Preparation and Release Characteristics of Cisplatin Albumin Microspheres Containing Chitin and Treated with Chitosan

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Cisplatin (CDDP) containing albumin microspheres and microcapsules incorporating biodegradable macromolecules, chitin and chitosan, were prepared, and their CDDP content and releasing ability and susceptibility to various enzymes were examined. Chitin was incorporated during preparation of the microspheres, while chitosan was used to treat preformed microspheres. CDDP content was remarkably increased by chitin; when chitin was incorporated at a concentration of 1.5%, the CDDP content of the microspheres was found to be 16.2% (1.8 times that with no addition of chitin). CDDP release was suppressed by chitin and chitosan. The 50% CDDP release time was about 1.5 h when no chitin was added, but about 16 h was required when chitin was incorporated into the microspheres at a concentration of 1.5%.

Chitin and chitosan suppressed the decomposition by protease. The microspheres treated with 70% deacetylated chitosan showed the greatest susceptibility to lysozyme. In conclusion, CDDP release can be controlled by the use of chitin or chitosan, and the microspheres should show no immunogenicity *in vivo* because of their susceptibility to lysozyme.

Keywords cisplatin; albumin microsphere; chitin; chitosan

There has been considerable progress recently in the application of microspheres or microcapsules for the delivery of anticancer and other drugs, and several treatment regimens have been examined.[1-6] In a previous study,[7] we prepared cisplatin (CDDP) albumin microspheres with albumin and ethyl cellulose, but obtained low CDDP content and inadequate slow release activity. In the present study we prepared CDDP albumin microspheres with biodegradable macromolecules, chitin and chitosan.

Experimental

Reagents CDDP powder was kindly supplied by Nippon Kayaku Co. In addition, the following agents were employed; human serum albumin (The Green Cross Co.), ethyl cellulose, chitin and chitosan (degree of deacetylation, 70, 80, 90, 100%) (Nakarai Tesque Co., Ltd.), protease and lysozyme (Sigma Co., Ltd.). All other reagents employed were commercial special-grade products.

Preparation of CDDP Albumin Microspheres and Chitin-Containing CDDP Albumin Microspheres Albumin microspheres were prepared as described in the previous report.[7] Chitin-containing CDDP albumin microspheres were prepared in the same way with CDDP and various concentrations of chitin dispersed in the albumin solution. The microspheres were filtered, and 74—177 μm microspheres were dried at 135 °C for 2 h.

Preparation of Chitosan-Treated CDDP Albumin Microspheres Albumin microspheres and chitin-containing albumin microspheres were added to various concentrations of chitosan–acetic acid (5% acetic acid) solution and mixed. The mixtures were washed with water and filtered. The chitosan-treated CDDP albumin microspheres were filtered off, and 74—177 μm microspheres were dried for 2 h at −25, 70, or 135 °C.

CDDP Content CDDP albumin microspheres (5 mg) were added to 5 ml of normal saline and homogenized. The mixture was centrifuged for 10 min at 2500 rpm, and the supernatant was decanted into a 25 ml measuring flask. This process was repeated 3 times and finally, the collected supernatant was diluted to 25 ml with normal saline. CDDP content in the solution was measured by an atomic absorption spectrophotometer (Hitachi Z-9000).

CDDP Release Test The test was conducted as described in the previous report.[7] Normal saline (100 ml) as a release solution was placed in a release cell, which was immersed in a thermostated tank maintained at 37 °C. A cell (nitrate cellulose membrane 3 μm) containing a suitable amount of the sample was immersed in this tank. The contents of the cell were stirred (50 rpm) with a stirring rod, and aliquots of the solution were serially taken. The CDDP content in the release solution was measured by atomic absorption spectrophotometry.

Observation by Scanning Electron Microscopy In order to examine the shape and surface character, CDDP albumin microspheres were examined under a scanning electron microscope. The microspheres were coated with platinum by a vacuum deposition apparatus (Hitachi HUS-5GB) and observed under a scanning electron microscope (Hitachi S-450) at an acceleration voltage of 5 kV.

Effect of Protease on Microspheres Protease was added to normal saline at a concentration of 1%. The CDDP released from the microspheres into the solution was measured by atomic absorption spectrophotometry, and the changes in the form of the microspheres were examined by microscopy (Nikon UFX).

Effect of Lysozyme on Microspheres Lysozyme was added to normal saline at a concentration of 1.5×10^{-2} mg/ml. The CDDP released from the microspheres into the solution was measured by atomic absorption spectrophotometry, and the changes of the form of the microspheres were examined by microscopy.

Measurement of the Viscosity Lysozyme was added to each chitosan–acetic acid solution at a concentration of 1.5×10^{-2} mg/ml, and changes of the viscosity of the solution was followed in order to examine the decomposition of chitosan by lysozyme. The concentration of chitosan was 1.5%, and the viscosity was measured at 37 °C by using a viscosity meter (Tokyo Keiki Visconic ED).

Results

CDDP Content and Chitin Concentration The relationship between chitin concentration and CDDP content is shown in Table I. CDDP content was found to be 16.2% at the chitin concentration of 1.5%, and the content was 1.8 times that when no chitin was added. The recovery rate increased with increase of chitin concentration, reaching 92% at the chitin concentration of 1.5%. No decrease of the content was noted when the microspheres were treated with chitosan.

CDDP Release from Albumin Microspheres and Chitin-Containing Albumin Microspheres The CDDP release profiles from albumin microspheres at various chitin concentrations are shown in Fig. 1. The time required for 50% release was about 1.5 h in the absence of chitin, but was 16 h at 1.5% chitin. The results indicated that the release rate was suppressed as the concentration of chitin was increased.

TABLE I. Effect of Chitin on CDDP Content

Concentration of chitin (%)	CDDP content (μg Pt/mg)
0	92.1
0.25	110.3
0.50	120.8
1.00	150.7
1.50	162.4

($n=5$).

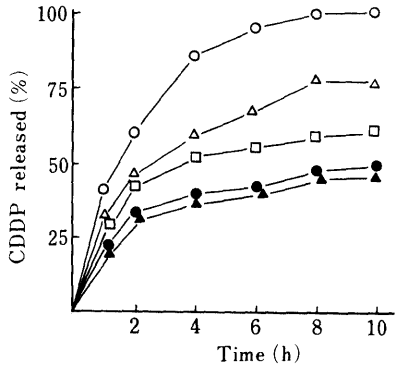

Fig. 1. Effect of Chitin on the Profiles of CDDP Release from Albumin Microspheres

Concentration of chitin: ○, 0%; △, 0.25%; □, 0.50%; ●, 1.00%; ▲, 1.50%.

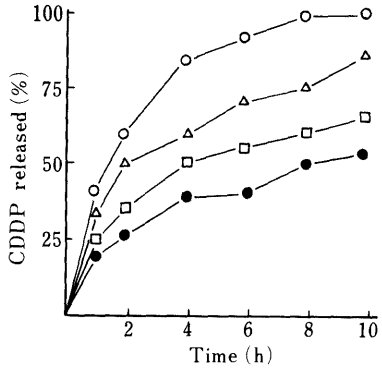

Fig. 2. Effect of Chitosan on the Profiles of CDDP Release from Albumin Microspheres Treated with Chitosan

Concentration of chitosan: ○, 0%; △, 0.5%; □, 1.0%; ●, 1.5%.

CDDP Release from Chitosan-Treated Albumin Microspheres The CDDP release profiles from chitosan-treated albumin microspheres (containing no chitin) at various chitosan concentrations are shown in Fig. 2 (70% deacetylated chitosan was used). The time required for 50% release was about 1.5 h in untreated microspheres reaching 8 h in the microspheres treated with 1.5% chitosan. The results indicate that the release rate was suppressed as the concentration of chitosan was increased.

CDDP release profiles from 1.5% chitin-containing chitosan-treated albumin microspheres are shown in Fig. 3 (70% deacetylated chitosan was used). Release of CDDP from the microspheres was suppressed by chitosan treatment. However, the effect of chitosan on CDDP release was less than that of chitin. Similar results were obtained when the microspheres were treated with chitosan deacetylated to various extents.

CDDP release from chitin-free chitosan-treated microspheres at various drying temperatures (−25, 70, or 135 °C) is shown in Fig. 4 (chitosan was 70% deacetylated and was used at a concentration of 1.5%).

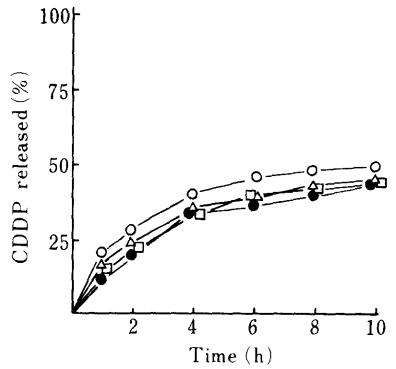

Fig. 3. Effect of Chitosan on the Profiles of CDDP Release from Chitin-Containing and Chitosan-Treated Albumin Microspheres

Concentration of chitosan: ○, 0%; △, 0.5%; □, 1.0%; ●, 1.5%.

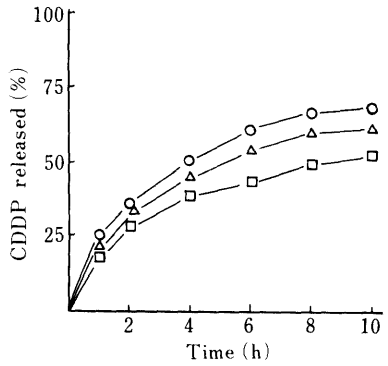

Fig. 4. Effect of Drying Temperature on the Profiles of CDDP Release from Albumin Microspheres treated with Chitosan

Drying temperature: ○, −25 C; △, 70 C; □, 135 C.

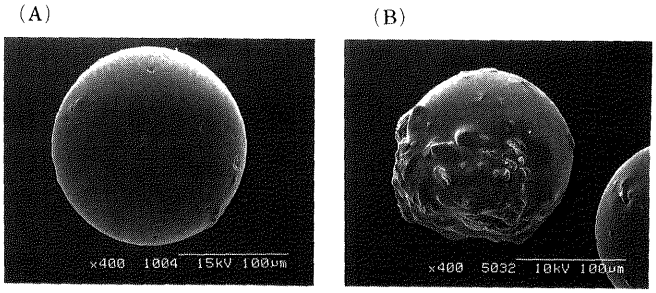

Fig. 5. Scanning Electron Micrographs of CDDP Albumin Microspheres

(A) chitinless, (B) containing 1.5% chitin.

Observation by Scanning Electron Microscopy Electron microscopic photographs of CDDP albumin microspheres and chitin-containing CDDP albumin microsphere are shown in Fig. 5. Rod-like chitin crystals can be seen at the surface of the 1.5% chitin-containing microsphere.

Effect of Protease on CDDP Release The effect of protease on CDDP release from albumin microspheres or chitin-containing albumin microspheres is shown in Table II. The time required for 50% CDDP release from chitinless microspheres decreased from about 1.5 to 0.5 h when protease was added to the released solution. The effect of

Table II. Effect of Protease on the CDDP Release from CDDP Microspheres

Chitin added (%)	Time of 50% release (h)	
	Physiological saline solution	Physiological saline solution containing protease
0	1.5	0.5
0.25	2.5	1.8
0.50	3.5	2.5
1.00	10.0	8.0
1.50	16.0	15.5

($n=5$).

Table III. Effect of Lysozyme on the CDDP Release from CDDP Microspheres

Chitin added (%)	Time of 50% release (h)	
	Physiological saline solution	Physiological saline solution containing lysozyme
0	1.5	1.4
0.25	2.5	2.1
0.50	3.5	2.0
1.00	10.0	6.0
1.50	16.0	6.5

($n=5$).

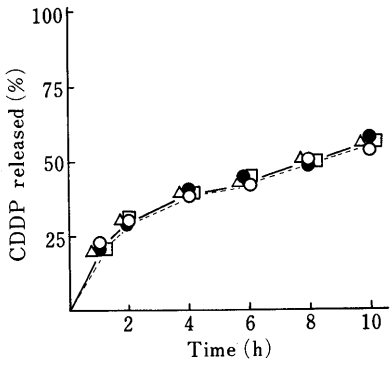

Fig. 6. Effect of Protease on the Profiles of CDDP Release from Albumin Microspheres Treated with Chitosan

Deacetylation: ○, 70%; △, 80%; □, 90%; ●, 100%. (------), normal saline; (——), normal saline containing protease.

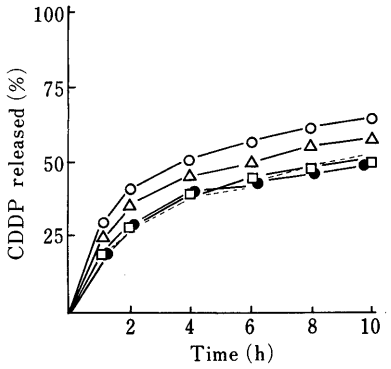

Fig. 7. Effect of Lysozyme on the Profiles of CDDP Release from Albumin Microspheres Treated with Chitosan

Deacetylation: ○, 70%; △, 80%; □, 90%; ●, 100%. (------), normal saline, (——), normal saline containing lysozyme.

protease decreased as the concentration of chitin was increased.

The effect of protease on CDDP release from chitosan-treated microspheres is shown in Fig. 6. No chitin was added. The effect of protease was suppressed by chitosan treatment. No deacetylation level-related effect was observed. However, the changes of the form of the microspheres in the releasing solution depended upon chitin concentration and chitosan treatment; although decomposition by protease was observed when chitinless microspheres were not treated with chitosan, decomposition was not observed in the case of chitosan treatment. Furthermore, although some cracking was observed on the surface of 1.5% chitin-containing microspheres, no decomposition was observed when the microspheres were treated with chitosan.

Effect of Lysozyme on CDDP Release The effect of lysozyme on CDDP release from albumin microspheres and chitin-containing albumin microspheres is shown in Table III. No significant effect was observed on the time required for 50% CDDP release when no chitin was added; 1.5 *vs.* 1.4 h. The release from chitin-containing albumin microspheres depended upon chitin concentration; the time required for 50% release increased as the chitin concentration was increased.

The effect of lysozyme on CDDP release from chitosan-treated albumin microspheres is shown in Fig. 7. No chitin was added. CDDP release from the microspheres was increased when lysozyme was added to the release solution; the increase was very large when the microspheres were treated with 70% deacetylated chitosan.

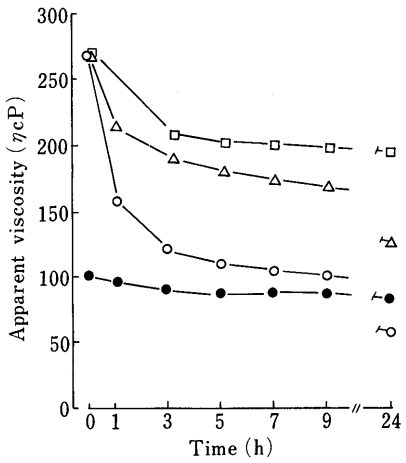

Fig. 8. Effect of Lysozyme on the Viscosity of Chitosan Solution

Deacetylation: ○, 70%; △, 80%; □, 90%; ●, 100%.

Changes in the form of the microspheres owing to decomposition by lysozyme were observed in chitin-containing or chitosan-treated microspheres.

Effect of Lysozyme on the Viscosity of Chitosan Solution The effect of lysozyme on the viscosity of 1.5% chitosan solution in 5% acetic acid is shown in Fig. 8. Decomposition of the microspheres by lysozyme depended upon the deacetylation levels of chitosan; 70% deacetylated chitosan was about 20% decomposed after 24 h. The results were consistent with the results of CDDP release tests and the changes of the microsphere form.

Discussion

Chemo-embolization may be an effective therapeutic modality for hepatocellular carcinoma, and various trials have been carried out. Microspheres prepared from albumin or cellulose can be used as carriers of thrombolytic drugs.[8-13] However, there are many problems concerning drug release and contents, and susceptibility to enzymes. In the present study, CDDP albumin microspheres were prepared with biodegradable macromolecules, chitin and chitosan. The *in vitro* results indicated that CDDP content was increased when chitin was added and the release of CDDP was suppressed from microspheres treated with chitosan. Therefore it is considered that the CDDP capacity of the matrix was increased when chitin was added. When CDDP microspheres were treated with chitosan, a hydrogen-bonded film of chitosan may be formed at the surface and may suppress the release of CDDP. When the drying temperature was increased during the preparation of chitosan-treated CDDP microspheres, CDDP release was suppressed, presumably because the degree of crystallinity of chitosan was increased, producing a more rigid surface membrane.

CDDP albumin microsphere susceptibility to enzymes and immunogenicity are potential problems, *in vivo*. These points were examined *in vitro*. The results indicated that the effect of protease decreased as the concentration of chitin was increased and with chitosan treatment. Decomposition by lysozyme was observed in chitin-containing or chitosan-treated microspheres. It was observed that the release depended upon the degree of deacetylation of chitosan. The decomposition was remarkable when the microspheres were treated with 70% deacetylated chitosan. It is therefore considered that chitin-containing albumin microspheres and chitosan-treated microspheres are unlikely to show marked immunogenicity. It has been reported that 70% deacetylated chitosan has excellent adjuvant activity,[14] and so CDDP albumin microspheres treated with 70% deacetylated chitosan might be a suitable formulation to enhance the antitumor activity of CDDP.

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