Vinyl Polymers Based on L-Histidine Residues. Part 2. Swelling and Electric Behavior of Smart Poly(ampholyte) Hydrogels for Biomedical Applications

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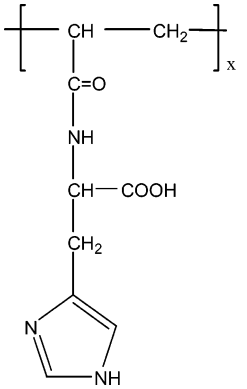
Hydrogels based on the uncharged *N*-isopropylacrylamide and the ionic ampholyte *N*-acryloyl-L-histidine showed a reversible multiple-responsive volume change and volume phase transition behavior in aqueous solution. The phase transition phenomenon was induced by the temperature, the pH, the salt-type concentration, and the electric potential. The kind of cation (Na^+, K^+, Cs^+, Mg^{2+}, Ca^{2+}, Sr^{2+}) and anion (Cl^-, ClO_4^-, NO_3^-, SO_4^{2-}) strongly influenced the critical concentration that improved the phase separation of the gels. The volume of the collapsed gel can be hundred times smaller than that of the swollen one. The oscillatory swelling of the gels in response to temperature and pH (4 and 9) changes was fast and reversible, while the contractile behavior in the electric field showed response only at pH 9, i.e., when the amount of negative charges on the L-histidine residues predominated. The electrically induced anisotropic gel deswelling was attributed to the syneresis of water from the gel. The nontoxicity against the RAW264 cell line and the low osmotic pressure exhibited by the swollen gels make these compounds useful scaffolds for human organs. The ability to load and release an ionizable drug molecular model (ferulic acid) from the hydrogels was shown also at different pH values.

Introduction

Cross-linked polymer networks (hydrogels) are studied for their widespread applications in the pharmaceutical, biomedical, and related fields.[1] In recent years, particular interest has been devoted to gels exhibiting reversible volume phase transitions in response to small external stimuli, such as temperature,[2] solvent composition,[3] pH,[4] or electric field.[5,6] Recently, we contributed in developing novel polymers containing, besides the carboxyl group, amido and isopropyl groups structurally related to the well-known pNIPAAm, poly(*N*-isopropylacrylamide).[7−10] The latter exhibits a temperature-dependent volume phase transition (lower critical solution temperature, LCST) behavior at 32 °C in aqueous solution.[11] The possibility of tuning the LCST of the pNIPAAm by incorporating comonomers with different hydrophilicity is an interesting feature to obtain smart polymeric materials for improved biocompatibility and potentiality in controlled delivery of drugs.[12,13] Moreover, charged materials that are responsive to electric stimuli highlight the potential of developing an electrically activated hydrogel to be used as artificial muscles and muscle actuators.[5,14] A discrete volume transition of the gel induced by an electric field may be of interest to make switches, memories, and mechanochemical transducers.[15]

The ampholytes are a special class of polyelectrolytes and they can serve as simple analogues to mimic the behavior of biopolymers and proteins.[16,17] In a previous paper we reported the synthesis and the protonation thermodynamics of a novel vinyl monomer carrying the imidazole and the carboxyl groups, as derived from L-histidine (Chart 1).[18] The monomer (Hist) was polymerized to form free polymer and cross-linked network hydrogels (polyHist).

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



The latter showed a polyelectrolyte behavior close to that of the free polymer analogue. The imidazole group of the histidine residue is responsible for most of the proteins buffering capacity, and it is also capable of combining with various metal ions, appearing to constitute the principal site for metal binding in proteins.[19,20] Among the characteristic features, the protonation study of the poly(ampholyte) revealed an isoelectric point (i.p.) at pH 5; at this pH, the free and the cross-linked polymer showed the minimum hydrodynamic coil and the lowest equilibrium degree of swelling (EDS), respectively. Moreover, at lower or at higher pHs than the i.p. the macromolecular coil increases

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Table 1. Feed Composition of Cross-Linked Hydrogels

compd	Hist, g/mmol	NIPAAm, g/mmol	EBA, mg/mmol	TEA, mmol	APS, mg/mmol	cross-link, mol %
CH1	0.553/2.20	3.00/25.7	51.2/0.30	1.35	20/0.086	1.1
CH9	0.561/2.23	3.02/25.9	500/2.91	1.35	30/0.129	9.4

for electrostatic reasons; this reflects, on a macroscopic scale, a greater and reversible EDS of the hydrogels. The reversible sensitivity to environmental conditions makes the amphoteric materials useful, for instance, as constituents of drug delivery systems.

Following the preliminary work,[18] based on hydrogels made of poly(Hist), we will investigate the amphoteric units' influence of hydrogels based on pNIPAAm to be used as scaffolds for tissue and organ repair. Loosely cross-linked hydrogels made of poly(NIPAAm-*co*-acrylic acid) have been recently studied as injectable polymer scaffolds for tissue engineering applications.[21] Unlike the anionic and the cationic gels that swell to a large extent only at high or low pH values, respectively, the amphoteric gels show a swelling degree that may be tunable before and after their i.p. The latter may be close to physiological conditions and can be tailored by the proper choice of the ampholyte residues. The aim of this work is to report the synthesis and the swelling properties of two hydrogels having a NIPAAm/Hist molar ratio of ca. 12 and different cross-linking densities. The effect of the temperature, the pH, and the salt-type, along with the swelling reversibility of the gel in different conditions, is reported. Moreover, we demonstrate here that a discrete and continuous volume change, due to the phase transition, is also induced by the application of an electric field across the gel. We believe that these materials, being nontoxic, may be of interest to design gels for biomedical applications because of the large range of responses.

Experimental Section

Materials. The *N*-Acryloyl-L-histidine (Hist) was synthesized as previously described.[18] The *N*-isopropylacrylamide (NIPAAm, 97%) was purchased from Aldrich Co. The *N*,*N*′-ethylenebis-acrylamide (EBA, 98%), the ammonium peroxo-disulfate (APS, 98%), and the triethylamine (TEA, 99.5%) were purchased from Fluka Co. and used as received. Tris, phosphate, and acetate buffer solutions were prepared at a concentration of 0.01 M either in twice-distilled water or in 0.15 M NaCl. Chloride, nitrate, perchlorate (hydrate), and sulfate salts of sodium, potassium, cesium, magnesium (hydrate), strontium (hydrate), and calcium (hydrate) were analytical grade reagents from Fluka and Riedel-deHaën Co. The ferulic acid was supplied by the Tsuno Rice Chem. Co. (Japan).

Synthesis of Hydrogels. The hydrogel H5, made of poly(Hist) and cross-linked with 5 mol % of EBA, was synthesized as previously reported.[18] The two poly(Hist-*co*-NIPAAm) hydrogels, at the NIPAAm/Hist molar ratio of 12, were synthesized by cross-linking with 1 (CH1) and 9 (CH9) mol % of EBA. Table 1 summarizes the feed composition. The preparation was carried out in a glass tube, under nitrogen atmosphere, by the following procedure. The monomers, Hist and NIPAAm, were dissolved in twice-distilled water, then a weighed amount of EBA was added with TEA, dissolved in water. The resulting mixture (25 mL) was first degassed at room temperature under vacuum for 30 min, then repeatedly flushed with nitrogen, and then APS was added. The concentration of the monomers was 15 wt %. The reaction mixture was kept at room temperature for 24 h even if the gelation was observed after 20 min. Afterward, the gels were removed, thoroughly washed with twice-distilled water for a week, and then cut in small disks. The latter were slowly dried at room temperature up to a constant weight in a desiccating cabinet. In both cases, the yield was >95%.

Scanning Electron Microscopy (SEM) and Spectroscopy (FT-IR, UV−Vis). FT-infrared spectra of the gel samples were recorded on a FTS 6000 Biorad spectrophotometer; their morphology was examined by the XL20 Philips scanning electron microscopy. Samples of the gels 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 CH1 and the CH9 was analyzed at 20 kV and at different magnifications. Absorbance measurements were done on a Perkin-Elmer UV−vis λ EZ201 spectrophotometer.

Potentiometric Measurements. The acid−base potentiometric titration data of the two hydrogels CH1 and CH9 were obtained at 25 °C in 0.15 M NaCl, according to a previously reported procedure,[18] by a TitraLab 90 titration system (Radiometer Analytical). For the titration experiments, samples of the dry gel were used after having grinded it to a fine powder, and a weighed amount of it (193.5 mg of CH1 and 181.3 mg of CH9) was dispersed in 100 mL of 0.15 M NaCl, containing a measured quantity of standard 0.1 M NaOH, by magnetic stirring and under nitrogen stream. The fine gel dispersion was kept to swell for 24 h and, when the equilibrium was established, the forward titration with standard 0.1 M HCl started at different time intervals (300, 900, 1800, and 3600 s) for each titration point. Rielable results were obtained at the longest time intervals (1800 and 3600 s) that reached the equilibrium pH. The two equivalent points of the titration curves allowed us to evaluate the imidazole nitrogen amount and the amount of free hydroxyl groups. Compared to the expected values, the amount of basic nitrogens was slightly greater since CH1 revealed 0.0812 mmol (10.3 wt %) and CH9 0.0785 mmol (10.6 wt %) of nitrogen being protonated. The basicity constants of the imidazole nitrogen was evaluated by the ApparK program,[22] and the results of two replicates were averaged.

Swelling Measurements. The hydrogel swelling was measured in a thermostated glass cell connected to a Haake DS thermostat and with a temperature probe controlled by the TimTalk 9 software. In a known volume (100 mL) of solution at the desired pH, a weighed amount of dry gel contained in a Strainer cell (100 μm pore size) was placed under stirring. The kinetics, the equilibrium, and the oscillatory swelling studies were carried out as functions of pH, temperature, ionic strength, and salt-type of the bathing medium. Samples of the dry hydrogels CH1 (21.9 mg) and CH9 (38.6 mg) were used throughout to evaluate the equilibrium degree of swelling (EDS) in the 0.01 M Tris buffer solution at different pHs (9.02, 5.01, and 2.0). The gel samples were kept at the desired conditions for 24 h. Thus, weighed portions of alkaline or earth-alkaline salts were added daily to the solution to produce the desired final concentration in the 0−3 M range. For the oscillatory swelling studies of CH1 (in 0.15 M NaCl), the temperature was varied between 24 and 35 °C, while the solution pH was buffered to pH 4 (0.01 M acetate) and 9 (0.01 M Tris). In all cases, the gel samples contained in the Strainer cell were removed from the bath at intervals, blotted with a tissue paper to remove any surface water, and weighed (wet weight, W_{wet}). The procedure was repeated daily. The EDS value was calculated as follows: $EDS = (W_{wet} - W_{dry})/W_{dry}$, where W_{dry} is the weight of the dry gel and its container.

Electric and Mechanical Measurements of the Osmotic Pressure. The contraction measurements of the hydrogels were made in a cylindrical nylon cell under a constant voltage between two gold electrodes (16 mm diameter), with a mobile cathode positioned on the gel sample. The latter was swollen in a buffered solution at pH 9.0 (0.01 M Tris/HCl) and/or 4.1 (0.01 M acetic acid/acetate) for 24 h, and a specimen of 5 mm thick was cut and used for contractile experiments. In all the experiments, the conducting electrodes were used in the absence of a conducting medium. When the electric field was applied, the hydrogel change in the thickness was measured using a digital comparator (Digimatic indicator 266-2745, Mitutoyo), which was sensitive to displacements of 10^{-2} mm. All the measurements were automatically controlled through a NI-DAQ driver software in Windows, from National Instruments. The graphical programming language LabView was used to create the application. To evaluate the contraction

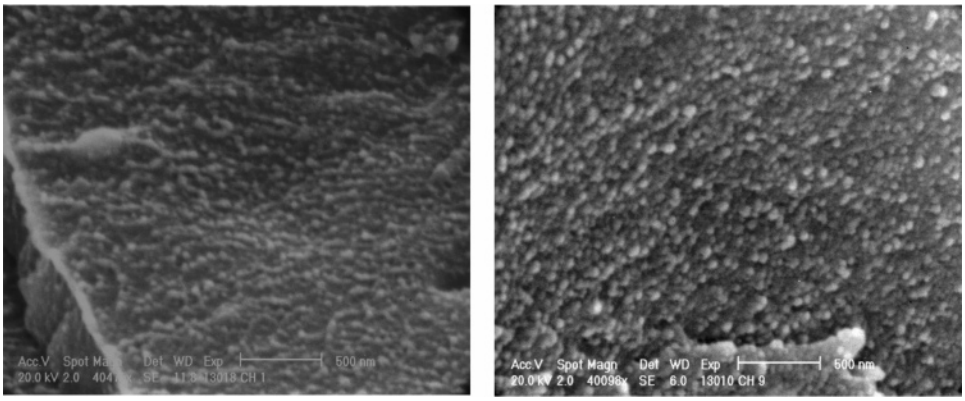

Figure 1. SEM images of the hydrogel networks: CH1 (left) and CH9 (right) at magnification 40000×.

reversibility, a sample of the gel CH9 (at pH 9) was held between the two gold electrodes for a period of 10 min and weighed. Then the sample was swollen in Tris buffer (0.01 M, pH 9) for 10 min before applying the same voltage. A voltage of 5 V and/or 7 V was applied. The operation was repeated at 10 min intervals for three times. The weight changes of the gel before and after the introduction of the electric field were measured by an analytical balance. The gel deformation was recorded at 1 s intervals under the applied voltage by a dc power supply.

The uniaxial expansion of the swelling gel CH1 (20 mg) was performed in the same cylindrical cell at room temperature between the two gold electrodes immersed in Tris (0.01 M, pH 9) buffered solution. The displacement (in millimeters) was measured after 24 h by the reading of the digital comparator connected to a pressure spring, at the equilibrium gel swelling. The same displacement value was applied as a vertical load on a digital balance and the weight recorded. Thus, the force acting on the gel was calculated from the reading balance m as $F = mg$, where g is the gravitational acceleration. The mass of 45.50 g was measured and the contrasting pressure, exerted by the swollen gel, calculated.

Drug Release Kinetics. The ferulic acid (FA) release from the hydrogels was conducted in a thermostated glass cell at 25 °C, at three different pHs (2, 5, and 9). For loading experiments, samples of the dry gel H5 (48.8 mg), of CH1 (64.9 mg), and of CH9 (58.4 mg), contained in a Strainer cell, were immersed in a buffered solution of ferulic acid (0.106 mg/mL) and allowed to reach the equilibrium swelling for 2 days, with magnetic stirring and under nitrogen stream. Then, after a quick washing with fresh buffer solution (50 mL), the gel was immersed in 50 mL of buffer solution to evaluate the amount of FA released. The absorbance at 321 and 310 nm,[23] the drug wavelength maxima, of the release medium was measured at time intervals. The same procedure was repeated at the three different pHs. Calibration curves, obtained at the same pHs, allowed the conversion of the observed absorbances in FA weighed amount (mg).

Evaluation of Cytoxicity. The hydrogels cytotoxicity was evaluated according to the previously reported procedure.[18,24] The RAW264 cells, derived from murine leukemoic monocytes, were provided by Riken (Tsukuba, Japan). The cells were cultured in a minimum essential medium (Sigma) with 10% FBS (fetal bovine serum) and 1% nonessential amino acids (Invitrogen Life Technologies); they 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; this suspension was added to the well of the 24-well plate in the presence of the gel CH1 or CH9 and was allowed to stand for 3 days at 37 °C. After the incubation, the number of cells was counted by microscopy. The results were expressed as viability (%) relative to a control containing no gel.

The means (±SD) of four experiments, each one containing three replicates, are reported.

Results and Discussion

Synthesis and Characterization. Two hydrogels of different composition were synthesized through the free-radical solution copolymerization of the corresponding monomers. The hydrogels containing Hist and NIPAAm units were synthesized in order to have a greater content of the temperature-responsive NIPAAm with respect to the pH-responsive Hist units. The samples, named CH1 and CH9, made at a NIPAAm/Hist molar ratio close to 12, were cross-linked respectively with 1 and 9 mol % of EBA. The water solution containing the monomer (14 wt % total monomer concentration) gelified at room temperature within 20 min, giving soft transparent products. The two hydrogels were cut in small disks, and the samples were repeatedly washed with twice-distilled water. Finally, they were dried at room temperature for 2 weeks and then under vacuum to constant weight.

The acid/base potentiometric titrations of the gels showed that the incorporated Hist was in agreement with the feed composition. Unlike the previously reported gels[18] made of only a Hist homopolymer and cross-linked with 5 mol % (H5) and 10 mol % (H10) of EBA, the potentiometric curves of the copolymer CH1 and CH9 gels, when carried with the same equilibration time of 300 s and 1500 s for each titrant addition, showed an hysteresis loop during the forward titration with hydrochloric acid and its back-titration with sodium hydroxide. Instead, increasing the equilibration time to 1800 s and 3600 s, the system under protonation reached the equilibrium condition and, from the two equivalent points observed, the amount of the imidazole nitrogen being protonated was evaluated to be in agreement with that expected. The different behavior may be attributed to the lower diffusive properties of the hydrated H^+ and OH^- ions, due to the more compact gel network structure. Unlike some reported[25] macroporous networks, made of pNIPAAm at increasing cross-linker content, the SEM analysis showed homogeneous and compact structures for CH1 and CH9. This similarity occurred even at different cross-link densities. In our case, the increasing cross-linker content in the copolymer gels revealed a structure consisting of poorly defined nanoaggregates of spherical domains (Figure 1). Moreover, the infrared spectroscopic analysis showed similar spectra for CH1 and CH9 but, due to the lower amount of Hist, their spectra are different from that of H5. Figure 2 shows the FT-IR spectra of the three hydrogels in the wavenumber range 1300−1800 cm^{-1}, and in Table 2 the main frequencies, along with the basicity constants relative to the protonation of the imidazole residues, are summarized.

Due to the different amount of monomers utilized in forming the gels, the principal stretching vibrational modes of the main

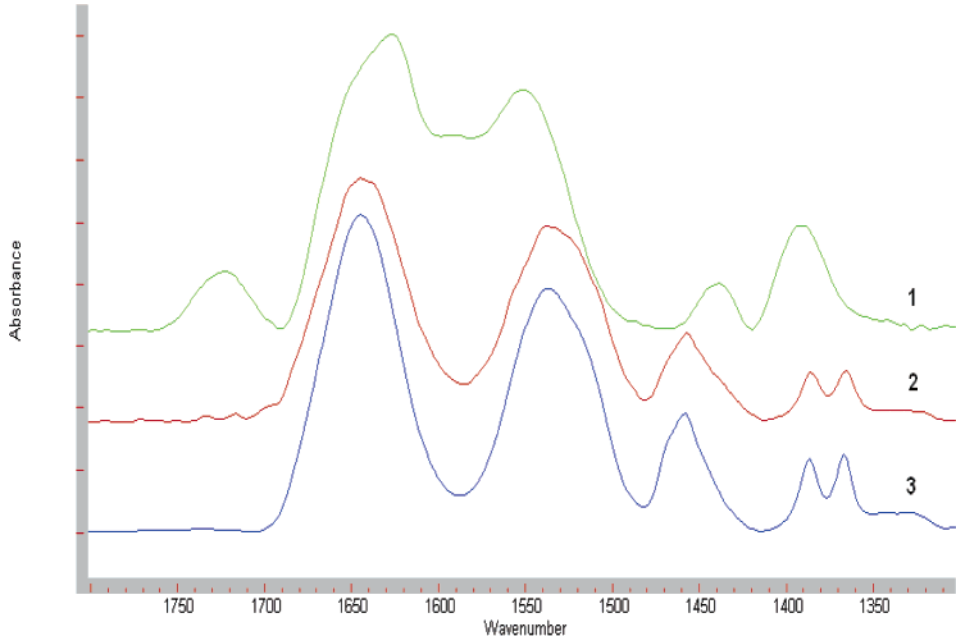

Figure 2. FT-IR spectra of the hydrogels H5 (1), CH1 (2), and CH9 (3).

Table 2. Main IR Frequencies (cm^{-1}) and Basicity Constants of the Hydrogels in 0.15 M NaCl at 25 °C

compd	COOH	COO^-	amide I	amide II	$-CH_3$	log K° [a]	n[a]	ref
CH1	–	1550 sh 1387 m	1645 vs	1537 vs	1459 s 1367 m	7.1	1.1	this work
CH9	–	1550 sh 1385 m	1643 vs	1534 vs	1457 s 1366 m	7.0	1.4	this work
H5	1723 s	1551 vs 1391 s	1652 sh	1535 sh	–	7.6	2.0	this work, 18
poly(Hist)	1717 vs	–	1646 vs	1542 vs	–	7.64	2.22	18
Hist	1729 vs	–	1658 vs	1543 vs	–	6.48	–	18

[a] $\log K = \log K^{\circ} + (n-1)\log[(1-\alpha)/\alpha]$.

functional groups of the gels lie at somewhat the same frequencies.[7,10,18,26,27] The methyl group, being present only on NIPAAm, becomes therefore a good marker for the CH1 and CH9 gels. On the other hand, the larger amount of Hist in the gel H5 revealed dominant peaks associated with the presence of the imidazole and the carboxyl groups, the latter being present in the ionized and in the neutral forms, as shown by the potentiometric titration curves. Compared to both the homopolymers in the free (polyHist) and in the cross-linked (H5) forms, the lower basicity constant (log K) of the cross-linked copolymers CH1 and CH9 is consistent with the randomly distributed Hist residues in the network with a lower charge density. The similar log K° value is close to that of the simple imidazole and slightly higher with respect to the monomer Hist.[18] In both cases, however, the linear log K pattern in relation to the degree of protonation α is well described by the modified Henderson−Hasselbalch equation (see footnote of Table 2). The lower n values, being a measure of the electrostatic interaction magnitude, are also consistent with the shielding effectiveness exhibited by the NIPAAm moiety. The larger amount of the latter greatly reduces the polyelectrolyte behavior of the short polyHist chain incorporated in the copolymer network.

Swelling Studies. The swelling behavior of the hydrogels has been studied in different conditions of pH, temperature, salt-type and ionic strength, electric current, and toward their ability to release small-molecular-weight molecules. Unlike the rigid and opaque CH9, the gel CH1 was extremely friable and transparent. Both hydrogels became more rigid and opaque when they underwent the phase transition phenomenon.

The swelling kinetics at pH 9 of the CH1 and CH9 gels in 0.15 M NaCl at 25 °C are reported in Figure 3. It is evident

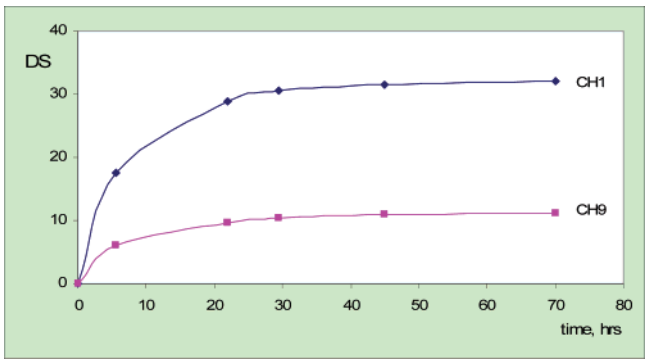

Figure 3. Swelling kinetics of the hydrogels in 0.15 M NaCl at 25 °C.

that the degree of swelling (DS) is greater for the gel CH1 because of its lower degree of cross-links. In both cases, however, the equilibrium DS was reached within 24 h.

Moreover, at pH 9 and with no salt added, this gel swelled to a large extent (more than 100-fold of its dry weight), by keeping a transparent and soft aspect (Figure 4). A slight opalescence and a lower swelling was shown in twice-distilled water. This was due to the neutral pH that led to a strong reduction of the net negative charges, because of the prevailing zwitterionic form.[18]

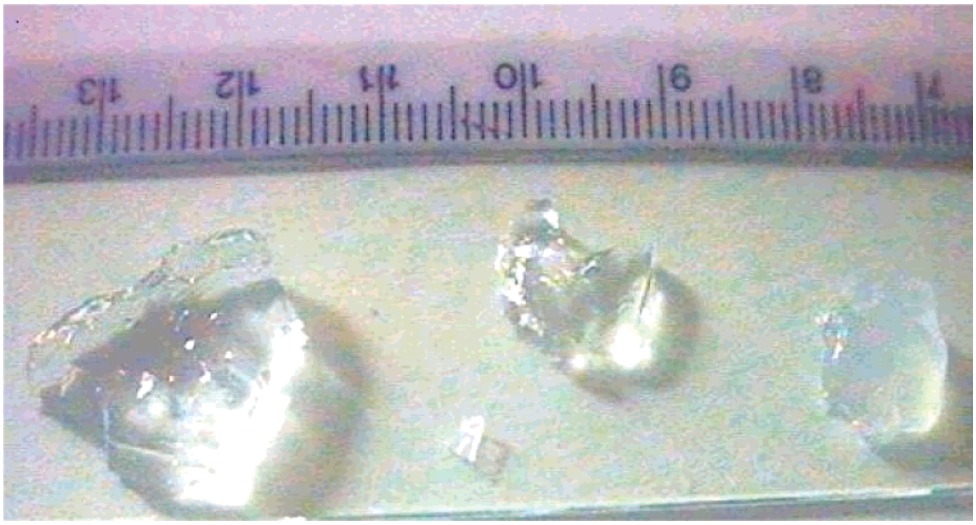

Figure 4. Hydrogel CH1 in the swollen and in the dry state. Samples of 6 mg in 0.01 M Tris buffer pH 9 (left), in 0.01 M Tris buffer pH 9 in 0.15 M NaCl (center), and in pure water pH 6 (right).

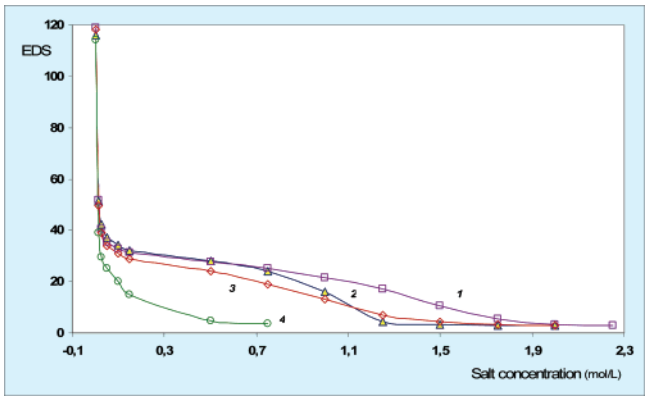

Figure 5. EDS of gel CH1 in relation to the concentration of salts: $NaNO_3$ (1), NaCl (2), $NaClO_4$ (3), and Na_2SO_4 (4); buffer solution (0.01 M Tris) pH 9.02 at 24.2 °C.

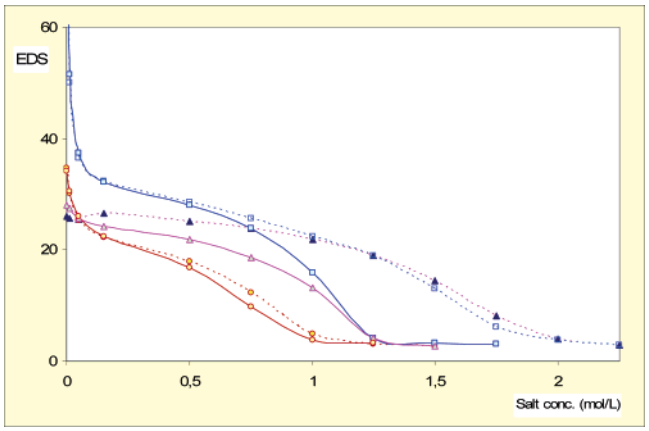

Figure 6. EDS of gel CH1 in relation to the concentration of NaCl (solid lines) and $NaNO_3$ (dotted lines) at different pH values (24.2 °C): 9.02 (square), 5.01 (triangle), and 2.0 (round).

Effect of the Salt-Type and Ionic Strength. The hydrogel CH1, having the largest swelling ratio, has been used to show the dependence on the ionic strength and the salt-type of the volume phase transition behavior in aqueous solution. In Figure 5 the equilibrium degree of swelling (EDS) in relation to the molar concentration of some simple sodium salts is reported, by varying its anionic nature.

Unlike the behavior of the nonionic polyNIPAAm in the free[28] and cross-linked gel forms,[29] in the case of polymers containing ionizable functional groups the first addition of salts provoked a sharp reduction of water into the gels. This happens because of the polyelectrolyte nature of the polymer network, where the salt shields the negative charges of the carboxylate groups. By increasing the salt concentration, the EDS slowly decreased and, depending on the anion-type, a critical concentration led to a volume phase transition phenomenon in aqueous solution. The gel collapsed at a critical sodium salt concentration, following the order: sulfate (0.50 M), chloride (1.25 M), perchlorate (1.75 M), and nitrate (2.00 M). This can be explained in terms of the extent of the ions binding to water. Thus, the effective concentration of the gel increased and it precipitated, so releasing low entropy surface water. The well-known Hofmeister series,[30,31] which originates from the ability of the ions to precipitate egg white proteins, for both anions and cations, will be taken into account for the observed salting-out of the gel. The SO_4^{2-} ion is the most stabilizing and strongly hydrated anion, and the Cl^- and NO_3^- ions are in line with the above-mentioned Hofmeister series. On the contrary, the anion ClO_4^- seems to have more hydration ability than the NO_3^- of the series. The differences of the relative order may be due to ion pair effects, as stated by the same series, and by the fact that we are considering a gel with different properties from that of proteins. The same Figure 5 shows a different EDS/concentration pattern for sodium perchlorate.

The effect of pH on the phase transition phenomenon of the CH1 gel and in the presence of salts is shown in Figure 6.

The addition of sodium chloride and/or sodium nitrate provokes a phase-transition from the swollen to a collapsed state of the CH1 at critical concentration of simple salts, strongly dependent on pH. At pH 9 this concentration is 2.0 M and 1.25 M, respectively, for $NaNO_3$ and NaCl. These critical concentrations are also obtained at pH 5. At this pH a flat curvature or a slight increase of the EDS in relation to the concentration of simple salt was observed because of the 'antipolyelectrolyte' effect. This effect was attributed to the unfolding of amphoteric macromolecules at the i.p. and in the presence of neutral salts. This effect is observed also in cross-linked polyampholytes.[6] On the other hand, at pH 2, the phenomenon appeared to be at the same lower concentration of 1 M for both the salts. It is evident that in the latter case the pH plays a role in the phase transition behavior of the gel CH1. Unlike the low pH responsivity of the gel CH1 in 0.15 M NaCl (physiological ionic strength), the 1 M or higher concentration led to a decreasing

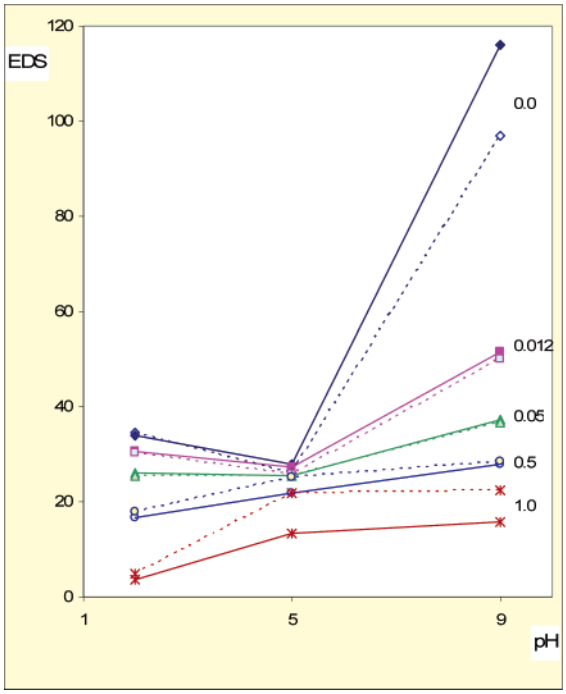

Figure 7. EDS of gel CH1 in relation to pH at different concentration of NaCl (solid lines) and $NaNO_3$ (dotted lines) at 25 °C.

Table 3. Critical Chloride Salt Concentration for the Swollen-Collapsed Phase Transition of the CH1 Gel in Tris (0.01 M) Buffer pH 9.02 at 24.2 °C

cation	ionic radius (pm)	molar concd (mol/L)
Na^+	102	1.25
K^+	138	1.50
Cs^+	170	1.75
Mg^{2+}	72	1.50
Ca^{2+}	100	1.25
Sr^{2+}	116	1.00

EDS/pH pattern. In Figure 7 is reported the EDS value at the three pHs investigated and for different concentrations of simple salts.

Without salt added or at a lower ionic strength, a lower EDS value is observed at pH 5. This pH, corresponding to the i.p. of the Hist unit, once again reveals the low EDS found in the H5 and H10 hydrogels.[18] The increase of the ionic strength provokes a gel shrinking that gives a phase transition at low pH and critical salt-type concentration. A very close behavior was found for the gel CH9. In the latter case, the greater cross-linking density slightly shifted the critical simple salt concentration to higher value, even the EDS values were one-third lower, and the EDS/concentration pattern was exactly the same as that reported in Figure 6.

When some simple chloride salts of different alkaline (Na^+, K^+, Cs^+) or earth-alkaline cations (Mg^{2+}, Ca^{2+}, Sr^{2+}) were considered to be added to an aqueous solution (pH 9), the EDS of the CH1 gel in relation to the molar salt concentration followed the same pattern as that shown in Figure 5. In Table 3 is reported the critical chloride salt concentration that induces the phase-transition of the gel CH1 at pH 9.

The first addition of salt drastically reduced the water into the gel for the screening effect of the charged carboxylate groups. The salting-out of the cations became clear in a 0.50 M range of the salt concentration. Small cations (Na^+) hydrated strongly than larger ones (Cs^+), and this led to an earlier collapse

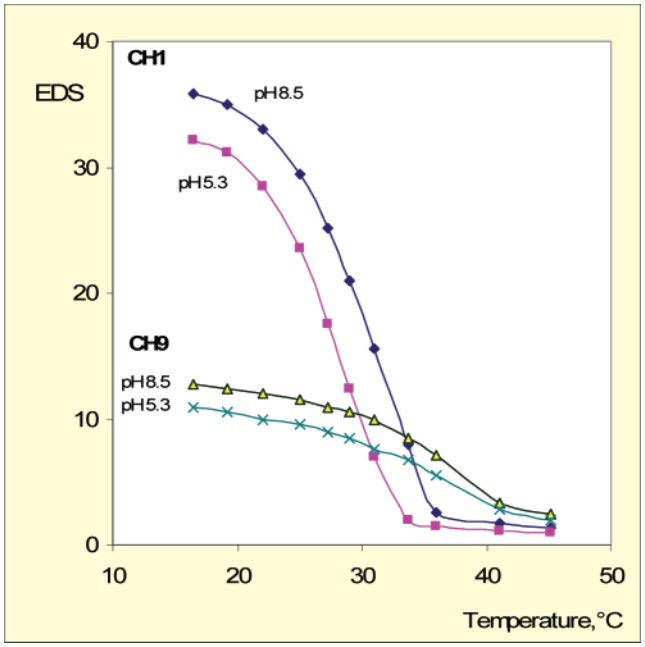

Figure 8. EDS of gels CH1 and CH9 in relation to the temperature at two different buffered pHs in 0.15 M NaCl.

of the CH1 gel. The behavior is in line with that expected by the Hofmeister series. On the other hand, when some chloride salts of different earth-alkaline cations were used in the same experimental conditions, the volume phase transition behavior of the gel CH1 showed different patterns in relation to the salt-type. In this case, small cations (Mg^{2+}) hydrated weakly with respect to larger ones (Sr^{2+}), resulting in a gel collapse to a great salt concentration. For these cations the Hofmeister series is not obeyed, probably for the great ability of the metal ions to form electrostatic complexes with the amphiphile residues.

Effect of pH and Temperature. Unlike the pH-responsive hydrogels H5 and H10, reported in the previous paper,[18] the hydrogels of the present study contain a larger amount of temperature-responsive NIPAAm moiety. At the physiological conditions (0.15 M NaCl), relevant for drug release, the two hydrogels CH1 and CH9 exhibit a greater temperature-dependent volume phase transition (lower critical solution temperature, LCST) behavior in aqueous solution and only a low pH responsivity. Figure 8 shows the effect of pH and temperature on the two hydrogels CH1 and CH9. The low degree of cross-links improved a greater swelling ability of CH1 and a lower LCST. Lowering the pH near to the isoelectric point of the histidine residues had the effect of slightly decreasing the LCST, which became closer to that of the polyNIPAAm.

The temperature-responsive behavior of the gels can be easily varied by the addition of small molecules, as these in general alter the polymer−water interactions. The presence of simple salts is known to disrupt the hydration structure surrounding the polymer chains, causing a decrease of LCST. This phenomenon was already reported for thermoresponsive polymers and recently investigated by means of powerful tools for characterizing LCST in polymer solutions.[28] In Figure 9 is reported the decreasing EDS pattern of the gel CH1 at pH 9 and at various concentrations of sodium chloride.

As the concentration of the latter increases, the LCST shifts to lower values, being in a greater temperature range at lower concentrations of the salt. This is in line with what happens for the soluble poly(NIPAAm) upon heating.

Oscillatory Swelling. We evaluated the swelling reversibility of the gel CH1 between two temperatures (24° and 35 °C)

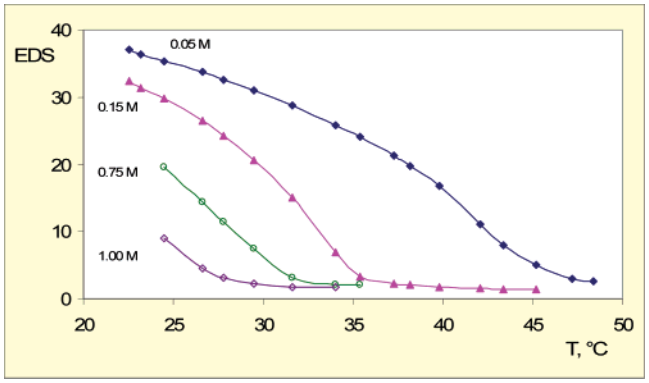

Figure 9. EDS of gel CH1 in relation to the temperature (°C) in buffered solutions (pH 9.02) and at different ionic strengths (NaCl).

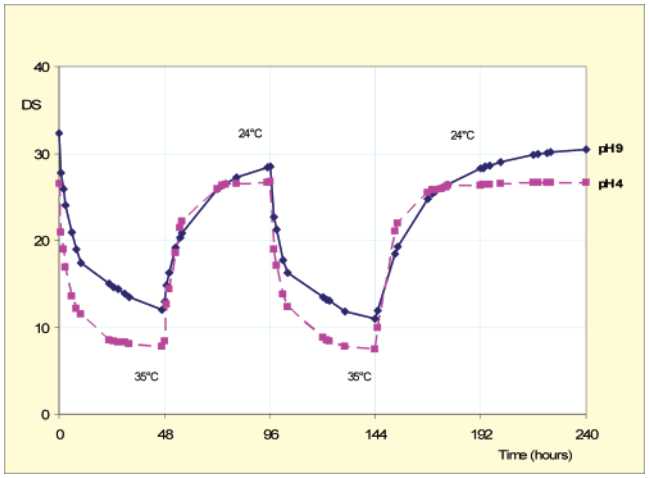

Figure 10. Oscillatory swelling of gel CH1 at two different pHs and temperatures in 0.15 M NaCl.

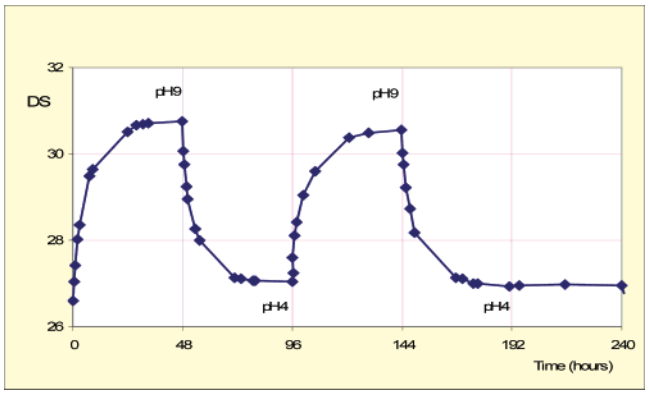

Figure 11. Oscillatory swelling of gel CH1 at two different pHs (0.15 M NaCl and 25 °C).

chosen to have a high an a low EDS in 0.15 M NaCl. The bathing medium was a buffer solution of pH 9 and 4. Figure 10 shows the swelling and the deswelling kinetics conducted over a 48 h cycle. In all cases, the oscillatory swelling was reversible, but it was faster at pH 4. At this pH the histidine residue is mostly in the zwitterionic form and thus the thermosensitive NIPAAm moiety works faster, reaching a plateau within 24 h irrespective of the temperature.

On the other hand, when the temperature was held constant to 25 °C the oscillatory swelling between the two pH values (9 and 4) was shown to be faster and reversible, even in the narrow range of the swelling degree of CH1 (Figure 11). The great reversible response is often desirable for the "on−off" type delivery systems.[24,32]

Effect of the Electric Current. We demonstrate here that the phase transition is also induced by the application of an electric field across the gel.[5,6,14,15,33] The contractile behavior in an electric field was examined for the hydrogels at 25 °C and at two different pHs, 4.1 and 9.0. At the higher pH a gel contraction with time was followed when a voltage of 2.50 V was applied. Figure 12 shows the results obtained for the gels H5 and CH1 at pH 9. In both cases, a linear contractile behavior was observed, and this was due to the electrically induced anisotropic gel deswelling between two parallel gold electrodes. The electric forces on the charged sites of the network produced a gel deswelling accompanied by an increase in gel opacity.

The electric field exercised a force not only on the small free ions in solution, causing a stationary current in the gel, but also on the negatively charged carboxylate groups in the polymer network. The motions of the ions inside the gels improved water-destructuring properties with a consequent release of water molecules from the gel network. Drops of water were in fact observed on the surface of the gel sample when the voltage was put on. It is worthwhile mentioning that the exudates appeared to 'drip out' of the anionic gel at the cathode end of the gel, which indicates a fluid movement in the gel toward the cathode. Moreover, when the voltage was put off, an additional weak electric current was recorded for a short time, meaning that the gel system works as a galvanic cell of opposite polarity. The different contraction/time slopes of the gels H5 and CH1 may be attributed to the different amount of charged ions inside the network, the gel H5 at pH 9 showing 70% of charged carboxylate anions (as evaluated by the log K values),[18] while the reduced number of charges in CH1 were due to its diluted amount in the network together with the noncharged NIPAAm co-monomer. The volume change at the transition was either discrete or continuous depending on the degree of ionization of the gel. A further evidence of the electrically induced ordering of the ions in the network arose from the fact that at pH 4.1 no contractile behavior was observed for all the studied hydrogels, because at this pH the swelling degree was the lowest, due to the maximum formation of the zwitterionic species. Near the isoelectric point and with an applied voltage, the charged positive and negative ions were aligned in the opposite direction between the two parallel electrodes. In the condition of a lowest degree of swelling, the amount of released water from the network became negligible and thus made the gels nonresponsive to electric signals. A similar behavior was reported for some amphoteric hydrogels based on vinyl 2-aminoethyl ether and sodium acrylate.[6] Because of the extremely friable cross-linked matrix of the transparent H5 and CH1, we performed electrostimulation of the more rigid and opaque gel CH9 at higher voltages. Upon the application of the electric field above a threshold value, the CH9 deswelled as water is syneresed from the gel. The gel, in the prevailingly anionic form (at pH 9), shrank at the anode. Moreover, upon electrical stimulation on a CH9 sheet, bulk fluid flowed toward the cathode. A strip of water molecules wetted the electrode surface. Meanwhile the hydrogel showed a bending response, with a curvature of the sheet toward the anode. This process showed fast reversibility as the polarity was inverted. Upon sequential switching 'on' and 'off' of the electric field, the gel CH9 deswelled and swelled, following the electric field protocol (Figure 13).

The magnitude of the gel response, as well as the degree of reversibility, often decreased with time and with increasing number of on−off cycles as the gel fatigues.[34] Although only the electric force was considered in describing the phase transition, other factors may play a role. The effects of an

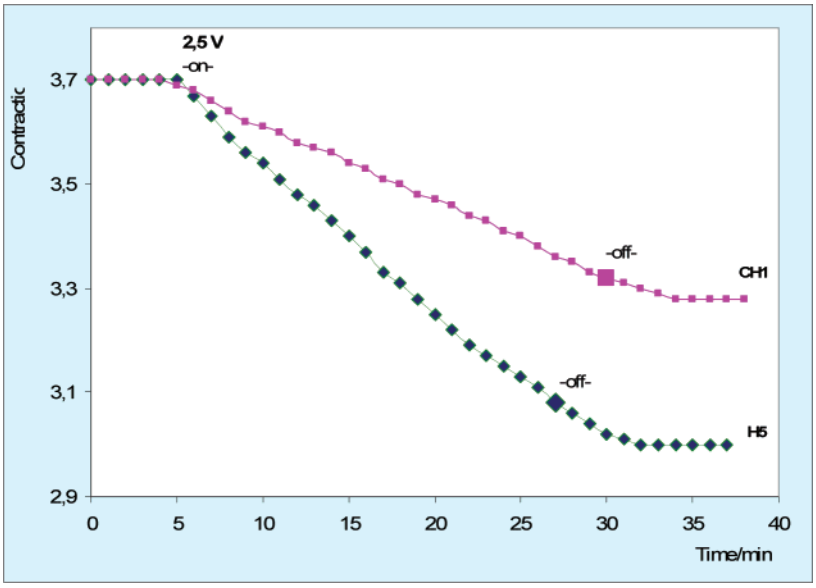

Figure 12. Contraction (mm) in relation to time (min) of the hydrogels H5 and CH1 at pH 9 and 25 °C with a potential of 2.50 V applied.

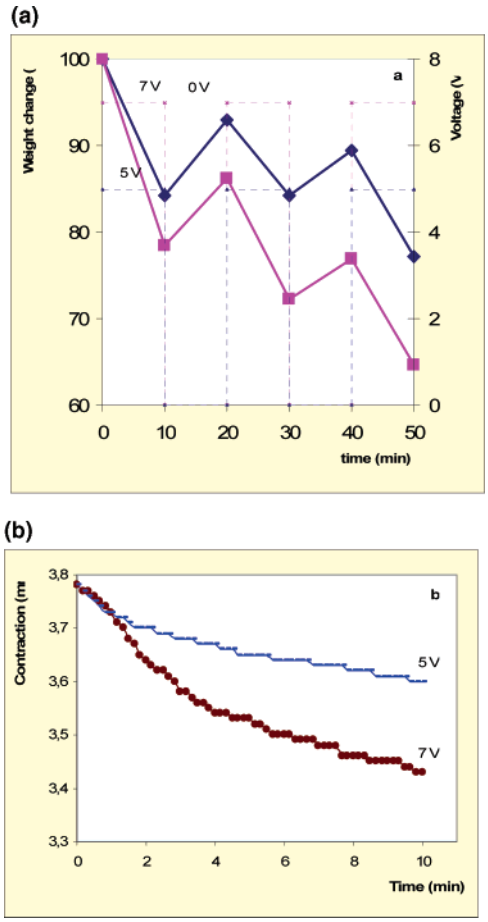

Figure 13. (a) Response (weight change, %) of the CH9 gel deswelling (pH 9) to pulse (10 min) of electrical stimulation (5 and 7 V). (b) Measured contraction (mm) of the CH9 gel (pH9) with time upon electrical stimulation.

inhomogeneous distribution of ions, current, and electrochemical reactions occurring at the electrodes need to be considered for a complete understanding of the phenomena.[5] This study is in progress.

Drug Release. Ferulic acid (FA) is a phenolic cinnamic acid derivative known to act as an in vivo substrate for peroxidase.[35] Its chemical structure shows an extended side chain conjugation, suitable to form a resonance-stabilized phenoxy radical under UV absorption, which accounts for its strong antioxidant potential.[36] The ability to form stable radicals and to show a radical scavenging activity[37,38] makes FA an interesting candidate as ingredient of many skin lotions and sunscreens designed for photoprotection.[36,38−40] Thus, FA was chosen as a small-molecular-weight drug model to load and release from hydrogels in different pH conditions. Figure 14 shows the releasing profile of FA from the gel H5 (at pH 2, 5, and 9) and the gels CH1 and CH9 at pH 5, relevant for the drug release.

The three pH values were chosen on the basis of the H5 gel log *K* values.[18] The predominant protonated, zwitterionic, and anionic species are LH_2^+, $LH^{\pm}$, and L^-, respectively (L is the monomer unit of the polymer). At pH 9, FA was not loaded because of the flat releasing plot. The negatively charged carboxyl groups of the gel exhibit a strong electrostatic repulsion toward the ionized FA molecule. At this pH both the hydroxyl and the carboxyl groups of FA were predominantly in the negative charged form, their log *K*'s being less than the pH.[40] On the other hand, at pH 2, the FA molecule, being in the protonated form, may diffuse more freely through the positively charged gel during the loading process. Then, the release of FA at pH 2 showed a characteristic releasing pattern, by a Fickian diffusion behavior. At the intermediate pH 5, the loading was even lower than that at pH 2 but higher than at pH 9. These differences may be found in the gel swelling ability.[18] At pH 5, the gel H5 showed its minimum EDS value because of the shrinking phenomena shown in the zwitterionic state, i.e., when the net charge on the polymer is zero. At the same pH 5, both the gels CH1 and CH9 showed loading capacities strongly dependent on the cross-linking degree. Moreover, a further electrostatic phenomenon may improve the loading capacity at pH 2. At this pH, in fact, the FA is prevailing in the neutral form, and this contributes to an increase in the diffusive loading process through the positively charged network. The initial releasing step may be attributed to a burst effect of drug release when in contact with water, which is related to the electrostatic process. The same releasing pattern was found for the gels CH1 and CH9. Indeed, in the latter case FA reached a greater loading capacity, more than three times higher in CH1 than that of the gel H5, at pH 5. This is a consequence of the above-described greater swelling ability of the gel CH1 in the prevailingly neutral

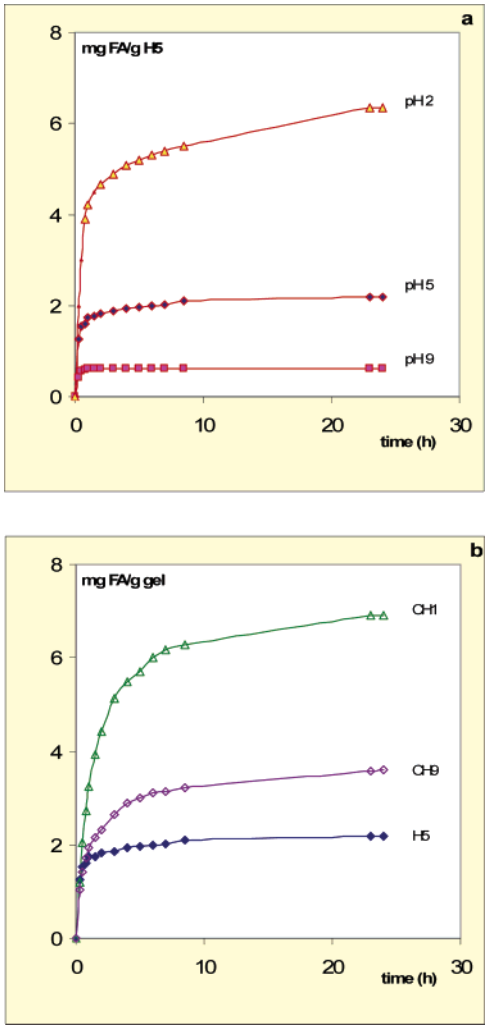

Figure 14. (a) Release profile of FA (mg FA/g H5) from the gel H5 in buffered solutions at different pHs. (b) Release profile of FA (mg FA/g gel) from the gels H5, CH1, and CH9 in buffered solution at pH 5.

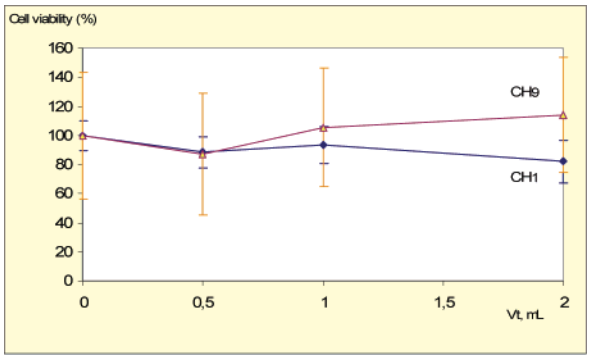

Figure 15. Cytotoxicity of the gels CH1 and CH9 against RAW264 cells.

state. Furthermore, the gel CH1 showed a slight increased FA release/time slope when the temperature was increased to 35 °C. The collapsing of the gel at the high temperature enhanced the release of water and its content.

In Vitro Cytoxicity. The cytotoxicity was evaluated by the viability of mammalian cells cultured in the presence of polymer gels (Figure 15). Neither polymer gels had significant toxicity for the cells after 3 days. In addition, no significant difference between CH1 and CH9 was found. Considering that it is known that neither soluble linear polymers of *N*-isopropylacrylamide and *N*-acryloyl-L-histidine has cytotoxicity,[18,41] it is consequent that neither gels has it. The same behavior was already reported for polymers in the free and in the cross-linked forms containing L-phenylalanine residues.[24]

Conclusions

The second paper of the series concerning the polymers based on L-histidine residues deals with the synthesis and the swelling properties of "intelligent" materials which exhibit multiple-responsive volume phase transition behavior in aqueous media. Besides the pH and the temperature, the gels CH1 and CH9 showed an unusual salt-induced phase transition and a dc electro-shrinking phenomenon, at high charge density. These properties may be of interest in the biomedical and in the pharmaceutical fields for novel polymer therapeutics for nanotechnologies.[33,42] When designed for biomedical applications at the physiological pH range, a gel with a greater ionic charge and a lower cross-linking density may show improved electrically induced ion properties. These kinds of gels are also available to solve problems of artificial organs and may be tailored as injectable polymer scaffolds for tissue engineering applications.[21] During the swelling phenomena, the gels contrast an external pressure of tens of millimeters of mercury. The gel CH1, for example, showed an osmotic pressure of 2.22 kPa (16.7 mmHg), which lies in the range of the human organs, like eyes and pulmonary blood circulation, as well as the kidney colloid pressure. The nontoxic effects, along with the slow drug release characteristics and the reversible swelling profile shown, enable the gel CH1 to be tailored in medical treatment as a scaffold for human organs.[43]

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