A Biodegradable Diblcok Copolymer Poly(ethylene glycol)-*block*-poly(L-lactide-*co*-2-methyl-2-carboxyl-propylene carbonate): Docetaxel and RGD Conjugation

Zhigang Xie,[1,2] Xiuli Hu,[1,2] Xuesi Chen,[1] Tiancheng Lu,[1,2] Shi Liu,[1] Xiabin Jing[1]

[1]*State Key Laboratory of Polymer Physics and Chemistry, Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, Changchun 130022, People's Republic of China*
[2]*Graduate School of Chinese Academy of Sciences, Beijing 100039, People's Republic of China*

Received 4 January 2008; accepted 15 June 2008
DOI 10.1002/app.28900
Published online 3 September 2008 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A diblcok copolymer monomethoxy poly (ethylene glycol)-*block*-poly(L-lactide-*co*-2-methyl-2-carboxyl-propylene carbonate) (MPEG-*b*-P(LA-*co*-MCC)) was obtained by copolymerization of L-lactide (LA) and 2-methyl-2-benzoxycarbonyl-propylene carbonate (MBC) and subsequent catalytic hydrogenation. The pendant carboxyl groups of the copolymer MPEG-*b*-P(LA-*co*-MCC) were conjugated with antitumor drug docetaxel and tripeptide arginine-glycine-aspartic acid (RGD), respectively. ^{1}H-NMR spectra confirmed the structure of the copolymer MPEG-*b*-P(LA-*co*-MCC/docetaxel) and MPEG-*b*-P(LA-*co*-MCC/RGD). *In vitro* antitumor assay indicates that the MPEG-*b*-P(LA-*co*-MCC/docetaxel) conjugate shows high cytotoxic activity against HeLa cancer cells. Cell adhesion and spreading experiment shows that copolymer MPEG-*b*-P(LA-*co*-MCC/RGD) is of benefit to cell adherence and is a promising biodegradable material for cell and tissue engineering. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 110: 2961–2970, 2008

Key words: biodegradable; block copolymers; docetaxel; RGD

INTRODUCTION

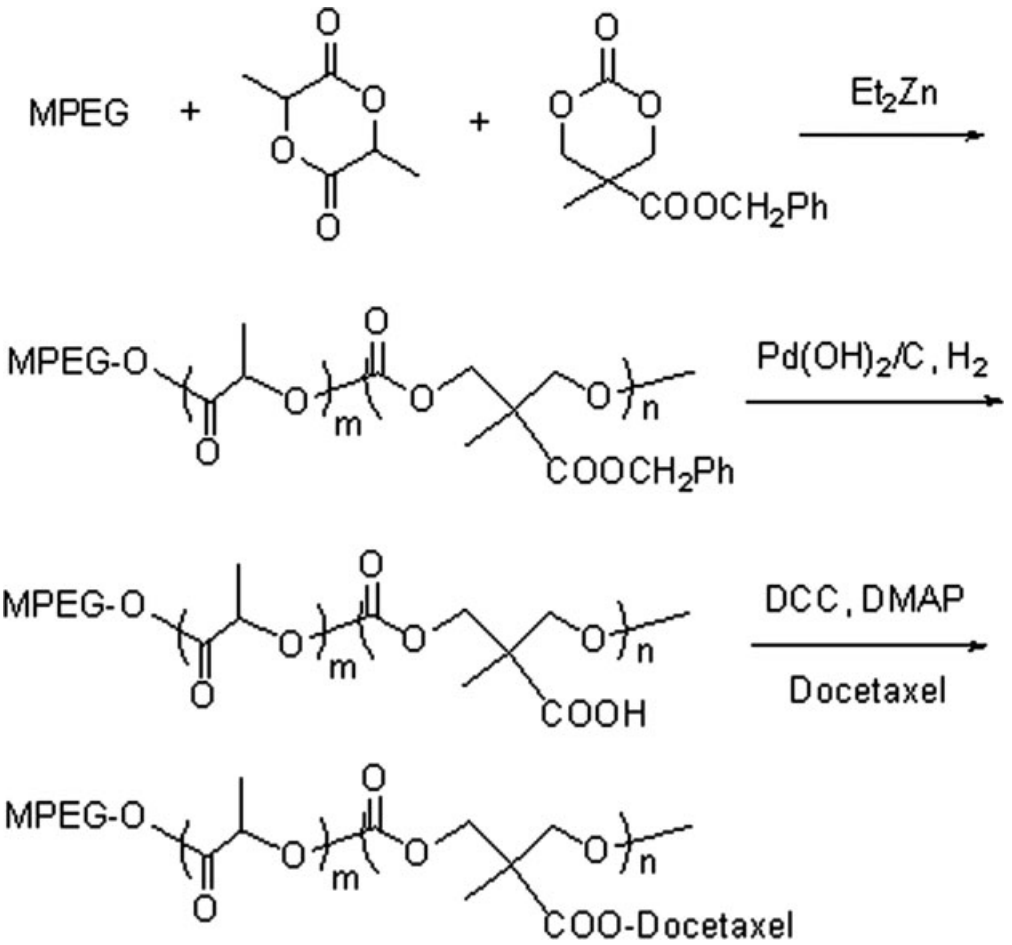
Recently, biodegradable polylactides (PLA) containing pendant functional or reactive groups have gained increasing interest in biomaterial fields.[1–6] Incorporation of functional pendant groups along the polymer backbone would be a highly efficient means of tailoring the properties of polyesters, such as hydrophilicity, biodegradation rates, bioadhesion, etc. Moreover, the functional or reactive groups could be reacted with biologically active moieties, such as antitumor drugs and short peptides.

In our previous works, several PLA-based copolymers with pendant functional groups have been prepared.[7–10] Furthermore, the functional groups were conjugated with drugs and short peptides for the use in drug delivery and tissue engineering, respectively.[11–13] For example, we grafted RGD peptide on triblock copolymers poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-glutamic acid) and poly(ethylene glycol)-*b*-poly(L-lactide)-*b*-poly(L-lysine acid), respectively, however, the block structure between PLA and poly(amino acid) may lead to partial distribution of RGD in the copolymer. We also conjugated the antitumor drug paclitaxel with the side carboxyl groups of copolymers poly{(lactic acid)-*co*-[(glycolic acid)-*alt*-(L-glutamic acid)]}-*block*-poly(ethylene glycol)-*block*-poly{(lactic acid)-*co*-[(glycolic acid)-*alt*-(L-glutamic acid)]}, but the parent copolymer is difficult to prepare because of low yield for preparation of (3s)-benzoxylcarbonylethyl-morpholine-2,5-dione.

As we pointed out in a previous article, compared to other approaches such as morpholine-2,5-dione and *N*-carboxyanhydride derivatives, functionalized cyclic carbonates are synthesized and polymerized more easily and more efficiently. So a diblock copolymer monomethoxy poly(ethylene glycol)-*block*-poly(L-lactide-*co*-2-methyl-2-carboxyl-propylene carbonate) (MPEG-*b*-P(LA-*co*-MCC)) was synthesized by copolymerization of LA and 2-methyl-2-benzoxycarbonyl-propylene carbonate (MBC) and subsequent deprotection.[14] Incorporation of PEG into PLA not only increase the hydrophilicity, but also minimize protein adsorption and decrease nonspecific cell adhesion.[15–18] The microstructural analysis of the copolymer by ^{13}C-NMR revealed the random sequence of LA and MBC units.

In this work, we studied the possibility of further chemical modification by using the pendant carboxyl groups. The antitumor drug docetaxel and tripeptide

Correspondence to: X. Jing (xbjing@ciac.jl.cn).
Contract grant sponsor: National Natural Science Foundation of China; contract grant number: 50373043.
Contract grant sponsor: National Fund for the Distinguished Young Scholars; contract grant number: 50425309.



Scheme 1 Synthesis of MPEG-*b*-P(LA-*co*-MCC) and MPEG-*b*-P(LA-*co*-MCC/docetaxel).

RGD were chosen as the target matter to do it. The ^{1}H-NMR spectra analysis and *in vitro* cell experiment was used to characterize the structure and cytotoxicity of the polymer, respectively.

EXPERIMENTAL SECTION

Materials

Docetaxel was purchased from Xi'an Baosai Biotechnology in China. *N*-hydroxylsuccinimide (NHS), and dicyclohexylcarbodiimide (DCC) from GL Biochem (Shanghai in China) Ltd., were used as received. (Dimethyl-amino)pyridine (DMAP, 99%) obtained from Acros, Palladium hydroxide on activated charcoal ($Pd(OH)_2/C$, 20%) supplied by Xi'an Kaida Chemical, Ltd. and RGD purchased from CL Bioscientific (Xi'an, China) were used without further purification. Dichloromethane was treated with hexamethylene diisocyanate for 5 h at 50°C and distilled to remove any traces of amine and alcohol. *N*,*N*-dimethylformamide (DMF) was dried over CaH_2 and then distilled under reduced pressure before use. Other reagents and solvents were commercially available and directly used without further purification.

Measurements

^{1}H-NMR spectra were measured by a Unity-400 NMR spectrometer at room temperature, with

TABLE I
Molecular Weight and Distribution of Copolymers MPEG-*b*-P(LA-*co*-MCC)

Samples	M_n (^{1}H-NMR)	MBC[a] (mol %)	M_n (GPC)	M_w/M_n (GPC)
MPEG5K-P(LA-*co*-MCC)20K	25,000	2.5	37,000	1.53
MPEG5K-P(LA-*co*-MCC)40K	45,000	4.0	56,000	1.48

[a] Molar ratio of MBC to (LA+MBC) in copolymer, determined by ^{1}H-NMR.

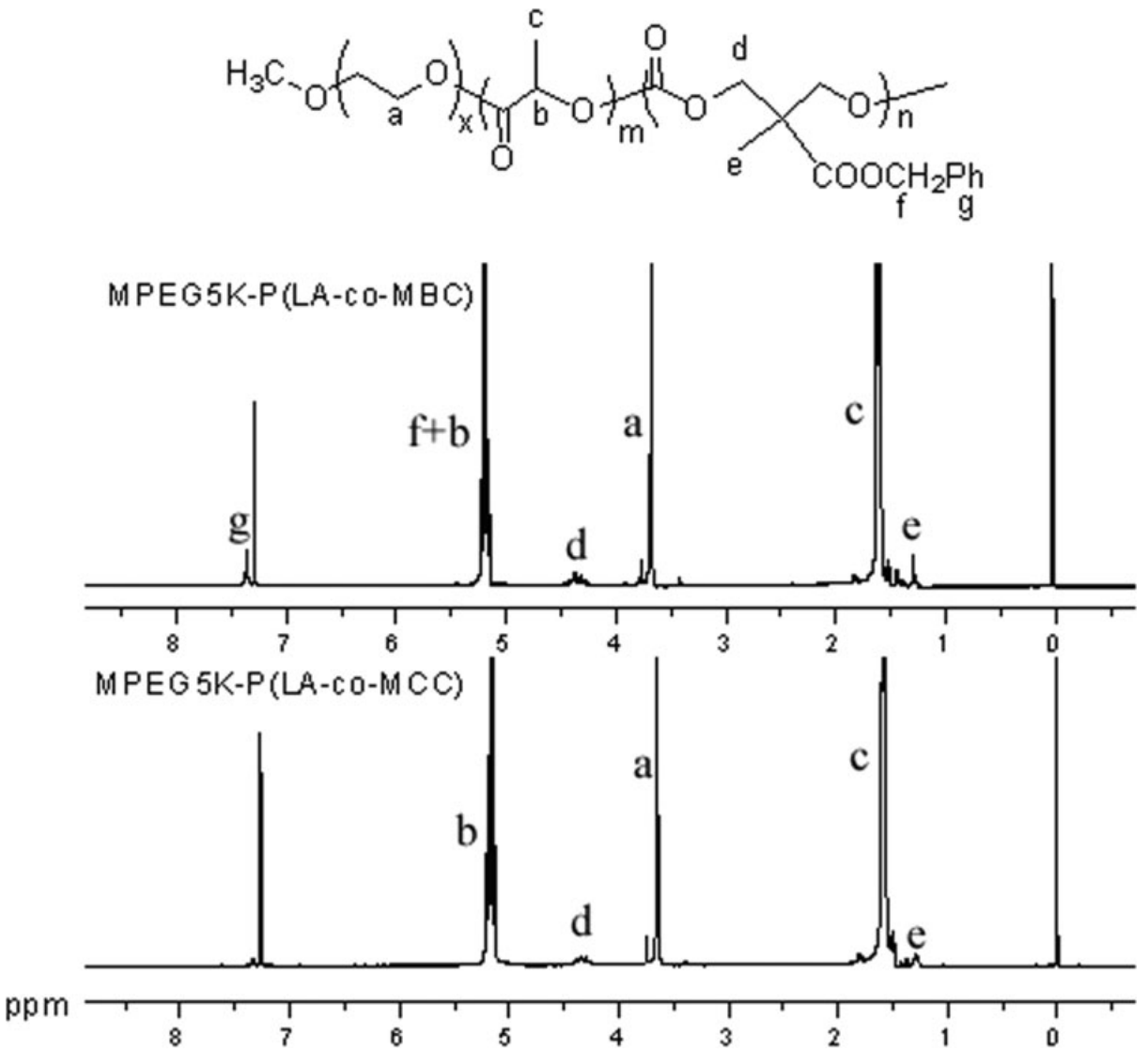


Figure 1 ^{1}H-NMR spectra of MPEG5K-P(LA-*co*-MBC) and MPEG5K-P(LA-*co*-MCC).

$CDCl_3$ or DMSO-*d*6 as solvent and TMS as internal reference. The GPC measurements were conducted at 35°C with a Waters 410 GPC instrument equipped with two Waters Styragel columns (HT6E, HT3) and a differential refractometer detector. $CHCl_3$ was used as eluent at a flow rate of 1.0 mL min^{-1}. The molecular weights were calibrated with polystyrene standards.

Synthesis of MPEG-*b*-P(LA-*co*-MCC)

The copolymer MPEG-*b*-P(LA-*co*-MCC) was prepared by ring-opening polymerization of LA and MBC in the presence of monomethoxy PEG (MPEG) as a macroinitiator and subsequent hydrogenation, as shown in Scheme 1. The experimental details have been described in a previous article.[14]

Synthesis of MPEG-*b*-P(LA-*co*-MCC/docetaxel)

In a dried flask, 0.21 g MPEG5K-P(LA-*co*-MCC)20K (Table I) was dissolved in 20 mL anhydrous dichloromethane and then 23 mg docetaxel, 5.7 mg DCC, and 3.5 mg DMAP were added into the above solution at 0°C. The reaction was carried out under stirring for 48 h at 0°C. The byproduct dicyclohexylurea was filtered out. The filtrate was condensed under a reduced pressure and poured into an excess amount of diethyl ether with stirring. The MPEG-*b*-P(LA-*co*-MCC/docetaxel) was collected by filtration and washed with methanol three times, and finally dried in vacuum at room temperature overnight. Purification of the conjugate from unreacted free docetaxel was done by dialysis in chloroform with a cellulose membrane (cutoff M_n = 5000) for 2 days (yield: 83%).

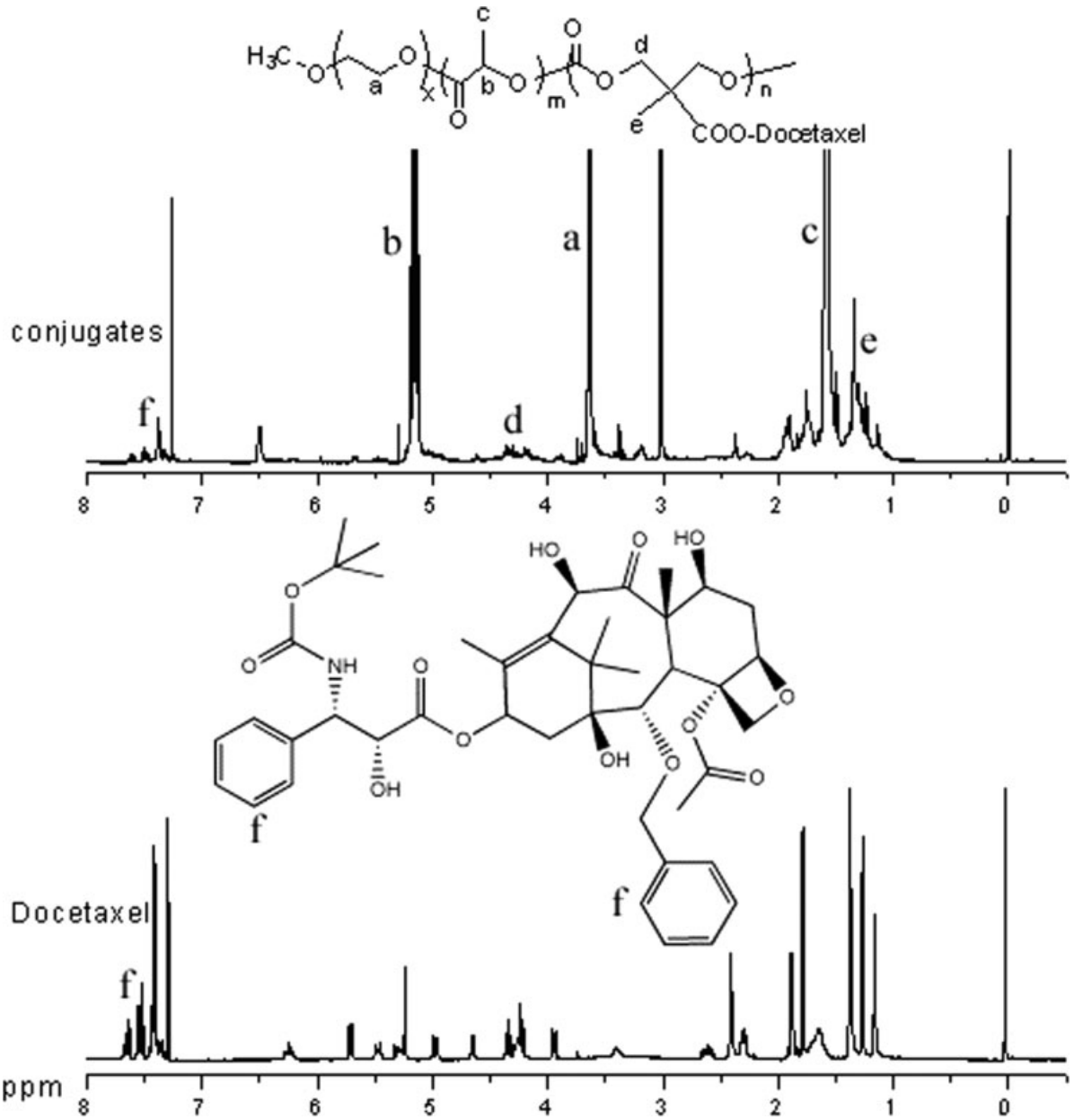


Figure 2 ^{1}H-NMR spectra of docetaxel and MPEG-*b*-P(LA-*co*-MCC/docetaxel) in $CDCl_3$.

In vitro cytotoxicity assay

The cytotoxicity activity of the docetaxel conjugate was evaluated by MTT method.[19] Woman HeLa cancer cells were chosen as target cells. They were cultured in the growth medium DMEM containing 10% fetal bovine serum (FBS), 2.0 m*M* glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin, and the cell density of the cell suspension obtained was adjusted to 5×10^4 cells/mL. Two hundred microliters of aliquots of this suspension were added to the wells in a 96-well plate and incubated for 24 h in a humidified atmosphere containing 5% CO_2 at 37°C. The conjugate used was made of MPEG-*b*-P(LA-*co*-MCC/docetaxel). It was dissolved in DMSO at a proper concentration. Diluted 200-folds with cell culture medium DMEM 200 μL of the aliquot was added into each well. After 48 h incubation, a 20 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/mL) was added to each well of the plate. The incubation was continued for another 4 h. Then the MTT derivative of the solution was dissolved in 150 μL of DMSO and the solution was determined by a Microplate Reader (Thermo, MK3) at 492 nm. The relative cell viability was calculated and averaged.

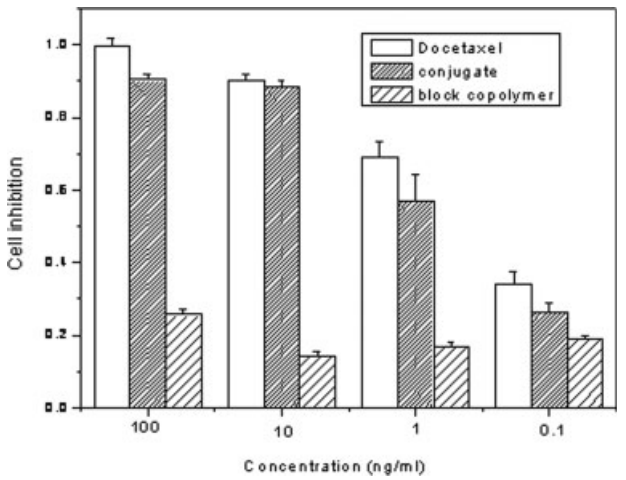


Figure 3 *In vitro* cytotoxicity of MPEG-*b*-P(LA-*co*-MCC/docetaxel) conjugate against human HeLa cancer cells. The general test procedures are described in the text. Cell density: 5×10^4 Cells/well.

Synthesis of MPEG-*b*-P(LA-*co*-MCC/RGD)

As shown in Scheme 2, the RGD was grafted onto the MPEG-*b*-P(LA-*co*-MCC) by first activating the side chain carboxyl groups of the MPEG-*b*-P(LA-*co*-MCC) with NHS and then coupling with the RGD. In a dried flask, 0.18 g MPEG5K-P(LA-*co*-MCC)40K (Table I) was dissolved in 10 mL anhydrous DMF and then 11 mg DCC and 6 mg NHS were added into the above solution at 0°C. The reaction was carried out under stirring for 48 h at 0°C. The byproduct dicyclohexylurea was filtered out, and the filtrate was cooled by ice-water. After adding 35 mg RGD, the reaction mixture was stirred at room temperature for another 48 h. The product mixture was precipitated in an excessive diethyl ether and then dialyzed in DMF with a cellulose membrane (cutoff $M_n = 5000$) for 48 h (yield: 78%).

Preparation of test films

The RGD-grafted copolymer was tested in the form of films. The MPEG-*b*-P(LA-*co*-MCC/RGD) was dissolved in DMF, and cast on round cover slides (15.5 mm in diameter). The copolymer films of MPEG-*b*-P(LA-*co*-MCC) and PLLA were prepared in a similar way and were used as control. The slides were kept for 48 h or more to remove the last traces of DMF and exposed to UV light for 30 min for sterilization.

Cell adhesion and spreading

Cell attachment and cell morphology on MPEG-*b*-P(LA-*co*-MCC/RGD), MPEG-*b*-P(LA-*co*-MCC), and PLLA films at different time intervals were studied. The cover slides coated with the polymer film were placed in each well of 96-well tissue culture plates

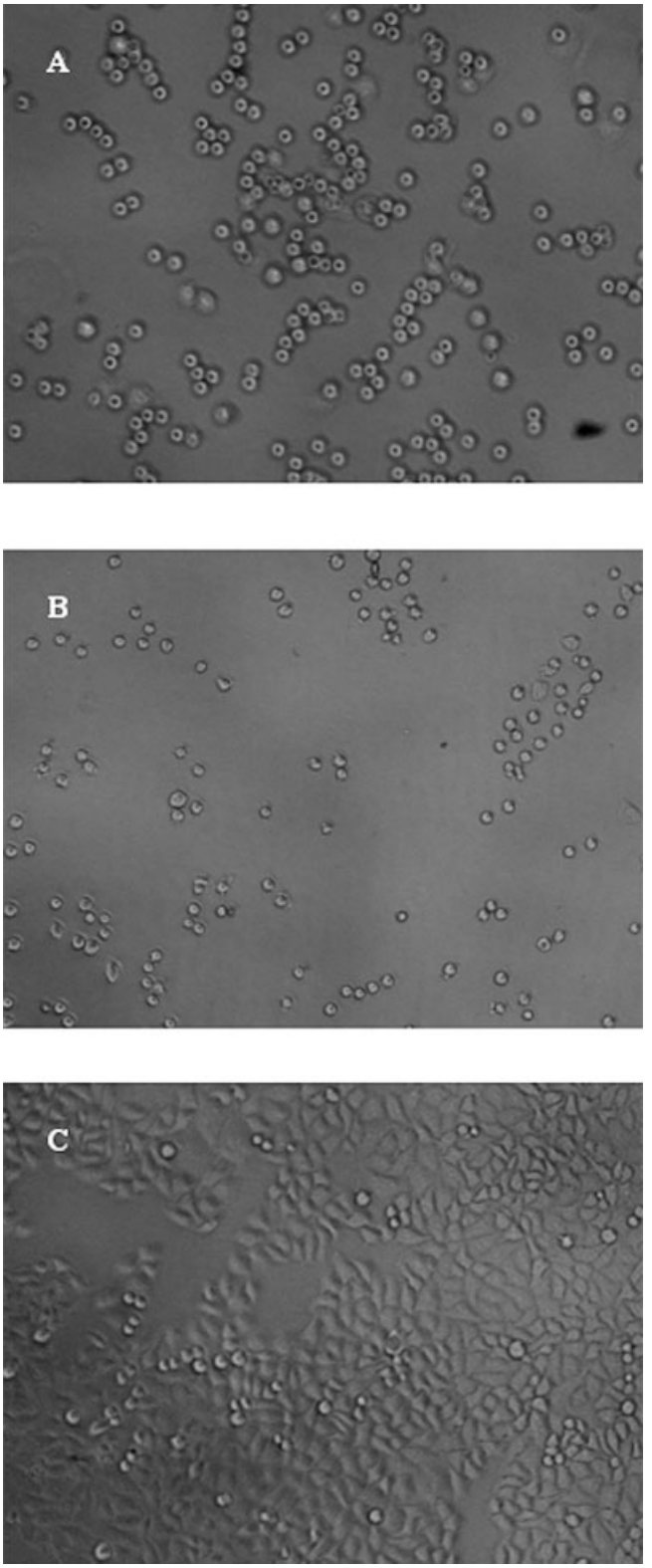
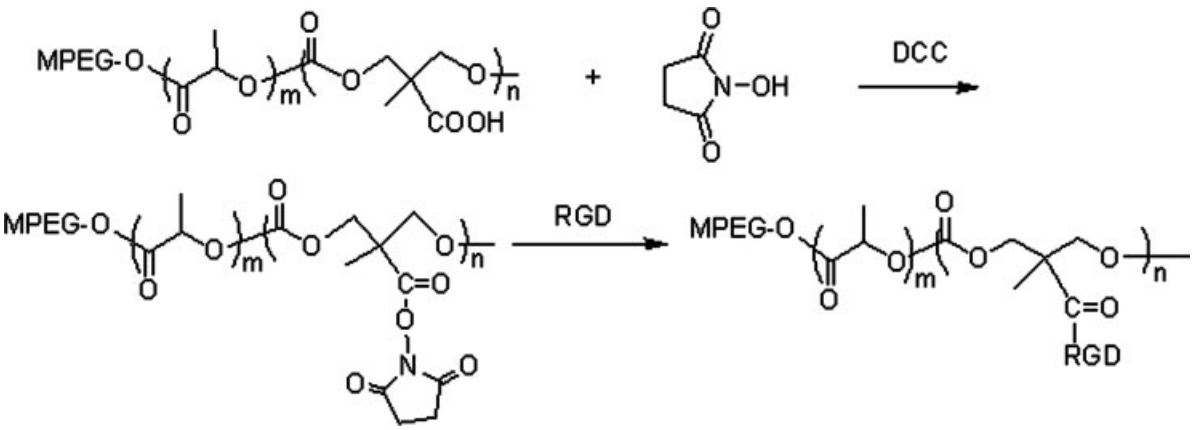


Figure 4 Typical micrographs of the cells after incubation for 48 h in the presence of (A) conjugates MPEG-*b*-P(LA-*co*-MCC/docetaxel); (B) pure docetaxel; (C) Copolymer MPEG-*b*-P(LA-*co*-MCC).



Scheme 2 Synthesis of copolymer MPEG-*b*-P(LA-*co*-MCC/RGD).

(NUNC). The ECV cells were seeded on the cover slides at a density of 2.5×10^4 cells/well, incubated in a humidified incubator at 37°C and 5% CO_2. The pictures of each cover slide were taken with a digital camera (DXM1200F, Nikon) after 3, 24, and 48 h, respectively.

RESULTS AND DISCUSSION

Synthesis of MPEG-*b*-P(LA-*co*-MCC)

As shown in Scheme 1, copolymers MPEG-*b*-P(LA-*co*-MCC) were prepared in following two step: (1) LA and MBC were copolymerized with diethyl zinc (Et_2Zn) as catalyst in the presence of MPEG with molecular weight 5000 as a macroinitiator. (2) The copolymer was deprotected by catalytic hydrogenolysis using H_2 over $Pd(OH)_2/C$ (20%) as catalyst in a mixture solvent of THF/methanol. The molecular structures of the copolymers MPEG-*b*-P(LA-*co*-MBC) and MPEG-*b*-P(LA-*co*-MCC) were confirmed by their ^{1}H-NMR spectra (Fig. 1). On comparing these two spectra it is very clear that the proton signals of C_6H_5 in the copolymer MPEG-*b*-P(LA-*co*-MCC) at 7.30 ppm disappeared, whereas other proton signals were little changed.

Here, two copolymers with different molecular weights were prepared by changing the feed composition. The block copolymers are denoted as MPEG5K-P(LA-*co*-MCC)20K and MPEG5K-P(LA-*co*-MCC)40K according to their molecular weights. They were used to conjugate with docetaxel and RGD, respectively. As shown in Table I, the molecular weight of copolymer calculated by ^{1}H-NMR is lower than that determined by GPC.

Synthesis of MPEG-*b*-P(LA-*co*-MCC/docetaxel)

As mentioned in previous articles,[13] the 2′-hydroxy in paclitaxel and docetaxel is more active than others, and thus esterification could take place between the pendant carboxyl groups of MPEG-*b*-P(LA-*co*-MCC) and the 2′-hydroxy of docetaxel. In fact, as shown in Scheme 1, the esterification was realized in the presence of DCC and DMAP at 0°C. Figure 2 shows the ^{1}H-NMR spectra of free docetaxel and the conjugates MPEG-*b*-P(LA-*co*-MCC/docetaxel). It shows that the characteristic peaks of docetaxel can all be found in MPEG-*b*-P(LA-*co*-MCC/docetaxel) conjugate. The signals from 7.3 to 7.8 ppm for phenyl ring protons can be obviously found in the ^{1}H-NMR spectra of MPEG-*b*-P(LA-*co*-MCC/docetaxel) conjugate. Because the unreacted docetaxel could be removed by dialysis, ^{1}H-NMR spectrum indicates that docetaxel had been conjugated successfully with copolymer MPEG-*b*-P(LA-*co*-MCC). As reported in previous work,[13,20] the docetaxel content in the conjugate calculated from the peak intensities of the phenyl proton signals (7.3–7.8 ppm) and methylene proton signal (3.6 ppm) of MPEG in the ^{1}H-NMR spectra was 6% by weight. Compared with the docetaxel weight in feed, we got the efficiency of docetaxel binding as 82%.

In vitro cytotoxicity assay

The cytotoxicity activity of MPEG-*b*-P(LA-*co*-MCC/docetaxel) against the woman HeLa cancer cells was evaluated by MTT method. Three different samples were studied in detail for comparison, including pure docetaxel, MPEG-*b*-P(LA-*co*-MCC/docetaxel) conjugate and block copolymer MPEG-*b*-P(LA-*co*-MCC). Figure 3 shows the cell inhibition rate after 48 h incubation at different concentrations. At the same drug content, the conjugate exhibits almost the same cytotoxicity activity as pure docetaxel. For example, the cell inhibition rates are 0.90 and 0.88 at a drug concentration of 10 ng/mL for the pure docetaxel and the conjugate, respectively. The pure

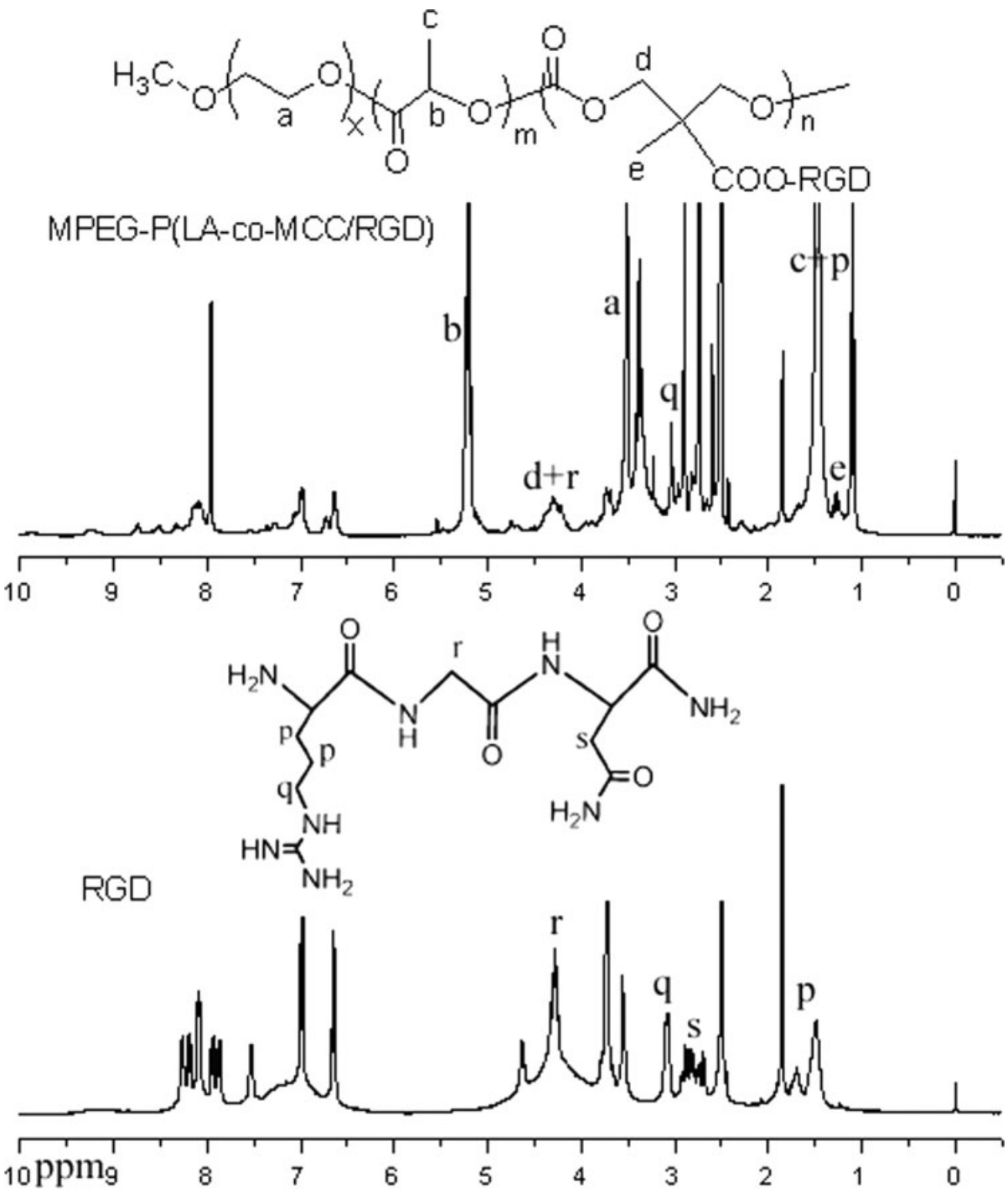


Figure 5 ^{1}H-NMR spectra of MPEG-*b*-P(LA-*co*-MCC/RGD) and RGD in DMSO-*d*6.

copolymer MPEG-*b*-P(LA-*co*-MCC) did not display obvious cytotoxicity to HeLa cells. Moreover, docetaxel and its conjugates show increasing cell inhibition rate with increasing docetaxel concentration from 0.1 to 100 ng/mL. At a docetaxel concentration of 1 ng/mL, both pure docetaxel and MPEG-*b*-P(LA-*co*-MCC/docetaxel) inhibit more than 55% of the HeLa cells. At 100 ng/mL, this rate reaches more than 85% and the difference between pure docetaxel and conjugates is only 5%. As far as cell inhibition rate is concerned, 10 ng/mL may be considered as the lowest effective docetaxel concentration. A similar conclusion has been reported for other polymer-paclitaxel conjugates.[21,22]

The cell morphologies of HeLa cancer cells treated by different samples are shown in Figure 4. The cells cultured with copolymer MPEG-*b*-P(LA-*co*-MCC) grow normally and occupy the whole field.

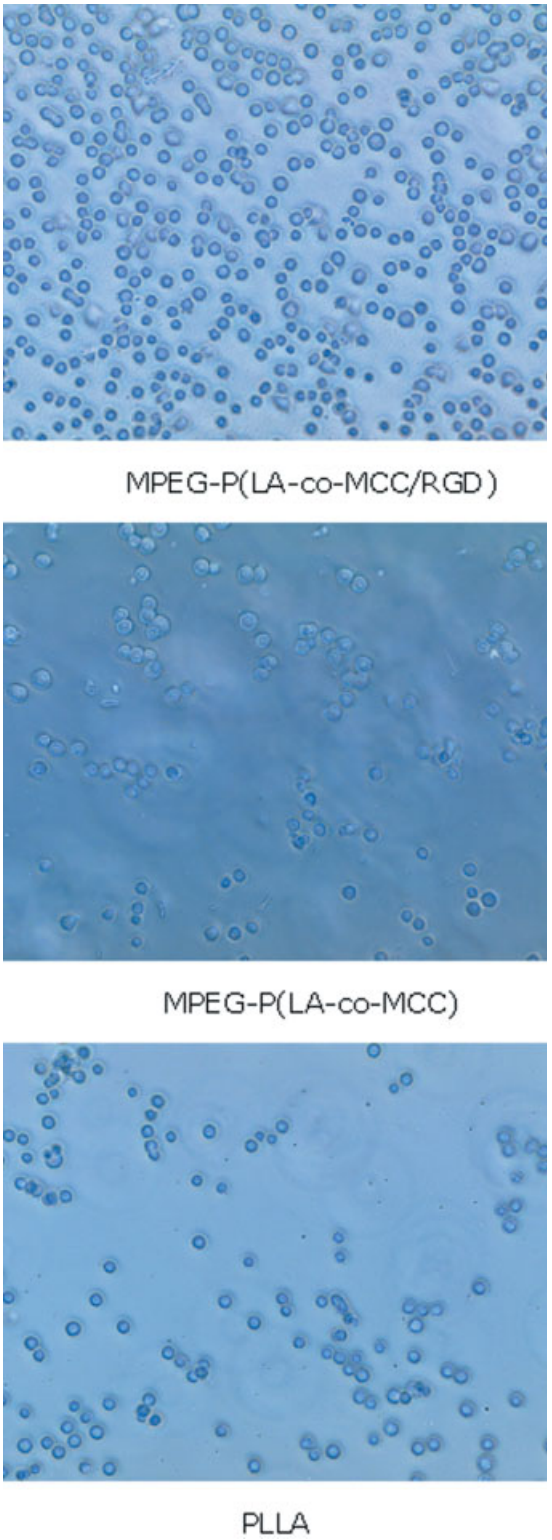


Figure 6 Microscopic images of adhered and spread ECV cells after 3 h of incubation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

However, almost all HeLa cancer cells died in the case of pure docetaxel or MPEG-*b*-P(LA-*co*-MCC/docetaxel) conjugate. From these results, it can be concluded that the MPEG-*b*-P(LA-*co*-MCC/docetaxel) conjugates show high cytotoxicity activity against HeLa cancer cells.

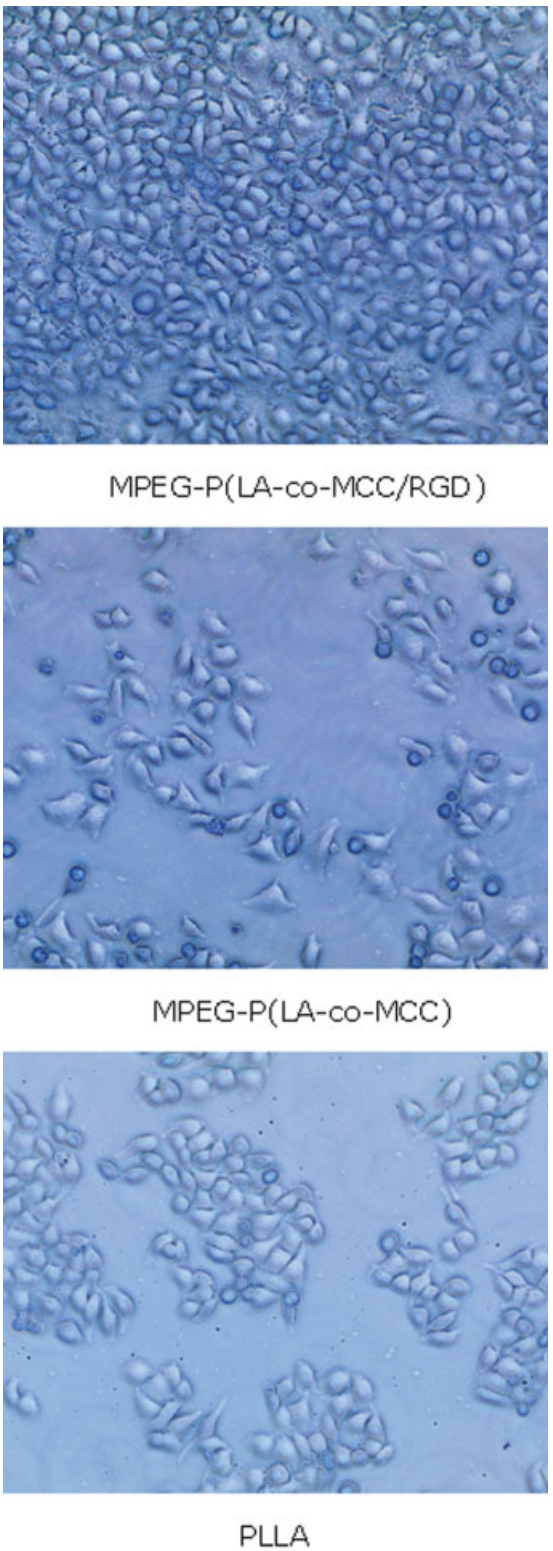


Figure 7 Microscopic images of adhered and spread ECV cells after 24 h of incubation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Synthesis of MPEG-*b*-P(LA-*co*-MCC/RGD)

It is well-known that RGD plays a central role in cell adhesion and has been widely used as modification matter for many polymer materials.[23–28] Jing et al. had reported that RGD can react with the activated carboxyl on the copolymer PEG-PLA-PGL.[11] Herein, we grafted RGD on the copolymer MPEG-*b*-P(LA-*co*-MCC) by using a similar method with previous work,[11] as shown in Scheme 2. It is reported that amino acid sequences containing RGD in a protein often display cell attachment activity, but for a short peptide, this activity can be retained only when the C-terminal of the RGD tripeptide is blocked.[29,30] So we chose RGD-NH_2 in the present study (but it is still abbreviated as RGD in the following text).

Without any protection, it was directly reacted with NHS-activated MPEG-*b*-P(LA-*co*-MCC) to obtain the final product MPEG-*b*-P(LA-*co*-MCC/RGD). Figure 5 shows the ^{1}H-NMR spectra of the free RGD and MPEG-*b*-P(LA-*co*-MCC/RGD) in DMSO-*d*6. It indicates that the characteristic peaks of RGD can all be found in the copolymer MPEG-*b*-P(LA-*co*-MCC/RGD). Especially, appearance of the two doublets at 6.6 and 7.0 ppm belonging to the characteristic protons on RGD confirms successful synthesis of MPEG-*b*-P(LA-*co*-MCC/RGD). The unreacted RGD had been removed by dialysis. Therefore, the RGD had been attached to the copolymer MPEG-*b*-P(LA-*co*-MCC). This result is similar to the previous work.[11]

Cell adhesion and spreading

Cell adhesion of various films was evaluated by culturing ECV cells in a culture medium of DMEM containing 10% of FBS. The test sample was copolymer MPEG-*b*-P(LA-*co*-MCC/RGD) and the control samples were copolymer MPEG-*b*-P(LA-*co*-MCC) and pure PLA. Figure 6 shows the cell morphology on different sample surfaces after 3 h of incubation. It is obvious that the cells on the copolymer MPEG-*b*-P(LA-*co*-MCC/RGD) are much more than those on pure PLA and polymer MPEG-*b*-P(LA-*co*-MCC), which indicates that MPEG-*b*-P(LA-*co*-MCC/RGD) is of benefit to cell adherence and cell spreading. The cell morphologies at the incubation time of 24 and 48 h are shown in Figures 7 and 8, respectively. After incubating for 24 h, almost all the cells on the MPEG-*b*-P(LA-*co*-MCC/RGD) film spread very well, and they are fatter and more than those on pure PLA and MPEG-*b*-P(LA-*co*-MCC) films. After incubating for 48 h, the cells on the MPEG-*b*-P(LA-*co*-MCC/RGD) film almost occupy the whole surface. In short, the cells adhere and spread better and proliferate faster on the RGD-grafted polymers than on the control films without RGD. A similar conclusion has been reported for other RGD-grafted polymers.[11,12] These results indicate that copolymer MPEG-*b*-P(LA-*co*-MCC/RGD) is a promising biodegradable material for cell and tissue engineering.

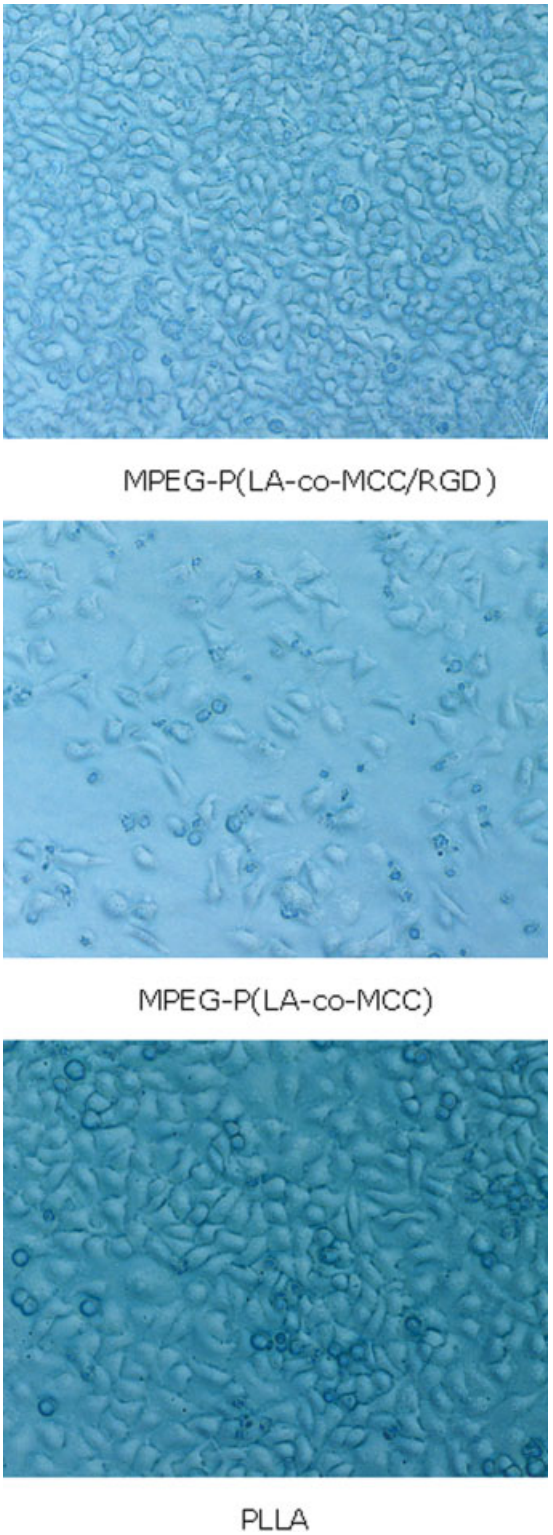


Figure 8 Microscopic images of adhered and spread ECV cells after 48 h of incubation. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CONCLUSIONS

Starting from PEG, a diblcok copolymer MPEG-*b*-P(LA-*co*-MCC) was obtained by copolymerization of LA and MBC and subsequent catalytic hydrogenation. The pendant carboxyl groups of the copolymer MPEG-*b*-P(LA-*co*-MCC) were conjugated with antitumor drug docetaxel and tripeptide RGD, respectively. ^{1}H-NMR spectra confirmed the structure of the copolymer MPEG-*b*-P(LA-*co*-MCC/docetaxel) and MPEG-*b*-P(LA-*co*-MCC/RGD). *In vitro* cytotoxicity assay indicated that the MPEG-*b*-P(LA-*co*-MCC/docetaxel) conjugate showed high cytotoxicity activity against HeLa cancer cells. Cell adhesion and spreading experiment found that copolymer MPEG-*b*-P(LA-*co*-MCC/RGD) is of benefit to cell adherence and might be a promising biodegradable material for cell and tissue engineering.

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