



RAFT-synthesized POEGMA-b-P4VP block copolymers: preparation of nanosized micelles for anticancer drug release

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Abstract

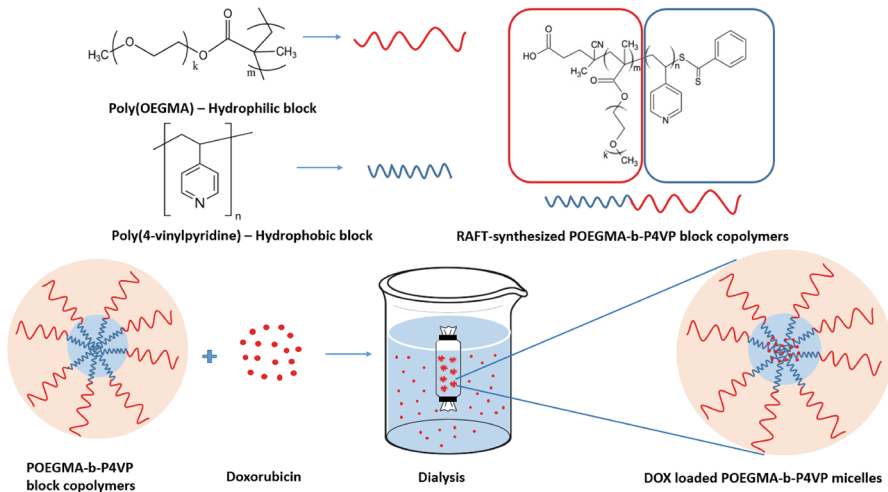
To achieve high stability and biocompatibility in physiological environment, oligoethyleneglycol methacrylate (OEGMA) and 4-vinylpyridine (4VP)-based amphiphilic block copolymers were prepared as micellar carriers to deliver doxorubicin into tumor cells. First, macroinitiator of OEGMA was synthesized by RAFT polymerization at $[M]_0/[CTA]_0/[I]_0$ ratio of 100/1/0.2 in dimethylformamide (DMF) at 70 °C, in the presence of 4,4'-azobis(4-cyanovaleric acid) (ACVA) as initiator and 4-cyano-4-(thiobenzoylthio)pentanoic acid (CTA) as chain transfer agent, respectively. It was followed by copolymerization with 4-VP at similar conditions. The formation of RAFT-mediated polymers was approved by FTIR, ¹H-NMR and GPC. For the preparation of drug-loaded micelles, a dialysis method was applied and hydrophobic doxorubicin, as a model drug, was entrapped into the micelles. Size distributions and morphologies of drug-loaded micelles were investigated by light scattering and scanning electron microscopy, respectively. Critical micelle concentration was estimated as 0.0019 mg/mL by measuring light scattering intensity in different polymer concentrations. Also, drug loading and entrapment efficiencies were calculated as 4.41% and 17.65% by measuring the DOX amount in the micelles, spectrophotometrically. At last, the drug-loaded micelles were applied to SKBR-3 breast cancer cell lines and revealed up to %40 cell inhibition at 48 and 72 h. As a result, these nanosized and biocompatible micelles can be used for the delivery of hydrophobic drugs, and they can also be modified for further targeting and imaging applications toward specific cancer cells.

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Graphical abstract



Keywords pH-responsive · Micelle nanocarrier · RAFT · Breast cancer

Introduction

Cancer is in the second rank of deaths after cardiovascular illnesses in the world. An estimated 18.1 million new cancer cases emerged and 9.6 million people were dead in 2018 [1]. Among the cancer types, breast cancer has the highest incidence rate in the world. Approximately 2.1 million women around the world were diagnosed with breast cancer for the first time in 2018 [2]. Current treatments against cancer, including breast cancer, are chemotherapy, radiotherapy and surgery. All these three treatments can be effective if the cancer is diagnosed early; however, only chemotherapy can be used if the cancer is spread to several tissues. The most significant limitation of the chemotherapy is the side effects of anticancer drugs [3, 4]. In particular, anticancer agents used in chemotherapy have cytotoxic and cytostatic properties, which cause destructive effects on healthy cells during treatment [5–7]. Besides their toxicity, these agents are pharmacologically insufficient due to their insolubility in aqueous solutions [8, 9]. To reduce the side effects of chemotherapeutics on the patient and provide effective treatment with the minimum dosage, researchers have been working on novel drug carrier systems, in which the anticancer agents' solubility is increased.

The most widely studied approach in administering anticancer agents to reduce the side effects and increase the efficiency for treating the disease is transporting the active substances with nanosized carriers, which is more advantageous than the naked drug administration. One of the essential advantages of nanocarriers is to target the drug molecule to tumors via passive targeting

(EPR effect), resulting in higher drug concentrations in the tumor site [10–12]. Liposomes, polymeric nanoparticles and micelles with diverse physicochemical properties and structures have been utilized for this purpose. Among these, polymeric micelles have held the advantages of increasing the solubility of the active agent, masking the toxicity of active agent, solvent, or adjuvant, and being modified with degradable or environment-sensitive groups easily according to the application [13–15]. Polymeric micelles are formed by the association of amphiphilic polymers generally due to the hydrophobic interactions between hydrophobic blocks of the polymers [16]. The micelle's shell is formed of hydrophilic segments that increase the residence time in the blood circulation compared to the naked nanocarriers owing to the “stealth” feature of hydrophilic segments [17, 18]. A nanocarrier without a hydrophilic coating may be inactivated by the reticuloendothelial system (RES) organs, particularly the liver. While this may be an advantage in targeting RES organs, it is problematic when it is desired to deliver drugs to areas outside the RES organs. Therefore, the hydrophilic coating has become a vital method to develop novel effective nanocarrier systems [19, 20]. The most commonly used approach for this purpose is coating with polyethylene glycol (PEG), which is a highly biocompatible FDA-approved polymer. Although alternative polymers such as poly(*N*-2-hydroxypropylmethacrylamide) (PHPMA) and poly(*N*-vinylpyrrolidone) (PVP) are also available, PEG is still the most commonly applied hydrophilic polymer. Clinical studies regarding the polymeric micelles having PEG-based hydrophilic layers are currently underway [21].

In our previous study [22], we exhibited a detailed synthesis of a novel block copolymer of 4-vinylpyridine (4VP) and oligo(ethylene glycol) methyl ether methacrylate (OEGMA). In this study, we aimed to investigate the drug delivery potential and biological efficacy of drug-loaded micelles of POEGMA-*b*-P4VP block copolymer. For this purpose, we used POEGMA-*b*-P4VP block copolymer synthesized by RAFT for the preparation of doxorubicin (DOX)-loaded micelles, and then the structure and biological efficacy of these micelles were investigated.

Materials and methods

Materials

OEGMA ($M_n = 500$ g/mol), 4VP, 4,4'-azobis(4-cyanovaleric acid) (ACVA), 4-cyano-4-(thiobenzoylthio)pentanoic acid (CTA) and sodium deoxycholate were purchased from Sigma USA. SKBR-3 (HER-2-positive human mammary gland/breast adenocarcinoma) cell lines were purchased from ATCC (HTB-30). CellTiter 96 Aqueous One Solution Cell Proliferation Assay was purchased from Promega, Madison, Wisconsin. *N,N*-dimethylformamide (DMF), diethyl ether, acetone, dichloromethane (DCM) and dimethyl sulfoxide (DMSO) were purchased from Merck, Germany. DOX.HCl was purchased from APEX-BIO, USA.

Synthesis of POEGMA-*b*-P4VP diblock copolymer

The copolymer synthesis was indicated in our previous study [22]. Briefly, POEGMA was synthesized by the RAFT polymerization of OEGMA and the $[M]_0/[CTA]_0/[I]_0$ ratio of 100/1/0.2 was used in the synthesis. OEGMA was dissolved in DMF with CTA, and the solution was purged with N_2 for 30 min. The mixture was heated to 70 °C, and then the reaction was initiated with ACVA. The polymer was precipitated with diethyl ether and dried at room temperature. Afterward, the polymer was characterized with FTIR and 1H -NMR spectroscopies and GPC. POEGMA was used as macroCTA in the polymerization of 4VP to obtain the second block of the copolymer. $[M]_0/[CTA]_0/[I]_0$ ratio is 200/1/0.2 for the RAFT polymerization of 4VP through the chain end of macroCTA (POEGMA). 4VP and macroCTA were dissolved in DMF, and the solution was purged with N_2 for 30 min. The mixture was heated to 70 °C, and the reaction was initiated with ACVA. The copolymer was precipitated with diethyl ether and dried at room temperature. The copolymer afterward was characterized with FTIR, 1H -NMR spectroscopies and GPC.

Preparation of drug-loaded POEGMA-*b*-P4VP micelles

The dialysis method was used for the preparation of drug-loaded micelles. Before drug loading, DOX.HCl was neutralized to obtain a hydrophobic form of DOX. Briefly, 30 mg of DOX.HCl was dissolved in 3 mL of DMSO and 21.63 μ L of trimethylamine was added to neutralize DOX. The mixture was stirred at room temperature overnight. The solution was dialyzed against water for 24 h using a dialysis tubing (MWCO: 3.5 kDa), and then the solution was lyophilized [23–27].

Drug-loaded micelles were prepared with DOX and DOX.HCl separately. For DOX-loaded micelles, copolymer (20 mg) and DOX (5 mg) were dissolved in 3 mL of DMF and stirred at room temperature overnight. The solution was dialyzed against water using a dialysis tubing (MWCO 3.5 kDa) for 8 h, and water was changed three times during dialysis. After dialysis, the sample was lyophilized and dried [23, 28, 29]. The same procedure was applied for DOX.HCl-loaded micelles; however, 5 mg DOX.HCl was used instead of DOX. DOX, and DOX.HCl-loaded micelles were characterized separately in terms of drug loading and loading capacity.

Characterizations

GPC analysis of polymers was accomplished by using the Viscotek TDA 302 GPC system. The Eprogen CatSEC300 column was used for separation, and the flow rate was 0.4 mL/min. 0.1 M acetate buffer (0.15 M NaCl) was used as the mobile phase. GPC system was calibrated with single standard of linear PEO ($M_w=21$ kDa, PDI=1.07).

$^1\text{H-NMR}$ spectra of the prepared polymers were acquired from Bruker 400 MHz (Germany) liquid NMR spectrometers using deuterated DMSO as the solvent.

Mean diameter and size distributions of drug-loaded micelles were determined by using Malvern Instruments Zetasizer Nano ZS (UK) instrument equipped with a 4 mV He-Ne laser operating at $\lambda = 633$ nm with a measurement backscattering angle of 173° . Samples were measured at 25°C in triplicate.

Critical micelle concentration (CMC) of the copolymer was determined by measuring the light scattering intensity of copolymer solutions prepared at different concentrations using dynamic light scattering spectroscopy.

Morphology of DOX-loaded polymeric micelles was evaluated using scanning electron microscopy (SEM) (Carl Zeiss EVO LS10, Germany). Micelle solutions were dropped on SEM stub and dried. Then, sputter coating with gold-palladium was applied onto the sample before the measurement.

Shimadzu UV-1800 UV-Vis spectrometer (Kyoto, Japan) was used to determine the absorbance of drug molecules. FTIR spectra of polymers and micelles were acquired from Thermo Scientific Nicolet 6700 Fourier Transform Infrared Spectrometer (Madison, USA).

Drug loading and encapsulation efficiencies

The micelles' drug content was determined by dissolving the micelle in DMSO with 1 mg/mL of concentration and measuring DOX absorbance at 496 nm using UV-Vis spectroscopy. The absorbance (A) of DOX in DMSO was introduced into the standard curve given in supporting information to calculate the DOX concentration (C, in mg/mL). The following equations determined drug loading and encapsulation efficiencies:

$$\begin{aligned} &\text{Loading efficiency (\%)} (\text{LE\%}) \\ &= (\text{Amount of DOX in micelles (mg)}/\text{Amount of the micelles (mg)}) \times 100 \end{aligned} \quad (1)$$

$$\begin{aligned} &\text{Encapsulation efficiency (\%)} (\text{EE\%}) \\ &= (\text{Amount of DOX in micelles (mg)}/\text{Initial amount of DOX (mg)}) \times 100 \end{aligned} \quad (2)$$

Drug release study

The drug release profile of the micelles was determined by using the dialysis technique. One milliliter of DOX-loaded micelle solutions was placed on the dialysis membrane (MWCO: 3.5 kDa) and dialyzed against 25 mL of PBS (pH 7.4) and citrate buffer (pH 5.0) in a shaker incubator at 37°C and 70 rpm. At certain time intervals (0, 4, 16, 32, 48 h), 1 mL of sample was withdrawn, and the fresh buffer was added to the solution. The release of the active substance was determined by measuring the absorption of the DOX molecule at 496 nm, and the cumulative release profiles were obtained using the following formula [30]:

$$\text{Cumulative release (\%)} (\text{CR}\%) = [100 \times ((V_m \times C_{\text{DOX}(n)}) + (1 \text{ ml} \times \sum C_{\text{DOX}(n-1)}))] / W_0 \quad (3)$$

In Formula (3), V_m is the emission media volume and W_0 is the loaded drug. $C_{\text{DOX}(n)}$ and $C_{\text{DOX}(n-1)}$ are the n th amount of DOX (mg/mL) in the sample taken from the release medium and $(n-1)$ th amount of DOX in the sample taken from the media, respectively.

Cytotoxicity assay

Breast cancer cells (SKBR-3) were cultured in growth medium McCoy supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Subculture was performed every 3 days. The cells were incubated overnight in a 100 μL nutrient medium to be 10^4 cells /well in a 96-well plate for the MTS experiment. SKBR-3 cell line was treated free doxorubicin, doxorubicin-loaded micelles and empty micelles. After the incubation for 48 and 72 h, cells were incubated with CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wis.) for 2 h, and proliferation levels were determined by measuring the absorbance at 490 nm in the microplate reader (Varioskan™ LUX, USA). Statistical comparison was made using two-way ANOVA with Sidak’s multiple comparisons test, and $p < 0.05$:*; $p < 0.01$:**; $p < 0.001$:***; $p < 0.0001$:**** were considered significant.

Results and discussion

In the study, DOX-loaded micelles of POEGMA-*b*-P4VP diblock copolymer were investigated to reveal these micelles’ potential in the breast cancer treatment. For this purpose, monodisperse POEGMA-*b*-P4VP diblock copolymer was synthesized with RAFT polymerization using our previous two-step method [22]. In the first step, the POEGMA block was synthesized with RAFT polymerization at $[M]_0/[CTA]_0/[I]_0$ ratio of 100/1/0.2 resulting in M_n of 27 kDa and PDI of 1.22. Then,

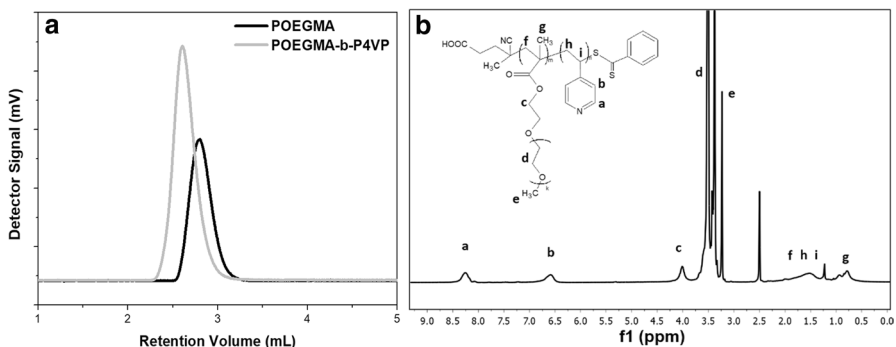


Fig. 1 GPC chromatograms of POEGMA and POEGMA-*b*-P4VP acquired from light scattering detector (a). $^1\text{H-NMR}$ spectrum of POEGMA-*b*-P4VP dissolved in deuterated DMSO (b) [22]

POEGMA was used as macroCTA in the polymerization of 4VP to obtain the POEGMA-*b*-P4VP diblock copolymer. The copolymer's molecular weight was 31 kDa (M_n), and the PDI value was 1.35. GPC chromatograms shown in Fig. 1a confirm the uniform distribution of both PEOGMA and POEGMA-*b*-P4VP. The $^1\text{H-NMR}$ spectrum of POEGMA-*b*-P4VP shown in Fig. 1b exhibits the chemical shifts belonging to the polymer's protons. While the pyridine ring protons are observed at 8.26 and 6.59 ppm, the protons of oligo(ethylene glycol) chains are seen at 4.01, 3.5 and 3.23. GPC and $^1\text{H-NMR}$ spectroscopy results reveal the successful synthesis of POEGMA-*b*-P4VP diblock copolymer [22].

The determination of the CMC value of micelle-forming copolymers is of great importance before their biological applications because it is an undesired issue if the micelle dissociates after dilution. Figure 2 shows the correlation between the concentration of POEGMA-*b*-P4VP and light scattering intensity. As seen, slope changes above a critical concentration, which gives the CMC value as 0.0019 mg/mL or 6.18×10^{-8} M. The determined value exhibits that the prepared copolymer micelle can maintain its stability under severe dilutions in biological applications.

After synthesis of the copolymer, DOX-loaded and DOX-free micelles were prepared using the dialysis method. In order to prepare DOX-loaded micelles, acetone, DCM, DMF and DMSO were tested as solvents. Since the solubility of DOX and DOX.HCl is better with DMF and DMSO, these solvents were used in the preparation of drug-loaded POEGMA-*b*-P4VP micelles. Chemical structures of micelles were characterized by FTIR spectroscopy and compared with the polymers. FTIR spectra of DOX, POEGMA, POEGMA-*b*-P4VP and DOX-loaded POEGMA-*b*-P4VP micelles are shown in Fig. 3. Figure 3a shows the FTIR spectrum of a pure DOX molecule in which the bands at 3525 and 3311 cm^{-1} belong to $-\text{OH}$ and $-\text{NH}_2$ groups in the DOX molecule. $\text{C}=\text{O}$ groups of DOX give bands at 1730 and 1616 cm^{-1} , while aromatic rings are observed at 1580 cm^{-1} . According to Fig. 3b-d,

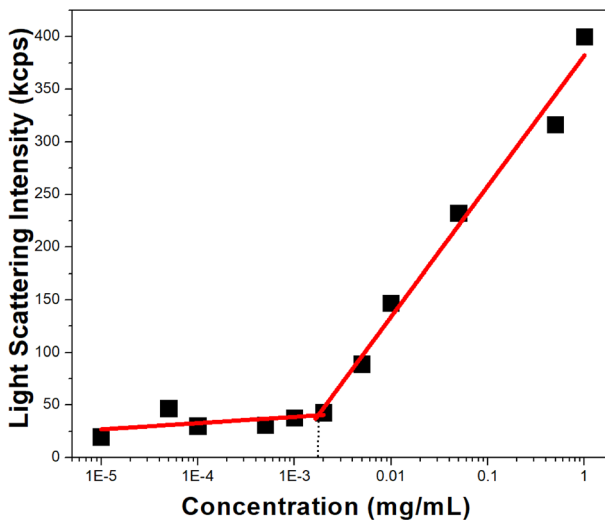


Fig. 2 Dependence of light scattering intensity on concentration of POEGMA-*b*-P4VP solutions at pH 7

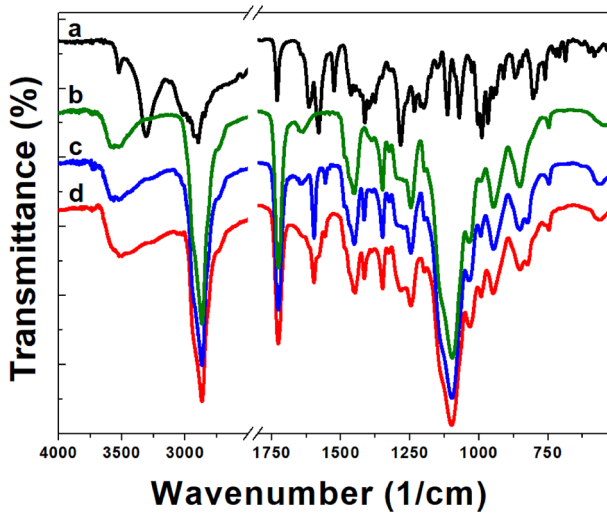


Fig. 3 FTIR spectra of DOX (a), POEGMA (b), POEGMA-*b*-P4VP (c) and DOX-loaded POEGMA-*b*-P4VP micelles (d)

C=O stretching signals of the carbonyl group were observed at 1726 cm^{-1} . C–O–C stretching of repeated $-\text{OCH}_2\text{CH}_2-$ units of POEGMA and the stretching vibrations of the $-\text{COO}-$ bonds are at 1098 and 1246 cm^{-1} , respectively. C=N stretching of the pyridine rings in the copolymer and the micelle are observed at 1596 cm^{-1} as shown in Fig. 3c, d. Furthermore, DOX-loaded POEGMA-*b*-P4VP micelles have a broader band at 3500 cm^{-1} than POEGMA-*b*-P4VP, which corresponds to the OH group of DOX [31]. Besides, small shoulders are observed at the band of 1596 cm^{-1} in the spectrum of DOX-loaded POEGMA-*b*-P4VP micelles, which corresponds to C=O and aromatic groups of DOX. It was evident that a part of DOX molecules exist on the surface of the micelle [32, 33].

The prepared DOX-free and DOX-loaded micelles were examined with dynamic light scattering (DLS) spectroscopy to reveal their hydrodynamic diameters, size distributions and zeta potential. While DOX-free micelle has a hydrodynamic diameter of 120 nm and unimodal size distribution (Fig. 4a), DOX-loaded

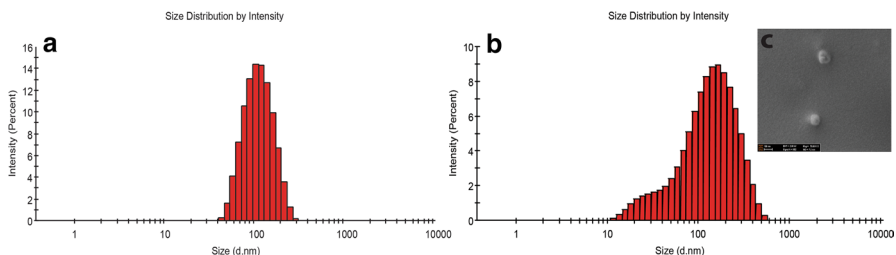


Fig. 4 The size distribution of DOX-free micelles (a) and DOX-loaded micelles (b) acquired from DLS spectroscopy. SEM image of DOX-loaded micelles (c)

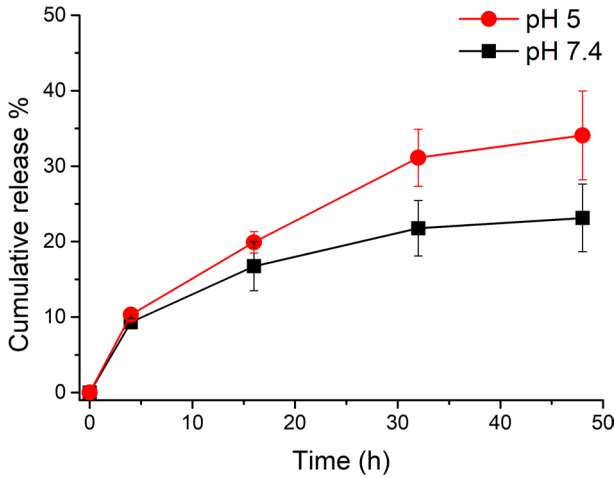


Fig. 5 In vitro drug release profile of DOX-loaded POEGMA-*b*-P4VP micelles in PBS at pH 7.4 and pH 5

Table 1 Loading and entrapment efficiencies of DOX-loaded and DOX.HCl-loaded POEGMA-*b*-P4VP micelles

| Sample | Encapsulation efficiency (EE) (%) | Loading efficiency (LE) (%) |
|------------------------|-----------------------------------|-----------------------------|
| DOX.HCl-loaded micelle | 1.29 | 0.32 |
| DOX-loaded micelle | 17.65 | 4.41 |

micelle’s hydrodynamic diameter is 154 nm and exhibits almost bimodal distribution (Fig. 4b). PDI values also confirm the broader size distribution of DOX-loaded micelle, of which PDI of DOX-free micelle is 0.21, while PDI of DOX-loaded micelle is 0.42. It is evident that the size of the addition of hydrophobic drugs into the micelle caused an expansion of the micelle size but added drug molecules increased hydrophobicity and thus increased the secondary aggregations. Also, zeta potentials of the free micelles and DOX-loaded micelles were investigated at neutral pH. According to our previous studies, copolymers form negatively charged micelles at neutral pH due to POEGMA blocks containing ionized carboxylic acid groups in the hydrophilic shell of micelles, making the micelles negatively charged [22]. In this study, the zeta potential of free and DOX-loaded micelles was -14.2 and -17.2 mV, respectively. Since the p(4-VP) moieties are not protonated in neutral medium, the negative charge of the POEGMA’s chain end group becomes more dominant and the micelle becomes negatively charged.

The size and morphology of DOX-loaded micelles were examined with SEM image (Fig. 4c), which is consistent with DLS measurement. The SEM image also reveals the spherical shape of the DOX-loaded micelles.

The hydrophobicity of a drug is a significant parameter affecting the loading into the micelles’ hydrophobic core. In our study, we evaluated the loading efficiencies of hydrophobic neutralized DOX and its hydrophilic variant of HCl salt

into POEGMA-*b*-P4VP micelles. Table 1 gives loading and encapsulation efficiencies of these drugs in which hydrophobic DOX exhibits significantly higher loading and encapsulation performance than the hydrophilic DOX.HCl. As predicted, hydrophilic DOX preferred to be dissolved in an aqueous solution besides loading into the micelle's hydrophobic core during dialysis. Thus, hydrophobic DOX-loaded micelles were preferred for investigation as the drug delivery system in this study.

The drug release profile of carrier systems directly affects drugs' bioavailability and depends on both the polymer and the drug's properties [34]. Therefore, it is critical to evaluate the drug release profile of the micellar carrier. In this study, the *in vitro* DOX release profile of the POEGMA-*b*-P4VP micelles was examined in PBS buffer (pH 7.4) and acetate buffer (pH 5) at 37 °C to evaluate the pH-sensitive DOX release from POEGMA-*b*-P4VP micelles. Figure 5 shows a biphasic cumulative release of DOX from the micelles. In the first 20 h, a burst release was observed due to the DOX molecules within the micelle's shell or at the interface between the core and shell [35]. According to Fig. 5, the DOX release reached 22% in 48 h hours in pH 7.4, while the release reached 35% in pH 5. The slight change in DOX release profile depending on pH can be explained by the inherent pH sensitivity of DOX and the increase in DOX release due to the protonation of the vinylpyridine groups through polymer chain [36]. The micelle's DOX release profile is similar to the DOX release profile from the block copolymer of poly(ethylene glycol)–poly(β -benzyl-L-aspartate) that revealed a biphasic drug release with a burst release phase and a long sustained release phase in pH 5 and pH 7.4 [37]. Possible π – π stacking between DOX and pyridine aromatic rings can cause the prolonged release of DOX molecule from the core of POEGMA-*b*-P4VP micelle [38].

In vitro evaluation of DOX-free and DOX-loaded micelles was accomplished by measuring their effect on viabilities of breast cancer cells (SKBR3) using MTS assay. Figure 6a, b shows cells' viability against empty micelles, and DOX-free micelles did not reveal any toxic effect on the SKBR3 cell line between the concentrations of 0.1 and 0.0001 mg/mL at different exposure times (48 and 72 h). This is an expected result because using PEG-based polymers on the shell part of the micelles increases the biocompatibility of the nanocarriers [39].

SKBR3 cells were treated with DOX-loaded micelles and free DOX at different concentrations of DOX (4–0.1 μ g/mL) for 48 and 72 h to evaluate the effect of using a micellar carrier on cell viabilities. After 24 h of treatment, the proliferation of SKBR3 cells did not change, revealing that the cells were not affected by drug and drug-loaded micelles. Figure 6c, d exhibits the change in proliferation of cells treated with DOX and DOX-loaded micelle for 48 and 72 h, respectively. As seen, the proliferation of SKBR3 cells decreases after 48 and 72 h of treatment with DOX-loaded micelles. At the beginning, free DOX exhibits higher toxicity on the cells probably because of instantaneous drug exposure. However, the toxicity of the DOX molecule was sustained when it was loaded into the micelle after 48 and 72 h, which was causing slower and controlled drug release up to 1 μ g/mL drug concentration. Since the micelles did not release all the DOX molecules within the 24 h, it confirms that lower concentration of DOX was released to the micelle's surroundings. Li et al.

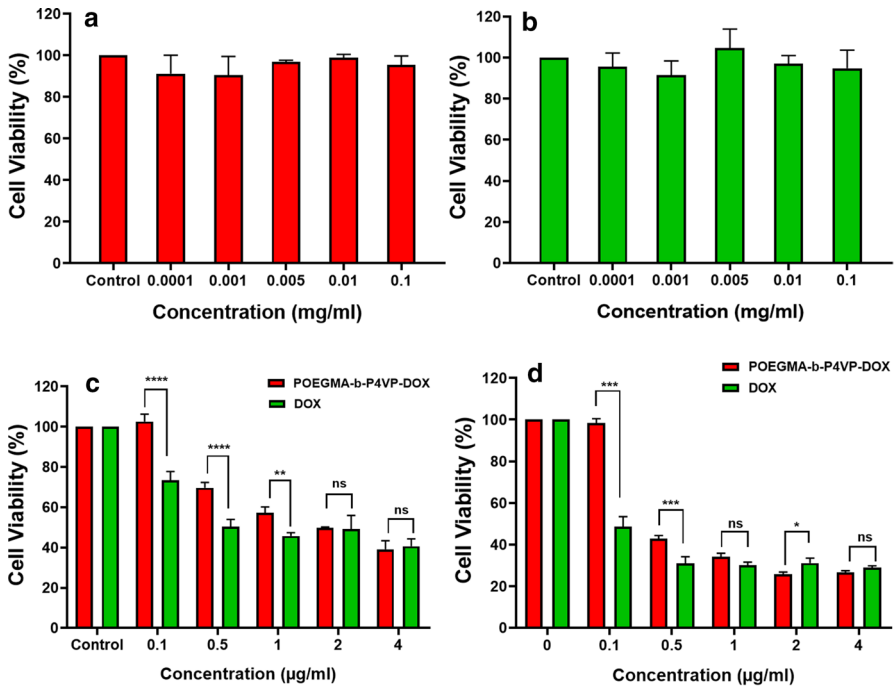


Fig. 6 Viability of SBKR3 cells at different concentrations of DOX-free micelles incubated for 48 h (a) and 72 h (b) and, DOX and DOX-loaded POEGMA-*b*-P4VP micelles incubated for 48 (c) and 72 (d) hours

reported a similar delayed drug release and inhibition effect on MCF-7 cells with redox-sensitive PEG-based nanomicelles [40]. In Jiang et al.’s study, DOX-loaded poly(caprolactone) and poly(carboxybetaine) containing micelles revealed similar inhibition effects on cancer cells [41]. In light of these results, our results are compatible with the literature [42].

Conclusion

In this study, the POEGMA-*b*-P4VP micelles were prepared for the delivery of DOX to cancer cells. First, POEGMA-*b*-P4VP diblock copolymer was synthesized with RAFT polymerization. The critical micelle concentration of the copolymer was determined by measuring the light scattering intensity, which showed a high stability of the micelle structure. After polymerization, doxorubicin-loaded micelles were prepared by the dialysis method using hydrophobic and hydrophilic doxorubicin to evaluate DOX loading capacity. The high loading capacity of hydrophobic DOX showed that the 4-vinylpyridine group, which has an internal structure and provides hydrophobicity of micelle, interacts with it. DOX-loaded micelles had particulate structures with almost 120 nm of diameter confirmed with DLS and SEM analysis. Besides, POEGMA-*b*-P4VP micelles exhibited an increased sustained release

of DOX molecules in the acidic medium compared to the neutral medium, which indicates that this micelle has a pH sensitivity. Also, *in vitro* cell studies showed that doxorubicin-loaded micelles inhibited the proliferation of breast cancer cells better than free DOX molecules after 48 h at 2 $\mu\text{g}/\text{mL}$ of DOX concentration. Therefore, the micelles obtained in this study can be used as nanosized carriers for effective delivery of anticancer agents due to their low PDI and good biocompatibility and can also be modified for further targeting and imaging applications toward specific cancer cells.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00289-021-03964-8>.

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