

Note

NOVEL pH-SENSITIVE DRUG DELIVERY SYSTEM BASED ON NATURAL POLYSACCHARIDE FOR DOXORUBICIN RELEASE*

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Abstract A novel pH-sensitive nanoparticle drug delivery system (DDS) derived from natural polysaccharide pullulan for doxorubicin (DOX) release was prepared. Pullulan was functionalized by successive carboxymethylation and amidation to introduce hydrazide groups. DOX was then grafted onto pullulan backbone through the pH-sensitive hydrazone bond to form a pullulan/DOX conjugate. This conjugate self-assembled to form nano-sized particles in aqueous solution as a result of the hydrophobic interaction of the DOX. Transmission electron microscope (TEM) and dynamic light scattering (DLS) characterization showed that the nanoparticles were spherical and their size was less than 100 nm. The DOX released from the nanoparticles in a pH-sensitive manner. *In vitro* cytotoxicity assay indicated the pullulan/DOX nanoparticles showed comparable cytotoxicity effect with free DOX on the 4T1 mouse breast cancer cells.

Keywords: Natural polysaccharide; pH-sensitive; Nanoparticle drug delivery system; Doxorubicin.

INTRODUCTION

An increasing research interest has been paid to nanoparticle DDS for their prominent advantages^[1]. Nanoparticle DDS could be tailor-made to keep highly stability in aqueous environment. Drugs encapsulated in nanoparticles could be released in controlled manner. For the limited uptake by the reticuloendothelial system (RES), the plasma half-time of nanoparticle DDS with hydrophilic surface become relatively longer. Furthermore, due to the enhanced permeability and retention (EPR) effect of tumor vasculature, nanoparticle DDS could accumulate passively in solid tumors thus led to high concentration to achieve site-specific drug localization. Some nanoparticle DDS could avoid induced multi-drug resistance of cancer cells and some could be injected locally in the peri-tumoral region^[2–4].

Doxorubicin (DOX) is one of the most effective anti-tumor drugs. However, the limitation of its applications is its systemic toxicities, primarily cardiotoxicity and immunosuppression. In recent years, many efforts have been afforded to reduce these side effects by using nanoparticle DDS. The targets for DOX delivery include tumor vasculature, extracellular space and tumor cells. As nanoparticles could be internalized through the endocytosis pathway, pH-sensitive nanoparticle DDS^[5] could be prepared to deliver DOX to tumor cells. In endocytic compartment, the DOX could be released from the nanoparticles by pH-controlled hydrolysis or by very specific enzymolysis (pH drop from physiological 7.4 to 5–6 in endosomes or 4–5 in lysosomes). Many approaches have been reported to prepared pH-sensitive nanoparticle DDS for DOX. The disadvantages of these methods are either of complicated and expensive synthetic route or of non-degradable of some synthetic materials^[6, 7].

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Pullulan is a natural polysaccharide with biocompatibility, non-toxicity and biodegradability. It can form nanoparticles after partly modified by hydrophobic molecules^[8–10]. In the present research, we reported a novel DDS based on pullulan for pH-sensitive release of DOX. Using a simple and easy synthetic route, the pullulan/DOX conjugate, which can self-assemble to form nanoparticles in aqueous solution, was synthesized. The morphology and size of the pullulan/DOX nanoparticles were characterized by transmission electron microscope (TEM) and dynamic light scattering (DLS). The *in vitro* DOX release behavior and *in vitro* cytotoxicity assay of the pullulan/DOX nanoparticles were also investigated.

EXPERIMENTAL

Materials and Methods

Pullulan (M_w 200000) was obtained from Hayashibara Biochemical Laboratories (Okayama, Japan). Doxorubicin hydrochloride (DOX·HCl) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd. China. Sodium chloroacetate, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and hydrazine hydrate were obtained from Asta Tech Pharmaceutical Co. Ltd. (Chengdu, China). All other chemicals were commercially available analytical grade reagents. Organic solvents are purified and dried through common standard methods. Dialysis tubing Spectra/Por (molecular weight cutoff 12000–14000) was purchased from Sigma.

Synthesis of Pullulan/DOX Conjugate and Fabrication of Nanoparticles

The pullulan/DOX conjugate was obtained by a three-step synthetic strategy, as shown in Scheme 1. Initially, pullulan was reacted with sodium hydroxide and sodium chloroacetate in mixture of water and isopropylalcohol^[11]. The synthesized carboxymethylpullulan (CMP) was freeze-dried and identified using FT-IR (PE Spectrometer, KBr plate) and ¹H-NMR (INOVA-400, solvent D₂O). The degree of substitution (DS) was determined using acid-base titration method. Then, the amidation between hydrazine hydrate and carboxyl group was carried out using EDC as catalyst. The amidated pullulan (AMP) was freeze-dried and identified by FT-IR and ¹H-NMR. The grafting efficiency of hydrazide was determined by oxidation reduction titration. Finally, DOX was grafted onto AMP through the formation of hydrazone bond. The pullulan/DOX conjugate was identified by FT-IR and ¹H-NMR. The DOX content was measured by a UV-spectrophotometer in acid DMSO^[12]. Nanoparticles formed spontaneously when the pullulan/DOX conjugate was dialyzed against PBS buffer. The diameter and size distribution of pullulan/DOX nanoparticles were evaluated by DLS (Malvern Nano-ZS) and TEM (H-600, Hitachi, Japan).

In vitro Release of DOX

Freeze-dried pullulan/DOX powder was re-suspended in PBS buffer and transported into dialysis tubing. The samples were placed in medium of pH 5.0 and pH 7.4 and stirred at 100 r/min, 37°C. The DOX release was evaluated from the decrease in the DOX content in the nanoparticles fraction using UV-spectrophotometer at 490 nm.

In vitro Cytotoxicity Assay

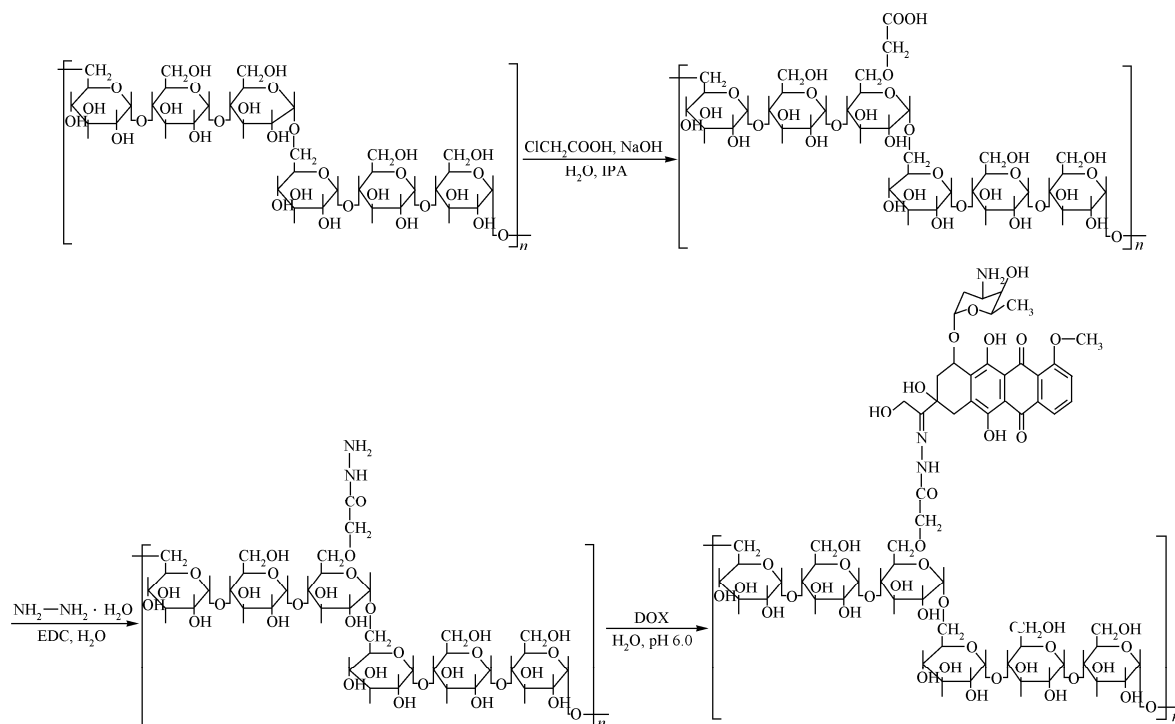
The *in vitro* cytotoxicity effect of free DOX, pullulan/DOX nanoparticles and blank AMP carriers on cultured 4T1 mouse breast cancer cells were evaluated by MTT assay method.

RESULTS AND DISCUSSION

The synthetic route was showed in Scheme 1. Two reactive groups in DOX structure can be employed to connect it with the carriers: the amino group that can form amide bond with carboxylic acid in the carrier molecules; and the carbonyl group in C13 that can form hydrazone bond with hydrazide group. The latter can be cleaved by weak acidic condition.

To graft DOX onto pullulan through the hydrazone bond, hydrazide must be introduced into pullulan backbone. Thus, pullulan was at first carboxymethylized in water/isopropylalcohol mixture and then condensed

with hydrazine hydrate using EDC as coupling agent. DOX was attached to pullulan through the pH-sensitive hydrazone bond formed between the hydrazide and carbonyl group of DOX.



Scheme 1 The route to prepare pullulan/DOX conjugate

The structures of pullulan after carboxymethylation, hydrazine hydrate condensation, as well DOX grafting were characterized using IR and $^1\text{H-NMR}$. In the IR spectra, characteristic peak at 1735 cm^{-1} for C=O group of carboxymethyl, 1636 cm^{-1} for amide C=O , and 1607 cm^{-1} in pullulan/DOX conjugate were observed, respectively. In the NMR spectra, specific signals corresponding to carboxymethyl at $\delta = 4.1\text{--}4.6$, and signals at $\delta = 7.2\text{--}7.4$, 0.9 , $1.2\text{--}1.4$ for DOX were observed. These results indicated the successful synthesis of carboxymethylized pullulan, pullulan hydrazine condensation, and the pullulan/DOX conjugate.

The DS was calculated by titration method because the application of NMR in the quantitative characterization of polysaccharides structure is limited^[13]. The proper DS of carboxymethyl group was found to be around 25%–30%. Higher DS results in lower water solubility of modified pullulan. Carboxymethylized pullulan was reacted with hydrazine hydrate to form hydrazide. After the amidation, the final grafting efficiency of hydrazide was around 10%. Different amount of DOX was reacted with AMP and the optimized DOX content in pullulan/DOX conjugate was 3.18 wt%.

The pullulan/DOX conjugate can self-assemble to form nanoparticles after dialysis against PBS buffer. The representative result of DLS was showed in Fig. 1, which indicated the size of the nanoparticles was mostly less than 100 nm in diameter with narrow size distribution. The pullulan/DOX nanoparticle displayed a negative surface zeta potential (-24.6 ± 1.1) mV).

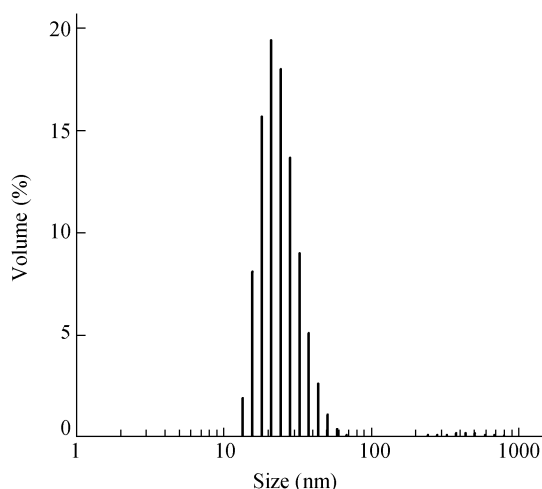


Fig. 1 The DLS result of pullulan/DOX nanoparticles

The pullulan/DOX nanoparticles observed by TEM showed spherical in shape with a diameter less than 100 nm (Fig. 2). The nanoparticles were well dispersed without obvious aggregation. Since no nanoparticles formation was found from empty AMP carrier before DOX was attached, it suggested the pullulan/DOX nanoparticles obtained by hydrophobic interactions of DOX. It has been reported that solid tumors show hypervascular permeability and impaired lymphatic drainage. As a result, nanoparticles (< 200 nm) could significantly accumulate in tumor by “filtration” mechanism^[14].

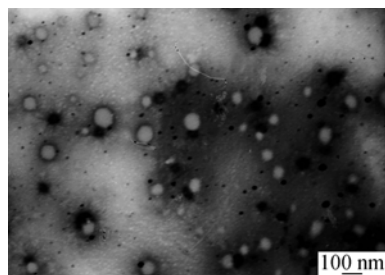


Fig. 2 The TEM result of pullulan/DOX nanoparticles

The *in vitro* release profiles of DOX from the conjugate nanoparticles were showed in Fig. 3. The release behavior was similar with the reported results of Hruby and co-workers^[15]. It was found that about 36% of DOX was released at pH 7.4 after 21 h incubation. When the pH decreased to 5.0, DOX release became much faster and 67% of DOX was released. One reason for the increase of release might be the shift of the equilibrium between the free DOX and the DOX bound to pullulan. Thus, at lower pH, more hydrazone bond was cleaved. In addition, re-protonation of amino group in DOX also was increased which resulted in the increase of DOX solubility. As a result, DOX was released faster at pH 5.0 (close to pH in endosomes) than at pH 7.4 (pH of blood plasma). This pH-sensitive release behavior is significant for tumor targeting. It was expected that more DOX could be released after the nanoparticles reached the tumor cell endosome or lysosome, where the pH is 5.0–6.0 and 4.5–5.5, respectively.

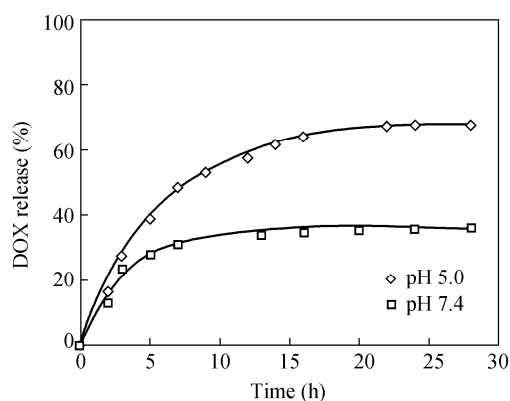


Fig. 3 *In vitro* DOX release from pullulan/DOX nanoparticles at pH 7.4 and pH 5.0 buffer

In vitro cytotoxicity of free DOX and pullulan/DOX nanoparticles on 4T1 was showed in Fig. 4. The IC_{50} value was 1.78 mg/L and 2.13 mg/L for free DOX and pullulan/DOX nanoparticles, respectively. Almost all the cells were killed when DOX concentration was above 10 mg/L, regardless whether DOX was in its free form or entrapped in nanoparticles ($p = 0.130$). No significant cytotoxicity was observed for blank AMP polymer carriers up to 600 mg/L concentration. Therefore, the comparable cytotoxicity effect of pullulan/DOX nanoparticles with free DOX indicated entrapped DOX can keep its biological activity.

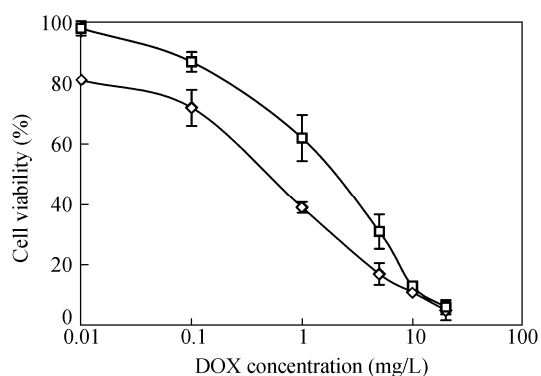


Fig. 4 *In vitro* cytotoxicity of free DOX (◇) and pullulan/DOX nanoparticles (□) against 4T1 mouse breast cancer cells

CONCLUSIONS

A pH-sensitive pullulan/DOX conjugate was prepared by a simple and easy synthesis route. The DOX content in the pullulan/DOX conjugate was 3.18 wt%. The conjugate can self-assemble to form nanoparticles less than 100 nm in diameter. *In vitro* DOX was released in a pH-sensitive manner and the pullulan/DOX nanoparticles showed comparable cytotoxicity effect on the 4T1 cells with free DOX.

REFERENCES

- 1 Langer, R., Nature, 1998, 392: 5
- 2 Maeda, H., Wu, J., Sawa, T., Matsumura, Y. and Hori, K., J. Controlled Release, 2000, 65: 271
- 3 Chavanpatil, M.D., Patil, Y. and Panyam, J., Int. J. Pharm., 2006, 320: 150
- 4 Wong, H.L., Bendayan, R., Rauth, A.M., Xue, H.Y., Babakhanian, K. and Wu, X.Y., J. Pharmacol. Exp. Ther., 2006,

317: 1372

- 5 Jung, J., Lee, I.H., Lee, E., Park, J. and Jon, S., *Biomacromolecules*, 2007, 8: 3401
- 6 Ulbrich, K. and Subr, V., *Adv. Drug Deliv. Rev.*, 2004, 56: 1023
- 7 Tabata, T., Murakami, Y. and Ikada, Y., *J. Controlled Release*, 1998, 50: 123
- 8 Sivakumar, P.A. and Rao, K.P., *Carbohydr. Polym.*, 2003, 51: 327
- 9 Hidaka, M., Kanematsu, T., Ushio, K. and Sunamoto, J., *J. Bioact. Compat. Polym.*, 2006, 21: 591
- 10 Constantin, M., Fundueanu, G., Bortolotti, F., Cortesi, R., Ascenzi, P. and Menegatti, E., *Int. J. Pharm.*, 2007, 330: 129
- 11 Bataille, I., Huguet, J., Muller, G., Mocanu, G. and Carpov, A., *Int. J. Biol. Macromol.*, 1997, 20: 179
- 12 Brigger, I., Dubernet, C. and Couvreur, P., *Adv. Drug Deliv. Rev.*, 2002, 54: 631
- 13 Glinel, K., Sauvage, J.P., Oulyadi, H. and Huguet, J., *Carbohydr. Res.*, 2000, 328: 343
- 14 Xu, Z.H., Gu, W.W., Huang, J., Sui, H., Zhou, Z.H., Yang, Y.X., Yan, Z. and Li, Y.P., *Int. J. Pharm.*, 2005, 288: 361
- 15 Hruby, M., Konak, C. and Ulbrich, K., *J. Controlled Release*, 2005, 103: 137