Preparation and characterization of potential doxorubicin-loaded mixed micelles formed from vitamin E containing graft copolymers and PEG-b-PLA diblock copolymers

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In this study, mixed micelles formed from biocompatible diblock copolymer methoxy poly(ethylene glycol)-b-poly(D,L-lactide) (mPEG-b-PLA) and vitamin E containing graft copolymer poly(hydroxypropyl methacrylamide)-g-\(\alpha\)-tocopheryl succinate (PHPMA-g-\(\alpha\)-TOS) were investigated to encapsulate anticancer drug doxorubicin (Dox) for evaluating anticancer capacity. Various graft and diblock copolymers were synthesized and characterized. Mixed micelles with optimal size and size distribution were successfully obtained from a fixed chain length and composition of graft and diblock copolymer. Dox was then loaded in the mixed micelles and the micelles were demonstrated an acid-induced rapidly drug release behavior. The mPEG-b-PLA in mixed micelles not only exhibited an excellent anti-protein adsorption ability, but also accelerated \(\alpha\)-TOS cleaved from graft copolymers and released from mixed micelles. From the cytotoxicity test, mixed micelles presented low risk to L929 normal cells but high toxicity to HCT116 colon cancer cells. Internalization study indicated that mixed micelles were accumulated more in HCT116 cells than micelles prepared from graft copolymer alone because mixed micelles had better stability in serum medium. 


eX\textsuperscript{vivo} study also showed that mixed micelles could largely accumulate in the tumor. Based on these results, this mixed micelle system has a great potential as a Dox-loaded carrier for use in cancer chemotherapy.

1. Introduction

Polymeric micelles which are formed by the self-assembly of amphiphilic polymers could be used to improve drug solubility and increase circulation half-life\textsuperscript{1, 2}. Among different architecture of micelles, mixed micelles have recently attracted much interest because of their beneficial properties, such as improving the physical stability, enhancing drug loading capacities of conventional polymeric micelles for drug delivery\textsuperscript{3, 4}. Due to these advantages, several mixed micelle systems have been developed as drug carriers for cancer therapy. For example, multi-functional mixed micelles made by Lo et al. which composed of poly(N-isopropylacrylamide-co-methacrylic acid)-graft-poly(d,l-lactide) (P(NIPAAm-co-MAA)-g-PLA) and methoxy poly(ethylene glycol)-b-poly(d,l-lactide) (mPEG-b-PLA) were demonstrated the good pH- and thermo-sensitivities for controlling drug release and overcame the limitation of graft copolymers used in drug delivery.\textsuperscript{5} Additionally, Lo et al. also applied a critical micelle concentration (Cmc) character’s diblock copolymer, mPEG-b-PLA (methoxy poly(ethylene glycol)-block-poly(d,l-lactide)), and temperature-sensitive character’s diblock copolymer, mPEG-b-P(NIPAAm-co-VIm) (methoxy poly(ethylene glycol)-block-poly(N-isopropylacrylamide-co-vinylimidazole)), to form mixed micelles to improve markedly micellar stability and extend the range of applications of micelles in controlled drug delivery.\textsuperscript{6} Otherwise, Lee et al. investigated mixed micelles by blending PolyHis-b-PEG (poly(L-histidiene) –block –poly(ethylene glycol)) with PLLA-b-PEG-b-PolyHis-biotin (poly(L-lactic acid)-block-poly(ethylene glycol)-block-poly(L-histidiene)-biotin). They concluded that their mixed micelles could expose biotin to enhance tumor specificity targeting and rapidly release of drugs under slightly acidic environmental conditions of various solid tumors.\textsuperscript{7}

In this study, a mixed micelle structure composed of \(\alpha\)-tocopheryl succinate (vitamin E analogue) containing graft copolymers, poly(hydroxypropyl methacrylamide-g-\(\alpha\)-tocopheryl succinate (PHPMA-g-\(\alpha\)-TOS) and diblock copolymers, methoxy Poly(ethylene glycol)-b-Poly(d,l-lactide) (mPEG-b-PLA) was prepared to encapsulate doxorubicin (Dox) for anticancer drug delivery, as shown in Fig. 1(A). The \(\alpha\)-tocopheryl succinate (\(\alpha\)-TOS) has been widely studied because of their anti-tumor properties.\textsuperscript{8, 9} It has been demonstrated that \(\alpha\)-TOS molecules could selectively kill cells with a malignant or transformed phenotype\textsuperscript{10-12}. Additionally, \(\alpha\)-TOS and Dox could exhibit cooperated effect to increase the anti-cancer efficacy via enhancing cellular uptake level of Dox\textsuperscript{13}. In our previous study, \(\alpha\)-TOS was conjugated onto mPEG-PHEMA (methoxy polyethylene glycol-block-poly(2-hydroxyethyl methacrylate)) by ester bonds and form polymeric micelles with Dox. The ester bonds between \(\alpha\)-TOS and mPEG-PHEMA could be degraded at acidic surroundings. Our study demonstrated that micelles not only reduced normal cell cytotoxicity but also enhanced chemotherapy efficacy by co-delivery of \(\alpha\)-TOS and Dox.\textsuperscript{14} Herein, poly(N-(2-hydroxypropyl) methacrylamide) (PHEMA) was instead of PHEMA because PHEMA has risk of thrombosis\textsuperscript{15, 16} and was conjugated with \(\alpha\)-TOS molecules by ester bonds to form amphiphilic graft
2. Materials and methods

Methoxypolyethylene glycol (mPEG, ACS degree) with M.W. 5K, N,N'-dicyclohexylcarbodiimide (DCC, ACS degree), 4-dimethylaminopyridine (DMAP, ReagentPlus), p-toluenesulfonic acid (PTSA, ACS degree), α-tocopheryl succinate (α-TOS, ACS degree) and McCoy's 5A Medium were purchased from Sigma-Aldrich (MO, USA). Doxorubicin hydrochloride (Dox-HCl, ACS degree) was purchased from LC Laboratories (MA, USA). Tin(II)-2-ethylhexanoate and D,L-lactide were purchased from Alfa Aesar (MA, USA). The D,L-lactide was purified by crystallization twice before use. N-(2-Hydroxypropyl) methacrylamide (HPMA, ACS degree) was purchased from Polysciences (PA, USA). 2,2'-Azobis(2-methylpropionitrile) (AIBN) was purchased from UniRegion Bio-Tech (Taiwan) and was purified by crystallization twice before use. Fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibico (NY, USA).

2.2 Synthesis of mPEG-b-PLA diblock copolymers

mPEG-b-PLA diblock copolymers were synthesized by ring-opening polymerization (in Fig. 1B)\(^4\), mPEG (MW 5000) and D,L-lactide with different molar ratios were dissolved in toluene. Stannous octoate as a catalyst was then added for ring-opening polymerization. The reaction was conducted at 110 °C for 24 h under nitrogen and terminated by 0.1 N methanolic KOH. The product was precipitated from diethyl ether twice at 0 °C. The chemical structure and molecular weight of mPEG-b-PLA were verified by \(^1\)H-NMR (DMSO-d6), FT-IR (KBr) and GPC. The \(^1\)H-NMR spectrum (400MHz) was recorded using Bruker Avance III400 spectrometer. Sample concentrations in DMSO-d6 were around 10 mg/mL. The FT-IR spectrum was obtained on a SHIMADZU IRAffinity-1 in transmittance mode in the range 4500 cm\(^{-1}\)–50 cm\(^{-1}\). The polymer sample for FT-IR was well mixed with KBr in a ratio of 1:10 (v/v) and pressed to form pellets. The molecular weight and polydispersity index of polymers was measured by gel permeation chromatography (GPC) using an organic solvent GPC system equipped with a model 501 solvent delivery system and a refractive index (RI) detector with Jordi Gel DVB 104 Å column and Shodex GPC KF-G column in series. The eluent was tetrahydrofuran at a flow rate of 1.0 mL/min. Sample concentrations were 5 mg/mL, and the injection volume was 20 µL. Polyacrylic acids with five molecular weights (MW= 1210, 6950, 15100, 34400 and 127000 g/mol) and a polydispersity of below 1.16 were used as standards.

2.2 Synthesis of PHPMA-g-α-TOS graft copolymers

The PHPMA-g-α-TOS was synthesized by free radical polymerization and DCC-mediated ester coupling reaction (in Fig. 1C). First, to synthesize PHPMA, HPMA monomer and initiator AIBN were dissolved in ethanol and purged with nitrogen for 30 min at room temperature. Polymerization was preceded at 70 °C for 24 h. The reacted solution was precipitated from diethyl ether twice to obtain PHPMA product. Then, PHPMA, α-TOS, DPTS, and DMAP were dissolved in DMF under nitrogen for coupling reaction. The reaction was conducted at 4 °C for 24 h. The final product was obtained by precipitation from diethyl ether twice and...
drying in vacuum. The chemical structure and compositions of PHPMA-g-α-TOS were also determined by \(^1\)H-NMR (DMSO-\(d_6\)), FT-IR (KBr) and GPC with the same equipment mentioned above.

2.4 Preparation and characterization of grafted micelles and mixed micelles

Various composition ratios of PHPMA-g-α-TOS graft copolymers and mPEG-\(b\)-PLA diblock copolymers were dissolved, individually or together, in DMSO (2.5 mL). The polymer solution was then dialyzed though a cellulose membrane bag (MWCO 6000-8000, SpectrumLabs, Inc.) against distilled water at 25 °C for 72 h. The water was replaced every 3 h. After dialysis, micelles prepared from graft copolymers alone (grafted micelles) and mixed micelles were collected to obtain dried products by freeze drying\(^5\), \(^18\), \(^19\). The distribution of particle sizes was determined using a zetasizer (Malvern 3000HSA) that was equipped with a 633 nm He-Ne laser and the corresponding dynamic light scattering (DLS) was detected using a detector that was set at 90° to the incident laser beam. The morphology of micelles were identified using a JEM-2000EX \(\text{II}\) transmission electron microscope (TEM) with an accelerating voltage of 100 keV. Micelles on a cooper grid were stained with 4 wt% uranyl acetate (UA).

2.5 Preparation and characterization of DOX-loaded grafted micelles and DOX-loaded mixed micelles

Doxorubicin hydrochloride, triethylamine and copolymers were dissolved in DMSO for 2 h to remove the hydrochloride. The mixture solutions were then dialyzed against water for 72 h. After dialysis, the solution of micelles was collected and removed water using a freeze dryer system. Then, the size distributions of DOX-loaded grafted micelles and DOX-loaded mixed micelles were determined by DLS.

To obtain drug content in micelles, weighted amounts of the DOX-loaded micelles were dissolved in DMSO and DOX was isolated by ultrafiltration (ultrafiltration membrane MWCO 10000, Millipore) and analysed using a UV-vis spectrometer (Perkin Elmer Lambda 35) at 485 nm. The drug content of micelles was calculated by the formula: drug content (% w/w) = (total mass of DOX in micelles)/ (total mass of DOX in micelles + total mass of copolymer in micelles) × 100\(^{20,21}\).

2.6 pH sensitivity

To evaluate pH sensitivity of micelles, micelles were suspended in pH 7.4 and 4.5 PBS buffer solutions at 37 °C. At each predetermined time point, the particle size and size distribution were analysed by DLS and the morphology of micelles was observed by TEM with 4 wt% uranyl acetate (UA) staining.

2.7 Drug release assay

Figure 2. Characteristics of graft and diblock copolymers. \(^1\)H-NMR (A) and FT-IR (C) spectra of mPEG-\(b\)-PLA copolymers; \(^1\)H-NMR (B) and FT-IR (D) spectra of P(HPMA)-g-α-TOS copolymers.
To determine the release behaviors of Dox and α-TOS from micelles, Dox-loaded micelles were suspended in PBS solution (1 mL) and placed in a dialysis bag (MWCO 6000-8000). The dialysis bags were then put into a fixed volume of pH 7.4 or 4.5 buffer slides for CLSM observation. Dox and FITC were excited at 488 nm with PBS twice and then fixed by 4 wt% paraformaldehyde for 30 min. The cells were then washed with PBS twice and covered by slides for CLSM observation. Dox and FITC were excited at 488 and 633 nm, respectively. The emission wavelength of Dox and FITC was detected at 590 nm and 693 nm.

To quantify the internalization behavior and investigate whether the serum protein influence endocytosis, FITC-labeled micelles were co-cultured with HCT116 cells in serum medium or serum free medium for 24 h. The cells were then washed by PBS and were dissociated using trypsin. The cells was analyzed by BD FACSCalibur flow cytometer (BD FACSCalibur; Becton Dickinson, San Jose, CA) using the 488 nm argon/krypton laser line and a 520 nm band pass FL1(H emission filter.

### 2.11 Noninvasive imaging and ex vivo fluorescence imaging
The HCT116 cells (1x10^6 cells) were transplanted subcutaneously to the female Balb-c/nude mice. Micelles were modified with fluorescence dye Cyanine 5.5 (Cy5.5) to prepare Cy5.5-labeled micelles. Cy5.5-labeled micelles were then injected into HCT116 tumor-bearing mice (tumor volume: approximately 1000 mm^3) by tail vein injection. To observe the biodistribution of Cy5.5-labeled micelles, tumor-bearing mice were sacrificed after 24 h post-injection and the major organs were harvested immediately. The optical images of organs were obtained by IVIS 50 imaging system.

### 3. Results and discussions

#### 3.1 Polymer characteristics
In this study, mPEG-b-PLA diblock copolymers were synthesized from mPEG (5000 M.W.) and D,L-lactide by ring-opening polymerization. The ^1H-NMR and FT-IR data were showed in Figs. 2A and C. Three mPEG-b-PLA diblock copolymers with different molecular weights were prepared: the repeating units of D,L-lactide in mPEG-b-PLA copolymers were 11, 35 and 48 by ^1H-NMR measurements. The polydispersity indexes for those copolymers were 1.08, 1.13 and 1.19 by GPC determination.
The grafting level of α(TOS in graft copolymers could make mixed copolymers. The experimental results indicated that the higher copolymers were used for comparison. The particle size and PDI of grafted micelles formed from high α(TOS contents of graft copolymers were chosen for the following experiments. Otherwise, from diblock copolymers (48 repeating units of LA) and graft copolymer with different diblock copolymers as shown in Figure 6 with 16 repeating units of α(TOS. Additionally, under a fixed graft especially for mixed micelles composed of the graft copolymer micelles with smaller size and better polydispersity index, composed of a fixed diblock copolymer with different graft micelles. Figure 3A shows the characterization of mixed micelles from graft and diblock copolymers. This graft-diblock mixed micelle also indicated that graft copolymers with lower cmc led co-micellization.

In order to determine the optimal composition of mixed micelles, several strategies were used: first, three different grafting levels of PHPMA-g-α-TOS copolymers (graft copolymers were abbreviated to E and repeating units of α-TOS were displayed in numbers) were co-micellized with a fixed mPEG-b-PLA diblock copolymers (35 repeating units of LA; L35) to find out which graft copolymer fitted co-micellization best; next, the optimal PHPMA-g-α-TOS copolymer were co-micellized with three mPEG-b-PLA diblock copolymers to clarify the optimal composition of mixed micelles. Figure 3A shows the characterization of mixed micelles composed of a fixed diblock copolymer with different graft copolymers. The experimental results indicated that the higher grafting level of α-TOS in graft copolymers could make mixed micelles with smaller size and better polydispersity index, especially for mixed micelles composed of the graft copolymer with 16 repeating units of α-TOS. Additionally, under a fixed graft copolymer with different diblock copolymers as shown in Figure 3B, the most appropriate size and PDI of mixed micelles formed from diblock copolymers (48 repeating units of LA) and graft copolymers at molar ratio of 4:1 were 44.8 nm and 0.28, respectively. Based on these results, mixed micelles with high α-TOS contents of graft copolymers and long PLA chain of diblock copolymers were chosen for the following experiments. Otherwise, grafted micelles from high α-TOS contents of graft copolymers were used for comparison. The particle size and PDI of grafted micelles were 127.4 nm and 0.521, respectively (data not show).

### 3.2 Micelle preparation

A report from Chu et al. demonstrated that copolymers with lower critical micelle concentration (cmc) could induce self-assembly of mixed micelles. The copolymers with lower cmc were firstly associated into micelles, and then the copolymers with higher cmc could gradually participate into micellization.

Our group has successfully prepared a multifunctional core-shell mixed micelle from graft and diblock copolymers. This graft-diblock mixed micelle was stable in PBS; mixed micelles and grafted micelles aggregated together tightly enough to resist hydrolysis. In contrast, size changes in grafted micelles were relatively insignificant until 48 h, perhaps because that graft copolymers released α(TOS from micelles by hydrolysis. In contrast, size changes in grafted micelles were significantly altered during 24 to 48 h owing to α-TOS released from mixed micelles. At pH 4.5, mixed micelles significantly changed their particle sizes during 24 to 48 h owing to α-TOS released from micelles by hydrolysis. In contrast, size changes in grafted micelles were relatively insignificant until 48 h, perhaps because that graft copolymers aggregated together tightly enough to resist hydrolysis of ester bonds. The similar results were also found in the TEM images. Figure 5 shows that grafted and mixed micelles remained...
their structures and sizes when they were suspended in neutral pH surroundings after 48 h. Additionally, the morphology of grafted micelles in low pH did not change at 48 h. In contrast, the structure of mixed micelles significantly swelled and disrupted when they were in low pH over 48 h. These results all indicated that mixed micelles had better pH-responsibility than grafted micelles.

Furthermore, figure 6 showed the GPC signal peak shift when mPEG-b-PLA incubated in acidic environment after 24 and 48 h. The loss of molecular weight demonstrated that polyester bonds in mPEG-b-PLA were pH-sensitive and could efficiently hydrolyze in acid. This result could also prove that mPEG-b-PLA diblock copolymer was important in mixed micelle system to improve stability and pH-sensitive behavior.

### 3.4 Dox loading and drug releasing

To evaluate the loading efficiency of Dox in mixed micelles, different concentrations of Dox were mixed with graft and diblock copolymers (1:4 w/w) to form Dox-loaded mixed micelles. Table 3 summarize the particle sizes, polydispersity indexes, and drug content of Dox-loaded mixed micelles. According to the results, when the feeding concentration of Dox was at 1 mg/mL, mixed micelles had the smallest particle sizes and PDI, which were approximately 108 nm and 0.213, respectively. Drug loading content in mixed micelles was around 1 wt%. In contrast, grafted micelles were also loaded with Dox with the particle size of ca. 195 nm, PDI of 1, and drug loading content of 2 wt%.

The absorption wavelength of free α-TOS and Dox were at 284 nm and 485 nm, respectively. For Dox release measurements, wavelength scanning was set from 450 nm to 500 nm. For α-TOS
release measurements, wavelength scanning was set from 250 nm to 350 nm. Figures 7A and B displayed one sets of the wavelength scanning of released Dox and α(TOS from mixed micelles in acidic surroundings, respectively. The experimental results indicated that the release of Dox or α(TOS was increased with time. In order to evaluate the effect of pH-responsibility of micelles, micelles were treated with pH 7.4 and pH 4.5 PBS and the released Dox were monitored by UV/Vis spectrometer. At pH 7.4, grafted and mixed micelles exhibited initial burst release at first 6 h, losing about 16 and 17 wt% of Dox, respectively. The release rate slowed down and remained constant after 24 h. The cumulated release of Dox after 96 h was about 45 wt% for mixed micelle and 35 wt% for grafted micelle. At pH4.5, Dox released from mixed micelles was over 50 wt% at first 24 h and exhibited sustained release behavior. After 96 h, cumulative release of Dox from mixed micelles was over 80 wt%, while the total release of Dox from grafted micelles was less than 70 wt%.

To further understand the relationship between the α-TOS releasing rate by hydrolysis and structural changes of grafted and mixed micelles, micelles were incubated in pH 7.4 and pH 4.5 PBS buffer solutions at 37 °C. According to the results, at pH 7.4, the amount of released α-TOS were all less than 5 wt% in both grafted and mixed micelles after 96 h because that ester bonds were more stable in neutral environment. Otherwise, at pH 4.5, ester bonds were degraded and α-TOS molecules were rapidly released from mixed micelles. After 6 h, the cumulated release of α-TOS from mixed micelles was increased with time. Almost 20 wt% of α-TOS was released after 96 h. In contrast, the α-TOS release behavior for grafted micelles in acidic surroundings was remained constant and total release was less than 5 wt%. These experimental results

Table 3. Characterization and evaluation of Dox content in Dox encapsulated micelles.

<table>
<thead>
<tr>
<th>Copolymer</th>
<th>Dox conc. (mg/mL)</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Drug contents (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E16</td>
<td>4</td>
<td>108.3±22.4</td>
<td>0.214±0.079</td>
<td>1.4</td>
</tr>
<tr>
<td>L48</td>
<td>4</td>
<td>169.0±22.0</td>
<td>0.145±0.040</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>361.4±45.6</td>
<td>0.259±0.025</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>306.3±201.5</td>
<td>0.260±0.058</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>269.8±81.9</td>
<td>0.209±0.059</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Figure 7. The absorption spectra of (A) free Dox at 450-500 nm and (B) α-TOS at 250-350 nm. (C) DOX release behaviors of mixed-micelles (graft : diblock =1:4 mole ratio) and grafted-micelles in pH 7.4 and pH 5.0 buffer solutions at 37 °C. (D) α-TOS release behaviors of mixed-micelles (graft : diblock =1:4 mole ratio) and grafted-micelles in pH 7.4 and pH 5.0 buffer solutions at 37 °C. Mean±SD (n=3). Asterisk indicate statistically differences (*p<0.05).
emphasized that more α-TOS released from micelles led more size and structure changes. Mixed micelles had faster α-TOS release ability, accompanying obvious structural changes and large Dox release.

3.5 Stability in BSA solution and protein adsorption

The process of serum protein adhesion is one of the most important biological barriers to limit drug delivery\textsuperscript{23, 24}. To understand whether a micelle system has promise of long blood circulation, the stability (size changes) of micelles in BSA solution was evaluated by DLS. Size and PDI change (%) were defined as the measured particle size or PDI divided by the particle size and PDI, respectively, which were measured at 0 h. In figure 8A, after 24 h incubation, size changes in grafted micelles increased significantly. Conversely, the particle sizes of mixed micelles were relatively stable under 96 h observations. It is known that surface hydrophobicity and surface charge of micelles played the dominant roles in the recognition by serum proteins\textsuperscript{25, 26}. Serum proteins, such as albumin, apolipoprotein, IgG, and etc, are likely absorbed onto micelles with positive charges or hydrophobic surface, which could lead to micelles aggregation and accelerated clearance of micelles from blood circulation by mononuclear phagocyte system (MPS)\textsuperscript{23, 27}.

To quantify the accumulation of micelles in cells before and after protein adsorption, FITC-labeled micelles were co-incubated with HCT116 cells for 24 h and the cells were harvested and detected the florescent intensity by flow cytometry. Figure 8B shows that the amounts of grafted micelles and mixed micelles in HCT116 cells were equal in serum free medium. However, the cellular uptake of grafted micelles was significantly decreased when micelles were incubated in FBS-containing medium, indicating that protein adsorption on grafted micelles decreased cell internalization. In this study, the surface charges of grafted micelles and mixed micelles in pH7.4 PBS buffer solution were -5.03±5.81 and -3.28±1.98 mV, respectively (Figs. 8C and D). Although the zeta-potentials for both micelles were negative and closed zero, the protein adsorption behavior for grafted micelles was more obviously. The possible reason was because the hydrophobic segments (such as α-TOS) in grafted micelles were exposed on the surface when self-assembling without PEG layer covering\textsuperscript{28}. The exposed α-TOS in grafted micelles were thereby bounded by BSA to increase particle sizes. In contrast, when mPEG-b-PLA copolymers were incorporated into micelles, PEG layer could successfully prevent micelles from serum protein adhesion.

3.6 \textit{In vitro} Cytotoxicity

HCT116 colon cancer cells and L929 mouse fibroblast cells were treated with empty micelles and Dox-loaded micelles to estimate the cytotoxicity of micelles. Free α-TOS and free Dox were utilized as negative controls. Figure 9 displayed the results of L929 cells

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**Figure 8.** The relationship between protein adsorption, size alteration, cell internalization and zeta potential of grafted and mixed micelles. (A) Evaluation of the size change of mixed-micelles (graft : diblock =1:4 mole ratio) and grafted-micelles in PBS contained 10% of BSA at 37°C by dynamic light scattering. Mean±SD (n=3). (B) Quantitative analysis of florescence intensity after HCT116 cells incubated in full serum medium or serum free medium treated with FITC-labeled micelles for 24 h(SF: serum free medium). Asterisk indicate statistically differences (*p<0.05; **p<0.001; ***p<0.005). The zeta-potential of (C) grafted micelles and (D) mixed micelles.
incubated with empty micelles after 24 and 96 h. Although free α-TOS caused L929 cell death at high concentration (50 µg/mL), neither grafted micelles nor mixed micelles were toxic to normal cells during 96 h observations. The experimental results indicated that both micelles were highly biocompatible to normal cells. Otherwise, free α-TOS also displayed anticancer ability at high concentration, as shown in Figs. 10 A and B. After 96 h incubation, less than 20% of HCT116 cells were survival at a concentration of

Figure 9. The cytotoxicities of α-TOS molecules and empty micelles after incubation with L929 cells for (A) 24h and (B) 96 h.

Figure 10. The cytotoxicities of α-TOS molecules and empty micelles after incubation with HCT116 cells for (A) 24h and (B) 96 h. The cytotoxicities of free Dox and Dox-loaded micelles after incubation with HCT116 cells for (C) 24h and (D) 96 h.
25 µg/mL. But, grafted micelles and mixed micelles also exhibited non-toxic to cancer cells in 24 or 96 h. In contrast, as shown in Figs. 10 C and D, Dox–loaded micelles, especially for Dox-loaded mixed micelles, were highly sensitive and toxic to HCT116 cells at 24 and 96 h. The high cytotoxicity for Dox-loaded mixed micelles as compared with free Dox and Dox-loaded grafted micelles was because of cell accumulation (internalization) and the cooperative effect of Dox and released α-TOS.

3.7 Internalization

Cellular uptake behaviors of grafted micelles and mixed micelles were observed by confocal laser scanning microscopy (CLSM). FITC dye was labelled on grafted and mixed micelles and the FITC-labelled micelles were incubated with HCT116 cells for 1 and 24 h. In Fig. 11, after 1 h incubation, free Dox rapidly diffused into HCT116 cells and accumulated in nucleus by simple diffusion. In contrast, micelles were internalized by endocytosis29. Micelles were first accumulated in endosomes and then released Dox from endosomes to cytosol. Furthermore, Dox-loaded mixed micelles were internalized more by HCT116 cells and faster released Dox than Dox-loaded grafted micelles in either 1 or 24 h. The CLSM results emphasized again that (1) the reducing protein adsorption in mixed micelles increased cell internalization and (2) mixed micelles were more sensitive in acidified endosomal environment to release Dox.

![Figure 11](image_url)
3.8 Ex vivo fluorescence observation

Figure 12 shows the tumor and organ accumulation by passive targeting and EPR effect after 24 h post-injection of grafted micelles and mixed micelles. The experimental result indicated that either grafted micelles or mixed micelles accumulated in the tumor, liver and kidney. The fluorescent intensities of both micelles in the tumor were larger than those in the liver. The accumulation of both micelles in the tumor was due to the EPR effect\(^\text{30}\), while the liver accumulation was because of the uptake of micelles by RES system\(^\text{31}\). Serum albumin bound on nanoparticles is considered to prolong blood circulation time of nanoparticles\(^\text{32}\). Although the distribution percentage of nanoparticles in tumor was size-dependent\(^\text{33}\), reports also indicated that particles larger than 500 nm in diameter resulted in the rapid clearance from the circulation system\(^\text{34, 35}\). The tumor distribution percentage for nanoparticles with 150 nm in diameter was no different than that with 300 nm\(^\text{35}\). Because the sizes of mixed micelles and grafted micelles after protein treatments were around 110 and 320 nm, respectively, the tumor accumulation behaviors for both micelles were similar. Although the ex vivo results seemed small difference between grafted micelles and mixed micelles, mixed micelles showed several advantages over grafted micelles to be a good candidate as a drug delivery system in cancer therapy, such as: relative uniform sizes, rapid pH-sensitivity in acidic surroundings, higher cytotoxicity to cancer cells, and higher internalization by cancer cells.

4. Conclusions

In this study, mixed micelles constructed from mPEG-\(b\)-(PLA diblock copolymers and PHPMA-\(g\)-\(\alpha\)-TOS graft copolymers were prepared to improve cancer cell uptake and enhance cancer cell cytotoxicity. Compared with grafted micelles, mixed micelles could simultaneously release Dox and \(\alpha\)-TOS at low pH, which could exhibit cooperative effect of cytotoxicity for HCT116 cancer cells in vitro. According to these experimental, we could conclude that mixed micelles composed of graft and diblock copolymers had better drug release behavior, protein adsorption resistance, cellular accumulation and toxicity, which could be recognized as a good candidate drug carrier rather than micelles composed of graft copolymers alone.

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