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Preparation, characterization, and in vitro efficacy of O-carboxymethyl chitosan conjugate of melphalan

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ABSTRACT

A series of melphalan-O-carboxymethyl chitosan (Mel-OCM-chitosan) conjugates with different spacers were prepared and structurally characterized. All conjugates showed satisfactory water-solubility (160-217 times of Mel solubility). In vitro drug release behaviors by both chemical and enzymatic hydrolysis were investigated. The prodrugs released Mel rapidly within papain and lysosomal enzymes of about 40–75%, while released only about 4–5% in buffer and plasma, which suggested that the conjugates have good plasma stability and the hydrolysis in both papain and lysosomes occurs mostly via enzymolysis. It was found that the spacers have important effect on the drug content, water solubility, drug release properties and cytotoxicity of Mel-OCM-chitosan conjugates. Cytotoxicity studies by MTT assay demonstrated that these conjugates had 52–70% of cytotoxicity against RPMI8226 cells in vitro as compared with free Mel, indicating the conjugates did not lose anti-cancer activity of Mel. Overall these studies indicated Mel-OCM-chitosan conjugates as potential prodrugs for cancer treatment.

1. Introduction

Melphalan is a cytotoxic drug that acts as a bifunctional alkylating agent on DNA and was introduced into clinic since the late 1950s. Mel can be administered orally or systemically to treat variety of cancers including myelomas, ovarian cancer and sarcomas. The main obstacles for Mel in clinical applications are due to its extremely poor aqueous solubility, relatively instability, rapid plasma clearance, poor bioavailability and biocompatibility (David, Roger, & Valentino, 1999), which would cause severe side effects.

To solve these problems, many efforts have been made to synthesize derivatives of Mel. The chemical structure of Mel invites to modification of both the N- and C-termini as well as incorporation into peptides (Morris, Atassi, Guilbaud, & Cordi, 1997; Peyrode et al., 2012; Sartania et al., 1999; Scutaru, Wenzel, & Gust, 2011; Zhao, Meng, Yuan, & Lan, 2010). For instance, beta-alanyl-Mel (Tsay & Lloyd, 1987) and proline prodrug of Mel (Mittal et al., 2004) have been prepared. These compounds showed much higher cytotoxic activity and had the potential for enhanced tumor selectivity. Nevertheless, these prodrugs were still water-insoluble and underwent unavoidable rapid elimination of the drugs from circulation.

Conjugating drug molecules with biocompatible water-soluble polymer is an alternative way to solve these problems. Polymeric prodrug have several advantages including: (1) an increase in water solubility of low soluble or insoluble drugs, and therefore, enhance drug bioavailability; (2) protection of drug from deactivation and preservation of its activity during circulation; (3) an improvement in pharmacokinetics; (4) the ability of provide passive targeting due to the enhanced permeability and retention (EPR) effect (Khandare & Minko, 2006; Mahato, Tai, & Cheng, 2011). Many polymeric prodrugs which contain alkylating agents have been synthesized, including poly amino acid (poly-L-lysine and poly-L-glutamic acid)-Mel (Yasunori et al., 1984), HPMA copolymer-Mel (Duncan et al., 1991), albumin-chlorambucil (Kratz et al., 1998), transferrin-chlorambucil (Beyer et al., 1998), PAMAM-chlorambucil (Bielawski, Bielawska, Muszyńska, Poplawska, & Czarnomysy, 2011) and fluorodeoxyglucose-chlorambucil (Miot-Noirault et al., 2011). These investigations proved that such conjugates incorporating a covalent linkage for attachment of drugs to carriers were suitable for alkylating agents delivery.

The appropriate polymeric carriers must be biocompatible and water-soluble. OCM-chitosan is a water soluble chitosan derivative, seems to be one of the most useful candidates for the drug carrier because of its non-toxicity, biodegradability (Fu et al., 2011), biocompatibility and high water solubility (Zheng, Han, Yang, Liu, 2011; Zheng, Rao, et al., 2011). Besides, OCM-chitosan can be easily derived from chitin, which is cheap and abundance in nature. OCM-chitosan contains a large number of —COOH and —NH_2 groups in the molecular that can be easily conjugated to drugs and proteins by either direct attachment or through a linker. Chitosan derivatives

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based prodrugs have gained significant interest for drug delivery (Lee, Kim, Lee, & Jon, 2009; Sabaa, Mohamed, Mohamed, Khalil, & Abd El Latif, 2010; Tokura, Miura, Johmen, Nishi, & Nishimura, 1994; Wang et al., 2011) to increase the longevity of therapeutic agents in the circulation and direct antitumor drugs to the tumor tissues through passive accumulation in the tumor.

In polymeric prodrug design, polymer linkage plays a crucial role in determining therapeutic potentials. Amino and small peptide linkers are widely used (Greenwald, 2001; Mitsunori, Hiroyuki, Toshiro, Takehiko, & Satoshi, 2000) due to their chemical versatility for covalent conjugation and biodegradability, which can be specific substrates for plasmin enzyme and proteinases whose concentration are much higher in various kinds of tumor mass (Ding et al., 2012).

In this work, for improving water-solubility, systemic circulation time, and pharmacokinetic profiles of Mel, Mel-OCM-chitosan conjugates linked with different amino acid spacers (including L-glycine (Gly), L-phenylalanine (Phe), L-leucine (Leu) and L-proline (Pro)) were synthesized. The solubility, in vitro drug release, cell cytotoxicity of Mel-OCM-chitosan conjugates were systematically investigated. It was expected that the conjugates would be soluble and stable during circulation, while in the targeted cells, the conjugates would lead to a controlled release of Mel.

2. Experimental

2.1. Materials

OCM-chitosan (molecular weight 8.6×10^4, degree of deacetylation 91% and degree of substitution 85%) was purchased from Qingdao Xunbo Biotechnology Co., Ltd. (China). Mel was purchased from Suzhou Lide Chemistry Co., Ltd. (China). Amino acids were purchased from Aladdin Reagent Co., Ltd. (China). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), 1,3-dicyclohexyl carbodiimide (DCC) and N-hydroxysuccinimide (NHS) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma–Aldrich Chemical Co., Ltd. (St. Louis, MO). Adult male Sprague-Dawley rats (200–225 g) were obtained from Hubei Experimental Animal Center (Wuhan, China). All other reagents were of analytical grade, and used as received without further purification. RPMI8226 cells were purchased from ATCC (USA) and grown in RPMI 1640 medium containing 12% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.2. Synthesis of Mel-OCM-chitosan conjugates

2.2.1. Synthesis of Fmoc-Mel

The synthesis of Fmoc-Mel was referred to a previous report (Zhao et al., 2010). 9-Fluorenylmethyl N-succinimidyl carbonate (Fmoc-Osu, 3.71 g, 11 mmol) in dioxane (20 mL) was added to an ice-cold solution of Mel (3.21 g, 10 mmol) in a mixture of dioxane (50 mL), distilled water (15 mL), and $NaHCO_3$ (0.93 g, 11 mmol). The mixture was stirred for 2 h at 0 °C and then for 20 h at room temperature. The reaction mixture was concentrated and partitioned between ethyl acetate and distilled water. The mixture was adjusted to pH 2 with hydrochloric acid, and then the aqueous phase was extracted with ethyl acetate (3× 50 mL). The combined organic phases were washed with distilled water and brine and dried over $MgSO_4$. The solid residue was purified by column chromatography (hexane:ethyl acetate = 3:1, v/v) to give the compound as white solid powder.

2.2.2. Synthesis of Fmoc-Mel-amino acid

A stirred solution of Fmoc-melphalan (10 mmol, 5.43 g) and NHS (1.05 equiv., 10.5 mmol, 1.21 g) in dry THF (100 mL) was treated at 0 °C with DCC (1.05 equiv., 10.5 mmol, 2.17 g). After 2 h, the mixture was allowed to warm to room temperature and stirred for 22 h. The precipitate was removed by filtration and washed with dry THF (2× 10 mL). The washes and filtrate containing Fmoc-melphalan anhydride were combined. Amino acid (10 mmol, the amino acid including Gly, Phe, Leu and Pro) was slowly added to a mixture solution of $NaHCO_3$ (1 equiv., 10 mmol, 0.84 g), water (50 mL) and THF (30 mL). After stirring for 15 min, the THF solution of Fmoc-melphalan anhydride prepared above was added to this suspension. The mixture was stirred at room temperature for 22 h, and then THF was removed as much as possible in vacuo at 30 °C, then the mixture was partitioned between ethyl acetate and water and adjusted to pH 2 with hydrochloric acid, the aqueous phase was extracted with ethyl acetate (3× 15 mL). The combined organic phases were washed with distilled water and brine and dried over $MgSO_4$. The resulting solution was concentrated and purified by column chromatography (hexane:ethyl acetate = 1:1, v/v) to give the compound as off-white solid.

Fmoc-Mel: ^{1}H NMR (CD_3Cl, ppm) d: δ = 2.88–3.14 (m, 2H, CH_2Ph), 3.48–3.78 (m, 8H, $N(CH_2CH_2)_2$), 4.2 (t, 1H, CH[Fmoc]), 4.33–4.53 (m, 2H, CH_2 [Fmoc]), 4.65 (m, 1H, CHCO), 5.4 (1H, m, NH), 6.6 (d, 2H, arom meta), 7.0 (d, 2H, arom ortho), 7.28–7.83 (8H, m, Ar[Fmoc]).

Fmoc-Mel-Gly: ^{1}H NMR (CD_3Cl, ppm) d: δ = 2.95–3.12 (2H, CH_2Ph), 3.45–3.72 (8H, $N(CH_2CH_2)_2$), 4.85–4.65 (6H, CH[Fmoc], CH_2[Fmoc], CHCO, CH_2[Gly]), 5.25–5.4 (2H, NH—CO), 6.5–7.0 (4H, benzene ring), 7.25–7.8 (8H, Ar[Fmoc]).

Fmoc-Mel-Phe: ^{1}H NMR (CD_3Cl, ppm) d: δ = 2.85–3.15 (4H, CH_2Ph), 3.51–3.75 (8H, $N(CH_2CH_2)_2$), 4.15–4.8 (5H, CH[Fmoc], CH_2[Fmoc], CHCO), 5.3–5.5 (2H, NH—CO), 6.6–7.25 (8H, benzene ring), 7.28–7.85 (8H, Ar[Fmoc]).

Fmoc-Mel-Leu: ^{1}H NMR (CD_3Cl, ppm) d: δ = 0.91 (6H, $CH(C\mathbf{H}_3)_\mathbf{2}$), 1.5–1.75 (1H, $C\mathbf{H}(CH_3)_\mathbf{2}$), 2.88–3.14 (2H, CH_2Ph), 3.48–3.78 (8H, $N(CH_2CH_2)_2$), 4.1–4.65 (7H, CH[Fmoc], CH_2[Fmoc]), CHCO, CH[Leu], CH_2[Leu]), 5.68 (2H, NH—CO), 6.6–7.1 (4H, benzene ring), 7.3–7.82 (8H, Ar[Fmoc]).

Fmoc-Mel-Pro: ^{1}H NMR (CD_3Cl, ppm) d: δ = 1.51–2.25 (4H, CH_2[Pro]), 2.85–3.25 (2H, CH_2Ph), 3.48–3.78 (10H, $N(CH_2CH_2)_2$, CH_2[Pro]), 4.15–4.75 (5H, CH[Fmoc], CH_2[Fmoc], CH[Pro], CHCO), 5.35 (1H, NH—CO), 6.5–7.2 (4H, benzene ring), 7.25–7.8 (8H, Ar[Fmoc]).

2.2.3. Synthesis of Mel-OCM-chitosan conjugates

Fmoc-Mel-amino acid (2 mmol) was mixed with EDC (383 mg, 2 mmol) in 20 mL N,N-dimethyl formamide (DMF) and reacted at room temperature for 4 h. Then, NHS (230 mg, 2 mmol) was added to the above mixture, and the whole mixture was stirred for 6 h at room temperature to obtain NHS-activated Fmoc-Mel-amino acid. OCM-chitosan was dissolved in 10 mL distilled water, and NHS-activated Fmoc-melphalan-amino was added drop-wise to solution of OCM-chitosan over 30 min. The mixture was reacted at room temperature for 48 h, and the water of the solution was removed in vacuo at 50 °C with adding suitable amount of toluene several times. Then the N-Fmoc derivatives of Mel were deprotected with piperidine.

Finally, the products were exhaustively dialyzed (MWCO 14,000) against DMF and deionized water, and then lyophilized to obtain the white, cotton wool-like products. Mel-OCM-chitosan conjugates without amino acid spacer was synthesized with the same method. The products were synthesized and named in Table 1.

Table 1
Synthesis of Mel-OCM-chitosan conjugates with different linkers and Mel contents.

Samples	Mel/OCM-chitosan (molar ratio)	Mel content (mg/100 mg)	Mel substitution degree (mol/mol)	Equivalent solubility (mg/mL)
Mel-OCM-chitosan	1:1	15.1	0.133	1.12
Mel-Gly-OCM-chitosan-1	1:1	15.9	0.147	1.43
Mel-Gly-OCM-chitosan-2	1:2	10.5	0.090	1.52
Mel-Phe-OCM-chitosan-1	1:1	14.7	0.141	1.35
Mel-Phe-OCM-chitosan-2	1:2	8.6	0.074	1.45
Mel-Leu-OCM-chitosan	1:1	11.9	0.107	1.39
Mel-Pro-OCM-chitosan	1:1	12.6	0.113	1.48

2.3. Instrumental analyses

FT-IR spectrum analysis: Samples were prepared as KBr pellet and scanned against a blank KBr pellet background at range of 600–4000 cm^{-1} and characterized by FT-IR spectrometer (Bruker Tesor 27, Germany).

^1H NMR spectroscopy analyses: The ^1H NMR spectrums were determined on a Varian 600 spectrometer (Varian, USA) using tetramethylsilane as internal standard at 25 °C. The samples of Fmoc-Mel, Fmoc-Mel-Gly, Fmoc-Mel-Phe, Fmoc-Mel-Leu, and Fmoc-Mel-Pro were dissolved in $CDCl_3$, while OCM-chitosan and Mel-OCM-chitosan conjugates were dissolved in D_2O.

2.4. Drug content of Mel-OCM-chitosan conjugates

Content of incorporated melphalan was determined by measurement of the UV absorption of the conjugates solution in distilled water at 261 nm. The Mel content was determined with the help of a calibration curve of Mel in mixture solution (methanol:water:acetic acid = 49.5:49.5:1) range from 2 to 50 μg/mL with $R^2 = 0.99636$. The Mel content was calculated as follows:

$$\text{Mel\%} = \left(\frac{m_{\text{Mel}}}{m_{\text{prodrug}}}\right) \times 100$$

2.5. Solubility of Mel-OCM-chitosan conjugates

To evaluate solubility of the conjugates and Mel, 20 mg Mel-*OCM-chitosan* conjugates or Mel was added into 1 mL deionized water respectively, and the mixtures were vortexed for 5 min, sonicated for 5 min, and centrifuged at 6000 rpm for 5 min. The supernatants were collected and analyzed using UV spectrophotometer. The amounts of Mel were quantified by measuring UV absorption at a wavelength of 261 nm.

2.6. In vitro drug release of Mel-OCM-chitosan conjugates

The chemical hydrolysis of the conjugates was carried out in triplicates in phosphate-buffered saline (PBS) at pH 7.4 and 5.8

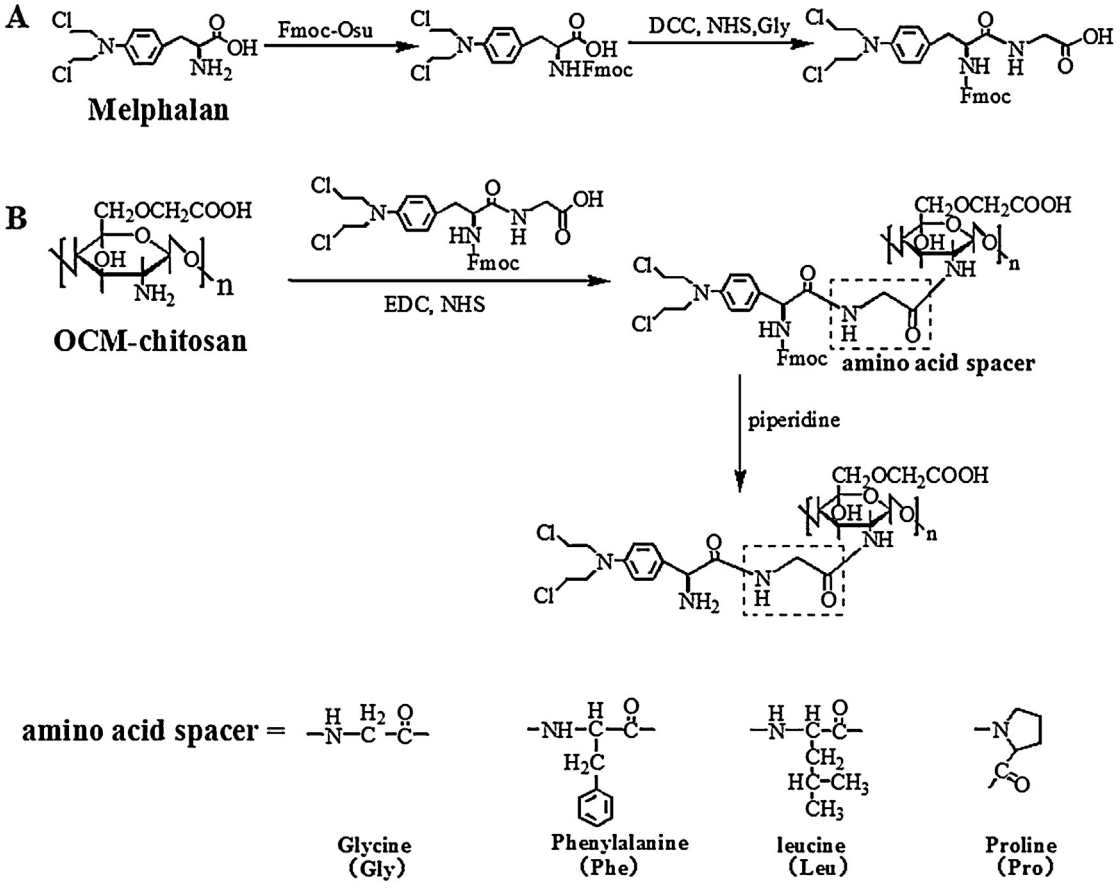

Fig. 1. (A) Synthesis of Fmoc-Mel-Gly, (B) synthesis of Mel-Gly-OCM-chitosan. Conjugates with other spacers (Phe, Leu, Pro) were synthesis with the same method.

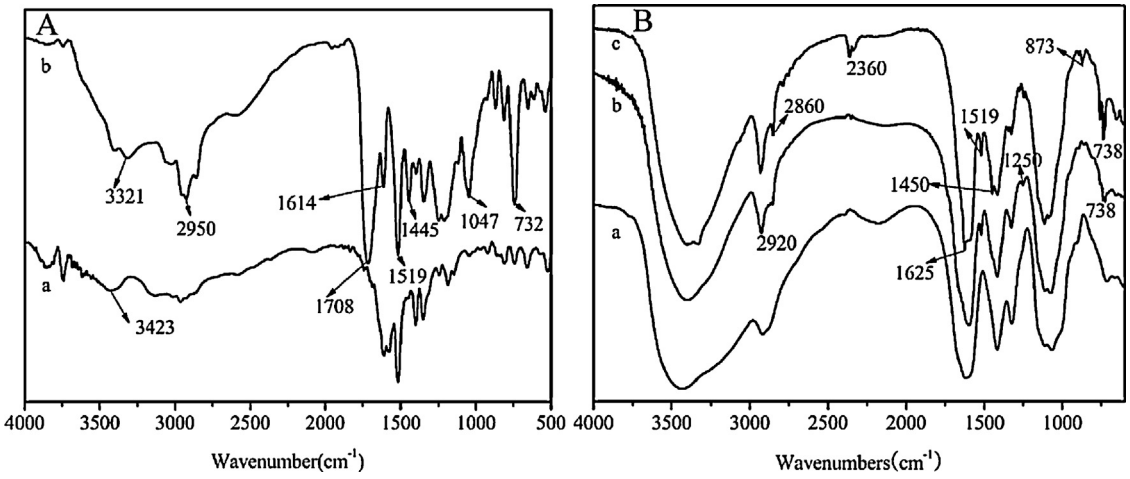

Fig. 2. FT-IR spectra: (A) – (a) Mel, (b) Fmoc-Mel; (B) – (a) OCM-chitosan, (b) Mel-OCM-chitosan (c) Mel-Pro-OCM-chitosan.

at 37 °C, respectively. The conjugates were dissolved in PBS and stirred mildly. At the scheduled time, 20 μL of the solution was removed and diluted with ice cold methanol (380 μL, containing 2% acetic acid). The mixture was vortexed for 5 min, sonicated for 1 min, and centrifuged at 6000 rpm for 5 min. 20 μL of the supernatants were injected and analyzed by HPLC method to determine the release of Mel from conjugates.

The method for analysis of Mel release in plasma and enzyme solution was similar to that of hydrolysis in PBS, except for the pretreatment process. Rat blood and liver was obtained from adult male Sprague-Dawley rats. Approximately 4 IU of heparin was added to each mL of blood to prevent coagulation (Mehvar, Dann, & Hoganson, 2000). The blood was immediately centrifuged at 6000 rpm, 4 °C for 5 min to collect the pale yellow supernatant as plasma. Rat liver lysosomal enzymes (tritosomes) were prepared by differential centrifugation of the liver homogenate solution according to the method of Bonifacino, Dasso, Harford, Lippincott-Schwartz, and Yamada (2009, chaps. 3.0.1–3.0.7). Papain and tritosomes solution need to be activated with 1 mM ethylenediaminetetra-acetic acid (EDTA) and 5 mM reduced glutathione at 37 °C for 15 min before used. In all cases, a bounded melphalan concentration of 1 mg/mL was used.

2.7. In vitro cytotoxicity

Evaluation of cytotoxicity of the conjugates was performed by MTT method. RPMI8226 cells were seeded into 96-well microtiter plates at a density of 5×10^4 cells/well in RPMI 1640 medium and incubation for 24 h. Mel or Mel-OCM-chitosan conjugates (corresponding Mel concentrate of 20–100 μg/ml) were added to the culture medium. After incubation 48 h, 10 μL MTT (5 mg/mL) solution in PBS (pH 7.4) was added to each well and further incubated in 5% CO_2 incubator at 37 °C for 4 h. After removal of the MTT containing medium, 100 μL DMSO was added to dissolve the formazan crystals formed in live cells. Finally, the absorbance was measured at 570 nm using a microplate reader (BIO-RAD, Model 550, USA). The relative cell inhibition ratio (%) was calculated by $(OD_{control} - OD_{sample})/OD_{control} \times 100$. The data were all corrected by the blank group with media in the absence of RPMI8226 cells.

3. Results and discussion

3.1. Synthesis and structural analysis of intermediates and prodrugs

In this study, we used different amino acid as spacers to synthesize a serial of Mel-OCM-chitosan conjugates and the synthesis route of Mel-OCM-chitosan are illustrated in Fig. 1. The $-NH_2$ of Mel were first protected with Fmoc-Osu. Then, the —COOH was activated and chemically coupled with the $-NH_2$ of different amino acid linkers by carbodiimide chemistry. In the following step, Fmoc-Mel and Fmoc-Mel-amino acid were grafted onto the $-NH_2$ of OCM-chitosan under the catalysis of EDC·HCl and NHS. The removal of the Fmoc moiety was achieved with piperidine treatment.

The FT-IR spectra of Mel, Fmoc-Mel are shown in Fig. 2A. The basic characteristics of Mel (see curve a) are shown at: 3423 cm^{-1} ($-NH_2$ stretch), 2950 cm^{-1} (—OH, $-CH_2$, C—H stretch), 1614 cm^{-1} (C=O stretch), 1519 and 1445 cm^{-1} (C=C stretch), 1350 cm^{-1} (C—N stretch), 807.7 cm^{-1} (C—H bend of benzene ring), 732 cm^{-1} (C—Cl stretch). In comparison with the Mel spectrum, the new absorption band appearing at 3321 cm^{-1} (amide N—H stretch), 1708 cm^{-1} (amide I band C=O stretch), 1047 cm^{-1} (C—O—C stretch) and the increased intensities at 2950.2 cm^{-1} (C—H stretch), 1641 cm^{-1} (amide II band C=O stretch), 1520 cm^{-1} (amide II band N—H bend) while the decreased intensity at 3423 cm^{-1} (primary amines) in the Fmoc-Mel spectrum (see curve b) indicated the formation of Fmoc-Mel. Fig. 2B shows the FT-IR spectra of OCM-chitosan, Mel-OCM-chitosan and Mel-Pro-OCM-chitosan. The characteristics of OCM-chitosan are shown at: 3500–3200 cm^{-1} (O—H stretch overlapped with N—H stretch), 1620 cm^{-1} and 1417 cm^{-1} (COO^- antisymmetric and symmetric stretch), 1060 cm^{-1} (C—O—C stretching vibration in the glucopyranose ring). Compared with OCM-chitosan, several new IR signals attributed to the Pro spacer and Mel structure are shown in IR spectrum of Mel-Pro-OCM-chitosan. The new absorption band appearing at 738 cm^{-1} (C—Cl stretch), 873 cm^{-1} (C—H bend of benzene ring), 1250 cm^{-1} (C—H stretch of benzene ring) and 1450 cm^{-1} (C—C stretch of benzene ring) can be attributed to Mel. And new absorbance at 1519 cm^{-1} and 1625 cm^{-1} (amide II band N—H bend) belong to the amide bond between OCM-chitosan and Pro and Mel. In addition, a new absorption band appearing at 2860 cm^{-1} and the increased intensity at 2920 cm^{-1} were corresponded to C—H stretching mode of the methylene in Mel and Pro.

^1H NMR spectra of OCM-chitosan, Mel-OCM-chitosan and Mel-Pro-OCM-chitosan are shown in Fig. 3F. The protons of OCM-chitosan are assigned as follows (ppm): 1.98 ($-COCH_3$), 2.78 (CH, carbon 2 of glucosamine ring), 3.3–3.9 (CH, carbon 2, 3, 4, 5 and 6 of glucosamine ring), 4.47 (CH, carbon 1 of glucosamine ring), 4.75 (O—CH_2—COOH). Comparing Mel-OCM-chitosan spectrum with OCM-chitosan spectrum, new peaks appears at 6.90 and 7.05 ppm were assigned to the protons of benzene rings of Mel. By comparison of Mel-Pro-OCM-chitosan conjugate with Mel-OCM-chitosan, additional chemical shifts at 4.3–4.5 and 2.2 ppm can be

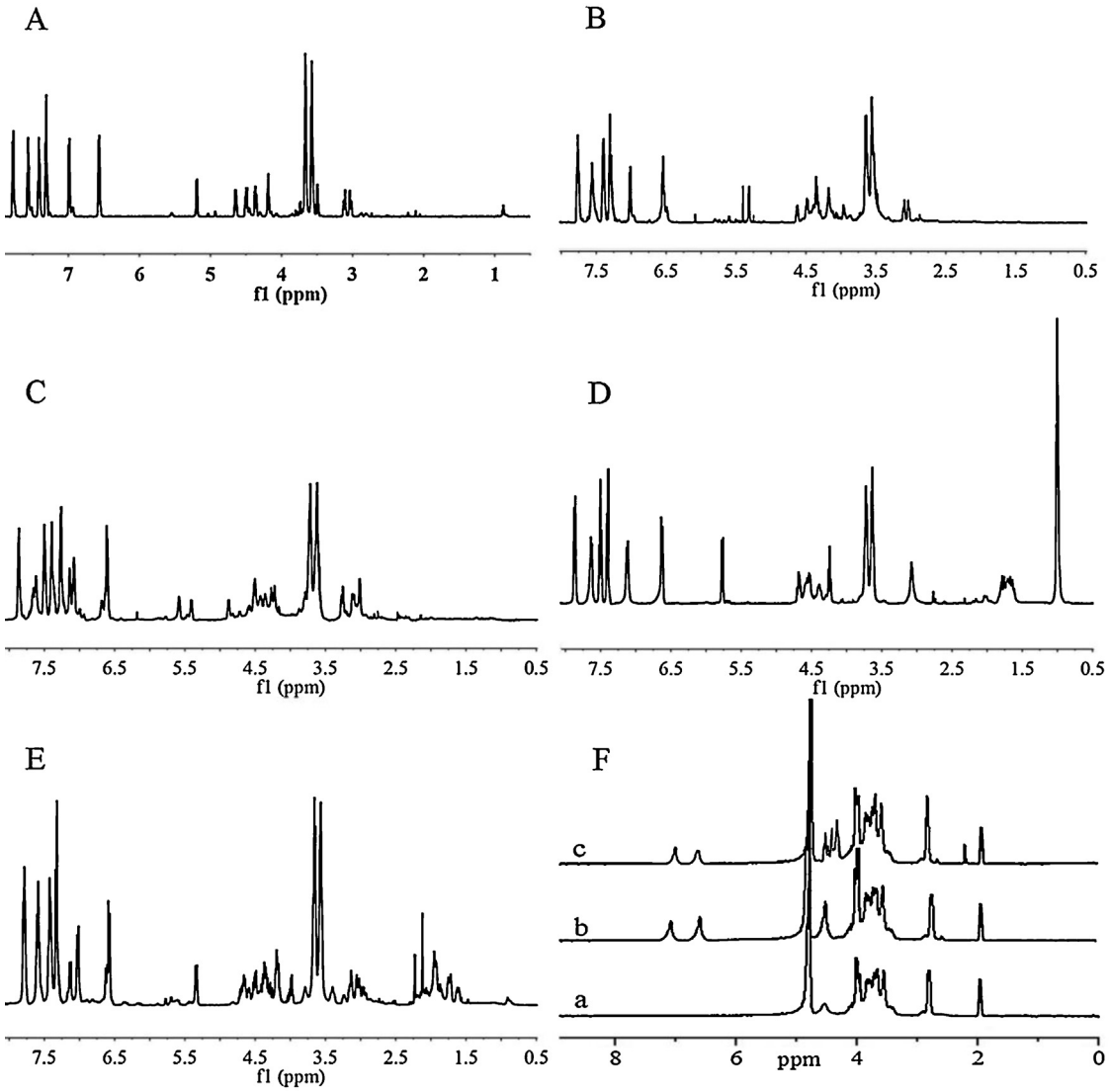

Fig. 3. ^{1}H NMR spectra: (A) Fmoc-Mel; (B) Fmoc-Mel-Gly; (C) Fmoc-Mel-Phe; (D) Fmoc-Mel-Leu; (E) Fmoc-Mel-Pro; (F) – (a) OCM-chitosan, (b) Mel-OCM-chitosan, (c) Mel-Pro-OCM-chitosan.

attributed to Pro spacer. In addition, the almost disappearance of the signals at 7.28–7.83 (8H, m, Ar[Fmoc]) indicated that Fmoc was completely de-protected by piperidine.

3.2. Drug content and water solubility

It was tested that free Mel and Mel-OCM-chitosan conjugates have the same UV absorption characteristic and both showed an absorption peak at around 261 nm. Table 1 shows the Mel content in all Mel-OCM-chitosan conjugates. Keeping OCM-chitosan feed ratio unchanged, it was found that Mel content in Mel-OCM-chitosan conjugates decreased from 15.9 to 11.9, Mel substitution degree decreased from 0.147 to 0.107, as the spacer changed from Gly to Leu. The result indicated that selection of amino acid spacer played an important role in adjusting Mel content.

The solubility of Mel in water was measured to be approximately 6–8 μg/mL. Compared with the extremely poor solubility of Mel, all of the tested conjugates showed satisfactory water solubility, which was 160-fold (Mel-OCM-chitosan, 1.12 mg/mL) at least and 217-fold (Mel-Gly-OCM-chitosan-2, 1.52 mg/mL) at most of Mel (as shown in Table 1). The solubility of the conjugates with same amino acid spacer decreased with Mel substitution degree increased. The conjugates showed good water solubility mainly due to the good aqueous solubility of OCM-chitosan and the possibility of micellar formation self-assembled by Mel-OCM-chitosan conjugates in the aqueous solution. It was reported that hydrophobic modified OCM-chitosan can self-assemble into nanoparticles with a hydrophobic core and a hydrophilic shell (Gong et al., 2012; Zheng, Han, et al., 2011; Zheng, Rao, et al., 2011). The hydrophilic shell could increase the water solubility of the prodrugs. In addition, the existence of spacers also slightly enhanced the water solubility of the conjugates although it showed no obvious regularity among different spacers.

3.3. In vitro drug release of Mel-OCM-chitosan conjugates

Chemical and enzymatic hydrolysis was carried out to evaluate the drug release behavior of Mel-OCM-chitosan conjugates by using HPLC method. The accumulative release rate of Mel from the different conjugates and hydrolysis conditions are shown in Figs. 4 and 5. The results displayed several important clues as follow.

Firstly, the drug release behavior toward chemical hydrolysis was evaluated at pH 7.4 and 5.8 in PBS (37 °C) to mimic the physiological and cell lysosomal pH environments, respectively. The release of Mel from Mel-OCM-chitosan conjugates in both PBS

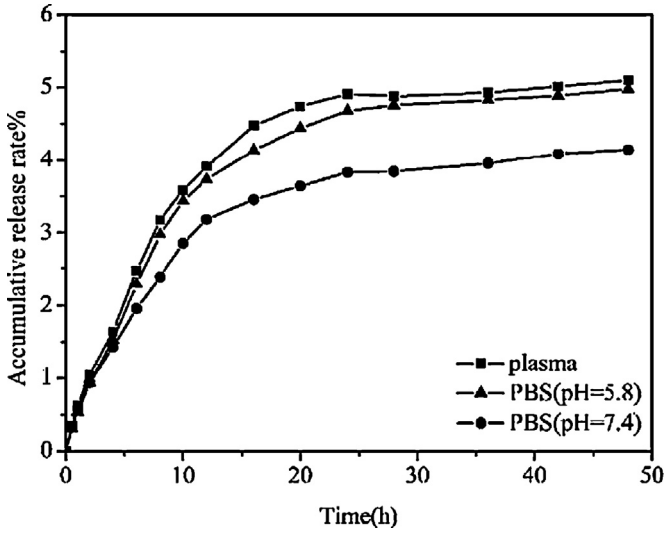

Fig. 4. Release of Mel from Mel-Gly-OCM-chitosan in plasma and PBS.

indicated a slow Mel release and which shown no apparent difference between all the conjugates. After 48 h, only 4.1% of the Mel was released from Mel-Gly-OCM-chitosan in PBS (pH 7.4) and 5.0% in PBS (pH 5.8). We can notice that lower pH environment led to faster releasing speed. The differences may be ascribed to that amido bond is more stable in neutral environment than in acid environment. The pH dependant release can be also attributed to the swelling property of the polymer matrix. At lower pH, the residual amine groups is protonated and creates a repulsive force between the adjacent positive charge, and thereby more amount of conjugated drug will be exposed to be hydrolyzed (Anitha et al., 2011). The rate of Mel release from Mel-OCM-chitosan conjugates in plasma showed no big different from those in buffer, which suggested that the few and low concentrate of the enzyme exist in plasma had no obvious effect on the drug stability in plasma.

Secondly, drug release by enzymatic hydrolysis was tested in the presence of a model enzyme (papain) and lysosome. It has reported a higher level of a number of enzymes expressing in tumor extracellular space for tumors to survive, grow and metastasize (Chau, Tan, & Langer, 2004). Among the many tumor-associated enzymes, we have focus on selected cathepsins (including Cathepsin B, Cathepsin H, Cathepsin X and so on), which belong to cysteine proteases and demonstrated over-expressing in malignant tumor (Repnik, Stoka, Turk, & Turk, 2012). Cysteine proteases degrade polypeptides share a common catalytic mechanism. Papain was used as a model of cysteine proteases. Comparing to the chemical hydrolysis, the obvious Mel release could be observed within an hour in both papain and tritosomes. These observations stand in contrasts with previous reports (Duncan et al., 1991) that HPMA-melphalan conjugates with Gly spacer was not degradable by thiol-proteases. So it is noteworthy that the nature of the polymeric backbone also serves to influence the rate and specificity of enzymatic cleavage of amino spacers. The rapid drug release in lysosome and papain while slow drug release in plasma ensure the effective release of bioactive molecules in tumor cells after the conjugates passive target the tumor cells through EPR effect.

Thirdly, connecting Mel and OCM-chitosan molecules with amide bond directly, displayed the highest stability toward both chemical and enzymatic hydrolysis. In contrast, conjugates with amino acid spacers showed relative fast and increased drug release velocity, which means that these conjugates have enzyme-responsive characteristic, and enzymatic release of melphalan from Mel-OCM-chitosan conjugates was clearly dependent on the amino acid used to link drug to polymer. In all cases, the rate of release was as follows: Mel-Gly-OCM-chitosan > Mel-Pro-OCM-chitosan > Mel-Phe-OCM-chitosan > Mel-Leu-OCM-chitosan. The results demonstrated that increased steric hindrance provided by spacers with large side chain could lead to a lower and slower free drug release under the same environmental condition. This can be attributed that the large side chain induced steric hindrance would become an obstacle for the enzymatic hydrolysis.

3.4. In vitro cytotoxicity

The RPMI8226 cells were selected as a model for in vitro cytotoxicity study of the Mel-OCM-chitosan conjugates. It was found in Fig. 6 that free Mel had the maximum inhibition ratio and all conjugates exhibited obvious cytotoxicity against RPMI8226 cells, indicating polymeric prodrugs did not lose anti-cancer activity of Mel. At the same time, we can notice a relatively lower cellular cytotoxicity induced by the polymeric derivatization of OCM-chitosan and amino acid, which probably due to the self-assembled behaviors of the amphiphilic polymers to nanoparticles. Mel was probably chemically embedded in the interior core of the self-assembled nanoparticles of the conjugates, which can protect Mel from interaction with the tumor cells. However, only slight activity loss was observed with the conjugated form, suggesting that free Mel was released from the conjugates through cleavage of the amino acid linker between Mel and OCM-chitosan. It is worth noticed that the conjugates with the amino acid linkers were more active than the conjugates without the linkers, suggesting the cell cytotoxicity was mainly due to the

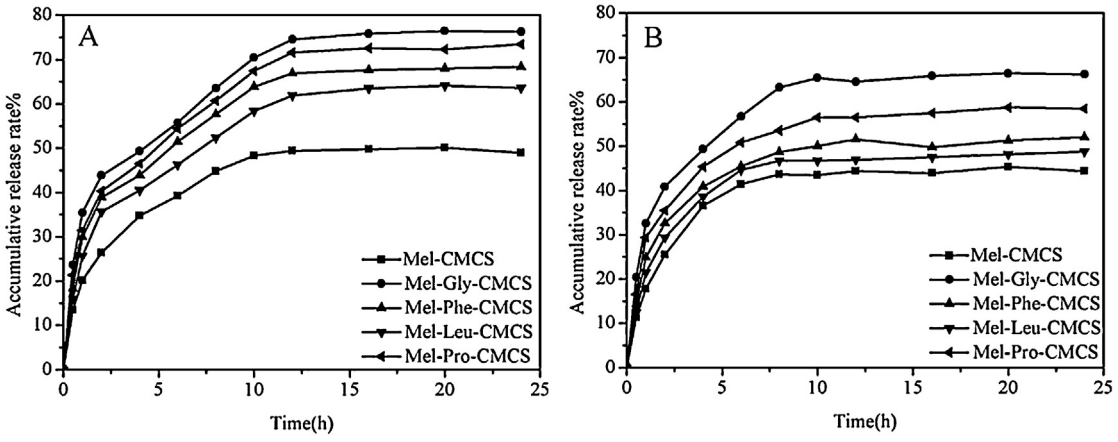

Fig. 5. Enzymatic degradation of Mel-OCM-chitosan conjugates. Degradation by (A) papain and (B) tritosomes is shown.

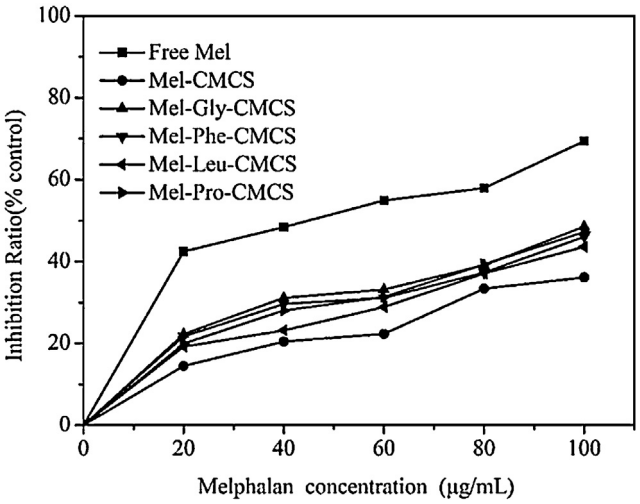

Fig. 6. MTT assay of in vitro cytotoxicity of free Mel and Mel-OCM-chitosan conjugates against RPMI8226 cells.

Mel released from the Mel-OCM-chitosan conjugates, which was consistent with the result of in vitro release.

4. Conclusions

A serial of Mel-OCM-chitosan conjugates with different amino acid spacers were synthesized and structures characterized by FT-IR, ^{1}H NMR. These Mel-OCM-chitosan conjugates showed satisfactory water solubility comparing with free melphalan. In vitro study showed that the conjugates are stable in plasma but rapidly degraded in enzyme solution. The selection of amino acid spacers had important influence on the solubility, drug content, and drug release behavior of polymeric prodrugs. The conjugates with Gly spacer was considered to be the best compound, as it had good water solubility and the highest antitumor activity compared with other conjugates. These results demonstrated that the polymeric prodrugs designed in this paper can solve many problems of melphalan and have the potential to be used as new polymeric prodrugs.

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