Incorporation of salicylic acid derivatives to hydrophilic copolymer systems with biomedical applications

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Hydrogels based on polymeric derivatives of salicylic acid have been prepared for biomedical applications by free radical copolymerization of 2-hydroxy-4-methacrylamidobenzoic acid, 4HMA, and 2-hydroxy-5-methacrylamidobenzoic acid, 5HMA, with 2-hydroxyethylmethacrylate, HEMA, in a wide range of compositions. The reactivity ratios of 4HMA and 5HMA with HEMA in radical copolymerization processes have been determined from their ^{1}H NMR spectra by applying linearization methods and non-linear least square treatments. Tgs of the corresponding copolymers were analyzed by DSC. The swelling behavior in water of the prepared copolymers was studied in comparison to poly-(HEMA), poly-(4HMA) and poly-(5HMA) hydration degrees, being in all cases superior to 35%. The hydrolytical behavior of the synthesized copolymers was studied at three different pHs (2, 7.4 and 10) determining the release percentage of the salicylic acid derivatives, 4-amino salicylic acid, 4ASA, and 5-amino salicylic acid, 5ASA, analyzed by high performance liquid chromatography (HPLC). The release analysis was followed during 230 days and a pH dependence was observed obtaining the highest release percentages at pH = 10, whereas at physiological pH (7.4) the release percentages were in range from 2 to 5% at that time for all copolymer systems. The hydrolytical stability is enough for long-term applications like bone cements, ionomers, etc.

1. Introduction

2-Hydroxyethyl methacrylate (HEMA) homopolymer and copolymers have been widely used in applications such as vascular grafts coating, vascular valves, vascular prosthesis and contact lenses due to their capability of forming biocompatible hydrogels with an excellent tolerance and good biostability, as well as a great bioadhesion [1–6].

The salicylic acid derivatives 4- and 5-aminosalicylic acids (4-ASA and 5-ASA) have been linked to several types of polymers and applied as pharmacological agents and anti-inflammatory drugs [7–9]. We previously reported the synthesis of the two methacrylic monomers derived from aminosalicylic acid, 2-hydroxy-4-*N*-methacylamidobenzoic acid, 4HMA, and 2-hydroxy-5-*N* methacylamidobenzoic acid, 5HMA[10] (see Fig. 1), which have been incorporated to acrylic bone cement formulations [11] due to their ability to form molecular complexes with calcium atoms [12] which can facilitate bone regeneration in these types of applications. In this sense, 5- HMA was added to the liquid phase of classical acrylic bone cement formulations with methyl methacrylate, MMA, and with HEMA, showing excellent curing parameters and a slightly higher hydration degree than PMMA formulations [13] also having the possibility of behaving as a control release systems of the salicylic acid side groups bonded to the macromolecular chains by amide links.

In addition, due to the intrinsic pharamacological activity of these compounds (they present pharmacological activity as macromolecules) HEMA derivatives might be quite interesting in alternative applications as hydrophilic coatings or hydrogel matrices. As it has been demonstrated for analogous polymers bearing salicylic acid or NSAIDs (non-steroidal anti-inflammatory drugs), HEMA copolymers not only act as drug carriers behaving as drug delivery systems in which the drug can be released in mild conditions by hydrolytical processes [13, 14] but also offer the possibility of being pharmacologically active in their macromolecular form [15].

The aim of this paper is the preparation of HEMA derivatives bearing the mentioned compounds by copolymerization reactions of 4HMA and 5HMA with HEMA. The copolymerization reaction as well as the

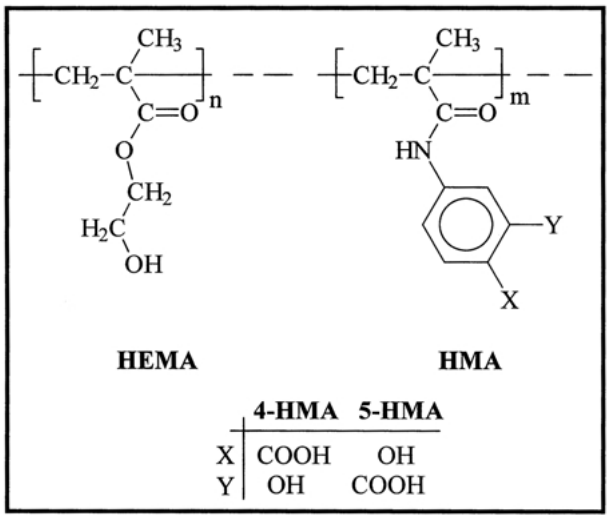

Figure 1 Schematic representation of units in copolymer chains, showing the functional groups of side residues.

aqueous behavior, chain flexibility and hydrolytical stability, have been characterized.

2. Materials and methods

2.1. Materials

The monomers 4HMA and 5HMA (see Fig. 1) syntheses have been described in a previous paper [12]. Azobisisobutironitrile (AIBN) (Merck) was recrystallized from methanol (melting point 104 °C). 2-Hydroxyethyl methacrylate, HEMA, was purified according to the literature [16]. The solvents diethyl ether (Quimicen), methanol (Quimicen) and dimethylformamide, DMF, (Scharlau) were purified by standard procedures.

2.2. Copolymerization

All copolymerization reactions were carried out in DMF solutions at 50 ± 0.1°C in Pyrex glass ampoules sealed under high vacuum. The monomer and initiator concentration were 1.0 and 1.5×10^{-2} mol l^{-1}, respectively. The sealed ampoules were vigorously shaken and immersed into a water bath regulated at the polymerization temperature. After reaction, the copolymers were precipitated into a large excess of diethyl ether, washed with water and dried under vacuum until constant weight was attained.

2.3. Characterization

The copolymers obtained from different mixtures of 4HMA and 5HMA with HEMA, were analyzed by ^{1}H nuclear magnetic resonance spectroscopy (NMR) with a Varian XLR-300 operating at 300 MHz. The spectra were recorded on 5% (w/v) deuterated methanol solutions (CD_3OD).

2.4. Differential scanning calommetry (DSC) analysis

The Tg values of the corresponding homopolymers and prepared copolymers were determinated with a Perkin-Elmer DSC-7 calorimeter. Measurements and calibration were carried out at a heating rate of 10 °C min^{-1}. Tg was taken as the midpoint of the transition region. Samples ($\sim$40 mg weight) were introduced into the aluminum pan, heated at 450 K. Compressed and quenched at room temperature before carrying out the measurements. Tg values determined at 5 and 15 °C min^{-1} heating rates, gave a deviation of $< 2\%$ with respect to those obtained at 10 °C min^{-1}.

2.5. Swelling behavior

Films of 0.4–0.5 thickness were prepared by slow evaporation of a solution of 0.5g of the copolymer samples in 5 ml of DMF/methanol (1 : 1). Copolymers prepared with 10 and 20% wt of 4HMA and 5HMA were used. In order to obtain homogeneous discs, a cylindrical mold of Teflon (20 mm diameter and 8 mm depth) was prepared. After evaporation and drying at reduced pressure, transparent and clear films were obtained. Homopolymers poly (4HMA) and poly (5HMA) films were also prepared to carry out comparative studies.

The study of the hydration degree of films was followed gravimetrically by measuring the weight with the time of immersion in 10 ml of distilled water at 37 °C. Measurements were taken until the equilibrium was reached, which was considered to be when three consecutive measurements gave the same value.

2.6. Hydrolytical behavior

The hydrolytical study of the homopolymers poly (4HMA), poly (5HMA), and the prepared copolymers, was carried out over a period of 230 days in buffer solutions of pH = 2, 7.4 and 10, analyzing the 4-ASA and 5-ASA release by high performance liquid chromotography (HPLC). The HPLC eluent was constituted by a buffer solution (pH = 7.8) of KH_2PO_4 (0.067 M)/acetonitrile/tetrahydrofuran, THF, (85/10/5), using a BIO-RAD Bio-Sil C18 HL 90–5 column connected to a Perkin-Elmer LC 250 pump, a UV/VIS detector Perkin-Elmer LC–95 and to a computer where the chromatograms were registered. The UV spectra gave maximum absorbance at 265 and 332 nm for the 4-ASA and 5-ASA solutions, respectively. Six standard solutions of 4-ASA and 5-ASA of known concentration between 750–25 μM were prepared to obtain the corresponding calibration lines. Solutions were injected onto the column using a 1 ml/min flow. On the other hand, the buffer solutions were prepared from HCl and glycine (pH = 2), KH_2PO_4 and Na_2HPO_4 (pH = 7.4) and NaOH and Glycine (pH = 10). 50 mg of the corresponding homopolymers and copolymers were added to 125 ml of each buffer solution. One ml samples were taken from each buffer at different times, precipitating the corresponding polymers by adding 0.4 ml of HCl 0.5 M, and analyzing the 4-ASA and 5-ASA solutions by adding the column eluent (1/1).

3. Results and discussion

The 4HMA-HEMA and 5HMA-HEMA copolymers were prepared by radical copolymerization reactions as described in the experimental section. The presence of

the polar carboxylic, phenolic (4HMA and 5HMA) and hydroxyl (HEMA) groups, together with the rigid character of the aromatic ring (4HMA and 5HMA) or the flexible oxyethylene residue (HEMA), impart to the material some particular properties as we will discuss later, specially those related with the behavior in aqueous media. The global flexibility as well as the polar interactions between the different units (4HMA-4HMA, HEMA-HEMA, etc) determine most of the characteristics of the hydrogel matrix. In this sense, a deep study of the copolymerization reactions, and mainly the knowledge of the sequence distribution, will be very helpful. Therefore, we have determined the reactivity ratios for both copolymerization reactions.

Copolymers at low conversion were prepared with molar fractions in the monomer feed, F_{4HMA} and F_{5HMA}, from 0.20 to 0.80 as shown in Table I. The reaction time was initially regulated to reach conversions of > 5 wt % in order to satisfy the differential copolymerization equation [17]. The molar fraction of monomer units in the copolymer chains was determined from the ^{1}H NMR spectra of the copolymer samples prepared with different monomer feed. The analysis was carried out by comparison of the integrated intensities of resonance signals with chemical shifts assigned to the aromatic protons (6.6–8.1 ppm) of monomers 4HMA and 5HMA, and from the signals with chemical shifts 3.7 ppm and 4.0 ppm corresponding to the -CH_2OH and -O-CH_2-HEMA groups as can be observed in Fig. 2 for the 4HMA-HEMA system.

The reactivity ratios of the monomers were determined according to the general copolymerization equation by application of the linearization methods proposed by Fineman and Ross [18] and Kelen and Tudos [19], as well as the non-linear squares treatment suggested by Tidwell and Mortimer [20]. The results obtained are shown in Table II and it can be observed that r_4, r_H and r_5, r_H reactivity ratio values are somewhat different depending on the applied method. In this sense, the Tidwell-Mortimer reactivity ratios are suggested to be the most probable because of the well-known higher accuracy of the non-linear method. Fig. 3 shows the 95% confidence limits defined by the area of the elliptical diagram determined by application of the mathematical treatment [19, 21], together with the points of the reactivity ratio values obtained by the three methods. These diagrams confirm the good approximation of the reactivity ratio values as indicated by the reduced dimensions of the ellipse. Fig. 4 shows the composition diagrams of both copolymer systems where the solid lines correspond to the theoretical diagrams obtained according to the Mayo-Lewis [17] equation, and the points are the experimental values obtained at low conversions. Both systems exhibit similar patterns and moreover, there is good agreement between the theoretical curve and the experimental data indicating again the reliability of the calculus and confirming the fitting to the terminal model. The reaction gives rise to random sequences with a slightly higher content of HEMA in the copolymer chains than in the feed. Thus, there is a tendency (mainly for the compositions rich in HEMA) to the formation of short HEMA sequences isolating 4HMA or 5HMA units. This fact can also be observed in Fig. 5 where the

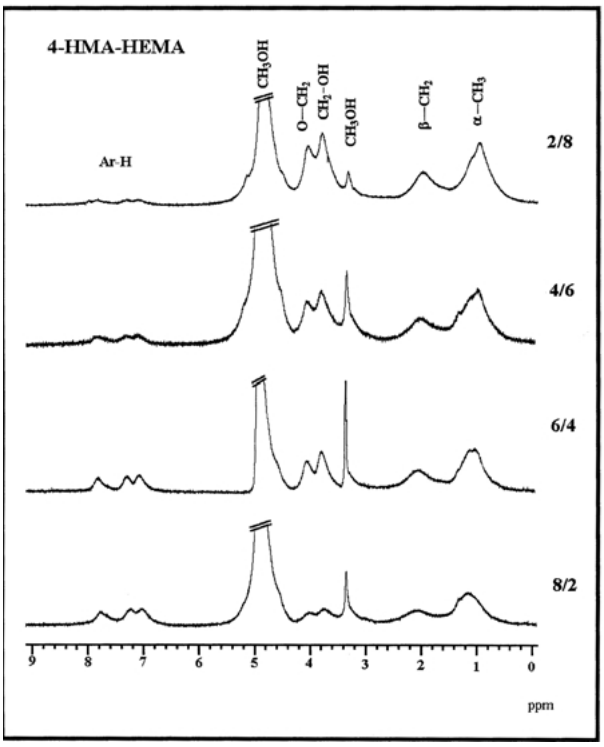

Figure 2 ^{1}H-NMR spectra of 4-HMA-HEMA copolymers registered in deuterated methanol solution at 30 °C.

TABLE I Molar and weight composition in the feed copolymer systems and Tgs of 4HMA-HEMA and 5HMA-HEMA prepared copolymers determined by differential scanning calorimetry

F_{4HMA} Feed	f_{4HMA} Copolymer	W_{4HMA} Copolymer	Tg, K
0	0	0	358
20	11	17	400
40	23	34	436
60	42	55	473
80	69	79	497
100	100	100	504
F_{5HMA} Feed	f_{5HMA} Copolymer	W_{5HMA} Copolymer	Tg, K
0	0	0	358
20	11	17	399
40	23	34	443
60	39	52	473
80	58	70	505
100	100	0	534

TABLE II Copolymerization parameters of the free radical copolymerization of 4HMA and 5HMA with HEMA

	Parameter	Method		
		Finemann-Ross	Kelen-Tudos	Tidwell-Mortimer
	r_H	2.32 ± 0.19	2.16 ± 0.16	2.42
4HMA-HEMA	r_4	0.67 ± 0.04	0.55 ± 0.24	0.62c
	r_H	1.88 ± 0.07	1.91 ± 0.04	1.88
5HMA-HEMA	r_5	0.25 ± 0.01	0.27 ± 0.05	0.26

instantaneous sequence distribution in terms of triads of 4HMA/HEMA and 5HMA/HEMA (determined from the reactivity ratio values using the terminal model) are plotted against the molar fraction of monomers 4HMA and 5HMA in the feed. It can be observed that for copolymers obtained at low conversion with feed composition rich in HEMA, the 4-HMA and 5-HMA monomer units are mainly present in H4H and H5H alternating triads. That means that samples rich in HEMA (which are the copolymers more interesting from a practical point of view) present the active units isolated between HEMA units and therefore the neighbor-to-neighbor interactions will be mainly 4HMA-HEMA or 5HMA-HEMA (in addition to the HEMA-HEMA), which might be important to understand the aqueous behavior. One interesting point is that H5H population increase with HEMA content faster than H4H (as a consequence of the lower value of r_5 with respect to r_4), which means that for the same composition the 5HMA units tend to be more isolated by HEMA blocks than the 4HMA units.

The previous discussion is referred to the character of the instantaneous copolymer formed during the reaction. However, form a practical point of view, high conversion materials are prepared. In this case, there is a scanning on feed compositions with conversion as a consequence of the higher consumption of the more reactive comonomer (HEMA). These particular copolymerization reactions do not change significantly the sequence distribution with conversion till the last steps of the reaction. Fig. 6 shows a contour plot of the H44* or H55* triad population (taken as an example because it will be used in the forthcoming discussion) as a function of the conversion and the initial feed composition, as determined using the well-known Skeist and Gallardo *et al.* [23] treatment. There is a shifting of the triad molar fraction towards higher values with conversion because of the higher consumption of HEMA and the increasing probability of the formation of H44 or H55 as the 4 or 5 monomer concentration increases. In any case, for

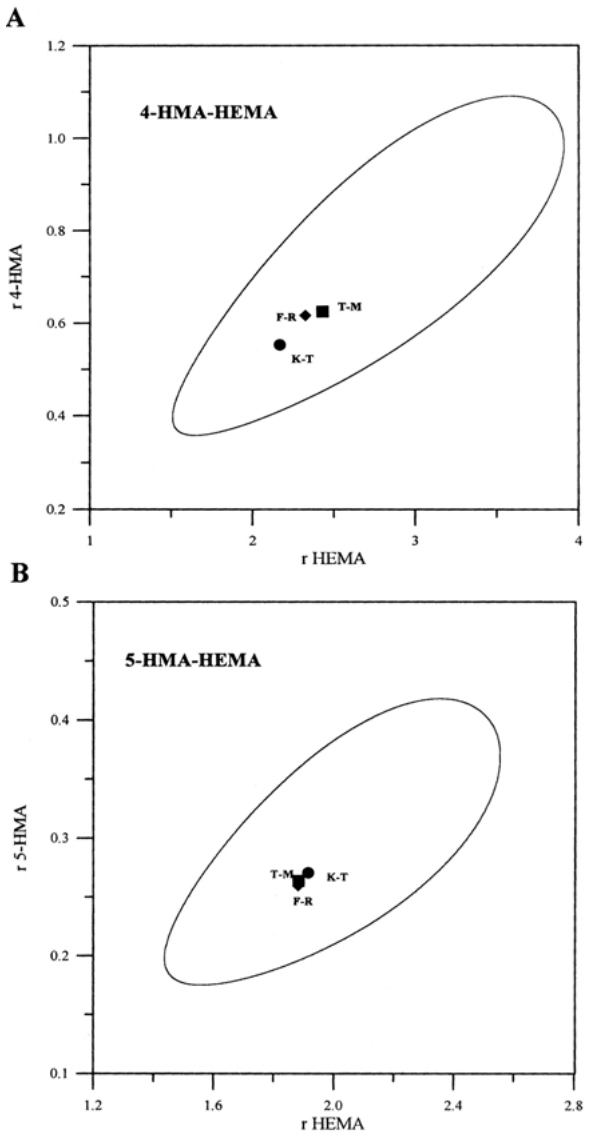

Figure 3 ''Confidence limits, 95%'' of the copolymerization parameters (reactivity ratios) of the systems 4-HMA-HEMA and 5-HMA-HEMA in free radical processes.

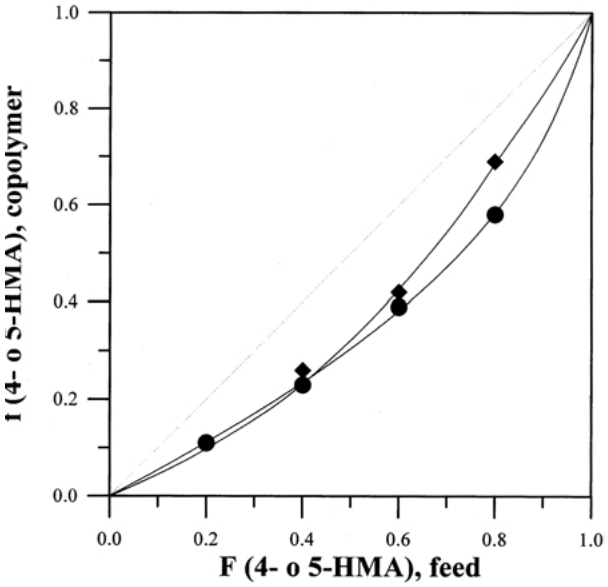

Figure 4 Average composition diagram for the free radical copolymerization of 4-HMA- HEMA (◆) and 5-HMA-HEMA (●) systems at low conversion.

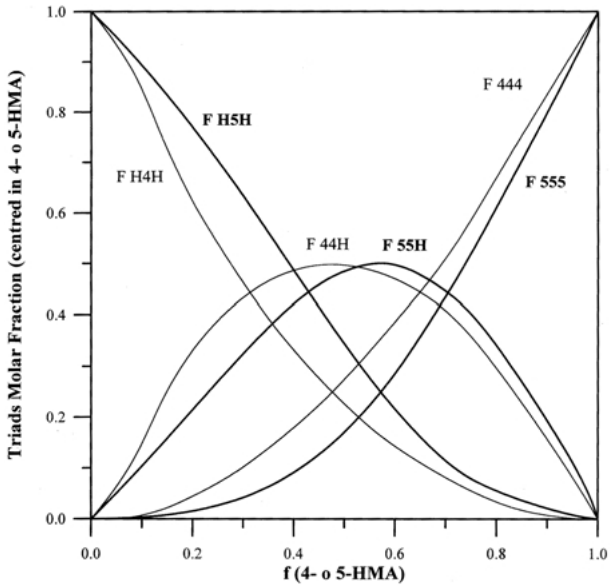

Figure 5 Statistical distribution of 4-HMA or 5-HMA centered triads in copolymers prepared by free radical reactions at low conversion.

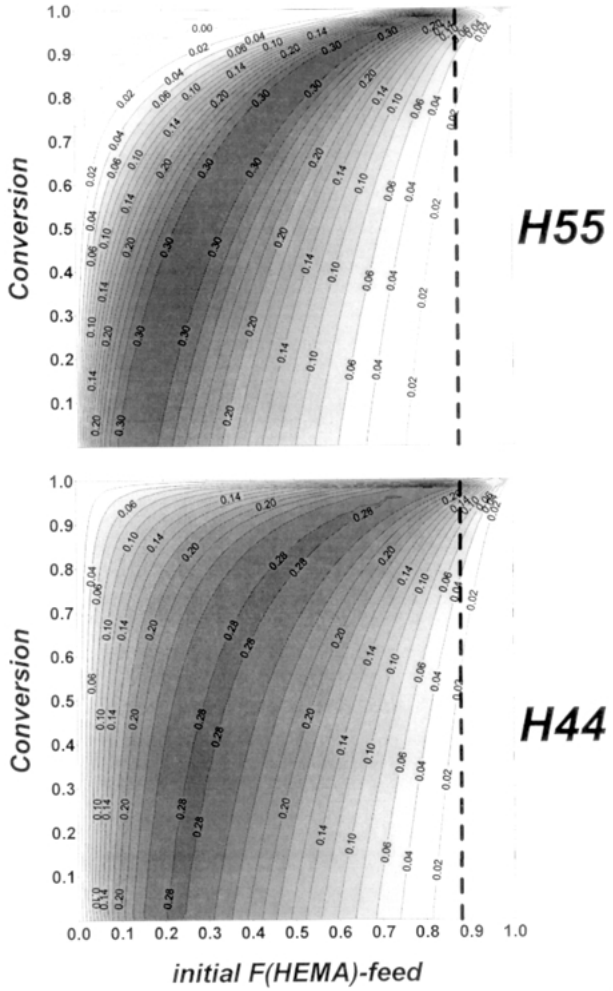

Figure 6 Effect of conversion degree on the distribution of 4-HMA or 5-HMA centered triads containing two neighboring units of the respective salicylic acid derivatives.

HEMA rich copolymers (the courses of the reactions for a initial feed composition of 0.88 of HEMA, which correspond to an 80 wt % are depicted as thick lines) there is a slightly higher population of cumulative H44 than H55 in most of the range of conversions indicating that the presence of neighbor 5–5 units is less probable than 4–4 in agreement with the instantaneous analysis.

3.1. *In vitro* studies

The preparation of the hydrogels was carried out by copolymerization reactions at total conversion using concentrations of 4HMA and 5HMA of 10 and 20 wt % in order to study the hydration and hydrolysis processes. Copolymer composition calculated by ^{1}H NMR spectroscopy was similar to that of the feed as expected for copolymers obtained at total conversion.

The high conversion materials were used to perform the *in vitro* assays. Fig. 7 shows the equilibrium swelling degrees of both copolymeric systems as a function of the average weight composition. The systems exhibit an interesting non-linear dependence with the composition. The equilibrium swelling degree of both homopolymers poly-4HMA and poly-5HMA are lower than the Heq of poly-HEMA. On the other hand, the swelling kinetics also depend on the composition: the initial water uptake decreases with the increasing 4HMA or 5HMA unit content in the copolymer chains (see Fig. 8).

These results agree with the DSC data obtained from the thermal analysis as depicted in Fig. 9 where the dependence of the inverse Tg is represented as a function of the weight composition in the copolymers. The Tgs of the homopolymers poly-4HMA and poly-5HMA are higher than the Tg of poly-HEMA because of the rigidity provided by the aromatic ring and the polar interactions compared with the flexible oxyethylene group of HEMA. On the other hand, the Tgs of the copolymers are strongly deviated from the Fox equation (the straight line) being higher than the weight average of the inverse of the

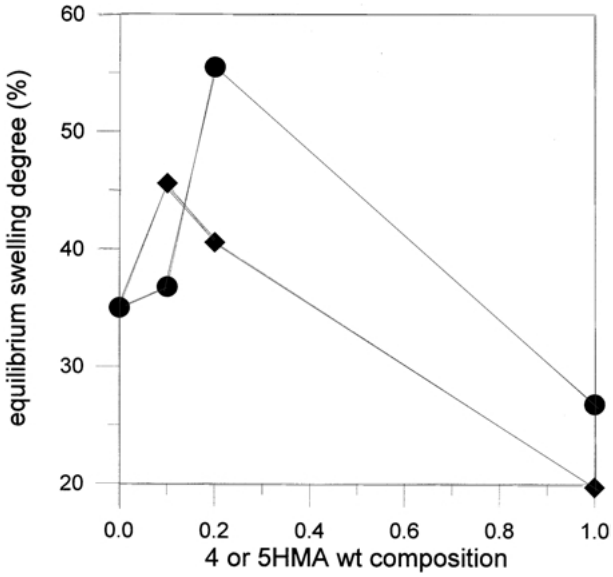

Figure 7 Effect of average weight composition on the hydration process for 4-HMA-HEMA (◆) and 5-HMA-HEMA (●) copolymer systems.

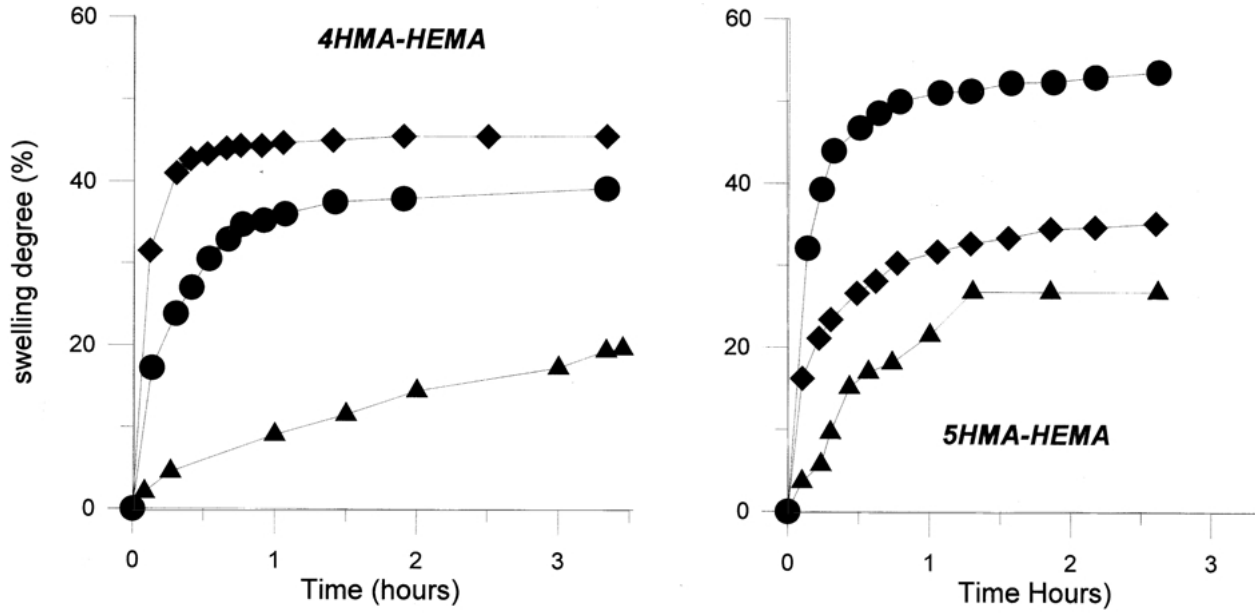

Figure 8 Kinetics of the swelling process for highly hydrophiclic copolymers prepared at total conversion. (▲) 4-HMA and 5-HMA respective homopolymer; (◆) 10% (w/w) of 4-HMA or 5-HMA; (●) 20% (w/w) of 4-HMA or 5-HMA in the corresponding copolymer systems.

homopolymers. This behavior has been well described for polar copolymeric systems with hydrogen atoms able to form H-bonds [24]. The schemes represented in Fig. 1 give us a good idea of the high possibility of H-bond formation because the presence of the polar -NH-, -COOH- and -OH (of both unit) groups. Strong polar interactions lead to a higher compact structure and to a lower flexibility. After the immersion in aqueous media, there is competition between the solvation phenomena and these strong polar interactions, being the swelling process governed kinetically by the comonomer composition and the related rigidity, as taken out from the thermal analysis. That might explain the differences observed in Fig. 8 because the richer in 4HMA or 5HMA the copolymer system is, the more compact is the original material. Finally, from the results obtained in Fig. 7, it seems that there is an optimum composition where the combination of the flexibility of the oxyethylene residue and the hydrophilia associated to the presence of such polar group as COOH, OH and NH in the 4HMA or 5HMA units, leads to a maximum equilibrium swelling degree (higher than the Heq of both homopolymers). This maximum appears at HEMA rich compositions but slightly different in both copolymer systems, at 10 and 20 wt % of 4HMA or 5HMA for 4HMA-HEMA and 5HMA-HEMA, respectively. From the previous discussion on sequence distribution, this displacement in the maximum could be related to the disappearance of the interaction neighbor-neighbor 5HMA-5HMA, which occurs at lower HEMA content than in the case of the 4HMA- 4HMA as a consequence of the analysis of the copolymerization reactions (see Figs 5 and 6). Figs 10 and 11 show the cumulative release profiles of both copolymer systems represented as released drug (in percentages) as a function of time. The hydrolysis rate is clearly dependent on pH, being higher at basic pH because of the well known basic catalysis of the amidolysis reaction. The release rate at physiological pH is very low, specially for the 4HMA-HEMA system. On the other hand, the amide character of the labil bond makes it sensitive to acid hydrolysis and as a consequence they exhibit some hydrolysis degree at acid pH, similar or higher than at neutral pH for the 5 or 4HMA's systems. In all the cases, the profiles, after a initial release, reach a zero order release at least during several months. The relatively hydrolytical stability of these hydrophylic formulations provides excellent characteristics for applications with associated anti-inflammatory properties, like bone cements, dental filling materials or even glass alkanoate cements and glass ionomers. However, analysis would be necessary of the enzymatic activation of the hydrolytical processes

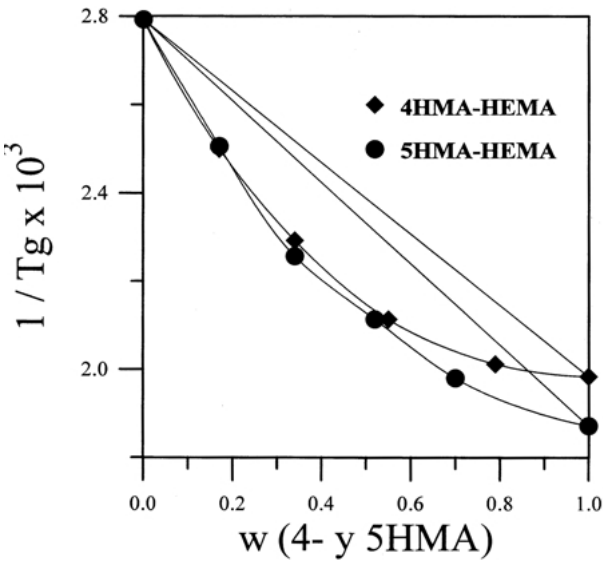

Figure 9 Variation of the glass transition temperature with the average weight composition of copolymer systems (◆) 4-HMA-HEMA and (●) 5-HMA-HEMA.

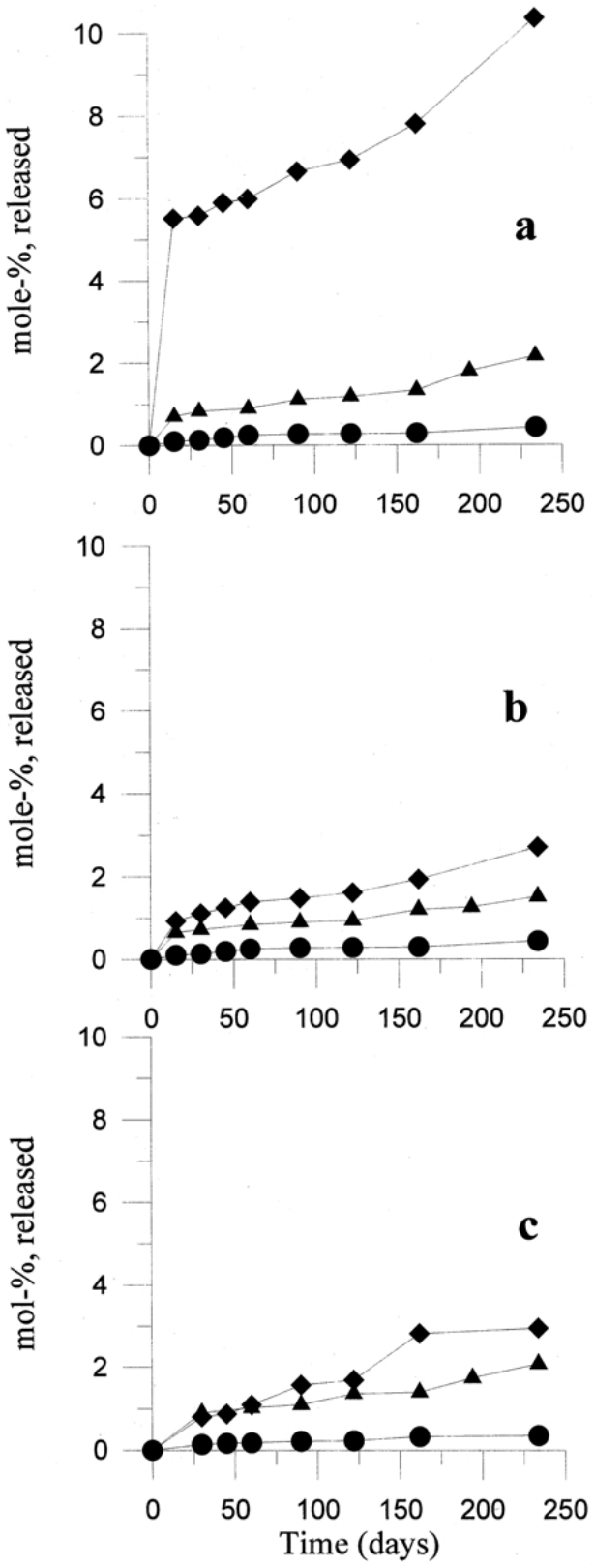

Figure 10 Hydrolytical behavior of 4-HMA-HEMA copolymers (a) 10% (w/w) of 4-HMA, (b) 20% (w/w) of 4-HMA and (c) 4-HMA homopolymer, at (●) pH = 7.4, (▲) pH = 2 and (◆) pH = 10.

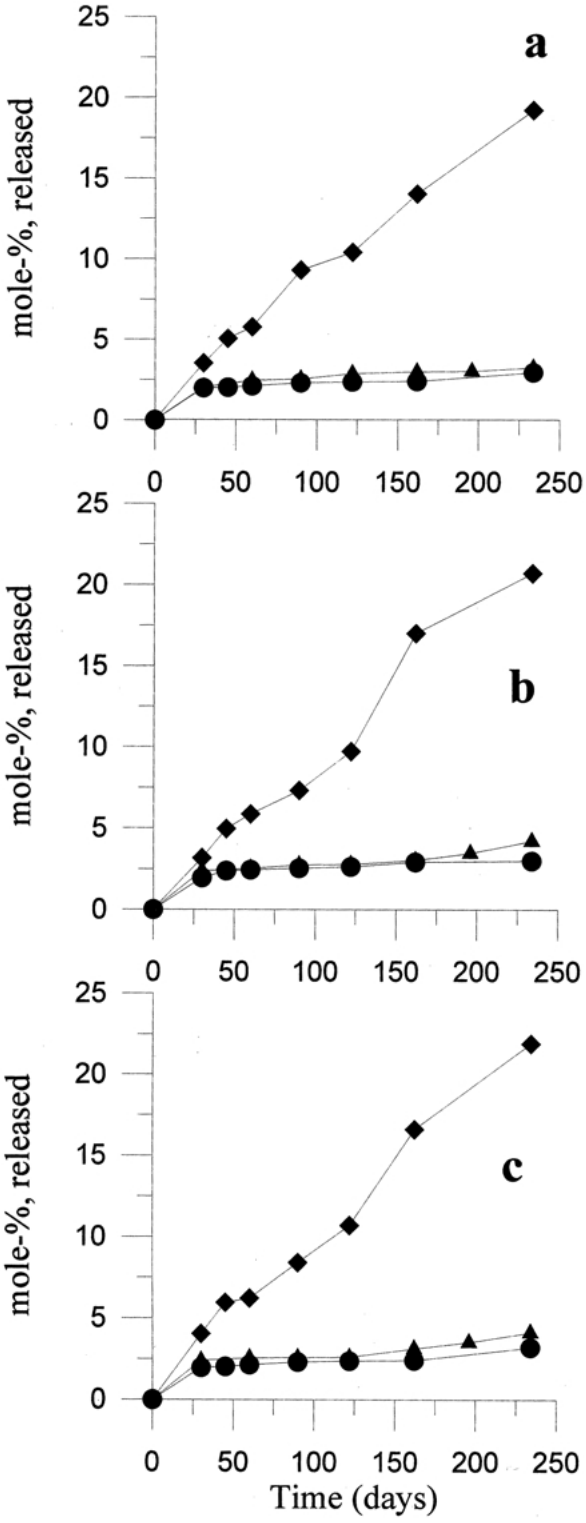

Figure 11 Hydrolytical behavior of 4-HMA-HEMA copolymers (a) 10% (w/w) of 5-HMA, (b) 20% (w/w) of 5-HMA and (c) 5-HMA homopolymer, at (●) pH = 7.4, (▲) pH = 2 and (◆) pH = 10.

mainly in physiological conditions, but the results obtained so far indicate the hydrolytical stability of the copolymers prepared with relatively high content in HEMA, is enough to guarantee the performance of applications with the additional pharmacological effect of the 4-ASA or 5-ASA residues.

The differences between 4- and 5- are related to their swelling behavior. It was discussed previously that 5HMA-HEMA samples, at that composition range, as well as the homopolymer poly-5HMA, swell significatively more than the 4HMA derivatives, leading to a higher water penetration and therefore to a relatively higher release rate which is in agreement with the obtained results. On the other hand, the 4HMA-HEMA 10 present the highest release rate for this system while the 5HMA-HEMA 20 has a much higher release rate than

the 4HMA-HEMA 20 in agreement with the swelling behavior.

A very slow hydrolysis rate can be an advantage in some specific applications such as hydrophilic coatings in vascular applications. San Roman *et al*. [25, 26] have successfully used similar polymeric systems for this particular application. The polymers are expected to exhibit activity in their macromolecular form as in other similar systems [15] because of the intrinsic antithrombogenic properties of 4-ASA and 5-ASA, and the hydrolysis of the amide group leads to (in addition to the release of the antithrombogenic agent) the formation of anionic carboxylate residues randomly distributed along the macromolecular chains (as was discussed in the first section of this paper). This fact might improve the vascular long-term performance.

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