Acta Biomaterialia 36 (2016) 241–253

Contents lists available at ScienceDirect

Acta Biomaterialia

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

Full length article

Redox and pH dual responsive poly(amidoamine) dendrimer-poly (ethylene glycol) conjugates for intracellular delivery of doxorubicin

Wen Hu [a,b], Lipeng Qiu [c], Liang Cheng [a], Qing Hu [a], Yang Liu [d], Ziyang Hu [a], Dawei Chen [a,*], Lifang Cheng [a,*]

[a] Department of Pharmaceutics, College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, China
[b] Yangtze River Pharmaceutical Group, Taizhou 225321, China
[c] Department of Pharmaceutics, School of Pharmaceutical Sciences, Jiangnan University, Wuxi 214122, China
[d] School of Pharmacy, Nanjing Medical University, Nanjing 210029, China

ARTICLE INFO

Article history:
Received 18 August 2015
Received in revised form 6 February 2016
Accepted 15 March 2016
Available online 16 March 2016

Keywords:
Poly(amidoamine) dendrimers
Poly(ethylene glycol)
Dual responsive
Cellular uptake
Antitumor activity

ABSTRACT

To solve the contradiction between long circulation time and effective intracellular drug release, redox and pH-responsive drug delivery system was developed by incorporated redox-sensitive disulfide linkage between poly(amidoamine) dendrimers (PAMAM) and poly(ethylene glycol) (PEG). Doxorubicin (DOX) was loaded into the hydrophobic core of the conjugates to prepare PAMAM-SS-PEG/DOX complexes (PSSP/DOX). *In vitro* release studies suggested that DOX release from PSSP/DOX complexes followed an redox and acid-triggered manner and increased with increasing PEGylation degree. *In vitro* cytotoxicity of PSSP/DOX complexes against B16 tumor cells increased with, while cellular uptake decreased with increasing PEGylation degree. Further, intracellular DOX release observation and measurement indicate that the intracellular DOX release played a critical role for the cytotoxicity of DOX-loaded PSSP conjugates. In addition, cellular entry mechanism of the PSSP/DOX study demonstrated that both clathrin- and caveolae-mediated endocytosis were the primary pathways for cellular entry of PSSP/DOX. Finally, *in vivo* study of PSSP/DOX complexes in B16 tumor-bearing mice indicate that PSSP/DOX could significantly improve antitumor efficiency and present a good safety. The redox and pH-responsive drug delivery system has been demonstrated to be a promising candidate for solid tumor therapy.

Statement of Significance

In previous research, pH-sensitive diblock polymer of poly(ethylene glycol)-poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PEG-PTMBPEC) was synthesized to facilitate the intracellular anticancer drug release. However, the nanoparticles based on PEG-PTMBPEC get into the tumor cells just relying on the EPR-mediated passive targeting resulting in the low drug accumulation. Therefore, cRGD peptide modified PEG-PTMBPEC polymeric micelles were developed for specific targeted delivery of doxorubicin (DOX) to neovascular cells and tumor cells simultaneously. The precise intracellular target site and effective drug concentration will contribute to enhancing the antitumor toxicity and reducing the systematic toxicity of DOX. The cRGD modified pH-sensitive micellar system is a promising vehicle for intracellular drug delivery to αvβ3 integrin receptor overexpressed tumor cells and neovascular cells.

1. Introduction

In the past decades, polymeric nanoparticles have emerged as one of the most promising platforms for targeted and controlled drug delivery [1,2]. Among many different polymeric nanoparticles, poly amidoamine (PAMAM) dendrimers have received much attention in the development of targeted drug delivery systems. In contrast to conventional polymers, PAMAM dendrimers are core-shell nanostructure with precise architecture and low polydispersity, which are synthesized in a layer-by-layer fashion around a core unit, and result in high level of control over size, branching points and surface functionality [3–5]. Such unique structural features make it widely used in biomedical applications, such as drug delivery, gene delivery and *in vivo* imaging [6–8].

* Corresponding authors at: College of Pharmaceutical Sciences, Soochow University, 199 Ren'ai Road, Suzhou 215123, China.
E-mail addresses: chendawei@suda.edu.cn (D. Chen), chenglifang0803@126.com (L. Cheng).

http://dx.doi.org/10.1016/j.actbio.2016.03.027
1742-7061/

Biocompatible polyethylene glycol (PEG) has been frequently used to modify PAMAM dendrimers to overcome charge-related cytotoxicity and hemolytic toxicity, prolong drug circulation time, improve the solubility of both drug and carrier system, decrease the opsonization by reticuloendothelial system (RES) and increase tumor accumulation via the enhanced permeability and retention (EPR) effect [9,10]. Unfortunately PEGylation is unfavorable for the drug release from PAMAM-PEG conjugates because it may form a closed structure on the periphery of PAMAM and greatly impeded the transport of encapsulated drug from the interior of PAMAM to the outer medium [11]. To overcome the dilemma, one ideal strategy is to introduce a cleavable PEG in PAMAM surface, that is keep PEG on the surface of PAMAM dendrimers during circulation but detach them from PAMAM dendrimers and quickly release its payload after entering tumor cells [12,13]. Thus, the hindrance effect of PEG on drug release can be avoided without sacrifice of long circulating properties.

To achieve this goal, redox-responsive drug delivery system had been developed by incorporated redox-sensitive disulfide linkage between hydrophilic PEG and PAMAM dendrimers. It has reported that the concentration of reductive glutathione (GSH) in blood is rather low, 10 μM, which is not high enough to cleave the disulfide linkage. However, after internalization of the nanoparticles by tumor cells, PEG can be cleaved from PAMAM due to the high concentration of GSH (10 mM) in the cytoplasm and cell nucleus [14–16]. Additionally, it has been reported that PAMAM dendrimers possess pH-sensitivity property result from loose structure under weakly acidic circumstance [17,18]. This unique feature is beneficial to tumor-specific release of loaded antitumor drugs, because the tumor microenvironment is considered more acidic than the normal tissues.

Based on these characteristics, redox and pH-responsive drug delivery system was successfully developed in our previous study to resolve the dilemma of extracellular stability *versus* intracellular drug release [19]. In the research, we found that the cell uptake of nanocarriers was decreased with the increasing of PEGylation degree. In general, PEGylation would lead to reduced cell uptake of nanocarriers due to the steric hindrance of PEG shell, which might impair the antitumor activity of the nanocarriers. However, it was interesting to note that the antitumor activity of the nanocarriers was proportional to the PEGylation degrees, and the similar phenomenon also was reported in Zhu's research [10]. Importantly, *in vitro* release studies revealed that drug release rate was also highly positively related to PEGylation degree. Taken together, we hypothesized that efficient intracellular drug release rather than cell uptake was the key factor to determine antitumor activity. In order to justify this hypothesis, the drug distribution in tumor cells was observed by confocal laser scanning microscopy (CLSM) and drug levels in whole tumor cells and tumor nuclei were also measured in this study. A further point of interest of this study was to elucidate the potential cellular entry mechanism of the nanocarriers, which would make us better understand the redox and pH-responsive drug delivery system how to exert the cytotoxicity in tumor cells.

In the present work, redox and pH-responsive PAMAM-SS-PEG conjugates (PSSP) were synthesized by incorporated redox-sensitive disulfide linkage between hydrophilic PEG and PAMAM dendrimers. Doxorubicin (DOX) was used as the model drug to prepare the PAMAM-SS-PEG/DOX (PSSP/DOX) complexes. The characteristics of the PSSP/DOX complexes such as zeta potential, particle size, drug loading and *in vitro* drug release were tested. The cellular uptake and the cytotoxicity of PSSP/DOX complexes were investigated with B16 cells. Moreover, the distribution of drugs in tumor cells was observed by CLSM and drug content in whole tumor cells and tumor nuclei were also measured in this study. The potential cellular entry mechanism of the nanocarriers was also elucidated. Finally, the B16-bearing mice model was established to further evaluate the curative effects of DOX-load conjugates *in vivo*.

2. Materials and methods

2.1. Materials

PAMAM dendrimers (Generation-4, —NH_2 terminated, M_w = 14,215) were purchased from Dendritech Inc (Michigan, USA). α-Mercaptoethyl-ω-methoxy polyoxyethylene (mPEG-SH, M_w = 5000) were purchased from JenKem Technology Co., Ltd. (Beijing, China). *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) was purchased from TCI development Co., Ltd. (Shanghai, China). Diethyl maleate (DEM) was obtained from Aladdin Chemistry Co. Ltd. (Shanghai, China) and chloroquine diphosphate was purchased from J&K Chemical Ltd. (Beijing, China). Triethylamine (TEA), glutathione (GSH) and sucrose were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Huafeng United Technology Co., Ltd. (Beijing, China). Hoechst 33342 and Lysotracker Green DND-26 were obtained from Molecular Probes (Eugene, OR, USA). Chlorpromazine, genistein, wortmannin, methyl-β-cyclodextrin and colchicine were obtained from Sigma (St Louis, MO, USA) and filipin was from Cayman Chemical Company (Ann Arbor, MI, USA). All other chemicals were of the analytic grade and used as received.

2.2. Synthesis and characterization of PSSP conjugates

PSSP conjugates with different PEGylation degrees were synthesized according to the method previously reported with some changes [20,21]. Briefly, various amounts of SPDP were added to the solution of PAMAM dendrimer (0.70 mmol, 10 mg) in 2 mL of methanol containing 10 μL of triethylamine, and the mixed solution was stirred at room temperature for 5 h. Then, different molar ratios of $mPEG_{5000}$-HSto PAMAM (8:1, 16:1 and 32:1) were added to the reaction solution and allowed to react overnight at room temperature. Finally, the products were purified by Sephadex G-50 column (φ 1 cm × 80 cm) using distilled water as the eluent. The products were collected and dried by vacuum freeze-dryer to obtain the final products denoted as PSSP conjugates. As control, PAMAM-PEG (PP) conjugates were synthesized as previously described with minor modification [22]. mPEG-SCM was added to the solution of PAMAM dendrimer (0.70 mmol, 10 mg) in 4 ml phosphate buffer (0.1 M, pH 8.2) and allowed to react at room temperature for 24 h with gentle stirring. The resulting solution was purified by Sephadex G-50 column to remove the unreacted PEG. After being freeze-dried, the final products were obtained denoted as PP conjugates.

The structure of PSSP conjugates was characterized by ^{1}H NMR and FT-IR spectroscopy, respectively. ^{1}H NMR spectra were recorded with a Unity Inova 400 spectrometer (400 Hz, Varian, USA) using deuteroxide (D_2O) as solvent. FT-IR spectra were recorded on a ProStar LC240 spectrometer (Varian, USA). The samples were pressed into transparent sheet with KBr.

The diameters and zeta potentials of PSSP conjugates were measured by (Dynamic Light Scattering) DLS using Nicomp™380 ZLS (PSS·Nicomp, Santa Barrara, USA). Samples were dissolved in 10 mM PBS (pH 7.4, 1 mg/mL) and filtered through 0.45 μm cellulose acetate membrane before measurement. All measurements were carried out at 37 °C.

The stability of PSSP conjugates in response to 10 μM and 10 mM reductive GSH in PBS (pH 7.4, 10 mM) was monitored by DLS. Briefly, 4 mg PSSP conjugates were dissolved in 4 mL of PBS, and then GSH was added to obtain the specified concentration.

ID	Title	Pages
73	Redox and pH dual responsive poly(amidoamine) dendrimer-poly(ethylene glycol) conjugates for intracellular delivery of doxorubicin	13