Living Polypeptides

ARTICLE *in* BIOMACROMOLECULES · SEPTEMBER 2004

Impact Factor: 5.75 · DOI: 10.1021/bm0497217 · Source: PubMed

Living Polypeptides

Thrasyvoulos Aliferis, Hermis Iatrou, and Nikos Hadjichristidis*

Department of Chemistry, University of Athens, Panepistimiopolis, Zografou, 15771 Athens, Greece

Received May 9, 2004; Revised Manuscript Received June 21, 2004

Block copolypeptides, which combine the self-assembly of block copolymers and the highly ordered 3D structures of proteins, are potential candidates for novel supramolecular structures and biotech applications, such as biosensors, tissue engineering, and selective drug delivery. The synthesis of model block copolypeptides through living nucleophilic/basic polymerization of α-amino acid *N*-carboxyanhydrides (NCAs) has been a challenge for more than fifty years, most probably due to traces of impurities in the system. This problem has been overcome, using high vacuum techniques in order to create and maintain the conditions necessary for the living polymerization of NCAs with primary amines. This method is a general one and opens avenues leading to novel, well-defined polypeptides with various architectures.

Natural polypeptides (proteins) are copolymers of α-amino acids linked together, through amide bonds, in a well-defined sequence, which characterizes their primary structure. Intra- and intermolecular interactions between the functional groups of the residual amino acids lead to organized higher order (secondary, tertiary, and quarternary) 3D structures,[1] which are responsible for the extremely selective and quantitative catalytic as well as mechanical performance of proteins. Tirrell and co-workers[2] have adapted bioengineering techniques to synthesize novel polypeptides that are not found in nature, whereas others[3] inspired by the seminal work of Merrifield[4,5] have attempted to employ the polymerization schemes used for synthetic polymers to make particular polypeptides.

On the other hand, incompatible polymeric chains joined together covalently into block copolymeric structures have the ability to self-assemble into ordered nanostructures,[6] which are responsible for their suitability in nanotechnology applications.[7,8] Consequently, block copolypeptides could lead to novel supramolecular assemblies which would impart tremendous potential not only for basic protein research but also for applications such as sensors, drug delivery systems, tissue engineering, and biomimetic synthesis of ordered inorganic nanostructures.[9−12]

In general, well-defined block copolymers can only be synthesized through living polymerization routes, which require that all reagents involved in the polymerization are free of any impurities. In addition, the environment in which the reactions take place must also be free of impurities, to avoid undesired side (different initiating sites) and termination reactions.

We suspect that these extremely restrictive conditions have led not only to poorly defined polypeptides and copolypeptides[13,14] but also to confusing interpretations of the ring opening polymerization (ROP) mechanisms[15−17] of α-amino acid *N*-carboxyanhydrides (NCAs) by nucleophile/base initiators. Resolving this problem has been a synthetic challenge for more than 50 years.[15, 18]

In 1997, Deming has succeeded in synthesizing well-defined block copolypeptides from the living polymerization of α-amino acid NCA using initiators resulting from the reaction of an NCA-monomer with the zerovalent nickel complex bipyNi(COD); bipy = 2,2′-bipyridyl, COD = 1,5-cyclooctadiene.[19−21] This initiator provides active sites, which are less accessible to side reactions due to steric and electronic reasons. Deming resolved the problem by using a different initiating system and thus a different polymerization mechanism. In this case, special care with regard to the elimination of the metal must be taken. In addition, to our knowledge, this method has not been applied by other groups. However, our approach of controlling the reaction conditions, resolved the existing for more than fifty-year-old challenge presented by the NCA/nucleophile system. Furthermore, it is also more efficient for the synthesis of block copolypeptides with complex macromolecular architectures.

Our strategy involves the polymerization of α-amino acid NCAs with primary amines, i.e., *n*-hexylamine and 1,6-diaminohexane, strong nucleophiles, which force the reaction to follow exclusively the "normal-amine route" (Figure 1A). Furthermore, we have employed our expertise in the high vacuum technique (HVT) in order to create and maintain the conditions necessary for the living polymerization of NCAs. In the past, we have successfully applied this technique to handle very sensitive organolithium initiators and living polymers in the synthesis of model complex macromolecular architectures.[22]

Until now, HVT is the only reported technique which ensures that all reagents and the reaction environment are completely impurity-free in all steps of the synthesis. For this purpose, reactors equipped with break-seals along with magnets covered with glass, and constrictions, for the addition of reagents and removal of the intermediate products are designed and employed. The sealed reactors are constructed only of glass without ground joints, which otherwise enable the leakage to the polymerization system of humidity

* To whom correspondence should be addressed. E-mail: hadjichristidis@chem.uoa.gr.

10.1021/bm0497217 CCC: $27.50
Published on Web 07/10/2004

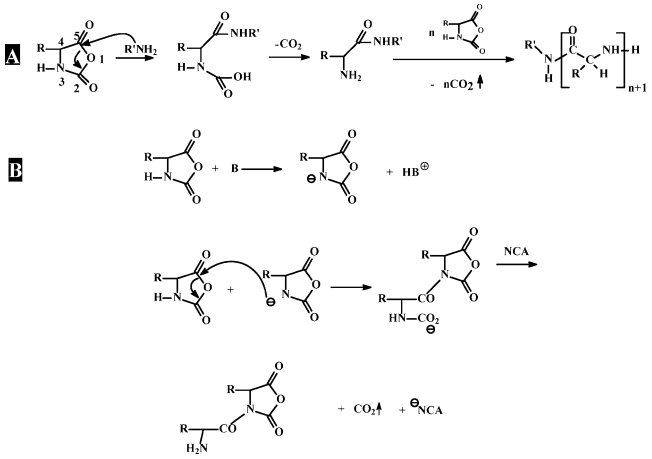

Figure 1. (A**)** "Normal-amine mechanism". The primary amines, strong nucleophiles, attack the 5-CO of the NCA monomer; the resulting carbamic acid releases CO_2 from the 1-O and 2-CO to give the free amino group, which continues the polymerization in the absence of active impurities. **(**B**)** The "monomer activated mechanism". In the presence of a strong base (e.g., alkoxide), deprotonation of the amine group of the NCA monomer takes place, and the resulting NCA anion acts as a nucleophile initiator.

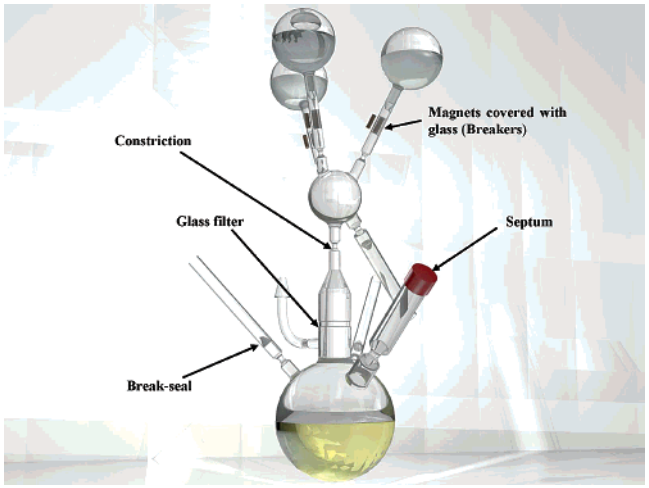

Figure 2. Apparatus used for the purification of the NCA monomers**.** The monomer solution (see text) is introduced through the rubber septum into the apparatus. Then, the apparatus is attached to the vacuum line, the solvent is distilled off under vacuum, and the solid monomer is pumped for 1 day to dryness. Three crystallizations are carried out at − 8 °C, using ethyl acetate (vacuum line) and *n*-hexane (three upper flasks) as the solvent/nonsolvent pair. After each crystallization, the solid monomer is separated from the mixture of solvent/nonsolvent through the glass filter, followed by removal of the liquid (by flipping the apparatus over and heat sealing the flasks) and drying of the monomer in the vacuum line. Finally, an appropriate amount of purified DMF is distilled from the vacuum line to the apparatus until a concentration of 10% w/w is reached. Similar kinds of apparatuses were used for purification of the reagents and for polymerizations.

or other contaminants from the air. All apparatuses are initially flame dried, under high vacuum ($<10^{-6}$ mmHg), followed by purging with dry benzene, before the addition of the reagents. Benzene was chosen because it can be made absolutely water free. It solubilizes and removes traces of water from the surface of the internal walls of the glass apparatuses. Elimination of water is critical because, depending on its concentration, it can act either as a slow initiator of NCA monomers or as a terminator.[16] The use of HVT for the exclusion of water and other impurities was the key parameter for the successful synthesis of living polypeptides, that has not been achieved so far with this initiating system. Full details concerning the HVT glass reactors and processes are given in our recent review.[23]

NCAs, the monomers, were synthesized from the corresponding α-amino acid and triphosgene in ethyl acetate at 70 °C. The unreacted species along with the HCl and the amino acid salts are removed by extraction with an aqueous alkali solution and water.[24] The organic phase was introduced into a specially designed homemade apparatus shown in Figure 2 for extreme purification by crystallization under high vacuum conditions.

Prior to use, the monomer solution was kept under vacuum in a sealed apparatus equipped with precalibrated ampules at −20 °C. It was found that at least three crystallizations were required in order to eliminate the tiny traces of impurities. This was necessary to synthesize high molecular weight polypeptides (>700 monomeric units).

DMF (99.9+ %, special grade for peptide synthesis with less than 50 ppm of active impurities), the polymerization solvent, was further purified by short-path fractional distillation under vacuum in a custom-made apparatus. The middle fraction was always used. The usual impurity is dimethylamine, which polymerizes NCA monomers according to the "normal-amine route", as well as the "activated monomer mechanism" (AMM) (Figure 1B)[16] due to its nucleophilic/basic character. The AMM leads to macromonomers and consequently to branched polypeptides. Recently Dimitrov and Schlaad[25] used the hydrochloric salt of the amino-initiators in order to avoid the AMM.

n-Hexylamine (99.9%) and 1,6-diaminohexane (99.9%), the initiators, which are highly hygroscopic compounds, were left to dry over a sodium mirror for 24 h and then were diluted with purified DMF, subdivided into ampules, and stored under high vacuum at room temperature.

The polymerization reactors were designed to have a volume at least three times larger than the volume of the CO_2 generated by each polymerization. The combination of high vacuum and large volume significantly influences the kinetics of the polymerization.

Characterization was performed by using size exclusion chromatography (SEC) and membrane osmometry (MO) for the determination of the polydispersity index and the absolute molecular weight of the synthesized polypeptides, respectively. Both analyses were performed at 60 °C with a 0.1 N LiBr solution in DMF. The SEC instrument was calibrated by low polydispersity PBLG samples, synthesized by HVT in our laboratory, with known number average molecular weights (M_n). The SEC instrument featured a diode-array UV−Vis detector operating at 267 nm in order to monitor the polypeptides containing UV absorbing groups.

The "living" nature of the ROP of NCAs was evaluated according to the following criteria:[26] (a) complete consumption of the monomer, (b) linearity of M_n with conversion, (c) stoichiometric control of the molecular weight, (d) narrow molecular weight distributions, and (e) synthesis of block copolypeptides by sequential monomer addition.

Complete consumption of the monomer was verified by the absence of the monomer peak in the SEC−UV chro-

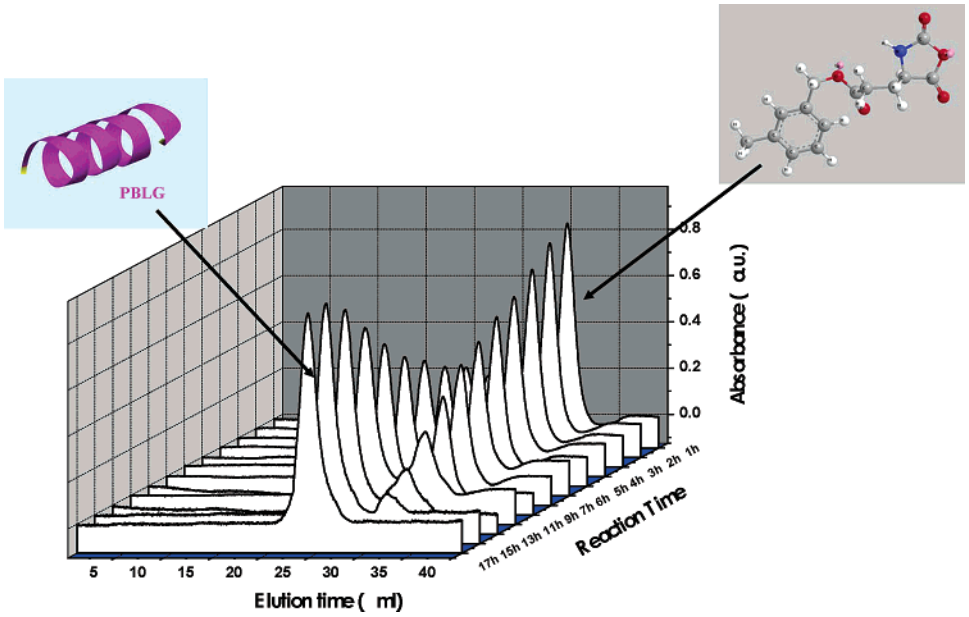

Figure 3. Monitoring the polymerization of the $PBLG_{438}$ using size exclusion chromatograms with UV detector. After 17 h, more that 99.9% of the monomer has been consumed. It is well-known that PBLG and PZLL with DP > 80 exhibit 100% the *a*-helix conformation.[27−29]

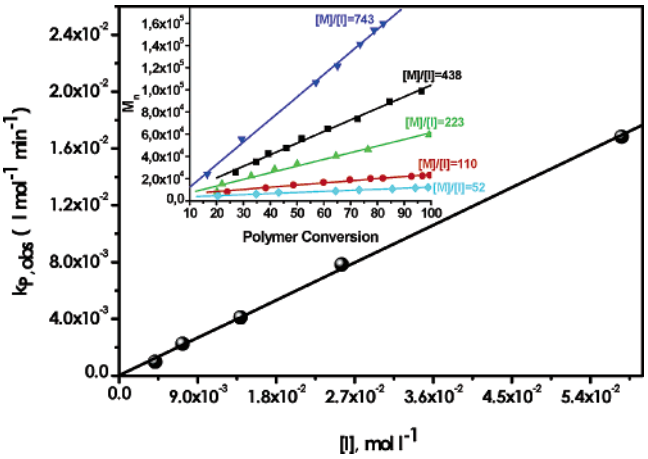

Figure 4. Linear dependence of $K_{p,obs}$ of the PBLG samples with initiator concentration proves that the chains grow without termination. At the inset is the dependence of the M_n as a function of polymer conversion for several [M]/[I] ratios.

matograms of the final products of all polymerizations. A representative example is given in Figure 3.

To confirm the linearity of M_n with conversion, a series of polymerizations of γ-benzyl-L-glutamate (Glu) NCA was performed, under identical conditions with the exception of the monomer [M] to initiator concentration [I] ratio, which was set at values of 52, 110, 223, 438, and 743. The polymer conversion was monitored by measuring the area of the peaks (SEC−UV) corresponding to the monomer and the growing polypeptide. The M_n of the growing chain at each conversion was obtained by SEC. A representative example corresponding to [M]/[I] = 438 is given in Figure 3. In all cases studied, the molecular weight varied linearly with the conversion up to eight half-life times (inset of Figure 4), proving that there is no chain transfer during polymerization.

The experiments carried out to verify the second criterion were also used to identify the polymerization mechanism. In the case of the "normal-amine" mechanism, the polymerization rate is given by the following equation:

$$-\frac{d[M]}{dt} = k_p[M][I] \Rightarrow -\frac{d\ \ln[M]}{dt} = k_{p,obs} \qquad (1)$$

where k_p and $k_{p,obs}$ are the propagation and the observed polymerization rate constants, respectively. It is clear from Figure 4 that $k_{p,obs}$ is a linear function of concentration of [I]. First order kinetics was also obtained in respect to [M], and consequently the polymerization follows exclusively the "normal-amine" mechanism.

The half-life times ($t_{1/2} = \ln 2/k_{p,obs}$) corresponding to different ([M]/[I]) ratios are 0.71 (52), 1.47 (110), 2.81 (223), 5.53 (438), and 11.8 h (743), and the rate constant $k_p = 5.12 \times 10^{-3}\ s^{-1}$ was practically the same for all polymerizations.

The control of molecular weight by stoichiometry is established as shown in Table 1 where the theoretical molecular weight, calculated from the [M]/[I] ratio, agrees very well with the membrane osmometry molecular weight (M_n).

The narrow molecular weight criterion is satisfied because the polydispersity indices were maintained at values from 1.02 to 1.12, for DP up to 223 and then increased slightly at DP 438 (1.18), and finally to 1.4 at DP 743 (see Table 1). For high molecular weights, the initiator concentration is lowered thus becoming comparable to that of the system's impurities, leading to relatively higher polydispersity indices.

The last criterion was checked with the synthesis of several diblock and triblock copolymers of various *a*-amino acid NCAs. The diblock and triblock copolypeptides were prepared by sequential addition of a-amino acid NCA to *n*-hexylamine or 1,6-diaminohexane, respectively. A wide variety of a-amino acids was employed, to verify that the "living" nature of the polymerization is independent of the monomer residue. It can be observed from Table 1 that the copolypeptides synthesized had low polydispersity indices and the molecular weights expected from the stoichiometry. Sample SEC chromatograms of a triblock and a diblock copolypeptide are given in Figure 5.

The yield of the polymerization was nearly 100% (Table 1). From the discussion above, it is clear that the polymer-

Table 1. Molecular Characteristics of Homo- and Block Copolypeptides Synthesized

	first polymerized block			final copolypeptide		
sample[a]	$M_n \times 10^3$ [b]	$M_{n,s} \times 10^3$ [c]	M_w/M_n [d]	$M_n \times 10^3$ [b]	$M_{n,s} \times 10^3$ [c]	M_w/M_n [d]
$PBLG_{57}$	12.7	12.5	1.02			
$PBLG_{110}$	24.3	24.1	1.07			
$PBLG_{223}$	48.4	48.8	1.12			
$PBLG_{438}$	96.6	95.9	1.18			
$PBLG_{743}$	166.3	162.7	1.40			
$PBLG_{130}-PZLL_{320}$	28.0	28.4	1.07	103.4	106.6	1.05
$PBLG_{110}-PZLL_{220}$	23.6	24.1	1.08	76.1	78.2	1.06
$PZLL_{90}-PBLG_{150}-PZLL_{90}$	30.8 [e]	32.9 [e]	1.07	75.2	77.1	1.07
$PBLG_{180}-PGLY_{15}$	37.1	39.4	1.15	38.1	40.3	1.20
$PBLG_{157}-PTYR_{20}$	32.8	34.4	1.16	37.8	39.3	1.17
$PBLG_{180}-PLEU_{22}$	37.1	39.4	1.15	41.0	41.8	1.17
$PZLL_{100}-PBLG_{60}$	23.1	24.6	1.05	36.1	37.7	1.06

[a] PBLG: poly(γ-benzyl-L-glutamate), PZLL: poly(ϵ-carbobenzoxy-L-lysine), PGLY: Poly(glycine), PTYR: poly(L-tyrosine), PLEU: poly(L-leucine); subscript denotes the theoretical DP of each block. [b] Membrane osmometry. [c] Stoichiometric molecular weight. [d] Size exclusion chromatography. [e] In this case, the first polymerized block is the middle block.

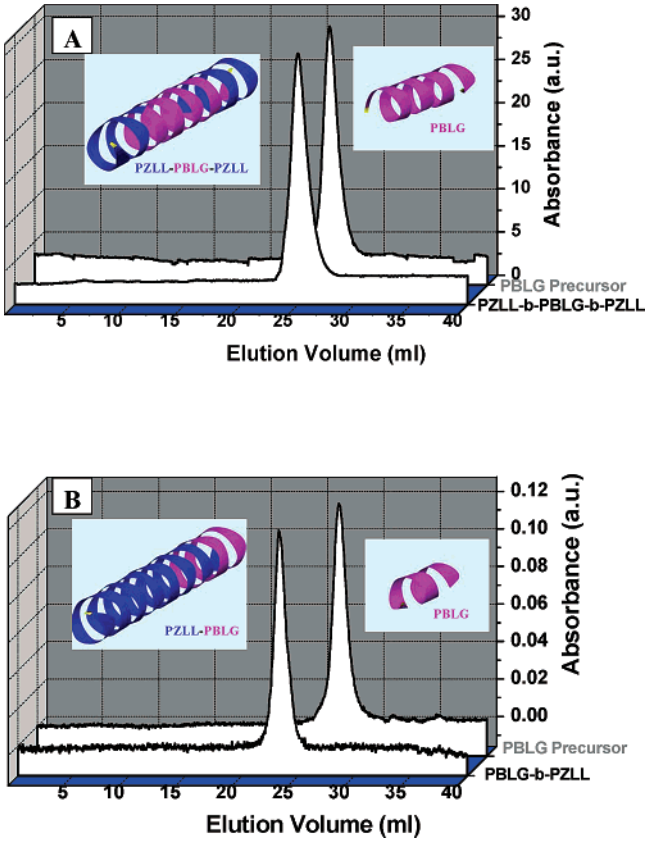

Figure 5. Monitoring the synthesis of $PZLL_{90}-PBLG_{150}-PZLL_{90}$ (A) and $PBLG_{130}-PZLL_{320}$ (B) by SEC with a UV detector.

ization fulfills all of the requirements to be characterized as "living". Consequently, the cause of the limited synthesis of polypeptides with the NCA/primary amines system was a question of impurity level.

In conclusion, we have solved a problem that has been a major synthetic challenge for more than 50 years by using high vacuum techniques which ensure not only high purity of all reagents involved but also create and maintain all necessary conditions for the living polymerization of NCAs. The livingness of the polypeptides under high vacuum conditions can be preserved indefinitely, thus making possible the use of linking chemistry for the synthesis of a wide variety of macromolecular architectures.

Preliminary experiments in this direction led to the successful synthesis of model 3-arm star homo- and block copolypeptides (linking agent triphenylmethane-4,4′,4″-triisocyanate). The synthesis of model polypeptides exhibiting complex architectures is currently under investigation.

References and Notes

(1) Alberts, B.; Bray, D.; Lewis, J.; Raff, M.; Roberts, K.; Watson, J. *Molecular Biology of The Cell* 2nd ed.; Garland Publishing: New York, 1989; pp 87−134, 820−830.
(2) Tirrell, D.; Fournier, M.; Mason, T. *MRS Bull.* **1991**, *16*, 23.
(3) Dawson, P.; Muir, T.; Clark-Lewis, I.; H. Kent, S. *Science* **1994**, *266*, 776.
(4) Merrifield, R. *Science* **1965**, *150*, 178.
(5) Merrifield, R.; Stewart, J. *Nature* **1966**, *207*, 522.
(6) Savic, R.; Luo, LB.; Eisenberg, A.; Maysinger, D. *Science* **2003**, *300*, 615.
(7) Sigel, R.; Fytas, G.; Vainos, N.; Pispas, S.; Hadjichristidis, N. *Science* **2002**, *297*, 67.
(8) Chan, N.; Hoffman, J.; Lee, V.; Iatrou, H.; Avgeropoulos, A.; Hadjichristidis, N.; Miller R.; Thomas, E. *Science* **1999**, *286*, 1716.
(9) Duncan, R. *Nat. Rev.* **2003**, *2*, 347.
(10) Griffith, L.; Naughton, G. *Science* **2002**, *295*, 1009.
(11) Cha, J.; Stucky, G.; Morse, D.; Deming, T. *Nature* **2000**, *403*, 289.
(12) Mao, C.; Solis, D.; Reiss, B.; Kottmann, S.; Sweeney, R.; Hayhurst, A.; Georgiou, G.; Iverson, B.; and Belcher, A. *Science* **2004**, *303*, 213.
(13) Hernandez, J.; Klok, H. *J. Polym. Sci. Part A: Polym. Chem.* **2003**, *41*, 1167.
(14) Cardinaux, F.; Howard, J.; Taylor, G.; Scheraga, H.; *Biopolymers* **1977**, *16*, 2005.
(15) Sekiguchi, H. *Pure Appl. Chem.* **1981**, *53*, 1689.
(16) Kricheldorf, H.; In *Models of Biopolymers by Ring Opening Polymerization*; CRC Press: Boca Raton, FL, 1990; pp 160−225.
(17) Kricheldorf, H.; In *α-Amino acid−N-Carboxyanhydrides and Related Materials*; Springer-Verlag: New York, 1987; pp 1−280.
(18) Woodward, R.; Schramm, C. *J. Am. Chem. Soc.* **1947**, *69*, 1551.
(19) Deming, T. *Nature* **1997**, *390*, 386.
(20) Deming, T. *J. Polym. Sci. Part A: Polym. Chem.* **2000**, *38*, 3011.
(21) Brzezinska, K.; Curtin, S.; Deming, T. *Macromolecules* **2002**, *35*, 2970.
(22) Hadjichristidis, N.; Pitsikalis, M.; Pispas S.; Iatrou, H. *Chem. Rev.* **2001**, *101*, 3747.
(23) Hadjichristidis, N.; Iatrou, H.; Pispas S.; Pitsikalis, M. *J. Polym. Sci. Part A: Polym. Chem.* **2000**, *38*, 3211.
(24) Poché, D.; Moore, M.; Bowles, J. *Synth. Commun.* **1999**, *29*, 843.
(25) Dimitrov, I.; Schlaad, H. *Chem. Commun.* **2003**, *23*, 2944.
(26) Fetters, L. *Encyclopedia of Polymer Science and Engineering*, 2nd ed.; Wiley-Interscience: New York, 1987; Vol. 10, pp 19−25.
(27) Lecommandoux, S.; Achard, M.; Langewalter, J.; Klok, H. *Macromolecules* **2001**, *34*, 9100.
(28) Koening, S.; Sutton, P. *Biopolymers* **1970**, *9*, 1229.
(29) Bird, G.; Idelson, M. *J. Chem. Phys.* **1956**, *25*, 798.

BM0497217