Novel Preparation of Zein Microspheres Conjugated with PS-K Available for Cancer Immunotherapy

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Microspheres which entrapped PS-K were prepared using Zein as a carrier matrix by a one-step or two-step process in dimethyl sulfoxide–H_2O media. Microspheres prepared by the latter process provided satisfactory recovery and uptake of PS-K. Use of a catalytic amount of *dl*-camphorsulfonic acid, glutaraldehyde and rapid addition of aqueous solution of polyvinylpyrrolidone into the reaction media are crucial for the preparation of fine and mono-dispersed microspheres. The release rate of PS-K could be controlled by altering the ratio of PS-K to Zein and the conditions of the medium. In the presence of actinase E, drug release was considerably increased to 70—80% after 24 h incubation. Sonication of the aggregated microspheres readily yielded a mono-dispersed preparation with a particle diameter of less than 1 μm, which would be a suitable size for phagocytosis by macrophages.

Keywords PS-K–Zein microsphere preparation; cancer immunotherapy; biodegradable Zein microsphere; PS-K colorimetric determination; macrophage phagocytosis

In an attempt to improve the low therapeutic index of cytotoxic drugs by targeting, a variety of drug–carrier complexes have been investigated. These include natural or synthetic macromolecules and particles such as globulin, deoxyribonucleic acid (DNA), SMA (polystyrene maleic acid anhydride), liposomes, microspheres and microcapsules with various constituents and sizes.[1] The same approach could be applied to biological response modifiers (BRMs). Fidler *et al.*[2] demonstrated that spontaneous lung metastases in a mouse tumor model were significantly inhibited by intravenous injection of liposome-entrapped lymphokines as compared with the non-encapsulated drugs. Ikada and Tabata[3,4] also found that protein-grafted cellulose microspheres containing muramyl dipeptide provided a much higher activation of macrophages than that obtained with the original dosage form of the drug. A protein polysaccharide, PS-K (Kureha Chemical Co., Ltd., Tokyo), extracted from *Basidiomyces*,[5] has been shown to exert antitumor effects by potentiating the host immune systems.[6] Currently PS-K is widely used as a BRM in the treatment of gastrointestinal or lung cancer by oral administration, giving some benefical effects in terms of patients' survival.[7,8] Modification of the dosage form is expected to increase the therapeutic effect of this drug. However, the preparation of BRM–carrier complexes usually requires complicated and subtle manipulations, often resulting in a low recovery rate of drugs and instability of the products. Herein we report a novel and simple preparation of PS-K microspheres. This method should be applicable to both hydrophilic and hydrophobic drugs with high yields.

Experimental

Materials PS-K was kindly supplied by Kureha Chemicals Industries, Ltd., Japan. Actinase E was purchased from Kaken Pharmaceutical Co., Ltd., Japan. Zein, 20% glutaraldehyde (GA), dimethyl sulfoxide (DMSO), polyvinylpyrrolidone (PVP), and *dl*-camphorsulfonic acid were purchased from Katayama Industries, Ltd., Maruishi Pharmacy, Ltd., Wako Pure Chemical Industries, Ltd., Japan, Nakarai Chemicals Ltd., Japan, and Aldrich Chemical Company, Inc., U.S.A., respectively.

Preparation of PS-K–Zein Microspheres Microspheres were prepared by a high dilution process at ambient temperature. Representative procedures were as follows.

Method Ia—d (One-Step Preparation) A mixture of 100 mg of PS-K and 200 mg of Zein in 5 ml of DMSO was heated at 80 °C for 5 min in order to allow the mixture to dissolve. The amount of 20% (w/w) aqueous glutaraldehyde indicated in Table I was added and the mixture was stirred for 5 or 30 min. Then 100 ml of 2% (w/w) aqueous solution of PVP was poured all at once into the above solution and the whole was vigorously stirred for 24 h. The resulting emulsion was centrifuged at 10000 rpm for 20 min and the supernatant was discarded. The obtained microspheres were washed three times with distilled water. The pellet was resuspended. Ultrasonication at 35 W followed by lyophilization of the pellet provided PS-K–Zein microspheres (Table I).

Method IIa—c (Two-Step Preparation) PS-K (100 mg) in 5 ml of DMSO was heated at 80 °C for 5 min and then 20% (w/w) aqueous glutaraldehyde as indicated in Table I was added. In method IIc, a catalytic amount of *dl*-camphorsulfonic acid was added with glutaraldehyde in order to enhance the rate of cross linkage between PS-K and Zein. The reaction mixture was stirred for 2 h, 200 mg of Zein was added, and the mixture was stirred for an additional 1 h. Exactly the same work-up as that of method I provided the microspheres (Table I).

Drug Release Test from Zein Microspheres *in Vitro* PS-K–Zein microspheres prepared according to method IIc were used in a drug release test. Zein microspheres (5 mg) were placed in a glass tube containing 5 ml of JP XI first solution (pH 1.2), JP XI second solution[9] (pH 6.8) or phosphate buffer (pH 7.2) containing actinase E (10^6 units/g, 0.017 mg/ml and 0.33 mg/ml) respectively. The mixture was shaken 100 times/min at 37 °C and then centrifuged at 3000 rpm for 5 min at 0.5, 1, 2, 3 or 24 h. PS-K released from microspheres was determined photochemically using the following analytical method.

Colorimetric Determination of PS-K in Microspheres Photospectrometric determination of drugs in biodegradable protein microspheres is usually difficult due to the interference of degradation products of the matrix produced by protease, or protease itself. It is known that a variety of sugars are easily determined spectrophotometrically by means of the phenol-sulfuric acid procedure.[10] Application of this method to the

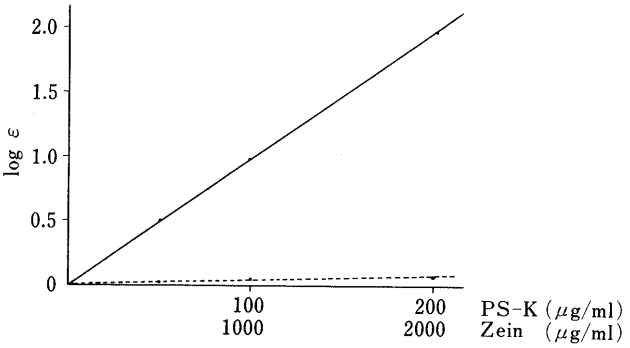

Fig. 1. Standard Curves of PS-K and Zein at 490 nm

●—●, PS-K, $y=0.009506x+0.0213$ ($r=0.9998$); ●---●, Zein, $y=0.06x+0.005$ ($r=0.9999$).

PS-K–Zein microspheres turned out to be useful for the quantitative analysis of PS-K.

Procedure Phenol (0.05 ml, 80% (w/w)) was added to a stirred suspension of 2 mg of the microspheres in 2 ml of distilled water and then 5 ml of concentrated sulfuric acid was added rapidly. The microspheres readily dissolved. The mixture was allowed to stand for 10 min, then shaken and placed for 20 min in a water bath at 30 °C before readings were taken. The absorbance of the characteristic color was measured at 490 nm.

Standard Curve Typical standard curves of PS-K and Zein at 490 nm are shown in Fig. 1. These standard curves clearly indicate that the absorption of Zein does not affect the colorimetric determination of PS-K at 490 nm.

Results and Discussion

As can be seen in Table I, satisfactory results were obtained by use of the two-step process (method II). Best results in terms of the diameter of microspheres were obtained by addition of a catalytic amount of *dl*-camphorsulfonic acid to the reaction medium. This may influence the rate of the cross linkage between PS-K and Zein to allow the formation of rigid microspheres which do not undergo aggregation. Consequently, PS-K-containing microspheres with a diameter of less than 1 μm were obtained.

Characterization of PS-K–Zein Microspheres Prepared at Various Ratios of PS-K and Zein

As can be seen in Table II increase of the ratio of Zein to PS-K resulted in a linear increase of the recovery of PS-K up to two-fold. This indicates that use of a three-fold amount of Zein should provide almost quantitative recovery of PS-K; the result actually obtained was 71.5%, and this is satisfactory. The best result was obtained at the ratio of Zein to PS-K of 2 : 1 as regards the uptake of PS-K into the microspheres.

Physical and Chemical Properties of PS-K–Zein Microspheres

Though the particles after lyophilization are considerably aggregated, sonication in distilled water readily gave a mono-dispersed preparation. The mono-dispersed and aggregated forms were observed by scanning electron microscopy (Fig. 3). The former consisted of regular spheri-

TABLE I. Characterization of PS-K–Zein MS Prepared under Various Conditions

	Methods						
	I (One step)				II (Two steps)		
	a	b	c	d	a	b	c
20% GA (ml)	0.2	0.2	0.4	0.4	0.1	0.2	0.2[b]
Reaction time	5 min	30 min	5 min	30 min	2 h	2 h	2 h
Weight of MS (mg)	167	244	231	235	185	203	199
Recovery of PS-K (%)	7.0	18.3	13.8	14.1	65.9	68.1	68.7
Uptake of PS-K into MS (%)[a]	4.2	7.5	6.0	6.0	35.6	33.6	34.5

a) $\frac{\text{PS-K (mg) incorporated into MS}}{\text{MS (mg)}} \times 100$. *b*) *dl*-Camphorsulfonic acid (10 mg) was added into the reaction mixture.

TABLE II. Characterization of PS-K–Zein Microspheres Prepared at Various Ratios of PS-K and Zein

Ratio of Zein to PS-K	Yield of MS (%)	Recovery of PS-K (%)	Uptake of PS-K into MS[a] (%)
0.5	63.7	27.3	28.6
1	94.5	38.5	20.4
2	66.3	68.7	34.5
3	98.3	71.5	18.2

a) $\frac{\text{PS-K (mg) incorporated into MS}}{\text{MS (mg)}} \times 100$.

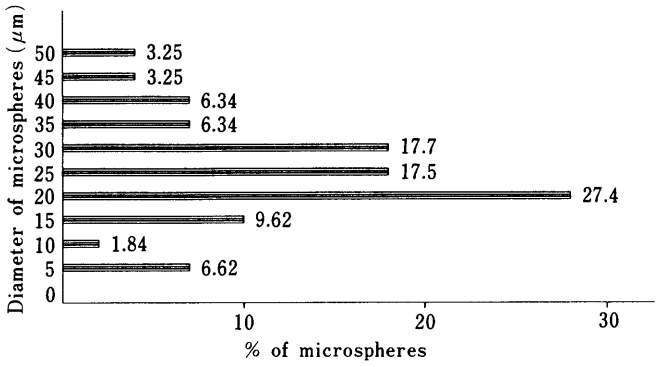

Fig. 2. Particle Size and Distribution of Aggregated Form of PS-K–Zein Microspheres

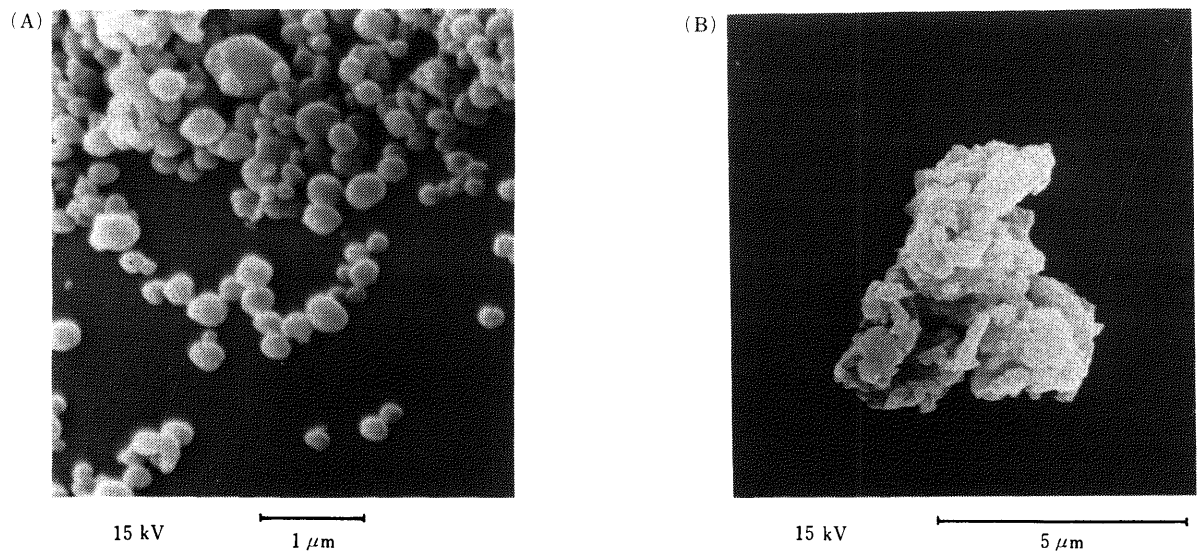

Fig. 3. Scanning Electron Microphotographs of PS-K–Zein Microspheres of Mono-dispersed Form (A) and Aggregated Form (B)

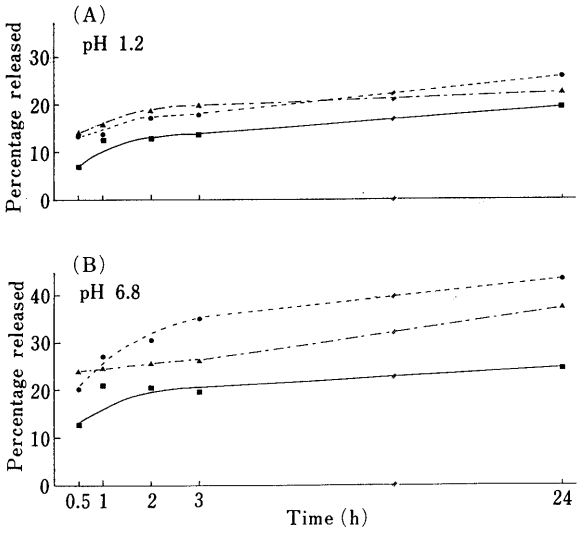

Fig. 4. PS-K Release from PS-K–Zein Microspheres in JP XI First Solution (A) and JP XI Second Solution (B)

●---●, PS-K : Zein = 1 : 1; ▲---▲, PS-K : Zein = 1 : 2; ■—■, PS-K : Zein = 1 : 3. The mixture was shaken 100 times/min at 37 °C.

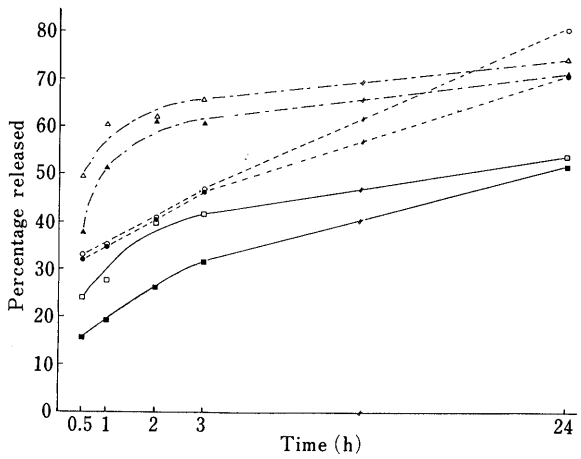

Fig. 5. PS-K Release from PS-K–Zein Microspheres in the Presence of Actinase E

Actinase E (0.017 mg/ml): ●---●, PS-K : Zein = 1 : 1; ▲---▲, PS-K : Zein = 1 : 2; ■—■, PS-K : Zein = 1 : 3. Actinase E (0.033 mg/ml): ○---○, PS-K : Zein = 1 : 1; △---△, PS-K : Zein = 1 : 2; □—□, PS-K : Zein = 1 : 3. Phosphate buffer (pH 7.2) was used in this experiment and the mixture was shaken 100 times/min at 37 °C.

cal particles with a diameter of less than 1 μm, appropriate for phagocytosis by macrophages, while the latter consisted of rough and invaginated particles. The particle size of the aggregated form measured with micron photo sizer ranged from 5 to 50 μm as shown in Fig. 2.

The slow release property of the microspheres was determined under various conditions. Extremely slow release and low cumulative amounts of PS-K in pH 1.2 solution turned out to be due to poor solubility of PS-K in acidic media, while the amount of release of PS-K at pH 6.8 increased by about 10% at each time as shown in Fig. 4. These results indicate that the release rate can be controlled by altering the ratio of PS-K to Zein and the enviromental conditions, and that PS-K in microspheres is released according to first-order kinetics through the semipermeable Zein-microspheres. The amounts of PS-K released from microspheres considerably increased in the presence of actinase E. The reason for the relatively slow release of PS-K from microspheres prepared at the ratio of PS-K–Zein of 1 : 1 may be the strong linkage of microspheres due to the large amount of glutaraldehyde present in the reaction medium when those compared to other conditions. The almost zero-order kinetics of these microspheres and the similarity of the release patterns suggest that the concentration of actinase E has little influence up to 3 h, presumably owing to the strong cross-linkage, but becomes important thereafter, presumably as the microsphere structure deteriorates increasingly. (Fig. 5)

The procedure described herein is entirely different from the previous procedures, and should be generally applicable to many drugs. In fact, we have already succeeded in the preparation of microspheres entrapping some antitumor drugs such as peplomycin, daunomycin, and mitomycin C as representatives of highly soluble, fairly soluble, and poorly soluble materials in H_2O, respectively.

Microspheres prepared by this procedure might be suitable for use as a sustained-release form, and the monodispersed microspheres were of adequate size for phagocytosis by macrophages. Microspheres with a diameter of 15—50 μm could also be useful for selective immunochemoembolization. Preliminary examination of the activity of PS-K–Zein microspheres on sarcoma 180 in ICR mice indicated a significant life-prolonging effect even at a thousandth of the usual dosage; these results will be described elsewhere.

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