Preparation of Gadopentetic Acid-Loaded Chitosan Microparticles for Gadolinium Neutron-Capture Therapy of Cancer by a Novel Emulsion-Droplet Coalescence Technique

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Biodegradable gadopentetic acid (Gd-DTPA)-loaded chitosan microparticles (Gd-μCPs) were prepared as a device for gadolinium neutron-capture therapy (Gd-NCT) by a novel emulsion-droplet coalescence technique: a water-in-oil (w/o) emulsion A containing chitosan and Gd-DTPA in droplets and a w/o emulsion B containing NaOH in droplets were mixed and stirred to solidify chitosan as a result of collision and coalescence between droplets of each emulsion. Gd-μCPs prepared by using 100% deacetylated chitosan in 25% Gd-DTPA solution were 4.1 μm (non-lyophilized) and 3.3 μm (lyophilized) in mass median diameter, and were 3.4% in gadolinium content, corresponding to 11.7% as Gd-DTPA. The particle size and gadolinium content of Gd-μCPs were not affected by Gd-DTPA concentration in the chitosan medium. However, the deacetylation degree of chitosan influenced the particle size; as the deacetylation degree of chitosan decreased, the particle size increased. The incorporated Gd-DTPA was not released entirely from Gd-μCPs in an isotonic phosphate buffered saline solution despite the high water-solubility of Gd-DTPA (less than 0.8% with every type of Gd-μCPs). These results indicated that ion-complex formation might be contributable to incorporation of Gd-DTPA. As a preliminary study, it was confirmed that the loss of γ-ray emission by gadolinium-loading in microparticle was negligible in the thermal neutron irradiation test *in vitro*. These results suggested that Gd-μCPs could be a useful device for intratumoral injection into solid tumor on Gd-NCT.

Key words chitosan; microparticle; gadopentetic acid; gadolinium neutron capture therapy; microsphere; emulsion-droplet coalescence

Neutron-capture therapy (NCT) is a cancer therapy which utilizes the nuclear neutron capture reaction of radiation-producing agents. The most common element for NCT is boron-10 at present. In clinical experiences, NCT with boron-10 (B-NCT) has achieved encouraging results using the mercaptoundecahydro-*closo*-dodecaborate dianion ($[B_{12}H_{11}SH]^{2-}$, BSH) in patients with grades III—IV glioma[1] and using the boronophenylalanine (BPA) in patients with malignant melanoma.[2] In B-NCT, the boron-10 compounds administered have to be delivered to tumor intracellularly in order to obtain an antitumor effect, because the boron-10 emits alpha-particles whose ranges are nearly equal (6—10 μm) to a cell diameter or shorter:[3] the success in clinical B-NCT trials depends on the selective accumulation of boron-10 compounds into individual tumor cells. The developers of NCT are still investigating other radiation-producing elements to make NCT more effective.

Gadolinium-157 is a radiation-producing element and causes the following neutron capture reaction by thermal neutron irradiation:[4]

$$^{157}Gd + n_{th} \rightarrow {}^{158}Gd + \gamma + 7.94\,MeV$$

NCT with gadolinium-157 (Gd-NCT) commonly has a few possible advantages over B-NCT: 1) gadolinium-157 has the highest thermal neutron capture cross section (255000 barns) among naturally occurring isotopes, 66 times larger than that of boron-10[5]; 2) gadolinium neutron capture reaction releases the long range (>100 μm) prompt γ-rays, internal conversion electrons, X-rays and Auger electrons[6]; consequently, Gd-NCT may increase the chance for photons to hit neoplastic cells and for electrons to damage cancer cells locally, intensively; 3) gadolinium has been used as a magnetic resonance imaging (MRI) diagnostic agent. It will be possible in the future to integrate Gd-NCT with MRI diagnosis by using gadolinium-loaded dosage forms. It has been suggested from past studies[7] that a key factor for success of Gd-NCT may be a device whereby gadolinium can be delivered efficiently and retained in tumor.

In a previous study, we reported a particulate system for Gd-NCT: the delayed-release type of gadolinium microcapsules was designed and prepared using Magnevist® (dimeglumine gadopentetate solution), an MRI contrast agent,[8] and applied in Gd-NCT trials. They were significantly effective on survival in the murine Ehrlich ascites tumor model.[9] Further, smaller gadolinium non-releasing microcapsules were prepared using Gd-DTPA distearylamide.[10]

Chitosan, a cationic polysaccharide obtained by *N*-deacetylation of chitin, is an appropriate material as a drug carrier, in particular for anionic drugs, because it is biodegradable and biocompatible, and its possibility for medical use has been widely reported. Various particles using chitosan have been prepared and evaluated so far. Most of them have been prepared using the cross-linking agent, glutaraldehyde,[11] since it is difficult to form micro- or nano-spheres or particles from chitosan alone. Using the cross-linking agent has some advantages such as hard particle formation and consequently strong drug-release control,[12] but it may lead to low loading of the anionic drug and to toxicity of the cross-linking agent.

In this study, gadolinium-loaded chitosan microparticles (Gd-μCPs) were prepared by a novel emulsion-droplet coalescence technique. The effects of various experimental conditions on the properties of Gd-μCPs were investigated and the potential of Gd-μCPs for Gd-NCT was discussed.

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Experimental

Materials Chitosans (Fig.1(A)), grade 10B (100% deacetylated; viscosity of 0.5% w/v chitosan/0.2 M acetic acid buffer (pH 4.0) solution at 20 °C, 53 mPa·s), 9B (91.4% deacetylated; viscosity, 240 mPa·s), 8B (84.9% deacetylated; viscosity, 150 mPa·s) and 7B (74.2% deacetylated; viscosity, 325 mPa·s) were provided by Katokichi Bio Co., Ltd., Japan. Gadopentetic acid (Gd-DTPA, Fig.1(B)) with natural gadolinium was obtained from Aldrich Chemical Company, Inc., Australia. Magnevist®, an MRI diagnostic agent, was purchased from Nihon Schering Co., Ltd., Japan. Sorbitan sesquioleate (Arlacel C), an emulsifier, and polyvinyl alcohol (PVA; degree of polymerization, about 500) were provided by Nacalai Tesque Inc., Japan. The other chemicals and solvents were of reagent grade and used without further purification.

Preparation of Gd-μCPs The preparation process for Gd-μCPs using an emulsion-droplet coalescence technique is shown in Fig. 2. Chitosan (2.5% w/v) was dissolved in an aqueous solution of Gd-DTPA (5—25% w/v). This solution (1 ml) was added to 10 ml of chloroform containing 5% v/v Arlacel C and saturated PVA, and was stirred using a high speed homogenizer, Polytron® with PTA-10S generator shaft (Kinematica AG, Switzerland), to form a water-in-oil (w/o) emulsion A. Similarly, w/o emulsion B consisting of 3 N sodium hydroxide solution (1.5 ml) and the 5% v/v Arlacel C chloroform solution saturated with PVA (10 ml) was prepared. The emulsions A and B were mixed and stirred by Polytron®. As a result of the coalescence of the droplets, the chitosan solidified as microparticles. Chloroform was removed by rotary evaporator (Type N-N, Tokyo Rikakikai Co., Ltd., Japan). The residue was dispersed thoroughly by ultrasonication (Branson 1200 (Bransonic®), Yamato Scientific Co., Ltd., Japan) . The dispersion was centrifuged at 3000 rpm for 30 min (KN-30F, Kubota, Japan) to separate Gd-μCPs and Gd-μCPs were successively washed with water by the centrifuging. The Gd-μCPs were resuspended in water and filtered through microsieve (105 μm). Finally the Gd-μCPs were obtained as water suspensions or powders lyophilized after suspension in isotonic mannitol solution.

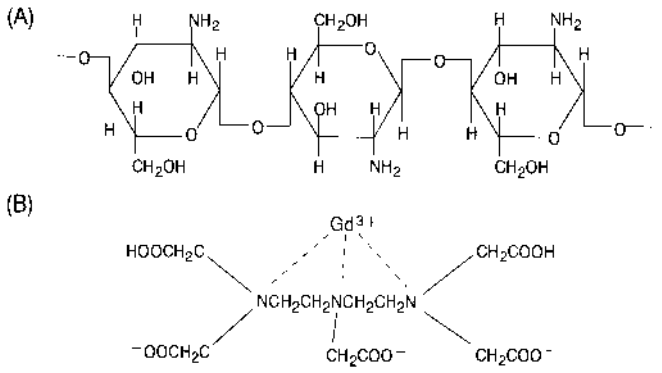

Fig. 1. Structures of Chitosan (100% deacetylated) (A) and Gadopentetic Acid (Gd-DTPA) (B)

Particle Size Analysis The particle size distribution of Gd-μCPs was measured in water using a laser scattering size analyzer (LDSA-2400A, Tonichi Computer Applications Co., Ltd., Japan).

Particle Morphology Study A few drops of the Gd-μCP water-suspension without mannitol were applied on a double-sided tape on the stage for scanning electron microscopy (SEM) and desiccated. The sample was observed using a SEM (JSM-5300LV, JEOL Ltd., Japan) after sputter-coating with gold (JEE-400, JEOL Ltd., Japan).

Determination of Gadolinium Content The water was completely removed from the Gd-μCP suspension without mannitol by heating (Heating Block HF-61, Yamato Scientific Co., Ltd., Japan). Dry weight of Gd-μCPs was measured exactly. The weighed Gd-μCPs was incinerated using nitric acid under heating. After the sample was dissolved in 6.6 N nitric acid, gadolinium concentration was determined using an inductively coupled plasma atomic emission spectrography (ICP-AES) (P-5200, Hitachi Co., Ltd., Japan). Gadolinium content (%) in Gd-μCPs was given on the dry base.

***In Vitro* Gadolinium Release Study** Dissolution tests of Gd-μCPs *in vitro* were performed in an isotonic phosphate buffer saline solution of pH 7.4 (PBS). The Gd-μCPs, corresponding to 300 μg of gadolinium, were dispersed in 40 ml of PBS. The suspension was incubated at 37 °C under intensive shaking (Taitec Incubator Personal and Thermo Minder Mini-80, Taiyo Scientific Industries, Japan). Sampling was carried out at predetermined time intervals for 7 d. The sample was filtered through a membrane (FM-45, Fuji Photo Film Co., Ltd., Japan) and incinerated to measure gadolinium using ICP-AES.

***In Vitro* Neutron Irradiation Study** The dosimetry of γ-ray born of gadolinium was carried out using the modified prompt γ-ray spectrometry based on the procedure for ^{10}B microanalysis in B-NCT. The experimental details of the prompt γ-ray spectrometry have been described by Kobayashi *et al.*[13] and ^{10}B concentration in tumor on hamster and human patient has been estimated in B-NCT research by Honda *et al.*[14] In this study, the prompt γ-ray spectrometry are based on the facts that the ^{157}Gd (n, γ) ^{158}Gd reaction releases a low energy prompt γ-ray of 181.9 keV and the H (n, γ) D reaction emits a prompt γ-ray of 2.2 MeV. Namely, since these two prompt γ-rays with different energy are generated simultaneously from the sample containing ^{157}Gd and H by thermal neutron, it is possible to assay the gadolinium concentration from the relative intensity of each detected prompt γ-ray independent of sample volume and thermal neutron irradiation conditions such as neutron energy spectrum, distribution, fluence rate and so on. In this experiment, the Gd-μCP suspensions of various gadolinium concentrations in isotonic mannitol solution or dilute Magnevist® solution were placed into pure teflon [$(C_2F_4)_n$] tubes and were irradiated with thermal neutron beams at a flux of 2×10^6 n/cm^2/s (operating power, 5 MW) for 300 s using a beam 5 mm in diameter collimated by ^{6}LiF tiles *via* thermal neutron guide tube (E-3) at Kyoto University Research Reactor Institute (Japan): the

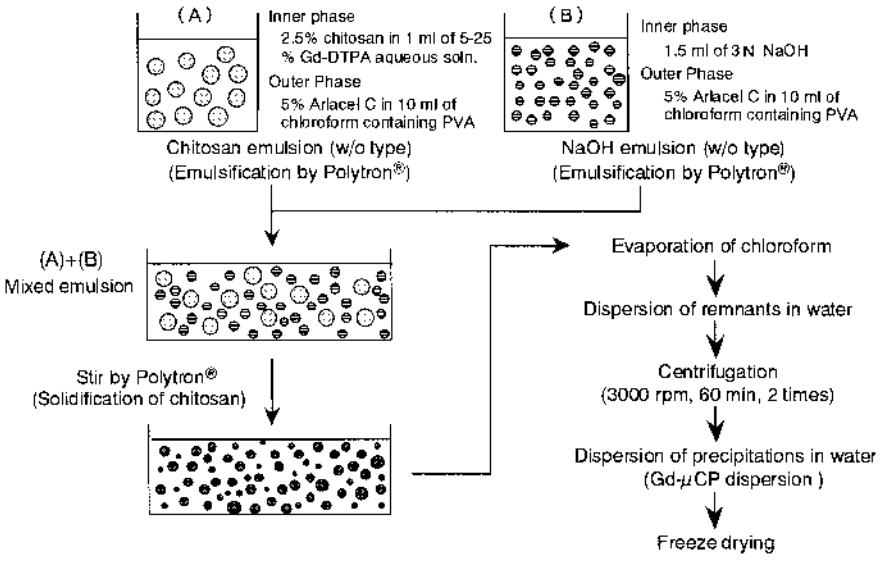

Fig. 2. Preparation Process of Gd-μCPs by Emulsion-Droplet Coalescence Technique

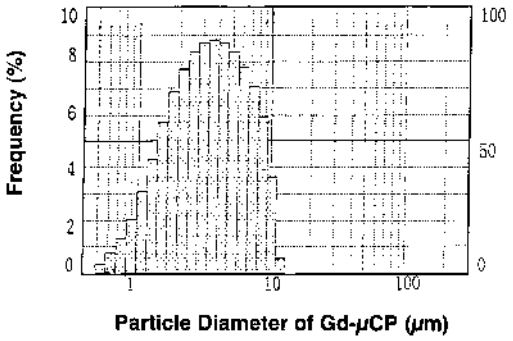

Fig. 3. Particle Size Distribution of Gd-μCPs

Experimental condition: emulsion A, chitosan 10B/25% Gd-DTPA solution.

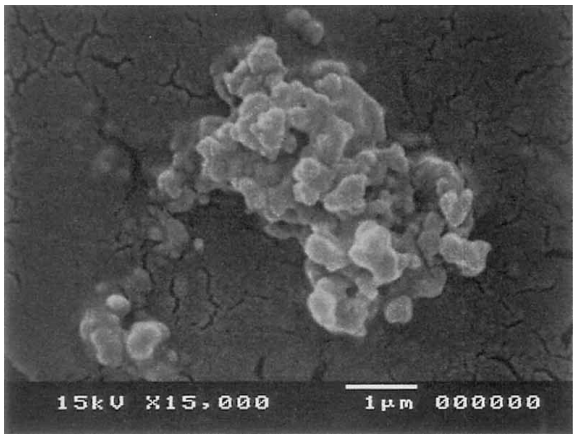

Fig. 4. Scanning Electron Micrograph of Gd-μCPs

Experimental condition: emulsion A, chitosan 10B/25% Gd-DTPA solution.

prompt γ-rays with each energy were counted by a high resolution-HPGe detector (IGC-30190, Princeton Gamma-Tech, Inc., U.S.A.). The gadolinium concentration was calculated from the ^{157}Gd/H intensity ratio using a standard curve by multi-channel analyzer (Canberra Series 20 Model 2802, Canberra Industries, Inc., U.S.A.). All measurements were performed three times per each sample.

Results

Particle Size Distribution, Morphology and Gadolinium Content Figures 3 and 4 show a typical particle size distribution and a scanning electron micrograph of non-lyophilized Gd-μCPs prepared with chitosan 10B at 25% Gd-DTPA concentration by the emulsion-droplet coalescence technique, respectively. The particle diameter was distributed between about 0.5 and 12 μm, and the mass median diameter ($\pm$S.D.) was 4.1 ($\pm$1.3) μm. Gd-μCPs were not spherical and seemed to be agglomerates consisting of small particles of 300—500 nm. By resuspending the sample after freeze-drying, the particle size distribution was shifted slightly toward a smaller range: mass median diameter ($\pm$S.D.) was 3.3 ($\pm$0.8) μm with no change for 10 d. Gadolinium content was 3.4 ($\pm$0.8)%, corresponding to 11.7 ($\pm$2.8)% as Gd-DTPA.

Effect of Gd-DTPA Concentration in Chitosan Medium The mass median diameter and gadolinium content of Gd-μCPs were evaluated in the 5—25% range of Gd-DTPA concentration using chitosan 10B. As shown in Fig. 5, the Gd-DTPA concentration did not significantly affect the particle size and the gadolinium content, which were about 4 μm and

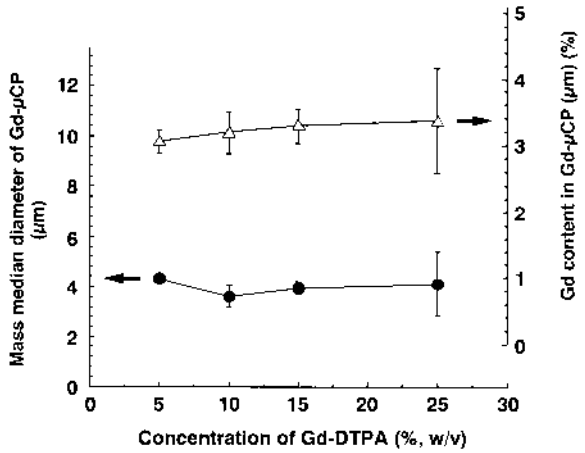

Fig. 5. Effects of Gd-DTPA Concentration in Chitosan Medium on the Mass Median Diameter and Gadolinium Content of Gd-μCPs

Experimental condition: chitosan 10B. ●, Mass median diameter; △, gadolinium content (%).

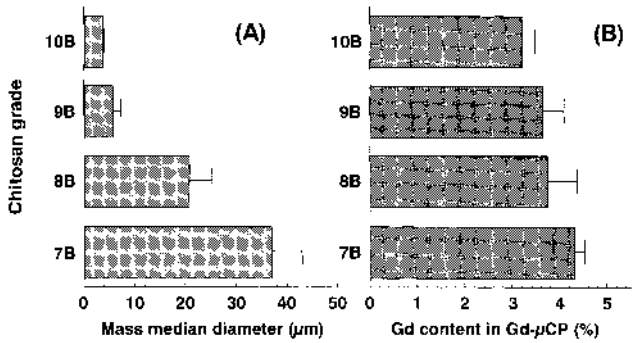

Fig. 6. Effects of Chitosan Grade on the Mass Median Diameter (A) and Gadolinium Content (B) of Gd-μCPs

Experimental condition: emulsion A, 2.5% chitosan 10B/10% Gd-DTPA solution.

3.1—3.4%, corresponding to 10.7—11.7% as Gd-DTPA, respectively.

Effect of Deacetylation Degree of Chitosan The Gd-μCPs were prepared at 10% Gd-DTPA concentration using different chitosan grades, 10B, 9B, 8B and 7B. Figure 6 shows the mass median diameter (A) and gadolinium content (B) with each chitosan grade. As the deacetylation degree of chitosan decreased, the particle size increased remarkably from 3.6 to 37.1 μm, while Gd content only increased from 3.2 to 4.3%, corresponding to 11.1—15.0% as Gd-DTPA.

***In Vitro* Release of Gd-DTPA in PBS** Four types of Gd-μCPs with different deacetylation degree of chitosan were examined by the dissolution test. A small amount of Gd-DTPA (0.4—0.8%) was released just after dispersing Gd-μCPs in PBS, but no more Gd-DTPA was released in PBS at 37 °C for 7 d for each type of Gd-μCPs, notwithstanding that Gd-DTPA has a high water solubility. The deacetylation degree of chitosan had no effect on Gd-DTPA release from Gd-μCPs.

***In Vitro* γ-Ray Emission Assay by Thermal Neutron Irradiation** This study was carried out to validate γ-ray emissions by gadolinium nuclear neutron capture reaction of Gd-μCPs as a preliminary study. The analysis was performed using the prompt γ-ray of 181.9 keV irradiated from gadolinium in Gd-μCP by thermal neutron irradiation. Figure 7

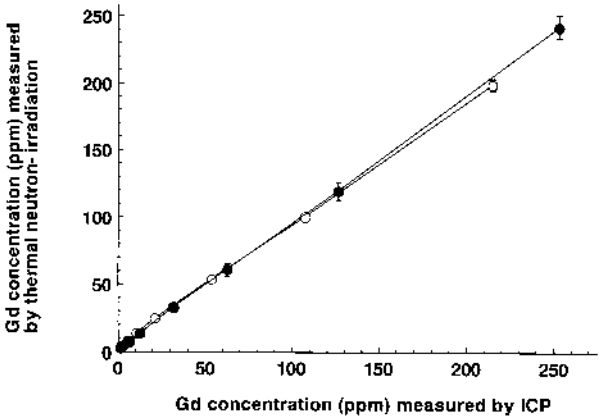

Fig. 7. Gadolinium Microanalysis on Gd-μCP Suspension and Dilute Magnevist® Solution Using Prompt γ-Ray Emission by Thermal Neutron Irradiation

●, Gd-μCP suspension; ○, dilute Magnevist® solution.

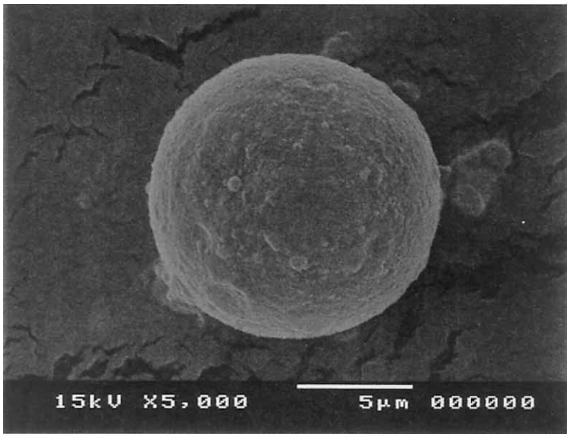

Fig. 8. Scanning Electron Micrograph of CMS

Experimental condition: emulsion A, 2.5% chitosan 10B/5% acetic acid.

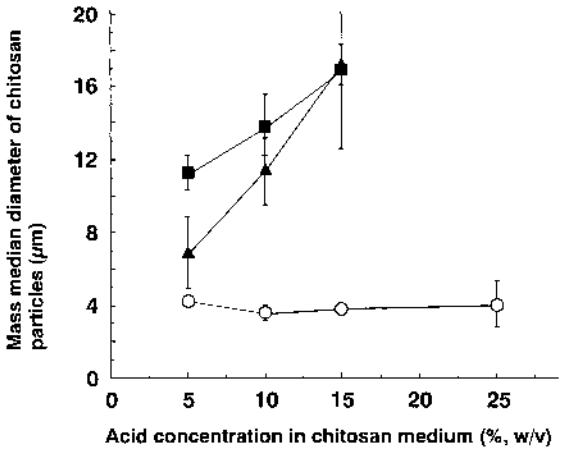

Fig. 9. Effects of Acid Type and Concentration in Chitosan Medium on the Mass Median Diameter of Chitosan Particles Prepared by an Emulsion-Droplet Coalescence Technique

Experimental condition: chitosan 10B. ○, Gd-DTPA solution; ■, acetic acid solution; ▲, citric acid solution.

shows the gadolinium concentration calculated from the intensity ratio of two prompt γ-rays which were emitted from gadolinium atoms and H atoms in Gd-μCP suspension or dilute Magnevist® solution. There was no difference between the Gd-μCP suspension and the dilute Magnevist® solution in the gadolinium concentration range studied here. This result indicated that the loss of the γ-ray emitted from gadolinium nuclear neutron capture reaction by gadolinium-loading in particulate device was negligible.

Discussion

One of the key factors to develop Gd-NCT is finding a drug carrier to deliver a sufficient amount of gadolinium to tumor cells or tissues and retain the gadolinium there during neutron irradiation. If gadolinium compounds and carriers were nontoxic, it would be possible to dose heavily in order to increase the absolute concentration of gadolinium in tumors because activation of gadolinium occurs only at the part of tumor irradiated with neutrons.

In the present study, a novel emulsion-droplet coalescence technique was developed in order to prepare biodegradable and highly gadolinium-loaded chitosan particulate carriers for Gd-NCT. As a gadolinium compound, Gd-DTPA was selected because its meglumine salt has been used widely as an MRI diagnosis agent (Magnevist®) and has been demonstrated to be nontoxic for organisms. Since a Gd-DTPA molecule has two-valent anionic charges, a high incorporation of gadolinium is anticipated by ionic interaction with the polyamino groups of chitosan molecules.

In the emulsion-droplet coalescence technique, when two kinds of the same type emulsion were mixed and stirred vigorously, droplets of each emulsion would reiterate random collision, coalescence and splitting; consequently, all of the droplets would be made uniform in their contents. The acidic chitosan-dissolving droplets of emulsion A would be neutralized by sodium hydroxide in droplets of emulsion B, leading to solidification of chitosan.

Gd-μCPs which were prepared using chitosan 10B by this technique did not have a spherical form (Fig. 4) and seemed to be agglomerates of nanoparticles, with a mass median diameter of 4.1 μm (Fig. 3). When blank chitosan (10B) microspheres (CMSs) were prepared by this technique without Gd-DTPA using acetic acid as a medium of chitosan, their shapes were almost spherical (Fig. 8) and the mass median diameter of CMSs was 9.4 μm: the size ranged from 2 to 20 μm. This result suggested that the generation of Gd-μCPs might be concerned with a special precipitation process in neutralization of chitosan medium, because the droplet size of emulsion A prepared by using Gd-DTPA or acetic acid was almost identical, about 4 μm.

Since the properties of Gd-μCPs might be affected by the acidity of droplets in emulsion A, Gd-DTPA concentration was changed. But the Gd-DTPA concentration did not influence the shape, size and gadolinium content of Gd-μCPs (Fig. 5). On the other hand, the mass median diameter of CMSs which were prepared using acetic acid or citric acid were strongly affected by the acid concentration: as the acid concentration increased, the diameter of CMSs increased remarkably (Fig. 9). CMSs might be enlarged by coalescence, followed by slow precipitation of chitosan inside the droplet, whereas precipitation from the medium containing Gd-DTPA would be very fast even at high Gd-DTPA concentration. This might be related to the fact that the mass median diameter of Gd-μCPs (10B) was kept at about 4 μm, corresponding

to the droplet size of emulsion A, though the details are not clear.

In this technique which utilized precipitation by alkali and subsequent ionic interaction of the amino groups of chitosan with Gd-DTPA, the deacetylation degree of chitosan influenced the particle size and gadolinium content of Gd-μCP (Fig. 6). From SEM observation, an increase in particle size with decreasing the deacetylation degree seemed to be induced by formation of hard agglomerates. Since the droplet size of emulsion A was not affected by the deacetylation degree of chitosan (about 4—5 μm), the degree of agglomeration might be due to the difference in precipitation properties of each chitosan inside the droplets.

Chitosan microspheres previously prepared by using glutaraldehyde incorporated gadolinium by electrostatic interaction between Gd-DTPA and chitosan, but the Gd-DTPA was completely released from the cross-linked particles in PBS.[15] However, Gd-DTPA was not released from all types of Gd-μCPs in PBS irrespective of chitosan's deacetylation degree, notwithstanding that Gd-DTPA had a very high water solubility. This suggested that Gd-DTPA might strongly interact with chitosan.

The Gd-DTPA non-releasing property of Gd-μCPs prepared in this study can be utilized effectively in some Gd-NCT trials. Magnevist® which has been used in Gd-NCT trials is not accumulated at all in tumors after intravenous injection and is eliminated rapidly from tumor tissues after intratumoral (i.t.) administration, as is well known; therefore, neutron has to be irradiated soon after i.t. administration[16] and the rapid elimination of gadolinium from tumor during irradiation depresses the therapeutic effect. On the other hand, Akine *et al*. demonstrated that the reservoir type of gadolinium microcapsules sustained-releasing Gd-DTPA was very useful as a gadolinium carrier for NCT.[11,12] If Gd-μCPs prepared in the present study were injected in the tumor directly, gadolinium would be retained for a long time without rapid elimination of Gd-DTPA. Namely, this would contribute to not only the enhancement of the Gd-NCT effect but also to greater flexibility in the treatment procedures, including the period and frequency of neutron irradiation and the timing of gadolinium administration. Furthermore, the validation of γ-ray which was emitted from Gd-μCPs by neutron irradiation *in vitro* suggested that gadolinium-incorporating particulate device, Gd-μCPs, could be used in Gd-NCT without considering loss of γ-ray emission.

Conclusion

The biodegradable Gd-μCPs were prepared by a novel emulsion-droplet coalescence technique as a device for Gd-NCT. Gd-μCPs prepared by using the 100% deacetylated chitosan were about 3—4 μm in mass median diameter and 3.1—3.4% in gadolinium content, corresponding to 10.7—11.7% as Gd-DTPA. They were agglomerates consisting of nanoparticles of 300—500 nm. The particle size and gadolinium content of Gd-μCPs were not affected by Gd-DTPA concentration in the chitosan medium. But, as the deacetylation degree of chitosan decreased, the particle size increased remarkably. Further, the release of Gd-DTPA from Gd-μCPs was less than 0.8% in PBS in spite of the high water solubility of Gd-DTPA, suggesting ion-complex formation. The γ-ray emission from Gd-μCP suspension by thermal neutron irradiation was identical to that from dilute Magnevist® solution. These results suggest that Gd-μCPs show promise as a device for i.t. injection in order to enhance the Gd-NCT effect.

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