Advances in the Synthesis and Characterization of Polypeptide-Based Hybrid Block Copolymers

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Summary: Linear polystyrene-*block*-poly(Z-L-lysine) copolymers with a very narrow molecular weight distribution (polydispersity index < 1.03) could be obtained *via* the ring-opening polymerization of Z-L-lysine-*N*-carboxyanhydride using ω-(primary amino hydrochloride)-polystyrenes as macroinitiators in *N*,*N*-dimethylformamide as the solvent at 40-80 °C. The block copolymer samples were analyzed by means of NMR, size exclusion chromatography, and analytical ultracentrifugation.

Keywords: analytical ultracentrifugation; block copolymers; liquid chromatography (SEC/GPC); polypeptides; ring-opening polymerization

Introduction

Amphiphilic block copolymers are known for their ability to form thermodynamically stable hybrid materials with a diverse ordering on the nanometer length scale.[1,2] Among the large number of block copolymers being investigated, the ones comprising a polypeptide and a synthetic segment (so-called "hybrids" or "molecular chimeras")[3] are of special interest as they may combine the advantageous properties of polypeptides, i.e. the structure formation, mutual recognition, high mechanical performance, and biodegradability, with the solubility, melt processability, and rubber elasticity of synthetic polymers. As a result, new materials with superior properties and complex hierarchical superstructures can be obtained.[3-5] Copolymers with polypeptide segments have as well a great potential in the field of biomedical applications and are used as model systems to study biophysical processes.[6,7]

Basically, there are two different ways to synthesize linear polypeptide-based block copolymers. The first one involves the solid-phase synthesis of a polypeptide (Merrifield synthesis),[8] followed by a coupling of the peptide chain with a carboxyl-endfunctional polymer. The second route is the primary amine-initiated ring-opening polymerization of amino acid-*N*-carboxyanhydrides (NCA, Leuchs Anhydride), which is the most frequently applied technique to produce polypeptides on a larger scale.[9-18] Here, chain growth should preferably proceed

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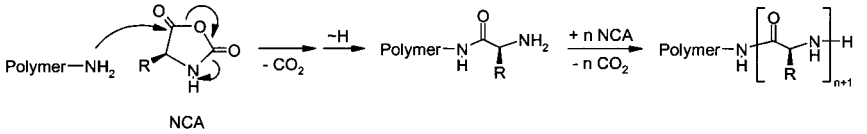
via the nucleophilic ring-opening of the NCA, the so-called "amine" mechanism, as depicted in Scheme 1.[19]



NCA

Scheme 1. Synthesis of polypeptide-based block copolymers by ring-opening polymerization of NCAs, initiated by primary amino-endfunctional polymers.

The so obtained polypeptides usually exhibit a very broad molecular weight distribution (polydispersity index, PDI > 2)[20] because NCA polymerization is not only proceeding *via* the "amine" mechanism but is suffering from side reactions. The most likely one is the "activated monomer" process, initiated by the deprotonation of an NCA, which then becomes the nucleophile and initiates by itself polymerization. Condensation of the produced *N*-aminoacyl NCA intermediates can finally give high-molecular weight polypeptides when monomer conversion approaches 100%. Since primary amines can act as both nucleophile and base, polymerization can switch back and forth between the "amine" and "activated monomer" mechanism.[8,19] However, side reactions can be avoided when metal-amine complex catalysts like bipyNi(COD) are used instead of primary amine initiators. Such a coordination polymerization of NCAs yields well-defined block copolypeptides with PDI < 1.2.[21]

For the characterization of polypeptides, methods like spectroscopy, viscometry, osmometry, light scattering, mass spectrometry, electrophoresis, and analytical ultracentrifugation (AUC) are commonly applied. Note, all these methods are well established to characterize natural proteins that are monodisperse. In order to gain information about molecular weight distributions, samples were submitted to time-consuming fractionation procedures prior to further analyses.[13,20,22,23] The characterization of amphiphilic polypeptide block copolymers demands even greater efforts and is complicated by the chemical dispersity of the samples and the presence of aggregates in most common organic solvents. To the best of our knowledge, the only applied methods to determine molecular weights of this kind of block copolymers were spectroscopy (NMR, IR, UV), elemental analysis, standard size exclusion chromatography (SEC), and AUC.[9-18] However, no adequate procedures have so far been reported that allow determination of absolute molecular weight distributions.

In the present contribution, we wish to describe our experiences in the polymerization of Z-L-lysine (ZLLys)-NCA initiated by ω-primary amino-functional polystyrenes (PS-NH_2) or corresponding amine hydrochlorides (PS-NH_2·HCl). Molecular weights and molecular weight distributions of the prepared PS-PZLLys block copolymer samples were determined using NMR, SEC, and AUC.

Experimental Part

Chemicals. All reagents were purchased from Aldrich or Fluka and were used as received; solvents were purified following standard procedures as described elsewhere in the literature. PS-NH_2(·HCl) macroinitiators were prepared by quenching polystyryl lithium with 1-(chlorodimethylsilyl)-3-[*N*,*N*-bis(trimethylsilyl)amino]-propane and subsequent hydrolysis of the trimethylsilyl protecting groups.[24] ZLLys-NCA was prepared from ZLLys and triphosgene as described by Poché and coworkers.[25,26] *Polymerizations.* Mixtures of PS-NH_2(·HCl) and ZLLys-NCA in *N*,*N*-dimethylformamide (DMF) were stirred for 3 days at 40-80 °C under a dry argon atmosphere. PS-PZLLys products were precipitated in petrolether or methanol/water 70:30 (v/v), extracted with cyclohexane, and finally dried in vacuum at 40 °C.

NMR. ^{1}H NMR spectra of PS-PZLLys copolymer samples were recorded with a Bruker DPX-400 spectrometer in DMF-d_7 (99.5% d, Euriso-top) at 25-100 °C. *Size Exclusion Chromatography.* SEC was performed in *N*,*N*-dimethylacetamide (DMA + 0.5 wt % LiBr; flow rate: 1.0 mL/min) at 70 °C on four 300 × 8 mm PSS GRAM 10-μm columns (30, 30, 100, 3000 Å). Detectors employed were TSP UV1000 (λ = 270 nm), Shodex RI-71, and Viscotek model H502B on-line viscometer. The comonomer-specific UV and RI detector response factors were determined by analyzing PS and PZLLys homopolymer samples. The universal calibration curve (log [η]M *vs.* elution volume) was recorded with commercial PS standards (PSS, Mainz, Germany). *Analytical Ultracentrifugation.* AUC measurements were performed on an Optima XL-I ultracentrifuge (Beckman-Coulter, Palo Alto, CA) with Rayleigh interference and UV/visible absorption optics. Sedimentation velocity experiments were done with 0.15 wt % polymer solutions in DMF at 40 °C and 60K rpm. Time-dependent concentration profiles were evaluated with correction for diffusion broadening using the SEDFIT 5 software (Peter Schuck).[27] Partial specific volumes of the copolymers in DMF solution were obtained with a density meter DMA5000 (Anton Paar, Graz, Austria).

Results and Discussion

In a first series of experiments, four different PS-PZLLys copolymer samples (**1-4**) were prepared by ring-opening polymerization of ZLLys-NCA in DMF at 40 °C. Polymerizations were initiated with PS_x-NH_2 (x = 52 (→ **1**, **2**) or 217 (→ **3**, **4**); PDI ~ 1.03), and the initial concentration of the NCA was ~8 wt %. The NCA was prepared from ZLLys and triphosgene in THF, re-crystallized three times from THF/petrolether 1:2 (v/v), and dried in high vacuum (method A).[25] As indicated by SEC and AUC analyses (see below), the isolated copolymer samples did not contain any homopolymer impurities.

^{1}H NMR was applied to confirm the chemical structure of the PS-PZLLys samples. The average composition of copolymers was determined from the peak intensities of meta phenyl protons of PS (*8*) and benzylic methylene protons of PZLLys (*19*, see Figure 1). From the mole fraction of ZLLys in the copolymer and the molar mass of the PS block segment (SEC), the absolute number-average molecular weight (M_n) was calculated—the results for samples **1-4** are listed in Table 1. Note that measurements were performed in DMF-d_7, and it was confirmed by temperature-dependent NMR experiments (25-100 °C) and by AUC that in DMF copolymers were dissolved on a molecular level and not forming aggregates.

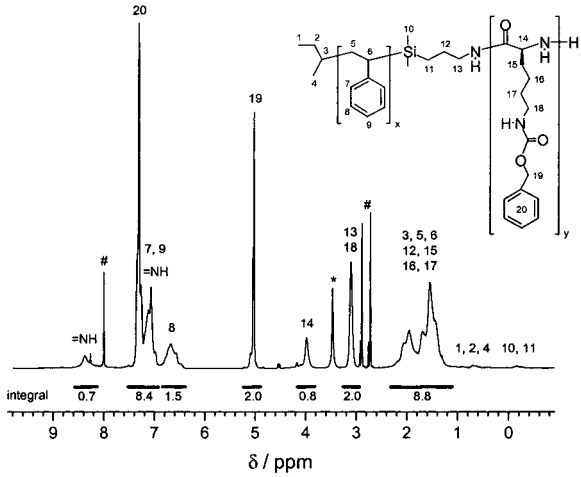


Figure 1. ^{1}H-NMR spectrum (400 MHz) of the PS-PZLLys sample **1** recorded in DMF-d_7 at 25 °C (# DMF, * water).

The samples were further characterized by SEC. For an adsorption-free fractionation of the samples, measurements were performed at 70 °C using DMA (+ 0.5 wt % LiBr) as the eluent and a polyester gel (PSS GRAM) stationary phase. The chromatograms obtained for **1-4** are shown in Figure 2. Also included in these plots are the results of compositional analysis of SEC fractions; the mole fractions of ZLLys were determined from the signal intensities of UV and RI detectors as described elsewhere.[28,29] Having this information and knowing the molar mass of the PS precursor, it was possible to determine the molar mass of every SEC fraction and thus the molecular weight distribution of the complete PS-PZLLys copolymer sample (SEC-UV/RI).[28] Molecular weight distributions were also determined by SEC with on-line tracing of the differential viscosity and application of the concept of universal calibration (SEC-DV/UC).[30,31] The M_n and PDI values of the PS-PZLLys samples **1-4**, as obtained by these two SEC methods, are summarized in Table 1. Evidently, the M_n values obtained by SEC agree well with the ones determined by NMR. PDI values were found to be in the range of 1.2-1.6 (SEC-UV/RI) or 1.4-1.8 (SEC-DV/UC), thus confirming that the polypeptide segments are rather polydisperse with respect to molar mass (cf. Introduction).

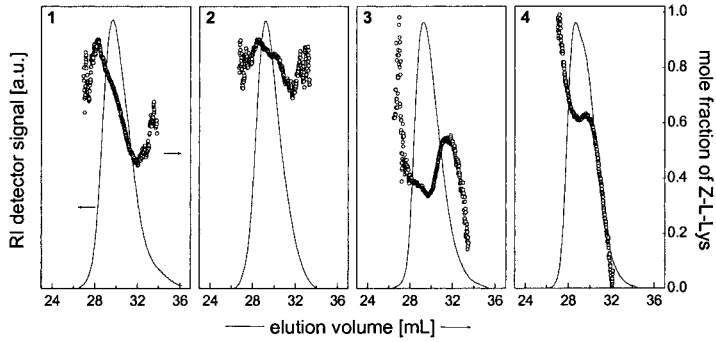


Figure 2. SEC chromatograms of the PS-PZLLys samples **1–4** (DMA + 0.5 wt % LiBr, 70 °C, PSS-GRAM); lines: RI detector signal, circles: mole fraction of ZLLys.

Table 1. Number-average molecular weights (M_n) and polydispersity indexes (PDI) of the PS-PZLLys samples **1-4** as determined by NMR and SEC.

sample	M_n [kg/mol]			PDI	
	NMR	SEC-UV/RI	SEC-DV/UC	SEC-UV/RI	SEC-DV/UC
1	23.7	23.6	21.7	1.6	1.7
2	34.7	38.4	35.8	1.3	1.6
3	47.2	54.6	51.0	1.3	1.4
4	100	101	76.4	1.5	1.8

Aiming to confirm SEC data, the PS-PZLLys samples were analyzed by AUC sedimentation-velocity. The sedimentation coefficient distributions obtained for **1-4** in DMF at 40 °C are shown in Figure 3. Note that the appearance of a single sedimenting species excludes the presence of homopolymer contaminants in the block copolymer samples. Also, sedimentation coefficients were found to be very low, which indicates that under the given experimental conditions copolymer chains were sedimenting as unimers.

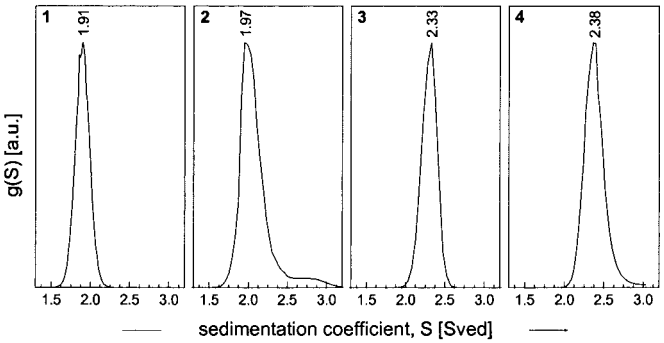


Figure 3. Sedimentation coefficient distributions, g(*S*), of the PS-PZLLys samples **1–4** (sedimentation-velocity; DMF, 40 °C, 60K rpm, UV absorption optics).

Assuming a constant partial specific volume and frictional ratio, evaluation of AUC data should provide absolute molar mass distributions and thus absolute values of the weight-average molecular weight (M_w).[27] However, the latter were about 10-60 % smaller than the corresponding M_n values obtained by NMR (or SEC). The reason for this nonsensical result is not known yet. It should be mentioned that the PZLLys segment might not only be poly-disperse with respect to molar mass but as well as with respect to conformation or shape. This should not affect NMR analyses but could have considerable impact on translational diffusion coefficients and partial specific volumes and thus on the modeling of the time-dependent concentration profiles to the Lamm equation.[27] Whether or not this is the case is subject of current investigations. In addition, samples shall be characterized by static light scattering (SLS). Note that the determination of the absolute molecular weight (M_w) of a polydisperse copolymer system requires the performance of SLS measurements in at least three different solvents (contrast variation method) to compensate the difference in specific refractive in-crements of the two block segments.[32]

Nevertheless, there seems to be good evidence that the applied SEC methods provide reliable information about the true molecular weight distribution of PS-PZLLys copolymers. This information is most important when aiming to optimize reaction conditions for a controlled polymerization of NCAs. The products, which were prepared throughout these studies, were characterized by means of NMR and SEC-UV/RI. The same polymerization conditions were applied as for the preparation of sample **1** (see above and Table 2). Mixtures of the PS_{52}-NH_2 macroinitiator and ZLLys-NCA in DMF were stirred for 3 days at 40-80 °C. In any case, conversion of the NCA went to completion as indicated by SEC analysis of the crude reaction solutions.

It is well known that acidic or other contaminants in the NCA sample can have severe impact on the polymerization results.[19] Recently, Poché et al.[26] reported an advanced procedure that makes it possible to obtain NCAs in a very high purity. Following this method B, the NCA was prepared from ZLLys and triphosgene in ethyl acetate as the solvent. The crude product was successively washed with cold water and aqueous $NaHCO_3$, precipitated from petrol-ether, and dried in high vacuum. The polymerization of the so obtained ZLLys-NCA using PS_{52}-NH_2 as the macroinitiator in DMF at 40 °C yielded PS-PZLLys sample **5** with M_n = 21.4 kg/mol (NMR) and PDI = 1.24 (SEC-UV/RI; cf. Figure 4, Table 2). This PDI value is considerably lower than that of reference sample **1** (PDI = 1.6), which was made using a ZLLys-NCA prepared by method A (see above). Performing the polymerization reaction at 80 °C produced a PS-PZLLys with even narrower molecular weight distribution (PDI = 1.12, sample **6**). It should further be noted that the initiator efficiency, as calculated from the ratio of the targeted molecular weight over the experimental molecular weight, was virtually the same for entries **1** (0.63), **5** (0.64), and **6** (0.66).

Hence, the NCA should be prepared according to method B in order to achieve a better control of the polymerization reaction. However, as mentioned in the Introduction, there is still the possibility that the polymerization proceeds not only *via* the desired "amine" mechanism but is competing with the "activated monomer" process. The key species of the latter process is an NCA anion (NCA^-), resulting from the deprotonation of the NCA by the basic amino moiety of the macroinitiator or growing peptide chain end. It is obvious that the suppression of this side reaction would require a chemical modification of the growing species, e.g. transformation into a transition metal-amine complex (Deming et al.).[8] We were thinking

of adding protons (HCl) to the reaction solution in order to shift the equilibrium back to the protonated NCA. Basically, this idea goes back to work of Knobler et al. published in the 1960s.[33,34] These authors employed the stoichiometric reaction between NCAs and the hydrochlorides of primary amines to synthesize α-aminoacyl compounds. It was emphasized that the nucleophilic substitution reaction proceeded smoothly without producing any polymeric by-products (presumably *via* an "activated monomer" process).

Since HCl could not be added as an aqueous solution (H_2O can promote polymerization of the NCA *via* the "activated monomer" mechanism),[19] we prepared a water-free hydrochloride of PS_{52}-NH_2. The polymerization of ZLLys-NCA (prepared by method B) with PS_{52}-NH_2·HCl in DMF at 40 °C afforded PS-PZLLys sample **7** with M_n = 17.9 kg/mol and a near monodisperse molecular weight distribution (PDI < 1.03; cf. Figure 4). Like in the previous runs, NCA conversion went to completion (SEC) after 3 days of stirring. According to SEC and AUC (not shown), the sample was free of PZLLys homopolymer, which might have eventually been formed by a chloride-initiated polymerization of the NCA.[19]

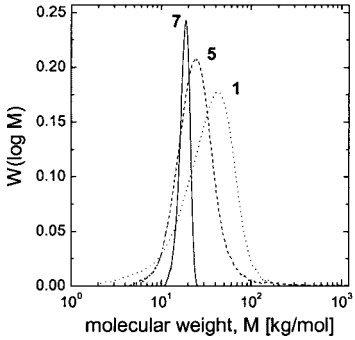
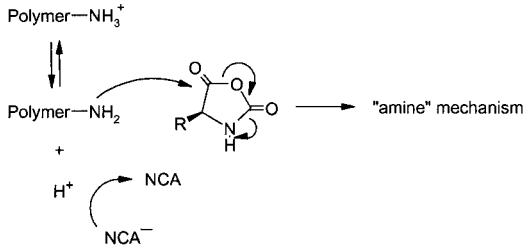


Figure 4. Mass distributions (SEC-UV/RI) of the PS-PZLLys samples **1** (PS_{52}-NH_2, ZLLys-NCA / method A, DMF, 40 °C), **5** (PS_{52}-NH_2, ZLLys-NCA / method B, DMF, 40 °C), and **7** (PS_{52}-NH_2·HCl, ZLLys-NCA / method B, DMF, 40 °C).

The tentative mechanism of the reaction is depicted in Scheme 2 (cf. Knobler et al.). The amine hydrochloride chain ends are considered as a dormant species, dissociating into the growing free primary amine and $H^+(Cl^-)$. The released protons should re-protonate any NCA^- present in solution. Note that this process should be faster than the nucleophilic attack of another NCA molecule to avoid chain growth *via* the "activated monomer" mechanism. How-

ever, kinetic studies have not yet been performed to confirm this mechanism. It is expected that the rate of polymerization will vastly depend on the position of the hydrochloride/amine equilibrium and thus on the polarity of the reaction medium and on the temperature.



Scheme 2. Tentative mechanism of the ring-opening polymerization of NCAs using primary amine hydrochlorides (chloride ions omitted).

Table 2. Experimental results of the polymerization of ZLLys-NCA (prepared by method B) with either PS_{52}-NH_2 (**5, 6**) or PS_{52}-NH_2·HCl (**7-9**) ($[NCA]_0/[\text{-}NH_2] = 31$, $[NCA]_0 \sim 8$ wt %) in DMF at 40-80 °C.

sample	T [°C]	M_n [kg/mol] NMR	PDI SEC-UV/RI	initiator efficiency
5	40	21.4	1.24	0.64
6	80	20.8	1.12	0.66
7	40	17.9	< 1.03	0.78
8	60	18.0	< 1.03	0.77
9	80	16.3	< 1.03	0.84

Polymerization of ZLLys-NCA with PS_{52}-NH_2·HCl in DMF proceeded in a controlled manner in the temperature range of 40-80 °C. All the prepared PS-PZLLys copolymers exhibit a very narrow molecular weight distribution, close to a Poisson distribution (PDI < 1.03; see Table 2). The initiator efficiency was found to be about 0.8, which is somewhat higher as for the free amine initiating system (< 0.7).

Conclusion

The synthesis of PS-PZLLys block copolymers by the NCA polymerization technique and their characterization with NMR, SEC, and AUC has been described. Advanced SEC analysis (SEC-UV/RI and SEC-DV/UC) was applied to determine the molecular weight distribution of the copolymers. SEC results (M_n) were found to be in good agreement with the ones obtained from NMR. However, AUC sedimentation-velocity runs provided M_w values lower than M_n,

which is presumably due to the fact that the applied standard algorithm to evaluate AUC data was not applicable to conformationally disperse copolymer systems.

Regarding the NCA polymerization reaction, it was found that whether or not polymerization was proceeding in a controlled manner depends very much on the quality of the NCA. It was further advantageous to employ PS-NH_2·HCl instead of the free amine macroinitiator. Dissociation of the hydrochloride produces the propagating primary amine and $H^+(Cl^-)$. The proton being released is supposed to re-protonate eventually formed NCA^-, thus eliminating a non-desired chain growth *via* the "activated monomer" process. The PS-PZLLys copolymers, which were so obtained in DMF solution at 40-80 °C, exhibited a very narrow molecular weight distribution, close to a Poisson distribution (PDI < 1.03; SEC-UV/RI).

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