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Preparation and evaluation of biodegradable albumin microspheres containing mitomycin C

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Summary

Albumin microspheres containing the antitumor agent, mitomycin C (MMC), were prepared by glutaraldehyde cross-linking and heat solidification in order to administer MMC to the tumor site and to maintain prolonged MMC release by a chemoembolization technique. Various emulsification conditions were utilized in order to ameliorate the drug release curve, the mean particle size and the yield of albumin microspheres. Drug release experiments revealed that entrapped drugs were released slowly from microspheres except for the initial burst effect. The digestion of albumin microspheres, when incubated in the pH 7.4 phosphate buffer containing various proteases, was indicated by determination of weight loss and by scanning electron microscopic observation. An in vivo biocompatibility test was carried out to assess the irritability of the microspheres prepared by the various methods. Microspheres prepared were found to cause acute inflammation, but elicited no severe inflammatory responses.

Introduction

Selective drug targeting to specific physiological sites will not only lead to a reduction in adverse reactions and toxic side effects, but could also increase therapeutic opportunities for use of highly toxic substances. For this purpose several drug carriers have been used especially in cancer chemotherapy; for example, drug–DNA complex and liposome.

Recently, Kato et al. (1980) reported that transcatheter intraarterial injection of ethylcellulose microcapsules containing MMC leads to substantial reductions in the size of many different primary and metastatic carcinomas due to chemoembolization. Recently, the development of improved catheters and progress in intraarterial infusion techniques enhanced their utility and effectiveness. It is considered that drug carriers in the chemoembolization therapy play two roles, embolization of the vessels in the target site and control of drug release. Since their functions are dependent on the properties of the carriers, the choice of them is important. We have also reported clinical application of albumin micro-

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spheres containing MMC for chemoembolization (Fujimoto et al., 1983), but basic evaluation was not done. Although methods for preparing albumin microspheres as drug carriers or imaging agents in sizes of approx. 1.0 μm diameter have already been established, preparation of them as a material for chemoembolization in diameter greater than 30 μm has not been established.

Therefore, in this paper, we present several new preparation methods for chemoembolization of albumin microspheres and assess their biodegradability and biocompatibility.

Materials and Methods

Materials

Human serum albumin (HSA, fraction V) was obtained from Sigma (St. Louis, MO). Mitomycin C (MMC) was supplied by Kyowa Hakko (Tokyo). Collagenase, papain, trypsin, and Protease® were obtained from Sigma and *n*-octane and 25% glutaraldehyde were purchased from Wako (Osaka). In this experiment 5% glutaraldehyde was prepared by diluting 25% glutaraldehyde with distilled water (or buffer). All other reagents used were commercial reagent-grade.

Animals

Male Wistar rats, weighing 130–150 g, were used in biocompatibility testing of albumin microspheres.

Preparation of albumin microspheres

Albumin microspheres as a material for chemoembolization were prepared using the following methods as outlined in Fig. 1. Method I was a modification of a method described in a previous paper (Sugibayashi et al., 1979). In this experiment, to prepare larger spheres (30–60 μm) for chemoembolization, stirring was performed at 500 rpm. Method II was developed by the modified method of Longs et al. (1982). HSA (300 mg) was dissolved in 2 ml of distilled water and mixed with 1 ml of MMC aqueous solution (30 mg/1.0 ml). Glutaraldehyde (5%, 0.6 ml) was then added to

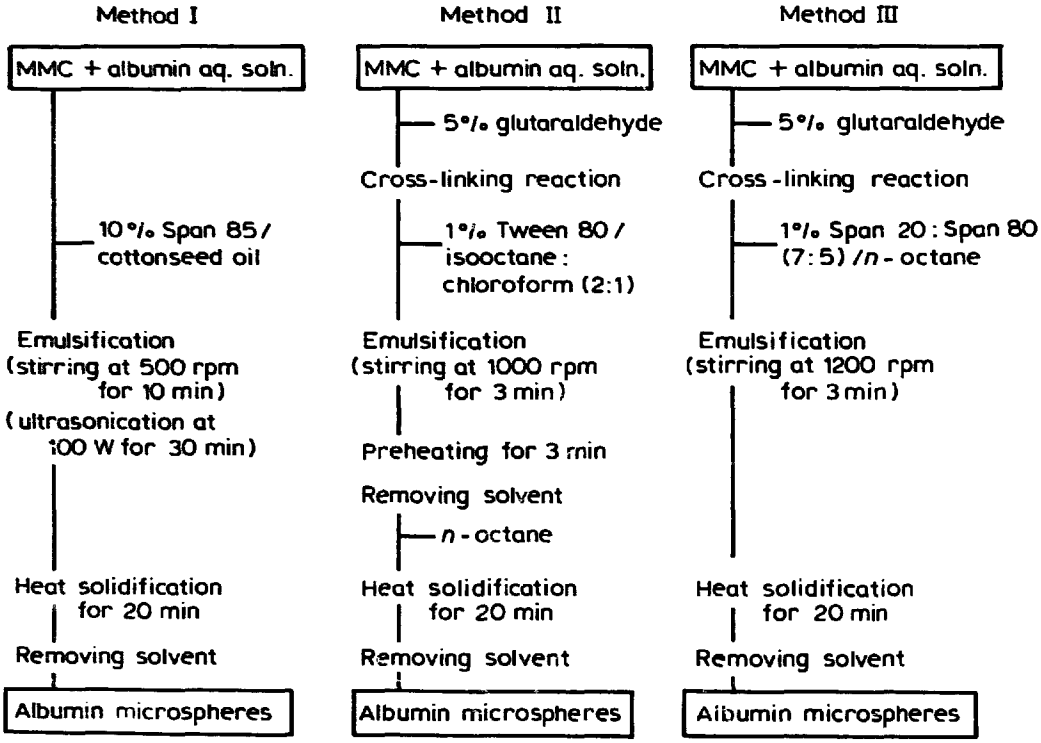

Fig. 1. Several preparation methods of albumin microspheres containing mitomycin C

the HSA–MMC mixture. This mixture was dropped in isooctane : chloroform (2 : 1, volumetric ratio, 100 ml total volume) containing 1% polysorbate 80 and emulsified with a motor-driven glass stirrer at 1200 rpm for 5 min. This emulsion was chemically transformed into a suspension during the stirring. The suspension was heated in an oil bath at 80–85 °C for 5 min. After cooling the suspension at room temperature, supernatant was decanted, *n*-octane (100 ml) was added to the residue, and the new suspension was heated in an oil bath at 120 °C for 20 min. After decanting *n*-octane, complete removal of residual solvent was achieved by washing the albumin microspheres in diethylether (3 times) followed by petroleum ether (once). After drying albumin microspheres under reduced pressure, they were stored in a desiccator. Method III was a modification of Method II. The identical procedure used in Method II was followed, except that after 5% glutaraldehyde was added to a HSA aqueous solution containing MMC, it was mixed with 100 ml of *n*-octane containing 1% surfactant (Span 80 and Span 20 were mixed volumetrically 7 : 5) and stirred at 1200 rpm for 3 min. Subsequently, the suspension was heated in an oil bath at 120 °C for 20 min, and washing and drying was performed in the same manner as described in Method II.

Furthermore, to clarify the effect of the HLB value of surfactants on the particle size of albumin microspheres, albumin microspheres were prepared using *n*-octane containing different ratios of Span 80 and Span 20 in accordance to Method III. HLB values ranged from 1.8 to 8.6.

Measurement of size distribution of albumin microspheres

Albumin microspheres prepared by Methods I and III were observed with a scanning electron microscope (X-650, Hitachi Seisakusho, Tokyo) and the size distribution of albumin microspheres in the resulting micrographs was analyzed with Mop Video Plan (Kontron Bildanalyse, Munich). Moreover, micrographs of several albumin microspheres prepared by altering surfactant HLB values were compared for size distributions.

In vitro MMC release from albumin microspheres

Albumin microspheres (10 mg) containing MMC prepared by Methods I, II, and III were put into 10 ml of pH 7.4 isotonic phosphate buffer containing Protease® (10 U/ml) or phosphate buffer alone. Suspensions were stirred with a magnetic stirrer at about 150 rpm and were shaken in an incubator at 37 °C. At each sampling time, these suspensions were separated by centrifugation at 3000 rpm for 10 min, 7.0 ml of supernatant was removed and replaced with 7.0 ml of pH 7.4 phosphate buffer to keep the volume constant. The drug concentration in the supernatant was determined spectrophotometrically at 365 nm using a spectrophotometer (model 100-60, Hitachi Seisakusho).

Degradation products of MMC and others did not interfere the determination of unchanged MMC in this wavelength; this was checked by HPLC. MMC content was estimated by dissolving albumin microspheres prepared in Method I, II and III in the Protease® solution for a week. As these microspheres were dissolved within a week, the total amount of MMC in solution was defined as the drug content.

Measurement of biodegradability of albumin microspheres by several proteases

Albumin microspheres (10 mg) prepared with Methods I, II, and III were mixed with 10.0 ml of pH 7.4 isotonic phosphate buffer containing several proteases (collagenase 10 units/ml, papain 10 units/ml, Protease® 10 units/ml, and trypsin 10^3 BAEE units/ml). These suspensions were stirred with a magnetic stirrer at 150 rpm and were shaken in an incubator at 37 °C for 96 h. Every 24 h these suspensions were separated by centrifugation at 10000 rpm for 15 min, 6.0 ml of supernatants were removed, and replaced with 4.0 ml of pH 7.4 phosphate buffers containing several proteases. After 96 h of incubation, these suspensions were separated by centrifugation at 10000 rpm and all supernatant was removed. Distilled water was added to the residues and stirred very well. These suspensions were separated by centrifugation again, and the supernatants were decanted. A washing procedure consisting of a single distilled water rinse followed by a methanol rinse was performed on the residues. Then the residues were sufficiently dried under N_2 gas and their

weights were measured using a chemical balance (Dickinson et al., 1981).

In addition, albumin microspheres prepared by Method III were treated with Protease® in the same manner as described above for 2 or 4 days and the resulting microsphere morphologies were observed with a stereomicroscope (Olympus, Model SZ-TR, Tokyo).

In vivo observation of biocompatibility of albumin microspheres

Isotonic NaCl solution (0.9%, 0.25 ml) containing 2.5 mg albumin microspheres prepared in diameters of approx. 42.5 μm (Methods II and III) and diameters of 1.0 μm (Method I) were injected into the annulus muscle of sodium pentobarbital (50 mg/kg)-anesthetized rats. NaCl solution only (0.9%) was injected as a control. The muscles were removed at 3-, 7- and 30-day periods, fixed in 10% formalin, sliced on a microtome and stained with a hematoxylin–eosin stain for histological evaluation (Lawravel et al., 1963; Wilson et al., 1981).

Results

Diameter distribution of albumin microspheres

Fig. 2 shows the size distribution for albumin microspheres prepared from Methods I and III. The mean diameter of albumin microspheres from Method I was 34.5 ± 19.3 μm and was broadly distributed. Overall yield of albumin microspheres within intended diameter range of 30–60 μm was approximately 40%. In contrast, mean diameters

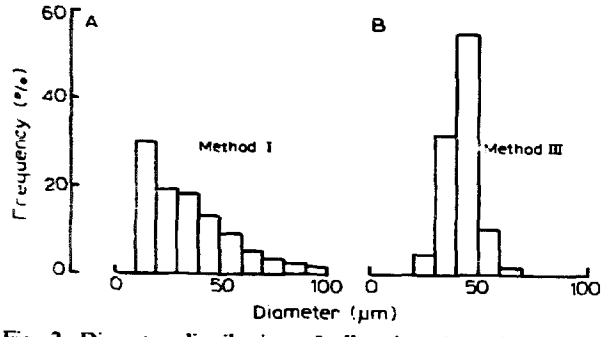

Fig. 2. Diameter distribution of albumin microspheres. (A) Albumin microspheres prepared by Method I; (B) albumin microspheres prepared by Method II.

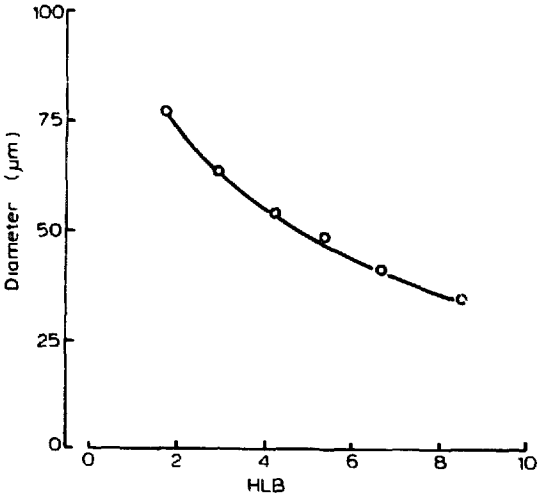

Fig. 3. Relationship between mean diameter of albumin microspheres and HLB value of surfactant in the oil phase at preparation.

of albumin microspheres from Method III was 42.4 ± 6.93 μm, demonstrating a narrow distribution curve. With Method III, more than 90% of the albumin microspheres produced were within the designated range. The mean diameter of albumin microspheres from Method II was approximately 46.8 μm, with a narrow size distribution and a yield in the desired size range of about 70% (data not shown). However, albumin microspheres prepared with Method II were not perfectly spherical and contained surface irregularities.

Regulation of albumin microsphere diameter with HLB values

Albumin microspheres sizing may be altered by changing the composition of the microspheres, the type and viscosity of continuous phase, stirring speed, and concentration of surfactant. Fig. 3 shows the change in mean diameters of albumin microspheres with changes in HLB values of surfactant. When the surfactant HLB value was 1.8, diameters of albumin microspheres were larger with a mean diameter of about 80 μm. By contrast, when the HLB value was 8.6, diameters of albumin microspheres decreased to a mean diameter of about 30 μm. In general, diameters of albumin microspheres become smaller with increasing HLB values of surfactants.

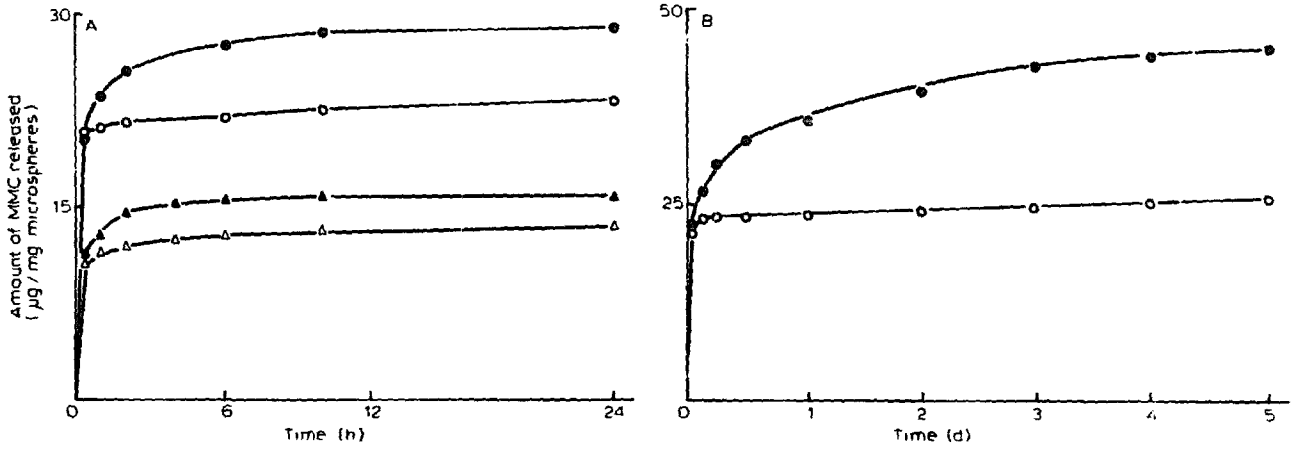

Fig. 4. In vitro mitomycin C release from albumin microspheres containing mitomycin C prepared by Methods I, II and III. (A) Method I (△, ▲) and Method II (○, ●); △, ○, without protease; ▲, ●, with protease; (B) Method III: (○) without protease; (●) with protease.

In vitro MMC release of albumin microspheres

MMC release from albumin microspheres prepared from Methods I and II is shown in Fig. 4A and MMC release from Method III is shown in Fig. 4B. Release of MMC from microspheres prepared by all 3 methods was negligible in pH 7.4 phosphate buffer after the initial burst effect. In pH 7.4 phosphate buffer containing Protease®, after the burst effect, MMC was released very slowly from albumin microspheres prepared by Method I and the release pattern of MMC was very similar to the release in phosphate buffer only. MMC release in phosphate buffer containing Protease® from albumin microspheres prepared by Method II showed a 70% burst effect followed by a slow, constant release for at least 10 h, whereas slow MMC release from the microspheres by Method III in phosphate buffer containing Protease® was observed up to 5 days following the release of 50% of the drug loaded by a burst effect. Drug contents in the albumin microspheres from Methods I, II and III, were determined by treatment with Protease® (refer to Methods) and were 15.6 ± 0.5, 28.6 ± 0.7, 45.6 ± 1.5 μg/mg microspheres, respectively, with highest drug contents in the albumin microspheres from Method III.

Biodegradability of albumin microspheres

Dry weights of albumin microspheres prepared from Methods I, II and III after treatment with several proteases are shown in Table 1. Since all microspheres in the control (no enzyme) were not recovered, this weight value (8.55 mg) was postulated to be 100% recovery, since the loss (1.45 mg) might be due to that occurring during filtration and might be consistent or equal in all weighing measurements. Albumin microspheres digested with collagenase appeared not to be digested by this enzyme. By contrast, treatment with papain shows a 58% digestion of albumin microspheres from Method I, yet albumin microspheres from Methods II and III were hardly digested at all. In the treatment with trypsin, albumin microspheres from Methods I and II were nearly completely digested while about 40% albumin microspheres from Method III were digested. Treatment with Protease® showed similar results to treatment by

TABLE 1

Biodegradability of albumin microspheres 96 h after treatment with several proteolytic enzymes

Data are shown as percentages of remaining weight against that of microspheres treated with buffer solution alone.

	Collagenase	Papain	Protease®	Trypsin
Method I	75	42	8	13
Method II	90	76	15	21
Method III	96	92	26	60

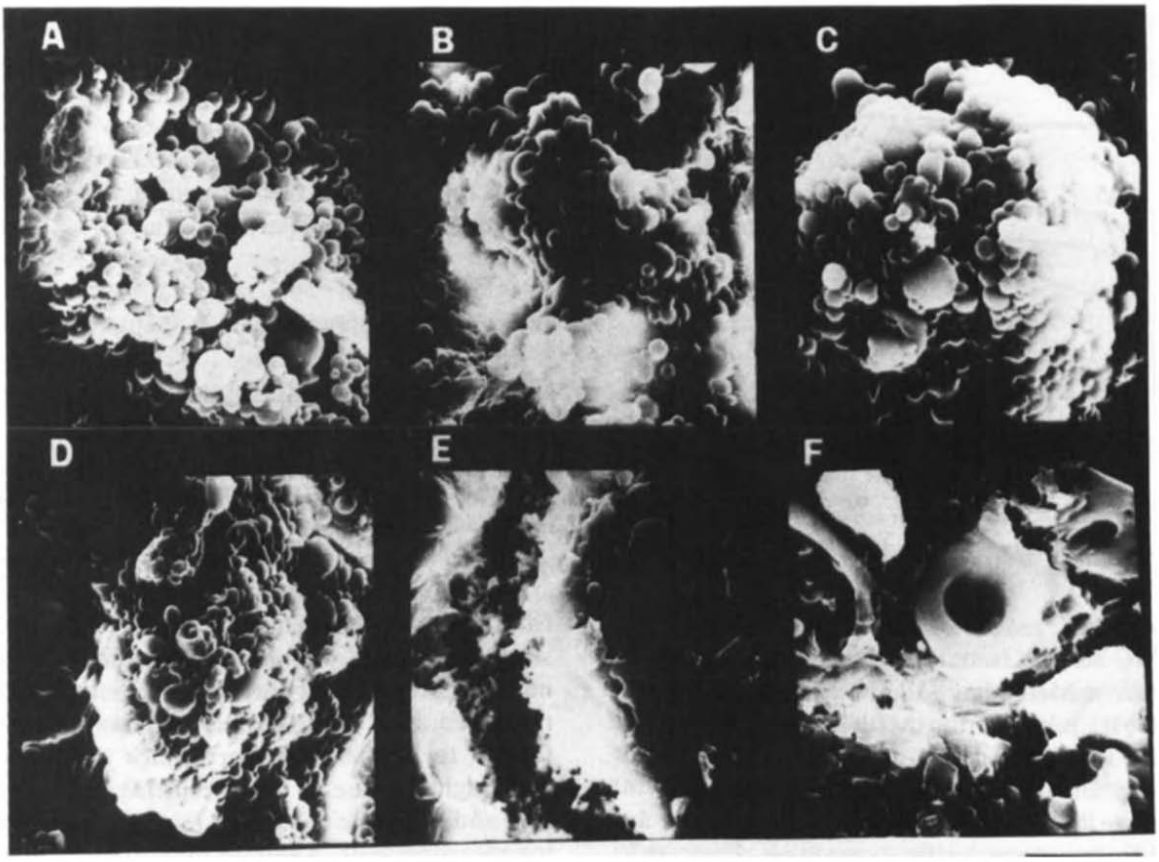

Fig. 5. Scanning electron micrographs of albumin microspheres prepared by Method I, 4 days after treatment with pH 7.4 phosphate buffer containing several proteases. (A) Non-treatment, (B) treatment with pH 7.4 phosphate buffer only, (C) treatment with pH 7.4 phosphate buffer containing collagenase, (D) treatment with pH 7.4 phosphate buffer containing papain, (E) treatment with pH 7.4 phosphate buffer containing Protease®, (F) treatment with pH 7.4 phosphate buffer containing trypsin. Bar = 10 μm.

trypsin except that albumin microspheres from Method III were also nearly completely digested.

Surface topographical changes in albumin microspheres prepared by Method I after treatment with each enzyme were observed using a scanning electron microscope (Fig. 5). Figure 5A shows untreated control microspheres, while Fig. 5B shows microspheres treated with saline. Both figures show no observed morphological changes in the shape of the albumin microspheres. Fig. 5C shows that microspheres treated with collagenase demonstrate little morphological changes. Figure 5D however, which shows microspheres treated with papain demonstrates that the surface of albumin microspheres has become irregular and uneven despite the fact that these microspheres have kept their spherical structure. In contrast, Fig. 5E and F which show microspheres treated with Protease® and trypsin, respectively, demonstrate cases where albumin microspheres were almost completely digested by proteolytic enzymes and failed to retain any resemblance of the original albumin microspheres.

Fig. 6 shows stereoscopic photographs of albumin microspheres prepared from Method III taken before treatment and 2 and 4 days after treatment with Protease®. Albumin microspheres which were not yet treated maintain a perfectly spherical structure (Fig. 6A). In contrast, albumin microspheres after 2 days of treatment with Protease®, show rough irregular surfaces advanced by proteolytic enzyme digestion (Fig. 6B). After 4

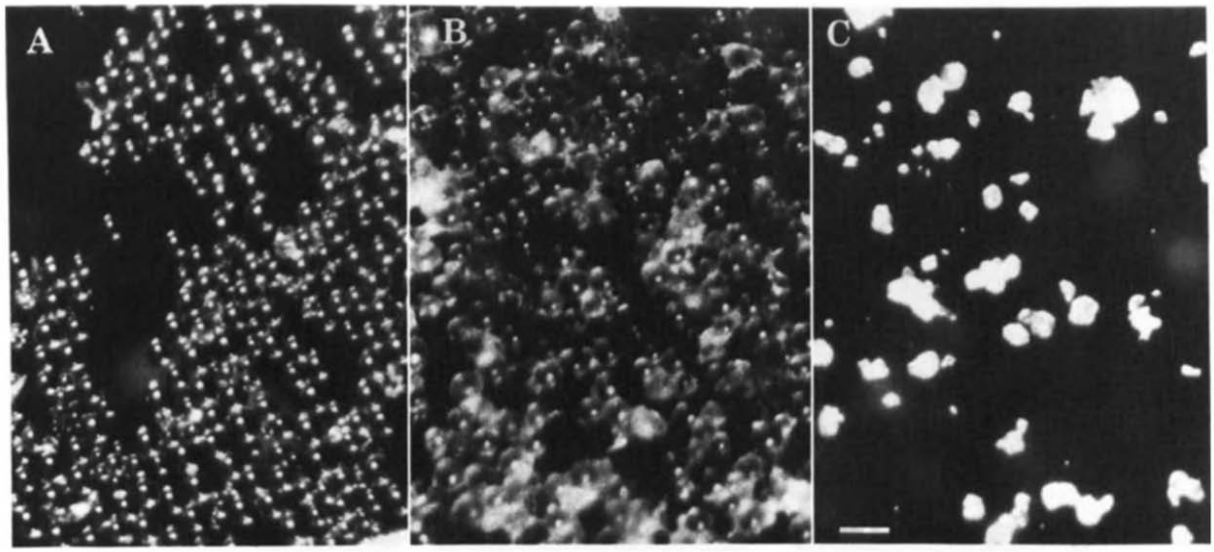

Fig. 6. Stereoscopic micrographs of albumin microspheres (Method III). (A) Non-treatment, (B) 2 days after treatment with Protease®, (C) 4 days after treatment with Protease®. Bar = 100 μm.

days treatment with the enzyme, albumin microspheres did not maintain a spherical structure and were aggregated with each other (Fig. 6C). The weight loss showed that the microspheres were nearly completely digested by Protease® (Table 1).

Biocompatibility of albumin microspheres

Fig. 7 shows light micrographs of albumin microspheres from Methods I, II and III taken after injection into annulus muscles of rats. Figure 7A shows results from control rats where saline was injected into the muscle. Here, no inflammation was observed after 3 days. Figure 7B and C show muscle samples 7 days after injection of albumin microspheres from Methods I and II. In this case a weak inflammation exists around the muscle tissue which has been invaded by neutrophils. Here a healing process may be observed. Fig. 7D, E and F show muscle tissue specimens after 3, 7 and 30 days, respectively, which have been injected with albumin microspheres from Method III. Three days after injection of albumin microspheres, a weak, acute inflammation was observed around the muscle tissues and many neutrophils had migrated around them. After 7 days, the inflammation becomes weak and the healing process appears to progress. After 30 days, the healing process has progressed further and neutrophils are not evidenced in the area.

Discussion

Consistent problems encountered in the development of new preparation methods for albumin microspheres are (1) sufficient yields of microspheres within a desired particle size for the intended purpose, (2) drug loading and (3) releasing character of the drug from the matrix. These problems may be overcome by development of Method III which can produce microspheres as materials for chemoembolization. Yields of albumin microspheres, prepared with Method III, were higher than those prepared with Method I. This may be due to the ability of Method III to keep emulsification constant since the viscosity of *n*-octane (which was used when the albumin solution was emulsified) was lower than that of cottonseed oil (0.5 and 70 cP, respectively). Further, diameter measurements of albumin microspheres demonstrate that the size can be regulated by utilizing various surfactants.

The release of glutaraldehyde from albumin microspheres was not observed using the Schiff

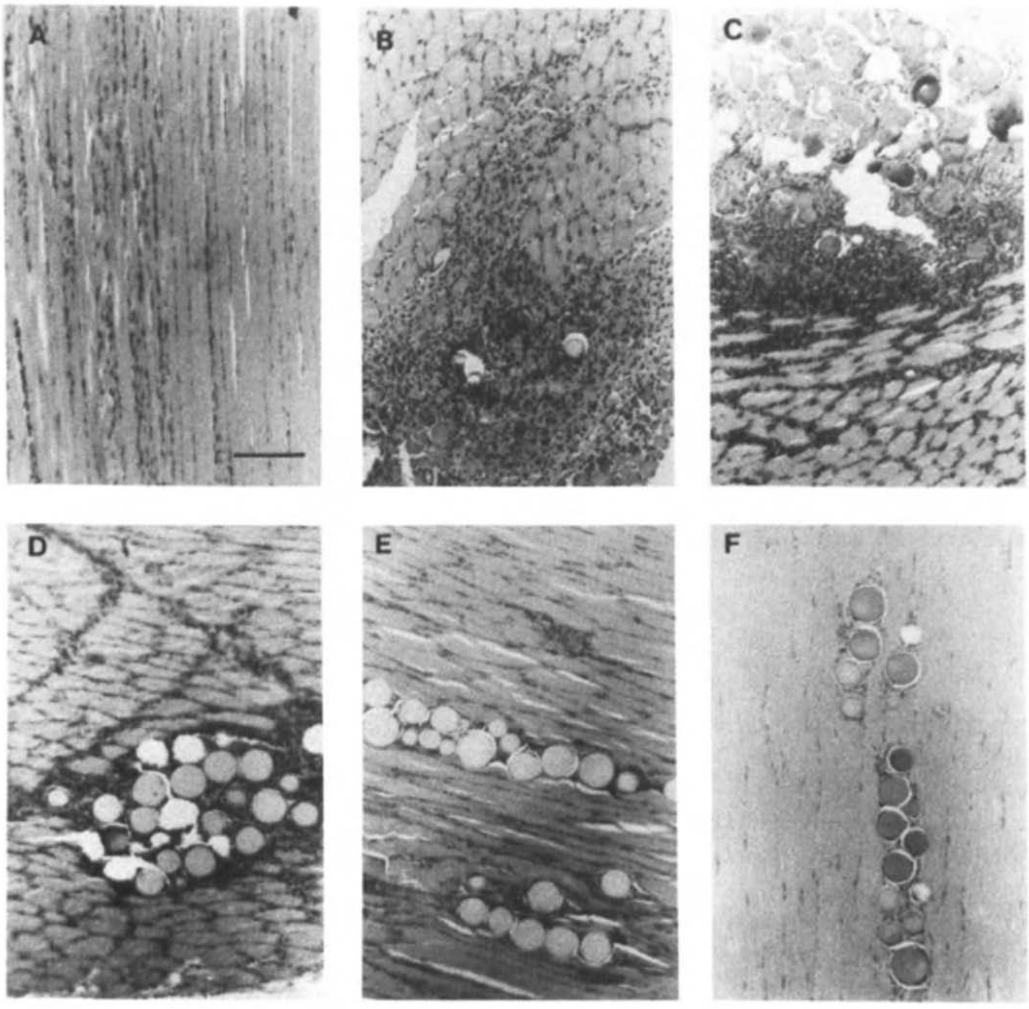

Fig. 7. Micrographs of the muscles after injection of saline or albumin microspheres. (A) 7 days after injection of saline, (B) 7 days after injection of albumin microspheres (Method I), (C) 7 days after injection of albumin microspheres (Method II), (D) 3 days after injection of albumin microspheres (Method III), (E) 7 days after injection of albumin microspheres (Method III), (F) 30 days after injection of albumin microspheres (Method III). Bar = 100 μm.

test. The amount of glutaraldehyde consumed for cross-linking with albumin microspheres in Methods II and III was not measured but it might be small from the result of Burgess' work (Burgess et al., 1987). Although no reduction step for removing free glutaraldehyde was included in Methods II and III, it would be clear that it was not present in the albumin microspheres prepared. It is thought that sufficient washing of microspheres with the solvent is effective for the removal of not only *n*-octane but also free glutaraldehyde.

Drug content in the microspheres, degraded with Protease® has been increased by adding glutaraldehyde as a cross-linking agent. Increased drug content might be due to improved drug–matrix interaction where drug uptake within the microsphere network structure is enhanced by the effects of a cross-linking agent. A difference in the drug contents in albumin microspheres prepared in Methods II and III may be due to a difference in hydrophobicity of solvents used at the emulsification and solidification steps for preparing al-

bumin microspheres. Although glutaraldehyde is the most efficient cross-linking agent, unfortunately the mechanism of its interaction with protein is quite complex (Sokoloski and Royer, 1984). Therefore, the structure of reaction products cannot be imaged. As cross-linking of albumin and drugs with glutaraldehyde not only affects the drug content in the microspheres but also drug release from the microspheres, it is well worthy to clarify its mechanism.

MMC release from albumin microspheres by Methods I, II and III was barely discernible after an initial burst effect. However, MMC in microspheres by Method III was released gradually over 5 days when using proteolytic enzymes and the magnitude of initial burst effect was suppressed to 50% and below. These results suggested that albumin microspheres prepared from Method III are better than other preparations in view of yield, drug loading and release character.

In vitro biodegradability of albumin microspheres by Methods I, II and III was especially accelerated by trypsin and Protease® and was also reported by Dickinson et al. (1981) who found that albumin microspheres were gradually digested by the same kind of proteases and mast cells in vitro. Although in vivo biodegradability of albumin microspheres was not yet examined, it was expected that they may have high biodegradability and digestibility. Before exposure to enzymes during the degradation process, albumin microspheres maintain their spherical structure. Upon exposure to enzymatic solutions, the microspheres digest irregularly to crumbling nonspherical structures.

When albumin microspheres from Methods I, II and III were injected into muscle tissues, a general weak inflammatory response was immediately observed in contrast to absence of inflammation observed in the controls (saline only), with only little further inflammation elicited thereafter. The same types of responses from all 3 microspheres suggest that albumin microspheres from the new Methods II and III elicit nearly identical physiological response and comparable biocompatibility to albumin microspheres from Method I which have been clinically applied in radiology as scanning agents. In addition, albumin microspheres, particularly those prepared by Method III show sustained release of model drugs for extended time. Thus, albumin microspheres prepared by Method III are promising as a candidate tool for clinical application as a drug carrier.

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References

Burgess, D.J., Davis, S.S. and Tomlinson, E., Potential use of albumin microspheres as a drug delivery system. I. Preparation and in vitro release of steroids. *Int. J. Pharm.*, 39 (1987) 129–136.

Dickinson, H.R., Hiltner, A., Gibbons, D.F. and Anderson, J.M., Biodegradation of A poly(-amino acid) hydrogel. *J. Biomed. Mater. Res.*, 15 (1981) 577–589.

Fujimoto, S., Endoh, F., Kitsukawa, Y., Okui, K., Morimoto, Y., Sugibayashi, K., Miyakawa, A. and Suzuki, H., Continued in vitro and in vivo release of an antitumor drug from albumin microspheres. *Experientia*, 39 (1983) 913–916.

Kato, T., Nemoto, R., Mori, H. and I. Kumagai, Sustained-release properties of microcapsulated mitomycin C with ethylcellulose infused into the renal artery of the dog. *Cancer*, 46 (1980) 14–21.

Lawravel, W.H., Mitchell, J.L., Guess, W.L. and Autian, J., Toxicity of plastics used in medical practice. I. *J. Pharm. Sci.*, 52 (1963) 958–963.

Longs, W.E., Iwata, H., Lindheimer, T.A. and Goldberg, E.P., Preparation of hydrophilic albumin microspheres using polymeric dispersing agent. *J. Pharm. Sci.*, 71 (1982) 1323–1328.

Sokoloski, T.D. and Royer, G.P., Drug entrapment within native albumin beads. In Davis, S.S., Illum, L., McVie, J.G. and Tomlinson, E. (Eds.), *Microspheres and Drug Therapy – Pharmaceutical, Immunological and Medical Aspects*, Elsevier, Amsterdam, 1984, pp. 295–307.

Sugibayashi, K., Morimoto, Y., Nadai, T., Kato, Y., Hasegawa, A. and Arita, T., Drug-carrier property of albumin microspheres in chemotherapy. II. Preparation and tissue distribution in mice of microsphere-entrapped 5-fluorouracil. *Chem. Pharm. Bull.*, 27 (1979) 204.

Wilson, J., Pigott, G.H., Schoen, F.J. and Hench, L.L., Toxicology and biocompatibility of bioglasses. *J. Biomed. Mater. Res.*, 15 (1981) 805–817.