Biomaterials 75 (2016) 193–202

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Doxorubicin encapsulated in stealth liposomes conferred with light-triggered drug release

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ARTICLE INFO

Article history:
Received 26 August 2015
Received in revised form
8 October 2015
Accepted 13 October 2015
Available online 23 October 2015

Keywords:
Liposomes
Chemotherapy
Phototherapy
Doxorubicin
Porphyrin-phospholipid
Chemophototherapy

ABSTRACT

Stealth liposomes can be used to extend the blood circulation time of encapsulated therapeutics. Inclusion of 2 molar % porphyrin-phospholipid (PoP) imparted optimal near infrared (NIR) light-triggered release of doxorubicin (Dox) from conventional sterically stabilized stealth liposomes. The type and amount of PoP affected drug loading, serum stability and drug release induced by NIR light. Cholesterol and PEGylation were required for Dox loading, but slowed light-triggered release. Dox in stealth PoP liposomes had a long circulation half-life in mice of 21.9 h and was stable in storage for months. Following intravenous injection and NIR irradiation, Dox deposition increased ~7 fold in treated subcutaneous human pancreatic xenografts. Phototreatment induced mild tumor heating and complex tumor hemodynamics. A single chemophototherapy treatment with Dox-loaded stealth PoP liposomes (at 5–7 mg/kg Dox) eradicated tumors while corresponding chemo- or photodynamic therapies were ineffective. A low dose 3 mg/kg Dox phototreatment with stealth PoP liposomes was more effective than a maximum tolerated dose of free (7 mg/kg) or conventional long-circulating liposomal Dox (21 mg/kg). To our knowledge, Dox-loaded stealth PoP liposomes represent the first reported long-circulating nanoparticle capable of light-triggered drug release.

Preferential accumulation of drugs at target sites in bioavailable form is a central goal of drug delivery systems [1–4]. Liposomes are applied for this purpose and are commonly used pharmaceutical carriers [5,6]. By incorporating a synthetic polyethylene glycol phospholipid (DSPE-PEG-2K) into stable liposomes, sterically stabilized "stealth" liposomes are obtained and can substantially prolong drug circulating time and increase drug accumulation in the tumors with enhanced antitumor efficacy [7–10]. However, once localized in the tumor, the drug should be released at an appropriate rate to ensure therapeutic concentrations reach target cells. Long circulating stealth liposomes release their cargo slowly, which limits efficacy in cancer treatment [11,12]. Exposure of large amounts of bioavailable drug in tumors is desirable, while ideally reducing exposure to healthy organs [1,13,14]. Remotely-triggered drug delivery systems hold potential to meet this need [15,16]. There has been interest in developing liposomes that effectively encapsulate anti-cancer agents and release them specifically within tumors. To this end, many liposome triggered-release strategies have been developed including activation methods based on pH [17–20], heat [13,21,22], enzymes [23,24], light [3,25,26] and magnetic pulses [27] and thermosensitive liposomes have entered clinical testing for multiple cancer indications [28].

Light, especially near infrared (NIR) light which can better penetrate tissues and is otherwise harmless itself, is an intriguing external trigger for drug release and can be applied with precise spatial and temporal control [3,26,29,30]. There has been much recent interest in development of light-triggered drug delivery systems [31–39]. Compared to thermosensitive liposomes, which have been extensively studied for more than three decades [40,41], light triggered release has seen less development and the release mechanisms, factors controlling the release rate and design strategies are still emerging. However, thermosensitive liposomes and other heat-triggered mechanisms (including photothermally

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http://dx.doi.org/10.1016/j.biomaterials.2015.10.027
0142-9612/

triggered) generally have limited stability in physiological conditions due to difficulty in developing materials that are stable at 37 °C but that can release their contents close to 42 °C. There have been only a few reports of long-circulating thermosensitive materials, and drugs loaded into such carriers only have a fraction of the circulation time of conventional stealth liposomes [42]. To our knowledge, until now there have not yet been any reports of long-circulating nanoparticles for light-triggered release.

Porphyrin-phospholipid (PoP) is a lipid-like molecule and can be used to form nanoparticles with theranostic character [43–48]. The structure of the PoPs used in this study comprises a monocarboxylic porphyrin derivative esterified to the central sn-2 hydroxyl of the glycerol backbone of phosphatidylcholine containing a palmitoyl group at the sn-1 position. The chlorophyll derived pyropheophorbide-*a* (Pyro) or related 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-*a* (HPPH) were used to generate Pyro-lipid or HPPH-lipid respectively. We previously reported that PoP-liposomes based on HPPH-lipid can release their contents in response to NIR light, via a mechanism that is still unknown [26]. However, relatively high amounts of HPPH-lipid were required, which in theory could lead to patient side effects such as sunlight-induced or treatment-induced cutaneous toxicity [49–51]. Furthermore, examination of HPPH-lipid liposomes revealed that the stability in 50% serum and light-triggered release rates were less than ideal. Here, we describe a systematic approach to develop stealth PoP liposomes with balanced lipid ratios to achieve both rapid light release rate and high storage and serum stability with long blood circulation.

1. Materials and methods

1.1. Preparation of PoP liposomes

Unless otherwise noted, lipids were obtained from Avanti and other materials were obtained from Sigma. HPPH-lipid and Pyro-lipid were synthesized as previously reported [26]. Various liposome formulations were all made using the same method. The finalized stealth PoP liposome formulation included 53 mol. % 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, Avanti #850365P), 40 mol. % cholesterol (Avanti #700000P), 2 mol. % Pyro-lipid and 5 mol. % 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG-2K, Avanti #880120P). To generate 100 mg of PoP liposomes (a 5 mL batch), 57.1 mg DSPC, 19.1 mg DSPE-PEG-2K, 2.76 mg Pyro-lipid and 21.1 mg cholesterol were fully dissolved in 1 mL ethanol at 60–70 °C, then 4 mL 250 mM ammonium sulfate (pH 5.5) buffer was injected into the mixed lipids (both mixed lipids and ammonium sulfate buffer were kept at 60–70 °C while injection). Lipids and buffer were fully mixed. The solution was passed 10 times at 60–70 °C through sequentially stacked polycarbonate membranes of 0.2, 0.1 and 0.08 μm pore size using a high pressure nitrogen extruder (Northern Lipids). Free ammonium sulfate was removed by dialysis in a 800 mL solution of 10% sucrose with 10 mM histidine (pH 6.5) with at least 2 buffer exchanges.

1.2. Cargo loading and release of PoP liposomes

Doxorubicin (Dox; LC Labs # D-4000) was loaded by adding the indicated ratio of drug and incubating at 60 °C for 1 h. Liposome sizes were determined by dynamic light scattering in PBS. Loading efficiency was determined by running 25 μL of liposomes diluted in 1 mL PBS over a Sephadex G-75 column. 24 x 1 mL fractions were collected and the loading efficiency was determined as the percentage of the drugs in the liposome-containing fractions (which elute in the in the first 3–10 mL). Dox was measured using fluorescence with an excitation of 480 nm and emission of 590 nm using a TECAN Safire fluorescent microplate reader. Light-triggered release experiments were performed using a power-tunable 665 nm laser diode (RPMC laser, LDX-3115-665) at a fluence rate of ~310 mW/cm^2 and Dox release was measured in real time in a fluorometer (PTI). Irradiations were performed in 50% bovine serum (Pel-Freez #37218-5) at 37 °C. Temperature was measured by inserting a K-type thermocouple probe directly into the irradiated solution. Dox release was assessed by measuring the release before and after treatment. Release was calculated using the formula: % Release=(F_{final}-$F_{initial}$)/($F_{X\text{-}100}$-$F_{initial}$) × 100%.

1.3. Cryo-electron microscopy

For cryo-EM, holey carbon grids (c-flat CF-2/2-2C-T) were prepared with an additional layer of continuous thin carbon (5–10 nm) and treated with glow discharge at 5 mA for 15 s. Then, 3.4 μL of liposome loaded with doxorubicin in buffer containing 10% sucrose solution and 10 mM histidine (pH 6.5) were applied to the grids for 30 s. The lipid concentration of the liposome solution was approximately 20 mg/mL. To perform the specimen vitrification, grids were blotted and plunged in liquid ethane at ~ -170 °C using a Vitrobot (FEI) with the blotting chamber maintained at 25 °C and 100% relative humidity. Liposomes were imaged in a JEOL2010F transmission electron microscope at 200 kV using a Gatan 914 cryo-holder. Images were collected at x 50,000 magnification and using a total dose of ~20 electrons per Å^2 and a defocus range between −7 and −11 microns. Images were recorded in SO-163 films. Micrographs were digitized in a Nikon Super Coolscan 9000 scanner.

1.4. Liposome storage stability

Dox loaded stealth PoP liposomes (drug to lipid molar ratio 1:5) were stored at 4 °C in closed amber vials without any other precautions and liposomes were periodically removed for routine analysis. Loading stability, size, polydisperity, serum stability and light triggered release rates were assessed every two weeks for 3 months with 3 separately prepared batches of liposomes. Liposomes sizes were determined in phosphate buffered saline (PBS) by dynamic light scattering. For serum stability measurements, liposomes were diluted 200 times (to 13.5 μg/mL Dox) in PBS containing 50% bovine serum (Pel-Freez #37218-5). Initial readings were taken and samples were incubated at 37 °C for 6 h. 0.5% Triton X-100 was used to lyse the liposomes and final fluorescence value were read. Dox release was calculated according to the formula: % Release= (F_{final}-$F_{initial}$)/($F_{X\text{-}100}$-$F_{initial}$) × 100%. Loading stability and light triggered release rates were determined as described above.

1.5. Pharmacokinetics

All procedures in this work performed on mice were approved by the University at Buffalo Institutional Animal Care and Use Committee. Female mice (female CD-1, 18–20 g, Charles River) were intravenously injected via tail vein with Dox-PoP-liposomes, sterically stabilized liposomal Dox or 10% HPPH liposomes (10 mol. % HPPH-lipid, 35 mol. % cholesterol, 5 mol. % DSPE-PEG-2K and 50 mol. % DSPC) at dose of 10 mg/kg Dox(n = 4 per group). Small blood volumes were sampled at sub-mandibular and retro-orbital locations at the indicated time points. Blood was centrifuged at 2000 × g for 15 min and 10 μL serum was added to 990 μL extraction buffer (0.075N HCl, 90% isopropanol) and stored for 20 min at −20 °C. The samples were removed and warmed up to room temperature and centrifuged for 15 min at 10,000 × g. The supernatants were collected and analyzed by fluorescence. Dox concentrations were determined from standard curves.