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Short communication

Combretastatin A4/poly(L-glutamic acid)-graft-PEG conjugates self-assembled to nanoparticles

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ABSTRACT

Combretastatin A4 (CA4) possesses varying ability to cause vascular disruption in tumors, while the short half-life, low water solubility and deactivation of many CA4 analogs during storage limited its antitumor efficacy and drug stability. A novel macromolecular conjugate of CA4 (CA4-PL) was synthesized by covalent bonding of CA4 onto poly(L-glutamic acid)-graft-polyethylene glycol (PLG-g-PEG) via Yamaguchi reaction. The obtained CA4-PL was characterized by ^{1}H NMR, GPC, and UV methods, and the properties of the nanoparticles composed of CA4-PL, including critical aggregation concentration, size and size distribution, and morphology, were investigated. CA4-PL can self-assemble to form micelle-like nanoparticles of 80~120 nm in diameter, which may have potential to improve the blood circulation period as well as the targetability of CA4, and find applications to treat various tumors when combined with traditional chemotherapy or radio therapy.

1. Introduction

Combretastatins are a class of natural phenols that are present in the bark of *Combretum caffrum*, commonly known as South African bush willow, and a variety of synthesis routes of the combretastatin skeleton are available [1]. Members of the combretastatin family possess ability to cause vascular disruption in tumors via binding to the β-subunit (known as the colchicine site) of tubulin, inhibiting tubulin polymerization, and thus preventing the synthesis of microtubules. CA4 is an effective antimitotic agent possessing potent cytotoxicity against

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a panel of cancer cells, including multi-drug resistant cancer cell lines, and previous studies also indicated that CA4 prodrug induced selective antivascular effects against tumor-associated endothelium [2]. In recent years, CA4 and its analogs were evaluated in clinical trials, including the phase II/III trials of phosphate prodrug of CA4 to treat neuroendocrine tumors or ovarian cancer as sponsored by OXiGENE Company. A phase I pharmacokinetic study revealed rapid dephosphorylation of the parent compound to CA4, with a short plasma half-life of only approximately 30 min [3].

The short half-life, as well as the low water solubility and deactivation of many CA4 analogs during storage greatly limited their antitumor efficacy and drug stability. Various CA4 analogous with increased water solubility, or cis-restriction property were synthesized for improving their bioavailability and efficiency [4]. Conjugation of drugs onto polymeric molecules to form macromolecular conjugates is a promising approach instead of formulating drugs with solubilizer(s) to circumvent the inadequate solubility of many anticancer drugs [5]. Pegylated polymeric nanoparticles or macromolecular conjugates also bear potentials of improving the stability and blood circulation period, and altering their *in vivo* distribution compared to the parent drugs.

Here, we reported a novel CA4 macromolecular conjugate (CA4-PL) by chemically linking CA4 molecule to the pegylated poly(glutamic acid) copolymer. As an amphiphilic macromolecule, CA4-PLs can self-assemble to form nanosized particles in aqueous media. Long circulation and improved tumor targeting depending on the pegylation modification and the well-known EPR effect were expected for such nanoparticles.

2. Materials and methods

2.1. Materials

CA4 was purchased from Great Forest Biomedical Ltd. (Hangzhou, China). Poly(glutamic acid) (PLG) was received from Changchun Institute of Applied Chemistry (Jilin, China). Monomethoxy PEG5000 (mPEG) was purchased from Sigma-Aldrich (Shanghai, China). 2,4,6-trichlorobenzoyl chloride (TCBC) was purchased from Heowns Biochemical Technology Co., Ltd. (Tianjin, China). Triethylamine (NEt_3), *N,N*-dimethyl formamide (DMF), ether, dichloromethane, deuterated water, and deuterated sodium hydroxide were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). DMF was dried with calcium hydride and distilled at reduced pressure before use. *N,N'*-Diisopropylcarbodiimide (DIC) and N-(4-Pyridyl) dimethylamine (DMAP) were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Sodium chloride (NaCl), sodium dihydrogen phosphate (NaH_2PO_4) and sodium hydrogen phosphate (Na_2HPO_4) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2. Synthesis of PLG-g-PEG

PLG-g-PEG was synthesized according to previously reported procedure [6]. Briefly, PLG was prepared by the ring-opening polymerization of BLG-NCA using n-hexylamine as the initiator at monomer/initiator molar ratio of 160. PLG and dried mPEG were the dissolved in anhydrous DMF by heating at 40 °C for 2 h. After the temperature was cooled down to 25 °C, DIC and DMAP were added in succession. After stirring at 25 °C for 2 d, the reaction mixture was precipitated by pouring into excess volume of ether and washed twice with ether. The precipitate was dried under vacuum and re-dissolved in DMF, placed in dialysis tube (MWCO 7000, Greenbird Technology Development Co. Ltd, Shanghai, China) and dialyzed against distilled water, and then freeze-dried to produce the PLG-g-PEG (white powder).

2.3. Synthesis of CA4-PL

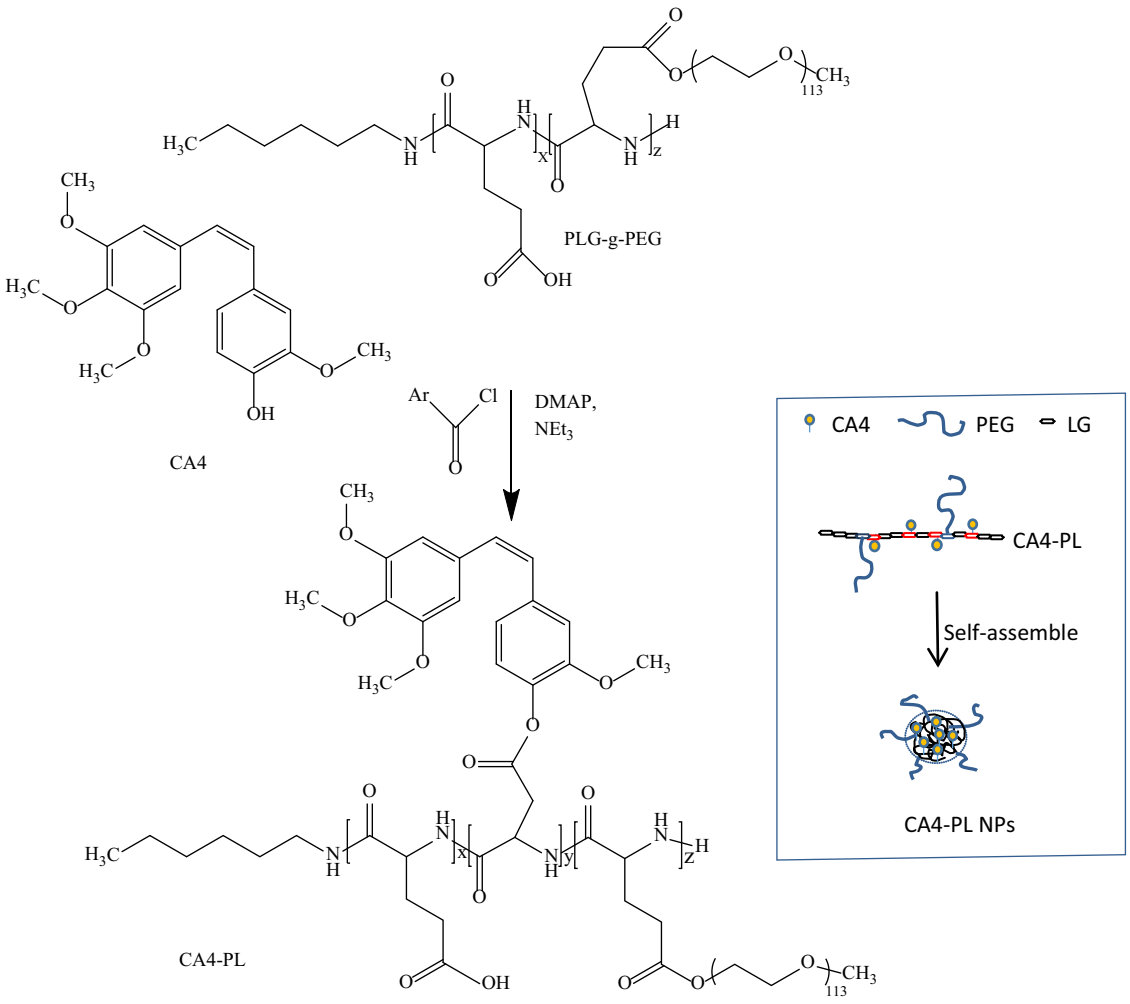
CA4-PL was synthesized by conjugating CA4 to PLG-g-PEG via Yamaguchi reaction with certain modification (see Scheme 1). Briefly, 600 mg of PLG-g-PEG was placed into dry flask and dissolved in 20 ml of dry DMF, and then 161 mg of NEt_3 dissolved in 1 ml of DMF and 390 mg of TCBC dissolved in 1 ml of DMF were added and mixed uniformly. 316 mg of CA4 dissolved in 2 ml of DMF, and 122.7 mg of DMAP dissolved in 2 ml of DMF were then added. The reaction was continued for 12 h under constant stirring. The reaction mixture was poured into excess volume of ether, centrifuged at 8000 rpm, discarded the supernatant. After drying under vacuum, the precipitate was re-dissolved in DMF and diluted with distilled water, ultra filtrated to remove DMF and impurities. The solution was then filtered and freeze-dried to produce the solid powder CA4-PL (yield: 60%).

2.4. Characterizations to CA4-PL

^{1}H NMR spectra were recorded on an AV 400 NMR Spectrometer (Bruker, Germany) in sodium deuteroxide/deuterium oxide ($NaOD/D_2O$) solution. GPC measurements were conducted on a GPC system (Ultrahydrogel Linear column, 1515 HPLC pump with 2414 Refractive Index Detector, Waters, USA) using phosphate buffer (0.2 M, pH 7.4) as the eluent (flow rate: 1 ml/min, 25 °C), and polyethylene glycol was used as standards. UV-Vis spectra of samples dissolved in DMF was scanned on a UV-5100 Spectrophotometer (Wanyee Science and Technology Co., Ltd, Anhui, China) at the wavelength range between 200 nm and 400 nm using DMF as the blank control.

2.5. Properties of the self-assembled nanoparticles

Critical aggregation concentration (*cac*) of CA4-PL was determined by fluorescence spectroscopy method with pyrene as the probe using a LS50B Luminescence Spectrometer (Perkin-Elmer, USA) with emission wavelength of 392 nm. The excitation fluorescence at 339 and 335 nm was monitored. Dynamic light scattering (DLS) measurements were performed on a Wyatt QELS instrument with a vertically polarized He-Ne laser (Wyatt Technology, USA) at 90° collecting optics. Zeta-potential was measured with a Zeta Potential/BI-90 Plus Particle Size Analyzer (Brookhaven, USA) at ambient temperature. A drop of the nanoparticle solution (about 0.1 mg/ml CA4-PL in water) was deposited onto a 230 mesh copper grid coated with carbon and allowed to dry in air at 25 °C before observation. TEM



Scheme 1 – Synthesis route of CA4-PL and illustration of self-assemble of CA4-PL into nanoparticles.

measurement was performed on a JEM-1011 Transmission Electron Microscope (JEOL, Japan) with an accelerating voltage of 100 kV.

2.6. *In vitro drug release*

For the drug release study, CA4-PL NPs aqueous suspension was introduced into a dialysis tube (MWCO 3500 Da, Greenbird Technology Development Co. Ltd, Shanghai, China), 50 ml of phosphate buffered saline of pH 7.4 or pH 5.5 was used as the release media, respectively. The samples were incubated at a shaking rate of 100 rpm in 37 °C constant temperature shock box (Fu Yi Electric Co., Ltd., Beijing). At predetermined intervals, 3 ml of release media was withdrawn and replaced with fresh PBS. The CA4 concentrations in the release media were determined by UV-5100 Spectrophotometer (Wanyee Science and Technology Co., Ltd, Anhui, China) at 309 nm. The λ_{max} of CA4 is 309 nm and a good linear range, so we use UV method in drug release study. The standard curve lines of pH 7.4 and pH 5.5 were determined, respectively. The drug release concentration can be calculated from the standard curves at pH = 5.5 and pH = 7.4.

3. Results and discussion

3.1. *Characterizations to PLG-g-PEG*

The ^{1}H NMR spectrum (400 M, NaOD/D_2O), GPC and UV spectra of PLG-g-PEG were shown in Fig. 1A. ^{1}H NMR (400 M, NaOD/D_2O): δ 4.12 ppm (t, -CH<), 3.52 ppm (s, -CH_2CH_2O-), 3.19 ppm (s, -OCH_3), 2.06 ppm (m, -CH_2COO-), 1.84 ppm and 1.74 ppm (m, -$CHCH_2$-). The PLG/mPEG weight ratio calculated as the ratio of the intensity of signal at 2.06 ppm (-CH_2COO-) to the intensity of signal at 3.52 ppm (-CH_2CH_2O-) in the ^{1}H NMR spectra was 1:2.05 that was approximate to the feed ratio of 1:2.

The synthesized PLG-g-PEG has a backbone of PLA of about 160 L-glutamic acid repeating units, and about 8.3 mPEG5000 chains on each backbone chain. It was calculated by hydrogen spectrum of hydrogen. Degree of polymerization of PLG and mPEG were 160 and 113, the mean molecular weight of PLG-g-PEG was 62140.

This result indicated that the condensation reactions of PLGs with mPEGs were highly efficient in DMF in the presence of DIC and DMAP.

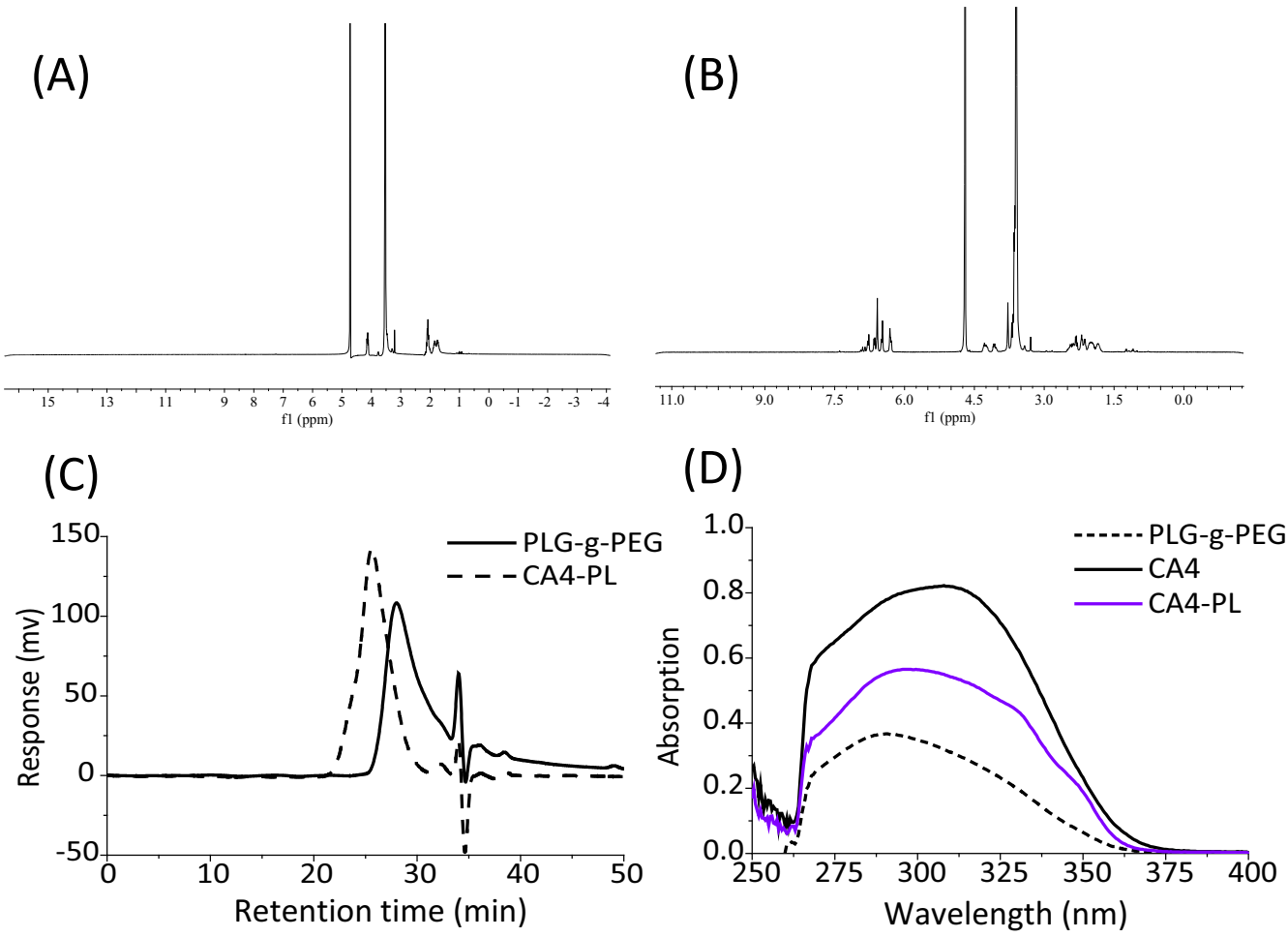


Fig. 1 – (A) ^{1}H NMR of PLG-g-PEG, (B) ^{1}H NMR of CA4-PL, (C) GPC of PLG-g-PEG and CA4-PL, and (D) UV spectra of CA4, CA4-PL and PLG-g-PEG (solvent: DMF).

3.2. *Characterizations to CA4-PL*

The ^{1}H NMR spectrum (400 M, NaOD/D2O), GPC and UV spectra of CA4-PL were shown in Fig. 1 B, C, D. ^{1}H NMR (400 M, NaOD/D_2O): δ 4.12 ppm (t, -CH<), 3.52 ppm (s, -CH_2CH_2O-), 3.19 ppm (s, -OCH_3), 2.06 ppm (m, -CH_2COO-), 1.84 ppm and 1.74 ppm (m, -$CHCH_2$-); δ 6.82~7.11 ppm (phenyl protons of CA4), and 3.73 ppm (methoxy protons of CA4). Drug loading (DL, %) was calculated as the percent of CA4 in CA4-PL to be 25 wt% by ^{1}H NMR.

$$\mathrm{DLC\%} = \frac{(\mathrm{MC}\times\mathrm{NC})}{(\mathrm{MC}\times\mathrm{NC}+\mathrm{MP}\times\mathrm{NP}+\mathrm{ME}\times\mathrm{NE})}\times 100\%$$

$$\mathrm{DLC\%} = \frac{(316\times 66)}{(316\times 66+160\times 129+5000\times 8.3)}\times 100\% = 25\%$$

NP, NE, and NC represented the number of moles of PLG, PEG, and CA4; MP, ME, and MC respectively represented the molar mass of PLG, PEG, and CA4.

Degree of polymerization of PLG and mPEG were 160 and 113, the mean molecular weight of CA4-PL was 82,996.

The GPC curves of PLG-g-PEG and CA4-PL had all exhibited unimodal peak (see Fig. 1C), the peaks of PLG-g-PEG and CA4-PL in the GPC spectra appeared in separate retention time, and the elution of CA4-PL was earlier than that of PLG-g-PEG. The UV-Vis spectra indicated maximum absorption peak of CA4, PLG-g-PEG and CA4-PL of 309 nm, 290 nm and 298 nm, respectively (Fig. 1D). These results demonstrated the chemical conjugation of CA4 to PLA-g-PEG.

3.3. *Properties of the self-assembled nanoparticles*

The self-assembling behavior of the CA4-PL was investigated for *cac* value, as well as particle size and morphology. The *cac* value was obtained from the plot of fluorescence intensity ratio of I339/I335 versus lgC, which was determined to be 1.04 ng/mL (Fig. 2A). The low *cac* value indicated good stability of CA4-PL self-assembles at extremely lower concentrations in the blood circulation. The hydrodynamic radii (R_h) of the CA4-PL self-assembles as measured by DLS were ranging between 80 and120 nm. The zeta-potential of CA4-PL self-assembling nanoparticles was –27.3 ± 1.0 mV. TEM image showed that CA4-PL aggregated to spherical-like nanoparticles with a narrow size distribution, which was in accordance to that determined by DLS (Fig. 2B, C).

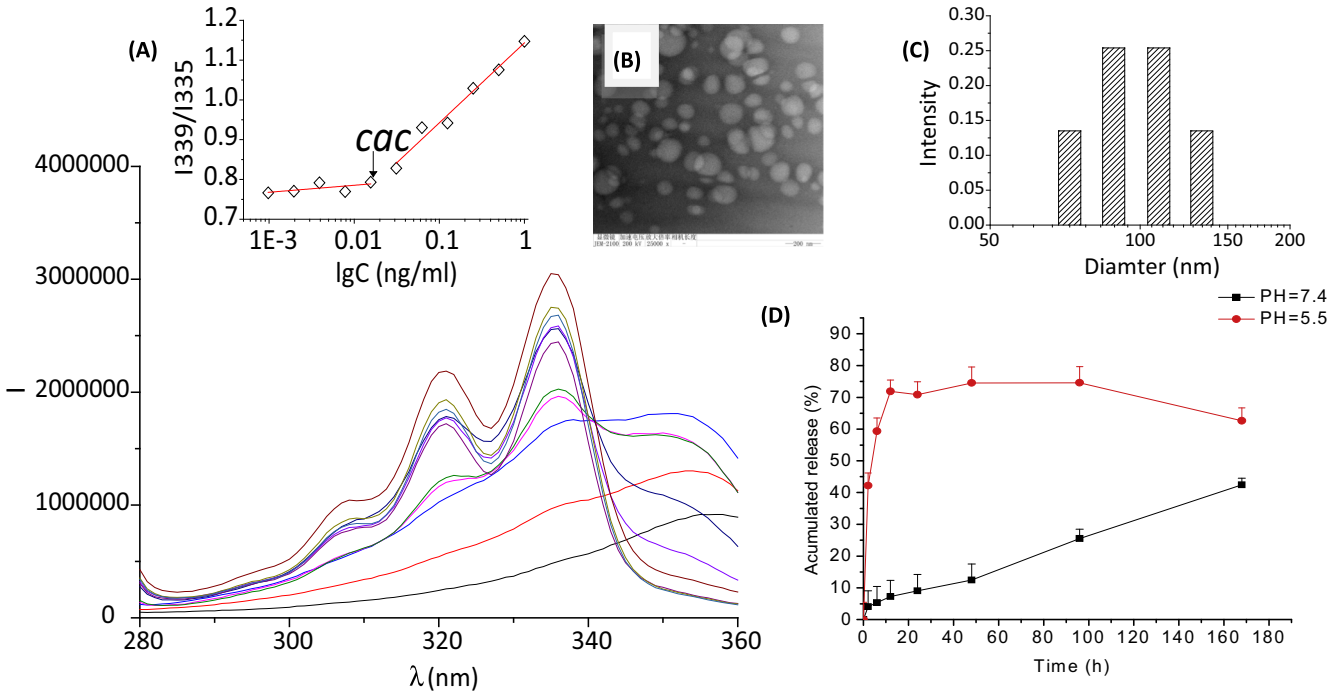


Fig. 2 – (A) Fluorescent spectra of pyrene in aqueous solutions of various CA4-PL concentrations (insert: I339/I335 versus lgC plot to calculate the *cac* value); (B) TEM image of CA4-PL self-assembled nanoparticles; (C) Size distribution of CA4-PL nanoparticles; (D) *In vitro* drug release profiles of the CA4-PL self-assembled nanoparticles in PBS of pH 7.4 and pH 5.5, respectively.

3.4. *In vitro drug release*

The release profiles of CA4 from CA4-PL self-assembled nanoparticles were shown in Fig. 2D. The *in vitro* CA4 release behavior of CA4-PL nanoparticle was investigated using a dialysis method with media of pH 7.4 and pH 5.5, respectively. The results showed that the release rate of CA4 was obviously higher at pH 5.5 than at pH 7.4, which is due to the higher degradation rate of the ester bond of the conjugate at lower pH media.

3.5. *Discussion*

Esterification between the carboxyl group of PLG and hydroxyl group of mPEG by DMAP/DIC reaction can conjugate mPEG chain onto PLG with a very high conversion rate [7–9], but the addition of DIC can lead to irreversible side reaction to the carboxyl group on PLG. As the feed of mPEG in our present study was very low, the mass of DIC required and the resulted side reaction was very limited. However, with a one-step method to synthesis CA4-PL using the mixture of mPEG, PLG, and CA4, larger amount of DIC must be added in order to obtain higher drug conjugation ratio, thus obvious side reaction occurred. Yamaguchi reaction provides another feasible synthesis procedure of the above esterification [10,11], but its disadvantage lies in the difficulty of conjugating PEG to PLG completely, and complicated purification procedures are needed to remove the un-reacted PLG and PEG. In this study, a two-step synthesis method was introduced. PLG-g-PEG was firstly synthesized by DMAP/DIC method, and then CA4 was chemically conjugated onto PLG-g-PEG via Yamaguchi reaction.

CA4-PL was an amphiphilic macromolecule composed of hydrophilic segment of mPEG and hydrophobic segment of CA4 conjugated LG links (Scheme 1), which will self-assemble into nanoparticles of micelle-like structure in aqueous media at relatively low concentration (*cac* value: 1.04 ng/ml).

As the ester bond that conjugate CA4 to PLG-g-PEG was liable to environment, such as breaking in aqueous media and degradation by enzyme, the drug is readily liberate from CA4-PL or the self-assembled nanoparticles of CA4-PL. According to the *in vitro* release results (Fig. 2D), CA4-PL is apt to be degraded, and then the small molecular weight CA4 can diffuse through the dialysis membrane and release into the media. Due to the lower pH of tumor extracellular micro-environment, the pH-responsive release of CA4-PL nanoparticles is beneficial to decrease unexpected drug release in blood circulation (pH ~7) and readily release in tumor region, thus greatly reduce the side effects to normal tissues and improve the overall therapeutic profits. The pH responsiveness is one of the most frequently used biological stimuli exploited for triggered drug release, because pH values vary in the different biological compartments and the cellular organelles. For example, the pH value at the tumor extracellular environment is more acidic than that in blood. The pH values in the endosomes and lysosomes are even lower. The pH-sensitive drug carriers not only greatly reduce the side effects to normal tissues in blood circulation by minimizing drug loss, but also undergo fast release during the endocytosis process after take-up by the tumor cells, which could improve the overall therapeutic efficacy.

The self-assembled nanoparticles of pegylated CA4-PL are expected to bear long circulation period in blood circulation, as well as tumor targeting via the well-known EPR effect and altering *in vivo* distribution compared to the parent drugs, thus induce antivascular effects against tumor-associated endothelium. Combined therapy of CA4-PL together with traditional chemotherapy (e.g. cis-platin) or radiotherapy may play a certain effect on tumor angiogenesis edge [12–15], thus find promising applications in tumor therapy.

4. Conclusion

CA4-PL was synthesized by chemical conjugation of CA4 to PLG-g-PEG via Yamaguchi reaction. This amphiphilic macromolecular conjugate can self-assemble to form nanosized particles crowned by hydrophilic PEG. CA4-PL and its self-assembled nanoparticles may find great application to treat various tumors, especially when combined with normal chemotherapy or radiotherapy.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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REFERENCES

[1] Singh R, Kaur H. Advances in synthetic approaches for the preparation of combretastatin-based anti-cancer agents. Synthesis 2009;2009:2471–91.
[2] Dark G, Hill S, Prise V, et al. Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. Cancer Res 1997;57:1829–34.
[3] Dowlati A, Robertson K, Cooney M, et al. A phase I pharmacokinetic and translational study of the novel vascular targeting agent combretastatin A-4 phosphate on a single-dose intravenous schedule in patients with advanced cancer. Cancer Res 2002;62:3408–16.
[4] Rajak H, Kumar Dewangan P, Patel V, et al. Design of combretastatin A-4 analogs as tubulin targeted vascular disrupting agent with special emphasis on their cis-restricted isomers. Curr Pharm Des 2013;19:1923–55.
[5] Dragojevic S, Ryu J, Raucher D. Polymer-based prodrugs: improving tumor targeting and the solubility of small molecule drugs in cancer therapy. Molecules 2015;20:21750–69.
[6] Yu H, Tang Z, Zhang D, et al. Pharmacokinetics, biodistribution and in vivo efficacy of cisplatin loaded poly(L-glutamic acid)-g-methoxy poly(ethylene glycol) complex nanoparticles for tumor therapy. J Control Release 2015;205:89–97.
[7] Iijima M, Nagasaki Y, Okada T, et al. Core-polymerized reactive micelles from heterotelechelic amphiphilic block copolymers. Macromolecules 1999;32(6):1140–6.
[8] Osada K, Christie RJ, Kataoka K. Polymeric micelles from poly(ethylene glycol)-poly(amino acid) block copolymer for drug and gene delivery. J R Soc Interface 2009;6:S325–39.
[9] La SB, Okano T, Kataoka K. Preparation and characterization of the micelle-forming polymeric drug indomethacin-incorporated polyfethylene oxide)-poly(β-benzyl L-aspartate) block copolymer micelles. J Pharm Sci 1996;85:85–90.
[10] Dhimitruka I, SantaLucia J. Investigation of the Yamaguchi esterification mechanism. Synthesis of a Lux-S enzyme inhibitor using an improved esterification method. Org Lett 2006;8:47–50.
[11] Ohmori K, Tamiya M, Kitamura M, et al. Regio- and stereocontrolled total synthesis of benanomicin B. Angew Chem Int Ed Engl 2005;44:3871–4.
[12] Dark GG, Hill SA, Prise VE, et al. Combretastatin A-4, an agent that displays potent and selective toxicity toward tumor vasculature. Cancer Res 1997;5:1829–34.
[13] Nabha SM, Mohammad RM, Wall NR, et al. Evaluation of combretastatin A-4 prodrug in a non-Hodgkin's lymphoma xenograft model: preclinical efficacy. Anticancer Drugs 2001;12:57–63.
[14] Zweifel M, Jayson GC, Reed NS, et al. Phase II trial of combretastatin A4 phosphate, carboplatin, and paclitaxel in patients with platinum-resistant ovarian cancer. Ann Oncol 2011;22:2036–41.
[15] Siemann DW, Chaplin DJ, Walicke PA. A review and update of the current status of the vasculature-disabling agent combretastatin-A4 phosphate (CA4P). Expert Opin Investig Drugs 2009;18:189–97.