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RELEASE BEHAVIOR OF 5-FLUOROURACIL FROM CHITOSAN-GEL NANOSPHERES IMMOBILIZING 5-FLUOROURACIL COATED WITH POLYSACCHARIDES AND THEIR CELL SPECIFIC CYTOTOXICITY

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ABSTRACT

Small-sized chitosan-gel nanospheres, CNSs (average diameter 250 nm), containing 5-fluorouracil (5FU) or immobilizing 5FU derivatives (aminopentyl-carbamoyl-5FU or aminopentyl-ester-methylene-5FU) were prepared by the glutaraldehyde crosslinking technique and the emulsion method. When chitosan was crosslinked with glutaraldehyde, these 5FU derivatives were simultaneously immobilized to CNSs by means of Schiff's base formation. The CNSs were coated with anionic polysaccharides, such as 6-*O*-carboxymethyl-*N*-acetyl-α-1,4-polygalactosamine/Na (CM-NAPGA/Na), 6-*O*-carboxymethyl-chitin/Na (CM-chitin/Na), and sodium hyaluronate, through formation of a polyelectrolyte complex membrane to give CNS/polyanion, i.e., CNS/G, CNS/C, and CNS/H, respectively. The polyelectrolyte complex of polysaccharide was employed to achieve the controlled release and effective targeting of 5FU by the CNSs. The release rate of 5FU from the CNSs could be controlled by immobilization of 5FU, degree of deacetylation of chitosan used and coating with polysaccharides. Since very few galactosamine residues are known to be able to cross-react with ligands for galactose, the galactosamine residues on the surface of CNS/Gs are expected to act as the targeting moieties for hepatocyte. The CNS/G showed the lectin-

mediated aggregation phenomenon by the addition of APA lectin. Moreover, CNS/G had the highest cytotoxic activity among the three kinds of CNS/polyanion and CNS in HLE human hepatoma cell culture system in vitro.

INTRODUCTION

The anticancer agents used clinically at present have not only high activities but also have strong side-effects. So, in cancer chemotherapy, the delivery of anticancer agent to the target tumor cells in a sufficient amount for a desired period of time is one of the most promising approaches to achieve an excellent clinical therapeutic effect and to overcome the problem of severe side-effects. In order to provide a drug delivery system which displays the slow and continuous release of anticancer agent, polymer microsphere or microcapsule systems using some kinds of biodegradable polymer such as polylactide [1], poly(lactide-*co*-glycolide) [2], albumin [3, 4], and fibrinogen [5] have been investigated. Moreover, some approaches to enable the site specific delivery of drugs in vivo using polymer microspheres containing magnetite were reported [6].

Some kinds of saccharide have been found to play an important role in such biological recognitions as receptor–ligand binding and cell–cell adhesion [7–11]. For example, liver parenchymal cells have the receptor which can specifically recognize galactose [8], phagocytic cells have the receptor to mannose [9], and fibroblasts have the mannose-6-phosphate specific receptor [10, 11]. From the standpoint of their application for drug delivery system (DDS), it was also reported that the liposome having mannose moieties exhibited a targetability to the phagocytic cells [12]. Therefore, the microspheres having saccharide moieties on their surface are expected to display the targetability to specific organ or cells.

5-Fluorouracil (5FU) has a remarkable antitumor activity [13–15] which is accompanied, however, by undesirable side-effects [16, 17]. Chitosan is noteworthy as a biogradable polymer carrier in DDS. Partially *N*-acetylated chitosan, which is reported to be selectively collected into tumor cells, is also interesting from the standpoint of targeting to tumor cells. As one study on DDS, we have previously reported on the design of chitosan-gel microspheres (CMSs) and the release behavior of 5FU from them [18].

In order to provide a device achieving the controlled release and the effective targeting of 5FU, a fundamental study on the release behavior of 5FU from small-sized chitosangel nanospheres (CNSs) coated with anionic polysaccharide (Fig. 1) and the appearance of cell specific recognition by them was carried out. The present paper is concerned with the preparation of CNSs coated with polysaccharide, the distributive stability of them, the release behavior of 5FU from them, the phenomenon of their specific aggregation induced by lectin, and the cytotoxic activity of them against HLE human hepatoma cells in vitro. Anionic polysaccharide, which could form a polyelectrolyte complex membrane with CNS on its surfaces, was employed to depress the release rate of 5FU from nonospheres and to increase the distributive stability of CNSs in aqueous solution owing to the excessive anion charge.

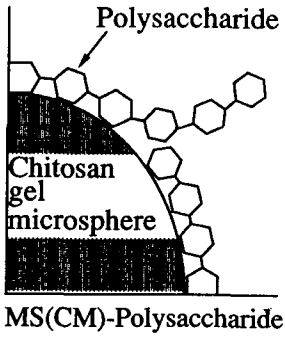


FIG. 1. Estimated structure of nanospheres.

EXPERIMENTAL

Materials

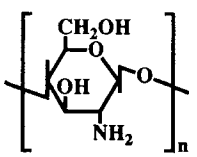
Chitosan [viscosity (η) of 0.5 wt% in acetic acid at 25°C = 5.1 cPs, degree of deacetylation = 80 mol% or η = 5.3 cPs, degree of deacetylation = 100 mol%] was supplied by Kimitsu Chemical Co. Sodium hyaluronate ($M_n = 1.4 \times 10^6$) was supplied by Shiseido Co. 6-*O*-Carboxymethyl-chitin/Na [$M_n = 1.3 \times 10^5$, degree of carboxymetylation (DCM) = 80 mol%] was supplied by Katokichi Co. 6-*O*-Carboxymethyl-*N*-acetyl-α-1,4-polygalactosamine ($M_n = 3-10 \times 10^3$, DCM = 60 mol%) was supplied by Higeta Shoyu Co.

Lectins, Abrus Precatorius Agglutinin (APA) and Concanavalin A (ConA), were purchased from Funakoshi Co. and used without further purification.

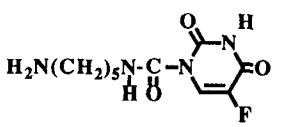
Toluene, acetone, and methanol were purified by the usual distillation methods. Water was ion exchanged, redistilled, and passed through a microfilter before use.

The other materials were of commercial grade and were used without further purification.

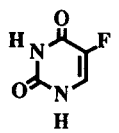
5FU was used in the forms of its sodium salt (5FU/Na), aminopentyl-carbamoyl-5FU (ApC-5FU) [19], and aminopentyl-ester-methylene-5FU (ApEM-5FU) [20] prepared by the methods reported previously.



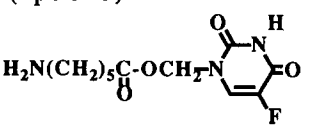
Chitosan



Aminopentyl-carbamoyl-5FU (ApC-5FU)



5FU



Aminopentyl-ester-methylene-5FU (ApEM-5FU)

Preparation of Chitosan-Gel Nanospheres (CNSs)

Chitosan-gel nanospheres crosslinked with glutaraldehyde, CNSs, were prepared according to a water-in-oil (W/O) emulsion method using ultrasonication and a crosslinking technique by glutaraldehyde [4, 18, 21, 22].

5FU derivatives (5FU/Na, ApC-5FU, or ApEM-5FU) (8×10^{-2} mmol) and 103 mg of chitosan (103 mg) were dissolved in 1 mL of 0.01 N HCl aqueous solution. Toluene (50 mL) containing 10 vol% Span 80 was added to the aqueous solution, stirred vigorously, and then sonicated at room temperature. The sonication was carried out with a bath-type sonicator (Bransonic 220, Yamato) for 10 minutes and a probe-type sonicator (UD-200, Tomy) of 100 W for 5 minutes. The emulsion obtained was poured into a three-necked, round-bottom flask. Glutaraldehyde solution (10 mL) saturated with toluene (11.0 mg/mL) containing 10 vol% Span 80 was added slowly to the emulsion by using a dropping funnel and then stirred for 8 hours. The suspension obtained with centrifuged at 1×10^4 rpm for 15 minutes and washed with toluene, acetone, and methanol twice. The CNSs obtained were dried under vacuum.

Preparation of CNS/Polyanion

The CNSs were coated with anionic polysaccharide through the formation of a polyelectrolyte complex membrane [18].

The CNSs (20 mg) were added to 20 mL of a sodium anionic polysaccharide aqueous solution as CM-NAPGA/Na, CM-chitin/Na, and sodium hyaluronate (6.23×10^{-3} saccharide unit mol/L) and incubated at room temperature for 10 minutes. The suspension obtained was centrifuged at 1×10^4 rpm for 15 minutes and then the precipitate was washed twice with water. The three kinds of CNS/polyanion (CNS/G, CNS/C, and CNS/H) obtained were dried under vacuum (Fig. 2).

Estimation of Size of Nanospheres

The size of the nanospheres obtained was estimated by using a JSM-6100 type (JEOL Co.) scanning electron microscope (SEM).

Nanospheres suspended in water were dried in vacuo and rendered electrically conductive with a 15-nm coating of gold films on the surface by using a ion sputter (JFC-1100 Fine Coat, JEOL Co.) after drying to be observed by SEM.

Observation of Distributive Stability of Nanospheres

The nanospheres were added to phosphate buffered saline solution (PBS) of pH 7.4, and the suspension (3 mg/mL) was poured into a quartz cell. The change in turbidity of the suspension was measured by transmittance at 600 nm by using a UV-Visible Recording Spectrophotometer (UV-240, Shimadzu).

Determination of 5FU Content

Nanospheres (2.0 mg) containing 5FU/Na or immobilizing 5FU derivatives (ApC-5FU, ApEM-5FU) were dissolved in 10 mL of 3 N NaOH aqueous solution and stirred for 2 days. The nanospheres were disintegrated, and the 5FU derivatives

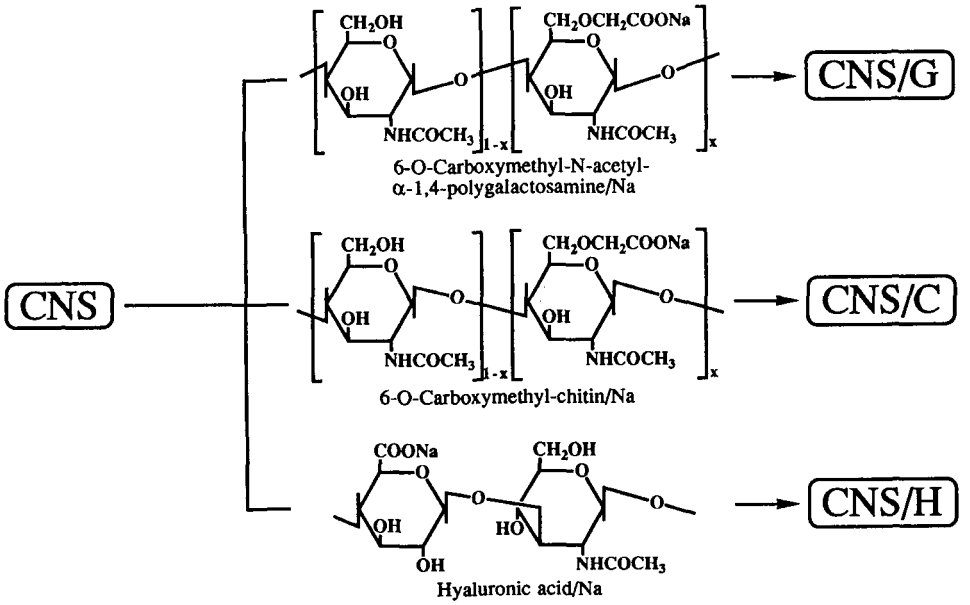


FIG. 2. Preparation of CNSs coated with anionic polysaccharides, CNS/G, CNS/C, and CNS/H.

were converted to free 5FU/Na by alkaline hydrolysis with 3 N NaOH aqueous solution for 2 days. The amount of 5FU encapsulated was determined by measurement of the absorbance of the solution obtained at 283 nm by using UV-240.

Measurement of Release Rate of 5FU from Nanospheres

The release rate of 5FU from the nanospheres was determined by a dialysis system with a cellulose tube [3, 4, 23, 24]. The nanospheres (20 mg), suspended in 5 mL of a physiological saline in a cellulose tube, were dialyzed in 10 mL physiological saline at 37°C. The amount of 5FU released from the nanospheres was examined by measurement of absorbance of the solution at 265 nm by using UV-240.

Observation of Lectin-Medicated Aggregation of CNS/G

APA and ConA were used as two kinds of lectin. Lectin solution (25 μL) in PBS (5.0 mg/mL) was added to 3 mL of the CNS/G suspension of PBS (0.4 mg/mL) of pH 7.4, and the change in turbidity was monitored by measurement of the transmittance at 600 nm by using UV-240 [4, 18].

Tumor Cell Lines

HLE human hepatoma cells were supplied from Shionogi Institute for Medical Science of Shionogi & Co.

Monolayer cultures of HLE were maintained on plastic in Dulbecco's minimum essential medium (D-MEM) supplemented with 10% fetal calf serum (FCS) with kanamycin (80 μg/mL) and amphotericin (2.5 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Measurement of Cytotoxic Activity in Vitro

The cytotoxic activity test was carried out while the cells were in a phase of exponential growth with a doubling time of 34 hours. Cells were diluted in 2 mL of culture medium to give a starting cell density of 1×10^5/2 mL/well in a Corning 24-well plate. The cytotoxic activity was evaluated in a HLE human hepatoma cell culture system in vitro by two methods.

One method was tested in the continuous exposure system. 5FU solution (10 μL) or a suspension of nanospheres immobilizing ApC-5FU in PBS was added to the aliquots of HLE cells by a 2-fold dilution system (starting 5FU converted concentration = 3.0×10^{-2} mol/L). HLE cells were exposed to free 5FU or nanospheres for 45 hours at 37°C without exchange of medium, and then the number of viable cells was counted by the trypan blue dye exclusion method. The cytotoxic activity (%) was calculated by the following equation:

$$\text{Cytotoxic activity (\%)} = (N_c - N_t)/N_c \times 100$$

where N_t and N_c are the number of surviving cells in the treated group and in the untreated group, respectively.

The other method was tested in the short time exposure system. Fifty μL of a suspension of CNS/polyanion immobilizing ApEM-5FU was added to the aliquots with a 5FU converted concentration of 2×10^{-2} mol/L. The HLE cells were exposed to CNS/polyanion for 4 hours at 4°C and then washed twice with fresh medium. Drug-treated HLE cells were incubated for 45 hours at 37°C, and then number of viable cells was counted by the trypan blue dye exclusion method. The cytotoxic activity (%) was estimated by using the formula given above.

RESULTS AND DISCUSSION

Size of Nanospheres

The SEM views of the nanospheres prepared are shown in Fig. 3. Nanospheres with round and nonporous surfaces were confirmed.

The distribution of particle sizes of each nanosphere is shown in Fig. 4. The average diameters of the nanospheres obtained by using two types of sonicator were estimated to be 250–300 nm by SEM. The coating with polysaccharides had no effect on the sizes and shapes of the nanospheres.

On the other hand, the average diameter of the chitosan-gel microspheres (CMSs) obtained by using only the bath-type sonicator was 1 μm [18].

Distributive Stability of Nanospheres

The results of the distributive stability test of CMS (average diameter 1 μm), CNS (average diameter 250 nm), and CNS/G (average diameter 290 nm) are shown in Fig. 5. The lower distributive stability of nanospheres results in increasing the

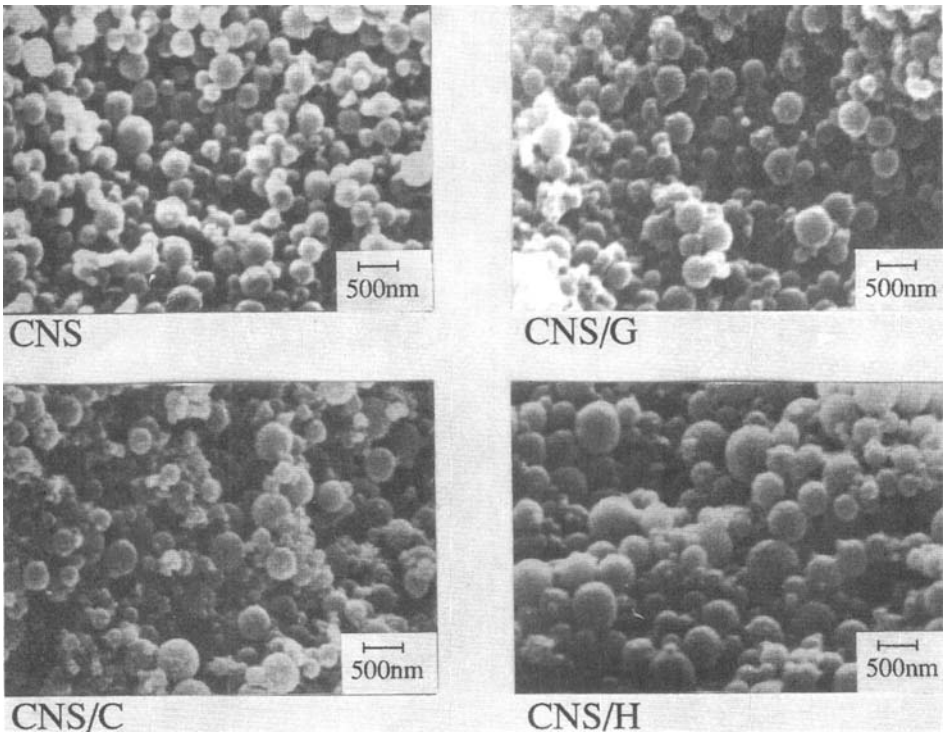


FIG. 3. SEM views of CNS, CNS/G, CNS/C, and CNS/H.

transmittance of the supsension. CNS has a high distributive stability in aqueous solution because of its small size. However, the distributive stability of CNS/G was higher than that of CNS because of electrostatic repulsion by the excessive anion charge on the surface of CNS/G. These results suggest the possibility of embolizing of blood stream was very low.

5FU Content in Nanospheres

In spite of the immobilization of 5FU, the degree of deacetylation of the chitosan used, and the coating with polysaccharides on the surface of CNSs, the amount of 5FU encapsulated in the nanospheres obtained could be controlled to 4–6 $\times$ 10^{-2} mg/mg.

Release Rate of 5FU from Nanospheres

The release rates of 5FU from nanospheres in physiological saline at 37°C are shown in Figs. 6–10.

The release rates of 5FU from the CNSs containing free 5FU and immobilizing ApC-5FU or ApEM-5FU are shown in Fig. 6. The slow release of 5FU was achieved by using ApEM-5FU as a drug because of its slower hydrolytic rate of ester bond via the monomethylene bond in ApEM-5FU compared to that of the carbamoyl

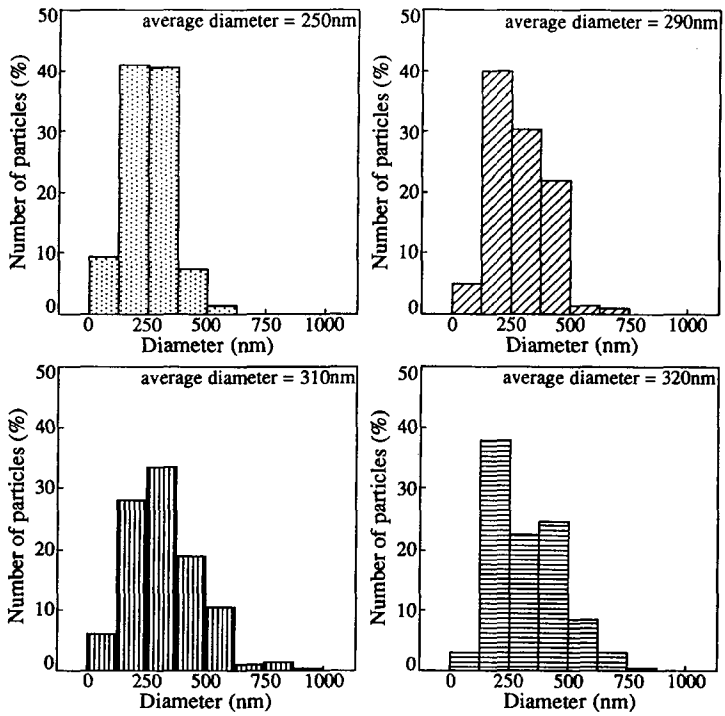


FIG. 4. The distributions of particle sizes of CNS, CNS/G, CNS/C, and CNS/H. Upper left: CNS. Upper right: CNS/G. Lower left: CNS/C. Lower right: CNS/H.

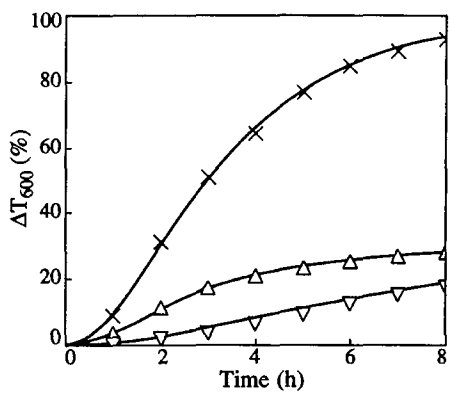


FIG. 5. Effects of particle size and coating polysaccharides on the distributive stability of CMS, CNS, and CNS/G in phosphate buffered saline solution. (△) CMS, average diameter = 1 μm. (×) CNS, average diameter = 250 nm. (▽) CNS/G, average diameter = 290 nm.

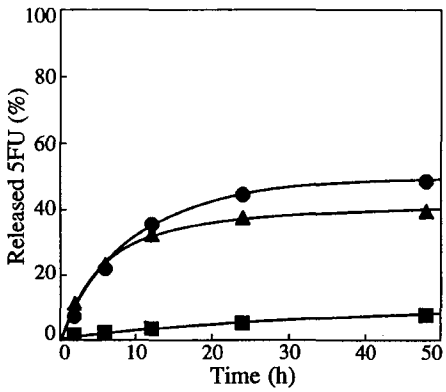


FIG. 6. Release rate of 5FU from CNSs (degree of deacetylation of chitosan = 100 mol%) containing free 5FU or 5FU derivatives in physiological saline at 37°C. (●) Free 5FU, (▲) ApC-5FU, (■) ApEM-5FU.

bond in ApC-5FU. 40% of 5FU from CNS-immobilizing ApEM-5FU was released in a period of 8 weeks (Fig. 7).

The effect of the degree of deacetylation of chitosan used on the release rate of 5FU from CNSs is shown in Fig. 8. The release rate of 5FU from CNSs prepared by using a high degree of deacetylation of chitosan was depressed owing to their high crosslinking density.

Depression of the release of 5FU was also observed by coating CNS with an anionic polysaccharide (Fig. 9). Compared with the release rate of 5FU from CNS after 48 hours, that from CNS/polyanion was depressed to one-third. These results mean that the polysaccharide polyelectrolyte complex membrane on the CNS acted as an effective barrier to the release of 5FU. The release rates of 5FU from the three kinds of CNS/polyanions were not very different because the nanospheres were almost perfectly coated with excess polysaccharides.

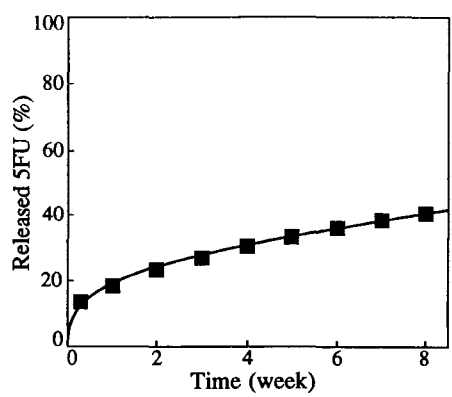


FIG. 7. Release rate of 5FU from CNS (degree of deacetylation of chitosan = 100 mol%) immobilizing ApEM-5FU in physiological saline at 37°C.

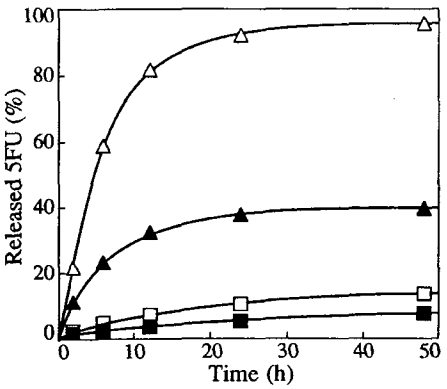


FIG. 8. Effect of degree of deacetylation of chitosan on the release rate of 5FU from CNSs in physiological saline at 37°C. (△) Immobilizing ApC-5FU, degree of deacetylation of chitosan = 80 mol%. (▲) Immobilizing ApC-5FU, degree of deacetylation of chitosan = 100 mol%. (□) Immobilizing ApEM-5FU, degree of deacetylation of chitosan = 80 mol%. (■) Immobilizing ApEM-5FU, degree of deacetylation of chitosan = 100 mol%.

Moreover, the release rate of 5FU from CNS prepared by using a high degree of deacetylation of chitosan was further depressed by coating it with polysaccharide (Fig. 10).

Only free 5FU was released from the CNSs in physiological saline. 5FU derivatives were not detected.

Lectin-Mediated Aggregation of CNS/Gs

Lectins are known to bind to certain saccharides or glycoproteins specifically and to aggregate some kinds of cell. Lectin APA is well known to bind specifically to the galactose or *N*-acetyl galactosamine unit [25].

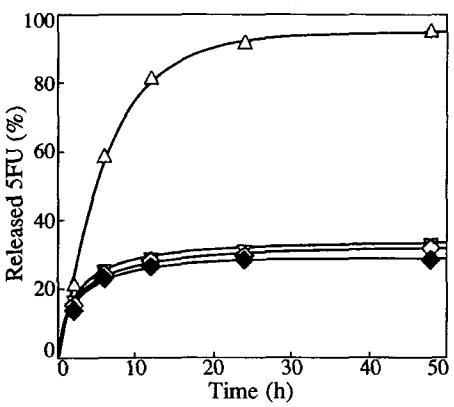


FIG. 9. Effect of coating with polysaccharides on the release of 5FU from CNS (degree of deacetylation of chitosan = 80 mol%) or CNS/polyanion immobilizing ApC-5FU in physiological saline at 37°C. (△) CNS, (▽) CNS/G, (◇) CNS/H, (◆) CNS/C.

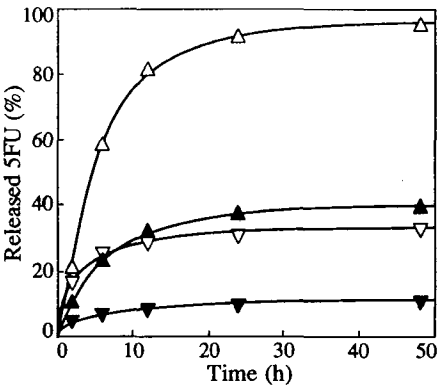


FIG. 10. Effect of deacetylation of chitosan on the release rate of 5FU from CNSs and CNS/Gs immobilizing ApC-5FU in physiological saline at 37°C. CNS: (△) degree of deacetylation of chitosan = 80 mol%, (▲) degree of deacetylation of chitosan = 100 mol%. CNS/G: (▽) degree of deacetylation of chitosan = 80 mol%, (▼) degree of deacetylation of chitosan = 100 mol%.

The addition of APA to the CNS/G suspension was found to induce a drastic decrease in the turbidity of the suspension (Fig. 11). Such a phenomenon indicates that aggregation occurs by the addition of APA.

A change in turbidity was not observed when APA was added to a suspension of CNS without CM-NAPGA coating. Moreover, the aggregation of CNS/Gs through the inter-CNS/G bridge by APA was dissociated by the addition of excess lactose.

A change in the turbidity of the CNS/G suspension was not observed upon the addition of ConA, which specifically bound to the α-D-mannose or the α-D-glucose unit [26].

Therefore, the occurrence of CNS/Gs aggregation is attributed to specific binding of APA to galactosamine residues located on their surfaces. These results

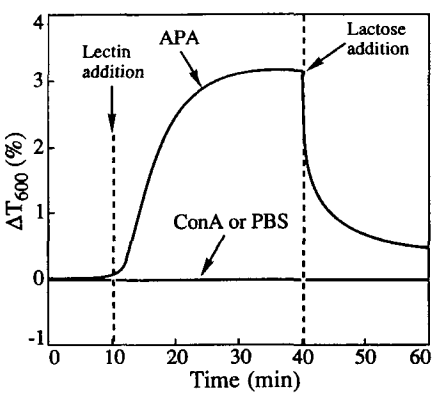


FIG. 11. Agglutination of CNS/G by addition of various kinds of lectin in PBS.

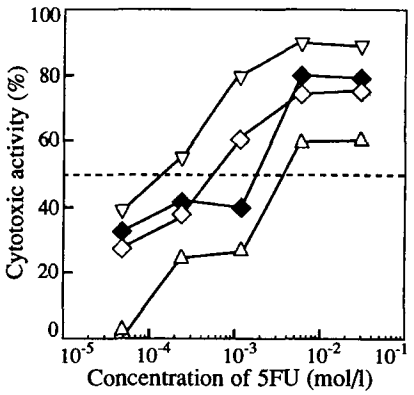


FIG. 12. Cytotoxicity of CNS/polyanion immobilizing ApC-5FU and free 5FU against HLE human hepatoma cells on the continuous exposure system for 45 hours at 37°C in vitro: (▽) CNS/G, (△) CNS/C, (◆) CNS/H, (◇) 5FU.

suggest that the saccharide chain on the surface of CNSs can be specifically recognized by lectin-like receptors of cells.

Cytotoxic Activity in Vitro

The possibility of targeting of 5FU to hepatoma cells by using CNSs coated with polysaccharide was investigated in vitro. The effect of dose on the cytotoxic activity in the continuous exposure system (37°C, 45 hours) by free 5FU or CNS/polyanion immobilizing ApC-5FU against HLE human hepatoma cells in vitro is shown in Fig. 12. The cytotoxic activity of CNS/G coated with CM-NAPGA was found to be higher than CNS/H and CNS/C coated with other polysaccharides against HLE cells. The 50% inhibition concentration (IC_{50}) value of CNS/G was comparable to that of free 5FU (Table 1).

TABLE 1. IC_{50} Values of CNS/Polyanion Immobilizing ApC-5FU or Free 5FU Against HLE Human Hepatoma Cells[a]

Sample	IC_{50} (mg/mL)
CNS/G	2.6×10^{-4}
CNS/C	2.9×10^{-3}
CNS/H	2.3×10^{-3}
Free 5FU	5.6×10^{-4}

[a]Cultured in 24-well multiplate in D-MEM medium (1 mL/well) supplemented with 10% fetal calf serum with kanamycin (80 μg/mL) and amphotericin (2.5 μg/mL) at 37°C in 5% CO_2 atmosphere. Incubation time: 45 hours.

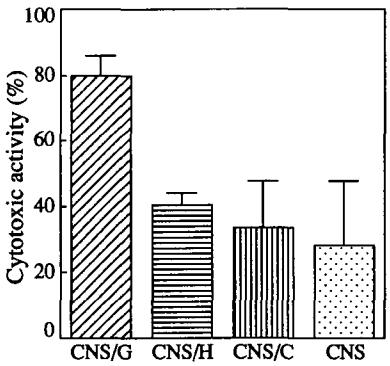


FIG. 13. Cytotoxicity of CNS and CNS/polyanion immobilizing ApEM-5FU against HLE human hepatoma cells in short time exposure system. The cells were cultured for 45 hours at 37°C after 4 hours exposure at 4°C against CNS immobilizing ApEM-5FU coated with various polysaccharides in vitro.

The results of cytotoxic activity in the short time exposure system (4°C, 4 hours) by CNS or CNS/polyanion immobilizing ApEM-5FU against HLE human hepatoma cells are shown in Fig. 13. CNS/G coated with CM-NAPGA exhibited the highest cytotoxic activity against HLE cells among the CNSs tested and the three kinds of CNS/polyanion. The hepatocyte has galactose receptors. Therefore, these results can be explained by the specific binding of *N*-acetyl-galactosamine residues located on the surface of the CNS/G to the galactose-receptor on the surface of the hepatoma cells and the uptake of the CNS/G into hepatoma cells via galactose-receptor mediated endocytosis.

The remarkable interaction of CNS/G with HLE could selectively supply a large amount of free 5FU to HLE. Therefore, the chitosan-gel nanospheres technique is available as a drug delivery system.

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