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Loading and releasing ciprofloxacin in photoactivatable liposomes

Sanjana Ghosh\textsuperscript{a}, Ruiquan Qi\textsuperscript{b}, Kevin A. Carter\textsuperscript{a}, Guojian Zhang\textsuperscript{b}, Blaine A. Pfeifer\textsuperscript{b}, Jonathan F. Lovell\textsuperscript{a,}\textsuperscript{*}

\textsuperscript{a} Department of Biomedical Engineering, University at Buffalo, State University of New York, Buffalo, NY, 14260, USA
\textsuperscript{b} Department of Chemical and Biological Engineering, University at Buffalo, State University of New York, Buffalo, NY, 14260, USA

HIGHLIGHTS

• Ciprofloxacin was loaded into photoactivatable liposomes containing porphyrin-phospholipid.
• A formulation was optimized for antibiotic loading, stability and fast light-triggered drug release.
• Liposomes released their content in less than 30 s under 665 nm irradiation.
• Antimicrobial activity of released ciprofloxacin was observe in vitro against Bacillus subtilis.

ABSTRACT

We demonstrate that ciprofloxacin can be actively loaded into liposomes that contain small amounts of porphyrin-phospholipid (PoP). PoP renders the liposomes photoactivatable, so that the antibiotic is released from the carrier under red light irradiation (665 nm). The use of 2 mole % PoP in the liposomes accommodated active loading of ciprofloxacin. Further inclusion of 2 mole % of an unsaturated phospholipid accelerated light-triggered drug release, with more than 90% antibiotic release from the liposomes occurring in less than 30 s. With or without laser treatment, ciprofloxacin PoP liposomes inhibited the growth of Bacillus subtilis in liquid media, apparently due to uptake of the liposomes by the bacteria. However, when liposomes were first separated from smaller molecules with centrifugal filtration, only the filtrate from laser-treated liposomes was bactericidal, confirming effective release of active antibiotic. These results establish the feasibility of remote loading antibiotics into photoactivatable liposomes, which could lead to opportunities for enhanced localized antibiotic therapy.

1. Introduction

An aim of drug delivery systems is for the selective accumulation of bioavailable drugs at target sites [1–3]. Several nanoparticles have shown their capacity for site-specific drug-release by means of intrinsic and extrinsic triggers [4]. Drug delivery at target sites by intrinsic triggers like pH [5–10], enzymes [11–13] or by external application of stimulus like heat [14–18], light [19–28] and magnetic pulses [29,30] has been proposed for improving local disease treatment, while protecting healthy organs from side-effects caused by the drug.

Liposomes are lipid-based, self-assembled nanostructures used as carriers for drug delivery [31–33]. The biocompatible and versatile cargo loading properties of liposomes make them potentially useful as a carrier for antibiotic drug delivery [34]. Several clinical liposome formulations show the properties of high drug-loading stability and long blood circulation times, which may often be attributed, in part, to inclusion of a polyethylene glycol (PEG) coating to the bilayer [35]. However, the use of stable liposomes may result in delivery of the drug in a form which is not released from the liposomes at the target site and hence is not bioavailable. Various strategies have been explored to enhance drug bioavailability by conferring stimuli-triggered cargo release to liposomes. Thermosensitive liposomes that have heat-triggered release mechanisms have limited stability in physiological conditions as it is difficult to develop materials that stay stable at 37 °C but release cargo around 42 °C [14,16,36]. Alternatively, studies on light trigger methods involving design strategies and new mechanisms are still emerging [37].

Photosensitive liposomes have been developed by altering the design of the lipidome structure. One strategy is by incorporating reactive unsaturated lipids which photopolymerize under ultraviolet light to
incite permeabilization of lipid bilayers [12,28,38,39]. Another way is by inducing reactive oxygen species generation that oxidizes unsaturated phospholipids resulting in membrane permeabilization [28,40–43]. Porphyrin phospholipid (PoP) is a lipid-like molecule that can be used to produce nanostructures with theranostic applications, owing to the fluorescence, singlet oxygen and metal chelation properties of the porphyrin [44]. PoP comprises of a glycerol backbone structure of phosphatidylcholine with a palmitoyl group at the sn-1 position and the central hydroxyl group at sn-2 position esterified to a monocarboxylic porphyrin derivative [30]. In previous work, the efficiency of PoP liposomes passively loaded with gentamicin on bacterial growth has been reported [45].

Phototherapeutic antimicrobial applications including photodynamic therapy (PDT) have been explored for a range of infectious disease targets. PDT is a non-invasive treatment method that involves a light induced oxygen dependent chemical reaction which activates a photosensitizing agent that generates cytotoxic oxygen species, mainly singlet oxygen. Since these reactive species can interact with bacterial components, result in bacteria killing, PDT is considered an effective and emerging domain for antimicrobial applications [46]. It is possible that combining light-triggered release of antibiotics could offer advantages for treating local infection by providing additional molecular mechanisms for bacterial cytotoxicity.

Ciprofloxacin (1-cyclopropyl-6-fluor-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid (CIP)) is a fluoroquinolone carboxylic acid derivative with a broad spectrum of antibacterial activity against both gram positive and gram negative bacteria [47,48]. It is administered by both intravenous infusion and oral dosing form [49]. CIP is an effective fluoroquinolone used and approved a wide range of infections, including pseudomonas associated with cystic fibrosis [50] and anthrax [51]. However, there are some concerns of adverse effects. Fluoroquinolones have been associated with heart abnormalities including Torsades de Pointus (TdP) which is a rare but lethal form of polymorphic ventricular tachycardia. There are reports on TdP associated with CIP [52]. It has also been suggested that CIP administration may contribute to tendon disorders [53]. Liposomal drug formulations can have altered toxicity profiles, as is the case for liposomal doxorubicin which has been shown to have reduced cardiotoxicity [54].

2. Materials and methods

2.1. Liposome preparation

Lipids were obtained from CordenPharma International and other materials were acquired from Sigma; unless stated otherwise. 1,2-distearoyl-sn-glycerol-3-phosphocholine (DSPC, CordenPharma # LP-R4-076), cholesterol (CordenPharma # CH-0355), 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine (DOPC, CordenPharma # LP-R4-070) and 1,2-Distearoyl-phosphatidylethanolamine-methyl-polyethylene glycol conjugate-2000 (MEG-2000-DSPE or PEG-lipid, CordenPharma # LP-R4-039) were used. Porphyrin phospholipid was synthesized as previously described [55]. Various liposome formulations were prepared by dissolving 100 mg of the lipids at indicated molar ratios in 1 mL of ethanol at 60 °C, then the lipid solution was injected with 4 mL of 250 mM ammonium sulfate (pH 5.5) buffer at 60 °C. To extrude the liposomes, the lipid solution was passed 10 times through a high pressure nitrogen extruder (Northern Lipids) with sequentially stacked 0.2, 0.1 and 0.08 µm pore size polycarbonate membranes at 60 °C. Removal of free ammonium sulfate and ethanol was done by dialysis (at least twice) in an 800 mL solution of 10% sucrose with 10 mM phosphate buffered saline (PBS; pH 5.8). Ciprofloxacin (CIP, Sigma Aldrich 17850-5G-F) was loaded into the liposomes by adding CIP in 1:10 drug:lipid (D:L) loading molar ratio and incubating the solution at 60 °C for 60 min.

2.2. Liposome characterization

Liposome sizes and polydispersity index were determined with dilution in PBS by dynamic light scattering in a NanoBrook 90 plus PALS instrument. Loading efficiency was estimated by passing 1 mL of loaded liposomes over a Sephadex G-75 column and collecting 24 × 1 mL fractions. The loading efficiency of the liposomes was measured as the percentage of the drug determined by the amount of drug fluorescence in the column fractions containing liposomes. CIP fluorescence was measured with an excitation of 277 nm and emission of 455 nm.

2.3. Cell viability

MIA PaCa-2 cells were cultured in Dulbecco’s modified eagle’s medium supplemented with 10% fetal bovine serum (FBS). Cells were always maintained at 37 °C and 5% CO₂. For cell viability assay, 1 × 10⁴ cells were seeded in a 96-well plate and placed in the incubator for 48 h. After 48 h, cells were incubated with either CIP liposomes or Dox liposomes at the indicated concentrations for 24 h. Dox liposomes were prepared as previously mentioned [30]. For CIP liposomes, the final optimized formulation DSPC:CHO:Lipid:CIP:PEG-lipid with a molar ratio of 58:33:2:2:5 was used. Cells were washed with PBS post incubation and were incubated in fresh media with PBS for 24 h. Cell viability was then assessed by removing the media from the 96-well plate and adding 100 µL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) stock solution into each well. 100 µg mL⁻¹ of XTT and 60 µg mL⁻¹ of N-methyl dibenzopryazine methyl sulfate were added to PBS. After adding XTT stock solution to 96-well plate, the plate was incubated at 37 °C for 2 h. Absorption was measured in a TECAN Safire 96-well plate at 450 nm, with 630 nm as background wavelength. Cell viability of treated cells was calculated relative to untreated control cells.

2.4. Fluorescence emission spectra and light release experiments

The fluorescence emission spectra of CIP in liposomes and liposomes alone with and without Triton X100 detergent was investigated with a fluorometer (PTI) at excitation of 277 nm. The CIP light release experiments were conducted by diluting liposome samples 1000 times in PBS and then irradiating it with a 665 nm diode laser (RPMC laser, LDX-3115-665) at a fluence rate of ~310 mW/cm². CIP release was determined in real time with PTI and the percentage of CIP release was calculated by the formula % Release = (Final−Initial)/(FTX-100−Initial) × 100%.

2.5. Antibacterial effect of liposomes

*Bacillus subtilis* (CmR) culture was inoculated in liquid LB media at 37 °C overnight. The CIP PoP liposomes were pre-treated with laser at fluence rate of 310 mW/cm² for 10 min. The CIP concentration in CIP PoP liposomes was 0.01 µg/mL. For experiments with filtrates of CIP PoP liposomes, laser treated or untreated CIP PoP liposomes were centrifuged and filtered using eppendorf tubes with 100 kDa membrane filters at 15,000 rcf for 15 min. *B. subtilis* was incubated in LB broth with empty PoP liposomes or laser treated/untreated CIP PoP liposomes with constant agitation of 250 rpm at 37 °C. The growth of the bacteria was monitored by measuring the Optical Density (OD) of the culture at 0, 1, 2, 4, 6, 8 and 10 h respectively.

3. Results and discussion

3.1. Drug loading and light-triggered release

Liposomal ciprofloxacin (CIP) formulations have previously been demonstrated using active loading techniques using ammonium sulfate [56,57]. To generate liposomes loaded with CIP, we prepared Pop...
liposomes with an ammonium sulfate gradient for active-loading, as we previously have done for doxorubicin [30]. Beyond a few molar percent PoP in the bilayer, doxorubicin loading into PoP liposomes was impeded, however other drugs such as irinotecan did not exhibit this limitation [58]. The reason for this is not clear, but might relate to the large size or crystalline-like properties of the actively-loaded doxorubicin fibrinous bundles, which can exert forces on the bilayer and de-form the liposomes, which might further be de-stabilized in these conditions in the presence of PoP. To evaluate how this phenomenon would apply to CIP, we prepared liposomes with DSPC:Cholesterol (molar ratio 67:33) and PoP (in different mole%) was titrated into the liposomes, replacing DSPC. Liposomes with 2 M % PoP showed effective loading of CIP, which was found to be 95% of the drug added (Fig. 1A). At higher PoP amounts, slightly lower loading efficiency was observed. However, CIP could still be loaded reasonably well into liposomes with a substantially higher PoP content of at least 15 M %, so there was no restriction that would prevent formation of CIP-loaded PoP liposomes with high porphyrin content.

The size and polydispersity index of the liposomes were evaluated at different stages of preparation. Fig. 1B shows that the size of PoP liposomes decreased to about 100 nm after extrusion and remained almost the same size until and after CIP was loaded. This observed size indicates the generation of well-formed liposomes free from larger aggregates. Fig. 1C shows that the polydispersity index of the PoP liposomes dropped after extrusion and remained close to 0.1 at all stages of the preparation. A polydispersity index of in this range generally indicates a liposome population with good monodispersity.

The fluorescence emission spectra of CIP loaded in PoP liposomes was examined. Fig. 2A shows the actively loaded drug was substantially quenched, and as a result, has a fluorescence emission spectra that is barely detectable. However, upon addition of detergent, released CIP had a strong emission spectra peak with a maximum intensity near 440 nm. PoP liposomes themselves did not exhibit background fluorescence at the CIP emission wavelength with or without detergent lysis (Fig. 2B). The mechanism for fluorescence quenching of CIP inside the liposome are not known, but likely involve electronic interactions of the drug at molecular high density that gives rise to contact quenching. These spectral properties of CIP in liposomal or free form provide a convenient method for monitoring the release of CIP from liposomes.

Light-triggered release of CIP from PoP liposomes containing 2 M % PoP was assessed in vitro, in PBS. As shown in Fig. 2C, CIP PoP liposomes were irradiated with 665 nm laser at four different laser fluence rates from 50 to 300 mW/cm² for 10 min. PoP liposomes released CIP at all the laser fluence rates, with the fastest release observed at the highest fluence rate of 300 mW/cm². At laser fluence rate of 300 mW/cm², more than 90% of the encapsulated CIP was released in less than 4 min. Without laser irradiation, no CIP release was observed (0 mW/cm²). Therefore, PoP liposomes that encapsulate CIP were suitable for light-triggered cargo release.

### 3.2. DOPC accelerates light-triggered drug release

To expedite release of CIP from CIP PoP liposomes, an unsaturated lipid DOPC was titrated into CIP loaded PoP liposomes of formulation DSPC:ChOL:PoP (molar ratio 65:33:2) replacing DSPC and the NIR light-triggered release of CIP was tested. In previous studies, it has been shown that light-triggered cargo release from PoP liposomes is greatly accelerated by the inclusion of a small amount of DOPC or other unsaturated lipids [28,59–61]. Accelerated light-triggered cargo coincides with oxidation of the unsaturated lipids, which presumably leads to faster bilayer destabilization. Hence, we conducted a comparative study on the NIR light triggered CIP release from CIP PoP liposomes containing different amounts of DOPC titrated into PoP liposome formulation replacing DSPC. This study was conducted in vitro in PBS under 665 nm laser at laser fluence rate of ~310 mW/cm² for 10 min. We observed that 2 M % DOPC titrated into PoP liposomes showed fastest release of 100% of the encapsulated drug as shown in Fig. 3A. Fig. 3B shows the influence of different laser fluence rates on the rate of CIP release from the liposome formulation [DSPC:ChOL:PoP:DOPC] molar ratio (63:33:2:2). Fig. 3C shows the rapid release of CIP as an effect of DOPC at different laser fluence rates as compared to the formulation without DOPC. Under 665 nm laser irradiation at fluence rate of 300 mW/cm², adding 2 mol% DOPC into PoP liposomes escalated the release rate to an extent such that more than 90% of the loaded CIP was released in less than 0.5 min whereas the formulation without DOPC took about 4 min. Fig. 3D shows that the drug loading capability of the new formulation of PoP liposomes with DOPC was not reduced much as compared to the previous formulation devoid of DOPC. Fig. 3E shows the size of the liposomes contracted after extrusion and remained close to 100 nm even after loading the drug. Fig. 3F shows that the polydispersity index of liposomes reduced after extrusion and remained lesser than 0.1 even after loading CIP which indicates that the liposomes were in a monodisperse pool of nanoparticles. However, when the CIP PoP liposomes were stored at 4 °C, they had a tendency to settle at the bottom of the storage tube. In a previous study, it was reported that inclusion of 5 M % of a PEG-lipid enabled them to have long blood circulation properties, with excellent storage stability and rapid light triggered release [30]. Therefore, to optimize the formulation, we substituted a portion of PEG-lipid into PoP liposomes (replacing DSPC), which resulted in improved colloidal stability as a homogenous suspension, without settling. Therefore, the finalized PoP liposome formulation developed was [DSPC:ChOL:PoP:DOPC:PEG-lipid] with a molar ratio of [58:33:2:2:5].

### 3.3. Antibacterial activity of CIP PoP liposomes

The antimicrobial efficiency of the CIP PoP liposomes was assessed by comparing the effects of laser treated or untreated CIP PoP liposomes and empty PoP liposomes on the growth patterns of *B. subtilis* (a

![Fig. 1. Loading CIP into PoP liposomes. A) Loading efficiency of liposomes consisting of DSPC:CHOL (molar ratio 2:1) including different amounts of PoP. B) Change in size (effective diameter) of liposomes at different stages of liposome preparation and loading. C) Change in Polydispersity index of liposomes at different stages of liposome preparation and loading. Data show mean +/− S.D. for n = 3.](image-url)
model gram positive bacteria. We observed that although empty liposomes did not show any inhibition in the growth of bacteria, laser treated and untreated CIP encapsulated liposomes showed nearly equivalent efficiency in inhibiting the growth of bacteria. The growth curve of *B. subtilis* with CIP-liposomes plus laser and minus laser showed similar growth patterns over 10 h incubation (Fig. 4A). To investigate the reason behind high bacteria killing efficiency of untreated CIP-liposomes, the ability of bacteria to internalize PoP liposomes was tested. We detected PoP fluorescence increasing in the bacterial cells over time (Fig. 4B). This showed that the bacteria took up the PoP liposomes effectively. To confirm that the light-triggered release of CIP still generated functional antibiotic, we separated small molecules from CIP PoP liposomes following laser treatment using microcentrifugal filtration. We treated bacteria with filtrates of laser treated

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**Fig. 2.** Fluorescence emission spectra of CIP and light-triggered cargo release: Emission spectra of CIP-loaded PoP liposomes (A) or empty PoP liposomes alone (B), with or without disruption by Triton X 100 detergent ("det."). An excitation wavelength of 277 nm was used to excite CIP. C) Light-induced release of CIP from liposomes consisting of DSPC:PoP:Chol (molar ratio 65:2:33) under 665 nm irradiation at indicated fluence rates. Data show the mean of three experiments.

**Fig. 3.** DOPC accelerates light-triggered CIP release from PoP liposomes. DOPC was included in PoP liposomes comprising DSPC:PoP:Chol (mole ratio 65:2:33). A) Laser-induced release of CIP under 665 nm irradiation from liposomes with different amounts of DOPC (added in place of DSPC). B) Laser-induced CIP release under 665 nm irradiation from liposomes with 2% DOPC at indicated fluence rates. C) Laser irradiation time required for 90% CIP release of indicated liposome formulations, measured in PBS. D) CIP loading into PoP liposomes with indicated amounts of DOPC. Liposome size (E). and polydispersity (F) at different stages of liposome preparation. Data show mean +/− S.D. for n = 3.
or untreated CIP loaded liposomes and measured growth over time. This showed that no inhibition of growth was found in bacteria with the filtrate of untreated CIP PoP liposome samples whereas ~100% inhibition of growth was seen in samples with filtrate of laser treated CIP PoP liposomes (Fig. 4C, D). Thus, the approach of light-triggered CIP release is effective in releasing the intact antibiotic from the liposomes. Cell viability assay of CIP PoP liposomes was carried out using XTT assay. This showed that at all concentrations CIP PoP liposomes showed minimal toxicity to mammalian cells, compared to doxorubicin-loaded PoP liposomes (Fig. S1).

Taken together, this work shows that CIP PoP liposomes can be used for light-triggered release of an actively loaded liposomal antibiotic. However, no functional differences with or without laser treatment were observed with respect to antimicrobial growth in the conditions assessed. It is possible that in vivo, the behavior of the free and liposomal drug would be different, as other parameters such as pharmakoekinetics and pharmacodynamics are at play. It has further been shown that a photodynamic effect in irradiated tissues leads first to enhanced vascular penetration of the liposomes followed by release of the cargo which could enhance in vivo biodistribution and bioavailability [62]. Further studies would be required examine in vivo behavior of CIP PoP liposomes to determine if greater local antibiotic concentrations can be achieved in response to target tissue irradiation with light.

4. Conclusion

We report a PoP lipidosome formulation of CIP with active loading and rapid light-induced CIP release. High CIP encapsulation and significant light-induced release of CIP was achieved by in liposomes containing 2 mole % PoP, while entrapment efficiency of CIP into PoP liposomes decreased slightly with increasing amounts of PoP. Inclusion of 2 mole % DOPC further accelerated light-triggered CIP release. Following light-triggered release from PoP liposomes, CIP was effective in inhibiting the growth of Bacillus subtilis, although similar bacterial inhibition was observed for lipidosome-entrapped CIP. Future studies should examine in vivo behavior of CIP PoP liposomes and elucidate experimental conditions in which the functional antimicrobial role of light-triggered antibiotic release is more apparent.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bej.2018.10.008.

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