Preparation and Evaluation of Glutaraldehyde Cross-Linked Chitosan Microspheres as a Gadolinium Reservoir for Neutron-Capture Therapy

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Chitosan microspheres (CMSs) containing water-soluble gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) were prepared by an emulsion method using Span 80 as a surfactant and glutaraldehyde (GA) as a cross-linker for the gadolinium neutron-capture therapy of cancer. When Gd-DTPA was applied at more than a 1 : 1 number ratio of its carboxylic groups to the amino groups of chitosan, the content of Gd in CMS was saturated at about 13% and the mass median diameter of CMS was increased beyond that of placebo CMS. The increase in the amount of Span 80 and GA applied led to a decrease in the CMS size and the Gd content. The electrostatic interaction between chitosan and Gd-DTPA and the preferential surface-hardening by GA contributed to the formation of fine, spherical, Gd-enriched and prolonged-releasing CMSs with a mass median diameter of 1.9 μm, a Gd content of 6.1% and a 50% dissolution time of 2.4 h.

Key words gadolinium neutron-capture therapy; microsphere; gadopentetic acid; chitosan

The success of gadolinium neutron-capture therapy (Gd-NCT) depends on a high accumulation of Gd in the tumor.[2] For Gd-NCT, ethylcellulose microcapsules containing a dimeglumine salt of gadolinium diethylenetriaminepentaacetic acid (Gd-DTPA) at a content of 27%, with a mass median diameter of 126 μm, were previously prepared.[3] These microcapsules have already been applied to *in vivo* experiments involving Gd-NCT using murine ascites tumor-bearing mice, leading to significantly prolonged survival.[2] Further, microcapsules containing hydrophobic Gd-DTPA stearylamide (Gd-DTPA-SA) coated with acrylic polymer were designed and prepared; they had a mass median diameter of 52 μm, a Gd-DTPA-SA content of 38% and a Gd content of 5.6%.[4]

Chitosan is biocompatible[5] and biodegradable,[6] in contrast to ethylcellulose and acrylic polymer. Therefore, chitosan microspheres (CMSs) have been investigated using many kinds of drugs as drug carriers.[7] Gd-DTPA, used as a Gd source in this study, contains two free carboxylic groups, and chitosan is a polycationic biopolymer. Therefore, it was expected that the electrostatic interaction between chitosan and Gd-DTPA would be able to create Gd-enriched CMSs.

The objective of the present study was to develop Gd-enriched CMSs as a depot in Gd-NCT by an emulsion technique using sorbitan monooleate (Span 80) as a surfactant and glutaraldehyde (GA) as a cross-linker.[7a] The effects of the amounts of Gd-DTPA, Span 80 and GA and the type of homogenizer on CMS size, Gd content and CMS morphology were studied.

Experimental

Materials Chitosan (10B, 100% deacetylated; the weight-average molecular weight, 950000[8]) was purchased from Katokichi Co., Ltd., Japan. Gd-DTPA was purchased from Aldrich Chemical Co., Inc., U.S.A. The other reagents were obtained from Nacalai Tesque, Inc., Japan, and used without further purification.

Preparation of CMSs The method reported by Ohya *et al.*[7a] was used with slight modification (see the caption of Fig. 1). As a homogenizer, Physcotron (NS-50, NITI-ON, Japan; open type NS-10 shaft, 22000 rpm, 1 h) or Clearmix (CLM-0.8S, M-Technique Co., Ltd., Japan; R2-rotor, 0.2 mm screen, open system, 14000 rpm, 15 min) was used to form primary w/o emulsions.

Characterization of CMSs To determine Gd content, 10 mg of CMSs were ashed in concentrated HNO_3 and then the ash was dissolved in 5 ml of 6.6 N HNO_3. The Gd concentration in the solution was determined by inductively coupled plasma emission spectrography (ICP-AES; P-5200 ICP system, Hitachi Co., Ltd., Japan) at 335.047 nm.

CMS size distribution was measured in methanol by a laser scattering size analyzer (LDSA-2400A, Tonichi Computer Applications Co., Ltd., Japan). The morphology of CMSs was assessed by scanning electron microscopy (SEM, JEOL JSM-5300LV, Japan).

The Gd release from CMS was studied using a dynamic dialysis system[9] with a cellulose tube; the cut off molecular weight was 12000—14000. Nineteen mg of CMSs were suspended in 4 ml of isotonic phosphate buffer (pH 7.4) in the cellulose tube and incubated at 37 °C against 46 ml of the same buffer solution under gentle stirring. Samples (4 ml) were withdrawn from the outer solution at certain time intervals and replaced by 4 ml of the buffer solution. The amount of Gd in the samples was measured by ICP-AES.

Results

A series of CMSs was prepared increasing the amount of Gd-DTPA. The Gd content sharply increased to 12.5% at 1.7 of the applied weight ratio of Gd-DTPA to chitosan (Gd-DTPA/chitosan ratio), followed by only a marginal increase (Fig. 1A). The mass median diameter (D50) of CMSs once became smaller than that of placebo CMS, but above a 1.7 ratio of Gd-DTPA/chitosan, it increased beyond that of the placebo CMS. SEM indicated that the placebo CMSs were entirely smooth-faced and spherical, but Gd-DTPA-CMSs partially contained nonspherical fragments and aggregates. Especially, all CMSs prepared at a 9.7 ratio of Gd-DTPA/chitosan were nonspherical, rough-faced, collapsed and/or aggregated. CMS prepared at a 1.7 ratio of Gd-DTPA/chitosan will be denoted below as Gd-DTPA-CMS(L).

With increasing amounts of Span 80 in the primary emulsion at the Gd-DTPA/chitosan ratio of 1.7, D50 decreased quickly, along with a comparatively small change in Gd content (Fig. 1B), and reached 3.1 μm when 20 g of Span 80 was used.

D50 decreased with increases in the volume of GA-saturated toluene (GST), and the size distribution became narrower (Gd-DTPA-CMS (P1, P10, P50), Table 1). SEM of Gd-DTPA-CMS (P50) showed that most CMSs were smooth-faced and spherical, but some with a nonspherical

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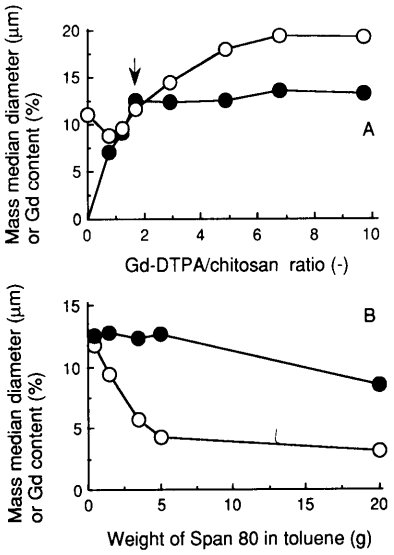

Fig. 1. Effects of the Applied Weight Ratio of Gd-DTPA to Chitosan (A) and the Amount of Span 80 (B) on the Size (○) and the Gd Content (●) of Gd-DTPA-CMS

(A) Various amounts of Gd-DTPA and 103 mg of chitosan were dissolved in 4 ml of 5% acetic acid aqueous solution. This solution was added to 60 ml of toluene containing 420 mg of Span 80 under vigorous stirring using a homogenizer, Physcotron, to form a w/o emulsion. Ten ml of GST, wherein Span 80 was added at 6% (w/v), was added to the w/o emulsion and stirred using a magnetic stirrer at room temperature overnight to provide cross-linked CMSs. The resultant suspension was centrifuged at 3000 rpm for 30 min. Finally, CMSs were washed twice with toluene, methanol, distilled water and acetone and dried on silica gel under a vacuum condition at room temperature. The arrow represents the formulation of Gd-DTPA-CMS(L). (B) The Gd-DTPA/chitosan ratio of 1.7 in 4 ml of 5% acetic acid aqueous solution, the volume of toluene (60 ml) dissolving various amounts of Span 80 and the volume of GST (10 ml) wherein Span 80 was added at 6% (w/v) were kept constant, respectively. GST was prepared by vigorously vortexing a mixture of 30 ml of toluene and 10 ml of 25% GA aqueous solution, and by phase-separating the mixture.[10]

Table 1. Effect of GST Volume on CMS Size and Gd Content and Comparison between Gd-DTPA-CMS(P) and Gd-DTPA-CMS(C), Prepared by Physcotron and Clearmix, Respectively[a]

	GST volume[b] (ml)	D10 (μm)	D50 (μm)	D90[c] (μm)	Gd content (%)
Gd-DTPA-CMS(P 1)	1	1.8	5.1	14.9	12.7
(P10)	10	1.5	3.1	8.5	8.5
(P50)	50	1.3	2.3	3.5	5.8
Gd-DTPA-CMS(C)	50	1.2	1.9	3.1	6.1

a) The applied weight ratio of 1.7 between Gd-DTPA and chitosan in 4 ml of 5% acetic acid aqueous solution, and the volume of toluene (60 ml) containing 20 g of Span 80 were kept constant, respectively. *b*) GST would contain 0.02 mol% GA[11]; that is, about 1.9 mg of GA in 10 ml. For all amino groups of 103 mg chitosan to be cross-linked, about 32 mg GA would be required. *c*) D10, D50 and D90 are the particle sizes at 10, 50 and 90% of cumulative size distribution, respectively.

shape remained.

A different homogenizer (Clearmix) was used instead of Physcotron for primary emulsification in the same formulation. Clearmix made CMSs which were similarly size-distributed, but more spherical and discrete (Table 1, Fig. 2).

From Gd-DTPA-CMS(L) having a Gd content of 11.6% and a D50 of 12.5 μm (Fig. 1A), 50% Gd was released within 50 min (T50 = 50 min) (Fig. 3A). However, Gd-DTPA-CMS (P50) and (C) prepared with 50 ml GST exhibited more prolonged release: T50 = 2.1 and 2.4 h,

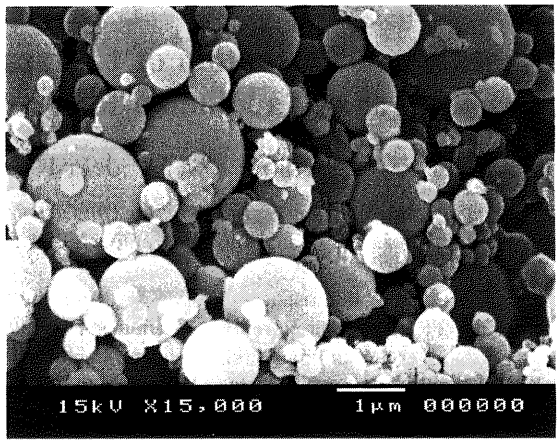

Fig. 2. Scanning Electron Micrograph of Gd-DTPA-CMS(C)

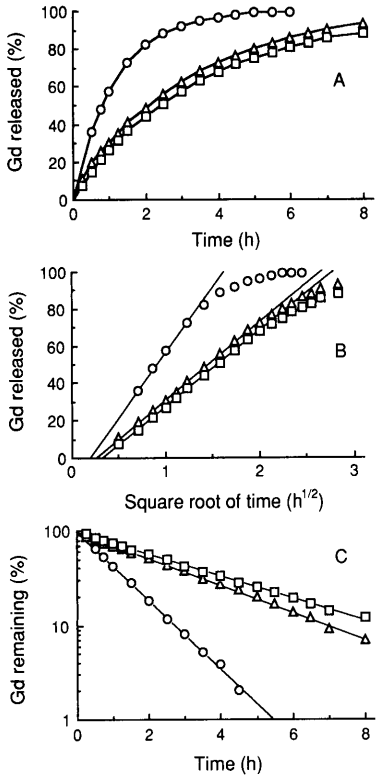

Fig. 3. *In Vitro* Release Profiles of Gd from Gd-DTPA-CMSs in an Isotonic Phosphate Buffer Solution (pH = 7.4) at 37 °C (A) and Plots of Gd % Released against the Square Root of Time (B) and Logarithm of Gd % Remaining against Time (C)

○, Gd-DTPA-CMS(L); △, Gd-DTPA-CMS(P50); □, Gd-DTPA-CMS(C).

respectively (Fig. 3A). When the dissolution data were plotted against the square root of time, linear relations were obtained below 70% released after a 2—5 min lag time (Fig. 3B). The relations between the logarithm of the percent remaining and time were more completely linear (Fig. 3C).

Discussion

There will always be competition between electrostatic interaction, arising between Gd-DTPA and chitosan,[7c] and cross-linking, arising between chitosan and GA, during emulsifying and hardening. With an increase in the amount of Gd-DTPA applied, an increased number of

amino groups of chitosan would presumably interact with Gd-DTPA in the aqueous droplets during emulsification (Fig. 1A). The calculated Gd content and Gd-DTPA/chitosan ratio were about 18% and 1.7, respectively, when the 1 : 1 ion-pair formation of the amino group and the carboxylic group completely occurred in the absence of Span 80 and GA. These would well explain the reason why the Gd content was saturated at a 1.7 ratio of Gd-DTPA/chitosan (Fig. 1A). GA dissolved in the continuous medium of toluene would be uniformly available for cross-linking mainly to the droplet surface.[7b,11] The presence of excess Gd-DTPA above a 1.7 ratio of Gd-DTPA/chitosan would prevent GA from cross-linking, leading to poor surface-hardening. In these cases, since only a small amount of Span 80 (420 mg) was used, the ability of the surfactant to prevent coalescence and aggregation of the poorly surface-hardened droplets would be poor (Fig. 1B); finally, growth of the droplets was not avoided during hardening (Fig. 1). These phenomena should be responsible for the aggregated and collapsed shape of CMSs when too much Gd-DTPA is introduced, thus indicating that the Gd-DTPA/chitosan ratio is a very important factor for the Gd content and the size and shape of Gd-DTPA-CMSs.

The least cross-linked Gd-DTPA-CMS (P1) had a wide size distribution in spite of the application of much Span 80 (20 g) (Table 1), meaning that very frequent aggregation would occur due to too poor surface-hardening. However, the size distribution had D50 of 2.3 μm and D90 of 3.5 μm at the highest cross-linking density (Gd-DTPA-CMS (P50), Table 1). Microscopy showed that most of the CMSs were smooth-faced and spherical. It was clear that sufficient amounts of GST and Span 80 were necessary for producing discrete spheres (Table 1).

The kinetics of Gd release seem to obey the first order mechanism (Fig. 3C) better than the Higuchi equation (Fig. 3B), suggesting that the CMSs might be a reservoir type. At a 1.7 ratio of Gd-DTPA/chitosan or below, the surface amino groups would preferentially react with GA from the organic phase. Consequently, displaced Gd-DTPA would be concentrated at the nucleus of the chitosan matrix,[7b,11] but excess Gd-DTPA, which could not intensely interact with the amino groups, would be washed out during subsequent cleaning. This would lead to a decrease in Gd content in spite of the improvement of CMS morphology and the size reduction (Table 1).

Microspheres smaller than 2 μm can be systemically circulated after intravenous (i.v.) injection, while i.v. or intraarterial (i.a.) injection of microspheres larger than 3 μm is intended to block the capillaries of the lungs, liver, kidney and spleen.[12] Meanwhile, the effectiveness of an intratumoral (i.t.) injection of albumin microsphere-entrapped anticancer drugs on the suppression of tumor growth has been demonstrated.[13] Although their particle sizes have to be more reduced for i.v. injection, CMSs which are prepared, according to the present study, to be finer, prolonged-releasing, biodegradable and biocompatible can be an alternative to the completely release-suppressing microcapsules which have been developed for i.a. or i.t injection in Gd-NCT by the present group.[3,4]

In conclusion, the electrostatic interaction between chitosan and Gd-DTPA, as well as the preferential surface-hardening by the cross-linker, contributed to the formation of fine (D50 of 1.9 μm), Gd-enriched (Gd content of 6.1%) and prolonged-releasing (T50 = 2.4 h) CMSs.

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