DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 20(4), 535-546 (1994)

FIBRIN-BASED DRUG DELIVERY SYSTEMS. II. THE PREPARATION AND CHARACTERIZATION OF MICROBEADS

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ABSTRACT

An emulsion method was employed to prepare fibrin beads having different sizes in this study. The oil phase of emulsion system was consisted of mineral oil with various amount of oleic acid as surfactant. Fibrin was converted from fibrinogen with thrombin in Tris buffer solution, then the mixture was emulsified into the oil phase forming droplets. After curing for one hour, 400 ul of glutaraldehyde solution (0.5% v/v) was added to minimize coagulation. The recovery of fibrin beads was simply done by decanting the oil phase and washing the residual with diethyl ether once and then with a mixture of isopropanol and n-hexane (1:3) containing 0.2% w/v Tween 80 twice. As expected, increasing the amount of oleic acid in the oil phase decreased the size of fibrin beads. It is due to the decrease of interfacial tension with increasing oleic acid amount. The presence of macromolecules showed no interference on the formation of fibrin beads except lysozyme. The diffusion characteristics of fibrin beads was

evaluated using macromolecules of different molecular weight as model. The size of fibrin beads affected the penetration rate, whereas the effect of molecular weight of macromolecules was inconclusive. An exponential equation was able to approximate the penetration of macromolecules into fibrin beads during the late-time period. The possibility of using fibrin beads as the carrier to deliver protein drugs was appreciated.

INTRODUCTION

The fibrin polymer represents a natural, biocompatible, biodegradable matrix. An approach utilizing the biochemical reaction between fibrinogen and thrombin to yield the fibrin polymer has been proposed (1). As a delivery system, drugs are entrapped within, or coated with, the fibrin polymer to form systems for parenteral injection, or they can be entrapped within sheets for surgical implantation (2). However, recent advances in biotechnology has made the production and use of proteins and peptides feasible, but such materials present a challenging formulation and drug delivery problem. The specificity of the fibrinogen/thrombin reaction is such that reactive molecules such as proteins and peptides may be incorporated without modification. Results of studies in numerous laboratories (3,4) indicate that a relatively large area is involved in the interaction between thrombin and its substrate, suggesting that there would be limited interactions with added proteins. Furthermore, the biochemical reaction between fibrinogen and thrombin is mild enough without denaturing macromolecules added. The diffusion characteristics of fibrin films has also been studied with macromolecules of different molecular weights (2). It suggested that the manipulation of formulation and process variables was possible to engineer into fibrin-based delivery system with a control characteristic for human application. The present study centers on the development of methods to produce fibrin beads and an evaluation of their potentials for delivering protein drugs.

EXPERIMENTAL

Materials

Fibrinogen (Type IV, from Bovine Plasma), Thrombin (Bovine plasma), Lysozyme (M.W., 14,300), Carbonic Anhydrase (M.W., 29,000) and Bovine Serum Albumin (M.W., 66,000) were obtained from Sigma Chemical Co.. Tris(hydroxymethyl)-aminoethane, Paraffin highly liquid (Light mineral oil), Tween 80 (Polyoxyethylene sorbitan monooleate), 2-Propanol, Glutaraldehyde and Sodium Azide were supplied by Merck Co.. Diethyl ether was provided by Riedel-de Haen and n-Hexane was obtained from Mallinkrodt.

Methods

Preparation of Fibrin Beads

Fibrin beads were prepared following a procedure previously reported for producing serum albumin microbeads (5). 60 mg of desalted, freeze-dried fibrinogen was dissolved in one gram of tris buffer (0.05 M, pH=7.4). 100 ul thrombin solution (containing 5 unit activity of thrombin in tris buffer, 0.05 M and pH 7.4) was placed in a syringe with a 22G x 1 1/2" needle, then one gram of fibrinogen solution was drawn into the syringe and mixing thoroughly. The resulting solution was emulsified into the oil phase consisted of light mineral oil and various amount of oleic acid and stirred at 480 rpm. After curing for one hour, 400 ul of glutaraldehyde solution (0.5% v/v) was added to minimize coaggulation of fibrin beads and kept stirring for 30 minutes. The recovery of fibrin beads from the emulsion system was simply done by decanting the oil phase and the residual was washed with diethyl ether for once and then with a mixture of isopropanol and n-hexane (1:3) containing 0.2% w/v Tween 80 for twice. Fibrin beads were placed in a desiccator for one day and then were used to proceed the diffusion study.

The Effect of Oleic Acid Amount on the Size Distribution of Fibrin Beads

The effect of oleic acid amount on the size distribution of fibrin beads was examined. The several ratios of mineral oil to oleic acid were dispensed and used as the oil phase to prepare fibrin beads following the general procedure as described above. Three batches were produced for each ratio. The size distribution of resulting fibrin beads was measured on twenty beads under a zoom lupe (Peak Zoom Lupe 816, Japan) with a scaled eyepiece. The mean and standard deviation were reported and compared.

The Stability of Macromolecules

Since most macromolecules were easily denatured (6), the stability of macromolecules in several conditions were compared and then a condition with minimal interference from denaturation of macromolecules was selected to study the diffusion characteristics of fibrin beads. The effect of mixing macromolecules either by rotation or shaking was examined at 37 °C. The preservability of sodium azide in various concentrations (0.1 or 0.5% w/v) were also compared. The concentration change of macromolecules was determined using a gradient HPLC system: solution A, 0.05% trifluoroacetic acid/deionized water; solution B, 0.05% trifluoroacetic acid/acetonitrile; mixed 24-76% B in A over 20 minutes. The flow rate was 1.5 ml/min. A Synchropak C4 column (4.6X150 mm) was used and UV detection was set at a wavelength of 220 nm.

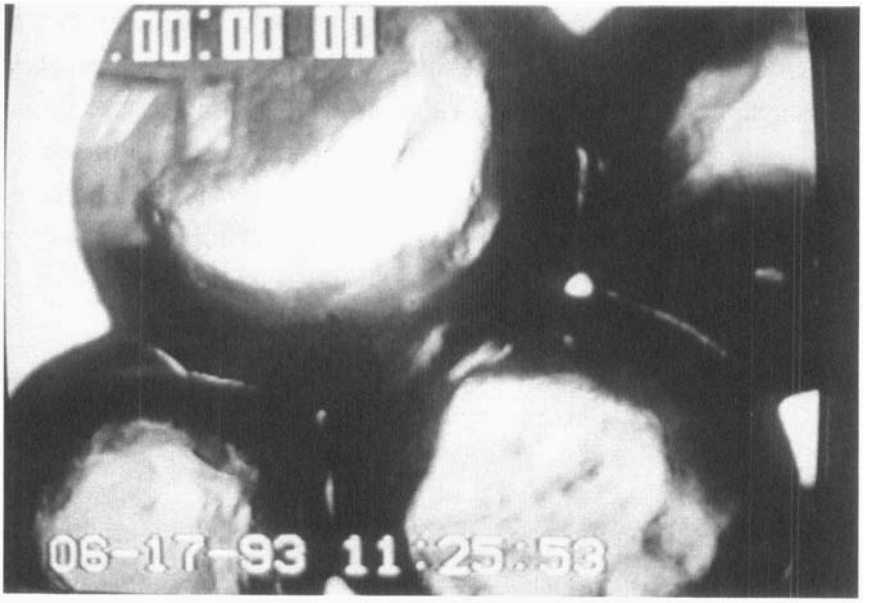
The Penetration Studies

In general, fibrin beads (either plain or containing macromolecules) were dispersed in 2 ml of tris buffer (0.05 M, pH=7.4) and kept in a shaking water bath at 37 °C. At predetermined intervals, 60 ul of the medium was taken for HPLC analysis of macromolecules. The medium was replenished with the same volume of fresh buffer solution. In this

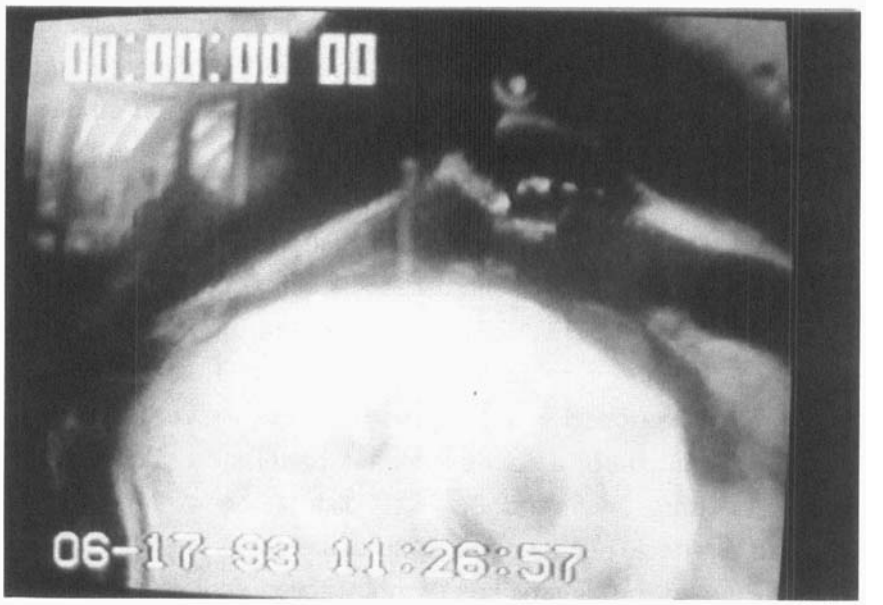
study, the diffusion characteristics of fibrin beads was evaluated by two ways. One of them, macromolecules was dissolved in the fibrinogen solution initially, and then the fibrin beads were prepared according to the general method. The release of macromolecules from fibrin beads into the medium was followed. Another way of studying the diffusion characteristics of fibrin beads was to examine the penetration of macromolecules from the medium into plain fibrin beads.

RESULTS AND DISCUSSION

Heat denaturation or chemical crosslinking with glutaraldehyde has been applied in the emulsion system to prepare microbeads for many biopolymers such as albumin and collagen. A similar emulsion method was employed in this study to prepare fibrin beads (5), but utilizing a mild condition of biochemical reaction between fibrinogen and thrombin. Basically, fibrinogen solution with either dissolved or dispersed drugs was mixed with thrombin thoroughly and the mixture was then emulsified into the oil phase. Fibrin beads were formed in situ. Since an emulsion method was used, fibrin beads obtained consist of a distribution of sizes. It has to be appreciated that the rate of drug released into a medium is not only dependent on the release mechanism for a single particle but on how the beads are distributed as well (7). This should be true for many particular systems whether it be pure solid drug , microcapsules, a microemulsion, or where drug is embedded in a solid matrix. If the delivery system is to be manipulated to engineer a desired release pattern, then both the physical properties of drug itself and the size distribution of beads has to be considered. There has several factors affecting the size of emulsion droplets including the dimensions of the reaction container and stirrer, the rate of stirring, the density and viscosity of the internal aqueous and external oil phases, and the interfacial tension between phases. Among them, the interfacial tension between phases is most significantly and can be easily controlled by varying the amount of surfactant added.



(A)



(B)

FIGURE 1

The Microscopic Examination of Fibrin Beads with enlargement of 100X (A) and 400X (B)

TABLE 1

The Effect of Added Amount of Oleic Acid on the Size and Its Distribution of Fibrin Beads

	Mineral Oil / Oleic Acid						
	0	500/1	400/1	300/1	200/1	100/1	50/1
Batch 1	1.335	0.805	0.640	0.485	0.330	0.240	0.170
	(0.489)	(0.157)	(0.094)	(0.093)	(0.086)	(0.060)	(0.066)
Batch 2	1.195	0.615	0.615	0.480	0.295	0.235	0.165
	(0.190)	(0.104)	(0.176)	(0.100)	(0.069)	(0.067)	(0.059)
Batch 3	1.030	0.720	0.615	0.495	0.285	0.210	0.145
	(0.232)	(0.120)	(0.131)	(0.110)	(0.067)	(0.091)	(0.051)
Mean	1.187	0.713	0.623	0.487	0.303	0.228	0.160
	(0.349)	(0.149)	(0.136)	(0.099)	(0.076)	(0.074)	(0.060)

*( ): Standard Deviation

In this study, oleic acid was selected as surfactant. Different ratio of oleic acid to mineral oil was used as the oil phase to examine its effect on the size distribution of fibrin beads. Figure 1 shows a typical picture of fibrin bead taken under microscopic examination with an 100 (A) or 200 (B) times of enlargement. The size and its distribution were measured on twenty beads. The results are shown on Table 1. It clearly reveals that increasing the ratio of oleic acid to mineral oil decreases the size of fibrin beads, but its distribution becomes wider. Since tris buffer of pH 7.4 was used to dissolve fibrinogen, it was able to neutralize oleic acid forming a surfactant within interfacial region to lower the tension between phases. The more oleic acid added, the more surfactant was converted making interfacial tension lower. As a result, smaller droplet size of aqueous phase was formed in the emulsion system giving smaller bead size. It would be useful for manipulating the release pattern by adjusting the size range of fibrin beads.

The stability of macromolecules was examined firstly in order to select a condition for studying the release or penetration of macromolecules. It was conducted for three macromolecules of lysozyme, bovine serum albumin and carbonic anhydrase at conditions of combining two mixing patterns of rotating and shaking and the addition of sodium azide at two concentrations as preservative. The results are illustrated in Figure 2. It demonstrates that lysozyme is stable in both conditions of shaking and rotating. The addition of sodium azide seems to improve the stability further. However, bovine serum albumin and carbonic anhydrase were gradually degraded when rotating. And the stability can be improved by shaking or keeping steady. It implies that both macromolecules are quite sensitive to shearing force. Nevertheless, the addition of sodium azide can significantly improve their stability. Also, it seems to be more stable at higher concentration of sodium azide. As a result, the penetration studies in the follow were conducted in a shaker, and adding 0.5% sodium azide as preservative.

In a preliminary study, it was found that there was no problem to prepare fibrin beads with the entrapment of lysozyme, carbonic anhydrase and bovine serum albumin. However, the release of those macromolecules from fibrin beads was too small to be detected. It was suspected that it was probably due to the crosslinking effect of glutaraldehyde, which was added to minimize the agglomeration of fibrin beads during preparation. However, the release of a solute from a spherical particles can be described by the same equation shown in the following as that for the penetration of a solute into a spherical particle mathematically if the solute is in a dissolved state (8). The only difference is they move in an opposite direction. Either way can be used to deduce the diffusion characteristics of fibrin beads. Since that, the diffusion of fibrin beads was characterized by the penetration study of macromolecules into the plain beads.

$$1 - M_t / M_{oo} = 6/\Pi^2 * EXP(-D * \Pi^2 * t / a^2) \qquad \text{Equation 1}$$

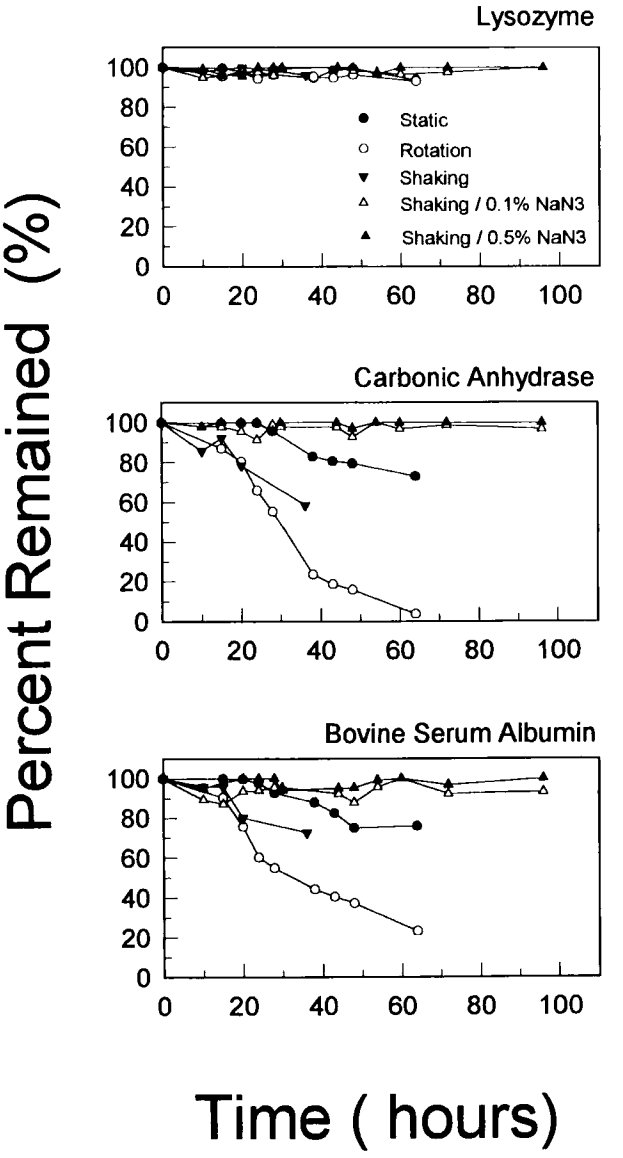


FIGURE 2

The Stability of Macromolecules in Various Conditions

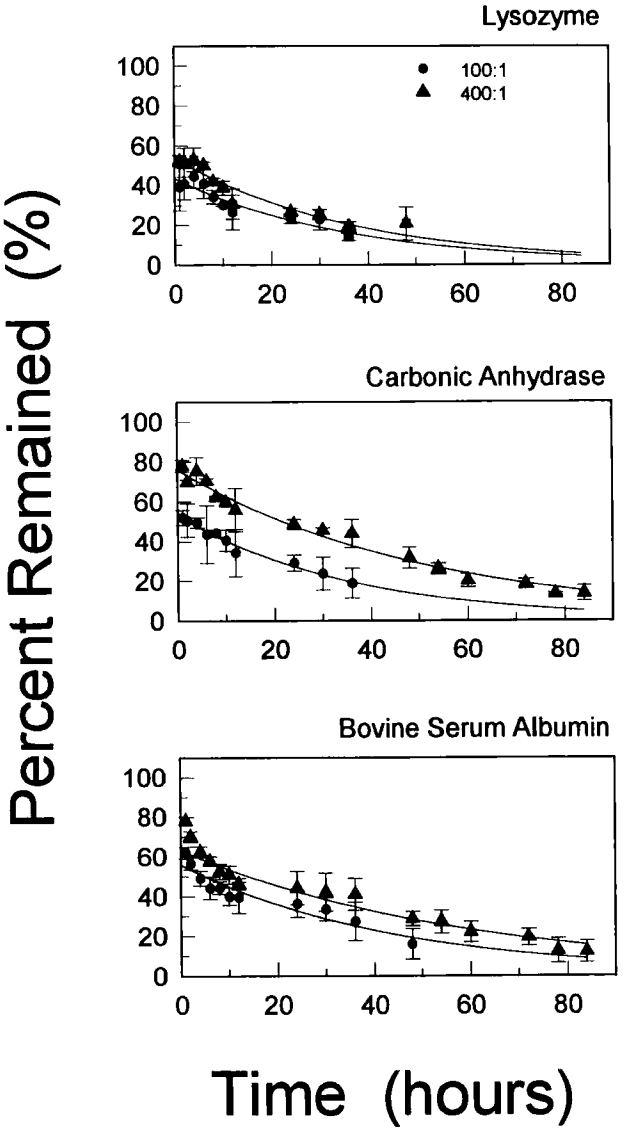


FIGURE 3

The Penetration of Macromolecules into Fibrin Beads of two Different Size

TABLE 2
The Regression Results According to Late Time Approximation.

	Lysozyme		Carbonic Anhydrase		Bovine Serum Albumin	
Mineral Oil/ Oleic Acid	400/1	100/1	400/1	100/1	400/1	100/1
Constant (a)	53.94	43.54	63.17	55.92	76.03	53.38
Exponential (b)	0.0269	0.0275	0.0166	0.0222	0.0193	0.0279
Diffusion Coefficient ($x10^{10}$ cm^2/sec)	7.3541	1.0088	4.5382	0.8143	5.2764	1.0235

In Equation 1, the term of $(1 - M_t / M_{oo})$ is the fraction remained to penetrate at time t, D is the diffusion coefficient in the medium of spherical particle and *a* is the radius of spherical particle. It is used to approximate the penetration rate of a solute into a spherical particle during late time. In this study, the penetration of lysozyme, carbonic anhydrase and bovine serum albumin into two size batches (prepared in the oil phases with a mineral oil/oleic acid ratio of 100/1 and 400/1) of fibrin beads were examined and the results are shown in Figure 3. It clearly illustrates that the penetration of macromolecules is dependent on the size of fibrin beads. The smaller the size of fibrin beads, the faster the penetration rate of macromolecules. It complies to the theoretical prediction by Equation 1. The solid lines in the plots of Figure 3 are the regression result of the fraction remained to penetrate versus time according to Equation 1 by assuming the radius of fibrin beads to be a constant and using data of less than 60%. Comparing the exponential terms in Table 2, it is also concluded that the smaller the size of fibrin beads, the faster the penetration of macromolecules. It is due to the larger surface area available for penetration.

The exponential term, which is equal to $(-D^*\Pi^2/a^2)$, was then used to calculate the diffusion coefficient of macromolecules in fibrin beads. The

results are also listed in Table 2. Theoretically, diffusion coefficient is a function of the molecular weight of macromolecules. Nevertheless, the dependence of diffusion coefficient on the molecular weight of macromolecules is inconclusive based on this result. A possible explanation is due to the assumption of all beads having the same size making the calculation of diffusion coefficient incorrect. Further elucidation of single beads releasing mechanism is necessary to more precisely predict the effect of molecular weight of macromolecules on diffusion coefficient.

ACKNOWLEDGEMENT

We would like to express our sincere thanks for the financial support of the National Science Council of the Republic of China (NSC 83-0412-B038-004).

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