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Rheological Characterization of Mechanical Properties of Chemically Crosslinked Microspheres

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ABSTRACT: This work presents a rheological method to characterize degradation of microspheres made of chemically crosslinked hydrogels. Conditions to measure rheological properties of microspheres, remaining as individual microspheres during measurement, were established. Relevant and reproducible measurements could be obtained with a rheometer equipped with a parallel plate geometry in which a homogenous and continuous monolayer of microspheres was inserted in a gap between the plates. The storage modulus of microspheres was determined under an imposed strain of 0.04% with an oscillatory measurement mode at various frequencies. The microspheres showed almost purely elastic behavior while their storage modulus was affected by the degree of crosslinking. In the second part of the work, the rheological method was applied to investigate the degradation of microspheres made of a hydrolyzable crosslinked hydrogel. The method was found suitable by measuring the storage modulus over time. A good correlation was identified between acidification of the incubation medium because of the release of degradation products and the decrease of the storage modulus of the microspheres indicating a reduction in the crosslinking of the hydrogel resulting from the degradation process. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci. 000: 000–000, 2012

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INTRODUCTION

Hydrogels are the synthetic biomaterial class most similar to natural living material.[1,2] They consist of a three-dimensional crosslinked macromolecular networks with a capacity of water absorption of up to thousand times their dry weight without being dissolved.[3,4] This unique property contributes to their soft consistency and biocompatibility. Therefore, they are receiving significant attention in developing biomaterials for medical applications including tissue engineering, cell encapsulation, and drug delivery.[5–9] For many of the applications in the biomedical field, the mechanical properties of hydrogels should be appropriate.

Rheology is one of the most employed methods used to characterize gels from their formation to their intimate properties. Indeed, rheological properties are sensitive to the degree of crosslinking of the material and were widely used to follow the kinetics of crosslinking reaction used during formation of chemically crosslinked hydrogels.[10–13] Various experimental conditions are suitable to study rheological properties of hydrogel. The most commonly used include time sweep at constant frequency and strain[10–12] or frequency sweep at constant shear stress amplitude.[13] Forming hydrogels can be also characterized by different rheological methods, that is, frequency sweep, strain sweep, or stress sweep mode.[14,15] It is noteworthy that these methods were mainly applied to characterize bulk gels, while their application to monitor the degradation of hydrogels including chemical crosslinks remains marginal. This is contradictory to the fact that rheological properties of hydrogels are sensitive to the degree of crosslinking and are suitable to follow gel formation during crosslinking reaction. Thus, it can be assumed that it could also be used more systematically to monitor a decrease of the crosslinking ratio occurring during degradation of a hydrolyzable crosslinked hydrogel. Only a few studies have actually applied rheological methods to follow degradation of hydrogels caused either from a biodegradable polymer network[16,17] or by the association of opposite charged microspheres.[18] In all cases, it was demonstrated that the rheological properties of the gels were modified during degradation and that the methods were suitable to monitor the degradation

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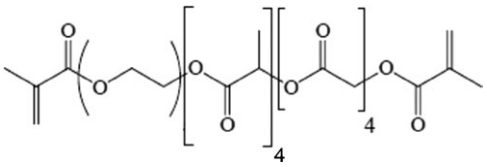

Figure 1. Chemical structure of the hydrolysable crosslinker Ethylene glycol-*co*-tetralactic-*co*-tetraglycolic dimethacrylate (HEMA4L4G) used in the synthesis of degradable microspheres.

of such materials. This is an advantage over all the other methods of characterization of polymers such as GPC, DSC, and ^{1}H NMR, which cannot be applied to characterize materials made of three-dimensional polymer networks as they are not soluble in any solvent.

Rheology is a powerful tool for the characterization of bulk hydrogels. However, it is noteworthy that its application to the characterization of hydrogels under the form of nanoparticles and microparticles has lead to a very limited number of works so far.[19] The aim of our work was to develop a method based on rheological measurements that would be suitable to characterize hydrogels occurring as individual microspheres and to follow their degradation by hydrolysis of their crosslinking bonds. The first part of the work describes the set up and experimental conditions resulting into reproducible measurements of the rheological characteristics of hydrogel microspheres with different degrees of reticulation. In the second part of the work, the rheological method was applied to monitor the degradation of hydrogel microspheres containing hydrolyzable crosslinking bonds.

EXPERIMENTAL

Materials

Poly(ethylene glycol methyl ether methacrylate) (PEGMMA) of number-average molecular weight 300 g/mol, poly(ethylene glycol dimethacrylate) (PEGDMA) of number-average molecular weight 575 g/mol, poly(vinyl alcohol) (PVA) (MW_n 89,000–98,000 g/mol 99 + % hydrolyzed) were purchased from Sigma-Aldrich (St. Louis, MO). Molar mass values were provided by supplier. Azobisisobutyronitrile (AIBN) used as polymerization initiator was obtained from Acros Organic (Geel, Belgium). Analytical grade solvents were supplied by Carlo Erba (Val de Rueil, France). All chemicals were used as purchased without further purification.

Phosphate buffer saline (PBS) (58 m*M*, 150 m*M* NaCl) at pH 7.4 was used as incubation medium for the microsphere degradation study.

^{1}H NMR analyses were performed with a 300 MHz Bruker apparatus. Deuterium solvents were purchased from Carlo Erba.

Methods

Synthesis of Ethylene glycol-co-tetralactic-co-tetraglycolic dimethacrylate (HEMA4L4G). The chemical structure of ethylene glycol-*co*-tetralactic-*co*-tetraglycolic dimethacrylate (HEMA4L4G) is given in Figure 1.

In a dry round flask containing a magnetic stir bar, lactide (8 mmol, 1.152 g), glycolide (8 mmol, 0.929 g), HEMA (4 mmol, 0.52 g), and $Sn(Oct)_2$ (0.025 mmol, 10 mg) were dissolved in toluene (5 mL) under an argon atmosphere. After 20 h at 90°C, chloroform (5 mL) was added to dilute the reaction mixture and the resulting polymer was purified by precipitating in a large volume of petroleum ether. The precipitate was then dried under vacuum.

^{1}H NMR ($CDCl_3$) d (ppm): 6.13 (s, 1H, CHH=), 5.58 (s, 1H, CHH=), 5.27 (m, 1H × PD_L, PLA), 4.73 (m, 2H × PD_G, PGA), 4.44 (m, 2 × CH_2, HEMA), 1.95 (s, 3H, methacrylate), 1.59 (m, 3H × PD_L, PLA). According to the NMR result, the polymerization degree of lactic PD_L was around 4. The same value was found for the polymerization degree of glycolic PD_G.

The dried polymer obtained from the first step was then subjected to an esterification of the hydroxyl group at the end of the PLGA chain by reacting with methacryloyl chloride. The total amount of the forming macromer was dissolved in CH_2Cl_2 (30 mL) under argon in a dry flask equipped with magnetic stir bar. The content of the flask was cooled to 0°C and TEA (12 mmol, 1.6 mL) was added in order to trap HCl formed during the esterification reaction. The solution was stirred and then methacryloyl chloride (12 mmol, 1.2 mL) was added dropwise to the solution during 30 min. The stirring was continued 1 h at 0°C and then 24 h at room temperature. The triethylamine chlorhydrate salt resulting from triethylamine (TEA) and HCl was removed by filtration and the polymer was precipitated in a large volume of petroleum ether. The crude product was then separated, dried and kept in an argon atmosphere for further reactions.

The methacrylate functionality *f* of the macromonomer, the polymerization degree of lactic acid and of glycolic acid (respectively PD_L and PD_G) were determined from ^{1}H NMR spectra of the obtained products. ^{1}H NMR ($CDCl_3$) d (ppm): 6.22 (m, 1H × *f*, CHH=), 6.12 (s, 1H, CHH=), 5.61 (m, 1H × *f*, CHH=), 5.57 (s, 1H, CHH=), 5.27 (m, 1H × PD_L, PLA), 4.73 (m, 2H × PD_G, PGA), 4.29 (m, 2 × CH_2, HEMA), 1.975 (m, 3H + 3H × *f*, methacrylate), 1.59 (m, 3H × PD_L, PLA). PD_L and PD_G were around 4. The *f* value was 97%.

Synthesis of Crosslinked Hydrogel Microspheres. A 0.75% solution of PVA (300 mL) was introduced into a 500 mL reactor and was purged with nitrogen atmosphere for 15 min. The dispersed phase containing the crosslinking agent and a comonomer, PEGMMA, at various molar ratios were solubilized in 12 mL of toluene and degassed by bubbling nitrogen through the solution for 15 min. Nondegradable microspheres were synthesized with PEGDMA as crosslinking agent at different molar ratios (expressed as percentage of the total monomers) ranging from 1% to 11% (Table I). Degradable microspheres were synthesized using 3% (in molar ratio regarding total amount of monomers) of HEMA4L4G as the crosslinking agent. Preparation of the microspheres was continued as followed in all cases. The dispersed phase containing the co-monomers dissolved in toluene was introduced into the aqueous phase at 30°C and agitated by means of a homemade glass 3-wing-propeller type stirrer at 250 rpm. AIBN (0.3 g) was solubilized in 2 mL of toluene and was then added into the reactor. The temperature was

Table I. Characteristics of Microspheres Obtained After Sieving Between 315 and 500 μm

Sample name	Crosslinking molar ratio (%)	Crosslinking agent	D[4,3] (μm)	Span 10^{-1}	G′ at 10 Hz 10^3 (Pa)	δ at 10 Hz (°)
PEGDMA-1%	1	PEGDMA (nondegradable)	462 ± 2	3.5 ± 0.1	1.1 ± 0.1	7.9 ± 1.8
PEGDMA-2%	2		439 ± 2	4.0 ± 0.1	2.8 ± 0.1	5.6 ± 0.4
PEGDMA-2.5%	2.5		418 ± 3	5.0 ± 0.1	3.8 ± 0.2	4.2 ± 0.2
PEGDMA-3%	3		419 ± 1	4.6 ± 0.1	5.1 ± 0.7	4.3 ± 0.2
PEGDMA-4%	4		391 ± 2	2.1 ± 0.1	8.0 ± 0.4	3.6 ± 0.6
PEGDMA-5%	5		389 ± 4	2.3 ± 0.3	8.7 ± 0.1	4.4 ± 0.4
PEGDMA-6%	6		412 ± 2	4.8 ± 0.1	12 ± 1	4.1 ± 0.3
PEGDMA-7%	7		423 ± 1	5.0 ± 0.1	15 ± 1	3.0 ± 0.3
PEGDMA-9%	9		406 ± 2	5.8 ± 0.1	16 ± 1	3.8 ± 0.2
PEGDMA-11%	11		376 ± 1	2.7 ± 0.2	17 ± 1	4.7 ± 0.6
HEMA4L4G-3%	3	HEMA4L4G (hydrolysable)	379 ± 2	7.3 ± 0.1	4.8 ± 0.2	7.7 ± 0.7

increased to 70°C and the reaction was allowed to proceed by stirring for 15 h at 70°C. The microspheres were washed with acetone and water before they were sieved over the following series of sieves (Inox sieve with mesh size 630, 500, 315, 100, 40 μm) (Fisher scientific, Illkirch, France). Particles retained by the sieve with a mesh size of 315 μm corresponding to microspheres with diameter ranging from 315 to 500 μm were kept for this study. They were freeze dried immediately after preparation and purification and stored at −20°C until use.

Degradation Studies. Samples of 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). Three essays were performed for each time point (0, 1, 3, 5, 10, 14, and 21 days). At the different incubation times, the supernatant and microspheres were separated for pH and rheological measurements, respectively.

Characterizations of Microspheres

Morphology and Size Analysis. The 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 and Olympus DPlan 10 objectives and a Mightex camera. At least 25 micrographs were taken for each sample.

Particle size distribution was determined by laser diffraction on 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. This time was sufficient to reach the swelling equilibrium with the type of microspheres considered in this study. 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 magnetically stirring. The quantity of microspheres was added in order to obtain an aperture between 5% and 10%. Each injection was analyzed three 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] eq. (1) and the span of their size distribution eq. (2)

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

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

With «n_i» et «d_i» represented the number of particles with a defined diameter

$D[v, 90]$ is the volume diameter above which it includes 90% of the distribution.

$D[v, 50]$ is the volume diameter above which it includes 50% of the distribution.

$D[v, 10]$ is the volume diameter above which it includes 10% of the distribution.

Mass Fraction Measurements of Microspheres. The mass fraction of the microspheres in concentrated suspensions obtained after sedimentation was determined as followed. A weighting boat was prepared with a preweighted piece of filter paper above which was placed a preweighted piece of organza. A sample of the concentrated suspension of microspheres obtained by sedimentation was placed over the organza. The mass measured, W_{sed}, corresponded to the weight of the wet microspheres (W_{WM}) and the weight of the surrounded liquid. As organza showed a mesh size much below the size of the smaller microspheres and it did not absorb water, it retained the microspheres while surrounded liquid was absorbed by the filter paper placed below. By weighting separately the preweighted piece of organza with the microspheres and the preweighted piece of filter paper having absorbed the liquid surrounding the microspheres, it was possible to determine the weight of wet microspheres (W_{WM}) and that of the surrounding liquid. The mass fraction of the microspheres (f_{wm}) expressed as a percentage can then be calculated from eq. (3).

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$$f_{wm} = \frac{W_{WM} \times 100}{W_{Sed}} (wt\%) \quad (3)$$

Determination of the Swelling Ratio of the Microspheres. The same procedure as described earlier was used to evaluate the weight of the wet microspheres (W_{WM}). Then the isolated wet microspheres were freeze dried and weighted again after drying to measure their dried weight (W_{DM}). The mass swelling ratio (Q_w) was then calculated from eq. (4):

$$Q_w = \frac{W_{WM} - W_{DM}}{W_{DM}} \quad (4)$$

pH Measurements. The pH of each supernatant obtained during degradation process was measured with a SevenMulti pH meter (Mettler Toledo) at 25°C.

Rheological Measurements. The rheological properties of microspheres were evaluated by a Haake RheoStress 600 rheometer (Thermo Electron) equipped with a 35 mm plate–plate geometry. Measurements were performed at 25°C (± 0.02) and regulated with a Peltier plate. A solvent trap placed on the geometry was used to prevent water evaporation during measurements. Microspheres were allowed to sediment by gravity for 30 min before sampling. The sediment with a mass fraction of microspheres of 65 ± 3% was deposited on the inferior plate of the measurement cell.

Rheological experiments were performed using the oscillatory modes with an imposed strain. The characterization of the microspheres included two steps that were carried out successively including a shear sweep and a frequency sweep. Three rheological parameters were monitored during each measurements, which included the storage (or elastic) modulus G′, the loss (or viscous) modulus G″, and the phase angle δ (defined as tan δ = G″/G′).

At first, a shear sweep was performed to define the stable zone (plateau) of G′ and G″ moduli as a function of the applied strain (from 0.0001 to 0.1) at a constant frequency of the oscillation (1 Hz). This experiment was performed on nondegradable microspheres with a crosslinking ratio ranging between 1% and 11%. It allowed choosing a strain value in the linear viscoelastic regime, which was common for all systems.

Then G′, G″ moduli, and phase angle δ were determined at different frequencies of the oscillation (0.01–100 Hz) for the different types of nondegradable microspheres. In the case of degradable microspheres, the three rheological parameters were recorded on different aliquots of the same batch of microspheres at various hydrolysis times.

All measurements were performed in triplicate for each sample. Results were given as the mean value and standard deviation of G′, G″, and δ which were calculated from the three determinations.

RESULTS AND DISCUSSIONS

Microsphere Synthesis

Degradable and nondegradable microspheres were obtained with good production yield. Indeed, 80% of the total mass of polymer produced during polymerization occurred as microspheres, whereas only 20% were discarded as aggregates.

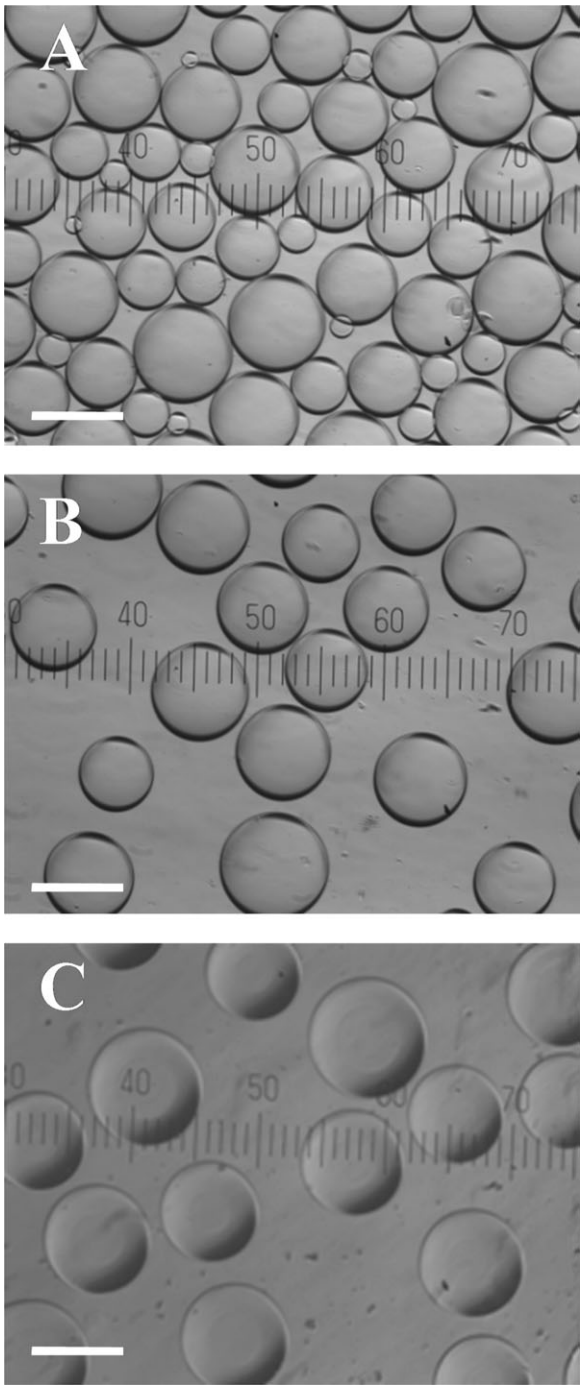

Figure 2. Morphological observations by optical microscopy of microspheres PEGDMA-1% in different stages of the study. A: before sieving. B: after sieving 315–500 μm, before rheological study. C: after sieving 315–500 μm, after rheological study. Scale bar: 400 μm.

Particles were spherical (Figure 2) and their diameter and size distribution were comprised within the range of the size of particles (315–500 μm) expected from the sieve used to isolate the microspheres after synthesis (Table I).

Swelling measurements were carried out for nondegradable microspheres with different crosslinking ratios. The swelling of

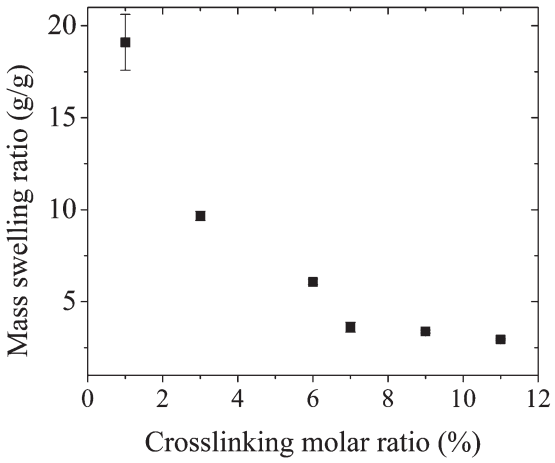

Figure 3. Swelling ratio of microspheres as the function of their crosslinking molar ratio.

the microspheres greatly depended on the crosslinking agent concentration used during the synthesis (Figure 3). Highly crosslinked microspheres contained less amount of absorbed water, whereas the lower the crosslinking the larger was the swelling. This result agreed with what is generally observed considering bulk hydrogels.[20,21]

Development of the Rheological Method for the Characterization of the Microspheres

The purpose of the work was to develop a rheological method to characterize mechanical properties of hydrogel microspheres, while they remained under individual microspheres during the entire process of the measurement. This was in contrast with many works in which the properties of the hydrogel forming microspheres or nanospheres were approached by measuring the rheological properties of a bulk hydrogel of the same composition.[22,23] Preliminary experiments included the determination of concentration in microspheres of the suspensions used for rheological measurement and determination of the gap between the plates of the measurement cell that needed to be used for providing relevant and reproducible experimental measurements.

Concentration in Microspheres in the Suspension

The key parameter for rheological measurements of a suspension is that it must form a homogeneous medium. In physical studies, in general and particularly in rheology, the volume fraction is frequently used to characterize the concentration of the dispersed phase in a suspension. It is an important factor that greatly influences the obtained moduli in rheological measurements.[24] This dependence is not only an issue for suspensions but also for bulk hydrogels.[19,25] As a consequence, it was important to verify that all measurements were performed at the same concentration in microspheres in the suspension throughout the study for obtaining relevant comparable results. The volume fraction can be calculated by the ratio of the volumes of all the particles (particles without the liquid entrapped in the void spaces subsisting between microspheres) to the total volume of the suspension (particles plus dispersing liquid). However, volume fraction is sometimes difficult to determine as it requires to know the density of the dispersed particles. This was the case with the hydrogel microspheres investigated in this study. A related factor can be used in the case that corresponds to the mass fraction. This parameter can be measured from the determination of the weight of the wet microspheres contained in a defined weight of the sample. The mass fractions measured for all the suspensions used in this study at their maximal concentration obtained after sedimentation for 30 min were 65 ± 3%. As the standard deviation was low between the different samples of microspheres, it was considered that rheological measurements were performed on suspensions at a constant mass fraction of the microspheres.

Choice of the Gap Between Plates of Measurement Cell

Sedimentation occurring during measurement is another parameter influencing results from rheological evaluations performed on microsphere suspensions. To avoid sedimentation phenomenon with the large size hydrogel microspheres dispersed in PBS, we have chosen a gap between plates of the measurement cell in which only one layer of microspheres can fit in (i.e., 300 μm). The gap was then slightly smaller than the mean size of the microspheres (microspheres sieved between 315 and 500 μm) assuming that the microspheres formed a reproducible homogenous monolayer of material between the plates of the cell measurement. This gap was also chosen to insure that only the elasticity of the microspheres will be investigated. It was verified that the measurements were conducted in the linear regime. Thus, the microspheres were reversibly deformed as confirmed by the observations under the optical microscope.

Rheological characterization of microspheres

Shear sweep studies. First, experimental conditions to perform rheological measurements on microspheres in relevant conditions needed to be identified. It was then verified that the material deposited in the measurement cell underwent reversible deformation during shear solicitations. In general, this condition is fulfilled in the linear regime that corresponds to plateau values of G′ and G″ shear moduli as a function of the strain.

In a first set of experiments, rheological behaviors of nondegradable microspheres were monitored under shear sweep to identify the range corresponding to the linear regime. Figure 4 presents the results of variation of G′ and G″ shear moduli for two of the nondegradable microspheres used in this study (Figure 4).

According to the results obtained from these experiments, G′ and G″ moduli were stable between values of the strain ranging from 0.02 to 0.3% indicating the limits of the linear regime for all types of microspheres. The imposed strain, γ, chosen for exploring the rheological behavior of the microspheres in the oscillatory shear mode used in the next step of the development of the method was 0.04%. It is noteworthy that this value of controlled strain is very low. It insured nondestructive microshear preserving microspheres from being damaged during rheological measurements. This was confirmed by a systematic observation of the microspheres by optical microscopy after rheological measurements. This controlled observation performed on the different microspheres after rheological measurements showed that all types of microspheres remained well

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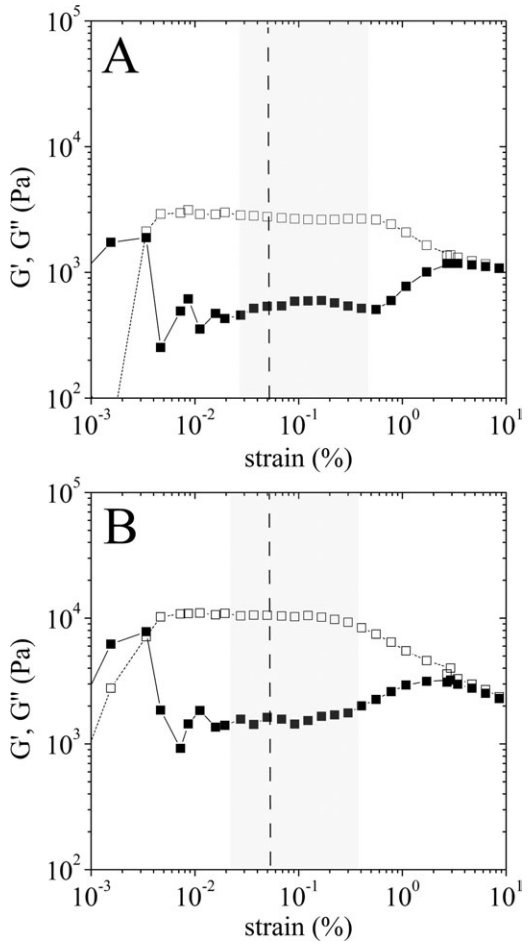

Figure 4. Determination of the range of linear regime under controlled strain in experiment with nondegradable microspheres with crosslinking molar ratio of 2% (A) and 6% (B). G′(open symbols) and G″(closed symbols) moduli were monitored as a function of strain sweeping from 0.001% to 10%. The grey zones correspond to the linear regime and the dotted lines indicate the positions of chosen controlled shear strain value (0.04%) for following experiments.

spherical and did not exhibit any fracture [All data not shown, See for an example Figure 2(C)].

Frequency sweep studies. Controlled strain mode with a constant strain at 0.04% was applied to study the viscoelastic characteristics of the nondegradable microspheres prepared with crosslinking ratios ranging between 1 and 11 %. Experiments repeated three times on the same sample of microspheres but by refilling the measurement cells with a new amount of sample always gave values in a low-range interval (ranging between 5% and 8%). An example of the curves obtained showing the mean value with standard deviation of the storage (G′) and loss (G″) moduli as a function of frequency was plotted in Figure 5(A) for the microspheres PEGDMA-1%. The two moduli G′ and G″ were almost independent on the variation of frequency in the whole range of the frequency sweep. The rheological behavior showed by the microspheres was typical of that of chemically crosslinked hydrogels. The ratio G″/G′ was roughly in the order of 0.1 and appeared rather independent on the frequency. In agreement with this, the phase angle, δ, was nearly constant over the range of frequencies comprised between 0.05 and 40 Hz [Figure 5(B)]. In those conditions, the value of the phase angle was around 8° indicating that the microspheres behaved like a gel material with nearly pure elastic properties.

As shown in Figure 6, the storage modulus G′, of the microspheres was almost independent on frequency whatever their crosslinking density was. The value of the storage modulus taken at a frequency of 10 Hz increased with the degree of crosslinking of the microspheres [Table I, Figure].

This was also the case considering the loss modulus, G″ [Figure 7. The phase angle deduced from these measurements ranged between 3° and 8° for the different microspheres (Table I). This indicated that all microspheres showed nearly pure elastic properties.

Both G′ and G″ increased linearly with the crosslinking molar ratio of the microspheres up to a crosslinking molar ratio of about 7%. Above this value, G′ reached a plateau value, while G″ kept increasing. The results obtained here can be discussed

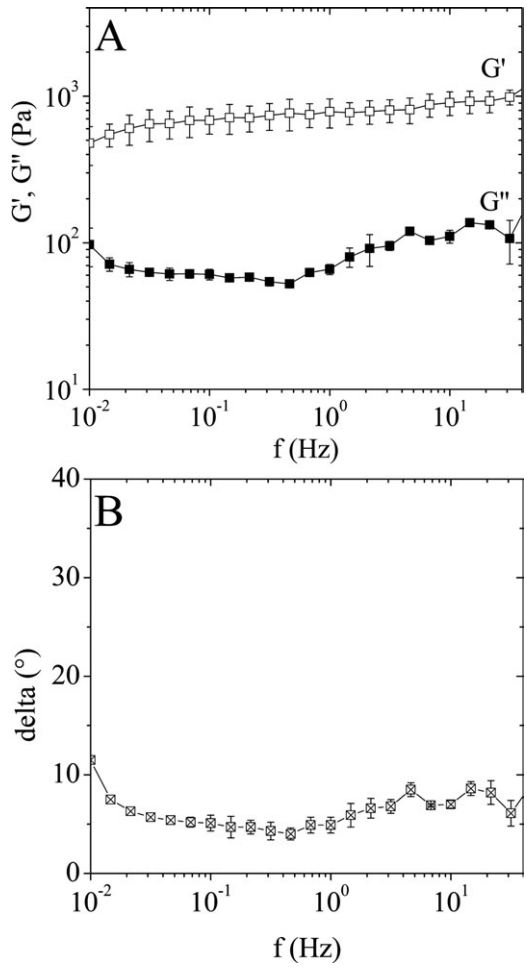

Figure 5. Influence of frequency on the rheological behavior of the microspheres PEGDMA-1%. Measurements were performed with the imposed shear strain at 0.04%. A: the storage (G′) and loss (G″) moduli; B: phase angle δ.

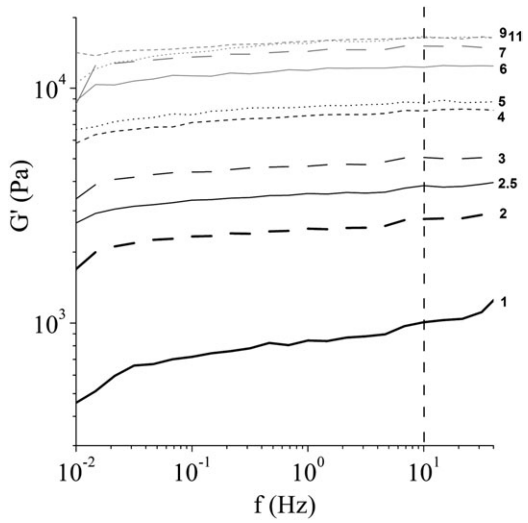

Figure 6. Determination of the storage modulus, G′, of non degradable microspheres with different crosslinking molar ratio (thick solid black line 1%, thick long dashed black line 2%, thin solid black line 2.5%, thin long dashed black line 3%, thin short dashed black line 4%, dotted black line 5%, solid grey line 6%, long dashed grey line 7%, short dashed grey line 9%, dotted grey line 11%) at the oscillatory frequency of 10 Hz and under an imposed shear strain of 0.04%.

considering the theory of the rubber elasticity proposed by Flory[26] in which the storage modulus G′ depends directly on the number of crosslink per elastically active chain, v_e, and on a constant characterizing the polymer solvent system, α. The relation appearing between these parameters and the storage modulus is given in eq. (5).

$$G' = \alpha v_e RT \tag{5}$$

where R is the universal gas constant and T the temperature.

The two parameters α and v_e are related to the characteristics of a given hydrogel. According to the Flory theory, they represent the swelling capacity of the gel. As a consequence, the aforementioned relation demonstrates the dependence between rheological moduli and swelling ratio of hydrogels. It can be pointed out that the Flory theory applied well with the microspheres considered in this study. Both the storage modulus, G′, and the swelling ratio of microspheres with degrees of crosslinking below 7% varied a lot (Figure 3). In contrast, with highly crosslinked microspheres (crosslinked density above 7%), very little differences in swelling capacities were observed and the storage modulus reached a plateau value. According to the above results, the storage modulus of microspheres varied a lot with crosslinking molar ratio only with microspheres crosslinked at a maximum ratio of 7%. Considering these microspheres, an almost linear relation was found between the two parameters [Figure 7]. This suggested that degradation of microspheres through hydrolysis of the crosslink bonds can be monitored by measuring the storage modulus of the microspheres during degradation experiments performed on microspheres with an initial crosslinking molar ratio below 7%.

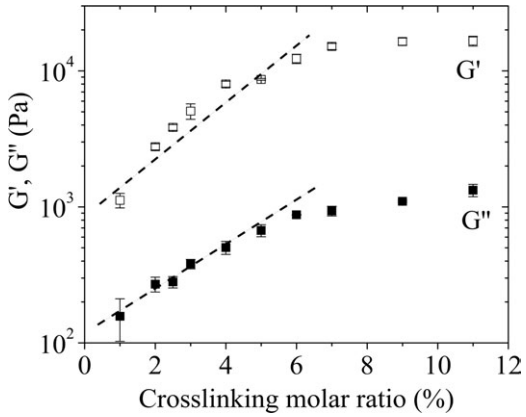

Figure 7. Storage modulus, G′, and loss modulus, G″, of hydrogel microspheres with different crosslinking molar ratio. Measurements were performed with an imposed shear strain at 0.04% and at an oscillation frequency of 10 Hz. The dashed line is only used as a guide to highlight the linear domains of the curve between the storage modulus and the crosslinking molar ratio of the microspheres.

Rheological Characterization of the Degradation of Hydrolysable Microspheres

Degradable microspheres were synthesized using a hydrolysable crosslinker. The degradation of these microspheres was believed to take place by hydrolysis of the crosslinking bonds, including lactic and glycolic acid segments occurring with contact of water. Thus, it was expected that the crosslinking density of the microspheres will be reduced during the degradation process, which in turn could be monitored by measuring the storage modulus of the microspheres over time. It was also expected that the hydrolysis of the microspheres would be accompanied by the release of carboxylic acids in the incubation medium coming from the hydrolysis of the ester bonds included in the lactic and glycolic acid containing segments of the crosslinker. This effect can be monitored by measuring the pH of the incubation medium. Thus, an acidification of the incubation medium would signify that the microspheres degraded through the expected hydrolytic mechanism, whereas a decrease in

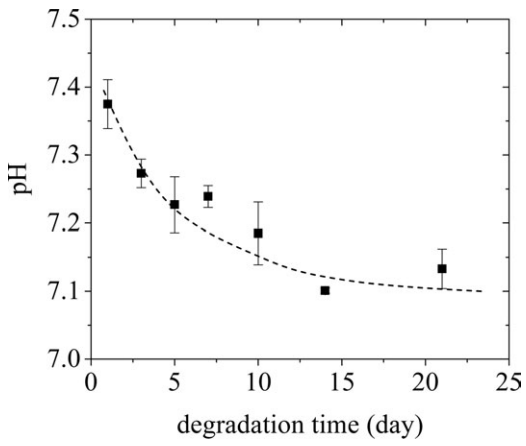

Figure 8. Variation of pH of the incubation medium during incubation of the microspheres HEMA4L4G-3% in PBS at 37°C.

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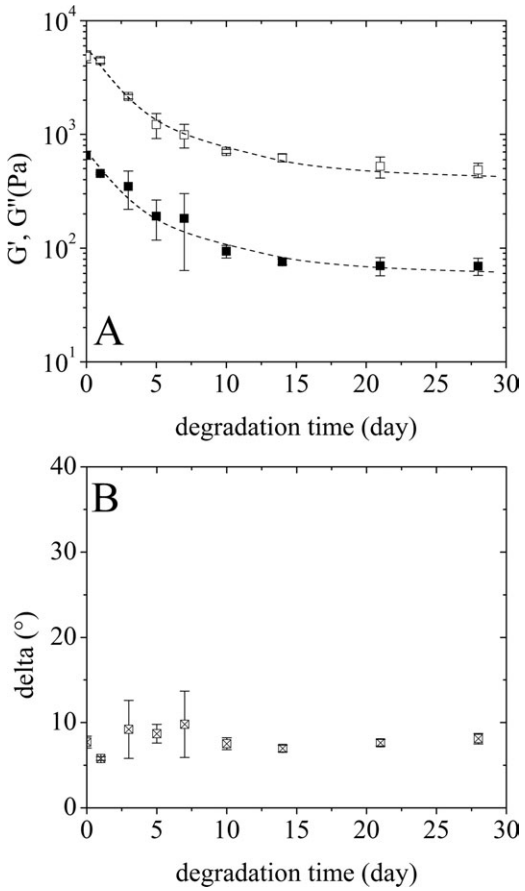

Figure 9. Evolution of rheological properties of microspheres HEMA4L4G-3% during incubation in PBS at 37°C. A: Storage (G′, open symbols) and loss (G″, closed symbols) moduli. B: Phase angle δ. Experimental conditions for the measurement of rheological properties of the microspheres were set with an oscillatory frequency at 10 Hz and a shear strain at 0.04%. The lines were drawn to serve as guides for the eyes.

storage modulus would indicate a loss of crosslinking bonds in the hydrogel structure of the microsphere.

Rheological measurements were performed in conditions established earlier on microspheres incubated in PBS. In agreement with the work presented in the first part of this article, microspheres with a low initial crosslinking density (3%) were selected for this study.

Figure 8 presents the results of the evolution of the pH of the incubation medium of microspheres HEMA4L4G-3% monitored during the experiment. It shows a significant decrease in pH value of the dispersing medium occurring from the start of the experiment up to the 10th day of the incubation time. The decrease of pH monitored over the first 10 days of the incubation indicated that the microspheres released acidic compounds in the incubation medium. This was in agreement with the hydrolytic mechanism expected from the chemical nature of the hydrogel composing the microspheres. After 10 days, the pH of the incubation medium remained constant indicating that the degradation of the microspheres was stopped.

Rheological measurements were performed on degradable HEMA4L4G-3% microspheres under the experimental conditions defined earlier: the oscillatory frequency was set to 10 Hz and the shear strain was set at 0.04% to remain in the linear regimen. Figure 9 presents the results obtained from the measurement of the storage, G′, and loss (G″) moduli of the degradable microspheres HEMA4L4G-3% incubated in PBS over a period of time of 21 days. A clear decrease of G′ was monitored between the start of the experiment (day 0) and day 10 where G′ reached a plateau value. The loss modulus, G″, followed exactly the same tendency with pH over the same period of time. The value of the phase angle, δ, remained constant and close to 8° during the whole period of time of the experiments indicating that the hydrogel remained as an almost purely elastic material.

The fact that the storage modulus of the microspheres was decreased with time during the incubation of the microspheres in PBS was in favor with a decrease in the crosslinking density of the hydrogel forming the microspheres. The timescale in which the modification of the rheological properties of the microspheres occurred correlated well with the time scale during which the pH of the incubation medium dropped down. This last result suggested that the modification of the rheological properties monitored during the incubation of the microspheres in PBS resulted from the degradation of the hydrogel through the hydrolysis of the crosslinking bonds.

CONCLUSIONS

Conditions to measure rheological properties of microspheres made of a chemically crosslinked hydrogel and remaining as individual microspheres were established. It was determined that relevant and reproducible measurements could be obtained with a rheometer equipped with a parallel plate geometry in which a homogenous and continuous monolayer of microspheres is inserted in the gap between the plates. The storage moduli of the microspheres followed the theory of rubber elasticity developed by Flory. It was affected by the degree of crosslinking of the hydrogel forming the microspheres in the same crosslinking density range than that affected the swelling ratio of the microspheres. Results from this work also showed that degradation of hydrolyzable crosslinked microspheres can be monitored by measuring their storage modulus over time by applying the rheological method of characterization of microspheres developed in this work. Further experiments will be conducted on hydrolyzable microspheres to investigate the influence of the crosslinking molar ratio and of the nature of the degradable crosslinking bond on both the rheological properties of the microspheres and their degradation rate.

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