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Hydrophilic Albumin Microspheres as Cytarabine Carriers

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The surface of cytarabine-entrapped albumin microspheres was modified by coupling with glycine. Compared with conventionally prepared albumin microspheres, the surface modified albumin microspheres showed remarkably increased hydrophilicity, good dispersability in aqueous medium and reduced aggregation during storage which met the requirements of injectable drug carriers in aqueous vehicle. *In vitro* cytarabine release from hydrophilic albumin microspheres (HAM) was a function of the cytarabine to albumin ratio, whereas no significant difference in the releasing capacity was observed between surface modified HAM within the small size range (2 to 5 μm) studied. HAM containing 15-23% drug were gradually degraded by protease and continuously released up to 60% of the total entrapped cytarabine for 6 h. These results thus suggest that HAM is a suitable cytarabine carrier which may be injected intraveneously with the benefits of a reduced risk of blood embolism induced by aggregates and prolonged cytarabine release.

Key words: Hydrophilic albumin microspheres, Incorporation efficiency, Drug release, Matrix degradation, Drug carrier, Cytarabine, Sustained release.

INTRODUCTION

Albumin microspheres have been studied as a potential drug carrier for specific organ targeting and controlled release (Widder *et al.*, 1979; Kim *et al.*, 1985; 1986; 1988; 1993). Albumin microspheres have been prepared by hardening albumin molecules in w/o emulsion by heat or chemical cross-linking agents. But the microspheres hardened by these methods are hydrophobic due to the interaction of albumin at the w/o interface with hydrophobic compounds like dispersion oil, hydrocarbon molecules, and the conformational change of albumin during preparation (Widder *et al.*, 1979; Gallo *et al.*, 1984). The albumin molecules at the w/o interface would unfold with the hydrophobic groups exposed to the non-aqueous medium and the hydrophobic groups oriented toward the aqueous phase. This hydrophobicity presented problems concerning the aggregation of microspheres during storage, the poor dispersability, and the physical stability of suspension for injection. Recently, several attempts have thus been made to solve such problems by preparing hydrophilic microspheres in polymer dispersion media (Longo *et al.*, 1982; Longo and Goldberg, 1985; Goldberg *et al.*, 1987). However, no study has yet been done on the comparison of physicochemical properties including hydrophilicity, stability during storage, hardness, and drug release pattern of hydrophillic albumin microspheres (HAM) with those of other conventionally made microspheres.

The purpose of this study was thus to compare these important physicochemical properties and drug release pattern of HAM with those of the conventionally made hydrophobic albumin microspheres to evaluate the suitability of HAM as injectable cytarabine carrier.

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MATERIALS AND METHODS

Materials

Cytarabine was supplied by Choong-Wae Pharm. Co. (Seoul, Korea). Bovine serum albumin, Fraction V (BSA) was purchased from Sigma Co. (St. Louis, MO, USA). All other chemicals were of reagent grade and used without further purification.

Preparation of microspheres

One milliliter aliquot of solution containing 250 mg of BSA and fixed amount of cytarabine (25, 50, 100 mg) was added to 20 ml of cotton seed oil, and dispersed for 3 min by vortex mixer. This coarse emulsion was added dropwise to another 30 ml

aliquot of cotton seed oil that had been constantly stirred (1000, 1600, 2300 rpm) by motor-driven glass stirrer for 10 min.

To the resultant emulsion, 1 ml of 25% glutaraldehyde was added for cross-linking. After the reaction was allowed to proceed for 4 h, 2 ml of 2 M glycine solution was dropped to these chemically cross-linked albumin microspheres (CAM) and the suspension was further stirred for 1 h. The products were separated by centrifugation, washed three times with anhydrous ether, two times with distilled water and once with ethanol. The washed microspheres were dried in vacuum desiccator. Thermally denatured albumin microspheres (TAM) were prepared by the method described in previous report (Kim *et al.*, 1985).

Determination of shape and size distribution

The shape and size of microspheres were observed by photomicroscopy and scanning electron microscopy (SEM: JEOL JSM-35, Japan). Mean diameter, size distribution and standard deviation were calculated by counting about 700 particles using scanning electron micrographs (×3000).

Measurement of hydrophilicity

The hydrophilicity of microspheres was measured by capillary rise technique (Longo and Goldberg, 1985). Capillary columns of equal size (7 cm×1.2 mm I.D.) were used in this study. The packed column was then placed in a flat-bottomed dish containing distilled water as a mobile phase. For 6 h, the hydrophilicity of microspheres was measured by the ratio of the height of the aqueous mobile phase at any time (H_t) to the height of the packed column (H_o) as a function of time.

Measurement of dispersability

Twenty milligrams of each microsphere was dispersed in 10 ml of distilled water. Three microsphere suspensions in 10 ml mass cylinders were kept undisturbed and then the sedimentation volume of each suspensions was measured at proper time intervals.

Measurement of aggregation

Aggregation of microspheres was observed with photomicroscope (Victer Instruments) and examined with Coulter Counter (Coulter Electronics Ltd., Luton, Beds, UK) by measuring the number and size of the microsphere aggregates. Twenty milligrams of each microspheres was suspended in 100 ml of three different dispersion medium (distilled water, pH 7.4 PBS solution, 0.9% NaCl). Suspensions were mildly shaken several times by hand and diluted with Isoton II solution (Coulter Electronics Ltd.) before measuring the size of aggregetes. At periodic day intervals for 30 days, the size change in the aggregates was determined.

Assessment of drug incorporation

Ten milligrams of microspheres suspended in 100 ml of PBS solution (pH 7.4) was sonicated at 30 μ for 4 min to remove the loosely adhered drug on the surface of microspheres and then centrifuged. The amount of drug associated near the surface of HAM was determined by measuring the amount of detached drug in the supernatant using HPLC (Hitachi, Japan). To measure the amount of the entrapped drug in the inner space, the resultant microsphere pellets were resuspended in 100 ml of pH 7.4 PBS solution containing 200 mg of protease (31,400 units/g, supplied by Dong-A Pharm. Co., Seoul, Korea). This suspension was stirred with a constant speed of 100 rpm at 37±1 ℃ for 20 h. Three milliliters of this solution was sampled and deproteinized with 2 ml of 10% trichloroacetic acid solution. After filtering the precipitated protein, the pH of the filtered solution was adjusted to 7.4. Then the solution (6 μl) was injected to HPLC and assayed on a C-18 column (μ-Bondapak) using 20% methanol as a mobile phase. Absorbance of the effluent from the column at 270 nm was monitored. Peak heights wereused for quantitation of the assay.

Drug release

Microspheres containing 450 μg of cytarabine were suspended in 100 ml of phosphate buffer (pH 7.4) and shaken in a 37±2℃ water bath using stirrer with a constant speed of 300 rpm. At scheduled time intervals, 4 ml aliquots were sampled and filtered through the membrane filters (0.22 μm). The amount of cytarabine released was assayed at 268 nm by UV spectrophotometer (LKB, Ultrospec 4050).

Degradation of microspheres

The measurement of matrix degradation was based on the fact that the turbidity of particle suspension varies with the particle size change (Campbell *et al.*, 1984). Fifty milliliters of homogeneous suspension containing 5 mg of albumin microspheres was incubated at 37℃ in the presence of proteolytic enzyme (63 units/ml). The change in turbidity was spectrophotometrically measured at 500 nm.

RESULTS AND DISCUSSION

Physicochemical properties

The color of HAM was yellowish brown, which

Table I. Mean diameter of albumin microspheres versus dispersion force

Dispersion force (rpm)	Diameter[a] (μm)
1000	4.36± 2.49
1600	2.61 ± 1.33
2300	1.92± 1.03

[a] Each value represents the mean ±SD for about 700 microspheres.

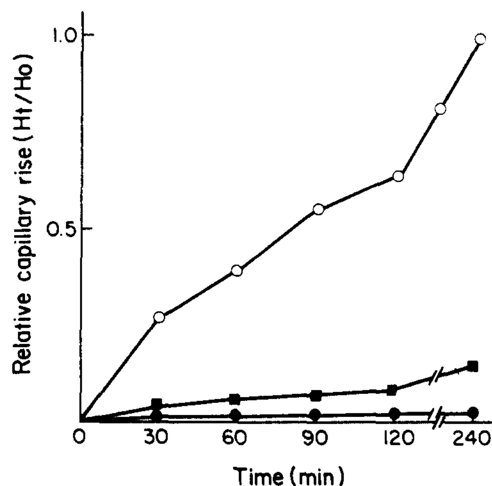

Fig. 1. Relative capillary rise (H_t/H_0) of three different types of albumin microspheres versus time.
○, HAM; ■, CAM; ●, TAM prepared at 120℃.

might had been due to the covalent binding of amino groups in glycine to the microsphere surface by forming Schiff base with the surface aldehyde groups (Longo *et al.*, 1982).

The size of the prepared microspheres was affected by the dispersion force applied in preparation. Table 1 shows that mean diameter and standard deviation were decreased, as the dispersion force increased. This indicates that thesize distribution of HAM mainly depends on the size distribution of the albumin solution droplets in emulsion just before adding the cross-linking agent, since the size of water phase in w/o emulsion is pricipally depended upon the emulsification power (Gallo *et al.*, 1984).

Evaluation of hydrophilicity

The hydrophilicity of the surface-modified microspheres containing cytarabine was compared with that of glycine-untreated microspheres. Fig. 1 shows that the hydrophilicity of the surface-modified albumin microspheres is much higher than those of the other chemically or thermally cross-linked albumin microspheres. In the case of the surface-modified microspheres, covalent binding of the amino group in glycine to the aldehyde groups on the surface of microspheres by Schiff base reaction resulted in the attachment of free carboxyl group of glycine on the surface of microspheres, increasing the polarity of the microsphere surface.

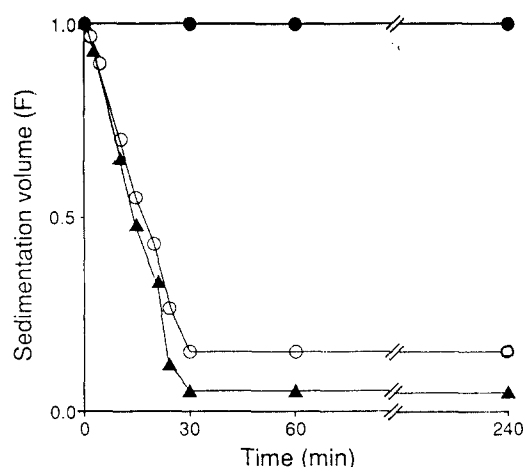

Fig. 2. Sedimentation volumes of three different types of albumin microspheres dispersed in distilled water.
○, HAM; ■, CAM; ●, TAM prepared at 120℃.

Compared with the results of Goldberg *et al.* (1987), the hydrophilicity of the newly developed microspheres was greater than that of their unquenched (i.e. glycine-untreated) microspheres, though we used vegetable oil as dispersion medium. It is thus thought that the hydrophilicity of microspheres depends comparatively more on the polarity of the surface than the stabilization of dispersion medium in preparation.

Evaluation of dispersability

The dispersability of microsphere suspensions was assessed by the sedimentation volume method (Lachman *et al.*, 1986). The sedimentation volume (F) was defined as the ratio of the ultimate sediment volume to the original suspension volume (Martin *et al.*, 1983). It was observed that CAM and TAM tended to settle immediately in the dispersion medium, producing a distinct boundary between the sediment and the supernatant liquid within 30 min. On the other hand, HAM showed no clear supernatant on standing for 240 min. This indicated that surface hydrophobicity of CAM and TAM had induced the forming of the aggregated microsphere clusters.

In Fig. 2, the F-values of HAM, CAM and TAM are 1, 0.15 and 0.05, respectively. Each values remained practically constant for 240 min. Compared with CAM and TAM, HAM showed good dispersability in the sense of content uniformity. Results above also revealed that CAM-and TAM-dispersed suspension which formed microsphere sediment shortly was not adequate for injection while suspension of HAM was stable enough for injectable suspension. Further, the

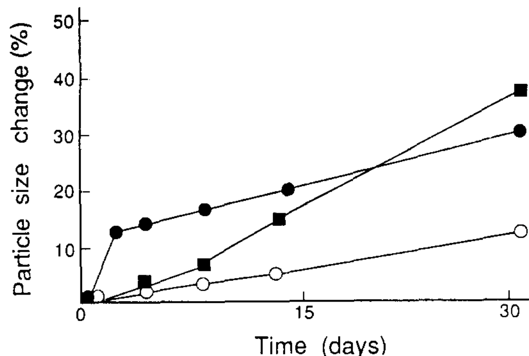

Fig. 3. Change in size of the aggregated albumin microsphere clusters.
○, HAM; ■, CAM; ●, TAM prepared at 120°C.

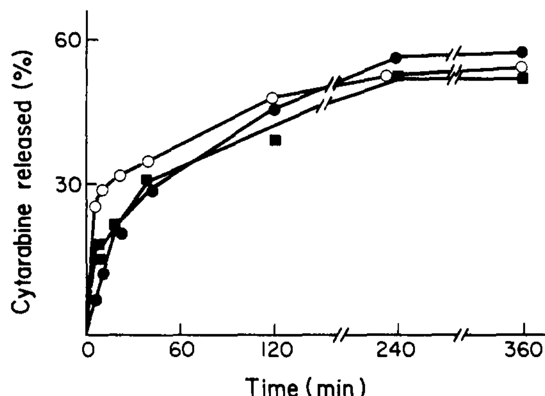

Fig. 4. Effect of HAM size on the release of cytarabine. ○, HAM I (mean diameter=1.92 μm); ■, HAM II (mean diameter=2.61 μm); ●, HAM III (mean diameter=4.36 μm).

HAMs might reduce the problem of blood embolism, one of the drawbacks of relatively hydrophobic microspheres.

Aggregation during storage

TAM and CAM were prepared by hardening albumin molecules in w/o emulsion by heat and cross-linking agent, respectively. It was observed that these microspheres were not easily dispersed in aqueous medium without wetting agent. The formation of microsphere aggregates during storage, which is one of the severe drawbacks in relatively hydrophobic microspheres such as TAM and CAM reduced considerably in HAM (Fig. 3). Photomicroscopic observation also showed that aggregation of CAM and TAM increased than that of HAM in different dispersion mediums (distilled water, phosphate buffer, 0.9% NaCl) (data not shown). It seemed that the enhanced repulsion between HAM with polar carboxyl groups on the surface (Longo *et al.*, 1982) reduced in tendancy to aggregate and increased physical stability during storage. It also indicated that TAM and CAM aggregated due to their hydrophobic surface and traces of reactive aldehyde groups on the surface of CAM might had been involved in the formation of aggregated clusters during storage.

Drug incc rporation efficiency

Drugs can be associated with albumin microspheres by adsorption onto the particle surface and/or inclusion in the microsphere matrix (Gupta *et al.*, 1986; Kim *et al.*, 1989). From our experiments, a total of 15-23% of cytarabine was incorporated; 6-11% was considered to be caused by cytarabine attached on the surface and/or entrapped in the near inner surface of HAM which were released after sonification at 30 μ for 4 min, and 9-12% by cytarabine entrapped in the more inner space of HAM which was released from the digested microsphere matrix in the presence of proteolytic enzyme. The total amount of drug incorporated in HAM usually depended upon the physical properties of drug (solubility, partitioning of drug into the oil or washing media), cross-linking density and preparation parameters (temperature, stirring rate, shape of the blade, etc). However, the amount of drug on the surface or in the near inner surface of HAM was mainlydue to solute migration inside the HAM under vacuum drying. It was highly dependent on the vacuum pressure, temperature and the time in drying procedure. To control the drug release from HAM over prolonged periods, solute migration in HAM during the drying process should be reduced by altering the drying procedure.

Drug release and degradation behavior

The effect of HAM size on cytarabine release was shown in Fig. 4. The amount of drug released was dependent on the microsphere size. During the initial incubation period, the smaller the mean diameter, the morethe drug release. But after 20 min, no significant

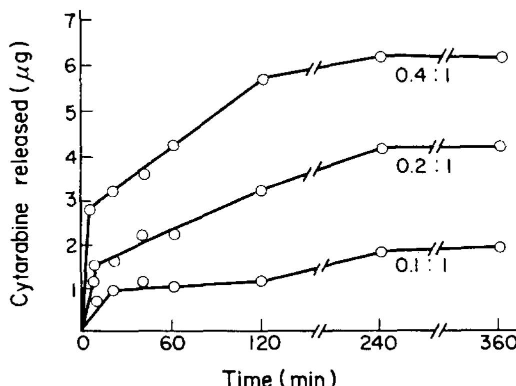

Fig. 5. Effect of cytarabine/albumine ratio on the drug release from HAM. All products were prepared using 25 w/v% BSA solution.

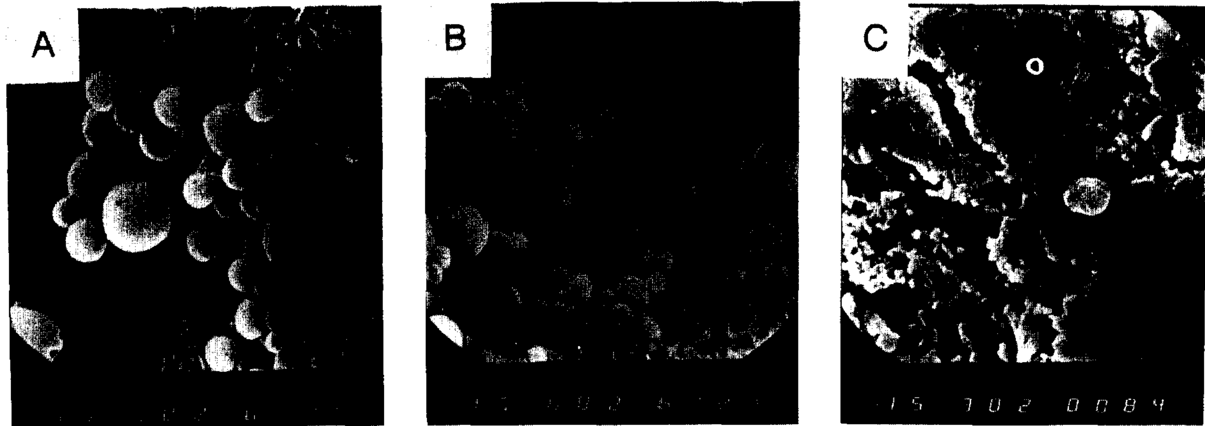

Fig. 6. Scanning electron micrographs (×3000) of HAM after incubation at 37℃. (A), HAM after incubation in the absence of protease for 2 h; (B), HAM after incubation in the presence of protease (20 units/ml) for 1 h; (C), HAM after incubation in the presence of protease (20 units/ml) for 2 h.

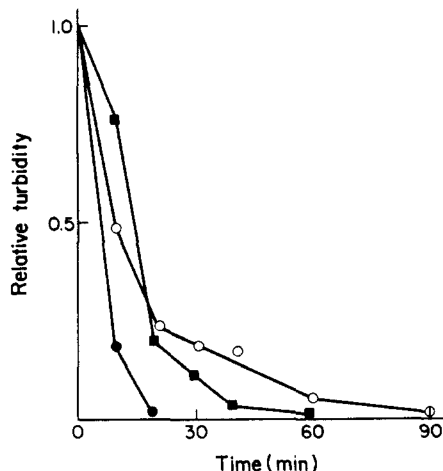

Fig. 7. Matrix degradation of albumin microspheres by proteolytic enzyme (63 units/ml).
○, HAM; ■, CAM; ●, TAM prepared at 120℃. All samples were prepared using 25 w/v% of BSA solution containing cytarabine.

difference in the releasing capacity was observed between HAMs within the small size range studied (2 to 5 μm). It was also shown that cytarabine release from HAM continuously increased to as much as 60% of the total entrapped amount by 6 h. The HAMs displayed an initial fast drug releasing period within few minutes and a much slower release rate thereafter. The release of drug fromalbumin microspheres depended on the location of drug in the carrier as well as on the properties of microsphere matrix (Yang, 1986; Gupta *et al.*, 1986; Kim *et al.*, 1989). The fast release of drug from HAMs at the initial phase was probably due to the release of drug attached on the surface and/or associated in the near inner surface of HAMs. The slower release throughout second phase might be due to the release of drug through the channel from the core of HAMs. It indicated that the sustained release of drug from HAM over a longer period could be achieved by entrapping a larger amount of drug in the core of HAM than that at the surface. This kind of HAM, having less cytarabine attached on the surface or in the near-surface space of HAM, may have the advantage of preventing side effects caused by those loosely adhered drug that are released before drug carrier reaches the target organ. Drug release rate could also be controlled by changing the degree of cross-linkage that affects microsphere porosity, hydration and swelling (Longo *et al.*, 1985; Kim and Oh, 1988).

In Fig. 5, it was shown that the drug released from HAM during the preceeding 4 h was proportional to the loading amount. This data suggested that in vivo drug release might be controlled rationally by encapsulating drug for loading and maintenance dose simultaneously within HAMs with varying the cytarabine/albumin ratio.

As shown in Fig. 6, the shape of HAM incubated in the absence and presence of protease (20 units/ml) were distinctly different. While HAM swelled but maintained their spherical shape in the absence of protease, theybecame gradually degraded and lost their initial shape in the presence of protease. The release of cytarabine from HAM in vitro, is thus thought to be controlled by the swelling of microspheres, whereas in vivo might be affected by the degradation of albumin matrix under the reaction of proteolytic enzymes (Yang, 1986).

Microsphere degradation rates by proteolytic enzyme are presented in Fig. 7. Degradation rate seemed to be determined by two steps; the first rate-determining step is hydration, dispersability and swelling of microspheres which enable the access of protease on the microsphere surface, and the second

rate-determining step is the enzymatic reaction on the microspheres. Of the three microsphere types, HAM was immediately dispersed and exposed to the enzyme, thus its initial degradation was sometimes faster than CAM. Nevertheless, it usually took the longest time for HAM to be completely digested, probably due to the hindrance in enzymatic reaction on HAM by the existence of glycine bound to the surface aldehyde groups (Longo *et al.*, 1982). This delayed degradation in vitro suggested that albumin matrix degradation by enzyme in vivo may bring about a retarded release of cytarabine. Therefore, the in vivo level of drug release from HAM, which is likely to be due to matrix degradation by lysosomal enzyme, might be controlled by varying the concentration of cross-linking agent and reaction time (Kim and Oh, 1988).

CONCLUSIONS

On the basis of these results, it is suggested that HAM is a useful cytarabine carrier which may be intravenously injectable with a lower risk of blood embolism caused by aggregates, and with desirable pharmaceutical properties such as prolonged cytarabine release and reduced aggregation during storage. To control the drug release from HAM over prolonged period, solute migration inside the HAM during drying process should be reduced by altering the drying procedure and cross-linking degree of albumin molecules must be controlled.

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