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Temperature and pH-Sensitive Polymers with Hydrophobic Spacers for the Controlled Delivery of Drugs

Angel Licea-Claverie,[*1] *José Manuel Cornejo-Bravo,*[2] *Rodolfo Salgado-Rodríguez,*[1] *Dolores del Rosario Santos-Rosas,*[1] *Eder Lugo-Medina,*[1] *Marco Antonio Ramos-Ibarra,*[2] *Karl-Friedrich Arndt*[3]

Summary: Acid methacrylates containing hydrophobic aliphatic and aromatic spacers were used to prepare pH-sensitive ampholytic hydrogels and bidimensional temperature- (T) and pH-sensitive hydrogels. Their swelling behaviour was studied by changing the pH and temperature of buffer solutions. Salicylamide, salicylic acid and green fluorescent protein (GFP) as model drugs were loaded into the gels and their release kinetics studied under simulated gastric and intestinal conditions. T- and pH-sensitive hydrogels containing aliphatic spacers show sustained release of analgesics depending on pH (e.g. 7.4); while longer aliphatic spacers resulted in drug release depending on pH and temperature ($T <$ transition T). GFP was released from temperature- and pH-sensitive ampholytic hydrogels after different lag times depending on hydrogel composition.

Keywords: drug delivery; green fluorescent protein; hydrogels; N-isopropylacrylamide; sensitive polymers

Introduction

Controlled release technology tries to solve two of the main problems encountered in traditional delivery systems of drugs: the low synchronization between the time necessary for therapeutic effectiveness of a drug and its real availability, and the site specific disponibility of a drug and its unwanted effect in other sites.[1] Sensitive ''smart'' polymers are among the most promising materials to tackle both problems since they respond in a predictable and reversible way to changes in the environment by modifying their solution properties and volume.[2–7] However, in spite of the interest and expectation that such sensitive materials have promoted, the mechanism of drug release from swellable matrix tablets continue to be a matter of debate.[8–10] Drug delivery systems for oral administration are usually prepared as tablets. Usually they consist of a mixture of a drug, glassy polymer(s), which may include acid groups for preventing release in the stomach, and excipients.[11–13] In our research group we have been developing new pH-sensitive polymers based on new acid monomers containing hydrophobic spacers with aliphatic and aromatic groups in their structure (Figure 1).[14,15] Linear and network polyelectrolytic polymers

[1] Instituto Tecnológico de Tijuana, Centro de Graduados e Investigación, A. P. 1166; 22000 Tijuana, B. C., México
Fax: (+52) 664 6233772
E-mail: aliceac@tectijuana.mx
[2] Facultad de Ciencias Químicas e Ingeniería, Universidad Autónoma de Baja California, Calzada Tecnológico 14418, Mesa de Otay, 22300 Tijuana, B. C., México
[3] Physikalische Chemie der Polymere, Technische Universität Dresden, D-01062 Dresden, Germany

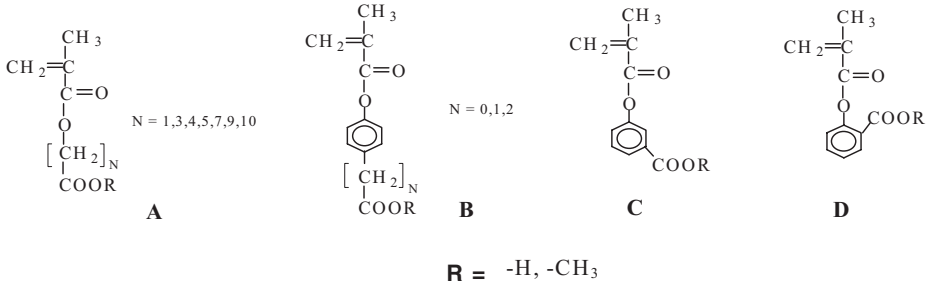


Figure 1.
Monomers with hydrophobic aliphatic (A) and aromatic (B, C and D) spacers. N represents the number of methylene units in the spacer.

from these monomers have shown coil to globule (linear polymers) or swelling-collapse transitions (swollen networks, gels) at very different pH values.[16,17]

Polyampholytic systems based on the same acid structures and using as basic comonomer commercially available methacrylates with tertiary amine groups, show chain expansion at high saline concentrations,[18] a property that may be exploited in a variety of technological applications.[19] For oral delivery of drugs in the gastrointestinal tract, pH-sensitive polyelectrolytic and polyampholytic polymers are very promising materials to study. We have recently shown that using poly(carboxyalkylmethacrylates) as base polymers in tablet formulations for propanolol, verapamil and diltiazem drugs, the release kinetics could be tailor-make by using a polyelectrolyte from the series (Figure 1A) with the suitable hydrophobicity.[20] Moreover, we used the same partially hydrophobic acid monomers developed to modify the temperature-sensitivity of poly(NIPAAm) to yield bidimensional temperature- and pH-sensitive polymers with different LCST values.[21] In this report we expand our investigation with these partially hydrophobic acid monomers for the preparation of ampholytic pH-sensitive hydrogels and modified temperature sensitive hydrogels, aiming to prepare in the last case, improved versions of the well-known poly[(N- isopropylacrylamide)-co-methacrylic acid] (NIPAAm-MAAc) hydrogels.[22–24] The hydrogels were tested for the delivery of conventional drugs and of a model protein drug: the green fluorescent protein (GFP).

Experimental Part

Materials

The free radical initiator 2,2″-azobisisobutironitrile (AIBN, Aldrich) was purified by re-crystallization from methanol. N,N′-dimethylaminoethyl methacrylate (DMAEM, Aldrich) was distilled using ETHANOX (Aldrich) as polymerization inhibitor during distillation. Crosslinking agent ethylenglycol dimethacrylate (EGDMA, Aldrich), was purified by passing it through a column of inhibitor-remover for hydroquinone and hydroquinone monomethyl ester (Aldrich). p-dioxane was further dried by using molecular sieves followed by distillation. Tetrahydrofuran was dried by reflux with sodium lump. N-isopropylacrylamide (NIPAAm, Aldrich) was re-crystallized from hexane. All other chemicals and solvents used were obtained either by Aldrich Chemicals or by Productos Químicos Monterrey and were used as received. Aliphatic (N3, N4, N5 and N10, where the number represents the numbers of methylenes in the spacer, Figure 1A) and aromatic monomers (B0, C and D) were prepared according to a previously reported methods.[14,15,21] Green Fluorescent Protein (GFP) was produced in house using recombinant DNA technology using plasmid pQHGF301 and the host bacteria *E. Coli XL1 Blue MRF*.

Synthesis of Gels

Amphoteric gels containing N3 or N5 and DMAEM were prepared by mixing the proper amounts of the monomers, EGDMA (2% molar with respect to the monomers), and AIBN (1% molar with respect to the monomers), in p-dioxane (1M total monomer concentration). The mixture was purged by vacuum and argon bubbling and then introduced between two silanized glass plates separated by silicone tubing (1 mm diameter). The polymerization occurred in a vacuum oven filled with argon at 60 °C. After 24 hours a polymer-gel sheet was obtained and cut to several 1 cm diameter discs. The discs were washed several times with p-dioxane and dried. Polyelectrolyte gels based on N5 and DMAEM (only) were also prepared using the same methodology.

Two types of NIPAAm-gels were also prepared: NIPAAm gels containing aliphatic acid units and NIPAAm gels containing ampholytic (aromatic acid + DMAEM) units. For the first set, the proper amounts of NIPAAm and N4 or N10 (as potassium salts), N,N′-methylene bisacrylamide (BIS) (1% molar), ammonium persulfate (APS, 1% molar) and N,N,N′,N′-tetramethylene ethylenediamine (TEMED, 1% molar) were dissolved in distilled water. Then the procedure described above to prepare gel sheets between glass plates, was followed but maintaining the temperature at 4 °C for 24 h. The so obtained polymer-gel sheet was then washed with large amounts of distilled water and cut to several 1 cm diameter discs. For the second set of gels (amphoteric-NIPAAm), aromatic acid monomers (B0, C and D) were mixed with equimolar amounts of DMAEM and the proper amount of NIPAAm with APS (1% molar) and BIS (1% molar) in dimethylsulfoxide (DMSO) (0.5 M monomer concentration). Each solution was gelled between glass plates following the procedure described above to prepare amphoteric gels sheets, but keeping the reaction at room temperature for 24 h. The gels sheets were then washed with DMSO several times and collapsed with mixtures of DMSO/water with decreasing proportion of DMSO until pure water. Finally, several 1 cm diameter discs were cut from the gel sheets.

The composition of all gels, ampholytic and non ampholytic, with and without NIPAAm was determined by elemental analysis (EA).

Swelling Experiments

pH dependent swelling experiments were performed using eleven buffered solutions with adjusted ionic strength (0.1 M) having nominal pH values from 2 to 12. The exact pH value was measured using a Corning pH meter 430. Pre-weighed dry gel-discs were placed in 11 vials, to each of which 15 ml of a particular buffer solution was added, and the gel-discs were allowed to swell for 24 h. Afterwards the buffer solutions were replaced and the gel-discs allowed to stand in the fresh solution for additional 24 h. The swelled gel disc were taken out from the buffer solution, the excess liquid taped out by using filter paper, and were weighted. The gels were placed again in the corresponding buffers solution. The procedure was repeated until swelling equilibrium was reached. The gel swelling, as mass swelling degree (Q_m), was evaluated according to following equation:

$$Q_m = [(W_s - W_d)/W_d] \times 100 \quad (1)$$

where W_s is the weight of the swollen gel and W_d is the weight of the dry gel. All swelling experiments were performed in triplicate and the average values were taken.

Drug Loading and Release

For loading of conventional drugs, the following methodology was employed: Gel discs were loaded by swelling the gel in a methanolic drug solution (0.2 M) for 24 h. Afterwards the disks were taken out, dried and rinsed for two minutes with a pH 7 phosphate buffer solution to remove excess crystalline drug from the surface and repeating the drying step. Amphoteric gels were loaded with salicylamide, while bidimensional gels (containing NIPAAm) were loaded with salicylic acid. Amphoteric gels

containing NIPAAM were loaded with GFP by soaking the gel in a 0.5 M phosphate buffer solution containing 2 mg/mL of GFP at 4 °C. After 3 days the gels were frozen in dry ice/acetone bath followed by liophylization. Drug release studies for salicylamide and salicylic acid were performed in a dissolutor (Sotax AT70) at 37 °C and 100 RPM. Loaded gels were placed in 300 ml of the buffer solution under study. At predetermined times, 4 ml aliquots were taken and the concentration of drug released was assayed by UV (salicylamide 299 nm, salicylic acid 295 nm). The volume of the sample was replaced by fresh buffer. The release of GFP was evaluated using a UV spectrophotometer with a flow cell (Varian Cary2). Gel was placed in a 50 ml jacketed beaker for temperature control. Stirring in the reservoir was performed with a magnetic stirring bar. The gel was protected with a plastic mesh. Releasing media was circulated trough the flow cell using a peristaltic pump. Absorbance at 475 nm was acquired every 5 min.

Results and Discussion

Ampholytic Hydrogels

Amphoteric hydrogels having basic and acid groups are known to show high swelling in saline solutions, the so called antipolyelectrolyte behaviour.[25] In solutions without salts their swelling behaviour is characterized by high swelling at low pH values when the basic groups are mainly ionized and the acid groups are in the unionized states giving rise to a net positive charge. The same occurs at high pH when the acid groups are ionized and the basic groups are unionized giving a net negative charge. A minimum in swelling is observed at the isoelectric point (IP), where the charges are balanced.[18] This point can be calculated approximately by using the following equation:[26]

$$\mathrm{IP} = (\mathrm{pKa})_b + \log\left\{1/2\left[(1 - R/R) + \left((1 - R/R)^2 + 4/R\ 10^{(\mathrm{pKa})_a - (\mathrm{pKa})_b}\right)^{1/2}\right]\right\} \quad (2)$$

where $(\mathrm{pKa})_b$ is the dissociation constant from the basic monomer, $(\mathrm{pKa})_a$ is the dissociation constant from the acid monomer and R is the molar ratio of acid to base. Table 1 present the composition of the gels studied and the observed and calculated IP. The behaviour of ampholyte gels N5 at different pH-values show the same trend regarding the isoelectric point as observed for the N3 gels (see Table 1).

However, the minimum swelling for gels containing approximately the same proportions of monomers is smaller for the N5 series of gels. This can be attributed to the expected higher hydrophobicity for N5 than for N3.

Figure 2A shows the release kinetics of salicylamide from ampholytic gels in solutions of pH 2 and 7 (phosphates) at I = 0.1 M. The ampholyte containing the monomer N3 releases the drug with near zero order kinetics and slightly pH dependent fashion. The amphoteric gel containing the monomer N5 present a slower

Table 1.
Isoelectric points of ampholytic gels.

Composition (Monomer Feed)	DMAEM Content (Mol% by EA)	Minimum Swelling [pH units]	IPa) [pH units]	IPb) [pH units]
N3-DMAEM (25–75)	73	8.1	8.2	6.9
N3-DMAEM (50–50)	59	5.1-7.1	7.7	6.5
N3-DMAEM (75–25)	19	5.1	5.3	5.3
N5-DMAEM (25–75)	72	8.0	8.2	6.9
N5-DMAEM (50–50)	55	6.1	7.5	6.6
N5-DMAEM (75–25)	6	5.1	5.1	5.1

pKa = 5.8/6.3 for N3/N5 respectively.[14]
[a] Calculated using pKa of DMAEM of 8.1;[26]
[a] Calculated using pKa of DMAEM of 6.6.[27]

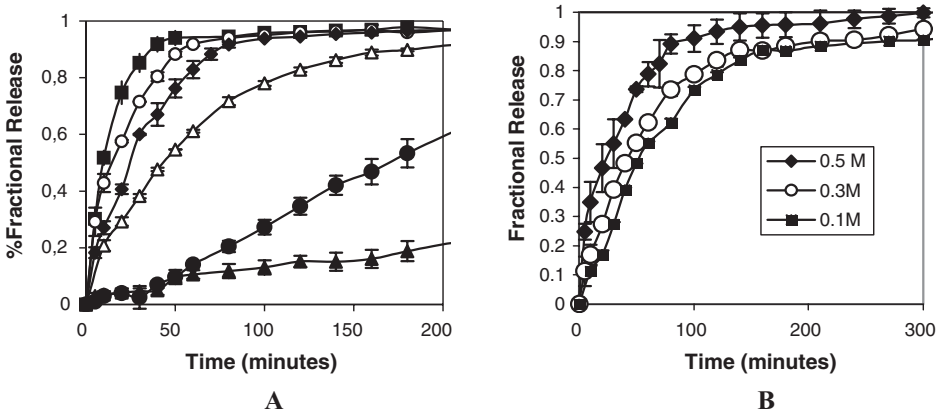


Figure 2.
A: Kinetics of salicylamide release from amphoteric gels: N3/DMAEM 41/59: pH 2 (■), pH 7 (○); N5/ DMAEM 45/55: pH 2 (◆), pH 7 (△); N5-gel: pH 7(●), pH 2 (▲). B: Effect of ionic strength on the kinetics of drug release form N3/DMAEM 45/55 at pH 7: (◆) I = 0.5M, (○) I = 0.3M, (■) I = 0.1M.

kinetics due to its higher hydrophobicity. Slower release is obtained at pH 7 which is closer to its minimum equilibrium swelling point due to charge balance.[18] However, pH dependency of the release is small when compared to the kinetics of the polyelectrolyte gel of N5. For this material rate of release is highly decreased at pH 2 since at this pH the gel is in the unionized, non-swollen state.

Figure 2B presents the effect of ionic strength on the rate of release from ampholytes containing 45 mol% of monomer N5. The rate of release increases as the ionic strength increases. This can be explained in terms of the antipolyelectrolytic effect presented by amphoteric polymers.[25] The charge screening produced by the ions in the medium facilitates the dissociation of the internal salt-bridge formed by the amine and carboxylic groups allowing a higher swelling of the gel and, as a consequence, a faster drug release.

Bidimensional Hydrogels

The incorporation of monomers containing acid groups into temperature sensitive NIPAAm-hydrogels has several well-established consequences. First of all it induces pH-sensitivity into the hydrogel.[22] Further, the swelling degree of the acid modified NIPAAm hydrogel will depend on the ionization degree of the acid groups: by high ionization degree, the swelling degree increases while at low ionization degree the swelling degree decreases.[24] The increase/decrease magnitude depends on the hydrophobic-hydrophilic balance of the acid group containing monomer.[17] Finally, the combination of acid groups in the monomer with amide groups of NIPAAm leads to the formation of hydrogen-bonding interactions at certain pH-values that influences the swelling behaviour.[28] Figure 3 shows Q_m with respect to the temperature for pure NIPAAm gels and the gels N4-NIPAAm(10–90) (containing 10% of N4) and N10-NIPAAm(5–95) (containing 5% of N10) at pH 1.2 and pH 7.4. It is observed that the swelling behaviour is very similar for the pure NIPAAm gel at both pH values. However, N4-NIPAAm(10–90) at pH 1.2 has a transition temperature below that of NIPAAm, while for N10- NIPAAm(5–95) gel a drastic reduction in the transition temperature at this pH is observed; this demonstrates the hydrophobic character of both monomers in the unionized state. On the other hand at pH 7.4, Q_m values are considerable higher for N4-NIPAAm(10–90)

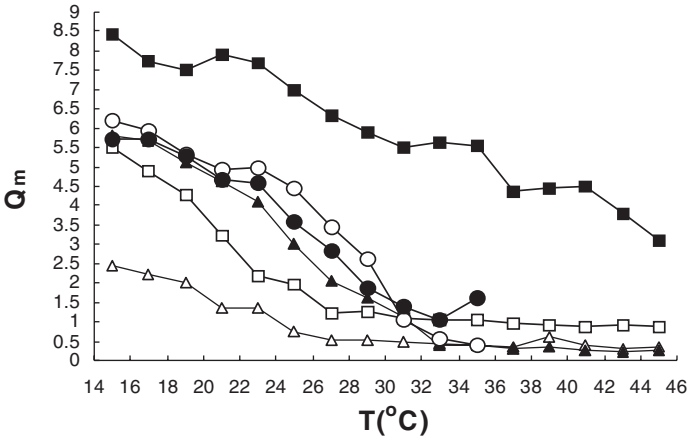


Figure 3.
Effect of temperature on swelling for bidimensional gels: (○) NIPAAm pH 1.2, (●) NIPAAm pH 7.4, (□) N4-NIPAAm(10–90) pH 1.2, (■) N4-NIPAAm(10–90) pH 7.4, (△) N10-NIPAAm(5–95) pH 1.2, (▲) N10-NIPAAm(5–95) pH 7.4.

while the transition temperature is practically lost.

However, gel N10-NIPAAm(5–95) shows a transition temperature still slightly below that of pure NIPAAm gels even at this pH value. This indicates that the ionization of the carboxylic groups in N4-NIPAAm(10–90) at pH 7.4 overcomes the hydrophobicity of the alkyl spacer while for N10-NIPAAm(5–95) the ionization of the carboxylic groups is not enough to overcome hydrophobic effect of the even longer alkyl spacer. In other words, at pH 7.4 the ionization of N10 monomer is below its critical ionization degree (α_{crit}) required for an hydrophilic influence into polyNIPAAm, while the N4 monomer at pH 7.4 is well ionized, far above α_{crit}.[21]

Figure 4A presents drug release results from N4-NIPAAm(10–90) gels. It is observed that at pH 7.4 release rates are very similar at 25 and 37 °C since the gels are in high swollen state at both temperatures. However, at pH 1.2 release kinetics are considerably slow since at both temperatures the gel is in its collapsed state. Figure 4B presents the kinetics of release for N10-NIPAAm(5–95). In this case drug release at pH 7.4 and 37 °C is very slow since the gel is above its transition tem-

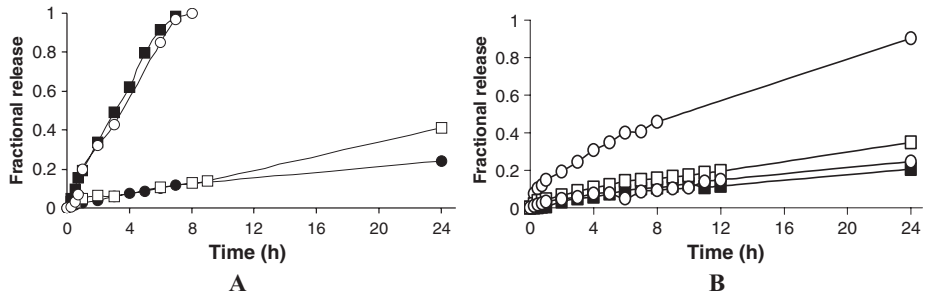
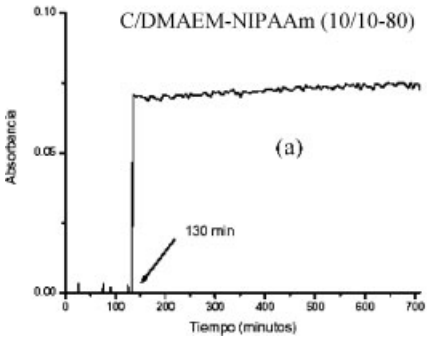


Figure 4.
A: Salicylic acid release from N4-NIPAAm(10–90) gels at: (●) T = 37 °C, pH 1.2, (■) T = 37 °C, pH 7.4, (○) T = 25 °C, pH 7.4, (□) T = 25 °C, pH 1.2. B: Kinetics of release from N10-NIPAAm(5–95) gels at: (●) T = 37 °C, pH 1.2, (■) T = 37 °C, pH 7.4, (○) T = 25 °C, pH 7.4, (□) T = 25 °C, pH 1.2.



Hydrogel	*At 37°C**	*At 25°C*
B0/DMAEM-NIPAAm (2.5/2.5-95)	No release	No release
C/DMAEM-NIPAAm (2.5/2.5-95)	No release	190 min
C/DMAEM-NIPAAm (10/10-80)	No release	130 min
D/DMAEM-NIPAAm (2.5/2.5-95)	No release	No release
NIPAAm (100)	45 min	-

*Stopped after 6 h

Figure 5.
Release behaviour of GFP at pH 7.4 from bidimensional amphoteric pH and temperature sensitive hydrogels.

perature (collapsed state). Similarly, at pH 1.2 there is almost no release at both temperatures. Only at pH 7.4 and at a temperature where this gel swells moderately, a release of the entrapped drug is observed. These preliminary results indicate that, selecting the proper carboxyalkylmethacrylate comonomer to incorporate into NIPAAm-gels in small amounts, we can tune the release for a target pH at constant body temperature with potential for site specific release (e.g. intestinal release).

In general, the observed release rates for salicylic acid are much slower at both pH values than the reported for NIPAAm-MAAc hydrogels of similar acid content by Sousa *et al.*[24] This is a further evidence that the hydrophobic effect introduced by the spacer is important both at simulated gastric and intestinal conditions, which could yield sustained release hydrogels if the proper comonomer type and content is well chosen.

In the case of the gels loaded with GFP, the fact that GFP has a nominal molecular weight of 27,000 Dalton[29] and the multiple possibilities of interactions arising from the aminoacids building this protein, with the acid and basic groups of the hydrogels, precluded a complex release behaviour. A typical experiment of GFP release from amphoteric gels containing NIPAAM is presented in Figure 5.

The time axis starts at zero after the temperature in bath was lowered from 37 to 25 °C. Before that, there was no release observed at 37 °C for 6 h in the same buffer. There is a considerable lag time to observe some protein release indicating that certain degree of swelling is required for the protein to diffuse out of the gel. In the same Figure 5 a table is included presenting the results obtained with the gels studied. The results indicate that protein release can be obtained after different time lags using these gels, however kinetics are considerably slow.

Conclusions

New acid monomers with hydrophobic spacers in hydrogel formulations bring a change in the pKa, in the hydrophilic-hydrophobic balance and in thermal and mechanical properties. This opens new possibilities for drug delivery purposes. Ampholytic hydrogels release salicylamide with close to zero-order kinetics (pH = 2 and 7). The release rate is increased with salt addition. Bidimensional (T- and pH-sensitive) hydrogels show sustained release of salicylic acid at pH 7.4 with minimum release at pH 1.2; while more hydrophobic hydrogels, including 10 methylenes as spacer, release it depending on pH (7.4) and temperature (T < transition T). Green fluorescent protein was released from T- and pH-sensitive ampholytic hydrogels after different lag times depending on hydrogel composition.

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[1] V. Sáez, E. Hernáez, L. Pérez, *Rev. Iberoamer. Polim.* **2003**, *4*(2), 111.
[2] N. A. Peppas, "*Hydrogels in Medicine and Pharmacy*", CRC Press, Boca Raton, Florida 1987.
[3] T. Okano, *Adv. Polym. Sci.* **1993**, *110*, 179.
[4] I. S. Kim, N. S. Kim, C. S. Cho, *Arch. Pharm. Res.* **1996**, *19*, 18.
[5] B. Jeong, S. W. Kim, Y. H. Bae, *Adv. Drug Deliv. Rev.* **2002**, *54*, 37.
[6] P. Gupta, K. Vermani, S. Garg, *Drug Discov. Today* **2002**, *7*, 569.
[7] E. S. Gil, S. M. Hudson, *Prog. Polym. Sci.* **2004**, *29*, 1173.
[8] J. E. Moeckel, B. C. Lippold, *Pharm. Res.* **1993**, *10*, 1066.
[9] A. T. Pham, P. I. Lee, *Pharm. Res.* **1994**, *11*, 1379.
[10] P. Colombo, R. Bettini, G. Massimo, P. L. Castellani, P. Santi, N. A. Peppas, *J. Pharm. Sci.* **1995**, *84*, 991.
[11] H. Kim, R. Fassihi, *J. Pharm. Sci.* **1997**, *86*, 323.
[12] U. Conte, A. Maggi, L. Colombo, A. Lamanna, *J. Control. Rel.* **1993**, *26*, 39.
[13] U. Conte, A. Maggi, *Biomat.* **1996**, *17*, 889.
[14] E. Rogel-Hernández, A. Licea-Claveríe, J. M. Cornejo-Bravo, K.-F. Arndt, *Desig. Monom. Polym.* **2001**, *4*, 343.
[15] A. Licea-Claveríe, E. Rogel-Hernández, J. A. López-Sánchez, L. A. Castillo, J. M. Cornejo-Bravo, K. F. Arndt, *Desig. Monom. Polym.* **2003**, *6*, 67.
[16] E. Rogel-Hernández, A. Licea-Claveríe, J. M. Cornejo-Bravo, K. F. Arndt, *Rev. Soc. Quim. Mex.* **2003**, *47*, 251.
[17] A. Licea-Claveríe, E. Rogel-Hernández, R. Salgado-Rodríguez, J. A. López-Sánchez, L. A. Castillo, J. M. Cornejo-Bravo, K. F. Arndt, *Macromol. Symp.* **2004**, *207*, 193.
[18] A. Licea-Claveríe, R. Santos-Rosas, L. S. Palazuelos-Armijo, J. M. Cornejo-Bravo, K.-F. Arndt, *Polym. Prep. (Amer. Chem. Soc, Div. Polym. Chem.)* **2005**, *46*(1), 597.
[19] A. E. English, T. Tanaka, E. R. Edelman, *Polymer* **1998**, *39*, 5894.
[20] J. M. Cornejo-Bravo, M. E. Flores-Guillén, E. Lugo-Medina, A. Licea-Claveríe, *Int. J. Pharm.* **2005**, *305*, 52.
[21] R. Salgado-Rodríguez, A. Licea-Claveríe, K. F. Arndt, *Eur. Polym. Jnl.* **2004**, *40*, 1931.
[22] C. S. Brazel, N. A. Peppas, *J. Control. Rel.* **1996**, *39*, 57.
[23] A. S. Hoffman, *Adv. Drug Deliv. Rev.* **2002**, *17*, 3.
[24] R. G. Sousa, A. Prior-Cabanillas, I. Quijada-Garrido, J. M. Barrales-Rienda, *J. Control. Rel.* **2005**, *102*, 595.
[25] S. Wen, W. T. K. Stevenson, *Colloid Polym. Sci.* **1993**, *271*, 38.
[26] C. S. Patrickios, W. R. Hertler, N. L. Abbott, T. A. Hatton, *Macromol* **1994**, *27*, 930.
[27] A. S. Lee, A. Gast, V. Bütün, S. P. Armes, *Macromol* **1999**, *32*, 4302.
[28] A. Prior-Cabanillas, I. Quijada-Garrido, G. Frutos, J. M. Barrales-Rienda, *Polym.* **2005**, *46*, 685.
[29] M. A. Perozzo, K. B. Ward, R. B. Thompson, W. W. Ward, *J. Biol. Chem.* **1988**, *263*, 7713.

