The effect of molecular weight on the material properties of biosynthesized poly(4-hydroxybutyrate)

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ABSTRACT:

Poly(4-hydroxybutyrate) (P4HB) is a bacterial polyhydroxyalkanoate with interesting biological and physicochemical properties for the use in biomedical applications. The synthesis of P4HB through a fermentation process often leads to a polymer with a too high molecular weight, making it difficult to process it further by solvent- or melt-processing. In this work P4HB was degraded to obtain polymers with a molecular weight ranging from $1,5 \cdot 10^3$ g/mol to $1,0 \cdot 10^6$ g/mol by using a method established in our laboratory. We studied the effect of the change in molecular weight on thermal and mechanical properties. The decrease of the molecular weight led to an increase in the degree of crystallinity of the polymer. Regarding the tensile mechanical properties, the molecular weight played a more prominent role than the degree of crystallinity in the evolution of the properties for the different polymer fractions. The method presented herein allows the preparation of polymer fractions with easier processability and still adequate thermal and mechanical properties for biomedical applications.

Keywords:P4HB, molecular weight, mechanical properties

1. Introduction

Poly(4-hydroxybutyrate) (P4HB) is a natural polyester that has been approved for use as an absorbable suture by the FDA in 2007. P4HB is a polyhydroxyalkanoate, a family of polymers synthesized by microorganisms as carbon and energy storage compounds.

The chemical synthesis of P4HB has been attempted; however, it is generally considered impossible to produce the polyester by this method with sufficiently high molecular weight necessary for most applications. [1] Furthermore, the chemically produced P4HB may contain residual metal catalysts that are used in the chemical synthesis of the polymer. Thus, P4HB is produced through a fermentation rather than a chemical process. To produce P4HB homopolymers, recombinant *Escherichia coli* strains were used. By introducing the PHB synthase gene (*phbC*) from *Ralstonia eutropha* and a 4-hydroxybutyric acid-coenzyme A transferase gene (*orfZ*) from *Clostridium kluyveri, E. coli* strains XL1-Blue and JM109 were able to produce P4HB when 4-hydroxybutyric acid (4HB) was supplied as a precursor in the culture medium. [2, 3] It was also reported that an *E. coli* JM109 mutant carrying two plasmids was able to synthesize P4HB using Lysogeny broth (LB) medium containing only glucose without P4HB related precursor such as 4HB. [4]

The interest on using P4HB in medical applications derives from its inherent biocompatibility and adequate physical properties. Research has focused on heart valves, vascular grafts, scaffolds, and sutures. [3, 5] Besides being biocompatible, the degradation process of P4HB is also milder than that of other biomedical polymers: P4HB degrades *via* surface erosion, which minimizes the burst release of acids [5]. Moreover, the degradation product, 4-hydroxybutyrate, is a metabolite commonly found in the human body. [6] Recent studies have shown that both fiber monofilaments [7] and fiber meshes [8] made of P4HB degraded without causing any adverse reactions to the surrounding soft tissue (muscle and abdominal wall, respectively). Regarding physical and chemical properties, P4HB provides a combination of properties that makes it very useful in biomedical applications: solubility in a range of polar solvents (e.g., acetone), elastomeric character at room and body temperature, low melt temperature (that is, easy melt processability), high molecular weight, very high ductility (>200 %), and a moderate resorption rate *in vivo*. [5]

Despite these characteristics and the large amount of data on *in vivo* animal studies, [5, 7–9] there has been little attention dedicated to the physical properties of P4HB. Specifically, no work has concentrated on studying changes in mechanical and thermal properties of P4HB as a function of molecular weight. Given that the bacterial synthesis of these polymers usually leads to very high molecular weight (Mn $\sim 10^6$ g/mol) and that at such high values the melt and solvent processability are compromised, we report in this study how the acid-catalyzed hydrolysis affects the mechanical and thermal properties of the biosynthesized P4HB.

2. Experimental

2.1. Biosynthesis of P4HB

All chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland). *Escherichia coli* JM109 carrying plasmid pKSSE5.3 was used in this study for P4HB production. pKSSE5.3 contains a PHA synthase gene (*phaC*) from

Ralstonia eutropha and a 4-hydroxybutyric acid-coenzyme A transferase gene (*orfZ*) from *Clostridium kluyveri*. [2] The JM109 recombinant cells were used to inoculate a 10 mL LB culture in a 50 mL flask. The cells were incubated at 37 °C and 150 rpm overnight. The culture was then used to inoculate 200 mL of preculture using modified E2 medium in a 1 L shake flask with a dilution of 1:20 (v v^{-1}). Modified E2 medium contained the following constituents: $NaNH_4HPO_4 \cdot 4H_2O$ 3,5 g L^{-1}, KH_2PO_4 3,7 g L^{-1}, and K_2HPO_4 7,5 g L^{-1}, dissolved in 1 L of water. One mL L^{-1} of 1 M $MgSO_4 \cdot 7H_2O$ was added to the medium. One mL L^{-1} of trace elements (TE) dissolved in 1 M HCl was also added. TE contained: $FeSO_4 \cdot 7H_2O$ 2,78 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 1,47 g L^{-1}, $MnCl_2 \cdot 4H_2O$ 1,98 g L^{-1}, $CoCl_2 \cdot 6H_2O$ 2,38 g L^{-1}, $CuCl_2 \cdot 2H_2O$ 0,17 g L^{-1}, $ZnSO_4 \cdot 7H_2O$ 0,29 g L^{-1}. 10 g L^{-1} of xylose and 4 g L^{-1} of Na-4HB were used as the growth substrate and the precursor for P4HB synthesis, respectively. 1 g L^{-1} of NZ-amines and 0,015 g L^{-1} of thiamine were supplemented to support the growth. 100 μg mL^{-1} of ampicillin was added to maintain the plasmid. The preculture was incubated at 150 rpm and 32 °C for 16 h. It was then transferred to 600 mL modified E2 medium in a total volume 1,4 L bioreactor (Infors AG, Bottmingen, CH) equipped with standard control units. The initial optical density (OD_{600}) value in the bioreactor was between 0,10 and 0,30. Temperature was controlled at 32 °C with an external circulating water bath, and pH was maintained at 7,0 $\pm$ 0,1 by automatic addition of 25 % NaOH or 30 % H_3PO_4. Dissolved oxygen tension was monitored continuously with an oxygen probe (Infors AG, Bottmingen, Switzerland) and kept always above 30 % oxygen saturation. The agitation was set at 500 rpm.

2.2. Extraction of P4HB

P4HB was extracted directly from the lyophilized cells (1 mbar, 48 to 144 h). Cells were transferred into pure dichloromethane (50 g dried cell biomass in 1,5 L solvent). After the suspension was stirred at 60 °C for 90 min or at room temperature for 16 hours, the solution was filtered with pressure and concentrated by distillation at 40 °C and 400 mbar in a rotary evaporator until the solution became viscous. The viscous solution was added dropwise under stirring to a 6-fold quantity of ice-cold methanol. P4HB was precipitated and dried in a vacuum dryer (VTR 5036, Heraeus, Hanau, Germany) for at least 24 h at 30 °C and 30 mbar. The polymer was stored at -20 °C.

2.3. Degradation procedure

A solution of 1 % P4HB in chloroform was prepared by dissolving the polymer overnight at room temperature in the solvent. The catalyst solution was prepared by adding 66 μL of sulphuric acid (95–97 %) in 10 mL methanol. Afterwards, the polymer solution was heated in reflux at 55 °C until evaporation of chloroform started, at which point the catalyst solution was added (t = 0). At each predefined degradation time point, 500 mL of the degradation solution were added to 500 mL pre-cooled water in a separation funnel, mixed, and allowed to separate. The bottom phase, containing the degraded polymer, was then dropped into 1 L of stirred, ice-cold methanol in order to precipitate it. The polymer was subsequently removed from the methanol, dried overnight under vacuum at 40 °C and stored at -20 °C until further use.

2.4. Characterization

The native and degraded polymers were characterized by gel permeation chromatography (GPC), differential scanning calorimetry (DSC) and tensile tests.

GPC was performed using a differential refractive index detector (Viscotek, Houston, USA). Each polymer sample was dissolved in chloroform (0,1 %), and aliquots of 100 μL of the polymer solution were injected and separated on three sequentially coupled size exclusion chromatography (SEC) columns (300 mm $\times$ 8 mm, pore sizes of $10^3, 10^5$, and 10^7 Å, Polymer Standard Services - PSS, Mainz, Germany) at 35 °C, applying a flow rate of 0,5 mL/min of chloroform. Calibration was performed with 10 narrow standard polystyrene (PS) samples supplied by PSS (from 2×10^3 g/mol to $2,13 \times 10^6$ g/mol). Both number-average (M_n) and weight-average (M_w) molar masses were determined, as well as the polydispersity index (PI = M_w/M_n).

DSC was performed using a Mettler-Toledo DSC822^e apparatus. The following 3-step program was applied to all specimens: first heating from -100 °C to 100 °C at 10 °C/min; cooling to -100 °C at a cooling rate of -10 °C/min; second heating to 100 °C at 10 °C/min. The glass transition temperature (T_g) was obtained during the cooling run, while the melting temperature (T_m) and the enthalpy of fusion (ΔH_m) were obtained from both the first and the second heating runs.

Mechanical tests were performed in tensile mode with dog-bone specimens in a Zwick Z100 equipped with a 100 N load cell. The specimens (3 mm width and 18 mm parallel length) were prepared by solvent casting solutions of the polymers in chloroform. Due to the high ductility of most specimens, two loading speeds were used: 8,33x10^{-5} m/s (corresponding to 5 mm/min) up to an elongation of 2 % for a more accurate determination of tensile modulus, and 8,33x10^{-4} m/s (corresponding to 50 mm/min) for higher elongations. The following mechanical properties were determined: tensile strength (σ_t), yield stress (σ_y), tensile modulus (E_t), elongation at yield (ε_y), and elongation at break (ε_b).

Statistical data analysis was performed with the "R" program and the "R-commander" package. [10, 11] One-way analysis of variance (ANOVA) was used to test for differences in means of groups of samples, with Tukey Contrasts being subsequently used for the multiple comparisons of means.

3. Results and Discussion

We have previously optimized the biosynthesis of P4HB. [3] However, the material properties of the polymer have not been investigated. In this paper, we measured thermal and mechanical properties of both the synthesized polymer and the degraded ones.

3.1. Evolution of the molecular weight

Table 1 displays the evolution of molecular weight of P4HB degraded for the specified period of time. Samples degraded for more than 16 h could not be collected at amounts sufficient to allow further testing. The main reason **was the increased difficulty in precipitating such short oligomers in methanol,** resulting in a too low yield of low molecular weight fragments. This is evidenced by the morphology changes and mass reduction of the obtained polymers with degradation (Figure 1).

As shown in Figure 2, the methodology is also sensitive to the operating conditions: changes in some of those lead to clear changes in the curve profile. However, the general tendency of decay of molecular weight with degradation

time was kept: the degradation followed a random chain scission mechanism, where degradation time is proportional to the reciprocal of the molecular weight ($t \propto 1/M_n$). [12] This mechanism is well described in literature for synthetic or bio-based polymers. [12, 13] We have investigated in detail the effects of process parameters (temperature, acid and/or methanol concentration) on the molecular weight evolution of medium-chain-length PHAs for such polymers, whose degradation products are more hydrophobic than ours, the linearity of a $t \times 1/M_n$ curve is kept for the whole degradation time. (P. Ketikidis, "Modeling molecular weight evolution of methanolyzed medium-chain-length Poly(3-hydroxyalkanoates)", personal communication) In the current study, and mainly due to **an increased methanol-solubility of short oligomers of P4HB when compared to mcl-PHA**, the curve deviates from the linearity for longer degradation times; accordingly, the curve profile was also found to be more sensitive to the process parameters for longer times. Nevertheless, the optimization of the degradation procedure was not the object of this study; the goal, instead, was to determine changes in thermal and mechanical properties of our P4HB as a function of molecular weight.

3.2. Change of thermal properties and crystallinity

Table 2 shows the thermal properties of polymers obtained during batch 2. P4HB is a rubbery polymer, with a T_g well below room temperature and **low** crystallinity. That means it may crystallize even when stored at sub-zero temperatures. To account for this effect, we extracted melting data (T_m and ΔH_m) from both the first and second heating runs, while T_g was measured during cooling.

In the polymer range, T_m is usually independent of molecular weight, because the contribution of the molecular weight-independent entropic and enthalpic terms are largely exceeded by those of each repeating unit. [14] For example, in the case of PHBs, Yu and Marchessault have shown that T_m is independent of the molecular weight of P3HB for $M_n > 30000$ g/mol. [15] In our case, the melting point was rather independent of the M_n for the whole range; there was only a tendency to lower T_m for the original polymer (highest molecular weight) during the second heating. Regarding the enthalpy of fusion (and, consequently, the degree of crystallinity), two important trends are clearly visible: a monotonic increase in the enthalpy of fusion for both heating runs, and a much higher value of the enthalpy in the first heating run as compared to the second one. This is also accompanied by higher values of T_m for the first run. The differences in T_m and ΔH_m between the first and second cycles may be explained by the preparation method: as described in section "Materials and Methods", specimens were prepared by precipitation in methanol, vacuum-drying at 40 °C, and storage at -20 °C. Therefore, enough time and thermal energy has been supplied to allow a much higher extent of crystallization and, simultaneously, the formation of crystals with less defects and/or thicker lamellae (what increases the T_m). On the other hand, crystals melting during the second heating had less than 25 min (approximately the total time expended between T_g and T_m during cooling and second heating) to be formed. Therefore, only a smaller amount of material could crystallize, resulting in lower ΔH_m. Moreover, during the melt crystallization, the chains do not have as high a mobility as when in the dissolved state, which contributes both to a lower degree of crystallinity (that is, lower enthalpy of fusion) and to the formation of crystals with thinner lamellae or more defects (lower T_m). This effect is especially relevant for polymers of high molecular weight, because the melt viscosity and chain entanglements are too high and chain mobility is too low. This results in imperfect packing of the chains and less perfect crystals and

could explain the slightly lower T_m for the original P4HB, with $M_n \sim 10^6$ g/mol. It also leads to the decrease of the enthalpy of fusion with increase in molecular weight for both heating runs: the easiness of the large-scale molecular motions needed for chain folding and lamellae formation decreases with increasing molecular weight.

3.3. Change of mechanical properties

We also determined the evolution of mechanical properties of degraded P4HB. Figure 3 shows a representative curve of each sample. Original P4HB (sample "t0") shows a typical behaviour for a semi-crystalline polymer, with a well defined yield point, followed by necking/cold-drawing and a last region of strain hardening. [16] With decreasing molecular weight both the yield point and the necking region become less evident. The most degraded sample ("t4") was very brittle due to inhomogenity in the specimens. In fact, it was not possible to prepare a defect-free film of this sample.

Table 3 displays a summary of the main mechanical parameters obtained from the curves. Different mechanical properties are influenced differently by the structure of the polymer. For example, tensile strength depends on the number of ends of polymer chains and should therefore follow a relation of the type

$$\sigma_t = a - b/M_n \tag{1}$$

even if M_n is in the polymer range. [14] The modulus, on the other hand, is mainly influenced by the degree of crystallinity; and elongation at break depends on both the degree of crystallinity and molecular weight. [14, 17] Our data in Table 3 indicates for P4HB a higher sensitivity of mechanical properties on the molecular weight than on the degree of crystallinity. Tensile strength, for example, agrees fairly well with a relation of the type shown in equation (2) (Figure 4). In fact, even the yield strength which, according to the discussion above should be more dependent on the degree of crystallinity, follows the same trend (Figure 4), being strongly influenced by the molecular weight. Moreover, the modulus was roughly constant for all degraded samples (no significant differences were observed among these samples). The main reason for this is **the small change in degree of crystallinity of degraded fractions when compared to the original P4HB**, as inferred from the enthalpy of fusion in Table 2. The increase in crystallinity achieved by the decrease of M_n from 10^6 to 3×10^4 g/mol was of only about 11 %. This relatively small increase in crystallinity was therefore masked by the 17-fold decrease in molecular weight of samples shown in Table 3.

3.4. Influence on solution and melt processing

As mentioned previously, the melt and solvent processability of bacterial synthesized P4HB is compromised by its ultra-high molecular weight. With a M_n close to or even above 10^6 g/mol, P4HB is only soluble at low concentration in chlorinated solvents, and the molten polymer does not flow, even at temperatures well above the T_m (Figure 5a). The degraded fractions, on the other hand, show a typical polymeric behaviour: a viscous fluid when molten (Figure 5aand Video in "Supporting Information") and solubility in common solvents such as acetone (Figure 5b). Fractions with M_n between ca. $50 \cdot 10^6$ g/mol and $300 \cdot 10^6$ g/mol had both suitable mechanical properties (Table 3) and melt/solvent processability to allow their processing by standard polymer processing techniques.

4. Conclusion

We showed here that the degradation of P4HB through random chain scission has clear effects on both thermal and mechanical properties. The decrease of molecular weight induced an increase in the degree of crystallinity, but neither the melt nor the glass transition temperature were affected. Despite this increase in crystallinity, the decrease of the molecular weight was the predominant factor controlling the mechanical properties of the degraded fractions: both the tensile strength and the modulus decreased with the decrease of molecular weight. By carefully controlling the molecular weight of the degraded polymer, materials with adequate mechanical, thermal and processability properties may be obtained to allow their use in biomedical applications as a strong yet ductile polymer.

5. Acknowledgements

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List of Tables

Table 1: **Evolution of the molecular weight (in 10^3 g/mol) during degradation**

Batch 1								
Time (h)	0	0,25	0,5	1	2	4	8	16
M_w	2500	920	170	93	55	30	17	9,5
M_n	870	290	89	49	30	17	10	6
Batch 2								
Time (h)	0	0,25	0,85	1,5	3	6		
M_w	2000	350	60	30	10	5		
M_n	1000	260	30	15	5	1,5		
Batch 3								
Time (h)	0	0,25	1	3	16	22		
M_w	2100	250	60	38	27	17		
M_n	520	170	29	19	51	9,2		

Table 2: **Thermal properties of original and degraded P4HB**

	First heating run		Second heating run		Cooling
M_n (10^3 g/mol)	T_m(°C)	-ΔH_m (J/g)	T_m(°C)	-ΔH_m (J/g)	T_g(°C)
1000	69	63,8	57	36,5	-52
260	69	64,7	61	37,6	-52
30	69	70,5	63	42,5	-51
15	66	78,5	63	51,1	-49
5	69	85,5	61	56,4	-50

Table 3: **Mechanical properties* of original and degraded P4HB**

Sample	M_n (10^3 g/mol)	σ_t (MPa)	E_t (GPa)	ε_b (%)	σ_y (MPa)	ε_y (%)
t0	520	28^a	$0,17^a$	520^a	13^a	17^a
t1	290	17^b	$0,12^b$	450^{ab}	$9,6^b$	12^b
t2	90	16^b	$0,12^b$	470^{ab}	$8,4^b$	10^{bc}
t3	50	11^{bc}	$0,13^b$	220^b	$7,8^{bc}$	10^{bc}
t4	30	$5,3^c$	$0,10^b$	10	$5,3^c$	$6,1^c$

The superscripts identify samples in the same column with significantly different values (at $p < 0,05$) for the corresponding property.
* Tensile strength (σ_t), tensile modulus (E_t), elongation at break (ε_b), yield stress (σ_y), and elongation at yield (ε_y).

List of Figures

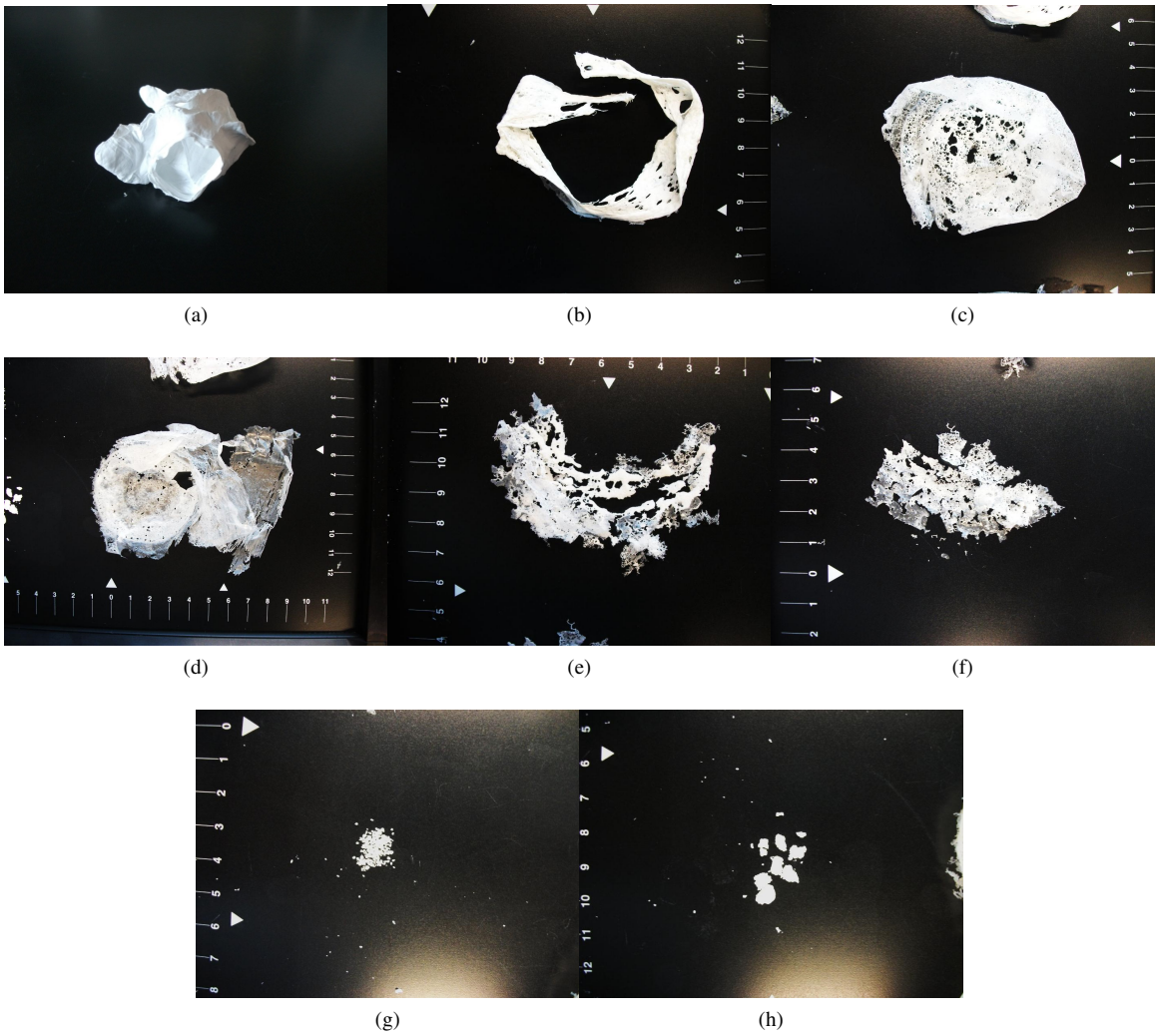


Figure 1: **Morphology change of the obtained P4HB after degradation.** A, B, C, D, E, F, G, and H represent the P4HB after degradation of 0, 0.25, 0.5, 1, 2, 4, 8, and 16 h, respectively.

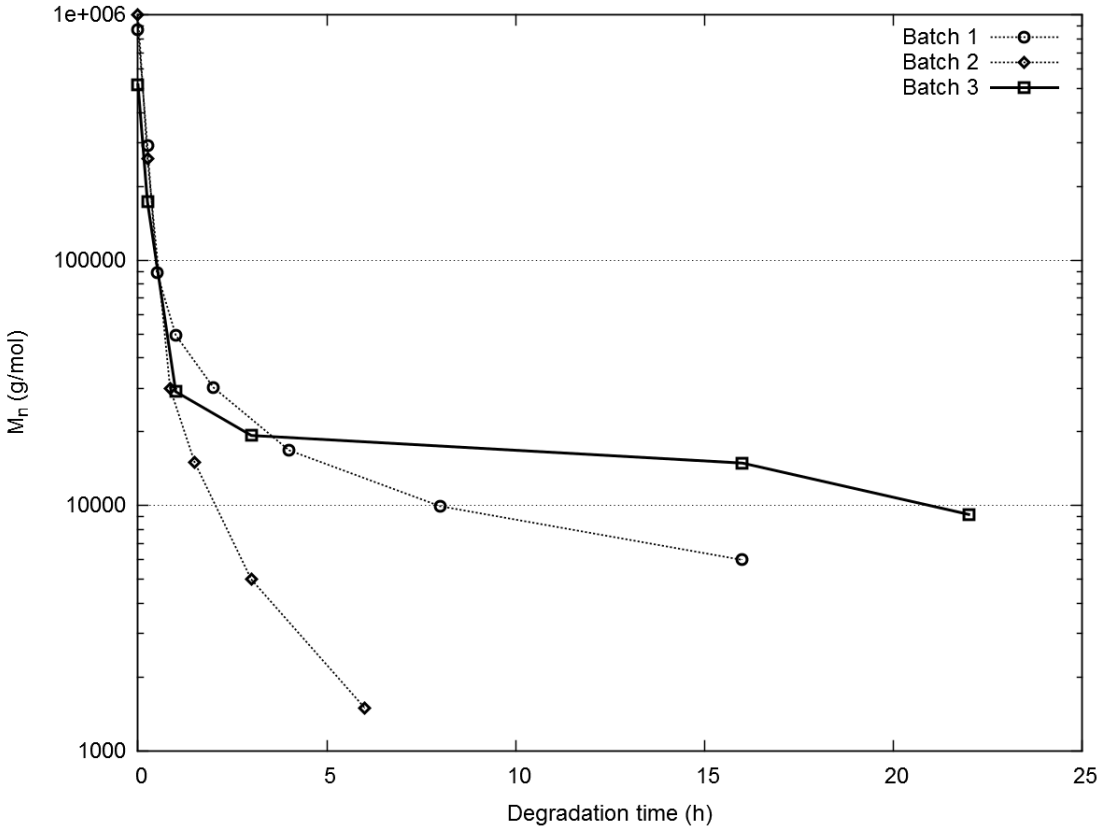


Figure 2: **Evolution of the molecular weight with degradation time.** Differences in the curve profile arise from differences in operator, volume of the aliquot removed at each time point, or in the method used to recover the polymer, showing the sensitivity of the degradation protocol to the process parameters. Lines are only to guide the eyes.

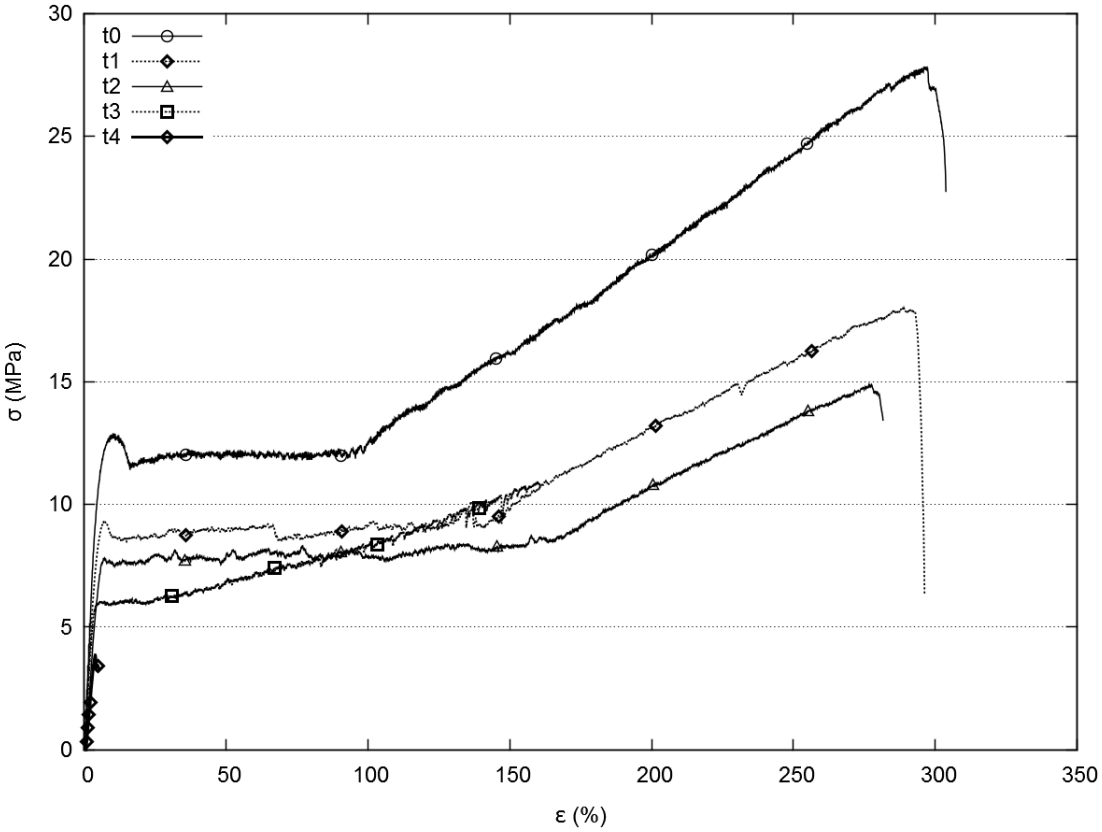


Figure 3: **Tensile curves of a representative specimen of each sample of original and degraded P4HB.**

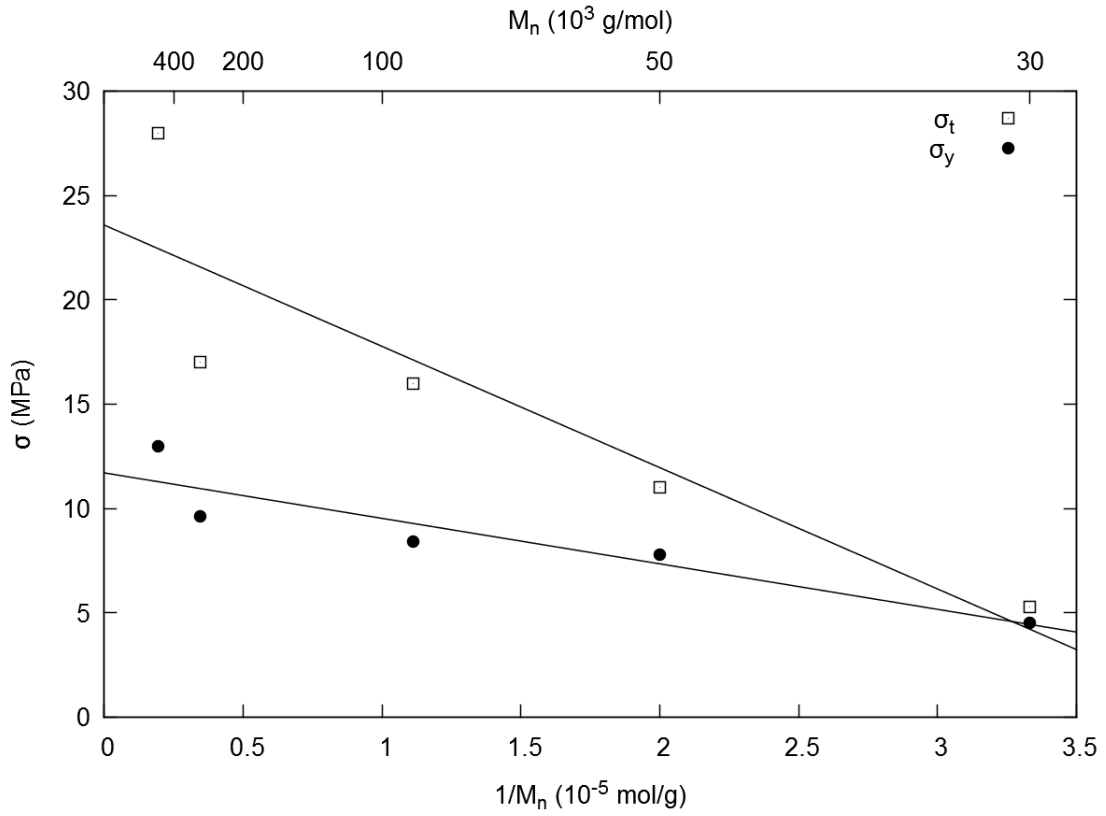
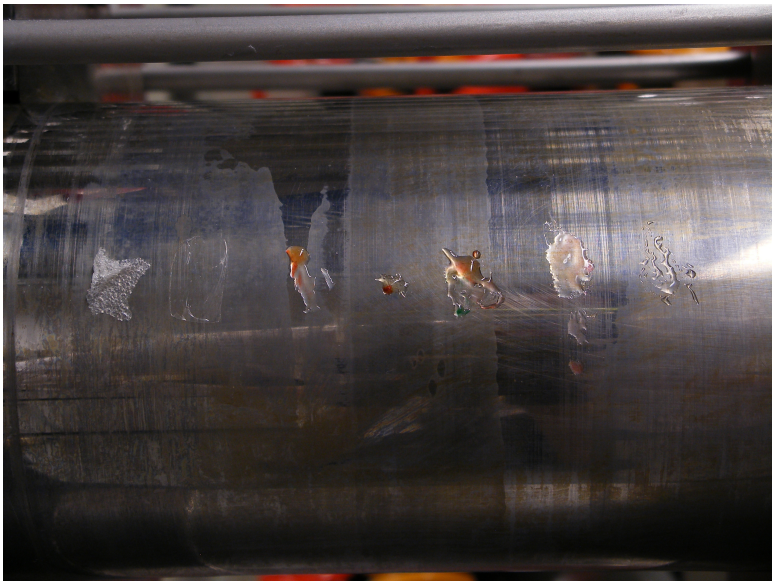
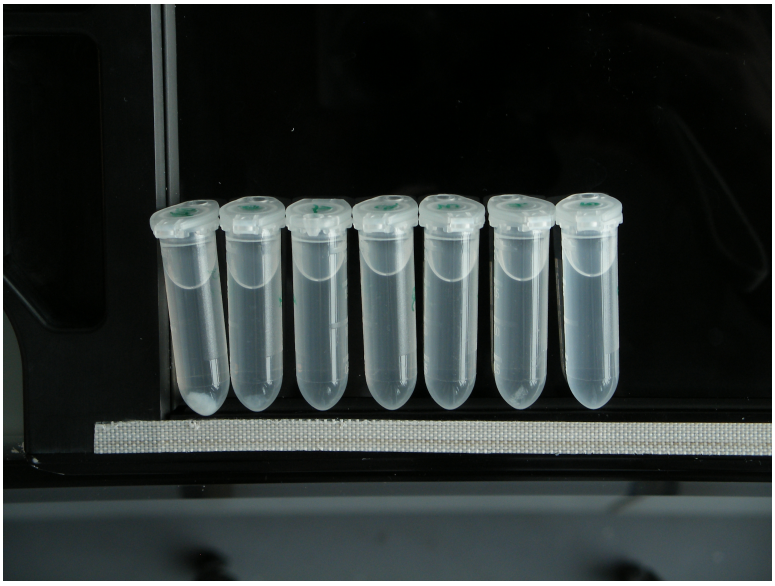


Figure 4: **Tensile (σ_t) and yield (σ_y) strength as a function of the molecular weight.** The fitting of each dataset with equation (2) is also shown: σ_t=23,574-581103*x ($r^2 = 0,81$), and σ_y=11,703-217927*x ($r^2 = 0,85$).



(a)



(b)

Figure 5: **(a) Molten original and degraded P4HB at 100 °C. (b) Solubility of original and degraded P4HB in acetone after 24 h at room temperature.** There is a clear precipitate in the solution of the two original P4HB specimens; all others are clear solutions. For both images, the molecular weight (M_n, in 10^3 g/mol) is (from left to right): 520 (foam), 520 (film), 290, 90, 50, 30, and 17.