DEGRADATION RATE OF γ-POLYGLUTAMIC ACID PROBED BY ^{1}H-NMR SPECTRAL ANALYSIS AND BY PFGSTE NMR – INTERNAL CONSISTENCY

[1]**Min Peng, Wei Liu, Qun Chen**[*] **& **[2]**Eddy W. Hansen**[*]

[1]Physics Department and Shanghai Key Laboratory for Functional Magnetic Resonance Imaging, East China Normal University, Shanghai 200062, P. R. China.

[2]Department of Chemistry, UiO, P. O. Box 1033 Blindern, N-0315 Oslo, Norway

E-mail: qchen@ecnu.edu.cn, eddywh@kjemi.uio.no

ABSTRACT

Pulsed Field Gradient Stimulated Spin-Echo (PFGSTE) NMR experiments on γ-Polyglutamic Acid (γ-PGA) dissolved in D_2O (pH = 3 and T = 353 K) was performed to monitor the degradation process as a function of time. After establishing the effect of molecular weight on relaxation (T_1 and T_2), the PFGSTE response curve (signal intensity as a function of gradient field strength squared) enabled the average or effective diffusivity to be determined, and hence the average molecular weight to be derived as a function of degradation time. The rate of degradation was determined to be $k_D = (2.3 \pm 0.4) \cdot 10^{-2} h^{-1}$. The corresponding rate determined by a single exponential (1. order rate law) fit to the time dependence of the signal intensity of resonance band δ = 4.0 – 4.1 ppm was found to be $k_I = (1.7 \pm 0.1) \cdot 10^{-2} h^{-1}$ showing that the two different and independent NMR approaches result in the same overall degradation rate. Finally, the time dependence of the PFGSTE NMR derived average molecular weight was shown to follow a random degradation process, as expected for a hydrolysis degradation process.

Keywords: *γ-Polyglutamic Acid, 1D-proton NMR, PFGSTE, degradation, Monte Carlo simulation*

1. INTRODUCTION

Over the past decade, a large numer of macromolecules have found considerable interest as drug carrier systems [1]-[3]. For instance, linking a drug onto a polymeric carrier has the effect of increasing the efficiency and prolongation of the blood circulation [4]. One of the most important requirements on macromolecular drug carriers is that they must not accumulate in the human body, since accumulation of polymer over years might cause severe organ damage. A significant and well-known way of polymer elimination is by degradation into smaller molecules having lower molecular weights [5]. Therefore, measurement of degradation rate and development of *in situ* techniques to monitor changes in molecular weight distribution (MWD) during degradation is of importance.

Among the biodegradable and biocompatible polymers, γ-PGA and its copolymers have been extensively applied as drug carriers. For instance, γ-PGA is a naturally occurring water-soluble polyamide that is synthesized from the genus Bacillus [6]. The polymer is made of glutamic acid units linked through the α-amino and the γ-carboxylic acid groups [7]. They are biocompatible, and do not elicit immune responses and have low toxicities in animal and human bodies. Recently, a series of biodegradable derivatives of γ-PGA, such as poly (γ-benzyl-L-glutamic acid), have been developed. These polymers showed great potential as drug delivery platforms [8] and suitable vectors for gene therapy [9]. While great attention has been paid to the improvement of γ-PGA derivatives performance through structure design, little is known about their fate inside the cell. Therefore, information regarding changes in the molecular weight distribution during degradation is important for such biodegradable polymers. In a previous study, we presented a new method suitable for elucidation of the degradation mechanism of polymers [10].

In this work we will monitor the diffusivity of γ-PGA during degradation with the objective to probe the average molecular weight as a function of degradation time. In order to decide whether the degradation process is random or not, the average molecular weight derived from NMR measurements will be compared to corresponding average molecular weight obtained by Monte-Carlo (MC) simulations.

2. EXPERIMENTAL

2.1 Materials

A γ-PGA polymer ($M_W \approx 10^5$ Da) supplied by Professor Wu (Department of Biology, ECNU, Shanghai) was vacuum dried and dissolved in a buffer solution (pH 3.0) with D_2O as a solvent. Its concentration was 1 g/L. The

sample was transferred into a 5 mm NMR tube and placed in an oven at 353 K at which temperature the polymer starts to degrade.

For MC calculation purposes, the MWD of the polymer was determined by Gel Permeation Chromatography (GPC) before degradation was initiated.

In order to establish a calibration curve between diffusivity and molecular weight some commercial samples (CAS: 26247-79-0; P1818-25MG, P4636-25MG, P4761-25MG, P4886-25MG) from SIGMA corporation were purchased

2.2 NMR measurements

Proton NMR spectra were acquired at 300 K on a Bruker Avance 500 spectrometer with a ^{1}H resonance frequency of 500.13 MHz. The parameters used were as follows; 32 scans, 90° pulse of 9.7 μs, a recycle delay of 5 s and 64 K of data points. The diffusion experiments were performed using a *bi*polar-*p*ulse *p*air *st*imulated *e*cho (BPPSTE) pulse sequence where the gradient amplitude g was successively increased up to 33 gauss cm^{-1}. The duration of the bipolar gradient pulse pair δ was set to 1.6 ms and the time T between the π/2 pulse and the π-pulse was fixed to 2.5 ms. The diffusion delay time Δ was set equal to 150 ms resulting in a diffusion time $t_D = \Delta-\delta/2-T/3 \approx 148.4$ ms.

At various times during the degradation process the sample was taken out of the oven and quickly cooled to 300 K at which temperature the NMR measurement was performed. Initial NMR measurements revealed that no degradation took place after cooling the sample to room temperature. The main reason for performing NMR diffusion measurements at room temperature was to avoid convective motion within the sample (due to formation of temperature gradients across the sample) which would otherwise affect the diffusion measurement.

3. RESULTS AND DISCUSSION

3.1 ^{1}H NMR

The molecular structure of the biodegradable γ-PGA polymer investigated in this work is illustrated in Figure1 together with its ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC spectra in a D$_2$O buffer solution (pH = 3.0).
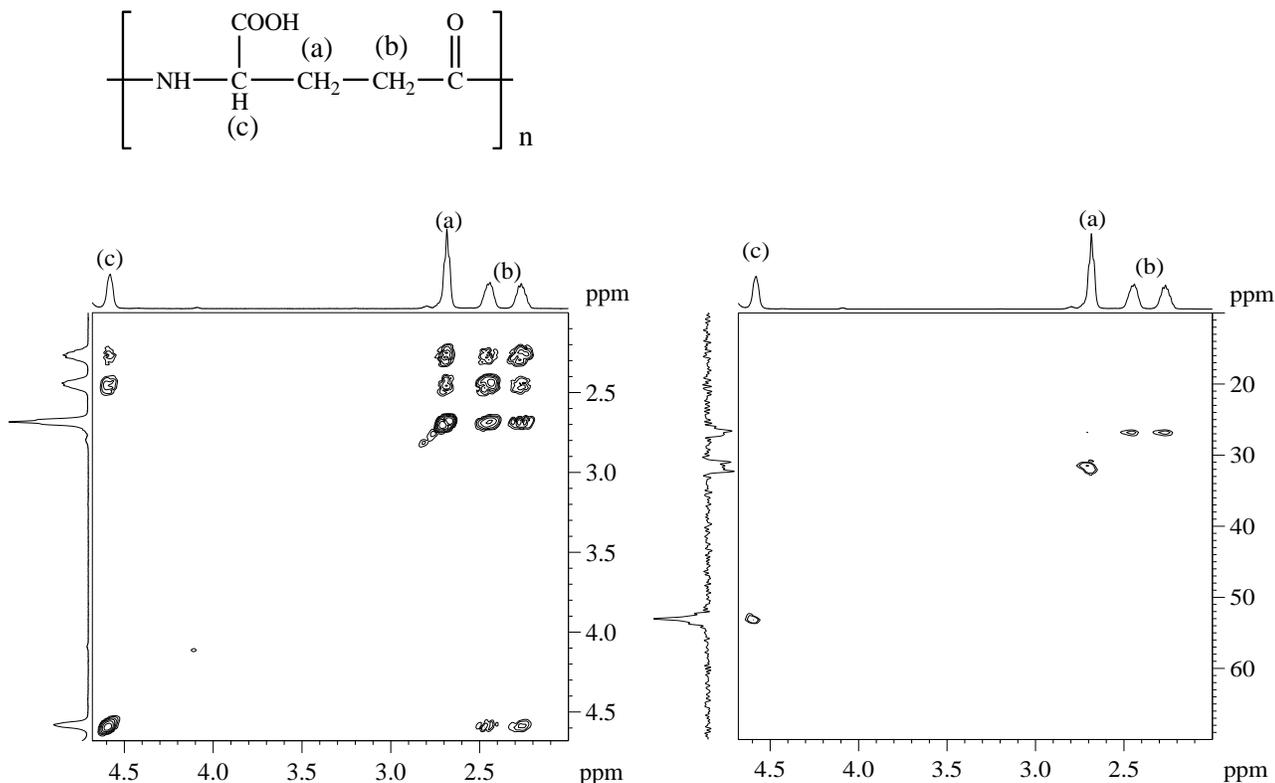
Figure 1. *Schematic description of the γ-polyglutamic acid (γ-PGA) structure and its ^{1}H-^{1}H COSY (left) and ^{1}H-^{13}C HSQC (right) spectra after dissolved in a D$_2$O buffer solution (pH = 3.0).*

Based on the correlation peaks in the COSY and in the HSQC spectra the proton resonances of γ-PGA were unambiguously assigned (Figure 1) and consistent with previous results published by Michiya Matsusaki et al. [0].

The specific resonance peaks of the -N$\underline{H}$ and -COO$\underline{H}$ protons could not be identified due to a fast exchange (on the NMR time scale) with deuterons of the D_2O solvent.

It is well known that under acidic conditions and at elevated temperature, the amide bonds of γ-PGA are susceptible to hydrolysis [2], [14]. Figure 2 shows the stacked ^{1}H NMR spectra of γ-PGA at different times during a five day exposure under hydrolysis conditions (pH = 3.0 and T = 353 K) and reveal some new peaks (a′, b′, c′) appearing in the spectra with increasing degradation time.
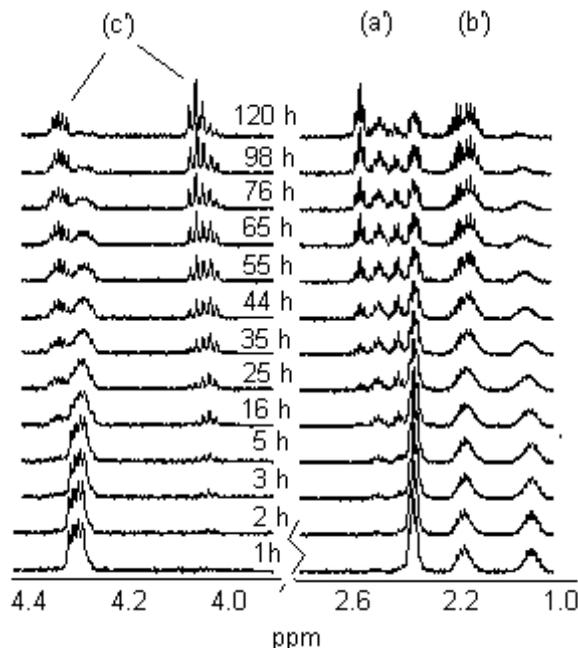

Figure 2. Stacked ^{1}H-NMR spectra of γ-PGA as a function of the hydrolysis time at 353 K and pH = 3.0. The resonance bands denoted a', b' and c' are formed during the degradation process. All spectra were acquired at 300 K, respectively.

The resonance assigned to c′ reveals two separate chemical shift regions in the spectrum, due to the existence of a chiral carbon in the molecule. A corresponding decrease in the intensity of the -C$\underline{H_2}$ (a), -C$\underline{H_2}$ (b) and -C$\underline{H}$ (c) protons of the non-hydrolyzed part of the γ-PGA is noted. The peak assignment was supported by the COSY spectrum of a fully hydrolyzed sample (pH = 3.0; not shown) in which the aforementioned three groups of resonances (a′, b′, c′) revealed the expected cross-peaks.

In order to estimate the rate of hydrolysis of γ-PGA, the intensity of the -C$\underline{H}$ (c′) protons relative to the intensity of all the -C$\underline{H}$ protons in the system was monitored as a function of time, as illustrated on Figure 3.
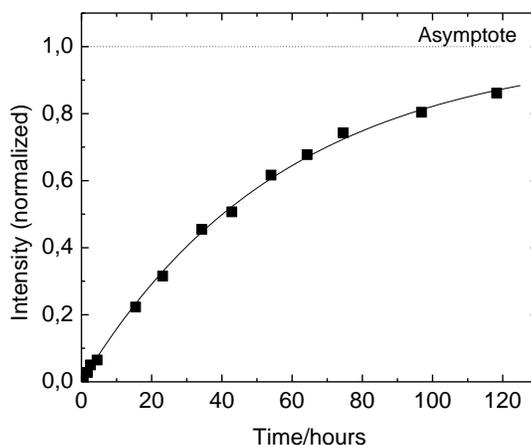

Figure 3. Relative change in the signal intensity $I_{C'}$ (■) of the CH proton resonance band c' ($\delta = 4.0 - 4.1$ ppm) as a function of hydrolysis time t. The solid curve was calculated by a non linear least squares fit to the equation;
$I_{c'} = [I_0 - I_\infty] \cdot \exp[-k_I t] + I_\infty$ *with $k_I = (1.7 \pm 0.1) \cdot 10^{-2}$ h^{-1}, $I_\infty = (1.00 \pm 0.02)$ and $I_0 = (0.01 \pm 0.02)$.*

If assuming the degradation process to be described by a first-order rate law, the observed signal intensity of the c′-peak as a function of degradation time was fitted to a single exponential function resulting in a degradation rate $k_I = (1.7 \pm 0.1) \cdot 10^{-2}$ h^{-1}

4. PFGSTE MEASUREMENTS

The pulse sequence applied in the diffusion experiment is illustrated in Figure 4 with a parameter list enclosed in the figure caption. Rather than plotting the signal intensity (I) of the PFGSTE response as a function of the gradient pulse strength squared (g^2) we choose $x = \gamma^2 \delta^2 t_D g^2$ as the independent variable, where γ (= $2.674 \cdot 10^4$ rad·s^{-1}·gauss^{-1}) is the gyromagnetic ratio, δ (= 1.6 ms) is the duration of the gradient pulse and T is the distance between the 90^0 and 180^0 pulses and t_D defines the diffusion time and equals (Δ-δ/2-T/3). This approach has the advantage that a single exponential response curve (on a semi-logarithmic plot) will be linear and will have a slope equal to some average self diffusion coefficient. Provided there are no complicating effects such as a distribution of molecular weights, the echo amplitude I for a simple molecule in an isotropic solution takes the form (using a stimulated spin-echo pulse sequence):

$$\frac{I}{I_0} = \exp\left[-\frac{4T}{T_2}\right] \cdot \exp\left[-\frac{\Delta'}{T_1}\right] \cdot \exp[-xD] \qquad (1)$$
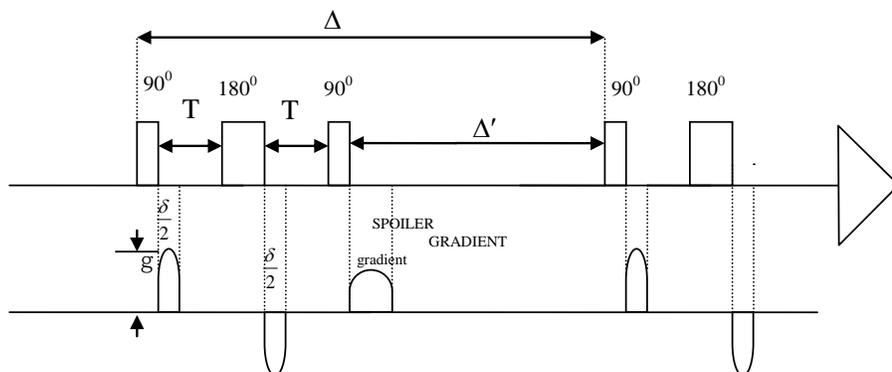

Figure 4. *Illustration of the bipolar-pulse pair stimulate echo (BPPSTE) pulse sequence for diffusion measurements, where gradient amplitude g is successively increased up to 33 gauss cm^{-1}. The duration δ of the bipolar gradient pulse pair is 1.6 ms and the time parameter T equals 2.5 ms. The diffusion delay time Δ was set equal to 150 ms and results in a diffusion time $t_D = \Delta-\delta/2-T/3$. The time interval during which T_1 is effective is denoted by Δ'. The symbol 'sp' is a short hand notation for "spoiler-pulse".*

Where I_0 is the signal intensity for x = 0, T_1 is the spin-lattice relaxation time and T_2 is the spin-spin relaxation time. The other symbols are defined in Figure 4. In the case of γ-PGA, the system is not initiation of the degradation process (Figure 5). The MWD characteristics were determined to be $M_W = 2.15 \cdot 10^4$ g/mol and $M_w M_n = 1.60$
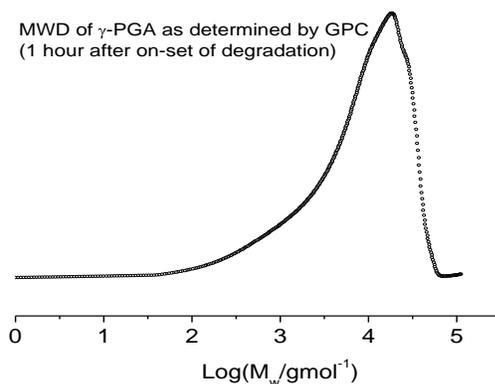

Figure 5. *MWD (as determined from GPC) of γ-PGA at the start of the experiment (t = 1 hour) with $M_w = 2.15 \cdot 10^4$ g/mol and $M_w/M_n = 1.60$*

D. von Meerwall [15] has reported significant departures from single exponential attenuation of the echo amplitude even in systems where the polydispersity (M_W/M_N) is as small as 1.1 suggesting that equation 3 in the general case must be replaced by a more generalized expression, taking the form:

$$\frac{I}{I_0} = \sum_i f(D_i)\exp\left[-\frac{4T}{T_{2i}}\right] \cdot \exp\left[-\frac{\Delta'}{T_{1i}}\right] \cdot \exp[-xD_i] \qquad (2)$$

where the distribution of diffusivities is represented by $f(D_i)$. D_i is related to the molecular weight M_{wi}, according to the general equation:

$$D_i = kM_{wi}^{-\alpha} \qquad (3)$$

where k and α are constants depending on the type of system investigated. For example, in entangled polymer systems the exponent α is predicted to be 2 [16]. At the other extreme, for a random coil polymer in an infinite dilute solution, the Flory theory predicts $0.5 \leq \alpha \leq 0.6$ [17] (in this work the parameters k and α were determined from PFGSTE measurements on four commercial γ-PGA samples possessing narrow MWDs, resulting in k = $9.53 \cdot 10^{-4}$ and $\alpha = 0.69$).

A subject of main concern relates to the dependence of the relaxation times T_1 and T_2 on diffusivity (or equivalently, molecular weight). It is frequently claimed (or assumed) that both T_1 and T_2 are independent on molecular weight. This may often be the case if one probes the polymer diffusivity by monitoring the peak assigned to a side chain proton. However, in the case of γ-PGA no such side chain protons exist. We therefore measured these relaxation times for one of the c'-peaks (the -**CH** proton assigned to the resonance peak with chemical shift $\delta = 4.0$-4.1 ppm; Figure 2). The results are shown in Figure 6 and shows that T_1 is constant (single exponential decay behavior) and independent on the molecular weight.
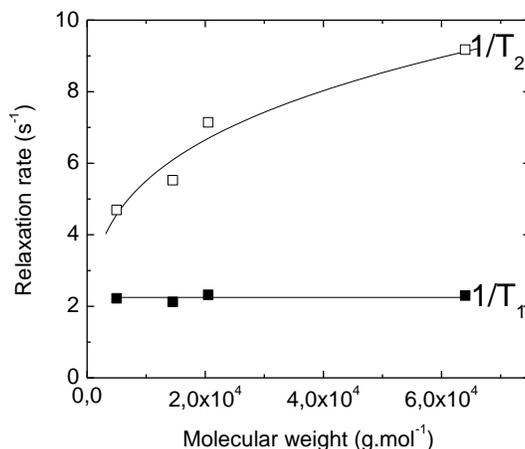

Figure 6. *The change in relaxation rates $1/T_1$ and $1/T_2$ of peak c' ($\delta = 4.0$-4.1 ppm; Figure 2) as a function of molecular weight of some commercial γ-PGA reference solutions characterized by narrow molecular weight dispersions.*

In contrast, T_2 (revealing single exponential decay behavior) was found to decrease rather significantly with increasing molecular weight. However, since T < 2.5 ms (see Figure4) and $T_2 > 100$ ms, we find that the term $\exp[-4T/T_2]$ in equation 1 is always larger than 0.90. Hence, we have approximated this term ($\exp[-4T/T_2]$) by 1, i.e., equation 2 takes the form;

$$\frac{I}{I_0} = \sum_i f(D_i) \cdot \exp[-xD_i]] = \int_D f(D) \cdot \exp[-xD)dD] \qquad (4)$$

in which the sum has been replaced by an integral (assuming a continuous distribution f(D) of the diffusivity D).

4.1 Average diffusivity - Ensemble averaging

Since the derivation of an average diffusivity (and thus the average molecular weight) from NMR diffusion measurements is of major concern in this work we find it necessary to discuss in somewhat more details how the PFGSTE data are analyzed. A typical PFGSTE response curve is depicted in Figure 7 and reveals a slight deviation from a pure and single exponential decay. Such a behaviour is symptomatic for poly disperse systems possessing a distribution of molecular weights, and hence a distribution F(D) of the diffusivity D. The solid curves in Figures 7 represent non-linear least squares model fits to the function;

$$\frac{I}{I_0} = \exp\left[-ax + 1/2bx^2\right] \tag{5}$$

where a and b are constants. It has been noticed that for many polymer systems the parameter b in equation 5 equals 0, which is ascribed to some undefined ensemble averaging process taking place in the solution. In order to calculate the average diffusivity of such a system we take the cumulant expansion of equation 4 to second order which results in $a = \langle D \rangle (= \int_D D \cdot f(D)dD)$ and $b = \left[\langle D^2 \rangle - \langle D \rangle^2\right]$. Strictly speaking, a represents the "true" average diffusivity provided the condition b/a << 1. Figure 8 shows a plot of a and b (equation 5) as a function of the degradation time and shows that b/a << 1 at and any time during degradation process. Consequently, the parameter $a = (\langle D \rangle (= \int_D D \cdot f(D)dD))$ represents a good estimate of the average diffusivity.
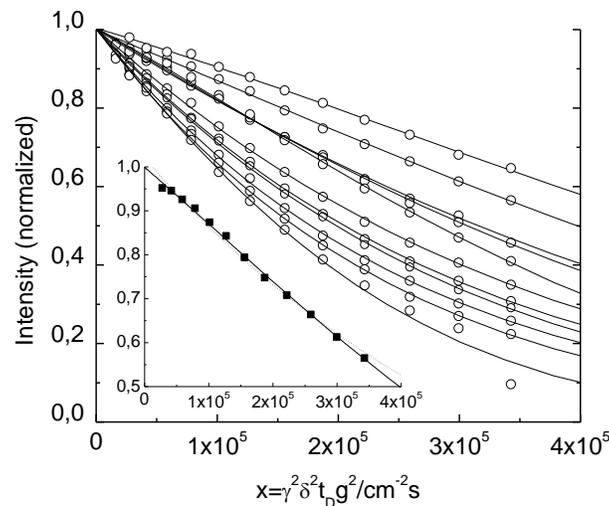

Figure 7. *Normalized signal intensity as a function of $x = \gamma^2\delta^2 t_D g^2$ during degradation time t (=3, 5, 16, 24, 35, 44, 55, 65, 78, 98 and 120 hours; from top to bottom). The solid curves represent non linear least squares fit of equation 5 ($I = \exp\left[-ax + bx^2/2\right]$) to the observed data (o) with the parameters a and b being adjustable. The insert shows two different model fits with i) b being fixed and equal to 0 (⋯) and ii) b being adjustable (—), respectively. The response curve was acquired 5 hours after the degradation process was initiated.*
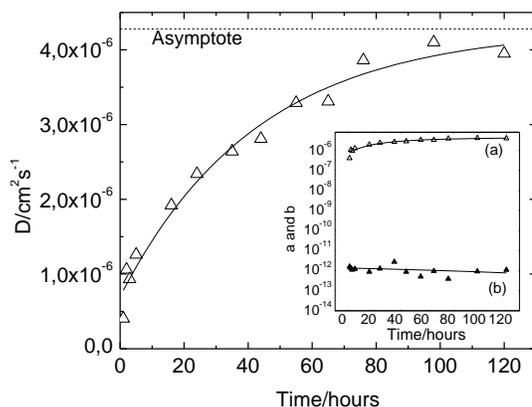
Figure 8. *Insert: Parameters $a = \langle D \rangle$ and $b = \left[\langle D^2 \rangle - \langle D \rangle^2 \right]$ (see equation 5) as a function of degradation time. For clarity, the solid curves represent simple eye-ball fits to the observed data. The average diffusivity <D> plotted as a function of degradation time. Main: The solid curve was calculated by a non linear least squares fit to <D> using a single exponential function $< D >= \left[< D >_0 - < D >_\infty \right] \cdot \exp \left[- k_D t \right] + < D >_\infty$ with k_D = (2.3 ± 0.4)·10^{-2} h^{-1}, $\langle D \rangle_\infty = (4.3 \pm 0.2) \cdot 10^{-2} cm^2 s^{-1}$ and $\langle D \rangle_0 = (7.0 \pm 0.2) \cdot 10^{-2} cm^2 s^{-1}$. The dotted curve represents the estimated limiting diffusivity $\langle D \rangle_\infty$ at long degradation time.*

It is interesting to notice that the rate of change of the diffusivity (k_D = (2.3 ± 0.4)·10^{-2} h^{-1}) equals - within experimental error - the rate of change k_I (= (1.7 ± 0.1)·10^{-2} h^{-1}) of the signal intensity of the CH resonance (δ = 4.0-4.1 ppm) as a function of time (see Figure 3).

5. AVERAGE MOLECULAR WEIGHT DURING DEGRADATION

The derived average diffusivity $\langle D \rangle$ as a function of degradation time is plotted in Figure 8 and shows that the diffusivity increases monotonically with degradation time. Actually, within the experimental time domain, the average diffusivity $\langle D \rangle$ is well represented by a single exponential function with respect to the degradation time. In order to determine whether the irreversible degradation process in random or not we performed MC-simulation to calculate the average molecular weight as a function of degradation for two different degradation processes; A) random scission and B) end group scission. It is assumed that only one bond is broken in each step implying that only molecules composed of more than a single monomer unit are considered, since monomers do not have any relevant bond that can be broken. Hence, there are two ways to structure the probability model:

2.3 Monte Carlo simulation

The degradation model for a linear polymer was designed from a Monte Carlo (MC) simulation, and involves a random scoring of events in a stepwise, successive manner. Starting from a specified mass-distribution (or distribution of molecular lengths), the degradation process is accomplished using a pseudo-random number generator to select randomly a bond within a molecule from the actual distribution by assuming that only one bond is broken at each unit step of time. In this work we have chosen two different scission models, a random scission model and an end-scission model *[11], [12]*.

2.3.1 Algorithm

Based on an experimental Molecular Weight Distribution (MWD) of the polymer in question, the corresponding number distribution (or molecular length) was determined using a discrete set of 10^5 molecules. To calculate the average molecular weight during degradation we followed a procedure detailed by Bose [12], so only a brief outline will be given here:

At any time (t) during the degradation process, the number-distribution (or length) of polymer chains is denoted by the vector $\bar{N}(t)$:

$$\bar{N}(t) = \{N(1,t), N(2,t),\ldots N(n,t)\ldots,N(n_0,t)\} \tag{6}$$

Where N(n,t) represents the number of polymer molecules containing n monomer units at time t. The term n_0 represents the upper limit (maximum) of the number of monomer units in a chain at any time t. As already pointed out, the initial distribution $\bar{N}(0)$ is determined from a specific experimental molecular weight distribution and can be expressed by:

$$\bar{N}(0) = \{N(1,0), N(2,0),\ldots N(n,0)\ldots,N(n_0,0)\} \tag{7}$$

Hence, the total number of molecules at any time t during the degradation process is:

$$T(t) = \sum_{n=1}^{N} N(n,t) \tag{8}$$

In the random scission model we assume a completely random bond-breaking process in which the probability P for breaking a bond takes the form [12]:

$$P(n,t) = \frac{(n-1)N(n,t)}{\displaystyle\sum_{i=1}^{n_0} (i-1)N(i,t)} \quad 1 \le n \le n_0 \; and\, 1 \le i \le n_0 \tag{9}$$

The other alternative considered is the end scission model in which the molecules are split only at the endpoint of the chain. As discussed in reference 12, the above models are rather general and can be used to derive other types of scission models.

Since the objective is to obtain a relationship between the average molecular weight and the NMR signal intensity as a function of the hydrolysis time, the degradation process was divided into a number of cycles in which $\bar{N}(t)$ was calculated for each cycle enabling the average molecular weight M_w and the corresponding signal intensity of the various peaks to be derived. The simulation was stopped when M_w reached 10^3 g mol^{-1}

The results of these simulations are shown in Figure 9 and shows that the NMR derived average molecular weight (as determined from the diffusion data via equation 3) is in excellent agreement with the average molecular weight determined from MC simulation, when assuming a random degradation process. The solid curve in Figure 9 was derived by scaling the time parameter t from the MC calculations such that a best fit to the observed data was obtained.
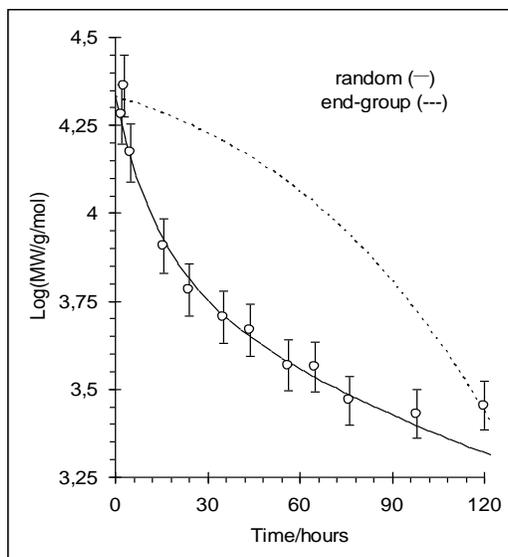

Figure 9. *Average molecular weight M_w (o) derived from diffusion measurement via equation 3. The dotted and solid curves represent model calculated M_w from MC simulations considering two different degradation models; random (—) and end group scission (---).*

One point of concern is that the estimated diffusivity $\langle D \rangle_\infty$ obtained from the exponential model fit in Figure 1 predicts an M_w equal to $2.53 \cdot 10^3$ g/mol at infinite degradation time and corresponds to an average molecular size of 16 monomer units of γ-PGA. However, after 14 days of degradation, the sample did not show any significantly smaller diffusivity. Actually, the diffusivity of a monomer unit of γ-PGA, as estimated from equation 3 amounts to $<D> = 2.9 \cdot 10^{-5}$ cm^{2}s^{-1}. However, such a fast diffusivity could not be observed. Hence, a γ-PGA sample which is initially composed of 460 monomer units (in average), seems to decompose to smaller fragments with an average size corresponding to approximately 16 monomer units. The simple exponential degradation behaviour (Figure 3) found by NMR is consistent with the results obtained from MC simulation (Figure 9), and suggests the scission process to be random.

6. CONCLUSION

A detailed analysis of the PFGSTE-NMR data of γ-PGA dissolved in deuterated water is presented and shows that the diffusivity of γ-PGA increases exponentially within the time domain investigated (120 hours). By applying an empirical equation ($<D> = kM_w^{-\alpha}$), which couples the average diffusivity ($<D>$) and the average molecular weight $<M_w>$, the latter was found to decrease exponentially with degradation time at a rate equal to $k_X = (2.3 \pm 0.4) \cdot 10^{-2}$ h^{-1}. By comparing the NMR derived M_w with the corresponding M_w obtained from Monte-Carlo simulation, the degradation of γ-PGA was shown to be random. In short, from an initial average molecular size of 460 monomer units, the degradation process was monitored until the average molecular size of γ-PGA approached approximately 16 monomer units.

7. ACKNOWLEDGEMENT

8. REFERENCES

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