THERMO-RESPONSIVE HYDROGELS BASED ON BRANCHED BLOCK COPOLYMERS

PROEFSCHRIFT

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Voorwoord

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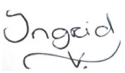

Contents

Chapter 1

General Introduction

Thermo-responsive hydrogels for biomedical applications

Hydrogels are hydrophilic polymer networks that are able to absorb large amounts of water or biological fluids[1]. Their properties do resemble those of natural living soft tissues and they show good biocompatibility due to their high water content, and are therefore interesting for applications such as drug delivery systems[1, 2] and tissue engineering[3, 4]. Hydrogels contain either chemical or physical crosslinks. Whereas in chemical hydrogels the network is covalently crosslinked, in physical hydrogels, molecular entanglements, or secondary forces, including ionic forces, hydrophobic forces and H-bonding, provide the crosslinks in the network[1, 5]. Because of the non-permanent character of these physical crosslinks, they may be broken by a change in environmental conditions, such as pH or temperature. Such a transition can cause the network to form a free flowing fluid, and in certain cases this transition is fully reversible[6, 7]. The transition of an aqueous polymer solution into a hydrogel by changing the temperature makes these materials very well applicable as 'in situ' forming injectable materials. By mixing an aqueous polymer solution with drugs or cells, which will gelate upon a change to body temperature, a method to introduce a local drug or cell depot into the body in a minimal invasive manner is provided[8-10]. When biodegradable polymers are used for the preparation of these hydrogels, an additional advantage is obtained. The hydrogels do not need to be explanted after their functional time, because they will be degraded in the body, and the degradation products will be excreted via natural pathways. A class of biodegradable copolymers that show thermo-responsive gelation behavior are copolymers based on poly(ethylene glycol) and aliphatic polyesters, such as poly(ε-caprolactone), poly(lactide), and poly(lactide-co-glycolide). These copolymers dissolved in water show a transition from a free flowing fluid, a sol, to a non-flowing gel upon a change in temperature, which, depending on their composition, can be close to body temperature.

Approach

The design of biodegradable thermo-responsive hydrogels known today is mainly based on amphiphilic block copolymers comprising aliphatic polyesters, as the hydrophobic block, and poly(ethylene glycol) as the hydrophilic block with a linear triblock or multiblock architecture[11-13]. The temperature at which the transition from a sol to a gel takes place depends on several parameters like the molecular weight and the composition of the copolymers, and the copolymer concentration in water. However, only a few studies provide information on the temperature dependent phase transition from sol to gel of polymers with non-linear architecture[14-16]. These polymers are mainly based on the combination of branched poly(ethylene glycol)s and linear aliphatic polyesters.

In this study, copolymers were prepared from branched polyesters and either linear or branched poly(ethylene glycol)s as the building blocks. The different functional groups present in the branched polyester provide a versatile method to prepare a variety of polymer architectures (Figure 1).

Aim of the study

The aim of this study was to prepare biodegradable hydrogels that have thermo-responsive gelation behavior, and have the potential to be used as injectable drug delivery systems. To meet these requirements, the sol to gel transition temperature should be close to body temperature and the hydrogels should be based on biodegradable polymers.

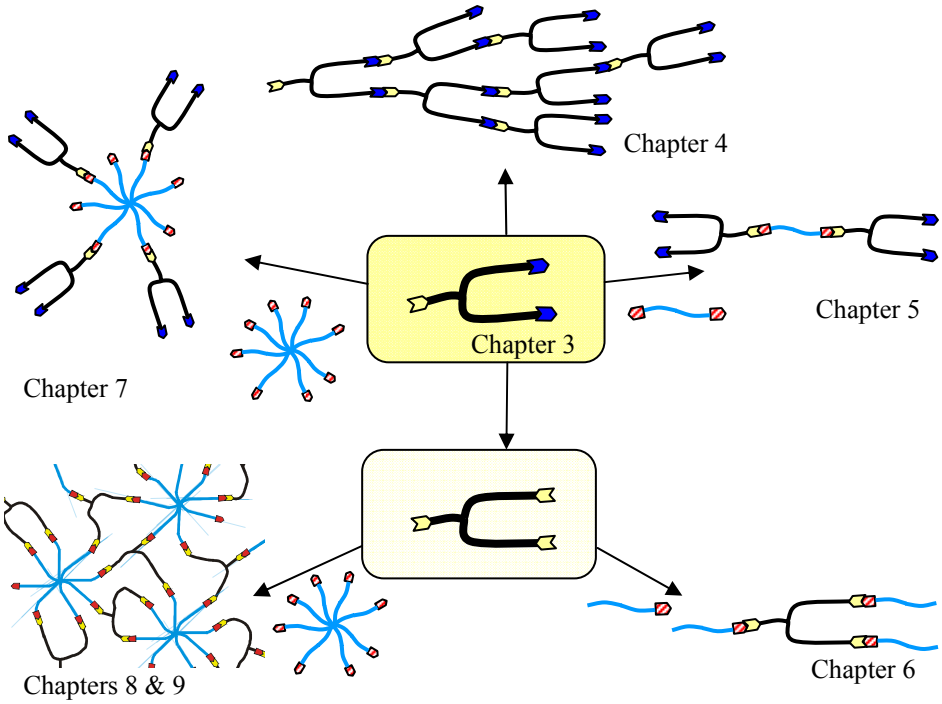

Figure 1. Schematic representation of copolymer architectures, starting from an AB_2 functional building block. (Black lines: polyester backbones, grey lines: poly(ethylene glycol) backbones, : (activated) carboxylic acid groups, : amine groups, : hydroxyl groups, : amide bonds, : ester bonds).

Outline of the thesis

In this thesis copolymers with branched polyester segments that were combined with either linear or branched poly(ethylene glycol) segments, and their thermo-responsive phase behavior are described. In **Chapter 2** a literature overview is given on currently known thermo-responsive hydrogels used in biomedical applications, with the emphasis on biodegradable hydrogels based on poly(ethylene glycol) and aliphatic polyesters. In **Chapter 3** the synthesis and characterization of branched polyesters are described. Branched AB_2 functional polyesters could be readily prepared by the stannous octoate catalyzed ring opening polymerization of L-lactide or ε-caprolactone using 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) as the initiator, with good control over molecular weight. The well-controlled polymerization reaction giving branched monomers with a carboxylic acid functional group and two hydroxyl functional groups provided a way to explore the

synthesis of hyperbranched poly(ε-caprolactone), which is described in **Chapter 4**. A facile method for the synthesis of hyperbranched poly(ε-caprolactone)s, consisting of ring opening polymerization of ε-caprolactone initiated from the AB_2 functional initiator bis-MPA, directly followed by polycondensation is presented in this chapter. In this 'one-pot' synthetic method hyperbranched polymers were prepared in which the number of branching points could be controlled by varying the polycondensation time. In **Chapter 5**, the synthesis and characterization of four-armed copolymers with a linear poly(ethylene glycol) (PEG) middle block, and branched poly(L-lactide) (PLLA) outer blocks are described. These copolymers had a low PEG content (≤ 44 wt%) and a relatively low molecular weight (≤ 6000 $g \cdot mol^{-1}$), analogously as PEG-PLLA triblock copolymers that show thermo-responsive gelation behavior in water. In **Chapter 6** branched PLLA-PEG copolymers having a higher PEG content (≥ 57 wt%) and a higher molecular weight (≥ 9200 $g \cdot mol^{-1}$) are described. These copolymers were prepared from branched PLLA with three N-hydroxysuccinimide (NHS) active ester groups as the core block and amine functionalized methoxy-PEG as the outer blocks. The thermo-responsive gelation behavior of these copolymers in water was studied using the vial tilting method and oscillatory rheology. In **Chapter 7** highly branched PEG-PLLA copolymers are described, prepared from star-shaped eight-armed amine functionalized PEG and branched PLLA. Their thermo-responsive gelation behavior in water was investigated. Furthermore, the degradation/dissolution of hydrogels placed in buffer (pH 7.4; 20 and 37 °C) was studied. A study on chemical hydrogels prepared from branched PLLA with three NHS active ester groups and linear or star-shaped PEG with amino end-groups is presented in **Chapter 8**. The degree of swelling of these hydrogels as a function of temperature was investigated. The degradation of the hydrogels by hydrolysis of ester bonds was evaluated by measuring the degree of swelling, and the mass loss of the dry network, in time. In **Chapter 9**, the release of lysozyme as a model protein, and of water-soluble and poorly water-soluble immuno-suppressant dexamethasone from chemically crosslinked networks of eight-arm star-shaped PEG amine and PLLA macromonomers with three NHS active ester groups, as described in chapter 8, is presented. Lysozyme and water-soluble dexamethasone were loaded by immersing the dry networks in PBS or water containing the active agent, whereas the poorly water-soluble dexamethasone was incorporated during network preparation.

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Chapter 2

Thermo-responsive hydrogels for biomedical applications

Introduction

Hydrogels have found numerous applications in tissue engineering and drug delivery, due to their good resemblance to natural living soft tissues, and their biocompatibility[1-6]. Hydrogels owe these properties due to the large amount of water or biological fluids that are imbibed in a polymeric network, without the network being dissolved in these fluids[7]. These networks are insoluble in water, due to the presence of chemical crosslinks, covalent bonds, or physical crosslinks, such as hydrogen bonds, hydrophobic interactions, or ionic interactions[8, 9]. Physical crosslinks however may be disrupted by a change in the environmental conditions, such as the temperature, pH, electric field, pressure, and the application of stress[1, 4, 10]. If the physical crosslinks are disrupted, a free flowing fluid, a sol, is formed. This sol to gel transition, which can be fully reversible, creates opportunities to use such materials as injectable systems[4, 6]. For example, temperature responsive hydrogels can undergo a sol-gel transition upon injection into the body. In this way, a local drug or cell depot can be transplanted into the body in a minimal invasive manner[11-14]. In the following sections, physically crosslinked hydrogels that respond to changes in temperature, and their gelation mechanisms will be discussed, with the emphasis on hydrogels based on biodegradable synthetic polymers.

Thermo-responsive gelation

Hydrogen bonding, hydrophobic interactions, and physical entanglements are the main features that form the junction zones in thermo-responsive physically crosslinked hydrogels. Hydrogen bonding occurs primarily at low temperatures and is disrupted by heating. Hydrogen bonding is the dominant cause for systems that gel upon cooling, and become soluble upon heating, such as gels based on the natural polymer gelatin. Hydrogen bonding provides the stable helical structures in natural occurring proteins and polysaccharides (Figure 1)[8, 12].

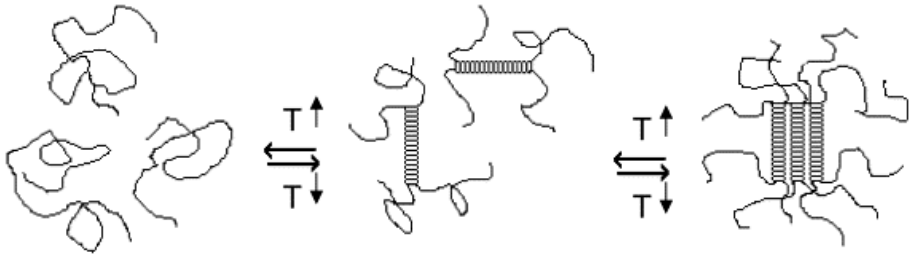


Figure 1. Gelation mechanism of polysaccharides in water. Random coils become helices, which subsequently aggregate to form the physical crosslinks in a gel. With permission from ref [12].

The gelation mechanism of hydrogels that gel upon heating, and become soluble upon cooling, is mainly a result of the enhanced hydrophobic interactions at elevated temperatures. Subsequently, the polymers self-assemble and form physical crosslinks.

The physical entanglements are the main reason for the transition from sol to gel upon lowering the temperature of some hydrogels based on synthetic polymers. For example, aqueous solutions of poly(ethylene glycol)-poly(L-lactide)-poly(ethylene glycol) (PEG-PLLA-PEG) copolymers showed this transition[15]. PEG-PLLA-PEG copolymers in water form micelles with a PLLA core, and a PEG corona. At elevated temperatures, PEG is in a shrunken state, because at higher temperatures PEG is dehydrated. At those high temperatures, it does not form entanglements with PEG chains of other micelles, resulting in a free flowing sol. At low temperatures, PEG becomes hydrated and swells. This allows physical entanglements to be formed between different micelles, and a sol to gel transition to occur.

Hydrogel materials

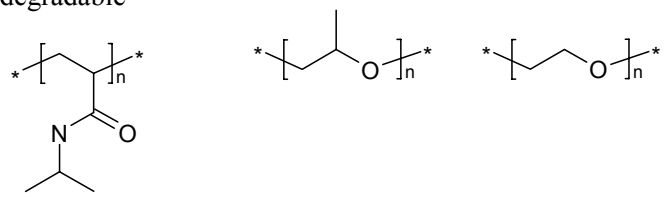
Both natural and synthetic polymers have been used for the preparation of temperature responsive hydrogels. Natural polymers include proteins, such as collagen and gelatin (produced by partial hydrolysis of collagen), and many polysaccharides, such as agarose, chitosan, and cellulose derivatives[8, 12, 16].

Thermo-responsive hydrogels prepared from synthetic polymers have at least one temperature sensitive component. The structures of some synthetic polymers or

copolymeric components that are used for the preparation of thermo-sensitive hydrogels are shown in Figure 2. Well-known synthetic polymers that show thermo-responsive gelation in water are PNIPAAM and its copolymers. PNIPAAM shows a lower critical solution temperature (LCST) at approximately 32 °C that can be adjusted to body temperature by the incorporation of comonomers. In general, the more hydrophobic the comonomer, the lower the LCST of the resulting copolymer[12, 17, 18]. However, the use of PNIPAAM has serious limitations because it is difficult to obtain FDA approval. Although several authors have reported good biocompatibility of PNIPAAM hydrogels[19, 20], PNIPAAM polymers themselves showed some cytotoxicity[21].

Another widely investigated class of temperature sensitive copolymers are copolymers consisting of poly(ethylene oxide) (PEO) and poly(propylene oxide) (PPO). These copolymers are known under the commercial names Pluronics (BASF) and Poloxamer (ICI). PEO-PPO-PEO copolymers show both a lower sol-gel transition and an upper gel-sol transition when heating an aqueous solution[22-26]. The phase diagram of these copolymers is rather complex and studies showed transitions from spherical micelles to lamellar and hexagonal packings and cubic liquid crystalline phases. For example, the temperature induced lower sol-gel transition in Pluronic F127 (PEO_{99}-PPO_{65}-PEO_{99}) from micellar solution to a (disordered) cubic liquid crystalline phase is extremely abrupt, and results in a dramatic thickening on increasing the temperature from room temperature to body temperature. The upper gel-sol transition is a result of the change from cubical to hexagonal packing of micelles leading to decreased intermicellar interactions[24]. A possible drawback of Pluronics is their non-biodegradability. Since these copolymers cannot be degraded, they have to be excreted trough natural pathways, which limit their molecular weight. Generally, only weak gels can be obtained from these relatively low molecular weight copolymers.

Synthetic non-degradable



Synthetic degradable

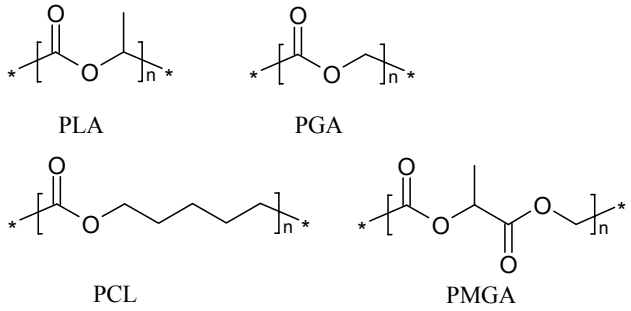


Figure 2. Molecular formulas of hydrogel forming polymers or copolymer building blocks.

An approach in the design of biodegradable hydrogels is the preparation of block copolymers that contain a biodegradable segment. Aliphatic polyesters, such as poly(lactide)s (PLA), poly(lactide glycolide)s (PLGA) and poly(ε-caprolactone)s (PCL) have found most interest as the hydrophobic segment, because these polyesters are both biocompatible and biodegradable, and can be easily synthesized via the ring opening polymerization of lactide, glycolide or ε-caprolactone, with good control over molecular weight. This type of biodegradable block copolymers will be reviewed in more detail in the following sections.

Thermo-responsive hydrogels based on biodegradable copolymers

Sol-gel-sol versus gel-sol transition behavior

Thermo-responsive hydrogels based on biodegradable copolymers with PEG as the hydrophilic block can be divided into two classes: (1) materials that give a sol at low temperatures and form a gel with increasing temperatures. Further increase leads to a sol phase again (Figure 3A); and (2) materials that give a gel at low temperatures and form a sol with increasing temperatures (Figure 3B).

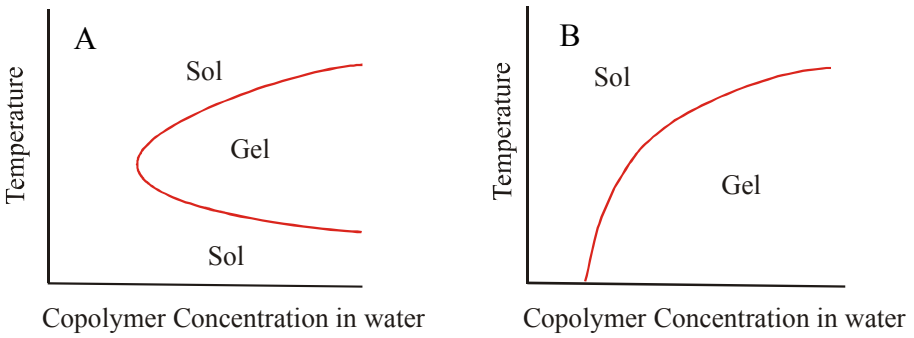


Figure 3. Schematic representation of the transition diagrams of copolymers in water that show (A) sol-gel-sol transitions (class 1), and (B) gel-sol transitions (class 2).

Whether a copolymer belongs to class 1 or 2 depends on the total molecular weight of the copolymer and its hydrophobic/hydrophilic balance. The hydrophilic content of copolymers belonging to class 1 is close to 33 wt%, and the molecular weight is approximately 5000 $g \cdot mol^{-1}$. For class 2 copolymers, the molecular weights are generally higher (> 10000 $g \cdot mol^{-1}$) and the hydrophilic content is often larger than 50 wt%. Aqueous copolymer solutions above a certain concentration form micelles at low temperatures, with a hydrophobic core and hydrophilic shell. In the case of the class 1 type materials, the block length of the hydrophilic blocks of the copolymer is below the critical entanglement length[27] and the micelles are free flowing, and form a sol state (Figure 4A). Upon increase in temperature, the hydrophobic interactions become stronger and the micelles start to aggregate.

Class 2 materials have hydrophilic block lengths above the critical entanglement molecular weight, and the micelles are connected to each other via these entanglements, resulting in a gel phase already at low temperatures (Figure 4B). In both class 1 and class 2 cases, the upper transition from gel to sol is considered to be caused by dehydration of the PEG, which causes the micelles to shrink, resulting in decreased interactions between the different micelles that are consequently able to form a sol phase. If the dehydration is more severe, the formation of the sol phase is accompanied by precipitation of the copolymer out of the water, forming water-rich and copolymer-rich phases.

Hydrogels of class 1 and 2 are discussed in the following two sections, with emphasis on the relation of their thermo-responsive behavior to their structure and architecture. After that, potential applications of these hydrogels as drug delivery systems are discussed.

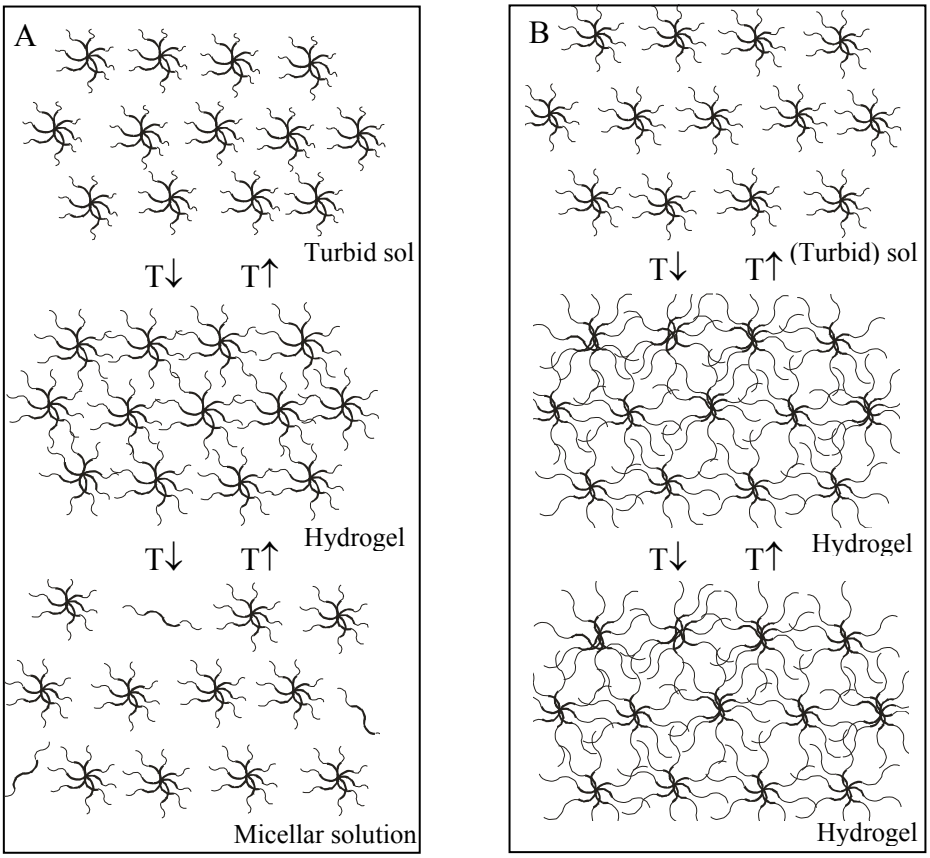


Figure 4. Schematic representations of copolymeric micelles in water. (A) Class 1 type copolymers that form free flowing micelles at low temperature, and show a sol-gel and gel-sol transition upon increase in temperature. (B) Class 2 type copolymers that form a gel at low temperature and show a gel-sol transition upon increase in temperature.

Hydrogels with sol-gel-sol phase transition behavior

Hydrogels prepared from biodegradable copolymers and showing a phase behavior with sol-gel-sol transitions were reported since 1999[28-30]. ABA type copolymers with PLGA as the outer block (A) and PEG as the middle block (B) were prepared via the ring opening polymerization of lactide and glycolide using a α,ω-dihydroxy

PEG as the initator. The inverse BAB triblock copolymers were prepared by the preparation of diblock PEG-PLGA copolymers by ring opening polymerization of lactide and glycolide, using methoxy-hydroxy PEG as the initiator. Subsequently, these diblock copolymers were coupled using hexamethylenediisocyanate as a spacer. The resulting triblock copolymers were water soluble at low temperatures, and transformed into a gel state at elevated temperatures. Further increase of the temperature led to phase separation, and this sol-gel-sol phase behavior is comparable to that of Pluronics. However, the proposed gelation mechanism differed from Pluronics since PLGA is more hydrophobic than PPO, and micelles are formed more readily. For these PEG-PLGA-PEG triblock copolymers the gelation mechanism is suggested to be a result of the increase in the size of the micelles, due to increased polymer-polymer attractions upon an increase in temperature. They can move relatively freely at low temperatures, and the sol-gel transition occurs with an increase in temperature when the total volume fraction of micelles is larger than the maximum packing fraction (Figure 5A).

For the ABA type PLGA-PEG-PLGA copolymer hydrogels an additional mechanism is proposed. Micelles are formed from a core of PLGA loops and a PEG shell (Figure 5B). Some copolymers form bridges between different micelles and form micellar groups. With increasing temperature, the number of bridging micelle groups increases abruptly, leading to gelation[28, 31]. These bridges lead to a decrease in the critical gelation concentration (CGC), the concentration necessary to form a gel. For example, aqueous solutions of PLGA-PEG-PLGA of a molecular weight of 3800 $g \cdot mol^{-1}$ and a PEG content of 34 wt% have a CGC of approximately 5 wt%, whereas aqueous solutions of a comparable PEG-PLGA-PEG copolymer have a CGC of approximately 25 wt%.

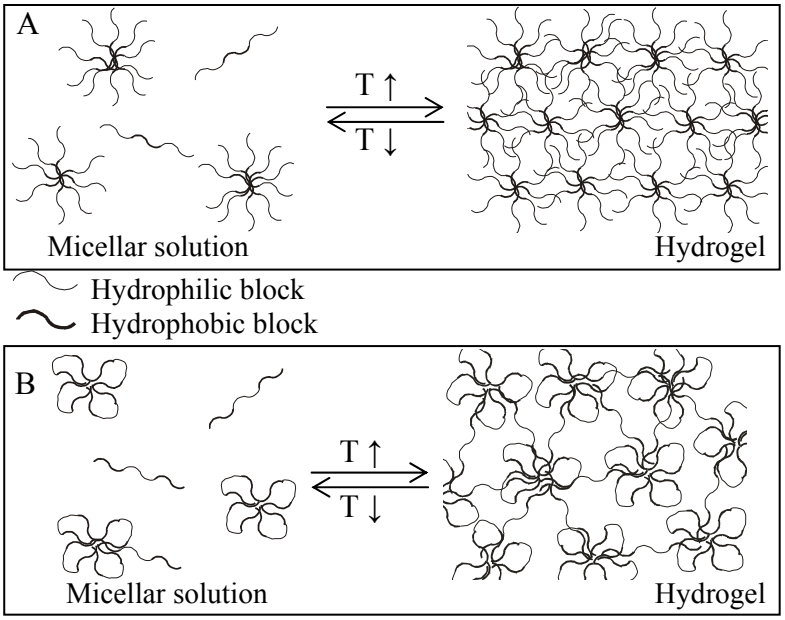


Figure 5. Schematic representation of self-assembly of amphiphilic (A) PEG-PLGA-PEG, and (B) PLGA-PEG-PLGA triblock copolymers in water upon a temperature change.

A similar tendency was observed for ABA versus BAB triblock copolymers in water, with PCL as the A-block [32, 33]. A 15 wt% aqueous solution of PCL-PEG-PCL in water showed a transition from sol to gel at 25 °C, whereas a PEG-PCL-PEG copolymer solution at the same concentration showed a transition at 33 °C (Figure 6). The upper gel-sol transition temperature was 5 °C higher for this PCL-PEG-PCL hydrogel than for the PEG-PCL-PEG hydrogel.

The effect of hydrophobicity of the PLGA block on the gelation behavior was investigated by changing the lactide to glycolide ratio for both PEG-PLGA-PEG and PLGA-PEG-PLGA copolymers [28, 30, 34]. An increase in the lactide to glycolide ratio lowered the sol-gel transition of the aqueous solutions due to larger hydrophobic interactions.

Next to changing the lactide to glycolide ratio in PLGA, the hydrophobicity was altered by using other aliphatic polyester segments in the copolymers, such as

PLA[31], PCL[32, 33, 35], and poly(D,L-3-methylglycolide) (PMGA)[36]. For example, the thermo-gelation behavior of PMGA-PEG-PMGA was investigated by Zhong et al.[36], using the vial tilting method and rheology. PMGA-PEG-PMGA copolymers have a uniform molecular structure of alternating lactyl and glycolyl units, instead of a blocky microstructure generally formed in PLGA-PEG-PLGA, due to the difference in ring opening reactivity of lactide and glycolide. The sol-gel-sol transition was observed in a smaller concentration window, because the PMGA-PEG-PMGA was less hydrophobic than the PLGA-PEG-PLGA, due to a smaller ratio of lactyl to glycolyl units. On the other hand, introducing a more hydrophobic polyester segment, such as PLA or PCL, results in a wider gel window. For example, a PEG-PCL-PEG triblock copolymer[32, 33] showed a CGC 10 wt% lower than the CGC of a PEG-PLGA-PEG triblock copolymer[28] of comparable molecular weight and PEG content (Figure 6).

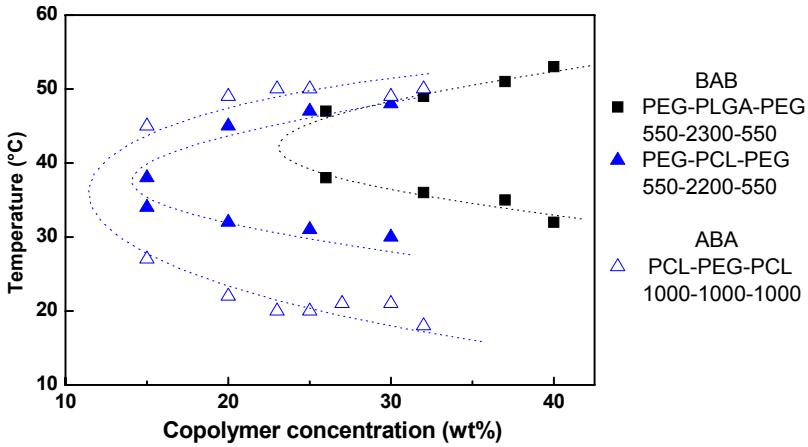


Figure 6. ABA type (open symbols) and BAB type (closed symbols) copolymers with PLGA (squares) or PCL (triangles) as the A blocks and PEG as the B blocks. Adapted with permission from ref. [28, 33].

Also copolymers with different architectures were investigated, and compared with the triblock copolymers[28-34, 36, 37] as discussed above. The various architectures include multiblock[38-41], grafted[42-45], and star-shaped copolymers[46] (Figure 7). Multiblock copolymers of alternating PEG and PLLA blocks were prepared by a coupling reaction of α,ω-dihydroxy PEG with α,ω-dicarboxy PLLA using DCC and DMAP as coupling agents[38, 39]. The total molecular weight could be controlled by using an excess of PEG. Multiblock copolymers of alternating PEG and PDLLA

were prepared via a similar route[39]. The molecular weight of the PEG block was 600 g·mol^{-1}, and the molecular weight of the PLLA block was varied between 1100 and 1500 g·mol^{-1}. The total molecular weight of the copolymers was in between 4400 and 6700 g·mol^{-1}[38]. The influence of the molecular weight of the PLLA, as well as of the total molecular weight, on the sol-gel transition temperature was investigated. Free flowing sols were obtained when the copolymers were dissolved at low temperatures. A sol-gel transition occurred upon heating the aqueous solutions. The sol-gel transition temperature was hardly influenced by the total molecular weight of the copolymer. A decrease in CGC was observed when the molecular weight of the PLLA blocks was increased from 1100 to 1300 g·mol^{-1}. Furthermore, the sol-gel transition temperature decreased by 5-7 °C as the PLLA molecular weight increased.

The effect of chain packing was investigated by comparing PLLA/PEG and PDLLA/PEG multiblock copolymers with the same molecular weight and prepared from polymer block with similar lengths[39]. The PLLA/PEG multiblock copolymers showed a larger gel window than the PDLLA/PEG copolymers. However, both the PLLA and PDLLA blocks were in the amorphous phase, and the difference in sol-gel transition properties was suggested to come from a higher aggregation tendency of the isotactic polymer.

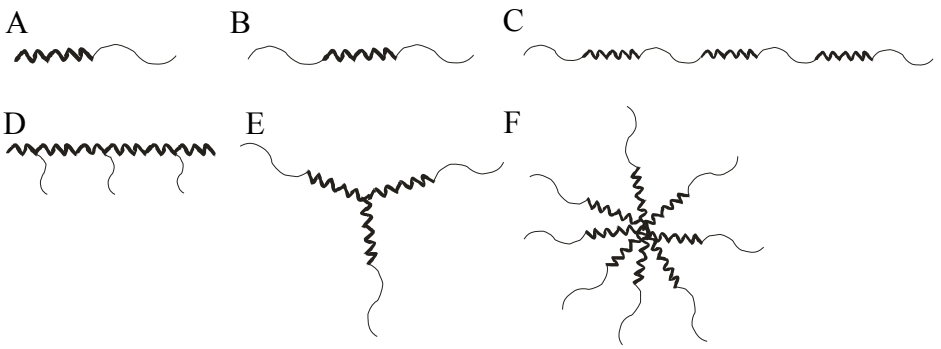


Figure 7. Various block copolymer architectures: (A) linear diblock copolymer; (B) linear triblock copolymer; (C) linear multiblock copolymer; (D) grafted copolymer; (E) three-arm star-shaped copolymer; (F) eight-arm star-shaped copolymer.

Grafted copolymers of PLGA and PEG were synthesized to overcome the molecular weight constraints of the triblock copolymers[42-45], and also for these systems a sol-gel-sol gelation behavior in water was observed. Interestingly, the mechanism for gel formation is different for both systems, based on the results of ^{13}C-NMR in D_2O and $CDCl_3$. It was suggested that PEG-g-PLGA had a micellar conformation in the sol state at low temperatures. With increasing temperature, the hydrophobic interactions increased and the association of the polymers decreased the PEG molecular motion, resulting in a long-range network, and thus a gel. On the other hand, the PLGA-g-PEG copolymers showed a micellar structure in the sol at low temperatures, as well as in the gel phase. The sol-gel transition is suggested to be a result of partial dehydration of PEG, causing micellar aggregation, as was confirmed with SANS and Raman spectroscopy[45].

Three-arm and four-arm star-shaped PLGA-PEG block copolymers with PLGA as the core moiety were prepared via the coupling reaction of star-shaped hydroxyl functional PLGA and α-carboxy-ω-methoxy PEG using DCC and DMAP[46]. The sol-gel transition behavior of these copolymers was investigated, and compared with that of linear PEG-PLGA-PEG. These star-shaped block copolymers showed a critical gelation concentration that was higher than that of the PEG-PLGA-PEG copolymers.

Hydrogels with gel-sol phase transition behavior

Thermo-responsive hydrogels based on PEG-polyester diblock copolymers, and triblock copolymers with PLLA as the central block were investigated by Kim and coworkers[15, 47, 48]. A single gel to sol transition was observed upon an increase in the temperature. The gel-sol transition could be adjusted by changing the copolymer concentration of the solution, and the composition of the block copolymer. In general, aqueous solutions of diblock copolymers with the same PEG content, and prepared from PEG with the same molecular weight, showed higher CGCs than aqueous solutions of the corresponding triblock BAB type PEG-PLLA-PEG copolymers. For PEG-PLLA-PEG triblock copolymer aqueous solutions, the CGC decreased from 20 to 10 wt% if the molecular weight of the PLLA block increased from 2000 to 5000 g·mol^{-1}, due to larger hydrophobic interactions. By varying the copolymer concentration from 10 to 30 wt%, the gel-sol transition temperature could be tuned from 2 to 80 °C.

The inverse ABA PLLA-PEG-PLLA triblock copolymers were conveniently prepared via the ring opening polymerization of L-lactide, using the α,ω-hydroxy PEG as the initiator[49-52]. Hiemstra et al.[52] investigated the thermo-responsive behavior of these PLLA-PEG-PLLA copolymers in water, by using the vial tilting method and oscillatory rheology. These hydrogels showed thermo-responsive gelation, and the gel-sol transition temperature increased with the copolymer concentration. A triblock PLLA-PEG-PLLA copolymer with 7.5 repeating lactide units at each side of the PEG (Mn_{total} = 14700 g·mol^{-1}), and a PEG content of 85 wt% showed a CGC of 15 wt% at room temperature. This is in the same concentration range as the inverse BAB copolymers. For example, a PEG-PLLA-PEG block copolymer with a comparable PEG content of 83 wt%, and a somewhat lower total molecular weight (Mn_{total} = 12300 g·mol^{-1}) showed a CGC of 17.5 wt%[15]. The gel-sol transition could be adjusted by changing the concentration of the solution and the composition of the block copolymer. In short, the gel-sol transition occurred at lower concentrations as the hydrophobicity of the polyester block increased, either by increasing the molecular weight or by changing the ratio of lactide to glycolide in the hydrophobic block[47, 48] or different polyesters, such as PCL and poly(δ-valerolactone) (PVL)[53].

Triblock copolymers with PCL or PVL as the hydrophobic outer blocks were also investigated[54]. A PCL-PEG-PCL copolymer at a copolymer concentration of 38 wt% in water showed a higher gel-sol transition temperature (42 °C) than a PVL-PEG-PVL copolymer at the same concentration (20 °C), which was attributed to a higher hydrophobicity of the PCL blocks, as compared to the PVL blocks.

Multiblock copolymers with alternating PEG and PLA or PCL segments were prepared by the condensation reaction of dicarboxylated polyesters with PEG diols[55, 56], by the coupling of PEG diols with PCL diols using hexamethylenediisocyanate as a spacer[57], or via the coupling reaction of triblock PLLA-PEG-PLLA copolymers using succinic anhydride or adipoyl chloride as difunctional spacer[58, 59]. The PEG/PDLLA multiblock copolymers prepared by Li[59] had a molecular weight of approximately 10000 g·mol^{-1} and showed a gel to sol transition upon an increase in temperature. The transition temperature increased with increasing molecular weight of the multiblock copolymer. PEG/PCL multiblock copolymers with relatively high PEG content (> 60 wt%) and molecular

weights between 15000 and 34000 g·mol^{-1} showed thermo-responsive gel-sol transitions. The gel-sol transition temperature increased with increasing molecular weight of the multiblock copolymer. Furthermore, the CGC decreased with increasing molecular weight. Phase separation between the PEG and PCL domains may induce gelation, instead of micellar gelation as is observed for the di- and triblock copolymers as described before.

Copolymers with star-shaped architectures were prepared[52, 60-63], and showed thermo-responsive gelation behavior in water, forming a gel at low temperatures that transformed into a sol at higher temperatures, comparable to the linear triblock copolymers of comparable molecular weight and PEG content. A three-arm star-shaped copolymer with PLLA as the core moiety and PEG as the outer blocks formed hydrogels at concentrations 5 wt% lower than a linear triblock PEG-PLLA-PEG copolymer with the same PEG content[60] (Figure 8). Furthermore, the gel-sol transition temperature increased to 70 °C for a 20 wt% hydrogel, and the critical gel concentration at room temperature decreased from 25 to 12 wt%, when the length of the PLLA blocks increased from 5 to 9 repeating lactide units.

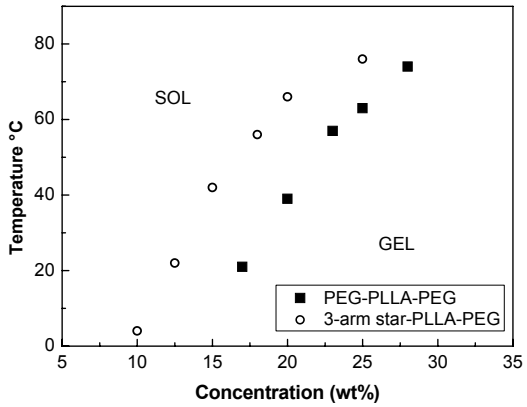


Figure 8. Gel-sol transition phase diagrams of (■) PEG_{5000}-$PLLA_{3000}$-PEG_{5000} (PEG content = 78 wt%)[15], and (○) 3-arm star shaped PLLA-PEG_{5000} (PEG content = 77 wt%) in water upon heating[60]. Adapted with permission from ref. [15, 60].

Three- and four-arm star-shaped PEG-PCL block copolymer solutions, with PEG as a core block, showed thermo-responsive gel-sol transitions[61, 63]. Unfortunately, a comparison between the gelation behavior of the three- and four-arm star-shaped

block copolymers was not made. Eight-arm star-shaped PEG-PLLA block copolymers were prepared by ring opening polymerization of L-lactide using an eight-arm star PEG with a molecular weight of 21800 g·mol^{-1} as the initiator[52, 62]. The thermo-responsive gelation behavior of these copolymers was compared with PLLA-PEG-PLLA triblock copolymers, and it was observed that an eight-arm star-shaped PEG-PLLA copolymer with a PEG content of 74 wt% and 7.5 repeating lactide units per arm showed almost the same gel-sol transition temperature as a triblock PLLA-PEG-PLLA copolymer with the same PLLA block length, but a 84 wt% PEG content. Furthermore, eight-arm PEG-PLLA hydrogels showed a decrease in the critical gelation concentration at room temperature from 40 to 15 wt% when the length of the PLLA blocks increased from 5 to 7 repeating lactide units. However, when the number of repeating lactide units per PLLA block was higher than 7, the copolymer was not water-soluble anymore.

Thermo-responsive hydrogels as drug delivery systems

The advantages of injectable drug delivery systems include easy application compared to implants, and localized delivery for a site-specific action[6, 64, 65]. The use of thermo-responsive hydrogels allows preparing injectable delivery systems and incorporating of bioactive agents by simple mixing in the fluid phase. The release of these bioactive agents may be controlled by diffusion, swelling and degradation, or a combination of these factors[11, 66, 67].

Diffusion, swelling and degradation

The release of an active agent from a polymeric matrix consists of the movement of the drug through the bulk of the polymer, known as diffusion. The diffusion through a polymer carrier can be described by Fick's law[11, 66, 67]:

$$J = -D \cdot \frac{dC}{dx} \qquad \text{Equation 1}$$

This law expresses the molar flux of a solute (J) as a function of the concentration gradient (dC) over a distance (dx) between the solute-rich interior and the solute-deficient surroundings of the matrix. D is the diffusion coefficient of the solute in the polymer matrix.

Formulations consisting of hydrophilic matrices, and from which the drug release is controlled by the inward flux of water from the outside environment, and

consequent swelling of the matrix, are referred to as swelling-controlled release systems. An example is the release of dispersed water-soluble agent of a dehydrated hydrogel when put in an aqueous environment. Initially the diffusion is low, but increases significantly as the gel absorbs water. The agent release involves the uptake of water from the surrounding media, and simultaneously, the rate of diffusion of the active agent into the surroundings. A complication is that the diffusion coefficient is dependent on the water uptake as well, which makes it more difficult to predict the release rate.

Degradation of a hydrogel network leads to a change in properties, for example an increased water-uptake, porosity, and/or hydrophilicity. As a result of this change in parameters, the drug permeability continuously changes during the degradation process, and makes it rather difficult to predict the release from degradable networks.

Protein and drug release from thermo-responsive hydrogels

The release of proteins and drugs from thermo-responsive hydrogels in vitro and in vivo was mainly investigated for PLGA/PEG class 1 type copolymers. Both ABA and BAB triblock copolymers with PLGA as the A block and PEG as the B block, and having relatively low molecular weight (< 5000 $g \cdot mol^{-1}$) have been claimed by Macromed as thermosensitive drug carrier systems with gelation properties[68]. PEG-PLGA-PEG triblock copolymer sols were injected subcutaneously in rats and the gel depots were found to last for more than a month, with little or no tissue irritation at the injection site[37]. Subcutaneously injected PLGA-PEG-PLGA hydrogels became progressively smaller over a 2 week period, after which it became a mixture of a gel in a viscous liquid[69].

A 23 wt% PLGA-PEG-PLGA solution in PBS buffer has entered the market under the name ReGel® (Macromed). A formulation containing paclitaxel at a concentration of 6 $mg \cdot g^{-1}$ is called OncoGel[69], and is designed to release paclitaxel into the tumor at a sustained rate over 4-6 weeks in order to achieve a higher concentration of paclitaxel in the tumor compared to intravenously administered drug. Intratumoral injections in nude mice were followed by a continuous drug release over a period of 6 weeks[69]. ReGel® also exhibits sustained release kinetics for therapeutic proteins. The proteins investigated included insulin, porcine growth

hormone (pGH), granulocyte colony-stimulating factor (G-SCF) and recombinant hepatitis B surface antigen (rHBsAG), and were evaluated both in vivo and in vitro[69]. The in vitro release data for G-SCF, pGH and insulin showed sustained release over 1 to 3 weeks. The controlled release of insulin from Zucker diabetic fatty (ZDF) rats was investigated by determining the blood glucose levels in time[70], after injecting a ReGel® formulation containing zinc-complexed insulin subcutaneously (Figure 9). Baseline insuline levels were achieved in vivo over 1 week by a single injection. The blood glucose level could be lowered over a 2 week period by injecting ReGel® with glucagon-like peptide 1(GLP-1) incorporated[71]. Similar type of PLGA-PEG-PLGA copolymers were investigated for the in vitro release of 5-fluorouracil, indomethacin[34] and lysozyme[72], and in vivo in rabbits for the potential treatment of superficial corneal burns[73].

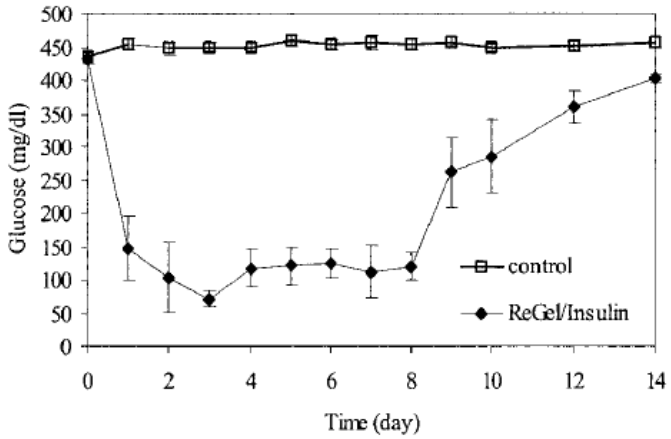


Figure 9. Blood glucose levels in ZDF rats. Time t=0 represents the time of the injection of the ReGel/insulin formulation. The control group consists of diabetic rats that were injected with ReGel without insulin at t=0. With permission from ref. [70].

The release of hydrophilic and hydrophobic model drugs, ketoprofen and spironolactone, respectively, from PEG-PLGA-PEG hydrogels was investigated in vitro[28, 74]. The release of ketoprofen was diffusion controlled over a period of 5 to 14 d, whereas the release of spironolactone was initially mainly diffusion controlled, followed by degradation controlled release at later stages, up to 55 d.

The stability of injectable hydrogels of grafted copolymers of PLGA and PEG was different whether the hydrogel was based on PLGA with PEG grafts or PEG with

PLGA grafts. Interestingly, subcutaneously injected gel depots of PLGA-g-PEG in rats persisted for a period of over 2 months (in vitro over a period of 3 months), whereas PEG-g-PLGA gel depots lasted for less than 1 week[42, 43, 75]. This difference is expected to be a result of the difference in gelation mechanism as discussed above. Sustained insulin delivery from a subcutaneously injected gel was investigated for diabetic type 2 rats[75]. Upon injection, the blood glucose level dropped in 1 h for both a 50/50 PEG-g-PLGA/PLGA-g-PEG hydrogel and a PLGA-g-PEG hydrogel. The duration of efficacy by one injection was 5 d and 16 d, respectively. One injection every 16 d compared to daily injection may improve patient compliance. The potential of this injectable hydrogel system for tissue engineering was proved by the appropriate filling of a cartilage defect in rabbits.

Conclusions

Thermo-responsive hydrogels as injectable drug delivery systems offer the advantage that they can be applied in a minimally invasive way, and locally can release therapeutic agents for a sustained period of time. In addition, the use of biodegradable polymers in the preparation of these hydrogels offers the advantage that they do not need to be explanted after their functional time, because they can be degraded in the body, and excreted via natural pathways. A major class of biodegradable copolymers that show thermo-responsive gelation behavior are copolymers based on poly(ethylene glycol) and aliphatic polyesters. These copolymers in water show a transition from a free flowing fluid, a sol, to a non-flowing gel upon a change in temperature. The total molecular weight, the composition as well as the architecture of these copolymers largely influence this sol to gel transition temperature. The achievements already reached and first products coming to the market show that thermo-responsive in situ gelating polymer systems are highly promising for a broad range of applications like drug delivery systems and tissue engineering. This offers opportunities for new designs of polymer systems that can be used for biomedical and pharmaceutical applications.

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Chapter 3

Synthesis and characterization of AB_2 functional polyesters prepared by ring opening polymerization

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Abstract

Aliphatic AB_2 functional polyesters, that can be used as macromonomers for the synthesis of hyperbranched polymers, were conveniently prepared by the ring opening polymerization of ε-caprolactone and L-lactide in the presence of the AB_2 functional initiator 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) and $Sn(Oct)_2$ as the catatyst. In L-lactide polymerization, both bis-MPA hydroxyl groups initiated the polymerization reaction, but for ε-caprolactone polymerization this depended on the monomer to initiator to catalyst ratio. At high monomer to initiator ([M]:[I]) ratios both bis-MPA hydroxyl groups initiated the ring opening polymerization, but at low [M]:[I] ratios, initiation by either one or two hydroxyl groups of bis-MPA occurred resulting in a mixture of polymers. Increasing the $Sn(Oct)_2$ to monomer ratio at low [M]:[I] ratios resulted in polymers in which both hydroxyl groups of the bis-MPA initiated the ring opening polymerization of ε-caprolactone. The melting temperatures of the AB_2-functional PLLA and PCL polymers were comparable to linear polymers with a DP equal to the DP per arm in the AB_2 polymer.

Introduction

Biodegradable aliphatic polyesters, such as polylactides and poly(ε-caprolactone)s have received much interest for their use in biomedical, pharmaceutical and environmental applications[1, 2]. For these specific applications, polymers with different properties are needed, and this has led to a still increasing interest in this field of research. These polymers are conveniently synthesized via ring opening polymerization of the corresponding (di)lactones, such as lactide, glycolide and ε-caprolactone. The development of new catalysts, especially coordination type catalysts, allowed controlling the polymerization and thereby a wide range of materials with different properties became available.

A method to adjust the polymer properties is to change the polymer architecture by using polyfunctional initiators to prepare star and graft (co)polymers. Such polymers are known to be less crystalline and have a high number of end-groups compared to their linear analogues[3-5]. The type and number of end-groups present, play an important role in the properties and degradation of biodegradable aliphatic polyesters[6-10]. As an example, carboxylic acid groups change the hydrophilicity of a polymer and can accelerate degradation by hydrolysis[6]. Moreover, functional end-groups allow further modification by coupling reactions, or can be used as initiators for ring opening polymerization of other lactones.

Initiators with different functional groups, so-called AB_x-functional initiators, have been applied for the ring opening polymerization of lactones and subsequent polycondensation to hyperbranched polymers[11-14]. Trollsås and Hedrick[11, 12] and Choi and Kwak[13] used the benzyl ester of 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) as a protected AB_2-initiator to prepare AB_2-functional polyesters. After deprotection of the carboxylic acid functional group, the AB_2 macromonomer containing one carboxylic acid and two hydroxyl functional groups was polycondensated to give a hyperbranched polymer.

In this paper, we describe an easy synthesis method for the preparation of AB_2 polyesters without the need of protection and deprotection. These AB_2 polyesters were prepared by ring opening polymerization starting from bis-MPA, and ε-caprolactone or L-lactide, using stannous octoate as the catalyst, and can be applied as starting materials for hyperbranched polymers. The macromonomers were analyzed for their structure, physical and thermal properties.

Experimental

Materials

2,2-Bis(hydroxymethyl)propionic acid (bis-MPA) was obtained from Acros (Geel, Belgium). Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) and ε-caprolactone were purchased from Aldrich (Zwijndrecht, the Netherlands). L-lactide was purchased from Purac (Gorinchem, the Netherlands). Diisopropyl ether and deuterated chloroform were obtained from Merck (Darmstadt, Germany). All other solvents were purchased from Biosolve (Valkenswaard, the Netherlands). Prior to use, ε-caprolactone was dried over calcium hydride (Aldrich) and distilled under vacuum. All other chemicals were used as received.

Synthesis

2,2-bis[ω-hydroxy poly(ε-caprolactone) methyl]propionic acid and 2,2-bis[ω-hydroxy poly(L-lactide) methyl]propionic acid were prepared with varying degrees of polymerization (DP), using bis-MPA as the initiator and $Sn(Oct)_2$ as the catalyst. The polymers are denoted as PCLn and PLLAn, where n is the average number of repeating ε-caprolactone or L-lactide units per arm. Polymerizations were performed using different monomer (ε-caprolactone or L-lactide) to initiator (bis-MPA) ratios as described below.

PCLn: Typical procedure for PCL24: ε-Caprolactone (40.0 g, 350 mmol) was added to a reaction vessel, which contained bis-MPA (1.17 g, 8.8 mmol) as the initiator and $Sn(Oct)_2$ (0.16 g, 0.40 mmol; 0.4 wt% based on ε-caprolactone) as the catalyst. The mixture was stirred and allowed to react for 7 h at 110 °C under an argon atmosphere. Subsequently, the product was cooled to room temperature and dissolved in dichloromethane. To this solution, a small amount of glacial acetic acid was added and the product was precipitated in an excess of cold diethyl ether. The product was collected by filtration and dried in vacuo to give a white powder. (Yield: 93 %)

PLLAn: Typical procedure for PLLA10: L-lactide (25.0 g, 174 mmol) was added to a reaction vessel, which contained bis-MPA (1.16 g, 8.7 mmol) as the initiator and $Sn(Oct)_2$ (0.10 g, 0.25 mmol; 0.4 wt% based on L-lactide) as the catalyst. The mixture was allowed to react for 3 h at 130 °C under an argon atmosphere. The product was subsequently cooled to room temperature and dissolved in dichloromethane. To this solution, a small amount of glacial acetic acid was added,

and the product was precipitated in an excess of cold diethyl ether. The product was collected by filtration and dried in vacuo to give a white powder. (Yield: 86 %)
In the synthesis of PLLA37 a reaction temperature of 140 °C was necessary to maintain a melt. The purification of PLLAn with n < 10, was performed by precipitation in diisopropyl ether instead of diethyl ether. PLLA7 was obtained as a white powder, whereas PLLA3, PLLA4 and PLLA5 were 'sticky' precipitates.

Characterization

NMR: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR spectra were recorded on a Varian Inova NMR spectrometer. Polymers were dissolved in $CDCl_3$ at a concentration of 0.015 g·ml^{-1} (^{1}H) or 0.2 g·ml^{-1} (^{13}C).
Viscometry: Intrinsic viscosities [η] were determined by single point measurements using a capillary Ubbelohde type 0C at 25 °C and a polymer solution with a concentration of 0.3 g·dl^{-1} in chloroform. The following empirical equation was applied:

$$[\eta] = \frac{\sqrt{2}}{c} \cdot \sqrt{\eta_{spec} - \ln \eta_{rel}} \qquad (1)$$

in which $\eta_{spec} = \eta_{rel} - 1$ and c is the polymer concentration in g·dl^{-1}. The relative viscosity ($\eta_{rel} = t/t_0$) was determined from the flow time of the polymer solution (t) and the flow time of the solvent (t_0).
The intrinsic viscosity was also determined by extrapolation of the inherent viscosities ($\eta_{inh} = \eta_{spec}/c$) and reduced viscosities ($\eta_{red} = (\ln \eta_{rel})/c$) to zero concentration of polymer solutions with different concentrations (0.1-0.7 g·dl^{-1}).
GPC: Molecular weights and molecular weight distributions of the polymers were determined with gel permeation chromatography (GPC) using chloroform as eluent. The GPC setup consisted of a Waters 510 pump, a HP Ti-Series 1050 auto sampler, a series of standard Waters Styragel HR columns, a Waters 410 differential refractometer, and a viscometer detector H502 (Viscotek Corp.). Polystyrene standards with narrow molecular weight distributions were used for calibration and the molecular weights were determined using the universal calibration principle.
MALDI-TOF: Matrix Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF) was performed using a Voyager-DE-RP 2010 MALDI-TOF mass spectrometer (Applied Biosystems/ PerSeptive Biosystems, Inc.) equipped with delayed extraction. A 337 nm UV nitrogen laser producing 2 ns pulses was used and the mass spectra of the polymers were obtained in the

reflection or linear mode. Samples were prepared by mixing ~2 mg polymer with 1 ml chloroform. After that, ~5 mg of 1,8,9-trihydroxyanthracene (dithranol) was added and the resulting solution was vigorously stirred. One µl of the solution was loaded on a gold sample plate. After evaporation of the solvent in air, the sample was transferred to the mass spectrometer for analysis.

DSC: Thermal analysis was carried out using a Perkin-Elmer Pyris 1 differential scanning calorimeter. PCLn samples (5-15 mg) were cooled to -100 °C and kept at this temperature for 1 min. The samples were then heated to 100 °C, annealed for 1 min, and cooled to -100 °C. Subsequently, the samples were kept isothermally for 5 minutes and heated again to 100 °C. The scanning rate of the heating and cooling scans was 20 °C·min^{-1}. PLLAn samples (5-15 mg) were treated similarly within the range -50°C to 200°C. Melting (T_m) and crystallization (T_c) temperatures were obtained from the peak maxima, melt (ΔH_m) and crystallization (ΔH_c) enthalpies were obtained from the area under the curve and the glass transition temperature (T_g) was taken at the inflection point. Data were taken from the second heating scan and the cooling scan.

Results and discussion

Poly(lactide) and poly(ε-caprolactone) AB_2 functional polymers were synthesized by ring opening polymerization in the melt using bis-MPA as the initiator and $Sn(Oct)_2$ as a catalyst (Figure 1). The number of repeating lactide or ε-caprolactone units per arm, n, was varied from 3 to 37 by varying the monomer to initiator ratio ([M]:[I]).

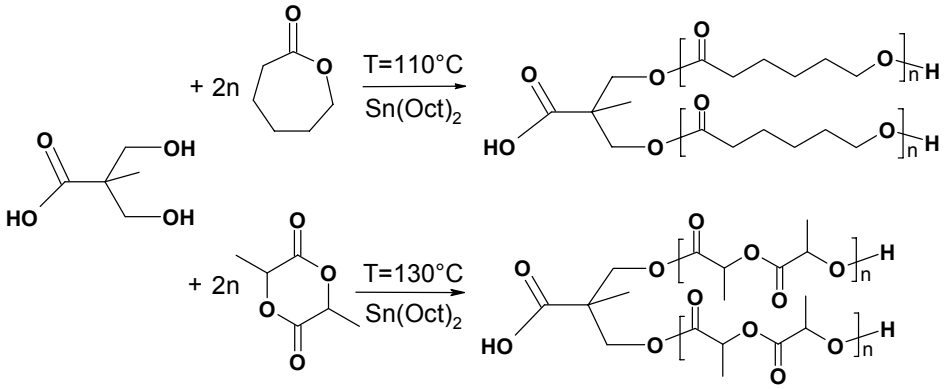

Figure 1. Bis-MPA initiated ring opening polymerization of L-lactide and ε-caprolactone.

Ring opening polymerization of L-lactide successfully resulted in AB_2 functional polymers. The integral ratio of the C*H*$_3$ protons of the monomer (1.59 ppm) to polymer (1.65 ppm) in the ^{1}H-NMR spectra of the crude samples was used to determine the conversion. In all cases, after 3 h reaction time, high monomer conversions of approximately 97 % were obtained. The ^{1}H-NMR spectra of the purified polymers (Figure 2) were used to calculate the degree of polymerization per arm (DP), and the number average molecular weight (Mn). The DP was determined from the ratio of the integrated areas of the C*H* protons of the lactide repeating units (c, 5.10 ppm) to the C*H*$_3$ protons of the bis-MPA moiety (a, 1.27 ppm). The results obtained (Table 1) are in good accordance with the theoretical values, based on the [M]:[I] ratio.

Table 1. Molecular weights, molecular weight distributions and intrinsic viscosities of bis-MPA initiated poly(L-lactide)s.

	[M]:[I]	Mn (g·mol^{-1})				PDI (-)		[η]^b
	(-)	Calc^a	NMR	MALDI	GPC	MALDI	GPC	(dl·g^{-1})
PLLA3	6	1000	1000	1200	1400	1.2	1.7	0.05
PLLA4	8	1290	1200	1200	2000	1.2	1.4	0.06
PLLA5	10	1580	1600	1700	2300	1.2	1.5	0.07
PLLA7	14	2150	2100	2300	2600	1.3	1.4	0.08
PLLA10	20	3020	2900	3500	4200	1.3	1.3	0.12
PLLA15	30	4460	4300	4300	5400	1.2	1.1	0.18
PLLA21	40	5900	6100	n.d.	6900	n.d.	1.3	0.20
PLLA25	50	7340	7500	n.d.	8200	n.d.	1.6	0.29
PLLA37	80	11660	10700	n.d.	11400	n.d.	1.5	0.39

[a] calculated from the [M]:[I] ratio; [b] chloroform, 25 °C; n.d. not determined

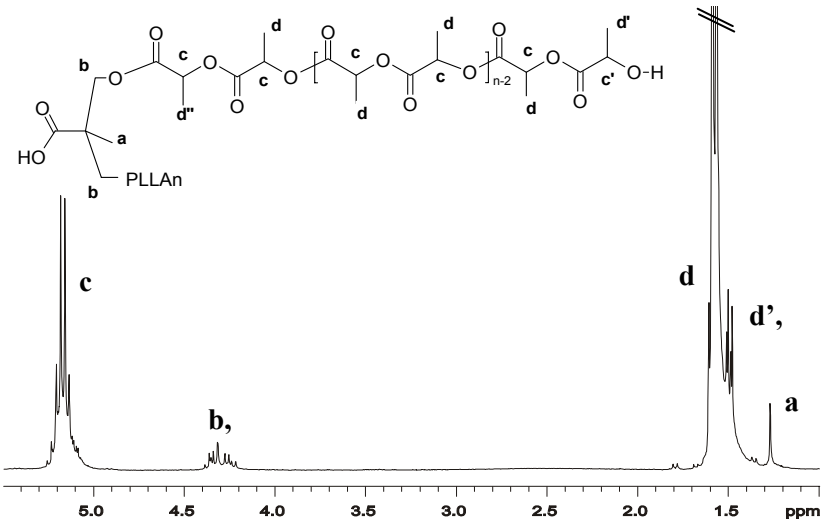

Figure 2. ^{1}H-NMR spectrum of PLLA10. Solvent: $CDCl_3$.

PCL polymers were successfully synthesized from ε-caprolactone by ring opening polymerization in the melt. Polymerization for 7 h gave complete monomer conversion as determined from the integral ratios of the C*H*$_2$ protons of the monomer (2.63 ppm) and the polymer (2.30 ppm) in the ^{1}H-NMR spectrum of the non-purified product. The ^{1}H-NMR spectra of the purified polymers were used to determine the DP (Figure 3), and was calculated from the ratio of the C*H*$_2$-OH protons of the terminal caprolactone unit (e', 3.63 ppm) to that of the methylene protons next to the carbonyl (a, 2.30 ppm). For all polymers, the DP calculated from the ^{1}H-NMR spectra were higher than the theoretical value based on the [M]:[I] ratio.

Table 2. Molecular weights, molecular weight distributions and intrinsic viscosities of bis-MPA initiated poly(ε-caprolactone)s.

	[M]:[I]	Mn ($g \cdot mol^{-1}$)				PDI (-)		$[\eta]^b$
	(-)	Calc[a]	NMR	MALDI	GPC	MALDI	GPC	($dl \cdot g^{-1}$)
PCL8	10	1280	2000	1500	1700	1.4	2.4	0.11
PCL13	20	2420	3100	2900	4000	1.2	1.3	0.12
PCL17	30	3560	4000	3700	4400	1.1	1.3	0.16
PCL24	40	4700	5600	n.d.	6100	n.d.	1.5	0.24
PCL25	42	4930	5900	n.d.	5300	n.d.	1.4	0.25
PCL28	50	5840	6400	n.d.	5800	n.d.	1.5	0.26

[a] calculated from the [M]:[I] ratio; [b] chloroform, 25 °C; n.d. not determined

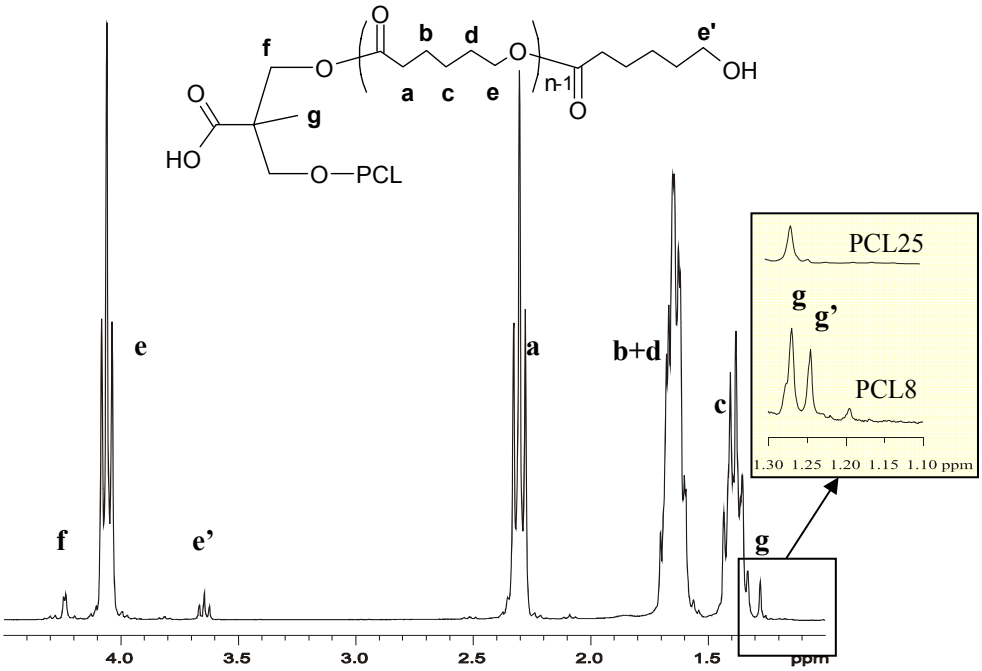

Figure 3. ^{1}H-NMR spectrum of PCL25. The insert shows the expanded 1.0-1.3 ppm region of PCL25 and PCL8, respectively. Solvent: $CDCl_3$.

Moreover, differences were observed in the structure of the polymers isolated, which could be related to the initiation reaction by the hydroxyl groups of bis-MPA. In the ^{1}H-NMR spectrum of PCL25 (Figure 3), the peak denoted as 'g' at 1.27 ppm corresponds to the methyl protons of the bis-MPA moiety. The insert in Figure 3

shows the 1.0-1.3 ppm region of PCL8 for comparison. Interestingly in this case, two peaks (g and g') are observed for the methyl protons of the bis-MPA unit at 1.27 and 1.24 ppm, respectively. The signal of the bis-MPA methyl protons shifts upfield to 1.24 ppm in case only one of the hydroxyl groups has initiated the ring opening polymerization of ε-caprolactone. The observed two signals at 1.27 ppm and 1.24 ppm in the spectrum of PCL8 reveals that a mixture of polymers with one (Figure 4A) or two (Figure 4B) polycaprolactone arms was obtained.

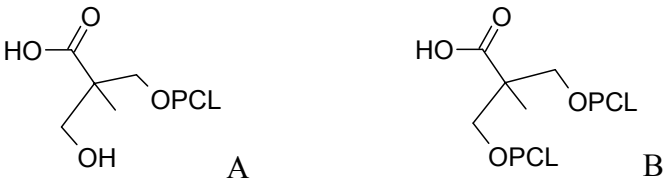

Figure 4. Schematic representation of a bis-MPA moiety that is (A) mono-substituted, and (B) di-substituted.

To study the observed differences in the initiation reaction, the progress of the polymerization of PCL13 and PCL25 was monitored in time by ^{1}H-NMR analysis. In Figure 5, the 1.0-1.5 ppm region of ^{1}H-NMR spectra of PCL13 at different time points is presented. After 30 minutes reaction time, the monomer conversion was 7 %, and two signals were observed at 1.27 ppm and 1.24 ppm, respectively. At longer reaction times the intensity of the signal at 1.27 ppm increased and simultaneously the intensity of the signal at 1.24 ppm decreased. These results show that with increasing reaction time, the amount of mono-substituted bis-MPA is decreasing, and the amount of di-substituted bis-MPA is simultaneously increasing. Up to the first two hours, an additional peak is observed at 1.10 ppm, which can be attributed to un-reacted bis-MPA. The disappearance of this peak at later time points, confirms that all bis-MPA has initiated the reaction giving polymers with either one or two arms.

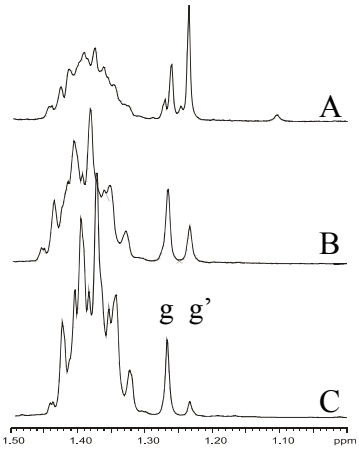

Figure 5. ^{1}H-NMR spectra of the 1.0-1.5 ppm region of PCL13 at different polymerization times: (A) after 2 h; (B) after 3 h; (C) after 7 h. Solvent: $CDCl_3$.

Monitoring the ring opening polymerization of ε-caprolactone to PCL25 at various stages of the reaction showed different results than described above for PCL13. From the start of the reaction, only one C*H*$_3$ proton signal appeared in the ^{1}H-NMR spectrum at 1.27 ppm. This indicates that both hydroxyl groups of the bis-MPA initiated the reaction and only two-arm PCL was formed (data not shown). From the results it is concluded that the amount of di-substituted bis-MPA units was dependent on the [M]:[I] ratio.

This prompted us to study the dependence of the catalyst to initiator ([C]:[I]) ratio of the bis-MPA initiated ring opening polymerization of ε-caprolactone. A reaction was performed at a [M]:[I] ratio as used in the preparation of PCL8, but at a [C]:[I] ratio ten times higher. The polymerization was now conducted at a [M]:[I]:[C] ratio of 10:1:0.1 and was monitored in time by taking samples from the reaction mixture and subsequent analysis by ^{1}H-NMR. In Figure 6, the 1.0-1.5 ppm region of the ^{1}H-NMR spectra at different time points is depicted. After 30 min, two peaks were visible in the ^{1}H-NMR spectrum that belong to mono- and di-substituted bis-MPA (1.24 and 1.27 ppm, respectively). Opposite to the results with a lower amount of catalyst (Figure 5A), mainly two hydroxyl groups of the bis-MPA moiety have initiated the reaction, because peak g is much larger than peak g'.

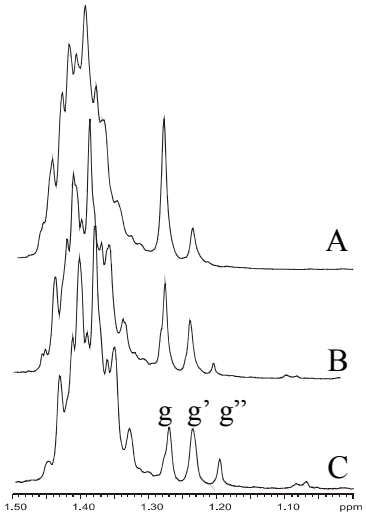

Figure 6. ^{1}H-NMR spectra of the 1.0-1.5 region of PCL prepared at a [M]:[I]:[C] ratio of 10:1:0.1, at different polymerization times: (A) after 30 min; (B) after 3 h; (C) after 7 h. Solvent: $CDCl_3$.

At later time points, esterification of the carboxylic acid group of the bis-MPA occurred, due to the large amount of $Sn(Oct)_2$ present. This became evident from the appearance of peak g" (1.20 ppm), belonging to a bis-MPA moiety of which the carboxylic acid group is esterified, and the shift in intensity of peaks g and g'. The large [C]:[I] ratio also resulted in a monomer conversion that reached 99 % after only 1 hour. In comparison, at a low [C]:[I] ratio (PCL8, Table 2) 99 % conversion required 6 h polymerization time.

In contrast with the above results for PCLn, the amount of di-substituted bis-MPA in PLLAn polymers was independent of the [M]:[I] ratio. The ^{1}H-NMR spectra of these polymers showed only a single peak at 1.27 ppm originating from the CH_3 protons of a bis-MPA moiety of which both hydroxyl groups are esterified. After the first ring opening reaction of a lactide, a secondary hydroxyl group is generated. Any un-reacted primary hydroxyl group of the bis-MPA moiety is more reactive towards the monomer than the secondary hydroxyl group of the lactide unit. In contrast, in the polymerization of ε-caprolactone a primary hydroxyl group is generated, which appears equally or more reactive than the sterically hindered bis-MPA hydroxyl group.

The ^{13}C-NMR spectral data confirmed the structural analysis by ^{1}H-NMR as described above. In Figure 7 the quaternary carbon region in the ^{13}C-NMR spectra of PLLA3 (A), PCL8 (B) and PCL25 (C) are presented. The chemical shift of the quaternary carbon of the bis-MPA moiety can be found at 46.5 ppm for di-substituted bis-MPA and at 48.5 ppm for mono-substituted bis-MPA[15]. The spectra of PLLA3 and PCL25 revealed a single signal at 46 ppm, corresponding to the quaternary carbon of di-substituted bis-MPA moieties. An additional peak was observed in the spectrum of PCL8 at 48 ppm, which belongs to the quaternary carbon of mono-substituted bis-MPA[15]. The appearance of this peak confirms the results from ^{1}H-NMR that PCL8 is a mixture of polymers with one and two caprolactone arms (Figure 4, structure A and B, respectively).

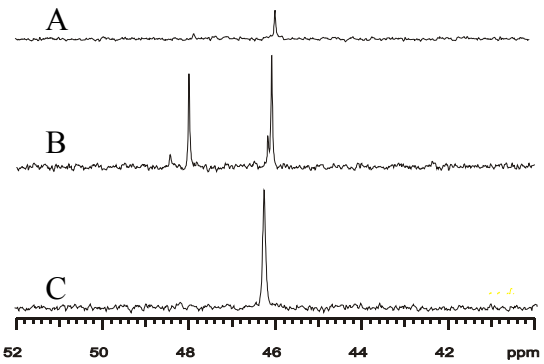

Figure 7. Quaternary carbon region of the ^{13}C-NMR spectra of (A) PCL25, (B) PCL8 and (C) PLLA3. Solvent: $CDCl_3$.

The molecular weight and molecular weight distributions (PDI) of the polymers prepared were determined with both GPC and MALDI-TOF mass spectroscopy. The results are listed in Table 1 and Table 2, for PCLn and PLLAn, respectively. The Mn's from GPC measurements of low molecular weight PLLAs are a factor ~1.4 larger than those calculated from ^{1}H-NMR. The results of the higher molecular weight PLLAs are in good accordance with those from ^{1}H-NMR. For PCL, the Mn's as determined by GPC are generally within 10 % difference with those from ^{1}H-NMR.

As typical examples, the MALDI-TOF mass distribution curves of PLLA7 and PCL13 are shown in Figure 8A and 8B, respectively. The expected masses can be

calculated using the molar masses of the initiator (bis-MPA: 134.13 g·mol^{-1}) and the repeating units (L-lactide: 144.13 g·mol^{-1} or ε-caprolactone: 114.14 g·mol^{-1}). It is known that polymer molecules become ionized with attachment of a sodium or potassium ion, originating from impurities in the polymer sample or matrix solution[16]. In these cases, the molecular weight of the attached ion (Na: 22.99 g·mol^{-1} or K 39.10 g·mol^{-1}) must be added.

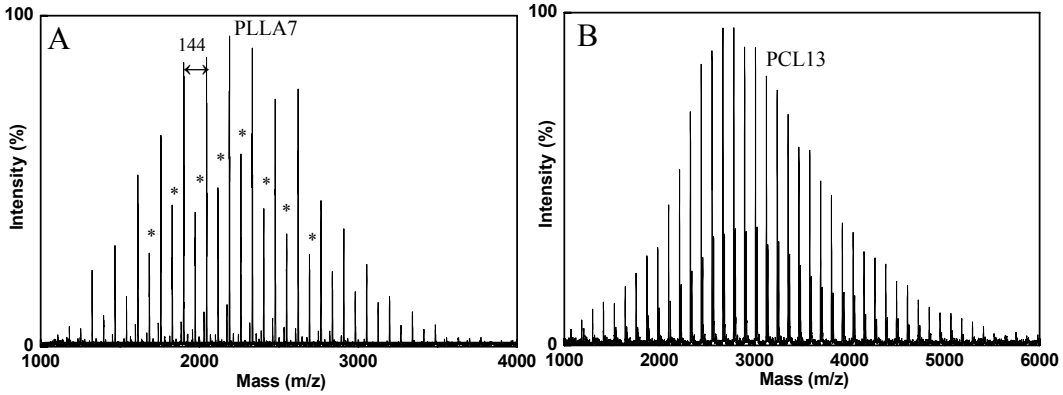

Figure 8. Maldi-tof mass distribution curves of (A) PLLA7 and (B) PCL13.

The polymer with 7 repeating L-lactide units per arm has a molar mass of 2191 g·mol^{-1} (K^+ as the attached ion). The peak corresponding to this polymer is labelled 'PLLA7' in Figure 8A. The mass difference of each adjacent peak is 144.1 g·mol^{-1}, which corresponds to the mass of one L-lactide unit. A second distribution of lower intensity is also observed. These peaks marked with an asterix (*) correspond to polymer chains with an odd number of lactic acid units, and result from transesterification, which is known to occur to some extent in ring opening polymerization reactions in the melt above 120 °C[17]. Similarly, in Figure 8B, the peak corresponding to a molar mass of 3125 g·mol^{-1} is labelled 'PCL13' and belongs to the polymer with 13 repeating ε-caprolactone units per arm (Na^+ as the attached ion). The mass difference between the peaks is 114 g·mol^{-1}, which corresponds to one ε-caprolactone unit.

Viscosity measurements of PLLA20 at varying concentrations (0.1-0.7 g·dl^{-1}) in chloroform gave the reduced and inherent viscosities, which are plotted as a function of concentration in Figure 9. The extrapolation of the inherent and reduced viscosities to zero concentration resulted in an intrinsic viscosity of 0.20 dl·g^{-1}.

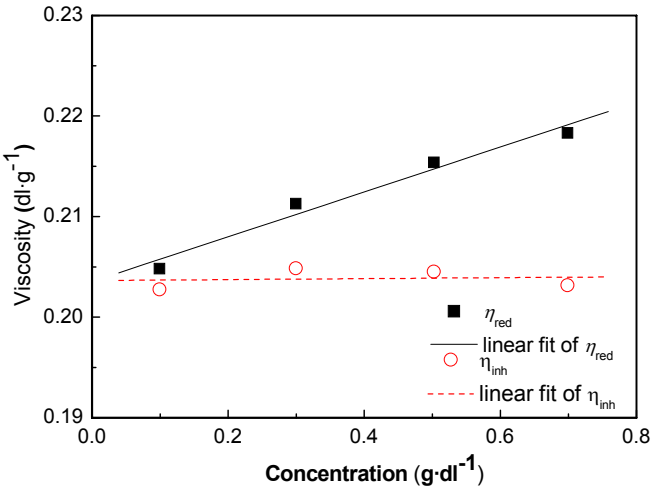

Figure 9. Reduced and inherent viscosities versus concentration for PLLA20 ($CHCl_3$, 25 °C).

From a single point measurement the same value of the intrinsic viscosity was obtained using equation 1. This indicates that the single point measurement can be used to determine the intrinsic viscosities of these polymers, and these data are presented in Tables 1 and 2. As expected, the intrinsic viscosity increases with increasing molecular weight.

When the intrinsic viscosities are plotted as a function of the Mn as determined with ^{1}H-NMR in a double logarithmic plot (Figure 10) the Mark-Houwink parameters k (dl·g^{-1}) and α (-) can be calculated, since:

$$[\eta] = k \cdot M_n^{\alpha} \tag{2}$$

and thus:

$$\ln[\eta] = \ln k + \alpha \cdot \ln M_n \tag{3}$$

in which [η] is the intrinsic viscosity (dl·g^{-1}) and M the molecular weight (g·mol^{-1}). A plot of the results presented in Tables 1 and 2 gives a straight line for both the PCL and PLLA polymers whose slopes equal α and whose intercepts equal ln k (Figure 10). The following Mark-Houwink relations can be deduced from the linear fits:

PCL: $$[\eta] = 3.01 \cdot 10^{-4} \cdot M_n^{0.77} \tag{4}$$

PLLA: $$[\eta] = 1.15 \cdot 10^{-4} \cdot M_n^{0.87} \tag{5}$$

The Mark-Houwink exponents (~0.8) show that chloroform is a good solvent for these polymers. The Mark-Houwink parameters of these PLLA and PCL polymers are similar to those determined by Eenink[18], Kim[19] (PLLA), Schindler[20] and Xi[21] (PCL) for linear polymers.

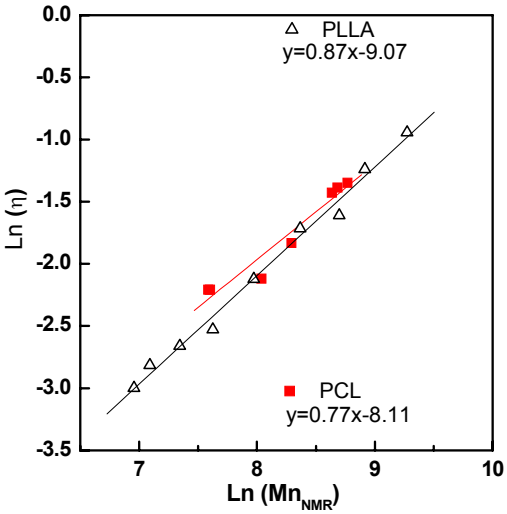

Figure 10. Double logarithmic plot of intrinsic viscosity versus Mn (from ^{1}H-NMR) for PCL (squares) and PLLA (triangles).

Thermal properties

The thermal properties of the PLLA and PCL polymers were determined using DSC. Second heating scans of selected PLLA and PCL polymers are presented in Figure 11A and B, respectively. For the PLLA polymers, the melting (T_m) and cold crystallization (T_{cc}) temperatures, their corresponding enthalpies (ΔH_m and ΔH_{cc}, respectively) and the glass transition temperatures (T_g) obtained from the second heating scan are reported in Table 3.

Table 3. Thermal properties of PLLA polymers.

	T_g (°C)	T_m (°C)	ΔH_m ($J \cdot g^{-1}$)	T_{cc} (°C)	ΔH_{cc} ($J \cdot g^{-1}$)
PLLA3	20	43/56	1	-	-
PLLA4	22	-	-	-	-
PLLA5	25	-	-	-	-
PLLA7	34	-	-	-	-
PLLA10	45	123/130	1	113	2
PLLA15	48	141	24	116	21
PLLA20	46	135/146	50	107	42
PLLA25	45	141/152	36	106	38
PLLA37	52	154/160	49	105	40

The thermal properties of the PCL polymers are reported in Table 4. Here, the crystallization temperature (T_c) and enthalpy (ΔH_c) were obtained from the cooling scan, while the T_g, T_m and ΔH_m were determined from the second heating scan.

Table 4. Thermal properties of PCL polymers.

	T_g (°C)	T_m (°C)	ΔH_m ($J \cdot g^{-1}$)	T_c (°C)	ΔH_c ($J \cdot g^{-1}$)
PCL8	-58	40	72	15	74
PCL13	-58	46	74	16	71
PCL17	-58	50	75	18	72
PCL24	-57	52	77	20	71
PCL25	-61	53	73	22	73
PCL28	-57	53	76	23	71

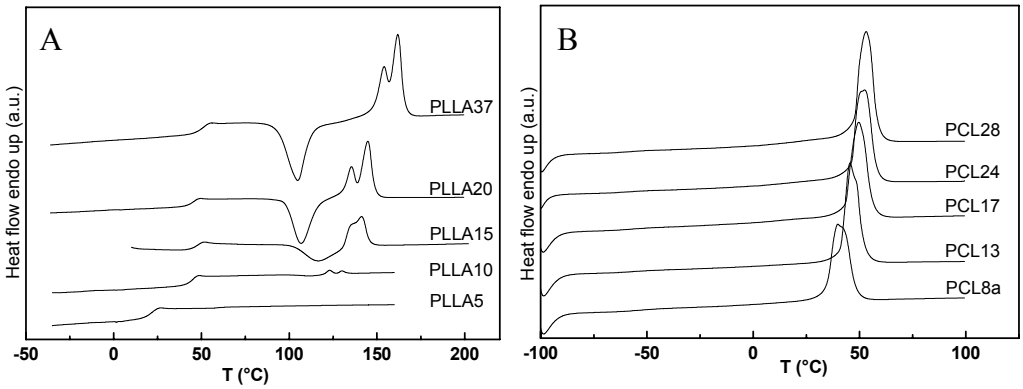

Figure 11. Second heating thermograms of PLLA (A) and PCL (B) with different DP.

Glass transition temperature

The DSC thermograms of the PLLA polymers show a T_g, which increases with PLLA arm length (Figure 11A). The low T_g's result from the larger contribution of chain end groups for low molecular weight PLLA's, and is also known for linear[22-24] and star-shaped[24] PLLA. The T_g of PLLA of infinite molecular weight can be determined from the following Flory-Fox equation[25]:

$$T_g = T_g^{\infty} - \frac{K}{M_n} \qquad (6)$$

in which T_g^{∞} is the T_g at infinite molecular weight, and K is a constant, that represents the excess free volume of the end groups of polymer chains. A plot of the measured T_g's versus the reciprocal molecular weight gives a straight line, whose slope equals K and whose intercept equals T_g^{∞}. This is shown in Figure 12. The T_g^{∞} value that is deduced from this plot is 54 °C, which is comparable to that of linear polylactide (T_g^{∞} is 57 °C)[22].

The PCL polymers show a T_g (Figure 11B) at -57 °C, independent of the molecular weight, and close to values found for linear PCL[26].

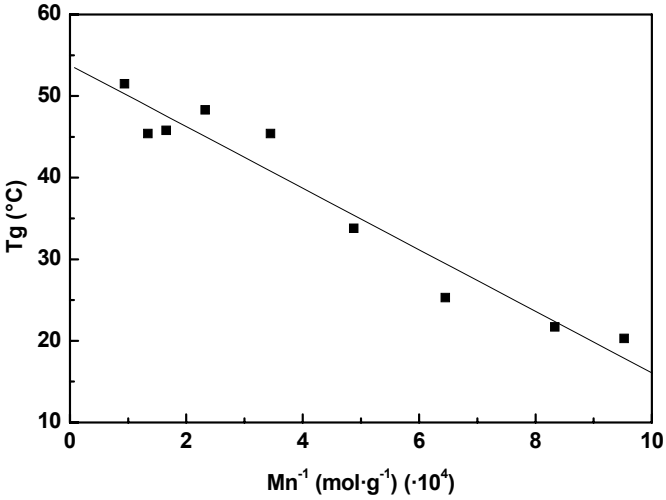

Figure 12. Tg versus Mn^{-1} Flory-Fox relationship for PLLA polymers. The intercept of the y-axis is T_g^{∞}.

Melting and crystallization temperature

Low molecular weight PLLAn ($n \leq 7$) did not show melting or cold crystallization in the second heating run. It is known that at least 5 repeating lactide units are required in the formation of crystals[23]. For the low molecular weight PLLA's, it is most likely that the lactide unit connected to the bis-MPA moiety cannot participate in the crystal formation. Apparently, due to the irregularity in the structure caused by the bis-MPA moiety, crystal formation is dependent on the DP of a single arm, and the thermal properties are comparable to linear polymers with a DP equal to the DP per arm in the AB_2 polymer[5, 22]. PLLAn's with $n > 7$ exhibit two overlapping melting peaks (Figure 11A) and a cold crystallization peak. The melting temperature increases with increasing molecular weight, as longer chains crystallize more efficiently. The cold crystallization temperature slightly decreases, because crystallization is more favored for longer PLLA chains than for shorter chains. The melting enthalpy increased with molecular weight to a value of ~50 $J \cdot g^{-1}$ for PLLA37. The melting enthalpy estimated for linear enantiopure PLA of 100% crystallinity is reported to be 93 $J \cdot g^{-1}$ [27].

All PCL samples showed a crystallization peak in the cooling scan and a melting peak in the second heating scan (Figure 11B). All melting peaks exhibited a small shoulder. With an increase in molecular weight, an increase was observed in both

the melting and crystallization temperature. Also, the melting enthalpy increased to ~75 J·g^{-1}. The melting enthalpy estimated for linear PCL of 100% crystallinity is reported to be 139 J·g^{-1} [28].

According to equation 7 as proposed by Flory, a linear relationship is expected when the reciprocal T_m is plotted versus the reciprocal Mn (Figure 13)[29].

$$\frac{1}{T_m} + \frac{1}{T_m^{\infty}} = -\frac{2 \cdot R \cdot M_0}{\Delta H_m} \cdot \frac{1}{M_n} \qquad (7)$$

in which T_m^{∞} is the T_m at infinite molecular weight, R is the gas constant, M_0 the molecular weight of the repeating unit and ΔH_m the melting enthalpy per mole repeating units. Low molecular weight polymers are excluded, because the influence of the end groups and bis-MPA moiety is too large. The Flory plot gives a T_m^{∞} of 182 °C for PLLA and 60 °C for PCL. This is comparable with linear PLLA (T_m^{∞} of 184 °C) [22] and linear PCL (T_m^{∞} of 60 °C)[22]. The slope of the linear fit for PLLA is similar as determined by Jamshidi and coworkers[22]. The calculated ΔH_m for PLLA is 1.8 kJ·mol^{-1} lactide units. The ΔH_m value for PCL is 4.3 kJ·mol^{-1}. The calculated values of ΔH_m of PLLA and PCL are lower than the values measured by others[27, 30]: 13.4 kJ·mol^{-1} and 16.4 kJ·mol^{-1}, respectively.

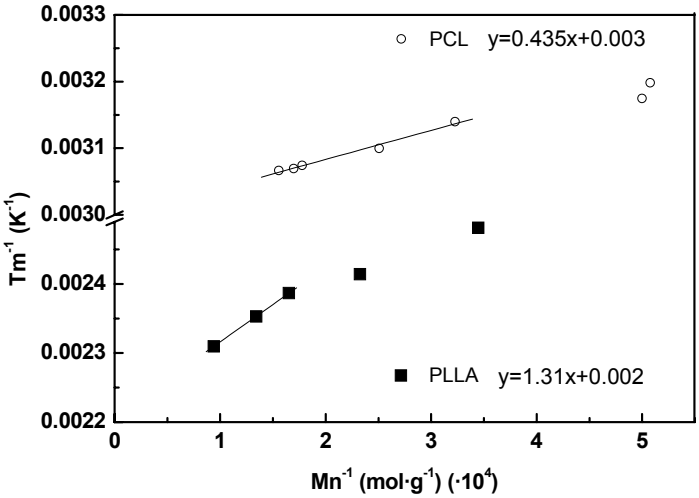

Figure 13. T_m^{-1} vs Mn^{-1} Flory relationship for PLLA and PCL. The intercept of the y-axis is $\frac{1}{T_m^{\infty}}$.

Conclusions

The $Sn(Oct)_2$ catalyzed ring opening polymerization of ε-caprolactone or L-lactide using 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) as the initiator allowed the preparation of polyesters with controlled molecular weight. In L-lactide polymerization, both bis-MPA hydroxyl groups initiated the polymerization reaction, but for ε-caprolactone polymerization this depends on the monomer to initiator ratio. At low [M]:[I] ratio, ε-caprolactone polymerization resulted in a mixture of polymers with one and two arms per initiating bis-MPA. Increasing the $Sn(Oct)_2$ concentration ([C]:[I] ratio) increased the formation of two-armed polymer. The melting temperatures of the AB_2-functional PLLA and PCL polymers were comparable to their linear analogues when the DP per arm was similar to the DP of the linear polymer. The method applied was highly efficient for the controlled synthesis of AB_2-functional macromonomers that can be used for the synthesis of hyperbranched polymers.

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Chapter 4

A facile method for the synthesis of hyperbranched poly-(ε-caprolactone)s

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Abstract

Hyperbranched poly(ε-caprolactone)s (HBPCLs) were prepared via a facile 'one-pot' synthesis method. This method consisted of the ring opening polymerization of ε-caprolactone in the presence of the AB_2 functional initiator 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) and stannous octoate as the catalyst, and subsequent polycondensation in the melt under reduced pressure. Polycondensation of a macromonomer with a degree of polymerization of 7 caprolactone units per arm for 16 hours resulted in HBPCL7 with an average of 4.5 branching points per molecule. ^{1}H- and ^{13}C-NMR spectral data and the intrinsic viscosity showed that upon increasing the polycondensation time, the molecular weight increased. Thermal analysis showed that the crystallinity of the hyperbranched polymers decreased with increasing number of branching points. A structural analysis of hyperbranched poly(ε-caprolactone)s, prepared via a 'two-step' procedure from purified PCL macromonomers at varying condensation times was performed. The structure of the hyperbranched polymer via the 'one-pot' preparation method was comparable to that prepared via the 'two-step' procedure.

Introduction

Hyperbranched polymers have received much interest for their use in coatings and thin film technology[1, 2], as well as biomedical applications[3-5]. These materials show several unique properties compared to their linear analogues, including lower viscosities, lower crystallinities and the presence of large numbers of functional end-groups that offer the opportunity for further modification[6].

Aliphatic hyperbranched polyesters have been synthesized by condensation of 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) as the AB_2 monomer and 2-ethyl-2-(hydroxymethyl)-1,3-propanediol as a core molecule[7]. These polyesters had a high degree of branching, molecular weight distributions lower than 2, and were fully amorphous. Hyperbranched polyesters were also prepared by the self-condensing ring opening polymerization of lactones with a pendant hydroxyl group[8-12].

In hyperbranched poly(ε-caprolactone)s crystallinity can be introduced by the incorporation of linear segments[13, 14]. Such polymers were synthesized by first ring opening polymerization of ε-caprolactone using the benzyl ester of bis-MPA as the initiator, and subsequent deprotection of the carboxylic acid group. The resulting macromonomers were then self-condensed to form hyperbranched poly(ε-caprolactone)s with well-defined spacers between the branching points. However, the synthesis of the macromonomers required protection and deprotection steps. A method to prepare hyperbranched polyesters as developed by Frey and coworkers[15-17] could overcome these protection and deprotection steps. They used concurrent ring opening polymerization of lactones and polycondensation using different catalysts, and 2,2-bis(hydroxylmethyl)butyric acid (BHB) as the AB_2 monomer. The degree of branching could be controlled by varying the lactone to BHB ratio. However, these methods have in common that the linear segments are randomly incorporated, and therefore do not give control over the segment length.

In this study, we report on a new 'one-pot' synthesis route to hyperbranched poly(ε-caprolactone)s, which avoids multi-step reactions, and is therefore more advantageous for industrial scale processing. Furthermore, this method allows incorporation of ε-caprolactone segments of controlled length, which allows good control over the properties of the hyperbranched polymers.

Experimental

Materials

2,2-Bis(hydroxymethyl)propionic acid (bis-MPA) was obtained from Acros (Geel, Belgium). Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$) and ε-caprolactone were purchased from Aldrich (Zwijndrecht, the Netherlands). Tetra-n-butyl orthotitanate ($Ti(OBu)_4$), glacial acetic acid, chloroform, and deuterated chloroform were obtained from Merck (Darmstadt, Germany). All other solvents were from Biosolve (Valkenswaard, the Netherlands). ε-Caprolactone was dried over calcium hydride (Aldrich) and distilled under vacuum, prior to use. All other chemicals were used as received.

Synthesis

Hyperbranched poly(ε-caprolactone)s were prepared via two different procedures. In the first method, materials were synthesized in a 'one-pot' procedure comprising the synthesis of a macromonomer by ring opening polymerization of ε-caprolactone from an AB_2 initiator and subsequent polycondensation. The hyperbranched polymers obtained are denoted as HB(1)PCLn-t, where (1) indicates the 'one-pot' procedure, n is the average number of repeating ε-caprolactone units per arm in the macromonomer and t is the condensation time in h. The second method consists of two steps. Firstly, a batch of 100 g of macromonomer is prepared by ring opening polymerization of ε-caprolactone, and subsequently purified by precipitation[18]. Secondly, 15 g batches of purified macromonomer were subjected to polycondensation for 6 up to 36 h. The polymers prepared are denoted HB(2)PCLn-t, where (2) indicates the 'two-step' process and PCLn is the starting macromonomer, with n repeating caprolactone units per arm, and t the condensation time in h.

Method 1: Hyperbranched poly(ε-caprolactone)s by a 'one-pot' procedure

The synthesis of HB(1)PCL7-16 is given as a typical procedure: ε-Caprolactone (15.0 g, 131 mmol) was added to a reaction vessel containing bis-MPA (1.76 g, 13.1 mmol) as the initiator and $Sn(Oct)_2$ (0.06 g, 0.15 mmol; 0.4 wt% based on ε-caprolactone) as the catalyst. The mixture was stirred and allowed to react for 6 h at 110 °C in an argon atmosphere. After ring opening polymerization, a sample was taken from the polymerization mixture for ^{1}H-NMR analysis. Subsequently, 1 ml of a 0.05 $g \cdot ml^{-1}$ $Ti(BuO)_4$ solution in dry toluene was added, and the temperature was

raised to 130 °C (t=0). The pressure was slowly reduced to 100 mbar, allowing the toluene to distill off. The pressure was further reduced to 0.2-0.05 mbar, and polycondensation was allowed to proceed for 16 h. The mixture was cooled to room temperature and the polymer was obtained by dissolution in dichloromethane and subsequent precipitation in an excess of cold diethyl ether. A white powder was obtained after drying in vacuo (Yield: 83 %).

Method 2: Hyperbranched poly(ε-caprolactone)s by polycondensation of purified macromonomers

The synthesis of hyperbranched HB(2)PCL8-18 is given as a typical procedure: The macromonomer PCL8 was prepared and purified as described previously[18]. PCL8 (15.0 g, 7.5 mmol) was placed in a reaction vessel and allowed to melt at 60 °C in an argon atmosphere. After 30 min, 1 ml of a 0.05 $g \cdot ml^{-1}$ $Ti(BuO)_4$ solution in dry toluene was added, and the temperature was raised to 130 °C. The pressure was slowly reduced to 100 mbar, allowing the toluene to distill off. Subsequently, the pressure was further reduced to 0.2-0.05 mbar. After 18 h, the product was cooled to room temperature, dissolved in dichloromethane and purified by precipitation in cold diethyl ether. HB(2)PCL8-18 was collected by filtration and dried in vacuo. The product was obtained as a white powder (Yield: 85 %).

Characterization

NMR: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR spectra were recorded on a Varian Inova NMR spectrometer. Polymers were dissolved in $CDCl_3$ at a concentration of 0.015 $g \cdot ml^{-1}$ (^{1}H) or 0.2 $g \cdot ml^{-1}$ (^{13}C).

Viscometry: Intrinsic viscosities [η] were determined by single point measurements (0.1 $g \cdot dl^{-1}$ polymer solutions in chloroform) using a capillary Ubbelohde type 0C at 25 °C. The following empirical relation was applied:

$$[\eta] = \frac{\sqrt{2}}{c} \cdot \sqrt{\eta_{sp} - \ln \eta_{rel}} \qquad (1)$$

in which $\eta_{spec} = \eta_{rel} - 1$ and c is the polymer concentration in $g \cdot dl^{-1}$. The relative viscosity ($\eta_{rel} = t/t_0$) was determined from the flow time of the polymer solution (t) and the flow time of the solvent (t_0).

GPC: Molecular weights and molecular weight distributions of the polymers were determined with gel permeation chromatography (GPC) using chloroform as eluent. The GPC setup consisted of a GPCmax VE-2001 GPC solvent/sample module, a

series of ViscoGEL I columns, and a TDA 302 triple detector array consisting of a light scattering detector (RALS and LALS), a differential refractive index detector, and a four-capillary differential viscometer. A polystyrene standard (Mn = 64000 $g \cdot mol^{-1}$) with narrow molecular weight distribution was used for calibration.

DSC: Thermal analysis of the macromonomers and hyperbranched polymers was carried out using a Pyris 1 differential scanning calorimeter connected with a liquid nitrogen cooling accessory. During a measurement, the polymer (5-15 mg) was cooled to -100 °C and kept at this temperature for 1 min. The samples were then heated to 100 °C, annealed for 1 min, and cooled to -100 °C. Subsequently, the samples were kept isothermally for 5 min and heated again to 100 °C. The scanning rate of the heating and cooling scans was 20 $°C \cdot min^{-1}$. Melting (T_m) and crystallization (T_c) temperatures were obtained from the peak maxima, melt (ΔH_m) and crystallization (ΔH_c) enthalpies were obtained from the area under the curve and the glass transition temperature (T_g) was taken at the inflection point. Data were taken from the second heating scan and the cooling scan.

Results and Discussion

The bulk ring opening polymerization of ε-caprolactone using $Sn(Oct)_2$ as a catalyst in the presence of an AB_2 functional initiator like bis-MPA was used to prepare two-armed PCLs having one carboxylic acid and two hydroxyl functional groups. Such macromonomers are potential precursors for the preparation of hyperbranched polymers by polycondensation (Figure 1).

To study hyperbranching by polycondensation, three types of macromonomers were prepared and purified. Macromonomers with two arms and a degree of polymerization per arm (DP) of either 6 or 25 caprolactone units were used. Also a macromonomer mixture consisting of single-arm and two-armed macromonomers and having an average DP of 8 caprolactone units per arm (Figure 2A and B, respectively) was prepared using low catalyst to initiator [C]:[I] ratios.

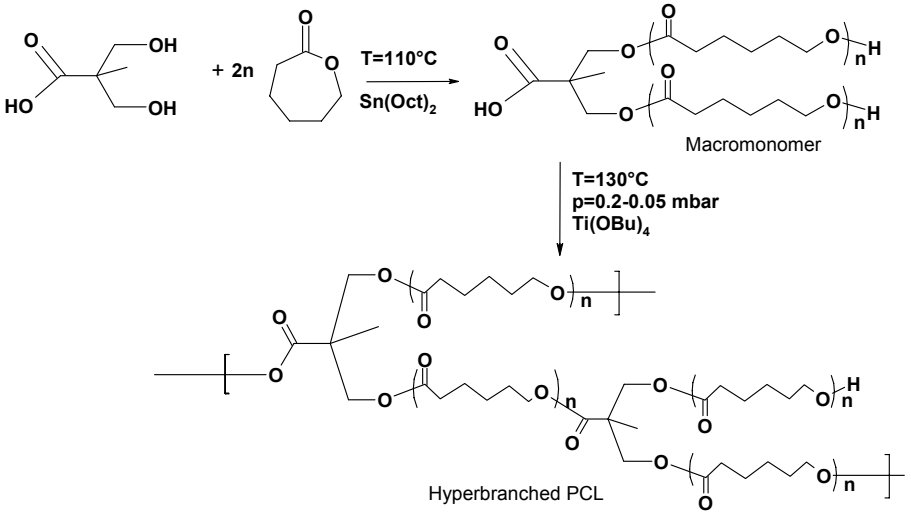

Figure 1. Synthesis of hyperbranched poly(ε-caprolactone)s: bis-MPA initiated ring opening polymerization and subsequent polycondensation.

Hyperbranching of poly(ε-caprolactone) macromonomers

The AB_2 functional macromonomers, 2,2-bis[ω-hydroxy poly(ε-caprolactone) methyl]-propionic acids, were prepared by ring opening polymerization using bis-MPA as an initiator and $Sn(Oct)_2$ as a catalyst (Figure 1). The DP of the polymer arms was controlled by the [M]:[I] ratio, and a macromonomer with 25 units per arm was conveniently synthesized. In a previous study it was shown that the polymerization at low [M]:[I] ratio depended on the [C]:[I] ratio[18]. At a low [C]:[I] ratio of 0.01:1, a mixture of two-armed and single-armed PCL8 was obtained. Increasing the [C]:[I] ratio to 0.1:1, and decreasing the reaction time from 6 to 1 h, afforded two-armed PCL6. At such a high [C]:[I] ratio, the DP became somewhat lower due to the presence of traces of water in commercially available $Sn(Oct)_2$ which induces homopolymer formation. The properties of the macromonomers that were used for the preparation of the hyperbranched polymers are listed in Table 1.

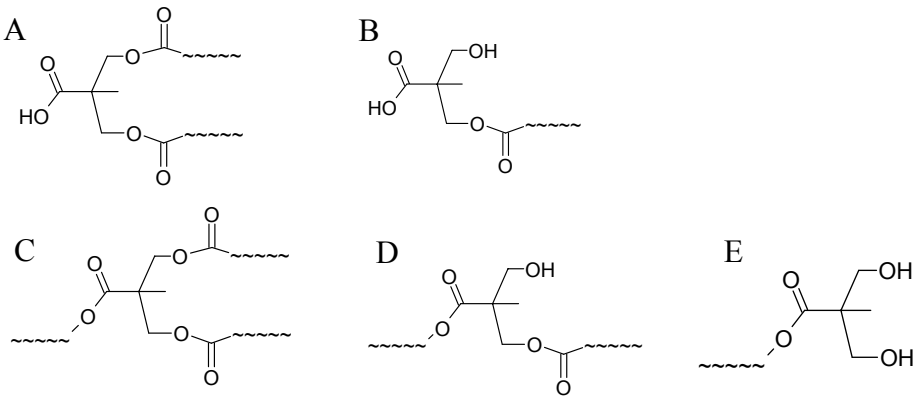

Figure 2. Schematic representation of a bis-MPA moiety that is (A) di-substituted, (B) mono-substituted; (C) esterified and di-substituted; (D) esterified and mono-substituted; or (E) esterified and unsubstituted.

Table 1. Molecular weights, molecular weight distributions and intrinsic viscosities of macromonomers used in the synthesis of hyperbranched poly(ε-caprolactone)s.

	[M]:[I]:[C]	DP	Mn ($g \cdot mol^{-1}$)		[η] [a]
	(mol:mol:mol)	(-)	^{1}H-NMR	MALDI	($dl \cdot g^{-1}$)
PCL6	10: 1: 0.10	6.1	1500	1400	0.10
PCL8	10: 1: 0.01	8.1	2000	1700	0.11
PCL25	42: 1: 0.05	25.4	5900	n.d.	0.25

[a] chloroform, 25 °C

n.d. not determined

Polycondensation of the purified macromonomers was performed at reaction times ranging from 6 to 36 h to give hyperbranched poly(ε-caprolactone)s with varying numbers of branching points (N_{BP}). During condensation, the polymer melt became more viscous, and in the case of HB(2)PCL25 after 30 h the reaction was stopped, because the melt was too viscous to stir. The notation HB(2) refers to the two-step procedure.

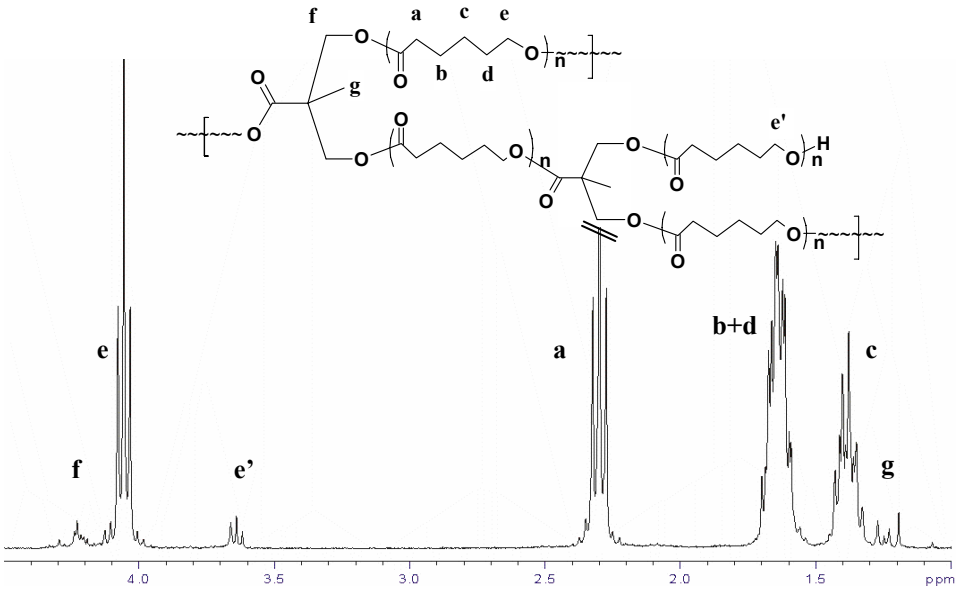

Figure 3. ^{1}H-NMR spectrum of HB(2)PCL8-18. Solvent: $CDCl_3$.

A ^{1}H-NMR spectrum of HB(2)PCL8-18 is shown in Figure 3. Hyperbranching is observed in the ^{1}H-NMR spectra from: (1) the increase of the relative integral ratio *r* of the C*H*$_2$ protons next to the carbonyl (a, 2.30 ppm) to the C*H*$_2$-OH protons of the terminal caprolactone unit (e', 3.63 ppm); (2) the increase of the relative integral ratio *q* of the C*H*$_2$ protons of the bis-MPA moiety (f, 4.23 ppm) to the C*H*$_2$-OH protons of the terminal caprolactone unit (e', 3.63 ppm); and (3) the increase in the number of signals belonging to the methyl protons of the bis-MPA moiety, compared to that of the macromonomer. The N_{BP} can now be calculated using equation 2[13, 14] or 3:

$$N_{BP1} = \frac{r}{2 \cdot DP - r} \tag{2}$$

in which DP is the average number of repeating units per arm in the macromonomer.

$$N_{BP2} = \frac{q}{2 - q} \tag{3}$$

Comparing signals of similar intensities has the advantage that a more accurate value of N_{BP} can be determined (Equation 3). However, for the hyperbranched poly(ε-caprolactone)s based on PCL6 and PCL8, this method could not be applied,

since the signals of the CH_2 protons of the bis-MPA moiety and the CH_2O protons of the caprolactone units overlapped. For these hyperbranched polymers, only equation 2 was used to calculate the N_{BP} (Table 2).

At similar reaction times, the N_{BP}s of the hyperbranched polymers based on PCL6 and PCL8 were higher than those based on PCL25 (Table 2), because the concentration of functional groups in the higher molecular weight macromonomer PCL25 was lower. Moreover, higher molecular weight macromonomers encounter more steric hindrance[14]. HB(2)PCL6 showed higher N_{BP}s than HB(2)PCL8 after the same reaction time, although the concentration of functional groups in both macromonomers is comparable. Apparently, the hydroxyl group at the end of a polycaprolactone arm is more reactive towards the carboxylic acid groups than the hydroxyl group at the bis-MPA moiety, due to steric hindrance. It has to be emphasized that the macromonomer PCL8 is a mixture of polymers consisting of single and two armed polymer molecules. Polycondensation of HB(2)PCL6 for 18 h at 130 °C resulted in network formation, due to side reactions such as etherification reactions[19]. Performing the condensation reaction for 18 h, but at 110 °C did not show formation of an insoluble network, but resulted in a hyperbranched PCL with a somewhat lower N_{BP} of 6 (Table 2) due to the lower reaction rate at 110 °C.

The molecular weights were also determined using GPC with triple detection. The molecular weights of HB(2)PCL6-t and HB(2)PCL8-t determined with GPC were lower than those determined from the ^{1}H-NMR spectra. This difference may partly be a result of side reactions, such as intramolecular esterification or etherification reactions[19]. In the case of these intramolecular side reactions, hydroxyl end-groups are consumed, but this does not lead to an increase in molecular weight. However, in ^{1}H-NMR, the consumption of hydroxyl end-groups is a measure to calculate the molecular weight. For HB(2)PCL25-t, the molecular weights obtained with GPC were lower than the molecular weights as obtained with ^{1}H-NMR using equation 2. However, they were in good agreement with the ^{1}H-NMR results as obtained using equation 3. The HB(2)PCL6-12 and HB(2)PCL25-30 GPC elution curves showed broad bimodal distributions, which resulted in very high PDI values.

Table 2. Number of branching points (N_{BP}), molecular weights, polydispersities and intrinsic viscosities of hyperbranched poly(ε-caprolactone)s.

Entry	NMR		NMR[b]		$GPC_{triple\ detect}$		
	N_{BP1} (-)	Mn ($g \cdot mol^{-1}$)	N_{BP2} (-)	Mn ($g \cdot mol^{-1}$)	Mn ($g \cdot mol^{-1}$)	PDI (-)	$[\eta]^a$ ($dl \cdot g^{-1}$)
HB(2)PCL6-6	7.4	11100			5800	1.7	0.17
HB(2)PCL6-12	11.7	17500	n.d.		8600	8.6	0.37
HB(2)PCL6-18[c]	6.2	9400			6600	1.3	0.15
HB(2)PCL8-6	2.4	4800			3600	2.0	0.14
HB(2)PCL8-12	6.6	13000			4000	2.2	0.18
HB(2)PCL8-18	8.2	16100	n.d.		5200	1.9	0.20
HB(2)PCL8-24	7.8	15400			6600	3.7	0.24
HB(2)PCL8-36	10.4	20500			10000	2.9	0.27
HB(2)PCL25-6	1.6	9500	1.0	6000	5300	2.1	0.31
HB(2)PCL25-12	1.9	11100	1.2	6900	6700	2.1	0.38
HB(2)PCL25-18	1.8	10600	1.3	7800	9300	1.7	0.39
HB(2)PCL25-24	3.5	20400	2.6	15600	15700	2.3	0.60
HB(2)PCL25-30	3.4	20000	4.9	28800	38300	9.0	1.61

[a] chloroform; 25°C

[b] Equation 3 could only be applied for the HB(2)PCL25 series

[c] Temperature during polycondensation was 110 °C

The intrinsic viscosities of the hyperbranched polymers listed in Table 2 showed that an increase in viscosity was found with increasing N_{BP} in both the HB(2)PCL8 and HB(2)PCL25 series. Furthermore, HB(2)PCL8 polymers have a lower viscosity than the HB(2)PCL25 polymers with a comparable molecular weight. For example, HB(2)PCL8-18 with a Mn of 16100 $g \cdot mol^{-1}$ has an intrinsic viscosity of only 0.20 $dl \cdot g^{-1}$, whereas the viscosity of HB(2)PCL25-24 with a Mn of 15600 $g \cdot mol^{-1}$ is 0.60 $dl \cdot g^{-1}$. This is due to the higher number of branching points of HB(2)PCL8. More branched structures possess a smaller hydrodynamic radius and a more spherical shape compared to their less branched analogues, resulting in lower viscosities[20].

The viscosity and molecular weight of polymers are related by the Mark-Houwink equation:

$$[\eta] = k \cdot M^{\alpha} \qquad (4)$$

in which, $[\eta]$ is the intrinsic viscosity in $dl \cdot g^{-1}$, M the molecular weight in $g \cdot mol^{-1}$, and k ($dl \cdot g^{-1}$) and α (-) are system specific parameters that depend on the constitution, configuration, and molar mass distribution of the polymer, as well as solvent and temperature[21]. Values of α in between 0.5 and 1.0 comprise random coiled polymers, whereas values < 0.5 represent polymer chains more globular in shape[2, 22, 23]. A double logarithmic plot of the viscosity and Mn (^{1}H-NMR) data of Table 2 is presented in Figure 4. The linear fits gave the Mark-Houwink parameters k as the intercept with the y-axis, and α as the slope of the line.

The PCLn macromonomers gave α = 0.8 and are plotted in Figure 4 for comparison[18]. The higher number of branching points of the HB(2)PCL8 polymers compared to the HB(2)PCL25 polymers is reflected by a difference in the value of α. The HB(2)PCL8-t polymers gave a value of α of 0.4, a value representing polymers with a more globular shape. The value of α = 0.6 of the HB(2)PCL25-t polymers having a low N_{BP} reveals that these polymers have a somewhat more linear structure.

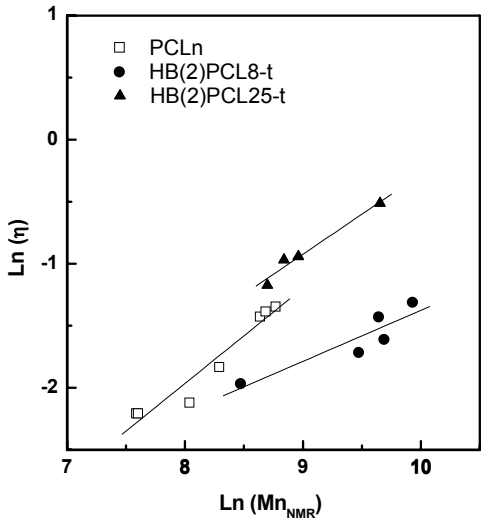

Figure 4. Double logarithmic plot of the intrinsic viscosities of the PCLn (□), HB(2)PCL8 (●), and HB(2)PCL25 (▲) series as a function of Mn as determined with ^{1}H-NMR.

Structure of HB(2)PCLn polymers

As was mentioned above, in the ^{1}H-NMR spectrum the number of signals belonging to the methyl protons of the bis-MPA moiety increased with increasing polycondensation time. For the PCL8 series, two peaks exist before condensation at 1.27 ppm and 1.24 ppm, corresponding to the methyl protons of the di- and mono-substituted bis-MPA moieties respectively (structure A and B in Figure 2). Upon condensation, the carboxyl groups present in the macromonomer are converted to esters by reacting with hydroxyl groups. This causes a chemical shift of the signal belonging to the methyl group of the bis-MPA in the ^{1}H-NMR spectra (Figure 5).

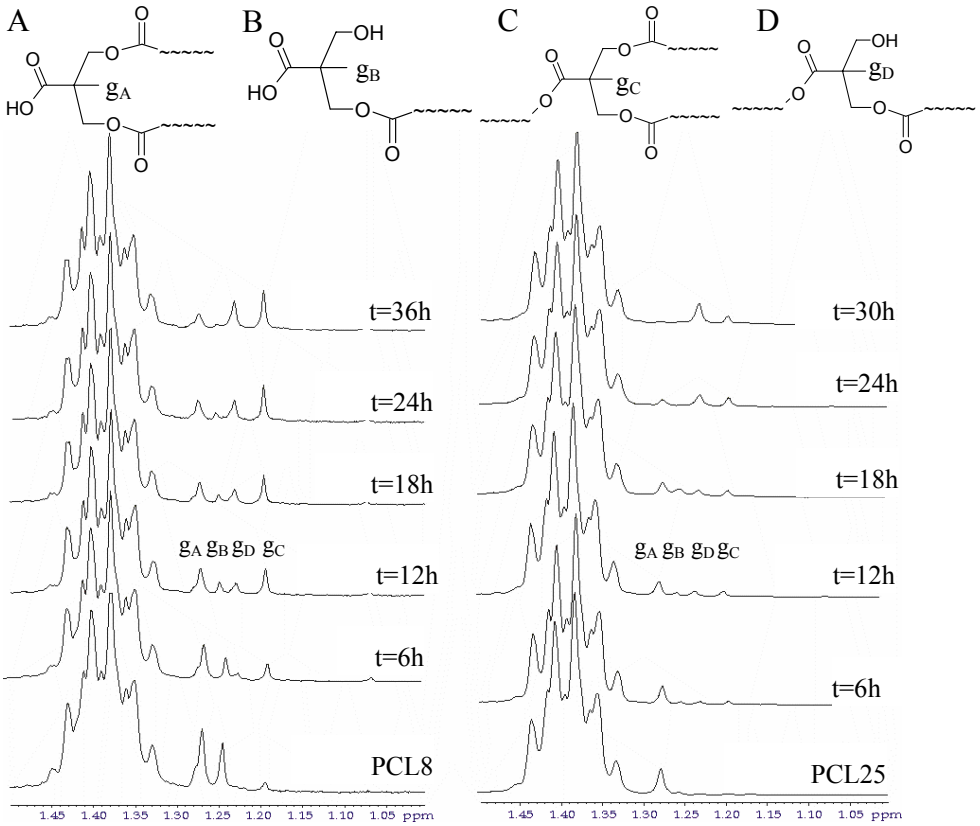

Figure 5. 1.0-1.5 ppm region of ^{1}H-NMR spectra of PCL8 (left) and PCL25 (right) and their hyperbranched polymers obtained after different polycondensation times (Solvent: $CDCl_3$). The notations g_A, g_B, g_C, and g_D refer to the methyl protons of the bis-MPA moiety in structures A, B, C, and D, respectively.

Upon condensation, the signals at 1.27 ppm and 1.24 ppm decreased, whereas new peaks appeared at 1.23 ppm and 1.20 ppm, belonging to the methyl protons of the bis-MPA moiety of structure C and D respectively. The remaining signal at 1.27 ppm corresponds to both the methyl protons of residual macromonomer and those next to the remaining carboxylic acid group in the hyperbranched polymer. The presence of small signals at 1.07 ppm is probably caused by end groups like structure E (Figure 2), which may result from transesterification reactions.

Upon condensation of PCL25, the signal at 1.27 ppm disappeared, and signals at 1.23 ppm and 1.20 ppm appeared, belonging to the methyl protons of esterified bis-MPA moieties. The fact that a signal appeared at 1.20 ppm, and at intermediate times at 1.25 ppm, indicates that transesterification occurred as well, since these peaks are attributed to methyl protons of mono-substituted bis-MPA moieties.
The ^{13}C-NMR spectral data confirmed the structural analysis by ^{1}H-NMR as described above. The quaternary carbon regions of the spectra of the hyperbranched polymers at different condensation times are shown in Figure 6. The spectrum of macromonomer PCL8 shows two quaternary carbon signals, indicating that both mono- and di-substituted bis-MPA are present (Figure 5, structure B and A, respectively). When the quaternary carbon is attached to an ester group, it resonates at a slightly higher chemical shift due to the less pronounced electron-withdrawing character of the ester group compared to that of the carboxylic acid group[24]. Upon condensation, two new peaks appeared at slightly higher chemical shifts: 46.4 ppm and 48.4 ppm. These peaks originate from esterified mono- and di-substituted bis-MPA moieties (structures C and D in Figure 5).

Similarly, upon condensation of PCL25, a new peak appears (46.4 ppm, Figure 6B) originating from the esterified di-substituted bis-MPA (structure C), which resonates at a slightly higher chemical shift. This happens simultaneously with the disappearance of the signal at 46.0 ppm, which indicates that the quaternary carbon next to a carboxylic acid carbon is converted to a quaternary carbon next to an ester carbon. The small signal at 47.9 ppm also disappears upon condensation and a new peak appears at 48.4 ppm, originating form esterified mono-substituted bis-MPA moieties (structure D, Figure 5).

The appearance and disappearance of the different methyl proton signals in the ^{1}H-NMR spectra occurred simultaneously with the changes in the chemical shifts and signal intensities of the quaternary carbon signals in the ^{13}C-NMR spectra (Figure 6). This structural analysis was used to determine the structure of hyperbranched polymers obtained via the 'one-pot' synthesis method as is described in the following section.

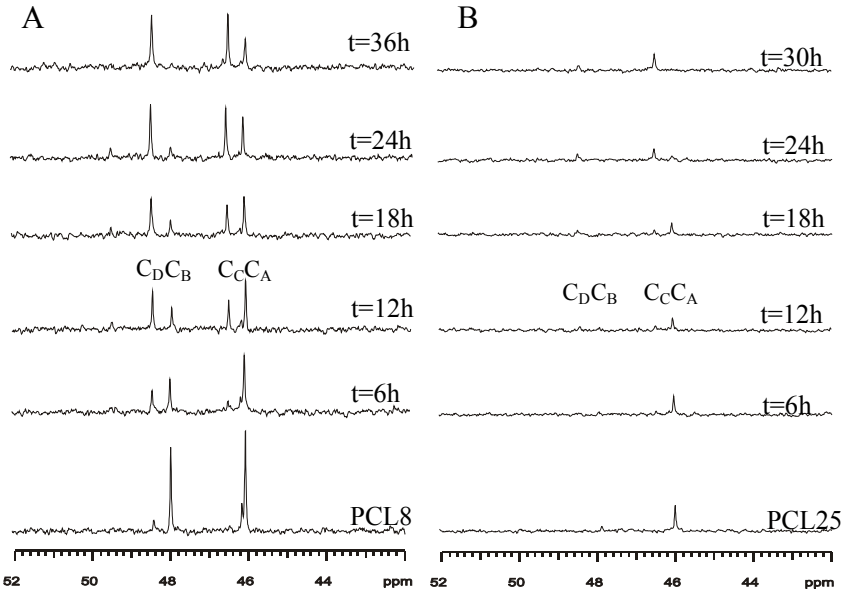

Figure 6. Quaternary carbon region of ^{13}C-NMR spectra of (A) PCL8 and HB(2)PCL8; and (B) PCL25 and HB(2)PCL25 at different condensation times (Solvent: $CDCl_3$). The notations C_A, C_B, C_C, and C_D refer to the quaternary carbon atoms of structures A, B, C, and D, respectively, in Figure 2.

Hyperbranched poly(ε-caprolactone) via the 'one-pot' synthesis

In the 'one-pot' synthesis of HBPCL the macromonomer synthesized was not isolated, but polycondensated in the reaction vessel for 16 h at reduced pressure. The [M]:[I]:[C] ratio was 10:1:0.01, providing a macromonomer with a DP of 7 caprolactone units per arm. A sample was withdrawn from the vessel before the polycondensation step to determine the degree of polymerization and the conversion at that time point. The ^{1}H-NMR spectrum revealed complete monomer conversion and a DP of 7 caprolactone units per arm. The macromonomer obtained is similar to that of the PCL8 macromonomer, a mixture of polymers having a

single or two arms. The hyperbranched polymer isolated after 16 h polycondensation (HB(1)PCL7-16 was structurally analyzed through the ^{1}H- and ^{13}C-NMR spectra and compared to the HB(2)PCL8-18 polymer (Table 3).

Table 3. Synthesis results of HB(1)PCL7 by direct condensation, and HB(2)PCL8-18h by a two step procedure.

Entry	NMR after ROP[a]		NMR[b]		GPC		
	DP (-)	Mn ($g \cdot mol^{-1}$)	N_{BP1} (-)	Mn ($g \cdot mol^{-1}$)	Mn ($g \cdot mol^{-1}$)	PDI (-)	$[\eta]^c$ ($dl \cdot g^{-1}$)
HB(1)PCL7-16	6.5	1650	5.8	9400	5600	1.6	0.18
HB(2)PCL8-18	8.1	1990	8.2	16100	5200	1.9	0.20

[a] calculated from the ^{1}H-NMR spectrum of the product after ring opening polymerization

[b] calculated from the ^{1}H-NMR spectrum of the product after polycondensation

[c] chloroform; 25 °C

In Figure 7A, the 1.0-1.5 ppm region of the ^{1}H-NMR spectrum, and in Figure 7B the quaternary carbon region of the ^{13}C-NMR of HB(1)PCL7-16 are shown. The chemical shifts and signal intensities of HB(2)PCL8-18 (Figure 5A and 6A) and HB(1)PCL7-16 (Figure 7A and B) are comparable. The Mn of the polymer prepared via the 'one-pot' procedure is somewhat lower, as well as the N_{BP}. The molecular weights and molecular weight distributions obtained with GPC are comparable. These first results show that the 'one-pot' synthesis is a very promising method.

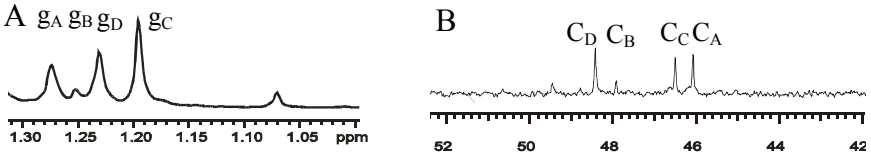

Figure 7. Detail of NMR spectra of HB(1)PCL7 by direct condensation: (A) 1.0-1.3 ppm region of the ^{1}H-NMR spectrum, (B) quaternary carbon region of ^{13}C-NMR spectrum (Solvent: $CDCl_3$). The notations g_A, g_B, g_C, and g_D refer to the methyl protons of the bis-MPA moiety of structures A, B, C, and D, respectively, in Figure 2, and the notations C_A, C_B, C_C, and C_D refer to the quaternary carbon atoms of structures A, B, C, and D, respectively, in Figure 2.

Thermal properties

Hyperbranched poly(ε-caprolactone)s with different condensation times

The thermal properties of the macromonomers and hyperbranched polymers were determined using DSC, and the results for the HB(2)PCL8-t and HB(2)PCL25-t polymers are presented in Tables 4 and 5. The crystallization temperature (T_c) and enthalpy (ΔH_c) were obtained from the cooling scan, while the T_g, T_m and ΔH_m were determined from the second heating scan (Figure 8).

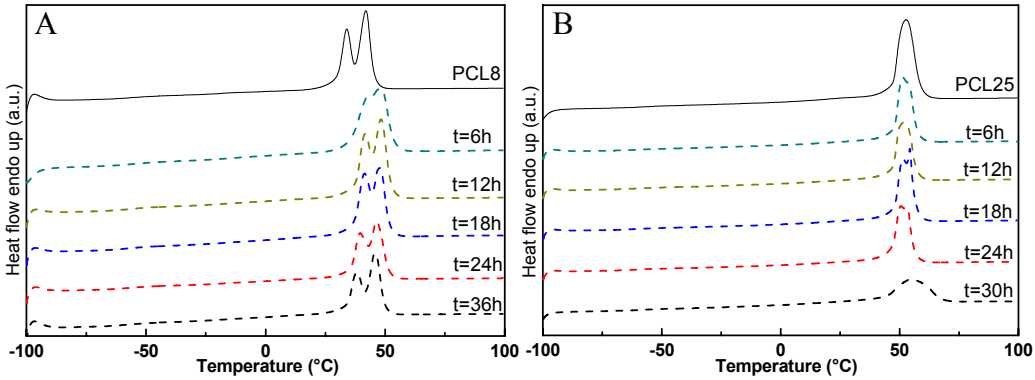

Figure 8. Second heating scans of (A) macromonomer PCL8 and its hyperbranched polymers HB(2)PCL8-t; and (B) macromonomer PCL25 and its hyperbranched polymers HB(2)PCL25-t.

All macromonomers and hyperbranched polymers exhibit a T_g around -60 °C, which is comparable with values of linear PCL[25]. The macromonomer PCL8 shows two overlapping melting peaks at 34 and 42 °C. This bimodal melting was reported previously for linear and branched PCL[26]. The hyperbranched polymers prepared from PCL8 also show two overlapping melting peaks, but at higher temperatures of approximately 41 and 48 °C. Both T_m and ΔH_m decreased with increasing condensation time. Furthermore, the T_c and the ΔH_c decreased with increasing condensation times. This decrease in crystallinity is due to the increased N_{BP} of the hyperbranched polymers at longer condensation times.

Table 4. Thermal properties of PCL8 and HB(2)PCL8-t, obtained after different condensation times t.

	T_g (°C)	T_m (°C)	ΔH_m ($J \cdot g^{-1}$)	T_c (°C)	ΔH_c ($J \cdot g^{-1}$)
PCL8	-57	34 / 42	74	7	70
HB(2)PCL8-6	-58	41 / 48	70	12	69
HB(2)PCL8-12	-60	42 / 48	65	11	63
HB(2)PCL8-18	-60	41 / 48	65	12	63
HB(2)PCL8-24	-60	40 / 46	59	10	59
HB(2)PCL8-36	-60	38 / 46	58	7	59

The macromonomer PCL25 had a T_m of 53 °C. Upon condensation, the T_m of the hyperbranched polymers remained constant, but the T_c showed a small decrease. The ΔH_m and ΔH_c also decreased, showing that upon hyperbranching the crystallinity of the polymers decreases.

Table 5. Thermal properties of PCL25 and HB(2)PCL25-t obtained after different condensation times t.

	T_g (°C)	T_m (°C)	ΔH_m ($J \cdot g^{-1}$)	T_c (°C)	ΔH_c ($J \cdot g^{-1}$)
PCL25	-61	53	73	22	73
HB(2)PCL25-6	-61	53	75	21	70
HB(2)PCL25-12	-62	53	73	21	69
HB(2)PCL25-18	-62	54	70	20	68
HB(2)PCL25-24	-63	53	68	17	61
HB(2)PCL25-36	-59	55	64	15	57

Hyperbranched poly(ε-caprolactone) via the 'one-pot' synthesis route

The thermal properties of the hyperbranched poly(ε-caprolactone) prepared via the 'one-pot' synthesis route were compared with the properties of HB(2)PCL8-18. The second heating scans of both hyperbranched polymers are plotted in Figure 9. A T_g of –56 °C was observed for HB(1)PCL7-16 and two T_m's at 39 and 47 °C, comparable with HB(2)PCL8-18. It can be concluded that the thermal properties were similar for polymers obtained via the two-step procedure and the 'one-pot' synthesis method.

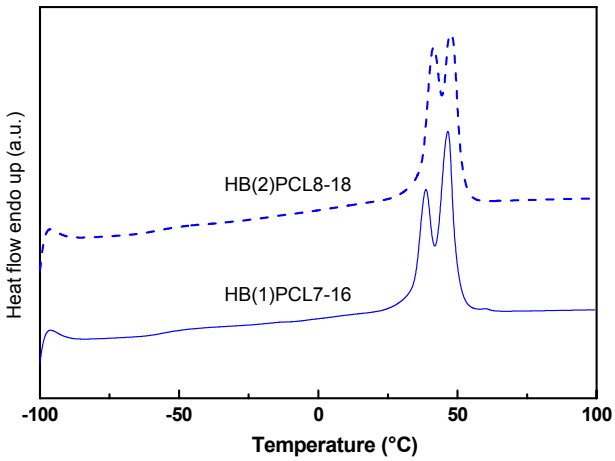

Figure 9. Second heating scans of HB(1)PCL7-16 via the 'one-pot' synthesis route and HB(2)PCL8-18 via the two-step procedure.

Conclusions

A facile 'one-pot' synthesis method was introduced for the preparation of hyperbranched poly(ε-caprolactone)s. This method consisted of the stannous octoate catalyzed ring opening polymerization of ε-caprolactone in the melt, in the presence of the AB_2 initiator 2,2-bis(hydroxymethyl)propionic acid (bis-MPA), and subsequent melt polycondensation under reduced pressure for 16 hours. This reaction resulted in hyperbranched poly(ε-caprolactone) with an average of 4.5 branching points per molecule, and an average DP of 7 caprolactone units per initiating bis-MPA hydroxyl group. Three series of hyperbranched poly(ε-caprolactone)s, based on macromonomers synthesized with different monomer to initiator to catalyst ratios, were prepared for structural analysis by ^{1}H- and ^{13}C-NMR. These results, together with the intrinsic viscosities, showed that upon increasing the condensation time, the number of branching points and the molecular weight increased. The number of branching points for macromonomer PCL25 was lower than for PCL6 and PCL8, prepared at the same condensation time, because the concentration of reactive groups is lower.

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Chapter 5

Synthesis and characterization of four-arm branched poly(L-lactide)-poly(ethylene glycol)-poly(L-lactide) copolymers

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Abstract

Four-arm branched poly(L-lactide)-poly(ethylene glycol)-poly(L-lactide) (PLLA-PEG-PLLA) block copolymers were prepared via the coupling reaction of α,ω-amine functionalized PEG and macromonomers containing two polylactide arms and a central carboxylic acid group. Aqueous solutions of these block copolymers (≥ 8 wt%) formed opaque hydrogels that showed a gel-sol transition upon heating. An increase in hydrophobic block length from 3 to 7 repeating lactide units per arm resulted in a higher gel-sol transition temperature. The critical gelation concentration decreased from 35 wt% for PLLA3-PEG1000-PLLA3 to 8 wt% for PLLA7-PEG2000-PLLA7. Furthermore, the gel-sol transition temperature increased with increasing copolymer concentration in water. Dilute aqueous solutions of the PLLA-PEG-PLLA block copolymers showed a critical association concentration of 0.032 - 0.035 w/v%, as determined with the hydrophobic dye solubilization method. Above this concentration, aggregates were formed with a Z-average diameter between 230 and 970 nm.

Introduction

Thermo-responsive hydrogels have attracted extensive attention as injectable drug delivery systems due to their transition from a fluid, free-flowing phase, the sol, at room temperature to a non-flowing state, the gel, at body temperature[1, 2]. Aqueous solutions of poly(ethylene glycol)-poly(propylene glycol)-poly(ethylene glycol)s (PEO-PPO-PEO), also known under the commercial name Pluronics, show both a lower sol-gel transition and an upper gel-sol transition upon heating[3, 4]. However, a drawback of Pluronics is its non-biodegradability, which limits its use in biomedical applications. This has challenged many researchers to design and investigate biodegradable copolymer systems which show a similar thermo-responsive gelation behavior. Block copolymers of poly(ethylene glycol) (PEG) and aliphatic polyesters, such as poly(lactide)s (PLA), poly(lactide-co-glycolide)s (PLGA), and poly(ε-caprolactone)s (PCL) resemble Pluronics in their structure and are biocompatible and biodegradable. The gelation behavior of copolymers containing PEG and aliphatic polyester segments was first reported in 1997[5, 6]. Copolymers that are water-soluble at low temperatures and give a phase transition at elevated temperatures were designed[6-11]. Further increase of the temperature led to formation of a sol phase and phase separation at higher temperatures. Such copolymers are characterized by their low molecular weight, generally below 5000 $g \cdot mol^{-1}$, and a PEG content of approximately 33 wt%. An increase in the lactide to glycolide ratio, while keeping the molecular weight of the PEG and the total molecular weight of the copolymer constant, led to a decrease in the lower sol-gel transition temperature and to an increase in the upper gel-sol transition temperature[8-11].

Besides triblock copolymers[6-11], copolymers with different architectures, such as grafted[12-14] and star-shaped copolymers[15] were investigated. Grafted PEG-g-PLGA and PLGA-g-PEG showed similar gelation behavior as triblock copolymers. Three-arm and four-arm star-shaped PLGA-PEG block copolymers, with PLGA as the core moiety, showed a critical gelation concentration in water that was higher than that of the linear PEG-PLGA-PEG copolymers[15].

Next to PLGA, other aliphatic polyesters were used in copolymers for the preparation of thermo-responsive hydrogels, such as poly(D,L-3-methyl glycolide)[16] and PCL[17-20]. PLA[21, 22] and poly[(*R*)-3-hydroxybuyrate][23] were used in the formation of multiblock copolymers. For example, a PEG-PCL-PEG triblock copolymer showed a lower CGC and a wider gel window than a PEG-

PLGA-PEG triblock copolymer of comparable molecular weight and PEG content[17], indicating that the nature of the hydrophobic block can alter the phase transition.

Applying the general design for the preparation of block copolymers that can show a thermo-responsive gelation behavior we synthesized PEG-PLLA block copolymers with a branched PLLA architecture. Polymers with a central linear PEG block and outer two-armed PLLA blocks with a maximal total molecular weight of 5000 g·mol^{-1} and a PEG content close to 33 wt% were synthesized and their thermo-responsive gelation behavior was studied.

Experimental

Materials

L-lactide (L-LA) was purchased from Purac (Gorinchem, the Netherlands). 2,2-Bis (hydroxymethyl)propionic acid (bis-MPA) and N,N'-dicyclohexylcarbodiimide (DCC) were obtained from Acros (Geel, Belgium). Dihydroxy poly(ethylene glycol)s, denoted as PEGy-OH (y is the molecular weight, and y is 1000, 1500 and 2000 g·mol^{-1}), tin(II) 2-ethylhexanoate ($Sn(Oct)_2$), mesyl chloride, and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Aldrich (Zwijndrecht, the Netherlands). 1-Hydroxybenzotriazole (HOBt), 1,8,9-trihydroxyanthracene (dithranol), and aqueous ammonia (25%) were obtained from Fluka (Buchs, Switzerland). Glacial acetic acid, triethylamine (TEA), and diisopropyl ether were obtained from Merck (Darmstadt, Germany). All other organic solvents were from Biosolve (Valkenswaard, the Netherlands). Prior to use, dichloromethane and toluene were dried over calcium hydride (Aldrich) and sodium wire, respectively, and subsequently distilled. All other chemicals were used as received.

Synthesis

PLLAn: PLLAn was synthesized as described previously[24]. In a typical procedure PLLA5 was prepared by adding L-lactide (25.0 g, 174 mmol) to a reaction vessel, which contained bis-MPA (2.33 g, 17 mmol) as the initiator and $Sn(Oct)_2$ (0.10 g, 0.25 mmol; 0.4 wt% based on L-lactide) as the catalyst. The mixture was allowed to react for 3 h at 130 °C under an argon atmosphere. The product was subsequently cooled to room temperature and dissolved in dichloromethane. To this solution, a small amount of glacial acetic acid was added, and the product was precipitated in an excess of cold diisopropyl ether. The product was collected by decantation, and

dried in vacuo to give a 'sticky' precipitate (Yield: 87 %). PLLA7 was obtained as a white powder.

PEGy-NH_2: Diamino PEG2000 (PEG2000-NH_2) was synthesized according to a procedure as described by Elbert and Hubbell[25]. In a typical procedure PEG2000-OH (25.0 g, 12.5 mmol) was dissolved in 700 ml of toluene and dried by the removal of 300 ml of solvent by azeotropic distillation. After the solution was cooled in an ice-bath, 50 ml of dichloromethane and TEA (10.6 ml, 75 mmol) were added. Subsequently, mesyl chloride (5.8 ml, 75 mmol) was added drop-wise under stirring and allowed to react overnight. The solution was filtered and precipitated in an excess of diethyl ether. After drying, the formed PEG2000-mesylate was reacted with 100 ml of an aqueous ammonia solution (25%) for 4 d at room temperature. Subsequently, the ammonia was allowed to evaporate and the pH of the solution was raised to 13, using 1 M NaOH. The solution was extracted with dichloromethane (50 ml) for 3 times. The dichloromethane extracts were combined and concentrated. The product was precipitated in cold diethyl ether, and dried in vacuo (Yield: 79 %).

PLLAn-PEGy-PLLAn: The synthesis of PLLA5-PEG2000-PLLA5 is given as a typical procedure: PLLA5 (3.0 g, 1.9 mmol) and PEG2000-NH_2 (1.9 g, 0.97 mmol) were dissolved in 60 ml of dichloromethane. To the resulting solution, DCC (0.48 g, 2.3 mmol) and HOBt (0.30 g, 1.9 mmol) were added. Subsequently, the reaction mixture was allowed to react for 24 h at room temperature under an argon atmosphere. The formed dicyclohexylurea was removed after the reaction by filtration. The clear solution was concentrated by partially evaporating the dichloromethane, and the polymer was precipitated in an excess of cold diisopropyl ether : methanol (10:1 v:v). The product was collected by decantation and dried in vacuo, and was obtained as a paste (Yield: 86 %). PLLA3-PEG1000-PLLA3 was a viscous oil, whereas PLLA7-PEG2000-PLLA7 was obtained as a powder.

Characterization

NMR: ^{1}H-NMR spectra were recorded on a 300 MHz Varian Inova NMR spectrometer. Polymers were dissolved in $CDCl_3$ at a concentration of 0.015 g·ml^{-1}.

Viscometry: Intrinsic viscosities [η] were determined by single point measurements using a capillary Ubbelohde type 0C at 25 °C and a polymer solution with a concentration of 0.3 g·dl^{-1} in chloroform. The following empirical relation was applied:

$$[\eta] = \frac{\sqrt{2}}{c} \cdot \sqrt{\eta_{sp} - \ln \eta_{rel}} \quad (1)$$

in which $\eta_{spec} = \eta_{rel} - 1$ and c is the polymer concentration in $g \cdot dl^{-1}$. The relative viscosity ($\eta_{rel} = t/t_0$) was determined from the flow time of the polymer solution (t) and the flow time of the solvent (t_0).

GPC: Molecular weights and molecular weight distributions of the polymers were determined with gel permeation chromatography (GPC) using chloroform as eluent. The GPC setup consisted of a Waters 510 pump, a HP Ti-Series 1050 auto sampler, a series of standard Waters Styragel HR columns, a Waters 410 differential refractometer, and a viscometer detector H502 (Viscotek Corp.). Polystyrene standards with narrow molecular weight distributions were used for calibration and the molecular weights were determined using the universal calibration principle.

MALDI-TOF: Matrix Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF) was performed using a Voyager-DE-RP 2010 MALDI-TOF mass spectrometer (Applied Biosystems/ PerSeptive Biosystems, Inc.) equipped with delayed extraction. A 337 nm UV nitrogen laser producing 2 ns pulses was used and the mass spectra of the polymers were obtained in the reflection mode. Samples were prepared by mixing ~2 mg polymer with 1 ml dichloromethane. After that, ~5 mg of dithranol was added as the matrix and the resulting solution was vigorously stirred. One µl of the solution was loaded on a gold sample plate. After evaporation of the solvent in air, the sample was transferred to the mass spectrometer for analysis.

DSC: Thermal analysis of the macromonomers and the copolymers was carried out using a Pyris 1 differential scanning calorimeter, calibrated with indium and gallium. During a measurement, the polymer (5-15 mg) was cooled to -50 °C and kept at this temperature for 1 min. The sample was then heated to 200 °C, annealed for 1 min, and subsequently cooled to -50 °C. Finally, the sample was kept at -50 °C for 5 min and heated to 200 °C again. The heating and cooling rate was 20 $°C \cdot min^{-1}$. Melting (T_m) and crystallization (T_c) temperatures were obtained from the peak maxima, melt (ΔH_m) and crystallization (ΔH_c) enthalpies were determined from the area under the curve and the glass transition temperature (T_g) was taken as the inflection point. The data presented are from the cooling and second heating step.

Aqueous solution properties

Vial tilting method: The phase behavior of aqueous polymer solutions was investigated by the vial tilting method. Block copolymers were dissolved in MilliQ water (6-40 wt%) in tightly capped 5 ml vials by repeatedly heating and stirring. The block copolymer solutions were kept at 4 °C overnight prior to measurement. The temperature was increased step-wise with 2 or 4 °C and the samples were left at the measuring temperature for 10 min to equilibrate. The sol-gel transition temperature was determined by tilting the vials 90° for 1 min. If there was no flow, the sample was regarded as a gel. In case of flow, the sample was regarded as a sol.

UV-VIS: The critical association concentration (CAC) of the copolymers in water was determined by the dye solubilization method at 20 °C. To 1.0 ml of the aqueous copolymer solutions with concentrations ranging from 1 to 0.0001 w/v%, 10 µl of a 0.5 mM DPH solution in methanol was added. The resulting mixture was stored in the dark and equilibrated over night. UV-VIS absorption spectra of the solutions were recorded in the 300 to 500 nm range. The difference in absorption at 378 nm relative to 403 nm was plotted against the polymer concentration and the intercept of the extrapolated straight lines was defined as the CAC of the copolymer.

DLS: Dynamic light scattering experiments were performed on a Malvern Zetasizer 4000 (Malvern Corp., Malvern, UK), using a laser wavelength of 633 nm and a scattering angle of 90° at 25 °C. The CONTIN method was applied for data processing.

Results and Discussion

Synthesis

Different methods to convert hydroxyl end-groups of PEGs into amine functional groups have been described in literature. One method is the Gabriel synthesis of primary amine end-groups by first converting the hydroxyl groups into p-toluenesulfonate esters, followed by nucleophilic displacement of tosyl groups with potassium phtalimide, and finally hydrazinolysis of the phtalimide groups into the corresponding amines[26]. In a second method, the hydroxyl groups are converted in their mesylates and then reacted with ammonia for 4 d at room temperature[25]. This latter method was applied in the synthesis of α,ω-diamino PEGs in high yields. The ^{1}H-NMR spectra of PEGy-NH_2's revealed that only amino end-groups were present, since only a signal of the CH_2-NH_2 protons was observed at 2.86 ppm (data not shown).

The $Sn(Oct)_2$ catalyzed ring opening polymerization of L-lactide in the presence of bis-MPA as an initiator is a convenient method to prepare 2,2-bis[ω-hydroxy poly(L-lactide) methyl]propionic acids, macromonomers that retain a carboxylic acid functional group (Figure 1). The conversion of lactide during the polymerization reaction was determined from the integral ratio of the C*H*$_3$ protons of the monomer (1.59 ppm) to polymer (1.65 ppm) in the ^{1}H-NMR spectra of the crude samples, and was always approximately 97 %, after a reaction time of 3 h. The degree of polymerization (DP), and the number average molecular weight (Mn) were calculated from the ^{1}H-NMR spectra of the purified PLLAn macromonomers (Figure 2A). The DP was determined from the ratio of the integrated area of the C*H* protons of the lactide repeating units (5.10 ppm) to the C*H*$_3$ protons of the bis-MPA moiety (1.27 ppm). The results obtained with ^{1}H-NMR were in good accordance with the theoretical values, based on the monomer to initatior [M]:[I] ratio (Table 1). Moreover, the ^{1}H-NMR spectral data gave no indication of remaining unreacted bis-MPA or bis-MPA in which only one of the hydroxyl groups had reacted.

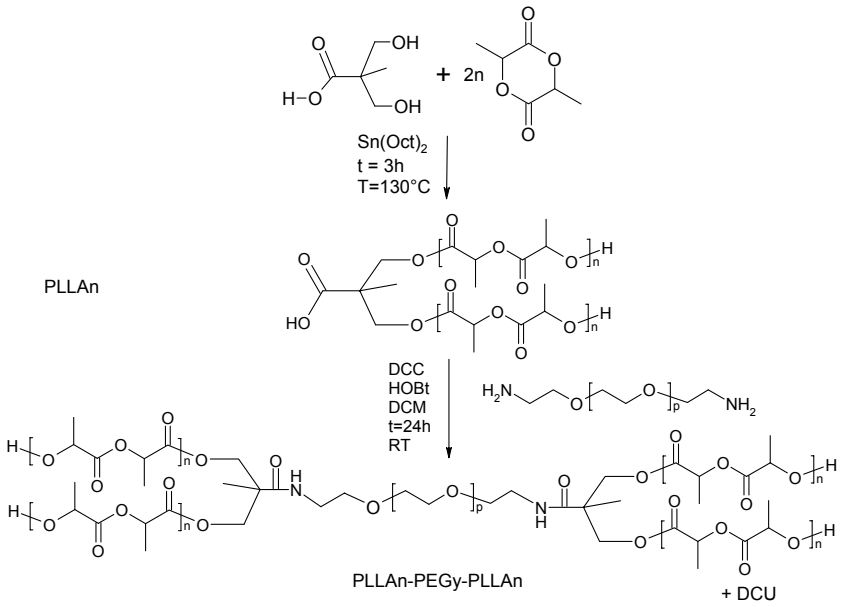

Figure 1. Synthesis scheme of macromonomers PLLAn, and the PLLAn-PEGy-PLLAn copolymers.

The molecular weights and molecular weight distributions (PDI) of the macromonomers with 3 to 7 repeating lactide units in both arms were determined with GPC and MALDI-TOF (Table 1). The Mn's determined by MALDI-TOF were in good agreement with the ^{1}H-NMR results. The molecular weights obtained by GPC measurements were a factor 1.4 higher than those determined from the ^{1}H-NMR spectra. This difference is also found for poly(lactide)s[27, 28]. The molecular weight distributions as measured with GPC ranged from 1.2 to 1.7 and were somewhat lower when determined with MALDI-TOF (PDI 1.2-1.3). The intrinsic viscosity was determined using single point measurements, and increased with increasing molecular weight.

Table 1. Molecular weight, molecular weight distribution and intrinsic viscosity of bis-MPA initiated PLLAn macromonomers.

	[M]:[I]	Mn (g·mol^{-1})				PDI (-)		$[\eta]^b$
	(-)	Calc[a]	NMR	MALDI	GPC	MALDI	GPC	(dl·g^{-1})
PLLA3	6	1000	1000	1200	1400	1.2	1.7	0.05
PLLA4	8	1290	1200	1200	2000	1.2	1.4	0.06
PLLA5	10	1580	1600	1700	2300	1.2	1.5	0.07
PLLA7	14	2150	2100	2300	2600	1.3	1.4	0.08

[a] calculation based on [M]:[I] ratio

[b] chloroform, 25 °C

The coupling of the amino end-functional PEGs with the carboxylic acid functional macromonomers (PLLAn) to yield the PLLAn-PEGy-PLLAn copolymers was performed in dichloromethane solution at room temperature, using HOBt and DCC as coupling agents. Structural analysis of the copolymers by ^{1}H-NMR revealed a quantitative conversion of amine and carboxylic acid end-groups. ^{1}H-NMR spectra showed a peak originating from the N*H* protons of the formed amide bonds at 6.59 ppm (g' in Figure 2B), and signals that belong to the PEG (h and i) and PLLA (a-e). Furthermore, the signal of the C*H*$_3$ protons of the bis-MPA moiety shifted from 1.27 to 1.22 ppm. The molecular weights as calculated from the ^{1}H-NMR spectral data (Table 2) are in good accordance with the theoretical values.

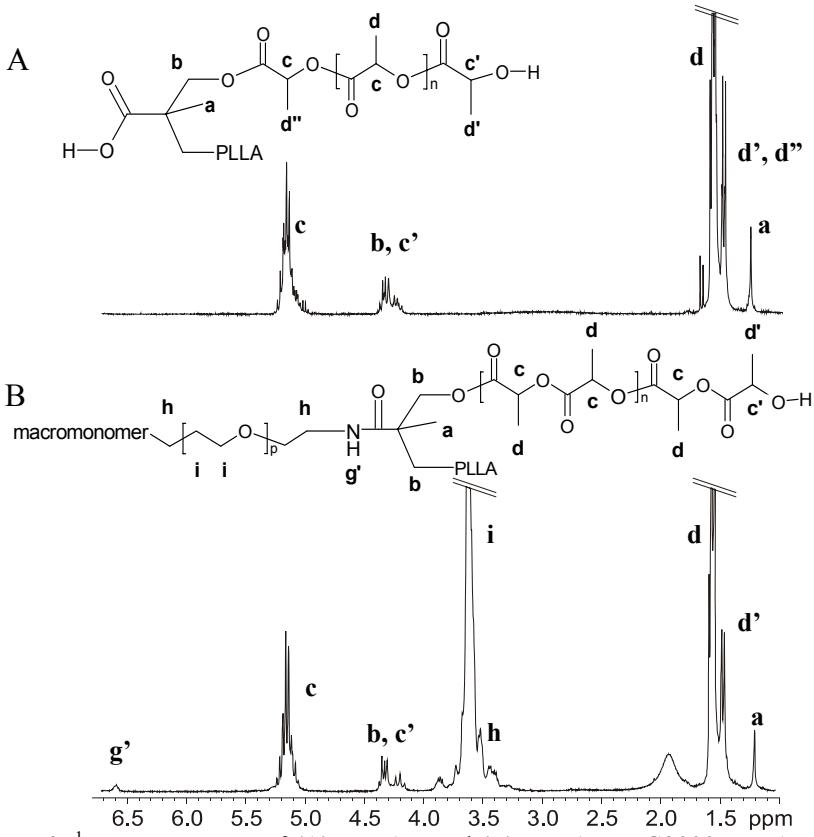

Figure 2. ^{1}H-NMR spectra of (A) PLLA5, and (B) PLLA5-PEG2000-PLLA5. Solvent: $CDCl_3$.

Table 2. Degree op polymerization (DP), molecular weight, and PEG content of PLLAn-PEGy-PLLAn copolymers.

PLLAn-PEGy-PLLAn		Theoretical		^{1}H-NMR		
y	n	Mn_{tot}	PEG cont.	DP_{PLLA}	Mn_{tot}[a]	PEG cont.[b]
(g·mol^{-1})	(-)	(g·mol^{-1})	(wt%)	(-)	(g·mol^{-1})	(wt%)
1000	3	3000	33	3.2	3100	37
1500	4	4050	37	4.3	4200	39
1500	5	4650	33	4.8	4500	37
2000	5	5150	39	4.8	5000	44
2000	7	6300	32	6.7	6100	35

[a] calculated as: $Mn_{tot} = 2 \cdot$ Mn PLLAn $+ 1 \cdot$ y

[b] calculated from the relative integral ratio of the peaks corresponding to the C*H* protons of PLLA (5.14 ppm) and the CH_2 protons of PEG (3.64 ppm)

Thermal properties

The thermal properties of the macromonomers and the copolymers were studied using DSC. The second heating scans and the cooling scans of the macromonomers PLLA5 and PEG2000-NH_2, and the block copolymer PLLA5-PEG2000-PLLA5 are plotted in Figure 3 as typical examples. The thermograms of the PLLA macromonomers showed the absence of crystallinity, due to the low degree of polymerization of the PLLA. Moreover, only a T_g was observed, which increased with increasing molecular weight from 20 to 34 °C with increasing DP of the PLLA arms from 3 to 7[24]. The amine end-functionalized PEGs showed a single crystallization and melting peak in the cooling and second heating scan, respectively (Table 3).

All copolymers showed a T_g (Table 3) that was lower than the T_g of the PLLA macromonomers, and higher than that of amorphous PEG (T_g ~ -60 °C)[29]. The measured T_g's are therefore considered to belong to a mixed amorphous phase of PEG and PLLA. In the PLLA3-PEG1000-PLLA3, PLLA4-PEG1500-PLLA4 and PLLA5-PEG1500-PLLA5 copolymers, crystallization of PEG was hampered by the coupled PLLA blocks. The T_m values of PLLA5-PEG2000-PLLA5 and PLLA7-PEG2000-PLLA7, 46 and 65 °C, respectively, suggest that crystallization of the PLLA blocks becomes possible in these block copolymers.

Table 3. Thermal properties of PEGy-NH_2 and PLLAn-PEGy-PLLAn copolymers.

	T_g (°C)	T_m (°C)	ΔH_m ($J \cdot g^{-1}$)	T_c (°C)	ΔH_c ($J \cdot g^{-1}$)
PEG1000-NH_2	-	45	140	19	128
PEG1500-NH_2	-	51	143	20	132
PEG2000-NH_2	-	55	147	20	133
PLLA3-PEG1000-PLLA3	-15	-	-	-	-
PLLA4-PEG1500-PLLA4	-14	-	-	-	-
PLLA5-PEG1500-PLLA5	-10	-	-	-	-
PLLA5-PEG2000-PLLA5	-22	46	1	-	-
PLLA7-PEG2000-PLLA7	-7	65	9	37[a]	10[a]

[a] observed as cold crystallization in the second heating scan

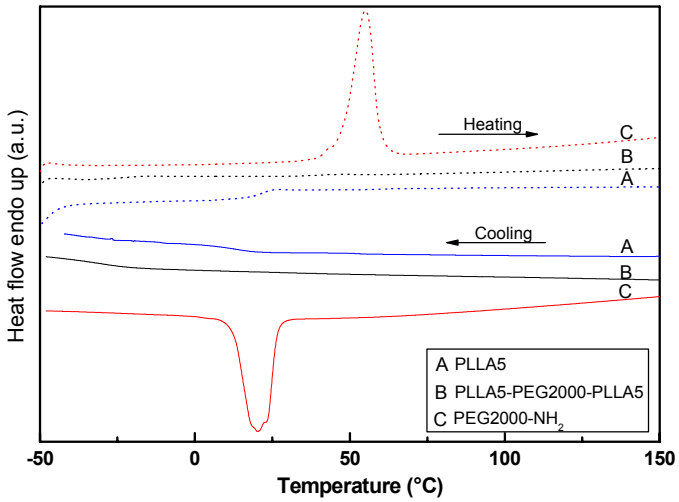

Figure 3. Second heating scans (dotted lines) and cooling scans (solid lines) of (A) PLLA5, (B) PLLA5-PEG2000-PLLA5 and (C) PEG2000-NH_2.

Aqueous solution properties

In different studies it was shown that aqueous solutions of triblock copolymers, with a central PEG block and outer polyester blocks, show sol-gel transitions at high concentrations upon heating[8, 16, 18]. Such copolymers are characterized by their low molecular weight (< 5000 g·mol^{-1}), and the PEG content is generally in between 25 and 42 wt%. The PLLAn-PEGy-PLLAn block copolymers described here have a PEG content in between 35 and 44 wt%. The main difference in the structures of the triblock copolymers and these four-armed PLLAn-PEGy-PLLAn copolymers is the branching and the length of the PLLA blocks, as a result of the bis-MPA central moiety.

The temperature dependent gelation behavior of aqueous solutions of PLLAn-PEGy-PLLAn copolymers was studied by the vial tilting method upon heating the copolymer solutions step-wise to 80 °C (Figure 4). At low temperatures, the solutions and hydrogels were either translucent or opaque, depending on the copolymer and concentration, whereas at high temperatures, they formed all opaque, phase-separated sols. The copolymer PLLA3-PEG1000-PLLA3 with a PEG content of 37 wt% formed a translucent solution in water, and only showed a gel-sol phase transition at low temperatures and concentrations (CGC) above 35

wt% (data not shown). Increasing the PLLA block length from 3 to 5 repeating lactide units per arm, and the PEG block length from 1000 to 1500 g·mol^{-1}, while retaining the PEG content at 37 wt%, shifted the CGC from 35 to 10 wt% (Figure 4). Moreover, the gel-sol transitions of PLLA5-PEG1500-PLLA5 were observed at much higher temperatures (40 to 74 °C) than those of PLLA3-PEG1000-PLLA3 (4 to 12 °C). Increasing the length of the PLLA block at the same PEG block length led to a decrease in CGC and an increase in gel-sol transition temperature. For example, a 16 wt% PLLA4-PEG1500-PLLA4 hydrogel showed a gel-sol transition at 46 °C, whereas the transition temperature of a 16 wt% PLLA5-PEG1500-PLLA5 hydrogel was at 52 °C. The CGC decreased from 12.5 to 10 wt%.

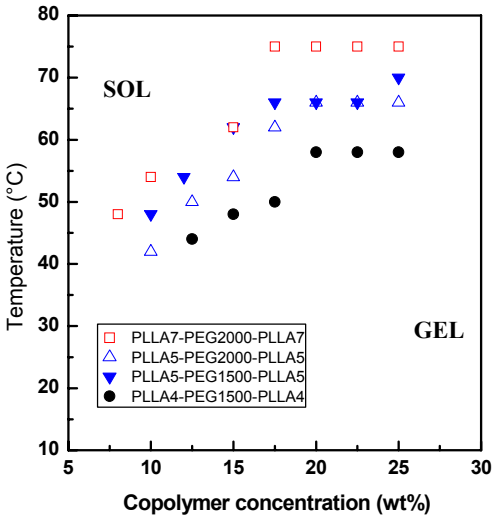

Figure 4. Gel-sol transition diagram of PLLAn-PEGy-PLLAn copolymers in water. Transitions were determined by heating the gel phase.

The lower CGC and higher transition temperature of copolymers with longer hydrophobic blocks is in accordance with the fact that copolymers with longer hydrophobic blocks exhibit a larger gel window than hydrogels from copolymers with shorter hydrophobic blocks[6, 15]. The transition temperature also increased with increasing copolymer concentration, up to approximately 20 wt%. At higher concentrations the gel-sol transition was hardly dependent on the concentration and the gel-sol transition occurred at the same temperature[18, 19, 30]. An increase in the molecular weight of the hydrophilic block only slightly affected the transition

temperature. For example, the PLLA5-PEG2000-PLLA5 showed a gel to sol transition at somewhat lower temperatures than PLLA5-PEG1500-PLLA5. Remarkably, the prepared four-arm copolymers did not show a sol to gel transition at low temperatures, as is observed for linear and star-shaped PLGA-PEG copolymers in water[8, 9, 15], although the PEG content and the total molecular weight of the copolymer is comparable.

A 12.5 wt% PLLA4-PEG1500-PLLA4 hydrogel was subjected to an oscillatory rheology experiment. The storage (G') and loss (G") moduli were measured upon heating (Figure 5). At 20 °C, the G' and G" were approximately 8 Pa. Upon heating, first an increase was observed in both G' and G", with maxima of 18 and 22 Pa, respectively, between 36 and 39 °C. A further increase in the temperature resulted in a decrease in both G' and G". This is comparable with the rheological behavior of linear and star-shaped PLGA-PEG copolymers. Furthermore, in the 36-55 °C region, G' is smaller than G", although visually a gel state is observed in the vial tilting experiment. This is contradictory to the mechanical properties of gels, which have a higher storage modulus than loss modulus[31]. However, a similar phenomenon was observed for PEG-PLGA-PEG copolymers[10].

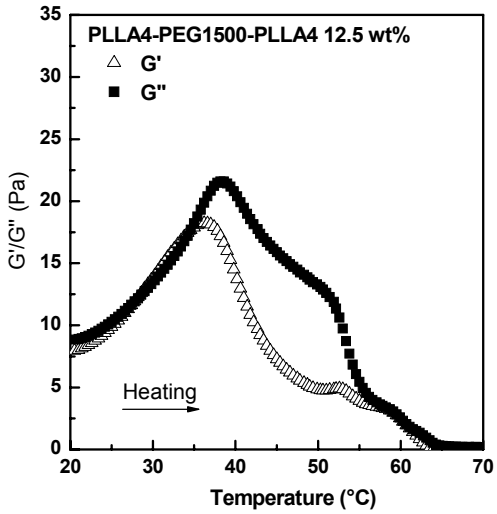

Figure 5. The storage (G') and loss (G") modulus of a 12.5 wt% aqueous solution of PLLA4-PEG1500-PLLA4, upon heating from 20 to 70 °C.

Because the solutions and hydrogels at concentrations between 5 and 25 wt% appeared translucent or even opaque, the critical association concentration (CAC) and particle sizes at higher dilution were determined. The CAC of the copolymers in water was determined with the hydrophobic DPH dye solubilization method using UV-VIS. At 20 °C, it was observed that the CACs of all copolymers were in between 0.032 and 0.035 w/v% (Table 4). Furthermore, the CAC only slightly decreased when longer PLLA blocks were attached to PEG. For example, PLLA7-PEG2000-PLLA7 had a lower CAC than PLLA5-PEG2000-PLLA5. The CAC also decreased when PEG of a lower molecular weight was used with the same PLLA block length. In summary, the CAC was always lower for the more hydrophobic copolymers, since their tendency to associate is higher.

Table 4. CAC at 20 °C for PLLAn-PEGy-PLLAn copolymers in water.

y ($g \cdot mol^{-1}$)	n (-)	$CAC_{20\,°C}$ (w/v%)
1000	3	0.032
1500	4	0.035
1500	5	0.033
2000	5	0.035
2000	7	0.032

Dynamic light scattering revealed that aqueous solutions with copolymer concentrations above the CAC formed relatively large aggregates with a Z-average particle size between 270 and 930 nm, and no micelles were detected. These relatively large particles explain the observed turbidity of the aqueous solutions and may be the reason for the relatively weak gels formed at high concentrations.

Conclusions

Four-armed branched poly(L-lactide)-poly(ethylene glycol)-poly(L-lactide) (PLLA-PEG-PLLA) copolymers were prepared via $Sn(Oct)_2$ catalyzed ring opening of L-lactide using bi-functional 2,2-bis(hydroxymethyl)propionic acid as the initiator, forming PLLA macromonomers, and subsequent coupling reactions of these macromonomers with α,ω-amine functionalized PEG, using HOBt and DCC as coupling agents. The resulting PLLA-PEG-PLLA copolymers in water gave turbid sols or gels, depending on the temperature and concentration. Above the critical gel

concentration (CGC) (≥ 8 wt%), the copolymers in water formed hydrogels at low temperature and showed a gel-sol transition when the temperature was raised. Increasing the hydrophobic block length resulted in a lower CGC and a higher transition temperature. A lower sol-gel transition was not observed. A critical association concentration was found at low concentrations (0.032-0.035 w/v%). Above this concentration, aggregates were formed with a Z-average diameter between 270 and 930 nm.

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Chapter 6

Thermo-responsive hydrogels based on branched poly(L-lactide)-poly(ethylene glycol) copolymers

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Abstract

Branched poly(L-lactide)-poly(ethylene glycol) (PLLA-PEG) block copolymers were synthesized from trifunctional PLLA and amine functionalized methoxy poly(ethylene glycol)s. The copolymers in water formed hydrogels that showed thermo-responsive behavior. The hydrogels underwent a gel to sol transition with increasing temperature as determined with the vial tilting method and oscillatory rheology. For all copolymers, the transition temperature increased with increasing copolymer concentration. The transition temperature of the branched copolymers also increased with increasing PEG molecular weight, and surprisingly decreased with increasing molecular weight of the PLLA branches. In general, the gel-sol transition is explained by disruption of micellar or aggregate interactions because of partial dehydration and shrinkage of the PEG chains. An increase in the molecular weight of the PLLA branches led to the formation of micelles and aggregates as observed with DLS at low concentrations. It is speculated that the non-uniform size distribution and possible crystallization of longer PLLA blocks may have a negative effect on the formation of micellar packing upon gelation, allowing the disruption of micellar or aggregate interactions to occur at lower temperatures. The transition temperature of the gels could be tuned closely to body temperature by varying the concentration of the solution or the molecular weight of the PEG block and the

PLLA blocks, which implies that these polymers may be used as injectable systems for in-situ gel formation.

Introduction

Thermo-responsive hydrogels have received much interest for their potential use in tissue engineering and as drug delivery systems[1, 2]. Upon injection of a thermo-sensitive polymer solution into the body the temperature change can cause a transition from the fluid state, the sol, to the immobile state, the gel. This temperature response is based on the formation or disruption of physical crosslinks, such as entanglements or hydrophobic interactions. The thermo-responsive behavior of poly(ethylene oxide-poly(propylene oxide)-poly(ethylene oxide) (Pluronics or Poloxamers) was widely investigated[3-5]. With increasing temperature, Pluronics first show a sol-gel transition, followed by a gel-sol transition. The mechanism of the sol-gel transition is based on the packing of micelles in a long-range order, such as a cubic crystalline phase. The gel-sol transition is caused by dehydration of poly(ethylene oxide) (PEG) at higher temperatures which decreases the interaction between micelles and/or aggregates. A possible drawback of the Pluronic systems is that they are non-biodegradable. This prompted investigators to design and evaluate thermo-responsive hydrogels based on block copolymers of PEG and biodegradable polyesters, such as poly(lactide)s (PLA), poly(lactide-glycolide)s (PLGA) and poly(ε-caprolactone)s (PCL). These copolymers can be conveniently synthesized by ring opening polymerization of lactide, glycolide or ε-caprolactone, using hydroxy-PEG's as initiators. Alternative preparation methods include the coupling of diblock copolymers using a difunctional spacer, such as hexamethylene diisocyanate, and the coupling of pre-synthesized polyesters to PEG, using DCC and DMAP as coupling agents[6, 7]. Thermo-responsive hydrogels based on PEG-polyester diblock copolymers, and triblock copolymers with PLLA or PLGA as the central block were investigated by the group of Kim[6, 8, 9]. A single gel to sol transition was observed when increasing the temperature. The gel-sol transition could be adjusted by changing the concentration of the solution and the composition of the block copolymer. In short, the gel-sol transition occurred at lower concentrations as the hydrophobicity of the polyester block increased, either by increasing the molecular weight or by changing the ratio of lactide to glycolide in the hydrophobic block. The gel to sol transition is caused by disruption of the micellar packing due to shrinkage of the PEG corona at higher temperatures. This shrinkage is caused by

partial dehydration of PEG, since water is a poorer solvent for PEG at higher temperatures[7, 8]. By changing the composition of the PEG-PLGA-PEG copolymers to copolymers with a PEG content close to 33 wt%, and a total molecular weight of approximately 5000 $g \cdot mol^{-1}$, triblock copolymer solutions were found to form a sol state at room temperature, and a gel at body temperature[10]. This sol-gel-sol transition behavior was comparable to the Pluronics systems. The low molecular weight inversed triblock copolymers with a central hydrophilic block were prepared by PEG-initiated ring opening polymerization. These PLGA-PEG-PLGA also exhibit sol-gel-sol transition behavior in water at concentrations between 10 and 30 wt%[11]. Ring opening polymerization using PEG as the initiator was also used to prepare high molecular weight copolymers with PLLA as the outer blocks[12-15]. These copolymers were able to form hydrogels at low temperature, and were investigated for their ability to form hydrogels via stereocomplexation with their corresponding PDLA-PEG-PDLA copolymer[13-15]. Hiemstra et al.[15] also investigated the thermo-responsive behavior of hydrogels of these PLLA-PEG-PLLA copolymers, by using the vial tilting method and oscillatory rheology. These hydrogels showed thermo-responsive gelation, and the gel-sol transition temperature increased with the copolymer concentration. A triblock PLLA-PEG-PLLA copolymer with 7.5 repeating lactide units per arm, and a PEG content of 85 wt% showed almost the same transition temperature as its corresponding eight-armed star-shaped PEG-PLLA copolymer having the same PLLA block length, but a PEG content of 74 wt%. This indicates that the gel-sol transition temperature is largely influenced by the PLLA block length. The influence of the copolymer architecture was also investigated for copolymers with PLLA as the central block. Star-shaped PLLA-PEG block copolymers with PLLA as the hydrophobic core were prepared by Park et al.[7]. Hydrogels of these three-arm star-shaped copolymers showed thermo-responsive gelation, with a gel to sol transition when the temperature was increased, as measured with the vial tilting method. The critical gelation concentration (CGC) decreased from 20 to 10 wt% when the PLLA block length of PLLA-PEG5000 copolymers increased from 5 to 9 repeating lactide units. The transition temperature increased with increasing copolymer concentration, and increasing molecular weight of the hydrophilic or hydrophobic block. The influence of the copolymer architecture was investigated by comparing the CGC of a star-shaped copolymer with that of a triblock copolymer with the same PEG content and

the same PEG molecular weight per block. It was found that the CGC of star-shaped PLLA-PEG was about 5 wt% lower than that of the linear copolymers.
In this paper, the synthesis and characterization of branched PLLA-PEG block copolymers are described, as well as the formation of thermo-responsive hydrogels from aqueous solutions of these block copolymers.

Experimental

Materials

L-lactide (L-LA) was purchased from Purac (Gorinchem, the Netherlands). 2,2-Bis (hydroxymethyl)propionic acid (bis-MPA), N,N'-dicyclohexylcarbodiimide (DCC) and succinic anhydride were obtained from Acros (Geel, Belgium). Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$), N-hydroxysuccinimide (NHS), mesyl chloride, and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Aldrich (Zwijndrecht, the Netherlands). Methoxy-hydroxy poly(ethylene glycol)s with molecular weights of 2 and 5 $kg \cdot mol^{-1}$ (mPEG2000-OH and mPEG5000-OH, respectively) and aqueous ammonia (25%) were obtained from Fluka (Buchs, Switzerland). Glacial acetic acid, 4-dimethylaminopyridine (DMAP), and triethylamine (TEA) were obtained from Merck (Darmstadt, Germany). All other organic solvents were from Biosolve (Valkenswaard, the Netherlands). Dichloromethane and toluene were dried over calcium hydride (Aldrich), and sodium wire, respectively, and distilled prior to use. All other chemicals were used as received.

Synthesis

Hydrophobic PLLA macromonomers (PLLAn, with n is the number of repeating lactide units per arm) were synthesized by first ring opening polymerization of L-lactide in the presence of bis-MPA as the initiator in the melt, and subsequent reaction of the hydroxyl end groups with succinic anhydride to afford carboxylic acid end-groups (CPLLAn), which were finally activated by conversion into their NHS-esters (NHS-CPLLAn). The hydrophilic PEG blocks with amine end-groups (mPEGy-NH_2, with y is the molecular weight in $g \cdot mol^{-1}$) were synthesized from the corresponding hydroxyl functional PEGs.

PLLAn: In a typical procedure PLLA10 was prepared by adding L-lactide (25.0 g, 174 mmol) to a reaction vessel, which contained bis-MPA (1.16 g, 8.7 mmol) as the initiator and $Sn(Oct)_2$ (0.10 g, 0.25 mmol; 0.4 wt% based on L-lactide) as the catalyst. The mixture was allowed to react for 3 h at 130 °C under an argon

atmosphere. The product was subsequently cooled to room temperature and dissolved in dichloromethane. To this solution, a small amount of glacial acetic acid was added, and the product was precipitated in an excess of cold diethyl ether. The product was collected by filtration, and dried in vacuo to give a white powder (Yield: 86 %).

CPLLAn: The synthesis of CPLLA10 is given as a typical procedure: PLLA10 (25.0 g, 8.3 mmol), succinic anhydride (1.99 g, 19.9 mmol), DMAP (1.22 g, 10.0 mmol), and TEA (1.68 g, 16.6 mmol) were dissolved in 200 ml of dichloromethane, and stirred for 24 h under an argon atmosphere at room temperature. The solvent was partially evaporated with a rotary evaporator and the polymer was precipitated in a diethyl ether : methanol (10:1 v:v) mixture and dried in vacuo over night. The product was obtained as a white powder (Yield: 88 %).

NHS-CPLLAn: The synthesis of NHS-CPLLA10 is given as a typical procedure: CPLLA10 (20 g, 6.5 mmol) was dissolved in 200 ml of dichloromethane. To the resulting solution, NHS (2.67 g, 23.2 mmol) and DCC (5.98 g, 29.0 mmol) were added. Subsequently, the reaction mixture was allowed to react for 18 h at room temperature under an argon atmosphere. The formed dicyclohexylurea (DCU) was removed after the reaction by filtration. The clear solution was concentrated by partially evaporating the dichloromethane, and the polymer was precipitated in an excess of cold diethyl ether : methanol (10:1 v:v). The product was dried in vacuo over night to give a white powder (Yield: 88 %).

mPEGy-NH_2: mPEGy-NH_2 was synthesized according to a procedure as described by Elbert and Hubbell[16]. In a typical procedure, mPEG2000-OH (25 g, 12.5 mmol) was dissolved in 700 ml of toluene and dried by the removal of 350 ml of solvent by azeotropic distillation. After the solution was cooled in an ice-bath, 25 ml of dichloromethane and TEA (5.3 ml, 37.5 mmol) were added. Subsequently, mesyl chloride (2.9 ml, 37.5 mmol) was added drop-wise under stirring and allowed to react overnight. The solution was filtered and the product was precipitated in a large excess of diethyl ether. After drying, the formed mPEG2000-mesylate was reacted with 100 ml of an aqueous ammonia solution (25%) for 4 d at room temperature. Subsequently, the ammonia was allowed to evaporate and the pH of the solution was raised to 13, using 1 M NaOH. The solution was extracted with dichloromethane (100 ml) for 3 times. The dichloromethane extracts were combined and concentrated. The mPEG2000-NH_2 was isolated by precipitation in cold diethyl ether, and drying in vacuo (Yield: 78 %).

PLLAn-PEGy: The synthesis of PLLA10-PEG5000 is given as a typical procedure: NHS-CPLLA10 (0.90 g, 0.22 mmol) and mPEG5000-NH_2 (3.24 g, 0.66 mmol) were dissolved in 80 ml of dichloromethane, and stirred for 24 h at room temperature under argon. The resulting solution was concentrated by partially evaporating the solvent, and precipitated in an excess of cold diethyl ether : methanol (10:1). The product was dried in vacuo and was obtained as a white powder (Yield: 95 %).

Characterization

NMR: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR spectra were recorded on a Varian Inova NMR spectrometer. Polymers were dissolved in $CDCl_3$ at a concentration of 0.015 g·ml^{-1} (^{1}H) or 0.2 g·ml^{-1} (^{13}C).

DSC: Thermal analysis of the macromonomers and the copolymers was carried out using a Pyris 1 differential scanning calorimeter, calibrated with indium and gallium. During a measurement, the polymer (5-15 mg) was cooled to -50 °C and kept at this temperature for 1 min. The sample was then heated to 200 °C, annealed for 1 min, and subsequently cooled to -50 °C. Finally, the sample was kept at -50 °C for 5 min and heated to 200 °C. The heating and cooling rate was 20 °C·min^{-1}. Melting (T_m) and cold crystallization (T_{cc}) temperatures were obtained from the peak maxima in the second heating scan. The melt (ΔH_m) and cold crystallization (ΔH_{cc}) enthalpies were determined from the area under the curve. The glass transition temperature (T_g) was taken as the inflection point. The crystallization temperature (T_c) and enthalpy (ΔH_c) were obtained from respectively the peak maximum and the area under the curve in the cooling scan.

Aqueous solution properties

Vial tilting method: The phase behavior of aqueous polymer solutions was investigated by the vial tilting method. Block copolymers were dissolved in MilliQ water (10-40 wt%) in tightly capped 5 ml vials by repeatedly heating to ~80 °C for 2 min and stirring while cooling. The block copolymer solutions were kept at 4 °C overnight prior to measurement. The temperature was increased step-wise with 2 or 4 °C and the samples were left at the measuring temperature for 10 min to equilibrate. The gel-sol transition temperature was determined by tilting the vials 90° for 1 min. If there was no flow, it was regarded as a gel state. In other cases it was regarded as a sol state.

Rheology: Rheology experiments were performed on a TA instruments AR1000 rheometer with a flat plate geometry (20 mm diameter, 0.5 mm gap) in oscillating mode. Aqueous polymer solutions were prepared by dissolving the appropriate amount of polymer by repeatedly heating to ~80 °C for 2 min and stirring while cooling. The polymer solutions were then applied on the rheometer and heated to 60 or 70 °C. To prevent evaporation of water, a solvent trap was placed over the geometry. A pre-shear was applied for 10 s, after which the polymer solution was allowed to equilibrate for 6 min. Subsequently, the polymer solution was cooled to 20 °C at 1 °C·min^{-1}, and then heated to 60 or 70 °C at 1 °C·min^{-1}. Gelation of the polymer solutions was monitored by measuring both the storage modulus G' and the loss modulus G" as a function of temperature. A system was considered a gel if G' was larger than G". The temperature at which G' and G" become equal is considered to be the transition temperature. A frequency ω of 1 Hz and a strain γ of 1 % were applied to minimize the influence of deformation on the formation of the hydrogels. After the cooling and heating cycles, an amplitude and frequency sweep were performed at γ 0.01-10 % (ω = 1 Hz) and ω 0.01-10 Hz (γ = 1 %) at 20 °C, to confirm that the applied ω of 1 Hz and the γ of 1 % was within the linear viscoelastic range.

UV-VIS: The critical association concentration (CAC) of the copolymers in water at 20 °C was determined by the dye solubilization method. To 1.0 ml of aqueous copolymer solutions with concentrations ranging from 1 to 0.0001 w/v%, 10 µl of a 0.5 mM DPH solution in methanol was added. The resulting mixture was stored in the dark and equilibrated over night. UV-VIS absorption spectra of the solutions were recorded in the 300 to 500 nm range. The difference in absorption at 378 nm relative to 403 nm was plotted against the polymer concentration and the intercept of the extrapolated straight lines was defined as the CAC of the copolymer.

DLS: Dynamic light scattering experiments were performed on a Malvern Zetasizer 4000 (Malvern Corp., Malvern, UK), using a laser wavelength of 633 nm and a scattering angle of 90°. The CONTIN method was applied for data processing. The micelle or aggregate size of copolymers in water was determined as a function of temperature in the 20 to 50 °C range. The aqueous solution was allowed to equilibrate at each measuring temperature for 45 min.

Results and discussion

Synthesis and characterization of PLLA-PEG copolymers

The synthesis of the mPEGy-NH_2's was performed according to a procedure as described by Elbert and Hubbel[16]. Hydroxy end-functionalized mPEGs were converted to their corresponding mesylates in high yield. The mesylates were quantitatively converted into amines by reaction with aqueous ammonia for 4 d at room temperature. The ^{1}H-NMR spectra of mPEGy-NH_2's revealed that only amino end-groups were present, since only a signal of the CH_2-NH_2 protons was observed at 2.86 ppm (data not shown).

PLLA macromonomers bearing one carboxylic acid group and two hydroxyl groups (Figure 1) were synthesized by the $Sn(Oct)_2$ catalyzed ring opening polymerization of L-lactide using bis-MPA as the initiator. The number of repeating lactide units per arm, n, was varied from 10 to 25 by varying the monomer to initiator ratio ([M]:[I]). The integral ratio of the CH_3 protons of the monomer (1.59 ppm) to polymer (1.65 ppm) in the ^{1}H-NMR spectra of the crude samples was used to determine the conversion. In all cases, after 3 h reaction time, high monomer conversions of approximately 97% were obtained.

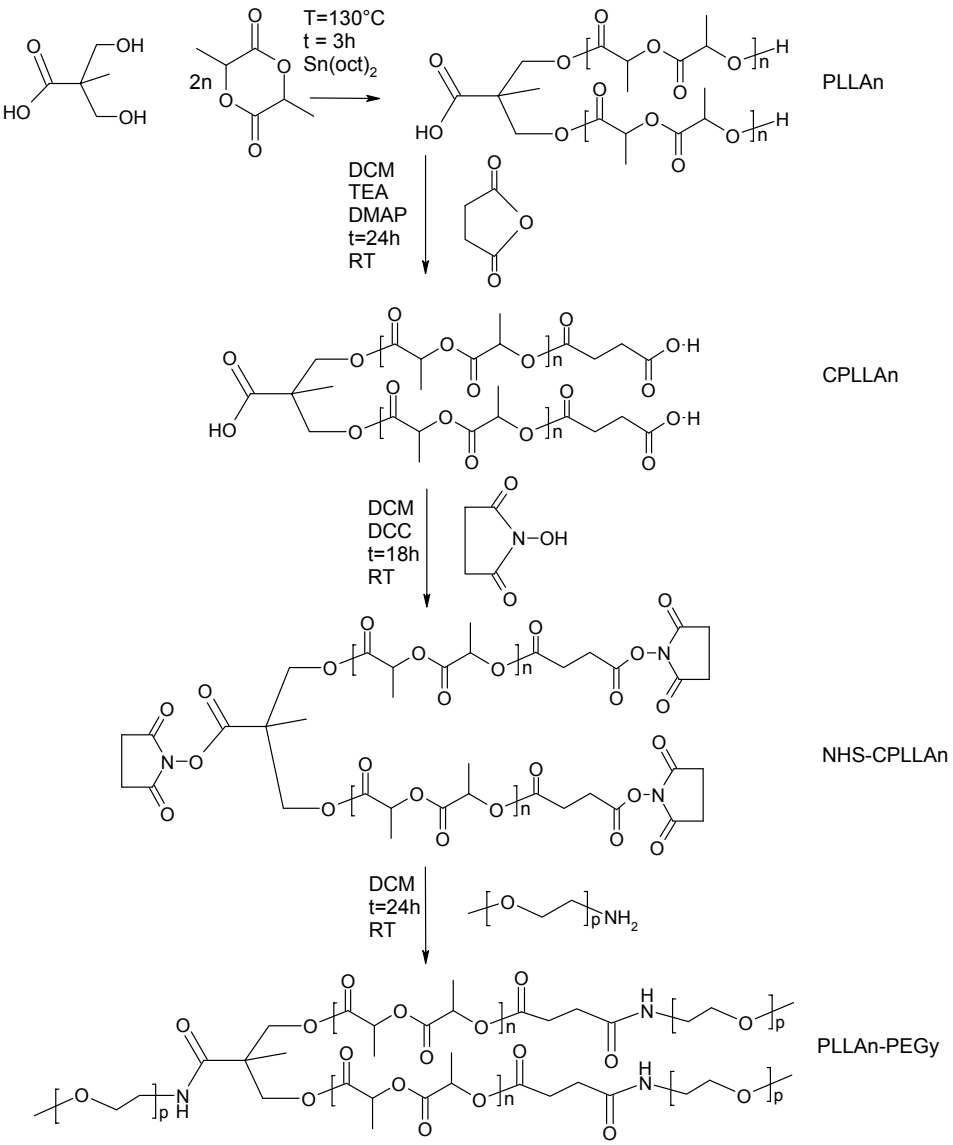

Figure 1. Schematic synthesis route for the preparation of PLLAn-PEGy copolymers.

The ^{1}H-NMR spectra of the purified PLLAn macromonomers (Figure 2A) were used to calculate the degree of polymerization (DP), and the number average molecular weight (Mn). The DP was determined from the ratio of the integrated area of the C*H* protons of the lactide repeating units (5.10 ppm) to the CH_3 protons of the bis-MPA moiety (1.27 ppm). The results obtained with ^{1}H-NMR (Table 1) are in good agreement with the theoretical values, based on the [M]:[I] ratio.

Table 1. ^{1}H-NMR results and calculated molecular weight of PLLAn , CPLLAn and NHS-CPLLAn.

	Calc[a]	^{1}H-NMR	
	Mn (g·mol^{-1})	DP (-)	Mn (g·mol^{-1})
PLLA10	3020	10	2900
PLLA15	4460	15	4300
PLLA20	5900	20	6000
PLLA25	7340	25	7500
CPLLA10	3220	11	3500
CPLLA15	4660	14	4400
CPLLA20	6100	19	5900
CPLLA25	7540	25	7400
NHS-CPLLA10	3560	12	4200
NHS-CPLLA15	5000	16	5300
NHS-CPLLA20	6450	21	6700
NHS-CPLLA25	7890	28	8700

[a] calculated from the [M]:[I] ratio

The hydroxyl end-groups of PLLAn were reacted with succinic anhydride to yield carboxylic acid end-groups (Figure 1). ^{1}H-NMR spectra (Figure 2B) showed the disappearance of the signals at 4.30 ppm (c', C*H*-OH) and 1.49 ppm (d', CH(CH_3)-OH), belonging to the protons of the terminal lactic acid unit, and the appearance of a new peak at 2.68 ppm (e), corresponding to the CH_2 protons of the succinic half ester. Furthermore, the signal belonging to the CH_3 protons of the bis-MPA moiety shifted from 1.27 ppm to 1.21 ppm. The integral ratio of the signals a, b, and e is 3:4:8 revealing a high conversion, and is in good accordance with the values based on the macromonomer structure (Table 1).

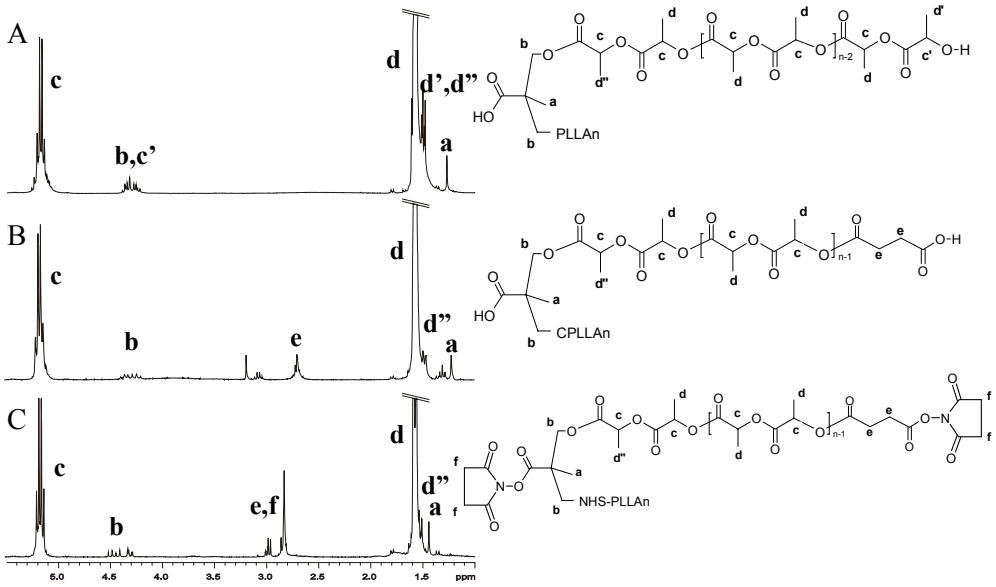

Figure 2. ^{1}H-NMR spectra of (A) PLLA10; (B) CPLLA10; and (C) NHS-CPLLA10. Solvent: $CDCl_3$.

Active esters were successfully generated by the reaction of the three carboxylic acid groups with NHS (Figure 1). ^{1}H-NMR spectra showed the appearance of a new signal originating from the CH_2 protons of the succinimide ring at 2.83 ppm (Figure 2C, peak f). The CH_2 protons of the succinic ester appeared as two triplets (e, 2.83 ppm and 2.95 ppm). Additionally, the signal corresponding to the CH_3 protons of the bis-MPA moiety (a) shifted from 1.21 ppm to 1.44 ppm, and the multiplet belonging to the CH_2 protons of the bis-MPA moiety shifted (b) from 4.20-4.40 to 4.25-4.55 ppm. High conversions were obtained, based on the integral ratio of the signals b, e, and f.

The ^{13}C-NMR spectral data of PLLAn, CPLLAn, and NHS-CPLLAn support the conclusions based on ^{1}H-NMR spectral data as discussed above. As a typical example, the spectrum of NHS-CPLLA20 is shown in Figure 3, and shows three major signals corresponding to the carbonyl, methine and methyl carbons (a, b, and c) of the poly(lactide) arms. The signals with low intensity correspond to the carbons of the bis-MPA (d, e and f), the succinic ester (h and i), and the succinimide moieties (j and k). The insert shows the 160-180 ppm carbonyl region of PLLA20 (A), CPLLA20 (B), and NHS-CPLLA20 (C). The signals at 176 and 172 ppm in

spectrum A and B belong to carboxylic acid carbon atoms. The disappearance of these peaks, and the appearance of multiple peaks around 170 ppm (Spectrum C) confirm that all carboxylic acid groups are converted to their active NHS-esters.

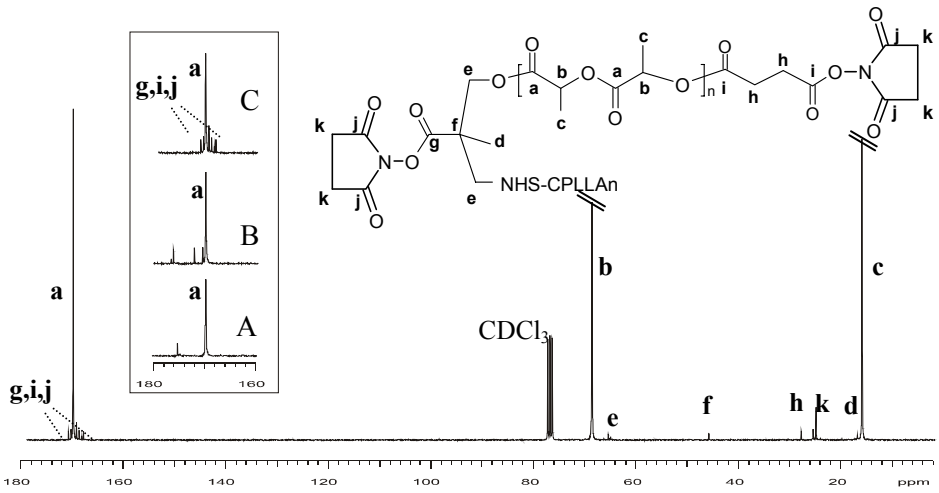

Figure 3. ^{13}C-NMR spectrum of NHS-CPLLA20. Solvent: $CDCl_3$. The insert shows the 160-180 ppm region of (A) PLLA20; (B) CPLLA20; and (C) NHS-CPLLA20.

The coupling reaction of NHS-CPLLAn and mPEGy-NH_2 in dichloromethane at room temperature afforded PLLAn-PEGy copolymers. ^{1}H-NMR spectra showed peaks originating from the N*H* protons of the formed amide bonds at 6.32 ppm and 6.45 ppm (g and g' in Figure 4), and signals that belong to the PEG (h, i, and j) and PLLA (a-e). Based on the complete disappearance of the signal belonging to the CH_2 protons of the succinimide ring, it is concluded that the coupling reaction was complete. Additionally, the CH_3 protons of the bis-MPA shifted from 1.44 ppm to 1.20 ppm (a), and the multiplet of the CH_2 protons of the bis-MPA shifted from 4.25-4.55 ppm to 4.10-4.35 ppm (b). Furthermore, the multiplets of the CH_2 protons of the succinic ester (e) shifted from 2.83 ppm and 2.95 ppm to 2.48 ppm and 2.70 ppm, respectively. The calculated and determined molecular weights based on the ^{1}H-NMR data are presented in Table 2.

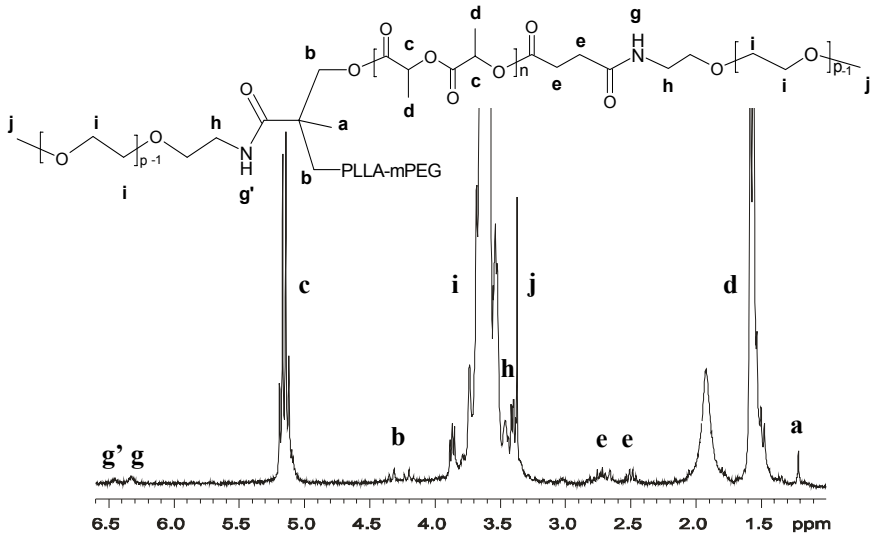

Figure 4. ^{1}H-NMR spectrum of PLLA10-PEG5000. Solvent: $CDCl_3$.

Table 2. Degree of polymerization (DP), molecular weight and PEG content of PLLAn-PEGy copolymers.

		Theoretical		^{1}H-NMR		
y	n	Mn_{tot}	PEG cont.	DP $_{PLLA}$	Mn_{tot}[a]	PEG cont.[b]
(g·mol^{-1})	(-)	(g·mol^{-1})	(wt%)	(-)	(g·mol^{-1})	(wt%)
2000	10	9200	65	9.8	9200	62
2000	15	10650	56	15.4	10800	57
2000	20	12100	50	21.3	12500	46
2000	25	13550	44	27.1	14100	41
5000	10	18200	82	10.8	18400	80
5000	15	19650	76	14.2	19400	75
5000	20	21100	71	21.1	21400	73
5000	25	22550	67	27.1	23100	69

[a] calculated as Mn_{tot} = Mn PLLA + 3·y

[b] calculated from the relative integral ratio of the peaks corresponding to the methine protons of PLLA (5.14 ppm) and the CH_2 protons of PEG (3.64 ppm)

The thermal properties of the macromonomers and the copolymers are listed in Tables 3 and 4, respectively. As typical examples, the second heating scans and the cooling scans of copolymer PLLA10-PEG2000, and the macromonomers NHS-

CPLLA10 and mPEG2000-NH_2 are shown in Figure 5. A T_g was observed for all NHS-CPLLAn macromonomers, which increased with increasing molecular weight. Melting and cold crystallization were observed for these macromonomers with $n \geq 15$, and increased with molecular weight. These T_m's, T_c's and T_g's are similar to values found for the corresponding AB_2 functional PLLAn, as obtained after ring opening polymerization (Figure 1) (data not shown). The mPEGy-NH_2 macromonomers showed melting and crystallization temperatures that were comparable to the corresponding hydroxyl functionalized mPEG's.

Table 3. Thermal properties of macromonomers NHS-CPLLAn and mPEGy-NH_2.

	T_g (°C)	T_m (°C)	ΔH_m ($J \cdot g^{-1}$)	T_c (°C)	ΔH_c ($J \cdot g^{-1}$)
NHS-CPLLA10	49	-	-	-	-
NHS-CPLLA15	53	140	2	119[a]	1
NHS-CPLLA20	54	146	6	119[a]	6
NHS-CPLLA25	54	154	13	125[a]	10
mPEG2000-NH_2	-	55	155	24	144
mPEG5000-NH_2	-	62	165	31	159

[a] observed as the cold crystallization peak in the second heating scan

In the second heating scan of the copolymers, a T_g was not observed, due to the low PLLA content in the block copolymers. A melting and crystallization peak was found for the PEG-rich phase in all cases and for the PLLA-rich phase in most cases (Table 4). The copolymer with the highest PEG content of 80 wt% (PLLA10-PEG5000) did not show crystallization of the PLLA. Interestingly, the PLLA10-PEG2000 copolymer exhibited both a T_m and T_c of the PLLA-rich phase, contrary to the corresponding NHS-CPLLA10 macromonomer (Figure 5). All other copolymers showed a T_m of the PLLA-rich phase, and of the PEG-rich phase that was lower than the T_m of the corresponding macromonomers, due to partial phase mixing.

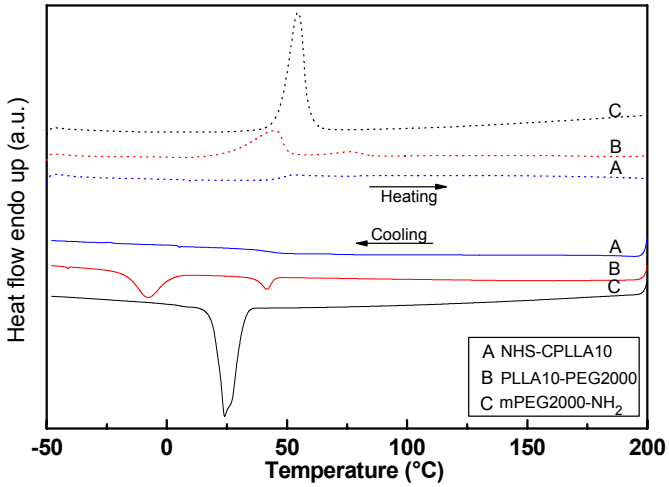

Figure 5. Second heating scans (dotted lines) and cooling scans (solid lines) of (A) NHS-CPLLA10; (B) PLLA10-PEG2000; and (C) mPEG2000-NH_2.

Table 4. Thermal properties of the PLLAn-PEGy copolymers.

		PLLA				PEG			
y	n	T_m	ΔH_m	T_c	ΔH_c	T_m	ΔH_m	T_c	ΔH_c
($g \cdot mol^{-1}$)	(-)	(°C)	($J \cdot g^{-1}$)	(°C)	($J \cdot g^{-1}$)	(°C)	($J \cdot g^{-1}$)	(°C)	($J \cdot g^{-1}$)
2000	10	77	7	42	9	45	55	-8	45
2000	15	87	10	49	12	43	46	3	35
2000	20	134	24	78	20	42	43	13	42
2000	25	150	26	87	22	40	34	14	32
5000	10	-	-	-	-	57	103	16	87
5000	15	81	4	42	5	52	84	19	83
5000	20	129	12	50	2	56	93	21	90
5000	25	147	11	51	2	57	79	24	80

Gel formation

All PLLAn-PEGy copolymers were soluble in water when n was 10 or 15. The PLLA20-PEG5000 copolymer was soluble, whereas the PLLA20-PEG2000 was not, due to its too high hydrophobic content. Both PLLA25-PEG2000 and PLLA25-PEG5000 copolymers were insoluble in water. All water-soluble copolymers provided transparent solutions or gels, depending on concentration and temperature. The temperature dependent gelation behavior of aqueous solutions of PLLAn-PEGy

copolymers was studied by the vial tilting method in a temperature range of 4-70 °C. The gel-sol transition diagram in Figure 6 shows that all hydrogels turned into a mobile phase, the so-called 'sol', upon heating. It was observed that for all copolymers the gel-sol transition temperature increased with increasing concentration, and that a copolymer concentration of at least 22.5 to 27.5 wt% in water was necessary to form a hydrogel. Furthermore, the gel-sol transition diagram in Figure 6 shows that the gel-sol transition temperature is dependent on the PEG block length. For example, a 30 wt% PLLA10-PEG2000 hydrogel exhibited a gel-sol transition at 22 °C, whereas this transition was found at 56 °C for a PLLA10-PEG5000 hydrogel. Previous studies on linear and star-shaped block copolymers with a central PLLA block and outer PEG blocks[6, 7], as well as block copolymers with a central multi-arm PEG block[15] revealed an increase in the gel-sol transition temperature with increasing hydrophobic block length. Surprisingly, the PLLAn-PEGy copolymers give a reversed behavior, a decreasing gel-sol transition temperature, with increasing PLLA block length. For example, the gel-sol transition temperatures of 30 wt% solutions of PLLAn-PEG5000 copolymer are 56, 46 and 34 °C for n is 10, 15 and 20, respectively.

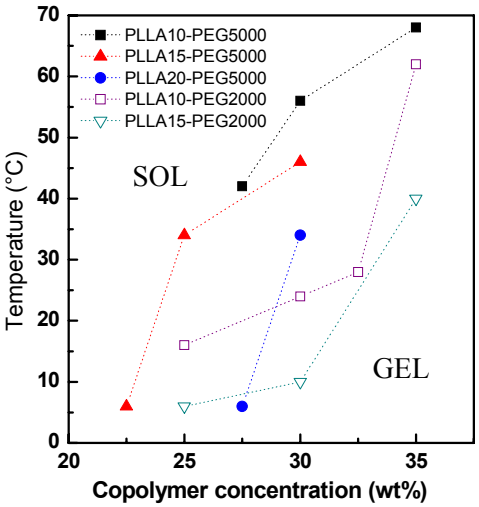

Figure 6. Gel-sol transition diagram of PLLAn-PEGy copolymers in water.

The thermo-responsive behavior of the hydrogels was also studied by oscillatory rheology experiments on aqueous copolymer solutions. The storage (G') and loss (G") moduli were monitored as a function of temperature, when cooling the

polymer solution from 70 °C to 20 °C. A system was considered a gel when G' was larger than G". The temperature of the cross-over point was considered as the transition temperature[17]. The storage and loss moduli of aqueous solutions of PLLA10-PEG5000 at concentrations of 25, 27.5 and 30 wt% are plotted as a function of temperature in Figure 7. At 70 °C, the G' of all solutions was lower than the G", and thus, the copolymer solutions were regarded as sol. While cooling, both G' and G" increased and showed a cross-over point, and thus, the solutions showed a transition from sol to gel. At 20 °C, all solutions formed hydrogels, with a G' increasing from 6 to 17 kPa, with increasing copolymer concentration. Moreover, the transition temperature increased with increasing copolymer concentration, which is in accordance with the vial tilting test results.

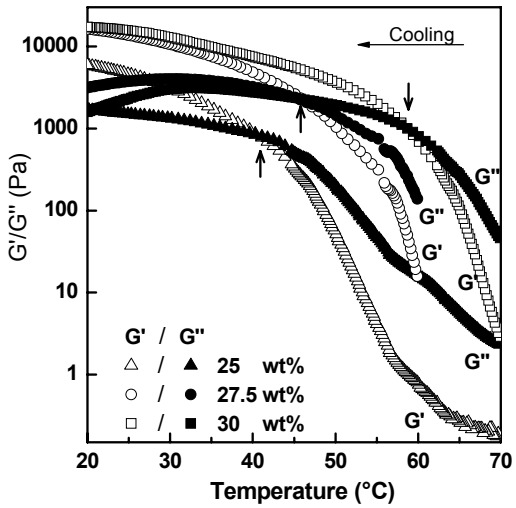

Figure 7. The storage (G') and loss (G") modulus of 25, 27.5 and 30 wt% aqueous solutions of PLLA10-PEG5000, upon cooling from 70 to 20 °C. The arrows indicate the sol-gel transition temperature.

A considerable higher temperature for the cross-over point of G' and the G" was found for a 30 wt% PLLA10-PEG5000 solution compared to a 30 wt% PLLA10-PEG2000 solution (Figure 8). Moreover, the storage modulus at 20 °C of the PLLA10-PEG5000 (17 kPa) was about three times higher than that of the PLLA10-PEG2000 (6.5 kPa) copolymer.

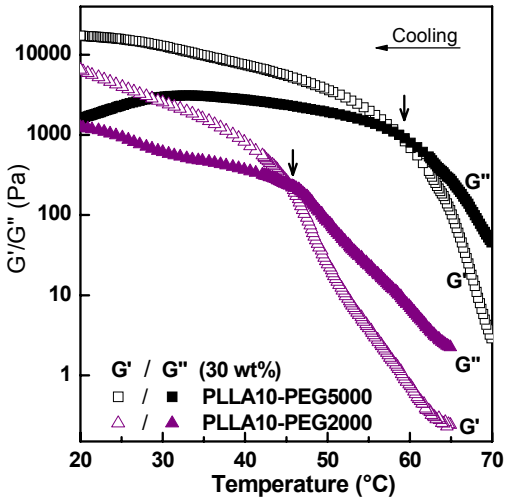

Figure 8. The storage (G') and loss (G") modulus of 30 wt% aqueous solutions of PLLA10-PEGy copolymers, upon cooling from 70 to 20 °C. The arrows indicate the sol-gel transition temperature.

The rheology experiments revealed a decreasing sol to gel transition temperature with increasing PLLA block length (Figure 9). It also has to be noted that at 20 °C, the storage modulus of gels with the same PEG chain length decreased almost two orders of magnitude when the PLLA block length was doubled.

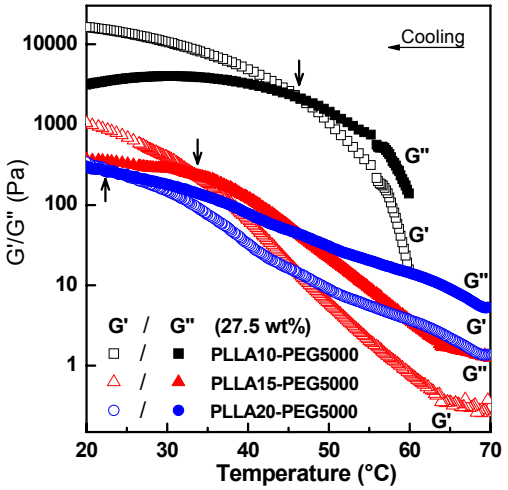

Figure 9. The storage (G') and loss (G") modulus of 27.5 wt% aqueous solutions of PLLAn-PEG5000 copolymers, upon cooling from 70 to 20 °C. The arrows indicate the sol-gel transition temperature.

Mechanism of the gel-sol transition

The critical association concentration (CAC) of the copolymers in water was measured by UV using the hydrophobic dye DPH. All CAC values of the water-soluble copolymers were in between 0.02 and 0.03 w/v% (Figure 10). The CAC decreased by increasing the hydrophobic block length, or decreasing the hydrophilic block length of the copolymer. Thus, the CAC decreases if the copolymers become more hydrophobic, and therefore tend to associate more readily. The CAC of copolymers PLLA10-PEG2000 and PLLA20-PEG5000 appeared to be independent on the temperature within the 20-50 °C temperature range (data not shown).

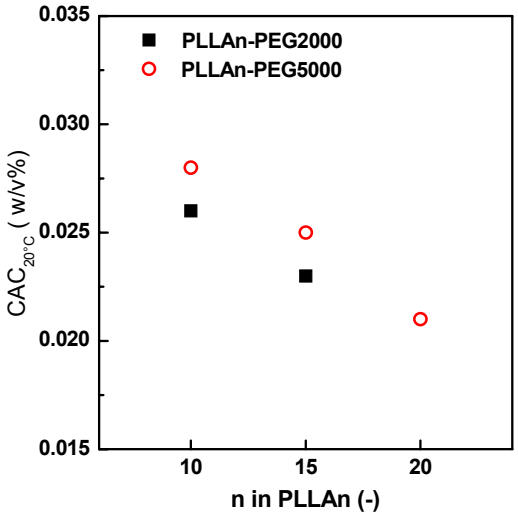

Figure 10. Critical association concentration (w/v%) of PLLAn-PEGy copolymers in water at 20 °C.

The aggregate size and aggregate size distributions of the copolymers in aqueous solutions were determined using dynamic light scattering (DLS) at a 90° angle, and the results of PLLA10-PEG2000 and PLLA20-PEG5000 at different temperatures are presented as typical examples in Figure 11A and B. The intensity plot of PLLA10-PEG2000 at 25 °C (Figure 11A) shows that mainly micelles with an average diameter of 14 nm are present. A second distribution was observed at 50 nm, which is attributed to small micellar aggregates. It should be emphasized that the intensity of scattered light can not directly be related to the number of particles, since the intensity of light scattered by larger particles is larger than that of smaller particles.

The PLLA20-PEG5000 solution consisted mainly of small micellar-like aggregates with an average diameter of 55 nm (Figure 11B). A second distribution of larger size aggregates of ~200 nm was also found. The shift in size to micellar-like aggregates results from both the higher molecular weight of the copolymer and the length of the hydrophobic block.

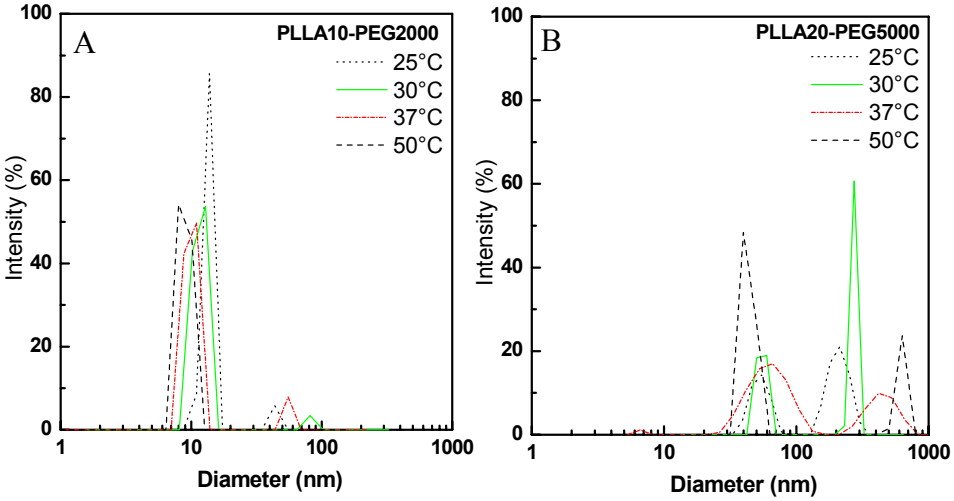

Figure 11. Intensity plot of aqueous solutions of (A) PLLA10-PEG2000 (1 w/v%) and (B) PLLA20-PEG5000 (0.3 w/v%) at various temperatures.

Furthermore, it was observed for all aqueous copolymer solutions that the size of the micelles or smaller aggregates was decreasing with increasing temperature, which is attributed to the shrinkage of the PEG corona upon partial dehydration[7]. On the other hand, the size of the large aggregates became larger, due to a more favored association of the micelles at higher temperatures that are no longer shielded off by the PEG corona. In Figure 12, the average diameter of the micelles and aggregates of the copolymers in water at 25 °C are plotted as a function of the PLLA block length. It is observed, that the PLLAn-PEG2000 copolymers in water formed smaller micelles and aggregates (Figure 12A) than the corresponding PLLAn-PEG5000 copolymers (Figure 12B), which can be attributed to the smaller PEG chains. Furthermore, an increase was observed in the micelle and especially the aggregate size when increasing the PLLA block length from 10 to 15 repeating lactide units.

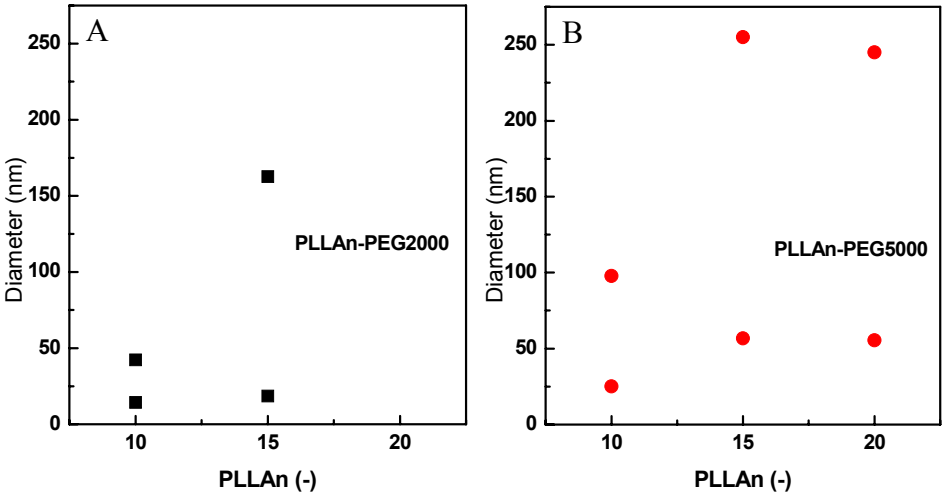

Figure 12. Diameter of the micelles and aggregates in copolymer solutions (0.3-1 w/v%) at 25 °C plotted as a function of the PLLA block length, with n is the average number of repeating lactide units per arm for (A) PLLAn-PEG2000, and (B) PLLAn-5000.

The gel-sol transition is proposed to be a result of breaking of the micelle packing structure, due to a decrease in effective diameter of the micelles, which is caused by partial dehydration of the PEG[7]. This mechanism is supported by the decrease in micellar size upon heating. Moreover, the higher molecular weight PEG chains have a higher degree of entanglement in the micellar packing, which hampers the transition to the sol phase, as observed from higher gel-sol transition temperatures. It further seems that the gel-sol transition temperature and the storage and loss moduli depend on the uniformity of micellar particles in solution. The presence of larger aggregates may have a negative influence on the packing of micelles into a gel state upon cooling, and irregular packing will result in lower storage and loss moduli than of a packing of micelles of a uniform size. This may be an explanation for the surprising decrease in transition temperature with increasing PLLA block length. Further, it can not be excluded that crystallization takes place in the aqueous state. The previously mentioned triblock and multi-arm copolymers[6, 7, 15], and the three-armed PLLAn-PEGy described here show a major difference in the degree of crystallization of the hydrophobic PLLA block in the solid state. At a similar PEG content in the block copolymers, the eight-arm PEG-PLLA copolymers are non-crystalline, whereas the three-arm PLLAn-PEG5000 copolymers show

crystallization of the PLLA segments (Table 4). It is speculated that the increased hydrophobic interactions upon increasing temperature may lead to crystallization of PLLA, for longer PLLA blocks, and apparently leads to lower transition temperatures of the gels. It is therefore suggested that crystallization may have a negative effect on the stability of the hydrogels as compared to the previous systems. It is further noted that no degradation is observed for all copolymers during the time period of the measurements.

Conclusions

Branched poly(L-lactide)-poly(ethylene glycol) (PLLA-PEG) copolymers were conveniently synthesized from trifunctional PLLA of controlled molecular weight and amine functionalized methoxy poly(ethylene glycol)s. These copolymers were able to form thermo-responsive hydrogels in water at high concentrations (> 22.5 wt%). The gel-sol transition behavior was investigated with the vial tilting method and oscillatory rheology. The transition temperature increased with increasing copolymer concentration, or increasing PEG molecular weight, for copolymers with corresponding PLLA block lengths. The gel-sol transition is considered to be due to partial dehydration of the PEG. Surprisingly, the transition temperature decreased when the molecular weight of the hydrophobic block increased. It is speculated that the non-uniform size distribution of the micelles and aggregates, for longer PLLA blocks at low concentrations, and possible PLLA crystallization may have a negative effect on the micelle packing, resulting in lower transition temperatures, and lower storage and loss moduli. The transition temperature could be tuned closely to body temperature by varying the concentration of the solution or the molecular weight of the PEG block and the PLLA block, which makes these hydrogels of interest as injectable systems for biomedical applications.

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Chapter 7

Thermo-responsive hydrogels based on highly branched poly(ethylene glycol)-poly(L-lactide) copolymers

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Abstract

Highly branched poly(ethylene glycol)-poly(L-lactide) (PEG-PLLA) copolymers were synthesized by a coupling reaction of eight-arm amine-functionalized PEG and macromonomers having 2 PLLA arms and a N-hydroxysuccinimide activated ester group at the branching point. A coupling efficiency of approximately 50 % was achieved, based on ^{1}H-NMR and the amount of free amine groups present after the reaction as determined with a TNBS assay. Copolymer solutions in water (≥ 4 wt%) showed a phase transition from gel to sol upon increasing the temperature. The critical gelation concentration at 20 °C decreased with increasing PLLA block length. The gel-sol transition of a 12 wt% aqueous copolymer solution increased from 4 °C for a copolymer with PLLA blocks with 3 repeating lactide units per arm to 38 °C for a copolymer with PLLA blocks with 5 repeating lactide units per arm. Furthermore, the gel-sol transition temperature increased with increasing copolymer concentration, and could be tuned closely to body temperature, which makes these hydrogels interesting as injectable systems for in-situ gel formation.

Introduction

Hydrogels that respond to stimuli of the environment are widely investigated for their use in drug delivery and tissue engineering[1, 2]. Among these stimuli sensitive hydrogels, thermo-responsive hydrogels are widely investigated[3, 4]. The best-known examples are hydrogels based on poly(ethylene oxide)-poly(propylene oxide)-(polyethylene oxide) (PEO-PPO-PEO), commercially known as Pluronics or Poloxamers, and hydrogels based on poly(N-isopropylacrylamide) (PNIPAAM) copolymers[5-8]. These copolymers in water form free flowing liquids at low temperatures, whereas they form gels at higher temperatures, which makes them interesting as in-situ forming drug delivery systems. However, a possible drawback of these systems is that they are non-degradable. This encouraged researchers to develop thermo-responsive hydrogels based on biodegradable block copolymers, consisting of poly(ethylene glycol) (PEG) and aliphatic polyesters, such as poly(lactide)s (PLA), poly(lactide-glycolide)s (PLGA), and poly(ε-caprolactone)s, that provide degradability by the hydrolysis of ester bonds.

Thermo-responsive hydrogels that are gels at low temperature and show gel-sol transitions upon increasing the temperature were prepared from BAB triblock copolymers with a central hydrophobic polyester (A) and hydrophilic PEG outer blocks (B), as described by the research group of Kim[9, 10]. On the other hand, ABA triblock copolymers with PEG as the hydrophilic core moiety were conveniently prepared by the ring opening polymerization of lactide, glycolide or ε-caprolactone, using α,ω-dihydroxy PEG as the initiator[11-15]. The thermo-responsive gelation behavior of such PLLA-PEG-PLLA copolymers as determined with the vial tilting method[14] revealed that the critical gel concentration at room temperature decreased from 80 to 10 wt% by increasing the length of the lactide blocks from 5 to 9.5 repeating lactide units. Triblock copolymers with PCL or poly(δ-valerolactone) (PVL) as the hydrophobic outer blocks were also investigated[15]. A PCL-PEG-PCL copolymer at a copolymer concentration of 38 wt% in water showed a gel-sol transition temperature of 42 °C, higher than that of a PVL-PEG-PVL copolymer (20 °C) at the same concentration, which was attributed to a higher hydrophobicity of the PCL blocks.

The triblock copolymer systems described above have a relatively high molecular weight (Mn > 10000 g·mol^{-1}) and a high PEG content (≥ 50 wt%). By changing the composition of the ABA and BAB triblock copolymers to a PEG content close to 33 wt%, and a total molecular weight of 5000 g·mol^{-1}, aqueous copolymer solutions

afforded a sol at low temperature, and a gel at body temperature[16-19]. This sol-gel transition behavior is comparable to that of Pluronics.

Recently, copolymers with star-shaped and branched architectures, a high PEG content and high molecular weight were prepared[14, 20-24]. These copolymers showed thermo-responsive gelation behavior in water, forming a gel at low temperatures that transformed into a sol at higher temperatures. This phase behavior is comparable to the phase behavior of linear triblock copolymers with similar molecular weight and PEG content. A three-arm star-shaped copolymer with PLLA as the core moiety and with PEG as the outer blocks formed hydrogels at somewhat lower concentrations than a linear triblock PEG-PLLA-PEG copolymer with the same PEG content[20]. Furthermore, the gel-sol transition temperature increased from 12 to 70 °C for a 20 wt% hydrogel when the length of the PLLA blocks increased from 5 to 9 repeating lactide units. Simultaneously, the critical gel concentration at room temperature decreased from 25 to 12 wt%.

Three- and four-arm star-shaped PEG-PCL block copolymer solutions, with PEG in the core of the copolymers, also showed thermo-responsive gel-sol transitions[22, 24]. Unfortunately, a comparison between the gelation behavior of the three- and four-arm star-shaped block copolymers is not available. Eight-arm star-shaped PEG-PLLA block copolymers, prepared by ring opening polymerization of L-lactide using an eight-arm star PEG with a molecular weight of 21800 $g \cdot mol^{-1}$ as the initiator were recently studied[14, 23]. The thermo-responsive gelation behavior of these copolymers was compared with PLLA-PEG-PLLA triblock copolymers. An eight-arm star-shaped PEG-PLLA copolymer with a PEG content of 74 wt% and 7.5 repeating lactide units per arm showed a gel-sol transition temperature at almost the same temperature as a triblock PLLA-PEG-PLLA copolymer with the same PLLA block length and a 84 wt% PEG content. Furthermore, eight-arm PEG-PLLA hydrogels showed a decrease in the critical gelation concentration at room temperature from 40 to 15 wt% when the length of the PLLA blocks was increased from 5 to 7 repeating lactide units. However, when the number of repeating lactide units per PLLA block was higher than 7, the copolymer was not water-soluble anymore.

In this paper, the synthesis and characterization of new highly branched PEG-PLLA block copolymers are described. The thermo-responsive gelation behavior of aqueous solutions of these copolymers was studied by the vial tilting method and oscillatory rheology.

Experimental

Materials

L-lactide (L-LA) was purchased from Purac (Gorinchem, the Netherlands). 2,2-Bis (hydroxymethyl)propionic acid (bis-MPA) and N,N'-dicyclohexylcarbodiimide (DCC) were obtained from Acros (Geel, Belgium). Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$), N-hydroxysuccinimide (NHS), mesyl chloride, and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Aldrich (Zwijndrecht, the Netherlands). 2-(4-Hydroxyphenylazo)benzoic acid (HABA), 1,8,9-trihydroxyanthracene (dithranol), 2,4,6-trinitro benzenesulfonic acid (TNBS), and aqueous ammonia (25%) were obtained from Fluka (Buchs, Switzerland). Hydroxyl functional, eight-arm star poly(ethylene glycol) 8PEG-OH ($Mn_{,H\text{-}NMR}$ = 21,800 $g \cdot mol^{-1}$) was purchased from Nektar (Huntsville, USA). Glacial acetic acid, triethylamine (TEA), chloroform, ethanol and diisopropyl ether were obtained from Merck (Darmstadt, Germany). All other organic solvents were from Biosolve (Valkenswaard, the Netherlands). Phosphate buffered saline (PBS) was obtained from Braun (Melsungen, Germany). Prior to use, dichloromethane and chloroform were dried over calcium hydride (Aldrich) and toluene over sodium wire, and subsequently distilled. All other chemicals were used as received.

Synthesis

PLLAn: PLLAn, where n is the number of repeating lactide units per arm, was synthesized as described previously[25]. In a typical procedure PLLA5 was prepared by adding L-lactide (25.0 g, 174 mmol) to a reaction vessel, which contained bis-MPA (2.33 g, 17 mmol) as the initiator and $Sn(Oct)_2$ (0.10 g, 0.25 mmol; 0.4 wt% based on L-lactide) as the catalyst. The mixture was allowed to react for 3 h at 130 °C under an argon atmosphere. The product was subsequently cooled to room temperature and dissolved in dichloromethane. To this solution, a small amount of glacial acetic acid was added, and the product was precipitated in an excess of cold diisopropyl ether. The product was collected by decantation, and dried in vacuo to give a 'sticky' precipitate (Yield: 87 %). PLLA7 was obtained as a white powder.

NHS-PLLAn: The synthesis of NHS-PLLA5 is given as a typical procedure: PLLA5 (12.0 g, 7.8 mmol) was dried by dissolution in 150 ml of toluene and subsequent azeotropic distillation. The dried material was dissolved in 200 ml of dichloromethane. To the resulting solution, NHS (1.07 g, 9.3 mmol) and DCC (2.39 g, 11.9 mmol) were added. The reaction mixture was allowed to react for 24 h at

room temperature under argon. The formed dicyclohexylurea (DCU) was removed after the reaction by filtration. The clear solution was concentrated by partially evaporating the dichloromethane, and the polymer was precipitated in an excess of cold diisopropyl ether : methanol (10:1 v:v). The product was decanted and dried in vacuo to give a white 'sticky' precipitate (Yield: 90 %).

8PEG: Amine functionalized eight-arm star 8PEG was synthesized according to a procedure as described for linear PEGs[26]. 8PEG-OH (10 g, 0.5 mmol) was dissolved in 300 ml of toluene and dried by the removal of 200 ml of solvent by azeotropic distillation. After the solution was cooled in an ice-bath, 30 ml of dichloromethane and TEA (1.7 ml, 12 mmol) were added. Subsequently, mesyl chloride (0.94 ml, 12 mmol) was added drop-wise under stirring and allowed to react overnight. The solution was filtered and precipitated in an excess of diethyl ether. After drying, the formed 8PEG-mesylate was reacted with 100 ml of an aqueous ammonia solution (25%) for 4 d at room temperature. Subsequently, the ammonia was allowed to evaporate and the pH of the solution was raised to 13, using 1 M NaOH. The solution was extracted with dichloromethane (50 ml) for 3 times. The dichloromethane extracts were combined and concentrated. The product was precipitated in cold diethyl ether, and dried in vacuo (Yield: 89 %).

8PEG-PLLAn: The synthesis of 8PEG-PLLA5 is given as a typical procedure: NHS-PLLA5 (1.83 g, 1.11 mmol) and 8PEG (3.17 g, 0.14 mmol) were dissolved in 100 ml of chloroform and allowed to react for 48 h at 60 °C. Subsequently, the solution was filtered to remove insoluble by-products, and the solvent was removed under reduced pressure. The copolymer was dissolved in dichloromethane and precipitated in an excess of cold diethyl ether. The product was collected by filtration and dried in vacuo, and was obtained as a powder (Yield: 81 %). 8PEG-PLLA3 was prepared by dissolving the macromonomers in toluene, and reacting for 6 h at 120 °C. The product was isolated as described above and obtained as a white powder (Yield: 22 %).

Characterization

NMR: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR spectra were recorded on a Varian Inova NMR spectrometer. Polymers were dissolved in $CDCl_3$ at a concentration of 0.015 $g \cdot ml^{-1}$ (^{1}H) or 0.2 $g \cdot ml^{-1}$ (^{13}C).

Free amine group content: The primary amine group content present in the copolymers after the coupling reaction, was determined by a TNBS assay[27, 28],

using 1.5 $mg \cdot ml^{-1}$ aqueous copolymer solutions. To 1 ml of the copolymer solution, 1 ml of a 4 wt% $NaHCO_3$ solution and 1 ml of a freshly prepared 0.5 wt% TNBS solution in water were added. After reacting for 2 h at 37 °C, 3 ml of a 6 M HCl solution was added and the samples were allowed to react for another 1.5 h at 37 °C. Finally, each sample was diluted with 2 ml of water, and the absorption was measured at 420 nm using a Cary 300 Bio UV-visible spectrophotometer (Varian). A calibration curve was derived from the measured absorption of various PEG-amines at different concentrations.

GPC: Molecular weights and molecular weight distributions of the polymers were determined with gel permeation chromatography (GPC) using chloroform as eluent. The GPC setup consisted of a Waters 510 pump, a HP Ti-Series 1050 auto sampler, a series of standard Waters Styragel HR columns, a Waters 410 differential refractometer, and a viscometer detector H502 (Viscotek Corp.). Polystyrene standards with narrow molecular weight distributions were used for calibration and the molecular weights were determined using the universal calibration principle.

MALDI-TOF: Matrix Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF) was performed using a Voyager-DE-RP 2010 MALDI-TOF mass spectrometer (Applied Biosystems/ PerSeptive Biosystems, Inc.) equipped with delayed extraction. A 337 nm UV nitrogen laser producing 2 ns pulses was used and the mass spectra of the polymers were obtained in the reflection mode. Samples were prepared by mixing ~2 mg polymer with 1 ml dichloromethane. After that, ~5 mg of dithranol or HABA was added as the matrix and the resulting solution was vigorously stirred. One µl of the solution was loaded on a gold sample plate. After evaporation of the solvent in air, the sample was transferred to the mass spectrometer for analysis.

DSC: Thermal analysis of the macromonomers and the copolymers was carried out using a Pyris 1 differential scanning calorimeter, calibrated with indium and gallium. During a measurement, the polymer (5-15 mg) was cooled to -50 °C and kept at this temperature for 1 min. The sample was then heated to 200 °C, annealed for 1 min, and subsequently cooled to -50 °C. Finally, the sample was kept at -50 °C for 5 min and heated to 200 °C again. The heating and cooling rate was 20 $°C \cdot min^{-1}$. Melting (T_m) and crystallization (T_c) temperatures were obtained from the peak maxima, melt (ΔH_m) and crystallization (ΔH_c) enthalpies were determined from the area under the curve and the glass transition temperature (T_g) was taken as

the inflection point. The data presented are from the cooling and second heating step.

Aqueous solution properties

Vial tilting method: The phase behavior of aqueous polymer solutions was investigated by the vial tilting method. Block copolymers were dissolved in MilliQ water (2-20 wt%) in tightly capped 5 ml vials by repeatedly heating to ~60 °C for 2 min and stirring while cooling. The block copolymer solutions were kept at 4 °C overnight prior to measurement. The temperature was increased step-wise with 2 or 4 °C and the samples were left at each measuring temperature for 10 min to equilibrate. The gel-sol transition temperature was determined by tilting the vials 90° for 1 min. If there was no flow, the contents were regarded as a gel. In other cases the contents formed a sol.

Rheology: Rheology experiments were performed on a TA instruments AR1000 rheometer with a flat plate geometry (20 mm diameter, 0.5 mm gap) in oscillating mode. Aqueous polymer solutions were prepared by dissolving the appropriate amount of polymer by repeatedly heating to ~60 °C for 2 min and stirring while cooling. The polymer solutions were then applied on the rheometer and heated to 60 °C. To prevent evaporation of water, a solvent trap was placed over the geometry. A pre-shear was applied for 10 s, after which the polymer solution was allowed to equilibrate for 6 min. Subsequently, the polymer solution was cooled to 20 °C and heated again to 60 °C at 1 °C·min^{-1}. Gelation of the polymer solutions was monitored by measuring both the storage modulus G' and the loss modulus G" as a function of temperature. A system was considered a gel if G' was larger than G". The cross-over point was considered as the transition temperature. A frequency ω of 1 Hz and a strain γ of 1 % were applied to minimize the influence of deformation on the formation of the hydrogels. After the cooling and heating cycles, an amplitude and frequency sweep were performed at γ 0.01-10 % (ω = 1 Hz) and ω 0.01-10 Hz (γ = 1 %) at 20 °C, to confirm that the applied ω of 1 Hz and the γ of 1 % was within the viscoelastic range.

Hydrogel dissolution/degradation: Copolymer solutions (0.5 ml) were prepared in tightly capped 5 ml vials by dissolving the appropriate amount of copolymer in water by repeatedly heating and stirring. After cooling, 3 ml of PBS was put on top of the hydrogels and the vials were stored at 20 or 37 °C. At regular time intervals, 2.5 ml samples of the PBS were taken and replaced by fresh PBS. The samples

were lyophilized and the mass of the solubilized copolymer fraction was determined gravimetrically.

UV-VIS: The critical association concentration (CAC) of the copolymers in water at 20 °C was determined by the dye solubilization method. To 1.0 ml of the aqueous copolymer solutions with concentrations ranging from 0.001 to 1 w/v%, 10 µl of a 0.5 mM DPH solution in methanol was added. The resulting mixture was stored in the dark and equilibrated over night. UV-VIS absorption spectra of the solutions were recorded in the 300 to 500 nm range. The difference in absorption at 378 nm relative to that at 403 nm was plotted against the polymer concentration and the intercept of the extrapolated straight lines was defined as the CAC of the copolymer.

DLS: Dynamic light scattering experiments were performed on a Malvern Zetasizer 4000 (Malvern Corp., Malvern, UK), using a laser wavelength of 633 nm and a scattering angle of 90° at 25 °C. The CONTIN method was applied for data processing.

Results and Discussion

Synthesis

The conversion of hydroxyl end-groups of an eight-arm PEG into amine end groups was performed according to a procedure as described for linear PEG's[26] (Figure 1). The hydroxy end-groups were first converted into the corresponding mesylates in high yield. The mesylates were quantitatively converted into amines by reaction with aqueous ammonia for 4 d at room temperature. The ^{1}H-NMR spectra of 8PEG revealed that only amino end-groups were present, since only a signal of the CH_2-NH_2 was found at 2.86 ppm (data not shown). A number average molecular weight (Mn) of 22,300 g·mol^{-1} was calculated from the ratio of the CH_2 protons in the PEG chains to the CH_2-NH_2 protons.

The $Sn(Oct)_2$ catalyzed ring opening polymerization of L-lactide in the presence of bis-MPA as the initiator afforded the PLLA macromonomers (Figure 1). The number of repeating units per arm was varied from 3 to 7, by varying the monomer to initiator ratio [M]:[I]. The integral ratio of the CH_3 protons of the monomer (1.59 ppm) to polymer (1.65 ppm) in the ^{1}H-NMR spectra of the crude samples was used to determine the conversion. In all cases, after 3 h reaction time, high monomer conversions of approximately 97 % were obtained. The ^{1}H-NMR spectra of the

purified PLLAn macromonomers (Figure 2A) were used to calculate the degree of polymerization (DP), and the Mn. The DP was determined from the ratio of the integrated area of the C*H* protons of the lactide repeating units (5.10 ppm) to the CH_3 protons of the bis-MPA moiety (1.27 ppm). The results obtained with ^{1}H-NMR are in good accordance with the theoretical values, based on the [M]:[I] ratio (Table 1).

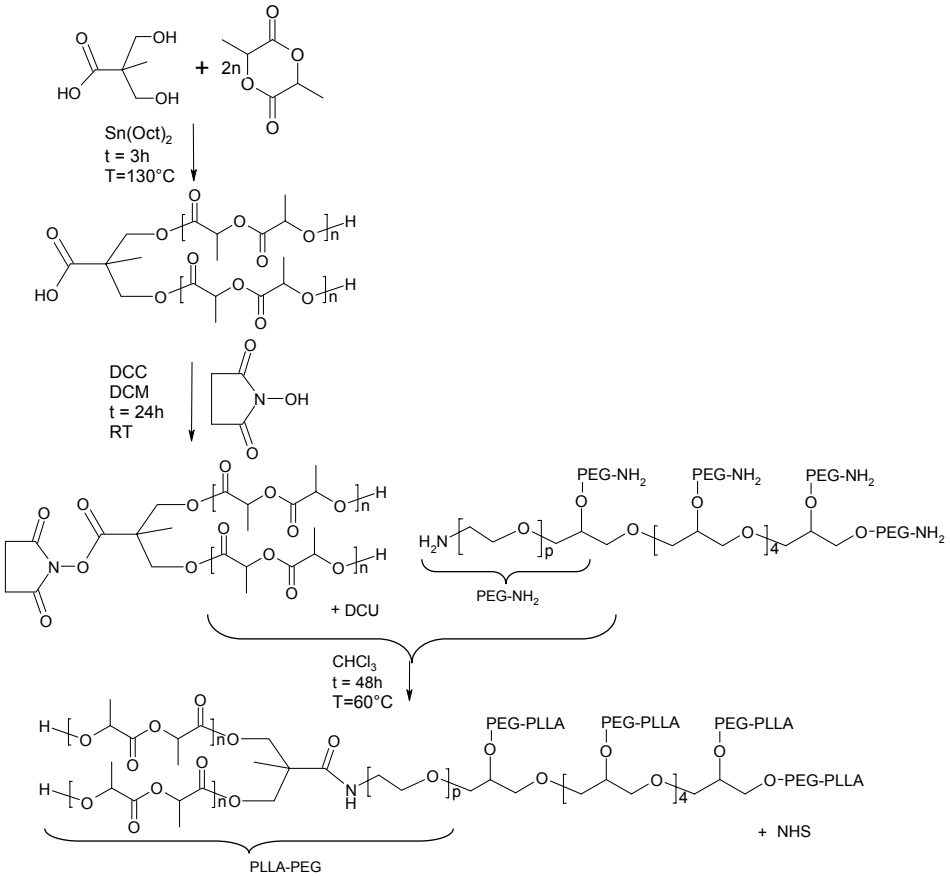

Figure 1. Synthesis scheme of NHS-PLLAn, and 8PEG-PLLAn copolymer.

The molecular weights and molecular weight distributions (PDI) of the PLLAn macromonomers were determined with GPC and MALDI-TOF (Table 1). The Mn's obtained by MALDI-TOF were in good agreement with the ^{1}H-NMR results. The molecular weights obtained by GPC measurements relative to polystyrene standards were approximately 1.4 times higher than those calculated from the ^{1}H-NMR

spectra. The molecular weight distributions of the macromonomers determined with GPC were ~1.5, and determined with MALDI-TOF ~ 1.1.

Table 1. Molecular weights and molecular weight distributions of bis-MPA initiated PLLAn macromonomers.

Macromonomer	Mn (g·mol^{-1})				PDI (-)	
	Calc[a]	^{1}H-NMR	GPC	MALDI	GPC	MALDI
PLLA3	1000	1100	1800	1300	1.6	1.1
PLLA4	1290	1300	1900	1500	1.5	1.1
PLLA5	1580	1600	2300	1700	1.5	1.2
PLLA6	1860	1800	2700	2000	1.5	1.1
PLLA7	2150	2100	2700	2200	1.5	1.1

[a] calculated from the [M]:[I] ratio

The carboxylic acid groups were converted in their corresponding NHS active esters. ^{1}H-NMR spectra showed the appearance of the CH_2 protons of the succinimide ring at 2.84 ppm (Figure 2B, f). The signal of the CH_3 protons of the bis-MPA moiety shifted from 1.27 to 1.44 ppm (a), and the multiplet of the CH_2 protons of the bis-MPA moiety shifted from 4.20-4.40 to 4.25-4.50 ppm (b). Comparing the ^{1}H-NMR spectra of starting compound and product showed that the conversion was complete. The molecular weights calculated from the [M]:[I] ratio and determined from the ^{1}H-NMR data are presented in Table 2. The molecular weight and PDI of the macromonomers were also determined with MALDI-TOF mass spectroscopy (Table 2). The Mn's as measured with MALDI-TOF are in good accordance with those calculated from ^{1}H-NMR, and the molecular weight distributions were 1.1.

Table 2. Molecular weights and molecular weight distributions (PDI) of NHS activated macromonomers.

Macromonomer	Mn (g·mol^{-1})			PDI (-)
	Calc[a]	^{1}H-NMR	MALDI	MALDI
NHS-PLLA3	1100	1200	1200	1.1
NHS-PLLA4	1390	1500	1400	1.1
NHS-PLLA5	1670	1800	1600	1.1
NHS-PLLA6	1960	2100	1800	1.1
NHS-PLLA7	2250	2400	2000	1.1

[a] calculated from the [M]:[I] ratio

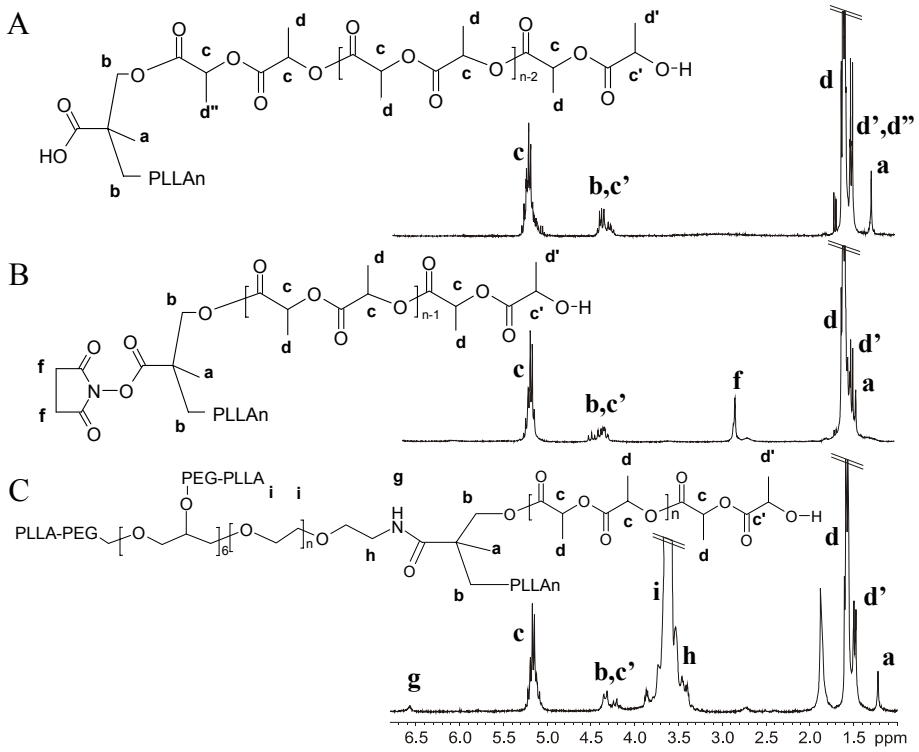

Figure 2. ^{1}H-NMR spectra of (A) PLLA5, (B) NHS-PLLA5 and (C) 8PEG-PLLA5. Solvent: $CDCl_3$.

The mass distribution curves obtained by MALDI-TOF support that the activation of the carboxylic acid end-groups was complete. The mass distribution corresponds to NHS-PLLAn macromonomers and a distribution that corresponds to the PLLAn macromonomers was absent. The mass distribution curve of NHS-PLLA5 is given as a typical example (Figure 3).

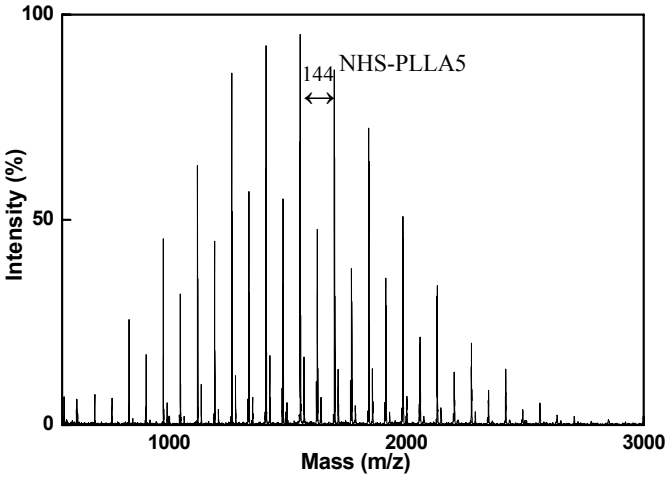

Figure 3. MALDI-TOF mass distribution curve of NHS-PLLA5.

The activated macromonomer with 5 repeating L-lactide units per arm has a molar mass of 1696 g·mol^{-1} (Na^+ as the attached ion). The peak corresponding to this activated macromonomer is labelled 'NHS-PLLA5' in Figure 3. The mass difference of each adjacent peak is 144.1 g·mol^{-1}, which corresponds to the mass of one L-lactide unit. A second distribution of lower intensity is also observed, and corresponds to macromonomer chains with an odd number of lactic acid units as a result of transesterification, which is known to occur to some extent in ring opening polymerization reactions in the melt above 120 °C[29].

The coupling reaction of the NHS-PLLAn macromonomers to the eight-armed PEG was performed in chloroform at 60 °C. ^{1}H-NMR spectra showed a peak belonging to the N*H* protons of the formed amide bonds at 6.42 ppm (g in Figure 2C), and signals that belong to PEG (h and i) and PLLA (a-e). The conversion was followed in time by ^{1}H-NMR analysis of samples drawn from the reaction mixture, and was calculated from the ratio of the CH_2 protons of the NHS-ester (f, 2.84 ppm), and free NHS (2.64 ppm). After 6 h, the conversion was 37-44 %, and increased to 47-

66 % after 48 h (Table 3). Longer reaction times did not result in higher conversions. Addition of an excess of macromonomer did also not bring the reaction to completion, which is probably due to increasing sterical hindrance with conversion.

Table 3. Conversion, molecular weight and PEG content of 8PEG-PLLAn copolymers.

Copolymer	Conversion (%)		Mn^{b}	PEG content[c]
	^{1}H-NMR[a]	TNBS	($g \cdot mol^{-1}$)	(wt%)
8PEG-PLLA3	53	60	26800	83
8PEG-PLLA4	47	44	27400	81
8PEG-PLLA5	63	58	30600	73
8PEG-PLLA6	52	n.d.	30500	73
8PEG-PLLA7	50	n.d.	31300	71

[a] calculated from the ratio of the CH_2 protons of the succinimide ring (f, 2.84 ppm) and NHS (2.64 ppm)

[b] calculated from $Mn_{NHS\text{-}PLLAn}$, Mn_{8PEG}, and the conversion

[c] calculated from the ratio of the C*H* protons of the PLLA (c, 5.10 ppm) to the CH_2 protons of the PEG (i, 3.64 ppm)

During the coupling reaction, an insoluble side product was formed. When the reaction of NHS-PLLA3 and 8PEG was performed in toluene at 110 °C for 6 h, a conversion of ~50 % was obtained. The formation of the side product appeared more severe at higher temperatures. From ^{1}H-NMR, the composition of the side product was analyzed and the PEG : PLLA ratio was similar to that of the 8PEG-PLLAn product. It is assumed that the insoluble side product is a result of a nucleophilic substitution reaction of the primary amine end-groups of the PEG on the bis-MPA methylene carbon atoms. Because of the high functionality of the PEG, this results in formation of a network.

Because no complete conversion could be reached, the final copolymer should still contain free amine groups. The amount of free amine groups in the copolymer was determined with a TNBS assay, and the values obtained appeared to be in good agreement with the values based on the conversion data (Table 3).

Thermal properties

The thermal properties of the macromonomers and the copolymers were studied using DSC. The second heating scans and the cooling scans of NHS-PLLA5, 8PEG, and 8PEG-PLLA5 are plotted in Figure 4 as typical examples. The NHS-PLLAn macromonomers showed a T_g in the second heating scan, that increased with increasing molecular weight from 26 °C for NHS-PLLA3 to 33 °C for NHS-PLLA7. These macromonomers did not show melting or crystallization. All copolymers and 8PEG showed a single melting peak in the second heating scan, and a crystallization peak in the cooling scan, due to the crystalline PEG-rich phase (Table 4). The T_m and the ΔH_m of the copolymers were lower than those of the 8PEG, because the PLLA blocks hamper the crystallization of PEG. The T_g of the PLLA blocks was not observed in the heating scans of the copolymers, due to the low PLLA content in the copolymers.

Table 4. Thermal properties of 8PEG and 8PEG-PLLAn copolymers.

	T_m (°C)	ΔH_m ($J \cdot g^{-1}$)	T_c (°C)	ΔH_c ($J \cdot g^{-1}$)
8PEG	48	100	28	95
8PEG-PLLA3	37	63	17	62
8PEG-PLLA4	38	67	18	64
8PEG-PLLA5	37	60	15	57
8PEG-PLLA6	39	60	15	62
8PEG-PLLA7	39	59	17	58

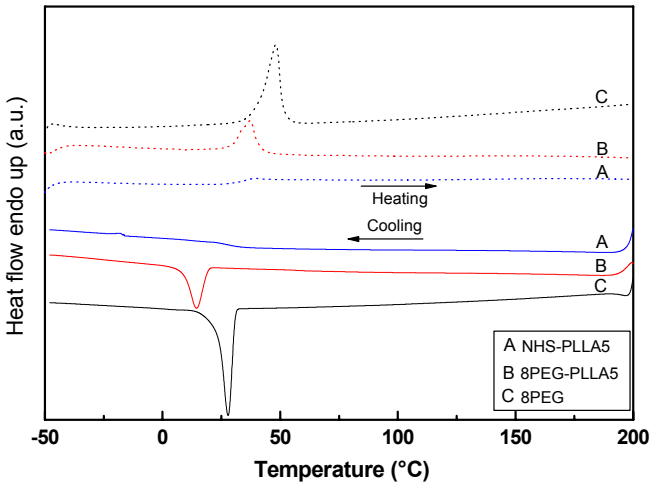

Figure 4. Second heating scans (dotted lines) and cooling scans (solid lines) of (A) NHS-PLLA5, (B) 8PEG-PLLA5 and (C) 8PEG.

Aqueous solution properties

Solubility

The solubility of the copolymers in water decreased with increasing PLLA block length. The copolymers 8PEG-PLLAn, with n is 3, 4, or 5 were soluble in water, and formed transparent solutions or hydrogels, depending on the temperature and concentration. At low concentrations, 8PEG-PLLA6 and 8PEG-PLLA7 block copolymers were not soluble, but swelled to a large extent. At higher concentrations (≥ 4 wt%), however, transparent hydrogels of 8PEG-PLLA6 could be prepared.

Vial tilting test

The temperature dependent gelation behavior of aqueous solutions of 8PEG-PLLAn copolymers was studied by the vial tilting method. Step-wise heating the solutions to 70 °C, and step-wise cooling gave the gel-sol transition diagrams as presented in Figure 5A and B. Figure 5A shows that all hydrogels turned into a mobile phase, the so-called 'sol' when heating the solution. For all copolymers, the gel-sol transition temperature increased with increasing concentration. For 8PEG-PLLA5 and 8PEG-PLLA4 hydrogels, the gel-sol transition temperature increased more sharply with increasing concentration than for 8PEG-PLLA3 hydrogels. This effect is also described for linear and star-shaped copolymers based on PLLA and PEG[10, 20]. A 2 wt% 8PEG-PLLA6 solution did not form a gel, but at 4 wt% a hydrogel was

obtained, which showed a gel-sol transition at 34 °C. At 6 wt%, no gel-sol transition was observed within this temperature regime and the copolymer stayed in the gel state (data not shown). The transition from sol to gel when cooling the solutions from 70 °C to 4 °C (Figure 5B) gave transition temperatures somewhat lower than the gel-sol transition upon heating.

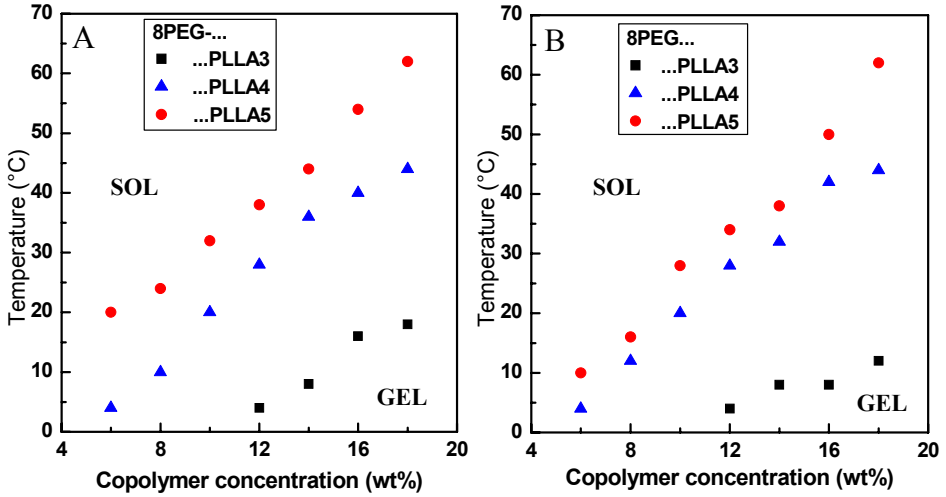

Figure 5. Gel-sol transition diagram of 8PEG-PLLAn copolymers in water, while (A) heating and (B) cooling.

From Figure 5 it can be seen that the gel-sol transition temperature is strongly influenced by PLLA block length. Increasing the length of the PLLA arms from 3 to 5 lactide units shifts the gel-sol transition temperature by more than 20 °C (8PEG-PLLA4), or even more than 30 °C for 8PEG-PLLA5. The critical gel concentration (CGC) at room temperature decreased from 18 wt% for 8PEG-PLLA3 to 6 wt% for 8PEG-PLLA5 possibly due to stronger hydrophobic interactions[10]. The CGC's of especially 8PEG-PLLA4 and 8PEG-PLLA5 are low compared to other linear and star-shaped copolymers based on PLLA and PEG[10, 20, 21, 23]. Star-shaped PEG-PLLA copolymers, prepared by ring opening polymerization of L-lactide on an eight-armed PEG (Mn =21800 g·mol^{-1}), and having 6 lactide units per arm, have a similar PEG content as the 8PEG-PLLA5[23]. The CGC at room temperature of such an eight-armed PEG with 8 PLLA arms was ~20 wt%, much higher than that of an 8PEG-PLLA5, which is 6 wt%. The reason for this large difference may be the stronger hydrophobic interactions in 8PEG-

PLLA5, because hydrophobic domains may be formed more easily, if only 4 out of 8 arms have to be folded into such a domain (Figure 6A) instead of 8 out of 8 arms (Figure 6B).

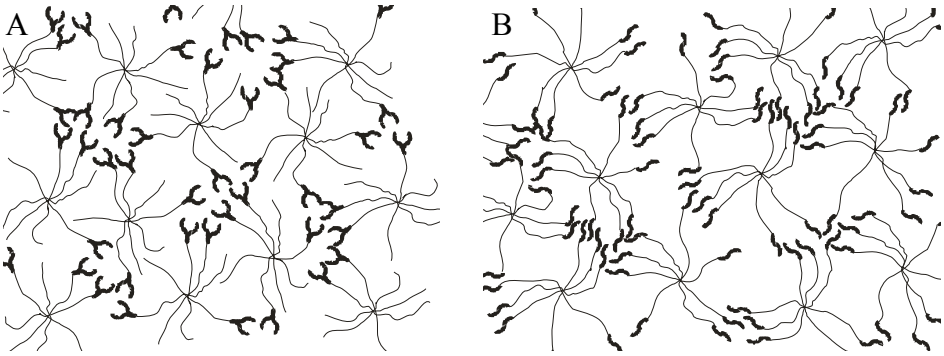

Figure 6. Schematic representation of a hydrogel of (A) 8PEG-PLLAn as described in this paper and (B) eight-armed star-shaped PEG-PLLA copolymer as described by Hiemstra et al.[23]

Rheology

The thermo-responsive gelation behavior was investigated by oscillatory rheology experiments upon cooling copolymer solutions. The storage modulus (G') and the loss modulus (G") were monitored as a function of temperature for 8PEG-PLLA4 and 8PEG-PLLA5 solutions with concentrations of 10, 12 and 14 wt%. Representative results are depicted for 8PEG-PLLA5 in Figure 7. At 60 °C, the G" was larger than the G', indicating that the solutions were in the sol state at all concentrations. When the solutions were cooled, both the G' and G" increased, and finally, the G' became larger than the G". Thus, the solutions underwent a transition to the gel state. At 20 °C, the hydrogels had storage moduli of 3.8 kPa (10 wt%), up to 10.3 kPa (14 wt%). The transition temperature slightly increased from 49 to 52 °C with increasing concentration. The same trends were observed for 8PEG-PLLA4 copolymer solutions in water. At 20 °C, hydrogels were obtained with storage moduli of 1.6 to 5.0 kPa, when increasing the concentration from 10 to 14 wt%. Furthermore, the transition temperature increased from 42 to 44 °C (data not shown). Comparing the sol-gel transitions as measured with rheology with those measured by the vial tilting method reveals a smaller dependence of the transition temperature on the copolymer concentration.

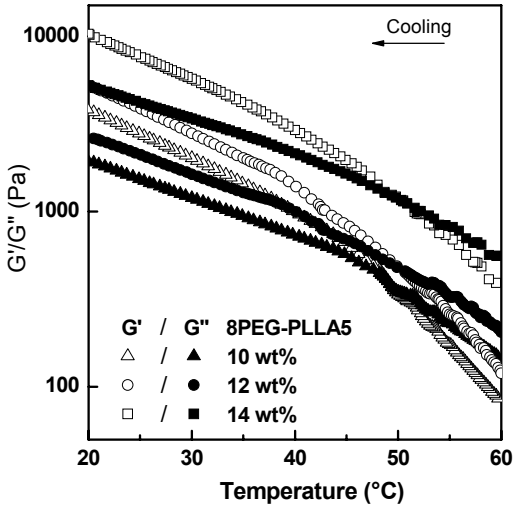

Figure 7. The storage (G') and loss (G") modulus of 10, 12, and 14 wt% 8PEG-PLLA5 aqueous solutions, upon cooling from 60 to 20 °C.

The temperature dependent G' and G" of 12 wt% 8PEG-PLLA4 and 8PEG-PLLA5 copolymer solutions and gels are presented in Figure 8. At 20 °C, the 8PEG-PLLA5 copolymer solution formed a hydrogel with higher G' and G" than the 8PEG-PLLA4 copolymer solution. The transition from sol to gel also occurred at a higher temperature. This suggests that the driving forces, such as the hydrophobic interactions, for gel formation in the 8PEG-PLLA5 hydrogel are larger than in the 8PEG-PLLA4 hydrogel.

A 4 wt% aqueous solution of 8PEG-PLLA6 formed a hydrogel with a G' and G" of 100 and 40 Pa at 20 °C, respectively, and exhibited a sol to gel transition at 31 °C. The relatively low G' and G" are considered to be due to the low copolymer concentration (data not shown).

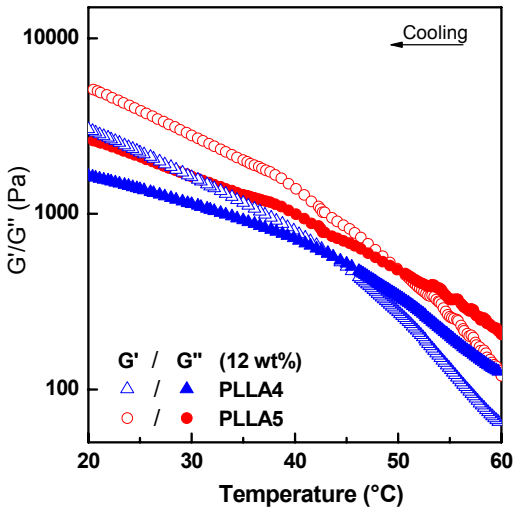

Figure 8. The storage (G') and loss (G") moduli of 12 wt% aqueous solutions of 8PEG-PLLA4 and 8PEG-PLLA5, upon cooling from 60 to 20 °C.

Hydrogel dissolution/degradation

PBS buffer (3ml) was placed on top of 0.5 ml of the hydrogels, and the dissolution/degradation of the hydrogels at 20 °C and 37 °C was monitored in time by measuring the mass of the solubilized copolymer. The cumulative mass loss as a function of time for various hydrogels at 20 and 37 °C is presented in Figure 9. At 20 °C, a 6 wt% 8PEG-PLLA5 hydrogel dissolved/degraded within 4 d, whereas a 6 wt% 8PEG-PLLA6 hydrogel required 39 d to dissolve/degrade. The same 8PEG-PLLA6 hydrogel at 37 °C was dissolved/degraded after 8 d, which shows that the temperature at which the experiment was performed had a large influence on the dissolution/degradation rate of the hydrogels. During the experiment, it was observed that the hydrogels swelled to a large extent, up to 200 % of their original volume. At 20 °C, this swelling started immediately after the start of the experiment for 8PEG-PLLA4 and 8PEG-PLLA5, whereas the 8PEG-PLLA6 hydrogel started to swell after 14 d. In all cases, the swelling was followed by dissolution/degradation of the hydrogels. It is suggested that the micellar structure packing avoids immediate solubilization of the copolymer into the PBS buffer. Moreover, the loss of the micellar structure packing is caused by the difference in copolymer concentration between the hydrogel and the PBS, and by decreasing hydrophobic interactions due to hydrolysis of the ester bonds in the PLLA blocks.

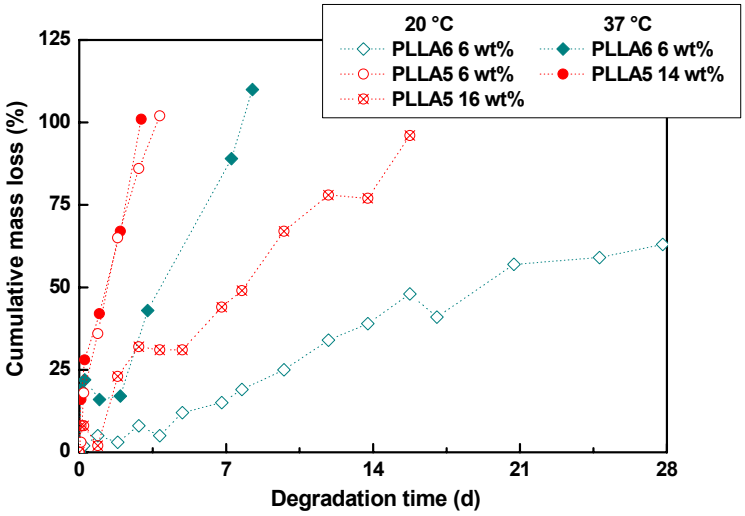

Figure 9. Relative mass loss as a function of time of 8PEG-PLLAn copolymer hydrogels placed under PBS at 20 °C (open symbols) and 37 °C (filled symbols).

Critical association concentration

The critical association concentration (CAC) of the copolymers in water was determined with the hydrophobic dye solubilization method. DPH was used as the hydrophobic dye and the absorption spectrum shows a characteristic triple band if the dye is partitioned in a hydrophobic environment such as a micellar core. At 20 °C, the CAC determined decreased from 0.092 to 0.083 w/v% with increasing hydrophobicity of the copolymer (Figure 10). The tendency to form micellar-like aggregates is higher if the copolymer is more hydrophobic. The temperature dependence of the CAC is determined from 20 to 50 °C. For all copolymers, the CAC was decreasing with increasing temperature, which can be explained by the increasing hydrophobicity of PEG at higher temperatures, since PEG dehydrates with increasing temperature. Furthermore, the influence of the temperature on the hydrophobic interactions of the 8PEG-PLLA5 copolymer in water was stronger than for the 8PEG-PLLA4 and 8PEG-PLLA3 copolymers. At higher concentrations, this can also be observed in the gel-sol transition diagrams as determined with the vial tilting method.

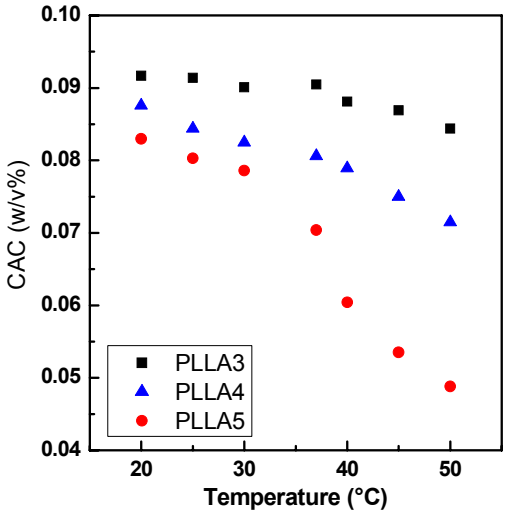

Figure 10. Critical Association Concentration (CAC) of 8PEG-PLLAn copolymers in water as a function of temperature.

Micelle formation

Dynamic light scattering revealed that 0.5 w/v% aqueous copolymer solutions at 25 °C formed micelles with a Z-average particle size between 24 and 39 nm, depending on the copolymer. The Z-average particle size was hardly depending on the temperature in the 25-50 °C temperature regime.

Conclusions

Highly branched poly(ethylene glycol)-poly(L-lactide) (PEG-PLLA) copolymers were conveniently synthesized from PLLA macromonomers and eight-armed PEG-amine. The copolymers obtained showed thermo-responsive gelation behavior with low critical gelation concentrations (≥ 4 wt%). The gel-sol transition was investigated with the vial tilting method and oscillatory rheology. Hydrogels could be designed with storage moduli varying from 0.1 to 10 kPa by changing the hydrophobic block length, and the copolymer concentration in water. The transition temperature could be tuned closely to body temperature by varying the copolymer concentration, and the molecular weight of the PLLA block.

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Chapter 8

Synthesis, characterization, and degradation of chemically crosslinked poly(ethylene glycol)-poly(L-lactide) hydrogels

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Abstract

Biodegradable poly(ethylene glycol)-poly(L-lactide) (PEG-PLLA) networks were prepared by the coupling of two armed PLLA macromonomers containing three N-hydroxysuccinimide active ester groups and α,ω-amine functionalized linear PEGs or amine end-group functionalized star PEGs. The degree of swelling (DS) in water varied from 11 to 1650 wt% and depended on the PEG/PLLA ratio of the network, and to a lesser extent on the crosslink density. The DS of the prepared hydrogels decreased with increasing temperatures, due to the partial dehydration of PEG in water at higher temperatures. Degradation of the networks in PBS buffer at 37 °C led to an increase of the DS and a decrease in the dry mass of the network with time. The networks with the highest PEG content and lowest crosslink density showed complete mass loss within 4 d, and the networks with the lowest PEG content showed a mass loss of 18 % after 28 d.

Introduction

Hydrogels have received much interest for their use in drug delivery and tissue engineering[1-3], because they are generally biocompatible due to their high water content, and their properties resemble those of natural, soft tissue. Hydrogels are highly water-swollen polymeric networks containing either physical crosslinks, such as entanglements, hydrophobic interactions or crystallites, or chemical crosslinks. Chemically crosslinked networks are generally more stable and have better mechanical properties compared to physically crosslinked networks. Poly(ethylene glycol) (PEG) is widely employed as a hydrophilic component in hydrogels, because of its good biocompatibility. Aliphatic polyesters, such as poly(lactide)s (PLA), poly(lactide-co-glycolide)s (PLGA) and poly(ε-caprolactone)s (PCL) are often used as the hydrophobic component, since they are biodegradable through the hydrolysis of ester bonds and thus eliminate the need for surgery after their functional time.

Many chemically crosslinked biodegradable networks have been prepared by photopolymerization of (meth)acrylate functionalized polymers[4-7]. Hydrogels containing 4 to 92 wt% of water were prepared using different synthetic approaches and different PEG and polyester ratios. The degradation of some of these networks was studied by placing them in PBS buffer at 37 °C and measuring the residual mass of the networks in time. The degradation varied from 0.7 to 45 d for PLGA-PEG networks. The more hydrophobic PLLA-PEG networks totally degraded within 35 to 120 d[5] depending on the molecular weight of the PEG and polyester block. A drawback of crosslinking by photopolymerization is that un-reacted gel precursors may be toxic, and (photo)initiators and co-crosslinkers are often required. Therefore, complete conversion of the end-groups, and complete removal of the additives is necessary. Another way to prepare chemical hydrogels is by reacting prepolymers with complementary reactive groups. Kricheldorf and coworkers[8] prepared cyclic triblock copolymers of PEG and aliphatic polyesters via the ring expansion polymerization of ε-caprolactone or D,L-lactide initiated from stannylated PEG. Biodegradable networks were then prepared by reacting these cyclic triblock copolymers with trimesoyl chloride, as a crosslinker molecule. With this method, the length of the hydrophobic and hydrophilic blocks could be varied independently by changing the feed ratio of monomer to PEG, and the crosslink density could be varied by changing the amount of trimesoyl chloride added. The degradation of networks based on PCL-PEG copolymers in PBS buffer

at 37 °C was examined by determining the mass loss in time. After 80 d, the mass loss was in between 0.5 and 7 wt%, depending on the composition of the network.
The examples mentioned require complete conversion of the functional groups, because of the toxicity of the compounds bearing un-reacted groups. Yoshida et al. reported the synthesis of hydrogels by reaction of N-hydroxysuccinimide modified poly(N-isopropyl acrylamide) (PNIPAAm) copolymers with amine terminated biodegradable poly(amino acid)-modified PNIPAAm[9]. The hydrogels showed temperature dependent swelling, due to the temperature dependent solubility of PNIPAAM. At 10 °C, a swelling ratio relative to the dry mass of the network up to 55 was observed, whereas at 37 °C, the swelling ratio was close to 1. The swelling ratios of the prepared hydrogels were determined over a period of 36 d. The hydrogels were enzymatically degraded in the presence of elastase at 25 °C after 30 d. Without elastase, no degradation was observed.
In this paper, the synthesis and characterization of amphiphilic networks obtained by a reaction of amine terminated poly(ethylene glycol)s and N-hydroxysuccinimide activated poly(L-lactide)s are described. The swelling and degradation behavior of these hydrogels are also presented.

Experimental

Materials

L-lactide (L-LA) was purchased from Purac (Gorinchem, the Netherlands). 2,2-Bis (hydroxymethyl)propionic acid (bis-MPA), N,N'-dicyclohexylcarbodiimide (DCC) and succinic anhydride were obtained from Acros (Geel, Belgium). Tin(II) 2-ethylhexanoate ($Sn(Oct)_2$), N-hydroxysuccinimide (NHS), mesyl chloride, and $NaHCO_3$ were purchased from Aldrich (Zwijndrecht, the Netherlands). 2-(4-Hydroxyphenylazo)benzoic acid (HABA), 1,8,9-trihydroxyanthracene (dithranol), 2,4,6-trinitro benzenesulfonic acid (TNBS), aqueous ammonia (25%), and poly(ethylene glycol) diols with molecular weights of 1, 2 and 10 $kg \cdot mol^{-1}$, respectively, were obtained from Fluka (Buchs, Switzerland). Tetra-functional and octa-functional poly(ethylene glycol) with molecular weights of 2 and 20 $kg \cdot mol^{-1}$, respectively, were purchased from Nektar (Huntsville, USA). Glacial acetic acid, 4-dimethyl-aminopyridine (DMAP), triethylamine (TEA), chloroform, ethanol and diisopropyl ether were obtained from Merck (Darmstadt, Germany). All other organic solvents were from Biosolve (Valkenswaard, the Netherlands). Phosphate buffered saline (PBS) was obtained from Braun (Melsungen, Germany). Prior to

use, dichloromethane was dried over calcium hydride (Aldrich) and toluene over sodium wire, and subsequently distilled. All other chemicals were used as received.

Synthesis

Trifunctional two armed PLLA macromonomers were synthesized in 3 steps. First L-LA was polymerized in the presence of bis-MPA as the initiator in the melt, forming PLLAn where n is the number of repeating lactide units per arm. Subsequently, the hydroxyl end-groups were reacted with succinic anhydride to afford carboxylic acid end-groups, which were finally activated by conversion to NHS-esters. PEGs with 2, 4, or 8 amine groups were synthesized from the corresponding hydroxyl functional PEGs, according to a procedure described by Elbert and Hubbel[10]. The amine functionalized PEGs are denoted as fPEGy, where f is the number of functional amine groups and y is the molecular weight in $kg \cdot mol^{-1}$.

PLLAn: PLLAn was synthesized as described previously[11]. In a typical procedure PLLA20 was prepared by adding L-lactide (25.0 g, 174 mmol) to a reaction vessel, which contained bis-MPA (0.58 g, 4.4 mmol) as the initiator and $Sn(Oct)_2$ (0.10 g, 0.25 mmol; 0.4 wt% based on L-lactide) as the catalyst. The mixture was allowed to react for 3 h at 130 °C under an argon atmosphere. The product was subsequently cooled to room temperature and dissolved in dichloromethane. To this solution, a small amount of glacial acetic acid was added, and the product was precipitated in an excess of cold diethyl ether. The product was collected by filtration, and dried in vacuo to give a white powder (Yield: 84 %). In the synthesis of PLLA40 a reaction temperature of 140 °C was necessary to maintain a melt. PLLA5 was precipitated in diisopropyl ether instead of diethyl ether.

CPLLAn: The synthesis of CPLLA20 is given as a typical procedure: PLLA20 (18.0 g, 3.1 mmol), succinic anhydride (0.74 g, 7.4 mmol), DMAP (0.45 g, 3.7 mmol), and TEA (0.62 g, 6.2 mmol) were dissolved in 200 ml of dichloromethane, and stirred for 24 h under an argon atmosphere at room temperature. The solvent was partially evaporated with a rotary evaporator and the polymer was precipitated in diethyl ether : methanol (10:1 v:v) and dried in vacuo over night. The product was obtained as a white powder (Yield: 95 %). The synthesis as well as the purification procedure were similar for CPLLA40. CPLLA5 was precipitated in diisopropyl ether : methanol (10:1 v:v), and subsequently extracted with diisopropyl ether using a Soxhlet set-up, to remove residual DMAP and TEA.

NHS-CPLLAn: The synthesis of NHS-CPLLA20 is given as a typical procedure: CPLLA20 (15.0 g, 2.5 mmol) was dried by dissolution in 150 ml of toluene and subsequent azeotropic distillation. Subsequently, the material was dissolved in 200 ml of dichloromethane. To the resulting solution, NHS (1.02 g, 8.9 mmol) and DCC (3.06 g, 14.8 mmol) were added. The reaction mixture was stirred for 18 h at room temperature under an argon atmosphere. The formed dicyclohexylurea was removed after the reaction by filtration. The clear solution was concentrated by partially evaporating the dichloromethane, and the polymer was precipitated in an excess of cold diethyl ether : methanol (10:1 v:v). The product was filtered and dried in vacuo over night to give a white powder (Yield: 91 %). NHS-CPLLA5 was precipitated twice in cold diisopropyl ether : methanol (10:1 v:v).

fPEGy: fPEGy was synthesized according to the procedure as described for linear PEGs[10]. In a typical procedure 8PEG20-OH (10 g, 0.5 mmol) was dissolved in 150 ml of toluene and dried by the removal of 75 ml of solvent by azeotropic distillation. After the solution was cooled in an ice-bath, 30 ml of dichloromethane and TEA (1.7 ml, 12 mmol) were added. Subsequently, mesyl chloride (0.94 ml, 12 mmol) was added drop-wise under stirring and allowed to react overnight. The solution was filtered and precipitated in a large excess of diethyl ether. After drying, the formed 8PEG20-mesylate was reacted with 100 ml of an aqueous ammonia solution (25%) for 4 d. Subsequently, the ammonia was allowed to evaporate and the pH of the solution was raised to 13, using 1 M NaOH. The solution was extracted with dichloromethane (50 ml) for 3 times. The dichloromethane extracts were combined and concentrated. The product was precipitated in cold diethyl ether, and dried under vacuum (Yield: 89 %).

fPEGy-PLLAn: The synthesis of 8PEG20-PLLA20 is described as a typical procedure for the synthesis of fPEGy-PLLAn networks: NHS-CPLLA20 (41 mg, $6.3 \cdot 10^{-3}$ mmol) was dissolved in 0.5 ml of dichloromethane and, under gentle shaking, added to a reaction vessel (Ø = 24 mm) that contained 8PEG20 (59 mg, $2.4 \cdot 10^{-3}$ mmol) dissolved in 0.5 ml dichloromethane. The reaction is allowed to proceed for at least 24 h. After the reaction, the dichloromethane was evaporated for 24 h under a nitrogen flow, and subsequently for 24 h under vacuum. The networks were subjected to extraction with ethanol for 24 h, followed by extraction with water for another 24 h.

Characterization

Gel fraction: The gel fraction (F_G) was calculated from:

$$F_G = \frac{W_{d,0}}{W_{d,e}} \cdot 100\% \quad (1)$$

in which $W_{d,0}$ is the weight of the dry network before extraction, and $W_{d,e}$ is the weight of the dry network after extraction.

NMR: ^{1}H (300 MHz) and ^{13}C (75.4 MHz) NMR spectra were recorded on a Varian Inova NMR spectrometer. Polymers were dissolved in $CDCl_3$ at a concentration of 0.015 g·ml^{-1} (^{1}H) or 0.2 g·ml^{-1} (^{13}C).

Free amine group content: The primary amine group content, present in a network after reaction, was determined using a TNBS assay[12, 13]. To a sample of the polymer network of 5.8 mg, 1 ml of a 4 wt% $NaHCO_3$ solution and 1 ml of a freshly prepared 0.5 wt% TNBS solution in water were added. After reaction for 2 h at 37 °C, 3 ml of a 6 M HCl solution was added and the temperature was raised to 60 °C. Degradation of the sample was achieved after 2 d vigorous stirring. Finally, the sample was diluted with 5 ml of water, and the absorption was measured at 420 nm using a Cary 300 Bio UV-visible spectrophotometer (Varian). A calibration curve was constructed from the measured absorptions of the PEG-amines.

Viscometry: Intrinsic viscosities [η] were determined by a single point measurement using a capillary Ubbelohde type 0C at 25 °C and a polymer solution with a concentration of 0.3 g·dl^{-1} in chloroform. The following empirical relation was applied:

$$[\eta] = \frac{\sqrt{2}}{c} \cdot \sqrt{\eta_{sp} - \ln \eta_{rel}} \quad (2)$$

in which $\eta_{spec} = \eta_{rel} - 1$ and c is the polymer concentration in g·dl^{-1}. The relative viscosity ($\eta_{rel} = t/t_0$) was determined from the flow time of the polymer solution (t) and the flow time of the solvent (t_0).

GPC: Molecular weights and molecular weight distributions of the polymers were determined with gel permeation chromatography (GPC) using chloroform as eluent. The GPC setup consisted of a Waters 510 pump, a HP Ti-Series 1050 auto sampler, a series of standard Waters Styragel HR columns, a Waters 410 differential refractometer, and a viscometer detector H502 (Viscotek Corp.). Polystyrene

standards with narrow molecular weight distributions were used for calibration and the molecular weights were determined using the universal calibration principle.

MALDI-TOF: Matrix Assisted Laser Desorption Ionization Time-Of-Flight mass spectrometry (MALDI-TOF) was performed using a Voyager-DE-RP 2010 MALDI-TOF mass spectrometer (Applied Biosystems/ PerSeptive Biosystems, Inc.) equipped with delayed extraction. A 337 nm UV nitrogen laser producing 2 ns pulses was used and the mass spectra of the polymers were obtained in the reflection mode. Samples were prepared by mixing ~2 mg polymer with 1 ml chloroform. After that, ~5 mg of HABA or dithranol was added and the resulting solution was vigorously stirred. One µl of the solution was loaded on a gold sample plate. After evaporation of the solvent in air, the sample was transferred to the mass spectrometer for analysis.

Degree of swelling: The degree of swelling (DS) of the PEG-PLLA networks was gravimetrically determined. Dry networks were weighed (W_0) and subsequently immersed in water or buffer solution until equilibrium was reached. Excess water or buffer solution was carefully wiped away with a tissue, and the hydrogel weight (W_s) was measured. The DS was measured in triplo, and was calculated from:

$$DS = \frac{W_s - W_0}{W_0} \cdot 100\% \qquad (3)$$

Network degradation: Network degradation was monitored in time by measuring the degree of swelling and the dry mass loss in time. Networks were immersed in 10 ml of PBS buffer at 37 °C under gentle shaking, and the degree of swelling was measured at regular time intervals. The samples were dried for 24 h under nitrogen flow and the mass of the dry network was measured. After measurement, the samples were re-immersed in PBS. All measurements were performed in duplo.

Results and discussion

Synthesis

Amphiphilic PEG-PLLA networks were prepared by coupling either linear or star poly(ethylene glycol)s with amine end-groups to PLLA macromonomers containing NHS-activated ester groups (NHS-CPLLAn) (Figure 1). The synthesis of amine functionalized PEGs was performed according to a procedure as described for linear PEG's[10]. Hydroxy end-functionalized linear, 4-armed and 8-armed PEGs were converted to their corresponding mesylates in high yield. Reaction with aqueous ammonia for 4 d afforded the corresponding amino terminated PEGs. The ^{1}H-NMR

spectra of the PEGs revealed that only amino end-groups were present, since only a signal of the CH_2-NH_2 was observed at 2.86 ppm (data not shown).

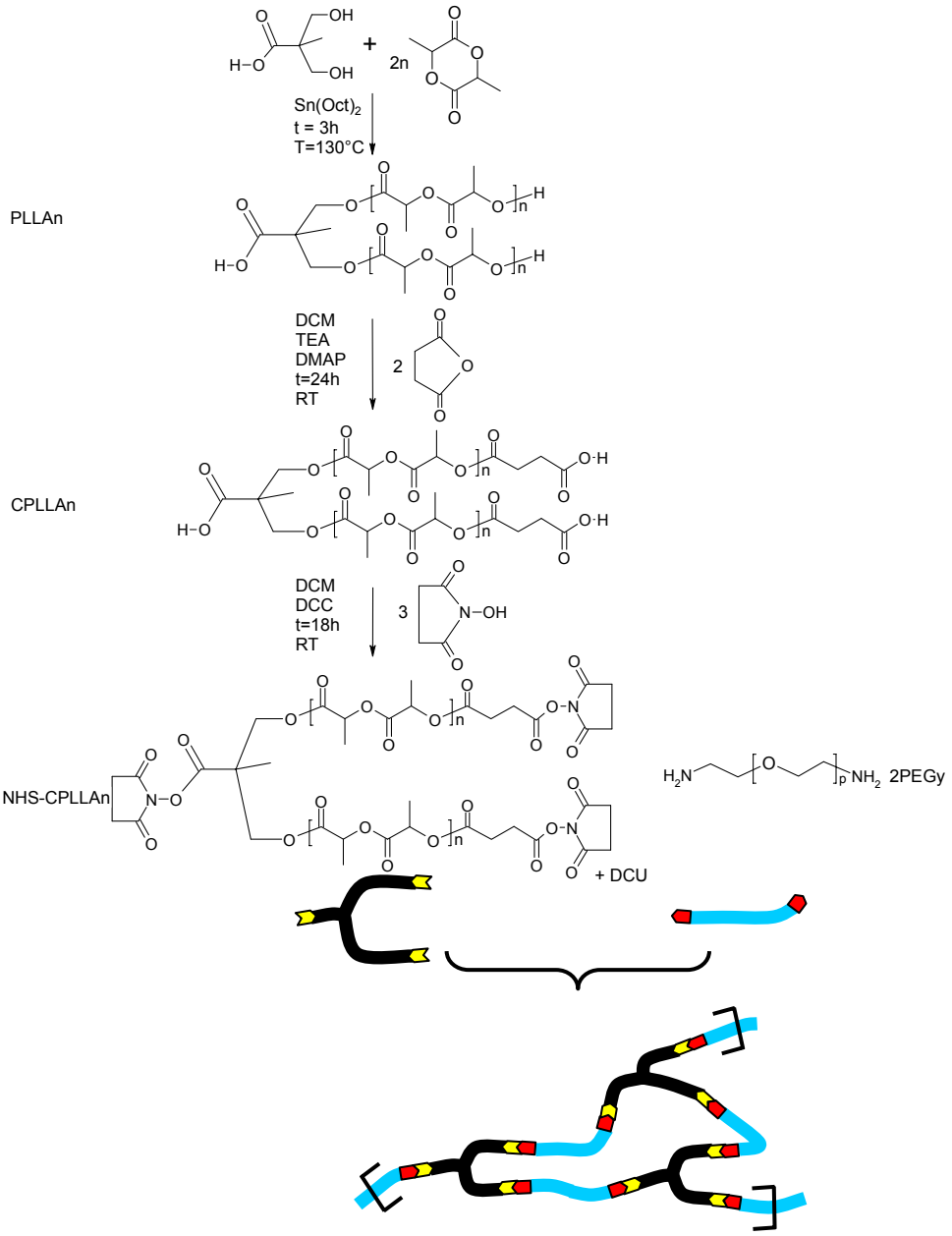

Figure 1. Synthesis route for the NHS-CPLLAn macromonomer, and schematic representation of the macromonomers and part of a PEG-PLLA network.

Ring opening polymerization of L-lactide using bis-MPA as the initiator resulted in PLLA macromonomers bearing one carboxylic acid group and two hydroxyl groups. High monomer conversions of approximately 97 % were obtained after 3 h reaction time as determined by the integral ratio of the C*H*$_3$ protons of the monomer (1.59 ppm) to polymer (1.65 ppm) in the ^{1}H-NMR spectra of the crude samples. The degree of polymerization (DP) and the number average molecular weight (Mn) were derived from the ^{1}H-NMR spectra of purified polymers (Figure 2A). The DP was determined from the ratio of the integrated area of the C*H* protons of the lactide repeating units (c, 5.10 ppm) to the C*H*$_3$ protons of the bis-MPA moiety (a, 1.27 ppm). The results obtained with ^{1}H-NMR (Figure 2) were in good agreement with the theoretical values, calculated from the monomer to initiator ratio [M]:[I].

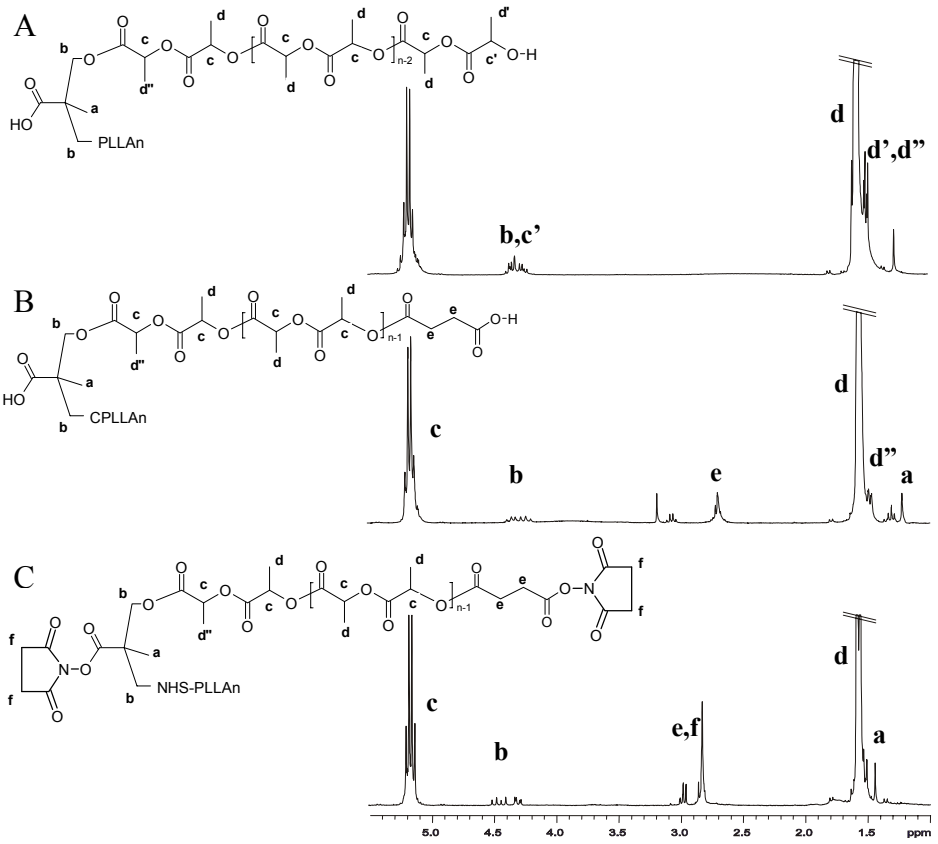

Figure 2. ^{1}H-NMR spectra of (A) PLLA20, (B) CPLLA20 and (C) NHS-CPLLA20. Solvent: $CDCl_3$.

Reaction of the hydroxyl end-groups of PLLAn with succinic anhydride resulted in CPLLAn having carboxylic acid end-groups. The ^{1}H-NMR spectra (Figure 2B) showed the appearance of a new peak at 2.68 ppm (e), corresponding to the C*H*$_2$ protons of the succinic half ester, and the disappearance of the signals at 4.30 ppm (c') and 1.49 ppm (d'), belonging to the protons of a terminal lactic acid unit. Furthermore, the signal belonging to the C*H*$_3$ protons of the bis-MPA moiety shifted from 1.27 ppm to 1.21 ppm (a). The ratio of the peaks corresponding to the C*H*$_3$ (a) and C*H*$_2$ (b) protons of the bis-MPA moiety, and the C*H*$_2$ protons of the succinic half ester (e) is 3:4:8 and reveals a high conversion of the hydroxyl end-groups.

The three carboxylic acid groups were subsequently converted to their N-hydroxysuccinimide activated esters. The ^{1}H-NMR spectrum of NHS-CPLLA20 showed the appearance of a new peak belonging to the C*H*$_2$ protons of the succinimide ring at 2.83 ppm (Figure 2C, peak f). Furthermore, the signal corresponding to the C*H*$_3$ protons of the bis-MPA moiety shifted from 1.21 ppm to 1.44 ppm (a), and the multiplet belonging to the CH_2 protons of the bis-MPA moiety shifted from 4.20-4.40 to 4.25-4.55 ppm (b). The C*H*$_2$ protons of the succinic ester appeared as two triplets (e, 2.83 ppm and 2.95 ppm). The 1:2:3 ratio of the signals that belong to the C*H*$_2$ protons of the bis-MPA moiety (b) and the C*H*$_2$ protons of the succinic ester and succinimide ring (e and f) revealed high conversions. An exception is a conversion of approximately 80 % of the carboxylic acid groups of CPLLA5. The macromonomers were further characterized by determining their intrinsic viscosities by single point measurements using chloroform as a solvent, and their molecular weights and molecular weight distributions by GPC (Table 1).

Table 1. Intrinsic viscosities, molecular weights and molecular weight distributions of PLLAn, CPLLAn and NHS-CPLLAn.

Macromonomer	$[\eta]^a$	Mn ($g \cdot mol^{-1}$)			PDI
	($dl \cdot g^{-1}$)	Calc [b]	^{1}H-NMR	GPC	(-)
PLLA5	0.07	1580	1600	2100	1.5
PLLA20	0.20	5900	6100	6900	1.3
PLLA40	0.39	11660	10700	11400	1.5
CPLLA5	0.08	1780	1700	1500	1.5
CPLLA20	0.22	6100	6200	5800	1.6
CPLLA40	0.39	11860	11200	9900	1.8
NHS-CPLLA5	0.12	2130	2000	2600	1.5
NHS-CPLLA20	0.24	6440	6500	7000	1.2
NHS-CPLLA40	0.40	12210	12300	12400	1.4

[a] chloroform, 25 °C

[b] calculated from the [M]:[I] ratio and 100% conversion

Since the conversion of the carboxylic acid groups to active NHS-esters for CPLLA5 was only 80 % based on ^{1}H-NMR, also ^{13}C-NMR and MALDI-TOF mass spectroscopy were performed on NHS-CPLLA5 to verify these results. The ^{13}C-NMR spectrum of NHS-CPLLA5 showed that a small signal was present at 176 ppm, which belongs to remaining carboxylic acid groups (data not shown).

Analysis of the mass distribution curve of NHS-CPLLA5 obtained with MALDI-TOF revealed 4 distributions. A detail of the mass distribution curve is shown in Figure 3. The 4 distributions are labeled as A_1, A_2, B_1 and B_2. The distributions A_1 and A_2 can be attributed to molecules possessing two NHS-groups (structure A), with either lithium (A_1) or sodium (A_2) as the attached ion. Distributions B_1 and B_2 can be attributed to molecules possessing three NHS groups (structure B) with lithium and sodium, respectively. The presence of the distributions corresponding to structure A confirm the ^{1}H-NMR results that the coupling of the carboxylic acid groups of CPLLA5 was incomplete.

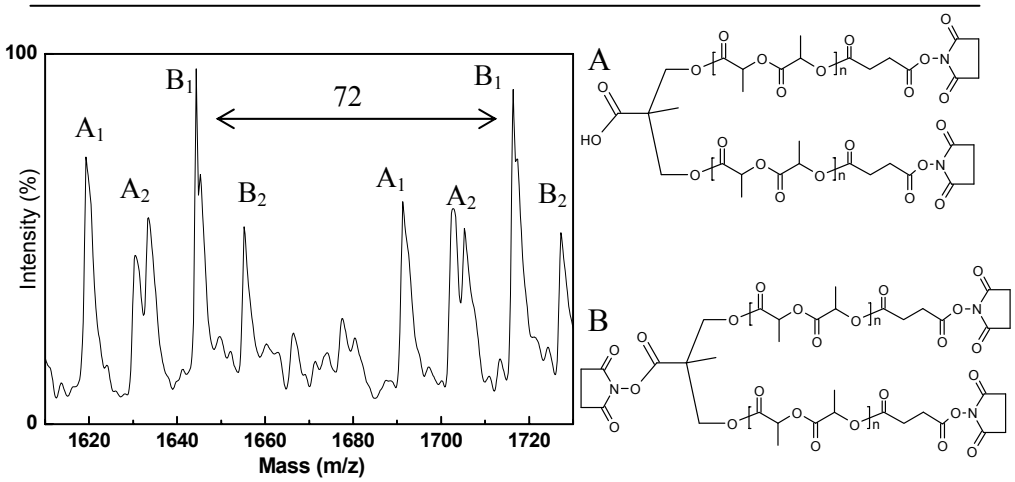

Figure 3. Detail of the MALDI-TOF mass distribution curve of NHS-CPLLA5 together with the structures of (A) NHS-CPLLA5 with only two NHS groups and (B) NHS-CPLLA5 with three NHS groups corresponding to distributions A and B, respectively.

Preparation of PEG-PLLA networks

The networks were prepared by reaction of the active ester groups of NHS-PLLAn with the amine groups of PEG in dichloromethane at room temperature. An increase in the viscosity of the reaction mixture was observed and the macromonomers were allowed to react until qualitatively no further increase in viscosity was observed, but at least for 24 h. In some cases, the reaction mixture was still free flowing after 24 h (Figure 4A). In these cases, the reaction was allowed to proceed for another 24 h at 37 °C. Cessation of the flow was regarded as an indication of network formation. The networks based on the hydrophilic macromonomer with the highest functionality (8PEG20) were formed most rapidly: within one min a highly viscous substance was obtained (Figure 4B).

Figure 4. Tilted reaction vials with 2PEG1-PLLA20 (A) and 8PEG20-PLLA20 (B) in dichloromethane after 24 h reaction time. 8PEG20-PLLA20 already formed a highly viscous substance (a network) after 1 min, whereas 2PEG1-PLLA20 was still free flowing after 24 h.

To investigate the network formation quantitatively, the extent of the reaction in one of the reaction mixtures, consisting of 2PEG1 and NHS-CPLLA20, was followed in time using ^{1}H-NMR (Figure 5). This reaction was performed in $CDCl_3$ instead of dichloromethane. The extent of the reaction could be followed in time, because of the difference in chemical shift of the succinimide protons in the NHS-ester and free NHS formed upon reaction of the amine group with the active ester. The extent of the reaction was calculated from the integral ratio of the methylene protons of the coupled succinimide ring (f, 2.83 ppm) and free NHS (f', 2.64 ppm). After 10 min, the extent of the reaction was already 62 %, and slowly increased during a following 2 d period to 73 %.

The extent of the reaction of the same network was also determined by measuring the amount of free amine groups after 2 d of reaction, using a TNBS assay, and was 68 %. This is in agreement with the results from ^{1}H-NMR.

For comparison, the extent of the reaction at which gelation takes place, the critical extent of the reaction p_c, can also be calculated using the statistical approach in case of an A_f +AA + BB system[14]:

$$p_c = \frac{1}{\{r \cdot [1 + \rho(f-2)]\}^{1/2}} \cdot 100\% \qquad (4)$$

in which f is the functionality of A_f, r is the ratio of the A and B groups initially present ($r \leq 1$), and ρ is the fraction of polyfunctional A ($f > 2$) of all A groups. In the system described above, no AA compound is present, and thus ρ is equal to 1. With r is equal to 1, and f is 3, this results in a p_c value of 71 %. This means that

with an extent of the reaction of 2PEG1 and NHS-CPLLA20 of 73 %, the reaction reached the gel point, and thus a network was formed.

The combination of NHS-CPLLA5 with any of the linear PEG amines did not result in an increase in viscosity, not after prolonged time, nor after elevation of the reaction temperature. It was concluded that network formation was far from complete for these mixtures. As described above the activation of the carboxylic acid groups of CPLLA5 was incomplete, and r and ρ are in this case both < 1. The extent of the reaction at which gelation occurs (p_c, equation 4) should then be higher than 88 %, which was apparently not reached, and no networks were formed.

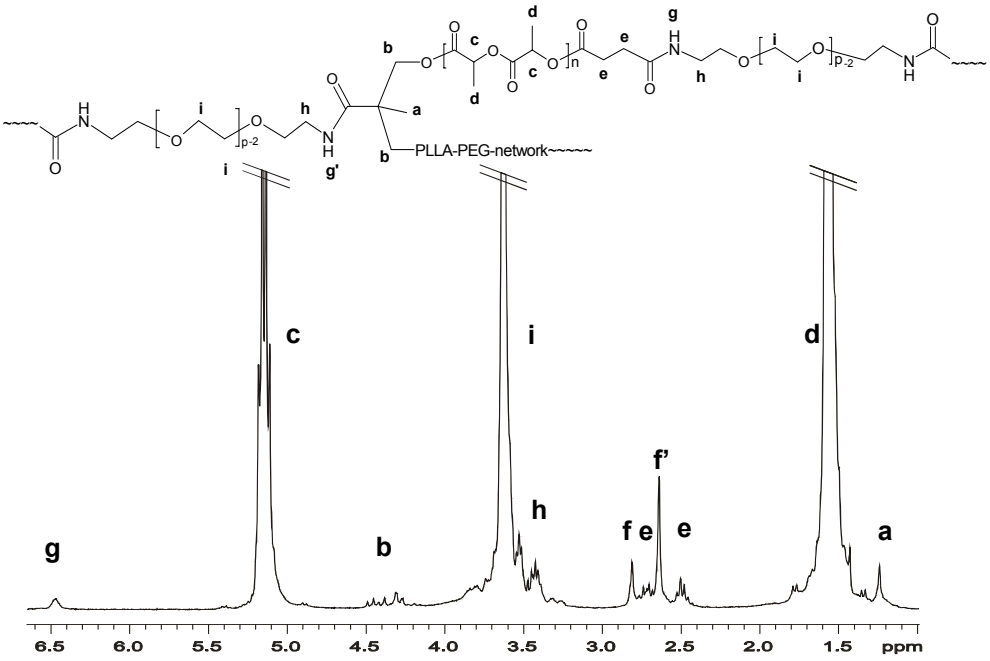

Figure 5. ^{1}H-NMR spectrum of the 2PEG1-PLLA20 network after 2 d reaction. Solvent: $CDCl_3$.

After evaporation of the organic solvent, the networks were extracted for 24 h with ethanol followed by 24 h extraction with water. The gel fractions (F_G) were determined from the dry weight of the network before and after extraction and are listed in Table 2. The gel fractions of all synthesized networks were highest when prepared from 8PEG20. The 8PEG20-PLLA5 network also had a F_G of 98 ± 1 wt%. The 4PEG2-PLLAn networks had the lowest F_G, although their functionality is

higher than that of the linear PEGs. This implies that the reactions with 4PEG2 had not reached the critical gel point, and that soluble fragments were extracted. Since the molecular weight per chain is relatively low in 4PEG2, increased sterical hindrance during the reaction may cause the low conversion and the critical gel point is not reached. When the F_G's of the networks based on linear PEGs are compared, the F_G decreases with increasing chain length of the PEG, possibly because the diffusion rate of PEG chains with higher molecular weight is lower than of PEG chains with lower molecular weight after the gel point is reached.

Table 2. Gel fractions (in wt% ± s.d.) of various networks after extraction with ethanol and water.

F_G (wt%)	NHS-CPLLA20	NHS-CPLLA40
2PEG1	94 ± 1	94 ± 1
2PEG2	91 ± 2	96 ± 0.4
2PEG10	78	95 ± 0.3
4PEG2	72 ± 7	92 ± 1
8PEG20	98 ± 1	98 ± 1

Network characterization

Degree of swelling

After extraction, the degree of swelling of the networks was determined by immersing the networks in water for 24 h at room temperature and measuring the weight of the hydrogel (Table 3).

Table 3. Degree of swelling (in wt% ± s.d.) in water at room temperature for various networks.

DS (wt%)	NHS-CPLLA20	NHS-CPLLA40
2PEG1	18 ± 1	11 ± 2
2PEG2	44 ± 3	23 ± 2
2PEG10	429 ±13	182 ±10
4PEG2	77 ± 4	17 ± 4
8PEG20	175 ± 5	104 ± 4

It was observed that the DS of the networks comprising the lower molecular weight PLLA20 block at similar PEG molecular weight and functionality are always higher. Two opposing effects influence the DS of these networks: (1) when the PEG content in the networks increases, this results in higher swelling; (2) when the hypothetic average number of atoms between branching points decreases, this results in reduced swelling. The calculation of the average number of atoms between branching points is made under the assumption of full conversion of end-groups (Table 4).

Table 4. PEG content and number of atoms between branching points of various networks.

	NHS-CPLLA20		NHS-CPLLA40	
	PEG cont. (wt%)	N[a] (-)	PEG cont. (wt%)	N[a] (-)
2PEG1	20	237	11	397
2PEG2	33	305	20	465
2PEG10	71	850	56	1010
4PEG2	20	118	11	198
8PEG20	55	252	39	332

[a] N = the average number of atoms between branching points, at full conversion of end-groups

A higher number of atoms between branching points is defined as a lower crosslink density, whereas a lower number of atoms between branching points is defined as a network with high crosslink density. The incorporation of smaller hydrophobic blocks resulted in a denser network, but also resulted in higher degrees of swelling. This indicates that the PEG content of the network may have a larger influence on the DS than the crosslink density. Comparing the networks synthesized from linear PEG-amines and both the NHS-CPLLA20 and NHS-CPLLA40 series showed that the DS increases when the molecular weight of the PEG was increased. This is due to the high PEG content in the network, and the formation of a less dense network. The 8PEG20-PLLA5 network had an exceptionally high DS of 1650 ± 180 wt%, which can be attributed to a combination of a very high PEG content and the loose network formed. The incomplete activation of the carboxylic acid groups of CPLLA5 is mainly responsible for the low crosslink density in this network.

The influence of the crosslink density on the DS can also be illustrated by comparing the swelling of networks with similar PEG content (Tables 3 and 4). For example, 2PEG1-PLLA20 and 2PEG2-PLLA40 both have a PEG content of 20 wt%, but the crosslink density of 2PEG1-PLLA20 is approximately two times higher. Nonetheless, it can be seen that the higher crosslink density in the 2PEG1-PLLA20 network only results in a slightly lower DS.

The influence of the PEG content of the networks on the DS can be illustrated by comparing networks with similar crosslink density. For example, the 2PEG1-PLLA20 and the 8PEG20-PLLA20 networks have the same crosslink density, but possess a PEG content of 20 wt% and 55 wt%, respectively (Table 4). The increase in PEG content results in a ten-fold increase in DS (Table 3).

Influence of temperature on degree of swelling

The temperature dependence of the network swelling was measured in the temperature range of 6 to 50 °C for fPEGy-PLLA20 networks (Figure 6). An increase in temperature led to a decrease of the DS for all networks due to dehydration of the PEG. For example, the DS of 2PEG10-PLLA20 decreased from 483 % at 6 °C to 359 % at 50 °C.

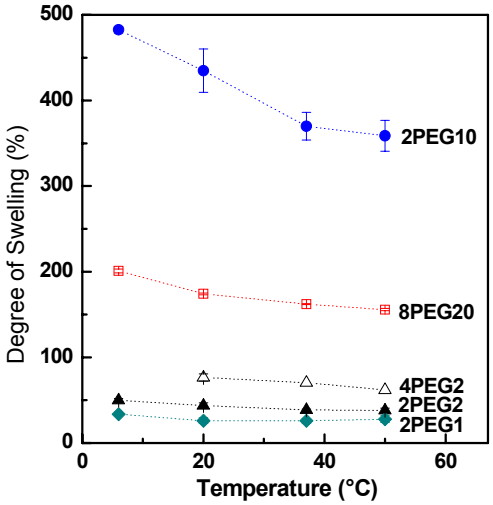

Figure 6. Degree of swelling at different temperatures of fPEGy-PLLA20 hydrogels.

The temperature dependence of the DS of the 8PEG20-PLLA40 hydrogels (data not shown) showed a similar dependency on temperature as the 8PEG20-PLLA20 hydrogels (Figure 6). The 8PEG20-PLLA5 hydrogels showed an increase in swelling due to severe degradation during the time of the swelling test.

Network degradation

The degradation of the fPEGy-PLLA20 hydrogels in PBS buffer (pH 7.4) at 37 °C was studied by monitoring the changes in DS (Figure 7) and dry mass loss (Figure 8) in time. As degradation proceeds through hydrolysis of ester bonds in the PLLA segments, the crosslink density decreases (Figure 7) The DS increased fastest for the 2PEG10-PLLA20 hydrogel having the highest PEG content. The 8PEG20-PLLA20 hydrogel showed an increase in DS from 175 wt% at the start of the experiment to 460 wt% after 21 d (Figure 7). At 28 d, it was not possible to measure the DS, due to severe fragmentation of the network. The network based on the four armed 4PEG2 degraded within 10 d. The low gel fraction of this network revealed that a less dense network was formed, leading to fast degradation.

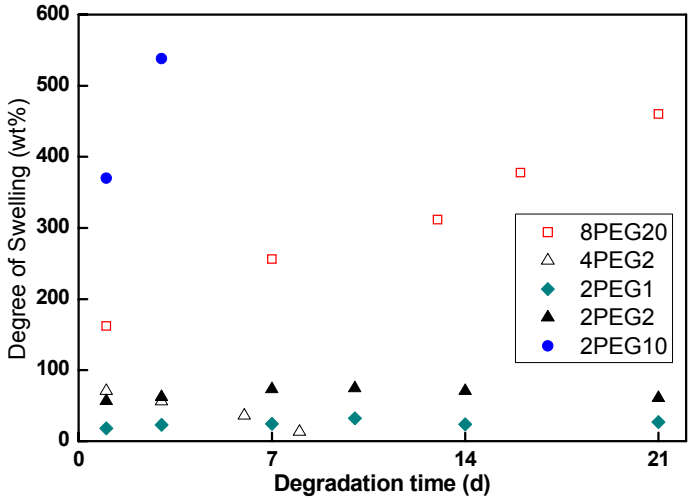

Figure 7. Degree of swelling of fPEGy-PLLA20 hydrogels as a function of degradation time (PBS, 37 °C).

The mass loss of the networks prepared from NHS-CPLLA20 and amine functionalized PEGs is shown in Figure 8. The networks comprising linear PEGs showed faster degradation at higher molecular weights of the PEG chains. The

2PEG1-PLLA20 networks showed a mass loss of 19 wt% after 28 d, whereas the 2PEG10-PLLA20 hydrogel was completely degraded within 10 d. The 8PEG20-PLLA20 showed 75 wt% mass loss after 28 d.

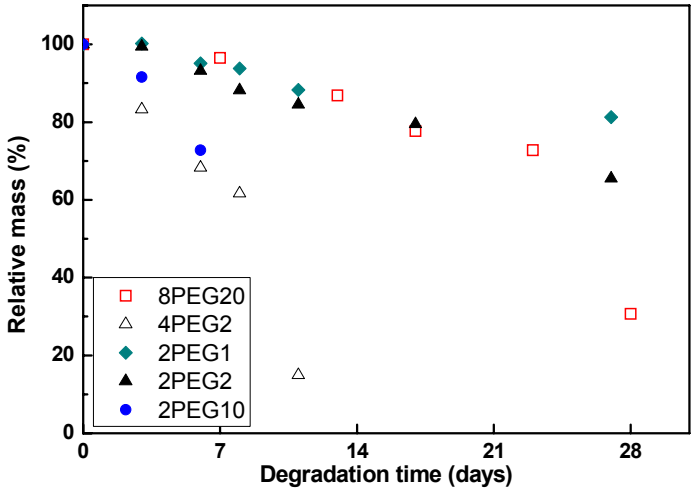

Figure 8. Mass (relative to the initial mass) of dried fPEGy-PLLA20 networks as a function of degradation time (PBS, 37 °C).

Conclusions

Amphiphilic biodegradable poly(ethylene glycol)-poly(L-lactide) (PEG-PLLA) networks were conveniently synthesized from linear and star-shaped poly(ethylene glycol)s having amine end-groups, and PLLA macromonomers of controlled molecular weight, bearing 3 NHS-activated carboxylic acid groups. Networks with high gel fractions were obtained. The gel fractions were highest for the networks based on the eight-arm star-shaped PEG. The degree of swelling ranged from 11 to 1650 wt% and mainly depends on the PEG/PLLA ratio. The hydrogels showed temperature dependent swelling in water. The degree of swelling decreased with increasing temperature, due to dehydration of PEG. In degradation experiments, the mass loss and the increase in degree of swelling were larger for hydrogels with a high PEG content and low crosslink density, as compared to hydrogels with a low PEG content and high crosslink density.

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Chapter 9

Initial studies on protein and drug release from chemically crosslinked poly(ethylene glycol)-poly(L-lactide) hydrogels

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Abstract

The release of lysozyme as a model protein and dexamethasone disodium phosphate (water-soluble, DEX-P) and dexamethasone (poorly water-soluble, DEX) from biodegradable PLLA-PEG hydrogels was studied. PLLA-PEG networks were prepared from an eight-arm star-shaped amine functionalized poly(ethylene glycol) (PEG) and two armed poly(L-lactide) (PLLA) macromonomers with three NHS active ester groups. The block length of each arm of the PLLA macromonomer block was 5, 20 or 40 lactide units. Lysozyme and DEX-P were loaded by swelling the dry networks in PBS buffer or aqueous solutions, respectively, containing the active agent. The poor water soluble DEX was loaded during network preparation. A fast release of DEX-P into PBS (pH 7.4) at 37 °C was observed from all networks, resulting in 100 % release after 1 d. The hydrophobic DEX was released within 1 d from the most hydrophilic network and within 3 d from a more hydrophobic network. Sustained release of lysozyme up to 8 d could be achieved, while retaining its enzymatic activity. The release of the first 50 % of lysozyme was linear with the square root of time, indicating diffusion controlled release. These preliminary results showed that PEG-PLLA hydrogels can be used as release systems for drugs or proteins, and that the release rate can be varied by changing the PEG : PLLA ratio.

Introduction

Hydrogels have received much interest for their use in drug and protein delivery[1-3], because they are generally biocompatible due to their high water content, and their properties resemble those of natural, soft tissue. Furthermore, proteins can be protected from denaturation in the body by incorporating them in hydrogels. The release of drugs and proteins is largely depending on the size of these drugs and proteins compared to the mesh size of the hydrogel[4, 5]. Amphiphilic hydrogels are of great interest, since their hydrophilic/hydrophobic ratio provides a way to control the swelling in aqueous solutions.

Block copolymers composed of poly(ethylene glycol) (PEG) and polyester blocks, like poly(lactide), have received much attention for the release of proteins and drugs. Such block copolymers are conveniently functionalized by their hydroxyl end-groups into vinyl end-groups through reaction with acyl halides. Sawhney et al.[6] used PEGs of various molecular weights and polyester blocks composed of lactide and glycolide. Crosslinking was accomplished using photopolymerization to produce highly swollen hydrogel networks. West and Hubbell studied the release of proteins and oligonucleotides from these type of hydrogels. The block copolymers had a central PEG block with a molecular weight of 10000 $g \cdot mol^{-1}$ and polylactide outer blocks (2.5 lactide units on either side) and were terminated with acrylate groups[7]. The equilibrium degree of swelling of such a network was 600 %. They incorporated various proteins, and observed that the release was highly dependent on the protein molecular weight. Release of insulin (MW = 6000 $g \cdot mol^{-1}$) was fastest with 47 % of the initial load released after 1 d, and immunoglobulin (MW = 150000 $g \cdot mol^{-1}$) was slowest. Lysozyme (MW = 14300 $g \cdot mol^{-1}$) was completely released in 2 d. Comparable networks were prepared by Anseth and coworkers[8-10]. They varied the content of degradable linkages to determine the effect on the overall swelling and mesh size of the network during degradation. It was found that a network with PEG (MW = 3400 $g \cdot mol^{-1}$) as the central block, showed a higher increase in swelling ratio and mesh size in time when the number of lactide units at each side of the PEG was increased from 0, to 1.5 and 2.5, with comparable degrees of crosslinking. Lysozyme was completely released from a network with 2.5 lactide units on each side of the PEG in 2 d. Lysozyme and bovine serum albumin (BSA) diffused from the network, since their sizes were smaller than the mesh-size of the network. The release of FTIC-dextran (77000 $g \cdot mol^{-1}$) was initially hindered, since

the size of FTIC-dextran was larger than the mesh-size. Upon degradation of the network, the mesh-size increased and FTIC-dextran was released.

Kricheldorf and coworkers[11] prepared cyclic triblock copolymers of PEG and aliphatic polyesters via the ring expansion polymerization of ε-caprolactone or D,L-lactide initiated from stannylated PEG. Biodegradable networks were then prepared by reaction of these cyclic triblock copolymers with trimesoyl chloride, as a crosslinker molecule. The release of dexamethasone (a hydrophobic steroidal anti-inflammatory drug) and 5-fluorouracil (a hydrophilic anti-neoplastic drug) from hydrogels based on PEG (MW = 1000 or 2000 $g \cdot mol^{-1}$) and 25 repeating ε-caprolactone units at each side of the PEG was studied. The release of the hydrophilic 5-fluorouracil was complete within 1 or 3 d for the network based on PEG with a molecular weight of 2000 and 1000 $g \cdot mol^{-1}$, respectively. Surprisingly, the total release of dexamethasone took 8 d for the PEG1000 based network, and 28 d for the PEG2000 based network.

We have previously reported on chemically crosslinked PEG-PLLA hydrogels prepared by the coupling of two-armed PLLA macromonomers and eight-armed PEG amine[12]. The degree of swelling varied from 104 to 1650 wt%. In addition, the degradation of these hydrogels was dependent on the degree of crosslinking and the number of repeating lactide units per arm. In this paper, the release of lysozyme as a model protein, as well as the release of the water-soluble dexamethasone disodium phosphate, and the more hydrophobic dexamethasone from these hydrogels are described.

Experimental

Materials

Eight-arm amine functionalized poly(ethylene glycol) with M_n = 20 $kg \cdot mol^{-1}$ (denoted as 8PEG20), poly(L-lactide) macromonomers containing three NHS activated ester groups (denoted as NHS-CPLLAn, where n is the number of repeating lactide units per arm), as well as networks prepared from 8PEG20 and NHS-CPLLAn, were synthesized as reported previously[12]. Lysozyme (from hen egg white M_w= 14kDa) was obtained from Fluka (Buchs, Switzerland). Dexamethasone and dexamethasone disodium phosphate were supplied by Sigma (St. Louis, USA). All organic solvents were from Biosolve (Valkenswaard, the Netherlands). Phosphate buffered saline (PBS) was obtained from Braun (Melsungen, Germany). Prior to use, dichloromethane was dried over calcium

hydride (Aldrich), and subsequently distilled. All other chemicals were used as received.

Loading and release

Dexamethasone disodium phosphate (DEX-P) loading: 8PEG20-PLLAn networks were loaded with DEX-P by swelling the networks (discs of ~ 50 mg) in an aqueous DEX-P (3.0 – 96 mg·ml^{-1}) solution for 24 h at room temperature. The DEX-P concentration was taken such that a loading of 5 wt% or 10 wt%, based on dry network mass, was achieved. After DEX-P loading, the networks were allowed to dry for 4 d at room temperature.

Dexamethasone (DEX) loading: 8PEG20-PLLAn networks were loaded with DEX during the preparation of the networks. Typically, NHS-CPLLA20 (41 mg, $6.3 \cdot 10^{-3}$ mmol) was dissolved in 0.5 ml of a dichloromethane : THF mixture (1:1 v/v) containing 5.0 mg DEX. The resulting solution was added to a reaction vessel (Ø = 24 mm) that contained 8PEG20K (59 mg, $2.4 \cdot 10^{-3}$ mmol) dissolved in 0.5 ml of a dichloromethane : THF mixture (1:1 v/v), under gentle shaking. The reaction was allowed to proceed for 24 h. After the reaction, the solvent was evaporated for 24 h under a nitrogen flow, and subsequently for 24 h under vacuum.

Lysozyme loading: 8PEG20-PLLAn networks (discs of ~ 50 mg) were loaded by swelling the networks in 0.6 - 48 mg·ml^{-1} lysozyme solutions in PBS for 24 h at room temperature. The lysozyme concentration was taken such that after swelling a loading of 1 wt% or 5 wt%, based on dry network mass, was achieved. After loading, the networks were allowed to dry for 4 d at room temperature.

***In vitro* release of active agents:** The loaded 8PEG20-PLLAn networks were placed in PBS at 37 °C. The volume of the release medium was chosen to ensure that the maximum concentration of the drug in the release medium would always be less than 10% of the maximum solubility, i.e., sink conditions[13]. Samples of 0.5 ml of the release medium buffer were taken at various time points, and replaced by an equal volume of fresh buffer. Measurements were performed in duplo. The concentration of DEX and DEX-P in the samples was determined from the absorbance at 243 nm (Varian Cary 300 UV-visible photospectrometer) and using calibration curves of DEX (concentration range 0.1 – 11.5 mg·l^{-1} in PBS) and DEX-P (concentration range 10 - 140 mg·l^{-1} in PBS). The concentration of lysozyme in the samples was determined using the BCA protein assay[22]. A calibration curve was prepared from lysozyme solutions in the concentration range from 0.02 to 0.6

mg·ml^{-1}. Samples (25 µl) were pipetted into a 96-microwell plate and 200 µl of working reagent (BCA reagent A : BCA reagent B, 50:1 vol:vol) was added. The plates were incubated for 30 min at 37 ºC followed by cooling to room temperature. Subsequently, the absorbance was determined at 550 nm with a Microplate Manager UV spectrophotometer (Bio-Rad Laboratories).
The release curves shown of DEX-P, DEX and lysozyme are the average of the release profiles of duplo measurements.
Lysozyme activity: The enzymatic activity of released lysozyme was determined for samples taken after 5 and 14 d. The assay is based on the lysis of the outer cell membrane of *Micrococcus lysodeikticus*, resulting in solubilization of the affected bacteria and consequent decrease of light scattering[14]. A sample of 10 µl was added to 1.3 ml of the bacteria suspension (0.2 mg·ml^{-1} in PBS). The decrease in turbidity was measured at 450 nm and the percent enzymatic activity was determined by comparing the activity of the sample with that of a freshly prepared reference lysozyme solution (0.05 mg·ml^{-1}).

Results and discussion

Hydrogel formation and degradation

In a previous study it was shown that amphiphilic PEG-PLLA networks could be conveniently prepared by the coupling reaction of star-shaped PEGs with 8 amine end-groups and two-armed PLLA macromonomers containing three NHS-activated ester groups (NHS-CPLLAn) (Figure 1)[12]. The reaction leads to chemically crosslinked networks by formation of amide bonds. In this study, disc-shaped PEG-PLLAn networks were prepared in which the number of repeating lactide units (n) in the NHS-CPLLAn macromonomers was 5, 20 or 40 (Table 1).

The networks are characterized by a large difference in their PEG content, which varies from 39 wt% for the 8PEG20-PLLA40 network to 81 wt% for the 8PEG20-PLLA5 network. The degree of swelling (DS) varies accordingly (Table 1). The exceptionally high DS of 903 ± 101 wt% of the 8PEG20-PLLA5 network, is due to a combination of a very high PEG content and a more loosely crosslinked network, compared to the networks 8PEG20-PLLA20 and 8PEG20-PLLA40. The loosely crosslinked network resulted from an incomplete activation of the carboxylic acid groups of NHS-CPLLA5[12]. The DS of all networks decreased with increasing temperature due to the lower solubility of PEG in water at higher temperatures.

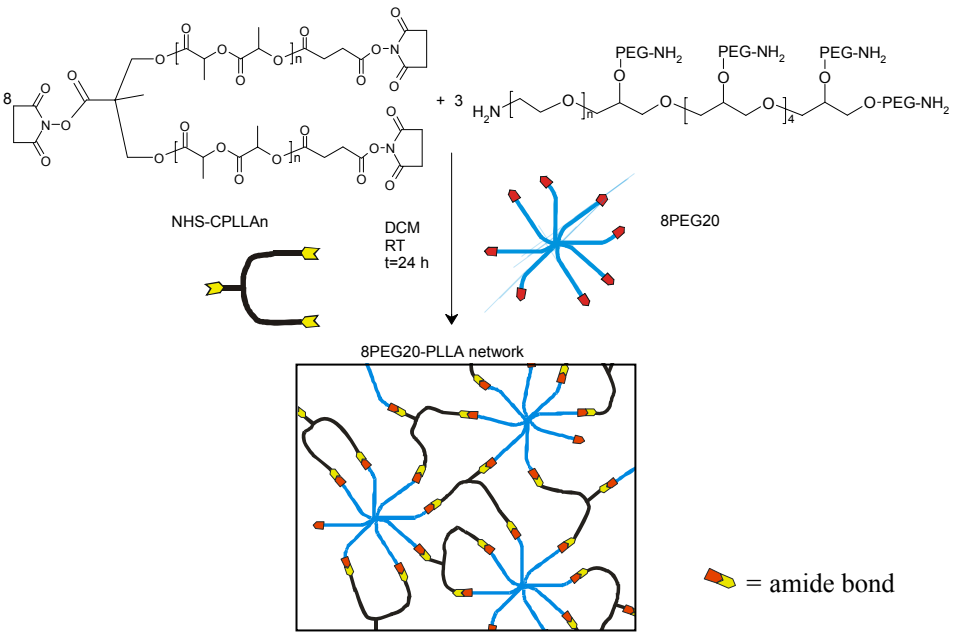

Figure 1. Formation of 8PEG20-PLLAn networks and schematic representations of the macromonomers and a part of a PEG-PLLA network.

Table 1. Characteristics of PEG-PLLA networks used in this study.

	Mn_{PLLA} [a] ($g \cdot mol^{-1}$)	$PEG_{cont.}$ [a] (wt%)	DS_{RT} [b] (wt% ± s.d.)
8PEG20-PLLA5	2000	81	903 ± 101
8PEG20-PLLA20	6500	55	171 ± 12
8PEG20-PLLA40	12300	39	80 ± 10

[a] calculated from ^{1}H-NMR

[b] Degree of swelling (DS) determined after swelling for 24 h at room temperature either using DEX-P containing water or lysozyme containing PBS. The standard deviation (s.d.) in the DS is based on combining the measurements using the two media.

Hydrogel degradation in PBS at 37 °C was monitored by measuring the increase in DS, and mass loss in time. For the 8PEG20-PLLA5 network which has a high PEG content and is loosely crosslinked, degradation occurred already to a large extent during the time scope of the experiments, resulting in an increased DS. The mass loss could not be determined accurately after 4 d, due to severe fragmentation of the

network. The degradation of 8PEG20-PLLA40 hydrogels was much slower and a mass loss of 10 wt% after 15 d was measured.

Loading and release

Loading and release of dexamethasone disodium phosphate

Dexamethasone (DEX) is well-known for its anti-inflammatory and immuno-suppressive properties. Dexamethasone disodium phosphate (DEX-P) is a hydrophilic pro-drug of DEX that can be converted into DEX in vivo, and is well soluble in water. Samples were loaded with DEX-P by immersing the dry network in a DEX-P solution in PBS at room temperature. The DEX-P concentration was taken such that after equilibrium swelling for 1 d an expected loading of ~5 or ~10 wt% was obtained. The actual loading (Table 2) was calculated from the total amount of drug released.

Table 2. Loading of water-soluble DEX-P in the 8PEG20-PLLAn networks.

	Low loading (wt%)		High loading (wt%)	
	Network 1	Network 2	Network 1	Network 2
8PEG20-PLLA5	4.2	8.4	9.0	9.2
8PEG20-PLLA20	6.9	7.5	13.1	9.1
8PEG20-PLLA40	3.8	3.7	7.1	6.4

The release of DEX-P from 8PEG20-PLLAn hydrogels in PBS buffer (pH 7.4) at 37 °C as a function of time is presented in Figure 2A. The release from 8PEG20-PLLA5 was almost complete (~90 %) within 30 min which is due to the high DS of this network. Furthermore, the mesh-size of the 8PEG20-PLLA5 network is expected to be much larger than the hydrodynamic diameter of the small DEX-P molecule. Similarly, a fast release of 90 % after 4 h from the 8PEG20-PLLA40 network was found. The release from 8PEG20-PLLA20 was more sustained and was complete within 24 h. The reasons for the differences of the release profiles of 8PEG20-PLLA20 and 8PEG20-PLLA40 have to be further investigated.

In Figure 2B the cumulative release profiles of DEX-P from 8PEG20-PLLAn hydrogels are presented as function of the square root of time. For the 8PEG20-PLLA20 hydrogel, the release is proportional to the square root of time. Because degradation of the networks is not taking place within the time scale of the

experiment, the profile implies that the release was diffusion controlled. At low loading the release of DEX-P appeared somewhat faster (Figure 3). Similarly, for the 8PEG-PLLA40 hydrogels profiles were found where the hydrogels with low loading show the fastest initial release (Data not shown). For the 8PEG20-PLLA5 hydrogels, the release rates are the same at both DEX-P concentrations.

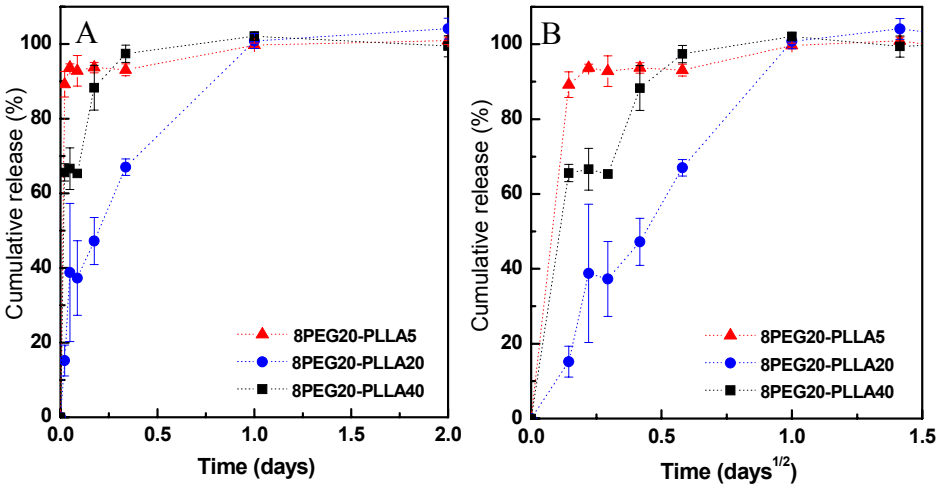

Figure 2. Cumulative release profiles of DEX-P from 8PEG20-PLLAn hydrogels in PBS (pH 7.4) at 37 °C as a function of (A) time and (B) square root of time. The curves are the average of measurements on networks at high loading (Table 2).

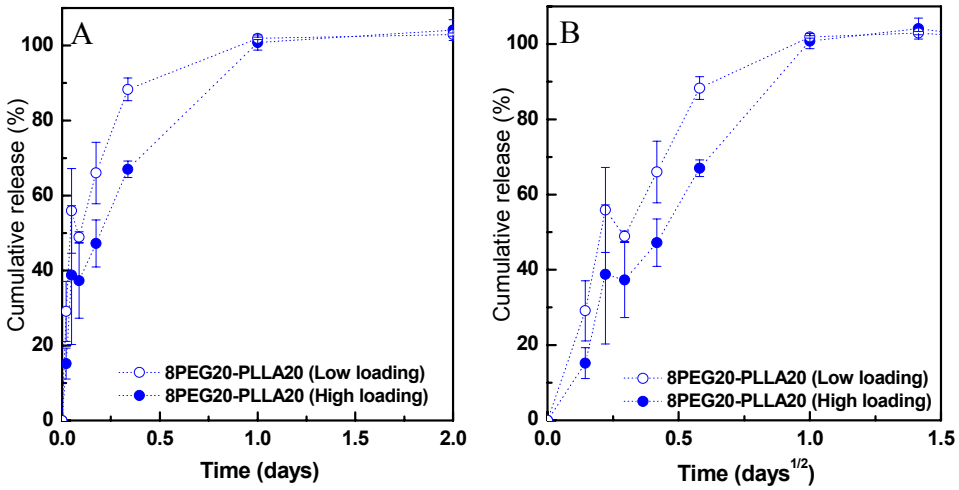

Figure 3. Cumulative release profiles of DEX-P from 8PEG20-PLLA20 hydrogels in PBS (pH 7.4) at 37 °C with different loadings, as a function of (A) time and (B) square root of time. The curves are the average of measurements on networks at low and high loading (Table 2).

Loading and release of dexamethasone

The hydrophobic DEX was loaded into the hydrogels during the network synthesis at a concentration of 5 wt% based on the dry network mass. After the preparation of the loaded hydrogel, the dichloromethane : THF mixture was allowed to evaporate, and the dry network was subsequently immersed in PBS. The release of DEX from 8PEG20-PLLAn hydrogel discs into PBS (pH 7.4) at 37 °C was subsequently determined. The cumulative release profiles of DEX from the 8PEG20-PLLAn hydrogels are plotted as a function of time (Figure 4A) and square root of time (Figure 4B), respectively.

It was observed that DEX was completely released from the 8PEG20-PLLA5 hydrogels within 1 d. The release from 8PEG20-PLLA20 and 8PEG20-PLLA40 was sustained over a period of 3 d. These release profiles show two distinct parts: a fast release during approximately 8 h, followed by more sustained release for the following 2.5 d. After swelling, a network with dissolved and dispersed drug is expected to be formed, since DEX is poorly soluble in water (100 $\mu g \cdot ml^{-1}$ [15]). The release up to 60 % of the initial loading is following a profile for a dispersed system as described by Higuchi[16].

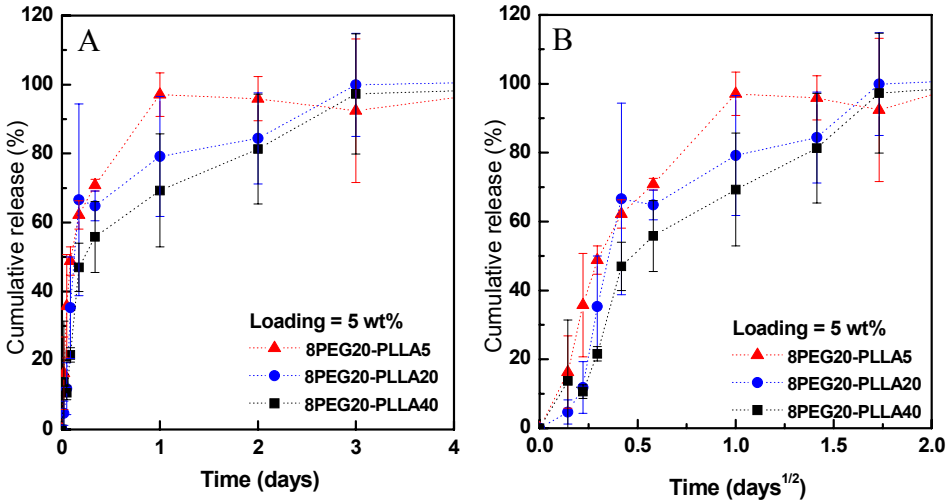

Figure 4. Cumulative release profiles of DEX from 8PEG20-PLLAn hydrogels in PBS (pH 7.4) at 37 °C as a function of (A) time and (B) square root of time. The curves are the average of duplo measurements.

Loading and release of lysozyme

Lysozyme was loaded into the hydrogel by immersing the dry network in a lysozyme solution in PBS at room temperature. The concentration of the solution was taken such that after swelling the loading would be approximately 1 wt% or 5 wt%. The actual loading was calculated based on the released lysozyme and is presented in Table 3. The amount of lysozyme loaded into 8PEG20-PLLA40 hydrogels was lower than into 8PEG20-PLLA5 and 8PEG20-PLLA20 hydrogels. Possibly, the 1 d loading time is not sufficient for the lysozyme to diffuse completely into the 8PEG20-PLLA40 hydrogel.

Table 3. Loading of lysozyme in the various 8PEG20-PLLAn networks.

	Low loading (wt%)		High loading (wt%)	
	Network 1	Network 2	Network 1	Network 2
8PEG20-PLLA5	1.2	1.3	4.0	3.8
8PEG20-PLLA20	1.2	1.1	1.6	1.8
8PEG20-PLLA40	0.2	0.1	0.3	0.6

The release of lysozyme (hydrodynamic diameter of 4.1 nm[17]) from 8PEG20-PLLA5 and 8PEG20-PLLA20 hydrogels, into PBS buffer (pH 7.4) at 37 °C was determined by sampling at regular time intervals. The release profiles of lysozyme from the hydrogel discs are plotted as a function of time (Figure 5A) and the square root of time (Figure 5B), respectively. The release profile of lysozyme from 8PEG20-PLLA5 showed a large burst (~50 %) in the first half h and after 8 h, 80 % was released. This fast release is due to the low crosslink density of the hydrogel. It is expected that the mesh-size of the 8PEG20-PLLA5 hydrogels is much larger than the hydrodynamic diameter of the lysozyme. Within the following ~8 d the remaining 20 wt% of the lysozyme was released. It has to be emphasized that the 8PEG20-PLLA5 network discs already loose their integrity after 4 d and were completely degraded after 8 d.

The release of lysozyme from the 8PEG20-PLLA20 hydrogel was more sustained. The profile shows two regions: in the first 8 h a linear profile with the square root of time (Figure 5B), indicating diffusion controlled release, is found. The release after 8 h was slower and showed a linear profile in time up to 8 d. The release profile of lysozyme from 8PEG20-PLLA40 is comparable with the profile of 8PEG20-PLLA20, and the lysozyme is completely released in 8 d.

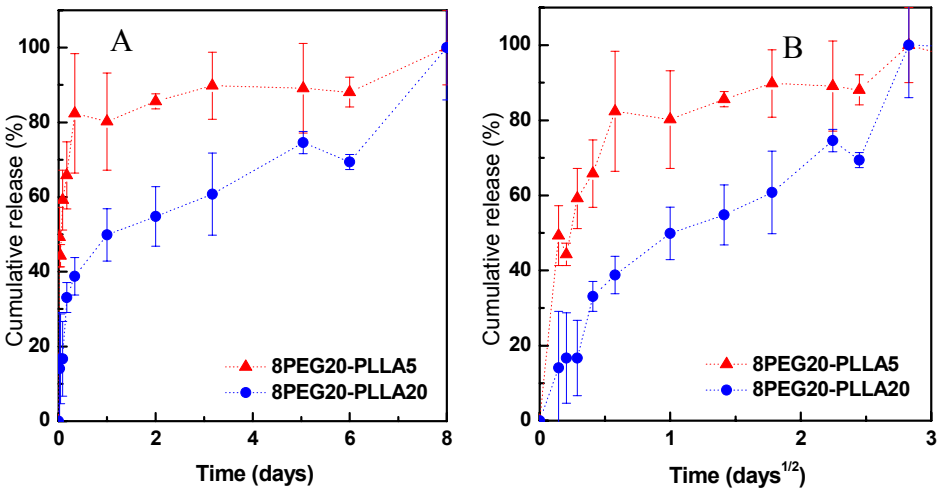

Figure 5. Cumulative release profile of lysozyme from chemically crosslinked 8PEG20-PLLAn hydrogels in PBS (pH 7.4) at 37 °C as a function of (A) time and (B) square root of time. The curves are the average of measurements on networks at high loading (Table 3).

The release profiles of lysozyme from 8PEG20-PLLA20 hydrogels with different loading are plotted in Figure 6 as function of time (A) and the square root of time (B), respectively. Although the initial release from the hydrogels with high loading was higher than for the hydrogels with low loading, the release rates were approximately the same for both hydrogels. In both cases the lysozyme was fully released in 8 d. The released lysozyme retained its activity as determined from bacteria lysis experiments performed on samples taken after 5 and 14 d (data not shown).

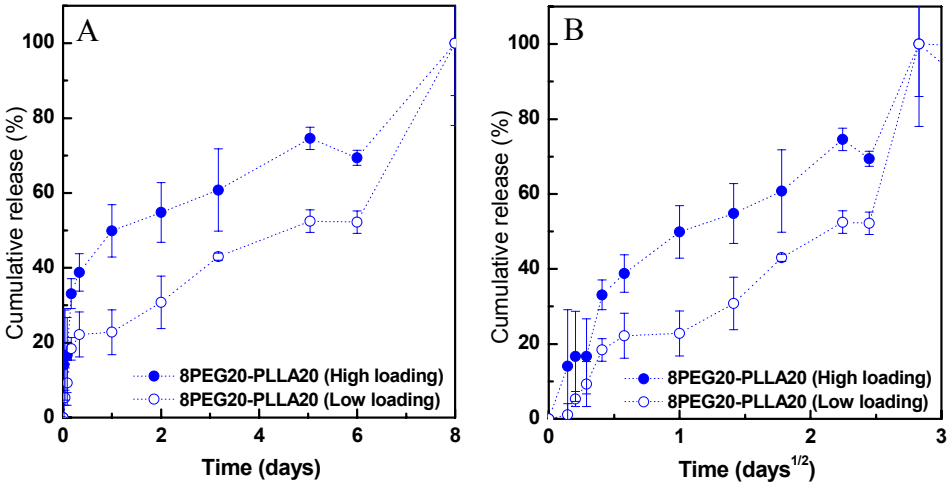

Figure 6. Cumulative release profiles of lysozyme from chemically crosslinked 8PEG20-PLLA20 hydrogels in PBS (pH 7.4) at 37 °C with different loadings as a function of (A) time and (B) square root of time. The curves are the average of measurements on networks at low and high loading (Table 3).

Conclusions

The release of lysozyme as a model protein and the anti-inflammatory and immunosuppressant drug dexamethasone in both hydrophilic and hydrophobic form from disc-shaped chemically crosslinked PEG-PLLA hydrogels was studied *in vitro*. The protein lysozyme was loaded into the hydrogels by swelling the networks in protein containing PBS buffer. Water soluble dexamethasone disodium phosphate (DEX-P) was loaded by swelling the networks in an aqueous solution of DEX-P. The hydrophobic dexamethasone (DEX) was loaded by preparing the network in a DEX containing dichloromethane : THF mixture. The hydrophilic pro-

drug DEX-P was released from the PEG-PLLA hydrogels within 1 d. After a fast release of 50 % of the hydrophobic DEX, the release of the remaining part was sustained over 3 d from the more hydrophobic hydrogels. Lysozyme was completely released from the hydrogels in 8 d, with full preservation of its enzymatic activity. These results show that biodegradable PEG-PLLA chemically crosslinked hydrogels can be used for the release of active agents such as proteins and hydrophobic drugs. The release rate can be adjusted by changing the PEG/PLLA ratio and the crosslink density of the network.

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Summary

The majority of thermo-responsive hydrogels currently investigated are based on amphiphilic polymers which give networks by the formation of physical crosslinks. Such physical crosslinks can be disrupted upon a change in temperature, which transfers the gel into a free flowing fluid, a sol. The transition of a gel to sol ideally is fully reversible. When this transition is close to body temperature these materials are potentially applicable as injectable systems for drug delivery and tissue engineering. Drugs or cells can be easily mixed into the free flowing sol, and upon injection into the body, the temperature change can cause a transition from sol to gel, forming a local drug or cell depot, in a minimally invasive manner. Preparing these hydrogels from biodegradable polymers offers the additional advantage that the hydrogels do not need to be explanted after their functional time, because the polymer will be degraded in the body, and the degradation products are excreted via natural pathways.

This thesis describes a study on the preparation of thermo-responsive hydrogels from biodegradable copolymers with branched architectures. In **Chapter 2** the current literature on thermo-responsive hydrogels for biomedical applications is reviewed. The emphasis is on physically crosslinked hydrogels based on synthetic biodegradable block copolymers with poly(ethylene glycol) (PEG) as one of the copolymeric components, and aliphatic polyesters as the other component. Depending on the block lengths, these copolymers in water show a transition from a free flowing fluid, a sol, to a non-flowing gel upon a change in temperature. The molecular weight, the composition as well as the architecture of these copolymers influence the sol-gel transition temperature. This sol-gel transition is discussed in relation to the copolymer architecture and composition.

The synthesis and characterization of branched polyesters is described in **Chapter 3**. Branched AB_2 functional polyesters could be readily prepared by the stannous octoate catalyzed ring opening polymerization of L-lactide and ε-caprolactone using 2,2-bis(hydroxymethyl)propionic acid (bis-MPA) as the initiator, with good control over molecular weight by varying the monomer to initiator ratio. In L-lactide polymerization, both bis-MPA hydroxyl groups initiated the polymerization

reaction, but for ε-caprolactone polymerization this depended on the monomer to initiator to catalyst ratio. The melting temperatures of the AB_2-functional poly(L-lactide) (PLLA) and poly(ε-caprolactone) (PCL) polymers were comparable to linear polymers with a degree of polymerization equal to the degree of polymerization per arm in the AB_2 polymer.

Chapter 4 describes a facile 'one-pot' method for the synthesis of hyperbranched poly(ε-caprolactone)s, consisting of ring opening polymerization of ε-caprolactone initiated from the AB_2 functional bis-MPA, followed by polycondensation at increased temperatures and under reduced pressure. The number of branching points and the molecular weight increased upon increasing the polycondensation time. Thermal analysis showed that the crystallinity of the hyperbranched polymers decreased with increasing number of branching points. A structural analysis of hyperbranched poly(ε-caprolactone)s, prepared via a 'two-step' procedure from purified PCL macromonomers at varying condensation times was performed. This analysis revealed that the structure of the hyperbranched polymer via the 'one-pot' preparation method was comparable to that of the hyperbranched poly(ε-caprolactone)s prepared via the 'two-step' procedure.

In **Chapter 5**, the synthesis and characterization of four-armed copolymers with a linear PEG middle block, and branched PLLA outer blocks are described. These copolymers have a relatively low molecular weight (< 6000 g·mol^{-1}) and a low PEG content (≤ 44 wt%). Above their critical gelation concentration (CGC ≥ 8 wt%) the copolymers formed turbid or translucent gels in water at low temperature, and showed a gel-sol transition upon an increase in temperature. An increase in hydrophobic block length resulted in a higher gel-sol transition temperature. Furthermore, the gel-sol transition temperature increased with increasing copolymer concentration in water. The storage and loss moduli of a 12.5 wt% hydrogel as measured with oscillatory rheology upon increasing the temperature showed maxima of 18 and 22 Pa, respectively, at 36 and 39 °C. This indicates that the gels formed are relatively weak.

In **Chapter 6** the thermo-responsive gelation behavior of PLLA-PEG copolymers, prepared from a branched PLLA containing three N-hydroxysuccinimide (NHS) active ester groups and amine functionalized methoxy-PEG, is presented. These copolymers with a hydrophobic PLLA core and PEG shell have a PEG content ≥ 57 wt%. Aqueous solutions of these branched block copolymers formed transparent gels at low temperatures above a CGC of ≥ 22.5 wt%. The hydrogels underwent a

gel to sol transition with increasing temperature as determined with the vial tilting method and oscillatory rheology. For all copolymers, the transition temperature increased with increasing copolymer concentration. The transition temperature of the hydrogels surprisingly decreased with increasing molecular weight of the PLLA branches. It is speculated that the non-uniform size distribution, as observed with dynamic light scattering, and possible crystallization of longer PLLA blocks may have a negative effect on the formation of micellar packing upon gelation, allowing the disruption of micellar or aggregate interactions to occur at lower temperatures. Oscillatory rheology experiments performed while cooling aqueous polymer solutions showed that both the storage and the loss moduli increased upon an increase in copolymer concentration. At 20 °C, hydrogels with relatively high storage moduli were obtained ranging from 0.2 to 17 kPa, depending on the copolymer composition, and the concentration of the aqueous solution.

In **Chapter 7** highly branched PEG-PLLA copolymers, prepared by the coupling of eight-armed amine functionalized PEG and PLLA macromonomer with a NHS-active ester group, are described. At low temperatures, copolymer solutions in water formed gels at low concentrations (≥ 4 wt%). These systems showed a phase transition from gel to sol upon increasing the temperature. The gel-sol transition temperature of an aqueous copolymer solution at a fixed concentration increased when the molecular weight of the PLLA block increased. Furthermore, the gel-sol transition temperature increased with increasing copolymer concentration. The transition temperature of the gels could be tuned closely to body temperature, which implies that these polymers may be used as injectable systems for in-situ gel formation.

In **Chapter 8** chemically crosslinked hydrogels, based on branched PLLA with three NHS active ester groups and linear or star-shaped PEG with two, four or eight amino end-groups, are described. The degree of swelling (DS) of these networks in water ranged from 11 to 1650 wt%, and depended mainly on the PEG/PLLA ratio. The DS of the prepared hydrogels decreased with increasing temperature, due to partial dehydration of PEG in water at higher temperatures. Degradation of the networks led to an increase of the DS and a decrease in the dry mass of the network with time. The degradation of the gels varied from complete mass loss within 4 d to a mass loss of 18 % after 28 d, depending on the polymer composition.

The chemically crosslinked networks prepared from eight-armed star-shaped PEG amine and branched PLLA with three NHS active esters as described in chapter 8,

were used to study the release of lysozyme as a model protein and dexamethasone in both water-soluble form (DEX-P) and poorly water-soluble form (DEX) (**Chapter 9**). Lysozyme and DEX-P were loaded by swelling the dry networks in PBS buffer or aqueous solutions, respectively, containing the active agent, and DEX was loaded during network preparation. A fast release of DEX-P in PBS (pH 7.4) at 37 °C was observed, resulting in 100 % release after 1 d. The hydrophobic DEX was released over a period of 1 to 3 d, depending on the PEG/PLLA ratio. Release of lysozyme could be achieved up to 8 d, after an initial burst. The enzymatic activity of the released lysozyme was retained. These preliminary results showed that PEG-PLLA hydrogels can be used as drug or protein release systems.

Samenvatting

De meeste temperatuur-gevoelige hydrogelen die momenteel veel aandacht krijgen, zijn gebaseerd op amfifiele polymeren die netwerken vormen door het ontstaan van fysische knooppunten ('crosslinks').
Deze fysische crosslinks ontstaan door een verandering in de temperatuur, waardoor de vloeistof, ook wel 'sol' genoemd, verandert in een gel. De overgang van sol naar gel is in het ideale geval volledig omkeerbaar. Wanneer deze overgang dichtbij lichaamstemperatuur plaatsvindt, zijn deze materialen potentieel toepasbaar als injecteerbare systemen voor medicijn afgifte en weefseltechnologie (tissue engineering). Medicijnen of cellen zijn eenvoudig te mengen in de vloeistof. Door injectie in het lichaam kan de temperatuursverandering een overgang veroorzaken van sol naar gel, zodat een lokaal medicijn- of celdepot onstaat, op een minimaal invasieve manier. De overgang van sol naar gel met natuurlijk wel snel zijn.
Het maken van deze hydrogelen op basis van biodegradeerbare polymeren heeft het extra voordeel dat de hydrogelen niet meer uit het lichaam verwijderd hoeven te worden nadat ze hun functie hebben volbracht, want het polymeer degradeert in het lichaam en de degradatieprodukten worden uitgescheiden via natuurlijke routes.
Deze thesis beschrijft een studie naar de bereiding van temperatuur-gevoelige hydrogelen op basis van biodegradeerbare copolymeren met vertakte architectuur.
In **Hoofdstuk 2** wordt een overzicht gegeven van de actuele literatuur over temperatuur-gevoelige hydrogelen voor biomedische toepassingen. De nadruk ligt op fysisch gecrosslinkte hydrogelen, gebaseerd op synthetische, biodegradeerbare blokcopolymeren met poly(ethyleen glycol) (PEG) als één van de copolymere componenten en alifatische polyesters als de andere component. Afhankelijk van de bloklengtes laten deze copolymeren in water een overgang zien van een vrij-vloeiende sol naar een niet-vloeiende gel door een verandering in temperatuur. Het molecuulgewicht, de compositie en de architectuur van deze copolymeren beïnvloeden de sol-gel overgangstemperatuur. De relatie van deze sol-gel overgang tot de copolymere architectuur en compositie is bediscussieerd.
De synthese en karakterisering van vertakte polyesters is beschreven in **Hoofdstuk 3**. Vertakte AB_2 gefunctionaliseerde polyesters zijn bereid door de tin-octoate

gekatalyseerde ring opening polymerisatie van L-lactide en ε-caprolacton, door gebruik te maken van 2,2-bis(hydroxymethyl)propion zuur (bis-MPA) als de initiator, met goede controle over het molecuulgewicht door de verhouding van monomeer tot initiator te variëren.

In de polymerisatie van L-lactide initieerden beide hydroxylgroepen van bis-MPA de polymerisatie reactie, maar voor de ε-caprolacton polymerisatie was dit afhankelijk van de monomeer tot initiator tot katalysator verhouding. De smelttemperaturen van de AB_2 gefunctionaliseerde poly(L-lactide) (PLLA) en poly(ε-caprolacton) (PCL) polymeren waren vergelijkbaar met lineaire polymeren met een polymerisatiegraad die gelijk was aan de polymerisatiegraad per arm in het AB_2 polymeer.

Hoofdstuk 4 beschrijft een gemakkelijke 'één-pots' methode voor de synthese van zeer sterk vertakte ('hyperbranched') poly(ε-caprolactone)s, bestaande uit de ring opening polymerisatie van ε-caprolacton geïnitieerd door AB_2 functioneel bis-MPA, gevolgd door polycondensatie bij verhoogde temperatuur en onder verlaagde druk. Het aantal vertakkingspunten en het molecuulgewicht namen toe bij toenemende polycondensatietijd. Thermische analyse liet zien dat de kristalliniteit van de hyperbranched polymeren afnam met toenemend aantal vertakkingspunten. Een structuur analyse was uitgevoerd voor hyperbranched poly(ε-caprolacton)s, gemaakt via een 'twee-staps' procedure vanuit gezuiverde PCL macromonomeren, bij verschillende condensatie tijden. Deze analyse liet zien dat de structuur van het hyperbranched polymeer via de 'één-pots' methode vergelijkbaar was met die van de hyperbranched poly(ε-caprolacton)s gemaakt via de 'twee-staps' methode.

In **Hoofdstuk 5** worden de synthese en karakterisering beschreven van vier-armige copolymeren met een lineair PEG middenblok en vertakte PLLA zijblokken. Deze copolymeren hebben een relatief laag molecuulgewicht (< 6000 $g \cdot mol^{-1}$) en een laag PEG gehalte (≤ 44 wt%). Boven hun kritische gelerings concentratie (CGC ≥ 8 wt%) vormen de copolymeren turbide of semi-transparante gelen in water bij lage temperatuur. Zij lieten een gel-sol overgang zien bij een toename in temperatuur. Een toename in de hydrofobe bloklengte resulteerde in een hogere gel-sol overgangstemperatuur. Bovendien nam de gel-sol overgangstemperatuur toe bij toenemende copolymeer concentratie in water. The opslag- en verliesmoduli van een 12.5 wt% hydrogel zijn gemeten met behulp van reologie bij toenemende temperatuur en lieten maxima zien van 18 en 22 Pa, respectievelijk, bij 36 en 39 °C. Dit geeft aan dat de gevormde gelen relatief zwak zijn.

In **Hoofdstuk 6** is het temperatuur-gevoelige gelerings gedrag gepresenteerd van PLLA-PEG copolymeren die gemaakt zijn van vertakte PLLA met drie N-hydroxysuccinimide (NHS) actieve ester groepen en amine gefunctionaliseerde methoxy-PEG. Deze copolymeren met een hydrofobe PLLA kern en PEG schil hebben een PEG gehalte ≥ 57 wt%. Oplossingen in water van deze vertakte blokcopolymeren vormden transparante gelen bij lage temperatuur boven een CGC van 22.5 wt%. De hydrogelen ondergingen een gel-sol overgang bij toenemende temperatuur, zoals is bepaald met de 'vial tilting' methode en reologie. Voor alle copolymeren gold dat de overgangstemperatuur toenam met toenemende copolymeer concentratie. De overgangstemperatuur van de hydrogelen nam onverwachts af met toenemend molecuulgewicht van de PLLA takken. Er is gespeculeerd dat de niet-uniforme grootte verdeling, zoals gevonden met dynamische licht verstrooiing, en mogelijk kristallisatie van langere PLLA blokken een negatief effect hebben op de vorming van een micellaire pakking tijdens gelering. Dit zorgt ervoor dat het verbreken van micellaire of aggregaat interacties kan plaatsvinden bij lagere temperatuur.

Reologie experimenten, uitgevoerd tijdens het afkoelen van polymeeroplossingen in water, lieten zien dat zowel de opslag- als de verliesmoduli toenamen bij een toename in copolymeer concentratie. Bij 20 °C werden hydrogelen met relatief hoge opslagmodi, varierend van 0.2 to 17 kPa, verkregen, afhankelijk van de copolymeer compositie en de copolymeer concentratie van de oplossing.

In **Hoofdstuk 7** zijn sterk vertakte PEG-PLLA copolymeren beschreven, die zijn gemaakt door het koppelen van acht-armige amine-gefunctionaliseerde PEG met PLLA die een NHS-actieve ester groep bevat. Bij lage temperaturen vormden copolymere oplossingen in water al gelen bij lage concentraties (≥ 4 wt%). Deze systemen lieten een fase overgang zien van gel naar sol bij toenemende temperatuur. De gel-sol overgangstemperatuur van een copolymere oplossing in water, bij een vaste concentratie, nam toe als het molecuulgewicht van de PLLA blokken toenam. Bovendien nam de gel-sol overgangstemperatuur toe als de copolymere concentratie toenam. De overgangstemperatuur van de gelen kon geregeld worden tot dichtbij lichaamstemperatuur, wat aangeeft dat deze polymeren gebruikt kunnen worden als injecteerbare systemen voor in-situ gel vorming.

In **Hoofdstuk 8** worden chemisch gecrosslinkte hydrogelen beschreven die gebaseerd zijn op vertakt PLLA met drie NHS actieve ester groepen en lineair of vertakt PEG met twee, vier of acht amino eindgroepen. De zwelgraad (DS) van

deze netwerken varieerde van 11 tot 1650 wt% en was met name afhankelijk van de PEG/PLLA ratio. De DS van de gemaakte hydrogelen nam af met toenemende temperatuur. Degradatie van de netwerken leidde tot een toename in de DS en een afname in de droge massa van het netwerk in de tijd. De degradatie van de gelen varieerde van volledig massa verlies binnen 4 d tot een massa verlies van 18 wt% na 28 d, afhankelijk van de polymeer compositie.

De chemisch gecrosslinkte netwerken, gemaakt van acht-armig stervormig PEG amine en vertakt PLLA met drie actieve ester groepen zoals beschreven in hoofdstuk 8, zijn gebruikt om de afgifte van lysozyme als model eiwit en dexamethason in zowel wateroplosbare vorm (DEX-P) als in slecht wateroplosbare vorm (DEX) te bestuderen (**Hoofdstuk 9**). Lysozyme en DEX-P zijn geladen door de droge netwerken te laten zwellen in, respectievelijk, PBS buffer of water dat de actieve stof bevatte. DEX was geladen tijdens de vorming van de netwerken. Een snelle afgifte werd gevonden voor DEX-P in PBS (pH 7.4) bij 37 °C, wat resulteerde in 100 % afgifte na 1 d. Het hydrofobe DEX werd afgegeven over een periode van 1 tot 3 d, afhankelijk van de PEG/PLLA verhouding. Afgifte van lysozyme kon worden verkregen tot een periode van 8 d, na een initiele ‘burst’. De enzymatische activiteit van de afgegeven lysozyme was behouden. Deze voorlopige resultaten laten zien dat PEG-PLLA hydrogelen kunnen worden gebruikt als medicijn- of eiwitafgifte systeem.

Curriculum Vitae

Ingrid Velthoen is geboren op 14 februari 1979 in Apeldoorn. Na het behalen van haar VWO diploma aan het Christelijk Lyceum te Apeldoorn in 1997, is zij chemische technologie gaan studeren aan de Universiteit Twente in Enschede met polymeerchemie en biomaterialen als hoofdrichting. In 2002 heeft zij stage gelopen bij het DaimlerChrysler onderzoekscentrum in Ulm, Duitsland. De stage opdracht was getiteld: "The effect of process conditions on the mechanical properties of uni-directional glass fiber reinforced thermoplastics". Na deze stage begon zij met afstuderen bij de vakgroep Polymeerchemie en Biomaterialen aan de Universiteit Twente van Prof. Dr. J. Feijen onder begeleiding van Dr. P.A.M. Lips. De afstudeeropdracht had als titel: "Preparation of biodegradable poly(ester amide) scaffolds for biomedical applications; scaffold preparation by gas foaming and particulate leaching". In 2003 behaalde zij haar universitair diploma. In dezelfde vakgroep ging zij vervolgens werken als assistent in opleiding onder begeleiding van Prof. Dr. J. Feijen en Dr. P.J. Dijkstra. Het promotieonderzoek was getiteld: "Thermo-responsive hydrogels based on branched block copolymers". De resultaten van dit onderzoek staan beschreven in dit proefschrift.