Drug Delivery, 7:45–53, 2000

1071-7544/00 $12.00 + .00

A Calorimetric Study on Diflunisal Release from Poly(Lactide-*co*-Glycolide) Microspheres by Monitoring the Drug Effect on Dipalmitoylphosphatidylcholine Liposomes: Temperature and Drug Loading Influence

Francesco Castelli
Dipartimento di Scienze Chimiche, Università di Catania, Catania, Italy

Paolo Giunchedi
Dipartimento di Scienze del Farmaco, Università di Sassari, Sassari, Italy

Olga La Camera
Dipartimento di Scienze Chimiche, Università di Catania, Catania, Italy

Ubaldo Conte
Dipartimento di Chimica Farmaceutica, Università di Pavia, Pavia, Italy

Diflunisal release from poly-Lactide-co-Glycolide (50:50, 34,000 MW) microspheres loaded with two different amounts of drug (2.5 ± 0.5% and 10 ± 0.5% w/w) was monitored by following the effects exerted by the drug on the thermotropic behavior of dipalmitoylphosphatidylcholine unilamellar vesicles at different temperatures. The effects of the drug released from the microspheres on the thermotropic behavior of lipid aqueous dispersion containing different molar ratios of drug was detected by differential scanning calorimetry and was compared with the effects exerted by the free Diflunisal. Diflunisal affects mainly the temperature (T_m) of the transition characteristic of phospholipid vesicles as model biomembrane, causing a shift toward lower values. This shift was modulated by the drug molar fraction with respect to the lipid concentration in the aqueous dispersion. Afterward, calorimetric measurements were performed on suspensions of blank liposomes added to weighed amounts of unloaded and differently Diflunisal-loaded microspheres as well as free powdered Diflunisal after incubation for increasing times at three different temperatures (25, 37, and 50°C). The T_m shifts of the lipid bilayer, caused by the drug released from polymeric system as well as by the free drug during incubation periods, were compared with that caused by free drug increasing molar fractions dispersed directly on the membrane, employed as a calibration curve to obtain the fraction of drug released. This in vitro study suggests that the kinetic process involved in drug release is influenced by the amount of drug loaded in the microspheres as well as by the temperature acting on drug solubility and membrane disorder. This drug release model, monitored by the calorimetric technique shows that a) the poly-Lactide-co-Glycolide microspheres are a good delivery system able to sustain the drug release; b) the differential scanning calorimetry technique applied on the drug interaction with biomembranes constitutes a good tool to follow the drug release; 3) this model, representing an innovative alternative in vitro model, should be used to determine the different kinetics involved in the drug transfer from a drug delivery system to a membrane as uptake site.

Keywords Differential Scanning Calorimetry, Diflunisal, Membranes, Microspheres, Phosphatidylcholine, Poly-Lactide-co-Glycolide

Microspheres, defined as solid, spherical particles ranging from 1–1000 μm in diameter, used for drug formulation have attracted much interest during the past two decades. They have been used as a protective measure against oxidation and/or degradation of the drug through its entrapment into the polymeric network of the micromatrix. Another important application has been based on biodegradable polymers to achieve drug targeting and/or controlled/prolonged drug delivery.

The most widely used biodegradable polymers in the pharmaceutical field are polyesters; in particular, those derived from lactic and glycolic acids and the corresponding copolymers have increased in importance. These polymers are characterized by the presence of hydrolyzable bonds: in aqueous environments the

Received 1 April 1999; accepted 5 May 1999.
This work was partially supported by the Italian C.N.R. and MURST (Progetto Nazionale "Tecnologie Farmaceutiche").
Address correspondence to Francesco Castelli, Dipartimento di Scienze Chimiche, Viale Andrea Doria 6, 95125 Catania, Italy.

degradation occurs by hydrolysis of their ester backbone, leading to the corresponding monomers (lactic and glycolic acids) (Giunchedi et al. 1998) that are metabolic substances.

A variety of techniques have been used for the preparation of microspheres. Emulsification followed by solvent evaporation is the most widely used (Whateley 1993); even if spray-drying is gaining increasing importance (Giunchedi and Conte 1995).

Nonsteroidal anti-inflammatory drugs (NSAIDs) are largely used not only for their therapeutic activity, but also as a model in investigations on drug delivery systems because of their easy capacity to be monitored (Bonina et al. 1994; Castelli et al. 1998a, 1998b; Hwang and Shen 1981; Lasonder and Weringa 1990; Raudino and Castelli 1998). Diflunisal, belonging to this group of active agents, has been chosen in this study to be dispersed in a microparticulate biodegradable form or system (poly-Lactide-*co*-Glycolide, PLGA, microspheres). This work studies the possible uses of such a system as drug controlled release systems to achieve a more favourable contact with the biological membranes (Kamijo et al. 1996; Lewis 1990; McGee, Davis, and O'Hagan 1994; O'Hagan, Jeffery, and Davis 1994; Okada and Toguchi 1995; Whateley 1993; Wise et al. 1979) minimizing, at the same time, drug side effects (Cotton and Hux 1985).

The literature cited studies the release of compounds from these systems using classical dissolution tests that, even if they are able to follow the release kinetics from the system to the surrounding medium, are not able to detect the capacity to migrate through the medium to reach the uptake site constituted by biomembranes. A model that permits movement of attention from the release device to the target should be near to an in vivo model, taking into account that drug absorption is affected by biological membranes that adsorb lipophilic molecules. The study of the drug transfer to a biological model membrane, by monitoring the amount transferred on/inside the lipidic membranes, gives more information on drug release behavior. Previous studies were carried out on the release of drugs from micromatrices following the drug transfer to biological model membranes made of dipalmitoylphosphatidylcholine (DPPC), or dimyristoylphosphatidylcholine (DMPC), large unilamellar vesicles, and multilamellar vesicles, whose thermotropic behavior is affected by molecules dissolved in their ordered structure (Castelli et al. 1994, 1996, 1997a, 1997b, 1998a, 1998b; Raudino and Castelli 1998).

These biomembrane models, when hydrated and heated, exhibit a phase transition between an ordered phase (gel phase) to a disordered phase (liquid crystalline phase), characterized by a transitional temperature (T_m) and an enthalpy change (ΔH) (Ringsdorf, Schlarb, and Wenzmer 1988; Taylor and Morris 1995). In the presence of drug molecules possessing the capacity to be dissolved in the ordered packing of the lipidic structure, a destabilization of the ordered structure of the lipid chains can occur, resulting in a shift of the transitional temperature toward lower values with respect to the lipid alone (Bach 1984; Castelli et al. 1989, 1992, 1994, 1996, 1997a, 1997b, 1998a, 1998b; Jain 1988; Lee 1977; Raudino and Castelli 1998; Sturtevant 1982).

The determination of transitional temperature and enthalpy changes can be performed by differential scanning calorimetry (DSC) and analysed by Van't Hoff model of the freezing point depression of a solution caused by a solute. This model has been verified for several classes of chemical compounds such as anaesthetics (Lee 1977; Suezaki et al. 1990), and it has been applied on a theoretical basis by some researchers (Lee 1977; Guggenheim 1952; Jorgensen et al. 1991). Van't Hoff's model was successfully applied in previous works (Castelli et al. 1994, 1996, 1998a, 1998b; Raudino and Castelli 1998; Inoue, Fukushima, and Shimozawa 1990).

The evaluation of melting temperature depression of pure DPPC liposomes caused by the transfer of Diflunisal from matrix systems to void liposomes is compared with the effects exerted by the increasing amount of Diflunisal in touch with empty lipidic vesicles; by this comparison, it is possible to follow the release kinetics.

The interaction of Diflunisal with model membranes has been previously described (Castelli et al. 1998a; Raudino and Castelli 1998). These previous studies are now extended by involving the release of Diflunisal from PLGA microspheres loaded with two different amounts of drug (2.5 ± 0.5 and 10 ± 0.5%) stored for 1 month at 20°C and 60% R.H., and relating this release to that shown by free Diflunisal. The release process is followed at three different temperatures to show that lipid structure and drug solubility modify the release profile. The acceptor site is represented by a lipid unilamellar vesicle, which like a biological membrane, accepts drug molecules showing saturation effects; the amount of drug released by the drug delivery system is so determined directly at the acceptor site.

The aim of our work, other than to confirm the suitability of DSC for the evaluation of in vitro release kinetics from PLGA microparticulate systems, is to evaluate the influence on polymer structure of different amounts of drug loaded and on the drug release process. We also investigated the temperature influence on the transfer processes influencing both the drug solubility and the lipidic structure. In fact, both morphological properties and amounts of drug loaded affect drug release from microspheres as well as the temperature of the process (Castelli et al. 1997b, 1998a; Rosilio et al. 1991; Pradhan and Vasavada 1994; Le Corre et al. 1994).

MATERIALS AND METHODS

Chemicals

Synthetic 1,2-dipalmitoyl-sn-glycero-3-phosphocholin e was obtained from Fluka Chemical Co. (Buchs, Switzerland). The lipid solution was chromatographically pure as assessed by two-dimensional thin-layer chromatography. Lipid concentrations were determined by phosphorous analysis with the method of Bartlett (Bartlett 1959). Fluka Chemical supplied Diflunisal. Poly-Lactide-co-Glycolide, (50:50), Resomer® RG503, Mw approx. 34,000 (GPC) was supplied by Boehringer Ingelheim (Ingelheim am Rhein, Germany).

Microsphere Preparation and Characterization

The microspheres were produced by a spray-drying technique, using a mini spray-dryer (Model 190, Büchi Laboratoriums-Technik AG, Flawil, Germany). The process consisted of spraying a solution of polymer and drug through the two-fluid pressure nozzle (0.7 mm size) of the spray-drying apparatus. The flow of feed was cocurrent with the direction of the drying air. The solvent quickly evaporated, leaving solid microparticles that settled into the final bottom collector, and then were harvested and kept under vacuum for 48 hr.

The process conditions were inlet air temperature 45–46°C; outlet air temperature 32–33°C; flow rate about 0.5 1/hr.

Blank microspheres were obtained from 2% w/v solution of polymer (PLGA) in dichloromethane. Drug-loaded microspheres were prepared by spraying a dichloromethane solution containing 0.05% w/v of Diflunisal and 1.95% w/v of PLGA, and 0.2% w/v of Diflunisal and 1.8% w/v of PLGA, respectively. Two batches of microspheres containing significantly different amounts of Diflunisal were morphologically characterized.

The PLGA microspheres drug content was determined by ultraviolet spectrophotometry. Weighed amounts (about 18 mg) of drug-loaded microspheres were dissolved in CH_2Cl_2, and Diflunisal was analysed with a Uvikon spectrophotometer (Kontron Instruments, Zurich, Switzerland) at 252 nm, after extraction from the microspheres. Microspheres drug content resulted in 2.5 ± 0.5% (batch 1) and 10 ± 0.5% (batch 2) w/w.

Microspheres were morphologically characterized by scanning electron microscopy, using a microscope Jeol JXA 840A (Jeol Italia, Milano, Italy). The samples were coated with a thin layer of Au and photographed under argon (Figure 1).

Physicochemical characterization of microspheres and polymer was performed by DSC, with a Mettler TA 4000 system at a scanning rate of 5°C/min between 5 and 220°C (Figure 2).

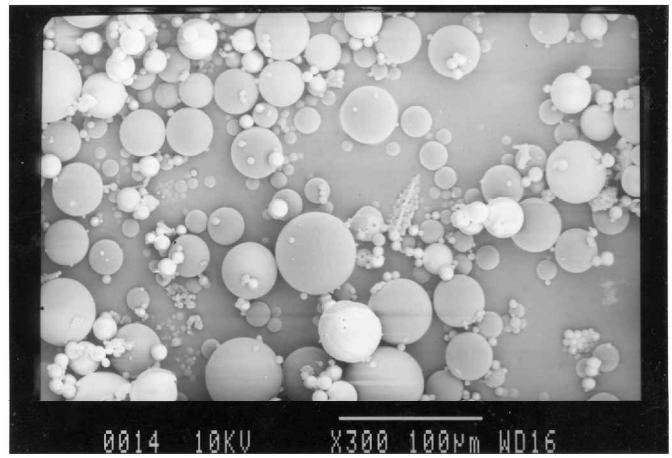
Liposome Preparation

In our study, we used unilamellar DPPC liposomes as model membranes. Multilamellar liposomes (MLV) were prepared in the absence of DFN by the following procedure: chloroform-methanol (1:1, v/v) lipid solution was transferred to a glass tube. The solvents were removed under a nitrogen flow in a rotoevaporator, and the resulting film was freeze-dried under vacuum to remove the residual solvents.

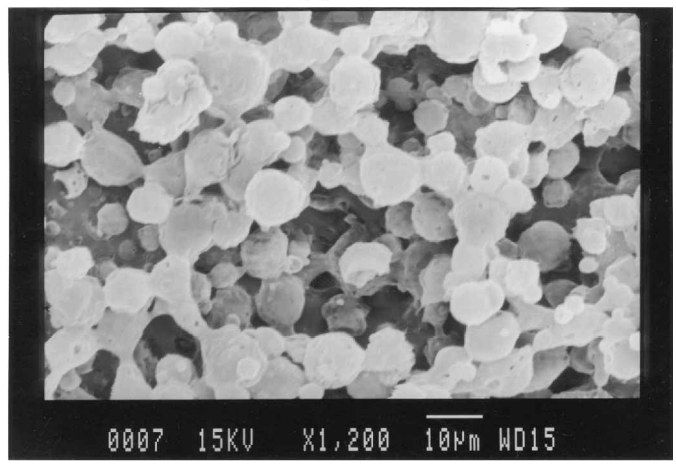
The sample (160 mg of lipid) of empty liposomes was obtained by adding 4800 μl of 50 mM Tris buffered (pH = 7.4) solution to the lipidic film. The sample was then heated at 60°C, above the gel-liquid crystalline phase transition, and vortexed three times for 1 min. The samples were shaken for 1 hr in a water bath (Dubnoff 721) at 60°C to homogenize the liposomes. This temperature was chosen to permit a better formation of multilamellar vesicles remaining in a disordered fluid state.

The unilamellar empty vesicles (LUV) were prepared by submitting empty MLV to extrusion through polycarbonate membranes of 100 nm in extruder system (Liposofast Avenstin) (Hope et al. 1985; MacDonald et al. 1991).

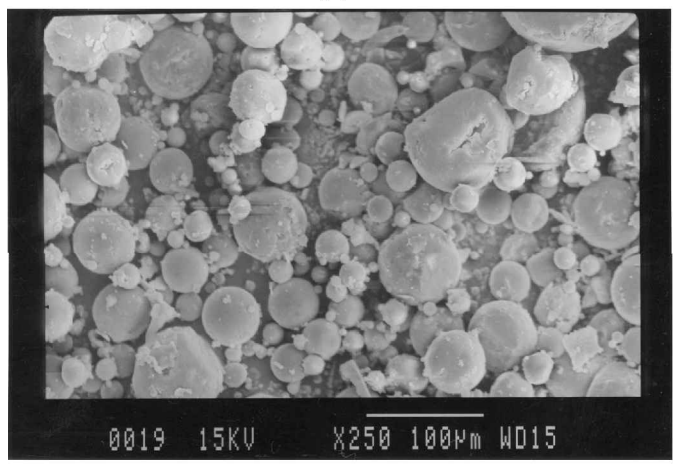
Aliquots of 120 μl (4 mg of lipid) of empty LUV were transferred to 150 μl DSC aluminium pans; afterward, the samples, sealed, were submitted to DSC analysis.



(A)



(B)



(C)

FIG. 1. Scanning electron micrograph of empty (A) and Diflunisal-loaded microspheres (B) 2.5% and (C) 10%.

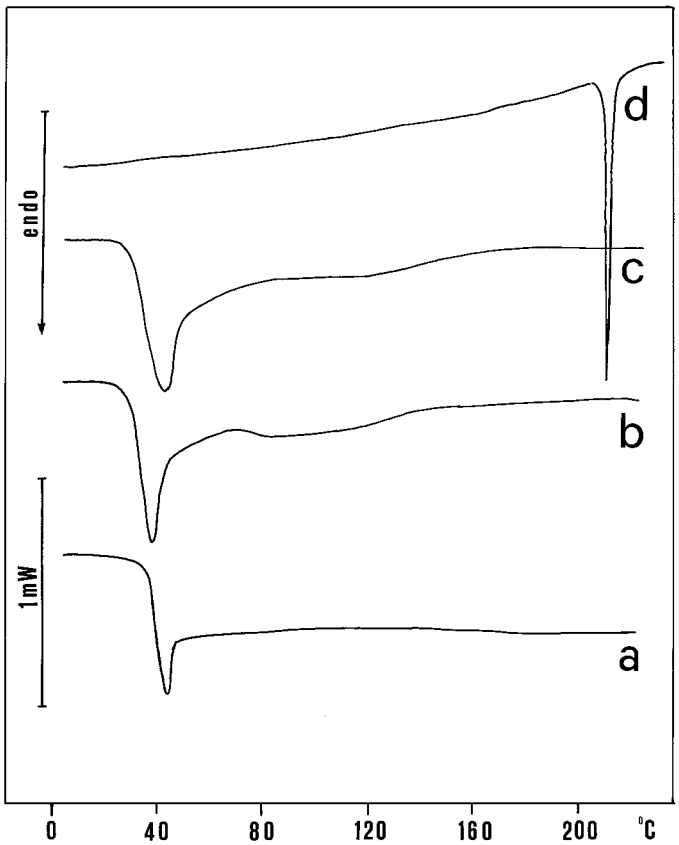


FIG. 2. Calorimetric scans of polymer and drug-loaded microspheres: (a) pure polymer microsheres; (b) 2.5% Diflunisal-loaded microspheres; (c) 10% Diflunisal-loaded microspheres; (d) Diflunisal alone.

Differential Scanning Calorimetry

DSC was performed using a Mettler TA 4000 system equipped with a DSC-30 cell and a TC-11 processor. The scanning rate employed was 2°C/min in temperature between 2–60°C. The sensitivity was 1.50 mW, and the reference pan was filled with Tris buffer solution. Indium was used to calibrate the transitional enthalpies (ΔH). Temperature and enthalpies were checked by using palmitic acid (61.82°C, 42.04 KJ/mol). After a routine temperature calibration in a large range, indium, palmitic acid, and water were used for better temperature calibration within a narrow range. Enthalpies were evaluated from the peak areas using the integration program of the TA processor. After the calorimetric scans, all samples were extracted from the pan and aliquots were used to determine the amount of phospholipid by the phosphorous assay (Bartlett 1959).

Diflunisal-DPPC Liposome Interaction

Different aliquots of Diflunisal, accurately grounded were weighed in DSC pans to ensure the presence of increasing molar fractions of drug and, after being added with the DPPC liposomes prepared as above, were incubated for 2 hr at 55°C and vortexed every 15 min to permit the best interaction between drug and liposomes. Afterward the samples were submitted to DSC analysis. The constancy in the DSC results showed the equilibrium was reached.

Drug Partition between Lipidic and Aqueous Phases

Samples (≈20 mg) of unilamellar vesicles loaded with different drug molar fraction (X = 0.03; X = 0.06; X = 0.12; X = 0.18) in Tris solution (pH = 7.4) were incubated at 55°C for at least 2 hr under gentle shaking. Aliquots were submitted to DSC analysis to check the complete interaction (partition equilibrium reached) with the liposomes, by comparing the calorimetric results with those obtained following the experimental reported in the previous section. The samples were transferred in a centrifuge tube and centrifuged at 60×10^3 *g* for 1 hr using a Beckman L8-60M centrifuge. The supernatant was separated by the lipidic *pellets*, then both were dried and lyophilized. The amount of drug present in the two fractions (aqueous and lipidic) was detected by ultraviolet spectroscopy in $CHCl_3$ at λ_{max} (252 nm), using a Perkin-Elmer 330 instrument equipped with a 3600 data station. The calculated partition coefficients were independent of the drug molar fractions. Each determination was made in triplicate and found to be corrected for the low fractions of water (carrying dissolved drug) remaining trapped within the lipid pellets. The amount of drug found in the lipid phase was 65 ± 1%.

Release Kinetics Experiments

Free-powdered Diflunisal, Diflunisal-loaded PLGA microspheres, as well as blank PLGA microspheres, have been added to the DPPC liposomes in known amounts to obtain the same relative molar fraction of drug and/or polymer with respect to the lipid.

The samples were analyzed immediately after preparation. Each sample was submitted to the following procedure:

- First scan (from 10 to 60°C) to detect drug release bringing the sample to 60°C.
- 60 min at the chosen temperature (25, 37, or 50°C) to detect the drug release after long incubation time.
- Subsequent scan from 10 to 60°C, after the incubation period at the chosen temperature followed by cooling the sample to 10°C.

The whole procedure has been performed on the two batches of microspheres loaded with different amounts of drug. This procedure has been repeated at least seven times until no further drug release or a slow release was observed.

RESULTS

Microsphere Characterization

Microspheres characterization was performed by SEM and DSC. By SEM characterization, it is possible to see only a small difference in the size and in shape between the pure microspheres (Figure 1A) with the two loaded matrices (Figure 1B,

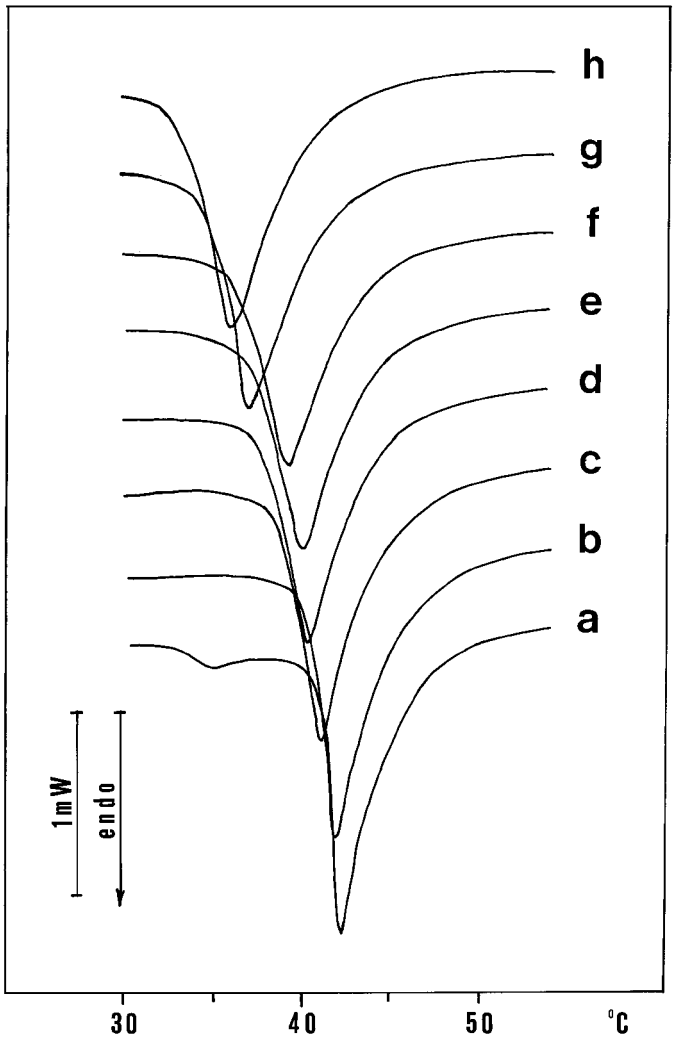


FIG. 3. Calorimetric curves of DPPC unilamellar vesicles in the presence of increasing molar fractions of Diflunisal: (a) = 0.0; (b) = 0.019; (c) = 0.038; (d) = 0.057; (e) = 0.083; (f) = 0.123; (g) = 0.186; (h) = 0.236.

2.5% and Figure 1C, 10%). But on their surface no crystals appear suggesting a homogeneous dispersion of DFN during PLGA microspheres preparation. Further information was obtained by submitting the samples to calorimetric investigation in comparison to the free drug and unloaded polymer. This knowledge is important to better understand how the nature of the drug dispersion in the polymeric matrix can influence the release rate. The DSC measurements show no differences in the nature of the drug dispersion throughout the polymeric matrices.

Figure 2 shows thermograms of polymer and drug-loaded microspheres. The change in slope of the polymer curve (curve a) can be assigned to the glass transition, typical for amorphous copolymers like PLGA 50:50. This phenomenon is characterized by a transitional temperature (T_g) defined as the temperature at which a rigid or "glassy" polymer converts to a softer "rubbery" polymer on heating (Hausberger and De Luca 1995). In the presence of drug (2.5% Diflunisal-loaded microspheres), this transition is slightly shifted toward a lower temperature (curve b). The lower T_g and the absence of a fusion event (that should correspond to a crystalline drug dispersed in the microspheres) suggest that Diflunisal is dissolved in the polymer as a solid solution, where drug and polymer strongly interact with each other, leading to a polymer plasticization (Dubernet 1995). When a higher amount of drug has been loaded into microspheres (10% Diflunisal), the behavior remains the same with only small changes in the shape of the glass transition of the polymer (curve c). The absence of drug fusion (curve d) confirms as the dispersions resulted as homogeneous. The endothermic process should be caused by a structural change (glass transition), on heating of the polymeric matrix, instead the fraction of drug is dispersed into microspheres as "amorphous." No melting process is observed at a temperature lower with respect to the pure drug, probably owing to the interaction between drug and polymer (Rosilio et al. 1991; Izumikawa et al. 1991; Bodmeier et al. 1989). These results show that the amount of the drug loaded in the matrix system slightly influences the nature of the dispersion; in fact, both lower and higher Diflunisal content (2.5 and 10%) leads to formation of a drug solid solution in the polymeric matrix.

Effect of Free Diflunisal on Thermotropic Behavior of DPPC Large Unilamellar Vesicles

The calorimetric curves of the free DPPC, compared with those obtained in presence of increasing amounts of Diflunisal on DPPC liposomes, (Figure 3) highlight Diflunisal as able to interact with lipidic model membranes by shifting the calorimetric peak toward lower values, depressing their transitional temperature (Table 1) but leaving the enthalpy change almost constant. This shift appears to be related to the molar fraction of drug present in the liposomial dispersion (Figure 4).

The interaction between drugs and DPPC liposomes is explained in terms of a "fluidifying" effect, due to the introduction of lipophilic drug molecules into the ordered structure of the lipidic bilayer. Drug molecules act as spacers in this structure causing a destabilization of the lipid mosaic with a decrease of

TABLE 1

Transitional temperatures and related temperature shifts obtained by considering the interaction of increasing amounts of Diflunisal in liposomal aqueous dispersion of DPPC vesicles or corrected by partition coefficient lipid/Tris solution really present inside the lipidic membranes

X_{DFN}	$T_m^\circ K$	$(\Delta T/T_m^\circ) \cdot 10^3$	$X_{DFN} \times 0.65$
0	314.9	0	0
0.019	314.6	−0.952	0.012
0.038	313.5	−4.445	0.025
0.057	312.8	−6.668	0.037
0.083	312.5	−7.620	0.054
0.123	311.6	−10.48	0.080
0.186	309.7	−16.51	0.121
0.236	308.6	−20.00	0.153

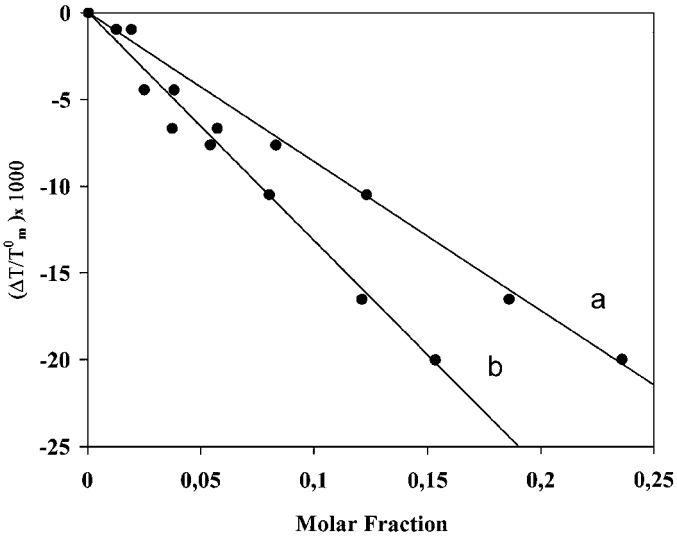


FIG. 4. Calibration curve relating the depression of DPPC unilamellar vesicles transition temperature (T_m) to concentration of Diflunisal: (a) present in the phospholipidic aqueous dispersion; (b) corrected by the partition coefficient to obtain the real amount of drug dispersed in the lipidic membrane.

T_m of the gel to liquid crystal phase transition. The negligible ΔH variation is explained by surface interaction between amphipatic molecules and DPPC polar heads, which occurs only at the surface of lipid layers without deeply interacting with acyl chains (Estep et al. 1978; Sturtevant 1982; Castelli et al. 1994, 1996).

Figure 4 curve a represents the $\Delta T/T_m^\circ$ plot versus drug mole fraction ($\Delta T_m = T_m - T_m^\circ$, where T_m° and T_m are the transition temperatures of pure DPPC and Diflunisal containing DPPC liposomes, respectively), indicating the effect of increasing amounts of Diflunisal. By relating T_m depression to the molar fraction of drug present on the membrane surface, a calibration curve ($Y = -0.7297 - 82.974X$; $r = 0.993$) is obtained to quantify and follow the transfer of Diflunisal, released from the microparticulate system, to empty membranes. The correlation curves can be modified by multiplying the molar fraction used for the partition coefficient (0.65, drug distribution between lipidic phase and aqueous one) to determine the real amount of drug (present in lipidic bilayer) exerting the fluidifying effect on thermotropic behavior of liposomes. The modified curve is reported in the Figure 4 curve b and the following equation is obtained: $Y = -0.73 - 127.60X$; $r = 0.992$.

Comparative Release between Free Diflunisal and Diflunisal-Loaded PLGA microspheres

In Figures 5, 6, and 7 (curves a and b), the transfer kinetics of Diflunisal from PLGA microspheres (batches 1 and 2) to void lipid unilamellar vesicles are reported and compared with the kinetic transfer observed for free drug (curve c). These transfer phenomena were monitored by observing the shift of DPPC

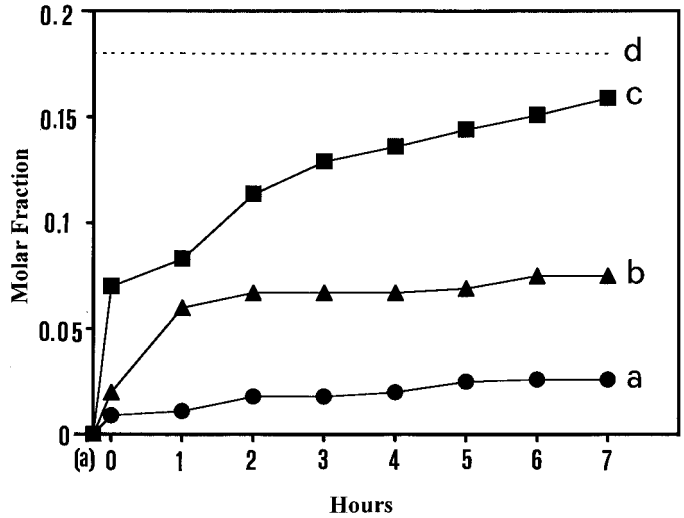


FIG. 5. Release kinetics at 25°C from free Diflunisal and Diflunisal-PLGA matrices to void LUV ($X_{Drug} = 0.18$): (a) batch A (2.5% drug-loaded sample); (b) batch B (10%-loaded drug sample); (c) free solid Diflunisal; (d) theoretical amount of Diflunisal to be released to DPPC unilamellar vesicles. The curves represent the concentrations of Diflunisal in the aqueous DPPC vesicle dispersions versus time. The (a) value on the X-axe represents the pure DPPC; the (0) first value represents the amount of Diflunisal released without incubating at 25°C; the successive values represent the release by the same samples heated at 60°C after successive 1-h incubation periods at 25°C.

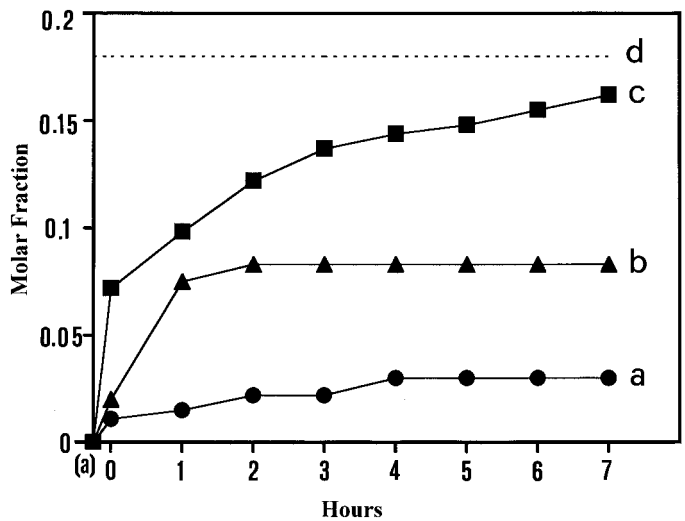


FIG. 6. Release kinetics at 37°C from free Diflunisal and Diflunisal-PLGA matrices to void LUV ($X_{Drug} = 0.18$): (a) batch A (2.5% drug-loaded sample); (b) batch B (10%-loaded drug sample); (c) free solid Diflunisal; (d) theoretical amount of Diflunisal to be released to DPPC unilamellar vesicles. The curves represent the concentrations of Diflunisal in the aqueous DPPC vesicle dispersions versus time. The (a) value on the X-axe represents the pure DPPC; the (0) first value represents the amount of Diflunisal released without incubating at 37°C; the successive values represent the release by the same samples heated at 60°C after successive 1-h incubation periods at 37°C.

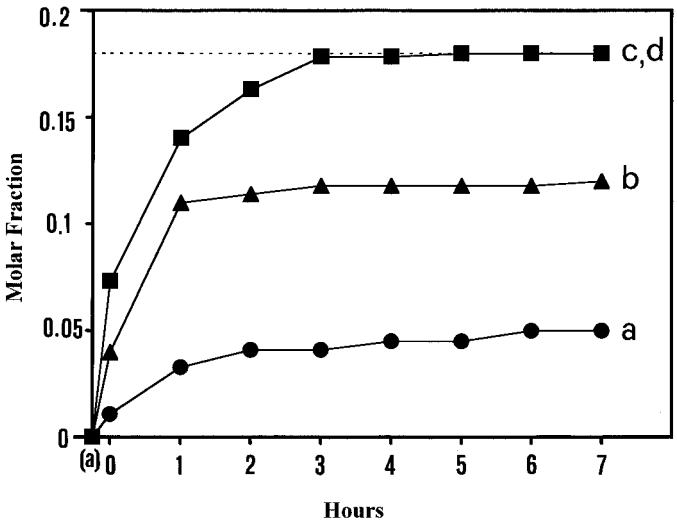


FIG. 7. Release kinetics at 50°C from free Diflunisal and Diflunisal-PLGA matrices to void LUV (X_{Drug} = 0.18): (a) batch A (2.5% drug-loaded sample); (b) batch B (10%-loaded drug sample); (c) free solid Diflunisal; (d) theoretical amount of Diflunisal to be released to DPPC unilamellar vesicles. The curves represent the concentrations of Diflunisal in the aqueous DPPC vesicle dispersions versus time. The (a) value on the X-axe represents the pure DPPC; the (0) first value represents the amount of Diflunisal released without incubating at 50°C; the successive values represent the release by the same samples heated at 60°C after successive 1-h incubation periods at 50°C.

calorimetric curves, as T_m shift, and by measuring the depression of melting temperature of void DPPC unilamellar vesicles after drug molecules uptaking from the matricial system or from the accurately powdered solid drug, comparing this shift with that observed by the interaction of increasing molar fractions of Diflunisal with the DPPC liposomes, reported as calibration curve in Figure 4.

A 0.18 molar fraction of drug (free or dispersed in PLGA microspheres) was chosen to follow the release. Then the maximum amount of drug to be transferred from the matrix to liposomes should cause an effect similar to that observed for the same X_{Drug} of free Diflunisal dispersed in DPPC liposomes and visualized in Figure 5 (curve d).

Release process at 25°C, 37°C, and 50°C are reported, respectively, in Figures 5, 6, and 7. By comparing the results shown in these figures it appears evident, as the sample loaded with the highest amount of drug, (batch 2), release the drug faster (curves b) with respect to the lowest loaded sample (batch 1, curves a). This pertains even if for both the samples the amounts released were every time lower than the pure DFN when this was left in contact with the DPPC vesicles. The release process also is strongly affected by the incubation temperature. In fact, at 50°C the release was faster than the other two temperatures (the release profile at 25°C is similar to that occurring at 37°C). This behavior is justified in the increased solubility of the drug other than into the membrane model present in a disordered phase that allows the drug to better permeate the lipidic bilayer. After 7 hr both samples reach a maximum in drug released, but only the batch 2 released almost 65% of drug at 50°C with respect to the higher amount releasable. The results obtained suggest that the process of drug diffusion through the polymeric matrix, before polymer erosion takes place, is affected by the amount of drug loaded, as well as by the temperature.

Batch 2 (10%) and to a lesser extent batch 1 (2.5%) are subjected for longer times (more than 7 hr) to polymer degradation and the effect of this phenomenon is reflected in the liposome T_m shift. The presence of free acids released from blank microspheres by polymer degradation should shift DPPC transition temperature to higher values, broadening the calorimetric peak.

DISCUSSION

Experiments carried out on unloaded PLGA microspheres with DPPC liposomes, in our experimental conditions (25, 37, and 50°C), gave no evidence of the presence of free lactic or glycolic acid, at least during the first 7 hr of the kinetic experiments (data not reported). By comparing these release profiles with those showed for the pure free drug, it appears evident that the release process is delayed, confirming that the polymeric system can constitute a good delivery system for all drugs possessing high activity.

The results represent the release kinetics of DFN from PLGA microspheres obtained by comparing the T_m depression of the pure phospholipids vesicles with the correlation curve a (Figure 4) ($\Delta T/T_m^\circ$ vs. molar fraction of the drug dispersed in the lipidic aqueous dispersion). If the values obtained are recalculated, by considering the curve b in (Figure 4) where the $\Delta T/T_m^\circ$ is reported, taking into account the partition of the drug between lipidic and aqueous phases as a function of the real amount of drug that reaches the target (biomembranes) surface, the kinetics are transformed to uptake processes such as an in vivo model. This represents the "innovative" aspect of this calorimetric investigation of a release process.

CONCLUSIONS

The release of Diflunisal from PLGA microspheres is by itself ruled by the diffusion process before that the erosion starts at least in large extent. The presence of higher amounts of drug loaded into microspheres seems to favour solvent penetration, drug dissolution rate and transfer to model membranes. The similar effect, increasing the release rate, is observed by increasing the temperature of the examined release process, but only when this overlaps the membrane phase transitional temperature, favouring the drug solubility and membrane permeability. SEM analysis shows no crystals on microspheres surface, so the drug seems to be dispersed inside the polymeric network. The information on the drug dispersion inside the microspheres obtained by DSC characterisation of polymer and drug loaded microspheres support the idea that the thermal behaviour is not modulated by percentage of drug present into polymer matrix.

These data show that high pay loads of the drug in the microspheres are not differently distributed into the matricial system with respect to low pay loads.

The different kinetics, for each temperature examined, obtained from the calorimetric experiments (slower for lowest loaded microspheres and faster for the highest), could be explained by a change in the matrices structure caused by the drug differently dispersed inside. A net should be constituted inside the microspheres where a drug should be dispersed as microcrystals or amorphous aggregates interconnected with each other. This net should constitute the preferential way for dissolution and diffusion of drugs through the polymeric matrix. In fact, when water dissolves a crystal makes a channel of water into the matrix and the consequent net of microcrystals (and aqueous porous) may favour the release process (Rosilio et al. 1991; LeCorre et al. 1994; Whateley 1993; O'Hagan et al. 1994).

It is worthwhile to highlight how this calorimetric technique can be applied to follow such slow kinetics directly at the site of the drug uptake instead of the usual "in vitro" determination. This technique is especially suitable to evaluate release processes that develop by diffusion mechanisms of the drug through the polymeric matrices.

The reported method represents a new approach compared to usual "in vitro" experiments, and it highlights how molecular weight, storage time and polymeric structure influence the release kinetics.

REFERENCES

Bach, D. 1984. Calorimetric studies of model and natural biomembranes. In *Biomembrane Structure and Function*, ed. D. Chapman, 1–41. London: MacMillan Press.

Bartlett, G. R. 1959. Phosphorous assay in column chromatography. *J. Biol. Chem.* 234:466–468.

Bodmeier, R., Oh, K. H., and Chen, H. 1989. The effect of addition of low molecular poly(DL-lactide) on drug release from biodegradable poly(DL-lactide) drug delivery system. *Int. J. Pharm.* 51:1–8.

Bonina, F. P., Castelli, F., Montenegro, L., and Mazzone, G. 1994. Partitioning and differential scanning calorimetry studies of N-alkyllactame ester dermal prodrugs of Indomethacin. *Int. J. Pharm.* 106:115–123.

Castelli, F., Puglisi, G., Pignatello, R., and Gurrieri, S. 1989. Calorimetric studies of the interaction of 4-biphenylacetic Acid and its β-cyclodextrin inclusion compound with lipid model membrane. *Int. J. Pharm.* 52:115–121.

Castelli, F., Puglisi, G., Giammona, G., and Ventura, C. A. 1992. Effect of the complexation of some nonsteroidal anti-inflammatory drugs with β-cyclodextrin on the interaction with phosphatidylcholine liposomes. *Int. J. Pharm.* 88:1–8.

Castelli, F., Conti, B., Puglisi, G., Conte, U., and Mazzone, G. 1994. Calorimetric studies on Tolmetin release from poly-DL-lactide microspheres to lipid model membrane. *Int. J. Pharm.* 103:217–223.

Castelli, F., Conti, B., Conte, U., and Puglisi, G. 1996. Effect of molecular weight and storage times on Tolmetin release from poly-D,L-lactide microspheres to lipid model membrane. A calorimetric study. *J. Controlled Release* 40:277–284.

Castelli, F., Pitarresi, G., Tomarchio, V., and Giammona, G. 1997a. pH effect on antiinflammatory release from polyaspartamide hydrogel to lipid model membrane. *J. Controlled Release* 45:103–111.

Castelli, F., Conti, B., Maccarrone, D. E., La Camera, O., and Conte, U. 1997b. Indomethacin-Dipalmitoylphosphatidylcholin e interaction. A calorimetric study on the drug release from poly(lactide-co-glycolide) microspheres to multilamellar vesicles. *Drug Del.* 4:273–279.

Castelli, F., La Camera, O., Pitarresi, G., and Giammona, G. 1998a. Temperature and polymer crosslinking degree influence on drug transfer from α, β, polyaspartahydrazide hydrogel to model membranes. A calorimetric study. *Int. J. Pharm.* 174:81–90.

Castelli, F., Conti, B., Maccarrone, D. E., Conte, U., and Puglisi, G. 1998b. Comparative study of "in vitro" release of antiinflammatory drugs from polylactide-co-glycolide microspheres. *Int. J. Pharm.* 176:85–98.

Cotton, M. L., and Hux, R. A. 1985. Diflunisal. In *Analytical Profiles of Drug Substances*, ed. K. Florey, 14:491–526. London: Academic Press.

Dubernet, C. 1995. Thermoanalysis of microspheres. *Thermochim. Acta* 248:259–269.

Estep, T. N., Mountcastle, D. B., Biltonen, R. L., and Thompson, T. E. 1978. Studies on the anomalous thermotropic behaviour of aqueous dispersions of dipalmitoylphosphatidylcholine-cholestero l mixtures. *Biochemistry* 17:1984–1989.

Giunchedi, P., and Conte, U. 1995. Spray-drying as a preparation method of microparticulate drug delivery systems: an overview, *S.T.P. Pharma Sciences* 5:276–290.

Giunchedi, P., Conti, B., Scalia, S., and Conte, U. 1998. In vitro degradation study of polyester microspheres by a new HPLC method for monomer release determination, *J. Controlled Release* 56:53–62.

Guggenheim, E. A. 1952. *Thermodynamics*. Amsterdam: North-Holland.

Hausberger, A. G., and De Luca, P. P. 1995. Characterisation of biodegradable poly(D,L-lactide-co-glycolide) polymers and microspheres. *J. Pharm. Biom. Anal.* 13:747–760.

Hwang, S. B., and Shen, T. Y. 1981. Membrane effects of antiinflammatory agents 2. Interaction of nonsteroidal antiinflammatory drugs with liposomes and purple membranes. *J. Med. Chem.* 24:1202–1211.

Inoue, T., Fukushima, K., and Shimozawa, R. 1990. Surfactant partition between bulk water and DPPC vesicle membrane: solid-gel vs. liquid-crystalline membrane. *Chem. Phys. Lipids* 52:157–161.

Izumikawa, S., Yoshioka, S., Aso, Y., and Takeda, Y. 1991. Preparation of poly(L-lactide) microspheres of different morphology and effect of crystalline morphology on drug release rate. *J. Controlled Release* 15:133–140.

Jain, M. K. 1988. Order and dynamics in bilayers and solute in bilayers. In *Introduction to Biological Membranes*, ed. M. K. Jain, 122–165 and references cited herein. New York: Wiley.

Jorgensen, K., Ipsen, J. H., Mouritsen, O. G., Bennet, D., and Zuckermann, M. 1991. The effects of density fluctuations on the partitioning of foreign molecules into bilayers: Application to anaesthetics and insecticides. *Biochim. Biophys. Acta* 1067:241–253.

Kamijo, A., Kamei, S., Saiikawa, A., Igari, Y., and Ogawa, Y., 1996. In vitro release test system of (D,L-lactic-glycolic) acid copolymer microcapsules for sustained release of LHRH agonist (leuprorelin). *J. Controlled Release* 40:269–276.

Le Corre, P., Le Guevello, P., Gajan, V., Chevanne, F., and Le Verge, R. 1994. Preparation and characterisation of bupivacaine-loade d polylactide and polylactide-co-glycolide microspheres. *Int. J. Pharm.* 107:41–49.

Lasonder, E., and Weringa, W. E. 1990. An NMR and DSC study of the interaction of phospholipid vesicles with some anti-inflammatory agents. *J. Coll. Interf. Science* 139:469–478.

Lee, A. G. 1977. Lipid phase transitions and phase diagrams. II. Mixtures involving lipids. *Biochim. Biophys. Acta* 472:285–344.

Lewis, D. H. 1990. Controlled release of bioactive agents from lactide/glycolide polymers. In *Biodegradable Polymers as Drug Delivery Systems*, eds. M. Chasin and R. Langer, 5. New York: Marcel Dekker.

MacDonald, R. C., MacDonald, R. I., Menco, B.Ph.M., Takeshita, K., Subbarao, N. K., and Hu, L. 1991. Small-volume extrusion apparatus for preparation of large, unilamellar vesicles. *Biochim. Biophys. Acta* 1061:297–303.

McGee, J. P., Davis, S. S., and O'Hagan, D. T. 1994. The immunogenicity of a model protein entrapped in poly(lactide-co-glycolide) microparticles prepared by a novel phase separation technique. *J. Controlled Release* 31:55–60.

O'Hagan, D. T., Jeffery, H., and Davis, S. S. 1994. The preparation and characterisation of poly-lactide-co-glycolide microparticles: III. Microparticles/polymer degradation rates and in vitro release of a model protein. *Int. J. Pharm.* 103:37–45.

Okada, H., and Toguchi, H. 1995. Biodegradable microspheres in drug delivery. *Crit. Rev. Ther. Drug Carriers Systems* 12:1–99.

Pradhan, R. S., and Vasavada, R. C. 1994. Formulation and in vitro release study on poly (DL-lactide) microspheres containing hydrophilic compounds: glycine homopeptides. *J. Controlled Release* 30:143–154.

Raudino, A., and Castelli, F. 1998. Modeling specific heat transient anomalies during permeation of liposomes by water-soluble substances. *J. Colloid Interf. Sci.* 200:52–58.

Ringsdorf, H., Schlarb, B., and Wenzmer, J. 1988. Molecular architecture of polymeric oriented systems: models for study of organization, surface recognition, and dynamics of biomembranes. *Angew. Chem. Int. Ed. Engl.* 27:113–158.

Rosilio, V., Benoit, J. P., Deyme, M., Thies, C., and Madelmont, G. 1991. A physicochemical study of the morphology of progesterone-loaded microspheres fabricated from poly(D,L-lactide-co-glycolide). *J. Biomed. Mater. Res.* 25:667–682.

Sturtevant, J. M. 1982. A scanning calorimetric study of small molecule-lipid binary mixtures. *Proc. Natl. Acad. Sci. USA* 79:3963–3967.

Suezaki, Y., Tatara, T., Kaminoh, Y., Kamaya, H., and Ueda, I. 1990. A solid-solution theory of anaesthetic interaction with lipid membranes: temperature span of the main phase transition. *Biochim. Biophys. Acta* 1029:143–148.

Taylor, M. G., and Morris, R. M. 1995. Thermal analysis of phase transition behaviour in liposomes. *Thermochim. Acta* 248:289–301.

Whateley, T. L. 1993. Biodegradable microspheres for controlled drug delivery. In *Encapsulation and Controlled Release*, ed. D. R. Karsa and R. A. Stephenson, 52–57, Cambridge: The Royal Society of Chemistry.

Wise, D. L., Fellman, T. D., Sanderson, J. E., and Wentworth, R. L. 1979. Lactide/glycolide polymers. Descriptions of the polymers used as surgical suture material, raw material for osteosynthesis and in sustained release forms of drugs. In *Drug Carriers in Medicine*, ed. G. Gregoriadis, 237–270. London: Academic Press.