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Lysosome-oriented, dual-stage pH-responsive polymeric micelles for β -Lapachone delivery

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Abstract

β -Lapachone (β -lap), a novel anticancer agent, is bioactivated by NADP(H):quinone oxidoreductase 1 (NQO1), an enzyme over-expressed in numerous tumors, including lung, pancreas, breast, and prostate cancers. Fast renal clearance and methemoglobinemia / hemolytic side-effects from the clinical formulation (β -lap-hydroxyl propyl- β -cyclodextrin complex) hindered its clinical translation. Here, we investigated a dual model pH responsive polymers for β -lap delivery. Three pH-sensitive linkages, including acylhydrazone, ketal and imine bonds for β -lap prodrug syntheses result in an aryl imine linkage the most optimal linkage. The conversion to β -lap was 2.8%, 4.5% and 100% at pH 7.4, 6.5 and 5.0 in 8 h, respectively. β -lap aryl imine prodrug conjugated ultra pH-sensitive (UPS) polymer reached high β -lap loading density (8.3%) and exhibited dual-stages responsiveness to pH variation. In pHs under pH_t , at stage I, micelle immediately dissociation and subsequently entering stage II, micelles start quickly release β -lap. *In vitro* release study showed that the micelles constantly release β -lap ($14.9 \pm 0.1\%$) at pHs above pH_t in 72 h, whereas boosted release of β -lap ($79.4 \pm 1.2\%$) at pH 5.0. Micelle intracellular distribution predominantly in the lysosome organelle guaranteed their pH responsive dissociation and subsequently β -lap controlled release. The M-P micelles retained NQO1-dependent cytotoxicity in A549 lung cancer cells, similar to free drug in both efficacy and mechanism of cell death. The lysosome-oriented dual-stage ultra pH responsive β -lap prodrug micelles potentially offer an alternative nanotherapeutic strategy for lung, as well as other NQO1+ cancer therapies.

Keywords

β -Lapachone (β -lap); prodrug micelles; staged pH responsive; Ultra pH-sensitive; drug release

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Introduction

Lung cancer is account for 28% males and 26% females cancer related death in the United States in 2014, while 80% of which is Non-small cell lung cancer (NSCLC)¹. Current clinical chemotherapies, such as combination of carboplatin and paclitaxel or small molecular kinase inhibitors, have low efficacy in NSCLC patients, and emergent drug resistance occurs after prolonged treatment. New therapeutics with high therapeutic efficacy and selectivity targeting lung cancer are in great need.

β -Lapachone (β -lap) is a natural product with significant anti-tumor efficacy that acts by a bioactivation mechanism dependent on expression of NADP(H):quinone oxidoreductase 1 (NQO1), a two-electron oxidoreductase overexpressed in numerous tumors, including breast², prostate³, pancreas⁴ and NSCLC⁵. β -Lap undergoes a futile redox cycle resulting in rapid formation of reactive oxygen species (ROS) and simultaneous poly(ADP-ribose) polymerase 1 (PARP1)-dependent degradation of NAD⁺ pools. Each mole of β -lap can reduce 60 moles NAD(P)H and produce 120 moles of H₂O₂ and other ROS in 2 mins. This causes DNA base damage and single-strand breaks⁶, hyper-activation of PARP1, loss of NAD⁺/ATP pools, and irreversible cell death⁷. Cell death by β -lap is independent of p53, cell cycle and Rb status, and no drug resistance has been found to date due to a potent bystander effect caused by H₂O₂ production⁸. Despite its unique mechanism of action, clinical utilization of β -lap (as ARQ501) is limited, although a new analog, ARQ761, is currently in Phase I clinical trials against NQO1+ solid cancers. Free β -lap has a low aqueous solubility of 0.038 mg/ml, which limits the direct administration in patients. In ARQ501, hydroxylpropyl β -cyclodextrin (HP- β -CD) was used to solubilize β -lap in the formation of inclusion complexes. However, low binding affinity (binding constant = $1.1 \times 10^3 \text{ M}^{-1}$)^{9, 10} resulted in the rapid dissociation of the complex, fast renal clearance and short half-life (0.4 h) in blood. In addition, hemolysis and methemoglobinemia were found to be major side-effects, causing withdrawal of ARQ501 from clinical trial¹¹. Poly(ethylene glycol)-*b*-poly(D,L-lactic acid) (PEG-*b*-PLA) micelles provided significant improvements to overcome hemolysis or methemoglobinemia side-effects. However, low drug loading density (~2.2%) limited delivery of β -Lap efficiency and precluded GMP-related scale-up for clinical translation¹².

The great application potential of stimuli responsive polymer in light, thermal and pH attracts huge interest into disease therapy^{13, 14}. pH, one of the most important physiological factors, plays a critical role both extracellularly and intracellularly. The acidic pH (pH=4.7–6.5) in endosome / lysosome is essential for cellular metabolites turnover¹⁵, and the slightly acidic tumor extracellular micro-environment pH (6.5–6.8) is an essential condition in cancer cell proliferation¹⁶. AB diblock amphiphilic copolymer with Carboxyl or amine groups have been reported to render polymeric micelles pH-sensitivity^{17–19}. Our previous investigations have shown alkylamine-based amphiphilic copolymers allowing ultra pH response in a broad and tunable pH range^{20, 21}, the monomers in ultra pH sensitive polymers (UPS) are critical to achieve a sharp pH transition and micelle morphology variation, different pH transition polymers were generated while tune the monomers of block copolymers, their application quickly extended to gene therapy and cancer imaging^{22, 23}.

Classical pH responsive drug delivery systems include either encapsulated anticancer payload in pH responsive carriers^{24–26} or conjugated drug with non-pH sensitive polymers via pH liable bonds^{27, 28}. In this study, to achieve β -lap precisely pH responsive release and improved loading, we investigated conjugation of pH sensitive β -lap prodrugs with UPS polymer. pH sensitivity evaluation to three prodrug linkages acyl hydrazone^{29–31}, ketal³² and imine bonds^{33, 34} (Scheme 1a) led to the selection of aryl imine linkage for the optimal β -Lap pH responsive prodrugs. In this study, monomer 2-(diisopropylamino) ethyl methacrylate (DPA) which has a transition $pH_t = 6.3$ was introduced to construct hydrophobic segment of UPS polymer, and by introducing multiple of β -lap aryl imine prodrug into the UPS polymer chain via chemical conjugation, a precisely pH sensitive drug delivery system was obtained. The β -lap loaded micelles exhibited lysosome-oriented, staged release profile (Scheme 1b) and the release kinetic of β -lap disclosed the polymer system achieve lysosome targeted and β -lap controlled release.

Materials and methods

Materials

All solvents and reagents were of analytical or HPLC grade and purchased from Sigma-Aldrich or Fisher Scientific unless otherwise stated. Deuterated solvents were obtained from Sigma. The 2-(diisopropylamino) ethyl methacrylate were recrystallized from ethyl acetate prior to use. Poly(ethylene glycol) methyl ether (mPEG-5000, Mn 5000) were purchased from Sigma Aldrich and purified before use by passing through a column filled with neutral alumina. Copper(I) bromide (CuBr, 99.99%), 2-bromo-2-methylpropionyl bromide, N,N,N',N'',N'''-Pentamethyldiethylenetriamine (PMDETA, 98%), p-Toluenesulfonic acid monohydrate (TsOH, 98%), 5-amino-1-pentanol (98%), 4-amino-2-methyl-phenol (98%), acylhydrazine (98%), N-(4-Aminophenyl)maleimide (Mal, 95%), Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), DL-Dithiothreitol (DTT), 2-(diisopropylamino) ethyl methacrylate (DPA, 99%) were all purchased from TCI America. Dialysis membrane (Molecular weight cut-off 3500, regenerated cellulose) was received from Fisher. β -Lap was synthesized from lapachol as described³⁵.

Syntheses of β -Lap prodrug analogues

β -Lap prodrug (E)-1-(4-(2,2-dimethyl-5-oxo-3,4-dihydro-2H-benzo[h]chromen-6(5H)-ylideneamino)phenyl)-1H-pyrrole-2,5-dione (IV) (Scheme 2) was synthesized following published procedures³⁶. Briefly, β -Lap (100 mg, 0.41 mmol), was dissolved in 3 mL anhydrous dichloromethane under Argon atmosphere with addition of 1 M $TiCl_4/CH_2Cl_2$ (206 μ L, 0.206 mmol), after stirring for 10 min, the solution of N-(4-Aminophenyl)maleimide (77.75 mg, 0.41 mmol) in 2 mL dichloromethane was added in and followed by dry triethylamine (345 μ L, 2.46 mmol). With 15 min interval, another portion of $TiCl_4 / CH_2Cl_2$ (206 μ L, 0.206 mmol) were added followed with (345 μ L, 2.46 mmol) Et_3N . The resulting mixture was extracted with 20 mL H_2O three times and the CH_2Cl_2 phase was dried with $MgSO_4$ and evaporated in vacuum. 123.6 mg (yield 73.2%) of prodrug (IV) was obtained by silicone gel chromatography with eluent Hexane/ EtOAc = 5:1.

The synthesis procedure of prodrug (I–III) (Scheme 2) are shown in the supplementary file. In the process of synthesis of β -lap imine prodrug, two β -lap derivatives (V) and (VI) were obtained via acid catalyzation, their chemical structure was shown in supplementary file Figure S1. The NMR and high-resolution mass spectrometer characterization of prodrug (Z)-N'-(2,2-dimethyl-5-oxo-3,4-dihydro-2H-benzo[h]chromen-6(5H)-ylidene)acetohydrazide (**I**), (2'r,5'r)-5'-(hydroxymethyl)-2,2,5'-trimethyl-3,4-dihydrospiro[benzo[h] chromene-6,2'-[1,3]dioxan]-5(2H)-one (**II**), (2's,5's)-5'-(hydroxymethyl)-2,2,5'-trimethyl-3,4-dihydro-spiro[benzo[h]chromene-6,2'-[1,3]dioxan]-5(2H)-one (**III**), (E)-1-(4-(2,2-dimethyl-5-oxo-3,4-dihydro-2H-benzo[h]chromen-6(5H)-ylideneamino)phenyl)-1H-pyrrole-2,5-dione (**IV**), (E)-6-(5-hydroxypentylimino)-2,2-dimethyl-3,4-dihydro-2H-benzo[h]chromen-5(6H)-one (**V**) and 3,3,11-trimethyl-2,3,13,13a-tetrahydrobenzo[a]pyrano[2,3-c]phenol-xazin-12(1H)-one (**VI**), see supplementary file Figure S2–13. The absolute structure conformation of prodrug (II) and Prodrug (III) were discussed in the supplementary file, see Figure S15–16.

Synthesis of mPEG_{5k}-Br and PDSM monomer

mPEG_{5k}-Br was synthesized through dissolved mPEG_{5k}-OH (10 g, 2 mmol) in 100 mL of toluene in a round bottom flask with reflux at 140 °C for 3 hours to remove H₂O. After removing toluene under vacuum, anhydrous CH₂Cl₂ (125 mL), Et₃N (0.55 mL, 8 mmol) was added to the residue, followed by adding 2-bromoisobutyl-yl-bromide (1 mL, 8 mmol) in 30 mL CH₂Cl₂ dropwise in 2 hours under nitrogen atmosphere. After 24 hours, 2 mL water was added into the vigorously stirring solution, rinsed with 1 M HCl aqueous solution, 1M NaOH and saturated NaCl solution each for three times individually. The dried crude product was condensed and dropped into 800 mL ethyl ester three times to obtain white solid^{29, 30} (Scheme 3a). 2-(pyridin-2-yl)disulfanyl ethyl methacrylate (PDSM) monomer was synthesized according to the literatures^{37, 38}.

Synthesis of Methoxy Polyethylene Glyco-b-poly(diisopropylaminoethyl methacrylate-co-(2-(2-pyridyl)disulfide)ethyl methacrylate) (mPEG-b-P(DPA-r-PDSM))

mPEG_{5k}-Br (300 mg, 0.6 mmol), DPA (642 mg, 30 mmol), PDSM (74.9 mg, 4.0 mmol), PMDETA (21 mg, 1.2 mmol) were dissolved in a mixture of 0.2 mL anisole, 0.3 mL DMF and 0.3 mL isopropanol³⁷. After the flask underwent 3 freeze-thaw processes in liquid nitrogen, CuBr (8.6 mg, 0.6 mmol) was added under N₂ atmosphere. The reaction was kept stirring at 65 °C for 24 hrs (Scheme 3b). Cu complexes were removed with a short neutral Aluminum oxide column with THF as eluent, followed by dialysis against DI water for 2 days and lyophilized to obtain a white powder.

Synthesis of Methoxy Polyethylene Glyco-b-poly(diisopropylamino ethyl methacrylate-co-2-(3-(4-(3,4,4a,5-tetrahydro-2,2-dimethyl-5-oxo-2H-benzo[h]chromen-6(10bH)-ylideneamino)phenyl)-2,4-dioxocyclopentylthio)ethyl methacrylate) (mPEG-b-P(DPA-r-PDSM(IV))

mPEG_{5k}-b-P(DPA₄₅-r-PDSM₆) (38.5 mg, 2.39 μ mol) was dissolved in 2 mL of anhydrous DMF, then 0.2 mL 1 mol / L TCEP solution in 0.1 mol / L NaOAc / HOAc buffer at pH 8.0 was added, after 30min, prodrug (IV) (9.2 mg, 22.3 μ mol) in 1 mL DMF was added. The

reaction was kept stirring for 4 h, followed by purification on Sephadex LH-20 column with CHCl_3 / MeOH = 3:1 as eluent, after removal of solvent, the gray solid was dissolved in anhydrous DMF and used as stock solution of mPEG-b-P(DPA-r-PDSM(IV) (M-P polymer). ^1H NMR analysis indicate about 6 prodrug IV were conjugated into each polymer chain (Scheme 3c).

Preparation and characterization of polymeric micelles

M-P polymeric micelle were prepared by solvent sonication method³⁵. Briefly, polymer in DMF solution was added drop-wise into deionized water under ultra-sonication. The micelle solution was purified by using the centrifugal filter device (Millipore, MWCO 3,000) and was set to a stock concentration of 10 mg / mL in deionized water.

In vitro β -Lap prodrug conversion and β -lap release from M-P polymeric micelle

To determine release kinetics of β -lap from prodrug IV and M-P polymeric micelles, prodrug IV was dissolved in a mixture solution of acetonitrile - NaOAc / HOAc (0.1 mol / L) buffered solutions (30–70, v/v) of pH 7.4, 6.5 and 5.0 at a final concentration of 20 μg / mL; M-P polymeric micelle was dissolved in NaOAc / HOAc (0.1 mol / L) buffered solution of pH 7.4, 6.5 and 5.0 at a final concentration of 200 μg / mL. At predetermined time points, 20 μL of sample solution was injected into HPLC to analyze content of β -lap converted from prodrug IV or from M-P polymeric micelle. The HPLC system was consisted of a quaternary pump, a vacuum degasser, an auto-sampler, a C18 column (4.6 mm $\times$ 250 mm, Agilent, CA) and UV detector at 257 nm (Agilent 1260, CA, USA). The mobile phase acetonitrile / water (70:30) at a flow rate of 1.0 mL / min were used. Prodrug IV conversion and β -lap release ratio in the samples were calculated from calibration curves. Total β -lap content in micelles was determined by disruption M-P micelle with 0.1 M HCl followed by analysis with HPLC.

The β -lap cumulative release ratio from prodrug (IV) and from β -lap loaded M-P micelles were calculated using the following formulae:

$$\text{Cumulative Release \%} = \frac{\text{AUC of } \beta\text{-lap released from samples}}{\text{AUC of all } \beta\text{-lap released from samples}} \times 100\%$$

Size and morphology of M-P polymeric micelles

The dynamic size and size distribution of the M-P polymeric micelle were determined by dynamic light scattering (DLS). The M-P polymeric micelle in different pH values (pH 7.4, 6.5, and 5.0) was determined over time at a concentration of 0.2 mg / mL on a Malvern zeta-sizer (Malvern Nano-ZS 90 laser particle size analyzer, Malvern Instruments Ltd, Malvern, Worcestershire, UK). After equilibration for 1 min, the particle size and distribution of micelles were determined with 633 nm laser at 25 $^\circ\text{C}$ and a scattering angle of 173 $^\circ$. All results were estimated from the mean of the six runs, and the intensity weighted mean diameter was expressed as mean $\pm$ standard deviation (SD). The particle morphology was observed on a Tecnai Spirit (120kV) transmission electron microscopy at an accelerating voltage of 120 kV. Micelles were deposited onto 400-mesh copper grids (Electron

Microscopy Sciences) that were pre-coated with a thin film of Formvar (poly (vinyl formal)) and carbon. Samples were allowed to sit on the grids for a few seconds and excess solution removed with a blotter. Samples were stained with uranyl acetate and the digital images were taken with a CCD camera.

Cellular distribution of M-P micelles

To determine the intracellular distribution of polymer, A549 cells at logarithm phase were seeded onto 6-well cell culture dishes at a cell density of 5×10^4 cells/well. After incubated for 24 h, tetramethyl-rhodamine-5-maleimide (Rho) conjugated Polymer (Rho-P), Rhodamine at 5 μM in cell culture DMEM medium with 10% FBS, after incubation for 1h or 4 h, the medium were removed and rinsed with PBS, the cellular nuclei were stained with 10 μM Hoechst 33342 for 10 min and lysosome were stained with 50nM LysoTracker Green for 10 min, respectively, followed by rinsed with PBS and further replenished with fresh medium and culture at 37°C. The dishes were observed with Confocal laser scanning microscopy (CLSM, Olympus FV1000, Japan), Hoechst 33342 were excited at 405nm, and the emission were recorded at 430–460nm, Rhodamine was excited at 543nm and the emission were recorded at 560–600 nm, LysoTracker Green was excited at 488 nm and the emission was recorded at 505–525nm. All images were obtained and processed with Fluoview software.

NQO1-mediated toxicity in A549 NSCLC cells

Long-term, relative survival was assessed based on DNA content as described³⁹. Briefly, A549 cells were seeded at 5×10^3 /well in 48-well tissue culture dishes. Twenty-four hours (24 h) later, cells were mock (0.01% DMSO or nanoparticles without drug)-treated or exposed to various doses of β -lap, M-P polymeric micelles or M-P polymeric residue for indicated times in the presence or absence of the fairly specific NQO1 inhibitor, dicoumarol (DIC, 40 μM) as indicated. After 2 h exposure, cells were exposed to drug-free medium and allowed to grow for 5–7 days until control cells reached ~100% confluence. DNA content was determined by freeze/thaw exposure and Hoechst 33258 staining in water and fluorescence detection using a plate reader (Perkin-Elmer, Boston, MA). Results were graphed as means $\pm$ SE in sextuplicate from independent experiments repeated three times.

Results and Discussion

Evaluation of prodrug linkages for β -lap

To achieve β -lap improved loading via ultra pH-sensitive polymer in NQO1-dependent and tumor-selective therapy. We chose the carbonyl group adjacent to the aromatic ring of β -lap with higher reactivity to evaluate several types of pH-sensitive linkages, including acylhydrazone, ketal and imine bonds, six β -lap prodrugs and analogues were synthesized with the possible pH liable linkers. Hydrolysis studies were performed at different pHs.

Acylhydrazone linker is liable in acidic conditions and was frequently used for construction of pharmaceutical agents or delivery of anticancer drugs in polymeric micelle system^{24, 31, 40, 41}. We used acetyl hydrazine as a model compound to prepare the acylhydrazone prodrug and evaluate its pH sensitivity. Acylhydrazine reacted with β -lap to

form a β -lap prodrug (I) with high yield. The conversion of prodrug (I) to parent drug was examined by UV-vis spectrum, the results indicate that at neutral and acidic condition (pH 5.0), β -lap was not converted. Even after 24 h in pH 1.0 buffer, there was no sign of β -lap formation (data not shown). This result was quite different from the data presented by Bae Y. et al.⁴¹, in a DOX delivery PEG-*b*-P(Asp-Hyd(ADR)) polymeric micelles system, DOX was conjugated into the polymer chain at its C-13 ketone position with an alkyl acylhydrazone linkage, where micelles remained stable at physiological pH, but released about 30% DOX from ADR at late endosomal / lysosomal pH within 72 h. Another study by Kwon et al.⁴⁰, also used the acylhydrazone strategy to deliver paclitaxel analogues by PEG-*b*-P(Asp-Hyd-LEV-PTX) and PEG-*b*-P(Asp-Hyd-4AB-PTX). Alkyl and aromatic linkers were introduced between the polymer acylhydrazine pendent group and PTX. The alkyl LEV acylhydrazone bond exhibited rapid release of LEV-PTX at pH 5.0, and slower release at pH 7.4. In contrast, the aromatic acylhydrazone bond, 4AB-PTX, did not show prodrug release at pH 5.0 or 7.4. These phenomena indicated the electron withdrawing effect (4AB linker) and the electron donating effect (LEV linker) were responsible for their C=N bond lability, the electron balance exist in both side of the C=N bond from electron donating group LEV linker and acylhydrazine group is responsible for their suspension to acid, however, the electron charge imbalance to two side of C=N bond from the electron donating group acylhydrazine and electron withdrawing group 4AB enhanced its stability. This was also verified by Amit. Et al.⁴² that hydrazone bond containing PEG-HZ-PE polymer derived from the aliphatic aldehyde was pH sensitive, and was irresponsive if which was derived from aromatic aldehyde. For the β -lap acylhydrazone prodrug (I), the imbalance of electron withdrawing effect between the aromatic rings in β -lap and electron donating effect from the acylhydrazine part enhanced the stability of the C=N bond, which reduced the tendency of cleaving the C=N bond even in pH 1.0 buffer. These data suggested that the acylhydrazone bond was too stable to convert to β -lap and was not suitable for polymer conjugation.

Another pH liable chemical bond, ketal linkage, was frequently used for protecting carbonyl groups³² and for pH-responsive polymer synthesis^{43, 44}. Conjugation of 1,1,1-trimethanolethane 2-(hydroxymethyl)-2-methyl-1,3-propanediol (Tri-OH) resulted in two β -lap ketal prodrugs, (II) and (III). The two ketal prodrugs resulted little conversion of β -lap at pH 7.4 in PBS buffer and pH 5.0 in 0.1 M NaOAc-HOAc buffer over 72 h. Even at pH 1.0, the half-lives of the two ketal prodrugs were ~4 h (see supplemental Figure S14). Their stability to pH condition were similar to the ketal-containing chemicals, an example was the study of ketal / acetyl containing polymer hydrogel by Griset et al.³², in which whether the ketal bond can be acid cleaved was critically depended on the substitute groups on both side of the ketal bond. With the electron donating group -OCH₃ on its 2, 4, 6 positions of benzaldehyde, the chemical was stable at neutral pH, but hydrolyzed at a mildly acidic pH: ~5.0. In contrast, with only hydrogen atom at these positions, hydrolysis only noted under acidic conditions (pH: <1.0). This is also verified in another research by Eric et al.⁴⁵ that Ac-DEX polymer hydrolyze in acidic environment (pH5.0) in 24 h, whereas kept stable in pH 7.4 buffer even after 72 h. Consequently, the stability of β -lap ketal prodrugs (II) and (III) was due to the electron donating -O-C-O- structure of ketal ring and the electron withdrawing aromatic ring of β -lap, the overall electron effects strengthened the stability of

the ketal bond and the two prodrugs. Due to the low pH responsive behavior, these results also excluded β -lap ketal linkages as candidates for β -lap pH-sensitive conjugation.

In the process of synthesizing β -lap imine prodrugs, an oxidative cyclization reaction occurred between the two carbonyl group of β -lap and amine reactant when two amine compounds 5-amino-1-pentanol and 4-amino-2-methylphenol reacted with β -lap in the existence of acid. The cyclization reaction stabilized the structure of β -Lap with five-membered ring in product (E)-6-(5-hydroxypentylimino)-2,2-dimethyl-3,4-dihydro-2H-benzo[h]chromen-5(6H)-one (**V**) and hexatomic ring in product 3,3,11-trimethyl-2,3,13,13a-tetrahydrobenzo[a]pyrano[2,3-c]phenol-xazin-12(1H)-one (**VI**) (see supplemental file, Figure S1), this lead to it is impossible for β -lap recovery in any condition (data not shown).

A β -lap aryl imine prodrug **IV** was obtained by conjugation of N-(4-aminophenyl) maleimide (**Mal**), which is bearing a maleimide structure, with β -lap successfully. The pH-responsiveness of prodrug **IV** was sensitive to acidic conditions, and stable under neutral pH (Fig. 1). Prodrug **IV** cumulative conversion to β -lap at different pH environments (7.4, 6.5 and 5.0) was determined by HPLC analyses. The release profile indicated that after 8 h only $4.5 \pm 1.1\%$ and $2.8 \pm 0.5\%$ of prodrug **IV** was converted into β -lap at pH 6.5 and pH 7.4, respectively. In comparison, conversion of β -lap was complete at the same time at pH 5.0 (Fig. 1a–b). Data were normalized by single HPLC tracings, and showed the content variation of prodrug **IV** and β -lap and the stacked HPLC chromatogram exhibited the quick hydrolysis of prodrug **IV** and arise of β -lap content. As shown that only trace of β -lap (5 min peak) and Mal linker (2.9 min peak) were detected immediately after blend the stock solution of prodrug **IV** with the hydrolysis buffer (pH 5.0), while most of prodrug **IV** (11.5 min peak) kept integral. After 40 mins, peaks of β -lap and Mal linker were greatly enhanced, while peak of prodrug **IV** was significantly decreased, indicating the half-process of prodrug **IV** conversion. After 6 h, only the Mal linker and β -lap peaks could be observed suggesting completion of **IV** hydrolysis. The half-life of prodrug **IV** at pH 5.0 was about 49 mins (Fig. 1–c). The slow release at neutral or slightly acidic pH and fast conversion of prodrug **IV** at pH 5.0 was due to the electron withdrawing effect from the aromatic structure at both sides of the C=N bond. When exposure of prodrug **IV** to acidic condition, protonation of C=N bond can easily break the weak balance from both side of C=N bond. This phenomena is consistent with the hydrolysis profile of 2,2-dimethyl-(Z)-6-(4-methoxyphenylimino)-3,4,5,6-tetrahydro-2H-naphtho[1, 2-b]oxin-5-one reported by Chenna et. al., which could be cleaved under acidic conditions while keep stable under neutral pH, The pH-responsive hydrolysis profile of prodrug **IV** suggested its potential utility for conjugation to polymer pmicelles³⁶.

Synthesis of Polymer mPEG_{5k}-b-P(DPA₄₅-r-PDSM(**IV**)₆)

UPS polymers with ionizable groups in the polymer hydrophobic chain could respond to the variation of proton concentration. Micelle formation and its thermodynamic stability were driven by the delicate balance between the hydrophobic and hydrophilic segments^{8, 22, 23}. Ionizable groups can act as tunable hydrophilic / hydrophobic blocks at different pH values, which directly affects the dynamic self-assembly of micelles. Amino groups have been incorporated into polymers as ionizable groups to render pH sensnizitivity. To achieve

staged, pH-dependent β -lap release, a block copolymer was synthesized by incorporating monomer 2-(diisopropylamino) ethyl methacrylate (DPA) into polymer as ionizable groups to render pH sensitivity^{46, 47}. This endow polymeric micelles a transit pH at of 6.3, which confer its micelles dissociation or assemblization once exposed to pH under or above this pH_t . To conjugate the β -lap imine prodrug IV into the polymer hydrophobic chain, monomers of PDSMs were also incorporated into the polymer hydrophobic chain, and by adjusting the number of PDSM monomers, the loading efficiency of β -lap could be tuned. Block copolymer $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM₆) was synthesized through atom transfer radical polymerization (ATRP) procedure with $mPEG_{5k}$ -Br as a macromolecular initiator. The numbers of DPA and PDSM were calculated as 45 and 6, respectively, by defining -CH₂-CH₂-proton number of $mPEG_{5k}$ as 450 ($\delta=3.64$) in ¹H NMR in CDCl₃. Polymer $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM(IV)₆) was obtained via high efficient conjugation of prodrug IV with polymer $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM₆) through thio-maleimide conjugation after removal of the protecting thiol-pyridine group. ¹H NMR showed the thiol groups in polymer $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM₆) were all depleted by the Maleic structure of prodrug IV. Proton assignment of ¹H NMR on $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM₆) polymer and drug loaded polymer $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM(IV)₆) indicated the successful construction of the target block copolymer. The number of prodrug IV on $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM(IV)₆) was also calculated by HPLC analysis after incubating $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM(IV)₆) polymer micelles under pH 1.0 HCl solution for 12 h to release all β -lap molecules (see supplemental file, Figure S17). In this defined structure, β -lap loading density reached 8.3% by weight, It is about 4 times higher than PEG-*b*-PDLLA (2.2%) encapsulation. ¹H NMR proton of $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM₆) and $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM(IV)₆) polymers were assigned in Fig. 2.

Characterization of β -lap prodrug micelles

Polymeric micelles of $mPEG_{5k}$ -*b*-P(DPA₄₅-*r*-PDSM(IV)₆) were fabricated by the solvent sonication method. The morphology of polymeric micelles was determined by transmission electron microscopy (TEM) in pH 7.4, 6.5 and 5.0 buffers. TEM images indicated that at pH 7.4 and 6.5, micelles were spherical particles with clear margin and diameter of 25 ± 5 nm (Fig. 3a and 3b). Size and morphology were quite stable and no significant differences were noted at the two pH conditions, even after 24 h incubation. In contrast, micelles dissociated immediately after incubation in pH 5.0 buffer. This phenomenon is consistent with previous observations²⁰. Dynamic light scattering (DLS) data showed that the M-P micelles had a hydrodynamic diameter of 27 ± 5 nm with narrow size distribution at pH 7.4 and pH 6.5 after incubation for 24 h. however, under pH 5.0 condition, after 5 min, the detectable dynamic size was only about 2.3 nm (Fig. 3d), which is consistent with the TEM size. The size and morphology variation at different pH conditions disclosed the ultra-pH responsiveness of the micelle system, and suggests micelle will be able to keep its payload in hydrophobic micelle core in pH conditions above their transition pH, while expose the pH labile linkages to protonation after their responsively dissociation in pH condition under that.

In vitro β -lap release from M-P micelles

The release kinetics of β -lap from M-P micelles was evaluated in buffer at pH 7.4, 6.5 and 5.0 over 72 h by HPLC analysis. During the hydrolysis process in pH 5.0 buffer, M-P micelles released β -lap continuously, lacking a burst release. Normalized M-P micelle hydrolysis HPLC tracings indicated that the β -lap peak increased constantly (Fig. 4a). The release speed of β -lap showed an accelerating inclination during the first 12 h followed by a subsequent reduction (Fig. 4b). Release of β -lap from M-P micelles reached 50% after 16 h incubation in pH 5.0 buffer (Fig. 4b). Compared to the hydrolysis at pH 5.0, release of β -lap from M-P micelles in buffers at pH 7.4 or 6.5 were only slightly noted, even after 72 h incubation, the cumulative release ratio of β -lap from M-P micelles was $14.9 \pm 0.1\%$ and $20.3 \pm 0.2\%$ respectively, whereas, $79.4 \pm 1.2\%$ of β -lap was released at pH 5.0 (Fig. 4b). Even though slightly release at pHs over transition pH to DPA was observed, the significant difference was observed between the lysosomal pH and the other two pHs (Fig. 4c). β -lap release from the M-P micelles was consistent with the release of β -lap from prodrug IV, the postponed half-life of β -lap from M-P micelles was probably because of the increasing hydrophobicity, which reduced the local proton concentration. These results indicated that the dual pH model ultra pH sensitive polymer system distinctly exhibited a dual stage responsiveness to pH conditions. In pHs under pH_t, at stage I, micelle immediately dissociation and followed by stage II, micelles quickly release payload drug β -lap. These phenomena suggest the pH responsiveness of M-P polymeric micelles to small pH variations may provide and proved a cancer therapeutic regimen that allows maintenance of long-lasting circulation of delivered β -lap via the blood without exposure to red blood cells avoiding hemolysis / methemoglobinemia, while allowing dissociation accompanied with fast release of β -lap in lysosomal pH (5.0–5.5).

M-P polymeric micelle cellular distribution

Lysosomes are the target cellular organelle in which our UPS polymer and the pH sensitive linkages response to the pH conditions. To trace the intracellular distribution of the polymeric micelle system, tetramethylrhodamine-5-maleimide (Rho) labeled polymer mPEG-*b*-P(DPA-*r*-PDMS) (Rho-P) was synthesized to simulate M-P micelles cellular distribution. Rho dye was primarily applied as control to distinguish from Rho-P micelles, Rho dye distributed into cytosol quickly but seldom co-localize with lysosome, even after 1 h incubation, as shown in Fig. 5a. After A549 cells were treated by Rho-P micelles for 1 h, 62.3% Rho-polymer micelles co-localized with the lysosome organelle (Figure 5b). and prolonged incubation to 4 h, the ratio increased to 78.6% (Figure 5b). This indicated that the Rho-P micelles predominantly enter cell lysosome. This high degree of cellular distribution in the lysosome organelle guaranteed the requirement of M-P micelles pH responsive dissociation and subsequently β -lap controlled release.

M-P micelles induced NQO1-mediated toxicity in A549 NSCLC cells

To determine the NQO1-dependent cytotoxicity effects of β -lap loaded M-P micelles, A549 cells were treated with β -lap, β -lap-loaded M-P micelles or the β -lap free M-P polymer residue. Within ~2 h exposure, a low dose of β -lap ($>4 \mu\text{M}$ for A549 cells; Fig. 6a) was sufficient to cause NQO1-specific lethality⁵. In contrast, exposure of cells to β -lap for 24 h

led to nonspecific death of cells independent of NQO1 expression (Fig. 6b), due to one-electron oxido-reduction by b5R and p450R⁵. Compared to free β -lap, β -lap-conjugated M-P micelles did not show significant lethality at up to 20 μ M after a 2 h treatment (Fig. 6c). However, with prolonged incubation time, noticeable lethality in both dose- and time-dependent manners were noted (Fig. 6d–f). Lethal doses of β -lap loaded M-P micelles decreased from 20 μ M after 4 h exposure (data not shown) to only 3 μ M after 24 h incubation (Fig. 6f), indicating that β -lap was gradually converted and released from prodrug micelles after cell uptake, consistent with the *in vitro* β -lap release study. When cells were treated with β -lap loaded M-P micelles in medium free of fetal bovine for 2 h, cytotoxicity to A549 cells was almost the same as that with FBS for 12 h (Fig. 6g). In contrast, no lethality was observed after treatment with M-P polymer residue alone for up to 24 h (Fig. 6h). To further demonstrate that the cytotoxicity caused by β -lap released from the β -lap-loaded M-P micelles was NQO1-specific, dicoumarol (a fairly specific NQO1 inhibitor) was used. These results confirmed that cell death noted in A549 cells after β -lap-loaded M-P micelle treatment was NQO1-dependent, caused by release of conjugated β -lap in the micelle particles. Similar to β -lap, the therapeutic window of β -lap-loaded M-P micelle particles decreased with a continuous treatment of 24 h period. The cytotoxicity studies *in vitro* showed that micelles possess similar NQO1-dependent tumor-selective lethality as β -lap alone, indicating that conjugated prodrug IV can be released from pH-responsive micelles and converted to parent drugs under acidic environments. However, compared to β -lap, the prodrug micelles showed less toxicity when incubating with cells at early time. Interestingly, similar IC₅₀s were observed after cells were incubated for a longer time. This would not be an issue if NQO1 overexpression tumor cells selectively take up the drug, as long as β -lap release in the blood stream was kept to a minimum. The delayed lethality is not an issue, since NQO1-dependency was maintained. The observed delayed toxicity is likely due to: *i*) lower efficiency of cell uptake for micelle nanoparticles; *ii*) endosome-engulfing dynamics for micelles and pH interference; *iii*) delayed drug release from micelles; and *iv*) the metabolic dynamic change of β -lap between intake directly from the extracellular environment into the cytosol and release from lysosome into the cytosol. A more detailed mechanism on the cell uptake of these prodrug micelles and β -lap escape from endosome / lysosome compartments remains to be investigated. Nevertheless, the unique therapeutic window of β -lap prodrug micelles, as an efficient and stable delivery vehicle, shed lights on the clinical application of this novel quinone drug for tumor-selective therapy. The delayed toxicity of micelles might also favor the flexibility on the design of different therapeutic strategies when combined with other chemotherapeutic agents or IR.

Conclusion

In summary, we report the evaluation of pH responsive β -lap prodrug loaded UPS polymer achieved improved drug loading and NQO1 mediated therapy via a lysosome-oriented, staged pH responsive manner. By evaluation of the possible linkages for construction of β -lap prodrugs, the aryl imine linkage exhibited superior responsiveness to pH-induced conversion to β -Lap. The prodrug IV conjugated M-P polymeric micelle system exhibited dual stage pH responsiveness to pH changes due to the occurrence of the two pH sensitive component - PDPA segment and the imine linkages in one polymer. M-P Micelle

intracellular distribution predominantly in the lysosome organelle guaranteed their pH responsive dissociation and subsequently β -lap controlled release. The toxicity studies in A549 NSCLC cells disclosed the M-P micelles appropriately reach its destination for taking effect and had the similar antitumor efficacy as free β -lap, causing NQO1-dependent mechanism of lethality. This lysosome-oriented dual stage UPS polymeric micelle system can be exploited as an effective therapeutic strategy against NQO1-overexpressing tumor cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Drug conversion of prodrug IV to β -lap in buffers at different pH values. (a) Cumulative release of β -lap from prodrug IV at pH 5.0, 6.5 and 7.4; (b) Normalized HPLC trace of prodrug IV conversion at 1, 20, 40, 60, 120 and 360 mins at pH 5.0; (c) Conversion of prodrug IV to β -lap at pH 5.0.

Fig. 2.

^1H NMR assignment of precursor and M-P polymers: (1) ^1H NMR spectra of precursor polymer $\text{mPEG}_{5\text{k}}\text{-}b\text{-P}(\text{DPA}_{45}\text{-}r\text{-PDSM}_6)$; (2) ^1H NMR spectra of β -lap conjugated polymer $\text{mPEG}_{5\text{k}}\text{-}b\text{-P}(\text{DPA}_{45}\text{-}r\text{-PDSM}(\text{IV})_6)$, the insets only showed the prodrug segment.

Fig. 3. β -Lap loaded M-P polymeric micelle size and morphology. TEM image of β -lap loaded M-P micelles at pH 7.4 (a); 6.5 (b) after incubation for 24 h; or pH 5.0 (c) for 5 min; (d) DLS size of micelles in buffers at pH 7.4, 6.5 or 5.0 after 5 min.

Fig. 4.

Release studies of β -lap from M-P micelles. (a) HPLC tracing of β -lap release over 72 h at pH 5.0; (b) β -Lap cumulative release in buffers at pH 7.4, 6.5 or 5.0; (c) Total release of β -lap in different pH-containing buffers after 72 h. *** $p < 0.001$.

Fig. 5.

Laser scanning Confocal Microscopy (LSCM) examination of Rhodamine and Rho-Polymer intracellular distribution: (a) after 1 h incubation of A549 cell with 5 μ M Rhodamine cellular distributions; (b) after 1h and 4 h incubation of A549 cells with 10 μ M Rho-polymer cellular distributions.

Fig. 6.

NQO1-dependent cytotoxicity of A549 NSCLC cells after treatment with M-P polymeric micelles. (a) NQO1-expressing A549 cells were treated with free β -lap for 2 h; (b) Cells were treated with free β -lap for 24 h; (c-g) Cells were treated with β -lap-loaded M-P polymeric micelles for (c) 2 h; (d) 8 h; (e) 12 h; and (f) 24 h; (g) Cells treated with micelles in culture medium without FBS; (h) Cytotoxicity of M-P polymer residue, cells were treated for 2, 12 and 24 h. Dicoumarol (DIC) is a fairly specific NQO1 inhibitor and blocked β -lap induced lethality.

Scheme 1.

(a) Schematic illustration of pH sensitive β -Lap prodrug linkages. (b) Schematic representation to micelle two stage drug release. At physiological pH, drug conjugated polymers mPEG-*b*-P(DPA-*r*-PDSM(IV)) micelles keep stable. Whereas in pHs under pH_t , at stage I, micelle immediately dissociation, and subsequently entering stage II, micelles quickly release payload drug β -lap.

Scheme 2.

Syntheses of β -lap prodrug analogues: (a) acylhydrazide, TsOH; (b) 2-(hydroxymethyl)-2-methylpropane-1,3-diol, TsOH, 4Å molecule sieves; (c) N-(4-Aminophenyl)maleimide, TiCl_4 , CH_2Cl_2 .

Scheme 3.

Synthesis route of polymer $mPEG-b-P(DPA_{45}-r-PDSM(IV)_6)$: (a) CH_2Cl_2 , Et_3N , 2-bromoisobutyl-bromide; (b) PDMETA, CuBr, isopropanol, DMF, Anisole, 65°; (c) TECP, prodrug IV.