Controlled Antibody Release from a Matrix of Poly(Ethylene-*co*-Vinyl Acetate) Fractionated with a Supercritical Fluid

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SYNOPSIS

A new method is presented for controlling the rate of antibody (Ab) release from an inert matrix composed of poly(ethylene-*co*-vinyl acetate) (EVAc), a biocompatible polymer that is frequently used to achieve controlled release. Using supercritical propane, a parent EVAc sample (M_n = 70 kDa, M_w/M_n = 2.4) was separated into narrow fractions with a range of molecular weights (8.7 < M_n < 165 kDa, 1.4 < M_w/M_n < 1.7). Solid particles of Ab were dispersed in matrices composed of different polymer fractions and the rate of Ab release into buffered saline was measured. The rate of Ab release from the EVAc matrix depended on molecular weight: > 90% of the incorporated Ab was released from low molecular weight fractions (M_n < 40 kDa) during the first 5 days of release, while < 10% was released from the high molecular weight fraction (M_n > 160 kDa) during 14 days of release. No significant differences in polymer composition, glass-transition temperature, or crystallinity were identified in the different molecular weight fractions of EVAc. Mechanical properties of the polymer did depend on the molecular weight distribution, and correlated directly with Ab release rates. Because it permits rapid and reproducible fractionation of polymers, supercritical fluid extraction can be used to modify the performance of polymeric biomaterials. © 1993 John Wiley & Sons, Inc.

INTRODUCTION

The development of new technologies for the delivery of protein drugs remains one of the major challenges of modern biotechnology. Controlled-release polymers represent one attractive method for providing long-term, continuous delivery of active macromolecules to living tissue.[1] For example, controlled-release polymers have been used to release macromolecules to the systemic circulation,[2,3] to localized regions of specific tissues like the brain,[4,5] or into the mucus secretions for localized[6] or systemic action.[7]

Two polymers have emerged as the most acceptable from the standpoints of reproducibility of protein release and biocompatibility: poly(ethylene-*co*-vinyl acetate) (EVAc) and poly(lactic-*co*-glycolic acid) (PLGA). PLGA is biodegradable and has been used in suture materials for many years, making it the obvious choice for applications where the polymer carrier must be resorbed.[8-10] Other classes of biodegradable polymers, which may be appropriate for release of macromolecules, are being developed as well.[11-13] On the other hand, devices composed of nondegradable polymers may be preferable in situations where the device might need to be withdrawn after implantation, like the Norplant® (Population Council),[14] for example, where contraception can be reversed by removal of the Silastic® (Dow Corning) device. Nondegradable polymers are preferred for topical applications, like the mucosal tissues of the eye or the vagina where a device can be inserted and removed by the patient. For example, we have recently demonstrated that vaginal rings composed of EVAc can be used to deliver biologically active

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antibody (Ab) continuously to the mucus secretions of the vagina,[6] suggesting new methods for passive and active immunization of mucosal tissues. For these applications, it is essential to develop new fabrication techniques that enhance the predictability and reproducibility of protein release from the polymer, with particular emphasis on techniques that can be implemented on a commercial scale.

In this article we report on the release of Ab dispersed from a polymer matrix of EVAc that has been fractionated with supercritical fluid (SCF) propane. SCF fractionation is a relatively new technique that uses a gas at temperatures and pressures above its critical point to fractionate a polymer sample with respect to molecular weight, chemical composition, and backbone structure.[15–28] Compared with solution fractionation, SCF fractionation is a more rapid technique that provides gram-sized samples of narrow molecular weight distribution. An overview of the techniques and underlying principles involved with SCF fractionation is presented by McHugh and Krukonis.[20] Nonpolar propane (T_c = 96.7°C, P_c = 42.5 bar) was the SCF of choice for the fractionation of EVAc (34 wt % vinyl acetate) since Hasch et al.[24] demonstrated that propane readily dissolves a similar copolymer, poly(ethylene-*co*-methyl acrylate) with 25 wt % acrylate, at modest pressures of ~ 550 bar and temperatures of 70–150°C. The fractionation of EVAc was performed at ~ 140°C which is slightly above the critical temperature of propane and is well above the melting temperature of the EVAc ($T_{melt} \approx$ 52°C).

EXPERIMENTAL

Materials

EVAc (ELVAX 40W, DuPont, Wilmington, DE) was used as received (parent polymer) or fractionated using supercritical propane (see below). Gamma globulin powder (Sigma Chemical, St. Louis, MO: 80% IgG, 10% IgM, < 10% other proteins) was sieved to obtain particles between 117 and 180 μm in size.

Fractionation of EVAc

The fractionation was performed using a dynamic flow apparatus capable of operating to 200°C and 650 bar (Fig. 1).[18] Glass wool was packed into the bottom of the first extraction column and into the top of the second column (1.8 cm I.D. × 30 cm long) and ~ 12 g of polymer were loaded into each column. Propane was supplied to a diaphragm compressor (Superpressure, model J46-14025-1), compressed, and delivered to a surge tank that was normally maintained at 690 bar. The SCF was then throttled through a pressure-reducing regulator (Tescom, model 26-1000) and delivered to the columns at a flow rate in the range of 2.5 to 7.0 ± 1.4 L/min (STP) (~ 6.0 ± 4.0 g/min). The system pressure through the columns was controlled to within ±10.0 bar using the regulator, and the flow rate was controlled by manipulating the heated valve (HIP Inc., model 30-12HF4-HT) at the outlet to the columns.

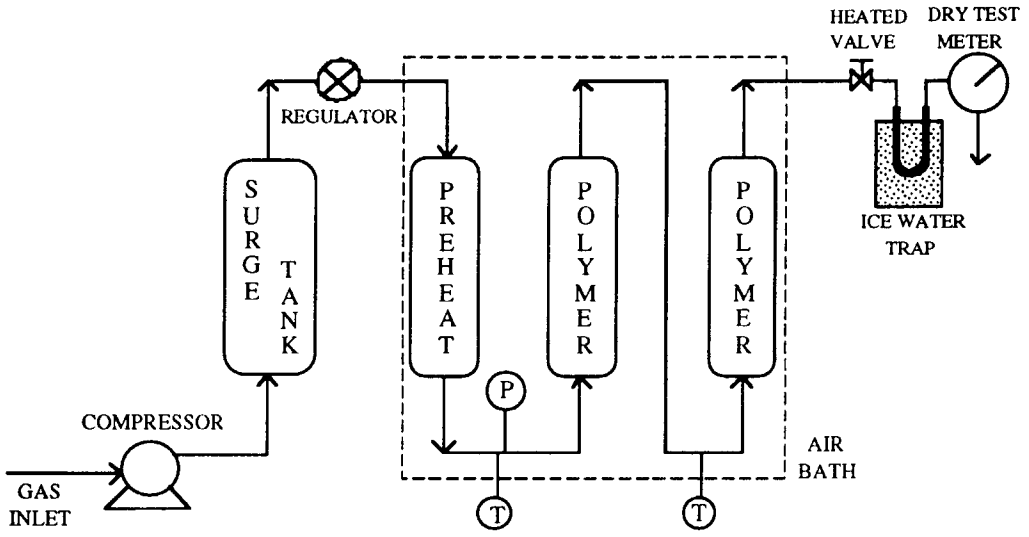

Figure 1 Schematic diagram of the high-pressure flow apparatus used for SCF fractionation.

Before entering the extraction columns, the SCF flowed through a preheater to reach thermal equilibrium with the air bath. The temperature of the gas was maintained to within ±1.0°C as measured with two platinum-resistance thermal devices located at the entrances of each extraction column.

The columns were first purged with nitrogen at room temperature to remove any air before introducing any propane to the columns. The system was heated to the desired system temperature and allowed to equilibrate for 30 minutes under a blanket of propane gas. The column pressure was then fixed at the desired level and the first sample was obtained. The loaded SCF exiting the column was expanded through a heated pressure let-down valve where polymer precipitated into a preweighed U-tube in an ice-water bath. Glass-wool filters at the exit of the U-tube trapped any fine mist entrained in the gas. The gas was routed to a dry-test meter (Singer American Meter Division, model DTM-200) to monitor the total volume passed through the extractors. After about 55 min the operating pressure was raised to the next desired pressure to obtain the next polymer fraction. The polymer samples in the U-tubes were weighed and analyzed as described below.

Incorporation of Proteins Into Polymer Matrices

Polymer matrices were formed by dispersing the particles of Ab in EVAc. Solid particles were added to a 10% (w/v) solution of EVAc in methylene chloride in sufficient quantity to obtain 40% particle mass per total mass (particles + polymer). This dispersion was vortexed to homogeneity and quickly poured into a prechilled glass mold (−80°C). After 10 min, the solid matrix was removed from the mold, maintained at −20°C for 48 h, and then at 25°C for another 48 h. Smaller discs (3 mm diameter, 1.3 mm thickness, 10 mg) were cut from the resulting slab.

Kinetics of Ab Release From Polymer Matrices

Polymer discs were continuously incubated in phosphate buffered saline (PBS with 0.02% gentamicin to inhibit bacterial growth) at 37°C with constant shaking to insure good mixing. At periodic intervals following immersion in PBS, the buffered saline solution was replaced with fresh solution (containing no protein) and the amount of protein released from the disc was determined by comparison to antibody standard solutions (0.1 to 50 μg/mL) using Coomassie Blue protein assay reagent (Pierce, Rockford, IL). Standard or sample solutions (150 μL) were mixed with protein assay reagent (150 μL) in the wells of a 96-well flat bottom plate. Bubbles were removed by degassing for 1–2 min. The absorbance in each well of the plate was determined at 595 nm using a ThermoMax microplate reader (Molecular Devices, Menlo Park, CA). The effective diffusion coefficient for Ab transport in the porous polymer matrix, D_{eff}, was determined by comparing the results from this experiment to:

$$\frac{M_t}{M_0} = \frac{4}{L}\sqrt{\frac{D_{eff}t}{\pi}} \qquad (1)$$

where M_t is the cumulative mass of Ab released at time t following immersion in PBS, M_0 is the total mass of Ab initially dispersed in the polymer, and L is the thickness of the polymer matrix. Equation (1) is an approximate solution to the equations describing desorption from a slab, and is valid for $0 < M_t/M_0 < 0.6$.[29]

Characterization of Polymer Fractions

Molecular weight and molecular weight distributions were determined by gel permeation chromatography using an HP1090 Liquid Chromatograph with three columns in series (100 Å, 1,000 Å, and 100,000 Å pore size; 5 μ, PL-gel, Hewlett Packard Corp.). Samples of approximately 0.5 wt % polymer in chloroform were injected into the columns with a mobile phase flow rate of 1 mL/min. Elution volumes were determined using a 1037A HP refractive index detector, and elution times were compared to polystyrene standards to determine molecular weights.

Glass-transition temperatures (T_g) were determined using a Seiko Instruments DSC-220C differential scanning calorimeter. The samples, ranging in size from 10 mg to 15 mg, were sealed in aluminum sample pans and scanned from −100°C to 180°C, cooled to −100°C, and reheated at a rate of 20°C/min. The T_g was taken as the inflection point in the heat flow versus temperature curve of the second heating.

A Seiko Instruments TMA-120C thermomechanical analyzer was used to determine the mechanical properties of the polymer fractions in tension. Films, nominally 0.8 mm thick, were prepared by solvent casting from a 10% methylene chloride

Table I Fractionation of EVAc With Propane at 139°C Obtained in This Study

No.	P (bar)	Weight (g)	M_w	M_n	M_w/M_n	wt % VAc	Crystalline (%)
1	258	0.50					
2	323	0.65	12,500	8,700	1.44		21
3	394	1.11	19,300	12,500	1.55		15
4	483	3.82	33,300	22,900	1.45		17
5	519	2.61	54,400	31,700	1.72		13
6	549	1.30	82,300	48,800	1.69	33	11
7	569	2.84	82,300	56,300	1.48	32	18
8	602	4.56	105,700	75,900	1.39	36	15
9	627	1.80	194,200	116,900	1.66	34	13
10	662	3.12	253,200	165,600	1.53	35	13
Parent			169,400	70,500	2.40	34	13

solution, as described above. These films were cut into strips 3 mm wide and mounted in the grips such that the sample length was 5 mm. The TMA furnace was replaced with a thermostated container so that the measurements could be conducted in PBS at 37°C. Measurements were made under length control, with a static strain of 1% and a peak-to-peak oscillatory strain of 0.2%. All measurements were made at 0.01 Hz. The storage and loss moduli (E' and E'') were computed from force versus time measurements using a correlation algorithm implemented in Fortran.

RESULTS AND DISCUSSION

Table I shows the experimental results from the fractionation of EVAc (34 wt % VAc) using propane at 139°C. Large pressure increases were taken between fractions 1, 2, and 3 since a small amount of material eluted from the columns. However, once gram-sized samples of EVAc were obtained, the pressure increment was reduced to approximately 30 bar to maintain a small molecular weight distribution for each sample. If larger pressure increments were taken between each pressure level, larger sam-

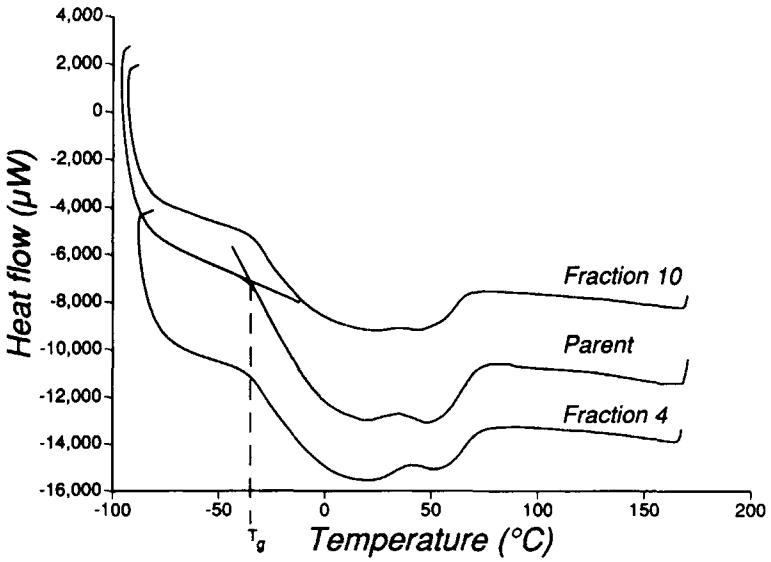

Figure 2 Typical DSC scans for several molecular weight fractions of EVAc. Our method for determining T_g (dashed line) is demonstrated by the straight lines drawn tangent to the heat flux trace before and after the inflection point.

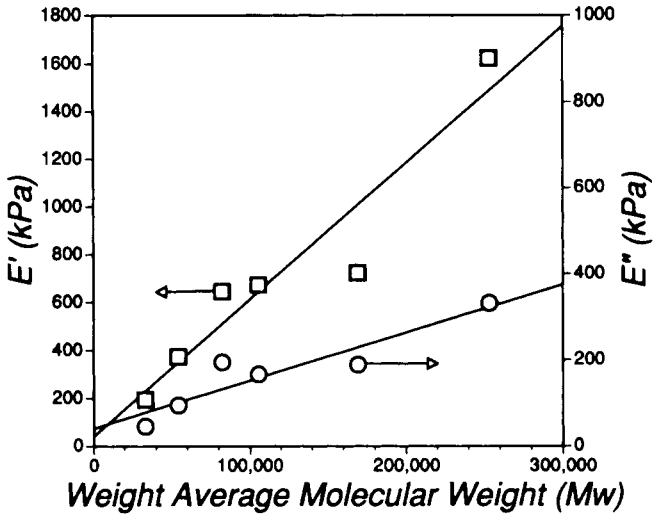

Figure 3 Correlation of storage and loss moduli (E' and E'') with weight average molecular weight. The solid lines indicate the best fit to the data, obtained by linear regression.

ples would have been recovered, but the samples would have had higher molecular weight dispersities. It is apparent that gram-sized quantities of reasonably monodisperse EVAc can be readily obtained by SCF fractionation. The vinyl acetate (VAc) content for fractions 6–10 were within ±2 wt % of the parent copolymer indicating that the copolymer was not fractionated with respect to backbone composition. Also the crystallinities of the fractions were within ±4% of the parent copolymer and the glass-transition temperatures of the fractions and the parent were virtually indistinguishable (Fig. 2), again indicating that the fractions and the parent material all contained similar amounts of VAc in the backbone. The mechanical storage and loss moduli (E' and E'') did vary significantly for the polymer fractions at 37°C (Fig. 3 and Table II).

Ab molecules were continuously released from polymer matrices fabricated from different molecular weight fractions of EVAc (Fig. 4). The rate of release depended on the molecular weight distribution: > 90% of the Ab was released from fraction 4 or 5 during the first several days of incubation in buffered saline, while only < 10% was released from fraction 10 matrices during that same time [Fig. 4(a)]. The extent of release was approximately linear with respect to the square root of time, suggesting that diffusion of Ab through the polymer matrix controlled the release of Ab [Fig. 4(b)]. To facilitate comparison between different matrix compositions, an effective diffusion coefficient for Ab transport in the porous polymer, D_{eff}, was calculated for each of the porous polymer matrices, according to eq. (1) (Table II). The rate of Ab release from these EVAc matrices decreased with increasing average molecular weight in the matrix. Interestingly, the rate of Ab release correlated with the mechanical properties of the polymer (Fig. 5).

Most of the previous experimental studies of protein release from EVAc matrices[30–34] are consistent with the following model: protein molecules diffuse through a network of water-filled pores that are formed within the polymer matrix as the initially incorporated solid protein particles dissolve. This simple model correctly predicts that the rate of protein release depends on particle loading (or total volume of the pore space), particle size, and molecular weight of the protein. In fact, for polymer matrices of a given molecular weight distribution (i.e., the parent polymer used here), the observed protein release rates can be predicted reasonably well by considering the microstructure of porous EVAc/protein matrices[33,35] and protein diffusion rates in highly constricted pores.[36] Since the polymer matrix is considered an inert scaffold of water-filled pores through which protein molecules can diffuse, this

Table II Rate of Ab Release from and Mechanical Properties of SCF-Fractionated EVAc Samples

EVAc Sample Preparation	Number Average (M_n)	Weight Average (M_w)	D_{eff} ($\times 10^{-9}$ cm^2/s)	E' (kPa)	E'' (kPa)
Fraction 10	165,600	253,200	0.01	1623	332
Fraction 8	75,900	105,700	0.9	674	167
Parent	70,500	169,400	0.9	724	189
Fraction 7	56,300	82,300	6	646	195
Fraction 5	31,700	54,400	10	374	95
Fraction 4	22,900	33,300	20	194	46

Samples are arranged in order of increasing Ab release rate.

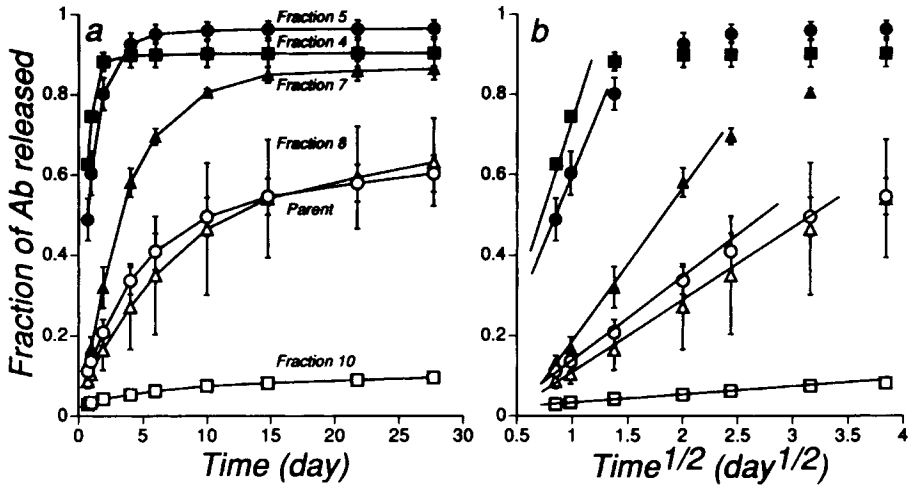

Figure 4 Ab release from matrices of EVAc into well-stirred PBS at 37°C. The fraction of protein released from the matrix (mean ± standard deviation) is plotted versus (a) time and (b) the square root of time. The straight lines in (b) indicate the best fit, determined by linear regression, to the experimentally determined points where fraction released < 0.6.

model cannot account for any differences caused by the molecular weight distribution within the polymer matrix.

The influence of polymer molecular weight on the release of incorporated proteins from EVAc was first demonstrated using polymer fractions obtained by solution fractionation.[37] These authors suggested that the continuous polymer phase actively resists the osmotic forces generated during protein dissolution and release. Mechanical properties of the polymer, therefore, are expected to influence the dynamic structure of the pore network, which has a direct impact on subsequent protein-release rates. Measurements of matrix swelling as a function of polymer molecular weight were used to support this hypothesis. A subsequent study examined the effects

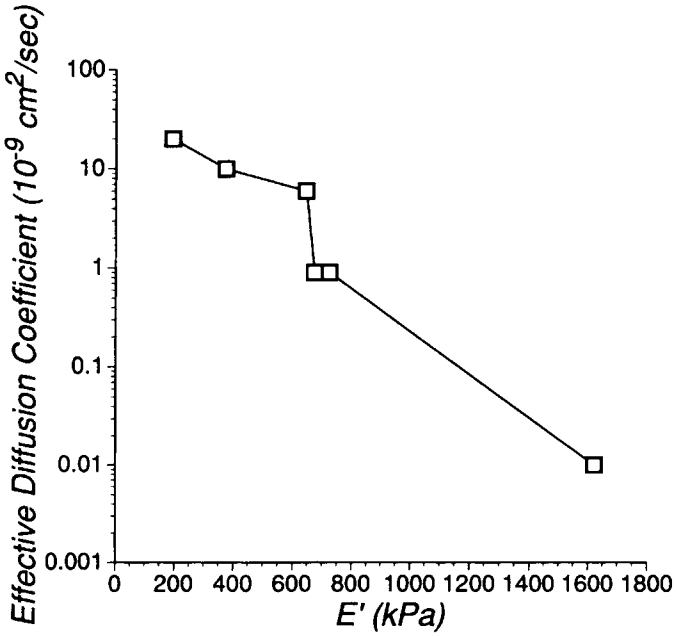

Figure 5 Correlation of storage and loss modulus (E' and E'') for the polymer fractions with the rate of diffusion within the polymer matrix (D_{eff}).

of temperature on polymer properties and protein release.[38] Thermal analysis revealed that melting of the crystallites in an EVAc matrix led to a significant increase in the swelling (water uptake) of the matrix, and supported the idea of osmotically-driven elastic deformation of the pore network during release. We have further supported that idea by demonstrating, over a wide range of protein release rates, a clear correlation between polymer molecular weight and the rate of protein release.

It is interesting to note that a relatively small change in the loss (E'') or storage (E') modulus leads to a substantial change in the D_{eff}. A tenfold change in either modulus produces a 1000-fold change in D_{eff} (see Table II). The pore network within the EVAc matrices has a characteristic geometry that consists of large pores ($\sim$ 100 μm radius) interconnected by highly constricted channels ($\sim$ 1–10 μm radius).[35] Previous investigators have demonstrated that the ratio of channel size to pore size can greatly influence the rate of pore-to-pore transport.[36,39,40] One possible explanation for our results is that the mechanical properties of the polymer determine the size of the channels. For high molecular weight polymers, where the modulus is low and the matrix relatively nondeformable, the channels will be small. For lower molecular weight polymers, with a higher modulus and greater deformability, the channels will expand in response to the high osmotic pressure within the pore space, which contains a high concentration of protein.

We have demonstrated that SCF fractionation is an important tool for modifying the properties of polymers for controlled release. The method of polymer fractionation used here is versatile, yielding large amounts of polymer fractions with narrow molecular weight distributions. Many of the principles that govern the fractionation of polymers with liquid solvents also are operative with SCF solvents. With SCF solvents, however, the solvent power can be finely tuned using pressure, giving an additional degree of freedom. The sharp decrease in polymer solubility with a decrease in pressure makes SCF solvents amenable for process recycle and the rapid disengagement of the gaseous SCF solvent at low pressure promotes facile recovery of a solvent-free polymer. SCF technology may become useful in other aspects of biomaterial production as well, such as the formation of uniform polymer microparticles[41] or the modification of polymer surfaces.

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