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Modification of Tricomponent and Dicomponent Poly(ε-caprolactone)-co-Poly(ethylene glycol) with Methotrexate and Folic Acid

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ABSTRACT: Regarding polymer-drug conjugation, the reaction and drug characteristics are of important because they reflect the possibility of conjugation. Tri- and dicomponent azido-functionalized copolymers were initially synthesized. Tricomponent copolymers consisted of caproyl, azido-substituted caproyl, and ethylene glycol repeating units, whereas dicomponent ones contained solely the last two repeating units. In parallel, the terminal alkyne derivatives of methotrexate (MTX) and folic acid (FOL) were synthesized by coupling reaction using *N,N*-dicyclohexylcarbodiimide and 4-dimethylaminopyridine with an additional *N*-hydroxysuccinimide for FOL coupling. By click reaction, MTX and FOL were successfully conjugated with tri- and dicomponent copolymers, respectively, without polymer chain degradation. The grafting efficiencies of MTX and FOL were higher than 77 and 68% by using CuI/1,8-diazabicyclo[5.4.0]undec-7-ene and $CuSO_4.5H_2O$/sodium ascorbate, respectively. According to the differential scanning calorimetry thermograms, MTX did not change the semicrystalline property of copolymers except for high % molar grafting, whereas the presence of FOL affected thermal properties of copolymer except at 5 molar grafting. The resultant copolymers could be further used as polymer-drug conjugate delivery system for cancer therapy. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 129: 721–734, 2013

KEYWORDS: PEGylated poly(ε-caprolactone); polymer-drug conjugation; click reaction; methotrexate; folic acid

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INTRODUCTION

Poly(ε-caprolactone) (P(CL)) is one of aliphatic polyester polymers which is biodegradable and biocompatible.[1] However, the high degrees of crystallinity and hydrophobicity make this polymer less attractive for biomedical use. This shortcoming can be overcome by copolymerization with nontoxic hydrophilic polymer, poly(ethylene glycol) (PEG).[2] The copolymerization product or PEGylated copolymer can be designed to gain desirable molecular properties by using different architecture, reactive functional group, and molecular weight of PEG.[3–6] In terms of its function after administration to the body, PEG exhibits a stealth effect to prevent nonspecific interaction with the blood component,[7] to prolong the plasma half-life, and to reduce the hepatic uptake.[8,9] From these advantages, PEGylated P(CL) (P(CL)-PEG) copolymers have been enormously studied to improve the hydrophilicity, biodegradability, and mechanical property of P(CL) being suitable for drug delivery system.[10–12] Nevertheless, for the purpose of polymer-drug conjugate system, the use of P(CL)-PEG copolymers has reached its limitation because of unavailable reactive sites for drug conjugation along the polymer backbone.[13] Additionally, an easily degradable ester linkage embedded along this polymer limits the use of various useful grafting reactions since such reactions may accelerate the polymer chain degradation and result in products with uncontrollable molecular characteristics.[14] To overcome such problems, copper-catalyzed Huisgen's 1,3-dipolar cycloaddition or click reaction has been received much attention as it can proceed under mild and fast condition. By this reaction, modified ε-caprolactone (ClCL) monomer was initially synthesized by Lenoir et al.[15] which was further copolymerized with ε-caprolactone (CL) monomer via ring opening polymerization. Following a conversion of chloride pendant to azide group, the desired ligand with terminal alkyne was grafted along the backbone at the azide position by click reaction.[16] With the use of beneficial method, P(CL)-PEG can be grafted with various molecules[17–20] and consequently developed as polymer-drug conjugate delivery systems.

Previously, our group has demonstrated the successful grafting of small bioactive molecules, nicotinic acid, and *p*-aminobenzoic acid, onto the P(CL) backbone at various amounts by click

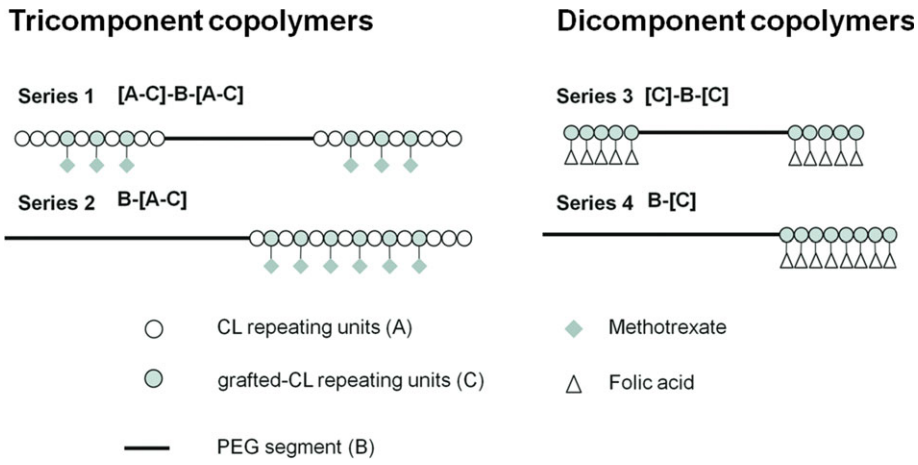


Figure 1. Schematic representation of tricomponent and dicomponent copolymers in triblock and diblock patterns. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

reaction.[21] It is of our interest to further investigate the grafting of various cytotoxic drugs and targeting ligands on the P(CL)-PEG copolymers which are intentionally used as targeting drug delivery systems for cancer therapy. To fulfill this purpose, methotrexate (MTX) and folic acid (FOL) were selected as model molecules to be chemically conjugated on the copolymers. MTX is antineoplastic drug that has been used for the treatment of various forms of cancers for several decades.[22] Typically, the systemic administration of MTX has some limitations such as systemic side effects, low plasma half-life, and resistance of cancer cells. Moreover, when MTX was physically entrapped in the nanoparticles, the premature release of MTX might be faced before reaching the target site of action. Therefore, the conjugation of MTX along macromolecular carriers was developed to overcome this drawback.[23,24] In case of FOL, it is a well-known targeting moiety and widely used in many efficient and precise carriers for cancer therapy.[25–28] Although both molecules are nearby similar in their chemical structures, they exhibit different solubilities in various solvents. Therefore, to successfully conjugate MTX and FOL on polymer backbone via click reaction, it is necessary to investigate an appropriate condition including solvent systems and catalysts used for grafting both molecules.[29,30]

In this study, the different series of copolymers were synthesized by using two types of PEG, namely α,ω-dihydroxyl PEG and α-methoxy-ω-hydroxyl PEG as an initiator because they provide different designing tailor-made block copolymers. When using α,ω-dihydroxyl PEG as an initiator, the copolymers resulted in triblock pattern ([A-C]-B-[A-C] and [C]-B-[C]), whereas the resultant copolymers using α,-methoxy-ω-hydroxyl PEG as an initiator would be in diblock pattern (B-[A-C] and B-[C]) as schematically illustrated in Figure 1. It is postulated that the different composition and the relative length of copolymer block may affect molecular and thermal characteristics of P(CL)-PEG copolymer and properties of P(CL)-PEG carriers including particle size and release characteristics and so forth.[31–33] Additionally, the different hydrophobicity of block copolymers could be fabricated by adjustment of monomer components in the polymerization step. The more hydrophobic tricomponent copolymers were synthesized at 100:1 molar ratio of monomer to PEG using ClCL and CL as monomers (series 1 and 2 in Figure 1). Meanwhile the more hydrophilic dicomponent copolymers were fabricated by the decrement in monomer to PEG molar ratio to 5:1, 10:1, and 20:1 and using sole ClCL as a monomer (series 3 and 4 in Figure 1). After fabrication, chloride pendant was converted to azide group. Afterwards, the terminal alkyne derivatives of MTX and FOL were individually grafted onto the hydrophobic segment at the azide position by click reaction. The feasibility of grafting and the molecular characteristics of copolymers were assessed by nuclear magnetic resonance spectroscopy (^{1}H NMR), Fourier transformed infrared spectroscopy (FT-IR), and gel permeation chromatography (GPC). Furthermore, the thermal behavior of the obtained grafted copolymers was evaluated by differential scanning calorimetry (DSC).

EXPERIMENTAL

Materials

α,ω-Dihydroxyl PEG (PEG_{4000}, MW 4000 g mol^{-1}, Aldrich, Germany) and α-methoxy-ω-hydroxyl PEG ($mPEG_{5000}$, MW 5000 g mol^{-1}, Fluka Chemie, Germany) were purified before use by recrystallization using a mixture of chloroform and diethyl ether (Et_2O). CL monomer (Aldrich, Germany) was dried over CaH_2 for 48 h and distillated under reduced pressure. MTX (Suzhou Rovathin, China) and FOL (Fluka, Germany) were used without purification. Propargylamine, copper (I) iodide (CuI), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), and stannous octoate ($Sn(Oct)_2$) were purchased from Aldrich Chemicals, Germany and used as received. Copper sulfate pentahydrate ($CuSO_4.5H_2O$), sodium ascorbate, and *N*-hydroxysuccinimide (NHS) were obtained from Sigma, Germany. ClCL monomer was synthesized according to our published method.[21] *N,N*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) were purchased from Fluka, Germany. Sodium azide (NaN_3) was bought from Asia Pacific Specialty Chemicals Limited, Australia. Dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), and dichloromethane (CH_2Cl_2) were dried over molecular sieve 4 Å overnight. All other organic solvents were used as received.

Table I. Fed Mole and Amount of Each Composition of Tricomponent and Dicomponent Copolymers used in the Polymerization Step

Copolymers	Fed molar ratio (mmol) ClCL:CL:PEG	Fed weight ratio (g) ClCL:CL:PEG
$P(CL)_2$-PEG_{4000}	0:100:1	0.00:2.28:0.8
P(CL)-$mPEG_{5000}$	0:100:1	0.00:2.28:0.8
Tricomponent copolymers		
Series 1		
10% $P(ClCLCL)_2$-PEG_{4000}	10:90:1	0.30:2.05:0.8
20% $P(ClCLCL)_2$-PEG_{4000}	20:80:1	0.59:1.82:0.8
30% $P(ClCLCL)_2$-PEG_{4000}	30:70:1	0.89:1.60:0.8
Series 2		
10% P(ClCLCL)-$mPEG_{5000}$	10:90:1	0.30:2.05:0.8
20% P(ClCLCL)-$mPEG_{5000}$	20:80:1	0.59:1.82:0.8
30% P(ClCLCL)-$mPEG_{5000}$	30:70:1	0.89:1.60:0.8
Dicomponent copolymers		
Series 3		
5 $P(ClCL)_2$-PEG_{4000}	5:0:1	0.74:0.00:0.80
10 $P(ClCL)_2$-PEG_{4000}	10:0:1	1.49:0.00:0.80
20 $P(ClCL)_2$-PEG_{4000}	20:0:1	2.97:0.00:0.80
Series 4		
5 P(ClCL)-$mPEG_{5000}$	5:0:1	0.74:0.00:0.80
10 P(ClCL)-$mPEG_{5000}$	10:0:1	1.49:0.00:0.80
20 P(ClCL)-$mPEG_{5000}$	20:0:1	2.97:0.00:0.80

Synthesis of Poly(ClCL-co-CL)$_2$-co-PEG$_{4000}$ (P(ClCLCL)$_2$-PEG$_{4000}$), Poly(ClCL-co-CL)-co-mPEG$_{5000}$ (P(ClCLCL)-mPEG$_{5000}$), Poly(ClCL)$_2$-co-PEG$_{4000}$ (P(ClCL)$_2$-PEG$_{4000}$), and Poly(ClCL)-co-mPEG$_{5000}$ (P(ClCL)-mPEG$_{5000}$)

The series of copolymers were prepared by ring opening polymerization method according to the previously reported method.[19] Shortly, the preset amount of ClCL, CL, and PEG (as listed in Table I) were added into the reaction flask containing 1.5% w/w of $Sn(Oct)_2$ and purged with argon gas. The polymerization was operated in an oil bath at 120°C for 24 h. Subsequently, the obtained copolymer was recovered by dissolving in chloroform and precipitated in an excess amount of cold hexane. The collected copolymer was dried and kept in vacuum desiccator.

Synthesis of Poly(N$_3$CL-co-CL)$_2$-co-PEG$_{4000}$ (P(N$_3$CLCL)$_2$-PEG$_{4000}$), Poly(N$_3$CL-co-CL)-co-mPEG$_{5000}$ (P(N$_3$CLCL)-mPEG$_{5000}$), Poly(N$_3$CL)$_2$-co-PEG$_{4000}$ (P(N$_3$CL)$_2$-PEG$_{4000}$), and Poly(N$_3$CL)-co-mPEG$_{5000}$ (P(N$_3$CL)-mPEG$_{5000}$)

The chloride pendant along the polymer was converted to azide by previously described method[20] with some modification. Briefly, the copolymer (1 equiv of chloride) was dissolved in DMF and followed by an addition of NaN_3 (1.02 equiv). The reaction was stirred under nitrogen atmosphere at room temperature overnight. The purification step of tricomponent and dicomponent copolymers was described individually in the following paragraph. After purification, the resultant copolymers were kept in vacuum desiccator before use.

For tricomponent copolymers $P(N_3CLCL)_2$-PEG_{4000} (series 1) and P(N_3CLCL)-$mPEG_{5000}$ (series 2), DMF was removed under reduced pressure. The dry copolymer was redissolved in toluene; afterwards the insoluble by-product was centrifuged at 3500 rpm, 25°C for 15 min. The supernatant was obtained and the solvent was removed under reduced pressure.

In case of dicomponent copolymers $P(N_3CL)_2$-PEG_{4000} (series 3) and P(N_3CL)-$mPEG_{5000}$ (series 4), the copolymer was redissolved in chloroform after the removal of DMF. The coinciding insoluble salt, sodium chloride, was filtered out through Whatman paper no.2 filter. The filtrate was precipitated in an excess amount of cold mixture of hexane and Et_2O (4:1). The obtained copolymer was dried under reduced pressure.

Synthesis of Propargyl Folamide

The synthesis of propargyl folamide (PFLA) was accomplished by the reported method with minor modification.[34] To the mixture of FOL (500 mg, 1.13 mmol) and triethylamine (TEA, 0.25 mL, 1.79 mmol) in DMSO (15 mL), NHS (250 mg, 2.17 mmol), and DCC (370 mg, 1.79 mmol) were added. The reaction mixture was stirred at 40°C in the dark environment for 6 h. Thin layer chromatography (TLC) was used to monitor the reaction. After 6 h, DMAP (280 mg, 2.29 mmol) and propargylamine (82 mg, 1.49 mmol) were added and stirred in the dark environment at ambient temperature for another 24 h. Afterwards, dicyclohexylurea (DCU) was removed by filtration through 0.22-μm syringe filter. The obtained yellow product was precipitated after adding an excess amount of cold Et_2O. The crude product was further purified by column chromatography. The collected fraction was evaporated by rotary evaporator to yield PFLA as a dark yellow solid: Yield: 65%. R_f. 0.35 (ethanol/CH_2Cl_2/28–30% v/v ammonia solution, 6:3:1). mp 67.67°C; ^{1}H NMR (400 MHz, DMSO-d_6, δ, ppm): 8.65 (s, 1H), 8.33 (d, J = 8.04 Hz, 1H), 8.10 (d, J = 7.83 Hz, 1H), 7.70 (d, J = 8.43 Hz, 2H), 7.50–7.40 (m, 1H), 6.99 (s, 2H), 6.64 (d, J = 8.51 Hz, 2H), 4.49 (d, J = 5.44 Hz, 2H), 4.45-4.30 (m, 1H), 3.78-3.83 (m, 2H), 2.98 (s, 1H), 2.25-1.71 (m, 4H); ^{13}C NMR (75.45 MHz, DMSO-d_6, δ, ppm): 174.6, 171.8, 165.8, 161.5, 154.3, 151.0, 150.6, 148.3, 166.3, 156.1, 111.3, 129.0, 121.8, 127.9, 111.2, 81.3, 72.6, 53.0, 45.9, 27.8, 31.8, 31.0; IR (KBr, thin film, cm^{-1}): v = 3264 (CONH), 2117 (C≡C), 1683 (C=O), 1608 (C=O—NH), 1511 (C=Cs), 1127 (C—N), 1102, 831, and 619 (C—Cb); MS (m/z): [M-H]$^+$ calcd for $C_{22}H_{22}N_8O_5$, 478.17; found, 477.83.

Synthesis of Propargyl MTX

Propargyl MTX (PMTX) was synthesized according to the reported method[34] with some modification. MTX (500 mg, 1.10 mmol) was dissolved in DMF (25 mL). Subsequently, DCC (270 mg, 1.31 mmol) was added to the clear yellow solution. After 6 h, the white precipitate of DCU was occurred. Propargylamine (82 mg, 1.49 mmol), DMAP (160 mg, 1.31 mmol), and an excess amount of TEA were added. The reaction was stirred in the dark for another 24 h and the reaction was monitored by TLC. Afterwards, the crude product was further purified using the same condition as PFLA to obtain PMTX as a yellow solid: Yield: 62%. R_f. 0.33 (ethanol/CH_2Cl_2/28–30 % v/v ammonia solution, 6:3:1). mp 65.87°C; ^{1}H NMR (400 MHz, DMSO-d_6, δ,

ppm): 8.65 (s, 1H), 8.40–3.50 (m, 1H), 8.01 (d, J = 6.77 Hz, 1H), 7.78 (d, J = 8.87, 2H), 7.50 (br s, 2H), 6.85 (d, J = 8.93, 2H), 6.65 (br s, 2H), 4.75 (s, 2H), 4.12–4.08 (m, 1H), 4.22–4.31 (m, 1H), 3.05 (s, 3H), 2.91 (s, 1H), 2.30–1.80 (m, 4H); ^{13}C NMR (75.45 MHz, DMSO-d_6, δ, ppm): 174.8, 172.7, 166.1, 165.2, 162.6, 155.0, 145.9, 146.5, 149.1, 162.7, 150.8, 111.1, 128.3, 121.3, 128.9, 111.0, 81.3, 72.6, 55.5, 54.7, 31.7, 27.9, 27.1. IR (KBr, thin film, cm^{-1}): ν = 3341 and 3205 (NH_2), 3327 (CONH), 2120 (C≡CH), 1643 (C=O), 1100 and 1003 (C—N), 614 (C≡C-H); MS (m/z): $[M\text{-H}]^+$ calcd for $C_{23}H_{25}N_9O_4$, 491.20; found, 490.56.

Conjugation of P(N_3CLCL)$_2$-PEG$_{4000}$ and P(N_3CLCL)-mPEG$_{5000}$ with PMTX

PMTX was conjugated to azide substituted copolymers, series 1 and 2, by click reaction. A hundred mg of azide substituted copolymer (1.0 equiv of azide) and PMTX (1.2 equiv) were dissolved in dry DMF. After well mixing, DBU (0.03 equiv) and CuI (0.03 equiv) were added consecutively into the reaction flask. The reaction mixture was stirred at 40°C under inert atmosphere. After 6 h, the product was purified by precipitating in an excess amount of hexane and Et_2O and the residual solvent was eventually evaporated under reduced pressure.

Conjugation of P(N_3CL)$_2$-PEG$_{4000}$ and P(N_3CL)-mPEG$_{5000}$ with PFLA

The copolymer series 3 and 4 were grafted with PFLA by the following method. A solution of copolymer (100 mg, 1.0 equiv of azide) in dry DMF was added into a solution of PFLA (1.2 equiv) in DMSO. After well mixing, an aqueous solution of sodium ascorbate (0.15 equiv) and $CuSO_4.5H_2O$ (0.05 equiv of $CuSO_4$) was added sequentially into the previous solution. The reaction mixture was allowed to stir overnight at room temperature. Subsequently, the product was purified by precipitating in Et_2O. The precipitates were washed several times with deionized water to remove the residual starting materials and by-products and finally washed with Et_2O. Then, the residual solvent was removed under reduced pressure.

Characterization

FT-IR spectra were recorded using a Nicolet FT-IR 6700 infrared spectrophotometer by KBr technique. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 at 300 and 75.45 MHz, respectively, with Bruker Avance 300 apparatus at 25°C. The weight- and number-average molecular weights (M_w and M_n) and molecular weight distribution (M_w/M_n) of the synthesized copolymers were determined by GPC apparatus equipped with a refractive index detector. The copolymer was eluted through Shodex GPC column using tetrahydrofuran as a mobile phase at a flow rate of 0.8 mL/min, 40°C. The M_w, M_n, and M_w/M_n values were calculated from calibration curve of polystyrene standards (Polymer laboratories Inc., USA), over the molecular weight range of 162–19,640 g/mol. DSC was conducted with DSC 7 Perkin Elmer differential scanning colorimeter calibrated with indium. Glass transition and melting temperatures were measured according to the running cycle: the sample was quenched to −80°C, heated to 100°C (first heating), cooled down to −80°C and heated again to 100°C (second heating). Thermograms were recorded during the second heating cycle at 10°C/min under a nitrogen atmosphere. Mass spectrum was recorded in electron spray ionization mode using the LCQ Fleet Ion Trap Mass Spectrometer (Thermo scientific, USA). Mass spectrum was scanned from 300 to 700 m/z.

RESULTS AND DISCUSSION

Synthesis of P(ClCLCL)$_2$-PEG$_{4000}$, P(ClCLCL)-mPEG$_{5000}$, P(ClCL)$_2$-PEG$_{4000}$, and P(ClCL)-mPEG$_{5000}$

Various amphiphilic copolymers were synthesized by ring opening polymerization using PEG$_{4000}$ or mPEG$_{5000}$ as a macroinitiator and Sn(Oct)$_2$ as a catalyst. As a result, four series of copolymers with different patterns were obtained. Tricomponent copolymers consisted of ClCL, CL, and ethylene glycol repeating units, designated as P(ClCLCL)$_2$-PEG$_{4000}$ (series 1) and P(ClCLCL)-mPEG$_{5000}$ (series 2) while, dicomponent copolymers contained ClCL and ethylene glycol repeating units, namely P(ClCL)$_2$-PEG$_{4000}$ (series 3) and P(ClCL)-mPEG$_{5000}$ (series 4). The synthetic pathway is illustrated in Scheme 1.

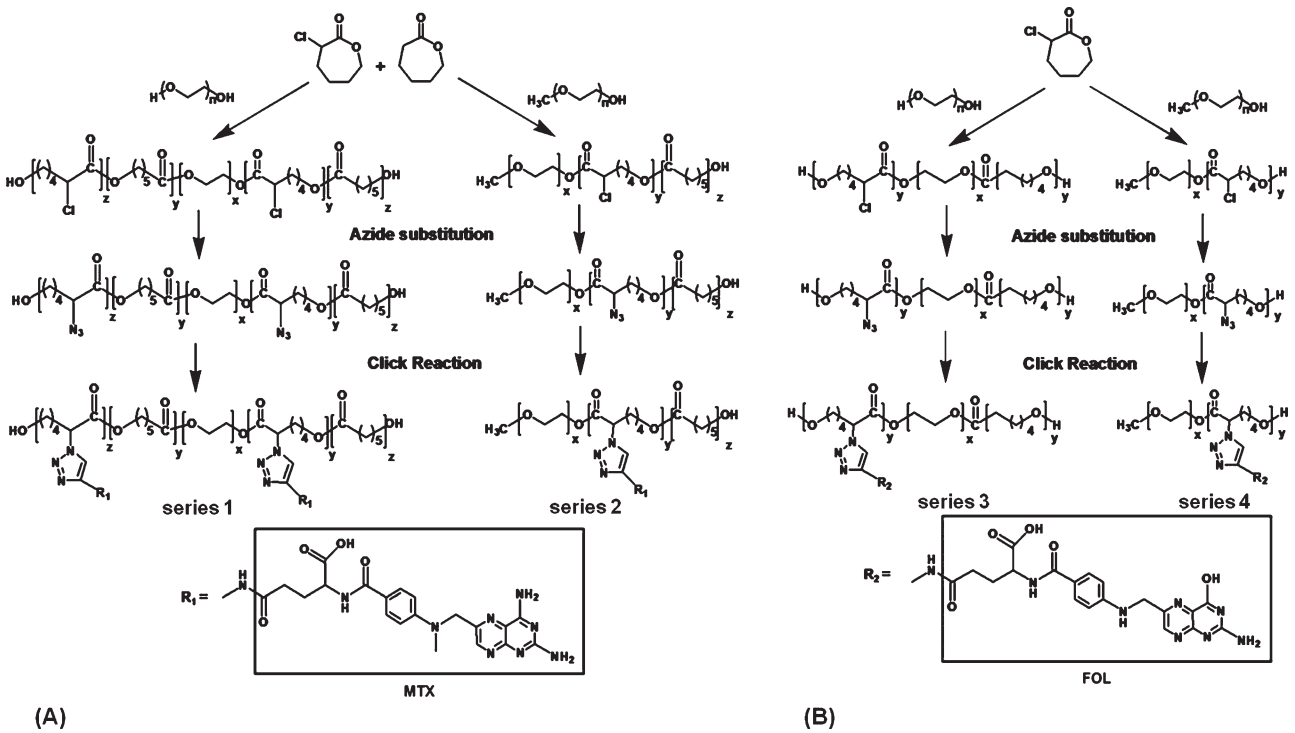
By setting molar ratio of monomer to initiator at 100, the tricomponent copolymers exhibited more hydrophobic due to higher amounts of hydrophobic ClCL and CL repeating units than those of ethylene glycol repeating units. On the contrary, the dicomponent copolymers showed more hydrophilic owing to much smaller extent of hydrophobic ClCL repeating units relative to those of ethylene glycol repeating units and an absence of CL repeating units. After polymerization, the yields of all copolymers were calculated by the weight of the synthesized copolymer compared with the theoretical weight. It was found that the yields of the copolymer series 1 and 2 were obtained at 82–94%.

However, the yields of the copolymer series 3 and 4 were found in lower extent in the range of 60–74%. The lower % yield of the copolymers in series 3 and 4 may be due to the fact that the homopolymerization of highly reactive ClCL monomer is more sensitive to the polymer degradation than the copolymerization.[16]

In this study, four series of copolymers were synthesized based on an assumption that the different architecture of macroinitiator would provide a different conformation of particulate carriers.[6] Hence, the copolymer modified by using two types of PEG polymerized with ClCL and CL at various ratios would provide the versatile applications of PEGylated copolymer. Additionally, achievement of polymer-drug conjugate could probably be affected by the compatibility between polymer and grafting molecules.[35] Therefore, the tricomponent copolymers with hydrophobic property were synthesized to conjugate with hydrophobic MTX, whereas the more hydrophilic dicomponent copolymers were fabricated for grafting with hydrophilic FOL.

Synthesis of P(N_3CLCL)$_2$-PEG$_{4000}$, P(N_3CLCL)-mPEG$_{5000}$, P(N_3CL)$_2$-PEG$_{4000}$, and P(N_3CL)-mPEG$_{5000}$

After fabrication of chloride pendent copolymers, chloride atom was converted to azide group as shown in Scheme 1. After conversion, azido-functionalized copolymers were characterized by FT-IR, ^{1}H NMR, and GPC techniques. In FT-IR spectra of all copolymer series [Figure 2A(b)–D(b)], the strong peak of azide was observed around 2107 cm^{-1} and the peak of C=O stretching of ester group was at 1750 cm^{-1}.



Scheme 1. Schematic approaching pathways of (A) engrafting MTX on tricomponent copolymers (copolymer series 1 and 2) and (B) engrafting FOL on dicomponent copolymers (copolymer series 3 and 4).

The $M_{n,NMR}$ values of copolymers were calculated from ^{1}H NMR spectra according to the reported method with minor adjustment.[19] Because the proton peak of C*H*Cl at 4.35 ppm was disappeared after conversion, the new peak at 3.95 ppm corresponding to proton of C*H*N$_3$ was used in the calculation of molar fraction of N_3CL repeating unit (FN_3). The calculation of FN_3 was based on the integrals of methyne proton of azide peak at 3.95 ppm, methylene proton of CL repeating units, and methylene proton of ethylene glycol repeating units. The molar fraction of substituted azide (FN_3) for all copolymer series were calculated from ^{1}H NMR according to the reported method[19] with some modification. Equations (1) and (2) illustrate the calculation of F^aN_3 based on hydrophobic segment for series 1 and 2, respectively whereas eqs. (3) and (4) demonstrate the computation of F^bN_3 with respect to polymer backbone. The results are summarized in Table II.

$$F^a_{N_3} = \frac{I_{A+b} - I_c}{(I_{A+b} - I_c) + \frac{1}{2}I'_A} \quad (1)$$

$$F^a_{N_3} = \frac{I_A}{I_A + \frac{1}{2}I_{A'}} \quad (2)$$

$$F^b_{N_3} = \frac{I_{A+b} - I_c}{(I_{A+b} - I_c) + \frac{1}{2}I_{A'} + \frac{1}{4}I_c} \quad (3)$$

$$F^b_{N_3} = \frac{I_A}{(I_{A+b} - I_c) + \frac{1}{2}I_{A'} + \frac{1}{4}I_b} \quad (4)$$

where I_{A+b} is an integral of methyne proton of N_3-substituted CL repeating units at 3.85 ppm and methylene proton in PEG end unit at 3.90, I_A is an integral of methyne proton of N_3-substituted CL repeating units at 3.85 ppm, $I_{A'}$ is an integral of methylene proton of CL repeating units at 2.30 ppm, I_c is an integral of methylene proton of ethylene glycol repeating units of PEG$_{4000}$, and I_b is an integral of methylene proton of ethylene glycol repeating units of mPEG$_{5000}$.

It can be seen from the results that the $M_{n,NMR}$ values of azido-functionalized copolymers of copolymer series 1 and 2 were in the range of 10,000 and 15,000 g mol^{-1} and those of copolymer series 3 and 4 ranged from 4000 to 4900 g mol^{-1}. In all cases, the $M_{n,NMR}$ and $M_{n,theo}$ values were higher than the $M_{n,GPC}$ values because the GPC results were relative values based on polystyrene standards having a different intrinsic viscosity.[36] When the amounts of azide substituted repeating units increased, the molecular weight distribution tended to increase which was in consistent with the previous report.[21]

The % substitution of azide group on the tricomponent copolymers was ranging from 60 to 100% compared with the theoretical molar fraction on the polymer backbone. For the dicomponent copolymers, the copolymer series 3 and 4 had % substitution of azide group varying from 68 to 84%.

Synthesis of PFLA and PMTX

To engraft FOL and MTX on azido-functionalized copolymers by click reaction, the terminal alkyne derivatives of FOL and MTX were essentially synthesized. PFLA and PMTX were synthesized by coupling between FOL or MTX with propargylamine via amide bond, as illustrated in Scheme 2.

Although the structures of FOL and MTX are relatively similar, the coupling reactions were slightly different. Both coupling

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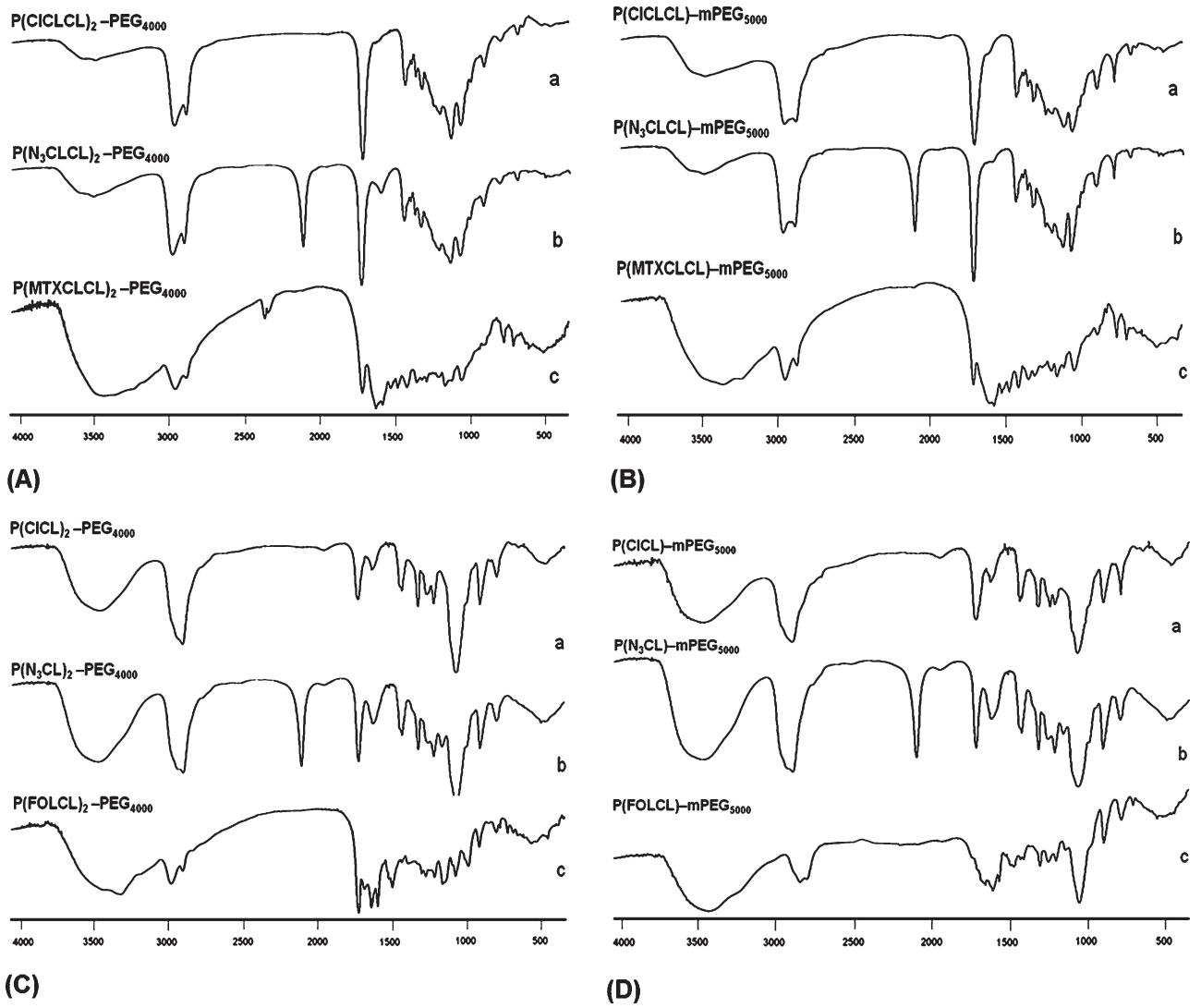

Figure 2. FT-IR spectra of copolymers, after polymerization (a), after conversion to azide (b), and after grafting with MTX or FOL (c) for series 1 (A), series 2 (B), series 3 (C), and series 4 (D).

reactions used DCC and DMAP as coupling reagents. In case of PMTX, only DCC and DMAP provided satisfied amount of the product at 62% yield. However, such coupling reagents gave low yield of PFLA under the same condition as PMTX. An addition of one more coupling reagent, NHS, increased % yield of PFLA up to 5%. It has been stated that, in an absence of NHS, DCC reacts with substrate to form amine-reactive intermediate which is unstable and easily reconverts to carboxyl group. The improved yield of PFLA by adding NHS was due to the fact that NHS forms amine-reactive succinate ester intermediate which is semistable to react with primary amine. Therefore, the presence of NHS could proceed the coupling reaction and raise the yield of final amide products.[37] Meanwhile, the addition of NHS in PMTX coupling reaction only resulted in an increased amount of by-products without the increment in % yield of PMTX. This finding is in accordance with the previous report.[38] Hence, the coupling reaction of PMTX was conducted by using solely DCC and DMAP as coupling reagents.

The structures of synthesized PFLA and PMTX were elucidated by FT-IR, ^{1}H NMR, ^{13}C NMR, and MS. The FT-IR spectra of both derivatives are illustrated in Figure 3. In FT-IR spectrum of PFLA [Figure 3(A)], the C-H stretching, C≡C stretching and C—H bending peaks of the terminal alkyne were observed at 3264, 2117, and 700–610 cm^{-1}, respectively. The peaks of N—H and C=O stretching of amide bond appeared at 3367 and 1683 cm^{-1}, respectively. The peak patterns of N—H, C=O, and C—N stretching of amide bond and those of O—H, C=O, and C—O stretching of carboxylic acid were slightly changed compared to those of FOL. Together with FT-IR spectrum, the characteristic peaks in ^{1}H NMR spectrum [Figure 3(C)] of the terminal alkyne proton and amide bonding proton were detected as singlet at 2.98 ppm and doublet at 8.10 ppm, respectively. The resultant amide bonding formation was confirmed by ^{13}C NMR of carbonyl peak at 171.8 ppm. From these results, it was suggested that FOL was successfully coupled with propargylamine resulting in the terminal alkyne derivative of FOL.

Table II. Molecular Characteristics of Tricomponent and Dicomponent Copolymers after Azide Conversion

Copolymers	F^aN_3 grafted		F^bN_3 grafted							
	Theoretical FN_3	Calculated[c] F^aN_3	Theoretical FN_3	Calculated[c] F^bN_3	% Substitution of N_3[d]	% Yield	$M_{n,theo}$[e]	$M_{n,NMR}$[c]	$M_{n,GPC}$[f]	$M_{w,GPC}/M_{n,GPC}$[f]
$P(CL)_2$-PEG_{4000}	-	-	-	-	-	94.33	15,400	13,182	9026	1.70
P(CL)-$mPEG_{5000}$	-	-	-	-	-	93.25	16,400	16,403	10,346	1.07
Tricomponent copolymers										
Series 1										
10% $P(N_3CLCL)_2$-PEG_{4000}	0.10	0.05	0.05	0.03	60.00	82.56	15,810	10,911	6021	1.23
20% $P(N_3CLCL)_2$-PEG_{4000}	0.20	0.15	0.10	0.07	70.00	84.01	16,220	13,335	7437	1.28
30% $P(N_3CLCL)_2$-PEG_{4000}	0.30	0.26	0.15	0.12	80.00	82.37	16,630	15,821	8000	1.63
Series 2										
10% $P(N_3CLCL)$-$mPEG_{5000}$	0.10	0.08	0.05	0.05	100.00	94.33	16,810	14,631	6313	1.31
20% $P(N_3CLCL)$-$mPEG_{5000}$	0.20	0.12	0.10	0.09	90.00	90.69	17,220	11,610	8878	1.32
30% $P(N_3CLCL)$-$mPEG_{5000}$	0.30	0.23	0.15	0.13	86.67	91.52	17,630	10,241	8612	1.40
Dicomponent copolymers										
Series 3										
5 $P(N_3CL)_2$-PEG_{4000}	-	-	5	4.20	84.00	74.07	4775	3968	2593	1.57
10 $P(N_3CL)_2$-PEG_{4000}	-	-	10	7.30	73.00	60.10	5550	4104	3076	2.07
20 $P(N_3CL)_2$-PEG_{4000}	-	-	20	13.60	68.00	59.91	7100	4851	3101	1.68
Series 4										
5 $P(N_3CL)$-$mPEG_{5000}$	-	-	5	3.59	71.80	69.01	5775	4151	2430	1.61
10 $P(N_3CL)$-$mPEG_{5000}$	-	-	10	7.50	75.00	60.01	6550	4934	3186	1.98
20 $P(N_3CL)$-$mPEG_{5000}$	-	-	20	15.01	75.05	62.31	8100	4838	4032	1.69

[a]Molar fraction of azide calculated based on hydrophobic segments,[b]Molar fraction of azide calculated based on polymer backbone,[c]Determined by ^{1}H NMR spectroscopy,[d]% substitution of N_3 calculated based on polymer backbone,[e]$M_{n,theo} = \left(\frac{[CL]}{[I]} \times 114\right) + \left(\frac{[N_3CL]}{[I]} \times 155\right) + M_{n,PEG}$ This value was calculated based on 100% conversion of monomer; where [*CL*] is the molar concentration of CL, [N_3CL] is the molar concentration of N_3CL, [*I*] is the molar concentration of the initiator (PEG_{4000} or $mPEG_{5000}$) and $M_{n,PEG}$ is the molecular weight of PEG_{4000} or $mPEG_{5000}$,[f]Determined by GPC.

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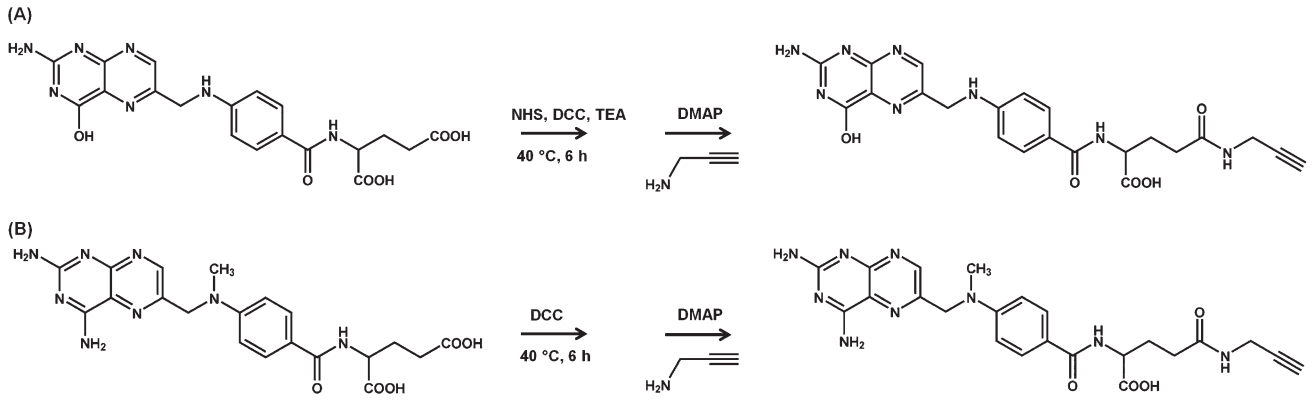

Scheme 2. Synthesis of propargyl folamide (A) and propargyl methotrexate (B).

Likewise, the FT-IR spectrum of PMTX [Figure 3(B)] also showed the characteristic peaks of the terminal alkyne at 3299, 2122, and 700–610 cm^{-1}, respectively, coinciding with the presence of N—H stretching peak at 3327 cm^{-1} and C=O stretching peak at 1643 cm^{-1} of amide bond. In NMR spectrum [Figure 3(D)], the characteristic peaks of the triplet of the terminal alkyne proton and the doublet of forming amide proton were observed at 2.91 and 8.01 ppm, respectively. The peak

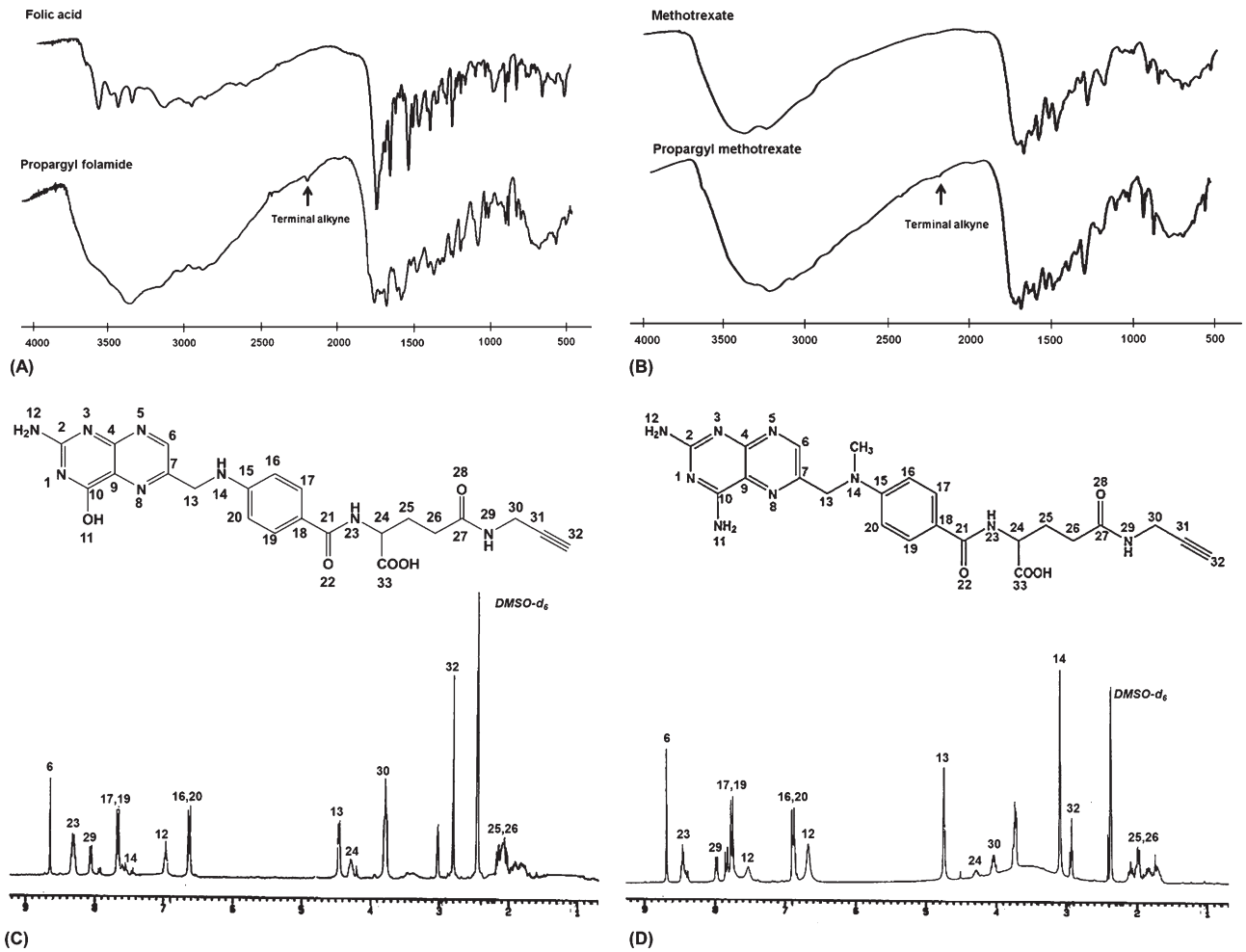

Figure 3. FT-IR (top) and ^1H NMR (bottom) spectra of PFLA (A and C) and PMTX (B and D), respectively.

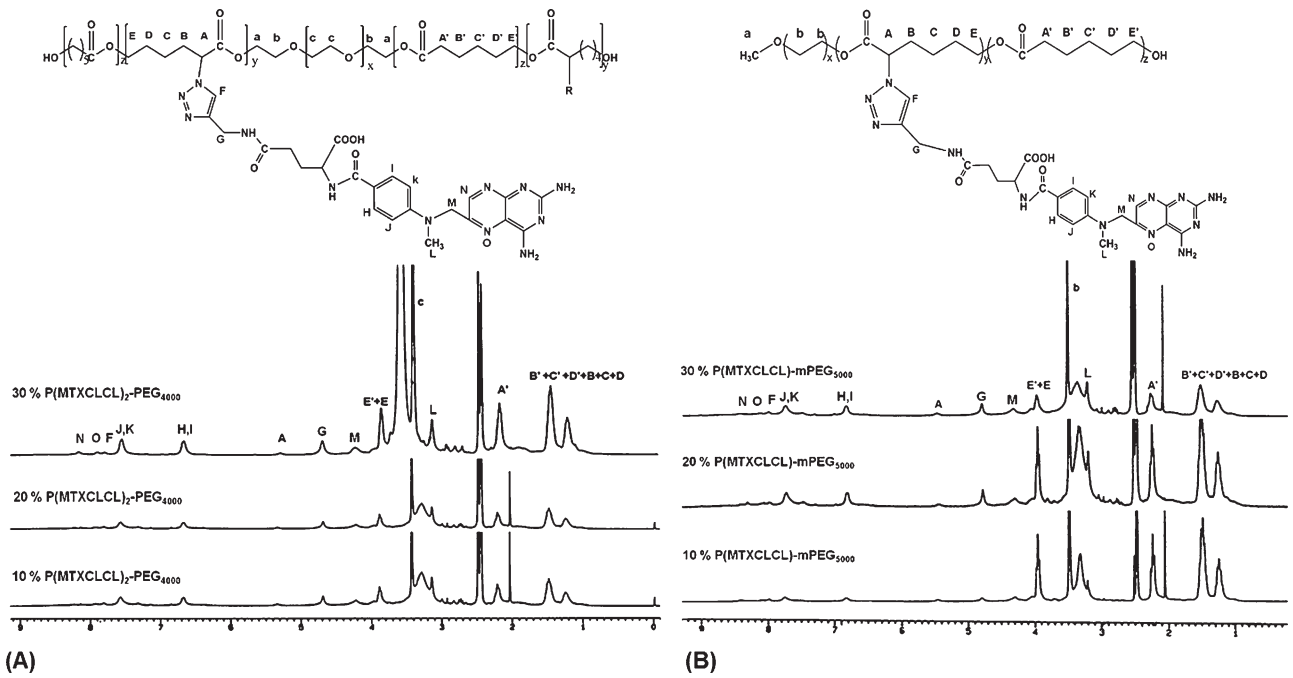

Figure 4. ^{1}H NMR spectra of grafting MTX on tricomponent copolymers, series 1 (A) and 2 (B) at the different amount of grafting MTX.

at 172.7 ppm in ^{13}C NMR spectrum was corresponded to carbonyl of amide bond.

In accordance with the previous report,[39] it has been described that the coupling reaction predominantly occurred at the γ-carboxylic group rather than the α-carboxylic group of FOL/MTX due to its higher reactivity. From our results, the assigned protons in NMR spectra and the molecular mass values from MS spectra of PFLA and PMTX agreed well with the theoretical structure. The integration ratio of terminal alkyne proton (2.98 ppm for PFLA and 2.91 ppm for PMTX) to the characteristic peak of methylene proton (H-6) in pteridine ring (8.65 ppm for PFLA and PMTX) was 1.10 indicating approximately equimolar ratio of propargylamine conjugated with FOL/MTX.

Conjugation of $P(N_3CLCL)_2$-PEG_{4000} and $P(N_3CLCL)$-$mPEG_{5000}$ with PMTX

According to our hypothesis, PMTX was conjugated with the tricomponent copolymer backbone through click reaction using CuI as a catalyst and DBU as a base. The achievement of the coupling reaction was confirmed by FT-IR, ^{1}H NMR, and GPC techniques. In Figure 2(A(c)–B(c)), FT-IR spectra showed the complete disappearance of strong absorption peak of azide together with the appearance of new characteristic peak of triazole ring at 1665 cm^{-1}. Moreover, the new absorption peaks were observed at 3327 cm^{-1} corresponding to N—H stretching of CONH of PMTX and 1643 cm^{-1} attributing to C=O stretching of polymer. In addition to FT-IR spectra, the new peak in ^{1}H NMR spectra at 5.30 ppm (I_A in Figure 4) was assigned to the methyne proton adjacent to triazole ring and that at around 7.55 ppm was contributed to the combined peaks of methyne proton in triazole ring and methyne protons in pteridine ring of MTX. The molar fraction of MTX (F_{MTX}) of copolymer series 1 and 2 was calculated from ^{1}H NMR according to the reported method[19] with minor modification. Equation (5) indicates the calculation of F^{a}_{MTX} based on hydrophobic segment while eqs. (6) and (7) show the computation of F^{b}_{MTX} regarding polymer backbone for copolymer series 1 and 2, respectively.

$$F^{a}_{MTX} = \frac{I_A}{I_A + \frac{1}{2} I_{A'}} \quad (5)$$

$$F^{b}_{MTX} = \frac{I_A}{I_A + \frac{1}{2} I_{A'} + \frac{1}{4} I_c} \quad (6)$$

$$F^{b}_{MTX} = \frac{I_A}{I_A + \frac{1}{2} I_{A'} + \frac{1}{4} I_b} \quad (7)$$

where I_A is an integral of methyne proton of MTX-grafted CL repeating units at 5.30 ppm, $I_{A'}$ is an integral of methylene proton of CL repeating units at 2.30 ppm, I_c is an integral of methylene proton of ethylene glycol repeating units of PEG_{4000}, and I_b is an integral of methylene proton of ethylene glycol repeating units of $mPEG_{5000}$.

The results of molecular characteristics of MTX-grafted tricomponent copolymers are listed in Table III. The molar fraction of MTX (F^{b}_{MTX}) values of copolymer series 1 were 0.03, 0.06, and 0.11 and those of copolymer series 2 were 0.04, 0.07, and 0.12. The % grafting efficiency could be calculated by comparing the molar fraction of grafting MTX (F^{b}_{MTX}) with that of azide group ($F^{b}N_3$). The grafting efficiency was ranged from 77 to 100% for both series. After grafting, the $M_{n,GPC}$ and $M_{w,GPC}/M_{n,GPC}$ values of MTX-grafted copolymers slightly increased as compared to those of azide-substituted copolymers. However, no small peak was observed in GPC chromatograms after grafting as seen in Figure 5. From the results, it was suggested that MTX was

Table III. Summarized Molecular Characteristics of MTX-Grafted Tricomponent Copolymers, Series 1 and 2

Grafted copolymers	Calculated F^{a}_{MTX}	Grafting efficiency (%)[b]	% Yield	$M_{n,GPC}$[c]	M_w/M_n[c]
Series 1					
10% P(MTXCLCL)$_2$-PEG$_{4000}$	0.03	100.00	79.50	7335	1.16
20% P(MTXCLCL)$_2$-PEG$_{4000}$	0.06	85.71	80.00	8600	2.10
30% P(MTXCLCL)$_2$-PEG$_{4000}$	0.11	91.67	78.50	8500	1.80
Series 2					
10% P(MTXCLCL)-mPEG$_{5000}$	0.04	80.00	80.12	7099	1.54
20% P(MTXCLCL)-mPEG$_{5000}$	0.07	77.78	82.11	9312	1.40
30% P(MTXCLCL)-mPEG$_{5000}$	0.12	92.31	78.50	7505	2.00

[a]Determined by ^{1}H NMR spectroscopy, [b]Calculated based on polymer backbone, [c]Determined by GPC.

successfully grafted along the polymer backbone by click reaction at various amounts of grafting MTX without the degradation of polymer backbone.

Conjugation of P(N$_3$CL)$_2$-PEG$_{4000}$ and P(N$_3$CL)-mPEG$_{5000}$ with PFLA

To approach our hypothesis, the short hydrophobic chain copolymers were fabricated and conjugated with PFLA as referred to the copolymer series 3 and 4. The short hydrophobic chains of these copolymers were decreased to 5, 10, and 20 moles relative to 1 mole of PEG resulting in a small hydrophobic part of caproyl repeating units and a large part of ethylene glycol repeating units leading to the copolymers with the predominant hydrophilic property. The engrafting reaction was also proceeded through click reaction using, however, the different catalyst and base system than those used in the grafting of MTX.

It was found that the grafting reaction by using CuI and DBU was incomplete within 6 h under the same condition as that of PMTX. Moreover, an increased amount of catalyst and base by 0.1–0.2 equiv in the reaction could not lead to complete grafting of PFLA and resulted in the products which were hardly redissolved in the solvent. The incomplete reaction was attributed to the solvent. Because solvent or solvent mixture is an important key factor affecting the rate and completeness of click reaction, the solvent must dissolve the substrates and Cu catalyst to insure rapid and complete reactions.[40,41] One of the polar solvent mixtures which could totally dissolve PFLA and azido-functionalized copolymer was DMSO/DMF rather than only DMF. Therefore, the catalyst/solvent system of the grafting reaction of PFLA was changed to $CuSO_4.5H_2O$ and sodium ascorbate in DMSO/DMF. The reaction was conducted at room temperature for 24 h. Under this condition, the reaction was completed as confirmed by FT-IR performance. In Figure 2(C(c)–D(c)), the characteristic peak of azide at 2107 cm^{-1} was entirely disappeared. The N—H stretching of CONH of PFLA, C=O stretching of polymer and PFLA, and C=N stretching of the overlapping vibrations of triazole ring and pteridine ring were found at 3264, 1683, and 1645 cm^{-1}, respectively. In ^{1}H

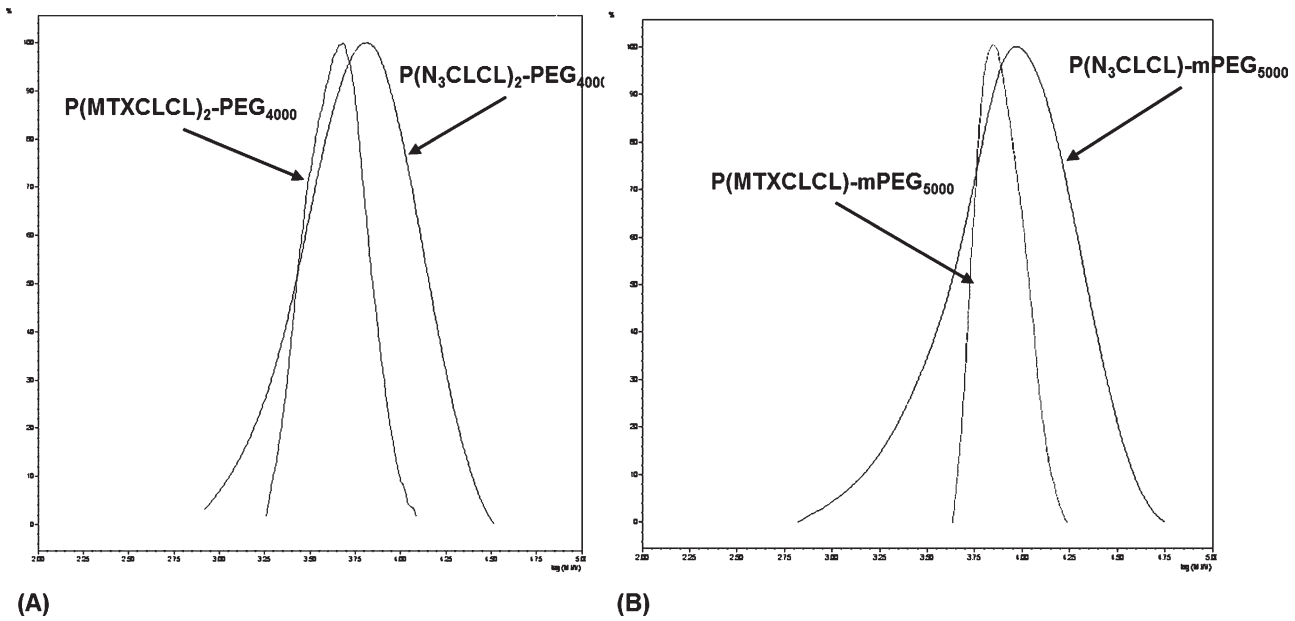

Figure 5. Examples of GPC chromatograms of MTX-grafted tricomponent copolymers, series 1 (A) and 2 (B) after grafting comparing with azido-functionalized tricomponent copolymers.

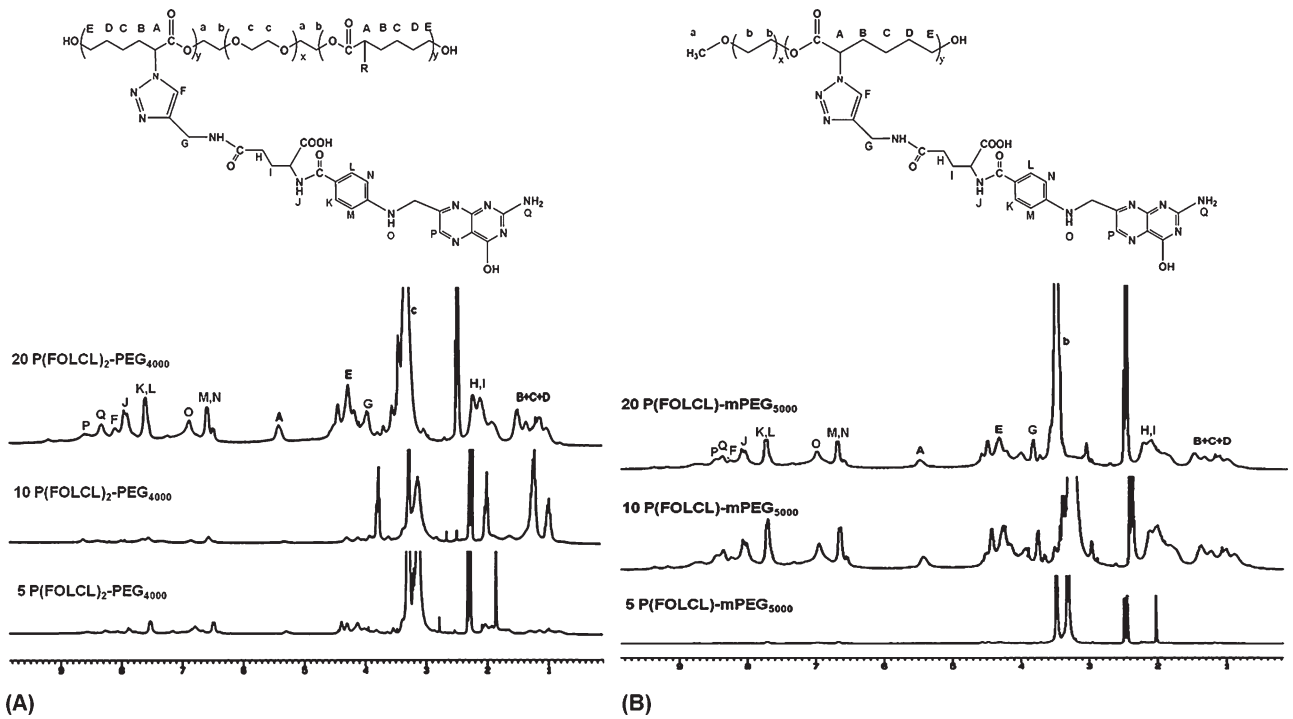

Figure 6. ^{1}H NMR spectra of FOL-grafted dicomponent copolymers, series 3 (A) and 4 (B) at various amounts of grafting FOL.

NMR spectra (Figure 6), the new methyne proton peaks were detected at 5.30 ppm corresponding to the methyne proton adjacent to triazole ring and at 7.55 ppm associated with the methyne proton in triazole ring. However, the latter was overlapped by the methyne protons in pteridine ring of FOL. Furthermore, the characteristic peaks of FOL were also presented in the spectra over the region of 2.0–2.5 ppm and 6.0–8.5 ppm. The molar fraction of FOL (F_{FOL}) of copolymer series 3 and 4 based on polymer backbone could be calculated from ^{1}H NMR spectra according to the published method[19] with some modification as shown in eqs. (8) and (9).

$$F_{FOL} = \frac{I_A}{I_A + \frac{1}{4} I_c} \quad (8)$$

$$F_{FOL} = \frac{I_A}{I_A + \frac{1}{4} I_b} \quad (9)$$

where I_A is an integral of methyne proton of FOL-grafted CL repeating units at 5.30 ppm, I_c is an integral of methylene proton of ethylene glycol repeating units of PEG_{4000}, and I_b is an integral of methylene proton of ethylene glycol repeating units of $mPEG_{5000}$.

Table IV demonstrates the molecular characteristics of FOL-grafted dicomponent copolymers. The F_{FOL} values were found to be 3.62, 6.67, and 10.19 for copolymer series 3 and 3.59, 6.87, and 10.29 for copolymer series 4. The grafting efficiencies calculated on the same basis as that of MTX were 86.19, 91.36, and 74.92% for copolymer series 3 and 100, 91.60, and 68.55% for copolymer series 4. The $M_{n,GPC}$ of FOL-grafted copolymers increased, whereas the molecular weight distribution were almost similar to those of azide-substituted copolymers. The GPC chromatograms showed the unimodal distribution peak after grafting with FOL as illustrated in Figure 7. These results

Table IV. Summarized Molecular Characteristics of FOL-Grafted Dicomponent Copolymers, Series 3 and 4

Grafted copolymers	Calculated F^a_{FOL}	Grafting efficiency (%)[b]	% Yield	$M_{n,GPC}$[c]	M_w/M_n[c]
Series 3					
5 P(FOLCL)$_2$-PEG$_{4000}$	3.62	86.19	75.11	3047	1.79
10 P(FOLCL)$_2$-PEG$_{4000}$	6.67	91.36	78.20	2605	1.70
20 P(FOLCL)$_2$-PEG$_{4000}$	10.19	74.92	69.15	3203	1.49
Series 4					
5 P(FOLCL)-mPEG$_{5000}$	3.59	100.00	79.12	2880	1.51
10 P(FOLCL)-mPEG$_{5000}$	6.87	91.60	72.55	3118	1.61
20 P(FOLCL)-mPEG$_{5000}$	10.29	68.55	75.30	4306	1.65

[a]Determined by ^{1}H NMR spectroscopy, [b]Calculated based on polymer backbone, [c]Determined by GPC.

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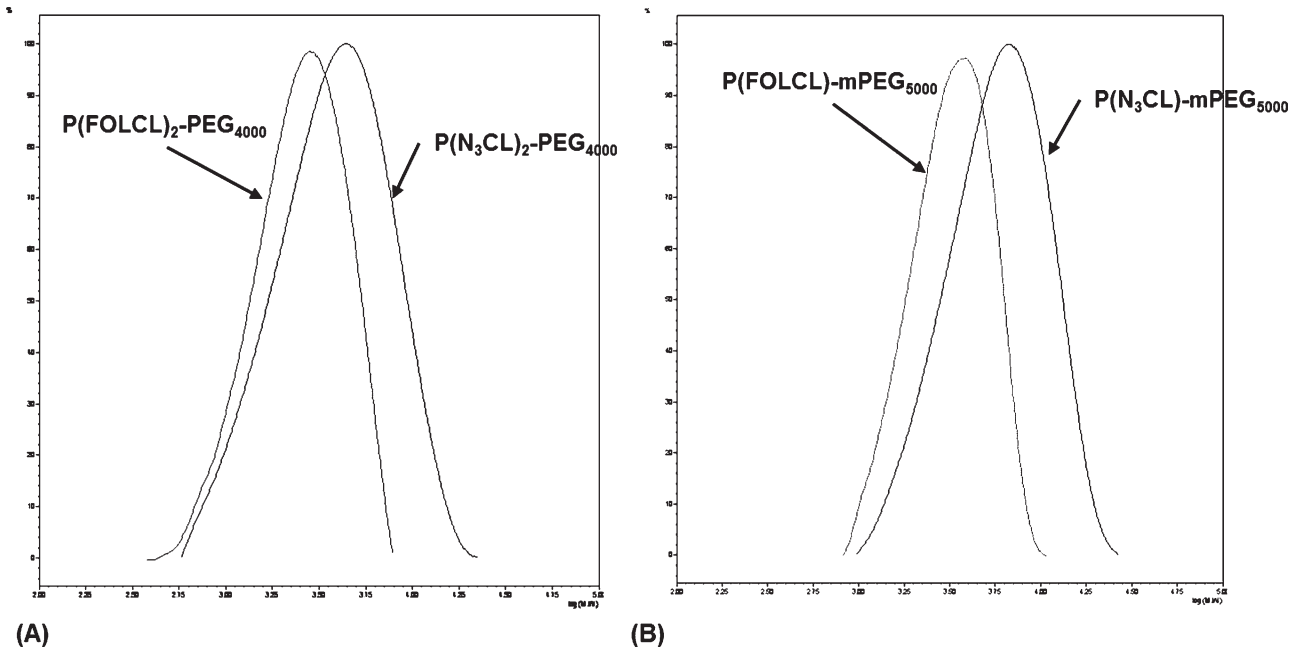

Figure 7. Examples of GPC chromatograms of FOL-grafted dicomponent copolymers, series 3 (A) and 4 (B) after grafting comparing with azido-functionalized dicomponent copolymers.

indicated that FOL could be grafted along the low molecular weight caproyl units of dicomponent copolymers with no degradation of the backbone.

Thermal Properties of Grafted Copolymers

To investigate the thermal behavior of the grafted copolymers, DSC technique was used. DSC thermograms of all copolymers are illustrated in Figure 8.

In general, P(CL) homopolymer usually has T_g around −60°C and T_m about 55–60°C.[42] PEG exhibits a high crystalline structure with the T_m around 56.2–69.7°C over the molecular weight range of 2000–20,000 g mol^{-1}.[17,43] When combining P(CL) with PEG, the thermal behavior of P(CL)-PEG copolymer differed from that of each homopolymer and depended on the ratio between P(CL) and PEG. Usually, P(CL)-PEG copolymer with the long hydrophobic P(CL) chain presented the same crystalline structure as P(CL) homopolymer, whereas PEG-bearing short hydrophobic P(CL) block retained the crystalline structure of PEG.[3,17,44] From the results, at the same ratio of P(CL) and PEG (100:1), two series of P(CL)-PEG copolymer were fabricated using PEG_{4000} and $mPEG_{5000}$ resulting in triblock and diblock copolymers, respectively. It was found that the T_g and T_m of $P(CL)_2$-PEG_{4000} were observed at −65.72 and 45.78°C and those of P(CL)-$mPEG_{5000}$ were detected at −63.20 and 48.61°C, respectively.

This slight difference in thermal behavior suggested that the different block of copolymers (triblock and diblock) had insignificant effect on the crystallinity of copolymer.

Before grafting, the DSC thermograms of grafting ligand, PFLA and PMTX, were also recorded to investigate a change in crystallinity of targeting molecule and drug. The T_m of PFLA and PMTX were observed at 67.67 and 65.87°C, respectively. The observed T_m of PFLA and PMTX were almost half less than their parent molecules.

All thermograms of grafted copolymers showed the endothermic patterns; however, in some cases, no endothermic peaks were recorded. In case of the copolymer series 1 (triblock pattern), the T_g and T_m values did not differ from $P(CL)_2$-PEG_{4000} with the increasing amount of grafting MTX. However, in case of 30% $P(MTXCLCL)_2$-PEG_{4000}, no endothermic peak was observed. For the copolymer series 2 (diblock pattern), the T_g and T_m values remained almost constant when increasing the amount of grafting MTX. Those values were also not different from those of P(CL)-$mPEG_{5000}$. The DSC thermograms of copolymer series 1 and 2 are shown in Figure 8(A–B). As seen in the thermograms, the triblock and diblock patterns of the grafted copolymers exhibited the different thermal behavior particularly at high % grafting as a result of the grafting MTX. In addition, it was due to the fact that the triblock copolymers bearing hydrophobic constituent on two arms of PEG had more profound disturbance on the crystallization of PEG segment as compared with the diblock copolymers which were affected by a single arm of the grafting hydrophobic portion. Consequently, the PEG chain of diblock copolymers was more flexible and thus less restricted to crystallize.[31,45]

In case of FOL-grafted copolymers in series 3 (triblock pattern) and 4 (diblock pattern), their thermal behaviors are shown in Figure 8(C–D). At 5 molar grafting, 5 $P(FOLCL)_2$-PEG_{4000} and 5 P(FOLCL)-$mPEG_{5000}$ had the similar T_g values at −60°C and the T_m values of about 50.07 and 54.69°C, respectively. However, at 10 and 20 molar grafting of FOL, no endothermic peak could be detected in both series. The thermal behavior of PEGylated P(CL) reportedly depended on the P(CL)/PEG ratio. The increasing P(CL) block length predominantly disturbed the

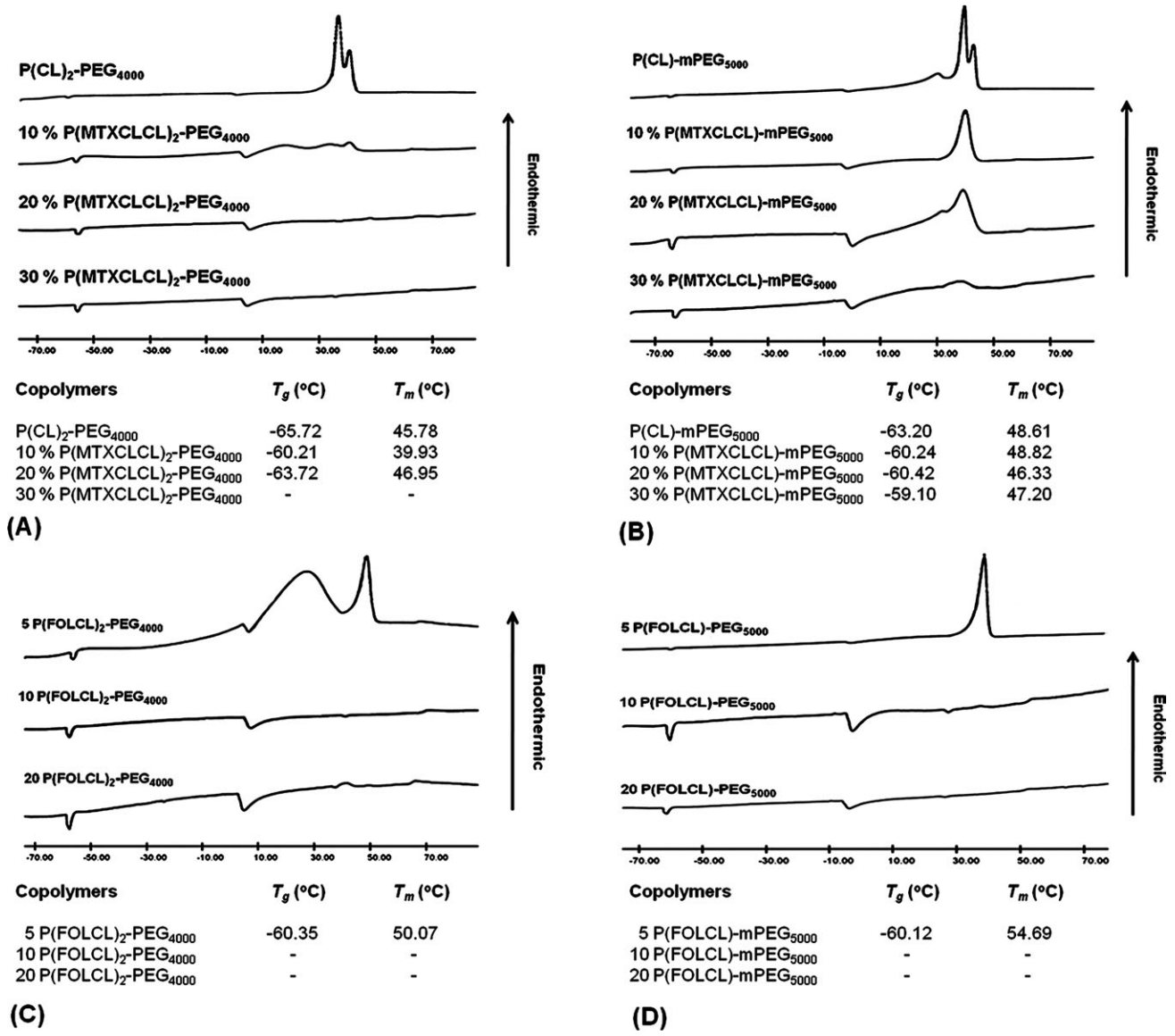

Figure 8. DSC thermograms of MTX-grafted tricomponent copolymer series 1 (A) and series 2 (B) and FOL-grafted dicomponent copolymer series 3 (C) and 4 (D).

crystallization of the PEG block.[31] In case of copolymer series 3 and 4, the increment in molar grafting from 5 to 10 and 20 led to dramatically increased P(CL) chain length due to the grafting FOL. Hence, the crystallinity of P(CL) segment was more pronounced compared with that of PEG chain. Because it was reported that the chloride pendants of P(ClCL) homopolymer turned the semicrystallinity of P(CL) homopolymer to amorphous state[16], the P(ClCL) chain as well as the grafted P(CL) homorepeating units copolymerized to PEG would also exhibit the amorphous state. Therefore, the grafted P(CL) chain of copolymers series 3 and 4 at high molar grafting more profoundly interfered the crystallization of PEG chain resulting in no observed endothermic peak in DSC thermograms. According to the results, the small amount of grafted repeating units (5 molar grafting) was incapable of hindering the crystallization of PEG segment. Nevertheless, the increased amount of FOL-grafted repeating units could impede the crystallization of PEG segment.

CONCLUSIONS

From the results, various copolymers with different compositions were synthesized by ring opening polymerization at 120°C for 24 h using different types of PEG as an initiator. Consequently, MTX and FOL were successfully grafted onto $P(CL)_2$-PEG_{4000} and P(CL)-$mPEG_{5000}$ backbones by click reaction without chain degradation. The grafting reaction of MTX was performed at 40°C for 6 h using CuI/DBU as a catalyst/base system whereas that of FOL was proceeded at room temperature for 24 h by using $CuSO_4.5H_2O$/sodium ascorbate. The established grafting system provided the satisfied grafting efficiency and could possibly be used for grafting other drugs.

The grafting MTX did not alter the semicrystalline property of the copolymers except for 30% molar grafting. However, an increase in FOL homogeneously grafted repeating units in the copolymers had changed the semicrystallinity of the copolymers to amorphous form. Of these results, it can be concluded that the MTX-grafted tricomponent copolymers and the FOL-grafted dicomponent copolymers could be successfully synthesized. Theses grafted copolymers can be further applied for preparation of targeting carriers for cancer therapy.

ACKNOWLEDGMENTS

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