JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 7 (1996) 161–166

Nanospheres prepared from poly(β-malic acid) benzyl ester copolymers: evidence for their *in vitro* degradation

S. STOLNIK, M. C. GARNETT, M. C. DAVIES, L. ILLUM, S. S. DAVIS
Department of Pharmaceutical Sciences, Nottingham University, University Park, Nottingham NG7 2RD, UK

M. BOUSTA, M. VERT
Centre de Recherche sur les Biopolymeres Artificiels, Universite Monpellier, 15, ave Charles Flahaut, 34060 URA CNRS 1465, Montpellier, France

The *in vitro* degradation of nanospheres perpared from three benzyl ester derivatives of poly (β-malic acid) containing 80, 90 and 100% of benzylated malic acid units was studied. The progressive decrease in the molecular weight of the copolymers was observed as the nanospheres degraded, demonstrating that the degradation under the experimental conditions occurred by a simple hydrolytic cleavage of the ester bond between the monomeric units. The degradation was slow, with the weight average molecular weight decreasing to about 70% of the initial value in 5 months for all the nanosphere systems. A comparison of degradation rates for benzyl ester copolymers with the degradation rate of poly (β-malic acid) homopolymer demonstrated a decreased degradation rate of benzylated copolymers which suggests that the introduction of a pendent benzyl ester function in proximity to the ester bond in the main chain, reduces the rate of the bond cleavage. No significant difference in the degradation behaviour of highly benzylated copolymers, containing 90 and 80% of benzylated malic acid units, and fully benzylated polymer could be detected to prove an autocatalytic role on degradation of the free pendent carboxyl group in the former two copolymers.

1. Introduction

Within the last two decades a variety of natural and synthetic materials has been extensively studied for possible biomedical use, such as bone or joint prosthesis, artificial blood vessels, surgical sutures, contact lenses or diagnostic reagents in clinical laboratory tests [1–3]. However, one of the most attractive potential uses of these materials is in the formulation of novel drug delivery systems for parenteral administration. Here the polymeric material is an essential part of a therapeutic system (or carrier) which is aimed at achieving either control over the rate of drug delivery or delivery of the drug selectively at its pharmacological site(s) of action within the body. Two types of polymeric carriers have been most often studied: macromolecular carriers, where the drug is covalently linked to a macromolecule soluble in body fluid, or colloidal carriers, where the drug is trapped inside a solid matrix formed from a macromolecule. The possibility of using colloidal carriers as targeting devices for site-specific delivery has been extensively tested in our and other research groups [4–7]. These investigations have demonstrated that by optimizing the particle size and surface properties of such carriers a certain degree of selectivity in delivery may be achieved. For instance, it has been shown in studies on model colloidal carriers that 60 nm polystyrene particles coated with amphipathic triblock or star-shaped copolymers (such as copolymers of polyoxyethylene-polyoxypropylene and polyoxyethylene-polyoxypropylene ethylene diamine, commercially available as Poloxamers and Poloxamines, respectively) can significantly alter particle biodistribution following intravenous injection [6, 8]. Hence, coating with Poloxamer 407 and Poloxamine 904 resulted in a reduction in the level of liver/spleen accumulation and in a redirection of a significant portion of the administered particles to the endothelial cells of the bone marrow, while Poloxamine 908 coated particles exhibited prolonged circulation time in the vascular compartment. Larger particles of 250 nm diameter coated with Poloxamer 407 were mostly sequestered by the liver and spleen and only a small fraction reached the bone marrow [9]. For such systems to have potential use in human therapy, it is important that the model polystyrene particles are replaced by carriers made from biodegradable and biocompatible materials.

Recently, the poly(β-malic acid) (PMLA) polymer has been synthesized and shown to be biodegradable

0957–4530

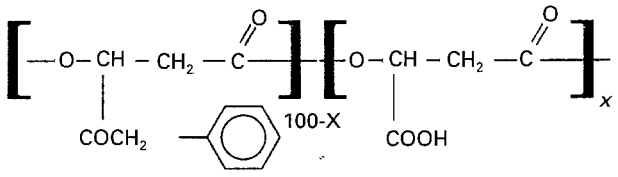

Figure 1 Structural formula of poly(β-malic acid-co-benzyl malate) copolymers (*x* indicates the percentage of malic acid units in the polymer chain).

and biocompatible [10, 11]. The PMLA homopolymer is hydrophilic, soluble in water and consequently not suitable for production of colloidal carriers. However, the synthesis of PMLA allows a range of benzyl ester derivatives with varying composition of repeating benzyl ester and malic acid units to be produced (Fig. 1). The physicochemical properties of these copolymers depend on the molecular composition, such that the hydrophobic nature of the copolymers increases with increasing content of benzylated malic acid units. Derivatives with more than approximately 25% of benzylated malic acid units are insoluble in water but soluble in common organic solvents.

Recently, we have produced colloidal carriers in the nanometre size range (nanospheres) from three benzyl ester copolymers of PMLA: fully benzylated ($PMLABe_{100}$) and two partly benzylated copolymers with 90 and 80% of benzylated monomeric units ($PMLABe_{90}H_{10}$ and $PMLABe_{80}H_{20}$, respectively) [12, 13]. It has been shown that the particle size, surface potential and surface chemistry of these nanospheres depend on the composition of the copolymer used in the preparation. The presence of Poloxamers and Poloxamines influenced the surface characteristics of the nanospheres. This effect was dependent on both the copolymer used in the nanosphere preparation and the coating polymer.

The degradation of nanospheres prepared from these highly benzylated PMLA derivatives has not been previously investigated. Accordingly, the present work describes the *in vitro* degradation of these nanosphere systems in an aqueous medium and forms an essential part of our current studies on the evaluation of their potential for site-specific drug delivery.

2. Materials and methods

2.1. Materials

The poly(β-malic acid-co-benzyl malate) copolymers ($PMLABe_{100-x}H_x$, with *x* indicating the percentage of malic acid repeating units in polymer chain, the entire range referred to in the text by acronym, PMLABeH) of differing composition were used: $PMLABe_{100}$ (weight average molecular weight, M_w 30 kD; polydispersity index M_w/M_n 1.4 as determined by gel permeation chromatography in tetrahydrofuran, calibrated using polystyrene standards), $PMLABe_{90}H_{10}$ (M_w 25.4 kD, polydispersity index 1.4) and $PMLABe_{80}H_{20}$ (M_w 27.1 kD, polydispersity index 1.7). The polymers were synthesised as described previously [10]. All other chemicals used were HPLC grade. Deionized water was chromatographically purified (Elgastat Ltd., UK).

2.2. Preparation of nanospheres

The nanospheres were prepared by a precipitation solvent evaporation method, as described previously [12]. The polymer was dissolved in acetone (5 mg ml^{-1}, 5 ml) and a mixture of deionized water and ethanol (1:1) was added dropwise (25G syringe needle) into the polymer solution stirred by a magnetic stirrer (Ika-Labortechnik, Germany), until turbidity indicative of copolymer precipitation was visually observed. The suspension of these preformed nanospheres was then added to water (10 ml) placed in a glass beaker (50 ml), and agitated by a magnetic stirrer at ambient temperature until complete evaporation of the organic solvent had taken place.

2.3. Morphological characterization of nanospheres by transmission electron microscopy (TEM)

Samples for microscopy were stained with phosphotungstic acid, and after drying, examined employing an electron microscope (Joel 1200 EX12, Japan).

2.4. Determination of particle size

Particle size and width of the size distribution were determined by photon correlation spectroscopy (PCS), as described previously [14]. The samples were diluted before measurement with deionized filtered water (0.2 μm membrane filter, Minisart MNL, Sartorius, Germany) and each sample was analysed a total of ten times to give an average value and standard deviation for particle diameter and polydispersity index.

2.5. Degradation study procedure

The PMLABeH nanospheres used in the degradation experiment were prepared in the absence of a surfactant to eliminate the possible effect of processing additives on the degradation process [15]. The degradation experiment was carried out in unbuffered water, which was advantageous for two reasons. First, surfactant-free $PMLABe_{100}$ nanospheres were found to have a low colloidal stability towards electrolytes and the presence of an electrolyte in the buffer would have caused an agglomeration and precipitation of nanospheres from the suspension, in contrast to the relatively stable suspensions of $PMLABe_{90}H_{10}$ and $PMLABe_{80}H_{20}$ nanospheres [13]. Secondly, such an experimental protocol enabled samples for gel permeation chromatography to be prepared by a simple freeze drying of the nanosphere suspension and subsequent dissolution of the dried powder in organic solvent without prior separation of the nanospheres from the degradation medium.

The nanospheres from several batches were collected and duplicate samples were placed in separate glass vials for each degradation time (15, 30, 45, 90 and 150 days). The vials were kept in a water bath at 37 °C and were constantly agitated. The initial pH values of the nanospheres suspensions were 6.1, 4.5 and 3.6 for the $PMLABe_{100}$, $PMLABe_{90}H_{10}$ and $PMLABe_{80}H_{20}$, respectively, as measured employing a Micro protein resistant combination electrode (Whatman, Maidstone, UK) connected to a pH meter (Corning, Halstead Essex, UK). The pH of the degradation medium was not adjusted to the same value for all the systems since it has been reported that, although strong acidic and alkaline media dramatically affect polyester degradation, pH values in the range 4 to 6 do not have a significant effect on degradation [16, 17]. Sodium azide (0.02% w/v) was added as a preservative. At appropriate time intervals, the nanosphere suspensions in the two vials were freeze dried. The dried samples were stored in desiccator at 4 °C until used for further analysis. The degradation study was terminated after 5 months since the $PMLABe_{100}$ nanospheres were seen to have coagulated forming agglomerates that could not be redispersed.

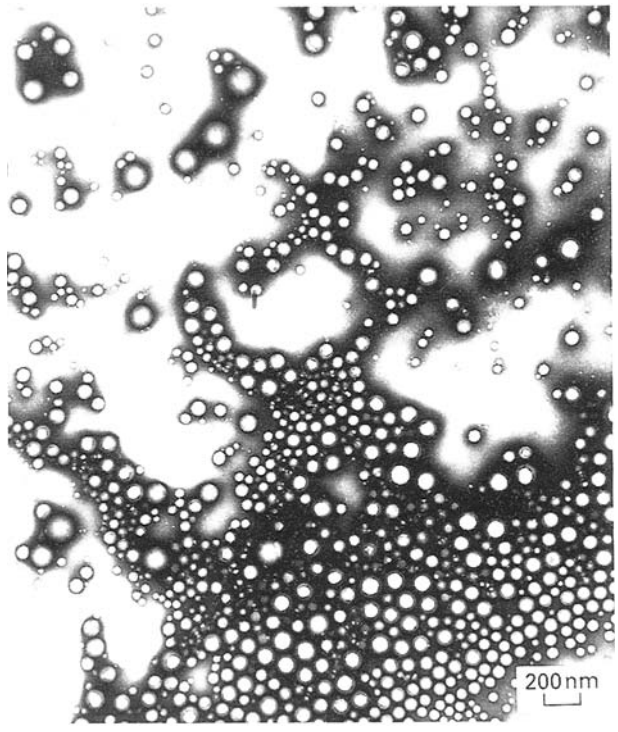

Figure 2 Transmission electron micrograph of the $PMLABe_{80}H_{20}$ nanospheres.

2.6. Gel permeation chromatography

In order to monitor degradation of the PMLABeH nanospheres, gel permeation chromatography (GPC) was employed. Samples for GPC were prepared by dissolving the freeze dried nanospheres in tetrahydrofuran (20 mg in 10 ml). The solutions were filtered through a 0.2 μm polyamide membrane filter prior to chromatography. The chromatography conditions were: PL gel mixed bed-D column, 30 cm, 5 μm packing; solvent tetrahydrofuran; flow rate 1.0 ml min^{-1}; ambient temperature; refractive index detector. The GPC system was calibrated with polystyrene standards, and the results are expressed as "polystyrene equivalent" molecular masses. For each degradation time the weight and number average molecular weight determined is the mean value of two runs performed on each of duplicate samples. A polydispersity index is expressed as a ratio between weight and number average molecular weight. All the samples in question were analysed on the same day. The measurements were performed by Rubber and Plastic Research Association (RAPRA), Shewsbury, UK.

3. Results and discussion

A transmission electron micrograph of the $PMLABe_{80}H_{20}$ formulation is shown in Fig. 2, and is typical of the images for all the systems produced from the PMLABeH copolymers. The micrograph illustrates that the formulation consists of discrete and spherical polymeric particles in the nanometre size range (nanospheres). The results of the particle size analysis confirm the microscopy observations, showing that the nanospheres from all the PMLABeH copolymers are in the sub-200 nm size range, with mean diameters of 89 ± 2 nm, 131 ± 3 nm and 137 ± 3 nm for $PMLABe_{100}$, $PMLABe_{90}H_{10}$ and $PMLABe_{80}H_{20}$ nanospheres, respectively. The polydispersity indices for all the nanosphere systems indicate a relatively narrow particle size distribution.

It should be noted that the $PMLABe_{100}$ nanospheres are smaller than $PMLABe_{90}H_{10}$ and $PMLABe_{80}H_{20}$ spheres. This difference in the size was not expected to cause significant difference in the nanospheres degradation, although it has been documented that the size and shape of a polymeric specimen undergoing degradation can affect the process, at least in the case of polylactide/glycolide copolymers [18, 19]. However, these findings are based on degradation studies on larger specimens (in milimetre size range), while no significant difference was detected in the degradation rates of small polylactide formulations with mean particle diameters ranging from 225 to 400 nm [15].

Representative gel permeation chromatograms for the degradation of the $PMLABe_{80}H_{20}$ nanospheres are shown in Fig. 3, and are illustrative for all the nanosphere systems studied. The chromatography profiles for all the degradation times appeared monomodal and show a broadening of the molecular weight distribution (with decreasing molecular mass of the polymers) as the nanospheres degrade with time. The appearance of a tail on the curves is indicative of formation of low or intermediate molecular weight fragments during the degradation process. This effect was followed by overall shifting of the profiles which indicates hydrolysis of the high molecular chains.

The degradation of PMLABeH nanospheres, represented by the plot of the weight and number average molecular weights and polydispersity indices versus time, is shown in Fig. 4. The results reveal that the molecular weight decreases with time almost linearly

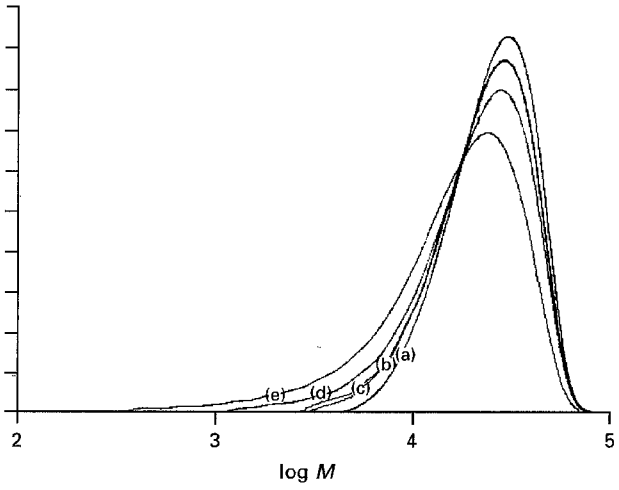

Figure 3 Gel permeation chromatograms of $PMLABe_{80}H_{20}$ nanospheres for different degradation times: (a) 0 day; (b) 30 days; (c) 45 days; (d) 90 days; (e) 150 days.

for all the nanosphere systems (r^2 0.996, 0.960, 0.962 for weight average molecular weights of $PMLABe_{100}$, $PMLABe_{90}H_{10}$ and $PMLABe_{80}H_{20}$, respectively). The broadening of the molecular weight distribution seen on the chromatograms is reflected in an increase in the polydispersity indices from 1.4 to 2.5. After 5 months of degradation the weight average molecular weight decreased to 72.6, 77.5 and 73.4% of the initial value for $PMLABe_{100}$, $PMLABe_{90}H_{10}$ and $PMLABe_{80}H_{20}$ nanospheres, respectively.

The molecular composition of the PMLABeH copolymers is characterized by the presence of benzylated malic acid units in the polymer chain. To assess the effect of the benzyl ester function on the degradation of the copolymers, the data were compared with those for the PMLA homopolymer, provided from previous studies [20, 21]. Prior to making such a comparison it should be mentioned that an exact comparison of the degradation data from different experiments is not possible due to effects from different experimental conditions on the degradation rate. However, general trends may be compared. The degradation of PMLA homopolymer in buffered aqueous medium is characterized by a rapid initial loss of molecular weight, whereby the weight average molecular weight decreased to 25% of its initial value in only 20 h. This was followed by the appearance of a whole series of oligomers and a continuous change in the molecular weight of the oligomers lasting several months. Hence, the results for the PMLABeH nanospheres reveal that the benzylated copolymers degraded at much slower rates than the PMLA homopolymer. This is in line with results obtained for $PMLABe_{22}H_{78}$ copolymer; another partly benzylated derivative with a low ratio of benzylated malic acid units [22]. This copolymer degraded gradually over a period of 19 days; the rate of degradation being in between those obtained for the degradation rate of PMLA homopolymer and highly benzylated PMLABeH copolymers. Hence, these findings suggest that the presence of the pendent benzyl ester function in the PMLABeH copolymers significantly decreases

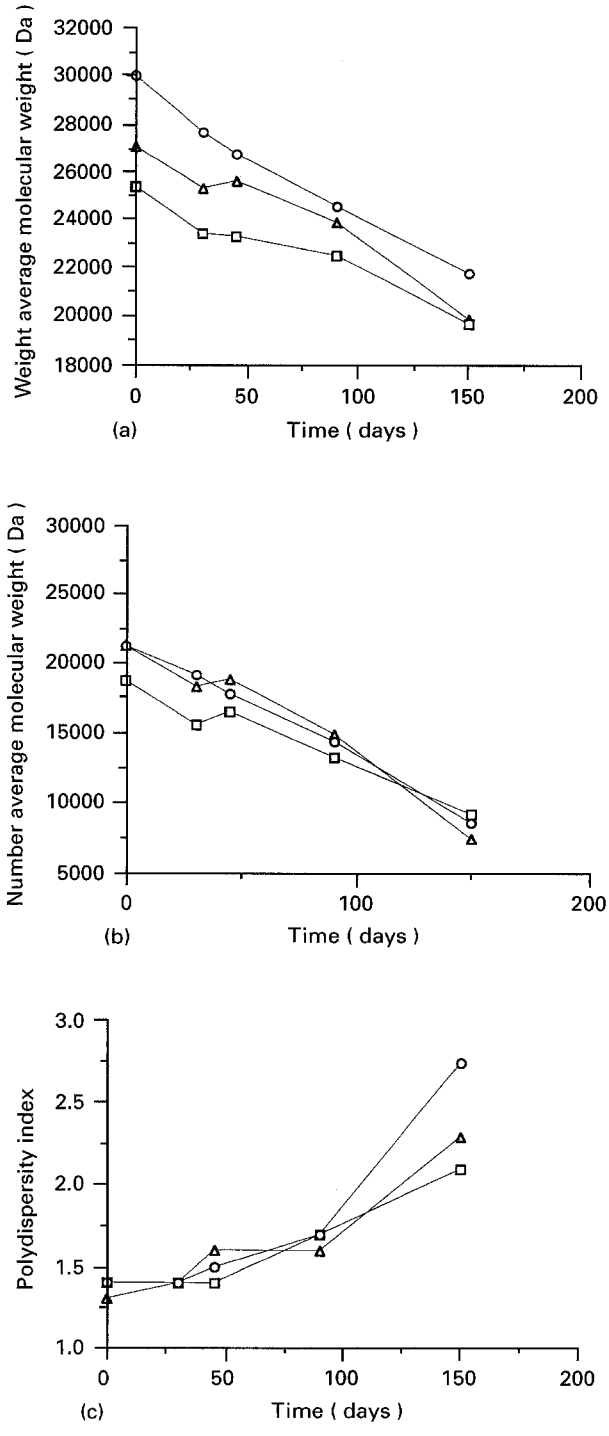

Figure 4 Molecular weight of the PMLABeH nanospheres as a function of degradation time: (a) weight average molecular weight, M_w; (b) number average molecular weight, M_n; (c) polydispersity index (ratio of average M_w to average M_n). -⊖- $PMLABe_{100}$; -□- $PMLABe_{90}H_{10}$; -△- $PMLABe_{80}H_{20}$.

the cleavage rate of the ester bond in the main copolymer chain relative to PMLA homopolymer. A comparison between three sets of degradation data, for PMLA homopolymer, $PMLABe_{22}H_{78}$ copolymer with low and $PMLABe_{80}H_{20}$ and $PMLABe_{90}H_{10}$ copolymers with high percentage of benzylated malic acid units, would indicate a decrease in the degradation rate with an increase in a level of benzylated malic acid units in the copolymers.

The reduction in degradation rate owing to the presence of the benzyl function in PMLABeH

copolymers is in agreement with findings for other polyesters showing that a substituent near the ester group influences the degradation rate. For instance, reduced reactivity of polylactic acid, relative to polyglycolic acid, is considered to be a reflection of the steric effect of the methyl chain [23], while alkyl or aryl substituents have been found to slow the hydrolysis rate of polyesters via steric hinderance and electron donation [24].

The second structural characteristic of the PMLA and PMLABeH polymers is the presence of a free pendent carboxyl group in the polymer chain. This carboxyl group may be expected to have an autocatalytic role in the hydrolysis of the copolymers, similar to that of a carboxyl end group formed during degradation of polylactic acid polymer [16, 24], leading to different degradation properties of partly benzylated in comparison with fully benzylated $PMLABe_{100}$ polymer. The results (Figs 3 and 4), however, reveal no significant differences in the molecular weight versus time profiles for degradation of $PMLABe_{100}$, $PMLABe_{90}H_{10}$ and $PMLABe_{80}H_{20}$ nanospheres.

One possible explanation of these findings may be the following. The degradation data for PMLA homopolymer and benzylated derivatives indicate that there is a balance in the effects that the pendent carboxyl group, benzyl ester function and water solubility of the copolymer can have on the degradation. For instance, in the case of $PMLABe_{22}H_{78}$ copolymer, which degraded slower than PMLA ,and faster than highly benzylated $PMLABe_{80}H_{20}$, $PMLABe_{90}H_{10}$ and $PMLABe_{100}$ polymers, the degradation appears to be a result of the combined effects of the high level of pendent carboxyl groups, a low level of benzyl ester function and the copolymer solubility in a water. The degradation of $PMLABe_{80}$ H_{20}, $PMLABe_{90}H_{10}$ and $PMLABe_{100}$ copolymers seems to be dominated by the effect of the steric hindrance of the benzyl ester function on the hydrolysis which, due to similarity in molecular composition of these copolymers, results in the degradation processes being very similar.

With respect to the possible use of the PMLABeH copolymers as degradable biomaterials for formulation of colloidal drug carriers, it is of interest to compare their degradation rate with that of other polymers used for the same purpose. Recently, Coffin and McGinity [15] reported on the *in vitro* degradation of a pseudolatex prepared from polylactic acid or polycaprolactone under experimental conditions similar to our study on the PMLABeH nanospheres. Their results show that for 200–241 nm diameter latex particles prepared from polylactic acid in the presence of a non-ionic surfactant and suspended in unbuffered aqueous medium at 37 °C, the weight average molecular weight dropped to 33% of its initial value in 4 months, while in the case of polycaprolactone the molecular weight was approximately 60% of the starting value after 2 months. Hence, a comparison with our data shows that the degradation of PMLABeH nanospheres is a relatively slow process.

In conclusion, the results of this investigation reveal that benzyl esters of PMLA degrade in aqueous medium by hydrolytic scission of the ester bond between monomeric units. The degradation proceeds by progressive decrease in the molecular weight of the copolymers over time. The reduced degradation rate of benzylated derivatives in comparison to PMLA homopolymer clearly demonstrate that the presence of pendent benzyl ester functions reduces the hydrolysis of the ester bond in the main chain. A significant difference in the degradation data for the two highly benzylated esters $PMLABe_{80}H_{20}$ and $PMLABe_{90}H_{10}$ and fully benzylated $PMLABe_{100}$ polymer could not be detected. Hence, an autocatalytic role of the free carboxyl group from malic acid units on the degradation and a catalytic effect of the degradation products, as seen for the degradation of large PMLABeH specimens [25], could not be proven. This is probably because the small size of the nanosphere systems allows easy exchange of soluble byproducts with surrounding aqueous medium. However, the results indicate that there is a balance between facilitated degradation, due to the presence of free carboxyl group, and reduced degradation, due to the presence of benzyl ester units. This would suggest that degradation of the PMLABeH copolymers can be controlled by optimization of the copolymer composition to produce nanospheres with the desired degradation properties.

Acknowledgements

This work was founded by a BRITE-EURAM project "Development of resorbable biodegradable polymers for the preparation of coated particles for drug delivery" BREU-0053-C, BE-3348-89. The authors wish to acknowledge the assistance of Dr Steve Holding of RAPRA.

References

1. T. TSURUTA, H. HAYASHI, K. KATAOKA, K. ISHIHARA and Y. KIMURA (eds), "Biomedical applications of polymeric materials" (CRC Press, Boca Raton, 1993).
2. C. G. GEBELEIN and R. L. DUNN (eds), "Progress in biomedical polymers" (Plenum Press, New York and London, 1990).
3. M. VERT, J. FEIJEN, A. ALBERTSSON, G. SCOTT, and E. CHIELLINI (eds), "Biodegradable polymers and plastics" (The Royal Society of Chemistry, Cambridge, 1992).
4. E. TOMLINSON and S. S. DAVIS (eds), "Site-specific drug delivery: cell biology, medical and pharmaceutical aspects" (John Wiley, Chichester, 1986).
5. G. GREGORIADIS, J. SENIOR and G. POSTE (eds) "Targeting of drugs with synthetic systems", NATO ASI Series, Series A: Life Sciences Vol. 113 (Plenum Press, New York, 1986).
6. L. ILLUM, S. S. DAVIS, R. H. MULLER, E. MAK and P. WEST, *Life Sci.* **40** (1987) 3647.
7. J. SENIOR, C. DELGADO, D. FISHER, C. TITLOK and G. GREGORIADIS, *Biochim. Biophys. Acta* **1062** (1991) 77.
8. I.S. MUIR, S. M. MOGHIMI, L. ILLUM, S. S. DAVIS and M. C. DAVIES, *Biochem. Soc. Trans.* **19** (1991) 329S.
9. J. PORTER, S. M. MOGHIMI, L. ILLUM and S. S. DAVIS, *FEBS Lett.* **305** (1992) 62.

10. M. VERT and R. W. LENZ, Malic acid polymers, US Patent, 4 265 247, July 1, 1981.
11. C. BRAUD, C. BUNEL and M. VERT, *Polym. Bull.* **13** (1985) 293.
12. S. STOLNIK, M. C. DAVIES, L. ILLUM, S. S. DAVIS M. BOUSTTA and M. VERT, *J. Controlled Release* **30** (1994) 57.
13. S. STOLNIK, M. C. GARNETT, M. C. DAVIES, L. ILLUM, M. VERT and S. S. DAVIS, *Colloids and Surfaces*, in press
14. S. J. DOUGLAS, L. ILLUM, S. S. DAVIS and J. KREUTER, *J. Colloid. Interf. Sci.* **101** (1984) 149.
15. M. D. COFFIN and J. W. McGINITY *Pharm. Res.* **9** (1992) 200.
16. K. MAKINO, H. OHSHIMA and T. KONDO, *J. Microencapsulation* **3** (1986) 203.
17. T. OUCHI and A. FUJINO, *Makromolecular Chemie* **190** (1989) 1523.
18. M. VERT, S. LI and H. GARREAU, *J. Controlled Release* **16** (1991) 15.
19. S. M. LI, H. GARREAU, M. VERT, M. THERIN, M and P. CRISTEL, in "Biodegradable polymers and plastics", edited by M. Vert, J. Feijen, A. Albertsson, G. Scott, G. and E. Chiellini (The Royal Society of Chemistry, Cambridge, 1992) p. 263.
20. C. BRAUD and M. VERT in "Biodegradable polymers and plastics", edited by M. Vert, J. Feijen, A. Albertsson, G. Scott and E. Chiellini (The Royal Society of Chemistry, Cambridge, 1992) p. 233.
21. C. BRAUD and M. VERT, *Trends Polym. Sci.* **3** (1993) 57.
22. A. CARON, C. BRAUD, C. BUNEL and M. VERT, *Polymer* **31** (1990) 1797.
23. S. J. HOLLAND, B. J. TIGLE and P.L. GOULD, *J. Controlled Release* **4** (1986) 155.
24. J. HELLER in "Medical applications of controlled release", edited by R. S. Langer and D. L. Wise (CRC Press, Boca Raton, 1984) p. 69.
25. J. MAUDUIT, M. BOUSTA and M. VERT, submitted for publication

Received 12 April
and accepted 11 May 1995