Macromolecular Research, Vol. 17, No. 3, pp 168-173 (2009)

Alginate Nanohydrogels Prepared by Emulsification-Diffusion Method

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Received June 2, 2008; Revised July 24, 2008; Accepted October 1, 2008

Abstract: This study reports the preparation and characterization of nanohydrogels by using sodium alginate as a model material. Alginate nanohydrogels (ANH) were prepared by emulsification-diffusion method in a w/o system with 1,2-diacyl-sn-glycero-3-phosphocholin as the lipophilic surfactant. The effects of the alginate to surfactant ratio and the remaining water contents on the mean particle size and swellability of ANHs were investigated in terms of the concentration, agitation speed, and agitation time. The feasibility of using nanohydrogels and their controllability were proved by the water the absorbency of ANHs during a 7-day evaluation by dynamic light scattering. In this work, the mean particle sizes of ANHs could be controlled from 49.2 nm (measured in ethanol phase) to 1.9 μm (measured in water phase, after 7 days of water absorption).

Keywords: alginate, nanohydrogel, emulsification-diffusion method, swelling, water absorption.

Introduction

Polymeric nanoparticles have been in focus of an increasing amount of recent research works because of their clinical usages especially for diagnostics and therapeutics including molecular imaging agents[1-4] and carriers for delivery systems[5,6] which urged nanoparticles to attach on and/or penetrate into the biological cells of over 10 mm in diameter. There are so many methodologies for preparing nanoparticles including emulsion polymerization, interfacial polymerization, solvent evaporation, solvent deposition, nanoprecipitation, desolvation of natural polymers,[7] and emulsification-diffusion.[8-12] Among these methods, emulsification-diffusion technique adopts thermodynamic principles of solvent mixing and diffusion to prepare polymeric nanoparticles of biocompatible polyester such as poly(D,L-lactic acid) and poly(D,L-lactide-*co*-glycolide). These researchers used hydrophilic surfactants to emulsify the polymer solution in water phase and then added excessive water to extract organic solvent from the inner polymer-rich phase. To obtain hydrophobic nanoparticles with this method, the use of partially water-miscible organic solvent is essential. Table I summerizes the characteristics of organic solvent used in emulsification-diffusion technique in literatures. With the consideration of the emulsification-diffusion mechanism mentioned above, we deduced that if we could reverse the o/w system of traditional emulsification-diffusion method to w/o counterpart, it would be possible to prepare polymeric nanohydrogels of hydrophilic polymers.

Hydrogel is a water-swellable network of polymers in which water is the dispersion medium. Hydrogels are important materials for biomedical applications because they possess hydrophilicity and a degree of flexibility very similar to that of natural tissue due to their significant water content. For these reasons, polymeric hydrogels have been used as scaffolds in tissue engineering,[13-15] sustained-release drug carriers,[16-18] biosensors,[19,20] and contact lenses.[21,22]

Alginate is probably one of the most frequently studied hydrogel-forming polymers. Alginate is a representative biocompatible natural polymer which is a linear copolymer of (1,4)-linked β-D-mannuronate and its C-5 epimer α-L-guluronate residues. It is insoluble in water but dissolves in some basic solutions by forming sodium salts. Due to its biocompatibility and simple gelation with divalent cations such as Ca^{2+}, it is widely used for biomedical applications including cell immobilization,[23] cell encapsulation,[24,25] wound dressing,[26-28] biomedical membrane,[29-31] and drug delivery system.[32]

In the present paper, modification of emulsification-diffusion technique is performed by using 1,2-diacyl-sn-glycero-3-phosphocholin (Cholin II) and chloroform as lipophilic surfactant and partially water-miscible organic solvent, respectively, to form a reversed w/o emulsion. Sodium alginate is adopted as a model polymer and lyophilized with

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Table I. Characteristics of Organic Solvents Used in Emulsification-Diffusion Method

Solvents	Emulsion System	Solubility in Water[a] (g/100 mL)	Density[b] (g/cm^3)	Viscosity[b] (cP)	References
Ethyl acetate	o/w	8.3	0.898	0.426	[8-12]
Propylene carbonate	o/w	8.3	0.897	0.426	[9]
Chloroform	w/o	0.8	1.480	0.550	[33]

[a]Measured at 20 °C. [b]Measured at 25 °C.

divalent calcium ions.

Experimental

Materials. Sodium alginate and lipophilic surfactant Cholin II were purchased from Junsei Chemical (Osaka, Japan) and Sigma (St. Louis, MO), respectively. Duksan Pure Chemical (Gyeonggido, Korea) supplied chloroform. Calcium chloride ($CaCl_2$) was purchased from Dong Yang Chemical (Seoul, Korea) and used as cross-linking and/or lyophilizing agent for sodium alginate. Distilled water was of Milli-Q quality (Millipore, USA). All the reagents were of either HPLC grade or American Chemical Society analytical grade.

Alginate Nanohydrogel Preparations. 20 mL of sodium alginate aqueous solution was saturated with equivalent amount of chloroform dissolving Cholin II to reach the state of thermodynamic equilibrium. W/o emulsion was prepared by agitation with a high speed homogenizer (UltraTurrex T24, IKA Labotecnik). In order to allow for diffusion of water into oil phase, 100 mL of chloroform was subsequently added to this w/o emulsion under the moderate magnetic stirring. To ensure the sufficient diffusion of water and, as a result, solidification of alginate nanohydrogel (ANH), this w/o emulsion was left at room temperature for 3 days. Detailed experimental conditions are presented in Table II.

100 mL of $CaCl_2$ aqueous solution (20 wt%) was then added to lyophilize the sodium ANH. Crosslinked ANPs were water insoluble and separated by ultracentrifugation for 10 min at 4 °C and 8,000 rpm by using a high speed refrigerated centrifuge (VL-2400, Vision Scientific). Separated calsium ANHs were isolated and washed thoroughly with distilled water; in detail, chloroform phase was removed from the water phase containing calcium ANHs followed by addition of excess water and ultracentrifugation as described above. These purification processes were repeated for 3 times. Freeze-drying of ANH was performed by using a freeze dryer (FreeZone 2.5, Labconco) at -50 °C under 0.1 mbar of pressure for a day.

Table II. Experimental Conditions for the Preparation of ANH

	Conditions
Concentration	
Sodium alginate (in water) (w/v%)	0.5; 1.0; 1.5
Cholin II (in chloroform) (w/v%)	0.5; 1.0; 1.5
Emulsification Conditions	
Agitating speed (rpm)	13,000; 16,000; 19,000; 22,000
Duration (min)	5; 10; 20; 30

Characterization. The size and morphology of ANHs were analyzed by transmission electron microscopy (TEM) (Hitachi-7600, Hitachi) at an accelerating voltage of 100 kV. TEM samples were prepared by dipping 200 mesh carbon-coated copper grid into an aqueous suspension of ANHs and air dried for 24 h prior to analysis. The mean particle diameters and their distributions of the ANHs were also determined using dynamic light scattering (DLS) (ELS-800, Photal Otsuka Electronics) equipped with vertically polarized light supplied by He-Ne laser, operated at 10 mW in room temperature. Water absorbance and saturation of ANHs were evaluated by the degree of swelling (DS) defines as follow;

$$DS(\%) = \frac{d - d_o}{d_o} \times 100$$

where d_o and d are initial and measured mean diameters of ANHs, respectively, obtained by DLS. To determine DS of ANH in water, freeze-dried ANHs were dispersed in water (2 mg/mL) and agitated with magnetic stirrer mildly for a week. DLS measurements were performed with time interval of a day. Before each analysis, the samples were dispersed in water and sonicated in an ultrasonicator bath for about 10 min. All the measurements were repeated three times.

Results and Discussion

Up to now, polymeric nanoparticles prepared from emulsification-diffusion method have been limited to the hydrophobic aliphatic polyesters due to the mechanism itself; diffusion of organic solvents from core of the micelle into outer water-rich medium. In this study we reversed the o/w system to w/o one to prepare hydrophilic polymeric nanoparticles, i.e., hydrogels. Firstly we adopted ethyl acetate and propylene carbonate as organic solvents for dispersion medium. Unfortunately, we couldn't obtain any nanoparticles by this modification; alginate micelles were completely collapsed during diffusion of water and sponge-like resultants were obtained. It was ascribed to the high solubilities of these organic solvents in water[33] which gave reason to the breakdown of the walls composing w/o micelles. To achieve

Table III. Mean Particle Size of ANH

Specimen Code	Conc. (w/v%)		Stirring Condition		Mean Particle in Water	Size (nm) in Ethanol
	Alginate	Cholin II	Speed (rpm)	Time (min)		
ANH-1	0.5	0.5	13,000	10	171.70	49.2
ANH-2	1.0	0.5	13,000	10	181.95	57.8
AMH-3	1.5	0.5	13,000	10	281.11	-
ANH-4	0.5	1.0	13,000	10	149.82	-
ANH-5	0.5	1.5	13,000	10	210.92	-
ANH-6	0.5	0.5	16,000	10	144.77	-
ANH-7	0.5	0.5	19,000	10	129.06	-
ANH-8	0.5	0.5	22,000	10	105.78	-
ANH-9	0.5	0.5	13,000	5	197.79	-
ANH-10	0.5	0.5	13,000	20	147.37	-
ANH-11	0.5	0.5	13,000	30	154.09	-

stable diffusion of water from micelle, chloroform was selected as an oil phase which showed 10 times less solubility in water.[33] Characteristics of organic solvents used in emulsification-diffusion methods were compared with those of chloroform in Table I. Concentration of surfactant was fixed to 0.5 w/v%. Because chloroform is much heavier than water, however, conditions for preparing ANHs were strictly limited as shown in Table II.

Table III shows the mean particle sizes of ANHs of this study. Dependence of mean particle size of ANH on alginate concentration can be deduced by comparing ANH-1, ANH-2, and ANH-3. These nanohydrogels were prepared with fixed surfactant concentration in chloroform, homogenizing speed, and emulsification time of 0.5 w/v%, 13,000 rpm, and 10 min, respectively. With increasing the concentration of alginate, the mean particle sizes of ANHs increased and their distributions were broadened. Actually there was not a significant difference of mean particle sizes and their distributions between 0.5 and 1.0 w/v% of alginate concentrations; however, when concentration of alginate aqueous solution reached 1.5 w/v%, there was a huge increase in mean particle size and broadening in its distribution. This result can be simply ascribed to the lack of surfactant comparing with the encapsulated sodium alginate; deficiency in surfactant leads to the larger micelles and, as a result, enlarged size of nanoparticle. Furthermore this enhanced size can restrict the water drainage from the micelle at diffusion process which is one of the reasons for particle size enhancement. For these DLS results, it should be mentioned that the sizes of ANHs obtained by TEM observation are much smaller as shown in Figure 1; this is attributed to the agglomeration of ANHs in water phase due to their water-swelling characteristics and interparticle crosslinking of nanohydrogels. When mean particle size of these ANHs were measured by

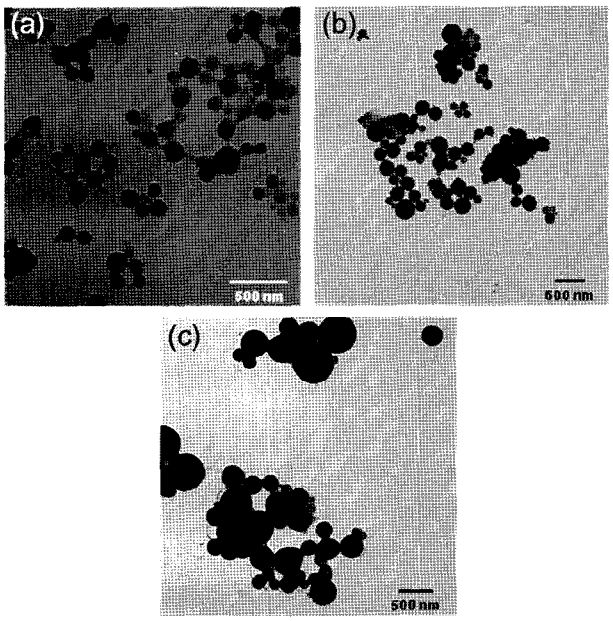

Figure 1. TEM photographs of ANHs: (a), ANH-1; (b), ANH-2; (c) ANH-3.

LS in ethanol phase for preventing water adsorption, we could obtain the values of 49.2 and 57.8 nm for 0.5 and 1.0 w/v% cases, respectively.

Effects of surfactant concentration on the mean particle size of ANHs were evaluated from the DLS results of ANH-1, ANH-4, and ANH-5, as shown in Table III. The mean particle diameters of ANHs decreased from 171.7 to 149.8 nm with increasing surfactant concentrations from 0.5 to 1.0 w/v%. ANH prepared at 1.5 w/v% of surfactant concentration showed some increase in the mean particle diameter of 210.9 nm. This can be ascribed to the formation of

vesicles due to the exceeding Choline II which takes after the structure of phosphoglyceride composing cell membranes.[34,35] Possibility of subsistence of vesicles even after freeze-drying was presumed by the traces of blank particles as shown in Figure 1(c). These is ascribed to the formation of vesicles that has not enough alginate polymers to form the solid nanoparticles. TEM image of Figure 1(c) was taken after freeze-drying followed by dispersion in water with sonicator for 10 min.

As mentioned above, chloroform is much less miscible with water than ethyl acetate and propylene carbonate. Therefore we could not be sure that the thermodynamic equilibrium between water and oil phases was achieved by emulsification-diffusion process in our work. With the settled conditions for concentrations of alginate and Cholline II, agitating speed and time will be the main parameters for emulsification-diffusion technique. These parameters will affect on amount of remaining water and swelling characteristics of ANHs, as well as the mean particle sizes. Variation of mean particle size of ANHs due to the agitating speeds can be evaluated by comparing the results of ANH-1, ANH-6, ANH-7, and ANH-8; the mean particle diameters are diminished with increasing agitation speeds; 171.7, 144.8, 128.9, and 105.8 nm for 13,000, 16,000, 19,000, and 22,000 rpm of agitation, respectively. This result is coincided with the result of Kwon, *et al.*[9] which showed that the higher agitation speed guaranteed the smaller particles in emulsification-diffusion method.

Mean particle sizes of ANH-1, ANH-9, ANH-10, and ANH-11 were measured and compared to reason out the effects of emulsification time on particle sizes of ANHs. Generally enhanced agitation induced the smaller ANHs. As shown in Table III, however, after 20 min of agitation there were not any significant decreases in the mean particle diameters of ANHs. From this result, it is deduced that the emulsification of this study can be fully performed within 20 min.

Figure 2 shows the effects of alginate concentration on DS values of ANHs maintained in water phase. ANH prepared at 1.5 w/v% of alginate concentration shows rapid growth in diameter about 6.7 times, which means that ANH swells to over 300 times in volume by water adsorption. But this is not fully affordable because of the above mentioned agglomeration of ANHs which may be enhanced with swelling and/or interparticle crosslinking though we performed 10 min of vigorous ultrasonification with deep type ultrasonicator before each measurement. On the other hands, ANHs obtained at 0.5 and 1.0 w/v% of alginate concentrations show maximum DS values of 11.1 and 54.5%, respectively.

Figure 3 shows the effects of surfactant concentration on DS values of ANHs. Apart from the ANH prepared at 0.5 w/v% of surfactant which showed almost same diameter in water, specimens prepared at higher surfactant concentrations swelled to about 60 and 75 % of DS, respectively, after 7 days. Particularly, ANH prepared at equivalent concentration, 0.5 w/v%, of alginate and surfactant shows almost nonswellable characteristics in water. Effects of crosslinking density should also be considered; substitution of sodium ion into calcium to crosslink and lyophilize the water-soluble sodium alginate nanoparticle was performed at fixed $CaCl_2$ concentration for all specimens. Water swellabilities of ANHs will decrease with the increase of crosslinking density of alginate on the surface of ANHs. Therefore low DS value can be a proof of the existence of dense outer shell of ANHs due to the higher crosslinking density. From these

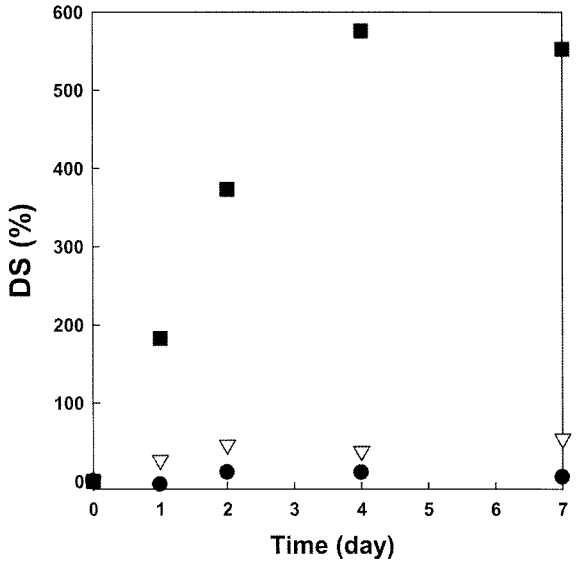

Figure 2. DS values of ANHs prepared at 0.5 w/v% of surfactant concentration in chloroform with varying alginate concentrations: ●, ANH-1; ▽, ANH-2; ■, ANH-3.

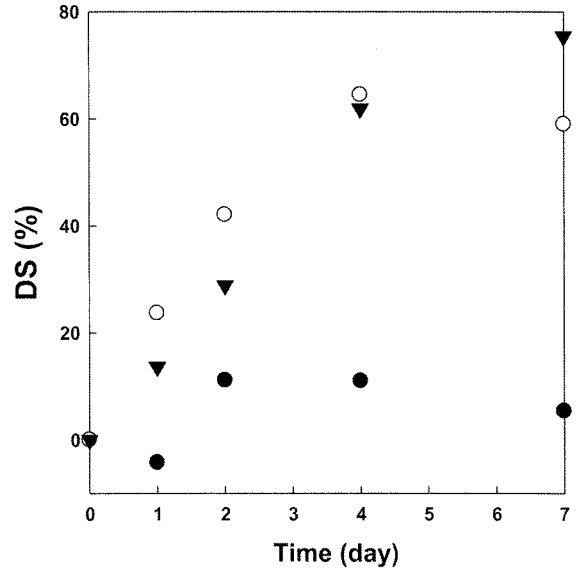

Figure 3. DS values of ANHs prepared at fixed concentration of alginate with varying surfactant concentrations: ●, ANH-1; ○, ANH-4; ▼, ANH-5.

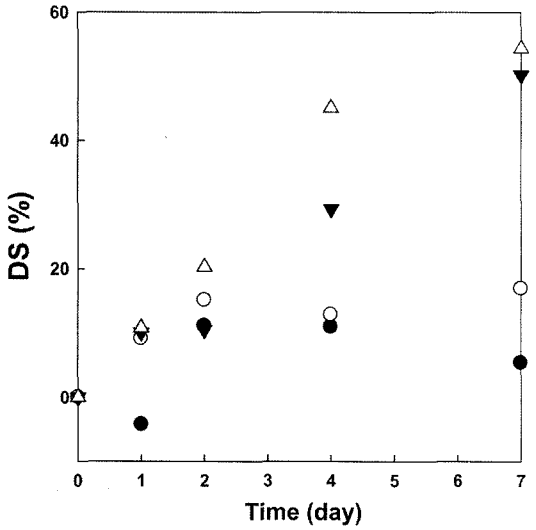

Figure 4. DS values of ANHs prepared at fixed concentrations of alginate and surfactant with varying agitation speeds; ●, ANH-1; ○, ANH-6; ▼, ANH-7; △, ANH-8.

results it is deduced that the water swellabilities of ANHs can be mainly restrained by controlling emulsion stabilities and surface crosslinking densities.

Effects of agitating speed on DS values were shown in Figure 4. DS values increased with increasing agitation speeds. However the diameters of ANHs after 7 days of swelling did not show considerable differences between specimens; 181.0, 169.4, 193.7, and 163.3 nm for 13,000, 16,000, 19,000, and 22,000 rpm of agitation, respectively. Furthermore, DS values of ANHs prepared at fast agitation had more risks of overestimation because of the smaller initial particle sizes. Similar particle sizes of ANHs after swelling can be explained if there are differences in water

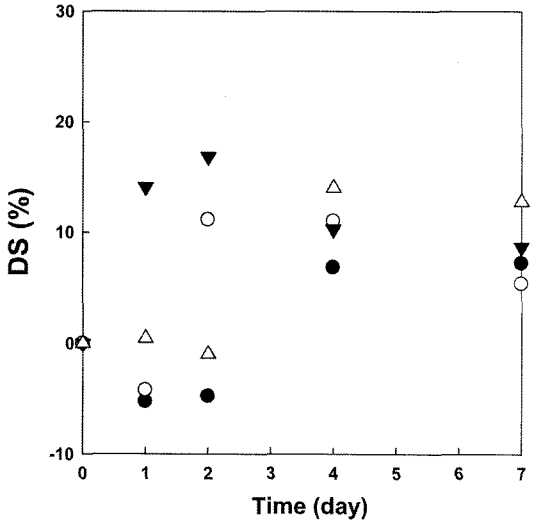

Figure 5. DS values of ANHs prepared at fixed concentrations of alginate and surfactant with varying agitation times: ● , ANH-9; ○, ANH-1; ▼, ANH-10; △, ANH-11.

contents of ANHs after emulsification. This indicates that diffusion of water will be accompanied with emulsification. Diffusion of water is accelerated at higher agitation speed and, as a result, ANHs prepared at rapid agitation have relative water deficiency in inner alginate-rich phase. If the ratios of alginate to surfactant are maintained for ANHs prepared at varying agitation speeds, ANHs will have similar mean particle diameters after saturation in water.

Figure 5 shows the emulsification time on DS values of ANHs. ANHs prepared at varying emulsification times showed almost no water absorbancy after 7 days of swelling. From these results, we can conclude that mechanical agitation cannot fully drain the water from the w/o emulsion and, as a result, prepared ANHs had limitation in additional water absorption during swelling.

Conclusions

It was shown that ANHs can be obtained by emulsification-diffusion method in w/o system by using lipophilic surfactant. To obtain the stable solid ANHs by freeze-drying, chloroform was adopted as an oil phase. Effects of alginate and surfactant concentrations, agitation speed, and agitation time were evaluated by the mean particle diameter, size distribution, and DS values obtained from DLS analysis. Swellability of ANH can be controlled by preparation condition. From the DLS analysis data, it can be concluded that swellability of ANH is mainly affected by alginate to surfactant ratio and remaining water contents. The mean particle diameters and DS values of ANHs increase with increasing alginate to surfactant ratio and with decreasing agitation speed. Swellabilities of ANHs are strictly restricted by the alginate to surfactant ratio and water contents and not by agitation time. Particularly ANHs with minimized water absorbancy, i.e., with relatively high degree of dimensional stability in water, seem to be effective for sustained-releasing in water-soluble drug and protein delivery.

Acknowledgment. This work was supported by grant No. R01-2006-000-10232-0 from the Basic Research Program of Korea Science & Engineering Foundation.

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