DOI: 10.1002/asia.201402524

Synthesis and Self-Assembly of Thermoresponsive Amphiphilic Biodegradable Polypeptide/Poly(ethyl ethylene phosphate) Block Copolymers

Qiuhua Wu, Dan Zhou, Renyu Kang, Xiuping Tang, Qi Yang, Ximing Song, and Guolin Zhang*[a]

Abstract: We report the design and synthesis of new fully biodegradable thermoresponsive amphiphilic poly(γ-benzyl L-glutamate)/poly(ethyl ethylene phosphate) (PBLG-*b*-PEEP) block copolymers by ring-opening polymerization of *N*-carboxy-γ-benzyl L-glutamate anhydride (BLG−NCA) with amine-terminated poly(ethyl ethylene phosphate) (H_2N−PEEP) as a macroinitiator. The fluorescence technique demonstrated that the block copolymers could form micelles composed of a hydrophobic core and a hydrophilic shell in aqueous solution. The morphology of the micelles as determined by transmission electron microscopy (TEM) was spherical. The size and critical micelle concentration (CMC) values of the micelles showed a decreasing trend as the PBLG segment increased. However, UV/Vis measurements showed that these block copolymers exhibited a reproducible temperature-responsive behavior with a lower critical solution temperature (LCST) that could be tuned by the block composition and the concentration.

Keywords: block copolymers · drug delivery · peptides · polymers · self-assembly

Introduction

Micelles fabricated from macromolecular species have attracted considerable interest in contemporary macromolecular science for both their diversiform morphologies and potential applications.[1] These nanosized assemblies demonstrate a series of attractive properties in drug-delivery systems, such as good biocompatibility and high stability in vitro and in vivo, and have been described as promising materials in applications such as biosensors, tissue engineering, or selective drug delivery.[2] Micelles formed by stimuli-responsive block copolymers in particular have received growing scientific interest in recent years.[3] Stimuli-responsive polymers that exhibit unique property changes in response to environmental stimuli, for example, temperature, pH, electric fields, and light, are promising for many biomedical applications, including smart drug/gene-delivery systems, injectable tissue engineering scaffolds, cell culture, and separation sheets.[4] Among all intelligent polymers studied, temperature-responsive polymeric systems have drawn more attention, because this is an important physiological factor in the body, and some disease states manifest themselves through changes in temperature.[5] Thermoresponsive polymers are soluble in cold water, although they precipitate with heating above a certain temperature, known as the lower critical solution temperature (LCST).[6] This phenomenon is reversible; upon cooling the thermosensitive polymers become soluble again.[7] This combination of biodegradability made the use of micelles self-assembled from thermoresponsive polymers possible as new functional biomaterials to be applied in the construction of novel controlled drug-delivery systems or in other related biomedical applications.[8]

Polyphosphoesters (PPEs) represent a class of biodegradable polymers with repeated phosphoester attachments in the backbone, which degrades under the physiological conditions by means of hydrolysis or enzymatic cleavage of the phosphoester bonds.[9] There is continuous interest in the development of PPEs for biomedical applications from drug and gene delivery to tissue engineering owing to their potential biodegradability, good biocompatibility, and functionality of the side chain as well as their structural similarities to naturally occurring nucleic and teichoic acids.[10] The degradation rates and other physicochemical properties of these polymers are controlled by the chemical structure in the backbone and side chains.[11] By choosing biocompatible building blocks of the polymer, degradation products of PPEs can have minimal toxic effects and good biocompatibility.[9c] It has been demonstrated that poly(ethyl ethylene phosphate) (PEEP), a typical hydrophilic polyphosphoester, also exhibits good biocompatibility in vitro and in vivo.[12] Recently, it has been reported that PEEP and its copolymers exhibit thermoresponsibility in aqueous solution.[13]

[a] Q. Wu, D. Zhou, R. Kang, X. Tang, Q. Yang, X. Song, G. Zhang
Liaoning Province Key Laboratory for Green Synthesis and Preparative Chemistry of Advanced Materials
College of Chemistry, Liaoning University
No. 66 Chongshan Mid-Road, Huanggu District
Shenyang, Liaoning Province (P.R. China)
Fax: (+86) 24-62202380
E-mail: glzhang@lnu.edu.cn

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/asia.201402524.

Synthetic polypeptides are known to be very important biodegradable materials. They have the potential to be degraded in biological environments. Moreover, since they have secondary conformations (e.g., α-helices, β-sheets, or turns), low immunogenicity, good biocompatibility, and excellent mechanical properties, they may be widely used in pharmaceuticals and other medical fields. Synthetic polypeptides such as poly(γ-benzyl-L-glutamate) (PBLG) assumed a rigid α-helical conformation stabilized by intramolecular hydrogen-bonding interactions, thereby guaranteeing it to be a versatile building block and a model for constructing superstructures and studying the phase behaviors of rod polymers.[14] But it is well known that most commonly used polypeptides such as poly(α-leucine), poly(α-alanine), poly(γ-benzyl glutamate), and so on are rather hydrophobic and degrade very slowly by simple hydrolysis under human body conditions. To improve the hydrophilicity and to control the biodegradable rate of these poly(α-amino acid)s, one might introduce hydrophilic functional groups into the polymer chain. Poly(ethylene glycol) (PEG) was usually introduced into the copolymer as hydrophilic segment to enhance the hydrophilicity of the copolymer because of its excellent hydrophilicity and biocompatibility.[15] There are many reports about copolymers of amino acids that were synthesized from PEG so as to regulate the hydrophilicity and biodegradable rate, such as poly(aspartic acid)/poly(ethylene glycol) block copolymer, poly(γ-benzyl L-glutamate)/poly(ethylene glycol) block copolymer, poly(L-lysine)/poly(ethylene glycol) block copolymer, poly(L-alanine)/poly(ethylene glycol) block copolymer, and so on.[16] These amphiphilic copolymers could be self-assembled into nanoscaled micelles in a suitable medium, and some have been used as carriers of drug-delivery systems.[17] However, there are few reports on fully biodegradable thermoresponsive amphiphilic copolymers that were synthesized from hydrophobic polypeptides with hydrophilic and biodegradable products. Poly(γ-benzyl L-glutamate) (PBLG) is one of these synthetic hydrophobic biodegradable polypeptides, and if the PBLG chain were combined with PEEP to prepare fully biodegradable thermoresponsive amphiphilic polymers, its hydrophilicity and biodegradability should be regulated, and thus its applications should be extended widely.

In this work, new thermoresponsive amphiphilic biodegradable poly(γ-benzyl L-glutamate)/poly(ethyl ethylene phosphate) (PBLG-*b*-PEEP) block copolymers were synthesized by the ring-opening polymerization of *N*-carboxy-γ-benzyl L-glutamate anhydride (BLG–NCA) by using amine-terminated poly(ethyl ethylene phosphate) (H_2N–PEEP) as macroinitiator. Their structures were characterized. Then the PBLG-*b*-PEEP micelles were prepared by means of the solvent evaporation method and their physicochemical characteristics and thermosensitivities were investigated.

Results and Discussion

Synthesis of Amine-Terminated Poly(ethyl ethylene phosphate) (H_2N–PEEP)

The ^{1}H NMR spectrum of H_2N–PEEP is shown in Figure 1A. The peaks at $\delta = 1.36$, 3.81, and 4.12 ppm are assigned to protons d, c, and e in the PEEP segment, respectively. The peaks at $\delta = 3.20$ and 4.25 ppm are assigned to protons a and b in the $H_2NCH_2CH_2O$– segment, respectively. The peak at $\delta = 3.75$ ppm is assigned to proton f in the methylene proton joined to the end hydroxyl group of the phosphoester unit of the block copolymer.[6]

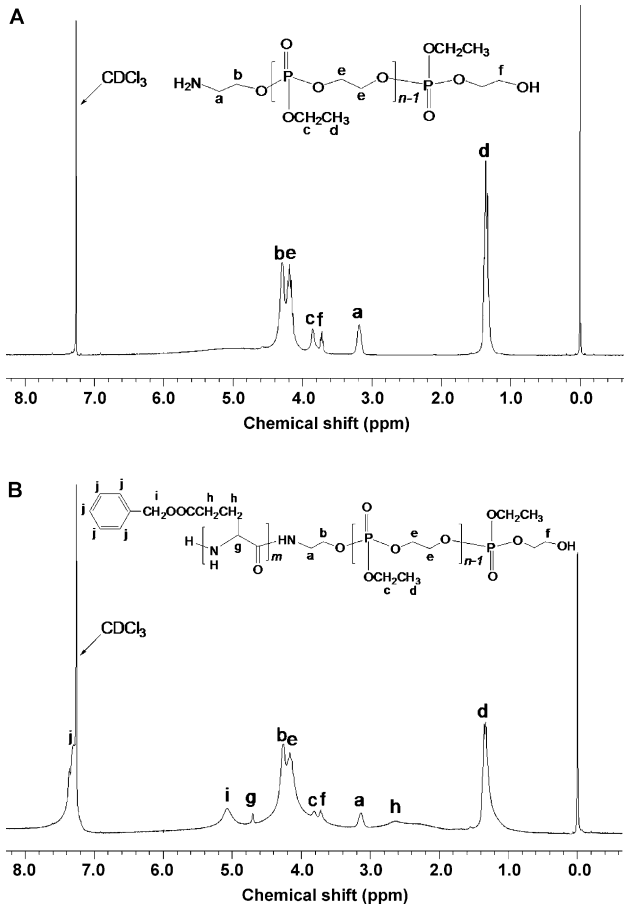


Figure 1. ^{1}H NMR spectra of A) H_2N–PEEP and B) the PBLG**1**-*b*-PEEP block copolymer in $CDCl_3$.

The gel permeation chromatography (GPC) trace of H_2N–PEEP is shown in Figure 2A. The sample showed unimodal molecular weight distribution, which further indicated that the polymerization was completed successfully and there was no another polymer in the product.

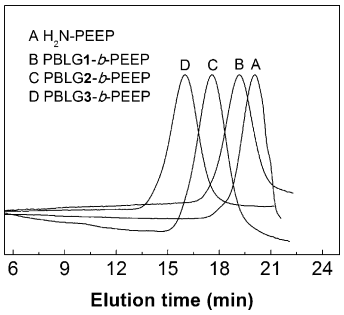


Figure 2. GPC curves of polymers.

Synthesis of Poly(γ-benzyl L-glutamate)/Poly(ethyl ethylene phosphate)/(PBLG-*b*-PEEP) Block Copolymer

It is well known that primary amines, which are more nucleophilic than basic, can be used as initiators for the ring-opening polymerization of NCA to prepare poly(α-amino acid)s by undergoing a nucleophilic addition to the carbonyl group of NCA.[18] Because H_2N–PEEP contains a primary amine group, it can initiate ring-opening polymerization of BLG–NCA to form block copolymer. A series of block copolymers with various molecular weights was synthesized and the results are summarized in Table 1. It was found that the total molecular weights of the copolymers increased along with an increase in the molar ratio of the feeding monomer BLG–NCA to H_2N–PEEP.

Table 1. Related data on PBLG-*b*-PEEP block copolymers.

Sample	BLG–NCA/H_2N–PEEP[a]	W_{PBLG}/W_{PEEP}[b]	M_n[b] [g mol^{-1}]	M_n[c] [g mol^{-1}]	M_w/M_n[c]
H_2N–PEEP	–	–	3040	2886	1.03
PBLG**1**-*b*-PEEP	10/1	37/63	4790	3989	1.10
PBLG**2**-*b*-PEEP	20/1	56/44	6980	6125	1.11
PBLG**3**-*b*-PEEP	30/1	67/33	9260	8167	1.13

[a] Molar ratio of H_2N–PEEP to BLG–NCA in feed. [b] Determined by ^{1}H NMR spectroscopy in $CDCl_3$. [c] Determined by GPC in THF at 30 °C.

The ^{1}H NMR spectrum of PBLG**1**-*b*-PEEP is shown in Figure 1B. The peaks at $\delta = 1.36$, 3.81, and 4.12 ppm are assigned to protons d, c, and e in the PEEP segment, respectively. The peaks at $\delta = 3.20$ and 4.25 ppm are assigned to protons a and b in the $H_2NCH_2CH_2O$– segment, respectively. The peak at $\delta = 3.75$ ppm is assigned to proton f in the methylene proton joined to the end hydroxyl group of the phosphoester unit of the block copolymer. The peaks at $\delta =$ 2.61, 4.65, 5.04, and 7.29 ppm are assigned to protons h, g, i, and j in the PBLG segment, respectively. No additional peaks were detected in the spectrum, thus implying that the block copolymer was prepared.

The GPC chromatograms of the block copolymers are shown in Figure 2, and three block copolymers show a unimodal peak with decreased retention times relative to H_2N–PEEP, thus demonstrating the formation of block polymers. Molecular weights and molecular weight distributions were measured by GPC and are summarized in Table 1.

Degradation

The in vitro degradations of PBLG-*b*-PEEP in phosphate buffer solution (PBS), pH 7.4, at 37 °C are shown in the Supporting Information. The introduction of the PEEP segment, a typical hydrophilic polyphosphoester, into the PBLG could accelerate the degradation rate. PBLG**1**-*b*-PEEP, PBLG**2**-*b*-PEEP, and PBLG**3**-*b*-PEEP showed a steady degradation rate, with a 51.1, 39.2, and 28.1 % mass loss over 30 days, respectively.

Conformation of Copolymer in Aqueous Solution

The PBLG chain in the copolymers is a synthetic polypeptide-like protein. Therefore, it would form a regular conformation such as an α helix or β sheet. The circular dichroism (CD) spectrum of the PBLG-*b*-PEEP copolymers in an aqueous solution is shown in the Supporting Information. Two negative peaks were clearly observed in the CD curve. The first one, near 222 nm, was ascribed to an n–π transition, and the second peak, close to 208 nm, was due to a π–π transition. Such a pattern of the double negative peaks in the CD spectrum is typical of an α helix in polypeptides held together by intramolecular hydrogen-bonding interactions.[19] The result suggested that the PBLG chains of the PBLG-*b*-PEEP copolymers existed in the α-helix conformation form in the aqueous medium.

Formation of Micelles

Pyrene has been widely used as a probe to monitor the association and micellization of macromolecules in solutions because its photophysical character changes with variation in the existing environment.[20] The micellar structures of PBLG-*b*-PEEP were confirmed by fluorescence techniques by using pyrene as a probe. The fluorescence excitation spectra of pyrene in the presence of PBLG**1**-*b*-PEEP at various concentrations are shown in Figure 3A. A redshift from 334 to 337 nm is observed with increasing concentration of PBLG**1**-*b*-PEEP, thus indicating that micellization takes place for the PBLG-*b*-PEEP copolymer. Such results can be attributed to the transfer of pyrene molecules from water to a hydrophobic environment within the core of the micelles.

The onset of micellization and the critical micelle concentrations (CMCs) could also be obtained from the studies of excitation spectra.[21] For copolymer PBLG**1**-*b*-PEEP, 334 and 337 nm were chosen as the peak wavelength of the (0,0) band in the pyrene excitation spectra in the aqueous phase and in the entirely hydrophobic core of the polymeric micelle, respectively. The pyrene fluorescence intensity ratios (I_{337}/I_{334}) were plotted against the logarithm of copoly-

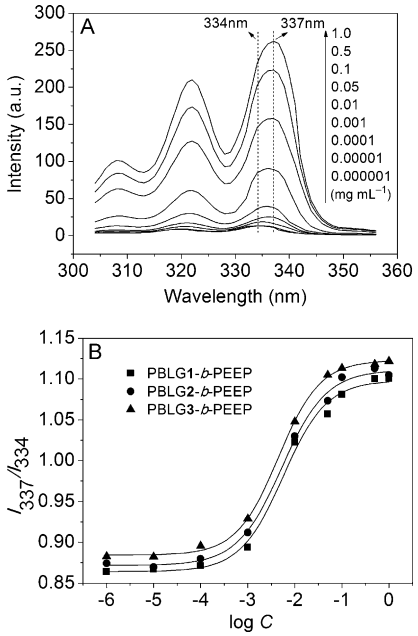


Figure 3. A) Excitation spectra of pyrene as a function of PBLG**1**-*b*-PEEP concentration in water and B) plots of I_{337}/I_{334} versus the logarithm of the PBLG-*b*-PEEP block copolymer concentrations.

mer concentration. The plots are shown in Figure 3B. Below a certain concentration, I_{337}/I_{334} is constant. Above this concentration, I_{337}/I_{334} increased with the increase of $\log C$ and finally reaches a plateau. From this plot, the critical micelle concentration of 5.75×10^{-4} mg mL^{-1} was obtained from the intersection of two straight lines: the base line and the rapidly rising I_{337}/I_{334} line. The CMCs of PBLG**2**-*b*-PEEP and PBLG**3**-*b*-PEEP were also obtained from the same method and listed in Table 2. The CMC values were in the magnitude of 10^{-4} mg mL^{-1} and reduced with the increase of the PBLG segment. This is reasonable since the higher content of the hydrophobic segments will result in stronger interactions between hydrophobic chains, thus leading to a more stable structure and therefore to a lower CMC value. This trend is in agreement with the results reported in the literature.[6,22] The low CMC values of PBLG-*b*-PEEP micelles might be due to the hydrophobicity and the α-helix conformation form of the PBLG block in aqueous medium. Therefore, it is believed that PBLG-*b*-PEEP micelles would be thermodynamically stable in aqueous media, and lower CMC values are surely a favorable indication when such micelles are used for systemic drug delivery. Thus, in this sense, PBLG-*b*-PEEP micelles should be suitable for pharmaceutical applications.

Table 2. Related data on PBLG-*b*-PEEP block copolymer micelles.

Sample	CMC [mg mL^{-1}][a]	Diameter [nm][b]	PDI[b]
PBLG**1**-*b*-PEEP	5.75×10^{-4}	119	0.12
PBLG**2**-*b*-PEEP	4.46×10^{-4}	102	0.15
PBLG**3**-*b*-PEEP	3.80×10^{-4}	87	0.14

[a] Critical micelle concentration (CMC) was determined by fluorescence measurements using pyrene as a probe. [b] Diameter and polydispersity index (PDI) of micelles were determined by DLS.

To further study the properties of the block copolymer micelles, both dynamic light scattering (DLS) and TEM measurements were performed. DLS results showed that all of the PBLG-*b*-PEEP micelles exhibited unimodal size distribution with mean diameters from 87 to 119 nm, and the detailed data are summarized in Table 2. The sizes of the micelles decreased with the increase of the proportion of hydrophobic segments. The PBLG chain in the copolymer was a polypeptide-like protein. Therefore, it would form an α helix by means of intramolecular hydrogen bonding in aqueous solution (see the Supporting Information). When the PBLG content increased, the intermolecular hydrogen bonding was enhanced. When the micellar aggregation number increased, the density increased and the particle size decreased.[23] Thus the size of the copolymer micelle could be adjusted by changing the proportion of the PBLG segment of the copolymers. The morphologies of the block copolymer micelles visualized by TEM are shown in Figure 4B. The copolymer aggregated into approximate spheri-

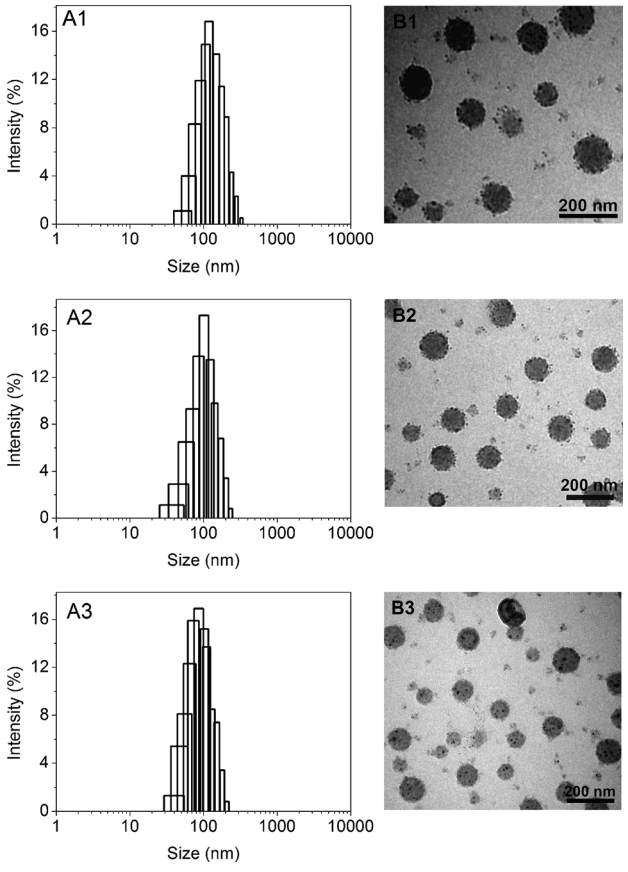


Figure 4. Characterization of the PBLG-*b*-PEEP micelles. A) The size distribution of PBLG-*b*-PEEP micelles determined by DLS: A1) PBLG**1**-*b*-PEEP, A2) PBLG**2**-*b*-PEEP, and A3) PBLG**3**-*b*-PEEP micelles. B) TEM images of self-assembled micelles: B1) PBLG**1**-*b*-PEEP, B2) PBLG**2**-*b*-PEEP, and B3) PBLG**3**-*b*-PEEP micelles ($c=0.1$ mg mL^{-1}, 25 °C).

cal micelles in aqueous solution, and the micelle sizes as determined by TEM were smaller than those determined by DLS in Figure 4A, because the micelle diameters determined by DLS represent their hydrodynamic diameter, whereas those obtained by TEM are related to the collapsed micelles after water evaporation.

Thermosensitivity of Polymers

The thermal phase transition temperature, expressed as the lower critical solution temperature (LCST), is one of the basic physical properties of thermoresponsive water-soluble polymers. The Supporting Information shows a typical photograph of aqueous solutions of PBLG**1**-*b*-PEEP. Below the LCST, PBLG-*b*-PEEP copolymers are amphiphilic and consist of a hydrophobic block (PBLG) and hydrophilic block (PEEP). The solution was transparent and colorless (see the Supporting Information). However, when heated close to the LCST, the solution gradually turned into a semitransparent emulsion (see the Supporting Information); above the LCST, the copolymers were hydrophobic and the solution became a white opaque suspension (see the Supporting Information); and finally the polymers precipitated from water if the solution was kept at a high temperature for enough time. When cooled, the semitransparent emulsion and transparent colorless solution were obtained again. Evidently, PBLG**1**-*b*-PEEP showed a reversible LCST phase transition in water. This phenomenon takes place on account of the different solvation of PEEP chains by water molecules at the temperatures below and above the phase-transition temperature.[24]

Figure 5A shows the temperature-dependent transmittance at a wavelength of 500 nm obtained from aqueous solutions of PBLG**1**-*b*-PEEP micelles at different concentrations. We can clearly see that LCST values increase as the block copolymer concentration decreases, and that the lower the polymer concentration is, the broader the temperature range exhibited by the decrease in transmittance. Figure 5B illustrates the effects of the block copolymer concentrations on LCST values for PBLG**1**-*b*-PEEP samples. As the polymer concentrations increase from 0.05 to 0.5 mg mL^{-1}, LCST values decrease from 37 to 29 °C for PBLG**1**-*b*-PEEP. In addition, it should be noted that the sharpness of the thermally induced phase transition was dependent on the polymer concentration. This finding is consistent with the generally accepted LCST principle for dilute solutions, which states that higher water content enhances the hydrogen-bonding interactions between water and the polymer chain, and this requires more thermal energy to break the water structure, thereby resulting in an increase in the LCST.[25]

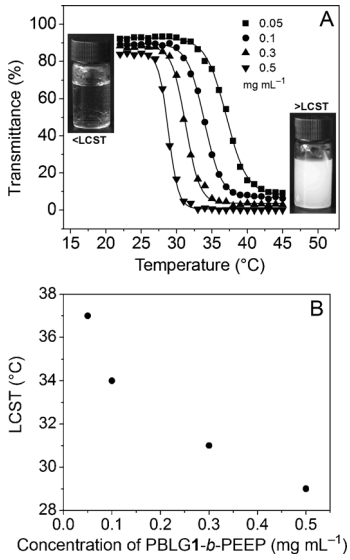


Figure 5. A) Temperature dependences of optical transmittance at 500 nm obtained for aqueous solutions of PBLG**1**-*b*-PEEP at varying polymer concentrations and B) LCST as a function of the concentration of PBLG**1**-*b*-PEEP.

It is reported that the LCST of thermoresponsive polymers can be controlled by compositions of hydrophobic and hydrophilic units.[26] In this study, the LCST transition behaviors of the PBLG-*b*-PEEP block copolymers were tuned by changing the lengths of the hydrophobic PBLG block: the larger the hydrophobic block content of the copolymer, the lower the LCST at a fixed PEEP molecular weight. Figure 6A shows the temperature dependence of optical transmittance of micellar solutions of the copolymers with different hydrophobic PBLG block lengths. The transmittance decreased significantly at a specific temperature upon heating solutions of all of the copolymers. Figure 6B illustrates the effects of the PBLG wt% on LCST values for PBLG-*b*-PEEP copolymers. As the PBLG wt% increased from 0 to

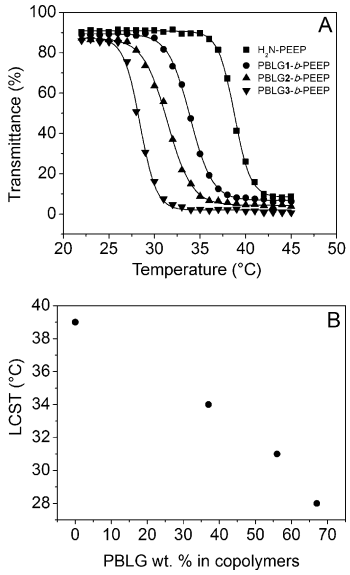


Figure 6. A) Effect of hydrophobic block length on the thermoresponsive behavior of copolymers in aqueous solution ($c = 0.1$ mg mL^{-1}) and B) LCST as a function of the PBLG block in the copolymers.

67%, LCST values decreased from 39 to 28°C, thus showing a decreasing trend as the PBLG block length increased. The LCSTs of PBLG-*b*-PEEP polymers were found to be lower than H_2N–PEEP homopolymers and to depend on their molecular weights. This was considered to result from hydrating contributions from polar terminal hydrophilic amino groups in the copolymers. In general, the LCST decreased as the hydrophilicity of the polymer decreased. Considering the fact that the PBLG block is more hydrophobic than the PEEP block, it is reasonable that the LCST decreases as the molar fraction of PBLG increases. These results clearly show that the phase transition of PBLG-*b*-PEEP can be controlled within a temperature range (28–39°C) by varying the block length of the PBLG block. Similar observations have been reported for several thermosensitive polymers.[27] Thermoresponsivity under physiological conditions is effective for drug delivery or tissue engineering applications. In fact, the LCST of thermoresponsive polymers can be controlled by compositions of hydrophobic and hydrophilic units.[13] Thus, LCST can also be further tuned by adjusting the composition of the PBLG block or by adding new hydrophobic blocks to find its application under physiological temperatures.

The phase-transition behavior of PBLG**1**-*b*-PEEP during the heating/cooling cycle was monitored by measuring the change of the transmittance with an increment of 1.0°C, as shown in Figure 7A. An interesting observation is the excellent reversibility and reproducibility displayed by the copolymer PBLG**1**-*b*-PEEP in the optical transmittance of the micellar solution during 10 cycles of temperature change between 22°C (below the LCST) and 45°C (above the LCST). The transparent micellar solution rapidly became cloudy when the temperature increased from 22 to 45°C and reverted to the transparent state when the temperature of the solution decreased from 45 to 22°C. The highest and lowest transmittance of the PBLG**1**-*b*-PEEP micellar solution remained almost constant during multiple heating/cooling cycles without any detectable hysteresis. However, a hysteresis was observed for copolymers PBLG**2**-*b*-PEEP and PBLG**3**-*b*-PEEP with a greater proportion of hydrophobic PBLG, as shown in Figure 7B. The hysteresis was due to the fact that greater hydrophobic character of the copolymer leads to the formation of more stable and larger aggregates by means of intermicelle association, in which the larger hydrophobic domain and closely compact structure could prevent PEEP rehydration by water upon cooling. Thus, the larger aggregates dissociated slowly to individual micelles.[28]

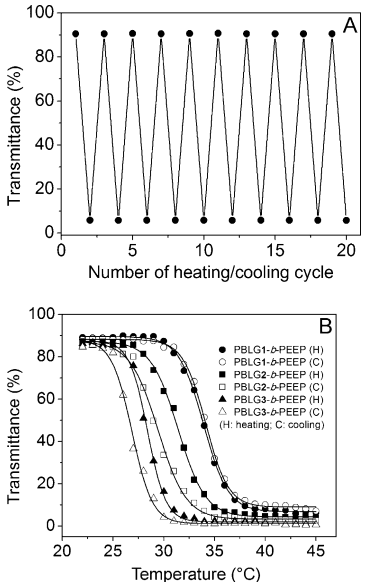


Figure 7. A) Reversible changes of optical transmittance in response to temperature fluctuations for PBLG**1**-*b*-PEEP micellar solution (c = 0.1 mg mL^{-1}) and B) transmittance versus temperature plots for various copolymer aqueous solutions (c = 0.1 mg mL^{-1}) during one heating–cooling cycle.

The phase-transition behaviors of thermoresponsive polymers in aqueous solution are known to be influenced by additives such as salts, surfactants, and alcohols. The reason is that these additives can alter the interactions between the polymer and water.[29] As a typical water structure-maker, sodium chloride can disrupt the hydration structure surrounding the polymer chains, thus leading to a salting-out effect and thereby lowering the LCST of the polymer solution. Figure 8 shows the influence of the NaCl concentration

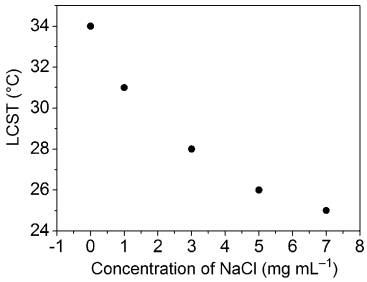


Figure 8. Effect of NaCl concentration on the LCST of a micellar solution of PBLG**1**-*b*-PEEP (c = 0.1 mg mL^{-1}).

on the thermosensitivity of PBLG**1**-*b*-PEEP by measuring the transmittance of polymer micelles in aqueous solution. With the increase in NaCl concentration, a decrease in LCST was observed, thus indicating a typical salting-out effect. It is explained by the fact that the presence of NaCl increases the hydrogen bonding among water molecules and decreases the hydrogen bonding among water and hydrophilic chains, which might lead to a partial dehydration of PEEP and results in a stronger tendency of association of PEEP that decreases its LCST. However, the addition of NaCl will undoubtedly increase the polarity of aqueous media and thus enhance the hydrophobic–hydrophobic interaction and subsequently lead to the stronger tendency for copolymer association, which is indicated by the decrease in LCST. This trend was in agreement with the result reported in the literature.[9e,28a]

Figure 9 shows the changes in the hydrodynamic diameter (d_H) for PBLG**1**-*b*-PEEP aqueous solution during the heat-

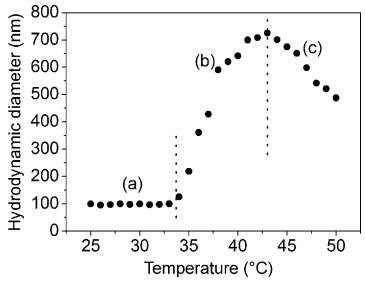
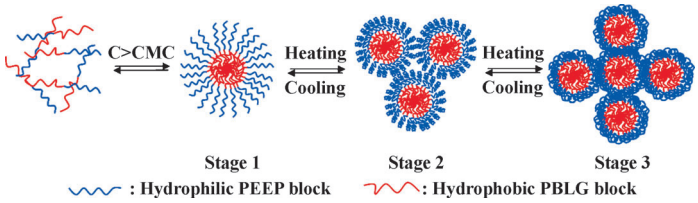


Figure 9. The changes in the hydrodynamic diameter (d_H) for PBLG**1**-*b*-PEEP aqueous solution during the heating process ($c = 0.1$ mg mL^{-1}).

ing process. Three clear regions can be observed: a) first the equilibrium stage, then b) the sharp increase, and c) the final decrease. At low temperature, the size of the micelle stays the same, almost without a clear response to the temperature. As the solution is heated to approximately 34 °C, a clear phase transition takes place, and d_H increases rapidly to 715 nm owing to the aggregation of the micelles, thereby revealing that the thermoresponsive PEEP shell probably leads to high assembly by means of the intra- and intermicelle hydrogen bonds. However, above the transition temperature, with the temperature increasing persistently, the d_H value goes through a decrease, which might result from the collapse of the aggregates by the removal of more water molecules and the formation of more compact and regular structures.[30]

Scheme 1 shows a schematic illustration of thermally induced aggregation and phase transition of PEEP-*b*-PBLG



Scheme 1. Schematic illustration of the thermally induced aggregation and phase transition of PBLG-*b*-PEEP copolymers in water.

copolymers in water. When the solution temperature was below the LCST (stage 1), spherical polymer micelles existed individually and the micellar solution was clear (see the Supporting Information). At temperatures close to the LCST (stage 2), intermicelle aggregation gave rise to the formation of larger aggregates with a multicore structure and associated with an abrupt increase in the aggregate radius. Moreover, the solution became cloudy (see the Supporting Information). When the temperature continued to increase, the collapse of the aggregates occurred by the removal of more water molecules and the formation of more compact and regular structures (Stage 3). The micelle dehydration certainly increased the solution turbidity on account of the scattering (see the Supporting Information).

Conclusion

In the present study, new thermosensitive amphiphilic biodegradable PBLG-*b*-PEEP block copolymers with various PBLG and PEEP block lengths were synthesized. The structures and compositions of the polymers were characterized by ^{1}H NMR spectroscopy and GPC. These copolymers had low CMC values that ranged from 3.80 to 5.75×10^{-4} mg mL^{-1}. The behaviors of the block copolymer in aqueous solution were also studied by DLS and TEM. These block copolymers were able to spontaneously self-assemble into micelles of around 87–119 nm in size in aqueous solution, which contained a hydrophobic PBLG core and hydrophilic PEEP shell. It has been revealed that the phase transition of PBLG-*b*-PEEP micelles is reversible, and the thermosensitivity is affected by the molecular weight, composition of the PBLG block, and the sodium chloride concentration in the medium, which in turn allows convenient adjustment of their thermosensitivity.

Experimental Section

Materials

L-Glutamic acid (biochemical grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China) and dried under vacuum for 24 h before use. Triphosgene (chemical reagent) was obtained from Haining Zhonglian Chemical Reagent Co., Ltd. (Zhejiang, China) and was used without any treatment. Stannous octoate ($Sn(Oct)_2$) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). *tert*-Butoxycarbonyl (Boc)-aminoethanol (Boc-$NHCH_2CH_2OH$; analytical reagent) was purchased from Tokyo Kasei Kogyo Co., Ltd. (TCI). Tetrahydrofuran (THF; analytical grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), dried, and distilled in the presence of sodium immediately before use. Phosphorus trichloride (analytical grade) was purchased from Guangfu Fine Chemical Research Institute (Tianjin, China) and distilled before use. Ethylene glycol (analytical grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), dried over $MgSO_4$, and distilled under vacuum before use. Toluene (analytical grade) was purchased from Baishi Chemical Reagent Co., Ltd. (Tianjin, China), dried, and distilled in the presence of sodium immediately before use. Triethylamine (TEA; analytical grade) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), dried over CaH_2, and distilled before use. Dichloromethane (CH_2Cl_2, analytical grade) was supplied by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), dried over P_2O_5, and distilled before use. Other chemicals are all analytical reagents made in China and used without further purification.

Synthesis of Ethyl Ethylene Phosphate (EEP)

Ethyl ethylene phosphate (EEP) was prepared according to the literature.[31] Briefly, ethylene glycol (124.14 g, 2 mol) was added dropwise to a stirred mixture of phosphorus trichloride (274.66 g, 2 mol) and dry CH_2Cl_2 (250 mL). After complete addition of ethylene glycol, the solution was stirred at room temperature for another 0.5 h, and CH_2Cl_2 was evaporated under reduced pressure. The residue was distilled under reduced pressure to give 2-chloro-1,3,2-dioxaphospholane (123.2 g). Yield: 49 %, b.p. 42–45 °C/1600 Pa.

The oxidation of 2-chloro-1,3,2-dioxaphospholane (123.2 g) was carried out by bubbling O_2 through the solution in toluene at 40 °C for 48 h. After removal of benzene, the residue was distilled under reduced pressure to give 2-chloro-2-oxo-1,3,2-dioxaphospholane (77.9 g) as a colorless liquid. Yield: 56 %, b.p. 88–90 °C/107 Pa.

A mixture of anhydrous ethanol (25.3 g, 0.55 mol) and anhydrous triethylamine (61.6 g, 0.605 mol) was added dropwise to a stirred and cooled mixture (−5 °C) that contained 2-chloro-2-oxo-1,3,2-dioxaphospholane (77.9 g, 0.55 mol) and anhydrous toluene (250 mL). The temperature of the reaction mixture was maintained at −5 °C. After complete addition, the resulting mixture was stirred at room temperature for another 2 h. Thereafter, the triethylamine hydrochloride was filtered off and the filtrate was concentrated. The residue was distilled under reduced pressure to give ethyl ethylene phosphate (EEP; 90.5 g) as a colorless liquid. Yield: 61 %, b.p. 95–97 °C/107 Pa.

Synthesis of N-carboxy-γ-benzyl l-glutamate Anhydride (BLG–NCA)

γ-Benzyl L-glutamate was prepared from L-glutamic acid and benzyl alcohol (Scheme 1). M.p. 172–174 °C;[32] yield: 44.1 %; elemental analysis calcd (%) for $C_{12}H_{15}NO_4$: C 60.75, H 6.37, N 5.90; found: C 60.48, H 6.62, N 5.97. *N*-Carboxy-γ-benzyl L-glutamate anhydride (BLG–NCA) was prepared by the reaction of γ-benzyl L-glutamate with triphosgene in dried THF at 50 °C according to a literature procedure. M.p. 96–97 °C;[33] yield: 42.2 %; elemental analysis calcd (%) for $C_{13}H_{13}NO_5$: C 59.31, H 4.98, N 5.32; found: C 59.54, H 4.87, N 5.26. ^{1}H NMR ($CDCl_3$): $\delta = 2.08$, 2.29 (2H; $-CH_2OOCCH_2CH_2$), 2.57 (2H; $-CH_2OOCCH_2CH_2$), 4.38 (1H; $-CH$), 5.13 (2H; $-CH_2OOCCH_2CH_2$), 6.52 (1H; N*H*), 7.37 ppm (5H; Ar−*H*). The reaction route is shown in Scheme 2.

Synthesis of H_2N–PEEP

EEP (3.003 g, 19.8 mmol) and THF (10 mL) were transferred to a 50 mL round-bottomed flask. After six cycles of evacuation purging with purified nitrogen, $Sn(Oct)_2$ (0.201 g, 0.49 mmol) and Boc-aminoethanol (0.074 g, 0.45 mmol) were added to the round-bottomed flask and kept at 40 °C for 3 h under stirring. After THF was removed under reduced pressure, the resulting polymer was dissolved in anhydrous CH_2Cl_2 (5 mL), precipitated in petroleum ether (100 mL), and kept at 2 °C for 12 h. The precipitate was dried under vacuum at 30 °C for 48 h to give the desired BocNH–PEEP. Yield: 40 %.

H_2N–PEEP was prepared by the removal of the Boc group from BocNH–PEEP. Typically, BocNH–PEEP was dissolved in CH_2Cl_2 (10 mL) and treated with trifluoroacetic acid (2 mL) at 0 °C for 1 h under stirring. Then the solution was poured into *n*-hexane (100 mL) and kept at 2 °C for 12 h. The precipitate was dried in vacuum at 30 °C for 48 h to give the desired H_2N–PEEP. Yield: 80 %. The reaction route is shown in Scheme 2.

Synthesis of Poly(γ-benzyl L-glutamate)/Poly(ethyl ethylene phosphate)/(PBLG-b-PEEP) Block Copolymer

Certain amounts of H_2N–PEEP and BLG–NCA were dissolved together in anhydrous CH_2Cl_2 (40 mL) and kept at 25 °C for 24 h under stirring. The reaction mixture was poured into ethyl ether (250 mL) to give a white precipitate. The precipitate was dried under vacuum at 30 °C for 12 h to give the desired PBLG-*b*-PEEP block copolymers. The yield was approximately 50 %. The reaction route is shown in Scheme 2. Different molar ratios of the feeding BLG–NCA to H_2N–PEEP resulted in the corresponding copolymers with various compositions as listed in Table 1.

In Vitro Degradations of Copolymers

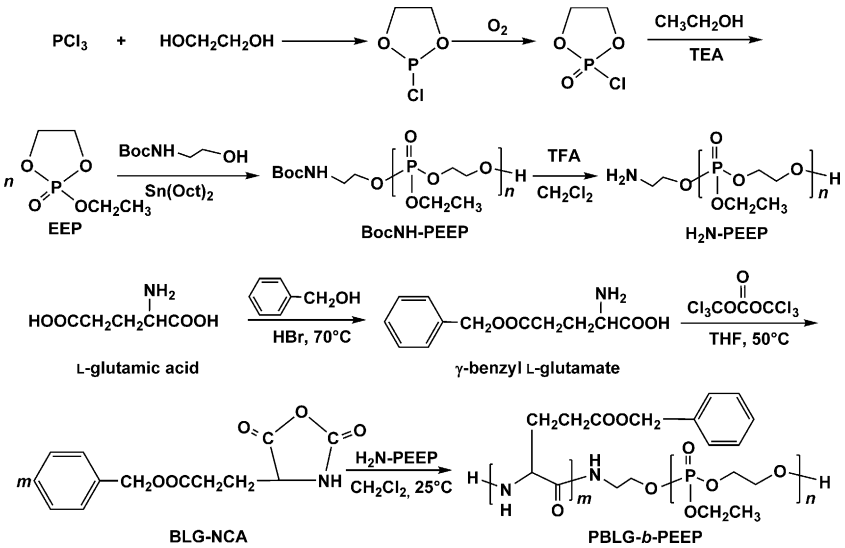
The in vitro degradation study was conducted by placing the disk samples described above in PBS (5 mL, 0.1 mol L^{-1}), pH 7.4 at 37 °C. The samples were with drawn at predetermined time points, washed three times with ultrapurified water, and dried to constant weight under vacuum.

Preparation of Micelles

The micelles were prepared using a solvent displacement method with a tetrahydrofuran/water (THF/H_2O) system.[34] A copolymer (50 mg) was first dissolved in THF (10 mL); thereafter the copolymer solution was slowly added into ultrapurified water (30 mL; Aquaplus 18.2 MΩ). THF was removed using a rotary evaporator at 25 °C for 2 h. The obtained solution was transferred into a 50 mL volumetric flask, followed by dilution to the calibration mark with ultrapurified water to obtain 1 mg mL^{-1} micelles.

Characterization

Elemental analysis was performed with a Thermo Electron Flash EA 1112 instrument. ^{1}H NMR spectra were measured with a Varian Mercury 300 NMR spectrometer at room temperature using $CDCl_3$ as solvent. Chemical shifts (δ) are given in ppm using tetramethylsilane (TMS) as an internal reference. GPC measurements were conducted with a Waters 1515 GPC instrument equipped with an HT4 and HT3 column (effective molecular-weight range: 5000 to 600000 and 500 to 30000) and a 2414 differential refractive index detector. THF was used as eluent at the flow rate of 1.0 mL min^{-1} at 30 °C, and the molecular weights were calibrated with polystyrene standards. The circular dichroism (CD) spectrum in an aqueous solution was measured with a JASCO 810 spectrophotometer. The CMCs of the copolymers were determined by fluorescence measurements using pyrene as a probe. A pyrene solution (in acetone) was added into a series of volumetric flasks in such an amount that the final concentration of pyrene in each solution was 6.5×10^{-7} mol L^{-1}; thereafter acetone was removed completely. The polymer solution was added into the volumetric flasks and diluted till the calibration mark using ultrapurified water to obtain the desired copolymer concentrations, which ranged from 1.0×10^{-6} to 1.0 mg mL^{-1}. The samples were maintained at 50 °C for 2 h and stored at room temperature overnight to equilibrate pyrene and micelles. Steady-state fluorescence excitation spectra were recorded with a Varian Cary Eclipse fluorescence spectrophotometer at 390 nm emission wavelength and 2.5 nm slit width. The scan rate was 120 nm min^{-1}. The size distribution of micelles was determined by dynamic light scattering with a Malvern Nano ZS instrument at 25 °C. The morphology of the micelles was investigated by TEM, which was carried out with a Hitachi H-7650 electron microscope operating at an accelerating voltage of 80 kV. Specimens were prepared by transferring a drop of the mi-



Scheme 2. The synthesis of the PBLG-*b*-PEEP block copolymer.

celle solution onto a 200 mesh copper grid coated with carbon and allowing the sample to dry in air before measurements. The lower critical solution temperature of the aqueous solution of the polymer was investigated with a Perkin–Elmer Lambda Bio 20 UV/Vis spectrophotometer together with a NESLAB RTE-111 temperature controller. Briefly, the polymers were dispersed in ultrapurified water (Aquaplus 18.2 MΩ). The transmittance of aqueous solutions of polymer at $\lambda = 500$ nm was recorded in a 1.0 cm path length quartz cell. In the heating/cooling cycle, the rate of heating or cooling was set at $1\,^{\circ}C\,min^{-1}$ with hold steps of 10 min at each temperature. Values for the LCST of aqueous solutions of the polymers were determined at a temperature with a half of the optical transmittance between the below and above transitions.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (grant no. 51373073), the Natural Science Foundation for Education Department of Liaoning Province of China (grant no. L2012007), the Program for Liaoning Innovative Research Team in University (grant no. LT2011001), and the Foundation of 211 Project for Innovative Talents Training, Liaoning University.

[1] a) H. Kuramochi, Y. Andoh, N. Yoshii, S. Okazaki, *J. Phys. Chem. B* **2009**, *113*, 15181–15188; b) J. X. Zhang, K. Ellsworth, P. X. Ma, *J. Controlled Release* **2010**, *145*, 116–123; c) C. H. Cai, J. P. Lin, T. Chen, X. H. Tian, *Langmuir* **2010**, *26*, 2791–2797.

[2] a) C. L. Peng, M. J. Shieh, M. H. Tsai, C. C. Chang, P. S. Lai, *Biomaterials* **2008**, *29*, 3599–3608; b) X. W. Zhang, M. Oddon, O. Giani, S. Monge, J. J. Robin, *Macromolecules* **2010**, *43*, 2654–2656; c) Y. Hu, Z. P. Jiang, R. Chen, W. Wu, X. Q. Jiang, *Biomacromolecules* **2010**, *11*, 481–488.

[3] a) J. Yao, Y. L. Ruan, T. Zhai, J. Guan, G. P. Tang, H. R. Li, S. Dai, *Polymer* **2011**, *52*, 3396–3404; b) Y. Y. Li, X. Z. Zhang, H. Cheng, J. L. Zhu, S. X. Cheng, R. X. Zhuo, *Macromol. Rapid Commun.* **2006**, *27*, 1913–1919; c) J. Virtanen, H. Tenhu, *J. Polym. Sci. Part A* **2001**, *39*, 3716–3725.

[4] a) Y. C. Wang, L. Y. Tang, Y. Li, J. Wang, *Biomacromolecules* **2009**, *10*, 66–73; b) J. Bigot, B. Charleux, G. Cooke, F. Delattre, D. Fournier, J. Lyskawa, L. Sambe, F. Stoffelbach, P. Woisel, *J. Am. Chem. Soc.* **2010**, *132*, 10796–10801; c) A. Kumar, A. Srivastava, I. Y. Galaev, B. Mattiasson, *Prog. Polym. Sci.* **2007**, *32*, 1205–1237.

[5] a) T. H. Qu, A. R. Wang, J. F. Yuan, J. H. Shi, Q. Y. Gao, *Colloids Surf. B* **2009**, *72*, 94–100; b) S. T. Sun, P. Y. Wu, *J. Phys. Chem. B* **2011**, *115*, 11609–11618.

[6] X. Liu, P. H. Ni, J. L. He, M. Z. Zhang, *Macromolecules* **2010**, *43*, 4771–4781.

[7] H. Vihola, A. Laukkanen, H. Tenhu, J. Hirvonen, *J. Pharm. Sci.* **2008**, *97*, 4783–4793.

[8] S. Ganta, H. Devalapally, A. Shahiwala, M. Amiji, *J. Controlled Release* **2008**, *126*, 187–204.

[9] a) Y. C. Wang, Y. Y. Yuan, F. Wang, J. Wang, *J. Polym. Sci. Part A* **2011**, *49*, 487–494; b) N. Koseva, A. Bogomilova, K. Atkova, K. Troev, *React. Funct. Polym.* **2008**, *68*, 954–966; c) D. P. Chen, J. Wang, *Macromolecules* **2006**, *39*, 473–475; d) C. S. Xiao, Y. C. Wang, J. Z. Du, X. S. Chen, J. Wang, *Macromolecules* **2006**, *39*, 6825–6831; e) Y. C. Wang, Y. Li, X. Z. Yang, Y. Y. Yuan, L. F. Yan, J. Wang, *Macromolecules* **2009**, *42*, 3026–3032; f) J. L. He, P. H. Ni, S. Wang, H. Y. Shao, M. Z. Zhang, X. L. Zhu, *J. Polym. Sci. Part A* **2010**, *48*, 1919–1930.

[10] a) J. Wang, H. Q. Mao, K. W. Leong, *J. Am. Chem. Soc.* **2001**, *123*, 9480–9481; b) C. Wachiralarpphaithoon, Y. Iwasakia, K. Akiyoshi, *Biomaterials* **2007**, *28*, 984–993; c) Q. Li, J. Wang, S. Shahani, D. D. N. Sun, B. Sharma, J. H. Elisseeff, K. W. Leong, *Biomaterials* **2006**, *27*, 1027–1034; d) Z. Zhao, J. Wang, H. Q. Mao, K. W. Leong, *Adv. Drug Delivery Rev.* **2003**, *55*, 483–499.

[11] L. S. Naira, C. T. Laurencina, *Prog. Polym. Sci.* **2007**, *32*, 762–798.

[12] a) Y. Iwasaki, E. Yamaguchi, *Macromolecules* **2010**, *43*, 2664–2666; b) J. Cheng, J. X. Ding, Y. C. Wang, J. Wang, *Polymer* **2008**, *49*, 4784–4790; c) Y. Y. Yuan, Q. Du, Y. C. Wang, J. Wang, *Macromolecules* **2010**, *43*, 1739–1746.

[13] Y. Iwasaki, C. Wachiralarpphaithoon, K. Akiyoshi, *Macromolecules* **2007**, *40*, 8136–8138.

[14] a) F. Rypáček, *Polym. Degrad. Stab.* **1998**, *59*, 345–351; b) H. Iwata, S. Matsuda, K. Mitsuhashi, E. Itoh, Y. Ikada, *Biomaterials* **1998**, *19*, 1869–1876; c) T. Fukuoka, H. Uyama, S. Kobayashi, *Biomacromolecules* **2004**, *5*, 977–983; d) A. Wamsley, B. Jasti, P. Phiasivongsa, X. L. Li, *J. Polym. Sci. Part A* **2004**, *42*, 317–325; e) C. Y. Chen, Z. H. Wang, Z. B. Li, *Biomacromolecules* **2011**, *12*, 2859–2863; f) Y. You, Y. Chen, C. Hua, C. M. Dong, *J. Polym. Sci. Part A* **2010**, *48*, 709–718.

[15] A. Lavasanifar, J. Samuel, G. S. Kwon, *Adv. Drug Delivery Rev.* **2002**, *54*, 169–190.

[16] a) A. Harada, K. Kataoka, *Macromolecules* **1998**, *31*, 288–294; b) K. Kugo, A. Ohji, T. Uno, J. Nishino, *Polym. J.* **1987**, *19*, 375–381; c) A. Harada, K. Kataoka, *Macromolecules* **1995**, *28*, 5294–5299; d) G. L. Zhang, J. B. Ma, Y. H. Li, Y. N. Wang, *J. Biomater. Sci. Polym. Ed.* **2003**, *14*, 1389–1400.

[17] a) Y. Li, G. S. Kwon, *Pharm. Res.* **2000**, *17*, 607–611; b) K. Kataoka, T. Matsumoto, M. Yokoyama, T. Okamo, Y. Sakurai, S. Fukushima, K. Okamoto, G. S. Kwon, *J. Controlled Release* **2000**, *64*, 143–153.

[18] Y. Yamashita, Y. Iwaya, K. Ito, *Makromol. Chem.* **1975**, *176*, 1207–1216.

[19] a) G. Holzwarth, P. Doty, *J. Am. Chem. Soc.* **1965**, *87*, 218–228; b) D. C. Dong, M. A. Winnik, *Can. J. Chem.* **1984**, *62*, 2560–2565.

[20] T. Li, J. P. Lin, T. Chen, S. N. Zhang, *Polymer* **2006**, *47*, 4485–4489.

[21] C. Deng, X. S. Chen, H. J. Yu, J. Sun, T. C. Lu, X. B. Jing, *Polymer* **2007**, *48*, 139–149.

[22] Y. C. Wang, L. Y. Tang, T. M. Sun, C. H. Li, M. H. Xiong, J. Wang, *Biomacromolecules* **2008**, *9*, 388–395.

[23] G. L. Zhang, F. Liang, X. M. Song, D. L. Liu, M. J. Li, Q. H. Wu, *Carbohydr. Polym.* **2010**, *80*, 885–890.

[24] J. Hain, M. Schrinner, Y. Lu, A. Pich, *Small* **2008**, *4*, 2016–2024.

[25] a) L. D. Shao, M. Q. Hu, L. Chen, L. Xu, Y. M. Bi, *React. Funct. Polym.* **2012**, *72*, 407–413; b) S. Verbrugghe, K. Bernaerts, F. E. Du Prez, *Macromol. Chem. Phys.* **2003**, *204*, 1217–1225.

[26] S. Shah, A. Pal, R. Gude, S. Devi, *Eur. Polym. J.* **2010**, *46*, 958–967.

[27] a) H. Vihola1, A. Laukkanen, L. Valtola, H. Tenhu, J. Hirvonen, *Biomaterials* **2005**, *26*, 3055–3064; b) Y. Maeda, M. Tsubota, I. Ikeda, *Macromol. Rapid Commun.* **2003**, *24*, 242–245.

[28] a) Q. L. Cui, F. P. Wu, E. J. Wang, *J. Phys. Chem. B* **2011**, *115*, 5913–5922; b) M. R. Berber, H. Mori, I. H. Hafez, K. Minagawa, M. Tanaka, T. Niidome, Y. Katayama, A. Maruyama, T. Hirano, Y. Maeda, T. Mori, *J. Phys. Chem. B* **2010**, *114*, 7784–7790.

[29] a) R. Freitag, F. Garret-Flaudy, *Langmuir* **2002**, *18*, 3434–3440; b) K. Van Durme, H. Rahier, B. V. Mele, *Macromolecules* **2005**, *38*, 10155–10163; c) Y. J. Zhang, S. Furyk, D. E. Bergbreiter, P. S. Cremer, *J. Am. Chem. Soc.* **2005**, *127*, 14505–14510.

[30] a) Z. W. Wang, H. J. Lai, P. Y. Wu, *Soft Matter* **2012**, *8*, 11644–11653; b) H. Mori, M. Yahagi, T. Endo, *Macromolecules* **2009**, *42*, 8082–8092.

[31] Q. H. Wu, C. Wang, D. Zhang, X. M. Song, D. L. Liu, L. P. Wang, G. L. Zhang, *React. Funct. Polym.* **2012**, *72*, 372–377.

[32] E. R. Blout, R. H. Karlson, *J. Am. Chem. Soc.* **1956**, *78*, 941–946.

[33] W. H. Daly, D. Poché, *Tetrahedron Lett.* **1988**, *29*, 5859–5862.

[34] Q. H. Wu, C. Wang, D. Zhang, X. M. Song, F. Verpoort, G. L. Zhang, *React. Funct. Polym.* **2011**, *71*, 980–984.

Received: May 19, 2014
Published online: August 21, 2014