RESEARCH COMMUNICATION

Folate-functionalized Polymeric Micelles based on Biodegradable PEG-PDLLA as a Hepatic Carcinoma-targeting Delivery System

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Abstract

Targeted delivery of anti-cancer drugs is a highly desirable strategy to improve therapeutic outcome because of the combination of enhanced efficacy and reduced toxicity. In this study, the anti-cancer drug doxorubicin (DOX) was accommodated in the cores of polymeric micelles self-assembled from amphiphilic block copolymers of poly(ethylene glycol)(PEGs) and poly(D,L-lactide) (PDLLA) with a targeting ligand (folate) attached to the distal ends of the PEG (Folate-PEG-PDLLA). In vitro tumor cell targeting efficacy was evaluated upon observing cellular uptake of these micelles by human hepatic carcinoma cells (Bel 7402 cells) overexpressing surface receptors for folate. In control release tests, DOX behavior of controlled release in folate receptor-mediated micellar folate-PEG-PDLLA-DOX-micelles was obvious, with pH sensitivity. Bel 7402 cells showed micelles to have low toxicity and suggested potential therapeustic application as a multifunctional platform for tumor management.

Keywords: Folate targeting - polymeric micelles - hepatic carcinoma

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Introduction

One of the most important features of anti-cancer drugs is how to increase its therapeutic effect to tumor cells but simultaneously reduce its toxic and side-effect to normal tissues(Ai et al., 2005; Torchilin et al., 2007). Thus, targeted delivery of anti-cancer drugs is a highly desirable strategy to improve the therapeutic outcome, provided the combination of enhanced efficacy and reduced toxicity (Nasongkla et al.,2006; Shuai et al.,2004).

Some polymers such as liposomes, conjugates and polymeric micelles have been tested for anticancer drug delivery over the past decade (Shuai et al., 2004;Khemtong et al., 2009). Folate is a kind of small molecular vitamin and the binding efficacy between folate and folate receptors is very high, which makes folate an excellent candidate that can be used to direct the delivery carrier to tumor cells. In our previous studies, Folate-PEG-PCL micelles, encapsulated with doxorubicin (DOX) and superparamagnetic iron oxide (SPIO), showed better targeting tropism toward hepatic carcinoma cells (Hong et al., 2008; Cheng et al., 2011). On the other hand, except targeted delivery, drug release is very important to achieve ideal therapeutic effect (Hruby et al., 2005). In recent years, a lot of studies have revealed the potential of stimuli-seneitive polymer micelles as a programmable delivery system in which the release of drugs can be easily triggered by responding to specific environmental or physical stimulus such as PH, temperature and redox etc (Xu et al., 2009; Chen et al., 2010).

In this study, DOX-contained polymeric micelles was prepared based on PEG and PDLLA and folate was used as a targeting ligand to functionalize the micelles. A human hepatic carcinoma cell line over-expressing surface receptors for folate, Bel 7402 cells, were used to test the targeting efficacy and cytotoxicity. The purposes of this study were to evaluate the tumor targeting of the folate-PEG-PDLLA-DOX-Micelles and to observe its control release and cytotoxicity.

Materials and Methods

Materials

D,L-lactide was purchased from Shenzhen Brightchina Industry (China) and recrystallized three times from ethyl acetate before use. Monoallyl-PEG was synthesized by a reaction as described (Shuai et al., 2004;Yang et al., 2008; Liao et al., 2010). 2-Aminoethanethiol hydrochloride, folic acid, N-hydroxysuccinimide (NHS), naphthalene, potassium persulfate ($K_2S_2O_8$), dicyclohexycarbodiimide (DCC), azobisisobutyronitrile (AIBN) and Stannous (II) octoate ($Sn(Otc)_2$) were purchased from Sigma-Aldrich and used as received. All other organic solvents are of analytic grade. Phosphate-buffered solutions (PBS, pH 7.4 and 5) were prepared in our laboratory. Doxorubicin hydrochloride (DOX·HCl) was purchased from Zhejiang

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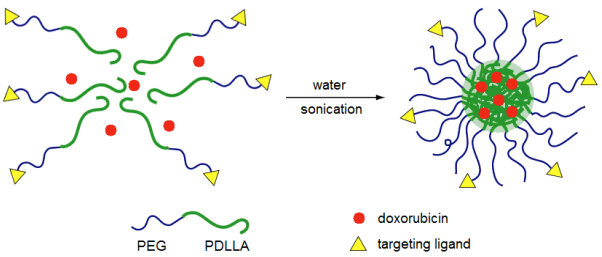

Figure 1. Schematic Formation of DOX-loaded Micelles by Adding THF Solution Containing DOX and Copolymer

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Synthesis of copolyers

The targeting and non-targeting copolymers, folate-PEG-PDLLA and allyl-PEG-PDLLA, were synthesized via multistep chemical reactions according to our previously reported methods (Nasongkla et al., 2004; Shuai et al., 2004; Yang et al., 2008). Briefly, allyl-terminated copolymer (allyl-PEG-PDLLA) was synthesized through a ring-opening polymerization of D,L-lactide with monoallyl-PEG as a macroinitiator. Subsequently, the allyl terminal groups of copolymers were converted into primary amino groups by a radical addition reaction, followed by conjugation of the carboxylic group of folic acid.

Preparation of DOX-loaded micelles and DOX-free micelles

The PEG-PDLLA micelles were formed self-assembly with DOX loading into the core (Hong et al., 2008; Yang et al., 2008). DOX-loaded micelles were prepared as follows: 10 mg of folate-PEG-PDLLA, 2 mg of doxorubicin hydrochloride and 1.3 mL of triethylamine were dissolved in a mixed solvent consisting of THF (1 mL) and DMSO (1 mL). Then, the solution was slowly added dropwise to 20 mL of pure water under sonication using a VCX 130 Sonic Dismembrator (Sonics & Materials Inc., USA) and then dialyzed against pure water for 2 days to allow the formation of DOX-loaded micelles and to remove organic solvents and unencapsulated DOX dissolved in the micelle solution (Figure 1). Afterwards, the micelle solution was filtered through a 0.22 μm membrane to eliminate the polymer and DOX aggregates. DOX-free copolymer micelles were prepared in the similar procedure without using doxorubicin hydrochloride.

Characterization of copolymers and micelles

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian 300-MHz NMR spectrometer in Deuterochloroform (CDCl3), or Dimethylsulfoxide deuterated (DMSO-d6) depending on sample solubility at room temperature. Gel permeation chromatography (GPC) was employed to determine molecular weight and molecular weight distribution. GPC analysis was carried out using a SHODEX 7.8 mm × 300 mm column with chloroform as an eluent (1 mL/min) and polystyrene standards for column calibration. Twenty microliter samples were injected with a microsyringe, and the eluent was analyzed with a differential refractive index (RI) detector Waters 2414 from Waters (USA). The micelles obtained were characterized with photon correlation spectroscopy, performed at 25 °C on a BI-200 SM dynamic laser scattering system from Brookhaven Instruments (Hong et al., 2008; Yang et al., 2008). Scattered light was detected at 90° angle and collected on an autocorrelator. Sizes and size distribution were obtained from the means of five measurements for each sample.

Determination of DOX-loading contents

The DOX-loading content (DLC), defined as the weight percentage of DOX in micelles, was quantified by UV–vis analysis using a Unico UV-2000 UV–vis spectrophotometer. First, DOX-loaded micelle solutions were lyophilized to yield the solid micelle samples. Then the dried micelle samples were weighed and dissolved in a mixture of chloroform and DMSO (1:1, v/v). The absorbance of DOX at 480 nm was measured to determine the drug content using a previously established calibration curve.

In vitro drug release

Freeze-dried micelle samples (10 mg each) were suspended in PBS (pH 7.4 or 5) and transferred into a dialysis bag (MW cut-off: 7000 Da). The bag was placed into 50 mL PBS (pH 7.4 or 5). The release study was performed at 37 °C in a Shanghai Zhicheng ZHWY-200B incubator shaker. At selected time intervals, solution outside the dialysis bag was removed for UV-Vis analysis and replaced with fresh buffer solution. DOX concentration was calculated based on the absorbance intensity of DOX at 480 nm. In the assessment of drug release behavior, the cumulative amount of the released drug was calculated, and the percentages of drug released from micelles were plotted against time.

Targeting study

Bel 7402 cells were seeded at 6-well plates and maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) in absence of folate. After incubation for 24 h at 37 °C in a humidified atmosphere with 5% CO2, a predetermined amount of targeted micelles (6k-2k) in PBS was added into each dish with a final DOX concentration of 5 μg/mL. The cells were incubated for 2 h and then washed twice with PBS. The DOX fluorescence was assessed on a Nikon TE2000-U inverted fluorescence microscope. In the control experiment, Bel 7402 cells were first incubated with free folate for 8 h and then co-incubated with folate-functionalized micelles (6k-2k) for 0.5 h. The free folate competed with the folate-functionalized micelles and decreased the micelles intake. Non-targeted micelle (6k-2k) with DOX (5 μg/ml) was used as controls. For the flow cytometry analysis, cells at 6-well plates were incubated with targeted micelles (6k-2k or 6k-4k) for 2h with a final DOX concentration of 5 μg/mL. Then, cells were washed with PBS, trypsinized, centrifuged, resuspended in 1 mL of PBS, and analyzed via flow cytometry. The completive group and non-targeted group (6k-2k or 6k-4k) were also tested.

In vitro cytotoxicity study

Bel 7402 cells were seeded at 96-well plates with a seeding density of 1,000 cells per well, and incubated in a humidified incubator (37 °C, 5% CO2) for 24 h. Cells were then incubated with targeted micelles (2k or 4k) containing DOX concentration: 1, 2, 5, 10 μg/mL for 24 h. The non-targeted micelles (2k or 4k) containing DOX concentration: 1, 2, 5, 10 μg/mL were used as negative controls. The PBS was used as the blank group. Free DOX (1, 2, 5, 10 μg/mL) and Free DOX (1, 2, 5, 10 μg/mL) mixed with non-drug micelles (2k or 4k) were also used as contrast in the other experiment. Afterwards, cells were washed twice with PBS, and incubated for 4 h in 200 μL RPMI-1640 medium containing 20 μL MTT (5 mg/mL in PBS). After the cells were washed by PBS, the precipitate was dissolved in 150 μL DMSO and analyzed on a Wellscan MK3 microplate reader. Then the cell inhibition ratio was obtained.

Statistical analysis

All data were expressed as mean values with standard deviations. Statistical analysis was performed using one-factor analysis of variance. Differences were considered statistically significant when P-values were less than 0.05. All statistical analyses were performed using the SPSS package,version 13.0 (SPSS, Chicago, Illinois,USA).

Results and Discussion

Synthesis of copolymers

Amphiphilic block copolymers, allyl-PEG-PDLLA and folate-PEG-PDLLA, were synthesized by multi-step chemical reactions as shown in Figure 2. The copolymer structures were confirmed by 1H NMR measurements in which the characteristic resonances of both allyl-terminated PEG and PDLLA were observed, indicating the coexistence of both blocks in the purified samples (Figure 3). By comparing the integrals of characteristic peaks of PEG blocks (e.g., the singlet of -OCH_2 at 3.6 ppm) and PDLLA blocks (e.g., the multiplet of -C(=O) $CH(CH_3)O$ at 5.1 ppm and -C(=O)$CH(CH_3)O$ at 1.5 ppm) with that of characteristic peaks of the terminal allyl groups (e.g., the multiplet of CH_2=CH at 4.9 ppm) in the 1H NMR spectrum, the length of PEG and PDLLA blocks was calculated and reported as molecular weights in Table 1. GPC measurements also demonstrated the successful synthesis of the diblock copolymer by revealing a unimodal molecular weight distribution in the GPC

Table 1. Characteristics of the Block Copolymers

Polymer	Mn[a]	Mn[b]	Mw[a]	Mw/Mn[a]
allyl-PEG6k-OH	6230	NA	6488	1.04
allyl-PEG6k-PDLLA2k	8154	8750	9266	1.14
NH2-PEG6k-PDLLA2k	8540	8930	9895	1.16
folate-PEG6k-PDLLA2k	8783	NA	10234	1.17
allyl-PEG6k-PDLLA4k	10439	11414	13342	1.28
NH2-PEG6k-PDLLA4k	10642	11726	12604	1.18
folate-PEG6k-PDLLA4k	10736	NA	12873	1.20

NA, data not available; [a]Determined by GPC with RI detector; bCalculated based on 1H NMR spectra; GPC-determined molecular weight of PEG block, 6230, was used for calculation

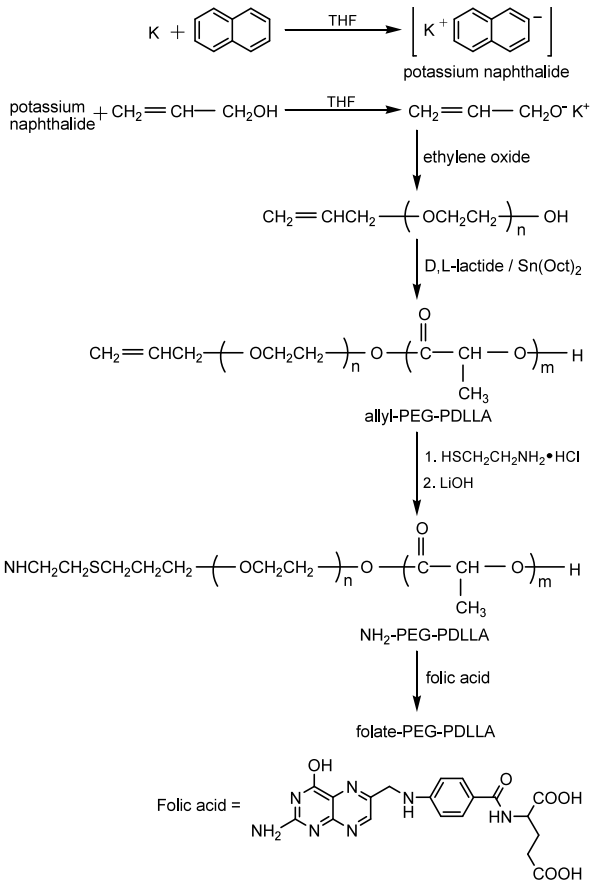

Figure 2. Schema for the Synthetic Approach for Folate-PEG-PDLLA

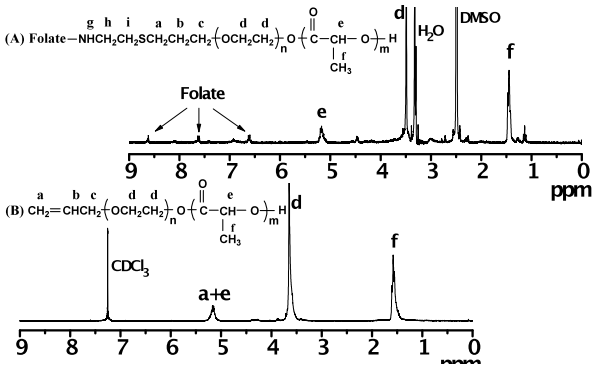

Figure 3. 1H NMR Spectra of Folate-PEG6k-PDLLA2k (A) in DMSO-d6 and allyl-PEG6k-PDLLA2k (B) in $CDCl_3$

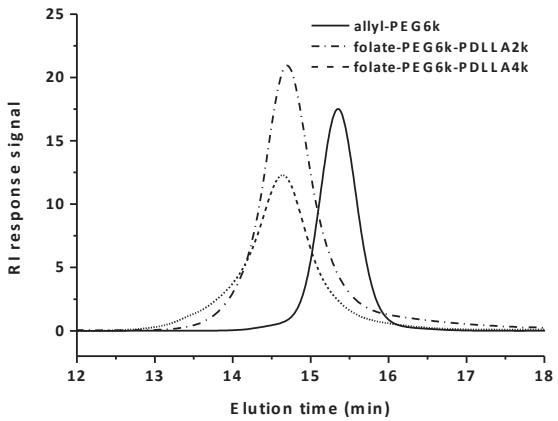

Figure 4. GPC Curves of Folate-PEG6k-PDLLA2k, Allyl-PEG6k-PDLLA2k and Allyl-PEG6k in THF

Table 2. Influence of Copolymer Composition on Micellar Properties

Copolymers	Micelles size (nm)		Drug content (%)
	DOX unloaded	loaded	
allyl-PEG6k-PDLLA2k	50.8±1.5	52.4±0.5	5.0±0.1
folate-PEG6k-PDLLA2k	63.7±1.0	60.6±1.8	4.8±0.2
allyl-PEG6k-PDLLA4k	76.9±2.3	78.6±1.9	6.5±0.2
folate-PEG6k-PDLLA4k	85.2±1.7	83.0±2.5	6.6±0.4

chromatograms (Figure 4 and Table 1).

Characterization of micelles

As shown in Table 2 and Figure 5, micelles predominantly less than 100 nm in diameter were successfully prepared, and the micelle size appeared dependent on copolymer composition. Folate functionalization of copolymers led to a subtle change in micelle size. The loading content of DOX (DLC) in

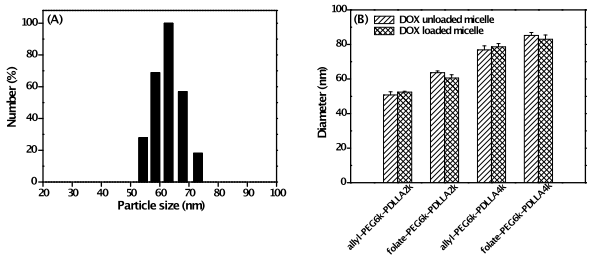

Figure 5. Dynamic Light Scattering Histogram of DOX-loaded and DOX-free M icelles. A for size distribution of DOX-loaded micelles based on folate-PEG6k-PDLLA2k, and B for four kinds of DOX-loaded and DOX-free micelles

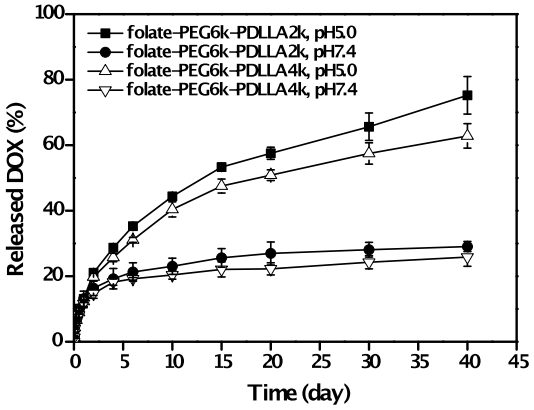

Figure 6. In vitro Dox-release Profiles for Two Micelle Formulations, Folate-PEG6k-PDLLA2k and Folate-PEG6k-PDLLA4k, under Neutral (pH 7.4) and Acidic (pH 5.0) Conditions at 37 °C. Data are presented as mean ±SD (n=3)

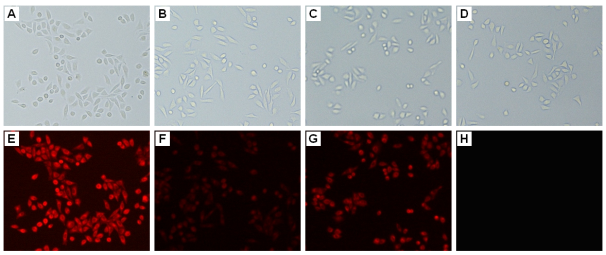

Figure 7. Fluorescence microscopy images of Bel 7402 cells incubated with DOX-loaded micelles. A, E for targeted group; B, F for non-targeted group; C, G for competitive group; D, H for blank group

folate-PEG-PDLLA micelles increased from 4.8% to 6.6% with an increase of PDLLA molecular weight from 2 to 4 kDa, likely due to the fact that longer PDLLA brings more micellar hydrophobic space for the drug to embed in, at the same time, causes stronger hydrophobic interaction between copolymer and DOX. Dynamic light scattering (DLS) measurements showed that the mean diameters were 63.7±1.5 nm and 85.2±1.7 nm for blank micelles (i.e. DOX-free micelles), and 60.6±1.8 nm and 83.0±2.5 nm for DOX-loaded micelles.

I*n vitro drug release*

As shown in Figure 6, Release of DOX from micelles was pH dependent. DOX release in the two media revealed a biphasic release pattern consisting of an initial burst release followed by a sustained and slow release over a prolong time up to several weeks. At pH 7.4, DOX release was less than 20 wt.% of DOX released in 5 days, and less than 30 wt.% of DOX released after 40 days. DOX release at pH 5.0 was much faster than that at pH 7.4 from both micelles ($p<0.05$). More than 30 wt.% and 60 wt.% of DOX was released in 5 and 40 days, respectively. It is likely due to the re-protonation of the amino group of DOX and faster degradation of micelle core at lower pH. This observed pH-dependent DOX release behavior is hypothesized to potentiate drug release from micelles once the micelles enter the tumor cells via endocytosis and are trapped within acidic endosomal compartments.

Study on cell uptake and cytotoxicity

Folate receptor-mediated cell uptake of DOX-loaded micelles was evaluated using fluorescence and flow cytometry analysis. In Figure 7, the most significant red DOX fluorescence can be seen in the targeted micelle group. This reason also resulted in a narrow fluorescence in the non-targeted group and competitive inhibition group. Although the free folate can compete against targted micelles, the cells can uptake few micelles. The fluorescence was stronger in completive group than in non-targeted group.

As is shown in Figure 8, the values of extinction of the folate-targeted group were higher than the non-targeted group and competitive inhibition groups. The blank contrast group was the lowest. These findings confirmed the folate-targeted functioning. In Figure 9, the cell inhibition ratios of targeted groups were all higher than the non-targeted groups. These results showed the function between folate and folate receptor again. When the concentration of DOX increased, the cell inhibition ratio increased due to the increased toxicity.

There was no statistically significant difference in the cell inhibition ratio between free DOX groups and free DOX mixed with non-drug micelle (6k-2k or 6k-4k), indicating that the non-drug micelles had no toxicity for cells. The DOX encapsulated in micelles resulted in the decrease of cell viability.

In this study, we have synthesized folate-functionalized nano-micelles, Folate-PEG-PDLLA-DOX-micelles. The synthesized nano-micelles had a uniform shape and size and high drug loading. Folate-PEG-PDLLA-DOX-micelles have better targeting tropism to the

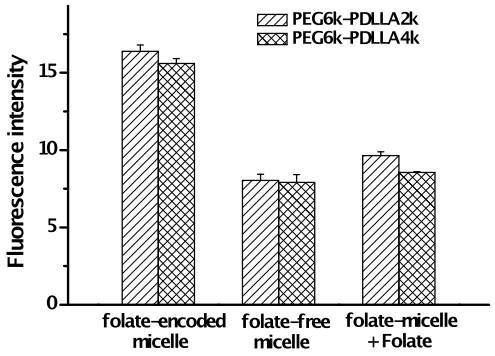

Figure 8. Fluorescence Intensities of Bel 7402 cells Incubated with DOX-loaded Micelles for 48 h (n = 3)

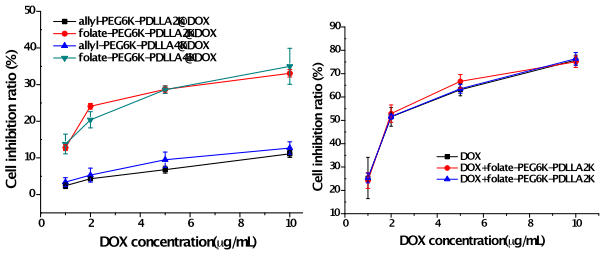

Figure 9. Cytotoxicity of DOX-loaded Targeted or Non-targeted Micelles to Bel 7402 cells after a 48 h Incubation (n = 3)

hepatic carcinoma cells in vitro than their non-targeting counterparts. The DOX behavior of controlled release in folate receptor-mediated Folate-PEG-PDLLA-DOX-micelles is obvious and has the pH dependent. Meanwhile, our experiment results showed that Folate-PEG-PDLLA-DOX-micelles have low cytotoxicity. The folate-functionalized PEG-PDLLA-DOX-micelles were demonstrated their potential as a powerful multifunctional platform for targeted delivery of anti-cancer drug in order to improve the therapeutic outcome.

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