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Filomicelles (worm-like micelles) possess high drug loading capacity and long circulation time in the bloodstream. A novel approach can be filomicelles with folic acid (FA) as a targeting moiety. Folate-drug delivery systems can target FA receptors (FAR) that are overexpressed in several human carcinomas, which can potentially maximize therapeutic efficacy while minimizing side effects. The aim of this study was to develop filomicelles from combination of poly(L-lactide)-Jeffamine-folic acid and poly(L-lactide)-poly(ethylene glycol) for delivery of betulin derivative. Phosphate derivative of betulin reveals high cytotoxicity against cancer cells, however its application is restricted due to poor solubility in water. Incorporation into hydrophobic core of micelles can effectively solubilize the drug. Three kinds of micelles were obtained with high drug loading capacity. Based on TEM analysis, the copolymers formed exclusively filomicelles or mixture of filomicelles and spherical micelles. All kinds of micelles provided release of betulin derivative for over 9 days and apart the very initial phase displayed similar release profile. The influence of PLA block on initial burst effect was revealed. The *in vitro* cytotoxicity of betulin derivative loaded micelles against FAR-positive HeLa cells was confirmed, which proves their usefulness for targeted delivery of cytostatic drug.

1. Introduction

Polymeric micelles have a core-shell structure formed by self-assembly of amphiphilic block copolymers which can solubilize poorly water soluble drugs. Advantages of the micelles include also ability to conjugate with targeting molecules via surface modification, and thus achieving the specific targeting and reducing the nonspecific uptake by the reticuloendothelial system (RES) (Chen et al., 2008; Jurenka, 2009; Wu and Li, 2013). Morphology of polymeric micelles influences their circulation time, biodistribution, cellular uptake, intracellular trafficking and overall efficiency (Truong et al., 2016c). Therefore, even more interesting are worm-like micelles that are also called filomicelles, which possess a long circulation time up to one week in the bloodstream and display almost twice higher drug loading capacity as compared to spherical micelles due to larger core volume per carrier (Cai et al., 2007; Loverde et al., 2011; Truong et al., 2017). The prolonged circulation results in higher accumulation of therapeutics at the targeted site of diseases, which increases drug efficiency and leads to reduced side effects (Truong et al., 2016c). Filomicelles gain an interest in a variety of fields including catalysis, material science, immunology, tissue engineering and drug delivery (Truong et al., 2016b). We have developed poly(lactide)/poly(ethylene glycol) (PLA-PEG) filomicelles

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for passive targeting of single or multidrug delivery (Jelonek et al., 2016; Jelonek et al., 2017; Jelonek et al., 2015). PLA-PEG has been widely studied as drug carrier in the form of micelles, nanoparticles and hydrogels as it is biocompatible, degradable and bioresorbable. For further progress we aimed to develop PLA-PEG filomicelles for targeted delivery of anticancer compounds. Folic acid (FA) was used as a targeting moiety. The use of small molecules like folic acid instead of peptides and antibodies as targeting ligands can provide several benefits including no risk of toxicity or immune reactions due to its function as a vitamin, low immunogenicity, unlimited availability and low cost, easy scale-up for clinical applications, facile chemical modification, stability during storage and high stability in acidic or basic media and at high temperature (Gruner and Weitman, 1998; Yameen et al., 2014). Folic acid has been known to target FA receptors (FAR) that are overexpressed in several human carcinomas including breast, ovary, endometrium, kidney, lung, head and neck, brain, colon and myeloid cancers while only minimally distributed in normal tissues (Gruner and Weitman, 1998; Lu and Low, 2002; Parker et al., 2005; Weitman et al., 1992). Thus, folate-drug delivery systems can enter cells by receptor-mediated endocytosis which can potentially maximize therapeutic efficacy while minimizing side effects. So far, only fructose-decorated worm-like micelles have been reported (Majdanski et al., 2018). Another novel approach is to use a betulin derivative (30-diethoxyphosphoryloxy-28-*O*-propynoylbetulin) as an anticancer agent (Fig. 1) (Boryczka et al., 2015).

The use of natural plant-derived compounds has been considered to be an interesting aspect for the treatment of human neoplastic diseases, because they are relatively easily available due to their common occurrence in nature (Krol et al., 2015; Laszczyk, 2009). Betulin has been shown to elicit anticancer properties by inhibiting cancer cells growth. Even better cytotoxicity was detected for betulin derivatives (Boryczka et al., 2013). However, their application is restricted because of poor water solubility and therefore, it is important to develop biocompatible dosage form that can effectively solubilize the drug. So far, some attempts to develop delivery system for betulin or betulin derivatives have been described, e.g. poly(D,L-lactide) nanovectors (Yadav et al., 2016), nanoemulsion (Dehelean et al., 2011), nanoparticles (Zhao et al., 2014). But there have not been studies on targeted filomicellar delivery systems of betulin derivatives.

2. Materials and methods

2.1. Materials

O,O'-Bis-(2-aminopropyl) poly(propylene glycol)-*b*-poly(ethylene glycol)-*b*-poly(propylene glycol) (Jeffamine) with number average molar mass (Mn) of 1900, folic acid with molar mass of 441.40, 4-dimethylaminopyridine (DMAP), N,N′-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich.

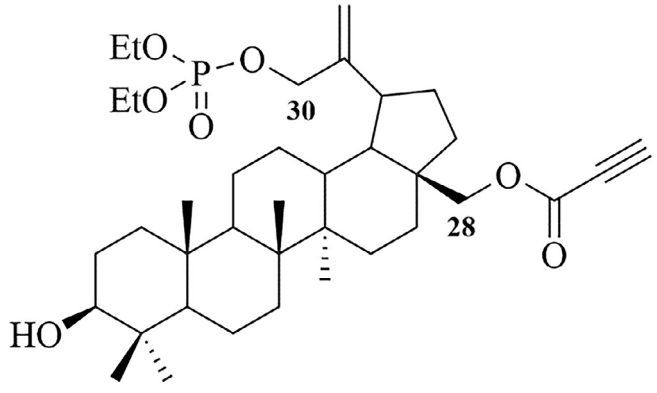

Fig. 1. Chemical structure of 30-diethoxyphosphoryloxy-28-*O*-propynoylbetulin (Mn = 646.85 g/mol).

Phosphate derivative of betulin (30-Diethoxyphosphoryloxy-28-*O*-propynoylbetulin; ECH160B) (Fig. 1) was synthesized at the Department of Organic Chemistry, Medical University of Silesia (Boryczka et al., 2015).

HeLa human cervix adenocarcinoma cell line was obtained from American Type Culture Collection (ATCC-LGC Standard). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific. All other organic solvents were of analytic grade from Sigma-Aldrich and used without further purification.

2.2. Synthesis and characterization of PLA-PEG copolymers

Three PLA-PEG diblock copolymers were synthesized by ring opening polymerization of L-lactide using monomethoxy PEG (mPEG) as macroinitiator and zinc lactate as non-toxic catalyst as previously described (Yang et al., 2010). Briefly, predetermined amounts of mPEG and lactide were introduced into a polymerization tube, and sealed under vacuum. Polymerization proceeded for 3 days at 140 °C. The product was recovered by dissolution in dichloromethane and precipitation in diethyl ether, and dried under vacuum to constant weight.

The composition of copolymers was determined by means of proton nuclear magnetic resonance (^{1}H NMR) recorded at Bruker spectrometer (600 MHz) using DMSO-d_6 as a solvent. Chemical shifts (δ) were given in ppm using tetramethylsilane as an internal reference.

The molar mass and molar mass distribution of block copolymers were measured by Waters 410 gel permeation chromatography (GPC) equipped with an RI detector. Tetrahydrofuran (THF) was used as the mobile phase at a flow rate of 1.0 mL/min. 20 µL of each polymer solution at a concentration of 10 g/L was injected for analysis.

2.3. Synthesis of PLA-Jeff-FA copolymer

PLA with a carboxyl chain end was first synthesized by ring opening polymerization of L-lactide using traces of water as initiator and zinc lactate as non-toxic catalyst. PLA was then coupled to Jeffamine to obtain PLA-Jeff block copolymer with active groups on the PEG end. Briefly, PLA (1 eq) was dissolved in dichloromethane, and NHS (2 eq) and DCC (2 eq) were added. The reaction proceeded under stirring for 24 h at room temperature in an argon atmosphere. The precipitate dicyclohexylurea (DCU) was filtered off. The filtrate was dropped into Jeffamine solution in dichloromethane (5 eq). After 24 h reaction in an argon atmosphere, the solution was precipitated and washed with methanol to remove Jeffamine in excess. The product was collected by centrifugation at 4000 rpm in 20 min and dried under vacuum.

0.25 mM of the obtained PLA-Jeff copolymer (1 eq) and 0.625 mM DCC (3 eq) were dissolved in DMSO containing folic acid (2.5 eq) and DMAP (0.5 eq). The solution was stirred for 6 h at room temperature in an argon atmosphere, and cooled down in ice water. The precipitate DCU was removed by filtration. Finally, the solution was transferred into a dialysis membrane with MWCO of 3500 Da, and dialyzed against distilled water for 48 h. The solution was freeze dried to yield PLA-Jeff-FA. ^{1}H NMR spectra of PLA-Jeff-FA and its precursors were recorded on a Bruker spectrometer (AMX500) operating at 500 MHz, using DMSO-d_6 as solvent.

2.4. Preparation and characterization of micelles

Blank micelles and betulin derivative loaded micelles were prepared by using co-solvent evaporation method (Supplementary data). PLA-PEG and PLA-Jeff-FA (2.5/1, w/w) were dissolved in methylene chloride, and the solution was added in distilled water to obtain a concentration of 1 mg/mL. The mixture was stirred vigorously at room temperature for 3 h and left for solvent evaporation for 24 h, yielding blank micelles. Betulin derivative dissolved in ethanol was added to the micelle solution with a drug to polymer ratio (w/w) of 1/4. The mixture

was stirred vigorously for 3 h, followed by centrifugation at 3 000 rpm for 5 min to eliminate unloaded drug. The supernatant was recovered, lyophilized and stored at 4 °C for further analysis.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijpharm.2018.12.033.

Transmission electron microscopy (TEM) was performed using a Tecnai F20 TWIN microscope (FEI Company, USA) equipped with field emission gun, operating at an acceleration voltage of 200 kV. Images were recorded on the Eagle 4 k HS camera (FEI Company, USA) and processed with TIA software (FEI Company, USA). Micellar solution (6 μL) was placed on a copper grid covered with carbon film, stained negatively with 2% phosphotungstic acid (PTA), and air dried at room temperature before measurements. ImageJ program was used to determine the distribution of micelles' length.

2.5. Quantification of drug loading content

Quantitative assessment of betulin derivative was conducted by means of reverse phase high performance liquid chromatography (RP-HPLC) using a VWR/Hitachi LaChrom Elite® chromatograph equipped with a LiChrospher® RP-18 column (4 mm × 250 mm, 5 μm). The mobile phase was a mixture of acetonitrile and water (86/14, v/v). Measurements were performed at 25 °C at a flow rate 1.0 mL/min. The UV detection was set at 210 nm.

For the determination of drug loading content (LC) and the encapsulation efficiency (EE), lyophilized micelles were dissolved in ethanol at a concentration of 1 mg/mL and analyzed by HPLC. The LC was defined as the ratio of the weight of loaded drug to that of drug-loaded micelles, and the EE as the ratio of the weight of loaded drug to that of added drug.

2.6. In vitro drug release

The *in vitro* release of drug from micelles was realized by dialysis method. Lyophilized betulin derivative-loaded micelles were dispersed in pH 7.4 phosphate buffered saline (PBS) at a concentration of 1 mg/mL, and added into a 3 mL Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) with a MWCO of 3 500 Da. Each cassette was placed in 400 mL PBS (pH 7.4), which was changed regularly to ensure sink conditions. A sample of 50 μL was drawn from each cassette at various time intervals up to 216 h, and replaced by the same volume of fresh PBS. The amount of released drug was measured by HPLC as per Section 2.5.

DDsolver, an Excel Ad in Program (Zhang et al., 2010) was used for modeling the kinetics of the drug release processes by fitting the profiles with time-dependent equations. The best fitting model is based on R^2 (adjusted coefficient).

2.7. In vitro cytotoxicity studies

HeLa cell line was used to assess the cytotoxicity of free drug and drug loaded micelles. The cells were allowed to grow in the RPMI-1640 culture medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 mM HEPES. The cell line was maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.7.1. Sulforhodamine B (SRB) assay

The sulforhodamine B (SRB) assay was used to measure the influence of betulin-derivative, drug-free micelles and drug loaded micelles on cellular growth. HeLa cells were seeded at a density of 2×10^3 cells per well in 200 μL of culture medium in 96-well plates, and incubated for 24 h to allow cell adhesion. Subsequently, the medium was replaced with a fresh one containing the tested formulations and the cells were incubated for 72 h. Untreated cells were used as control. Blank micelles and drug-loaded micelles were dissolved directly in culture medium before analysis. The free drug was dissolved in DMSO as a stock solution (20 mM) from which dilutions in culture medium were prepared before use. The final DMSO concentration in culture medium in all experimental groups was adjusted to 0.2%. Betulin derivative (free drug and drug in micellar solution) was studied in the range of concentration from 1 μM to 30 μM. The blank micelles were added at a concentration required for the particular drug concentration (2.4–74 μg/mL). Each concentration was tested at least six times, and experiments were repeated three times. At the end of incubation period, the medium was withdrawn and the cells were fixed with 100 μL of 10% trichloroacetic acid, washed three times with deionized water and finally stained with 0.4% sulforhodamine B (dissolved in 1.0% acetic acid). After triple rinsing the unincorporated dye was removed out using 1.0% acetic acid, the incorporated stain was solubilized in 200 μL of 10 mM unbuffered Tris base solution. Absorbance was measured at 570 nm and 690 nm (reference wavelength) using the MRX Revelation plate reader (Dynex Technologies). For statistical analysis of the results one-way ANOVA was used.

2.7.2. Lactate dehydrogenase (LDH) assay

The cytotoxic effect of betulin derivative-loaded micelles was assessed by means of lactate dehydrogenase (LDH) release assay as a reliable measure of cell death. In Vitro Toxicology Assay Kit, Lactate Dehydrogenase Based (Sigma Aldrich) was used. HeLa cells were seeded at a density of 5×10^3 cells per well in 200 μL of culture medium in 96-well plates, and incubated for 24 h to allow cell adhesion. Some wells contained only culture medium and no cells (background control). Subsequently, the medium was replaced with a fresh one containing the tested formulations and the cells were incubated for 24 h. The final concentration range of drug in micellar solution was 1–30 μM. The blank micelles were added at a concentration required for the particular drug concentration (2.4–74 μg/mL). Each concentration was tested at least six times, and experiments were repeated three times. In selected wells of each plate, lysis buffer was added before assay, to determine maximal LDH release (high control). Wells with untreated cells were used as the low control of the assay. LDH activity in the culture medium was determined according to the manufacturer's instruction. The absorbance was measured at wavelengths of 490 nm and 690 nm (background absorbance) using a microplate reader (MRX Revelation, Dynex Technologies). Subsequently, the percentage of liberated enzyme was calculated for cells growing in the presence of micellar solution according to the formula:

$$\text{Cytotoxicyty}\,[\%] = (A_T - A_L/A_H - A_L) * 100$$

where: A_T – absorbance of treated wells; A_L – low control; A_H – high control (maximal LDH release). For statistical analysis of the results One-way ANOVA was used.

2.7.3. Cell apoptosis assay

To study cellular apoptosis, fragmentation of genomic DNA was assessed using the Cell Death Detection ELISA^{PLUS} kit (Roche). This method is based on immunoenzymatic detection of nucleosomal DNA fragments (formed as the result of apoptotic cell death) in the cytoplasmic cell fraction. Cells were cultured as described above for LDH release assay. After 24 h incubation of cells with the tested drug, plates were centrifuged (200 g; 10 min.), culture medium was removed and cells were lysed for 30 min. at room temperature. Then, plates were centrifuged again and 20 μL of supernatant (cytoplasmic fraction) was transferred to the streptavidin coated 96-well plate. DNA fragments were detected using ELISA technique according to the manufacturer's instructions. Absorbance was measured at 405 nm and 490 nm (reference wavelength) and enrichment factor (EF) was calculated:

$$EF = A_S/A_C$$

where A_S is absorbance of the sample and A_C – absorbance of control. For statistical analysis of the results One-way ANOVA was used.

2.8. Cellular uptake

Flow cytometry and confocal laser scanning microscopy (CLSM) were used to study the cellular uptake of PLA-PEG/PLA-Jeff-FA micelles.

2.8.1. Flow cytometry

HeLa cells (5×10^5) were seeded in T25 flasks and cultured for 24 h in standard conditions. Subsequently, the medium was replaced with a fresh one containing fluorescein encapsulated in PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ micelles or free fluorescein. The final concentration of fluorescein was 30 µg / mL and the cells were incubated for 24 h. Untreated cells were used as control. Cells were washed 4 times with PBS (pH 7.4), detached by trypsin treatment and collected by centrifugation. The final concentration of fluorescein was 30 µg / mL. Untreated cells were used as control. Cells were washed 4 times with PBS (pH 7.4), detached by trypsin treatment and collected by centrifugation. After resuspension in PBS, cells were washed again 3 times, and stained with 7-AAD (7-Aminoactinomycin D, BioLegend). The fluorescence intensity was measured on a BD FACSCanto flow cytometer (BD Biosciences). For determination of the uptake efficiency, 7-AAD^- viable cells containing fluorescein were investigated after using gates of forward scatters to exclude debris and cell aggregates. The untreated cells were set as negative control gates in the analysis. 50,000 events were analyzed for each sample. The raw data were analyzed with the BD FACSDiva Software v.6.1.2.

2.8.2. Confocal laser scanning microscopy

FAR-positive HeLa cells and normal human connective tissue cells (Lonza) were seeded for 24 h at a density of 2×10^4 and 1×10^4 cells/well, respectively, in 8-well chambered coverglass (Nunc™ Lab-Tek™, Thermo Scientific™). After 24 h of initial culture, cells were incubated with fluorescein encapsulated in PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ micelles at the concentration of 30 µg / mL for another 24 h. Untreated cells were used as control. The excess of micelles was removed by 4 washings with PBS (pH 7.4). Images were acquired on living cells using an LSM 710 Zeiss confocal microscope (Carl Zeiss Microscopy GmGB, Gottingen, Germany).

3. Results

3.1. Synthesis and characterization of PLA-PEG copolymers

Three PLA-PEG diblock copolymers were synthesized using mPEG of Mn 2000 or 5000 as macro-initiator and zinc lactate as non-toxic catalyst. The composition of the copolymers or the EO/LA molar ratio was obtained from the integrations of NMR resonances belonging to the methylene protons of PEG at 3.6 ppm and to the methine proton of PLA at 5.2 ppm, as previously described (Li and Vert, 2003). The degree of polymerization (DP) of PEG and PLA blocks, as well as the M_n the copolymers was determined from the following equations:

$$DP_{PEG} = M_{nPEG}/44 \quad (1)$$

$$DP_{PLA} = DP_{PEG}/(EO/LA) \quad (2)$$

$$M_n = M_{nPEG} + DP_{PLA} \times 72 \quad (3)$$

where 44 and 72 are the molar mass of the ethylene oxide and lactate moieties, respectively.

The molecular characteristics of PLA-PEG diblock copolymers are summarized in Table 1. Two PLA-PEG copolymers obtained from PEG2000 have DP_{PLA} = 32 and 42, respectively, and the third copolymer obtained from PEG5000 has a longer hydrophobic block (DP_{PLA} = 77). The $M_{n(NMR)}$ of the copolymers ranged from 4300 for $PLA_{2300}PEG_{2000}$ to 10,500 for $PLA_{5500}PEG_{5000}$. GPC was also used to determine the molar mass and dispersity ($Đ = M_w/M_n$) of the copolymers. The $M_{n(GPC)}$ ranged from 5500 for $PLA_{2300}PEG_{2000}$ to 11,900 for $PLA_{5500}PEG_{5000}$, and the dispersity from 1.4 for $PLA_{2300}PEG_{2000}$ to 1.3 for $PLA_{5500}PEG_{5000}$. The weight fraction of EO (f_{EO}) is 0.47, 0.40 and 0.48 for $PLA_{2300}PEG_{2000}$, $PLA_{3000}PEG_{2000}$, and $PLA_{5500}PEG_{5000}$, respectively. According to the literature, copolymers with f_{EO} around 0.45 are susceptible to form worm-like micelles (Dalhaimer et al., 2003).

3.2. Synthesis and characterization of PLA-Jeff-FA copolymer

The synthesis of PLA-Jeff-FA was realized via a 3 step procedure: 1) synthesis of PLA with hydroxyl and carboxyl chain ends by ring opening polymerization of L-lactide using traces of water as initiator, 2) reaction of PLA-COOH with Jeffamine having amino chain ends in excess to obtain PLA-Jeff copolymer with an amine group at the Jeffamine chain end using DCC/NHS as coupling agents, and 3) reaction of PLA-Jeff-NH_2 with folic acid in excess to obtain PLA-Jeff-FA using DCC/DMAP as coupling agents.

Fig. 2 shows the ^{1}H NMR spectra of PLA-COOH, Jeffamine and PLA-Jeff-NH_2. Signals at 1.5 and 5.2 ppm were assigned to main chain methyl and methine groups (Fig. 2A), respectively. The methine group at the hydroxyl and carboxyl chain ends was detected at 4.2 and 5.0 ppm, and the methyl group at the hydroxyl and carboxyl chain ends was detected at 1.3 and 1.4 ppm, respectively. The DP of PLA was determined from the integration ratio of signal c (5.2 ppm) and b (4.2 ppm). A value of DP_{PLA} = 17 was obtained.

The spectrum of Jeffamine with Mn = 1900 is shown in Fig. 2B. Signal at 3.55 ppm belongs to the methylene of PEO, and methine and methylene of PPO, whereas signals at 1.05 and 0.90 ppm are assigned to the main chain and chain end methyl groups of PPO, respectively. On the spectrum of PLA-Jeffamine (Fig. 2C), signals of both components are detected, suggesting the successful coupling of PLA and Jeffamine chains.

Folic acid was attached to PLA-Jeffamine copolymer through an amidation reaction using DCC and DMAP as coupling agent and catalyst. Fig. 3 presents the ^{1}H NMR spectra of folic acid and PLA-Jeff-FA. Folic acid exhibits a number of signals in the 1.0 to 9.0 ppm range. In particular, signals **a**, **b** and **c** detected at 2.3, 2.0 and 4.4 ppm are assigned to the two methylene groups and methine group between the two carboxyl groups (Fig. 3A). After the coupling reaction, signals **a** and **c** adjacent to the carboxyl groups almost disappeared (Fig. 3B), in agreement with the successful attachment of folic acid to the PLA-Jeffamine chain.

3.3. Characterization of drug loaded micelles

Micelles for targeted cancer therapy were obtained from 2.5/1 (w/w) mixture of PLA-Jeff-FA and PLA-PEG copolymers.

The morphology of micelles was observed using TEM. As shown in Fig. 4A–A1, the mixture of PLA-Jeff-FA and $PLA_{2300}PEG_{2000}$ formed

Table 1
Characteristics of PLA-PEG diblock copolymers.

Copolymer	M_{nPEG}	EO/ LA[a]	DP_{PEG}[b]	DP_{PLA}[c]	$M_{n(NMR)}$	$M_{n(GPC)}$	$Đ$	f_{EO}
$PLA_{2300}PEG_{2000}$	2000	1.38	45	32	4300	5500	1.4	0.47
$PLA_{3000}PEG_{2000}$	2000	1.06	45	42	5000	5600	1.3	0.40
$PLA_{5500}PEG_{5000}$	5000	1.50	114	76	10,500	11,900	1.3	0.48

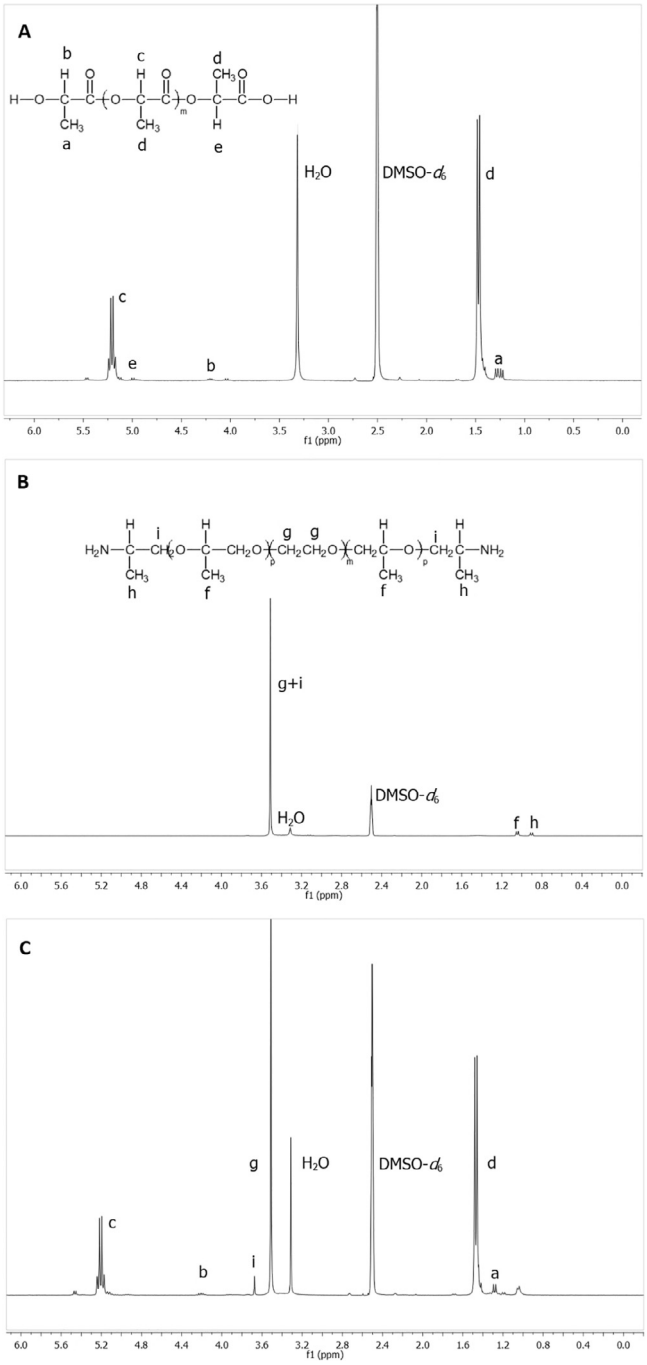

Fig. 2. ^{1}H NMR spectra of PLA-COOH (A), Jeffamine (B) and PLA-Jeff-NH_2 (C) in DMSO-d_6.

both filomicelles of 100–400 nm length and spherical micelles of 15–50 nm diameter. Long filomicelles of 300 – over 1900 nm length were exclusively obtained from PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ (Fig. 4B–B1). In the case of PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$, filomicelles of 50 – over 650 nm length were obtained (Fig. 4C–C1).

The encapsulation properties of PLA-PEG/PLA-Jeff-FA micelles were evaluated by physical loading of betulin derivative, a novel anticancer agent derived from natural plants. The chemical structure of betulin derivative is presented in Fig. 1. A theoretical drug content of 20% was applied. Nearly quantitative encapsulation of the drug was obtained for all the three micelle systems with encapsulation efficiency close to 100% and loading content close to 20% (Table 2).

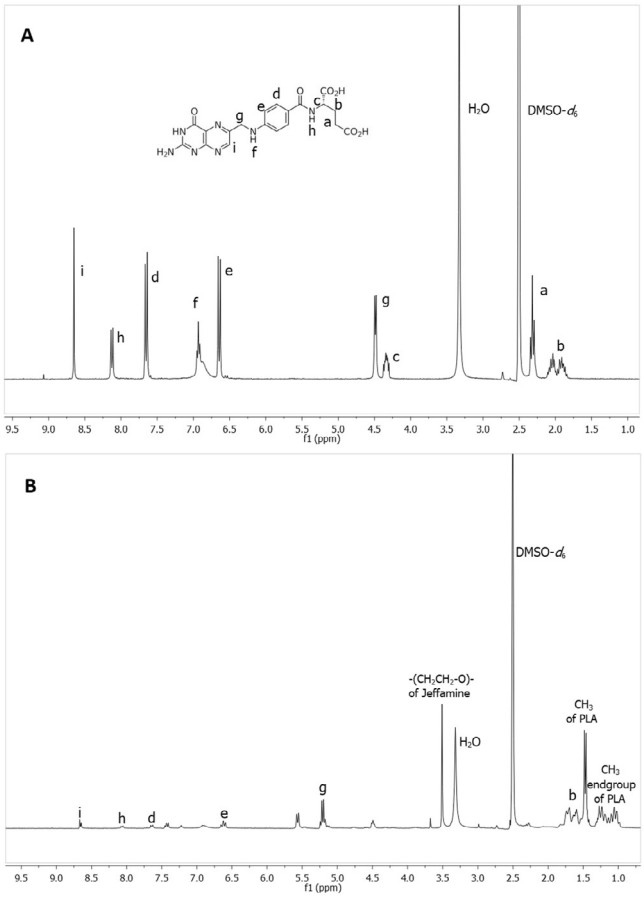

Fig. 3. ^{1}H NMR spectra of folic acid (A) and PLA-Jeff-FA (B) in DMSO-d_6.

3.4. In vitro release

The release of betulin derivative from micellar systems was studied under *in vitro* conditions. Fig. 5 presents the release profiles from different PLA-PEG/PLA-Jeff-FA micelles. In the case of micelles obtained from $PLA_{2300}PEG_{2000}$ and $PLA_{3000}PEG_{2000}$, a burst effect was observed. 52% and 48% of betulin derivative were released after 1 h, respectively. Beyond, the amount of drug released from $PLA_{2300}PEG_{2000}$ gradually increased (62% after 24 h, 73% after 72 h, 90% after 168 h, and 99% after 216 h). In the case of $PLA_{3000}PEG_{2000}$, similar drug release profile was observed although the drug release was slower till 48 h. Contrary to $PLA_{2300}PEG_{2000}$ and $PLA_{3000}PEG_{2000}$, only 20% of betulin derivative was released from $PLA_{5500}PEG_{5000}$ micelles after 1 h. However, apart from the initial difference in drug release, the further process proceeded similarly to $PLA_{2300}PEG_{2000}$ and $PLA_{3000}PEG_{2000}$. Almost all drug was released after 216 h.

The release data were fitted to Higuchi and Peppas-Sahlin kinetic models in order to determine the drug release mechanism from micelles. The Higuchi model is described by the following expression:

$$M_t = K_H \cdot t^{1/2}$$

where M_t is the amount of released drug at time t, K_H is the Higuchi dissolution constant. And the Peppas-Sahlin model is described by the following expression:

$$M_t/M_\infty = k_1 t^m + k_2 t^{2m}$$

where k_1 and k_2 are the Fickian kinetic constant and the erosion rate constant, respectively. The coefficient *m* is the purely Fickian diffusion exponent (Peppas and Sahlin, 1989). The values of model parameters and the regression coefficients are summarized in Table 3. It was found that the obtained drug release data fitted well with the Peppas-Sahlin

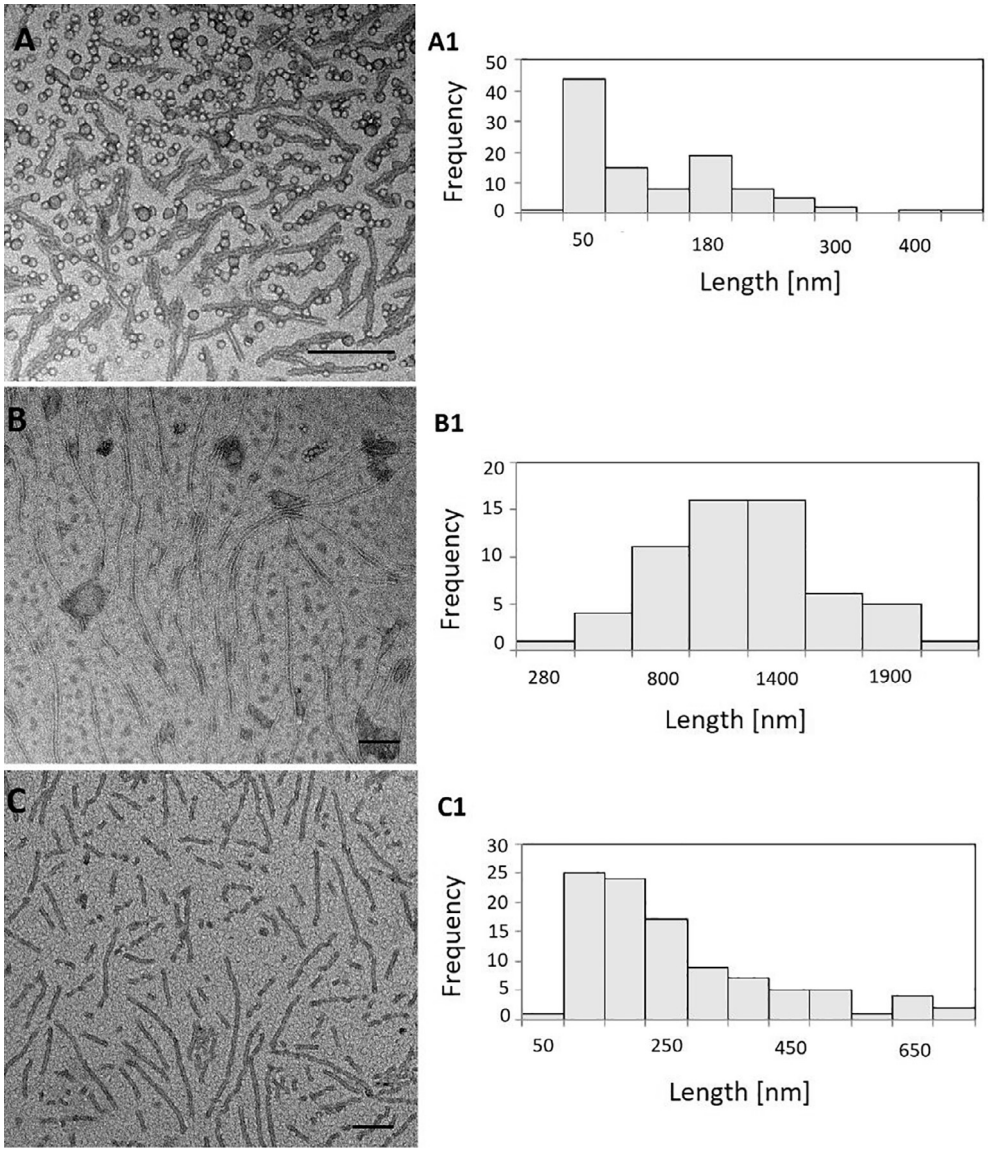

Fig. 4. TEM images and histograms of length of micelles obtained from mixture of PLA-Jeff-FA and $PLA_{2300}PEG_{2000}$ (A, A1), $PLA_{3000}PEG_{2000}$ (B, B1) or $PLA_{5500}PEG_{5000}$ (C, C1). Scale bars indicate 200 nm.

Table 2
Encapsulation efficiency and loading content data of betulin derivative in PLA-PEG/PLA-Jeff-FA micelles (Data represent mean value ± S.D., n = 3).

Copolymer	Initial loading (wt-%)	LC [%]	EE [%]
$PLA_{2300}PEG_{2000}$	20	19.7 ± 1.2	99.5 ± 2.2
$PLA_{3000}PEG_{2000}$	20	20.0 ± 0.3	100 ± 1.3
$PLA_{5500}PEG_{5000}$	20	20.0 ± 0.7	100 ± 1.5

model for all micelles. Also, the value of *m* was lower than 0.43 in the each case, which indicates that drug release was controlled mostly by Fickian diffusion (Peppas and Sahlin, 1989).

3.5. Biocompatibility of PLA-PEG/PLA-Jeff-FA micelles

The effect of drug-free micelles on the growth of HeLa cells is shown in Fig. 6. The blank filomicelles were analyzed at concentrations corresponding to the drug loaded micelles (2.4–74 μg/mL). Proliferation of cells cultured with micelles was comparable with the control. Even the highest concentration of all PLA-PEG/PLA-PEG-FA micelles did not affect cell proliferation.

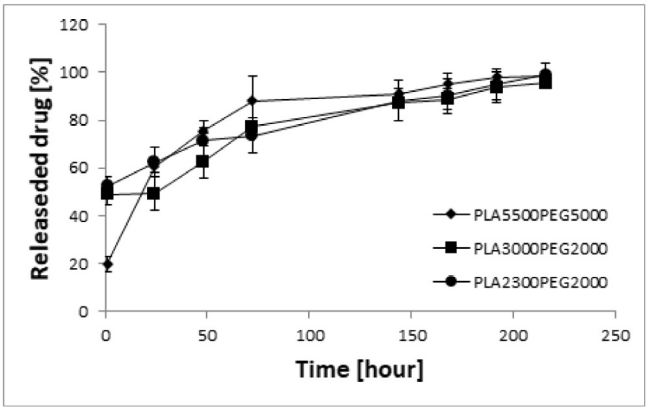

Fig. 5. *In vitro* release of betulin derivative from PLA-PEG/PLA-Jeff-FA micelles (S.D. shown as error bars, n = 3).

Table 3
Model parameters of betulin derivative release from PLA-PEG/PLA-Jeff-FA micelles.

Model	Type of micelles			
	Parameter	$PLA_{2300}PEG_{2000}$	$PLA_{3000}PEG_{2000}$	$PLA_{5500}PEG_{5000}$
Higuchi	$R^2_{adjusted}$	0.5437	0.6710	0.8105
Peppas-Sahlin	$R^2_{adjusted}$	0.9656	0.9279	0.9950
	m	0.10	0.13	0.41

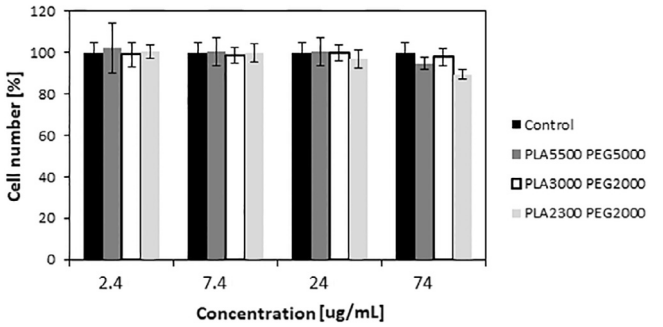

Fig. 6. Effect of PLA-PEG/PLA-Jeff-FA drug-free micelles on proliferation of HeLa cells ($P < 0.05$ versus the control group).

The lack of cytotoxicity of drug free micelles was also confirmed by means of lactate dehydrogenase (LDH) assay.

3.6. In vitro cytotoxic activity of free betulin derivative

The effect of free betulin derivative on the proliferation of HeLa cells was analyzed in the range of 1 μM–30 μM by means of sulforhodamine B based assay. As shown in Fig. 7, the lowest concentration (1 μM) did not affect the number of cells. Higher concentrations (2.5 μM–30 μM) significantly reduced cell proliferation. Very strong inhibition of cell growth was observed for drug concentrations above 10 μM. Nevertheless, the cytotoxic effect of 10–30 μM of betulin derivative was comparable.

3.7. In vitro cytotoxic activity of betulin derivative-loaded micelles

The effect of betulin derivative-loaded micelles on cell growth was tested by means of the SRB assay (Fig. 8). In each group, the decrease of cell proliferation was observed in the presence of 3–30 μM of drug. Density of cells cultured with 1 μM of drug in micelles was comparable to the untreated cells, which was similar to free drug (Fig. 7).

The cytotoxicity of drug-loaded micelles was analyzed also with the use of LDH assay, which determines cell membrane damage and the release of LDH into the extracellular environment - a phenomenon typical for cell death (Fig. 9). In the case of PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ micelles loaded with betulin derivative (Fig. 9A), the cytotoxicity significantly increased with increasing concentration from 1 μM to 30 μM. PLA-PEG-FA/$PLA_{2300}PEG_{2000}$ and PLA-PEG-FA/$PLA_{3000}PEG_{2000}$ micelles showed also the lowest effect at 1 μM of betulin derivative (Fig. 9B–C). The cytotoxic effect was significantly higher at 3–30 μM, but there were no significant differences at higher concentrations (3–30 μM).

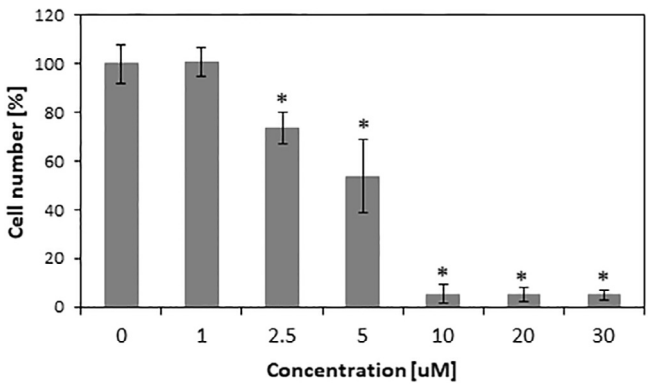

Fig. 7. Effect of free betulin derivative on proliferation of HeLa cells ($P < 0.05$ versus the control group).

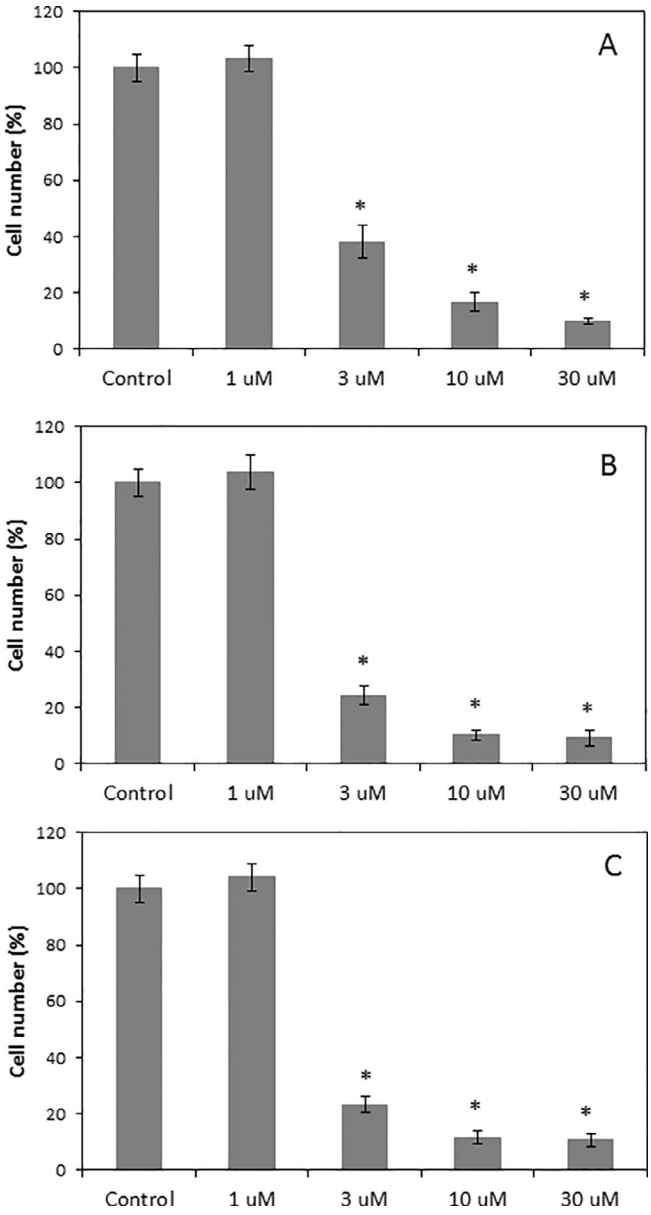

Fig. 8. Effect of betulin derivative-loaded PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ (A), PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ (B) and PLA-Jeff-FA/$PLA_{2300}PEG_{2000}$ (C) micelles on proliferation of HeLa cells ($P < 0.05$ versus the control group).

Activation of HeLa cells apoptosis by all kinds of betulin derivative-loaded PLA-Jeff-FA/PLA-PEG micelles was concentration-dependent (Fig. 10). The most significant apoptotic effect was observed for 10 μM–30 μM of drug.

3.8. Cellular uptake

Fig. 11 shows cellular uptake of PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$

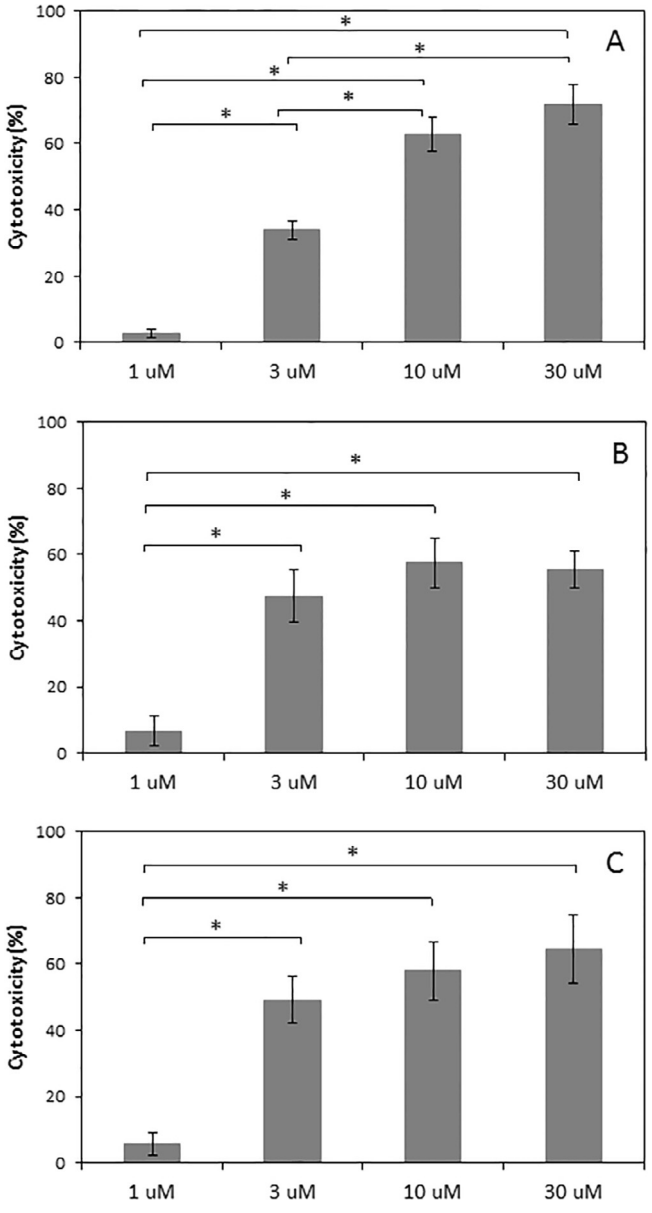

Fig. 9. Cytotoxicity of betulin derivative-loaded PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ (A), PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ (B) and PLA-Jeff-FA/$PLA_{2300}PEG_{2000}$ (C) micelles to HeLa cells (*$P < 0.05$).

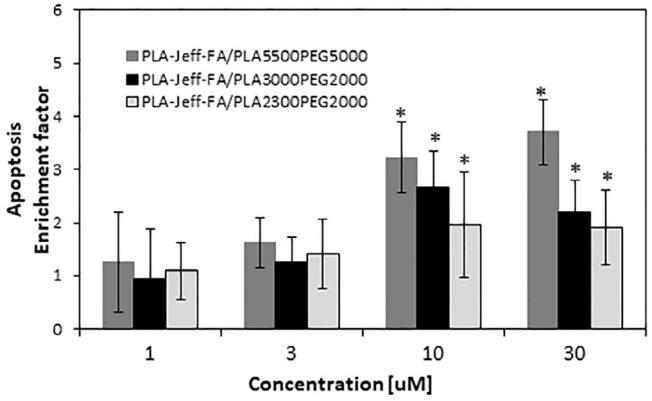

Fig. 10. Effect of betulin derivative-loaded PLA-Jeff-FA/PLA micelles on apoptosis induction in HeLa cells (*$P < 0.05$ *vs.* Control).

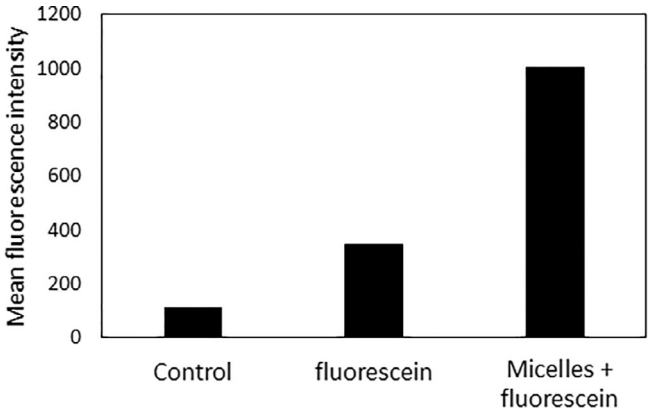

Fig. 11. Cellular uptake of PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ micelles with fluorescein or free fluorescein into HeLa cells after 24 h measured by flow cytometry. Untreated cells were used as control.

micelles with fluorescein or free fluorescein into HeLa cells determined by flow cytometry. The mean fluorescence intensity of the HeLa cells incubated with PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ micelles with fluorescein reached more than 1000, which is much higher than that of cells cultured in the presence of free fluorescein (about 300) and the control with untreated cells (about 100). This proves the successful internalization of PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$.

Intracellular localization of PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ micelles was also evidenced by confocal laser scanning microscopy. As shown in Fig. 12, HeLa cells treated with folate-targeted filomicelles exhibited intense intracellular fluorescence. In contrast, almost no fluorescence was observed in the case of FAR negative cells.

4. Discussion

Filomicelles are very attractive drug delivery systems due to long circulation time and high drug loading capacity (Cai et al., 2007; Loverde et al., 2011). Even more advantageous may be fillomicelles with folic acid as a targeting ligand which should enhance the selectivity and effectivity of the systems (Gruner and Weitman, 1998; Lu and Low, 2002; Parker et al., 2005; Weitman et al., 1992).

There are many methods for preparation of micelles, including particle replication in nonwetting templates, film stretching, self-assembly, crystallization-driven self-assembly, polymerization-induced self-assembly, temperature-induced morphological transformation, and reversible addition-fragmentation chain transfer (RAFT) emulsion polymerization (Esser et al., 2016; Truong et al., 2016a; Truong et al., 2017; Truong et al., 2016b). In the present study, the micelles were obtained by self-assembly using co-solvent evaporation method. In this procedure, diblock copolymer is first dissolved in a good solvent for both blocks. Then, a poor solvent that solubilizes only solvophilic block is added, thus leading to aggregation of solvophobic block and formation of micelles. This is a versatile method widely used for many types of polymers (Truong et al., 2016b).

Filomicelles were obtained from mixture of PLA-Jeff-FA and one of the PLA-PEG copolymers ($PLA_{2300}PEG_{2000}$, $PLA_{3000}PEG_{2000}$, $PLA_{5500}PEG_{5000}$). Exclusively filomicelles were obtained from PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ and PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ (Fig. 4B–C), whereas coexistence of filomicelles and spherical micelles was observed for PLA-Jeff-FA/$PLA_{2300}PEG_{2000}$ (Fig. 4A). In fact, the weight fraction of EO (f_{EO}) is lower for PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ (0.40) compared to $PLA_{2300}PEG_{2000}$ (0.47) (Table 1). With decrease of the f_{EO}, the morphology turns from spherical micelles to filomicelles, and then to bilayered polymerosomes. Thus, coexistence of several morphologies is a common phenomenon in transition regions (Rajagopal et al., 2010; Wu et al., 2013). PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ also exclusively forms

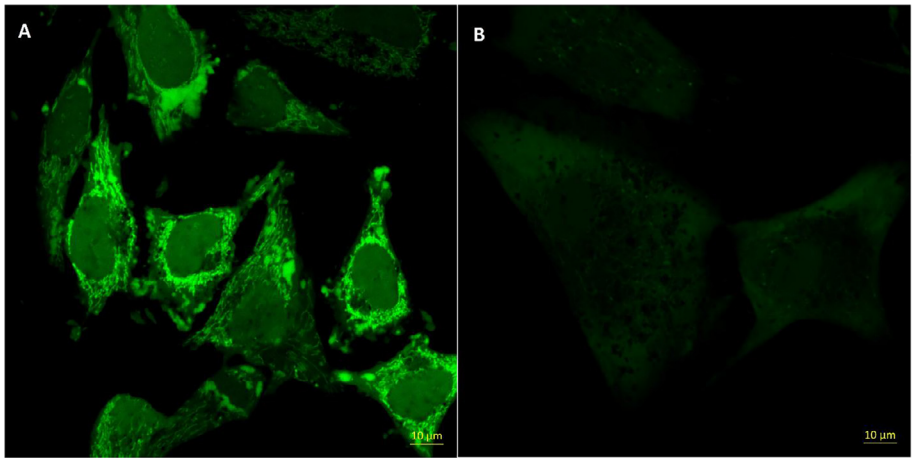

Fig. 12. Confocal images showing the subcellular distribution of PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ micelles with fluorescein after 24 h incubation with HeLa cells (A) and normal human connective tissue cells (B).

filomicelles, although it has similar f_{EO} to PLA-Jeff-FA/$PLA_{2300}PEG_{2000}$. In fact, apart from the weight fraction of PEG block, the molar mass of PEG is also an important factor that affects self-assembling structures (Jelonek et al., 2015). These findings well agree with our previous study on the self-assembled micelles from PLA-PEG copolymers (Jelonek et al., 2015), which suggests that addition of PLA-Jeff-FA does not change micelles' morphology.

Phosphate betulin derivative was incorporated into the micelles (Boryczka et al., 2015). All micelles exhibited quantitative encapsulation efficiency (≈100%) (Table 2). The EE is much higher than that observed for paclitaxel under similar conditions (Jelonek et al., 2015), which could be attributed to the lower molar mass of betulin derivative (Mn = 646.85 g/mol) compared to that of paclitaxel (Mn = 853.91 g/mol). Although a very rapid release of the active agent from all micelles was observed after 1 h, the release process lasted for over 216 h (Fig. 5). The lowest burst effect was observed for PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ (20%) compared to the PLA-Jeff-FA/$PLA_{2300}PEG_{2000}$ (52%) and PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ (48%). It has been demonstrated that micelles with longer PLA block length present higher encapsulation efficiency, drug loading and slower drug release process compared to micelles with shorter hydrophobic block (Jelonek et al., 2015; Yang et al., 2010). Therefore, PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ micelles with longer PLA chain exhibit slower initial release of paclitaxel. Beyond 24 h, the release profile of betulin derivative proceeds similarly for all micelles. All release profiles fit well to Peppas-Sahlin kinetic model (Table 3). This model is generally used to analyze the release of pharmaceutical dosage forms when the release mechanism is not well known or when more than one type of release phenomena could be involved (Costa et al., 2001). The release profiles from the three micelle systems are characterized by different diffusion exponents (m). The m value was lower than 0.43 for all micelles, which indicates drug release mostly controlled by Fickian diffusion (Peppas and Sahlin, 1989). The lowest diffusion exponent was observed for PLA-Jeff-FA/$PLA_{2300}PEG_{2000}$, and the highest for PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ (m = 0.414) probably due to contribution of longer PLA block to the release process. In fact, drug release from micelles occurs through mechanisms such as diffusion and core degradation and therefore, the properties of the hydrophobic block (e.g., its composition and affinity to a drug) control both drug entrapment and drug release (Glavas et al., 2013).

The successful internalization of PLA-Jeff-FA/PLAPEG micelles by FAR-positive HeLa cell line was confirmed by flow cytometry and confocal laser scanning microscopy (Figs. 11–12).

Cytotoxicity of free betulin derivative and betulin derivative-loaded micelles was analyzed on HeLa cell line (Figs. 7–9). The lowest concentration of free betulin derivative did not affect the viability of cells and its toxicity increase with concentration from 2.5 µM to 10 µM (Fig. 7). Very strong inhibition of cell growth was observed at concentrations of 10–30 µM, but the cytotoxic effect was comparable in this range and did not increase with concentration.

Similar to the effect of free drug (Fig. 7), also the betulin derivative-loaded micelles did not affect cell viability at the concentration of 1 µM (Fig. 8). In each group, the decrease of cell proliferation was observed in the presence of drug at concentrations of 3–10 µM (Fig. 8). However, some differences were observed between micelles in their effect to HeLa cells. The effect of 3 µM of betulin derivative was stronger when delivered in PLA-Jeff-FA/$PLA_{2300}PEG_{2000}$ and PLA-Jeff-FA/$PLA_{3000}PEG_{2000}$ compared to PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ (Figs. 8 and 9). This fact may be caused by differences in drug release (Fig. 5) – slower release of betulin derivative from PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$ in the initial period may result in lower cytotoxicity. This difference is observed only for lower drug amount, because cytotoxicity of betulin derivative at concentrations above 3 µM is similar for all micelles (Figs. 8 and 9).

All kinds of betulin derivative-loaded PLA-Jeff-FA/PLA-PEG micelles induced significant apoptosis at concentration 10 µM–30 µM (Fig. 10), which explains their cytotoxic activity against HeLa cells.

Importantly, all drug-free micelles do not affect cell viability (Fig. 6), which confirms their usefulness for targeted cancer therapy.

5. Conclusion

This work aimed to develop filomicelles for targeted delivery of phosphate betulin derivative. Folic acid was successfully attached to PLA-Jeff copolymer, and the resulting PLA-Jeff-FA was used in combination with PLA-PEG copolymers to prepare micelles. TEM analysis revealed that filomicelles were exclusively obtained from PLA-Jeff-FA and $PLA_{5500}PEG_{5000}$ or $PLA_{3000}PEG_{2000}$, whereas coexistence of filomicelles and spherical micelles was observed from PLA-Jeff-FA/$PLA_{2300}PEG_{2000}$. Quantitative encapsulation of betulin derivative was obtained for all micelles. The drug release profiles well fitted to the Peppas-Sahlin kinetic model, showing that drug release was mostly controlled by Fickian diffusion. Significantly lower burst release was observed for PLA-Jeff-FA/$PLA_{5500}PEG_{5000}$. This finding indicates that longer PLA chain favors drug entrapment inside the micelles, in agreement with the higher diffusion coefficient as compared to the other two copolymer micelles.

Drug-free micelles do not affect the viability of cells. In contrast, the cytotoxicity of betulin derivative loaded micelles against FAR-positive HeLa cells was confirmed at concentrations above 3 µM. Effective cell uptake should benefit efficient and targeted delivery of betuline derivative.

Acknowledgement

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