Immobilization of Active Urokinase on Albumin Microspheres: Use of a Chemical Dehydrant and Process Monitoring

Kumaril Bhargava[1,2] and Howard Y. Ando[2,3]

Received April 9, 1991; accepted December 23, 1991

A method of immobilizing urokinase on albumin microspheres has been developed. Laser scattering, which was used to follow particle size from the initial emulsification stage to the final aqueous resuspension of the microsphere stage, showed that particle coalescence and crosslinking were critical parameters in manufacturing the microspheres. Chemical dehydration with 2,2-dimethoxypropane was used to convert an albumin emulsion into an albumin suspension and to reduce coalescence. An optimal amount of dehydrant produced 0.3-μm particles which resisted a 50°C temperature challenge. Since oil/glutaraldehyde emulsion resulted in large particles with no urokinase activity, the cross-linking concentration of glutaraldehyde was reduced by solubilizing 25% (w/v) glutaraldehyde in the oil phase with *n*-propanol. A concentration of 0.015% (v/v) glutaraldehyde effectively immobilized urokinase and stabilized albumin microspheres. Amidolytic activity using the specific chromogenic substrate for urokinase, S-2444, showed that enzyme activity could be retained during this glutaraldehyde cross-linking.

KEY WORDS: chemical dehydration; solubilized glutaraldehyde; process monitoring; urokinase; immobilization.

INTRODUCTION

Albumin microspheres have been widely used for controlled or localized delivery of protein molecules (1–4). The most widely used method to manufacture these albumin microspheres is chemical cross-linking (4–7,17). Chemical cross-linking needs to be delicately balanced between the cross-linking agent and the activity of the enzyme, as an excessive amount of cross-linking agent may denature the immobilizing enzyme, while a reduced amount may not produce structurally sound microspheres.

Several studies have evaluated the variables which may affect the characteristics of entrapped drug as well as the size and size distribution of albumin microspheres, however, opinions vary on the effect of different manufacturing stages on particle size (1,8–11). In most of the studies, particle size was determined only on the final water phase suspension of microspheres or dried microspheres. This does not show the relationship of process and final particle size. Preliminary studies in our laboratory indicated that albumin emulsion stabilization by chemical dehydration and particle size monitoring in the oil phase might be possible. In this report, particle size reduction and monitoring are explored using 2,2-dimethoxypropane (DMP) and the Coulter N4 MD particle size analyzer, respectively, without compromising the activity of urokinase.

MATERIALS AND METHODS

Albumin Solution. Aqueous bovine serum albumin solution, 12.5% (w/v) (fraction V powder, Cat. No. A 7906; Sigma Chemical Co., St. Louis, MO), was prepared using distilled water on the day of an experiment.

Urokinase. Lyophilized low molecular weight urokinase (250,000 IU, Abbokinase; Abbot Laboratories, North Chicago, IL) was reconstituted with 10 ml of sterile 10% aqueous glycerin solution. The solution was stored at −70°C in aliquots of 22,000 IU/ml. Prior to use, the tubes were thawed to room temperature by immersing them in a 37°C water bath.

Glutaraldehyde/Propanol Cottonseed Oil Solution. A clear solution of 1.25 ml of 25% (w/v) glutaraldehyde (Cat. No. P 8648; Eastman Kodak Chemical Co., Rochester, NY), 250 ml of cottonseed oil (Cat. No. C 7767; Sigma Chemical Co., St. Louis, MO), and 5 ml of propanol (Cat. No. A 414; Fisher Scientific, Fair Lawn, NJ) was made by mixing with a magnetic stirrer.

Preparation of Albumin or Albumin/Urokinase and Oil Emulsion. Fifteen milliliters of cottonseed oil was cooled to 0°C in a 50-ml polycarbonate tube using a refrigerated water bath (Neslab Instruments Inc., Newington, NH). After addition of 0.5 ml of the albumin solution or albumin mixed with urokinase solution, the mixture was sonicated (Branson Sonic Power Co., Danbury, CT) for 23 sec (120 W, 30% duty cycle). Finally, the emulsion was cooled back to 0°C and resonicated for 1 min (30 W, 10% duty cycle).

Formation of Albumin or Albumin/Urokinase Suspension. During the final sonication stage, the emulsion formed by the above step was converted to a suspension by dehydrating it with DMP (Cat. No. D-8761; Sigma Chemical Co., St. Louis, MO). The reaction products acetone and methanol were phase separated under vacuum and removed by aspiration.

Dilution. Seven and one-half milliliters of the albumin suspension or albumin mixed with urokinase suspension was added to 100 ml of cottonseed oil and stirred at room temperature with a stainless-steel sigmoidal shaped paddle stirrer at approximately 60 rpm.

Cross-Linking and Capping. After proper mixing was assured in the dilution stage, the glutaraldehyde/propanol/cottonseed oil solution was added for cross-linking. After allowing the cross-linking to proceed for 15, 30, or 45 min (4,17), free aldehyde groups were derivatized using 0.2 ml of ethanolamine (Cat. No. A 7177; Sigma Chemical Co., St. Louis, MO). The suspension was then centrifuged for 30 min at 4200*g* in 50-ml centrifuge tubes (Cat. No. 05 528; Fisher Scientific, Fair Lawn, NJ). The pellet so obtained was resuspended in 6 ml of DMP, agitated, and recentrifuged for 5 min at 4200*g*. Final resuspension was in 2 ml of water. Non-cross-linked albumin was determined by recentrifuging and assaying the supernatant for protein using the BCA method

[1] Current address: Health & Personal Care Technology Division, The Proctor & Gamble Co., Cincinnati, Ohio.

[2] Department of Pharmaceutics, Philadelphia College of Pharmacy and Science, Woodland at 43rd Street, Philadelphia, Pennsylvania 19104.

[3] To whom correspondence should be addressed.

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(Pierce Albumin Kit, Pierce Chemical Co., Rockford, IL). A flow diagram of the manufacturing process is shown in Fig. 1.

Determination of Urokinase Immobilized on Microspheres. Microspheres obtained from the above step were washed with water by centrifuging them at 12,000*g* for 4 min in an Eppendorf centrifuge (Model 5414, Eppendorf Co., Hamburg, West Germany). The supernatant obtained was discarded and the pellet was resuspended in 0.5 ml of water. The procedure was repeated five times, after which no more urokinase was obtained in the supernatant. The assay for urokinase was done using S-2444 (pyro-Glu-Gly-Arg-pNA, KabiVitrum Diagnostica, Sweden), which is a specific substrate for urokinase. Urokinase cleaves the pNA ring, which gives a yellow color readable on a UV spectrophotometer. Microspheres obtained after the final washing were added to 1.5 ml phosphate buffer (0.05 *M*, pH 7.4). A 10-μl sample of this suspension was withdrawn at the desired time and added to a cuvette holding 800 μl of Tris buffer, pH 8.8. After 100 μl of S-2444 solution was added to the cuvette, the amidolytic reaction was allowed to proceed in a temperature-controlled, automated spectrophotometer (Beckman DU-7, Beckman Instrument Co., Somerset, NJ) for 30 min, after which the microspheres were centrifuged at 12,000*g* for 5 min and the absorbance of the supernatant was measured at 405 nm.

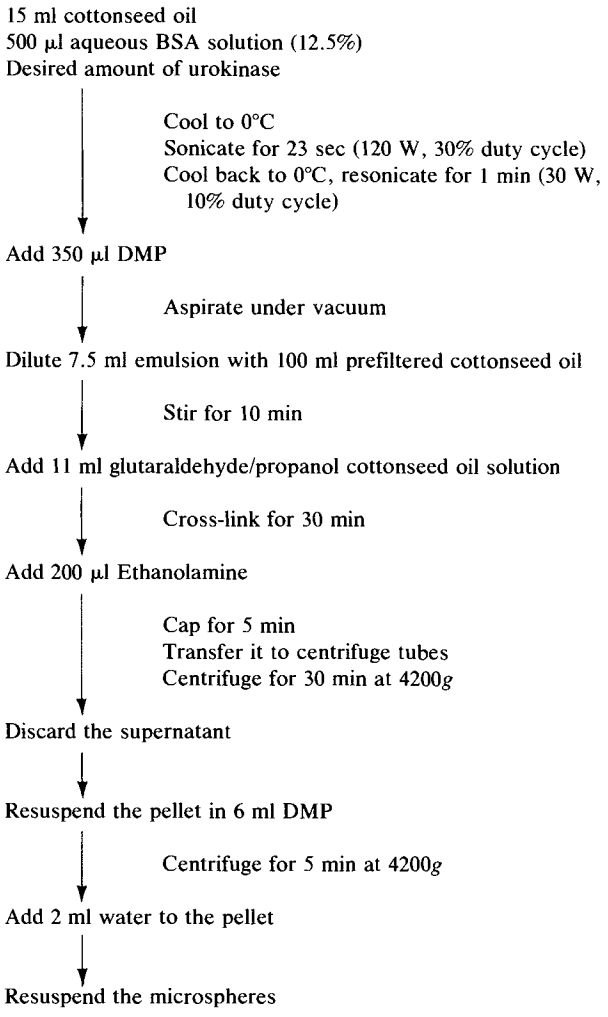

Fig. 1. Flow diagram of the microsphere manufacturing process.

Particle Size Determinations. These studies were carried out on albumin microspheres manufactured without urokinase. This was justified, as the ratio of urokinase to albumin was extremely low (5×10^{-8}:1, calculated on the basis of the specific activity of urokinase being 100,00 IU/mg). Particle size measurements were carried out with a Coulter N4 MD particle size analyzer (Coulter Electronics, Hialeah, FL). The size of the microspheres at different stages of the production process was determined by withdrawing a 2-ml sample and diluting it with 5 ml of filtered (0.22-μm Millipore filter, Millipore Corp., Bedford, MA) cottonseed oil. Diluted samples were degassed until gas ebullition ceased. Particle size determinations were done in triplicate in polystyrene cuvettes (Cat. No. CMS-0068-668; Curtis Mathes Co., Wayne, NJ) at 90° scattering (12). Each run was continued until a smooth auto correlation curve was obtained. Electron microscopy was done on the dried finished product in order to confirm the spherical shape (Fig. 2).

Effect of Temperature on Dehydrated Emulsion. Diluted samples were heated with the Peltier unit of a Coulter N4 MD particle size analyzer and were allowed to equilibrate at the desired temperature for at least 20 min before readings were obtained.

RESULTS

Effect of Emulsion Dehydration. Figure 3 shows the relationship between temperature and particle size of albumin microspheres at different concentrations of DMP in the oil phase prior to cross-linking. DMP decreased the particle size at all temperatures and stabilized the emulsion to temperature stress. When no DMP was added to the emulsion, a gradual increase in particle size occurred as the temperature was raised from 20 to 50°C. In contrast, emulsion with 65% DMP maintained constant particle size for all temperatures. Although DMP causes a progressive particle size reduction up to 65% DMP (Fig. 4), larger amounts of DMP caused the albumin to aggregate and precipitate from the oil. No decrease in the amidolytic activity of urokinase was observed at any of the above DMP concentrations.

Cross-Linking of Albumin Microspheres in the Oil Phase. The balance between the efficiency of glutaraldehyde cross-linking and particle size is shown in Fig. 5. Particle size of the microspheres as well as microsphere-associated albumin increased as glutaraldehyde concentration increased. At 0.015% (v/v) glutaraldehyde, approximately 60% of the albumin is microsphere-associated. Increasing the concentration of glutaraldehyde to 0.02% appears to cause more than a 40% particle size increase.

Monitoring of the Manufacturing Process. Figure 6 shows the particle size for the emulsion/microspheres at different stages of the cross-linking process with and without DMP; in the absence of DMP, longer cross-linking times are associated with larger particle size (Fig. 7). When these microspheres were resuspended in water, a much smaller dependence on cross-linking time was observed (Fig. 8).

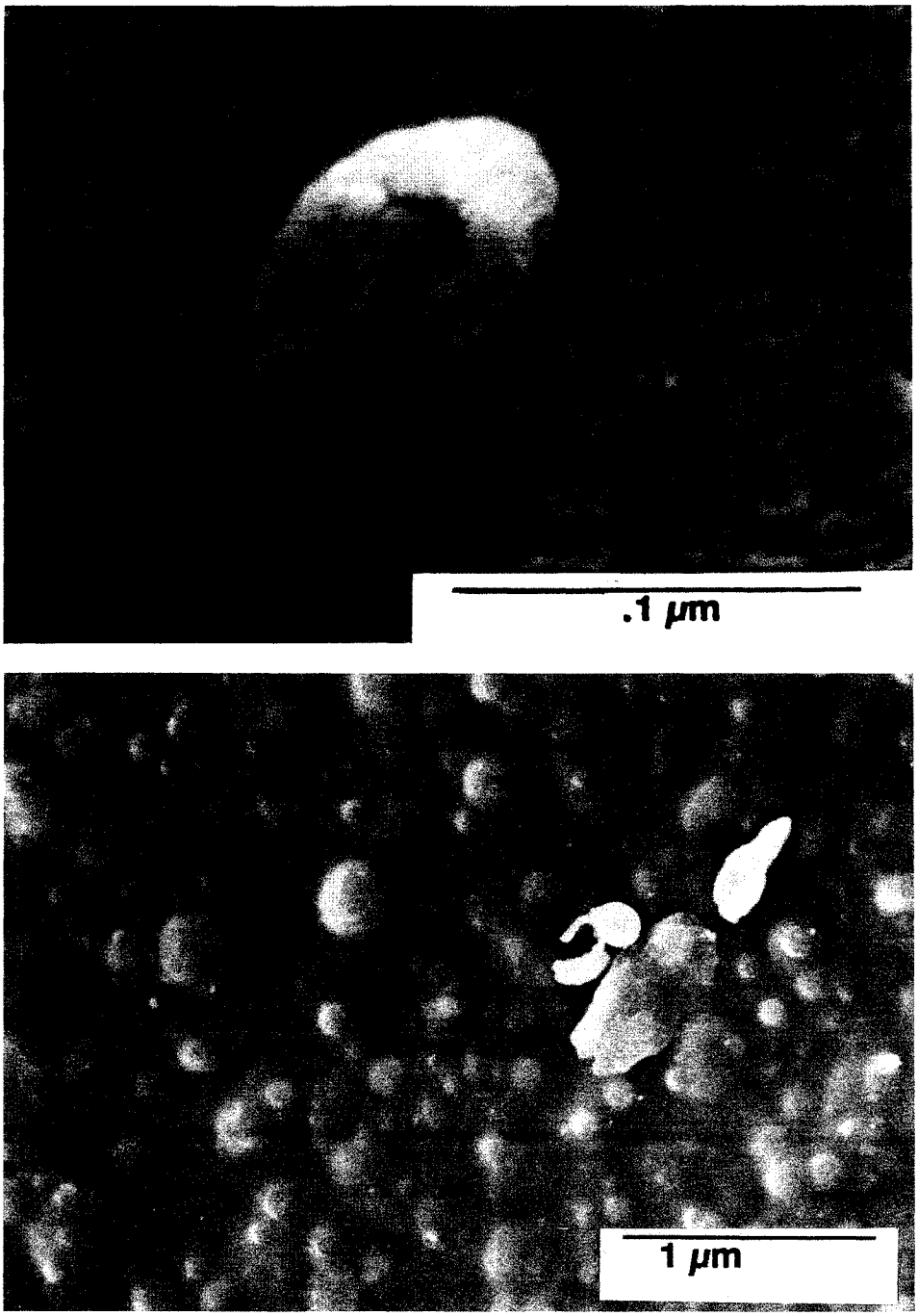

Fig. 2. Electron photomicrographs of albumin microspheres showing their spherical shape.

Effect of Dehydration and Cross-Linking on Particle Size of Albumin Microspheres. Since DMP reduces microsphere size, studies were initiated to investigate its effect on the cross-linking of microspheres. The addition of DMP dramatically reduced the size of cross-linked microspheres (Fig. 6); 65% DMP produced microspheres that were one-third of the size of control microspheres after resuspension in water. Figure 9 shows the size of microspheres after various time intervals of cross-linking with 0.015% (v/v) glutaraldehyde in the oil phase and dehydration with different amounts of DMP. Twenty percent DMP appears to give the smallest particle size for all times up to 45 min. This concentration of DMP also gives the smallest aqueous resuspended microspheres for oil-phase crosslinking times up to 30 min (Fig. 10).

Effect of 65% (v/v) DMP and Glutaraldehyde on the Activity of Immobilized Urokinase. Once the amounts of DMP and glutaraldehyde necessary to dehydrate and crosslink the microspheres, respectively, were determined, an effort was made to study their effects on urokinase. Figure 11 shows immobilized urokinase on microspheres made with 65% (v/v) DMP and 0.009% (v/v) or 0.015% (v/v) glutaralde-

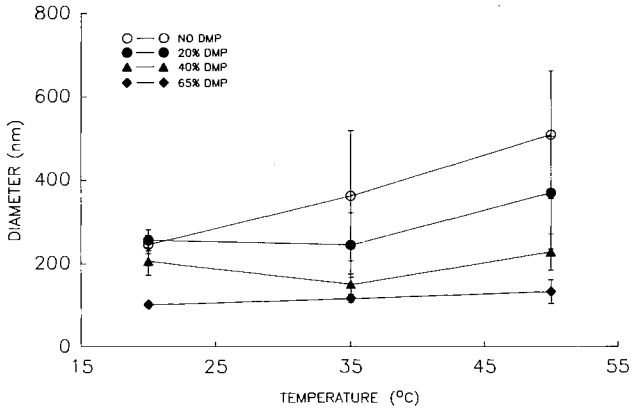

Fig. 3. Temperature stabilization of the emulsion due to dehydration. DMP concentration is as the percentage (v/v) of the total aqueous albumin phase. Each data point represents the average of three replicates and bars represent the standard error of the mean.

hyde. The absorbance shown on the *y* axis represents the amidolytic activity of urokinase obtained by hydrolysis of its specific substrate S-2444. The immobilization of urokinase was found to be inversely related to the concentration of glutaraldehyde used. No change from the initial reading was observed over 120 hr.

DISCUSSION

A modified method to manufacture submicron-size albumin microspheres has been developed. The method uses 65% (v/v) DMP in the aqueous phase to reduce the particle size prior to cross-linking. We have investigated the possibility of stabilizing the emulsion with DMP by converting an albumin emulsion (w/o) into an albumin suspension prior to cross-linking to prevent the emulsion droplets from coalescing into larger droplets. The use of temperature challenge as a method to evaluate emulsion/suspension stability appears to be useful (13). Particle size variability decreased as DMP concentration was increased from 0% to 65%. However, particle size reduction with DMP is limited. If concentrations of

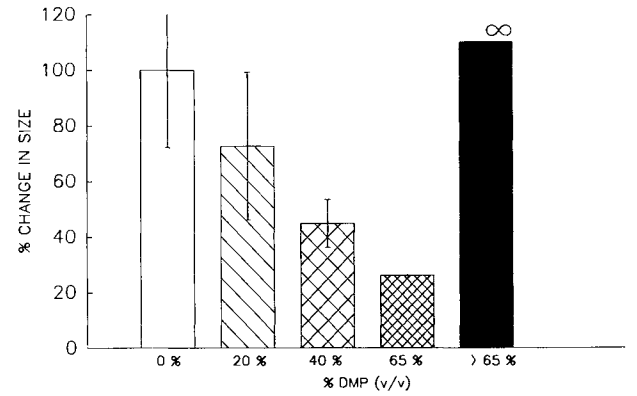

Fig. 4. Particle size changes due to dehydration. DMP concentration is as the percentage (v/v) of the total aqueous albumin phase. Percentage change is calculated with respect to initial emulsion size. ∞ indicates particle size increase beyond the measurement range of instrument capability. Each data point represents the average of three replicates and bars represent the standard error of the mean. Temperature, 50°C.

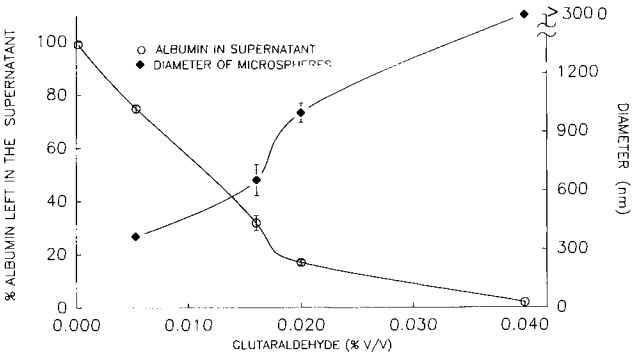

Fig. 5. Tradeoff between cross-linking efficiency and particle size. Cross-linking time, 30 min. DMP concentration = 65% (v/v). Temperature, 25°C. Each data point represents the average of three replicates and bars represent the standard error of the mean.

DMP over 65% are added, the resultant suspension aggregates and precipitates out of solution. Apparently a minimum amount of water is necessary to prevent particle–particle aggregation. Since water can act as a dielectric shield, the removal of the water may enhance particle–particle interactions in the continuous hydrophobic phase. Our goal was to explore the use of albumin microspheres as a delivery device for fibrinolytic enzymes (14). In our studies, exposure of urokinase to DMP in the emulsion showed no effect on its activity.

The use of glutaraldehyde as a cross-linking agent in manufacturing albumin microspheres is well documented (15,16). The concentration of glutaraldehyde plays a vital part in the integrity of the microspheres. It has been shown that particle size increases with increase in the concentration of glutaraldehyde (17–19). It has also been demonstrated that a variation in the concentration of glutaraldehyde from 1 to 4% leads to a modification of the lysine residues in albumin from 40 to 89%, respectively (20). Therefore very narrow constraints exist with respect to the extent of cross-linking that can be introduced while still resulting in a biologically useful product.

Glutaraldehyde is generally added directly to the oil/aqueous albumin emulsion (4,17,21–23). The current method of cross-linking albumin emulsions with glutaraldehyde em-

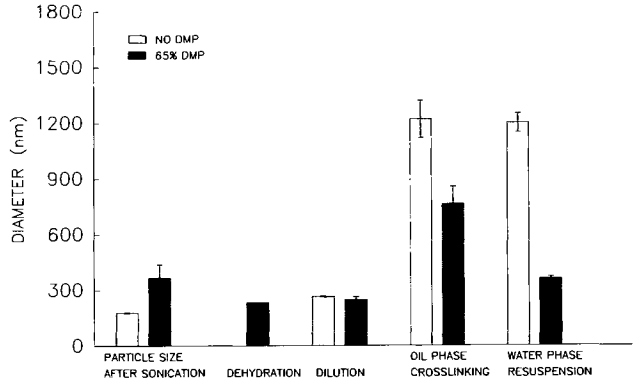

Fig. 6. Production stages with and without 65% (v/v) DMP. Cross-linking time, 30 min. Glutaraldehyde, 0.015% (v/v). Each data point represents a replicate of at least three batches and bars indicate the standard error of the mean.

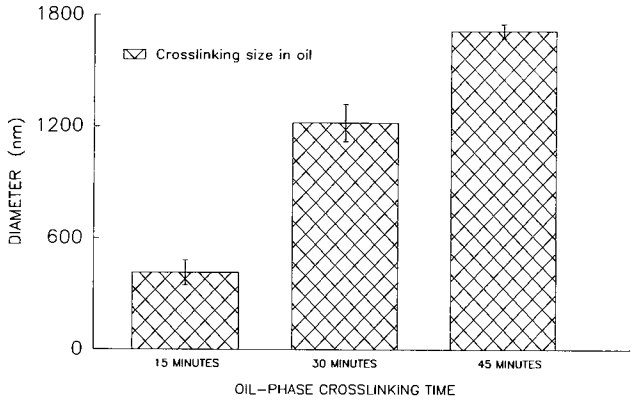

Fig. 7. Oil-phase cross-linking time versus particle size curve without using DMP. Glutaraldehyde, 0.015% (v/v). Temperature, 25°C. Each data point represents replicates of a minimum of three batches. Bars denote the standard error of the mean.

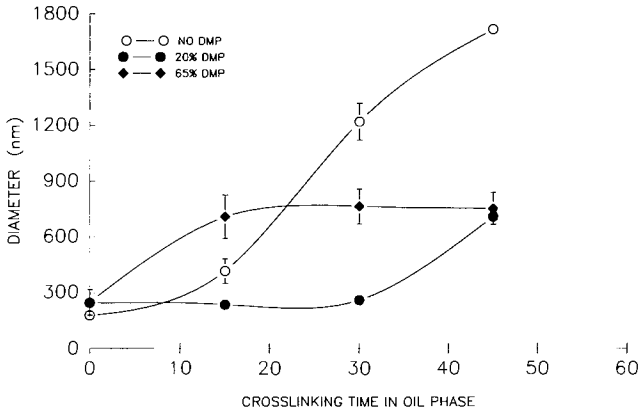

Fig. 9. Oil-phase particle size and cross-linking time. Data point at zero time indicates particle size after dilution stage. Glutaraldehyde used, 0.015% (v/v). Time is in minutes. Each data point represents replicates of at least three batches. Bars denote standard error of the mean.

ploys the addition of 0.2–5% glutaraldehyde to the oil phase while agitating for 15 min to 5 hr (4,17,24). This has the potential to produce variable results and raises the question of how an aqueous glutaraldehyde–oil emulsion reacts during agitation with an albumin–oil emulsion. If particle–particle collision is important, then agitation rate may be an important factor in cross-linking. Particle–particle collision might also imply a nonuniform cross-linking over the surface of a given microsphere. Urokinase activity has been reported to be affected by the glutaraldehyde concentration (25). Preliminary studies carried out in our laboratory by adding glutaraldehyde directly to the oil phase produced microspheres of particle size greater than 500 μm and caused significant denaturation of urokinase.

To avoid these concerns, we solubilized glutaraldehyde in cottonseed oil using *n*-propanol. Similar efforts have been reported using *n*-butanol as a dewatering agent during the cross-linking step (4,17). In our studies, the microspheres were chemically dehydrated prior to cross-linking, thus a smaller individual surface area and a reduced total surface area was offered to the glutaraldehyde for cross-linking. This enabled us to reduce the concentration of glutaraldehyde from 1 to 0.015% and the cross-linking time from 180 to 30 min. Reduction in the concentration of glutaraldehyde may also be due to the solubilization of glutaraldehyde, which resulted in an increased interaction probability between the dehydrated microspheres and the solution-phase glutaraldehyde in the continuous oil phase. This reduction in glutaraldehyde concentration for cross-linking of microspheres helped preserve the activity of urokinase immobilized on the microspheres.

Constant process monitoring with a Coulter N4 MD particle size analyzer helped in identifying the stages where particle growth occurs. The stages identified were dilution and cross-linking. In the absence of a dehydrating agent, at 15 min of cross-linking, particle size in the water phase was found to be greater than in the oil phase. This may be due to incomplete cross-linking in the oil phase and aggregation of these poorly cross-linked particles in the water phase. Similar particle size in the oil and water phases at 30 min may be attributed to proper cross-linking which occurred in the oil phase. The addition of ethanolamine to this solution made the microspheres hydrophilic and dispersable in the aqueous phase. Results obtained at 45 min in the oil phase reflect

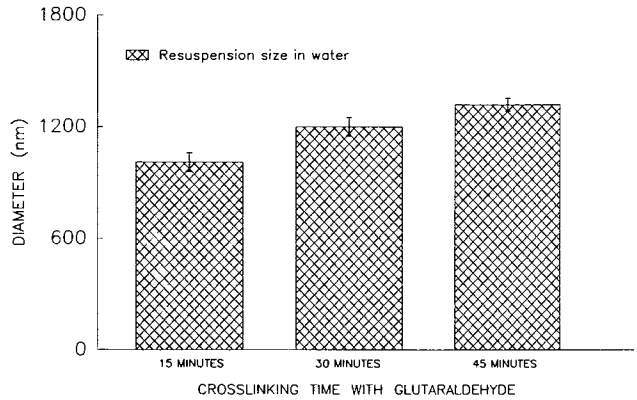

Fig. 8. Aqueous resuspendibility and oil-phase cross-linking time without using DMP. Temperature, 25°C. Each data point indicates a minimum of three batches and the bars denote the standard error of the mean.

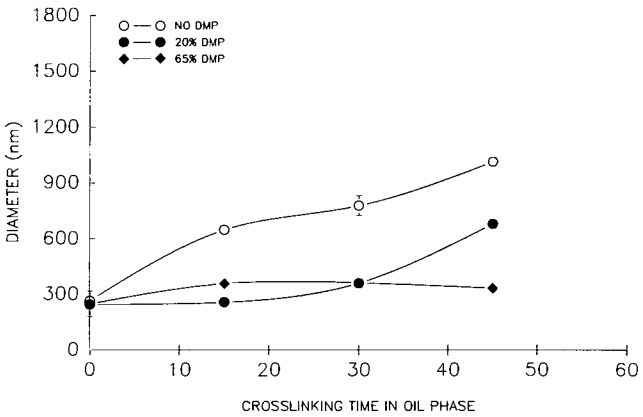

Fig. 10. Water-phase resuspended particle size. Data point at zero time indicates particle size after dilution stage. Time is in minutes. Each data point represents replicates of at least three batches. Bars denote standard error of the mean.

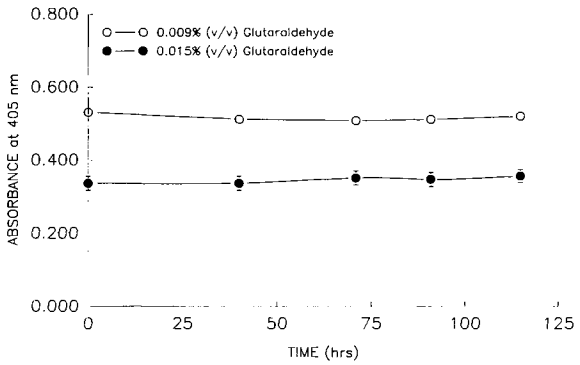

Fig. 11. Immobilization of urokinase due to different concentrations of glutaraldehyde. Absorbance on *y* axis represents the amidolytic activity of urokinase to its specific substrate S-2444. The *x* axis represents the time points at which the samples were withdrawn. Each data point represents the average of three replicates and bars represent the standard deviation of the mean.

increased particle size, which could be due to enhanced particle–particle aggregates or particle–particle cross-linking after 30 min. Similar particle sizes observed in the aqueous phase at both 30 and 45 min of cross-linking indicate that interparticle bonds formed after 45 min in the oil phase were absent upon resuspension in water. Thus, aggregates were most likely formed in the oil phase during longer cross-linking times. It appears that 30 min is sufficient time to cross-link microspheres using 0.015% glutaraldehyde.

No change in particle diameter using 65% (v/v) DMP at 15, 30, or 45 min in the oil phase shows that particle cross-linking reached a saturation stage within 15 min. This may be due to decreased surface area of the spheres. Particles obtained at these time periods showed a decreased and uniform particle size in the aqueous phase. This supports our finding that cross-linking was complete in 15 min; and additional incubation time contributed to weak interparticle cross-linking which was broken during resuspension in the aqueous phase, revealing the true particle size.

ACKNOWLEDGMENTS

The work reported here is based on research performed in partial fulfillment of the requirements for the degree of doctor of philosophy in pharmaceutics at the Philadelphia College of Pharmacy and Science. The work was supported by NIH Grant HL 38858-01 and the Johnson & Johnson Focused Giving Program.

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