Biodegradation and Drug Release of Chitosan Gel Beads in Subcutaneous Air Pouches of Mice

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Chitosan (CS) gel beads were prepared in 10% amino acid solution (pH 9) and implanted into air pouches (AP) prepared subcutaneously on the dorsal surface of mice. No inflammatory response was observed, and degradation of the beads in the AP increased as their degree of deacetylation decreased. Degradation could be altered by changing the nature of the CS or by increasing the CS concentration. The release of prednisolone (PS) *in vivo* from CS gel beads was similar to the release *in vitro*. When a suspension of PS was injected into the AP, the PS had almost completely disappeared 24 h after injection. Retention of PS in the AP was not increased by using a viscous CS solution. Alginate (Alg) gel beads, which were not degraded, released PS slowly into the AP over 3 d. The *in vitro* release profile of PS using 1% CS (deacetylation: 70% (7B) and 80% (8B)) and 1.5% CS (deacetylation: 90% (9B)) gel beads was similar to that with Alg gel beads. However, the *in vivo* release of PS was affected by the degradability of the gel beads. CS7B and 8B (1%) gel beads had released PS into the AP earlier than 3 d according to their rate of degradation. CS9B (1.5%) gel beads were not degraded after 3 d and went on to release PS into the AP for 3 d similar to the release profile of Alg gel beads. CS9B (2%) gel beads were also not degraded after 3 d and the release of PS from these beads into the AP was sustained; 76% and 27% of administered PS remained in the gel beads after 1 and 3 d, respectively. Therefore, degradation and drug release of CS gel beads can be controlled by changing the structure of the gel matrix, which appears to make these beads a promising biodegradable vehicle for sustained drug delivery.

Key words chitosan; biodegradation; sustained release; gel; amino acid; air pouch

The polysaccharide chitosan (CS) is known to be an excellent material for drug preparation. CS is a plentiful natural biopolymer and is non-toxic, biocompatible and biodegradable.[1—3] These properties are important for materials that are implanted in the body, because such materials must avoid the host's defense system during their long-term contact with living structures. CS has been investigated as a unique vehicle for the sustained delivery of drugs,[4,5] in particular, the preparation of CS microspheres has been studied.[5—7] We investigated the preparation of a suitable vehicle, such as microspheres, for intra-articular injection in rheumatoid arthritis to allow sustained drug delivery. Lu *et al.*[8] reported that CS could act on the epiphyseal cartilage and augment wound healing of articular cartilage. Therefore, it might be useful in healing wounds in articular cartilage after fulfilling a role as a vehicle for drug delivery. In a previous study, CS was found to form gel spheres at around pH 9 in amino acid solution, despite usually forming a gel in solutions with a pH >12.[9] This is thought to be due to coacervation. Preparations made at a lower pH are preferable due to their effect on the solubility or stability of the drug contained in the gel beads and on the tissue into which they are injected. Furthermore, the release of drug from CS gel beads could be controlled by the formation of a complex between chondroitin sulfate and CS.[10] Intra-articular injection of steroids is used commonly in the treatment of rheumatoid arthritis. Under such conditions, it is difficult to achieve a sustained intra-articular drug level on the basis of drug solubility. During *in vivo* degradation, drug release is governed by both diffusion and biodegradation of the matrix. It is necessary to clarify the relationship between *in vivo* biodegradability and drug release profiles of CS gel beads under different conditions (enzymes, dissolution medium, stirring strength, *etc.*), and those *in vitro*. In this study, this relationship was investigated by implanting novel CS gel beads into subcutaneous air pouches (AP) prepared on the dorsal surface of mice.

MATERIALS AND METHODS

Materials CS with varying degrees of deacetylation (70% (7B), 80% (8B), 90% (9B), 100% (10B)) was purchased from Katokichi Co., Ltd., Japan. Prednisolone (PS) and sodium alginate (300 cps) (Alg) were purchased from Nacalai Tesque Inc., Japan. The other reagents were obtained from Wako Pure Chemical Industries and Nacalai Tesque Inc., Japan.

Preparation of CS Gel Beads CS gel beads were prepared as follows: CS (1% w/w) was dissolved in 0.1 M acetate buffer (pH 4.5) and 1% PS was then added to the CS solution. One gram of this suspension, theoretically containing 10 mg of PS, was dropped slowly into 20 ml of 10% glycine solution (pH 9.0) using a pipette and left at room temperature for 25 min. Hydrogel beads were formed spontaneously. Dried gel beads were obtained by drying the hydrogel beads at 37 °C for 24 h in a dish, before holding them under vacuum in a desiccator in the presence of P_2O_5. The dried gel beads retained PS above 95% of the theoretical total amount and had a diameter of about 3.5 mm and glycin content of about 51% of their dry weight. The beads were then implanted into mice as follows: the dried beads were washed with distilled/demineralized water before implantation to remove glycine because of the effect of glycine on living tissue. The hydrogel beads retained PS to about 80% of the theoretical total amount and had a diameter of 4.3 mm and glycine content of $<1.56\ \mu$g (limit of detection). Glycine was detected by a modification of the method of Watanabe and Imai.[11]

Preparation of Alg Gel Beads Alg (1% w/w) was dis-

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Table 1. Relationship between CS Properties and Gel Formation

CS species	Degree of deacetylation (%)	Molecular weight[a]	Concentration of CS (%)			
			0.5	1.0	1.5	2.0
7B	70	2210000	− (66)	+ (610)	++ (8750)	++ (34940)
8B	80	2140000	− (52)	+ (400)	++ (3340)	++ (33600)
9B	90	900000	− (24)	− (170)	+ (740)	+ (1210)
10B	100	950000	− (9)	− (25)	− (21)	− (18)

a) Ref. 1. Gel formation: − no gelation; + gelation; ++ difficult to drop (high viscosity). ( ):Viscosity (cps), determined on CS solution at 37 °C using a B type viscometer (Tokyokeiki).

solved in distilled/demineralized water, and 1% PS was then added to the Alg solution. One gram of the suspension, theoretically containing 10 mg of PS, was dropped slowly into 0.1 M $CaCl_2$ using a pipette and left at room temperature for 1 h. Hydrogel beads were formed spontaneously and retained PS to about 90% of the theoretical total amount, with a diameter of about 4.0 mm. The hydrogel beads were then implanted into mice. Dried gel beads were useful from the point of view of preservation. However, dried Alg gel beads did not swell well, even after soaking in water. Thus, in the case of Alg, hydrogel beads were used for implantation.

Dissolution Test The release of PS from the various types of gel beads into 0.1 M phosphate buffer (pH 7.2) was determined. Dried CS gel beads, corresponding to 1 g of hydrogel, and 1 g of Alg hydrogel beads were added to 500 ml of dissolution medium in a JP XIII dissolution test apparatus (paddle method, 100 rpm, 37 °C). A 4 ml aliquot of the solution was removed periodically for analysis and replaced with 4 ml of the dissolution medium (pre-warmed to 37 °C) to maintain a constant volume. The absorbance of each sample was determined with a Hitachi model 200-20 spectrophotometer at 246 nm. All the dissolution tests were performed in triplicate.

***In Vivo* Degradation of CS Gel Beads** The biodegradation of CS gel beads implanted into mice and the *in vivo* release of PS were investigated as follows. Air (3 ml) was injected subcutaneously into the dorsal surface of mice (ddy, male, 5—6-weeks-old) to form AP. An oval AP was formed after an additional 1 ml of air was injected at 1 and 4 d. A grain of beads was implanted into the AP under anesthesia with ether 7 d after the first injection of air. The beads were retrieved from the AP after 1 and 3 d, and contents of the AP were collected by washing with 1 ml of physiological saline three times under anesthesia with pentobarbital sodium. The abdominal skin was then opened and blood was collected from the *vena cava caudalis*. The PS concentration in plasma and the amount of PS in the gel beads and AP were determined. The protein content of the AP was also determined by the method of Lowry.[12]

Determination of Plasma PS Concentration Blood samples were centrifuged at 3500 rpm for 5 min. Supernatant (100 μl) was then added to 500 μl of 20% trichloroacetic acid aqueous solution to remove protein, 200 μl of 4 μg/ml methyl *p*-hydroxybenzoate aqueous solution as internal standard, and 200 μl of distilled/demineralized water. The mixture was centrifuged at 3500 rpm for 5 min, and the supernatant was then loaded into a sample extraction product (OASIS HLB, Waters). The extracted sample was subjected to high-performance liquid chromatography (HPLC). A 20 μl aliquot of the sample was loaded onto a column (YMC-Pack Pro C18; 150 mm×3.0 mm), and eluted with 25% tetrahydrofuran (THF) aqueous solution as the mobile phase at a flow rate of 0.3 ml/min (Shimadzu LC-10AS). PS in the effluent from the column was detected at 246 nm with a UV spectrophotometer (Shimadzu SPD-10AVVP).

Determination of PS in the Gel Beads CS gel beads were dissolved in 2 ml of 0.1 M acetate buffer (pH 4.5), and Alg gel beads were dissolved in 2 ml of 1% tetra-sodium ethylenediaminetetraacetate. These samples were added to 50 μl of 200 μg/ml internal standard made up in 25% THF aqueous solution, and made up to 5 ml with 25% THF aqueous solution. The mixture was centrifuged at 3500 rpm for 5 min, and the supernatant was filtered (Cosmonice Filter S, 0.5 μm, Millipore) and subjected to HPLC as described above.

Determination of PS in the AP The AP contents were added to 50 μl of 200 μg/ml internal standard made up in 25% THF aqueous solution, and made up to 5 ml with physiological saline. The protein concentration was then determined by the method of Lowry using 350 μl of the mixture. One milliliter of the mixture was added to 1 ml of 50% MeOH and centrifuged at 3500 rpm for 5 min. The supernatant was filtered (Cosmonice Filter S, 0.5 μm, Millipore) and subjected to HPLC as described above.

RESULTS AND DISCUSSION

Preparation of CS Gel Beads Preparation of the CS gel beads was affected by the properties of the CS (Table 1). The optimum concentrations for the formation of gel spheres were 1% for both CS7B and CS8B, and 1.5% and 2% for CS9B. Lower concentrations of CS did not form gel spheres and higher concentrations were difficult to drop by pipette due to their high viscosity.

Evaluation of Biocompatibility The amount of protein in the AP 3 d after implantation of CS gel beads without PS was determined to evaluate the inflammatory response (Fig. 1). No increase in protein was observed in the AP compared with injection of 30 mg carrageenin with 1 ml of physiological saline which is known to induce inflammation. This may indicate that the CS gel beads were biocompatible.

***In Vivo* Degradation of CS Gel Beads** It is known that mucopolysaccharides are degraded by hydrolysis by enzymes

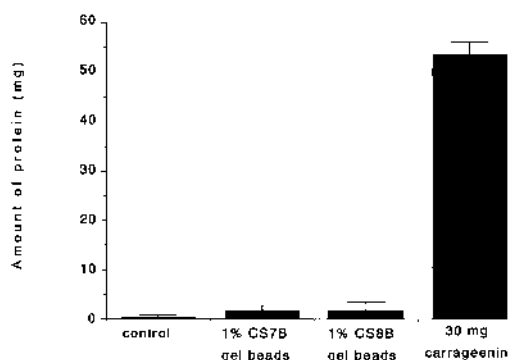

Fig. 1. Evaluation of Biocompatibility on Inflammatory Response 3 d after Implantation Determined from the Amount of Protein in the AP (n=3—8)

Control: only incision (no implantation).

such as lysozyme. In a previous study, CS gel beads were degraded in 0.1 M phosphate buffer containing lysozyme. CS7B gel beads began to degrade after 3 d, although with CS8B only slight degradation was noted after 28 d.[10] In this study, *in vivo* degradation of CS gel beads in the AP occurred faster than that *in vitro*. Degradation of the CS gel beads was accelerated as their degree of deacetylation decreased. Some CS7B gel beads in the AP were degraded within 1 d, although with CS8B degradation was not observed this quickly. Both CS7B and CS8B gel beads in the AP were degraded in less than 3 d after implantation, however Jameela and Jayakrishnan[5] described that CS (deacetylation: 74%) microspheres, cross-linked by glutaraldehyde, were not degraded 12 weeks after implantation in rat muscle. On the other hand, Tomihata and Ikada[3] reported that CS films, prepared by the solution casting method, were degraded within 4 weeks in the case of chitin and CS (deacetylation: 68.8%), and CS films which were >73% deacetylated were not degraded after 12 weeks subcutaneously on the dorsal surface of rats. Degradation may be affected by enzymes, body constituents, the conditions of application and the structure of the gel matrix, although the reasons for this are not clear. It was possible, however, to control the *in vivo* degradability of CS gel beads by considering the differences in degradability of the various types of CS with different degrees of deacetylation. CS9B (1.5%) gel beads were not degraded in the AP after 3 d. The degradability of CS gel beads could also be controlled by changing the CS concentration. CS9B (2%) gel beads degraded less than 1.5% beads after 3 d.

***In Vivo* Release of PS from CS Gel Beads** The *in vivo* release of PS from gel beads was evaluated by measuring the percentage of residual PS in the gel beads and AP compared with the amount administered. When 1% PS suspended in physiological saline was injected into the AP, PS disappeared immediately from the site and the residual amount was 0.2% after 24 h (Table 2). The plasma level also changed gradually according to the amount of PS in the AP, indicating that PS in the AP passed rapidly into the blood stream. This rapid disappearance was also recognized when PS was suspended in CS7B acetate buffer solution (pH 4.5) and injected into the AP. As shown in Table 3, the reduction in PS was faster because the CS solution was more viscous than saline. A similar result was obtained with CS8B solution. No retention of PS in the AP occurred even with viscous CS. These results

Table 2. Percentage of Residual PS in the AP after Injection of PS Suspension

After implantation (h)	Residual PS in the AP (%)	Plasma PS concentration (mg/ml)
1	19.7±2.8	2.3±0.5
3	7.2±2.8	1.2±0.3
5	2.4±2.9	0.3±0.1
24	0.2±0.1	N.D.

N.D.: not determined (percentage of residual PS in the AP <0.02%, plasma PS concentration <0.1 mg/ml). Residual PS in the AP (%): residual/administered. Data represent the mean±S.D. (n=5).

Table 3. Percentage of Residual PS in the AP after Injection of PS Suspended in CS Solution

After implantation (d)	CS species	Residual PS in the AP (%)	Plasma PS concentration (μg/ml)
1	7B	0.5±0.9	0.2±0.1
	8B	0.4±0.6	0.2±0.2
3	7B	N.D.	N.D.
	8B	N.D.	N.D.

N.D.: not determined (percentage of residual PS in the AP <0.02%, plasma PS concentration <0.1 mg/ml). Residual PS in the AP (%): residual/administered. Data represent the mean±S.D. (n=5).

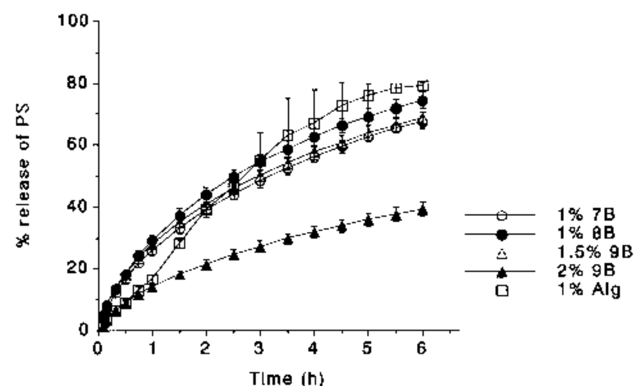

Fig. 2. The *in Vitro* Release Profile of PS from Gel Beads

show that when PS powder is dissolved in the exudate in AP it does not remain there for long. To reduce the side-effects and prolong the duration of activity of a drug it is therefore necessary to employ a vehicle to administer the drug. PS was released more gradually from Alg gel beads and little remained after 3 d, although the beads did not degrade over 7 d in the AP. Thus, control of drug release is as important as control of gel degradation. As shown in Fig. 2, 1% CS7B, CS8B and 1.5% CS9B gel beads showed similar PS release profiles to Alg gel beads. However, as shown in Table 4, the release of drug in the AP was affected by degradability of the CS gel beads. In the case of 1% CS7B gel beads, about 70% of the PS was released after 1 d, and all of the PS had been released from 1% CS7B and CS8B gel beads before 3 d as degradation accelerated. In contrast, release of PS from 1.5% CS9B gel beads in the AP was similar to that of Alg gel beads. In addition, the *in vivo* release of PS from CS9B gel beads reflected the *in vitro* release profile as there was no degradation. Changing the type of CS or increasing the CS concentration decreased the *in vivo* release of PS from the

Table 4. Effect of Gel Species on the Percentage of Residual PS in the Gel Beads

After implantation (d)	Gel species	Residual PS in the gel beads (%)	Residual PS in the AP (%)	Plasma PS concentration (μg/ml)
1	1% 7B	31.5±20.0	6.23±5.74	0.1±0.1
	1% 8B	52.3± 8.3	0.24±0.04	N.D.
	1.5% 9B	64.4± 5.7	0.48±0.71	N.D.
	2% 9B	76.5± 9.2	0.15±0.03	N.D.
	Alg	48.0±11.1	0.12±0.03	N.D.
3	1% 7B	Degraded	N.D.	N.D.
	1% 8B	Degraded	N.D.	N.D.
	1.5% 9B	1.3± 1.8	N.D.	N.D.
	2% 9B	27.2± 9.7	0.05±0.05	N.D.
	Alg	2.3± 2.1	0.03±0.04	N.D.

N.D.: not determined (percentage of residual PS in the gel beads and the AP <0.02%, plasma PS concentration <0.1 mg/ml). Residual PS in the gel beads and the AP (%): residual/administered. Data represent the mean±S.D. ($n=5$).

gel beads. The *in vivo* release of PS from 2% CS9B was sustained, and 76% and 27% of administered PS remained in the gel beads after 1 and 3 d, respectively.

These results show that PS release in the AP is governed by both diffusion and degradation of the gel matrix. The control of biodegradation and drug release from a vehicle will make possible the supply of the minimum requirement of a dose by local application, and may result in a prolongation of the duration of drug activity and a reduction of the side effects. In a previous study, control of drug release from CS gel beads *in vitro* was achieved by application of an electrostatic complex formed between chondroitin sulfate and CS. Degradation and drug release of the CS gel beads can be controlled by modifying the structure of the gel matrix. Thus, these beads appear promising as a biodegradable vehicle for sustained drug delivery.

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