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Release of proteins via ion exchange from albumin-heparin microspheres

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Albumin-heparin and albumin microspheres were prepared as ion exchange gels for the controlled release of positively charged polypeptides and proteins. The adsorption isotherms of chicken egg and human lysozyme, as model proteins, on microspheres were obtained. An adsorption isotherm of chicken egg lysozyme on albumin-heparin microspheres was linear until saturation was abruptly reached.

The adsorption isotherms of human lysozyme at low and high ionic strength were typical of adsorption isotherms of proteins on ion exchange gels. The adsorption of human lysozyme on albumin-heparin and albumin microspheres fit the Freundlich equation suggesting heterogeneous binding sites. This was consistent with the proposed multivalent, electrostatic interactions between human lysozyme and negatively charged microspheres. Scatchard plots of the adsorption processes of human lysozyme on albumin-heparin and albumin microspheres suggested negative cooperativity, while positive cooperativity was observed for chicken egg lysozyme adsorption on albumin-heparin microspheres.

Human lysozyme loading of albumin-heparin microspheres was 3 times higher than with albumin microspheres, with long term release occurring via an ion exchange mechanism. Apparent diffusion coefficients of 2.1×10^{-12} and 3.9×10^{-11} cm^2/sec were obtained for the release of human lysozyme from albumin-heparin and albumin microspheres, respectively. The release was found to be independent of diffusion, since the rate determining step was likely an adsorption/desorption processes. An apparent diffusion coefficient of 4.1×10^{-12} cm^2/sec was determined for the release of chicken egg lysozyme from albumin-heparin microspheres.

Low release of the lysozymes from albumin-heparin microspheres was observed in deionized water, consistent with the proposed ion exchange release mechanism. Overall, albumin-heparin microspheres demonstrated enhanced ion exchange characteristics over albumin microspheres.

Key words: Microsphere; Protein release; Ion exchange

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Introduction

Recently, polymeric microspheres have been investigated as drug delivery systems (DDS) for

polypeptides and proteins [1]. Polymeric microspheres exhibit properties such as injectability, site specific drug delivery and controlled drug release which make them amenable for drug delivery [2,3]. The release of solutes from nanoparticles and microspheres occurring by Fickian diffusion is usually not a suitable release mechanism for long term delivery, due to the high surface area and short diffusional path length of these DDS. Other release mechanisms (e.g., polymer degradation, self diffusion through pores) for polymeric microspheres have been able to achieve long term release of proteins [1].

A viable release mechanism for the loading and controlled release of proteins and polypeptides from polymeric microspheres is via ion exchange. The ion exchange mechanism has recently been investigated for the delivery of low molecular weight ionic drugs from biodegradable, polymeric microspheres, and higher loading and slower release rates, relative to pure diffusion have been achieved [4–6]. However, the low diffusivity of proteins in ion exchange gels used for liquid chromatography has been observed [7].

In this study, the viability of ion exchange as a release mechanism for macromolecular delivery from microspheres was studied. Both chicken egg and human lysozymes were used as model, positively charged proteins to examine the effects of their dissimilar adsorption behavior [8] on the release from the microspheres.

High loading and long term release of positively charged lysozymes were obtained from albumin-heparin microspheres. The ion exchange properties of albumin microspheres, which have previously been noted [9], were also evident in the release of lysozyme. In general, albumin-heparin microspheres exhibited enhanced ion exchange properties, relative to albumin microspheres.

Materials

Human serum albumin (lyophilized and crystallized), olive oil, chicken egg lysozyme and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma, St. Louis, MO. Heparin (167 IU/mg, lot number LPP060143), from porcine intestinal mucosa, was obtained from Diosynth, Oss, The Netherlands. Purified glutaraldehyde (25% w/v) was purchased from Fisher, Pittsburgh, PA. Sodium cyanoborohydride was obtained from Aldrich, Milwaukee, WI. Blue Sepharose CL-6B and diethylaminoethyl (DEAE) Sepharose CL-6B were obtained from Pharmacia, Piscataway, NJ. Human lysozyme was obtained from United States Biochemical, Cleveland, Ohio. Cellulose acetate (molecular weight cutoff 1000 and 3500) dialysis membranes and polytetrafluoroethylene membranes (PTFE) (3.0 μm pore size) were obtained from Gelman, Ann Arbor, MI. Cellulose acetate membranes (8.0 μm pore size) were obtained from Millipore, Bedford, MA. Latex particles (100 μm) were purchased from Coulter Counter, Hialeah, FL. All other chemicals were reagent grade.

Methods

Preparation of albumin-heparin and albumin microspheres

Albumin-heparin and albumin microspheres (3% w/v crosslinking) were prepared using a protocol described previously [10].

Size analysis

The diameter of albumin-heparin and albumin microspheres (n=300) was determined by phase contrast light microscopy (Biophot, Nikon, Tokyo, Japan) using graticules calibrated against latex particles of known diameters. The diameters of albumin-heparin and albumin microspheres were measured in the swollen state at 25°C in isotonic phosphate buffered saline (PBS) (2.0 mM potassium phosphate, 8.0 mM disodium phosphate, 0.145 M NaCl), pH 7.0 with 0.01% NaN_3.

Swelling

The equilibrium swelling of albumin-heparin and albumin microspheres was determined in is-

otonic PBS (0.01% NaN_3, pH 7.0) as previously described [10]. The swelling ratio

$$q=\frac{V_{\text{swollen}}}{V_{\text{dried}}} \quad (1)$$

was determined by measuring the diameter of albumin-heparin and albumin microspheres and assuming a spherical geometry.

Adsorption isotherms

Adsorption isotherms were obtained for both chicken egg and human lysozyme on albumin-heparin and albumin microspheres. Chicken egg and human lysozyme (37.5 mg) were separately dissolved in 15.0 ml of 67 mM PBS (28 mM potassium phosphate, 39 mM disodium phosphate), pH 7.0 at 25°C and diluted to make 5 ml solutions of concentrations ranging from 0.078 mg/ml to 5.00 mg/ml. The albumin-heparin and albumin microspheres were separately swollen and washed with 3 portions of 25 ml 67 mM PBS, pH 7.0 on a cellulose acetate filter membrane (pore size 8.0 μm). Vacuum was applied to remove buffer solution from the interstitial spaces of the microspheres. The microspheres were then weighed out in 100 mg portions and added to the lysozyme solutions. The experiments were carried out for 25 h at 25°C under constant agitation using a shaking apparatus (Burrell, Pittsburgh, PA). The amount of adsorbed lysozyme was determined by solution depletion by measuring the absorbance of the loading solutions at 280 nm with a UV/VIS spectrophotometer (Lambda 7, Perkin Elmer, Norwalk, CT). The concentration of the lysozyme adsorbing to the microspheres was calculated by [11]

$$m_p=(W_s/W_g)(m_i-m_f) \quad (2)$$

where m_p is the molarity of the protein in the microspheres, m_i and m_f are the molarities of the lysozyme in the loading solution at the initial and equilibrium states respectively, W_s is the weight of the lysozyme solution and W_g is the weight of the swollen microspheres. The adsorption of human lysozyme on albumin-heparin microspheres was also carried out in 67 mM PBS, 0.50 M NaCl, pH 7.0 using identical experimental conditions.

In vitro release studies

The lysozyme loaded microspheres (50.0 mg) were placed in 10.0 ml isotonic PBS with 0.01% NaN_3, pH 7.0 at 37°C. The vials were then placed in a shaking water bath (BT-47, American Scientific Products, McGaw Park, IL) and agitated at 100 strokes/min. The release medium was assayed at appropriate times by withdrawing 1.00 ml samples and replacing the volume with 1.00 ml isotonic PBS with 0.01% NaN_3, pH 7.0 to maintain a constant volume of 10.0 ml. The amount of chicken egg and human lysozyme released was quantitated by measuring the absorbance at 280 nm by UV spectroscopy.

To investigate the effect of release media ionic strength, the release of human lysozyme from albumin-heparin microspheres was examined using 67 mM PBS, 0.50 M NaCl, pH 7.0 as the release medium. In addition, the release of human and chicken egg lysozyme from albumin-heparin microspheres was also carried out in deionized water (0-ionic strength).

The total amount of adsorbed lysozyme was determined by placing the microspheres in 67 mM PBS, 0.50 M NaCl, pH 7.0 for 100 h and assaying the released amount, as previously described.

Results

Albumin-heparin and albumin microspheres prepared were spherical, with an average diameter of 100 ± 50 μm ($n=300$) in the hydrated state. The swelling ratios, q, of the albumin-heparin and albumin microspheres were 1.8 and 1.6, respectively. Swelling of the microspheres was rapid (i.e., <15 min) and was assumed not to influence the kinetics of lysozyme release after 15 min. The heparin content of the albumin-

heparin microspheres was determined to be 10% w/w [10]. The microspheres consisting of albumin-heparin conjugate were stable due to the crosslinking density used for these studies.

The main driving force for the adsorption of lysozymes onto the microspheres was through electrostatic interactions. The adsorption isotherms of human lysozyme on albumin-heparin

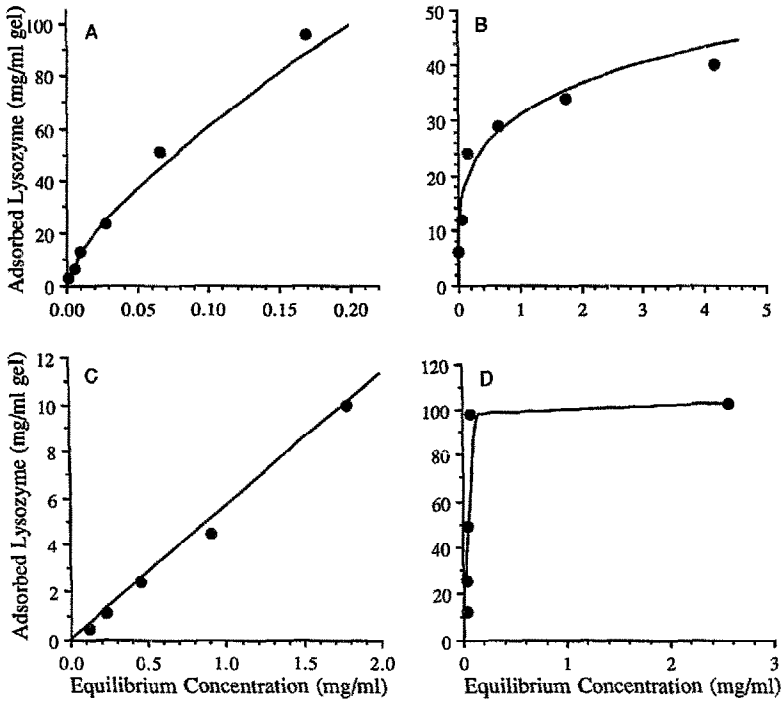

Fig. 1. Adsorption isotherms of (A) human lysozyme to albumin-heparin microspheres, (B) human lysozyme to albumin microspheres, (C) human lysozyme to albumin-heparin microspheres at high ionic strength (0.50 M NaCl), and (D) chicken egg lysozyme to albumin-heparin microspheres at 25°C in 67 mM PBS (pH 7.0). The solid lines in A and B were calculated from the Freundlich equation and parameters in Table 1.

and albumin microspheres are shown in Figures 1A and 1B, respectively, with albumin-heparin microspheres adsorbing the larger amount of lysozyme. The adsorption of human lysozyme onto albumin-heparin microspheres did not reach a distinct plateau at the initial concentrations used

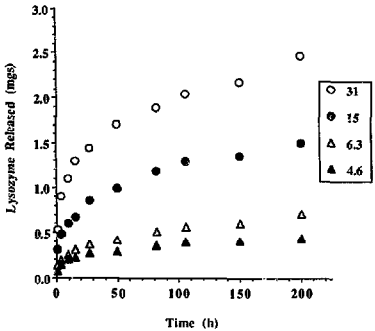

Fig. 2. Release of human lysozyme from albumin-heparin microspheres of varying loading content in isotonic PBS, pH 7.0 at 37°C.

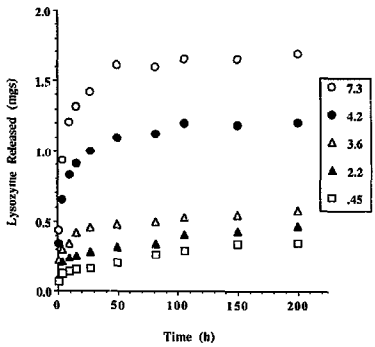

Fig. 3. Release of human lysozyme from albumin microspheres of varying loading content in isotonic PBS, pH 7.0 at 37°C.

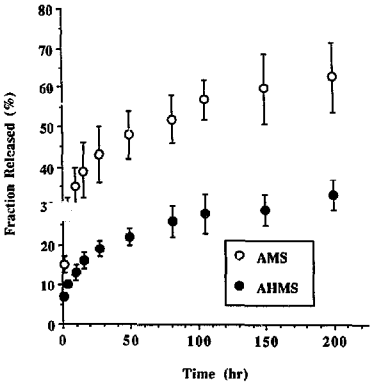

Fig. 4. Fractional release of human lysozyme from albumin-heparin and albumin microspheres in isotonic PBS, pH 7.0 at 37°C; mean ± SD.

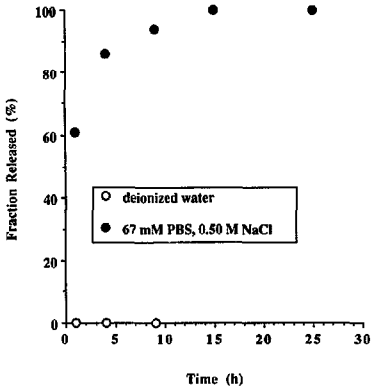

Fig. 5. Release of human lysozyme from albumin-heparin microspheres at 37°C in 67 mM PBS, 0.50 M NaCl, pH 7.0, and deionized water.

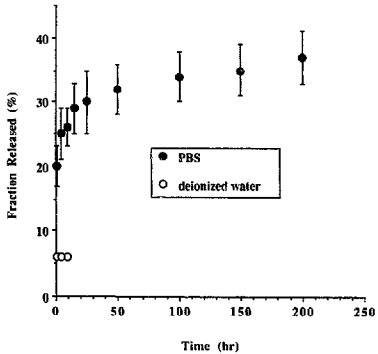

Fig. 6. Release of chicken egg lysozyme from albumin-heparin microspheres at 37°C in isotonic PBS, pH 7.0 and deionized water.

for loading the microspheres. The adsorption of human lysozyme on albumin microspheres appeared to increase rapidly at low equilibrium concentrations, then plateau at higher equilibrium concentrations to approximately 40 mg/ml gel.

The effect of higher ionic strength on human lysozyme adsorption on albumin-heparin microspheres is shown in Figure 1C. At higher ionic strength, a linear isotherm was observed. No plateau was reached at the initial concentrations of human lysozyme used for this experiment. The amount of adsorbed human lysozyme at 67 mM PBS, 0.50 M NaCl, pH 7.0, was much less than in 67 mM PBS, pH 7.0.

The adsorption isotherm of chicken egg lysozyme on albumin-heparin microspheres is shown in Figure 1D. The adsorption of chicken egg lysozyme onto albumin-heparin microspheres also exhibited a linear isotherm, but abruptly reached a plateau at 100 mg/ml gel at an equilibrium concentration less than 0.2 mg/ml.

The release kinetics of human lysozyme from albumin-heparin and albumin microspheres, and the effect of loading on release are illustrated in Figures 2 and 3. The loading of human lysozyme into albumin-heparin and albumin microspheres ranged from 4.6 to 31% and from 0.45 to 7.3% (w/w), respectively. With increased loading of human lysozyme, the absolute amount released versus time was greater for both albumin-heparin and albumin microspheres.

In Figure 4 the release of human lysozyme at varying loading contents from albumin-heparin and albumin microspheres was plotted as fraction released versus time. At the loading levels used, there was no significant effect of loading on the fraction released versus time for either albumin-heparin or albumin microspheres. The release of human lysozyme from albumin microspheres was initially rapid (up to ~30% release) and then slowed considerably. For albumin-heparin microspheres, release of human lysozyme exhibited a slower release rate relative to albumin microspheres. In both situations, long term release of human lysozyme was attained under isotonic conditions.

Figure 5 illustrates the release of human lysozyme from albumin-heparin microspheres in 67 mM PBS, 0.50 M NaCl, pH 7.0 and in deionized water. The release of human lysozyme was very rapid at high ionic strength. Practically no release of human lysozyme from albumin-heparin microspheres was observed when the release medium was deionized water.

Chicken egg lysozyme (10% w/w loading) release from albumin-heparin microspheres was similar to the release of human lysozyme, except for an initial burst effect not observed for human lysozyme release from albumin microspheres (Figure 6). Very little release of chicken egg lysozyme was observed from albumin-heparin microspheres in deionized water.

Discussion

Drugs incorporated into polymeric microspheres are usually loaded concurrently during microsphere preparation. This mechanism of drug loading is possible only for drugs which are stable during polymeric microsphere preparation. The drug should not contain functional groups which may react during the crosslinking

procedure or be chemically modified, and the drug should not be heat sensitive. This is especially relevant with peptides and proteins.

Recently, approaches have been developed to load drugs into microspheres after their preparation [4–6]. Drug loading after microsphere preparation is advantageous in that impurities associated with the microsphere may be removed prior to loading, and loading may be done under aqueous conditions.

In previous studies [12], the amount of drug loaded into albumin microspheres was shown to be limited by the aqueous solubility of the drug. To achieve high payloads of hydrophobic drugs during albumin microsphere preparation, the drug was added as a suspension prior to cross-linking [13,14].

In this report, a negatively charged polyelectrolyte (heparin) was incorporated into the albumin microspheres. This increased the electrostatic interactions between the negatively charged microsphere and the positively charged drugs, thereby increasing the loading capacity. The ionic capacity of albumin microspheres is 1.51 meq/g [15], and for albumin-heparin microspheres, assuming a low number of bonds between albumin and heparin, is 1.90 meq/g.

The loading and release of positively charged proteins from albumin-heparin microspheres is illustrated in Figure 7. Positively charged proteins, having an electrochemical valence, Z_P, may bind through electrostatic interactions with the albumin-heparin microsphere's fixed negative charges displacing counter ions. The electrochemical valence is dependent on the isoelectric point of the protein and pH. For albumin microspheres, interactions may occur through the carboxyl groups (e.g., glu, asp) of albumin. Both albumin-heparin and albumin microspheres are cation exchange gels. Because of the multiple charges on both the protein and the microsphere multivalent adsorption is possible.

In these studies, both chicken egg and human lysozymes were used as a model proteins. Both lysozymes have been well characterized [16]. They each have a molecular weight of 14,000 and an isoelectric point of 10.0–11.0, therefore being positively charged at pH 7.0. The amino acid compositions of the lysozymes are different at 40% of the positions. There are approximately one third more negatively charged groups as there are positively charged groups on the surfaces of both lysozymes [8]. In previous studies, chicken egg and human lysozyme exhibited dissimilar adsorption behavior on polymer surfaces [8]. Human lysozyme exhibited a typical protein adsorption isotherm on model negatively charged surfaces. In contrast, chicken egg lysozyme adsorption exhibited a linear isotherm; this was also the case for adsorption of chicken egg lysozyme on albumin-heparin microspheres.

The adsorption of human lysozyme on albumin-heparin and albumin microspheres corresponded to the Freundlich type of isotherm [17]

$$a = \alpha c^{1/n} \qquad (3)$$

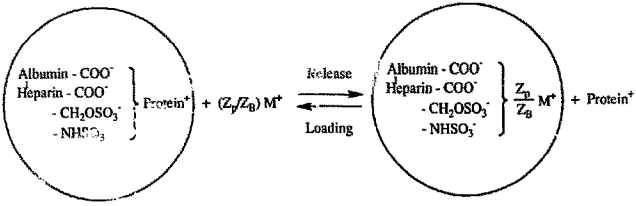

Fig. 7. Schematic illustration of protein loading and release from albumin-heparin microspheres via ion exchange mechanism. Z_P and Z_B are the electrochemical valences of the protein and counter ion, respectively.

where a is the amount of adsorbed human lysozyme in mg/ml gel, α is the adsorption constant in mg/ml gel, $1/n$ is the adsorption exponent and c is the equilibrium concentration in mg/ml. The adsorption parameters and coefficients of determination are shown in Table 1. The adsorption constant is a measure of the adsorption capacity, which for albumin-heparin microspheres is approximately 100 times the value of albumin microspheres. The adsorption exponent indicates the intensity of the adsorption process and was greater for albumin-heparin microspheres relative to albumin microspheres.

The adsorption data fit the Freundlich equation which suggested the presence of heterogeneous binding sites [17] due to multivalent adsorption. Electrostatic interactions between human lysozyme and the microspheres may be univalent, bivalent, etc., and thus exhibiting varying adsorption energies.

Protein adsorption isotherms may be analyzed in terms of multiple equilibria models, as discussed by Andrade [18]. Negatively charged groups associated with the microspheres may be considered as ligands at a fixed concentration. The adsorption may be studied at varying protein concentrations, using Scatchard plots for analysis.

The Scatchard plots of human lysozyme on albumin-heparin and albumin microspheres are illustrated in Figures 8A and 8B. The upward concavity suggested negative cooperativity of the adsorption process for both microspheres. This was consistent with multivalent adsorption, in that nascent human lysozyme adsorbed to fixed negative charges of the microspheres decreased the likelihood of subsequent lysozyme adsorption. Jennissen has termed such phenomena as sequential adsorption [19]. For these adsorption processes, the maximum binding sites are available for nascent proteins; subsequent proteins would find fewer sites for multivalent adsorption. Clearly this would account for the non-independence of the adsorption phenomenon.

TABLE 1

Freundlich parameters for human lysozyme adsorption to albumin-heparin and albumin microspheres

Microsphere	α	$1/n$	r^2
Albumin-heparin	316	0.72	0.99
Albumin	30.9	0.24	0.96

At high ionic strength the Scatchard plot of human lysozyme on albumin-heparin microspheres was linear with a slope equal to zero (Figure 8C). For Scatchard plots, the slope is typically the binding constant and at high ionic strength the adsorption energy of human lysozyme with albumin-heparin microspheres was negligible. Thus, at 67 mM PBS, 0.50 M NaCl, pH 7.0 albumin-heparin microspheres behaved as an uncharged gel.

Figure 8D shows the Scatchard plot of chicken egg on albumin-heparin microspheres. The downward concavity indicated positive cooperativity for the adsorption process; adsorption of nascent chicken egg lysozyme facilitated the subsequent adsorption of chicken egg lysozyme. To account for the linear adsorption isotherm of chicken egg lysozyme on model negatively charged surfaces, protein-protein interaction in the form of lysozyme multilayers has been postulated [8].

The long term release kinetics of lysozyme from albumin-heparin and albumin microspheres was likely due to an ion exchange mechanism. For the ion exchange of two counter ions A and B in a gel the following expression of Fick's First Law has been derived [20]

$$J_B = -\bar{D}_{AB}\frac{\partial C_B}{\partial x} \tag{4}$$

where

$$\bar{D}_{AB} = \left[\frac{\bar{D}_A \bar{D}_B (z_A^2 \bar{C}_A + z_B^2 \bar{C}_B)}{z_A^2 \bar{C}_A \bar{D}_A + z_B^2 \bar{C}_B \bar{D}_B}\right] \tag{5}$$

The counter ion A is considered to be in the solution and the counter ion B bound to the ion exchanger. The flux of B is dependent on the individual diffusion coefficients of the counter ions A and B, the valences of the counter ions, the concentrations of the counter ions in the ion exchanger and the concentration gradient of B. The over bars indicate that the parameters are of the

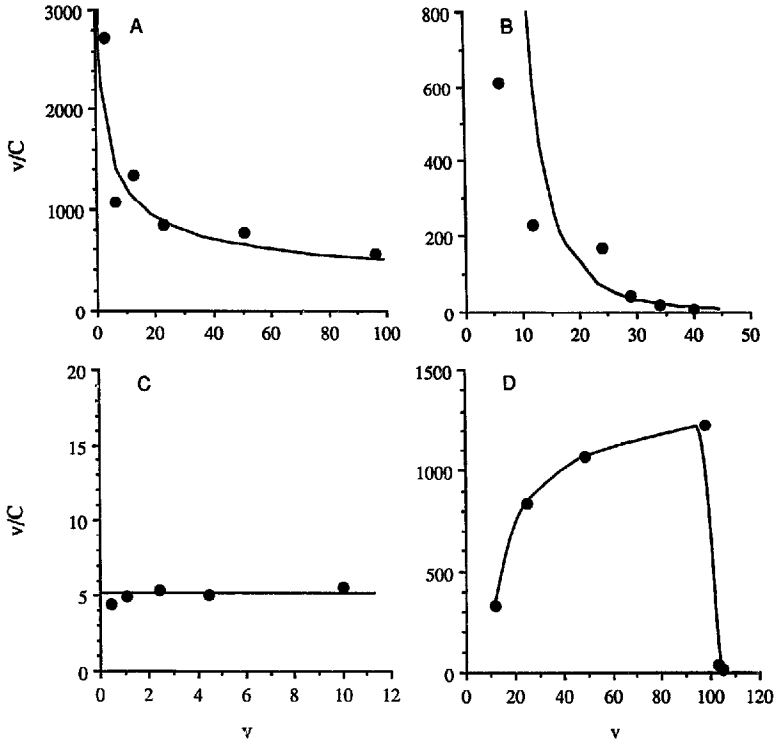

Fig. 8. Scatchard plots of the adsorption isotherms of (A) human lysozyme to albumin-heparin microspheres, (B) human lysozyme to albumin microspheres, (C) human lysozyme to albumin-heparin microspheres at high ionic strength (0.50 M NaCl), and (D) chicken egg lysozyme to albumin-heparin microspheres at 25°C in 67 mM PBS, pH 7.0. The solid lines in A and B were calculated from the Freundlich equation and parameters in Table 1. ν denotes the amount of adsorbed lysozyme in mg/ml gel, and C is the equilibrium concentration in mg/ml.

ion exchanger. The counter ion B may be considered to be the adsorbed lysozyme. The equation suggests that the ion in the smaller concentration has a larger effect on the rate of interdiffusion.

Even though the diffusion process is not a rate-limiting step, the flux of ion B was expressed in Fick's First Law with the interdiffusion coefficient [20]. Thus, Fick's Second Law could be applied for sphere geometry. A short time solution (i.e., <40% release) may be obtained [21]

to determine values for the apparent diffusion coefficients of lysozyme release from albumin-heparin and albumin microspheres.

$$\frac{M_t}{M_\infty}=6\left[\frac{Dt}{\pi r^2}\right]^{1/2}+\frac{3Dt}{r^2} \qquad (6)$$

The apparent diffusion coefficients of human lysozyme in albumin-heparin and albumin microspheres and other release parameters are given in Table 2. The apparent diffusion coefficients are much less than the self diffusion coefficient of human lysozyme ($D_0=1.12\times10^{-6}$ cm^2/s) [22]. The apparent diffusion coefficient of human lysozyme in albumin-heparin microspheres was about 20 times less than in albumin microspheres, attributed to the difference in the number and availability of negatively charged groups associated with albumin-heparin compared to albumin microspheres. In addition, diffusion was not the main release mechanism, since there was no concentration effect of drug loading on fractional release versus time for human lysozyme from both albumin-heparin and albumin microspheres.

The heparin associated with the albumin-heparin microspheres was not crosslinked during synthesis of the microsphere network, but was grafted onto the network. This 'pendant-free' nature of heparin may exhibit greater accessibility for lysozyme adsorption. This suggests that the rate determining step of release was the adsorption/desorption of the human lysozyme on the microspheres. With the albumin-heparin microspheres, stronger multivalent adsorption occurred due to the higher charge density. The increased binding sites for multivalent adsorption with proteins resulted in higher adsorption affinity [23].

An apparent diffusion coefficient of 4.1×10^{-12} cm^2/s was determined for chicken egg lysozyme release from albumin-heparin microspheres (Table 2). This indicated that the initial rapid release from albumin-heparin microspheres was perhaps due to the multilayers of chicken egg lysozyme being released from the surface. The low amount of chicken egg lysozyme release in deionized water from albumin-heparin microspheres supports ion exchange as the primary release mechanism.

TABLE 2

Release parameters for human and chicken egg lysozyme from albumin-heparin and albumin microspheres

Microsphere	Apparent diffusion coefficient (cm^2/s)	Diameter (μm)	Swelling ratio
Albumin-heparin	2.1×10^{-12}	100 ± 46^b	1.8
Albumin-heparin	4.1×10^{-12a}	100 ± 46^b	1.8
Albumin	3.9×10^{-11}	100 ± 50^b	1.6

[a]Chicken egg lysozyme.
[b]Mean ± SD ($n=300$).

Conclusions

The loading of lysozymes into albumin-heparin and albumin microspheres was accomplished after fabrication of the microspheres. The adsorption isotherm of human lysozyme on the microspheres fit the Freundlich equation suggesting, heterogeneous binding sites. Scatchard plots of the adsorption processes of human lysozyme on albumin-heparin and albumin microspheres suggested negative cooperativity, consistent with multivalent, electrostatic interactions.

The Scatchard plot for the adsorption of chicken egg lysozyme on albumin-heparin microspheres suggested positive cooperativity. This was possibly due to the formation of chicken egg lysozyme multilayers on negatively charged surfaces.

The long term release of human and chicken lysozyme was obtained from albumin-heparin and albumin microspheres. These studies indicated that the release kinetics were not dependent on diffusion, but the rate determining step was likely adsorption/desorption processes. The low release rate of lysozyme from albumin-heparin microspheres via ion exchange suggested that this was a viable mechanism for the controlled release of polypeptides and proteins, and important in the design of DDS for proteins.

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