Albumin Microspheres as a Drug Delivery System: Relation Among Turbidity Ratio, Degree of Cross-linking, and Drug Release

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The degree of cross-linking of albumin microspheres, with and without drug, was assessed using turbidity measurements carried out in the presence of water and the protein denaturant guanidine hydrochloride (GuHCl) at a concentration that disrupted noncovalent bonds while having no effect on covalent bonds. The measurements allowed calculation of a turbidity ratio (T_G/T_W), expressed as the ratio of the turbidity of albumin microspheres in 6 *M* GuHCl (T_G) divided by that in water (T_W). A linear relation existed between T_G/T_W and the (i) temperature at which the microspheres were prepared, (ii) concentration of the cross-linking agent glutaraldehyde, and (iii) time of exposure to a second cross-linking agent, formaldehyde vapor, three conditions that increase the degree of cross-linking. The turbidity ratio also increased as the concentration of the albumin solution used to prepare the microspheres increased from 25 to 50%. Drug release from the microspheres consisted of an initial, rapid, burst followed by a second, slower, phase. The rates in both release phases were inversely related to the turbidity ratio, suggesting that this parameter has utility as an indicator of the degree of cross-linking in albumin microspheres.

KEY WORDS: albumin microspheres; drug delivery; turbidity ratio; degree of cross-linking, drug release.

INTRODUCTION

Albumin microspheres, especially human serum albumin microspheres (HSA-MSs), have potential as a prolonged release drug delivery system (1–3). They possess advantages over other types of polymeric microspheres. Thus they are nonantigenic, are metabolizable, and can accommodate a variety of drugs in a relatively nonspecific fashion (1). They have been studied as carrier systems for proteins and enzymes (2,3), anticancer agents (4), steroids (5), and highly water-soluble drugs (6) and for drug delivery into the lungs (7).

According to Tomlinson (8), factors influencing drug release from microspheres include the (i) size and density of the sphere, (ii) physicochemical properties of the drug, (iii) percentage loading and distribution of the drug within the sphere, (iv) interactions between the drug and the matrix, (v) type and amount of the matrix, (vi) release environment, including the presence of enzymes, and (vii) extent and nature of cross-linking of the matrix. As used in this paper, the term "cross-linking" describes the formation of covalent bonds and is distinguished from weaker, noncovalent, interactions that contribute to protein conformation. Cross-linking, which can be achieved by either heat or chemical methods, is believed to play a major role in the stability of albumin microspheres as well as influencing drug release thereform. The work presented here relates the degree of cross-linking, as indicated by turbidity determinations, to drug release from microspheres.

Heat affects albumin conformation, causing denaturation at about 60–70°C and cross-linking at temperatures above 100°C. In investigating the latter effect, Asquith and Ottenburn (9) found that heat treatment of proteins, including albumin, at alkaline pH's caused degradation of cystine residues to dehydroalanyl residues that then reacted with alanine and cysteine to form lysinoalanine and lanthionine cross-links, respectively.

Chemical cross-linking is achieved most frequently by glutaraldehyde, available as a 25% aqueous solution containing free glutaraldehyde in equilibrium with both monomer and polymer forms of its cyclic hemiacetal (10). A number of mechanisms have been proposed to account for the chemical cross-linking of proteins, including albumin, and these have been summarized by Sokoloski and Royer (11). While there is some uncertainty as to the precise mechanism(s) involved, lysine is the only residue within the albumin molecule modified in the presence of glutaraldehyde (12), the extent being directly proportional to the concentration of glutaraldehyde.

Currently, there is no absolute method to determine the degree of cross-linking in a microsphere, although it is assumed that this property increases with an increase in (i) the temperature and/or time of heating and (ii) the concentration and/or time of exposure to glutaraldehyde and formaldehyde. Swelling has been investigated as an indicator of the degree of cross-linking. However, the results are not consistent, either for heat-cross-linked HSA-MSs (13,14) or for glutaraldehyde-cross-linked HSA-MSs (15,16).

Solubility has been used to assess the degree of cross-linking in such proteins as wool (17). By using solvents that disrupted either noncovalent bonds (e.g., urea and sodium dodecyl sulfate) or disulfide covalent bonds (e.g., mercaptoethanol), Van Kleef (18) obtained information on crosslinking in thermally induced ovalbumin gels. We have extended Van Kleef's approach in the present work by the use of turbidity that provides a simple method to estimate the concentration of dispersions. Aguiar *et al.* (19) used the turbidity measured at 650 nm to determine the deaggregation rate of particles of benzoic acid derivatives. Crommelin *et al.* (20) employed this approach to monitor changes in the particle size of liposomes, while Sheu *et al.* (21) followed the time-dependent loss in turbidity of albumin microspheres in α-chymotrypsin solutions to assess biodegradability.

When determining the turbidity of a dilute dispersion spectrophotometrically, the influencing variables are particle size and the refractive index of the medium. The latter has an

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insignificant effect on the turbidity of HSA-MSs (22) and, in the present study, the microsphere particle size was held constant at either 5–10 or 60–90 μm. Therefore, a change in turbidity will be a direct result of a change in concentration of the HSA-MSs. A turbidity ratio, T_G/T_W, was calculated using the turbidities from the partial or total dissolution of HSA-MSs in an aqueous solution of 6 *M* GuHCl (T_G) divided by the turbidity of the same batch of microspheres in water (T_W). Both systems contained 0.1% (w/v) Tween 80 as a wetting agent. At this strength, GuHCl acts as a protein denaturant to disrupt noncovalent bonds but yet has no effect on covalent bonds or cross-links (23). The turbidity of HSA-MSs in 6 *M* GuHCl is related to the presence of covalent bonds or cross-links while the turbidity in water is related to the presence of both covalent and noncovalent bonds stabilizing the microspheres. This ratio is used as an indicator of the degree of cross-linking produced in both nonloaded and drug-loaded HSA-MSs when the conditions of preparation (temperature, concentration of glutaraldehyde, and exposure time to formaldehyde vapor) are varied. The relationship between turbidity ratio and drug release from drug-loaded HSA-MSs was also evaluated.

MATERIALS AND METHODS

Materials

Human serum albumin (Fraction V) and cottonseed oil used in the preparation of microspheres were obtained from Sigma Chemical Co. Amiloride hydrochloride was a gift of Merck Sharp and Dohme Laboratories. Tartrazine (FD&C Yellow No. 5) was obtained from Warner Jenkinson Co. These, and other chemicals and reagents of analytical grade, were used as received.

Methods

Preparation of HSA-MSs. The process of preparation was based on methods described elsewhere (4,6). Cottonseed oil (120 mL) was placed in a 300-mL flat-bottomed glass beaker fitted with four baffles positioned against the sides and stirred at 1400 rpm for 30 min. Human serum albumin [25 or 50% (w/v) HSA solution, 0.5 mL] was added dropwise to the oil and stirring was continued for a further 10 min. To produce nonstabilized microspheres, the HSA-MSs were separated from the oil phase by the addition of 120 mL anhydrous ether followed by centrifugation at 2400 rpm for 5 min. The supernatant was decanted and discarded. To remove all traces of oil, the HSA-MSs were washed in 80 mL anhydrous ether, and the dispersion was sonicated for 2 min in an ultrasonic bath (Model B-220, Branson) and again centrifuged. The supernatant was discarded and the previous washing step repeated. The dispersion of HSA-MSs in ether was vacuum filtered onto a 0.22-μm polycarbonate membrane (Nuclepore, CA) and air-dried.

To produce heat-cross-linked HSA-MSs, the dispersion of HSA-MSs was immersed in an oil bath at the desired temperature (125–185 ± 0.5°C) for 75 min with continuous stirring at 1400 rpm for the 5- to 10-μm fraction and 350 rpm for the 60- to 90-μm fraction. The dispersion was cooled to room temperature (~21°C) in an ice bath, and anhydrous ether (120 mL) added. Following centrifugation at 4000 rpm (1300 *g*) for 5 min, the supernatant was decanted and discarded. The HSA-MSs were further washed and collected as previously described.

Microspheres chemically- cross-linked with glutaraldehyde were prepared as follows. Cottonseed oil (120 mL), *n*-butanol (13 mL), and 25% (w/v) glutaraldehyde solution (0.05–0.25 mL to give 0.1–0.5 mg glutaraldehyde/mg HSA) were stirred at 1150 rpm (5- to 10-μm fraction) or 350 rpm (60- to 90-μm fraction) for 30 min. An aqueous solution of HSA [25% (w/v), 0.5 mL] was added dropwise to the oil phase and stirring was maintained at the appropriate speed for a further 1 hr. Anhydrous ether (120 mL) was added to the dispersion, which was centrifuged, washed, and dried as described above. Formaldehyde was also used to achieve chemical cross-linking. In this case, nonstabilized HSA-MSs were exposed at room temperature (~21°C) to formaldehyde vapor produced by covering the bottom of a vacuum desiccator with 37% (w/v) formaldehyde solution. The period of exposure ranged from 0.5 to 3 hr.

Drug-containing HSA-MSs were prepared as described for nonloaded HSA-MSs, the drug being added directly to the HSA solution. In preparing tartrazine-loaded HSA-MSs, the HSA-tartrazine solution [2.5% (w/v) tartrazine in 25% (w/v) HSA, 0.5 mL] was used as the aqueous phase. The theoretical loading was 90.9 μg tartrazine/mg of HSA-MSs. For amiloride-loaded HSA-MSs, drug was incorporated in 25% (w/v) HSA solution (0.5 mL) as (i) a saturated solution to give a loading of 23.37 μg amiloride/mg HSA-MSs or (ii) a suspension to produce a loading of 111.11 μg amiloride/mg HSA-MSs.

Following preparation, all microspheres were wet sieved to the desired size range using microsieves (Buckbee-Mears, MN). Approximately 100 mg of the HSA-MSs was dispersed in 100 mL of anhydrous ether or heptane and the dispersion passed through either a 10-μm or a 90-μm microsieve sitting on top of a 5- or 60-μm microsieve, respectively. An ultrasonic probe (Sonic Dismembrator, Model 300, Fisher) was used to facilitate sieving. The HSA-MSs were collected on a 0.22-μm polycarbonate membrane by vacuum filtration, air-dried, and stored in a screw-capped vial kept in a desiccator.

Turbidity Measurements. HSA-MSs (1 mg) were placed into a screw-capped vial and 4 mL of either 6 *M* GuHCl in 0.1% (w/v) Tween 80 or 0.1% (w/v) Tween 80 alone was added. The mixture was sonicated for 2 min to form a uniform dispersion, at which time the turbidity of each sample was measured at room temperature using a Beckman DU40 Spectrophotometer at a wavelength of 630 nm. Triplicate samples were determined.

Extent of Drug Loading into HSA-MSs. With nonstabilized drug-containing HSA-MSs, known weights (2–10 mg) containing amiloride or tartrazine were dissolved in either 4 or 100 mL, respectively, of release media (see below). Cross-linked amiloride-containing HSA-MSs were digested by sonication in 10 mL of 5 *M* acetic acid. Tartrazine was determined spectrophotometrically at 425 nm using a calibration curve that was linear in the concentration range of 5–16 μg/mL. Amiloride was determined using reversed-phase HPLC with *p*-nitroaniline as an internal standard. Separation was achieved on a C-18 column (Econosil, 10 μm) using a water:methanol:pH 3.0 phosphate buffer (61.5:35.0:3.5) at a

flow rate of 2.0 mL/min and a pressure of 2000 psi at ambient temperature. Ultraviolet detection was used at 254 nm. Under these conditions the retention times of amiloride hydrochloride and *p*-nitroaniline were 4.6 and 7.3 min, respectively. The limit of detection was 4 μg/mL, with a peak area ratio greater than 0.3.

Drug Release Studies. Known weights of HSA-MSs (2–5 mg) were placed into vials containing 4 mL of release medium (0.04 *M* phosphate buffer with 0.04 *M* NaCl and 0.03 *M* KCl at pH 7.0) which were rotated at 30 rpm while held at 37°C. Samples, filtered through a 0.22-μm filter membrane, were taken at appropriate time intervals and analyzed as described above.

RESULTS AND DISCUSSION

Turbidity Studies

Initial studies confirmed the existence of a linear relationship between turbidity and HSA-MS concentration over the range 0 to 0.4 mg/mL. In water, the slope was 1.477 absorbance units/mg/mL (r^2 = 0.992), while in 6 *M* GuHCl it was 0.784 absorbance units/mg/mL (r^2 = 0.987). Absorbance readings remained constant during the period of measurement, indicating that sedimentation of microspheres was not a problem. All subsequent turbidity determinations were carried out at a concentration of 0.25 mg HSA-MS/mL of water. Turbidities of HSA-MSs in GuHCl were all lower than that in water alone, indicating that the presence of GuHCl leads to partial dissolution of the microspheres by disruption of noncovalent bonds. It was also observed that HSA-MSs prepared by different methods have different surface characteristics. For example, heat-cross-linked HSA-MSs viewed through an optical microscope had a wrinkled surface (Fig. 1a) compared to glutaraldehyde-cross-linked microspheres, which had a smooth surface (Fig. 1b). It was to take account of any possible changes in absolute turbidity that might arise from such differences that a turbidity *ratio* was used rather than the absolute turbidity in GuHCl. The effects of various cross-linking conditions on the turbidity ratio of HSA-MSs are presented in Table I. The results are discussed according to the cross-linking method used.

Heat-Cross-linked HSA-MSs. Microspheres used as a drug delivery system should dissolve slowly in an aqueous environment. However, it was found that both the nonstabilized HSA-MSs prepared at room temperature and the heat-cross-linked HSA-MSs prepared between 70 and 105°C for 75 min completely dissolved upon contact with water. HSA-MSs prepared between 105 and 120°C partially dissolved, becoming transparent at the edges. As a result, all subsequent studies were undertaken using HSA-MSs prepared in the range of 125 to 185°C.

It has been reported that albumin solutions coagulate above 60°C (24) as a result of denaturation. The finding that water-insoluble microspheres are formed only at or above 125°C suggests that the denaturation process by itself is insufficient to form insoluble HSA-MSs. The higher temperature required to coagulate albumin when dispersed as microspheres may be due to a change in conformation of albumin molecules when present as droplets in the oil phase during the HSA-MSs preparation. This explanation is consistent with the observation that adsorption of albumin at the oil/

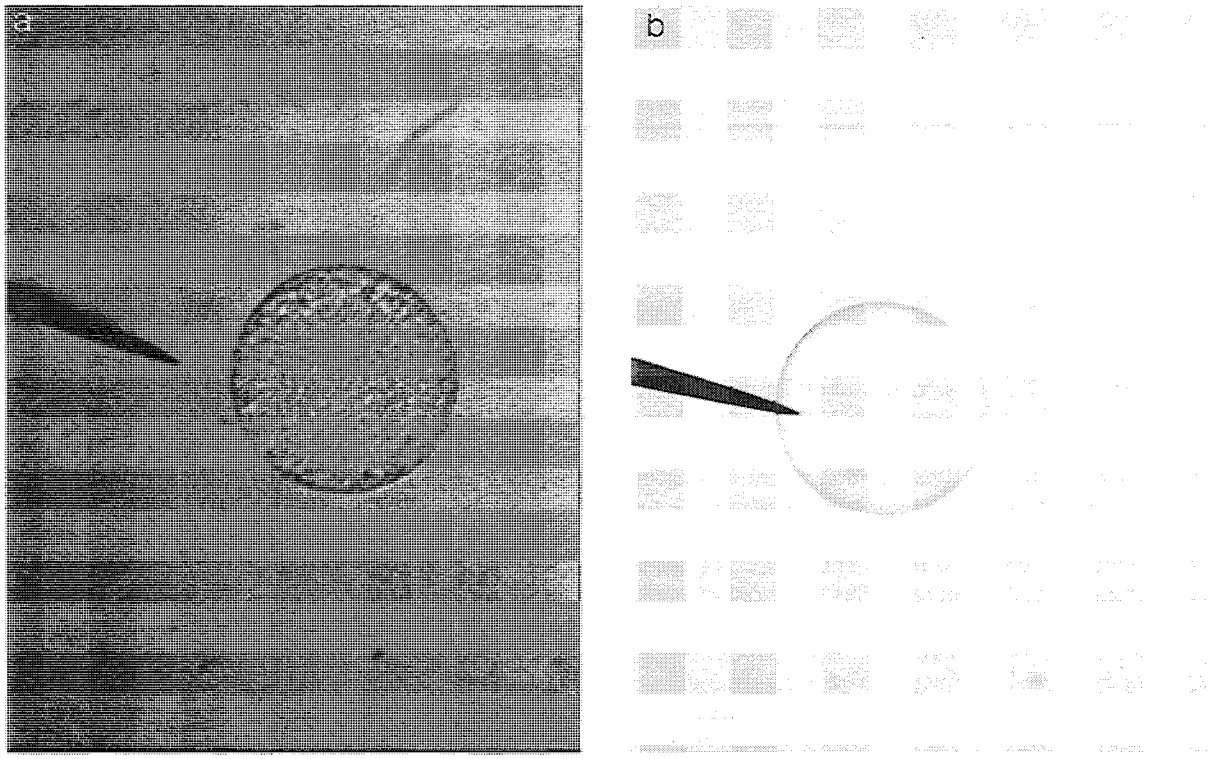

Fig. 1. Surface appearance of (a) heat-cross-linked HSA-MS and (b) glutaraldehyde-cross-linked HSA-MS, as viewed by an optical microscope. ×450.

Table I. Turbidities and Initial Drug Bursts of HSA-MSs Prepared by Various Cross-linking Conditions

Conditions of preparation and cross-linking of HSA-MSs	Average T_G (SD)[a]	Average T_W (SD)	T_G/T_W	% drug released as initial burst
a. Nonloaded				
25% (w/v) HSA, heat-cross-linked for 75 min at a temperature of (±0.5°C)				
125	0.087 ± 0.010	0.335 ± 0.009	0.260	n/a
128	0.112 ± 0.009	0.412 ± 0.030	0.272	
135	0.168 ± 0.022	0.461 ± 0.009	0.364	
145	0.177 ± 0.008	0.466 ± 0.017	0.380	
148	0.203 ± 0.017	0.374 ± 0.023	0.543	
155	0.185 ± 0.016	0.333 ± 0.017	0.556	
160	0.213 ± 0.006	0.367 ± 0.030	0.580	
175	0.231 ± 0.009	0.398 ± 0.010	0.579	
185	0.202 ± 0.019	0.261 ± 0.010	0.774	
50% (w/v) HSA heat-cross-linked for 75 min at a temperature of (±0.5°C)				
130	0.070 ± 0.005	0.184 ± 0.005	0.380	n/a
145	0.086 ± 0.006	0.157 ± 0.001	0.548	
155	0.116 ± 0.010	0.188 ± 0.015	0.617	
175	0.164 ± 0.016	0.222 ± 0.012	0.739	
b. Tartrazine-loaded				
25% (w/v) HSA, heat-cross-linked for 75 min at a temperature of (±0.5°C)				
125	0.062 ± 0.006	0.277 ± 0.015	0.224	96.64 ± 8.64
145	0.096 ± 0.004	0.265 ± 0.013	0.362	89.17 ± 1.87
165	0.146 ± 0.010	0.263 ± 0.008	0.555	82.51 ± 2.15
185	0.173 ± 0.010	0.251 ± 0.019	0.690	55.17 ± 1.69
c. Amiloride-loaded				
25% (w/v) HSA, glutaraldehyde-cross-linked for 1 hr at a conc. of (mg glutaraldehyde/mg HSA)				
0.1	0.186 ± 0.001	0.412 ± 0.024	0.451	68.17 ± 3.32
0.2	0.205 ± 0.012	0.333 ± 0.020	0.616	52.57 ± 2.93
0.5	0.236 ± 0.004	0.290 ± 0.001	0.814	34.18 ± 1.35
25% (w/v) HSA, formaldehyde vapor-cross-linked at 21°C for a period of (hr)				
0.5	0.023 ± 0.027	0.239 ± 0.027	0.098	73.00 ± 2.21
1.0	0.149 ± 0.010	0.355 ± 0.025	0.420	59.78 ± 4.50
2.0	0.211 ± 0.019	0.323 ± 0.029	0.653	49.80 ± 1.46

[a] SD, standard deviation ($n = 3$).

water interface can lead to surface denaturation, while mixing an albumin solution in oil at high speed can result in shear denaturation (25). Such denaturation may then cause the coagulation process to occur at higher temperature.

Figure 2 shows the relationship between the preparation temperature and T_G/T_W for some of the different HSA-MS systems studied. All three types of HSA-MSs listed show a direct relationship between the temperature of preparation and the turbidity ratio, and this is independent of whether drug is present or absent. At the same temperature, the turbidity ratios of HSA-MSs prepared with 50% HSA are higher than those prepared with 25% HSA. This suggests a higher degree of cross-linking in microspheres prepared at higher HSA concentrations, a not-unexpected result since there are more amino acid residues available to form cross-links. While it may be desirable to use as high a concentration of HSA as possible, such systems are viscous and lead to the formation of relatively large HSA-MSs or aggregates. Furthermore, with drug-containing HSA-MSs, increasing the HSA concentration results in a lower percentage drug loading. For these reasons, and because such a level has been used by others (13,26), a concentration of 25% HSA was used in all subsequent studies.

When the data plotted in Fig. 2 are linearly regressed, the three slopes fall in the range $0.783–0.796 \times 10^{-2}$ and show no significant differences as determined by a t test. Even though the HSA-MSs prepared with 50% HSA have higher turbidity ratios than those prepared with 25% HSA, these results show that the *change* in T_G/T_W with temperature is constant, a situation that is also true for tartrazine-loaded HSA-MSs. This suggests that heat cross-linking depends mainly on the preparation temperature and less on the drug incorporated or the HSA concentration used.

Other workers (14,26) have shown that HSA-MSs prepared at higher temperatures release entrapped drug slower than those prepared at lower temperatures, implying a higher degree of cross-linking at higher temperatures. The direct correlation between T_G/T_W of HSA-MSs and the tempera-

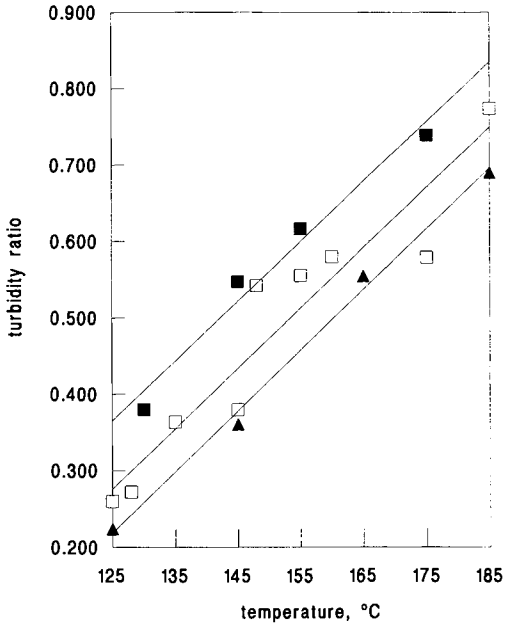

Fig. 2. Effect of the temperature of HSA-MSs preparation on the turbidity ratio (T_G/T_W). (□) Nonloaded HSA-MSs with 25% HSA; (■) nonloaded HSA-MSs with 50% HSA; (△) tartrazine-loaded HSA-MSs with 25% HSA.

ture of preparation found in this work suggests that T_G/T_W is a useful indicator of the degree of cross-linking.

Glutaraldehyde-Cross-linked HSA-MSs. The relation between glutaraldehyde concentration and turbidity ratio is presented in Table I. The higher turbidity ratios arising from the use of increasing glutaraldehyde concentrations are not surprising since glutaraldehyde-induced cross-linking has been shown to be concentration dependent (27). It has also been reported (5,11) that an increase in glutaraldehyde concentration causes a decrease in drug release from albumin microspheres. Using amino acid analysis, Lee *et al.* (5) also showed that the number of modified lysine residues increased with glutaraldehyde concentration. Since cross-linking by glutaraldehyde involves lysine residues, this implies the formation of a greater number of cross-links. As noted earlier, microspheres prepared by this method (Fig. 1b) differ in appearance to those prepared by heat cross-linking (Fig. 1a).

Formaldehyde Vapor-Cross-linked HSA-MSs. Table I shows that the turbidity ratios of amiloride-loaded HSA-MSs prepared by exposing nonstabilized amiloride-loaded HSA-MSs to formaldehyde vapor increased with time of exposure, suggesting that more cross-links were formed. The cross-linking mechanism of formaldehyde vapor remains unclear, and so there is no definite explanation for dependency of the turbidity ratio on the exposure time.

Turbidity Ratio and the Degree of Cross-linking

All three methods of cross-linking studied (heat, glutaraldehyde, and formaldehyde vapor) demonstrate a direct relationship between the turbidity ratio and properties known to influence the extent of cross-linking, namely, the temperature of preparation, glutaraldehyde concentration used, and time of exposure to formaldehyde vapor. It therefore appears that the turbidity ratio is a potentially valuable, albeit indirect, indicator of the degree of cross-linking present in HSA-MSs, regardless of the method of cross-linking and whether or not the microspheres contain drug. The simplicity of the technique and the use of standard laboratory equipment enhance the appeal of the turbidity ratio method as a tool to assess the degree of cross-linking in HSA-MSs and, thereby, facilitate evaluation of the role of different cross-linking methods and conditions in controlling drug release from drug-containing albumin microspheres.

Drug Release from Albumin Microspheres

Under the conditions of study used, a biphasic pattern of drug release was observed from all HSA-MSs that was independent of the method of cross-linking or the drug used. Thus, in all cases studied, there was an initial, brief period of rapid release, followed by a second, more prolonged, period during which slower release took place. Typical data showing both of these phases for amiloride-containing HSA-MSs are plotted in Fig. 3. The presence of an inverse relationship between the magnitude of the initial release and the turbidity ratio for systems containing tartrazine or amiloride is also apparent from Table I.

Raising the temperature of preparation from 145 to 155°C (Fig. 3) decreases the amount released in the first 2 min from 61.1 to 47.7%. Under the same conditions of preparation, the second, more prolonged, rate of release was

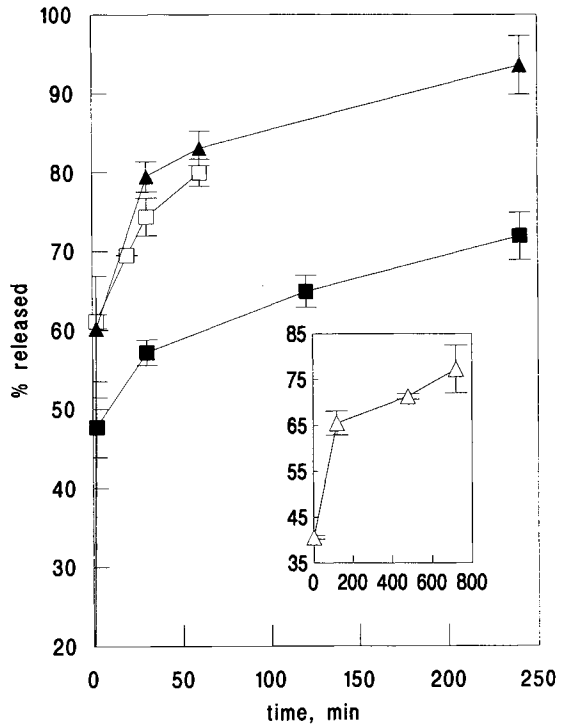

Fig. 3. Release profiles of amiloride from HSA-MSs (60–90 μm in diameter and containing 111.11 μg amiloride/mg HSA) prepared using a variety of cross-linking conditions: (□) 145°C; (■) 155°C; (▲) formaldehyde vapor at 21°C for 1 h; (△) 0.1 mg glutaraldehyde/mg HSA for 1 hr. Error bars = ±SD.

reduced more than threefold from 0.2414% min^{-1} at 145°C to 0.0694% min^{-1} at 155°C. Changing the cross-linking agent from formaldehyde vapor (1-hr exposure) to glutaraldehyde (1-hr exposure at a concentration of 0.1 mg/mg HSA) caused the amount released in the first 2 min to decrease from 60.2 to 40.6%. The rate of release during the second phase was reduced more than fivefold, falling from 0.0641 to 0.0125% min^{-1}.

This pattern of release from albumin microspheres has been observed by other workers, who have suggested that the drug broadly exists in two locations, namely, (i) on or at the surface, release from which causes the initial rapid phase, and (ii) entrapped in the inner matrix, where release is slow and is brought about by diffusion. According to Widder *et al.* (28), the initial rapid release from the surface involves about 40% of the drug regardless of the method of preparation. In contrast, Tomlinson *et al.* (29) found that the rapidly releasing fraction could vary from 40 to 93% and was dependent on the degree of cross-linking, a finding consistent with the work reported here based on turbidity ratios. This suggests that there may be factors besides the surface area of the microsphere that influence the initial rapid release fraction. This view is supported by simple surface area calculations and the assumption that the surface drug exists as an adsorbed monolayer. Under these conditions, rapid release of all drug on the surface of 5- to 10-μm microspheres would only contribute 1.3% of the total amount available: with 60- to 90-μm microspheres, 0.13% would come from the surface. Since Table I shows initial release amounts ranging from 34 to 96%, either excessive multilayer formation must take place or release occurs from regions below the surface of the microsphere. The latter situation is thought more likely. Again, simple calculations show that, for the rapid release of 50% drug from a 60- to 90-μm microsphere, it is necessary to deplete not only the surface but also the region adjacent to the surface and extending 7.7 μm into the microsphere. Presumably, drug that is located farther toward the core experiences greater difficulty in reaching the surface and so leads to the second, slower, release profile observed. Diffusional forces and hydrolysis of covalently bound drug are likely to control this phase of the release profile. In an attempt to investigate the depletion mechanism(s), fluorescence microscopy studies are currently under way to monitor drug distribution throughout the microsphere as the release process occurs. This will be the topic of a future publication.

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