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Formulation and Characterization of Formaldehyde Cross-linked Degradable Starch Microspheres Containing Terbutaline Sulfate

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ABSTRACT Preparation of starch microspheres using epichlorohydrin is a time consuming method and requires around 18 hr for cross-linking reaction. To reduce reaction time, terbutaline sulfate (TBS) loaded degradable starch microspheres (DSM) were prepared using formaldehyde as the cross-linking agent. All microspheres were spherical in shape and had a porous, rough surface with a mean particle size of 18–24 μm. Whatever the cross-linking time, it was seen that the release of the TBS was not complete during the release experiments. The influence of enzyme on the degradation of microspheres was moderate. Following intravenous administration, initial uptake of microspheres by the lung was higher than those of other organs.

KEYWORDS Terbutaline sulfate, Formaldehyde, Degradable starch microspheres, Degradation, Biodistribution

INTRODUCTION

Terbutaline sulfate (TBS) is a synthetic β-adenoreceptor stimulant that is mainly used for the treatment of bronchial asthma, chronic bronchitis, and emphysema (Ahuja & Ashman, 1990), and is available in formulations for oral intake, inhalation and injection. However, it has a short biological half-life of about 3–4 hr (Ahuja & Ashman, 1990), and requires frequent dosing (e.g., for oral route: 5 mg three times at approximately 6 hr intervals during waking hours; Manekar et al., 1991). Therefore, a long acting formulation is desirable to improve not only the treatment of lung disorders effectively but also patient compliance.

Prolongation of TBS release from cellulose acetate phatalate (Manekar et al., 1991), ethylcellulose (Manekar et al., 1992), and Eudragit RS (Kim et al., 1994) microparticulate systems has been demonstrated by means of in vitro dissolution studies. After oral administration, the main advantage with slow release tablets is the twice-daily dosage regimen and better maintains therapeutic levels during the whole-night interval without significant advantage for the

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reduction of objectively measured side effects (Nyberg & Kennedy, 1984). Yet, terbutaline is incompletely absorbed from the gastrointestinal tract (average bioavailability is 10% in healthy subjects; Nyberg, 1984), and is also subjected to fairly extensive first-pass metabolism by sulfate conjugation in the liver and the gut wall (Sweetman, 2002). This first-pass metabolism can be avoided by intravenous administration of the compound. Furthermore, following the intravenous administration of terbutaline, particles greater than 7 μm are deposited in the lungs by means of mechanical entrapment (Hamdi et al., 2001). When the particle size is controlled in the range of 12–44 μm, 95% of microparticles can be concentrated in the lung after intravenous injection (Lu et al., 2003). Therefore, intravenous administration of a TBS microparticulate system not only avoids the first-pass effect but also provides a higher tissue concentration of the active compound, and hence more effective treatment of lung associated diseases. High deposition of TBS loaded liposomes (6–8 μm; Palakurthi et al., 2000), albumin (22–30 μm; Sahin et al., 2002), and L-PLA (9–21 μm; Selek et al., 2003) microspheres in the lung were indeed demonstrated after intravenous administration.

The need for using biodegradable and biocompatible carriers for controlled drug delivery systems leads to use of specific polymers as the base materials for preparation of such systems. Many materials such as albumin, PLA, PLGA, and starch have been proposed as biodegradable carriers. Of these, starch possesses many desirable characteristics such that they can be degraded by α-amylase, which has been shown to be present in the bronco-alveolar fluids extracted from the human pulmonary tract (Nandapalan et al., 1995). Moreover, their ability to entrap a wide variety of drug molecules has been demonstrated in various studies. In addition, the use of starch microspheres has been suggested for embolization (Edman & Sjoholm, 1992; Lindberg et al., 1984), parenteral administration (Teder & Johansson, 1993), and nasal administration (Bjork & Edman, 1988; Mao et al., 2004) and they do not stimulate albumin-like antigen response in vivo (Mao et al., 2004).

The purpose of this study was to develop and characterize TBS containing degradable starch microspheres (DSM) for passive lung targeting. In general, starch microspheres are produced from hydrolyzed potato starch and are cross-linked by a common emulsion polymerization process using epichlorohydrin. However, it is a time-consuming method and requires around 18 hr for the cross-linking reaction (Hamdi et al., 1998, 2001). Therefore, the initial aim of this study was to reduce reaction time using formaldehyde as the cross-linking agent, and then to investigate the effect of cross-linking time on preparation, release properties, sensitivity to degradation and morphology of the microspheres, and finally to determine the body distribution of labeled starch microspheres in mice.

MATERIALS AND METHODS

Materials

TBS was a generous gift from Eczacibasi (Turkey). Extra pure soluble starch and the cross-linking agent formaldehyde were purchased from Merck (Germany). Corn oil, which acted as the oil phase was supplied by the Komili Oil Company (Turkey). Pancreatin was purchased from Sigma (St. Louis, MD, USA) and Tween 80 from J.T. Baker Chemical Co. (Phillipsburg, NJ, USA). Technetium-99m (^{99m}Tc) as pertechnetate was purchased from Amersham International, England. All other chemicals were of analytical grade and used without further purification.

Preparation of Microspheres

The DSM containing TBS were prepared using a modified emulsion polymerization technique developed by Gurkan et al. (1986). A total of 10 mL of the starch solution (7.5%, w/v) containing 0.5 mg TBS 0.5 was added dropwise into continuously stirred (1500 rpm) corn oil (100 mL) containing Tween 80 (0.1%) at 25°C. After 10 min of stirring, the obtained emulsion (water in oil) was cooled in an ice bath (0°C), washed with 100 mL of acetone, and then centrifuged for 10 min at 3200 rpm to remove the oil phase. The residue was re-suspended in 100 mL of acetone containing formaldehyde (20%, v/v) as the cross-linking agent. The suspension was stirred (1500 rpm) for 15, 30, and 60 min at 25°C. After this stabilization process, excess of carbonyl reagent was removed by immediately adding 100 mL of acetone, centrifuging for 5 min at 3200 rpm and decanting the supernatant. The resulting microspheres were washed four times in this manner, filtered and subsequently air-dried.

Preparation of blank microspheres was the same as the drug containing microspheres except that they did not contain TBS.

Characterization of Microspheres

Morphological Properties of Microspheres

The surface morphology of starch microspheres was examined by scanning electron microscopy (Jeol-SEM ASID-10 Device operating at 80 KV, Japan) before and after the TBS release experiments. Microspheres were mounted on metal stubs with conductive silver paint and then sputtered with a 150 Å-thick layer of gold using a BIO-RAD apparatus.

Determination of Particle Size Distribution

The particle size distribution of DSM formulations was determined by a Malvern Mastersizer (Malvern Instrument, UK). Size analysis was performed after dispersing 10 mg of microspheres in 5 mL of deionized water containing Tween 80 (0.01%) and then sonicating for 2 min.

Encapsulation Efficiency of Microspheres

For each batch, TBS containing DSM formulations (25 mg) were mixed with 6 N HCl solution (25 mL) and kept in an ultrasonic bath for 15 min. Following the centrifugation at 5300 rpm for 30 min, it was filtered through a 0.22 μm filter and absorbance of the clear supernatant was measured on a UV spectrophotometer (Shimadzu model, 160 A, Japan) at 276 nm wavelength. The amount of encapsulated TBS was determined using a calibration curve constructed over the range of 20–120 μg/mL.

In Vitro Release Studies

An incubation method was used for investigation of in vitro TBS release from the starch microspheres. Accurately weighed microspheres (50 mg) were suspended in 25 mL of isotonic phosphate buffer (pH 7.4), and then immersed in a constant temperature water bath (37 ± 0.5°C) with agitation at 50 counts per minute in a horizontal laboratory shaker (Nuve, Turkey). At various time intervals (5, 10, 15, 30, 60, 120, 180, 240 min), samples (1 mL) were withdrawn and replaced by an equal volume of fresh medium. UV absorbencies of the samples were measured at 276 nm wavelength after processing the samples with 1 mL of 6 N HCl in an ultrasonic bath for 15 min and then centrifuged. The amount of TBS released was determined using a calibration curve constructed over the range of 20–140 μg/mL. The release profiles were evaluated according to zero order, first order, Higuchi square-root of time, and Hixon-Crowell cube root models.

Determination of Microsphere Degradation

Degradation of microspheres by pancreatin was assessed by a method based on microvolume measurements during the time course of enzymatic degradation by a Coulter counter method as described by Hamdi et al. (1998). Briefly, a suspension of N15-coded microspheres (2 mg in 100 mL Isoton® II solution containing one drop of Tween 80) was agitated and placed in the stand of Counter Multisizer II (Counter Electronics Ltd, UK) with a 100 μm orifice tube. Measurements were performed in manometer mode by analyzing 100 μL of particle suspension. Counting was done before and after the addition of an enzymatic solution of pancreatin (1, 2, 4, 16 mg/mL) which was filtered through 0.22 μm filter, prior to mixing with the suspension. The initial volume of the particles (Vo) was measured before the degradation, and remaining volume during degradation (V*t*) was measured as a function of time (10, 20, 40, 60, 75, and 150 min). The volumetric degradation profiles were prepared from the volume distributions (V*t*/Vo) obtained during the time course of degradation. Degradation rates were calculated as the slope of the linear portion of the linearized volume degradation profiles by employing the following equation (Hamdi & Ponchel, 1999):

$$\sqrt[3]{\frac{\mathrm{Vt}}{\mathrm{Vo}}} = 1 - \mathrm{bt}$$

where t stands for the degradation time (min) and b for the degradation rate (1/min). The time of half degradation (T_{50}) was also estimated using this equation.

In Vivo Studies

^{99m}Tc labeling of starch microspheres

The method used for ^{99m}Tc labeling of starch microspheres was the modification of that of Ridley et al. (1995). Two drops of Tween 80 (5%) and 2 mL of $SnCl_2 \cdot 2H_2O$ solution (1 mg/mL) were added on the accurately weighed TBS containing microspheres (100 mg) and then placed in an ultrasonic bath for one minute. Two milliliters of saline containing 20 mCi ^{99m}Tc were added on the precipitate formed by centrifuging at 5000 rpm for 3 min. The suspension was centrifuged 20 min after incubation, and then the precipitate was washed twice with 2 mL of distilled water. Quality control of the labeling was checked by miniaturized ITLC^{TM}/SG plates (Gelman Sci, Ann Arbor, MI, USA) by using an acid citrate dextrose solution as running solvent (Ercan, 1992). At the end of the development procedure, the cut strips were measured in a well type gamma counter for radioactivity. Bound and free fractions of ^{99m}Tc activities were calculated.

Biodistribution Studies

A suspension of labeled microspheres (0.2 mL) was administered into the tail vein of Swiss albino male mice (25–30 g, $n = 18$). At predetermined time intervals (0.25, 0.5, 1, 3, 6, and 24 hr), animals were sacrificed by cervical dislocation (3 mice at each time point). Selected organs (heart, intestine, kidneys, liver, lungs, stomach, spleen) were excised and then weighed. As the organ counts were too high for detection at the time of the death, all tissue samples were stored for 24 hr, and then the activities were determined by a well type gamma counter (Wallac 1480, WIZARD 3 Finland). Percent uptake per gram tissue was calculated for each organ. To estimate the extent of uptake for each tissue, area under the curve (AUC_{0-t}) was estimated using the linear trapezoidal approximation from the pooled biodistributional data of the tissue of interest. Mean residence time (MRT) was calculated from the ratio of area under the first moment curve ($AUMC_{0-t}$), estimated by the linear trapezoidal approximation, to AUC_{0-t} ($MRT = AUMC_{0-t}/AUC_{0-t}$; Sahin & Rowland, 1998).

RESULTS AND DISCUSSION

Preparation and Characterization of Microspheres

There are two general methods for preparation of starch microspheres. The so-called soaking/lyophilization procedure involves in swelling of a preformed microspheres (e.g., Spherex®, Pharmacia and Upjohn, Erlangen, Germany) to equilibrium in a suitable drug solution before being further dried, whereas in the other method, the polymerization and/or cross-linking is achieved in the presence of drug, resulting in the trapping of drug within the matrix. Various cross-linking agents (e.g., epichlorohydrin, terephthaloyl chloride) have been used for the preparation of these particles (Hamdi et al., 2001; Mao et al., 2004). In general, DSM is produced from hydrolyzed potato starch and is cross-linked by a common emulsion polymerization process using epichlorohydrin. However,cross-linking time is rather long (18 hr at 40°C) when epichlorohydrin is used (Hamdi et al., 2001). Therefore, in the present study, formaldehyde was selected as the cross-linking agent to reduce the reaction time. Although it has been widely used as the stabilization agent for preparation of various microparticulate systems such as albumin (Katti & Krishnamurti, 1999; Widder et al., 1979), gelatin (Iseri et al., 1991; Leucuta, 1990; Lu et al., 2003) and chitosan (Ganza-Gonzalez et al., 1999) microspheres in a concentration range of 5–50% (Ganza-Gonzalez et al., 1999), to our knowledge, its use has not been reported for preparation of starch microspheres.

In this study, effect of cross-linking time (15, 30, 60 min) on the fabrication of starch microspheres was investigated at a fixed formaldehyde concentration (20%). Regardless of the cross-linking time, microspheres were formed easily and the yield value was in the range of 52–56% (Table 1). Furthermore, morphological examination of microspheres by scanning electron microscopy (SEM) indicated that they were spherical in shape and had a porous, rough surface (Fig. 1) with a mean particle size of 18–24 μm (Table 1). Although an attempt was made to take SEM photographs of the microspheres after the release experiments, this was not possible as they displayed a gel like structure corresponding to a loss of individualized structure.

The encapsulation of TBS in the starch microspheres was low for all DSM formulations (2–4%) and influenced by the cross-linkling time: an increase in the stabilization period from 15 to 60 min resulted in a decrease in the encapsulation (Table 1).

In Vitro Release Studies

Starch microspheres are not only biodegradable but also show a high degree of swelling when in contact with aqueous media. They then form a gel-like system. The release of a drug from hydrogels is controlled by the rate and degree of formation of swelling-induced porous structure, degradation of the polymeric network and hydrophobic interaction between drug and

TABLE 1 **Characterization of Starch Microspheres**

Codes	Cross-linking agent (%)	Cross-linking time (min)	Particle size* (μm)	Yield value* (%)	Encapsulated TBS (%)
N15	20	15	19.67 ± 1.50	51.9 ± 2.3	3.59
N30	20	30	23.95 ± 1.71	56.4 ± 0.4	3.23
N60	20	60	18.07 ± 1.19	55.7 ± 2.9	2.29

*mean ± SD, $n = 3$.

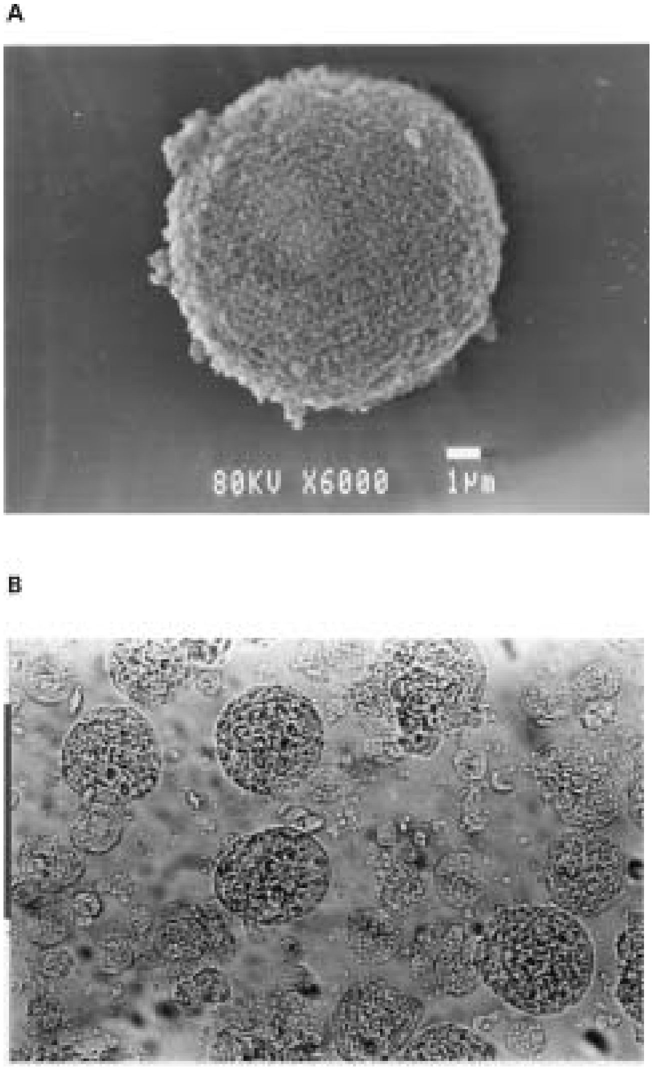

FIGURE 1 **Scanning Electron Micrograph (A) and Optical Microscopic Image (×100) (B) of Degradable Starch Microspheres Cross-linked With Formaldehyde.**

hydrogel network (Kim et al., 1992; Zhang & Chu, 2002).

TBS release profiles described by the Higuchi square-root time model ($r > 0.97$), were very similar for all microsphere formulations indicating that the cross-linking time did not have a significant effect on the released amount ($p > 0.05$). All profiles were characterized by an initial burst release phase followed by a slow and sustained release phase. TBS release from starch microspheres was not complete over the time course of experiment (60–66% in 4 hr). About 50–56% of loaded TBS was released from DSM within 15 min, whereas an additional 10% was released during the slow phase (Fig. 2). The initial burst release could be attributed to both rapid swelling of starch microspheres reflecting the contribution of rapid diffusion of water into the microspheres leading to formation of porous structure quickly upon contact with water, and rapid loss of pore and/or surface associated drug. The slow phase is most likely to be controlled by the degradation rate of DSM and hydrophobic interaction between the drug and polymer matrix.

Degradation of Microspheres

Alpha amylase has been widely used for the degradation of starch microspheres (Civalleri et al., 1989; Russell, 1983). However, use of pancreatin for this purpose has also been reported (Levy & Andry, 1992) because of the presence of alpha amylase in pancreatin. In the present study, the influence of pancreatin concentration on the volumetric degradation profiles was investigated by varying the amount of pancreatin in the range 1 to 16 mg/mL. Degradation profiles of N15-coded microspheres were hyperbolic (Fig. 3) and could be characterized by determining the time of half degradation (T_{50}) which was necessary for observing the disappearance of one half of the initial volume. When the pancreatin concentration was increased from 1 to 16 mg/mL, the half time of degradation was moderately decreased from 17 to 13 min. On the other hand, degradation rates calculated as the slope of the initial linear portion of the profiles were increased from 0.013 to 0.016, 1/min for the corresponding enzyme concentrations (Table 2). The microvolume

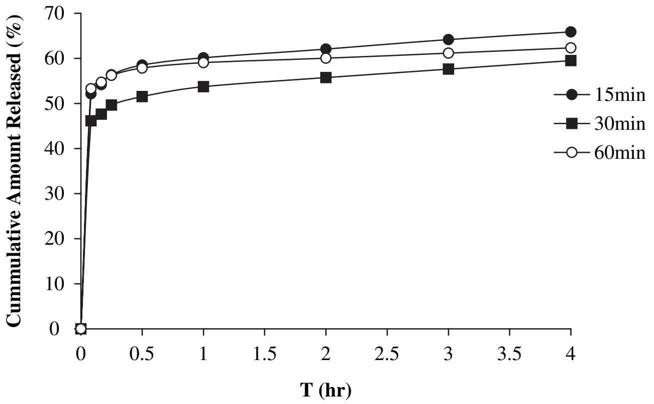

FIGURE 2 In Vitro Release Profiles of Terbutaline Sulfate (TBS) From Degradable Starch Microspheres. Formulations Were Cross-linked With Formaldehyde for 15, 30, and 60 min.

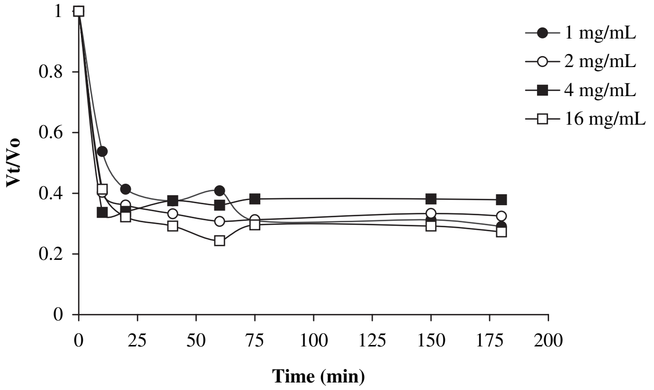

FIGURE 3 Volumetric Degradation Profiles of N15-coded Microspheres (Cross-linked for 15 min Using Formaldehyde) as a Function of Pancreatin Concentration (1, 2, 4, 16 mg/mL).

TABLE 2 Degradation Rates and T_{50} Values as a Function of Pancreatin Concentration

Pancreatin (mg/mL)	Degradation rate* × 10^{-2} (1/min)	$T_{\%50}$* (min)
1	1.313 ± 0.467	16.91 ± 5.24
2	1.444 ± 0.178	14.43 ± 1.77
4	1.513 ± 0.120	13.69 ± 1.09
16	1.582 ± 0.221	13.20 ± 1.72

*mean ± SD; *n* = 3.

method assumes that the degradation process of the starch microspheres is controlled by the surface erosion. The relationship between the degradation rates and pancreatin concentrations was not linear, indicating that saturation of the microsphere surface was reached under the experimental conditions used.

Biodistribution Studies

Based on the encapsulation efficiency and release studies, N15-coded formulation was selected for the biodistribution studies. The labeling efficiency of ^{99m}Tc was over 90% as determined by ITCL. The results of the biodistribution studies are summarized in Fig. 4, in which the organ uptake per gram tissue (% uptake/g) are plotted as a function of time. As the particles greater than 7 µm are trapped in the capillary bed of the lungs by a simple process of mechanical entrapment (Hamdi et al., 2001), it can be expected that initial uptake following the administration would be higher for this organ. Although initial uptake was higher for the lungs (14% of the administered dose), it was not the case for total uptake (AUC_{0-24}). Total uptake was higher for the elimination organs (kidneys:149% uptake.hr/g and liver:123% uptake.hr/g) followed by lungs (97% uptake.hr/g), spleen (70% uptake.hr/g) and stomach (50% uptake.hr/g). This could be attributed to the rapid degradation and hence rapid removal of the microspheres from the lungs, resulting in higher degree of accumulation in the elimination organs. So that, mean residence time can be expected to be shorter in the lungs. Evaluation of MRT values indicated that this value was slightly shorter in the lungs (9 hr) in comparison to the other organs (10–14 hr). This observation is in agreement with our previous results obtained by intravenous injection of the L-PLA microspheres into the mice (MRT: 9 hr for lungs and 12 hr for the liver; Selek et al., 2003). In the case of other organs (intestines and heart), the AUC values were in the range of 12–13% (uptake.hr/g)

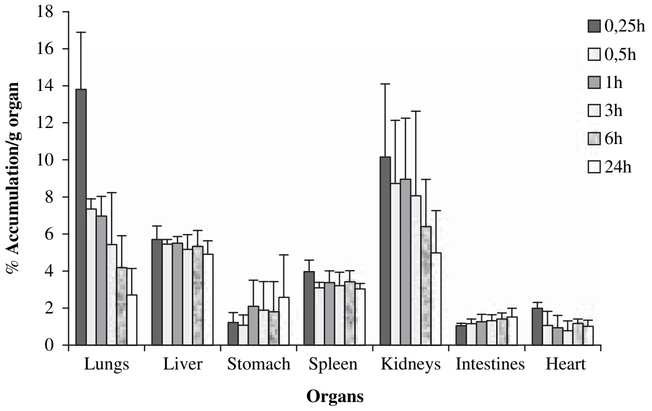

FIGURE 4 Biodistribution of N15-coded Microspheres. Tissue Samples Were Collected 0.25, 0.5, 1, 3, 6, and 24 hr After the Administration of ^{99m}Tc-labeled Microspheres into the Tail Vein of Swiss Albino Male Mice.

indicating that the degree of uptake by these organs is minimal.

CONCLUSIONS

The results of the present study suggest that formaldehyde can be used as a stabilization agent for the preparation of the degradable starch microspheres. For a given formaldehyde concentration (20% v/v) and cross-linking time (15, 30, 60 min), microspheres were formed easily with a yield value of 52–56%. They were spherical in shape and had a porous, rough surface. Although the size of microspheres (18–24 μm) were suitable for passive targeting to the lung, because of the rapid release of drug from microspheres (50–56% within 15 min) as a result of rapid swelling and also enzymatic degradation, initial uptake of DSM by this organ was lower than expected (14% vs. 35 and 40% for L-PLA and albumin microspheres, respectively Sahin et al., 2002; Selek et al., 2003). As the cross-linking density is likely to modulate degradation and swelling rates, drug release especially during the initial phase, and organ uptake can be modified by varying the concentration of cross-linking agent used.

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