Journal of Controlled Release 194 (2014) 71–81

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Localized delivery of doxorubicin *in vivo* from polymer-modified thermosensitive liposomes with MR-guided focused ultrasound-mediated heating

Terence Ta [a,*], Elizabeth Bartolak-Suki [a], Eun-Joo Park [b,1], Kavon Karrobi [a], Nathan J. McDannold [b], Tyrone M. Porter [c]

[a] Department of Biomedical Engineering, Boston University, 44 Cummington Street, Boston, MA 02215, USA
[b] Department of Radiology, Harvard Medical School, Brigham and Women's Hospital, 221 Longwood Avenue, Boston, MA 02115, USA
[c] Department of Mechanical Engineering, Boston University, 110 Cummington Street, Boston, MA 02215, USA

ARTICLE INFO

Article history:
Received 17 February 2014
Accepted 13 August 2014
Available online 23 August 2014

Keywords:
Drug delivery
Thermosensitive liposomes
Smart polymers
MR-guided focused ultrasound
NIPAAm
Chemotherapy

ABSTRACT

Thermosensitive liposomes have emerged as a viable strategy for localized delivery and triggered release of chemotherapy. MR-guided focused ultrasound (MRgFUS) has the capability of heating tumors in a controlled manner, and when combined with thermosensitive liposomes can potentially reduce tumor burden *in vivo*. However, the impact of this drug delivery strategy has rarely been investigated. We have developed a unique liposome formulation modified with p(NIPAAm-*co*-PAA), a polymer that confers sensitivity to both temperature and pH. These polymer-modified thermosensitive liposomes (PTSL) demonstrated sensitivity to focused ultrasound, and required lower thermal doses and were more cytotoxic than traditional formulations *in vitro*. A set of acoustic parameters characterizing optimal release from PTSL *in vitro* was applied in the design of a combined MRgFUS/PTSL delivery platform. This platform more effectively reduced tumor burden *in vivo* when compared to free drug and traditional formulations. Histological analysis indicated greater tumor penetration, more extensive ECM remodeling, and greater cell destruction in tumors administered PTSL, correlating with improved response to the therapy.

1. Introduction

Traditional thermosensitive liposomes (TSL) for triggered release of chemotherapy are comprised of lipids that undergo temperature-dependent phase transitions, becoming more permeable at elevated temperatures [1–4]. More recently, lysolipids have been employed to modify formulations, greatly increasing the rate and amount of drug release while reducing the thermal dose threshold for release [5–7]. However, lysolipids have been shown to desorb from the membrane in the presence of plasma proteins and cellular membrane pools [8,9], resulting in a reduction in thermosensitivity [9] and premature drug leakage at physiological conditions (up to 80% within 30 min at 37 °C) [10–13].

An alternate approach is to incorporate synthetic polymers that are membrane-disruptive and trigger drug release in response to heating. Recently we copolymerized *N*-isopropylacrylamide (NIPAAm) and propylacrylic acid (PAA) to produce a membrane-disruptive copolymer that is sensitive to both temperature and pH (Fig. 1) [11], the latter component aiming to take advantage of the tumor microenvironment which is known to be mildly acidic [12]. We showed that liposomes modified with NIPAAm-*co*-PAA stably entrapped drug in serum-containing media and released more than 50% of entrapped doxorubicin rapidly when heated ($T \geq 40$ °C) or subjected to mild acidity (pH ≤ 6.5) [11]. Thus, these polymer-modified thermosensitive liposomes (PTSL) can be used for localized deposition of chemotherapy *in vivo* provided that solid tumors can be heated in a localized and controlled manner.

Focused ultrasound (FUS) has emerged as a leading modality for non-invasive, heat-triggered drug release from thermosensitive liposomes [14]. Studies with TSL have shown improved tumor response with alternative modalities such as microwave antennas and applicators [15]. These suffer from limited therapeutic depth and are thus only suitable for superficial tumors, and offer less spatial and temporal control of heating compared to FUS [14,15]. Furthermore, the emergence of MR-compatible FUS transducers has allowed for the adoption of MR thermometry as a tool to noninvasively monitor the spatial distribution of heat deposited by FUS [16–18]. Recent studies have shown that MR-guided focused ultrasound (MRgFUS) can be utilized to deposit heat and trigger local drug release from thermosensitive liposomes within

* Corresponding author at: 881 Massachusetts Avenue, Apt. 34, Cambridge, MA 02139, USA. Tel.: +1 808 741 6313.
E-mail addresses: terence.ta@gmail.com (T. Ta), ebartola22@gmail.com (E. Bartolak-Suki), eunjoo.park@gmail.com (E.-J. Park), kkarrobi@bu.edu (K. Karrobi), njm@bwh.harvard.edu (N.J. McDannold), tmp@bu.edu (T.M. Porter).
[1] Present address: 101 Daehak-ro, Jongno-gu Seoul 110-744, South Korea.

http://dx.doi.org/10.1016/j.jconrel.2014.08.013
0168-3659/

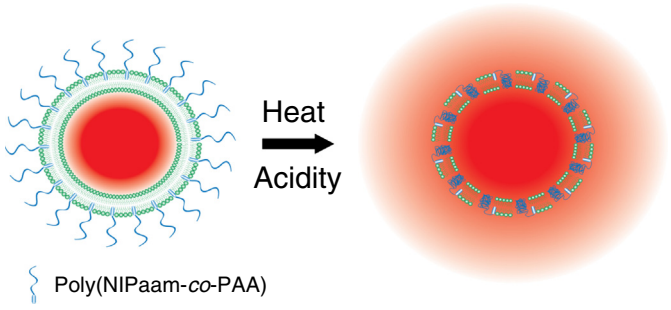
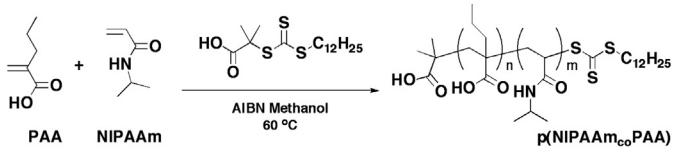


Fig. 1. DOX release from p(NIPAAm-*co*-PAA)-modified thermosensitive liposomes (PTSL). Release is triggered by both heating and reduction in pH.



Scheme 1. RAFT copolymerization of *N*-isopropylacrylamide (NIPAAm) and propylacrylic acid (PAA), originally published in [11].

solid tumors [19–22]. However, the impact of this drug delivery strategy on tumor growth has rarely been investigated.

Therefore, the main objective of this study was to evaluate the response of solid tumors to DOX released from PTSL via sustained MRgFUS heating. First, DOX release from PTSL was assessed as a function of acoustic intensity and pulsing scheme. Knowledge gained from these experiments was used to tailor the output from an MRgFUS transducer to sustain a desired elevated temperature in tumors *in vivo*. PTSL were designed to release DOX in mildly acidic and/or hyperthermic conditions, both of which are achievable in solid tumors. Thus, we conducted *in vitro* studies to assess the cytotoxicity of DOX released from our dual-sensitive PTSL as a function of temperature and pH. Finally, we evaluated the response of solid tumors to DOX released from PTSL heated with MRgFUS by measuring tumor growth and through histological analysis of tumor remodeling at the cellular level.

2. Materials and methods

2.1. Materials

N-isopropylacrylamide (NIPAAm), 2,2′-azobis(2-methylpropionitrile) (AIBN), propylacrylic acid (PAA), doxorubicin hydrochloride (DOX), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and manganese sulfate ($MnSO_4$) were purchased from Sigma (St. Louis, MO, USA). NIPAAm was recrystallized in hexane and AIBN recrystallized in methanol prior to use. 2-Methyl-2-[(dodecylsulfanylthiocarbonyl)sulfanyl] propanoic acid (DMP) was purchased from Strem Chemicals, Inc. (Newburyport, MA, USA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), L-a-phosphatidyl-choline(soy-hydrogenated) (HSPC), cholesterol (CHOL), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG-2000) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). MTT cell proliferation assay kits, McCoy's 5A medium, human mammary adenocarcinoma MCF7 cells, and rat mammary adenocarcinoma 13762 MAT B III cells were obtained from American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was purchased from Life Technologies (Grand Island, NY). Dulbecco's Modified Eagle Medium (DMEM), Penicillin–Streptomycin (PS), and L-Glutamine (L-Glu) were purchased from Corning (Manassas, VA).

2.2. Polymer synthesis and characterization

p(NIPAAm-*co*-PAA) was synthesized using RAFT chemistry with initiator AIBN, chain transfer agent DMP, and monomers NIPAAm and PAA (Scheme 1), as described in [11] with minor modifications. NIPAAm (2.18 g, 19.3 mmol), PAA (0.138 g, 1.21 mmol), DMP (60.8 mg, 167 μmol), and AIBN (15.3 mg, 93.2 μmol) were reacted in degassed methanol at 60 °C for 17 h. Alternatively, DMP and AIBN concentrations were halved to study the effect of degree of polymerization (DP, $DP = M_0 / (CTA_0 + 2 \cdot I_0)$, where M_0, CTA_0, and I_0 are initial concentrations of monomer, chain transfer agent, and initiator, respectively). Following precipitation into pentane, dried polymer was dissolved in ethanol and dialyzed against deionized water to remove unreacted monomer and impurities. Samples were then frozen and lyophilized. Polymer molecular weights were determined using a gel permeation chromatograph (Viscotek) with dimethylformamide (DMF) as eluent (1 ml/min) and polystyrene calibration standards. Copolymer composition was determined via ^{1}H NMR (Varian 400 MHz FT-NMR, deuterated chloroform as solvent) and analysis of peaks as in [11].

2.3. Preparation and characterization of DOX-loaded liposomes

DOX-loaded liposomes were prepared by the lipid film hydration and extrusion method followed by pH-gradient-driven DOX loading as previously described [11], with slight modifications. Nonthermosensitive liposome (NTSL), traditional thermosensitive liposome (TSL), and polymer-modified thermosensitive liposome (PTSL) were generated, with compositions as follows: NTSL = HSPC:CHOL:DSPE-PEG-2000 at 75:50:3 molar ratio, TSL = DPPC:HSPC:CHOL:DSPE-PEG-2000 at 100:50:30:6, and PTSL with identical composition as TSL but with the addition of copolymer (see below). Chloroform was removed by rotary evaporation (Buchi R-210), leaving a dried film that was then rehydrated in 300 mM $MnSO_4$ (pH 3.5), giving a suspension with lipid concentration of 10 mg/ml. The suspension was extruded [11] after which unentrapped $MnSO_4$ was removed and pH gradient established by dialysis (1 kDa MWCO) against 20 mM HEPES buffer (pH 7.5). DOX was added to the dialyzed suspension at a 1:10 drug-to-lipid mass ratio and the suspension incubated at 38 °C for 1 h and then at room temperature overnight. Unentrapped DOX was removed by dialysis (1 kDa MWCO) against 20 mM HEPES. For PTSL, polymer at 2.5% molar polymer:lipid was added to DOX-loaded liposomes and liposomes were incubated at 30 °C for 1 h and then at room temperature overnight. A final dialysis (50 kDa MWCO) against HEPES removed unincorporated polymer. For the *in vitro* cytotoxicity and *in vivo* studies, suspensions were sterile-filtered (0.2 μm) prior to administration. For the *in vivo* study, a greater concentration of lipid (50 mg/ml) was used to provide more highly concentrated drug suspensions. Liposome size and zeta potentials were measured via dynamic light scattering (Brookhaven Instruments, 90Plus Zeta). DOX:lipid mass ratios were calculated by taking advantage of the self-quenching properties of DOX [11] and quantifying lipid content via total phosphorus assay [23].

2.4. Measurement of FUS-triggered release

Drug release from the various formulations was studied as a function of time, temperature, and pH. Two heating modalities were compared: conventional heating with a circulating water bath and heating from focused ultrasound. Drug release from liposomes heated via bath was measured as in [11], and compared to release behavior observed in liposomes exposed to FUS-mediated heating from a 5-MHz transducer (Sonic Concepts).

Prior to the release studies, a custom-assembled radiation force balance was used to calibrate the transmitted acoustic intensity (*I*, source of heating) from the transducer as a function of peak-to-peak excitation voltage (V_{pp}) (Supplementary material, Fig. S1). The setup illustrated in Fig. 2 was then used to measure temperature elevation as a function of *I*. A mixture of 50% glycerol in 20 mM HEPES (v/v)