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RESEARCH

# Gadolinium diethylenetriaminopentaacetic acid-loaded chitosan microspheres for gadolinium neutron-capture therapy

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**Abstract**—In order to provide a suitable device that would contain water-soluble drugs, highly water-soluble gadolinium diethylenetriaminopentaacetic acid-loaded chitosan microspheres (CMS-Gd-DTPA) were prepared by the emulsion method using glutaraldehyde as a cross-linker and Span 80 as a surfactant for gadolinium neutron-capture therapy of cancer. The gadolinium content and the mass median diameter of CMS-Gd-DTPA were estimated. The size and morphology of the CMS-Gd-DTPA were strongly influenced by the initial applied weight ratio of Gd-DTPA:chitosan. FTIR spectra showed that the electrostatic interaction between chitosan and Gd-DTPA accelerated the formation of gadolinium-enriched chitosan microspheres. Sufficient amounts of glutaraldehyde and Span 80 were necessary for producing discrete CMS-Gd-DTPA. The CMS-Gd-DTPA having a mass median diameter 11.7 μm and 11.6% of gadolinium could be used in Gd-NCT following intratumoral injection.

*Keywords:* Chitosan; Microspheres; Gadolinium diethylenetriaminopentaacetic acid; Gadolinium neutron-capture therapy

## 1. Introduction

Chitosan and its partially N-acetylated derivatives are biocompatible[1,2] as well as biodegradable[3–5] polymers. The high proportion of amino functions in chitosan (Fig. 1a) has been found to provide novel binding properties for many metal ions, and thus a number of studies using raw and chemically modified chitosans for heavy metal removal from wastewater have been extensively conducted.[6–10] The possibility of using chitosan as a material to remove dyes from water and wastewater has also received considerable attention.[11–15] As a drug carrier, chitosan has been widely used in the preparation of various biomedical products, and their therapeutic activities have also been evaluated.[16–23] The organ distribution of injected particles primarily depends on their size and injection route. For example, microspheres of a diameter smaller than 2 μm can be injected intravenously. However, the intravenous injection of microspheres ranging 3–12 μm has blocked the capillaries of the lungs, liver, and spleen.[24] It was demonstrated that adriamycin-loaded albumin microspheres of 14–17 μm in diameter displayed superior antitumor

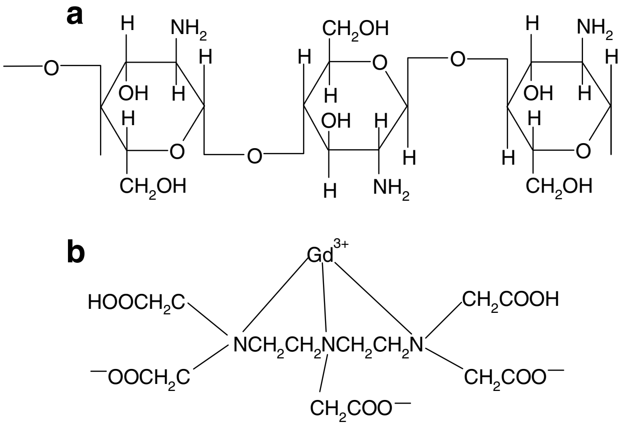

**Figure 1.** Structures of (a) chitosan and (b) Gd-DTPA.

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activity following the direct intratumoral injection in comparison with that of free drug at the same dose in solution.[25]

On the other hand, gadolinium neutron-capture therapy (Gd-NCT) is considered to be a powerful tool for the treatment of certain incurable types of cancers located in the liver, brain, and other organs. The success of Gd-NCT depends on a high concentration of gadolinium and its retention in the tumor during neutron irradiation. Firstly ethyl cellulose microcapsules containing dimeglumine gadopentate were prepared to act as a gadolinium reservoir for Gd-NCT.[26] These microcapsules have already been used in in vivo experiments using murine Ehrlich ascites tumor-bearing mice for Gd-NCT.[27] The survival of the mice receiving microcapsules containing dimeglumine gadopentate with neutron irradiation was considerably prolonged in comparison with that observed for the mice receiving other treatments. These results encouraged us to design gadolinium-enriched chitosan particles for Gd-NCT. We prepared chitosan microparticles containing gadolinium diethylenetriaminopentaacetic acid (Gd-DTPA) (Fig. 1b), which was applied in Gd-NCT trails using melanoma solid tumor-bearing mice.[28–31] The tumor growth in the microparticles-treated mice was significantly suppressed compared to that of the mice treated with only Gd-DTPA solution. These results encouraged us to develop more gadolinium-enriched chitosan microspheres and to investigate their mechanism of formation, which would give insight into the molecular mechanism of the Gd-DTPA-chitosan interactions.

In this study, Gd-DTPA was considered as a gadolinium source because it is clinically used as a magnetic resonance imaging (MRI) contrast enhancement agent.[32] Gd-DTPA is stable in the bloodstream and it is relatively nontoxic, although the gadolinium ion ($Gd^{3+}$) is itself toxic. The pharmacokinetics, biodistribution, and tolerance of Gd-DTPA and other gadolinium compounds used for MRI are well documented.[33–35] Gd-DTPA contains two free carboxylic groups, and chitosan is a polycationic biopolymer.[36] Therefore, it was expected that the electrostatic interaction between chitosan and Gd-DTPA could be able to make gadolinium-enriched chitosan microspheres. Here, we report the complete preparation of gadolinium-enriched chitosan microspheres (CMS-Gd-DTPA) by an emulsion technique using sorbitan monooleate (Span 80) as the surfactant and glutaraldehyde-saturated toluene (GST) as the cross-linker.[16] In addition, the effects of the initial applied weight ratio of Gd-DTPA:chitosan, Span 80, and GST on the size and gadolinium content in CMS-Gd-DTPA were studied. Finally, the morphology of the microspheres and the in vitro release of the gadolinium from CMS-Gd-DTPA were studied, and infrared spectra were measured to assess the Gd-DTPA–chitosan interactions.

## 2. Experimental

### 2.1. General

Chitosan (10B, 100% degree of deacetylation; the weight-average molecular weight[37] 950,000) was purchased from Katokichi Co., Ltd., Japan. Gadolinium diethylenetriaminopentaacetic acid (Gd-DTPA) was purchased from Aldrich Chemical Company, Inc, USA. Glutaraldehyde, sorbitan monooleate (Span 80) and all other solvents were commercially available and were of the highest grade of purity; hence, they were used without purification.

### 2.2. Preparation of chitosan microspheres

CMS-Gd-DTPA was prepared according to the method of Ohya et al.[16] with a slight modification. Chitosan (103 mg) and various amounts of Gd-DPTA were dissolved in 4 mL of 5% aqueous acetic acid solution. This solution was added to 60 mL of toluene containing 420 mg of Span 80 under vigorous stirring using a homogenizer (Physcotron NS-50, NITI-ON, Japan; open type NS-10 shaft, 22,000 rpm, 1 h) water-in-oil (w/o) emulsion. Ten milliliters of glutaraldehyde-saturated toluene (GST) containing 600 mg of Span 80 was added to the w/o emulsion and stirred using a magnetic stirrer at room temperature overnight to provide cross-linked chitosan microspheres. The resultant suspension was centrifuged at 3000 rpm for 30 min. Finally, microspheres (CMS-Gd-DPTA) were washed twice with toluene, MeOH, distilled water, and acetone and then dried over silica gel under vacuum for 24 h at room temperature. Placebo chitosan microspheres (PCMS) were also prepared in the absence of Gd-DPTA according to the same procedure as described above. GST was prepared by vigorously vortexing a mixture of 30 mL of toluene and 10 mL of 25% aqueous glutaraldehyde solution, and by phase-separating the mixture.[38] GST would contain 0.02 mol % glutaraldehyde;[39] that is, about 1.9 mg of glutaraldehyde in 10 mL.

### 2.3. Characterization of CMS-Gd-DTPA

To determine gadolinium content, 10 mg of CMS-Gd-DPTA was burned in concentrated $HNO_3$ to make ash, and then the ash was dissolved in 5 mL of 6.6 N $HNO_3$. The amount of gadolinium 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. Particle size distribution of microspheres was measured in MeOH by a light-scattering size analyzer (LDSA-2400A, Tonichi Computer Applications Co., Ltd., Japan). The morphology of dried microspheres was assessed using scanning electron microscopy (SEM) (JEOL JSM-5300LV scanning electron microscope, JEOL Ltd., Japan). The infrared (IR) spectra of the solid samples (chitosan, PCMS, Gd-DTPA and CMS-Gd-DPTA) compressed with KBr disks were measured using a Shimadzu FTIR-8101 spectrophotometer (Shimadzu, Kyoto, Japan).

### 2.4. In vitro release of gadolinium from CMS-Gd-DTPA

The in vitro release of gadolinium from CMS-Gd-DPTA was carried out using a dynamic dialysis system[40] with a cellulose tube having a molecular weight cut-off of 12,000–14,000. CMS-Gd-DPTA was suspended in 4 mL of isotonic phosphate buffer (pH 7.4) in a cellulose tube and incubated at 37 °C against 46 mL of the same buffer solution. The inner solution was stirred with a stirrer attached to an electric motor, and the outer solution was stirred using a magnetic stirrer. A small aliquot (4 mL) was withdrawn from the outer solution at particular time intervals, and 4 mL of buffer solution was added to the system, keeping the volume unchanged. The amount of gadolinium in the samples was measured by ICP-AES in the same manner as described above.

## 3. Results

### 3.1. Formation and characterization of chitosan particles loaded with Gd-DTPA

CMS-Gd-DPTA was prepared by the emulsion technique using glutaraldehyde as the cross-linker and Span 80 as the surfactant. To find the optimum weight ratio between Gd-DTPA and chitosan for gadolinium-enriched CMS-Gd-DPTA, a series of samples were prepared using various amounts of Gd-DPTA with a specific amount of chitosan. The effects of initial applied weight ratio of Gd-DPTA/chitosan on both the gadolinium content and the size of the CMS-Gd-DPTA are shown in Figure 2. The results demonstrated that the gadolinium content was increased to 11.6 wt % at 1.7 of the weight ratio of Gd-DPTA/chitosan, followed by only a slight increase in gadolinium content for the additional amount of Gd-DPTA. Similarly, the mass median diameters of PCMS and CMS-Gd-DPTA were almost same up to 1.7 (Fig. 2), and then they sharply increased with increasing applied weight ratio of Gd-DPTA/chitosan.

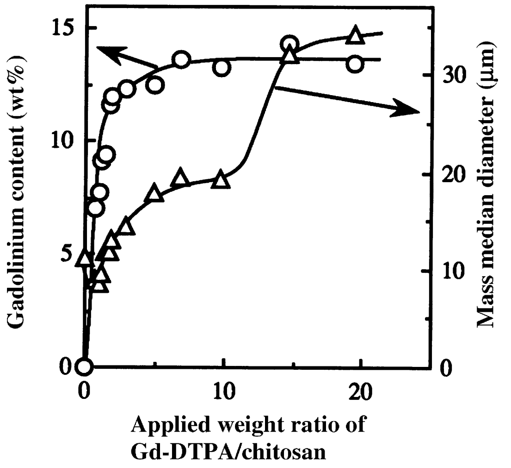

**Figure 2.** The effects of initial applied weight ratio of Gd-DTPA:chitosan on the gadolinium content (○) and the size (△) of CMS-Gd-DTPA. The amount of chitosan (103 mg), toluene (60 mL) containing 420 mg of Span 80, and the volume of glutaraldehyde-saturated toluene (GST, 10 mL) containing 600 mg of Span 80 were kept constant, respectively.

Figure 3 shows the scanning electron micrographs (SEM) of PCMS and CMS-Gd-DPTA prepared at various applied weight ratios of Gd-DPTA/chitosan. All PCMS were smooth-faced spherical (Fig. 3a). Similarly, CMS-Gd-DTPA prepared at ratios of 1.5:1 and 1.7:1 of Gd-DTPA:chitosan was fairly smooth and spherical (Fig. 3b and c); however, all CMS-Gd-DTPA prepared at a ratio of 15:1 Gd-DTPA:chitosan were nonspherical, rough-faced, collapsed and/or aggregated (Fig. 3d). These results indicate that the initial applied weight ratio of Gd-DTPA:chitosan is very important for the spherical size and smooth morphology of CMS-Gd-DTPA. Therefore, CMS-Gd-DTPA was prepared at a 1.7:1 ratio of Gd-DTPA:chitosan for the following experiments.

In order to confirm Gd-DTPA-chitosan interactions in CMS-Gd-DTPA, samples were analyzed by FTIR spectroscopy. Figure 4 shows the spectra of chitosan (a), PCMS (b), Gd-DTPA (c), and CMS-Gd-DTPA (d). The chitosan spectrum showed a broad peak of a hydroxyl group at 3450 cm$^{-1}$ and a strong peak of an amino group at 1560 cm$^{-1}$. The spectrum of PCMS showed a strong peak at 1660 cm$^{-1}$, which provides proof that the cross-linking in chitosan had taken place through a Schiff base (–C=N–) reaction.[23] However, the peak of the amino group at the region 1540–1560 cm$^{-1}$ partially remained, indicating that a part of the amino groups is still vacant for interaction. The Gd-DTPA spectrum showed a shoulder peak at 1715 cm$^{-1}$ for the stretching vibration band due to the C=O in the COOH group and the strong absorption bands of the asymmetrical and symmetrical stretching vibrations due to the –COO$^-$ group at around 1594 and 1400 cm$^{-1}$, respectively. The spectrum of CMS-Gd-DTPA exhibited the following absorption bands: a broad and strong $NH_3^+$ stretching band at 3100–2600 cm$^{-1}$, and strong absorption bands of the asymmetric and symmetric stretching vibrations due to the –COO$^-$ group at around 1602 and 1400 cm$^{-1}$, respectively. Moreover, the shoulder at 1715 cm$^{-1}$ for the C=O in the free carboxylic groups of Gd-DTPA disappeared in CMS-Gd-DTPA. The broad band centered at 1602 cm$^{-1}$ might be a convoluted one due to the Schiff

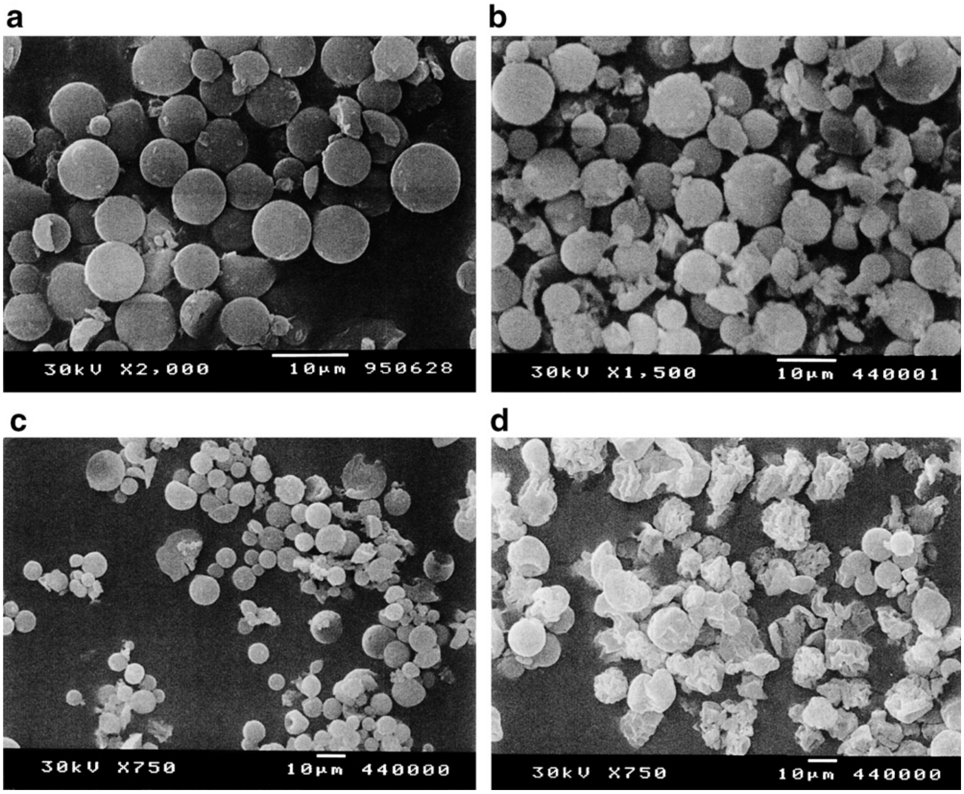

**Figure 3.** The scanning electron micrographs (SEM) of CMS-Gd-DTPA prepared at various Gd-DTPA:chitosan ratio: (a) placebo chitosan microspheres (PCMS), (b) 1.5:1 (gadolinium content, 9.1%), (c) 1.7:1 (gadolinium content, 11.6%), and (d) 15:1 (gadolinium content, 13.3%).

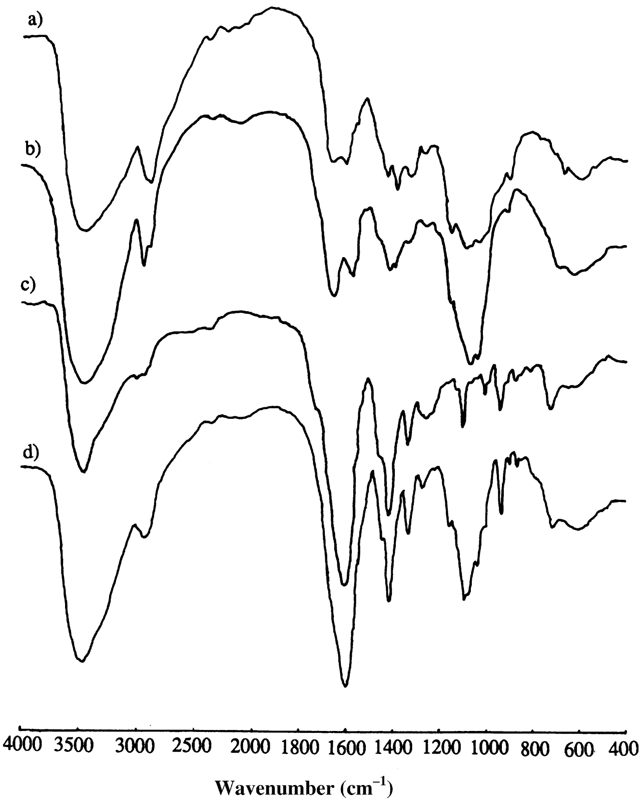

**Figure 4.** Infrared (IR) spectra of (a) chitosan, (b) PCMS, (c) Gd-DPTA, and (d) CMS-Gd-DTPA in KBr.

base (–C=N– stretching)[23] at 1660 cm$^{-1}$ and the asymmetric stretching vibration might be due to the –COO$^{-}$ group at ~1594 cm$^{-1}$.[41]

### 3.2. Effects of Span 80 and glutaraldehyde-saturated toluene (GST)

To evaluate the effects of Span 80 on the size and gadolinium content in CMS-Gd-DPTA, a number of samples were prepared using various amounts of Span 80 in the organic phase of the basic primary w/o emulsion, in which an aqueous phase with a 1.7:1 ratio of Gd-DPTA: chitosan was included. The mass median diameter of CMS-Gd-DTPA quickly decreased with increasing Span 80 and reached 2.6 μm when 20 g of Span 80 was used; however, the gadolinium content was only slightly changed (Fig. 5).

The effects of glutaraldehyde-saturated toluene (GST) on the size and gadolinium content in CMS-Gd-DPTA were also investigated. The size distributions of CMS-Gd-DTPA prepared using various amounts of GST are shown in Figure 6. The particle size distribution range of CMS-Gd-DPTA became very narrow upon increasing the volume of GST. The mass median diameters of CMS-Gd-DPTA were 5.1 μm for 1 mL, 3.1 μm for 10 mL, and 2.3 μm for 50 mL of GST, respectively. Moreover, the gadolinium contents of those micro-

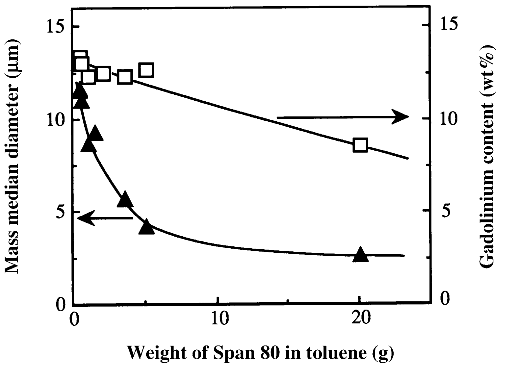

**Figure 5.** Effect of Span 80 on the size (mass median diameter; ▲) and gadolinium content (☐) in CMS-Gd-DTPA of the basic system. The applied weight ratio of Gd-DTPA:chitosan was 1.7:1 in 4 mL of 5% aq AcOH. The volume of toluene (60 mL containing various amounts of Span 80) and the volume of glutaraldehyde-saturated toluene (GST; 10 mL) containing 600 mg of Span 80 were kept constant, respectively.

spheres were found to be 12.7%, 8.5%, and 5.8%, respectively.

### 3.3. In vitro release of gadolinium from CMS-Gd-DTPA

The in vitro release of gadolinium from CMS-Gd-DPTA prepared with 10 mL of GST having 11.6% of gadolinium and 11.7 µm of mass median diameter was studied in isotonic phosphate buffer (pH 7.4) at 37 °C (Fig. 7a). The release profile showed a $t_{50\%}$ (the time for 50% release of gadolinium) of 0.83 h. The release of gadolinium was virtually completed within 5–6 h. Moreover, the linear relation was observed below 70% release after a 5-min lag time when the dissolution data were plotted against the square root of time (Fig. 7b). The relation between the logarithm of the percent remaining amount of gadolinium in the microspheres and time was completely linear (Fig. 7c). These results indicated that the release data obeyed first-order kinetics. However, CMS-Gd-DPTA prepared with 50 mL of GST exhibited more prolonged release with $t_{50\%}$ of 2.1 h (data not shown).

## 4. Discussion

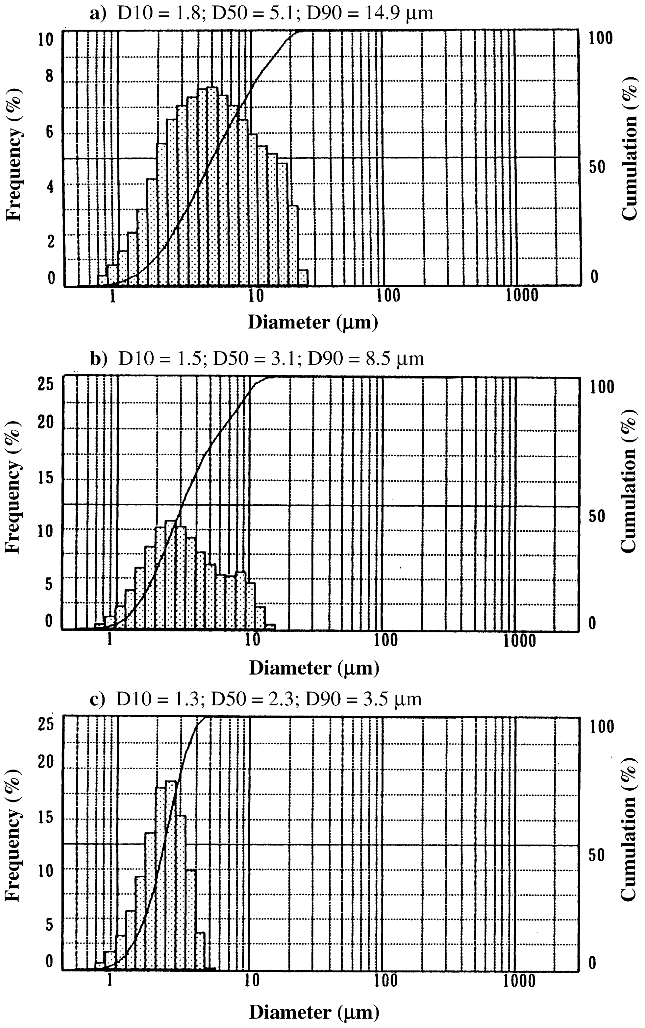

**Figure 6.** Effects of glutaraldehyde-saturated toluene (GST) on the size distribution of CMS-Gd-DTPA. The volumes of GST were as follows: (a) 1 mL, (b) 10 mL and (c) 50 mL containing 600 mg of Span 80. D10, D50, and D90 are the particles sizes at 10%, 50%, and 90% of cumulative size distribution, respectively. The applied weight ratio of Gd-DTPA:chitosan (1.7:1) in 4 mL of 5% aq AcOH and the volume of toluene (60 mL) containing 20 g of Span 80 were kept constant.

The necessary conditions for successful Gd-DTPA-loaded chitosan microspheres have been established by the emulsion technique using glutaraldehyde as the cross-linker and Span 80 as the surfactant. There is always a competition between electrostatic interaction arising between Gd-DTPA and chitosan, and cross-linking arises between chitosan and glutaraldehyde during the hardening process w/o emulsion droplets.[42] The cross-linking is accomplished via an organic phase due to the solubility of GST in the dispersion medium. As a result, GST would be uniformly available for cross-linking mainly to the surface of the droplets in the dispersion.[39,43] The surface hardening of the droplets by cross-linking thus fixes the shape and surface morphology of the microspheres. However, in the presence of excess Gd-DTPA, large numbers of $-NH_2$ groups of chitosan presumably are protonated and strongly interact with $-COO^-$ groups of Gd-DTPA in the emulsion droplets. This could be intended to prevent glutaraldehyde cross-linking of chitosan and to influence interdroplets binding in the hardening process. Finally, these phenomena would be responsible for a marginal increase in gadolinium content (Fig. 2), large aggregation, and the collapsible shape of CMS-Gd-DTPA (Fig. 3d) for higher ratios of Gd-DTPA:chitosan. The

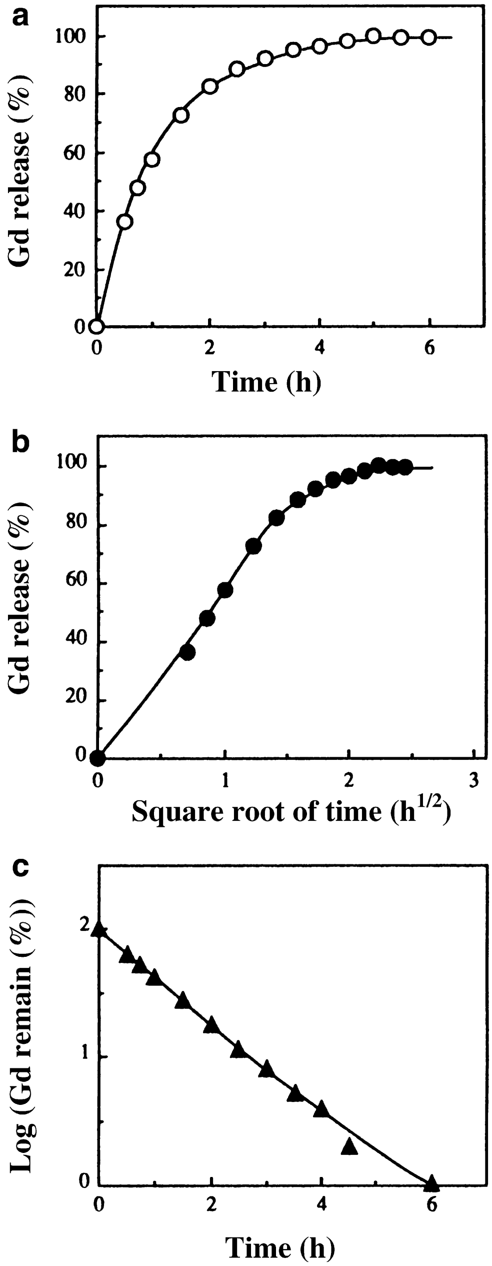

**Figure 7.** (a) In vitro release profile of gadolinium from CMS-Gd-DTPA cross-linked with 10 mL GST having 11.6% of gadolinium and 11.7 μm of the mass median diameter in isotonic phosphate buffer solution (pH 7.4) at 37 °C; (b) a plot of gadolinium release versus square root of time (data were taken from (a)); and (c) a plot of log (% of remaining gadolinium) against time (data were taken from (a)).

results suggest that the applied weight ratio of Gd-DTPA:chitosan is a very important factor for gadolinium content, size and shape of CMS-Gd-DTPA. Therefore, it is assumed that the initial applied weight ratio of Gd-DTPA:chitosan is 1.7:1 needed for the preparation of spherical gadolinium-enriched CMS-Gd-DTPA.

The FTIR spectra have shown a strong absorption band around 1594–1602 $cm^{-1}$ that belongs to the asymmetric stretching vibration of $–COO^{-}$, and a band around 1400 $cm^{-1}$ for the $–COO^{-}$ symmetric stretching vibration[41] in both the Gd-DTPA and CMS-Gd-DTPA (Fig. 4c and d). It has been reported that the amino groups of chitosan are capable of interacting with anionic polymers, which have carboxyl groups such as sodium alginate and sodium polyacrylate by ionic interaction.[44] Accordingly, it can be considered that the ionic interaction is a primary binding force for the Gd-DTPA-loaded chitosan microspheres.

The cross-linking density of the microspheres influences their size distribution (Fig. 6) while in the presence of a sufficient amount of Span 80. The least cross-linked CMS-Gd-DTPA has a wide size distribution (Fig. 6a), indicating that frequent aggregation would occur due to too poor surface hardening. However, the size distribution has a D50 of 2.3 μm and D90 of 3.5 μm at the highest cross-linking density (Fig. 6c). It is clear that sufficient amounts of GST and Span 80 are necessary for producing discrete CMS-Gd-DTPA.

The in vitro release of gadolinium from CMS-Gd-DTPA is significantly delayed (Fig. 7a) in comparison with the Gd-DTPA alone (data not shown), which is almost instantaneously dissolved in saline solution. The kinetics of gadolinium release seems to obey the first-order kinetics (Fig. 7c) better than the Higuchi equation (Fig. 7b), suggesting that CMS-Gd-DTPA is a reservoir type rather than a matrix type. At a 1.7:1 ratio of Gd-DTPA:chitosan or below, the surface amino groups would preferentially react with glutaraldehyde from the organic phase. Consequently, displaced Gd-DTPA would be concentrated at the nucleus of the chitosan matrix,[39,43] but excess Gd-DTPA, which could not strongly interact with the amino groups, would be washed out during subsequent cleaning. This would lead to a decrease in gadolinium content along with an improvement of CMS-Gd-DTPA morphology and size reduction (Fig. 6c).

The use of particulate microspheres is perhaps one of the most promising systems for drug delivery to a specific organ in the body. The organ distribution of injected particles primarily depends on their size and injection route. Microspheres of a diameter smaller than 2 μm can be injected intravenously. However, the intravenous injection of microspheres ranging 3–12 μm is found to block the capillaries of the lungs, liver, and spleen.[24] On the other hand, the direct intratumoral (i.t.) injection of adriamycin-loaded albumin microspheres of 14–17 μm diameter shows superior antitumor activity over that of the free drug at the same dose given in solution.[25]

On the basis of the above results, it is concluded that the electrostatic interaction between chitosan and Gd-DTPA accelerates the formation of gadolinium-enriched chitosan microspheres. The CMS-Gd-DTPA having a mass median diameter 11.7 μm and 11.6% of gadolinium might be useful for i.t. injection in Gd-NCT.

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