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Amphiphilic peptide dendritic copolymer-doxorubicin nanoscale conjugate self-assembled to enzyme-responsive anti-cancer agent

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ABSTRACT

Peptide dendrimer drug conjugate based nanoparticles are recently developed as a potential candidate for drug delivery vehicle. In this study, we prepared and characterized the enzyme-sensitive amphiphilc mPEGylated dendron-GFLG-DOX conjugate via two-step highly efficient click reaction. Dynamic light scattering (DLS) and transmission electron microscope (TEM) studies demonstrated the mPEGylated dendron-GFLG-DOX conjugate self-assembled into compact nanoparticles with negatively charged surface. The nanoparticles with 9.62 wt% (weight percent) of DOX showed enzyme-sensitive property by drug release tests. The nanoparticles were shown to effectively kill cancer cells *in vitro*. The fluorescent image indicated that the nanoparticles could accumulate and retain within tumor for a long time. Moreover, the nanoparticles substantially enhanced antitumor efficacy compared to the free DOX, exhibiting much higher effects on inhibiting proliferation and inducing apoptosis of the 4T1 murine breast cancer model confirmed as the evidences from tumor growth curves, tumor growth inhibition (TGI), immunohistochemical analysis and histological assessment. The nanoparticles reduced DOX-induced toxicities and presented no significant side effects to normal organs of both tumor bearing and healthy mice as measured by body weight shifts and histological analysis. Therefore, the mPEGylated dendron-GFLG-DOX conjugate based nanoparticle serves as a potential drug delivery vehicle for breast cancer therapy.

1. Introduction

Chemotherapy has great advantage as one of therapeutic routes for cancer therapy while many antitumor drugs have to face the problems of low antitumor efficiency and rebarbative toxic side effects [1,2]. To overcome those challenges, in consideration of the special tumor microenvironment which is different from the normal tissues, some nanoscale drug delivery systems have been designed and employed to enhance their accumulation into tumor via enhanced permeability and retention (EPR) effects, resulting in increased antitumor efficacy and lower side effects [3–6]. Various nanoscale systems, including lipids (liposomes), polymeric nanoparticles, micelles and dendrimers, have been reported for cancer therapeutic applications [7–10]. However, the searching for drug delivery systems with high drug accumulation in tumor tissues and fast drug release in tumor cells still remains challenges.

Peptide dendrimers or dendrons composed of amino acids have many wonderful properties, such as well-defined architectures, highly-branched structures, high density of functional terminal groups, good biocompatibility, water solubility and resistance to proteolytic digestion [11–14]. These advantages promote those dendritic formulations as promising drug delivery vehicles [15,16]. However, dendrimer and dendron based vehicles less than 10 nm in size may be rapidly cleared by renal or through extravasation in the blood circulation [17,18]. Simultaneously, the high generation dendrimers (over 5 generations) may cause cytotoxicity [19,20]. It's notable that the PEGylated dendrimers or dendrons with low generation were designed, resulting in longer blood circulation time and fewer side effects [21–25]. Our previous studies showed that amphiphilic dendron-drug conjugates could self-assemble into nanoscale drug delivery vehicles with ideal sizes, and led to higher antitumor efficacy as well as low side effects [26–28].

In addition to the framework, the compositions of the dendritic vehicles are also of great importance to their performances as drug delivery vehicles [29,30]. Since the nanoscale vehicles are required to have long time lasting in blood circulation, high stability in circulation system, high accumulation in tumor tissues and selectively

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drug release features [6,7,31]. The environment-responsive polymer-drug conjugates have been prepared and employed as potential drug delivery vehicles for cancer therapy due to their high stability in blood circulation and selectively drug release in tumor tissues/cells [9,32–35]. The enzyme of cathepsin B as a lysosomal cysteine protease was found to be overexpressed in many tumor cells and tumor endothelial cells, such as the breast cancer cell line, providing the possibility of designing enzyme-responsive drug delivery vehicles [36–38]. The glycylphenylalanylleucylglycine tetra-peptide spacer (Gly-Phe-Leu-Gly, GFLG), as an appropriate substrate of cathepsin B, has been used to link antitumor drugs to polymers [28,39–41], and the polymer-drug conjugate therapeutics demonstrated good stability in plasma and serum during transportation and permitted intralysosomal drug release after endocytosis [41,42].

Previously, we prepared and characterized mPEGylated peptide dendrimer-doxorubicin conjugates as enzyme-responsive drug delivery vehicles, whereas the drug DOX was conjugated to the periphery of dendrimer via an enzyme-responsive tetra-peptide linker GFLG. The dendrimer-DOX conjugates can self-assemble into compact nanoparticles, and showed good biosafety and wonderful anti-cancer efficacy. However, for the polymer-DOX conjugates, including our designed peptide dendrimer-DOX conjugates [27] and other reported linear polymeric conjugates [43], the drug release under the intracellular environment was very slow due to the high steric hindrance [12,44], which may prevent their performance as efficient drug delivery vehicles, since the tumor intracellular drug concentration is a crucial factor of antitumor efficiency [45,46]. Based on above careful consideration, the question raised here was if optimization of the dendritic structures and compositions can increase the DOX release rate from the dendritic conjugates, and if the optimized conjugates can be suitable as nanoscale drug delivery vehicles with good biosafety as well as significant antitumor efficacy.

In this study, we aimed to prepare mPEGylated peptide dendron-GFLG-DOX conjugate as enzyme-sensitive drug delivery vehicle for breast tumor therapy. The synthesis route of conjugate was shown in Scheme 1-2, the DOX was conjugated to the mPEGylated peptide dendron using GFLG as an enzyme-sensitive linker via click reaction. Through DLS and TEM studies, the size, zeta potential and morphology of conjugate were observed. The enzyme-responsive drug release features of the conjugate were studied. The *in vitro* and *in vivo* anti-cancer efficacy of conjugate based drug delivery system was also evaluated. The biosafety was evidenced by a series of *in vivo* assays of normal mice. Meanwhile, *ex vivo* images were studied to reveal the details of these conjugates in tumor tissue and normal organs. That the mPEGylated peptide dendron-GFLG-DOX conjugate based nanoparticle could act as a promising drug delivery vehicle for breast cancer therapy.

2. Materials and methods

2.1. Materials and measurements

N,*N*-Diisopropylethylamine (DIPEA), 5-hexynoic acid, 1-hydroxybenzotriazole (HOBt), *N*,*N*,*N*′,*N*′-tetramethyl-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), 1-Hydroxy-7-azabenzotriazole (HOAT), 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), trifluoroacetic acid (TFA), methoxy poly(ethylene glycol) (mPEG, 2 kDa), sodium ascorbate, 4-azidobenzoic acid and 2-thiazolidinethione were purchased from Sigma–Aldrich and used without further purification.

Boc-L-Lys(Boc)-OH and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl) were purchased from GL Biochem (Shanghai) Ltd. Azido methoxy poly(ethylene glycol) (azido-mPEG) and N_3-GFLG-DOX were synthesized as previous reports [44]

Characterization and structural identification of dendritic intermediates and products were performed by ^{1}H NMR, electrospray ionization mass spectrometry (ESI MS, TSQ Quantum Ultra LC-MS/MS) and matrix assisted laser desorption ionization time-of-light (MALDI-TOF, Autoflex MALDI-TOF/TOF) mass spectrometry. Generally, 10 mg/mL 2, 5-dihydroxybenzoic acid (DHB) (water/acetonitrile = 2/1, 0.1% TFA) was mixed with 125 μL of a diammonium hydrogen citrate distilled aqueous solution (18 mg/mL) or 375 μL ethanol solution of 2, 5-dihydroxyacetophenon (DHAP) (20.2 mg/mL) and used as matrix solution for sample preparation for MALDI analysis. ^{1}H NMR data was obtained using a 400 MHz Bruker Advanced Spectrometer at room temperature, and chemical shifts were recorded in ppm on the δ scale. DLS and zeta potential measurements were performed in MilliQ water using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The *ex vivo* fluorescent images were obtained using Maestro *In-Vivo* Imaging System (Cri, USA).

2.2. Synthesis of materials

2.2.1. Synthesis of dendrimer G1L

Under a nitrogen atmosphere, Z-Lys(Z)-OH (14.5 *g*, 35 mmol) and *N*-Boc-ethylenediamine (7.9 *g*, 42 mmol) were dissolved in anhydrous DMF (100 mL). The solution was stirred for 5 min in ice bath, and DIPEA (29 mL, 175 mmol) was added. HBTU (16.1 *g*, 47 mmol) and HOBT (6.7 *g*, 47 mmol) were added to the solution. The solution was stirred in ice bath for 30 min and at room temperature for 24 h. After reaction, the solvents were removed, EtOAc (300 mL) was added and the organic solution was washed with $NaHCO_3$ aq. (satd.), HCl (1 M) and NaCl aq. The solution was dried ($MgSO_4$) and concentrated to 15 mL by rotary evaporation. The final product was obtained by recrystallization with EtOAc and ether, giving white solid (17.8 *g*) in 91.4% yield. ^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.34 (s, 10H, $NHCOOCH_2$Ph-H), 5.09 (s, 4H, OCH_2–Ph), 4.08 (m, 1H, CH(NHCbz)–CH_2), 3.33–3.19 (t, 6H, CH_2CH_2–NH), 1.25–1.83 (m, 6H, CH_2–Lys and s, 9H, CH_3–Boc). MALDI-TOF MS: *m*/*z* = 558.526 [$(M + H)^+$, $C_{29}H_{41}N_4O_7^+$].

2.2.2. Synthesis of G1L-G1L

Under a nitrogen atmosphere, product G1L (14.4 *g*, 26 mmol) was dissolved in anhydrous DCM (20 mL). The solution was stirred for 5 min in ice bath and TFA (20 mL, 260 mmol) was added. The solution was stirred for 4 h and the solvents were removed, then anhydrous ether was added, giving white solid. Under a nitrogen atmosphere, the white solid, Boc-Lys(Boc)-OH (10.9 *g*, 31 mmol), HBTU (12.1 *g*, 36 mmol) and HOBT (5.0 *g*, 36 mmol) were dissolved in anhydrous DMF (100 mL). DIPEA (22 mL, 130 mmol) was added by dropwise under ice bath. The solution was stirred in ice bath for 30 min, and at room temperature for 24 h. After reaction, the solvents were removed, EtOAc (300 mL) was added and the organic solution was washed with $NaHCO_3$ aq. (satd.), HCl (1 M) and NaCl aq. The solution was dried ($MgSO_4$) and concentrated to 15 mL by rotary evaporation. The final product was obtained by recrystallization from EtOAc and ether, giving white solid 17.7 *g*, 86.8% yield. ^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.34 (s, 10H, $NHCOOCH_2$Ph-H), 5.09 (s, 4H, OCH_2-Ph), 4.08 (m, 2H, CH(NHCbz)–CH_2), 3.33-3.19 (t, 8H, CH_2CH_2–NH), 1.25–1.83 (m, 12H, CH_2–Lys and s, 18H, CH_3-Boc). MALDI-TOF MS: *m*/*z* 785 [$(M + H)^+$, $C_{40}H_{61}N_6O_{10}^+$], 807 [$(M + Na)^+$, $C_{40}H_{60}N_6O_{10}Na^+$].

2.2.3. Synthesis of G1L-G2L

Under a nitrogen atmosphere, the Boc groups on the product G1L-G1L (10.6 *g*, 14 mmol) were deprotected as description in "*Synthesis of G1L-G1L*", giving white solid. Under a nitrogen atmosphere, the white solid, Boc-Lys(Boc)-OH (11.3 *g*, 32 mmol) HBTU (14.4 *g*, 38 mmol) and HOBT (5.2 *g*, 38 mmol) were dissolved in anhydrous DMF (100 mL). The DIPEA (23 mL, 135 mmol) was added by drops under ice bath. The solution was stirred in ice bath for 30 min, and at room temperature for 24 h. After reaction, solvent was removed; then, EtOAc (300 mL) was added and the organic solution was washed with $NaHCO_3$ aq. (satd.), HCl (1 M) and NaCl aq. The solution was dried ($MgSO_4$) and concentrated to 15 mL by rotary evaporation. The final product was obtained by recrystallization from EtOAc and ether, giving white solid 16.1 *g*, 92.4% yield. ^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.33 (s, 10H, $NHCOOCH_2$Ph-H), 5.08 (s, 4H, OCH_2–Ph), 4.08 (m, 4H, COCH(NH)–CH_2), 3.00–3.20 (t, 12H, CH_2CH_2–NH), 1.25–1.80 (m, 24H, CH_2–Lys and s, 36H, CH_3-Boc). MALDI-TOF MS: *m*/*z* 1264 [$(M + Na)^+$,$C_{62}H_{100}N_{10}O_{16}Na^+$].

2.2.4. Synthesis of G2L-G2L

Under a hydrogen atmosphere, product G1L-G2L (14.5 *g*, 12 mmol) was dissolved in methanol (60 mL, HPLC). Pd/C (6 *g*) dissolve in methol (HPLC) was added. Under 0.8 MPa hydrogen pressure, the solution was stirred at room temperature for 48 h. After reaction, Pd/C and the solvent were removed; then, the product was transferred into branch bottle. Under a nitrogen atmosphere, Z-Lys(Z)-OH (11.5 *g*, 28 mmol), HBTU (12.1 *g*, 32 mmol) and HOBT (4.4 *g*, 32 mmol) were added into that branch bottle and dissolved in anhydrous DMF (100 mL). The DIPEA (19 mL, 115 mmol) was added by drops under ice bath. The solution was stirred in ice bath for 30 min, and then at room temperature for 24 h. After reaction, solvent was removed; then, EtOAc (300 mL) was added and the organic solution was washed with $NaHCO_3$ aq. (satd.), HCl (1 M) and NaCl aq. The solution was dried ($MgSO_4$) and concentrated to 15 mL by rotary evaporation. The final product was obtained by recrystallization from EtOAc and ether, giving white solid 16.6 *g*, 78.2% yield. ^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.33 (s, 20H, $NHCOOCH_2$Ph-H), 5.06 (s, 8H, OCH_2-Ph), 4.08 (m, 6H, COCH(NH)–CH_2), 2.95–3.25 (t, 16H, CH_2CH_2–NH), 1.25–1.73 (m, 36H, CH_2-Lys and s, 36H, CH_3-Boc). MALDI-TOF MS: *m*/*z* 1788.591 [$(M + Na)^+$, $C_{90}H_{136}N_{14}O_{22}Na^+$].