Optimization of Conditions for Preparing 2- to 5-Micron-Range Gelatin Microparticles by Using Chilled Dehydration Agents

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INTRODUCTION

Gelatin, a widely commercially available degraded animal collagen, is extensively employed as a pharmaceutical adjuvant and excipient because of its biocompatibility and biodegradability (1). Gelatin has been used as a drug delivery system in a number of forms, including microcapsules, or as a means of encapsulating drug materials. Microparticles containing 5-fluorouracil, mitomycin, or adriamycin have been reported to be successfully prepared using a process in which aqueous solutions of gelatin and drug, together with an appropriate emulsifier, have been emulsified in an inert oil, hardened by a suitable cross-linking agent such as glutaraldehyde, and collected by centrifugation or filtration, and the extraneous materials removed with solvents (2–5).

Quite apart from the necessity of having to use considerable amounts of environmentally undesirable solvents such as diethyl ether or chloroform, which make these processes difficult to scale up from the laboratory to an industrial process, the external state of the gelatin surface is not always ideal and the generally elevated temperatures employed can be detrimental to any drug incorporated initially into the microparticles.

With a view to employing gelatin as an immunological drug delivery system in its own right (6; Olson and others, in preparation), a simple desolvation process was explored in which warm aqueous gelatin solutions were added to an excess volume of a cold water-miscible solvent to producing small gelatin microspheres. The fact that relatively large volumes of water or dehydrating solvents are used generally precludes incorporation of drug *ab initio* since this is washed out during processing. However, the drug can be loaded into the completed microparticles, and this will be the subject of a further communication.

Exploratory studies have demonstrated the feasibility of this concept. The present communication describes the basic process and optimization of some of the process variables by using analysis of variance.

MATERIALS AND METHODS

Materials

Gelatin-bovine skin, lime cured (Type B), with bloom strengths of 60 and 225, and porcine skin, acid cured (Type A), with bloom strengths of 60 and 300, were from Sigma Chemical, St. Louis, MO. Acetone, 2-propanol, and ethanol [anhydrous reagent grade denatured with 2-propanol (5%)], glutaraldehyde (50%, w/w, in water), and Isoton (buffered saline) were from Fisher Scientific, Itasca, Illinois. All were used as received.

Preparation of Microparticles

Preliminary experimentation was sufficient to establish the feasibility of the concept and define some of the experimental parameters.

A flask containing 500 mL of dehydrating solvent was immersed in a dish containing granules of solid carbon dioxide in order to maintain the temperature at −15°C. This dehydrating solvent was stirred using a rotating bar magnet at a speed of 200 rpm. Microparticles were prepared by slowly adding volumes of 5.0 to 25 mL of 1% (w/v) aqueous gelatin at ambient room temperature (~25°C) into the flask. The temporary microparticles were maintained at −15°C for 15 min. The droplets were then hardened by adding 4% (w/v) glutaraldehyde and stirred uniformly at −15°C for 45 min. The flask containing the microparticles was then transferred to a refrigerator (~4°C) for 24 or 48 hr to allow the completion of the cross-linking process. This process was then stopped by adding the contents of the flask to 1500 mL of 5% (w/v) sodium metabisulfite solution at 4°C, and the suspension concentrated by filtration with an Amicon 8200 YM100 membrane system. The microspheres were washed three times with aqueous 0.01 *M* phosphate buffer, pH 7.0. They were then suspended in 5% aqueous mannitol, frozen and lyophilized (Labconco freeze-drier system) for 48 hr. The freeze-dried product was stored at 4°C.

It should be noted that other workers have used appreciably shorter reaction times. For example, Tabata and Ikeda (9) found that 6 hr was optimal for gelatin. However, Olson (6) demonstrated that material prepared under cross-linking conditions of less than 24 hr softened, swelled, or even dissolved while being measured with the Coulter counter.

Size Characterization

Microparticles were sized using a Coulter Multisizer II (Coulter Electronics Inc., Hialeah, FL), fitted with a 50-μm orifice. One milliliter of the solvent–glutaraldehyde mixture containing microparticles was dispersed in Isoton prior to measurement at a dilution of 1:150 or as required. Typical size distributions are shown as log-probit plots in Figs. 1 and 2.

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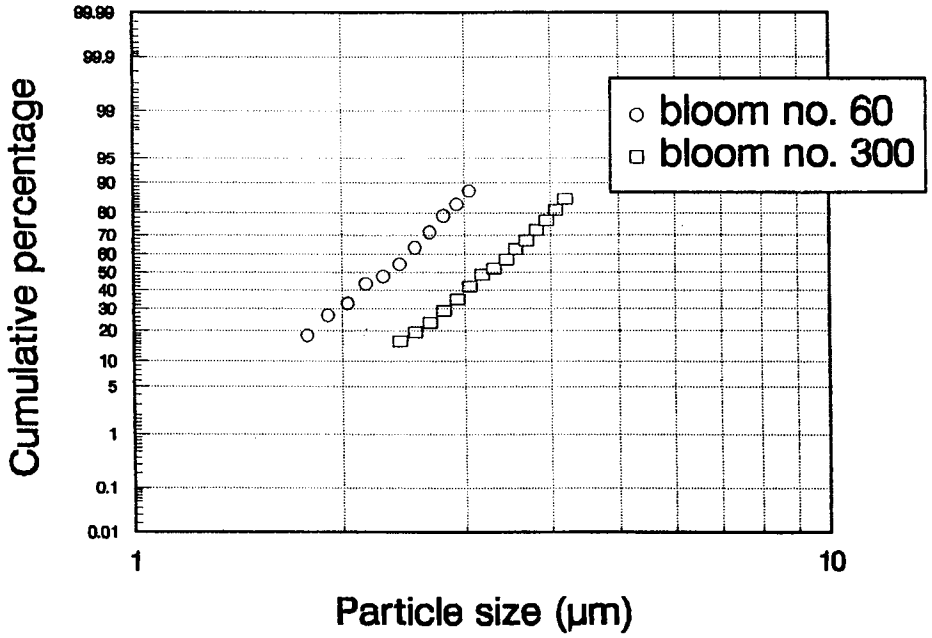

Fig. 1. Cumulative size distribution of gelatin (type A) microparticles prepared using ethanol and plotted as a log-probit relationship.

Effect of pH

Solutions, 25 mL, of gelatin (1%, w/w) in 0.01 *M* phosphate buffer (at pH 3, 4, 5, 6, 7, or 8) were dispersed into chilled ethanol at −15°C. The droplets were then hardened by adding 4% (w/v) glutaraldehyde and treated as above.

Factorial Designs

The overall objective of factorial analysis is to obtain a general picture of how the results are affected by changing the parameters or factors involved (7).

Dehydration Agent, Bloom Strength, and Duration of Cross-Linking Process

In this study a 2^3 factorial design was utilized. The effects of three factors—dehydration agent (*A*), Bloom strength of gelatin (*B*), and duration of the cross-linking pro-

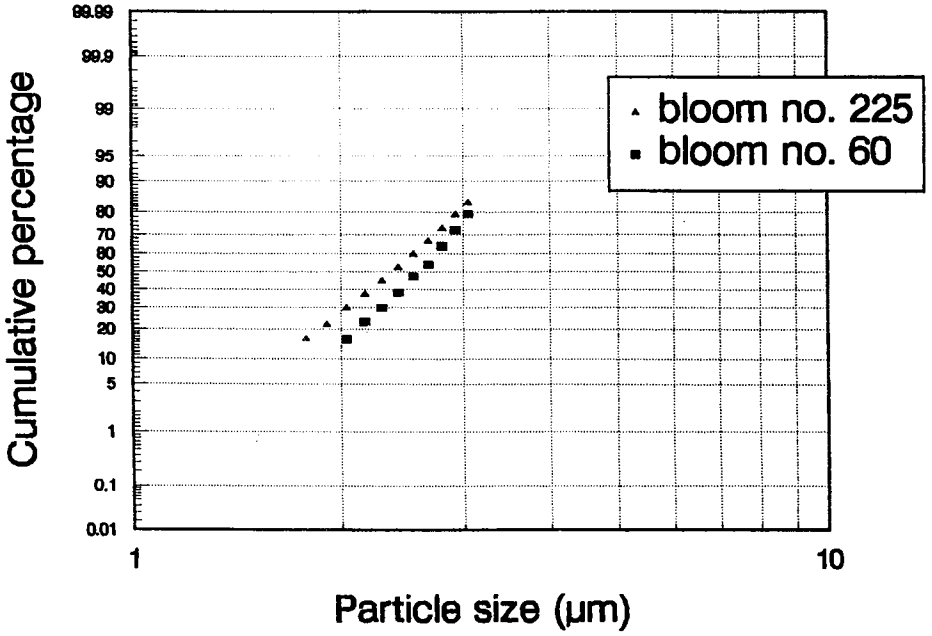

Fig. 2. Cumulative size distribution of gelatin (type B) microparticles prepared using ethanol and plotted as a log-probit relationship.

Table I. The Independent Variables and Their Levels Investigated in the Gelatin Microparticles

Factor	Low level	High level
A (dehydration agent)	Ethanol	2-Propanol
B_1 (bloom strength of type A gelatin)	60	300
B_2 (bloom strength of type B gelatin)	60	225
C (duration of cross-linking)	24 hr	48 hr

cess (*C*)—were studied at two levels. The various combinations for the eight trials used are presented in Table I. Two types of gelatin, acid-cured tissue (B_1) and lime-cured tissue (B_2), were used for the studies on the particle size of the gelatin microparticles. Thus, two similar 2^3 factorial experiments were performed in duplicate, the only difference between them being the type of gelatin. Table II is the calculation matrix for a 2^3 factorial design, with the following combinations of factors *A*, *B*, and *C* at two levels: (1), *a*, *b*, *ab*, *c*, *ac*, *bc*, and *abc*. In these combinations (1) refers to all factors at their low level, *a* refers to the experiment with factor *A*, at the high level and *B* and *C* low levels, and so forth.

Volume and Concentration of Gelatin (Type B, Bloom Strength 225) Solution in 0.01 M *Phosphate Buffer (pH 4) Investigated at Three Levels*

Based on the observed data, quadratic equations were generated to establish the correlation between the independent variables [volume and concentration of gelatin (type B, bloom strength 225) solution in 0.01 *M* phosphate buffer (pH 4)] and the dependent variable (the mean particle size of gelatin microparticles). The independent variables and their levels investigated in this study are shown as a response surface (Fig. 5).

The regression coefficients for the quadratic equations were calculated by computer software developed by 3-D Visions Corporation. Graftool, Version 3.3 (3-D Visions, Torrance, CA) was employed to produce the response surface diagram.

Table II. Calculation Matrix for a 2^3 Factorial Design

Factor combination	Level of factor in experiment[a]			Level of the interactions[b]			
	A	*B*	*C*	*AB*	*AC*	*BC*	*ABC*
(1)	−	−	−	+	+	+	−
a	+	−	−	−	−	+	+
b	−	+	−	−	+	−	+
ab	+	+	−	+	−	−	−
c	−	−	+	+	−	−	+
ac	+	−	+	−	+	−	−
bc	−	+	+	−	−	+	−
abc	+	+	+	+	+	+	+

[a] −, factor at low level; +, factor at high level.
[b] Multiply signs of factors to obtain signs for interaction terms in combination [e.g., *AB* at (1) = (−)(−) = (+)].

Table III. Results and Analysis of Variance for a 2^3 Factorial Experiment (Run in Duplicate): The Effect of Dehydration Agent, Bloom Number of *Acid-Cured* Tissue Gelatin, and Duration of Cross-Linking on the Particle Size of Microparticles

Source of variation	Particle size (μm) Expt 1	Particle size (μm) Expt 2	df	Mean square	*F*
(1)	2.3	2.8	1		
a	3.1	3.2	1	6.24	173.52*
b	2.5	2.6	1	3.32	89.70*
ab	4.7	4.8	1	1.24	33.59*
c	2.3	2.7	1	0.08	2.16
ac	3.2	3.4	1	0.13	3.71
bc	3.2	3.3	1	0.04	1.08
abc	4.5	4.7	1	0.19	5.24
Exp. error			8	0.037	

* Significance level based on 1 df; $P < 0.01$.

Table IV. Results and Analysis of Variance for a 2^3 Factorial Experiment (Run in Duplicate): The Effect of Dehydration Agent, Bloom Number of *Lime-Cured* Tissue Gelatin, and Duration of Cross-Linking on the Particle Size of Microparticles

Source of variation	Particle size (μm) Expt 1	Particle size (μm) Expt 2	df	Mean square	*F*
(1)	2.7	2.5	1		
a	4.3	3.8	1	3.73	91.05*
b	2.6	2.4	1	2.07	50.44*
ab	2.7	2.8	1	1.44	35.19*
c	2.6	2.5	1	$3.36 * 10^{-3}$	0.08
ac	4.5	3.9	1	0.02	0.54
bc	2.4	2.4	1	$5.26 * 10^{-3}$	0.13
abc	2.8	2.9	1	$5.62 * 10^{-3}$	0.01
Exp. error			8	0.04	

* Significance level based on 1 df; $P < 0.01$.

Table V. The Size Characteristics of Gelatin (Type A, Bloom Strength 60) Microparticles as Measured by Coulter Counter

pH	Mean volume diameter[a] Expt 1 μm	Expt 1 σ	Expt 2 μm	Expt 2 σ	Type of distribution
3	2.6	1.24	2.8	1.25	Unimodal
4	2.7	1.17	2.9	1.21	
5	3.4	1.19	3.1	1.28	
6	3.4	1.42	3.2	1.29	
7	1.1	1.36 (15%)[b]	1.1	1.36 (16%)	Bimodal
	4.7	1.30 (85%)	4.8	1.29 (84%)	
8	1.1	1.36 (18%)	1.2	1.42 (16%)	
	5.0	1.28 (82%)	5.1	1.28 (84%)	

[a] The mean values and standard deviations (σ) are geometric and geometric standard deviations, respectively.
[b] Percentage of total distribution.

Table VI. The Size Characteristics of Gelatin (Type B, Bloom Strength 225) Microparticles as Measured by Coulter Counter

	Mean volume diameter[a]				
	Expt 1		Expt 2		
pH	μm	σ	μm	σ	Type of distribution
3	3.2	1.22	3.1	1.29	Unimodal
4	2.4	1.28	2.4	1.29	
5	2.9	1.24	3.0	1.20	
6	3.0	1.26	3.0	1.30	
7	1.1	1.36 (15%)[b]	1.1	1.27 (17%)	Bimodal
	4.7	1.24 (85%)	4.9	1.25 (83%)	
8	1.1	1.49 (20%)	1.1	1.52 (19%)	
	5.9	1.32 (80%)	5.7	1.30 (81%)	

[a] The mean values and standard deviations (SD) are geometric and geometric standard deviations, respectively, of distribution.
[b] Percentage of total distribution.

RESULTS AND DISCUSSION

The initial experimentation enabled a number of experimental parameters to be identified that clearly influenced the size and state of the final gelatin microparticles. These included the temperature, concentration, and volume of the gelatin solution. Compared to −15°C and ambient room temperature (~25°C), the use of B225 lime-washed gelatin showed, for example, that elevated temperature gave a significantly larger mean particle size, 2.4 vs 9.2 μm.

Anhydrous ethanol and 2-propanol were more effective than acetone in producing dispersed, small particles. However, comparing ethanol:2-propanol, the former is optimal (Tables III and IV). In addition, it was noted that, at concentrations of glutaraldehyde lower than 4% (w/v), the cross-linking process produced microparticles that showed subsequent swelling or solution behavior when transferred to the aqueous dispersion medium used for the Coulter size analysis.

The value for the effect of each factor or interaction can be obtained from the matrices in Tables III and IV.

Acid-Cured Tissue Gelatin Microparticles

The effects of dehydration agent, bloom strength, and duration of cross-linking are summarized in Table III. With factorial analysis, the factor effects and interaction phenomena can be interpreted easily and accurately. In Table III the average particle sizes were analyzed according to Yates' method (7). From the total effect, corresponding to the particular combination of treatments, an analysis of variance was performed.

With an F test, significance of the observed effects is tested. Based on the mean squares in Table III, the type of dehydration agent (A), bloom number of gelatin, and interaction of dehydration agent and bloom number (AB_1) are significant ($P < 0.01$), in that order.

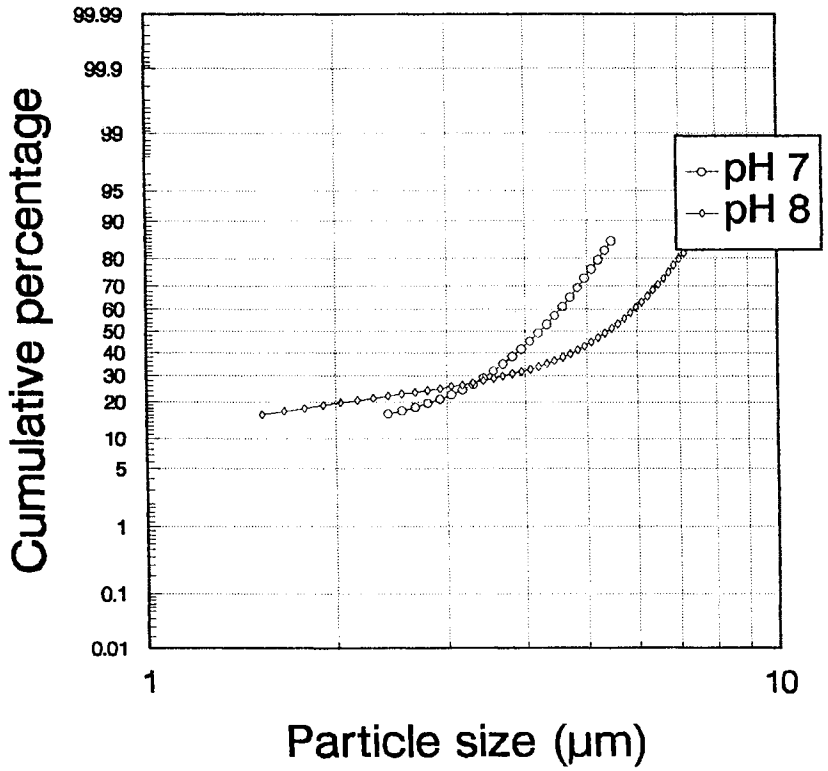

Fig. 3. Cumulative size distribution of gelatin (lime-cured type B, bloom number 225) microparticles prepared at high pH obtained using a Coulter Counter and plotted as a log-probit function.

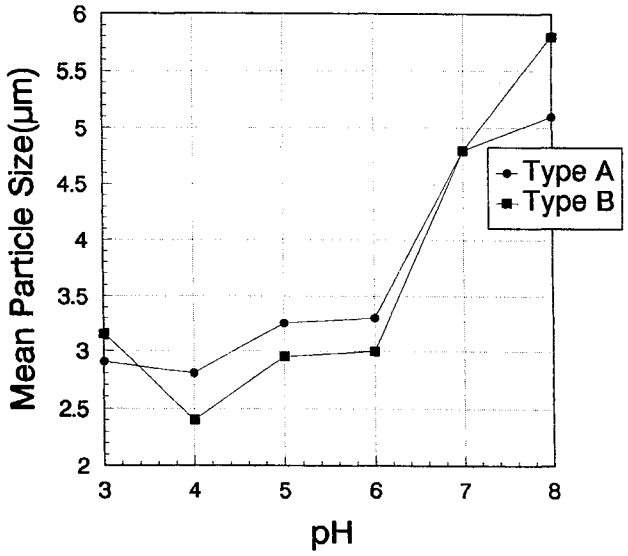

Fig. 4. The relationship of pH of gelatin solutions and particle size. Type A, Bloom 60; type B, Bloom 225.

Lime-Cured Tissue Gelatin Microparticles

The mean particle size results and mean squares of dehydration agents, bloom strength, and duration of cross-linking process are summarized for lime-cured gelatin in Table IV.

The F test for the significance of the observations demonstrated significant ($P < 0.01$) effects for the type of dehydration agent (A) and bloom strength of gelatin (B_2). Furthermore, the AB_2 interaction was significant at the 99% level ($P < 0.01$).

The factorial experiment suggests that there is a relationship among the size of the resulting microparticles, the gelatin bloom number, and the dehydrating solvent. However, the smallest microparticles are produced with the lowest bloom number for acid-cured tissue gelatin (Gelatin type A, bloom number 60) and the highest bloom number for lime-cured tissue gelatin (Gelatin type B, bloom number 225) when collected in anhydrous ethanol.

The Effect of pH

The size characteristics of microparticles made under different conditions are summarized in Tables V and VI. Material prepared at acid pH showed unimodal distributions (Figs. 1 and 2). However, samples prepared at pH 7 and 8 showed a bimodal distribution (Fig. 3) in which the parent distributions intersected on a log-probability plot. The geometric means of the parent distributions were obtained by plotting relative percentage frequency against particle size on log-linear paper (8). The biomodal distributions found at high pH could be separated and demonstrated that the small particulate system found at pH $\leq$6.0 was apparently conserved, but a second, larger, population was produced with a mean diameter of approximately 5 μm (Tables V and VI).

The optimal pH in terms of minimal particle size was found to be between pH 3 and pH 4 for both acid-cured (bloom strength 60) gelatin and lime-cured (bloom strength 225) gelatin respectively (Fig. 4).

The response surface (Fig. 5) demonstrates the effect of concentration and volume of the gelatin solution on the mean particle sizes of the gelatin microparticles. The data in Table IV indicate that the mean particle size of the gelatin microparticles increases with increases in concentration and volume of the gelatin solution.

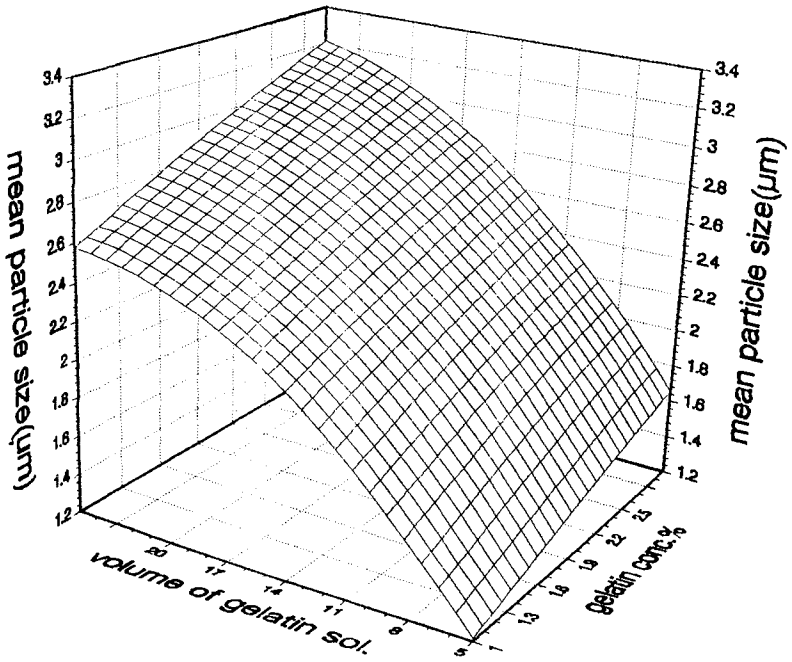

Fig. 5. Response surface diagram for the mean particle size of gelatin microparticles prepared with variation in concentration and volume of type B, Bloom 225, gelatin solutions.

The quadratic equation relating the mean particle size of microparticles, as a function of concentration and volume of gelatin solution ($r^2 = 0.993$, SD of estimation = 0.059), was

$$Z = 0.235 + 0.292*X + 0.153*Y - 0.025*X^2 - 0.003*Y^2 + 0.005*X*Y$$

where Z is the mean volume diameter of gelatin microparticles, and X and Y are the concentration and volume of the gelatin solution, respectively.

In conclusion, this study design allowed the investigation of effects on various types of gelatin produced by a range of pH, concentrations, and volumes of the primary aqueous gelatin solution for the development of microparticles within the micron range. Use of low gelatin concentrations and volumes of gelatin solution at pH 4 are required for micron-range microparticles. The microparticles prepared under optimum conditions by this process may have a mean size as low as 1.2-μm mean diameter. These gelatin systems may be useful as carriers for chemotherapeutic agents but, because of the relatively large volumes of ethanol and water used, require loading after stabilization. However, the process uses water and ethanol as solvents, which may be attractive alternatives in any manufacturing process to the chlorinated or flammable solvents widely used in the emulsion processes.

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REFERENCES

1. R. T. Jones. Gelatin: Structure and manufacture. In K. Ridgeway (ed.), *Hard Gelatin, Development and Technology,* Pharmaceutical Press, London, 1987, pp. 13–30.
2. N. Tanaka, S. Takino, and I. Utsumi. A new oral gelatinized sustained-release dosage form. *J. Pharm. Sci.* 52:664–667 (1963).
3. H. O. Sjogren, K. Nilsson, P. Malmstrom, and B. Axelsson. Column separation of monocytes by adherence to gelatin beads. *J. Immunol. Methods* 56:285–294 (1983).
4. T. Yoshioka, M. Hashida, S. Muranishi, and H. Sezaki. Specific delivery of mitomycin C to the liver, spleen and lung: Nano- and microspherical carriers of gelatin. *Int. J. Pharm.* 81:131–141 (1981).
5. J. H. Ratcliffe, I. M. Hunneyball, A. Smith, C. G. Wilson, and S. S. Davis. Preparation and evaluation of biodegradable polymeric systems for the intra-articular delivery of drugs. *J. Pharm. Pharmacol.* 36:431–436 (1984).
6. W. P. Olson. Ph.D. thesis, University of Illinois at Chicago, Chicago, 1992.
7. S. Bolton. *Pharmaceutical Statistics, Practical and Clinical Application,* Marcel Dekker, New York, 1990.
8. T. Allen. *Particle Size Measurement,* 4th ed., Chapman and Hall, London, 1990.
9. Y. Tabata and Y. Ikeda. Synthesis of gelatin microspheres containing interferon. *Pharm. Res.* 6:422–427 (1989).