Degradation of poly(ester) microspheres

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Biodegradable polymeric microspheres have been prepared by spray drying, precipitation, rotary evaporation and press grinding methods. Erosion of microspheres of poly(lactide), poly(3-hydroxybutyrate), copolymers of lactide and glycolide, and copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate at 85°C and 37°C have been studied using ion chromatography, nuclear magnetic resonance, residual mass measurements, viscometry and gel permeation chromatography. Such studies demonstrated that these polyester matrices degraded via (1) random chain scission and (2) release of soluble monomeric and oligomeric products. Protein release from microspheres prepared by these methods indicated that most of the protein is released before the polymer matrix loses weight.

Keywords: Microspheres, biodegradation, poly(esters)

Biodegradable polymers have been used for the controlled delivery of drugs for the past two decades[1-11]. These polymers do not require removal after drug delivery is complete due to their ability to be hydrolysed to soluble monomeric or oligomeric units[12]. In addition, the reaction between the tissue and the implanted polymer is ameliorated as the polymer biodegrades[13]. Toxicity of the polymer and all decomposition products must be low for the use of these polymers in drug delivery applications.

Recently, there has been much interest in developing new methods to deliver both vaccines and adjuvants[14,15]. In many instances delivery of an immunogen or accompanying adjuvant must be delayed or sustained over a prolonged period of time[16]. Such sustained delivery of immunogen might result in a heightened immune response eliminating the need for vaccination boosters[17].

Poly(esters) were chosen for a vaccine delivery vehicle because they are currently widely used in suture materials[18-23] and both the polymer and their decomposition products have demonstrated low toxicity[24-26].

This research focusses on biodegradable poly(esters) as potential carriers of vaccines. The matrix characteristics during degradation of a variety of poly(esters) have been determined. Studies on the change in properties of poly(DL-lactide : glycolide, 50 : 50) during degradation have been performed to better understand the erosion mechanism of poly(esters). Finally, poly(ester) microspheres containing protein have been prepared and *in vitro* protein release has been examined.

MATERIALS

Poly(DL-lactide : glycolide, 50 : 50 and 85 : 15) (Medisorb, bioresorbable polymers) were obtained from Dupont (Wilmington, DE, USA). Poly(3-hydroxybutyrate : 3-hydroxyvalerate, 93 : 7 and 80 : 20) and poly(3-hydroxybutyrate) were obtained from Goodfellow (Cambridge, UK) and used in original powder form. Poly(esters) including poly(L-lactide) mol. wt 50 000, 100 000 and 200 000; poly(DL-lactide : glycolide, 80 : 20 and 70 : 30), poly(3-hydroxybutyrate) mol. wt 50 000, and polystyrene molecular weight standards were obtained from Polysciences Inc. (Warrington, PA, USA). Lactate, glycolate, chlorobutanol, Lubrol PX, bovine serum albumin (BSA), sodium oleate, Span 85, and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (Chaps) were from Sigma (St Louis, MO, USA). 3-Hydroxybutyrate, 3-hydroxyvalerate, deuterium oxide (99.96% D) and tetra-*n*-butylammonium hydroxide were from Aldrich (Milwaukee, WI, USA). Heptafluorobutyric acid (Fluka, Ronkonkoma, NY, USA), octanesulphonic acid (Dionex, Sunnyvale, CA, USA), sodium azide (Fisher, Pittsburgh, PA, USA), Coomassie dye reagent (Bio-Rad, Richmond, CA, USA), sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS, Merck Sharp & Dohme Inc. Quebec, Canada), silicone oil (Silicone Fluid 500, Spectrum Chemical

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Co., Gardena, CA, USA) were used. All other chemicals and solvents were reagent grade.

METHODS

Preparation of microspheres

Four methods of microsphere preparation were investigated: spray drying, solvent extraction precipitation, rotary evaporation and press grinding.

Spray drying. Bovine serum albumin (BSA) microspheres were prepared by dissolving 1 g of BSA in 30 ml of deionized water. This solution was then pumped by a peristaltic pump (Masterflex, Cole-Parmer, Chicago, IL, USA) at 5 ml/min into a Yamato Pulvis Mini-spray GA-32 spray drier (Yamato Scientific Co., Chicago, IL, USA) using 49 kPa atomizing air pressure with an inlet temperature of 100°C and drying air at 0.5 cm^3/min. The BSA microspheres coated with a surfactant (Lubrol PX or Chaps) were similarly prepared by mixing BSA (1 g) and surfactant (100 mg) with 30 ml deionized water and spray drying. Poly(L-lactide) and poly(DL-lactide : glycolide) microspheres were prepared by dissolving 5 g of polymer in 250 ml organic solvent (methylene chloride or tetrahydrofuran). In the case of microspheres containing protein, an additional 250 mg of spray-dried BSA microspheres (with or without surfactant) were suspended with agitation in the organic solvent. The polymer microspheres were spray-dried under the same conditions used to prepare protein microspheres, except the inlet air temperature was 37°C.

Solvent extraction precipitation. This method used a TTA60 Titration Assembly (Radiometer, Copenhagen, Denmark). Poly(DL-lactide : glycolide) (1 g) was dissolved in 10 ml methylene chloride. BSA (50 mg) was then dispersed in this solution by sonification (Sonifier, Branson, Banbury, CT, USA) with 125-W, 40% duty cycle, pulsed mode for 10 min. This solution containing suspended protein was passed through a syringe equipped with a 22G needle into silicone oil (30 ml) containing Span 85 (1 ml). The silicone oil was stirred during addition of BSA and stirring was continued for 30 min. Petroleum ether (20 ml) was then added to extract methylene chloride and precipitate the microspheres which were filtered, dried and sieved to 20–60 mesh (250–850 μm) using Fisher Scientific US standard sieves.

Rotary evaporation. A solution of poly(DL-lactide : glycolide) (0.5 g) in methylene chloride (5 ml) was emulsified with an aqueous solution of sodium oleate (0.5 g) in deionized water (50 ml). To prepare protein–polymer microspheres, spray-dried BSA (25 mg) was added to the poly(DL-lactide : glycolide)–methylene chloride solution. The resulting dispersion was rotary-evaporated at 635 Torr (10 min), 510 Torr (10 min), 385 Torr (20 min), and 260 Torr (50 min) to remove the methylene chloride. The microspheres obtained were filtered, washed with water and vacuum dried at room temperature.

Press grinding. Spray-dried poly(DL-lactide : glycolide) (400 mg) was mixed with spray-dried BSA (20 mg) and pressed at ambient temperature or 60°C in a cylindrical die under 6.9×10^7 Pa using a Carver press (Pasadena Hydraulics, El Monte, CA, USA). The pellet (13 mm diameter; 3 mm thick) formed was ground with dry-ice in a grinder for 15 min. The particles produced were collected and sieved to 20–60 mesh.

Characterization of polymer and microsphere

Molecular weight. Polymer molecular weights were determined by gel permeation chromatography (GPC) using a Waters M-6000A pump (Waters, Milford, MA, USA), 50 μl injection loop, a 7.8 mm internal diameter × 30 cm column packed with Ultrastyragel® (<10 μm, mixed bed resin), and a Shimadzu RID-6A refractive index detector (Shimadzu Inc. Columbia, MD, USA). Polymers dissolved in either methylene chloride or tetrahydrofuran at 0.5(w/v)% were analysed by GPC at a 1 ml/min flow rate at 30°C. Polystyrene molecular weight standards were used to calibrate the column.

Intrinsic viscosity. A Cannon-Ubbelode viscometer (Cannon, State College, PA, USA) was used to determine the intrinsic viscosity of polymer–methylene chloride solutions at 25°C. Polymer solutions with concentrations, 0.075, 0.15, 0.3, 0.45 and 0.6 g/dl were used in the viscosity study. The specific viscosity of polymer was determined at each concentration and the intrinsic viscosity was calculated by extrapolating the specific viscosities to infinite dilution.

Scanning electron microscopy (SEM). Microspheres were fixed on copper-taped metal studs, coated with gold, and analysed by SEM (Model S-570, Hitachi, Tokyo, Japan). Particle sizes, distribution and morphology were obtained.

^{1}H-n.m.r. spectroscopy. ^{1}H-n.m.r. spectra were obtained on hydrolysis products in deuterium oxide on a Bruker WM-360 n.m.r. spectrometer using 0.5% DSS as an internal standard.

Degradation of microspheres

Degradation studies were conducted by two methods: cumulative and differential.

Cumulative method. Microspheres (20 mg) were placed into 50 ml of phosphate buffered saline (PBS, 150 mM anhydrous sodium phosphate (dibasic) and 0.9 (w/v)% sodium chloride at pH 7.2) and gently agitated at 37°C and 100 rev min^{-1} in a G-24 environmental incubator shaker (New Brunswick Scientific Co., Edison, NJ, USA). Degradation studies at 85°C were performed in an oil bath without agitation. Soluble products were obtained by periodically removing all the PBS from the vessel containing polymer and replacing it with the same volume of fresh PBS. Analysis for soluble degradation products was conducted with a Dionex QIC ion-chromatography system on an HPICE-AS1 column eluted with octanesulphonic acid (2 mM) for 3-hydroxybutyrate, and 3-hydroxyvalerate or an HPICE-AS5 column eluted with heptafluorobutyric acid (1.6 mM) for DL-lactate and glycolate. A post-column AMMS-ICE membrane suppressor column run with 10 mM tetra-*n*-butylammonium hydroxide at a flow rate of 1 ml/min was used with ion-detection by conductance (100 micro Siemens Full Scale, μSFS).

Differential method. Ten screw-top test tubes were prepared, each containing microspheres (200 mg) dispersed in PBS (2.5 ml). These tubes were incubated at 37°C or 85°C. At various time intervals, one of the sample tubes was removed

and the solution and the residual mass were separated. The solution was analysed by Dionex HPLC system and the polymer residue was washed with water and dried under vacuum. The residue mass was recorded and dissolved in methylene chloride for analysis by viscometry and GPC.

In vitro release studies

Protein release studies were conducted using poly(DL-lactide : glycolide, 50 : 50) microspheres containing BSA. The microspheres (200 mg) were placed in 2.5 ml of PBS at 37 °C. Every 24 h the PBS was completely removed and the protein content was measured using a Coomassie dye binding assay[27]. Fresh PBS (2.5 ml) was then added to replenish that which had been removed. The presence and identity of low concentrations of protein recovered late in the release studies was also confirmed using SDS polyacrylamide gel electrophoresis[28] with Coomassie staining.

RESULTS AND DISCUSSION

Characterization of polymers and microspheres

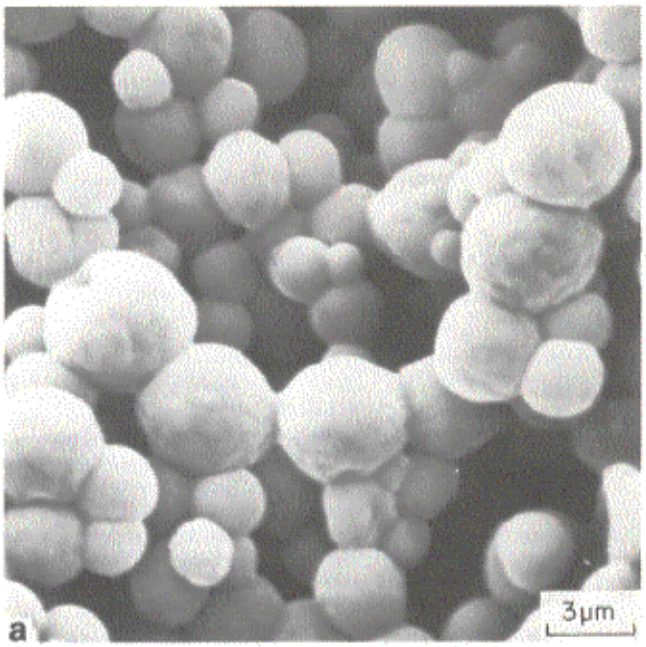
A number of commercially available polyesters of different molecular weights and composed of various hydroxyacid monomers including lactide, glycolide, 3-hydroxybutyrate and 3-hydroxyvalerate were studied (*Table 1*). The molecular weight of each polymer was determined in a suitable solvent by GPC. The poly(esters) chosen for study had molecular weights ranging from <10 000 to >600 000 Da.

Microspheres prepared by spray-drying poly(DL-lactide : glycolide, 50 : 50) and containing BSA in the presence of a non-ionic surfactant (Lubrol PX) had a particle size distribution of <0.5 μm (2%), 0.5–1.0 μm (53%), 1.0–1.5 μm (30%), 1.5–2.0 μm (13%), > 2 μm (2%), and had a tendency to aggregate (*Figure 1a*). Microspheres prepared by the solvent extraction precipitation method gave larger particle sizes. These were predominantly between 250 and 850 μm (63%) with 22% <250 μm and 15% >850 μm. These microspheres did not aggregate as those prepared by spray drying and they resuspended well in PBS. Rotary evaporation of poly(DL-lactide : glycolide, 50 : 50) formed microspheres with a particle size distribution of 1–5 μm (51%), 5–10 μm (39%), 10–15 μm (6%), 15–20 μm (1%), >20 μm (3%) (*Figure 1b*). This method produced microspheres with low protein content due to protein dissolution into the aqueous medium. Particles prepared by press grinding had broad particle size distributions. About 34% (60 °C) or 45% (room temperature) were particles below 250 μm.

Table 1 Properties of various biodegradable polymers

Polymer	Monomer[a] composition	Molecular weight: Reported kDa	Molecular weight: Determined kDa	Time to half-erosion[e] in PBS (days): 37 °C	Time to half-erosion[e] in PBS (days): 85 °C
1	1.0L	50	88	nd	2
2	1.0L	100	106	nd	11
3	1.0L	200	nd[d]	nd	50
4	0.85L, 0.15G	60–100	63[b]	100	nd
5	0.8L, 0.2G	-	8[b], 6[c]	nd	1
6	0.7L, 0.3G	-	4[b], 4[c]	nd	0.1
7	0.5L, 0.5G	60–100	60[b], 60[c]	40	1
8	1.0B	50	nd	nd	110
9	0.93B, 0.07V	-	610	nd	150
10	0.8B, 0.2V	-	400	nd	130

[a]Monomers (preceded by mole fraction) are: L, lactide; G, glycolide; B, 3-hydroxybutyrate; V, 3-hydroxyvalerate.
[b]Molecular weight determined by GPC in methylene chloride.
[c]Molecular weight determined by GPC in tetrahydrofuran.
[d]nd, not determined.
[e]Calculated by measuring the cumulative monomer released.



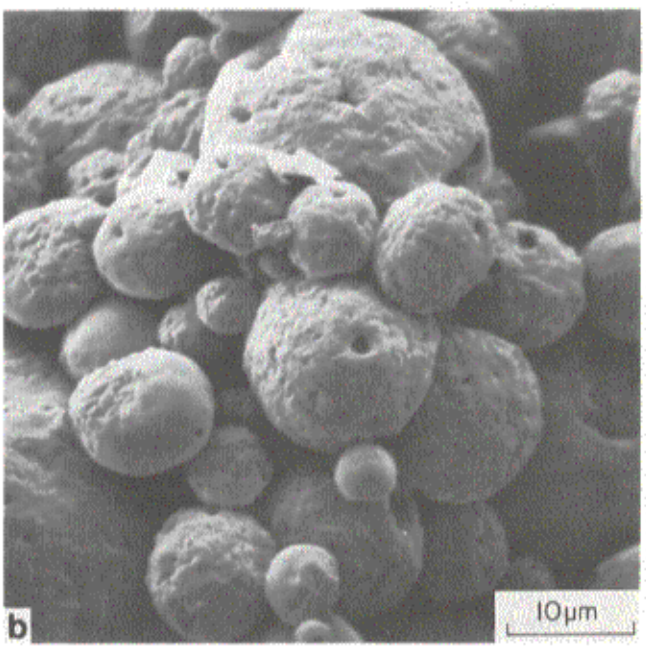


Figure 1 SEM photographs of (a) spray-dried poly(DL-lactide : glycolide, 50 : 50) microspheres containing 5% BSA and 5% Lubrol PX and (b) rotary-evaporated poly(DL-lactide : glycolide, 50 : 50) microspheres.

Degradation of microspheres

Degradation studies on polymers, performed in PBS, demonstrated a range of hydrolysis rates. Poly(3-hydroxybutyrate) and copolymers of 3-hydroxybutyrate and 3-hydroxyvalerate hydrolysed slowly over 5 month at 85 °C (*Figure 2*)[29]. Copolymers having a higher fraction of 3-hydroxyvalerate were more labile to hydrolysis. The lower molecular weight poly(3-hydroxybutyrate : 3-hydroxyvalerate, 80 : 20) mol. wt 400 000 (Goodfellow) and poly(3-hydroxybutyrate) mol. wt 50 000 (Polysciences), degraded faster than the higher molecular weight polymer (Goodfellow, mol. wt >600 000).

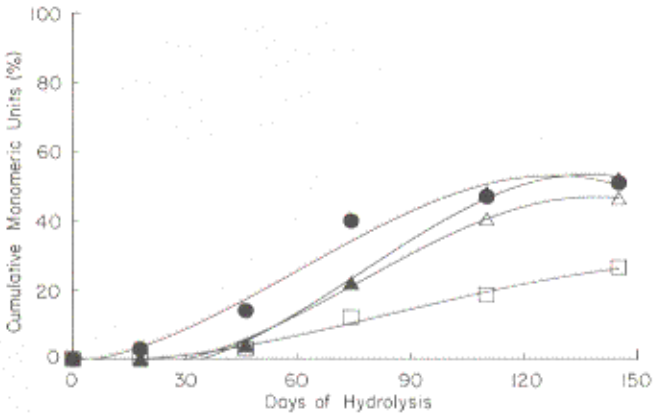


Figure 2 Monomer-release profiles at 85°C of poly(3-hydroxybutyrate : 3-hydroxyvalerate, 80 : 20 (▲) and 93 : 7 (△)) obtained from Goodfellow, and poly(3-hydroxybutyrate), ((●) obtained from Polysciences, and (□) obtained from Goodfellow). Cumulative monomer released is the total soluble monomeric products formed on polymer degradation at any given time. If all the polymer hydrolyses to soluble monomer(s) then a value of 100% cumulative monomeric units is expected.

Poly(L-lactide) polymers showed decreasing half-erosion times (time in which the cumulative monomeric units reach 50%) at 85 °C with decreasing molecular weight (*Figure 3*). This is probably the result of faster solubilization of low molecular weight poly(L-lactides)[23].

As previously reported[22], copolymers of lactide and glycolide show increasing rates of hydrolysis as the glycolide to lactide ratio approaches 50 : 50 (*Figure 4*). Again lower molecular weight copolymers (mol. wt ≈ 10 000, Polysiences) degraded faster than higher molecular weight copolymers (*Figure 4*). Again lower molecular weight copolymers (mol. wt ≈60 000, Dupont, *Figure 4a*). Initially, little monomeric product could be detected. It is clear, however, that hydrolysis is occurring during this period from molecular weight measured by GPC and from viscosity measurements made on polymer residues. Degradation of spray-dried microspheres (with or without protein) and ground polymer gave similar erosion profiles. Addition of 0.04% sodium azide or 1% chlorobutanol into polymer/PBS suspensions to prevent microbial growth did not change the degradation profile. Degradation at 85°C resulted in similarly shaped curves for the 85 : 15 and 50 : 50 copolymers.

The formation of both soluble monomeric products, lactic acid and glycolic acid, were measured over time during studies of poly(DL-lactide : glycolide, 50 : 50) degradation (*Figure 4b*). Glycolic acid was formed initially at a higher rate than lactic acid suggesting that the glycolide units are

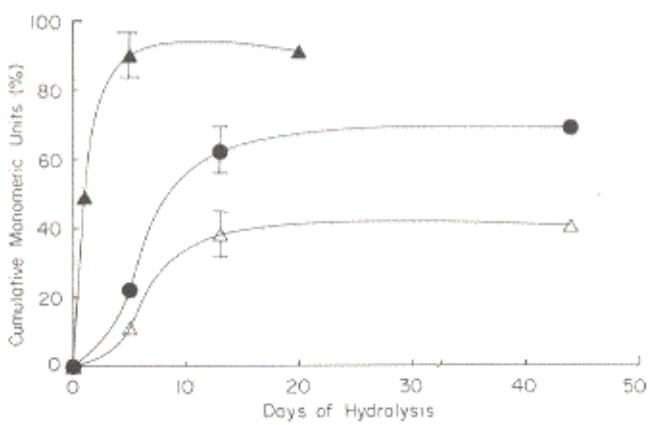


Figure 3 Monomer-release profiles at 85°C of poly(L-lactide) with mol. wt 50 000 (▲), 100 000 (●), and 200 000 (△).

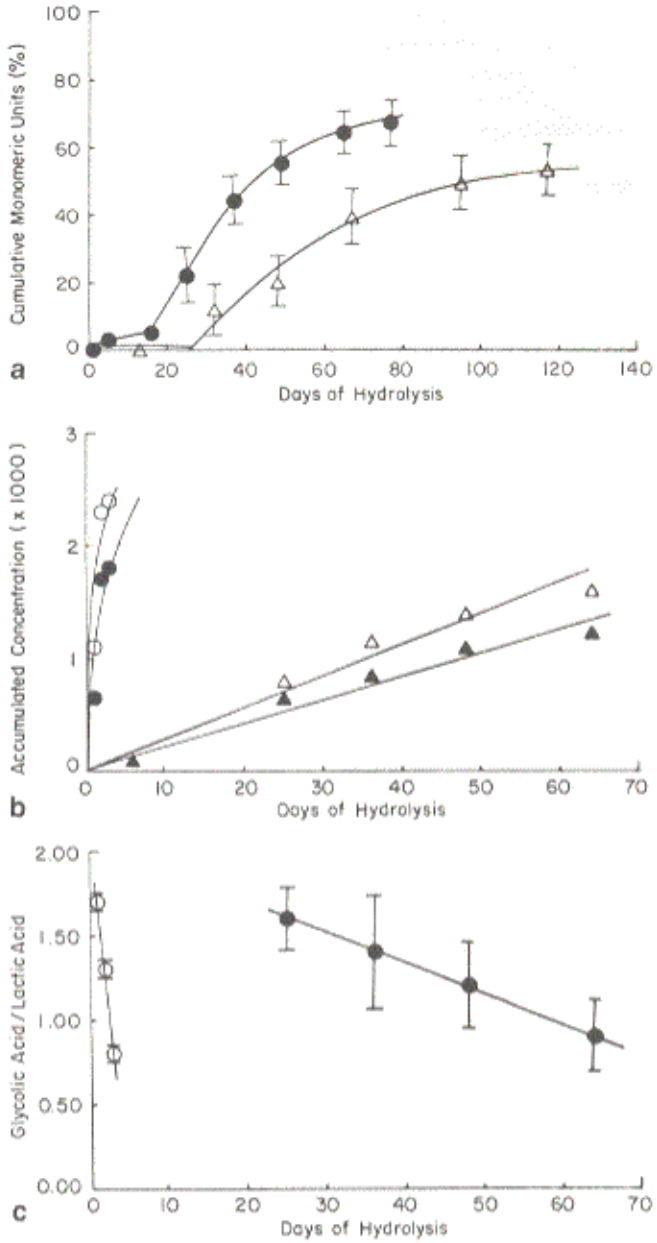


Figure 4 (a) Monomer-release profiles of poly(DL-lactide : glycolide, 85 : 15 (△), and 50 : 50 (●)) degraded at 37°C. (b) The accumulated millimolar concentration of glycolate ((△) 37°C, (○) 85°C) and lactate ((▲) 37°C, (●) 85°C) products for degraded poly(DL-lactide : glycolide, 50 : 50) is plotted as a function of hydrolysis time in days. (c) The ratio of glycolate to lactate formed is plotted as a function of time at 37°C (●) and 85°C (○).

preferentially hydrolysed or that lactide units exist in blocks which are more resistant to hydrolysis. Later, the release of lactic acid predominates. The ratio of glycolate/lactate released in each time interval of *Figure 4b* changes linearly over the time course of degradation (*Figure 4c*).

After residual mass loss had begun for poly(DL-lactide : glycolide, 50 : 50) polymer, a sample of soluble product was examined by n.m.r. (*Figure 5a*). The major monomeric products (≈80%) were detected as well as evidence for the presence of small quantities (≈ 20%) of soluble oligomeric products. Additional minor signals between 1.4 and 1.5 p.p.m. corresponding to the methyl resonance signal of these oligomeric products (*Figure 5a* inset) were observed. These minor signals decreased after sitting overnight in deuterium oxide indicating that they arise from transient water-soluble oligomeric products which are themselves labile. The same type of minor oligomeric

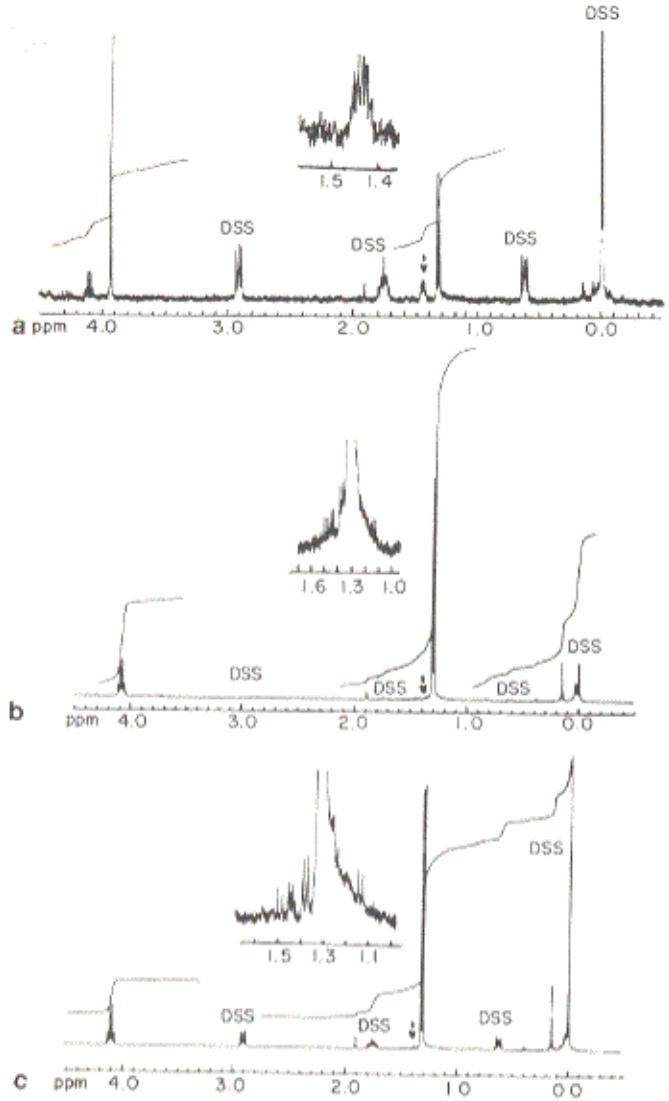


Figure 5 360 MHz ^{1}H-n.m.r. spectra of degradation products for poly(DL-lactide : glycolide, 50 : 50) (a), and poly(L-lactate) mol. wt 100 000 (b), and 200 000 (c). The arrow indicates the resonances assigned to oligomeric products shown in the expanded spectra. These spectra are assigned as follows: methyl proton resonances (1.0–1.6 p.p.m.), methine proton resonances (3.9–4.0 p.p.m.), and methylene proton resonances (4.0–4.2 p.p.m.).

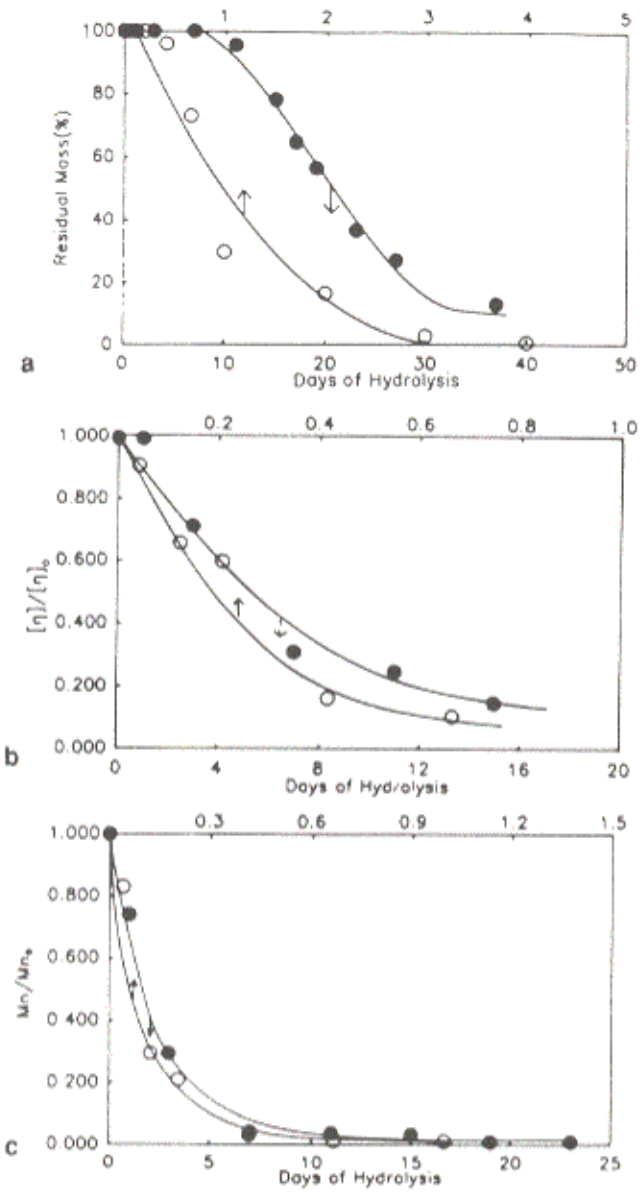


Figure 6 (a) The residual mass (%) is plotted as a function of time for poly(DL-lactide : glycolide, 50 : 50) degradation at 85°C (○) and 37°C (●). (b) The ratio of intrinsic viscosities $([\eta]/[\eta]_o)$ is plotted as a function of time for poly(DL-lactide : glycolide, 50 : 50) degradation at 85°C (○) and 37°C (●). (c) The ratio of the number average molecular weights (M_n/M_{no}) is plotted as a function of time for poly(DL-lactide : glycolide, 50 : 50) degradation at 85°C (○) and 37°C (●).

products were observed in n.m.r. spectra of the water-soluble products obtained from degradation studies of poly(L-lactide) (mol. wt 100 000 and 200 000). Five pairs of doublets were observed both upfield and downfield from the major doublet signal assigned to the lactic acid methine proton at 1.3 p.p.m. (*Figure 5b, c*). These signals correspond to at least five minor soluble oligomeric species. Studies are in progress aimed at elucidating the relationship between methyl proton chemical shift and oligomer size.

Poly(DL-lactide : glycolide, 50 : 50) was chosen for further detailed study as it demonstrated a sufficiently rapid rate of hydrolysis to be used for certain vaccine delivery applications. Little or no polymer mass loss could be detected during the first 5 h at 85°C or 7 d at 37°C of hydrolysis in PBS (*Figure 6a*). The polymeric residue viscosity decreased to 30% of its original value (*Figure 6b*) while the number average molecular weight decreased from 60 000 to 2000 (*Figure 6c*). Further decrease in viscosity (30 to 10%) or decrease in molecular weight (2000 to 700) occurred from 5–24 h at 85°C or 7–23 d at 37°C. During this same period, the residual polymer mass dropped to 30% of its initial value. Finally, it took 3 d at 85°C to degrade all the remaining polymer and 15 d at 37°C to decrease the residual polymer mass from 30 to 10% of its original value. Similar results have been reported by Schindler *et al.*[23] in their study of the biodegradation of poly(DL-lactide) films (2 by 1 cm, 100 μm thick) implanted in rabbits.

These results indicate that the copolymer degradation occurs in three stages. The initial stage of degradation has been shown to proceed through a random-chain scission process. While the molecular weight of the polymer decreased significantly, there is no weight loss in polymer and no soluble monomeric products were produced throughout this stage. In the middle period, decrease in molecular weight was accompanied by rapid loss in the mass of polymer residue and soluble monomeric and oligomeric products are detected. In the final stage, weight loss slowed and soluble monomeric products were produced. It appears that chain scission occurs in the insoluble polymer and the formed soluble oligomeric products at different rates.

According to the Mark–Houwink equation, $[\eta] = KM^a$, the intrinsic viscosity of polymer in solution is proportional to the molecular weight. From the $\log[\eta]$ *versus* $\log[M]$ relationship, the exponent 'a' obtained from the slope has a value of 0.61 ($K = 6.31 \times 10^{-4}$) indicating that the polymer chain is a random coil.

In vitro release studies

The BSA-release profiles at 37°C from poly(DL-lactide : glycolide, 50 : 50) microspheres prepared by spray drying, solvent extraction precipitation and press grinding are shown in *Figures 7a* and *b*. Most of the BSA (70–80%) was released during the first day. Only 1–3% of the BSA remained in the polymer after 3 d. Small but detectable quantities of BSA are still released after 7 d.

The initial release of BSA from the polymeric matrix could be due to:

(1) The presence of surface-bound or loosely associated BSA at high loading may have created a significant burst effect.
(2) Based on degradation studies conducted on spray-dried poly(DL-lactide : glycolide, 50 : 50), in the absence of loaded protein, the molecular weight of the polymer was found to drop quickly during the first 3 d of the hydrolysis at 37°C. This could create a porous structure throughout the polymer matrix facilitating BSA (mol. wt 65 000) release.
(3) Insufficient coverage of protein by the polymer or cracks or pores formed during the preparation may faciliate rapid release of protein.

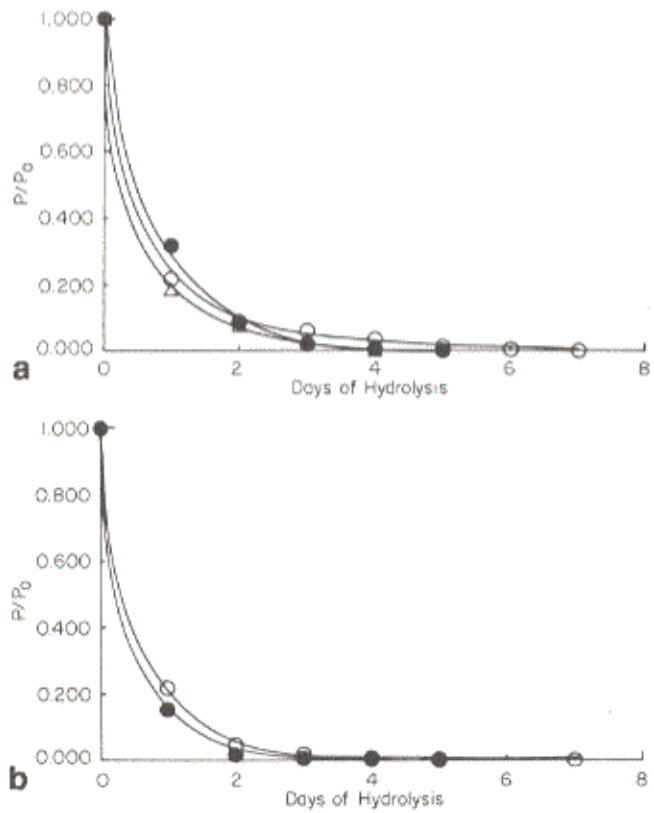


Figure 7 (a) The ratio of unreleased (P) and loaded (P_0) protein is plotted as a function of time for spray-dried microspheres. (Protein-incorporated microspheres are prepared in methylene chloride (○), tetrahydrofuran (△), or with Chaps (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate) in methylene chloride (●).) (b) The ratio of unreleased (P) and loaded (P_0) protein is plotted as a function of time for solvent extraction precipitated (○) and press-ground (●) microspheres.

(4) Microsphere preparation methods were a contributing factor in the initial release of protein. *Figure 7a* shows that microspheres prepared by suspending BSA in polymer/methylene chloride solutions released protein over 1 wk. Microspheres prepared by suspending BSA in polymer/tetrahydrofuran solutions gave 4–5 d of protein release as did microspheres made by suspending BSA in polymer/methylene chloride in the presence of surfactant and spray drying. Microspheres prepared by solvent extraction precipitation or press grinding also gave BSA-release profiles for 4–5 d (*Figure 7b*). This may have been due to the high porosity of the microspheres prepared by solvent extraction precipitation and press grinding methods.

CONCLUSIONS

Poly(esters) can be prepared as microspheres with spray drying, solvent extraction precipitation, rotary evaporation and press grinding methods. Hydrolysis of microspheres proceeds by random chain scission and the hydrolysis rate is a function of monomer structure, polymer molecular weight and copolymer ratio. Molecular weight decreases before any significant mass loss of polymer is observed. This indicates that hydrolysis must proceed to a high degree before soluble oligomers or monomers are produced.

We have shown that ion chromatography with conductivity detection proved useful in following poly(DL-lactide : glycolide) hydrolysis. We also demonstrated that glycolate was formed at a faster rate early in the degradation of poly(DL-lactide : glycolide, 50 : 50) while lactate lagged behind glycolate. Ion chromatography may prove useful in following the hydrolysis of other biodegradable polymers. This work represents the first direct evidence for the production of soluble oligomers from poly(esters) during hydrolysis.

Protein release (BSA) studies showed that most of the protein was released during the random chain scission stage of polymer matrix erosion before the loss of polymer mass. An increase in the molecular weight of the polymer or its hydrophobicity may result in a slowing of random chain scission and an extension of protein release time. In addition, new approaches of formulation to completely entrap protein by polymer matrix are needed to decrease the rate of protein release.

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