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Synthesis and Studies of *N*-Vinylcaprolactam/*N*-Vinylimidazole Copolymers that Exhibit the “Proteinlike” Behavior in Aqueous Media

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ABSTRACT: Proteinlike copolymers were first predicted by the computer-aided biomimetic design (*Physica A* **1998**, *249*, 253−261). These copolymers consist of comonomer units of differing hydrophilicity/hydrophobicity. Heterogeneous blockiness, characteristic for such copolymers, facilitates chain folding with the formation of specific spatial packing: a dense core consisting of hydrophobic units and a polar shell formed by hydrophilic units. This paper describes the synthesis of *N*-vinylcaprolactam/*N*-vinylimidazole copolymers via the redox-initiated radical copolymerization in the medium of 10% aqueous DMSO at the temperatures both below and above the phase separation threshold. The synthesized macromolecular products were separated into thermally precipitating and nonprecipitating fractions. Their molecular weight characteristics were evaluated using size-exclusion chromatography; their comonomer composition was determined from ^{1}H NMR spectra of copolymers dissolved in DMSO-d_6. The temperature-dependent behavior of copolymer macromolecules in water was investigated by thermonephelometry, high-sensitivity differential scanning calorimetry, and ^{1}H NMR spectroscopy of the copolymers dissolved in D_2O. It was shown that thermally nonprecipitating copolymer fractions obtained at initial comonomer molar ratios of 85:15 and 90:10 can be identified as proteinlike copolymers.

1. Introduction

A new way of computer-aided biomimetic design of functional copolymers was suggested a few years ago.[1−3] It was based on a well-known fact that functioning of many globular proteins in the living systems depends on the following: (i) these proteins are globular; (ii) the proteins are soluble in aqueous medium. For synthetic polymers, the combination of these factors is nontrivial, since for the water-soluble homopolymers and random copolymers, the coil−globule transition usually results in a marked decrease in solubility. The protein globules, however, are soluble in water. This is due to their specific conformation, where most of the hydrophobic pendant groups are hidden in the core of the globule, while hydrophilic and charged pendant groups form the shell of this globule. Certainly, the attribution of 20 types of amino acids that belong to globular proteins to only two types of monomer units (hydrophobic and hydrophilic) is rather approximate. However, a dense conformation consisting of hydrophobic core and hydrophilic shell is a good model of a globular protein. The question is: is it possible to create such a sequence of comonomer units in a synthetic HP copolymer (the copolymer consisting of monomer units of two types, hydrophobic H and polar P) that the hydrophobic H-units are in the core of the globule, while hydrophilic P-units form the shell of this globule?

This problem was first addressed in 1998,[1] and the corresponding copolymers were called “*proteinlike*” copolymers.

The proteinlike HP-sequences were first obtained in the following computer experiments. The procedure starts with a homopolymer in an arbitrary globular conformation formed due to the strong attraction of monomer units. Then “coloring” is applied, namely the monomer units in the globule center are called H units, and the units belonging to the globule surface are assigned to P type. This primary structure is fixed, and the attraction of monomer units is turned down. Thus, the generated primary structure can be used for subsequent investigations.

Theoretical studies and computer simulation of such proteinlike HP copolymers were described in several papers.[1,2,4] The heterogeneous nonalternating sequence of H and P units (heterogeneous blockiness) in these copolymers was shown and the effect of memorizing of the “parent” spatial conformation (i.e., the conformation prior to coloring) was found. This can be regarded as one of the possible mechanisms of molecular evolution: polymer acquires a specific sequence of monomer units

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in the initial state, and then under other conditions the primary structure turns out to be "tuned to perform certain functions" connected with the parent conformation. It was also shown[4] that the described design procedure leads to long-range correlations in primary structure, which obey the so-called Levy flight statistics.[5]

To test these ideas, the attempts were made to synthesize the proteinlike copolymers. Two approaches were examined:

(i) Chemical modification of reactive pendant groups in certain macromolecules.

(ii) Formation of proteinlike conformation of HP copolymers directly in the course of their synthesis from appropriate comonomers.

To realize the first approach, grafting of oligo(ethylene oxide) chains to thermosensitive poly(*N*-isopropylacrylamide-*co*-glycidyl methacrylate) in aqueous solutions was performed.[6-8] Grafting at room temperature yielded the copolymer in the coil state, and grafting was random along the chain. At elevated temperature the transition to globule occurred, and grafting occurred mostly at the globule surface. This resulted in the hydrophilization of outer regions of the globule and thus creation of proteinlike copolymer. Such proteinlike copolymer exhibited phase transition at somewhat higher temperatures than the random one, and gave smaller aggregates at the temperatures higher than the lower critical solution temperature (LCST).[7]

An example of the second approach is the copolymer synthesis from amphiphilic (hydrophilic/hydrophobic) monomer *N*-vinylcaprolactam (NVCl) and essentially hydrophilic monomer *N*-vinylimidazole (NVIAz). The synthesis was performed via redox-initiated copolymerization in aqueous DMSO at the temperature above the phase separation threshold (PST). At a certain initial ratio of the comonomers, three fractions of NVCl/NVIAz copolymers with similar content of monomer units, but possessing different temperature-dependent solution behavior, were isolated from the reaction system.[9] One of the fractions exhibited typical (theoretically predicted[1]) proteinlike behavior in aqueous media. It did not precipitate from aqueous solution upon heating to 70 °C (at least), but underwent conformation transition at around 35 °C (registered by DSC). Two other copolymer fractions precipitated at temperatures in the vicinity of LCST for NVCl homopolymer synthesized in the same solvent under identical thermal conditions.[10]

Similar (qualitative) results were later reported[11] for another pair of monomers, *N*-isopropylacrylamide and *N*-vinylpyrrolidone. The copolymers synthesized in aqueous medium at 60 °C (i.e., higher than the PST) had denser globular packing of macromolecules in solutions as compared to the copolymers fabricated from the same initial comonomer mixtures at 30 °C (i.e., below the transition point). However, no attempt was made to separate the synthesized copolymers into the thermally precipitating and nonprecipitating fractions.

The possibility to obtain the proteinlike copolymers via copolymerization of the comonomers differing in hydrophilicity/hydrophobicity at temperatures above the PST has also been confirmed by computer simulations.[12]

Yet another example of proteinlike copolymers obtained from the monomer precursors is the *N*-isopropylacrylamide/NVIAz copolymers synthesized in aqueous solution.[13] The copolymer with virtually random distribution of NVIAz units along the chains did not interact with the metal chelate adsorbent Cu^{2+}−iminodiacetate−Sepharose. On the other hand, the resin specifically absorbed the copolymer possessing the proteinlike sequence of these monomer units. This was explained by the accumulation of the hydrophilic pendant imidazole groups (which are responsible for complexing ability) at the hydrophilic periphery of the macromolecular coil.

The present work deals with the continuation of our studies of NVCl/NVIAz copolymers. We shall describe the experimental results, in more detail than in a letter communication.[9] Our main objectives were the following: (1) to clarify the influence of the specific synthesis conditions on the production of the copolymers with proteinlike behavior in aqueous milieu; (2) to investigate the properties of the synthesized proteinlike copolymers.

2. Experimental Section

2.1. Materials. The following chemicals were used in the work. *N*-vinylcaprolactam was a gift from Gunnar Nord (ISP Norden AB). The monomer was purified by dissolution in benzene, refluxing with powdered active charcoal, filtration, and crystallization of the target substance by the addition of hexane. The product obtained (gross transparent crystals) had the melting point of 34−35 °C (vs $T_m = 34.5$ °C lit.[14]) and showed virtually theoretical results of elemental analysis. *N*-vinylimidazole (Aldrich,) was distilled in vacuo (78−80 °C/13 mmHg).

Ammonium persulfate (APS) and L-tryptophan were obtained from Serva; *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) was supplied by Aldrich. Blue dextran was purchased from Loba Chemie , PEG 400 was received from Merck. DMSO ("chemically pure" grade, Reakhim) was additionally purified by the freezing-out with subsequent distillation under argon. All aqueous solutions were prepared with deionized water.

Deuterosolvents employed in NMR studies, D_2O and DMSO-d_6, were purchased from Reakhim and Euriso-Top (Gif-sur-Yvette, France) and used as received.

2.2. Methods. 2.2.1. Synthesis of NVCl/NVIAz Copolymers. The weighed amount of NVCl was initially dissolved in such a volume of DMSO that should correspond to 10% (v/v and further on throughout the paper for the DMSO concentration) of this solvent in the reaction media. The required amounts of NVIAz and deionized water were added afterward. The final sum concentration of comonomers in the solution thus prepared was 0.35 mol/L. The solution was bubbled with air-free argon for 15 min, then 0.2 mL of TMEDA and 0.2 g of APS were added per 100 mL of the reaction solution. The reaction system was then stirred in argon atmosphere at a temperature of either 25 or 65 °C for 18 or 3 h, respectively. Subsequent processing of the resulting system depended on its phase state (Figure 1).

(i) Upon completion of polymerization at 25 °C the system was a transparent solution. It was put into a cellophane sack (Reakhim) and dialyzed for 72 h against the 100-fold excess of deionized water; the water was exchanged every 24 h. The polymer solution thus purified from the substances with the molecular weight less than ca. 20 kDa (the cutoff limit for the cellophane used) was freeze-dried using 77400 FreeZone freeze-dry system (Labconco Corp.). Further on we will call this solid matter product **t** (total). Product **t** was then separated into the thermally precipitating (**p**) and thermally nonprecipitating or soluble (**s**) fractions (for more details see section 2.2.2).

(ii) Upon completion of polymerization at 65°C, the system looked like an opaque liquid, and it was cooled to room temperature. The system became transparent, and then it was dialyzed and freeze-dried as in the previous case. Further on this nonfractionated matter is also designated as product **t**, which was then thermally separated into the **p** and **s** fractions.

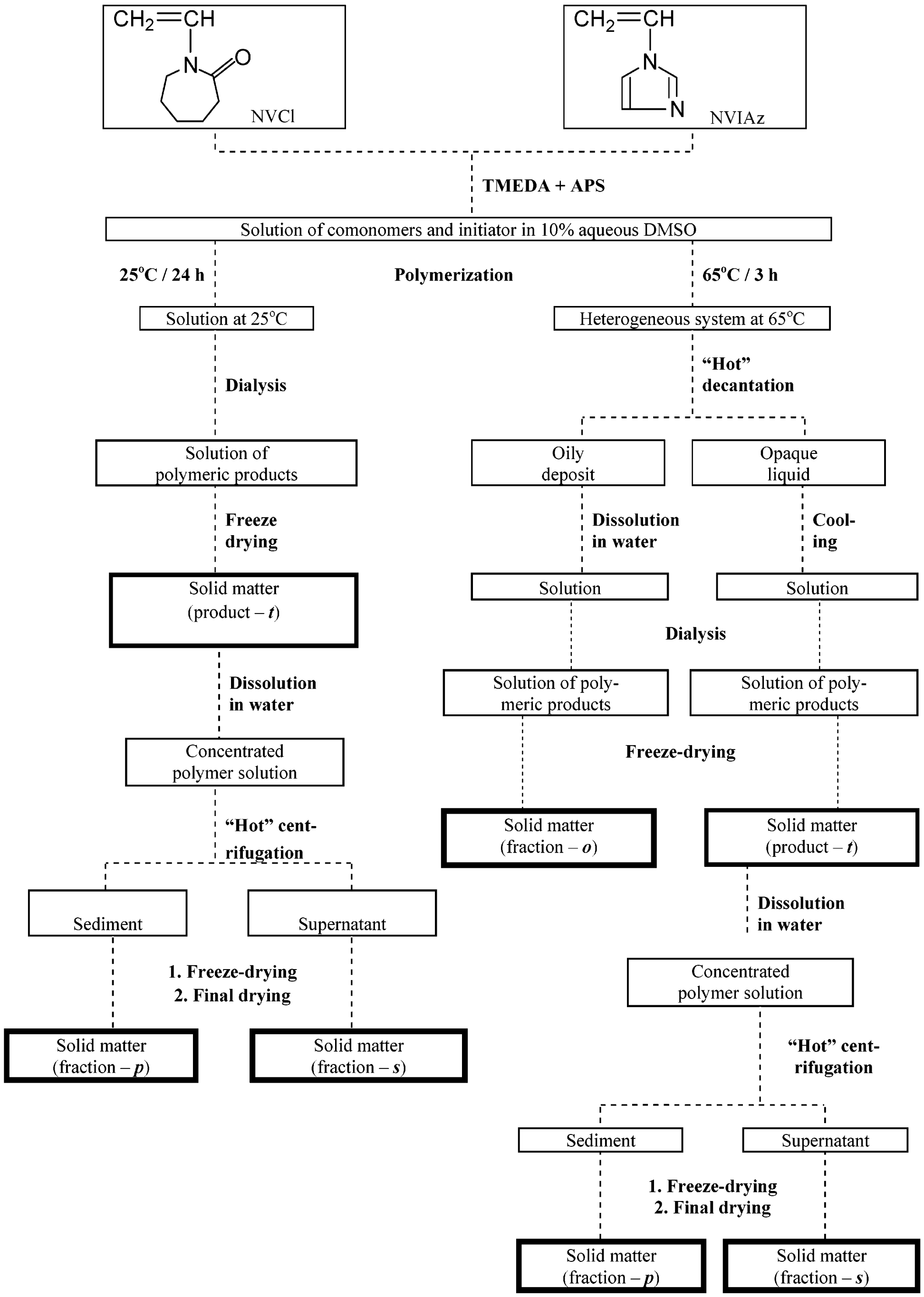

Figure 1. Schematic diagram describing the synthesis conditions, and the operations for isolation and purification of different fractions of NVCl/NVIAz copolymers.

(iii) In yet another case, an oily deposit (along with opaque liquid) was formed on the walls of the reaction vial in the course of polymerization at 65 °C. These two phases were, while hot, separated by decantation followed by rinsing the oily fraction with hot water. The opaque liquid portion and washings were combined, cooled to room temperature, dialyzed and freeze-dried. The obtained solid matter is further designated as product **t**, since it included **p** and **s** fractions as the product **t** in the case (ii) did. The oily deposit was dissolved in water at room temperature, dialyzed, and freeze-dried, yielding a white solid. Later in this paper, this polymer product is designated as the **o** fraction (isolated from the oily matter).

2.2.2. Fractionation of Copolymers in Respect of Their Solubility/Precipitation at Elevated Temperatures. The polymers of the **t**-type obtained as described above (section 2.2.1), were further processed as follows.

The solid matter was redissolved in deionized water at concentration of 5 g/L; the dissolution was performed overnight at 4−6 °C. The resulting system was afterward centrifuged at 65 °C and 8000 rpm for 30 min with a centrifuge TsLN-2 (Medpribor) installed into an air thermostat WS-50 (MLW). The obtained white sediment and virtually transparent supernatant were separated, while being hot. The sediment was dissolved in pure water at room temperature. The resultant transparent solution was frozen, freeze-dried, and the solid matter was finally dried up to a constant weight in a vacuum-desiccator over the water-free $CaCl_2$ granules. Further on such thermally precipitating matter is designated as the **p** fraction. The supernatant, on cooling to room temperature, was processed and dried in the same fashion. Further on, such thermally nonprecipitating matter is called the **s** fraction.

2.2.3. Size-Exclusion Chromatography (SEC) of the Copolymers. SEC was carried out using the column (15 × 450 mm) packed with CL-Sepharose 6B resin (Pharmacia Fine Chemicals). The elution curves were recorded at 220 nm using the flow-through UV/vis–spectrophotometer model 260-10 (Hitachi). It was earlier found[9,10] that pure water was not appropriate as an eluent liquid, since certain nonspecific interactions between the polysaccharide resin and NVCl copolymers were observed. Introduction of 0.2 M NaCl eliminated such interactions. The eluent pumping rate through the column was 18 mL/h. The copolymer sample to be analyzed was dissolved in 0.2 M NaCl-solution at a concentration of 2 g/L, and a 1 mL aliquot was applied to the column. Calibration of the column was performed with PEG molecular weight standards (Scientific Polymer Products, Inc.) with M_w of 113.2, 157, 300, 630, and 917 kDa. Blue dextran (MW ca. 2000 kDa) and oligomeric ethylene glycol (MW 400 Da) were used for the determination of working volume of the column.

2.2.4. Thermonephelometry (TNM) Measurements. The TNM studies were performed with a Specol vis spectrophotometer (Carl Zeiss, Jena, former GDR) equipped with a nephelometric unit of the Ti model connected with ultrathermostat U 4 (MLW). The nephelometric unit was supplied with a vacuum photocell F3MO-19 and an additional photomultiplier Pho M3/19. The light-scattering measurements for the 1 g/L copolymer aqueous solutions were carried out at 500 nm wavelength under the angle of 90°. The result was expressed as a ratio $I_{90}(T)/I_{90}(20\ °C)$ in percent light-scattering intensity of a polymer solution at the temperature T to the light-scattering intensity of a polymer-free solvent at 20°C. The heating rate in the course of the experiments was about 1 °C/min.

2.2.5. High-Sensitivity Differential Scanning Calorimetry (HS-DSC). DASM-4 differential scanning microcalorimeter (Biopribor) was used in HS-DSC experiments. The procedure (for the case of NVCl homopolymers) was described in ref 9. The measurements were performed over the temperature range 10−70 °C for the copolymers synthesized at 25 °C and over the range 10−130 °C for the copolymers synthesized at 65 °C. The scans were performed at a heating rate of 1 °C/min and excess pressure of 2.5 atm. Aqueous solutions of the copolymers with solute concentration of about 5 g/L were used in the calorimetric measurements. As a rule, two subsequent scans were done for each sample. The first scan was required to eliminate the prehistory of the sample. The results of the second scan were considered to characterize the thermodynamic properties of the system under study. Specific partial heat capacity functions of the polymers were calculated under assumption that their specific partial volume is equal to that of PNVCl (0.788 cm^3/g) determined elsewhere[15] by precision vibration densitometry. These functions were converted into specific excess heat capacity functions using the spline approximation of the transition baseline. The peak temperature of the excess heat capacity functions was assumed to be the transition temperature. Specific transition enthalpies were determined by integration of the excess heat capacity functions.

2.2.6. NMR Spectroscopy Studies. ^1H NMR spectra were recorded with WM-250 spectrometer (Bruker). The following parameters of registration were used: pulse width 4 μs; time delay between the pulses 10 s; the number of scans from 40 to 100. Since the spectral lines were broad, the induction signal was multiplied by an exponent with power equal to 2−3 Hz to smooth out the high-frequency noise. Suppression of ^1H NMR signals of H_2O and HDO admixtures in the D_2O solutions of respective polymers was performed in a "broad band" regime during the accumulation of impulses.

The spectra were recorded for the D_2O and DMSO-d_6 solutions of the polymers with solute concentration of 5 g/L. This was done over the temperature range from 20 to 55 °C, and the temperature was maintained with an accuracy of ±0.5 °C. The chemical shifts at 4.70 and 2.49 ppm of the proton signals from the H_2O admixture in D_2O and in DMSO-d_6, respectively, were used as the standard signals. ^1H NMR spectra of the studied polymers were compared with the ^1H NMR spectrum of N-methylpyrrolidone in order to attribute the proton signals of $-NCH_2-$ and $O{=}CCH_2-$ groups in the caprolactam cycle.

3. Results

3.1. Synthesis of NVCl/NVIAz Copolymers at Temperatures below and above the PST. 3.1.1. Prerequisites for the Choice of Synthesis Conditions. The main idea of the synthesis, which could, from our point of view, lead to the formation of proteinlike copolymers originating from a mixture of NVCL and NVIAz comonomers dissolved in aqueous medium, was as follows.[9]

If the polymerization of a nonequimolar mixture of these comonomers (with a necessary excess of more hydrophobic NVCl) in aqueous solution is initiated at a temperature higher than the phase separation threshold, one can suppose the fast formation of oligomeric NVCl chains with length sufficient for the realization of LCST behavior. For instance, similar critical lengths of the most studied thermoresponsive polymer, poly(N-isopropylacrylamide), do not exceed 10−15 monomer units.[16] The growing, but still short, oligoNVCl chain should collapse already at the early stages of the polymerization process (when the concentration of such chains is still relatively low) with the formation of a small dense globule. This globule can be a "germ" for future hydrophobic core of a proteinlike macromolecule. Therefore, subsequent polymerization will proceed under heterogeneous conditions. The further sequence of comonomer units in the chains to be formed will depend on (i) the ratio of reactivities of the comonomers at given temperature (ratio of r_1 and r_2 parameters) and (ii) the instant position of the end of a growing macroradical. The growing end can be located either inside the core or on the periphery of the globule. In the more hydrophobic core more hydrophobic molecules of NVCl should preferably be attached, while on the periphery of the globule hydrophilic NVIAz molecules should preferably react, thus enriching these segments with hydrophilic units. Therefore, formation of proteinlike sequences of NVCl and NVIAz units in the macromolecular chains is possible, at least, for a part of the produced chains. Theory[1] predicts a significant difference in temperature-dependent solution behavior of such proteinlike macromolecules in aqueous medium and random temperature-sensitive copolymers. Aqueous solutions of proteinlike copolymers should exhibit higher phase transition temperatures, whereas coil to globule conformational transition of such macromolecules should not lead to the loss of water-solubility. However, the growth of some copolymer chains might not follow the "favorable" scenario described above. These macromolecules will then possess more random distribution of the comonomer units. Therefore, their solution behavior should not differ significantly from that of the "traditional" temperature-sensitive copolymers (provided the amount of hydrophilic units incorporated in to the chains is not high enough to inhibit at all the heat-induced phase separation).

The synthesis conditions in this study have been chosen by following the considerations discussed above.

3.1.2. Influence of Synthesis Conditions on the Polymerization Features and the Yield of Final Products. The formation of NVCl/NVIAz copolymers in solutions initially containing the comonomers with molar ratios over the range from 90:10 to 75:25 has been

Table 1. Summary of Reaction Conditions and Compositions of NVCl/NVIAz Copolymers

	polymerization conditions			yields (%)		
sample	initial comonomer ratio (mol/mol)	*T*, °C	appearance of the reaction system	total product	fractions of the total product	found comonomer ratio[a] (mol/mol)
75/25−25(**t**)	75:25	25	transparent solution	43.0		
75/25−25(**p**)					66.1	84.4:15.6
75/25−25(**s**)					33.9	79.0:21.0
75/25−65(**t**)		65	opaque heterogeneous dispersion	12.3		
75/25−65(**p**)					65.9	78.0:22.0
75/25−65(**s**)					34.1	73.0:27.0
80/20−25(**t**)	80:20	25	transparent solution	39.6		
80/20−25(**p**)					55.0	79.0:21.0
80/20−25(**s**)					45.0	72.0:28.0
80/20−65(**t**)		65	opaque heterogeneous dispersion	17.0		
80/20−65(**p**)					65.4	72.7:27.3
80/20−65(**s**)					34.6	67.0:33.0
85/15−25(**t**)	85:15	25	transparent solution	36.2		
85/15−25(**p**)					16.1	62.0:38.0
85/15−25(**s**)					83.9	61.5:38.5
85/15−65(**t**+**o**)		65	opaque heterogeneous dispersion and oily deposit	32.1		
85/15−65(**o**)				39.5		81.0:19.0
85/15−65(**t**)				60.5		
85/15−65(**p**)					69.2	74.0:26.0
85/15−65(**s**)					30.8	73.0:27.0
90/10−25(**t**)	90:10	25	transparent solution	3.0		
90/10−25(**p**)					4.4	79.0:21.0
90/10−25(**s**)					95.6	78.0:22.0
90/10−65(**t**)		65	opaque heterogeneous dispersion	10.3		
90/10−65(**p**)					67.5	88.0:12.0
90/10−65(**s**)					32.5	81.0:19.0

[a] Determined from the ^{1}H NMR spectra; experimental accuracy was ±10% from a value measured.

explored. Preliminary experiments revealed that, at higher than 25 mol % content of hydrophilic NVIAz, virtually random copolymers were usually formed under the thermal conditions employed. On the other hand, at higher than 90 mol % content of hydrophobic NVCl, a very low yield of the thermally nonprecipitating **s** fraction (Figure 1) was obtained.

The 10% aqueous DMSO solution was used as a reaction medium. First, this was necessarily because of insufficient water-solubility of NVCl. Second, it was shown earlier that in this solvent mixture the NVCl homopolymers and NVCl/NVIAz copolymers did not lose their LCST behavior.[9,10] Hence, such amount of DMSO in the composition of reaction solvent did not suppress significantly the hydrophobic interactions of NVCl units.

The redox-initiated copolymerization of these monomers was carried out at two temperatures: (i) at 25 °C, that is, in the solution below the PST; (ii) at 65 °C, that is, above the PST, when the reacting system 2−3 min after the addition of initiator already became a heterogeneous opaque dispersion.

The formation of oily deposit on the walls of the reaction vessel was observed at 65 °C for certain NVCl/NVIAz ratios. Therefore, any further strategy of isolation and purification of polymer fractions possessing different solution behavior depended on the phase state of the reaction bulk. All the operations (their features are discussed below in section 3.2) of such processing are shown as a diagram in Figure 1. Note that syntheses at 25 °C were initially performed only to compare the properties of resulting copolymers with those of copolymers synthesized at higher temperature. We intended to trace the difference between the results of precipitation polymerization (at 65 °C) and those of solution polymerization. However, some unexpected effects were observed for the formation of NVCL/NVIAz copolymers at 25 °C. These results will be also discussed though they are not related directly to proteinlike copolymers.

Table 1 summarizes the results of these syntheses and of subsequent isolation of the copolymer fractions. The samples in Table 1 are specified by the abbreviations showing, first, the initial molar ratio of the comonomers, second, the temperature of the synthesis and, third, the fraction type. For instance, the abbreviation 80/20−25(**s**) means the **s** fraction of the copolymer synthesized at 25 °C from the 80:20 (mol/mol) NVCl/NVIAz mixture.

Total yields of the copolymers synthesized were rather low under the conditions used in this work (see Table 1). The yields did not exceed 43% for the synthesis temperature of 25 °C and 32% for polymerization at 65 °C. However, the dependence of the yield on the NVCl/NVIAz ratio was different for these two temperatures of polymerization process (and therefore for different phase states of the reacting systems).

In the case of solution polymerization (25 °C) the yield of polymeric products **t** decreased monotonically with increase in the portion of hydrophobic NVCl in the initial feed. This was obviously due to a decrease in hydrophilicity of the copolymer formed in aqueous solution. The precipitation polymerization of the same comonomers at 65 °C gave a bell-shaped yield dependence on the fraction of NVCl in the initial feed. The exact reason for this dependence is not yet clear.

In any case, the efficiency of the chain growth in the course of copolymerization of given pair of monomers in aqueous medium was markedly influenced not only by the phase state of reacting system, but also by the NVCl/NVIAz ratio in the feed.

3.2. Temperature-Dependent Fractionation of Synthesized Copolymers. 3.2.1. Strategy of Fractioning.

A principal point in the “fabrication” of proteinlike copolymers through the polymerization reaction (that is, via the second approach to their preparation discussed in Introduction) was the search for the presence of proteinlike macromolecules among the synthesized polymeric products. As was pointed out in section 3.1.1, the probability of formation of the proteinlike sequences is not absolute and depends on many factors. Therefore, the scheme shown in Figure 1 was based on dialysis (contrary to the iterative precipitation-dissolution routinely used in polymer chemistry for the separation of high and low molecular weight products) and on the temperature-dependent solubility/insolubility of the copolymers in water.

The content of **p** and **s** fractions in each product **t** (as well as the total yield) depended on both the phase state of the reacting system and the initial comonomer ratio, and the trends observed were rather unusual (Table 1).

The polymeric products of **t**-type synthesized at 65 °C contained thermally precipitating (**p**) and nonprecipitating (**s**) fractions. The latter ones were water-soluble under the "hot" centrifugation conditions (the properties of these fractions shall be discussed below). The surprising thing was the presence of **p** fraction in the polymeric products **t** synthesized in solution at 25 °C, i.e., below the PST (see Figure 1 and Table 1), without the phase separation. In this case, the most probable products should be random copolymers with a rather short oligoPNVCl blocks (accounting for the comonomer ratios in the initial feeds). Such copolymers are hardly capable of heating-induced cooperative transitions. Nonetheless, upon "hot" centrifugation, we obtained respective **p** fractions with a high yield (Table 1). Moreover, a decrease in the amount of hydrophilic NVIAz in the initial feed from 25 to 10 molar % caused an unexpected increase in the content of the **s** fraction from 33.9 to 95.6%. This occurred in parallel with the overall decrease in the yield of **t**-type polymeric products from 43 to 3%. This allows one to conclude that NVIAz during the solution polymerization in aqueous medium has a higher reactivity than that of NVCl.

At the same time, precipitation polymerization above the PST, yielded almost constant portions of **s** and **p** fractions (34−36% and 66−64%, respectively) in the composition of product **t** (Table 1). However, the **o** fraction produced at certain comonomer ratios should also be taken into account because this fraction also consists of a thermally precipitating copolymer. Therefore, the amount of **o** and **p** species should be added in order to estimate the total yield of thermally precipitating copolymers. Note that the summary yield of **o** and **p** fractions has a maximum. This is similar to the trend for the total yield of products **t** vs. NVCl/NVIAz ratio in the synthesis performed at 65 °C. Also note that apart from the case of 85/15−65 samples, the formation of oily deposits during the polymerization at 65 °C was also detected for the initial comonomer molar ratios of 82.5:17.5 and 87.5:12.5. However, the latter two cases are not shown in Table 1, because respective samples were not studied in detail (their yields were lower than that of 85/15−65(**o**) copolymer).

In general, we conclude that a certain optimum of initial comonomer ratio exists for an efficient formation of both thermally precipitating and nonprecipitating copolymers in the course of precipitation copolymerization of NVCl/NVIAz mixtures at temperatures higher than PST.

3.2.2. Composition of Isolated o, p, and s Fractions. 3.2.2.1. NMR Data. The quantitative content of NVCl and NVIAz units in the thermally separated copolymer fractions was determined from their NMR spectra. The attribution of the signals is presented in the scheme of chemical structure of these NVCl/NVIAz copolymers in Figure 2. In Figure 3, the ^{1}H NMR spectra of the 5 g/L solutions of 85/15−65(**p**) and 85/15−65(**s**) copolymers in DMSO-d_6 (Figure 3, parts A and C) and D_2O (Figure 3, parts B and D) are depicted as the examples.

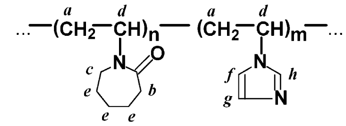

Figure 2. Chemical structure of NVCl/NVIAz copolymers (small letters identify the position of protons corresponding to the lines in NMR spectra).

The signals of imidazole protons were very broad in the spectra of D_2O solutions of the copolymers. Therefore, the estimation of their true intensities was difficult. In the spectra of DMSO-d_6 solutions of these copolymers the signals of protons of the imidazole ring at 6.8−7.8 ppm were narrower, and this allowed determining their integral intensities. The spectra of D_2O solutions of the copolymers were used in the study of effects associated with the temperature-dependent solution behavior of respective polymeric fractions.

The signals related to the three chemically nonequal imidazole protons (*f*, *g*, and *h*) consisted of a narrow singlet and two broader multiplets with the relation of intensities equal to 1:1:1 (Figure 3, parts A and C). This difference of the line widths was obviously due to the partial protonisation of nitrogen atom N(3) in the heterocycle by the water protons present in the solvent, DMSO-d_6. As a result, the charge on the N(3) atom changes with a rate determined by proton exchange. Thus, the spectral lines of the *g* and *h* protons became broader, while the width of the NMR signal related to the *f* protons was only slightly affected.

The splitting of spectral lines of the *g* and *h* protons was not due to the spin−spin interaction, but rather reflected the difference between the nonprotonated and protonated states of imidazole ring. This conclusion is supported by narrowing of the signals, and disappearance of the splitting upon heating of the samples to 70 °C (the latter is due to the acceleration of proton exchange).

The sum of integral intensities of the *c* and *d* spectral lines equals the intensities of $4n$H protons ($-NCH_2-$ and $O{=}CCH_2-$) of a caprolactam cycle, where n is the number of NVCl monomer units in the copolymer. The sum of integral intensity of the *f*, *g*, and *h* lines equals the intensities of $3m$H protons, where m is the number of NVIAz monomer units. Therefore, measuring the integral intensities of corresponding NMR signals allowed evaluation of the relative amount of the comonomers in the respective polymeric fractions. Thus, for the 85/15−65(**p**) and 85/15−65(**s**) copolymers the molar ratios were 74:26 and 73:27 (error ±10%), accordingly. The data obtained for other copolymers synthesized in this study are given in Table 1.

Relatively wide and intense lines $a + e$ in the ^{1}H NMR spectra of the copolymers were related to the *a* protons of the $-CH_2$ groups in the polymer chain and to the *e* protons of the $-C-CH_2-C$ groups in caprolactam cycle. The absence of any pronounced multiplicity of this signal pointed to the fact that syndiotactic triad was the major configurational motive in these copolymers.

3.2.2.2. Influence of the Synthesis Conditions on the Copolymer Composition. NVCl/NVIAz Copoly-

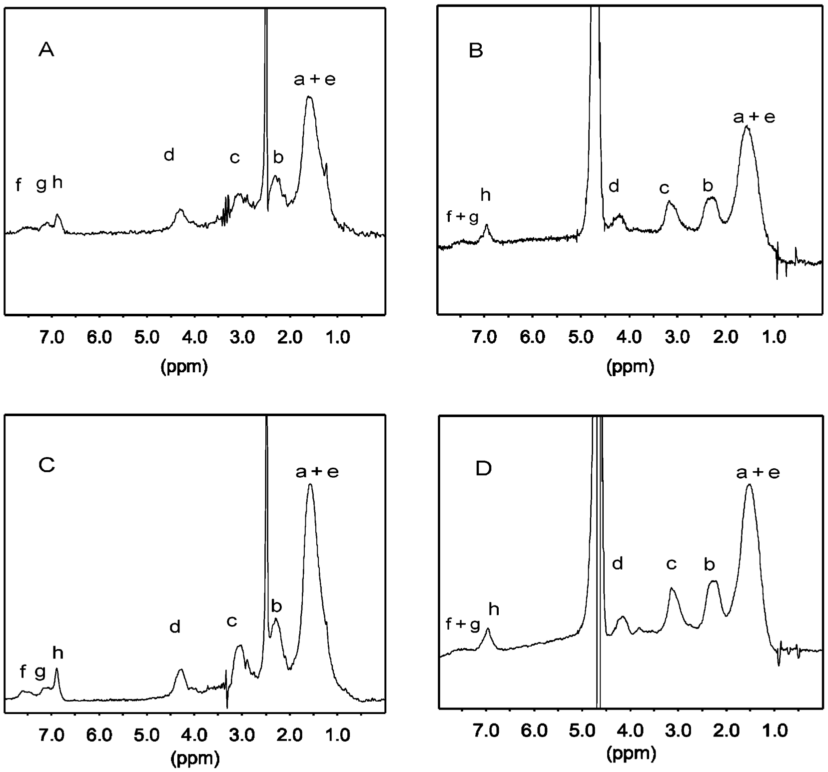

Figure 3. ^{1}H NMR spectra of the 85/15−65(**p**) (A, B) and 85/15−65(**s**) (C, D) copolymers dissolved in DMSO-d_6 (A, C) and D_2O (B, D). The assignment of each resonance peak corresponds to the assignment of protons in Figure 2.

mers Synthesized below the PST. The data on the composition of obtained copolymers (Table 1) confirm the conclusion on a higher reactivity of NVIAz during its copolymerization with NVCl in aqueous solution at 25 °C. For both separated fractions, i.e., **s** and **p**, an enrichment of resulting copolymers with NVIAz units vs their content in the initial comonomer mixture was registered. For instance, the portion of hydrophilic monomer units in the **s**-type macromolecules synthesized at 25 °C increased from 21 to 42 molar % with a decrease in content of NVIAz in the initial mixture from 25 to 10 molar %. Such enrichment was more pronounced in the cases of nonprecipitating copolymers (**s**) than for the thermally precipitating ones (**p**).

NVCl/NVIAz Copolymers Synthesized above the PST. Dependence of the content of NVCl and NVIAz units in such copolymers on the initial comonomer ratio was (according to NMR data) nonmonotonic. The highest amount of NVCl units and, hence, the lowest amount of NVIAz units (67 and 33 molar %, respectively) was found in the **s** copolymer formed from the 80:20 (mol/mol) NVCl/NVIAz initial mixture. Among the **p** fractions, the highest extent of enrichment was also found at the same initial comonomer ratio, but the enrichment was somewhat less (27.3 molar %) than that for the **s** fraction. Even in the composition of the 85/15−65(**o**) specimen we found a higher content of hydrophilic units (19 molar %) than in the initial mixture of the comonomers. These results pointed to the higher reactivity of NVIAz as compared with NVCl in aqueous media not only during the above-discussed solution polymerization below the PST but also during the precipitation polymerization under the conditions used.

3.3. Molecular Characteristics of the Copolymer Fractions. The **o**, **t**, **p**, and **s** fractions, isolated in accordance with the scheme in Figure 1, were studied by SEC (Figures 4 and 5). This study could reveal possible differences in MW of the fractions caused by differing synthesis conditions.

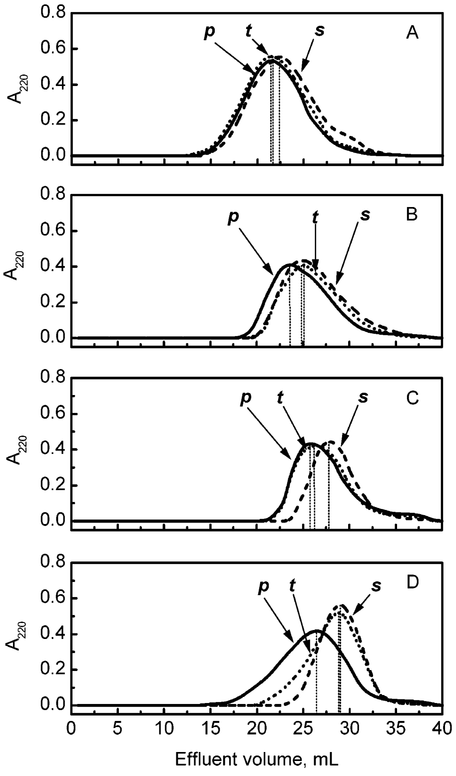

Figure 4. Size-exclusion chromatography profiles of the **t**, **p**, and **s** fractions of NVCl/NVIAz copolymers synthesized at 25 °C (i.e., below the PST). Molar ratios of comonomers in the initial feed: 75:25 (A), 80:20 (B), 85:15 (C), and 90:10 (D).

NVCl/NVIAz Copolymers Synthesized below the PST. The main feature of all such copolymers was a

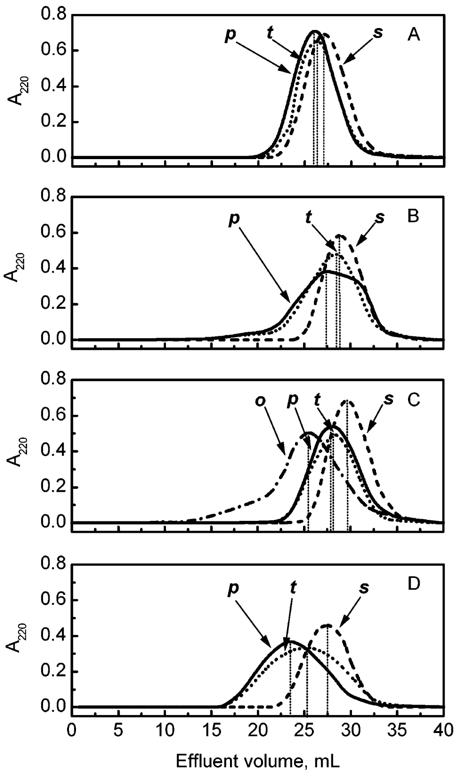

Figure 5. Size-exclusion chromatography profiles of the **t**, **o**, **p**, and **s** fractions of NVCl/NVIAz copolymers synthesized at 65 °C (i.e., above the PST). Molar ratios of comonomers in the initial feed: 75:25 (A), 80:20 (B), 85:15 (C), and 90:10 (D).

Table 2. Molecular Weights of the o, p, and s Fractions of NVCL/NVIAz Copolymers (SEC Data) and Cloud Points of Their Aqueous Solutions (TNM Data)

sample	MW,[a] kDa	T_{cp},[b] °C
75/25−25(***p***)	660	39
75/25−25(***s***)	610	>70
75/25−65(***p***)	225	44
75/25−65(***s***)	160	65
80/20−25(***p***)	370	32
80/20−25(***s***)	290	60
80/20−65(***p***)	150	35
80/20−65(***s***)	85	66
85/15−25(***p***)	240	46
85/15−25(***s***)	120	>70
85/15−65(***o***)	270	33
85/15−65(***p***)	105	34
85/15−65(***s***)	40	>70
90/10−25(***p***)	210	47
90/10−25(***s***)	65	>70
90/10−65(***p***)	390	37
90/10−65(***s***)	160	64

[a] The values correspond to effluent volumes of SEC peaks. [b] Measured in aqueous medium at solute concentration equal to 1.0 g/L; experimental accuracy was ±1°C.

rather broad molecular-weight distribution and higher MW of **p** fractions as compared with **s** fractions (dotted vertical lines show the peaks of effluent volumes). The molecular weights estimated from the elution peaks are presented in Table 2. For copolymers prepared below the PST, the differences in the elution volumes gradually increased with decreasing the NVIAz portion in the initial feed (cf., for example, Figure 4, parts A and D). However, more pronounced distinctions were registered not between the **p** and **s** fractions with the same initial comonomer ratio but instead between the copolymers synthesized at different NVCl/NVIAz ratios. Thus, the SEC peaks for the samples 75/25−25(**p**) and 75/25−25(**s**) corresponded to MW of about 660 and 610 kDa, whereas the MWs diminished until 210 and 60 kDa for the 90/10−25(**p**) and 90/10−25(**s**) copolymers. This trend, as well as the decrease in the polymer yield (Table 1), was, obviously, due to the decreasing content of more reactive hydrophilic comonomer in the initial mixture.

NVCl/NVIAz Copolymers Synthesized above the PST. The following tendencies were observed for the variation of MW for such copolymers (Figure 5).

The **p** fractions had higher MW than the **s** fractions, as in the previous case (i.e., Figure 4). When decreasing NVIAz portion in the feed, the difference between the SEC peaks of respective **p** and **s** fractions also increased. However, the MW values decreased with decreasing concentration of hydrophilic monomer, reached a minimum at [NVCl]/[NVIAz] = 85:15 (mol/mol), and then increased (Table 2).

Also, an interesting feature of SEC patterns in Figures 4 and 5 was a rather marked distinction between the areas under the peaks for different samples, although all the samples were dissolved at equal concentration before the SEC analyses, and the aliquots applied on the column were identical. For instance, these distinctions are well seen from the comparison of respective curves in parts A and B of Figure 4 or parts A and D of Figure 5. Such effects can depend at least on two of the following factors. The first one is unequal content of *N*-vinylimidazole units in these samples (Table 1) (because imidazole rings make major contribution to the UV absorbance by these copolymers at the wavelength of 220 nm used in SEC detector). The second factor is (possibly) different exposure of these units inside and on the periphery of macromolecular coils, which can be responsible for the different UV-absorption properties of these copolymer fractions, as is sometimes observed for proteins in their native and denatured conformations.[17] In the case of NVCl/NVIAz copolymers this could be the evidence of the differences in structural organization of respective macromolecules in aqueous solution.

3.4. Temperature-Dependent Solution Behavior of the Copolymer Fractions. Conformational and phase transitions can potentially be indicative of primary structure of thermosensitive macromolecules. Therefore, the study of the solution behavior of the **o**, **t**, **p**, and **s** fractions of NVCl/NVIAz copolymers in aqueous medium at different temperatures was of importance. The temperature-dependent solution behavior of these copolymers was studied by thermonephelometry (TNM) (Figures 6 and 7). The right column of Table 2 presents the cloud point temperatures (T_{cp}) determined in these experiments.

All the **p** fractions of NVCl/NVIAz copolymers produced at both below and above the PST exhibited pronounced phase separation effects upon heating of their aqueous solutions (Figures 6 and 7). The light scattering of the systems increased sharply at temperatures higher than threshold temperature. This indicated the phase separation behavior typical for LCST-exhibiting polymers like the NVCl homopolymers.[10,18]

NVCl/NVIAz Copolymers of p-Type Synthesized below the PST. The dependence of cloud point temperatures on the initial comonomer ratio for such copolymers passed through a minimum (see Table 2). The lowest T_{cp} equal to 32 °C was found for the 80/20−25(**p**) copolymer. No positive correlation was found

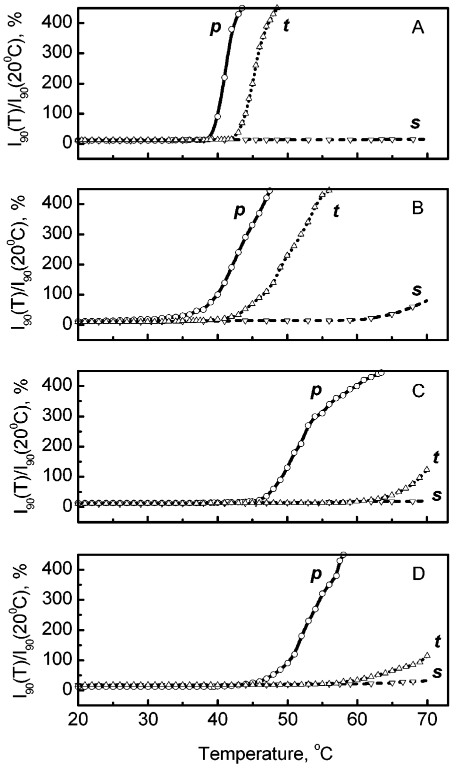

Figure 6. Thermonephelometry curves of aqueous solutions of the **t**, **p**, and **s** fractions of NVCl/NVIAz copolymers synthesized at 25 °C. The molar ratios of comonomers in the initial feed: 75:25 (A), 80:20 (B), 85:15 (C), and 90:10 (D).

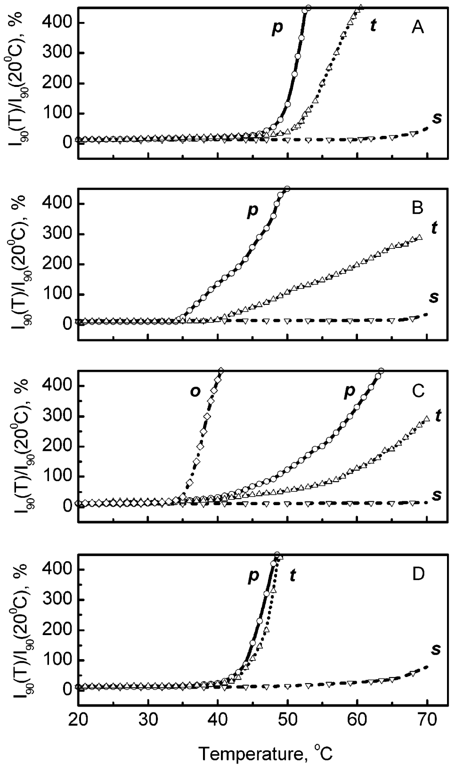

Figure 7. Thermonephelometry curves of aqueous solutions of the **o**, **t**, **p**, and **s** fractions of NVCl/NVIAz copolymers synthesized at 65 °C. Molar ratios of comonomers in the initial feed: 75:25 (A), 80:20 (B), 85:15 (C), and 90:10 (D).

between the T_{cp} and the NVCl/NVIAz composition or between the T_{cp} and the MW of the copolymers. Thus, the samples 80/20−25(**p**) and 90/10−25(**p**) had the same composition 79:21 (mol/mol) (Table 1), but their cloud points were 32 and 47 °C, respectively. At the same time, the MWs of these two copolymers differed not so drastically (370 and 210 kDa) to cause the 15° difference in T_{cp} values. Note that the 75/25−25(**p**) sample with T_{cp} at 39 °C had the highest MW of about 660 kDa, whereas a lower T_{cp} of 32 °C was found for the 80/20−25(**p**) copolymer with MW of only 370 kDa. We believe that these data indicate certain differences in the primary structure of respective chains.

NVCl/NVIAz Copolymers of p-Type Synthesized above the PST: The dependence of T_{cp} values on the initial comonomer ratio for such copolymers (Table 2) was also nonmonotonic with a minimum at 34 °C (85/15−65(**p**) copolymer). This dependence was not so sharp as in the case of 25(**p**) fractions. The temperature-dependent solution behavior and $T_{cp} = 33$ °C found for the **o**-type copolymer fraction was very similar to those of respective **p** fraction (Figure 7C and Table 2). The differences in MW values probably affected a higher ability of the **o** fraction to the heat-induced precipitation, since the comonomer composition of these two samples, that is, 85/15−65(**o**) and 85/15−65(**p**), did not differ significantly (81:19 and 76:24 (mol/mol), respectively) (Table 1), and the **o** fraction had a MW around 2.5 times higher than the **p** fraction.

The properties of the 80/20−65(**p**) copolymer deserve special attention. This specimen (MW ~150 kDa) had the lowest content of NVCl units (72.7 molar %, Table 1) among the other 65(**p**) species. This value was closer to the content of NVCl units in the 75/25−65(**s**) and 85/15−65(**s**) copolymers than that of other 65(**p**) samples. However, $T_{cp} = 35$ °C (Table 2) and TNM curve (Figure 7B) of the 80/20−65(**p**) copolymer did not differ considerably from those of the "typical" **p** fractions of the NVCl/NVIAz copolymers synthesized at 65 °C (see, e.g., Figure 7, parts A, C, and D). The latter contained larger amount (76−88 molar %) of hydrophobic comonomer and had higher MWs (225−390 kDa). All this allows one to conclude that for the products of **p**-type their monomer composition and MW values were not the only factors influencing the LCST behavior of these thermoresponsive copolymers. Most likely, in this case we dealt with the influence of different sequence of hydrophilic and hydrophobic units and their blocks in the **p**-type copolymers. Otherwise, such differences in the properties of the samples with very similar overall comonomer composition can hardly be explained, if one assumes that the distribution of comonomer units along the polymer chains follows always the classical theories of copolymerization.[19]

NVCl/NVIAz Copolymer Products of t-Type. The heat-induced precipitation (Figure 6) of the **t** samples formed at 25 °C was in a function of their **p**−**s** composition (Table 1). The higher the portion of **s** fraction, the higher was the phase separation temperature (cf. **t** curves in Figure 6A−D). On the other hand, the precipitation behavior varied for the **t** samples synthesized at 65 °C (Figure 7), although in them the amount of **s** fractions was nearly the same, 31−34% (Table 1). These variations depended on the "properties" of **s** fractions. For instance, the heat-induced precipitation of the 75/25−65(**t**) and 90/10−65(**t**) samples (Figure 7A and 7D) was suppressed to a markedly less extent as compared with 85/15−65(**t**) and 80/20−65(**t**) specimens (Figure 7, parts B and C). Obviously, such differences were due to the divergent influence of respective **s**

fractions on the coagulation processes in the thermoprecipitating parts of the total products **t**. This could be because of different surface hydrophilicity of the **s**-type coils with different spatial packing of hydrophobic and hydrophilic blocks.

NVCl/NVIAz Copolymers of s-Type. Not all of the solutions of such **s** copolymers remained almost transparent upon heating to 70 °C (see, e.g., **s** curves in Figures 6B, 7A, 7B, 7D), although their separation from the **p** fractions and isolation was performed at 65 °C (Figure 1). The detected increase in light scattering by these **s** samples at the temperatures of TNM experiments was rather small as compared with that registered for the samples capable of the heat-induced phase separation (**o** and **p** fractions). A slight increase in turbidity of the system at elevated temperatures was found in the copolymers of **s**-type possessing significantly dissimilar MW values (Table 2) and comonomer compositions (Table 1).

3.5. The Search for Proteinlike Properties of Isolated Copolymer Fractions. 3.5.1. Preliminary Remarks. Our interests were mainly focused on the **s** fractions, since we hoped to detect proteinlike copolymers exactly among them. Water solubility at elevated temperatures is an essential property of proteinlike macromolecules (see Introduction). However, other structural features can be also responsible for such behavior, including the following:

(i) A random distribution of a significant number of hydrophilic NVIAz units along the polymer chain could result in its uniform hydrophilisation. This, in turn, could lead to a loss of ability for the coil−globule transition, which is caused by the hydrophobic interactions. As a result, such copolymer should be water-soluble over a wide temperature range.

(ii) The primary structure of alternating block copolymer could have the hydrophobic blocks too short to facilitate efficient cooperative hydrophobic interactions. Such interactions could be capable of promoting the phase separation at temperature not far from the temperature of heat-induced precipitation detected for **p** fractions.

(iii) The proteinlike mode of distribution of the blocks of hydrophobic and hydrophilic units could facilitate the temperature-dependent folding of the chains with the formation of a dense hydrophobic core surrounded by a hydrophilic shell. The latter can protect the hydrophobic interior of the globules against the intermolecular hydrophobic interactions and subsequent phase separation.

Therefore, to identify the copolymers possessing the proteinlike structure, it was required to choose such a property that could distinguish them unambiguously from random copolymers or alternating block copolymers. One of such properties, as was demonstrated earlier,[9] is the change in partial heat capacity of the copolymer solution upon the heat-induced conformational transition of macromolecules. Such a change can be detected by the high-sensitivity differential scanning calorimetry (HS-DCS).

3.5.2. HS-DSC studies. Data of HS-DSC for copolymers synthesized at 25 and 65 °C are shown in Figures 8 and 9, respectively. The scale of the temperature axis is the same in both Figures to make the comparison easier. The **p** and **s** fractions of the 75/25−25 and 75/25−65 copolymers were not studied, since the data did not indicate the proteinlike properties of these copolymers.

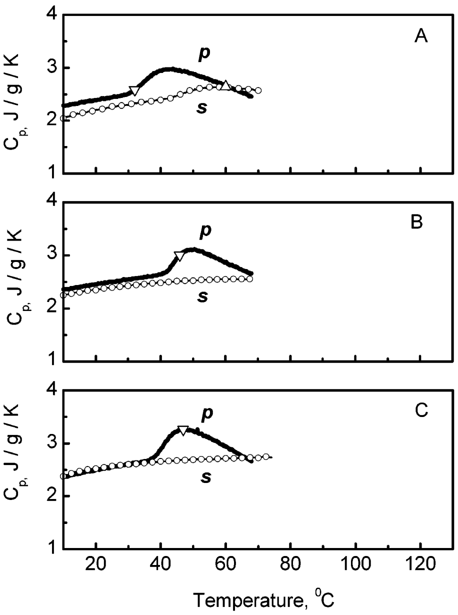

Figure 8. Partial heat capacity functions of NVCl/NVIAz copolymers synthesized at 25 °C for the following molar ratios of comonomers in the initial feed: 80:20 (A), 85:15 (B), and 90:10 (C) (▽, the cloud point of the **p** fraction, △, the cloud point of the **s** fraction; part of the points in **s** curves were omitted for a more clear presentation).

The functions of partial heat capacity for all the **p** fractions had a similar profile typical of polymer solutions with LCST. The curves went through a maximum within the temperature range of phase separation of the system. However, its cloud point (Table 2) did not necessarily coincide with the onset of the heat capacity peak. This discrepancy arose, probably, from the fact that TNM curves had a smoothed character (Figures 6 and 7) in the phase transition area. They were highly diffused. In most cases, they do not display a well-defined end point of the transition. For this reason, it was difficult to obtain reliable data on the transition enthalpy. However, some tendencies in the enthalpy change were found depending on the synthesis conditions and on composition of the copolymer fractions.

The transition enthalpy decreased regularly with decreasing in content of hydrophobic NVCL units in the copolymers. This tendency was more pronounced for the samples synthesized at 65 °C (Figure 9) than at 25 °C (Figure 8).

The comparison of HS-DSC data for the **p**-type copolymers obtained from the 90/10 initial feed revealed (Figures 8C and 9C) that the changeover from the solution polymerization to the precipitation polymerization led to almost a 3-fold increase in the transition enthalpy, namely, from 9.4 to 27.3 J/g. This also caused a marked decrease in the transition temperature (from ∼48 to 41.8 °C).

Generally, the transition enthalpy can be regarded as a measure of cooperativity of hydrophobic interaction of the oligoNVCl blocks: the higher the cooperativity, the larger the transition enthalpy. On the other hand, it is evident that cooperativity of interaction of the blocks is determined by their length. On this basis it is possible to evaluate changes in the length of thermoresponsive blocks in **p** fractions by examining the enthalpy of phase transition. Apparently, an average

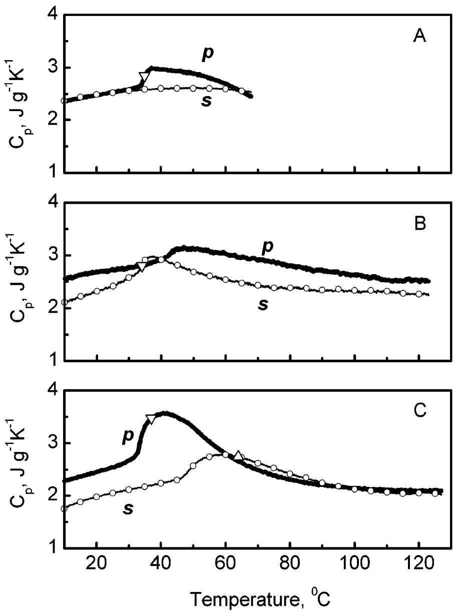

Figure 9. Partial heat capacity functions of NVCl/NVIAz copolymers synthesized at 65 °C for the following molar ratios of comonomers in initial feed: 80:20 (A), 85:15 (B) and 90:10 (C) (▽, the cloud point of the **p** fraction, △, the cloud point of the **s** fraction; part of the points in **s** curves were omitted for a more clear presentation).

length of such blocks decreases with increasing in content of the NVIAz units. In this regard, the calorimetric data for the 85/15−25(**s**), 90/10−25(**s**), and 80/20−65(**s**) samples (Figures 8B, 8C and 9A, respectively) showed that these copolymers did not undergo any cooperative conformational changes upon heating. It is highly probable that this fact reflected an insufficient length of the hydrophobic oligoNVCl blocks. In fact, at a uniform (random) distribution of the NVIAz units along the chains in the **s** fractions with the comonomer molar ratios from 62:38 to 78:22 (Table 1), the length of the oligoNVCl-blocks would vary only from ∼2 to ∼4 units. It is evident that so short NVCl-containing sequences cannot exhibit cooperative conformational changes.

The thermal behavior of the thermally nonprecipitating copolymers 80/20−25(**s**) (Figure 8A), 85/15−65(**s**) and 90/10−65(**s**) (Figure 9, parts B and C) was of special interest. For these specimens, the functions of partial heat capacity revealed a maximum. Since no phase separation was observed in these systems (Figures 6B, 7C, and 7D), we suppose that such experiments give evidence of cooperative conformational changes in these copolymers. Only slight increase in light scattering observed for the 80/20−25(**s**) and 90/10−65(**s**) samples at temperatures higher than 60 °C (Figures 6B and 7D), as well as a specific asymmetry of their heat capacity peaks (Figures 8A and 9C, respectively), imply that these changes could be indicated as cooperative micellization conjugated with the "coil−globule" transition. The heat capacity peak for the 80/20−25(**s**) copolymer was too small for a reliable estimation of the transition enthalpy.

The transition enthalpy of the 85/15−65(**s**) copolymer was equal to 7 J/g, which was close to the enthalpy of the 85/15−65(**p**) sample (6 J/g). Both these fractions had a very similar comonomer composition: 73:27 and 74:26 (mol/mol), respectively (Table 1). Hence, they should be characterized by almost the same average length of oligoNVCl blocks. However, the solution and, possibly, conformational properties of the 85/15−65(**s**) and 85/15−65(**p**) copolymers were drastically different. No phase separation upon heating to 70 °C (Figure 7C) was found for the 85/15−65(**s**) copolymer, while DSC detected a cooperative transition with $T_{\max}$ in the vicinity of 37 °C (Figure 9B). This apparently was due to a specific distribution of hydrophobic and hydrophilic residues along the polymer chains. In fact, such properties of the 85/15−65(**s**) sample lead us to the conclusion that this **s** fraction of the NVCl/NVIAz copolymer can be considered as a proteinlike copolymer.

The enthalpy of conformational transition of the 90/10−65(**s**) copolymer amounted to 11 J/g, which was about a third of the enthalpy (27.3 J/g) of phase transition of the 90/10−65(**p**) sample. Thus, we suppose that 90/10−65(**s**) copolymer differed from the corresponding **p** fraction by shorter average length of hydrophobic oligoNVCl blocks. This was also in agreement with a higher average content of hydrophilic NVIAz units in the **s** fraction (Table 1). The HS-DSC-detected transition in aqueous solution of this copolymer started at ∼47 °C and showed $T_{\max}$ at 61.3 °C (Figure 9C), whereas the cloud point of the system was observed at around 64 °C (Figure 7D and Table 2). These facts can be indicative of the proteinlike properties for the 90/10−65(**s**) specimen. At least, such behavior was characteristic for this copolymer over the temperature range from the onset of HS-DSC peak to T_{cp}.

An important common property of the 80/20−25(**s**), 85/15−65(**s**), and 90/10−65(**s**) copolymers was that their partial heat capacity in the pre-transition region was lower than that of the corresponding **p** fractions (Figures 8A, 9B, and 9C). The partial heat capacity of a polymer provides information about the accessibility of hydrophobic groups to water molecules.[15] The higher the accessibility, the larger is the partial heat capacity. Macromolecules have much larger accessibility of hydrophobic groups to water in the coil conformation than in the globule conformation. Therefore, the **s** fractions seem to have a more compact conformation in the pre-transition region than the **p** fractions did. A possible origin of local folding in these copolymers of **s**-type can be the intramolecular segregation of hydrophobic and hydrophilic blocks. A necessary condition for such segregation seems to be an appropriate (critical) length of the hydrophobic and hydrophilic sequences.

3.5.3. NMR Studies. The conformational and phase transitions of water-soluble temperature-responsive polymers influence the molecular dynamics of water molecules.[20−22] Therefore, it was important to study the temperature dependence of the parameters reflecting such mobility. It is known[23] that NMR signal width at half-height of spectral line ($\Delta_{1/2}\nu$) of water protons is inversely proportional to the spin−spin relaxation time T_2. Therefore, the $\Delta_{1/2}\nu$ parameter can characterize the molecular mobility. In the case of polymer solutions, the simplest model of water dynamics is a two-phase model implying the coexistence of low mobility protons of the polymer-bound water molecules and of high mobility protons of the bulk water molecules. The water protons are exchanged between these "phases" with the rate of $k_c = \tau_c^{-1}$, where τ_c is the correlation time. In turn, the width of NMR lines of highly mobile protons depends on the ratio of the correlation time and the frequency of spectrum registration (ω). If the proton exchange rate is small in comparison with the ω value, $\omega\tau_c \gg 1$, and

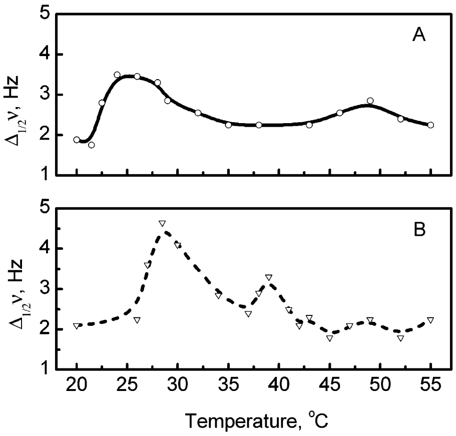

Figure 10. Temperature dependences of the full width of NMR signal ($\Delta_{1/2}\nu$) at half-maximum (fwhm) measured for water protons in D_2O solutions of the 85/15−65(**p**) (A) and 85/15−65(**s**) (B) copolymers.

only the NMR signal of bulk water is observed. Upon heating, the exchange rate increases, and τ_c decreases ($\omega\tau_c \sim 1$) resulting in broadening of spectral lines. Further temperature rise leads to $\omega\tau_c \ll 1$, and the value of $\Delta_{1/2}\nu$ becomes smaller; i.e., the line becomes narrower.

We measured $\Delta_{1/2}\nu$ for water protons in the D_2O solutions of the 85/15−65(**s**)- and 85/15−65(**p**) copolymers over the temperature range from 20 to 55 °C. Both samples had very close overall chemical composition (Table 1), but exhibited different solution behavior upon heating (Figure 7). The heat-induced variations of $\Delta_{1/2}\nu$ parameter for these two copolymer fractions were also different (Figure 10). For instance, in the solution of 85/15−65(**p**) copolymer the line width increased from 1.88 to 3.5 Hz at 20−24 °C, decreased further to 2.25 at 34−35 °C, was unchanged up to 43 °C, rose again to 2.85 Hz at 49 °C, and then diminished to 2.25 Hz at 55 °C (Figure 10A). The cloud point of the 85/15−65(**p**) copolymer was around 34 °C (Table 2 and Figure 7C). These facts lead us to the conclusion that the increase in $\Delta_{1/2}\nu$ over the temperature range of 20−34 °C was mainly connected with changes of proton exchange rate between the polymer-bound and bulk water. At the same time, broadening of the water proton signal at temperatures 43−49 °C was evidently caused by the phase separation in the system and dehydration of polymer chains. At further heating, due to increasing mobility of bulk water molecules, only the signals of their protons were registered (the condition of $\omega\tau_c \ll 1$). This was manifested in the descending branch of the curve in Figure 10A at the temperatures from 49 to 55 °C. Note that similar changes of water proton mobility was observed in the D_2O solutions of poly(*N*-isopropylacrylamide).[24] In that case, more pronounced effects were registered. This was owing to the homopolymeric nature of poly(*N*-isopropylacrylamide) and high cooperativity of phase transition.

The heat-induced variations of $\Delta_{1/2}\nu$ values for the 85/15−65(**s**) copolymer (Figure 10B) were even more pronounced compared with those for the **p** fraction (Figure 10A). The first peak on the $\Delta_{1/2}\nu$ (T) curve for the **s** fraction was higher and detected at a higher temperature. Such "temperature shift" could testify that this **s**-NVCl/NVIAz copolymer interacted with water molecules somewhat stronger than the **p** copolymer, because the condition $\omega\tau_c \sim 1$ was reached at higher temperature for the **s** fraction.

The second peak on the $\Delta_{1/2}\nu$ (T) curve in Figure 10B was observed over the temperature range of the descending branch of the heat capacity peak (Figure 9B). Note that the conformational transitions in thermoresponsive polymers is accompanied by a collapse of such macromolecules.[25] Such a collapse has to result in a partial dehydration of the chains and in a decrease in mobility of the polymer-bound water and, thereby, in broadening of the whole NMR signal from the water protons. Exactly this was observed for the 85/15−65(**s**) copolymer at 37−39 °C, thus indicating a compaction of these macromolecules at the temperatures, where the transition effect was registered by HS-DSC, but no macroscopic phase separation took place. At further heating the proton mobility increased, and this caused some narrowing of NMR signal.

Since D_2O was the solvent employed in NMR experiments instead of H_2O used in optical and calorimetric studies, some difference in the temperatures of the effects observed can be attributed to the isotopic effects.

4. Discussion

The results of the studies of NVCl/NVIAz copolymers synthesized in aqueous medium at the temperatures below and above the phase separation threshold have demonstrated rather different temperature-dependent behavior of polymers depending on the initial comonomer ratio in a feed and the phase state of a reacting system. Some of the copolymers exhibited typical LCST properties, whereas other copolymers remained water-soluble upon heating of their solutions, but showing the transition effects detected by HS-DSC and NMR. From the experimental data obtained the following major issues can be formulated:

1. What are the main features of the primary structure of **o**, **p**, and **s** fractions of these copolymers?

2. What are the possible reasons for the simultaneous formation of so distinct fractions in the course of polymerization reaction and how this peculiarity depends on the polymerization conditions?

3. Which of the copolymers, among those synthesized and studied in this work, can be classified as proteinlike copolymers and why?

These problems are discussed below in the same order.

4.1. Primary Structure of o, p, and s Fractions of the NVCl/NVIAz Copolymers. Thermally Precipitating o and p Fractions. The data of Table 1 demonstrate that molar ratios of NVCl and NVIAz units found for such copolymers of **o**- and **p**-types varied from 62:38 (85/15−25(**p**) sample) to 88:12 (90/10−65(**p**) sample). Therefore, assuming a random distribution of the comonomers along the chains, the length of hydrophobic oligoNVCl blocks should be from ~2 to ~7 units. Such a length is too short to cause pronounced heat-induced phase separation effects typical for the thermoresponsive NVCl copolymers. The onset of the phase transition for such copolymers was observed at the temperatures from 32 °C (80/20−25(**p**) sample) to 47 °C (90/10−25(**p**) sample) (Table 2), i.e., in the region of T_{cp} values characteristic of the PNVCl homopolymers (32−45 °C, depending on the polymer MW).[10,14,18,22] We believe this proves that all the obtained NVCl/NVIAz copolymers of the **o**- and **p**-types were not the random copolymers. They did possess a primary structure of the block copolymers, and the length of some of the blocks was large enough to cause the pronounced LCST behavior of such macromolecules. At the same time, the distribution of hydrophilic NVIAz units and oligomeric blocks in the polymer chains was such that hydrophilic segments were unable to “shield” the expanded NVCl-rich parts against the intermolecular hydrophobic interactions upon heating. In other words, each macromolecule of the **o-** and **p**-type should contain at least several long enough oligoNVCl blocks.

Thermally Nonprecipitating s Fractions. The samples 75/25−25(**s**), 85/15−25(**s**), and 90/10−25(**s**) formed at 25 °C in the solution polymerization process were most likely random NVCl/NVIAz copolymers. These specimens did not precipitate from aqueous solutions up to 70 °C (Figure 6, Table 2) and did not show transition peaks in DSC thermograms (Figure 8), though molar ratios in these copolymers were similar to those of **p**-type copolymers (61.5:38.5 in 85/15−25(**s**) sample to 79:21 in 75/25−25(**s**) sample) (Table 1). A slight increase in the system turbidity upon heating of the 80/20−25(**s**) copolymer solution ($T_{\mathrm{cp}} \sim 60$ °C; Table 2) and small DSC peak for this sample (Figure 8A) could be due to the micellization phenomena (see the discussion in section 3.5.2). This means that the chains of given copolymer had, nevertheless, a certain amount of oligoNVCl blocks “nonprotected by the hydrophilic microenvironment” and thus capable of participating in the associative intermolecular processes at elevated temperatures.

Finally, the primary structure of the 85/15−65(**s**) and 90/10−65(**s**) copolymers can be that of block copolymers with, probably, a heterogeneous blockiness (see the Introduction). The basis for such conclusion is the lack of macroscopic heat-induced phase separation at elevated temperatures (Figure 7, parts C and D) and, at the same time, the transition effects revealed by the HS-DSC (Figure 9, parts B and C) and NMR (Figure 10B). This means that primary structure of these specimens included, apart from the irregular sequences of comonomer units, both long enough hydrophobic oligoNVCl blocks and the long enough hydrophilic oligoNVIAz blocks. The former were responsible for the cooperative transitions within the macromolecular coils, while the latter were capable of protecting the chains against the intermolecular aggregation. According to the computer simulation data,[1] exactly such a primary structure should be characteristic of the proteinlike HP copolymers.

4.2. **Possible Reasons for the Parallel Formation of Thermally Precipitating and Nonprecipitating Fractions of NVCl/NVIAz Copolymers and Influence of Polymerization Conditions on That Process.** Another problem concerns the reasons for the formation of the thermally precipitating **p**-type fractions during the solution co-polymerization of NVCl and NVIAz in aqueous medium at temperatures below the PST and, vice versa, the formation of the thermally nonprecipitating **s** fractions during the precipitation copolymerization of the same comonomers at temperatures above the PST.

Formation of the p-Type Fractions during the Solution Polymerization. The 10% DMSO aqueous solution is a good solvent for the macromolecules formed in the process at 25 °C. Why then the polymeric products with a nonrandom distribution of comonomer units (i.e., 25(**p**) fractions containing the long enough hydrophobic oligoNVCl blocks) were produced in parallel with the statistical copolymers (i.e., 25(**s**) fractions)?

This indicates that the copolymerization of NVCl and NVIAz at the their initial molar ratios used at room temperatures was not a classical free-radical solution polymerization. The simultaneous formation of the **p** and **s** fractions with sometimes very similar average comonomer composition (Table 1), testifies to the microheterogeneity of the reacting system even below the temperature of macroscopic phase separation. A similar inhomogeneity was also mentioned in our recent work[26] dealing with the *N*-isopropylacrylamide polymerization in aqueous medium at different phase states of a reacting system. It was assumed that in aqueous solutions such amphiphilic monomers as *N*-alkylacrylamides or NVCl tend to form the micellelike clusters. Similar association of water-soluble vinyl monomers is known to influence on the course of polymerization in aqueous media.[27] Owing to such microheterogeneity of the initial solution at 25 °C, the initiation of the copolymerization of NVCl/NVIAz pair should result in a parallel formation of, at least, two sorts of chains. The first are those that started to grow and then propagate in a molecular-dispersed (dissolved) state. The second type macromolecules are formed mainly from the monomers organized in clusters, thus memorizing, to a certain extent, their ordered structure. This mechanism can explain the presence of **p** and **s** fractions in the polymeric products **t** synthesized at temperatures below the PST. A similar assumption allows also understanding the presence of two fractions in the *N*-isopropylacrylamide/NVIAz copolymers synthesized in aqueous solution at room temperature,[13] when the fractions had very different affinity to Cu^{2+}−chelate−resin (see Introduction).

Formation of the s-Type Fractions during the Precipitation Polymerization. During the copolymerization of NVCl and NVIAz at 65 °C, the thermal conditions promote a strong attraction between the hydrophobic entities and therefore should have favored the formation of thermally precipitating copolymers of the **o-** or **p**-types. What mechanisms can be responsible for the formation of **s** fractions under such conditions?

One of the most significant factors for this process is the instant location of the reactive end of growing macroradical during the polymerization process. Possible variants of such location can be as follows: (i) either in a dissolved propagating chain, which is in a coil conformation, or (ii) either inside or at the surface of a dense hydrophobic core formed already at the early stages of precipitation polymerization from an excessive amount of more hydrophobic comonomer, NVCl, due to coil−globule transition.

MW and overall comonomer ratio, as well as the sequence of comonomer units along the chains of synthesized copolymers, should be different in these two cases.

We believe that the chains of **s** fractions that did not exhibit conformational transition (Figures 8B, 8C, and 9A) started to grow after the reaction of the initiator and NVIAz. Thus, the propagation of more hydrophilic chains with predominantly random distribution of comonomer units takes place as predicted theoretically for the solution copolymerization.[19,28]

On the contrary, the reactive macroradicals, whose growing ends are located within the hydrophobic interior of collapsed chains, should predominantly react with NVCl molecules. Thus, the formation of long enough oligoNVCl segments is facilitated; i.e., such a mechanism favors the formation of the **o**- and **p**-type copolymers.

Finally, the scenario with the formation of a heterogeneous blockiness characteristic of proteinlike copolymers can be realized upon the fast formation of dense hydrophobic core enriched with NVCl units and subsequent "exit" of growing end onto the surface of this collapsed nanoglobule, where then the hydrophilic NVIAz molecules can easily react. The elevated temperature is the factor maintaining the hydrophobic interior of such chains in a collapsed state, preventing the macromolecules (at least, their dense cores) from acquiring loose conformation as the copolymerization of the comonomers with differed hydrophobicity/hydrophilicity occurs. Apparently, this is the only variant of the polymer chain growth capable of forming thermally nonprecipitating **s** fractions with the "hydrophobic core—hydrophilic shell" structure inherent in the proteinlike HP copolymers.

Other Factors Affecting the Simultaneous Formation of Thermally Precipitating and Nonprecipitating Fractions. There is a number of factors that determine which of the above-discussed ways of polymerization goes. One of them is the already-discussed enrichment of final copolymers with the more hydrophilic comonomer, NVIAz, due to its higher reactivity. Higher (than for NVCl) reactivity of NVIAz is determined by a stronger polarization of double bond of vinyl group by imidazole moiety as compared with caprolactam cycle. Furthermore, a good solubility of hydrophilic NVIAz can also contribute to its higher reactivity in aqueous media.

The temperature of the synthesis is a very significant factor determining the phase state of a reacting system. It had a pronounced effect on the properties of NVCl/NVIAz copolymers, although this influence was ambiguous. Thus, a decrease in MW of the polymers with rise of the synthesis temperature is a well-known regularity for a free radical solution polymerization.[28] An increase in MW is frequently encountered for the precipitation polymerization due to a reduced mobility of growing macroradicals in the precipitate phase and a sharp decrease of the recombination rate.[29] We observed the latter effect for the precipitation polymerization in the system with high initial content of NVCl. The MWs of 90/10−65(**p**) and 90/10−65(**s**) samples were considerably higher than those of the 90/10−25(**p**) fractions synthesized at 25 °C (Table 2). A similar tendency was also observed for precipitation homopolymerization of NVCl.[10] However, all the copolymers synthesized in the precipitation polymerization process had lower MWs than macromolecular products of solution polymerization, when the concentration of NVCl in the feed was less (Table 2). In other words, in the presence of a greater amount of hydrophilic comonomer, NVIAz, the influence of the decreased mobility of growing macroradicals in the precipitate phase on the polymerization efficiency was only moderate. This can also be regarded as the manifestation of high enough mobility of growing chains in this phase with the increase in amount of hydrophilic comonomer in the polymerization system.

4.3. Why Can the 85/15—65(s) and 90/10—65(s) Fractions of NVCl/NVIAz Copolymers Be Attributed to the Proteinlike Copolymers? Theory predicts that the main feature of the primary structure of synthetic proteinlike copolymers is a sequence of heterogeneous blocks possessing distinct relative hydrophilicity/hydrophobicity. With a "favorable" combination of such blocks, a specific segment packing of a macromolecule occurs in aqueous medium: the dense core (enriched with hydrophobic (H) monomer units) surrounded by a shell enriched with polar (P) comonomer. If the H blocks are long enough to exhibit the LCST behavior, an increase in temperature higher than a certain critical point should cause a collapse of these blocks. This occurs for thermoresponsive water-soluble polymers in dilute solutions.[18,25,30,31] However, hydrophilic polar blocks on the periphery of the collapsed core of proteinlike copolymers should prevent further aggregation of such collapsed cores, thus "preserving" these copolymers against the heat-induced phase separation. Hence, only the combination of properties, namely, the temperature-induced conformational transition with retention of water solubility is a reliable indication of proteinlike HP copolymers.

In fact, among the products synthesized (Table 1) such behavior was found only for the 80/20−25(**s**), 85/15−65(**s**), and 90/10−65(**s**) fractions. The "indicators" of conformational transition were the changes in the partial heat capacity of the copolymers detected by HS-DSC (Figures 8A, 9B, and 9C) in combination with a transparency (or a very slight turbidity) of the solutions (Figures 6B, 7C, and 7D) at temperatures of DSC peaks. However, the thermal behavior of these (**s**) copolymers was not identical.

80/20—25(s) Copolymer. Some slight changes in the behavior of this copolymer were observed at markedly higher temperatures than the transitions detected by HS-DSC for the 85/15−65(**s**) and 90/10−65(**s**) samples. However, the corresponding turbidity effects were not significant, and respective heat capacity peak (Figure 8A) had a specific asymmetry. For these reasons, the behavior of the 80/20−25(**s**) copolymer in aqueous solution at elevated temperatures was identified above (section 3.5.2) as a cooperative micellization.

90/10—65(s) Copolymer. Apparently, a similar micellization took place upon heating of the solution of this copolymer above its T_{cp} (64 °C; Table 2), when a slight increase in light scattering (Figure 7D) coincided with the diffuse descending branch of heat capacity curve (Figure 9C). However, the onset of this HS-DSC peak has been registered at the temperature about 17 degrees lower (~47 °C), when the polymer solution remained still transparent (Figure 7D). So, the behavior of the 90/10−65(**s**) copolymer in aqueous solution over the temperature range from 47 °C to T_{cp} (i.e., prior to micellization) was identified above (section 3.5.2) as the behavior typical of the proteinlike copolymers.

85/15—65(s) Copolymer. The most pronounced effects were found for this fraction. Its aqueous solution remained transparent upon heating to at least 70 °C (Figure 7C), whereas endothermic transition was clearly observed by HS-DSC at temperatures from ~30 to ~60 °C (T_{max} 37.3 °C; Figure 9B). In addition, NMR studies revealed (Figure 10B) broadening of the water proton signal (caused by the decreased mobility of bound water) just in vicinity of the temperature of HS-DSC peak. These data indicate the compactisation of the interior of polymer coils, as it should be for the proteinlike macromolecules. Thus, the 85/15−65(**s**) copolymer can be attributed to the proteinlike copolymers. Naturally, the same conclusion is also related to the proteinlike structure acquired by the above-discussed 90/10−65(**s**) copolymer at temperatures from 47 °C to T_{cp}.

In the pre-transition temperature region similar macromolecules (i.e., of the 85/15−65(**s**)- and 90/10−65(**s**)-type), possess less dense interiors, but the prerequisites for their compactisation (folding) with rise of temperature already exist. Such prerequisites can lead (as it was pointed out in section 3.5.2) to intramolecular microsegregation of the blocks of different hydrophilicity/hydrophobicity.

5. Conclusions

The task of the synthesis of proteinlike HP copolymers from the mixture of appropriate comonomers through the polymerization reactions is a novel problem for polymer chemistry. The alternative ways are biosynthesis, or directed chemical synthesis like the step-by-step solid-phase peptide synthesis or sequential coupling of the blocks of preassigned composition. In the case of ribosomal biosynthesis of proteins, the fidelity of amino acid sequence is governed by the gene-stored information and the enzymes involved in the process. In the case of directed chemical synthesis, the “program” specified by a chemist determines the exact primary structures of the resulting chains. On the other hand, during the free radical copolymerization, one deals with the chain growth process. Such a process obeys the laws of chemical statistics, so the primary structure of the formed synthetic macromolecules depends on the relative reactivities of the comonomers used, composition of initial feed, phase state of reacting system, rate of generation of initiating radicals, temperature regimes, and a series of other factors. When the polymerization process is also additionally complicated by the macro- or microinhomogeneity of the reaction medium, this causes deviations from simple statistics. As a result, it is impossible to obtain the copolymer chains having definite MW and primary structure. The set of final polymer products differs not only in degree of polymerization but also in the sequence of comonomer units along the chains. The formation of different copolymers proceeds simultaneously. Therefore, an additional problem of the isolation of target compounds arises. Indeed, all such problems have been met in our work on the synthesis, characterization and study of behavior in aqueous medium of the NVCl/NVIAz copolymers, among which the proteinlike fractions have been identified.

The continuation of these studies is in progress. In particular, we are attempting to obtain similar proteinlike HP copolymers originating from other comonomers. This study is performed in order to reveal common regularities of the formation of such macromolecular products, as well as to determine characteristic features of different comonomer pairs. The insertion in respective chains of additional probe monomer units carrying special chromophor groups can presumably allow detecting (by spectral methods) the stimuli-dependent variation of chain conformation. The use of comonomers with pH-dependent chargeable functions allows tracing the influence of charge effects on the properties of proteinlike copolymers. All these efforts should, as we hope, help to formulate certain general approaches to the preparation of such copolymers, as well as to understand which of the factors of polymerization process affects principally the formation of HP copolymers with the desired proteinlike primary structure.

List of Abbreviations

APS = ammonium persulfate
DMSO = dimethyl sulfoxide
HS-DSC = high sensitivity differential scanning calorimetry
LCST = lower critical solution temperature
NMR = nuclear magnetic resonance
NVCl = *N*-vinylcaprolactam
NVIAz = *N*-vinylimidazole
PST = phase separation threshold
SEC = size-exclusion chromatography
TMEDA = *N*,*N*,*N*′,*N*′-tetramethylethylenediamine
TNM = thermonephelometry

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