Polymer xxx (2014) 1–11

Contents lists available at ScienceDirect

Polymer

journal homepage: www.elsevier.com/locate/polymer

PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA

Junping Lai [a], Zhangyan Xu [a], Rupei Tang [a,b,*], Weihang Ji [c], Rui Wang [a], Jun Wang [b], Chun Wang [c,**]

[a] School of Pharmaceutical Science, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu Province 214122, PR China
[b] School of Life Science, Anhui University, 111 Jiulong Road, Hefei, Anhui Province 230601, PR China
[c] Department of Biomedical Engineering, University of Minnesota, 7-105 Hasselmo Hall, 312 Church Street S. E., Minneapolis, MN 55455, USA

ARTICLE INFO

Article history:
Received 18 February 2014
Received in revised form
16 April 2014
Accepted 20 April 2014
Available online xxx

Keywords:
PEGylation
Ortho ester
Gene delivery

ABSTRACT

A new type of acid-labile cationic copolymer consisting of a hydrophilic poly(ethylene glycol) (PEG) block and a polymethacrylamide block bearing tertiary amines linked by acid-labile ortho ester rings in side chains (PAOE), with defined chain length, had been synthesized via RAFT polymerization. The copolymers could efficiently bind and condense plasmid DNA at neutral pH into narrowly dispersed nanoscale polyplexes. The hydrolysis of ortho ester group in the side-chains of PAOE followed a distinct exocyclic mechanism and the rate of hydrolysis was much accelerated at mildly acidic pH, resulting in the accelerated disruption of polyplexes and the release of intact plasmid DNA. The three polymers were not toxic to cultured COS-7 cells as measured by MTT assay. As expected, PEG segments of the PEG-*b*-PAOE copolymers prevented nonspecific transfection of COS-7 cells. Once conjugated to a targeting ligand to enhance cell-specific entry, PEG-*b*-PAOE with its pH-triggered DNA release properties may achieve efficient intracellular delivery of DNA or other nucleic acid therapeutics.

1. Introduction

Viral vectors and non-viral vectors are two major approaches that have been widely utilized in gene therapy. Non-viral vectors based on cationic polymers have been widely investigated over the past decades due to potential advantages in large DNA loading capacity, low immunogenicity, improved safety profile, easy fabrication, and more tailorable structure for functionalization, in vivo which has been a key issue associated with the use of viral vectors [1]. Cationic polymers such as poly(ethylenimine) (PEI) [2], poly(L-lysine) (PLL) [3], chitosan [4], dendritic poly(amidoamine) (PAMAM) [5] are a few representative polymers that have shown potential as gene carriers. However, compared with viral vectors, the major disadvantages of these cationic polymers are their poor stability and low transfection efficiency in vivo [6–8]. Thus, development of non-viral vectors with better efficiency and stability for systemic in vivo applications remains a major challenge [9].

Specific challenges for in vivo applications of cationic polymers include their rapid clearance from the circulation system and activation of the complement system, which lead to inefficient delivery to the target cells/tissues. To address these challenges, poly(ethylene glycol) (PEG) modification of cationic polymers has been proven a useful method for gene delivery to provide biocompatibility and charge shielding effect to reduce nonspecific interactions of the polyplexes with blood components, prolonged circulation, and low toxicity in vivo [10–13]. Notably, the properties of PEGylated polycations could be tuned by applying PEG with different molecular weight and different PEGylation density [14–16].

Endosomal escape, in particular, is one of the major barriers in the process of gene delivery [17,18], since DNA trapped in endosomes is typically trafficked into lysosomes where DNA is degraded [19–22]. To facilitate the endosomal escape, a variety of stimuli-responsive non-viral vectors have been developed [23–26]. Among those, pH-responsive polymers that exploit the pH

* Corresponding author. School of Life Science, Anhui University, 111 Jiulong Road, Hefei, Anhui Province 230601, PR China.
** Corresponding author.
E-mail addresses: tangrp99@iccas.ac.cn (R. Tang), wangx504@umn.edu (C. Wang).

http://dx.doi.org/10.1016/j.polymer.2014.04.036
0032-3861/

gradients formed in the intracellular vesicular trafficking pathways have been developed to show improved gene transfection. There are two methods frequently used to design the polymers. In one approach, a amine-functional group of low pKa value is incorporated into the main-chain or side-chain of a polymer gene carrier, which can buffer the endosomes, resulting in endosomal disruption and subsequent release of polyplexes into cytoplasm [27–29]. Another promising approach is to develop the polymers of amine groups with pH-sensitive linkages into polymer main-chains or side-chains, such as β-amino esters [30,31], acetal [32,33], hydrazine [34,35], ketal [27,36], silyl ether [37], and vinyl ether [38,39]. They can degrade into short fragments in the endosomal acidic environment that might facilitate the endosome disruption, causing leakage and release of cargos. Thus, we can design a wide variety of pH-sensitive polymers with different acid-lable linkages, which can hydrolyze into distinct products according to respective hydrolysis kinetics and possess unique structures and characters that other polymers are unmatched.

Poly(ortho esters) (POEs) represent an important family of acid-sensitive polymers with good biocompatibility [40–43]. Our group has a lasting interest in designing pH-sensitive polymers through the incorporation of ortho ester linker in the side chains or main chains with functional groups [26,44]. In the present work, we report a new type of pH-labile cationic copolymer consisting of a hydrophilic poly (ethylene glycol) (PEG) block and a polymethacrylamide block bearing tertiary amines linked by acid-labile ortho ester rings in side chains (PAOE), PEG-*b*-PAOE (Scheme 1). These copolymers were synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization. The pH-sensitive polyplexes are stable at pH 7.4 and have lower cytotoxicity in comparison to the non-PEGylated polyplexes. The ortho ester group in side-chains of PAOE hydrolyzed at mildly acidic pH (e.g. at endosomal pH), resulting in accelerated disruption of polyplexes and release of intact plasmid DNA (Scheme 1). PEGylation reduces undesired interactions with serum proteins and prevents nonspecific transfection of cells by decreasing the electrostatic association with cell membrane, resulting in low cellular uptake [45].

2. Experimental section

2.1. Chemicals and general methods

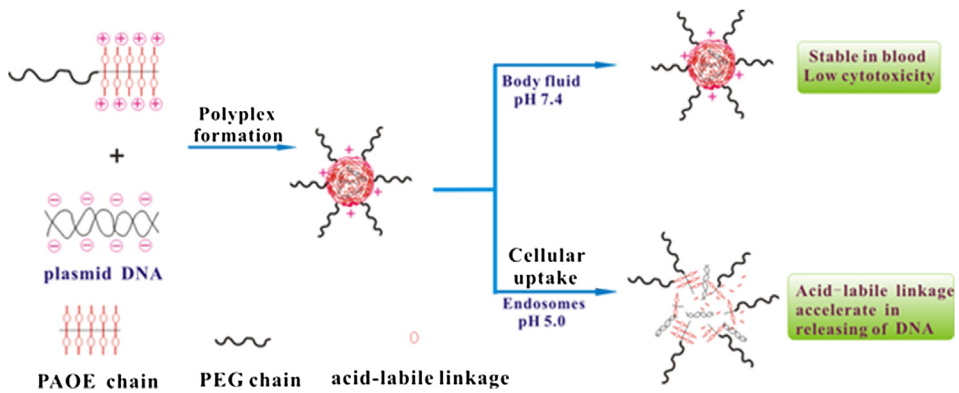
Acetonitrile was dried from CaH_2 before use. Dimethylformamide (DMF) was dried over $MgSO_4$ followed by distillation under reduced pressure. 2,2,2-Trifluoro-N-(2-methoxy-[1,3]-dioxolan-4-ylmethyl)acetamide was synthesized as described previously [26]. Branched PEI (M_W 25,000), 2,2′-azobis(2-methyl propionitrile), 3-amino-1,2-propanediol, 2-dimethylamino ethanol, N-succinimidyl methacrylate, poly(ethylene glycol) methyl ether (4-cyano-4-pentanoate dodecyl trithiocarbonate) (M_n = 5400), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazodium bromide (MTT) were purchased from Sigma–Aldrich. Green fluorescent protein (pEGFP-N1) DNA plasmids were obtained from Promega. Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Gibco. COS-7 cells (ATCC) were cultured in DMEM containing 10 mM HEPES, 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin 5% CO_2 at 37 °C. Other chemicals and solvents were purchased commercially and used without further purification. High-resolution Electrospray Ionization Mass Spectrometry (ESI-MS) experiments were conducted on a Bruker MicrOTOF-Q mass spectrometer. The ^1H and ^{13}C NMR spectra were recorded on a Bruker Advance III 400 MHz Digital NMR spectrometer using deuterated dimethylsulfoxide or chloroform as solvents. Chemical shifts were recorded in ppm and referenced against tetramethylsilane (TMS). Gel Permeation Chromatography (GPC) experiments to analyze PEGylation copolymers were performed 35 °C in DMF with a flow rate of 1 mL/min, a Waters 1512 equipped with three Waters Styragel HR1, HR2, and HR3 columns. The GPC instrument was calibrated with polyethylene glycol (PEG) standards. All sample solutions were filtered through 0.22 μm filters before analysis.

2.2. Synthesis of 2-dimethylamino ethanol p-toluene sulfonate

p-toluene sulfonic acid (10.70 g, 56.25 mmol) was placed in three-necked flack with a magnetic stirrer, and benzene (150 mL) was added under nitrogen, then the mixture was heated until no volatile component was distilled. 2-dimethylamino ethanol (5.01 g, 56.25 mmol) was added after cooling to 60 °C, the mixture was vigorously stirred overnight at room temperature, filtered and then dried under vacuum for 16 h to yield 14.09 g (96%) of 2-dimethylamino ethanol *p*-toluene sulfonate as a white solid. The solid was used without further purification in the following reaction.

2.3. Synthesis of 2-(4-(aminomethyl)-1,3-dioxolan-2-yloxy)-N,N-dimethylethylamine (3)

2-(4-(Aminomethyl)-1,3-dioxolan-2-yloxy)-N,N-dimethylethylamine was synthesized by a two-step reaction. In the first step, a mixture of 2-dimethylamino ethanol *p*-toluene sulfonate (11.98 g, 45.84 mmol), pyridinium *p*-toluene sulfonate (78.00 mg, 0.29 mmol, Py-PTSA) and 2,2,2-trifluoro-N-(2-methoxy-[1,3]-dioxolan-4-ylmethyl) acetamide (10.01 g, 43.68 mmol) was heated in an oil-bath at 130 °C for 2 h under vacuum. On cooling to room



Scheme 1. Illustration of pH-sensitive PEGylated block copolymers and pH-triggered release of DNA.

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036

temperature the residue was dissolved in tetrahydrofuran (200 mL) and sodium hydroxide (2.0 M, 200 mL). The mixture was stirred overnight, evaporated, then extracted with dichloromethane and dried over anhydrous $MgSO_4$, and concentrated to yield 4.47 g (54%) of 2-(4-(aminomethyl)-1,3-dioxolan-2-yloxy)-N,N-dimethylethylamine as an oil. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 1.48 (b, 2H, NH_2), 2.27 (s, 6H, CH_3—N—CH_3), 2.51–2.54 (t, 2H, N—CH_2), 2.75–2.95 (m, 2H, CH_2—NH_2), 3.60–3.67 (m, 2H, N—CH_2—CH_2), 3.68–4.37 (m, 2H, O—CH_2—CH), 4.13–4.22 (m, 1H, O—CH—CH_2), 5.85–5.88 (d, 1H, CH—O—CH_2). ^{13}C NMR ($CDCl_3$, δ ppm): 44.66, 46.04, 46.04, 58.85, 62.60, 66.55, 78.54, 115.66. ESI-MS Calcd for ($C_8H_{18}N_2O_3$), 190.2; Found m/z 191.2 ($M + H^+$).

2.4. Synthesis of poly(ethylene glycol)-block-poly(2-(4-(aminomethyl)-1,3-dioxolan-2-yloxy)-N,N-dimethylethylamine) (PEG-b-PAOE)

The three diblock copolymers poly(ethylene glycol)-block-poly(N-succinimidyl methacrylate) (PEG-*b*-PNSM) were synthesized by RAFT. A two-neck flask was charged with poly(ethylene glycol) methyl ether (4-cyano-4-pentanoate dodecyl trithiocarbonate) (PEG-CTA) and N-succinimidyl methacrylate (NSM, 1 g, 5.46 mmol), then dried DMF (2.5 ml) and 2,2′-azobis(2-methyl propionitrile) as an initiator were added. The mixture was degassed for 30 min and the flask was sealed and then heated at 70 °C for 20 h. Then the reaction was terminated, diluted by DMF and precipitated in diethyl ether twice, then filtered and dried under vacuum at room temperature for 24 h. To obtain PNSM blocks with varying chain length, three different NSM-to-chain transfer agent (PEG-CTA)-to-initiator feed ratios (40:1:0.2, 60:1:0.2, 90:1:0.2) were used. To remove the succinimidyl groups, 0.30 g of PEG-*b*-PNSM was dissolved in 4 mL of anhydrous DMF, $n_{(\text{compound } \mathbf{3})}$: $n_{(\text{repeating units of PNSM block})} = 1{:}1.5$ and triethylamine (a trace amount) were added, the mixture were vigorously stirred for 72 h at 45 °C. Then the reaction mixture was cooled to room temperature and placed in a semi-permeable dialysis membrane (MWCO 3500), and dialyzed against distilled water (triethylamine, a trace amount) for 72 h. The obtained product was lyophilized, and analyzed by NMR and GPC. The average molecular weight (M_n) of PEG-*b*-PNSM were calculated to be 0.98, 1.52, and 1.83×10^4 Da with the polydispersity index (PDI) of 1.27, 1.26, and 1.27, respectively. The average molecular weight (M_n) of PEG-*b*-PAOE were calculated to be 1.15, 1.85, and 2.36×10^4 Da with the polydispersity index (PDI) of 1.26, 1.23, and 1.22, respectively.

2.5. Determination of mechanism and rate of hydrolysis of PEG-b-PAOE bearing ortho ester side-chain

The three copolymers were dissolved in 50 mM *d*-phosphate buffer (pH 7.4, 6) and *d*-acetate buffers (pH 5, 4) at 5 mg/mL, and ^{1}H NMR spectra were recorded on a Bruker Advance-400 spectrometer (400 MHz) at different time points. The rate of hydrolysis of ortho ester side-chains were calculated by ^{1}H NMR.

2.6. Preparation of polyplexes and agarose gel retardation assay

Gel electrophoresis was done to examine DNA binding ability of the polymers. General procedures for polyplex formation were as follows: 25 μL of DNA plasmid solution (0.2 mg/mL) was mixed with 25 μL of polymer solutions at various N/P ratios in HEPES buffer (20 mM, pH 7.4). After vortexing, the samples were incubated for 30 min at room temperature. Then polyplex solution (10 μL) was mixed with 6× loading buffer (2 μL) and loaded onto a 1.0% agarose gel containing 0.5 mg/mL ethidium bromide (EB). The polyplexes were electrophoresed at 100 V for 50 min in 1× TAE running buffer. The migration of DNA was observed under a GelDoc-It^{TM} imaging system (UVP Corporation) and photos were taken.

2.7. DNase I degradation assay

To further ascertain DNA binding ability and whether polyplexes could protect DNA from enzymatic degradation, DNase I degradation assay was performed. Plasmid solution was mixed with polymer solutions with varying N:P ratios and incubated for 30 min. DNase I (1 μL, 0.6–0.8 U/μL) and DNase I buffer (10 μL) were added to 90 μL polyplexes. After vortexing, the samples were incubated for 8 min at room temperature. Polyplex solutions were then analyzed by gel electrophoresis as described in Part 2.6.

2.8. Heparin displacement assay

To determine the strength of DNA binding by the three copolymers, polyplexes at N:P ratio of 8 were incubated with increasing concentrations of heparin (0.1–1.0 IU per μg of DNA), then all the samples were left for 8 min at room temperature. The dissociation of polyplex at different heparin levels was detected by electrophoresis as described in Part 2.6.

2.9. Dynamic light scattering (DLS) and zeta potential measurements

Particle size and zeta potential of polyplexes were determined using a Zetasizer dynamic light scattering detector (Malvern Zetasizer Nano ZS, 6 mW laser; 633 nm incident beam, 173° scattering angle, 25 °C). Polyplexes with various N:P ratios were prepared as described above and diluted 10 times to a final volume of 1 mL in HEPES buffer (20 mM, pH 7.4) before measurement. The diameter and zeta-potential of particles were done in triplicates for each sample.

2.10. Stability of polyplexes and pH-triggered release of DNA

To determine whether hydrolysis of ortho ester rings in side-chains at acidic pH would trigger the release of DNA from polyplexes, polyplexes at N:P ratio of 8:1 were exposed to phosphate and acetate buffers (50 mM, pH 7.4, 6, 5, and 4) at room temperature for 1 h, 3 h, 8 h, and 24 h, respectively, and followed by agarose gel electrophoresis at corresponding time points.

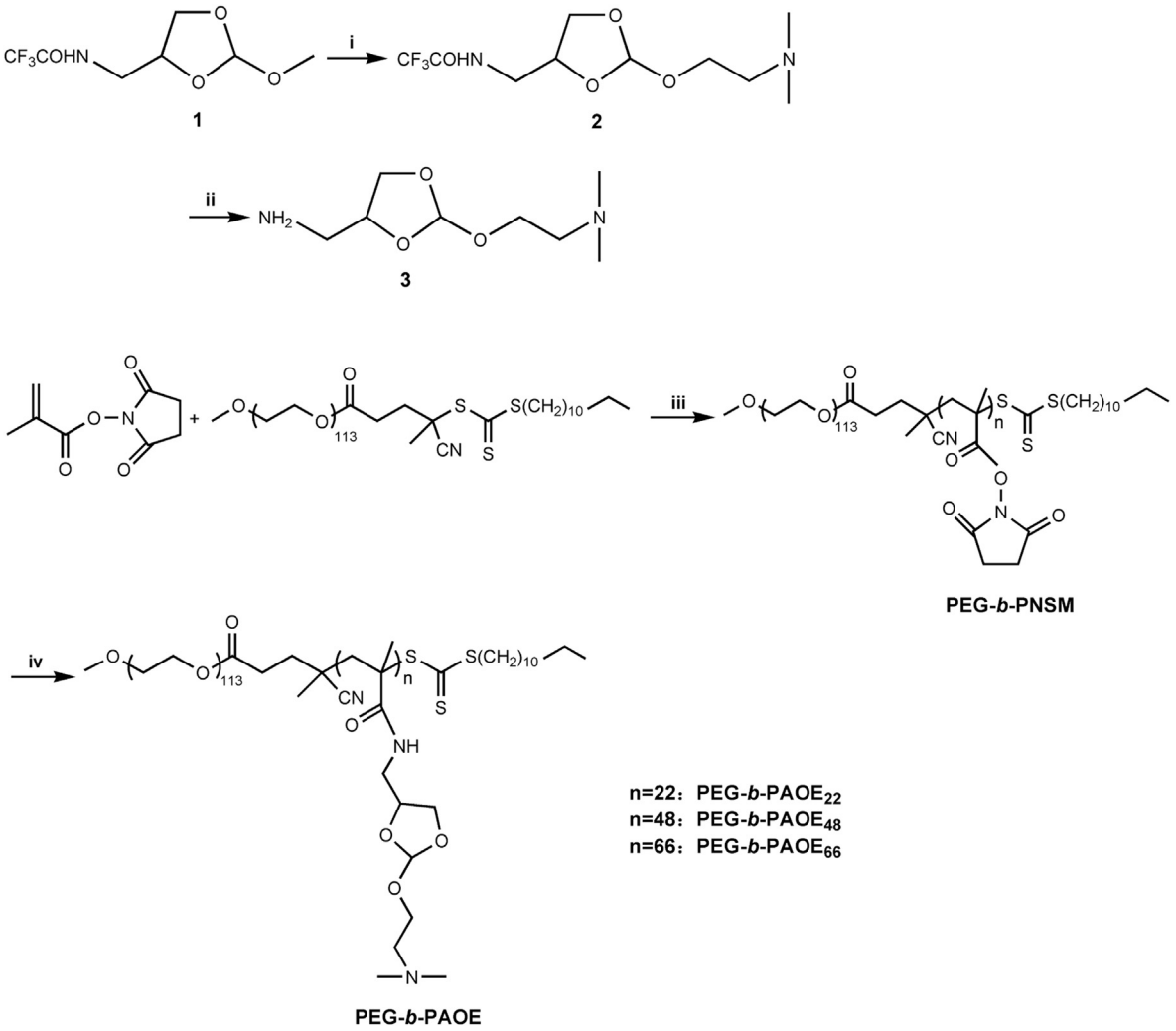
2.11. Cytotoxicity assay

The cytotoxicity of copolymers was determined by MTT assay. COS-7 cells were seeded into 96-well plates at 1×10^4 cells/well and incubated for 24 h in DMEM (10% FBS, 10 mM HEPES, 100 U/mL penicillin/streptomycin). All cultured cells were grown at 37 °C in 5% CO_2 humidified atmosphere. To each well, 20 μL of polymer solutions was added and cultured for another 24 h, the final polymer concentration in growth media varied from 1 to 1000 μg/mL. MTT in PBS (20 μL, 5 mg/mL) was then added to each well and incubated for another 4 h, solutions in the wells were removed by aspiration, and the formazan crystals were dissolved in 150 μL of DMSO. After 10 min of shaking, the absorbance was measured at 570 nm using a Thermo scientific Multiskan GO 1510 plate reader. The viability of cells treated with PBS only was taken as 100% and the relative cell viability of polymer treated cells (mean % ± S.D., $n = 3$) was calculated.

2.12. In vitro gene transfection

COS-7 cells were seeded into 12-well plates at 5×10^4 cells/well, and cultivated for 24 h. Afterwards, the media was removed and the

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036



Scheme 2. Reagents and conditions: (i) 2-Dimethylamino ethanol *p*-toluene sulfonate, Py-PTSA; 2 h, 130 °C, under vacuum; (ii) NaOH/H_2O/THF; overnight, room temperature; (iii) 2,2′-Azobis(2-methyl propionitrile) (AIBN), DMF; 20 h, 70 °C; (iv) Compound **3**, triethylamine, DMF; 72 h, 45 °C.

cells were washed twice by warm PBS. Polyplexes at various N/P ratios were then added in 1 mL of serum-free DMEM, 2.0 μg of DNA was used per well. Each N/P ratio had 6 replications and cells treated with naked DNA acted as the negative control. After incubation for 4 h, the polyplex containing media was discarded and the cells were washed with 500 μL of PBS and cultured in serum-containing DMEM for 44 h. The transfected cells were subjected to flow cytometry or observed under Nikon Eclipse TE2000-u fluorescence microscope for GFP fluorescence signal visualization. For flow cytometry, the cells were trypsinized and harvested in FACS buffer, and analyzed using a FACSCalibur 501 flow cytometer (Beckman Coulter). In addition, COS-7 cells were transfected by polyplexes of luciferase plasmid under the same conditions. The GFP positive gate was set based on polyplexes containing firefly luciferase to avoid autofluorescence of cells.

3. Results and discussion

3.1. Synthesis and characterization of PEG-b-PAOE

The synthetic routes and chemical structures of PEG-*b*-PAOE bearing tertiary amines linked by acid-labile ortho ester rings in side-chains and the corresponding compound **3** are shown in Scheme 2. The compound **3**, containing a five-membered ortho ester ring, was synthesized in two steps. The starting material of 2,2,2-Trifluoro-N-(2-methoxy-[1,3]-dioxolan-4-ylmethyl)acetamide (compound **1**) was synthesized using similar procedures reported by our groups [26]. Compound **2** was obtained by transesterification in the presence of compound **1** and 2-dimethylamino ethanol *p*-toluene sulfonate. The TFA-protecting group of compound **2** was removed by aqueous sodium hydroxide in THF, and the acid-labile functional compound **3** was obtained with total yield of approximately 54%. The ^1H and ^{13}C NMR spectra confirmed that the compound **3** was pure and structurally correct (Fig. 1).

Here we synthesized PEG-*b*-PAOE copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages using reversible addition-fragmentation chain transfer (RAFT) polymerization. In the first step, PEG-*b*-PNSM was obtained by RAFT of methacrylate monomer (NSM) reacted with a monofunctional PEG (M_n = 5400) chain-transfer agent (PEG-CTA) using 2,2′-azobis(2-methyl propionitrile) as the initiator. The molar ratio of NSM, PEG-CTA, and initiator was varied to achieve PEG-*b*-PNSM blocks with different lengths. The structure and molecular weight of PEG-*b*-PNSM were determined by GPC and NMR, and the results summarized in Table 1. Three PEG-*b*-PNSM copolymers with different NSM block lengths were synthesized with over 80% yield

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036

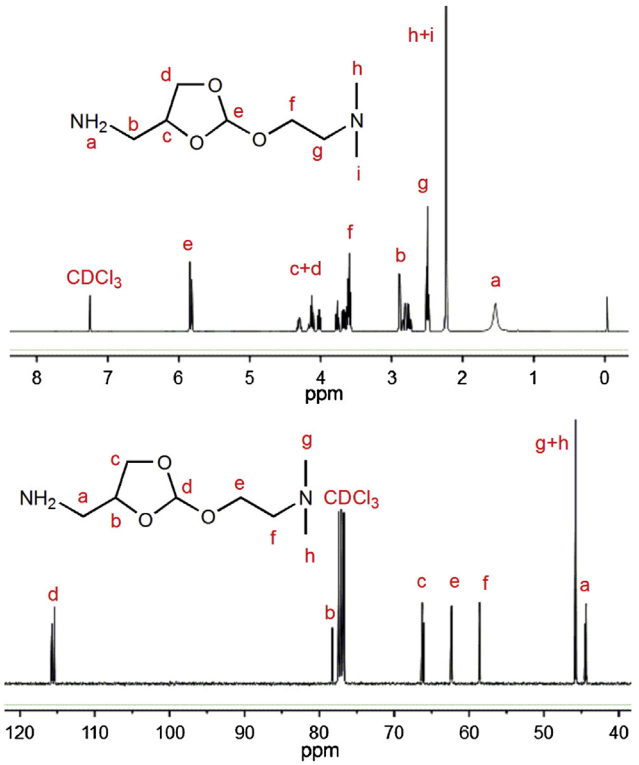


Fig. 1. ^{1}H and ^{13}C NMR spectra of compound **3** in $CDCl_3$.

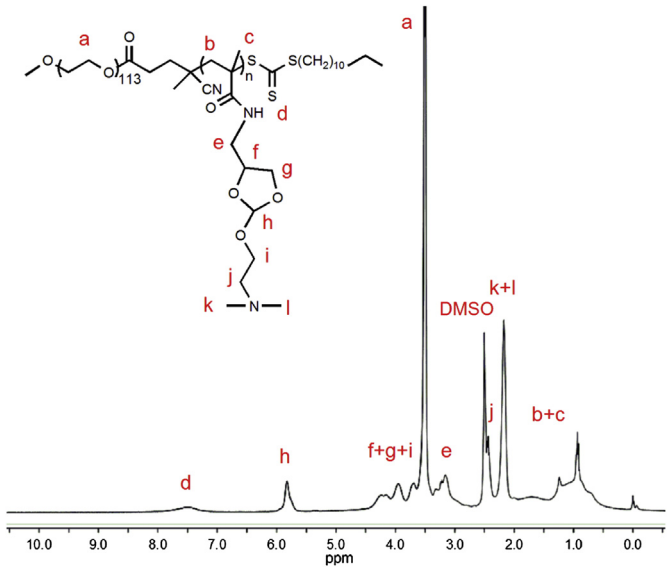


Fig. 2. ^{1}H NMR spectra of PEG-*b*-$PAOE_{48}$ in d_6-DMSO.

by varying NSM/PEG-CTA/initiator feed ratio. Based on GPC analysis, the number average molecular weight (M_n) of the three polymers was calculated to be 0.98, 1.52, and 1.83 $\times$ 10^4 Da with the polydispersity index (PDI) of 1.27, 1.26, and 1.27, respectively (Table 1). The narrow molecular weight distribution of the polymers suggested that controlled polymerization by RAFT was successful. The average numbers of repeating units of the PNSM block were estimated to be 22, 48, and 66 by ^{1}H NMR analysis (Fig. S1 in Supporting Information), which were thus denoted as PEG-*b*-$PNSM_{22}$, PEG-*b*-$PNSM_{48}$, and PEG-*b*-$PNSM_{66}$, respectively. In the second step, the succinimidyl groups of PEG-*b*-PNSM were removed by an amidation reaction with 2-(4-(aminomethyl)-1,3-dioxolan-2-yloxy)-N,N-dimethylethylamine (compound **3**), yielding the final polymer PEG-*b*-PAOE. As shown in Fig. 2, the ^{1}H NMR spectra of PEG-*b*-PAOE acquired in d_6-DMSO showed complete disappearance of the diagnostic ethyl proton signal of the succinimidyl group at δ 2.80 ppm, confirming that all succinimidyl groups had been completely removed. The obtained copolymers bearing ortho ester side-chains were thus denoted as PEG-*b*-$PAOE_{22}$, PEG-*b*-$PAOE_{48}$, and PEG-*b*-$PAOE_{66}$, respectively. The average M_n of PEG-*b*-PAOE was determined by integrating the area of characteristic peaks of PEG protons (peak "a") and PAOE protons (peaks "h"), which agreed well with the average M_n values determined by GPC using DMF as eluent and PEG as standard (Table 1). As shown in Fig. 3, all three copolymers had single symmetric peaks and narrow PDI values of 1.26, 1.23, and 1.22, respectively.

Table 1
Condition of polymerization and characterization of PEGylated copolymers.

Polymer	NSM:PEG-CTA: initiator (molar feed ratio)	Yield (%)	M_n[a] ($\times$ 10^4, Da)	M_n[b] ($\times$ 10^4, Da)	PDI[a]
PEG-*b*-$PNSM_{22}$[b]	40:1:0.2	85	0.98	1.12	1.27
PEG-*b*-$PNSM_{48}$[b]	60:1:0.2	89	1.52	1.78	1.26
PEG-*b*-$PNSM_{66}$[b]	90:1:0.2	82	1.83	2.11	1.27
PEG-*b*-$PAOE_{22}$	\	97	1.15	1.31	1.26
PEG-*b*-$PAOE_{48}$	\	97	1.85	2.09	1.23
PEG-*b*-$PAOE_{66}$	\	94	2.36	2.66	1.22

[a] Average molecular weight and polydispersity index were determined by GPC.
[b] Average degree of polymerization was determined by ^{1}H NMR in d_6-DMSO.

3.2. Kinetics of pH-dependence copolymer hydrolysis

Ortho ester structures are easily cleavable in acidic aqueous condition [46]. There are two possible pathways for hydrolysis of cyclic ortho esters that involve exocyclic and endocyclic breakage of the ortho ester bond [42,47]. In a recent report, 2-(5,5-Dimethyl-1,3-dioxan-2-yloxy) ethyl acrylate, a monomer containing a six-membered cyclic ortho ester, was copolymerized to produce a family of block copolymers [48]. This ortho ester ring hydrolyzed through endocyclic pathways, giving rise to formic acid and 2,2-dimethyl-1,3-propanediol monoformate [49]. Our group has recently developed novel poly(ortho ester amidine) copolymers (POEAmd), the five-membered cyclic ortho ester main-chains of POEAmd hydrolyzed through endocyclic pathways and shows pH-dependent hydrolysis kinetics confirmed by ^{1}H NMR [26].

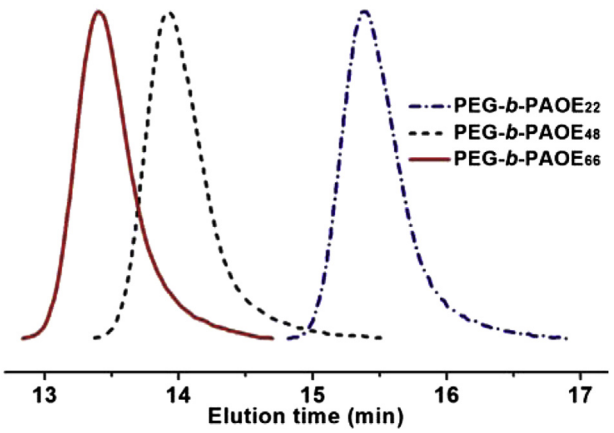


Fig. 3. GPC chromatograms of PEG-*b*-PAOE copolymers.

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036

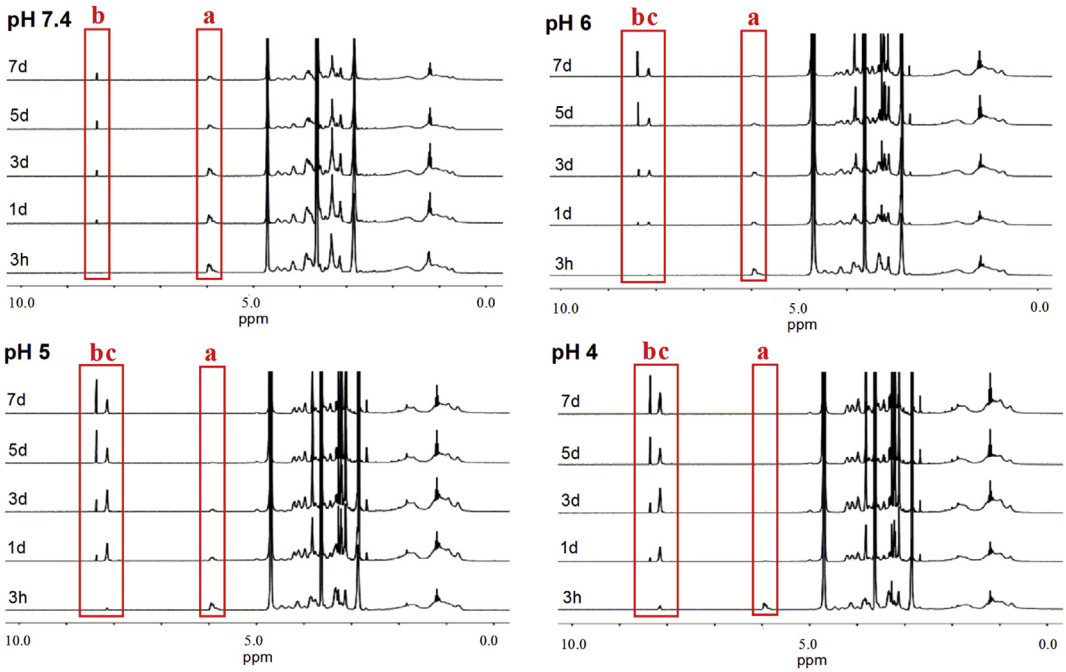
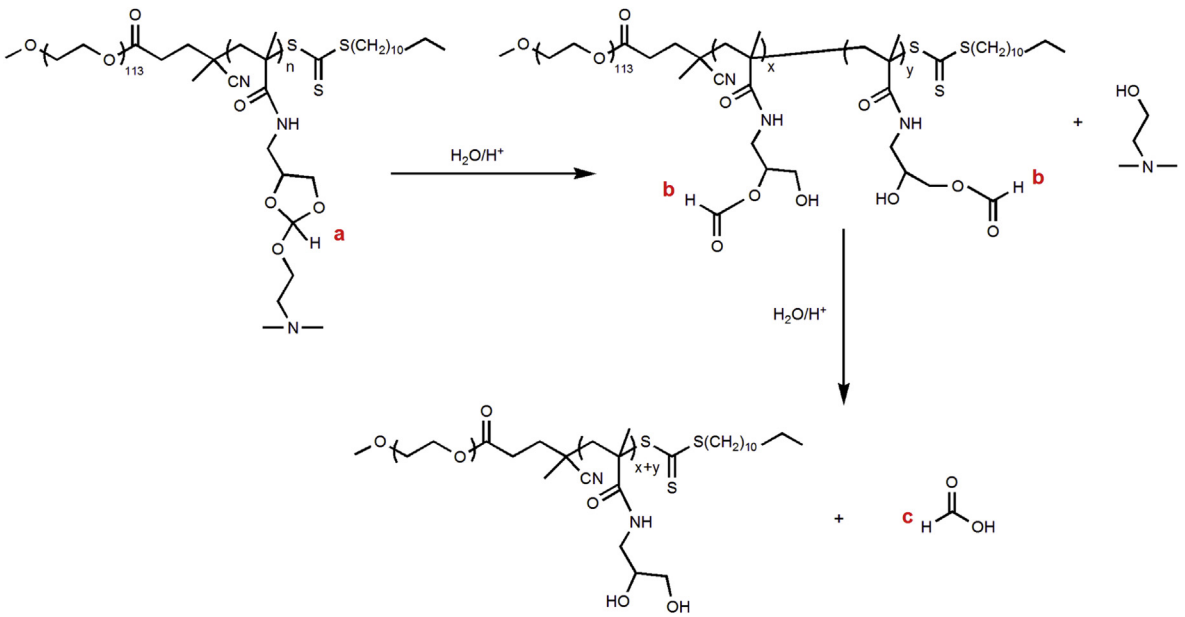


Fig. 4. ^{1}H NMR spectra of PEG-*b*-PAOE$_{48}$ (5 mg/mL) in *d*-buffers of pH 7.4, 6, 5, and 4. Peaks labeled with "a" "b" and "c" are characteristic protons of the cyclic ortho ester, formate groups, and formic acid, respectively (Scheme 3).

To understand the hydrolytic mechanism of PEG-*b*-PAOE, NMR studies were carried out to track the time-course of hydrolysis for up to 7 d in aqueous buffers at physiological pH and mildly acidic pHs (6, 5, and 4), and the results were shown in Fig. 4. The peak at 5.90 ppm (denoted as "a") is the characteristic peak of the ortho ester proton before hydrolysis (Scheme 3). At pH 7.4 over the course of 7 d, the area of peak "a" decreased slowly, suggesting that most of the ortho ester groups remained intact. Concurrently, at pH 7.4, a new peak at 8.37 ppm (denoted as "b") appeared after approximately 24 h, which corresponded to the proton of formate. But for other pHs (6, 5, and 4), another new peak at 8.14 ppm (denoted as "c") appeared. The peak "c" corresponded to the proton of formic acid, which was the hydrolysis product of polymeric formate (Fig. 4). The exocyclic pathway of hydrolysis would produce two different kinds of formate, which constituted a copolymer by various degrees of polymerization, and a small molecule, 2-dimethylamino ethanol (Scheme 3). With prolonged degradation of PEG-*b*-PAOE, part of formate further decomposed to formic acid (Scheme 3). Here we observed a formate peak at 8.37 ppm, a formic acid peak at 8.14 ppm and small molecule peaks between 3 and 4.5 ppm after extensive hydrolysis at pH 4 for 7 d (Fig. 4), demonstrating that the hydrolysis of PEG-*b*-PAOE followed



Scheme 3. pH-Dependence hydrolysis of PEG-*b*-PAOE side-chains.

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036

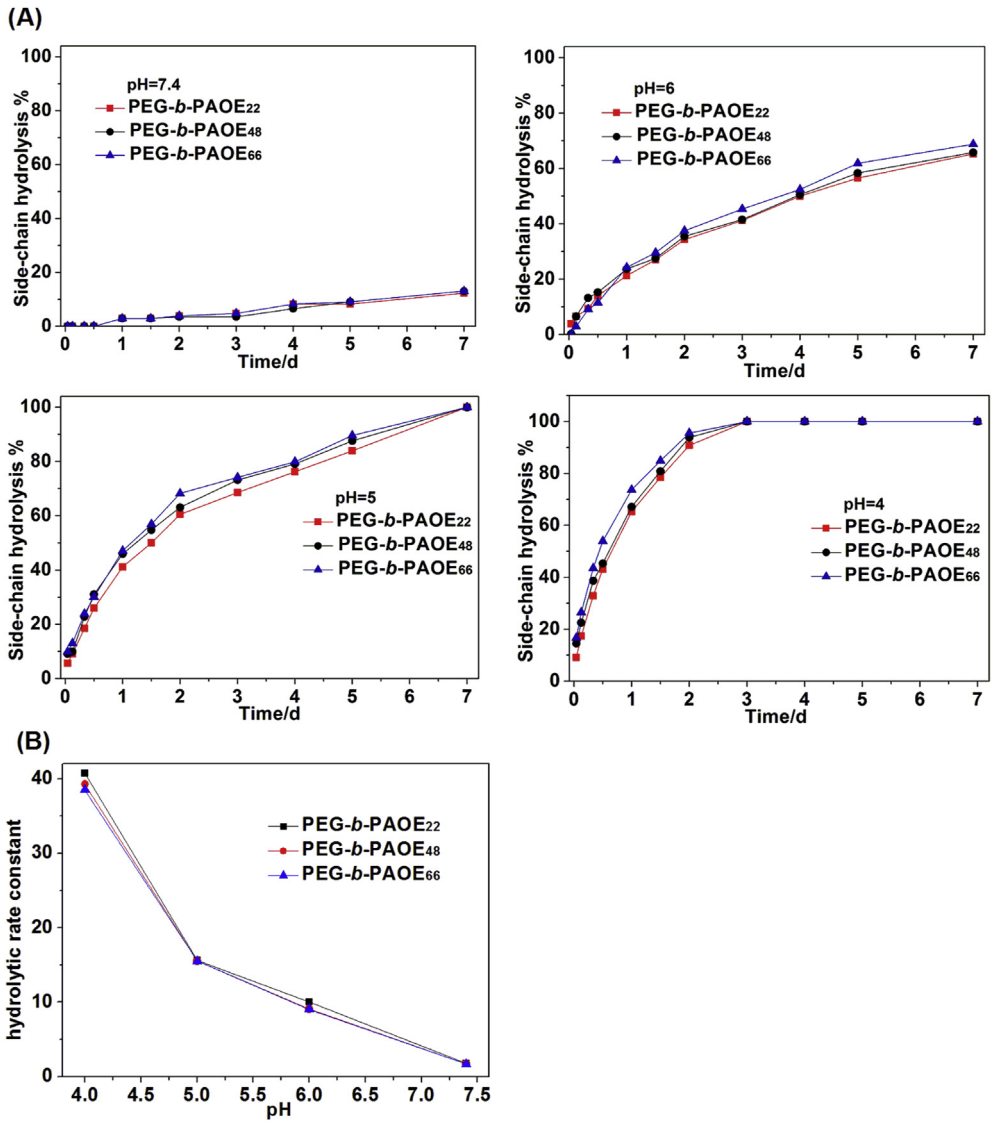


Fig. 5. (A) Time and pH-dependence of ortho ester hydrolysis in PEG-*b*-PAOE based on NMR analysis. (B) Hydrolysis rate constant of PEG-*b*-PAOE as a function of pH based on Fig. 5A.

exocyclic pathway exclusively, unlike the six-membered ortho ester ring reported before [47]. This finding agrees well with previous studies on other polymers with cyclic ortho esters in the main chains or side chains [26,42].

Furthermore, we found that at all time points and all pHs, the increase in the integral area of peaks "b" and "c" were equal to the decrease of the area of peak "a", and the total integral area of the three peaks remained constant (Fig. 4). Taken together, the NMR data confirm that the ortho ester structure of PEG-*b*-PAOE undergo exocyclic cleavage, and are converted quantitatively to a copolymer composing of a pair of stereo-isomers containing a hydroxyl and a formate (Scheme 3). The progression of ortho ester hydrolysis was revealed by NMR analysis, and could be quantified based on the changing integral area of peak "a" (representing the ortho ester proton before hydrolysis), peak "b" (representing the formate proton as a result of hydrolysis) and peak "c" (representing the formic acid proton as a result of hydrolysis of formate). We define the degree of hydrolysis to be the percentage of peak "b" and peak "c" relative to the summed area of peaks "a" "b" and "c". The time/pH-dependence of hydrolysis is shown spectroscopically in Fig. 4, and PEG-*b*-$PAOE_{48}$ was chosen as an example and quantitatively analyzed in Fig. 5.

As expected, hydrolysis of the ortho ester structure is highly sensitive to mildly acidic environments, and is much accelerated at more acidic pHs. For example, for PEG-*b*-$PAOE_{22}$, at pH 7.4 and 6, 12% and 65% of the ortho esters were hydrolyzed in 7 d, whereas the hydrolysis was essentially complete in 7 d and 3 d when pH was lowered to 5 and 4, respectively (Fig. 5). Comparing PEG-*b*-$PAOE_{48}$, PEG-*b*-$PAOE_{66}$ with PEG-*b*-$PAOE_{22}$ (Fig. 5), the percentage of hydrolysis remained almost the same at pH 7.4, whereas the hydrolysis rate of ortho ester followed the general order of PEG-*b*-$PAOE_{22}$ > PEG-*b*-$PAOE_{48}$ > PEG-*b*-$PAOE_{66}$ when pH was lowered to 6, 5, and 4. The hydrolytic rate constant for each pH has been calculated from Fig. 5A, and the rate constant vs pH was ploted, as shown in Fig. 5B. At the same pH value, PEG-*b*-PAOE with different hydrophobic chain length showed almost the same hydrolytic rate constant with a ExpDec2 correlation.

This result indicates that the chain length of PAOE block may influence the rate of polymer hydrolysis in that the degradation

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036

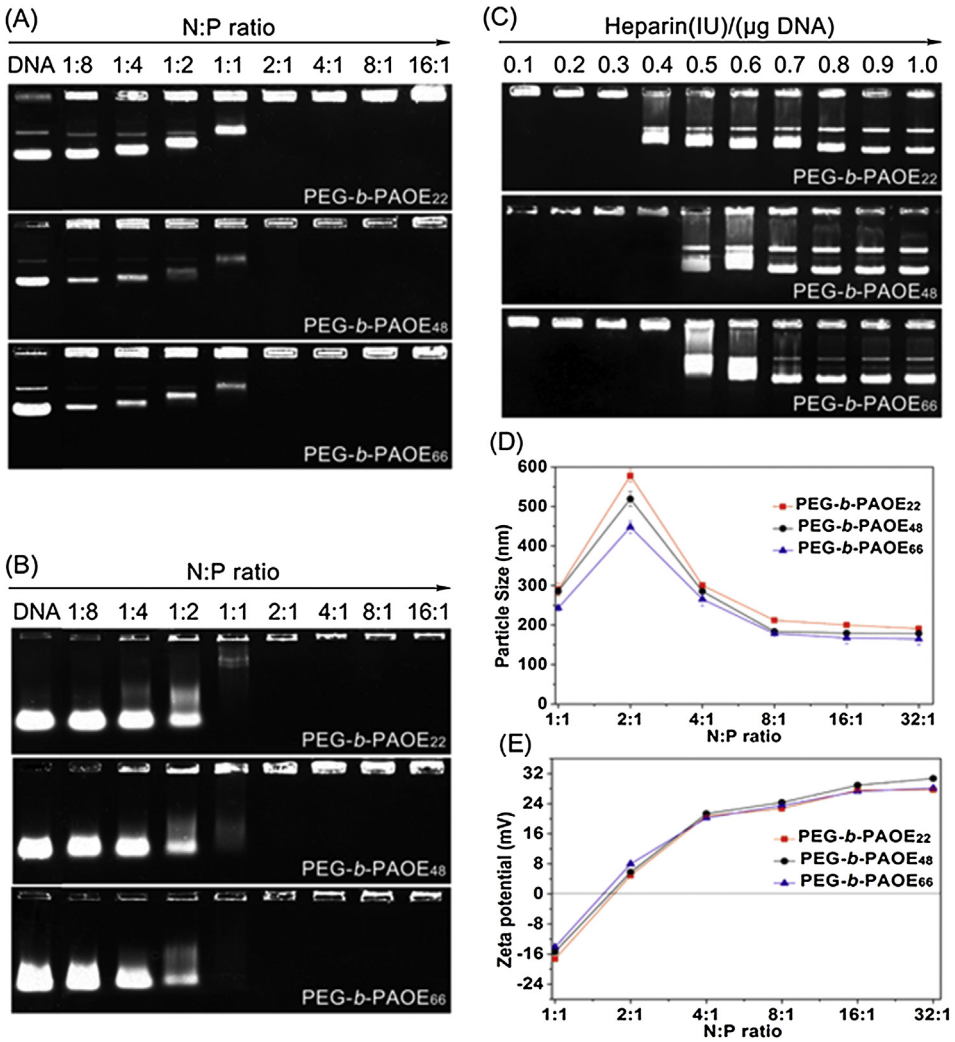


Fig. 6. Characterization of plasmid DNA binding and condensation by PEG-*b*-PAOE. (A) Gel retardation assay of polyplexes prepared at N:P ratios ranging from 1/8 to 16; (B) DNase I degradation assay of polyplexes prepared at N:P ratios ranging from 1/8 to 16; (C) Destabilization of polyplexes induced by increasing amount of heparin; (D) DLS measurement of average particle size of polyplexes in aqueous buffer (20 mM HEPES, pH 7.4) as a function of N:P ratio; (E) Zeta potential of polyplexes in aqueous buffer (20 mM HEPES, pH 7.4) as a function of N:P ratio.

rate decreases with the increase of their repeating units. The ability of achieving markedly different hydrolysis kinetics from hours to days over a fairly narrow pH range (from 4 to 7.4), as we have demonstrated here, bodes well for the potential use of such polymer systems for pH-triggered gene delivery.

3.3. DNA binding and condensation

Agarose gel retardation assay qualitatively revealed the difference in DNA binding affinity among the three PEG-*b*-PAOE copolymers. For all the three copolymers, complete retardation of plasmid DNA occurred when the N/P ratio was 2:1, regardless of the chain-length of PAOE block (Fig. 6A). Such results were further supported by the DNase I degradation experiment where DNA in compact polyplexes was protected from enzyme degradation (Fig. 6B). In addition, polyplexes at N:P ratio of 8 were selected to evaluate polyplex stability in physiological conditions. The polyplexes were analyzed by agarose gel electrophoresis after incubation in the presence of increasing amount of heparin, a polyanion that could compete with DNA for the binding to polycations. As shown in Fig. 6C, the threshold concentration of heparin at which polyplex disruption occurred were 0.4, 0.5, and 0.5 IU/μg of DNA for PEG-*b*-PAOE$_{22}$, PEG-*b*-PAOE$_{48}$, and PEG-*b*-PAOE$_{66}$, respectively. The findings suggest that polyplexes formed with longer PAOE chains were more stable than shorter ones. This conclusion agrees well with findings of other PEGylated cationic copolymer carriers, such as PEG-*b*-PAEM [50].

DLS reveals that average particle size of polyplexes in aqueous buffer spanned a range of 160−580 nm with much dependence on the N:P ratio (Fig. 6D). The size distribution of each type of polyplex was narrow with PDI ranging from 0.13 to 0.29. The particle size for all three polyplexes was most prominent at N:P ratio of 2:1, and gradually decreased as an increasing or decreasing of the N:P ratio (Fig. 6D). Average particle size stabilized around 200 nm for all three polyplexes at N/P ratios higher than 4 (Fig. 6D). As shown in Fig. 6E, There was no distinct difference in zeta potential of three types of polyplexes, zeta potential of polyplexes raised with an increase of the N:P ratio. At N:P ratio of 1:1, all the tested complexes shared similar zeta potential values at around −16 mV (Fig. 6E), suggesting that PEG moieties might serve as a hydrophilic corona on the complex surface to partially shield the cationic charges of the PAOE segment. Taken together, these results confirm that PEG-*b*-PAOE can effectively condense DNA into narrowly dispersed nanoparticles, and that polymer chain length has an important

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036

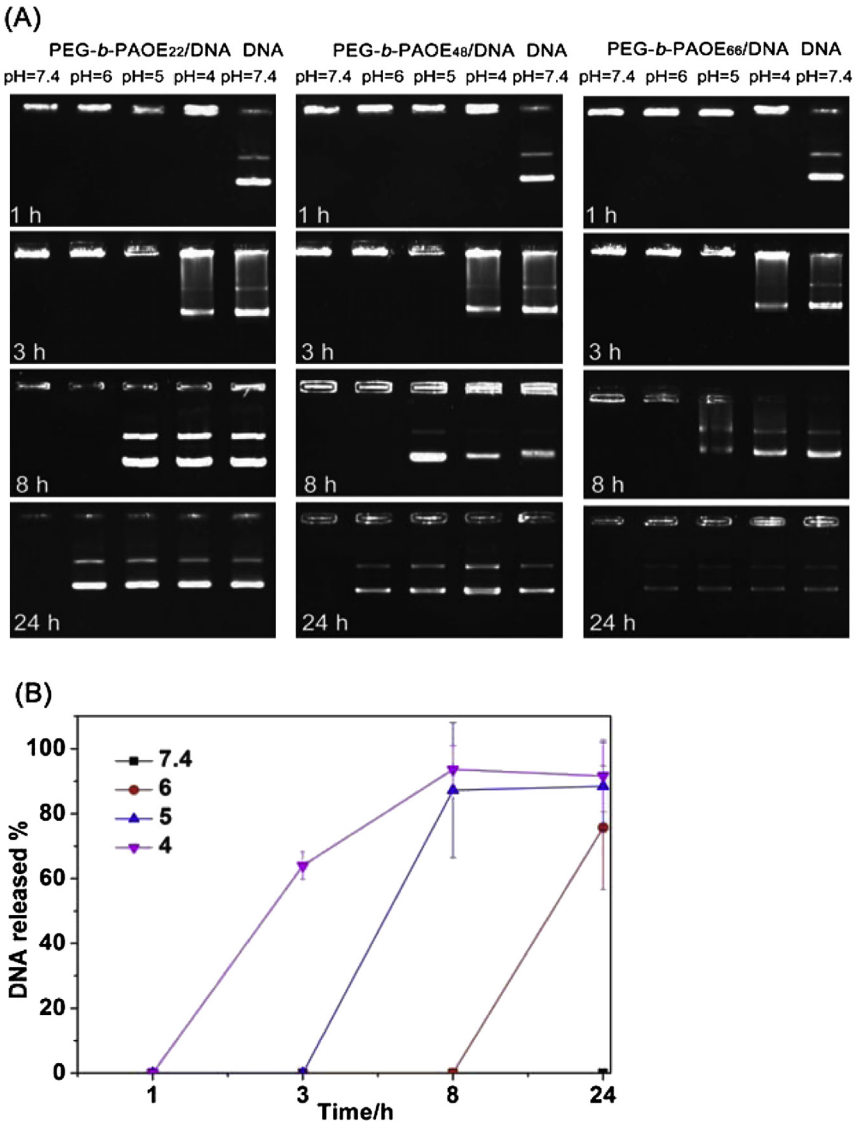


Fig. 7. (A) pH-Triggered release of DNA from PEG-*b*-PAOE polymers at N:P ratio of 8:1; (B) Time and pH-dependence of DNA release from PEG-*b*-PAOE polymers by densitometry based on Fig. 7A.

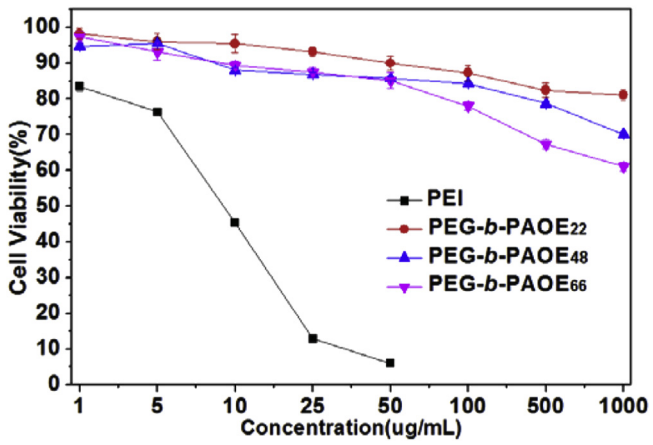


Fig. 8. Cytotoxicity of PEG-*b*-PAOE on COS-7 cells measured by MTT assay.

influence on the ability to condense DNA and polyplex stability against competition of polyanions.

3.4. pH-Triggered DNA release

To determine whether the copolymer hydrolysis at acidic pH triggered the release of DNA, polyplexes at N:P ratio of 8:1 were exposed to phosphate and acetate buffers (50 mM, pH 7.4, 6, 5, and 4), and analyzed by agarose gel electrophoresis. The three acid-labile PEG-*b*-PAOE copolymers bound to DNA stably at pH 7.4 and released free DNA at pH 6, 5, and 4 (Fig. 7A). Migration of free DNA occurred after 3 h, 8 h and 24 h when pH was lowered to 4, 5, and 6 respectively. PEG-*b*-PAOE with different PAOE chain length showed similar DNA release profiles, and time and pH-dependence of DNA release based on Fig. 7A was plotted as Fig. 7B. As shown in Fig. 7B, all DNA could be completely released in 24 h at pH 6, and in 8 h at pH 5 and 4. This was presumably due to the hydrolysis of ortho ester side chains and the subsequent loss of DNA condensing cationic charges. At pH 4 after 3 h there was at least 20% polymer hydrolysis (Fig. 5A) and DNA release was observed (Fig. 7), but at pH

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036

of 7.4–5, polymer hydrolysis was less than 20% (Fig. 5) and there was no DNA release (Fig. 7). This suggests that achieving at least 20% polymer hydrolysis might be necessary to trigger DNA release. The hydrolysis of PEG-*b*-PAOE caused the polymer chains to lose their cationic charges and be converted to a neutral hydroxylated structure (Scheme 3), thereby losing the ability to bind DNA. Taken together, these experiments established that the acid-catalyzed hydrolysis of the ortho ester linkages of PEG-*b*-PAOE side-chains were indeed capable of controlling the release of DNA at different pHs.

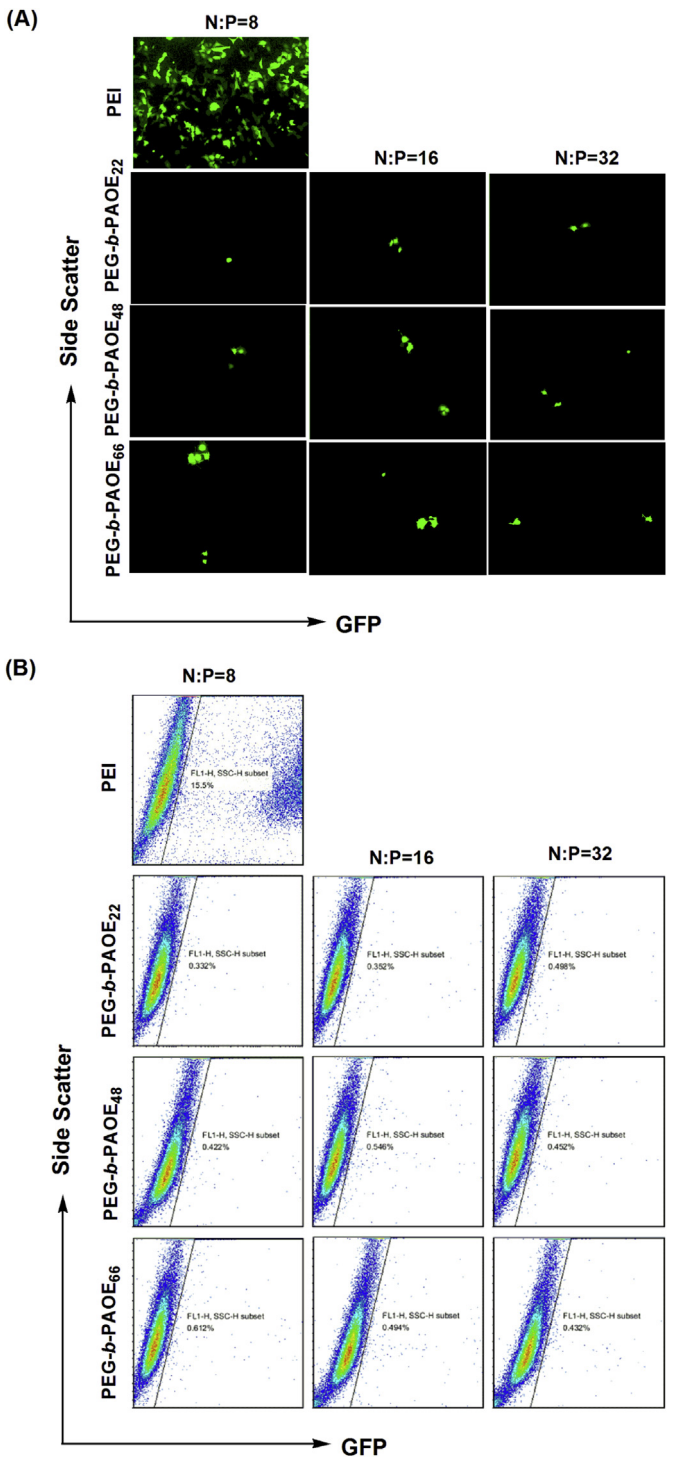


Fig. 9. In vitro transfection results on COS-7 cells determined by the fluorescence microscopy observation and flow cytometry. (A) Fluorescent microscope images of COS-7 cells transfected by pEGFP containing polyplexes; (B) Representative dot plots of transfected cells.

3.5. In vitro cytotoxicity and gene transfection

To evaluate the effect of PEG shielding on biological properties of polyplexes, in vitro cytotoxicity and transfection efficiency were tested on COS-7 cells. Branched polyethylenimine (PEI) (M_w = 25 kDa) was used as a control. The three PEG-*b*-PAOE copolymers regardless of chain length were not toxic to COS-7 cells at concentrations as high as 0.1 mg/mL (Fig. 8). In contrast, branched PEI was more toxic, killing almost all of the COS-7 cells. The low cytotoxicity of PEG-*b*-PAOE was expected and could be largely due to the presence of PEG shielding layer. Nonetheless, at a rather high concentration of 1 mg/mL, moderate toxicity was observed for all three PEG-*b*-PAOE copolymers, and the level of toxicity correlated with the chain-length of the PAOE block, where polymers bearing longer PAOE block were more toxic (Fig. 8), however, cell viability was still above 60%.

To validate low nonspecific gene transfection, in vitro transfection efficiency on COS-7 cells was assessed using GFP as the reporter gene. Images of transfected cells by fluorescence microscopy were showed in Fig. 9A, and the expression level of GFP was determined by flow cytometry (Fig. 9B). Luciferase plasmid was used as negative controls to set the GFP+ gate (Fig. S2 in Supporting Information). At N:P ratio of 8, tansfection efficiency of PEI polyplexes measured by the percentage of GFP^+ cells around 16% (Fig. 9B). Compared to PEI, transfection efficiency of PEG-*b*-PAOE polyplexes ranged from around 0.33% to 0.61% (Fig. 9B). Therefore, these results imply that the PEG shielding layer lowered zeta potential of the polyplexes and reduced their interaction with cell membrane, resulting in reduced cellular uptake and low nonspecific transfection efficiency [51,52].

Overall, we demonstrated the synthesis of a new type of acid-labile block copolymer bearing tertiary amines in side chains, PEG-*b*-PAOE, and the potential for such polymers as non-viral gene carriers. We also comprehensively investigated the physical–chemical properties of PEG-*b*-PAOE/DNA complexes as well as their biological performance including cytocompatibility and lack of nonspecific gene transfection. This structure–function relationship study focused on the influence of polycation chain length and acid-sensitive side chain hydrolysis on properties of the polyplexes and on pH-triggered DNA release. Our findings could help design more efficient and sophisticated polymeric gene carriers, due to the high flexibility and controllability of the synthetic routes of this type of polymers. In addition, PEG-*b*-PAOE could be re-engineered in the future with targeting ligands to serve as cell-specific gene carriers for particular medical applications. For example, T-cell-specific antibodies could be conjugated onto PEG-*b*-PAOE to transfect T lymphocytes, where receptor-mediated endocytosis and minimum off-target transfection on other cell types are highly desired [53].

4. Conclusions

We reported a new type of acid-labile block copolymer, PEG-*b*-PAOE, which was synthesized by RAFT polymerization. These PEGylated block copolymers were designed to bear tertiary amines linked by ortho ester rings in side chains of PAOE blocks to impart the capability of condensing DNA at physiological pH and releasing DNA at mild acid pH. At physiological pH, PEG-*b*-PAOE could efficiently bind and condense plasmid DNA into narrowly dispersed nanoparticles, whose size and colloidal stability depended on the length of PAOE blocks. These polyplexes were stable at pH 7.4, but in mildly acidic media, DNA was released due to hydrolysis of the

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036

ortho ester linkages. In general, shorter PAOE block and more acidic pH resulted in quicker DNA release. PEG-*b*-PAOE polymers had no obvious cytotoxicity on COS-7 cells and the corresponding polyplexes resulted in minimal level of non-cell-specific gene transfection. This comprehensive structure–function relationship study on PEG-*b*-PAOE demonstrates the feasibility of pH-triggered release of DNA using a simple but versatile family of acid-labile polymers, which serve as promising prototypes for further functionalization to achieve cell-specific DNA delivery.

Acknowledgments

This work is financially supported by the Chinese Program for New Century Excellent Talents in Universities (No. NCET-11-0661), the National Natural Science Foundation of China (No. 21174054, 21004030), the Scientific Research Foundation for Returned Scholars from Ministry of Education of China, the Natural Science Foundation of Anhui Province of China (No. 1408085MB26), and the Academic and Technology Introduction Project of Anhui University of China (AU02303203). W. Ji and C. Wang acknowledge the support by the US National Institutes of Health (Grant No. R01CA129189.)

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.polymer.2014.04.036.

References

[1] De llarduya CT, Sun Y, Duezguenes N. Eur J Pharm Sci 2010;40(3):159–70.
[2] Peng SF, Tseng MT, Ho YC, Wei MC, Liao ZX, Sung HW. Biomaterials 2011;32(1):239–48.
[3] Zhang X, Ma GH, Su ZG, Benkirane-Jessel N. J Control Release 2011;152(1):182–4.
[4] Mishra D, Kang HC, Bae YH. Biomaterials 2011;32(15):3845–54.
[5] Yu HJ, Nie Y, Dohmen C, Li YQ, Wagner E. Biomacromolecules 2011;12(6):2039–47.
[6] Ewert KK, Ahmad A, Evans HM, Safinya CR. Expert Opin Biol Ther 2005;5(1):33–53.
[7] Haensler J, Szoka FC. Bioconjug Chem 1993;4(5):372–9.
[8] Tang MX, Redemann CT, Szoka Jr FC. Bioconjug Chem 1996;7(6):703–14.
[9] Morille M, Passirani C, Vonarbourg A, Clavreu LA, Benoit JP. Biomaterials 2008;29(24–25):3477–96.
[10] Zhang W, Cheng Q, Guo S, Lin D, Huang P, Liu J, et al. Biomaterials 2013;34(27):6495–503.
[11] Rungsardthong U, Ehtezazi T, Bailey L, Armes SP, Garnett MC, Stolnik S. Biomacromolecules 2003;4(3):683–90.
[12] Merdan T, Kunath K, Petersen H, Bakowsky U, Voigt KH, Kopecek J, et al. Bioconjug Chem 2005;16(4):785–92.
[13] Park K. J Control Release 2010;142(2):147–8.
[14] Venkataraman S, Ong W, Ong ZY, Joachim Loo SC, Rachel Ee PL, Yang Y. Biomaterials 2011;32(9):2369–78.
[15] Mao S, Neu M, Germershaus O, Merkel O, Sitterberg J, Bakowsky U. Bioconjug Chem 2006;17(5):1209–18.
[16] Zhang X, Pan SR, Hu HM, Wu GF, Feng M, Zhang W, et al. Biomed Mater Res 2008;84(3):795–804.
[17] Khalil K, Kogure K, Akita H, Harashima H. Pharmacol Rev 2006;58(1):32–45.
[18] Forrest ML, Pack DW. Gene Ther 2002;6(1):57–66.
[19] Pecot CV, Calin GA, Coleman RL, Lopez-Berestein G, Sood AK. Nat Rev Cancer 2011;11(1):59–67.
[20] Juliano R, Alam MR, Dixit V, Kang H. Nucleic Acids Res 2008;36(12):4158–71.
[21] Thomas M, Lu JJ, Chen J, Klibanov AM. Adv Drug Deliv Rev 2007;59(2–3):124–33.
[22] Whitehead KA, Langer R, Anderson DG. Nat Rev Drug Discov 2009;8(2):129–38.
[23] Kang HC, Samsonova O, Bae YH. Biomaterials 2010;31(11):3071–8.
[24] Mok H, Lee SH, Park JW, Park TG. Nat Mater 2010;9(3):272–8.
[25] Shim MS, Kwon YJ. Adv Drug Deliv Rev 2012;64(11):1046–58.
[26] Tang RP, Ji WH, Wang C. Polymer 2011;52(3):921–32.
[27] Shim MS, Kwon YJ. Biomaterials 2010;31(12):3404–13.
[28] Park SJ, Na K. Biomaterials 2010;33(27):6485–94.
[29] Minchin RF, Yang S. Expert Opin Drug 2010;7(3):331–9.
[30] Tzeng SY, Guerrero-Cazares H, Martinez EE, Sunshine JC, Quinones-Hinojosa A, Green JJ. Biomaterials 2011;32(23):5402–10.
[31] Yin Q, Shen JA, Chen LL, Zhang ZW, Gu WW, Li YP. Biomaterials 2012;33(27):6495–506.
[32] Cohen JA, Beaudette TT, Cohen JL, Brooders KE, Bachelder EM, Frechet JMJ. Adv Mater 2010;22(32):3593–7.
[33] Liu ZH, Zhang N. Curr Pharm Des 2012;18(23):3442–51.
[34] Kale AA, Torchilin VP. Bioconjug Chem 2007;18(2):363–70.
[35] Sawant RM, Hurley JP, Salmaso S, Kale A, Tolcheva E, Levchenko TS, et al. Bioconjug Chem 2006;17(4):943–9.
[36] Shim MS, Kwon YJ. Polym Chem 2012;3(9):2570–7.
[37] Parrott MC, Luft JC, Byrne JD, Fain JH, Napier ME, DeSimone JM. J Am Chem Soc 2010;132(50):17928–32.
[38] Kim HK, Thompson DH, Jang HS, Chung YJ, Van den Bossche J. Appl Mater Interfaces 2013;5(12):5648–58.
[39] Boomer JA, Qualls MM, Inerowicz HD, Haynes RH, Patri VS, Kim JM, et al. Bioconjug Chem 2009;20(1):47–59.
[40] Tang RP, Ji WH, Panus D, Palumbo RN, Wang C. J Control Release 2011;151(1):18–27.
[41] Tang RP, Ji WH, Wang C. Macromol Chem Phys 2011;212(11):1185–92.
[42] Tang RP, Palumbo RN, Ji WH, Wang C. Biomacromolecules 2009;10(4):722–7.
[43] Tang RP, Ji WH, Wang C. Macromol Biosci 2010;10(2):192–201.
[44] Wang C, Ge Q, Ting D, Nguyen D, Shen HR, Chen JZ, et al. Nat Mater 2004;3(3):190–6.
[45] Lin S, Du FS, Wang Y, Ji SP, Liang DH, Yu L, et al. Biomacromolecules 2008;9(4):109–15.
[46] Heller J. Adv Polym Sci 1993;107(1):41–92.
[47] Heller J, Barr J, Ng SY, Abdellauoi KS, Gurny R. Adv Drug Deliv Rev 2002;54(7):1015–39.
[48] Qiao ZY, Du FS, Cheng J, Zhang R, Liang DH, Li ZC. Macromolecules 2010;43(15):6485–94.
[49] Song CC, Ji R, Du FS, Liang DH, Li ZC. ACS Macro Lett 2013;2(3):273–7.
[50] Tang RP, Palumbo RN, Nagarajan L, Krogstad E, Wang C. J Control Release 2010;142(2):229–37.
[51] Deshpande MC, Davies MC, Garnett MC, Williams PM, Armitage D, Bailey L, et al. J Control Release 2004;97(1):143–56.
[52] Oupicky D, Ogris M, Howard KA, Dash PR, Ulbrich K, Seymour LW. Mol Ther 2002;5(4):463–72.
[53] Chen GH, Chen WJ, Wu ZG, Yuan RX, Li H, Gao JM, et al. Biomaterials 2009;30(10):1962–70.

Please cite this article in press as: Lai J, et al., PEGylated block copolymers containing tertiary amine side-chains cleavable via acid-labile ortho ester linkages for pH-triggered release of DNA, Polymer (2014), http://dx.doi.org/10.1016/j.polymer.2014.04.036