Synthesis and Characterization of Saccharide-Functionalized Polymer–Gemcitabine Conjugates Based on Chemoenzymatic Selective Strategy

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ABSTRACT: An efficient protocol for the synthesis of polymer–gemcitabine conjugates with variable composition and potential hepatoma-targeting property was achieved by combining selectively enzymatic transesterification with radical polymerization. Four polymerizable vinyl gemcitabine esters were first prepared by highly selective transesterification of gemcitabine with divinyl dicarboxylates using CAL-B as catalyst in acetone and characterized by IR, ^{1}H-NMR, ^{13}C-NMR, and ESI-MS. The effects of enzyme sources, organic solvents, and molar ratio of substrates on the enzymatic transesterification were investigated in detail. Then α,α′-azobis-(isobutyronitrile)-initiated homopolymerization of the resultant gemcitabine monomers was performed and three polymer–gemcitabine conjugates with high gemcitabine content (>55 wt %) were synthesized. Moreover, radical copolymerization of the gemcitabine monomer 5′-*O*-vinyladipyl-gemcitabine with different saccharide comonomers was performed, and three saccharide-functionalized polymer–gemcitabine conjugates with 5.7–15.3 wt % gemcitabine content were obtained, among which the conjugates with galactose or lactose as pendants had potential hepatoma-targeting function. All the resultant polymer–gemcitabine conjugates were characterized by IR, NMR, and gel permeation chromatography. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 1840–1847, 2012

Key words: drug delivery systems; enzymes; radical polymerization; gemcitabine; conjugates

INTRODUCTION

In recent years, the design and fabrication of polymer–drug conjugates has been an intense field of research as the drug system shows numerous advantages over conventional low-molecular-weight drugs including prolonged drug release, improved site-specificity, and reduced side effects.[1–7] Especially, the introduction of targeting ligands effectively achieves the specific recognition of polymer–drug conjugates to such tissue or organ, and thus enhances the pharmacological effect of given drugs.[8–15] Among the ligands, saccharides have attracted the extensive attention as biological recognition signals and functional biomolecules.[16–18] Moreover, it is particularly effective that covalently linking saccharides to conjugates for the improvement of water-solubility.[19,20]

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine) is a deoxycytidine nucleoside analog with broad spectrum cytotoxicity against a variety of solid tumors including pancreatic cancer, non-small cell lung cancer, breast cancer, and so on.[21–25] However, gemcitabine has very short plasma half-life and greater hematological toxicity, thus exhibits a narrow therapeutic index.[26–28] To circumvent these limitations and improve the biopharmaceutical features of gemcitabine, several polymer–gemcitabine conjugates have been successfully reported. For example, Chung and coworkers[29] synthesized poly-L-glutamic acid–gemcitabine conjugate. Pasut et al.[30] described the synthesis of PEG–gemcitabine conjugates and folate–PEG–gemcitabine conjugate. Cavallaro et al.[31] prepared folic acid-functionalized PHEA–gemcitabine conjugates. The reported conjugates were almost constructed by conjugating gemcitabine, targeting ligands, or their derivatives to polymer carriers, which required additional postpolymerization multistep modification for gemcitabine, targeting ligands, or polymer precursors. Therefore, the synthetic routes of numerous functional conjugates were

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tedious, and the composition of obtained conjugates was difficult to be precisely controlled.

Polymerization of drug with targeting ligand monomers offered a facile route to synthesize functional polymer–drug conjugates with well-defined composition.[32–34] Nevertheless, the drug or ligand molecules usually contain a variety of functional groups. It was challenging to high-selectively synthesize polymerizable monomers of the multifunctional molecules by one-step chemical reaction. Enzymes showed particular advantages in selectively structural modification of multifunctional substrates including high regioselectivity, chemoselectivity, stereoselectivity, and mild reaction conditions.[35–37] By selectively enzymatic acylation, we have successfully synthesized polymerizable monomers of some drugs and saccharides.[38–40]

Herein, we wish to achieve the facile synthesis of polymer–gemcitabine conjugates with different saccharides by combining enzymatic reaction with radical polymerization. First, four polymerizable gemcitabine monomers were easily prepared by CAL-B-catalyzed selective transesterification of gemcitabine with divinyl dicarboxylates. The effects of enzymes, organic solvents, and molar ratio of substrates on the enzymatic reaction were investigated in detail. Then, homopolymerization of the resultant gemcitabine monomers and copolymerization of the gemcitabine monomer with galactose, glucose, or lactose monomer were achieved, and six polymer–gemcitabine conjugates with or without saccharides were synthesized and characterized by IR, NMR, and gel permeation chromatography (GPC).

EXPERIMENTAL

Materials and instruments

Lipase immobilized on acrylic resin from *Candida antarctica* (CAL-B, 10,000 U/g) and Lipase Type VII from *Candida rugosa* (CRL, 706 U/mg) were purchased from Sigma (USA). Lipozyme immobilized from *Mucor miehei* (Lipozyme, 42 U/g), lipase from *hog pancreas* (HPL, 2.4 U/mg), lipase from *porcine pancreas* (PPL, 30–90 U/mg), and lipase from *Candida cylindracea* (CCL, 2.8 U/mg) were purchased from Fluka (Switzerland). Amano Lipase M from *Mucor javanicus* (MJL, 10 U/mg) was purchased from Aldrich (USA). Lipase AY30 (AY30) was purchased from Acrõs (USA). Immobilized penicillin G acylase from *Escherichia coli* (PGA, immobilized on acrylic beads) was purchased from Hunan Flag Biotech Co. (Hunan, PR China). D-Aminoacylase from *E. coli* (DA, 10,000 U/mg) and Acylase "Amano" from *Aspergillus oryzae* (AA, ≥30,000 U/g) were purchased from Amano Enzyme (Japan). Alkaline protease from *Bacillus subtilis* (Subtilisin, 100 U/mg) was purchased from Wuxi Enzyme Co. (Wuxi, PR China). Divinyl succinate, divinyl adipate, divinyl azelate, and divinyl sebacate were produced and purified as described in the patent.[41] 6-*O*-Vinylsebacoyl-D-glucose (VGL), 6-*O*-vinylsebacoyl-D-galactose (VGA), and 6-*O*-vinylsebacoyl-lactose (VLA) were prepared and purified according to the literature.[39] α,α′-Azobis-(isobutyronitrile) (AIBN) was purchased from Fluka and purified by recrystallization in ethanol. Organic solvents used in enzymatic reactions were analytical grade and were dried by storing over activated 3 Å molecular sieves for 24 h before use.

All reactions were monitored by thin-layer chromatography (TLC) on silica gel plates. IR spectra were measured in the form of a KBr disk using a Nicolet Nexus FTIR 670 spectrophotometer at room temperature. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AMX-400 MHz spectrometer using tetramethylsilane as an internal standard in DMSO-d_6 solutions. Mass spectrometry data were obtained on Bruker Esquire-LC for electrospray (ESI-MS) measurements. Analytical HPLC was performed using an Agilent 1100 series with a reversed-phase Shim-Pack VP-ODS column (150 × 4.6 mm) and a UV detector (267 nm). Gel permeation chromatography (GPC) was performed with a system equipped with refractive-index detector and Styragel HR columns. The GPC columns were standardized with PMMA, and DMF was used as the mobile phase.

General procedure for enzymatic synthesis of vinyl gemcitabine esters

The reaction was initiated by adding CAL-B (20 mg/mL) to acetone (10.0 mL) containing gemcitabine (4 mmol) and divinyl dicarboxylate (16 mmol). The suspension was then kept at 50°C and shaken under 250 rpm. The reaction process was monitored by TLC (ethyl acetate/methanol/water = 17/2/1, by vol), and reaction was terminated by filtering off the enzyme. The filtrate was evaporated under reduced pressure. The product was separated by silica gel chromatography.

Synthesis of 5′-*O*-vinylsuccinyl-gemcitabine (SUG)

The reaction time was 1 h and the product yield was 62%. IR (KBr, cm^{-1}): 1746, 1652, 1524, 1495, 1072. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.57 (1H, d, 6-H), 7.49, 7.43 (2H, —NH_2), 7.19 (1H, dd, J = 6.3, 13.9 Hz, —CH=), 6.45 (1H, 3′-OH), 6.18 (1H, 1′-H), 5.82 (1H, d, 5-H), 4.90 (1H, d, J = 14.0 Hz, CH_2=), 4.65 (1H, d, J = 6.3 Hz, CH_2=), 4.41–3.99 (4H, m, 3′-H, 4′-H, 5′-H), 2.54 (4H, m, —CH_2—C=O). ^{13}C-NMR (DMSO-d_6, δ, ppm): 172.3, 170.2 (C=O), 141.7, 98.9 (—CH=CH_2), 166.3 (C-4), 155.1 (C-2), 141.7 (C-6), 123.2 (C-2′), 95.6 (C-5), 84.4 (C-1′), 78.0 (C-4′), 70.6 (C-3′), 63.4 (C-5′), 28.9, 28.8 (—CH_2—). ESI-MS (m/z): 390.1 $[M+H]^+$.

Synthesis of 5′-*O*-vinyladipyl-gemcitabine

The reaction time was 4 h and the product yield was 78%. IR (KBr, cm^{-1}): 1745, 1654, 1647, 1523, 1496, 1076. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.51 (1H, d, 6-H), 7.44, 7.42 (2H, —NH_2), 7.19 (1H, dd, J = 6.3, 13.9 Hz, —CH=), 6.45 (1H, 3′-OH), 6.16 (1H, 1′-H), 5.80 (1H, d, 5-H), 4.88 (1H, d, J = 14.0 Hz, CH_2=), 4.64 (1H, d, J = 6.3 Hz, CH_2=), 4.39–3.97 (4H, m, 3′-H, 4′-H, 5′-H), 2.44 (2H, t, —CH_2—C=O), 2.39 (2H, t, —CH_2—C=O), 1.56 (4H, m, —CH_2—). ^{13}C-NMR (DMSO-d_6, δ, ppm): 172.9, 170.7 (C=O), 141.6, 98.5 (—CH=CH_2), 166.1 (C-4), 155.0 (C-2), 141.6 (C-6), 123.1 (C-2′), 95.3 (C-5), 84.3 (C-1′), 77.7 (C-4′), 70.5 (C-3′), 63.0 (C-5′), 33.2, 33.1, 24.0, 23.8 (—CH_2—). ESI-MS (m/z): 418.1 $[M+H]^+$.

Synthesis of 5′-*O*-vinylnonanedioyl-gemcitabine (VNG)

The reaction time was 3 h, and the product yield was 37%. IR (KBr, cm^{-1}): 2934, 1744, 1651, 1524, 1494, 1084. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.50 (1H, d, 6-H), 7.44, 7.38 (2H, —NH_2), 7.20 (1H, dd, J = 6.0, 14.0 Hz, —CH=), 6.45 (1H, 3′-OH), 6.15 (1H, 1′-H), 5.80 (1H, d, 5-H), 4.87 (1H, d, J = 14.0 Hz, CH_2=), 4.63 (1H, d, J = 6.0 Hz, CH_2=), 4.38–3.98 (4H, m, 3′-H, 4′-H, 5′-H), 2.39 (2H, t, —CH_2—C=O), 2.34 (2H, t, —CH_2—C=O), 1.52 (4H, m, —CH_2—), 1.25 (6H, m, —CH_2—). ^{13}C-NMR (DMSO-d_6, δ, ppm): 173.1, 170.9 (C=O), 141.6, 98.4 (—CH=CH_2), 166.0 (C-4), 155.0 (C-2), 141.6 (C-6), 123.1 (C-2′), 95.3 (C-5), 84.2 (C-1′), 77.8 (C-4′), 70.5 (C-3′), 62.8 (C-5′), 33.6, 33.4, 28.7, 28.6, 28.5, 24.6, 24.3 (—CH_2—). ESI-MS (m/z): 482.1 $[M+Na]^+$.

Synthesis of 5′-*O*-vinylsebacoyl-gemcitabine (SEG)

The reaction time was 2 h, and the product yield was 24%. IR (KBr, cm^{-1}): 2932, 1744, 1651, 1524, 1494, 1085. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.49 (1H, d, 6-H), 7.43, 7.38 (2H, —NH_2), 7.19 (1H, dd, J = 6.3, 14.0 Hz, —CH=), 6.44 (1H, 3′-OH), 6.15 (1H, 1′-H), 5.79 (1H, d, 5-H), 4.86 (1H, d, J = 14.0 Hz, CH_2=), 4.62 (1H, d, J = 6.3 Hz, CH_2=), 4.37–3.99 (4H, m, 3′-H, 4′-H, 5′-H), 2.38 (2H, t, —CH_2—C=O), 2.29 (2H, t, —CH_2—C=O), 1.51 (4H, m, —CH_2—), 1.23 (8H, m, —CH_2—). ^{13}C-NMR (DMSO-d_6, δ, ppm): 173.1, 170.9 (C=O), 141.6, 98.4 (—CH=CH_2), 166.0 (C-4), 155.0 (C-2), 141.6 (C-6), 123.0 (C-2′), 95.3 (C-5), 84.2 (C-1′), 77.8 (C-4′), 70.5 (C-3′), 62.8 (C-5′), 33.6, 33.4, 28.9, 28.8, 28.7, 28.6, 24.7, 24.4 (—CH_2—). ESI-MS (m/z): 474.2 $[M+H]^+$.

Synthesis of poly(VAG)

The homopolymerization of 5′-*O*-vinyladipyl-gemcitabine (VAG) was performed as follows: VAG (300 mg) was dissolved in DMSO (0.5 mL), and AIBN (15.0 mg) was added as initiator. The mixture was sealed in a small flame-dried flask and stirred at 70°C under nitrogen for 24 h. The resultant product was repeatedly precipitated in acetone and dried under vacuum to give a light yellow solid poly (VAG) PVAG (69.0 mg, 23%). M_w = 1.1 × 10^4 g/mol, M_w/M_n = 1.3. IR (KBr, cm^{-1}): 1736, 1655, 1493, 1084. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.50–7.38 (3H, 6-H, and —NH_2 of gemcitabine), 6.44 (1H, 3′-OH of gemcitabine), 6.13 (1H, 1′-H of gemcitabine), 5.80 (1H, 5-H of gemcitabine), 4.71 (—CH—O— of the main chain), 4.35–3.97 (4H, 3′-H, 4′-H, and 5′-H of gemcitabine), 2.35–2.14 (4H, —CH_2—C=O), 1.69–1.48 (—CH_2—).

Synthesis of poly(VNG) (PVNG)

The homopolymerization of VNG was achieved by the same method as the synthesis of PVAG. The light yellow solid PVNG was obtained in 47% yield. M_w = 1.2 × 10^4 g/mol, M_w/M_n = 1.1. IR (KBr, cm^{-1}): 2935, 1736, 1655, 1493, 1084. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.45–7.38 (3H, 6-H and —NH_2 of gemcitabine), 6.44 (1H, 3′-OH of gemcitabine), 6.12 (1H, 1′-H of gemcitabine), 5.79 (1H, 5-H of gemcitabine), 4.69 (—CH—O— of the main chain), 4.32–3.95 (4H, 3′-H, 4′-H, and 5′-H of gemcitabine), 2.27–2.13 (4H, —CH_2—C=O), 1.65–1.18 (—CH_2—). ^{13}C-NMR (DMSO-d_6, δ, ppm): 173.1, 172.4 (C=O), 166.1 (C-4), 155.1 (C-2), 141.6 (C-6), 123.1 (C-2′), 95.4 (C-5), 84.2 (C-1′), 77.9 (C-4′), 70.5 (C-3′), 67.6 (—CH— of the main chain), 63.0 (C-5′), 34.0, 33.6, 28.8, 24.7 (—CH_2—).

Synthesis of poly(SEG) (PSEG)

The homopolymerization of SEG was also achieved by above method, and the light yellow solid PSEG was obtained in 29% yield. M_w = 1.1 × 10^4 g/mol, M_w/M_n = 1.1. IR (KBr, cm^{-1}): 2931, 1736, 1655, 1524, 1493, 1084. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.48–7.38 (3H, 6-H, and —NH_2 of gemcitabine), 6.48 (1H, 3′-OH of gemcitabine), 6.15 (1H, 1′-H of gemcitabine), 5.82 (1H, 5-H of gemcitabine), 4.73 (—CH—O— of the main chain), 4.33–3.98 (4H, 3′-H, 4′-H, and 5′-H of gemcitabine), 2.30–2.15 (4H, —CH_2—C=O), 1.71–1.20 (—CH_2—).

Synthesis of poly(VAG-*co*-VGL)

Poly(VAG-*co*-VGL) was prepared by adding VAG (100 mg) and VGL (374 mg) into a 10-mL polymerization tube containing DMSO (0.6 mL) and AIBN (24.0 mg). The mixture was degassed by three freeze-thaw cycles, and then stirred under nitrogen at 70°C for 24 h. The resultant product was repeatedly precipitated in acetone and dried under

vacuum to afford a light yellow solid (294 mg, 62%). $M_w = 1.0 \times 10^4$ g/mol, $M_w/M_n = 2.2$. IR (KBr, cm^{-1}): 3423, 2930, 2857, 1736, 1653, 1080. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.46–7.32 (6-H and —NH_2 of gemcitabine), 6.64, 6.31 (1-OH of D-glucose), 6.40 (3′-OH of gemcitabine), 6.12 (1′-H of gemcitabine), 5.79 (5-H of gemcitabine), 5.30–2.88 (—CH—O— of the main chain; 1-H, 2-H, 3-H, 4-H, 5-H, 6-H, 2-OH, 3-OH, and 4-OH of D-glucose; 3′-H, 4′-H, and 5′-H of gemcitabine), 2.31–1.12 (—CH_2—). ^{13}C-NMR (DMSO-d_6, δ, ppm): 173.6, 172.8 (C=O), 166.2 (C-4 of gemcitabine), 155.2 (C-2 of gemcitabine), 141.7 (C-6 of gemcitabine), 123.1 (C-2′ of gemcitabine), 97.4, 92.9 (C-1 of D-glucose), 95.6 (C-5 of gemcitabine), 84.3 (C-1′ of gemcitabine), 78.1 (C-4′ of gemcitabine), 70.7 (C-3′ of gemcitabine), 63.2 (C-5′ of gemcitabine), 77.0, 75.2, 74.1, 73.5, 72.8, 71.1, 70.8, 69.7, 64.5 (C-2, C-3, C-4, C-5, and C-6 of D-glucose), 34.0, 33.5, 29.3, 29.1, 25.1, 24.3 (—CH_2—).

Synthesis of poly(VAG-*co*-VGA)

The copolymerization of VAG with VGA was achieved as the synthesis of poly(VAG-*co*-VGL), and the molar ratio of VAG to VGA was 1 : 4. The light yellow solid poly(VAG-*co*-VGA) was obtained in 61% yield. $M_w = 1.2 \times 10^4$ g/mol, $M_w/M_n = 2.2$. IR (KBr, cm^{-1}): 3425, 2931, 2858, 1736, 1654, 1076. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.47–7.31 (6-H and —NH_2 of gemcitabine), 6.56, 6.19 (1-OH of D-galactose), 6.39 (3′-OH of gemcitabine), 6.11 (1′-H of gemcitabine), 5.78 (5-H of gemcitabine), 5.27–3.23 (—CH—O— of the main chain; 1-H, 2-H, 3-H, 4-H, 5-H, 6-H, 2-OH, 3-OH, and 4-OH of D-galactose; 3′-H, 4′-H, and 5′-H of gemcitabine), 2.28–1.18 (—CH_2—). ^{13}C-NMR (DMSO-d_6, δ, ppm): 173.5, 173.0, 172.7 (C=O), 166.3 (C-4 of gemcitabine), 155.3 (C-2 of gemcitabine), 141.7 (C-6 of gemcitabine), 123.2 (C-2′ of gemcitabine), 98.0, 93.3 (C-1 of D-galactose), 95.5 (C-5 of gemcitabine), 84.3 (C-1′ of gemcitabine), 78.1 (C-4′ of gemcitabine), 70.7 (C-3′ of gemcitabine), 63.2 (C-5′ of gemcitabine), 73.7, 73.6, 72.5, 70.2, 69.9, 69.7, 69.3, 69.2, 64.6 (C-2, C-3, C-4, C-5, and C-6 of D-galactose), 34.1, 33.5, 29.4, 25.1, 24.4 (—CH_2—).

Synthesis of poly(VAG-*co*-VLA)

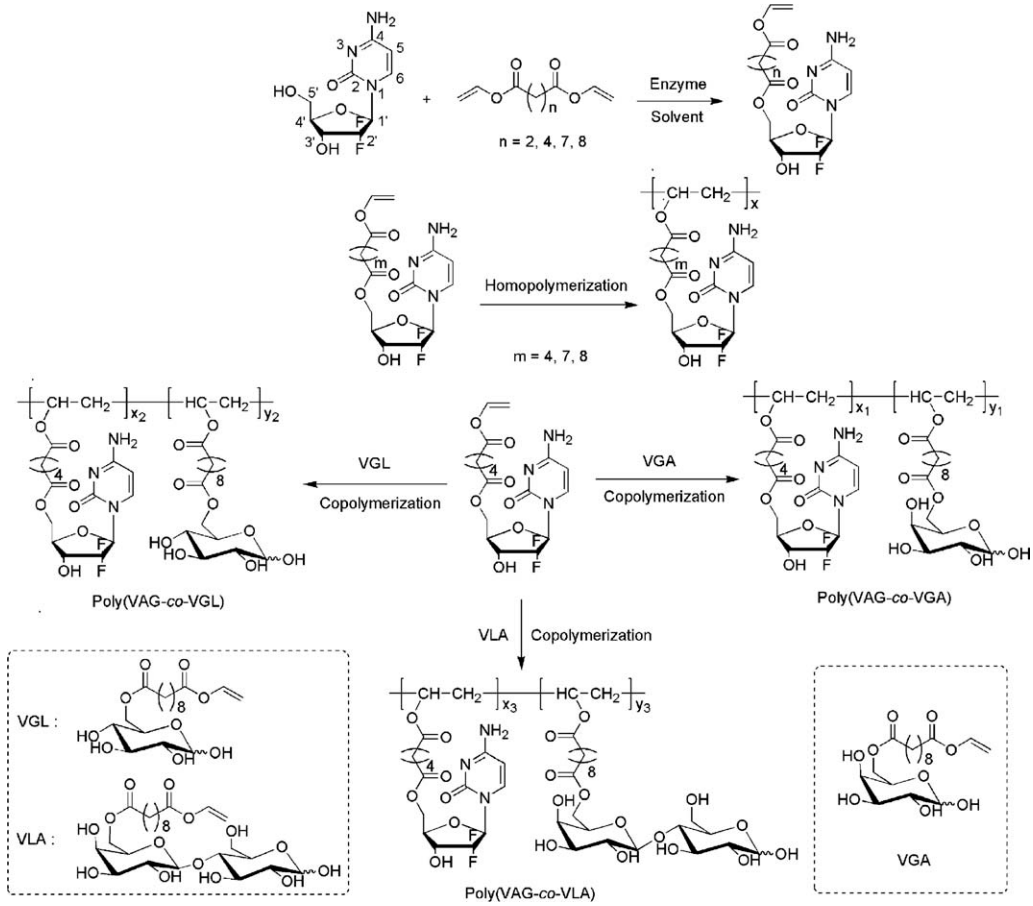
The copolymerization of VAG with VLA was achieved by the similar method, and the molar ratio of VAG to VLA was 1 : 8. The light yellow solid poly(VAG-*co*-VLA) was obtained in 54% yield. $M_w = 1.1 \times 10^4$ g/mol, $M_w/M_n = 2.1$. IR (KBr, cm^{-1}): 3425, 2930, 2858, 1736, 1655, 1076. ^{1}H-NMR (DMSO-d_6, δ, ppm): 7.46–7.29 (6-H and —NH_2 of gemcitabine), 6.67, 6.33 (1-OH of lactose), 6.43 (3′-OH of gemcitabine), 6.11 (1′-H of gemcitabine), 5.79 (5-H of gemcitabine), 5.35–2.92 (—CH—O— of the main chain; 1-H, 2-H, 3-H, 4-H, 5-H, 6-H, 2-OH, 3-OH, 6-OH, 2′-OH, 3′-OH, 4′-OH, 1′-H, 2′-H, 3′-H, 4′-H, 5′-H, and 6′-H of lactose; 3′-H, 4′-H, and 5′-H of gemcitabine), 2.24–1.18 (—CH_2—). ^{13}C-NMR (DMSO-d_6, δ, ppm): 173.6, 173.2, 172.7 (C=O), 166.4 (C-4 of gemcitabine), 155.5 (C-2 of gemcitabine), 141.8 (C-6 of gemcitabine), 123.3 (C-2′ of gemcitabine), 104.1 (C-1′ of lactose), 97.3, 92.6 (C-1 of lactose), 95.7 (C-5 of gemcitabine), 84.4 (C-1′ of gemcitabine), 78.2 (C-4′ of gemcitabine), 70.8 (C-3′ of gemcitabine), 63.2 (C-5′ of gemcitabine), 81.6, 81.2 (C-4 of lactose), 75.4, 75.3, 75.2, 73.5, 73.0, 71.9, 70.9, 70.4, 68.9, 63.9, 61.1 (C-2, C-3, C-5, C-6, C-2′, C-3′, C-4′, C-5′, and C-6′ of lactose), 34.1, 33.9, 29.2, 25.0, 24.4 (—CH_2—).

RESULTS AND DISCUSSION

In this study, a facile method for the synthesis of polymer–gemcitabine conjugates was developed by combining enzymatic transesterification with radical polymerization. Vinyl gemcitabine esters were first prepared by selectively enzymatic transesterification of gemcitabine with divinyl dicarboxylates, and then were chosen as polymerizable monomers for AIBN-initiated radical homopolymerization or copolymerization with different saccharide monomers to offer a series of polymer–gemcitabine conjugates without or with saccharides. The entire synthetic route is shown in Scheme 1.

Enzymatic synthesis and characterization of vinyl gemcitabine esters

By choosing appropriate enzymes and organic solvents, selective acylation at the primary hydroxyl of gemcitabine was successfully realized, and four polymerizable vinyl gemcitabine esters (SUG, VAG, VNG, SEG) were easily synthesized by the selectively enzymatic acylation of gemcitabine with divinyl dicarboxylates (Scheme 1). The resultant gemcitabine monomers were fully characterized by ^{1}H-NMR, ^{13}C-NMR, IR, and MS. Taking VAG for example, the structure of the vinyl gemcitabine ester was preliminarily determined by the appearance of vinyl proton signals at δ = 7.19, 4.88, 4.64 ppm in the ^{1}H-NMR spectrum of VAG (Fig. 1). The broader peaks at δ = 2.44, 2.39, 1.56 ppm were attributed to the protons of the —CH_2—C=O and —CH_2— groups in the vinyl adipate moiety, respectively. Other proton signals assigned to the gemcitabine moiety (6-H at δ = 7.51 ppm, —NH_2 at δ = 7.44, 7.42 ppm, 3′-OH at δ = 6.45 ppm, 1′-H at δ = 6.16 ppm, 5-H at δ = 5.80 ppm, 3′-H, 4′-H, and 5′-H at δ = 4.39–3.97 ppm) were also observed in the ^{1}H-NMR spectrum of VAG. The acylation position of gemcitabine was determined by ^{13}C-NMR spectrum according to the general strategy described by Yoshimoto et al.[42] that



Scheme 1 Chemoenzymatic synthesis of polymer–gemcitabine conjugates.

acylation of a hydroxyl group of the substrate resulted in a downfield shift of the peak corresponding to the *O*-acylated carbon and an upfield shift of the peak corresponding to the neighboring carbon. In the ^{13}C-NMR spectrum of VAG (see Supporting Information), the peak at $\delta = 63.0$ ppm corresponding to the downfield shift of C-5′ from 59.4 ppm and the peak at $\delta = 77.1$ ppm corresponding to the upfield shift of C-4′ from 80.8 ppm were observed. Therefore, the selectively enzymatic acylation occurred at the C-5′ primary hydroxyl position of gemcitabine. The result of MS was 418.1 $[M+H]^+$, which further confirmed the successful preparation of VAG. The structures of SUG, VNG, and SEG were also determined by the same methods.

Enzyme screening for synthesis of vinyl gemcitabine esters

One of the most important parameters for enzymatic reactions is the selection of enzyme sources. Enzymes derived from various sources usually show different stability, activity, and specificity. To identify the enzymes with high catalytic activity and selectivity for gemcitabine, 12 kinds of commercially available enzymes were tested for the reaction of gemcitabine with divinyl adipate in acetone. At the same time, two control experiments were designed to demonstrate that the reaction was an enzymatic process. The results are shown in Figure 2. As expected, no product was detected when enzyme was absent from the system, and BSA did not show catalytic ability for this reaction. Among the 12 enzymes screened, two kinds of lipases could catalyze the acylation of gemcitabine with divinyl adipate in acetone. CAL-B showed the highest catalytic activity for the transesterification, and the reaction high selectively occurred at the C-5′ primary hydroxyl position of gemcitabine. Therefore, we selected CAL-B as catalyst for further investigation.

Influence of organic solvents on synthesis of vinyl gemcitabine esters

Generally, organic solvents are also one of the important parameters affecting enzymatic reactions. A good solvent for biocatalysis should not only maintain the enzyme activity, but also dissolve substrates. To optimize the reaction conditions for enzymatic synthesis of vinyl gemcitabine esters, nine kinds of conventional organic solvents with log *P* value ranging from −1.3 to 3.9 were screened for CAL-B-catalyzed

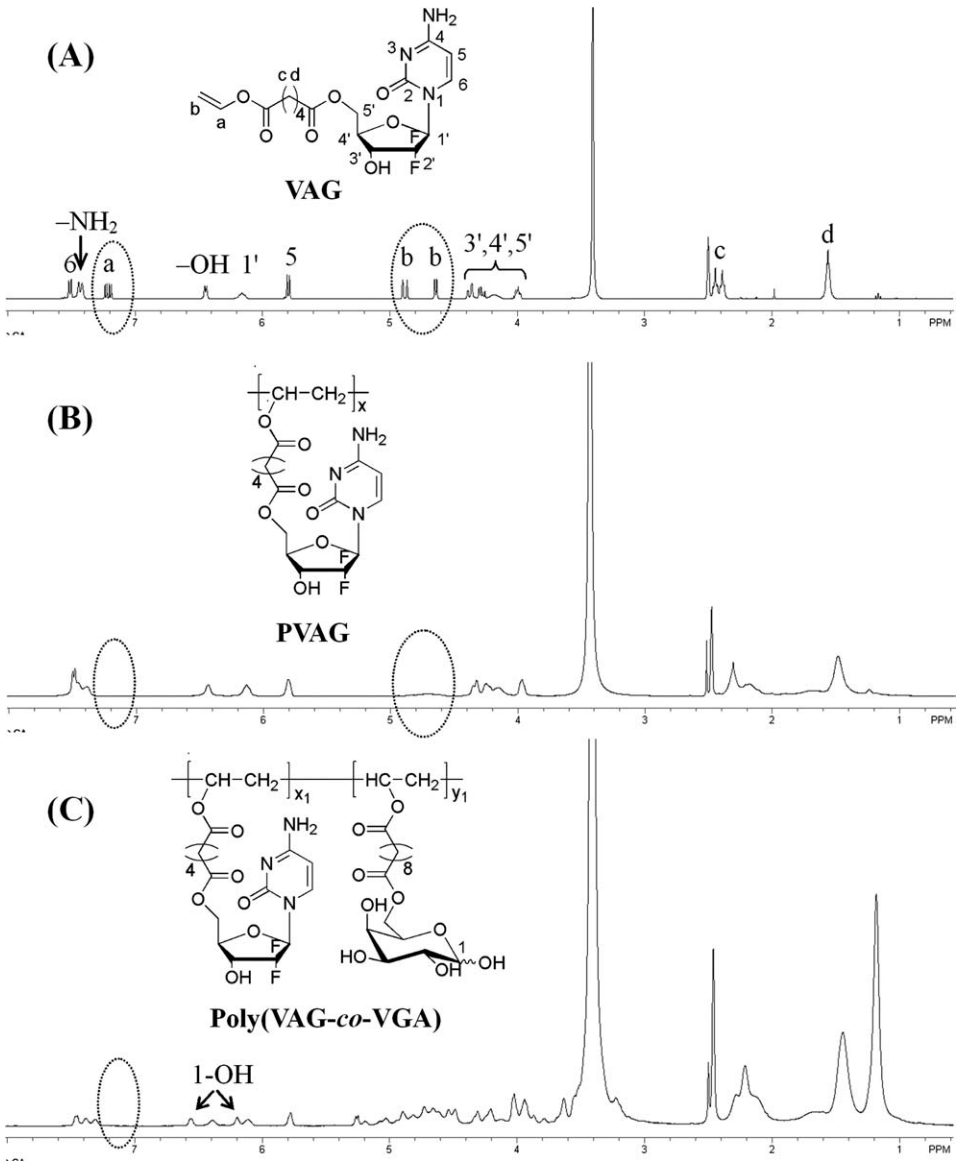


Figure 1 ^{1}H-NMR spectra of (A) VAG, (B) PVAG, and (C) poly(VAG-*co*-VGA).

transesterification of gemcitabine with divinyl adipate. The results are indicated in Table I (entry 1–9). It could be found that CAL-B showed good activity only in the organic solvents with the log *P* from −0.39 to 0.46, and yields of the resultant VAG ranged from 58% to 83%. When acetone was chosen as reaction medium, the highest yield of 83% was obtained. However, the hydrophobic organic solvents with log P greater than 1.9 were unfavorable for the transesterification, which may be relative to the poor solubility of gemcitabine in these solvents. Very low yields were also observed in the strong hydrophilic solvents such as DMF and DMSO. The result may be due to the denaturing effects of the organic solvents on the enzyme activity.[43] Therefore, acetone was selected as the reaction medium for CAL-B-catalyzed transesterification of gemcitabine with divinyl dicarboxylates.

Influence of molar ratio on synthesis of vinyl gemcitabine esters

Molar ratio of substrates was one of the necessarily investigated factors in selective reactions of multifunctional reagents. Therefore, influence of substrate molar ratio on the enzymatic transesterification of gemcitabine with divinyl adipate was further investigated after the screening of enzyme resources and organic solvents. Table I (entry 10–14, entry 6) shows yields of the resultant VAG at different molar ratios of divinyl adipate to gemcitabine. It could be found that the yield of VAG considerably increased from 49% to 93% when the molar ratio of divinyl adipate to gemcitabine was increased from 2 : 1 to 8 : 1, and the plateau was reached at the molar ratio of 4 : 1. Thus, the molar ratio of 4 : 1 was chosen as the feed

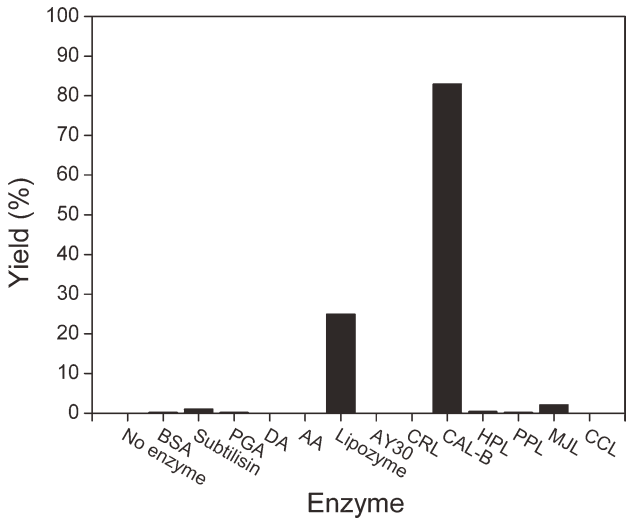


Figure 2 Influence of enzymes on the transesterification of gemcitabine with divinyl adipate. Conditions: enzyme (15.0 mg), gemcitabine (10.0 mg, 0.038 mmol), divinyl adipate (22.5 mg, 0.114 mmol), acetone (1.0 mL), 50°C, 250 rpm, 1 day. Yields were determined by HPLC.

ratio of divinyl adipate to gemcitabine for enzymatic synthesis of vinyl gemcitabine esters.

Synthesis and characterization of homopolymers with gemcitabine as pendants

Homopolymerization of the resultant vinyl gemcitabine esters was facilely achieved by radical polymerization using AIBN as initiator in DMSO and three corresponding homopolymers with gemcitabine as pendants were respectively obtained (Scheme 1). The synthetic polymer–gemcitabine conjugates were characterized by NMR and IR. Taking PVNG for example, the peaks of vinyl group existing in monomer VNG (^{1}H-NMR: δ 7.20, 4.87, 4.63 ppm; ^{13}C-NMR: δ 98.4 ppm) were absent in ^{1}H-NMR spectrum and ^{13}C-NMR spectrum of the homopolymer PVNG (see Supporting Information), but the signals assigned to the gemcitabine moiety and —CH_2— groups of the vinyl azelate moiety still appeared. Moreover, an evident bathochramic shift of the characteristic peak attributed to the carbonyl group from 1744 cm^{-1} in IR spectrum of monomer VNG to 1736 cm^{-1} in IR spectrum of homopolymer PVNG was observed (see Supporting Information). Analysis of the NMR and IR spectra confirmed the successful preparation of the polymer–gemcitabine conjugate PVNG. The molecular weight of PVNG was determined by GPC, and the result further confirmed achievement of the homopolymerization (see Supporting Information). The structure of conjugates PVAG and PSEG was also confirmed by the same methods. All three conjugates had high gemcitabine content (>55 wt %).

Synthesis and characterization of copolymers with gemcitabine as pendants

The AIBN-initiated radical copolymerization of the vinyl gemcitabine ester VAG with different saccharide comonomers was also performed to endow the polymer–gemcitabine conjugates with more variability in their composition and properties. Vinyl glucose ester VGL, vinyl galactose ester VGA, and vinyl lactose ester VLA were chosen as comonomers to synthesize poly(VAG-*co*-VGL), poly(VAG-*co*-VGA), and poly(VAG-*co*-VLA), respectively. The resultant saccharide-functionalized polymer–gemcitabine conjugates were characterized by IR, NMR, and GPC. Taking poly(VAG-*co*-VGA) for example, IR spectrum (see Supporting Information) of the galactose-functionalized polymer–gemcitabine conjugate revealed that vinyl group absorption (1647 cm^{-1}) present in VAG and VGA monomers was difficult to be observed in the corresponding conjugate. The characteristic absorption assigned to —CH_2— groups of D-galactose comonomer and that assigned to gemcitabine ester moiety appeared in IR spectrum of the conjugate poly(VAG-*co*-VGA). Also, ^{1}H-NMR (Fig. 1) and ^{13}C-NMR data of poly(VAG-*co*-VGA) proved the disappearance of vinyl group existing in monomers (^{1}H-NMR: δ 7.19 ppm; ^{13}C-NMR: δ 98.5 ppm) and the existence of gemcitabine and D-galactose moieties. From ^{1}H-NMR spectrum of poly(VAG-*co*-VGA), the molar ratio of VAG to VGA in the resultant conjugate could be approximately calculated according to the ratio between the integral of —CH_2— of VGA (δ 1.18 ppm) and the integral of —CH_2—C=O of VAG and VGA (δ 2.28–2.12 ppm).

TABLE I
Influence of Organic Solvents and Molar Ratio on the Transesterification of Gemcitabine with Divinyl Adipate

Entry	Organic solvent	Log *P*	Molar ratio[a]	Yield[b] (%)
1	Hexane	3.9	3/1	n.d.
2	Cyclohexane	3.4	3/1	n.d.
3	Isopropyl ether	1.9	3/1	n.d.
4	*Tert*-butyl alcohol	0.79	3/1	33
5	THF	0.46	3/1	63
6	Acetone	−0.23	3/1	83
7	Acetonitrile	−0.39	3/1	58
8	DMF	−1.0	3/1	n.d.
9	DMSO	−1.3	3/1	n.d.
10	Acetone	−0.23	2/1	49
11	Acetone	−0.23	4/1	92
12	Acetone	−0.23	5/1	90
13	Acetone	−0.23	6/1	92
14	Acetone	−0.23	8/1	93

[a] The feed molar ratio of divinyl adipate to gemcitabine.
[b] Yields were determined by HPLC.
n.d. means no product was detected.
Conditions: CAL-B (15.0 mg), gemcitabine (10.0 mg), divinyl adipate (15.1–60.2 mg), organic solvent (1.0 mL), 50°C, 250 rpm, 1 day.

Therefore, content of gemcitabine in the conjugate could be evaluated and was found to be ~ 14.1 wt %. At the same time, content of galactose in the conjugate could be also determined, and the result was about 35.9 wt %. Furthermore, the structure of glucose-functionalized or lactose-functional polymer–gemcitabine conjugates was determined by the same methods, and the contents of gemcitabine in two conjugates were 15.3 wt % and 5.7 wt %, respectively. The existence of galactose or lactose endowed the polymer–gemcitabine conjugates with potential hepatoma-targeting function.[44,45]

CONCLUSIONS

In this study, we described a highly selective enzymatic approach for the preparation of polymerizable gemcitabine monomers through the CAL-B-catalyzed transesterification of gemcitabine with divinyl dicarboxylates in acetone. Four vinyl gemcitabine esters were synthesized and characterized by IR, ^{1}H-NMR, ^{13}C-NMR, and ESI-MS. The effects of enzyme sources, organic solvents, and molar ratio of substrates on the enzymatic reaction were systematically investigated. By combining the selectively enzymatic transesterification with radical polymerization, a facile route to synthesize polymer–gemcitabine conjugates was further achieved. Three saccharide-free conjugates with high gemcitabine content (>55 wt %) and three saccharide-functionalized conjugates with 5.7–15.3 wt % gemcitabine content were, respectively, prepared and characterized by IR, NMR, and GPC. In these conjugates, galactose-functionalized and lactose-functionalized polymer–gemcitabine conjugates were potential hepatoma-targeting drug delivery systems. Further studies about the targeting function of the two conjugates to hepG2 human hepatoma cells and their release behaviors are being conducted in our laboratory.

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