JOURNAL OF Applied Polymer SCIENCE

Application of Microwave-Assisted Click Chemistry in the Preparation of Functionalized Copolymers for Drug Conjugation

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ABSTRACT: The aim of this study is to develop azido-carrying biodegradable polymers and their postfunctionalization with alkynyl compounds via click chemistry and to investigate their potential use in drug delivery. Azido polymers were prepared by ring-opening polymerization of cyclic carbonate monomer, 2,2-bis(azidomethyl)trimethylene carbonate (ATC) with lactide using stannous octoate as catalyst. Several alkynyl compounds were selected to investigate the feasibility and reaction condition of click chemistry. With microwave-assisting, the reaction time of click chemistry was shortened to 5 min. By using poly(ethylene glycol) (PEG) as macroinitiator, amphiphilic block copolymer mPEG-*b*-P(LA-*co*-ATC) was obtained and it could self-assemble into micelles by solvent replacement method. The pendant groups were used for conjugating anticancer drugs gemcitabine and paclitaxel and fluorescent dye Rhodamine B. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was used to assay the cytotoxicity of the conjugate micelles against SKOV-3 and HeLa cell lines. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

KEYWORDS: biodegradable; polyesters; functionalization of polymers; polymer–drug conjugate; click chemistry

Received 20 October 2011; accepted 9 March 2012; published online
DOI: 10.1002/app.37662

INTRODUCTION

Over the last few decades, block copolymers based on aliphatic polycarbonates have attracted great attention because of their biocompatibility, biodegradability, nontoxicity, and potential use in biomedical and pharmaceutical applications.[1,2] Of particular interests are amphiphilic block copolymers, which can self-assemble into polymeric micelles with core-shell architectures.[3] In the course of using polymeric micelles for drug delivery, polymeric prodrug method provides a powerful means for solubilization of hydrophobic drugs, elimination of initial burst drug release, and tuning of drug pharmacokinetics.[4] Thus, various functional monomers were designed and synthesized to construct functional polymers for drug conjugation. Various functional groups such as carboxyl,[5–7] hydroxyl,[8–12] amino,[11,13] propargyl groups,[14] etc., have been incorporated into cyclic carbonate monomers for ring-opening copolymerization.

Click chemistry is proposed by Sharpless and coworkers in 2001[15] and has the following characteristics: (1) high yields, (2) regiospecificity and stereospecificity, (3) insensitivity to oxygen or water, (4) mild reaction conditions, and (5) amenability to a wide variety of readily available starting compounds.[16] The Cu(I)-catalyzed Huisgen 1,3-dipolar cycloaddition reaction between terminal alkynes and azides is one of the most popular and commonly used click chemistry.[17,18] Extensive literature has reported the use of azide/alkyne chemistry to prepare biomedical libraries, dendrimers, functional block copolymers, crosslinking adhesives, derivatization of cellular surfaces, and many others.[19,20] For example, various polymer architectures have been developed by ring-opening polymerization and click chemistry. We have synthesized block copolymers with pendant alkynyl groups by ring-opening copolymerization of lactide and 5-methyl-5-propargyloxycarbonyl-1,3-dioxan-2-one. Sugars and proteins were immobilized on the copolymers via Huisgen's click reaction.[21,22] Recently, microwave-assisted click chemistry has emerged as a powerful means of shortening the reaction time of click polymerization.[23,24]

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine) (GEM) is a nucleoside analogue of deoxycytidine. It is relatively well tolerated when used as a single agent in the treatment of a wide variety of cancers, including lung, colon, head and neck, and ovarian cancers. However, it has obvious drawbacks, particularly its very short plasma half-life after intravenous administration

(8–17 min in human plasma and 9 min in murine plasma),[25] which necessitates the administration of high doses to achieve a required therapeutic response and thus results in significant side effects. To improve the intravenous delivery of GEM and enhance its antitumor activity, various nanoparticles were used for GEM incorporation.[26,27] Paclitaxel (PTX) is one of significant antineoplastic agents, derived from the bark of the Pacific yew tree Taxus brevifolin.[28] It has been shown to exhibit significant activity against various solid tumors, including ovarian, breast, nonsmall cell lung cancer, head and neck carcinomas, etc.[29] But, its low water-solubility (0.25 μg/mL) requires coinjection in a vehicle composed of 1 : 1 blend of Cremophor EL® (polyethoxylated castor oil) and ethanol, which was proved to cause hypersensitivity reactions.[30]

In the present work, we synthesized an azido-carbonate monomer, 2,2-bis(azidomethyl)trimethylene carbonate (ATC). Block copolymers or amphiphilic block copolymers based on ATC and L-lactide (LA) were synthesized. The pendant azido groups on these copolymers were reacted with alkynyl compounds via click reaction to obtain various functional groups. Anticancer drug GEM, PTX, and fluorescent dye Rhodamine B (RhB) were then conjugated to the functionalized mPEG-*b*-P(LA-*co*-ATC). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was used to assay the cytotoxicity of the conjugate micelles against SKOV-3 and HeLa cell lines. These functionalized polymers have potential use in biomedical applications such as drug delivery and diagnosis.

EXPERIMENTAL SECTION

Materials

Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$, Strem Chemicals, 90% in 2-ethylhexanoic acid), ethyl chloroformate (Sigma-Aldrich, 97%), monomethoxyl poly(ethylene glycol) (mPEG, average M_n ~2000 and 5000, Sigma-Aldrich in China) were used as received. L-Lactide was prepared in our own laboratory and recrystallized from ethyl acetate for three times before use. Propargyl alcohol (98%), propiolic acid (98%), propargyl amine (98%), and 4-nitrophenyl chloroformate (NPC) were obtained from Sigma and used as received. Triethylamine (TEA), methylene chloride (CH_2Cl_2), and dimethylformamide (DMF) were dried over CaH_2 and distilled before use. Tetrahydrofuran (THF) and toluene were purified by distillation with sodium and benzophenone. MTT was purchased from Sigma. Trypsin and RPMI 1640 medium were purchased from Gibco BRL, Maryland. Fetal bovine serum (FBS) was purchased from Sijiqing Biologic, China. All other chemicals were of analytical or chromatographic grade.

Synthesis of Azido Carbonate Monomer 2,2-Bis(azidomethyl)trimethylene Carbonate (ATC)

The ATC was prepared according to the literature.[31,32] The final products were white crystals (yield: 78%). ^{1}H-NMR ($CDCl_3$, 300 MHz, ppm with respect to TMS): 4.23 (s, CH_2O, 4H), 3.53(s, CH_2N_3, 4H).

Synthesis of Copolymer P(LA-*co*-ATC) and mPEG-b-P(LA-*co*-ATC)

A mixture of ATC and LA with a specified molar ratio was placed in a thoroughly dried glass flask. The reaction vessel was sealed, evacuated, and purged with nitrogen three times. Next, the reaction vessel was immersed in a thermostated oil bath preheated to 120°C. After 5 min of stirring, a certain amount of the catalyst ($Sn(Oct)_2$ in toluene) was added via a syringe. The reaction was continued at 120°C for 20 h, and then terminated by cooling the vessel to room temperature. The copolymer P(LA-*co*-ATC) obtained was dissolved in dichloromethane and precipitated into excessive cold diethyl ether, isolated by filtration, and dried under vacuum at room temperature.

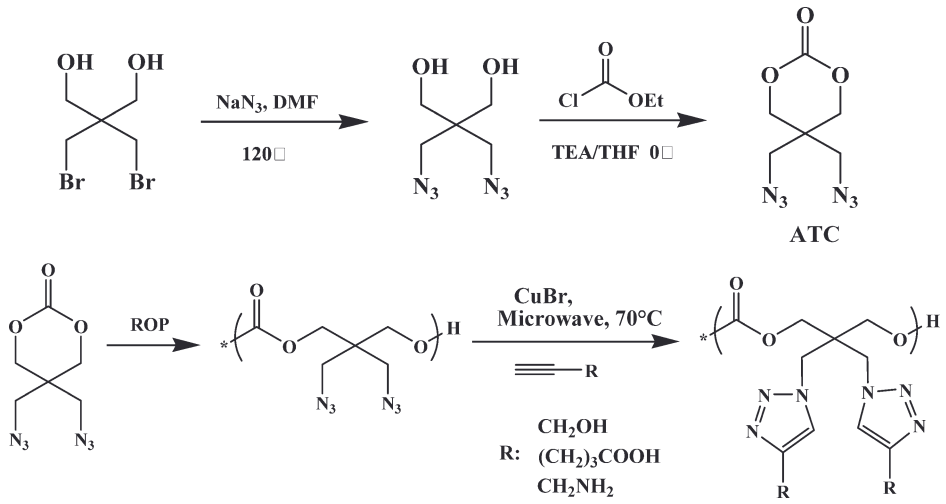
Amphiphilic block copolymer mPEG-*b*-P(LA-*co*-ATC) was synthesized through ring-opening polymerization of LA and ATC in the presence of mPEG as the macroinitiator, with $Sn(Oct)_2$ as the catalyst. A certain amount of mPEG (1.0 g, 0.2 mmol) was dried by azeotropic distillation of toluene for 1 h, a mixture of LA (1.0 g, 6.9 mmol) and ATC (0.368 g, 1.7 mmol) was added into the above system, followed by nitrogen-purging three times. After sealing the system, prescribed amount of $Sn(Oct)_2$ (0.5 mol % of the total monomers) was added using a glass syringe. The reaction mixture was then heated to 120°C and stirred at this temperature for 20 h. Purification was performed by precipitating the reaction mixture against large excess of diethyl ether. The block copolymer was collected and dried *in vacuo*.

Micelle Formation

Micellization was achieved by solvent replacement method. In brief, the synthesized block copolymer mPEG-*b*-P(LA-*co*-ATC) (50 mg) was dissolved in THF (1 mL). This solution was added to 5 mL of doubly distilled water in a dropwise-manner under moderate stirring at room temperature, followed by dialyzing against water with a cellulose membrane (cutoff M_n = 3500) for 2 days. The prepared micellar solution was then lyophilized.

Modification of mPEG-*b*-P(LA-*co*-ATC) Block Copolymer via Microwave-Assisted Click Reaction

Click reaction was carried out in a microwave reactor using CuBr as catalyst according to literature.[33] Four propargyl-containing molecules (propargyl alcohol, propiolic acid, 5-hexynoic acid, and propargyl amine) were selected to investigate the universality of this reaction. In a typical reaction, a solution of mPEG-*b*-P(LA-*co*-ATC) (1 g, 1.65 mmol N_3), propargyl alcohol (0.136 g, 1.5 equiv.), TEA (32.9 mg, 0.2 equiv.), and CuBr (23.4 mg, 0.1 equiv.) in N_2-purged DMF (10 mL) was placed in the microwave reactor (MCR-3, Yuhua Instruments, Zhengzhou, China, AC: 220 V ± 10%, $P \leq$ 800 W, Microwave frequency: 450MHz ± 50Hz) and was irradiated (70°C and 90 W) for 20 min under a nitrogen atmosphere. The solution was poured into an amount of cold diethyl ether and the precipitate was isolated by filtration, and dried under vacuum at room temperature (yield: 90%). The product is coded as mPEG-*b*-P(LA-*co*-ATC/OH). Similar reactions were performed for 5-hexynoic acid and propargyl amine, the products were coded as mPEG-*b*-P(LA-*co*-ATC/COOH) and mPEG-*b*-P(LA-*co*-ATC/NH_2), respectively. Unexpectedly, propiolic acid cannot react with mPEG-*b*-P(LA-*co*-ATC), which will be illustrate in the following text. To determine the reaction time needed for complete assumption of azido groups, a small fraction of the product was taken out at different reaction times for FTIR analysis.



Scheme 1. Synthetic route of ATC monomer and its polymer functionalization.

Conjugation of Gemcitabine onto mPEG-*b*-P(LA-*co*-ATC/OH) Copolymer

GEM has free amino and hydroxyl groups that can be used for conjugation with polymers. Cavallaro et al. reported the synthesis of two macromolecular prodrugs by conjugating GEM to poly(*N*-2-hydroxyethyl)-DL-aspartamide (PHEA) through succinyl or diglycolyl hydrolysable spacers via its hydroxyl group.[34]

In the present study, amino group of GEM was selected for its high efficiency. Firstly, mPEG-*b*-P(LA-*co*-ATC/OH) copolymer was activated with NPC. Briefly, to an mPEG-*b*-P(LA-*co*-ATC/OH) solution in methylene chloride (0.8 g/10mL), NPC (0.195 g) and TEA (0.3 mL) were added dropwise at 0°C (molar ratio of DHP/NPC/TEA is 1/1.2/4). The reaction mixture was stirred for 4 h at 0°C, and finally the NPC-activated mPEG-*b*-P(LA-*co*-ATC) was obtained by precipitating in cold diethyl ether, and dried *in vacuo*. Then the NPC-activated mPEG-*b*-P(LA-*co*-ATC) (0.9 g) dissolved in DMF (10 mL) was reacted with GEM (400 mg) in the presence of TEA (60 μL) for 48 h at room temperature under nitrogen. The product mPEG-*b*-P(LA-*co*-ATC/GEM) was purified as polymeric micelles by adding doubly distilled water to the organic phase, followed by dialyzing against water with a cellulose membrane (cutoff M_n = 3500) for 3 days. After dialysis, mPEG-*b*-P(LA-*co*-ATC/GEM) was lyophilized and stored. The GEM content was determined

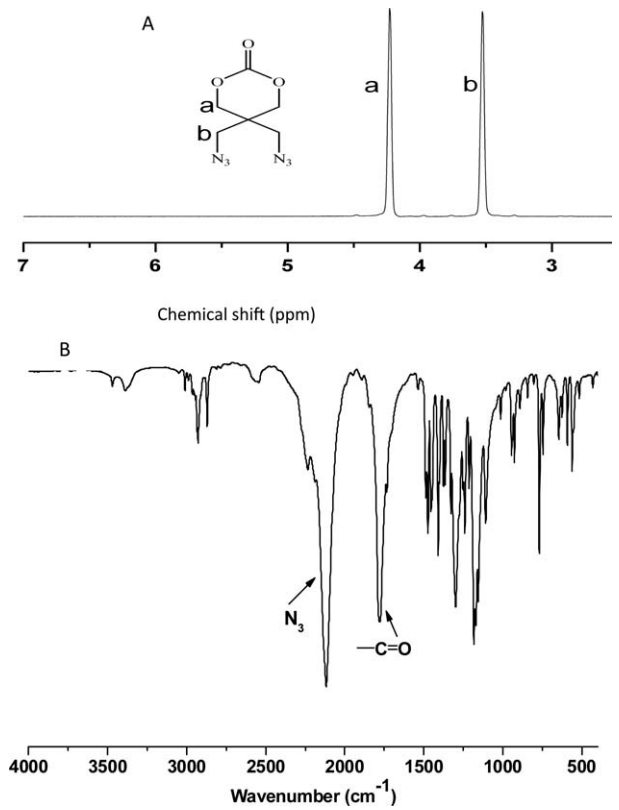


Figure 1 A. ^{1}H-NMR spectrum of ATC monomer in $CDCl_3$ and peak assignments; (B) FTIR spectrum of ATC: The arrows indicate the presence of characteristic groups of N_3 and C=O.

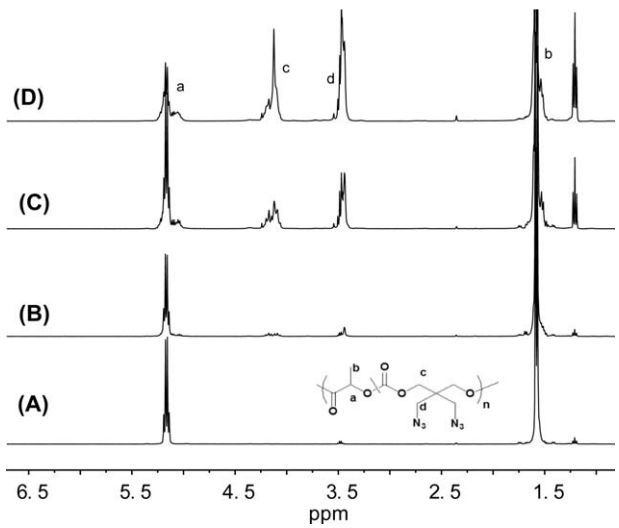


Figure 2. ^{1}H-NMR spectra of P(LA-*co*-ATC) with different molar ratios: (A) 0%, (B) 5%, (C) 20%, and (D) 40%.

Table I. Information About Copolymerization of LA and ATC in Bulk

Entry	Mol % of ATC in feeding	Mol % of ATC in product[a]	$M_n/10^4$ [b]	M_w/M_n [b]	T_g (°C)
1	0	0	5.77	1.60	59
2	5	5	2.37	1.81	55.6
3	10	8	3.20	1.93	50.1
4	20	16	4.70	1.62	40.9
5	60	58	5.79	1.61	28.6

[a]Determined by ^{1}H-NMR, [b]Determined by GPC.

to be 48% by measuring the UV absorbance of a DMSO solution of the conjugate at 268 nm. A calibration curve was constructed using a series of concentrations of free GEM in DMSO.

Conjugation of Paclitaxel onto mPEG-*b*-P (LA-*co*-ATC/COOH) Copolymer

The copolymer mPEG-*b*-P(LA-*co*-ATC/COOH) with free carboxyl groups (0.2 g) was dissolved in 10 mL CH_2Cl_2, and the solution was cooled in an ice/water bath. Then, PTX (80 mg, 0.118 mmol) in 5 mL CH_2Cl_2 was added, which was followed immediately by dicyclohexylcarbodiimide (DCC, 20.6 mg, 0.1 mmol) and 4-dimethylaminopyridine (DMAP, 12 mg, 0.1 mmol). The mixture was stirred for 48 h at 0°C. The dicyclohexylurea (DCU) formed was filtered out and the filtrate was condensed in vacuum. The condensation product was precipitated into a large amount of diethyl ether, filtered, and washed with diethyl ether several times. The precipitates were dried under vacuum at room temperature (yield: 65%). The mPEG-*b*-P(LA-*co*-ATC/PTX) micelles were prepared by solvent replacement method mentioned above.

Conjugation of Rhodamine B onto mPEG-*b*-P (LA-*co*-ATC/NH_2) Copolymer

RhB was first activated with *N*-hydroxysuccinimide and then reacted with the amino group of mPEG-*b*-P(LA-*co*-ATC/NH_2). In brief, RhB (0.1 g) was added into a mixture of anhydrous dimethyl sulfoxide (DMSO, 10 mL) and TEA (0.06 mL) and dissolved in the mixture with stirring. Then dicyclohexylcarbodiimide (DCC, 51.6 mg) and *N*-hydroxysuccinimide (NHS, 28.9 mg) were added, and stirred in the dark for 24 h. The side product dicyclohexylurea (DCU) precipitated was removed by filtration. DMSO and TEA were evaporated under vacuum. Vacuum dried RhB-NHS was then dissolved into 10 mL of DMSO. 0.4 g mPEG-*b*-P(LA-*co*-ATC/NH_2) and 0.02 mL TEA were added. The reaction was conducted under an anhydrous condition overnight and then dialyzed against water with a cellulose membrane (cutoff M_n = 3500) for 2 days and then lyophilized to obtain RhB-labeled micelles.

Characterization

^{1}H-NMR (300 MHz, field strength: 7.05 T) spectra were recorded on a Bruker AV300M in $CDCl_3$ at 25°C. Chemical shifts were given in parts per million from that of tetramethylsilane (TMS) as an internal reference. Gel permeation chromatography (GPC) measurements were conducted with a Waters 410 GPC instrument equipped with a Waters Styragel HT3 column (bead size 10 μm; molecular weight range, 500−30,000) and a differential refractometer detector. $CHCl_3$ was used as eluent at a flow rate of 1 mL/min at 35°C. The molecular weights were calibrated with polystyrene standards (molecular weight range: 1790−200,000).

Measurement of the Micellar Size

Size distribution of the micelles was determined by dynamic light scattering (DLS) with a vertically polarized He–Ne laser (DAWN EOS, Wyatt Technology, USA). The scattering angle was fixed at 90° and the measurement was carried out at 25°C.

TEM Observation

The morphology of the micelles was observed by transmission electron microscopy (TEM) performed on a JEOL JEM-1011 electron microscope operating at an acceleration voltage of 100 kV. To prepare specimens for TEM, a drop of micelle solution (1 mg/mL) was deposited onto a copper grid with a carbon coating. The specimens were air-dried, sputter-coated with a layer of gold, and measured at room temperature.

Cell Lines

Two cell lines including SKOV-3 (human ovarian cancer cell line) and HeLa (human cervical carcinoma cell line) were

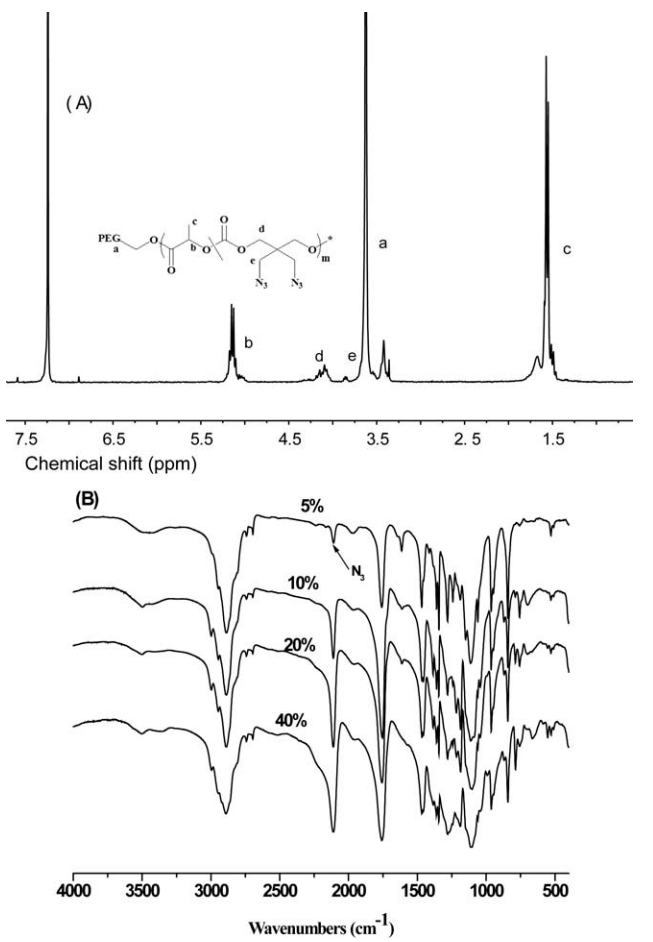


Figure 3 A. ^{1}H-NMR spectra of mPEG-*b*-P(LA-*co*-ATC20%) in $CDCl_3$ and peak assignments; (B) FTIR spectra of mPEG-*b*-P(LA-*co*-ATC) with different ATC contents.

Table II. Information About Copolymerization of LA and ATC Initiated by mPEG

Entry	Copolymer	Monomer conv[a] %		$M_n/10^3$		
		LA	ATC	^{1}H-NMR[b]	GPC[c]	PDI[d]
1	mPEG-P(LA-*co*-ATC 5%)[e]	95	90	9.2	10.2	1.10
2	mPEG-P(LA-*co*-ATC10%)	92	87	9.8	11.0	1.15
3	mPEG-P(LA-*co*-ATC20%)	92	82	10.2	11.3	1.21
4	mPEG-P(LA-*co*-ATC40%)	93	85	10.8	13.1	1.21

[a]Determined by ^{1}H-NMR, [b]Calculated based on the known PEG molecular weight, [c]The molecular weights were calibrated with polystyrene standards, [d]Determined by GPC, [e]The molecular fraction of ATC to total monomers in feeding.

chosen for *in vitro* tests. They were purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China. SKOV-3 cells were grown in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% FBS (Life Technologies), 0.03% L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2 at 37°C. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% heat-inactivated FBS (GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). The culture medium was replaced once very day.

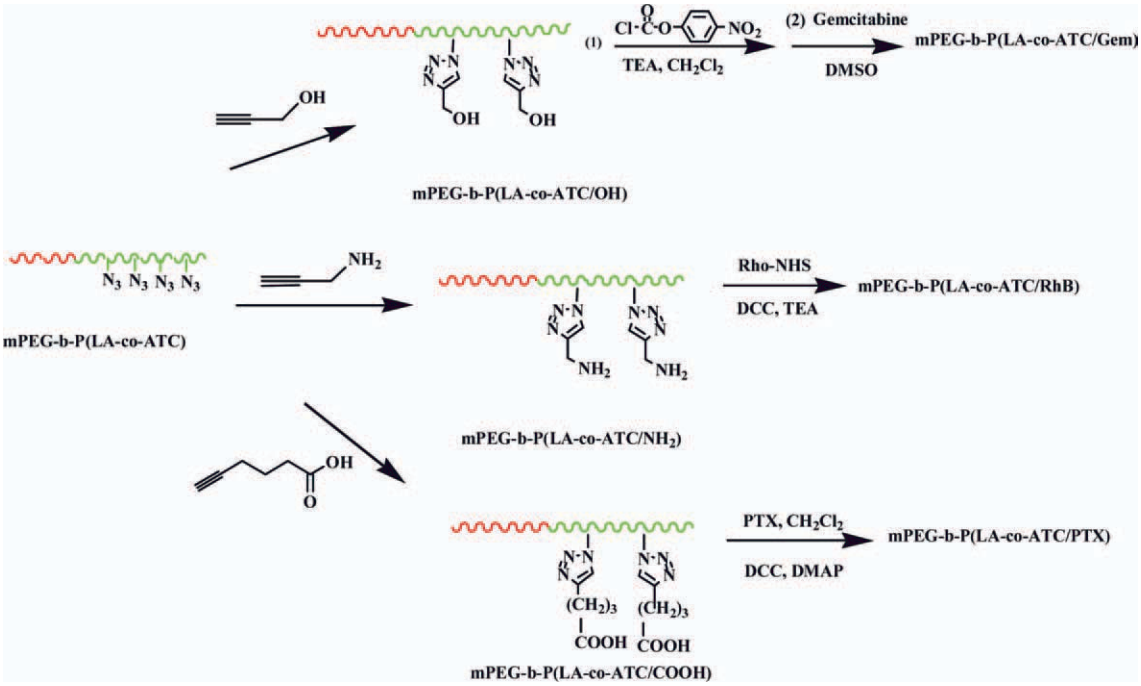
In Vitro Cytotoxicity

The MTT assay was used to evaluate *in vitro* antitumor activity of the mPEG-*b*-P(LA-*co*-ATC/GEM) and mPEG-*b*-P(LA-*co*-ATC/PTX) conjugates using free GEM and PTX as a control. Briefly, SKOV-3 (or HeLa) cells harvested in a logarithmic growth phase were seeded in 96-well plates at a density of 10^5 cells/well and incubated in RPMI 1640 (DMEM for HeLa) for 24 h. The medium was then replaced with the conjugate micelles or free GEM solution at various GEM concentrations from 0.05 to 50 μg/mL. At the designated time intervals (72 h), 20 μL of MTT solution (5 mg/mL) in PBS was added and the plate was incubated for another 4 h at 37°C. After that, the medium containing MTT was removed and 150 μL of DMSO was added to each well to dissolve the formazan crystals formed. Finally, the plates were shaken for 10 min, and the absorbance of formazan product was measured at 492 nm by a microplate reader.

RESULTS AND DISCUSSION

Synthesis of Block Copolymer mPEG-*b*-P(LA-*co*-ATC)

First, cyclic carbonate monomer ATC containing azido groups was synthesized according to Ref. 31 with the synthetic route as shown in Scheme 1. The structure of ATC was confirmed by ^{1}H-NMR spectrum [Figure 1(A)] and FTIR [Figure 1(B)]. In ^{1}H-NMR spectrum, corresponding proton peaks were observed



Scheme 2. Schematic route of mPEG-*b*-P(LA-*co*-ATC) postfunctionalization with propargyl alcohol, propargyl amine, 5-hexynoic acid, and their drug or fluorescent dye RhB conjugation. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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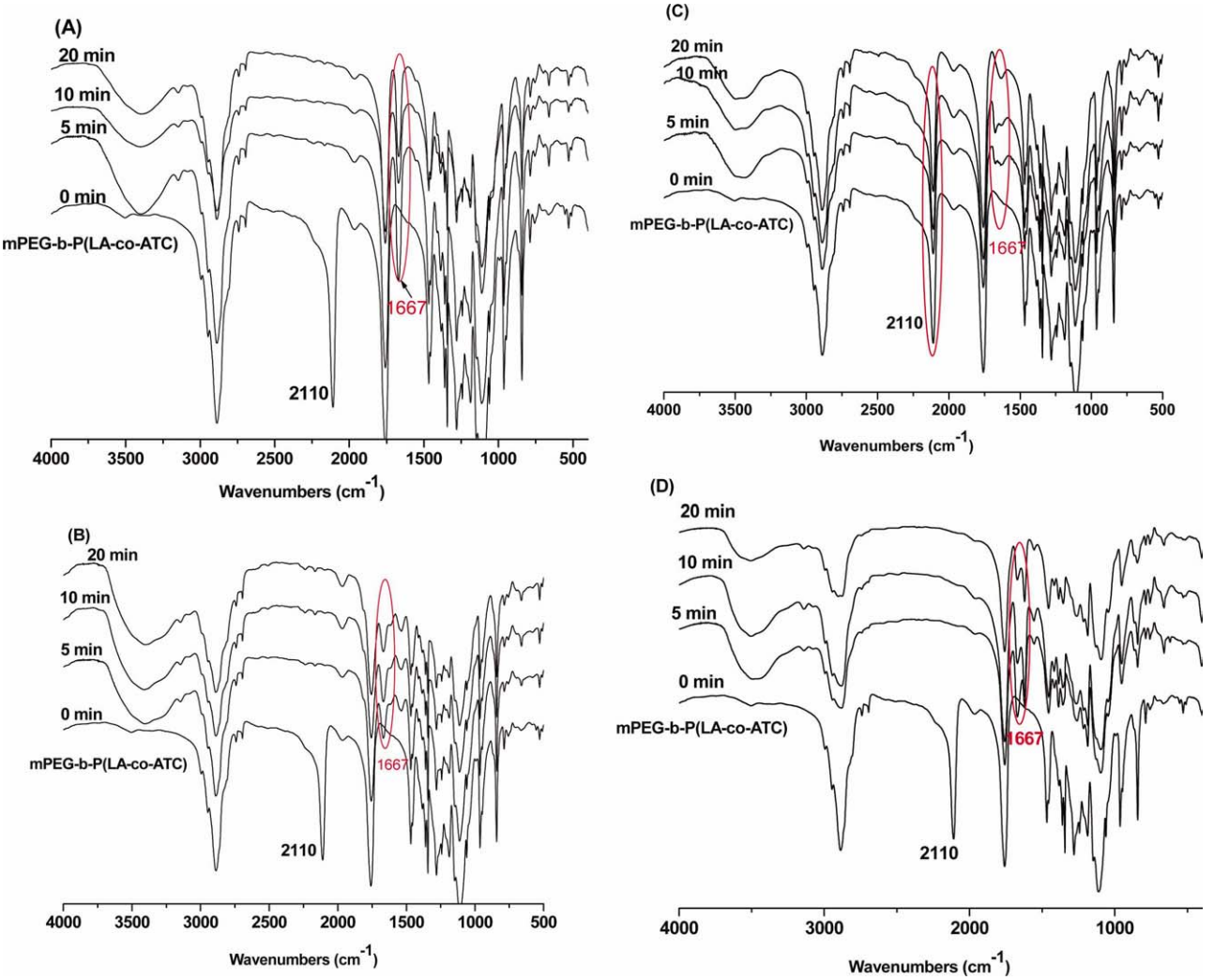

Figure 4. FTIR spectra of mPEG-*b*-P(LA-*co*-ATC) during its reaction with propargyl alcohol (A), proparyglamine (B), propiolic acid (C), and 5-hexynoic acid (D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

at δ: 4.23 (s, C—CH_2—O, 4H); δ: 3.53 (s, C—CH_2—N_3, 4H) [Figure 1(A)]. The strong peak at 1750 cm^{-1} in the FTIR spectra corresponds to the carbonyl groups and strong peak at 2110 cm^{-1} corresponds to the azido (N_3) group [Figure 1(B)], also indicating successful synthesis of the monomer.

The copolymerization of LA and ATC was conducted in bulk at various molar ratios of LA and ATC. Molecular weights were measured using GPC and thermal data were recorded using DSC. Figure 2 gives the typical ^{1}H-NMR spectra of copolymer P(LA-*co*-ATC) with different ATC molar contents. The C*H* and CH_3 protons of LA repeat unit show chemical shifts at 5.1 and 1.56 ppm according to homopolymer PLA. Signal c from 4.2 to 4.3 ppm is assigned to —CO—O—CH_2— protons of repeat units of ATC. Peaks d at 3.5 ppm are assigned to N_3—CH_2—C— protons of repeat unit of ATC. The fact that both LA and ATC repeat units can be found in the copolymers suggests the successful copolymerization. With ^{1}H-NMR, copolymer composition was calculated from the relative areas of 4.2−4.3 ppm and 1.56 ppm peaks. The good agreement between the monomer feed and the polymer composition (Table I) indicated that the copolymer composition could be adjusted by changing the feed ratio. The GPC curves of the copolymers with different contents of ATC all showed unimodal peaks. Copolymer composition, molecular weights, and thermal data of the copolymers are summarized in Table I.

Then mPEG was utilized to initiate the ring-opening copolymerization of LA and ATC with the catalyst $Sn(Oct)_2$. The copolymer structure of mPEG-*b*-P(LA-*co*-ATC) was confirmed by combined analysis of ^{1}H-NMR [Figure 3(A)] and FTIR [Figure 3(B)]. In 300 MHz ^{1}H-NMR spectrum, every proton of the polymer can find its characteristic signal. Typically, the signal at 3.6 ppm is due to the CH_2CH_2 protons in the PEG block. The signal at 5.2 ppm is attributed to the O—C*H*—CH_3 proton in LA units. The signal at 4.07–4.35 ppm is attributed to the O—CH_2—C protons in ATC units. Relative proportions of the three blocks can be then calculated from their intensities. On the basis of these relative proportions and the known molecular weight of mPEG, the number-average molecular weight of

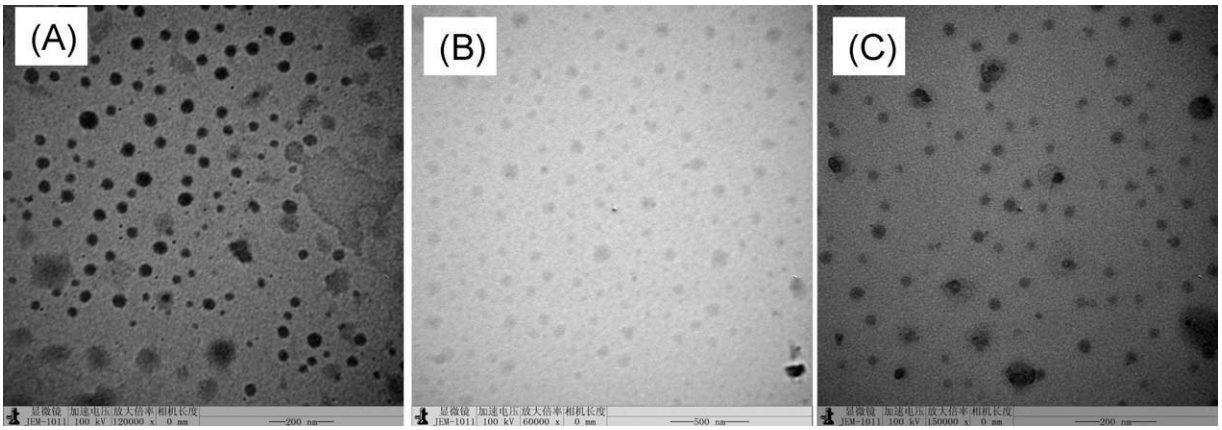

Figure 5. TEM images of mPEG-*b*-P(LA-*co*-ATC) (A), mPEG-*b*-P(LA-*co*-ATC/GEM) (B), and mPEG-*b*-P(LA-*co*-ATC/PTX) (C) micelles.

mPEG-*b*-P(LA-*co*-ATC) was calculated. The strong FTIR peaks at 1750 cm^{-1} corresponds to the carbonyl groups and the strong peak at 2110 cm^{-1} corresponds to the azido groups from ATC, indicating successful synthesis of copolymer mPEG-*b*-P(LA-*co*-ATC). With increasing ATC content in the monomer feeds, the azido peak intensity in the FTIR spectrum increases as shown in Figure 3(B). Table II summarizes related data of the polymerization reactions. From Table II, the yield of copolymerization is quite high. Every product shows a unimodal peak in GPC curve (data not shown), implying that the copolymerization is completed successfully and no homopolymerization of LA or ATC occurs. These data suggest that different copolymerization ratios of ATC with LA can be achieved by varying the feeding ratio and the molecular weight was controllable over a large range of composition.

Click Reactions of mPEG-*b*-P(LA-*co*-ATC) with Functional Propargyl Compounds

The copolymer mPEG-*b*-P(LA-*co*-ATC) and P(LA-*co*-ATC) with pendant azido groups could be used to introduce various functional groups on the side chains via azide–alkyne click reaction. The reaction mechanism of azide–alkyne click reaction has been illustrated in detail in the Refs. [35] and [36]. And in the present study, four propargyl-containing compounds (propargyl alcohol, propiolic acid, 5-hexynoic acid, and propargyl amine) were selected to obtain OH, COOH, and NH_2 groups, respectively. The reaction was conducted in a microwave reactor using CuBr/Et_3N as a catalyst. The synthetic route is shown in Scheme 2. To determine the reaction time needed for complete assumption of azido groups, FTIR was used to monitor the conversion of azido groups at different reaction time. As an example, Figure 4 shows the FTIR spectra of mPEG-*b*-P(LA-*co*-ATC) during its reaction with propargyl alcohol at 0, 5, 10, and 20 min. After 5 min, the azido characteristic peak at 2110 cm^{-1} almost disappears, which indicates the complete reaction. A new peak at 1667 cm^{-1} assignable to the triazole groups formed was observed [Figure 4(A)]. As for propargyl amine, the same phenomena were observed as shown in Figure 4(B). Five minutes is enough for azido conversion. Unfortunately, under the same conditions, the reaction does not happen between propiolic acid and mPEG-*b*-P(LA-*co*-ATC). As shown in Figure 4(C), the azido characteristic peak at 2110 cm^{-1} still remains after 20 min. This is because the triple bond in propiolic acid is directly connected to the electron-drawing COOH group and its reaction activity is lowered. This explanation is supported by following experimental facts: propargyl alcohol and propargyl amine are capable of click reaction under the same conditions; longer alkynyl acids, such as 5-hexynoic acid, can react with azido compounds [Figure 4(D)].

In conclusion, microwave irradiation is helpful to azido–alkynyl click reactions and 5 min is sufficient for the click reactions between mPEG-*b*-P(LA-*co*-ATC) with propargyl alcohol, 5-hexynoic acid, or propargyl amine with the microwave-assistance. Another benefit of this click reaction for polymer functionalization is there is no need for protection and deprotection of the functional groups, because the click reactions are not interfered by these groups.

Construction and Physicochemical Properties of Drug- and RhB-Conjugated Micelles

The anticancer drug GEM was chemically conjugated to the pendant hydroxyl groups in two steps (Scheme 2): (1) Reacting the OH groups with NPC to obtain carbonate linkage; (2) reacting the carbonate with GEM to obtain carbamate linkage. The GEM content was determined to be 48 wt % in the polymer–GEM conjugate by measuring the UV absorbance of a DMSO solution of the conjugates at 268 nm. The anticancer drug PTX was chemically conjugated to the pendant carboxyl groups with its hydroxyl group via ester linkage using DCC and DMAP as the coupling reagents. The PTX content was determined to be 15 wt % in the polymer–PTX conjugate by ^{1}H-NMR. For polymer–drug conjugation, its biodistritution *in vitro* or *in vivo* is very important. However, most drugs are not fluorescent, so RhB was conjugated to the copolymer as a model drug for subsequent intracellular or excised organ's fluorescence detection. RhB-grafted copolymer mPEG-*b*-P(LA-*co*-ATC/RhB) was prepared in two steps: (1) activation of RhB with NHS; (2) reaction with the NH_2 group of mPEG-*b*-P(LA-*co*-ATC/NH_2) to yield mPEG-*b*-P(LA-*co*-ATC/RhB). The amount of RhB linked onto the polymer was calculated to be 9.5 wt % on the basis of UV–vis measurement and the calibration curve of free RhB in DMSO.

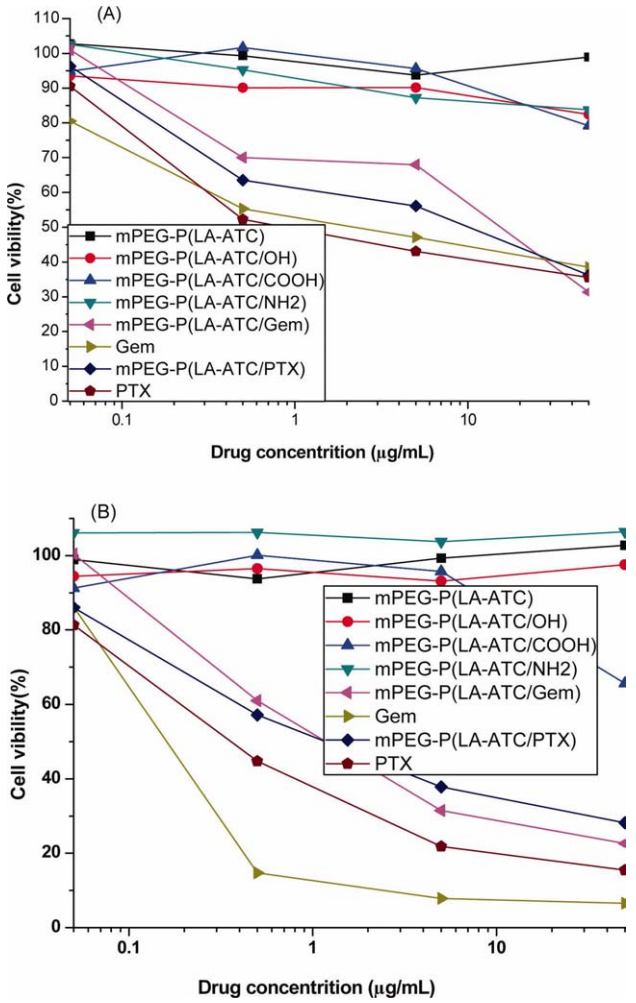

Figure 6. Cell viabilities of HeLa (A) and SKOV-3 cells (B) after 72-h incubation with mPEG-*b*-P(LA-*co*-ATC), mPEG-*b*-P(LA-*co*-ATC/OH), mPEG-*b*-P(LA-*co*-ATC/COOH), mPEG-*b*-P(LA-*co*-ATC/NH2), mPEG-*b*-P(LA-*co*-ATC/GEM), GEM, mPEG-*b*-P(LA-*co*-ATC/PTX), and PTX as a function of equivalent drug concentration. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Because of the amphiphilic nature of copolymer mPEG-*b*-P(LA-*co*-ATC) and its drug-conjugate, they can self-assemble into micelles using a solvent replacement method. The hydrophobic P(LA-*co*-ATC), P(LA-*co*-ATC/GEM) or P(LA-*co*-ATC/PTX) segment constitutes the core of the micelles, and the hydrophilic PEG segment forms the shell of the micelles, which improves the stability and circulation half-life of these drug-delivering micelles. Transmission electron microscopy observation showed that both micelles had a spherical shape and had a diameter of 30−50 nm (Figure 5).

In Vitro Antitumor Activity

The HeLa and SKOV-3 cell lines were employed to investigate the cytotoxicity of functionalized polymer and drug conjugate micelles with free drug as a positive control and blank culture medium as a negative control. Figure 6 shows the cell viability after 72-h culture. The results showed that the block copolymers mPEG-*b*-P(LA-*co*-ATC), mPEG-*b*-P(LA-*co*-ATC/OH), mPEG-*b*-P(LA-*co*-ATC/COOH), and mPEG-*b*-P(LA-*co*-ATC/NH_2) have low cytotoxicity, indicating great potential use in biomedical applications such as drug encapsulation and drug conjugate. For mPEG-*b*-P(LA-*co*-ATC/GEM), mPEG-*b*-P(LA-*co*-ATC/PTX), and their free drugs, the cytotoxicities were all concentration dependent. The cytotoxicity of mPEG-*b*-P(LA-*co*-ATC/GEM) micelles was lower than that of free GEM, and this may be because of the delayed release of the drug from the conjugate. However, the cytotoxicity of mPEG-*b*-P(LA-*co*-ATC/PTX) micelles was similar to that of free PTX, indicating the easier release of PTX linking with ester bond to the copolymer than GEM linking with carbamate to the copolymer. Of the two cell lines, SKOV-3 is more sensitive than HeLa to GEM and PTX.

CONCLUSION

Biodegradable block copolymers mPEG-*b*-P(LA-*co*-ATC)s were successfully synthesized via ring-opening polymerization of LA and cyclic carbonate monomer ATC using mPEG as macroinitiator. Functional alkynyl compounds were introduced into the ATC units via microwave-assisted Cu(I)-catalyzed 1,3-Huisgen cycloaddition click chemistry in only 5 min to afford various functional groups. The pendant groups were conjugated with anticancer drugs (GEM, PTX) and fluorescent dye (RhB). Well-defined micelles based on the mPEG-*b*-P(LA-*co*-ATC) and its anticancer drug and dye conjugates were obtained. The copolymer and its micelles were characterized by ^{1}H-NMR, FTIR, and TEM. The cytotoxic activity of mPEG-*b*-P(LA-*co*-ATC/GEM) and mPEG-*b*-P(LA-*co*-ATC/PTX) micelles was evaluated against SKOV-3 and HeLa cell lines. These results suggested that the synthesized copolymer could be a promising drug delivery carrier.

ACKNOWLEDGMENTS

Financial support was provided by the National Natural Science Foundation of China (Project Nos. 21004062, 50733003), and by the Ministry of Science and Technology of China ("973 Project", No. 2009CB930102; "863 Project", No. 2007AA03Z535).

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