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Preparation and Characterization of 5-Fluorouracil-loaded Microparticles as Biodegradable Anticancer Drug Carriers

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Abstract

To provide a device releasing 5-fluorouracil in a controlled manner and injectable into the brain by stereotaxy, biodegradable poly ((±)-lactide-co-glycolide) (PLAGA) microparticles were prepared by an emulsion-extraction process.

Although the solubility profile of the drug was not suitable for its encapsulation by the aforementioned method, careful choice of process variables allowed significant drug loading, reaching 30%. Thus, the size of the 5-fluorouracil crystals, the organic phase/aqueous phase ratio, the theoretical drug loading and the microparticle size played a predominant role. The microsphere size was adjusted to 20–40 μm by selecting the appropriate PLAGA and polyvinylalcohol concentrations, and the stirring rate of the initial emulsion.

It was shown that the microparticle structure depended directly on the experimental conditions governing the precipitation rate of the coating material: two types of microparticles, I and II, were characterized. The morphology of the particles influenced the 5-fluorouracil-release patterns, as did other process parameters, such as the 5-fluorouracil crystal size and the PLAGA concentration.

It was possible to sustain the 5-fluorouracil release over 18 days.

Malignant glial brain tumours develop rapidly and are invariably fatal. Patients with the most common human malignant glioma, glioblastoma, have a median survival time from the day of surgery of 4 to 7·5 months and a 90% mortality rate within two years (Ammirati et al 1987).

5-Fluorouracil is a highly water-soluble antimetabolite used in cancer therapy (Bécouarn et al 1989). Unfortunately, due to its hydrophilic nature, it does not readily cross the blood-brain barrier (Neuwelt et al 1983) and is consequently of little interest in the treatment of brain tumours when given systemically. Promising results have been reported by using intracerebral-implanted BCNU-loaded wafers both in animal models and in clinical studies (Brem et al 1991, 1993). Previous work has shown that blank poly ((±)-lactide-co-glycolide) (PLAGA) microspheres implanted in rat brains are biocompatible and degrade totally within two months (Menei et al 1993). The local administration of biodegradable microparticulate 5-fluorouracil delivery systems made from PLAGA therefore appears feasible, allowing sustained high local drug concentrations at the site of a resected brain tumour and adjacent tissues; this would also prevent significant systemic toxicity. This strategy would be consistent with the short biological half-life of 5-fluorouracil (ca 10 min, Diasio & Harris (1989)). The present study was carried out to show how various process parameters affect the preparation and properties of 5-fluorouracil-loaded PLAGA microspheres, which may be implanted stereotactically in the brain, to treat cerebral tumours.

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Materials and Methods

Materials

The poly ((±)-lactide-co-glycolide) designated PLAGA (25%(−)-lactic units, 25%(+)-lactic units and 50% glycolic units) was supplied by B. I. Chimie (Resomer RG 506, Le Vésinet, France). Size exclusion chromatography in chloroform (1%) indicated mean molecular weights of 116·5 ($\overline{Mw}$) and 58·5 kDa ($\overline{Mn}$), giving a polydispersity index of 1·99 ($\overline{Mw}/\overline{Mn}$).

5-Fluorouracil was donated by Roche laboratories (Neuilly-sur-Seine, France) as a crystalline powder. The mean size of the particles as determined by a laser size analyser (Coulter LS, Coultronics, Margency, France) was 247 ± 95 μm. The melting point and heat of fusion determined by differential scanning calorimetry (DSC 30, Mettler-Toledo, Viroflay, France) were respectively 282°C and 220 J g^{-1}. Solubility in water and in dimethylsulphoxide was 12·2 and 112·6 mg mL^{-1}, respectively.

The 88% hydrolysed polyvinylalcohol (PVA), Rhodoviol 4/125, was obtained from Prolabo (Paris, France).

Methylene chloride, acetone and dimethylsulphoxide were used without further purification.

5-Fluorouracil grinding and size distribution analysis

Three methods of grinding were used.

5-Fluorouracil powder was ground in a ball mill (KU 1, Erweka, Apparatebau Gmbh, Heusenstamm, Germany) for 28 or 32 h. The powder was resuspended in acetone, and the organic solvent was removed by evaporation at 50°C. The resulting powders were designated RG28 and RG32 5-fluorouracil.

5-Fluorouracil obtained by freeze-drying an aqueous solution (Freeze-dryer RP2V, Sérail, Argenteuil, France)

was ground using a ball mill for 1, 15 or 30 h. The 5-fluorouracil was resuspended in acetone which was removed by evaporation (LG1, LG15 and LG30 5-fluorouracil).

A further sample of freeze-dried 5-fluorouracil was suspended in 150 mL acetonitrile and ground using a high pressure homogenizer (ALM 2, Guérin, Mauzé, France) for 20 min, at 160 bar. The powder was obtained after solvent evaporation (LH 5-fluorouracil).

The micrometric analyses were performed with a laser size analyser (Coulter LS) on a suspension of 100–300 mg powder sonicated for 5 min in Fréon 113 (Branson Sonifier 250D, OSI, Paris, France).

Microsphere preparation

Microspheres were produced by an emulsion-extraction process. Such a microencapsulation technique was based on the formation of an oil/water emulsion under controlled stirring. A solution of 3 mL PLAGA in methylene chloride/acetone (8·3% w/v, 80/20 v/v), in which 5-fluorouracil crystals were suspended, was poured into a polyvinylalcohol aqueous phase (10% w/v). A stable emulsion was obtained by mechanical stirring (800 rev min^{-1}) at room temperature (21°C); after a given time, the emulsion was added to 500 mL de-ionized water. The resulting solvent extraction allowed the formation of microspheres which were collected by filtration, washed with de-ionized water, suspended in water, frozen with liquid nitrogen and freeze-dried.

Five protocols were used for which the organic phase/polyvinylalcohol aqueous phase ratio and the emulsion-extraction time varied. The theoretical drug content within the microspheres was 50% unless otherwise stated.

Microsphere characterization

5-Fluorouracil content. Ten milligrams of 5-fluorouracil-loaded microspheres was dissolved in 50 mL dimethylsulphoxide. Samples were agitated at room temperature and the antineoplastic agent was analysed by UV spectrophotometry at 266 nm (Uvikon 930, Kontron Instrument, Montigny-le-Bretonneux, France). Results were based on triplicate determinations and expressed in terms of the weight of 5-fluorouracil per weight of microspheres.

Size-distribution analysis. Microsphere sizes were determined using a Coulter counter (Multisizer, Coultronics, Margency, France). Microparticles (20 mg) were suspended by sonication for 5 min in an aqueous solution of Tween 80 (0·02% w/v) and assayed after dilution in Isoton II (Coultronics, Margency, France).

Microscopy studies. Optical microscopy was performed using an Olympus BH2 microscope (OSI, Paris, France). The external surface and the internal structure of microsphere samples were viewed by scanning electron microscopy (Service Commun de Microscopie Electronique, Université d'Angers) (SEM) (Jeol JSM 35 C, Jeol, Paris, France) after a layer of gold was deposited by evaporation (Ion Sputter JFC 1100, Jeol, Paris, France). Microspheres were embedded in epoxy resin (Epon 812, Fluka, Saint Quentin Fallavier, France). Cross-sections were obtained using an ultramicrotome (Reichert Ultracut E, Reichert-Jung, Austria).

5-Fluorouracil dissolution and in-vitro release studies

5-Fluorouracil powder or microsphere samples (50 mg) were placed into dialysis bags (molecular weight cut-off 6–8 kDa; Polylabo, Strasbourg, France), then placed into glass vials containing 500 mL 0·1 M phosphate buffer (pH 7·4) and shaken in a USP dissolution apparatus (100 rev min^{-1}, 37°C (Sotax AT7, OSI, Paris, France). The apparatus was protected from light. Supernatant was sampled over time and 5-fluorouracil concentrations were measured by UV spectrophotometry at 266 nm. Each in-vitro release study was performed in triplicate.

Results

Optimization of the core loading

Effect of the size of the 5-fluorouracil crystals. In its raw state, the drug has a mean size of 247 ± 95 μm, incompatible with the formation of 20–40 μm microspheres. In a classical emulsion-extraction process, when the active ingredient is not soluble in the dispersed phase, the use of a comminuted drug is a prerequisite to assuring adequate distribution of the particles within the matrix, ensuring significant loadings. Pulverization techniques were therefore used to divide the active ingredient finely. The results are reported in Table 1.

If the 5-fluorouracil crystals were too large (raw powder), they could not be correctly incorporated into the polymeric matrix which was reflected by very low core-loading; the

Table 1. Effect of different formulation parameters on the core loading of microspheres (crystal size, emulsion-extraction time (E- E), organic phase/aqueous phase ratio).

State of the drug	Crystal average size (μm)	Core-loading % ± s.d.		
		Phase ratio 1:33		Phase ratio 1:57
		E-E time 8,8 min	E-E time 2,2 min	E-E time 2,2 min
Lyophilized, homogenized	19 ± 13	15 ± 2·08		
Lyophilized, ground 1 h	13 ± 8·4	9·3 ± 0·03	13·7 ± 1·02	
Lyophilized, ground 15 h	9·8 ± 7	9·7 ± 1·21	12·5 ± 0·69	
Lyophilized, ground 30 h	6·6 ± 4·4	5·2 ± 0·55	7·5 ± 0·75	
Raw, ground 28 h	11 ± 9	17 ± 0·15	21·9 ± 0·80	30 ± 1·23
Raw, ground 32 h	6·5 ± 4·4	11 ± 0·89	15 ± 1·19	21·5 ± 0·66

Microsphere size, 27 ± 10 μm.

Table 2. Comparison of the theoretical and actual drug loadings for microspheres prepared using an organic phase/aqueous phase ratio of 1:57, an emulsion-extraction time of 2,2 min and RG32 5-fluorouracil.

Theoretical drug content (%)	27·5	33	50	60
Actual drug content (%)	14·6 ± 0·49	20·8 ± 0·91	20·6 ± 0·95	23·9 ± 0·74

5-fluorouracil crystal size needed to be below 20 μm to obtain significant loading. However, discrepancies could be noted and drug loading was seen to decrease with decreasing crystal size in this size range. When the drug pulverization was processed through freeze-drying, the core-loading fell significantly. Freeze-drying modifies the surface energy of drug crystals, thus favouring their dissolution in water. This might explain the observed reduction in entrapment yield, due to the dissolution of 5-fluorouracil crystals when the microparticles are in prolonged contact with water (Table 1).

Effect of the emulsion-extraction time. The effect of the emulsion-extraction time on the final drug content is shown in Table 1. Decreasing the microparticle production time from 16 to 4 min improved the entrapment, for a given size distribution and physical state of the drug. The microparticles were prepared according to an organic phase/aqueous phase ratio of 1:33 and an emulsion-extraction time of 8,8 or 2,2 min.

Effect of the organic phase/aqueous phase ratio. The following study was conducted with RG28 and RG32 5-fluorouracil. A reduction of the organic phase/aqueous phase was obtained by increasing the aqueous phase volume in the emulsion step. The microparticles were prepared using an emulsion-extraction time of 2,2 min. Increasing the volume of the external aqueous phase led to an increase in 5-fluorouracil content (Table 1).

In the next two series of experiments, the selected operating conditions were among the most favourable in terms of drug content: phase ratio 1:57, emulsion-extraction time 2,2 min and RG32 5-fluorouracil.

Comparison of the theoretical and actual drug loadings. The comparison of the theoretical and actual drug loadings is shown in Table 2. When the theoretical drug content varied from 27·5 to 60%, the actual drug content increased concomitantly with the theoretical drug loading between 27·5 and 33% for microspheres having a mean diameter of 25 μm; the encapsulation efficiency (actual drug content/theoretical drug content ratio) subsequently decreased showing that the entrapment process within the microspheres is saturated.

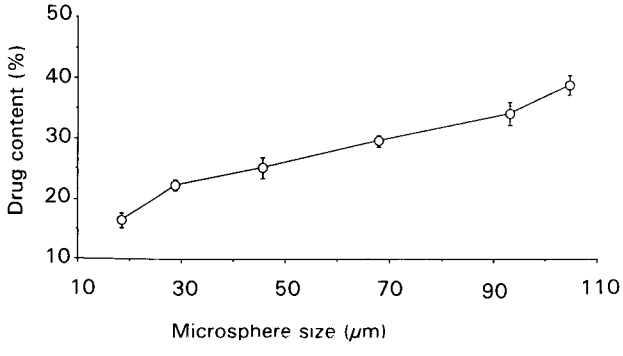

FIG. 1. Influence of the microparticle size on actual drug content for a phase ratio of 1:57, emulsion-extraction time of 2,2 min and RG32 5-fluorouracil.

Effect of the microsphere size. The particle mean diameter was increased by modifying the PLAGA and PVA concentrations in both organic and aqueous phases. The actual drug content approached the theoretical value, 50%, for microspheres having a mean diameter above 100 μm (Fig. 1). A linear relationship between the actual drug content and the microsphere size could be observed.

Adjustment of the microsphere size

This study was performed to obtain microparticles with a size ranging from 20 to 40 μm, essential for injection into the rat brain. The microspheres were prepared according to a phase ratio of 1:57 and an emulsion-extraction time of 2,2 min with RG32 5-fluorouracil.

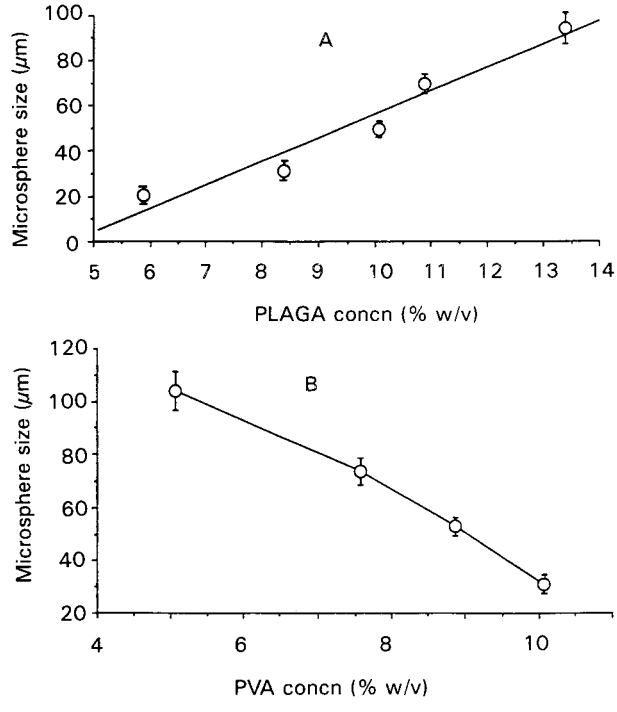

FIG. 2. Effect of polymer concentrations on the 5-fluorouracil microparticle size (phase ratio 1:57, emulsion-extraction time 2,2 min, RG32 5-fluorouracil). A. PLAGA concentration (respectively 5·8; 8·3; 10·0; 10·8; 13·3% (w/v)) in the organic phase. B. Polyvinylalcohol concentration (respectively 10; 8·8; 7·5; 5% (w/v)) in the external aqueous phase.

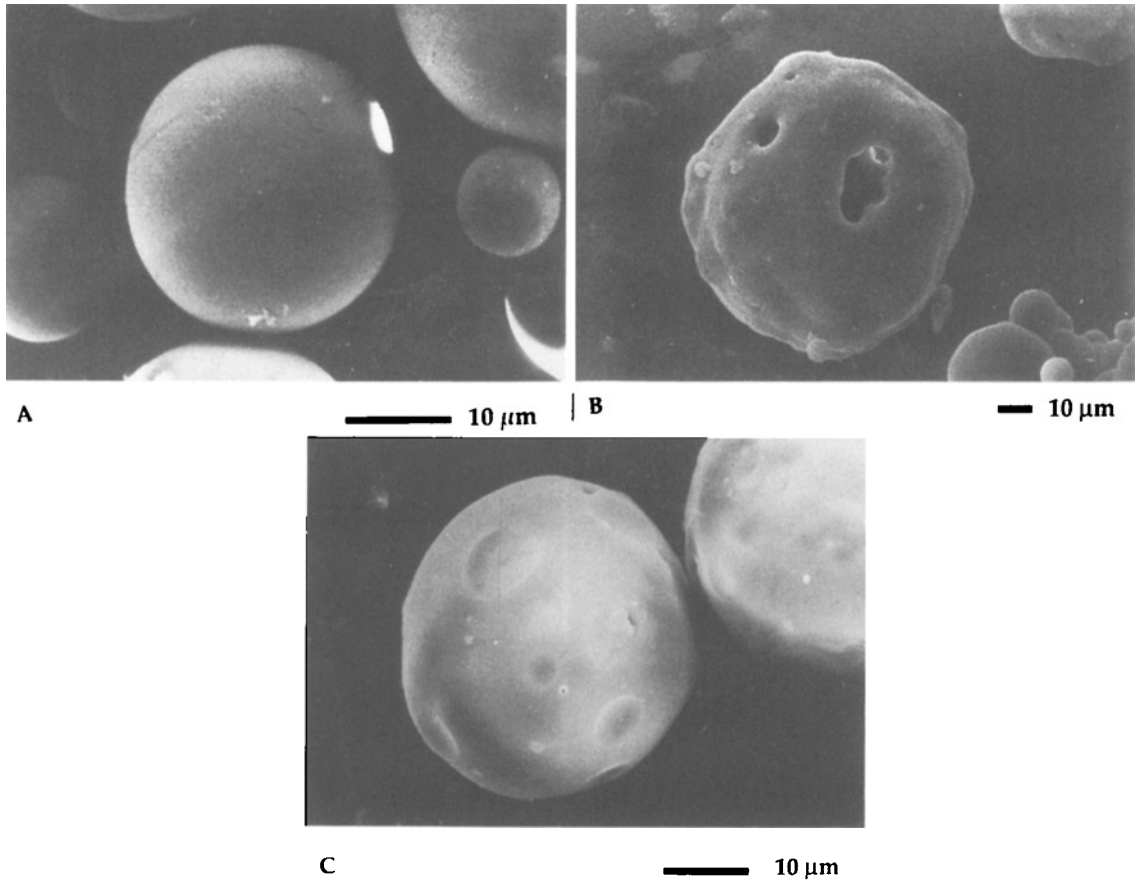

FIG. 3. Scanning electron microscopy of microparticles prepared using the following conditions: A, phase ratio 1:33 and emulsion-extraction time 8,8 min, blank microspheres; B, phase ratio 1:33, emulsion-extraction time 8,8 min and LH 5-fluorouracil; C, phase ratio 1:33, emulsion-extraction time 2,2 min and RG28 5-fluorouracil.

Effect of the PLAGA concentration in the organic phase. The effect of the coating polymer concentration on the microsphere size is shown in Fig. 2. A modification of the polymer concentration in the range 5·8–13·3% allowed an increase on the loaded microsphere size from 18 ± 7 to $93 \pm 42\,\mu$m. A quasi-linear relationship between the two parameters was observed.

Effect of the PVA concentration in the aqueous phase. Decreasing the PVA concentration in the continuous phase from 10 to 5% (w/v) significantly increased the microsphere size from 20 ± 9 to $104 \pm 42\,\mu$m. Below 5%, microsphere formation was extremely difficult.

The effect of the stirring rate was studied in parallel. As would be predicted, the microparticle size decreased when the stirring rate of the emulsion increased (data not shown).

Morphological studies of microparticles

Microparticle external structure. Blank microspheres appeared perfectly spherical with a smooth surface, as viewed by SEM (Fig. 3A). When 5-fluorouracil was incorporated, their aspect changed as a function of the pulverization mode of the starting powder. Microencapsulation of LH 5-fluorouracil led to misshapen microspheres with visible pores (Fig. 3B). These pores might be attributed to the presence of 5-fluorouracil crystals on the surface which were dissolved at the end of the manufacturing process and during the washing step. With methods of grinding leading to smaller drug particles ($11 \pm 9\,\mu$m), the surface aspect was almost unaltered by the presence of the drug (Fig. 3C).

Microparticle internal structure. Using SEM, the cross-sections of blank microparticles appeared dense and homogeneous. When 5-fluorouracil particles with a mean diameter of 11 μm were encapsulated, optical microscopic studies with polarized light showed that the microparticle internal structure varied according to the method of preparation. Using a phase ratio of 1:33 and an emulsion-extraction time of 8,8 min, most of the smaller microparticles were empty and in most of the larger particles, the 5-fluorouracil crystals disappeared from the periphery and tended to gather in the centre of each reservoir (Fig. 4A). By decreasing the preparation time to 4 min (2 min emulsion and 2 min extraction), the drug particle distribution was more regular in the larger particles. When the organic phase/aqueous phase ratio was decreased to 1:57, all particles were uniformly loaded irrespective of the preparation time (Fig. 4B). The results of 5-fluorouracil distributions were confirmed by SEM observations of cross-sections immersed for 2 h in water to dissolve the 5-fluorouracil crystalline domains. When the organic phase/aqueous phase ratio was 1:33 and the total preparation time was 16 min, larger cavities

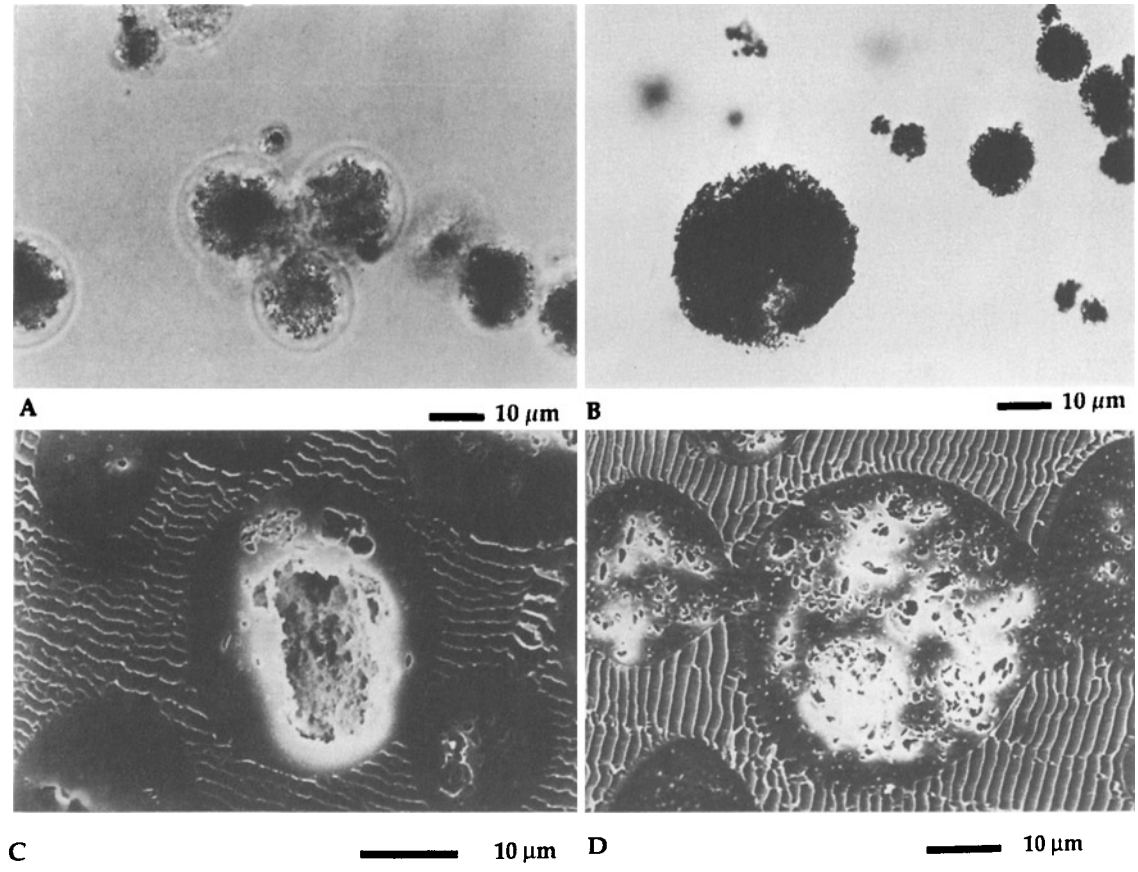

FIG. 4. Optical microscopy with polarized light of microparticles prepared with RG28 5-fluorouracil according to: A, phase ratio 1:33 and an emulsion-extraction time 8,8 min; B, phase ratio 1:57, emulsion-extraction time 2,2 min. Cross-sections (SEM) of microparticles after immersion for 2 h in water are also shown: C, phase ratio 1:33 and an emulsion-extraction time 8,8 min; D, phase ratio 1:57, emulsion-extraction time 2,2 min.

appeared within the particles (Fig. 4C). In contrast, a more even distribution of cavities was observed for shorter preparation times. For a ratio of 1:57, the preparation time did not affect the internal morphology of the loaded microspheres. In each case, a uniform dispersion of cavities left by 5-fluorouracil crystals in the polymeric matrix was obtained (Fig. 4D).

In-vitro release study

The dissolution profiles of three types of 5-fluorouracil are shown in Fig. 5. LG 5-fluorouracil dissolved much more quickly than the two other drug preparations, and was not investigated further.

A number of factors influenced the drug release when 5-fluorouracil was encapsulated, including crystal size. As expected, when the crystal size decreased from 11 (RG28) to 6·5 μm (RG32), the burst effect was more marked (Fig. 5). This result was consistent with the morphology of the microspheres prepared with a phase ratio of 1:57 and an emulsion-extraction time of 2,2 min (PLAGA concentration 10·8%), where the drug stayed in the form of crystalline domains, uniformly dispersed in the polymeric matrix.

The coating material concentration in the dispersed phase of the medium used for preparation also had a strong bearing on the drug release. As shown in Fig. 5, a PLAGA concentration increase favoured a more sustained drug release. For a 5·8% polymer concentration, the majority of encapsulated 5-fluorouracil was liberated within 48 h, whereas, for a 10·8% concentration, total release of the drug required 18 days. We suggest that an increase in PLAGA concentration in the organic phase led to a decrease in the microsphere porosity as suggested by other studies. In all cases, the microsphere size was 29 ± 10 μm.

The organic phase/aqueous phase ratio also contributed to the modulation of the release rate of 5-fluorouracil. Increasing the ratio from 1:61 to 1:33 slowed the release of the drug (Fig. 6).

These experiments were carried out with RG32 5-fluorouracil. As previously seen in the morphology studies, the phase ratio determined the crystal distribution in the polymeric matrix which, in turn, influenced the release kinetics. The drug content could to some extent influence the 5-fluorouracil release pattern when the microparticles were prepared with a phase ratio of 1:33 and a total preparation time of 16 or 4 min. The burst effect became more marked when the encapsulation ratio rose from 15 to 22% (Fig. 7A). On the other hand, no modification of the release profiles accompanied a drug content increase for microparticles prepared with a phase ratio of 1:57 and a total preparation time of 16 or 4 min (Fig. 7B).

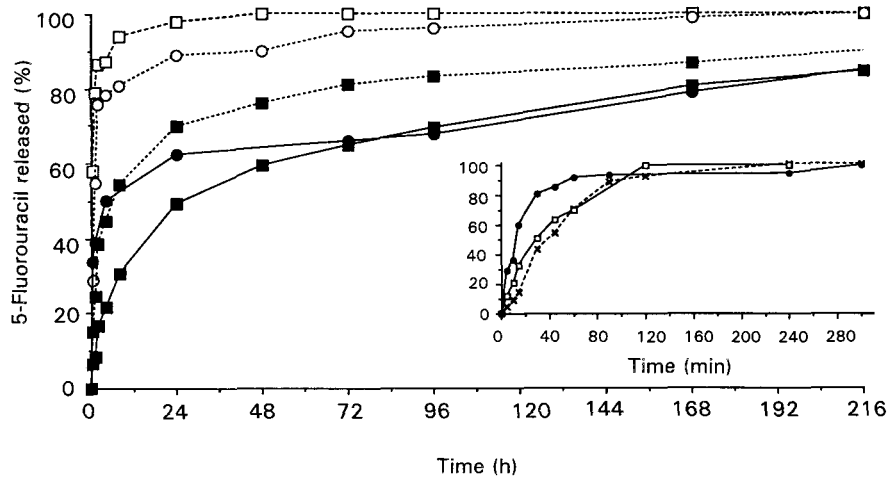

FIG. 5. Effect of the crystal size (-■- RG28, -●- RG32) with a PLAGA concentration of 10·8% in the organic phase and effect of the PLAGA concentration (– –□– – 5·8, – –○– – 8·3 and – –■– – 10·8%) in the dispersed phase with RG28 5-fluorouracil on drug release for microparticles prepared using a phase ratio of 1:57 and an emulsion-extraction time of 2,2 min. Inset: dissolution profiles of three types of 5-fluorouracil: raw powder (×), RG32 (□) and LG1 (●) 5-fluorouracil.

Discussion

5-Fluorouracil is a water-soluble drug and is therefore not a good candidate for encapsulation by the classical emulsion-extraction process using an aqueous phase as the dispersing phase (Thies 1992). Nevertheless, by adjusting the process variables and using a finely ground drug powder, it was possible to reach an encapsulation ratio of 30%. Factors favouring the PLAGA precipitation in the dispersed phase such as the emulsion-extraction time, the organic phase/aqueous phase ratio, the use of acetone as co-solvent of methylene chloride, enhanced the microencapsulation yield, thus preventing drug diffusion across the phase boundary as shown in other studies (Bodmeier & McGinity 1988).

It was previously reported that, when the drug was not soluble in the dispersed phase, process parameters could govern different drug crystal distributions in the microspheres (Spenlehauer et al 1988). In this work, the morphology study revealed that the ratio of organic and aqueous phases and the preparation times could modify the drug distribution. Experimental conditions which quenched the system led to microsphere-type structures where the crystals were homogeneously distributed in the polymeric mass (Type I). By contrast, when the dispersed phase remained sufficiently fluid for a longer time, crystals tended to gather in the central regions of dispersed droplets; subsequently, a microcapsule-type structure was obtained (Type II). In this case, it must be noted that a few drug crystals remained embedded in the polymeric membrane of the system. Fig. 8 illustrates the two different structures obtained by varying the microencapsulation parameters.

The characterization of these morphologies allowed a better understanding of the release profiles of 5-fluorouracil from differently loaded microparticle batches. For the highest phase ratio (1:33), the burst effect obtained could be attributed to the presence of drug crystals spread over the

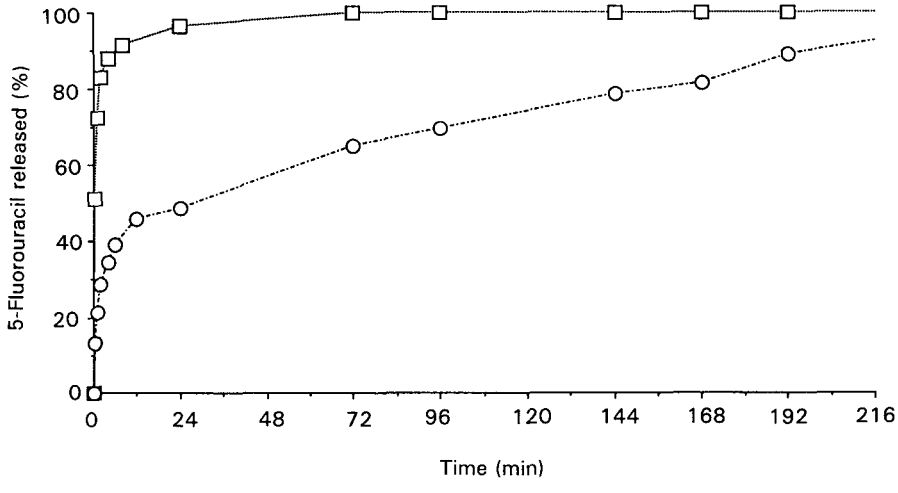

FIG. 6. Effect of the phase ratio (□ 1:61, ○ 1:33) on drug release for microparticles prepared using an emulsion-extraction time of 2,2 min and RG32 5-fluorouracil (19 and 16%).

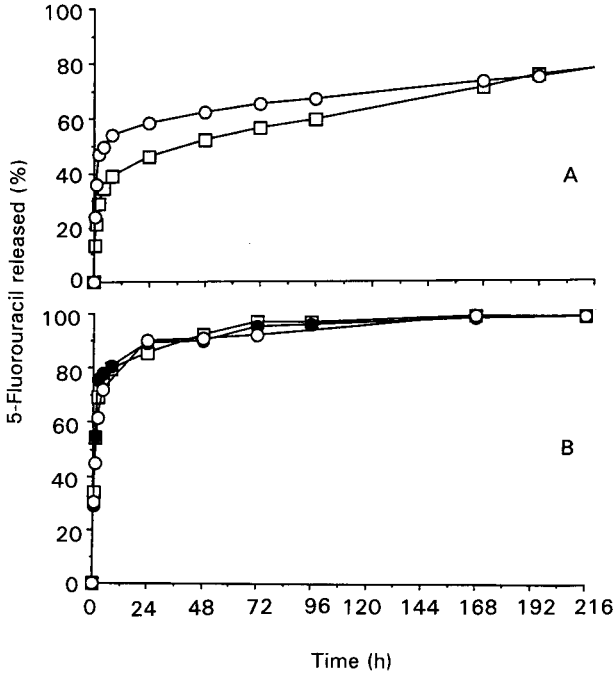

FIG. 7. Influence of the encapsulation rate on drug release: A, microparticles prepared using a phase ratio of 1:33 and respectively a total preparation time of 16 and 4 min (□ 15, ○ 22%); B, microparticles prepared using a ratio of 1:57 and respectively a total preparation time of 16 (□ 20·9%) and 4 min (● 25 and ○ 30%) with RG28 5-fluorouracil.

periphery of the particles, representing half of the carrier content. The other half was released progressively over 18 days, due to the presence of a diffusion barrier slowing the passage of the drug towards the external medium. For the lower phase ratios (1:57, 1:61), the crystals were more evenly distributed in the polymeric matrix. Microsphere-like microparticles resulted from repartition of the drug. The fast release obtained (90% in 48 h) led us to believe that a percolation phenomenon occurred (Deyme et al 1992). This was confirmed by the finding that the drug release was influenced by the encapsulation ratio. For high phase ratio, a difference in the intensity of the burst effect could be noted when the drug content rose; the remaining amount of encapsulated drug then diffused according to similar patterns, supporting the presence of a PLAGA membrane regulating the 5-fluorouracil flux. At lower ratios and with high drug content, the encapsulation ratio did not seem to affect the drug release, which was consistent with the assumed percolation mechanism (Spenlehauer et al 1988). One could argue that differences in release patterns were simply due to differences in drug content. The encapsulation ratio, the internal morphology and the release profile were, to some extent inter-related. For instance, it was not possible to exceed 22% with a phase ratio of 1:33 and an emulsion-extraction time of 2,2 min. However, it was clear that 22 and 20·9%-loaded microparticles prepared according to two different protocols, exhibited two distinct release patterns, which demonstrated the predominant role of the microparticle internal morphology on the 5-fluorouracil

TYPE I TYPE II

FIG. 8. 5-Fluorouracil microparticle structures.

liberation. Finally, the entire formulation study allowed us to obtain a variety of 5-fluorouracil-loaded microparticulate systems made from a biodegradable material, exhibiting different release profiles.

Acknowledgements

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