ELSEVIER

Polymer 42 (2001) 3373–3381

polymer

www.elsevier.nl/locate/polymer

Thermosensitive copolymer of *N*-vinylcaprolactam and 1-vinylimidazole: molecular characterization and separation by immobilized metal affinity chromatography

A.E. Ivanov[1], S.V. Kazakov, I.Yu. Galaev, B. Mattiasson*

Department of Biotechnology, Center for Chemistry and Chemical Engineering, Lund University, P.O. Box 124, S-221 00 Lund, Sweden

Received 30 May 2000; received in revised form 28 August 2000; accepted 7 September 2000

Abstract

Thermosensitive copolymer of *N*-vinylcaprolactam (VCL) and 1-vinylimidazole (VI), a new reagent for metal–chelate affinity separation of proteins, was characterized by static and dynamic light scattering and ^{1}H NMR spectrometry. The copolymer of $M_w = 11\,700$, $M_w/M_n = 1.1$ and VI-unit molar fraction of 11% was prepared by free radical co-polymerization of the monomers followed by dialysis of the product. The copolymer solution in 10 mM Na-phosphate buffer containing 0.15 M NaCl, pH 8.5, at 25°C turned out to be close to θ-conditions. Its diffusion coefficient in these conditions was 6×10^{-7} cm^2 s^{-1} indicating the hydrodynamic diameter of 8 nm. The copolymer was separated by immobilized metal affinity chromatography on Cu^{2+}-IDA-Sepharose into the non-adsorbing fraction (5 molar% VI-units) and the adsorbing fraction further eluted with EDTA and thermally precipitated at 48°C (8.5 mol% VI-units). The combination of IMAC and thermal precipitation of the copolymer enables isolation of its high-affinity, relatively homogeneous fraction most promising for bioseparations, including displacement chromatography of proteins. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Thermosensitive polymers; θ-conditions; Immobilized metal affinity chromatography

1. Introduction

Water-soluble polymers with the combined properties of thermosensitivity and binding to transition metal ions are efficient reagents for metal–chelate affinity precipitation of proteins [1,2] and promising displacers for biochromatography [3]. Copolymers of *N*-vinylcaprolactam (VCL) and 1-vinylimidazole (VI) are examples of such reagents capable of complex formation with Cu^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+}-ions and, further, with proteins containing nucleophilic residues of histidine, methionine and tryptophane. Facile purification of biomolecules achieved by thermal precipitation of the thus obtained protein–polymer complexes was demonstrated earlier in our laboratory [4–6].

Although (VCL–VI)-copolymers with different average ratios of the monomer units were prepared and their metal-binding capacities were evaluated in detail [4,6], there is still a lack of information about the molecular characteristics of the copolymers, including their molecular weight and molecular weight distribution as well as divergence from the average unit ratio in their chains. Obviously, due to the different co-polymerization rate constants of the monomers, the copolymers prepared by free radical polymerization at high polymerization yields [6] would necessarily contain chains with different molar percentage of monomer units. These chains may behave differently in the processes of metal chelating, protein binding and thermal precipitation. The present work was, therefore, aimed at the study of heterogeneity of the copolymers and preparation of more homogeneous fractions with well defined molecular characteristics and properties.

On the other hand, the state of the copolymer chains in aqueous solution may also be important for their complex formation either with metal ions or with proteins. Molecular dimensions of poly(*N*-vinylcaprolactam) chains in pure water and their interaction parameters with the solvent

---

* Corresponding author. Tel.: +46-46-222-8264; fax: +46-46-222-4713. *E-mail address:* bo.mattiasson@biotek.lu.se (B. Mattiasson).

[1] On leave from Shemyakin & Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, 117871 Moscow V-437, Russia.

Abbreviations: Cu^{2+}-IDA-Sepharose: iminodiacetate-Sepharose loaded with Cu^{2+}-ions; EDTA: ethylenediaminetetraacetic acid, disodium salt; IDA: iminodiacetic acid; IDA-Sepharose: iminodiacetate-Sepharose; IMAC: immobilized metal affinity chromatography; VCL: *N*-vinylcaprolactam; VI: 1-vinylimidazole; (VCL–VI)-copolymer: copolymer of *N*-vinylcaprolactam and 1-vinylimidazole.

PII: S0032-3861(00)00666-2

were recently reported in Ref. [7]. In this respect, nothing is known yet about (VCL–VI)-copolymers, reagents of even wider potential applications. The aim of the present work was, therefore, to obtain the physico-chemical characteristics of (VCL–VI)-copolymers: their molecular weight, dimensions and diffusivity in aqueous solution.

2. Experimental

2.1. Materials

1,4-Dioxane was dried over potassium hydroxide and distilled (101–102°C). *N*-vinylcaprolactam was a generous gift from ISP (Guildford, UK) and was recrystallized from hexane, m.p. 38–39°C. *N*-vinylimidazole (Aldrich, Steinheim, FRG) was distilled under vacuum (74–76°C, 10 mm Hg). 2,2′-Azobis(2-methylpropionitrile) was purchased from ACROS (Malmö, Sweden). Deterium oxide was from Euriso-Top, Gif-sur-Yvette, France. Copper sulfate, EDTA disodium salt, sodium chloride, sodium dihydrogen phosphate and potassium dihydrogen phosphate were of analytical grade and supplied by Merck (Darmstadt, Germany). Iminodiacetate Sepharose CL-6B (IDA-Sepharose) was prepared as described by Porath and Olin [8]. Spectra/Por Dialysis Membrane MWCO 6.000–8.000 was purchased from KEBO (Lund, Sweden).

2.2. Methods

2.2.1. Copolymerization of 1-vinylimidazole (VI) with N-vinylcaprolactam (VCL)

VCL (3.75 g, 0.02 mol), VI (0.273 ml, 2×10^{-3} mol) and 180 mg AIBN were dissolved in 60 ml 1,4-dioxane. The prepared solution was placed into a two-necked 100 ml round bottom flask, equipped with reversing cooler and gas-supplement tube for nitrogen bubbling. Free radical polymerization was started by heating the reaction mixture to 80°C under nitrogen bubbling in a water bath and carried out for 20 h (10 + 10 h with an overnight break). Two portions of AIBN (80 + 80 mg, each in 2 ml dioxane) were added as the reaction proceeded, in 5 h and 24 h after the start. The volume of the reaction mixture was then reduced to 6 ml by evaporation of the solvents at reduced pressure; the solution of the copolymer became viscous. Two portions of the solution (3 ml) were dropped into each 60 ml diethyl ether at vigorous magnetic stirring, separated from the solvent by filtration through paper, washed by ca. 50 ml ether on the filter and dried in air. The yield of the copolymer was 75%.

To prepare the copolymer samples for further investigation by means of light scattering and NMR, the substances were dissolved in distilled water at concentration of ca. 10 mg ml^{-1}, dialyzed against distilled water using a Spectra/Por Dialysis Membrane and freeze-dried.

2.2.2. Thermal precipitation of (VCL–VI)-copolymer

(VCL–VI)-copolymer solution (10 mg ml^{-1}) in 10 mM Na-phosphate, containing 0.3 M NaCl, pH 8.5 (10 ml) was heated to 48°C in a water bath at vigorous shaking until the copolymer precipitated on the walls of the test-tube, while the supernatant became almost clear (8–10 min). The supernatant was collected, dialyzed against distilled water and freeze-dried. The precipitate was dissolved in 5 ml distilled water. Further it was dialyzed and freeze-dried for preparation of the samples for NMR and light scattering, or adjusted to the conditions of repeated precipitation and affinity re-chromatography by addition of salts, distilled water and sodium hydroxide.

2.2.3. Immobilized metal affinity chromatography of (VCL–VI)-copolymer on Cu^{2+}-IDA-Sepharose

Cu^{2+}-IDA-Sepharose placed in a standard “Varian Bond Elut LPC Reservoir” column (1.6 ml) was washed with 15 ml distilled water. Solution of (VCL–VI)-copolymer in distilled water (1 mg ml^{-1}) was applied in a frontal mode to the column at flow rate of 0.5 ml min^{-1}. Fractions of about 1.6 ml were collected, diluted twofold by distilled water and monitored at 220 nm (amide group absorption; $\epsilon_{220} = 7.2$ ml mg^{-1} cm^{-1}). The non-bound fractions (from 7 to 18th, see Fig. 6) were joined, reduced in volume to ca. 10 ml by vacuum evaporation, dialyzed against distilled water and freeze-dried. The weakly bound portions of the copolymer were removed by washing with distilled water and 0.2 M NaCl in 10 mM Na-phosphate, pH 7.0. The specifically adsorbed copolymer was eluted by 0.02 M EDTA in 0.15 M NaCl, pH 8.0, the volume of the eluate was reduced twofold by vacuum evaporation and its pH was adjusted to 8.5. Thermal precipitation of the copolymer was performed as described above, the precipitate was dissolved in distilled water, dialyzed against diluted hydrochloric acid (pH 2.5) and freeze-dried.

Re-chromatography of the purified copolymer fraction was performed in 10 mM Na-phosphate, 0.15 M NaCl, pH 7.0 on a 1×5.5 cm column with Cu^{2+}-IDA-Sepharose. For the detection of copolymer the collected fractions (1 ml) were heated up to 48°C and absorbance was measured at 450 nm to detect the turbidity of the thermosensitive polymer.

2.2.4. Light scattering

Light scattering measurements were done with a Malvern 4700c System. An argon ion laser (Uniphase 2213-75 SL) operating at 488 nm wavelength and 30 mW output power was used as a light source. The scattering angle was varied from 30 to 150° in 13 steps. The spectrometer was calibrated with distilled water and toluene to make sure that the scattering intensity from water and toluene had no angular dependence in the used range. All measurements were performed at 25.0 ± 0.1°C. The specific refractive index increment ($\partial n/\partial c = 0.206$ ml g^{-1}) was taken as for poly-(*N*-vinylcaprolactam) from the paper [7] with corresponding

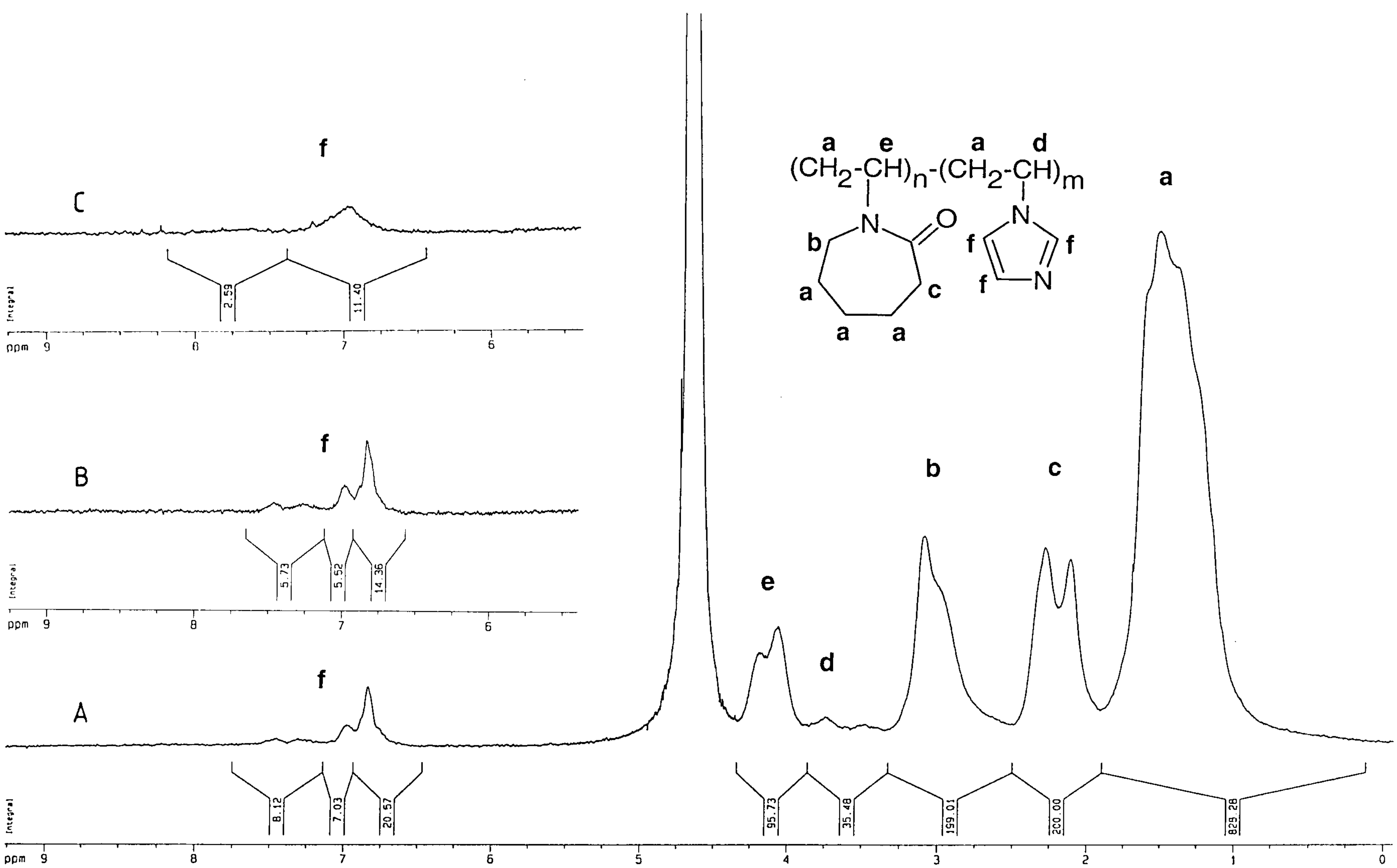

Fig. 1. 500 MHz ^{1}H NMR spectrum of: (A) the parent (VCL–VI)-copolymer, (B) the copolymer purified by IMAC, and (C) its fraction non-adsorbing to Cu^{2+}-IDA-Sepharose. The samples were dialyzed against distilled water and freeze dried. Solvent: D_2O.

wavelength correction. The pure toluene (>99.5%, pro analysis, MERCK, Germany) with known value of the excess Raleigh ratio, $R(q) = 4 \times 10^{-5}\ cm^{-1}$ [9], was used as a standard.

Na-phosphate buffer (10 mM) pH 8.5, containing 0.15 M NaCl was used in light scattering experiments. Stock solution of (VCL–VI)-copolymer was prepared at concentration of 24 mg ml^{-1} and filtered using 0.02 μm ANOTOP® 25 PLUS filter (MERCK, Germany). Filtered solution (0.5 ml) was poured into the ∅10 mm quartz sample cell. Solutions with lower concentration were obtained by addition of the corresponding amount of filtered buffer.

For measurements of the light scattering intensity at different temperatures Malvern 4700c System was used with the detector installed at 90°. Temperature control was provided with the precision of ±0.1°C. Installation of the equilibrium temperature was controlled on the stable level of the scattering intensity. An average value of the stabilized intensity was calculated over 100 measured values at each temperature.

The quality of dynamic light scattering measurements was checked over the signal-to-noise ratio and the range of the correlation function. The intensity autocorrelation function was collected in 128 channels and in a so-called far point, a special group of correlator channels pushed out in time by a special extension of the memory. The difference between the measured and the calculated baselines was taken into account.

2.2.5. NMR measurements

NMR spectra were registered on Bruker DRX500 spectrometer operating at 500.2 MHz for ^{1}H at 27°C. The samples for NMR-spectrometry measurements were prepared by dissolution the freeze-dried copolymer fractions in 99.9 at.% D deuterium oxide at 2 mg ml^{-1}. Chemical shifts are reported as ppm downfield from tetramethyl silane (TMS).

3. Results and discussion

3.1. Molecular characterization of (VCL–VI)-copolymer: chemical composition

Chemical composition of (VCL–VI)-copolymer prepared as described in Section 2.2.1 is proven by its ^{1}H NMR-spectrum given in Fig.1(a). The average molar share of VI monomer units was calculated from f and e peak areas (S) as follows: $S_f : 3/(S_f : 3 + S_e)$ and yielded 11% for the parent copolymer. Some portions of the copolymer contain, however, a much lower share of VI monomer units, as discussed below in Section 3.3. Obviously, a hydration of (VCL–VI)-copolymer and molecular dimensions of its chains should strongly depend on ionization of the pendant imidazole groups. To diminish the effects of polyelectrolyte swelling and, therefore, the influence of polymer heterogenity on light scattering, we have chosen the composition of the aqueous solvent corresponding to the non-protonated form of VI-units ($\mathrm{p}K\mathrm{b} \approx 7$) : 10 mM Na-phosphate buffer, containing 0.15 M NaCl, pH 8.5.

3.2. Molecular weight determination by static light scattering

The angular and concentration dependence (Zimm plot) of the excess Rayleigh ratio, $R(q,c) = r^2 I(q)/I_0$, i.e. the excess absolute time-averaged scattering intensity of a macromolecular solution at a relatively low scattering angle Θ and concentrations c (mg ml^{-1}) can be related to the weight-average molecular weight M_w [10] as follows:

$$Kc/R(q,c) \approx M_w^{-1}(1 + R_g^2 q^2/3 + 2A_2 M_w c), \tag{1}$$

where I_0 is the intensity of incident beam, $I(q)$ the measured scattering intensity, r the distance from scattering volume to detector, $K = 4\pi^2 n_0^2 (\partial n/\partial c)^2/(N_A \lambda_0^4)$, q the magnitude of the scattering vector related to the scattering angle, $q = (4\pi n_0/\lambda_0)\sin(\Theta/2)$, n_0 the refractive index of the solvent, λ_0 is the wavelength of light used, N_A and $\partial n/\partial c$ being Avogadro's number and the specific refractive index increment, respectively. A_2 is the second-order virial coefficient. $R_g \equiv [\langle S^2 \rangle_z]^{1/2}$, where S is the time independent static structure factor. The angle bracket in $\langle S^2 \rangle_z$ denotes the average over all possible conformations and the index z indicates that this value is the z-average over the molar mass distribution. Measuring $R(q,c)$ at a set of c and q allows calculation of M_w, R_g, and A_2 from Zimm-plot, which incorporates the extrapolation of $q \to 0$ and $c \to 0$ on a single grid. It follows from Eq. (1) that if the limits

$$qR_g \ll \sqrt{3} \qquad \text{and} \qquad A_2 M_w c \ll 0.5 \tag{2}$$

are fulfilled, the only molecular weight M_w can be detected from

$$R(q,c)/Kc \approx M_w. \tag{3}$$

Fig. 2 shows q^2-dependences of the intensity of light scattered from (VCL–VI)-copolymer solution of different concentrations. The latter are indicated near each line in mg ml^{-1}. One can observe that in the whole measured angular range the scattering intensity is independent on the magnitude of wave vector. Solid lines are the average values of $I(q)$ for a given concentration. According to these results Eq. (3) is fulfilled and can be used for determination of the polymer molecular weight.

Fig. 3 shows that $R(q,c)/Kc$ is independent of concentration and Eq. (3) gives the M_w valid within the experimental errors whose limited values are indicated by dotted lines. The molecular weight of (VCL–VI)-copolymer appeared to be $M_w = (11.7 \pm 0.4) \times 10^3$ g/mol, which corresponds to the degree of polymerization $n = 85$–90 and the amount of VI-units per chain ca. 10. The above results also show that the second virial coefficient A_2 is very close to zero (at

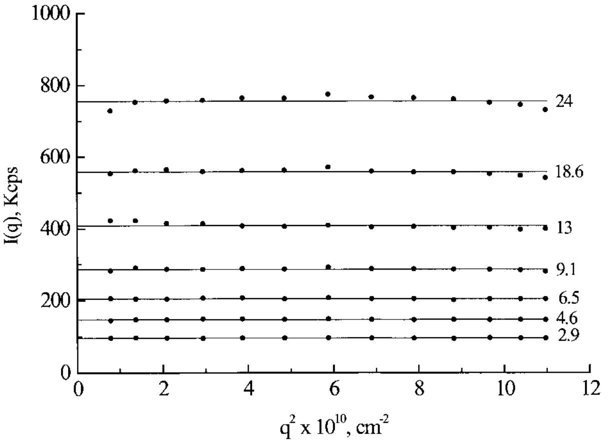

Fig. 2. q^2-Dependencies of the intensity of light scattered from the (VCL–VI)-copolymer solution with different concentrations. The numbers near each line indicate the corresponding concentration in mg ml^{-1}. Solid lines are the average values of $I(q)$ for given concentration.

least less than 0.5×10^{-4} mol ml g^{-2}), indicating that the copolymer solution exists close to θ-conditions.

Recently, the solutions of poly(*N*-vinylcaprolactam) in pure water were studied by light scattering and exhibited the well-expressed angular dependences of $R(q,c)/Kc$ yielding the corresponding second virial coefficients in the range from 1.5 to 2.0×10^{-4} mol ml g^{-2} obtained for the polymer samples of higher molecular weights [7]. Moreover, the discrepancy with the results obtained in the present study probably arises from the different composition of the solvent. The presence of sodium chloride may enhance the intrachain hydrophobic interactions in the copolymer. According to the results of the static light scattering, the angular dependence of $I(q)$ (see Fig. 2) does not allow evaluation of the molecular dimensions, but the dynamic light scattering provides information of the diffusion coefficients and hydrodynamic radii of the copolymer molecules.

3.3. Diffusion coefficient and hydrodynamic radius of the copolymer coils

The intensity–intensity time autocorrelation function of the scattered intensity in the self-beating mode can be related to the normalized first-order electric field time correlation function $g^{(1)}(t,q)$ as follows [11]:

$$G^{(2)}(t) = \langle I(0)I(t)\rangle = B[1 + \beta|g^{(1)}(t,q)|^2], \tag{4}$$

where β is a parameter depending on the coherence of the detection, t the delay time, and B the measured baseline.

For polydisperse small particles $g^{(1)}(t,q)$ is given by $g^{(1)}(t,q) = (\sum c_i M_i \exp(-\Gamma_i t))/(\sum c_i M_i)$, where the initial slope yields the z-average inverse decay constant:

$$\Gamma_z \equiv \left[-\frac{\mathrm{d}\ln g^{(1)}(t,q)}{\mathrm{d}t}\right]_{t\to 0} = \frac{\sum c_i M_i \Gamma_i}{\sum c_i M_i},$$

where c_i and M_i are the concentration and molecular weight of each component, respectively. In integral form $|g^{(1)}(t,q)|$ can be connected with the normalized characteristic linewidth distribution $G(\Gamma)$ by

$$|g^{(1)}(t,q)| = \int_0^{\infty} G(\Gamma)\,\mathrm{e}^{-\Gamma t}\,\mathrm{d}\Gamma. \tag{5}$$

Logarithmic correlation function can be expanded in a power series in terms of the delay time (cumulant analysis)

$$\ln g^{(1)}(t) = -\Gamma_1 t + (\Gamma_2/2!)t^2 - (\Gamma_3/3!)t^3 + \ldots, \tag{6}$$

where Γ_1, Γ_2, etc. are the first, second, etc. cumulants. The first cumulant can be calculated by equilibrium statistical thermodynamics and at low q related to the apparent diffusion coefficient $D : \Gamma_1 = Dq^2$. "Z-average particle size" can be found using well-known Stokes–Einstein relationship, which is a definition for a hydrodynamically effective sphere diameter $\langle d_\mathrm{h}\rangle$

$$\langle d_\mathrm{h}\rangle = \mathrm{k_B}T/3\pi\eta D, \tag{7}$$

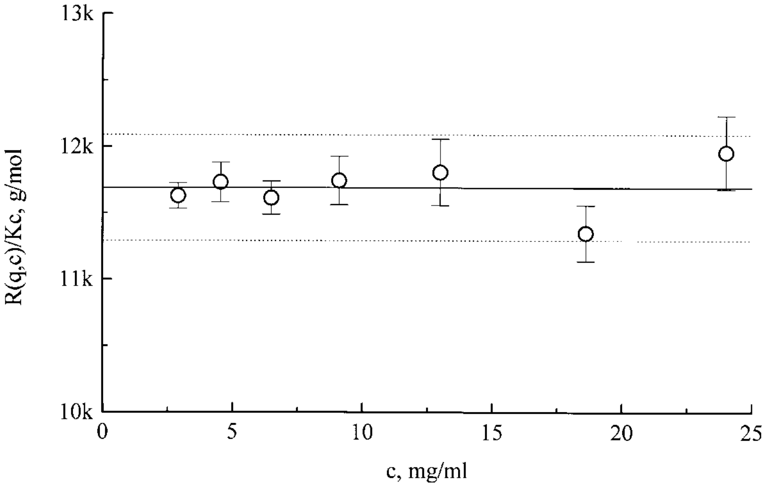

Fig. 3. Reduced excess absolute time-averaged scattering intensity $R(q,c)/Kc$ vs. concentration. Solid line is the average value of the molecular weight of the (VCL–VI)-copolymer studied. Dotted lines indicate the limits of maximal errors for M_w obtained.

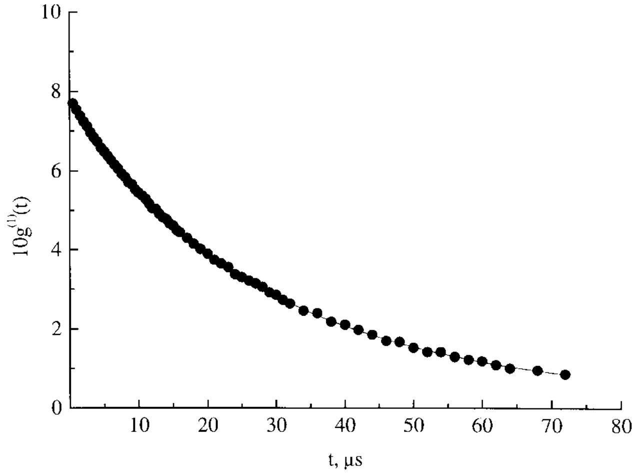

Fig. 4. Typical field–field correlation function $g^{(1)}(t)$ of the (VCL–VI)-copolymer aqueous solution at 90°.

where η is the viscosity of the solvent, T the absolute temperature, k_B the Boltzmann's constant. If the sample is polydisperse, the value $2\Gamma_2/\Gamma_1^2 = \mathrm{PI}$, the polydispersity index (the width of the distribution), which is the dimensionless measure of the broadness of the distribution. The polydispersity index is related with the polydispersity M_w/M_n as follows:

$$M_w/M_n = 1 + \mathrm{PI}. \quad (8)$$

The typical field–field correlation function at 90° is presented in Fig. 4. It was found out that the field–field correlation function is a single-exponential for the polymer studied within the angular range from 30 to 150°. The data were processed by the cumulant analysis, using the PCS software program (version 1.35) supplied by Malvern Instruments Ltd. The average polydispersity index was PI = 0.115 ± 0.020. Fig. 5(a) shows angular independence of the measured hydrodynamic diameter of average value $\langle d_h \rangle = (8.03 \pm 0.08)$ nm (the corresponding diffusion coefficient was $6 \times 10^{-7}\ \mathrm{cm^2\ s^{-1}}$). Fig. 5(b) shows that $\langle d_h \rangle$.does not depend on concentration. These results confirm once more the evidence of conditions (2).

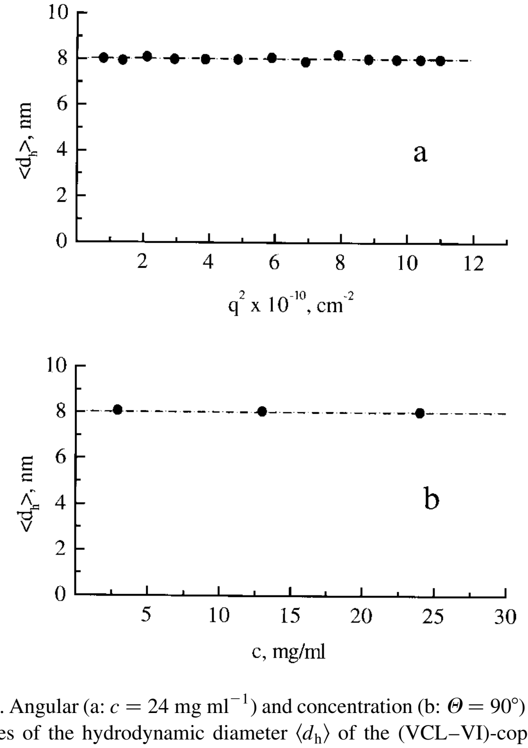

Fig. 5. Angular (a: $c = 24\ \mathrm{mg\ ml^{-1}}$) and concentration (b: $\Theta = 90°$) dependencies of the hydrodynamic diameter $\langle d_h \rangle$ of the (VCL–VI)-copolymer solutions. Dotted lines indicate the average value of hydrodynamic diameter $\langle d_h \rangle$.

To rationalize the molecular-weight characteristics of the prepared copolymer given above one should bear in mind the conditions of its synthesis and purification (see Section 2.2.1). Apparently, the high concentration of the initiator taken for the copolymer synthesis (see Section 2.2.1) has led to its low molecular weight (11 700 g mol^{-1}). In combination with dialysis removing oligomeric fractions of $M_w < 6000\ \mathrm{g\ mol^{-1}}$ it resulted in the (VCL–VI)-copolymer preparation of rather narrow molecular weight distribution ($M_w/M_n = 1.1$). One should point out that similar values of M_w/M_n of 1.07–1.10 were obtained for homopolymer, poly(*N*-vinylcaprolactam) produced by radical polymerization in benzene followed by precipitation in diethyl ether and fractionation by thermoprecipitation at 80 °C from acetone/water mixture [12].

Further, we applied immobilized metal affinity chromatography (IMAC) for the separation of the copolymer according to the molar share of its VI-units in order to prepare its fractions more homogeneous also in sense of their chemical composition.

3.4. Separation of (VCL–VI)-copolymer using immobilized metal affinity chromatography and thermal precipitation

Immobilized metal affinity chromatography was used widely for separation and purification of proteins carrying histidine, cysteine and tryptophan residues capable of chelating the transition metal ions immobilized on chromatographic carriers [8]. Interaction of thermosensitive VI-copolymers with Cu^{2+}, Co^{2+}, Ni^{2+} and Zn^{2+}-ions in solution was previously studied in our laboratory. Two to three imidazole residues of the copolymer bind to a metal ion in solution while the VI-unit molar fraction in the studied copolymer was 26% [2]. The question however arises whether the (VCL–VI)-copolymers with lower imidazole content (11 mol%) would bind to the immobilized metal ions exhibiting more sterical hindrance towards the imidazole ligands.

The frontal chromatogram of (VCL–VI)-copolymer on Cu^{2+}-IDA-Sepharose is shown in Fig. 6. Obviously, the synthesized copolymer contained some adsorbing as well as some non-adsorbing fractions. Judging by the absorbance ($\lambda = 220$ nm) of the joined non-adsorbing fractions (see Section 2.2.2) one could presume the content of ca. 5 mg or 11% of the applied copolymer therein. After those

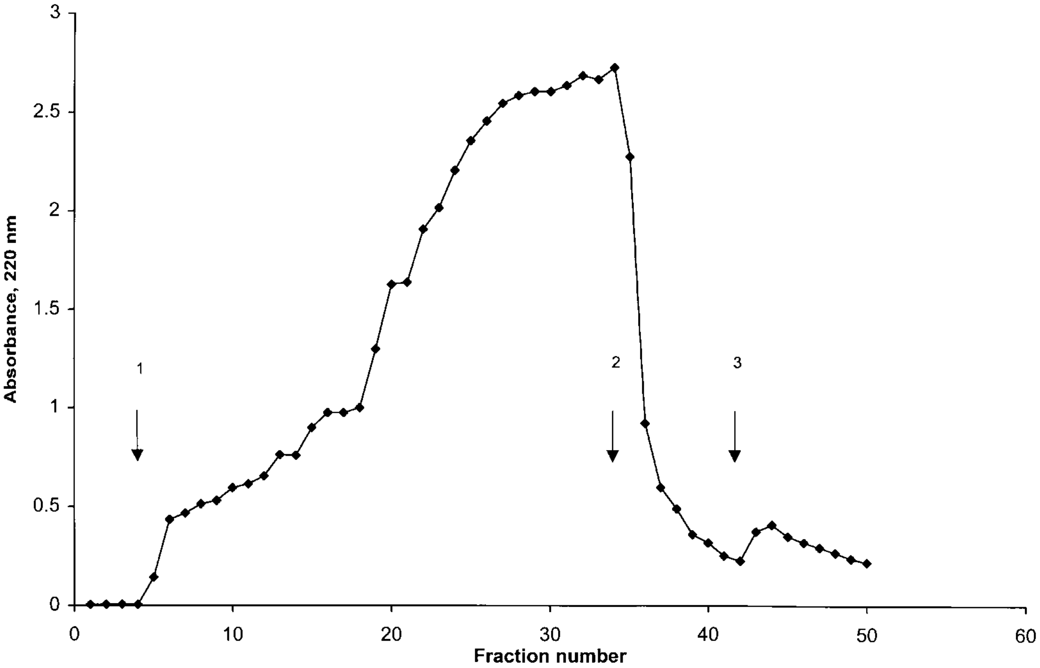

Fig. 6. Breakthrough curve of (VCL–VI)-copolymer (1 mg ml^{-1} solution in distilled water) on a Cu^{2+}-IDA-Sepharose column (1.6 ml) at a flow rate of 0.5 ml min^{-1}. Fractions of 1.6 ml were collected, diluted twofold by distilled water and monitored at 220 nm (amide group absorption). Arrow 1: application of the copolymer solution, arrow 2: washing with distilled water, and arrow 3: washing with 0.2 M NaCl in 10 mM Na-phosphate, pH 7.0.

fractions were dialyzed and freeze-dried, their actual mass turned out to be only 2 mg. Apparently, some lower molecular weight chains of the copolymer did not interact with the sorbent and were removed by dialysis. The higher molecular weight fraction of the non-interacting copolymer was isolated and studied as described below.

The specific binding capacity of the affinity sorbent was estimated as 15 mg copolymer/ml gel. This corresponds to ca. 10 μmol bound vinylimidazole group per ml gel. Taking into account the Cu^{2+}-binding capacity of the used IDA-Sepharose (8 μmol ml^{-1}) one can very roughly evaluate that ca. 1.2 VI-units, as average, bind to the immobilized Cu^{2+} -ion, i.e. somewhat less than what bind to Cu^{2+}-ions in solution. This may be explained by the smaller number of spare valences displayed by the immobilized Cu^{2+}-ions. Such evaluation neglects, however, by the conformational state of the adsorbed macromolecules: some non-interacting imidazoles might be located on the loops and tails of the adsorbed copolymer, while some immobilized Cu^{2+}-ions might be inaccessible for the pendant imidazole groups.

After the weakly bound portions of the copolymer were removed by washing with distilled water and 0.2 M sodium chloride in 10 mM sodium phosphate, pH 7.0 (see Fig. 6), the specifically adsorbed copolymer was eluted from the column with 0.02 M EDTA in 0.15 M NaCl, pH 8.0.

Separation of the desorbed copolymer from Cu^{2+}-ions, EDTA and the salts may be done by thermal precipitation of the copolymer. The success of the copolymer isolation strongly depends, however, on its concentration and pH. At the copolymer concentration of 10 mg ml^{-1} and pH 8.5 (most imidazoles in the monomer units are deprotonated, $pK_b \approx 7$) its preparative isolation is feasible at sodium chloride concentration of ca. 0.3 M at 48°C. In these conditions the fraction of the high-affinity (VCL–VI)-copolymer was obtained with the yield of ca. 60% from the parent copolymer sample after its repeated precipitation. Its analysis by ^1H NMR has shown a spectrum similar to that shown in Fig. 1 while the content of VI-units was 8.5 mol%. In contrast, the non-adsorbing fraction of the copolymer (Fig. 1(b)) contains much less imidazole monomer units (5 mol%) compared to the purified copolymer (8.5 mol%) and the parent copolymer (11 mol%). Moreover, the relevant resonance band of the imidazole ring protons (the chemical shift of 6.95 ppm) is poorly resolved and is somewhat wider than that of the purified copolymer. This testifies to the longer relaxation times and restricted mobility of these protons. Although it is hard to presume what structural features underlie this phenomenon, it is likely that a certain portion of vinyl imidazole units in the copolymer suffers from constrained reactivity, so that the relevant chains could be separated from the rest of copolymer by means of IMAC.

Thermal precipitation of the studied polymeric reagents is a key property allowing their function and purification. We applied static light scattering technique for the study of precipitation transitions in the aqueous solution of the parent and purified copolymers. It is well known [13] that scattered intensity is proportional to the spatial fluctuations of concentrations $I \sim \langle \Delta c^2 \rangle$ and one can expect that this value could be extremely sensitive to attainment of the cloud point of polymer.

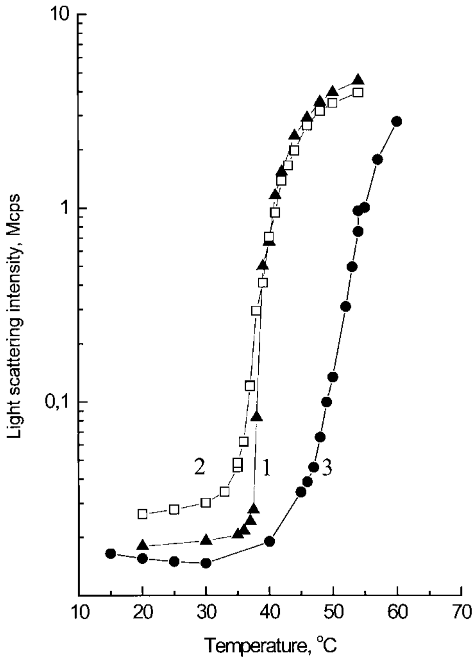

Fig. 7. Intensity of static light scattering by 0.15 mg ml^{-1} copolymer solutions as a function of temperature. 1 — the parent (VCL–VI)-copolymer; 2 — the copolymer purified by IMAC and thermal precipitation; 3 — the copolymer fraction remained soluble in the course of thermal precipitation at 48°C. All samples were dissolved in 10 mM Na-phosphate, 0.3 M NaCl, pH 8.5.

Fig. 7 shows the light scattering intensities registered with different fractions of (VCL–VI)-copolymer as a function of temperature. The phase transitions of the parent copolymer (1) and its high affinity precipitated fraction (2) occur under similar conditions (cloud point of ca. 38°C), whereas the fraction that remained in solution in the course of thermal precipitation at 48°C (3, see Section 2.2.2) exhibit a much higher cloud point.

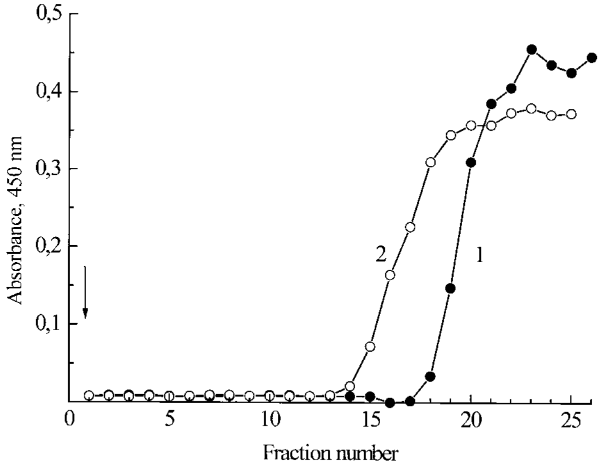

Fig. 8. Frontal re-chromatography of the high-affinity, thermally precipitated (VCL–VI)-copolymer (5 mg ml^{-1}) on Cu^{2+}-IDA-Sepharose column (1 × 5.5 cm). Eluent: 10 mM Na-phosphate, containing 0.15 M NaCl, pH 7.0. Flow rate: 0.25 ml min^{-1} (1); and 1 ml min^{-1} (2). Arrow indicates the application of the copolymer to the column. Detection of the copolymer in the eluate was performed by turbidity measurements at 48°C (see Section 2.2).

Thus, IMAC combined with thermal precipitation allows isolation of the (VCL–VI)-copolymer fraction with well-defined chemical composition and phase transition properties. Besides, as discussed in Sections 3.2 and 3.3, the copolymer has a narrow molecular weight distribution. This set of properties makes the thus prepared polymeric reagent a convenient tool for studies of metal–chelate complex formation between proteins and synthetic polymers. The hydrodynamic radius of the copolymer (4 nm) is ca. fourfold lower than the contour length of its chain and may be compared to the dimensions of protein globules. The number of pendant imidazole groups in the chain (ca. 8) is enough for its interaction with two or more metal ions. Thus, depending on the conditions, one may conceive different modes of polymer–protein interactions: binding of several protein globules to a single polymer chain in its dilute solution or bridging the globules by the excessive amount of the copolymer at higher concentrations.

Affinity frontal re-chromatography of the purified (VCL–VI)-copolymer carried out in the conditions typical of the displacement mode of IMAC [14] was carried out as shown in Fig. 8. Obviously, the resultant breakthrough curves are much more sharp compared to the parent copolymer (see Fig. 6). This may be ascribed, first, to the high molecular compactness of the solute and, secondly, to its high diffusivity, which allows its fast penetration into pores of the gel followed by affinity binding. Although the shape of the breakthrough curve shows a dependence on the flow rate, the dynamic adsorption capacity of the copolymer is still high enough even at flow rate of 1 ml min^{-1}, which is much higher than those commonly used in displacement chromatography on analytical columns [14]. The fast mass-transfer of the metal-chelating copolymer in the affinity sorbent seems promising for its function as a chromatographic displacer in IMAC [3].

We have obtained a detailed picture of molecular properties of the new functional thermosensitive copolymer useful as a reagent for metal–chelate affinity precipitation and displacement chromatography of proteins. The combination of immobilized metal affinity chromatography and thermal precipitation allows isolation of the high affinity copolymer fraction with defined chemical composition and phase-transfer properties. These characteristics of the polymeric reagent together with its narrow molecular weight distribution make it a convenient tool for study of the metal–chelate complex formation between proteins and synthetic polymers including modeling of the relevant processes.

Acknowledgements

This research was supported by Wenner-Gren Foundations, Swedish Competence Center for Bioseparation and

EC project within 4 FP in the Industrial and Materials Technologies Programme (contract BRPR — CT98-0652) and INTAS project 97-1676. The Knut and Alice Wallenberg Foundation is acknowledged for providing light scattering machine to the Department of Biotechnology where the work had been carried out.

References

[1] Galaev IYu, Kumar A, Agarwal R, Gupta MN. Appl Biochem Biotechnol 1997;68:121.

[2] Kumar A, Galaev IYu, Mattiasson B. Bioseparation 1999;7:185.

[3] Freitag R, Vogt S, Moedler M. Biotechnol Prog 1999;15:573.

[4] Kumar A, Galaev IYu, Mattiasson B. Biotechnol Bioengng 1998;59:695.

[5] Kumar A, Galaev IYu, Mattiasson B. Bioseparation 1998;7:129.

[6] Pietruzska N, Galaev IYu, Kumar A, Brzozowski ZK, Mattiasson B. Biotechnol Prog 2000;16:408.

[7] Lau ACW, Wu C. Macromolecules 1999;32:581.

[8] Porath J, Olin B. Biochemistry 1983;22:1621.

[9] Bender Th, Lewis RJ, Pecora R. Macromolecules 1986;19:244.

[10] Zimm BH. J Chem Phys 1948;16:1093.

[11] Brown W, editor. Dynamic light scattering Oxford: Clarendon, 1993.

[12] Eisele M, Burchard W. Makromol Chem 1990;191:169.

[13] Kazakov SV, Chernova NI. Static and dynamic light scattering in phase-separating systems. In: Pike ER, Abbiss JB, editors. Light scattering and photon correlation spectroscopy, NATO ASI series. The Netherlands: Kluwer Academic, 1997. p. 401–22.

[14] Kim YJ, Cramer SM. J Chromatogr 1991;549:88.