6-*O*-Carboxymethyl-chitin (CM-chitin) as a Drug Carrier

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Gel was prepared from 6-*O*-carboxymethyl-chitin (CM-chitin) by the addition of iron(III) chloride under mild conditions without any organic solvent. The optimal conditions for the gel formation were 15 to 30 mM iron(III) chloride and 0.5 to 0.8 degree of substitution in CM-chitin. The amounts of bovine serum albumin (BSA) and the anticancer drug doxorubicin (DOX) incorporated into CM-chitin gels were more than 80% and 30%, respectively under the conditions described above. The release of BSA or DOX from the gels was observed to be increased by lysozyme digestion in a time-dependent manner. This result indicates that CM-chitin might prove useful as a carrier gel for the sustained release of drugs and cytokines, including vaccines.

Keywords 6-*O*-carboxymethyl-chitin; 6-*O*-carboxymethyl-chitosan; sustained release; gel; drug carrier; doxorubicin

Some anticancer agents or lymphokines in general have a very short half life in the circulation, which may result in a decrease of the therapeutic potential. Therefore the control of drug release *in vivo* may allow more effective expression of the biological effects. For this purpose, some attempts have been made to incorporate drugs into high-molecular membranes, matrices or vehicles composed of polysaccharides, protein or lipids.

Chitin, an-(1→4) linked polysaccharide composed of 2-acetamido-2-deoxy-β-D-glucopyranose residues, is distributed widely in nature, and has been reported to have some beneficial medicinal[1] and pharmaceutical[2] applications. For example, chitin has been used as a blood anticoagulant,[3] a wound-healing accelerator,[4] and a surgical suture. Recently, 6-*O*-carboxymethyl-chitin (CM-chitin) has been shown to induce macrophage activation for a short period *in vivo* compared with other chitin derivatives such as phosphorylated-, sulfonated-, and dehydroxypropyl-chitin,[5] and to have weak mitogenic activities on normal spleen cells.[6] Chitin and CM-chitin are also known to be susceptible to lysozyme in the human body.[7,8]

In the present study, we examined the applicability of chitin derivatives in the design of dosage forms for sustained release of drugs. We selected CM-chitin because of its biological and chemical properties, and tried to prepare a gel carrier for sustained release of drugs under mild conditions using iron(III) chloride. We preliminarily investigated whether or not bovine serum albumin (BSA) or doxorubicin (DOX) are able to be efficiently incorporated into CM-chitin gels, and whether the release of BSA or DOX from the gels can occur continuously by lysozyme digestion at the physiological concentration *in vitro*.

Experimental

Chitin Derivatives CM-chitin, 6-*O*-carboxymethyl-chitosan (CM-chitosan) and 70% deacetylated chitin (DAC-70, acetate type) were prepared by the procedures reported previously.[9,10] The degree of substitution (D.S.) was estimated by potentiometric titration with 0.1 M HCl in 0.1 M NaCl under a nitrogen atmosphere at room temperature as described elsewhere.[9]

Reagents BSA was purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Lysozyme (egg white) was obtained from Seikagaku Kogyo Co., Ltd., Tokyo. DOX was purchased from Kyowa Hakko Co., Ltd., Tokyo, and dissolved in sterile distilled water at 2 mg/ml before use. Other reagents of guaranteed grade were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Cell Line B16-BL6, a cultured cell line derived from murine melanoma, was kindly provided by Dr. I. J. Fidler, M. D. Anderson Cancer, Center, Houston, Texas, U.S.A. Cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine.

Preparation of Chitin Derivative Gels Chitin derivatives (30 mg) were dissolved in 10 ml of distilled water in a 50 ml centrifugal tube. To make the viscous solution uniform, it was vigorously stirred with a magnetic stirrer for 1 h at room temperature. Then 5 ml of 90 mM iron(III) chloride was dropped into the solution, and the mixture was stirred for an additional 30 min. The resulting gels of chitin derivatives were centrifuged at 10000 rpm for 5 min, washed twice with distilled water by centrifugation and lyophilized. The lyophilized gels were ground uniformly and stored at 4 °C. The gel particle size in this study was approximately 10—20 μm in diameter as measured with a particle counter (Electrozone/Celloscope, Particle Data, Inc. Elmhurst, Illinois).

Preparation of CM-chitin Gel Containing BSA or DOX Either 1.5 ml of BSA (10 mg/ml) or 1 ml of DOX (2 mg/ml) was added to 10 ml of CM-chitin solution, and the mixture was stirred for 1 h at 4 °C. Then, 5 ml of 90 mM iron(III) chloride solution was added and the mixture was stirred overnight at 4 °C. The resulting gel suspension was washed twice with distilled water by centrifugation and resuspended in a minimal amount of distilled water. The suspension thus obtained was homogenized by gentle grinding with a Teflon homogenizer to make uniform gels. Again the gel suspension was washed twice with distilled water and lyophilized.

Determination of the Amount of Materials Incorporated into CM-chitin Gel Lyophilized CM-chitin gels containing BSA or DOX (1 mg) were suspended in 1 ml of 50 mM Tris–HCl buffer containing 0.1% sodium azide (pH 7.4) and incubated for 2 d at 37 °C to allow uniform dissolution. The amount of incorporated BSA was determined by the BIO-RAD PROTEIN ASSAY (Bio-Rad Laboratories, Richmond, CA). The amount of incorporated DOX was determined by measuring the absorbance at 485 nm.

Release of BSA or DOX from CM-chitin Gel by Lysozyme Digestion The suspension of BSA-containing gel in saline (1 mg/ml) was incubated with 500 or 10000 U/ml of egg white lysozyme (EC 3.2.1.17) at 37 °C for 10 d. At various time points, the mixture was centrifuged at 10000 rpm for 5 min and the amount of BSA released into the supernatant

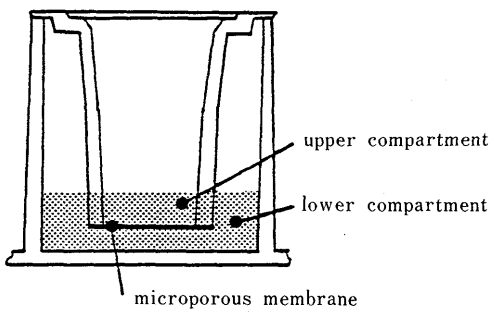

Fig. 1. Transwell Cell Culture Chamber Equipped with Microporous Membranes (8 μm Pore Size)

was measured by BIO-RAD PROTEIN ASSAY. Then saline with lysozyme was added to the remaining gel pellets and incubation was continued at 37 °C. This process was repeated at each time point. The results were expressed as accumulative amounts of released BSA at various time points. Similarly, DOX-containing gel was incubated with or without lysozyme in the upper compartment of a Transwell cell culture chamber equipped with microporous membrane filter (8 μm, pore size) (Costar No. 3422, Broadway/Cambridge) as illustrated in Fig. 1.

Briefly, the gel suspension (100 μl) of CM-chitin containing DOX in MEM supplemented with 2% FBS was added to the upper compartment with or without 500 or 10000 U/ml lysozyme, and 600 μl of MEM containing 2% FBS was added to the lower compartment. At various times after incubation, medium in the lower compartment was collected and the amount of DOX released into the medium was determined by *in vitro* assay for growth inhibition of tumor cells. Media containing DOX thus obtained (0.1 ml) were transferred to a monolayer of B16-BL6 melanoma cells (5×10^3/0.1 ml/well) in 96-well tissue culture plate (Falcon No. 3072, Lincoln Park, New Jersey) and the cells were incubated for 48 h at 37 °C. The cultures were pulsed with 0.5 μCi/well of [^{3}H]thymidine ([^{3}H]TdR, specific activity 23 Ci/mmol, Amersham International, Buckinghamshire, UK) for the last 4 h before the termination of culture, and collected on a glass filter by an automatic cell harvester. The radioactivity was measured in a liquid scintillation counter. The percentage inhibition of the cell growth was calculated from the cumulative radioactivity (cpm) at each time point as follows:

$$\text{growth inhibition (\%)} = \left[1 - \frac{\text{cpm (tumor cells + media containing DOX)}}{\text{cpm (tumor cells alone)}}\right] \times 100$$

Results

Preparation of Chitin Derivative Gel In the first experiment, we tried to prepare gel suspensions of carboxymethylated chitin derivatives (CM-chitin and CM-chitosan) and DAC-70 by the addition of iron(III) chloride (Table I).

Iron(III) chloride solution at final concentrations ranging from 7.5 to 60 mM was added by dropping to a solution of CM-chitin (D.S. = 0.80), CM-chitosan (D.S. = 0.66) or DAC-70. As soon as one drop of iron(III) chloride solution was added to CM-chitin solution, the gel began to form. In the case of DAC-70, however, the gel was not formed by the addition of iron(III) chloride under these conditions. CM-chitin gel was recovered to the extent of more than 80% on a dry weight basis by the addition of more than 15 mM iron(III) chloride, whereas CM-chitosan gel was recovered to the extent of approximately 30% even by the addition of 60 mM iron(III) chloride. We therefore selected CM-chitin for the gel formation in the following experiments, and investigated the effect of the degree of carboxymethylation in CM-chitin on the gel formation. Iron(III) chloride solution (30 mM) was added to solutions of CM-chitin with different degrees of substitution ranging from 0.25 to 1.20.

As shown in Table II, the formation of CM-chitin gel was observed when the degree of carboxymethylation in CM-chitin was more than 0.4. The optimal degree of substitution for gel formation by the addition of 30 mM iron(III) chloride was found to be between 0.5 and 0.8. At the same time, more than 80% of CM-chitin was recovered as gel.

Release of BSA from CM-chitin Gel Containing BSA We next tried to prepare CM-chitin gel containing BSA according to the procedure described above.

Iron(III) chloride at concentrations ranging from 7.5 to

TABLE I. Gel Formation of Chitin Derivatives with Various Concentrations of Iron(III) Chloride

$FeCl_3$[a] (mM)	Recovery[b] (%)		
	CM-chitin	CM-chitosan	DAC-70
0	0	0	0
7.5	38.7±4.7	19.0±2.1	0
15	81.2±1.5	24.4±1.3	0
30	83.6±0.9	23.1±3.6	0
60	89.2±2.9	27.2±2.7	0

a) Various concentrations of $FeCl_3$ were dropped into a solution of CM-chitin (D.S. = 0.80) CM-chitosan (D.S. = 0.66) or DAC-70 (30 mg). The resulting gel was lyophilized and weighed. D.S., degree of substitution. *b*) Recovery is expressed as the weight of the lyophilized gel per that of initially added chitin derivative.

TABLE II. Effects of D.S. in CM-chitin on the Formation of Gel Preparation

D.S.	Weight (mg)	Recovery[a] (%)
0	0	0
0.25	0	0
0.35	2.0±0.8	8.3±2.8
0.40	4.9±0.2	16.2±0.7
0.56	29.7±0.4	99.0±1.4
0.70	29.3±0.4	97.9±1.2
0.80	24.8±1.9	82.7±6.6
1.20	12.6±0.6	41.8±2.1

$FeCl_3$ (30 mM) was added to a solution of CM-chitin (30 mg) with different degrees of carboxymethylation ranging from 0.25 to 1.20. The resulting gel was lyophilized and weighed. *a*) Recovery is expressed as the weight of the lyophilized gel per that of initially added CM-chitin. The value represents mean ± standard deviation of the three independent experiments.

TABLE III. Effect of Concentration of Iron(III) Chloride on Incorporation of BSA into CM-chitin Gel

$FeCl_3$ (mM)	Weight[b] of incorporated BSA (mg)	Recovery[c] (%)
0	0	0
7.5	9.1±1.6	60.9±10.9
15	13.6±0.2	90.5± 1.1
30	12.7±0.6	84.8± 4.0
40	10.5±0.3	69.8± 1.9

a) Various concentrations of $FeCl_3$ solution were added to a mixture of CM-chitin (D.S. = 0.8) and 15 mg of BSA. *b*) The results are expressed as the amount of BSA incorporated into the gel measured by Bio-Rad protein assay. *c*) Recovery is expressed as the amount of BSA incorporated per initially added BSA.

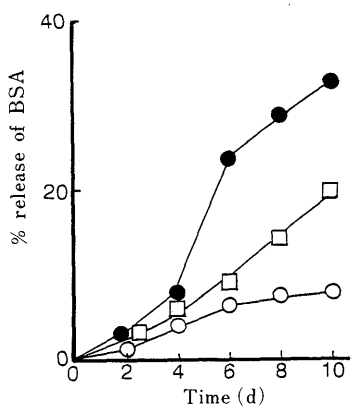

Fig. 2. Release of BSA from CM-chitin Gel by Lysozyme Digestion

CM-chitin gel containing BSA was incubated with medium (○), 500 U/ml (□) or 10000 U/ml (●) of lysozyme for the indicated time in days. The amount of BSA released into culture media was measured.

40 mM were added to the solution containing 50 mg of CM-chitin (D.S. = 0.8) and 15 mg of BSA. The amount of BSA incorporated into gel was observed to be more than 80% when iron(III) chloride solution was added at concentrations from 15 to 30 mM (Table III). As shown in Table I, more than 80% of CM-chitin was recovered as gel by the addition of iron(III) chloride at the same concentration. In order to examine whether or not BSA could be released from CM-chitin gel by lysozyme digestion *in vitro*, CM-chitin gel containing BSA (1 mg gel) was incubated with lysozyme for various times at 37 °C.

As shown in Fig. 2, BSA was scarcely released from the gel in the absence of lysozyme (less than 10%). The release of BSA from BSA-containing gel in the presence of 500 and 10000 U/ml lysozyme was observed to increase in a time-dependent manner after 4 d of incubation, but not within 4 d. On day 10 after the start of incubation, about 20% of BSA was released from the gel by lysozyme treatment at the concentration of 500 U/ml, which is approximately equivalent to the physiological concentration in the human body. When CM-chitin gel without BSA was incubated with 500 U/ml of lysozyme for 4 d, the amount of reducing groups on the sugar residue was shown to increase in a time-dependent manner and was approximately 5% of CM-chitin on day 4 as determined by the method of Park and Johnson with *N*-acetylglucosamine as a reference standard.[11] This indicates that CM-chitin gel alone (without BSA) can be degraded by lysozyme during that period. The appearance of gel changed from viscous and rigid to soft and smooth after 10-d incubation.

Release of DOX from CM-chitin Gel Containing DOX

We tried to prepare CM-chitin gel containing the anticancer drug DOX instead of BSA according to the conditions described previously. The amount of BSA incorporated into the gel was more than 80% of the initial amount added (Table III), whereas the incorporation of DOX into the gel was lower (approximately 30%) under the same conditions (data not shown). We investigated whether or not DOX can be released from CM-chitin gel containing DOX by lysozyme digestion and whether DOX released in the medium is active for the inhibition of tumor cell growth *in vitro* (Fig. 3).

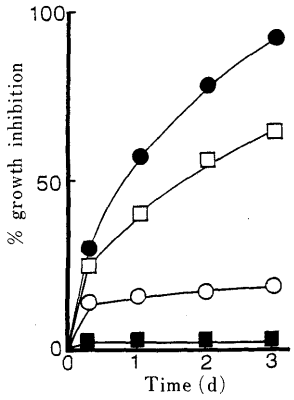

Fig. 3. Inhibition of Tumor Growth by DOX Released from CM-chitin Gel Containing DOX

DOX-containing CM-chitin gel was incubated with medium (○), 5000 U/ml (●) or 500 U/ml (□) of lysozyme for the indicated time in days. The gel without DOX was also incubated with 500 U/ml of lysozyme (■). B16-BL6 cells (5×10^3/well) were cultured with the media containing the released DOX for 48 h at 37 °C. The growth inhibition was assessed in terms of the incorporation of [^{3}H]TdR.

CM-chitin gel containing DOX (10 μg DOX/2.7 mg gel) was incubated with 500 and 5000 U/ml lysozyme in the upper compartment of a Transwell cell culture chamber for various times at 37 °C. The media containing released DOX in the lower compartment was transferred to a monolayer of B16-BL6 melanoma cells (5×10^3) and incubated for 48 h at 37 °C. The growth inhibition of tumor cells was estimated by assay of the uptake of [^{3}H]TdR into the cells. The results were expressed as cumulative inhibition of tumor growth at each time point. When the gel was incubated without lysozyme, the growth-inhibitory effect of the media in the lower compartment appeared rapidly within 12 h (approximately 10% inhibition), but thereafter did not increase. This may be due to spontaneous (nonspecific) release of DOX from the gel. On the other hand, when the gels were incubated with 500 or 5000 U/ml lysozyme, the growth inhibition of B16-BL6 cells by the media containing released DOX was observed to increase in a time-dependent manner. Free DOX (10 μg/ml) corresponding to the total DOX incorporated within the CM-chitin gel caused the complete inhibition of tumor cell growth (approximately 98%) *in vitro* (data not shown). DOX-containing media after a 3-d incubation with 500 U/ml of lysozyme showed 65% growth inhibition of B16-BL6 cells, while DOX-containing media after a 3-d incubation with 5000 U/ml of lysozyme almost completely inhibited the growth of B16-BL6 cells. When CM-chitin gel without DOX was treated with 500 U/ml of lysozyme for 3 d, the media containing both lysozyme and digested CM-chitin gel did not affect the growth of tumor cells, as in the case of media alone. In addition, the amount of iron(III) chloride incorporated within the gel was less than 3% of gel weight as measured by atomic absorption spectrometry and the growth-inhibitory effect of DOX was not affected by the addition of iron(III) chloride (data not shown).

Discussion

The applicability of natural polysaccharides in the design of dosage forms for sustained release has been examined. Some hydrophilic macromolecules such as agar, konjac and pectin are known to form gels in which drugs may be incorporated by means of the formation of matrices. Nakano *et al.* have reported that agar is useful for the preparation of beads that exhibit sustained release of drugs.[12] Pectin was shown to form water-insoluble complexes with nonsteroidal antiinflammatory drugs such as benzydamine hydrochloride and ketoprofen and to give sustained release.[13] Miyazaki *et al.* reported that the sustained release of indomethacin and papaverine hydrochloride was obtained from dried gels of chitin and chitosan.[14]

In the present study, we tried to prepare gels using soluble chitin derivatives under mild conditions. Chitin is insoluble in general in organic solvents as a result of its rigid crystalline structure supported by hydrogen bonds between hydroxy groups and acetamido groups of *N*-acetylglucosamine residues.[15] The introduction of *O*-carboxymethyl groups into chitin was found to increase the solubility in water.[16] The carboxymethylation of chitin led to a decrease in the adsorption of blood components such as serum albumin, γ-globulin and fibrinogen as compared with chitin,[17] and allowed it effectively to bind with metal

ions such as calcium ion.[16] Also CM-chitin showed increased sensitivity to lysozymic degradation as compared with chitin.[7,8] Recently the conjugation of CM-chitin with haptens such as methamphetamine has been reported to facilitate the induction of hapten-specific antibody, but little antibody induction against CM-chitin as a carrier was detected.[18] Thus, taking into consideration the low toxicity, lack of antigenicity and other beneficial properties of CM-chitin, CM-chitin should be a useful material as a sustained-release carrier of drugs. Both efficient incorporation of BSA into the gel and high recovery of the gel were obtained (more than 80%, respectively) when 15 to 30 mM iron(III) chloride was added to a solution of CM-chitin with a degree of carboxymethylation from 0.5 to 0.8 (Tables II and III). The mechanism of gel formation by the addition of iron(III) chloride may involve tetrahedral chelation of CM-chitin on iron(III) ion, assisted by the acetamido, hydroxyl and carboxyl groups. A preliminary small- angle X-ray scattering study has revealed that the radius of gyration was 9.5 Å for iron(III) chelated gel, but 6.3 Å for iron(II) chelated CM-chitin (data not shown). These data might suggest that a larger cavity was formed by iron(III) ion and a much smaller one by iron(II). In this study, the formation of a very loose gel was observed with iron(II) and a rigid gel with iron(III). Low or no formation of gel with CM-chitosan or DAC-70 seems to imply that efficient chelation between iron(III) ion and each of reactive groups can not occur.

In the case of the small-molecular anticancer agent DOX, however, the incorporation rate into the gel was approximately 30% of the initial amount of DOX. The reason for the difference in the incorporation is still unclear. It may be partially due to low affinity between DOX and CM-chitin, high permeability of DOX, *etc.* The incorporated BSA was shown to be continuously released from the gel by lysozyme digestion at a physiological concentration after 4 d of incubation, but not before 4 d. In contrast, the release of DOX was continuously observed in proportion to the incubation time. These results may indicate that the degradation of CM-chitin gel containing high-molecular BSA by lysozyme tends to decrease because of steric hindrance based on the adsorption of BSA on the gel, or the much slower diffusion velocity of BSA in the gel than that of DOX. DOX thus released showed increased inhibition of tumor cell growth *in vitro* (Fig. 3). Therefore, it is expected that the application of CM-chitin as a sustained release-carrier for drugs might provide a promising modality for the therapy of some diseases. We are now applying gel preparations containing various drugs such as anticancer drugs in *in vivo* experiments.

Acknowledgement This work was supported in part by Grants-in-Aid from the Japanese Ministry of Education, Science and Culture; from the Japanese Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control; and for Scientific Research and for Developmental Scientific Research (No. 62870023) from the Japanese Ministry of Education, Science and Culture. Funds were also provided by the Osaka Foundation for Promotion of Clinical Immunology, by Yamanouchi Foundation for Research on Metabolic Disorders, and by CIBA-GEIGY Foundation for the Promotion of Science. We also gratefully acknowledge Grants-in-Aid for Special Project Research from Hokkaido University, Japan.

We thank Ms. M. Sato for her secretarial assistance.

References

1) R. A. A. Muzzareill, "Chitin," Pergamon Press, Inc., New York, 1978, p. 139.
2) S. Miyazaki, K. Ishii and T. Nadai, *Chem. Pharm. Bull.*, **29**, 3067 (1981).
3) R. J. Whistler and M. Kosik, *Arch. Biochem. Biophys.*, **142**, 106 (1971).
4) J. F. Prudden, P. Migel, P. Hanson, L. Friedrich and L. Balassa, *Am. J. Surg.*, **119**, 560 (1970).
5) K. Nishimura, S. Nishimura, N. Nishi, I. Saiki, S. Tokura and I. Azuma, *Vaccine*, **2**, 93 (1984).
6) K. Nishimura, S. Nishimura, N. Nishi, F. Numata, Y. Tone, S. Tokura and I. Azuma, *Vaccine*, **3**, 379 (1985).
7) L. R. Berger and R. S. Wiser, *Biochim. Biophys. Acta*, **26**, 517 (1957).
8) N. Nishi, S. Nishimura, O. Somorin and S. Tokura, *Sen-i Gakkaishi*, **39**, 45 (1983).
9) S. Tokura, N. Nishi, A. Tsutsumi and O. Somorin, *Polymer J.*, **15**, 485 (1983).
10) S. T. Horowitz, S. Roseman and H. J. Blumenthal, *J. Am. Chem. Soc.*, **79**, 5046 (1957).
11) J. T. Park and M. Johnson, *J. Biol. Chem.*, **181**, 149 (1949).
12) M. Nakano, Y. Nakamura, K. Takikawa, M. Kouketsu and T. Arita, *J. Pharm. Pharmacol.*, **31**, 869 (1979).
13) Y. Takahashi, N. Nambu and T. Nagai, *Chem. Pharm. Bull.*, **26**, 3836 (1978).
14) S. Miyazaki, K. Ishii and T. Nadai, *Chem. Pharm. Bull.*, **29**, 3067 (1981).
15) O. Carlstrom, *J. Biophys. Biochem. Cytol.*, **3**, 669 (1957).
16) S. Tokura, S. Nishimura and N. Nishi, *Polymer J.*, **15**, 597 (1983).
17) S. Nishimura, Y. Ikeuchi and S. Tokura, *Carbohydr. Res.*, **134**, 305 (1984).
18) S. Tokura, O. Hasegawa, S. Nishimura, N. Nishi and T. Takatori, *Anal. Biochem.*, **161**, 117 (1987).