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Degradation of poly(β-malic acid) – monitoring of oligomers formation by aqueous SEC and HPCE*

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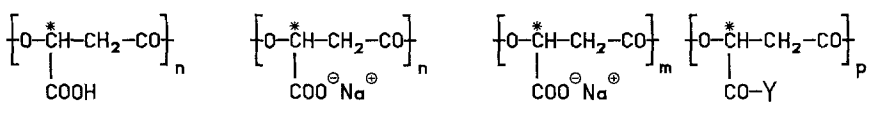
Summary

Poly(β-malic acid) is a functional aliphatic polyester which can serve as parent compound to make systems aimed at temporary therapeutic applications. Degradation of the polyester backbone of poly(β-malic acid) leads to the formation of oligomers which can be hardly separated by conventional Size Exclusion Chromatography. In order to monitor the formation of oligomeric degradation by-products and to quantify their relative amount, the potential of two separation techniques has been investigated, namely Aqueous Size Exclusion Chromatography using a Bio-Gel P2 gel column and High Performance Capillary Electrophoresis. Aqueous SEC allowed separation of oligomers smaller than hexamer whereas HPCE successfully separated oligomers smaller than pentadecamers. These techniques were used to control the efficiency of oligomer fractionation by the dissolution/precipitation method applied to the sodium salt of poly(β-malic acid).

Introduction

For the last two decades, biodegradable polymers (BDP's) have been regarded as compounds of great practical interest with respect to surgical and pharmacological therapies (CHIELLINI and ST PIERRE 1986 & 1987a,b).

Upon degradation, BDP's produce low molecular weight fragments which soon or later become water-soluble. The monitoring of the formation of these water-soluble oligomeric compounds can provide worthwhile information on the actual degradation mechanism of high molecular weight parent molecules.



$PMLA^{X}H_{100}$ $PMLA^{X}Na_{100}$ $PMLA^{X}Na_{(100m/(m+p))}Y_{(100p/(m+p))}$

Poly(β-malic acid) is a poly(carboxylic acid) with an aliphatic polyester backbone which is composed of malic acid repeating units linked via hydroxyl and β-carboxyl groups. Malic acid is a chiral compound and optically active poly(β-malic acid)s have been

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synthesized with different enantiomeric excesses (GUERIN et al 1985). For the sake of clarity, the acronyms $PMLA^{x}H_{100}$ and $PMLA^{x}Na_{100}$ are now currently used to name poly(β-malic acid) stereocopolymers and their sodium salts, x being the percentage of L-malic acid units present in the main chains. The backbone of these water-soluble polymers is sensitive to simple hydrolysis and molecular weight decreases rapidely with time in aqueous solution (BRAUD et al 1985). The degradation rate depends on both temperature and pH, greater degradation rates being observed at low pH and high temperature (BRAUD et al 1988).

In order to correlate the mechanism of degradation of poly(β-malic acid) with mathematical models, the possibility of collecting accurate data on the formation of oligomeric and monomeric degradation by-products is essential.

In this paper, we wish to report on the results obtained by using two different separation techniques namely an Aqueous Size Exclusion Chromatography (Aqueous SEC) performed on a Bio-Gel P2 gel column and High Performance Capillary Electrophoresis (HPCE). UV absorption was used to detect oligomeric materials. Because of the fact that the selected wavelenght (214 or 200 nm respectively) reflected both carboxylic and ester carbonyl chromophores which have different absorption characteristics, the extinction coefficient of each oligomer depends on its molecular weight, and thus data had to be normalized in order to allow significant quantitative comparison. This was done for Aqueous SEC by measuring the radioactivity of collected fractions of a radiolabelled racemic poly(β-malic acid), namely $P^{*}MLA^{50}Na_{100}$.

Experimental

Materials : $PMLA^{95}H_{100}$ was synthesized by using a method previously described (GUERIN et al 1985). Before use, the crude polyacid was dissolved in distilled water and neutralized by NaOH. The resulting neutral solution was dialyzed by using a Spectrapor 3 dialysis tube (molecular weight cutoff = 3500) against pure water at 4°C for 24 hours. Finally, the solution was freeze-dried to yield solid $PMLA^{95}Na_{100}$.
1 g of the polysalt was then dissolved in 3 cm^3 of distilled water in a 10 cm^3 beaker. Analytical grade ethanol was added droply, the solution being magnetically stirred. The addition of ethanol (3 cm^3) was stopped when the mixture turned cloudy. Phase separation was then obtained when stirring was stopped. The upper phase which contained primarily oligomers was removed and then 6 cm^3 of ethanol were added to the visquous coacervate bottom phase in order to precipitate the high molecular weight molecules. A waxy material was separated which was then dried under vaccum at room temperature. The resulting powder was stored in a vaccum desiccator in the presence of phosphoric anhydride.
Radiolabelled oligomers of $P^{*}MLA^{50}Na_{100}$ were obtained from the upper phase obtained during the solution/precipitation purification of the main chain unit-radiolabelled poly(β-malic acid) sample whose synthesis was described before (FOURNIE 1991).
L-malic acid used as a standard was purchassed from Iwata Chemical Co. and used without further purification.

Aqueous SEC analysis on CM-Sepharose CL-6B gel : The technique used was described in a previous paper (BRAUD et al 1988).

SEC analysis on Bio-Gel P2 gel : A Pharmacia FPLC apparatus, equipped with a UV detection at 214 nm, was connected to a column (0.4 cm i.d., 2.7 m length) filled with Bio-Gel P2 gel (Bio-Rad). A mixture of 1M NaCl + 0.15 M sodium phosphate buffer (pH=7.4) was used as the mobile phase at a flow rate of 0.05 $cm^3.min^{-1}$. For radiolabelled assays, 0.25 cm^3 fractions were collected by using a Pharmacia FRAC 200 fraction collector.

HPCE analysis : Experiments were performed by using an automated capillary electrophoresis instrument (Beckman P/ACE system 2000). A capillary of 50 mm i.d. and 0.5 m length was filled with a 0.1 M sodium borate buffer (pH=9.2) solution. 5 $mg.cm^{-3}$ aliquots were injected at 35°C. UV absorption at 200 nm was recorded.

Radioactivity determination : Measurements were carried out at the "Laboratoire de Technologie Enzymatique" of the University of Compiègne, France by using a method already described (FOURNIE, 1990).

Results and Discussions

Detection of oligomers in samples of Poly(β-malic acid)

$PMLA^{95}H_{100}$ was obtained via the classical synthesis route which is based on the final hydrogenolysis of the parent poly(benzyl malolactonate) (GUERIN et al 1985). The molecular weight of the crude polyacid was first characterized by SEC on CM-Sepharose CL-6B gel according to a method previously reported (BRAUD et al 1988). The chromatogram was constituted of a broad peak in the separation domain of the column (MW_{sec}= 48000). A small peak located at the permeation volume of the column was due to low molecular weight molecules remaining from hydrogenolysis; these molecules did not appear after dialysis of the sodium salt against water. $PMLA^{95}Na_{100}$ was characterized by MW_{sec}= 45000, a value which was of the same order than the weight-average molecular weight of the sodium salt of poly(β-malic acid) (BRAUD et al 1988).

Figure 1 shows the chromatogram obtained by SEC on Bio-Gel P2 gel for $PMLA^{95}H_{100}$. This gel is characterized by an operation range between 10^2 and 10^3 daltons in SEC (BIO-RAD 1971). The large peak centered at 23 cm^3 can be assigned to the excluded macromolecules. This peak is followed by very small peaks located at V_e = 25.4, 26.6, 28.4, 31.0 and 35.5 cm^3 respectively. The last peak can be assigned to malic acid produced by degradation of poly(β-malic acid) in agreement with the location of the peak observed when L-malic acid was injected solely.

Figure 2 shows the $\log(MW)=f(V_e)$ plot obtained when one assumed that peaks at V_e= 31.0, 28.4, 26.6 and 25.4 cm^3 were due to the presence of di-, tri-, tetra- and pentamers respectively. A linear relationship was observed that confirms the above assumption on the peak assignment.

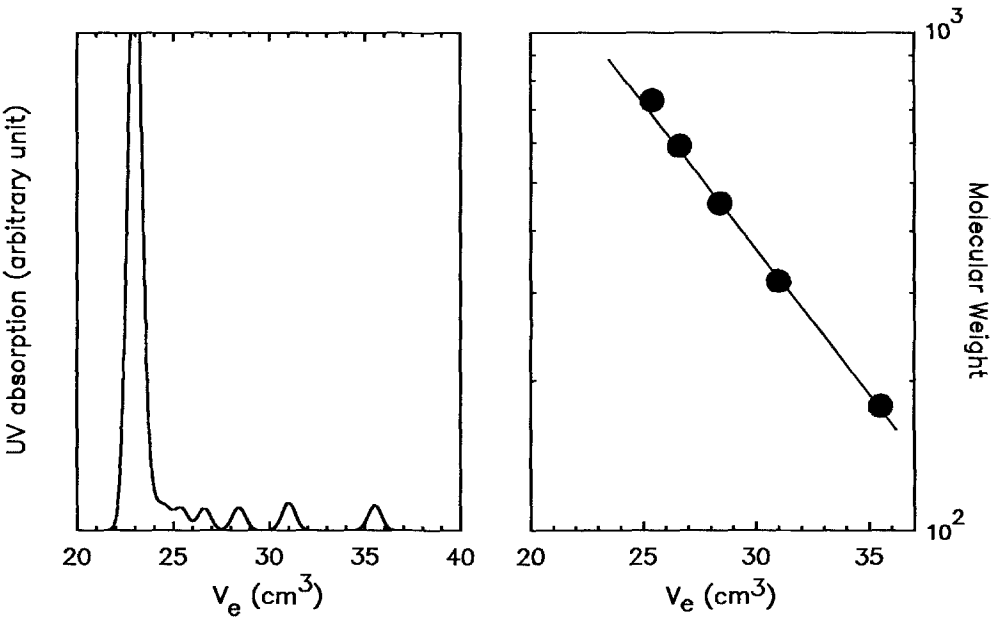

Figure 1 (left) UV-detected SEC chromatogram (Bio-Gel P2 gel) of 0.5% $PMLA^{95}Na_{100}$

Figure 2 (right) Plot of log(MW) versus V_e (MW is the molecular weight of the i-mer assigned to the peaks of Figure 1)

Elimination of oligomers from the polysalt

In order to eliminate the oligomeric species detected in Figure 1, fractionation was achieved by adding methanol to a concentrated aqueous solution of $PMLA^{95}Na_{100}$. A coacervate system was formed. After separation of the bottom phase followed by drying, only 60% of the initial sample was recovered. After this purification process, the absence of peaks in the 24-36 cm^3 range of the Bio-Gel P2 chromatogram allowed us to ascertain that the recovered polysalt was free from oligomers. The oligomer-free sample was then kept in a vaccum desiccator in the presence of desiccant. Analyses carried out from time to time showed that no evolution occured over a period of one year under this storage condition.

Weight calibration of UV-detected chromatograms from SEC on Bio-Gel P2 gel

Detection by UV absorption was used in SEC analysis. This technique does not give a signal which is proportional to the weight of the oligomer which is eluted since the UV signal depends on the molecular weigh of the polysalt (BRAUD et al 1985).

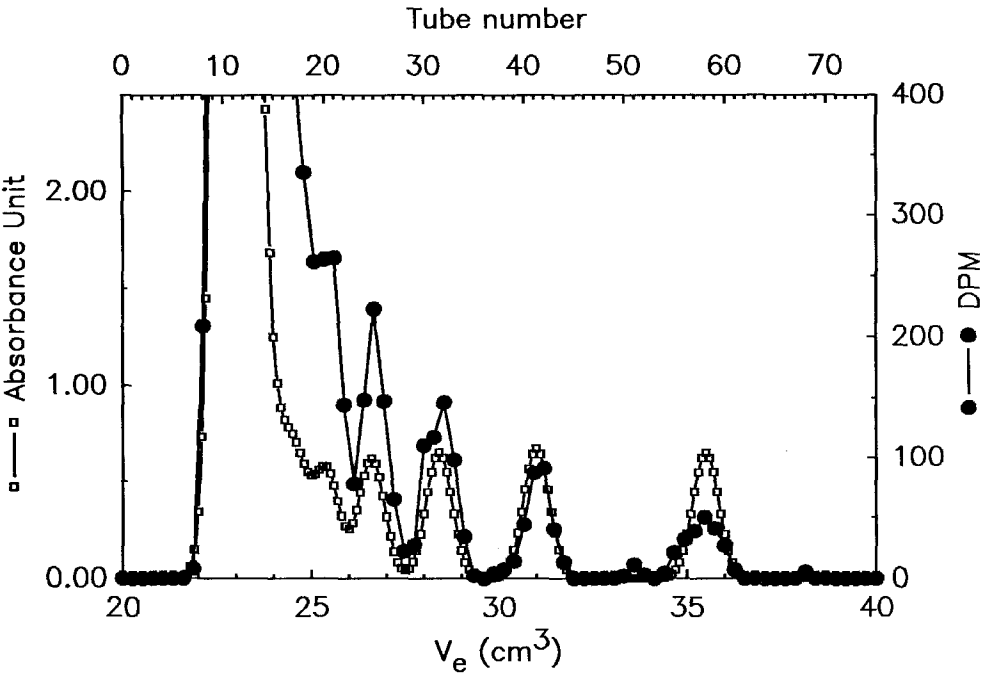

Figure 3 Comparison of the UV-detected SEC chromatogram (Bio-Gel P2 gel) and the related counts of collected fractions of 0.5% $P^*MLA^{50}Na_{100}$

Table 1 Calibration of UV detection with counts from Figure 3 for P^*MLA^{50} Na_{100} oligomer fraction

i	V_e (cm^3)	AU at V_e	DPM at V_e	DPM/AU	CF_i
1	35.5	0.65	50	77	1
2	31.0	0.67	90	134	1.74
3	28.4	0.65	132	203	2.64
4	26.6	0.62	223	360	4.67
5	25.3	0.58	264	455	5.91

The use of a fraction of $P^*MLA^{50}Na_{100}$ which is rich in oligomers allowed us to normalize the various peaks given by UV detection.

Figure 3 shows the comparison of the UV signal with the related counts of the collected fractions. The DPM values being proportional to the content in malic acid moieties, the comparison

of these values with the UV absorbance showed that the molecular absorbance of a given oligomer decreases with its degree of polymerization. A correction factor (CF_i) was calculated from the comparison between UV and DPM data (Table 1). The experimental UV absorbance of i-mers were then corrected according to :

$$(\text{UV absorption})_{true} = (\text{UV absorption})_{observed} \cdot CF_i$$

to get its relative weight in the set of the resolved oligomers.

HPCE separation of oligomers

For routine analysis, SEC on Bio-Gel P2 gel is a time-consuming technique since it requires more than 15 hours for each run. Figure 4 shows the electropherogram obtained for the recovered upper phase during coacervation of $PMLA^{95}Na_{100}$ by using a free solution capillary electrophoresis. More than 15 peaks appeared separated together with a broad band centered at 9.2 minutes, the whole run being carried out within 15 minutes. The peak at t=14.0 minutes was assigned to malic acid and the others can be assigned to the 15 first oligomers as shown by the linear variation of log(MW)=f(time) plot (Fig. 5).

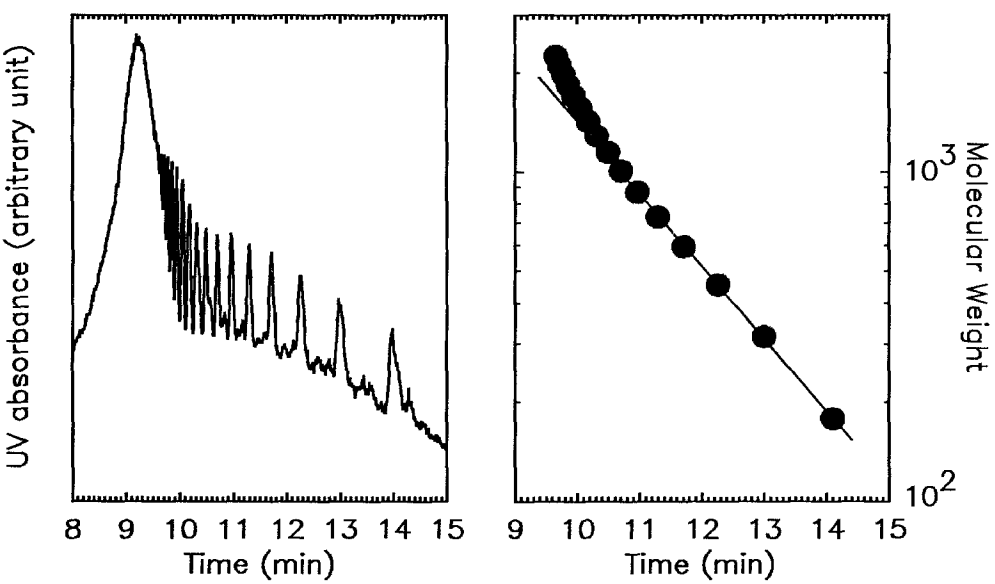

Figure 4 (left) Electrophoregram of 0.5% oligomer fraction of $PMLA^{95}Na_{100}$ dissolved in 0.1 M sodium borate buffer (pH=9.2)

Figure 5 (right) Plot of log(MW) versus time (MW is the molecular weight of the i-mer assigned to the peaks of Figure 4)

In conclusion, detection of oligomers of poly(β-malic acid) can be currently done that allowed us to consider the controlled degradation in model aqueous solutions (BRAUD et al 1992). Finally, it must be noted that poly(β-malic acid) is very sensitive to degradation since dialysis is not sufficient to remove oligomers. Only coacervation gives a polysalt free from oligomers.

Acknowledgements

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