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Casein microspheres: preparation and evaluation as a carrier for controlled drug delivery

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Abstract

A method for the preparation of smooth, spherical, glutaraldehyde-crosslinked microspheres of the amphiphilic protein, casein, is reported. Casein microspheres (CAS/MS) were found to be even more hydrophilic than comparable microspheres made from bovine serum albumin by the method developed in this laboratory several years ago and were readily dispersed in water without aggregation. Enzymatic degradation studies with the enzyme protease suggested that the CAS/MS with lower crosslink density degraded faster than those with higher crosslink density. Methotrexate (MTX) was incorporated into the CAS/MS during microsphere synthesis to the extent of 15 wt% loading, and in vitro release was examined in phosphate buffer at 37°C. Drug release of 35% was observed in 24 h, suggesting that most of the incorporated drug was strongly associated with the CAS/MS. Similar release was observed for MTX loaded albumin spheres prepared in the same manner. Based on these studies, casein appears to be a highly promising, low-cost, bioacceptable protein for the synthesis of drug conjugates and microsphere carrier systems for targeted drug delivery.

Key words: Casein microspheres; Albumin microspheres; Methotrexate; Controlled release; Glutaraldehyde crosslinking

1. Introduction

A method to achieve efficient and selective delivery of therapeutic agents to the site of action to enhance tissue localization and at the same time reduce the toxicity of the drugs to healthy tissues has been a subject of research for several years (Goldberg, 1983). Among the various drug delivery systems investigated to achieve this goal, microsphere based systems have received considerable attention (Davis et al., 1984). Although various protein, polysaccharide and other polymeric microspheres have been investigated as drug carriers, microspheres based on albumin have received considerable attention (Morimoto and Fujimoto, 1982; Gupta and Hung, 1989a,b).

Surface characteristics of microspheres play a significant role in their clinical utility and fate in

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vivo. Davis and Illum (1988) used coatings of absorbed or grafted copolymers to create more hydrophilic polystyrene particles and provide steric barriers to interactions with macrophages and plasma components. In general, hydrophobic particles appear to be removed more rapidly from circulation than hydrophilic particles (Illum et al., 1986). Hydrophilicity of albumin microspheres has also been shown to be important for post-synthesis drug loading as well as for easy wetting and rapidly reconstituting an injectable dispersion of microspheres in a suitable aqueous vehicle (Longo et al., 1982). These hydrophilic albumin microspheres were prepared by the glutaraldehyde crosslinking of an aqueous dispersion of albumin at room temperature by a steric stabilization technique using polymeric dispersing agents and with addition of the crosslinking agent via the organic phase. Nearly perfect, highly hydrophilic spheres of various sizes were produced by this method.

Recent work by Chen et al. (1987) showed that the carrier matrix can play an important role in the in vivo behavior of microspheres. To compare the characteristics of two different protein microsphere systems, they attempted the preparation of casein microspheres and compared their performance in vivo with microspheres based on albumin. With casein, however, their method of preparation yielded particles which were irregular in shape and also of wide particle size distribution. Nevertheless, they observed that the casein system showed greater potency per unit amount of the drug doxorubicin than did the albumin system in a mammary carcinoma in Wistar rats.

Since the casein system was reported to show improved anti-tumor properties with the cytotoxic drug doxorubicin and since casein is a much less expensive protein than serum albumin, it was felt worthwhile to attempt the preparation of casein microspheres with good spherical structure and good control of particle size; microspheres which could also be readily loaded with various drugs. This paper reports the successful synthesis of such CAS/MS and presents results for the in vitro evaluation of CAS/MS containing the cytotoxic drug methotrexate (MTX).

2. Materials and methods

2.1. Preparation of casein microspheres

1 ml of a 20% solution of casein (Sigma, U.S.A) in 0.5 M NaOH was added dropwise to 50 ml of a mixture of *n*-hexane (Fisher, U.S.A) and light mineral oil (Sigma, U.S.A) in the ratio 4 : 1 containing 1 g of an oil soluble surfactant, sorbitan sesquioleate (Arlacel® C, ICI Americas Inc., U.S.A). A reverse emulsion was formed by the dispersion of the aqueous phase in the organic phase. The droplet size of the emulsion and thus the resulting microsphere size was determined by the nature of the dispersion treatment. Microspheres in the size range of 5–35 μm were produced by stirring the dispersion in a 100 ml round bottomed flask using a Teflon® paddle stirrer at 5000 rpm. In order to prepare spheres of smaller size (1–2 μm), the dispersion was placed in a 100 ml beaker, cooled in ice and sonicated using the Q-horn of a sonicator (Heat Systems-Ultrasonics Inc., Model W-375, U.S.A) at a power setting of 9 for 5 min. After sonication, the contents were transferred into a 100 ml round-bottomed flask and stirred using the Teflon® paddle stirrer at 2000 rpm. Crosslinking the protein microspheres was accomplished via the organic phase according to the method of Longo et al. (1982) by the addition of glutaraldehyde saturated toluene (GST). The contents were stirred for 1 h at room temperature. Varying the amount of GST added resulted in different crosslink densities. Microspheres of low crosslink density were prepared by adding 0.4 ml of GST whereas medium and high crosslink density was achieved using 5 and 10 ml of GST, respectively. After the reaction, the microsphere dispersion was centrifuged at 5000 rpm for 10 min in a Beckman centrifuge (Model J2 21), the supernatant was discarded and the pellet resuspended in petroleum ether (Fisher, U.S.A) with the aid of a metal spatula and mild sonication (Cole-Parmer, Model 8851, U.S.A). The petroleum ether wash was repeated three times followed by three acetone washes to completely remove all oil and surfactant. The microspheres thus obtained were then vacuum dried (30 mm Hg) at room temperature. Spherical, smooth, uni-

form CAS/MS of 1–2 μm size were readily prepared in 78% yield.

2.2. Drug incorporation during synthesis

Incorporation of MTX in the microspheres was accomplished by dissolving 50 mg of the drug (Sigma, U.S.A.) in the casein solution, dispersing and crosslinking the emulsion as before. Maximum drug loading possible based on the initial casein : drug weight ratio was 20%. Actual loadings were approx. 15%, indicating a drug loading efficiency of 75%.

2.3. Optical and scanning microscopy

A Nikon optical microscope equipped with an ocular micrometer was used to determine the size and swelling behavior of the microspheres. Scanning electron microscopy (SEM) was performed using a Jeol instrument (JSM 35 CF). Microspheres were dusted onto double sided tape on an aluminum stub, sputter coated with gold-palladium and examined in the microscope at 10–15 kV. At least 100 spheres were counted from each specimen to determine the number average diameter and size distribution of the particles in the dry state.

2.4. Analysis of the drug content

The drug content of the drug loaded microspheres was determined by digesting the microspheres with protease (Campbell et al., 1984). 5 mg of the drug loaded microspheres were suspended in 20 ml of phosphate buffer (Sigma, U.S.A., pH 7.4, 0.1 M) containing 5 mg protease (Sigma, U.S.A., type VIII, 7.8 units/mg) in a 50 ml screw-capped test tube. The suspension was incubated at 37°C for 4 h or until all the microspheres were digested. An aliquot was diluted with an equal volume of 10% trichloroacetic acid (Sigma, U.S.A) to precipitate the proteins. After centrifugation at 1500 rpm for 10 min, the supernatant was filtered through a 0.45 μm Acrodisc® CR filter (Gelman, U.S.A), and the filtrate was analyzed spectrophotometrically in a UV-Vis spectrophotometer (Perkin Elmer 552). Absorbance values were recorded at 307 nm for MTX (Merck Index, 1976). Controls were performed with pure drug incubated with protease and pure drug plus unloaded microspheres incubated with protease. No degradation of the drug due to the enzyme nor association of the drug with precipitated protein from the digested microspheres was observed.

2.5. Matrix degradation

The effect of crosslink density on the degradation of microspheres was studied by digesting them with protease and monitoring the turbidity of the suspension spectrophotometrically. Microspheres (5 mg) were suspended in 5 ml of phosphate buffer and dispersed by ultrasonication with a microtip attachment (Heat Systems-Ultrasonics Inc, U.S.A). After the addition of 5 ml of PB containing 2.5 mg of protease (activity 19.5 U), the samples were incubated at 37°C. The percent transmission at 600 nm was recorded at various time intervals.

2.6. In vitro drug release

Approx. 10 mg of the MTX loaded microspheres were suspended in 300 ml phosphate buffer in stoppered Erlenmeyer flasks. The flasks were placed in a bath-incubator shaker (Blue M, U.S.A.) thermostatted at 37 ± 1°C at a speed setting of 10 (20 cycles/min). Aliquots of 2 ml were withdrawn at various time intervals, filtered through a 5 μm filter and analyzed spectrophotometrically for MTX. A constant volume in each flask was maintained by replacing the aliquots with fresh buffer.

3. Results and discussion

Chen et al. (1987) reported that when casein was dispersed and crosslinked like albumin using Span 80 as the surfactant, the microspheres obtained were somewhat irregular in shape with a perforate and discontinuous appearance. CAS/MS prepared by the technique presented here, however, were obtained in higher yields (> 75%)

and were smooth, uniform, and spherical by SEM (Fig. 1), and resembled hydrophilic albumin microspheres prepared by the synthesis developed in this laboratory (Longo et al., 1982). An important difference in our method of MS synthesis as compared with others is our addition of the crosslinking agent via the organic phase as glutaraldehyde saturated toluene. Both supermicron

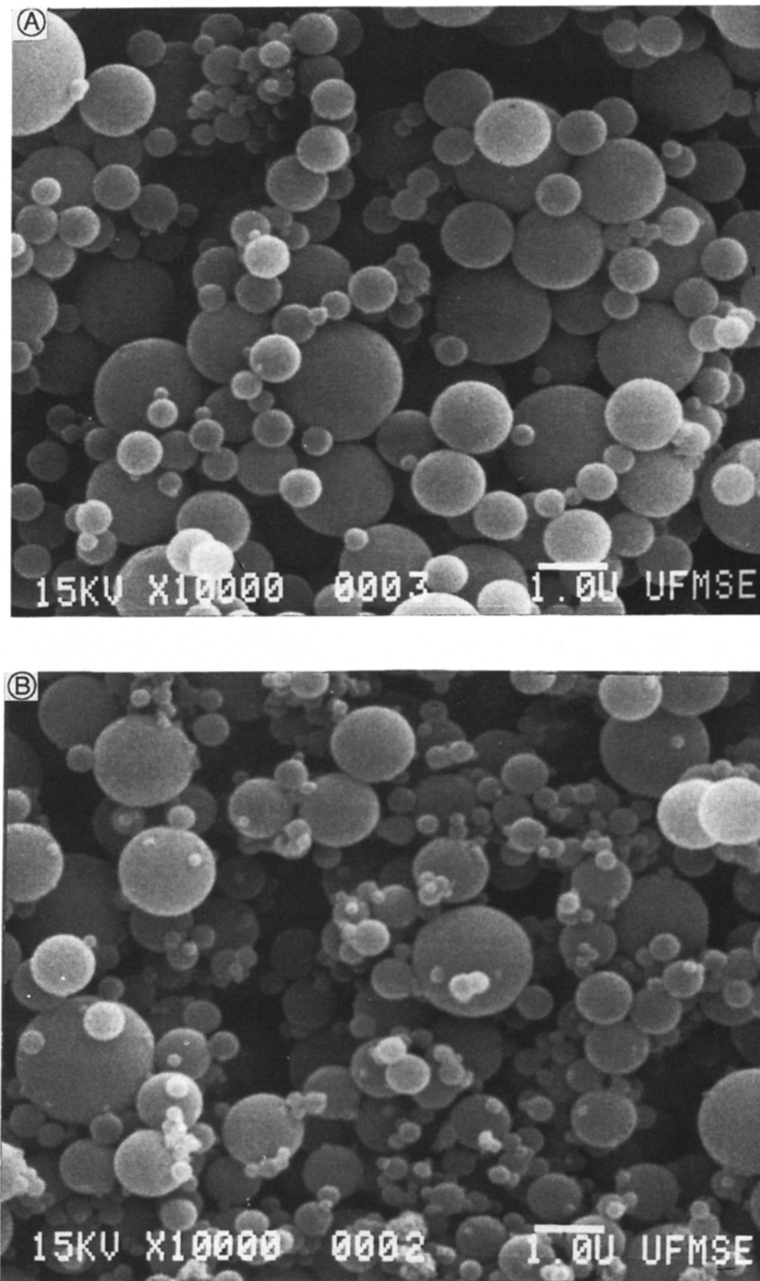

Fig. 1. SEM of (a) casein and (b) albumin microspheres showing their similar spherical structure and smooth surface morphology.

and submicron CAS/MS were readily prepared. The typical particle size distribution of 0.7 μm spheres (average diameter – dry) prepared by ultrasonication is shown in Fig. 2. CAS/MS obtained by paddle stirring (large spheres) as well as by ultrasonication (smaller spheres) were brown free flowing powders and were readily dispersed in water without the aid of surfactants. In fact, the casein microspheres were even more readily dispersed in water than hydrophilic albumin spheres produced by the same technique. The increased affinity of casein microspheres for water could be attributed to the more ionic nature of the protein and the sodium salt form used in this method of preparation.

In order to determine the extent of swelling,

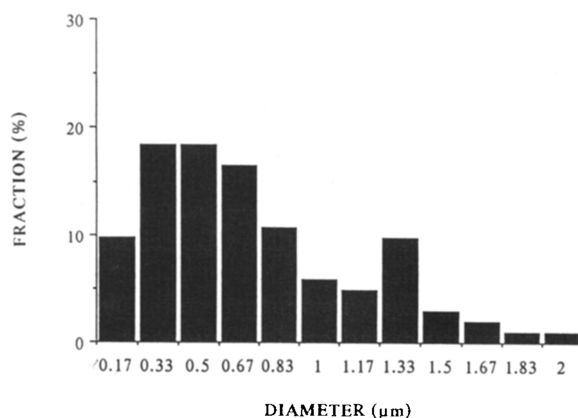

Fig. 2. Particle size distribution measured by SEM of 0.7 μm average diameter high crosslink density casein microspheres (prepared by ultrasonication) in the dry state.

Fig. 3. Optical photomicrograph of casein microspheres (prepared by paddle stirring) (a) in acetone and (b) swollen in water.

microspheres prepared by paddle stirring were dispersed in acetone and examined under the optical microscope. Fig. 3a shows an optical photomicrograph of high crosslink density CAS/MS (6.7 μm average diameter) in acetone where there was little swelling. Examination of the spheres after equilibration in a drop of water following the evaporation of acetone showed that swelling occurred in the aqueous medium (Fig. 3b). Quantitative determination of the extent of swelling by counting at least 100 spheres before and after equilibrium swelling showed an average increase in diameter of approx. 25% in water. Similar experiments performed with 6.3 μm average diameter albumin microspheres prepared by the same technique and with comparable high crosslink density showed an average diameter increase of 14%. Thus, CAS/MS appear somewhat more hydrophilic than albumin. This may be potentially advantageous in reconstituting and maintaining stable injectable dispersions in aqueous vehicles and may also help prolong circulatory lifetimes for submicron MS.

3.1. Matrix degradation

The ease with which the microspheres were degraded by the enzyme protease depended on the crosslink density of the protein. Proteolysis was measured by a turbidometric method and Fig. 4 shows the turbidity values obtained when microspheres with three different crosslink densities were digested with protease. As can be seen, a direct relationship exists between the crosslink density and the ease of degradation; the lower the crosslink density, the faster the rate of degradation of the protein matrix. This suggests that the residence time of the microspheres in tissue or blood might be controlled by changing the crosslink density of the casein matrix.

3.2. Drug release from microspheres

The MTX concentration in CAS/MS prepared for this study was approx. 15 wt%. The incorporation efficiency based on concentrations used for synthesis was 75%, which was comparable to the incorporation efficiency in albumin

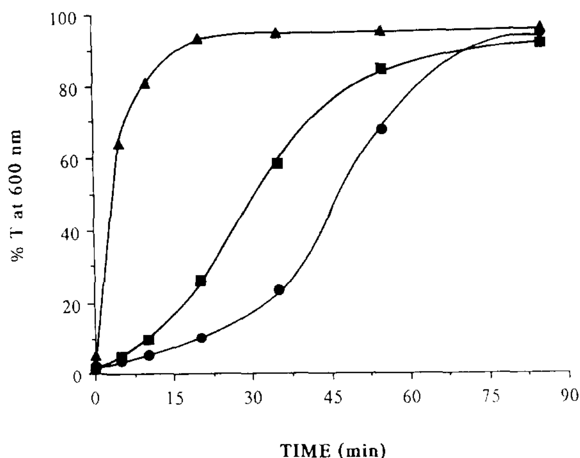

Fig. 4. Degradation of casein microspheres of different crosslink density prepared with various amounts of glutaraldehyde saturated toluene (GST). All points represent the average of at least three measurements. Lines are drawn for clarity but do not indicate curve fitting. (▲) 0.4 ml, low; (■) 5.0 ml, medium; and (●) 10.0 ml GST, high crosslink density.

spheres. By altering the protein : drug ratio during synthesis, even higher drug payloads appear readily feasible. In vitro release profiles of the drug from casein microspheres with high and low crosslink densities are shown in Fig. 5. For comparison, the drug release from albumin spheres prepared under the same conditions as the highly crosslinked casein spheres is also shown in Fig. 5. Interestingly, the rate of release from both matrices was virtually identical in spite of the fact that the CAS/MS were slightly more hydrophilic and swollen than the albumin spheres. As expected, the higher the crosslink density, the slower the rate of release. This was demonstrated for casein spheres with two different crosslink densities. In the case of high crosslink density casein spheres, the cumulative release over a 24 h period was only about 35% of the total drug incorporated. This suggests that MTX is strongly bound to the matrix, presumably by glutaraldehyde coupling through the primary amino groups of the drug, and would release the major payload of MTX during degradation of the matrix.

Chen et al. (1987) did not report the preparation of smooth, uniform spherical CAS/MS by their method. However, the technique we have

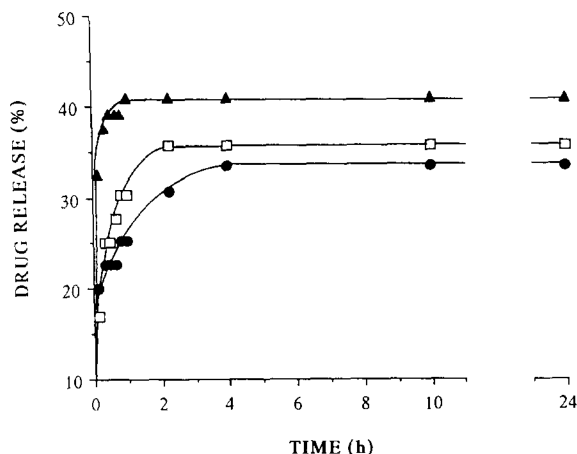

Fig. 5. In vitro release profiles of MTX from casein and albumin microspheres containing 15 wt% mixture: (●) from albumin microspheres crosslinked with 10 ml GST, (□) from casein microspheres crosslinked with 10 ml GST and (▲) from casein microspheres crosslinked with 0.4 ml GST. All points represent the average of at least three measurements. Lines are drawn for clarity but do not indicate curve fitting.

developed yielded CAS/MS of smooth, spherical geometry which were comparable to albumin spheres prepared by the same method. Chen et al. used Span 80 as the suspension stabilizer and observed foaming which could have produced the large pores and discontinuities observed in their microsphere preparations after crosslinking with aqueous glutaraldehyde. In contrast, we used a low HLB surfactant such as Arlacel®C and introduced glutaraldehyde via the organic phase.

Estimation of the drug content in our albumin and casein microspheres by protease digestion showed that the MTX loading was virtually the same for both proteins; approx. 15 wt%. In vitro release from albumin and casein spheres crosslinked to the same extent were also similar. This observation is not consistent with that of Chen et al., who found the rate of release of doxorubicin from casein to be much slower than from albumin. This slow release was considered to be the determining factor for the greater potency observed per unit amount of the drug with the casein system. The slower doxorubicin release reported may be due to a surfactant effect and/or the morphology of the irregular CAS/MS. The comparable in vitro behavior of 1–2 μm casein-MTX and albumin-MTX microspheres in the present investigation suggests that the protein matrices are in fact quite similar in behavior. Thus, casein, which is a readily available, low cost, relatively non-immunogenic protein appears to be a highly promising carrier for future investigation of microsphere drug carrier systems and drug conjugates for targeted drug delivery. Syntheses of CAS/MS with a variety of drugs and localized intratumoral chemotherapy studies with CAS/MS containing MTX and other drugs have been initiated in a mouse Lewis lung carcinoma model and will be reported at a later date.

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