Highly Spherical and Deformable Chitosan Microspheres for Arterial Embolization

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Received May 18, 2009; accepted November 11, 2009; published online December 18, 2009

The aim of this study was to fabricate deformable chitosan (CS) microspheres for arterial embolization. CS microspheres containing poly(ethylene glycol) (PEG) were prepared by ionotropic gelation; PEG was then removed from the CS microspheres to produce the highly porous structure to allow deformability. The porosity was controlled by blending ratios of CS/PEG polymers (CS/PEG=from 100/0 to 15/85) and the effect of porosity on microcatheter delivery was examined. The size range of porous microspheres was 500—600 μm with sphericity between 1.012—1.041. Scanning electron microscope observation confirmed that microporous networks were effectively obtained by PEG extraction proportional to the initial amount of PEG. Water retention capacities, indicative of internal porosities of microspheres, increased with increasing initial amounts of blended PEG. CS microspheres with water retention values of greater than 28% exhibited noticeable deformation and smooth passage through the microcatheter tip. Novel deformable microspheres are, therefore, expected to be clinically applicable for arterial embolization.

Key words chitosan microsphere; arterial embolization; deformation; poly(ethylene glycol)

Arterial embolization is an intra-arterial chemotherapy carried out by introducing embolic materials into a tumor-feeding artery through a microcatheter. These embolic materials (the emboli) block the blood flow toward tumor tissues, and thereby obstruct the supply of oxygen and nutrition necessary for tumor survival.[1] Arterial embolization has widely been investigated with a variety of microparticular systems prepared with poly(D,L-lactide/glycoside) copolymer,[2] starch,[3] dextran,[4] trisacryl gelatin,[5] albumin[6] and poly(vinyl alcohol).[7] For almost 30 years, non-spherical poly(vinyl alcohol) particles and gelatin sponge have been most common systems used to perform various types of therapeutic embolization. Poly(vinyl alcohol) particles vary in size and are generally irregular in shape, which results in unpredictable proximal vascular occlusion and catheter obstruction.[8,9] Gelatin sponge is absorbed within 7 to 21 d,[10] and is therefore only used for temporary devascularization. Furthermore, since gelatin sponges must be cut manually before infusion into the microcatheter, the fragment sizes are not uniform.[11]

Ideal devascularization was reported to be achieved by distal and permanent blood vessel occlusion. To efficiently reach the distal vessel, embolic particles should be spherical, deformable, and homogeneous in size.[12] Furthermore, deformability is also essential for delivery through the narrow catheter tip.[13]

Chitosan (CS), a linear polysaccharide composed of β-(1-4)-linked D-glucosamine, has been used extensively in the medical and pharmaceutical fields due to its non-toxic, biocompatible and biodegradable nature.[14,15] However, CS microspheres have never been successfully developed as emboli because of their rigid and brittle properties.[16]

It has been reported that the deformation of polymeric microparticles is greatly dependent on porosity.[17] In practice, trisacrylgelatin (Embosphere®, BioSphere Medical Inc.), poly(vinyl alcohol) (Contour SE®, Boston Scientific), and polyvinyl alcohol–sodium acrylate copolymer (HepaSphere™, Biosphere Medical Inc.) microspheres organized with numerous cavities exhibit outstanding catheter deliverability and devascularization ability.[18] Based on these reports, we assumed that a highly porous internal structure should allow the temporary deformation of CS microspheres needed for the embolization procedure.

In our study, highly spherical CS microspheres were prepared by nitrogen gas-assisted dropping and ionotropic gelation method. A poly(ethylene glycol) (PEG) extraction technique was employed to form porous structures within the CS microspheres. PEG is biocompatible and has hydrophilic heads comprised of hydroxyl groups, allowing good water-solubility.[19] We expected that the removal of PEG from CS microspheres containing PEG initially blended would produce numerous micropores in the microspheres. The pore structure was controlled by the blending ratio of CS/PEG polymers and the influence of PEG extraction on porosity and deformability of the microspheres was examined.

Experimental

Materials Chitosan (CS) from crab shells (over 85% deacetylated, practical grade), poly(ethylene glycol) (PEG) with molecular weight of 20000 g/mol were purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.). Glacial acetic acid, ethyl alcohol, petroleum ether, acetone and sodium hydroxide (NaOH) were obtained from Duksan Pure Chemical Co. (Seoul, Korea), and genipin was purchased from Wako Pure Chemical Industries (Tokyo, Japan). Distilled and deionized water was used for the preparation of all solutions.

Preparation of Deformable CS Microspheres CS was dissolved in 1% (v/v) acetic acid solution to a concentration of 1.5% (w/v). PEG was then added to the viscous CS solution at various weight percentage ratios, and the mixtures were stirred overnight at room temperature. The ratios (%, w/w) of CS/PEG tested were 100/0, 65/35, 50/50, 35/65, 25/75 and 15/85, respectively. The CS/PEG solution was then drawn into a syringe (31 g) and the syringe was emptied *via* a syringe pump at a rate of 600 μl/h. The solution was dropped into coacervation medium in nitrogen gas (1.0 kgf/cm^2) as shown in Fig. 1A. The coacervation medium was a mixture of ethanol (50 ml) and 10% NaOH aqueous solution (5 ml). Before introduction into the coacervation medium, the drop was allowed to pass through a petroleum ether (specific gravity=0.64 at 20 °C) layer formed on top of the coacervation media. The solidified microspheres were dehydrated in acetone and cross-linked

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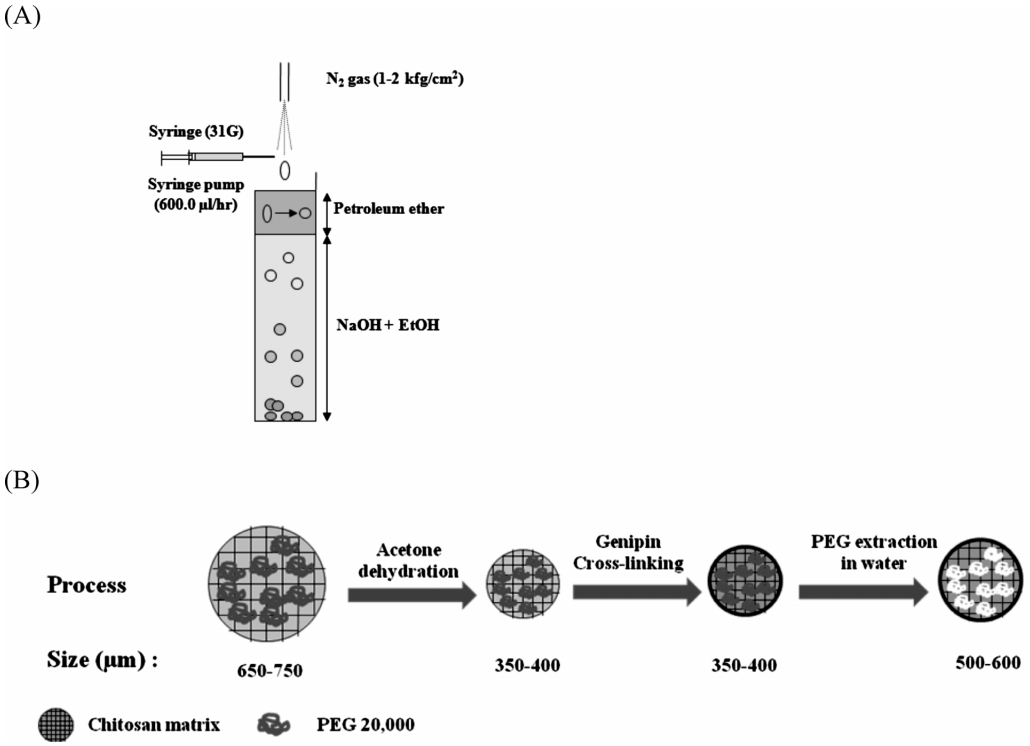

Fig. 1. Schematic Illustration of Procedures for Fabricating Deformable CS Microspheres; (A) Ionotropic Gelation; (B) Genipin Cross-Linking and PEG Extraction

with 0.05% genipin solution for 5 min (Fig. 1B). The acetone was removed by evaporation under reduced pressure at room temperature. The CS/PEG microspheres were then incubated in hot water for 24 h to extract the PEG, washed with water and stored in normal saline.

Visual Observation, Particle Size and Sphericity Measurement The appearance of CS microspheres was visually examined with an optical microscope (BX60 F5; Olympus Optical, Tokyo, Japan). The size distribution of the prepared microspheres was determined by image analysis software (Optimas 6.1, VSG, U.K.) and the sphericity was calculated by taking the ratio between the larger and smaller diameters.

Scanning Electron Microscope (SEM) Observation The surface morphology and internal structure of the microspheres were examined by SEM (Model S-3400N, Hitachi, Japan). Samples were coated with gold by sputtering (Polaron E 5000 vacuum unit) prior to examination. Some microspheres were cross-sectioned for visualization of internal structures.

Water Retention Capacity Water retention capacity was measured as an indicator of porosity.[20] After measurement of the weight of microspheres in wet state, the microspheres were dried at 60 °C for 24 h and the weight was re-measured. The degree of water uptake was calculated with the following equation:

$$\text{water retention (\%)} = [(W_1 - W_2)/W_2] \times 100$$

where W_1 and W_2 represent the weight of hydrated and dried CS microspheres, respectively.

Microcatheter Deliverability Microcatheter pass-through ability of microspheres was evaluated with commercially available microcatheter (Progreat®, 2.4 Fr, inner diameter 570 μm, Terumo Corp., Tokyo, Japan). Fifty milligrams of microspheres suspended in normal saline/X-ray contrast agent (Visipaque™, GE Healthcare, U.K.) (50/50 v/v) were drawn into a syringe and was then connected to the catheter. The size range of the microspheres examined was 550±70 μm. The deliverability of microspheres was evaluated by examining the ease of passage through the microcatheter when injected and by microscopically observing changes in the appearance during the transition through the microcatheter tip.

Storage Stability The physical changes of CS microspheres were examined both in normal saline and in normal saline/Visipaque™ mixture (50/50 v/v). Characterization parameters such as morphology, particle size, sphericity and suspendibility of the microspheres were evaluated 4 weeks after preparation.

Results and Discussion

Design and Preparation of Deformable CS Microspheres Ionotropic gelation and PEG-leaching procedures were employed to fabricate deformable CS microspheres (Fig. 1). In our study, a double layered-hardening medium was uniquely devised; this medium consisted of an organic layer of petroleum ether and an aqueous layer of NaOH solution (10% w/v) and ethanol (specific gravity=0.81 at 20 °C). Petroleum ether placed above the aqueous layer was used to induce a change in the appearance of CS droplets from oblong to spherical shape during passage through the organic layer. This shape change was attributed to the generation of interfacial tension against CS drops.[21] Such interfacial tension between the surface of the aqueous CS droplet and the organic solvent (petroleum ether) could provide each part with a tendency to minimize its surface area. This tension became large enough to overcome the strain energy of the oblong shape of the CS droplets caused by the gravitational force as they fell into the coacervation medium from the syringe needle, and finally gave the droplets their spherical shape. The CS droplets that passed through the petroleum ether layer were coacervated in the aqueous NaOH layer to form a solid state network. The difference in specific gravity between petroleum ether and NaOH solution is great, thus when a CS droplet was first introduced into petroleum ether layer, the drop could not proceed toward the NaOH layer and was arrested between the organic and aqueous layers. Ethanol reduced the difference in specific gravity between petroleum ether and NaOH solution; thus, the droplets could be introduced into the NaOH solution, causing microsphere formation.

PEG-leaching procedure was employed to form micropores and to control the porosity of CS microspheres and

allow deformability of the microspheres. Genipin cross-linking procedure was improved the mechanical strength of CS microspheres.[22,23] In our previous study, CS microspheres without genipin cross-linking were fragile during passage through the microcatheter due to extremely poor mechanical strength. Thus, it was necessary to obtain the appropriate mechanical strength. The cross-linking reaction with genipin improved the strength of CS microspheres by covalent cross-linking interactions. It was reported that genipin cross-linked with amino groups in CS and underwent further secondary polymerization to improve the mechanical strength.[24]

Using the fabrication procedure developed for our study, we produced CS microspheres with sizes ranging from 200 to 1200 μm with a rather narrow size distribution. The key parameters for controlling the size of CS microspheres were the flow rate of nitrogen gas and the internal diameter of the syringe needle. Among the CS microspheres with varying size ranges, microspheres 550±70 μm in size, one of the sizes being used commercially for embolotherapy, were chosen for further experimental evaluation.

Optical Microscopic Observation Photomicrographs demonstrated that the microspheres had smooth surface, spherical shape and yellow-brown color, which might originate from the genipin cross-linking process (Fig. 2).[25] The mean diameter of the microspheres initially prepared by ionotropic gelation was about 650—750 μm. The acetone dehydration and cross-linking procedures considerably reduced the particle size to about 350 to 400 μm. During the PEG extraction process, the CS microspheres swelled and the mean particle size increased up to about 500—600 μm. The amount of PEG added initially did not significantly change the particle size of CS microspheres (Table 1). The sphericity of the microspheres was between 1.012—1.041. Most of the microspheres prepared were spherical and largely homogeneous in size.

SEM Observation Several methodologies have been reported for fabricating films or particles with porous structures including phase inversion technique,[26,27] freeze-drying or cryogenic-induced phase separation,[28,29] and casting/salt-leaching.[30,31] Of these, the casting/salt-leaching method is considered to offer the easy control of pore structures and has been widely employed in the fabrication. Among porogens that have been utilized to generate porous structure, PEG was selected due to good water solubility and reproducibility.

In our work, CS microspheres containing PEG were obtained, and then the PEG was extracted from the microspheres to produce the porous structure. SEM observation confirmed that PEG was efficiently extracted and more porous networks were thereby successfully constructed (CS/PEG=25/75, Fig. 3B; CS/PEG=15/85, Fig. 3C) compared to the intact CS microspheres (CS/PEG=100/0, Fig. 3A). PEG extraction from the microspheres gave rise to mi-

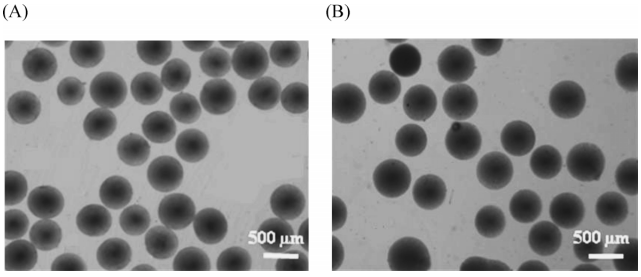

Fig. 2. Morphological Features of CS Microspheres (50× Magnification); (A) Intact CS Microspheres (CS/PEG=100/0); (B) CS Microspheres with PEG Blended and Extracted (CS/PEG=25/75)

Table 1. Physical Characteristics of CS Microspheres (Mean±S.D., n=3)

CS/PEG (w/w)	Diameter (μm)	Sphericity	Water retention (w/w, %)
100/0	545.5±67.5	1.036±0.005	18.0±2.1
65/35	541.4±72.8	1.030±0.004	22.7±3.3
50/50	545.5±83.2	1.041±0.010	25.5±4.1
30/70	543.2±85.6	1.016±0.008	28.1±3.3
25/75	535.5±94.5	1.032±0.003	30.0±2.8
20/80	545.5±81.1	1.021±0.005	32.0±4.2
15/85	553.1±85.1	1.012±0.006	35.2±5.6

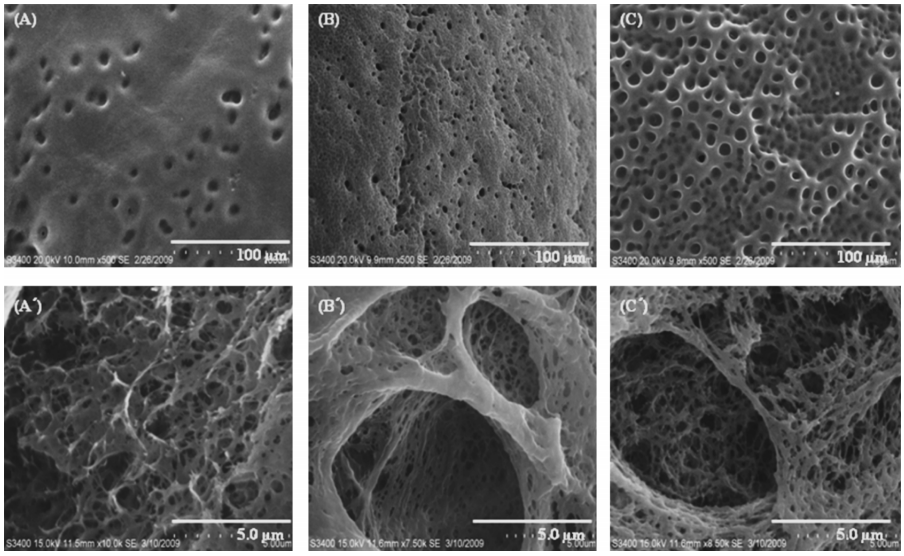

Fig. 3. SEM Images of the Microspheres Produced from CS/PEG 100/0 (A, Surface; A′, Cross-Sectioned), 25/75 (B, Surface; B′, Cross-Sectioned) and 15/85 (C, Surface; C′, Cross-Sectioned)

cropores in the microspheres and the pore size of the biggest pores was estimated to be between 5—10 μm. The number of pores formed proportionally increased with increasing PEG content blended with CS. The cross-section images further demonstrated the highly porous networks formed within the PEG-blended microspheres (Fig. 3B′ or 3C′) compared to intact CS microspheres (Fig. 3A′).

Some empty spaces were also observed in CS/PEG (100/0) microspheres. This occurrence might be attributed to the initial existence of water in the production of CS solution. CS drops were hardened in coacervation medium, forming a polymeric network and water was entrapped between CS chains in the microspheres. The water retained in CS microspheres was evaporated before SEM experiment and thereby empty spaces could be observed in SEM examination. However, more and larger micropores were certainly observed in the CS microspheres treated with PEG.

Water Retention Capacity Comparison of porosity between microspheres prepared with various amounts of PEG was performed by measuring water retention capacity, as it has been well demonstrated that water retention depends primarily on porosity and pore size distribution.[32] The water retention value obtained from intact CS microspheres (100/0) was 18% and was proportionally increased up to 35% (CS/PEG=15/85) with increasing PEG amount. As shown in SEM observation, the PEG extraction process led to the creation of micropores in the CS networks and provided further spaces for water retention compared to that in intact CS microspheres. From the SEM observation and water retention measurement data, it was noted that PEG extraction generated the porous structure in CS matrices and porosity could be increased by increasing the amount of PEG.

Microcatheter Deliverability Several particulate embolic materials are currently available in the market, but they are not necessarily satisfactory for clinical use. One of the major problems encountered during embolization intervention is the frequent incidence of microcatheter clogging; indeed, smooth injection of the embolic microparticles is greatly limited.[33]

Comparably, rigid CS microspheres (CS/PEG=100/0) devoid of PEG spaces accumulated in the microcatheter hub spaces due to lack of enough flexibility for introduction into the microcatheter tube (Fig. 4). However, the CS microspheres with more than 70% PEG blended and extracted (*i.e.*, water retention capacities exceeding 28%) could easily pass through the microcatheter without clumping. These microspheres were introduced into the narrow microcatheter tube (or microcatheter tip) (Fig. 5A), proceeded through the inner lumen (Fig. 5B) and reached the distal tip. The microspheres were finally squeezed out of the microcatheter tip with noticeable elastic deformation (Figs. 5C, D). The particles recovered their original spherical shape after passing through the catheter tip (Table 2). The changes in sphericity of the microspheres before and after the passage were less than 3%. The deformation of microparticles was reported to rely largely on composition, stress, temperature, and porosity.[17] An increased porosity of CS microspheres significantly reduced the rigidity of CS microspheres and, thus, provided the ability for alteration of the shape under pressure. The increased deformability also reduced the clogging phenomenon at the microcatheter hub and provided unproblematic passage through a narrow distal tip.

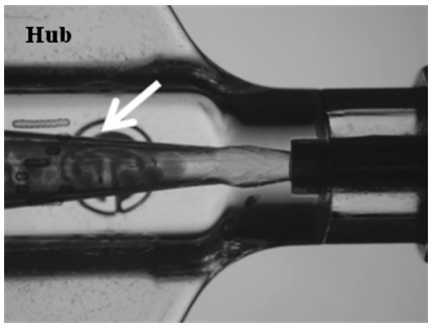

Fig. 4. Clogging of CS Microspheres (CS/PEG=100/0) in the Hub of Progreat™ (2.4 Fr) Microcatheter Due to Lack of Deformability Required to Pass through the Microcatheter

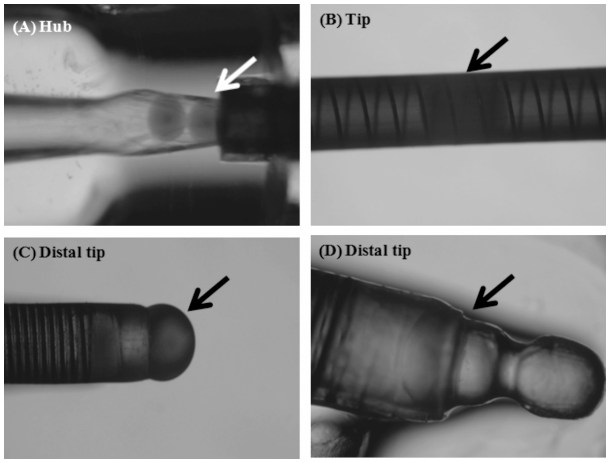

Fig. 5. Microcatheter Delivery of Deformable CS Microspheres

The microspheres (CS/PEG=25/75) were introduced into the hub easily (A), passed through the inner lumen (B), and squeezed out of the distal tip (C). Photomicrograph showing great deformability of the microspheres (D). The arrow points to the microspheres.

Table 2. Delivery of CS Microspheres in Progreat™ (2.4 Fr) Microcatheter

CS/PEG	Ease of injection	Deformation in the catheter	Sphericity changes before and after passage	
			Before	After
100/0	Difficult	Slightly deformable	1.036	1.051
65/35	Difficult	Slightly deformable	1.030	1.051
50/50	Difficult	Moderately deformable	1.041	1.054
30/70	Easy	Markedly deformable	1.016	1.042
25/75	Easy	Markedly deformable	1.032	1.057
20/80	Easy	Markedly deformable	1.021	1.059
15/85	Easy	Markedly deformable	1.012	1.048

Table 3. Comparison of Physical Stability of Deformable CS Microspheres (CS/PEG=25/75) in Normal Saline and X-Ray Contrast (Visipaque™)-Mixed Normal Saline for 4 Weeks (Mean±S.D., n=3)

	Initial		4th week	
	Saline	Saline/Visipaque™	Saline	Saline/Visipaque™
Diameter (μm)	535.5±94.5	526.6±85.8	540.3±83.0	535.3±81.5
Sphericity	1.032±0.003	1.026±0.004	1.029±0.003	1.023±0.004
Aggregation	ND[a)]	ND	ND	ND

a) Not detected.

Storage Stability Generally, embolic materials are stored with radiopaque contrast materials to allow monitoring of the embolic materials by X-ray upon injection into the target blood vessel. Therefore, testing the physical changes of the microspheres in X-ray contrast medium is very important. The size, morphology and suspendibility of the microspheres were evaluated both in normal saline and in a mixture of saline and Visipaque™. As shown in Table 3, after 4 weeks storage in normal saline and in saline/Visipaque™ mixture, no size, sphericity changes or aggregation were detected in any microspheres tested. Storage in contrast agent medium did not result in noticeable differences in physical properties compared to normal saline.

Conclusion

In our study, homogeneous and deformable CS microspheres were efficiently prepared by ionotropic gelation and PEG extraction methods. Their physical characteristics and suitability as an embolic material were evaluated. The mean size of the microspheres was 550 μm. Microspheres having more than 28% water retention capacity easily passed through the microcatheter with temporary deformation. The deformable CS microspheres could therefore be useful for arterial embolization.

Acknowledgements This work was supported by the Seoul R&BD Program (10543). This research was financially supported by the Ministry of Knowledge Economy (MKE) and Korea Industrial Technology Foundation (KOTEF) through the Human Resource Training Project for Strategic Technology (MJK, JMP, WSC). This work was supported by National Research Foundation of Korea Grant funded by the Korean Government (2009-0087953).

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