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Stepwise targeted drug delivery to liver cancer cells for enhanced therapeutic efficacy by galactose-grafted, ultra-pH-sensitive micelles

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ABSTRACT

To promote drug accumulation and cell-killing ability at tumor tissue, we have prepared a stepwise targeted drug delivery system that can remain stealthy and long-circulating in the blood vessels, improve drug retention at extracellular stimuli, enhance cellular uptake through special targeting ligands, and then achieve rapid drug release to improve toxicity to tumor cells at intracellular stimuli. Herein, galactose-grafted, ultra-pH-sensitive drug carriers (POEAd-*g*-LA-DOX micelles), which could respond to both extracellular and intracellular pH, and combine with galactose-receptors in cell membrane, were constructed by a facile method, therefore achieving: (i) remaining stable at pH 7.4; (ii) responding to tumoral extracellular pH following gradually larger nanoparticles (NPs); (iii) conjugating receptors in the cell membrane of liver cancer through surface galactose-ligands of micelles; (iv) being sensitive to tumoral intracellular pH following further swelling for rapid drug release. In vitro cytotoxicity and cellular uptake measurement showed that POEAd-*g*-LA20-DOX micelle was more easily internalized and more toxic effect on tumor cells than free DOX. Moreover, in vivo biodistribution and tumor inhibition examinations demonstrated that POEAd-*g*-LA20-DOX formulation had more superior efficacy to significantly enhance drug accumulation in tumor, and then restrain tumor growth while decreasing drug concentration in heart.

Statement of Significance

Chemotherapeutic efficacy is limited by poor tumor selectivity, which also causes severe toxicity in normal tissues and organs, although many targeted drug delivery systems have been developed by passive targeting strategies or active targeting strategies with specific targeting ligands in recent years. Herein, galactose-grafted, ultra-pH-sensitive, ortho ester-based drug carriers, which can respond to both extracellular and intracellular pH, and target to galactose-receptors in cell membrane, have been successfully constructed by facile method, therefore achieving stepwise targeting to microenvironment of liver cancer and then enhancing drug accumulation and tumor inhibition. The strategy of designing dual-stimuli-responsive copolymers can be potentially useful, and extrapolated to synthesizing other categories of highly labile drug carriers in a range of biomedical applications.

1. Introduction

Polymeric nanoparticles (NPs) are widely used as promising drug carriers to introduce anticancer drugs into tumor cells [1]. Unfortunately, the clinical application of these drug carriers for cancer treatment has not been realized in patients due to their limited efficacy by physiological barriers in the tumor microenvironment, although some of them approve so far minimal toxicity [2]. To overcome the barriers, it is crucial to make these NPs precisely target tumors from blood vessels [3,4]. Targeted NPs by passive targeting strategies or active targeting strategies with specific targeting ligands, have been successfully used for variously targeted anticarcinogen delivery [5]. However, the physiological barriers in the tumor tissues and nonspecific interactions and recognition in normal tissues are still not overcome for many in vivo application. Therefore, it is in much demand to develop

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multi-functional NPs with more precise target to tumor microenvironment for enhancing drug accumulation, and cell-killing ability.

Acid-responsiveness, as the most frequently used approach among tumor environmental stimuli-triggered strategies in recent years, is a desirable choice to achieve precise tumor target based on its intrinsic feature to the tumor microenvironment and the existence of evident pH gradient among blood vessels (pH ~7.4), extracellular (pH ~6.5–7.2) and intracellular (pH ~5.0–6.0) space in tumors [6–8]. So far, many ultra-pH-sensitive NPs have been designed to achieve physical and chemical changes for improved tumor penetration or/and cellular uptake upon arrival at target tumor sites such as a large-to-small size transition [9,10], PEGylation and dePEGylation [11,12], a negative-to-positive charge transition [13–15], or disintegration to release drug in the tumor vicinity [16]. While high therapeutic efficacy would be yielded by an ideal drug delivery system only if it efficiently overcome obstacles from blood vessels to tumor targeting site [2,9]. So, there has been great interest in utilizing ultra-pH-sensitive NPs to design a stepwise targeted drug delivery system, that can respond to extracellular pH for improved drug retention, subsequently conjugate to receptors in the cell membrane for enhanced cellular internalization by special targeted ligand, and finally be sensitive to intracellular pH for rapid drug release to kill tumor cells.

Therefore, we are trying to prepare ultra-pH-sensitive drug carriers by optimizing surrounding structures of ortho esters, whose hydrolysis rates are higher than these of such acid-labile linkages as acetal, ketal and hydrazine in response to mildly acidic condition [8,17–29]. Previously, our groups have developed various types of acid-labile polymeric drug carriers with ortho ester linkages in their side-chains or backbones, which have shown great potential as drug carriers with excellent biocompatibility and preferable drug release [21–26]. Moreover, the hydrolyzed rates of ortho ester linkages vary from several hours to a few days depending on hydrophobicity of its surrounding environments at pH 5.0–6.0. In addition, some are easily acid-sensitive to perform size changes under mildly acidic conditions. The changes may promote tumor accumulation and retention through well-known "enhanced permeability and retention" (EPR) effect [21–26]. Hence, we hypothesize that the introduction of ortho ester into galactose-grafted main-chains of NPs may make ultra-pH-sensitivity possible at tumor tissues (pH 6.5–7.2) by optimizing hydrophilic property of surrounding structures of ortho ester linkages, so that the ultra-pH-sensitive NPs will concurrently achieve stepwise targeted process from blood circulation to tumor cells: (i) remaining stealthy and long-circulating in the blood vessels; (ii) responding to tumoral extracellular pH following gradually larger nanoparticles (NPs) for improved drug retention; (iii) conjugating receptors in the cell membrane of liver cancer through surface galactose-ligands of NPs for enhanced cellular uptake; (iv) being sensitive to tumoral intracellular pH following further swelling for rapid drug release to improve toxicity to tumor cells (Scheme 1).

In this work, ortho esters were introduced into backbone as hydrophobic segments and lactobionic acid (LA) as both hepatic targeting ligand and hydrophilic segments were grafted with different value as the side chains. The grafted copolymers (POEAd-*g*-LA) would form micelles in normal physiological environment and deprotonated DOX was embedded. Herein, we focus on the investigation of ultra-pH-sensitivity-LA-driven drug accumulation, retention, cellular uptake and antitumor activities in human liver carcinoma cell line (HepG2), human neuroblastom cancer cell line (SH-SY5Y) and murine hepatic cancer cell line (H22) (in vitro), and hepatic H22 subcutaneous tumor model (in vivo) based on POEAd-*g*-LA-DOX micelles.

2. Material and methods

2.1. Materials

Acetonitrile and pyridine were dried from CaH_2 before used. N, N-Dimethylformamide (DMF) was dried over CaH_2 and distilled under reduced pressure. Triethylamine (TEA) was refluxed with benzoic anhydride and then dried from CaH_2. 2-Amino-1,3-propanediol, ethyl trifluoroacetate (ETFA), lactobionic acid (LA), succinic anhydride (SA), N,N'-disuccinimidyl carbonate (DSC), 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), trinitrobenzene sulfonic acid (TNBSA), doxorubicin hydrochloride (DOX·HCl) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Tokyo Chemical Industry Co., Ltd. (Shanghai, China). 2,2′-((4,4′-(Oxybis(methylene)) bis(1,3-dioxolane-4,2-diyl)) bis(Oxy)) diethanamine (OEN) was synthesized by our group [30]. Human neuroblastom cancer cell line (SH-SY5Y), human liver carcinoma cell line (HepG2), murine hepatic cancer cell line (H22) and human embryonic kidney cell line (293T) were purchased from Shanghai Institute of Cell Biology (Shanghai, China). Male ICR mice (18–22 g) were obtained from Animal Center of Anhui Province (Hefei, China).

2.2. Synthesis of 2,2,2-trifluoro-N-(1,3-dihydroxypropyl) acetamide (NF)

Ethyl trifluoroacetate (34.10 g, 0.24 mol) was added dropwise to a solution of 2-amino-1,3-propanediol (18.20 g, 0.20 mol) in acetonitrile (200 mL). After 12 h, the solvent was evaporated and the residue was dissolved in ethyl acetate. The solution was washed with 5% $KHSO_4$ and saturated NaCl aqueous solution respectively, then dried over $MgSO_4$, filtrated and concentrated to yield 32.00 g (85.5%) of 2,2,2-trifluoro-N-(1,3-dihydroxypropyl) acetamide as a white solid. ^{1}H NMR (400 MHz, D_2O): δ (ppm) 3.61–3.78 (m, 4H, -CH-CH_2-OH), 4.14 (m, 1H, -NH-CH-CH_2OH).

2.3. Synthesis of 4,4′-((2-(2,2,2-trifluoroacetamido)propane-1,3-diyl) bis(oxy)) bis(4-oxobutanoic acid) (NFC)

In a nitrogen atmosphere, NF (18.71 g, 100.03 mmol), succinic anhydride (25.03 g, 250.12 mmol), and pyridine (100 mL) were placed in a 250 mL three-necked flack with a magnetic stirrer. The mixture was vigorously stirred overnight at room temperature and concentrated to afford the crude product. The product was dissolved in ethyl acetate (250 mL), washed once respectively with 5% HCl and saturated NaCl water solution. The organic layer was dried over $MgSO_4$, and concentrated to yield 4,4′-((2-(2,2,2-trifluoroacetamido)propane-1,3-diyl)bis(oxy)) bis(4-oxobutanoic acid) as white powder (35.62 g, 92%). ^{1}H NMR (400 MHz, Acetone-d_6): δ (ppm) 2.61 (s, 8H, -CH_2-CH_2-), 4.24–4.37 (m, -COO-CH_2-CH-, 4H), 4.46–4.54 (m, 1H, -HN-CH-CH_2-), 8.50 (d, -NH-, 1H), 10.67 (s, -COOH, 2H). ^{13}C NMR (100 MHz, Acetone-d_6, δ): 29.2, 29.6, 49.6, 63.0, 115.6, 157.8, 172.7, 173.8.

2.4. Synthesis of bis(2,5-dioxopyrrolidin-1-yl) O,O'-(2-(2,2,2-trifluoroacetamido) propane-1,3-diyl) disuccinate (BOD)

NFC (15.48 g, 40.0 mmol) was dissolved in acetonitrile (200 mL), and N,N′-disuccinimidyl carbonate (30.74 g, 120 mmol) was added under nitrogen. Triethylamine (22.18 mL, 160 mmol) was added dropwise to the solution while stirring and then the mixture was unceasingly stirred overnight at room temperature. After reaction, the mixture was evaporated to get the crude