Synthesis and Drug-Release Characteristics of the Conjugates of Mitomycin C with *N*-Succinyl-chitosan and Carboxymethyl-chitin

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By condensation of mitomycin C (MMC) with *N*-succinyl-chitosan (Suc-chitosan) and carboxymethyl-chitin (CM-chitin) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, Suc-chitosan–MMC conjugate (Suc-chitosan–MMC) and CM-chitin–MMC conjugate (CM-chitin–MMC) were prepared, respectively. The reaction conditions for 45 min at pH 5 and for 2 h at pH 5 were selected as the most appropriate for the preparations of Suc-chitosan–MMC and CM-chitin–MMC, respectively. Suc-chitosan–MMC was obtained as a water-insoluble product, while CM-chitin–MMC was partially water-soluble. When the ratio of MMC to the polymer supports changed in the conjugation reaction, the conjugates with 33% (w/w) and 23% (w/w) MMC contents were obtained as those most highly drug-loaded for Suc-chitosan–MMC and CM-chitin–MMC, respectively. At pH 7.4 at 37 °C, Suc-chitosan–MMC regenerated MMC very slowly, while the release of MMC from CM-chitin–MMC was relatively fast. Each drug release followed very nearly pseudo-first order kinetics, in which the apparent drug release rate constants (k_{app}s) of Suc-chitosan–MMC and CM-chitin–MMC were 3.9×10^{-3} and 1.1×10^{-1} (h^{-1}), respectively.

Keywords mitomycin C; *N*-succinyl-chitosan; carboxymethyl-chitin; conjugate; reaction conditions; drug-release

Although mitomycin C (MMC) is widely used in cancer chemotherapy, it presents such side effects as severe bone marrow depression and gastrointestinal damage.[1] One of the possible approaches for overcoming these disadvantages and improving chemotherapeutic activity is the method using a macromolecular prodrug, which is known to be useful in order to concentrate the cytotoxicity at the tumor site and to achieve prolonged duration of the activity.[2] Dextran–MMC,[3-8] albumin–MMC,[9,10] polyamino acid–MMC[11] and antibody–MMC[12,13] have been reported to act as prodrugs of MMC.

Chitin and chitosan can be expected to be utilized as drug carriers because they are inexpensive, have low toxicity and are degradable.[14-16] *N*-Succinyl-chitosan (Suc-chitosan) and carboxymethyl-chitin (CM-chitin) are applicable as drug carriers for MMC because they have many carboxyl groups, which are functional and useful for conjugation reaction. Further, they are expected to have relatively low toxicity because they are anionic polymers.

Because of the above considerations, the present study attempts to prepare macromolecular prodrugs of MMC using Suc-chitosan and CM-chitin as polymer supports, and the *in vitro* characteristics of the obtained conjugates are investigated.

Experimental

Materials MMC, produced by Kyowa Hakko Kogyo Co., was used throughout this work. Suc-chitosan sodium salt (Suc-chitosan: molecular weight, 3×10^5; degree of *N*-succinylation per hexosamine unit of chitosan, 0.72) was obtained from Katakura Chikkarin Co., Ltd. CM-chitin sodium salt (CM-chitin: molecular weight, 6×10^5; degree of carboxymethylation per hexosamine unit of chitin, 0.65) was purchased from Ichimaru Pharcos Co., Ltd. All other chemicals were of a reagent grade.

Preparation of Conjugates 1) Reaction Conditions: Suc-chitosan (48 mg) or CM-chitin (48 mg), MMC (6 mg) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (120 mg) were mixed and stirred in 15 ml of purified water, and the mixture pH was adjusted and maintained at 5.0, 6.0, or 7.0, using 1% HCl and 1% NaOH. During the reaction at room temperature, 0.3 ml of the sample was withdrawn at fixed time intervals. Immediately after withdrawal of the aliquot, it was diluted by the addition of 3 ml of purified water. Unbound MMC was filtered out from the diluted sample using an ultrafilter unit, USY-5 (Advantec Toyo), with a molecular weight cut-off limit of 5×10^4, which was applied to all the ultrafiltration studies in this work; at the same time, the bound MMC was estimated from the unultrafiltered MMC. The binding ratio of MMC was determined as a ratio of the bound MMC to the total used MMC. The condition of pH and reaction time to show the highest binding ratio was selected as a preparation condition of the conjugate.

2) Binding Mode: The binding mode of MMC to the polymer support was checked for the selected reaction condition. Namely, a mix of MMC and the drug carrier was carried out both with and without EDC under the selected condition. By checking unbound MMC by ultrafiltration of the mixture, condensation with EDC was estimated.

3) Preparation Procedures: Suc-chitosan–MMC conjugate (Suc-chitosan–MMC) and CM-chitin–MMC conjugate (CM-chitin–MMC) were prepared under the selected reaction conditions as follows. MMC (60 mg), Suc-chitosan (480 mg), and EDC (1.2 g) were mixed in 150 ml of purified water, and the mixture pH was adjusted to 5.0 using 1% HCl. The mixture was stirred for 45 min at room temperature. After that, the resulting precipitate was isolated by filtration and washed sufficiently with water. After that, by drying it *in vacuo*, Suc-chitosan–MMC was obtained, and it was used for the *in vitro* drug release study. MMC (60 mg), CM-chitin (480 mg) and EDC (1.2 g) were mixed in 150 ml of purified water, and the solution pH was adjusted to 5.0 using 1% HCl. The mixture was stirred for 2 h at room temperature. The product was precipitated by the addition of cold acetone. The precipitate was washed sufficiently with a mixture of acetone and purified water (3 : 1, v/v). After that, by drying it *in vacuo*, CM-chitin–MMC was obtained, and it was used for the *in vitro* drug release study. In each conjugation, bound MMC was determined by the ultrafiltration method, and the MMC content of the obtained conjugate was estimated as a ratio of the bound MMC to the sum of the bound MMC and the used polymer support.

***In Vitro* Drug Release Experiment** The conjugate (10 mg) was suspended in 1/15 M phosphate buffer (20 ml) of pH 7.4 and stirred sufficiently at 37 °C. As for Suc-chitosan–MMC, the aliquot samples were withdrawn after stirring for 4, 8, 24, 48 and 72 h. Concerning CM-chitin–MMC, the aliquot samples were withdrawn after stirring for 1, 2, 4, 8 and 12 h. The amount of MMC released was determined by high performance liquid chromatography (HPLC). The drug release rates were estimated from the MMC release profiles.

Analytical Methods The binding ratio of MMC to the drug carriers, the content of MMC in the conjugates and the released MMC from Suc-chitosan–MMC were spectrophotometrically determined from UV absorption at 364 nm, using a Jasco Ubest-30 UV/VIS spectrophotometer. The amount of MMC released from CM-chitin–MMC was determined by HPLC, which was done using a Shimadzu LC-5A apparatus equipped with a NEO-Pack $5C_{18}$ reversed phase column (4.6 × 250 mm) and an SPD-6A UV detector set operated at 364 nm. The mobile phase was a mixture of 0.01 M phosphate buffer, pH 6.0, and methanol (63 : 35, v/v).

Results and Discussion

Reaction Conditions Suc-chitosan (48 mg) was easily soluble in 15 ml of purified water. When the solution pH

was lowered by the addition of 1% HCl, Suc-chitosan was soluble at pH 6—7, but became partially colloidal at pH 5. CM-chitin (48 mg) was also easily soluble in 15 ml of purified water. When the solution pH was changed by 1% HCl and 1% NaOH, CM-chitin was soluble at pH 5—7. As Suc-chitosan and CM-chitin scarcely passed the USY-5 membrane, unbound MMC could be separated from bound MMC by a USY-5. The binding ratio of MMC was estimated by the ratio of the bound MMC to the total used MMC. Since the fast degradation of MMC was spectrophotometrically recognized at pH 4, the conjugation reaction at pH 4 was discarded. At pH 5, the reaction mixture of MMC, Suc-chitosan and EDC showed the highest binding ratio of more than 95% after the 45 min reaction, and the mixture of MMC, CM-chitin and EDC showed the highest binding ratio of more than 90% after 2 h reaction. After the time at which the highest binding ratio was produced, a gradual decrease in the binding ratio was observed. Moreover, at the highest binding reaction conditions at pH 5, MMC was spectrophotometrically recognized not to be degraded. The time at which the highest binding ratio was produced was investigated at pH 6 and 7 in the same manner. However, the highest binding ratio was much smaller at pH 6 and 7 than at pH 5, as shown in Fig. 1. Since the preparation conditions were desirable, being simple and giving high yields, the conjugation reaction conditions for Suc-chitosan–MMC and CM-chitin–MMC were selected to be carried out at pH 5 for 45 min and at pH 5 for 2 h, respectively. These reaction conditions were used to study the coupling mode, the drug contents and the *in vitro* drug release.

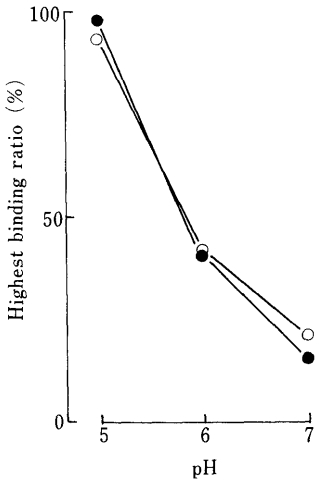

Fig. 1. Influence of pH of Reaction Medium on the Highest Binding Ratio of MMC

●, Suc-chitosan–MMC; ○, CM-chitin–MMC.

TABLE I. Binding Characteristics of MMC with and without EDC

Suc-chitosan (mg)	CM-chitin (mg)	MMC (mg)	EDC (mg)	MMC in the ultrafiltrate (mg)	Dissolution property[a] of the product
48		6	120	0.12	Insoluble
48		6		5.25	Soluble
	48	6	120	0.40	Partially soluble
	48	6		4.54	Soluble

a) Measured in 1/15 M phosphate buffer, pH 9.

Conjugates MMC bound to the polymer support was considered to be composed of MMC covalently bound and MMC adsorbed. Therefore, in order to check the adsorption of MMC to Suc-chitosan and CM-chitin, ultrafiltration was carried out for the reaction mixtures of MMC and macromolecules with and without EDC under the selected reaction conditions. As described in Table I, MMC scarcely appeared in the filtrate for the mixtures with EDC, while for the mixtures without EDC, the filtrate showed almost the same MMC concentration as that for the mixtures before ultrafiltration. Especially, regarding the mixture of Suc-chitosan and MMC without EDC, most of the MMC was filtered out. Thus, the adsorption of MMC to the polymer supports was recognized to be small. These results demonstrated that most of the MMC in each conjugate was combined with the polymer supports by amide linkage based on EDC condensation, and most free MMC was filtered out using a USY-5. Thus, the conjugate was estimated as the sum of non-ultrafiltered MMC (bound MMC) and the used polymer support, and the MMC content was estimated as a ratio of the bound MMC to the sum of the bound MMC and the used polymer support. Since the amount of EDC remaining affected the estimation, it was simply checked as follows for the remaining EDC to be washed out. After the incubation of CM-chitin–MMC in 1/15 M phosphate buffer, pH 9, at 37 °C for 8 h, MMC was regenerated by almost 100%. This demonstrated that EDC was washed out almost completely and justified the estimation of the drug content of CM-chitin–MMC. After the reaction of Suc-chitosan and EDC under the same reaction condition, but with no MMC as a conjugation reaction condition, ultrafiltration by a USY-5 was executed. After the residue was washed sufficiently through the USY-5 membrane, its dried weight was measured. Then, since no increase in the polymer weight was observed, EDC could be considered to be washed out. This supported the finding that EDC was washed out in the preparation of Suc-chitosan–MMC and that the estimation of the drug content was adequate.

Suc-chitosan–MMC was obtained as a water-insoluble material. On the other hand, CM-chitin–MMC was obtained as a partially water-soluble material. CM-chitin–MMC was considered to consist of a water-soluble part and a water-insoluble one. Concerning the water-insoluble products, the intra or inter-crosslinking among the polymer supports was considered to be formed as follows. Since the deacetylation degree of chitosan used as a starting material for the synthesis of Suc-chitosan was 0.96 and the

TABLE II. Contents of MMC in the Conjugates Prepared under Various Conditions

Suc-chitosan (mg)	CM-chitin (mg)	MMC (mg)	EDC (mg)	Binding ratio %(w/w)	Content of MMC in the conjugate %(w/w)	Number of MMC in 10 glucosamine units
16		10	200	71	33	3.9
16		4	80	96	21	2.1
16		2	40	98	12	1.1
	48	20	120	59	23	2.0
	48	12	120	73	16	1.5
	48	6	120	95	11	1.0

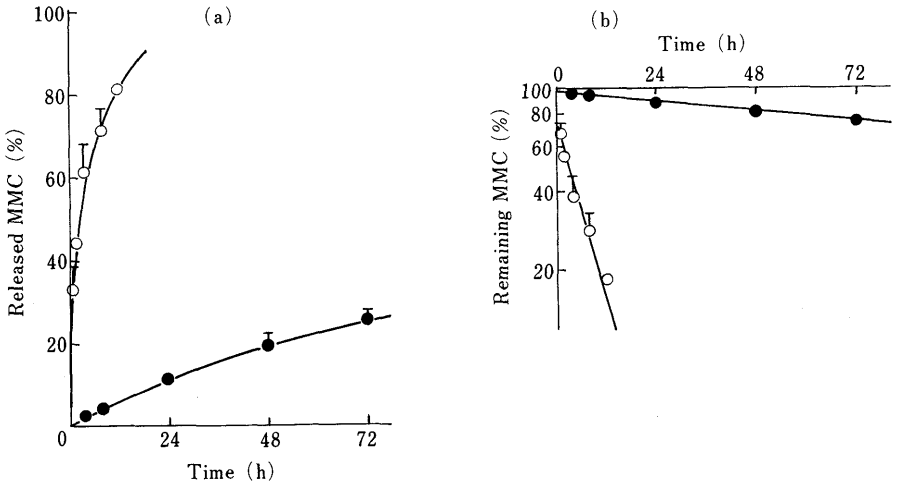

Fig. 2. Release Profiles of MMC (a) and Semi-logarithmic Plots of Remaining MMC (b) for Suc-chitosan–MMC (●) and CM-chitin–MMC (○) in 1/15 M Phosphate Buffer of pH 7.4

Every point represents the mean ± S.D. ($n=3$). For every point, S.D. is described when larger than the point plotted. On the percent remaining MMC (x) and the incubation time, h, (t), the linear regression was executed between t and $\log x$, and the correlation coefficient (r) was calculated. The results are as follows. Suc-chitosan–MMC, $\log x = 1.992 - 0.001712t$, and $r = -0.9974$; CM-chitin–MMC, $\log x = 1.838 - 0.04839t$, and $r = -0.9879$.

N-substitution degree was 0.72, simple calculation showed that the amino groups remained in the amount of 0.24 per glucosamine unit. Therefore, crosslinking was considered to be caused easily by carbodiimide-catalyzed condensation between carboxyl groups and remaining amino groups in Suc-chitosan, and this would make the conjugate insoluble. On the other hand, the precipitate was not formed easily on CM-chitin–MMC. Since the chitin used as a starting material for the synthesis of CM-chitin possessed few amino groups (< 10%), CM-chitin would also contain few amino groups. Therefore, the crosslinking by carbodiimide-catalyzed condensation would occur only slightly for the preparation of CM-chitin–MMC, and consequently CM-chitin–MMC would be obtained as a material composed of a water-insoluble part and a water-soluble one. For checking these suggestions, simple tests were carried out by mixing Suc-chitosan or CM-chitin with EDC in the same manner, except without the use of MMC as in the case of the conjugation reaction. Then, a white and fluffy precipitate was formed rapidly in a mix of Suc-chitosan and EDC, while a jellied material was formed quickly in a mix of CM-chitin and EDC. These results suggested that the precipitation during the conjugation reaction was caused by the crosslinking among Suc-chitosan or CM-chitin by EDC.

When the ratio of MMC to the polymer supports was changed in the conjugation reaction, the MMC contents of the conjugates were obtained as shown in Table II. As the ratio of MMC to macromolecules was increased, the contents of MMC in the conjugates were observed to increase but the ratio of bound MMC to unbound MMC decreased. In this approach, the highest contents of MMC were found to be 33% (w/w) for the conjugate of MMC and Suc-chitosan, and 23% (w/w) for the conjugate of MMC and CM-chitin.

***In Vitro* Drug Release** The liberated MMC from Suc-chitosan–MMC was checked by both UV spectroscopy and HPLC to the ultrafiltrate of the aliquot samples in the first of three repeated experiments. At that time, both methods showed similar results. Owing to a long incubation period (72 h) for Suc-chitosan–MMC, it was convenient to measure the UV absorption of the ultrafiltrate of the aliquot sample at every sampling. Thus, the drug release from Suc-chitosan–MMC was checked by UV spectroscopy. Concerning CM-chitin–MMC, since the incubation period (12 h) was short, it was convenient to analyze all the aliquot samples at once using HPLC. The drug release from Suc-chitosan–MMC was much slower than that from CM-chitin–MMC as shown in Fig. 2a. Each conjugate exhibited monoexponential liberation of MMC at pH 7.4 as described in Fig. 2b, which showed semi-logarithmic plots for the data in Fig. 2a. The MMC degradation was considered to be slight under these incubation conditions, pH 7.4, as was found under similar conditions in other reports.[5,10,11,17] The apparent drug release rate constant (k_{app}) was calculated from the slope of the linear equation fitted to the plot in Fig. 2b using the least-squares technique (Table III). Hashida *et al.*[5] and Kaneo *et al.*[10,17] had applied the non-linear least squares program MULTI to the estimation of the conversion of macromolecular conjugates of MMC, described in Fig. 3. Analysis using

TABLE III. Apparent Release Rate Constants (k_{app}s) and Apparent Halflives ($t(1/2)_{app}$s) in 1/15 M Phosphate Buffer, pH 7.4, at 37 °C

Conjugate	k_{app} (h^{-1})	$t(1/2)_{app}$ (h)
Suc-chitosan–MMC	3.9×10^{-3}	180
CM-chitin–MMC	1.1×10^{-1}	6.2

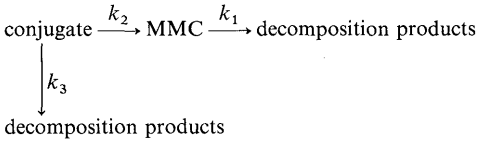

Fig. 3. Conversion Reaction Scheme for Conjugates

$$(\mathrm{MMC}) = (k_2 A_0/(k_1 - k_2 - k_3))\exp(-(k_2 + k_3)t) + (100 - (k_1 - k_3)A_0/(k_1 - k_2 - k_3))\exp(-k_1 t)$$

In which (MMC) means percent of free MMC in the incubation medium to the initial contained MMC and A_0 means 100 minus percent of burst MMC.

MULTI was also carried out in this study. For each conjugate, the change in the initial burst became too large when A_0 was set as a variable parameter, and k_3 was converged to a minus value when k_3 was a variable. Thus, A_0 and k_3 were fixed to the value obtained from the linear equation in Fig. 2b and zero, respectively. At that time, k_1 and k_2 for Suc-chitosan–MMC were converged to 2.9×10^{-3} and 4.4×10^{-3} (h^{-1}), respectively, while k_1 and k_2 for CM-chitin–MMC were converged to 3.5×10^{-3} and 1.2×10^{-1} (h^{-1}), respectively. The obtained k_1 values were close to the values at pH 7.4 reported by Hashida *et al.*[5] and Kaneo *et al.*[10,17] The obtained k_2 value was similar to the k_{app} value for each conjugate. Concerning the application of MULTI to the drug release from Suc-chitosan–MMC, although the drug release profile by UV spectroscopy was almost the same as that by HPLC, a minute difference was possible. The values of k_1 and k_2 for Suc-chitosan–MMC might be estimated more exactly by determination using HPLC. Anyway, since the MMC degradation was slight during the experimental periods, the k_{app} and $t(1/2)_{app}$ were appropriate as the MMC release parameters for each conjugate.

The present results indicate that Suc-chitosan–MMC can be characterized as a conjugate, being water-insoluble but swelled in aqueous solution and gradually regenerating MMC at a physiological pH. Thus, Suc-chitosan–MMC is expected to be useful in order to concentrate MMC to the target site by local administration and to maintain the action of MMC by infrequent administration. On the other hand, CM-chitin–MMC was partially water-soluble and exhibited relatively fast drug release. Therefore, concerning localization of MMC at the administered site and prolongation of the drug release, CM-chitin–MMC was considered to be less available than Suc-chitosan–MMC. It can be proposed that CM-chitin–MMC is available as a conjugate for delivering MMC to target sites *via* such affinity ligands as antibodies and hormones, *etc.* Each conjugate should be further studied for *in vivo* characteristics.

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