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Dendrimer-doxorubicin conjugate as enzyme-sensitive and polymeric nanoscale drug delivery vehicle for ovarian cancer therapy

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Peptide dendrimer based nanoparticle has presented significant potential to be a candidate for drug delivery system. In this study, we synthesized and characterized enzyme-responsive mPEGylated peptide dendrimer-GFLG-doxorubicin conjugate (dendrimer-GFLG-DOX) as chemotherapeutic drug delivery nano-carrier via two-step highly efficient copper-catalyzed alkyne-azide click cycloaddition (CuAAC) reaction. The tetra-peptide sequence Gly-Phe-Leu-Gly (GFLG) was explored as an enzyme-responsive linker to connect the doxorubicin (DOX) to the periphery of mPEGylated peptide dendrimer. The dendrimer-GFLG-DOX was capable of self-assembling into nanoparticle, which was proved by dynamic light scattering (DLS) and transmission electron microscope (TEM) assays. Compared to free drug DOX at the equal dose, the dendrimer-GFLG-DOX conjugate based nanoparticle demonstrated higher accumulation and retention within SKOV-3 ovarian tumor tissue, resulting in higher antitumor activity, as the evidences from tumor growth curves, tumor growth inhibition analysis, immunohistochemical assessment and *in vivo* imaging. Meanwhile, no obvious systemic toxicity was observed via histological assessment. Thus, the mPEGylated peptide dendrimer-DOX conjugate based nanoparticle may be a promising candidate of nanoscale and enzyme-sensitive drug delivery vehicle for ovarian cancer therapy.

Introduction

Ovarian cancer has been reported as the most common lethal female gynecological malignancy.[1] To treat ovarian cancer, although tremendous developments of conventional drug have emerged in recent years, some limitations still remain.[2, 3] With the developments of nanotechnology, nanoscale drug delivery vehicles have presented the potential to revolutionize cancer therapy by improving the therapy efficacy and reducing the side effects of chemotherapeutics owing to the enhanced permeability and retention (EPR) effect.[4-9] Polymeric nanoscale carriers, such as micelles and dendritic polymers, likely have the great potential clinical impact for the foreseeable future.[9-11] Recently, due to the water-solubility, biodegradability, good biocompatibility and immunocompatibility, peptide dendrimer based nanoparticle has been investigated as the anti-tumor drug vehicle, demonstrating great potential for cancer therapy.[12, 13]

To enhance the specificity of drug delivery and reduce side effects, the stimuli-responsive nanoscale drug delivery systems have received extensive attention for their unique advantages that the release of anti-tumor drug from nano-carriers is triggered by the environment of tumor tissues/cells, such as the lower pH, overexpressed particular sorts of enzymes.[10, 14] Enzyme-sensitive nanoparticles have exploited frequently and it has been becoming one of the most important branches of the drug delivery systems.[15-17] Cathepsin B, as a lysosomal cysteine protease overexpressed in many tumor cells and tumor endothelial cells,[18] has been recruited extensively as a central target in various cancer therapies. The glycylphenylalanylleucylglycine tetra-peptide spacer (Gly-Phe-Leu-Gly, GFLG) considered as the appropriated substrate of the cathepsin B, as its drug conjugates exhibit stability in plasma and serum during transport and permit intralysosomal drug liberation after endocytosis.[19, 20] The features of GFLG peptide provide a possibility of the combination of enzyme-sensitive ability and peptide dendrimer based nano-carrier might be a promising strategy to decrease the side effect, and such particular sensitive drug delivery system was barely reported in the previous studies.

Another challenge to the application of sensitive peptide dendrimer based drug delivery systems for *in vivo* cancer treatment is the small size of dendrimers, leading to rapidly clear from the circulation.[4, 21] Although longer blood circulation can be induced by increased size via increasing the generation of dendrimer, some issues, such as difficult synthesis and relevantly increased toxicity, will be involved.[22] To overcome these problems, the covalent conjugation of poly(ethylene glycol) (PEG) chains to the surface of dendrimers to form dendrimer based nano-carriers is an appropriate method for increasing molecular weight and size of dendrimer based drug delivery vehicles, resulting in decreased renal filtration, longer blood circulation, enhanced accumulation in tumor by EPR

effect and lower side effects.[23] In addition, PEGylation of dendrimers can assist in minimizing premature clearance by the reticuloendothelial system (RES). The general approach to construct PEGylated dendrimers is coupling activated PEG chain to the end group of dendrimer.[24, 25] However, it is not easy to synthesize PEGylated dendrimers as the low effective conjugation associated with the high steric hindrance. Fortunately, the highly efficient copper-catalyzed alkyne-azide click cycloaddition (CuAAC) reaction, is an attractive alternative in this context, remarkably offering minimized undesired side reactions and maintained low polydispersity of dendrimers.[26] In our previous work, we have prepared mPEGylated dendron-DOX conjugate, via click reaction, and the conjugate can be self-assembled into nanoscale particles and used as a pH-sensitive drug delivery system for breast cancer therapy.[26]

In this study, we prepared mPEGylated peptide dendrimer-DOX conjugate based nanoparticle as an enzyme-responsive drug delivery system for ovarian tumor therapy through two-step copper-catalytic click reaction. The surface of peptide dendrimer was modified with alkynyl groups twice. Azido mPEG chains were firstly conjugated to dendrimer via click reaction (**Fig. 1**), and then GFLG modified DOX was conjugated to surface of dendrimer through click reaction. The size and morphology, zeta potential, *in vitro* and *in vivo* antitumor efficacy and toxicity, were evaluated, suggesting the enzyme-responsive mPEGylated peptide dendrimer-DOX conjugate may be a potential drug delivery vehicle for ovarian cancer chemotherapy.

Experimental

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), trifluoroac etic acid (TFA), methoxy poly(ethylene glycol) (mPEG, 2 kDa), 4-azidobenzoic acid, 2-thiazolidinethione, 5-hexynoic acid and capthesin B were purchased from Sigma-Aldrich and used without further purification. Boc-L-Lys(Cbz)-OH, 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) was prepared as previous work.[26] Characterization and structural confirmation 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. 10 mg/mL 2, 5-dihydroxybenzoic acid (DHB) (water/acetonitrile = 2/1, 0.1% TFA) and mixture of 125 μL of a diammoniumhydrogen citrate distilled aqueous solution (18 mg/mL) or 375 μL ethanol solution of 2, 5-dihydroxyacetophenon (DHAP) (20.2 mg/mL) was used 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 are reported 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). All samples of 100 mg/mL were measured at 20 ^{o}C. *In vivo* fluorescent imaging of nude mice bearing SKOV-3 tumor was performed using a Maestro *in vivo* imaging system (CRi, U.S.).

The synthesis of PEGylated dendrimer-DOX conjugate

The synthesis of N_3-GFLG-OMe Boc-GFLG-OMe (2.0 g, 3.94 mmol) was dissolved in 10 mL mixture of anhydrous DCM/TFA (1:1, v/v) and the solution was stirred under nitrogen at 0 ^{o}C for 24 h. After the solvent was removed by rotary evaporation, anhydrous diethyl ether was added and white precipitate appeared. The precipitate was collected by centrifugation and washed 3 times with anhydrous diethyl ether. The sample was dried under high vacuum for 0.5 h and 30 mL anhydrous DMF was added followed by DIPEA (4.2 mL, 23.69 mmol), HOBt (798 mg, 5.91 mmol) and HBTU (2.24 g, 5.91 mmol). The solution was stirred under nitrogen in ice bath for 1 h and another 48 h at room temperature. After woke up, 500 mL EtOAc was added into the solution and the new solution was washed by $NaHCO_3$ aq. (satd.), 1 M HCl aq., $NaHCO_3$ aq. (satd.) and then NaCl aq. (satd.). The solution was dried by anhydrous $MgSO_4$ and the solvent was removed by rotary evaporation. The residue was recrystallized from ethyl ether/pet ether mixture at 4 ^{o}C to give brown solid in 56% (1.22 g). ^{1}H NMR (400 MHz, DMSO): δ = 0.83-0.89 (m, C***H***$_3$), 1.47-1.63 (m, C***H***$_2$ and C***H***$_3$C***H***C***H***$_3$), 2.76-3.04 (m, ArC***H***$_2$), 3.61 (s, OC***H***$_3$), 3.73-3.82 (m, NHC***H***$_2$CO), 4.32-4.55 (m, NHC***H***CH$_2$), 7.22-8.77 (m, Ar***H*** and N***H***). ESI-MS: m/z= 552.37 ([M+H]$^+$).

The synthesis of N_3-GFLG-TT N_3-GFLG-OMe (1.0 g, 1.81 mmol) was dissolved into 2 mL MeOH and 9.0 mL 1.0 M NaOH (aq.) was added into this solution. This mixture was stirred at 4 °C for 24 h and then the pH was adjusted to 2-3 by 1 M HCl aq. The solution was diluted by 500 mL ethyl ether and washed by NaCl aq. (satd.) for 3 times, and then the solvent was removed. White solid N_3-GFLG-OH was collected and waited for using further.

Under nitrogen, N_3-GFLG-OH (924.37 mg, 1.72 mmol) was dissolved into 10 mL anhydrous DCM followed by thiazolidine-2-thione (307.5 mg, 2.58 mmol) and DIPEA (1.8 mL, 10.32 mmol). 5 mL anhydrous DCM solution of EDC (494.6 mg, 2.58 mmol) was added into this solution by drop-wise at 0 ^{o}C. This mixture was stirred for 30 h at 4 ^{o}C and then diluted by 500 mL EtOAc. The solution was washed by $NaHCO_3$ aq. (satd.), 1 M HCl aq., $NaHCO_3$ aq. (satd.) and then NaCl aq. (satd.). The solution was dried by anhydrous $MgSO_4$ and the solvent was removed by rotary evaporation. The residue was recrystallized from ethyl ether/pet ether mixture at 4 ^{o}C to give light yellow solid in 53.7 % (590 mg). ^{1}H NMR (400 MHz, DMSO): δ = 0.62-0.90 (m, C***H***$_3$), 1.52-1.63 (m, C***H***$_2$ and C***H***$_3$C***H***C***H***$_3$), 2.77-3.05 (m, ArC***H***$_2$), 3.44 (m, SC***H***$_2$), 3.73-3.87 (m, CONC***H***$_2$), 4.35-4.68 (m, NHC***H***$_2$CO and NHC***H***CH$_2$), 7.16-8.79 (m, Ar***H*** and N***H***). ESI-MS: m/z= 661.35 ([M+Na]$^+$).

The synthesis of N_3-GFLG-Dox N_3-GFLG-TT (500 mg, 0.93 mmol) and DOX•HCl (592.8 mg, 1.02 mmol) were dissolved into 10 mL anhydrous DMSO followed by 5 mL distilled pyridine. This solution was stirred at room temperature in darkness for 30 h. After woke up, the solution was added into

iced EtOAc and dark red solid precipitated. The precipitate was collected via centrifuged and waited for using later without further purification. ESI-MS: m/z=1085.57($[M+Na]^+$), 1101.55($[M+K]^+$)

The synthesis of mPEGylated Boc-dendrimer Under nitrogen, Alkyne-Boc-dendrimer (150 mg, 29.02 μmol), $CuSO_4$•$5H_2O$ (87.5 mg, 0.35 mmol), N_3-mPEG (1.06 g, 0.52 mmol) and sodium ascorbate (69 mg, 0.35 mmol) were added into the 20 mL mixture of DMF and H_2O (3:1, V/V). The mixture was stirred for 3 days in darkness. After woke up, the solution was dialyzed under darkness. The solvent was removed by freeze-dry and the residue was further fractionated/purified by size exclusion chromatography using a Superose 12 HR/16/30 column on an ÄKTA FPLC system (GE Healthcare) column with sodium acetate buffer containing 30% acetonitrile (pH 6.5) asmobile phase. After dialysis against water and freeze-drying, the final product was obtained in 68% yield (445.5 mg). MALDI-TOF MS: m/z=27481($[M+K]^+$).

The synthesis of mPEGylated Alkyne-dendrimer Under nitrogen, mPEGylated Boc-dendrimer (3.0 g, 0.11 mmol) was dissolved into 10 mL mixture of anhydrous DCM and TFA(1/1, v/v). The solution was stirred at 0 ^oC for 24 h and the solvent was removed by rotary evaporation, anhydrous diethyl ether was added and white precipitate appeared. The precipitate was collected by centrifugation and washed 3 times with anhydrous diethyl ether. The sample was dried under high vacuum for 0.5 h and 10 mL anhydrous DMF was added followed by DIPEA (1.0 g, 7.92 mmol), HOBt (303 mg, 1.98 mmol), HBTU (750 mg, 1.98 mmol) and 5-hexynoic acid (222 mg, 1.98 mmol). The solution was stirred under nitrogen in an ice bath for 1 h and another 72 h at room temperature. After woke up, the solution was dialyzed in 0.1 M $NaHCO_3$ (aq.) at 4 ^oC for 24 h. The solvent was removed by freeze-dry and the residue was further fractionated/purified by size exclusion chromatography using a Superose 12 HR/16/30 column on an ÄKTA FPLC system (GE Healthcare) column with sodium acetate buffer containing 30% acetonitrile (pH 6.5) asmobile phase. After dialysis against water and freeze-drying, the final product was obtained in 83.7 %. MALDI-TOF MS: m/z=27407 ($[M+K]^+$).

The synthesis of mPEGylated dendrimer-GFLG-DOX conjugate Under nitrogen, mPEGylated dendrimer-Alkyne (150 mg, 29.02 μmol), $CuSO_4$•$5H_2O$ (87.5 mg, 0.35 mmol), N_3-GFLG-DOX (1.06 g, 0.52 mmol) and sodium ascorbate (69.03 mg, 0.35 mmol) were added into the 20 mL mixture of DMSO and H_2O (3:1, V/V). The mixture was stirred for 3 days in darkness. After woke up, the solution was dialyzed under darkness. The solvent was removed by freeze-dry and the residue was further fractionated/purified by size exclusion chromatography using a Superose 12 HR/16/30 column on an ÄKTA FPLC system (GE Healthcare) column with sodium acetate buffer containing 30% acetonitrile (pH 6.5) asmobile phase. After dialysis against water and freeze-drying, the final dark red product was obtained in 67.2% (mg). MALDI-TOF MS: m/z=30312($[M+K+H]^+$).

The size, shape and the zeta potential

The hydrodynamic size and zeta potential of the nanoparticle were characterized using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). The mPEGylated dendrimer-DOX conjugate was diluted to 10 mL with distilled water to a final concentration of 100 μg/mL. The TEM samples (concentration of 100 μg/mL) were prepared by dipping a copper grid with formvar film into the freshly prepared nanoparticles solution.

Tumor cell lines, cell culture and Animals

The human ovarian cancer cell line SKOV-3 and Vervet Monkey kidney cell lines (COS-7) was purchased from Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China). The cell line was maintained as monolayer cultures in RPMI 1640 medium (Life Technologies), supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS, Hyclone), 1% penicillin and streptomycin mixture, and incubated in 5% CO_2/95% air at 37°C. Female BALB/c nude mice (15 ± 2 g, 4-5 weeks old) were selected for tumor model. The animals were purchased from West China Animal Culture Center of Sichuan University, which were randomly divided and housed in specific pathogen-free conditions and controlled temperature room with regular alternating cycles of light and darkness. Animals were performed in line with national regulations and approved by the animal experiments ethical committee.

***In vitro* cytotoxicity assays**

Cytotoxicity of the drug free dendrimer (mPEGylated dendrimer-GFLG), mPEGylated dendrimer-GFLG-DOX conjugate (dendrimer-GFLG-DOX) based nanoparticle and free DOX were evaluated by the percentage of cell viability via Cell Counting Kit-8 (CCK-8) assay. 5.0×10^3 cells/well SKOV-3 cells and COS-7 cells were seeded in 96-well plates. After 24 h culture, mPEGylated dendrimer-GFLG, dendrimer-GFLG-DOX and free DOX solutions containing RPMI 1640 culture medium were added to each well, respectively. After incubation for further 48 h, 10 μL CCK-8 was added to each well and the plates were incubated at 37 °C for another 2 h. Then, the absorbance of each sample was measured using a microplate reader Varioscan Flash (ThermoFisher SCIENTIFIC). The cell viability (%) was obtained according to the manufacturer's instruction.

***In vivo* efficacy**

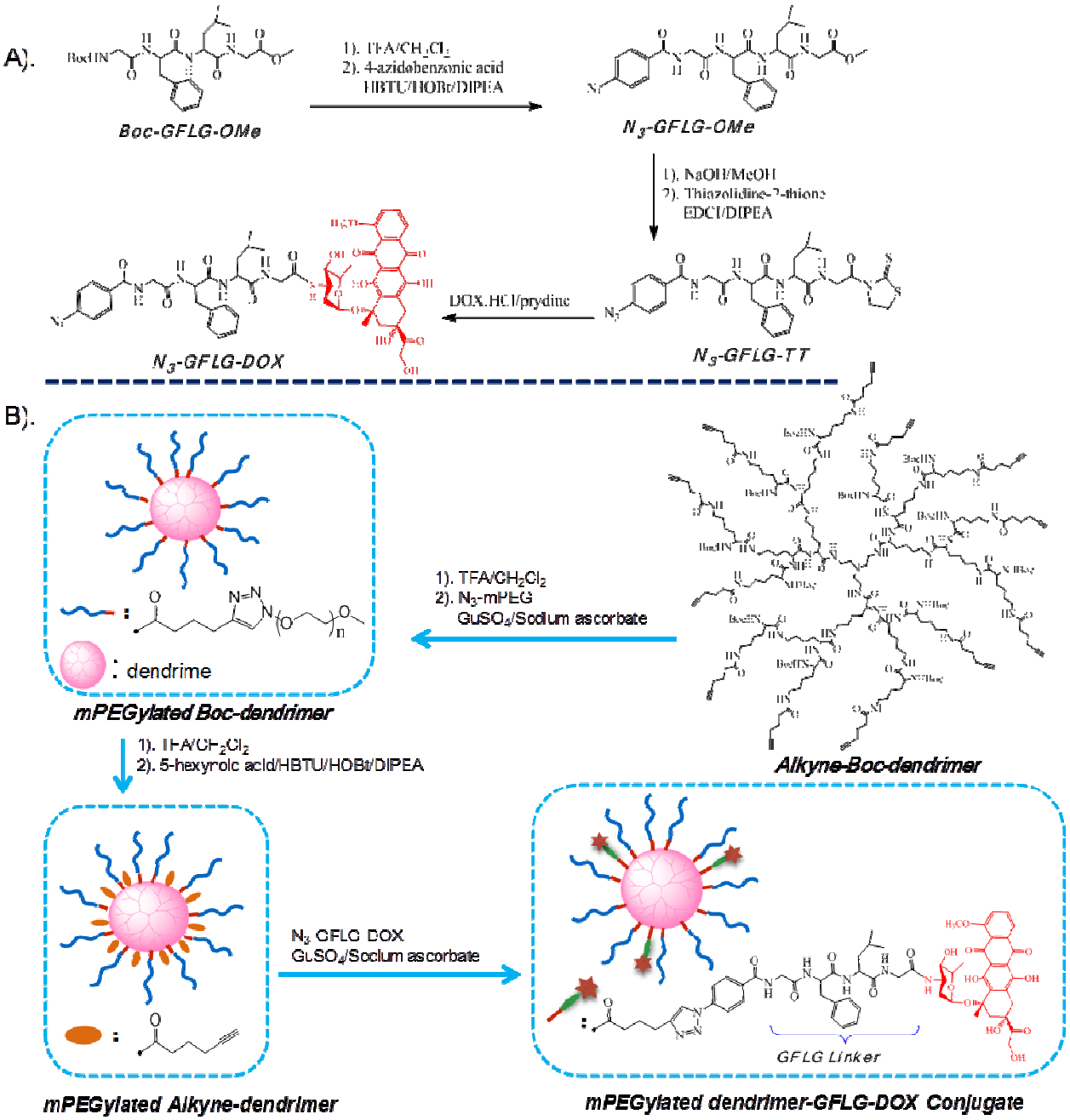

Fig. 1. The synthesis of mPEGylated dendrimer-GFLG-DOX Conjugate

In vivo antitumor efficacy was investigated in subcutaneous SKOV-3 model. To produce tumors, female BALB/c nude mice were inoculated with SKOV-3 cells suspension via the following procedures. Mice were anaesthetized and then 2×10^6 SKOV-3 cells suspended in 50 μL PBS were subcutaneously injected into the previously treated region of each mouse. Solid tumors were allowed to form over a period of 1 week to reached a volume ranging from 50 to 100 mm^3, then tumor bearing mice were randomized to receive different treatment with saline (control), DOX (5 mg DOX/kg mouse) and dendrimer-GFLG-DOX (5 mg DOX/kg mouse) in a final volume of 200 μL via the tail vein every 4 day for 5 times. The tumor sizes and the mouse body weights were measured every 2 days. Tumor volumes were normalized to 100% at day 0, and calculated by the following formula: tumor volume V (mm^3) = 1/2 × length (mm) × width (mm^2). At day 21, all the animals were euthanized and sacrificed, the tumors were excised for calculating tumor growth inhibition (TGI) and immunohistochemical study to evaluate apoptosis in tumor. Major organs, such as liver, heart, spleen, lung and kidney were separated and fixed for histological examination. TGI was calculated as: TGI = (1-(Mean tumor weight of treatment group)/(Mean tumor weight of control group))×100%.[27]

In vivo imaging study

In vivo fluorescent imaging was performed using a Maestro *in vivo* imaging system (CRi, U.S.). Female Balb/C nude mice bearing SKOV-3 tumor were fasted for 12 h before imaging. DOX and dendrimer-GFLG-DOX groups (equivalent to free DOX) were injected by intravenous tail vein, while saline was injected as control. The optical images of the whole body were taken at 1 h, 6 h, and 24 h after injection.

Statistical analysis

Comparison between groups was analyzed by the Student's *t*-test was used to address statistical significance. All data are presented as mean ± SD, and p values were below 0.05 indicating statistical significance.

Results and discussion

Design and preparation of mPEGylated dendrimer-DOX conjugate based enzyme-sensitive drug delivery system

Because of great biocompatibility, multifunctionable and low dispersity, peptide dendrimers have been investigated as drug/gene nanocarriers.[28, 29] Based on these understandings and our previous studies, we designed and prepared the mPEGylated dendrimer-DOX conjugate based enzyme-responsible drug delivery system via two-step click-reaction. The oligopeptide GFLG was extensively utilized as enzyme-sensitive spacer between the antitumor drug and the carrier in varies of drug delivery systems,[30] as this peptide can be specifically hydrolyzed by cathepsin B highly expressed in lysosome of most of the tumor cells and tumor endothelial cells, including the ovarian cancer.[30-35] For decreasing the influence of steric hindrance to the loading of drug,[36] "click reaction" was exploited owing to its high efficiency. As there are -OH and -NH_2 in the structure of DOX, both of the two groups can be reacted with -COOH via condensation reaction. Therefore, for increasing the selective reactivity of conjugation between DOX and GFLG sequence, the N_3-GFLG-OMe was deprotected and activated by thiazolidine-2-thione (-TT) group, resulting in activated intermediate of N_3-GFLG-TT. The high selectivity of N_3-GFLG-TT allowed it reacted with -NH_2 to form amide group avoiding of the formation of ester bone,[37] and the DOX was conjugated to N_3-GFLG segment via amide group. Compared to the ester bond formed form the reaction of –OH and –COOH, the GFLG-DOX linked with amide groups may be more stable in blood circulation due to the avoided hydrolysis and low rates of amiolysis. The successful synthesis of the new compounds of N_3-GFLG-OMe, N_3-GFLG-TT and N_3-GFLG-DOX were verified by ^{1}H NMR (**Fig. 2A and 2B**) and ESI-MS (**Fig. 2C**), and the purity was validated by a single peak in HPLC (**Fig. 2D**), which confirmed the expected structures of the new compounds. As the **Fig. 1** illustrated, for increasing the efficiency of modification of dendrimer, the two-step click-reaction was exploited to prepare the mPEGylated dendrimer-GFLG-DOX conjugate. Mass spectrometry was utilized to confirm the mPEGylated dendrimer conjugate. For MALDI-TOF MS of mPEGylated dendrimer conjugate (m/z = 27442), the most abundant peak (m/z = 27481) was assigned as $[M+K]^+$ (**Fig. 3A**), suggesting eleven mPEG chains were conjugated to the dendritic scaffold. After attachment of N_3-GFLG-DOX to mPEGylated dendrimer via the second step click reaction, the trace amount of copper which may cause undesired toxicity was cleared by dialysis in EDTA dilute solution. The mPEGylated dendrimer-GFLG-DOX conjugate was purified further by size exclusion chromatography using a Superose 12 HR/16/30 column on an ÄKTA FPLC system and dialysis. The mass of mPEGylated dendrimer-GFLG-DOX

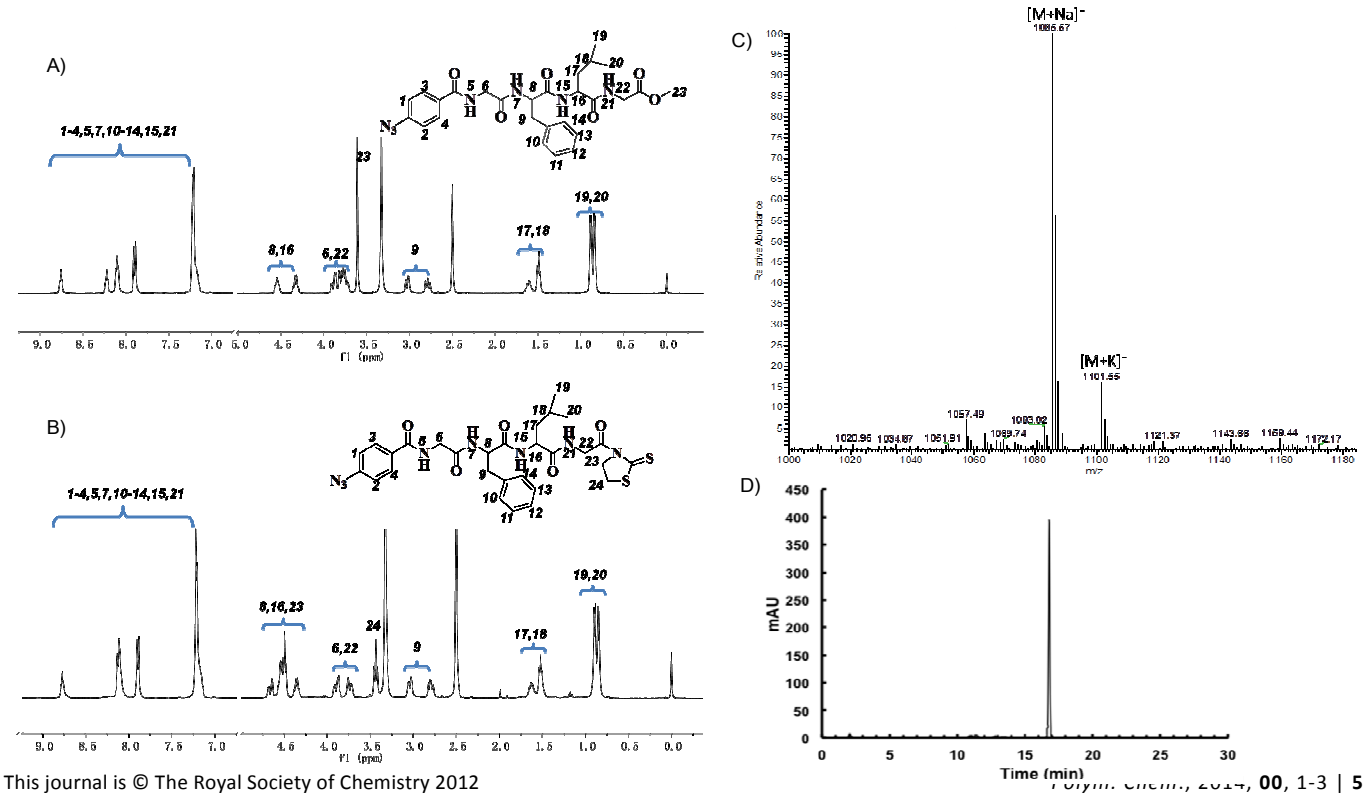

Fig. 2. The ^{1}H NMR Spectrum of N_3-GFLG-OMe (A) and N_3-GFLG-TT (B); The ESI-MS (C) and HPLC profile (D) of N_3-GFLG-DOX

conjugate was measured by MALDI-TOF MS, and the most abundant peak (m/z = 30312) was assigned as a potassium and proton adduct $[M+K+H]^+$ (**Fig. 3B**), indicating that the average number of GFLG-DOX attached to a single one mPEGylated dendrimer was three, less than the theoretical number (twelve). The possibility was the high steric hindrance to chemical reaction, despite the click reaction with highly efficient. UV-vis spectrophotometry analysis was utilized to determine the content of drug, resulting in 4.1 wt% (weight percent).

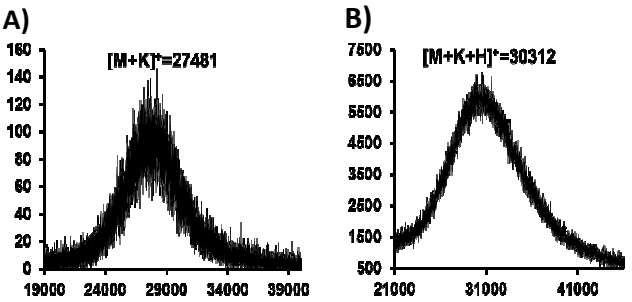

Fig. 3 MALDI-TOF mass spectrum of mPEGylated dendrimer (A) and mPEGylated dendrimer-DOX conjugate (B). The observed mass were 27481 ($[M + K]^+$) and 30312 ($[M + K + H]^+$), respectively.

Size and zeta potential of nanoparticle

The results of dynamic light scattering (DLS) demonstrated that the mPEGylated dendrimer-DOX conjugate was capable of assembling into particle with nanoscale size in water (pH = 7.4), giving size of 86 nm (PDI = 0.161) (**Fig. 4A**). That was attributed to the minimization of the interfacial energy governed by the balance between the hydrophilic interaction of the PEG moieties and the hydrophobic interaction of the dendrimer core.[38] Additionally, the self-assemble behaviour of the dendrimer-GFLG-DOX should be due to the driving forces, which were endowed by the different chemical composition of DOX, such as π-π stacking, dipole interactions, and H-bonding.[39] The zeta potential of the nanoparticle was -8 mV, suggesting that the interaction between nanoparticle and serum proteins as well as recognition and capture of macrophage in the circulation system could be significantly reduced or even prevented owing to the negative charge of the nanoscale carrier.[40] Thus, the *in vivo* antitumor efficacy may be increased, as the probable enhanced accumulation into target tissue (tumor) of this mPEGylated dendrimer-GFLG-DOX conjugate.

As presented in **Fig. 4B**, the size and shape of the mPEGylated dendrimer-GFLG-DOX conjugate based nanoparticle were measured by the TEM. The nanoparticles were compact, which may be resulted from the strong aggregation of dendrimer-GFLG-DOX conjugate because of the noncovalent forces mediated by DOX. The size of particles measured by TEM was around 60 nm, which was mildly smaller than that of observed by DLS. Since the diameter of nanoparticles assayed by DLS in pure water (pH=7.4) is the hydrodynamic one, while the TEM image displayed the actual size of samples at the dried state. It is currently accepted that the nanoscale drug delivery systems may be capable of aggregating in tumor tissues efficiently by EPR effect.[41] In this study, the prepared nanoparticles have the diameter of 60-90 nm measured by DLS and TEM, suggesting that the mPEGylated dendrimer-GFLG-DOX conjugate based nanoparticle might be large enough to have accessibility within solid tumours via EPR effect and prevent their rapid leakage into blood capillaries, but also small enough to escape from the capture of macrophages.[42]

In vitro cytotoxicity

To compare the cytotoxicity of free drug DOX and mPEGylated dendrimer-GFLG-DOX conjugate based nanoparticle against the SKOV-3 human ovarian cell line, cells were exposed to the antitumor agents for 48 hours, and cell viability was observed by CCK-8 assay. As the results shown in **Fig. 5A**, the IC50 (half maximal inhibitory concentration) of free DOX against SKOV-3 cells was 0.39 μg/mL, while that of dendrimer-GFLG-DOX conjugate based nanoparticle (IC50, 8.69 μg/mL) was found to be approximately 22-fold of that of free drug DOX. The possible reason was the free DOX could pass through the cell membrane easily with free diffusion and being uptaken by tumor cells. On the other hand, the DOX linked to dendrimer via GFLG linker can only be released after endocytosis and entering lysosome.[20, 34, 35] Another reason may be the slow release of DOX from the carriers under the intracellular environment (See **Fig. S1**), that is due to the high steric hindrance of GLFG-DOX.[43] Simultaneously, no

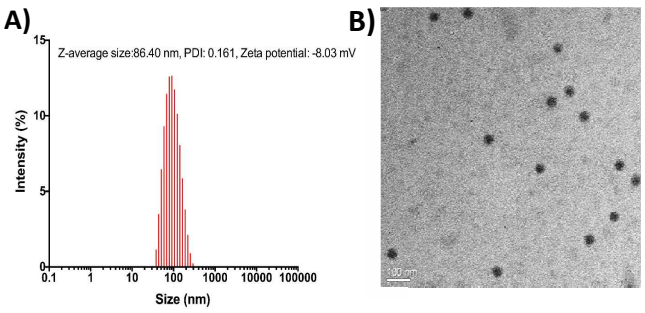

Fig. 4. The size and zeta potential measured by DLS (A) and TEM (B) of mPEGylated dendrimer-DOX conjugate based nanoparticle. The hydrodynamic size of the particle was around 80 nm, and TEM showed around 60 nm.

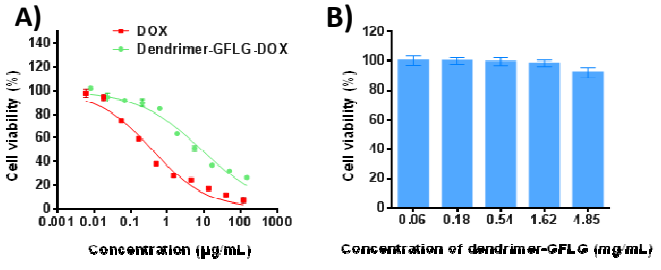

Fig. 5. The *in vitro* antitumor efficacy of free doxorubicin (DOX), mPEGylated dendrimer-DOX conjugate based nanoparticle (Dendrimer-GFLG-DOX) at DOX-equivalent concentrations against SKOV-3 cell line (A) and the cytotoxicity of drug-free mPEGylated dendrimer conjugate (dendrimer-GLFG) against COS-7 cell line (B). Values represent mean ± SD (n = 3).

obviously cytotoxicity of the drug-free mPEGylated dendrimers conjugate (dendrimer-GFLG) against COS-7 normal cell was observed at the concentrations below 4.85 mg/mL as illustrated in **Fig. 5B**, which means the mPEGylated dendrimer-GFLG carrier was biocompatible and the potential candidate for chemotherapeutic drug delivery system. Obvious *in vitro* cytotoxicity of dendrimer-GFLG-DOX conjugate based nanoparticle also demonstrated that DOX can be released from the nanoparticle to result in cytotoxicity against tumor cells, and the *in vivo* antitumor efficacy may be observed once the nanoparticle arrives at the tumor tissues and enters tumor cells.

In vivo efficacy

To evaluate the *in vivo* antitumor efficacy and toxicity of the mPEGylated dendrimer-GFLG-DOX conjugate based nanoparticle against SKOV-3 human ovarian cancer, nude mice bearing SKOV-3 tumor was injected with saline (control), nanoparticle and free DOX via the tail vein every four days for 17 days, while the saline was explored as control. The dose of free DOX was 5 mg/kg, which is extensively applied in mice models. As shown in **Fig. 6A** and **Fig. 6B**, free DOX treatment only showed moderate therapeutic effect on tumor growth inhibition, which resulted in relative tumor volume of 256.6 ± 52.8 % and presented tumor growth inhibition (TGI) of 18 %. In contrast, the treatment of the dendrimer-GFLG-DOX conjugate (5 mg DOX/kg mice, equivalent to free drug DOX) demonstrated much higher anticancer efficacy compared to either the DOX-treated group or saline control ($p < 0.01$ versus saline, $p < 0.05$ versus free drug DOX), resulting in relative tumor volume of 191.7 ± 24% and presenting a significant increased TGI of 38.55 %, which demonstrated that the mPEGylated dendrimer-GFLG-DOX based nanoparticle was a drug delivery system with more significant efficient to inhibit tumor growth in SKOV-3 model. Additionally, as the results of deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) immunohistochemical (IHC) staining of tumor tissues (**Fig, S2**), the dendrimer-GFLG-DOX induced higher apoptosis level in the tumors than that of free DOX. Therefore,

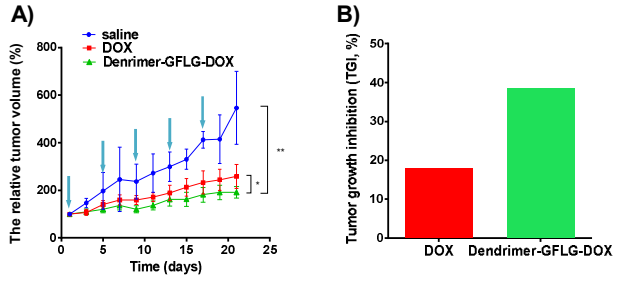

Fig. 6 In vivo tumor growth inhibition of dendrimer-GFLG-DOX. Comparison of the tumor inhibition effect of nanoparticle (5 mg DOX /kg mouse) versus free drug DOX (5 mg DOX /kg mouse) and saline in the human ovarian tumor SKOV-3 model (n = 5). The dendrimer-GFLG-DOX demonstrated significant tumor inhibition at equal dose (**p < 0.01, compared to saline; * p < 0.05, compared to free drug DOX) (A). At the end of this trial, tumor tissues were collected from each sacrificed animal, and the tumor growth inhibition (TGI, %) was calculated (B).

for the dendrimer-GFLG-DOX conjugate based polymeric nanoscale drug delivery vehicle, the much more effective on inducing apoptosis of tumor cells resulted in higher anticancer efficacy. Meanwhile, compared to the saline (control) group, no obvious toxicity was observed from histological analysis of dendrimer-GFLG-DOX treatment group (**Fig. S3**), indicating the dendrimer based nanoparticles may be as efficient and safe drug delivery vehicles for cancer therapy.

Significant *in vivo* antitumor efficacy of the nanoscale drug delivery vehicle probably resulted from negative charged surface, prolonged blood circulation, potentially more efficient accessibility within tumor tissue via EPR effect and intralysosomal release of DOX. Additionally, the conjugate based drug delivery vehicle was stable (See **Fig. S1**), since no released drug was observed at the physiological condition. In contrast, the drug released from the vehicle at the presence of enzyme Cathepsin B (a lysosomal cysteine protease overexpressed in tumor cells), resulting in cytotoxicity and anticancer efficacy. To determinate the *in vivo* accumulation in tumor tissue of the dendrimer-GFLG-conjugate based nanoparticle via EPR effect, the *in vivo* fluorescent imaging of nude mice bearing SKOV-3 tumor was analysed at different time points after injection of therapy agents through the tail vein. As shown in **Fig. 7**, at 1 h post-injection, obvious fluorescence signals were observed at the tumor site in both of the DOX and dendrimer-GFLG-DOX group, while the signal in saline group was invisible.

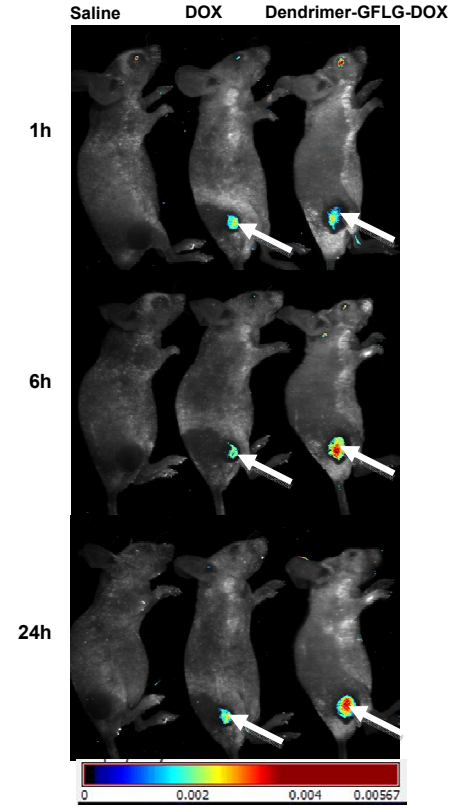

Fig. 7 *In vivo* fluorescent imaging of nude mice bearing SKOV-3 tumor was analysed at different time points (1h, 6h, 24h) after injection of dendrimer-GFLG-DOX through caudal vein. (n=3 mice per group)

At 6 h after injection, the signals from the tumor of nanoparticle group increased significantly and the maximum at 24 h while that of DOX group was barely changed. The results illustrated that the dendrimer-GFLG-DOX conjugate based nanoparticle was capable of more rapidly aggregating at tumor site via EPR effect than that of free DOX due to the appropriate nanoscale size. All those *in vivo* antitumor trial results suggested that the dendrimer-GFLG-DOX conjugate based drug delivery vehicle with mPEGylated, nanoscale and enzyme-sensitive features was obviously capable of aggregating within tumor tissue obviously, inducing the apoptosis of tumor cells and inhibiting the growth of tumor efficiently without increased toxicity. Thus, the mPEGylated dendrimer-GFLG-DOX conjugate based polymeric nanoscale drug delivery vehicle might be a promising candidate for cancer therapy.

Conclusions

In this study, we designed and synthesized mPEGylated peptide dendrimer-drug conjugate as a promising candidate of drug delivery system via two-step click reaction with excellent characteristics and functionalities. A tetra-peptide sequence GFLG, which was cleavable at the presence of cathepsin B overexpressed in the tumor cells, was employed as linker to connect the anticancer drug DOX to mPEGylated peptide dendrimer. Due to the controlled release of drug and the particular nanoscale size, mPEGylated dendrimer-GFLG-DOX conjugate was found to have obviously improved *in vivo* antitumor efficacy over commercial DOX formulation at an equal dose, as well as low side effects measured by changes in body weight and histological analysis. Overall, the structural design of mPEGylated peptide dendrimer-DOX conjugate based nanoparticle in this study may provide useful strategy for design and preparation of peptide dendrimer as safe and effective drug delivery system.

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Notes and references

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Graphical Abstract

Dendrimer-doxorubicin conjugate prepared via click chemistry was employed as enzyme-sensitive nanoscale drug delivery system for ovarian cancer therapy, showing high accumulation in tumor tissue, significantly increased antitumor activity and good biosafety.