 1.26 (br s, 50H. 25 × CH_2), 0.88 (t, 6H, 2 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.3 (d, CH_2COO), 155.9 (NCO), 79.6 ($C(CH_3)_3$), 71.8 ($C_{15}H_{31}CH_2O$), 70.5 (CH_2CHCH_2, J = 7.4 Hz), 68.2 (CH_2CHCH_2), 67.1 (POCH_2CH_2N, J = 5.9 Hz), 66.1 (dd, CH_2CHCH_2, J = 4.9 Hz), 54.4 (CH_3OP, J = 4.8), 40.8 (POCH$_2$$CH_2N$, J = 5.1 Hz), 34.1 (CH_2COO), 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.0, 24.9, 22.5, (CH_2 aliphatic), 28.2 ($(CH_3)_3$), 14.0 (2 × CH_3).

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-*O*-methylethanolamine (9). To **8** (100 mg, 0.128 mmol) in dichloromethane (3 mL) at 0 °C was added CF_3COOH (3 mL), and the reaction mixture was stirred 35 min at 0 °C. TLC showed 100% conversion, and the solvent was removed under reduced pressure. Toluene was added to remove CF_3COOH by azeotropic distillation. The resulting viscous oil was used without purification in the next reaction. R_f = 0.05 (CH_2Cl_2/EtOAc 7:3).

***N*-Succinimide Poly(ethylene glycol)$_{350}$ Carbonate (12).** Poly(ethylene glycol)$_{350}$ (100 mg, 0.286 mmol) was dissolved in acetonitrile (1.5 mL) in a flame-dried flask under N_2. To this solution was added triethylamine (120 μL, 0.86 mmol) and disuccinimide carbonate (146 mg, 0.571 mmol). The clear solution was stirred for 4 h at room temperature and then concentrated to give the crude product as a white solid. The crude product was purified by flash chromatography (8% MeOH/CH_2Cl_2), which gave 226 mg (80%) of the title compound as a white solid. R_f = 0.54 (eluent, 10% MeOH/toluene). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.47 (ABX_2, 2H, J = 4.6 Hz, OCH_2CH_2OCOO), 3.81 (ABX_2, 2H, J = 4.6 Hz, OCH_2CH_2-OCOO), 3.72−3.61 (m, 26H, $(CH_2CH_2O)_n$), 3.59−3.51 (m, 2H, CH_3OCH_2), 3.39 (s, 3H, CH_3O), 2.85 (s, 4H, 2 × CH_2CO).

***N*-Succinimide Poly(ethylene glycol)$_{2000}$ Carbonate (13).** Poly(ethylene glycol)$_{2000}$ (1.15 g, 0.572 mmol) was dissolved in acetonitrile (4 mL) in a flame-dried flask under N_2. To this solution was added triethylamine (240 μL, 1.72 mmol) and disuccinimide carbonate (292 mg, 1.14 mmol). The clear solution was stirred for 4 h at room temperature and then concentrated to give the crude product as a white solid. The crude product was purified by flash chromatography (16% MeOH/Toluene), which gave 1.0 g (82%) of the title compound as a white solid together with an impurity of *N*-hydroxysuccinimide. R_f = 0.15 (eluent, 16% MeOH/toluene). ^{1}H NMR (300 MHz, $CDCl_3$): δ 4.47 (ABX_2, 2H, J = 4.6 Hz, OCH_2CH_2OCOO), 3.81 (ABX_2, 2H, J = 4.6 Hz, OCH_2CH_2OCOO), 3.71−3.61 (m, 180H, $(CH_2CH_2O)_n$), 3.59−3.53 (m, 2H, CH_3OCH_2), 3.38 (s, 3H, CH_3O), 2.85 (s, 4H. 2 × CH_2CO).

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-*O*-methylethanolamine Poly(ethylene glycol)$_{350}$ (10). Activated polymer **12** (126 mg, 0.256 mmol) was added to a solution of **9** (87 mg, 0.128 mmol) and Et_3N (75μL, 0.54 mmol) in dry $CHCl_3$ (5 mL), and the reaction mixture was stirred for 2 h at 40 °C. The reaction mixture was concentrated to give a yellow solid, which was redissolved in toluene (50 mL) and washed with water (10 × 10 mL) to remove uncoupled polymer. Toluene was removed under reduced pressure and the residue was purified by column chromatography (16% MeOH/toluene), resulting in 104 mg (74% over two steps) of a white solid. R_f = 0.35 (16% MeOH/toluene). >99% pure by HPLC. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.15 (m, 1H, CH), 4.28−4.04 (m, 6H, POCH_2CH_2N $NCOOCH_2$ CH_2CHCH_2), 3.76 (dd, 3H, J = 11.2 Hz 3.7 Hz, CH_3OP), 3.70−3.59 (m, 20H, $(CH_2CH_2O)_n$), 3.57−3.50 (m, 4H, $NCOOCH_2CH_2O$ CH_3OCH_2), 3.49−3.37 (m, 4H, CH_2CHCH_2 $C_{15}H_{31}CH_2O$), 3.36 (s, 3H, CH_3O), 2.32 (t, 2H, J = 7.4 Hz, CH_2COO), 1.60 (m, 2H, β-CH_2), 1.52 (m, 2H, β-CH_2), 1.26 (br s, 50H. 25 × CH_2), 0.88 (t, 6H, 2 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.2 (COO), 156.5 (NCO), 71.9 (CH_2-OCH_3), 71.8 ($C_{15}H_{31}CH_2O$), 70.9−70.1 ($(CH_2CH_2O)_n$ CH_2CHCH_2), 69.4 ($NCOOCH_2CH_2O$), 68.2 (CH_2CHCH_2), 66.8 (POCH_2CH_2N, J = 5.7 Hz), 66.1 (t, CH_2CHCH_2, J = 5.9 Hz), 64.1 ($NCOOCH_2$-CH_2O), 59.0 (CH_3O), 54.4 (CH_3OP, J = 6.0 Hz), 41.2 (CH_2NCO, J = 6.3 Hz), 34.1 (CH_2COO), 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.0, 24.9, 22.5 (CH_2 aliphatic), 13.9 (2 × CH_3).

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphoethanolamine Poly(ethylene glycol)$_{350}$ (2). Phospholipid (**10**) (88 mg, 0.081 mmol) was dissolved in 2-butanone (5 mL), and sodium iodide (122 mg, 0.81 mmol) was added. The reaction mixture was stirred for 2 h at 75 °C with the flask immersed to its neck in an oil bath, after which it was concentrated to give a yellow residue. The residue was redissolved in dichloromethane (50 mL) and washed with 1 M Na_2SO_3 (5 × 10 mL) and then with water (5 × 10 mL). The crude product was purified by column chromatography (12% MeOH/CH_2Cl_2) to give 85 mg (95%) of **2** as a white solid. R_f = 0.31 (12% MeOH/CH_2Cl_2). >99% pure by HPLC; phospholipase A_2 shows >95%. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.13 (m, 1H, CH), 4.21 (m, 2H, $NCOOCH_2$), 3.94 (m, 4H, POCH_2CH_2N CH_2CHCH_2), 3.80−3.61 (m, 22H, $(CH_2CH_2O)_n$), 3.60−3.50 (m, 4H, $NCOOCH_2CH_2O$ CH_3OCH_2), 3.48−3.27 (m, 4H, CH_2CHCH_2 $C_{15}H_{31}CH_2O$), 3.37 (s, 3H, CH_3O), 2.30 (t, 2H, J = 7.9 Hz, CH_2COO), 1.59 (m, 2H, β-CH_2), 1.51 (m, 2H, β-CH_2), 1.26 (br s, 50H. 25 × CH_2), 0.88 (t, 6H, 2 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.7 (COO), 156.9 (NCO), 72.0 (CH_2OCH_3), 71.7 ($C_{15}H_{31}CH_2O$), 70.9−69.1 ($(CH_2CH_2O)_n$ CH_2CHCH_2 $NCOOCH_2CH_2O$ CH_2CHCH_2), 64.4 (POCH_2CH_2N), 63.9 (CH_2CHCH_2), 63.1 ($NCOOCH_2CH_2O$), 59.2 (CH_3O), 41.4 (CH_2NCO), 34.4 (CH_2COO), 32.0, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.0, 24.9, 22.5 (CH_2 aliphatic), 14.0 (2 × CH_3).

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phospho-*O*-methylethanolamine Poly(ethylene glycol)$_{2000}$ (11). Activated polymer **13** containing *N*-hydroxysuccinimide (1 g, 0.467 mmol) was added to a solution of **9** (87 mg, 0.128 mmol) and Et_3N (150μL, 1.076 mmol) in dry $CHCl_3$ (10 mL), and the reaction mixture was stirred for 2 h at 40 °C. The reaction mixture was concentrated to give a yellow solid, which was redissolved in dichloromethane (50 mL) and was washed with water (10 × 10 mL). Dichloromethane was removed under reduced pressure and the residue was purified by column chromatography (8% MeOH/CH_2Cl_2), resulting in a white solid, which was used without further purification in the next reaction. R_f = 0.45 (10% MeOH/CH_2Cl_2).

1-*O*-Hexadecyl-2-palmitoyl-*sn*-glycero-3-phosphoethanolamine Poly(ethylene glycol)$_{2000}$ (3). Phospholipid **11** (0.128 mmol) was dissolved in 2-butanone (50 mL), and sodium iodide (180 mg, 1.28 mmol) was added. The reaction mixture was stirred 2 h at 75 °C with the flask immersed to its neck in an oil bath, after which the yellow solution was concentrated to give a yellow residue. The residue was redissolved in dichloromethane (40 mL) and washed with 1 M Na_2SO_3 (5 × 10 mL) and then with water (5 × 10 mL). The crude product was purified by column chromatography (12% → 50% MeOH/CH_2Cl_2) to give 245 mg (70% over three steps) of **3** as a white solid. R_f = 0.25 (10% MeOH/CH_2Cl_2). >99% pure by HPLC; phospholipase A_2 shows >95% conversion. ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.13 (m, 1H, CH), 4.21 (m, 2H, NCOOCH_2), 4.00−3.86 (m, 4H, POCH_2CH_2N CH_2CHCH_2), 3.78−3.60 (m, 168H, $(CH_2CH_2O)_n$), 3.60−3.50 (m, 4H, $NCOOCH_2CH_2O$ CH_3OCH_2), 3.48−3.28 (m, 4H, CH_2CHCH_2 $C_{15}H_{31}CH_2O$), 3.38 (s, 3H, CH_3O), 2.29 (t, 2H, J = 6.9 Hz, CH_2COO), 1.59 (m, 2H, β-CH_2), 1.51 (m, 2H, β-CH_2), 1.26 (br s, 50H, 25 × CH_2), 0.88 (t, 6H, 2 × CH_3). ^{13}C NMR (75 MHz, $CDCl_3$): δ 173.4 (COO), 156.7 (NCO), 71.9 (CH_2OCH_3), 71.5 ($C_{15}H_{31}CH_2O$), 70.9−69.0 ($(CH_2CH_2O)_n$ CH_2CHCH_2 $NCOOCH_2CH_2O$ CH_2CHCH_2), 64.0 ($POCH_2CH_2N$), 63.7 (CH_2CHCH_2), 63.1 ($NCOOCH_2CH_2O$), 59.2 (CH_3O), 42.3 (CH_2NCO), 34.3 (CH_2COO), 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 26.0, 24.9, 22.5 (CH_2 aliphatic), 14.0 (2 × CH_3).

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