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Development of Superparamagnetic Iron Oxide Nanoparticles (SPIOs)-Embedded Chitosan Microspheres for Magnetic Resonance (MR)-Traceable Embolotherapy

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Abstract – Superparamagnetic iron oxide nanoparticles (SPIOs)-embedded chitosan microspheres were developed for magnetic resonance (MR)-traceable embolotherapy. SPIOs-loaded chitosan microspheres were prepared by emulsion and cross-linking technique and 100-200 μm sized spherical microsparticles were obtained. Loading efficacy and loading amount of SPIOs in microspheres were about 40% and 0.26-0.32%, respectively, when measured by inductively coupled plasma atomic emission spectroscopy. Within 30 days, about 60% of the incorporated SPIOs were released from low cross-linked microspheres, whereas only about 40% of SPIOs was released from highly cross-linked microspheres. Highly cross-linked microspheres were more efficient for lower degree of swelling leading to secure entrapment of SPIOs in matrix. Prepared novel embolic microspheres are expected to be practically applicable for traceable embolotherapy with high resolution and sensitivity through magnetic resonance imaging (MRI).

Keywords: Embolotherapy, Chitosan microspheres, Superparamagnetic iron oxide nanoparticles, Magnetic resonance imaging (MRI)

INTRODUCTION

Embolotherapy is one of the intra-arterial anti-cancer therapies of the selective occlusion of the blood vessel around tumor by purposely introduced materials. Introduction of embolic materials into the blood vessels blocks the blood flow toward tumor tissues, thus starves the tumor (Liu *et al*., 2001).

Several types of materials have been investigated as embolic materials, such as gelfoam (Huang *et al*., 2004), small coils (Furuse *et al*., 1997), metal sponges (Tarazov *et al*., 1993), polyvinyl alcohol particles (Lee *et al*., 1999) and various polymeric microparticles (Forsberg, 1978; Dion *et al*., 1986; Flandroy *et al*., 1990; Laurent *et al*., 1996). Particularly, precisely calibrated spherical microparticles were reported to be more suitable for chemoembolization as it could be transported more distally than irregular materials and produced more homogeneous and complete occlusion of blood vessel (Flandroy *et al*., 1990). Of these, chitosan microspheres have been attempted as promising embolic materials (Eroglu *et al*., 2006; Kim *et al*., 2007). Chitosan is a linear polysaccharide composed of β-(1-4)-linked *D*-glucosamine (deacetylated unit) and N-acetyl-*D*-glucosamine (acetylated unit). It has been extensively used in medical and pharmaceutical areas due to its abundance, non-toxicity, biocompatibility and biodegradability (Badawy *et al*., 2004; Sinha *et al*., 2004).

In embolotherapy, to exactly place the embolic material to desired sites and monitor the changes in its structures are essentially needed to observe the occlusion position of injected embolic materials and prevent the serious complications caused by adverse embolization. However, it is difficult to monitor the changes in its structure over time.

Superparamagnetic iron oxide nanoparticles (SPIOs, Fe_3O_4, magnetite) are nanocrystals of iron oxides coated with hydrophilic polymers. SPIOs are currently used as a contrast agent for magnetic resonance imaging (MRI) developed for clinical applications with high spatial-resolution MR sequences (Muller *et al*., 1991; Roch *et al*., 1999).

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These are characterized by a large magnetic moment in the presence of a static external magnetic field and are mostly used because of their negative enhancement effect on T2-and T2-weighted sequences (Canet *et al*., 1993; Chambon *et al*., 1993). Therefore, the incorporation of SPIOs into embolic materials is expected to be applicable for traceable embolotherapy with high resolution and sensitivity through magnetic resonance imaging (MRI).

The aim of this study was to design a novel MR-traceable embolic material. SPIOs-embedded chitosan microspheres were fabricated by emulsion and cross-linking technique and the microspheres were evaluated through *in vitro* studies.

MATERIALS AND METHODS

Materials

Chitosan (minimally 84% deacetylated, obtained from crab), glutaraldehyde (25% aqueous biological grade), sorbitan sesquioleate, tris (hydroxymethyl)-aminomethan, hydrochloride and iron standard solution for ICP (10,000 μg/ml of Fe in 4.2% (w/w) nitric acid) were purchased from Sigma Aldrich Chemical Company (St. Louis, USA). SPIO nanoparticles, hydrodynamic diameter of 80 nm, were kindly supplied in the suspension at a concentration of 3.33 mg of iron per milliliter by Chung-Ang University Medical Center (Seoul, Korea). Liquid paraffin with viscosity of 18 cP at 20°C, petroleum ether, acetic acid, toluene, sodium bisulfite, acetone and other chemicals were purchased from Duksan Chemicals (Seoul, Korea). Distilled and deionized water was used for the preparation of all solutions.

Preparation of SPIOs-embedded chitosan microspheres

SPIOs-loaded chitosan microspheres were prepared by emulsion and cross-linking method (Jameela *et al*., 1998). A 4% (w/v) aqueous chitosan solution was prepared by dissolving chitosan in 5% (v/v) acetic acid solution. Then SPIO suspension (approximately 1.0 mg/300 μl) was added to 3 ml of viscous chitosan solution and dispersed homogeneously. The SPIOs-dispersed chitosan solution was added to the mixture of liquid paraffin and petroleum ether (7:5, 30 ml) containing sorbitan sesquioleate (1.5 ml), an emulsifier, in a 100 ml flask. The dispersion was stirred using an over-head stirrer (Servodyne, model 50,000-30) for 10 min at 2,000 rpm to form w/o emulsion. Then 25% aqueous glutaraldehyde solutions (1 and 2 ml) or glutaradehyde-saturated toluene (GST, 5 and 10 ml) was added into the flask and stirred for 1 h and incubated for another 1 h. GST used was prepared by adding 25% aqueous glutaraldehyde solutions to toluene and stirring for 6 h. The hardened microspheres were then separated by centrifugation at 3,000 rpm, washed three times with petroleum ether and three times with sodium bisulfite, acetone with one time and three times with water. Washed microspheres were dried at 40°C and stored in a desiccator until next study.

Solubility of glutaraldehyde in toluene

To elucidate the amount of glutaraldehyde added as toluene saturated form in the preparation procedure, solubility of glutaraldehyde in toluene was measured. The concentration of glutaraldehyde was measured by UV/VIS spectrometer (CARY WinUV3, Varian Inc.) at 280 nm (Fahimi *et al*., 1965) and calibration curve was plotted against 0.25-2.50% (w/v). Glutaraldehyde saturated toluene (GST) was evaporated to dryness under a gentle stream of nitrogen at 40°C. Then it was reconstituted with water and analyzed by UV/VIS spectrometer.

Morphological features of SPIOs-embedded chitosan microspheres

Micrographs of the microspheres cross-linked with GST or glutaraldehyde aqueous solutions were taken with a scanning electron microscope (SEM, Philips XL30) for visual inspection of the surface morphology and internal structure of microspheres. To observe the internal structure of the microspheres, the microspheres were crushed and examined using SEM. Samples were mounted on metal stubs using double-sided adhesive tape and vacuum-coated with gold film.

Particle size distribution of SPIOs-embedded chitosan microspheres

Particle size distribution was measured by sieving technique. Particles that passed through upper sieve, but retained on the bottom were collected, weighed in an analytical balance and the percent weight distribution was plotted against particle size ranges.

Measurement of SPIOs loading amount and efficiency

Loading amount and efficiency of SPIO nanoparticles in microspheres were measured by determining iron concentration using inductively coupled plasma atomic emission spectrometer (ICP-AES; P-5200 ICP system, Hitachi Co. Ltd., Japan) at 259.940 nm (Hamoudeh *et al*., 2007). SPIO-chitosan microspheres (10 mg) were digested in a medium containing H_2SO_4 (95%) and HNO_3 (65%) and heated at 70°C for 3 h to solubilize the iron oxide. Filtered solution was then diluted with distilled water and assayed

by ICP-AES. Calibration curve of Fe was plotted against in the range of 1-50 μg/ml. The encapsulation amount and efficiency was calculated by following equation.

$$\text{SPIO loading amount (\%)} = \frac{\text{Mass of SPIO in particles}}{\text{Total mass of particles}} \times 100$$

$$\text{SPIO loading efficiency (\%)} = \frac{\text{Mass of SPIO in particles}}{\text{Initially added mass of SPIO}} \times 100$$

Swelling test

The dried SPIOs-loaded microspheres (20 mg) were immersed in simulated body fluid (50 mM tris (hydroxymethyl)-aminomethane and 45 mM HCl, pH 7.4) and were shaken for 24 h at room temperature. Then swollen samples were collected by centrifugation, and then blotted with filter paper to remove the water on the surface, and immediately weighed. The degree of swelling was calculated using the following equation:

$$\% \text{ degree of swelling} = (W_2 - W_1)/W_1 \times 100$$

In the above equation, W_1 and W_2 represent the weight of dry and swollen chitosan microspheres, respectively.

Release test

Twenty milligrams of the microspheres were immersed into 10 ml of simulated body fluid and stirred at 100 rpm at room temperature in shaking incubator. The release test was performed for 4 weeks and the samples were collected periodically at 0.5, 1, 2, 5, 10, 15, 20 and 30 day. Samples were digested using acidic medium and the concentration of iron was determined by ICP-AES.

RESULTS AND DISCUSSION

Solubility of glutaraldehyde in toluene

The solubility of glutaraldehyde in toluene measured by UV/VIS spectrometer was 10.52 ± 1.27% (w/v) and this value was significantly greater than previously reported value (2.02%) (Latha *et al*., 1995). The solubility of water in toluene was reported in the range of 3.16-5.72% (w/w) at

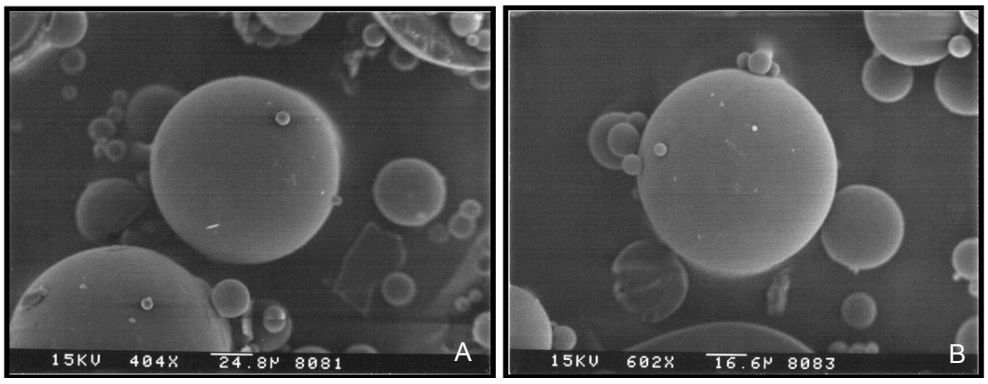

Fig. 1. Scanning electron micrograph of (A) SPIO-embedded chitosan microspheres cross-linked with glutaraldehyde aqueous solution; (B) SPIO-embedded chitosan microspheres cross-linked with glutaraldehyde saturated toluene.

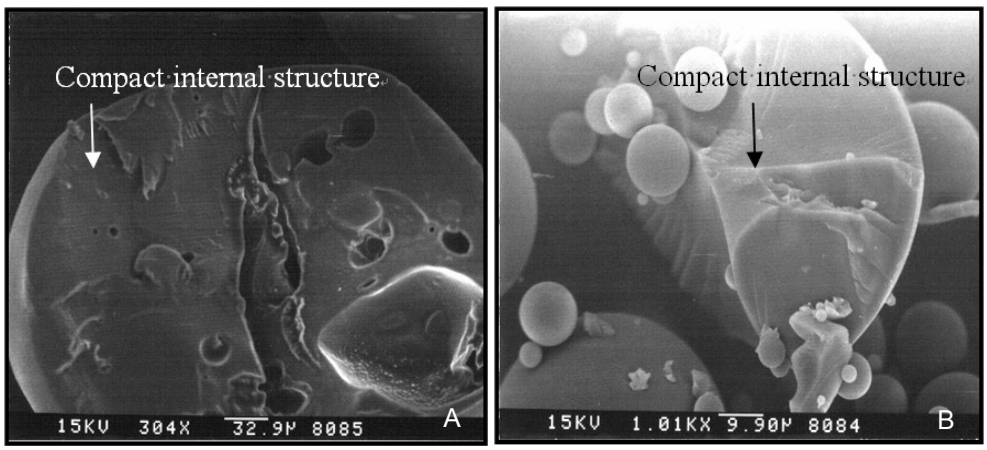

Fig. 2. Scanning electron micrograph of internal structure of (A) SPIOs-embedded chitosan microspheres with cross-linked with glutaraldehyde aqueous solution; (B) SPIOs-embedded chitosan microspheres cross-linked with glutaraldehyde saturated toluene.

20-25°C (Gross and Saylor, 1931; Klevens, 1950) and glutaraldehyde dissolved in water was considered to be moved with water in saturation procedure. Chitosan:glutaraldehyde ratio were 1:2, 1:4, 1:4.38 and 1:8.77, respectively, when aqueous glutaraldehyde solutions 1 ml and 2 ml, GST 5 ml and GST 10 ml were added in the preparation procedure.

Morphological features of SPIO-loaded chitosan microspheres

Morphological features of microspheres were observed by SEM. SPIO-loaded microspheres were highly spherical in appearance as seen in SEM both aqueous glutaraldehyde and GST added microspheres (Fig. 1). Morphologies of the internal structure of the microspheres observed by SEM showed that the internal structure of the microspheres was solid and greatly compact regardless of glutaraldehyde adding methods (Fig. 2).

The addition of aqueous glutaraldehyde was reported to lead the formation of microspheres with rough surface (Gohel *et al*., 1994), but there were little differences between aqueous form and toluene saturated added form in our study.

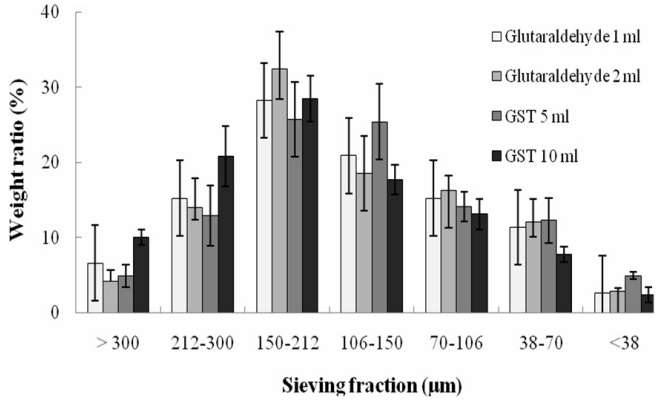

Fig. 3. Size distributions of SPIO-embedded chitosan microspheres (Mean ± SD., n=3).

Table I. Loading amount and loading efficiency of SPIOs in chitosan microspheres (Mean ± SD., n=3)

Samples	Loading amount (%)	Loading efficiency (%)
Glutaraldehyde 1 ml	0.27 ± 0.03	39.97 ± 4.36
Glutaraldehyde 2 ml	0.26 ± 0.03	41.60 ± 5.14
GST 5 ml	0.32 ± 0.06	41.57 ± 5.27
GST 10 ml	0.31 ± 0.05	43.85 ± 3.71

GST: glutaraldehyde saturated toluene.

Size distribution of SPIO-loaded chitosan microspheres

The particle size distribution of microspheres measured by sieving technique was shown in Fig. 3. All microspheres showed the similar particle size distribution regardless of the amount of glutaraldehyde. Over 70% of microspheres was fabricated between 70-212 μm.

SPIO loading amount and loading efficiency

Loading amount and loading efficiency of SPIO in the microspheres were shown in Table I. Loading efficiency of SPIO in microspheres were approximately 40% and loading amounts were in the range of 0.26-0.32%. There were little noticeable differences between each formulation.

Sufficient amounts of SPIOs were seemed to be embedded into chitosan microspheres for MR-detection. In early report, lower amount of SPIOs (approximately 0.2 mM as iron concentration) were detected in MR images in rabbit (Lee *et al*., 2005). Therefore, the microspheres containing more SPIOs over ten folds than those prepared by Lee *et al.* (2005) are expected to be sufficient for MR detection, although the potency of SPIOs should be thoroughly considered.

Swelling and release test

Chitosan is readily hydrated in water since it contains hydroxyl and amino groups. After cross-linking reaction of glutaraldehyde with primary amino groups of chitosan, the number of hydroxyl and amino groups were changed and thus, degree of hydration could be changed. Glutaraldehyde can react with a primary amine group and forms a water-insoluble imine bond (Bachtsi and Kiparissides, 1995).

It is well demonstrated that the higher cross-linking degree of chitosan shows the lower water absorption capacity (Monteiro and Airoldi, 1999). Therefore, the degree

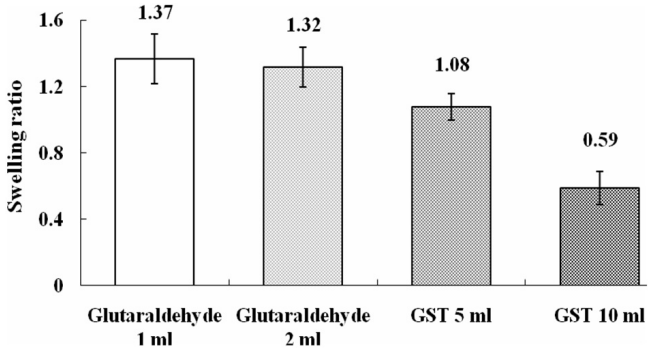

Fig. 4. Swelling ratios of the SPIO-embedded chitosan microspheres in simulated body fluid at room temperature. GST means the glutaraldehyde saturated toluene (Mean ± SD., n=3).

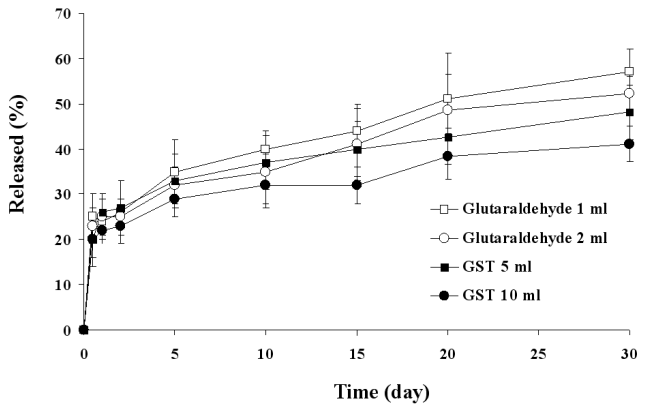

Fig. 5. The accumulated release profiles of SPIOs from microspheres at room temperature depend on the type of cross-linking agent. GST means the glutaraldehyde saturated toluene (Mean ± SD., n=3).

of cross-linking can be elucidated through the degree of water uptake capacity. The swelling ratios of chitosan microspheres examined were shown in Fig. 4. The swelling ratios of chitosan microspheres significantly decreased with increasing the amount of glutaraldehyde. The swelling ratios of the microspheres cross-linked with 1 and 2 ml of glutaraldehyde and 2 and 5 ml of GST were 1.37, 1.32, 1.08 and 0.59, respectively.

The *in vitro* release profiles of SPIOs from microspheres depend on the type of cross-linking agent were shown in Fig. 5. The SPIOs release was characterized by an initial burst followed by sustained gradual release. The 'burst effect' shown from all preparations were approximately 20-25% at 12 h then slow release profiles were observed.

The SPIO release from highly cross-linked microspheres was slower compared to that of low cross-linked microspheres. Within 30 days, about 60% of the incorporated SPIO was released from low cross-linked microspheres, whereas only about 40% of SPIOs was released from highly cross-linked microspheres. After burst release of SPIO within 12 h, about 33% of the incorporated SPIO was released from low cross-linked microspheres, whereas only about 19% was released from highly cross-linked microspheres after 1 month. The cross-linking degree of microspheres effectively controlled the diffusion of SPIOs from microspheres. It has been well demonstrated that the increase of cross-linking degree of polymer increases the density of the polymer network which decreases available free space for drug diffusion, results in a decrease in drug release rates (Fahimi and Drochmans, 1965).

In conclusion, highly cross-linked microspheres were more efficient for lowering the degree of swelling of matrix of microsphere leading to secure entrapment of SPIO. Lower degree of SPIO release was obtained from the microspheres cross-linked with 10% of GST and considered to be more promising for long-term MR-imaging due to the slow SPIO release rate.

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