PEGylated Dendrimer-Doxorubicin Conjugates as pH-Sensitive Drug Delivery Systems: Synthesis and In Vitro Characterization

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To achieve liver-specific delivery of antitumor drug doxorubicin (DOX), PEGylated dendrimer-DOX conjugates were designed and synthesized, whereas DOX was conjugated to dendrimers via hydrazone bonds and the dendrimers were functionalized with galactose moieties. The release rates of DOX from the conjugates at pH 5.0 were much faster than those at pH 7.4 due to the pH-sensitive cleavage of the hydrazone bonds. The conjugates were shown to effectively kill HepG2 cells in vitro. Compared to other conjugates, the PEGylated dendrimer-DOX one with multiple galactose moieties (Dendrimer-DOX-PEG-Gal) demonstrated HepG2 cells specificity, higher efficacy and good biosafety due to the lower IC50 value and higher cellular uptake confirmed by in vitro cytotoxicity assays, confocal laser scanning microscopy and flow cytometric studies. These results suggest that Dendrimer-DOX-PEG-Gal is an efficient and biocompatible candidate for the specific delivery of antitumor drug to HepG2 cells and could be used as liver cancer specific drug delivery system.

KEYWORDS: Dendrimer, Drug Delivery, PEGylation, Cancer Therapy, Cytotoxicity.

INTRODUCTION

Many anticancer chemotherapy drugs are effective for cancer treatment and tumor suppression. However, some of the challenges to chemotherapy are the non-specific cytotoxicity, poor water-solubility and unfavorable pharmacokinetics.1 Highly efficient and safe drug delivery vehicles may have potential to overcome these shortcomings. Among the emergent drug delivery platforms,2 polymeric technologies likely have the potential clinical impact for the foreseeable future.3 Currently, natural polymers and synthetic polymers such as dendritic polymers have been widely prepared as drug delivery vehicles for cancer therapy.4–7

Dendrimers are attractive drug delivery vehicles due to their structural features such as their precise and monodisperse size, low polydispersity, nanometer dimensions, modifiable surface functionality, water solubility and multivalency,8,9 resulting in advantages such as pharmacokinetic controls.10 Recently, polylsine dendrimers with attractive characteristics are under investigation as drug/gene delivery systems due to their structural flexibility, good biocompatibility,11,12 degradation and resistance to proteolytic digestion.11 The fate of injected drug delivery systems suitable for cancer therapy depends on their sizes, structure and surface characteristics.13–19 However, dendrimers based drug delivery vehicles with ideal antitumor properties have not been sufficiently achieved, since dendrimers with small sizes are rapidly cleared from circulation through extravasation or renal clearance.20

In order to increase the sizes of dendrimers based drug delivery systems and their biocompatibility, polyethylene glycol (PEG) has been used to modify the surface of dendrimers to form a new kind of dendritic structures.21–24 PEGylation can extend retention time in blood by decreasing non-specific interactions with endogenous components and macrophages. PEGylated dendrimer also enhances its drug loading and solubility, and increase the accumulation of drug delivery system in tumor tissue via the effect of enhanced permeability and retention (EPR).25,26 However, it’s not easy to prepare PEGylated dendrimers due to the steric hindrance to chemical reactions. The emergence of click chemistry has provided a powerful tool to address the challenges due to its high efficiency, quantitative yield.
and technical simplicity. It thus provides a better route for preparation of PEGylated dendrimers.27

On the other hand, drug delivery systems with covalently grafted receptor to their peripheries are capable of delivering the drug to specific sites, thus dramatically improving cytotoxicity in tumor cells.28,29 Galactose moieties, which can be recognized by asialoglycoprotein receptors (ASGP-R) on the surface of hepatocytes, have served as efficient liver cell targeting ligands for drug delivery and can be internalized via the receptor-mediated endocytosis.30 However, the interactions of isolated carbohydrates and proteins are typically weak.31 Recently, the gadolinium (Gd³⁺)-based dendrimers bearing multiple galactosyl moieties as magnetic resonance imaging (MRI) probes lead to a much-higher hepatocyte-cell uptake after intravenous injection, holding promises as liver targeting MRI probes.32–34 Glycopeptide dendrimers bearing multiple galactose moieties on a single dendritic scaffold showed excellent capacities for ASGP-R, termed as “cluster effect.”33 Although PEGylated dendritic polymers as drug vehicles shown high antitumor efficacy,21–24 the studies on PEGylated dendrimers with targeting moieties are very limited.20 One curious question here is if the PEGylated and DOX loaded dendrimer conjugates with multiple galactosyl moieties can be employed as liver targeting drug delivery systems by combining the features of PEG, dendrimer and galactosyl moieties.

In this study, we described the preparation and characterization of dendrimer based conjugates bearing DOX and galactosyl-PEG (PEG-Gal), and their potential as liver specific drug delivery systems. The PEG-Gal or PEG was attached to dendrimers via the click reaction; DOX was conjugated to the surface of dendrimers through pH-sensitive hydrazone bonds, resulting in galactose-poly(ethylene glycol)-dendrimer-doxorubicin conjugate (Dendrimer-DOX-PEG-Gal), amido-poly(ethylene glycol)-dendrimer-doxorubicin conjugate (Dendrimer-DOX-PEG-amino) and methoxy-poly(ethylene glycol)-dendrimer-doxorubicin conjugate (Dendrimer-DOX-mPEG), as shown in Schemes 1–2. The in vitro characteristics of conjugates as pH-sensitive drug delivery systems, such as drug release, toxicity and cellular uptake were evaluated. The results showed the great potential of Dendrimer-PEG-Gal as drug delivery vehicle for liver cancer therapy.

**MATERIALS AND METHODS**

**Materials**

N,N-Diisopropylethylamine (DIPEA), 1-hydroxybenzotriazole (HOBT), N,N,N’,N’-tetramethyl-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU), propargylamine, trifluoroacetic acid (TFA), poly(ethylene glycol) (PEG, 400) and methoxy-poly(ethylene glycol) (mPEG, 750) were purchased from Sigma-Aldrich and used without further purification. Boc-L-Lys(Cbz)-OH and Boc-L-Lys(Boc)-OH were purchased from GL Biochem.

![Dendrimer-DOX-mPEG Synthesis](image-url)
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(Shanghai) Ltd. Azido methoxy poly(ethylene glycol) (mPEG-N₃), 4-(N-tert-butoxycarbonyl-hydrazino)-4-oxo-butryric acid and tert-butyl-hydrazincarboxylate were synthesized according to the literatures.

**Methods**

Characterization and structural confirmation of dendritic intermediates and products were performed by ¹H NMR, electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS, Waters Q-TOF Premier) and matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. ¹H NMR data was obtained using a 400 MHz Bruker Advanced Spectrometer, and chemical shifts are reported in ppm on the δ scale. The content of DOX conjugated to the mPEGylated dendrimer was determined by measuring the absorbance with UV-vis spectrophotometry (Perkin-Elmer). The polydispersity (PD) of PEGylated dendrimers was measured using size-exclusion chromatography (SEC) on an ÅKTA/FPLC system (GE Healthcare) using GE Healthcare columns: Superoxer 12 10/300 with sodium acetate buffer containing 30% acetonitrile (pH 6.5) as mobile phase (flow rate: 0.4 mL/min). The released DOX was detected by a fluorescence detector (F700, HITACHI, Japan). The laser confocal images were captured on a LEICA TCS SP5.

**Synthesis of Compound 1**

4-(N-tert-butoxycarbonyl-hydrazino)-4-oxo-butryric acid (2.32 g, 10 mmol), HOBt (1.35 g, 10 mmol) and HBTU (830 mg, 2.2 mmol), HOBt (300 mg, 2.2 mmol) and DIPEA (1.41 g, 11 mmol) were added and solution was stirred under nitrogen in an ice bath for 30 min. The reaction mixture was allowed to warm to room temperature and stirred for another 24 h. The solvent was removed by rotary evaporation and the mixture purified by column chromatography (silica, CH₂Cl₂: MeOH, 15:1) to give the product 840 mg in 90% yield. ¹H NMR (400 MHz, CDCl₃): δ = 1.11–1.86 (m, 15 H, CH₃-Lys and CH₂-Boc), 2.40–2.72 (m, 8 H, COCH₂CH₂CO), 3.22 (m, 3 H, COCH(R)CH₂CH₂ and NCH₂CH₂), 3.72 (s, 3 H, OCH₃), 4.0 (m, 2 H, CHC(CH₂)₂NHCO), 4.53 (s, 1 H, COCH(R)). ESI-TOF MS: m/z 534.26 [(M+Na)⁺, C₂₂H₁₅N₃O₈Na⁺].

The product (1.0 g, 1.96 mmol) above was dissolved in 20 mL MeOH, 1 M NaOH (10 mL) was added under ice bath for 30 min. The reaction mixture was allowed to warm to room temperature and stirred for another 8 h. The solvent was removed by rotary evaporation and the mixture was added 1 M HCl until the pH is about 3. The solvents were extracted with EtOAc (50 mL × 3). The combined organic solvents were dried over Na₂SO₄, and evaporated to give the product 820 mg in 85% yield. ¹H NMR (400 MHz, CD₃OD): δ = 1.30–1.89 (m, 15 H, CH₃-Lys and CH₂-Boc), 2.45–2.67 (m, 8 H, CH₂CH₂CO), 3.17 (m, 3 H, COCH(R)CH₂CH₂CH₂ and NCH₂CH₂), 3.93 (s, 2 H, NCH₂CH₂), 4.34 (m, 1 H, COCH(R)). ESI-TOF MS: m/z 520.24 [(M+Na)⁺, C₂₂H₁₅N₃O₈Na⁺].

**Synthesis of Compound 3**

A solution of NaHCO₃ (pH 8.5, 0.8 mL) followed by 1.7 mL H₂O was added to a solution of tert-butyl bromoacetate (200 mg, 1.03 mmol) and 2,3,4,6-tetra-O-acetyl-b-D-thiogalactopyranose (364 mg, 1 mmol) in DMF (4 mL). The solvents were stirred at room temperature for 3 h and diluted with EtOAc. The mixture was washed successively with saturated aqueous NaHCO₃ and brine. The organic layer was dried over MgSO₄ and concentrated in vacuum to give a residue which was purified by flash-column chromatography (petroleum ether: EtOAc, 1:1) to afford galactosylthiohexoic acid tert-butyl ester 410 mg in 83% yield as a white amorphous solid. ¹H NMR (400 MHz, CDCl₃): δ = 1.21–1.87 (m, 18 H, OC(CH₃)₃), 1.98 (s, 3 H, COCH₃), 1.95–2.75 (m, 3 H, COCH₂CH₂CO), 3.15 (s, 3 H, COCH₂CH₂), 3.72 (s, 3 H, OCH₃), 4.34 (m, 1 H, COCH(R)). ESI-TOF MS: m/z 520.24 [(M+Na)⁺, C₂₂H₁₅N₃O₈Na⁺].

**Synthesis of Compound 5**

Compound 3 (480 mg, 1 mmol) was first dissolved in anhydrous dichloromethane (2 mL). Trifluoroacetic acid (TFA, 2 mL) was then added. The mixture was stirred for...
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30 min under nitrogen. After the solvents were removed by rotary evaporation, the sample was dried under high vacuum for 1 h. Then the sample, HBTU (380 mg, 1 mmol) and HOBt (135 mg, 1 mmol) were dissolved in anhydrous DMF (5 mL) under nitrogen. DIPEA (650 mg, 5 mmol) was added and the solution was stirred for 30 min in ice bath, and NH2-PEG(400)-N3 (400 mg, 0.94 mmol) was then added. The solution was stirred under nitrogen in ice bath for 30 min and at room temperature for another 24 h. After work-up, the solvent was removed. This crude product was purified by column chromatography (silica, CH2Cl2; CH3OH, 20:1) to give colourless amorphous solid 600 mg in 75% yield. 1H NMR (400 MHz, CDCl3): δ = 3.14–3.19 (t, OCH2CH2N2), 3.36–3.40 (t, OCH2CH2NH2), 3.5–3.8 (m, OCH2CH2O), 3.85–3.95 (t, OCH2CH2NH2).

Synthesis of 4-(N-propynylamine)-4-Ox0-Butyric Acid

Succinic (6.0 g, 60 mmol) and propynylamine (7.93 g, 60 mmol) were dissolved in 100 mL DMF. Triethylamine (60 mL, 430 mmol) was dropped to the reaction mixture. The solvents were warmed up to 40 °C and stirred for 8 h. The solvents were removed by rotary evaporation and the mixture was purified by recrystallisation in EtOAc/petroleum ether to give the product 7.0 g in 75.2% yield. 1H NMR (400 MHz, DMSO): δ = 2.30–2.43 (m, 4 H, COCH2CH2CO), 3.08 (t, 1 H, NHCH2CH2CH2), 3.83 (m, 2 H, NHCH2CH2CH2), 8.31 (s, 1 H, NHCO), 12.07 (s, COOH). ESI-TOF MS: m/z 178.0509 [(M + Na)+, C6H13NO3 Na].
mixture was allowed to warm to room temperature and stirred for another 24 h. The solvents were removed by rotary evaporation. The mixture was dissolved in 100 mL EtOAc and washed successively with saturated aqueous NaHCO$_3$, 1 M HCl and brine. The organic solvents were dried over MgSO$_4$ and concentrated, giving the product 310 mg in 81.6% yield as white solid. $^1$H NMR (400 MHz, DMSO): $\delta = 1.11–1.68$ (m, CH$_2$-Lys and CH$_2$-Boc), 2.20–2.40 (m, COCH$_2$CH$_3$CO), 2.89–3.17 (m, CH$_2$NH-Lys and NHCH$_2$CCH), 3.8 (m, COCH(R)NH and NHCH$_2$CCH), 4.99 (s, CH$_2$C$_6$H$_5$), 6.70 (s, NHCO), 6.90 (s, NHCO), 7.60–8.31 (m, NHCO). MALDI-TOF MS: $m/z$ 2743.75 [(M + Na)$^+$]. $C_{152}H_{216}N_{28}O_{33}$Na$^+$. 

**Synthesis of Dendrimer 4**

Dendrimer 3 (0.56 g, 0.21 mmol) was first dissolved in anhydrous dichloromethane (4 mL). 4 mL TFA was then added, and the solution was stirred for 30 min under nitrogen. After the solvents were removed by rotary evaporation, diethyl ether was added and precipitates appeared. The precipitate was collected by centrifugation and washed 3 times with anhydrous diethyl ether. The sample was dried under high vacuum for 1 h and 15 mL DMF was added followed by DIPEA (0.80 g, 6 mmol), 4-(N′-tert-butoxycarbonyl-hydrazino)-4-oxo-butyric acid (0.94 g, 1.89 mmol), HBTU (0.72 g, 1.89 mmol) and HOBt (0.24 g, 1.8 mmol) were then added simultaneously and the solution was stirred under nitrogen in ice bath for 30 min and another 24 h at room temperature. The solvents were removed by rotary evaporation and the mixture was dissolved in EtOAc (20 mL), giving the product (0.73 g) in 71.6% yield. $^1$H NMR (400 MHz, DMSO): $\delta = 1.11–1.72$ (m, CH$_2$-Lys and CH$_2$-Boc), 2.20–2.45 (m, COCH$_2$CH$_3$CO), 2.98 (s, CH$_2$NH-Lys), 3.03 (m, CH$_2$NH-Lys), 3.43–3.63 (m, OCH$_2$CH$_2$O), 3.84 (s, CH$_3$COCH$_2$NH), 4.03–4.13 (m, COCH(R)NH), 4.33 (s, OCH), 4.39 (s, OCH), 5.01–5.17 (m, CH$_2$COCH$_2$CH), 5.39 (s, CH$_3$), 7.85 (s, CH$_3$CCH$_2$NH).

In the MALDI-TOF mass spectrum of Dendrimer 5, the most abundant peak was observed at 12400 (Fig. 1(a)), indicating that the about 9 galactosyl-PEG(400)-N$_3$ had been attached to the dendrimer. K$_2$CO$_3$ (100 mg, (EDTA-Na$_2$) aqueous solution using dialysis membrane (molecular weight cut-off (MwCO) = 3500). The retenate was lyophilized to give a yellow solid 220 mg in 73.9% yield. $^1$H NMR (400 MHz, D$_2$O): $\delta = 1.05–1.78$ (m, CH$_2$-Lys), 1.91 (s, COCH$_3$), 1.99 (s, COCH$_3$), 2.02 (s, COCH$_3$), 2.11 (s, COCH$_3$), 2.42 (s, COCH$_3$CH$_2$CO), 3.03 (m, CH$_2$NH-Lys), 3.43–3.63 (m, OCH$_2$CH$_2$O), 3.84 (s, CH$_3$COCH$_2$NH), 4.03–4.13 (m, COCH(R)NH), 4.33 (s, OCH), 4.39 (s, OCH), 5.01–5.17 (m, CH$_2$COCH$_2$CH), 5.39 (s, CH$_3$), 7.85 (s, CH$_3$CCH$_2$NH).

**Synthesis of Dendrimer 5**

Dendrimer 4 (0.1 g, 20 $\mu$mol) was first dissolved in anhydrous dichloromethane 2 mL, TFA 2 mL was added and the solution was stirred under nitrogen for 30 min. The solvents were removed from the solution by rotary evaporation and the sample was then dried under high vacuum for 1 h. Diethyl ether was added and a precipitate appeared. The precipitate was collected by centrifugation and washed 3 times with anhydrous diethyl ether. The sample was dried under high vacuum for 1 h. The sample and galactosyl-PEG(400)-N$_3$ (238 mg, 0.29 mmol) were dissolved in DMSO-H$_2$O (4:1, 8 mL) in the presence of 5 mol% CuSO$_4$·5H$_2$O with 10 mol% sodium ascorbate, the solution was stirred under nitrogen at room temperature for 24 h. The solution was dialyzed exhaustively with 1 $\mu$M ethylenediaminetetra-acetic acid disodium salt (EDTA-Na$_2$) aqueous solution using dialysis membrane (molecular weight cut-off (MwCO) = 3500). The retenate was lyophilized to give a yellow solid 220 mg in 73.9% yield. $^1$H NMR (400 MHz, D$_2$O): $\delta = 1.05–1.78$ (m, CH$_2$-Lys), 1.91 (s, COCH$_3$), 1.99 (s, COCH$_3$), 2.02 (s, COCH$_3$), 2.11 (s, COCH$_3$), 2.42 (s, COCH$_3$CH$_2$CO), 3.03 (m, CH$_2$NH-Lys), 3.43–3.63 (m, OCH$_2$CH$_2$O), 3.84 (s, CH$_3$COCH$_2$NH), 4.03–4.13 (m, COCH(R)NH), 4.33 (s, OCH), 4.39 (s, OCH), 5.01–5.17 (m, CH$_2$COCH$_2$CH), 5.39 (s, CH$_3$), 7.85 (s, CH$_3$CCH$_2$NH).

**Figure 1.** MALDI TOF mass spectrum (a) and $^1$H NMR spectrum (b) of protected dendrimers and its deprotected one’s $^1$H NMR spectrum (Dendrimer 5, (c)).
0.72 mmol) was added to a stirred solution of the product (200 mg, 15.7 μmol) in 5 mL methanol/H2O, the reaction was carried out at room temperature for 2 h. Then the solution was dialyzed exhaustively with MilliQ water using dialysis membrane (MW cut-off, 3500 Da). The retentate was lyophilized to give a yellow solid in 75.1% yield (130 mg, 13.5 μmol). 1H NMR (400 MHz, D2O): δ = 1.10–1.84 (m, CH2-Lys), 2.45 (s, COCH2CH2CO), 3.05 (s, CH2NH-Lys), 3.43–3.75 (m, OCH2CH2O), 3.87 (s, CH=CCH2NH), 4.07–4.19 (m, COCH2(R)NH), 4.36 (s, OCH), 4.52 (s, OCH), 7.89 (s, CH=CCH2NH).

Synthesis of Dendrimer 6
Dendrimer 4 (0.1 g, 20 μmol) was first dissolved in anhydrous dichloromethane 2 mL, TFA 2 mL was added, and the solution was stirred under nitrogen for 30 min. The solvents were removed from the solution by rotary evaporation and the sample was then dried under high vacuum for 1 h. Diethyl ether was added and a precipitate appeared. The precipitate was collected by centrifugation and washed 3 times with anhydrous diethyl ether. The precipitate was collected by centrifugation and the sample was then dried under high vacuum for 1 h. The sample was dried under high vacuum for 1 h. The sample and NH2-PEG(400)-N3 (122 mg, 0.29 mmol) were dissolved in DMSO-H2O (4:1, 8 mL) in the presence of 5 mol% CuSO4·5H2O with 10 mol% sodium ascorbate, the solution was stirred under nitrogen at room temperature for 24 h. The solution was dialyzed exhaustively with 1 mM EDTA-Na2 aqueous solution using dialysis membrane (MwCO = 3500). The retentate was lyophilized to give a yellow solid 0.35 g in 81.5% yield. 1H NMR (400 MHz, DMSO): δ = 1.11–1.71 (m, CH2-Lys and CH2-Boc), 2.20–2.40 (m, COCH2CH2CO), 2.98 (s, CH2NH-Lys), 3.06 (s, NHCH2CH2), 3.83 (m, COCH2(R)NH and NHCH2CH2), 4.13 (s, COCH2(R)NH), 6.70 (s, NHCO), 6.90 (s, NHCO), 7.62–7.9 (m, NHCO). MALDI-TOF MS: m/z 3405.35 [(M + H)+, C156H252N40O45H+].

Synthesis of Dendrimer 8
Dendrimer 7 (0.2 g, 59 μmol) was first dissolved in anhydrous dichloromethane 2 mL, trifluoroacetic acid 2 mL was added and the solution was stirred under nitrogen for 30 min. The solvents were removed by rotary evaporation and the sample was then dried under high vacuum for 1 h. Diethyl ether was added and a precipitate appeared. The precipitate was collected by centrifugation and washed 3 times with anhydrous diethyl ether. The sample was dried under high vacuum for 1 h. The sample and m-PEG(750)-N3 (0.29 g, 0.39 mmol) were dissolved in DMSO-H2O (4:1, 8 mL) in the presence of 5 mol% CuSO4·5H2O with 10 mol% sodium ascorbate, the solution was stirred under nitrogen at room temperature for 24 h. The solution was dialyzed exhaustively with 1 mM EDTA-Na2 aqueous solution using dialysis membrane (MwCO = 3500). The retentate was lyophilized to give a yellow solid 0.35 g in 81.5% yield. 1H NMR (400 MHz, D2O): δ = 1.11–1.95 (m, CH2-Lys), 2.32–2.68 (m, COCH2CH2CO), 3.01–3.15 (m, CH2NH-Lys), 3.43–3.70 (m, OCH2CH2O), 3.89 (s, CH=CCH2NH), 4.0–4.53 (m, COCH2(R)NH), 7.91 (s, CH=CCH2NH). In the MALDI-TOF mass spectrum of Dendrimer 8, the most abundant peak was observed at 5600, indicating that the about 4 m-PEG(750)-N3 had been attached to the dendrimer.

Synthesis of PEGylated Dendrimer-DOX Conjugates
Dendrimer-PEG-amino (100 mg, 11.6 μmol) was dissolved in 100 mL methanol, a drop of TFA was added as a catalyst and DOX·HCl (48 mg, 83 μmol) was added. The reaction mixture was stirred overnight at room temperature. The solvent was removed by rotary evaporation and the mixture was purified by size exclusion chromatography using sephadex LH-20 resin and H2O as the mobile phase. The water was lyophilized to get red solid 80 mg. The content of DOX conjugated to the PEGylated dendrimers was determined by measuring the absorbance at 485 nm with UV-vis spectrophotometry, giving 7.5% (Wt%) and 1.2 DOX per conjugate. Dendrimer-DOX-PEG-Gal was synthesized by the same procedure as Dendrimer-DOX-PEG-amino. The water was lyophilized to get red solid 83 mg. The content of DOX conjugated to the PEGylated dendrimers was determined by measuring the absorbance at 485 nm with UV-vis spectrophotometry, giving 7.7% (Wt%) and about 1.5 DOX per conjugate.
Dendrimer-DOX-mPEG was synthesized by the same procedure as Dendrimer-DOX-PEG-amino. The water was lyophilized to get red solid 78 mg. The content of DOX conjugated to the PEGylated dendrimers was determined by measuring the absorbance at 485 nm with UV-vis spectrophotometry, giving 11.0% (Wt%) and about 1.2 DOX per conjugate.

In Vitro Release of DOX from Conjugates
The release studies were performed at 37 °C in the phosphate buffer with different pH values (pH 5.0 and 7.4). Dendrimer-DOX-mPEG, Dendrimer-DOX-PEG-amino and Dendrimer-DOX-PEG-Gal conjugates (0.4 mg) were dissolved in 2 mL PBS and were placed in dialysis bags with a molecular weight cutoff of 3500 Da. The dialysis bags were then immersed in 30 mL of phosphate buffered saline solution with different pH values and kept in a horizontal shaker maintained at 37 °C for 170 rpm. 5 mL of the medium was removed periodically, and the same volume of fresh PBS was added. The released DOX was detected by a fluorescence detector with excitation wavelength at 480 nm and emission wavelength at 550 nm.

In Vitro Cytotoxicity Assays
HepG2 cells obtained from the Shanghai Institute for Biological Science (Shanghai, China) were used for the cytotoxicity study. HepG2 cells were cultured in DMEM containing penicillin 100 U/mL, streptomycin 100 μg/mL, and fetal bovine serum 10% at 37 °C and 5% CO2. The cells were harvested with 0.02% EDTA and 0.025% trypsin and rinsed. The resulted cell suspension was used in the following experiments. HepG2 cells were cultured on 96-well plates at a density of 5000 cells/well. The cells were incubated for 24 h to allow for attachment to the culture vessel. The medium was DMEM and 1.5 ng/mL to 30 μg/mL doxorubicin equivalents (as DOX, dendrimer-DOX conjugates) or medium alone added to each of wells. Cells were incubated with doxorubicin for 2 days before being assayed for viability via cell counting kit-8 (CCK-8; Dojindo laboratories, Kumamoto, Japan) assay as described. Cytotoxicity tests of the complexes were carried out by determining the percentage of cell viability using CCK-8 assay. After incubation for 48 h, 10 μL CCK-8 was added to each well and the plates were incubated at 37 °C for another 4 h. Absorbance was measured at wavelength of 490 nm and a reference wavelength of 630 nm using a microplate reader 550 (Bio-Red).

Cytotoxicity of Dendrimer-mPEG, Dendrimer-PEG-amino and Dendrimer-PEG-Gal conjugates was evaluated at concentrations from 100 to 400 μg/mL for 48 h at 37 °C. After incubation for 48 h, 10 μL CCK-8 was added to each well and the plates were incubated at 37 °C for another 4 h. Absorbance was measured at wavelength of 450 nm and a reference wavelength of 650 nm using a microplate reader 550 (Bio-Red).

Intracellular Drug Release
For confocal laser scanning microscopy (CLSM), HepG2 cells (5 × 10^4) were seeded on 22 mm glass coverslips in a six-well plate on the day before the experiment. The cells were treated with DOX, Dendrimer-DOX-mPEG and Dendrimer-DOX-PEG-Gal (those three with the same DOX concentration: 5 μg/mL) for 3 h, washed with PBS three times and watched under confocal laser scanning microscopy. Cell images were taken by CLSM (excitation/emission wavelengths: 488 and 510 nm). The laser confocal images were captured on a LEICA TCS SP5 using a 40× water-immersion objective lens and processed by Leica Confocal Software. DOX was excited at 488 nm with emissions at 595 nm.

Flow Cytometric Assay
HepG2 cells were seeded in 6 well plates at 5 × 10^4 cells per well in 1 mL of complete DMEM medium and incubated at 37 °C in 5% CO2 humidified atmosphere for 24 h. The medium was replaced with fresh medium containing free DOX, Dendrimer-DOX-mPEG and Dendrimer-DOX-PEG-Gal conjugates with equivalent doxorubicin at a concentration of 5 μg/mL. The cells were further incubated for specific periods of times, and washed with PBS twice, harvested, and suspended in 1 mL of PBS for analyses using flow cytometer. Cell with PBS treatment were used as control. Files were collected of 20,000 events and analyzed by Flowjo to generate each histogram.

RESULTS AND DISCUSSION
Dendrimer carriers for drug delivery have been already proven to be a valuable alternative to other vehicles. In our previous studies, peptide dendrons/dendrimers have been used as drug/gene delivery vehicles and magnetic resonance imaging probes taking advantage of dendrimer’s multivalency. Factors such as molecular size, chemical architecture, surface charge, structural flexibility and functional patterns should be taken into consideration during the design and synthesis of dendrimers. However, dendrimer based drug delivery system can be easily cleaned up from the body due to the small size. Although high generation dendrimers showed greater sizes, they result in toxicity in vitro and in vivo, and are also associated with synthesis difficulties. Recently, PEGylation provides a possibility to prepare dendritic biomaterials due to the features of PEG, such as excellent solubility in water, high flexibility, low protein absorption, lack of immuno-gencity and toxicity. PEGylated dendrimers not only drastically augment hydrophobic drug loading and their kinetic stability, but also eliminate the naked dendrimer scaffold drawbacks of hemolytic toxicity, uncontrolled drug outflow, macrophageal uptake and short half-life. In our previous study, the MPEG modified dendrimers as drug delivery systems and MRI probes showed much longer blood circulation, which was benefit for its use.
as an antitumor drug delivery vehicle. Thus, in this work asymmetric and amphiphilic peptide dendrimers with different functional groups at the surface were synthesized to provide alternatives to the classical dendrimer-based drug carriers, as shown in Scheme 1–2. To the best of our knowledge, these new dendrimers have not previously been reported.

However, it’s nontrivial to conjugate PEG moieties to dendrimers, because their high molecular weights tend to lead to relatively large stereospecific blockade. In an alternative way, the tail of PEG was functionalized with one azido group. The dendrimer was functionalized with an alkylnyl group. The azido-PEG was covalently attached to the dendrimer via Cu(I)-catalyzed azide–alkyne cyclo-addition (CuAAC) click chemistry due to highly specific and quantitative yield, resulting in the final water–soluble PEGylated dendrimer. It should be noticed that the product was washed and dialysed with EDTA solution to fully remove trace amounts of copper, because the chelated copper may cause unwanted toxicity. For $^1$H NMR spectrophotometry, the presence of the triazole proton of 7.85–8.06 ppm indicated the formation of the 1,4-regioisomer exclusively (Figs. 1(b)–(c), 2(b), 3(b)), indicating that PEG moieties were attached to one dendritic fraction. The yield was about 80% due to the high efficiency of click chemistry.

Major challenges in the field of dendritic drug delivery systems include the synthesis of functional structures for targeting/specific systems. In order to synthesize liver tumor targeting/specific dendritic drug delivery system, the D-thioglycosyl derivative as a liver-specific ligand was conjugated to PEGylated dendrimer, because dendrimers containing galactose can be recognized by the galactose particle receptor on Kupffer cells in vivo. The ligand was modular and it is comprised of three units: a potential biological functional group (galactosyl) and azido group appended at the end of a short PEG chain, where PEG promotes water solubility and azido group provides conjugation onto dendrimers. These PEG derivatives not only increase the stability of dendrimer, but also reduce nonspecific adsorption. The ligand will also promote water solubility and biocompatibility of dendrimer. The galactosyl moieties was conjugated to dendrimer through flexible PEG linkage, which was expected to enhance the water solubility of dendrimer drug conjugate and extend the length of the ligand and spread outside the dendrimer, in order to enhance targeting efficiency.

Characterization of multifunctional dendrimers, however, still remains a great challenge due to the complicated architectures. Therefore, $^1$H NMR, ESI-TOF MS, MALDI-TOF MS and SEM were used to determine the degree of dendrimer surface decoration, as shown in Table I. Mass spectrometry confirmed that each generation of synthesized dendrimers had the expected molecular weights. The ESI-TOF MS spectra of Dendrimer 1 and 2 showed peaks corresponding only to the fully substituted materials.

The result of MALDI-TOF MS for Dendrimer 7 revealed the detection of the desired number of 6 alkylene groups at the surface. For Dendrimer 7 ($M = 3405.35$), the most abundant peak ($m/z = 3405.9175$) was assigned as a hydroxide adduct [M+H]+. In the MALDI-TOF MS spectrum of Dendrimer 4 ($M = 4996.72$), the most abundant peak was observed at $m/z = 5020.84$, which corresponds to the [M+Na]+ signal of Dendrimer 4, indicating that the designed G2.5 peptide dendrimer with 12 alkylene groups at the surface was successfully synthesized. For Dendrimer 5 before deprotection, the most abundant peak was observed at $m/z = 12400$ (Fig. 1(a)), suggesting 9 galactosyl-PEG(400)-N$_2$ was attached to the dendrimer. In addition, once the protected groups of galactosyl moieties were depoorted, the peaks of acetyl group (1.85–2.18 ppm, 4 single peaks, Fig. 1(b)) in $^1$H NMR spectrophotometry of Dendrimer 5 were observed and disappeared after deprotection (Fig. 1(c)). In addition, the result of MALDI-TOF MS for Dendrimer 6 and 8 indicated 9 and 4 PEG moieties were attached to the designed dendrimers (Figs. 2(a) and (b)), respectively. These results indicated that the average numbers of PEG moieties conjugated to a single dendritic molecular were less than the designed ones. The possible reason could be the high steric hindrance to chemical reaction, despite excess of PEG moieties was used in the reaction.

The antitumor drug DOX was covalently linked to PEGylated dendrimers via hydrazone bond. To further estimate the conjugation efficiency and the number of DOX on each dendrimer, UV-vis spectrophotometry analysis was employed to determine the DOX conjugation, the results showed dendrimer-DOX conjugation (denoted as Dendrimer-DOX-nPEG, Dendrimer-DOX-PEG-amino and Dendrimer-DOX-PEG-Gal), corresponding to drug contents of 11.0%, 7.5%, and 7.7% respectively, approximately a DOX attached to each dendrimer molecule, far less than the theoretical ones due to the high steric hindrance to chemical reaction. Additionally, the low yields may be related to the hydrazone reaction which is a dynamic covalent chemistry reaction.
She et al. PEGylated Dendrimer-DOX Conjugates as pH-Sensitive Drug Delivery Systems: Synthesis and In Vitro Characterization

The characterization of drug release from carriers, especially in the intracellular environment, should be taken into consideration when designing drug delivery systems. For a drug delivery system with long blood circulation, high accumulation in tumor tissue and high antitumor efficacy, high stability in body circulatory system and fast release in tumor cell are required. To quantitatively determine release from the dendrimer based DOX conjugates, the dendrimer-DOX conjugates were dissolved in PBS at pH 7.4 (corresponding to the pH of blood) and PBS at pH 5.0 (corresponding to the pH of endosome) at 37°C, respectively. The amount of released doxorubicin at different predetermined time points was measured by fluorescence detector with excitation wavelength at 480 nm and emission wavelength at 550 nm. For the release of doxorubicin from Dendrimer-DOX-mPEG, as shown in Figure 4, approximately 80% of drug was released after 24 h and approximately 90% after 56 h at pH 5.0. In contrast, only 20% of drug was released after 56 h at pH 7.4. Very similar DOX release profiles were observed for Dendrimer-DOX-PEG-amino and Dendrimer-DOX-PEG-Gal. The results indicate that hydrazine linkers provided good stability over several days at the physiological pH of 7.4, whereas the cleavage of hydrazine linkers accelerated the release of the drug at lower pH values. The in vitro release results suggested the stability of dendrimer-DOX conjugates in circulation system (pH 7.4) and their ability to release DOX in the acidic endosomes and/or lysosomes where the pH range was 4.0–6.0, indicating that the prepared dendrimers would be used as pH-responsive drug delivery system.

In vitro cytotoxicity of the dendrimer-DOX conjugates was estimated in HepG2 cells using CCK–8 assay with various equivalent DOX concentrations from 1.5 ng/mL to 30.0 μg/mL for 48 h, compared with free DOX. As shown in Figure 5(a), the IC_{50} (inhibitory concentration to produce 50% cell death) of free DOX was 0.13 μg DOX equiv/mL. The IC_{50} values of Dendrimer-DOX-mPEG, Dendrimer-DOX-PEG-amino and Dendrimer-DOX-PEG-Gal conjugates were 1.58 μg/mL, 0.56 μg/mL and 0.95 μg/mL, respectively. The IC_{50} results suggested that these dendrimer-DOX conjugates based on PEGylated polylysine dendrimers exhibited less toxic effects than free DOX on HepG2 cells, because free DOX as small molecule can rapidly cross the cell membranes and preferentially concentrate within the cellular nuclei where it exerts its cytotoxic actions via DNA intercalation and inhibition of topoisomerase. Release of DOX from the dendrimer-DOX conjugates is slow in cells and PEGylated carriers are poorly internalized into cell.

In order to examine the cytotoxicity of drug-free dendrimers, the HepG2 cells were incubated with Dendrimer-mPEG, Dendrimer-PEG-amino and Dendrimer-PEG-Gal at various concentrations (100, 200 and 400 μg/mL) for 48 h. The cell viability was determined by CCK-8 assay.
Figure 4. *In vitro* drug releases from PEGylated dendrimer-DOX conjugates at pH 7.4 and 5.0 (n = 3). (a) Dendrimer-DOX-mPEG; (b) Dendrimer-DOX-PEG-amino; (c) Dendrimer-DOX-PEG-Gal. Values represent average ± s.d. (n = 3).

As shown in Figure 5(b), the cell viability of Dendrimer-PEG-Gal was above 98% across all the tested concentration range. The cell viability of Dendrimer-mPEG was above 94% at all tested concentrations. In contrast, the cell viability of Dendrimer-PEG-amino was about 38% at 400 μg/mL concentration. These results suggested that dendrimer-DOX conjugates were less toxic than the free drug, but more toxic than PEGylated polyllysine dendrimers alone. The observed *in vitro* cytotoxicities of the dendrimer-DOX conjugates were likely due to the drug released from the dendrimer-DOX conjugates in the course of the incubation. The *in vitro* cytotoxicity results

Figure 5. *In vitro* efficacy. (a) HepG2 cells upon incubation with free doxorubicin and with Dendrimer-DOX Conjugates (doxorubicin-equivalent concentrations). (b) The cytotoxicity of drug-free dendrimer (Dendrimer-mPEG, Dendrimer-PEG-amino, and Dendrimer-PEG-Gal). The concentrations (100, 200 and 400 μg/mL) of drug-free dendrimer were calculated. (c) The cytotoxicity of Dendrimer-DOX-mPEG, Dendrimer-DOX-PEG-Gal and DOX at the normalized DOX concentration (5 μg/mL) after 3 h of incubation. Values represent average ± s.d. (n = 5).
also indicated that DOX could release from the dendrimer based conjugates in the acidic environment of endosomes. Another observed phenomenon was that the cytotoxicity of Dendrimer-PEG-amino was more toxic than Dendrimer-mPEG and Dendrimer-PEG-Gal, which could be attributed to the cytotoxicity caused by the amino group on the PEG, because dendrimers with cationic surfaces generally showed cytotoxic activity. The cationic surface groups (e.g., primary amines) can disrupt cell membranes through initial adhesion by electrostatic attraction to the negative cell surface groups, followed by either hole formation or endocytosis. The formation of holes and channels in the cell wall will cause the cells to lyse. Therefore, although the Dendrimer-DOX-PEG-Gal showed a slightly higher IC50 than that of Dendrimer-DOX-amino, it’s a good candidate for drug delivery for liver therapy due to the specificity to liver tumor cells and the much lower cytotoxicity of drug-free carriers.

In addition, further cytotoxicity studies were performed to determine whether or not the high DOX concentration of the investigated conjugates could significantly influence the cell viability and then influence the results of the intracellular uptake studies. Considering the observed cytotoxicity of the drug free Dendrimer-PEG-amino has been examined, only Dendrimer-DOX-mPEG, Dendrimer-DOX-PEG-Gal and DOX were tested. As shown in Figure 5(c), after 3 h of incubation, the cell viability of all tested samples was higher than 90%, indicating no noticeable cytotoxicity was observed towards HepG2 and HEK 293 at the normalized DOX concentration of 5 µg/mL. The results demonstrated no significant influence could be caused by the tested DOX concentration in the intracellular uptake studies, since 3 h of incubation did not result in obvious cytotoxicity due to the short time interaction with cells.

To confirm intracellular drug release and uptake by cells, DOX fluorescence in HepG2 cells was followed by confocal laser scanning microscopy (CLSM). According to the in vitro cytotoxicity studies before, Dendrimer-DOX-mPEG, Dendrimer-DOX-PEG-Gal and DOX were investigated by CLSM. Fluorescence was observed on HepG2 cells after 3 h of incubation as shown in Figure 6. However, the observed weak fluorescence at the nuclei and perinuclei regions suggested that a small amount of molecular free drug was taken up by cells and entered nuclei and perinuclei. In contrast, significant fluorescence was observed at perinuclei of HepG2 cells after 3 h of incubation with Dendrimer-DOX-PEG-amino and Dendrimer-DOX-PEG-Gal conjugates, suggesting higher cell uptake than Dendrimer-DOX-mPEG. However, little fluorescence was observed in cell nucleus with Dendrimer-DOX-PEG-amino and Dendrimer-DOX-PEG-Gal conjugates, due to the slow release of DOX from the dendrimer-DOX conjugates at the time of evaluation. In addition, Dendrimer-DOX-mPEG resulted in much less detectable fluorescence. Modification of dendrimers with cell specific moieties is one of the strategies to improve efficiency and enhance specific cellular uptake via receptor mediated endocytosis. Therein, the Dendrimer-DOX-PEG-Gal with galactosyl moieties could recognize asialoglycoprotein receptors (ASGP-R) on HepG2 cells effectively and led to a higher uptake. So, the galactosyl functionalized dendrimer conjugates may be utilized as specific drug delivery system for liver tumor.

For further characterization of dendrimer-DOX conjugates in their interaction with cells, cell association and internalization experiments were performed by flow cytometry using ASGP-R positive HepG2 cells and ASGP-R negative HEK 293 cells. Incubation of conjugates was performed at 37 °C for cellular association and cellular uptake. According to Figure 7, for HepG2 cells, Dendrimer-DOX-PEG-Gal conjugates displayed high intracellular uptake ratio of 94.4% after 1 h of incubation, which was slightly lower than free DOX of 96.3%. The faster cellular association and high cellular uptake governed by free DOX may be due to the features of small molecule. In contrast, Dendrimer-DOX-mPEG conjugates resulted in uptake ratio of 40.8%, indicating much less uptake than Dendrimer-DOX-PEG-Gal conjugates and free DOX. Similar results were obtained after

Figure 6. CLSM images of HepG2 cells. The cells were incubated with (a) free DOX; (b) Dendrimer-DOX-mPEG; (c) Dendrimer-DOX-PEG-Gal; for 3 h with the same DOX concentration of 5 µg/mL.
3 h of incubation. Those results were consistent with those of detectable fluorescence and in vitro cytotoxicity assays. For HEK 293 cells, all tested samples presented high intracellular uptake ratio (> 80%) either 1 h of incubation or 3 h of incubation, and no significantly different uptake ratio was detected for all samples. It may be caused by the characteristics of different cell type, because it has been reported that the cell-line-specific differences were relevant to the uptake of the different nanoparticles.50,51 Meanwhile, HEK 293 cells may result in much effective uptake since HEK 293 cells were wildly used in the transfection studies and presented high transfection effect.52–55 In addition, for both HepG2 cells and HEK 293 cells, geometric mean fluorescence intensity of Dendrimer-DOX-PEG-Gal was higher than Dendrimer-DOX-mPEG, however, different geometric mean fluorescence intensity ratio of Dendrimer-DOX-PEG-Gal to Dendrimer-DOX-mPEG was analyzed after 3 h of incubation. For HepG2 cells, the ratio was 3.04, which was higher than the ratio (2.22) for HEK 293 cells, suggesting the non-specific interaction.
with cells caused by cationic groups increased the intracellular uptake of Dendrimer-DOX-PEG-Gal conjugates, but further increased uptake found in HepG2 cells should be induced by the introduction of HepG2 cell specific moieties, because the dendrimer based conjugates bearing multiple galactose moieties on a single dendritic scaffold showed excellent capacities for ASGP-R, termed as “cluster effect,” consistent with our previous similar studies. Therefore, the PEGylated dendrimer-DOX conjugates with multiple galactose moieties may be used as specific drug delivery for liver tumor therapy. The in vivo pharmacokinetics, antitumor efficacy, toxicity and biodistribution studies are in progress and will be published in next paper.

CONCLUSIONS

In this study, three PEGylated polylysine dendrimer-DOX conjugates were synthesized and the in vitro features were evaluated. The anticancer drug DOX was conjugated to the dendrimers via an acid–labile hydrazone bond, resulting in a pH-sensitive drug release property. The galactosyl moieties were conjugated to dendrimers through flexible PEG linkage, which enhanced water solubility of drug delivery system, as well as displayed tumor cell specificity, due to the higher cellular association and cellular uptake of Dendrimer-DOX-PEG-Gal. The drug free Dendrimer-PEG-Gal showed promising biosafety, whereas its drug conjugate demonstrated high cytotoxicity in vitro by combination of the features of dendrimer, polymer and galactosyl moieties, which was confirmed by in vitro IC50 study. The overall structural design of dendrimer-drug conjugate may provide useful design and preparation strategies for dendriic macromolecules as tumor specific, safe and efficient drug delivery systems.

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