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Properties of Human Albumin Microparticles Prepared by a Chilled Cross-linking Technique

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Abstract—Examination of conditions needed to form albumin microparticles (in the 1–5 μm diam. size range) by glutaraldehyde cross-linking, at low temperature (−15°C), suggested that the particles formed very rapidly over a short time scale and at low concentrations of cross-linking reagent. Detailed analysis showed that the particles increased in size with time of reaction and with an increase in glutaraldehyde concentration. Evaluation of the particle matrix using a dilute trypsin digestion process suggested that the above factors may influence the internal composition since the particles dissolved less rapidly than might be anticipated from a consideration of particle diameter alone.

The ideal dosage form in cancer chemotherapy is the one that provides a specific delivery of anticancer agent to the tumour site in a sufficient amount, for a long period of time with no interaction with the normal tissue (Gregoriadis 1977). Targeting of microparticulate drug carriers to extravascular tumours is considered to require particles in the submicrometer size range (Illum & Davis 1982) and these colloidal drug carriers have been described by others (Ratcliffe et al 1984; Laakso et al 1987). Microspheres as sustained release targeting agents for antitumour drugs have received attention during recent years, and albumin, gelatin and polylactic acid have all been evaluated (Yoshioka et al 1981; Tsai et al 1986; Willmott & Harrison 1988). The use of albumin microspheres in drug delivery was first suggested by Kramer (1974). Several methods have been reported in the literature for the preparation of albumin microspheres (Gallo et al 1984; Burgess & Davis 1986; Gupta et al 1986a, b). Most methods involve the application of suspension and emulsion technology. Factors involved in the formation of beads in the emulsification process were studied by Lee et al (1981), Sheu et al (1986) and Sheu & Sokolowski (1986). However, studies of the in-vitro disintegration of microspheres are limited (El-Samaligy & Rohdewald 1983; Ratcliffe et al 1984; Tabata & Ikada 1989).

In this study, albumin microparticles were prepared by a method recently described by us for gelatin microparticles (Öner & Groves 1993). A 4 × 4 factorial design was used to optimize the formulation of the albumin microparticles. The process involves relatively large volumes of ethanol and aqueous reagents, and for this reason, it is not possible to preload the microspheres with drug. However, since only ethanol and water are used, which are environmentally acceptable, this process appeared to offer the prospect of being readily scaled-up. The loading of albumin microspheres has been discussed by Lewis et al (1992).

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The factors involved in preparing unloaded microspheres between 1 and 5 μm diameter were evaluated and the nature of the internal matrix examined using a tryptic digestion technique. The emulsification process described by Lewis et al (1992) produced albumin microspheres that were completely digested within 1 h at 37°C. Here we used a lower concentration of enzyme in order to slow down the digestion process, thereby enabling differences between samples to be determined.

Materials and Methods

Materials

Human albumin was from Sigma (St Louis, MO, USA), trypsin (0·25%) was from Gibco Laboratories (Grand Island, NY, USA), ethanol (anhydrous reagent grade denatured with 5% 2-propanol), glutaraldehyde (50% w/w in water), Isoton (buffered saline) and sodium metabisulphite were from Fisher Scientific (Itasca, IL, USA) and were used as received.

Preparation of microparticles

Albumin microparticles were prepared by the method described for gelatin microparticles (Öner & Groves 1993). In brief, 5 mL solutions (1% w/w) of albumin in deionized water were slowly dispersed into 500 mL chilled anhydrous ethanol at −15°C. The droplets were then hardened by adding up to 8% w/v glutaraldehyde. The flask containing the microparticles was then transferred to a refrigerator (~4°C) for 12, 24, 36 or 48 h.

Cross-linking was stopped by addition to 1000 mL 5% w/v sodium metabisulphite solution at 4°C. The suspension was vortexed before concentration by filtration with a YM100 membrane (Amicon 8200). Microparticles were washed three times in 0·01 M phosphate buffer (pH 7·2). At this point, they were suspended in deionized water containing 5% mannitol, frozen at −20°C and lyophilized for 48 h (Labconco Freeze Dry System). The freeze-dried product was stored at 4°C.

Size characterization of microparticles

Freeze-dried albumin microparticles were sized using a Coulter-Multisizer II (Coulter Electronics Inc., Hialeah, FL, USA), fitted with a 50 μm orifice. Each microparticle sample was reconstituted and suspended to the appropriate concentration with Isoton before analysis.

In-vitro disintegration of albumin microparticles

Albumin microparticles (50 mg) were dispersed in 20 mL 0·01 M phosphate buffer (pH 7·2) containing 0·1 mg trypsin in screw-capped tubes (20 × 150 mm). The samples were shaken horizontally at the rate of 40 strokes min^{-1} in a shaker bath (Precision Scientific) at 37 ± 0·1°C. The turbidity of the samples was determined as normalized nephelometric turbidity units (NTU%) using a turbidimeter (Cole Parmer Instrument Co., Chicago, IL, USA) after shaking for appropriate time intervals.

Without trypsin the NTU values remained unchanged over a 5-h period, suggesting no disintegration of the microparticles under these conditions.

Experimental design

A 4 × 4 factorial design was used to optimize the formation of albumin microparticles. The two independent variables considered in this study were glutaraldehyde (X_1) and duration of the cross-linking process (X_2). The dependent variables include the mean particle size and the time for 100% disintegration (t_{100}) of the in-vitro disintegration process. The regression coefficients for the equations were calculated by the Graftool, Version 3.3 computer software program (3-D Visions, Torrance, CA, USA), and this program was employed to produce the response-surface diagrams.

Results and Discussion

Conventional disintegration profiles of albumin microparticles in 0·01 M phosphate buffer (pH 7·2) containing trypsin are shown in Fig. 1 and profiles based on a square root time basis are shown in Fig. 2. Table 1 lists the effects produced by different cross-linking times and glutaraldehyde concentrations on the time for 100% disintegration and the mean particle diameter of the formed albumin microparticles.

The significant formulation variables affecting the mean particle size of the droplets investigated here are the time of cross-linking and concentration of the glutaraldehyde cross-linking agent. Surprisingly, the response-surface diagram for these three variables, Fig. 3, suggests that the cross-linking process is essentially dynamic, with the particles becoming larger with both time and increased concentrations of glutaraldehyde. This was unlike the situation observed when making gelatin microparticles under the same conditions (Öner & Groves 1993) where, once formed, the particles remained constant in size. This may be a function of the higher molecular weight of the globular albumin and the subsequent greater sensitivity to structural changes induced by the cross-linking reagent. In the case of gelatin, unless particles had been hardened for a sufficient time, they swelled and dissolved on addition to water. Albumin microparticles, on the other hand, formed very rapidly at low concentrations of reagent and the size increased over a twofold range at the extremes of reagent concentration and reaction time evaluated here. This would therefore allow albumin microparticles of a predetermined size to be readily made by careful selection of conditions.

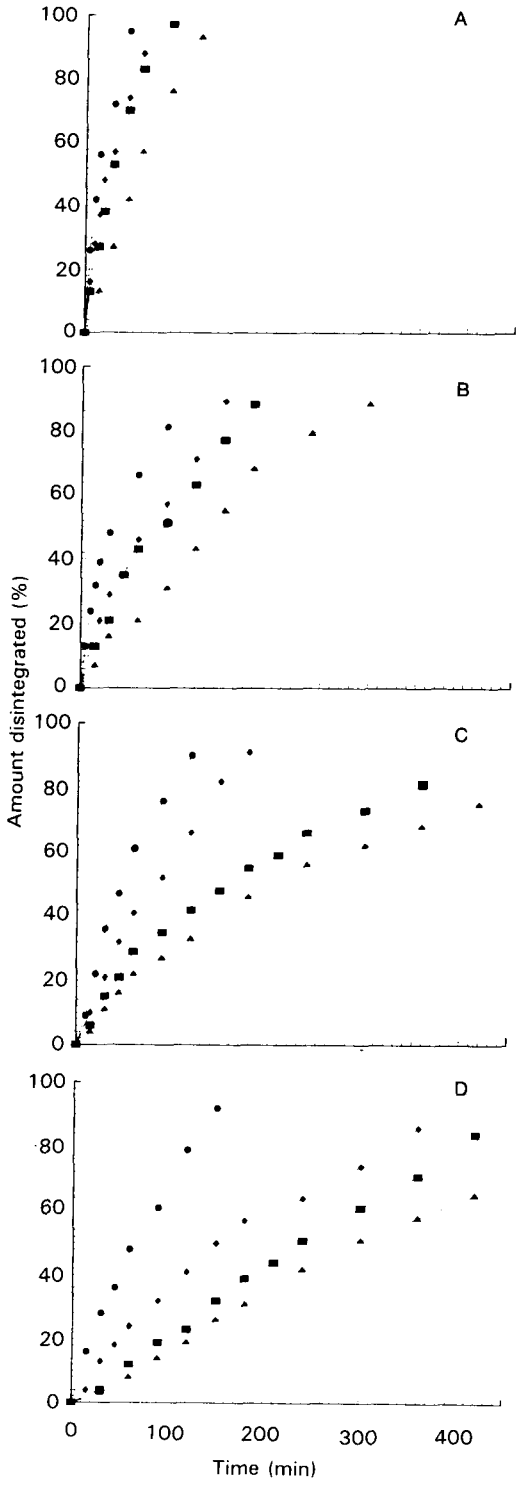

FIG. 1. Disintegration of albumin microparticles in trypsin in 20 mL 0·01 M phosphate buffer (pH 7·2). Duration of cross-linking: A 12, B 24, C 36, D 48 h. ● 2, ◆ 4, ■ 6, ▲ 8% glutaraldehyde.

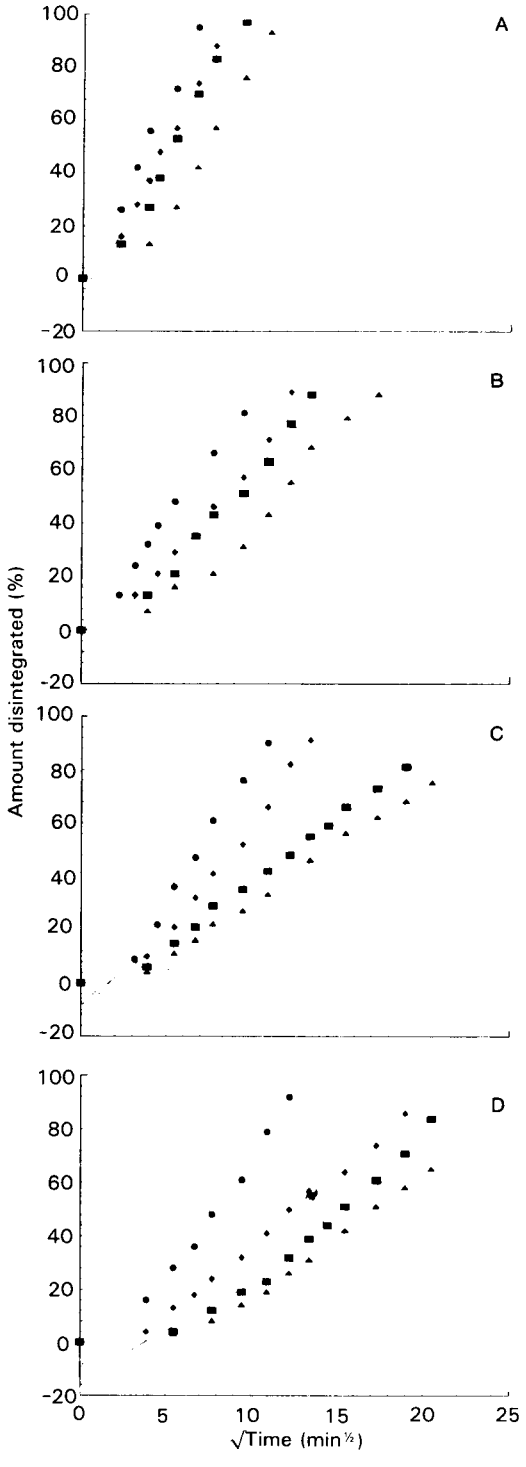

FIG. 2. Square root time plots of the disintegration of albumin microparticles in 0·01 M phosphate buffer (pH 7·2) containing trypsin, 1 mg mL^{-1}. Duration of cross-linking: A 12, B 24, C 36, D 48 h. ● 2, ◆ 4, ■ 6, ▲ 8% glutaraldehyde.

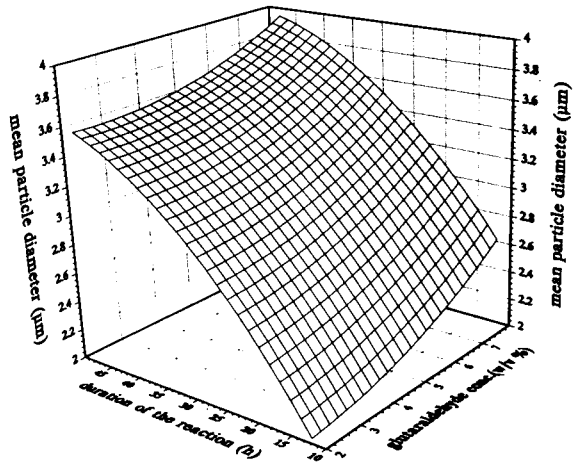

FIG. 3. Response-surface diagram for the mean particle diameter of albumin microparticles prepared by variation of glutaraldehyde concentration and duration of cross-linking.

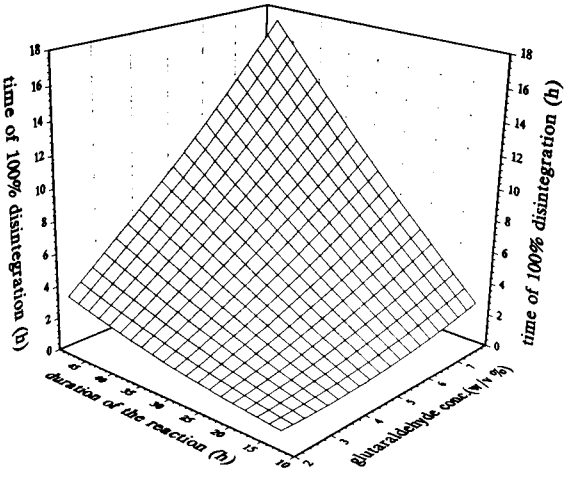

FIG. 4. Response-surface diagram for the time of 100% in-vitro enzymic disintegration of albumin microparticles prepared by variation of glutaraldehyde concentration and duration of cross-linking.

Enzymic disintegration or dissolution of formed particles, on the other hand, appears to be affected by a number of associated factors, including particle diameter. The enzyme would be expected to attack sites at the surface and, to a lesser degree, in the interior of the particles, suggesting a square root time relationship commonly seen with Fickian transport phenomena (Gehrke & Lee 1990).

In turn this might suggest a relationship between Q, a measure of the albumin dissolved, and square root of time and this is substantially demonstrated when comparing data shown in Figs 1 and 2. However, the relationship between initial size and time for the solution process to go to completion (estimated from the square root time plots (Zarrintan & Groves 1991)) is clearly more complex, suggesting, all other factors being equal, that the porosity or tortuosity of the particle matrix could be influential on the overall digestion process.

The response surface relating the time for 100% disintegration, the duration of the cross-linking process and concentration of reagent is shown in Fig. 4. This strongly indicates that the more intense the reaction (longer time and higher concentration of glutaraldehyde) the greater is the degree of

Table 1. Properties of the albumin microparticles.

Formation conditions			Disintegration	
Cross-linking time (h)	Glutaraldehyde concn (w/v%)	Mean particle diameter ± s.d. (μm)	t_{100}[a] (h)	Correlation coefficient
12	2	2·0 ± 2·15	0·80	0·992
	4	2·1 ± 2·47	1·36	0·986
	6	2·2 ± 2·05	1·57	0·976
	8	2·7 ± 2·11	2·65	0·937
24	2	2·8 ± 1·78	2·23	0·993
	4	3·0 ± 2·27	3·75	0·969
	6	3·0 ± 1·77	4·34	0·968
	8	3·2 ± 1·93	6·85	0·947
36	2	3·2 ± 1·97	2·59	0·962
	4	3·3 ± 2·06	4·03	0·957
	6	3·4 ± 1·88	9·09	0·987
	8	3·6 ± 1·81	12·24	0·984
48	2	3·6 ± 1·83	3·36	0·964
	4	3·6 ± 1·61	8·66	0·976
	6	3·7 ± 1·78	11·79	0·923
	8	4·0 ± 1·87	17·17	0·923

[a] The time of 100% disintegration was estimated from the square root time plot (Zarrintan & Groves 1991).

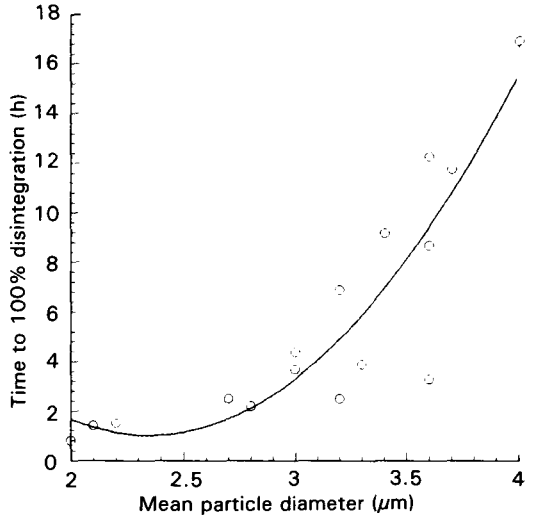

FIG. 5. The relationship between mean particle diameter and the time for 100% in-vitro enzymic disintegration for all conditions under which the microparticles were made.

cross-linking and, therefore, the slower the disintegration under enzymic attack. These conditions clearly affect both the porosity (degree of cross-linking) and the tortuosity (resistance to the penetration of the trypsin) of the matrix and, again, allows predetermined performance characteristics to be designed into the albumin microparticles.

Some indication of the relative importance of these matrix factors can be seen in the relationship between the time for 100% disintegration (Y) and mean particle diameter (X), irrespective of the conditions of manufacture. The data, for all samples, made under a variety of conditions, are shown in Fig. 5 and fit a quadratic equation ($r^2 = 0{\cdot}908$, s.e. $= 2{\cdot}301$) in the form:

$$Y = 30{\cdot}779 - 25{\cdot}33\,X + 5{\cdot}39\,X^2$$

This suggests that both diffusional penetration (dependent on X), and erosion of the surface of the particle (dependent on X^2) are taking place simultaneously. The shape of the graph also indicates that both factors, together or individually, are not acting consistently. The smaller particles appear to dissolve more slowly than might be anticipated initially. This could be due to the matrix becoming more resistant to movement by development of a denser cross-linked system at an early stage in the process.

This present investigation suggests that albumin microparticles are readily made by a chilled desolvation procedure which has some potential for scaling up to an industrial process. The study has also shown that the formation of the insoluble albumin particles can be achieved at low temperatures by a process which appears to be dynamic or that, as the volume of the initial aqueous solution of albumin is added to an excess of the dehydrating solvent the particles appear to grow in size as the reaction continues. The process probably involves precipitation of albumin as water surrounding the albumin molecules is removed by the anhydrous ethanol. However, as glutaraldehyde is added, cross-linking is induced and aggregates are rendered insoluble in water. It is these aggregates that become larger with time and are measured as microspheres. This process needs to be compared with the literature reports on the formation of albumin particles made by the emulsification process. For example, Lee et al (1981), Sheu et al (1986) and Sheu & Sokolowski (1986) all reported parameters such as process variables, including stirring rates and temperature on bead sizes and concluded that parameters of the emulsifying oil phase were the main size-controlling factors. Torrado et al (1989) compared the properties of albumin microspheres prepared by either glutaraldehyde or heat denaturation and confirmed the influence of the oil phase on the subsequent size of the particles.

Studies of the properties of the particles themselves remain relatively few. Gupta et al (1986b) demonstrated that the release rates of doxorubicin from albumin beads denatured

at temperatures over the range 105–150°C were different, decreasing with an increase in denaturation temperature. Albumins in general are readily denatured at temperatures of 60–65°C, suggesting that the properties of the beads may be considerably affected at temperatures of up to 150°C. Lewis et al (1992) found complete disintegration of the microparticles (prepared at 125°C) within 1 h in 1% trypsin solution but differences under different conditions of preparation were not reported. The dynamic process detected under the conditions of our experiments could not be completely correlated with the trypsin digestion process, as shown by comparison of the two response surfaces (Figs 3, 4). The relationship between time for complete enzymic distintegration and particle size (Fig. 5), is not linear, although there is a strong indication that particles made under different conditions do have different properties, probably related to the cross-linking activity and the nature of the particulate matrix. We did not, for example, see evidence of the swelling and dissolution behaviour seen by Willmott et al (1992) for doxorubicin-loaded casein microspheres.

One possible reason for the differences is the effect induced by lyophilization of the microparticles which, for ease of handling, is an essential element of our process. Willmott et al (1992) reported substantially no difference in properties of their doxorubicin-loaded casein microparticles, and Willmott & Harrison (1988) found the same for doxorubicin-loaded albumin particles. These results have been substantially confirmed for both unloaded albumin and gelatin microparticles (Öner, unpublished data).

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