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Development of Specific Organ Targeting Drug Delivery System II: Physico-pharmaceutical study on the cross-linked albumin microspheres containing cytarabine

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Abstract□Bovine serum albumin microspheres containing cytarabine were prepared using cross-linking agent, formaldehyde. The shape and the size distribution of them were observed. The shape of them was spherical and the surface was compact and smooth. The size distribution of them was affected by dispersion forces during emulsification. The release of cytarabine from albumin microspheres was dependent upon cross-linking time, amount of cross-linking agent and drug/albumin ratio. However, the difference of drug release by the dispersion forces was not great. After release test, the shape of albumin microspheres was non-spherical and the albumin matrix seemed to be a little relaxed. The degradation tests of albumin microspheres by the proteolytic enzyme showed that albumin microspheres were progressively digested according to the cross-linking degree.

Keywords□Preparation of albumin microspheres, Drug release from albumin microspheres, Cytarabine, Formaldehyde, Size distribution Dispersion forces, Cross-linking time, Cross-linking agent.

It has been reported that albumin microspheres are chemically and physically stable, non-immunogenic[1] and biodegradable,[2] amenable to preparation in large batches and capable of accomodating a wide variety of water-soluble drug molecules in relatively nonspecific fashion.[3] And it has been investigated that microspheres are rapidly removed from the vascular system by phagocytosis,[4] preferentially localized in the reticuloendothelial system and found within the cytoplasm of tumour cells.[5] Several research groups investigated albumin microspheres as the prominent drug carrier in the treatment of cancer and fungal or bacterial infestations.[5-8]

Albumin microspheres were currently prepared by either thermal denaturation at elevated temperature or chemical cross-linking in vegetable oil or isooctane emulsions.[9-13] The low temperature conditions employing either cross-linking agent or no deliberate denaturating step at all would permit encapsulation of many temperature-sensitive drugs.

In this study, bovine serum albumin microspheres containing cytarabine were prepared by the cross-linking method. The shape and surface characteristics of albumin microspheres were determined by scanning electron microscopy, size distribution and drug release behavior were investigated. The degradation of albumin matrix by the proteolytic enzyme was studied according to the cross-linking degree.

EXPERIMENTAL METHODS

Materials

Cytarabine (assay: 98.7%) was supplied by Choong Wae Pharm. Co.. Bovine serum albumin (BSA), Fraction V was purchased from Sigma Co.. Cottonseed oil (Kokusan Chem. Work Ltd.) was used. Protease was supplied by Dong-A Pharm. Co.. Formalin 40 (minimum 37% solution of formaldehyde) of reagent grade was used as the cross-linking agent and pH 7.4 phosphate buffered saline (PBS) solution was used as a medium for release test. All other chemicals used were of reagent grade. Membrane filter (Millipore GS 0.22 μ) and Toyo membrane filter (Type NC, pore size 0.2 μ) were used.

Apparatus

Scanning electron microscope (SEM: JEOL JSM-35) was used for the observation of the shape and size of albumin microspheres. Cytarabine was analyzed by UV spectrophotometers (LKB and SP1750 Pye Unicam). Motor-driven galss stirrer was used in the preparation and the voltage was fixed constantly by automatic

voltage stabilizer (NHS3000). The rpm was measured by hand tachometer (Fuji Kogyo Co., Ltd). Sonicator (Branson Cleaning Equipment Co.) for suspending microspheres and centrifuge (Kokusan, Type H-36A) were used in the preparation.

Preparation of Cross-linked Albumin Microspheres

Albumin microspheres containing cytarabine were prepared by the modified emulsion polymerization method.[14] Fig. 1 shows the schematic diagram of pre paration of crossed-linked albumin microspheres. Stabilization of albumin matrix was accomplished by the cross-linking with carbonyl compound in an ether phase reaction.

An aqueous solution was prepared containing 250mg of BSA and 50mg of cytarabine per milliliter. 0.8ml aliquot of this solution was added to 60 ml of cottonseed oil and constantly stirred (1200, 1800, 2500 rpm) for 10 min by motor-driven glass stirrer. The resultant emulsion was added dropwise into 70ml of cottonseed oil with continuous stirring and stirring was maintained for 10 more min. The albumin microspheres were then washed free of oil by adding anhydrous ether, centrifuging for 15 min at 4,000 rpm and decanting the supernate of centrifuge.

And the microspheres were resuspended in ether containing formaldeyde* as the cross-linking agent and stirred for certain period (5, 15, 30 min) with fixed rpm. Excess cross-linking agent was removed by decanting the supernate of centrifuge at 2000 × g for 5 min. The microspheres were washed four times in this manner and subsequently dried in a vacuum desiccator.

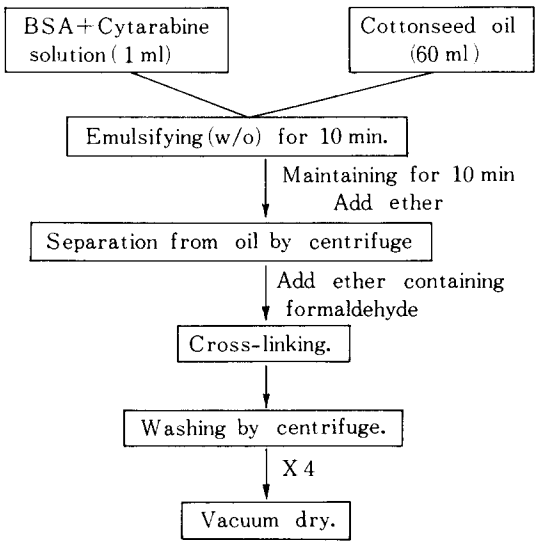


Fig. 1. Schematic diagram of preparation of cross-linked bovine serum albumin microspheres.

Determination of Morphology and Size Distribution

Dried albumin microspheres were coated by gold with an ion-coater (250-300Å, Ion Sputtering), and observed with scanning electron microscopy. Then, mean diameter, size distribution and its standard deviation were calculated.

Determination of Drug Released

pH 7.4 PBS solution was used as the drug release medium. 8 mg of dried albumin microspheres was added to 80 ml of PBS solution and agitated by magnetic stirrer at constant speed. At periodic time intervals for 48 hrs, 5 ml of this solution were removed and filtered through the membrane filter (pore size 0.22 μ). The amount of released cytarabine was assayed by UV-spectrophotometer at 274 nm.

Determination of Matrix Degradation in Proteolytic Enzyme Solution

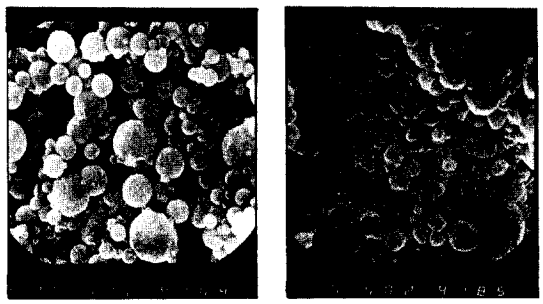
10mg of albumin microspheres was added to 100 ml of the PBS solution (pH 7.8) and ultrasonicated for 10 min in order to suspend. Then, 50mg of protease was added and incubated with slow stirring by magnetic bar at 37 ± 2°C.

At periodic time intervals, 5 ml of the solution containing albumin microspheres was removed and its turbidity was measured at 500 nm by UV-spectrophotometer.

RESULTS AND DISCUSSION

Physical Shape and Size Distribution

The geometry of albumin microspheres was spherical and the surface of them was compact and smooth as shown in Fig. 2A. It is probably due to the prepara-



(A) (B)

Fig. 2. Scanning electron microphotograph of cross-linked bovine serum albumin microspheres prepared at 1,200rpm(A) and 2,500rpm(B) (x 6,000).

*Prepared by the vigorous shaking for 10 min with 1:5 solution of formalin-ether with the addition of a saturating amount of ammonium sulfate.

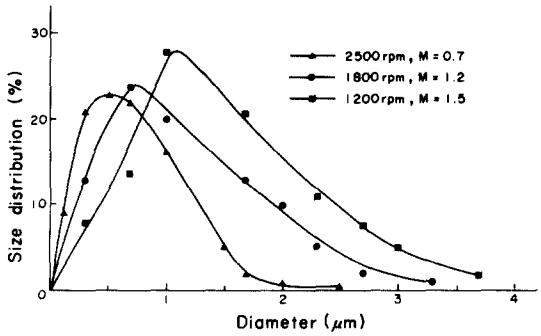


Fig. 3. Effect of dispersion force on the size distribution of bovine serum albumin microspheres.

tion procedure in which the spherical shape of the aqueous phase of w/o emulsion was formed with constant stirring and became firm matrix by means of adding the cross-linking agent. Cross-linking time, concentration of cross-linking agent, albumin concentration affected the geometry of micropheres. Excess amounts of cross-linking agent resulted in the aggregation of albumin microsphere. Also, excess dispersion force (2500 rpm) during the preparation caused the microsphere to form nonspherical and irregular shape as shown in Fig. 2B.

The effect of three dispersion forces on the size distribution of microspheres is shown in Fig. 3 and Table I. As the dispersion force was increased, the size distribution became narrower and the mean diameter became smaller. It is assumed that their size distribution depended mainly on the size distribution of albumin droplets dispersed in the oil phase during emulsification.

Drug Release and Degradation Behavior

The temperature of release medium (PBS solution) did not affect the drug release as shown in Fig. 4. Cytarabine was released during the first 10 minutes and was hardly released thereafter. The initial burst out is probably resulted from the released cytarabine loosely held on the surface of albumin microspheres. The difference in the release of cytarabine from albumin microspheres was negligible amount (0.1 μg) when it was most.

Table I. Size distribution of albumin microspheres versus dispersion force.

Dispersion force	Diameter, μm	
	Mean	S. D.
1200 rpm	1. 5	0. 809
1800 rpm	1. 2	0. 679
2500 rpm	0. 7	0. 429

All albumin microspheres were prepared with 25w /v% BSA solution and stabilized by the vigorous shaking for 10 min with 1:5 solution of formalin 40%-ether with the addition of a saturating amount of ammonium sulfate.

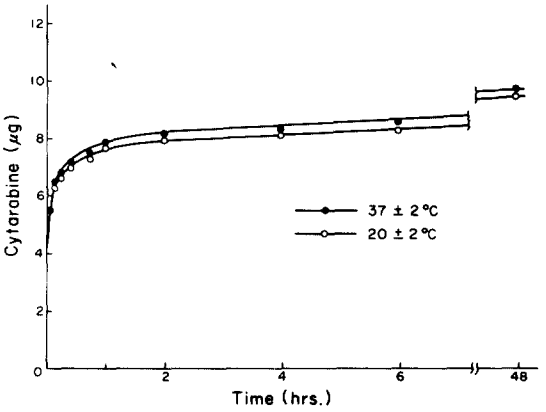


Fig. 4. Effect of temperature on the release of cytarabine.

Albumin microspheres prepared at 1,200rpm in Table I were tested.

The effect of size distribution by dispersion force on the release of cytarabine is shown in Fig. 5. The smaller the mean diameter was and the narrower the size distribution was, the greater the drug release was. The increase may be due to an increase of the total surface area as the mean size become smaller. But the release difference by dispersion forces was little.

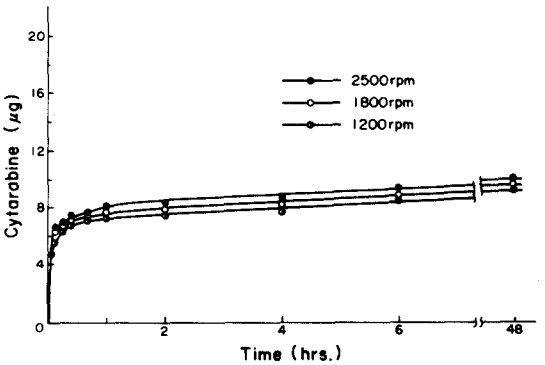


Fig. 5. Effect of dispersion force on the release of cytarabine.

Three different bovine serum albumin microspheres in Table I were tested.

The effect of cross-linking time and concentration of cross-linking agent on the cytarabine release is shown in Fig. 6. As the cross-linking time was longer and the formalin concentration was higher, the drug release was decreased. It may be due to the increased tightness of albumin matrix according to the cross-linking degree.

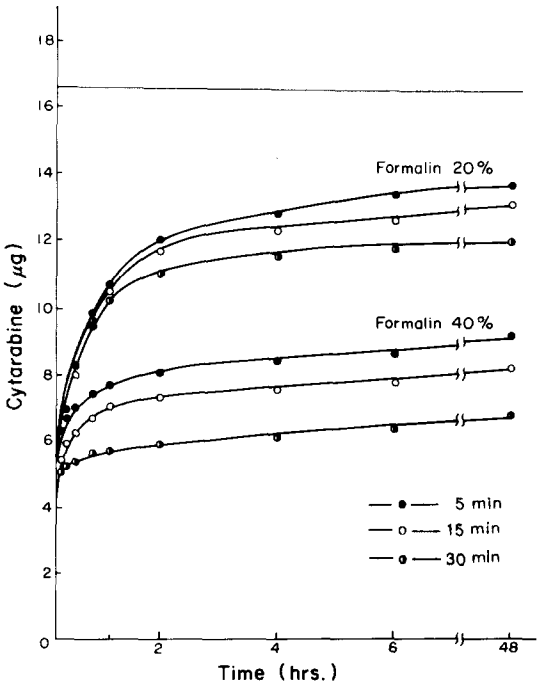


Fig. 6. Effect of cross-linking time and formalin concentration on the release of cytarabine.

The highest straight line represents the amount of completely released cytarabine.

Also, the level of drug release during the initial burst out may be dependent upon the cross-linking degree.

The effect of drug content (25, 50, 100 mg) per albumin 250 mg in 1 ml of the aqueous solution of albumin is shown in Fig. 7. As the drug/albumin ratio was increased, the cytarabine release was proportionally increased.

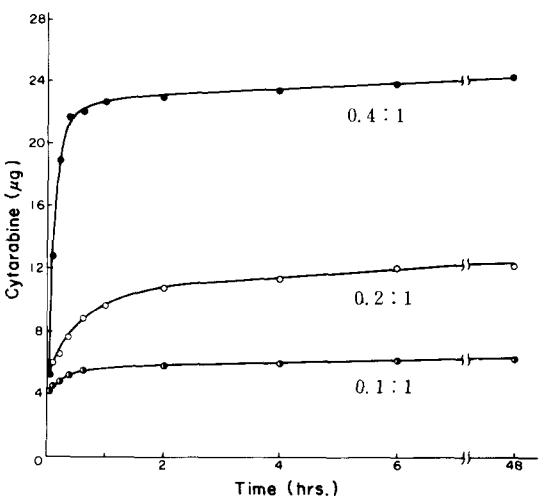


Fig. 7. Effect of the drug/albumin ratio on the release of cytarabine.

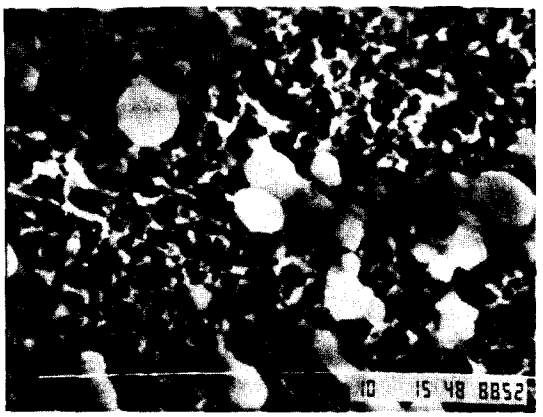


Fig. 8. Scanning Electron Microphotograph of cross-linked Bovine Serum Albumin Microspheres after Drug Release test. (x 4,600).

Drug content per mg of microspheres can be controlled by varying the drug concentration in the aqueous albumin solution. The effect of drug content (2.5, 5, 10 w/v % in 25 w/v % BSA solution) on the release of drug is shown in Fig. 7. As the drug/albumin ratio was increased, the cytarabine release was proportionally increased. This indicates that the added cytarabine has been entrapped as much as that.

After drug release test, the morphology of albumin microspheres was changed as shown in Fig. 8. The shape of microspheres was non-sperical and albumin matrix was little bit relaxed after incubation for 48 hours in pH 7.4 PBS solution at 37°C.

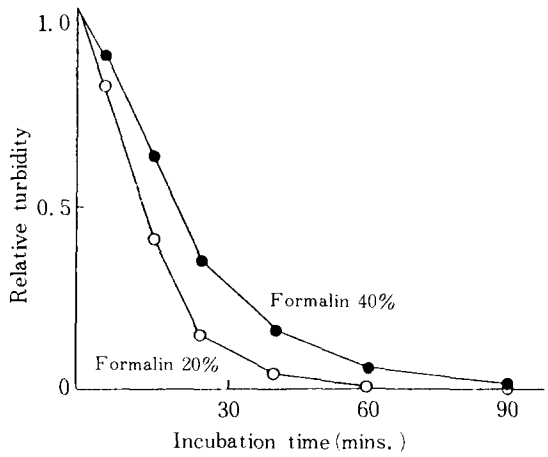


Fig. 9. Effect of the concentration of cross-linking agent on the albumin matrix degradation. Key: —● Formalin 40%; —○ Formalin 20%.

As the results of these drug release tests, most of cytarabine was released during the first one hours and then it was released slowly thereafter. The initial burst out is probably resulted from the release of cytarabine attached on the surface of albumin microspheres, and the next slow release may be resulted from the diffusion of drug in the inner space of microspheres by the slow relaxation or the degradation of albumin matrix.

Fig. 9 shows the effect of concentration of cross-linking agent on the albumin matrix degradation in proteolytic enzyme solution. Albumin matrix was progressvely destroyed according to the cross-linking degree.

It implies a digestion of albumin microspheres by the lysosomal enzyme in the reticuloendothelial system, when albumin microspheres are injected intravascularly.

As the results of current study, it is thought that the amount and rate of drug release could be controlled by varying the conditions of preparation such as cross-linking time, type of cross-linking agents, concentration of cross-linking agent, drug/albumin ratio, stirring speed, etc.

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