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Medicinal Chemistry

Drug Delivery by an Enzyme-Mediated Cyclization of a Lipid Prodrug with Unique Bilayer-Formation Properties**

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The development of advanced biomaterials and drug-delivery systems has had a significant impact on our ability to treat severe diseases.[1] In the design of nanoparticle-based drug-delivery systems for intravenous administration, the objective is to create a particle that is stable during blood circulation, accumulates to a high degree in the diseased tissue, and is able to release the drug after accumulation at a rate that matches the pharmacodynamic profile of the drug.[2,3] Current strategies include the use of lipid-based micelles and liposomes,[1,3] hydrocolloids,[4] and more recently, polymersomes.[5] Herein, we report the development of a novel drug-delivery system, whereby lipid-based prodrugs are formulated as liposomes, and drug release is affected by an enzymatically triggered cyclization reaction. We illustrate the idea with secretory phospholipase A_2; however, the principle can equally well be used with other enzymes, for example, matrix metalloproteases.

The use of liposomes for targeted drug delivery to tumor tissue has attracted increasing attention in the last decade, with a particular focus on the development of trigger mechanisms for site-specific drug release.[2,6] We have been particularly interested in liposomal drug-delivery systems activated by the enzyme secretory phospholipase A_2 ($sPLA_2$).[7] This enzyme is overexpressed in cancerous and inflammatory tissue and is present in the extracellular matrix.[8] $sPLA_2$ hydrolyzes the ester group in the *sn*-2 position of glycerophospholipids and shows dramatically increased activity when absorbed onto a lipid membrane–water interface, such as a liposome.[2] We have shown previously that it is possible to construct long circulating liposomes that can carry encapsulated drugs to cancerous tissue and release the drugs upon activation by human type IIA $sPLA_2$ ($sPLA_2$-IIA).[2] However, it would be very useful if the activity and elevated levels of $sPLA_2$-IIA in cancerous tissue could be exploited to activate prodrugs specifically at the diseased target site. To investigate this possibility, we studied the versatility of $sPLA_2$ for the hydrolysis of different lipid structures.[9] This study showed that $sPLA_2$ tolerates a number of structural changes in the *sn*-1 position of glycerophospholipids. On the basis of these results, we envisioned that a drug could be attached covalently to the *sn*-1 position of a glycerophospholipid derivative in such a way that the hydroxy group liberated at the *sn*-2 position by $sPLA_2$ hydrolysis of the lipid prodrug would react subsequently with an ester group at the *sn*-1 position to form a five-membered lactone and release the carried drug (Scheme 1).

To investigate this new concept in liposomal drug delivery, we used the anticancer drug capsaicin[10] as a model drug. The capsaicin prodrug **8** was synthesized from the commercially available lactone **1** (Scheme 2). Benzyl protection of lactone **1**, followed by reduction with lithium borohydride, gave diol **3**. The primary alcohol group of diol **3** was protected with a silyl group, and the secondary alcohol was coupled with octadecanoic acid. After removal of the benzyl group of the resulting ester **4** by hydrogenolysis, the phosphate head group was introduced by the phosphoramidite method[11] with 5-phenyl-1*H*-tetrazole as the proton donor.[12] The silyl protecting group in phosphate **5** was removed with TBAF, and the alcohol generated was oxidized to the corresponding acid, which was coupled directly to capsaicin without intermediate purification. Treatment of the resulting phosphate **7** with trimethylamine, followed by exposure to acidic conditions, led to the removal of the protecting groups on the phosphate moiety to afford the desired prodrug **8**.

The capsaicin prodrug **8** was hydrated in a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.5). Its thermodynamic phase behavior was investigated by differential scanning calorimetry (DSC); however, no phase transitions were detected in the range 10–70 °C. The lipid solution was investigated by dynamic light scattering (DLS), which revealed a population of vesicles with an average diameter of 66 nm and a low polydispersity. Furthermore, cryo-TEM images revealed that prodrug **8** self-aggregates into small unilamellar vesicles (SUVs; Figure 1). The vesicles had a very uniform appearance, and no multilamellar structures were observed, which was surprising, as it

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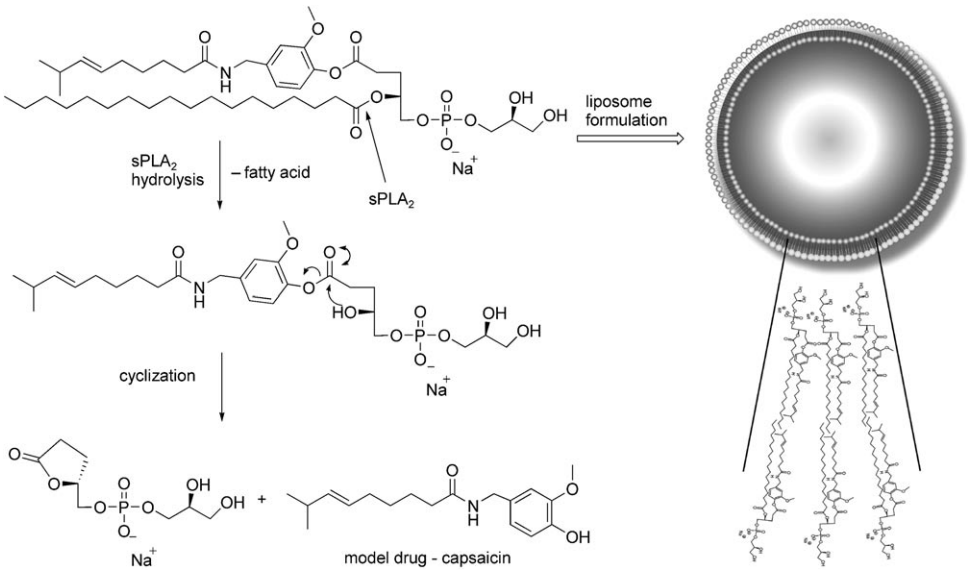
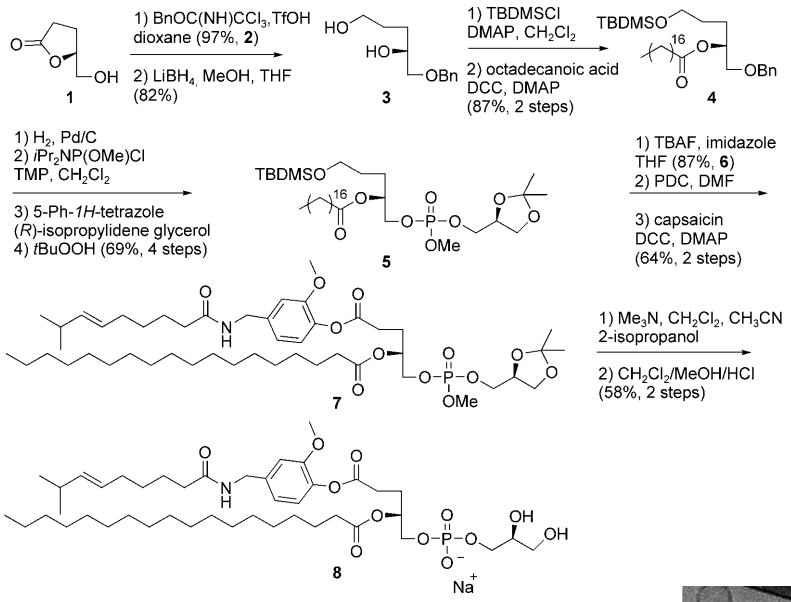


Scheme 1. The lipid prodrug forms the liposome membrane and is hydrolyzed by $sPLA_2$ to liberate the drug after a cyclization reaction.



Scheme 2. Synthesis of the capsaicin prodrug **8**. Bn = benzyl, DCC = *N*,*N*′-dicyclohexylcarbodiimide, DMAP = 4-dimethylaminopyridine, DMF = *N*,*N*-dimethylformamide, PDC = pyridinium dichromate, TBAF = tetrabutylammonium fluoride, TBDMS = *tert*-butyldimethylsilyl, Tf = trifluoromethanesulfonyl, TMP = 2,2,6,6-tetramethylpiperidine.

was not necessary to extrude or sonicate the lipid solution. We have never previously encountered natural or synthetic lipids that exclusively form uniform bilayer vesicles directly upon dispersion in a buffer, as observed for prodrug **8**, nor have we been able to find reports of such lipid behavior in the literature. An understanding of the basis of this behavior would be of the highest interest in liposomal and drug-delivery research, as it would dramatically change the requisites for liposomal preparation for medical use. We speculate that this behavior may be a consequence of the phenyl group of capsaicin lying in the interface region in the lipsome bilayer, possibly in combination with the high net negative charge of the liposomes.

The absence of a main phase transition in the scanned temperature range indicates that the phospholipids do not seem to be organized in a highly ordered fashion in the lipid bilayer, possibly as a consequence of the reduced hydrophobicity of capsaicin relative to that of the fatty-acid chain in naturally occurring phospholipids. To further characterize the SUVs, we carried out calcein-encapsulation studies, which revealed that the prodrug **8** does not form vesicles with the capacity to encapsulate water-soluble compounds. Finally, an attempt to determine the critical aggregation concentration (CAC) of the prodrug by isothermal titration calorimetry (ITC) was not successful, as we found that the CAC value was below the ITC detection limit and thus below 10^{-8} M. From this result, we conclude that the prodrug will be present exclusively as aggregated structures.

The vesicles composed of the capsaicin prodrug were investigated for their susceptibility to $sPLA_2$ activation and degradation. We used purified snake-venom $sPLA_2$ (*Agkistrodon piscivorus piscivorus*), which is known to be active towards a large variety of substrates; we have

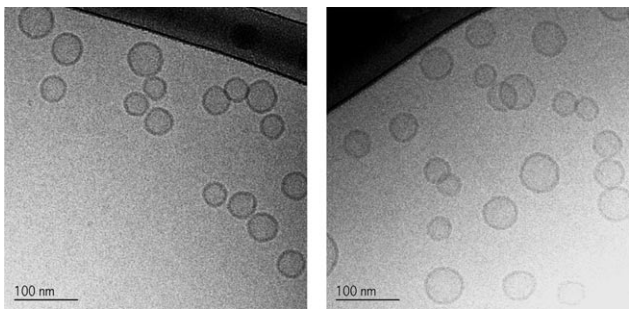


Figure 1. Cryo-TEM images (two representative images shown) clearly revealed that the prodrug lipids formed small unilamellar vesicles (SUVs); the bilayer thickness was measured to be approximately 4 nm. The specimens were prepared as thin liquid films (< 0.3 μm thick) on lacey carbon films (black area) supported by a copper grid and plunged into liquid ethane at −180 °C. A few liposomes are lying on top of one another owing to the thickness of the amorphous water film.

previously observed that the substrate specificity of this enzyme is comparable to that of human $sPLA_2$-IIA.[13a] We used static light scattering at 90° as an indirect measurement of enzymatic hydrolysis and used HPLC and MALDI-TOF to quantify the degree of hydrolysis. After the addition of $sPLA_2$, there is a dramatic change in the static light scattering (Figure 2a). This result strongly indicates a change in vesicle

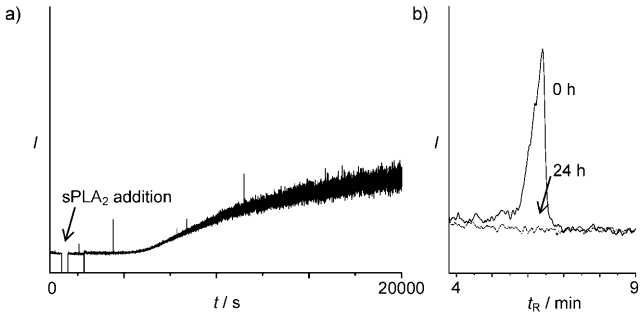


Figure 2. a) Static light scattering at 90° as a measurement of the action of $sPLA_2$ on vesicles composed of the capsaicin prodrug. b) HPLC chromatogram showing the amount of prodrug before the addition of $sPLA_2$ (0 h) and the amount of prodrug 24 h after the addition of $sPLA_2$ (I = intensity, t_R = retention time).

morphology as a consequence of $sPLA_2$ hydrolysis. HPLC also indicated hydrolysis of the lipids by $sPLA_2$ (Figure 2b).

The main focus of the present study was to show that capsaicin can be released through $sPLA_2$ activation of the prodrug. The hydrolysis experiments were therefore analyzed further by MALDI-TOF. For the measurements, we used a DHB-KCl matrix, which did not interfere with the regions of interest for the prodrug and free capsaicin (Figure 3a–c). Figure 3c shows the MALDI-TOF measurements before the addition of snake-venom $sPLA_2$; it is evident that free capsaicin is not present in the samples. The prodrug is therefore stable in the vesicles and does not degrade in the buffer solution for at least two weeks after the preparation of the vesicles.

Snake-venom $sPLA_2$ was added to the vesicles, and the samples were analyzed at different time intervals. The MALDI-TOF data show clearly that the prodrug is hydrolyzed after $sPLA_2$ addition. The rate of hydrolysis shows that the prodrugs are good substrates for $sPLA_2$. Furthermore, it is evident from the MALDI-TOF measurements in the region with the molecular weight of capsaicin that capsaicin is released from the phospholipids after hydrolysis. This result is highly interesting, as it shows that the cyclization takes place at a relatively fast rate. Moreover, it was not possible to detect any lysophospholipids (lipids that had not cyclized after hydrolysis by the enzyme), which indicates that the cyclization is highly favored.

We also investigated the activity of human $sPLA_2$-IIA towards the prodrug vesicles by adding human $sPLA_2$-IIA to the vesicle solution and analyzing the mixture by MALDI-TOF during the experiment (Figure 3d,e). The measurements showed that the capsaicin prodrugs are indeed hydrolyzed by human $sPLA_2$-IIA and that capsaicin is released after

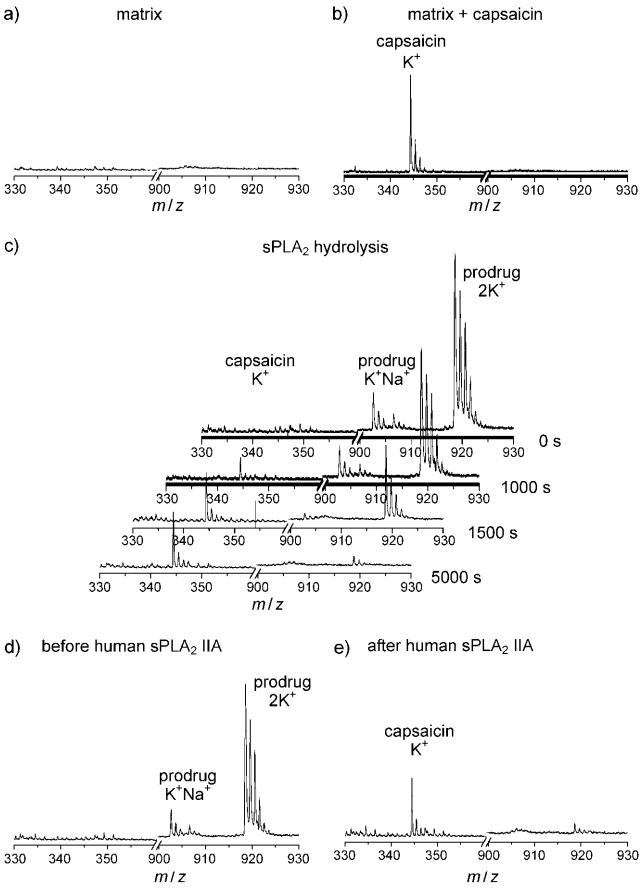


Figure 3. MALDI-TOF measurements of the hydrolysis of capsaicin prodrug vesicles by $sPLA_2$. a) Matrix noise in the regions of interest. b) Matrix and capsaicin, K^+. c) The amount of prodrug and capsaicin before (0 s) and 1000 s, 1500 s, and 5000 s after the addition of $sPLA_2$. d,e) MALDI-TOF measurement of the hydrolysis of prodrug vesicles by human $sPLA_2$-IIA before (d) and 24 h after (e) the addition of the enzyme.

enzymatic activation. The amount of capsaicin released through hydrolysis by human $sPLA_2$-IIA was found to be $(90 \pm 11)\%$ ($n = 3$) after 24 h, and no uncyclized lysophospholipid was detected. We used $sPLA_2$-IIA from human tear fluid as a convenient source,[13a] but verified that the hydrolysis of the prodrug in conditioned medium from Colo 205 colon cancer cells (Colo 205 cells secrete $sPLA_2$-IIA), with an enzyme concentration of $(75 \pm 20)\ ng\,mL^{-1}$,[13b] also led to full conversion of the prodrug within 24 h.

In summary, we have developed a novel class of lipid-based prodrugs with unique properties. The prodrugs spontaneously form SUVs in water and upon enzymatic activation release the drug by a cyclization reaction. We illustrated the concept by using secretory phospholipase A_2 as the prodrug activator; however, the cyclization principle can equally well be used with other disease-associated enzymes. We expect that the SUV-formation properties of prodrug **8** is specific to this structure, and that prodrugs of drugs other than capsaicin would not express this unique behavior. However, even if new prodrugs do not form liposomes but other types of aggregates, the hydrolysis of these aggregates by $sPLA_2$ would still be

possible and would result in drug release by the described cyclization principle. Investigations into anticancer efficacy and the attachment of other drug molecules are now in progress.

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