In-vitro cytotoxic activities of poly(2-ethyl-2-oxazoline)-based amphiphilic block copolymers prepared by CuAAC click chemistry

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Abstract. Synthesis and characterization of well-defined amphiphilic block copolymers containing poly(2-ethyl-2-oxazoline) as hydrophilic block and poly(ε-caprolactone) or poly(L-lactide) as hydrophobic block is achieved by copper-catalyzed azide-alkyne cycloaddition (CuAAC) click chemistry. The clickable precursors, α-alkyne-functionalized poly(ε-caprolactone) and poly(L-lactide) and ω-azido-functionalized poly(2-ethyl-2-oxazoline) are simply prepared and joined using copper sulfate/ascorbic acid catalyst system at room temperature. The structures of precursors and amphiphilic block copolymers are characterized by spectroscopic, chromatographic and thermal analyses. The cytotoxic activities of resulting amphiphilic block copolymers and their precursors are investigated in the prostate epithelial and cancer cells under *in-vitro* conditions. The treatment of the healthy prostate epithelial cell line PNT1A reveals that no significant cytotoxicity, whereas some significant toxic effects on the prostate cancer cell lines are observed.

Keywords: polymer synthesis, molecular engineering, amphiphilic block copolymers, copper-catalyzed azide-alkyne cycloaddition click chemistry, in-vitro cytotoxic activity, polyoxazolines

1. Introduction

Amphiphilic block copolymers that consist of hydrophilic and hydrophobic segments, represent a new class of functional materials serving a number of applications mainly drug delivery, pharmaceutics, separation, surfactant and coating [1]. By changing various parameters such as hydrophilic/hydrophobic block ratio, polymer concentration, temperature and polymer-solvent interaction, they can self-assemble into a wide variety morphologies including bilayer-type, cylindrical or spherical micelles, vesicles and nanotubes [2]. Since the amphiphilic block copolymers deliberately require precise molecular

characteristics, only controlled polymerization methods are able to prepare these type of copolymers [3]. The combination of these methods with highly efficient 'click' chemistry reactions are great opportunities for the synthesis of complex macromolecular architectures [4]. This combination is very useful to join the chemically incompatible blocks in a single molecule, such as a block and a graft copolymer, a star-shaped polymer, a hybrid material, and a bioconjugate [5–10].
After the discovery of the CuAAC click reaction [11], it has become a universally used method in chemical sciences, especially in drug discovery, medicinal,

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polymer and material sciences [12]. Due to its high efficiency, less by-products and high tolerance of functional groups, many complex macromolecular architectures can be easily prepared with tailored topologies, compositions and microstructures [13]. One of the first examples of this CuAAC click reactions was demonstrated by Van Hest and coworkers for the modular synthesis of amphiphilic block copolymers [14, 15]. And then, many groups have extensively used this 'click' reaction to synthesize a wide variety of block copolymers (such as AB, ABC, ABA, (AB)$_n$, etc.) [16-22]. To take advantage of this efficient strategy, recently, the poly(2-alkyl-2-oxazoline)-based amphiphilic block copolymers can be simply prepared by combination of coordination-insertion ROP (ring opening polymerization) and living CROP (cationic ring opening polymerization) with CuAAC click reactions [23, 24]. Both hydrophilic and hydrophobic blocks are biocompatible, therefore, these block copolymers have promising potentials for drug delivery applications.

Recently, owing to the their unique nontoxicity, biodegradable and biocompatible features, aliphatic polyesters, such as poly(ε-caprolactone) (PCL), polylactide (PLA) and their block copolymers are intensively studied in the biomedical applications, such as in controlled drug release systems and absorbable sutures [25]. However, these aliphatic polyesters have several disadvantages including their low solubility and lack of reactive sites for further functionalization, which limit their usage in biomedical applications [26]. The introduction of pendent functional groups or hydrophilic segments along these aliphatic polyester chains enables to adjust their physicochemical properties, such as crystallinity, hydrophilicity, bioadhesion, biological activity and biodegradation rate [27]. Up to now, various modification strategies for example, functional initiators or monomers, copolymerization and post-modification reactions are applied for the functionalization aliphatic polyesters [28, 29]. Recently, poly(2-oxazoline)s have drawn attention with the finding of the potential biological and chemical favorability due to their some advantageous structural properties such as pH and temperature sensitive, ionic strength, chemical and biological stimuli [30–34]. These polymers can be obtained via living cationic ring opening polymerization (CROP) of 2-alkyl-oxazolines and functionalized by using either functional initiator or nucleophilic terminating agents [35–39]. Among them,

poly(2-oxazoline)s with short side chains (ethyl, *n*- and *iso*-propyl), except for 2-methyl-2-oxazoline, permit easy access to amphiphilic and well-defined block copolymers owing to the lower critical solution temperature (LCST) behavior in aqueous solutions and thermoresponsive properties [35–39].

To date, poly(2-oxazoline)s are obtained utilizing various initiators including halides such as benzyl bromide, alkyl esters such as methyl tosylate, methyl triflate and Lewis acids such as boron trifluoride (BF$_3$-OEt$_2$) [40–42]. Moreover, poly(2-oxazoline)s are functionalized with many terminating agents such as water, hydroxyl ion in methanolic sodium hydroxide solution, ammonia, piperidine, sodium azide etc [43–47].

In this work, we reported the synthesis and characterization of amphiphilic block copolymers containing poly(2-ethyl-2-oxazoline) as hydrophilic segment and poly(ε-caprolactone) or poly(L-lactide) as hydrophobic segments by combination of coordination-insertion ring opening polymerization (ROP) and living CROP with CuAAC click chemistry. With this aim, the clickable azide-functionalized poly(2-ethyl-2-oxazoline) (PEtOx-N$_3$) was prepared by living CROP of 2-ethyl-2-oxazoline, which terminated by sodium azide. Whereas, clickable alkyne-poly(ε-caprolactone) (PCL-alkyne) and alkyne-poly(L-lactide) (PLA-alkyne) were synthesized via coordination-insertion ROP of ε-caprolactone and L-lactide using propargyl alcohol as an initiator via tin (II) 2-ethylhexanoate as a catalyst. In the final step, the CuAAC of these azide and alkyne functionalized polymers was simply carried out at room temperature by using copper sulfate and ascorbic acid catalyst system [48, 49]. All precursor polymers and resulting amphiphilic block copolymers were characterized by spectroscopic, chromatographic and thermal analyses. The cytotoxic activities of these three polymers were evaluated on the prostate epithelial cell line PNT1A, and two prostate cancer lines 22RV1 (androgen independent) and LnCAP (androgen dependent) by using WST1 (cell proliferation reagent) cell viability assay for 24, 48 and 72 hours [50].

2. Experimental
2.1. Materials
2-Ethyl-2-oxazoline (EtOx, ≥99%, 137456, Aldrich, Germany) and ε-caprolactone (CL, 99%, 173442500, Acros, Japan) were dried over calcium hydride (95%, 208027, Aldrich, Germany) overnight before

distillation and then were purified by vacuum distillation. These monomers were stored under nitrogen atmosphere. L-Lactide (LA, 98%, 367044, Aldrich, Netherlands) was purified by recrystallization from tetrahydrofuran (THF, ≥99.7%, 28559.320, VWR, EC). Methyl para-toluenesulfonate (MeTos, ≥98%, 158992, Aldrich, China) and propargyl alcohol (PA, 99%, 131452500, Acros, Germany) were purified via vacuum distillation. Tin (II) 2-ethylhexanoate $(Sn(Oct)_2$, 92.5-100%, S3252, Aldrich, Japan) was used directly. Acetonitrile (ACN, ≥99.8%, 8149, J. T. Baker, US) and toluene (≥99.7%, 32249, Aldrich, France) were distilled from calcium hydride under reduced pressure. All other reagents for synthesis were purchased commercially and used without further purification.

2.2. Synthesis of azide end-functionalized poly(2-ethyl-2-oxazoline) (PEtOx-N₃)

To obtain PEtOx-N_3, the published method was used [51, 52]; firstly, a flask equipped with a stirring bar was preheated via a heat gun. After being closed by a rubber, it was again heated under vacuum. After cooling under vacuum, PEtOx was prepared by living CROP of the monomer 2-ethyl-2-oxazoline (EtOx, 5 mL, 49.531 mmol) and with the methyl *p*-toluenesulfonate initiator (MeTos, 112,28 µL, 0.744 mmol) dissolved in acetonitrile (ACN, 15 mL) under an inert atmosphere at room temperature. After polymerization for 15 hours at 130 °C, the reaction mixture was again cooled at room temperature and sodium azide (NaN_3, 193.47 mg, 2.976 mmol) was added for termination of the polymerization. After termination for 24 hours at 65 °C in the dark, the solvent was removed under reduced pressure. Then, the product was dissolved in dichloromethane and precipitated in cold diethyl ether and dried under vacuum for overnight. (Yield = 4.35 g, 88.6%; $M_{n,theo}$ = 5850 Da; $M_{n,NMR}$ = 5400 Da; $M_{n,GPC}$ = 9200 Da; polydispersity index (PDI) = 1.07.)
^{1}H-NMR (600 MHz, CDCl₃): δ 3.5–3.3 (4H, –N–C$\underline{H}_2$–C$\underline{H}_2$–), 3.0–2.9 (3H, C$\underline{H}_3$–N–CH₂–CH₂–N–), 2.4–2.2 (2H, –N–CO–C$\underline{H}_2$–CH₃), 1.1–0.9 (3H, –N–CO–CH₂–C$\underline{H}_3$).
FT-IR (ATR): ν [cm⁻¹] 2100 (azide) and 1630 (carbonyl).

2.3. Synthesis of alkyne end-functionalized poly(ε-caprolactone) (PCL-alkyne)

To obtain PCL-Alkyne, the published method was used; a ROP of epsilon-caprolactone using $Sn(Oct)_2$ as a catalyst and propargyl alcohol as an initiator was conducted [53]. Typically, CL (5 mL, 45.12 mmol) and propargyl alcohol (PA, 148,39 µL, 2.512 mmol) were added in a flask with a magnetic stirring bar, and a solution of $Sn(Oct)_2$ (12.63 µL, 0.039 mmol) in 5 mL of toluene was introduced using a syringe. The mixture was deaerated with nitrogen and then immersed in a thermostated oil bath at 120 °C for 5 h. After removing the solvent under reduced pressure, the resulting solid was dissolved in dichloromethane, and then precipitated into an excessive amount of cold methanol. The product was subsequently dried under vacuum oven for overnight at room temperature. (Yield = 4.2 g, 81.6%; $M_{n,theo}$ = 1700 Da; $M_{n,NMR}$ = 2500 Da; $M_{n,GPC}$ = 5100 Da; polydispersity index (PDI) = 1.35.)
^{1}H-NMR (600 MHz, CDCl₃): δ 4.66 (s, 2H, C$\underline{H}_2$–C≡CH), 4.00 (m, C$\underline{H}_2$O on PCL), 3.65 (t, 2H, C$\underline{H}_2$OH), 2.50 (s, 1H, CH₂–C≡C$\underline{H}$), 2.35–2.27 (m, C$\underline{H}_2$C=O on PCL), 1.67–1.57 (m, C$\underline{H}_2$ on PCL), 1.40–1.38 (m, C$\underline{H}_2$ on PCL).
FT-IR (ATR): ν [cm⁻¹] 3265, 2945, 2865, 1730, 1460, 1410, 1390, 1365, 1295, 1245, 1165, 1105, 1045, 1005, 960, 730.

2.4. Synthesis of alkyne end-functionalized poly(L-lactide) (PLA-alkyne)

The PLA-alkyne was obtained according to the published method; a ROP of LA using $Sn(Oct)_2$ as a catalyst and propargyl alcohol as an initiator [54]. Typically, propargyl alcohol (PA, 210,89 µL, 3,57 mmol) and LA (5 g, 34,69 mmol) were added in a flask with a magnetic stirring bar, and a solution of $Sn(Oct)_2$ (9,64 µL, 0,03 mmol) in 5 mL of toluene was introduced using a syringe. The reactive mixture was deaerated with nitrogen and then immersed in a thermostated oil bath at 120 °C for 5 h.
After removing the solvent under reduced pressure, the residue was dissolved in dichloromethane and precipitated in cold methanol. The product was subsequently dried under vacuum oven for overnight at room temperature. (Yield = 3,85 g, 77%; $M_{n,theo}$ = 1100 Da; $M_{n,NMR}$ = 1450 Da; $M_{n,GPC}$ = 3200 Da; polydispersity index (PDI) = 1.41.)

^{1}H-NMR (600 MHz, CDCl$_3$): δ 5.16 (m, (CO)–C$\underline{H}$–(CH$_3$)–O on PLA), 4.71 (s, 2H, C$\underline{H_2}$–C≡CH), 4.34 (m, (CO)–C$\underline{H}$–(CH$_3$)OH end-group of PLA), 2.50 (s, 1H, CH$_2$–C≡C$\underline{H}$), 1.48–1.69 (m, (CO)–CH–(C$\underline{H_3}$)O on PLA).
FT-IR (ATR): ν [cm^{-1}] 3290, 2980, 2945, 1740, 1460, 1380, 1260, 1180, 1090, 1050, 860, 750, 660.

2.5. General procedure of CuAAC click reactions

Firstly, a flask equipped with a stirring bar was pre-heated via a heat gun. After being closed by a rubber, it was again heated under vacuum and cooled. The PCL-alkyne or PLA-alkyne, PEtOx-N$_3$, copper sulfate (CuSO$_4$) and ascorbic acid were dissolved in *N,N*-dimethylformamide (DMF) and were added into the flask by a syringe. And then, the mixture was deaerated with nitrogen and stirred at the room temperature in the dark for 24 hours. After the completion of the reaction duration, the reaction mixture was purified by passing through a silica column and the solvent was removed under reduced pressure. Finally, the residue was dissolved in dichloromethane and precipitated in cold diethyl ether and dried under vacuum for overnight.

2.6. Cytotoxic activities of amphiphilic block copolymers

22RV1, LnCAP and PNT1A cells were plated into 96 microculture well plates at a density of 2500 cells/well. Polymers were added at various concentrations; 10, 20 and 30 µL/mL and incubated for 24, 48 and 72 h. Cytotoxic effect was then assessed by WST1 assay, which was performed according to the manufacturer's instructions. Absorbance value obtained for each sample was used to calculate the percentage of cell viability normalized to non-treated control cells, which was set to 100%.

2.7. Characterization

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy measurements were recorded using a Perkin-Elmer Spectrum BX FT-IR spectrometer over the range of 4000–500 cm^{-1} with a maximum OPD resolution of 1 cm^{-1}. The spectrum BX can operate in ratio, single-beam or interferogram mode. An electronics system based on the Motorola 68340 Integrated Processor. All proton nuclear magnetic resonance (^{1}H-NMR) measurements were carried out on a Varian 600 Spectrometer operating at 599.90 MHz. The used probe was a Varian's One NMR. The following parameters were employed for acquisition of spectra: spectral width, 16 ppm; acquisition time, 3.4 s; relaxation delay, 1 s; 90° pulse width, 7.2 µs; time domain, 64K data points; 32 scans; temperature, 298.15 K. The gel permeation chromatography (GPC) measurements were carried out in two different systems: an Agilent instrument (Model 1100) was used for PCL-alkyne, PLA-alkyne and resulting amphiphilic block copolymers, whereas a Viscotek TDA302 GPC instrument was only used for PEtOx-N$_3$. The Agilent systen included a pump, refractive index (RI), and ultraviolet (UV) detectors and four Waters Styragel columns (guard, HR 5E, HR 4E, HR 3, and HR 2), (4.6 mm internal diameter, 300 mm length, packed with 5 µm particles). The effective molecular weight ranges are 2000–4000 000, 50–100 000, 500–30 000 and 500–20 000 respectively. Tetrahydrofuran (THF) was used as eluent at a flow rate of 0.3 mL/min at 30 °C and as an internal standard respectively. The apparent molecular weights ($M_{n,GPC}$ and $M_{w,GPC}$) and polydispersity indexes (PDI) were determined with a calibration based on linear polystyrene (PS) standards using PL Caliber Software from Polymer Laboratories. The Viscotek system equipped with a Viscotek GPCmax pump, refractive index and right angle light scattering detectors and a Tosoh TSKGel G3000PWxl (300 mm×7.8 mm) column at 25 °C for poly(2-ethyl-2-oxazoline) as hydrophilic segment. Phosphate buffer saline (PBS, pH = 7.0) with 0.05% NaN$_3$ was used as mobile phase and flow rate was 0.8 mL/min. The detectors were calibrated with poly(ethylene oxide) (10 kDa standard solutions). All samples were filtered with 0.2 µm regenerated cellulose syringe filters. Differential scanning calorimetry (DSC) was recorded on a Perkin Elmer Diamond DSC with a heating rate of 10 °C·min^{-1} under nitrogen flow (20 mL·min^{-1}). The method of heat-cool-heat was used in the DSC measurements.

3. Results and discussion

3.1. Synthesis and characterization of clickable precursors

The clickable azide- and alkyne-functionalized precursors were prepared according to literature procedure Figure 1. The living CROP of 2-ethyl-2-oxazoline was initiated by methyl *p*-toluenesulfonate (monomer to initiator concentration [M]:[I] was 67:1) and conducted in acetonitrile at 130 °C and terminated
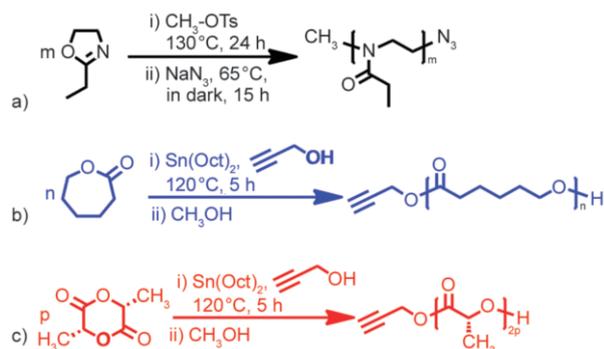
Figure 1. Synthesis of clickable precursors (PEtOx-N₃, PCL-alkyne and PLA-alkyne) via living CROP and co-ordination-insertion ROP; a) PEtOx-N₃ , b) PCL-alkyne, c) PLA-alkyne

with excess sodium azide to obtain an azide-terminated poly(2-ethyl-2-oxazoline) (PEtOx-N₃) [51, 55, 56]. After the successful synthesis, its macromolecular structure was confirmed by FT-IR and ^{1}H-NMR spectroscopies. In the ^{1}H-NMR spectrum, the peaks around 0.9–1.1, 2.2–2.4 and 3.3–3.5 ppm were assigned to methyl (C$\underline{H}_3$–CH₂–C=O–), methylene (CH₃–C$\underline{H}_2$–C=O–) and (–N–C$\underline{H}_2$–C$\underline{H}_2$–N–) protons of the repeating units. Whereas, the peak at 2.98 ppm was from methyl protons (C$\underline{H}_3$–N–CH₂–CH₂–N–) of the initiator. Moreover, absence of the aromatic signals of the tosyl group demonstrated the quantitative degree of azide functionalization. In addition, the antisymmetric stretching vibration band of PEtOx-N₃ was clearly detected at 2103 cm^{-1} in the FT-IR spectrum. The number-average molecular weight (M_n) and polydispersity index (PDI) were measured by gel permeation chromatography and found as 9200 Da and 1.07, respectively.

On the other hand, clickable PCL-alkyne and PLA-alkyne precursors were prepared by coordination-insertion ROP of ε-caprolactone and L-lactide, using tin (II) 2-ethylhexanoate as catalyst and propargyl alcohol as initiator (with [M]/[I] ratio = 17.96 for PCL and 9.72 for PLA) in toluene at 120 °C. The structure of the obtained polymers were also confirmed by FT-IR, ^{1}H-NMR and GPC analyses. According to the ^{1}H-NMR analysis of PCL-alkyne, the methylene protons (O–C$\underline{H}_2$–C$\underline{H}_2$–C$\underline{H}_2$–C$\underline{H}_2$–C$\underline{H}_2$–C=O) of the repeating unit were observed around 1.3–1.4, 1.5–1.7 and 3.9–4.0 ppm. The singlet peaks at 4.6 and 2.3 ppm were attributed to the methylene (C$\underline{H}_2$–C≡CH) and methine (CH₂–C≡C$\underline{H}$) protons of propargyl alcohol, whereas the triplet peaks at 3.7 ppm were assigned to methylene (C$\underline{H}_2$OH) protons of PCL end-groups. The characteristic ester and alkyne bands of PCL-

alkyne were determined at 1105 (C–O–C=O), 1730 (C=O), 2100 (C≡C) and 3265 (C≡C–H) cm^{-1} in the FT-IR spectrum. According to GPC analysis, the M_n and PDI values of PCL-alkyne were found as 5100 Da and 1.35. Additionally, the PLA-alkyne was prepared and characterized by using similar procedure. In the ^{1}H-NMR spectrum, the characteristic methine (CH₂–C≡C$\underline{H}$) and methylene (C$\underline{H}_2$–C≡CH) protons of alkyne group were observed at 2.5–2.6 and 4.7–4.8 ppm, respectively. On the other hand, the methyl ((C=O)–CH–C$\underline{H}_3$) and methine ((C=O)–C$\underline{H}$–CH₃) protons of the repeating units of PLA were detected around 1.5–1.7 and 5.2–5.6 ppm. Its structure was further confirmed by FT-IR spectroscopy, in which the alkyne and ester groups were detected at 1095 (C–O–C=O), 1750 (C=O), 2090 (C≡C) and 3280 (C≡C–H) cm^{-1}. The M_n and PDI values of PLA-alkyne were found as 3200 Da and 1.41 by GPC analysis.

3.2. Synthesis and characterization of amphiphilic block copolymers

Finally, the targeted amphiphilic block copolymers, PEtOx-*b*-PCL and PEtOx-*b*-PLA were formed by CuAAC click reactions of PEtOx-N₃ with PCL-alkyne or PLA-alkyne ([PEtOx-N₃]:[PCL-alkyne] or [PLA-alkyne] = 1:1). All reactions were catalyzed with copper sulfate as a catalyst and ascorbic acid as a reductant and conducted at room temperature for 24 h Figure 2. The yields and the molecular weight characteristics of precursors and resulting copolymers were summarized in Table 1.

The successful formation of PEtOx-*b*-PCL and PEtOx-*b*-PLA block copolymers were also monitored

Figure 2. Synthesis of amphiphilic block copolymers (PEtOx-*b*-PCL and PEtOx-*b*-PLA) via CuAAC click reactions

Table 1. The data of precursor polymers (PEtOx-N$_3$, PCL-alkyne, PLA-alkyne) and block copolymers (PEtOx-*b*-PCL and PEtOx-*b*-PLA)

Entry	Yield[a] [%]	$M_{n,theo}$[b] [Da]	$M_{n,NMR}$[c] [Da]	$M_{n,GPC}$[d] [Da]	PDI[d]	T_g[e] [°C]	T_m[e] [°C]	x_{PEtOx}[f]
PEtOx-N$_3$	88.6	5 850	5 400	9 200[i]	1.07[i]	57	n.d.	–
PCL-alkyne	81.6	1 700	2 500	5 100	1.35	n.d.	51	–
PLA-alkyne	77.0	1 070	1 450	3 200	1.41	37	112	–
PEtOx-*b*-PCL	94.6	7 550[g]	7 100[h]	13 100	1.22	n.d.	49	68.1
PEtOx-*b*-PLA	93.2	6 950[g]	6 600[h]	11 300	1.28	58	n.d.	76.7

[a]Determined by gravimetrically;
[b]$M_{n,theo}$ = mmonomer·[M]$_0$/[I]$_0$·conversion;
[c]Calculated according the ^{1}H-NMR analysis;
[d]Determined by GPC with linear polystyrene standards;
[e]Determined by DSC with a heating rate of 10 °C/min under nitrogen flow (10 mL/min);
[f]Copolymer compositions (x) were calculated according the ^{1}H-NMR analysis by comparing the integration of the methyl protons of PEtOx (d) with methylene protons (n) of PCL or methyl protons (k+n) of PLA;
[g]Sum of $M_{n,theo}$ of precursor polymers;
[h]Calculated according to ^{1}H-NMR analysis by comparing the integration of triazole proton (g) with methylene protons (n) of PCL or methyl protons (k+n) of PLA;
[i]Determined by GPC with linear poly(ethylene oxide) standard;

Figure 3. FT-IR spectra of PEtOx-N$_3$, PCL-alkyne and PEtOx-*b*-PCL samples

Figure 4. FT-IR spectra of PEtOx-N$_3$, PLA-alkyne and PEtOx-*b*-PLA samples

by FT-IR spectroscopy. The characteristic azide band of PEtOx-N$_3$ at 2103 cm^{-1} and alkyne bands (C≡C and C≡C–H) of PCL-Alkyne and PLA-Alkyne at 2125 and 3320 cm^{-1} were clearly disappeared, whereas new peaks corresponding to C=O and C–O–C bonds of ether groups were appeared at 1728 and 1240 cm^{-1} for PCL, and 1758 and 1048 cm^{-1} for PLA. In addition, the characteristic amide, methine, methylene and methyl bands of PEtOx were still presented in both block copolymer samples Figure 3 and 4. After the click reaction, in ^{1}H-NMR spectrum of PEtOx-*b*-PCL, a new peak corresponding to triazole ring (*g*) was observed at around 8.1 ppm Figure 5. Although, the methine (CH$_2$–C≡C$\underline{H}$) proton of PCL-alkyne protons at 2.3 ppm was overlapped with methylene protons (c) of PEtOx, the methylene (C$\underline{H}_2$–C≡CH) protons (h) at 4.6 ppm were clearly shifted to 5.1 ppm. On the other hand, the similar results were obtained for PEtOx-*b*-PLA, in which triazole proton was clearly detected, the characteristic methylene protons were quantitatively shifted to 5.2 ppm Figure 6. In addition, the characteristic protons of both PEtOx with PCL or PLA segments were detected and the copolymer composition was calculated using ^{1}H-NMR spectroscopy data by comparing the integration of the methyl protons (d) of PEtOx with methylene protons (*n*) of PCL or the methyl protons (k+n) of PLA. By using similar method, the molecular weights ($M_{n,NMR}$) of obtained copolymers could be also calculated by using integral ratio of the triazole proton (g) with the methylene protons (n) of

Figure 5. ^{1}H-NMR spectrum of PEtOx-*b*-PCL sample

Figure 6. ^{1}H-NMR spectrum of PEtOx-*b*-PLA sample

PCL or the methyl protons (k+n) of PLA. The obtained molecular weights results were summarized in Table 1 and discussed as follows. According to ^{1}H-NMR and FT-IR results, successful block copolymer synthesis was achieved by CuAAC click reaction under mild condition.

Successful block copolymer synthesis via CuAAC click reaction was also confirmed by GPC analysis. The GPC chromatograms of initial precursors demonstrated unimodal patterns with narrow molecular weight distributions, which implied that control over molecular weight was achieved by either coordination-insertion ROP or living CROP. After block copolymer formations, the GPC traces of PEtOx-*b*-PCL and PEtOx-*b*-PLA were monomodal and shifted to higher molecular weight regions Figure 7. The increase of M_n of PEtOx-*b*-PCL was higher than

PEtOx-*b*-PLA due to the initial block length. On the other hand, both block copolymers had slightly high molecular weight distributions below than 1.28 compared to PEtOx-N$_3$ homopolymer. Based on GPC and ^{1}H-NMR results, the ^{1}H-NMR results were in good agreement with theoretical molecular weights of block copolymers. Since the GPC equipment was calibrated with polystyrene standards, the molecular weights of copolymers were calculated higher than theoretical molecular weights. However, an increase of molecular weights in both PEtOx-*b*-PCL and PEtOx-*b*-PLA block copolymers were clearly observed in GPC chromatograms. The composition of block copolymers were calculated by the ^{1}H-NMR analysis by comparing the integration of the methyl protons of PEtOx (d) with methylene protons (n) of PCL or methyl protons (k+n) of PLA. The ratio of PEtOx
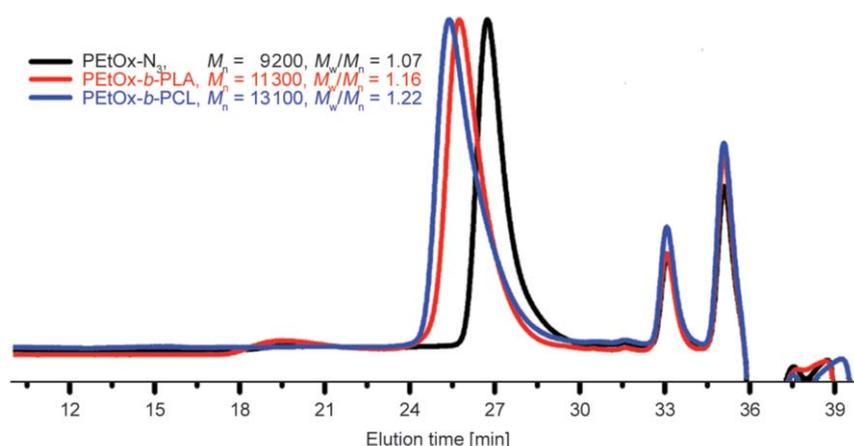
Figure 7. GPC traces of PEtOx-N$_3$, PEtOx-*b*-PCL and PEtOx-*b*-PLA block copolymers

block was determined as 68.1% in PEtOx-*b*-PCL, whereas 76.7% in PEtOx-*b*-PLA.

3.3. Thermal behavior of amphiphilic block copolymers

Thermal transitions of amphiphilic block copolymers and their linear counterparts were investigated to show the influence of chain compositions on these transitions by DSC analysis. The PEtOx was an amorphous polymer that did not have any crystallization or melting transitions, but it exhibited a glass transition temperature (T_g) at 57 °C. Conversely, the PCL and PLA, were semi-crystalline polymers, exhibited both glass transition and melting points, which were below –60 and 51 °C for PCL (Due to the cooling limitation of our DSC instrument, T_g of PCL was not determined), and 37 and 112 °C for PLA [57]. In the case of PEtOx-*b*-PCL, T_g of PEtOx was not detected due to overlapping with the melting peak of PCL, which was shifted from 51 to 49 °C [58]. Since the PLA block had a short block length, its thermal transitions in the PEtOx-*b*-PLA in such block copolymers strongly depends on the molecular weight of the blocks, were not observed [38]. However, the T_g of PEtOx was slightly improved by addition of semi-crystalline PLA block [37].

3.4. Cytotoxic activities of amphiphilic block copolymers

The cytotoxicity activities of resulting amphiphilic block copolymers and their precursors were evaluated under *in-vitro* conditions. For that reason, three different cell types were used to investigate the cytotoxic effect of these polymers on the prostate epithelial and cancer cells. In order to compare the effect of polymers with respect to androgen dependency the hormone-sensitive cell line LnCAP and hormone-resistant cell line 22RV1 was used in the cytotoxicity studies. The PEtOx-N$_3$ polymer significantly induced the proliferation of PNT1A cell line with the percentage of 40 to 50% Figure 8a. Interestingly, this polymer led to a slight toxic effect on the viability of 22RV1 cells in the first 24 hours, which was pulled back to control levels for the later time points Figure 8b. A similar trend in the proliferation rate for LnCAP was detected in that PEtOx-N$_3$ polymer treatment resulted in a 40 to 50% decreased on the cell viability of the LnCAP cell line at 24 and 48 hours, while at 72 hour time point LnCAP cell viability was detected around 90% in the presence of 10 and 30 μL/ml PEtOx-N$_3$ Figure 8c.

Characterization of PCL-alkyne polymer toxicity for PNT1A cells showed an induction in cell proliferation in a dose-dependent manner at all-time points, while treatment of 22RV1 and LnCAP with this polymer led to a decrease in cell viability at early time points Figure 9a. In the presence of PCL-alkyne, the cell viability of 22RV1 cells was decrease to 20% at 24 hours, 30% at 48 hours and 35% at 72 hours Figure 9b. Albeit following a similar trend at the early time points, LnCAP cell viability was returned to control levels at 72 hour-treatment with PCL-alkyne at all-concentrations Figure 9c.

Exposure to PLA-alkyne polymer at all concentrations resulted in an approximate 20% of toxicity in PNT1A cells at 24 and 48 hours, albeit with no detectable toxic effect was evident at 72 hours Figure 10a. Regardless of the treatment concentration PLA-alkyne displayed slight toxicity with a percentage of 20% to 22RV1 cells Figure 10b. PLA-alkyne
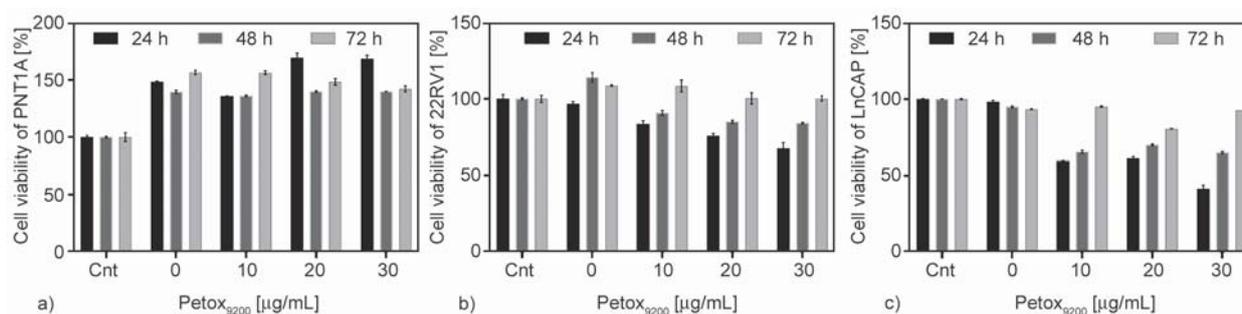
Figure 8. Cytotoxic effect of PEtOx-N$_3$ on PNT1A, 22RV1 and LnCAP cell lines. Percentage of cell viability of PNT1A (a), 22RV1 (b), and LnCAP (c). Each data point represents to the mean percentage of viable cells at different time points with three different concentrations (0–30 µL/mL) of at least three separate experiments performed for each concentration. The percentage of cell viability was calculated by assigning the absorbance value obtained from non-treated cells as 100% for each time point (Cnt: control experiment).
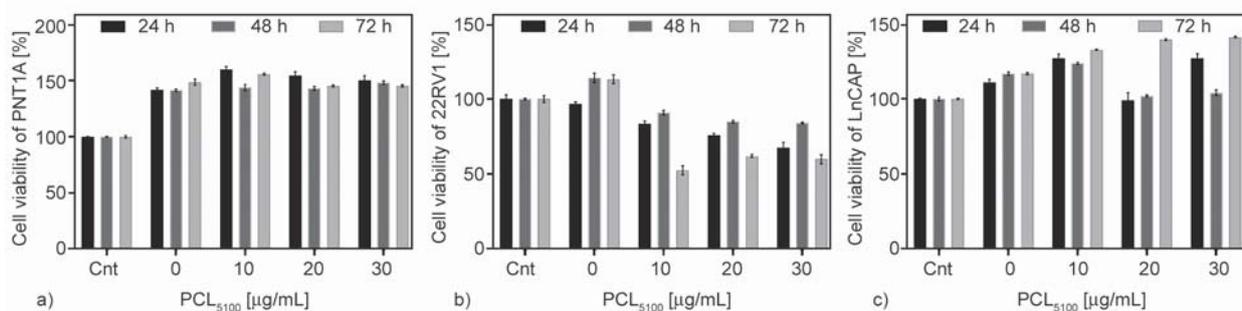
Figure 9. Cytotoxic effect of PCL-alkyne on PNT1A, 22RV1 and LnCAP cell lines. Percentage of cell viability of PNT1A (a), 22RV1 (b), and LnCAP (c). Similar method in Figure 8 was used.
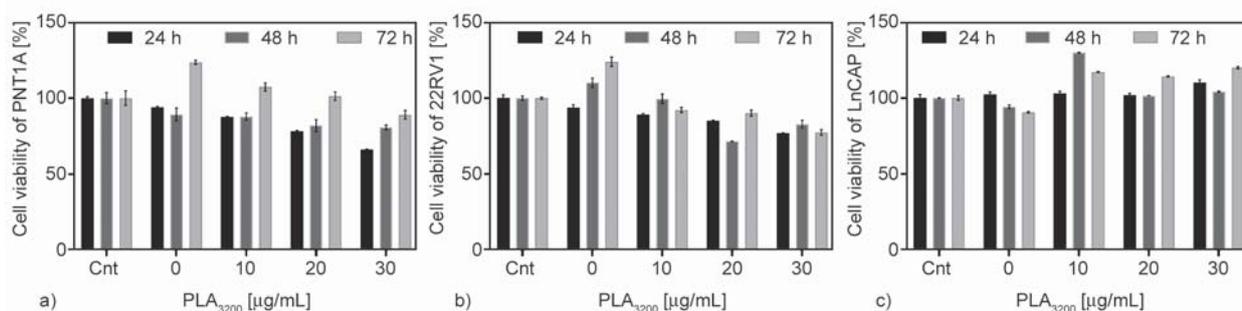
Figure 10. Cytotoxic effect of PLA-alkyne on PNT1A, 22RV1 and LnCAP cell lines. Percentage of cell viability of PNT1A (a), 22RV1 (b), and LnCAP (c). Similar method in Figure 8 was used.

polymer exhibited was not only a non-toxic but also slight proliferative effect the LnCAP cell line in time and dose independent manner Figure 10c.

The treatment of PNT1A cells with PEtOx-*b*-PCL polymer resulted in an 30% increase of cell proliferation at all concentrations for the three time points tested Figure 11a. Similarly, a significant cell proliferation (40–50%) was evaluated for 22RV1 cells at 72 hour time point Figure 11b. For LnCAP cell line this polymer displayed a slight toxicity with a 10% at the 10 and 20 µL/mL, while a 30% decrease in LnCAP cell viability at 30 µL/mL for 48 and 72 hours Figure 11c.

The PEtOx-*b*-PLA led to increase cell proliferation rate in PNT1A cells at all the doses tested Figure 12a. Although this polymer showed no toxicity at the 10 and 20 µL/mL on 22RV1 cells, a moderate toxicity of this polymer at 30 µL/mL was evident for these androgen independent cells Figure 12b. Likewise, on the androgen dependent cell line LnCAP, the PEtOx-*b*-PLA showed moderate toxicity effect by decreasing the cell viability by 15% at the tested doses Figure 12c.

To summary, the treatment of the healthy prostate epithelial cell line PNT1A with 5 different polymers revealed that no significant cytotoxicity instead the
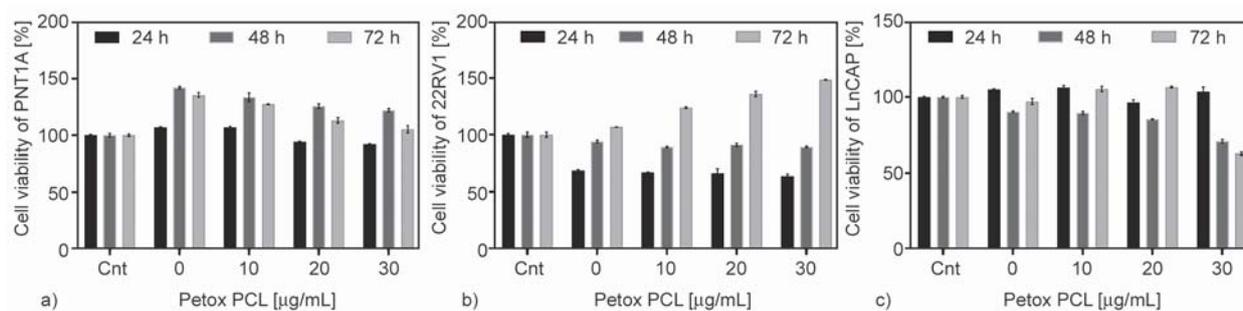
Figure 11. Cytotoxic effect of PEtOx-*b*-PCL on PNT1A, 22RV1 and LnCAP cell lines. Percentage of cell viability of PNT1A (a), 22RV1 (b), and LnCAP (c). Similar method in Figure 8 was used.
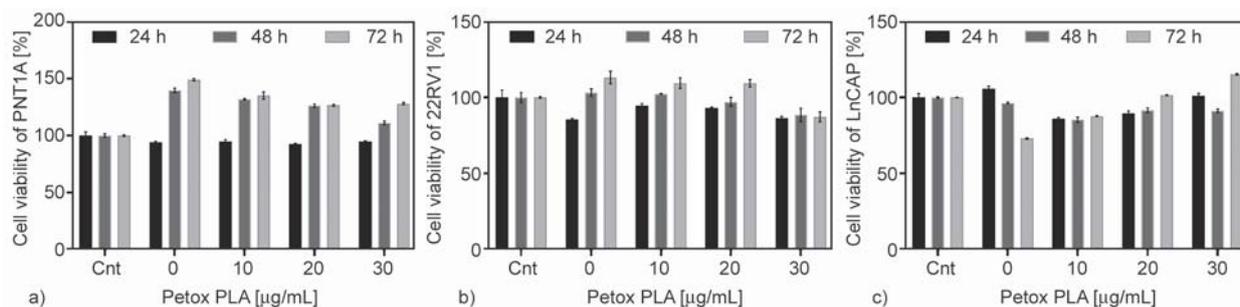
Figure 12. Cytotoxic effect of PEtOx-*b*-PLA on PNT1A, 22RV1 and LnCAP cell lines. Percentage of cell viability of PNT1A (a), 22RV1 (b), and LnCAP (c). Similar method in Figure 8 was used.

cell proliferation rate of PNT1A cells significantly increased once exposed to these polymers. On the other hand, these polymers showed some significant toxic effects on the prostate cancer cell lines. The PEtOx-*b*-PLA revealed a moderate toxicity in both prostate cancer lines while PLA-alkyne sample possessed significant cytotoxic effect only on 22RV1 cells suggesting that the androgen dependency might have a role in the effect of cytotoxicity of PLA-alkyne sample. Similarly, in cytotoxic studies carried out on human dermal fibroblast (HDF) cells with PEtOx-*b*-PLA, a slight cytotoxic effect was found at lower concentrations [59]. Given that uptake routes of homo- and co-polymers differs between cancer and healthy cells, and cationic polymers were known to distrupt the integrity of the plasma membrane, cancer cells with their high rate of cellular uptake and fragile plasma-membrane integrity were expected to exhibit increased cytotoxicity after polymer treatment than their healty counterpart [60]. In contrast, a study carried by Hsiue and coworkers found a marked cytotoxicity for PEtOx-*b*-PLA at higher concentrations on non-small cell lung carcinoma [61]. Although PEtOx-N$_3$ and PCL-alkyne led to decrease cell viability at early time points for 22RV1 and LnCAP cells at later time points this effect was compensated and the viable cell number for 22RV1 and LnCAP cells was comparable to the non-treated con-

trol pointing at a possible cytostatic effect of these polymers. According to the study conducted by Luxenhofer and coworkers, the cytotoxicity of PEtOxs on healthy and cancer cells may have vary due to their chemical and physical properties [62].

4. Conclusions

In conclusion, well-defined amphiphilic block copolymers containing poly(2-ethyl-2-oxazoline) as hydrophilic block and poly(ε-caprolactone) or poly(L-lactide) as hydrophobic block were successfully synthesized by combination of CuAAC click chemistry with either coordination-insertion or living cationic ring-opening polymerization. The results indicated that the CuAAC click reactions can be used to combine mechanistically incompatible blocks (PEtOx, PCL and PLA) in a single molecule under mild conditions. The cytotoxicity activities of resulting amphiphilic block copolymers and their precursors were investigated in the prostate epithelial and cancer cells under *in-vitro* conditions. The results indicated that PEtOx-*b*-PCL and PEtOx-*b*-PLA displayed no toxic effect on the healthy prostate epithelial cells but on prostate cancer cell lines, therefore, it will be an excellent candidates in biomedical applications such as pharmaceutics, drug delivery systems, stabilizing agents, cosmetics and processing aids.

Acknowledgements

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