NANO · MICRO small
www.small-journal.com

Optimized Photoactivatable Lipid Nanoparticles Enable Red Light Triggered Drug Release

Nisha Chander, Johannes Morstein, Jan S. Bolten, Andrej Shemet, Pieter R. Cullis, Dirk Trauner,* and Dominik Witzigmann**

Encapsulation of small molecule drugs in long-circulating lipid nanoparticles (LNPs) can reduce toxic side effects and enhance accumulation at tumor sites. A fundamental problem, however, is the slow release of encapsulated drugs from these liposomal systems at the disease site resulting in limited therapeutic benefit. Methods to trigger release at specific sites are highly warranted. Here, it is demonstrated that incorporation of ultraviolet (UV-A) or red-light photoswitchable-phosphatidylcholine analogs (AzoPC and redAzoPC) in conventional LNPs generates photoactivatable LNPs (paLNPs) having comparable structural integrity, drug loading capacity, and size distribution to the parent DSPC-cholesterol liposomes. It is shown that 65–70% drug release (doxorubicin) can be induced from these systems by irradiation with pulsed light based on *trans*-to-*cis* azobenzene isomerization. In vitro it is confirmed that paLNPs are non-toxic in the dark but convey cytotoxicity upon irradiation in a human cancer cell line. In vivo studies in zebrafish embryos demonstrate prolonged blood circulation and extravasation of paLNPs comparable to clinically approved formulations, with enhanced drug release following irradiation with pulsed light. Conclusively, paLNPs closely mimic the properties of clinically approved LNPs with the added benefit of light-induced drug release making them promising candidates for clinical development.

1. Introduction

Lipid nanoparticles (LNP) are the leading drug delivery platform in the clinic for systemic applications.[1–3] Unilamellar LNPs with diameters less than 100 nm are favored for delivery of small molecular drugs. Solid core systems are better suited for delivery of macromolecular genetic drugs, such as siRNA or mRNA.[4–6] More than 10 LNP therapeutics have been approved by the US FDA and other regulatory agencies. Most of these are liposomes containing anticancer drugs that exhibit reduced toxicity and enhanced efficacy compared to the free drug.[4,7–9]

Robust techniques exist for achieving efficient drug encapsulation in <100 nm diameter liposomal systems that exhibit long half-lives in the circulation (up to 24 h in humans) and preferential accumulation at tumor sites following intravenous injection.[9–11] However, a major limitation is that they do not selectively leak their contents after arrival at the target site. This severely limits the improvement in therapeutic index that can be gained by liposomal delivery.[1,10] Technologies that trigger release of liposomal contents either at or near the target site would have significant benefits. This is particularly true given that liposomes containing cancer drugs can exhibit maximum tolerated doses that are up to five times higher than those of free drug and thus are systemically much less toxic.[10–12]

There have been many attempts to develop triggered release systems for liposomal systems containing anticancer drugs.[13] To this end, thermosensitive lipids or metallic nanoparticles (such as or gold nanoparticles or iron oxide nanoparticles) tethered to the liposome have been employed.[14–16] These systems give rise to liposomes that leak contents in response to local heating or irradiation. Many systems, however, are quite complex, limiting their manufacturing scalability or require the development of a specific device to trigger release. In addition, several reported systems exhibit poor drug loading/retention and relatively short circulation lifetimes resulting in off-target release and reduced ability to access the desired target tissues.[13,17] As a result, only one triggered release technology has progressed to late stage clinical trials to date: ThermoDox—a liposomal doxorubicin (Dox) formulation for the treatment of

N. Chander, Dr. P. R. Cullis, Dr. D. Witzigmann
Department of Biochemistry and Molecular Biology
University of British Columbia
2350 Health Sciences Mall, Vancouver, BC V6T 1Z3, Canada
E-mail: pieterc@mail.ubc.ca; dominik.witzigmann@ubc.ca
J. Morstein, Dr. A. Shemet, Dr. D. Trauner
Department of Chemistry
New York University
100 Washington Square East, Room 712, New York, NY 10003, USA
E-mail: dirktrauner@nyu.edu
J. S. Bolten
Department of Pharmaceutical Technology
University of Basel
Klingelbergstrasse 50, Basel 4056, Switzerland
Dr. P. R. Cullis, Dr. D. Witzigmann
NanoMedicines Innovation Network (NMIN)
University of British Columbia
2350 Health Sciences Mall, Room 5451, Vancouver, BC V6T 1Z3, Canada
The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/smll.202008198.

DOI: 10.1002/smll.202008198

inoperable hepatocellular carcinoma, where drug release is stimulated by a mild hyper-thermic trigger.[17,18] The lipid composition of ThermoDox is significantly different as compared to the approved Doxil formulation to ensure a relatively sharp transition temperature. However, this resulted in a formulation with different pharmacokinetics and a relatively short circulation lifetime.[19,20] Despite more than 30 years of efforts, the only triggered release system (in response to local heating) that had made it into the clinic failed in phase III.[20,21]

Alternative attempts to develop approaches for light-triggered drug release from liposomal targets have not progressed to a clinical setting due to inefficient drug encapsulation and release, lack of straightforward and scalable methods of manufacture, and difficulty in selecting the clinical entry point.[22,23] In addition, there is a range of competitive technologies including simple and effective device-only ablative methods such as microwave and radiofrequency ablation, as well as surgery and radiotherapy. The implementation of light-triggered drug release systems, however, could benefit from approved phototherapies and photodynamic therapies (PDT) as well as emerging technologies to deliver light deep within patients.[24,25]

Ideally, new systems for light-triggered release should closely mimic the composition and properties of clinically approved LNPs in terms of composition, size, loading, and stability. The approved systems Doxil, Ambisome, and Marqibo, all use saturated lipids that contain choline headgroups at approximately equimolar levels with cholesterol as the primary lipid constituents.[9,18] Such phosphatidylcholine lipid-cholesterol compositions can be readily formulated into liposomal systems with diameters <100 nm that can be efficiently loaded with weakly basic drugs, such as Dox, and display long circulation lifetimes following i.v. administration. With the constantly evolving field of photo pharmacology, approaches to include photosensitizers like porphyrin into stealth liposomes have helped advance the light-triggered drug release concept to have more clinical translatability. These types of systems take advantage of the well-established field of PDT.[26,27] The investigation of non-porphyrin based photosensitizers as novel PDT agents has been considerably less extensive than porphyrin-based compounds.

Here, we report a liposomal light-triggered release system containing photoswitchable phosphatidylcholine analogs with azobenzenes incorporated into the lipid tail, compounds termed AzoPC and red-AzoPC.[28,29] This strategy enables the design of an exterior lipid composition that allows long circulation lifetimes, incorporation of an agent responsive to light, and an aqueous interior offering small molecule drugs to be encapsulated. We show that DSPC-cholesterol systems incorporating AzoPC (in the trans-form) at low (10 mol%) levels result in liposomes that have similar size and drug (Dox) loading properties as parent LNPs. When stimulated to adopt the cis-form, the AzoPC containing liposomes exhibit triggered release properties resulting in enhanced cytotoxic effects in vitro. The responsiveness of photoactivatable LNPs (paLNPs) to a different wavelength is easily tuned by substituting AzoPC with a red-shifted variant red-AzoPC. In vivo studies confirmed long blood circulation half-lives and triggered release properties of paLNP system. This proof-of-concept study demonstrates the potential therapeutic utility of liposomal systems containing AzoPC.

2. Results and Discussion

2.1. Design of Dox-Loaded paLNP Liposomes

In order to design a light-triggered release system enabling efficient drug loading and long-circulation properties, we first modified conventional DSPC-cholesterol liposomes (55 mol% DSPC, 45 mol% Chol) by incorporating a UV-A photoswitchable AzoPC (**Figure 1**A).[22] The use of such azobenzene photoswitches in biology and medicine is well established.[23,30]

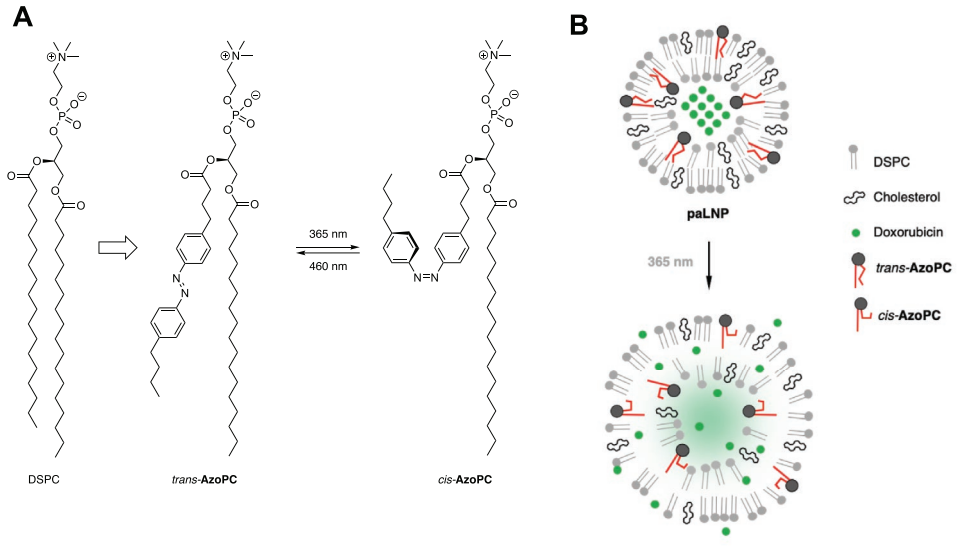

Figure 1. Design of a light-triggered drug release system. A) Chemical structure of DSPC and its photoswitchable analog AzoPC. The azobenzene can be isomerized from the trans to the cis form at 365 nm. B) Schematic for light induced drug release from paLNPs. Low levels of trans AzoPC are incorporated in a DSPC-cholesterol liposomal system loaded with Dox. Photoisomerization of AzoPC induces drug release.

Stimulation of paLNPs at 365 nm should trigger trans to cis isomerization resulting in Dox release (Figure 1B).

2.2. Synthesis and Characterization of Dox-Loaded paLNP Liposomes

DSPC-cholesterol liposomes were prepared employing ethanol dilution-rapid mixing techniques and subsequent dialysis steps to contain 300 mM ammonium sulphate in their aqueous core and phosphate buffered saline (PBS) as an exterior buffer and were used as control formulations (control-LNP). A range of paLNP liposomes were synthesized by titrating varying amounts of AzoPC (2.5, 5, 10, 15, 20, 30 mol%) into the control DSPC-cholesterol liposomes where the added AzoPC substituted for DSPC. Dynamic light scattering (DLS) analysis of the particles showed a monodisperse population (PDI <0.1) of uniformly sized ≈55–60 nm particles (**Table 1**). The trans to cis photoswitching kinetics of AzoPC incorporated into paLNPs was comparable to that observed in the stock ethanol solution as observed by measuring absorbance at 340 nm at 30 s intervals (λ = 365 nm) (**Figure 2**A).

The LNP formulations were loaded with Dox using the pH gradient (interior acidic) generated by encapsulated ammonium sulphate. The LNP were incubated at 65 °C for 30 min with free drug to achieve a maximum encapsulated drug:lipid ratio (wt/wt) of 0.1 after which unentrapped Dox was removed via dialysis. Drug:lipid ratios were assayed for samples taken before and after drug loading and used to calculate percent entrapment. Efficient loading was achieved for liposomes containing up to 10% Azo-PC. Formulations containing higher mol% of AzoPC (15–30 mol%) were unable to maintain the ammonium sulphate gradient effectively, which resulted in lower drug loading (Figure 2B). The dependence of drug loading efficiency on the ammonium sulphate gradient in the paLNP system was confirmed by switching the AzoPC conformation to the cis form prior to drug loading which resulted in a significant reduction in entrapment efficiency in cis-paLNPs (≈20% entrapment) as compared to trans-paLNPs (≈100% entrapment) (Figure 2C,D). The control LNP did not show any change in encapsulation efficiency upon irradiation (Figure 2E).

Table 1. Physicochemical characterization of LNPs. Hydrodynamic diameter and size distribution (PDI) of control-LNP (DSPC-Chol system) and paLNPs containing various amounts of AzoPC.

Lipid composition	Mean diameter ±SD [nm]	PDI
Control-LNP	52.83±2.588	0.038
AzoPC 2.5%	55.49±0.732	0.064
AzoPC 5%	56.33±1.811	0.083
AzoPC 10%	57.89 ±1.150	0.072
AzoPC 15%	59.20±1.689	0.097
AzoPC 20%	59.86±4.118	0.105
AzoPC 30%	63.44±2.988	0.101

2.3. Liposomes containing 10 mol% AzoPC Exhibit up to 80% Light-Triggered Release of Dox when AzoPC is Switched to the cis Form

Drug release from paLNPs following irradiation was measured in PBS at room temperature. paLNPs loaded with Dox at a drug:lipid ratio of 0.1 (wt/wt) and a concentration of 3 mg mL^{-1} total lipid were irradiated with the UV-A light source (365 nm) for 5 min followed by storage in the dark for 1 h. Samples were assayed for drug release by measuring absorbance at 492 nm. Limited release was observed in paLNPs containing 2.5–5 mol% AzoPC while paLNPs containing 10–30 mol% AzoPC showed up to 20% drug release at the 1 h timepoint (**Figure 3**A). paLNP containing 10% AzoPC in the trans form showed drug loading and drug release properties that were similar to the control LNP system (DSPC-cholesterol), whereas paLNP systems with higher trans AzoPC contents exhibited decreased drug loading capabilities that may be attributed to increased permeability of the liposome bilayer. We therefore chose to move forward with the paLNP systems containing 10 mol% AzoPC for subsequent experiments.

Initial work was performed to determine whether triggered release could be achieved in response to irradiation to switch the azo-PC from the trans to the cis form. It was found that significant triggered release of up to 40% could be achieved for paLNP containing 10 mol% Azo-PC following 5 min irradiation at 365 nm at room temperature when the paLNP were suspended in PBS (Figure 3B). We next tested whether light triggered release was influenced by the presence of serum proteins. It is well known that serum proteins adsorb to liposomal surfaces forming a protein corona that can influence membrane permeability and other properties such as accessibility of light to membrane surface.[31,32] We therefore evaluated light-triggered release from paLNPs containing 10 mol% AzoPC following dilution into cell culture medium (DMEM) containing serum (10% fetal bovine serum, FBS). It was found that the presence of serum proteins inhibited light-triggered drug release significantly compared to paLNP in PBS. After an initial irradiation time of 5 min at 365 nm followed by storage in the dark, ≈25% of Dox was released after a 24 h time period as compared to 40% of Dox released in PBS (Figure 3B).

Of note, AzoPC in the cis form will spontaneously revert to its trans form over time, potentially reducing leakage from the irradiated paLNP. We therefore investigated whether the amount of drug release could be increased through pulsed irradiation following the 5 min initial irradiation to prevent re-isomerization keeping the AzoPC in its cis form for a longer period of time. The paLNP were subjected to pulses of LED light (wavelength 365 nm) of 75 ms duration every 15 s over a 24 h period (**Figure 4**A,B). We found that this pulsed irradiation protocol led to increased drug release within 24 h compared with initial irradiation. This result could be explained by thermal relaxation and repeated switching over time. While constant irradiation over the same time frame could potentially produce a similar effect in terms of release, the pulsed method allows for successful switching and maintenance of the AzoPC in cis conformation at >100 times less irradiation time, preventing any side effects associated with prolonged exposure to

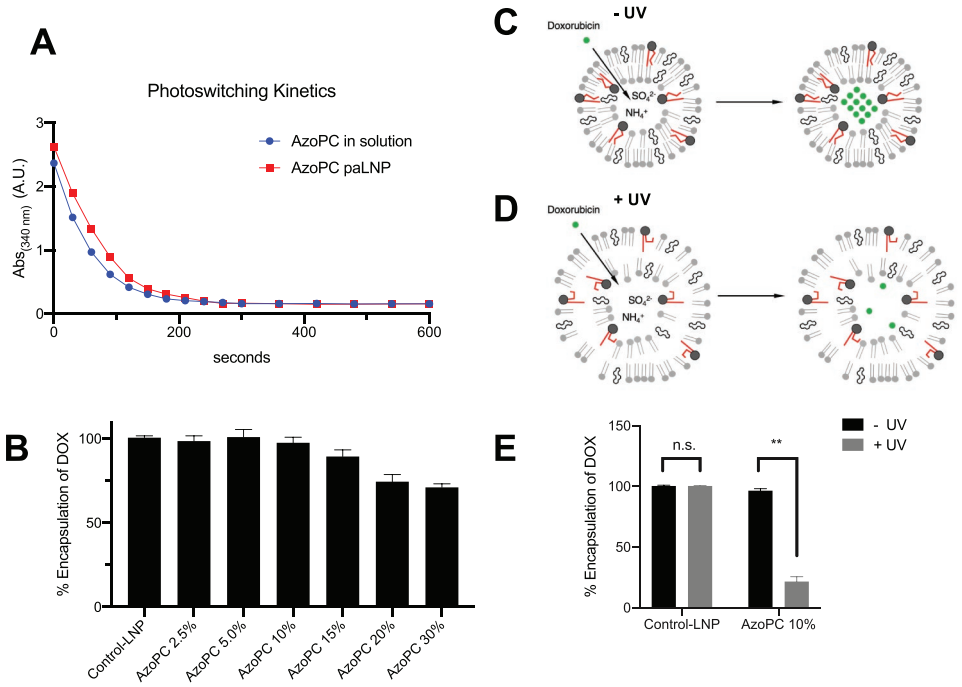

Figure 2. Substitution of up to 10 mol% trans AzoPC for DSPC in DSPC-cholesterol liposomes allows for efficient loading of Dox. A) AzoPC photoswitching kinetics in paLNPs versus free dissolved AzoPC. Samples at 3 mg mL^{-1} AzoPC concentration were used for absorbance measurements. B) Dox entrapment efficiency of paLNP formulations with increasing amounts of AzoPC. C) Schematic for remote loading of Dox into paLNPs incorporating AzoPC in the trans form. paLNPs containing 300 mM ammonium sulphate in the aqueous core and suspended in PBS were mixed with Dox at a drug to lipid (wt/wt) ratio of 0.1. The mixture was heated in a water bath at 65 °C for 30 min, following removal of unentrapped Dox via dialysis. D) Schematic for remote loading of Dox into paLNPs with AzoPC in the cis form. paLNPs were subject to UV irradiating at 365 nm for 5 min, following the same drug loading procedure as above. E) Comparison of Dox loading efficiency in paLNP formulations containing 10% AzoPC before and after photoswitching via irradiation with UV-A light (365 nm). Error bars represent SEM $^{**}p < 0.01$, n.s., not significant, Student's *t*-test.

UV-A light such as increase in temperature of irradiated area or cytotoxicity.

Correspondingly, the in vitro drug release experiments were repeated on paLNP in PBS and DMEM, where the initial irradiation time of 5 min was followed by pulsed irradiation. This pulsed irradiation enabled significantly improved drug release of 75–80% from paLNPs in PBS (Figure 4C) and 65–70% from paLNPs in serum containing medium (DMEM with 10% FBS) (Figure 4D). The control LNP demonstrated a relatively low Dox release and did not show any change in drug release on irradiation (Figure 4C,D).

2.4. The Morphology of Doxorubicin-Loaded Liposomes Containing AzoPC Following Pulsed Irradiation is Consistent with Drug Release

Liposomes loaded with Dox employing pH loading techniques exhibit characteristic "coffee bean" morphology as detected by

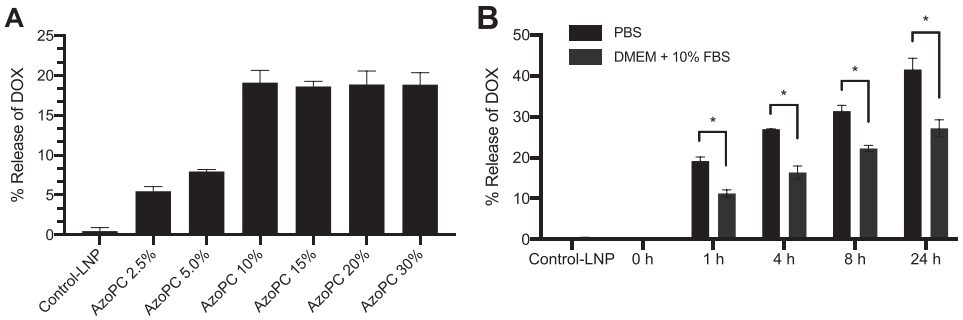

Figure 3. Presence of serum proteins significantly inhibited drug release from paLNP compared to paLNP in PBS. A) paLNP particles containing a substitution of 10–30 mol% trans AzoPC for DSPC in control DSPC-cholesterol liposomes suspended in PBS were irradiated with a UV-A light source (365 nm) for 5 min followed by storage in the dark at room temperature for 1 h. Samples were assayed for drug release (Dox by measuring absorbance at 492 nm. B) paLNP particles containing 10 mol% AzoPC suspended in PBS or DMEM media containing 10% FBS were irradiated with a UV-A light source (365 nm) for 5 min followed by storage in the dark at room temperature for 24 h. Samples were assayed for drug release (Dox) by measuring absorbance at 492 nm at 0, 1, 4, 8, and 24 h timepoints. Error bars represent SEM $^{*}p < 0.1$, Student's *t*-test.

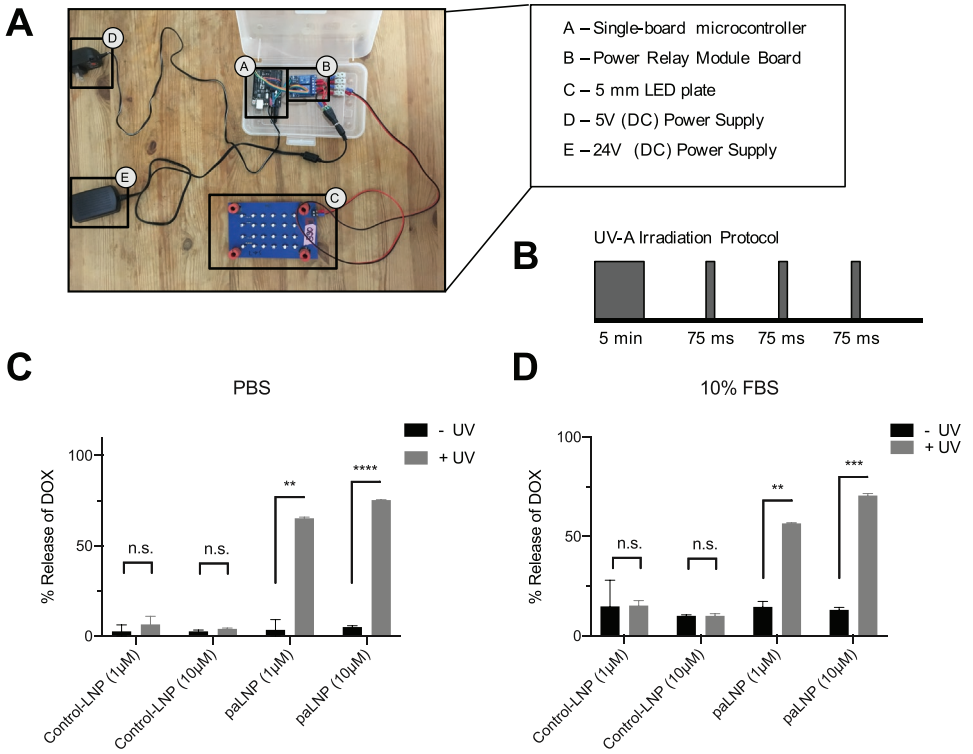

Figure 4. Pulsed irradiation (365 nm) of paLNP (10 mol% AzoPC) results in triggered release of Dox both in the absence and presence of serum. A) "Cell-DISCO" setup for pulsed irradiation using a single-board microcontroller (e.g., Arduino), power relay module board, LED plate, and power supplies. B) Irradiation protocol for pulsed LED starting with a 5 min initial irradiation followed by 75 ms irradiation pulses. C) Light-triggered Dox release from control-LNP and paLNP (10 mol% AzoPC) using pulsed LED irradiation (365 nm) for 24 h at 20 °C in PBS or D) DMEM media containing 10% FBS. Error bars represent SEM $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, n.s., not significant, Student's *t*-test.

cryo-TEM due to the formation of nano-sized crystals of precipitated Dox in the center of the liposomes.[33] It is of interest to determine whether similar morphology is exhibited by the loaded paLNP and whether this morphology is affected by the light-triggered release of Dox. We performed structural evaluation of the paLNP formulation using cryo-TEM imaging.

As shown in **Figure 5**A, paLNPs showed a typical bilayer structure, indistinguishable from the control DSPC-cholesterol liposomes, with sizes in agreement with those obtained via DLS (≈55–60 nm). Control-LNPs and paLNPs loaded with Dox showed Dox crystalized within the liposome interior. After light-triggered release using pulsed irradiation at 365 nm over 24 h, there is a clear decrease in the number of entrapped drug crystals within the paLNPs, while the control DSPC-cholesterol liposomes show no visible changes. This was also confirmed through quantification of crystal thickness within the various samples (Figure 5B).

2.5. Dox Released from Loaded paLNP Following Irradiation is Biologically Active

Dox is a cytotoxic agent and its release from paLNP following irradiation would be expected to result in cytotoxic effects on nearby tissues. In order to demonstrate this, we investigated the effects of light-released Dox on the viability of a human derived liver cancer cell line (i.e., HuH7 cells) in vitro. We compared the cell viability effects of Dox in its free versus liposome encapsulated forms with and without light-triggered drug release. HuH7 cells were treated with either free Dox (dissolved in PBS), Dox-loaded control-LNP or paLNP at drug concentrations up to 100 μM. Cells were subjected to irradiation at 365 nm for 5 min at 6 h post exposure to trigger drug release, followed by pulsed irradiation at 365 nm for 24 h to keep the AzoPC in its cis form. As expected, control-LNP (with or without UV irradiation) did not result in a decrease in viable cells due to their limited drug release properties. In contrast, treatments using paLNPs were highly dependent on the light-trigger. Whereas paLNP without UV irradiation did not affect cell viability, photoswitched paLNP resulted in a dose-dependent decrease in the number of viable cells 24 h after treatment similar to that of free Dox (**Figure 6**A). To confirm the significant light-triggered release of Dox from paLNP, HuH7 cells were treated with control-LNP or paLNP at a Dox concentration of 10 μM as stated above and analyzed through confocal microscopy with and without UV treatment. The confocal images demonstrate that only paLNPs result in effective release of Dox after irradiation. Once released, Dox accumulates in the nucleus and is fluorescent. (Figure 6B).

2.6. Formulation and Characterization of Red-Shifted paLNP Incorporating Tetra-Ortho-Chloro-Azobenzene Modified AzoPC

Incorporation of AzoPC sensitive to 365 nm could be perceived as a challenge for in vivo translation due to the limitation in

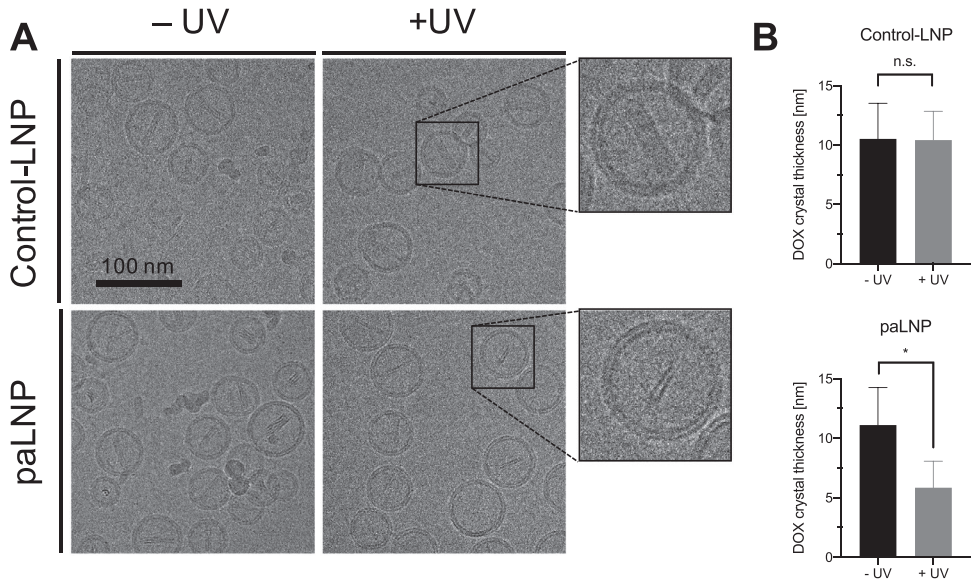

Figure 5. Dox loaded paLNP show evidence of drug release following pulsed irradiation (365 nm) as detected employing cryo-TEM. A) Representative cryo-TEM images of control DSPC-cholesterol liposomes and paLNP containing 10 mol% AzoPC prior to UV irradiation and post UV irradiation using a pulsed LED at 365 nm over a 24 h period. B) Comparison of thickness of Dox crystal within the liposomes pre and post UV irradiation. Error bars represent SEM $*p < 0.1$, n.s., not significant, Student's *t*-test.

tissue penetration depth and low tolerance to UV-A light. To extend the applicability of our light-triggered release system, we developed a red-shifted version of AzoPC, termed redAzoPC (synthesis described elsewhere), that undergoes a switch from the cis to the trans conformation at 660 nm (**Figure 7**A). Red-paLNPs were prepared using the previously established procedures (Section 2.1) by adding 10 mol% redAzoPC into the control DSPC-cholesterol liposomes (substituting DSPC for redAzoPC). Characterization of the red-paLNP particles showed a monodisperse population (PDI <0.1) of uniformly sized ≈56–58 nm nanoparticles with >98% entrapment efficiency of Dox, matching the physicochemical characteristics of control and paLNPs.

Drug release from red-paLNPs following irradiation was measured in PBS and cell culture medium (DMEM) containing serum (10% FBS) at room temperature. The red-paLNP were subjected to pulses of LED light (wavelength 660 nm) of 75 ms duration every 15 s over a 24 h period. This pulsed irradiation enabled a 75–80% Dox release from red-paLNPs in PBS (Figure 7B) and 70–75% release from red-paLNPs in serum containing medium (Figure 7C). In contrast, control-LNPs did not show any change in drug release following deep-red light irradiation (Figure 7B,C).

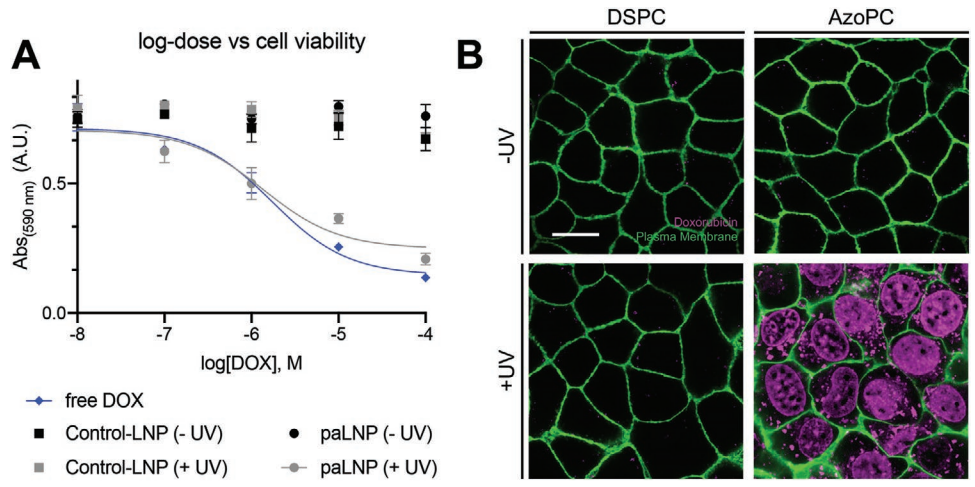

Figure 6. Dox released from drug loaded paLNP following irradiation is biologically active. A) Influence of irradiation on the viability of HuH7 cells incubated with increasing concentrations of Dox in free form or encapsulated in control-LNP or paLNP. Cells were incubated in the presence of 0, 0.1, 1, 10, and 100 μM Dox concentrations and were irradiated for 5 min at 365 nm and then subjected to pulsed irradiation at 365 nm for 24 h. Drug release was reflected by decreased cell viability. B) Confocal images of HuH7 cells treated with control-LNP and paLNP with and without UV irradiation. Samples with 10 μM Dox concentrations were irradiated for 5 min at 365 nm following, after which samples were subjected to pulsed irradiation at 365 nm for 24 h.

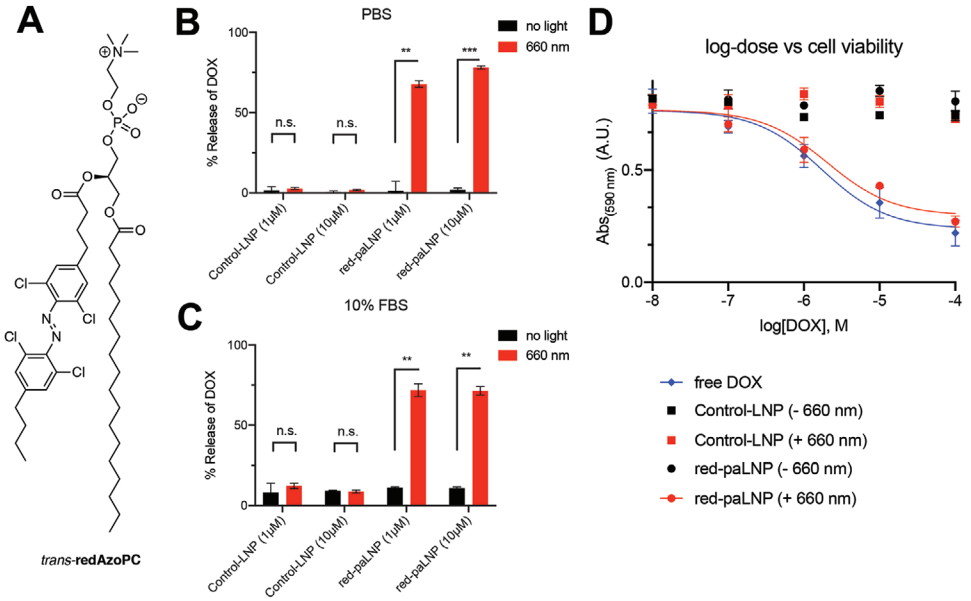

Figure 7. Pulsed deep-red light irradiation (660 nm) of red-paLNP (10 mol% redAzoPC) results in triggered release of Dox. A) Chemical structure of photoswitchable analog redAzoPC. The tetra-ortho-chloro-azobenzene can be isomerized from the trans to the cis form at 660 nm. B) Light-triggered Dox release from control-LNP and red-paLNP (10 mol% redAzoPC) using pulsed LED irradiation (660 nm) for 24 h at 20 °C in PBS or C) DMEM media containing 10% FBS. Error bars represent SEM $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant, Student's *t*-test. D) Influence of irradiation on the viability of HuH7 cells incubated with increasing concentrations of Dox in its free form or encapsulated in control-LNP or red-paLNP. Cells were incubated in the presence of 0, 0.1, 1, 10, and 100 μM Dox concentrations and were irradiated for 5 min at 660 nm and then subjected to pulsed irradiation at 660 nm for 24 h. Dox release was reflected by decreased cell viability.

To assess the effects of Dox in its free versus red-paLNP encapsulated forms (with and without light-trigger) on the viability of a human derived liver cancer cell line (i.e., HuH7 cells), an in vitro study was performed (similar to Section 2.4). HuH7 cells were treated with either free Dox (dissolved in PBS), Dox-loaded control-LNP or red-paLNP at drug concentrations up to 100 μM. Cells were subjected to irradiation at 660 nm for 5 min at 6 h post exposure to trigger drug release, followed by pulsed irradiation at 660 nm for 24 h to keep the redAzoPC in its cis isoform. As seen previously, control-LNP (with or without irradiation) did not result in a decrease in viable cells due to their limited drug release properties. In contrast, cytotoxic effects of red-paLNPs were highly dependent on the light-trigger. Similar to the results seen with the paLNP (UV-A light), the red-paLNP did not affect cell viability without irradiation whereas the photoswitched red-paLNP resulted in a dose-dependent decrease in the number of viable cells 24 h after treatment similar to that of free Dox (Figure 7D).

2.7. paLNP and Red-paLNP Display Long Circulation Lifetimes In Vivo Following I.V. Administration

To assess the pharmacokinetic properties of developed paLNP systems (i.e., influence of incorporating AzoPC analogs into conventional liposomes), we used the zebrafish embryo model. As demonstrated previously by our team, zebrafish embryos are a reliable and predictive in vivo tool to investigate liposomal circulation behavior and clearance mechanisms.[34,35] First, we intravenously injected fluorescently labeled control-LNP, paLNP, and red-paLNP (1 nL) into transgenic zebrafish expressing green fluorescent protein in their vascular endothelial cells (Tgkdrl:EGFP) at total lipid concentrations of 10 mg mL^{-1}. Next, we performed confocal microscopy imaging of the tail region 2 and 24 h post-injection (hpi). All liposomal systems (with and without irradiation) demonstrated excellent circulation properties within blood vessels 2 hpi (**Figure 8**A,B and Figure S3, Supporting Information) without any signs of agglomeration within the intersegmental vessels (ISV) and dorsal longitudinal anastomotic vessels. At 24 hpi, significant extravasation of LNPs into surrounding tissue and accumulations in the posterior caudal vein region (indicating macrophage clearance) was observed (Figure 8A–C). These pharmacokinetic characteristics, that is, excellent systemic circulation resulting in pronounced tissue extravasation, are typical for long-circulating liposomes. Importantly, incorporation of photoswitchable AzoPC analogs into LNP did not affect the pharmacokinetic properties thereby confirming the ideal characteristics for a light-triggered release system.

2.8. Light-Triggered Release of Doxorubicin from paLNP Systems In Vivo

In assessing the light-triggered release of Dox from paLNPs, various factors must be considered to enable its detection. Upon i.v. injection, Dox is entrapped in circulating LNPs (fluorescence is quenched). Release of Dox into circulation does not result in increased fluorescence due to low quantum efficiency and rapid clearance. Release into tissue however, is detectable due to fluorescence de-quenching and enhanced penetration (fluorescent area). Based on these considerations, we injected

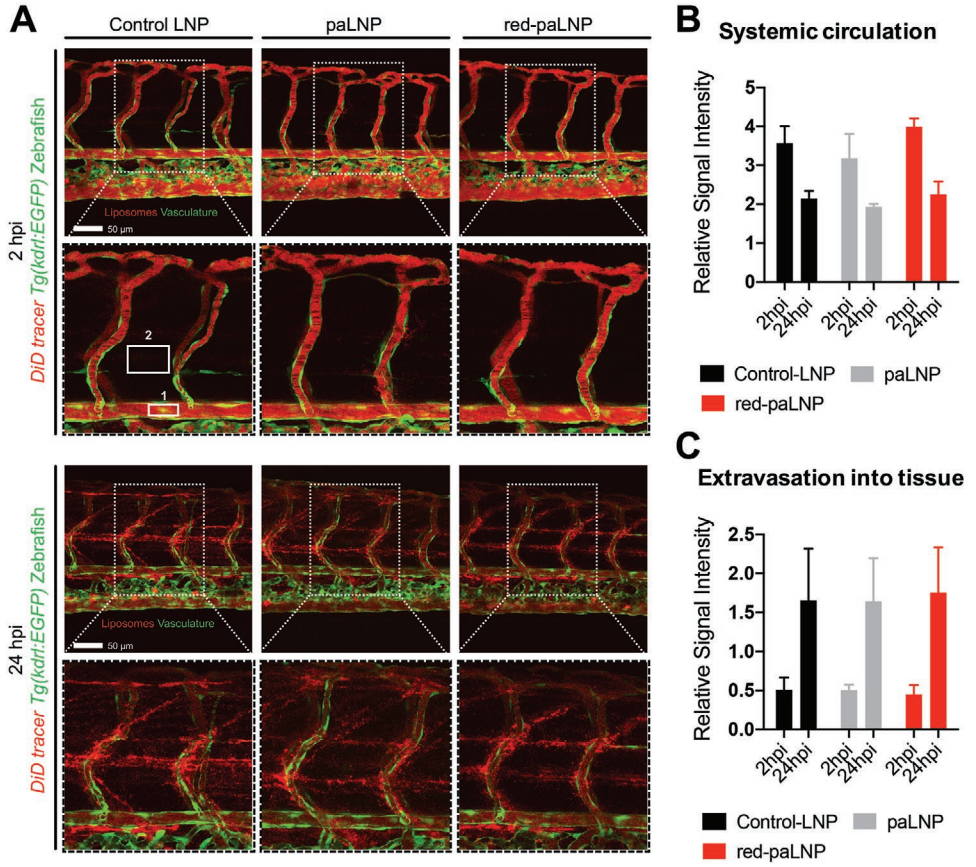

Figure 8. Assessment of LNP pharmacokinetics in vivo in transgenic zebrafish. Control LNP and paLNPs were injected intravenously into transgenic Tg(kdrl:EGFP) zebrafish expressing enhanced green fluorescent protein in their vasculature. LNPs were fluorescently labeled with DiD. A) Confocal images of tail region were acquired at 2 and 24 hpi. B) Systemic circulation properties were quantified based on fluorescence signals in the dorsal aorta (box 1). C) LNP extravasation into tissue was quantified between the ISV (box 2). Error bars represent SEM.

control-LNP, paLNP, and red-paLNP (2 nL) at a Dox concentration of 3 mg mL^{-1} into wildtype Tg(abc/tübingen) zebrafish embryos. Following tissue extravasation and accumulation, we exposed one set of zebrafish to pulsed UV-A (365 nm) or deep-red light (660 nm) irradiation for 24 h. Next, we performed confocal microscopy imaging of the tail region and analyzed the penetration area of free Dox.

All tested LNPs released Dox 48 hpi (**Figure 9**A,B). Although similar Dox signals in the absence of light-trigger were detected for all LNPs, paLNP, and red-paLNP triggered with UV-A or deep red pulsed light, respectively, demonstrated significantly enhanced Dox release in zebrafish embryos (Figure 9C). This result highlights the potential of paLNPs for triggered drug release in vivo.

3. Conclusion

This work demonstrates that DSPC-cholesterol liposomes containing 10 mol% photoswitchable phosphatidylcholines (substituting for DSPC) enable light-triggered Dox release in a physiological context. These paLNPs exhibit similar size-distribution, stability, and loading efficiencies as the parent DSPC-cholesterol systems which are clinically approved and widely used in human cancer therapy. They have the added benefit of being able to release contents upon UV-A or deep-red light irradiation inducing a trans to cis isomerization in photoswitchable phosphatidylcholine analogs. This results in up to 80% release of encapsulated Dox over 24 h. The triggered release could potentially be made more rapid in response to a higher intensity light source.[22] The described characteristics in combination with their long blood circulation half-lives make the paLNPs interesting and promising candidates for clinical development. It will be an important task in the future to demonstrate the local release in specific regions using rodent models. With regard to the utility of irradiation at 365 nm, the field of optoelectronics is currently undergoing significant advances, for example, through the development of fully implantable optoelectronic systems[36] that could help overcome the issue of limited tissue penetration. This limitation can also be overcome by incorporating red-shifted analogs of AzoPC as demonstrated in our work or by using methods for upconversion of light.[37]

In summary, in addition to the triggered release properties exhibited by the paLNP systems developed here, an important feature is their compositional simplicity and similarity to the well-studied clinical formulations. This allows us to be able to predict their physicochemical properties, nano-bio interactions, and pharmacokinetics in vivo. As such, this proof-of-concept

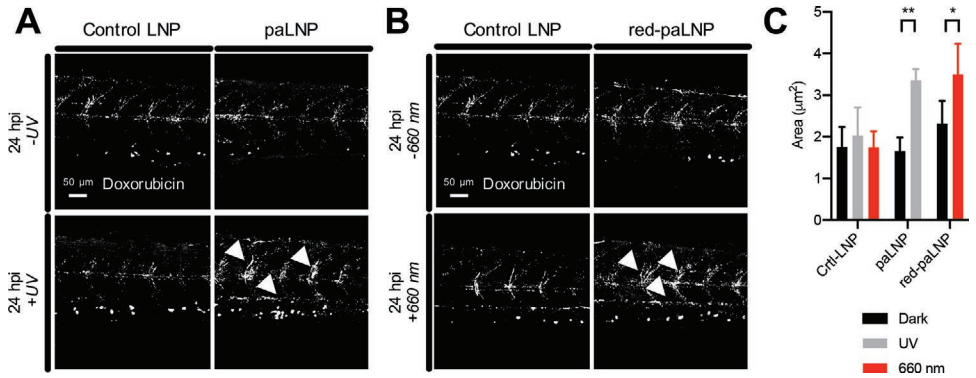

Figure 9. Dox release in zebrafish embryos in presence or absence of pulsed light trigger. Control LNP and paLNPs were injected intravenously into wildtype zebrafish embryos 48 hpf (2 nL, 3 mg mL^{-1} Dox). After 24 h, zebrafish were exposed to pulsed irradiation and confocal images of tail region were acquired at 48 hpi. A,B) Representative images of Dox release (white signal) from control LNP, paLNP, or red-paLNP in presence or absence of pulsed UV-A (365 nm, A) or deep-red light (660 nm, B) irradiation. White arrows indicate exemplified areas with enhanced Dox release. C) Quantitative image analysis of Dox release. Errors bars represent SEM. *$p < 0.1$, **$p < 0.01$.

study, and in particular the tools and methods employed, will potentially allow for rational design of new, simple, and effective systems with enhanced drug release capabilities that will have a significant impact on the therapeutic index gained by liposomal delivery of cytotoxic drugs.

4. Experimental Section

Materials: Phospholipids used for liposome preparation 1,2-distearolyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol and MS-222 (Tricaine) and agarose was purchased from Sigma-Aldrich (Saint Louis, MO). AzoPC and redAzoPC was provided by the lab of Dr. Dirk Trauner (New York University). Ammonium sulphate, Dulbecco's PBS, FBS, and Triton ×-100 were purchased from Sigma-Aldrich (Saint Louis, MO). Doxorubicin hydrochloride (Dox) was purchased from Cayman Chemicals (Ann Arbor, MI).

The Cell-DISCO was engineered in the Trauner lab and used as described.[38] A 365 nm/660 nm LED (Roithner Lasertechnik) plate was used at one 75 ms flash per 15 s.

Liposome Preparation: Lipid stocks of cholesterol and DSPC were co-dissolved in ethanol at appropriate molar ratios. In some cases, AzoPC or redAzoPC was incorporated into the lipid mix at varying molar ratios, keeping the DSPC to cholesterol ratio constant. All the LNPs were made using the T-tube formulation method at total flow rate of 20 mL min^{-1} and flow rate ratio of 3:1 aqueous: organic phases (v/v) with an initial lipid concentration of 10 umol in 25% ethanol and 300 mM ammonium sulphate. Following formulation, particles were dialyzed against 300 mM ammonium sulphate using 12–14 kDa regenerated cellulose membranes (Spectrum Labs, Rancho Dominguez, 38 CA) overnight to remove residual EtOH. Prior to drug loading (see Section 4.3 Remote loading of Dox into preformed liposomes), particles were dialyzed against Dulbecco's PBS (pH 7.4) overnight using 12–14 kDa regenerated cellulose membranes. Cholesterol concentration of the particles was measured using the Wako Cholesterol E assay (Mountain View, CA) and used to determine the total lipid concentration.

Remote Loading of Dox into Preformed Liposomes: Prepared liposomes with ammonium sulphate gradient (see previous section) were combined with Dox dissolved in PBS to a final concentration of 3 mg mL^{-1} total lipid and a drug:lipid (molar) ratio of 0.1. Loading mixture was incubated at 65 °C for 30 min before being dialyzed against PBS overnight to remove any unencapsulated Dox. Dialyzed particles were sterile filtered using a 0.2 μm syringe filter (Pall, Ville St. Laurent, QB).

Characterization of Dox-paLNP and Dox-Red-paLNP: Particle size and polydispersity index (PDI) were determined through DLS using the Malvern Zetasizer NanoZS (Worcestershire, UK). Reported values correspond to number mean diameters. Cholesterol concentration of the Dox-LNP particles was determined using the Wako Cholesterol E assay (Mountain View, CA) and used to determine the total lipid concentration. The concentration of Dox in the loaded particles was measured using absorbance at 492 nm. Dox-control LNP, dox-paLNP or dox-red-paLNP samples were collected prior to the incubation step in the loading procedure as well as post-loading and dialysis. The samples were mixed with 0.5% Triton ×-100 in PBS at a dilution 1:20 dilution in a 96 well plate. After shaking briefly and incubating at room temperature for 5 min, absorbance values were measured at 492 nm. Encapsulation efficiency (percent encapsulation) was determined through comparison of the drug:lipid ratio of Dox-LNP pre-loading and post-loading and dialysis to remove unencapsulated Dox (see Section 2.3 Remote loading of Dox into preformed liposomes). Drug:lipid ratios were determined using the molar concentrations of Dox and total lipid determined through A492 and Wako Cholesterol E assay, respectively.

Cryo-TEM Imaging of Dox-Control LNP and Dox-paLNP: Control-LNP and paLNPs loaded with Dox (0.1 drug:lipid, molar) were concentrated (Amicon Ultra-15 Centrifuge Filter Units, Millipore, Billerica, MA) to a total lipid concentration of ≈25 mg mL^{-1} prior to analysis. Some samples were subject to light-triggered drug release (see Section 4.6) and compared to non-UV treated samples. Morphological liposome characteristics and Dox loading were investigated by Cryo-TEM as described previously.[39–41] In brief, liposome formulations were deposited onto glow-discharged copper grids and vitrified using a FEI Mark IV Vitrobot (FEI, Hillsboro, OR). Cryo-TEM imaging was performed using a 200 kV Glacios microscope equipped with a Falcon III camera at the UBC High Resolution Macromolecular Cryo-Electron Microscopy facility (Vancouver, BC).

Drug Release Assay of Dox-LNP Incubated in PBS: Control-LNP and paLNP particles diluted to a final concentration of 3 mg mL^{-1} total lipid in PBS were irradiated with a UV-A light source (365 nm) for 5 min followed by storage in the dark at room temperature. At the 1 h time point, the incubated sample was passed down a size exclusion column to remove free drug. The drug:lipid ratio of purified LNPs was determined as detailed in the previous section and percent retention calculated relative to the $t = 0$ h time point. Release experiments were repeated using pulsed irradiation at 365 nm over a 24 h period in the dark at room temperature, following which drug release was determined based on drug:lipid ratio as detailed in the previous section and percent retention calculated relative to the $t = 0$ h time point.

Drug Release Assay of Dox-LNP Incubated in DMEM Media Containing 10% FBS: Control-LNP and paLNP particles loaded with Dox

(0.1 drug:lipid ratio) at a final concentration of 3 mg mL^{-1} total lipid in DMEM media containing 10% FBS were irradiated with a UV-A light source (365 nm) for 15 min followed by pulsed irradiation at 365 nm over a 24 h period in the dark at room temperature. After 24 h, an aliquot was passed down a size exclusion column to remove free drug, followed by addition of a fixed ratio of isopropanol to precipitate the proteins. The supernatant was analyzed to determine the drug:lipid ratio as detailed in the previous section and percent retention calculated relative to the $t = 0$ h time point.

In Vitro Cell Viability Assay: Cell viability assay was performed using HuH7 cells—hepatocyte derived carcinoma cell line. Growth media was composed of DMEM with FBS (10%). Cells were plated in 96-well cell culture treated plates (Falcon/Corning Inc., Corning, NY) at a density of 12 500 cells/well ≈24 h prior to treatment. Either free Dox, control-LNP, paLNPs, or red-paLNPs (0.1 drug:lipid ratio) in PBS were diluted as necessary with PBS and added to the appropriate volume of media to obtain final treatment concentrations of 0, 0.1, 1, 10, and 100 µM Dox (free drug, control-LNP or paLNP). Treated cells were subject to irradiation with UV-A light (365 nm) or deep-red light (660 nm) for 15 min following by pulsed irradiation (365 or 660 nm) incubated at 37 °C and 5% CO_2 for a total of 24 h. At the 24 h time point, cell viability was analyzed using an MTT assay (Abcam Inc.) comparing UV irradiated and non-UV irradiated cells.

Confocal Imaging: Imaging was performed using HuH7 cells—hepatocyte derived carcinoma cell line. Growth media was composed of DMEM with FBS (10%). Cells were plated in confocal imaging plates at a density of 40 000 cells/well 24 h prior to treatment. Either free Dox, control-LNP or paLNPs (0.1 drug:lipid ratio) in PBS were diluted as necessary with PBS and added to the appropriate volume of media to obtain final treatment concentrations of 10 µM Dox (free drug, control-LNP, or paLNP). Treated cells were subject to irradiation with UV-A light (365 nm) for 15 min following by pulsed irradiation (365 nm) incubated at 37 °C and 5% CO_2 for a total of 24 h. At the 24 h time point, cell membranes were stained with cell mask deep red plasma membrane stain (1.0 mg mL^{-1}, Thermo Fisher Scientific). Live cell imaging comparing UV irradiated and non-UV irradiated cells was conducted using a Leica TCS SP8 laser scanning confocal microscope (Leica, Germany), equipped with a 60× oil-immersion objective (numerical aperture 1.40). Dox was excited at 488 nm argon laser and CellMask was excited with a 633 nm.[42]

Zebrafish Pharmacokinetic Studies: Embryos from Tg(kdrl:eGFP) and Tg(AB/Tübingen) adult zebrafish (*Danio rerio*) were breaded at 28 °C in zebrafish culture media containing 30.4 ug mL^{-1} 1-phenyl-2-thiourea (PTU) and maintained according to Swiss animal welfare regulations. Embryos were embedded in 0.3% agarose containing tricaine and PTU and injected with a calibrated volume of 1 nL into Duct of Cuvier (48 h post fertilization, hpf) and cardinal vein (72 hpf) using a micromanipulator (Wagner Instrumentenbau KG), a pneumatic Pico Pump PV830 (WPI) and a Leica S8APO microscope (Leica). A portion of the injected fish was exposed, starting 0.16 hpi, to the corresponding pulsed light trigger at 28 °C for 24 h. The tail region of zebrafish embryos was imaged 2 and 24 hpi using an Olympus FV3000 confocal laser scanning microscope equipped with a 30× UPIanSApo oil-immersion objective (NA 1.35). Quantitative image analysis was performed as previously described.[34,42]

Dox Release Studies in Zebrafish Embryos: 48 hpf embryos from Tg(abc/tu) were injected with 2 nL of 3 mg mL^{-1} Dox containing LNPs into the duct of Cuvier and incubated at 28 °C in PTU containing zebrafish culture media. 24 hpi, one set of zebrafish was irradiated with pulsed-light for additional 24 h. For quantitative analysis, confocal images of five fishes were processed applying a defined threshold, followed by area calculation in Fiji (ImageJ) 2.1.0.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

N.C. and J.M. contributed equally to this work. The authors acknowledge Dr. Natalie Strynadka and Dr. Claire Atkinson from the UBC High Resolution Macromolecular Cryo-Electron Microscopy facility for support. Further, the authors acknowledge Dr. M. Affolter and Dr. H.G. Belting (Biozentrum, University of Basel, Switzerland) for providing zebrafish eggs, Dr. Jörg Huwyler (Pharmazentrum) for support with zebrafish injection and imaging, and Dr. Kai Schleicher (IMCF) for supporting quantitative image analysis of zebrafish studies (all University of Basel, Switzerland). J.M. thanks the NCI for a F99/K00 award (1F99CA253758-01). D.W. was supported by the Swiss National Science Foundation (#183923). This work was supported by NMIN (the NanoMedicines Innovation Network), a member of the Networks of Centres of Excellence Canada program.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

cancer, doxorubicin, liposome, photoswitch, triggered drug release

Received: December 30, 2020
Revised: February 25, 2021
Published online: April 20, 2021

[1] T. M. Allen, P. R. Cullis, *Adv. Drug Delivery Rev.* **2013**, *65*, 36.
[2] A. Wicki, D. Witzigmann, V. Balasubramanian, J. Huwyler, *J. Controlled Release* **2015**, *200*, 138.
[3] J. Buck, P. Grossen, P. R. Cullis, J. Huwyler, D. Witzigmann, *ACS Nano* **2019**, *13*, 3754.
[4] G. Bozzuto, A. Molinari, *Int. J. Nanomed.* **2015**, *10*, 975.
[5] J. A. Kulkarni, D. Witzigmann, S. Chen, P. R. Cullis, R. van der Meel, *Acc. Chem. Res.* **2019**, *52*, 2435.
[6] A. Akinc, M. A. Maier, M. Manoharan, K. Fitzgerald, M. Jayaraman, S. Barros, S. Ansell, X. Du, M. J. Hope, T. D. Madden, *Nat. Nanotechnol.* **2019**, *14*, 1084.
[7] A. Gabizon, R. Catane, B. Uziely, B. Kaufman, T. Safra, R. Cohen, F. Martin, A. Huang, Y. Barenholz, *Cancer Res.* **1994**, *54*, 987.
[8] R. T. Chlebowski, *West. J. Med.* **1979**, *131*, 364.
[9] D. Bobo, K. J. Robinson, J. Islam, K. J. Thurecht, S. R. Corrie, *Pharm. Res.* **2016**, *33*, 2373.
[10] D. Papahadjopoulos, T. M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S. K. Huang, K. D. Lee, M. C. Woodle, D. D. Lasic, C. Redemann, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 11460.
[11] A. Gabizon, H. Shmeeda, Y. Barenholz, *Clin. Pharmacokinet.* **2003**, *42*, 419.
[12] L. D. Mayer, L. C. Tai, D. S. Ko, D. Masin, R. S. Ginsberg, P. R. Cullis, M. B. Bally, *Cancer Res.* **1989**, *49*, 5922.
[13] S. Bibi, E. Lattmann, A. R. Mohammed, Y. Perrie, *J. Microencapsulation* **2012**, *29*, 262.
[14] A. Dabbagh, B. J. J. Abdullah, H. Abdullah, M. Hamdi, N. H. A. Kasim, *J. Pharm. Sci.* **2015**, *104*, 2414.

[15] M. E. Lorkowski, P. U. Atukorale, K. B. Ghaghada, E. Karathanasis, *Adv. Healthcare Mater.* **2020**, *10*, e2001044.
[16] N. Forbes, A. Pallaoro, N. O. Reich, J. A. Zasadzinski, *Part. Part. Syst. Charact.* **2014**, *31*, 1158.
[17] D. Needham, G. Anyarambhatla, G. Kong, M. W. Dewhirst, *Cancer Res.* **2000**, *60*, 1197.
[18] U. Bulbake, S. Doppalapudi, N. Kommineni, W. Khan, *Pharmaceutics* **2017**, *9*, 12.
[19] C. D. Landon, J.-Y. Park, D. Needham, M. W. Dewhirst, *Open Nanomed. J.* **2011**, *3*, 24.
[20] Y. Dou, K. Hynynen, C. Allen, *J. Controlled Release* **2017**, *249*, 63.
[21] Celsion, *A Phase III, Randomized, Double Blind, Clinicaltrials.Gov* **2018**.
[22] C. Pernpeintner, J. A. Frank, P. Urban, C. R. Roeske, S. D. Pritzl, D. Trauner, T. Lohmüller, *Langmuir* **2017**, *33*, 4083.
[23] K. Hüll, J. Morstein, D. Trauner, *Chem. Rev.* **2018**, *118*, 10710.
[24] D. Miranda, J. F. Lovell, *Bioeng. Transl. Med.* **2016**, *1*, 267.
[25] R. H. Bisby, C. Mead, C. G. Morgan, *Photochem. Photobiol.* **2000**, *72*, 57.
[26] D. Luo, K. A. Carter, A. Razi, J. Geng, S. Shao, D. Giraldo, U. Sunar, J. Ortega, J. F. Lovell, *Biomaterials* **2016**, *75*, 193.
[27] K. A. Carter, D. Luo, J. Geng, S. T. Stern, J. F. Lovell, *Mol. Cancer Ther.* **2019**, *18*, 592.
[28] J. Morstein, A. C. Impastato, D. Trauner, *ChemBioChem* **2021**, *22*, 73.
[29] D. B. Konrad, J. A. Frank, D. Trauner, *Chem. Eur. J.* **2016**, *22*, 4364.
[30] M. M. Lerch, M. J. Hansen, G. M. van Dam, W. Szymanski, B. L. Feringa, *Angew. Chem., Int. Ed.* **2016**, *55*, 10978.
[31] R. Pattipeiluhu, S. Crielaard, I. Klein-Schiphorst, B. I. Florea, A. Kros, F. Campbell, *ACS Cent. Sci.* **2020**, *6*, 535.
[32] G. Caracciolo, *Nanoscale* **2018**, *10*, 4167.
[33] Y. Barenholz, *J. Controlled Release* **2012**, *160*, 117.
[34] S. Bleher, J. Buck, C. Muhl, S. Sieber, S. Barnert, D. Witzigmann, J. Huwyler, M. Barz, R. Süss, *Small* **2019**, *15*, 1904716.
[35] S. Sieber, P. Grossen, J. Bussmann, F. Campbell, A. Kros, D. Witzigmann, J. Huwyler, *Adv. Drug Delivery Rev.* **2019**, *151–152*, 152.
[36] S. H. Yun, S. J. J. Kwok, *Nat. Biomed. Eng.* **2017**, *1*, 0008.
[37] J. Morstein, D. Trauner, *Curr. Opin. Chem. Biol.* **2019**, *50*, 145.
[38] M. Borowiak, W. Nahaboo, M. Reynders, K. Nekolla, P. Jalinot, J. Hasserodt, M. Rehberg, M. Delattre, S. Zahler, A. Vollmar, *Cell* **2015**, *162*, 403.
[39] D. Witzigmann, S. Sieber, F. Porta, P. Grossen, A. Bieri, N. Strelnikova, T. Pfohl, C. Prescianotto-Baschong, J. Huwyler, *RSC Adv.* **2015**, *5*, 74320.
[40] S. Sieber, P. Grossen, P. Detampel, S. Siegfried, D. Witzigmann, J. Huwyler, *J. Controlled Release* **2017**, *264*, 180.
[41] J. A. Kulkarni, D. Witzigmann, J. Leung, R. van der Meel, J. Zaifman, M. M. Darjuan, H. M. Grisch-Chan, B. Thöny, Y. Y. C. Tam, P. R. Cullis, *Nanoscale* **2019**, *11*, 9023.
[42] D. Witzigmann, P. Uhl, S. Sieber, C. Kaufman, T. Einfalt, K. Schöneweis, P. Grossen, J. Buck, Y. Ni, S. H. Schenk, *eLife* **2019**, *8*, e42276.