[Chem. Pharm. Bull. 34(8)3341—3347(1986)]

In Vitro Release of Poly(ethylene oxides) from Serum Albumin Microspheres

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(Received December 19, 1985)

The *in vitro* release of poly(ethylene oxides) from bovine serum albumin microspheres was found to be biphasic. When the release of poly(ethylene oxides) was analyzed by using two theoretical equations, it was suggested that the poly(ethylene oxides) diffused in the albumin matrix of the microspheres without resistance, and the release rate was dependent only on the concentration gradient. This result can be ascribed to the large channels in the albumin matrix (larger than 55 Å). When the pH of the albumin solution used for microsphere preparation was changed from 5.0 to 7.4, the poly(ethylene oxide) release pattern was greatly modified.

Keywords——drug delivery system; drug carrier; serum albumin microsphere; drug release rate; release control; poly(ethylene oxide); molecular weight effect

One approach to minimize systemic side effects of a chemotherapeutic agent is to target the drug to the desired site. From this point of view, Kramer prepared biodegradable serum albumin microspheres containing anticancer agents and suggested their *in vivo* use.[1] Albumin microspheres provide a potentially useful means of delivering drugs to tumor sites because microspheres injected into the vascular system as a suspension in a suitable liquid are rapidly removed from it by phagocytosis, and are entrapped at the target sites. Kramer and Burnstein reported that microspheres containing an anticancer agent were entrapped in human tumor cells.[2] In addition, delivery of microsphere can be controlled by using a novel technique. Magnetically responsive albumin microspheres which contain magnetite have been developed and tested.[3] The microspheres could be delivered to the target site by using extracorporeal magnets and were retained there for a prolonged period after removal of the magnetic field.

Drug-release characteristics are also important in site-specific drug delivery systems. To attain desired therapeutic effects, the microspheres entrapped in the target sites must retain the majority of the drug originally incorporated in them and must gradually release the drug to the surroundings over a certain period. Over 90 drugs have been incorporated into albumin microspheres and their release profiles have been reported, as reviewed by Tomlinson.[4] Drugs are generally released from albumin microspheres in a biphasic manner, with an initial fast release (called the 'burst effect') followed by a slower release.[5] The initially incorporated drug is mostly released from the microspheres in the first release phase. However, the desired therapeutic effects will not be attained if the drug is mostly released before entrapping of the microspheres at the target sites, and therefore, the mechanisms of the initial large and fast release phase need to be established. Some investigators have attempted to control the drug release from albumin microspheres.[6] However, in spite of these studies, the details of drug release from serum albumin microspheres remain obscure. Much work needs to be carried out to collect information about the mechanism and controlling factors of drug release from albumin microspheres.

Experimental

Materials——Bovine serum albumin (BSA; Fraction V, A-8022 from Sigma Chemical Co., St Louis, MO, U.S.A.) was used without further purification. Three poly(ethylene oxide) fractions, 1500, 3060 and 8700 in average molecular weight (PEO-I, II and III; PEO-I and III from Wako Pure Chemical Industries, Ltd., Osaka, Japan, PEO-II from Kokusan Chemical Works, Ltd., Tokyo, Japan), were used as model drugs for a study on the effect of drug molecular weight upon drug release from albumin microspheres, and a narrow-distribution poly(ethylene oxide) fraction (PEO-S; $M_n = 2671$, $M_w/M_n = 1.038$, Nippon Oil & Fats Co., Ltd., Tokyo, Japan) was also used to examine the effect of the pH of the BSA solution. Cottonseed oil (Okamura Oil Mill, Ltd., Osaka, Japan) was of commercial origin. The other chemicals used were of analytical reagent grade.

Preparation of BSA Microspheres——BSA microspheres were prepared in a manner similar to that described by Widder *et al.*[3b] BSA solutions at a concentration of 25% (pH 5.0) were obtained by dissolving BSA in aqueous PEO solutions. Adjustment of the pH with aqueous sodium hydroxide solution was necessary to prepare a pH 7.4 BSA solution. A 0.6 ml aliquot of BSA solution was dispersed into 100 ml of cottonseed oil with a magnetic stirrer. Then, the resulting w/o-type emulsion was added with stirring to 200 ml of cottonseed oil which had been preheated to 180 ± 5 °C. As Widder *et al.* had reported that increased stabilization of the microspheres by the use of increased temperature decreased the drug release rate,[6a] BSA microspheres were stabilized only at 180 ± 5 °C. The stirring was further continued for 20 min at 180 ± 5 °C. Then, the dispersion was cooled to room temperature, 200 ml of ethyl ether was added to it and BSA microspheres were separated by filtration with a membrane filter (pore size-1 μm, Nuclepore Corp., Pleasanton, CA, U.S.A.). The microspheres were washed four times with ethyl ether to remove cottonseed oil, and then lyophilized.

The microspheres containing PEO-III were prepared by emulsifying the BSA solution into cottonseed oil saturated with water to prevent phase separation of PEO in the aqueous droplets.

For experimental convenience in the release test, the microspheres were prepared to be 1—40 μm in diameter. The size distribution of microspheres was determined by a method similar to that described in a previous paper.[7] More than one thousand microspheres were photographed under an optical microscope and printed images of the spheres were measured. The scale of the micrometer was used for calibration.

The shape and surface appearance of microspheres were observed with an optical microscope and a scanning electron microscope (JSM-T20; JEOL, Tokyo, Japan).

Release Test——The PEO release rate of the microspheres was deternmined as follows. A given weight of BSA microspheres was dispersed into 300 ml of a test medium maintained at 37 ± 0.5 °C in a cylindrical glass cell. The dispersion was sonicated for a few minutes in an ultrasonic bath (Bransonic 32; Branson Ultrasonic Corp., Shelton, CT, U.S.A.) to disperse the microspheres homogeneously, and then it was immediately placed in a water bath thermostated at 37 ± 0.5 °C and agitated with a six-bladed impeller at a constant rate of 124 rpm. The cell was equipped with a plastic cover to prevent water evaporation during the test period. Without stopping the agitation, aliquots of the dispersion were withdrawn with syringes at appropriate time intervals, and immediately filtered through a membrane filter (pore size-0.45 μm, Millipore Co., Bedford, MASS, U.S.A.) to remove the microspheres.

A phosphate buffer solution (pH 7.4) adjusted to be isotonic with sodium chloride was used as the test medium. As the microspheres prepared using the pH 7.4 BSA solution had poor dispersibility in comparison with those prepared using the pH 5.0 BSA solution, an ionic surfactant, sodium dodecyl sulfate (SDS), was added to the test medium used to evaluate the PEO release from the microspheres prepared using the pH 7.4 BSA solution.

A modification of the method of Stevenson, which was reported by Hasegawa *et al.*,[8] was employed to determine the PEO concentration, as follows. A 3-ml aliquot of the filtrate was mixed with an equal volume of acetone, and the mixture was allowed to stand for 30 min to precipitate free albumin molecules. The precipitate was removed by centrifugation, then 2 ml of water was added to 3 ml portions of the supernate and 0.1 ml portions of 7.3 N aqueous hydrochloric acid, 10% (w/v) barium chloride and 10% (w/v) phosphomolybdic acid solutions were added successively. The resultant precipitate was separated by centrifugation, washed twice with water and dissolved in concentrated sulfuric acid. Next, 1 ml of 10% (w/v) aqueous ammonium thiocyanate and 0.5 ml of 2% (w/v) aqueous zinc chloride were added, and the total volume of the solution was adjusted to 10 ml with water. The absorbance was determined at 460 nm after 20 min. The presence of SDS did not interfere with the determination of PEO.

Each experiment was performed in triplicate, and mean values are reported. Reproducibility was adequate.

Results and Discussion

PEO Release from BSA Microspheres

Drug release from albumin microspheres seems to be dependent on many factors, such as (1) the density of the albumin matrix, (2) the extents of denaturation, polymerization and cross-linking of albumin molecules, (3) the size and the surface area of the microspheres, (4)

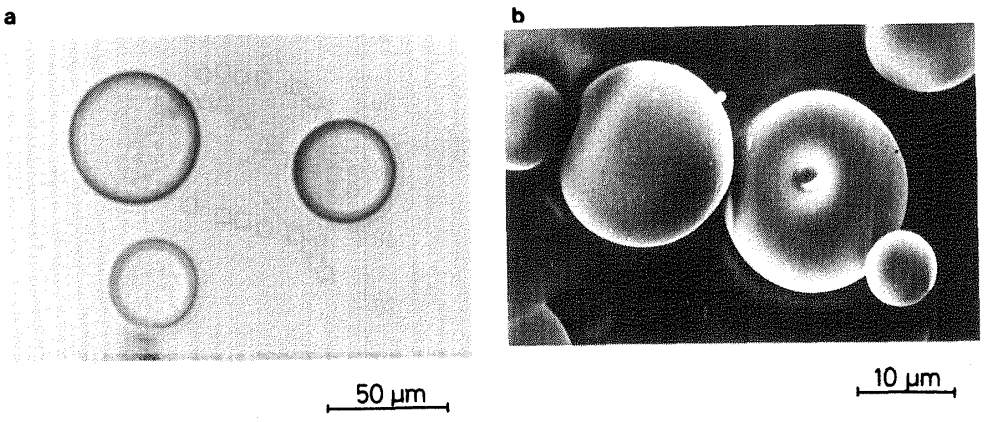

Fig. 1. Optical and Scanning Electron Micrographs of BSA Microspheres Containing Poly(ethylene oxides)

(a) Optical micrograph of microspheres prepared using a pH 7.4 BSA solution (3% PEO-S); (b) Scanning electron micrographs of microspheres prepared using a pH 5.0 BSA solution (3% PEO-III).

TABLE I. Mean Diameters and Specific Surface Areas of BSA Microspheres

Polymer	Mean diameter (μm)	Ratio of specific surface area
PEO-I	$13.2 \pm 7.5^{a)}$	1.02
PEO-II	$12.7 \pm 7.9^{a)}$	1.00
PEO-III	$11.3 \pm 7.2^{a)}$	1.11

a) Standard deviation.

physical and physicochemical properties of the drug, (5) the molecular weight of the drug, (6) the level of drug incorporated in the microspheres, (7) the location of the drug in the microspheres, (8) physical and physicochemical interactions between the drug and the albumin matrix.[9] In this study, the effect of drug molecular weight on the drug release from albumin microspheres was examined by using hydrophilic and water-soluble PEOs as model drugs.

Optical and scanning electron micrographs of the resultant albumin microspheres containing PEO are shown in Fig. 1. Optical microscopy did not reveal any aggregates in the aqueous dispersion of the microspheres during the release rest. It was shown by scanning electron microscopy that some large microspheres (above 15 μm) had a single large hollow, which may have been created when water molecules escaped from the aqueous droplets to the oil phase. However, no differences were found among the microspheres, irrespective of whether they contained PEO or not and whether they had been tested or not.

Size and specific surface area are important factors in studying drug release from pharmaceutical products. For albumin microspheres, Tomlinson *et al.* reported that drug release from small microspheres was faster than from large microspheres.[9] The mean diameter and ratio of specific surface area of BSA microspheres containing PEO are summarized in Table I. The size distribution of microspheres was scarcely affected by the molecular weight of PEO because the PEO levels in the microspheres were very low. The initial PEO levels in microspheres were calculated to be 3.0% on the assumption that water-soluble PEO had not distributed itself to the oil phase in the process of microsphere preparation.[9] Though, as described above, some of the large microspheres had a large hollow, the existence of the hollows would not affect the calculation of the specific surface area from the size distribution of microspheres.

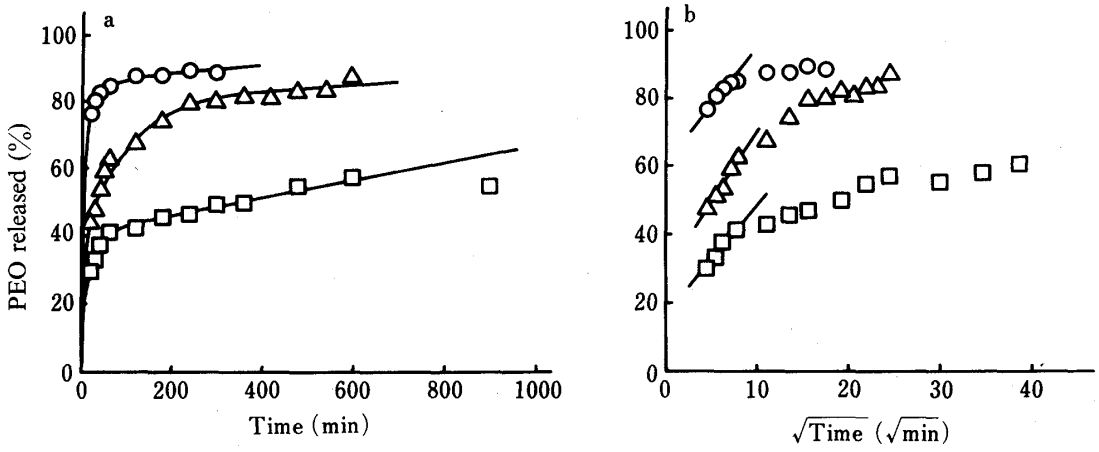

Fig. 2. Effect of Molecular Weight of Poly(ethylene oxides) on Their Release from BSA Microspheres

(a) Plots of the percentage of PEO released against time; (b) Plots of the percentage of PEO released against the square root of time. (○) PEO-I; (△) PEO-II; (□) PEO-III.

TABLE II. Effect of Molecular Weight of PEO on Release Rate

Polymer	$D_b \times 10^{13}$ (cm²/min)	Radius[a] (Å)	Amount of polymer released-time plot			Amount of polymer released-square root of time plot		
			$V_i \times 10^9$ (mol/min)	$R_{exp.}$	$R_{theo.}$	$K_h \times 10^8$ (mol/min$^{1/2}$)	$R_{exp.}$	$R_{theo.}$
PEO-I	13.4	10.9	15.2	1.36	2.99	17.7	1.30	3.00
PEO-II	9.30	15.8	11.2	1.00	1.00	13.6	1.00	1.00
PEO-III	5.45	26.9	2.66	0.24	0.23	3.28	0.24	0.23

a) Hydrodynamic radius.

Release profiles of PEO from microspheres are shown in Fig. 2. In the plots of the percentage of PEO released against time (Fig. 2a), rapid initial release was also observed. The release of PEO continued gradually in the later stage. The amount of PEO released rapidly in the initial stage decreased with increase in the molecular weight. As BSA microspheres have a matrix structure, the Higuchi equation can be used to evaluate PEO release from BSA microspheres.[10] In the plots of the percentage of PEO released against the square root of time (Fig. 2b), linear relationships were obtained in the initial release stage, though negative deviations were found in the later stage. These deviations could be attributed to exhaustion of the polymer suspension phase in the albumin matrix, as reported previously.[7] That is, the number of vacant microspheres may increase in the later release phase.

To consider the data in detail, the initial release rate constants were calculated from the linear parts of the respective plots (Table II). The experimental and theoretical ratios of each rate constant to that for PEO-II are also listed in Table II. The theoretical ratios were calculated by using the following equations. Equation 1 was derived on the assumption that the release rate in the initial stage is simply dependent on the concentration gradient,

$$Q = K \cdot S \cdot G \cdot D_m \cdot t \qquad (1)$$

where Q is the amount of PEO release in time t, D_m is the diffusion coefficient of PEO in the albumin matrix, G is the concentration gradient between a point on a microsphere surface and a certain point in the matrix (near the surface), S is the total surface area of BSA

microspheres, and K is a coefficient dependent on the porosity and tortuosity of channels in the matrix. The concentration gradient, G, was assumed to be constant for a short period at the beginning of the release test. Equation 2 is a modification of the Higuchi equation, which was derived by Fessi *et al.* under the condition that $C/\varepsilon < S_o$, where C is the drug concentration in the matrix, ε is the porosity of the matrix, and S_o is the solubility of the drug in the dissolution medium.[11]

$$Q = S \cdot C\sqrt{D_m \cdot t} \tag{2}$$

Accordingly, the initial release rate, V_i, and the initial release rate constant, K_h, are given by the following equations.

$$V_i = K \cdot S \cdot G \cdot D_m \tag{3}$$

$$K_h = S \cdot C\sqrt{D_m} \tag{4}$$

If there are physical and physicochemical interactions between the molecules and the matrix, the diffusion rate of the molecules in the matrix should be smaller than that in a bulk solution. Therefore, D_m is given by the following equation,

$$D_m = R \cdot D_b \tag{5}$$

where D_b is the diffusion coefficient in a bulk solution and R is the resistance coefficient arising from the physical and physicochemical interactions of PEO with the albumin matrix. Inserting Eq. 5 into Eqs. 3 and 4 gives:

$$V_i = K \cdot S \cdot G \cdot R \cdot D_b \tag{6}$$

$$K_h = S \cdot C\sqrt{R \cdot D_b} \tag{7}$$

Values of the diffusion coefficient used for the calculation and the hydrodynamic radii of the polymers were estimated from the data and the relationship described by Brown and Johnsen ($\log D_b = a \cdot \log M_w + b$, where a and b are constants, and M_w is the molecular weight of PEO.).[12]

In the case of PEO-I, the experimental ratios ($R_{exp.}$) of V_i and K_h were smaller than the theoretical ratios ($R_{theo.}$). On the other hand, the experimental ratios of V_i and K_h for PEO-III were in fair agreement with the theoretical ratios. For PEO-II and -III, the release profiles in the plots of the percentage of PEO released against the square root of time could be divided into three areas, but for PEO-I, the first area was not detected. Namely, the disagreement in the case of PEO-I was probably caused by the use of the V_i and K_h values obtained in the secondary release area for the calculation.

As the theoretical ratios were calculated on the assumption that there was no resistance to PEO diffusion through the matrix ($R=1$), the agreements of the experimental ratios with the theoretical ratios for PEO-III imply that the diffusion coefficients of the polymers in the matrix were the same as those in bulk solutions; in other words, PEO was able to diffuse through the matrix without resistance. It is suggested that the PEO release rate is not influenced by the molecular weight and is dependent only on the concentration gradient.

This result can be ascribed to the existence of large channels in the matrix. The diameter of channels was estimated to be more than 55 Å from the fact that PEO-III having a hydrodynamic radius of 26.9 Å could diffuse freely through the matrix. Judging from the results obtained in this study, it is reasonable that low-molecular-weight drugs, such as 5-fluorouracil (2.26 Å in hydrodynamic radius[13]), were released from albumin microspheres faster than high-molecular-weight substances. Further, the results would explain why the biphasic release properties of the microspheres are less apparent when the drug is very slightly water-soluble or when drug loading exceeds about 30%.[5] Namely, the high concentration

gradient probably makes distinction between the fast release phase and the slow release phase difficult when drug loadings exceed about 30%, and the drug release from albumin microspheres would depend on the drug dissolution process as a rate-limiting step when the drug is very slightly water-soluble.

Effect of the pH of BSA Solution on Drug Release

Control of drug release from carriers is necessary to attain the desired therapeutic effects in site-specific drug delivery systems. The drug release from albumin microspheres is influenced by the density of the albumin matrix and the extents of denaturation, polymerization and cross-linking of albumin molecules, as described above. As it is common knowledge that the three-dimensional conformation of albumin molecules changes with pH in solution, the density of the albumin matrix and the extents of denaturation, polymerization and cross-linking of albumin molecules must be dependent on it. Albumin molecules extend due to dissociation of carboxyl groups at a pH higher than the isoelectric point. Therefore, the albumin matrix prepared at higher pH (7.4) than the isoelectric point (4.7) probably contains more intermolecular disulfide bonds than that prepared near the isoelectric point (5.0). This means that the former is probably denser than the latter.

The effect of the pH of the BSA solution used for preparation on the drug release from albumin microspheres is clearly shown in Table III. The initial release rate of PEO-S from the microspheres was reduced to 1/11 of its initial value by changing the pH of the BSA solution from 5.0 to 7.4. In addition, the secondary release rate (V_s) calculated from the slow release phase was greatly influenced by the pH of the BSA solution used for microsphere preparation, being reduced to 1/735.

The reason for the reduction of V_i and V_s can be examined by using Eq. 6, as follows. Equation 6 can be rewritten as follows:

$$(V_i)_{5.0} = K_{5.0} \cdot R_{5.0} \cdot S_{5.0} \cdot G \cdot D_b \tag{8}$$

$$(V_i)_{7.4} = K_{7.4} \cdot R_{7.4} \cdot S_{7.4} \cdot G \cdot D_b \tag{9}$$

Hence, the ratio of the product of the coefficient dependent on the matrix density and the resistance coefficient for the microspheres prepared using a pH 7.4 BSA solution ($K_{7.4} \cdot R_{7.4}$) to that for the microspheres prepared using a pH-unadjusted BSA solution ($K_{5.0} \cdot R_{5.0}$) can be calculated by means of the following equation.

$$K_{7.4} \cdot R_{7.4} / K_{5.0} \cdot R_{5.0} = (V_i)_{7.4} \cdot S_{5.0} / (V_i)_{5.0} \cdot S_{7.4} \tag{10}$$

The ratio calculated from the data was 0.0763. Therefore, it is concluded that the decrease in the drug release rate due to the change in the pH of a BSA solution was caused by the increase in the density of albumin matrix and/or the occurrence of interactions of PEO with the albumin matrix.

Compacting the matrix and the use of interactions between the drug and the matrix

TABLE III. Effect of pH of BSA Solutions on PEO[a] Release from Microspheres

Microspheres	Mean diameter (μm)	Ratio of specific surface area	V_i (mol/min)	V_s[b] (mol/min)
Prepared using pH 5.0 BSA soln.	10.2±6.2[c]	1.00	3.25×10^{-9}	1.55×10^{-11}
Prepared using pH 7.4 BSA soln.[d]	9.0±5.3[c]	1.19	2.95×10^{-10}	2.11×10^{-14}

a) PEO-S. *b*) Release rate in the secondary phase. *c*) Standard deviation. *d*) Test medium contained 0.05% sodium dodecyl sulfate to disperse the microspheres homogeneously.

material may be effective means for controlling drug release from albumin microspheres, as described above. Change in the pH of the albumin solution used for microsphere preparation and addition of such denaturants as inorganic salts, organic solvents, guanidinium chloride and urea, which influence the three-dimensional conformation of albumin molecules dissolved in water, are certainly useful in compacting the matrix. Indeed, it was demonstrated in this study that the change in pH of the albumin solution from 5.0 to 7.4 delayed the PEO release from albumin microspheres. Interactions useful in controlling drug release might include ionic bonds, protein binding, hydrolyzable chemical bonds, *etc.* For example, as reported by Widder *et al.*,[6a] doxorubicin, which forms complexes with serum albumin in a pH 7.3 aqueous solution,[14] is slowly released from albumin microspheres as compared to 5-fluorouracil, which does not physicochemically interact with albumin.[15]

Further studies to obtain information about factors controlling drug release are required to improve the delivery of various kinds of drugs in albumin microspheres to the desired sites.

Acknowledgment The authors thank Nippon Oil & Fats Co., Ltd. for supplying a poly(ethylene oxide).

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