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Release of macromolecules from albumin-heparin microspheres

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Summary

Hydrophilic microspheres based on albumin-heparin conjugates have been prepared as a macromolecular delivery system. The soluble albumin-heparin conjugate was synthesized and crosslinked in a water-in-oil emulsion with glutaraldehyde to form microspheres in the same manner as for albumin microsphere preparation. The microspheres were characterized in terms of their size and swelling properties. The loading of macromolecules into albumin-heparin microspheres was carried out concurrently and after microsphere preparation. FITC-dextran was applied as a model macromolecule. A higher loading content was achieved when loading was carried out concurrently with microsphere preparation than when loaded subsequently. Prolonged release of FITC-dextran from albumin-heparin microspheres was achieved and attributed to the high molecular weight of the macromolecule. The release of FITC-dextran was modulated by crosslinking density, loading content and the method of drug incorporation. Apparently, the mechanism of FITC-dextran release from albumin-heparin microspheres was dependent on the method of drug incorporation. For release of FITC-dextran from the microspheres, assuming negligible interactions, a diffusion coefficient of 1.7×10^{-9} cm^2/s was determined.

Introduction

Albumin microspheres have been widely investigated as biodegradable drug carriers due to their potential for site-specific drug delivery in vivo by direct injection into discrete anatomical compartments and by targeting within the vascular system (Tomlinson, 1987). The biodegradation of albumin microspheres results in nontoxic byproducts (i.e., amino acids). Microspheres based on serum albumin display a high water content and a rubbery nature. Albumin microspheres may be classified as hydrogels (Heller, 1987) and are highly biocompatible (Lee et al., 1981; Ratcliffe et al., 1984). While many different drugs have been incorporated into albumin microspheres, a vast majority have been of low molecular weight (e.g., antineoplastics) (Gupta et al., 1988). Currently, there exists much interest in the delivery of genetically engineered macromolecules due to their low oral bioavailability and short plasma half-lives. A seminal investigation by Goosen et

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al. (1982) demonstrated the possibility of long-term insulin release from albumin microspheres after subcutaneous injection in vivo. The parameters influencing insulin release from albumin microspheres were not examined.

The preparation, characterization and in vitro release of adriamycin from hydrophilic microspheres of albumin-heparin conjugates have been described previously (Cremers et al., 1990; Kwon et al., 1991). Albumin-heparin microspheres are hydrogels which exhibit enhanced hydrophilicity relative to albumin microspheres, greater colloidal stability and pH- and ionic-strength-dependent, stimulus-sensitive swelling. Drugs, in principle, can be loaded into the microspheres both during and after microsphere preparation through a swelling/deswelling process or ion exchange. The objective of this work was to study the release of FITC-dextrans from loaded microspheres in vitro in order to examine the viability of albumin-based microspheres to deliver macromolecules in a controlled manner. FITC-dextran was chosen as a model macromolecule in order to simplify the system and to study release behaviors of non-interacting solutes with the microsphere matrix.

Materials and Methods

Materials

Human serum albumin (lyophilized and crystallized), olive oil, fluorescein isothiocyanate (FITC) dextrans and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Sigma, St. Louis, MO. Heparin (lot no. LPP 060143), 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. Microconcavity slides were received from Clay Adams, Parsippany, NJ. Cellulose acetate (molecular weight cut-off 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. Latexes (10 and 100 μm) were purchased from Coulter Counter, Hialeah, FL. All other chemicals were reagent grade.

Methods

Preparation of albumin-heparin microspheres

Albumin-heparin conjugates were synthesized and purified using a slightly modified protocol of Hennink et al. (1983). Briefly, serum albumin and heparin were coupled via amide bonds using a water-soluble carbodiimide (EDC). In this experiment, a 0.067 M phosphate buffer, pH 5.1 was used to maintain the pH of the reaction mixture constant in order to preclude the addition of concentrated HCl which had resulted in precipitation during the course of the reaction. The conjugate was then purified from free serum albumin and heparin by Blue Sepharose CL-6B and DEAE anion-exchange liquid chromatography, respectively. The resulting conjugate was composed of about 10% heparin by weight (Kwon et al., 1991). Albumin-heparin microspheres were prepared using a method similar to that of Burger et al. (1985). A 140 ml volume of olive oil was placed in a baffled cell and stirred at 300 rpm for 30 min to stabilize the stirring rate. Albumin-heparin conjugate (100 mg), dissolved in 400 μl distilled water at 4°C, was injected dropwise into stirred olive oil at 25°C and stirring continued for 15 min. A predetermined amount of purified glutaraldehyde (25% w/v) to make 1–3% (w/v) of the added albumin-heparin conjugate solution was then added along with twice the equimolar amount of sodium cyanoborohydride solution. The crosslinking reaction was allowed to proceed for 4 h. Subsequently, 50 ml acetone was added, and the emulsion stirred for 1 min. The microspheres were isolated by centrifugation (MSE, VWR, Philadelphia, PA) at 1000 rpm for 10 min. The supernatant was then decanted. The microspheres were resuspended in acetone, collected on a 3.0 μm PTFE membrane and washed and

dehydrated with acetone. The microspheres were air dried for 24 h, vacuum dried for an additional 24 h and stored frozen in the dark.

Size analysis

The diameter of albumin-heparin microspheres ($n = 300$) was determined by phase contrast light microscopy (Biophot, Nikon, Tokyo, Japan) using a calibrated graticule. Calibration was performed with latexes of known diameter. The diameters of albumin-heparin microspheres were measured in the swollen state in isotonic phosphate buffer (PBS) (2.00×10^{-3} M potassium phosphate, 8.00×10^{-3} M disodium phosphate, 0.145 M NaCl), pH 7.4 with 0.01% sodium azide at 25°C for longer than 1 h to ensure equilibrium swelling.

Swelling

The equilibrium swelling of albumin-heparin microspheres was determined in isotonic PBS, 0.01% sodium azide, pH 7.4 as previously described (Kwon et al., 1991). The swelling ratio

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

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

Drug loading

For in vitro studies, a model macromolecule, FITC-dextran ($M_W = 17\,200$), was loaded concurrently with albumin-heparin microsphere preparations. Varying amounts of FITC-dextran (2.50–10.0 mg) were dissolved along with the albumin-heparin conjugate, and the microspheres were prepared as described. The FITC-dextran was not expected to participate in the crosslinking reaction during the formation of the albumin-heparin microspheres. The amount of FITC-dextran incorporated into the microspheres was determined by the method of Tomlinson and Burger (1985). Albumin-heparin microspheres were prepared with FITC-dextran added as described without crosslinking agent. The marginally stable microspheres were placed in isotonic PBS, pH 7.4, 0.01% sodium azide, where they readily underwent dissolution and released the loaded FITC-dextran. The extent of loading was quantitated by measuring the absorbance at 494 nm with a UV/Vis spectrophotometer (Lambda 7, Perkin Elmer, Norwalk, CT).

The potential for loading macromolecules into albumin-heparin microspheres after microsphere preparation was investigated. To examine the loading via a swelling/deswelling process, albumin-heparin microspheres (100 mg, 2% w/v glutaraldehyde) with a relatively low degree of crosslinking of which swelling was sensitive to ionic strength were swollen in 10.0 ml solution of FITC-dextran in deionized water. The concentration of FITC-dextran used was 2.50 mg/ml. The loading experiments were carried out for 50 h at 25°C with constant agitation. The loading of FITC-dextran into albumin-heparin microspheres (100 mg, 3% w/v glutaraldehyde), of which swelling was relatively insensitive to ionic strength, was also carried out in 10.0 ml isotonic PBS, with 0.01% sodium azide, pH 7.4 under otherwise identical experimental conditions. This loading procedure was utilized to obtain an estimate of the diffusion coefficient of the dextran under identical conditions with loading and release studies. Albumin-heparin microspheres were then isolated by vacuum filtration on a cellulose acetate membrane (pore size 8.0 μm), dehydrated with acetone on a PTFE membrane, air dried and then vacuum dried. The incorporation of FITC-dextran into albumin-heparin microspheres (50.0 mg) was measured by determining the amount released in 10 ml deionized water during a period of 100 h.

In vitro release studies

Albumin-heparin microspheres (50.0 mg) loaded with FITC-dextran were placed in 10.0 ml isotonic PBS with 0.01% sodium azide, pH 7.4 at 37°C. The vials were then placed in a shaking waterbath (BT-47, American Scientific Products, McGaw Park, IL) and agitated at 100 strokes/min. The release medium was assayed at appropriate time intervals by withdrawing 1.00 ml sam-

ples and replacing with 1.00 ml isotonic PBS with 0.01% sodium azide, pH 7.4 to maintain a constant volume of 10.0 ml. This dilution was accounted for when establishing the release kinetics. The amount of FITC-dextran released was quantitated by measuring the absorbance at 494 nm by UV/Vis spectrophotometry.

Results and Discussion

Albumin microspheres are formed by chemical- and heat-induced crosslinking, and drugs may be incorporated into albumin microspheres concurrently with microsphere preparation. For this study, albumin-heparin microspheres were chemically crosslinked. This route was quite facile and has been routinely used for the drug loading of albumin microspheres (Tomlinson and Burger, 1985). Swollen albumin-heparin microspheres in isotonic PBS, pH 7.4, prepared as described above had average diameters of 140 ± 90, 120 ± 80 and 85 ± 50 μm on crosslinking at 1, 2 and 3% w/v glutaraldehyde, respectively. No significant batch to batch variation was observed. The size of microspheres was normally distributed and was due to the use of a baffled cell (Tomlinson and Burger, 1985). The preparation parameters (e.g., oil phase volume, stirring rate) were adjusted in order to prepare relatively larger microspheres to achieve long-term release of the FITC-dextrans.

For this study, FITC-dextran was used as a model macromolecule. This model compound had a low polydispersity ($M_w/M_n < 1.5$) and was readily assayed. The FITC-dextrans had a weight average molecular weight of 17200. Interactions of the FITC-dextran with the microspheres were expected to be weak. Macromolecules are good candidates for sustained release from albumin-heparin microspheres due to their lower diffusivity (i.e., on the order of 10^{-6}–10^{-7} cm^2/s) (Tyn et al., 1990) relative to low molecular weight solutes which have diffusivities on the order of 10^{-5} cm^2/s (Bird et al., 1960). The release of low molecular weight drugs from albumin microspheres is typically biphasic with an initial rapid release followed by a much slower first-order release (Tomlinson, 1985). Within 5 min, up to

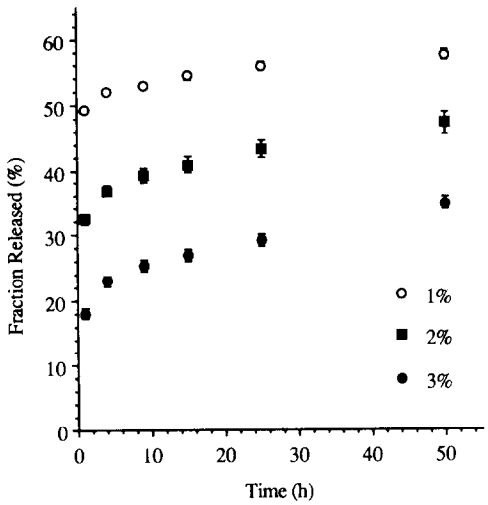

Fig. 1. Release of FITC-dextran (4% w/w loading content) from albumin-heparin microspheres of varying crosslinking density. Loading during preparation of microspheres. Mean ± SD ($n = 3$).

95% drug release has been observed from albumin microspheres during that nascent period of release. Heat-crosslinked albumin microspheres exhibit substantially slower release than chemically crosslinked microspheres. Transport mechanisms of macromolecules from albumin microspheres have not been clearly elucidated. Macromolecular transport mechanisms through hydrogel membranes have been investigated (Sato and Kim, 1984; Gilbert et al., 1988; Pitt, 1990), and macromolecules were observed to permeate predominantly via the 'pore' mechanism.

Tomlinson (1985) has discussed the parameters influencing the release of drugs from albumin microspheres. The effects of crosslinking density, actual loading content and loading process on macromolecule release from albumin-heparin microspheres were ascertained. In Fig. 1, the effects of crosslinking of the albumin-heparin microspheres on the release of FITC-dextran are illustrated. A nascent period of rapid release was observed followed by a period of sustained release. The nascent release of FITC-dextran decreased as the crosslinking density increased. The latter phase of sustained release was apparently independent of crosslinking density. Peppas and Reinhart (1983) showed that by increasing the

crosslinking density of a hydrogel network, the effective mesh size (i.e., pore size) of the hydrogel decreased, and solute transport became hindered. At high crosslinking density, proteins may be physically immobilized in hydrogel networks (Keyes and Saraswathi, 1985). Albumin-heparin microspheres crosslinked at 1, 2 and 3% w/v glutaraldehyde had swelling ratios of 7.5, 5.0 and 2.6, respectively. The swelling of the microspheres was rapid (i.e., < 15 min) and was assumed to have no influence on the release kinetics after that time period. From Fig. 1 it is apparent that loading FITC-dextran concurrently with microsphere preparation resulted in FITC-dextran physically immobilized in the microspheres, the amount being dependent on the crosslinking density. This may be due to the high molecular weight of the FITC-dextran which resulted in chain entanglement within the microsphere network and would account for the latter period of prolonged release.

The amount of drug loading was adjusted to alter the release of FITC-dextran from albumin-heparin microspheres (Fig. 2). When the loading content of FITC-dextran was increased from 2 to 7% w/w, the initial release of FITC-dextran from albumin-heparin microspheres (2% w/v glutaraldehyde) increased. Subsequent sustained release did not vary with increased drug loading.

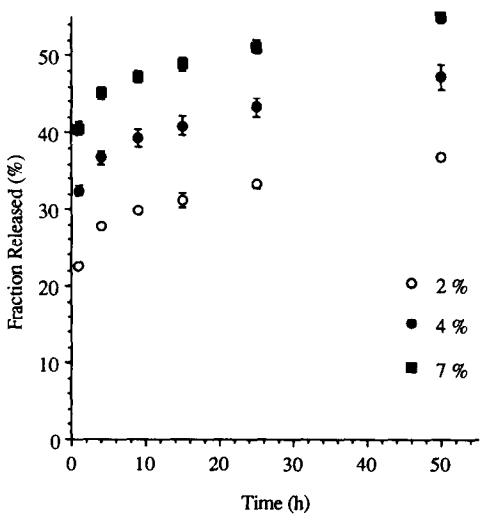

Fig. 2. Release of FITC-dextran from albumin-heparin microspheres (2% w/v glutaraldehyde) of varying loading content. Loading during preparation of microspheres. Mean ± SD ($n = 3$).

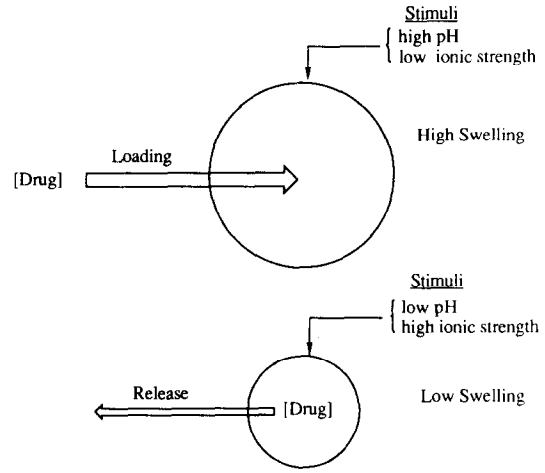

Fig. 3. Schematic illustration of drug loading and release using the stimulus-sensitive swelling properties of albumin-heparin microspheres.

Another way of loading drugs into synthetic hydrogels has been through use of their stimulus-sensitive swelling properties. Modulation of swelling for the drug loading of hydrogels has been carried out by varying the solvent composition (Forni et al., 1989). Temperature and pH have also been utilized as external stimuli to modulate the swelling of hydrogels in aqueous solutions in order to load drugs (Hoffman et al., 1986; Kou et al., 1988). External stimuli such as high pH and low ionic strength may induce the albumin-heparin microspheres to swell considerably (Kwon et al., 1991). This results in drugs being able to diffuse quickly into the matrix (Fig. 3). The release of drug from the albumin-heparin microspheres was carried out at a lower swelling state (i.e., physiological pH and ionic strength) where the diffusivity through the microsphere matrix of the drug was lower due to a reduction in swelling level. The pH-dependent swelling of albumin-based microspheres may also be used for site-specific delivery within the gastrointestinal tract (Farhadieh, 1975). The microspheres exhibit a low degree of swelling in the stomach and protect the drug, releasing the drug at the higher pH of the small intestine. By carrying out drug loading in an aqueous system, the stability of

macromolecules to be loaded may be increased compared to loading with organic solvents. For albumin-based microspheres, drug loading after microsphere preparation may be advantageous if drugs are unstable when incorporated concurrently with microsphere preparation. Proteins and peptides may be covalently modified if the molecules contain functional groups which react with the crosslinking agent. A loading content of 0.53% w/w of FITC-dextran into albumin-heparin microspheres (2% w/v glutaraldehyde) was achieved by a swelling/deswelling process. The kinetics of FITC-dextran release from albumin-heparin microspheres loaded via a swelling/deswelling process is shown in Fig. 4. Compared to the case when FITC-dextran was loaded concurrently with albumin-heparin microsphere preparation, lower loading was attained, a greater fraction of the FITC-dextran was released and release was relatively fast. This was likely due to the FITC-dextran being largely associated with the 'pore' domain of the microspheres when loading was carried out after microsphere preparation. The low loading content may also be caused by the dehydration process after the microspheres have been collected from the FITC-dextran loading solution. FITC-dextran was possibly

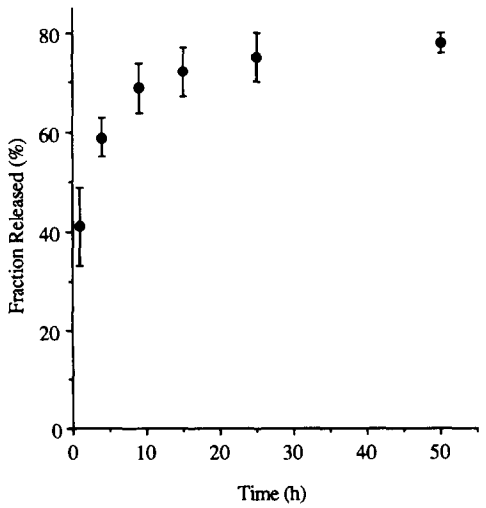

Fig. 4. Release of FITC-dextran (0.53% w/w loading content) from albumin-heparin microspheres (2% w/v glutaraldehyde) loaded via swelling/deswelling process. Mean + SD ($n = 3$).

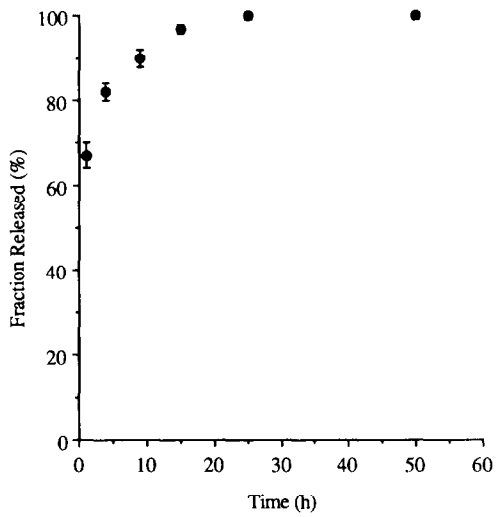

Fig. 5. Release of FITC-dextran (0.58% w/w loading content) from albumin-heparin microspheres (3% w/v glutaraldehyde) loaded after microsphere preparation in isotonic PBS, pH 7.4, 25°C. Mean ± SD ($n = 3$).

removed, along with water, when the microspheres were dehydrated with acetone. Similar results were observed for the release of low molecular weight drugs from polyvinylalcohol particles loaded concurrently and after microparticle preparation (Gander et al., 1990), and it was postulated that when drugs were loaded concurrently with microparticle preparation, drug could be associated with regions of high crosslinking, with release occurring slowly. When drugs were loaded after microparticle preparation, drugs were likely to diffuse into regions having a lower degree of crosslinking (i.e., pore domain) and to be released more rapidly.

Fig. 5 shows the release of FITC-dextran loaded after microsphere preparation (0.58% w/w) under isotonic conditions. Albumin-heparin microspheres at 3% w/v glutaraldehyde exhibit swelling which is largely insensitive to pH and ionic strength. The release was rapid, and all of the FITC-dextran was released. Release occurred more rapidly than when the microspheres were loaded by a swelling/deswelling process and was ascribed to less physically entangled FITC-dextran. Presumably all the FITC-dextran was released from the albumin-heparin microspheres by diffusion via the pore mechanism (Sato and Kim,

1984; Gilbert et al., 1988). The time for half-fraction release of solutes assuming diffusion with low extent of interaction with microspheres may be determined from

$$t_{50\%} = 0.030r^2/D \quad (2)$$

where r is the radius of the sphere, and D denotes the diffusion coefficient (Baker and Kim, 1974). Assuming a radius of 100 μm and a $t_{50\%}$ of 7.5 min, an estimate for a diffusion coefficient of 1.7×10^{-9} cm^2/s was obtained which was similar to values determined for the transport of macromolecules through hydrogels (Sato and Kim, 1984; Gilbert et al., 1988). This suggested that the rapid release was due to the high specific surface area of the microspheres. Therefore, the nascent period of rapid release observed from albumin-heparin microspheres in the case of FITC-dextran loaded concurrently with microsphere preparation may be due to diffusion through the pore pathway, and the sustained release from FITC-dextran physically entrapped in the microspheres taking place more slowly.

Conclusions

The loading of FITC-dextran into albumin-heparin microspheres was accomplished both concurrently with and after microsphere preparation. A higher loading content of FITC-dextran was achieved when loading was carried out concurrently with microsphere preparation (7.0% w/w) than when performed subsequently (0.58% w/w). When loaded concurrently, an initial period of rapid release from albumin-heparin microspheres of FITC-dextran was observed which most likely occurred by diffusion via the pore mechanism. The initial release of FITC-dextran was modulated by crosslinking and drug loading. A longer period of sustained release was then observed and was attributed to the release of entangled FITC-dextran. The FITC-dextran, when loaded after microsphere preparation, was released more quickly, predominantly occurring via the pore mechanism. The release of FITC-dextran from albumin-heparin microspheres was observed to be prolonged to a greater extent than the release of low molecular weight drugs from chemically crosslinked albumin microspheres and was due to the high molecular weight of the dextran. Albumin-heparin microspheres, by virtue of their hydrophilicity and release properties, may be used as drug carriers and warrant further investigation.

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