International Journal of Pharmaceutics, 22 (1984) 63–74
Elsevier

IJP 00743

Analysis of albumin microsphere preparation

J.M. Gallo, C.T. Hung and D.G. Perrier

Department of Pharmacy, University of Otago, Dunedin (New Zealand)

(Received December 1st, 1983)
(Modified version received June 16th, 1984)
(Accepted June 22nd, 1984)

Summary

Numerous variables which could affect the size of albumin microspheres were evaluated. Albumin microspheres were prepared at multiple levels of each variable and a particle size analysis was done on the resultant microspheres by electron microscopy. The effect of each variable on microsphere size is discussed and an optimal method for microsphere preparation presented.

Introduction

Albumin microspheres were first prepared for the detection of abnormalities in the reticuloendothelial system and the blood circulation (Rhodes et al., 1968; Rhodes et al., 1969; Zolle et al., 1970a). Radioactive microspheres with diameters ranging from 5 to 65 μm were produced by these investigators. About the same time Pasqualini et al. (1969), using a different technique than the above investigators, prepared albumin microspheres in the 30–60 μm range. Further developments produced microspheres with a mean diameter of about 1 μm (Zolle et al., 1970b; Scheffel et al., 1972).

Albumin microspheres, both magnetized and unmagnetized, have also been prepared for use as drug carriers (Kramer, 1974; Sugibayashi et al., 1977; Widder et al., 1978), and their preparation is based on a method developed by Scheffel et al. (1972). Preparation of microspheres includes numerous variables due to the fact that both emulsion and suspension technology are involved. There has been no complete,

Correspondence: D.G. Perrier, Department of Pharmacy, University of Otago, P.O. Box 913, Dunedin, New Zealand.

systematic and quantitative investigation of these variables and how they may affect the ultimate microsphere size distribution. This study was designed to identify which variables are important in the preparation of albumin microspheres. From this information, standard guidelines can be given for the preparation of albumin microspheres.

Experimental

Materials

Bovine serum albumin was obtained from Sigma Chemicals, No. A-60003. Cottonseed oil was purchased from Real Food, Dunedin, New Zealand, and B.P. grade light paraffin oil and maize oil were purchased from H.F. Stevens, Christchurch, New Zealand. 1-Octanol was supplied by Ajax Chemicals, Sydney, Australia, while anhydrous ether was obtained from Baker Chemicals.

Ultrasonic equipment consisted of a Dawe Soniprobe, type 7530A and a Bransonic model 220 ultrasonic water bath. Heidolph stirrers RZR1 were used. Heating mantles were obtained from Electrothermal, London, and Isopad, Borehamwood, Herts, U.K. The scanning electron microscope used was a Siemans Autoscan. A Polaron unit E3000 critical point dryer and a unit E5000 sputter coater were used.

Methods

An existing technique was adopted as a starting point to investigate the variables involved in the preparation of albumin microspheres (Widder et al., 1979). This initial method for microsphere preparation consisted of dissolving 250 mg of bovine serum albumin in 1.0 ml of deionized distilled water. One half of this aqueous solution was added to 30 ml of cottonseed oil at 4°C and ultrasonicated for 2 min at 125 W power setting. The resultant emulsion was added dropwise (40 ± 10 drops/min) to 100 ml of cottonseed oil at 125 ± 5°C which was being stirred at 1500 rpm. After the emulsion was added heating was continued for 10 min. The suspension was then allowed to cool to 25 ± 3°C. Once this temperature had been reached stirring was stopped and 60 ml of anhydrous ether was added to the microsphere–cottonseed oil suspension. The mixture was transferred to a centrifuge vessel and centrifuged at $3000 \times g$ for 15 min. The supernatant was decanted and 60 ml of anhydrous ether was added to the microsphere pellet. The microsphere pellet was resuspended in the ether using an ultrasonic water bath. This suspension was then centrifuged at $3000 \times g$ for 15 min. The ether phase was decanted and this washing step was repeated two times. After decanting the final ether wash, the microspheres were resuspended in 10 ml of anhydrous ether, and stored at 4°C in an air-tight container. The apparatus used to heat-stabilize the albumin microspheres is illustrated in Fig. 1.

From this initial method, a number of variables were identified which might effect the final microsphere size. These variables were: albumin concentration, emulsification time, emulsification power, and aqueous-to-oil phase volume ratio. In

addition to the above emulsification variables, the following variables relating to heat stabilization were evaluated: stirring rate, time of heat stabilization, emulsion drop rate, temperature of heat stabilization, and method of cooling the microsphere suspension. Finally, three other non-aqueous dispersed media were studied in addition to cottonseed oil.

Table 1 gives the order of the studies and level at which each each variable was studied. Each variable was evaluated sequentially by preparing one batch of microspheres for each level listed in Table 1. All levels for each variable were prepared on the same day and photomicrographs taken shortly (normally < 1 day) after preparation. From the particle size analysis the optimal level of each variable was de-

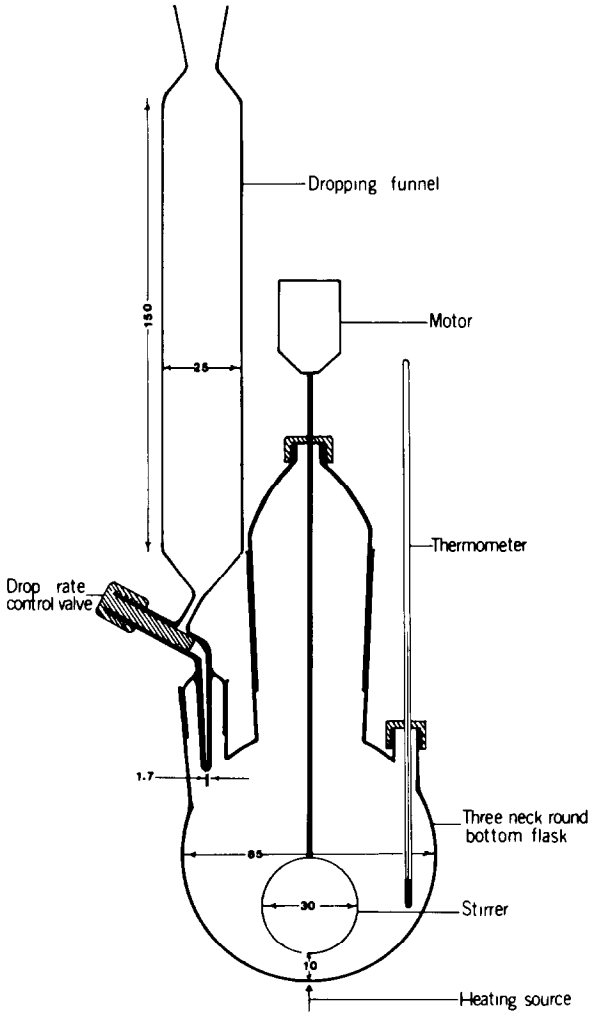

Fig. 1. Apparatus used for the heat stabilization of albumin microspheres (dimensions in mm).

TABLE 1

MEAN DIAMETERS AND STANDARD DEVIATIONS FOR ALBUMIN MICROSPHERES PREPARED UNDER DIFFERENT CONDITIONS

Order of evaluation	Variable	Level	Number of particles counted	Mean particle diameter ($\mu m \pm$ S.D.)
First	Albumin concentration (mg/ml)	500	564	0.44 ± 0.30
		400	581	0.46 ± 0.37
		300	542	0.51 ± 0.31
		200	632	0.52 ± 0.36
Second	Emulsification time (min)	10	689	0.52 ± 0.30
		5	755	0.52 ± 0.31
		2	647	0.51 ± 0.28
		1	498	0.61 ± 0.48
Third	Power of emulsification (W)	125	584	0.54 ± 0.32
		109	494	0.63 ± 0.39
		94	414	0.72 ± 0.43
		78	384	0.67 ± 0.40
Fourth	Aqueous-to-non-aqueous phase volume ratio (v/v)	0.5 : 30 [a]		
		1.0 : 30	231	0.61 ± 0.45
		2.0 : 30 [b]		
Fifth	Stirring rate (rpm)	1900	483	0.54 ± 0.32
		1500	755	0.48 ± 0.29
		950	683	0.51 ± 0.32
		460	603	0.53 ± 0.31
Sixth	Miscellaneous	Standard [c]	346	0.58 ± 0.42
		Heat stabilized (30 min)	468	0.59 ± 0.40
		Ice-cooled	441	0.63 ± 0.44
		Drop rate of 10 ± 5 drops/min	343	0.68 ± 0.44
Seventh	Heat stabilization temperature (°C)	165	441	0.58 ± 0.36
		145	507	0.49 ± 0.37
		125	503	0.54 ± 0.35
		105	405	0.60 ± 0.37
Eighth	Non-aqueous phases	Cottonseed oil	462	0.56 ± 0.33
		Maize oil	304	0.71 ± 0.45
		Paraffin oil	218	0.82 ± 0.82
		Octanol [d]		

[a] Due to the problems encountered using a 1 : 30 and 2 : 30 ratio, the preparation of microspheres using a 0.5 : 30 ratio was not warranted on this day.

[b] Microspheres were not recovered at this phase volume ratio.

[c] Standard conditions were: emulsion added at 40 ± 10 drops/min, heat stabilized for 10 min and allowed to cool at room temperature.

[d] Particle size was not determined due to the erratic nature of the microspheres.

termined at that level which produced the smallest arithmetic mean diameter.

As albumin concentration was the first variable studied, four batches of microspheres were prepared by adding 0.5 ml of a 500 mg/ml, 400 mg/ml, 300 mg/ml or 200 mg/ml albumin solution to 30 ml of cottonseed coil at 4°C. At this point the initial method of preparation (see above) was followed. Since an albumin concentration of 500 mg/ml produced the smallest mean particle diameter (see Table 1), it was used for all subsequent variables studied. Therefore, to study the next variable, emulsification time, four batches of microspheres were prepared using 0.5 ml of a 500 mg/ml albumin solution and emulsification times of 1, 2, 5 or 10 min with a 125 W power setting. As each new variable was studied, the initial method of microsphere preparation (see above) was altered in accordance with the results on the optimum level of each preceding variable. This type of experimental design ignores interactions between the variables. For example, microspheres were not prepared using a 300 mg/ml albumin concentration and emulsification times of 1, 5 and 10 min since the 500 mg/ml albumin concentration was optimum.

Particle size analyses were done with a scanning electron microscope. About 3 drops of the microsphere suspension in ether were placed on an electron microscope stub and the ether allowed to evaporate. The stubs were placed briefly in a critical point dryer briefly and then coated with 300 Å of gold. Pictures of the microspheres were taken by random scanning of the stub. The diameter of the microspheres were manually measured from the resultant photomicrographs, about 500 particles were counted per batch. Particle size distributions were calculated according to a method by Allen (Allen, 1981).

Results and Discussion

Table 1 summarizes the arithmatic mean particle size diameter and standard deviations for all batches prepared. In general, the differences in the mean diameter and standard deviations between batches are small regardless of the variable studied. Although a monodisperse particle size distribution may be considered as important as the absolute size, it can be seen that in most cases the smallest mean diameter batch has the smallest standard deviation.

The original interest in studying albumin concentration stemmed from the fact that the emulsifying agent concentration has an effect on emulsion droplet size and stability (Ishizaka et al., 1981; Higuchi et al., 1962; Florence et al., 1975). Specifically, various proteins have been used as stabilizing agents for o/w emulsions due to their ability to be adsorbed at the interface (Graham and Phillips, 1976; Kitchner and Mussellwhite, 1968). In our system, it was believed that, upon emulsification, the albumin at the w/o interface would unfold with the hydrophobic groups exposed to the non-aqueous medium, and the hydrophillic groups oriented towards the aqueous phase. Such a protein denaturation process will render the microspheres hydrophobic in nature. In this case the albumin would act as an emulsifying agent and thus its concentration might affect the droplet size and stability of the emulsion. Fig. 2 shows the particle size distribution of the resultant microspheres at different

albumin concentrations. An increase in albumin concentration tends to produce a smaller microsphere. Although the differences are minor (see Table 1), the use of a higher albumin concentration has the added advantage of yielding a greater quantity of microspheres per batch.

It is well documented that the power and time of emulsification, and the phase volume ratio will affect the droplet size of emulsions, and therefore, the resultant microsphere size (Eberth and Merry, 1983; Li and Fogler, 1978; Becher, 1965). Figs. 3 and 4 show the particle size distributions for different emulsification times and powers. The largest effect on the particle size distribution for different emulsification times was seen in going from 1 to 2 min. It was felt that longer emulsification times would produce a narrower size distribution, but this was not seen. The power of emulsification had a relatively large effect on the particle size distribution, with the highest available power producing the smallest particle size (see Table 1).

Higher phase volume ratios were studied to see if larger amounts of microspheres could be recovered without increasing the particle size relative to the 0.5 : 30 phase volume. At the higher phase volume ratios the emulsion appeared to form, however, microspheres were not recovered at the 2 : 30 level. The problem with the higher ratios became apparent in the washing procedure. In the 2 : 30 phase volume ratio batch, the 'microsphere' pellet contained large pieces of protein material seemingly with some entrapped oil. This pellet could not be resuspended. At the 1 : 30 level, a microsphere pellet was obtained but large proteinaceous pieces were lost upon decanting the ether. No doubt these pieces also contained some oil.

Stirring of the oil phase during the addition of the emulsion, heat stabilization

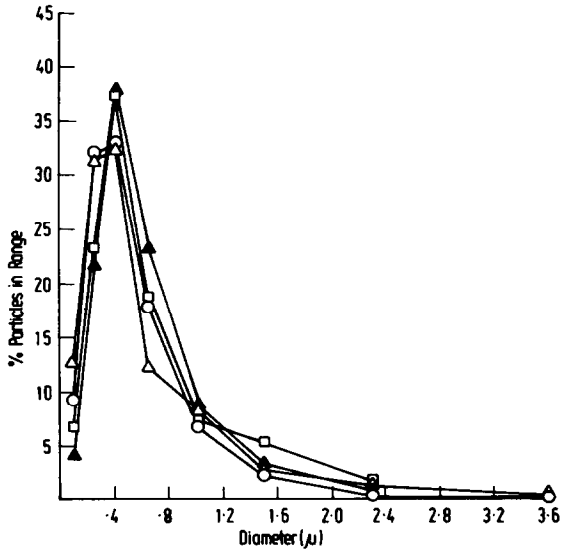

Fig. 2. Particle size distribution of albumin microspheres prepared at different albumin concentrations. ○, 500 mg/ml; △ 400 mg/ml; ▲, 300 mg/ml; □, 200 mg/ml.

and the cooling process are necessary to prevent agglomeration of individual aqueous albumin droplets and the resultant microspheres. The mean diameters and standard deviations are the most similar of all the variables studies (see Table I). Under the heat stabilization variables, more subtle factors were examined. An

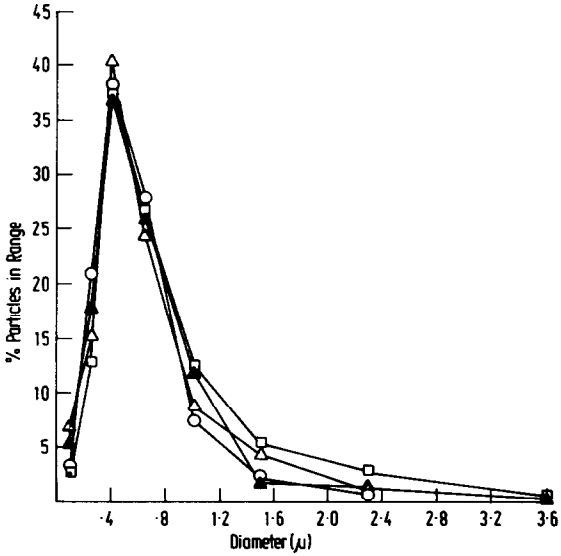

Fig. 3. Particle size distribution of albumin microspheres prepared at different emulsification times. □, 1 min; ○, 2 min; △, 5 min; ▲, 10 min.

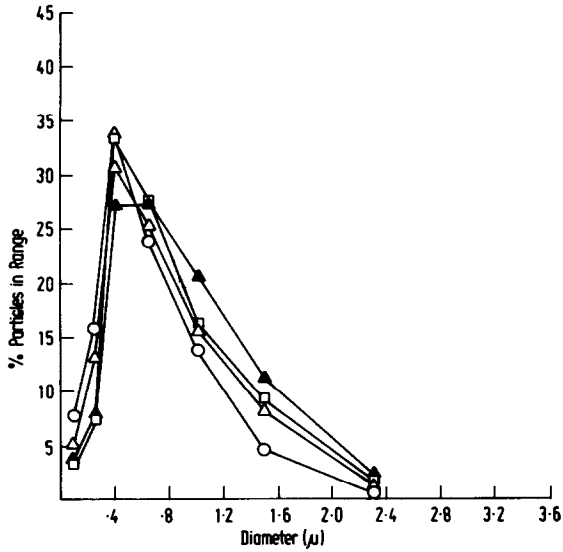

Fig. 4. Particle size distribution of albumin microspheres prepared at different emulsification powers. ○ 125 W; △, 109 W; ▲, 94 W; □, 78 W.

increase in the heat stabilization time from 10 to 30 min produced a negligible change in the mean particle size. A slower drop rate of 10 drops/min caused a relatively large increase in the mean size compared to the standard drop rate of 40 drops/min. The lower drop rate increases the time required to add the emulsion to the heated cottonseed oil allowing the emulsion temperature to rise by heat transfer from the heated flask. As the emulsion temperature increases, coalescence of the dispersed albumin droplets will occur leading to larger microspheres. A cold water-jacketed dropping funnel might prevent temperature-dependent aggregation of the internal phase. A higher drop rate would make temperature control of the non-aqueous phase difficult.

Ice cooling of the heat stabilized microsphere suspension was tried in an attempt to save time. The normal cooling process takes about 90 min while ice cooling only took 15 min. Except for the small difference in mean particle size the characteristics of the ice-cooled microspheres were the same. However, a smaller diameter was achieved by allowing the microspheres to cool via room temperature.

The effect of the heat stabilization temperature on microsphere size is probably due to the rate and extent of water evaporation from the internal phase. Fig. 5 shows the particle size distribution for the different temperatures. A heat stabilization temperature of 105 °C produced the largest mean particle size indicating that water may still be trapped in the albumin matrix (see Table 1). The relatively larger particle size at 165°C is difficult to explain. One possible explanation is that the albumin molecules at the w/o interface solidify instantaneously upon contacting the heated oil while the water evaporates. This may prevent any albumin shrinkage that could occur at lower temperatures.

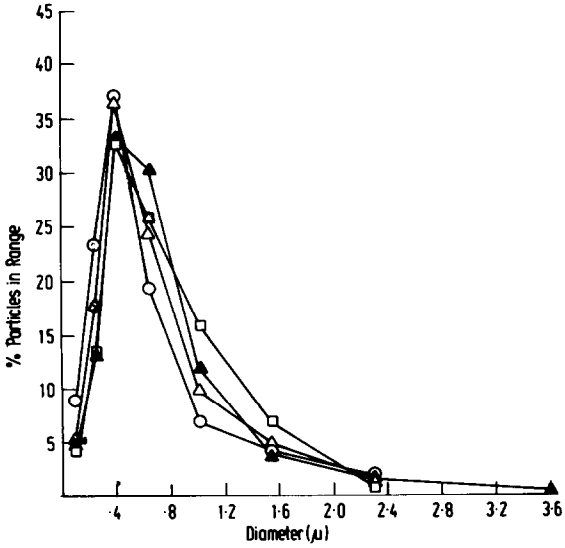

Fig. 5. Particle size distribution of albumin microspheres prepared using different heat stabilization temperatures. ▲, 165°C; ○, 145°C; △, 125°C; □, 105°C.

Alternate non-aqueous phases were tried to demonstrate if, in fact, other phases could be utilized for microsphere preparation. The non-aqueous phases were substituted for cottonseed oil in both the emulsion and heat stabilization processes. The choice of maize oil, paraffin oil and 1-octanol represent a range of viscosities and all are available at a reproducible quality. Also, the use of a more hydrophilic non-aqueous phase such as octanol was appealing from the standpoint that its hydrophilicity might be imparted to the microspheres. Longo et al. (1982) have suggested that part of the hydrophobic nature of albumin microspheres is due to surface binding of the oil at elevated temperatures. If more hydrophilic microspheres could be produced with octanol then the use of surfactants to suspend the microspheres in aqueous solutions may not be necessary. Fig. 6 shows the particle size distributions for the different non-aqueous phases and Fig. 7 shows octanol-prepared microspheres. The microspheres obtained with octanol appeared larger and more variable than those produced by the other non-aqueous phases. There also appears to be some non-spherical denatured protein material, possibly due to the greater solubility of the albumin solution in octanol compared to the other non-aqueous phases. Due to the erratic nature of the octanol microspheres, no particle count was done. Larger particle sizes and standard deviations were obtained with maize oil and paraffin oil, relative to cottonseed oil (see Table 1). Although it might be easy to disregard the use of non-aqueous phases differing in nature to cottonseed oil, it should be remembered that all variables listed optimized microsphere production using cottonseed oil.

The optimal procedure for preparing albumin microspheres differs from the

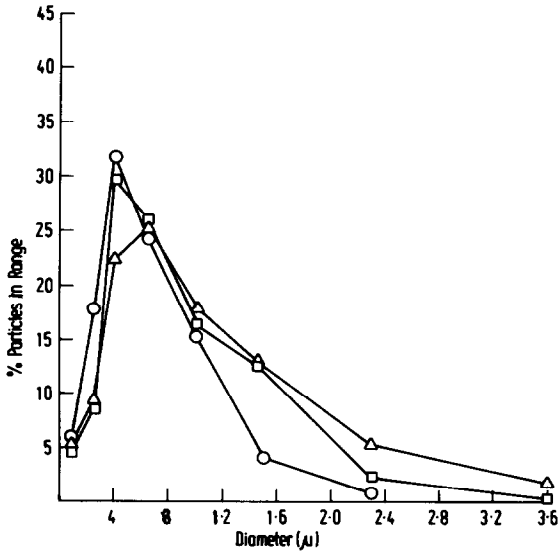

Fig. 6. Particle size distribution of albumin microspheres prepared using different non-aqueous phases. ○, cottonseed oil; □, maize oil; △, light paraffin oil.

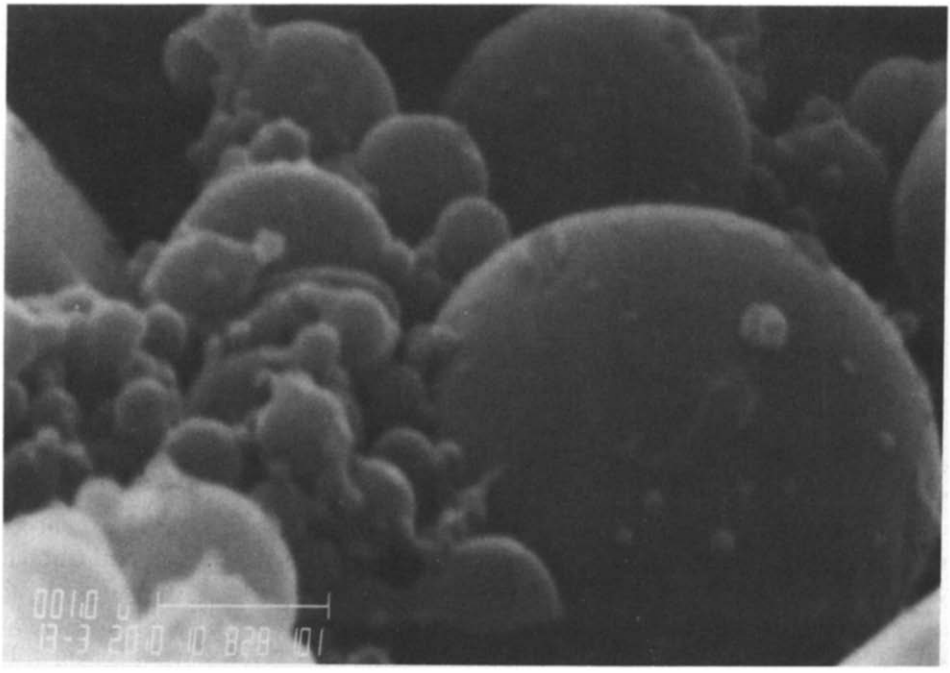

Fig. 7. Scanning electron microscope photograph of albumin microspheres prepared using octanol. (magnification = 13,000×; note 1.0 μm line.)

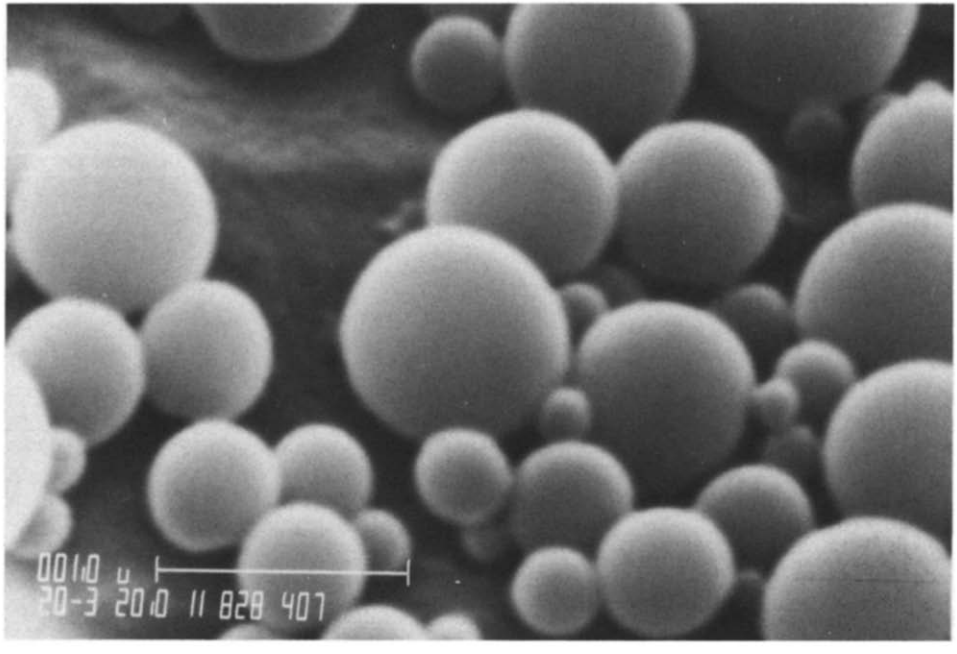

Fig. 8. Scanning electron microscope photograph of albumin microspheres prepared by optimal method. (magnification = 20,000×; note 1.0 μm line.)

initial procedure only by using a 500 mg/ml albumin solution and a heat stabilization temperature of 145°C. Fig. 8 shows microspheres prepared by this method. During this analysis, there were 7 batches prepared by the same method. The mean particle diameter and standard deviation among these seven batches is 0.51 ± 0.05 μm, which gives a between day coefficient of variation of 9.8%.

Overall, it can be said that the procedure for preparing albumin microspheres can withstand quite a large range of variability. It should be realized that this method and criteria for optimal microsphere production may need to be altered depending on the physical set-up of the apparatus and the intended use of the product.

Acknowledgements

We would like to thank the Department of Pathology for the use of their scanning electron microscope laboratory and the Department of Clinical Biochemistry for the use of their laboratory facilities.

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