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Particle size and size distribution of albumin microspheres produced by heat and chemical stabilization

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Summary

Albumin microspheres of sizes ranging from 1 to 32 μm were prepared by an oil-in-water emulsification method. The effect on mean size and size distribution of different variables at two levels was studied according to a factorial design of experiments. The following variables were studied: (1) type of albumin ; (2) albumin concentration; (3) speed of agitation; (4) chemical cross-linking or heat denaturation; (5) glutaraldehyde concentration or temperature; (6) addition or absence of surfactant (Span 85); (7) type of oil; and (8) mixing-cell with or without baffles. Particle size analysis was performed on the resultant microspheres using a laser diffraction technique. The effect of each variable and possible interactions between the variables on microsphere size are discussed.

Introduction

The selective delivery of drugs to specific target sites or organs in the body would reduce the systemic dose of a given drug while still achieving an effective local concentration. This would result in a reduction of unwanted side-effect and adverse reactions (Davis et al., 1985). Drug targeting can be achieved by a variety of different approaches. One approach to targeting chemotherapeutic agents is the inclusion of the agents within carriers (Illum and Davis, 1982). Colloidal carriers have the advantage of being able to entrap relatively large amounts of pharmacological agent and are relatively easy to prepare.

The factors which can modify the localisation and distribution of particles in the body are (Davis and Illum, 1986): the route of administration; the particle size; and the particle surface characteristics.

Depending on the surface characteristics, colloidal carriers can be recognised and subsequently taken up by various cells of the reticuloendothelial system, especially those residing in the liver. Consequently, the possibility of altering the surface characters of particles in order to influence the targeting ability is an attractive area of research (Illum and Davis, 1987; Illum et al., 1987a).

Colloidal drug delivery systems have been proposed for application to various anatomically discrete sites to include: intra-articular, subcutaneous and intramuscular injection of therapeutic and diagnostic agents, ocular and nasal therapy and chemoembolization (Tomlinson, 1983; Li et al., 1987; Ratcliffe et al., 1987; Illum et al., 1987b; Douglas et al., 1985). There is an optimal particle

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size for each proposed application and route of administration. For example, after intravenous administration, large particles (≥ 7 μm) will be trapped by mechanical filtration in the capillary beds in the lungs while particles smaller than about 5 μm normally will be taken up mainly by the phagocytic cells of the liver and spleen (Kanke et al., 1980; Illum and Davis, 1987; Illum et al., 1987a). Furthermore, it is important to control the size of the particles and their size distribution because both can have an important effect on drug release (Sheu and Sokoloski, 1986).

Delivery systems based upon albumin microspheres seem particularly advantageous since they utilize a natural product, which, if not altered to any significant extent, should result in a product that is both biocompatible and biodegradable (Sokoloski and Royer, 1984).

Different factors, which can modify the size of the albumin microspheres produced by the emulsification method, have been studied recently (Ishizaka and Koishi, 1981; Tomlinson et al., 1982; Tomlinson and Burger, 1985; Gallo et al., 1984; Sheu and Sokoloski, 1986). Unfortunately, the results of these different studies are not always consistent because different workers have used different techniques and conditions to produce the albumin microspheres. If albumin microspheres of defined sizes are required for medical application, it is first necessary to study, in a systematic way, the relevant variables. To this end we have chosen a factorial design. Factorial designs are widely used in experiments involving several factors where it is necessary to study the joint effect of these factors on a response. The differences between such a factorial arrangement and the more usual "one-factor-a-time" method is that in the factorial design of experiment the variables can be changed concurrently. Thus, if the variables act additively, the factorial design provides a more precise answer. If the variables do not act additively, the factorial design can detect and estimate the interactions (Box et al., 1978).

Emulsion and suspension technology are employed as techniques for the manufacture of albumin microspheres (Gallo et al., 1984) and the variables are so numerous that it is very difficult to perform a complete investigation. Consequently in the present work the following 8 factors have been selected as being the most important variables in the production of albumin microspheres.

(1) Type of albumin.
(2) Albumin concentration.
(3) Speed of mechanical agitation.
(4) Chemical cross-linking with glutaraldehyde or physical modification by heat.
(5) Different glutaraldehyde concentration or different temperature.
(6) Surfactant (with or without Span 85).
(7) Type of oil.
(8) Mixing-cell with or without baffles.

Other variables, not studied in the present work that could have an influence include: concentration of added drug and the size of the drug particle if it is added as a suspension ("seed effect"), oil and aqueous phase proportion, the use of organic polymer solutions (i.e. PEG solutions) proposed for the production of relatively hydrophilic albumin microspheres (Longo and Goldberg, 1985) and added organic solvents.

Materials and Methods

Materials

Ovalbumin (grade II) was purchased from the Sigma Chemical Co. (Dorset, U.K.). Human serum albumin was a gift from Rhone-Poulenc Farma S.A.E. (Madrid, Spain). All other chemicals were of reagent grade.

Methods

Production of albumin microspheres

Albumin microspheres stabilized by chemical denaturation. Albumin microspheres were produced by a modification of the method described by Ratcliffe et al. (1984). One ml of different concentrations (5% or 15%, w/v) of human serum albumin or ovalbumin at pH 6.8 was added to 25 ml of olive oil or light mineral oil with or without 0.25 ml of Span 85. The mixture was stirred in a baffled or non-baffled mix-cell for 10 min, under turbulent flow conditions to form a w/o emulsion, using a mechanical stirrer (Heidolph) at 180 or 775 rpm (Tachometer DOT 1, Compact Instru-

ments). Glutaraldehyde solution 25% (w/v) was added to 1% or 3.6% (v/v) of aqueous phase and the emulsion stirred for a further 30 min to denature and cross-link the albumin. The microspheres were collected by centrifugation at 2500 *g* for 20 min. The oil was then removed and the spheres washed with diethyl ether followed by ethanol. The microspheres were collected by decantation.

Heat-stabilized albumin microspheres. Microspheres were prepared by the same method described above, but instead of glutaraldehyde the reaction vessel was immersed in a thermostatted bath maintained at 70 °C or 100 °C for 30 min in order to denature the albumin.

Particle size analysis. All particles were sized on the same day of manufacture using a laser diffraction technique (Malvern Particle Sizer Type 2600D, Malvern Instruments, U.K.). The particles were resuspended in water and sonicated for 2 min at 60 W before counting. The size distribution (polydispersity) was measured as in terms of a SPAN factor expressed as:

$$\text{SPAN} = \frac{D_{90\%} - D_{10\%}}{D_{50\%}}$$

where $D_{90\%}$, $D_{50\%}$ and $D_{10\%}$ are the diameters where the given percentage of particles is smaller than that size.

A high value of SPAN indicates a wide distribution in size and a high polydispersity.

Viscosity. A rheometer model RM 15 from Contraves A.G. (Switzerland) was used to measure the viscosity of the oil phase at either room temperature (20 °C) or 100 °C.

Factorial design of experiments

In order to perform a general factorial design, a fixed number of "levels" for each of a number of chosen variables (factors), and then experiments, were conducted with all possible combinations.

The election of the factors (variables) and levels was important. The 8 variables listed previously were chosen, with each variable occurring at two suitable levels. This design requires relatively few runs per factor studied and although it is unable to explore fully a wide region in the so-called "factor space", it can indicate major trends and so determine a promising direction for further experimentation (Daniel, 1976; Montgomery, 1976; Box et al., 1978).

TABLE 1

Factors and levels studied in the preparation of albumin microspheres using the emulsion technique

Factors	Code of factors	Levels	Levels coded *
Type of albumin	(1)	Ovalbumin	(−)
		Human albumin	(+)
Albumin concentration	(2)	5%	(−)
		15%	(+)
Speed	(3)	180 ± 25 rpm	(−)
		775 ± 40 rpm	(+)
Denaturation process	(4)	Chemical cross-linking with glutaraldehyde	(−)
		Physical – with heat	(+)
Conditions Glutaraldehyde	(5)	1.5% (w/v)	(−)
		5.3% (w/v)	(+)
Heat		70 °C	(−)
		100 °C	(+)
Surfactant **	(6)	–	(−)
		1%	(+)
Type of oil	(7)	Olive oil	(−)
		Light mineral oil	(+)
Mixing-cell	(8)	Without baffles	(−)
		With baffles	(+)

* For the quantitative variables a minus sign represents the low level, and a plus sign the high level.
** Span 85 expressed as % of the oil phase.

The factors and levels that were studied in the preparation of albumin microspheres produced using the emulsion technique are summarized in Table 1. This experimental design required 2^8 runs (in duplicate to provide standard errors). Thus $2 \times 2^8 = 512$ experiments are required to perform a complete study.

In order to decrease the number of experiments, the study was divided in 4 blocks. In each block some variables were held as constants and the effect of others was then studied. The experi-

TABLE 2

Experimental conditions and summary of results for albumin microspheres produced by chemical or physical denaturation

Experimental conditions										Results			
Block	Variables								Number of experiments in each block	Size (μm)		SPAN	
	1	2	3	4	5	6	7	8		Mean *	Range ***	Mean	Range
1	−	−	−	−	−	−	−	−	64	4.2 (0.7)	1.9– 9	2.3 (0.6)	1.1–8
	+	+	+		+	+							
2	−	−	−	+	−	−	−	−	64	4.7 (1.1)	2.1–11	3.4 (0.7)	1 –9
	+	+	+		+	+							
3	+	−	−	−	−	−	−	+	32	13 (2.5)	1.4–32	2.8 (0.6)	1.6–5.6
		+	+			+	+						
4	+	−	−	+	+	−	−	+	32	6.1 (0.9)	1.3–13	3.8 (0.5)	1.4–8
		+	+			+	+						

* The variables are given in Table 1.

** In parentheses are the standard errors calculated according to Box et al. (1978) for factorial design of experiments using replicated runs.

*** Maximum and minimum results obtained in the experimental conditions of each block of experiments.

mental conditions are shown in Table 2. For example, in the first block of experiments, the following were held constant: chemical denaturation, olive oil as oil phase and mixing-cell without baffles (factors 4, 7 and 8) while the type and concentration of albumin, speed of agitation, concentration of glutaraldehyde and the effect of addition of surfactant (variables 1, 2, 3, 5 and 7) were studied in 64 experiments.

Results and Discussion

A summary of results from the 192 experiments, in terms of the mean size (diameter) of the resultant microspheres, the SPAN and the range obtained in each block, is given in Table 2. The standard errors for the mean sizes have been calculated from Box et al. (1978) for a factorial design of experiments using replicated runs.

The effect of factors and interactions between the different factors in the factorial design of experiments can be calculated using a so-called table of contrast coefficients (Daniel, 1976; Montgomery, 1976; Box et al., 1978). The simple effects and their interactions can be plotted on normal probability paper and a test of significance can be applied directly (Figs. 1, 2). If there are no significant effects, a straight line should be obtained. The distribution of the effects indicates that if they are on the right side of the line then the change of level in each factor (from minus to plus) (see Table 1) increases the size of the microspheres and if the effects are on the left side they indicate a decrease of size. The statistical differences were assessed using the Student's *t*-test. For instance, in Fig. 1A the change of albumin concentration (factor 2) from 5% to 15% significantly increased the size of the microspheres ($P < 0.01$).

In our results the factors that modify the SPAN (polydispersity) are the same as those that can lead to an alteration in size.

The various studied factors that could modify the mean size and size distribution will be discussed in turn.

The type and concentration of albumin (factors 1 and 2)

We have used ovalbumin (mol. wt. 44,000) and human serum albumin (mol. wt. 67,000) which are proteins with different structures and some differences in their properties. The sizes of the microspheres produced with human albumin are some-

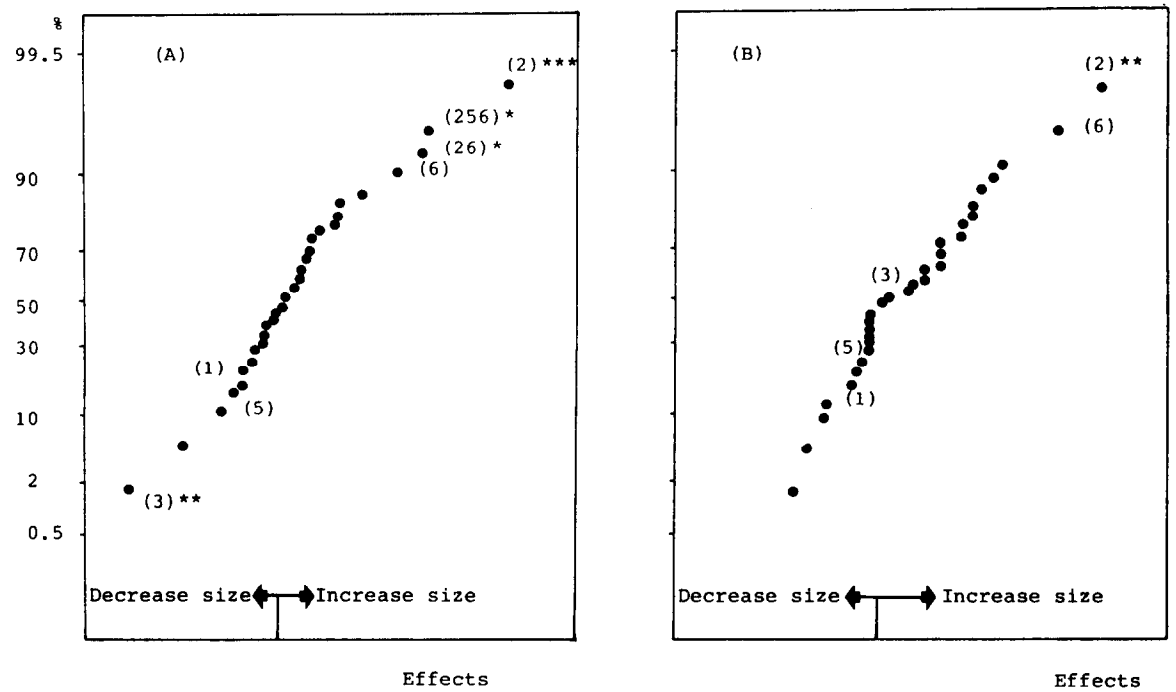

Fig. 1. Plot of effects on normal probability paper of albumin microspheres produced with olive oil as the oil phase and a non-baffled mixing-cell under the experimental conditions (see Table 1) of: (A) block 1, by chemical cross-linking; (B) block 2, by heat denaturation. Key of factors: (1) type of albumin; (2) albumin concentration; (3) speed; (5) glutaraldehyde concentration (Fig. 1A) or temperature (Fig. 1B); (6) surfactant. Significance: *** $P < 0.01$; ** $P < 0.05$; * $P < 0.1$.

what smaller (factor 1 in Fig. 1A and B) and the size distribution narrower, than those prepared with ovalbumin. These small differences are probably due to the better dissolution properties of the human albumin.

An increase in albumin concentration (factor 2 in Fig. 1A and B) gives rise to an increase in both the mean size and the size distribution of the prepared microspheres by cross-linking reaction and heat denaturation in non-baffled systems. It is known that albumin microspheres increase in size with increasing albumin concentration (Tomlinson and Burger, 1985; Burger et al., 1985). This effect can probably be attributed to a higher relative viscosity of the protein solution (Ishizaka and Koishi, 1981).

There are two aspects to consider when attempting to exploit these results to obtain larger microspheres. One is the broad size distribution obtained with high albumin concentration, especially in non-baffled systems, and the other is the observed experimental "interactions" in a non-baffled system between the presence or absence of surfactant and albumin concentration when the chemical cross-linking method is applied (factors 256 and 26 in Fig. 1).

In a baffled system the influence of albumin concentration is less important (factor 2 in Fig. 2) than in a non-baffled system (Fig. 1). So, in order to scale-up the process and increase the yield of the albumin microspheres by employing higher amounts of albumin, it is preferable to work with baffled systems to avoid undesirable modifications of size and size distribution.

Speed (factor 3)

Previously, a faster stirring speed has been used to produce smaller albumin microspheres (Ratcliffe et al., 1984; Tomlinson and Burger, 1985; Tomlinson et al., 1982; Burger et al., 1985). In order to obtain large albumin microspheres we have chosen relatively slow speeds (180 and 775 rpm). Figs. 1A, 2A and 2B show that a faster stirring speed significantly decreases microsphere

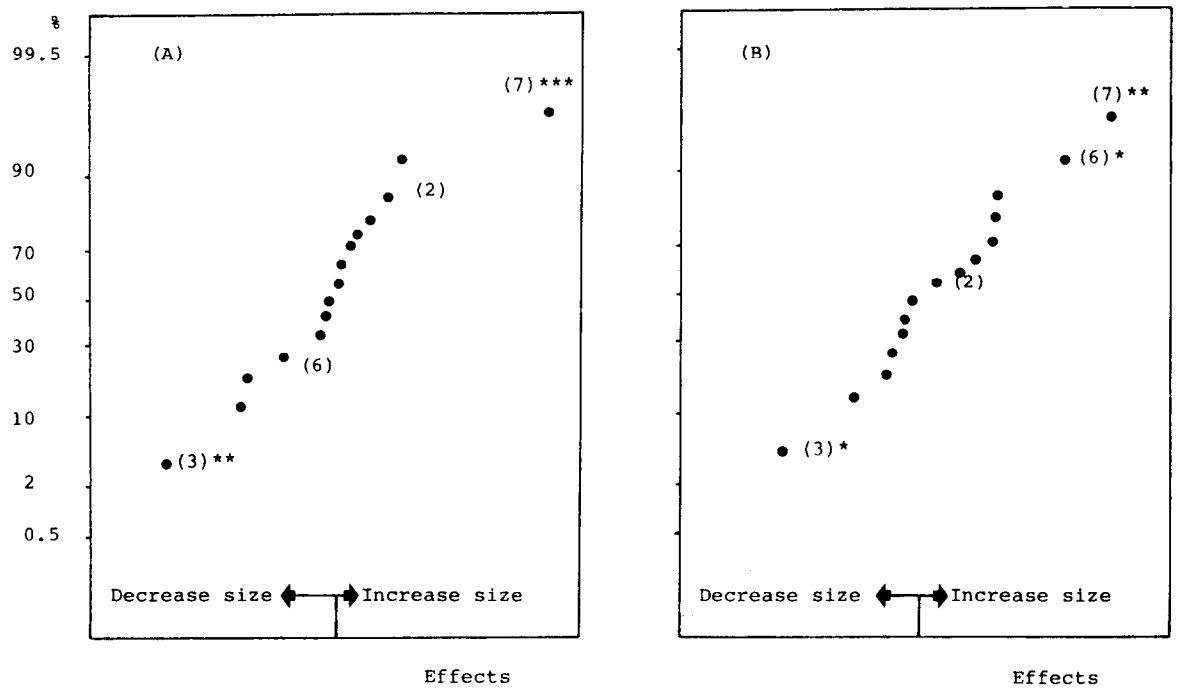

Fig. 2. Plot of effects on normal probability paper of albumin microspheres produced with human serum albumin, 1% glutaraldehyde as denaturating agent and a baffled mixing-cell under the experimental conditions (see Table 1) of: (A) block 3, by chemical cross-linking; (B) block 4, by heat denaturation. Key of factors: (2) albumin concentration; (3) speed; (6) surfactant; (7) type of oil. Significance: *** $P < 0.01$, ** $P < 0.05$, * $P < 0.1$.

size. Only for heat-stabilized microspheres in a non-baffled system (Fig. 1B) was size not modified significantly when the speed was altered. A similar effect has also been mentioned by Gallo et al. (1984).

Fig. 3 shows the different size and size distributions of albumin microspheres produced at different agitation speeds. In our experimental conditions the agitation speed was the most critical factor affecting size distribution. The size distribution curve became narrower and sharper with increased stirring speed as has been reported previously (Ishizaka and Koishi, 1981; Sheu and Sokoloski, 1986).

The denaturation process (heat or cross-linking) (factor 4) and the condition of the denaturation process (glutaraldehyde concentration or temperature) (factor 5)

It is possible to compare the effect of different denaturation processes (heat or chemical cross-linking) by examining the mean and range of size in the different experimental blocks (Table 2). The condition of the denaturation process is the only variable that is different between blocks 1 and 2, and between blocks 3 and 4 of the experiments. The experimental results of block 1 (chemical

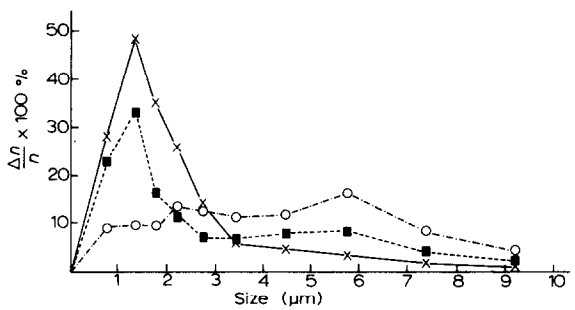

Fig. 3. Different size and size distribution of heat-stabilized albumin microspheres produced at different stirring speeds in a baffled system, Δn is the number of particles in each size increment and n is total number of particles measured. Speeds: * = 1000 rpm; ■ = 775 rpm; ○ = 176 rpm.

cross-linking) are very similar to block 2 (heat denaturation) but the results of block 3 are different to block 4. These differences are particularly important in the highest values of the size range of experimental block 4 which are smaller than those of block 3 (13 and 32 μm, respectively). The difference is probably attributable to the use of light mineral oil in the production of microspheres at different temperatures (20 °C in chemical and 100 °C in heat denaturation process). The change in temperature not only affects the aqueous phase, in which the albumin denatures and becomes solid, but also modifies the oil phase. In our experimental conditions the viscosity of light mineral oil was 25 cps at 20 °C but changed to 6 cps at 100 °C. These combined changes make the dispersion of the aqueous phase in the system (emulsion at the beginning and suspension at the end of the process) more difficult and lead to the formation of a precipitate of small particles (< 5 μm) in the bottom of the mixing cell. The size of these particles is very different to the size of the microspheres produced by chemical cross-linking in the more homogeneous system obtained at 20 °C.

The use of different glutaraldehyde concentrations to produce microspheres (factor 5 in Fig. 1A) did not have a significant effect on the size. The same observation has been mentioned by Tomlinson and Burger (1985) and Sheu and Sokoloski (1986). However, it is important to note that the use of a high glutaraldehyde concentration to modify the release of the drug from the microspheres can produce problems in the subsequent redispersion of albumin microspheres stored as a dry solid.

Different temperatures (70–100 °C) did not produce significant differences in size (factor 5 in Fig. 2) but it was observed that 70 °C was possibly too low a temperature to stabilize human albumin microspheres and higher temperatures (between 90 and 170 °C) are proposed for further experiments.

Surfactant (factor 6)

It is not easy to compare the present results with those of other authors since the nature of the surfactant, its concentration and other experimental conditions were not the same. It is known that alteration of the particle size of the dispersed phase during manufacture leads to a corresponding alteration in the size of the formed microspheres (Burger et al., 1985; Fujimoto et al., 1985). Added surfactants decrease the size of the albumin solution droplets in the emulsification step and this effect has been exploited in order to obtain smaller albumin microspheres (Ishizaka and Koishi, 1981; Ratcliffe et al., 1984). However, the effect of surfactant on albumin microsphere production is not straightforward since surfactants can cause nucleation and aggregation effects.

Fig. 1A shows how, in a non-baffled system, factor 6 (Span 85) can increase slightly (not significantly) the size of the albumin microspheres, but that it can also be the origin of significant ($P < 0.1$) interactions with albumin and glutaraldehyde (factors 256 and 26 in Fig. 1).

Fig. 2B shows a significant increase in size ($P < 0.1$) and size distribution ($P < 0.1$) when Span 85 is used in heat-stabilized albumin microspheres produced in a baffled system.

In order to clarify this unexpected effect of the Span 85 in the production of albumin microspheres, the size of the original emulsion drops was measured using an optical microscope for different Span 85 concentrations (unpublished data). As was expected, the emulsion droplet size decreased with increase in the surfactant concentration. However, the size and size distribution of the resultant albumin microspheres increased with the surfactant concentration. This discrepancy can probably be attributed to an aggregation effect caused by the Span 85. A similar effect has been reported recently for casein microspheres produced using Span 80 as the surfactant (Chen et al., 1987).

Type of oil (factor 7)

Under our experimental conditions the oil was the most critical factor affecting the size of the albumin microspheres produced by the emulsion technique (factor 7 in Fig. 2A and B). When light mineral oil was used instead of olive oil as the oil phase of the emulsion, the size increased significantly for both: chemically stabilized ($P < 0.01$) and heat-stabilized microspheres ($P < 0.05$). In our experiments the viscosity at 20 °C was 76 cps for

olive oil and 25 cps for light mineral oil. Data on interfacial tension have been given by Sheu and Sokoloski (1986). Decreasing viscosity and increasing interfacial tension (through the use of light mineral oil) brings about a larger particle size for the albumin microspheres, as would be expected for a disruptive manufacturing process. Similar results have been reported earlier (Gallo et al., 1984; Burger et al., 1985; Tomlinson and Burger, 1985).

Mixing-cell with or without baffles (factor 8)

A non-baffled mixing-cell system was used in the experiments in blocks 1 and 2 (factor 8 in Table 2) and a baffled mixing-cell in blocks 3 and 4. Fig. 1 (without baffles) and Fig. 2 (with baffles) show how the effect of the different variables is not the same with the different cells. For example, the albumin concentration (factor 2) had a critical effect on mean size and polydispersity for a non-baffled mixing-cell (Fig. 1) but was not so important for the baffled system (Fig. 2).

It is also interesting to note that a mixing-cell with baffles has been found by others to be critical for the preparation of uniform and reproducible microspheres (Tomlinson et al., 1982; Tomlinson and Burger 1985; Burger et al., 1985). In our studies the advantage of using a mixing-cell with baffles, as compared to one without baffles, was that the size distribution was narrower at faster stirring speeds and it was possible to work at higher albumin concentrations without producing microspheres with a wide size distribution.

It can be concluded from the present studies that by adjusting different experimental variables it is possible to obtain albumin microspheres with a mean size between 1 and 3 μm. Under our experimental conditions the nature of the oil was found to be the main factor which can affect the size of microspheres and the most critical factors affecting the size distribution were the design of the mixing-cell, agitation speed and protein concentration.

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