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Albumin-Heparin Microspheres as a Drug Delivery Carrier

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Albumin microspheres (AMS) are usually prepared using an emulsion process. An aqueous phase containing albumin and a drug is emulsified in an organic phase, such as olive oil or cottonseed oil. Stable microspheres are obtained by chemical crosslinking or thermal denaturation.

The AMS surface obtained in this process are hydrophobic. Albumin molecules, aligning at the oil-water interface, undergo conformational changes such that hydrophobic regions of the molecules are preferentially exposed to the oil phase.[1)] The hydrophobic surface requires the use of a surfactant to prepare stable resuspension. Hydrophobicity may also influence the *in vivo* biodistribution. Tabata and Ikada,[2)] for instance, showed that phagocytosis of modified cellulose microspheres increased with enhanced hydrophobicity. To obtain hydrophilic AMS, Longo *et al.*[3)] used a solution of poly(methyl methacrylate) in chloroform/toluene as an organic phase. Glutaraldehyde (a chemical crosslinker) was extracted in toluene or chloroform and added after emulsification of the aqueous albumin solution. In addition to hydrophilicity of the surface, a high concentration of unreacted aldehyde groups was present at the surface. These groups could be used for further surface modification, such as coupling antibodies or bioactive agents to the surface.

Drugs incorporated into polymeric microspheres used to be loaded concurrently with microsphere preparation. This mechanism of drug loading is possible only for drugs which are stable during microsphere preparation. If the drug contains functional groups, the drug may be covalently modified during the crosslinking procedure,[4)] and the drug may be altered if it exhibits heat instability.[1,5)] This is especially relevant with peptides and protein drugs.

Another disadvantage of AMS are low payload which is generally obtained. The payload was improved by incorporating negatively charged polymers, such as poly (β-aspartic acid)[6)] and poly (α-L-glutamic acid),[3)] in the polymer matrix, thus resulting in ion-exchange microspheres. Recently, it has been shown that ion-exchange microspheres can be loaded with positively charged various drugs, yielding relatively high payloads.[7)] An additional advantage of drug loading after the microsphere preparation is that unreacted aldehyde groups can be quenched with glycine prior to the drug loading, thus preventing covalent coupling of drugs containing amino groups.

The objective of the study is the development of biodegradable ion-exchange microsphers based on the serum albumin and heparin for the loading

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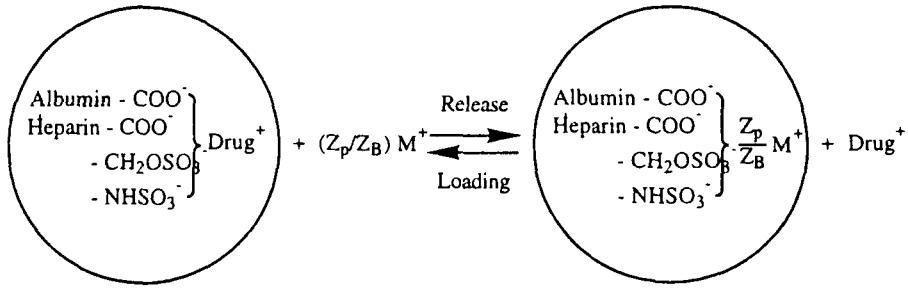

Figure 1—Shematic illustration of drug loading and release from albumin-heparin microspheres via ion-exchange mechanism. Z_P and Z_B are the electrochemical valances of the drug and counter ion, respectively.

and controlled delivery of a cytotoxic agent, adriamycin and macromolecules. Heparin was incorporated into albumin microspheres to increase its biocompatibility, hydrophilicity, and enhanced ion-exchange property for drug loading and release.

The loading and release of positively charged drugs from albumin-heparin microspheres is illustrated in Figure 1.

Materials and Methods

Synthesis of Albumin-heparin Conjugate

The protocol used to synthesize soluble albumin-heparin conjugates was based on the method of Hennick *et al.*[8] The procedure was slightly modified to inhibit denaturation of the serum albumin during the conjugation process. A buffer was used to keep the pH constant during the course of the reaction without the addition of HCl, and all steps were carried out at 4℃.

Two different conjugates were prepared from human serum albumin and heaprin; an unfrac tionated conjugate with a heaprin content of 10. $7 \pm 0.9\%$ w/w and a high affinity conjugate (heparin content of $10.2 \pm 1.2\%$) prepared by fractionation of an unfractionated conjugate by ion-exchange chromatography using a DEAE-sepharose column. Furthermore albumin-heparin conjugates, prepared from porcine albumin with heparin contents of 5.9%, 16.0%, and 21% as indicated by the manufacturer, were obtained from Holland Biomaterials Group (HBG), Enschede, The Netherlands.

Preparation of Albumin-heparin and Albumin Microspheres (AHMS)

Microspheres were synthesized using a protocol similar to Burger *et al.*[9] A 125 mL volume of olive oil was placed in a baffled cell and stirred at a predetermined rate for 30 min. The 100 mg of albumin-heparin conjugate or serum albumin, which had been dissolved in 400 μL of distilled water at 4℃, was added dropwise into stirred olive oil at 25℃, and stirring was continued for 15 min. A predetermined amount of purified glutaraldehyde (25%, w/v) to make 1 to 4% (w/v) of the albumin-heparin solution was then added, and if required, twice the equimolar amount of sodium cyanoborohydride based on the added glutaraldehyde was added as a reducing agent. The crosslinking reaction was allowed to proceed for 3.5 h. A 100-mL aliquot of glycine (10%, w/v) was then added to quench unreacted aldehyde groups and allowed to proceed for 30 min. Subsequently 60 mL of acetone was added to dilute the oil phase and the emulsion were isolated by centrifugation at 1000 rpm for 15 min. The supernatant was then decanted. The microspheres were resuspended in acetone, collected on a Teflon membrane (0.45 μm pore size), and washed

and dehydrated with acetone. The collected microspheres were resuspended in acetone, and washed with isotonic phosphate buffered saline (PBS). The microspheres were air-dried for 24 h, vacuum-dried for an additional 24 h, and stored frozen. In the preparation of AHMS, crosslinking density, reduction and quenching did not change the size distribution. Using the high affinity conjugate, a more viscous aqueous solution was obtained at the same conjugate concentration, resulting in larger microsphere with a broader distribution.

Carrier for Adriamycin (ADR)

In a typical experiment, 10 mg of AHMS (5 ~35 μm size) was ultrasonically suspended in 1 mL of a ^{14}C-adriamycin solution (5 mg/mL water). The ratio of adriamycin to AHMS was varied. This suspension was gently rotated for 20 h at 4℃ in the dark. The suspension was then centrifused, the loading solution was decanted and 1 mL of distilled water was added to rinse the microspheres. After centrifugation, the washing solution was decanted again. This procedure was repeated three more times and was subsequently repeated four more times with 1 mL of acetone to dehydrate the microspheres. The microspheres were left overnight at room temperature in the dark to evaporate the acetone, and subsequently dried *in vacuo* for 3 h at room temperature. The loading kinetics were determined by monitoring the ^{14}C-adriamycin levels in the solution during the drug loading. The maximal payload of the microspheres was determined by increasing the concentration of the loading solutions (0.5~10 mg/mL).

Results

Compared with AMS, the AHMS are more hydrophilic and relatively easy to resuspend in aqueous medium. This enables resuspension of AHMS in the ADR containing loading solution. Due to the negatively charged heparin moieties in the AHMS, the loading of the positively charged ADR is a fast and effcient precess, as can be seen Figure 2. If an ADR/AHMS ratio of 1/2 (w/w) was used, drug loading was completed after 2-3 h and approximately 90% of the drug initially present in the loading solution was entrapped in the AHMS.

The binding capacity of ADR to AHMS was limited. At higher concentrations the ADR binding became saturated. Increasing the concentration in the loading solution to an ADR/AHMS ratio of 1/1 (w/w) gave only a small increase in payload. This is demonstrated in Figure 3 in which the maximum ADR payload of the microspheres as a function of the concentration of the loading solution is given.

The loading efficiency, expressed as the relative ADR depletion from the loading solution, was almost 100% up to the level, at which the maximal loading capacity was reached. This also indicates that under these circumstances, no unbound ADR is present. Unbound ADR, removed during the washing and dehydration procedure would give a lower efficiency.

Figure 4 shows the influence of the heparin

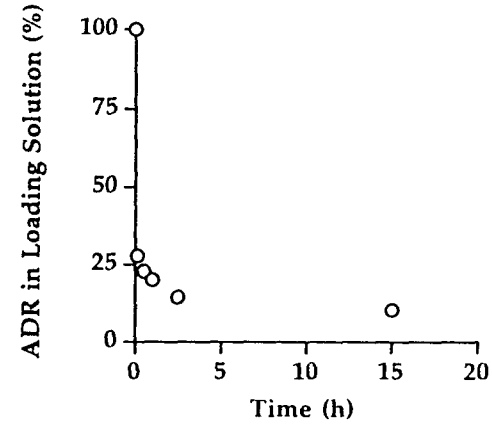

Figure 2—Relative amount of ADR in the loading solution during ADR loading of 10 mg of a typical AHMS (crosslinked with 0.5% glutaraldehyde) using 1 mL of an ADR solution of 5 mg/mL. ADR/AHMS ratio=0.5 mg/mg.

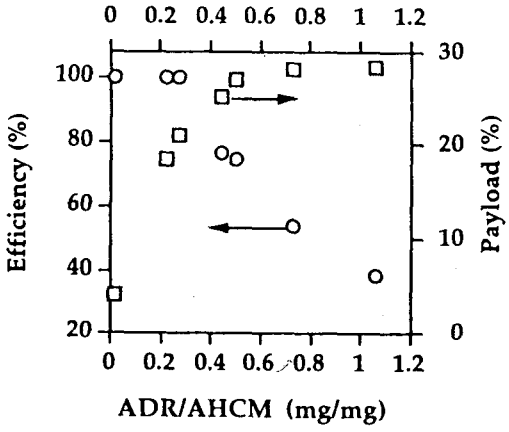

Figure 3—Influence of the ADR/AHMS ratio on the payload and the loading efficiency after 24 h of drug loading. AHMS (10 mg) were suspended in 1 mL of ADR solutioon (0.5~10 mg/mL).

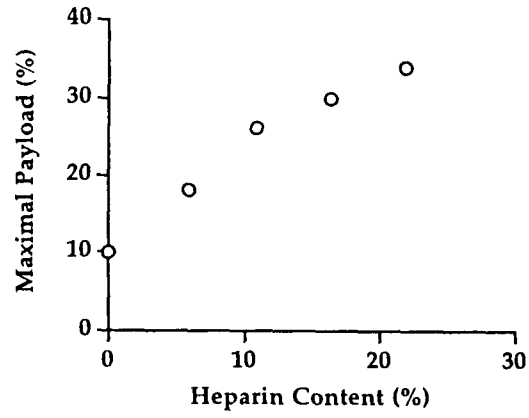

Figure 4—Maximal ADR payloads as a function of heparin content of AHMS.

content on the drug loading capacity of AHMS. With increasing heparin content, higher payload can be achieved. From this experiment, it can be concluded that the heparin content may be the most important parameter determining the payload. Previously, Menozzi and Arcamone[10] have determined the ADR-binding capacity of heaprin. They found that 3.3moles of ADR could be bound per mole of hexosamine residues of heparin. It was determined that, in solution, the unfractionated albumin-heparin conjugate had an ADR binding capacity of 3.2 moles of ADR/mole of hexosamine residues. Therefore the ADR-heparin binding appears not to be influenced by the covalent coupling of albumin and heparin.

The ADR-heparin binding experiments with the drug solution suggest that several types of drug binding accur. Next to an initial electrostatic binding between the basic group of the aminosugar moiety of the drug and the polyanions, the drug binding may be enhanced by self association of ADR due to hydrophobic interactions between ADR molecules.[10] Formation of ADR aggregate in the concentrated solution is a well known phenomenon, which was described previously.[11]

The fractionation of the conjugate based on ionic interaction with DEAE sepharose, yields a conjugate which shows more affinity towards ADR compared to the unfractionated conjugate. Accordingly the ADR payloads of microspheres prepared from the high-affinity conjugate were higher than that of micropheres prepared from the unfractionated conjugate (32% instead of approximately 27%). The concentration of the crosslinking agent, quenching and reduction had virtually no influence on the payload (data not presented). From these results, it can be concluded that the charge density of the matrix material is the most important factors determining the final payload. The drug loading process is a very attractive one since it allows effective drug loading with little or no drug loss during the procedure.

Adriamycin release experiments were carried out using a batch system. The loaded AHMS were weighed on an aluminium foil basket, which was then put into a 20 mL polypropylene vial. Two hundred mL ethanol was added and the AHMS were ultrasonically resuspended. At t=0, 20 mL of various release media was added and the counting vials were put into a shaking bath at 37℃. The amplitude of the shaking was 2.5 cm and the frequency, approximately 120 strokes/min. At

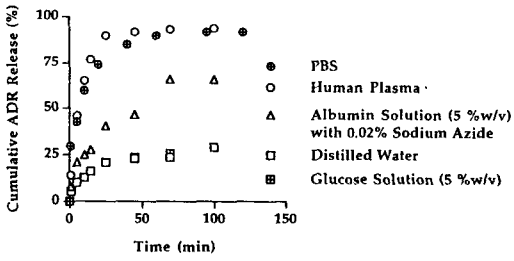

Figure 5—Cumulative ADR release from AHMS (0.5% glutaraldehyde) in several release media at 37℃.

predetermined intervals, the vials were taken from the bath and shaken vigorously to homogenize the suspension. A 0.5 mL sample was pipetted from the vial into a 1.5 mL Eppendorf cup®. After centrifugation, two 200 μL samples were taken from the supernatant, and tranferred into 6 mL counting vials. Scintillation medium (4 mL) was added and the activity was determined. The release profile was calculated from the activity of the samples.

ADR release from AHMS is controlled by the ionic strength of the medium, as shown in Figure 5. The release in ion-containing media, such as PBS and human plasma, was fast. Almost all of the drug was released within two hours. In non-ionic media, however, only 30% of the drug was released. The ion controlled release provides a method to prepare an ADR-AHMS suspension containing little drug. This is demonstrated in Figure 6. Maximally 30% of the drug may be released prior to 'injection'. Upon increasing the salt concentration up to the physiological level by addition of salt solution, thus imitating injection, the ramaining ADR was released immediately. Another important feature is that ADR remaining in the AHMS after the initial release is not released in fresh glucose solution, indicating that the ADR released prior to the injection can be removed using a simple washing step with non-ionic medium. The drug release in non-ionic medium (30%) was unexpected since the ADR ought

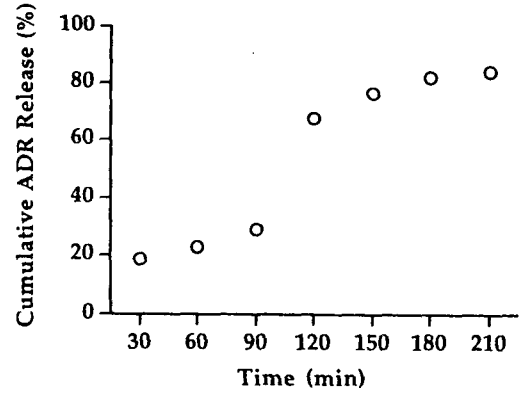

Figure 6—Cumulative ADR release from AHMS (1.0% glutaraldehyde) in 5% w/v glucose solution at 20℃. At t=90 min, warm saline was added to increase the NaCl concentration of the release medium to a physiological level and temperature to 37℃.

to be bound ionically to the matrix. Moreover, no drug was released in water during the washing steps in the drug loading procedure. It was already mentioned, however, that the electrostatic interaction of the ADR and the matrix is not the only mode of binding in this system. Apart from this type of binding, a substantial amount of ADR will be bound by self association of ADR. It is suggested that due to the dehydration steps with acetone during the drug loading procedure, some of the hydrophobically bound ADR is detached from the ionically bound ADR. Previously, it has been shown that the dimerization equilibrium of antracyclines, such as ADR and daunomycin, is shifted towards free drug in the presence of organic solvents.[12, 13] This drug remain trapped in the dehydrated microspheres as an unbound drug. Upon swelling in water, this drug is released, regardless of the ionic strength of the medium. Figure 7 shows the ADR release profiles from several types of AHMS. As can be seen in the figure, the ADR release profile was not influenced by the quenching and reduction steps, nor by fractionation of the conjugate. All microspheres crosslinked with 1.0% glutaralhehyde

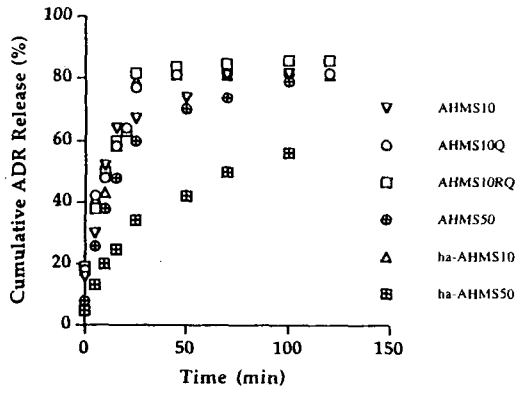

Figure 7—Cumulative ADR release from several types oof AHMS in Phosphate buffered saline at 37℃.

(AHMS 10) released the drug within 45 min. There was an influence of corsslinking density, i.e., the drug release from microspheres cross-linked with 5.0% (AHMS50) glutaraldehyde was slower.

Figure 7 also shows that not all of the drug was released from the microspheres. In the best case, approximately 90% was released. If this is due to covalent binding of ADR to the unreacted aldehyde groups, quenching should result in more complete drug release. The maximal release of these microspheres quenched with glycine was not different from that of unquenched microspheres. Quenching in the presence of a reducing agent, however, did result in more drug release. This indicates that adequate quenching may only be achieved if a reducing agent is present. It also indicates that some of the drug remaining in the non-quenched microsphers after drug release, may be covalently bound to the matrix. Since ADR release from the microspheres quenched in the presence of a reducing agent is only 90%, covalent coupling of ADR to the albumin-heparin conjugate matrix does not account for all the drug remaining in the microspheres. An explanation for the drug remaining in the microsphere is the stabilization of electrostatically bound ADR by hydrophobic interactions with other ADR molecules, which was already memtioned before. Menozzi and Arcamone[10] found that in solution, only 50% of the ADR could be displaced from the heparin by Na^+ at physiolosical concentrations. ADR-conjugate binding studies carried out with solution showed that, in PBS, only 80% of the drug was displaced from the conjugate. Based on these results it is likely that some of the ADR aggregate, electrostatically bound to the matrix cannot be detached by counterions in the release medium.

Carrier for a Model Protein, Lysozyme

In these studies, both chicken egg and human lysozymes were used as model proteins. Both lysozymes have been well chararacterized[14]; the protein has a molecular weight of 14,000 and an isoelectric point of 10.0~11.0, therefore being po sitively charged at pH 7.0. The amino acid composition of the lysozymes is different at 40% of the positions. There exist approximately one third as many negatively charged groups as there are positively charged groups on the surfaces of both lysozymes.[15] In previous studies chicken egg and human lysozymes exhibited dissimilar adsorption behavior on polymer surfaces.[15] Human lysozyme exhibited a typical protein adsorption isotherm on model negatively charged surfaces.

The larger of AMS and AHMS prepared from unfractionated albumin-heparin conjugate (heparin content; 10 w/w%) were used for lysozyme loading and release experiments, the diameter of which ranged from 50 to 150 μm in the hydrated state.

Lysozyme adsorption isotherms were obtained for both chicken egg and human lysozyme on AHMS and AMS. Chicken egg and human lysozymes (37.5 mg) were separately dissolved in 15.0 mL of PBS (28 mM potassium phosphate, 39 mM disodium phosphate), pH 7.0 at 25℃ and diluted to make 5 mL solution of concentrations ranging from 0.078 mg/mL to 5.00 mg/mL. The AHMS and AMS were separately swollen and washed with 3 portions of 25 mL 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 portion and added to the lysozyme solutions. The experiments were carried out for 25 h at 25℃ under constant agitation using a shaking apparatus. The amount of adsorbed lysozyme was determined by the solution depletion method, where the absorbance of the loading solution was measured at 280 nm with UV/Vis spectrophotometer. The concentration of the lysozyme adsorbing to the microspheres was calculated by[16]

$$m_p=(W_s/W_g)(m_i-m_f)$$

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 AHMS was also carried out in 67 mM PBS, 0.50 M NaCl, pH 7.0 using identical experimental conditions.

The adsorption isotherms of human lysozyme on AHMS and AMS are shown in Figures 8A and 8B, respectively. The adsorption of human lysozyme onto AHMS did not reach a distinct plateau at the initial concentrations of human lysozyme used for loading of the microspheres. The adsorption of human lysozyme on AMS appeared to increase rapidly at low equilibrium concentrations of human lysozyme and then reached to plateau at higher equilibrium concentrations to approximately 40 mg/mL gel. The effect of higher ionic strength on human lysozyme adsorption on AHMS is shown in Figure 8C. 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

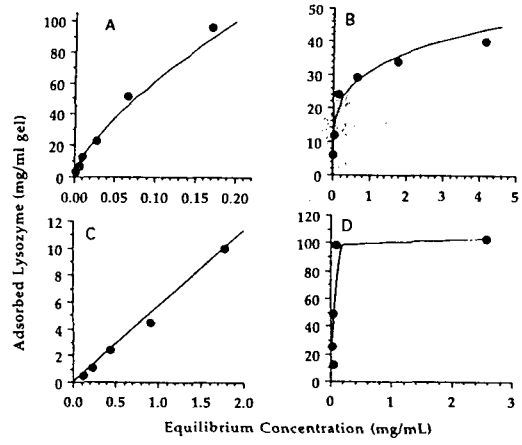

Figure 8—Adsorption isotherms of (A) human lysozyme to AHMS, (B) human lysozyme to AMS, (C) human lysozyme to AHMS at high ionic strength (0.50 M NaCl), and (D) chicken egg lysozyme to AHMS at 25℃ in 67 mM PBS (pH 7.0). The solid lines in A and B were calculated from the Freundlich equation and parameters in Table 1.

67 mM PBS, pH 7.0. The adsorption isotherm of chicken egg lysozyme on AHMS is shown in Figure 8D. The adsorption of chicken egg lysozyme onto AHMS 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 adsorption of human lysozyme on AHMS and AMS corresponded to the Freundlich type of isotherm[17]

$$a=\alpha c^{1/n}$$

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 and for AHMS is approximately 100 times the value of AMS. The adsorption exponent indicates the intensity of the adsorption process and was greater for AHMS to AMS. The adsorption data

Table 1—Freundlich parameters for human lysozymes adsorption to AHMS and AMS

Microspheres	α	1/n	r^2
AHMS	316	0.72	0.99
AMS	30.9	0.24	0.96

fit the Freundlich equation which suggested the presence of heterogeneous binding sites[17] due to multivalent adsorption. Multivalent adsorption may account for the heterogeneous binding sites. 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 of the microspheres may be considered as ligands at a fixed concentration, and the adsorption may be studied at varying protein concetrations as was carried out for these studies. The Scatchard plots of human lysozyme on AHMS and AMS are illustrated in Figures 9A and 9B. The concavity upward suggested negative cooperativity of the adsorption process in both cases. 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 a phenomenon as sequential adsorption.[19] For such 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 nonindependence of the adsorption phenomenon. At high ionic strength the Scatchard plot of the human lysozyme on AHMS was linear with zero slope (Figure 9C). For Scatchard plots the slope is typically the binding constant and at high ionic strength the adsorption energy of human lysozyme with AHMS was negligible. Thus at 67 mM PBS, 0.50 M NaCl, pH 7.0 AHMS behaved as an uncharged gel. Figure 9D shows the Scatchard plot of chicken egg on AHMS. 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 lysozyme multilayers has been postulated.[15]

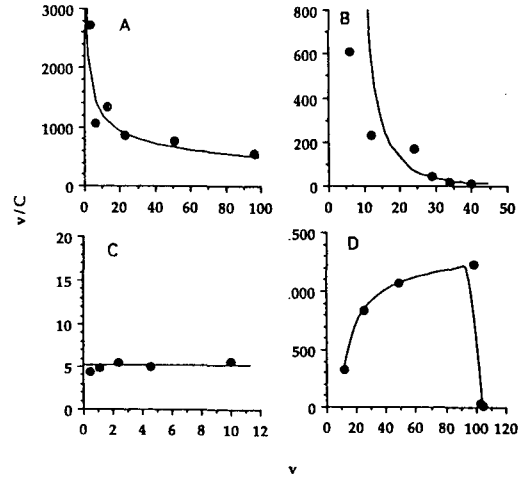

Figure 9—Scatchard plots of the adsorption isotherms of (A) human lysozyme to AHMS, (B) human lysozyme to AMS, (C) human lysozyme to AHMS at high ionic strength (0.50 M NaCl), and (D) chicken egg lysozyme to AHMS at 25℃ in 67 mM PBS (pH 7.0). The solid lines in A and B were calculated from the Freundlich equation and parameters in Table 1. v denotes the amount of adsorbed lysozyme in mg/mL gel, and c is the equilibrium concentration in mg/mL.

The lysozyme loaded microspheres (50.0 mg) were placed in 10 mL isotonic PBS with 0.01% sodium azide, pH 7.0 at 37℃. The vials were then placed in a shaking water bath 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 iso-

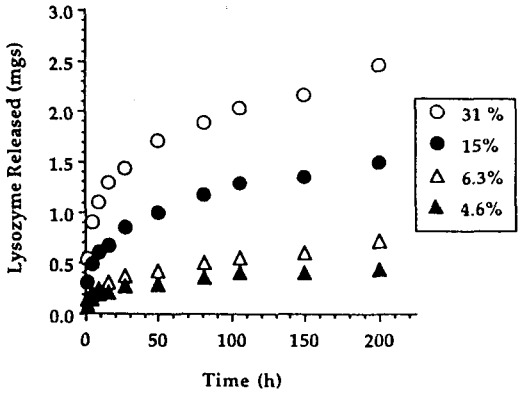

Figure 10—Release of human lysozyme from AHMS of varying loading content in isotonic PBS, pH 7.0 at 37℃.

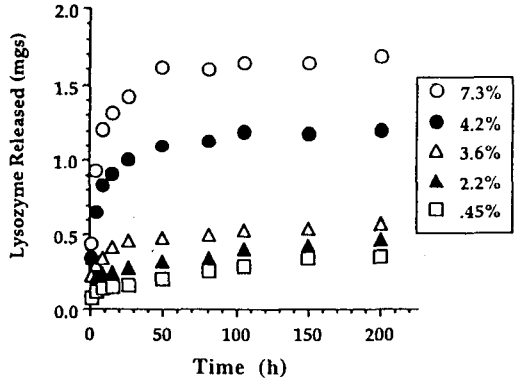

Figure 11—Release of human lysozyme from AMS varying loading content in isotonic PBS, pH 7.0 at 37℃.

tonic PBS with 0.01% sodium azide, pH 7.0 to maintain 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/Vis spectrophotometer.

To investigate the effect of release media ionic strength, the release of human lysozyme from AHMS 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 AHMS was also carried out in deionized water (0-ionic strength).

The total amount of incorporated lysozyme in the microspheres was determined by placing the microspheres in 67 mM PBS, 0.50 M NaCl, pH 7.0

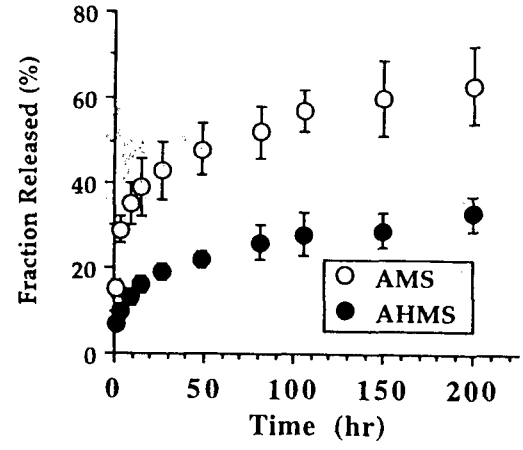

Figure 12—Fractional release of hyman lysozyme from AHMS and AMS in isotonic PBS, pH 7.0 at 37℃.

for 100 h and then assaying the released amount.

The release kinetics of human lysozyme from AHMS and AMS and the effect of loading on release are illustrated in Figures 10 and 11. The loading of human lysozyme into AHMS and AMS 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 AHMS and AMS. In Figure 12 the release of human lysozyme at varying loading contents from AHMS andAMS was plotted as fraction released versus time. Apparently at the loading levels used there was no significant effect of loading on the fractional release for either AHMS and AMS. The release of human lysozyme from AMS was initially rapid (up to ~30% release) and then slowed considerably. For AHMS release of human lysozyme exhibited a slower release rate relative to AMS. For both cases long term release of human lysozyme was attained under isotonic conditions. Figure 13 illustrates the release of human lysozyme from AHMS 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 AHMS was observed when the release medium

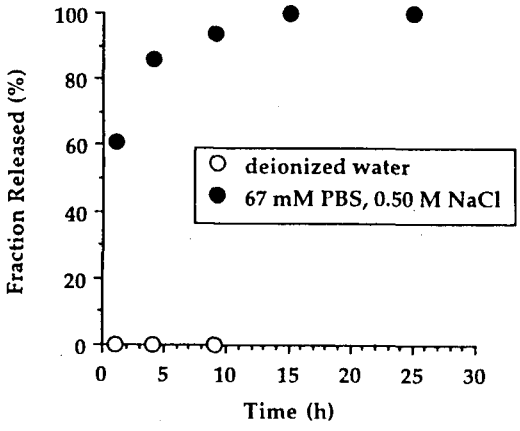

Figure 13—Release of human lysozyme from AHMS at 37℃ in 67 mM PBS, 0.50 M NaCl, pH 7.0, and deionized water.

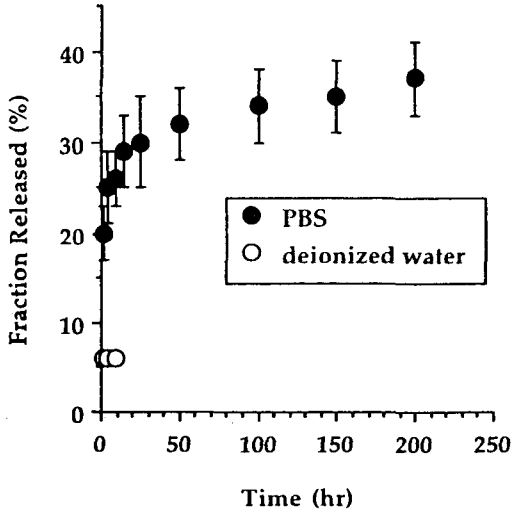

Figure 14—Release of chicken lysozyme from AHMS at 37℃ in 67mM PBS, 0.50 M NaCl, pH 7.0, and deionized water.

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 prevalent for human lysozyme release from AMS (Figure 14). Very little release of chicken egg lysozyme was observed from AHMS in deionized water.

The long term release kinetics of lysozyme from AHMS and AMS 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]

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

where

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

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 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 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]

$$\frac{M_t}{M_\infty} = 6\left[\frac{D_t}{\pi r^2}\right]^{1/2} - \frac{3Dt}{r^2}$$

to determine values for the apparent diffusion coefficents of lysozyme release from AHMS and AMS. The apparent diffusion coefficents of human lysozyme in AHMS and AMS and other release parameters are given in Table 2. The apparent diffusion coefficents are much less than the self diffusion coefficient of human lysozyme ($D_o = 1.12 \times 10^{-6} cm^2/sec$).[22] The apparent diffusion coefficient of human lysozyme in AHMS was about 20 times less than in AMS and was attributed

Table 2—Release parameters for human and chicken egg lysozyme from AHMS and AMS

Microspheres	Apparent diffusion coefficient(cm^2/s)	Diameter (μm)	Swelling ratio ($V_{swollen}/V_{dried}$)
AHMS	2.1×10^{-12}	100 ± 46^b	1.8
AHMS	4.1×10^{-12} [a]	100 ± 46^b	1.8
AMS	3.9×10^{-11}	100 ± 50^b	1.6

[a], Chicken egg lysozyme; [b], mean ± SD (n=300).

largely to the difference in the number of negatively charged groups between AHMS and AMS and the availability of charged sites. Heparin of the conjugate does not participate in the cross-linking reaction in the formation of the microsphere network, but is grafted onto the network and 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 probably due to the higher charge density. The increased binding sites for multivalent adsorption with proteins results in higher adsorption affinity.[23] That diffusion was not likely the main release mechanism was also indicated by the absence of effect of drug loading on fractional release versus time of human lysozyme from both albumin-heparin and albumin microspheres.

An apparent diffusion coefficient of chicken egg lysozyme release from AHMS of $4.1\times10^{-12}cm^2$/sec was determined (Table 2). This indicated that the initial rapid release of chicken egg lysozyme from AHMS was perhaps due to the release from the multilayers of chicken egg lysozyme at the surface of the AHMS. The low amount of chicken egg lysozyme release in deionized water from albumin-heparin microspheres support ion exchange as the primary release mechanism.

Conclusion

Albumin-heparin conjugate microspheres are biodegradable ion-exchange particles prepared from endogeneous materials. The AHMS could easily loaded with the positively charged adriamycin up to payloads of 10 to 34%, depending on the type of conjugate used. Drug release was ionically controlled and this enabled us too prepare injectable suspension in which the drug release could be delayed until contact with body fluids.

The adsorption of human lysozyme on the microspheres was fit to the Freundlich equation suggesting heterogeneous binding sites. Scatchard plots of the adsorption processes of human lysozyme on AHMA and AMS suggested negative cooperativity which was consistent with multivalent, electrostatic interactions. The Scatchard plot for the adsorption of chicken egg lysozyme on AHMS suggested positive cooperativity. This was accounted for by the formation of chicken egg lysozyme multilayers at negatively charged surfaces and suggested that protein-protein interactions are important in the design of DDS for proteins.

The long term release of human and chicken lysozyme was obtained from AHMS and AMS. 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.

References

1) K. Widder, G. Flouret and A. Senyei, *J. Pharm. Sci.*, **68**, 79(1979).
2) Y. Tabata and Y. Ikata, *Biomaterials*, **9**, 356 (1988).
3) W.E. Longo, H. Iwata, T.A. Lindheimer and E.P. Goldberg, *J. Pharm. Sci.*, **71**, 1323.
4) E.P. Goldberg, H. Iwata and W. Longo, in: S.S.

Davis, L. Illum, J.G. McVie, and E. Tomlinson (Eds.), Microspheres and Drug Therapy. Pharmaceutical, Immunological and Medical Aspects, Elsvier, *Amsterdam*, 309-325(1984).
5) S.L. Law, C.H. Chiang, F.M. Lin, F.M. Lin and G.W. Teh, Biomater., Art. Cells & Immob. *Biotech.*, **19**, 6133(1991).
6) N. Willmott, Y. Chen and A.T. Florence, *J. Control. Rel.*, **8**, 93(1988).
7) C. Jones, M.A. Burtoon, B.N. Gray and J. Hodgkin, *J. Control. Rel.*, **8**, 251(1989).
8) W.E. Hennik, J. Feijen, C.D. Ebert and S.W. Kim, *Thromb. Res.*, **29**, 1(1983).
9) J.J. Burger, E. Tomlinson, E. Mulder and J.G. McVie, *Int. J. Pharm.*, **23**, 333(1985).
10) M. Menozzi and F. Acramone, *Biochem. Biophys. Res. Comm.*, **80**, 313(1978).
11) E. Yayakawa, K. Furuya, T. Kuroda, M. Moriyama and A. Kondo, *Chem. Pharm. Bull.*, **39**, 1282(1911).
12) S.R, Martin, *Biopolymers*, **19**, 713(1980).
13) M. Nukui, C. Hoes, H. van den Berg and J. Feijen, *Makromol. Chem.*, **192**, 2925(1991).
14) D.C. Phillips, in: E.F. Osserman, R.E. Canfield, and S. Blychok (Eds.), *Lysozyme*, Academic Press, New York, 9-30, (1974).
15) D. Horsey, J. Herron, V. Hlady and J.D. Andrade, in: J.L. Brash and T.A. Horbett (Eds.), Proteins at Interfaces: Physicochemical and Biochemical Studies, ACS, Wasgington, D.C., 290-305(1987).
16) S. Yamamoto, K. Nakanishi and R. Matsuno. Ion-Exchange Chromatography of Proteins, *Marcel Dekker*, New York (1988).
17) A.W. Adamson, Physical Chemistry of Surfaces, John Wiley and Sons, New York (1976).
18) J.D. Andrade, in : J.D. Andrade (Ed.), Surface and Interfacial Aspects of Biomedical Polymers, *Plenum Press*, New York, 1-80(1985).
19) H.P. Jennissen, *Biochemistry*, **15**, 5683(1976).
20) F. Helfferich, Ion-exchange, McGraw-Hill, New York, (1962).
21) R.W. Baker and H.K. Lonsdale, in: A.C. Tanquary and R.E. Lacey (Eds), Controlled Release of Biologically Active Agent, *Plenum Press*, New York, 5-71(1974).
22) M.T. Tyn and T.W. Gusek, *Biotech. Bioeng.*, **35**, 327-338(1990).
23) H.P. Jennissen and L.M. Heilmeyer, *Biochemistry*, **14**, 754(1975).