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Investigation of thermo-sensitive amphiphilic micelles as drug carriers for chemotherapy in cholangiocarcinoma *in vitro* and *in vivo*

Xuefeng Wang[a,1], Songgang Li[a,1], Ziwei Wan[a], Zhiwei Quan[a,**], Qinggang Tan[b,c,*]

[a] *Department of General Surgery, Xinhua Hospital, School of Medicine, Shanghai Jiaotong University, 1665 Kongjiang Road, Shanghai 200092, PR China*
[b] *Institute of Nano and Bio-polymeric Materials, School of Material Science and Engineering, Tongji University, Cao An Road 4800, Shanghai 201804, PR China*
[c] *Key Laboratory Advanced Civil Engineering Materials, Ministry of Education, School of Material Science and Engineering, Tongji University, Cao An Road 4800, Shanghai 201804, PR China*

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ABSTRACT

Cholangiocarcinoma is an epithelial cancer of the bile ducts with poor prognosis and, in recent years, a rapidly increasing incidence. In this study, nano-sized thermo-sensitive micelles were investigated as drug carriers to improve chemotherapy in cholangiocarcinoma. Thermo-sensitive amphiphilic block copolymer, P-(*N*,*N*-isopropylacrylamide-co-*N*-hydroxymethylacrylamide)-b-caprolactone [P-(NIPAAm-co-NHMAAm)-b-PCL] with lower critical solution temperature (LCST) at about 38 °C was synthesized. Doxorubicin (DOX)-loaded micelles were prepared by dialysis method. The micelles exhibited a sustained and temperature-dependent DOX release. Toxicity of the blank micelles for human cholangiocarcinoma (QBC939) cells was minimal both *in vitro* and *in vivo*. In contrast, the DOX-loaded micelles effectively inhibited proliferation and induced apoptosis of QBC939 cells *in vitro* ($p < 0.05$) and inhibited tumor growth in nude mice by 21.49%. These results indicated that thermo-sensitive amphiphilic micelles are a promising and effective drug carrier, and show potential for improving chemotherapy for cholangiocarcinoma.

1. Introduction

Over the past decade, the worldwide incidence of cholangiocarcinoma has shown a sharp increase. Complete resection with negative histological resection margins is accepted as the best possible option for long term survival (Silva et al., 2005; Khan et al., 2005). Unfortunately, less than 30% of patients with cholangiocarcinoma are suitable for resection at the time of diagnosis (Jarnagin et al., 2001). The survival of unresectable cholangiocarcinoma is approximately 3 months without intervention and 4–6 months with biliary decompression (Kahaleh et al., 2008). Patients with advanced cholangiocarcinoma typically present with obstructive jaundice. Biliary stent has become the standard palliative treatment to improve life quality or prolong survival period of patients, but it improves survival time only slightly (Lai et al., 2001). Therefore, other more effective methods need to be developed for the treatment of cholangiocarcinoma.

Chemotherapy, i.e. the use of drugs to kill cancer cells is, sometimes, the only available cancer treatment. However, chemotherapy has not been shown to improve quality of life or prolong survival in patients with cholangiocarcinoma (Khan et al., 2002). Thus, improvement to chemotherapy for cholangiocarcinoma is urgently needed. Targeted and controlled delivery nano-vehicles are able to achieve a high local drug concentration at the treatment site and thus increase efficacy of chemotherapy while reducing systemic side effects (Bertrand and Leroux, 2012; Kataoka et al., 2001; Peer et al., 2007; Tong and Cheng, 2007). Nanosized amphiphilic copolymer micelles have attracted much attention as such a drug delivery system (Mikhail and Allen, 2009; Lu and Park, 2013; Jeong et al., 2009; Soliman and Winnik, 2008; Rosler et al., 2001; Xun et al., 2011). These micelles consist of hydrophilic and hydrophobic segments and significantly improve solubility for poorly water-soluble drugs. The enhanced permeability and retention effect (EPR effect) allows for passive targeting of the drug to solid tumor tissues due to the leaky vasculature in these tissues. More importantly, by introducing a stimulus responsive moiety into the copolymer structure, nano-micelles can also achieve active drug release at the target site (Zhang et al., 2012). Such amphiphilic copolymer micelles utilizing temperature stimulus responsive poly(*N*-isopropylacrylamide) (PNIPAAm) as shell have been studied intensively because of the passive targeting and

* Corresponding author at: Institute of Nano and Bio-polymeric Materials, School of Material Science and Engineering, Tongji University, Cao An Road 4800, Shanghai 201804, PR China. Tel.: +86 21 69580234.
** Corresponding author. Tel.: +86 21 65790000 7900.
E-mail addresses: zhiwquan@yahoo.cn (Z. Quan), qgtan@tongji.edu.cn (Q. Tan).
[1] These authors contributed equally to this work.

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thermo-induced active release mechanisms (Yan and Tsujii, 2005; Wei et al., 2009; Liu et al., 2005). The main advantage of PNIPAAm is that it can undergo a sharp coil-globule phase transition in water at a critical solution temperature (LCST) (about 32 °C), transforming from an expanded hydrophilic structure below LCST to a compact hydrophobic structure above LCST (Schild, 1992). Recently, a variety of thermo-sensitive amphiphilic polymers using PNIPAAm as a hydrophilic outer shell and other hydrophobic moieties as hydrophobic inner core, have been synthesized (Liu et al., 2005; Chung et al., 1997; Kohori et al., 1998; Wei et al., 2008; Chang et al., 2011). The balance between hydrophilic and hydrophobic segments in a thermo-sensitive polymer determines the LCST and increasing hydrophilic components of polymer backbone generally leads to higher LCSTs. Therefore, the LCST of PNIPAAm based micelles could be increased by increasing the hydrophilicity to meet practical demands.

Because of its high metabolic rate, the temperature inside the liver is usually around 38 °C. Since the bile duct is fully covered by liver tissue, its inner temperature is generally the same. Therefore, micelles with a LCST of 38 °C would be mostly suitable to ensure constant temperature-dependent drug release in the bile duct. Additionally, due to the lower temperature of the intestine, drug release from micelles that are transported through the bile duct to the intestine, would be decreased leading to lower side effects.

In this study, a more hydrophilic monomer *N*-hydroxymethylacrylamide (HMAAm) was selected to copolymerize with NIPAAm monomer improving the LCST as a hydrophilic outer-shell; then, poly (NIPAAm-co-HMAAm) precursor polymer was polymerized with caprolactone (CL) to obtain the amphiphilic thermo-sensitive block copolymer, P-(NIPAAm-co-NHMAAm)-b-PCL. A thermo-sensitive amphiphilic block copolymer with LCST of about 38 °C was successfully synthesized *via* described above method and could self-assemble to form nanosized micelles. DOX-loaded micelles show a sustained and temperature-dependent DOX release and could effectively inhibit the growth of human cholangiocarcinoma cells (QBC939) *in vitro* and tumor in xenografts in Balb/c nude mice *in vivo*.

These findings demonstrated that the potential of P-(NIPAAm-co-NHMAAm)-b-PCL micelles as drug delivery system for chemotherapy in cholangiocarcinoma.

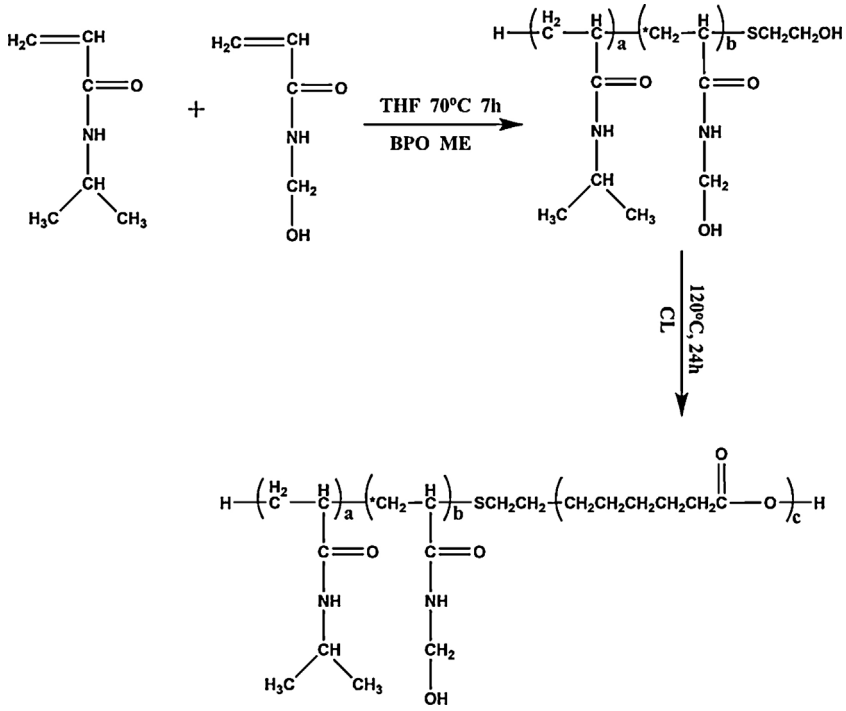
2. Materials and methods

2.1. Materials

Tetrahydrofuran (THF), 2-mercaptoethanol, stannous octoate (SnOct), *N*,*N*′-dimethylformamide (DMF) and benzoyl peroxide (BPO) were purchased from Shanghai Chemical Reagent Co., LTD. *N*,*N*′-Isopropylacrylamide (NIPAAm) was purchased from TCI. *N*-Hydroxymethylacrylamide (HMAAm) was obtained from Sinopharm Chemical Reagent Company and purified by recrystallizing it twice in ethanol. Caprolactone (CL) and 3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma. NIPAAm was purified by recrystallization in hexane and toluene. THF and CL were dried over CaH_2 and distilled before used. A human cholangiocarcinoma cell line (QBC939) was donated by the Renji hospital, School of Medicine, Shanghai Jiaotong University. BAL B/c nude mice were purchased from Shanghai SLAC laboratory animal Co. Ltd. All other reagents were commercially available and were used as received.

2.2. Synthesis of P-(NIPAAm-co-HMAAm)-b-PCL

The amphiphilic block copolymer was synthesized according to the similar method reported previously (Wei et al., 2008) (Scheme 1). Briefly, hydroxyl-terminated poly (NIPAAm-co-HMAAm) was synthesized by radical polymerization. The ratio of NIPAAm/HMAAm in the feed (mol %) was set at 7:1, in order to improve the LCST to about 40 °C (Cheng et al., 2008). The procedure was briefly described as following: NIPAAm monomer (7 mmol), HMAAm monomer (1 mmol), 2-mercaptoethanol (0.08 mmol) as a chain transfer agent, and benzoylperoxide (0.04 mmol) as an initiator were dissolved in 10 mL of tetrahydrofuran; the solution then was degassed by bubbling with nitrogen for 20 min; polymerization was carried out at 70 °C for 7 h, followed by precipitation in diethyl



Scheme 1. Synthesis diagram of P-(NIPAAm-co-HMAAm)-b-PCL.