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In vitro and in vivo evaluation of PLAGA (50/50) microspheres containing 5-fluorouracil prepared by a solvent evaporation method

Kadriye Ciftci[a,*], H. Süheyla Kas[a], A. Atilla Hincal[a], T. Meral Ercan[b], Olgun Güven[c], Sevket Ruacan[d]

[a]*Faculty of Pharmacy, Department of Pharmaceutical Technology, University of Hacettepe, 06100 Ankara, Turkey*
[b]*Faculty of Medicine, Department of Nuclear Medicine, Univeristy of Hacettepe, 06100 Ankara, Turkey*
[c]*Faculty of Chemical Engineer, Department of Chemistry, University of Hacettepe, 06100 Ankara, Turkey*
[d]*Faculty of Medicine, Department of Pathology, University of Hacettepe, 06100 Ankara, Turkey*

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Abstract

Poly (DL-lactide-co-glycolide) PLAGA (50/50) microspheres containing an antineoplastic drug, 5-fluorouracil (5-FU) were prepared by a solvent evaporation process in order to passively target liver carcinomas. The microspheres were spherical with diameters 2–5 μm and encapsulated more than 70% (w/w) of the 5-FU. In vitro release patterns of 5-FU from microspheres were determined for various systems. It was found that drug release depended upon the amount of entrapped drug, the polymer molecular weight and pH of the dissolution medium. The in vitro release mechanism was diffusion controlled and followed a square-root of time relationship. In vivo distribution of ^{99m}Tc labeled microspheres after intravenous injection into mice was characterized by an initially high uptake by organs of the mononuclear phagocyte system (MPS). Following i.v. administration of fluorescein-labeled PLAGA microspheres, accumulation was into the MPS, mainly the Kupffer cells cytoplasm and near the liver sinusoids.

Keywords: 5-fluorouracil; Biodegradable microspheres; In vivo distribution; Poly (DL-lactide-co-glycolide)

1. Introduction

Hepatocellular carcinoma is one of the most common human malignancies and also the most lethal cancer, with a fatality rate higher than 94% (Skolnick, 1994). In the past, chemotherapy has not been effective in targeting non-resectable cancers of the liver. However, novel ways of using chemotherapy to destroy these and some other solid tumors are showing promise in phase I clinical trials (Shepherd et al., 1992). In addition, by obtaining the maximum anticancer effects of anticancer drugs with minimum side effects, it would be desirable that the systemic concentration be kept low but higher in the vicinity of targeted tumors (Gupta, 1990).

* Corresponding author. The University of Michigan Medical School Kresge II, R5014 Ann Arbor, MI 48109-0576. Tel.: +1 313 936-2850; Fax: +1 313 747-3270.

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For this purpose, attempts have been focused on the development of drug delivery systems containing antineoplastic drugs (Skolnick, 1994; Kim et al., 1993; Duncan et al., 1992). Promising results have been reported for cisplatin loaded biodegradable particles in clinical studies (Skolnick, 1994). Cisplatin was found to provide 60- to 100-fold higher levels into the tumor than had previously been recorded by an infusion method. In our study, 5-flourouracil (5-FU) was chosen as a model antineoplastic drug and poly (DL-lactide-co-glycolide) (PLAGA) copolymer as a matrix drug delivery vehicle. Biodegradable polymers such as PLAGA have been extensively studied in controlled release technology with respect to their biodegradability and biocompatability (Jalil, 1990; Kulkarni et al., 1966). These copolymers eventually undergo hydrolytic scission, producing by-products that can be metabolized in the body (Schindler et al., 1977).

Recently, we have described the preparation of biodegradable polyester microspheres containing 5-FU (Ciftci et al., 1992a,b; Ciftci et al., 1994). In this present work, we have evaluated the physicochemical properties of PLAGA microspheres prepared by a solvent evaporation process. Attention has been paid to the effects of various factors on release profiles such as drug loading, pH of the dissolution medium and polymer molecular weight. In addition, we have studied the tissue distribution of ^{99m}Tc or fluorescein labeled PLAGA microspheres after i.v. administration into swiss albino mice.

2. Materials and methods

2.1. Materials

Different molecular weight grades of PLAGA were supplied from different manufacturers; the 74 000 Da weight average molecular weight (Mw) was supplied by Birmingham Polymer (Birmingham, USA) and the 23 100 Da (Mw) was a gift from Sandoz (Basel, Switzerland). 5-FU was purchased from Roche (Basel, Switzerland), methylene chloride, chloroform and fluorescein were from E. Merck (Darmstadt, Germany). Polymeric emulsifier, 99% hydrolyzed polyvinyl alcohol (PVA), was obtained from J.T. Baker Chemical (Phillipsburg, USA), polyoxyethylene sorbitan monooleate (Tween-80) was purchased from Sigma (St. Louis, USA) and technetium (99m-Tc) was supplied by Amersham (Amersham, UK). All other chemicals were reagent grade purity. Swiss albino mice were obtained from the Experimental Animal Division of University of Hacettepe (Ankara, Turkey).

2.2. Methods

2.2.1. Preparation of microspheres

PLAGA microspheres containing 5-FU or fluorescein were prepared by a solvent evaporation method as described previously (Ciftci et al., 1994). Typically, different amounts of 5-FU (100 mg or 175 mg) were dispersed in 10 ml of methylene chloride containing 600 mg of PLAGA copolymer. This organic suspension was then poured into 175 ml of aqueous phase containing 0.2% (w/v) of PVA and continuously stirred (1700 rpm) at room temperature until the methylene chloride had evaporated. The microspheres were collected by filtration, washed several times with distilled water and separated by vacuum filtration. Thereafter, the microspheres were dried in a desiccator for 24 h at room temperature.

2.2.2. Physicochemical characteristics of microspheres

The appearance of the microspheres was observed by optical microscopy and scanning electron microscopy (SEM) (Camebax-Socíeté Camera). The particle sizes of PLAGA microspheres were measured by using a Coulter Counter (Coulter Multisizer-II). The particles were suspended in Isoton-II together with 1% Tween-80 and sonicated before counting. Results are indicated as a mean volume number diameter (d_{vn}) and geometric standard deviation (σ_g).

2.2.3. Determination of 5-FU content of the microspheres

30 mg of 5-FU loaded microspheres were dissolved in 5 ml of methylene chloride to which distilled water (10 ml) was added in order to

Table 1
Characteristics of PLAGA microspheres

Polymer (Mw)[a]	Theoretical 5-FU content (%)	Microspheres recovery (%)	Actual 5-FU content (%)	Encapsulation efficiency (%)	$d_{vn} \pm \sigma_g^b$ (μm)
PLAGA1	9.5	72.9 ± 2.5	6.71 ± 1.5	75.0 ± 2.1	3.79 ± 1.6
(74000 Da)	15.5	77.8 ± 0.5	12.9 ± 1.1	79.3 ± 1.2	4.02 ± 1.1
PLAGA2	9.5	71.8 ± 1.9	5.24 ± 2.3	70.5 ± 1.7	2.97 ± 2.2
(23100 Da)	15.5	75.9 ± 1.8	10.1 ± 3.2	71.9 ± 2.9	3.11 ± 1.2

[a] Weight average molecular weight given by company.
[b] Mean volume-number diameter and geometric standard deviation.
$n = 6$ samples for each experiment.

extract the 5-FU. The 5-FU content in the aqueous layer was then assayed spectrophotometrically at 266.2 nm (UV-160A-Shimadzu).

2.2.4. In vitro release studies

Drug release from PLAGA microspheres was studied under sink conditions in pH 1.2, pH 9 buffered aqueous solutions (USP XXII) and saline. The release medium contained 0.01% (w/v) Tween-80 in order to prevent the microspheres from clumping. Microspheres (30 mg) were suspended in 100 ml release medium in a glass vial placed in a shaker bath at 50 cycles/min and at 37°C. The samples were collected at various time points, filtered through a 0.45 μm filter (Millipore) and assayed by using a UV-spectrophotometer at 266 nm.

2.2.5. Differential scanning calorimetry (DSC)

The glass transition temperatures (Tg) of PLAGA polymers were determined using a Dupont-910 (DSC) system interfaced with a thermal analysis data station computer. Samples (5 mg) were weighed and put into aluminum pans and heated at 10°C/min. The system was calibrated using an indium standard.

2.2.6. In vitro polymer degradation studies

Microspheres (25 mg) of PLAGA containing 5-FU or drug free were suspended in 10 ml saline solution in a glass culture tube placed in a thermostated bath at 37°C. Tubes were removed at 0, 7, 14 and 30 days, microspheres were centrifuged, washed three times with distilled water and air-dried. Microspheres (10 mg) were dissolved in 5

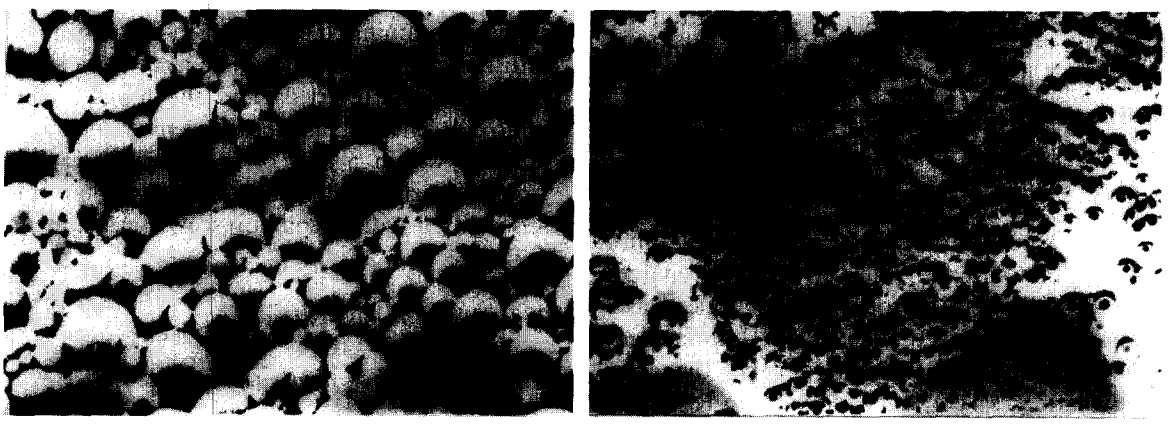

Fig. 1. SEM of PLAGA microspheres. (A) PLAGA1 microspheres (15.5% 5-FU), Bar: 5 μm, (B) PLAGA2 microspheres (15.5% 5-FU), Bar: 10 μm. Magnification: × 2000.

Table 2
Intrinsic viscosity and glass transition temperature data of PLAGA microspheres

Polymer type	5-FU (%)	Tg (°C)[a]		Intrinsic viscosity (dl/g)				Rate ($\times 10^{-3}$)
		I	II	0 day	7 day	14 day	30 day	(day^{-1})
PLAGA1	9.5	43.5	41.5	1.358	1.292	1.249	1.188	5.435
(74000 Da)	15.5	43.5	42.1	1.358	1.293	1.249	1.187	5.434
PLAGA2	9.5	40	39.2	1.121	1.057	1.001	0.930	6.219
(23100 Da)	15.5	40	38.9	1.122	1.056	1.004	0.932	6.220

I: Before preparation.
II: After preparation.
[a] 5-FU melting point 283.5°C.

ml chloroform. Intrinsic viscosity (η_i) was determined by measuring the flow times of solutions of copolymer in chloroform through a Ubbelohde capillary viscometer at 25°C as described in an earlier study (Ciftci et al., 1992b).

2.2.7. Radiolabeling of free 5-FU or microspheres with ^{99m}Tc

Free 5-FU was labeled with 99m-Tc by the tin reduction method (Saha, 1984). Briefly, a weighed amount of 5-FU was dissolved in water and 0.2 ml of a 0.1% w/w aqueous solution of stannous chloride added. The solution was filtered by a membrane filter (Millipore Corporation) and 1 mCi technetium pertecnetate (99m-TcO_4^-) added (Ercan, 1989). PLAGA microspheres containing unlabeled 5-FU were labeled by the above mentioned method. 1.5 mCi 99m-TcO_4^- solution in saline solution was mixed with PLAGA microspheres and 0.1% (w/w) Tween-80 solution was added into this mixture. The suspension was diluted to 6 ml with saline. The pH of this suspension was 4.3 (Ciftci et al., 1994). The labeling efficiencies of 99m-Tc labeled microspheres and free 5-FU were determined by impregnated thin-layer chromatography (ITLC), using prepared plates and methylethylketone as the mobile phase (Ercan, 1989; Ciftci et al., 1992b). The labeled compound stayed at the origin and the free pertechnetate ($^{99m}TcO_4^-$) moved with an R_f of 1.0 (Ercan, 1976). The amount of $^{99m}TcO_4^-$ was less than 2%.

2.2.8. In vivo distribution studies

The in vivo distribution studies of free 5-FU and PLAGA microspheres were carried out as described previously (Ciftci et al., 1992b; Ciftci et al., 1994). For each system, 42 swiss albino mice were injected via the tail vein with the solution of ^{99m}Tc labeled free 5-FU (20 μCi, 0.2 ml) or a suspension of PLAGA microspheres in saline. The mice were sacrificed at predetermined intervals and the liver, lungs, heart, spleen, stomach and kidneys were removed. The radioactivity in the organs was counted using a well-type gamma counter (Berthold, Model 5300) against a standard solution (Ciftci et al., 1994).

2.2.9. Histologic evaluation

PLAGA microspheres containing fluorescein (10% w/w), (10 mg/0.2 mL) were suspended in saline containing 0.01% Tween-80 and injected into the tail vein of mice. The mice were killed at predetermined intervals and the lungs and liver removed. The organs were frozen to −18°C in a cryostat within 5 min of sacrifice. Sections of 6 μm were examined with a Zeiss-Axioplan microscope fitted with a epifluorescence attachment.

3. Results and discussion

3.1. Microspheres characteristics

The characteristics of PLAGA microspheres, prepared by the solvent evaporation process are

shown in Table 1. The mean particle size was slightly reduced ($p < 0.05$) when low molecular weight polymer and low concentrations of 5-FU were used. The percentage yield of PLAGA microspheres was similar when different molecular weight polymers were used (Table 1). The drug encapsulation and loading in microspheres increased with increasing molecular weight of the polymer and 5-FU loading employed (Table 1). This can be explained by the increased deposition rate of polymer due to the decrease in solubility of polymer with increasing molecular weight and viscosity effects on the system.

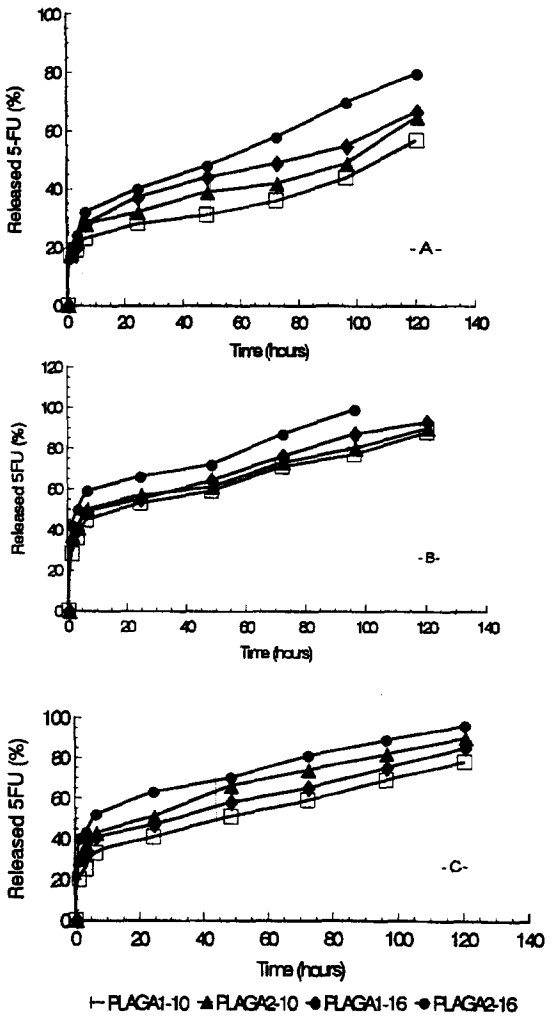

Fig. 2. Release profiles of 5-FU from PLAGA microspheres. (A) in pH 1.2 buffer solution, (B) in pH 9 buffer solution and (C) in saline solution. Data from six separate runs.

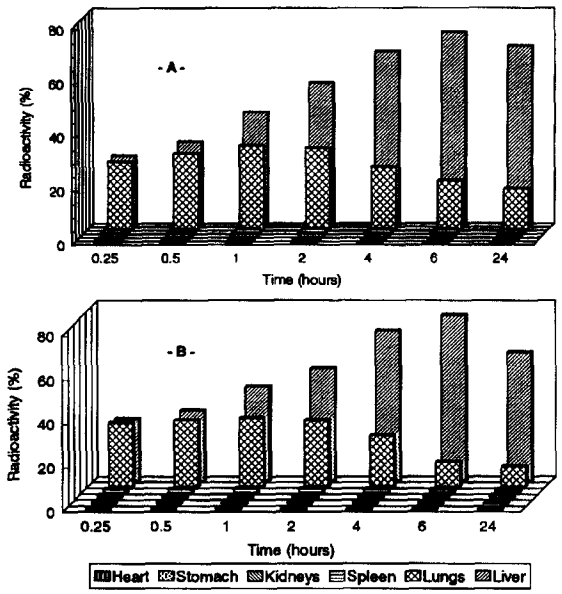

Fig. 3. In vivo distribution of ^{99m}Tc labeled PLAGA1 microspheres. (A) 9.5% 5-FU loading and (B) 15.5% 5-FU loading.

The scanning electron photomicrographs of PLAGA microspheres with 5-FU are shown in Fig. 1. The surface of microspheres appeared to be smooth and there were no drug crystals on the surface even at high 5-FU loading (15.5% w/w).

DSC thermograms of the microspheres showed only a glass transition and melting endotherm (Table 2). There was no melting endotherm in the region of 283.5°C attributable to crystalline 5-FU at two different loadings, suggesting that it was either totally dissolved in the polymer or dispersed as an amorphous solid.

In vitro degradation data showed that the rates of polymer molecular weight reduction over a month followed first order kinetics (Table 2). Previous studies (Vert et al., 1991) have indicated that the presence of dispersed molecules as a solid solution or suspended particle would be able to play the role of plasticizer or filler respectively. Those compounds affecting chemical degradation of polymer depend on their chemical and physicochemical properties (Vert et al., 1991; Heller, 1984). In our study, the initial 5-FU loading was not affected on polymer degradation for at least

Table 3
Release rate constants and correlation coefficients

Polymer type	5-FU content[a] (%)	pH[b]	Rate constants ± SD (correlation coefficients, $p > 0.05$)		
			Zero order $\times 10^{-2}$	First order ($k = h^{-1}$) $\times 10^{-2}$	Higuchi ($k = h^{-1/2}$) $\times 10^{-2}$
PLAGA1 (74 000 Da)	9.5	1.2	2.3 ± 0.5 (0.846)	1.0 ± 0.2 (0.908)	26.0 ± 2.1 (0.878)
		S[c]	3.6 ± 0.2 (0.865)	1.1 ± 0.5 (0.801)	40.8 ± 3.5 (0.940)
		9	3.6 ± 0.4 (0.783)	1.0 ± 0.5 (0.658)	42.8 ± 3.8 (0.882)
	15.5	1.2	5.6 ± 0.3 (0.844)	1.3 ± 0.2 (0.667)	65.2 ± 4.6 (0.932)
		S	6.5 ± 0.2 (0.0812)	1.2 ± 0.3 (0.531)	75.2 ± 7.1 (0.895)
		9	7.1 ± 0.4 (0.771)	1.7 ± 0.4 (0.487)	83.4 ± 7.9 (0.874)
PLAGA2 (23 100 Da)	9.5	1.2	2.6 ± 0.2 (0.830)	1.1 ± 0.3 (0.839)	29.8 ± 3.2 (0.881)
		S	3.9 ± 0.3 (0.804)	1.5 ± 0.2 (0.676)	45.6 ± 4.3 (0.905)
		9	4.1 ± 0.5 (0.728)	1.6 ± 0.7 (0.574)	41.1 ± 4.0 (0.829)
	15.5	1.2	6.6 ± 0.4 (0.884)	6.5 ± 0.4 (0.684)	75.9 ± 6.8 (0.940)
		S	7.1 ± 0.3 (0.736)	1.4 ± 0.3 (0.440)	84.1 ± 7.4 (0.852)
		9	8.6 ± 0.6 (0.677)	1.3 ± 0.2 (0.384)	91.7 ± 8.8 (0.789)

[a] Initial 5-FU content.
[b] pH of the dissolution medium.
[c] Saline solution.

the monitored period. This may be due to use of polymers with higher molecular weights than those used by other workers (Ruiz et al., 1990; Cohen et al., 1991).

Degradation in saline solution is not enzyme mediated and likely occurs by simple hydrolytic cleavage of ester groups. Low molecular weight PLAGA2 polymers are accompanied by elevated amounts of -COOH and -OH end groups which leads to an enhanced degree of hydrophilicity and, therefore, an increased degradation rate. Consequently, molecular weight and surrounding medium characteristics might be effective on in vitro hydrolytic degradation of PLAGA copolymers in our study.

3.2. *In vitro release of 5-FU from microspheres*

The release profiles of 5-FU from PLAGA microspheres are illustrated in Figs. 2 and 3. The drug was released from microspheres in different mediums following biphasic kinetics (Fig. 2, Table 3) which depended upon the molecular weight of polymer employed, initial 5-FU loading and pH of the dissolution medium. An initial burst is often observed due to the release of the drug from near the surface and solubility of 5-FU in dissolution medium. In addition, the burst effect could be attributed to the presence of drug crystals spread over the periphery of the 5-FU microspheres as explained in previous research (Boisdron-Celle et al., 1995). The extend of 5-FU burst

decreased with increasing polymer molecular weights or at low 5-FU loading (Fig. 2).

During the second phase, the degradation rate of the polymer per se was not a critical factor over the monitored period, Table 2. This slow release phase however was found to be affected by variations in the polymer molecular weights, drug loading and pH of the dissolution medium. The amount of 5-FU released from PLAGA microspheres increased with decreasing polymer molecular weight and increasing 5-FU loading. A higher 5-FU content would result in a more porous polymer matrix and release occurs by self-diffusion through drug filled porous in the matrix. In addition, 5-FU release from all microspheres increased with higher pH of dissolution medium due to higher solubility of 5-FU in pH 9 buffer solution, as we described in the previous study (Ciftci et al., 1994). The release data were investigated by using zero order, first order and Higuchi release kinetics and application of the method of residuals. The details of this statistical technique are explained by Bamba et al. (1979). The experimental data indicated that Higuchi's diffusion kinetics (Higuchi, 1963) showed a significantly better fit than the other mechanisms by the F-test. Release rate constants and determination coefficients are illustrated in Table 3.

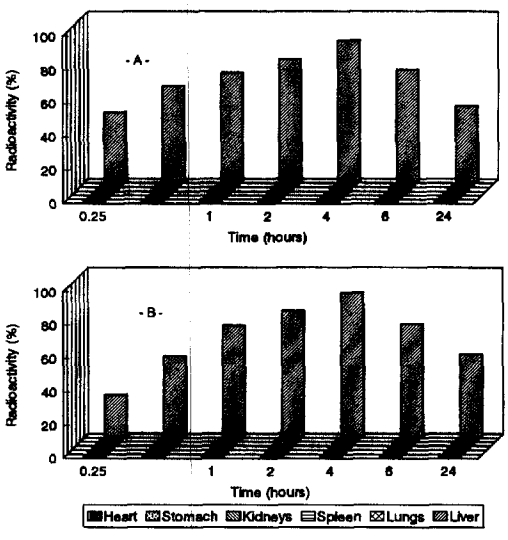

Fig. 4. In vivo distribution of ^{99m}Tc labeled PLAGA2 microspheres. (A) 9.5% 5-FU loading and (B) 15.5% 5-FU loading.

3.3. In vivo distribution of PLAGA microspheres

The in vivo administration of ^{99m}Tc labelled free 5-FU compound showed accumulation in the liver at 1 h. The initial radioactivity value, 58% fell rapidly to 41% by the second hour as described in the previous study (Ciftci et al., 1994). PLAGA1 microspheres at 9.5% and 15.5% 5-FU loading accumulated both in the lungs and liver in 1 h (Fig. 3). The lung and liver uptakes were similar during this stage. After 1 h, a decrease of lung uptake and increase of liver uptake was observed. This may be explained because some of the smaller microspheres although smaller than the lung capillaries might form agglomerates thus becoming larger and accumulating in the lungs. This concept has been investigated by various researchers (Edman and Sjoholm, 1983; Illum et al., 1985). As time progressed, the smaller amount of microspheres observed 24 h ($< 18\%$) after injection was due to a mechanical action of the blood flow and movement of the lungs, dispersing the particles, allowing them to be eventually taken up by the liver.

The PLAGA2 microspheres at 9.5% or 15.5% 5-FU loading were passively targeted to the liver, the main organ of the mononuclear phagocyte system (MPS) (Fig. 4). The rate of hepatic elimination was slower than that of free 5-FU or PLAGA1 microspheres. Thus, the PLAGA2 microspheres were still present in the liver over 24 h (Fig. 4). The liver uptake of the studied PLAGA2 microspheres was relatively slow when compared to other studies (De Keyser et al., 1991). The differences in the kinetics could be explained by particle diameter. On the other hand, larger particles would be eliminated more rapidly from the blood compartment and taken up more rapidly by the liver and the spleen than smaller particles which would remain longer in the blood circulation. Apart from the particle size, other factors should be considered to precisely predict liver uptake, i.e. surface nature, chemical structure which is explained in previous studies (Müller and Wallis, 1993; Illum and Davis, 1987). The spleen accumulation of PLAGA microspheres was very low ($> 2.5\%$ over 24 h). The differences between liver and spleen uptake could be explained by the

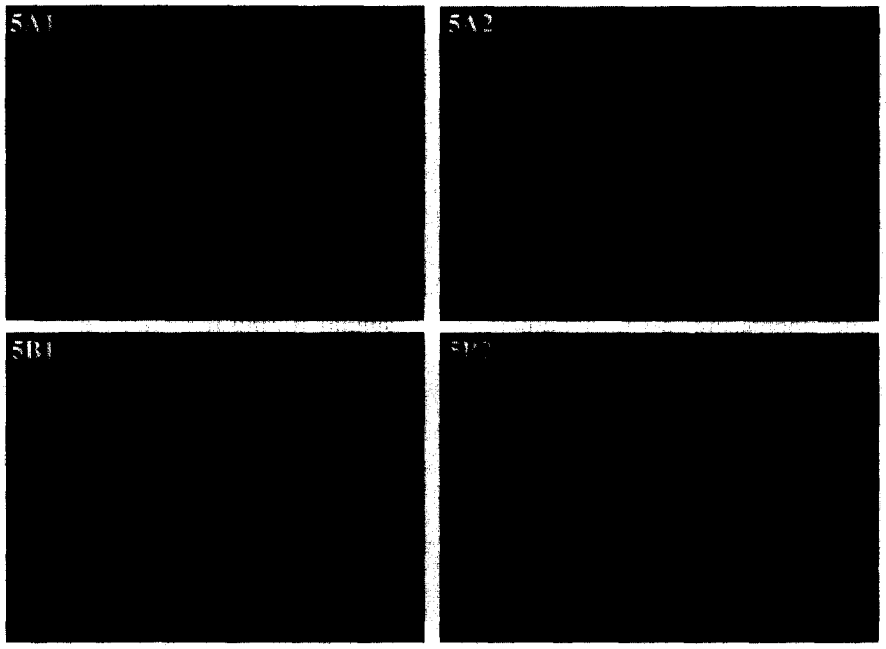

Fig. 5. Fluorescent photomicrographs of liver tissues 2 h after i.v. administration of PLAGA microspheres. (A) PLAGA1 microspheres (1: 9.5% 5-FU and 2: 15.5% 5-FU). (B) PLAGA2 microspheres (1: 9.5% 5-FU and 2: 15.5% 5-FU). Magnification: × 200.

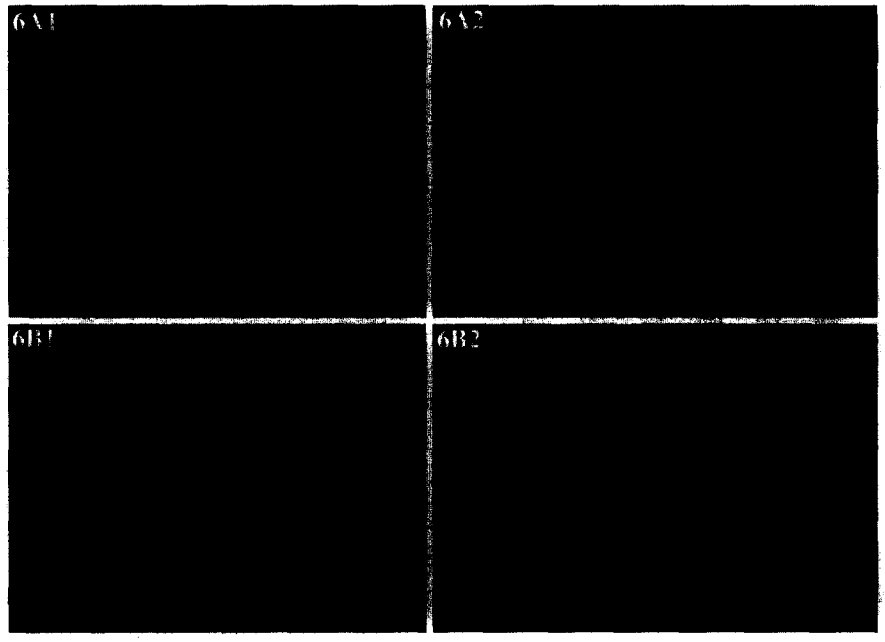

Fig. 6. Fluorescent photomicrographs of liver tissues 4 h after i.v. administration of PLAGA microspheres. (A) PLAGA1 microspheres (1: 9.5% 5-FU and 2: 15.5% 5-FU). (B) PLAGA2 microspheres (1: 9.5% 5-FU and 2: 15.5% 5-FU). Magnification: × 200.

weight differences of these organs. The radioactivity found in the other organs was probably the result of the presence of microspheres in the intravascular compartment of these organs. Radioactivity was also found in the elimination organs, i.e., the kidneys at a low level for all microspheres. The radioactivity did not accumulate in the stomach and heart ($< 0.4\%$) (Figs. 3 and 4). These results were in accordance with those found in the literature (Edman and Sjoholm, 1983). In vivo distribution studies of radiolabeled microspheres were confirmed · by histologic examination of lung and liver tissues at 1, 2, 4, 6 and 24 h after i.v. injection of fluorescein labeled microspheres. The liver photomicrographs of fluorescein labeled particles at 2 and 4 h are illustrated in Figs. 5 and 6, respectively. The maximum accumulation in the liver was observed in 4 h for all microspheres (Fig. 6). The MPS accumulation of particles is the function of particle size. This appears to correlate with the in vivo distribution data of PLAGA microspheres. The fluorescent particles were mainly localized within the liver sinusoids or in the Kupffer cells cytoplasm. As expected, the fate of microspheres was characterized by high uptake by MPS organs.

In conclusion, 5-fluorouracil was encapsulated in PLAGA microspheres (diameter 2–5 μm) by the solvent evaporation method using polyvinyl alcohol as a surfactant. The drug was released from the microspheres in different media following the biphasic kinetics of an initial burst and a subsequent slow release which strongly depended on initial drug loading, pH of the dissolution medium and the molecular weight of the polymer employed. Drug release was not due to the degradation of polymer, at least for the following period. In addition, the results of PLAGA microspheres in vivo distribution indicate that most particles below 4 μm are localized in the liver. The information obtained using 4 μm microspheres indicates an initial presumably mechanical retention by the lungs with subsequent relocation by phagocytosis in the liver for 24 h periods. Organ targeting may be controlled by adjusting the size distribution of the microspheres.

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