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Article

Synthesis and Characterization of Biodegradable Amphiphilic Star and Y-Shaped Block Copolymers as Potential Carriers for Vinorelbine

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Abstract: Two amphiphilic block copolymers using hydrophobic poly(ε-caprolactone) (PCL) and hydrophilic poly(ethylene glycol) (PEG) were successfully synthesized. One of them is an (A-*b*-B)$_4$ type star polymer [(PCL-*b*-PEG)$_4$] and the other one is a Y-shaped PEG–(PCL)$_2$. A star-shaped polymer (PCL-*b*-PEG)$_4$ was prepared by ring-opening polymerization (ROP) of ε-caprolactone continued by click reaction of (PCL-azide)$_4$ and PEG-alkyne. The synthesis of Y-shaped PEG–(PCL)$_2$ block copolymer was carried out via Diels-Alder click reaction of a furan protected maleimide end-functionalized PEG (PEG-MI) with an anthracene end-functionalized PCL following the ROP of ε-caprolactone. The characterization of micelles is carried out using both materials in aqueous media as drug delivery vehicles, which showed satisfying results and enhanced the cytotoxic effect of the

anti-cancer drug vinorelbine (VLB). However, micelles consisted of Y-shaped unimers were found to be more convenient for delivery of hydrophobic drugs such as VLB because they formed in lower concentration, carrying a higher amount of drugs and owing a monomodal distribution. We concluded that the free tails of hydrophobic chains in Y-shaped block copolymer facilitate the assembly of amphiphilic material in water to form micelles.

Keywords: polymeric drug delivery systems; micelle; nano formulation; targeted cancer therapy; vinorelbine; amphiphilic block copolymer; star-shaped polymer; Y-shaped polymer

1. Introduction

Continuous efforts are being made to develop new nanosized drug delivery systems to enhance the therapeutic effects of traditional drugs and to overcome the side effects associated with them. Biodegradable and biocompatible polymeric materials have received widespread attention over other materials for developing new delivery vehicles because of their convenient and easily tailored structure for chemical synthesis and their favorable degradation kinetics. Amphiphilic block copolymers with the ability of forming self-assembled micelles in aqueous solutions have received the most attention [1,2]. The spherical shape observed most often for micelles consists of a core part made of the hydrophobic block and a shell part made by hydrophilic block. Not all micelles are spherical in shape. The structures are determined by insoluble/soluble ratio. The very well known equation for this ratio is:

$$\rho = \frac{v}{a_0 d} \quad (1)$$

The dimensionless packing parameter ρ was originally developed for small amphiphiles in water but can be generalized and used to define the relative size of the non-soluble region of a copolymer. The balance between hydro-phobic and hydrophilic interactions gives rise to an optimal surface area of the hydrophobic block at the interface between the hydrophobic and hydrophilic blocks (a_0). This, together with the length and the volume of the non-soluble domain, contributes to the packing parameter. v is the volume and d is the length of the solvent-phobic block. The packing parameter is the ratio between the insoluble chain molecular volume and the volume actually occupied by the copolymer in the assembly. As a general rule, spherical micelles are formed when $\rho \leq 1/3$, cylindrical micelles are formed at $1/3 < \rho \leq 1/2$ and membranes arise when $1/2 < \rho \leq 1$ [3].

The spherical micelle is of interest to drug delivery research since it is able to entrap the non-soluble hydrophobic drug molecules in the core part. In addition, the hydrophilic part (e.g., PEG) which generally carries polar functional groups is able to entrap water molecules between its tails by making hydrogen bonds. The entrapped water molecules all around the micelle give a huge water droplet appearance to the drug delivery system. As a result the drug delivery system carries the hydrophobic drug molecule in the blood circulation at concentrations many times above its water

solubility while the whole particle is undetectable by reticuloendothelial system and this property prolongs the blood circulation period of the payload.

Enhanced permeability and retention (EPR) effect is the effect by which nano drug delivery systems penetrate through the enhanced gaps between vein epithelial cells and enter the tumor tissue [4]. This is what is referred to as passive targeting to the cancer site and is the most known phenomena targeted drug delivery studies and is the most utilized tool for targeted drug delivery. The general explanation that is given for this phenomenon is that, in order for tumor cells to grow quickly, they must stimulate the production of blood vessels. Tumor cell aggregates of size as small as 150–200 μm, start to become dependent on blood supply carried out by neovasculature for their nutritional and oxygen supply. These newly formed tumor vessels are usually abnormal in form and architecture. They are poorly aligned defective endothelial cells with wide fenestrations, lacking a smooth muscle layer, or innervation with a wider lumen. Furthermore, tumor tissues usually lack effective lymphatic drainage. All these factors will lead to abnormal molecular and fluid transport dynamics, especially for macromolecular drugs. Namely, this phenomenon was coined "enhanced permeability and retention (EPR)-effect" of macromolecules and lipids in solid tumors [5]. Taking advantage of EPR effect becomes possible only by using particles with the hydrodynamic diameter less than 200 nm [6]. Controlling the chain length of amphiphilic block copolymers and synthesizing the particles with desired diameter to use the EPR effect is another attractive feature of using these materials for targeted drug delivery area.

However, it has been shown that utilizing all the above-mentioned advantages of polymeric drug delivery systems is dependent on the morphology of unimers, the assembly of which results in the formation of micelles [7]. The major factor that influences the performance of polymeric micelles is their stability upon dilution in the blood stream. Critical micellar concentration (CMC) is the concentration above which micelles start to form. The lower the CMC the stronger the micelles upon dilution. Until now the lowest CMC value for micellar particles made of hyper branched amphiphilic materials have been obtained using star-shaped block co-polymers.

Star-shaped block copolymers with a core consisting of hydrophobic inner branches and multiple hydrophilic poly(ethylene glycol) (PEG) tails have been synthesized in the range of 3 to 32 by Wang *et al.* [7,8], Zheng *et al.* [9] and some other groups [10–16]. The unimers with arm numbers of 16 or 32 have shown the high stability, drug delivery efficacy and physicochemical properties compared to unimers with a smaller arm number. Star-shaped polymers with a smaller arm number have been found to either form a relatively loose outer PEG shell, or to have a weak capacity of carrying drug molecules [7,17]. Although poly(ε-caprolactone) (PCL) and PEG are very well known biodegradable and biocompatible polymeric materials, they are not allowed to be used above a certain concentration because of toxicity considerations [18]. The important point is to synthesize drug delivery systems with optimal properties and minimal toxicity.

In this study we attempted to synthesize and to study the self-assembly of star and Y-shaped block copolymers using click chemistry to attach hydrophobic drugs. The star-shaped polymers were synthesized with only four arms to achieve all above mentioned performance criteria together with high drug delivery opportunity and less toxicity.

Y-shaped block copolymers are another interesting vehicle for drug delivery since they exhibit different micellization behavior (especially stability) compared to the traditional amphiphilic materials [19–22]. However, it is still a challenge to prepare biodegradable Y-shaped block

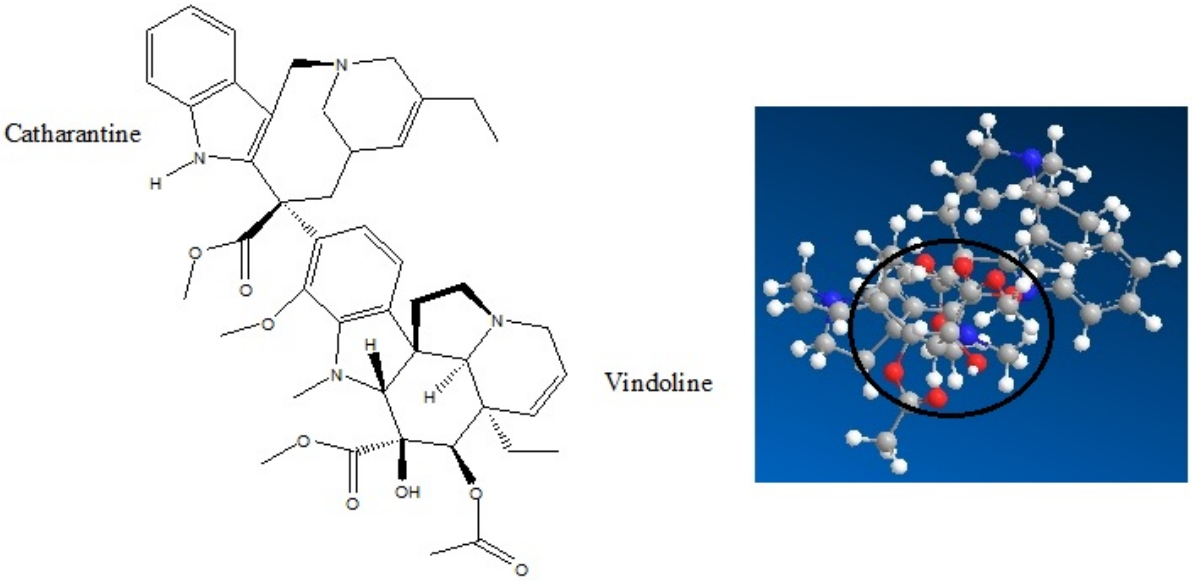
copolymers. Herein, we also report the first synthesis and characterization of the Y-shaped block copolymer consisting of PCL and PEG.

The incorporation of a very well-known, hydrophobic, anti-cancer agent Vinorelbine (VLB), prone to serious side effects was studied with both micelles consist of star and Y-shaped block copolymers.

VLB is currently used in salt form (Bitartarate) in chemotherapy under the commercial name; Navelbine. However, it has been reported to cause venous irritation, chemical phlebitis, localized rashes and urticarial at the site of injection [23]. Phlebitis is the major side effect of this drug and is believed to occur when vein epithelial cells contact with the drug on the molecular level. Based on this observation, encapsulating the drug in a micellar nano drug delivery system will provide protection of vein epithelial cells against contact with drug molecules and should result in a reduction in side effects of the chemotherapeutic agent. The reduced toxicity, together with passive targeting via the EPR effect, should result in an overall improved therapeutic effect.

Vinorelbine has an ideal structure to study the behavior and capacity of a newly synthesized drug delivery system. It is a dimeric compound and consists of catharanthine and vindoline moieties. Together with its poor water solubility, drawing its 3D structure and calculating the conformation in which the molecule is minimum energy level, shows that VLB carries almost all polar functionalities in a localized manner (Figure 1). In other words, vinorelbine is an amphiphilic molecule. Similar behavior was observed by others for another poorly soluble molecule, Amphotericin B [24]. Similarly, it has been found that when vinorelbine insertes phospholipid bilayers, the most favored topographical position of vinorelbine is at the interface of polar head groups and alkyl chains of lipid bilayers [25]. When a drug molecule carries all polar functionalities at one surface, this property helps it to incorporate with the hydrophobic core of the micelle with its other non-charged face. Charge distribution will lead to interactions with the polar shell part and the drug will be carried on the corona. Replacement of drug in the core of the micelle is a more firm way of drug delivery and causes a longer release period compared to replacement on the shell.

Figure 1. Structure of Vinorelbine and 3D illustration of its structure showing the localization of its functional groups.



2. Experimental Section

2.1. Materials and Instrumentation

ε-Caprolactone (ε-CL, 99%, Aldrich, Taufkirchen, Germany) was distilled from CaH_2 under vacuum. Poly(ethylene glycol monomethyl ether) (Me-PEG-OH) (M_n = 550 g/mol, Acros, Geel, Belgium) was dried by azeotropic distillation in toluene. 9-Anthracenemethanol (97%, Aldrich), *N*,*N*′-dicyclohexylcarbodiimide (DCC, 99%, Aldrich), 4-dimethylaminopyridine (DMAP, 99%, Acros), *p*-toluene sulfonyl chloride (*p*-TsCl, 99%, Aldrich) and CuBr (99.9%, Aldrich) were used as received. Dichloromethane (CH_2Cl_2) was purchased from Aldrich and used after distillation over P_2O_5. Tetrahydrofuran (THF; 99.8%, J.T. Baker, Deventer, The Netherlands) was dried and distilled over benzophenone-Na. Solvents unless specified here were purified by conventional procedures. Acetonitrile, methanol, trifluoro acetic acid (TFA, HPLC grade) and other solvents were bought from Fischer scientific (Geel, Belgium) and used as received without further purification. Vinorelbine (Cat# AL-4176) obtained from Altan Biochemicals (Orange, CT, USA), M_W: 778.932. Amicon® Ultra-4 Centrifugal Filter Tubes (Millipore, Darmstadt, Germany) and the dialysis membrane Spectra/Por 3 (molecular porous spectrum, MWCO 6000–8000), part no. 132720 and Spectra/Por 1 (molecular porous spectrum, MWCO 3500), part no. 132650 were purchased from Fisher Scientific. Fisher brand Syringe Filters nylon membrane; with pore size: 0.2 μm, (Catalog No: 09-719C) was used in filtration of polymeric micelles. MCF-7 cells (#HTB-22), fetal bovine serum, trypsin-EDTA and Eagle's Minimum Essential Medium (EMEM) with Earle's Balanced Salt System (BSS) were obtained from American Type Culture Collection (Manassas, VA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Aldrich (Cat# M2003).

Phosphate buffered saline (1× PBS, pH = 7.4) was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 800 mL of distilled H_2O and pH was adjusted to 7.4 with HCl. Final volume was fixed to 1 L by adding H_2O and subsequently sterilized by autoclave.

Particle size distribution and mean diameter of the prepared aqueous dispersions of VLB were determined by Dynamic Light Scattering (DLS) using a quasi-elastic light scattering using a NICOMP 380 Submicron Particle Sizer (Brookhaven Instrument, Holtsville, NY, USA) equipped with a 100 mW helium-neon laser at 658 nm and a temperature controlled cell holder. The concentration of VLB in supernatants was measured by reading the absorbance in ELISA Reader Spectrophotometer (Molecular Devices Spectro Max340 PC, Biberach an der Riss, Germany) and comparing with the previously recorded concentration curve. Vortex IKA MS3 basic and Ultrasonic Bath (Fisher Scientific FB 15051) used in uploading VLB to polymeric micelles. The conventional gel permeation chromatography (GPC) measurements were carried out with an Agilent instrument (Model 1100, Santa Clara, CA, USA) consisting of a pump, refractive index (RI), and ultraviolet (UV) detectors and four Waters Styragel columns (guard, HR 5E, HR 4E, HR 3, HR 2, Santa Clara, CA, USA), (4.6 mm internal diameter, 300 mm length, packed with 5 μm particles). The effective molecular weight ranges are 2000–4,000,000, 50–100,000, 500–30,000, and 500–20,000 g/mol, respectively. The triple detection GPC (TD-GPC) setup with an Agilent 1200 model isocratic pump, four Waters Styragel columns (guard, HR 5E, HR 4, HR 3, and HR 2), and a Viscotek TDA 302 triple detector including RI, dual laser light scattering (λ = 670 nm, at 90° and 7°), and a differential pressure

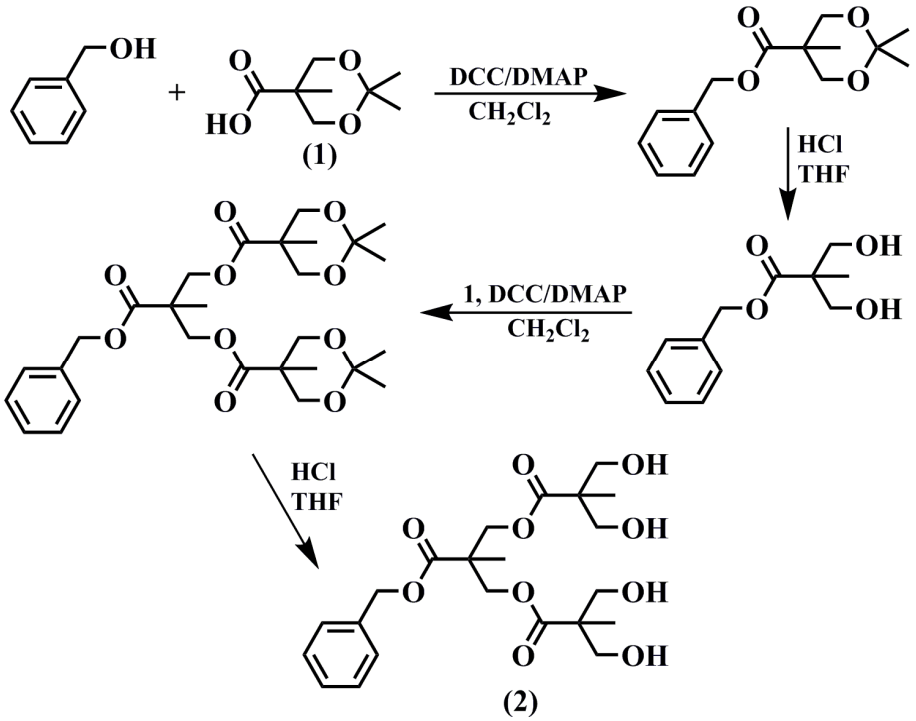
viscometer was conducted to measure the absolute molecular weights ($M_{n,TD\text{-}GPC}$ and $M_{w,TD\text{-}GPC}$) in THF with a flow rate of 0.5 mL/min at 35 °C. Three detectors were calibrated with a PS standard with narrow molecular weight distribution (M_n = 115,000 g/mol, M_w/M_n = 1.02, [η] = 0.519 dL/g at 35 °C in THF, d*n*/d*c* = 0.185 mL/g) provided by Viscotek company (Worcestershire, UK).

THF was used as eluent at a flow rate of 0.3 mL/min at 30 °C and toluene was used as an internal standard, to monitor the constant flow of the pump and for flow-correction as needed. The apparent molecular weights ($M_{n,GPC}$ and $M_{w,GPC}$) and polydispersities (M_w/M_n) were determined with a calibration based on linear PS standards using PL Caliber Software from Polymer Laboratories (Viscotek, Worcestershire, UK). UV-Vis spectra were recorded on a Shimadzu UV-1601 spectrophotometer (Kyoto, Japan) in CH_2Cl_2. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AC-250 spectrometer (250 MHz for proton, Coventry, UK).

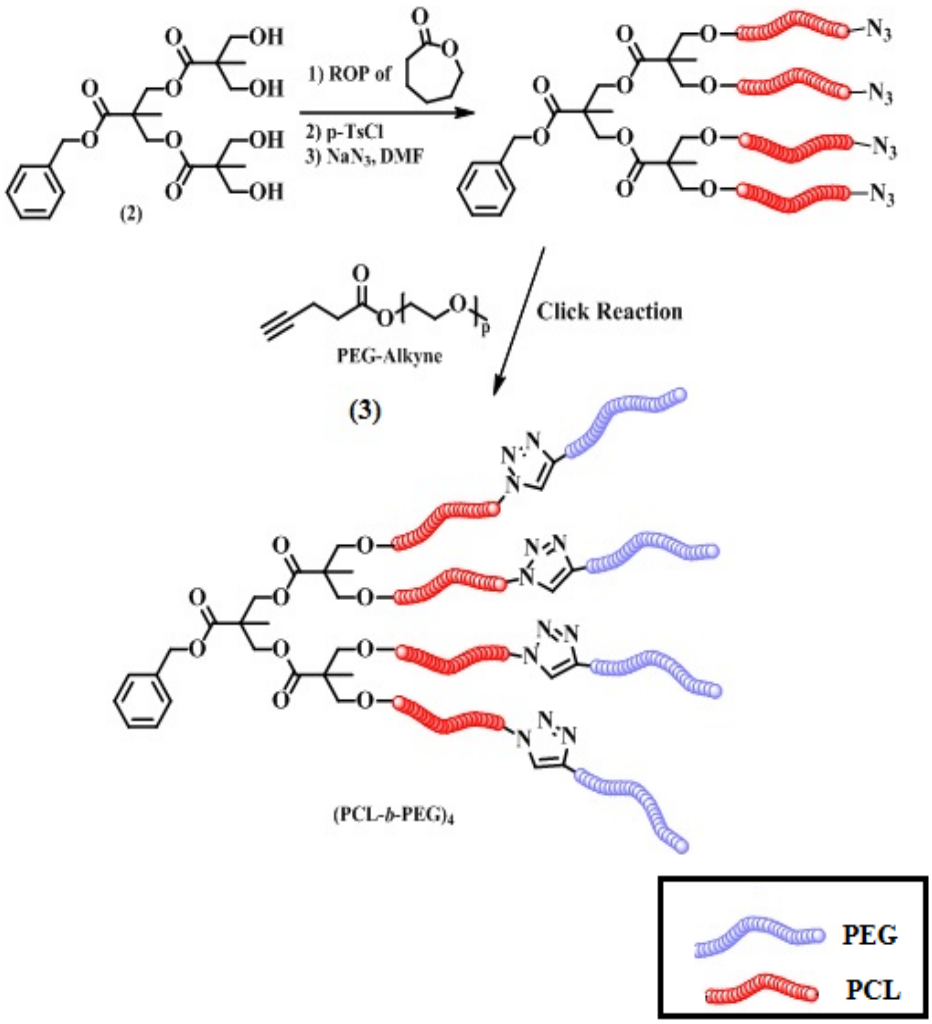
2.2. Syntheses and Polymerizations

2,2,5-Trimethyl-1,3-dioxane-5-carboxylic acid [26] **1**, mono alkyne terminated PEG (PEG-alkyne) [27] **3** (Scheme 1), anthracen-9-ylmethyl 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate [28] **4**, furan protected maleimide end-functionalized PEG (PEG-MI) [29] **5** (Scheme 2), were synthesized according to previously reported procedures.

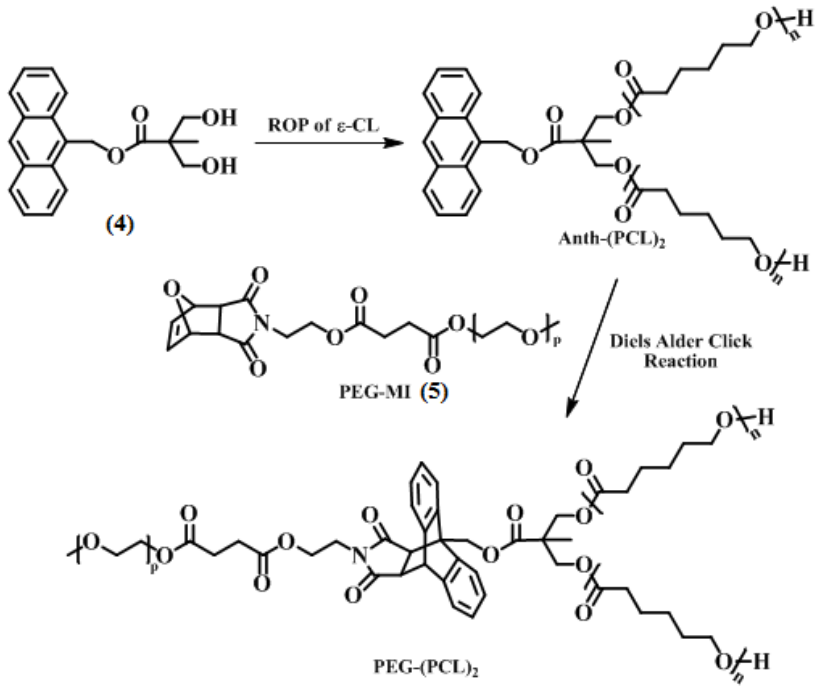
Scheme 1. Synthesis of (PCL-*b*-PEG)$_4$.



Scheme 1. *Cont.*



Scheme 2. Synthesis of PEG–$(PCL)_2$.



2.2.1. Synthesis of 2-(Benzyloxycarbonyl)-2-methylpropane-1,3-diyl bis[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate] (**2**)

Synthesis of 2-(benzyloxycarbonyl)-2-methylpropane-1,3-diyl bis[3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate] **2** was prepared within four steps according to literature procedure [30]. In first step, 2,2,5-trimethyl-1,3-dioxane-5-carboxylic acid **1** (3.54 g, 20.32 mmol) in 60 mL of dry CH_2Cl_2 along with compound benzyl alcohol (2.00 g, 18.50 mmol) and DMAP (1.12 g, 9.23 mmol) were added in the order mentioned, after stirring 5 min at room temperature, DCC (4.19 g, 20.32 mmol) dissolved in 25 mL CH_2Cl_2 was added. Reaction mixture was stirred at room temperature overnight, then filtered, and the solvent was evaporated. After that, ethyl acetate was added to the residue and was kept in a refrigerator overnight. After filtration of the urea byproduct, the solvent was removed, and the remaining product was purified by column chromatography over silica gel eluting with ethyl acetate/hexane (1/4) to give benzyl 2,2,5-trimethyl-1,3-dioxane-5-carboxylateas, a viscous colorless oil (Yield = 3.18 g, 65%). In second step, benzyl 2,2,5-trimethyl-1,3-dioxane-5-carboxylate (2.80 g, 10.59 mmol) was dissolved in THF (30 mL), and 1 M HCl solution (30 mL) was added. The reaction mixture was stirred overnight at room temperature and evaporated to remove THF. CH_2Cl_2 (100 mL) was added and the reaction mixture was extracted two times with distilled water. The organic layer was dried with anhydrous Na_2SO_4, and the solvent was removed *in vacuo*. The product (benzyl 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate) was obtained as a white solid (Yield = 2.32 g, 98%). **1** (4.46 g, 25.7 mmol) was dissolved in 60 mL of dry CH_2Cl_2 along with the compound; benzyl 3-hydroxyl-2-(hydroxymethyl)-2-methylpropanoate (2.3 g, 10.2 mmol) and DMAP (1.24 g, 8.71 mmol) were added in the mentioned order. After stirring for 5 min at room temperature, DCC (4.18 g, 20.3 mmol) dissolved in 25 mL CH_2Cl_2 was added. Reaction mixture was stirred at room temperature for overnight, then filtered and the solvent was evaporated. After that, ethyl acetate was added to remaining product and was stored in freezer overnight. After filtration of the urea byproduct, the solvent was removed, and the remaining product was purified by column chromatography over silica gel eluting with ethyl acetate/hexane (1:10) and then (1:9) to give 2-(benzyloxycarbonyl)-2-methylpropane-1,3-diyl bis(2,2,5-trimethyl-1,3-dioxane-5-carboxylate) as a viscous colorless oil. (Yield = 2.7 g, 46%).

In forth step, 2-(benzyloxycarbonyl)-2-methylpropane-1,3-diylbis(2,2,5-trimethyl-1,3-dioxane-5-carboxylate) (2.6 g, 4.84 mmol) was dissolved in THF (30 mL), and 1 M HCl solution (30 mL) was added. The reaction mixture was stirred overnight at room temperature and evaporated to remove THF. CH_2Cl_2 (100 mL) was added, and the reaction mixture was extracted two times with distilled water. The organic layer was dried with anhydrous Na_2SO_4, and the solvent was removed *in vacuo*. The product **2** was obtained as a white solid. (Yield = 2.10 g, 95%). ^{1}H-NMR (250 MHz, $CDCl_3$, δ) 7.40–7.30 (m, 5H, Ar*H*), 5.17 (s, 2H, Ph–C*H*$_2$O), 4.45 (d, 2H, CC*H*$_2$OC=O), 4.29 (d, 2H, CC*H*$_2$OC=O), 3.80–3.70 (m, 4H, CC*H*$_2$OH), 3.69–3.71 (m, 4H, CC*H*$_2$OH), 3.22 (bs, 4H, O*H*), 1.00 (s, 9H, C=OCC*H*$_3$). ^{13}C-NMR ($CDCl_3$, δ) 175.2 (CH$_2$O*C*=O), 172.1 (Ph-CH$_2$O*C*=O), 135.1 (Ar*C* of Ph), 128.6 (Ar*C* of Ph), 128.5 (Ar*C* of Ph), 128.3 (Ar*C* of Ph), 67.8 (C*C*H$_2$OH), 67.1 (Ph–*C*H$_2$O), 64.8 (C*C*H$_2$O), 49.6 (*C*CH$_3$), 46.4 (*C*CH$_3$), 18.0 (C*C*H$_3$), 17.4 (C*C*H$_3$).

2.2.2. Preparation of Azide End-Functionalized PCL Star Polymer (PCL-Azide)$_4$

The PCL star polymer was prepared by ROP of ε-CL (5.00 mL, 0.045 mol) in bulk using tin(II) 2-ethylhexanoate as a catalyst and **2** (0.205 g, 0.451 mmol) as an initiator at 110 °C for 14 h. Typical procedure is as follow; To a previously flame-dried Schlenk tube equipped with a magnetic stirring bar, the degassed monomer, catalyst, and initiator were added in the order mentioned. The tube was degassed with three freeze–pump–thaw cycles, left *in vacuo*, and placed in a thermostated oil bath. After polymerization, the mixture was diluted with THF, precipitated into an excess amount of methanol, and then isolated by filtration and dried at room temperature in a vacuum oven for 2 days. (*Conversion* = 60%). ($M_{n,theo}$ = 6250 g/mol; $M_{n,NMR}$ = 7200 g/mol; $M_{n,GPC}$ = 10,600 g/mol; M_w/M_n = 1.02; relative to polystyrene (PS) standards). ^{1}H-NMR ($CDCl_3$, δ): 7.40–7.30 (m, Ar*H*), 5.13 (s, Ph–CH_2O), 4.41 (s, CCH_2OC=O of **2**), 4.23 (bs, CCH_2OC=O of **2**), 4.06–4.01 (br, CH_2OC=O of PCL), 3.71–3.60 (m, CCH_2O of **2** and CH_2OH of PCL end group), 2.50–2.20 (br, C=OCH_2 of PCL), 1.80–1.00 (m, CH_2CH_2CH_2 of PCL and C=OCCH_3 of **2**).

The obtained PCL star polymer (2.4 g, 0.33 mmol) was dissolved in CH_2Cl_2 (100 mL). Then DMAP (0.080 g, 0.66 mmol) and Et_3N (1.85 mL, 0.0130 mol) were added to the reaction mixture. After stirring for 5 min at 0 °C, *p*-TsCl (2.5 g, 0.013 mol) dissolved in 20 mL CH_2Cl_2 was added to the reaction mixture and further stirred overnight (room temperature). The extraction of the organic layer was done by adding CH_2Cl_2 and water three times and drying over Na_2SO_4. The tosylated product was recovered after evaporation of excess amount of CH_2Cl_2 and precipitation in excess amount of cold MeOH. The polymer was dried for 24 h in a vacuum oven at 25 °C. (Yield = 2.42 g, 91%) ($M_{n,GPC}$ = 10,300 g/mol; M_w/M_n = 1.05; relative to PS standards). ^{1}H-NMR ($CDCl_3$, δ): 7.70 (bs, Ar*H*), 7.40–7.30 (m, Ar*H*), 5.13 (s, Ph-CH_2O), 4.41 (s, CCH_2OC=O of **2**), 4.23 (bs, CCH_2OC=O of **2**), 4.06–4.01 (br, CH_2OC=O of PCL), 3.71–3.60 (m, CCH_2O of **2** and CH_2OH of PCL end group), 2.50–2.20 (br, C=OCH_2 of PCL), 1.80–1.00 (m, CH_2CH_2CH_2 of PCL and C=OCCH_3 of **2**).

To afford an azide end-functionalized PCL star polymer, the tosylated PCL (2.2 g, 0.28 mmol) was dissolved in *N*,*N*-dimethyl formamide (DMF) (20 mL) and NaN_3 (1.1 g, 0.017 mol) was added. The reaction mixture was kept at room temperature for overnight. The extraction of organic layer and the process for obtaining the product is as described above (Yield = 1.97 g, 95%). ($M_{n,GPC}$ = 9950 g/mol; M_w/M_n = 1.05; relative to PS standards). ^{1}H-NMR ($CDCl_3$, δ): 7.40–7.30 (m, Ar*H*), 5.13 (s, Ph–CH_2O), 4.41 (s, CCH_2OC=O of **2**), 4.23 (bs, CCH_2OC=O of **2**), 4.06-4.01 (br, CH_2OC=O of PCL), 3.71–3.60 (m, CCH_2O of **2** and CH_2OH of PCL end group), 3.20 (bs, CH_2N$_3$), 2.50–2.20 (br, C=OCH_2 of PCL), 1.80–1.00 (m, CH_2CH_2CH_2 of PCL and C=OCCH_3 of **2**).

2.2.3. Synthesis of (A-*b*-B)$_4$ Type Star Polymer

Mono alkyne terminated PEG (PEG-alkyne**)** **3** was prepared via an esterification reaction of polyethyleneglycol monomethyl ether (Me-PEG), (M_n = 550 g/mol) with 4-pentynoic acid ($M_{n,theo}$ = 630 g/mol; $M_{n,NMR}$ = 685 g/mol; $M_{n,GPC}$ = 550 g/mol; M_w/M_n = 1.11; relative to PS standards). The general procedure for the preparation of (PCL-*b*-PEG)$_4$ via click reaction of (PCL-azide)$_4$ and PEG-alkyne is as follow: (PCL-azide)$_4$ (0.880 g, 0.119 mmol, based on $M_{n,NMR}$) was added to a 25 mL of Schlenk tube and dissolved in 3 mL of DMF. PEG-alkyne (0.359 g, 0.571 mmol based on $M_{n,theo}$) **3**

was added to the polymer solution along with PMDETA (0.10 mL, 0.48 mmol) and CuBr (0.068 g, 0.48 mmol). The reaction mixture was degassed by three freeze-pump-thaw (FPT) cycles, left in vacuum and allowed to stir at ambient temperature overnight. The solution was diluted with THF and filtered through a column filled with neutral alumina to remove copper complex. Volatiles were then removed and the crude polymer solutionwas purified by precipitation in cold methanol two times and dried in a vacuum oven at 40 °C for 24 h. (Yield = 1.03 g, 87%) ($M_{n,theo}$ = 9850 g/mol; $M_{n,GPC}$ = 8500 g/mol; M_w/M_n= 1.14; relative to PS standards). ^{1}H-NMR ($CDCl_3$, δ): 4.05–4.02 (br, C*H*$_2$OC=O of PCL), 3.62 (br, repeating unit of PEG), 3.4 (bs, OC*H*$_3$ of PEG), 2.2 (br, C=OC*H*$_2$ of PCL), 1.80–1.20 (m, C*H*$_2$C*H*$_2$C*H*$_2$ of PCL).

2.2.4. Synthesis of Anthracene End-Functionalized Poly(ε-caprolactone) [Anth-(PCL)$_2$]

A procedure for the synthesis of anthracen-9-ylmethyl 3-hydroxy-2-(hydroxymethyl)-2-methylpropanoate **4**, was described in our published procedure [28]. Anth-(PCL)$_2$ was prepared by ROP of ε-CL (5.0 mL, 0.047 mol) in bulk using tin(II)-2-ethylhexanoate as a catalyst and **4** (0.30 g, 0.94 mmol) as an initiator at 110 °C for 9 h. The degassed monomer, catalyst, and initiator were added to a previously flamed schlenk tube equipped with a magnetic stirring bar in the order mentioned. The tube was degassed with three FPT, left in argon, and placed in a thermo stated oil bath. After the polymerization, the mixture was diluted with THF, and precipitated into an excess amount of cold methanol. It was isolated by filtration and dried at 40 °C in a vacuum oven for 24 h. (*Conversion* = 85%; $M_{n,theo}$ = 4750 g/mol; $M_{n,NMR}$ = 5350 g/mol; $M_{n,GPC}$ = 6000 g/mol; M_w/M_n = 1.14; relative to PS standards). ^{1}H-NMR ($CDCl_3$, δ) 8.50 (s, Ar*H* of anthracene), 8.30 (d, Ar*H* of anthracene), 8.03 (d, Ar*H* of anthracene), 7.60–7.47 (m, Ar*H* of anthracene), 6.2 (s, *CH*$_2$–anthracene), 4.05–4.02 (br, C*H*$_2$OC=O of PCL), 3.60 (bs, C*H*$_2$OH of PCL end-group), 2.20 (br, C=OC*H*$_2$ of PCL), 1.80–1.20 (m, C*H*$_2$C*H*$_2$C*H*$_2$ of PCL).

2.2.5. Synthesis of Y-shaped PEG–(PCL)$_2$ Block Copolymer via Diels-Alder Click Reaction

The PEG-MI **5** was synthesized according to the protocols previously reported [29] ($M_{n,theo}$ = 2300 g/mol; $M_{n,NMR}$ = 2800 g/mol; $M_{n,GPC}$ = 3000 g/mol; M_w/M_n = 1.05; relative to PS standards). ^{1}H-NMR ($CDCl_3$, δ) 6.50 (s, C*H*=C*H* as bridge protons), 5.25 (s, –C*H*O, bridge-head protons), 4.23 (m, C*H*$_2$OC=O), 3.75–3.51 (m, OC*H*$_2$C*H*$_2$ repeating unit of PEG, C=ONC*H*$_2$, and C*H*$_2$–PEG repeating unit), 3.36 (s, PEG–OC*H*$_3$), 2.87 (s, C*H*–C*H*, bridge protons) 2.61–2.56 (m, C=OC*H*$_2$C*H*$_2$C=O).

A mixture of anth-(PCL)$_2$ (1.0 g, 0.19 mmol, based on $M_{n,NMR}$) and PEG-MI (0.51 g, 0.22 mmol, based on $M_{n,theo}$) **5** was dissolved in toluene (75 mL) in a 100 mL of two-necked round bottom flask. The mixture was bubbled with nitrogen to remove residual traces of oxygen. The solution was then placed in an oil-bath preheated at 110 °C and the reaction was carried out for 48 h in the dark. After that time, the solution was concentrated in a rotary evaporator and re-dissolved in THF followed by precipitation in cold methanol. The polymer precipitate was filtered and re-precipitated in cold methanol two more times. The obtained product was dried in a vacuum oven at 40 °C for 24 h. (Yield = 0.98 g, 68%). ($M_{n,theo}$ = 8000 g/mol; $M_{n,NMR}$ = 6450 g/mol; $M_{n,GPC}$ = 7700 g/mol; M_w/M_n = 1.10; relative to PS standards). ^{1}H-NMR ($CDCl_3$, δ) 7.20 (m, Ar*H* of cycloadduct), 5.40

(br, cycloadduct–CH_2OC=O), 4.83 (s, C*H*, bridge-head proton), 4.05–4.02 (br, CH_2OC=O of PCL), 3.62 (br, repeating unit of PEG and NCH$_2$CH_2OC=O), 2.4 (m, C=OCH_2CH_2C=O), 2.30 (br, C=OCH_2 of PCL), 1.80–1.20 (m, CH_2CH_2CH_2 of PCL).

2.3. Preparation of Micelles

2.3.1. Preparation of Free Polymeric Micelles

The star-(PCL-*b*-PEG)$_4$ and PEG–(PCL)$_2$ polymers were dissolved in DMF at a concentration around 100 mg/mL. The mixture was stirred overnight at room temperature. The day after, water was added in a drop wise manner. Addition of water was continued until the ratio of 67/33 (water/DMF) was achieved. After one more night of stirring the mixture was dialyzed against double distilled water using Spectra/Por 1 Molecular porous Dialysis Membrane. The water was exchanged at first, second, third, sixth and twelfth hours [9,17,31,32]. Obtained micelles were filtered using Fisher brand Syringe Filters with a nylon membrane with pore size: 0.2 μm.

Its noteworthy that the amount of water that must be added to DMF solution of polymers prior to dialysis has been determined by trying different ratios of water/DMF and the ratio 67/33 water/DMF has been found to afford the polymeric micelles with the best size and stability.

Filtered micelles were lyophilized and re-hydrated prior to preparation of formulations or characterization assays using Phosphate Buffer Saline (PBS) (1× PBS, pH = 7.4).

2.3.2. Preparation of Drug Loaded Polymeric Micelles and Determination of Vinorelbine Concentration Associated with Polymeric Micelles, Using Elisa Reader Spectrophotometer

Solid Extraction: A solid film of Vinorelbine (VLB) was obtained by dissolving certain amounts of it in MeOH and evaporating under vacuum. A pre-prepared polymer micelle solution (by using Dialysis) was agitated with VLB for 15 min [Vortex (5 min) and Ultrasonic Bath, (10 min)] and shaken mechanically overnight. The day after drug loaded micelles were incubated in 25 °C for 2 h [9,32].

To determine the saturated amount of VLB associated with polymeric micelles, above mentioned solid extraction method was followed using increasing concentrations of VLB while the concentration of polymeric micelles were kept constant at ~CMC × 1000 and ~CMC × 5000 in case of using star and Y shape polymers respectively. The prepared formulations were centrifuged at 6000 rpm for 30 min. The supernatants transferred to clean holders by filtration through 0.2 μm nylon syringe filters and diluted 10 times with methanol to prevent any aggregation of the drug in PBS. Previously we had prepared the concentration curve of VLB by reading UV absorbance at 286 nm.

The concentration of VLB in supernatants was measured by reading the absorbance in ELISA Reader Spectrophotometer (Molecular Devices Spectro Max340 PC) and comparing with the concentration curve.

As a second trial, centrifuge tubes with filters were used [33]. Dispersions of different concentrations of VLB in the above-mentioned constant concentrations of polymeric micelles (1 mL) were prepared and transferred to centrifuge tubes with filters. The optimum centrifugation time was calculated based on the radius of the centrifuge tube holder. A short period, 2 min at 2700 RCF (*g*), was used. This only allowed free VLB dissolved in PBS pass through filter and the VLB associated

with micelle stayed stable in the supernatant compartment. Filtrates were analyzed for drug content by ELISA Reader.

2.4. Particle Size Determination

Particle size distribution and mean diameter of the prepared aqueous dispersions of VLB were determined by Dynamic Light Scattering (DLS) using a NICOMP 380 Submicron Particle Sizer as described previously [34,35]. Data were analyzed by volume and intensity-weighted distributions [36–40].

2.5. Determination of the Critical Micelle Concentration (CMC)

The same method has been applied to determine CMC of both star- and Y-shaped polymers. The method is briefly described below.

The apparent critical micelle concentration was determined using pyrene as a fluorescent probe. Pyrene in the final concentration of 6×10^{-7} M was dissolved in acetone in a series of empty vials. After the evaporation of acetone, solutions of star-(PCL-*b*-PEG)$_4$ and Y-shaped PCL$_2$-PEG of various concentrations in phosphate buffered saline (PBS) were added to the vials. Entrapment of pyrene in polymeric micelles was accomplished by vortexing and sonicating for 2 and 5 min respectively. Steady-state fluorescence spectra were recorded at 336 nm for emission i_{ex}, and for excitation spectra, i_{em} was 390 nm. From the obtained fluorescence spectra, the values I_1/I_3 has been calculated by dividing the height of the peak at 339 by that of 336 in the case of the star-shaped polymer and 338/333 in the case of the Y-shaped polymer.

The first point in which an increase in the fluorescence yield of pyrene is observed is the concentration that micellar structure forms and the probe settles in the hydrophobic environment of the core. As it is explained in Results and Discussion, Section 3.4., this point could be calculated by finding the point of intersection of the tangents of fluorescence curve. However, this value must be confirmed by using other evidence as will be explained in the results [9,17,31,32].

2.6. Stability of Vinorelbine Associated with Polymeric Micelles Formulation after Lyophilization

When proposing VLB-Star or Y shape polymeric micelles formulations as new cancer therapy agents to the pharmaceutical industry, it is important to emphasize the method of storing the material. We suggest lyophilizing the formulation and rehydrating it before use. The objective of this experiment is to measure the amount of Vinorelbine before and after lyophilization and measure the size of polymeric micelles in the same conditions to determine the stability of formulation.

The amount of Vinorelbine incorporated with SSM has been measured by using ELISA Reader Spectrophotometer, before and after lyophilization. The related method is mentioned above. The stability of size of both formulations has been determined by using DLS. The latter assay has been repeated 2, 3, 5, 7 and 15 days after lyophilization and rehydration.

2.7. Drug Release Studies by Dialysis of Polymeric Micelles Containing Vinorelbine (Pol-VLB)

To perform dialysis, 3 mL of the VLB-polymeric micelle dispersion (5 mg VLB in 100 mg polymer in both cases) was transferred into dialysis membrane tubing, which was then be placed into 300 mL of

dialysis medium (PBS buffer) at 37 °C under constant slow stirring and kept in dark throughout the experiment. Two milliliters of dialysis medium was drawn at 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 20, 22 and 24 h of the experiment. One milliliter was used for immediate drug measurement while the other milliliter was saved in case of necessary lyophilization to concentrate the sample more for Elisa Reader measurement. Samples were also taken from the dialysis membrane tubing before and after the experiment. Release studies were conducted for 24 h without any change or replacement of dialysis medium [9,41–44].

2.8. Investigation of Cytotoxic Activity of Polymeric Nano-VLB Formulations

The cell line used to evaluate the *in vitro* activity of the formulations was MCF-7. The cell line was maintained in RPMI 1640 medium containing 10% fetal bovine serum and 1.0% antibiotics (penicillin and streptomycin) in a 5% carbon dioxide humidified atmosphere at 37 °C.

Optimum solutions of VLB-SSM chosen from the solubilization studies were used as the test solutions. As will be mentioned in Section 3.5. of this paper, the most stable concentration of VLB in polymeric drug delivery systems have been found as 50 and 350 μg VLB in 5 mg of star and Y shape polymers respectively. The same ratio has been followed in preparing the samples (test solutions) to apply to the well plates. A 10% dimethyl sulfoxide (DMSO) solution of vinorelbine also was tested as a control. Drug-free polymeric micelles in 1× PBS buffer, pH 7.4, also were prepared at the same concentrations as the test solution (calculated using the final concentration of polymeric micelles in above mentioned most stable formulations) and were used as controls. Solvents, 10% DMSO and 1× PBS buffer, were tested at the highest concentration used in the formulations. All the samples were prepared and tested in triplicate. The procedure used to test the *in vitro* cytotoxic activity of the formulation is as previously described [38]. Samples were prepared as described earlier and serial dilutions were made to obtain final VLB concentrations ranging from 16 to 8 ($\times 10^{-4}$) μg/mL using the respective solvent that is either PBS buffer or 10% DMSO. 190 μL of cell suspension at a density of 7×10^3 cells/well was plated in a 96-well plate. After that, 8 μL/well of the test solutions and 10 μL/well controls were added to the microtiter plates. Before adding VLB-delivery system, drug-free SSM have been applied to well plates in amount of 2 μL to keep the drug delivery system above CMC. The concentrations of polymeric drug delivery systems have been fixed to 100 times above its CMC. Control groups with 10 μL of the solvents also were added. Each sample was evaluated in triplicate. The plates were then incubated for 3 days in a 5% CO_2 humidified atmosphere at 37 °C [36,37,45]. Reduction of the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan is possible only by the bioactivity of viable cells which is detectable by measuring the absorbance at 570 nm. Before applying MTT, the medium has been aspirated from wells and all wells have been washed by PBS for three times [46,]. MTT has been applied in the concentration of 1 mg/mL in the volume of 100 μL to each well to determine the cell survival in each well. The readings obtained for the solvent controls were used to define 100% growth after correcting for the value obtained for the zero day control. These values were then expressed as % survival and IC_{50} values calculated using nonlinear regression analysis (percent survival *vs.* concentration) [38].

3. Results and Discussion

In the current work, we studied copper catalyzed azide-alkyne cyclo addition (CuAAC) reaction and Diels-Alder reaction for the preparation of amphiphilic A4-B4 type star- and Y-shaped block copolymers consisting of poly(ε-caprolactone) and poly(ethylene glycol).

3.1. Synthesis of (A-b-B)$_4$ Type Star Polymers

In the first part of this work, we present the synthesis of (PCL-*b*-PEG)$_4$ star polymer containing exactly one focal benzyl group, accessible by four synthetic steps using a tetra functional initiator.

The properties of the synthesized A_4-B_4 type polymer is summarized in Table 1 and the synthetic routes, employed for the preparation of (PCL-*b*-PEG)$_4$ star polymer, are shown in Scheme 1.

Table 1. The conditions and characterization data of the A4-B4 type star polymer and its precursors.

Polymer	$M_{n,theo}$	$M_{n,NMR}$	GPC	
			M_n	M_w/M_n
PEG-alkyne [a]	630	685	550	1.11
(PCL)$_4$ [b]	6,250 [c]	7,200	5,400 [d]	1.02
(PCL-OTs)$_4$	6,900	7,850	5,250 [d]	1.05
(PCL-azide)$_4$	6,400	7,350	5,000 [d]	1.05
(PCL)$_4$-(PEG)$_4$	10,100	9,850	8,500	1.14

[a] Obtained by an esterification reaction between 4-pentynoic acid and Me-PEG (550); [b] Synthesized by ROP of ε-CL in bulk using tin(II)-2-ethylhexanoate as a catalyst and **2** as an initiator at 110 °C. $[M]_0$:$[I]_0$ = 100; [c] Determined gravimetrically; [d] Determined by conventional GPC using linear PS standards, after applying a correction formula ($M_{n,PCL} = 0.259 \times M_{n,GPC}^{1.073}$).

Firstly, well-defined star-shaped PCL terminated with four hydroxyl end groups (PCL–OH)$_4$ was synthesized by the ring-opening polymerization of ε-CL monomer according to our previous publication [27]. The degree of polymerization of (PCL–OH)$_4$ could be easily determined by means of ^{1}H-NMR spectra and was found to be 15 for each PCL arm. By two consecutive terminal modification reactions, the (PCL–OH)$_4$ precursor was subsequently transformed into first (PCL-OTs)$_4$ with tosyl end groups and then into (PCL-azide)$_4$ with azide end groups. Comparing the ^{1}H-NMR of the resulting (PCL-azide)$_4$ with that of the (PCL–OH)$_4$ precursor, the proton signals at 3.65 ppm assignable to the primary hydroxyl methylene end group (–CH_2OH) of the (PCL–OH)$_4$ precursor, wholly disappeared while new signals corresponding to methylene protons adjacent to the azide end group appeared at 3.20 ppm for the obtained (PCL-azide)$_4$ (Figure 2).

Moreover, the integral ratio of the proton signal on the -CH_2N_3 end group to the repeating methylene unit of (PCL-azide)$_4$ was very close to the theoretical value. These results show that the hydroxyl end groups of the (PCL–OH)$_4$ precursor were quantitatively converted into azide end groups within (PCL-azide)$_4$. GPC analysis revealed monomodal peaks with low poly dispersity indices after functionalization of the (PCL–OH)$_4$ (Table 1).

The CuAAC click reaction of (PCL-azide)$_4$ with PEG-alkyne was conducted in DMF using Cu(I) catalyst at room temperature for 20 h to afford star polymer, (PCL-*b*-PEG)$_4$. A 20% molar excess

amount of PEG-alkyne to (PCL-azide)$_4$ for each arm was deliberately chosen to easily remove this polymer precursor from the final product by dissolution–precipitation procedure (THF-methanol). As confirmed by ^{1}H-NMR and GPC measurements, successful click reaction had occurred. The ^{1}H-NMR spectroscopy of the (PCL-*b*-PEG)$_4$ star polymer indicated the appearance of the triazole C*H* proton at 7.6 ppm, along with the CH_2O and C=OCH_2 protons of PCL at 4.0 and 2.2 ppm, respectively, and the CH_2CH_2O protons of PEG at 3.6 ppm (Figure 3).

Figure 2. ^{1}H-NMR spectrum of the (PCL-azide)$_4$ in CDCl3.

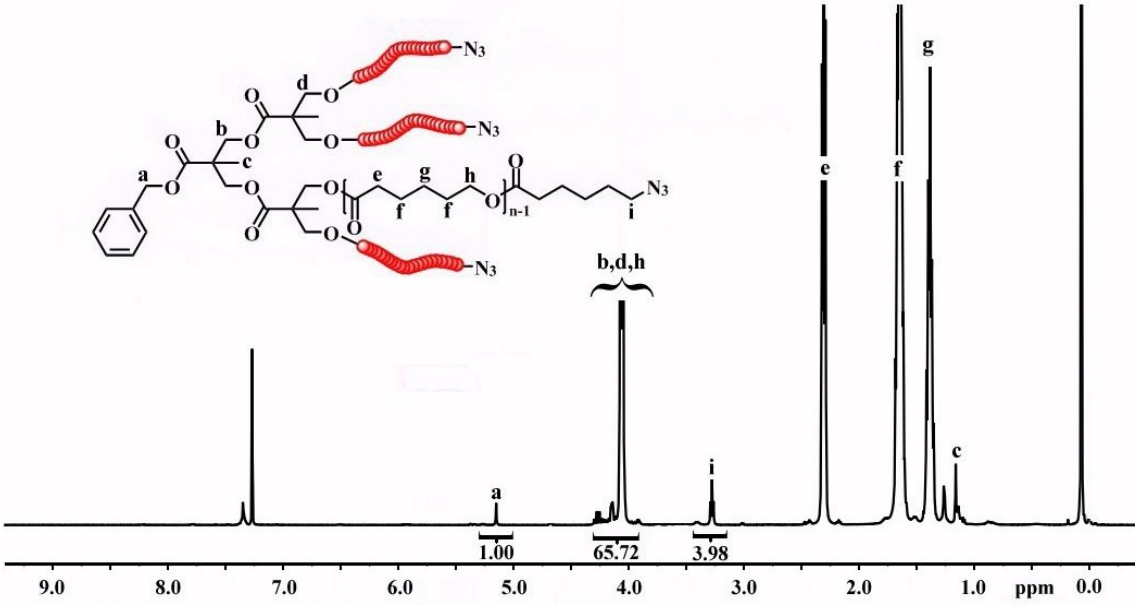
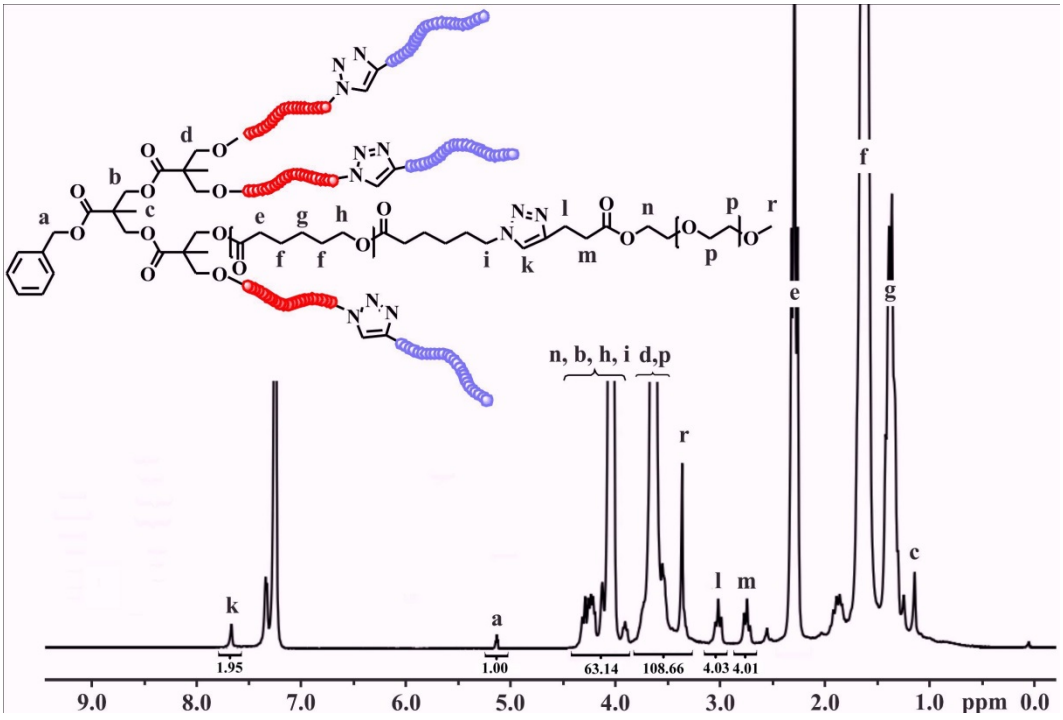
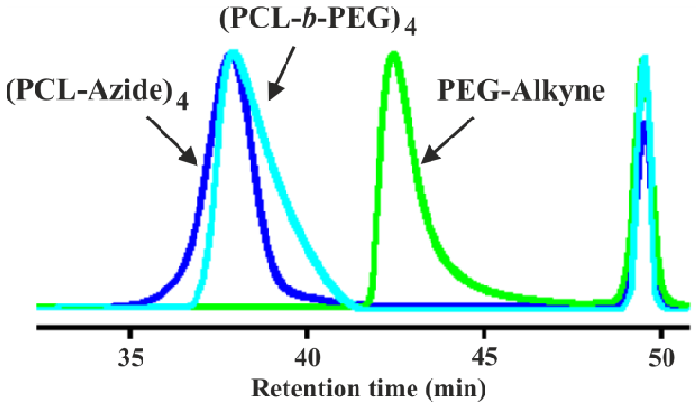


Figure 3. ^{1}H-NMR spectrum of the (PCL-*b*-PEG)$_4$ in $CDCl_3$.



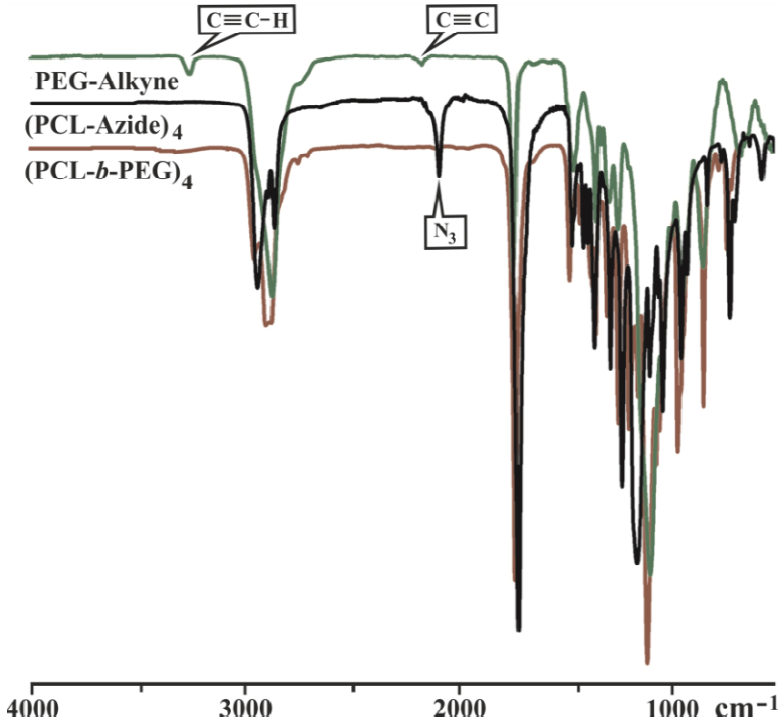
GPC analysis of (PCL-*b*-PEG)$_4$ polymer showed a monomodal trace with no trace of the PEG-alkyne, which shifted to higher retention time with respect to that of (PCL-azide)$_4$ polymer (Figure 4). This may be due to adsorption of the PEG segment for which a stationary phase might have caused a shift to a lower molecular weight region.

Figure 4. Gel permeation chromatography (GPC) overlay of linear poly(ethylene glycol) (PEG)-alkyne, (PCL-azide)$_4$ and the resulting (PCL-*b*-PEG)$_4$ polymer in tetrahydrofuran (THF) at 30 °C.



The CuAAC click reaction was further confirmed by FT-IR spectroscopy. From Figure 5, we can observe the complete disappearance of characteristic azide absorbance peak at 2100 cm^{-1} for (PCL-azide)$_4$, as compared to that of (PCL-*b*-PEG)$_4$. Moreover, the relative intensity of the absorbance peak at 3280 cm^{-1}as a characteristic for terminal alkyne groups of PEG-alkyne also disappeared. This further confirmed that the CuAAC click reaction was complete.

Figure 5. FT-IR spectra of PEG-alkyne, (PCL-azide)$_4$ and the resulting (PCL-*b*-PEG)$_4$ polymer.

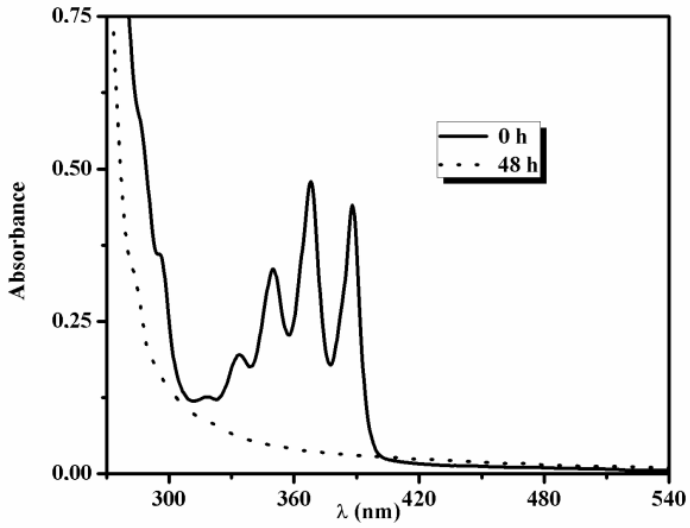


3.2. Synthesis of Y-Shaped Block Copolymer

In the second part of this work, we described the synthesis and self-assembly of a well-defined Y-shaped block copolymer. It should be mentioned that Diels-Alder click reaction is a more biocompatible method for the synthesis of a wide variety of biocompatible and biodegradable polymers, since it does not contain toxic copper catalyst used in the CuAAC click reaction. Synthetic routes, employed for the preparation of PEG–$(PCL)_2$ block copolymer, are shown in Scheme 2.

The Diels-Alder reaction was monitored by UV spectroscopy by following the disappearance of the characteristic five-finger absorbance of the anthracene at 300–400 nm (Figure 6). After 48 h, it was observed that five-finger absorbance completely disappeared due to formation of Y-shaped block copolymer. The Diels-Alder reaction efficiency (DA_{eff}) was calculated to be >99, and thus, displaying a quantitative Diels-Alder reaction.

Figure 6. UV spectra of anth-$(PCL)_2$ before and after reacting with PEG-MI under standard Diels-Alder reaction conditions (Toluene, 110 °C, 48 h) ($C_0 = 1.48 \times 10^{-6}$ mol/L in CH_2Cl_2).



Following isolation, the ^{1}H-NMR spectroscopy analysis obviously showed the presence of both CH_2OC=O of the PCL and the CH_2CH_2O of the PEG segments at 4.10–3.90 and 3.80–3.00 ppm, respectively (Figure 7). Remarkably, ^{1}H-NMR revealed complete disappearance of Ar*H* signals assignable to anthracene at 8.29, 8.16 and 7.84 ppm and the occurrence of new signals corresponding to the CH_2 linked to the adduct and the C*H* (bridge-head) at 5.47 and 4.74 ppm, respectively. Complete disappearance of the original signals for the PEG-MI at 6.5 and 5.25 ppm were also observed by NMR spectroscopy, supporting the quantitative purification after click reaction.

Moreover, Figure 8 shows the GPC diagrams of PEG-MI, anth-$(PCL)_2$ and the corresponding Y-shaped PEG–$(PCL)_2$ block copolymer. The significant shift in elution volume confirms a successful clicking of PEG-MI to anth-$(PCL)_2$, which is also supported by an increase in molecular weight, causing an increase of the hydrodynamic radius detected by GPC. No residual signal of the remaining PEG-MI can be seen, which is in agreement with the assumption of complete transformation of the reactive anth-$(PCL)_2$ as a building block for the synthesis of PEG–$(PCL)_2$. The conditions and characterization data of the Y-shaped block copolymer and its precursors are shown in Table 2.

Figure 7. ^{1}H-NMR spectrum of the Y-shaped PEG–$(PCL)_2$ block copolymer in $CDCl_3$.

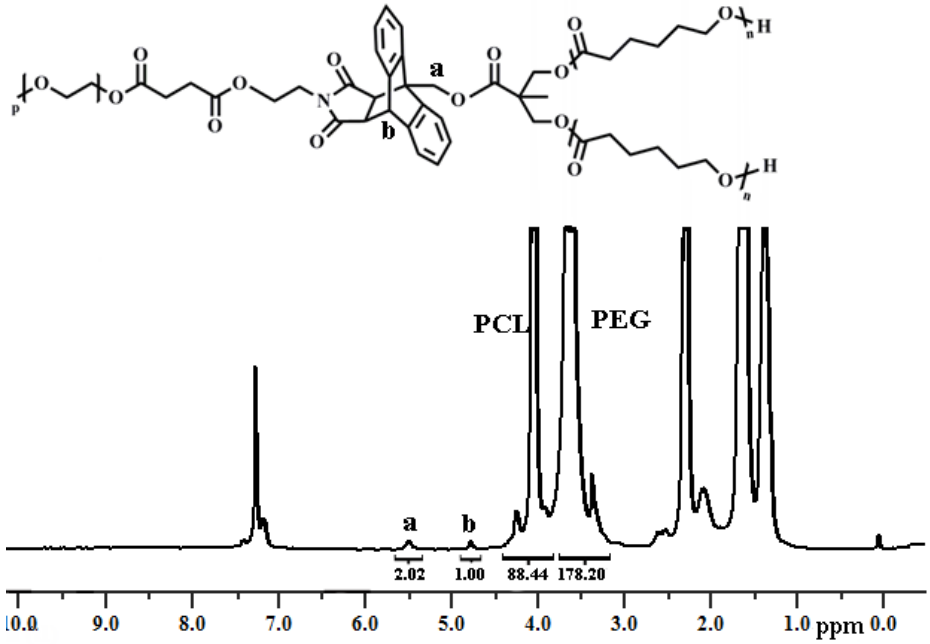


Figure 8. GPC overlay of linear PEG-MI, anth-$(PCL)_2$ and the resulting Y-shaped PEG–$(PCL)_2$ block copolymer in THF at 30 °C.

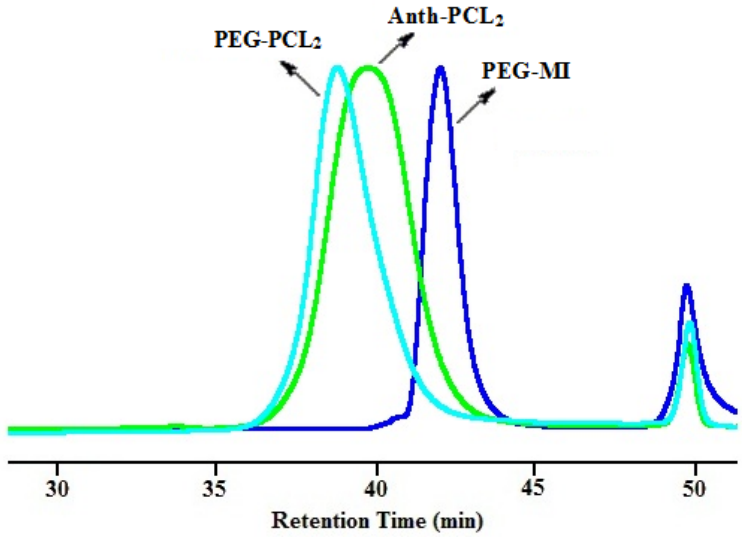


Table 2. The conditions and characterization data of the Y-shaped block copolymer and its precursors.

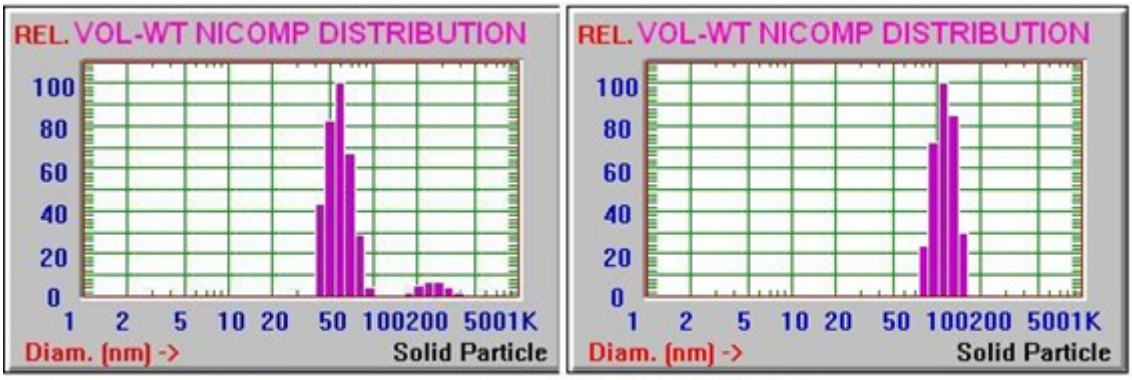
Polymer	Ini.	Time (h)	*Conversion* [b] (%)	$M_{n,GPC}$ (g/mol)	M_w/M_n	$M_{n,theo}$ (g/mol)	$M_{n,NMR}$ (g/mol)	$M_{n,TD\text{-}GPC}$ (g/mol)
PEG-MI	-	-	-	3000	1.05	2350	2600	-
Anth-$(PCL)_2$ [a]	4	12	85	6000	1.14	4750 [c]	5350	5100
PEG–$(PCL)_2$	-	48	-	7700	1.10	8000 [d]	6450	5500

[a] Synthesized by ROP of ε-CL in bulk using tin(II)-2-ethylhexanoate as a catalyst and **4** as an initiator at 110 °C; $[M]_0:[I]_0 = 50$; [b] Determined by gravimetrically; [c] $M_{n,theo} = ([M]_0/[I]_0) \times$ conversion% $\times M_W$ of monomer + M_W of **3**; [d] $M_{n,theo}$ = Sum of $M_{n,NMR}$ of the precursor polymers.

3.3. Particle Size Determination

The micelle size distributions of polymeric micelles of both star and Y-shaped polymers with a concentration of 20 mg/mL [8] were determined by DLS and is shown in Figure 9. The micellar size of particles made of star-shaped micelle has a bimodal distribution with a size of 59 and 278 nm and the micelles formed from Y-shaped polymer shows a monomodal distribution with the effective hydrodynamic diameter of 114 nm.

Figure 9. Results of dynamic light scattering (DLS) investigation of star-(PCL-*b*-PEG)$_4$ (**A**) and Y-shaped PEG–(PCL)$_2$ (**B**).



A **B**

The reason for existence of aggregation for star-(PCL-*b*-PEG)$_4$ is discussed in conclusion part of the paper. In a summary, it is possible that the star-shaped unimers interact with each other on the exposed hydrophobic PCL sides and make amorphous particles [7,8].

3.4. Determination of the Critical Micelle Concentration (CMC)

The micellar behavior of star and Y-shaped PEG–(PCL)$_2$ block copolymers in aqueous solutions was further characterized by a fluorescence technique using pyrene as a probe. This method is based on the changes in the spectroscopic properties of pyrene upon its transfer from aqueous environment to the nonpolar environment of the micellar core [8,47]. Introducing pyrene to a nonpolar environment (the core of the micelle) causes an increase in the intensity of the fluorescence maximum (I_1) in the emission spectrum of pyrene. Also, the excitation spectra of pyrene shows a red shift of I_3 (336 nm in case of hydrophobic environment of micelles consisted of star-shaped polymer and 333 nm in case of that of Y-shaped polymer). These changes are best characterized by the decrease in the ratio of the intensity of the band one to the band three (I_1/I_3), which is in agreement with the decrease in polarity of the environment of the probe. In case of star-shaped polymeric micelle I_{339}/I_{336} *vs.* concentration gave the critical micelle concentration (CMC) curve. This ratio was I_{338}/I_{333} in case of evaluation of micelles consisting of Y-shaped PEG–(PCL)$_2$ polymers. CMC values have been calculated as 50 and 1 mg/L for star- and Y-shaped block copolymers respectively using below graphics (Figures 10 and 11). It is noteworthy that since drawing the tangents of fluorescence curve is very person dependent on the obtained CMC values must be checked with the point of which I_1 band intensity starts increasing and the I_3 band starts shifting to the red.

Figure 10. Dependence of intensity ratio I_{339}/I_{336} (from pyrene excitation spectra) as a function of star-(PCL-*b*-PEG)$_4$ concentration. [Py] = 6.0 × 10^{-7} μM, λ_{em} = 390 nm, T = 37 °C.

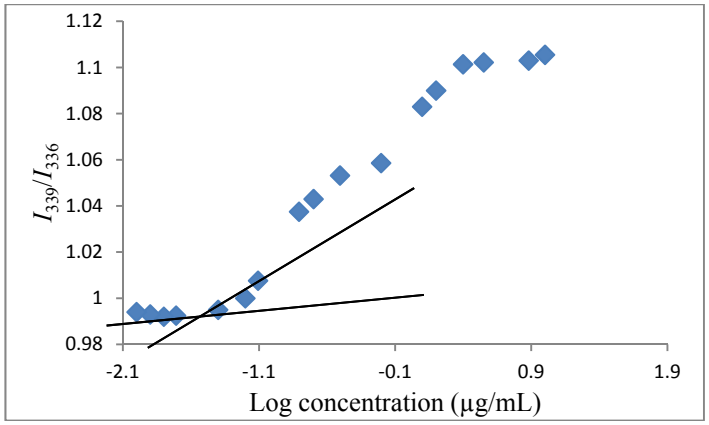
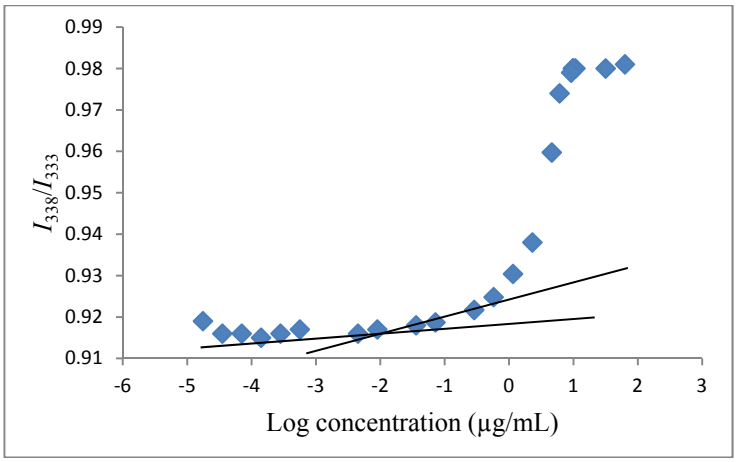


Figure 11. Dependence of intensity ratio I_{338}/I_{333} (from pyrene excitation spectra) as a function of Y-shaped-PEG–(PCL)$_2$ conc. [Py] = 6.0 × 10^{-7} μM, λ_{em} = 390 nm, T = 37 °C.



3.5. Determination of Vinorelbine Concentration Associated with Polymeric Micelles by Using ELISA Reader Spectrophotometer

Currently commercially available Vinorelbine is in salt form (Bitartarate), so it is sufficiently water soluble. However, VLB in base form is poorly water soluble. We used VLB in base form to have it delivered more safely by micelles encapsulation within their core. As is shown in Tables 3 and 4, VLB was used in concentrations between 50 to 300 and 150 to 450 μg for star and Y shape polymers respectively, while the concentration of pre-prepared polymeric micelles was kept constant in 5 mg in both cases. The associated amounts of VLB with polymeric micelles were measured following the method mentioned in the experimental section.

Table 3. Maximum solubility of vinorelbine (VLB) in 5 mg of micelles made of star-shaped polymer.

Total VLB used in the assay (μg/mL)	Drug loading efficacy calculated using regular centrifuge tubes, %/μg·mL^{-1}, (Standard Deviation)	Drug incorporated with micelles calculated using centrifugal filter tubes %/μg·mL^{-1}, (Standard Deviation)	Free VLB dissolved in PBS (μg/mL)
25	100/25 (±0.5)	20/5 (±0.3)	20
50	100/50 (±0.5)	60/30 (±0.7)	20
100	70/70 (±1.0)	70/70 ±0.4	30
150	63.3/94.5 (±1.5)	50/75 (±0.4)	75
200	47.5/95 (±1.5)	37.5/75 ±0.7	125
250	32/80 (±2.5)	24/60 ±0.9	190
300	23.3/69.9 (±1.5)	16.6/50 ±0.9	250

Table 4. Maximum solubility and the incorporation percentage of VLB in 5 mg of micelles made of Y-shaped block copolymer.

Total VLB used in the assay (μg/mL)	Drug loading efficacy calculated using regular centrifuge tubes %/μg·mL^{-1}, (Standard Deviation)	Drug incorporated with micelles calculated using centrifugal filter tubes %/μg·mL^{-1}, (Standard Deviation)	Free VLB dissolved in PBS (μg/mL)
150	100/150 (±4.6)	86.6/130 (±0.8)	20
200	100/200 (±5.8)	90180 (±0.8)	20
250	100/250 (±4.6)	92/230 (±0.4)	20
300	100/300 (±3.2)	93.3/280 (±0.9)	20
350	100/350 (±3.2)	94.2/330 (±0.1)	20
400	87.5/350 (±3.6)	80/320 (±0.5)	80
450	76.7/345 (±6.4)	68/305 (±0.6)	145

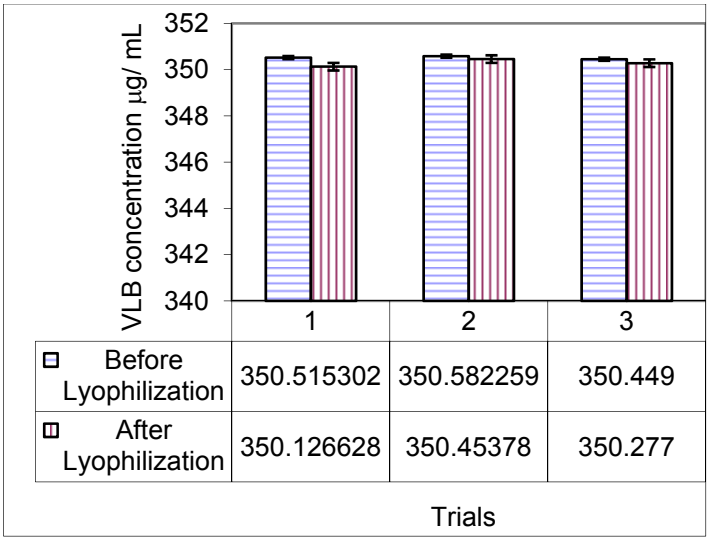
As seen in Table 3 and 4, the solubility of VLB in polymeric micelles increases until a maximum level and then incrementally decreases. As mentioned before, VLB is an amphiphilic molecule. So it is always possible for VLB to make its own micelles. We hypothesize that, after the drug reaches its maximum incorporation value within the drug delivery system, the added excess amount of drug, starts making aggregates at the aqueous media. Since the affinity of drug molecules to their own aggregates is higher than to that of drug and polymeric molecules, further amounts of added drug prefer to join aggregates than to incorporate within polymeric drug delivery system. So the incorporated amount of drug within drug delivery system decreases after a certain concentration. The most stable polymeric micelle-VLB formulations we accept as 50 and 350 μg VLB incorporated with 5 mg of each star and Y-shaped polymeric micelles respectively. At this point it is important to make sure that these amounts are incorporated with the polymeric drug delivery systems and are not making their own soluble micelles due to the amphiphilic character of VLB. To this end, we carried out a second experiment using centrifugal filter tubes. In this experiment the amounts of VLB which passes throw filters are the free (or self-assembled) VLB molecules. As it could be seen in Tables 3 and 4, in low concentrations a constant amount of the drug dissolves in PBS and an increasing amount of drug is incorporated with

micelle. Above concentrations of 50 and 350 μg/mL VLB in case of using star and Y shape polymers respectively, the micellar delivery systems are saturated with the drug and the excess amount prefers to make its own assemblies (aggregates) in PBS than to incorporate with micelle. This result is in good agreement with the high release rate observed in the first minutes of release study shown in Section 3.7.

3.6. Stability of Vinorelbine Associated with Polymeric Micelle Formulations after Lyophilization

Lyophilization of polymeric drug delivery systems incorporated with VLB resulted in obtaining a very fluffy material making a thick bed at the bottom of holder. This fluffy material was kept for 2, 3, 5, 7 and 15 days and rehydrated. The percentage difference of particle size and /or decrease in the amount of drug incorporated with both polymeric micelles, was measured before and after lyophilization and there was no significant difference (Figure 12 shows the data obtained from VLB-Y-shaped polymer lyophilization studies). Therefore, our polymeric micellar nano-VLB formulations are stable enough to be stored in freeze-dried form and rehydrated prior to use.

Figure 12. The difference in concentration of VLB incorporated with micelles made of Y-shaped polymer before and after lyophilization (350 μg VLB/5 mg polymer).



3.7. Drug Release Studies by Dialysis of Polymeric Micelles Containing Vinorelbine (Pol-VLB)

The release rate and time is a very important characteristic of a nano drug carrier used in targeting cancer therapy. To maximize the efficacy of anticancer drugs and minimize unwanted side effects drug release mechanism must afford local high-dose in cancerous tissues and at intracellular compartments. Such technology requires strong incorporation of the drug and a carrier system which performs the desired chemical/physical functions.

As shown in Figures 13 and 14, a high release rate occurs in the first minutes of release. It is a desired feature for a nano formulation to show a strong effect at the first moments of therapy. Together with this, a very slow release continues for 48 h which is enough time for an anti-cancer drug to be sufficiently effective on the cancer tumor site.

Figure 13. Drug release study of micelles made of star-shaped polymer-VLB formulation.

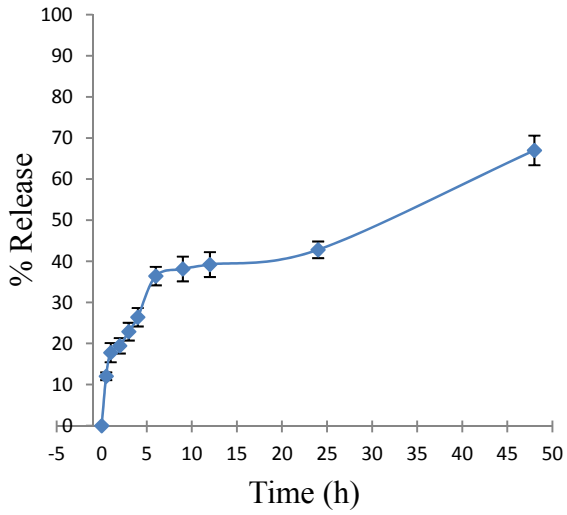
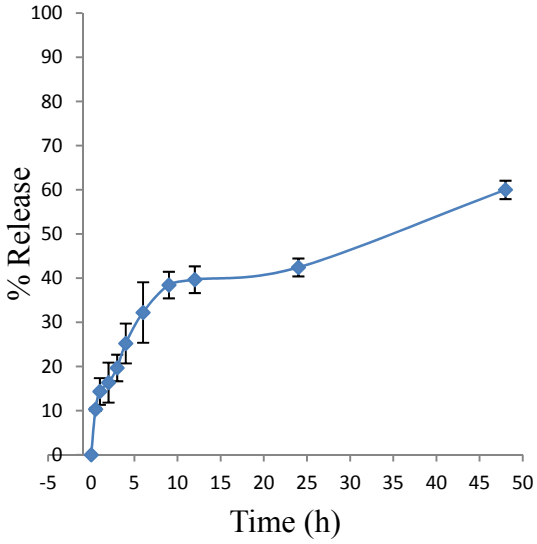


Figure 14. Drug release study of micelles made of Y-shaped polymer-VLB formulation.



3.8. Cytotoxic Activity of Polymeric Nano-VLB Formulations

Although the cytotoxic activity of commercial formulation of Vinorelbine (in Bitartarate form) is well-known, this experiment was carried out to understand whether VLB incorporated in the polymer micelles can exhibit cytotoxic effects in cell culture and to compare the mentioned activity of nano formulations with the commercial drug. The data suggest that both polymers alone are not toxic (Figure 15), whereas VLB incorporated in the micelles displays significant cytotoxic activity, almost 5 times enhanced compared to that of the free VLB (Figure 16).

Figure 15. Cytotoxic activity of positive and negative controls on MCF-7 cells in 24, 48 and 72 h.

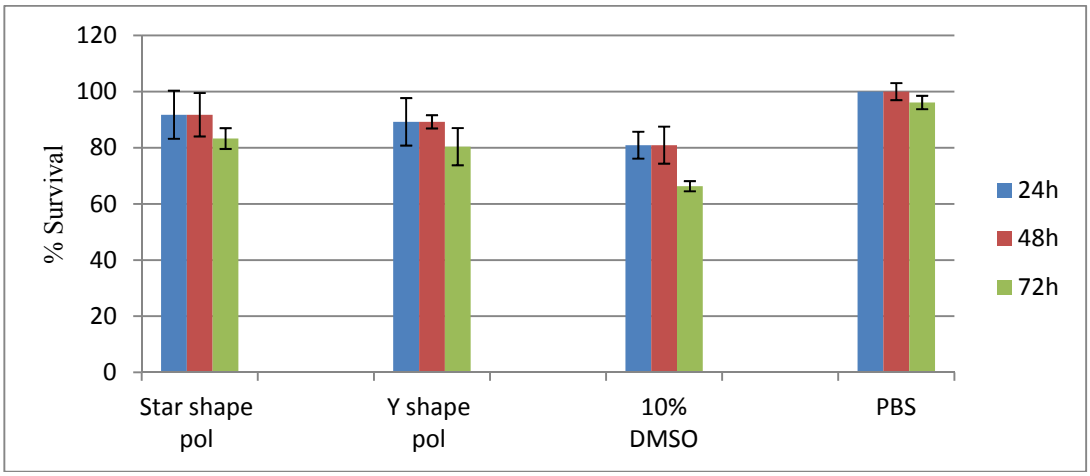
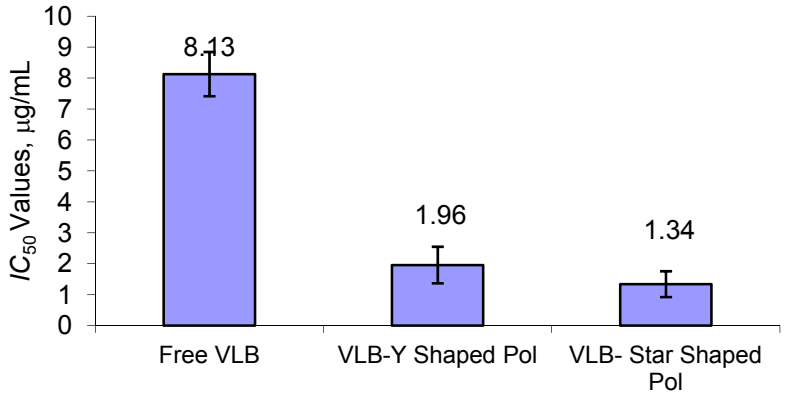


Figure 16. IC_{50} values of two polymeric formulations of Vinorelbine in 72 h compared to free VLB.



4. Conclusions

Numerous micellar drug delivery systems that consist of biodegradable polymeric materials have been synthesized and reported as potential carriers for hydrophobic drugs. However, problems associated with stability of micelles made of fewer branched star-shaped polymers led us to investigate an appropriate structure for a star-shaped block co-polymer with only four arms but acceptable micellar stability. Y-shaped block copolymers are relatively less known amphiphilic materials in the aspect of synthesis methods, but are well known for their different micellizaton behavior. Although synthesis steps of star-shaped polymer are short enough, Y-shaped block copolymer is being synthesized in even fewer steps (three) which is favorable for industrial production. Overall, the incorporation of a highly hydrophobic molecule, pyrene, during CMC determination assays, prior to VLB association, reveals the suitability of these micellar systems as potential carriers for lipophilic compounds [48]. However, CMC of micelles from Y-shaped block copolymer is much more favorable (1 mg/L) than that of star-shaped polymer (50 mg/L). One of the groups involved in this study has

previously reported synthesis and characterization of micelles consisted of PEGylated phospholipid materials which have shown highly satisfying properties such as low CMC, convenient micellar size for targeting tumor tissue using EPR effect (less than 200 nm), high drug loading capacity and efficient preparation [36,38,45]. We synthesized Y-shaped block copolymers in which the hydrophobic parts of the amphiphilic material, the PCL chains, are completely free at one end. This may help hydrophobic blocks to make a strong core by forming a network at the center of micellar structure. In this structure branching of PCL and junction of PCL and PEG takes place on the same point, anthracene unit. The strength of micellar aggregates results in a considerable decrease in CMC value.

On the other hand, it has been very well established that star-shaped block co-polymers show bimodal distribution [49]. The existence of aggregation for star-(PCL-*b*-PEG)$_4$ which was observed in the DLS data in our study, suggested that some star-shaped unimers interact with each other from their hydrophobic PCL sides via hydrophobic-hydrophobic or van der Waals attractions and this causes the occurrence of some particles other than spherical micelles [7,8]. As it has been well demonstrated, these large associations form in the organic solvent of polymeric materials with the adding of first drops of water [50] during the micelle preparation process. That is why optimization of water/organic solvent ratio is very important in minimizing the amount of these aggregates. This ratio has been determined as 67/33, water/organic solvent in our study.

Comparing the physicochemical properties of star and Y-shaped block copolymers we can conclude that although micelles consist of Y-shape polymer are larger than those of star-shaped polymer (114 nm in case of Y-shape and 50 nm in case of star-shaped polymers) the synthesized Y-shape block copolymer is more favorable to produce a drug delivery system because of its monomodal size distribution in the aqueous media.

Although the molecular weight ratio of PCL/PEG block in star-shaped polymer is slightly higher than that of Y shape polymer (2.5 and 2.17 respectively) which makes star shape polymer more hydrophobic, Y-shaped polymer shows higher capacity for carrying this chemotherapeutic agent by carrying the drug in a ratio equal to 7% of its own weight, where, carrying capacity of star-shaped polymer decreases to 1%, *w*/*w*.

Combination of the above results suggests that the micelles of star-shaped polymers consist of less polymer units compared to that of Y-shape polymer. Considering that micelles start to form above CMC, high CMC value must cause formation of micelles consisted of more polymer units and consequently larger particles. In spite of that, small size and low drug delivery capacity of micelles consisting star-shaped block copolymers could be related to low aggregation number of these self-assembly systems. However, this assumption needs to be confirmed by experimental results.

Based on MTT assays which was made on MCF-7 breast cancer cell line, we can deduce that the cytotoxic property of VLB-polymeric drug delivery formulations shows 6 and 4 times enhancement in case of using star and Y shape polymers, respectively compared to free VLB (IC_{50} values: free VLB = 8.13, VLB-Star-shaped polymer = 1.34 and VLB-Y shape polymer = 1.96 μg/mL). The shelf-life of both formulations could be prolonged using lyophilization method and both are stable enough to carry the payload to the cancer side.

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Conflicts of Interest

The authors declare no conflict of interest.

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