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Modern Synthesis and Thermoresponsivity of Polyphosphoesters

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1. Introduction

There has been a great deal of interest in polyphosphoesters, which are biodegradable through hydrolysis and possibly through enzymatic digestion of phosphate linkages under physiological conditions (Renier et al., 1997). Biodegradable polyphosphoesters appear interesting for biological and pharmaceutical applications because of their biocompatibility and structural similarities to naturally occurring nucleic and teichoic acids. Recently, there have been interesting studies of polyphosphoesters used in biomedical applications (Wang et al., 2009). In particular, the advantages of polyphosphoesters for use in the field of tissue engineering as scaffolds and gene carriers was elucidated (Wan et al., 2001; Wang et al., 2002; Huang et al., 2004; Ren et al., 2010).

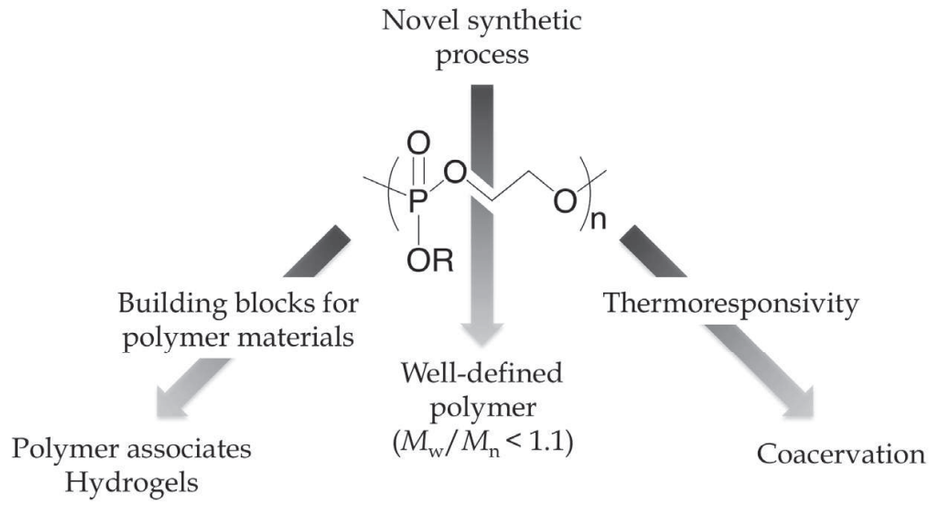


Fig. 1. Schematic contents of this chapter.

Figure 1 is a schematic representation of the contents of this chapter describing current research on polyphosphoesters. Although polyphosphoesters have a relatively long history, well-defined synthesis of the polymers has not been well explained. For use in medical applications such as drug delivery systems, understanding the synthetic process of

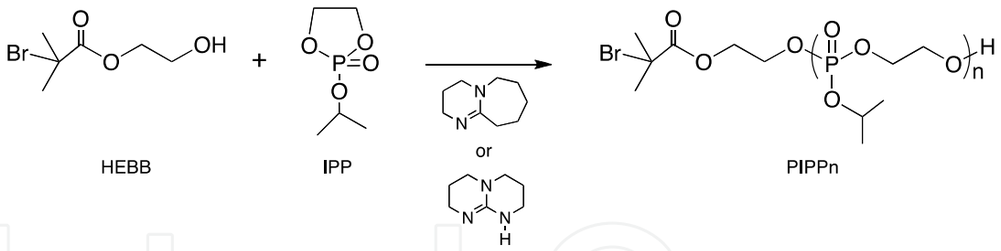
polymers with narrow molecular weight distribution may be quite important to obtain reproducibility. The first part of this chapter discusses the controlled synthesis of polyphosphoesters.
In comparison with conventional biodegradable polymers, the molecular functionalization of polyphosphoesters is easier because varied cyclic phosphoesters, which work as monomers, can be obtained by a simple condensation reaction between alcohol and chloro cyclic phosphoesters. That is, theoretically, any alcohol can be introduced into polyphosphoesters. Here, a biodegradable macroinitiator and macrocrosslinker based on polyphosphoesters are described. They can be used as building blocks for preparing polymer blends and hydrogels.
We have also recently found that polyphosphoesters show thermoresponsivity in aqueous media. This polymer solution makes a lower critical solution temperature (LCST) type coacervate. The phenomenon is strongly influenced by the structure and molecular weight of the polymers and the solvent condition. The basic thermoresponsive properties of polyphosphoesters are summarized in this chapter. Enzyme-responsive polyphosphoesters are also introduced.

2. Synthesis of well-defied polyphosphoesters and incorporation of functional groups into polymers

A variety of synthetic routes for polyphosphoesters have been reported including ring-opening polymerization (ROP) (Libiszowski et al., 1978; Pretula et al., 1986), polycondensation (Richard et al, 1991), transesterfication (Pretula et al., 1999; Myrex et al., 2003), and enzymatic polymerization (Wen et al., 1998). Since the pioneering experiments by the Penczek group (Penczek & Klosinski, 1990), the ROP of cyclic phosphate has been studied for more than three decades and various polymers having a phosphoester backbone have been designed. The ROP of cyclic phosphoesters is the most common process used to obtain polyphosphoesters. This is because a variety of polyphosphoesters can be designed in comparison with conventional biodegradable polymers because cyclic phosphoesters are obtained as monomers from the condensation of alcohol and 2-chloro-2-oxo-1,3,2-dioxaphospholane (Katuiyhski et al., 1976).

2.1 Synthesis of polyphosphoesters using organocatalysts

For the ROP of cyclic phosphoesters, metallic compounds are commonly used as initiators or polymerization catalysts (Penczek et al., 1990; Libiszowski et al., 1978; Pretula et al., 1986; Xiao et al., 2006). Although the polymerization processes are very successful in producing polyphosphoesters, the metal compounds are environmentally sensitive and a lack of residual metal contaminants is required in biomedical applications. Recently, organocatalysts have been the focus of the modern synthetic processes of polyesters, polycarbonates, and silicones (Kamber et al., 2007). One of the most successful procedures for making biodegradable polymers is polymerization using guanidine and amidine bases, both in bulk and in solution. Nederberg and Hedrick prepared poly(trimethylene carbonate (TMC)) (PTMC) with the base catalysts in the presence of benzyl alcohol (Nederberg et al., 2007). Excellent controlled polymerization conditions were present with several catalysts, and PTMCs with relatively high molecular weight, narrow distribution, and high yield were obtained. We have recently recognized that organocatalysts have high potency for the ROP of cyclic phosphoesters (Iwasaki et al., 2010).



Scheme 1. Synthetic route of PIPP. (Reproduced from Iwasaki et al., (2010) *Macromolecules*, Vol. 40, No. 23, pp. 8136-8138, Copyright (2010), with permission from the American Chemical Society)

Poly(2-isopropoxy-2-oxo-1,3,2-dioxaphospholane) (PIPPn, n is degree of polymerization) was synthesized by ROP using an organocatalyst as an initiator in the presence of 2-hydroxyethyl-2′-bromoisobutyrate (HEBB) (Scheme 1). In the case of 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), polymerization was homogeneously performed in a solvent-free condition. In contrast, a small amount of toluene was used for dissolving 1,5,7-triazabicyclo[4,4,0]dec-5-ene (TBD) to make a homogeneous solution. The results of PIPP synthesis are summarized in Table 1. Twenty mmoles of IPP was first introduced into a polymerization tube under an argon gas atmosphere at 0°C, and then a given amount of HEBB was added to the tube. Finally, a given amount of organocatalyst was introduced. Polymerization was carried out at 0°C. The range of molecular weights was approximately 2.0 x 10^3 to 3.0 x 10^4 g/mol by gel-permeation chromatography (GPC) using a calibration curve based on linear polystyrene standards with chloroform as the mobile phase. In every case, the molecular weight distribution was lower than 1.10. Under each condition, the molecular weights of the synthetic polymers agreed with the theoretical values.

Code	Catalyst	$[M]_0/[I]$	HEBB (mmol)	Catalyst (mmol)	Time (min)	Conv. (%)	M_n x 10^{-3}	M_w/M_n	$M_{n(Theo)}$ x 10^{-3}
PIPP13	DBU	25	0.80	1.20	60	52.8	2.4	1.03	2.2
PIPP32	DBU	50	0.40	0.60	90	52.7	4.7	1.07	4.4
PIPP50	DBU	100	0.20	0.30	300	50.8	7.7	1.09	8.4
PIPP48	TBD	50	0.40	0.20	20	81.2	8.2	1.06	6.7
PIPP77	TBD	100	0.20	0.20	20	80.7	13.0	1.09	13.4
PIPP117	TBD	150	0.13	0.20	20	75.5	16.9	1.07	18.8
PIPP174	TBD	200	0.10	0.20	20	90.3	28.9	1.05	30.0

Table 1. Synthetic results of $PIPP_n$. (Reproduced from Iwasaki et al., (2010) *Macromolecules*, Vol. 40, No. 23, pp. 8136-8138, Copyright (2010), with permission from the American Chemical Society)

Figure 2 shows the number-averaged molecular weight (M_n) versus monomer conversion for the polymerization of IPP by using DBU as a catalyst. The plot of M_n vs. conversion was linear up to 60% conversion. The linearity of the plot suggests that the number of macromolecules in the reaction system was constant during polymerization. The molecular weight distribution of PIPP was narrow and stable during polymerization. The mechanism of ROP with organocatalysts was characterized using ^{1}H NMR by Hedrick and co-workers (Nederberg et al., 2007; Pratt et al., 2006). They indicated that DBU and TBD form hydrogen bonds to the alcohol of an initiator. ROP of IPP with DBU then occurs through a quasi-

anionic polymerization mechanism by activation of the alcohol of the initiator. In contrast, the increase in monomer conversion for the polymerization of IPP between DBU and TBD was significantly different. When TBD was used as a catalyst, the conversion of PIPP reached a level of more than 75% within 20 min. The heightened activity of TBD for the polymerization of lactone and TMC was also observed (Nederberg et al., 2007).

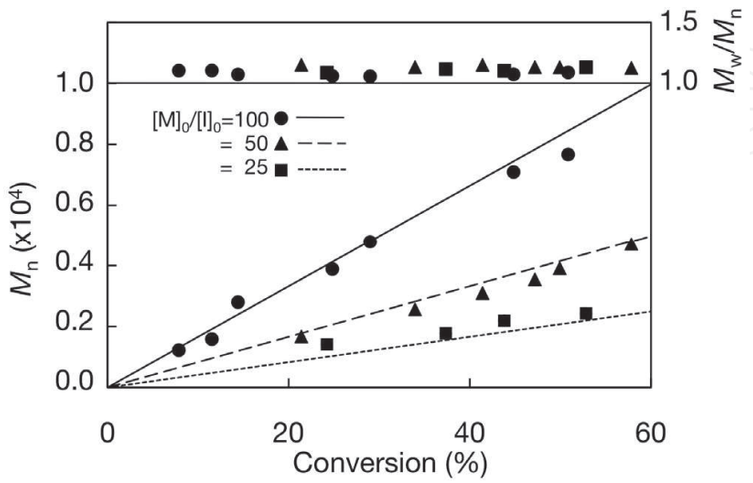


Fig. 2. Plot of M_w/M_n and M_n versus monomer conversion for the polymerization of 2-isopropoxy-2-oxo-1,3,2-dioxaphospholane by using 1,8-diazabicyclo[5,4,0]undec-7-ene as a catalyst. Lines suggest the theoretical amount of each polymerization condition.
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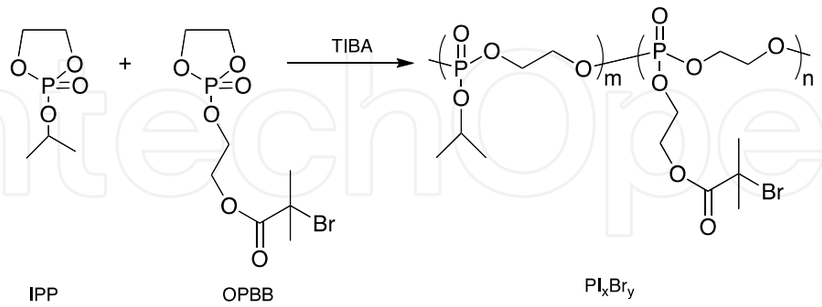
2.2 Polyphosphoester macroinitiators

Atom transfer radical polymerization (ATRP) has great ability to control the molecular architecture of synthetic polymers and is an exceptionally robust method of producing block or graft copolymers (Matyjaszewski & Xia, 2001). However, the still limited design of biodegradable amphiphilic polymers has been performed via ATRP. Polyphosphoesters bearing 2-bromo-isobutyryl groups as novel biodegradable macroinitiators for ATRP were then synthesized and amphiphilic polymers with well–defined hydrophilic graft chains were prepared (Iwasaki & Akiyoshi, 2004).

A cyclic phosphoester bearing bromoisobutyrate, 2-(2-oxo,1,3,2-dioxaphospholoyloxy) ethyl-2′-bromoisobutyrate (OPBB), was obtained from the reaction of HEBB and 2-chloro-2-oxo-1,3,2-dioxaphosphorane (COP). Poly(IPP-co-OPBB) (PI_xBr_y (Scheme 2); x:IPP (mol%), y: OPBB (mol%)) was synthesized by ring-opening polymerization using triisobutyl aluminum (TIBA) as an initiator. The chemical structure and synthetic results of the polyphosphoesters are shown in Scheme 2 and Table 2, respectively. Polymerization was homogeneously performed by a solvent-free reaction. As indicated in Table 2, the composition of each monomer unit could be controlled by the feed. The M_w of the polyphosphoester was 3.1 x 10^4 to 3.9 x 10^4 g/mol. The absolute molecular weights of $PIBr_2$ and $PIBr_5$ determined by MALLS were 3.4 x 10^4 and 3.7 x 10^4, respectively.

ATRP of 2-methacryloyloxyethyl phosphorylcholine (MPC) from macroinitiator polyphosphoesters was carried out in an ethanol solution. Figure 3 shows the number of

MPC units in a graft chain of $PIBr_2$-g-PMPC and $PIBr_5$-g-PMPC as determined by ^1H NMR. The numbers were linearly increased with an increase in the duration of polymerization. The slope of the $PIBr_2$-g-PMPC was much greater than that of $PIBr_5$-g-PMPC. The rates of polymerization decreased with graft density.



Scheme 2. Synthetic route of polyphosphoester bearing bromoisobutyrate (PIBr). (Reproduced from Iwasaki et al., (2004) *Macromolecules*, Vol. 37, No. 20, pp. 7637-7642, Copyright (2004), with permission from the American Chemical Society)

Polyphosphoesters	OPBB/IPP (mol%)		Yield (%)	Mw x 10^{-4}	M_w/M_n	No. of OPBB per PIBr molecule
	In feed	In copolymer				
$PI_{97}Br_3$	2.0/98.0	1.5/98.5	76.1	3.9 3.4	1.4	3.0
$PI_{95}Br_5$	6.0/94.0	5.0/95.0	49.2	3.1 3.7	1.4	10.5

Table 2. Synthetic results of PIBr. (Reproduced from Iwasaki et al., (2004) *Macromolecules*, Vol. 37, No. 20, pp. 7637-7642, Copyright (2004), with permission from the American Chemical Society)

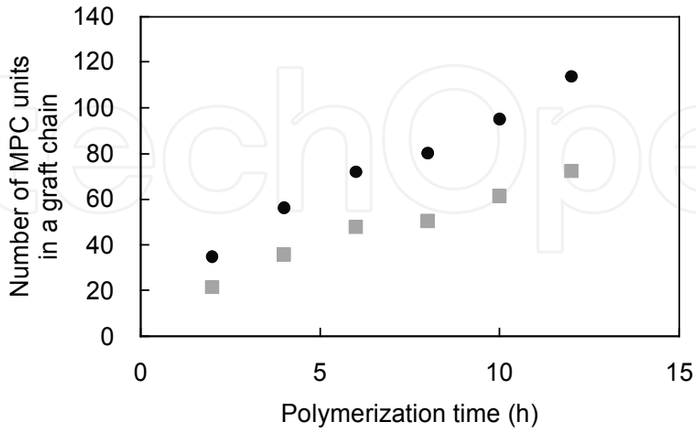


Fig. 3. Change in number of units of MPC in a graft chain during ATRP. (Circle): $PIBr_3$-g-PMPC; (Square): $PIBr_5$-g-PMPC. (Reproduced from Iwasaki et al., (2004) *Macromolecules*, Vol. 37, No. 20, pp. 7637-7642, Copyright (2004), with permission from the American Chemical Society)

The transition point of the surface tension increased with an increase in the molecular weight and density of PMPC. Typical examples for the concentrations of $PIBr_3$-g-PMPC71[1] and $PIBr_5$-g-PMPC115 were 8.6 x 10^{-3} g/dL and 2.3 x 10^{-3} g/dL, respectively. A decrease in surface tensions was observed on every graft copolymer. The surface tensions were influenced by the density and molecular weight of PMPC.

Based on MALLS analysis for associative $PIBr_3$-g-PMPC71, the molecular weight of the polymeric associate was 91.1 x 10^4. From the data in Figure 3, the molecular weight of $PIBr_3$-g-PMPC71 can be estimated at 13.6 x 10^4. Thus, the association number of the $PIBr_3$-g-PMPC71 was 6.7. For $PIBr_5$-g-PMPC, the association number was 1.5, that is, it is almost a "unimer-micelle." Figure 4 shows schematic representations of the polymeric associates of $PIBr_2$-g-$PMPC_{12}$ and $PIBr_5$-g-PMPC115.

In an acidic medium, the loss of molecular weight of the graft copolymer was observed as being less; degradation remarkably occurred after 50 days of soaking. Under physiological pH conditions, the molecular weight of the PIBr-g-PMPC decreased from 15.6 x 10^4 (GPC data) to 12.7 x 10^4 after 50 days. Under a basic condition, the polyphosphoester degraded almost completely within 3 days. After soaking in pH11.0, the $PIBr_2$-g-PMPC71 and $PIBr_5$-g-PMPC115 polymers had molecular weights of 2.4 x 10^{-4} and 3.1 x 10^{-4} (M_w/M_n=1.2), respectively, as determined by GPC. These polymers were identified as PMPC by ^{1}H NMR (data not shown). Although a basic condition (pH11.0) is not a physiological condition, we chose the optimal pH to characterize the degradation behavior of polyphosphoesters in a relatively short period. Under an acidic condition (pH 4.0), the hydrolysis of PIBr was slow. In contrast, under a basic condition (pH 11.0), the PIBr was completely degraded in only 3 days.

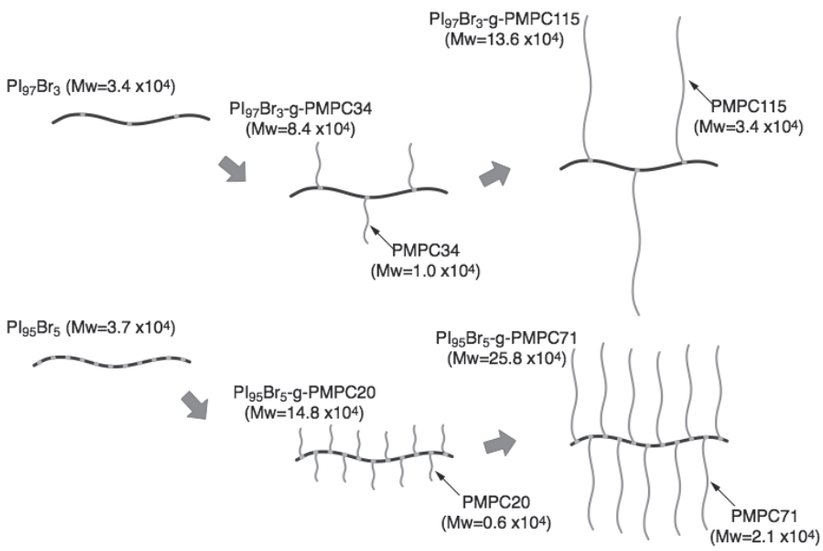


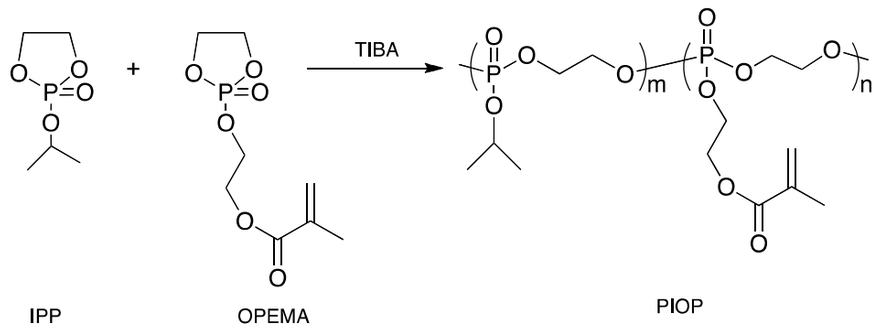
Fig. 4. Schematic representation of PIBr and PIBr-g-PMPC. (Reproduced from Iwasaki et al., (2004) *Macromolecules*, Vol. 37, No. 20, pp. 7637-7642, Copyright (2004), with permission from the American Chemical Society)

[1] The number after PMPC is degree of MPC polymerization in each graft chain.

The PIPPn shown in Scheme 1 also works as a macroinitiator because it has bromoisobutyrate at the end. Using PIPPn, well-defined block copolymers can be obtained by ATRP (Iwasaki et al., 2010).

2.3 Polyphosphoester macrocrosslinkers

Biomaterials have an enormous impact on human health care. They are widely used in biomedical applications, including drug delivery devices and tissue engineering matrices (Lin et al., 2003). Specifically, hydrogels are included in the more recent development of biomaterials because they can absorb significant amounts of water and are as flexible as soft tissue, which minimizes their potential for irritating surrounding tissue. In order to obtain synthetic cellular matrices offering both biocompatibility and biodegradability, a novel porous biodegradable MPC polymer hydrogel crosslinked with polyphosphoesters was prepared with a gas-forming technique (Iwasaki et al., 2003; Iwasaki et al., 2004; Wachiralarpphaithoon et al., 2007).



Scheme 4. Synthetic route of PIOP. (Reproduced from Wachiralarpphaithoon et al., (2007) *Biomaterials*, Vol. 28, No. 6, pp. 984-993, Copyright (2007), with permission from Elsevier)

Code	PIOP:MPC (%)	Potassium hydrogen carbonate size range (μm)	Swelling ratio (%)	Elastic modulus (x 10^4 Pa)	Porosity (%)
G1	0.5:99.5	-	1519±208	2.47±0.47	95.0±0.3
G1A	0.5:99.5	500-300	1576±191	0.06±0.01	98.4±0.4
G1B	0.5:99.5	300-250	1549±502	0.05±0.01	98.2±0.1
G1C	0.5:99.5	250-150	1547±665	0.04±0.00	97.8±0.2
G2	1:99	-	804±128	3.08±0.77	92.7±0.6
G2A	1:99	500-300	963±129	0.18±0.01	96.4±0.3
G2B	1:99	300-250	957±153	0.21±0.02	96.5±0.1
G2C	1:99	250-150	977±26	0.26±0.01	96.7±0.2
G3	2.5:97.5	-	357±103	10.10±3.26	86.0±1.3
G3A	2.5:97.5	500-300	518±40	2.61±0.23	96.2±0.1
G3B	2.5:97.5	300-250	523±183	2.61±0.25	95.8±0.1
G3C	2.5:97.5	250-150	512±133	2.65±0.01	94.8±0.2

Table 3. Synthetic condition and properties of hydrogels. (Reproduced from Wachiralarpphaithoon et al., (2007) *Biomaterials*, Vol. 28, No. 6, pp. 984-993, Copyright (2007), with permission from Elsevier)

The synthetic route of the macrocrosslinker, PIOP, was also synthesized using TIBA as an initiator (Scheme 4). The molecular weight of PIOP was 1.1 x 104 (M_w/M_n=1.1). The calculated number of 2-(2-oxo-1,3,2-dioxaphosphoroyloxy) ethyl methacrylate (OPEMA) units in a PIOP chain was 2.02.

The synthetic conditions and characterizations of the hydrogels are summarized in Table 3. Figure 5 shows macroscopic pictures of the swollen hydrogels prepared in this study. The hydrogels (G1, G2, and G3) shown in picture a) were prepared without porogen salts. When the crosslinking density is low, the hydrogels have a highly stretched network, which was experimentally observed as a large transparent appearance. With an increase in the composition of PIOP, the size of the hydrogels decreased and the transparency became poor because of the close distance of the PIOP molecules. Picture b) shows porous hydrogels (G1A, G2A, and G3A) prepared with the largest porogen salts (ϕ = 300-500 μm). The effect of PIOP composition on the macroscopic form was similarly observed as in picture a). This result indicates that PIOP works as a macromolecular crosslinking reagent in the preparation of hydrogels. Many small bubbles are observed in the hydrogels prepared with porogen salts. Macroscopic observation clarifies the difference in the inner structure between G1 and G1A.

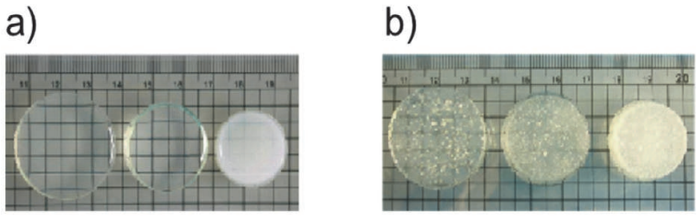


Fig. 5. Macroscopic pictures of swollen hydrogels. a) Hydrogels without porogen salts (G1, G2, and G3) b) Hydrogels with porogen salts (G1A, G2A, and G3A) after 24 h equilibration in water. (Reproduced from Wachiralarpphaithoon et al., (2007) *Biomaterials*, Vol. 28, No. 6, pp. 984-993, Copyright (2007), with permission from Elsevier)

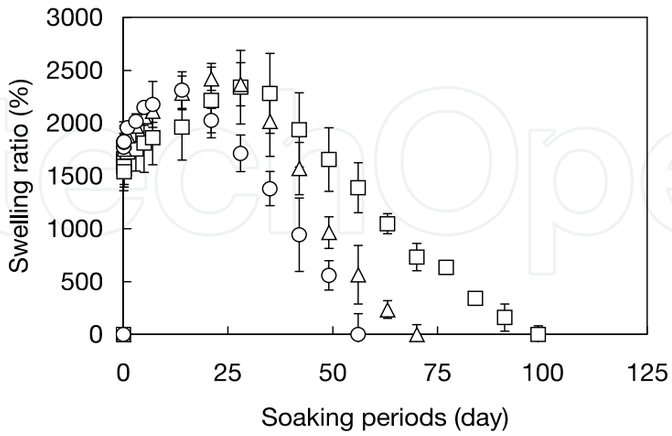


Fig. 6. Enzymatic degradation as a function of time for hydrogel G1A in ALP aqueous solution at 37°C; [ALP]= 0 U/L (□), 72.5 U/L (△), 220 U/L (○). Each point represents the average of three samples. (Reproduced from Wachiralarpphaithoon et al., (2007) *Biomaterials*, Vol. 28, No. 6, pp. 984-993, Copyright (2007), with permission from Elsevier)

Alkaline phosphatase (ALP) is an important enzyme produced in bone and liver cells. It catalyzes the hydrolysis of phosphate groups from monophosphate ester substrates mostly found in an alkaline state with a pH of 9 (Coburn et al., 1998). Although Zhao and co-workers reported that synthetic polyphosphoesters and polyphosphoesters are enzymatically degradable (Zhao et al., 2003), the process was not described in detail. The concentration of ALP for the degradation study was adjusted to 72.5 and 220 U/L, which is the concentration in healthy adults and children, respectively (Takeshita et al., 2004; Rafan et al., 2000). Figure 6 is an enzymatic degradation profile of G1A hydrogels by changing the concentration of ALP. G1A took about 100 days to reach complete dissolution at pH 9.0. The degradation was accelerated with a higher concentration of ALP; G1A completely degraded after 60 days in 220 U/L of ALP. The degradation period was shortened with an increase in the concentration of the enzyme. The digestion of a hydrogel might be regulated by varying the density of cells secreting an enzyme in the hydrogel.

MC3T3-E1 is a clonal osteogenic cell line derived from neonatal mouse calvaria. The cells are well characterized and provide a homogeneous source of osteoblastic cells for study. They were encapsulated in various biomaterial networks and remained viable (Burdick et al., 2005). MC3T3-E1 cells express high levels of alkaline phosphatase and differentiate into osteoblasts that can form calcified bone tissue *in vitro* (Choi et al., 1996). The response of MC3T3-E1 cells to many growth factors and hormones mimics that of primary cultures of rodent osteoblastic cells.

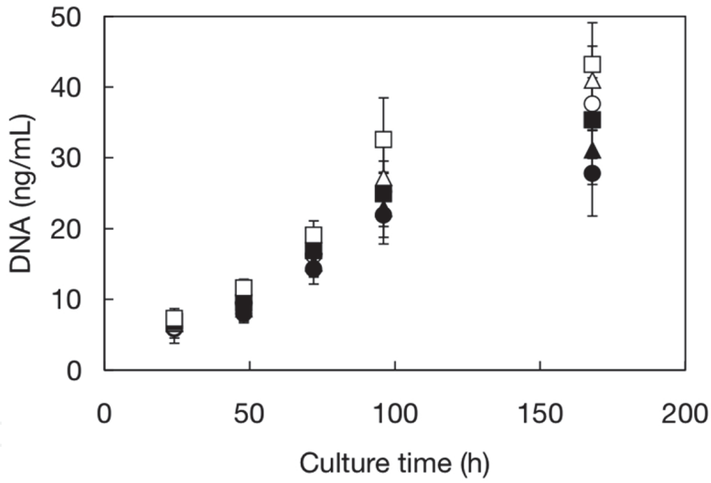


Fig. 7. Kinetics of MC3T3-E1 cell proliferation in hydrogels. (○) G1A, (△) G2A, (□) G3A with bFGF; (●) G1A, (▲) G2A, (■) G3A without bFGF. (Reproduced from Wachiralarpphaithoon et al., (2007) *Biomaterials*, Vol. 28, No. 6, pp. 984-993, Copyright (2007), with permission from Elsevier)

Figure 7 shows the time-dependent concentration of the DNA produced from the MC3T3-E1 cells in porous hydrogels. The concentration increment of DNA corresponds to the proliferation of cells in a hydrogel. Under every sample condition, the amount of DNA significantly increased ($p < 0.05$) with increased cultivation time. After culture for 168 h, the amount of DNA collected was significantly higher from G3A ($p = 0.036$) in comparison to G1A. Therefore, the density of PIOP influenced cell proliferation. When the bFGF was incorporated into a hydrogel, the rate of cell proliferation relatively increased with an

increase in the concentration of PIOP ($p = 0.017$ and $p = 0.107$ G1A vs. G3A after culture for 96 h and 168 h, respectively). While MPC polymer provides a suitable condition for maintaining cell viability, this polymer is not effective for inducing cell adhesion on the surface (Wachiralarpphaithoon et al., 2007). Polyphosphoester might induce cell adhesion and proliferation in a hydrogel. Wang and co-workers have recently reported that poly(ethylene glycol) (PEG) hydrogel having a phosphoester linkage promotes gene expression of bone-specific markers and secretion of alkaline phosphatase, osteocalcin, and osteonectin protein from marrow-derived mesenchymal stem cells (Wang et al., 2005).

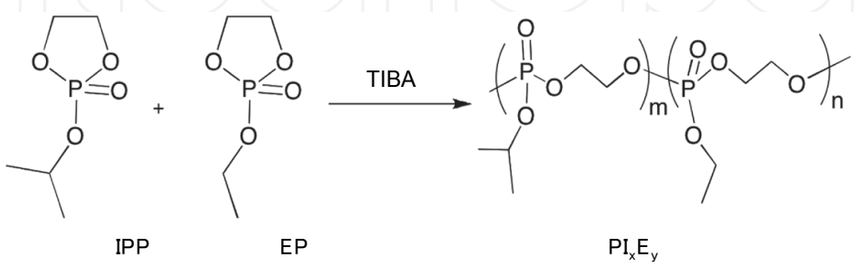
3. Thermoresponsive polyphosphoesters

Thermoresponsive polymers are widely studied in both research and technology because of their versatility in many fields. Recent trends in the application of polymer materials are drug delivery (Kikuchi & Okano, 2002), separation of bioactive molecules (Kobayashi et al., 2003), and tissue engineering (Kikuchi & Okano, 2005). *N*-Substituted acrylamide polymers have been found to have a phase separation characteristic with changes occurring in their properties upon heating above a certain lower critical solution temperature (LCST) (Monji et al., 1994; Yamazaki et al., 1999; Idziak et al., 1999). In particular, *N*-isopropyl acrylamide (NIPAAm) is one of the best monomers for accomplishing this; the homopolymer has LCST at 32°C in aqueous solution (Heskins et al., 1968). Although NIPAAm is a robust monomer for obtaining thermoresponsive polymer materials such as stimuli-responsive surfaces, particles, and hydrogels, the polymers are not biodegradable.

Besides the stimuli-responsive nature, biodegradability and biocompatibility are important characteristics for polymeric materials used in biomedical fields. While the thermoresponsivity of some biodegradable polymers such as aliphatic polyester block copolymers or polypeptides was recently advanced (Fujiwara et al., 2001; Kim et al., 2004; Tachibana et al., 2003; Shimokuri et al., 2006), the molecular design and synthetic processes of thermoresponsive biodegradable polymers are still limited.

3.1 Thermoresponsivity of polyphosphoesters

In current research, thermoresponsive polyphosphoesters are now being synthesized with simple copolymerization of cyclic phosphoester compounds and their properties are being investigated (Iwasaki et al., 2007). Poly(IPP-co-EP) (PI_xE_y (Scheme 4); x:IPP (mol%), y: EP (mol%)) was synthesized by ring-opening polymerization using TIBA as an initiator. The range of weight-averaged molecular weights was 1.2×10^4 to 1.5×10^4 g/mol (GPC analysis).



Scheme 4. Synthetic route of PI_xE_y (Reproduced from Iwasaki et al., (2007) *Macromolecules*, Vol. 40, No. 23, pp. 8136-8138, Copyright (2007), with permission from the American Chemical Society)

Figure 8 shows the LCST-type phase separation of $PI_{24}E_{76}$ aqueous solution. From the optical microscopic image, it is clear that the polymer solution was separated at the liquid-liquid phase above the cloud point. This appears to be coacervation. After several hours, the turbid solution spontaneously separated into two phases. The cloud point could be controlled by the ratio of IPP and EP, that is, it decreased with an increase in the molar fraction of hydrophobic IPP.

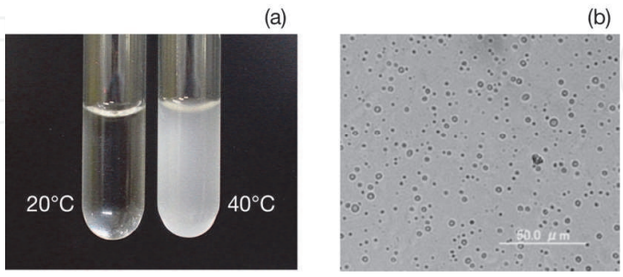


Fig. 8. LCST-type phase separation of polyphosphoester aqueous solution. (a) 1%- $PI_{24}E_{76}$ aqueous solution at 20 and 40°C. (b) Optical micrograph of 1%-$PI_{24}E_{76}$ aqueous solution at 40°C. (Reproduced from Iwasaki et al., (2007) *Macromolecules*, Vol. 40, No. 23, pp. 8136-8138,Copyright (2007), with permission from the American Chemical Society)]

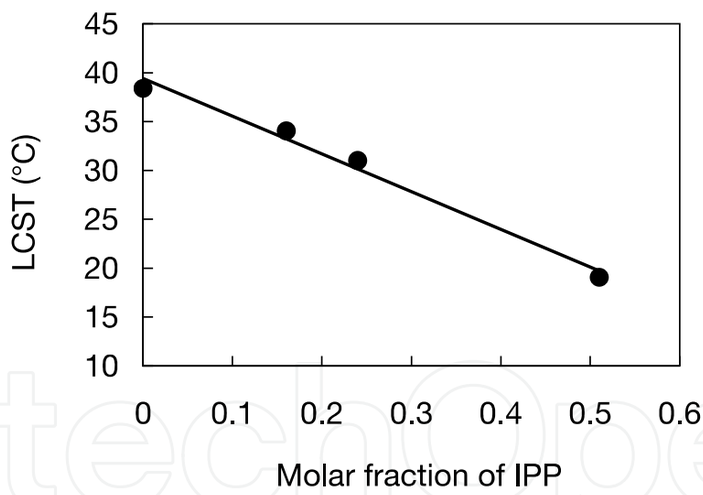


Fig. 9. Effect of molecular fraction of IPP on LCST of PIxEy (Reproduced from Iwasaki et al., (2007) *Macromolecules*, Vol. 40, No. 23, pp. 8136-8138. Copyright (2007), with permission from the American Chemical Society)

Figure 9 shows the effect of the composition of the monomer unit on the LCST of the copolymers. The LCST of poly(EP) (PEP) was 38°C and it linearly decreased with an increase in the ratio of IPP. IPP is relatively hydrophobic; the homopolymer of IPP is not soluble in water above 5°C. Dehydration of the polymer then preferably occurred with the addition of the hydrophobic IPP unit. It is reported that the LCST of thermoresponsive polymers can be controlled by the ratio of the hydrophobic and hydrophilic units (Takei et al., 1993; Tachibana et al., 2003). Thermoresponsivity under physiological conditions is

effective for drug delivery or tissue engineering applications (Okuyama et al., 1993; Nishida et al., 2004). The thermoresponsivity of polyphosphoesters can also be observed under physiological temperatures. Thus, the polymers are applicable in the biomedical field.

The effect of NaCl concentration on the cloud point on PEP and $PI_{24}E_{76}$ is shown in Figure 10. The cloud point of the polymer solution decreased with an increase in the concentration of NaCl in aqueous media. Under physiological conditions ([NaCl] = 100 mM), the cloud point of PEP and $PI_{24}E_{76}$ was 28 and 26°C, respectively. The solution property of nonionic polymer in water is sensitively influenced by the addition of salt because salt can alter polymer-water interaction (Foss et al., 1992).

Figure 11 shows the dependence of the cloud point of $PI_{24}E_{76}$ on polymer concentration in distilled water. The cloud point decreased with an increase in polymer concentration. Furthermore, the change in the transmittance of the polymer solution was more abrupt at a higher concentration. The effect of polymer concentration on phase separation temperature was also observed on poly(acryl amide) derivatives (Miyazaki & Kataoka, 1996). In their report, coacervate droplets could be condensed with centrifugation; the polymer concentration of the coacervate phase was much greater than that of the homogeneous solution.

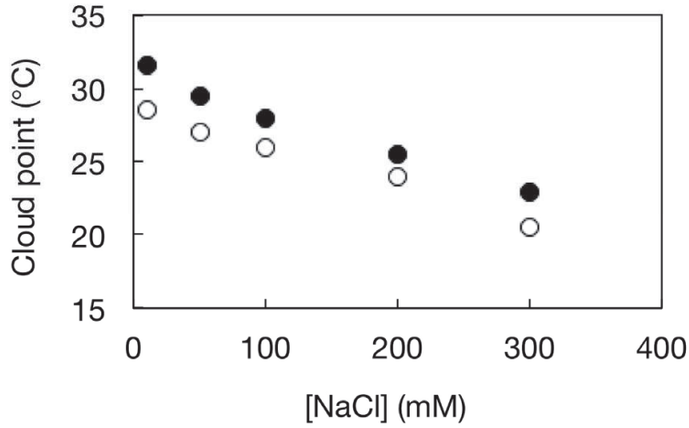


Fig. 10. Effect of NaCl concentration ([NaCl]) on cloud point of polyphosphoester aqueous solution. (●) PE, (○) $PI_{24}E_{76}$, [Polymer] = 1.0 wt%.

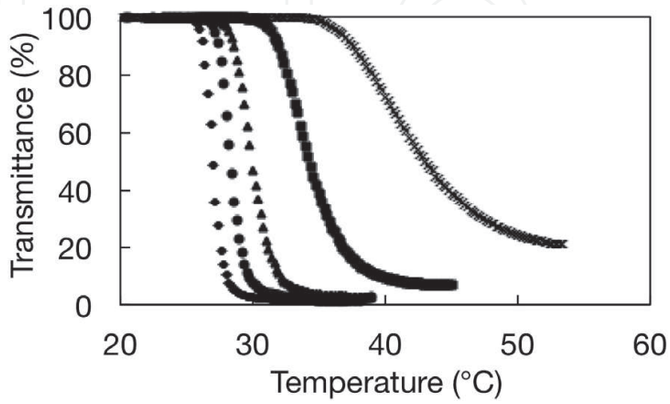


Fig. 11. Effect of polymer concentration on cloud point of $PI_{24}E_{76}$ aqueous solution. [Polymer] = 1.0 (◆), 0.75 (●), 0. 5 (▲), 0.25 (■), and 0.1 wt% (×).

Temperature (°C)	Relaxation time (ms) 4.1 ppm (main chain CH_2)	Relaxation time (s) 4.0 ppm (side chain CH_2)	Relaxation time (s) 1.2 ppm (side chain CH_3)
T_1			
19.0	577.314	1.721	1.493
39.0	671.000	2.022	1.889
T_2			
19.0	314.293	1.084	1.187
39.0	438.944	1.233	1.380

Table 4. Spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) of proton in $PI_{24}E_{76}$.

To understand the molecular phenomenon for creating coacervates, we measured T_1 and T_2 of the protons in the main and side chains of $PI_{24}E_{76}$. Table 4 summarizes the typical data for relaxation times. It can be considered that a polymer in solution behaves as a liquid molecule with high mobility (Mao et al., 2000). As shown in Table 4, T_1 and T_2 of every proton contained in the main and side chains of $PI_{24}E_{76}$ increase as the temperature increases. Furthermore, a significant change of these relaxation times at the cloud point of $PI_{24}E_{76}$ was not observed. T_2 of the protons is mostly influenced by the dipole-dipole interaction of nuclear spin. The shorter the distance between protons, the slower the motion of the polymer chains and the stronger the interaction of the proton-proton dipolar coupling; thus the smaller T_2. The experimental results indicated that the mobility of the polymer thermodynamically increased with an increase in temperature regardless of the phase separation.

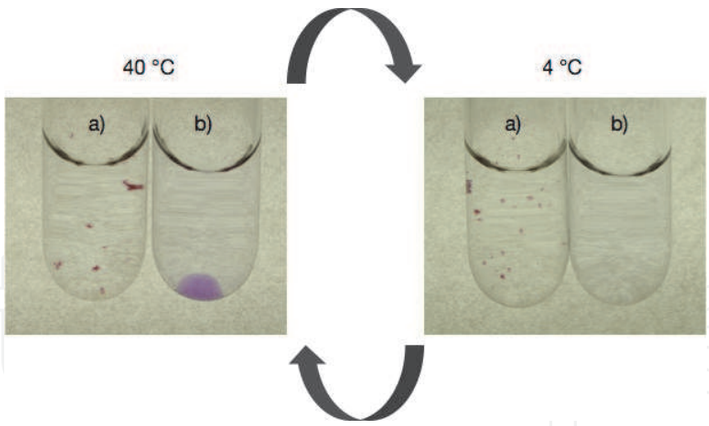


Fig. 12. Condensation of hydrophobic compound (Nile Red) from aqueous media. a) 150 mM NaCl aqueous solution, b) 150 mM NaCl aqueous solution containing 1-wt% $PI_{24}E_{76}$. [Nile Red] = 5 μg/mL.

The relaxation times of the protons of associated trigger groups normally decrease because of a decrease in mobility (Hsu et al., 2005). However, the results did not show this. In the coacervate phase with enriched polymers, solvent remained above the cloud point. Then, the polymers might loosely associate and their mobility was not reduced with an increase in temperature. While the mobility of the polymers in the coacervate phase was clarified, further study will be needed to show the molecular mechanism of coacervation.

We demonstrated the separation of hydrophobic molecules with thermoresponsive polyphosphoesters from aqueous media. Nile Red was used as a model compound; its solubility in water is very low. Nile Red dissolved in acetone was added to Dulbecco's phosphate buffered saline (PBS, calcium chloride- and magnesium chloride-free, Sigma). Polyphosphoester was then immediately introduced into the solution. Both PBS and that containing the polymer appear homogeneous before heating. When the solutions were incubated at 40°C, significant differences in solution behavior were observed, as shown in Figure 12. At 40°C, the polymer solution became turbid and then separated into two phases. Nile Red selectively condensed at the bottom layer, which contains the concentrated polymers. In contrast, the aggregation of Nile Red was observed in PBS at 40°C because the acetone evaporated and the Nile Red could not then disperse in the aqueous solution. After a decrease in temperature back to 4°C, the polymer solution appeared clear and homogeneous, but the aggregation of Nile Red remained in the PBS. The polyphosphoesters interact with hydrophobic Nile Red and help its dispersion. Furthermore, the precipitation of Nile Red was not observed even after the polymer solution was diluted 100 times with PBS. By using polyphosphoesters, we were able to improve the solubility of hydrophobic molecules in aqueous media and separate them with temperature increments.

Wang and co-workers also observed the thermoresponsivity of polyphosphoesters. They have synthesized well-defined block copolymers of poly(ethylene glycol) and polyphosphoester (Wang et al., 2009). Block copolymers can form core-shell type polymeric micelles in an aqueous medium with the effect of temperature caused by self-association of the polyphosphoester block. Although it is clear that polyphosphoester is the new candidate thermoresponsive polymer, its properties have only been partially evaluated. The effect of molecular weight on the cloud point of PIPPn (Scheme 1) has not been discussed. Figure 13 shows the dependence of the phase separation temperature of PIPP in phosphate buffered saline (PBS) on molecular weight.

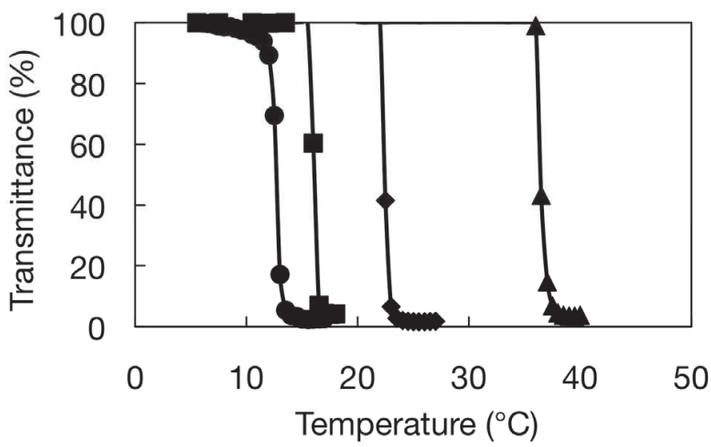
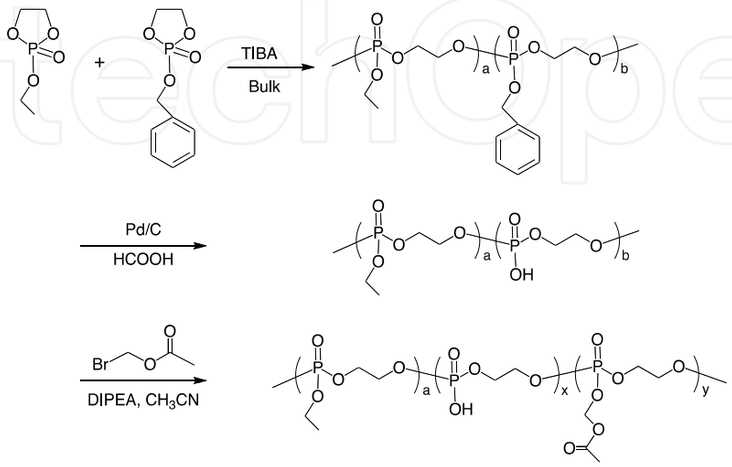


Fig. 13. Effect of molecular weight on cloud point of poly(2-isopropoxy-2-oxo-1,3,2-dioxaphospholane) (PIPP) (1 wt %) in PBS. (●) PIPP50(DBU), (■) PIPP48(TBD), (◆) PIPP32(DBU), (▲) PIPP13(DBU). (Reproduced from Iwasaki et al., (2010) *Macromolecules*, Vol. 40, No. 23, pp. 8136-8138, Copyright (2010), with permission from the American Chemical Society)

The cloud point of the polymer solution decreases with an increase in the molecular weight of PIPP. The result indicates that the type of organocatalyst does not influence the phase separation temperature. The phase separation temperature of polyphosphoesters is influenced by the chemical structure of the side chains, the concentration, and the ion strength of the aqueous media. In our previous report, PIPP that was synthesized using TIBA as an initiator was not soluble in water even when the molecular weight was less than 1.0×10^4 (Iwasaki & Akiyoshi, 2004). An uncontrolled reaction might occur when a metallic catalyst was used. Wang reported that long-term polymerization of cyclic phosphoesters with $Sn(Oct)_2$ makes some branch structures with high conversion rates (Xiao et al., 2006). In addition, some side reactions might occur in ring-opening polymerization of five-membered cyclic phosphoesters at high temperature (Liu et al., 2009). Furthermore, the molecular weight distribution of polyphosphoesters synthesized with an organocatalyst was significantly narrow compared with polymers that used metallic catalysts. The advantages of using organocatalysts can be observed on the synthesis of well-defined polymers with high conversion rates.

3.2 Polyphosphoester macroinitiators

Thermoresponsive polymers have great potential in bioscientific applications (Alarcon et al., 2005; Klouda et al., 2008). In particular, the selective delivery of drugs to target sites through hyperthermia could be performed (Chikoti et al., 2002). However, heat treatment might induce adverse effects on normal tissue and limitations remain in terms of selectivity. A polymer that can change its thermoresponsivity after contact with esterase has been synthesized. As shown in Scheme 5, polyphosphoesters bearing benzyl groups were synthesized. The synthetic results are listed in Table 5. The polymerization ability of BP and EP was similar. The ^{1}H NMR spectra of the polymers at each reaction step are summarized in Figure 14. After treatment with Pd/C in formic acid, a signal caused by the aromatic group at around 7.2 ppm disappeared. Deprotection of benzyl groups from PEB was completely accomplished and PEH was obtained. Then, PEH reacted with acetoxymethyl bromide in the presence of ethyldiisopropylamine. The ^{1}H NMR spectrum of PEHA clarified that the acetoxymethyl group was introduced at the deprotected position. No decrease in molecular weight was observed. No polymer degradation occurred during the introduction of the AM groups.



Scheme 5. Synthetic route of polyphosphoester bearing acetoxymethyl groups

The enzymatic digestion of acetoxymethyl esters from PEHA was evaluated in contact with porcine liver esterase for a specific time. Figure 15 shows the time dependence of the relative fraction of the acetoxymethyl groups on the EP units. The data are represented as the mean from 4 samples. When the enzyme was treated with PEHA, the decrease in the fraction of AM groups was dramatic compared to that soaked in PBS for 24 h. The fraction then gradually decreased over time. Esterase activity might influence this data. Geurtsen and co-worker reported that the activity of porcine liver esterase decreased during the first 24 h to approximately 40% and then remained constant for up to 6 days (Geurtsen et al., 1999). Even in synthetic polymer systems, the effect of esterase has been observed. The AM groups spontaneously degraded in PBS. The degradation rate at the early stage was much slower than that of the esterase treatment.

Polymer	Molar fraction		M_n x 10^{-3}	M_w/M_n
	In feed	In copolymer		
	0.90/0.10	0.92/0.08	6.31	1.43
		0.92/0.02/0.06	6.93	1.77

Table 5. Synthetic results of polyphosphoester bearing acetoxymethyl groups

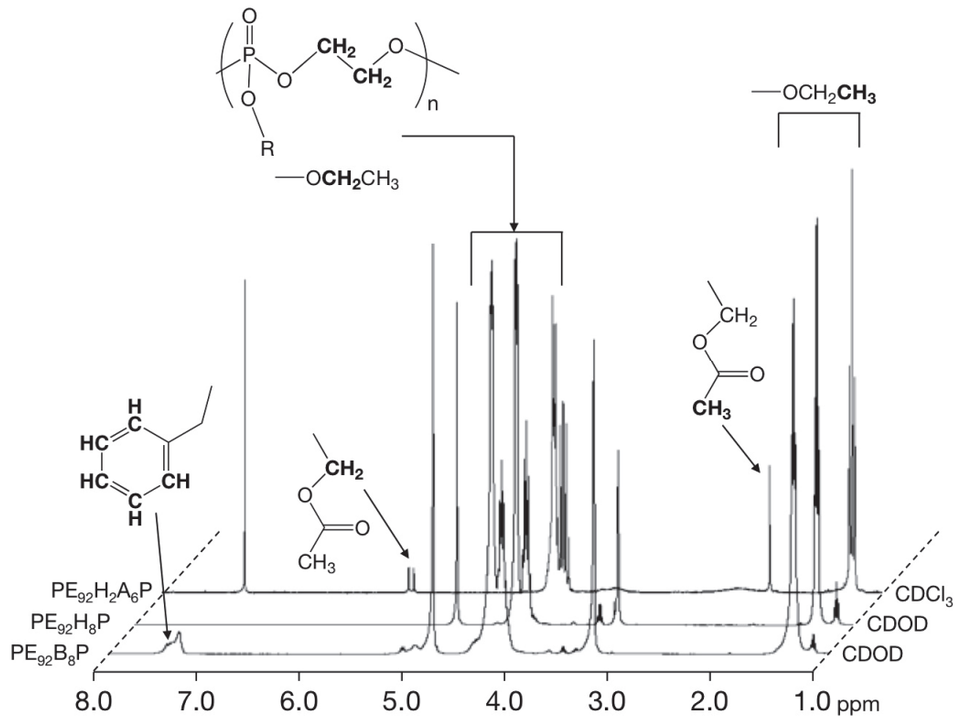


Fig. 14. ^{1}H NMR spectra of polyphosphoester bearing acetoxymethyl ester groups and the prepolymers.

Figure 16 shows the change in the number-averaged molecular weight (M_n) of PEHA incubated in PBS and that containing esterase. The decrease in molecular weight of PEHA was remarkable when the polymer was in contact with esterase. Digestion of the main chain was also accelerated with the esterase treatment.

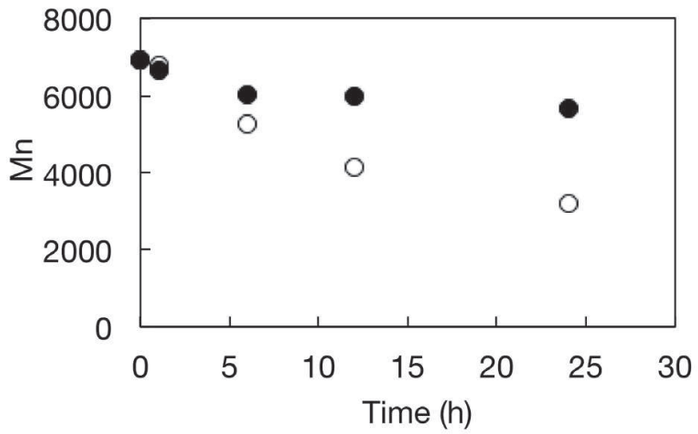


Fig. 15. Change in unit mole fraction of acetoxymethyl ester group of PEHA in contact with porcine liver esterase. (●) in PBS, (○) in esterase solution [Esterase] = 40 U/mL.

The thermoresponsivity of PEHA before and after contact with protease is shown in Figure 17. The PEHA/PBS showed LCST-type liquid-liquid phase separation and the cloud point was 40°C. In both PBS and that with esterase, the temperature of the phase separation increased with an increase in incubation time. In particular, the PEHA treated with esterase for 24 h did not have a cloud point between 20 and 65°C. The degree of AM groups on the polymer influenced its thermoresponsivity. That is, the phase separation phenomena could be controlled by acetoxymethylation of the polyphosphoesters. In addition, PEH, the polymer before acetoxymethylation, did not show any LCST-type liquid-liquid phase separation (data not shown). The influence of the change in molecular weight of PEHA with esterase treatment should also be of concern. While the cloud point of PEHA synthesized in this study was not in physiological conditions (>40°C), it could be adjusted by introducing more hydrophobic units into the polymer as described in previous literature (Iwasaki et al., 2004). Because the block copolymers composed of polyphosphoesters and poly(ethylene glycol) form a micelle structure above phase separation temperature (Wang et al., 2009), PEHA will work as building blocks for making enzyme-responsive micelles.

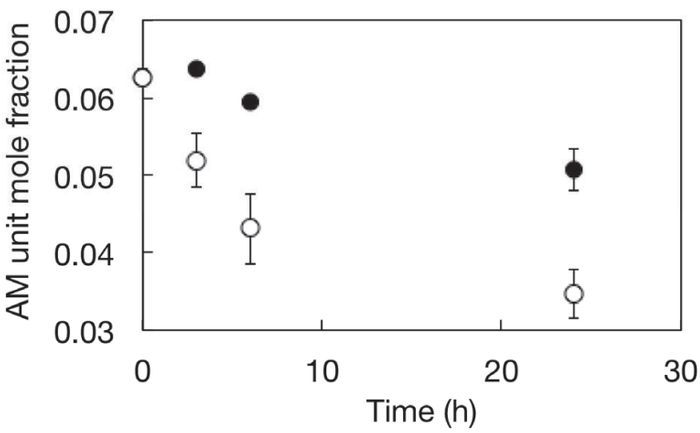


Fig. 16. Change in number-averaged molecular weight (Mn) of PEHA in contact with porcine liver esterase. (●) in PBS, (○) in esterase solution [Esterase] = 40 U/mL.

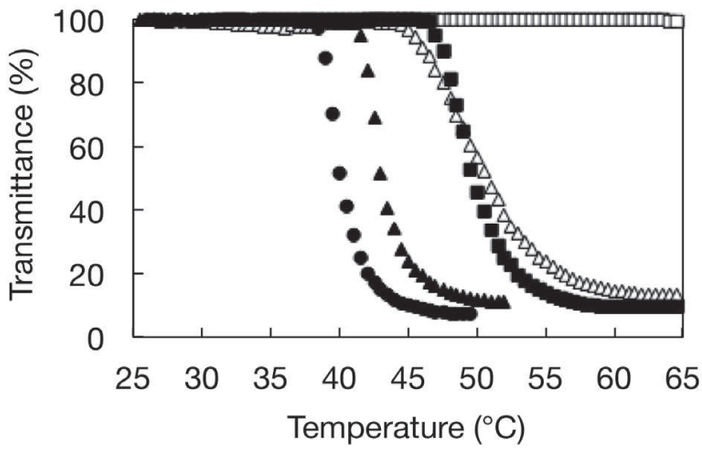


Fig. 17. Thermoresponsivity of PEHA in PBS before and after incubation with porcine liver esterase for 6 and 24 h. (●) 0, (▲) 6, and (■) 24 h in PBS; (△) 6 h and (□) 24 h in esterase solution.

The AM group is widely used for prodrugs and for fluorescence probes for cell imaging (Hecher et al., 2008; Takakusa et al., 2003). This group effectively induces cell membrane penetration and is rapidly cleaved intracellularly (Shultz et al., 1993; Yogo et al., 2004). Figure 18 is a fluorescence micrograph of HeLa cells in contact with Nile Red for 60 min with or without PEHA. The localization of Nile Red into the cells was improved by the presence of PEHA. At this concentration of PHEA, the polymer does not have a cloud point around 37°C. The solubilization capacity for hydrophobic molecules and the amphiphilic nature of the polymer might be improved by the cytoplasmic penetration of Nile Red. Although the mechanism of delivery of Nile Red into cells has not been fully clarified, the polyphosphoester bearing AM groups has the potential to induce penetration of hydrophobic drugs through the cell membrane.

To understand the interaction of PEHA and the cell membrane, we investigated the cytotoxicity of PEHA using Chinese hamster fibroblasts (V79), as described in a previous report (Iwasaki et al., 2004). There was no adverse effect of PEHA on cell viability when the PEHA concentration was below 0.01 g/dL (see supporting data). On the other hand, the cytotoxicity of PEHA was observed when the concentration was more than 0.1 g/dL. From the nature of this cytotoxicity test, it can be assumed that a high concentration of PEHA might damage the cell membrane. That is, that PHEA has an affinity for cell membrane.

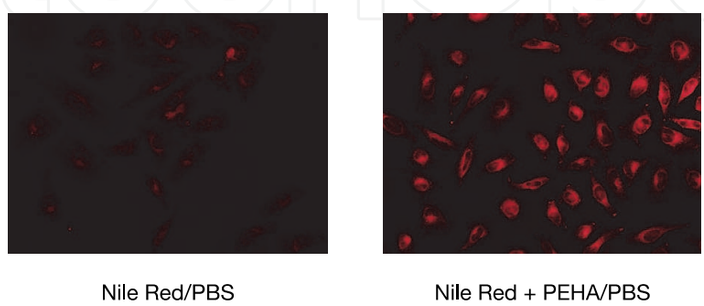


Fig. 18. Fluorescence micrographs of HeLa cells in contact with Nile Red in culture medium. a) Nile Red, b) Nile Red with PEHA. [PEHA] = 0.0025 mg/mL, [Nile Red] = 0.0125 μg/mL.

4. Conclusion

This chapter described current studies of new methods of syntheses and the characteristics of polyphosphoesters. Polymerization with a narrow molecular weight distribution is important to obtain the reproducible properties of polymers. In addition, the functionalization of the end or side groups of the polymers results in producing various types of polymer materials. The robustness of polyphosphoesters as biomedical materials has been clarified during the past decade (Zhao et al., 1003; Wang et al., 2009). However, the molecular and material designs of polyphosphoesters for biomedical applications are still limited. Polyphosphoesters have been explored as biomimetic to nucleic and teichoic acids. The study of the biological activity of polyphosphoesters will prove to be interesting.

As one of the unique properties of polyphosphoesters, LCST-type liquid-liquid phase separation of polyphosphoesters in aqueous media was introduced with a difference in the structure of their side chains. The aqueous solution of the polymers bearing alkyl groups became turbid with increments in temperature. From microscopic observation, liquid-liquid phase separation was observed in the turbid solution. The cloud points of the polymer solutions were influenced by polymer concentration, copolymerization ratio, and NaCl concentration. In addition, the copolymer effectively improved the solubility of the hydrophobic molecules in an aqueous medium and enabled separation of the molecules from the solution with increments in temperature.

Furthermore, thermoresponsive polyphosphoesters bearing AM groups as side chains were demonstrated as enzyme-responsive polymers. The thermoresponsivity of polymers in aqueous solution depended on the concentration of AM units and their molecular weight. Cleavage of the AM units and degradation of the polymer chain were accelerated with esterase treatment. The solubility of hydrophobic molecules and localization of the molecules into living cells were also improved by the synthetic polymers. To use polyphosphoesters bearing AM groups as drug carriers, further molecular design to achieve self-assembly, stealth, and targeting characteristics will be needed. However, the newly designed structure is interesting as a basic motif for applications.

5. Acknowledgments

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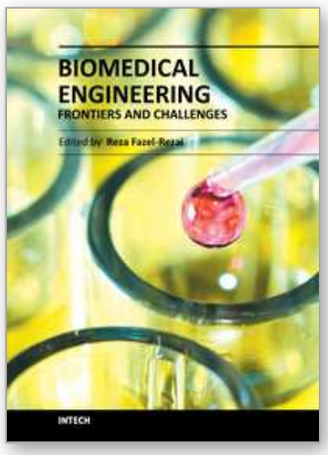

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In all different areas in biomedical engineering, the ultimate objectives in research and education are to improve the quality life, reduce the impact of disease on the everyday life of individuals, and provide an appropriate infrastructure to promote and enhance the interaction of biomedical engineering researchers. This book is prepared in two volumes to introduce recent advances in different areas of biomedical engineering such as biomaterials, cellular engineering, biomedical devices, nanotechnology, and biomechanics. It is hoped that both of the volumes will bring more awareness about the biomedical engineering field and help in completing or establishing new research areas in biomedical engineering.

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