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RESEARCH PAPER

Preparation and In Vitro Properties of *N*-Succinylchitosan– or Carboxymethylchitin–Mitomycin C Conjugate Microparticles with Specified Size

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ABSTRACT

The preparation of cross-linked conjugate microparticles of N*-succinyl-chitosan (Suc) or 6-*O*-carboxymethylchitin (CM) with mitomycin C (MMC), which showed an adequate size for liver targeting (0.2–3 μm), was attempted by a combination of water-soluble carbodiimide (EDC) coupling and emulsification technique. As for Suc, microparticles with a diameter less than a few micrometers could be obtained easily, while the preparation of CM microparticles (CM-MPs) of the same diameter was not necessarily easy. First, preparation conditions were compared for CM-MPs, and some conditions gave CM-MPs with a diameter less than a few micrometers. As to CM-MMC conjugate microparticles, the method by addition of EDC after emulsification using CM with low molecular weight (CM_L) gave more appropriate microparticles with a mean diameter of 0.97 μm (CM_L-MP-MMC). Suc-MMC conjugate microparticles adequate for liver targeting could be produced by the addition of EDC both before and after emulsification; especially, the conjugate microparticles with a mean diameter of 0.45 μm (Suc-MP-MMC) were derived by the addition of EDC before emulsification.*

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Suc-MP-MMC exhibited a higher drug content than CM_L-MP-MMC. CM_L-MP-MMC and Suc-MP-MMC exhibited 50% drug release times of 2.87 h and 42.1 h, respectively.

Key Words: *6-O-Carboxymethylchitin; Cross-linked conjugate microparticles; Mitomycin C;* N-*Succinyl-chitosan*

INTRODUCTION

N-Succinyl-chitosan (Suc) and 6-*O*-carboxymethylchitin (CM) are anionic chitosan and chitin derivatives with many carboxyl groups. Further, since they have low toxicity, they have been utilized as macromolecular carriers of the conjugates of mitomycin C (MMC) (1–3), which were produced as water-soluble or water-insoluble conjugates (3–9). As to the water-insoluble conjugates, microparticles could be obtained by mechanical grinding (6). However, this grinding technique did not give small size microparticles with a narrow size distribution; that is, the mean particle diameter was more than several micrometers, and the particle diameter ranged from 1 μm to a few tens of micrometers (6).

The size of the microparticles is known to be an important factor that affects biodistribution characteristics (10–12). For example, the particles with a diameter of 0.2–3 μm are accumulated mainly in the liver and spleen due to trapping by the reticuloendothelium (11). The particles with a diameter of more than 5 μm are subjected to trapping by the lungs; especially, those with a diameter of more than 7 μm stay for a long time in the lung due to trapping by the pulmonary vascular bed (11).

The localization to the liver based on the particle size has been examined as a targeting method against diseases that appear in the liver. Namely, such targeting could make the drug locate specifically in the liver. For example, adriamycin-containing microspheres with an appropriate size for liver targeting, 0.2–3 μm, were reported to be effective for liver metastatic tumor (13).

Therefore, conjugate microparticles of Suc or CM with MMC that show specific localization to the liver are attractive as a targeting system of the antitumor agent MMC to the liver. In the present study, a preparation method of the Suc-MMC conjugate microparticles and CM-MMC microparticles with a particle diameter of 0.2–3 μm were examined by a combination of the water-soluble carbodiimide (EDC) coupling and emulsification technique.

EXPERIMENTAL

Materials

At present study, the polymer molecular weight was determined by size exclusion chromatography–multiangle light scattering (SEC-MALS), and the chemical structure was checked by ^{1}H-NMR (nuclear magnetic resonance) spectra.

N-Succinylchitosan sodium salt (Suc: molecular weight [MW] 3.4×10^5, *N*-succinylation degree of 0.81 mol/sugar unit, free amino groups of 0.15 mol/sugar unit) was supplied by Katakura Chikkarin Company, Limited (Tokyo, Japan). 6-*O*-Carboxymethylchitin (MW 4.3×10^5, carboxymethylation degree of 0.66 mol/sugar unit) was obtained from Ichimaru Pharcos Company, Limited (Gifu, Japan), and it was treated at room temperature for 50 h with 10% (w/v) sodium hydroxide aqueous solution to make increase the deacetylation degree (14,15). The resultant deacetylated 6-*O*-carboxymethylchitin (CM), exhibited a MW of 1.4×10^5, carboxymethylation degree of 0.66 mol/sugar unit, and deacetylation degree of 0.39 mol/sugar unit. CM was treated at 40°C for 60 min with 5-*N*-hydrochloric acid to decrease the molecular weight. The product, carboxymethylchitin with low molecular weight (CM_L), showed a MW of 2.1×10^4, carboxymethylation degree of 0.66 mol/sugar unit, and deacetylation degree of 0.39 mol/sugar unit. Suc, CM, and CM_L were used for the experiments described below.

Mitomycin C (MMC) was purified as a powder by solvent evaporation after extraction with acetone from Mitomycin Kyowa S (Kyowa Hakko Kogyo Co., Tokyo, Japan), which contains only NaCl in addition to MMC. This purified MMC was used for the experiments. Sorbitan sesquioleate (SO-15) was purchased from Nikko Chemicals Company, Limited, and was used as a surfactant. 1-Ethyl-3-(3-

dimethylaminopropyl)carbodiimide hydrochloride (EDC) was purchased from Wako Pure Chemical Industries, Limited, and was used as a coupling agent. All other chemicals were reagent grade.

Preparation of Carboxymethylchitin Microparticles

The CM microparticles (CM-MPs) were prepared as follows: The preparation was executed at room temperature. Five ml of aqueous solution of CM (12.5, 25, or 50 mg) was prepared, and the pH of the solution was adjusted to 6.0 with 0.1 N hydrochloric acid. The solution was added to 75 ml of *n*-heptane containing SO-15 (1%, 2%, or 4% w/v), which was stirred at 600 rpm. The mixture was sonicated at 28 kHz (100 W) with stirring (600 rpm) for 10 min to emulsify. Then, 1 ml of aqueous solution of EDC (80 or 40 mg) with the pH adjusted to 5 by 0.1 N hydrochloric acid was added to the emulsion. After that, the sonication (28 kHz) with stirring (600 rpm) was continued for 5 min, and then stirring was performed at 300 rpm without sonication for 30 min. Further, the sonication (28 kHz) with stirring (300 rpm) for 5 min was carried out, and then stirring was executed at 300 rpm without sonication for 30 min. After that, the mixture was centrifuged at 3000 rpm for 10 min, and the precipitate was taken. The precipitate was washed twice using 50 ml of methanol and further washed twice with 50 ml of water. The product was suspended in water for size measurement. Figure 1A summarizes the procedure for CM-MP.

Preparation of Conjugate Microparticles

The conjugate microparticles of MMC with Suc, CM, and CM_L, called Suc-MP-MMC, CM-MP-MMC, and CM_L-MP-MMC, respectively, were prepared by addition of EDC to the mixture of polymer and MMC after (method 1) and before (method 2) emulsification.

For method 1, EDC was added after the emulsification of the solution of the polymer and MMC (Fig. 1B). Briefly, 10 mg of MMC was added to aqueous polymer solution (25 mg in 5 ml of water), and the solution pH was adjusted to 6 with 0.1 N hydrochloric acid. The solution was added to 75 ml of *n*-heptane containing 2% (w/v) SO-15. The rest of the procedure for EDC addition, stirring, and sonication was the same as stated in the preparation

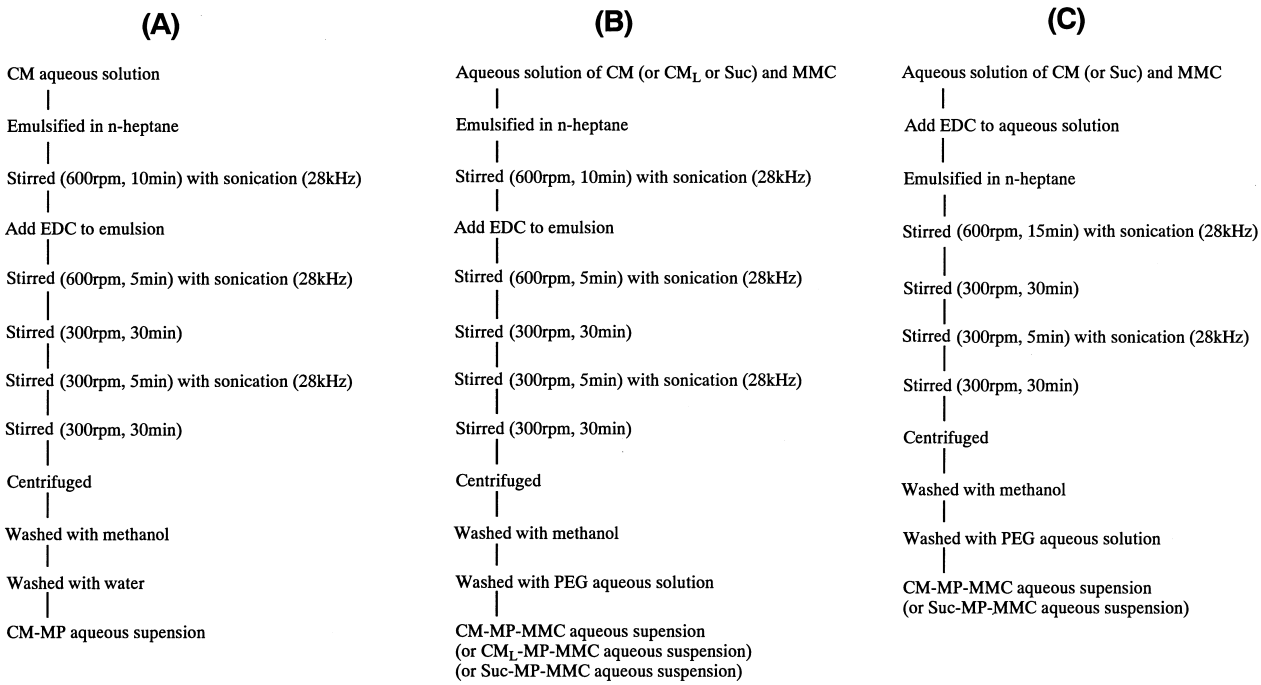

Figure 1. Preparation procedures of CM-MP, CM-MP-MMC, CM_L-MP-MMC, and Suc-MP-MMC: A, procedure for CM-MP; B, method 1 for conjugate microparticles; C, method 2 for conjugate microparticles.

of CM-MP. The conjugate microparticles were washed twice by 50 ml of methanol and subsequently twice by 50 ml of aqueous 1% (w/v) polyethylene glycol 6000 (PEG6000) solution. The obtained microparticles were suspended in 5 ml of water containing 50 mg of PEG6000. For the size measurement, 1 ml was used. Four ml of suspension was lyophilized to yield the powder of the mixture of conjugate microparticles and PEG6000, which was named Suc-MP-MMC/PEG or CM-MP-MMC/PEG. CM_L-MP-MMC conjugate microparticles (CM_L-MP-MMC) and the mixture with PEG (CM_L-MP-MMC/PEG) were also prepared in the same way except using CM_L instead of CM.

For method 2, EDC was added to the mixture of polymer and MMC before emulsification in this preparation (Fig. 1C). Briefly, the aqueous solution (5 ml) of polymer (25 mg) and MMC (10 mg) was prepared, and its pH was adjusted to 6 using 0.1 N hydrochloric acid. One ml of water containing 80 mg of EDC was prepared, and its pH was adjusted to 5 using 0.1 N hydrochloric acid. After both the aqueous solutions were mixed and stirred shortly, the mixture was added to 75 ml of *n*-heptane containing SO-15 at 2% (w/v) and emulsified by sonication with stirring. The sonication (28 Hz) with stirring (600 rpm) was done for 15 min. The rest of the procedure was the same as method 1. Finally, the size measurement and production of Suc-MP-MMC/PEG or CM-MP-MMC/PEG were performed as in method 1.

Physicochemical Characteristics

The particle size and its distribution of CM-MP, Suc-MP-MMC, CM-MP-MMC, and CM_L-MP-MMC were measured by dynamic light scattering using an ELS-800 (Ostuka Electronics Co., Ltd.) after appropriate dilution of their suspension with water. Further, the particle shape, drug content, and drug release were examined for Suc-MP-MMC and CM_L-MP-MMC, which were selected as adequate conjugate microparticles. The particle shape was examined by scanning electron microscopy (SEM) using a Jeol JSM T200 scanning electron microscope.

The drug content of Suc-MP-MMC and CM_L-MP-MMC was measured by high-performance liquid chromatography (HPLC) after complete liberation of MMC from the microparticles by treatment in 1/15 M phosphate buffer, pH 9.0, at 85°C. Namely, 10 mg of Suc-MP-MMC/PEG or CM_L-MP-MMC/PEG were put in 7 ml of 1/15 M phosphate buffer, pH 9. The suspension was heated at 85°C, and the aliquot samples (200 µl each) were withdrawn at 0, 20, 40, 60, and 90 min. After each sample was filtered by membrane (pore diameter 0.45 µm), the filtrate was analyzed by HPLC to determine the regenerated MMC. Complete release was decided from the maximum release point.

The drug release from Suc-MP-MMC and CM_L-MP-MMC was examined in 1/15 M phosphate buffer, pH 7.4, at 37°C. Three mg of Suc-MP-MMC/PEG or 5 mg of CMs-MP-MMC/PEG were suspended in 1/15 M phosphate buffer, pH 7.4, and incubated at 37°C at 60 rpm. The supernatant (100 µl) was withdrawn at 0, 1, 2, 4, 7, 24 and 48 h after the start of incubation. It was analyzed by HPLC after filtration by membrane (pore diameter 0.45 µm).

High-Performance Liquid Chromatographic Assay

The HPLC was performed at room temperature. Twenty µl of the sample were injected on an HPLC column Sumipax Nucleosil $5C_{18}$ reversed-phase column (4 mm in inner diameter by 250 mm long), which was set to a Shimadzu LC-6AD apparatus and a Shimadzu SPD-10AV ultraviolet detector adjusted at 365 nm. The mixture of 0.01 M phosphate buffer, pH 6.0, and methanol (65 : 35 v/v) was used as a mobile phase.

RESULTS AND DISCUSSION

Particle Size Characteristics of 6-*O*-Carboxymethylchitin Microparticles

The particle size of 0.2–3 µm, which is adequate for passive trapping in the liver by the reticuloendothelial system, was selected as a criterion for judgment of preparation conditions. In the preliminary study, Suc-MP-MMC with an adequate particle size and size distribution were readily obtained, while CM-MP-MMC with such appropriate particle characteristics was not necessarily easy to prepare (data not shown). Therefore, first, preparation conditions were examined for the microparticles obtained using CM as a polymeric carrier, although here MMC was not used.

The preparation conditions were checked stepwise; that is, full combinations of various factors

Table 1

Effect of Preparation Condition on the Particle Size of CM-MP

Formation	Outer Solvent	SO-15 in Outer Solvent (% w/v)	CM (mg)	EDC (mg)	Mean Diameter (μm)	Range of Particle Diameter: Minimum–Maximum (μm)
A	*n*-Heptane	2	12.5	80	6.03	2.47–21.0
B	*n*-Heptane	2	25	80	1.82	1.71–2.41
C	*n*-Heptane	2	50	80	21.9	8.96–45.9
D	*n*-Heptane	2	25	40	2.49	2.07–2.91
E	*n*-Heptane	1	25	80	6.29	1.77–55.0
F	*n*-Heptane	4	25	80	3.28	2.67–3.87
G	Liquid paraffin	2	25	80	5.21	4.33–6.24

Table 2

Effect of Polymer Species and Conjugation Method on the Particle Size of CM-MP-MMC and Suc-MP-MMC

Formation	Polymer Species	Method	Mean Diameter (μm)	Range of Particle Diameter: Minimum–Maximum (μm)
H	CM	Method 1	2.30	0.87–4.72
I	CM	Method 2	13.2	3.06–55.0
J	CM_L	Method 1	0.97	0.69–1.42
K	Suc	Method 1	1.00	0.23–2.66
L	Suc	Method 2	0.45	0.32–0.66

were not necessarily checked. Each formation obtained is described in Table 1. First, 25 mg of CM were selected as a fairly good particle size (formation B). When CM was fixed to 25 mg, reduction of the EDC amount to 40 mg hardly influenced the particle size (formation D). When CM and EDC were fixed to 25 and 80 mg, respectively, 2% (w/v) of SO-15 was best for the appropriate particle size. The concentration of CM in the aqueous phase was related to the viscosity of the aqueous phase, and the concentration of SO-15 in the organic phase was considered to influence the size of the emulsion. The concentration of EDC was supposed to be related to the rate and degree of particle formation and further might influence the aggregation of the individual microparticles. As a result, as far as this experiment was concerned, formation B was chosen as a good one. When liquid paraffin was used as a solvent instead of *n*-heptane, the size distribution was narrow, but the mean diameter increased (formation G). Although the viscous solvent is effective for suppression of aggregation of emulsion particles, this property appeared to be ineffective for the production of fine microparticles. Namely, it was considered that the fine emulsion was not easy to produce with liquid paraffin due to its high viscosity.

Particle Size Characteristics of 6-*O*-Carboxymethylchitin Microparticles–Mitomycin C and *N*-Succinyl-Chitosan Microparticles–Mitomycin C

Conditions to provide good CM-MP were obtained as stated above (formation B in Table 1). Method 1 followed the same procedure, except that the mixture of CM and MMC was used instead of CM. The particle size characteristics are described in Table 2. Method 1 presented Suc-MP-MMC with a satisfactory particle size distribution, but made the size of CM-MP-MMC a little larger than ideal (formations H and K). The CM-MP-MMC exhibited a little larger size than expected from CM-MP, which might be related to a change in the emulsion size by MMC incorporation or to structural change by polymer-MMC conjugate formation. When CM_L was used instead of CM, satisfactory conjugate microparticles were obtained (formation J). CM_L has a lower molecular weight than CM; therefore,

finer emulsion might be formed, resulting in formation of smaller microparticles.

As to preparation of conjugate microparticles, the timing of EDC addition was examined. Namely, EDC was added to the mixture of the polymer and MMC immediately before emulsification as shown in method 2. This method allowed EDC to function more effectively for conjugation and cross-linking because EDC coexisted with the polymer and MMC in the aqueous phase before emulsification. CM-MP-MMC were obtained with a much larger size (formation I). As to Suc-MP-MMC, although some part was precipitated as a nonparticulate form, the microparticles with a smaller particle size (formation L) were obtained after filtration with the fine filter (pore diameter 10 μm). Probably, method 2 exhibited aggregation favorably in the preparation of CM-MP-MMC. On the other hand, smaller Suc-MP-MMC were considered to be produced in method 2, probably because Suc had the tendency to donate conjugate microparticles more easily than CM, as recognized preliminarily.

Judging from the particle size characteristics, CM_L-MP-MMC (condition J) and Suc-MP-MMC (condition L) were recognized as the best candidates as microparticles for the passive liver targeting. Their size distribution profiles and scanning electron micrographs are shown in Fig. 2.

Characteristics of Drug Content and Drug Release

The microparticles of polymer-drug conjugate, CM_L-MP-MMC (formation J) and Suc-MP-MMC (formation L), were investigated for drug content and drug release. MMC was reported to be released faster from CM-MMC conjugate than from Suc-

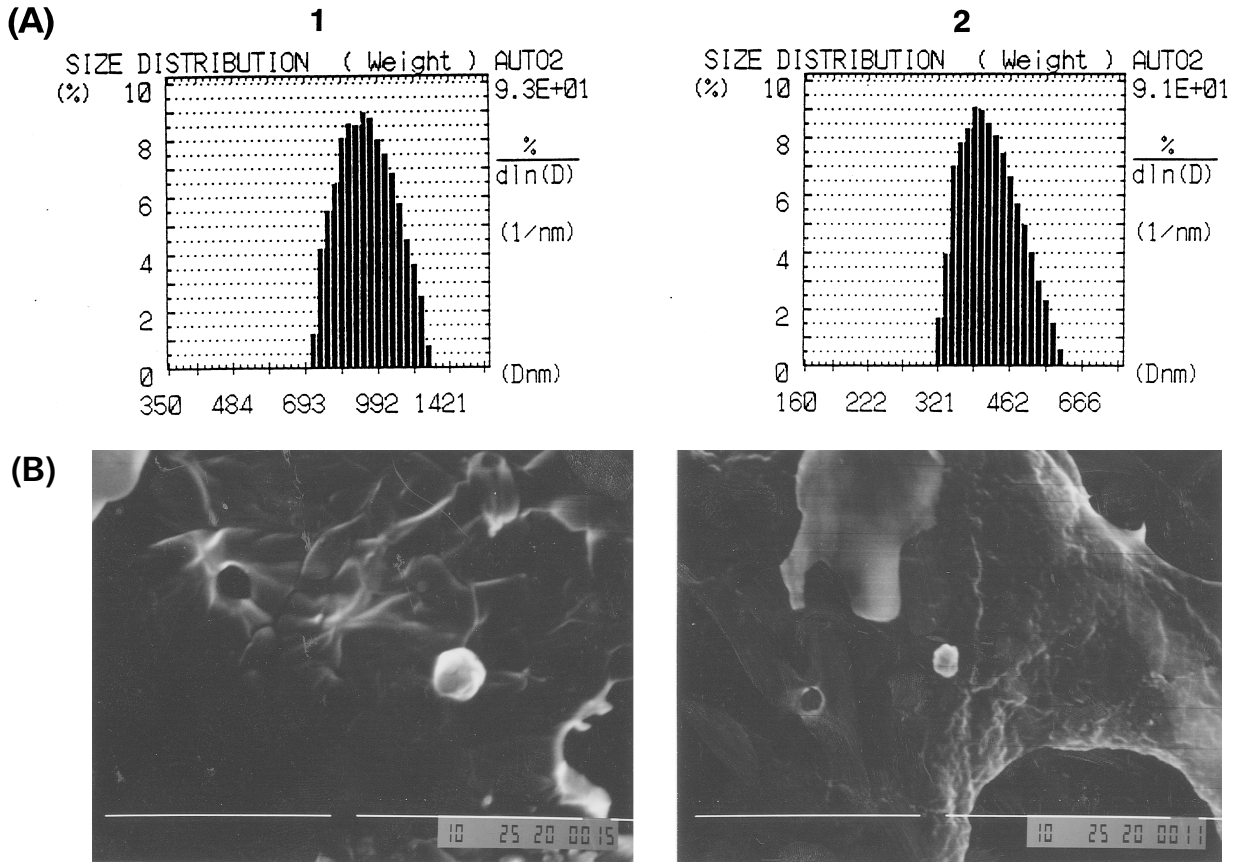

Figure 2. (A) Particle size distributions and (B) scanning electron micrographs of CM_L-MP-MMC and Suc-MP-MMC: 1, CM_L-MP-MMC (formation J); 2, Suc-MP-MMC (formation L). The horizontal and vertical axes mean the particle diameter and weight percentage of distribution (A), respectively. The length of the bar corresponds to 10 μm (B).

MMC (4). Such characteristics were true for CM_L-MP-MMC and Suc-MP-MMC. The release of MMC was accelerated with the increase of pH. The drug content was determined by the complete liberation of MMC from the microparticles at the accelerated condition (85°C and 1/15 M phosphate buffer, pH 9), in which the content could be obtained because MMC was relatively stable (6,9) (Fig. 3A). The amount of CM_L-MP-MMC (or Suc-MP-MMC) produced was calculated by subtraction of the calculated PEG amount (40 mg) from the amount of CM_L-MP-MMC/PEG (or Suc-MP-MMC/PEG) obtained. These results are shown in Table 3.

CM_L-MP-MMC exhibited a drug content of less than 1% (w/w). On the other hand, Suc-MP-MMC showed a high drug content, 18% (w/w). The polymer recovery was calculated as the ratio of the polymer amount included in the product to the polymer amount added initially. The recovery in CM_L-MP-

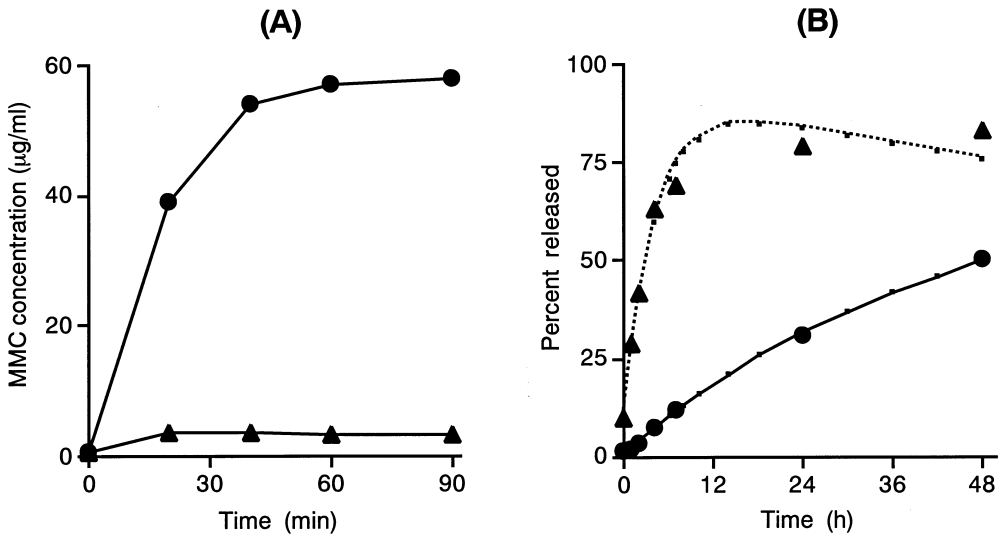

Figure 3. (A) Liberation of MMC in 1/15 M phosphate buffer, pH 9, at 85°C and (B) release profiles of MMC in 1/15 M phosphate buffer, pH 7.4, at 37°C. ▲, CM_L-MP-MMC (formation J); ●, Suc-MP-MMC (formation L). In Fig. 3B, broken and solid lines represent calculated curves for CM_L-MP-MMC and Suc-MP-MMC, respectively.

Table 3

Chemical Characteristics and Stability Parameters in 1/15 M Phosphate Buffer, pH 7.4, of CM_L-MP-MMC and Suc-MP-MMC

	Conjugate Particle	
Parameter	CM_L-MP-MMC	Suc-MP-MMC
Drug content (% w/w) in conjugate particle/PEG	0.25	4.1
Drug content (% w/w) in conjugate particle	0.68	18
Recovery of polymer (% w/w)	117	48
$k_1(h^{-1})$[a]	0.004	0.004
$k_2(h^{-1})$	0.217	0.016
$k_3(h^{-1})$	0.024	0.0[b]
$t_{1/2}$(h)[c]	2.87	42.1

[a]The value previously reported (Ref. 9) was used.
[b]This value was fixed at 0.0 in profile fitting due to being less than 0.
[c]This value was calculated by $\ln 2/(k_2 + k_3)$.

MMC/PEG was over 100% (w/w), which meant that most CM was recovered as the microparticles, and some water might remain after drying. On the other hand, as to Suc-MP-MMC/PEG, the polymer recovery was less than half of the amount added initially, which was considered due to the nonparticulate precipitate observed.

Figure 3B shows the drug release profiles from both conjugate microparticles. In 1/15 M phosphate buffer, pH 7.4 at 37°C, CM_L-MP-MMC released MMC much more quickly than Suc-MP-MMC. The release of MMC from CM_L-MP-MMC reached a plateau after incubation for 12 h, while approximately half the content was released from Suc-MP-MMC after the 2-day incubation. The release rate was analyzed using the following scheme:

$$\begin{array}{c}\text{Conjugate particles} \xrightarrow{k_2} \text{MMC} \xrightarrow{k_1} \text{Degradation products} \\ \downarrow k_3 \\ \text{Degradation products}\end{array}$$

where k_1 is the decomposition rate constant of MMC, k_2 is the release rate constant, and k_3 represents the decomposition rate constant of the drug moiety in the conjugate form. At that time, the percentage of MMC generated at time t after the start of the incubation $M(t)$ was given by the following equation:

$$\begin{aligned} M(t) = {} & ((k_2 \times M_b)/(k_1 - k_2 - k_3)) \\ & \times (\exp(-(k_2 + k_3)t) - \exp(-k_1 t)) \\ & + (100 - M_b) \times \exp(-k_1 t) \end{aligned} \quad (1)$$

where M_b represents the percentage of the initial bound MMC.

The release parameter k_2 and the degradation parameters k_1 and k_3 were obtained by fitting the curve given by Eq. 1 to the observed one, when the nonlinear least squares program MULTI was used (16) (Table 3). The half-life $t_{1/2}$ of MMC conjugated in the microparticles was calculated by $\ln 2/(k_2 + k_3)$ (Table 3). The values of $t_{1/2}$ were 2.87 and 42.1 h for CM_L-MP-MMC and Suc-MP-MMC, respectively.

These results suggest that CM_L-MP-MMC should be appropriate for rapid exposure of drug after targeting, but that Suc-MP-MMC should be adequate for prolonged drug exposure after targeting. The conjugate microparticles of Suc- or CM-doxorubicin microparticles with similar size (1 μm to a few micrometers) were obtained by the same method, and they exhibited a liver-specific localization of doxorubicin (data not shown), which suggested the localization of the conjugate microparticles following the general concept of size-dependent liver passive targeting. Anyway, the in vivo examination of the present conjugate microparticles will elucidate their biological features. However, as to CM_L-MP-MMC, the drug content may have to be improved for the in vivo approach.

CONCLUSION

The preparation of Suc- or CM-MMC conjugate microparticles with a particle size (0.2–3 μm) adequate for passive liver targeting was attempted by the combination of chemical coupling and an emulsification technique. The particle size was dependent on the preparation condition, and the conjugate microparticles with the satisfactory particle size were obtained in some conditions. This preparation method may be applicable for preparation of other small conjugate microparticles. Suc-MMC conjugate microparticles showed relatively high drug content, while CM-MMC conjugate particles need improved drug content. The in vivo approach will be needed to elucidate their biological properties in the future.

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