Preparation and evaluation of poly(\(L\)-histidine) based pH-sensitive micelles for intracellular delivery of doxorubicin against MCF-7/ADR cells

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In this study, a pH-sensitive micelle self-assembled from poly(\(L\)-histidine) based triblock co-polymers of poly(ethylene glycol)–poly(\(L\),\(\alpha\)-lactide)–poly(\(L\)-histidine) (mPEG-PLA-Phis) was prepared and used as the intracellular doxorubicin (Dox) delivery for cancer chemotherapy. Dox was loaded into the micelles by thin-film hydration method and a Box–Behnken design for three factors at three levels was used to optimize the preparations. The optimized mPEG-PLA-Phis/Dox micelles exhibited good encapsulation efficiency of 91.12%, a mean diameter of 45 nm and narrow size distribution with polydispersity index of 0.256. In vitro drug release studies demonstrated that Dox was released from the micelles in a pH-dependent manner. Furthermore, the cellular evaluation of Dox loaded micelles displayed that the micelles possessed high antitumor activity in vitro with an IC_{50} of 35.30 \(\mu\)g/ml against MCF-7/ADR cells. The confocal microscopy and flow cytometry experiments indicated that mPEG-PLA-Phis micelles mediated efficient cytoplasmic delivery of Dox with the aid of poly(\(L\)-histidine) mediated endosomal escape. In addition, blank mPEG-PLA-Phis micelles were shown to be nontoxic to MCF-7/ADR cells even at a high concentration of 200 \(\mu\)g/ml. The pH-sensitive mPEG-PLA-Phis micelles have been demonstrated to be a promising nanosystem for the intracellular delivery of Dox for MDR reversal.

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1. Introduction

Doxorubicin (Dox), an anthracycline cytotoxic drug, is commonly used as a primary and first-line chemotherapeutic agent in breast cancer therapy. However, therapeutic efficacy of Dox is far from perfect due to its poor tumor selectivity, cardiotoxicity and the emergence of multidrug resistance (MDR) during chemotherapy [1-3]. One way to resolve these problems is to encapsulate the drug into biodegradable nano drug delivery systems (Nano-DDS) [4-6]. As antitumor drug carriers, Nano-DDS exhibited unique properties such as high drug...
loading capacity, good stability and alteration of in vivo pharmacokinetic profile of incorporated payloads. Moreover, one of the most attractive property is that the Nano-DDS can prolong the systematic circulation of drugs, preferentially accumulate in tumors via the enhanced permeability and retention (EPR) effect (so-called “passive tumor-targeting”) [7–9]. The EPR effect mediated tumor targeting reduced the drug distribution in other organs and side effects, leading to the enhanced therapeutic efficacy.

Despite the enhanced distribution in the tumor cells via EPR effect, the encapsulation of payloads in endothelium after endocytosis was the major barrier during the intracellular transport. This would lead to a decreased effective concentration of payloads in the tumor cells. To further increase the effective concentration of payload, pH sensitive copolymer micelles have been developed for intracellular delivery of anticancer drug. [10] poly(ε-caprolactone)–poly(L-histidine) (PHis) based copolymers have attracted considerable attention because of their biocompatibility, low toxicity, and more importantly, appropriate responsive pH as well as endolysosomal escape properties [11,12]. According to our previous studies [13], the endolysosomal escape property of PHis based copolymers could be attributed to the “proton sponge” effect. When the micelles were endocytosing into the acidic endolysosome, the PHis blocks in the copolymers became protonated and kept the proton pump functioned, leading to the influx of Cl− ions and water molecules. This caused an increase of osmotic pressure in endolysosomes and swelling up of endolysosomes, resulting in the release of incorporated drug into the cytoplasm with the endosome membrane remaining intact. PHis-based micelles have great potential in enhancing the effective payload concentration in tumor cells due to the pH triggered payload release and endosomal escape. Noteworthy, PHis based micelles have been demonstrated to overcome multidrug resistance (MDR) of various tumors because of the enhanced intracellular delivery of anticancer drugs [14,15].

In this study, the copolymer of poly(ethylene glycol)–poly(ε-caprolactone)–poly(L-histidine) (mPEG-PLA-Phis) was used to construct a pH sensitive micelle for intracellular delivery of Dox against MCF-7/ADR cells. Box–Behnken technique with three-factor three-level was applied to optimize the incorporation of Dox into the micelles because it can depict the relationship between responses and independent variables [16–18]. The in vitro release, cell cytotoxicity, cellular uptake and subcellular distribution of the mPEG-PLA-Phis/Dox micelles were further evaluated.

2. Materials and methods

2.1. Materials

Doxorubicin hydrochloride (Dox·HCl) was purchased from Meilunbio (Dalian, China). Nα-CBZ-Nim-DNP-L-histidine was purchased from GLBiochem (Shanghai, China). ε-caprolactone was obtained from GLACO (Beijing, China). Poly(ethylene glycol) methyl ether (mPEG, MW: 2000 g/mol), Pyrene and Hoechst 33258 were purchased from Sigma-Aldrich (MO, USA). N,N′-Carbonyldimidazole (CDI) was supplied by J&K Ltd. (Beijing, China). 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and Lysotracker Green were purchased from Beyotime Biotechnology Co. Ltd (Nantong, China). Culture plates and dishes were purchased from Corning Inc. (NY, USA). RPMI 1640 medium and penicillin-streptomycin solution were purchased from Gibco BRL (Maryland, USA). Fetal bovine serum (FBS) was purchased from PAN Biotech (Aidenbach, Germany). Purified deionized water was prepared via the Milli-Q plus system (Millipore Co., Billerica, MA). All the other reagents and chemicals were of analytical or chromatographic grade and were purchased from Concord Technology (Tianjin, China).

2.2. Synthesis and characterization of mPEG-PLA-Phis copolymer

The mPEG-PLA-Phis copolymers were synthesized according to our previous research [13]. The molecular weights of the copolymers were determined by 1H NMR at 400 MHz and GPC, respectively.

2.3. Preparation of mPEG-PLA-Phis/Dox micelles

Thin-film hydration method was used to incorporate Dox to the copolymer micelles. Briefly, Dox·HCl was first dissolved in deionized water and extracted with CHCl3 by slowly adding triethylamine until the water phase became clear. The CHCl3 phase was collected and rotary evaporated to obtain the Dox base [19]. Then 7.03 mg of mPEG-PLA-Phis was mixed with 1 mg of Dox in 10 ml of dichloromethane and sonicated for 30 min. The solvent was removed by rotary evaporation at 25 °C to obtain a thin film. Residual dichloromethane remaining in the film was further evaporated overnight at room temperature under vacuum. The resultant thin film was hydrated with 10 ml of PBS (pH 7.4) for 30 min to obtain a micellar solution at 40 °C.

2.4. Optimization of Dox-loaded micelles using Box–Behnken design

The single factor study was first conducted to reveal the key factors affecting the encapsulation efficiency and drug loading. Three main technological factors were chosen, i.e. the amount of mPEG-PLA-Phis (X1, mg), the film-forming temperature (X2, °C), and the hydration temperature (X3, °C), to evaluate the interaction effects of those factors in the formulations. The encapsulation efficiency (Y1, %) and drug loading content (Y2, %) were chosen as the responses. Then, the Box–Behnken design (3-factor, 3-level) was used to optimize the test and a total of 17 experimental runs were generated by Design Expert software. All the variables were investigated at three different levels (Table 1).

| Table 1 – Variables in the Box–Behnken design. |
|----------------|----------------|----------------|
| Level          | X1             | X2             | X3             |
| Low (−1)       | 5              | 20             | 30             |
| Medium (0)     | 10             | 25             | 40             |
| High (1)       | 15             | 30             | 50             |


2.5. Characterization of mPEG-PLA-Phis/Dox micelles

2.5.1. Measurement of particle size, size distribution and zeta potential
The average particle size, size distribution and zeta potential of the copolymer micelles were measured by a Zetasizer Nano ZS instrument (Malvern, U.K.) at 25 °C after equilibration for 5 min. All the values were the average of at least three independent samples.

2.5.2. Transmission electron microscopy (TEM) observation
The morphology of the copolymer micelles was observed by JEOL JEM-2100 electron microscope (Jeol Ltd., Tokyo, Japan) with an accelerating voltage of 100 kV. Before visualization, a drop of each sample was first deposited on a carbon-coated copper grid. After drying at room temperature, phosphotungstic acid (2.0%) was used for negative dyeing, and the morphology and size of the micelles were observed under the transmission electron.

2.5.3. Encapsulation efficiency and drug loading content
The obtained micellar solution was centrifugation at 12,000 rpm for 10 min. Then the supernatant was filtered through a 0.45 μm film to remove the unincorporated aggregates. The amount of Dox encapsulated in the micelles was measured by a multifunctional microplate reader (Tecan, Austria) with excitation wavelength at 470 nm and emission wavelength at 585 nm. The encapsulation efficiency (EE, %) and drug loading content (DL, %) were calculated by the following equations, respectively:

\[
\text{EE} = \frac{\text{Weight of encapsulated drug}}{\text{Weight of feeding drug}} \times 100%
\]

\[
\text{DL} = \frac{\text{Weight of encapsulated drug}}{\text{Weight of feeding drug} + \text{Weight of copolymers}} \times 100%
\]

2.6. In vitro release of mPEG-PLA-Phis/Dox micelles
Release studies were performed to investigate the release profiles of Dox from the micelles. Dox-loaded micelles and free Dox were placed into a dialysis bag (molecular weight cutoff (MWCO) 3500 Da) and immersed into 50 ml of phosphate buffer saline (PBS, 0.01 M, pH 7.4, 5.0) containing 0.5% w/v Tween 80 at 37 °C in a shaking incubator at 100 rpm. At predetermined time intervals, 2.0 ml of release medium was sampled from the vials and the same volume of fresh buffer was added to maintain the volume. The concentration of Dox in the release medium was measured by a multifunctional microplate reader as mentioned in Section 2.5.3.

2.7. Cell culture
Doxorubicin resistant human breast cancer cell line (MCF-7/ADR) was established from parental MCF-7 and was supplied from KeyGen Biotech. Co., LTD. (Nanjing, P.R. China). MCF-7/ADR cells were cultured in RPMI 1640 medium containing 20% FBS, 1% penicillin–streptomycin solution and 1000 ng/ml Dox at 37 °C in a humidified atmosphere with 5% CO2. All the experiments were performed on the cells in the logarithmic phase of growth.

2.8. In vitro cytotoxicity assays
The in vitro cytotoxicity of free Dox and mPEG-PLA-Phis/Dox micelles in MCF-7/ADR cells was evaluated by the standard MTT assay. Briefly, MCF-7/ADR (1.4 × 104 per well) cells were seeded in 96-well plates and incubated for 24 h to allow cell attachment. The cells were then treated with free Dox, mPEG-PLA-Phis aqueous solutions and Dox-loaded micelles in a concentration gradient in separate plates for 48 h. At the end of the incubation, the cells were incubated with 20 μl of MTT (5 mg/ml) for an additional 4 h before adding 150 μl of DMSO to dissolve the MTT formazan crystals. The absorbance at 570 nm was recorded using a multifunctional microplate reader (Tecan, Austria). The wells containing untreated cells served as blank controls. Results were shown as the average cell viability of triplicate wells. The cell viability (%) was calculated using the following equation:

\[
\text{Cell viability} = \left(\frac{\text{OD}_{\text{treat}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}}\right) \times 100\%
\]

where ODblank and ODtreat are the absorbance in the absence and in the presence of sample treatment respectively, and ODcontrol is the absorbance of the medium.

2.9. Intracellular influx of Dox
The intracellular accumulation of Dox was measured by flow cytometry. The MCF-7/ADR cells (1 × 105 per well) were pre-treated with free Dox and mPEG-PLA-Phis/Dox micelles with equivalent Dox concentration (5.0 μg/ml) for 6 h. After incubation, the media were removed and the cells were carefully washed 3 times with ice-cold PBS. The cells were then harvested by trypsinization, centrifuged at 1000 rpm for 5 min, re-suspended in 500 μl of PBS medium and analyzed using a FACS Calibur BD II flow cytometry (BD Biosciences, USA).

2.10. Confocal laser scanning microscopy (CLSM) observation
Confocal laser scanning microscopy (CLSM) was used to observe the subcellular localization and intracellular release of Dox from micelles. The MCF-7/ADR cells (2 × 104 per well) were cultured on microscope slides in a 6-well plate and incubated for 24 h. Dox and mPEG-PLA-Phis/Dox micelles were added to each well and incubated for a given time (Dox concentration was kept at 2 μg/ml, respectively. The cells were then washed 3 times with ice-cold PBS and stained with LysoTracker green (60 min) and Hoechst 33342 (20 min) to visualize endolysosomes and nucleus, respectively. In the end, the cells were fixed with 4% paraformaldehyde for 30 min. The microscope images were captured using a confocal laser scanning microscope (CLSM, Olympus FV1000-IX81, Japan).
3. Results and discussion

3.1. Characterizations of mPEG-PLA-PHis copolymer

The chemical structure of mPEG-PLA-PHis copolymer was confirmed by $^1$HNMR spectra in Fig. 1A. The typical $^1$H NMR spectrum (CDCl$_3$) of mPEG-PLA-PHis copolymers showed peaks at $\delta$ 5.24 ppm (—COCH(CH$_3$)O—), $\delta$ 3.67 ppm (—OCH$_2$CH$_2$O—), $\delta$ 3.47 ppm (—OCH$_3$) and $\delta$ 1.83 ppm (—COCH(CH$_3$)O—), which were attributed to mPEG-PLA. The degree of polymerization of PLA block was about 20, which was calculated from the intensity ratio of the characteristic peaks at $\delta$ 5.24 ppm (lactic acid CH) and $\delta$ 3.47 ppm (mPEG CH$_2$). The characteristic peaks of poly(L-histidine) were located at $\delta$ 1.55 ppm (—C(CH$_3$)$_2$—), $\delta$ 4.35 ppm (—CH—NH—), $\delta$ 7.11 ppm (N—CH$=\text{N}$ of imidazole ring) and $\delta$ 8.02 ppm (N—CH$=\text{C}$ of imidazole ring). The degree of polymerization of PHis was 6, which was calculated from the intensity ratio of the isopropyl proton peaks ($\delta$ 1.55 ppm) and N-acetyl peaks ($\delta$ 4.35 ppm). The $^1$H NMR spectra of the products suggested that mPEG-PLA-PHis copolymers were synthesized. The typical GPC chromatogram of the copolymer is shown in Fig. 1B. The copolymer showed unimodal distribution with a polydispersity less than 1.2.

3.2. Preparation and optimization of DOX incorporated micelles

In the present study, the mPEG-PLA-PHis copolymers and the drugs self-assembled in aqueous condition to form micelles by a thin-film hydration method. Box–Behnken design method was used to optimize the preparation conditions. This design was suitable for exploring quadratic response surfaces and constructing second order polynomial models [20,21]. Based on
Table 3 – The composition and observed responses in Box–Behnken design.

| Run | Independent variables | Dependent variables |
|-----|-----------------------|---------------------|
|     | $X_1$ | $X_2$ | $X_3$ | $Y_1$ | $Y_2$ |
| 1   | −1    | −1    | 0     | 85.00 | 14.50 |
| 2   | 0     | 0     | 0     | 96.20 | 8.78  |
| 3   | 0     | 1     | 1     | 90.56 | 8.30  |
| 4   | 1     | −1    | 0     | 93.12 | 5.85  |
| 5   | 0     | −1    | −1    | 91.00 | 8.34  |
| 6   | 1     | 0     | 1     | 98.00 | 6.13  |
| 7   | 0     | 0     | 0     | 95.45 | 8.71  |
| 8   | 0     | 0     | 0     | 95.00 | 8.68  |
| 9   | −1    | 1     | 0     | 88.00 | 15.00 |
| 10  | 0     | 0     | 0     | 96.88 | 8.83  |
| 11  | 1     | 1     | 0     | 97.00 | 6.07  |
| 12  | 0     | 1     | 1     | 93.32 | 8.54  |
| 13  | −1    | 0     | −1    | 87.00 | 14.80 |
| 14  | 1     | 0     | −1    | 97.25 | 6.09  |
| 15  | 0     | 1     | −1    | 92.00 | 8.42  |
| 16  | −1    | 0     | 1     | 86.25 | 14.70 |
| 17  | 0     | 0     | 0     | 96.00 | 8.76  |

single factor study, three main technological factors, the amount of mPEG-PLA-Phis ($X_1$, mg), the film-forming temperature ($X_2$, °C), and the hydration temperature ($X_3$, °C), were chosen to evaluate the interaction effects in the formulations. Encapsulation efficiency and drug loading content are the key indices in assessing the drug loading capacity of micelles. Therefore, the encapsulation efficiency ($Y_1$, %) and drug loading content ($Y_2$, %) were chosen as the responses for the optimization. Each factor was assigned to three different levels as low, middle and high, respectively (Table 1). Then, 17 experimental runs were generated by Design Expert and the results were shown in Table 2. Analysis of variance for the response surface and the quadratic model was shown in Table 3, respectively.

In this case, the value of the Model $F$ was 33.99, implying that the model was significant. There was only a 0.01% chance that a “Model $F$-Value” this large could occur due to noise. Values of “Prob > $F$” less than 0.0500 indicate that the model terms were significant. The “Lack of Fit $F$-value” of 2.76 implies the Lack of Fit is not significant relative to the pure error, indicating that the quadratic model was adequate. There was a 17.58% chance that a “Lack of Fit $F$-value” this large could occur due to noise. As shown in Table 3, $X_1$, $X_2$, $X_3$, $Y_1$, $Y_2$ were significant model terms. The equation relating to responses of particle size by the design was given as below:

$$Y_1 = -8.27150 + 2.34640X_1 + 5.35700X_2 + 8.85240X_3 + 8.80000E^{-003}X_1 + 7.50000E^{-003}X_1 + 8.80000E^{-003}X_1 - 0.094420X_2^2 - 0.11062X_2^2 - 0.014205X_2^3 \quad (R^2 = 0.9776 \ P < 0.0001)$$

$$Y_2 = 16.89950 - 2.28270X_1 + 0.44990X_1 + 0.84050X_1 - 2.80000E^{-003}X_1 + 7.00000E^{-004}X_1 + 8.00000E^{-004}X_1 + 0.072660X_2^2 - 8.54000E^{-003}X_2 - 1.38500E^{-003}X_2^3 \quad (R^2 = 0.9997 \ P < 0.0001)$$

The regression equations described quantitatively the effects of three independent variables ($X_1$, $X_2$, $X_3$) on index and their correlations. Based on the equations above, fixed the variable that affected the responses least, the 3D surface response surfaces for the effect of other variables were obtained by using the Design Expert software (Fig. 2).

According to the results above, the optimized experimental conditions were shown as below: the amount of mPEG-PLA-Phis was 7.03 mg, the film-forming temperature was 26.16 °C, and the hydration temperature was 39.94 °C. Optimized micelle formulation showed encapsulation efficiency of 91.12% and drug loading content of 11.42%, which were close to the predicted values (92.30% and 12.01%). The results suggested that the optimization technique was reliable for the optimization of Dox incorporation into the micelles.

3.3. Characterization of mPEG-PLA-Phis/Dox micelles

The Dox incorporated micelles showed mean diameter of (45 ± 3.32) nm and a narrow size distribution with polydispersity index of 0.256, as determined from the DLS (Fig. 3A). This small particle size of the micelles (<200 nm) was helpful to evade the detection and uptake by the reticuloendothelial system, resulting in prolonged circulation time [22]. The EE% and DL% of the Dox incorporated micelles were 91.12% and 11.42%, respectively, indicating a good encapsulation of Dox in the micelles. The values of the zeta potentials of all the formulations were about (−15.42 ± 0.82) mV.

TEM was used to observe the morphology of the Dox incorporated micelles (Fig. 3B). The image revealed that the micelles were individual particles with a near-spherical structure homogeneously distributed around a size of 30 nm. The average diameter of micelles appeared to be inconsistent with the results obtained from DLS due to the different conditions of the micelles. The diameters detected by DLS were 'hydrated diameters', which were usually larger than their authentic diameters. TEM images obtained the size at the dried state of the sample, which was smaller than their genuine diameters.

3.4. In vitro release of mPEG-PLA-Phis/Dox micelles

To investigate the in vitro release behavior of Dox incorporated micelles, pH 7.4 and pH 5.0 phosphate buffer solutions...
Fig. 2 – 3D surface response diagrams. (A) The effect of mPEG-PLA-PHis amount ($X_1$) and the film-forming temperature ($X_2$) on the encapsulation efficiency ($Y_1$). (B) The effect of the film-forming temperature ($X_2$) and the hydration temperature ($X_3$) on the encapsulation efficiency ($Y_1$). (C) The effect of mPEG-PLA-PHis amount ($X_1$) and the hydration temperature ($X_3$) on the drug loading content ($Y_2$). (D) The effect of the film-forming temperature ($X_2$) and the hydration temperature ($X_3$) on the drug loading content ($Y_2$).

Fig. 3 – Particle size distribution (A) and transmission electron microscopy (TEM) image of mPEG-PLA-PHis/Dox micelles. Scale bar represents 50 nm (B).
were selected to simulate the physiological conditions and weakly acidic circumstance, respectively. In addition, prior to the conduct of release assays, free Dox release from stock solution was investigated as control. It was found that non-encapsulated drug was almost fully released in approximately 4 h. This suggested that Dox molecules could freely diffuse through the dialysis membrane. Fig. 4 represented the in vitro cumulative release profile of the mPEG-PLA-Phis/Dox micelles. The release of Dox was pH-dependent and increased with the decrease of pH value in releasing medium. At pH 7.4, the release of Dox was in a sustained pattern with only 42% of Dox released after 24 h. It also suggested that the micelles were stable at the normal physiological condition and maintained structure integrity before reaching the tumor site. Nevertheless, when the pH was decreased to 5.0, the Dox release was remarkably accelerated. More than 70% of Dox was released from the micelles after 24 h. This was attributed to the protonation of Phis chains of the copolymer, which disrupted the micellar structure, leading to a burst release of Dox.

3.5. In vitro cytotoxicity assays

The in vitro cytotoxicity of blank micelles, free Dox and mPEG-PLA-Phis/Dox micelles were tested against MCF-7/ADR cells by MTT assay. As shown in Fig. 5A, no obvious growth inhibition effect was observed after incubation with blank micelles (<20% inhibition), demonstrating that the mPEG-PLA-Phis copolymers were atoxic and safe for biomedical applications. Dox solutions inhibited the proliferation of MCF-7/ADR cells in a dose-dependent manner. Improvement was observed when Dox was loaded into mPEG-PLA-Phis micelles (Fig. 5B). The IC\textsubscript{50} of Dox and mPEG-PLA-Phis/Dox micelles were 120 \mu g/ml and 35.30 \mu g/ml, respectively. This indicated that the pH sensitive micelles could enhance the cytotoxicity of Dox against MCF-7/ADR cells.

3.6. Intracellular delivery of Dox by the micelles

To investigate the potential mechanism by which micelles sensitized Dox-induced cytotoxicity, the effect of mPEG-PLA-Phis/Dox micelles on the intracellular accumulation of Dox was examined using flow cytometry. As Dox is fluorescent itself, the intracellular Dox amount is in direct proportion to its fluorescence. The cellular uptake of Dox after incubation for 6 h was presented in Fig. 6. Compared to free Dox group, the intracellular levels of Dox of the micelles were significantly increased. This indicated that the copolymer was capable of increasing the concentration of Dox in MCF-7/ADR cells, resulting in the enhanced cytotoxicity.

3.7. Confocal laser scanning microscopy (CLSM) observation

The cellular internalization and intracellular distribution of Dox were evaluated by CLSM with free Dox as control. The CLSM photos of the micelles and free Dox incubated with MCF-7/ADR cells for different time intervals were presented in Fig. 7. The cell nuclei and endo-lysosomes were labeled with blue (Hoechst 33324) and green (LysoTracker DND-26) fluorescent dyes, respectively. The mPEG-PLA-Phis/Dox micelles were promptly uptaken by the cells and located in the primary

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**Fig. 4** – In vitro release profiles of Dox from the mPEG-PLA-Phis/Dox micelles in PBS (pH 7.4 and 5.0) at 37 °C. The data are presented as means (%) ± SD from three independent experiments.

**Fig. 5** – In vitro cytotoxicity of mPEG-PLA-Phis copolymer against MCF-7/ADR cells (A) and mPEG-PLA-Phis/Dox micelles (B). All experiments were tested on MCF-7/ADR cells after 48 h of incubation. Cell viability (%) was expressed as a percentage compared to the untreated control cells.
endosome, which revealed a yellow fluorescence in merged images after 1 h. The red fluorescence was increased in the cytoplasm and orange fluorescence in merged images was enhanced (the overlap of LysoTracker with Dox) after 4 h of incubation, suggesting the continuous accumulation of the micelles in the endosome. After incubation for 6 h, a purple fluorescence (the overlap of Dox with Hoechst 33258) was observed, indicating the escape of the Dox from endosome and the accumulation in the nuclei. This result indicated that the acidic endosomal pH triggered Dox release and PHis facilitated endo-lysosomal escape accounted for the high Dox intracellular delivery efficiency of Dox.

4. Conclusion

The copolymer of poly(ethylene glycol)–poly(D,L-lactide)–poly(L-histidine) (mPEG-PLA-PHis) was used to construct a pH sensitive micelle for intracellular delivery of Dox. The incorporation of Dox to the micelles was optimized using the Box-Behnken
design. The Dox incorporated micelles showed small particle size, uniform distribution and high encapsulation efficiency. The micelles demonstrated pH dependent Dox release and high toxicity against MCF-7/ADR cells. The enhanced toxicity was attributed to the endosomal pH triggered Dox release and PHis facilitated endo-lysosomal escape. The copolymer micelles have been demonstrated to be a potential nanocarrier for effective intracellular delivery of Dox to reverse tumor MDR.

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Conflicts of interest

The authors declare that there is no conflicts of interest.

REFERENCES

[1] Cho H-J, Yoon I-S, Yoon HY, et al. Polyethylene glycol-conjugated hyaluronic acid-ceramide self-assembled nanoparticles for targeted delivery of doxorubicin. Biomaterials 2012;33:1190–1200.
[2] Khasraw M, Bell R, Dang C. Epirubicin: is it like doxorubicin in breast cancer? A clinical review. Breast 2012;21:142–149.
[3] Kim JO, Kabanov AV, Bronich TK. Polymer micelles with cross-linked polyanion core for delivery of a cationic drug doxorubicin. J Control Release 2009;138:197–204.
[4] Pradeepkumar P, Govindaraj D, Jeyaraj M, et al. Assembling of multifunctional latex-based hybrid nanocarriers from Calotropis gigantea for sustained (doxorubicin) DOX releases. Biomed Pharmacother 2017;87:461–470.
[5] Li Y, Zhi X, Lin J, et al. Preparation and characterization of DOX loaded keratin nanoparticles for pH/GSH dual responsive release. Mater Sci Eng C Mater Biol Appl 2017;73:189–197.
[6] Du H, Cui C, Wang L, et al. Novel tetrapeptide, RGDF, mediated tumor specific liposomal Doxorubicin (DOX) preparations. Mol Pharm 2011;8:1224–1232.
[7] Tanaka T, Shiramoto S, Miyashita M, et al. Tumor targeting based on the effect of enhanced permeability and retention (EPR) and the mechanism of receptor-mediated endocytosis (RME). Int J Pharm 2004;277:39–61.
[8] Allen TM, Cullis PR. Drug discovery systems: entering the mainstream. Science 2004;303:1818–1822.
[9] Marchal S, Hor A, Millard M, et al. Anticancer drug delivery: an update on clinically applied nanotherapeutics. Drugs 2015;75:1601–1611.
[10] Qiu L, Qiao M, Chen Q, et al. Enhanced effect of pH-sensitive mixed copolymer micelles for overcoming multidrug resistance of doxorubicin. Biomaterials 2014;35:9877–9887.
[11] Lee ES, Shin HJ, Na K, et al. Poly(l-histidine)–PEG block copolymer micelles and pH-induced destabilization. J Control Release 2003;90:363.
[12] Chen A-Z, Chen M-Y, Wang S-B, et al. Poly(L-histidine)-chitosan/alginite complex microcapsule as a novel drug delivery agent. J Appl Polym Sci 2012;124:3728–3736.
[13] Zhang X, Chen D, Ba S, et al. Poly(l-histidine) based triblock copolymers: pH induced reassembly of copolymer micelles and mechanism underlying endolysosomal escape for intracellular delivery. Biomacromolecules 2014;15:4032–4045.
[14] Oh KT, Yin H, Lee ES, et al. Polymeric nanovehicles for anticancer drugs with triggering release mechanisms. J Mater Chem 2007;17:3987–4001.
[15] Hong W, Chen D, Zhang X, et al. Reversing multidrug resistance by intracellular delivery of Pluronic(R) P85 unimers. Biomaterials 2013;34:9602–9614.
[16] Shao Q, Deng Y, Shen H, et al. Optimization of polysaccharides extraction from Tetrastigma hemsleyanum Diels et Gilg using response surface methodology. Int J Biol Macromol 2011;49:958–962.
[17] Ferreira SLC, Bruns RE, Ferreira HS, et al. Box-Behnken design: an alternative for the optimization of analytical methods. Anal Chim Acta 2007;597:179–186.
[18] Abu-Izza KA, Garcia-Contreras L, Robert Lu D. Preparation and evaluation of sustained release AZT-loaded microspheres: optimization of the release characteristics using response surface methodology. J Pharm Sci 1996;85:144–149.
[19] Lince F, Bolognesi S, Stella B, et al. Preparation of polymer nanoparticles loaded with doxorubicin for controlled drug delivery. Chem Eng Res Des 2011;89:2410–2419.
[20] Shaikh MV, Kala M, Nivsarkar M. Formulation and optimization of doxorubicin loaded polymeric nanoparticles using Box-Behnken design: ex-vivo stability and in-vitro activity. Eur J Pharm Sci 2017;100:262–272.
[21] Abbas G, Hanif M, Khan MA. pH responsive alginate polymeric rafts for controlled drug release by using Box Behnken response surface design. Des Monomers Polym 2017;20:1–9.
[22] Gill KK, Kadoumai A, Nazzal S. Mixed micelles of PEG2000-DSPE and vitamin-E TPGS for concurrent delivery of paclitaxel and parthenolide: enhanced chemosensitization and antitumor efficacy against non-small cell lung cancer (NSCLC) cell lines. Eur J Pharm Sci 2012;46:64–71.