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RESEARCH PAPER

# Block Copolymer Micelles for Controlled Delivery of Glycolytic Enzyme Inhibitors

Shanjida Akter • Brian F. Clem • Hyun Jin Lee • Jason Chesney • Younsoo Bae

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## ABSTRACT

***Purpose*** To develop block copolymer micelles as an aqueous dosage form for a potent glycolytic enzyme inhibitor, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO).

***Methods*** The micelles were prepared from poly(ethylene glycol)-poly(aspartate hydrazide) [PEG-p(HYD)] block copolymers to which 3PO was conjugated through an acid-labile hydrazone bond. The optimal micelle formulation was determined following the screening of block copolymer library modified with various aromatic and aliphatic pendant groups. Both physical drug entrapment and chemical drug conjugation methods were tested to maximize 3PO loading in the micelles during the screening.

***Results*** Particulate characterization showed that the PEG-p(HYD) block copolymers conjugated with 3PO (2.08~2.21 wt.%) appeared the optimal polymer micelles. Block copolymer compositions greatly affected the micelle size, which was 38 nm and 259 nm when 5 kDa and 12 kDa PEG chains were used, respectively. 3PO release from the micelles was accelerated at pH 5.0, potentiating effective drug release in acidic tumor environments. The micelles retained biological activity of 3PO, inhibiting various cancer cells (Jurkat, He-La and LLC) in concentration ranges similar to free 3PO.

***Conclusion*** A novel micelle formulation for controlled delivery of 3PO was successfully prepared.

S. Akter · H. J. Lee · Y. Bae (✉)
Department of Pharmaceutical Sciences, College of Pharmacy
University of Kentucky
789 South Limestone
Lexington, Kentucky 40536-0596, USA
e-mail: younsoo.bae@uky.edu

B. F. Clem · J. Chesney
Department of Medicine, James Graham Brown Cancer Center
University of Louisville
505 South Hancock Street
Louisville, Kentucky 40202, USA

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## INTRODUCTION

Rapid proliferation of cancer cells causes several metabolic transformation events for cell survival, suggesting that metabolic pathways might be promising therapeutic targets for cancer treatment (1). Tumors take up significantly more glucose than adjacent normal tissues *in vivo* and oncogenic proteins converge on glycolysis by activating the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatases (PFKFB) to produce fructose-2,6-bisphosphate, an allosteric activator of 6-phosphofructo-1-kinase (PFK1) (2–4). A recent study showed that the suppression of glycolytic flux induced cytostatic effects on neoplastic cells (5,6). 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO) has been previously identified as an inhibitor of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 3 (PFKFB3), leading to suppression of glycolytic flux and tumor cell growth *in vitro* and *in vivo* (7). Despite its therapeutic potential, 3PO has limitations in clinical applications due to poor water solubility and a relatively high pre-clinical effective dose (7). Dimethylsulfoxide (DMSO), Cremophore EL and surfactants are dosage forms commonly used for hydrophobic drugs. These toxic formulations act as therapeutic factors that cause side-effects (8), and would narrow the therapeutic index of 3PO. Therefore, there is an urgent need to develop an optimal and safe 3PO dosage form that can be used in aqueous solutions, readily sterilized and lyophilized while improving drug accumulation in tumors.

Polymer micelles, nano-scaled drug carriers from self-assembling block copolymers, have been studied as partic-

ulate formulations for the tumor-targeted delivery of various therapeutic agents (9–11). Polymer micelles possess a hydrophobic core enveloped with a hydrophilic shell. The hydrophobic core provides a nano compartment for drug-loading while the hydrophilic shell prevents the drug-loaded core from precipitation (12). The core-shell structure allows the micelles to stay longer in the blood stream by suppressing protein adsorption that triggers the immune response and leads to phagocytic clearance of foreign materials from the body (13,14). While the shell is important to keep the micelles biocompatible, the core plays a more crucial role in drug entrapment and release (15). Drugs are normally loaded in polymer micelles through two approaches: (a) physical drug entrapment and (b) chemical drug conjugation. Drug-loading through physical drug entrapment is dependent primarily on miscibility between drug molecules and the core-forming segment of block copolymers (16). Hydrophobicity and lipophilicity of drugs are two major factors that determine the drug miscibility in the micelle core. For these reasons, pendant groups that have similar chemical structures to drug molecules are frequently introduced to block copolymers to enhance the drug loading. Polymer micelle formation is known to be an entropy-driven process, yet having the core tightly packed with drug molecules contributes to the micelle stability (17). The most practical method to achieve the maximum miscibility and micelle stability is to covalently conjugate drug molecules to the micelle-forming block copolymers through degradable linkers (18). The chemical drug conjugation approach allows the micelles to entrap drug molecules stably and to regenerate the drugs as active pharmaceutical ingredients at the disease lesions.

Among various micelle formulations, the polymer micelles from functional poly(ethylene glycol)-poly(aspartate) [PEG-p(ASP)] block copolymers are versatile platforms to design multi-functional drug carriers by introducing degradable drug-binding linkers to the micelle core (19–22). Our previous studies demonstrated that the PEG-p(ASP) based block copolymer micelles achieved prolonged plasma retention time, tumor-specific accumulation, *in vivo* stimuli-responsive release, reduced systemic toxicity, and enhanced therapeutic efficacy of several anticancer drugs (23). In this study, we prepared PEG-p (ASP) polymer micelles to improve the bioavailability of 3PO and to preferentially deliver the drug to solid tumors. Tumor-preferential delivery of 3PO would not only potentially lower the effective dose of 3PO but also achieve controlled inhibition of glycolytic pathways in tumors, providing a safe and effective therapy to cancer patients.

To maximize 3PO loading in the micelles, both physical drug entrapment and chemical drug conjugation approaches were tested in this study. For physical entrapment drug-loading, various aromatic and aliphatic chemicals were introduced as pendant groups to PEG-p(ASP) block copolymers. For chemical drug conjugation, hydrazide groups were introduced to PEG-p(ASP), producing poly(ethylene glycol)-poly(aspartate hydrazide) [PEG-p (HYD)] block copolymers. 3PO was conjugated at its ketone moiety to PEG-p(HYD) block copolymers through an acid-labile hydrazone bond. The hydrazone bond, involved in many biomolecular events, is stable at physiological pH (7.4), yet is cleaved in acidic conditions (pH < 7.0) in a pH-dependent manner. Such a unique degradation pattern has been employed to design pH-controlled drug delivery systems that can trigger drug release in intracellular lysosomal compartments (pH 5.0), following the cellular uptake of drug carriers (24). Tumor tissues are also acidic (pH 6.5~7.0) due to the inefficient glucose consumption that massively produces lactic acids (25). Low pH in tumors is also attributed to high flux at the glyceraldehydes-3-phosphate dehydrogenase step of glycolysis (26). These two factors contribute to tumor acidosis in combination. Therefore, the polymer micelles entrapping 3PO through the hydrazone bond may achieve pH-sensitive drug release in tumor tissues. This hypothesis was tested by drug release experiments at different pHs (7.4 and 5.0). Biological activity of the 3PO micelles was subsequently evaluated *in vitro* using human cervical cancer (HeLa), human T-cell leukemia (Jurkat), and mouse Lewis lung carcinoma (LLC) cell lines.

## MATERIALS AND METHODS

### Materials

α-Methoxy-ω-amino poly(ethylene glycol) (PEG: 5 or 12 kDa) was purchased from NOF Corporation (Japan). L-aspartic acid β-benzyl ester (BLA), triphosgene, anhydrous tetrahydrofuran (THF), anhydrous hexane, anhydrous dimethylsulfoxide (DMSO), anhydrous dichloromethane (DCM), anhydrous N,N-Dimethylformamide (DMF), dimethylsulfoxide-*d6* (DMSO-*d6*), anhydrous diethyl ether, acetonitrile (ACN), chloromethyl phenyl sulfoxide, 1,3-diphenyl-2-bromo-propane, 3-bromobiphenyl, 2-hydroxyethyl dodecyl sulfoxide, 2-hexanone, 4-acetylbutyric acid, 4-oxohexanoic acid, anhydrous hydrazine, *N,N'*-diisopropyl carbodiimide (DIC), 4-(dimethylamino)pyridine (DMAP) were purchased from Sigma-Aldrich (USA). Chemicals were used as obtained without further purification. Regenerated cellulose dialysis bags with molecular weight cut off (MWCO) 6–8 kDa and Slide-A-Lyzer® G2 dialysis cassettes with MWCO 10 kDa were purchased from Fisher Scientific (USA). Amicon-Ultra centrifugal ultrafiltration devices with MWCO 100 kDa

were purchased from Millipore (USA). 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO), was purchased from ChemBridge Corporation (USA).

### Cell Culture

Human cervical cancer (HeLa: CCL2) and mouse Lewis lung carcinoma (LLC: CRL-1642) cells were obtained from American Type Culture Collection (ATCC), and cultured in DMEM (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS: Hyclone, USA) and 50 μg/mL gentamicin sulfate (Invitrogen, USA). Human T-cell leukemia (Jurkat: TIB-152) cells were cultured in RPMI (Hyclone, USA) supplemented with 10% FBS and 50 μg/mL gentamicin sulfate. Cells were incubated a humidified atmosphere with 5% $CO_2$ at 37°C.

### Synthesis of PEG-p(BLA) Block Copolymers

Poly(ethylene glycol)-poly(β-benzyl L-aspartate) [PEG-p (BLA)] block copolymers **4** were synthesized as the starting material to introduce various pendant groups for 3PO entrapment, following the synthesis protocol previously established (27). β-benzyl L-aspartate N-carboxy-anhydride (BLA-NCA) **2** was synthesized as a monomer by reacting BLA **1** with triphosgene (1.3 eq) in THF (50 mg/mL), followed by recrystallization in anhydrous hexane under nitrogen atmosphere at −20°C overnight. PEG-p(BLA) was then prepared by ring-opening polymerization of BLA-NCA using PEG **3** as an macroinitiator in DMSO at 40°C for 2 days. The block copolymers were collected by ether precipitation and freeze drying.

### Synthesis of Block Copolymer Library

PEG-p(BLA) was modified with various functional groups either by conjugation or aminolysis reactions. For conjugation reactions, all benzyl esters on PEG-p(BLA) were deprotected with 0.1N NaOH in advance to prepare poly (ethylene glycol)-poly(aspartate) [PEG-p(ASP)] block copolymers **5**. The carboxyl groups of PEG-p(ASP) were then conjugated with 3-bromobiphenyl **8**, 1,3-diphenyl-2-bromo-propane **9** and chloromethyl phenyl sulfoxide **10** at the 1:1.5 mixing ratio in DCM at room temperature overnight. The products were filtered, precipitated in ether, and collected by freeze-drying form benzene. 2-hydroxyethyl dodecyl sulfoxide **11** was conjugated to PEG-p(ASP) in the presence of DIC and DMAP. For aminolysis reactions, anhydrous hydrazine (5 folds) was reacted with dry PEG-p(BLA) in DMF at 50°C for 1 h, producing poly(ethylene glycol)-poly(aspartate hydrazide) [PEG-p(HYD)] block copolymers **6**. Aliphatic compounds, such as 2-hexanone **12**, 4-acetylbutyric acid **13** and 4-oxohexanoic acid **14** were conjugated with PEG-p (HYD) in DMSO at 40°C for 3 days. The products were purified by ether precipitation and dialysis prior to freeze drying.

### Polymer Micelle Preparation and 3PO Entrapment

Polymer micelles were prepared by either dialysis or freeze-drying method to maximize 3PO entrapment in the micelles. For the dialysis method, block copolymers and 3PO were dissolved in DMSO at 5~10 mg/mL. The solution was titrated in deionized water under vigorous stirring, which was further diluted to adjust the DMSO level 5%. After removing DMSO completely, the micelle solutions were freeze-dried. For the freeze-drying method, block copolymers and 3PO were dissolved in ACN at 10 mg/mL first, and then, the solution was mixed with deionized water to obtain 20% ACN solutions. The mixed solutions were quickly frozen in a dry ice bath and freeze-dried. In both methods, freeze-dried micelle powders were reconstituted in deionized water and filtered through 0.22 μm prior to the following characterization. Only the dialysis method was used to prepare the micelles for block copolymer micelles to which 3PO was covalently conjugated [PEG-p(HYD)/3PO conjugates] **15**. The absence of free drugs was confirmed by TLC ($CHCl_3$:MeOH = 95:5). Prepared micelles were stored as freeze-dried powders at −20°C under nitrogen until use.

### Particulate Characterization

The particle size and its distribution were analyzed by dynamic light scattering (DLS) measurements (Zetasizer Nano-ZS, Malvern, UK). Light scattering intensity was used to observe dissociation of the micelles. Drug loading was determined by using a UV/VIS spectrometer (Spectra Max M5, Molucular devices, USA) equipped with variable spectrum filters and Softmax Pro software. 3PO molecules in the micelle solutions were measured at 310 nm. The drug loading yields were determined by weight percentage (wt%=mg 3PO/mg micelle). Potential formulations for further study were selected from the micelles that showed drug loading greater than 1 wt.%, retained the particulate structure after filter sterilization (0.22 μm), and underwent no burst drug release in aqueous solutions (pH 7.4, 37°C). We determined the minimally acceptable drug loading yield (1 wt.%) based on the fact that our empty micelles showed no cellular toxicity at up to 100 mg/mL (data not shown). Polymer micelles with 1 wt.% drug loading at 100 mg/mL can allow us to

prepare 1 mg/mL drug solutions, which are high enough to test drug cytotoxicity and future pilot studies *in vivo*. Drug release patterns from the selected micelles were subsequently investigated. The micelles were added in dialysis cassettes (MWCO 10 kDa), followed by dialysis at 37°C against 10 mM buffer solutions at pH 7.4 or 5.0 for 48 h. Drug release experiments were conducted under the sink condition in which the dialysis medium volume was maintained >1,000 times the micelle solution volume in the dialysis cassette. 3PO remaining in the dialysis cassettes was quantified at 0, 1, 3, 6, 24 and 48 h. Data were collected in triplicate and converted to 3PO released by subtracting the amount of drug remaining from the initial drug concentration.

### Cytotoxicity Assays

Exponentially growing Jurkat, HeLa or LLC cells were seeded in 24-well plates (100,000 cells/well) in 1 ml of media containing 10% FBS. For suspension cells (Jurkat), samples (free 3PO and micelles) were added immediately to each well at different concentrations (3PO based). For adherent cells (HeLa and LLC), samples were added 24 h after cell seeding. Cells were incubated for 48 h total, followed by cell viability measurement using trypan blue exclusion. The drug dose–response curves were fitted to a variable of Hill Slope to determine the inhibitory concentrations for 50% cell viability (IC50). Data are reported as the mean ± standard deviation (SD) from three independent experiments.

## RESULTS AND DISCUSSION

### Synthesis of PEG-p(BLA) Block Copolymers

Compositions of block copolymers (Fig. 1) are described as X–Y, where 'X' and 'Y' indicate the molecular weight of PEG $\times 10^{-3}$ **3** and the number of BLA **1** on the PEG-p(BLA) **4** starting material, respectively. $^{1}$H-NMR (500 MHz) showed that PEG-p(BLA) compositions were 5–33 and 12–35, which were determined by comparing the peak area of benzyl groups (7.4 ppm) with respect to that of PEG (3.5 ppm). Deprotection of benzyl esters on PEG-p(BLA) produced PEG-p(ASP) block copolymers **5**. Absence of benzyl groups were confirmed by $^{1}$H-NMR in $D_2O$. Neither unreacted PEG nor p(ASP) homopolymers were observed by GPC (Shimadzu LC20, PEG standard, 100 mM PBS mobile phase at 40°C). $^{1}$H-NMR also confirmed that the aminolysis reaction between PEG-p(BLA) and hydrazine produced PEG-p(HYD) block copolymers **6**. All benzyl esters were successfully replaced with hydrazine, which was consistent with our previous observations (28).

### Synthesis of Block Copolymer Library

PEG-p(BLA), PEG-p(ASP) and PEG-p(HYD) 5–33 were used to prepare our block copolymer library for initial screening of polymer micelles. Figure 2 shows chemical structures of all block copolymers modified with various pendant groups. Block copolymer library was prepared to investigate effects of micelle core structures on 3PO entrapment. The polymer library can be categorized into two groups according to pendant groups conjugated to block copolymers. The first group is an aromatic pendant group in which various aromatic pendant groups were tested to improve the miscibility between the micelle core and 3PO molecules possessing two pyridine rings. The second group is an aliphatic group, which include pendant groups with linear carbon chains that can improve drug entrapment in the micelles by interacting with the propenone portion of 3PO. Unmodified PEG-p(BLA) was used as PEG-p(ASP) block copolymers modified with benzyl alcohol as a pendant group **7**. Either PEG-p(Asp) or PEG-p(Hyd) was used to complete the synthesis of the polymer library. Conjugation yields between PEG-p(ASP) and pendant groups appeared less than 20% in all compositions, indicating not more than 7 pendant groups were attached to PEG-p(ASP) 5–33. Limited conjugation yields suggest that there was steric hindrance among carboxyl groups on the block copolymers.

### Polymer Micelles and Drug Entrapment

PEG-p(ASP) 5–33 block copolymers modified with pendant groups were used to test both physical entrapment and chemical conjugation of 3PO in polymer micelles. Carboxyl groups that remained intact on the block copolymers were considered as a factor that can cause adverse effects on the micelle formation and stability. Aqueous GPC and DLS analyses showed that all block copolymers formed nanoaggregates (<1,000 nm) even in the absence of 3PO. Formation of nanoaggregates indicates that the block copolymers conjugated with 20% pendant groups were still capable of self-assembling. However, nanoaggregates larger than 200 nm unlikely have a distinctive core-shell structure of polymer micelles. For this reason, only the nanoaggregates that successfully went through 0.22 μm filters were tested further for drug entrapment experiments. 1,3-dephenyl-2-bromo-propane **8** and 3-bromobiphenyl pendant **9** groups made block copolymers too hydrophobic to disperse in aqueous solutions forming micelles without vigorous sonication. Block copolymers modified with 2-hydroxyethyl dodecyl sulfoxide **11** and 4-oxohexanoic acid **12** pendant groups dissolve slightly in aqueous solutions, yet the cloudy solutions failed to go through 0.22 μm filters for sterilization. Nanoaggregates from block copolymers with

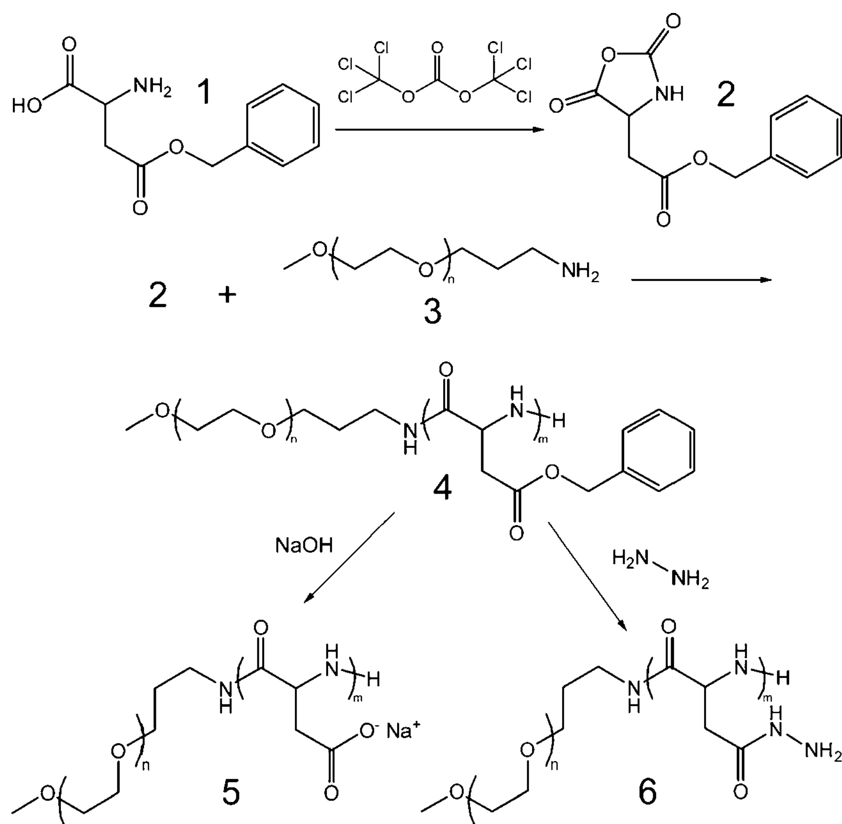


**Fig. 1** Synthesis of block copolymers.

benzyl alcohol **7** (micelle candidate 1: MC1), chloromethyl phenyl sulfoxide **10** (MC2) and 4-acetylbutyric acid **13** (MC3) pendant groups were successfully sterilized by 0.22 μm filters. These nano-scaled aggregates (<200 nm) in aqueous solutions were considered as polymer micelles entrapping 3PO through physical entrapment.

There was no significant difference in the micelle size between the dialysis and freeze-drying methods. Drug-loading measurements revealed that 0.7, 2.6 and 4.3 wt.% of 3PO were present in MC1, MC2 and MC3 (or 0.39, 1.27 and 3.77 drug molecules per each polymer chain), respectively. Drug loading yields indicate that short

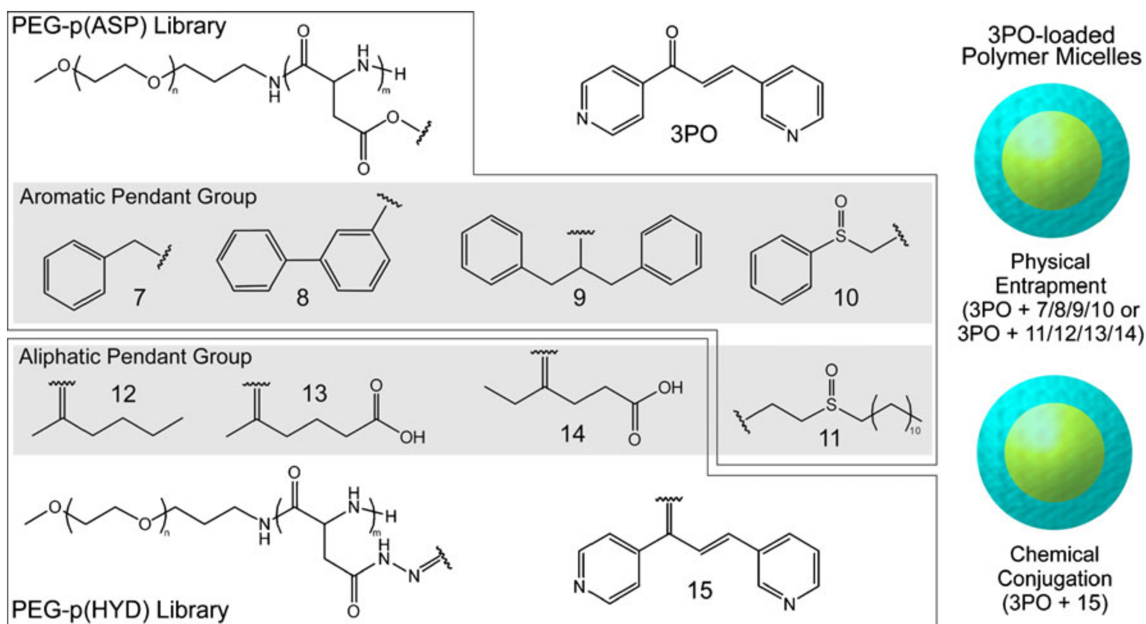


**Fig. 2** Block copolymer library and micelle preparation.

lipophilic pendent groups would more effective to increase 3PO entrapment in the micelles in comparison to hydrophobic pendant groups. Particle sizes of MC2 (68.1 nm) and MC3 (37.8 nm) were smaller than MC1 (164.2 nm). However, particle size distribution was broad as MC2 and MC3 underwent burst release of 3PO. MC2 and MC3 solutions turned turbid gradually and 3PO precipitates were observed in 1 h.

These results were unexpected because polymer micelles that possess pendant groups similar to drug molecules typically improve drug solubility by enhancing miscibility between the micelle core and drug molecules. One possible reason is that the number of pendant groups introduced was insufficient. Despite empty micelle formation, free carboxyl groups remaining unreacted on block copolymers would have caused charge repulsion, which could make the micelle core unfavorable to 3PO entrapment. This hypothesis, however, does not fully explain why PEG-p(BLA), which has all carboxyl groups protected by hydrophobic benzyl groups, did not improve 3PO loading. The most likely explanation is that there are stronger and more thermodynamically stable interaction between individual 3PO molecules than between 3PO and the prepared polymers. 3PO is poorly water soluble, yet contains polar functional groups such as a ketone and pyridinyl moieties. Aggregation between 3PO molecules would be more thermodynamically favorable than molecular interactions between the drug and polymers. Although the detailed mechanism is uncertain, our findings suggest that physical entrapment of 3PO in PEG-p(ASP) micelles would be unsuitable to prepare particulate delivery formulations with clinically relevant stability and 3PO loading.

Chemical conjugation of 3PO was subsequently tested by using PEG-p(HYD) block copolymers **15**. The ketone group of 3PO was covalently conjugated to the hydrazide groups of PEG-p(HYD) block copolymers through acid-labile hydrazone bond. Pilot tests confirmed that the polymer micelles from 3PO-conjugated PEG-p(HYD) block copolymers went through 0.22 μm filters, remained clear for 1 h, and contained mono-dispersed nanoparticles. For further investigation, polymer micelles were prepared using 3PO-conjugated PEG-p(HYD) 5–33 and 12–35 block copolymers. These micelles were denoted as MC4(5–33) and MC4(12–35), respectively. The 3PO entrapment yields were 2.08 and 2.21 wt.% with respect to the micelle mass for MC4(5–33) and MC4(12–35) (or 1.52 and 1.47 drug molecules per each polymer chain), respectively. The particle size of MC4(5–33) at 20 mg/mL was smaller than that of MC4(12–35) (38 *vs* 259 nm) as shown in Fig. 3. These results indicate that PEG-p(HYD) block copolymers with 5 and 12 kDa PEG chains showed similar drug

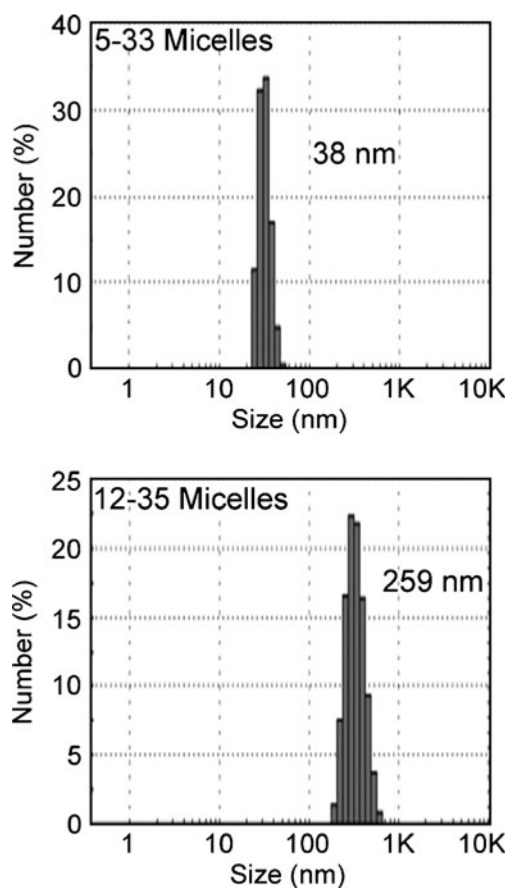


**Fig. 3** Particle size distribution of 3PO-conjugated polymer micelles.

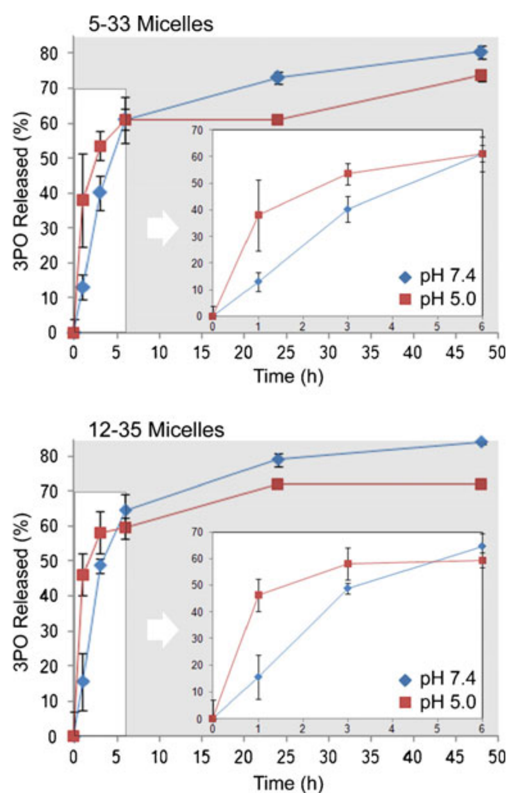


**Fig. 4** 3PO release patterns from the micelles at different pHs.

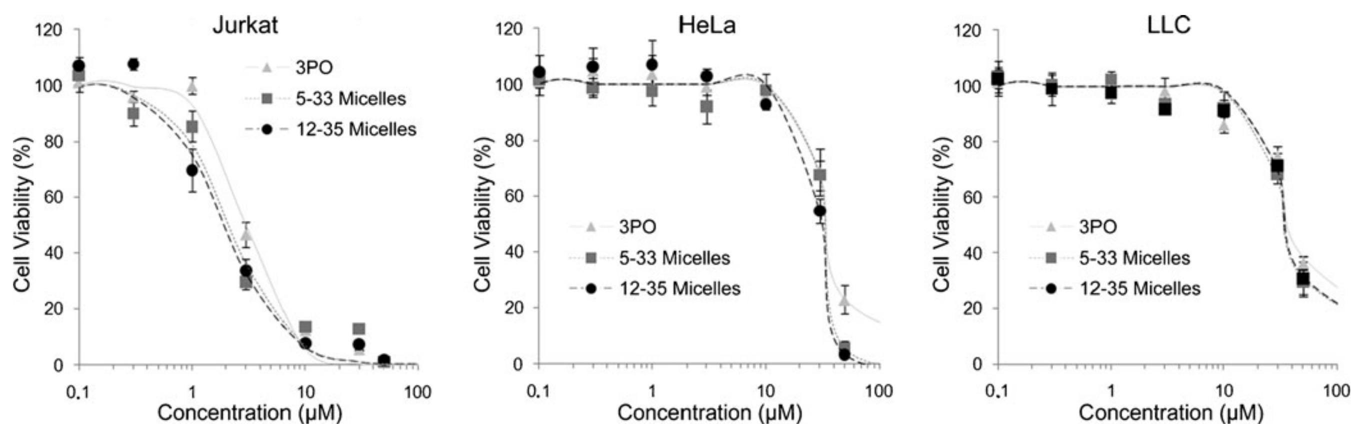


**Fig. 5** Concentration-dependent inhibition of various cancer cells by the micelles.

entrapment yields, although the particle sizes were different. In the meantime, MC4(5–33) and MC4(12–35) showed relatively low drug entrapment yields (~2 wt.%). Even though the drug loading was higher than the minimally acceptable drug loading (1 wt.%) we determined in the experimental section, it would need further improvement to reduce the amount of total block copolymers necessary for drug delivery.

A mono-dispersed narrow particle size distribution implies that MC4(5–33) formed a single core-shell type structure, which is ideal for particulate drug delivery in the body. Interestingly, both micelles went through 0.22 μm filters, indicating that MC4(12–35) would have formed nanoaggregates from block copolymers weakly bound each other. It is also surmised that MC4(12–35) dissociated into smaller micellar structures and assembled back to large nanoaggregates after filtration. Further DLS analyses revealed that the particle size of MC4(12–35) decreased to 181.7 nm at 10 mg/mL, supporting weak interactions between 3PO-conjugated block copolymer chains. No concentration-dependent change was observed in the particle size of MC4(5–33). Even though the particle sizes were variable, MC4 solutions remained clear at all concentrations before and after filter sterilization irrespective of block copolymer compositions. Based on these results, MC4 were selected as our prototype micelle formulation for subsequent biological evaluation as 5–33 and 12–35 micelles.

## Drug Release from Polymer Micelles

Figure 4 shows drug release patterns of the selected 5–33 and 12–35 micelles. Both micelles showed significant drug release at pH 7.4 (>10% in 1 h and >35% in 3 h). These results indicate that the micelles may retain 3PO in blood stream (pH 7.4) only for a short time. However, in comparison to free drugs that normally get removed from the body in a few hours, these micelles are still expected to increase 3PO concentrations in the blood. Drug release from 5–33 to 12–35 micelles was accelerated at pH 5.0, suggesting that these micelles will release the drug as pH decreases in tumor tissues (pH < 7) and the intracellular lysosomes (pH 5.0). Such pH-dependent drug release patterns are favorable not only to improve tumor-preferential delivery of 3PO but also to reduce non-specific drug distribution in normal organs. In addition, drug-conjugated micelles are advantageous over prodrugs because the micelles can protect both drugs and drug-binding linkers more effectively from the *in vivo* environment.

Rapid drug release in the early stage (0~6 h, Fig. 4 insets) was followed by sustained release (>6 h). Two-step drug release patterns would effectively inhibit cells growing in tumor tissues after injection and extend the period of therapy, thereby avoiding frequent drug injections (29,30). The PEG shells of polymer micelles are normally considered a physical barrier to drugs entrapped in the core. Thick micelle shells can reduce drug release rates because drug molecules need to go through the PEG layer. It is intriguing that the micelles with 12 kDa PEG released 3PO

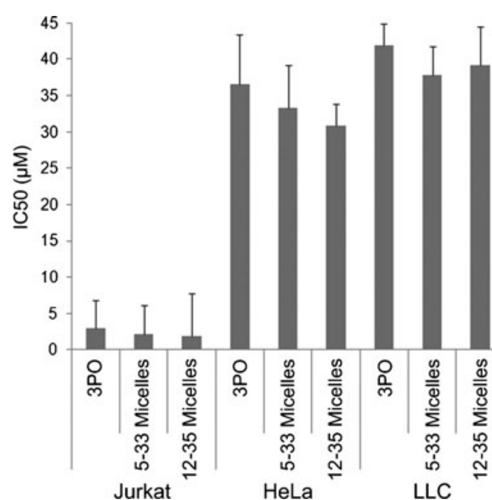


**Fig. 6** IC50 comparison between the micelles and free 3PO.

more quickly at both pH 7.4 and 5.0 than the micelles with shorter 5 kDa PEG. The PEG shell in the 3PO-conjugated micelles seems to assist drug release from the micelles probably due to attracting more water molecules close to the micelle core for hydrolysis of the hydrazone bond between 3PO and the polymer chains.

### Cytotoxicity of 3PO-Conjugated Polymer Micelles

To demonstrate the efficacy of 3PO-conjugated micelles, Jurkat, HeLa and LLC cell lines were incubated with the micelles or free 3PO alone for 48 h. As summarized in Fig. 5, the dose response curves demonstrate that Jurkat cells were the most sensitive to free 3PO, followed by HeLa and LLC. These cellular responses remained the same irrespective of 3PO formulations. The IC50 values of the micelles were equivalent to or slightly higher than those of free 3PO in all cell lines tested (Fig. 6), which is likely a consequence of the continuous release of fresh 3PO from the micelles after 6 h. We also observed no significant difference in cytotoxicity between the 5–33 and 12–35 micelles. These results are encouraging because the polymer micelles can retain the cytotoxicity of 3PO while controlling drug release patterns in pH-dependent manners even with shorter PEG chains (5 kDa *vs* 12 kDa PEG). Smaller 5–33 micelles (38 nm) would also facilitate tumor targeting *in vivo*. Previous studies demonstrated that polymer micelles favorably change the pharmacokinetic profiles of drugs (31–34). The micelles in this study are also expected to preferentially carry 3PO to tumor tissues in an active form and control the drug release patterns, potentially achieving reduced toxicity and improved efficacy of 3PO.

## CONCLUSION

In this study, block copolymer micelles were successfully prepared as an aqueous dosage form of the glycolytic enzyme inhibitor, 3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO). The micelles were prepared from poly (ethylene glycol)-poly(aspartate) [PEG-p(ASP)] block copolymers to which various pendant groups were attached to improve drug loading yields. Physical entrapment of 3PO in the micelles was unsuccessful, suggesting that the interaction between drug molecules was more thermodynamically favorable than between the drug and micelle-forming block copolymers. Chemical conjugation of 3PO to PEG-p(ASP) possessing hydrazide linkers [PEG-p(HYD)] achieved approximately 2 wt.% drug-loading in polymer micelles. These 3PO-conjugated polymer micelles released the drug in a pH-dependent manner, demonstrating that the micelles can accelerate 3PO release favorably in tumor tissues as well as intracellular lysosomes. 3PO released from the micelles retained its biological activity, causing effective or considerable cell death in Jurkat, HeLa and LLC cell lines. Cellular responses to free 3PO and micelles with different polymer compositions (5 or 12 kDa PEG) remained almost identical. These results indicate that the 3PO-conjugated polymer micelles prepared in this study are a promising formulation for controlled delivery of 3PO to tumors.

## ACKNOWLEDGMENTS & DISCLOSURES

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## REFERENCES

1. Berdanier CD. Drugs, nutrients, and hormones in mitochondrial function. Oxid Stress Dis. 2005;16:455–506.
2. Vizan P, Boros LG, Figueras A, Capella G, Mangues R, Bassilian S, *et al*. K-ras codon-specific mutations produce distinctive metabolic phenotypes in human fibroblasts. Cancer Res. 2005;65:5512–5.
3. Hue L, Rousseau GG. Fructose-2,6-bisphosphate and the control of glycolysis by growth-factors, tumor promoters and oncogenes. Adv Enzyme Regul. 1993;33:97–110.
4. Ramanathan A, Wang C, Schreiber SL. Perturbational profiling of a cell-line model of tumorigenesis by using metabolic measurements. Proc Natl Acad Sci. 2005;102:5992–7.
5. Mazurek S, Zwerschke W, Jansen-Durr P, Eigenbrodt E. Metabolic cooperation between different oncogenes during cell transformation: interaction between activated ras and hpv-16 e7. Oncogene. 2001;20:6891–8.
6. Telang S, Yalcin A, Clem AL, Bucala R, Lane AN, Eaton JW, *et al*. Ras transformation requires metabolic control by 6-phosphofructo-2-kinase. Oncogene. 2006;25:7225–34.
7. Clem B, Telang S, Clem A, Yalcin A, Meier J, Simmons A, *et al*. Small-molecule inhibition of 6-phosphofructo-2-kinase activity suppresses glycolytic flux and tumor growth. Mol Cancer Ther. 2008;7:110–20.
8. Cukierman E, Khan DR. The benefits and challenges associated with the use of drug delivery systems in cancer therapy. Biochem Pharmacol. 2010;80:762–70.
9. Matsumura Y, Kataoka K. Preclinical and clinical studies of anticancer agent-incorporating polymer micelles. Cancer Sci. 2009;100:572–9.
10. Kataoka K, Kwon GS, Yokoyama M, Okano T, Sakurai Y. Block-copolymer micelles as vehicles for drug delivery. J Control Release. 1993;24:119–32.
11. Kwon GS, Yokoyama M, Okano T, Sakurai Y, Kataoka K. Biodistribution of micelle-forming polymer-drug conjugates. Pharm Res. 1993;10:970–4.
12. Kwon GS, Naito M, Kataoka K, Yokoyama M, Sakurai Y, Okano T. Block copolymer micelles as vehicles for hydrophobic drugs. Colloids Surf B Biointerfaces. 1994;2:429–34.
13. Matsumura Y. Poly (amino acid) micelle nanocarriers in preclinical and clinical studies. Adv Drug Deliv Rev. 2008;60:899–914.
14. Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv Drug Deliv Rev. 2001;47:113–31.
15. Yokoyama M, Kwon GS, Okano T, Sakurai Y, Naito M, Kataoka K. Influencing factors on *in-vitro* micelle stability of

adriamycin-block copolymer conjugates. J Control Release. 1994;28:59–65.
16. Gao ZS, Eisenberg A. A model of micellization for block-copolymers in solutions. Macromolecules. 1993;26:7353–60.
17. Allen C, Maysinger D, Eisenberg A. Nano-engineering block copolymer aggregates for drug delivery. Colloid Surf B. 1999;16:3–27.
18. West KR, Otto S. Reversible covalent chemistry in drug delivery. Curr Drug Discov Technol. 2005;2:123–60.
19. Cammas S, Kataoka K. Functional poly[(ethylene oxide)-co-(beta-benzyl-l-aspartate)] polymeric micelles—block-copolymer synthesis and micelles formation. Macromol Chem Phys. 1995;196:1899–905.
20. Lavasanifar A, Samuel J, Kwon GS. Poly(ethylene oxide)-block-poly(l-amino acid) micelles for drug delivery. Adv Drug Deliv Rev. 2002;54:169–90.
21. Lee HJ, Ponta A, Bae Y. Polymer nanoassemblies for cancer treatment and imaging. Ther Deliv. 2010;1:803–17.
22. Bae Y, Jang WD, Nishiyama N, Fukushima S, Kataoka K. Multifunctional polymeric micelles with folate-mediated cancer cell targeting and ph-triggered drug releasing properties for active intracellular drug delivery. Mol Biosyst. 2005;1:242–50.
23. Bae Y, Kataoka K. Intelligent polymeric micelles from functional poly(ethylene glycol)-poly(amino acid) block copolymers. Adv Drug Deliv Rev. 2009;61:768–84.
24. Jones AT, Gumbleton M, Duncan R. Understanding endocytic pathways and intracellular trafficking: a prerequisite for effective design of advanced drug delivery systems. Adv Drug Deliv Rev. 2003;55:1353–7.
25. Vander Heiden MG, Thompson CB, Cantley LC. Understanding the warburg effect: the metabolic requirements of cell proliferation. Science. 2009;324:1029–33.
26. Gillies RJ, Robey I, Gatenby RA. Causes and consequences of increased glucose metabolism of cancers. J Nucl Med. 2008;49:24s–42s.
27. Yokoyama M, Kwon GS, Okano T, Sakurai Y, Seto T, Kataoka K. Preparation of micelle-forming polymer-drug conjugates. Bioconjugate Chem. 1992;3:295–301.
28. Ponta A, Bae Y. Peg-poly(amino acid) block copolymer micelles for tunable drug release. Pharm Res. 2010;27:2330–42.
29. Jain RK. Delivery of molecular and cellular medicine to solid tumors. Adv Drug Deliv Rev. 1997;26:71–90.
30. Wexler EJ, Gravallese EM, Czerniak PM, Devenny JJ, Longtine J, Wong MK, *et al*. Tumor biology: use of tiled images in conjunction with measurements of cellular proliferation and death in response to drug treatments. Clin Cancer Res. 2000;6:3361–70.
31. Bae Y, Nishiyama N, Fukushima S, Koyama H, Matsumura Y, Kataoka K. Preparation and biological characterization of polymeric micelle drug carriers with intracellular ph-triggered drug release property: tumor permeability, controlled subcellular drug distribution, and enhanced *in vivo* antitumor efficacy. Bioconjugate Chem. 2005;16:122–30.
32. Kataoka K, Matsumoto T, Yokoyama M, Okano T, Sakurai Y, Fukushima S, *et al*. Doxorubicin-loaded poly(ethylene glycol)-poly (beta-benzyl-l-aspartate) copolymer micelles: their pharmaceutical characteristics and biological significance. J Control Release. 2000;64:143–53.
33. Suzuki H, Nakai D, Seita T, Sugiyama Y. Design of a drug delivery system for targeting based on pharmacokinetic consideration. Adv Drug Deliv Rev. 1996;19:335–57.
34. Takakura Y, Hashida M. Macromolecular carrier systems for targeted drug delivery: pharmacokinetic considerations on biodistribution. Pharm Res. 1996;13:820–31.