ACID-LABILE CORE CROSS-LINKED MICELLES **FOR pH-TRIGGERED RELEASE OF DOXORUBICIN**

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Abstract. A acid-labile core cross-linked micelle system of amphiphilic triblock copolymer of poly(oligo(ethylene glycol) methyl ether methacrylate)-*block*-poly(styrene-*alt*-maleic anhydride)-*block*-poly(styrene) (POEGMA-*b*-PSMA-*b*-PS) was prepared. The POEGMA-*b*-PSMA-*b*-PS triblock copolymers were synthesized by the reversible addition-fragmentation chain transfer (RAFT) polymerization. Doxorubincin (DOX) was entrapped into micellar structure with POEGMA block as a shell and PSMA-*b*-PS block as a core. Then, core cross-linking was carried out by amidation reaction between 2,2-bis(aminoethoxy)propane and maleic anhydride group inner core. DOX loading content and efficiency were calculated to be 18 and 90 (wt. %), respectively. Core cross-linked micelles illustrated good stability under physiological condition but disassociated rapidly under acidic pH. The DOX release experiments indicated a rapid DOX release at pH 5.0 compared to pH 7.4.
Keywords. triblock copolymer, RAFT polymerization, acid-sensitive, core cross-linked, doxorubicin.

NỐI MẠNG NHÂN MICELLE NHẠY MÔI TRƯỜNG ACID TRONG DẪN TRUYỀN THUỐC DOXORUBICIN

Tóm tắt. Một hệ thống nối nhân mạng micelle dựa trên triblock polymer của poly(oligo(ethylene glycol) methyl ether methacrylate)-*block*-poly(styrene-*alt*-maleic anhydride)-*block*-poly(styrene) (POEGMA-*b*-PSMA-*b*-PS) được tổng hợp. POEGMA-*b*-PSMA-*b*-PS được tổng hợp bằng phương pháp RAFT polymer hóa. Thuốc doxorubicin (DOX) được đưa vào trong nhân của hệ micell với POEGM như một vỏ và PSMA-*b*-PS như một nhân. Sau đó phản ứng nối mạng nhân được tiến hành dựa trên phản ứng amide hóa giữa tác nhân nối mạng 2,2-bis(aminoethoxy)propane và nhóm chức maleic anhydride trong nhân. Hàm lượng và hiệu quả thuốc DOX có thể đưa vào nhân được tính toán lần lượt là 18 và 90% khối lượng. Micell nối mạng nhân chứng tỏ có một độ bền cao dưới điều kiện sinh lý nhưng phân rã nhanh trong môi trường acid. Thực nghiệm dẫn truyền thuốc DOX chỉ ra rằng thuốc được thoát ra nhanh chóng tại điều kiện pH 5.0 so với pH 7.4.
Từ khóa. triblock polymer, RAFT polymer hóa, nhạy acid, nối mạng nhân, doxorubicin

1 INTRODUCTION

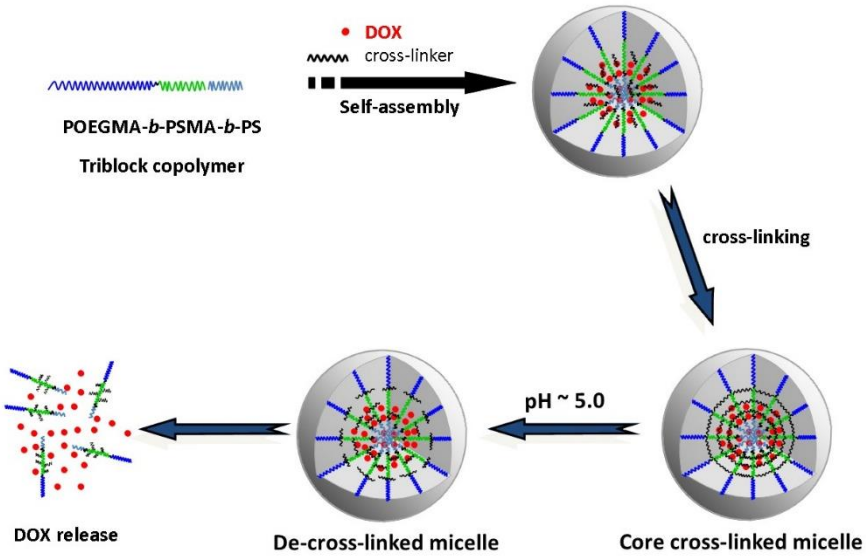
Over the past decade, polymeric micelles have been extensively explored for in vivo application as drug nanocarriers [1]. Various polymeric nanoparticle-based drug delivery systems including liposomes, dendrimers, micelles, and protein aggregates, have been developed for the delivery of chemotherapeutics agents. Among them, polymeric micelles may provide greater therapeutic advantage and more effective in targeting tumor [2]. Additionally, the benefits of polymeric micelle formulations include their ability to encapsulate hydrophilic therapeutic agents at high loading efficiency, superior biocompatibility, and favorable drug release profiles [3]. Nevertheless, the practical applications of micelles are limited since the dynamic nature of micelles and intravenous administration may lead to destabilize in vivo, which significantly affects their cellular uptake and also biodistribution [4]. The polymeric micelles can hardly keep their structural stability due to certain factors such as low concentration, ionic strength, changes in pH, temperature, and high shearing force. To overcome this bottleneck, there are many ways through cross-linking, which locks the self-assembled structure of the micelle. The stability of micellar could be enhanced through core cross-linked (CL) [5], shell CL [6], and intermediary layer CL micelles [7]. In order to avoid undesirable intermicellar cross-linking, shell cross-linking is carried out at high dilution, which leads to a low efficiency. Moreover, shell CL micelles may have some disadvantages coming from the stealthiness

and mobility of the hydrophilic moiety [8]. In contrast, core CL micelle is a really versatile strategy which can demolish all hindrance of shell CL micelles. In this light, various stimulus-responsive core CL micelles have been reported including acid-labile cross-linking polymerization [9], dimethylmaleide photo-cross-linking [10], phototriggered disulfide cross-link [11], and metal-coordination complexes [12].

In recent years, controlled radical polymerization has become a powerful tool for synthesizing macromolecules with controlled molecular weight and well-defined architectures which involving reversible addition fragmentation chain transfer (RAFT) polymerization, atom transfer radical polymerization (ATRP), and nitroxide-mediated radical polymerization (NMRP) [13-15]. Among them, RAFT polymerization has been considered as one of the most successful methods since it can be applied to a wide range of functional monomers (styrenics, alkyl (meth)acrylates, acrylic acid, vinyl acetate etc.), which allows polymers with precisely controlled structural parameters to be prepared such as random, block, gradient, grafted and star copolymers its easy setup, tolerance to functional groups, mild conditions, and wide range of applications [16].

The formation of CL polymeric micelles using the amidation reaction of amphiphilic diblock copolymers has been reported recently [17-19]. Furthermore, shell cross-linking [20,21], core cross-linking [22], and nexus cross-linking [23] were studied by amidation process with a ketal cross-linker. The hydrolysis of ketal linkages in CL micelles can be promoted by an acidic circumstance (pH ~ 5.0) to trigger drug release.

In the present work, a facile route for preparation of core CL micelles with ketal cross-linking was proposed by amidation process for pH trigger release of doxorubicin (DOX). Firstly, amphiphilic triblock copolymers of poly(oligo(ethylene glycol) methyl ether methacrylate)-*block*-poly(styrene-*alt*-maleic anhydride)-*block*-poly(styrene) (POEGMA-*b*-PSMA-*b*-PS) were prepared by RAFT polymerization. The anticancer drug doxorubicin (DOX) was entrapped in the hydrophobic micellar core. Then, the amidation reaction was allowed to take place between maleic anhydride groups located along POEGMA-*b*-PSMA-*b*-PS backbone and 2,2-bis(aminoethoxy)propane as a ketal cross-linker to form pH-responsive core CL micelles. Ketal core CL micelles illustrated good stability in selective solvent under physiological condition but disassociated rapidly under acidic condition leading to a rapid DOX release at pH 5.0 compared to pH 7.4 (Scheme 1).



Scheme 1: The formation of reverisble core CL micelles following by DOX release.

2 EXPERIMENTAL DETAILS

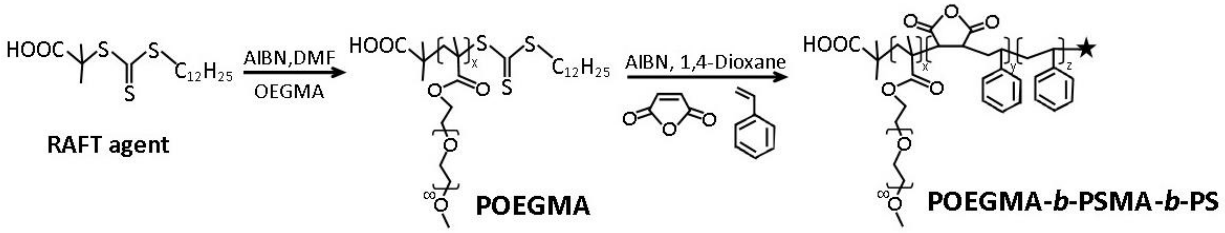
2.1 Materials

Oligo(ethylene glycol) methyl ether methacrylate (OEGMA, M_n=500, Polysciences, Inc.) was purified by passing through a basic alumina column. Styrene (99%, Alfa) was passed through a neutral alumina column.

2,2'-Azobis(isobutyronitrile) (AIBN) (98%, Sigma-Aldrich) was recrystallized in methanol prior to use. N, N'-Dimethylformamide (DMF) (HPLC grade) and 1,4-dioxane (TCI) were distilled prior to use. Maleic anhydride (≥99%, Sigma-Aldrich) and 2,2'-(ethylenedioxy)bis(ethylamine) (98%, TCI) were used without further purification. Other solvents and chemicals of analytical grade were used as received. Doxorubicin hydrochloride (DOX.HCl) was kindly provided from Boryung Pharm. Co. (Korea). S-dodecyl-S′-(α,α′-dimethyl-α″-acetic acid) trithiocarbonate (DDMAT) was synthesized according to the our previous work [24]. ^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 3.27 (t, 2H, S-CH_2-$(CH_2)_{10}$-CH_3), 1.71 (s, 6H, C-$(CH_3)_2$), 1.66 (m, 2H, S-CH_2-CH_2-$(CH_2)_9$-CH_3), 1.33 (m, 18H, S-CH_2-CH_2-$(CH_2)_9$-CH_3), 0.87 (t, 3H, SCH_2-$(CH_2)_{10}$-CH_3). 2,2-Bis(aminoethoxy)propane (ketal cross-linker) was prepared as described in the previous literature [22,23].^{1}H NMR (400 MHz, $CDCl_3$, δ ppm) 1.35 (s, 6H, CH_3–C), 1.63 (bs, 4H, NH_2), 2.81 (t, 4H, CH_2–NH_2), 3.42 (t, 4H, t, CH_2–O). IR (cm^{-1}): 3377 (br), 1573 (s), 1486 (s), 1383 (m).

2.2 Synthesis of poly(oligo(ethylene glycol) methyl ether methacrylate) (POEGMA)

The homopolymer of OEGMA was synthesized by RAFT method using DDMAT as a chain transfer agent. OEGMA (8.00 g, 16 mmol), DDMAT (0.365 g,1.0 mmol), and AIBN (0.030 g, 0.183 mmol) were dissolved in 6 mL of dried DMF in a round bottom flask and purged with nitrogen for 1 h. The polymerization was conducted in oil bath at 80 °C for 24 h. The reaction mixture was mixed with 10 mL water and purified by dialysis against distilled water using a dialysis membrane (MWCO 13 kDa). The water was removed under rotary vacuum and product was dried in vacuum oven at 40 °C for 24 h, yielding a yellow viscous liquid (7.42 g; yield: 89%) (Scheme 2).



Scheme 2: Schematic illustration of the synthesis of POEGMA-b-PSMA-b-PS triblock copolymers.

2.3 Synthesis of poly(oligo(ethylene glycol) methyl ether methacrylate)-*block*-poly(styrene-*alt*-maleic anhydride)-*block*-polystyrene (POEGMA-*b*-PSMA-*b*-PS)

POEGMA-*b*-PSMA-*b*-PS triblock copolymers were prepared as follows: POEGMA (0.76 g, 0.1 mmol), styrene (1.55 g, 15 mmol), maleic anhydride (0.20 g. 2.0 mmol), AIBN (8 mg, 0.025 mmol) and 1,4-dioxane (7 mL) were added to a 25 mL round-bottom flask with a magnetic stirrer. The flask was then carefully degassed with nitrogen for 1 h and placed in a preheated oil bath at 80 °C. After stirring for 20 h, the mixture was cooled in ice bath and precipitated twice in 200 mL of cold diethyl ether. The product was collected by filtration and dried under vacuum oven at 40 °C, yielding a yellow solid (1.5 g, yield: 60%).

2.4 Synthesis of core CL micelles of POEGMA-*b*-PSMA-*b*-PS

The block copolymer POEGMA-*b*-PSMA-*b*-PS (15 mg, 0.02 mmol maleic anhydride group) and 2,2-bis(aminoethoxy)propane (3.3 mg, 0.02 mmol) were dissolved in 1.2 mL DMF homogeneously. Then, 15 mL of phosphate buffer saline (PBS, 10 mM, pH 7.4) was added dropwise (5 mL/h) to the solution under stirring. The micelle solution was stirred for additional 12 h and was dialyzed against distilled water (pH 9.0) for 48 h using dialysis membrane (MWCO 13 kDa) to remove unreacted cross-linker, DMF and salt.

The micelles with acid non-degradable cross-linker was prepared following the same process above, except that 2,2'-(ethylenedioxy)bis(ethylamine) was used as non-degradable cross-linker instead of 2,2-bis(aminoethoxy)propane.

2.5 Preparation of DOX-loaded Core CL micelles of POEGMA-*b*-PSMA-*b*-PS

Firstly, DOX.HCl (40 mg, 0.07 mmol) was stirred with triethylamine (30 µL, 0.22 mmol) in DMF (4 mL) overnight in the dark. A mixture of DOX (370 µL, 10 mg/mL), 2,2-bis(aminoethoxy)propane (200 µL, 16.5

mg/mL in DMF, 0.02 mmol), and POEGMA-*b*-PSMA-*b*-PS block copolymer (1 mL, 15 mg/mL in DMF) was added to a 25 mL round-bottom flask with a magnetic stirrer. Then, PBS (15 mL, pH 7.4, 10 mM) was added dropwise to the solution under vigorous stirring for 3 h. The micelle solution was kept stirring for additional 12 h to induce cross-linking reaction. The CL micelle solution was transferred into a dialysis bag (MWCO 13 kDa) and dialyzed against 2 L of distilled water at pH 9.0. Unloaded DOX was remove by centrifugation at 2500 rpm for 20 min. To determine the drug loading content (DLC) and drug loading efficiency (DLE), DOX loaded CL micelles was measured by using UV-Visible spectroscopy with a standard calibration curve at 485 nm. The DLC and DLE were calculated by the following equations: DLC (%) = (weight of loaded drug in micelles/total weight of drug-loaded micelles)x100 and DLE (%) = (weight of loaded drug in micelles/weight of drug in feed)x100. The DLC and DLE were calculated to be 18 and 90 (wt. %), respectively. The CL micelle with acid non-degradable cross-linker was prepared following the same process above, except that 2,2'-(ethylenedioxy)bis(ethylamine) was used as non-degradable cross-linker instead of 2,2-bis(aminoethoxy)propane.

2.6 In vitro drug release

DOX release experiments were conducted at 37 °C at different pH values. In a typical procedure, 2 mL of DOX loaded CL micelles (1 mg/mL) was transferred in a dialysis bag (MWCO 13 kDa) and immersed into 20 mL of acetate buffer (0.1 M, pH 5.0) or PBS (0.1M, pH 7.4) in a water bath at 37 ^{o}C with shaking rate of 80 rpm. At selected time intervals, 3 mL of release media was taken out and an equal volume of fresh buffer at the same condition was replenished after each sampling. The amount of DOX release was measured using UV/Vis absorbance at 485 nm. The experiments were carried out in triplicate and average values were taken.

2.7 Characterization

^{1}H NMR spectra were measured on a JNM-ECP 400 (JEOL) instrument. Molecular weight and molecular weight distribution were determined by gel permeation chromatography (GPC) Agilent 1100 GPC with differential refractive index (RI) detector (THF as an eluent at flow rate of 1.0 ml/min). The system was calibrated with commercial polystyrene standards. Fourier transform infrared (FTIR) spectra were measured on an Agilent Cary640 spectrometer in the 4000-400 cm^{-1} range. The micelles were deposited on copper grids without staining. Dynamic light scattering (DLS) was carried out on Otsuka ELS-8000 with He-Ne laser 633 nm and 90^{o} collecting optics.

3 RESULT AND DISCUSSION

Synthesis and characterization of core CL micelles

The triblock copolymer of POEGMA-*b*-PSMA-*b*-PS was synthesized by two-step sequential RAFT polymerization as shown in Scheme 2. POEGMA homopolymer was first synthesized in DMF using DDMAT as the chain transfer agent and AIBN as an initiator at 80 ^{o}C.^{1}H NMR analysis shows the characteristic peaks of POEGMA at 4.05, 3.62, 3.35, 1.7-1.9 and 0.64-1.07 ppm attributed to C$\boldsymbol{H_2}$O ester, C$\boldsymbol{H_2}$O ether, C$\boldsymbol{H_3}$O, C$\boldsymbol{H_3}$−C, and C$\boldsymbol{H_2}$−C backbone, respectively (Figure 1a). In addition, dodecyl group of DDMAT was confirmed by the presence of peak at 1.22 ppm [24]. The degree of polymerization was calculated to be 14 by comparing the integration of peak at 3.35 ppm to the aliphatic proton 1.15-1.39 ppm. Styrene (S) and maleic anhydride (MA) were copolymerized via RAFT method using POEGMA homopolymer as a macro-initiator at ratio of [S]: [MA]: [macro-initiator]: [AIBN] = 150: 20: 1: 0.25. After all MA units were consumed to form diblock copolymers of POEGMA-*b*-PSMA, the reaction was continued with remaining styrene for PS chain extension. The number average molecular weight $M_{n,\ NMR}$ of block copolymer was calculated by comparing the integration of methylene peak (3.6 ppm) in POEGMA block with phenyl peak of styrene block (6.35-7.7 ppm) (Figure 1b). The calculated $M_{n,\ NMR}$ is 14,900 g/mol which is good agreement with theoretical value 14,700 g/mol.

The reaction between MA group and ketal cross-linker induced CL micelles. The formation of the CL micelles was evidenced by the ^{1}H NMR spectrum in DMSO-d_6. As shown in Figure 1d, the protons of the two germinal methyl groups are observed at 1.34ppm and the protons -C$\boldsymbol{H_2}$−NH- are observed at 2.81 ppm.

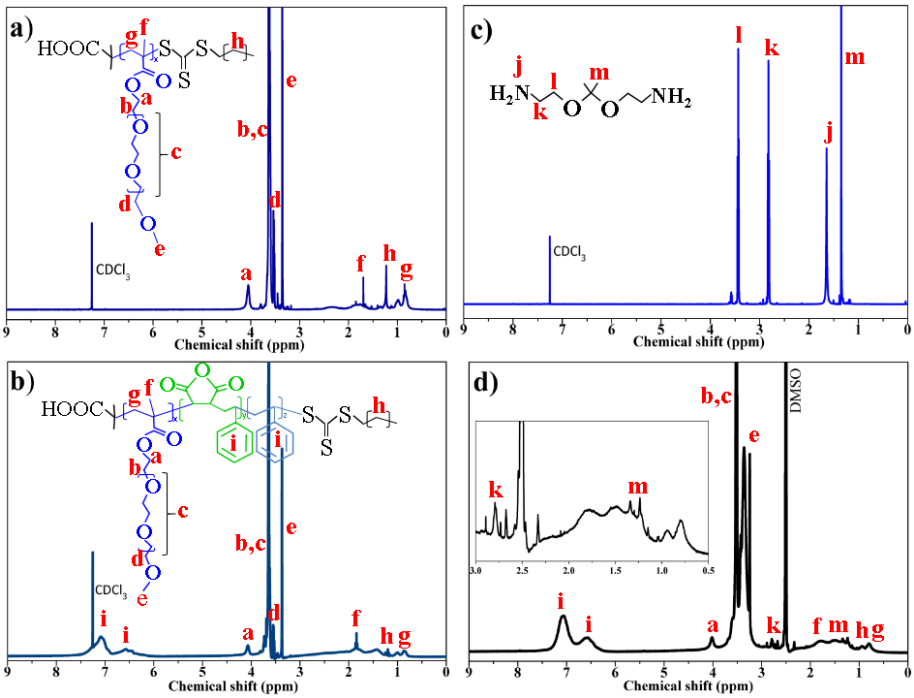


Figure 1: 1H NMR spectra of (a) POEGMA homopolymer, (b) POEGMA-b-PSMA-b-PS, (c) Ketal cross-linker in CDCl3, and (d) POEGMA-b-PSMA-b-PS CL micelles.

For further verification, IR spectroscopy was performed. The FTIR spectra of homopolymer and block copolymer are shown in Figure 2. The two peaks at 1728 cm^{-1} and 1107 cm^{-1} corresponding to the stretching vibrations of carbonyl group and ether (Figure 2a). After forming block copolymer, the spectrum in Figure 2b displays the characteristic anhydride peaks at 1777cm^{-1}, 1856 cm^{-1} and styrene peaks at 702cm^{-1}, 3027 cm^{-1} and 3061 cm^{-1}, confirming the existence of styrene and maleic anhydride in the block copolymer [24]. In the spectrum of CL micelles Figure 2d**,** the anhydride peaks have disappeared and the new peaks of amide carbonyl and carboxylic acid at lower frequency. In addition, the absorption at 1570 cm^{-1} and 1486 cm^{-1} of ketal cross-linker exits in product shows the evidence of successfully CL micelles.

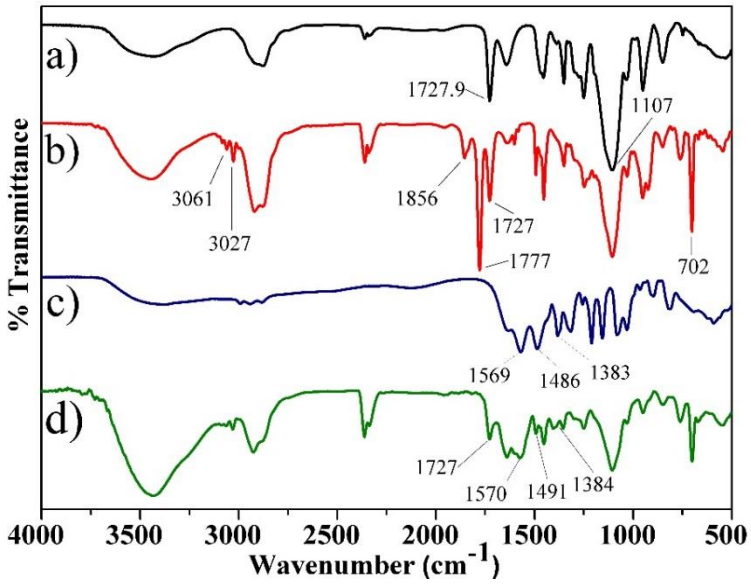


Figure 2: FTIR spectra of (a) POEGMA homopolymer, (b) POEGMA-b-PSMA-b-PS, (c) Ketal cross-linker , and (d) POEGMA-b-PSMA-b-PS CL micelles.

Gel permeation chromatography (GPC) traces of block copolymer were examined as shown in Figure 3. POEGMA-*b*-PSMA-*b*-PS triblock copolymers showed unimodal curves without shoulder in the direction of low molecular weight, indicating high initiation efficiency of POEGMA macro-RAFT agent without inactive precursor. The block was obtained with monomer conversion 60% ($M_{n\ theo}$ = 14700 g/mol, M_n GPC = 11800 g/mol and polydispersity (PDI) 1.38). It is relatively low PDI value after two sequential RAFT polymerization indicated a good level of controlled radical polymerization [14]. However, the M_n GPC is smaller than theoretical values. This is due to the difference between nature of polystyrene standard and the MA containing polymer.

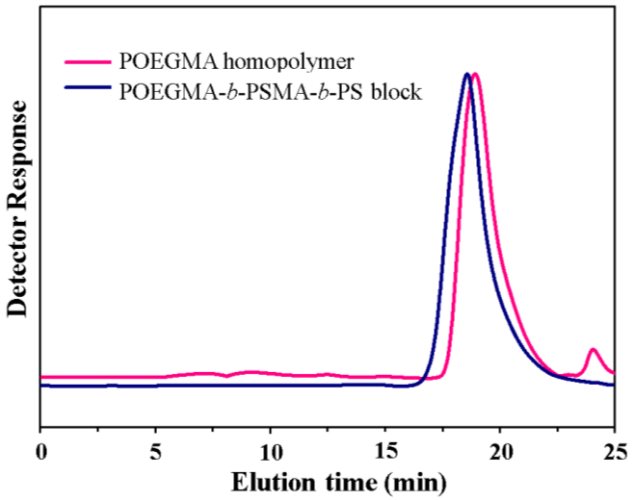


Figure 3: GPC chromatogram of POEGMA homopolymer and POEGMA-b-PSMA-b-PS triblock copolymers.

The DOX was loaded into the micelle of POEGMA-*b*-PSMA-*b*-PS blocks which consists of a POEGMA hydrophilic corona and a PSMA-*b*-PS hydrophobic core. The cores contain maleic anhydride groups which are used to react with cross-linkers to form core CL micelles with or without acid-degradable linkage. The micelles were diluted in a large amount of solvent. DMF was selected as a good solvent for both POEGMA and PSMA-*b*-PS blocks. The hydrodynamic diameter was used to evaluate the stability of micelles before and after crosslinking. An average hydrodynamic diameter of non-CL micelles was 67 nm in water, but it was below 5 nm in DMF indicating an unimer solution (Figure 4a and b). The CL micelles showed average diameter at 49 and 76 nm in water and DMF, respectively (Figure 4c and d). The results illustrated that the CL micelles were much stable than non-CL micelles [5]. In order to examine whether the core CL micelles are stimuli-responsive in an acidic environment, pH value of micelle solution was controlled to 5.0. As shown in Figure 4e, the hydrodynamic signal of CL micelles nearly disappeared, whereas the size distributions of non-ketal CL micelles were consistent before and after acid treatment in both solvents (Figure 4f and g). The results could be explained by the fact that the core CL micelles having ketal bonds in micellar cores were acid-susceptible and cleaved by an acidic reagent.

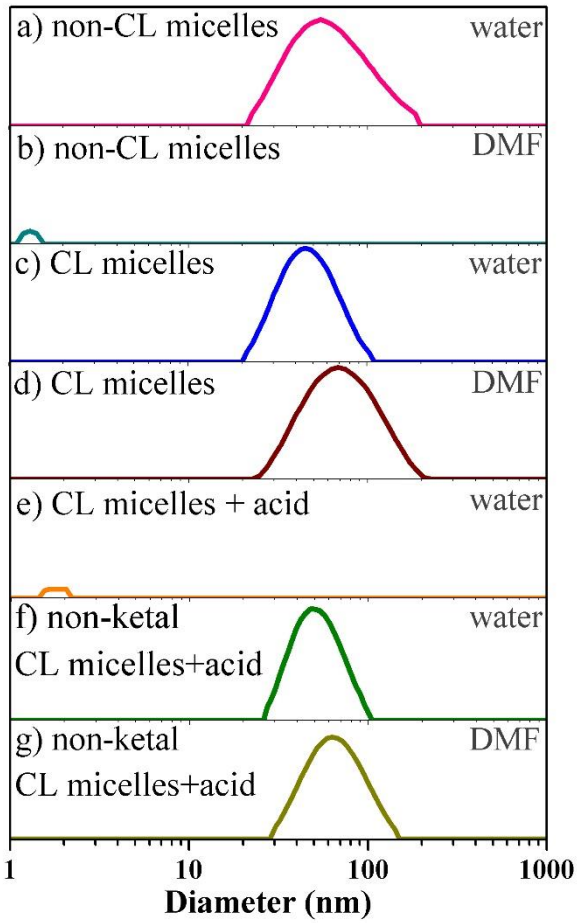


Figure 4: Hydrodynamic diameters measured by DLS: non-CL micelles in water (a) and DMF (b); CL micelles in water (c) and DMF (d); CL micelles after treatment with acid in water (e); non-ketal CL micelles after treatment with acid in water (f) and DMF (g).

In vitro release

The pH-responsive drug release was investigated in buffer solution at pH 5.0 and 7.4. As shown in Figure 5, the DOX release from non-CL micelles presented 53 and 48% of cumulative drug release corresponding to pH 5.0 and 7.4. There is only 5% drug release difference from pH 7.4 and 5.0 indicating that the effectiveness of pH on DOX release could be ignored for non-CL micelles. On the other hand, CL micelles exhibited pH-responsiveness properties. DOX release from CL micelles is 18% at pH 7.4 after 48 h but only 37% at pH 5.0. In the same condition at pH 7.4, the drug leakage from CL micelles was reduced by 2.7-fold compared to non-CL micelles illustrating the enhanced stability of CL micelles. It is important to consider that fewer drug is leaked from drug carrier system during circulation in blood stream leading to more drugs are specified on cancer cells [20]. The result suggested that the CL micelles could be not only stable during blood circulation but also effective for drug delivery in cancer cells.

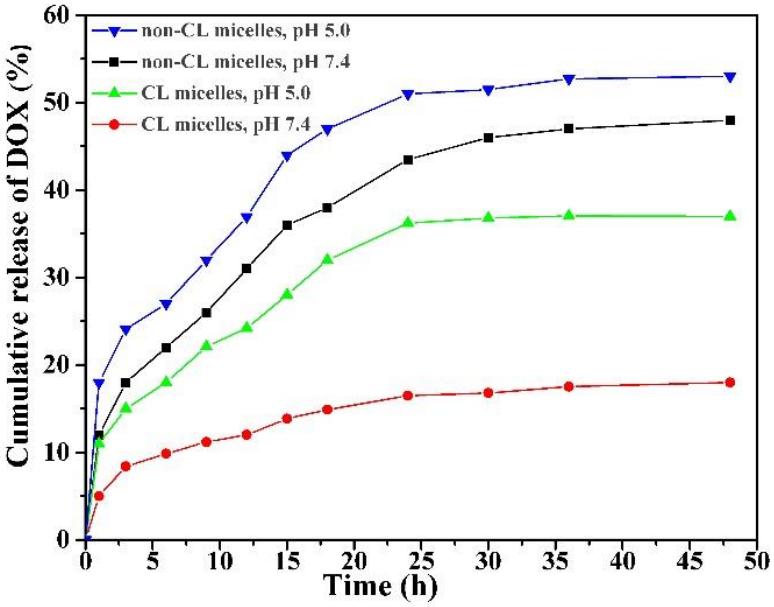


Figure 5: In vitro DOX release in PBS at 37 ºC from non-CL and CL micelles at pH 5.0 and 7.4

4 CONCLUSIONS

In this study, a drug delivery system of DOX based on pH-responsive core CL micelles has been constructed. Triblock copolymers of POEGMA-*b*-PSMA-*b*-PS were prepared by RAFT polymerization. Core CL micelles of POEGMA-*b*-PSMA-*b*-PS triblock copolymers were carried out by amidation process in the presence of an acid-cleavable ketal cross-linker. Ketal core CL micelles were more stable than non-CL micelles in selective solvent under physiological circumstance. Ketal core CL micelles have a high DOX encapsulation efficiency up to 90 (wt. %). Furthermore, ketal core CL micelles could be degraded in an acidic environment which facilitates for drug triggered release. The DOX loaded core CL micelles showed burst release behavior at pH 5.0 compared to pH 7.4 in PBS at 37 ºC. This ketal cross-linking strategy is promising a versatile route in the future development for nanomedical applications.

ACKNOWLEDGMENT

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NỐI MẠNG NHÂN MICELLE NHẠY MÔI TRƯỜNG ACID TRONG DẪN TRUYỀN THUỐC DOXORUBICIN

Tóm tắt. Một hệ thống nối nhân mạng micelle dựa trên triblock polymer của poly(oligo(ethylene glycol) methyl ether methacrylate)-*block*-poly(styrene-*alt*-maleic anhydride)-*block*-poly(styrene) (POEGMA-*b*-PSMA-*b*-PS) được tổng hợp. POEGMA-*b*-PSMA-*b*-PS được tổng hợp bằng phương pháp RAFT polymer hóa. Thuốc doxorubicin (DOX) được đưa vào trong nhân của hệ micell với POEGM như một vỏ và PSMA-*b*-PS như một nhân. Sau đó phản ứng nối mạng nhân được tiến hành dựa trên phản ứng amide hóa giữa tác nhân nối mạng 2,2-bis(aminoethoxy)propane và nhóm chức maleic anhydride trong nhân. Hàm lượng và hiệu quả thuốc DOX có thể đưa vào nhân được tính toán lần lượt là 18 và 90% khối lượng. Micell nối mạng nhân chứng tỏ có một độ bền cao dưới điều kiện sinh lý nhưng phân rã nhanh trong môi trường acid. Thực nghiệm dẫn truyền thuốc DOX chỉ ra rằng thuốc được thoát ra nhanh chóng tại điều kiện pH 5.0 so với pH 7.4.

Từ khóa. triblock polymer, RAFT polymer hóa, nhạy acid, nối mạng nhân, doxorubicin

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