Stimuli-Responsive Polymers Based on L-Phenylalanine Residues: Protonation Thermodynamics of Free Polymers and Cross-Linked Hydrogels

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ABSTRACT: Vinyl polymers carrying L-phenylalanine residues were synthesized in the free and in the cross-linked hydrogel form, as homo- and copolymers with *N*-isopropylacrylamide (NIPAAm). The protonation reaction thermodynamics of the COO^- group were studied in aqueous media, at different temperatures and at increased concentrations of sodium chloride, mainly by potentiometry and solution calorimetry. The soluble polymer, namely poly(*N*-acryloyl-L-phenylalanine), and its copolymers with low NIPAAm content displayed characteristic features. Their basicity constants (log *K*), as well as the enthalpy ($-\Delta H°$) changes in relation to α (degree of protonation) showed an abrupt drop at $\alpha = 0.5$. This was ascribed to the formation of hydrogen bonds between the protonated and the ionized neighboring COO^- groups. The process was driven by the side-chain aromatic rings that improved hydrophobic interactions. The entropy ($\Delta S°$) changes sharply increased as a result of the increased macromolecular conformational freedom and the release of water molecules surrounding the hydrophilic groups of the polymer. The corresponding cross-linked polymers formed hydrogels that were responsive to pH, temperature, and ionic strength. The two hydrogels, P9 (homopolymer with 9 mol% cross-links) and CP2 (copolymer with 90 mol% of NIPAAm and 2 mol% cross-links), were characterized for their pH- and temperature-responsive behavior by equilibrium and oscillatory swelling studies. They demonstrated a strong pH-dependent volume phase-transition and an unusual sodium chloride phase-transition phenomenon. Moreover, the hydrogel CP2 exhibited a temperature-dependent volume phase-transition (LCST, lower critical solution temperature) behavior in aqueous solution, where the LCST decreased by lowering the pH. It was nontoxic against the RAW264 cell line.

Introduction

In previous papers of recent years, several stimuli-responsive polymers were synthesized and studied from a thermodynamic point of view.[1−7] The polymers contained, besides the carboxyl group, amido and isopropyl groups structurally related to the poly(*N*-isopropylacrylamide) (PNIPAAm).[8] The latter is a well-known nonionic polymer that exhibits a sharp, thermoreversible phase-transition (lower critical solution temperature, LCST) behavior at 32°C in aqueous solution.[9] The possibility of tuning the LCST of PNIPAAm by incorporating comonomers with different hydrophilic characters is an interesting feature to obtain "intelligent" polymer systems for many biomedical and pharmaceutical applications,[10] including controlled delivery of drugs,[11−14] molecular separation,[15] tissue culture substrates,[16] and materials for improved biocompatibility.[17] Among these "intelligent" materials, the pH- and temperature-responsive light-cross-linked polymers (hydrogels) are the most widely investigated.[8,18−26]

Previously, we studied the protonation thermodynamics of a series of (meth-) acrylate polymers containing α-amino acid residues (L-valine and L-leucine) that showed a close structure to PNIPAAm and had a carboxyl acid group in the monomer unit.[1−4,27,28] The polymers were studied in aqueous media as homo- and copolymers with NIPAAm, either in the soluble and hydrogel forms.[29] Recently, a poly(ampholyte) polymer, containing L-histidine residues was also reported.[30] The latter polymer was studied because its LCST may be changed by tuning pH at low or high values, i.e., below and above the isoelectric point.[31,32]

The purpose of this work is to synthesize a novel vinyl monomer containing L-phenylalanine residues. From this, a series of acrylate polymers (Chart 1) and copolymers with NIPAAm were obtained in the free and in the cross-linked hydrogel form.

L-Phenylalanine is an essential amino acid and tyrosine precursor that works on the central nervous system as an anti-depressant and mood elevator.[33] The phenyl group of the phenylalanine residue should improve a rather large hydrophobic domain in which the cohesive forces play a major role during the conformational transition of the macromolecules. Morcellet et al. reported that the methacrylate analogue, poly(*N*-methacryloyl-L-phenylalanine), showed a pH-induced conformational transition due to the hydrophobic interactions between the aromatic side chains.[34]

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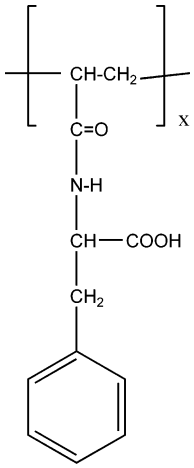
Chart 1. Structure of the Monomer Unit of Poly(Phe)



The solution properties of this polymer, as well as of the polymers with L-valine and L-leucine residues previously reported, are those of polyelectrolytes with a compact conformation in water due to hydrophobic forces between phenyl or isopropyl groups.[1,3,4,27,28] These attractive forces outweigh the repulsive electrostatic interactions between the negatively charged COO^- groups when the polymer reaches a critical degree of protonation, α. This critical α value strongly depends on the nature of the α-amino acid residues and on the kind of acrylate or methacrylate polymer.[1,3,27] The magnitude of the hydrophobic character may be revealed also by calorimetric data. Thus, the thermodynamic study is of special interest because it enables a correlation to be established between the increasing hydrophobicity and the appearance of compact structure.[34,35] Moreover, the results of this preliminary investigation should be of interest in designing slightly cross-linked polymer networks (hydrogels) that should be responsive to different external stimuli. We evaluated the thermodynamic functions of poly(Phe) and described the protonation mechanism of the polymer in the free and in the cross-linked form and the corresponding copolymers with NIPAAm by using mainly potentiometric and solution calorimetric techniques. Moreover, the swelling behavior of the hydrogels with respect to ionic strength, pH, and temperature was investigated. A copolymer hydrogel, which is responsive to both pH and temperature, would be able to respond to conditions where both variables are coupled.

Experimental Section

Materials. L-Phenylalanine (99%), acryloyl chloride (97%), α,α′-azobisisobutironitrile (AIBN), 2,6-di-*tert*-butyl-*p*-cresol, ammonium peroxodisulfate (APS, 98%), triethylamine (TEA, 99%), and *N*,*N*′-ethylene-bis-acrylamide (EBA, 98%) were purchased from Fluka Co. *N*-Isopropylacrylamide (NIPAAm, 97%) was purchased from Aldrich Co.

Syntheses. *Monomer.* *N*-Acryloyl-L-phenylalanine (Phe) was obtained by the usual synthetic route to introduce the vinyl group in the monomers.[1,3,4,28,30,36] To a well-stirred aqueous solution of L-phenylalanine (54.5 g, 0.33 mol), sodium hydroxide (26.7 g, 0.67 mol) and 2,6-di-*tert*-butyl-*p*-cresol (0.10 g) in twice-distilled water (100 mL) was added dropwise acryloyl chloride (29.3 g, 0.31 mol) over a 30 min period while the reaction mixture was kept below 0 °C by external ice-bath cooling. After the addition was completed, the stirring was continued for additional 1 h with the temperature raising to room temperature. The mixture was acidified to pH 2 with concentrated hydrochloric acid (28 mL). A voluminous white product was separated; it was filtered with suction and purified by recrystallization from water. The yield of the dry product was 38 g (56%). The compound was characterized by spectroscopy (proton NMR and infrared spectra), and elemental analysis.

Polymers. Poly(*N*-acryloyl-L-phenylalanine) [poly(Phe)] and poly(*N*-acryloyl-L-phenylalanine-*co*-*N*-isopropylacrylamide) [poly(Phe-*co*-NIPAAm)] at different Phe/NIPAAm molar ratios were prepared by a radical polymerization of the corresponding monomers.[3,4,29,30] The poly(Phe) was prepared in two different solvents, in methanol and in ethanol/benzene (1/1) mixture, dissolving 2.00 g of Phe in 20 mL of a well-degassed and nitrogen-purged solution. To each solution, 30 mg of recrystallized (from methanol) AIBN was added. The mixture was allowed to react in a thermostated water bath at 65 °C for 24 h. In the methanol solution, the polymer started to precipitate after 30 min. It was washed with fresh methanol and treated with ethyl ether three times. The polymer from the ethanol/benzene mixture was precipitated in ethyl ether (200 mL), under magnetic stirring. The voluminous white polymer was repeatedly washed with fresh ethyl ether and dried in vacuo. The yield was 1.75 g (87%) and 1.10 g (55%) for the poly(Phe)s, respectively, from methanol and an ethanol/benzene mixture. Both compounds were characterized by proton NMR and infrared spectra, along with elemental analysis. The poly(Phe-*co*-NIPAAm)s were obtained with a similar procedure at three different Phe/NIPAAm molar ratios (Table 1). In all cases, the Phe and the NIPAAm monomers were dissolved in ethanol/benzene (1:1) solution. The mixture was purged with nitrogen and was allowed to react at 60 °C in a thermostated water bath for 24 h. The copolymer was precipitated in ethyl ether (300 mL), washed with fresh ether, and dried in vacuo. The amount of COOH groups incorporated into the compounds was determined by potentiometric titrations.

Cross-Linked Polymers (Hydrogels). Two polymers were obtained with different amounts of cross-linking *N*,*N*′-ethylene-bis-acrylamide (EBA).[30] The first one (P9) was a poly(Phe) containing 9 mol% of EBA, and the second one (CP2) was a poly(Phe-*co*-NIPAAm) with a NIPAAm/Phe molar ratio of 10 and cross-linked with 2 mol% of EBA. The synthesis was carried out at room temperature in a glass tube and under nitrogen atmosphere by the following procedure. *Hydrogel P9* (14.5 wt% monomer concentration): the monomer Phe (2.00 g, 9.12 mmol) was dissolved in twice-distilled water (15 mL) containing TEA (93 mg) and EBA (156 mg, 0.91 mmol). *Hydrogel CP2* (14 wt% monomer concentration): the monomer Phe (0.57 g, 2.58 mmol) was dissolved in twice-distilled water (27 mL) containing TEA (275 mg) and EBA (97 mg, 0.56 mmol). Both mixtures were repeatedly flushed with nitrogen, and then APS (20 mg) was added. The reaction mixtures were kept at room temperature (r.t.) for 24 h even if the gelation was observed within 15 min. Afterward, the hydrogels were removed, thoroughly washed with twice-distilled water for 1 week, and then slowly dried at r.t. up to constant weight in a desiccating cabinet. Unlike the hydrogel CP2, which was cut in small disks, the hydrogel P9 was treated with acetone to give a fine powder for potentiometric analysis.

Spectroscopic Measurements. Proton NMR spectra of the monomer and the polymers dissolved in DMSO-d_6 were recorded on a Bruker AC200 spectrometer using tetramethylsilane as internal reference. The FT-IR spectra of the samples were recorded on a FTS 6000 Biorad spectrophotometer.

Molecular Characterization. The molecular characterization of the poly(Phe) homopolymer and of the poly(Phe-*co*-NIPAAm) copolymer (*co*-2) was performed by a multi-angle laser light scattering (MALS) Dawn DSP−F photometer from Wyatt (Santa Barbara, CA). As the *co*-2 did not elute at all from the usual aqueous size exclusion chromatography (SEC) columns, both the homopolymer and the copolymer were characterized by means of a hydrodynamic chromatography (HC) method. In such a system, the classical SEC column was replaced with a long capillary tube. Obviously, the separation performance of this HC-MALS system was not adeguate in obtaining the whole molar mass distribution but sufficient in

Table 1. Feed Composition for the Copolymerization of NIPAAm with Phe

	Phe		NIPAAm					COOH group content (wt%)[b]	
compd	g	mmol	g	mmol	AIBN mg	vol.[a] mL	yield g	theor	exp
co-10	2.04	9.30	0.11	0.94	37	25	1.34	94.9	83.4
co-2	2.04	9.30	0.55	4.71	43	25	2.07	78.8	71.8
co-0.5	2.14	9.76	2.06	17.4	53	40	2.89	51.3	46.5

[a] Ethanol/benzene (1:1) solution; [b] From the acid−base titration.

obtaining a reliable weight-average molar mass, M_w, value of the polymers. The HC-MALS experimental conditions were the following: 0.2 M NaCl + 0.1 M Tris buffer pH 8.0 as mobile phase, 35 °C temperature, 0.4 mL/min flow rate, and 50 μL injection volume. The wavelength of the MALS laser was 632.8 nm. The light scattering signal was simultaneously detected at 15 scattering angles ranging in the solvent from 14.5° to 151.3°. The calibration constant was calculated using toluene as standard assuming a Rayleigh Factor of 1.406×10^{-5} cm^{-1}. The angular normalization was performed by measuring the scattering intensity of a concentrated solution of a BSA globular protein in the mobile phase assumed to act as an isotropic scatterer. The MALS photometer was described in detail elsewhere.[37,38] The refractive index increment, d*n*/d*c*, of the poly(Phe) and of the *co*-2, with respect to the used solvent, was measured by a KMX-16 differential refractometer from LDC Milton Roy (Riviera Beach, FL). The d*n*/d*c* values for poly(Phe) and for *co*-2 were, respectively, 0.215 and 0.202 mL/g.

Scanning Electron Microscopy (SEM). The morphology of the hydrogels P9 and CP2 was examined by the XL20 Philips scanning electron microscopy. Samples were mounted on SEM stubs with Leit-C Conductive Carbon Cement and sputtered with 20 nm gold by a Balzers Med 01 sputter-coater. The morphology of the hydrogels was analyzed at 20 kV and at different magnifications.

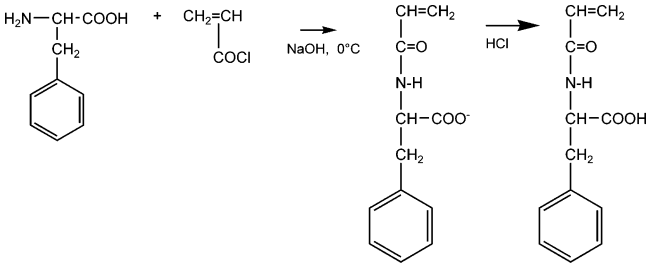
Potentiometric Measurements. Potentiometric titrations were performed according to a previously described procedure.[30] The measurements were carried out in aqueous solution at 25 °C by using a TitraLab 90 titration system from Radiometer Analytical. TitraLab 90 consists of three components: the powerful Titration Manager (TIM900), the high-precision autoburet, and a convenient sample stand (SAM7). The TimTalk 9, Windows-based software, was used in connection with the TIM900 Titration Manager for remote control. The titrations of the compounds were performed in the thermostated glass cell filled with 100 mL of 0.15 M NaCl in which a weighed amount of solid material (monomer, 0.18−0.35 mmol; polymer, 0.17−0.21 mmol; copolymers, 0.20−0.34 mmol; hydrogel, 0.19−0.26 mmol) and a measured amount of standard sodium hydroxide solution were dispersed by magnetic stirring. A presaturated nitrogen stream was maintained over the surface of the solution to avoid CO_2 contamination. Unlike the monomer, which was soluble over the whole range of pH, the forward titrations of the equilibrated alkaline solution containing the polymer, the copolymer, or the hydrogel were performed with standard 0.1 M hydrochloric acid solution at the equilibration time of 300 s for each titration step (0.04 mL). The backward titrations with standard 0.1 M NaOH solution showed rielable results. The endpoint of the potentiometric titration curve was taken to calculate the excess of sodium hydroxide equivalents. The difference between the initial amount and the equivalents of hydroxide ions was attributed to the COOH proton equivalent of the polymer and the copolymers. The basicity constants (log *K*'s) of the monomer and the polymers were evaluated with the Superquad[39] and the ApparK[40] programs, respectively. The results of three replicates were averaged.

Calorimetric Measurements. Calorimetric titrations were performed following a previously reported procedure.[30] The measurements were carried out in aqueous solution at 25 °C with a Tronac 1250 titration calorimeter, operating in the isothermal mode by using a stainless steel reaction vessel of 25 mL capacity. The water bath was thermostated by means of a PTC 40 (precision temperature controller, from Tronac, Inc.). The aqueous solution (25 mL of 0.15 M NaCl), containing a weighed amount of compound (monomer, 0.20−0.25 mmol; polymer, 0.18−0.21 mmol; copolymer 0.17−0.22 mmol) and a measured volume of standard sodium hydroxide, was titrated with standard 0.1 M HCl solution with a constant BDR (buret delivery rate) of 0.0837 mL/min through a Gilmont buret. A chemical calibration (Tris/HCl) of the instrument and corrections for the heats of the titrant dilution were made before each titration experiment. All of the experiments were automatically controlled by the Thermal program from Tronac, Inc., which was renewed to operate through a NI-DAQ driver software in Windows, from National Instruments. The graphical programming language LabVIEW was used to create the application. The enthalpy changes were evaluated with the Fith program[40] by taking into account the dependence of the log *K*'s on the degree of protonation, α, for the polymer and the copolymers. The entropy change values were calculated by the relation $\Delta S° = (\Delta H° - \Delta G°)/T$ with $-\Delta G° = RT \ln K$. The results of at least two replicates were averaged.

Swelling Measurements. The equilibrium and oscillatory swelling studies were carried out on the two hydrogels P9 and CP2 as functions of environmental pH, temperature, and ionic strength of the bathing medium. The hydrogel CP2 had a dried thin-disk form. In the equilibrium swelling experiments, a dried CP2 sample was weighed (W_{dry}) and placed in a Stainer cell (100 μm pore size); then, the cell was immersed in a buffer solution of known pH and was allowed to swell to equilibrium at the desired pH value, temperature, or ionic strength. A similar procedure was followed for the hydrogel P9, even if in the form of a fine powder. The degree of swelling (DS) was monitored at intervals, until a plateau of DS/time plot was reached. Afterward, for swelling studies in buffer solutions (0.01 M Tris, 0.01 M phosphate, 0.01 M acetate, and 0.01 M hydrochloric acid) contained in a thermostated glass cell (100 mL of 0.15 M NaCl) and having pH values ranging from 3 to 9, the gel sample was placed in the proper medium and allowed to equilibrate for a further 24 h under stirring. The temperature changes were monitored by the Haake D8 thermostat. To study the effect of sodium chloride concentration, both the gels P9 and CP2 were swollen at high pHs in Tris buffer (0.01 M). Thus, weighed portions of NaCl were consecutively added to the solution to have the desired final concentration of the simple salt. The degree of swelling was taken after 24 h. In the oscillatory swelling experiments of CP2, the pH was kept constant at 4.80 by a buffer solution (0.01 M acetate in 0.15 M NaCl) and the temperature was varied between 25 and 35 °C. In all cases, the sample was removed from the bath at intervals, quickly blotted with tissue paper to remove any surface water, and weighed (wet weigh, W_{wet}). The procedure was repeated to a constant weight. The equilibrium degree of swelling (EDS) was calculated as: $EDS = (W_{wet} - W_{dry})/W_{dry}$.

Evaluation of Cytotoxicity. The evaluation of cytotoxicity was performed as previously reported.[30] The RAW264 cells, derived from murine leukemoic monocyte and having a macrophage-like morphology, were provided by Riken (Tsukuba, Japan). They were cultured in a minimum essential medium (Sigma) with 10% fetal bovine serum (FBS) and 1% nonessential amino acids (Invitrogen Life Technologies). The cells were harvested with a 0.25% trypsin solution containing 0.5 mM EDTA. The recovered cells were washed with the culture medium and suspended in the medium. The cell suspension was added to the well of the 24-well plate in the presence of the gel CP2 and was allowed to stand for 3 days at 37 °C. After the incubation, the cell number was counted by microscopy. The results were expressed as viability (%) relative to a control

Scheme 1. Synthetic Route to Phe



containing no polymer. The means (±SD) of four experiments, each one containing three replicates, are reported.

Results and Discussion

Synthesis and Characterization. *Monomer and Polymers*. The vinyl monomer *N*-acryloyl-L-phenylalanine (Phe) was obtained following the usual acylation reaction of acryloyl chloride with the L-phenylalanine, dissolved in aqueous solution and containing 2 equiv of sodium hydroxide (Scheme 1).[1,3,4,28,30,36]When the solution was neutralized with concentrated hydrochloric acid, a white solid formed. The crystallization from water gave rise to white crystals of analytical grade, as revealed by acid−base titrations, elemental, and spectroscopic analysis. It was freely soluble in water and in most organic solvents. The proton NMR (Figure 1) and the FT-IR spectra were consistent with the proposed structure. The main IR frequencies observed (COOH, 1711 cm^{-1}; amide I, 1650 cm^{-1}; −C=C−, 1596 $cm^{-1;}$ amide II, 1530 cm^{-1}) were lower than those found in the previously synthesized *N*-acryloyl derivatives containing L-valine, L-leucine, or L-histidine residues,[1,3,4,30] so the benzene ring provided a great inductive effect. The elemental analysis was found to be in satisfactory agreement: found (%) C, 65.2; N, 6.1; H, 6.2; $C_{12}H_{13}NO_3$; required C, 65.7; N, 6.4; H, 6.0. The monomer could be cold-stored for a long time and showed no sensitivity to the atmospheric moisture.

Unlike the monomer, which was soluble over the wide pH range investigated, the corresponding polymer and copolymers dissolved in aqueous solution only in the ionized form. They formed a cloudy solution when a stoichiometric amount of hydrochloric acid was added to neutralize the COO^- groups. The poly(*N*-acryloyl-L-phenylalanine), the corresponding copolymers with NIPAAm, and the hydrogels were synthesized by the free-radical polymerization of the corresponding monomers. The poly(Phe) was prepared in methanol and in an ethanol/benzene (1:1) mixture using AIBN as a radical initiator. Unlike the polymer obtained by the ethanol/benzene mixture, which was recovered from precipitation in ethyl ether, the compound from methanol precipitated out during the polymerization. In both cases, the purified compound showed a similar structure, as confirmed by spectroscopy (^{1}H NMR and FT-IR) and thermodynamic (potentiometry and solution calorimetry) data. The elemental analyses were in substantial agreement: found (%) C, 62.2; N, 5.9; H, 6.1; $(C_{12}H_{13}NO_3{\cdot}0.75H_2O)_x$; required C, 61.9; N, 6.0; H, 6.3. Moreover, the FT-IR spectra revealed the disappearance of the vinyl double bond at 1596 and 990 cm^{-1} and a slight wavenumber shift of the COOH to 1725 cm^{-1}, while the other IR frequencies remained almost the same as those in the monomer. The ^{1}H NMR spectra also showed the complete absence of the vinyl double bond and the presence of broad lines for backbone and side-chain resonances, consistent with the presence of a slowly tumbling macromolecular species in solution. The used HC-MALS method was able to recover a rielable weight-average molar mass, M_w, value of 47.6 kDa.

A series of copolymers with NIPAAm were prepared at different Phe/NIPAAm comonomer compositions. The composition was determined by potentiometric titrations of the carboxyl acid groups. Compared with the feed composition, a slight decreasing of the Phe unit content was revealed in all cases (Table 1). These results suggest that the comonomer feed ratios almost reflected the relative comonomer incorporation levels. The radical polymerization probably obeyed Bernouillian statistics, forming copolymers with a random distribution of COOH groups along the chain. This was confirmed by the thermodynamic data, as the increasing amounts of uncharged comonomer led to a decrease of the polyelectrolyte effect because the NIPAAm units shielded the charges.[41] The disappearance of the 1596 and 990 cm^{-1} very strong bands of the IR spectrum, in conjunction with the NMR results, indicated a total conversion of the monomers into the corresponding polymers and copolymers. In all cases, the copolymers of acidic monomers with NIPAAm showed a greater M_w value;[28] with the HC-MALS method, it was possible to recover a rielable weigh-average molar mass, M_w, of 146.1 kDa for the *co*-2 copolymer.

Two hydrogels were prepared to study the effect of the pH and the pH/temperature sensitivity on the swelling properties of P9 and CP2, respectively. The gel P9 was a homopolymer of Phe cross-linked with 9 mol% of EBA, while the gel CP2 was a copolymer of Phe and NIPAAm containing 10 mol% of Phe and cross-linked with 2 mol% of EBA. The water solution containing the monomers gelled at r.t. within 15 min, giving a soft and clear product. The two cross-linked polymers were treated in different ways. Unlike CP2, which was cut in small disks, the hydrogel P9 was treated with acetone to give a dispersed powder for the potentiometric study. In both cases, the samples were slowly dried at r.t. for 15 days and then under vacuum to a constant weight.

Protonation Study. The protonation behavior of the polymers in the free form and in the hydrogel form was studied at 25 °C in aqueous media (0.15 M NaCl) by potentiometric and solution calorimetric techniques.

Basicity Constants. The potentiometric titrations allowed for the evaluation of the carboxylic acid groups amount and of the basicity constants (log *K*). The values of the latter, evaluated at the degree of protonation $\alpha = 0.5$, are reported in Table 2, along with all the thermodynamic functions.

The peculiar polyelectrolyte behavior of the polymeric compounds are depicted in Figures 2 and 3. It is evident that the log *K* for the protonation of the Phe ionized carboxyl group is greater than that of the corresponding simple L-phenylalanine[42] but more than 2 orders of magnitude lower with respect to the poly(Phe) (Table 2). Moreover, the basicity of the acrylate monomer is lower compared to the methacrylate analogue,[34] while the corresponding polymers revealed the greatest basicity in the poly(acrylate) compound. This is the usual trend already observed in vinyl polymers containing α-amino acid residues.[1,3,4,30] In the present case, the log *K* of the monomer was lower than that of the other monomers previously studied and the basicity of the corresponding polymers was higher. The different in-

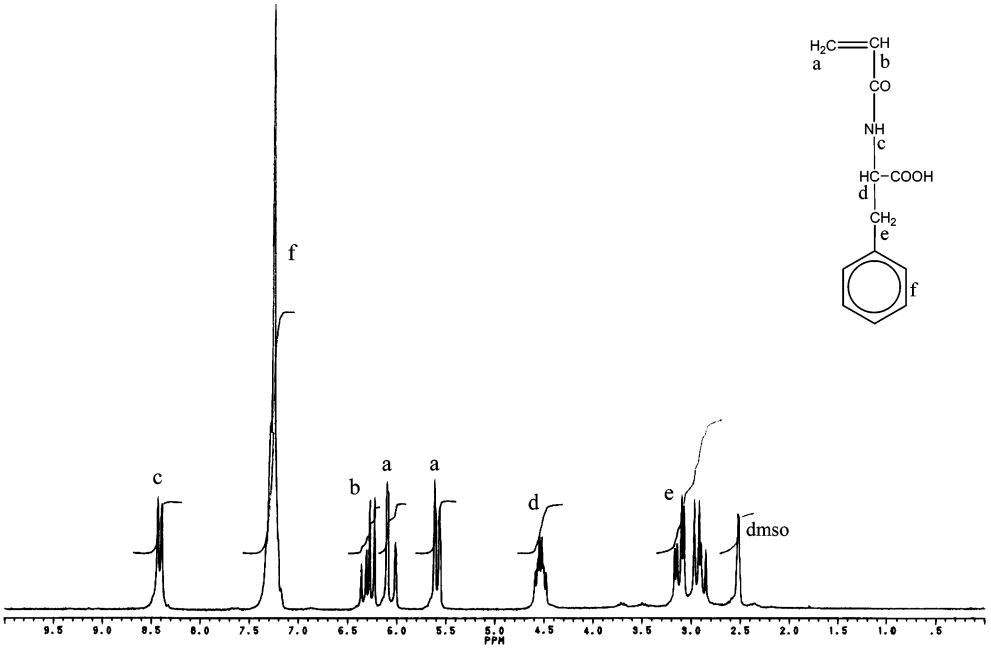


Figure 1. ^{1}H NMR spectrum of Phe in DMSO-d_6.

Table 2. Basicity Constants and Thermodynamic Functions for the Protonation of the COO$^-$ Group in Vinyl Compounds at 25 °C in 0.15 M NaCl (the Reported Values Were Evaluated at α = 0.5)[a]

compd	log *K*	−ΔG° kJ/mol	−ΔH° kJ/mol	ΔS° J/mol K
Phe	3.338 (2)	19.05 (1)	−1.67 (2)	69.5 (7)
poly(Phe)	5.45	31.1	2.6	95
co-10	5.11	29.2	1.6	93
co-2	4.96	28.3		
co-0.5	4.75	26.8	−15.6	142
P9	4.82	27.5		

[a] Values in parentheses are standard deviations.

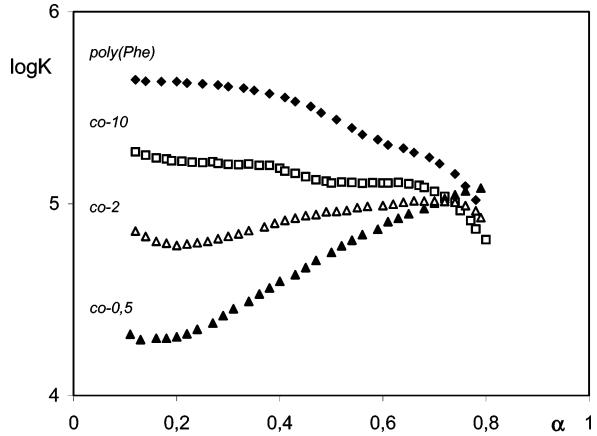


Figure 2. Basicity constants in relation to α for the protonation of the COO$^-$ group in the polymers at 25 °C in 0.15 M NaCl.

ductive effect was due to the presence of the phenyl group, which enabled a peculiar polyelectrolyte behavior in the polymers and in the copolymers, as shown in Figure 2. The log *K* trend of poly(Phe) in relation to α was almost flat during the first half of the COO$^-$ groups' protonation, beyond which a decreasing pattern was evidenced. A more strongly decreasing log *K*/α slope was observed at α > 0.7 according to Morcellet's work where a compact conformation of the polymer was shown to occur in pure water.[34] A similar behavior, even if lower, was observed also for *co*-10 and *co*-2. The shift of the latter compounds to lower log *K* was attributed to a shielding effect of the uncharged groups inserted between the adjacent monomer units.[3,30] The observed log *K*/α pattern was quite peculiar and very different from that observed in other vinyl polymers previously studied, where the log *K* linearly decreased with α in a wide range.[1,27,30] The only similarity was found with the copolymers containing a larger amount of NIPAAm moiety. In fact, the *co*-0.5 showed an increasing log *K*/α trend, similar to that observed in the copolymers of NIPAAm with L-valine or L-leucine residues.[3,4] These polymers, bearing the isopropyl group, hydrophobically interacted to form a micelle-like structure and to make easier the protonation of the remaining COO$^-$ groups in the more hydrophilic environment. These interactions led to an increased electrostaticity and, consequently, to a greater log *K*. This occurred in poly(Phe-*co*-NIPAAm)s containing a larger amount of NIPAAm content.

The linear log *K*/α pattern of P9 may be well described by the modified Henderson−Hasselbalch equation:[43] log $K = \log K^0 + (n - 1)\log[(1 - \alpha)/\alpha]$

In this case log $K^0 = 4.82$ and $n = 1.1$ in the wide α-range 0.10−0.63, beyond which a strong inflection was observed, as in the free polymer. The low *n* value seems to indicate a low polyelectrolyte behavior in the wide reported α-range. The uncharged cross-links act as shields, thus reducing electrostatic and inductive effects exerted by the phenyl groups.

At the critical value of α > 0.7 for poly(Phe), and slightly lower for *co*-10 and P9, the hydrophobic interactions between the aromatic side chains overcame the electrostatic repulsion of the negative ionized group and brought the monomer units close in a compact conformation. This caused a more difficult protonation of the residual COO$^-$ groups, lowering their basicity. Indeed, the opposite occurred for *co*-2 and *co*-0.5 or other copolymers with larger NIPAAm content (*co*-0.1).

Enthalpy and Entropy Changes. To elucidate the peculiarity of the poly(Phe) and its copolymers with NIPAAm, a calorimetric study was undertaken in

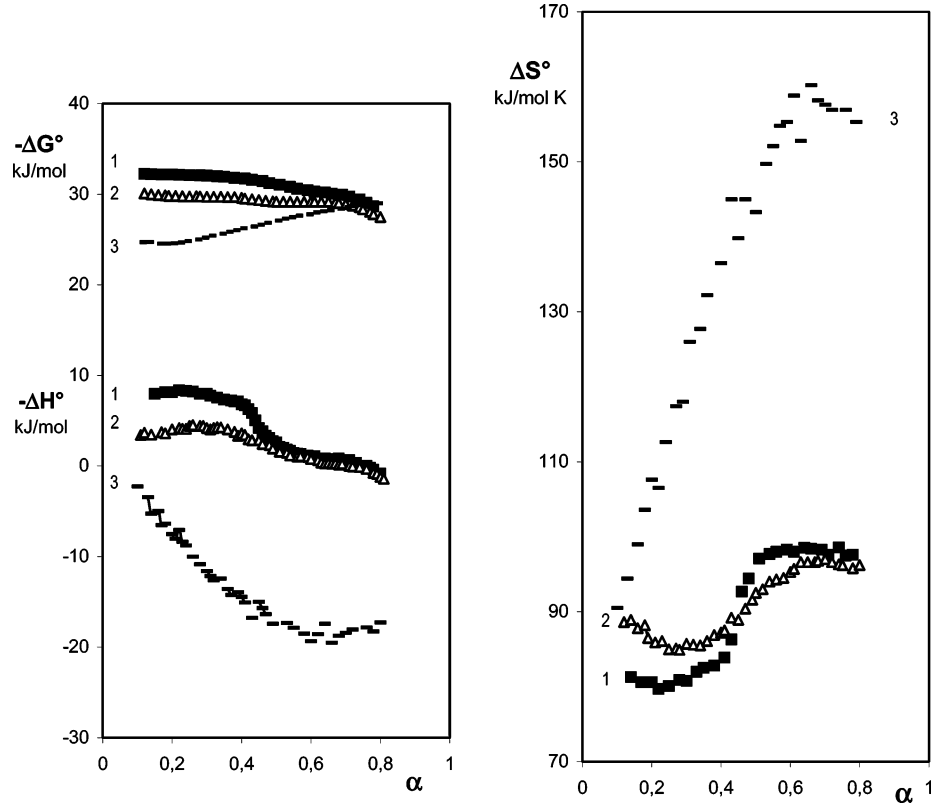


Figure 3. Free energy ($-\Delta G°$), enthalpy ($-\Delta H°$), and entropy ($\Delta S°$) changes in relation to α for the protonation of poly(Phe) (1), *co*-10 (2), and *co*-0.5 (3) in 0.15 M NaCl and 25 °C.

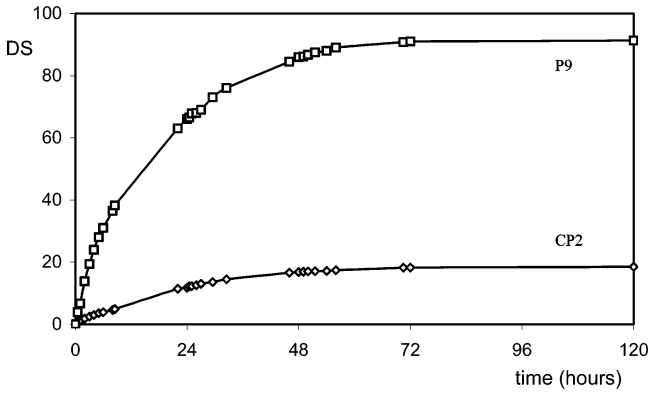


Figure 4. Swelling kinetics of the hydrogel P9 (pH 9.0) and CP2 (pH 8.5) in 0.15 M NaCl (0.01 M Tris/HCl buffer).

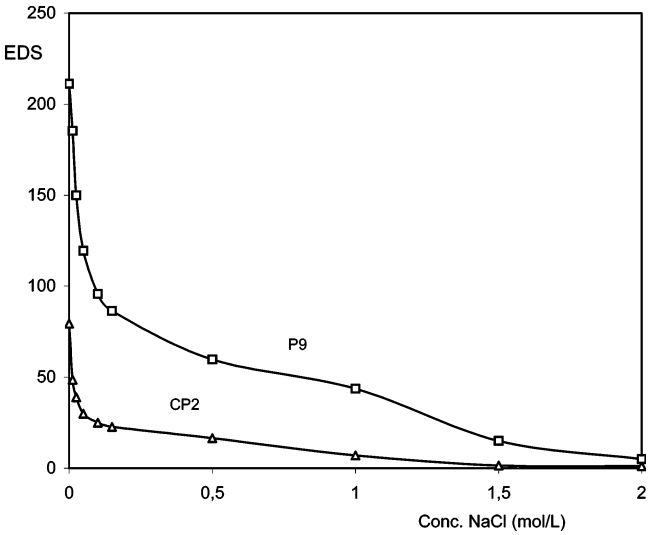


Figure 5. Equilibrium degree of swelling (EDS) in relation to the molar NaCl concentration of the hydrogels P9 (pH 7.4) and CP2 (pH 8.3) at 25 °C in 0.01 M Tris/HCl buffer.

solution. Figure 3 shows the enthalpy ($-\Delta H°$) and the entropy ($\Delta S°$) changes of the protonation of poly(Phe), *co*-10, and *co*-0.5. A characteristic S-shaped $-\Delta H°/\alpha$ and $\Delta S°/\alpha$ plot was observed only for poly(Phe) and *co*-10. At low α values, the protonation of poly(Phe) showed an exothermic enthalpy changes, unlike the endothermic one observed for the simple Phe. As α approached a value of 0.5, $-\Delta H°$ sharply decreased to reach a value close to that of the monomer at a high degree of protonation. Contemporaneously, the almost flat $\Delta S°$ trend at lower α suddenly increased at $\alpha = 0.5$ and then leveled off. This behavior, observed also for *co*-10 with lower entity, was not shown for *co*-0.5. In this latter case, the protonation became progressively endothermic due to the release of water molecules surrounding the hydrophilic amido and the carboxyl groups, even more than for the hydrophobic isopropyl groups of the NIPAAm moiety. This is the already reported behavior of the copolymers with a large amount of NIPAAm in copolymers containing COOH groups,[3] which lead the $\Delta S°$ value to increase during the charge neutralization, and is in line with the basicity constants data reported above. A convenient explanation of the S-shaped $-\Delta H°/\alpha$ plot may be found in the likely hypothesis of the H-bonded structure through a spatial approach. The protonation of the COO^- group in the monomer unit led to a H-bonded structure with a similar group close to it. The proton was thus shared between two adjacent monomer units via spatial approach. This implied an exothermic contribution until $\alpha = 0.5$ was reached. Beyond this value, the further protonation of the COO^- led the H-bonds to break, a process that is an endother-

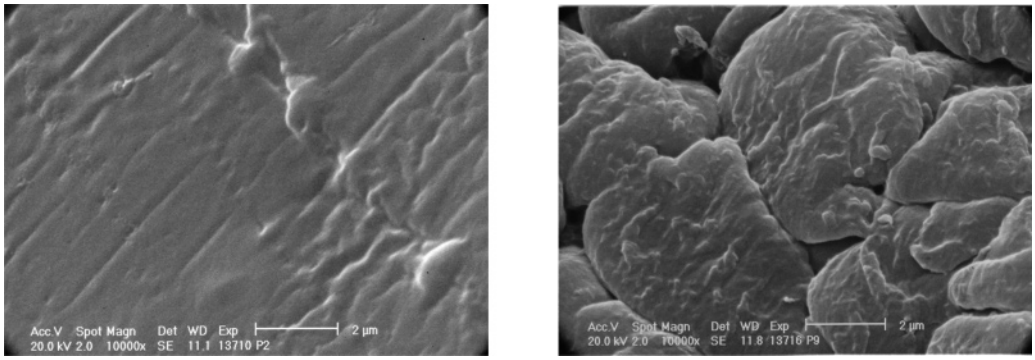


Figure 6. SEM images of CP2 (left) and P9 (right) hydrogel networks at magnification of 10 000×.

mic one, and increases the $\Delta S°$ for the more conformational freedom of the whole macromolecule. Besides the well-known poly(vinylamine) system,[44] this may be considered a further example of such spatial approaching systems that involve carboxyl groups, whereas we previously reported systems containing basic nitrogen in ring structures, such as piperazine[45] and imidazole[30] residues in the side chain of vinyl polymers.

Hydrogel Swelling. The two hydrogels P9 and CP2 showed different swelling kinetics. The degree of swelling (DS) of P9 was much greater than that of CP2. Although the latter showed a lower amount of cross-links, the observed difference may be attributed to the larger number of carboxyl groups in P9. These groups retain more water molecules because of their complete ionization at pH > log *K*. In Figure 4 is reported the swelling rate from the dried state at 25 °C of the two hydrogels P9 and CP2, respectively, at pH 9.0 and 8.5.

In both cases, the equilibrium degree of swelling (EDS) was reached within 3 days. This means that the water slowly diffused into the hydrogel network. It is worthwhile mentioning the peculiar behavior of P9 at high pHs, which showed an EDS value about five times larger than the more hydrophilic poly(ampholyte) hydrogel containing L-histidine residues.[30] Moreover, low concentrations of simple salts sharply increased the swelling capacity, reaching an EDS value higher than 200 in pure water (Figure 5). The EDS of P9 was always higher than that of CP2, and it decreased at increasing the sodium chloride concentration. The observed decrease of EDS was not regular because a sharper drop in swelling was observed at a concentration of simple salt greater than 1 M. A similar behavior was observed for CP2. In both cases, however, the higher concentration of simple salt led to a collapse of the hydrogels at their minimum hydration state. The evidence of this peculiar behavior was already reported by Hoffman, who studied uncharged hydrogels based only on NIPAAm.[46] Besides the charge state and the cross-linking density of P9 and CP2, the different behavior of the gels may be seen by the morphological analysis. Figure 6 shows the different morphologies of the two hydrogels, CP2 being more homogeneous while P9 forms an evident shrunk shape.

The H-bonding interactions between adjacent monomer units, which led to rather large hydrophobic domains in poly(Phe), or the greater cross-linking amount in P9, may be responsible of the different behavior.

Hydrogel P9. The swelling behavior of the hydrogel P9 was also studied at different pHs and temperatures. Unlike the negligible influence of the temperature, the

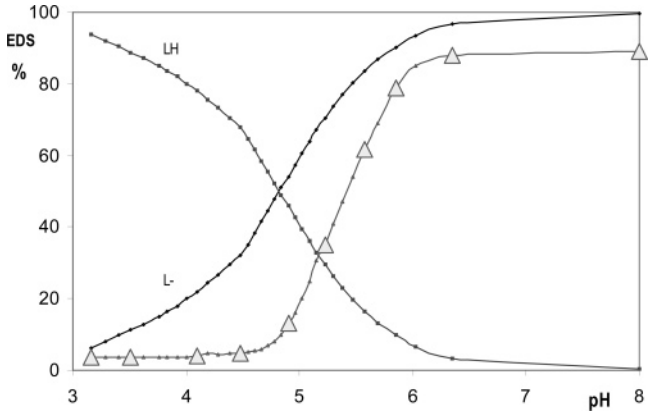


Figure 7. Equilibrium degree of swelling (EDS) and species distribution curves (%) in relation to pH of the gel P9 in 0.15 M NaCl at 25 °C.

EDS values in relation to pH evidenced that the hydrogel collapsed sharply at the critical pH of 4.8 (Figure 7). This pH value is close to the log *K* of P9 (Table 2, Figure 2). At pH > log *K*, the hydrogel was highly swollen owing to the ionization of the carboxyl acid groups on the polymer chain. In Figure 7, the distribution curves are superimposed to the EDS/pH relation. A swelling transition that occurred between pH 6 and 5 was due to the neutralization of the COO^- groups in the polymer. At pH = log *K*, both COOH/COO^- species (LH/L^- form) were present at the same concentration and the gel exhibited much lower swelling.

This means that the H-bonds between the protonated COOH and the adjacent COO^- groups formed in P9 in a way similar to that described for the corresponding soluble poly(Phe). The further protonation of the COO^- groups in P9 had no appreciable effects on the EDS decrease. The hydrogel probably shrank because the flat structure of the aromatic rings hydrophobically interacted in a compact conformation.

Hydrogel CP2. Unlike P9, the swelling behavior of the hydrogel CP2 was more responsive to the temperature, owing to the presence of the NIPAAm moiety. The CP2 became a multiple-responsive material that showed volume phase transitions at characteristic pHs and temperatures. In Figure 8 is reported the EDS in relation to pH and/or temperature in 0.15 M NaCl by keeping constant the temperature or the pH, respectively.

Oscillatory Swelling. With respect to the applications in drug delivery, the kinetics and the reversibility of the swelling must also be evaluated.[47] It is often desirable

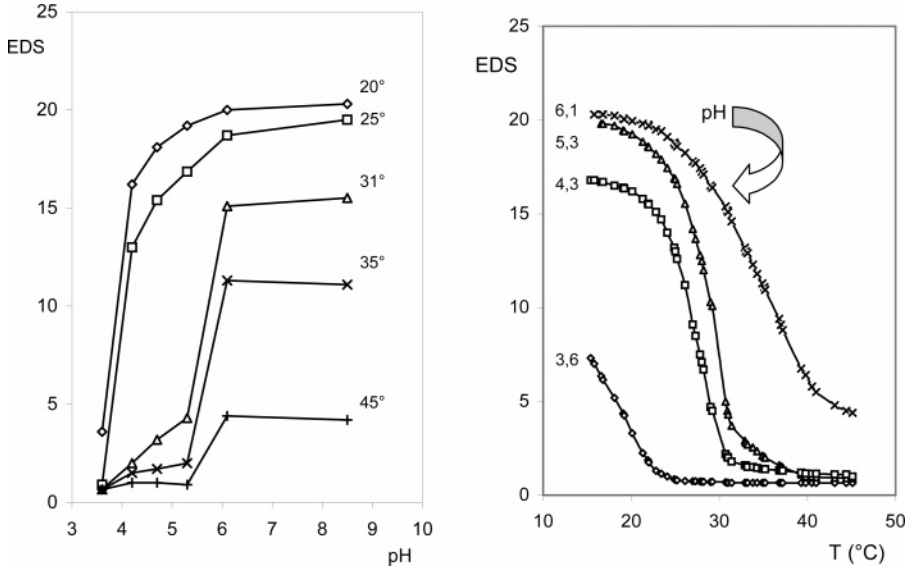


Figure 8. Equilibrium degree of swelling (EDS) in relation to pH and temperatures (°C) of the hydrogel CP2 in 0.15 M NaCl.

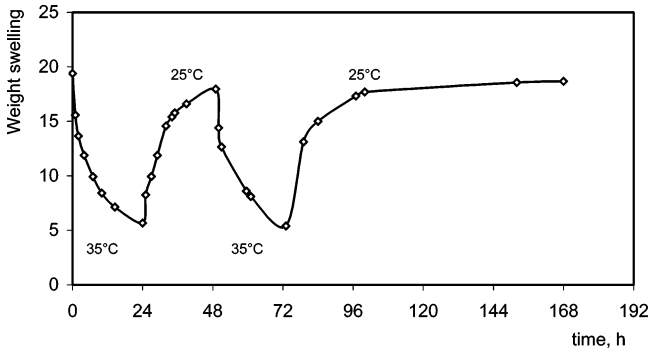


Figure 9. Oscillatory swelling behavior as a function of time and temperature at pH 4.8 (0.01 M acetate buffer) for the CP2 sample in 0.15 M NaCl.

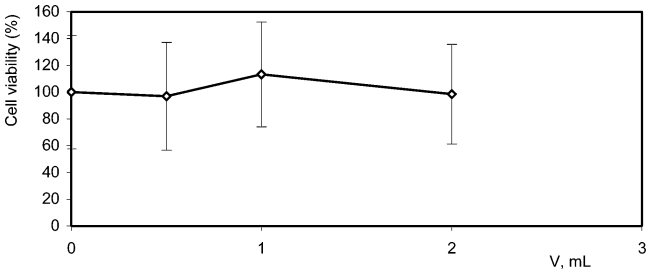


Figure 10. Cytotoxicity of CP2 against RAW264 cells.

that the swelling changes rapidly in response to external stimuli. For "on−off" type delivery systems, the swelling must be reversible. It was shown that, for the hydrogel CP2, the oscillatory swelling in response to a temperature change was fast and reversible. The following swelling abrupt change in temperature is shown in Figure 9. Here, a sample of CP2 was stored at the constant pH of 4.8 and the solution temperature was varied between 25 and 35 °C from time to time. These conditions were chosen on the basis of the data reported in Figure 8. The hydrogel CP2 was able to respond in a reversible manner to temperature pulses.

In Vitro Cytotoxicity. The cells exposed to the polymer gel CP2 proliferated at the same rate as the cells grown in polymer gel-free solution up to 48 h. The cytotoxicity was not observed at any amount up to 2 mL (Figure 10). It is known that the gel based on NIPAAm has no cytotoxicity and is used as a cell culture material.[48] The copolymer gel CP2 synthesized in the present study also has no cytotoxicity.

Conclusions

The thermodynamic data for the protonation of the carboxylate group in polymers containing L-phenylalanine residues showed that at $\alpha = 0.5$ and $\alpha > 0.7$ the macromolecule underwent a conformational transition from a random coil to a compact conformation. The transition was mainly driven by hydrogen bonds and hydrophobic interactions, the compact conformation occurred when the cohesive forces overcame the electrostatic repulsions of the charged groups and the side-chain aromatic rings led to an hydrophobic domain. The peculiar conformational transition behaved differently in copolymers with NIPAAm because the isopropyl groups contribution provided a greater hydrophobic character beyond critical α values. These findings are supported by the CD preliminary data, revealing as *co*-2 shows a molar ellipticity three times higher than that shown by poly(Phe). The CD spectrum, a random coil signal at 220 nm recorded at pH 8, was insensitive to the temperature changes (in the range 10−30 °C).[49] Moreover, the copolymer *co*-2 hydrophobically interacted with synthetic corticosteroids (prednisone, prednisolone), and this interaction was stronger than that shown by the poly(Phe) that, in turn, was close to that shown by the bovine serum albumin.[50]

The thermodynamic and conformational analysis data of the soluble compounds should be always considered when studying the cross-linked analogues (hydrogel) designed for technological applications. It was shown that P9 and CP2 hydrogels possess unique swelling properties. The mutual influence of pH and temperature on the swelling of the polyelectrolyte hydrogels was closely related to the ionization state of the polymer. The temperature range in which the temperature-induced swelling transition occurred was related to the amount of ionizable monomer. The pH-induced swelling transition was shown to be controlled by temperature due to the influence of the NIPAAm on the ionization of the Phe moiety COOH. These features may be very useful in designing hydrogels with specific temperature- or pH-induced swelling transitions. The absence of

cytotoxicity, along with the reversible and fast responses to the temperature variations, makes these kind of hydrogels suitable candidates for many biomedical and pharmaceutical applications, expecially in controlled drug delivery systems.

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