ORIGINAL ARTICLE

Synthesis of diblock functional poly(ϵ -caprolactone) amphiphilic copolymers grafted with bioactive molecules and characterization of their micelles

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In this study, the grafting of an alkynyl bioactive compound to poly(α -azido- ϵ -caprolactone)-*b*-poly(ϵ -caprolactone) (P α N₃CL-*b*-PCL) was performed using Huisgen's 1,3-dipolar cycloaddition, also known as click chemistry. The grafted copolymers were successfully obtained at various ratios, as confirmed by nuclear magnetic resonance, gel permeation chromatography and Fourier transform infrared spectroscopy. The graft-block poly(α -azido- ϵ -caprolactone-*graft*-bioactive molecule) ((P α N₃CL-*g*-BioM)-*b*-PCL) copolymers were semicrystalline, with the melting temperature (T_m) depending on the type and the amount of grafting compounds. Grafting of 1-dimethylamino-2-propyne, pent-4-ynyl nicotinate and propargyl *N*-benzyloxycarbonyl-4-hydroxy proline onto the P α N₃CL-*b*-PCL caused these amphiphilic copolymers to self-assemble into micelles in the aqueous phase. The critical micelle concentration (CMC) ranged from 4.6 to 20 mg l⁻¹, and the average micelle size ranged from 105 to 162 nm. The hydrophilicity and the unit of the grafting compounds influenced the stability of the micelle. This study describes the drug-entrapment efficiency and drug-loading content of the micelles, which were dependent on the composition of the graft-block polymers. The results from *in vitro* cell viability assays showed that (P α N₃CL-*g*-BioM)-*b*-PCL possessed low cytotoxicity. *Polymer Journal* (2013) 45, 962–970; doi:10.1038/pj.2012.233; published online 6 March 2013

Keywords: amphiphilic; bioactive molecules; click chemistry; graft-block functional polyester; micelles

INTRODUCTION

Over the past decade, an increasing amount of attention has been paid to environmentally friendly thermoplastics and biomaterials.^{1,2} Aliphatic polyesters, such as poly(glycolide), poly(lactide) and poly(ϵ -caprolactone) (PCL) combine biodegradability and biocompatibility, and are produced on an industrial scale. Nevertheless, a lack of pendent functional groups along these polyester chains is a major limitation to their potential applications.³ The introduction of pendent functional groups along these polyester chains is highly desirable to tailor and modulate their physicochemical properties, such as hydrophilicity, biodegradation rate, bioadhesion, crystallinity and biological activity.^{4–6}

Amphiphilic block copolymers are known for their self-assembly into micelles or larger aggregates in solvents that are selective for one block. In aqueous solutions, a core-shell structure commonly forms consisting of a hydrophobic core surrounded by a hydrated hydrophilic shell. Previous research has extensively studied the properties of block copolymer micelles in biomedical applications, namely the efficacious delivery of hydrophobic drugs sequestered within micellar cores.^{7–10}

The parameter that principally governs the self-organization of amphiphilic block copolymers is the hydrophilic–hydrophobic balance, although other experimental factors, such as concentration, pH,

ionic strength, temperature and sample preparation, may also influence aggregation mechanisms.¹¹ Hence, the macromolecular structure of block copolymer aggregates relatively predetermines their nano-scale morphology.^{12,13} The hydrophilic–hydrophobic balance of diblock copolymers can be potentially modified by coupling them with additional hydrophilic or hydrophobic moieties.^{14,15}

Recently, the possibility of grafting functional groups onto the chain of PCL through Huisgen's 1,3-dipolar cycloaddition, known as 'click chemistry,' was reported. This reaction has received significant attention because of its feasibility and mild conditions. Recent research has extensively examined copper-catalyzed azido-alkyne cycloadditions in biological and material sciences. Riva *et al.*,^{16,17} Zednik *et al.*,¹⁸ Suksiriworapong *et al.*¹⁹ and other reports^{20,21} have shown that small functional groups (for example, benzoate, triethyl ammonium bromide, nicotinic acid, *p*-aminobenzoic acid and hexyne) and macromolecules (such as poly(ethylene oxide)) could be successfully grafted onto a polymer backbone using a click reaction. Although numerous attempts have been made to attach either functional groups or drug molecules onto polymer backbones, few studies have been concerned with attaching drugs onto polyester backbones, in particular PCL.^{22,23}

This study investigates using copper-catalyzed azido-alkyne cycloaddition as a tool for varying the hydrophilic–hydrophobic balance of

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diblock copolymers in an aqueous medium. This approach ‘clicks’ a model AB amphiphilic block copolymer, composed of a PCL hydrophobic segment and a poly(α -azo- ϵ -caprolactone-*graft*-bioactive molecule) (P α N₃CL-*g*-BioM) hydrophilic segment, with additional hydrophilic BioM (Scheme 1). The nitrogen atoms in the grafted BioM serve as bases and hydrogen bond acceptors. The grafting of BioM onto AB amphiphilic block PCL copolymers has not been reported previously. This work studies the influence of the hydrophilic/hydrophobic chain lengths of the block copolymers, and the grafted BioM on micelle sizes, drug-entrapment efficiency and drug-loading content. Fluorescence spectroscopy, dynamic light scattering (DLS) and transmission electron microscopy were used to evaluate the micellar characteristics of these graft-block copolymers in an aqueous solution.

EXPERIMENTAL PROCEDURE

Materials

Benzyl alcohol, 2-chlorocyclohexanone, pyrene, 1-dimethylamino-2-propyne (DMAP), nicotinic acid, 4-pentyn-1-ol, 1,3-dicyclohexyl-carbodiimide, 4-dimethylamino-pyridine, Z-1-4-hydroxyproline, propargyl bromide, indomethacin (IMC) and sodium azide were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). *m*-Chloroperoxybenzoic acid was purchased from Fluka Chemical Co. (Buchs SG1, Switzerland). Stannous octoate was purchased from Strem Chemical Co. (Newburyport, MA, USA). ϵ -Caprolactone was dried and vacuum-distilled over calcium hydride. α -Chloro- ϵ -caprolactone (α ClCL) was prepared using a previously reported method.²⁴ Pent-4-ynyl

nicotinate (PNIC) and propargyl *N*-benzyloxycarbonyl-4-hydroxy proline (PBHP) were prepared as reported previously.¹⁹ Organic solvents such as tetrahydrofuran, methanol, chloroform, toluene, *N,N*-dimethylformamide and *n*-hexane were high-pressure liquid chromatography grade and were purchased from Merck Chemical (Darmstadt, Germany). Ultrapure water was obtained by purification with a Milli-Q Plus system (Waters, Milford, MA, USA).

Typical click reaction

P α N₃CL-*g*-b-PCL₁₆ (27.9 mmol, 1.06 mol equiv. of azide) was transferred into a glass reactor containing tetrahydrofuran. The alkynyl BioM (1.06 mol), CuI (2.8 mmol) and triethyl amine (2.8 mmol) were then added to the reactor. The solution was stirred at 60 °C for 24 or 48 h. The cold reaction product was precipitated in diethyl ether. The purified polymer was dried under vacuum at 50 °C for 24 h and then analyzed. Figures 1a–c show the proton nuclear magnetic resonance (¹H-NMR) spectra of (P α N₃CL-*g*-DMAP)-*b*-PCL, (P α N₃CL-*g*-PNIC)-*b*-PCL and (P α N₃CL-*g*-PBHP)-*b*-PCL, respectively.

Characterization

¹H-NMR spectra were recorded at 500 MHz (with a WB/DMX-500 spectrometer; Bruker, Ettlingen, Germany) using chloroform (δ 7.24 p.p.m.) as an internal standard in chloroform-*d* (CDCl₃). Thermal analysis of the polymer was performed on a DuPont 9900 system using differential scanning calorimetry (DuPont, Newcastle, DE, USA). The heating rate was 20 °C min^{−1}. Glass-transition temperatures (*T*_gs) were recorded in the middle of the heat capacity change and were taken from the second heating scan after quick cooling. Number- and weight-average molecular weights (*M*_n and *M*_w, respectively) of the polymers were determined by a gel permeation

Scheme 1 The synthesis of grafted bioactive molecules diblock (P α N₃CL-*g*-BioM)-*b*-PCL copolymers.

Figure 1 Representative proton nuclear magnetic resonance (^1H -NMR) spectra of (a) $(\text{P}\alpha\text{N}_3\text{CL-g-DMAP})\text{-b-PCL}$, (b) $(\text{P}\alpha\text{N}_3\text{CL-g-PNIC})\text{-b-PCL}$ and (c) $(\text{P}\alpha\text{N}_3\text{CL-g-PBHP})\text{-b-PCL}$. DMAP, 1-dimethylamino-2-propyne; PBHP, propargyl *N*-benzyloxycarbonyl-4-hydroxy proline; PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotine.

chromatography (GPC) conducted on a Jasco HPLC system equipped with a model PU-2031 refractive-index detector (Jasco, Tokyo, Japan) and Jordi Gel DVB columns (Jordi, Bellingham, MA, USA), with pore sizes of 100, 500 and 1000 Å. Chloroform was used as an eluent with a flow rate of 0.5 ml min^{-1} . PEG standards with low dispersion (Polymer Sciences, Mainz, Germany) were used to generate a calibration curve. The data were recorded and manipulated with a Windows-based software package (Scientific Information Service, Augustine, Trinidad and Tobago). UV-vis spectra were obtained using a Jasco V-550 spectrophotometer (Jasco, Tokyo, Japan). The pyrene fluorescence spectra were recorded on a Hitachi F-4500 spectrofluorometer (Hitachi, Tokyo,

Japan) with square quartz cells of $1.0 \times 1.0\text{ cm}^2$ used as substrates. For fluorescence excitation spectra, the detection wavelength (λ_{em}) was set at 390 nm.

Preparation of polymeric micelles

Polymeric micelles of $(\text{P}\alpha\text{N}_3\text{CL-g-BioM})\text{-b-PCL}$ copolymers were prepared using the oil-in-water emulsion technique. In all, 30 mg of each polymer were dissolved in dichloromethane (5 ml). The solution was added dropwise into deionized water (100 ml) that was stirred vigorously at ambient temperature. The emulsion solution was then ultrasonicated for 1 h and stirred overnight at

Table 1 Results of the grafting of P α N₃CL-*b*-PCL with BioM by click reaction

Entries	Copolymer	[P α N ₃ CL- <i>b</i> -PCL]/[BioM] molar ratio in the feed	[P α N ₃ CL- <i>b</i> -PCL]/ [BioM] molar ratio ^a	Grafting effi- ciency ^b (%)	M _{n,th} ^c (g mol ⁻¹)	M _{n,GPC} ^d (g mol ⁻¹)	M _w / M _n ^d	M _{n,NMR} ^a (g mol ⁻¹)	T _m ^e (°C)	T _g ^e (°C)
1	(P α N ₃ CL- <i>g</i> -DMAP)- <i>b</i> -PCL (P α N ₃ CL ₁₁ - <i>g</i> - DMAP ₁₁)- <i>b</i> -PCL ₃₀	1/11	1/11	100	6228	7300	1.67	6228	52.1/ 55.2	— ^f
2	(P α N ₃ CL ₃₅ - <i>g</i> - DMAP ₃₃)- <i>b</i> -PCL ₁₂	1/35	1/33	94	9884	8830	1.71	9405	44.1/ 48.1	−34.1
3	(P α N ₃ CL ₃₀ - <i>g</i> - DMAP ₃₀)- <i>b</i> -PCL ₃₃	1/30	1/30	100	11 127	13 220	1.59	11 127	52.1	−13.3
4	(P α N ₃ CL ₃₁ - <i>g</i> - DMAP ₂₆)- <i>b</i> -PCL ₆₄	1/31	1/26	84	14 968	13 220	1.56	13 771	53.9	−17.9
5	(P α N ₃ CL- <i>g</i> -PNIC)- <i>b</i> -PCL (P α N ₃ CL ₇ - <i>g</i> - PNIC ₄)- <i>b</i> -PCL ₂₆	1/7	1/4	57	5554	4000	1.59	4514	55.3	— ^f
6	(P α N ₃ CL ₁₇ - <i>g</i> - PNIC ₁₁)- <i>b</i> -PCL ₄₀	1/17	1/11	65	10 646	9230	1.87	8567	55.1	— ^f
7	(P α N ₃ CL ₃₅ - <i>g</i> - PNIC ₁₇)- <i>b</i> -PCL ₃₄	1/35	1/17	49	16 185	10 020	1.56	9948	50.1	13.9
8	(P α N ₃ CL ₅₅ - <i>g</i> - PNIC ₂₀)- <i>b</i> -PCL ₃₀	1/55	1/20	36	22 651	10 130	1.78	10 523	43.4	−39.9
9	(P α N ₃ CL- <i>g</i> -PBHP)- <i>b</i> -PCL (P α N ₃ CL ₁₁ - <i>g</i> - PBHP ₅)- <i>b</i> -PCL ₃₅	1/11	1/5	45	9097	7680	1.66	6412	50.6	−27.3
10	(P α N ₃ CL ₄₁ - <i>g</i> - PBHP ₆)- <i>b</i> -PCL ₄₅	1/41	1/6	14	23 683	7990	1.82	8021	47.5/ 51.6	−33.3

Abbreviations: BioM, bioactive molecules; DMAP, 1-dimethylamino-2-propyne; DSC, differential scanning calorimetry; GPC, gel permeation chromatography; ¹H-NMR, proton nuclear magnetic resonance; PBHP, propargyl *N*-benzyloxycarbonyl-4-hydroxy proline; PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotinate.

^aDetermined by ¹H-NMR spectroscopy of (P α N₃CL-*g*-alkyne)-*b*-PCL.

^bThe grafting efficiency was calculated by comparing the grafting units of the experimental with the theoretical. An experimental alkyne-graft unit based on ¹H-NMR integration of the singlet at δ 7.63 p.p.m. (the methine of the triazole ring) against the resonance at δ 4.03 p.p.m. (−CH₂OCO− polyester backbone).

^c $M_{th} = M_{n, P\alpha N_3 CL-b-PCL} + M_{BioM} \times [BioM]/[P\alpha N_3 CL-b-PCL]$, (where $M_{n, P\alpha N_3 CL-b-PCL}$ is the number-average molecular weight of P α N₃CL-*b*-PCL, M_{BioM} is the molecular weight of bioactive molecule, [BioM] is the bioactive molecule molarity concentration and [P α N₃CL-*b*-PCL] is the polymer P α N₃CL-*b*-PCL molarity concentration).

^dDetermined by GPC.

^eDetermined by DSC thermograms.

^fNo peak observed.

ambient temperature. A polymeric micelle solution was achieved after complete evaporation of dichloromethane.

Fluorescence measurements

To confirm micelle formation, fluorescence measurements were conducted using pyrene as a probe.²⁵ Fluorescence spectra of pyrene in aqueous solution were recorded at room temperature with a fluorescence spectrophotometer. The sample solutions were prepared by first adding known amounts of pyrene in acetone to a series of flasks. After the acetone had evaporated completely, measured amounts of micelle solutions with concentrations of (P α N₃CL-*g*-BioM)-*b*-PCL ranging from 0.0183 to 300 mg l⁻¹ were added to each flask and mixed with a vortex. The pyrene concentration in the final solutions was 6.1 $\times$ 10⁻⁷ M. The flasks were allowed to stand overnight at room temperature for equilibration of the pyrene and the micelles. The emission wavelength for the excitation spectra was 390 nm.

DLS measurements

The size distributions of the micelles were estimated by DLS using a particle-size analyzer (Zetasizer Nano ZS, Malvern, UK) at 20 °C. Scattered light intensity was detected 90° to the incident beam. Measurements were made after the aqueous micellar solution ($C = 300$ mg l⁻¹) was passed through a microfilter with an average pore size of 0.2 μ m (Advantec MFS, Dublin, CA, USA). The average size distribution of the aqueous micellar solution was determined with the CONTIN programs of Provencher and Hendrix.²⁶

Transmission electron microscopy measurements

The morphology of the micelles was observed using transmission electron microscopy (JEM 1200-EXII, Tokyo, Japan). Drops of a micelle solution ($C = 300$ mg l⁻¹, not containing a stain agent) were placed on carbon film coated on a copper grid and were then dried at room temperature. The observation was conducted with an accelerating voltage of 100 kV.

Drug-loading content and drug-entrapment efficiency

Using oil-in-water solvent evaporation, (P α N₃CL₃₀-*g*-DMAP₃₀)-*b*-PCL₃₃ (135 mg, 50-fold CMC) was dissolved in 6 ml of methylene chloride followed by the addition of IMC, which served as a model drug, at various weight ratios to the polymer (1/10–2/1). The solution was added dropwise to 150 ml of distilled water containing 1 wt% poly(vinyl alcohol) under vigorous stirring. Poly(vinyl alcohol) was used as a surfactant to reduce micelle aggregation. Sonication at ambient temperature for 60 min reduced the droplet size. The emulsion was stirred overnight at ambient temperature to evaporate the methylene chloride. The unloaded residue of IMC was removed by filtration using a Teflon filter (Whatman, Maidstone, UK) with an average pore size of 0.45 μ m. The micelles were obtained by vacuum drying. The addition of a 10-fold excess volume of *N,N*-dimethylformamide disrupted a weighed amount of the micelle. The drug content was assayed spectrophotometrically at 320 nm using a Diode Array UV–vis spectrophotometer (Jasco, Tokyo, Japan). Equations (1) and (2) were used to calculate the drug-loading content and drug-entrapment efficiency as follows:

$$\text{Drug-loading content (\%)} = (\text{weight of the drugs in micelles/the weight of micelles}) \times 100 \quad (1)$$

$$\text{Drug-entrapment efficiency (\%)} = (\text{weight of the drugs in micelles/the weight of the drug fed initially}) \times 100 \quad (2)$$

In vitro drug release

The appropriate amounts of the IMC-loaded micelles (110.2 mg) were precisely weighed and suspended in 10 ml of phosphate-buffered saline (0.1 M, pH 7.4). The micellar solution was introduced into a dialysis membrane bag (molecular weight cutoff = 3500), and the bag was placed in 50 ml of phosphate-buffered saline release media. The media were then shaken (30 r.p.m.) at 37 °C. At predetermined time intervals, 3 ml of aliquots of the

Figure 2 Infrared (IR) spectra of (a) $P\alpha N_3CL_{35}\text{-}b\text{-PCL}_{13}$, (b) $P\alpha N_3CL_{35}\text{-}b\text{-PCL}_{12}$ and (c) $(P\alpha N_3CL_{35}\text{-}g\text{-DMAP}_{33})\text{-}b\text{-PCL}_{12}$. DMAP, 1-dimethylamino-2-propyne; αCICL , α -chloro- ϵ -caprolactone; PCL, poly(ϵ -caprolactone).

aqueous solution were withdrawn from the release media, and the same volume of a fresh buffer solution was added. The concentration of released IMC was monitored with a UV-vis spectrophotometer at a wavelength of 320 nm. The rate of controlled drug release was measured by the accumulative released weight of IMC according to the IMC calibration curve.

Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and antibiotics (100 IU ml⁻¹ penicillin, 100 μ g ml⁻¹ streptomycin and 0.25 μ g ml⁻¹ amphotericin B) at 37 °C in a humidified 5% CO₂ atmosphere.

Cellular viability assay

A Promega CellTiter 96 Aqueous One Solution kit (Promega, Madison, WI, USA) was used to determine cellular viability by following the manufacturer's instructions with slight modifications. In brief, HeLa cells were seeded in a 24-well plate (3×10^4 cells per well) overnight, treated with either polymers at various concentrations or dimethylsulfoxide vehicles, and then placed in the Dulbecco's modified Eagle's medium containing 1% fetal bovine serum at 37 °C in a humidified 5% CO₂ atmosphere. After 24 h, the medium in each well was removed and replaced with 350 μ l of warm phosphate-buffered saline and 35 μ l of CellTiter 96 Aqueous One Solution kit, and then incubated at 37 °C for 1–4 h. After incubation, 110 μ l of supernatant from each well was transferred to a 96-well plate, and the absorbance at 485 nm was measured using a Hidex Plate Chameleon V (Hidex, Turku, Finland) multilabel microplate reader.

RESULTS AND DISCUSSION

Click reaction of $P\alpha N_3CL\text{-}b\text{-PCL}$ with BioM

$P\alpha N_3CL\text{-}b\text{-PCL}$ was synthesized following previously published procedures with some modifications.²⁷ To functionalize the aliphatic polyesters, chemical transformation using click reaction between alkynes and azides is facile because of the mild conditions and quantitative yields. Copper iodide and Et₃N in *N,N*-dimethylformamide catalyzed the click reaction at 60 °C over 24 or 48 h. The bioactive compounds nicotinic acid, 4-hydroxy-L-proline and dimethylamino propyne were chosen as model compounds for grafting onto PCL. Table 1 shows the cycloaddition results. $M_{n,GPC}$

Figure 3 Gel permeation chromatography (GPC) curves of (a) $(P\alpha N_3CL_{35}\text{-}g\text{-DMAP}_{33})\text{-}b\text{-PCL}_{12}$, (b) $P\alpha N_3CL_{35}\text{-}b\text{-PCL}_{12}$ and (c) $P\alpha N_3CL_{35}\text{-}b\text{-PCL}_{13}$. αCICL , α -chloro- ϵ -caprolactone; DMAP, 1-dimethylamino-2-propyne; PCL, poly(ϵ -caprolactone).

Figure 4 Differential scanning calorimetry (DSC) curves of (a) $(P\alpha N_3CL_{11}\text{-}g\text{-DMAP}_{11})\text{-}b\text{-PCL}_{30}$, (b) $(P\alpha N_3CL_{30}\text{-}g\text{-DMAP}_{30})\text{-}b\text{-PCL}_{33}$, (c) $(P\alpha N_3CL_{31}\text{-}g\text{-DMAP}_{26})\text{-}b\text{-PCL}_{64}$, (d) $(P\alpha N_3CL_{35}\text{-}g\text{-DMAP}_{33})\text{-}b\text{-PCL}_{12}$, (e) $(P\alpha N_3CL_{17}\text{-}g\text{-PNIC}_{11})\text{-}b\text{-PCL}_{40}$, (f) $(P\alpha N_3CL_{35}\text{-}g\text{-PNIC}_{17})\text{-}b\text{-PCL}_{34}$ and (g) $(P\alpha N_3CL_{11}\text{-}g\text{-PBHP}_5)\text{-}b\text{-PCL}_{35}$ for the second run. DMAP, 1-dimethylamino-2-propyne; PBHP, propargyl *N*-benzyloxycarbonyl-4-hydroxy proline; PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotinate.

Figure 5 Excitation spectra of the $(P\alpha N_3CL_{17}\text{-}g\text{-PNIC}_{11})\text{-}b\text{-PCL}_{40}$ copolymer monitored at $\lambda_{\text{max}} = 390$ nm (pyrene concentration $= 6 \times 10^{-7}$ M). PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotinate.

was in agreement with $M_{n,\text{NMR}}$ or $M_{n,\text{th}}$. The grafting efficiency was between 84 and 100% for the $(P\alpha N_3CL\text{-}g\text{-DMAP})\text{-}b\text{-PCL}$ series of polymers. For the grafted nicotinic acid and 4-hydroxy-L-proline at low percent grafting, the $M_{n,\text{GPC}}$ value was remarkably lower than the $M_{n,\text{th}}$, which we attribute to reduced grafting caused by steric hindrance.

The disappearance of the azide signal at 2104 cm^{-1} , and the appearance of a new absorption peak at 1608 cm^{-1} characterized the vibration of the triazole unsaturated group in the Fourier transform infrared spectroscopy spectrum, and confirmed the success of the click reaction, yielding an alkyne-grafted polyester (Figure 2c). Figure 3 shows typical GPC plots of $(P\alpha N_3CL_{35}\text{-}g\text{-DMAP}_{33})\text{-}b\text{-PCL}_{12}$ compared with those of the original $P\alpha N_3CL_{35}\text{-}b\text{-PCL}_{12}$ and $P\alpha ClCL_{35}\text{-}b\text{-PCL}_{13}$. The GPC traces show a bimodal distribution of the block copolymer, with a shoulder on the low-molecular-weight side, and the peak shift toward the higher-molecular-weight region compared with the original $P\alpha N_3CL\text{-}b\text{-PCL}$ and $P\alpha ClCL\text{-}b\text{-PCL}$ peaks. A shoulder bimodal molecular weight distribution was observed as result of contamination of the copolymer by unreacted macromonomers. The cycloaddition was confirmed by the disappearance of the CHN_3 proton resonance at 3.87 p.p.m. and the appearance of a new peak at 7.65 p.p.m. in the ^1H -NMR spectrum, which represented a triazole proton. Additional new peaks, assigned to the protons of grafted bioactive compounds, were also observed. The $[\text{BioM}]/[P\alpha N_3CL\text{-}b\text{-PCL}]$ molar concentration ratio of the addition products, as calculated by $2I_{\text{m}}/I_{\text{b}}$, was close to the molar ratio in the feed.

Thermal properties of the grafted copolymers

The thermal properties of the grafted copolymers were measured with differential scanning calorimetry and compared with those of the original $P\alpha N_3CL\text{-}b\text{-PCL}$ and $P\alpha ClCL\text{-}b\text{-PCL}$. The glass transition temperature, T_g , and melting temperature, T_m , characterizing the polymorphic properties of the copolymers, are shown in Table 1, and indicated semicrystalline formation. In some instances, the T_g of the destructible polymers was not detected.

The graft-block $(P\alpha N_3CL\text{-}g\text{-BioM})\text{-}b\text{-PCL}$ copolymers were semicrystalline with T_m in the range of $43\text{--}56^\circ\text{C}$ (see Figure 4). As the hydrophobic segment remained constant, T_m decreased with the increase in the hydrophilic segment because of the steric effect of the grafted compound that reduced the crystalline properties of PCL. The T_m of the nicotinic acid, PNIC and the grafted copolymer

Figure 6 Plot of I_{338}/I_{334} intensity ratio (from the pyrene excitation spectra; pyrene concentration $= 6.1 \times 10^{-7}$ M) versus the logarithm of the concentration ($\text{Log } C$) for: (a) $(P\alpha N_3CL\text{-}g\text{-DMAP})\text{-}b\text{-PCL}$ series of graft-block copolymers: (●) $(P\alpha N_3CL_{35}\text{-}g\text{-DMAP}_{33})\text{-}b\text{-PCL}_{12}$, (▲) $(P\alpha N_3CL_{30}\text{-}g\text{-DMAP}_{30})\text{-}b\text{-PCL}_{33}$, (▼) $(P\alpha N_3CL_{31}\text{-}g\text{-DMAP}_{26})\text{-}b\text{-PCL}_{64}$; (b) $(P\alpha N_3CL\text{-}g\text{-PNIC})\text{-}b\text{-PCL}$ series of graft-block copolymers: (●) $(P\alpha N_3CL_{17}\text{-}g\text{-PNIC}_{11})\text{-}b\text{-PCL}_{40}$, (■) $(P\alpha N_3CL_7\text{-}g\text{-PNIC}_4)\text{-}b\text{-PCL}_{26}$, (▼) $(P\alpha N_3CL_{55}\text{-}g\text{-PNIC}_{20})\text{-}b\text{-PCL}_{30}$, (▲) $(P\alpha N_3CL_{35}\text{-}g\text{-PNIC}_{17})\text{-}b\text{-PCL}_{34}$. DMAP, 1-dimethylamino-2-propyne; PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotinate. A full color version of this figure is available at *Polymer Journal* online.

decreased from 55.3 to 43.4°C when the number of grafting units was increased from 4 to 20. The grafted PNIC and PBHP copolymers demonstrated a lower T_m than the grafted DMAP polymer because of the larger size and steric effect of the PNIC and PBHP compared with the DMAP, which reduced the crystalline properties of PCL.

For T_g , as the length of hydrophilic segment was similar, a decreased T_g was observed when the hydrophobic segment was increased. In addition, a lower T_g was also observed when the number of grafted units was increased. Particularly with grafting PNIC, the effect was more pronounced than the grafting DMAP or PBHP. The results show that grafting the aliphatic or heterocyclic amine compounds reduced the crystallinity of the polymers. This may have occurred because the steric bulk of the molecules interrupted the orientation of the polymer chain, thereby restricting crystallization. The reduction of crystallinity may have affected the permeability of the drug through the matrix of copolymers when the nanoparticle systems were formed.

Micelles of graft-block copolymers

The amphiphilic nature of the graft-block copolymers, consisting of hydrophilic $P\alpha N_3CL\text{-}g\text{-DMAP}$, $P\alpha N_3CL\text{-}g\text{-PNIC}$ or $P\alpha N_3CL\text{-}g\text{-PBHP}$

Table 2 Properties of IMC-loaded ($P\alpha N_3CL$ -*g*-BioM)-*b*-PCL copolymer micelles

Entries	Copolymer	CMC ($mg\ l^{-1}$)	Feed weight ratio IMC/polymer	Drug-entrapment efficiency (%)	Drug-loading content (%)	Micelle size (nm)		
						Without drug	Zeta potential (mV)	With drug
1	($P\alpha N_3CL$ - <i>g</i> -DMAP)- <i>b</i> -PCL							
2	($P\alpha N_3CL_{11}$ - <i>g</i> -DMAP ₁₁)- <i>b</i> -PCL ₃₀	12	1/1	26.7	13.4	120.6	−12.5	125.2
3	($P\alpha N_3CL_{35}$ - <i>g</i> -DMAP ₃₃)- <i>b</i> -PCL ₁₂	20	1/1	96.0	58.9	105.2	−7.9	109.1
	($P\alpha N_3CL_{30}$ - <i>g</i> -DMAP ₃₀)- <i>b</i> -PCL ₃₃	18	2/1	86.9	48.0			
			1/1	88.4	43.3	108.4	−6.2	111.2
			1/2	95.6	31.9			
			1/5	96.8	16.1			
			1/10	97.9	8.9			
4	($P\alpha N_3CL_{31}$ - <i>g</i> -DMAP ₂₆)- <i>b</i> -PCL ₆₄	4.6	1/1	71.6	35.8	110.3	−1.7	114.5
	($P\alpha N_3CL$ - <i>g</i> -PNIC)- <i>b</i> -PCL							
5	($P\alpha N_3CL_7$ - <i>g</i> -PNIC ₄)- <i>b</i> -PCL ₂₆	19	1/1	23.6	11.8	137.5	−24.0	141.3
6	($P\alpha N_3CL_{17}$ - <i>g</i> -PNIC ₁₁)- <i>b</i> -PCL ₄₀	13	1/1	35.6	17.8	129.4	−22.3	135.2
7	($P\alpha N_3CL_{35}$ - <i>g</i> -PNIC ₁₇)- <i>b</i> -PCL ₃₄	11	1/1	89.4	44.7	113.5	−25.6	123.0
8	($P\alpha N_3CL_{55}$ - <i>g</i> -PNIC ₂₀)- <i>b</i> -PCL ₃₀	6.7	1/1	89.8	44.9	110.3	−19.2	117.5
	($P\alpha N_3CL$ - <i>g</i> -PBHP)- <i>b</i> -PCL							
9	($P\alpha N_3CL_{41}$ - <i>g</i> -PBHP ₆)- <i>b</i> -PCL ₄₅	5.0	1/1	16.4	8.21	162.3	−11.8	169.0
10	($P\alpha N_3CL_{11}$ - <i>g</i> -PBHP ₅)- <i>b</i> -PCL ₃₅	8.8	1/1	25.6	12.8	151.5	−12.3	153.4

Abbreviations: BioM, bioactive molecules; CMC, critical micelle concentration; DMAP, 1-dimethylamino-2-propyne; IMC, indomethacin; PBHP, propargyl *N*-benzyloxycarbonyl-4-hydroxy proline; PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotinate.

blocks and hydrophobic PCLs, provided the potential for the formation of micelles in water. Fluorescence techniques were used to investigate the characteristics of the graft-block copolymer micelles in the aqueous phase, including the CMCs, by using pyrene as a probe.

Figure 5 shows the excitation spectra of pyrene in the ($P\alpha N_3CL_{17}$ -*g*-PNIC₁₁)-*b*-PCL₄₀ solution at various concentrations. The findings demonstrate that the fluorescence intensity increased as the ($P\alpha N_3CL_{17}$ -*g*-PNIC₁₁)-*b*-PCL₄₀ concentration was increased. A characteristic feature of the pyrene excitation spectra, the red shift of the (0,0) band from 334 to 338 nm upon uptake of pyrene into micellar hydrophobic core, was used to determine the CMC values of the ($P\alpha N_3CL_{17}$ -*g*-PNIC₁₁)-*b*-PCL₄₀ graft-block copolymers. Figure 6 shows the intensity ratios (I_{338}/I_{334}) of the pyrene excitation spectra versus the logarithm of concentration of the ($P\alpha N_3CL$ -*g*-DMAP)-*b*-PCL, and ($P\alpha N_3CL$ -*g*-PNIC)-*b*-PCL series of graft-block copolymers. The CMC was determined by intersecting straight-line segments drawn through the points at the lowest polymer concentrations, which lay on a nearly horizontal line, and the points on the rapidly rising part of the plot. Table 2 shows the CMC values of the graft-block copolymers with various block compositions. The CMC of the graft-block copolymers ranged from 4.6 to 20 $mg\ l^{-1}$, and from 6.7 to 19 $mg\ l^{-1}$, for the ($P\alpha N_3CL$ -*g*-DMAP)-*b*-PCL and ($P\alpha N_3CL$ -*g*-PNIC)-*b*-PCL series of polymers, respectively. Generally, if the hydrophilic block remained constant for a series of copolymers, an increase in the molecular weight of the hydrophobic block reduced the CMC. To a lesser extent, if a constant length of the hydrophobic block was maintained, then an increase in the length of the hydrophilic block caused an increase in the value of the CMC.²⁸ Consistent results were observed for the ($P\alpha N_3CL$ -*g*-DMAP)-*b*-PCL series of polymers. However, contrasting results were shown for the ($P\alpha N_3CL$ -*g*-PNIC)-*b*-PCL series of polymers. At similar hydrophobic block lengths, when the length of the hydrophilic segment was increased, the CMC values decreased. This may be because the hydrophilicity decreased when the number of grafted PNIC units was increased, as nicotinic acid is only slightly water-soluble.²² The zeta potential of the grafted PNIC polymers was in the range of −19.2 to −25.6 mV, which was more negative than that of the grafted DAMP or PBHP polymers. The resulting micelles of the grafted PNIC

were more stable than the micelles of the grafted DAMP or PBHP. The negative surface charge prevented micelles from aggregating, which can be caused by serum protein absorption when administered in the blood plasma.²⁹

The mean hydrodynamic diameters of the micelles without IMC, as determined by DLS, ranged from 105 to 162 nm. Fixing the concentration at 50 times the CMC value ($50 \times CMC$), the mean diameter of the micelles increased as the ratio of the lengths of the hydrophobic segment to the hydrophilic segment was increased. In general, as the hydrophobic portion of the polymer chain increased, the size of the particles became larger. When the drug was incorporated, the size of the micelle increased marginally. Figure 7 shows a similar trend in micelle morphology (not containing a stain agent). The blank and IMC-loaded micelles were nearly spherical. The diameter was marginally smaller than the hydrodynamic diameter obtained from the DLS experiment: this may have been caused by shrinkage and collapse of micelles after drying. The mean hydrodynamic diameter of IMC-loaded micelles was in the range of 109–169 nm, which was less than the 200 nm that reduced the reticuloendothelial system uptake.

Evaluation of drug-loading content and drug-entrapment efficiency

In this study, IMC, a common non-steroidal anti-inflammatory drug, was chosen as a model drug to investigate the effects of different structures and the amount of grafting compounds on the loading efficiency of these nanoparticles. Regarding the efficiency of these carriers, the percentages of drug-loading content (% DLC) and drug-entrapment efficiency (% DEE) were evaluated according to Equations (1) and (2). The amount of IMC incorporated into graft-block ($P\alpha N_3CL$ -*g*-DMAP)-*b*-PCL micelles, which was defined by the ratio of the weight of IMC in the nanosphere to the pre-weighted IMC-loaded micelles, was calculated by absorbance measurement after removing free IMC by filtration. Table 2 shows the amount of IMC introduced into the micelle by controlling the weight ratio between the drug and the polymer. Drug-entrapment efficiency increased as the weight ratio of drug-to-polymer decreased. For example, for ($P\alpha N_3CL_{30}$ -*g*-DMAP₃₀)-*b*-PCL₃₃, the feed weight ratio of IMC-to-polymer was decreased from 2 to 0.1, and the DEE increased from

Figure 7 Transmission electron microscopy (TEM) photograph of the micelles formed by $(P\alpha N_3CL_{35}-g-PNIC_{17})-b-PCL_{34}$, $(P\alpha N_3CL_{30}-g-DMAP_{30})-b-PCL_{33}$ and $(P\alpha N_3CL_{11}-g-PBHP_5)-b-PCL_{35}$. Blank: (a, c, e); loading indomethacin (IMC): (b, d, f); scale bar = 0.5 μm . DMAP, 1-dimethylamino-2-propyne; PBHP, propargyl *N*-benzyloxycarbonyl-4-hydroxy proline; PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotinate.

86.9 to 97.9%. A contrasting result was observed when the DLC was decreased from 48.0 to 8.9%, and the DEE and DLC, depending on the composition of the block polymer, were also decreased. At a constant feed weight ratio (1/1), the drug-entrapment efficiency and drug-loading content increased with an increase in the ratio of hydrophilic/hydrophobic segments. Increasing the micelle stability also increased the amount of drug entrapped in the micelles. The study showed that the DEE and DLC of the grafted DMAP and PNIC polymer were higher than those of the grafted PBHP polymer.

In vitro release of IMC

Drug release tests, performed at 37 °C with different IMC-loaded $(P\alpha N_3CL-g-DMAP)-b-PCL$ and $(P\alpha N_3CL-g-PNIC)-b-PCL$ nanoparticles, were used to investigate the effects of polymer compositions on drug-release behavior. Figure 8 shows that the IMC-loaded $(P\alpha N_3CL-g-DMAP)-b-PCL$ and $(P\alpha N_3CL-g-PNIC)-b-PCL$ nanoparticles exhibited well-developed, sustained drug-release patterns, with a trend for faster drug release by increasing the hydrophilicity of the hydrophilic blocks or decreasing the lengths of the hydrophobic block. As the length of the hydrophilic block decreased, the water solubility increased and caused the hydrophilicity of micelles to increase. The drug release of the IMC-loaded $(P\alpha N_3CL-g-DMAP)-b-PCL$ was faster than $(P\alpha N_3CL-g-PNIC)-b-PCL$, and can be attributed to DMAP being more water-soluble than PNIC. These findings indicate that varying the length of the hydrophilic and grafted molecules modulates the extent of drug release from the micelles.

Figure 8 Indomethacin (IMC) released from the micelles of $(P\alpha N_3CL_{11}-g-DMAP_{11})-b-PCL_{30}$ ($\blacktriangle$, a), $(P\alpha N_3CL_{30}-g-DMAP_{30})-b-PCL_{33}$ ($\blacklozenge$, b), $(P\alpha N_3CL_{17}-g-PNIC_{11})-b-PCL_{40}$ ($\bullet$, c) and $(P\alpha N_3CL_{35}-g-PNIC_{17})-b-PCL_{34}$ ($\bullet$, d). DMAP, 1-dimethylamino-2-propyne; PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotinate. A full color version of this figure is available at *Polymer Journal* online.

Beyond the initial burst, the release profile reached a plateau, suggesting that the IMC-loaded micelles are stable under physiological conditions.

Cell viability study

The cytotoxic effects of the $(P\alpha N_3CL_{17}-g-PNIC_{11})-b-PCL_{40}$ polymer were investigated using the MTS method in HeLa cancer cells, and the

Figure 9 The cell viability of HeLa cells with various concentrations of $(P\alpha N_3CL_{17}\text{-}g\text{-}PNIC_{11})\text{-}b\text{-}PCL_{40}$. The data are presented as the mean $\pm$ s.e. ($n=4$). PCL, poly(ϵ -caprolactone); PNIC, pent-4-ynyl nicotinate.

in vitro cytotoxicities of the $(P\alpha N_3CL_{17}\text{-}g\text{-}PNIC_{11})\text{-}b\text{-}PCL_{40}$ polymers were quantified at various concentrations. The wells containing only media without polymers were treated as the positive controls, with a cell viability of 100%. The equation $[\text{Abs}]_{\text{sample}}/[\text{Abs}]_{\text{control}} \times 100$ was used to calculate the relative cell viability. Figure 9 shows the cell viability of HeLa cells at various $(P\alpha N_3CL_{17}\text{-}g\text{-}PNIC_{11})\text{-}b\text{-}PCL_{40}$ polymer concentrations. The viability of HeLa cells after 24 h incubation was 100%, even with polymer concentrations up to $100 \mu\text{g ml}^{-1}$. The polymer demonstrated no significant cytotoxicity. The cell viability was higher than 100%, most likely due to variations in the cells.

CONCLUSIONS

Graft-block $(P\alpha N_3CL\text{-}g\text{-}BioM)\text{-}b\text{-}PCL$ copolymers with various molar ratios of grafting units were successfully obtained through copper-catalyzed Huisgen's 1,3-dipolar cycloaddition. The difference in the molecular structure of the grafting compounds influenced the feasibility of the grafting reaction and the physicochemical and thermal properties of the polymers. In addition, the nanoparticles prepared from the grafting compounds exhibited different characteristics in particle size, surface charge, drug-loading content and drug-entrapment efficiency. A toxicity study of the grafted nanoparticles demonstrated the safety of the nanoparticles at concentrations lower than $100 \mu\text{g ml}^{-1}$. These polymeric micelles were designed to be biodegradable and possess a sufficiently low molecular weight (<40 kDa) to be eliminated through renal clearance. The size of the micelles (<200 nm) reduced non-selective uptake by the reticuloendothelial system.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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