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Degradation of hydrolysable hydrogel microspheres

Van Nga Nguyen,^{a,b} Christine Vauthier,^{*ac} Nicolas Huang,^{ac} Jean-Louis Grossiord,^{ac} Laurence Moine^{ac} and Florence Agnely^{ac}

^a Univ Paris-Sud, Faculté de Pharmacie, 5, rue J.B. Clément, 92296 Chatenay-Malabry, France

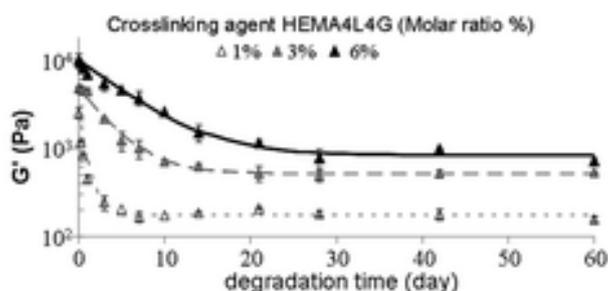
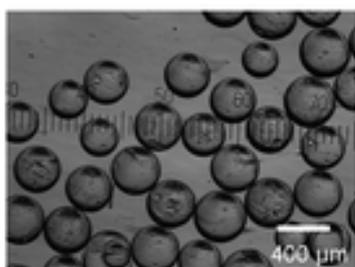
^b Occlugel S.A.S., 12 Rue Charles de Gaulle, 78350 Jouy en Josas, France

^c CNRS UMR 8612, Institut Galien Paris-Sud, 5, Rue J.B. Clément, 92296 Chatenay-Malabry, France.

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Correspondance : Christine Vauthier, CNRS UMR 8612, Institut Galien Paris-Sud, 5, Rue J.B. Clément, 92296 Chatenay-Malabry, France, Email : christine.vauthier@u-psud.fr; Fax: +33 146835312



Abstract

This work aims to monitor the hydrolysis of microspheres made of synthetic degradable hydrogels. Well spherical microspheres incorporating hydrolysable crosslinkers inside their structure were first synthesized by direct suspension polymerization. Their elastic properties evaluated prior to degradation by a rheological method show that microspheres behave like nearly pure elastic material with an elastic modulus varying from 2 to 11 kPa according to their composition. The pH, swelling and rheological properties were used to follow microsphere modifications during hydrolysis. Results obtained over a period of 60 days revealed that microspheres were only partially degraded under the experimental conditions used. After this period, microspheres were highly swollen with a low elastic modulus (ranging from 0.17 to 0.83 kPa) compared to the starting material. The hydrolytic degradation rate could be tuned by modulating the crosslinking degree and the chemical structure of the degradable crosslinker included in the hydrogel network.

1 Introduction

Over the last decade, biodegradable hydrogels have been widely investigated for medical applications including drug delivery, tissue engineering and cell encapsulation.¹⁻⁹ Because of their high-water content, hydrogels are considered as the synthetic biomaterials most similar to natural living.^{10,11} They were used as delivery systems for macromolecules such as proteins, peptides and RNA. In the field of tissue engineering, biodegradable hydrogels form suitable scaffolds for the development of tissue and cells.¹² When implanted *in vivo*, their *in-situ* degradation is a tremendous advantage because they avoid post-surgical interventions aiming to remove the implant.¹³ However, the rate of degradation is a critical issue which needs to be finely tuned according to the final application. For instance, in a controlled drug eluting system, the time of degradation should suit the time scale of the drug delivery. In tissue regeneration, the time of degradation of the scaffolds must be adjusted to the time required for the tissue to reconstitute. Adjustment of the degradation time of hydrogels can be achieved by choosing appropriate monomers and crosslinkers while designing the implantable device.

In the present work, we have developed novel degradable microspheres composed of a hydrolysable hydrogel obtained from the polymerization of a macromer of poly(ethylene glycol) methyl ether methacrylate (PEGMMA) including hydrolysable crosslinkers. The general structure of the crosslinkers was based on the model initially proposed by Sawhney et al.¹⁴ Such crosslinkers are known to be cleaved by a simple hydrolysis occurring in aqueous media.^{15,16} Thus, it is expected that the degradation rate of such microspheres would be only defined by the chemical composition of the biomaterial, while it will be far less sensitive to conditions found at the site of implantation in patients and to the physiopathology of the patient. It was postulated that the degradation rate of the microspheres can be modulated by varying the chemical composition and the molar ratio of the crosslinking agent added to the principal monomer PEGMMA. The present study also aimed to evaluate the degradation of the microspheres and to identify parameters that can be adjusted to tune their degradation time in order to develop new degradable biomaterials suitable for various clinical indications including implantable controlled eluting drug delivery systems. A new method based on rheological measurement of a densely packed microsphere assembly was applied to evaluate the degradation of the microspheres.¹⁷

2 Materials and methods

2.1 Materials

Poly(ethylene glycol) methyl ether methacrylate (PEGMMA) (number-average molecular weight (M_n) \approx 300 g mol⁻¹), poly(ethylene glycol) dimethacrylate (PEGDMA) (M_n \approx 575 g mol⁻¹), and poly(vinyl alcohol) (PVA) (M_n \approx 89 000–98 000 g mol⁻¹, 88%) were purchased from Sigma-Aldrich (St Louis, USA). M_n of the different compounds were given by the supplier. 2,2'-Azobisisobutyronitrile (AIBN) was obtained from Acros Organic (Geel, Belgium). Analytical grade solvents were supplied by Carlo Erba (Val de Rueil, France). All chemical products were used as received.

Phosphate buffer saline (PBS) (58 mM, 150 mM NaCl) at pH 7.4 was used as the incubation medium for the microsphere degradation study. The PBS solutions were prepared following the protocol previously described.¹⁸

2.2 Synthesis of degradable microspheres

Three degradable crosslinking agents were synthesized as described elsewhere.¹⁷ They were: ethylene glycol-co-tetralactico-tetraglycolic dimethacrylate (HEMA4L4G), ethylene glycol-cododecalactic-cododecaglycolic dimethacrylate (HEMA12L12G), and poly(ethylene glycol)-co-tetralactico-tetraglycolic dimethacrylate (PEGMA4L4G) ($M_n \approx 1046 \text{ g mol}^{-1}$ as determined by NMR) (see chemical formulae in Figure 1).

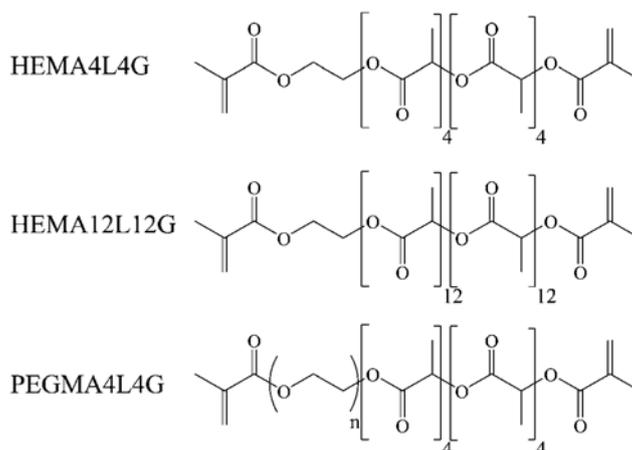


Figure 1. Crosslinking agents used for preparing different types of degradable microspheres.

Degradable microspheres were obtained by direct suspension polymerization. A PVA solution (300 mL at 0.75% wt/v PVA, NaCl 0.1 M) was introduced into a 500 mL reactor and was purged with nitrogen for 15 min. The dispersed phase was prepared by dissolving the two co-monomers (13 g) including the degradable crosslinking agent and PEGMMA in various molar ratios (1/99, 3/97, 6/94%) in 12 mL of toluene. The solution was also purged with nitrogen for 15 min. Then, the dispersed phase was introduced into the aqueous phase at 30 C under agitation at 250 rpm. The initiator, 0.3 g AIBN solubilized in 2 mL of toluene, was then introduced into the reactor. The temperature was raised to 70 C and the reaction was allowed to continue for 15 h under continuous stirring. Microspheres were washed with acetone and water. The obtained microspheres were then sieved to collect fractions with a homogenous size distribution ranging from 100 to 315 mm and from 315 to 500 mm. After sieving, the microspheres were immediately freeze-dried and kept at 20 C until use to preserve them from hydrolysis.

2.3 Degradation study

Dried microspheres (0.100 - 0.002 g) were suspended in 15 mL of PBS pH 7.4 and incubated at 37°C under lateral stirring at 100 rpm (Incubator shaker KS4000i – IKA). At different time points (0, 1, 3, 7, 14, 21, 28, 42 and 60 days) and for each type of microsphere, the supernatant and microspheres were separated and characterized by various methods to evaluate changes in microsphere morphology (optical microscopy), physical properties (rheology studies) and release of degradation products (pH measurements of the supernatants). Each time point was performed in triplicate and the corresponding characterizations were also performed in triplicate.

2.4 Methods of characterizations

2.4.1 Observation of the microsphere morphology.

Microspheres obtained after synthesis or during the degradation process were observed by optical microscopy. The optical microscope (OLYMPUS BH2 Microscope) was equipped with Leitz PL2.5/0.08, Olympus DPlan 10 objectives and a Mightex camera. At least 25 micrographs were taken for each sample.

2.4.2 Size measurements of microspheres.

Particle size distribution was determined by laser diffraction on a Mastersizer® S apparatus (Malvern Instrument, Orsay, France) at 25 C. Dry beads were dispersed in water and were allowed to swell for 15 min before measurement. They were then introduced in the QSpec small volume sample dispersion unit. Homogenous circulation between the latter and the measurement cell was performed by means of 1000 rpm magnetic stirring. The quantity of microspheres was added in order to obtain an obscuration between 5 and 10%. Each injection was analyzed 3 times. Granulometry was analyzed using the Fraunhofer optical model. Results were presented in % volume distribution using the volume/mass moment mean diameter $D[4,3]$ (equation (1)) and the *Span* of their size distribution (equation (2))

$$D[4,3] = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (1)$$

$$Span = \frac{D[v,90] - D[v,10]}{D[v,50]} \quad (2)$$

where n_i represents the number of particles with a defined diameter d_i . $D[v, 90]$ is the volume diameter below which is included 90% of the distribution. $D[v, 50]$ is the volume diameter below which is included 50% of the distribution. $D[v, 10]$ is the volume diameter below which is included 10% of the distribution.

2.4.3 Determination of the swelling ratio of microspheres before degradation.

The swelling ratio of the microspheres, Q_m , was expressed according to the water uptake. It was deduced from the ratio between the weight of the wet microspheres at t_0 (W_{WM0}), evaluated 15 min after dispersion in PBS at 37°C over the weight of dried microspheres (W_{DM}) (equation (3)).

$$Q_{m0} = \frac{V_{WM}}{W_{DM0}} \quad (3)$$

2.4.4 Swelling kinetic of microspheres during degradation experiment.

In a 1 mL graduated test tube, 0.010 g (0.0002 g) of dried microspheres was dispersed in 1 mL of PBS pH 7.4. The test tube was then sealed to avoid evaporation of water. The volume occupied by the sedimenting swollen microspheres was taken after 15 min and was considered as the volume at time 0, V_{sed0} . Modification of the swelling of the microspheres during degradation was followed by

measuring V_{sed} at different time intervals over a period of 60 days. Although the microspheres were maintained at 37°C under orbital agitation for the whole duration of the study, the agitation was stopped 30 min prior to the measurement to allow complete sedimentation of the microspheres before direct reading of the value of V_{sed} on the graduations of the graduated tube. All readings were done in triplicate on each graduated tube and pictures of the tubes were also taken for records. Results were expressed as the ratio between the volume of the sediment at time t , $V_{sed,t}$, over that at time 0, $V_{sed,0}$.

2.4.5 Measurement of the pH of the degradation medium.

At defined incubation times, samples were let to sediment and their supernatants were collected for pH measurements. The pH of each supernatant was measured with a Seven-Multi pH meter (Mettler Toledo, Viroflay, France) at 25 C.

2.4.6 Rheological characterization of microsphere.

Rheological properties of the microspheres were evaluated at different incubation times according to a method recently developed in our group.¹⁷ Measurements were performed using a Haake controlled stress RS600 rheometer (Thermo Fisher Scientific, Villebon sur Yvette, France) equipped with a 35 mm plate–plate geometry maintained at constant temperature, $25 \pm 0.02^\circ\text{C}$, with a Peltier plate. A solvent trap placed on the geometry was used in order to prevent water evaporation during measurements. Microspheres were deposited on the plateau at a mass fraction which corresponded to the random close packing obtained after allowing the suspension to settle in the incubation medium for 30 minutes (mass fraction 65%). To ensure reproducible measurements, the microspheres were deposited as a monolayer covering the entire surface of the lower plate of the measurement device. Rheological properties of the microspheres were characterized by oscillatory measurements in the linear regime. Frequency sweeps from 0.01 to 40 Hz were performed under a constant strain of 0.04%. The gap between plates was chosen as 200 mm for beads having diameters ranging between 100 and 315 mm and as 300 mm for microspheres with diameters ranging from 315 to 500 mm beads. Rheological parameters monitored on each densely packed assembly of microsphere samples and at each time point were: the storage (or elastic) modulus G' , the loss (or viscous) modulus G'' and the phase angle δ (defined by $\tan \delta \approx G''/G'$). Rheological measurements were performed in triplicate at each time point. Means and standard deviations of G' , G'' and δ were calculated.

3 Results

3.1 Characterization of the microspheres

Degradable microspheres were obtained with a good production yield (>80%). Particles appeared spherical with a homogenous size distribution shown by a single peak with a Gaussian shape (Figure 2). The mean diameter and size distribution of the different microspheres are listed in Table 1. Results agreed with size distribution expected from the size fraction selected during the sieving which was applied during the preparation of the microspheres (i.e. either 100–315 mm or 315–500 mm). The initial swelling ratio of the microspheres crosslinked at 3% was roughly the same, indicating that it was not influenced by the nature of the crosslinking agent. However, the initial swelling ratio varied a lot with the degree of crosslinking, in agreement with general observations made on hydrogels.^{19,20}

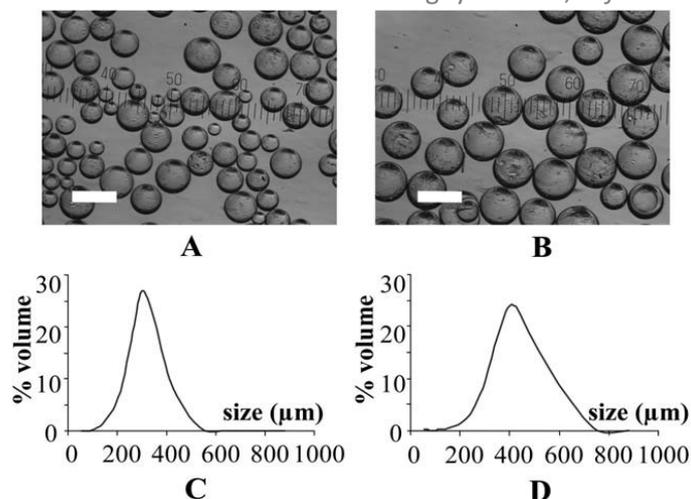


Figure. 2 Optical micrographs of microspheres HEMA4L4G-3% obtained after sieving on 100–315 mm (A) and 315–500 mm (B) and their respective size distributions as evaluated by laser light diffraction with a Mastersizer® S (C) and (D). Bar scale $\frac{1}{4}$ 400 mm.

Rheological measurements performed on the microspheres showed that G^0 varied with the degree of crosslinking for a given crosslinker, the size of the microspheres and the chemical nature of the crosslinker. The evaluation of the elastic properties revealed that all microspheres behaved like nearly pure elastic material under the measurement conditions as indicated by the very low value of d (<10).

3.2 Monitoring of the microsphere degradation

Degradation of the microspheres was expected to take place through the hydrolysis of the PLGA segment introduced in the structure of the crosslinking agent used to synthesize the hydrogel microspheres. Such hydrolysis would transform ester groups in the PLGA chain into acid functions, which in turn would decrease the pH of the incubation medium.²¹ Thus, a first method of monitoring the microsphere degradation was based on the measurement of the pH of the incubation medium over a period of 60 days. In parallel, the degree of crosslinking of the hydrogel microspheres was assumed to decrease as a consequence of the hydrolysis of the PLGA segments of the crosslinking bonds. This was expected to modify rheological and swelling properties of the microspheres as these properties are generally highly sensitive to the degree of crosslinking of hydrogels.^{17,22–24} Therefore, the swelling and the rheological behavior of the microspheres were also evaluated during the course of the experiment. Finally, the morphology of the microspheres was observed using optical microscopy over the same period of time.

Table 1. Initial characteristics of the microspheres prepared for the degradation studies

Microspheres	Sieving (μm)	$D[4,3]$ (μm)	Span $\times 10^1$	Q_{m0}	G' at 10 Hz (10^3 Pa)	δ at 10 Hz ($^\circ$)
HEMA4L4G-1%	315–500	417	4.5	8.32 ± 0.20	2.5 ± 0.4	7.0 ± 0.5
HEMA4L4G-3%		379	7.3	4.91 ± 0.15	4.8 ± 0.2	7.7 ± 0.7
HEMA4L4G-6%		414	5.7	3.77 ± 0.06	11 ± 1	8.0 ± 0.7
HEMA12L12G-3%		342	5.4	4.20 ± 0.08	8.4 ± 0.6	8.0 ± 0.2
PEGMA4L4G-3%		390	6.1	4.57 ± 0.03	8.1 ± 0.4	4.8 ± 0.8
HEMA4L4G-3%	100-315	277	6.6	4.47 ± 0.11	11 ± 1	4.2 ± 0.2

3.2.1 Monitoring of the pH of the degradation medium.

The pH evolution over time of the incubation media of the different microspheres is plotted in Figure 3.

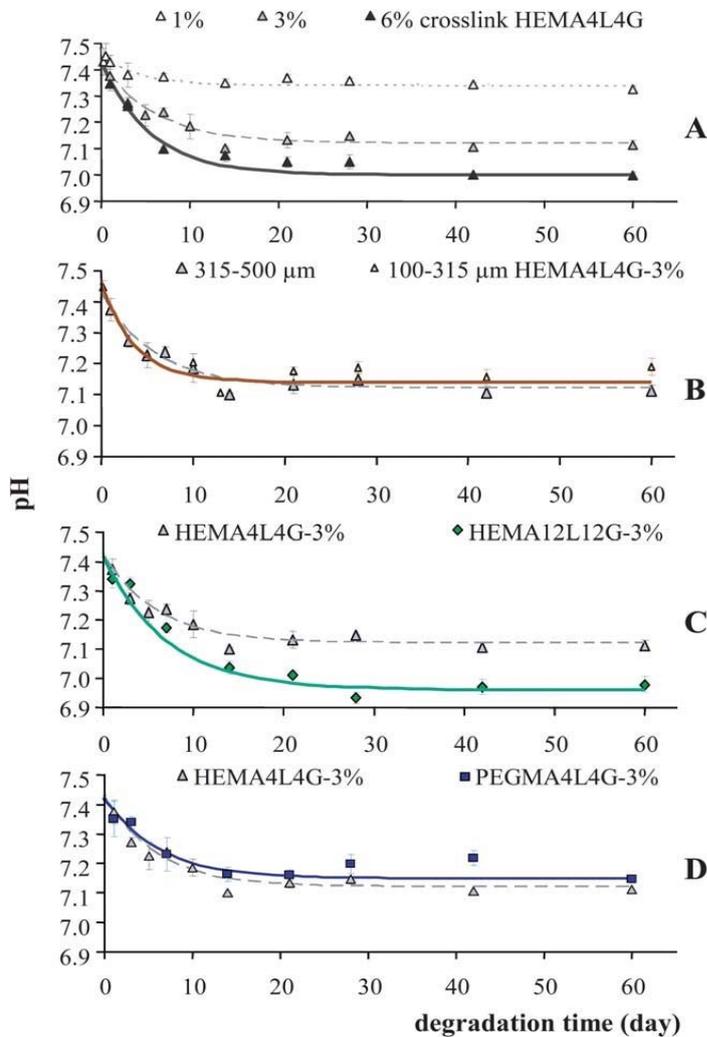


Figure 3. Effect of crosslinking density (A), of microsphere size (B), of crosslinker PLGA segment length (C) and of the PEG/PLGA balance in the crosslinker (D) on the variation of the pH of the incubation medium. Lines were exponential curve fits.

All curves showed a decrease of the pH, which indicated a release of acid compounds from the microspheres in agreement with the degradation of ester bonds included in the PLGA segments of the crosslinker. As shown in Figure 3A, the higher the crosslinking percentage in the hydrogel, the more the pH decreased. The comparison of the two classes of size distribution of the same microspheres did not reveal differences in pH variation during the degradation process (Figure 3B). Microspheres prepared with a crosslinker having a long PLGA chain (HEMA12L12G) have produced a higher pH decrease than those obtained with the HEMA4L4G crosslinker in which the PLGA segment was shorter (Figure 3C). This agreed with the fact that the content in lactic acid and glycolic acid in the microspheres HEMA12L12G-3% was higher than that of the microspheres HEMA4L4G-3%. Microspheres with different PEG chains in the structure of the crosslinker were also compared:

microspheres HEMA4L4G-3% with only one ethylene glycol unit and microspheres PEGMA4L4G-3% with an average of 5 ethylene glycol units per chain. The total pH variation was the same for the two types of microspheres. This indicated that the hydrophilic/hydrophobic balance of the crosslinker did not significantly influence the production of acid compounds resulting from the hydrolysis of the hydrogel (Figure 3D).

3.2.2 Evolution of the morphology of microspheres during incubation.

Microspheres were observed by optical microscopy at different times during the degradation experiment. As an example, Figure 4 shows the morphology evolution of microspheres HEMA4L4G-3%. Microspheres remained spherical during the whole duration of the experiment (i.e., 60 days). An obvious swelling of the microspheres can be observed up to day 21, while, at longer incubation times, the microspheres became less visible because of a progressive contrast match of the refractive index of the microspheres on that of the surrounding medium. This effect can easily be explained by the combination of two effects. During microsphere degradation, the amount of crosslinking decreased as well as the polymer concentration in the microspheres. As the second effect, the swelling of the microspheres tended to increase the amount of incubation medium composing the hydrogel.

Thus, the composition of the microspheres tended to that of the dispersion medium as the degradation proceeded, hence the refractive index of the microspheres tended to match that of the incubation medium. It is noteworthy that even after 60 days of incubation the microspheres remained, indicating that they were not fully degraded. The same observations were made with the other microspheres.

3.2.3 Swelling behavior of microspheres during the degradation.

The volume taken by a defined amount of swelled microspheres in the incubation medium was measured at different time intervals. Results are reported in Figure 5. In agreement with optical microscopy observations, the volume taken by the microspheres increased during incubation in PBS. The curves showed two parts. The first part corresponded to an increase of the volume occupied by the microspheres over several days before reaching a plateau value corresponding to the second part of the curve. For a given crosslinker (HEMA4L4G), the degree of crosslinking of the microspheres greatly influenced the rate of the variation of swelling (Figure 5A). Microspheres crosslinked at 3% showed almost the same variation of swelling independent of their size and of the nature of the crosslinking agent (Figure 5B and D). The microspheres obtained with the more hydrophobic crosslinker HEMA12L12G displayed a slower rate of swelling during incubation (Figure 5C).

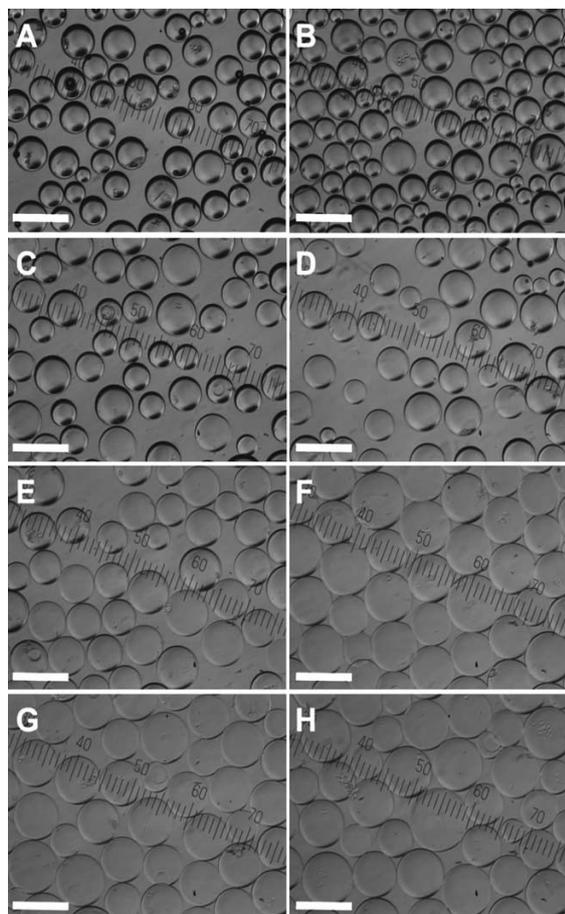


Figure 4. Morphology of HEMA4L4G-3% microspheres at different incubation time ((A): before start of the experiment, (B): 1 day, (C): 3 days, (D): 7 days, (E): 14 days, (F): 21 days, (G): 41 days, (H): 60 days) in PBS pH 7.4 at 37°C as observed by optical microscopy. Scale bar 400 mm.

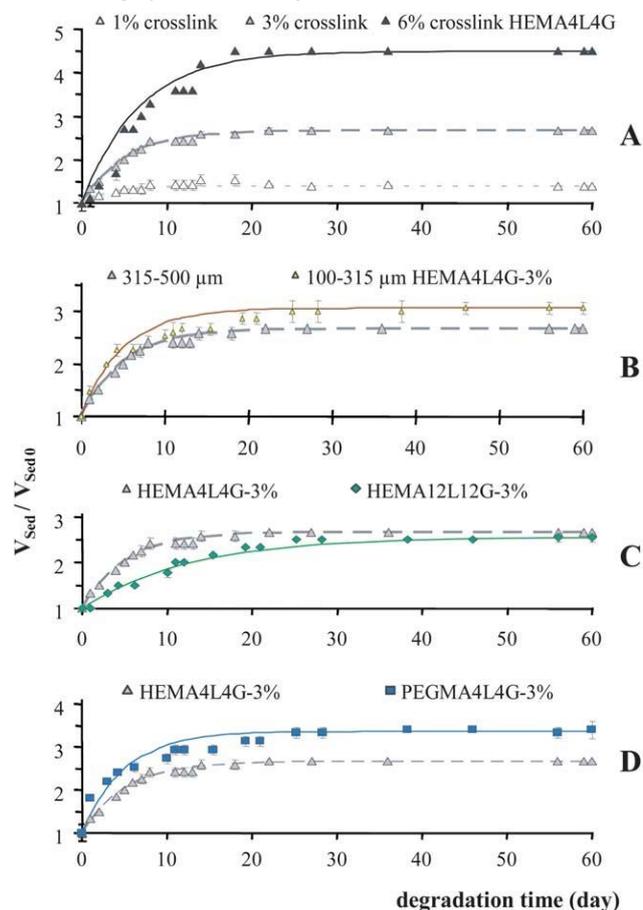


Figure 5. Monitoring of the variation of the swelling of the microspheres during incubation in PBS at 37°C. Microspheres differing from their crosslinking density (A), their size (B), the length of the hydrophobic segment in the crosslinker length (C), and the PEG/PLGA balance in the crosslinker (D). Lines were exponential curve fits.

3.2.4 Monitoring the rheological properties of the microspheres during the degradation.

Results from the monitoring of the rheological properties of the microspheres during degradation are plotted in Figure 6. As mentioned above, G' of microspheres varied with their degree of crosslinking, the nature of the crosslinker and the size. The initial values ranged from 2500 to 11 000 Pa (Table 1). During the incubation in PBS, a dramatic decrease of G' was monitored with all types of degradable microspheres whatever the initial value was. In contrast, when the same experiments were performed on nondegradable microspheres, the G' values remained almost constant ($G' = \pm 2$ to 8% of the G' value at time 0) after 1 and 2 months of incubation. Therefore, it was concluded that the decrease of G' observed on the degradable microspheres resulted from the hydrolysis of the microsphere hydrogel crosslinking. The curves in Figure 6 show two distinct parts similarly to those monitoring the swelling of the microspheres during degradation. The decay shown in the first part of the curve was more pronounced for the most crosslinked microspheres (Figure 6A) and the smaller microspheres (Figure 6B). In contrast, the decay of the first part of the curves appeared very similar

between the microspheres obtained with crosslinkers of different nature and composition (Figure 6C and D).

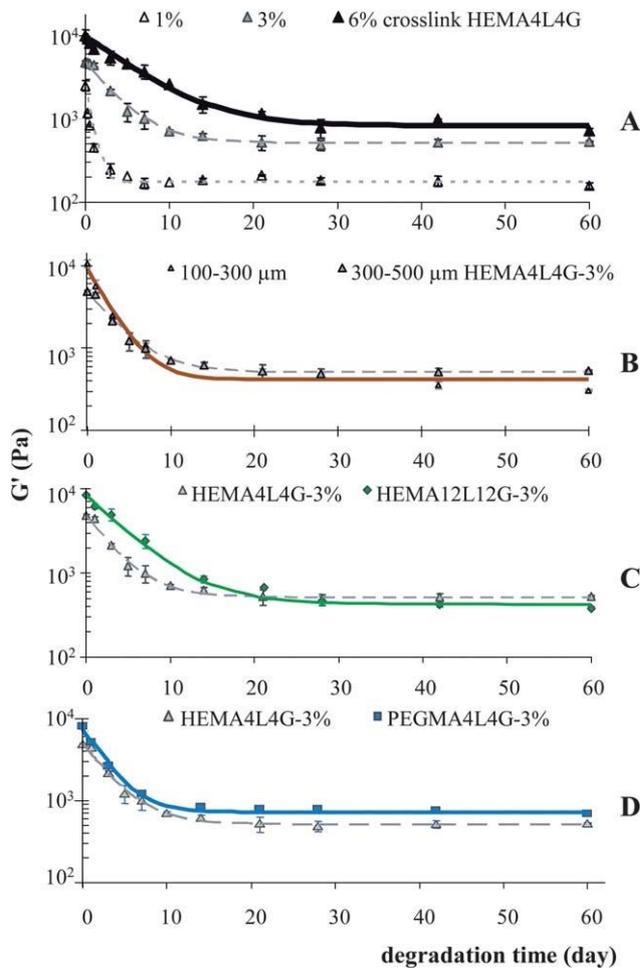


Figure 6 Monitoring of the variation of G' of the microspheres during incubation in PBS. Microspheres differing from their crosslinking density (A), their size (B), the length of the hydrophobic segment in the crosslinker (C) and the PEG/PLGA balance in the crosslinker (D). Lines were exponential curve fits.

The decrease in G' was monitored until it reached a plateau value which ranged between 150 and 800 Pa. This plateau corresponded to the second part of the curve. This indicated that the degradation of the hydrogel composing the microspheres was stopped or that the rate of degradation dropped down considerably. These results were in agreement with observations above showing that microspheres still remained after 60 days of incubation in PBS. The time at which the G' reached the plateau value depended on the degree of crosslinking (Figure 6A) and on the length of the hydrolysable PLGA segment introduced in the crosslinker (Figure 6C). It was rather not dependent on the size range of the microspheres and on the nature of the crosslinker (comparing crosslinkers with the same content in PLGA in their structure). The difference between initial G' and G' at the plateau greatly depended on the type of microspheres. This agreed with the fact that the initial G' of the microspheres varied over a wide range of values (2500–11 000 Pa) while it was comprised in a quite narrow range at the plateau value (200–800 Pa) obtained during incubation of the microspheres in PBS. Finally, it can be pointed out that the hydrogel microspheres which showed

almost ideal elastic properties at the beginning of the experiment ($d < 10$) kept their elasticity properties throughout the duration of the incubation period (data not shown).

4 Discussion

Hydrogel microspheres were synthesized by copolymerization in emulsions of PEGMMA with different hydrolysable crosslinkers and at different degrees of crosslinking. PEGMMA was used to obtain a highly swellable and elastic hydrogel forming the microspheres. The hydrolysable crosslinkers were included in the hydrogel structure to introduce degradable bonds making possible a degradation of the hydrogel forming the microspheres by a simple hydrolytic mechanism. The synthesized microspheres were sieved to obtain fractions with homogenous size distribution ranging from 100 to 315 μm and from 315 to 500 μm . The initial swelling and rheological behaviors of the different microspheres were evaluated prior to the degradation. The evolution of the swelling and the rheological properties observed with different degrees of crosslinking and with different crosslinkers agreed with what is generally observed with other hydrogels independent of the nature and the shape of the device.^{23,25}

Once the microspheres were incubated in PBS they started swelling and their rheological properties were greatly modified. The G' decreased dramatically, and the swelling proceeded until a steady state was reached. In parallel to these modifications of the microsphere properties, the pH of the degradation medium decreased, which indicated that there was a release of acid compounds. This agreed with the expected degradation mechanism of the hydrogels composing the microspheres. Indeed, during the degradation, the diminution of the pH of the incubation medium could correspond to the release of carboxylic acid containing components resulting from the hydrolysis of the ester bonds of the oligo (lactic and glycolic acid) segments included in the structure of the crosslinkers. Based on this hypothesis and on results reported in the literature, the decrease of pH expected should depend on the composition of the crosslinker in lactic and glycolic acid residues.²⁶ The higher the amount of lactic and glycolic acid in the microspheres, the higher the quantity of carboxylic acid containing compounds that may be released, hence the higher the decrease in pH of the incubation medium.²¹ Results from the experiments (Figure 3) agreed well with what was expected from the degradation mechanism. Thus, the modification of the swelling and the rheological properties of the microspheres occurring after the start of the incubation in PBS can be correlated with a hydrolytic degradation of the hydrogel. Due to the hydrolysis, the G' of the microspheres decreased as the density of the crosslink was reduced, implying a higher swelling.²⁷ This is also in agreement with the assumed chemical structure of the hydrogel and with data reported in the literature on similar types of hydrogels.²³ However, it is noteworthy that, contrary to what was expected, none of the microspheres were totally degraded in the framework of our experimental conditions. All curves showed a first part in which the studied parameters varied, which can be attributed to modifications of the microsphere properties due to the hydrolysis of the crosslinking bonds. Then, while no more variations of pH were recorded in the incubation medium, the swelling of the microspheres and their G' value remained also constant after a rapid evolution stage. This was in contrast with the exponential increase of the swelling curves followed by a sudden drop attributed to the dissolution of the hydrogel reported with fully hydrolysable hydrogels.²² Samples having reached the plateau values were observed by optical microscopy. They revealed the presence of highly swollen microspheres. To explain the presence of the remaining microspheres, it can be postulated that the remaining particles were still composed of a crosslinked hydrogel. While the rate of crosslinking of this hydrogel was expected to be low, the polymer network was strong enough to hold the material together within the shape of the microspheres. A similar result was reported earlier

considering the same type of hydrogels.²² The authors assumed that it included a very low fraction of non-hydrolysable crosslinks due to an incomplete modification of the PEG during the synthesis of the hydrolysable PEG crosslinker. As it was reported that very low non-degrading crosslinks may induce the formation of relatively permanent hydrogel networks, this hypothesis should be considered to explain the only partial degradation of the microspheres observed here. The method used for the synthesis of the microspheres was based on a radical co-polymerization of PEGMMA with the hydrolysable crosslinker. It was reported that the monomer PEGMMA can reticulate during a radical polymerization even when the reaction is performed in the total absence of crosslinking agents.^{28,29} According to the authors, the crosslinking occurred through a chain transfer or a coupling reaction which follows the formation of reactive functions (radicals, hydroxyl and aldehyde groups) due to the degradation of the PEG segment of the macromer by a radical. The occurrence of such a crosslinking side reaction during microsphere preparation was confirmed by performing a polymerization of PEGMMA under the same conditions as those used to prepare the microspheres of this study but without adding a crosslinking agent in the polymerization medium. Well spherical particles could be obtained under these conditions of polymerization. The microspheres were totally insoluble in many common solvents for polymers (i.e., chloroform, THF, DMSO, DMF, acetone, ethanol, water), indicating that they were composed of a crosslinked network. Their rheological characteristics ($G' = 100$ Pa) and swelling properties ($Q_{mo} = 29.19 \pm 0.66$) suggested that the cross-linking rate was very low but sufficient to form a hydrogel of a well-defined spherical shape. This supported the assumption that microspheres remaining after complete hydrolysis of the PLGA ester bonds were formed by a cross linked hydrogel. The persisting crosslinks resulted from a side reaction which occurred during the synthesis of the microspheres by radical polymerization of PEGMMA. The latter contained at least one or two ester bonds in their structure and thus were also susceptible to hydrolysis. But their localization close to the main chain rendered them far less sensitive to hydrolysis.

It was possible to quantify the degradation rate through the hydrolytic mechanism of the microspheres by fitting the experimental data plotted in Figure 3, 5 and 6 with an exponential equation (equation (4))

$$Y = A + B \exp\left(\frac{-t}{\tau}\right) \quad (4)$$

where A is the value at the plateau, $A + B = C$ is the initial value at time 0, t is the time and s is the characteristic time of the rate of degradation. A small value of s indicated a fast degradation while a high value of s indicated a slow degradation. The same exponential trend was previously observed during the degradation of bulk hydrogels obtained from similar crosslinking agents.^{15,16} Parameters of the equation fitted the results of the variation of G' with the incubation time are given in Table 2 for the different microspheres considered in this study. Results from the variation of G' were taken for this analysis because of the high sensitivity of this parameter monitoring microsphere degradation compared with other methods used in this study. The analysis revealed that the degradation rate of microspheres depended on their degree of crosslinking, on the composition of the crosslinking agents and, to a lesser extent, on their size. Results were in agreement with the literature considering hydrogels made with similar crosslinkers independently of their shape.^{14,30} Interestingly, equation (4) could be used to calculate the time needed to reach completeness of the hydrolysis. It was defined as t_{99} , the time at which the decrease of G' reached 99% of the total variation monitored between the initial value ($t = 0$) and that of the plateau. This calculation revealed that the time required to hydrolyze the hydrogels composing the microspheres ranged from 7 to 49 days.

Microspheres showing the shortest time of hydrolysis were the less reticulated, while the microspheres with the highest degree of crosslinking showed the longest hydrolysis time.

Table 2 Parameters of the exponential equation (4) obtained by fitting the experimental curves monitoring the variation of G^0 of the microspheres during their incubation in PBS

Sieving (mm)	Microspheres	Fit equation of G' (Pa)			
		A	B	s	t_{99} (days)
315–500	HEMA4L4G-1%	172	2340	0.7	7
	HEMA4L4G-3%	511	4334	3.1	28
	HEMA4L4G-6%	830	9248	5.3	49
	HEMA12L12G-3%	423	7945	4.6	45
	PEGMA4L4G-3%	724	7340	2.5	24
100–315	HEMA4L4G-3%	418	9980	2.1	21

The hydrolysis time was greatly affected by the composition of the hydrolysable crosslinker as suggested by the comparison between the microspheres prepared with the crosslinkers PEGMA4L4G-3% and HEMA4L4G-3% ($t_{99} = 24$ and 28 days respectively) and those prepared with a crosslinker having a longer segment of lactic and glycolic acid unit HEMA12L12G (HEMA12L12G-3%, $t_{99} = 45$ days). This can be explained by a higher hydrophobicity of the crosslinker. The hydrolysis time of the hydrogel composing the microspheres can be tuned by adjusting the degree of crosslinking as well as the nature and structure of the hydrolysable crosslinker.

Conclusion

Microspheres were prepared by radical copolymerization of PEGMMA with a hydrolysable crosslinker. They were composed from a hydrogel. The microspheres degraded by hydrolysis in a few days during incubation in PBS. The rate of hydrolysis can be tuned by varying the length of the hydrolysable hydrophobic segment introduced in the chemical structure of the crosslinker and by the degree of crosslinking of the hydrogel forming the microspheres. In contrast with the initial assumption, full degradation of the microspheres could not be observed within the time frame considered in this work. It was concluded that part of the crosslinking bonds included in the structure of the hydrogel was far less sensitive to hydrolysis. Their formation was attributed to a side reaction due to the polymerization of PEGMMA occurring under the conditions of polymerization used in this work to prepare the microspheres. More work is now needed to improve the method of synthesis of the microspheres in order to avoid the occurrence of this side reaction. This should lead to the obtaining of rapidly and fully hydrolysable hydrogel microspheres which will be suitable for various applications in the biomedical area.

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