Macromolecular Research, Vol. 20, No. 3, pp 327-334 (2012)
DOI 10.1007/s13233-012-0063-9

www.springer.com/13233
pISSN 1598-5032 eISSN 2092-7673

pH-Sensitive Degradable Hydrophobe Modified 1.8 kDa Branched Polyethylenimine as "Artificial Viruses" for Safe and Efficient Intracellular Gene Transfection

Meng Zheng[1], Chunmei Yang[2], Fenghua Meng[1], Rui Peng[3], and Zhiyuan Zhong*[,1]

[1]Biomedical Polymers Laboratory, and Jiangsu Key Laboratory of Advanced Functional Polymer Design and Application, Department of Polymer Science and Engineering, College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, P. R. China
[2]Lanzhou Military Command Center for Disease Control and Prevention, Lanzhou 730020, P. R. China
[3]Jiangsu Key Laboratory for Carbon-Based Functional Materials & Devices, Institute of Functional Nano & Soft Materials (FUNSOM), Soochow University, Suzhou 215123, P. R. China

Received November 30, 2011; Revised January 28, 2012; Accepted January 30, 2012

Abstract: 1.8 kDa branched polyethylenimine (PEI) was modified with pH-sensitive degradable acetal containing hydrophobe, 2,4,6-trimethoxybenzylidene-tris(hydroxymethyl)ethane (TMB-THME), to enhance its DNA condensation under extracellular conditions as well as to achieve active DNA release inside cells. PEI-(TMBTHME)$_n$ conjugates in the amount of 1.8 kDa were prepared with varying degrees of substitution (DS) from 3.0, 5.7 to 10.1. Notably, dynamic light scattering (DLS) measurements showed that all three 1.8 kDa PEI-(TMB-THME)$_n$ conjugates could effectively condense DNA into nano-sized particles (189-197 nm) at N/P ratios ranging from 20/1 to 80/1. The surface charges of PEI-(TMB-THME)$_n$ polyplexes depending on DS and N/P ratios varied from +22 to +28 mV, which were comparable to or slightly higher than the unmodified 1.8 kDa PEI counterparts (~+22 to +23 mV). Under a mildly acidic condition mimicking that of endosomes, interestingly, 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes were quickly unpacked to release DNA because of the pH-induced acetal degradation that transforms hydrophobic modification into hydrophilic modification. MTT assays demonstrated that all PEI-(TMB-THME)$_n$ polyplexes displayed low cytotoxicity (>80%) to 293T, and HeLa cells at N/P ratios ranging from 20/1 to 60/1. The in vitro gene transfection studies showed that the transfection activity of 1.8 kDa PEI was significantly enhanced by modifications with TMB-THME, in which transfection efficiencies increased with increasing DS. For example, 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes displayed 250-fold and 80-fold higher transfection efficiencies than those of the unmodified 1.8 kDa PEI counterparts in 293T and HeLa cells, respectively, which were approximately 4-fold and 2-fold higher than that of 25 kDa PEI control. The superior transfection activity of 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes was also confirmed by confocal laser scanning microscopy (CLSM), which showed efficient delivery of DNA into the nuclei of 293T cells following 4 h transfection. Modification of low molecular weight PEI with pH-sensitive degradable hydrophobe has appeared to be highly promising in the development of "artificial viruses" for safe and efficient gene transfer.

Keywords: polyethylenimine, hydrophobic modification, pH-sensitive, degradable, gene delivery.

Introduction

In the past decade, cationic polymers due to their many advantages including lack of specific immune response, ease of large scale production and handling, and facile vector modifications have emerged as an important and versatile non-viral gene delivery platform.[1-3] Polyethylenimine (PEI) with unique combination of high charge density and proton sponge effect is among the most studied polymeric gene carriers.[4,5] As a matter of fact, 25 kDa branched PEI and 22 kDa linear PEI are currently applied as golden standards for non-viral gene transfection.[6,7] However, these high molecular weight PEIs are often associated with pronounced *in vitro* and *in vivo* toxicity. In comparison, low molecular weight PEIs such as 1.8 kDa PEI with favorable cytotoxicity profiles display significantly lower transfection activity than 25 kDa PEI.[8] In the past decade, in an effort to develop non-toxic and efficient gene vectors, various types of hydrolytically or reductively degradable PEI polymers and networks have been prepared and investigated for *in vitro* gene transfection.[9-21] These degradable PEIs while maintaining low cytotoxicity often showed enhanced transfection activ-

*Corresponding Author. E-mail: zyzhong@suda.edu.cn

ity as compared to the parent low molecular weight PEIs, in a few cases approaching that of 25 kDa PEI. The synthesis of degradable PEI polymers and networks, however, usually involves complex coupling reactions which yield ill-defined macromolecular structures as well as broad molecular weight distributions.

Hydrophobic modification represents another effective approach to improve the transfection activity of low molecular weight PEI through enhancing its DNA complexation and protection as well as cellular uptake.[22,23] For example, low molecular weight PEI has been modified with different hydrophobic molecules including cholesterol,[24] dodecane,[25] aliphatic lipids (such as caprylic, myristic, palmitic, stearic, oleic, and linoleic acids),[26] phenylboronic acid,[27] lipoic acid,[28] dexamethasone,[29] and pluronic.[30] The transfection activity has reportedly been enhanced, but to varying degrees depending on nature of hydrophobic ligand and degree of substitution. It should be noted that hydrophobic modification usually yields PEI derivatives with defined structures and molecular weights. Recently, we reported that 10 kDa PEI following modification with a pH-sensitive degradable acetal containing hydrophobe, 2,4,6-trimethoxybenzylidene-tris(hydroxymethyl)ethane (TMB-THME), exhibits superior gene transfection to 25 kDa PEI, as degradation of acetals in endo/lysosomal compartments reverses hydrophobic modification into hydrophilic modification leading to active DNA release inside the cells.[31] The absence of active intracellular DNA release mechanism is an important factor limiting gene transfection activity of cationic polymers including 25 kDa PEI.[32]

In this paper, we report on pH-sensitive degradable TMB-THME hydrophobe modified 1.8 kDa PEI, denoted as 1.8 kDa PEI-(TMB-THME)$_n$, as "artificial viruses" for non-toxic and potent *in vitro* gene transfection. As the cytotoxicity of PEI is known to be inversely associated with its molecular weights and PEI is not degradable, it is highly desirable to develop gene carriers based on low molecular weight PEIs such as 1.8 kDa PEI. Notably, our results showed that pH-sensitive degradable TMB-THME hydrophobe modified 1.8 kDa PEI while maintaining low cytotoxicity displayed markedly enhanced transfection activity in 293T and HeLa cells, with transfection efficiencies up to four times exceeding that of 25 kDa PEI control. These pH-sensitive reversibly hydrophobilized 1.8 kDa PEI derivatives could be regarded as kind of "artificial viruses" in that they like viruses were designed to effectively encapsulate and protect DNA from degradation, transport DNA into cells, and actively release DNA inside cells to achieve efficient transfection. The DNA complexation, pH-triggered DNA release, cytotoxicity, and *in vitro* gene transfection activity of 1.8 kDa PEI-(TMB-THME)$_n$ were investigated.

Experimental

Materials. 2,4,6-Trimethoxybenzaldehyde-tris(hydroxymethyl)ethane-nitrophenyl chloroformate (TMB-THME-NC) was prepared according to a pervious report.[31] 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Amresco), dextran sulfate sodium (DSS, Sigma), Cy-5 (Mirus), and Hoechst 33342 (Sigma) were used as received. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and trypsin were obtained from Invitrogen. Luciferase assay kits and Micro BCA protein assay kits were obtained from R-Protagen Co., Ltd and Pierce, respectively.

Synthesis of 1.8 kDa PEI-(TMB-THME)$_n$ Conjugates. 1.8 kDa PEI-(TMB-THME)$_n$ derivatives were synthesized similar to our previous report.[31] In brief, to a solution of 1.8 kDa PEI (0.20 g, 4.7 mmol nitrogen) in 2 mL CH_2Cl_2 was added dropwise a solution of TMB-THME-NC (0.43 g, 0.94 mmol, TMB-THME/nitrogen mole feed ratio of 1/5) in 2 mL CH_2Cl_2 at room temperature (RT). The reaction was allowed to proceed for 24 h. The product was isolated by precipitation in cold diethyl ether for three times, dialysis against water to remove *p*-nitrophenol, and freeze-drying. Yield: 50%, ^{1}H NMR (400 MHz, $CDCl_3$): δ 6.07, 5.93, 4.50, 3.97, 3.81, 3.75, 3.57, 3.25, 0.74, and 2.40-2.95 (PEI). The degree of substitution (DS) could be calculated by comparing signals at δ 2.40-2.95 and δ 4.50, which showed a value of 10.1 (accordingly denoted as 1.8 kDa PEI-(TMB-THME)$_{10.1}$). In the same way, 1.8 kDa PEI-(TMB-THME)$_{3.0}$ and 1.8 kDa PEI-(TMB-THME)$_{5.7}$ derivatives were obtained at TMB-THME/nitrogen mole feed ratios of 1/25 and 1/10, respectively.

Acid-Base Titration. The buffer capability of 1.8 kDa PEI-(TMB-THME)$_n$ conjugates was determined by acid-base titration assays over a pH range from 11.0 to 2.0. Briefly, the polymer (0.1 mmol nitrogen atoms) was dissolved in 5 mL of 150 mM NaCl solution. The solution was brought to a starting pH of 11.0 with 0.1 M NaOH, and then was titrated with 0.1 M HCl using a pH meter (DELTA 320). For comparison, 1.8 kDa PEI also titrated in the same way. The buffer capacity, defined as the percentage of amine groups becoming protonated from pH 5.1 to 7.4, was calculated according to the following equation:

$$\text{Buffer capacity (\%)} = \frac{(\Delta V_{HCl} \times 0.1\text{M})}{N \text{ mol}} \times 100$$

wherein ΔV_{HCl} is the volume of HCl solution (0.1 M) required to bring the pH value of the polymer solution from 7.4 to 5.1, and N mol is the total moles of protonable amine groups in the tested polymer (0.1 mmol).

Particle Size and ζ-Potential Measurements. The polyplexes were prepared at varying N/P ratios from 20/1 to 80/1 by adding a (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer solution (20 mM, pH 7.4) of polymer (600 μL, varying concentrations) to a HEPES buffer solution (20 mM, pH 7.4) of plasmid DNA (150 μL, 37.5 μg/mL), followed by vortexing for 5 s and incubating at r.t. for 30 min. The surface charge and size of polyplexes were measured

at 25 °C with a Zetasizer Nano ZS instrument (Malvern) equipped with a standard capillary electrophoresis cell and dynamic light scattering (DLS, 10 mW He-Ne laser, 633 nm wavelength), respectively. The measurements were performed in three independent replicates. The data are presented as mean value±standard deviation.

Gel Retardation Assays. The DNA binding ability of 1.8 kDa PEI-(TMB-THME)$_n$ conjugates was studied by agarose gel electrophoresis. The polymer/DNA complexes prepared as above at varying N/P ratios from 1/1 to 4/1 were electrophoresed through a 0.8% agarose gel containing ethidium bromide at 100 V in TAE solution (40 mM Tris-HCl, 1 v/v% acetic acid, and 1 mM EDTA).

pH-Dependent Acetal Hydrolysis of 1.8 kDa PEI-(TMB-THME)$_n$ Polyplexes. The acetal hydrolysis was determined by UV/vis spectroscopy by measuring the absorbance at 290 nm according to the previous reports.[33,34] The polyplex suspensions prepared as above were divided into four aliquots and adjusted to pH 4.0, 5.0, 6.0, and 7.4, respectively, by adding 22.5 μL of 4.0 M pH 4.0, 5.0, and 6.0 acetate buffer or 7.4 phosphate buffer, while keeping the salt concentration the same. At desired time points, 80 μL aliquot was removed and diluted with 3.5 mL of phosphate buffer (0.1 M, pH 7.4) and the absorbance at 290 nm was measured. In the end, all the samples were completely hydrolyzed by adding two drops of concentrated HCl and were measured again to determine the absorbance at 100% hydrolysis, which was used to calculate extent of acetal hydrolysis.

Acid-Triggered Unpacking of 1.8 kDa PEI-(TMB-THME)$_n$ Polyplexes. 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplex suspension prepared at an N/P ratio of 20/1 as above was divided into two aliquots. One sample was adjusted to pH 5.0 by adding 4.0 M pH 5.0 acetate buffer while the other sample was added with the same volume of 4.0 M 7.4 phosphate buffer, to keep the same ionic strength. At different time intervals, the samples incubated at pH 5.0 were adjusted back to pH 7.4 with 4 M pH 7.4 phosphate buffer, and then polyplex sizes were measured. The samples incubated at pH 7.4 were also added with equal volume of 4 M pH 7.4 phosphate buffer before measurements.

Acid-triggered DNA release from 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes was also studied by agarose gel electrophoresis. The suspension pH of 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes prepared at an N/P ratio of 20/1 was adjusted to 5.0 using 4.0 M pH 5.0 acetate buffer. For the control sample at pH 7.4, same volume of 4 M pH 7.4 PB buffer was added. After 4 h, 5 μL of DSS was added to give DSS/DNA charge ratios of 0/1, 4/1, 8/1, and 12/1, respectively, and incubated for additional 0.5 h before gel electrophoresis assay.

Cytotoxicity Assays. The cytotoxicity of 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes prepared at N/P ratios of 20/1, 40/1, and 60/1 was evaluated in HeLa and 293T cells by MTT assays. 1.8 kDa PEI polyplexes were used as a control. To facilitate comparison, the experiment was performed using the same protocols as transfection studies. In brief, HeLa and 293T cells were seeded in a 96-well tissue culture plate at 6,000 cells/well in 100 μL DMEM medium containing 10% FBS for 1 day. 1.8 kDa PEI-(TMB-THME)$_n$ polyplex dispersions were added and the cells were cultured for 4 h at 37 °C. Next, the polyplexes were removed, 200 μL of fresh culture medium containing 10% serum was added, and the cells were cultured for 2 days. The medium was replaced with 100 μL of fresh medium containing 100 μg of MTT and cells were further incubated for 4 h at 37 °C. The medium was aspirated, the MTT-formazan generated by live cells was dissolved in 150 μL of DMSO, and the absorbance at a wavelength of 570 nm of each well was measured using a microplate reader. The relative cell viability (%) was determined by comparing the absorbance at 570 nm with that of control wells containing only cell culture medium. Data are presented as average standard deviation (n = 4).

***In vitro* Gene Transfection.** Transfection experiments were performed in HeLa and 293T cells using the plasmid pGL3 as a reporter gene. Transfections were conducted using polyplexes formed at N/P ratios of 20/1, 40/1, and 60/1. The cells were plated in 24-well plates (6×10^4 cells/well) and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere containing 5% CO_2 until 70% confluency. In a standard transfection experiment, the cells were rinsed with PBS and incubated with 100 mL of polyplex dispersions (1 μg of plasmid DNA per well) and 400 mL of culture medium with or without 10% serum for 4 h at 37 °C. Next, the polyplexes were removed, 500 μL of fresh culture medium containing 10% serum was added, and the cells were cultured for 2 days. Luciferase quantification was done using a commercial luciferase assay kit (R-Protagen Co., Ltd) and a multifunctional microplate reader (Multiskan Flash, Thermo). The total protein was measured according to a BCA protein assay kit (Pierce). Transfection efficiency was expressed as relative light unit (RLU) per mg of protein. 25 kDa PEI/DNA formulation prepared at an optimal N/P ratio of 10/1 was used as a reference. All the experiments were carried out in triplicate.

Confocal Microscopy. The cellular uptake and intracellular DNA release behaviors of 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes at an N/P ratio of 40/1 were studied in 293T cells with CLSM using Cy5-labeled DNA. 25 kDa PEI polyplexes at an N/P ratio of 10/1 were used as a control. In brief, 293T cells were plated on coverslips in 24-well plates (cell density 6×10^4 cells/well) and maintained in DMEM supplemented with 10% FBS at 37 °C in a humidified atmosphere containing 5% CO_2 for 1 day. 100 μL of polyplex dispersions (1 μg of Cy-5 labeled plasmid DNA per well) were added. The cells were cultured for 4 h at 37 °C. The polyplexes were removed and the cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 min. The nuclei were stained with 200 μL of Hoechst 33342 (20 μg/

mL) for 15 min at room temperature (RT). The cells following three times rinsing with PBS were observed with a confocal laser scanning microscope (TCS SP5 Leica).

Results and Discussion

Synthesis of 1.8 kDa PEI-(TMB-THME)$_n$ Conjugates. The aim of this study was to develop bioresponsive nonviral carriers based on nontoxic 1.8 kDa PEI for efficient intracellular gene transfer. To this end, 1.8 kDa PEI was modified with pH-sensitive degradable TMB-THME hydrophobe, which was hypothesized to not only improve its DNA condensation and cellular interactions but also facilitate intracellular DNA release due to reversal of hydrophobic modification as a result of acetal hydrolysis in acidic endo/lysosomal compartments. 1.8 kDa PEI-(TMB-THME)$_n$ derivatives were readily prepared by treating 1.8 kDa PEI with TMB-THME-nitrophenyl chloroformate in CH_2Cl_2 at RT. ^{1}H NMR showed that besides resonances owing to PEI (δ 2.40-2.95) signals assignable to TMB-THME (δ 6.07, 5.93, 4.50, 3.97, 3.81, 3.75, 3.57, and 0.74) were detected (Figure 1). Moreover, there appeared also a new signal at δ 3.25 attributable to the methylene protons of PEI linking to TMB-THME (Figure 1), confirming successful synthesis of 1.8 kDa PEI-(TMB-THME)$_n$ conjugates. The degrees of substitution (DS) could be calculated by comparing signals at δ 2.40-2.95 (methylene protons of PEI) and δ 4.50 (methylene protons of TMB-THME neighboring to the urethane bond). The results showed that 1.8 kDa PEI-(TMB-THME)$_n$ conjugates with DS of 3.0, 5.7, and 10.1 were obtained at theoretical DS of 4, 10, and 20, respectively (Table I). Acid-base titrations revealed that buffer capacities of 1.8 kDa PEI-(TMB-THME)$_n$ conjugates decreased from 13.8% to 13.0% with increasing DS from 3.0 to 10.1, which were slightly lower than that of the parent 1.8 kDa PEI (14.1%) (Table I).

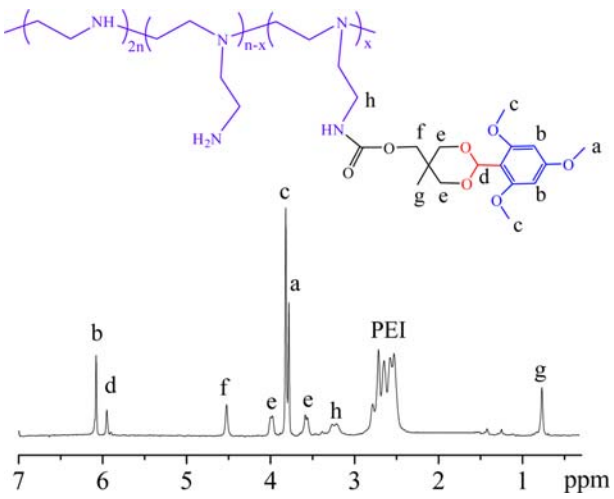

Figure 1. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of 1.8 kDa PEI-(TMB-THME)$_{10.1}$ (Table I, Entry 3).

Biophysical Characterization of 1.8 kDa PEI-(TMB-THME)$_n$ Polyplexes. The DNA complexation behaviors of 1.8 kDa PEI-(TMB-THME)$_n$ conjugates were investigated by gel electrophoresis, dynamic light scattering (DLS) and zeta-potential measurements. Gel retardation assays showed that the DNA binding ability of 1.8 kDa PEI was slightly impaired by conjugation with TMB-THME, in which 1.8 kDa PEI-(TMB-THME)$_{3.0}$, 1.8 kDa PEI-(TMB-THME)$_{5.7}$, and 1.8 kDa PEI-(TMB-THME)$_{10.1}$ completely inhibited DNA migration at an N/P ratio of 3/1, 3/1, and 4/1, respectively (Figure 2). The reduced DNA binding ability of 1.8 kDa PEI-(TMB-THME)$_n$ conjugates is likely due to their decreased cationic charge density, as TMB-THME links to PEI mostly via its

Table I. Characteristics of 1.8 kDa PEI-(TMB-THME)$_n$ Conjugates[a]

Entry	Polymer	DS (theory)	DS[b] (^{1}H NMR)	Buffer Capacity[c] (%)
1	1.8 kDa PEI-(TMB-THME)$_{3.0}$	4	3.0	13.8
2	1.8 kDa PEI-(TMB-THME)$_{5.7}$	10	5.7	13.5
3	1.8 kDa PEI-(TMB-THME)$_{10.1}$	20	10.1	13.0

[a]1.8 kDa PEI-(TMB-THME)$_n$ wherein n represents degree of substitution (DS), defined as number of TMB-THME substituents per 100 nitrogen of PEI. [b]Determined by ^{1}H NMR. [c]Determined by acid-base titration.

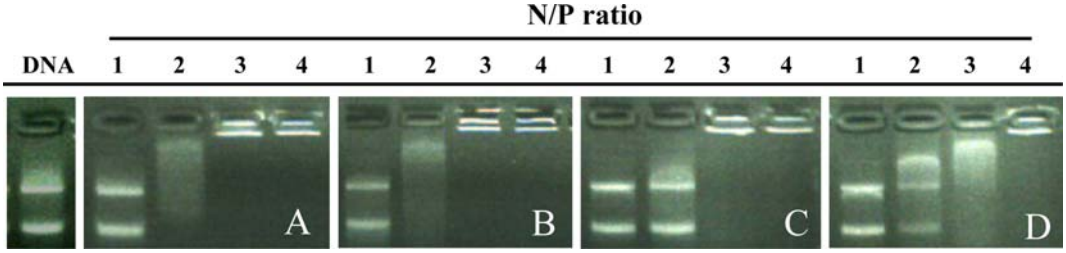

Figure 2. Gel retardation assays of 1.8 kDa PEI and 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes formed at N/P ratios ranging from 1/1 to 4/1. (A) 1.8 kDa PEI; (B) 1.8 kDa PEI-(TMB-THME)$_{3.0}$; (C) 1.8 kDa PEI-(TMB-THME)$_{5.7}$; (D) 1.8 kDa PEI-(TMB-THME)$_{10.1}$.

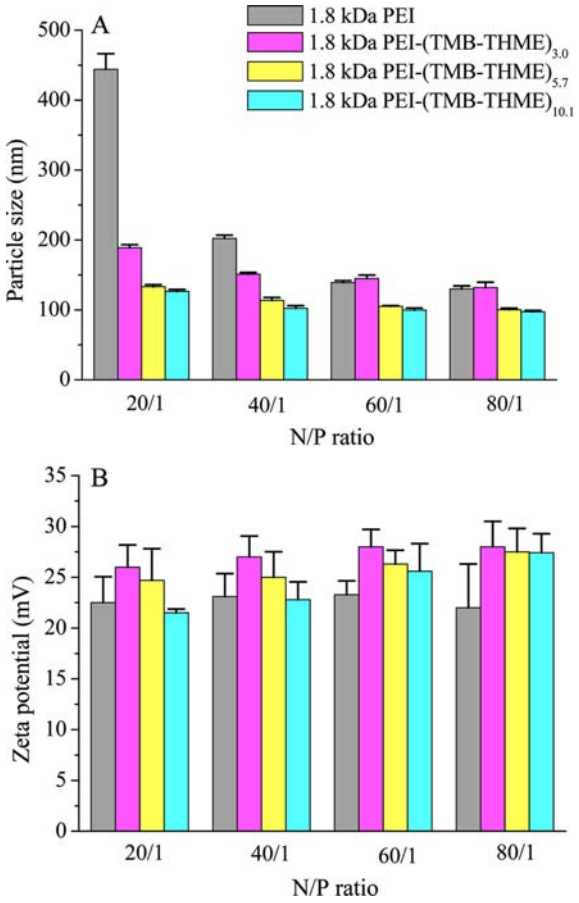

Figure 3. Average particle size (A) and ζ-potential (B) of 1.8 kDa PEI-(TMB-THME)$_n$/DNA polyplexes at N/P ratios ranging from 20/1 to 80/1. 1.8 kDa PEI was used as a control. Data are shown as mean±SD (n=3).

primary amine group.

DLS showed that all 1.8 kDa PEI-(TMB-THME)$_n$ conjugates could effectively condense DNA into nano-sized particles (average diameters < 200 nm) at and above an N/P ratio of 20/1 (Figure 3(A)). At the same N/P ratio, the polyplex sizes decreased with increasing DS. For example, polyplexes of 1.8 kDa PEI-(TMB-THME)$_{3.0}$, 1.8 kDa PEI-(TMB-THME)$_{5.7}$, and 1.8 kDa PEI-(TMB-THME)$_{10.1}$ formed at an N/P ratio of 40/1 had average sizes of approximately 151, 114, and 102 nm, respectively, which were smaller than polyplexes of the unmodified 1.8 kDa PEI (202 nm). Zeta potential measurements revealed that the surface charges of 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes increased slightly with increasing N/P ratios (Figure 3(B)). It is interesting to note that 1.8 kDa PEI-(TMB-THME)$_{3.0}$ polyplexes had considerably higher surface charges (+26~28 mV) than those of the unmodified 1.8 kDa PEI (+22~23 mV). However, at the same N/P ratio of 20/1, 40/1, or 60/1, further increasing DS resulted in decreased surface charges of polyplexes (Figure 3(B)). At a high N/P ratio of 80/1, all 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes had similar surface charges of *ca.* +28 mV. It appears, therefore, that modification of 1.8 kDa PEI with TMB-THME would impair its DNA binding ability at low N/P ratios (*i.e.* low polymer concentrations) as a result of reduced charge density but would enhance its DNA condensation ability at high N/P ratios (*i.e.* high polymer concentrations) possibly due to increased surface charge density resulting from micellization.

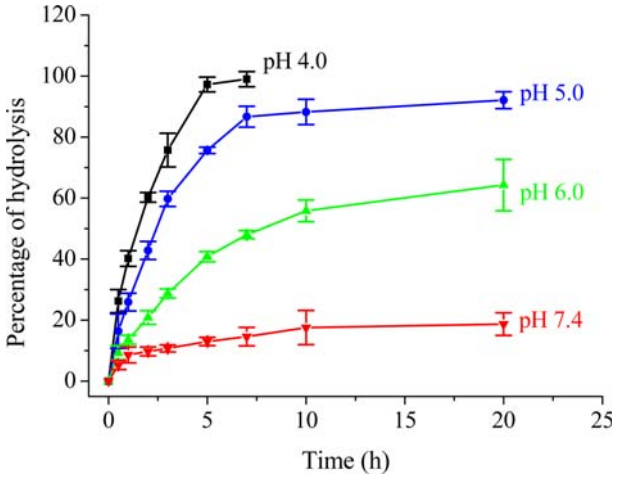

Figure 4. pH-Dependent hydrolysis of acetals in 1.8 kDa PEI-(TMB-THME)$_{10.1}$/DNA complexes (N/P ratio=20/1).

pH-Triggered Acetal Degradation and Unpacking of Polyplexes. The extent of acetal degradation was conveniently determined using UV/vis spectroscopy by monitoring the absorbance at 290 nm, which is the characteristic absorbance of the hydrolysis product, 2,4,6-trimethoxybenzaldehyde.[33,34] The pH-dependent hydrolysis of acetals in 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes formed at an N/P of 20/1 was studied at pH 4.0, 5.0, 6.0, and 7.4, respectively. The results showed clearly that the lower the pH, the faster the degradation of acetals, in which degradation half lives of 1.5, 2.4, and 7.8 h were observed at pH 4.0, 5.0, and 6.0, respectively (Figure 4). In contrast, acetal hydrolysis (< 20%) was minimal in 20 h at pH 7.4 (Figure 4).

The hydrolysis of acetals would result in reversal of hydrophobic modification, which was hypothesized to trigger unpacking of polyplexes. Gel retardation assays revealed that DNA was released from 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes formed at an N/P ratio of 20/1 following 4 h incubation at pH 5.0 in the presence of 4-fold excess of negatively charged dextran sodium sulfate (DSS) relative to DNA phosphate groups (Figure 5). In contrast, no free DNA was detected following 4 h incubation at pH 7.4 under otherwise the same conditions. In accordance, DLS studies showed that both size and polydispersity of 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes formed at an N/P ratio of 20/1 increased significantly in 2 h at pH 5.0, while little changes in size and size distribution were observed at pH 7.4 (Figure 6). Taking

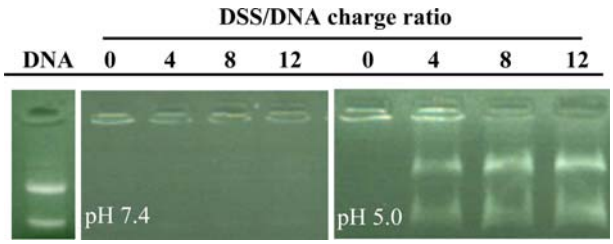

Figure 5. Agarose gel electrophoresis of 1.8 kDa PEI-(TMB-THME)$_{10.1}$/DNA polyplexes formed at an N/P ratio of 10/1 under the competition of dextran sodium sulfate (DSS) after 4 h incubation at pH 7.4 and 5.0. The lanes correspond to DSS/DNA charge ratios of 0, 4, 8, and 12, respectively.

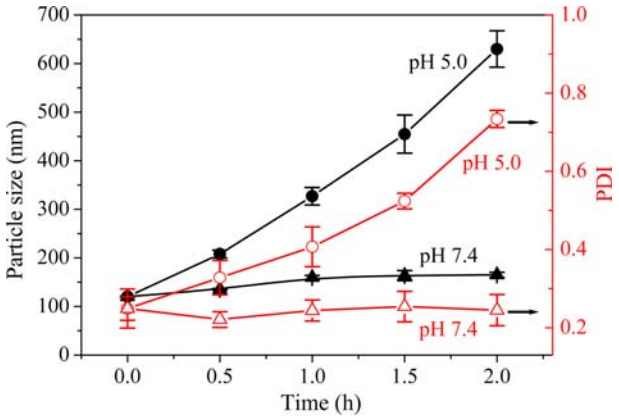

Figure 6. Change of average particle size and polydispersity index (PDI) of 1.8 kDa PEI-(TMB-THME)$_{10.1}$/DNA polyplexes (N/P ratio=20/1) over incubation time at pH 5.0 and 7.4.

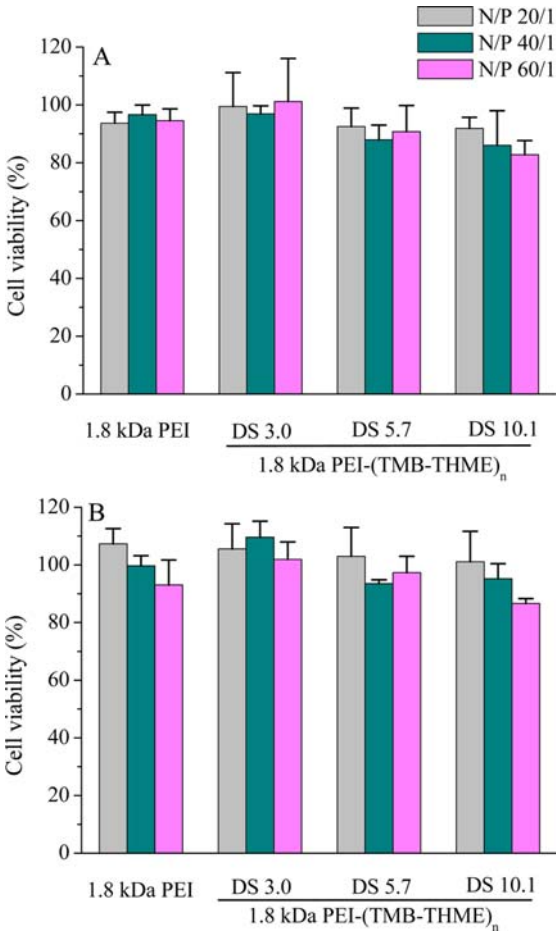

Figure 7. Cytotoxicity of 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes at N/P ratios of 20/1, 40/1, and 60/1. The polyplexes of 1.8 kDa PEI were used as a control. Cell viabilities are shown as mean±SD (n=4). (A) 293T cells; (B) HeLa cells.

advantage of pH-sensitive degradation of the acetal bonds in TMB-THME group, we have previously prepared endosomal pH-responsive micelles and polymersomes for triggered release of anticancer drugs.[35,36] These results confirm that pH-sensitive acetal-containing hydrophobe modified 1.8 kDa PEI form smart nano-sized DNA complexes that are sufficiently stable at pH 7.4 but are readily dissociated under endosomal pH conditions.

Cytotoxicity and *In vitro* Gene Transfection. The cytotoxicity of 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes was investigated in 293T and HeLa cells using MTT assays. Interestingly, the results showed that 1.8 kDa PEI-(TMB-THME)$_{3.0}$ polyplexes were nontoxic to both 293T (cell viabilities 96.9%~101.2%) and HeLa cells (cell viabilities 101.9% ~109.6%) at N/P ratios ranging from 20/1 to 60/1 (Figure 7). The increase of DS to 5.7 and 10.1 caused slight toxicity to cells, in particular at high N/P ratios of 40/1 and 60/1. Moreover, the cytotoxicity of 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes was shown to increase with increasing N/P ratios from 20/1 to 60/1. It should be noted, however, that all 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes exhibited in general low cytotoxicity (cell viability > 80%) at varying N/P ratios from 20/1 to 60/1 (Figure 7).

The *in vitro* transfection activity of 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes formed at N/P ratios of 20/1, 40/1, and 60/1 was evaluated in HeLa and 293T cells in the presence of 10% serum using pGL3 as a reporter gene. Interestingly, 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes displayed markedly enhanced transfection efficiencies in both HeLa and 293T cells as compared to the unmodified 1.8 kDa PEI controls (Figure 8). The transfection efficiency increased with increasing DS. Notably, 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes at an N/P ratio of 40/1 showed 250-fold increase of transfection efficiency as compared to the parent 1.8 kDa PEI polyplexes in 293T cells under otherwise the same conditions, which corresponded to approximately 4-fold higher transfection level than 25 kDa PEI formulation at its optimal N/P ratio of 10/1 (Figure 8(A)). In HeLa cells, 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes exhibited the best transfection activity at an N/P ratio of 60/1, which revealed approximately 80-fold higher transfection efficiency than that of the unmodified 1.8 kDa PEI counterpart, 2-fold exceeding that

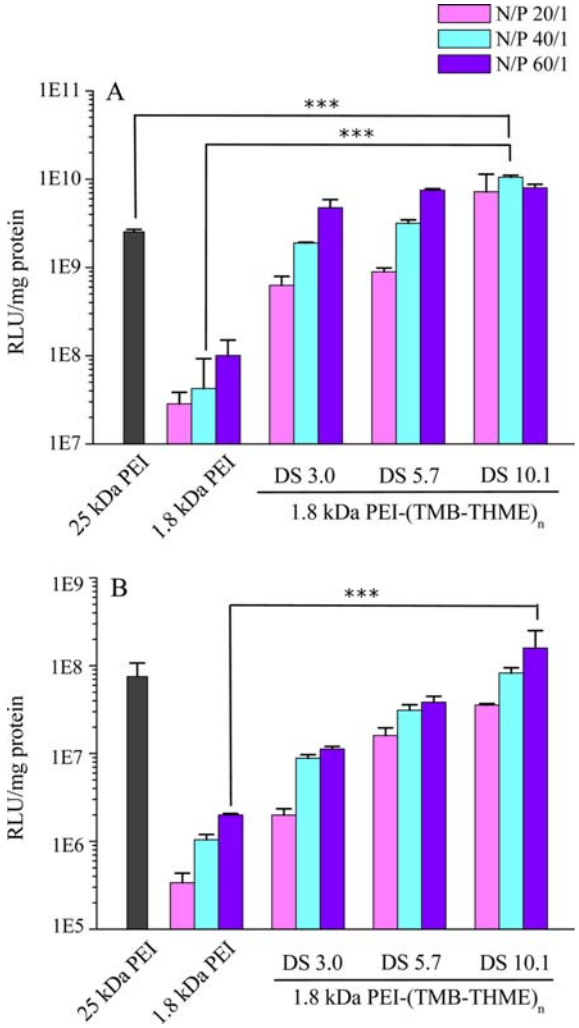

Figure 8. Transfection efficiencies of 1.8 kDa PEI-(TMB-THME)$_n$ polyplexes in 293T (A) and HeLa (B) cells at N/P ratios of 20/1, 40/1, and 60/1 in 10% serum condition. 25 kDa PEI formulation at its optimal N/P ratio of 10/1 and 1.8 kDa PEI formulations at N/P ratios of 20/1, 40/1, and 60/1 were used as controls. Data are shown as mean±SD (n=3) (Student's *t*-test, $^{***}p<0.001$).

of 25 kDa PEI control (Figure 8(B)).

To obtain insight into the intracellular fate of DNA complexed with 1.8 kDa PEI-(TMB-THME)$_n$ conjugates, transfection experiments were studied in 293T cells with confocal laser scanning microscope (CLSM) using Cy-5 labeled DNA. The cell nuclei were stained with Hoechst 33242 (blue). Interestingly, the results showed that 1.8 kDa PEI-(TMB-THME)$_{10.1}$ polyplexes formed at an N/P ratio of 40/1 effectively delivered DNA into the nuclei of 293T cells following 4 h transfection (Figure 9(A)). In comparison, much less DNA fluorescence was detected in the nuclei of 293T cells transfected with 25 kDa PEI control under otherwise the same conditions (Figure 9(B)). These results confirm that 1.8 kDa PEI-(TMB-THME)$_{10.1}$ mediates superior intracellular DNA delivery to 25 kDa PEI control.

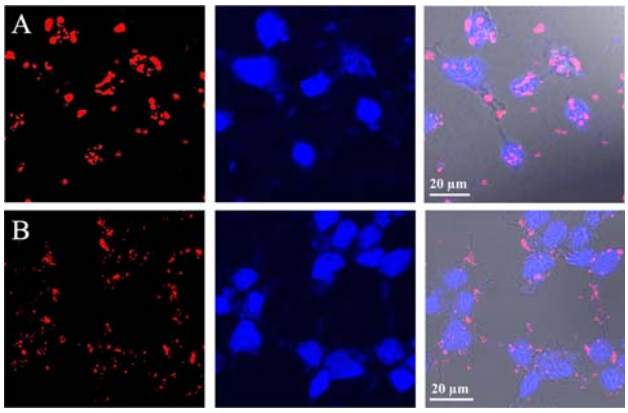

Figure 9. CLSM images of 293T cells transfected with Cy5-labeled pDNA polyplexes of 1.8 kDa PEI-(TMB-THME)$_{10.1}$ at an N/P ratio of 40/1 in the presence of 10% serum (1 μg DNA/well). 25 kDa PEI at an N/P ratio of 10/1 was used as a control. Cells were incubated with Cy5-labeled pDNA polyplexes for 4 h. For each panel, images from left to right show Cy5 labeled pDNA, cell nuclei stained by Hoechst 33342 (middle, blue), and overlays of both images. The bar represents 20 μm. (A) 1.8 kDa PEI-(TMB-THME)$_{10.1}$; (B) 25 kDa PEI.

The markedly enhanced transfection activity of 1.8 kDa PEI following modification with pH-sensitive degradable hydrophobe arises from its virus-mimicking features, *i.e.* condensing DNA into virus-sized nanoparticles under extracellular conditions while efficiently unpacking polyplexes to release DNA inside cells. The absence of an active DNA release mechanism in cells is one of the major barriers limiting transfection activity of non-viral gene carriers.[37,38] These virus-mimicking polymeric carriers are highly promising for the development of safe and efficient gene transfer agents.

Conclusions

We have demonstrated that modification of 1.8 kDa PEI with an endosomal pH-sensitive degradable acetal containing hydrophobe, 2,4,6-trimethoxybenzylidene-tris(hydroxymethyl) ethane (TMB-THME), provides efficient and low toxic virus-mimicking non-viral gene carriers. This unique modification method has elegantly combined hydrophobic modification of 1.8 kDa PEI that enhances its DNA condensation under extracellular conditions and pH-responsive degradation that facilitates DNA release inside the transfected cells. It should be noted that synthesis of PEI-(TMB-THME)$_n$ conjugates is straightforward, resulting in vectors with defined structures and compositions. Importantly, PEI-(TMB-THME)$_n$ conjugates maintain a low cytotoxicity and likely will not pose long-term toxicity due to its low molecular weight and degradation property. The bioresponsive reversible hydrophobic modification of non-toxic low molecular

weight cationic polymers has appeared to be a particularly attractive approach to develop "artificial viruses" for safe and efficient gene transfection.

Acknowledgments. This work was supported by National Natural Science Foundation of China (NSFC 20874070, 50803043, 50973078, 20974073, and 31070707), a Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions, and Program of Innovative Research Team of Soochow University.

References

(1) J. H. Jeong, S. W. Kim, and T. G. Park, *Prog. Polym. Sci.*, **32**, 1239 (2007).
(2) M. A. Mintzer and E. E. Simanek, *Chem. Rev.*, **109**, 259 (2008).
(3) D. W. Pack, A. S. Hoffman, S. Pun, and P. S. Stayton, *Nat. Rev. Drug Discov.*, **4**, 581 (2005).
(4) T. G. Park, J. H. Jeong, and S. W. Kim, *Adv. Drug Deliv. Rev.*, **58**, 467 (2006).
(5) D. Schaffert and E. Wagner, *Gene Ther.*, **15**, 1131 (2008).
(6) L. Bonetta, *Nat. Methods*, **2**, 875 (2005).
(7) U. Lungwitz, M. Breunig, T. Blunk, and A. Gopferich, *Eur. J. Pharm. Biopharm*., **60**, 247 (2005).
(8) W. T. Godbey, K. K. Wu, and A. G. Mikos, *J. Biomed. Mater. Res.*, **45**, 268 (1999).
(9) R. Arote, T. H. Kim, Y. K. Kim, S. K. Hwang, H. L. Jiang, H. H. Song, J. W. Nah, M. H. Cho, and C. S. Cho, *Biomaterials,* **28**, 735 (2007).
(10) R. B. Arote, S. K. Hwang, M. K. Yoo, D. Jere, H. L. Jiang, Y. K. Kim, Y. J. Choi, J. W. Nah, M. H. Cho, and C. S. Cho, *J. Gene Med*., **10**, 1223 (2008).
(11) J. H. Jeong, L. V. Christensen, J. W. Yockman, Z. Y. Zhong, J. F. J. Engbersen, W. J. Kim, J. Feijen, and S. W. Kim, *Biomaterials,* **28**, 1912 (2007).
(12) D. Jere, H. L. Jiang, R. Arote, Y. K. Kim, Y. J. Choi, M. H. Cho, T. Akaike, and C. S. Cho, *Expert Opin. Drug Deliv.,* **6**, 827 (2009).
(13) Y. H. Kim, J. H. Park, M. Lee, Y. H. Kim, T. G. Park, and S. W. Kim, *J. Control. Release,* **103**, 209 (2005).
(14) J. Liu, X. L. Jiang, L. Xu, X. M. Wang, W. E. Hennink, and R. X. Zhuo, *Bioconjug. Chem.*, **21**, 1827 (2010).
(15) J. Luten, C. F. van Nostruin, S. C. De Smedt, and W. E. Hennink, *J. Control. Release,* **126**, 97 (2008).
(16) M. R. Park, K. O. Han, I. K. Han, M. H. Cho, J. W. Nah, Y. J. Choi, and C. S. Cho, *J. Control. Release,* **105**, 367 (2005).
(17) Q. Peng, Z. L. Zhong, and R. X. Zhuo, *Bioconjug. Chem*., **19**, 499 (2008).
(18) H. Petersen, T. Merdan, F. Kunath, D. Fischer, and T. Kissel, *Bioconjug. Chem.,* **13**, 812 (2002).
(19) V. Russ, H. Elfberg, C. Thoma, J. Kloeckner, M. Ogris, and E. Wagner, *Gene Ther.*, **15**, 18 (2008).
(20) V. Russ, M. Gunther, A. Halama, M. Ogris, and E. Wagner, *J. Control. Release,* **132**, 131 (2008).
(21) Y. X. Wang, P. Chen, and J. C. Shen, *Biomaterials,* **27**, 5292 (2006).
(22) V. Incani, A. Lavasanifar, and H. Uludag, *Soft Matter*, **6**, 2124 (2010).
(23) Z. H. Liu, Z. Y. Zhang, C. R. Zhou, and Y. P. Jiao, *Prog. Polym. Sci.*, **35**, 1144 (2010).
(24) S. O. Han, R. I. Mahato, and S. W. Kim, *Bioconjug. Chem.*, **12**, 337 (2001).
(25) M. Thomas and A. M. Klibanov, *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 14640 (2002).
(26) A. Neamnark, O. Suwantong, K. C. R. Bahadur, C. Y. M. Hsu, P. Supaphol, and H. Uludag, *Mol. Pharm.*, **6**, 1798 (2009).
(27) Q. Peng, F. J. Chen, Z. L. Zhong, and R. X. Zhuo, *Chem. Commun.*, **46**, 5888 (2010).
(28) M. Zheng, Y. N. Zhong, F. H. Meng, R. Peng, and Z. Y. Zhong, *Mol. Pharm.*, **8**, 2434 (2011).
(29) Y. M. Bae, H. Choi, S. Lee, S. H. Kang, Y. T. Kim, K. Nam, J. S. Park, M. Lee, and J. S. Choi, *Bioconjug. Chem*., **18**, 2029 (2007).
(30) K. C. Cho, S. H. Choi, and T. G. Park, *Macromol. Res*., **14**, 348 (2006).
(31) Z. Z. Liu, M. Zheng, F. H. Meng, and Z. Y. Zhong, *Biomaterials*, **32**, 9109 (2011).
(32) Y. H. Wang, M. Zheng, F. H. Meng, J. Zhang, R. Peng, and Z. Y. Zhong, *Biomacromolecules,* **12**, 1032 (2011).
(33) E. R. Gillies and J. M. J. Frechet, *Bioconjug. Chem*., **16**, 361 (2005).
(34) E. R. Gillies, T. B. Jonsson, and J. M. J. Frechet, *J. Am. Chem. Soc*., **126**, 11936 (2004).
(35) W. Chen, F. H. Meng, R. Cheng, and Z. Y. Zhong, *J. Control. Release,* **142**, 40 (2010).
(36) W. Chen, F. H. Meng, F. Li, S. J. Ji, and Z. Y. Zhong, *Biomacromolecules,* **10**, 1727 (2009).
(37) C. L. Grigsby and K. W. Leong, *J. R. Soc. Interface*, **7**, S67 (2010).
(38) T. Wang, J. R. Upponi, and V. P. Torchilin, *Int. J. Pharm.* (2011), DOI: 10.1016/j.ijpharm.2011.07.013.