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A Polyrotaxane-based pH-labile Drug Delivery System

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RESEARCH ARTICLE

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Abstract

A novel polyrotaxane (PR)-based triblock copolymer was exploited as polymeric carrier for doxorubicin (DOX). A sample holding a feed molar ratio of Pluronic F127 to β-CD to poly(ethylene glycol) methyl ether methacrylate (PEGMA) equal to 1:30:20 was synthesized via the in situ atom transfer radical polymerization (ATRP) and then conjugated with DOX using pH sensitive hydrazone linker. The resulting PR-DOX conjugates enabled to self-assemble into nano-particles of about 70 nm in diameter in aqueous solution as evidenced by TEM. The release of DOX was varied from 10 % to 37 % over 72 h at physiological and acidic pH, respectively. The PR-based triblock copolymer without DOX conjugation showed almost non toxicity, while these nano-particles with DOX conjugation presented increased toxicity.

Keywords

polyrotaxane · self-assembly · DOX · pH sensitive · nanoparticle

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1 Introduction

During the past decade there has been a growing interest in the investigation of polymeric conjugates or polymer-drug conjugates as potential drug delivery systems [1]. When poorly water- soluble drugs are covalently linked to water-soluble polymers or amphiphlic block copolymers to give rise to the polymeric conjugates, they generally enable to self-assemble into nano-sized polymeric micelles with a size of about 50-150 nm in diameter in aqueous solution. These micelles usually show a unique core-shell structure, a hydrophobic core surrounded by a hydrophilic outer shell [2], which can easily escape from the blood vessel and accumulate in tumor tissues–a phenomenon known as "enhanced permeability and retention (EPR) effect" [3,4]. Consequently, these micelles can be passively targeted to tumor site with the modified pharmacokinetics at the body and cellular level.

An ideal drug-loaded nano-particle should be stable in both blood circulation and healthy tissues, but release free drug once it is internalized by the cancer cells. For a drug to be delivered at the target site in a controlled manner, the development of pH-sensitive polymeric conjugates for cancer chemotherapy has recently attracted tremendous attention due to the mildly acidic pH intrinsically encountered in tumor (pH=5) other than in the healthy tissues (pH=7.4) [5]. To this end, such acid-labile moieties as cis-aconitic acid [6] and hydrazine [7] are extensively exploited in polymeric conjugate chemistry.

Cyclodextrins (CDs) constitute a series of cyclic oligosaccharides composed of 6, 7 and 8 D-glucose units linked by α-1,4 linkage and are named as α-, β- and γ-CD, respectively. In addition to small molecules, they can include polymers to give access to polypseudoro-taxanes (PPRs) and polyrotaxanes (PRs) after end-capping with polymers or oligomers via the atom transfer free radical polymerization (ATRP) [8]. Since CDs are nontoxic and biodegradable, the CD-based PRs have been explored not only for fundamental supramolecular polymer research [9], but also for polymer therapeutics [10]. On one hand, this is because there are a large number of hydroxyls on the CD rings which could be easily modified by chemical

reactions, thus allowing the conjugation of bioactive agent, for example, DOX as an anti-tumor drug to synthesize polymeric conjugates. On the other hand, as threaded CDs can freely slide and rotate along the included polymer chain, this configurational flexibility is beneficial to the formation of polymeric micelles *via* the self-aggregation of polymeric conjugates because the grafted bioactive agent on the CD rings is decoupled with the main chain restriction. Several investigations on CD-based PRs as drug nano-carriers were reported in the literature [11]. However, their releasing strategies were mostly focused on the dissociation of supramolecular structure via hydrolysis of the terminal bulky groups, which would lead to the release of the drug along with the CD slipping off the polymer main chain, other than the cleavage of the grafted drugs off the CD rings. The research on stimuli-triggered release of drug from the CD rings is still an unfinished task.

In this report, we prepared a pH-triggered target delivery system of DOX conjugated to β-CDs entrapped on the Pluronic F127 block of a PR-based triblock copolymer *via* acid-labile hydrazone linkage. The obtained PR-DOX conjugates were self-assembled into around 70 nm-diameter nano-particles in aqueous solution. The release behavior of DOX was evaluated at pH=7.4 and 5, respectively. The *in vitro* experiments toward Hela cells showed these nano-particles having lower cytotoxicity than the free drug and enabling to efficiently transfer and release the drug into the cells.

2 Experimental part

2.1 Materials

N,N′-Carbonyldiimidazole (CDI) was purchased from Alfa Aesar, USA. Doxorubicin·HCl (DOX·HCl) was available from HuaFeng United Technology Co., Ltd, Beijing, China. Pluronic F127 comprising a central block of 65 PPO units and two PEO blocks of 100 units (M_n=12600 Da), poly(ethylene glycol) methyl ether methacrylate (PEGMA) (M_n=1100 Da) and *N,N,N',N",N"*-pentamethyldiethylenetriamine (PMDETA) were purchased from Sigma, USA. PEGMA was passed over a short basic alumina column to remove the inhibitor before polymerization. Copper(I) chloride (Cu(I)Cl) was prepared from $CuCl_2$, purified by stirring in acetic acid, washed with methanol and finally dried under vacuum prior to use. All other solvents and reagents were of analytical grade.

2.2 Synthesis of PR-based block copolymer

As previously reported [12], selected PR-based triblock copolymer was synthesized via the in situ ATRP of poly(ethylene glycol) methyl ether methacrylate (PEGMA) initiated with a PPR comprising a distal 2-bromopropionyl end-capped Pluronic F127 and β-CDs. The PR-based triblock copolymer was designated as F-30-20, where F means Pluronic F127, and 30 and 20 are the feed molar ratios of β-CD and PEGMA to Pluronic F127, respectively. The found molar ratio of Pluronic F127 to β-CD to PEGMA was 1:21.4:93.1 as measured by ^{1}H NMR. The number-average molecular weight was 4.47×10^5 Da and the polydispersity index was 1.07 as determined by GPC analysis.

2.3 Synthesis of polyrotaxane having amino pendant group (PR-HYZ)

As a typical sample, F-30-20 (200 mg, 1.44 μmol) was dissolved in 4 mL DMF and then mixed with CDI (74 mg, 0.462 mmol) dissolved in 2 mL DMF. After stirring for 2 h at 40 °C, the mixture was dropped into 80 % hydrazine hydrate (184 mg, 3.7 mmol) and continuously stirred at room temperature for 2 h. The mixture was dialyzed using a dialysis bag (MWCO 3500) for 24 h with water changing every 8 hours, and finally freeze-dried to give rise to the hydrazine containing polyrotaxane, yield 84.5 %.

2.4 Synthesis of PR-DOX

To conjugate DOX to β-CDs entrapped on the Pluronic F127 backbone of the PR-based triblock copolymer, PR-HYZ (100 mg, 0.724 μmol) was dissolved in 7 mL of DMSO, and the certain amount of DOX•HCl was added. The mixture was stirred at room temperature for 72 h under protection from light. The crude product was dialyzed using a dialysis bag (MWCO 3500) for 24 h with water changing every 8 hours, followed by gel purification by using Sephadex LH-20 to completely remove unbound DOX using DMF as elution solvent to yield the PR-DOX conjugate (PR-DOX), yield 82.7 %.

2.5 Preparation of PR-DOX micelles

The PR-DOX micelles were prepared by directly dissolving the PR-DOX conjugate in water and allowing it to stand overnight. The resulting solution was then diluted to the desired concentration with the proper amount of distilled water.

2.6 In vitro release of DOX from polymeric micelles

A release study was performed at 37 °C in a Guo Hua THZ82 incubator shaker (Jiangsu, China). A dialysis bag (MWCO 3500) containing 3 mL PR-DOX micelle solution was placed into a flask containing 60 mL 0.1 M citrate/phosphate buffers at pH=5 and 7.4, respectively. The flask was shaken at 100 rpm at 37±0.5 °C. At selected time intervals, 3 mL solution outside the dialysis bag was removed for UV-Vis analysis and replaced with the same amount of fresh buffered solution. The DOX concentration was determined based on the absorbance intensity at 480 nm according to the standard line obtained from a series of solutions with different DOX concentrations. The release experiments were conducted in triplicate and take the arithmetic mean as the results.

2.7 Evaluation of in vitro cytotoxicity of PR-DOX

The cytotoxicity of the PR-DOX nano-particles against Hela cells (ATCC, USA) was assessed by using the

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Hela cells were seeded in 96-well plates and incubated for 24 h. The media were replaced with fresh medium containing free DOX and PR-DOX micelles with certain DOX concentrations, and incubated in a humidified atmosphere containing 5 % CO2 at 37 ºC for 48 h. Thereafter, MTT solution was added. The plates were incubated at 37 °C for another 4 h. The intracellular metabolized product formazan of MTT was retrieved by addition of DMSO. The plates were read at 550 nm (Biotek synergy2, USA), and the cell viability was calculated.

2.8 Characterization of the drug-conjugated polyrotaxanes

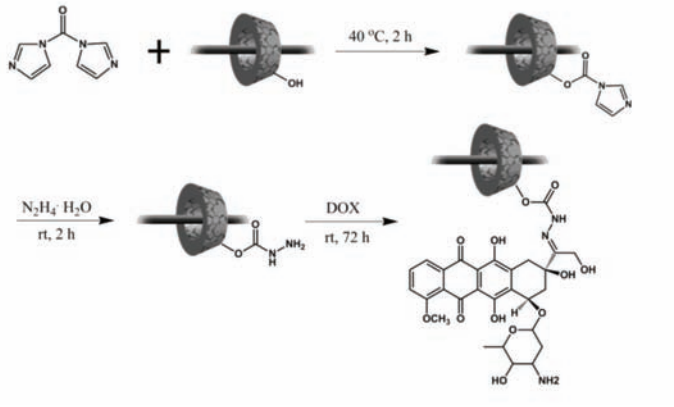
^{1}H NMR (400 MHz) spectra were recorded on a Bruker ARX-400 spectrometer at room temperature using DMSO-d_6 as solvent and tetramethylsilane (TMS) as internal standard. UV-vis spectra were performed on a Hitachi U-2800 spectrophotometer. Samples for trans-mission electron microscopy (TEM, Tecnai G220 S-TWIN, FEI, Netherlands) observation were prepared by dropping a few microliters of the aqueous solution (0.5 mg/mL) of the sample on a copper grid coated with amorphous carbon. FTIR spectra were measured using Shimadzu IR Prestige-21 FTIR spectrometer at room temperature in the range between 4000 and 500 cm^{-1}, with a resolution of 2 cm^{-1} and 20 scans. Samples were prepared by dropping their corresponding chloroform solutions on KBr plate and drying at 50 °C. Gel Permeation Chromatographic (GPC) measurements were carried out at 50 ºC on a Waters 2410 instrument using DMF and LiBr (0.05 M) as a mixing eluent at a flow rate of 1.0 mL/min. All the GPC data were calibrated by using polystyrene (PS) standards.

3 Results and discussion

3.1 Synthesis and characterization of PR-DOX

According to our previous report [12 a], a PR-based triblock copolymer sample F-30-20 was synthesized via the *in situ* ATRP of PEGMA initiated with a PPR made from a distal 2-bromopropionyl end-capped Pluronic F127 with β-CDs at 25 °C. Its number-average molecular weight was 4.47×10^5 Da and polydispersity index was 1.07 as determined by GPC. The found molar ratio of Pluronic F127 to β-CD to PEGMA was 1:21.4:93.1 as measured by 1H NMR. About two thirds of β-CDs were found to be still entrapped on the Pluronic F127 main chain. However, a substantially higher number of PEGMA was incorporated to attach to two ends of the PPR most likely due to the fact that the resulting PPR is insoluble in aqueous solution and the end-capping ATRP was carried out heterogeneously.

F-30-20 was further conjugated with a varying amount of DOX. The preparation pathway of the PR-DOX conjugates is illustrated in Scheme 1. At first the hydroxyl groups of β-CDs entrapped on the Pluronic F127 backbone were activated by



Sch. 1. Preparation pathway of the PR-DOX conjugates.

Tab. 1. Composition and drug loading content of PR-DOX conjugates

Entry	Sample Name	Molar composition (DOX:β-CD)		DOX (wt. %)
		Feed ratio	Found ratio[a]	
1	PR-DOX-2	2:1	1.1:1	4.8
2	PR-DOX-4	4:1	1.5:1	8.3
3	PR-DOX-7	7:1	1.7:1	10.5

a. Determined by UV measurement

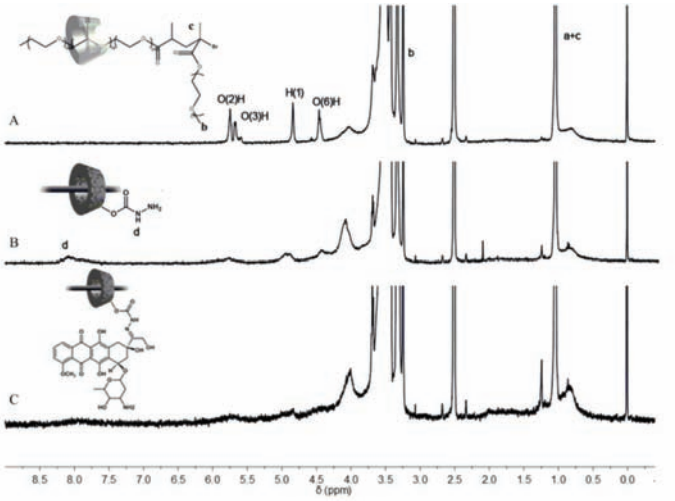


Fig. 1. ^{1}H NMR spectra of (A) unmodified F-30-20, (B) PR-HYZ and (C) PR-DOX-7.

CDI, followed by reacting with hydrazine hydrate. The products were characterized by 1H NMR analysis. As compared with the 1H NMR spectrum of F-30-20 (Figure 1 (A)), the signals of both β-CD and hydrazine moiety were observable in the spectrum of PR-HYZ (Figure 1 (B)), and the proton resonance peaks of β-CD were heavily broadened. However, the characteristic peaks of DOX were not seen in the spectrum of PR-DOX-7 (Figure 1 (C)) due to the high molecular weight of the PR-based block copolymer and the trace amount of the DOX in this study. In fact, the vibration signals of DOX conjugated onto the

resulting PR sample were observed in the FTIR spectrum. As compared to the FTIR spectrum of F-30-20 (Figure 2 (C)), the peaks at 535, 567 and 790 cm^{-1} in the spectrum of PR-DOX-7 (Figure2 (B)) are attributed to the bending vibrations of C-H bondings on the aromatic rings of DOX, and the peaks at 1029 and 1150 cm^{-1} are assigned to the characteristic peaks of β-CD. It clearly indicated that the DOX was successful conjugated to β-CDs entrapped on the Pluronic F127 of the PR-based triblock copolymer. Furthermore, the UV-Vis spectroscopy is an appropriate analytical method to quantitatively determine the DOX content. As shown in Table1, the drug loading content range is 4.8-10.5 %, about each β-CD grafted with 1.1-1.7 DOX. The PR-DOX-7 sample was used for the following testings.

3.2 Micelle properties of PR-DOX in aqueous solution

As can be seen in Figure 3, the PR-DOX-7 conjugates were indeed self-assembled into well-defined nano-particles with regard to the micelles of its precursor F-30-20 in aqueous solution. The diameter of the particles observed is about 300 nm for F-30-20 and around 70 nm for the PR-DOX-7 conjugates. The diameter shrinkage may be caused by the strong π-π stacking between the grafted DOX molecules as well as the flexibility of the β-CDs moving along the Pluronic F127 chain, enhancing the hydrophobicity of the core in the self-assembly process and leading to the nano-particles more compact and smaller.

3.3 Effect of pH on in vitro DOX release rate

The pH sensitivity is crucial for a delivery system to release drug at tumor cells (acidic environment) in a controlled manner. Figure 4 depicts the release behavior of DOX from the PR-DOX-7 conjugates assessed by UV-Vis spectroscopy in two buffered solutions at pH 5.0 and 7.4, respectively. The amount of released DOX from the nano-particles incubated at pH 5.0 and pH 7.4 for the first 12 h was 10 % and 7 % of the total drug loaded. After 72 h, however, the release of DOX at pH 5.0 reached to 37 %, while the release at pH 7.4 was only about 10 %. It indicated that the nano-particles self-assembled from the PR-DOX conjugates exhibit our desired pH sensitive character. Most of the conjugated-DOX can remain in the micelle cores for a considerable period of time when the micelles stay in the plasma at normal physiological conditions (pH 7.4), while a faster release occurs once the micelles are taken by the tumor cells because the pH values of the endocytic compartments generally ranges from 4.5 to 6.5, much lower than the pH value of the normal physiological conditions.

3.4 Cytotoxicity study

Figure 5 (A) illustrates the viability (%) of Hela cells treated with F-30-20 and PR-HYZ, respectively. The cells incubated with both F-30-20 and PR-HYZ exhibited the viabilities above 50 % in the concentration range of 0.9-2000 μg/mL, meaning

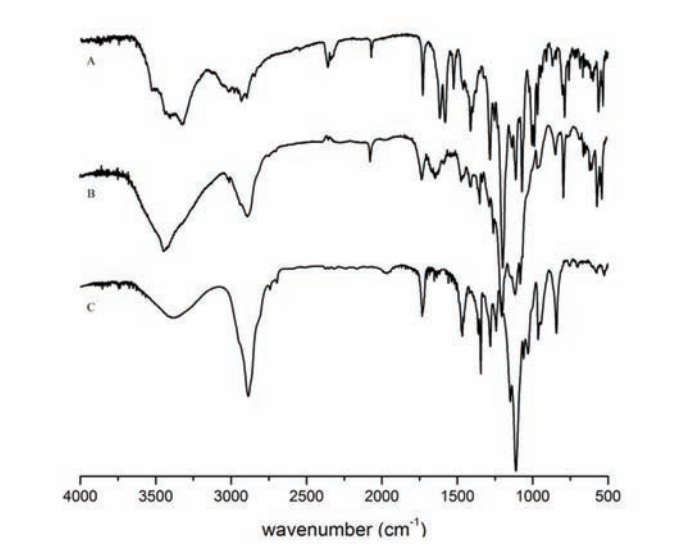


Fig. 2. The FTIR spectra of DOX (A), PR-DOX-7 (B) and F-30-20 (C).

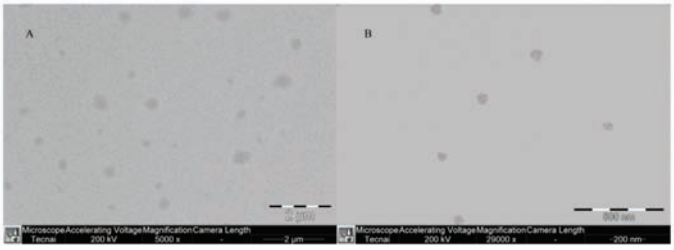


Fig. 3. TEM micrographs of the F-30-20 (A) and PR-DOX-7 (B).

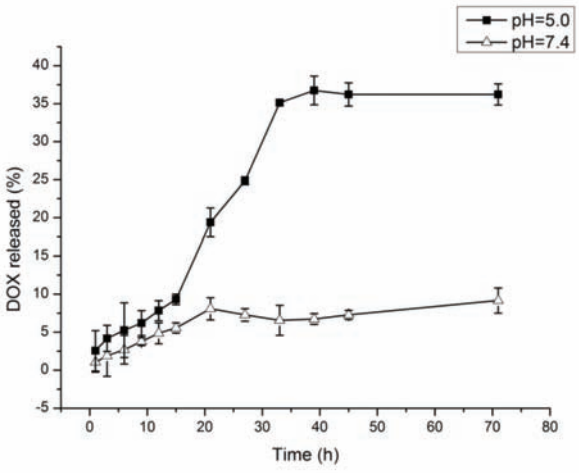


Fig. 4. Release profiles of DOX from the PR-DOX-7 nano-particles at different pH values.

the PR-based triblock copolymer and its HYZ derivative have nearly no significant toxicity to Hela cells even in a very high concentration of 2000 μg/mL. Figure 5 (B) shows the cytotoxic effects of free DOX and PR-DOX-7 nano-particles with different DOX concentrations incubated with Hela cells for 72 h. The concentration of free DOX required to inhibit cell viability by 50 % (IC50) was 1.23 μg/mL. The IC50 of PR-DOX-7 was

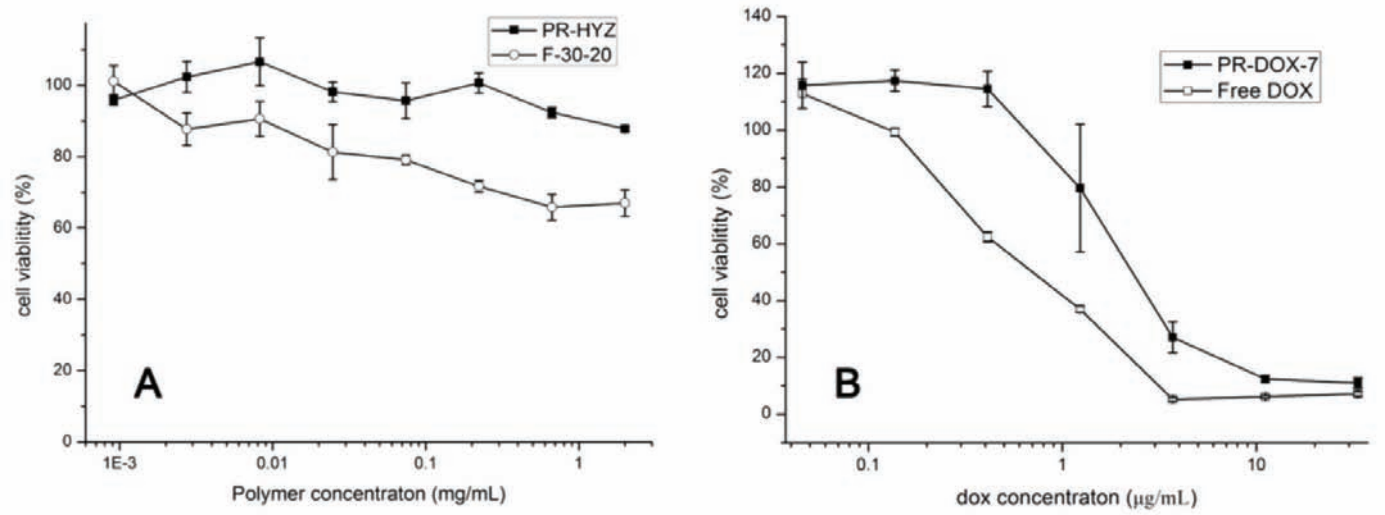


Fig. 5. Cytotoxicity of (A) F-30-20 and PR-HYZ with different polymer concentration, and (B) free DOX and PR-DOX-7 nano-particles with different DOX concentrations against Hela cells (incubation time 72 h).

3.7 μg/mL, three-fold more than that of free DOX. The relatively low cytotoxicity of the PR-DOX correlates well with its drug delivery process as it is common for the reducing of in vitro toxicity of polymer-anticancer drug conjugates as compared to free drug due to the long-term drug release process from the nano-particles.

4 Conclusions

In this study, we prepared a PR-based triblock copolymer composed of β-CDs being threaded onto Pluronic F127 end-capped with polymers formed by the in situ ATRP of PEGMA. The anticancer drug DOX was conjugated onto the entrapped β-CDs via acid-sensitive hydrazone linkage. The resulting PR-DOX conjugates enabled to self-assemble into well-defined nano-particles with size of around 70 nm in aqueous solution. There was only little DOX release at pH=7.4 in the initial period of time and three-times more DOX released at pH=5.0 over a period of longer than 3 days, indicating that the conjugates would be stable during blood circulation and in the healthy tissues, but release the loaded DOX in the acid endocytic compartments. The assay of anticancer activity in vitro revealed that the PR-DOX was effective for inhibiting the growth of the Hela cells, while the intact PR-based triblock copolymers possessed no significant toxicity. This conjugate offers a great potential for intracellular drug delivery into tumor cells in achieving highly effective and safe drug therapy.

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