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Journal of Macromolecular Science, Part A
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597274

Preparation of Albumin Microspheres Grafted Galactose Residues Through Polyethylene-Glycol Spacers, Release Behavior of 5-Fluorouracil from Them, and Their Lectin-Mediated Aggregation

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To cite this Article Ohya, Yuichi , Takei, Toshiaki , Fukushima, Haruya and Ouchi, Tatsuro(1991) 'Preparation of Albumin Microspheres Grafted Galactose Residues Through Polyethylene-Glycol Spacers, Release Behavior of 5-Fluorouracil from Them, and Their Lectin-Mediated Aggregation', Journal of Macromolecular Science, Part A, 28: 8, 743 — 760
To link to this Article: DOI: 10.1080/00222339108054054
URL: http://dx.doi.org/10.1080/00222339108054054

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J. MACROMOL. SCI.—CHEM., A28(8), pp. 743–760 (1991)

PREPARATION OF ALBUMIN MICROSPHERES GRAFTED GALACTOSE RESIDUES THROUGH POLYETHYLENE-GLYCOL SPACERS, RELEASE BEHAVIOR OF 5-FLUOROURACIL FROM THEM, AND THEIR LECTIN-MEDIATED AGGREGATION

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ABSTRACT

Small-sized albumin gel microspheres, MSs, containing 5-fluorouracil (5FU) with targeting moieties on their surfaces (average diameter: 1.5 μm) were prepared by the glutaraldehyde crosslinking method and suspension technique. Since galactose is known to interact specifically with the asialoglycoprotein receptor on hepatocyte, the galactose residues were introduced on the surface of MSs as the targeting moieties for *hepatoma* through polyethylene glycol (PEG) spacers. PEG spacers were employed to depress the immunogenicity of albumin, to keep the mobility of the galactose residues, and to heighten the distributive stability of the MSs in aqueous solution. It was confirmed by ESCA analysis that the PEG chains were introduced onto the surfaces of MSs. The amount of galactose residues introduced to MS were estimated to be 0.013 wt%. The intra-MSs aggregation was observed by the addition of

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Ricinus Communis Agglutinin I (RCA120) into the MS suspension, and then the aggregation of MSs was dissociated by addition of free lactose. Moreover, by incubation of the MSs with human *hepatoma* HLE cells, the phenomena of MS's specific binding onto HLE cell surfaces and phagocytosis of MSs by HLE cells were observed. These results suggested that the galactose residues on the surface of MSs were recognized with the galactose receptors on *hepatoma* cell surfaces. The release rate of 5FU from MSs was investigated *in vitro* in physiological saline at 37°C. About 90% of encapsulated 5FU were found to be released from MSs through incubation for 8 h.

INTRODUCTION

It is well known that 5-fluorouracil (5FU) has a remarkable antitumor activity which is accompanied, however, by side effects [1, 2]. As one study on the drug delivery system, the design of albumin microspheres, MSs, which release the antitumor drugs slowly, has been investigated. To achieve an excellent clinical therapeutic effect against cancer, the construction of MSs having cell-specific targetability is desired.

In order to design MSs having targetability to hepatocyte, the present paper deals with the preparation of albumin MSs having galactose residues grafted on their surfaces. Some kinds of saccharide have been found to play important roles in such biological recognitions as receptor–ligand binding and cell–cell adhesion [3–6]. For example, liver parenchymal cells have a receptor which can specifically recognize galactose [4], phagocytic cells have a receptor to mannose [5], and fibroblasts have a mannose-6-phosphate specific receptor [6]. Recently, novel drug delivery systems employing liposome and having the ability for recognition via the saccharide unit were reported by Sunamoto's group [7–9].

Biodegradable albumin, which is one of the most popular plasma proteins, has been used as a microsphere material in many previous studies on drug delivery systems [10–14]. Thermally denaturated albumin was crosslinked with glutaraldehyde to give MSs. The unreacted aldehyde groups on the surface of MSs were employed to graft galactose residues through polyethylene-glycol (PEG) spacers. Since galactose is cell-specifically recognized by hepatocyte, MSs having galactose residues can be expected to have a targetability to liver. PEG was employed to decrease the immunogenicity of albumin, to increase the mobility of

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galactose residues, and to increase the distributive stability of MSs in aqueous solution.

The present paper is concerned with the preparation of small-size albumin MSs with grafted galactose residues on their surface, MS(Alb-PEG-Gal)s, the release behavior of 5FU from them, the phenomenon of their specific aggregation induced by lectin, and the phenomenon of their phagocytoses by human *hepatoma* HLE cells.

EXPERIMENTAL

Materials

Bovine serum albumin was purchased from Wako Chemical Co. and used without further purification. PEO-amine [MW = 1000 (n = 20), 6000 (n = 134)] was obtained from Kawaken Fine Chemical Co. Such lectins as Ricinus Communis Agglutinin I (RCA120) and Concanavalin A (Con A) were purchased from Funakoshi Co. and used without further purification. Toluene, methanol, acetone, and chloroform were purified by the usual distillation methods. Water was ion exchanged, redistilled, and passed through a microfilter before use. Lactose, glutaraldehyde, and the other reagents were commercial grade and used without further purification.

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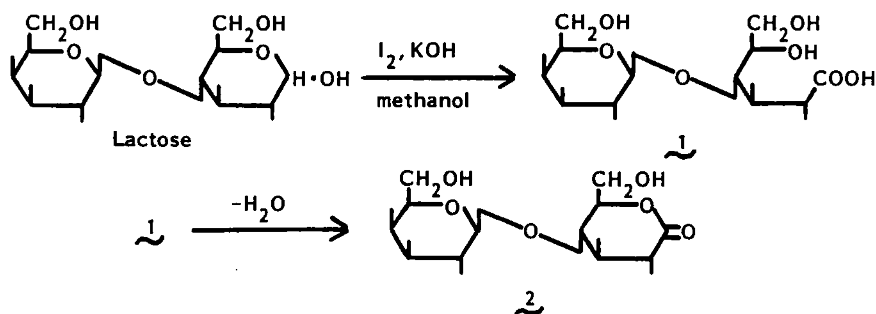
Preparation of Albumin Microsphere (MS(Alb))

Albumin microspheres crosslinked with glutaraldehyde, MS(Alb)s, were prepared according to the thermal denaturation procedure of Sugibayashi et al. [10] and the glutaraldehyde crosslinking technique with a minor modification of the method of Longo et al. [11]. The sodium salt of 5-fluorouracil (5FU/Na) (100 mg) and 1.0 g bovine serum albumin (BSA) were dissolved in 2.0 mL distilled water. Cottonseed oil (100 mL) containing 10 vol% Span 80 was added to the aqueous solution, stirred vigorously, and then sonicated at room temperature. The emulsion obtained was heated to 180°C and stirred for 15 min. After cooling the emulsion to room temperature, the glutaraldehyde solution saturated with toluene was added slowly to the emulsion and then stirred for 6 h. The suspension obtained was centrifuged at 3500 rpm for 10 min and washed 3 times with toluene, 3 times with acetone, and once with methanol. The MS(Alb)s obtained were dried under vacuum.



SCHEME 1.

Preparation of MS(Alb-PEG-Gal)

Albumin gel microspheres grafted galactose residues through polyethylene-glycol spacers, MS(Alb-PEG-Gal)s, were prepared according to the routes shown in Schemes 1-3.

Synthesis of Lactonolactone 2 [15]. Lactose (3.77 g) was dissolved in a 50-mL solution of water/methanol (2:3, v/v). An iodine solution in methanol (5.7 g/80 mL) was added to the lactose solution, and then the mixture was kept at 40°C. A KOH solution (4 wt%) in methanol was added dropwise into the mixture with stirring until the iodine color faded. The crystalline product, precipitated by cooling in an ice-bath, was filtered off and washed with cold methanol, cold ether, and then recrystallized from a mixed solvent of methanol/water (9:1, v/v). The potassium lactonoate obtained was converted to lactonoic acid by pass-

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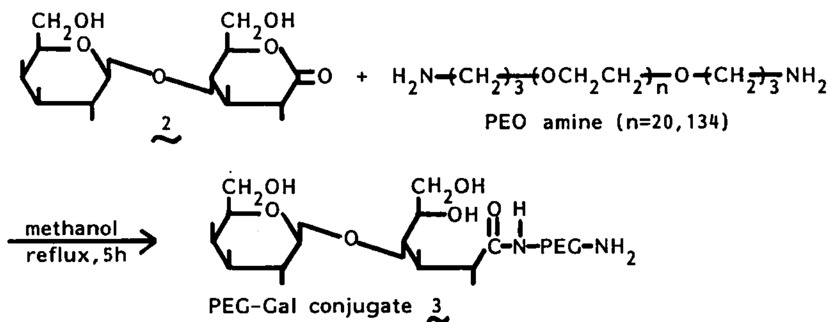
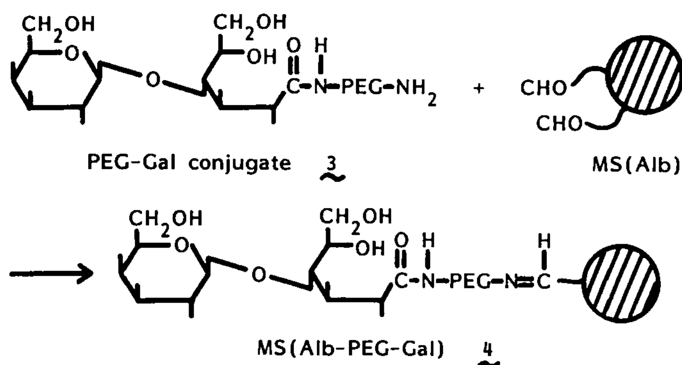


SCHEME 2.



SCHEME 3.

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ing the aqueous solution through a column of ion-exchange resin (Amberlite IR-120B). The conversion of potassium lactonate to free acid was confirmed by the shift of the IR peak of C=O (1630 to 1738 cm^{-1}). The acidic eluent was collected and concentrated in a rotary evaporator. Repeated evaporations of methanol and ethanol solutions of the lactonoic acid gave lactonolactone containing a small amount of water in quantitative yield. The yield was 2.5 g (67.2%). IR(KBr): 3356 (OH), 2935 (CH), 1738 (C=O), and 1078 cm^{-1} (C—O—C).

Synthesis of PEG-Gal Conjugate 3. Lactonolactone (0.5 g) and PEO amine (n = 20 or 134) (molar ratio, 1.2:1) were dissolved in 7.0 mL methanol and then refluxed for 6 h at 70–80°C to afford the PEG-Gal conjugate **3**. Conjugate **3** was freeze-dried for use in further syntheses.

PEG-Gal conjugate **3**: ^1H NMR (DMSO-d_6) δ3.56 (m, C$\underline{H}$(OH)) and 3.67 ppm (m, CH_2). IR(KBr): 3382 (OH); 2871 (CH); 1651, 1537 (CONH); and 1105 cm^{-1} (C—O—C).

Immobilization of PEG-Gal Conjugate to MS(Alb). The solution of PEG-Gal conjugate **3** in chloroform was added dropwise into the suspension of MS(Alb)s in the mixed solvent of toluene/chloroform (1:1, v/v). Unreacted aldehyde groups on the surface of MS(Alb)s were allowed to react with the terminal amino group of conjugate **3**. After stirring for 18 h, the MS(Alb-PEG-Gal)s obtained were centrifuged at 4000 rpm for 10 min and then washed 3 times with a mixed solvent of toluene/chloroform (1:1, v/v), 3 times with acetone, and once with methanol, and then dried under vacuum.

Estimation of Size of Microsphere

The size of the MSs obtained was estimated by using JEOL JSM-35 scanning electron microscopy (SEM).

Estimation of Amount of Introduced Saccharide Residues

The amount of saccharide residues introduced on the surface of MSs was determined by the orcinol-sulfuric acid method [16]. About 10 mg of MSs was dissolved in 0.1 *N* NaOH aqueous solution and cooled in an ice-bath. The orcinol solution [a mixture of orcinol aqueous solution (64 mg/4 mL) and 30 mL of 60 vol% H_2SO_4 solution] was added to the MS's solution with cooling. The mixture was heated to 80°C and kept there for 15 min, and then cooled to room temperature in the water bath. The UV absorbance of the mixture solution obtained was measured at 425 nm to estimate the amount of saccharide residue.

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Analysis of Surface of Microsphere by ESCA

Electron spectroscopy for chemical analysis (ESCA) of the MS(Alb)s and MS(Alb-PEG-Gal)s obtained was measured by using a JEOL X-ray photoelectron spectrometer JPS-90MX micro. An anode of Mg was used as the x-ray (MgK_α) source. The pass energies (ES) were 50 and 10 eV for measurements of total region spectra and narrow region spectra, respectively. The 1s peak of aliphatic carbon was assigned a binding energy of 285.0 eV to correct the energy shift due to electrification. Wave analysis was carried out by using the mixed Gaussian–Lorenzian (80:20) function.

Observation of Lectin-Mediated Aggregation of MS(Alb-PEG-Gal)s

RCA120 and ConA were used as two kinds of lectin. Lectin solution (25 μL, 5.0 mg/mL) was added to 3 mL of the MS(Alb-PEG-Gal) suspension in phosphate buffer solution (0.3 mg/mL) of pH 7.0, and the change in turbidity was examined by measurement of transmittance at 600 nm.

Observation of Uptake of MS(Alb-PEG-Gal) into *Hepatoma*

MS(Alb-PEG-Gal)s were added to the PBS of HLE *hepatoma* cells and incubated at 37°C. After incubation for 0, 2, 8, 24, or 48 h, the cells were washed with PBS, freeze-dried, and rendered electrically conductive with a 15-nm coating of gold to observe the uptake of MS(Alb-PEG-Gal)s to *hepatoma* cells by SEM.

Measurement of Release Rate of 5FU from MS(Alb-PEG-Gal)

The release rate of 5FU from the microspheres was determined by a dynamic dialysis system with a cellulose tube [12]. MS(Alb-PEG-Gal)s (50 mg) suspended in 5.0 mL physiological saline or 0.4 wt% trypsin aqueous solution in a cellulose tube were dialyzed in physiological saline or 0.4 wt% trypsin aqueous solution at 37°C. The amount of 5FU released from the MSs was measured by using GPC (column: Shodex OHpack B-805; eluent: 1/75 *M* KH_2PO_4-Na_2HPO_4 buffer solution of pH 7.0; detector: UV_{265}).

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RESULTS AND DISCUSSION

Size of Microsphere

An SEM view of the prepared MS(Alb-PEG(n = 134)-Gal)s is shown in Fig. 1. MSs of round and nonporous shapes were confirmed to have been prepared. The distribution of particle sizes of MSs is shown in Fig. 2. The average diameter of the MSs obtained was estimated to be about 1.5 μm, based on SEM. This small size is usable for intravenous injection.

Amount of Saccharide Residues Introduced onto Microspheres

The amount of saccharide residues introduced on the surface of MSs was estimated by the orcinol–sulfuric acid method [13]. This method is often used to estimate the saccharide residue of glycoprotein. Because bovine serum albumin is a simple protein, there is no saccharide residue in native albumin molecules. Therefore, all of the detectable saccharide residues are attributable to PEG-Gals introduced to the MSs. Measurement was carried out by using lactonolactone as the standard reagent. The amount of lactose residues attached to 1 g MS(Alb-PEG(n = 20)-

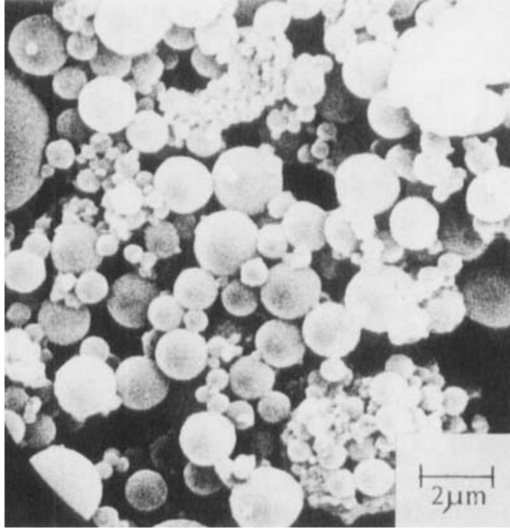

FIG. 1. The SEM view of MS(Alb-PEG(n = 134)-Gal)s.

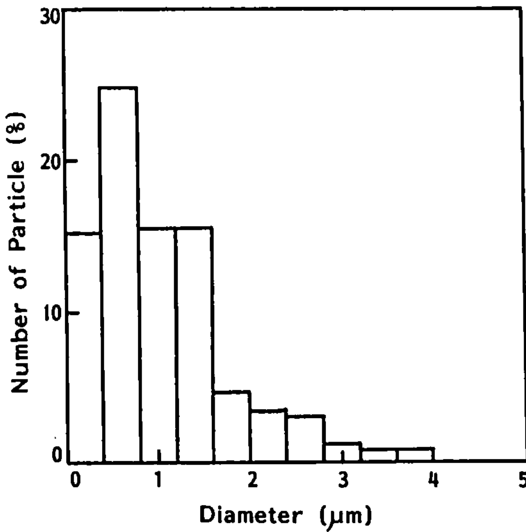

FIG. 2. Distribution of particle size of MS(Alb-PEG(n = 134)-Gal)s.

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Gal) was estimated to be 0.133 mg. The amount of saccharide residues introduced onto the MSs was evaluated as 0.013 wt%. Although the exact molar ratio of saccharide residue and albumin on the surface of the MSs obtained could not be calculated by this method, the degree of introduction of PEG-Gals to the MSs was presumed to be high.

ESCA Analysis of Surface of Microsphere

ESCA data provide information with regard to the surface abundance of each element from the topmost ~50 Å. The total ESCA spectrum region of MS(Alb-PEG(n = 20)-Gal) is shown in Fig. 3, and the O_{1s}, N_{1s}, and C_{1s} spectra of MS(Alb-PEG-Gal) and MS(Alb) are shown in Fig. 4. The atom contents of C, N, and O on the surface of MS(Alb-PEG-Gal)s were estimated to be 74.47 mol% (69.55 wt%), 8.38 mol% (9.12 wt%), and 17.14 mol% (21.33 wt%), respectively. On the other hand, the atom contents of C, N, and O of MS(Alb) were estimated to be 74.72 mol% (69.86 wt%), 8.59 mol% (9.36 wt%), and 16.68 mol% (20.78 wt%), respectively. Trace amounts of S from cystein and methionine in albumin and F from 5FU were detected in both MSs. The molar ratios of N/C and O/C of MS(Alb-PEG-Gal) were 0.115 and 0.233, and

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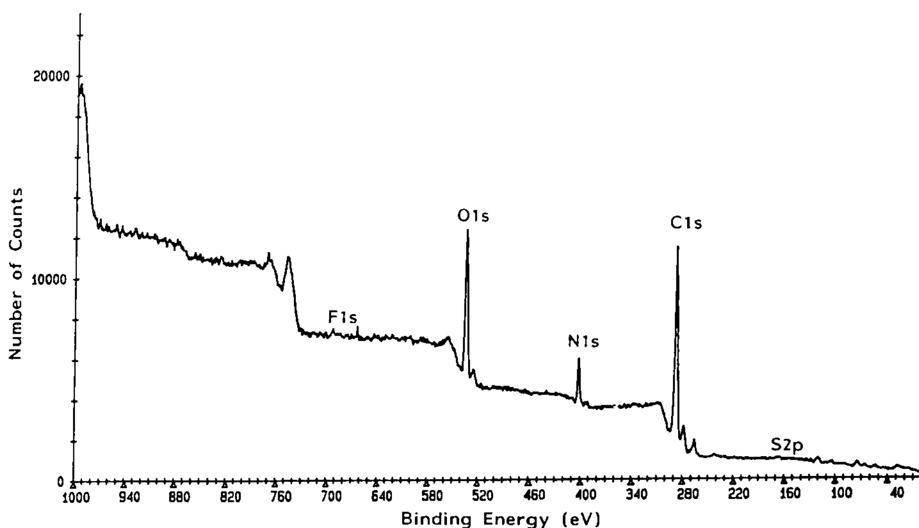

FIG. 3. Total ESCA spectrum region of MS(Alb-PEG-Gal).

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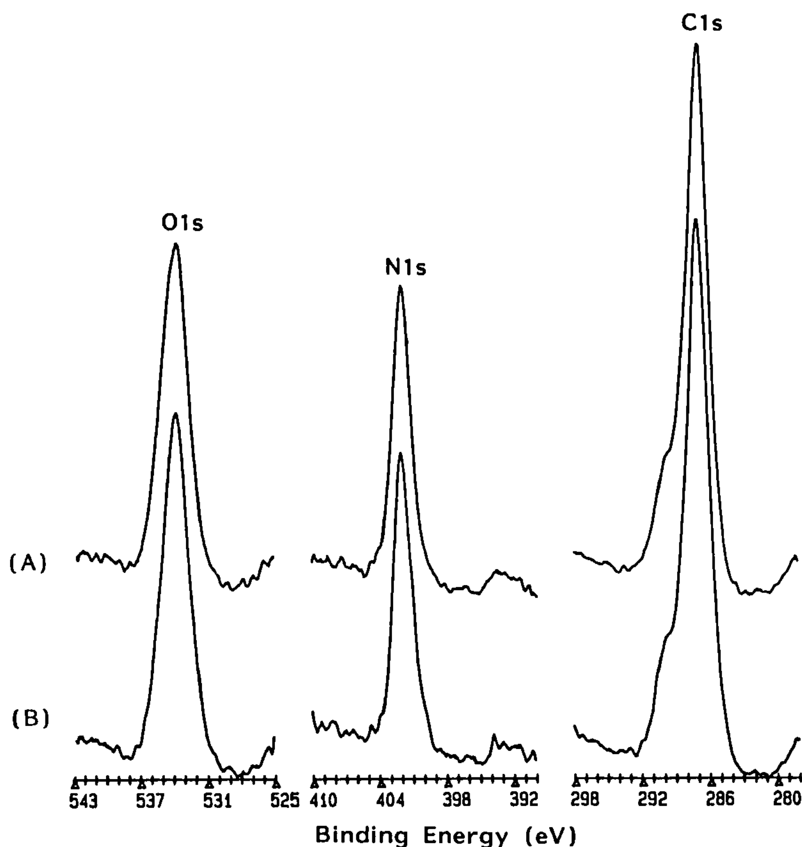

FIG. 4. ESCA spectra of the O_{1s}, N_{1s}, and C_{1s} regions of MS(Alb-PEG(n = 20)-Gal) (A) and MS(Alb) (B).

those of N/C and O/C of MS(Alb) were 0.113 and 0.230, respectively. The MS(Alb-PEG-Gal) had a higher nitrogen content and a lower oxygen content than MS(Alb). These results suggest that PEG and saccharide residues were introduced on the surface of MS by the method of chemical modification with PEG-Gal conjugate **3**. The C_{1s} spectra of MS(Alb-PEG-Gal) are shown in Fig. 5. The results of wave analysis show that the C—C, C—O (or C—N), and C=O contents in the total carbon were estimated to be 68.0, 17.3, and 14.7%, respectively.

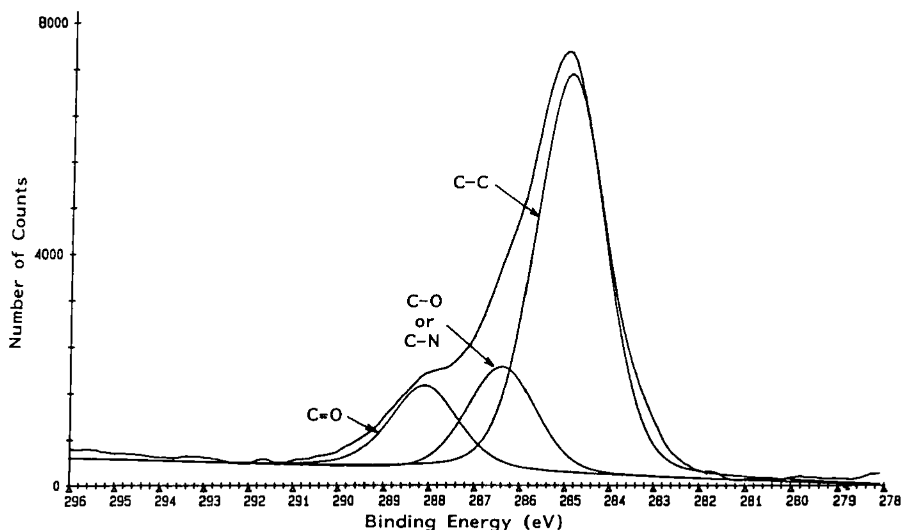

FIG. 5. ESCA spectrum of the C_{1s} region of MS(Alb-PEG(n = 20)-Gal). The wave analysis was carried out by using the mixed Gaussian-Lorenzian (80:20) function.

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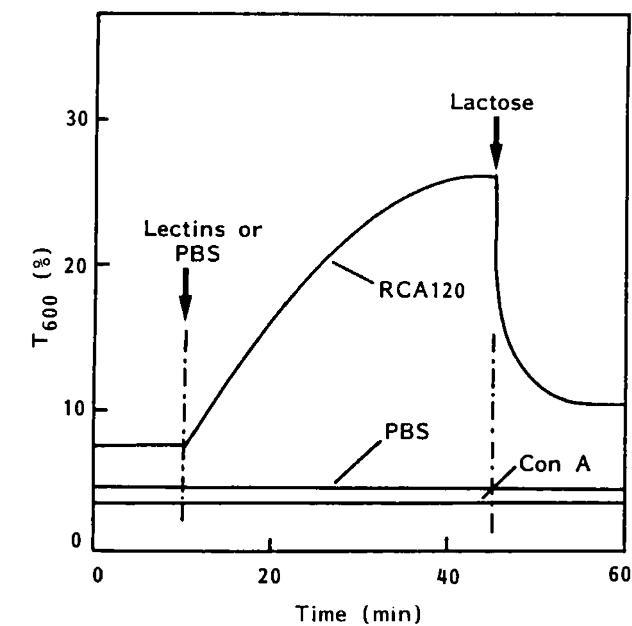
Lectin-Mediated Aggregation of MS(Alb-PEG-Gal)s

Lectin RCA120 is well known to bind specifically to the galactose unit. The addition of RCA120 to the MS(Alb-PEG-Gal) suspension was found to induce decreased turbidity of the suspension (Fig. 6); that phenomenon meant the occurrence of aggregation by the addition of RCA120. In the case of its addition to a MS(Alb) suspension with no grafted Gal, the change in turbidity was not observed. Moreover, the aggregation of MS(Alb-PEG-Gal)s through the intra-MS bridge of RCA120 was dissociated by the addition of excessive lactose. On the contrary, a change in the turbidity of the MS(Alb-PEG-Gal) suspension was not observed upon the addition of ConA, which specifically binds to the α-D-mannose or the α-D-glucose unit. Therefore, the occurrence of the aggregation of MS(Alb-PEG-Gal)s is attributed to specific binding of RCA120 to galactose residues located on their surfaces.



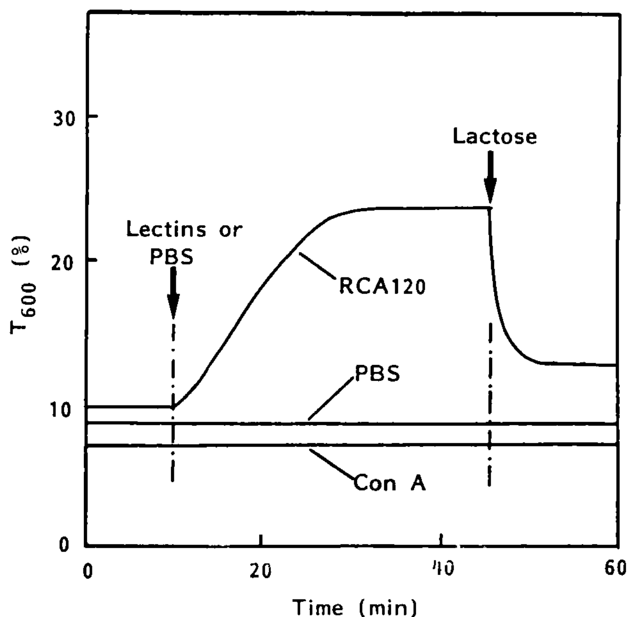


FIG. 6. Aggregation of MS(Alb-PEG-Gal)s in PBS by lectin addition. (A): MS(Alb-PEG(n = 20)-Gal), (B): MS(Alb-PEG(n = 134)-Gal).

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Uptake of MS(Alb-PEG-Gal) into *Hepatoma*

The results of SEM of HLE *hepatoma* phagocytosing MS(Alb-PEG-Gal)s are shown in Fig. 7. A few MSs added to the cell culture were observed on the surfaces of *hepatoma* cells after incubation for 2 h. Furthermore, most of the MSs added to the cell culture were observed to be taken up into the *hepatoma* cell after incubation for 24–48 h.

These results suggest that galactose residues located on the surface of MS(Alb-PEG-Gal)s could be recognized by receptors on the surface of hepatocytes, and that MSs were taken up into hepatocytes via galactose-receptor mediated phagocytosis.

Release Rate of 5FU from MS(Alb-PEG-Gal)

The amount of 5FU encapsulated in MS(Alb-PEG-Gal) was estimated to be about 0.01 mg/mg. The results of the release rate of 5FU from MS(Alb-PEG(n = 20)-Gal)s in physiological saline and 0.4 wt% trypsin aqueous solution at 37°C are shown in Fig. 8. Although the release rate of 5FU in the trypsin solution tended to be higher than that in physiological saline, the remarkable increase in the release of 5FU was not recognized, regardless of the presence of enzyme. The amounts of 5FU released in physiological saline for 1 and 8 h were about 50 and 90%, respectively. The release rate of encapsulated material from the small-sized MSs obtained *in vitro* was more rapid than that from the general-sized MSs (diameter: 30–50 μm) of albumin reported previously [12–14]. When cell-specific targeting to hepatocytes is achieved by galactose moieties, such a relatively rapid release of 5FU from MS(Alb-PEG-Gal)s is presumed to be effective *in vivo*.

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CONCLUSION

(1) Small-sized albumin gel microspheres grafted galactose residues through polyethylene-glycol spacers, MS(Alb-PEG-Gal)s, were prepared by a suspension technique.

(2) The location of galactose residues on the surface of MSs was confirmed through ESCA analysis, and the occurrence of intra-MSs aggregation was determined by the addition of RCA120 lectin.

(3) The phenomena of specific binding of MSs onto human *hepatoma* HLE cell surfaces and phagocytosis of MSs by HLE cells were observed by SEM.

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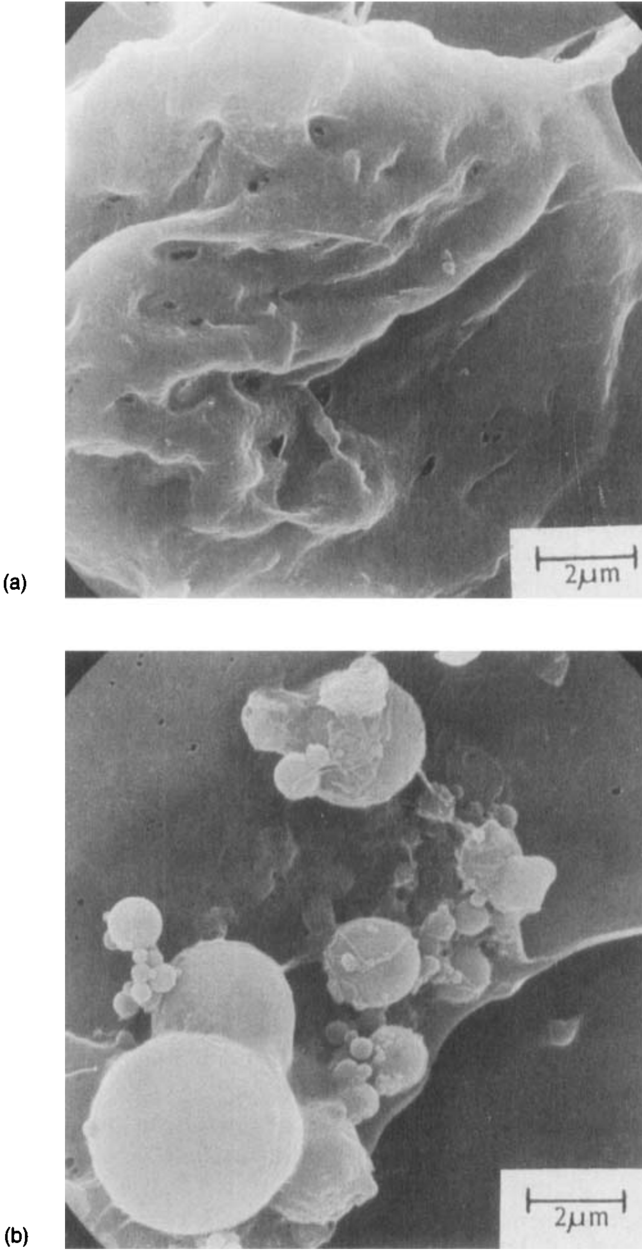
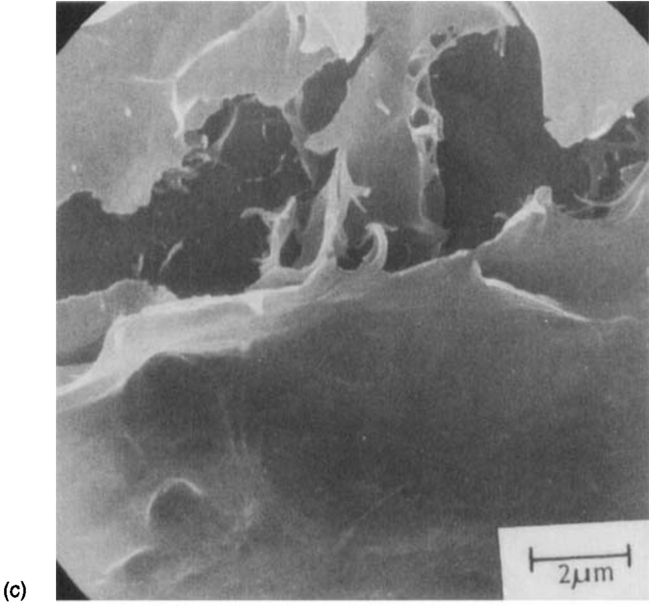
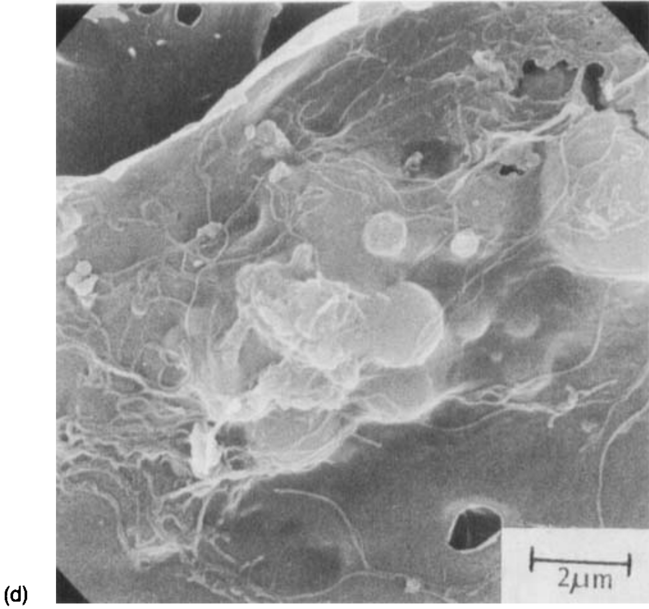


FIG. 7. The SEM views of HLE *hepatoma* phagocytosing MS(Alb-PEG-Gal)s after incubations for (a) 0, (b) 2, (c) 8, (d) 24, and (e) 48 h. Photograph (f) is the control.



(c)



(d)

FIG. 7 (continued).

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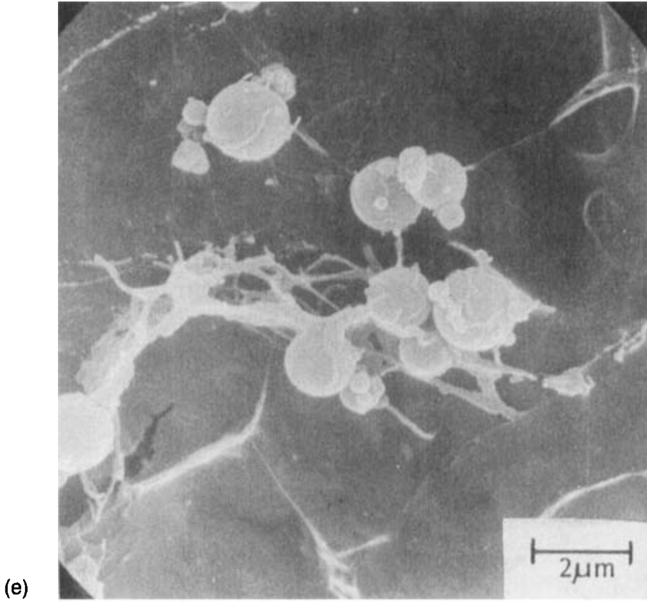
(e)



(f)

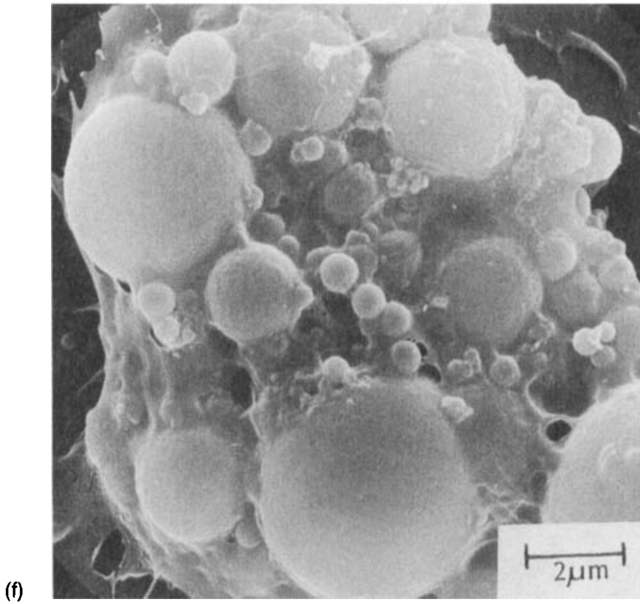


FIG. 7 (continued).

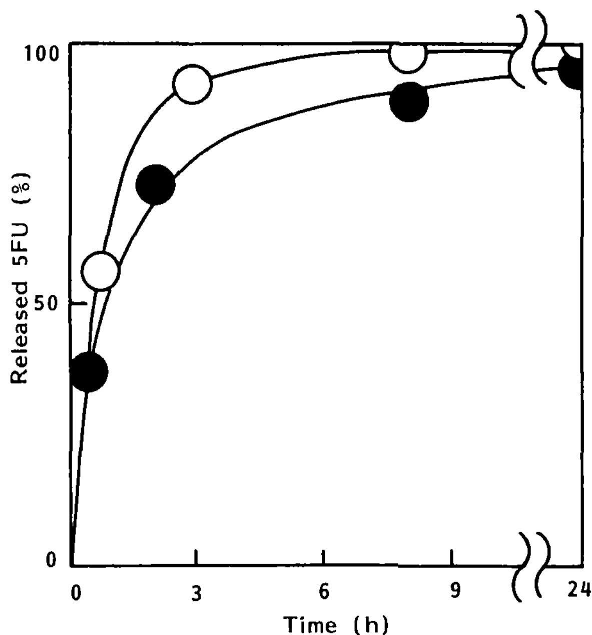


FIG. 8. Release rate of 5FU from MS(Alb-PEG(n = 20)-Gal)s in two kinds of solution at 37°C: (○) 0.4 wt% trypsin aqueous solution, (●) physiological saline.

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(4) The release rate of 5FU from MSs was not always very slow *in vitro*.

ACKNOWLEDGMENTS

The authors wish to express their sincere appreciation to JEOL (Nihon Denshi Co.) for the measurement of ESCA spectra of MS(Alb) and MS(Alb-PEG-Gal). The authors also express their thanks to Kawaken Fine Chemical Co. for providing PEO-amine.

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Received December 28, 1990
Revision received March 6, 1991

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