Polymer Bulletin 45, 389–396 (2000)

Polymer Bulletin

Solvent effect on the lower critical solution temperature of biodegradable thermosensitive poly(organophosphazenes)

Sang Beom Lee[1], Soo-Chang Song[1,*], Jung-Il. Jin[2], Youn Soo Sohn[1,*]

[1] Inorganic Chemistry Laboratory, Korea Institute of Science and Technology
Seoul 130-650, Korea
e-mail: scsong@kist.re.kr, Tel.: +82-2-958-5123, Fax: +82-2-958-5089
[2] Department of Chemistry, Korea University, Seoul 136-701, Korea

Received: 8 September 2000/Revised version: 6 November 2000/Accepted: 9 November 2000

Summary

The solvent effect on the lower critical solution temperature (LCST) of poly(organophosphazenes) with methoxy-poly(ethylene glycol) (MPEG) and amino acid esters as side groups was examined in terms of the structure of polyphosphazenes in aqueous solutions containing one of the organic solvents selected from monoalcohols, ethylene glycol derivatives, alkylamines, and other common solvents. When such a solvent was added to the aqueous solutions of the polymers, their LCST was found to be mainly dependent on the hydrophobic and hydrophilic properties of the solvents. Most of the alcohols and amines with shorter alkyl chains increased the LCST of the polymers but those with longer chains decreased the LCST. Trifluoroethanol (TFE) showed a strong LCST decreasing effect in spite of its short chain, which seems to be due to its strong hydrophobicity. Temperature-induced molecular weight fractionation of the polymer bearing MPEG350 (M_w = 350) and L-aspartic acid ethyl ester as a side group was carried out by using the LCST decreasing effect of TFE, and the fractionated samples were characterized by gel permeation chromatography (GPC) and ^{1}H- and ^{31}P NMR spectroscopies. Thus it has been shown that a polymer may be fractionated to the higher and lower molecular weight fractions with smaller polydispersity indices (PDI): the polymer with the weight-average molecular weight (M_w) of 73,500 with PDI of 5.56 was fractionated to those of 106,000 with PDI of 4.37 and 11,000 with PDI of 1.86.

Introduction

Thermosensitive polymers are promising candidates for application as stimulus-sensitive biomaterials since they have a variety of phase transition temperatures in aqueous solution. Many applications of these systems were described for membranes, separation, drug delivery systems (DDS), and immunoassay technology [1-4].

When a transparent solution of a high molecular weight poly(ethylene glycol) or amphiphilic polymer is heated gradually, it becomes turbid at a certain temperature, and the polymer is separated out from the solution as solid-like fine

* Corresponding author

drops. This phase transition temperature is termed a lower critical solution temperature (LCST) or cloud point, which is an important factor for applications of thermosensitive polymers. For biological purpose, suitable thermosensitive polymers may be designed by either control of their molecular weight or hydrophilic/hydrophobic balance in their structure. The LCST may also be controlled by addition of a third component such as a salt [5-7], saccharide [8,9], surfactant [10-11], or solvent [6,12].

Most of the synthetic or natural polymers for biomaterials were designed to be degradable in a suitable rate in aqueous media. Thus the polymers need various chemical modifications such as insertion of a hydrolytic labile spacer between the drug and polymer backbone, allowance of backbone degradability, control of molecular weight, and so on. For this reason, their separation or fractionation processes have to be carried out under mild conditions. The solvent effect on the LCST of thermosensitive polymers can serve a methodology for separation or fractionation of biomaterials. The LCST of thermosensitive polymers for such processes must be low, but some of the polymers do not have low LCSTs. Thus, it is necessary to lower the LCST of the polymers by addition of a third component. The solvent effect has several advantages, which include experimental conveniences, lower cost, and ease of waste disposal: some solvents have low boiling points, and thereby they can be eliminated conveniently by low temperature evaporation or freeze-drying after the bioseparation process. Also, many solvents are miscible with water, and the bioactive materials may be obtained simply by precipitation.

Recently, we have reported that the poly(organophosphazenes) bearing methoxy-poly(ethylene glycol)(MPEG) and amino acid esters as side groups exhibited a wide variety of LCST depending on the compositions and kinds of side groups [13], and their hydrolytic degradability and salt effect [14] were examined in aqueous solution. In this study, solvent effect on the LCST of poly(organophosphazenes) was investigated depending on several factors such as kinds of amino acid esters, mole ratio of side groups, and the MPEG chain length. In addition, the molecular weight fractionation of a poly(organophosphazene) was performed by lowering its LCST by addition of trifluoroethanol (TFE) showing a strong LCST decreasing effect in the polymer solution.

Experimental

Polymers

Various thermosensitive poly(organophosphazenes) with MPEG and amino acid esters as side groups were employed in this study. Polymers **1 - 7** (see Table 1) were prepared in the previous work [13]. The characteristics of all polymers are listed in Table 1. In addition, a high molecular weight poly(dichlorophosphazene) was obtained from thermal polymerization of hexachlorocyclotriphosphazene with 3 wt% $AlCl_3$ at 230°C for 5hrs for the preparation of polymer **8**. Polymer **8**, $[NP(MPEG350)_{1.19}(AspEt)_{0.81}]_n$, was prepared and characterized by the same previous method [13]: MPEG350 (17.3mmol) and aspartic acid ethyl ester

(34.5mmol) were used. Yield: 78%. ^{31}P- NMR(Acetone-d_6), δ (ppm): 21.90. ^{1}H-NMR(D_2O), δ (ppm): 1.2-1.4(m, 6H), 2.9-3.2(b, 2H), 3.4(s, 3H), 3.6-3.9(b, 26H), 4.0-4.4(b, 7H). elem. anal. (%) calcd: C, 46.38; H, 7.79; N, 5.01; P, 5.57. found: C, 46.32; H, 7.67; N, 5.18; P, 5.59.

Materials

Guaranteed reagent grade methanol, ethanol, 2,2,2-trifluoroethanol, n-propanol, isopropanol, and n-butanol from Junsei Chemical and ethylene glycol, diethylene glycol, tetraethylene glycol, poly(ethylene glycol) (M_n = ca. 300), 2-methoxyethanol, 2-(2-methoxyethoxy)ethanol, 2-(2-ethoxyethoxy)ethanol, 2-(2-buthoxyethoxy)ethanol, and 2-methoxyethyl ether from Fluka were used without further purification. Acetone, acetonitrile, tetrahydrofuran, 1,4-dioxane, *N*,*N*-dimethylformamide, and dimethyl sulfoxide were used as received from Kanto. n-Butylamine, amylamine, and n-hexylamine were also used as received from Acros.

Measurements of LCST

The phase transition of the polymer solutions (5 wt%) containing different kinds and concentrations of solvents (0 - 2.0 M) was detected visually in a closed glass tube immersed in an oil bath. The LCST was identified as the temperature at which the solution became turbid.

LCST-Fractionation

A 5ml portion of a polymer **8** solution (5wt % in water) was pipetted into a 10mL long tapered centrifuge tube. To this was added a trifluoroehanol (2 - 3M), and the content was precipitated due to its lowered LCST. The tube was moved into an ice-bath to make a transparent solution, and then incubated in a thermostated shaking water bath for 15min at the desired temperature (20-30°C). The precipitated polymer was obtained by centrifugation at 3500 rpm for 5 min. The clear water layer was decanted to a flask and subjected to freeze-dry, and the polymer rich phase was vacuum-dried.

Results and Discussion

In order to examine the effect of various solvents on the LCST of poly(organophosphazenes) depending on the polymer structure and solvent concentration, four different groups of solvents, alcohols (linear, branched, or fluorinated types), ethylene glycols (diol, alkoxyalcohol, or dialkoxyalcohol types), amines (different chain lengths), and other common solvents (different structure and polarity) were employed.

Figure 1 shows the effect of monoalcohols with different chain lengths and structures on the LCST of polymer **5** (5 wt %) in aqueous solution. The LCST was found to change remarkably depending on both alkyl chain length and structure of alcohols. n-Butanol decreased remarkably the LCST of the polymer with

increasing its concentration whereas methanol, ethanol, n-propanol, and isopropanol exhibited a negligible effect on the LCST. Such alcohol effects on the LCST were similarly observed in case of the block copolymer of ethylene oxide-propylene oxide in aqueous solution [6]. The effect of alcohols on the LCST of thermosensitive polyphosphazenes can be understood by assuming that the short chain alcohols prefer a water environment while the longer chain alcohols are effectively attracted by the hydrophobic part of the polymers, decreasing the interaction of the polymers with water to the extent that depends on the chain length of alcohol. Interestingly, TFE of a short-chain alcohol exhibited the greatest LCST decreasing effect compared with other alcohols. TFE is a very hydrophobic ethanol analogue with three fluorine atoms and therefore, seems to interact effectively with the polymers resulting in decrease of the LCST of the polymers.

Such a strong LCST decreasing effect of TFE may be applied to bioseparation, fractionation of molecular weight, and LCST (or cloud point) extraction [15-17]. LCST-extraction is a separation or preconcentraion method of major products from an aliquot by using thermosensitive polymers or surfactants and has been studied in such fields as biochemistry, environmental chemistry, and protein extraction [3,15-17]. In most cases, "salting-out" (LCST decrease, water structure maker) salts such as ammonium sulfate or sodium chloride, are added to decrease the LCST and to induce precipitation of the polymers [3,17]. However, most of

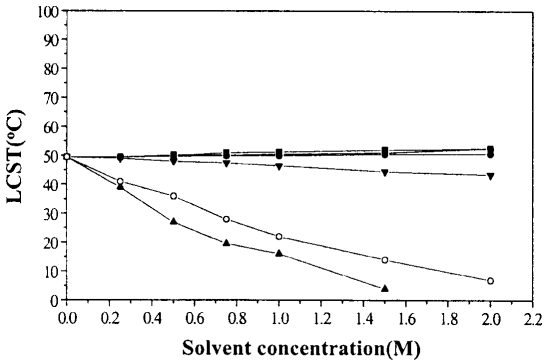

Figure 1. Change in LCST of polymer 5 in aqueous solution by addition of monoalcohols: methanol(●); ethanol(■); 2,2,2-trifluoroethanol(▲); n-propanol(▼); isopropanol(◆); n-butanol(○)

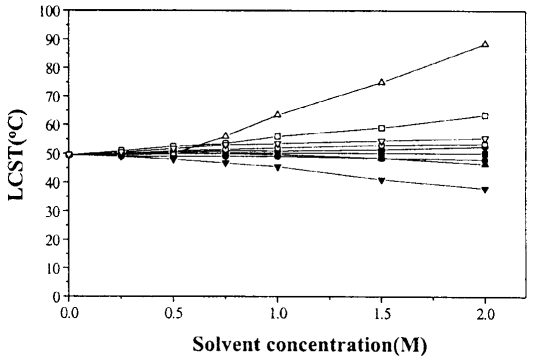

Figure 2. Change in LCST of polymer 5 in aqueous solution by addition of ethylene glycol and its derivatives: ethylene glycol(●); diethylene glycol(■); tetraethylene glycol(▲); PEG300(▼); 2-methoxyethanol(◆); 2-(2-methoxyethoxy)ethanol (○); 2-(2-ethoxyethoxy)ethanol(□); 2-(2-buthoxyethoxy)ethanol(Δ); 2-methoxyethyl ether(∇)

these salts are non-volatile and have to be eliminated by extraction. In some cases salts may be complexed to the biological active sites. In contrast, most of the solvents are volatile, and may be easily eliminated by evaporation.

Table 1 shows the n-butanol effect on the LCST of the polymers with different amino acid esters. All polymers showed decrease in LCST by addition of n-butanol, which was found to be dependent on the composition of substituents and kinds of amino acids and ester groups of the polymers. The $^{\Delta}$LCST ($T_{1.0M}$-T_{0M}), a

change of LCST of polymers by addition of 1.0M n-butanol, was found to be -49.5°C, -44.0°C, and –30.0°C for polymers **1**, **2**, and **3** respectively. Such a trend was similarly observed for polymers **4** and **5** as seen in the table. The more hydrophilic ester groups resulted in the more LCST decrease. These results

Table 1. Characteristics of poly(organophosphazenes)

polymer	formula	LCST(°C)[a]	$M_w(\times 10^{-4})$[b]	ΔLCST(°C) $(T_{1.0M}-T_{0M})$[c]
1	$[NP(MPEG350)_{1.42}(GlyEt)_{0.58}]_n$	93.2	4.73	-49.5
2	$[NP(MPEG350)_{0.99}(GlyEt)_{1.01}]_n$	77.5	3.84	-44.0
3	$[NP(MPEG350)_{0.58}(GlyEt)_{1.42}]_n$	64.5	1.77	-30.0
4	$[NP(MPEG350)_{1.03}(GlyMe)_{0..97}]_n$	88.5	3.08	-58.0
5	$[NP(MPEG350)_{1.00}(GlyBz)_{1.00}]_n$	49.5	2.13	-27.5
6	$[NP(MPEG750)_{1.09}(GlyEt)_{0.91}]_n$	98.5	4.14	-40.5
7	$[NP(MPEG350)_{1.01}(AspEt_2)_{0.99}]_n$	60.2	4.40	-38.0
8	$[NP(MPEG350)_{1.19}(AspEt_2)_{0.81}]_n$	63.5	7.35	

[a,b] Data from ref. 13. [c] The change of LCST by addition of 1.0M n-butanol.

indicate that n-butanol interacts with MPEG more favorably than amino acid esters.

Figure 2 shows the effect of ethylene glycol and its derivatives on the LCST of polymer **5**. The short-chain or polar alcohols such as ethylene glycol, diethylene glycol, tetraethylene glycol, 2-methoxyethanol and 2-(2-methoxyethoxy)ethanol, and 2-methoxyethyl ether increased slightly the LCST of the polymer. On the other hand, 2-(2-ethoxyethoxy)ethanol (EEE) and 2-(2-buthoxyethoxy)ethanol (BEE) increased remarkably the LCST of the polymer, and such an increased effect on the LCST was shown to be more prominent for BEE having the more hydrophobic butyl chain. BEE seems to have a surfactnat-like hydrophobic interaction [10,11] with the hydrophobic part of the polymer: increase in LCST of the polymers by adding a surfactant was further elevated with increasing the chain length of the hydrophobic alkyl group of the surfactant. However, PEG-300 showed a slight decrease on the LCST of the present polymers similarly to the effect of PEG on the LCST of the conventional thermosensitive organic polymers [12]. A decrease in the LCST of the polymer by addition of PEG implies some interaction between the polymer and PEG although such an interaction may be very weak.

In Figure 3, LCSTs were plotted against the concentration of alkylamines for polymer **5**. In general, the short-chain amines and their salts play a salting-in effect in the aqueous solution of thermosensitive polymers [18]. Especially, tetraalkylammonium halide shows a strong salting-in effect, and the effect is stronger with increased length of the alkyl chain. However, the present amines have shown to decrease the LCST with increased alkyl chain length: n-butylamine exhibited a strong LCST increasing effect in the whole range of its concentration whereas n-hexylamine exhibited a strong LCST decreasing effect. n-Amylamine decreased the LCST with increasing concentration up to 0.5M, but beyond this

concentrations, the LCST increased with increasing concentration of n-amylamine. n-Butylamine seems to interact with water as "water structure breaker", thereby increasing the interaction between the polymer and water molecules, which resulted in increase of LCST. On the other hand, n-hexylamine is likely to associate with the polymer through hydrophobic interaction, which caused to decrease the LCST.

Figure 4 shows the effect of common solvents on the LCST of polymer **5**. The LCST of the polymer shows to be nearly independent on the kinds of common

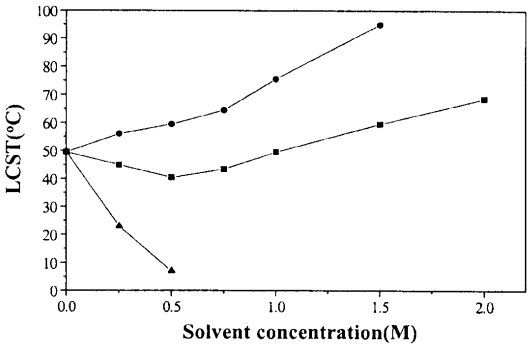

Figure 3. Change in LCST of polymer 5 in aqueous solution by addition of amines: n-butylamine(●); n-amylamine(■); n-hexylamine(▲)

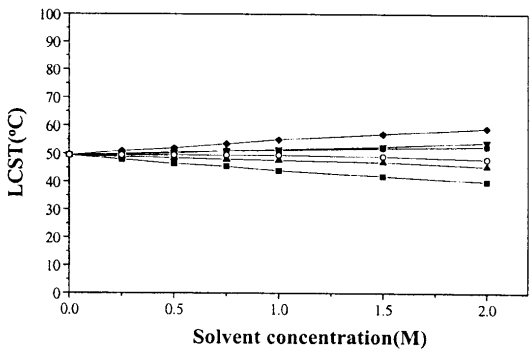

Figure 4. Change in LCST of polymer 5 in aqueous solution by addition of common solvents: acetone(●); acetonitrile(■); tetrahydrofuran(▲); 1,4-dioxane(▼); *N,N*-dimethylformaide(◆); dimethyl sulfoxide(○)

solvents, implying that these common solvents do not strongly interact either with the polymer molecules or with water to make hydrogen bonding.

The fractionation of polymers has been mostly executed by methods such as, fractional precipitation, GPC, sedimentation, and so on [20]. Synthetic polymers are not chemically pure substances in the strict sense, and they are molecular mixtures of various components. Especially, the molecular weight distribution of polyphosphazenes is generally very broad since they usually contain small amounts of cyclic oligomers and partially cross-linked polymers [21]. Therefore, in case of polyphosphazenes, it is important to obtain a narrow molecular weight distribution by fractionation. For effective fractionation, the process must be executed at low temperature in order to prohibit degradation of polymers. Thus, thermosensitive polymers have to be designed to have a low LCST, but sometimes the polymers may have a high LCST due to introduction of hydrophilic groups during the chemical modification for their biological purpose. The LCST-fractionation of $[NP(MPEG350)_{1.19}(AspEt_2)_{0.81}]_n$ (**8**) was attempted by using trifluoroethanol (TFE) showing a strong LCST decreasing effect in aqueous solution.

To induce a phase separation in aqueous solution of the polymer, one can use either n-butanol or TFE with a strong LCST decreasing effect. We have selected TFE with a stronger LCST decreasing effect and a lower boiling point (77-80°C). Polymer **8** has shown to undergo a phase separation in aqueous solution containing 2 – 3 M TFE. The GPC profiles, ^{31}P NMR spectra, and characteristic

properties of the fractions are presented in Figure 5 (a), (b), and Table 2, respectively. Polymer **8** has the weight-averaged molecular weight (M_w) of 7.35×10^4 with PDI of 5.56 before separation. The molecular weight of polymer **8-1** which is the precipitated fraction increased to $M_w = 10.6 \times 10^4$ with its PDI decreased to 4.37. The residue polymer **8-2** obtained by freeze-dry of the water layer shows a narrower distribution of molecular weight of 1.10×10^4 with PDI of 1.86, which seems due to the absence of the shoulder of polymers **8** and **8-1** as seen in the figure. However, as shown in Figure 5 (b), the ^{31}P NMR spectrum of polymer **8-2** was broad due to the increased intensity of P(O,O) resonance. Polymer **8-2** was characterized to exhibit a higher LCST and to involve higher content of MPEG, resulting in more P(O,O) units instead of P(N,O) units in the polymer back-bone compared with polymers **8** and **8-1**, as shown in Table 2. Such a result implies that the polymer can be purified to some extent by using LCST fractionation, which has advantages in requiring small amount of water for processing, short processing time, and mild conditions (lower temperature)

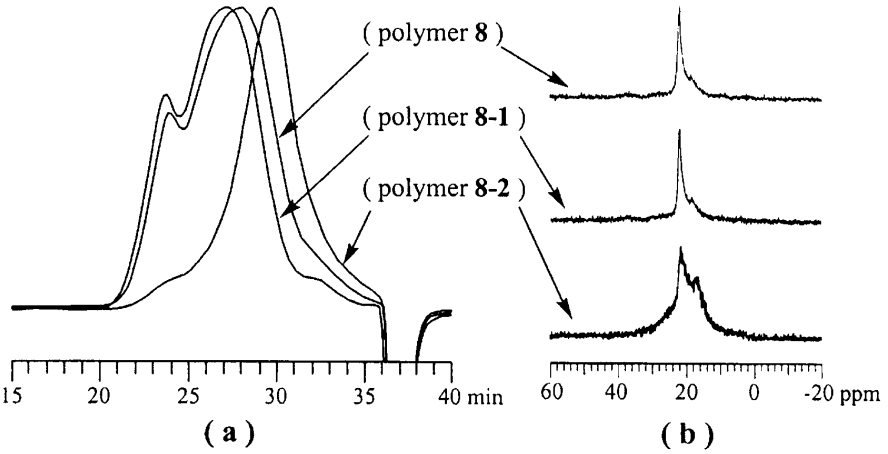

Figure 5. GPC chromatogram(a) and ^{31}P-NMR spectra(b) before and after fractionation of polymer 8. Peak identification: polymer 8, before fractionation; polymer 8-1, precipitated fraction; polymer 8-2, unprecipitated fraction

Table 2. Characteristics of polymer **8** before and after fractionation

polymer	weight(%)	LCST(°C)	M_w(×10^{-4})	PDI	relative mole ratios MPEG : $AspEt_2$
8		63.5	7.35	5.56	1.19 : 0.81
8-1	89	61.5	10.6	4.37	1.12 : 0.79
8-2	11	71.0	1.10	1.86	1.64 : 0.36

compared with the dialysis process by membrane.

Conclusion

The LCST of thermosensitive poly(organophosphazenes) in aqueous solution was found to be affected by kinds of solvents as well as composition of substituents and the structures of amino acids and ester groups of the polymers. Most of the monoalcohols play a LCST decreasing effect: the LCST decreased more sharply with increasing chain length of the monoalcohols, but the derivatives of ethylene glycol increased the LCST of the polymer solutions except

for PEG. In the case of alkyl amines, the chain length of their alkyl groups played an important role: an increase in the chain length of the alkyl group lead to a decrease in the LCST of the polymer solutions but those of shorter chain length increased the LCST. The molecular weight of the present polymer was fractionated by LCST-fractionation. Such a simple fractionation method may offer a wide range of applications such as separation, purification, or fractionation of biomolecules.

Acknowledgements. This research was financially supported by the Ministry of the Science and Technology in Korea. J.-I. Jin expresses his thanks to the Center for Electro- and Photo-Responsive Molecules and Research Division of Chemistry and Molecular Engineering, Korea University, for support.

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