Acta Biomaterialia 54 (2017) 227–238

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Full length article

Legumain-cleavable 4-arm poly(ethylene glycol)-doxorubicin conjugate for tumor specific delivery and release

Huicong Zhou [a,b,1], Huanjiao Sun [a,b,1], Shixian Lv [b], Dawei Zhang [b], Xuefei Zhang [a,*], Zhaohui Tang [b,*], Xuesi Chen [b]

[a] Key Laboratory of Environmentally Friendly Chemistry and Applications of Ministry of Education and Key Laboratory of Polymeric Materials & Application Technology of Hunan Province, Xiangtan University, Xiangtan 411105, PR China
[b] Key Laboratory of Polymer Ecomaterials, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, PR China

ARTICLE INFO

Article history:
Received 7 December 2016
Received in revised form 3 March 2017
Accepted 13 March 2017
Available online 15 March 2017

Keywords:
Legumain
PEG
Doxorubicin
Conjugate
Drug delivery

ABSTRACT

Traditional chemotherapy strategy exists undesirable toxic side-effects to normal tissues due to the low selectively to cancer cells of micromolecule cytotoxic drugs. One considered method to realizing the targeted delivery and increasing the specificity to tumor tissues of the cytotoxic drug is to transporting and discharging it through an environment-sensitive mechanism. In this study, a novel enzyme-sensitive polymer-doxorubicin conjugate was designed to delivery chemotherapeutic drug in a tumor-specific behavior and selectively activated in tumor tissue. Briefly, doxorubicin (DOX) was conjugated to carboxyl-terminated 4-arm poly(ethylene glycol) through a tetrapeptide linker, alanine-alanine-asparagine-leucine (AANL), which was one of the substrates of legumain, an asparaginyl endopeptidase that was found presented in plants, mammals and also highly expressed in human tumor tissues. Hereinafter, the polymer-DOX conjugate was termed as 4-arm PEG-AANL-DOX. Dynamic laser scattering (DLS) and transmission electron microscopy (TEM) measurements indicated that the 4-arm PEG-AANL-DOX could self-assemble into micelles in aqueous solution. Drug release and *in vitro* cytotoxicity studies revealed that the 4-arm PEG-AANL-DOX could be cleaved by legumain. *Ex vivo* DOX fluorescence imaging measurements demonstrated that the 4-arm PEG-AANL-DOX had an improved tumor-targeting delivery as compared with the free DOX·HCl. *In vivo* studies on nude mice bearing MDA-MB-435 tumors revealed that the 4-arm PEG-AANL-DOX had a comparable anticancer efficacy with the free DOX·HCl but without DOX-related toxicities to normal tissues as measured by body weight change and histological assessments, indicating that the 4-arm PEG-AANL-DOX had an improved therapeutic index for cancer therapy.

Statement of Significance

Herein we describe the construction of a novel tumor environment-sensitive delivery system through the instruction of a legumain-cleavable linkage to a polymer-DOX conjugate (4-arm PEG-AANL-DOX). This particular design strategy allows for polymer-DOX conjugates to be delivered in a tumor-specific manner and selectively activable in tumor microenvironment so that it can combine the advantages of tumor-specific delivery and tumor intracellular microenvironment-triggered release systems.

1. Introduction

Chemotherapy is among the most commonly used treatments for cancer therapy [1]. However, chemotherapeutic drugs aggressively kill both tumor and normal cells, thereby causing numerous undesirable severe side effects such as cardiotoxicity, renal toxicity, or the digestive tract toxicity and so on, these all are because of their non-specific distribution *in vivo*. In order to selectively eradicate tumor cells while minimizing toxicity to neighboring tissues, it is urgently needed to develop new chemotherapeutic agents which are delivered in a tumor-specific manner and selectively activated in tumor tissue.

* Corresponding authors.
E-mail addresses: zxf7515@163.com (X. Zhang), ztang@ciac.ac.cn (Z. Tang).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.actbio.2017.03.019
1742-7061/

Doxorubicin hydrochloride (DOX·HCl) is commonly used for the treatment of a variety of malignancy tumors, including sarcomas, leukemia and solid tumors [2]. However, the side effects of DOX·HCl, such as myelosuppression and cardiotoxicity, greatly impair its usages [3]. In order to enhance the therapeutic index of DOX·HCl, one frequently-used approach is to exert the tumor-specific delivery of DOX·HCl using nanocarriers which can primarily accumulate in tumor tissues *via* the "passive targeting" or "active targeting" after intravenous injection administration [4–16]. However, DOX·HCl must have access to target deoxyribonucleic acid (DNA) of tumor cells, not just at the tumor tissue lesion level. The "passive targeting" or "active targeting" can deliver DOX·HCl to tumor tissues, but the delivery of DOX·HCl to DNAs is still a challenge for nanomedicine. Generally speaking, DOX·HCl that is tightly encapsulated or conjugated in nanocarriers is difficult to liberate free active principle in tumor tissues. For example, PEGylated liposomal DOX (Doxil) is highly stable and releases active free DOX·HCl very slowly, hence its anticancer activity is only moderate [17]. To solve this problem, a number of tumor extracellular and intracellular microenvironment-triggered drug release systems were explored. Because the tumor extracellular and intracellular lysosomal environment is relatively acidic [18], acid-cleavable linkages, including hydrazone, maleyl and aconityl amide linkages, are widely used to conjugate DOX [19–23]. In addition, peptide linkers that are cleavable by tumor-associated endogenous proteases such as matrix metalloproteinases and cathepsin B, have attracted considerable attention [24–28].

Legumain/asparaginyl endopeptidase is a lysosomal/vascular cysteine protease and has a strict specificity for the hydrolysis of peptide bonds with asparagine or aspartic acid at the P1 position [29,30]. Legumain has an important role in the endosomal/lysosomal degradation system [31]. Under physiological conditions, legumain is mainly expressed in kidney [32,33]. Accumulating evidence shows that legumain is overexpressed on the surface of tumor-associated macrophages [34] and in a variety of solid tumors, such as carcinomas of the breast, colon, ovarian, prostate, central nervous system tumors, lymphoma and melanoma [35]. The level of legumain is positively correlated with the degree of malignancy. Overexpression of legumain is significant in tumors with high invasion and metastasis [36–42]. Because of the unique function and overexpression in many human tumors, legumain represents a promising target for the design of prodrugs and anticancer drug carriers [43]. Several legumain-cleavable small molecular anticancer drugs conjugates for tumor-specific active agent release have been reported [32,35,44–48], however, these small molecular conjugates could not deliver cytotoxic drugs in a tumor-specific manner.

Herein, we describe the construction of a novel tumor intracellular microenvironment-sensitive delivery system through the introduction of a legumain-cleavable tetrapeptide linkage to a polymer-DOX conjugate. This particular design strategy allows for polymer-DOX conjugates to be delivered in a tumor-specific manner and selectively activable in tumor intracellular microenvironment so that it can combine the advantages of tumor-specific delivery and tumor intracellular microenvironment-triggered release systems. For the proof of concept, a polymer-DOX conjugate (4-arm PEG-AANL-DOX) was prepared here by using AANL, a substrate of legumain [35], to link 4-arm PEG and DOX. The 4-arm PEG-AANL-DOX is expected to accumulate in a treated solid tumor *via* the enhanced permeability and retention (EPR) effect after injection *via* the tail vein, and specifically release active cytotoxic leucine-DOX molecules [49] at the tumor site where legumain is often highly upregulated. The 4-arm PEG-AANL-DOX was synthesized, characterized and evaluated *in vitro* and *in vivo* in detail.

2. Materials and methods

2.1. Materials

4-arm poly(ethylene glycol) (4-arm PEG-OH, M_n = 100 K) was purchased from the Pharmicell Co. Ltd, Korea and implemented as received. 3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma–Aldrich. Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng United Technology Corporation, China. Alanine-Alanine-Asparagine-Leucine (AANL) was custom-made by BAM Biotech Co., Ltd., Xiamen, China. Recombinant mouse legumain was purchased from Sino Biological Inc., Beijing, China. Benzotriazol-1-yl-oxytripyrrolidino-phosphonium hexafluorophosphate (PyBOP) and *N*-(3-dimethyl aminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from Aladdin Industrial Corporation, Shanghai, China. *N*,*N*-Dimethylformamide (DMF), dichloromethane (DCM), pyridine (Py) and triethylamine (TEA) were dried by distillation over calcium hydride (CaH_2) before use. All the other reagents and solvents were purchased from Sinopharm Chemical Reagent Co. Ltd. and used without further purification.

2.2. Synthesis of 4-arm PEG-COOH

Succinic anhydride (4.00 g, 40.0 mmol) and 4-arm PEG-OH (20.0 g, 2.00 mmol) were dissolved in dry pyridine (50 mL), the solution was stirring at 25 °C for 48 h, then 250 mL DCM was added, after the pH of the reaction solution was tuned to 1.0 with aq. HCl (1 M), the mixture was then washed with saturated sodium chloride solution three times, dried with Na_2SO_4 and evaporated. The product was isolated by precipitation in excessive ice diethyl ether and dried under vacuum, yielding a white solid. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ (ppm) 4.26 (t, 2H, —OCH$_2$C*H*$_2$O(CO)—), 3.64 (s, 226H, —OC*H*$_2$C*H*$_2$O—), 3.41 (s, 2H, C(C*H*$_2$O—)), 2.64 (m, 4H, —(CO)C*H*$_2$C*H*$_2$(CO)—).

2.3. Synthesis of 4-arm PEG-NHS

4-arm PEG-COOH was activated with *N*-hydroxysuccinimide (NHS) in the presence of EDC·HCl at room temperature. Briefly, 4-arm PEG-COOH (15.0 g, 1.29 mmol), NHS (2.07 g, 18.0 mmol) and EDC·HCl (3.44 g, 18.0 mmol) were dissolved into dry DCM (150 mL). The solution was stirred overnight. Then the mixture was diluted with DCM, washed with saturated brine, dried over Na_2SO_4 and evaporated, precipitated in excessive ice diethyl ether and dried under vacuum. 4-arm PEG-NHS was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$, 298 K): δ (ppm) 4.28 (t, 2H, —OCH$_2$C*H*$_2$O(CO)—), 3.64 (s, 225H, —OC*H*$_2$C*H*$_2$O—), 3.41 (s, 2H, C(C*H*$_2$O—)), 2.96 (t, 2H, —(CO)CH$_2$C*H*$_2$(CO)OSu), 2.84 (s, 4H, —C*H*$_2$-C*H*$_2$— from NHS), 2.78 (t, 2H, —(CO)C*H*$_2$CH$_2$(CO)OSu).

2.4. Synthesis of 4-arm PEG-AANL

The 4-arm PEG-AANL was prepared by the conjugation of 4-arm PEG-NHS to the N-terminus of peptide AANL. Briefly, the 4-arm PEG-NHS (2.00 g, 0.177 mmol), AANL (0.486 g, 1.20 mmol) and TEA (24.0 mg, 2.40 mmol) were dissolved in 20 mL of dry DMF. The conjugation reaction was carried out at 30 °C for 48 h. The solution was dialyzed with distilled water. The 4-arm PEG-AANL was obtained by lyophilization. ^{1}H NMR (400 MHz, trifluoroacetic acid-*d*, 298 K): δ (ppm) 4.97 (br, 1H, —C*H*< of Asn unit), 4.52 (br, 2H, —C*H*< of Ala unit), 4.42 (br, 1H, —C*H*< of Leu unit), 4.22 (br, 2H, —OCH$_2$C*H*$_2$O(CO)—), 3.70 (s, 227H, —OC*H*$_2$C*H*$_2$O—), 3.43 (s, 2H, C(C*H*$_2$O—)), 2.91 (m, 2H, —C*H*$_2$— of Asn unit), 2.66 (br, 2H,