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Photocleavable Membranes

Triggered Liposomal Release through a Synthetic Phosphatidylcholine Analogue Bearing a Photocleavable Moiety Embedded within the *sn*-2 Acyl Chain

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Abstract: Liposomes represent promising carriers for drug delivery applications. To maximize this potential, there has been significant interest in developing liposomal systems encapsulating molecular cargo that are highly stable until their contents are released remotely in a controlled manner. Herein, we describe the design, synthesis, and analysis of a photocleavable analogue of the ubiquitous lipid phosphoatidylcholine (PC) for the development of highly stable and controllable photodisruptable membranes. Our strategy was to develop a lipid that closely mimics the structure of PC to optimize favorable properties including biocompatibility and stability of subsequent liposomes when mixed with lipids possessing a broad range of physicochemical properties. Thus, **NB-PC** was designed, which contains a photocleavable 2-nitrobenzyl group embedded within the acyl chain at the *sn*-2 position. Following the synthesis of **NB-PC**, liposome disruption efficacy was evaluated through photolysis studies involving the detection of nile red release. Studies performed using a range of liposomes with different percentages of **NB-PC**, PC, phosphatidylethanolamine (PE), cholesterol, and polyethylene glycol-PE (PEG-PE) demonstrated minimal background release in controls, release efficacies that correlate directly with the amount of **NB-PC** incorporation, and that release is only minimally impacted by the inclusion of the lipids PE and cholesterol that possess disparate properties. These results demonstrate that the **NB-PC** system is a highly stable, flexible, and tunable system for photoinitiated release from liposomal systems.

Introduction

The development of disease-specific delivery vesicles for drug administration represents a critical emerging avenue for therapeutic intervention. Current research in this field faces several obstacles, including rapid clearance of drugs and drug carriers from the bloodstream, difficulty obtaining localization at the diseased site, and challenges associated with efficient triggering of drug release at the diseased area. Liposomes have emerged as promising biocompatible chaperones for medicinal agents in which properties can be tuned to circumvent these challenges.[1] For example, liposomal surfaces can be decorated with polyethylene glycol (PEG) chains, creating "stealth" liposomes with significantly enhanced circulation times.[2] In addition, chemical moieties can be introduced onto the liposome surface to aid in the targeting of these containers to diseased cells.[3] In order to maximize the potential for liposomal drug delivery, it is of significant interest to control the release of molecular contents such that this occurs on a relatively quick timescale at the target location. In the triggering of liposomal content release, approaches exploiting light,[4] redox conditions,[5] enzymes,[6] temperature,[7] and pH[8] changes have been developed. As opposed to passive stimuli that are dependent on internal factors, such as temperature and pH changes in the local environment, light-mediated release is beneficial as it can be utilized in an external manner with control over both positioning and duration.

Light-mediated control of organic molecules is typically achieved using a photocleavable protecting group. There are many existing photolabile groups, such as dithianes,[9] disulfides,[10] coumarin,[11] diazirine,[12] and 4-hydroxyphenacyl,[13] as well as the 2-nitrobenzyl group,[14] the most widely studied moiety in which near UV irradiation leads to decomposition of the benzylic position to yield an aldehyde and a leaving group. Several strategies have been reported involving disruption of liposomes or photolabile amphiphiles. Zhang and Smith developed a phosphatidylethanolamine (PE) analogue bearing a nitrobenzyl protecting group on the amino moiety of the headgroup.[15] Upon irradiation of liposomes bearing 50 mol% PE analogue, release was achieved, due to the phase change driven by the preference of PE for the inverted hexagonal phase. Nagasaki and co-workers utilized cationic amphiphiles bearing two hydrophobic chains linked through a nitrobenzyl group to enhance transfection efficiency of the pGL3 plasmid into COS-1 cells.[16]

Chandra and co-workers designed and synthesized several amphiphiles containing a nitrobenzyl moiety separating a long hydrophobic tail from a polar amino acid headgroup.[17] This system was used for successful release of encapsulated fluores-

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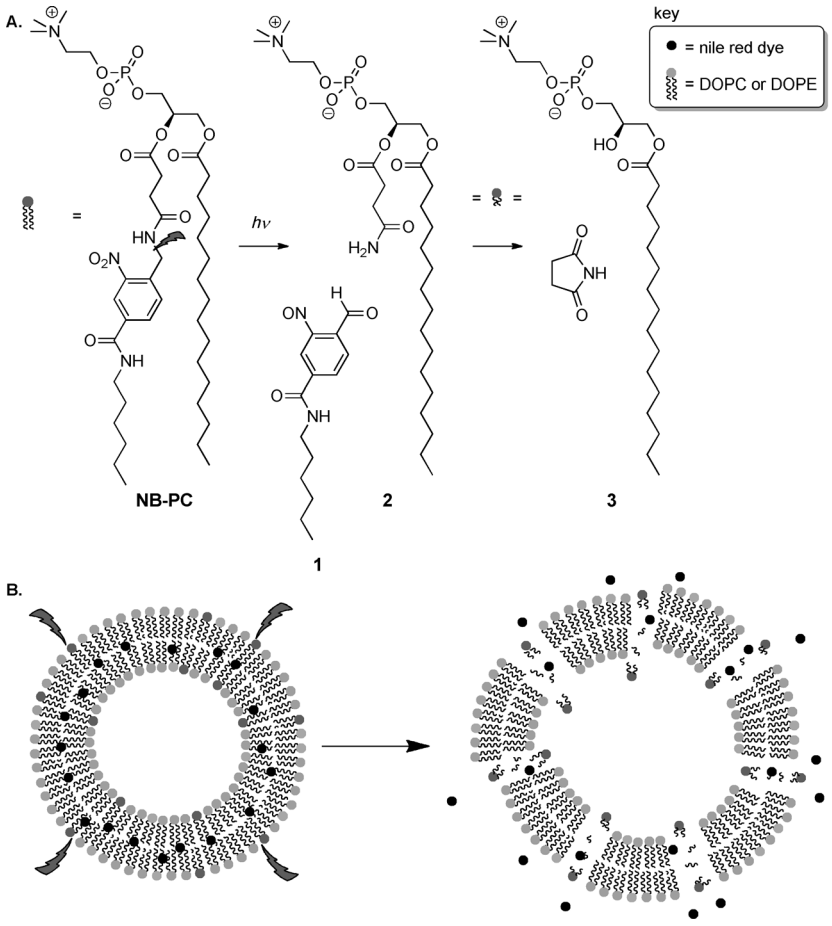
cein from PC liposomes containing 5% photocleavable amphiphile. Similar amino acid-based amphiphiles were described by Subramaniam and co-workers in which 6-bromo-7-hydroxycoumarin-4-methyl (Bhc) was employed as both the hydrophobic backbone and the photocleavable moiety.[18] Recently, Dong and co-workers used polymerized vesicles containing lipids bearing a cleavable moiety for release using two-photon absorption.[19] Photodisruption of liposomes has also been carried out by dithiane lipids, as described by Wan et al.,[9] which release the phosphocholine headgroup under UV light. In addition, Liang and co-workers have incorporated the azobenzene functional group in a silica-modified liposome, or cerasome, to induce dye permeability induced through *cis*-to-*trans* isomerism.[20] Lipid cross-linking initiated by irradiation has also been employed as a strategy for photochemical membrane destabilization and release.[21] It should be noted that synthetic lipids bearing photocleavable moieties have also been developed as caged analogues of biologically active lipids such as diacylglycerol,[22] phosphatidic acid,[23] and phosphatidylinositol-(3,4,5)-trisphosphate.[24]

While this prior work has resulted in a range of successful strategies for triggered liposome release, further study is likely needed to optimize properties associated with delivery. In particular, the development of analogues that bear close resemblance to robust and commonly occurring lipids would seem to be ideal for enhancing biocompatibility and thus stability during circulation. For this reason, we elected to pursue a photocleavable analogue of PC, since this lipid is the primary component of cellular membranes. Furthermore, our design incorporates the photocleavable group within a lipid acyl chain, leaving the natural PC headgroup intact. In this way, our analogues were designed to form stable membrane bilayers with naturally occurring aqueous interfaces to maximize stability and biocompatibility. Herein, we describe the design and synthesis of photocleavable PC analogue **NB-PC** along with photolysis studies aimed at characterizing the release efficiency of this compound when incorporated into liposomes composed of different lipids that affect the properties of the membrane bilayer.

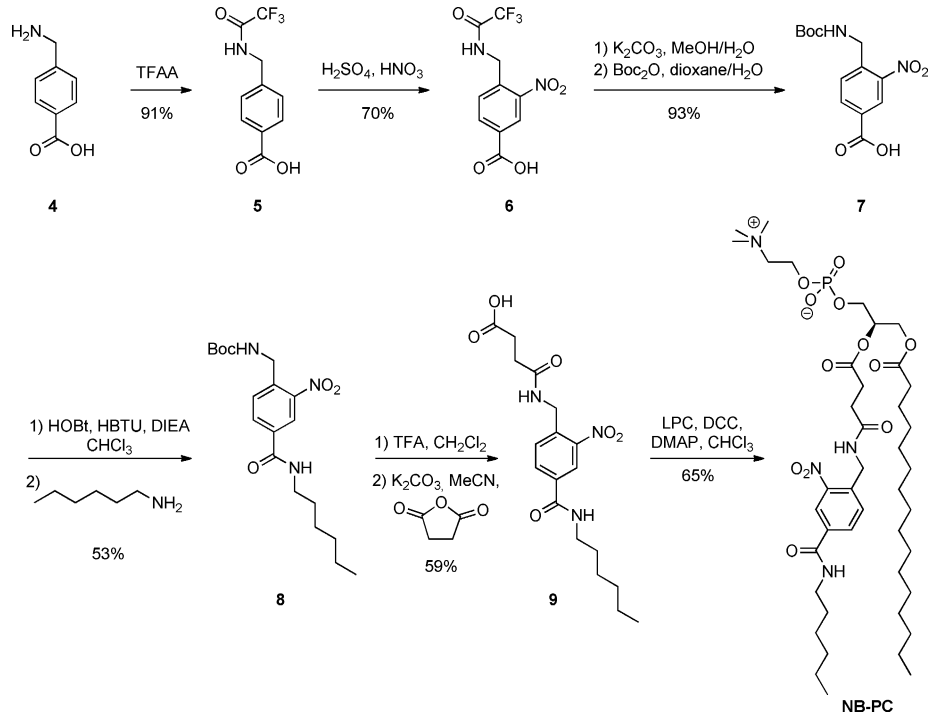
Results and Discussion

Our design for a biologically inert photocleavable analogue (**NB-PC**) of the common structural lipid PC is depicted in Scheme 1 A. This compound includes a 2-nitrobenzyl moiety embedded within the *sn*-2 acyl chain of PC in order to generate a stable bilayer lipid in which membrane properties would be significantly modulated through photocleavage. This acyl chain also includes a succinyl linker to the PC headgroup as well as a hexyl group to add hydrophobic character at the terminus of the acyl chain. This was designed such that irradiation would cause the breakage of the bond between the benzylic position and amide nitrogen, releasing the hydrophobic terminus of the acyl chain in the form of aldehyde **1**, as well as a shortened PC analogue bearing a polar terminal amide functional group (**2**). Furthermore, it was envisaged that this release could stimulate the amide nitrogen to attack the succinyl ester group, leading to the release of succinimide and thus the further truncation of the lipid structure, potentially producing the nonbilayer lipid lysophosphatidylocholine (LPC, **3**).

The synthesis of **NB-PC** was completed as described in Scheme 2. Towards this end, Boc-(aminomethyl)nitrobenzoic



Scheme 1. Approach for membrane disruption using photocleavable PC analogue **NB-PC**. a) Irradiation of **NB-PC** is expected to directly produce aldehyde **1** and amide **2** through C–N bond breakage. Subsequent intramolecular cyclization could also produce succinimide and LPC product **3**. b) Cartoon depicting the disruption of membrane structure upon photolysis of **NB-PC**.



Scheme 2. Synthesis of **NB-PC**. 4-Aminomethylbenzoic acid (**4**) was protected with a trifluoroacetate group to **5**, nitrated to **6**, and underwent protecting group exchange to **7** following a previously reported procedure.[17a] Coupling with hexylamine to **8** was followed by chain extension through nucleophilic attack of succinic anhydride to form photocleavable fatty acid **9**. Finally, coupling onto the *sn*-2 chain of LPC (**3**) yielded **NB-PC**. TFAA = trifluoroacetic anhydride.

acid **7** was first synthesized from aminomethylbenzoic acid **4** as previously described[17a] through amine protection to **5**, nitration to **6** and protecting group exchange to **7**. Next, an amide bond coupling reaction with hexylamine was used to introduce the hydrophobic terminus of compound **8**. The succinyl linker was then introduced through deprotection of the Boc protecting group of **8**, followed by opening of succinic anhydride to produce photocleavable fatty acid **9**. Finally, this fatty acid was introduced onto commercial palmitoyl-lysophosphatidylcholine through a coupling reaction in a manner similar to a prior report.[25]

Following the completion of the synthesis of **NB-PC**, we set out to characterize the efficacy of this compound for photoinitiated release of encapsulated cargo with properties similar to typical drugs. In doing so, we exploited an assay involving the release of nile red reported by Liang and co-workers (Scheme 1 B).[20] Nile red mimics the properties of common drugs by inserting into the hydrophobic core of the membrane bilayer interior, and thus the liposome solubilizes the encapsulated dye. Upon triggered release, the nile red is rendered insoluble, which can be detected by the decrease in emission of the solution. In studies, one of the benefits of using an analogue of the robust bilayer lipid PC is that this compound can be incorporated into liposomes at a broad range of percentages to form stable membranes. As such, Figure 1 shows the release of nile red from liposomes composed entirely of either commercially available dioleyl-PC (DOPC) or photocleavable analogue **NB-PC**. In these studies, liposomes were generated using standard procedures including hydration, extrusion with a 200 nm filter, and characterization using dynamic light scattering (DLS). After this, the resulting liposomes were irradiated with 350 nm light, during which nile red emission at 612 nm was tracked with excitation at 595 nm. As can be seen in Figure 1, irradiation of **NB-PC** led to a significant time-dependent decrease (≈80%) in nile red emission, which was attributed to fluorophore release over a period of approximately one hour. These data were then fitted with a kinetic decay equation, providing figures including fraction of original fluorescence (y_0), first order rate constant (k), half-life ($t_{1/2}$) fit parameters (A) and correlation (R^2) shown in Table 1 (see Experimental Section for further details). In control studies, liposomes composed of DOPC that were irradiated (light) and untreated (dark)

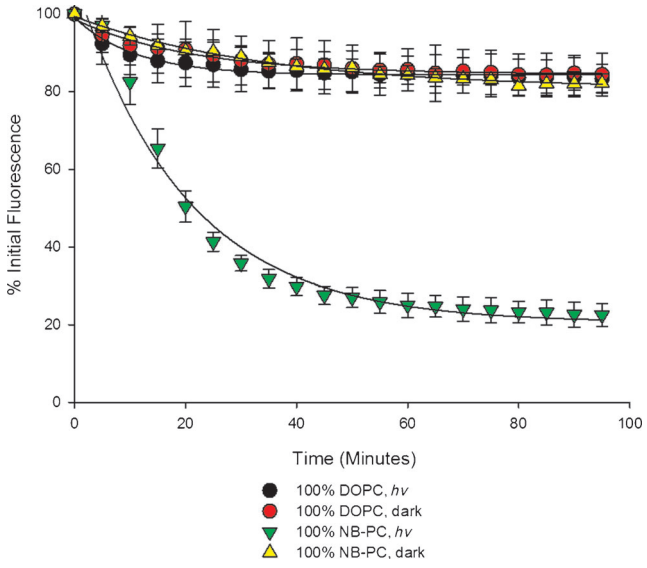


Figure 1. Nile red release upon irradiation of liposomes containing 100% **NB-PC.** Photocleavage led to significant release from **NB-PC** liposomes compared to controls including **NB-PC** left in the dark and irradiation of DOPC liposomes.

as well as those composed of **NB-PC** that were not photolyzed (dark) all led to minimal decreases in nile red emission (≈16–19%), confirming that release was directly caused by liposome photolysis and that this only occurred in the presence of compound **NB-PC**. These studies additionally indicate that photo-

Table 1. Data resulting from curve fitting of release from **NB-PC** liposomes and controls.

Sample	**NB-PC** [%]	DOPC [%]	y_0	k [s^{-1}]	$t_{1/2}$ [min]	A	R^2
Light	100	0	0.2053	8.45E-04	13.7	0.8848	0.9628
Light	0	100	0.8432	1.53E-03	7.6	0.1481	0.4233
Dark	100	0	0.8074	4.83E-04	23.9	0.1863	0.8597
Dark	0	100	0.8454	7.93E-04	14.6	0.1412	0.3813

Table 2. Data resulting from curve fitting of release from liposomes consisting of 10–100% **NB-PC** mixed with DOPE and DOPC.

Sample	**NB-PC** [%]	DOPE [%]	DOPC [%]	y_o	k [s^{-1}]	$t_{1/2}$ [min]	A	R^2
Light	10	50	40	0.6450	1.35E-03	8.6	0.3592	0.9569
Light	25	50	25	0.5180	1.13E-03	10.2	0.4980	0.9614
Light	50	50	0	0.3845	7.55E-04	15.3	0.6302	0.9853
Light	100	0	0	0.2053	8.45E-04	13.7	0.8848	0.9628
Dark	10	50	40	0.8278	1.08E-03	10.7	0.1628	0.7013
Dark	25	50	25	0.8725	9.43E-04	12.2	0.1194	0.8070
Dark	50	50	0	0.8738	6.95E-04	16.6	0.1159	0.4473
Dark	100	0	0	0.8074	4.83E-04	23.9	0.1863	0.8597

bleaching of the dye is not occurring since light and dark controls yielded comparable results.

With this successful result, we next set out to characterize the effects of lipid composition on release efficacy to determine the flexibility of the system. In the next batch of studies, we decided to include 50% dioleylphosphatidylethanolamine (DOPE) in the liposomes, which may affect release due to the nonbilayer properties of this lipid.[15,26] Thus, liposomes containing DOPE fixed at 50%, varying **NB-PC** at 10, 25, and 50%, and the remaining percentage filled in with DOPC were next analyzed. Data from liposomes containing 100% **NB-PC** are also included with these results for the sake of comparison. As can be seen in Figure 2 and Table 2, the extent of nile red release was found to directly correlate with the percentage of **NB-PC** included in the liposome, and dark controls once again yielded minimal release. Inclusion of 10, 25 and 50% of **NB-PC** resulted in ≈36, 48 and 62% decreases in nile red emission, respectively, all lower than liposomes completely composed of **NB-PC** (≈80%). These data show that release can be carried out using a broad range of percentages of **NB-PC** within liposomes, and that the percentage can be used to tune the release properties of the vesicles. It should also be noted that variations in the percentage of **NB-PC** did not have as significant of an effect on background release, as dark controls ranged from ≈12–19% emission decrease, indicating that incorporation of **NB-PC** does not destabilize the membrane bilayer.

We next set out to vary the percentage of DOPE in liposomes used for studies, while keeping the percentage of **NB-PC** fixed. This series of studies was explored to evaluate how DOPE inclusion would affect release due to the nonbilayer properties of this lipid, which may be pronounced after irradiation of **NB-PC** and release of photolysis products, by increasing the percentage of DOPE in the product liposomes. As can be seen in Figure 3 and Table 3, the effect of DOPE variation was less pronounced compared to that of **NB-PC** percentages. Liposomes with fixed percentages of **NB-PC** (50%) and DOPE at 0, 10, 25, and 50% led to emission decreases of ≈68, 67, 66, and 62%, respectively. The extent of release in control liposomes was comparable to prior studies involving the variation of **NB-PC**. These data suggest that DOPE inclusion does not pro-

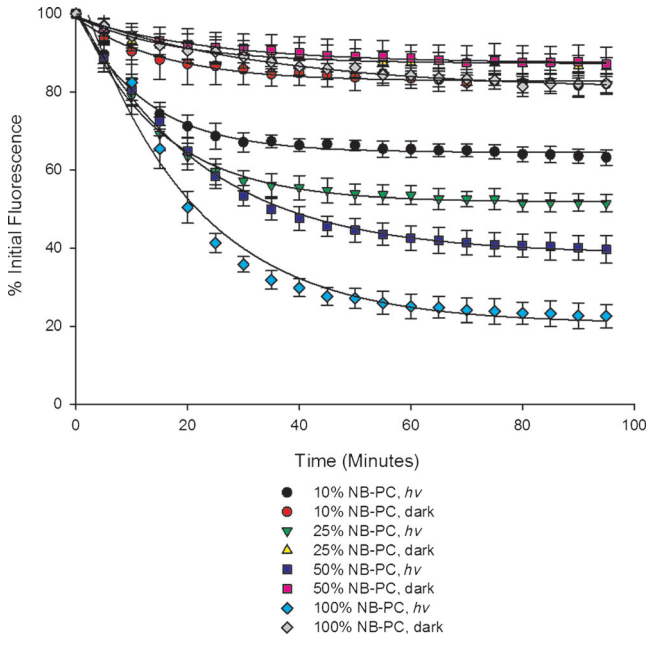


Figure 2. Nile red release upon irradiation of liposomes containing 10–100% **NB-PC** mixed with DOPE and DOPC. Release efficacy was found to increase directly with the percentage of **NB-PC**, while controls remained relatively static.

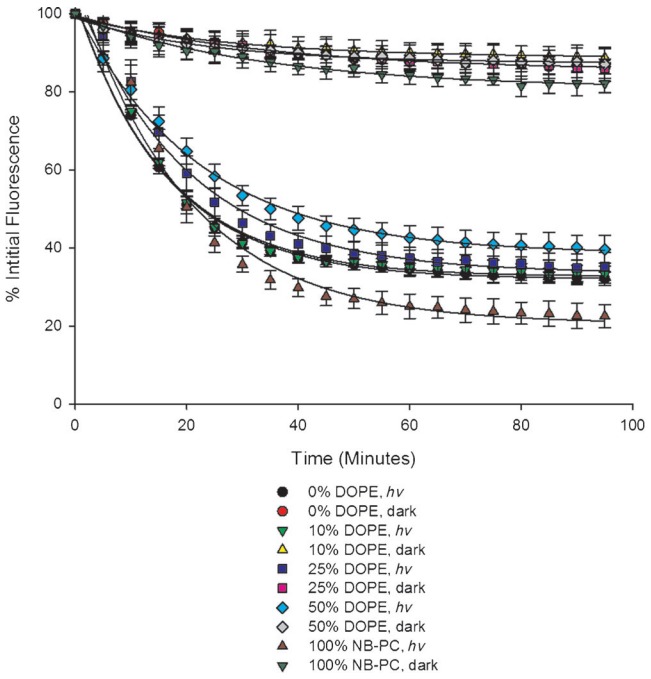


Figure 3. Nile red release upon irradiation of liposomes containing 50% **NB-PC** mixed with 0–50% DOPE along with DOPC. Release efficacy was not significantly modified by large changes in the percentage of DOPE.

Table 3. Data resulting from curve fitting of release from liposomes consisting of 50% **NB-PC** mixed with 0–50% DOPE along with DOPC.

Sample	**NB-PC** [%]	DOPE [%]	DOPC [%]	y_o	k [s^{-1}]	$t_{1/2}$ [min]	A	R^2
Light	50	0	50	0.3202	1.02E-03	11.3	0.7140	0.9877
Light	50	10	40	0.3268	1.03E-03	11.2	0.7112	0.9844
Light	50	25	25	0.3358	8.43E-04	13.7	0.7226	0.9616
Light	50	50	0	0.3845	7.55E-04	15.3	0.6302	0.9853
Light	100	0	0	0.2053	8.45E-04	13.7	0.8848	0.9628
Dark	50	0	50	0.8542	4.80E-04	24.1	0.1418	0.8591
Dark	50	10	40	0.8877	5.95E-04	19.4	0.1037	0.6430
Dark	50	25	25	0.8074	4.83E-04	23.9	0.1863	0.8597
Dark	50	50	0	0.8738	6.95E-04	16.6	0.1159	0.4473
Dark	100	0	0	0.8074	4.83E-04	23.9	0.1863	0.8597

Table 4. Data resulting from curve fitting of release from liposomes consisting of 50% **NB-PC** mixed with 0–50% cholesterol along with DOPC.

Sample	**NB-PC** [%]	Chol [%]	DOPC [%]	y_o	k [s^{-1}]	$t_{1/2}$ [min]	A	R^2
Light	50	0	50	0.3202	1.02E-03	11.3	0.7140	0.9706
Light	50	10	40	0.3024	1.00E-03	11.6	0.7350	0.9827
Light	50	25	25	0.3189	9.42E-04	12.3	0.7241	0.9829
Light	50	50	0	0.2821	9.73E-04	11.9	0.7808	0.9589
Light	100	0	0	0.2053	8.45E-04	13.7	0.8848	0.9628
Dark	50	0	50	0.8542	4.80E-04	24.1	0.1418	0.8591
Dark	50	10	40	0.8718	5.58E-04	20.7	0.1168	0.5715
Dark	50	25	25	0.8564	4.97E-04	23.3	0.1281	0.5379
Dark	50	50	0	0.8707	3.07E-04	37.7	0.1211	0.6046
Dark	100	0	0	0.8074	4.83E-04	23.9	0.1863	0.8597

foundly affect triggered release and further point to the versatility of this compound, since significant variation in the properties of the lipids incorporated into liposomes does not have a great effect on release.

To further characterize the tolerance of this system for different lipids with varying properties, we next sought to determine how release is affected by the inclusion of cholesterol, which increases the fluidity and stability of membranes. As shown by the data in Figure 4 and Table 4, inclusion of cholesterol at 0, 10, 25 and 50%, with **NB-PC** fixed at 50%, led to nile red emission decreases of ≈68, 70, 68 and 72%, respectively. Thus, once again broad fluctuations in membrane properties caused by the inclusion of cholesterol did not significantly affect the ability of **NB-PC** to induce the release of nile red from liposomes.

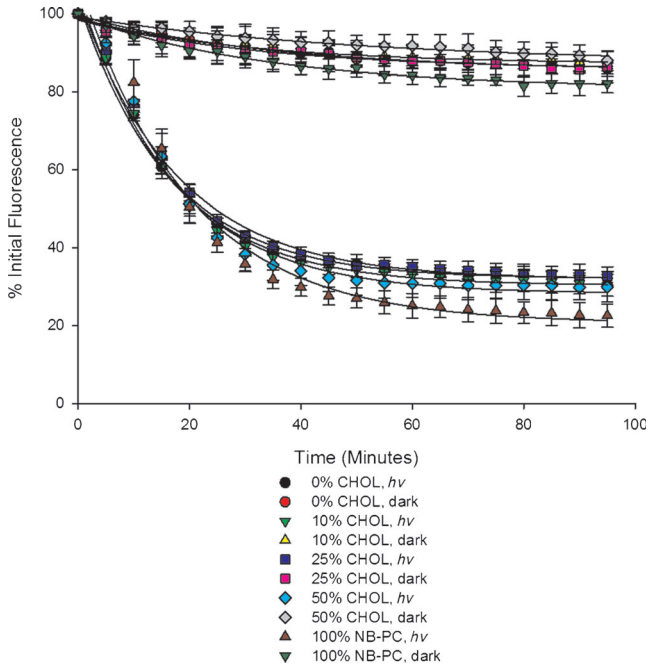


Figure 4. Nile red release upon irradiation of liposomes containing 50% **NB-PC** mixed with 0–50% cholesterol (CHOL) along with DOPC. Release efficacy was not significantly modified by large changes in the percentage of cholesterol.

PEGylated lipids are commonly added to liposomes to increase circulation in samples used for clinical applications. As a result, we next sought to assess whether the inclusion of such lipids affects release in this system. Towards this end, liposomes composed of 15% distearoyl-PEG(2000) PE-amine (DSPE-PEG(2000) amine), which is on the high end of amounts typically used, and 85% **NB-PC** were compared to 100% **NB-PC** liposomes. Both behaved similarly during irradiation and in the dark, as can be seen in Figure 5 and Table 5. The PEG sample showed fluorescence decreases of 79 and 14% in UV light and in the dark, respectively, compared to 79 and 19% for the pure **NB-PC** sample. Thus, the inclusion of PEGylated lipids made little difference in controlled release in this system, and thus this approach could be implemented to improve in vivo survival in the pursuit of future medicinal applications.

Finally, with an effective release system in hand, we sought to characterize the system further by determining the products generated from membrane photocleavage. As previously discussed, irradiation of **NB-PC** was expected to directly generate

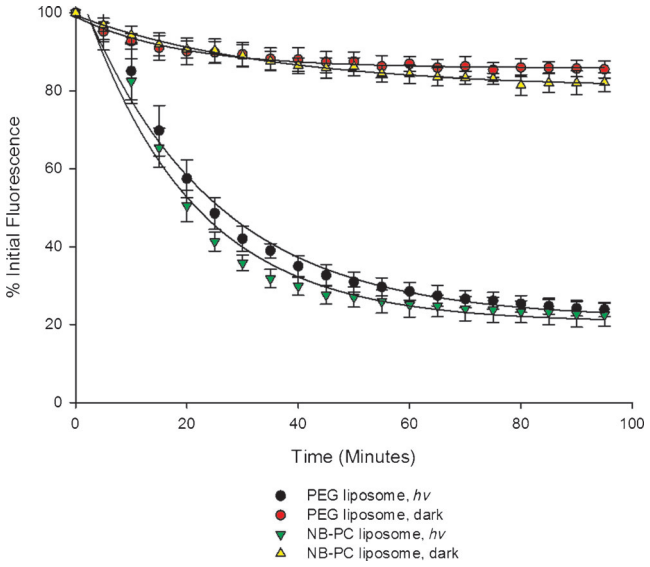


Figure 5. Nile red release upon irradiation of liposomes containing **NB-PC** compared to those containing 15% DSPE-PEG(2000) amine. Release efficacy was not significantly modified by the addition of DSPE-PEG(2000) amine.

Table 5. Data resulting from curve fitting of release from liposomes consisting of **NB-PC** alone or when mixed with DSPE-PEG(2000) amine.

Sample	**NB-PC** [%]	PEG-PE [%]	y_o	k [s^{-1}]	$t_{1/2}$ [min]	A	R^2
Light	100	0	0.2053	8.45E-04	13.7	0.8848	0.9628
	85	15	0.2150	7.07E-04	16.3	0.8561	0.9700
Dark	100	0	0.8074	4.83E-04	23.9	0.1863	0.8597
	85	15	0.8581	8.83E-04	13.1	0.1317	0.7304

compound **2** through traditional cleavage of the benzylic C–N bond following nitrophenyl excitation (Scheme 1). Subsequently, the released amine of the terminal amide group of **2** could potentially undergo nucleophilic attack of the proximal ester linkage at the *sn*-2 position, which would liberate succinimide and LPC (**3**). To evaluate this possibility, mass spectrometric analysis of the products following irradiation of liposomes composed of 100% **NB-PC** was performed using a JEOL AccuTof DART instrument with an ESI source. The resulting mass spectra indicated significant peaks corresponding to both PC-amide **2** as well as LPC **3**, providing evidence for the formation of both of these products (see representative mass spectrum in the Supporting Information).

Conclusion

Herein, we have described the design, synthesis, and analysis of a novel photocleavable analogue of PC, **NB-PC**, which enables remote light-triggered release of hydrophobic compounds from liposomes. Our strategy in this endeavor was to design a compound that closely resembles the structure of PC, since this is the most common lipid in cellular membranes and it forms highly stable heterogeneous membranes when mixed with a broad range of lipids with different properties. This was done to enhance favorable properties including biocompatibility and stability, thus ensuring that nonspecific background release would be minimized and content release would be limited to membrane disruption caused by photolysis of **NB-PC**.

The data obtained from the release of nile red from **NB-PC** liposomes indicate the benefits of this system. First, since **NB-PC** can be incorporated into stable liposomes at any percentage, and release efficacy directly correlates with the amount of **NB-PC**, this enables broad tunability of release properties to achieve a desired outcome. On the other hand, major fluctuations in **NB-PC** composition do not significantly affect background release rates, providing further evidence that any composition of **NB-PC** can be functional. Subsequent studies involving fixed amounts of **NB-PC** with percentages of DOPE and cholesterol ranging from 0–50% did not lead to major modifications in liposome release efficacy, further demonstrating that the **NB-PC** system is highly stable and effective for controlling release using liposomes composed of lipids with widely varying physicochemical properties. The ultimate goal of triggered drug release presents a number of technical challenges involving the logistics of delivering light to desired locations in living organisms, as well as other issues pertaining to circulation and targeting. Since incorporation of PEG did not produce significant differences in release, stealth liposomes are a viable route for future usage of this compound. With this information, the stability and adaptability of the **NB-PC** system provides a beneficial starting point for building a more complex delivery system.

Experimental Section

General experimental

Reagents and solvents were generally purchased from Acros, Aldrich, or Fisher Scientific and used as received. Palmitoyl-lysophosphatidylcholine (**3**) and DSPE-PEG(2000) amine were purchased from Avanti Polar Lipids, Inc. and (4-aminomethyl)benzoic acid was purchased from Chem Impex International. Dry solvents were obtained from a Pure Solv solvent delivery system purchased from Innovative Technology, Inc. Column chromatography was performed using 230–400 mesh silica gel purchased from Sorbent Technologies and a C18 (17%) reverse phase column (6 mL, 2 g) purchased from Silicycle. NMR spectra were obtained using a Varian Mercury 300 MHz or Varian VNMRS 500 MHz spectrometer. Mass spectra were obtained with a JEOL AccuTof DART or a Waters Quattro II triple quadrupole spectrometer with high-resolution capabilities. Optical rotations were measured with a PerkinElmer 241 Polarimeter using the sodium D line. Ultrapure water was purified via a Millipore water system ($\geq$18 MΩ cm) triple water purification system. 4-(((*Tert*-butoxycarbonyl)amino)methyl)-3-nitrobenzoic acid (**7**) was synthesized from (4-aminomethyl)benzoic acid (**4**) according to a prior literature procedure.[17a] Detection of nile red emission decreases attributed to release were performed using a PerkinElmer LS55 fluorescence spectrometer. Samples were irradiated with a Rayonet Preparative Type RS photoreactor while suspended in a cuvette with Pyrex as a filter.

tert-Butyl (4-(hexylcarbamoyl)-2-nitrobenzyl)carbamate (8)

To 1.67 g (5.64 mmol) of compound **7**, dissolved in 350 mL of chloroform, was added diisopropylethylamine (DIEA, 2.95 mL, 16.9 mmol), hydroxybenzotriazole (HOBt, 0.229 g, 1.69 mmol,), and *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU, 2.78 g, 7.33 mmol,). After 30 min, hexylamine (2.24 mL, 16.9 mmol) was added. The reaction was then allowed to stir overnight, after which it was washed with water, and the aqueous portion was extracted with chloroform (2×100 mL). The organic layers were then combined and washed with saturated sodium chloride, dried with magnesium sulfate, filtered and concentrated by rotary evaporation. Column chromatography using gradient elution of 30–50% ethyl acetate-hexanes gave 1.13 g of orange-yellow product (53%). R_f=0.18 (25% ethyl acetate-hexanes); ^{1}H NMR (300 MHz, $CDCl_3$): δ=8.33 (d, J=1.8 Hz, 1H), 7.92 (dd, J=8.0, 1.9 Hz, 1H), 7.54 (d, J=8.1 Hz, 1H), 7.01 (t, J=5.9 Hz, 1H), 5.55 (t, J=6.5 Hz, 1H), 4.53 (d, J=6.5 Hz, 2H), 3.40 (q, J=7.0 Hz, 2H), 1.58 (m, 2H), 1.40 (s, 9H), 1.35–1.22 (m, 6H), 0.86 ppm (t, J=6.8 Hz, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ=165.01, 156.07, 147.69, 137.61, 135.15, 132.17, 130.89, 123.68, 80.16, 42.26, 40.51, 31.56, 29.51, 28.40, 26.75, 22.62, 14.10 ppm; HRMS-DART $[M-H]^-$ calcd for $C_{19}H_{29}N_3O_5$, 378.2029; found 378.2018.

4-((4-(Hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoic acid (9)

To compound **8** (1.19 g, 4.01 mmol), which was placed in an ice bath, was added a solution of trifluoroacetic acid (TFA) in dichloro-

methane (15 mL, 20%). After one hour of stirring, starting material was no longer detected with TLC, and the solution was then concentrated and dried under high vacuum. To the resulting residue was then added acetonitrile (40 mL), dry potassium carbonate (1.67 g, 12.0 mmol), and succinic anhydride (0.442 g, 4.42 mmol), and the mixture was allowed to stir at RT. After 24 h, citric acid (50 mL, 10%) was added, and the reaction was extracted with ethyl acetate (3×50 mL). The organic layers were then combined and washed with saturated sodium chloride, dried with magnesium sulfate, filtered, and concentrated using rotary evaporation. Column chromatography with gradient elution of 50–100% ethyl acetate–hexanes including 0.2% acetic acid gave product **10** as a light yellow solid (893 mg, 59% yield). R_f=0.36 (10% methanol–dichloromethane); ^{1}H NMR (300 MHz, CD_3OD): δ=8.45 (d, J=1.7 Hz, 1H), 8.04 (dd, J=8.1, 1.6 Hz, 1H), 7.68 (d, J=8.1 Hz, 1H), 4.68 (s, 2H), 3.35 (t, J=7.2 Hz, 2H), 2.64–2.50 (m, 4H), 1.59 (m, 2H), 1.44–1.24 (m, 6H), 0.89 ppm (t, 6.6 Hz, 3H); ^{13}C NMR (75 MHz, CD_3OD): δ=176.20, 175.10, 173.45, 149.33, 138.38, 135.85, 132.88, 131.05, 124.67, 74.12, 43.82, 41.22, 32.64, 30.29, 29.79, 27.74, 23.61, 14.36 ppm; HRMS-DART: $[M-H]^-$ calcd for $C_{18}H_{25}N_3O_6$, 378.1665; found 378.1652.

1-Palmitoyl-2-(4-((4-(hexylcarbamoyl)-2-nitrobenzyl)amino)-4-oxobutanoyl)-*sn*-glycero-3-phosphocholine (NB-PC)

In a vial, compound **9** (211 mg, 0.556 mmol) was combined with dicyclohexylcarbodiimide (DCC, 0.86 mL, 0.556 mmol) and *N,N*-dimethylaminopyridine (DMAP, 0.068 g, 0.556 mmol) in 4 Å-molecular sieve-dried ethanol-free chloroform (2 mL) under argon, along with crushed glass, similar to a method described by Rosseto and Hadju.[27] After 30 min, palmitoyl-lysophosphatidylcholine (**3**, LPC, 69 mg g, 0.138 mmol) was added in one portion and argon atmosphere was then reestablished. After 6.5 h of sonication, Dowex 50Wx8 residue was added and the mixture was sonicated for 30 min before filtration through a fritted filter. After filtration and concentration, normal phase column chromatography was carried out through elution with 15% methanol–dichloromethane containing 0.2% acetic acid to remove any unreacted acid, followed by 65:25:4 chloroform–methanol–water to remove the product. Reverse phase chromatography using a C18 column with gradient elution from water to methanol was then used to separate the product from any residual dimethylaminopyridine and silica gel. Residual water was removed by repeatedly concentrating with acetonitrile. The solution was then concentrated to provide a slightly yellow lipidlike substance in 65% yield. R_f=0.32 (65:25:4 chloroform-methanol-water); $[\alpha]_D^{22.5}+4.3^\circ$ (c=2.9, $CHCl_3$); ^{1}H NMR (500 MHz, 60% $CDCl_3$-CD_3OD) δ=8.58 (s, 1H), 8.51 (s, 1H), 8.47–8.40 (m, 1H), 8.11 (d, J=8.0 Hz, 1H,), 7.67 (d, J=7.8 Hz, 1H), 5.27–5.20 (m, 1H), 4.73 (s, 2H), 4.36 (d, J=11.3 Hz, 1H), 4.27–4.15 (m, 3H), 4.08–3.97 (m, 2H), 3.58 (s, 2H), 3.45–3.37 (m, 2H), 3.27–3.16 (m, 9H), 2.80–2.53 (m, 4H), 2.32 (t, J=7.0 Hz, 2H), 1.63 (m, 4H), 1.43–1.22 (m, 30H), 0.95–0.85 ppm (m, 6H); ^{13}C NMR (125 MHz, 60% $CDCl_3$-CD_3OD): δ=174.45, 173.55, 172.81, 166.31, 148.43, 137.34, 135.37, 132.33, 130.35, 124.44, 71.53, 66.78, 64.23, 62.72, 59.48, 54.34, 41.16, 40.86, 34.34, 32.30, 31.93, 30.73, 30.68, 30.05, 30.02, 30.01, 29.99, 29.91, 29.87, 29.72, 29.66, 29.50, 27.11, 25.21, 22.94, 14.22 ppm; ^{31}P NMR (125 MHz, 60% $CDCl_3$–CD_3OD): δ=−1.35 ppm, referenced to triphenyl phosphate at −17.70 ppm; MS: $[M+Na]^+$ calcd for $C_{42}H_{73}N_4O_{12}P$, 879.4855; found 879.4869.

Fluorescence-based liposome release studies

Stock solutions were initiated by weighing out samples of DOPC, DOPE, cholesterol, DSPE-PEG(2000) amine, and/or **NB-PC** along with nile red in vials. To the appropriate lipid components corresponding to each liposome sample was added ethanol-free chloroform (500 μL), and after brief vortexing, proper volumes of each lipid were pipetted into a clean vial per calculations on a 5 mM, 500 μL total lipid scale, to obtain the desired molar percentage of each component. Next, a solution of nile red was added per calculations on a 250 μM, 500 μL scale. As an example, for liposomes composed of 50% **NB-PC** and 50% DOPE, ethanol-free chloroform (500 μL) was added to separate vials containing **NB-PC** (4.9 mg), DOPE (3.6 mg), and nile red (4.2 mg), producing stock solutions of 11, 9.7, and 26 mM concentrations, respectively. After 30 seconds of vortexing, **NB-PC** (109.4 μL), DOPE (129.2 μL), and nile red (4.74 μL) stock solutions were combined in a new vial. The chloroform was dried with a nitrogen stream, and the lipids were subsequently dried overnight under vacuum. The next day, the lipids were hydrated with MilliQ purified water (500 μL), vortexed, and incubated on a rotary evaporator at 60 °C for 3 sets of 20 min, with vortexing after each set. Liposomes were then freeze-dried between a −40 °C dry ice bath and a 60 °C water bath for 10 cycles and extruded through a 200 nm membrane for 21 passes using a LiposoFast extruder (Avestin, Inc.), placing the uniform-sized vesicles into a fresh vial. DLS scans were performed to confirm the formation of stable liposomes.

Next, 70 μL of this liposomal solution was diluted to 7 mL with ultrapure water. Two identical samples were made by placing 3 mL of this dilute solution into quartz cuvettes and were sealed with parafilm to minimize atmospheric exposure. After an initial fluorescence scan (λ_{ex}=595 nm; λ_{em}=612 nm), one sample was irradiated with 350 nm light while suspended and covered by Pyrex beakers between four 350 nm bulbs in a Rayonet Preparative Type RS photoreactor. The other sample was placed in a dark container. For each fluorescence scan (every 5 min), the sample was removed from the reactor or dark container and placed in the fluorimeter for scanning. Total amounts of time plotted for release experiments represent the amount of time the sample spent in the photoreactor. Experiments were run at least four times each, including runs with different batches of liposomes, and averaged to obtain the results shown in Figures 1–5, with error bars included to depict standard error. Data were then curve fit using an exponential decay equation in SigmaPlot to obtain the values shown in Tables 1–5, according to the function [Equation<(1)]:

$$Y = y_o + Ae^{-kt} \quad (1)$$

in which k is the first order rate constant, t is time (in minutes), Y is the % of initial fluorescence, and y_o and A are fit parameters. Values were tabulated in Tables 1–5, along with $t_{1/2}$, the irradiation half-life.

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