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Synthesis, characterization, and cytotoxicity analyses of ABA-type block copolymer bearing p-xylene-bis(2-mercaptoethyloxy) core

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Keywords:
Amphiphilic block copolymer, Cytotoxicity, Cancer cells

Abstract – ABA–type amphiphilic novel poly(*N*-vinyl pyrrolidone)-*block*-poly(D,L-lactide)-*block*-poly(*N*-vinyl pyrrolidone) (PNVP-*b*-PDLLA-*b*-PNVP), triblock copolymer was synthesized via combination of ring-opening polymerization (ROP) and reversible addition-fragmentation chain transfer (RAFT) polymerization using novel bifunctional PDLLA based RAFT macro chain transfer agent (CTA) bearing *p*-xylene-bis(2-mercaptoethyloxy) core. For this goal, bifunctional initiator *p*-xylene-bis-(1-hydroxy-3-thia-propane) **(1)** was synthesized by the reaction of α,α'-dibromo-*p*-xylene with 2-mercaptoethanol in the presence of KOH in ethanol. PDLLA-diol was synthesized by metal-free organocatalyzed ROP of D, L-lactide (D,L-LA) in presence of 4-(dimethylamino) pyridine (DMAP) as catalyst using **(1)** as initiator Dibromoester end-functionalized PDLLA-based macroinitiator **(3)** was prepared by esterification of hydroxyl end groups of PDLLA-diol **(2)**. PDLLA macro-CTA (4) was then synthesized via a substitution reaction of **(3)** with potassium ethylxanthate (KEX). Finally, PNVP-*b*-PDLLA-*b*-PNVP triblock copolymer **(5)** was synthesized RAFT polymerization of NVP using **(4)**. Characterization of the molecular structures for synthesized novel polymers was made by spectroscopic (FTIR, ^{1}H NMR) methods. In this study's application phase, polymers' effectiveness was examined on cervical cancer cells. Cytotoxicity and metastatic effects were evaluated in vitro on HeLa cell lines. In our study, we observed no toxic effect of the block copolymer below 600 µg/mL on the HeLa cervical cancer cell line and in future studies on drug delivery studies of cancer treatments.

1. Introduction

Among the biocompatible and biodegradable polymers used in nanoparticle formulation, amphiphilic copolymers facilitate the straightforward creation of nanostructures with various morphologies (polymersomes, micelles, and nanospheres) owing to their self-assembling ability in aqueous environments [1, 2]. The synthesis of amphiphilic block copolymers consisting of a biodegradable hydrophobic portion and a hydrophilic portion by a combination of ring-opening polymerization (ROP) and controlled radical polymerizations (CRPs) has significantly contributed to the studies in the field of nanomedicine. ROP is one of the polymerization methods that can be performed depending on the initiator/catalyst system variations and the monomer in the syntheses of various aliphatic polyesters [3]. The hydrophobic biodegradable polyesters, such as poly(ε-caprolactone) (PCL) and poly(lactic acid) (PLA), used for controlled drug delivery studies were generally prepared via ROP as the most preferred technique [4, 5]. Among these polyesters, PLA is a bio-based and biocompatible polymer used in many applications, such as biomedicine, agriculture, and packaging. It is used because of its renewability, biocompatibility, and excellent processability and because it decomposes to H2O and CO2 when degraded, forming non-toxic and non-carcinogenic products [6, 7].

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Poly(*N*-vinyl-pyrrolidone) (PNVP) is a hydrophilic polymer that can be used in the pharmaceutical, biomedical, and nutraceutical fields due to its properties such as water solubility and low toxicity [8−10]. The polar lactam groups in the pyrrolidone part increase its water solubility. In addition, nonpolar methylene groups add lipophilic characters to PNVP. PNVP is also used in industrial applications such as food, cosmetics, adhesives, and textiles. PNVP has been used extensively in pharmaceutical tablets and hydrogels [11,12].

The studies focusing on the synthesis of AB- or ABA-type block copolymers, including PLA and PNVP, using many polymerization routes have been investigated extensively by many researchers in recent years. AB-type poly(*N*-vinyl-pyrrolidone)-*block*-poly(D,L-lactide) PNVP-*b*-PDLLA diblock copolymers were synthesized using reversible addition-fragmentation chain transfer (RAFT) first method followed by ROP of D,L-LA using hydroxyl PNVP macro-chain transfer agent (CTA). Le Garrec et al. [10] have reported the preparation of PNVP-*b*-PDLLA via ROP of D,L-LA was initiated by the hydroxyl groups of poly(vinyl-pyrrolidone) (PVP) using potassium hydride and investigated the evaluation of this polymer as a novel polymeric solubilizer for drugs. Gaucher et al. [13] have reported that oligomeric PVP having hydroxyl group was utilized as an initiator to synthesize PVP-*b*-PDLLA via anionic polymerization of D,L-LA and studied the in vitro release of two hydrophobic anticancer drugs (paclitaxel and etoposide). Ravenelle et al. [14] investigated the incorporation of propofol into polymeric micelles obtained from amphiphilic block copolymers of PNVP-*b*-PDLLA.

The ABA-type block copolymers can be prepared by sequential polymerization of monomers using a bifunctional initiator [15]. Many studies have been published on synthesizing and applying block copolymers consisting of PDLLA and PNVP segments. For example, Ramesh et al. [16] have reported the preparation of ABA-type amphiphilic block copolymers via ROP and RAFT polymerization methods and investigation of properties on drug loading and antibacterial activity. Leroux et al. [17] studied the synthesis of ABA and (BA)4-type block copolymers via radical polymerization of *N*-vinyl pyrrolidone (NVP) using PDLLA dithiol (HS-PDLLA-SH) and star-poly(D,L-lactide) tetrakisthiol (star-(PDLLA-SH)4) and evaluation of delivery of hydrophobic drugs. Xiong et al. [18] have reported the synthesis, characterization, and degradation of PDLLA-*b*-PNVP-*b*-PDLLA triblock copolymer prepared through the ROP of D,L-LA initiated by dihydroxy PNVP as macro-initiator in presence of dibutyl tin dilaurate (DBTDL) as catalyst. Hira et al. [19] synthesized well-defined 4-arm star (PDLLA-*b*-PNVP)$_4$ block copolymers and investigated their self-assembly properties and application as nanocarriers for drug delivery.

This work prepared a novel ABA-type block copolymer in four stages via a combination of ROP and RAFT polymerization of DL-lactide and NVP, respectively. Firstly, PDLLA-diol **(2)** was synthesized by metal-free organocatalyzed ROP of D,L-LA using 4-(dimethylamino) pyridine (DMAP) as a catalyst in dichloromethane at 35 °C using **(1)** as initiator. Secondly, dibromoester end-functionalized Br-PDLLA-Br macroinitiator **(3)** was obtained by esterifying hydroxyl groups of PDLLA-diol **(2)**. Thirdly, PDLLA macro-CTA **(4)** was then synthesized via a substitution reaction of **(3)** with potassium ethylxanthate (KEX). Finally, novel PNVP-*b*-PDLLA-*b*-PNVP **(5)** block copolymer was synthesized RAFT polymerization of NVP using **(4)**. Characterization of the molecular structures for synthesized novel polymers was made by spectroscopic (FTIR and ^{1}H NMR) methods. In this study's application phase, copolymer's effectiveness was examined on cervical cancer cells. Cytotoxicity and metastatic effects were evaluated in vitro on HeLa cell lines.

2. Materials and Methods

2.1. Materials

Reactions were performed under an argon atmosphere using standard Schlenk techniques unless otherwise specified. *N*-vinyl pyrrolidone (NVP, Sigma-Aldrich, >99%, UK) was dried over anhydrous magnesium sulfate and distilled under reduced pressure. D,L-lactide (D,L-LA, TCI, >98%, Japan) was recrystallized from a toluene/ethyl acetate mixture. Dichloromethane (DCM, Sigma-Aldrich, ≥99.5%, Germany) was dried over calcium hydride (CaH_2) and stored over molecular sieves (4 Å). 2-Bromopropionyl bromide (Sigma-Aldrich,

97%, Germany), triethylamine (TEA, Sigma-Aldrich, ≥99%, Sweden), potassium ethylxanthate (TCI, >95%, Japan), *α,α′*-dichloro-*p*-xylene (Acros, 98%, Belgium) and DMAP (Sigma-Aldrich, >99%, UK) were used as received. Conventional procedures were used for the purification of all solvents [20].

2.2. Measurement

Transmission IR spectra were recorded on an FTIR-ATR spectrophotometer (Perkin-Elmer 1600) in the spectral range 4000–400 cm^{-1} with samples. ^1H NMR spectra were recorded on the Bruker AVANCE III 400 MHz NMR instrument to characterize the Varian Mercury 400 MHz spectrometer with $CDCl_3$ as solvent at ambient temperature.

2.3. Synthesis of (1)

p-Xylene-bis(1-hydroxy-3-thia-propane) **(1)** was synthesized according to our previously published procedure [21]. (Yield: 87 %,; FTIR (ATR, cm^{-1}): 3318 (OH), 3042 (ArH), 2949-2814 (CH_2); ^1H NMR ($CDCl_3$): δ= 7.28 (s, 4H, Ar***H***), 3.71 (s, 4H, S−C***H***$_2$−Ar), 3.66-3.60 (q, 4H, S−CH_2−C***H***$_2$−OH), 2.66-2.59 (t, 4H, S−C***H***$_2$−CH_2−OH), 2.24 (s, 2H, −O***H***).

2.4. Synthesis of (2) in the Presence of Initiator (1) via ROP

PDLLA **(2)** was synthesized as follows: into a dried Schlenk tube equipped with a magnetic stirrer, ROP initiator **(1)** (0.129 g, 0.5 mmol), D,L-LA (1.47 g, 10 mmol), and DMAP (0.123 g, 1 mmol) were added and dried in vacuo for 2 h. Dry DCM (5 mL) was introduced into the flask under an argon atmosphere. The Schlenk flask was immersed in an oil bath at 35 ℃ for 30 h. The reaction mixture evaporated half of the volume and precipitated into cold methanol. The resulting polymer was collected by filtration and drying under vacuum at 35 ℃ for 48 h until constant weight.

Yield: 1.13 g, Conversion: 68%. M_n (theo.); 2218 g/mol; M_n (NMR); 2245 g/mol; FTIR (ATR, cm^{-1}): 3503, 2995, 2943, 2882, 1749, 1452, 1381, 1271, 1182, 1081, 863, 745 in Figure 1A; ^1H NMR ($CDCl_3$, δ) = 7.24 (Ar***H***, H^{a}), 5.22–5.16 (main chain, –C***H***(CH_3)OCO) H^{e}), 4.35 (terminal, –C***H***(CH_3)OH, $H^{e'}$), 4.22 (S−CH_2−C***H***$_2$−OH, H^{d}), 3.71 (Ar−C***H₂***−S, (H^{b})], 2.64 (−S−C***H***$_2$−CH_2−O, H^{c}), 1.58 (main chain, –CH(C***H***$_3$)OCO-, H^{f}), 1.49 (terminal, –CH(C***H***$_3$)OH, $H^{f'}$) in Figure 2A.

2.5. Synthesis of Dibromoester-Ended PDLLA (3)

PDLLA-diol **(2)** was converted to bromo-ester functionalized PDLLA macroinitiator **(3)** using 2-bromopropionyl bromide. **(2)** (0.4 g, 0.176 mmol, M_n (NMR) =2245 g/mol) was charged into a round-bottom two-necked flask and dissolved in dry DCM (15 mL). Then, TEA (0.12 mL, 0.88 mmol) was added to the mixture under an inert atmosphere. The reaction was cooled down to 0 ℃, and 2-bromopropionyl bromide (0.076 mL, 0.704 mmol) dissolved in dry DCM (5 mL) was added dropwise for 30 min. The reaction continued at room temperature for 48 h with stirring. After removing the precipitated salt, the filtrate was diluted with 30 mL of DCM, washed with 5% aqueous $NaHCO_3$ (3x20 mL), then water (3x20 mL), dried over $MgSO_4$, and filtered. The concentrated solution was precipitated into cold methanol, and the product **(3)** was dried in a vacuum at 40 °C.

M_n (NMR): 2605 g/mol; ^1H NMR ($CDCl_3$, δ) = 5.21–5.14 (–C***H***(CH_3)OCO) H^{e})], 4.42 (–C***H***(CH_3)Br, H^{g}), 4.23 (S−CH_2−C***H***$_2$−O, H^{d}), 3.71 (Ar−C***H₂***−S, (H^{b})], 2.64 (−S−C***H***$_2$−CH_2−O, H^{c}), 1.84 (–CH(C***H***$_3$)Br, H^{g}), 1.58 (–CH(C***H***$_3$)OCO-, H^{f}) in Figure 2B.

2.6. Synthesis of Dixanthate Terminated PDLLA (4)

The functional polyester **(3)** was converted into a PDLLA-based RAFT-CTA **(4)** via a substitution reaction of **(3)** with potassium ethylxanthate (KEX). In a typical synthesis process, **(3)** (0.11 g, 0.043 mmol, M_n(NMR) =2605 g/mol) and KEX (0.053 g, 0.33 mol) were taken in a dried and argon purged round-bottom flask, and the flask was immersed in an ice bath. In another dried flask, pyridine (0.48 mL, 5.90 mol) dissolved in 20 mL DCM and added dropwise to the first reaction mixture during stirring for 30 min. The reaction mixture continued at room temperature for 48 h with stirring. The reaction mixture diluted with 60 mL of DCM was washed successively with saturated NH_4Cl solution (3x30 mL), saturated $NaHCO_3$, solution (3x30 mL), and water (3x50 mL), dried over $MgSO_4$ and filtered. After the filtrate was dry, the residue was dissolved in THF and precipitated into hexane. The macro-CTA **(4)** was dried overnight under vacuum.

M_n (NMR); 2900 g/mol; ^{1}H NMR ($CDCl_3$, δ) = 5.21–5.14 (–C***H***(CH_3)OCO), H^e), 4.64 (O–C***H***$_2$$CH_3$, H^i), 4.42 (–C***H***(CH_3)Br, H^g), 4.23 (S−CH_2−C***H***$_2$−O, H^d), 3.72 (Ar−C***H***$_2$−S, H^b), 2.65 (−S−C***H***$_2$−CH_2−O, H^c), 1.57 (–CH(C***H***$_3$)OCO-, H^f), 1.42 (O–CH_2C***H***$_3$, H^j) in Figure 2C.

2.7. Synthesis of PNVP-*b*-PDLLA-*b*-PNVP (5)

In a dried Schlenk flask, **(4)** (37 mg, 0.0128 mmol, M_n (NMR) =2900 g/mol) was dissolved in 1 mL THF, and then NVP (0.14 mL, 1.28 mmol) and AIBN (1.05 mg, 0.0064 mmol) were added to the solution. The homogeneous solution was degassed with argon and continued for 0.5 h with stirring. The flask was immersed in an oil bath at 80 °C, and the reaction was allowed to proceed for 24 h. The reaction mixture was diluted with THF (4 mL), precipitated with 250 mL hexane and the precipitated polymer was separated by centrifugation. The obtained polymer **(5)** was purified by dissolution/precipitation procedure two more times and dried overnight under a vacuum at 30 °C.

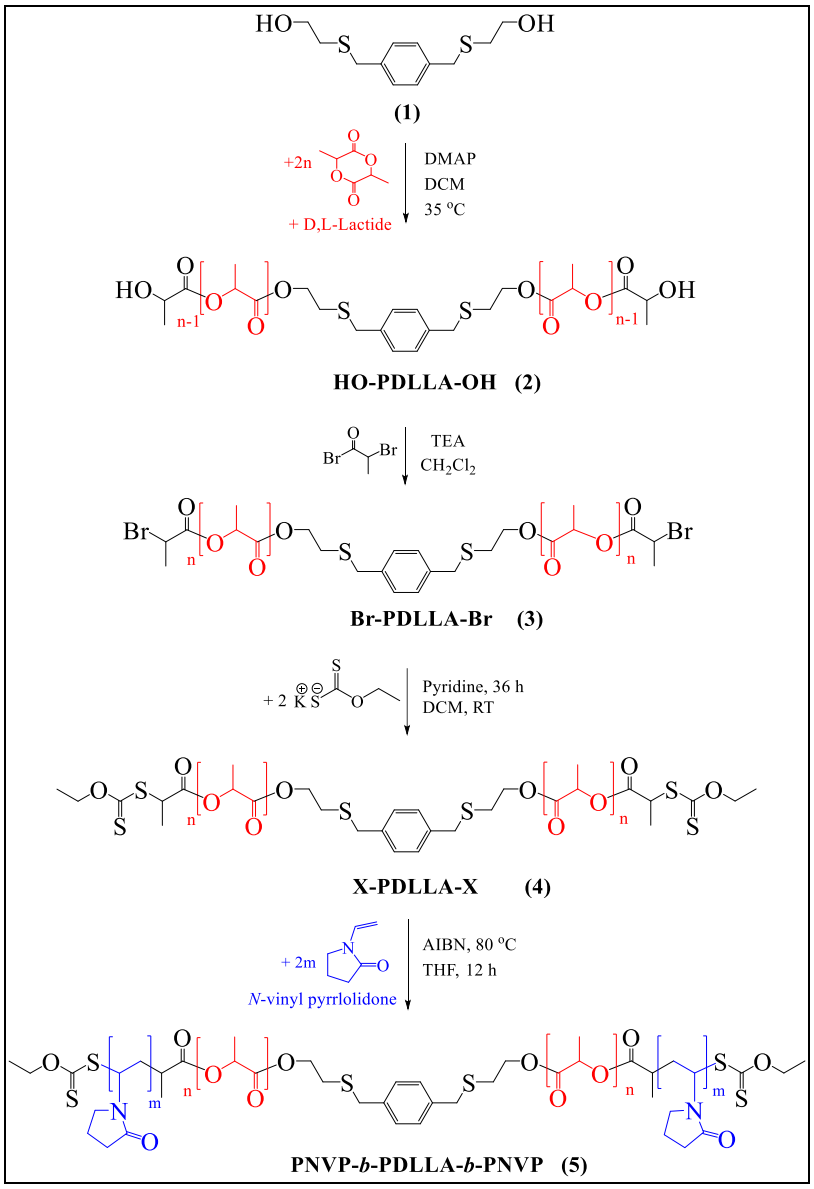
M_n (theo.); 4970 g/mol; M_n (NMR): 5220 g/mol; FTIR (ATR, cm^{-1}): 3435, 2974, 2946, 2925, 2886, 1755, 1650, 1421, 1289, 1182, 1084, 842, 735 in Figure 1B; ^{1}H NMR ($CDCl_3$, δ) = 5.22–5.15 (H^e, in PDLLA), 4,02–3.55 (CH_2C***H***, H^l in PNVP backbone), 3.55–3.02 (–NC***H***$_2$$CH_2$$CH_2$, H^m, in PNVP ring), 2.64 (−S−C***H***$_2$−CH_2−O, H^c, initiator), 2.54–2.22 (–NCH_2CH_2C***H***$_2$, H^p, in PNVP ring), 2.22–1.81 (–NCH_2C***H***$_2$$CH_2$, H^n, in PNVP ring), 1.67–1.14 ($H^{f+k+h+i}$) in Figure 2D.

2.8. Cell Culture

This study used human cervical cancer (HeLa) cell lines obtained from Kırşehir Ahi Evran University, Department of Medical Pharmacology. The cells were cultured at 37 °C with 5% CO_2 in RPMI medium supported with 10% Fetal Bovine Serum and 1% penicillin-streptomycin.

2.9. Cytotoxicity Analyses

The cytotoxic impact of molecules HeLa cells was evaluated using the XTT assay kit (Biological Industries, USA). 8000 cells were seeded per well in a 96-well plate. Following a 24-hour incubation period, the cells were exposed to copolymer. The polymer was in powder form and dissolved in DMSO. Then, starting from the highest dose of 850 ug/ml, it was applied to the cells. After 72 h of incubation, the solutions from the XTT kit were introduced to the cells. Cell viability was then measured using a microplate reader (BIOTEK ELX808, USA) at a wavelength of 450 nm. The IC50 value was determined. As a result of the readings of the obtained values, the inhibition rates of cells were calculated using the following formula: inhibition%: (A450 nm test - A450 nm control/A450 nm control) ×100. In the XTT analysis, the polymer was dissolved in DMSO, and only the highest dose of 0.5% DMSO was used as a control. No toxic effects were observed at this highest dose and serial dilutions. The only medium was applied as a negative control to cells.



Scheme 1. Synthesis of PNVP-*b*-PDLLA-*b*-PNVP.

2.10. Wound Healing Assay

1.5×10^5 cells were seeded in 6-well plates and incubated at 37 ℃ with 5% CO_2 for 48 h. When the cells reached 80% confluence, wounds were created using a 20–200 µL pipette tip [22]. Subsequently, 4 mL of fresh medium and 1 mL of copolymer were added. The closure of the wounds was monitored and captured using an inverted microscope.

2.11. Invasion Assay

Cell culture inserts were positioned in 24-well plates, and 100 µL of a pre-prepared stock of 1:10 matrigel was applied. The plate was then incubated for 48 h at 37 ℃ to achieve a gel-like consistency. Once the specified structure was formed in the matrigel, RPMI-1640 medium containing fetal bovine serum (FBS) was added to the wells. Serum-free RPMI-1640 medium was added to the matrigel-containing insert, and approximately $1x10^6$ cells were inoculated. Copolymers were put in wells containing medium with serum and incubated at 37 ℃ for 48 h. After incubation, the cells were treated with 10% formaldehyde for 10 min, followed by staining with Giemsa [23].

2.12. Statistical Analyses

SPSS 29 was used in the study. Differences between groups were determined using the Student T test and ANOVA analysis. Experiments were performed with three repetitions. A value of $p<0.05$ was defined as significant.

3. Results and Discussion

The synthetic route is depicted in Scheme 1. Firstly, PDLLA **(2)** ([monomer]:[initiator]=20:1) was synthesized by ROP of D,L-LA using **(1)** as the initiator and DMAP as a catalyst. FTIR and ^{1}H NMR elucidated the structure of HO-PDLLA-OH. A metal-free organocatalyzed controlled ROP proposed by [4,24] was applied to synthesize bifunctional PDLLA using **(1)** as an initiator. FTIR spectra of PDLLA and PNVP-*b*-PDLLA-*b*-PNVP are depicted in Figure 1. In Figure 1A, the absorption band of the carbonyl group of the PDLLA block was observed at 1749 cm^{-1}. The spectrum shows two bands representing symmetric and asymmetric C-H stretching at 2995-2882 cm–1. The bands at 1452 and 1381 cm^{-1} represent the stretching of C–H in the methyl group of PDLLA. The bands at 1182 and 1081 cm^{-1} are attributed to carbon-oxygen stretching. The spectrum also showed the lactide ring vibration of PDLLA vibration at 936 cm^{-1} was lost entirely due to polymerization [25].

Figure 2A shows a ^{1}H NMR spectrum of bifunctional PDLLA. The peaks of methine (H^e) and methyl (H^f) protons, corresponding to PDLLA repeating units, were detected at 5.19 and 1.58 ppm, respectively. In the ^{1}H NMR spectrum of **(2),** the signal (H$^{e'}$) of the terminal methine group of PDLLA was observed at 4.35 ppm. The signals attributed to methylene protons of initiator **(1)** at δ =4.22 (S−CH$_2$−C***H***$_2$−O), 3.71 (Ar−C***H***$_2$−S), 2.64 (−S−C***H***$_2$−CH$_2$−O) ppm are observed in the spectrum. The aromatic protons of the initiator gave a signal at δ =7.28 ppm. M_n (NMR) of polymer **(2)** was determined by using the integral ratio of the methine proton peaks of PDLLA (δ = 5.18 ppm) to the methylene proton peaks of the initiator (peak c in Figure 2A). NMR spectra's calculated molecular weight value is close to the theoretical number-average molecular weight M_n (theo.). Integral area of related peak displacements calculated Mn (NMR) of PDLLA according to (3.1)

$$M_n\,(\mathrm{NMR}) = \frac{I_e}{I_c} M_{monomer} + M_{initiator} \qquad (3.1)$$

Here, $M_{monomer}$ and $M_{initiator}$ are molecular weights of the DL-lactide and initiator, respectively.

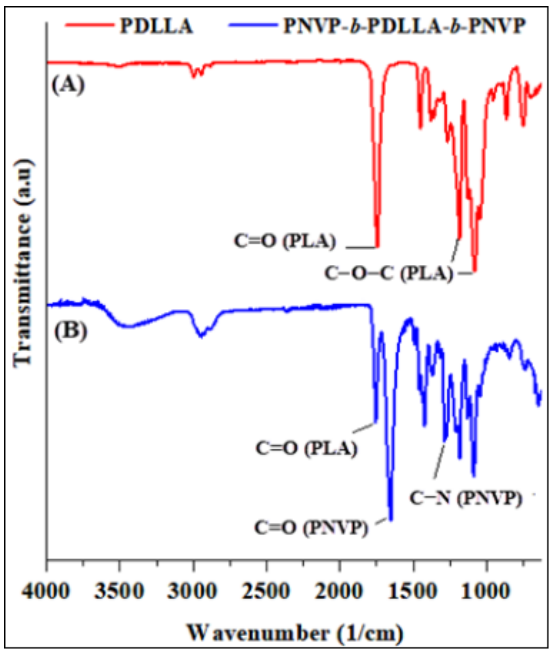


Figure 1. FTIR spectra of PDLLA diol (A) and PNVP-*b*-PDLLA-*b*-PNVP (B)

The theoretical number-average molecular weight M_n (theo.) was calculated based on (3.2)

$$M_n(\text{theo.}) = \frac{[M]}{[I]} M_{monomer}\ \text{Conversion\%} + M_{initiator} \quad (3.2)$$

In the second step, dibromoester functionalized PDLLA macroinitiator **(3)** was synthesized by esterifying PDLLA (2) end groups with 2-bromopropionyl bromide. ^{1}H NMR spectrum of **(3)** is displayed in Figure 2B. After the esterification of terminal hydroxyl groups of PDLLA, two novel signals appeared at 4.42 and 1.84 ppm. These signals were attributed to methine (H^g**)** and methyl (H^h) protons of bromo propionate end, respectively. The disappearance of the peak at 4.35 ppm, corresponding to the methine protons adjacent to the terminal hydroxyl end groups of the PDLLA end, indicates that the esterification was successful. The peaks of methine and methyl protons of the repeating unit of PDLLA ppm were detected at 5.18 (H^e) and 1.57 (H^f), respectively. The methylene protons of (1) gave signals at 4.23 (H^d) and 2.64 (H^c) ppm as a triplet, while the other methylene protons (H^b) were observed at 3.71 ppm as a singlet.

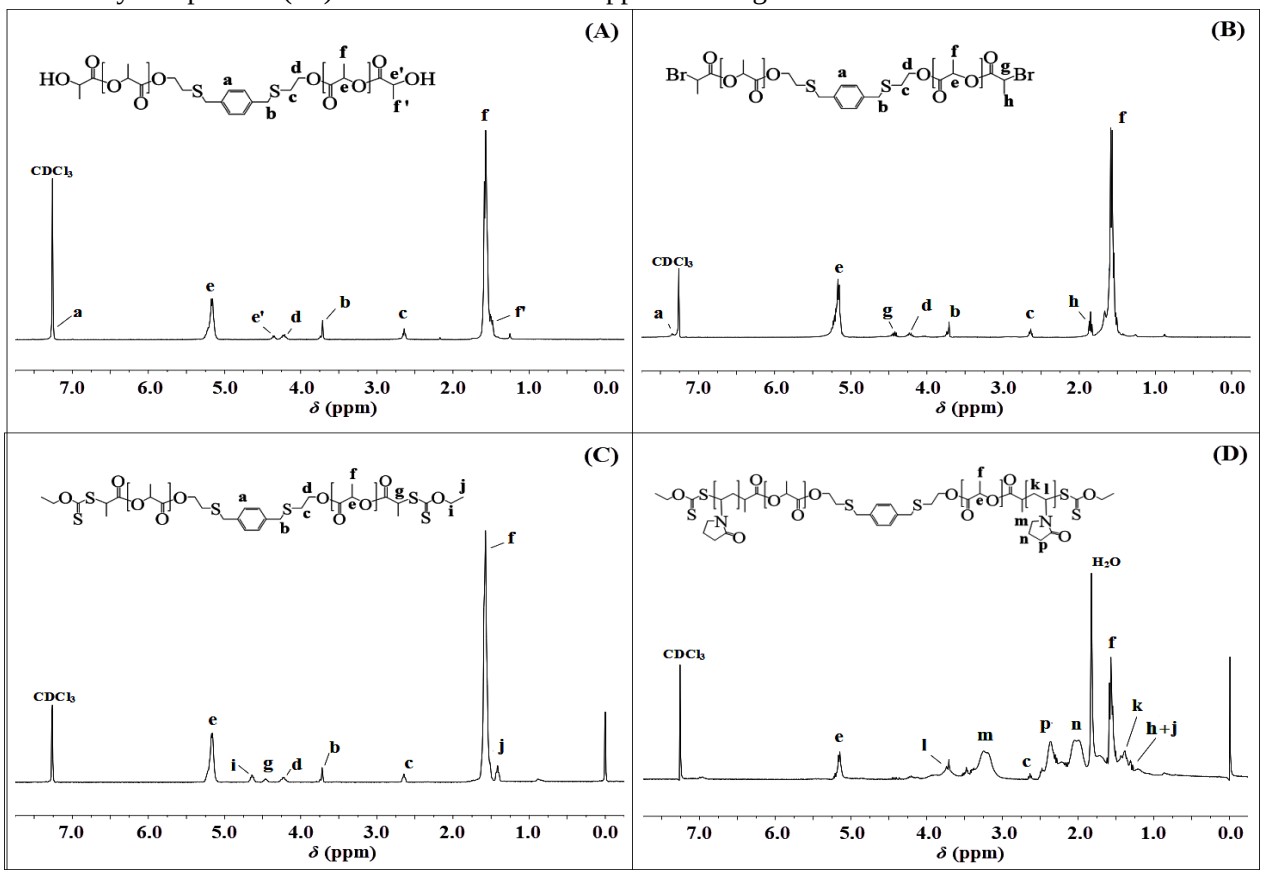


Figure 2. ^{1}H NMR spectra of PDLLA-diol (A), PDLLA macroinitiator (B), PDLLA macro-CTA (C), and PNVP-*b*-PDLLA-*b*-PNVP block copolymer (D)

In the third step, PDLLA macro-CTA **(4)** was synthesized via substitution reaction of **(3)** with KEX. ^{1}H NMR spectrum of **(4)** is displayed in Figure 2C. Following the substitution reaction, two novel signals appeared at 4.64 and 1.42 ppm, corresponding to methyl (H^i**)** and methyl (H^j) protons of the xanthate end group. The peaks of methine and methyl protons of the PDLLA at 5.17 (H^e) and 1.57 (H^f) ppm were detected, respectively. The signals for the methylene protons of initiator fragment **(1)** in macro-CTA agree with the values recorded in the previous spectrum (Figure 2B).

Finally, PNVP-*b*-PDLLA-*b*-PNVP triblock copolymer **(5)** was synthesized RAFT polymerization of NVP using **(4)**. FTIR spectrum of **(5)** is shown in Figure 1B. As seen in the FTIR spectrum of **(5)**, the bands corresponding to PVP can be detected by the appearance of C=O and C−N peaks at 1650 and 1289 cm^{-1}, respectively. The broad band around 3435 cm^{-1} corresponds to the hydroxyl group arising from absorbed

moisture due to the hygroscopic nature of PNVP. In addition to these values, of C=O band at 1755 cm^{-1}, attributable to the PDLLA block, supports block copolymer formation.

^{1}H NMR spectrum of **(5)** is shown in Figure 2D. In Figure 2D, methylene (H^m), (H^p), and (H^n) protons of the pyrrolidone ring, corresponding to the characteristic peaks of the PVP backbone, were detected at 3.55–3.02, 2.54–2.22, and 2.22–1.81 ppm respectively. The other peaks of methine proton (H^l) of the PNVP chain appeared at 4.02–3.55 ppm. The observation of the peak at 5.19 of the methine (H^e) protons of PDLLA block and the overlapped peaks at 1.81–1.28 ppm methylene (H^k) of PNVP block with methyl (H^f) protons of PDLLA indicates the formation of block copolymer.

M_n (NMR) of PNVP-*b*-PDLLA-*b*-PNVP was calculated by comparing the peak integrals derived from the methine proton peaks of PNVP (δ =3.24 ppm, peak m) and the methine proton peaks of PDLLA (δ = 5.19 ppm, peak e in Figure 2D) according to (3.3) and (3.4)

$$M_n\,(\text{NMR}) = DP_{NVP}\, M_{monomer} + M_{n,PDLLA} \tag{3.3}$$

$$DP_{PNVP} = \frac{I_m}{I_e}\, DP_{PDLLA} \tag{3.4}$$

Here, DP_{PNVP} and DP_{PDLLA} are degrees of polymerization for PNVP and PDLLA segments, respectively. $M_{monomer}$ is also the molecular weight of the NVP.

In vitro cytotoxicity studies in cancer cell lines are essential to evaluate new drug carriers' potential activity and toxicity [26]. XTT analysis was performed to investigate the cytotoxic effect of synthesized amphiphilic block copolymer on cervical cancer (HeLa) cells. As a result of the cytotoxicity analysis showed cell death in tissues at 800 and 600 ug/mL, but no toxic effect of block copolymer was observed below 450 ug/mL (%100 cell proliferation) on the cervical cancer (HeLa) cell line. The results obtained are shown in Figure 3.

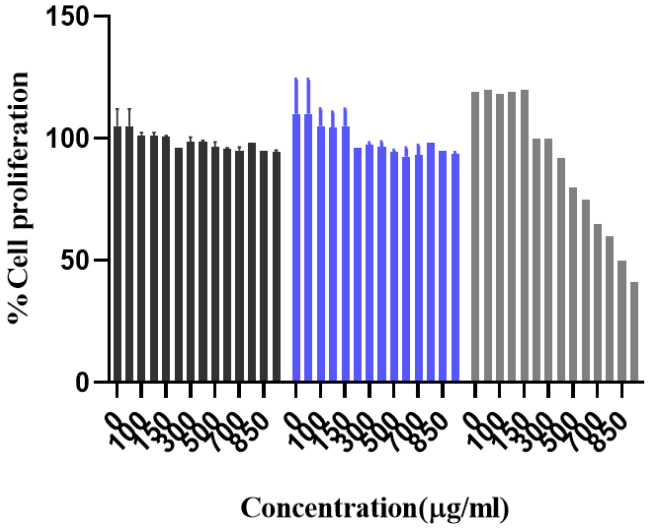


Figure 3. Cytotoxicity assay of PNVP-*b*-PDLLA-*b*-PNVP block copolymer, DMSO(%05) and Control (not treated) in HeLa cells

According to the wound assay results obtained, it is seen that the HeLa cells in the control group started to close the wound at the 48th hour (Figure 4). In the wound assay evaluation of block copolymer, it was applied to the cells at the doses used in the cytotoxicity analysis. In HeLa cell lines applied at 800 ug/mL (Figure 5A), a decrease in the wound closure rate and death of cells were observed. However, at the 400 ug/mL dose, the wound closure rate was comparable to the control group (Figure 5B).

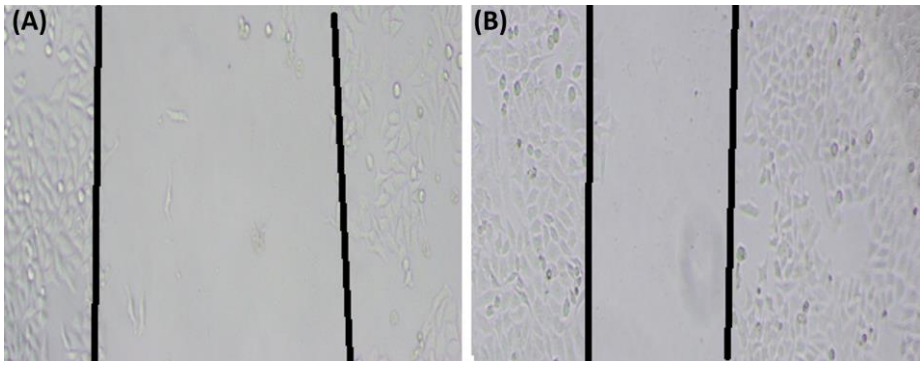


Figure 4. Control group (Noncopolymer treated) HeLa cell line (A: 24 h; B 48 h)

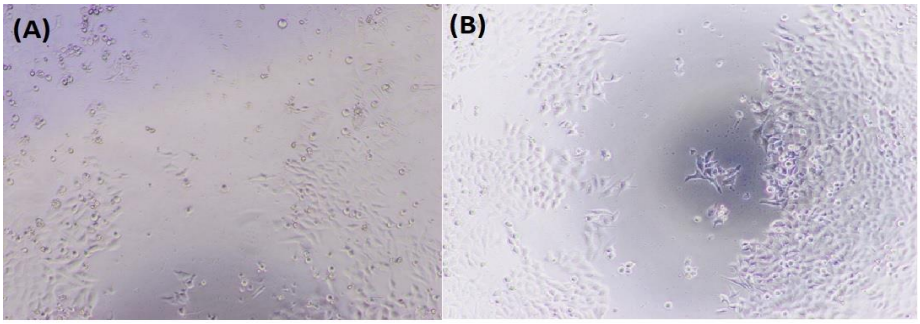


Figure 5. A; PNVP-*b*-PDLLA-*b*-PNVP block copolymer treated cells (48 h) (800 ug/mL), B; PNVP-*b*-PDLLA-*b*-PNVP block copolymer treated cells (48 h) (400 ug/mL)

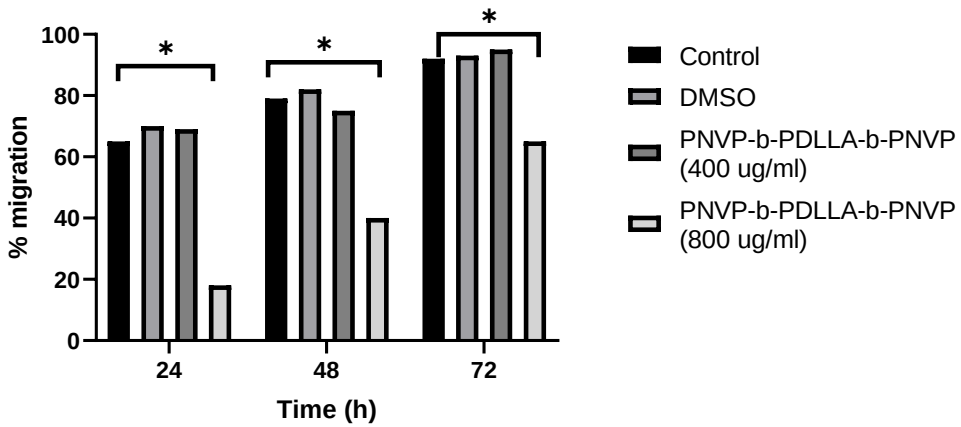


Figure 6. Graphical representation of migration assay

In Figure 6, the control group, DMSO treated group, PNVP-b-PDLLA-b-PNVP block copolymer (400 ug/mL), and PNVP-b-PDLLA-b-PNVP block copolymer (800 ug/mL) were applied at 24, 48, and 72 hours. As a result of the analysis, the dose of PNVP-b-PDLLA-b-PNVP block copolymer (800 ug/mL) was found to be statistically significant compared to the other groups ($p < 0.05$).

According to invasion assay results, the control group of HeLa cells can be seen for 48 h (Figure 7A). In the invasion assay evaluation of block copolymer, it was applied to the cells at the doses used in the cytotoxicity analysis. It was observed that the invasion rate of the cells decreased in HeLa cell lines treated with 800 ug/mL (Figure 7B). However, at the dose of 400 ug/mL, it was determined that the rate of invasion of the cells was seen to be parallel to the control group (Figure 7C).

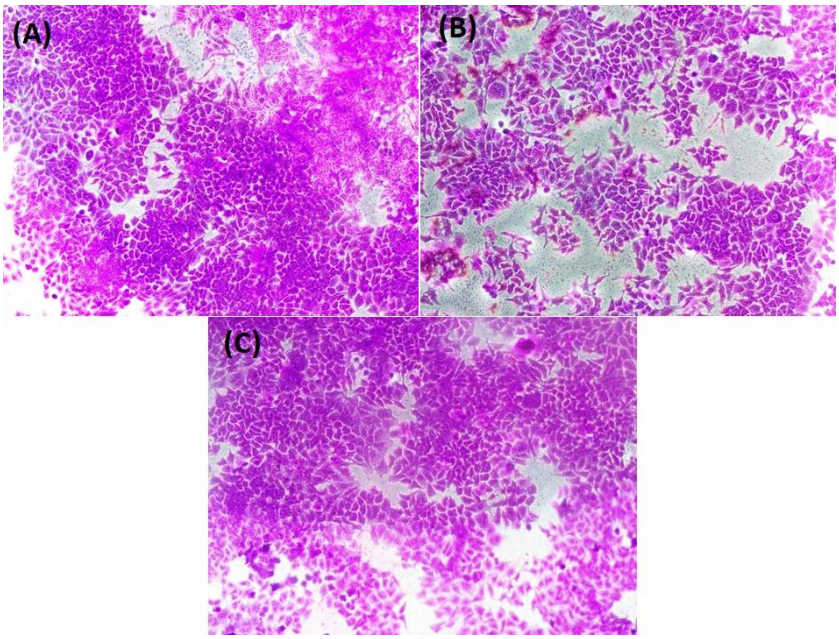


Figure 7. A. Control group HeLa cells (48h) (Noncopolymer treated), B. PNVP-*b*-PDLLA-*b*-PNVP block copolymer treated cells (48 h) (800 ug/mL), C. PNVP-*b*-PDLLA-*b*-PNVP block copolymer treated cells (48 h) (400 ug/mL)

This polymer class offers numerous advantages as drug delivery depots: biocompatibility, low toxicity, and metabolites easily processed or excreted [27]. This study showed no toxic effect of the block copolymer below 600 µg/mL on the HeLa cervical cancer cell line. This situation is also seen in different polymeric or dendrimer structures [28, 29]. In future animal experiments, drug delivery studies will be conducted using doses below the specified dose.

In Figure 3, the toxicity analysis of the cell group without any substance, the DMSO group, and the copolymer were evaluated with XTT analysis. DMSO was evaluated as a positive control. PNVP-*b*-PDLLA-*b*-PNVP block copolymer was dissolved in DMSO. The small molecule DMSO shows polarity, aprotic nature, and amphiphilicity. This solvent is widely used in medical applications and scientific research because it dissolves numerous polar and nonpolar molecules. However, it has a toxic effect on living cells. There are many studies to determine the non-toxic dose of DMSO on living organisms. Studies indicate that DMSO can be widely used as a solvent for pharmacological agents and is usually used at concentrations between 0.5% and 1.5% [30, 31].

4. Conclusion

In this work, an ABA-type block copolymer (PNVP-*b*-PDLLA-*b*-PNVP) was successfully synthesized via the combination of ROP and xanthate-mediated RAFT polymerization using novel bifunctional PDLLA-based RAFT macro-CTA bearing *p*-xylene-bis(2-mercaptoethyloxy) core. The synthesis utilized a novel bifunctional PDLLA-based macroinitiator, created using a bifunctional initiator, *p*-xylene-bis (1-hydroxy-3-thia-propane), not previously used in polymerization of D,L-LA. The molecular structures of the compounds were confirmed using FTIR and ^{1}H NMR methods. The M_n of the obtained polymers was calculated by comparing the peak integrals of related NMR spectra and agreed with theoretical values. In vitro analyses, synthesized block copolymer's cytotoxicity and metastatic properties were investigated in cervical cancer cell lines at 0-800 ug/mL doses. According to the obtained analysis results, it was observed that the block copolymer had toxic properties on cells at doses above 600 ug/mL and that the block copolymer at doses below 600 ug/mL could be used in future studies on drug delivery studies of cancer treatments.

Author Contributions

The author read and approved the final version of the paper.

Conflict of Interest

The author declares no conflict of interest.

Ethical Review and Approval

No approval from the Board of Ethics is required.

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References

[1] S. Rawal, M. M. Patel, *Threatening cancer with nanoparticle aided combination oncotherapy*, Journal of Controlled Release. 301 (2019) 76-109.

[2] L. Zhao, N. Li, K. Wang, C. Shi, L. Zhang, Y. Luan, *A review of polypeptide-based polymersomes*, Biomaterials 35 (4) (2014) 1284-1301.

[3] T. Biela, A. Kowalski, J. Libiszowski, A. Duda, S. Penczek, *Progress in polymerization of cyclic esters: mechanisms and synthetic applications*, Macromolecular symposia 240 (1) (2006) 47-55

[4] D. Babikova, R. Kalinova, I. Zhelezova, D. Momekova, S. Konstantinov, G. Momekov, D. Ivaylo, *Functional block copolymer nanocarriers for anticancer drug delivery*, RSC Advances 6 (88) (2016) 84634-84644.

[5] T. K. Dash, V. B. Konkimalla, *Polymeric modification and its implication in drug delivery: Poly-ε-caprolactone* (*PCL*) *as a model polymer*, Molecular Pharmaceutics 9 (9) (2012) 2365-2379.

[6] M. Hussain, S. M. Khan, M. Shafiq, N. Abbas, *A review on PLA-based biodegradable materials for biomedical applications*, Giant 18 (2024) Article Number 100261 31 pages.

[7] C-F. Yu, S-P. Rwei, S-J. Yang, W-C. Tsen, L-H. Lin, *Synthesis and characterization of poly (DL-lactide) containing fluorene structures*, Polymers 15 (11) (2023) Article Number 2555 16 pages.

[8] P. Franco, I. De Marco, *The use of poly (N-vinyl pyrrolidone) in the delivery of drugs: A review*, Polymers 12 (5) (2020) Article Number 1114 29 pages.

[9] D. Gigmes, T. Trimaille, *Advances in amphiphilic polylactide/vinyl polymer based nano-assemblies for drug delivery*, Advances in Colloid and Interface Science 294 (2021) Article Number 102483 13 pages.

[10] D. Le Garrec, S. Gori, L. Luo, D. Lessard, D. C. Smith, M.-A. Yessine, M. Ranger, J-C. Leroux, *Poly (N-vinylpyrrolidone)-block-poly (D, L-lactide) as a new polymeric solubilizer for hydrophobic anticancer drugs: in vitro and in vivo evaluation*, Journal of Controlled Release 99 (1) 2004 83-101.

[11] X. Liu, Y. Xu, Z. Wu, H. Chen, *Poly (N-vinylpyrrolidone)-modified surfaces for biomedical applications*, Macromolecular Bioscience 13 (2) (2013) 147-154.

[12] R. K. Mishra, M. Datt, A. K. Banthia. *Synthesis and characterization of pectin/PVP hydrogel membranes for drug delivery system*. Aaps Pharmscitech 9 (2008) 395-403.

[13] G. Gaucher, M. Poreba, F. Ravenelle, J-C Leroux, *Poly (N-vinyl-pyrrolidone)-block-poly (D, L-lactide) as polymeric emulsifier for the preparation of biodegradable nanoparticles*, Journal of Pharmaceutical Sciences 96 (7) (2007) 1763-1775.

[14] F. Ravenelle, S. Gori, D. Le Garrec, D. Lessard, L. Luo, D. Palusova, J. R. Sneyd, D. Smith, *Novel lipid and preservative-free propofol formulation: properties and pharmacodynamics*, Pharmaceutical Research 23 (2008) 313-319.

[15] H. Feng, X. Lu, W. Wang, N-G. Kang, J. W. Mays, *Block copolymers: Synthesis, self-assembly, and applications*, Polymers 9 (10) (2017) Article Number 494 31 pages.

[16] K. Ramesh, R. K. Gundampati, S. Singh, K. Mitra, A. Shukla, M. V. Jagannadham, D. Chattopadhyay, *Self-assembly, doxorubicin-loading and antibacterial activity of well-defined ABA-type amphiphilic poly (N-vinylpyrrolidone)-b-poly (d, l-lactide)-b-poly (N-vinyl pyrrolidone) triblock copolymers.* RSC Advances 2016 6 (31) 25864-25876.

[17] N. Kang, J-C. Leroux, *Triblock and star-block copolymers of N-(2-hydroxypropyl) methacrylamide or N-vinyl-2-pyrrolidone and d, l-lactide: synthesis and self-assembling properties in water*, Polymer 45 (26) (2004) 8967-8980.

[18] L. Xiong, H. W. Jiang, D. Z. Wang, *Synthesis, characterization and degradation of poly (dl-lactide)-block-polyvinylpyrrolidone-block-poly (dl-lactide) copolymers*, Journal of Polymer Research 2009 16:191-197.

[19] S. K. Hira, K. Ramesh, U. Gupta, K. Mitra, N. Misra, B. Ray, P. P. Manna, *Methotrexate-loaded four-arm star amphiphilic block copolymer elicits CD8+ T cell response against a highly aggressive and metastatic experimental lymphoma*, ACS Applied Materials & Interfaces 7 (36) (2015) 20021-20033.

[20] D. D. Perrin, W. L. Armarego, Purification of laboratory chemicals, 2nd Edition, Pergamon Press, Oxford, 1988.

[21] M. Mısır, S. S. Yılmaz, A. Bilgin, *Synthesis and characterization of ABA-type triblock copolymers using novel bifunctional PS, PMMA, and PCL macroinitiators bearing p-xylene-bis (2-mercaptoethyloxy) core*, Polymers 15 (18) (2023) Article Number 3813 25 pages.

[22] K. Kamenova, E. Haladjova, G. Grancharov, M. Kyulavska, V. Tzankova, D. Aluani, K. Yoncheva, S. Pispas, P.Petrov, *Co-assembly of block copolymers as a tool for developing novel micellar carriers of insulin for controlled drug delivery*, European Polymer Journal 104 (2018) 1-9.

[23] L. M. Shaw, Tumor cell invasion assays, Vol 294 of Cell Migration. Methods in Molecular Biology, 2005, Humana Press, pp. 97-105.

[24] F. Nederberg, E. F. Connor, M. Möller, T. Glauser, J. L. Hedrick, *New paradigms for organic catalysts: The first organocatalytic living polymerization.* Angewandte Chemie (International ed in English). 40 (14) (2001) 2712-2715.

[25] J. Burke, R. Donno, R. d'Arcy, S. Cartmell, N. Tirelli, *The effect of branching (star architecture) on poly (D, L-lactide)(PDLLA) degradation and drug delivery*, Biomacromolecules 18 (3) (2017) 728-739.

[26] A. Ali, R. Bhadane, A. A. Asl, C-E. Wilén, O. Salo-Ahen, J. M. Rosenholm, K.K. Bansal, *Functional block copolymer micelles based on poly (jasmine lactone) for improving the loading efficiency of weakly basic drugs*, RSC Advances 12 (41) (2022) 26763-26775.

[27] M. Stanković, C. Hiemstra, H. de Waard, J. Zuidema, R. Steendam, H.W. Frijlink, W. L. Hinrichs, *Protein release from water-swellable poly(D,L-lactide-PEG)-b-poly(ϵ-caprolactone) implants*, International Journal of Pharmaceutics 480 (1-2) (2015) 73–83.

[28] K. Khodadust, P. Mutlu, S. Yalcın, G. Unsoy, U. Gunduz, *Doxorubicin loading, release, and stability of polyamidoamine dendrimer-coated magnetic nanoparticles.* Journal of Pharmaceutical Sciences 102 (6) (2013) 1825-1835.

[29] K. Khodadust, G. Unsoy, S. Yalcın, G. Gunduz, U. Gunduz, *PAMAM dendrimer-coated iron oxide nanoparticles: synthesis and characterization of different generations*, Journal of Nanoparticle Research 15 (2013) 1-13.

[30] C. Yuan, J. Gao, J. Guo, L. Bai, C. Marshall, Z. Cai, L. Wang, M. Xiao, *Dimethyl sulfoxide damages mitochondrial integrity and membrane potential in cultured astrocytes*, PloS One 9 (9) (2014) Article Number e107447.

[31] F. Hajighasemi, S. Tajic, *Assessment of cytotoxicity of dimethyl sulfoxide in human hematopoietic tumor cell lines.* Iranian Journal of Blood and Cancer 9 (2) (2017) 48-53.