JPP
Journal of
Pharmacy and Pharmacology

JPP 2001, 53: 23–31

Received May 17, 2000
Accepted September 19, 2000
ISSN 0022-3573

Formulation and release characteristics of poly(lactic-*co*-glycolic acid) microspheres containing chemically modified protein

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Abstract

Chemical modification of proteins may influence their formulation into and release from polymeric microspheres. Three chemical modifications of rat serum albumin (RSA) were effected on the amine groups of this protein: conjugation with a polyanion using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, intermolecular cross-linking using glutaraldehyde, and reductive alkylation using propyl aldehyde. The modified proteins had different physicochemical properties as well as improved encapsulation efficiencies compared with native RSA microspheres.

The microspheres were incubated at 37°C for over one month to investigate the influence of protein modification on the release profiles. Microsphere degradation accelerated from the ninth day of the release studies and this coincided with an increase in the release rates. The degradation rates of poly(lactic-*co*-glycolic acid) microspheres containing either native or cross-linked RSA were more rapid than those containing either heparin conjugated or propylated RSA. This was in agreement with the release data, since the release of the native and cross-linked RSA were more rapid than those of the other modified proteins. The release profiles of the RSA-heparin conjugates and the propylated RSA were approximately zero rather than first order between the tenth and thirtieth day of study.

Chemical modification of protein may be a useful method to increase encapsulation efficiency and to decrease release rates of proteins that are to be used in microsphere formulations of potent therapeutic proteins.

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Acknowledgement and funding: The authors wish to acknowledge Professors J. Koberstein and D. Kreutzer, and Drs F. Moussy and F. Papadimitrakopoulos for useful discussions about this research. This research was supported by a grant from National Institute of Health (#1R01RR14171).

Introduction

Protein delivery has become an important area of research as a large number of recombinant proteins are now being investigated for many therapeutic applications. Most of these proteins have short in-vivo half-lives due to physical and chemical instability and enzymatic degradation (Abuchowski et al 1977; Stahl et al 1976; Stahl & Diment 1988) and, as a consequence, multiple injections are required to achieve desirable therapy. In addition, proteins that are administered parenterally are recognized by the body as specific or non-specific antigens, which can result in anaphylaxis. One way to increase the therapeutic efficiency of these proteins is by encapsulating them in a sustained dosage form that is capable of releasing the macromolecule continuously, at a controlled rate, over a period of weeks or even months.

These considerations have led to the development of numerous new delivery technologies, including particulate controlled release systems such as liposomes

(Alving et al 1992), emulsions and microspheres (Tomiya et al 1985) and chemical modification of proteins (Mean & Feeney 1995). Coon & Hunter (1973, 1975) have shown that the attachment of an aliphatic chain onto a protein can modulate or decrease the humoral immune response of the body to administered proteins.

Polymer microspheres are a promising delivery system for the protection and release of proteins (Cohen et al 1991). They have many advantages for protein delivery including single injection administration requiring no surgical incision, low dose dumping potential and the possibilities of controlled and targetted release. Through the application of different processing methods and the use of a wide diversity of natural and synthetic polymers, microspheres can be prepared with a variety of physicochemical properties, such as biodegradability and release characteristics.

Poly(lactic-*co*-glycolic acid) (PLGA) is one of the most frequently used polymers to encapsulate proteins. It is biocompatible, biodegradable and bioresorbable (Wise et al 1979; Gilding 1981). PLGA has been utilized commercially in the preparation of microspheres containing leuprolide (Lupron Depot) (Soloway et al 1995). When proteins are incorporated into PLGA microspheres, various formulation factors can affect their stability and release kinetics, such as the proportion of lactic and glycolic acid, polymer molecular weight, microsphere size distribution, microsphere morphology, protein loading, moisture content and the presence of excipients (Crotts & Park 1998). Protein physicochemical properties (such as hydrophobicity, molar mass and isoelectric point) can affect release rates from microspheres. In addition, release methods and experimental conditions affect protein release rates in-vitro and similarly the site of injection can affect in-vivo release rates (Crotts & Park 1998). Maintenance of protein activity during microsphere preparation and release studies is a further complicating factor.

PLGA degradation results in an increase in the number of carboxyl groups and this has been reported to decrease the pH in the interior of the microspheres (Mäder et al 1998; Brunner et al 1999). Usually proteins are unstable in acidic conditions and therefore it may be necessary to use a buffering system to conserve protein stability (Cremers et al 1994). Furthermore, encapsulation of hydrophilic molecules, such as proteins, in PLGA microspheres is problematic, since these molecules tend to partition to the external aqueous phase during preparation. Consequently, an increase in hydrophobicity may improve encapsulation efficiency as well as modulate release rates through hydrophobic interactions.

The formulation of potent therapeutic proteins into microspheres often requires the use of a carrier protein as a diluent, protective agent and to enhance encapsulation efficiency. Many authors (Cohen et al 1991; Cao & Shoichet 1999) have used this strategy. For example, bovine serum albumin (BSA) has been used as a carrier protein in the formulation of PLGA microspheres containing nerve growth factor (Cao & Shoichet 1999).

We report here a modification of this method. Rat serum albumin (RSA) was chemically modified to improve its encapsulation efficiency, buffer the acidic environment within PLGA microspheres and alter the release profile from PLGA microspheres. RSA was self-cross-linked, conjugated with heparin and propylated. The effect of chemical modification of RSA on its release kinetics from microspheres and degradation of PLGA microspheres were compared with those of native RSA. RSA was selected because it is our intention that subsequent animal studies will be conducted using a rat model. In future studies we aim to use the modified RSA as a carrier protein for potent therapeutic proteins to facilitate formulation in PLGA microspheres.

Materials and Methods

Materials

Poly(lactic-*co*-glycolic acid) (PLGA, 50:50, RESOMER RG504, MW 60 kDa, polydispersity 1.72) was a gift from Boerhinger Ingelheim (Germany). Rat serum albumin (RSA), heparin from porcine intestinal mucosa (179 USP units mg^{-1}, 17–19 kDa), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, glutaraldehyde (50% solution of grade I), *N*-2-hydroxyethylpiperazin-*N*′-2-ethanesulfonic acid (HEPES), nickel chloride ($NiCl_2$), cyanogen sodium borohydride ($NaBH_3CN$), hydro-soluble polyvinyl alcohol (PVA, MW 30–70 kDa) and dialysis bags (50 000 MW cut-off) were obtained from Sigma (St Louis, MO). Propanal was purchased from Aldrich (Milwaukee, WI).

Protein modification (RSA-heparin conjugate)

Albumin-heparin conjugates were prepared by a modified Henink's method (Henink et al 1983). Briefly, RSA (200 mg, 3.03 μmol) and heparin (98 mg, 5.44 μmol) were dissolved in 14 mL distilled water. This solution was adjusted to pH 5.0 with HCl (0.2 M) to activate the

carboxyl function of heparin. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (100 mg in 640 μL water) was added in a dropwise manner over 1 h, the pH was adjusted to pH 7.6 with NaOH solution (1 M). The resulting mixture was stirred for 24 h at 4°C. The solution was dialysed against 0.025 M Tris-HCl buffer (pH 7.5) using a 50 kDa dialysis bag to eliminate unreacted heparin. The protein content of the conjugate was measured by a bicinchoninic acid (BCA) method (Smith et al 1985) using native RSA as a standard. The heparin content was determined using a metachromic assay (Hennink et al 1983) and the amino group content was measured using a modified Fields' method (Fields 1971; Snyder & Sobocinski 1975). The apparent molecular weight of the modified protein was measured using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% acrylamide concentration (Laemmli 1970). The isoelectric point of each modified protein was determined by polyacrylamide gel isoelectric focusing (Giulian et al 1984; Warlow et al 1988) using pI markers as a standard (pI standard: 6.8, myoglobin (horse heart); 6.6, carbonic anhydrase I (human erythrocyte); 5.9, carbonic anhydrase I (bovine erythrocyte); 5.4, carbonic anhydrase II (bovine erythrocyte); 4.6, trypsin inhibitor (soybean); 4.2, glucose oxidase (*Aspergillus niger*)).

Protein modification (cross-linked RSA and propylated RSA)

RSA (100 mg, 1.515 μmol, 71.2 μmol of NH_2 group) was dissolved in HEPES buffer (0.2 M, pH 7.4) containing 15 mM $NaBH_3CN$ and 10 mM $NiCl_2$ (Fager et al 1972). To this solution was added 16 μL 50% glutaraldehyde solution (80 μmol) or 97 μL (78.3 μmol) propanal to cross-link or propylate RSA, respectively. These mixtures were gently stirred for 24 h at room temperature in the dark. The resulting solutions were dialysed against water for 24 h and lyophilized. The protein and the amino group contents were measured as described above.

Size exclusion chromatography

Size exclusion chromatography was conducted to compare the size of modified proteins. A Sephacryl S-200 (10–300 kDa range, Pharmacia, Sweden) column was used with acetate buffer (0.1 M, pH 5.2) as the mobile phase and a UV detector was set at 280 nm. The modified proteins were eluted using an isocratic method for 20 min at 25°C.

Hydrophobicity determination

The hydrophobicity of each modified protein was determined by HPLC. Two mobile phases (A: Tris-HCl, 0.025 M, pH 7.0; B: CH_3CN containing 0.1% of trifluoroacetic acid) were used in an automatic linear gradient system, 100% of solvent A to 100% of solvent B for 50 min. The retention times of each preparation and the peak areas were determined using a Nova-Pak (Waters, C18, 60 Å, 4 μm, 3.9 × 150 mm) column. The detection wavelength was 254 nm. The temperature was adjusted to 37°C and the injection volume was 20 μL.

Microsphere preparation

Each protein (100 mg) in powder form (native RSA, RSA-heparin conjugate, cross-linked RSA and propylated RSA) was dispersed in 4 mL poly(lactic-*co*-glycolic acid) (PLGA, 50:50) solution (25%, w/v) in CH_2Cl_2 using a homogenizer at 7500 rev min^{-1} for 2 min (Cao & Shoichet 1999). Polyvinylalcohol (PVA) solution (20 mL, 1%) was added to this mixture and homogenized at 7500 rev min^{-1} for a further 2 min. This emulsion was poured into 300 mL of a 0.1% (w/v) PVA solution and stirred for 1 h under vacuum to evaporate the organic solvent. The hardened microspheres were centrifuged, washed three times with distilled water, and dried for 24 h under vacuum.

Particle size analysis

The mean particle size and size distribution of the microspheres were measured using an Accusizer (model 770, Particle Sizing Systems, CA). Each measurement was the mean of three samples per batch and three different batches (n = 3) (50 mg in 20 mL 0.1% w/v PVA solution).

Encapsulation efficiency

The encapsulation efficiency was determined by a solvent dissolution method with dimethylsulfoxide (DMSO) (Sah 1997). Briefly, microsphere samples (50 mg) were dissolved in 10 mL DMSO containing 2% w/v SDS and 0.1 M NaOH. The protein concentration in the mixed DMSO solution was measured by a BCA method using RSA in mixed DMSO solution as a standard. Finally, the measured protein was compared with the amount of protein initially added during microsphere preparation.

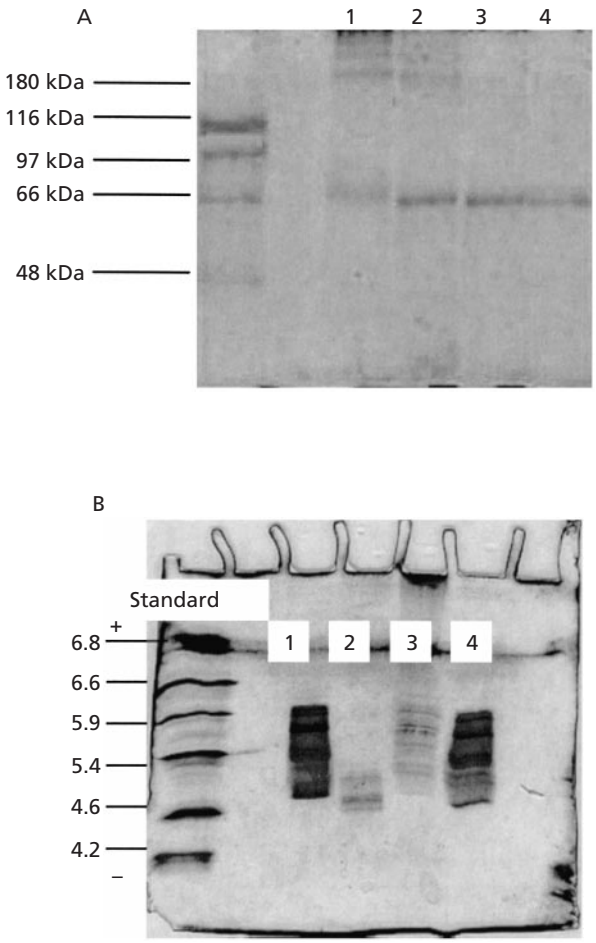

Figure 1 SDS-PAGE and isoelectric focusing of native and modified RSA. A. SDS-PAGE: lane 1, cross-linked RSA; lane 2, RSA-heparin conjugate; lane 3, propyl RSA; lane 4, native RSA. B. Isoelectric focusing: lane 1, native RSA; lane 2, RSA-heparin conjugate; lane 3, cross-linked RSA; lane 4, propyl RSA. pI standard: 6.8, myoglobin (horse heart); 6.6, carbonic anhydrase I (human erythrocyte); 5.9, carbonic anhydrase II (bovine erythrocyte); 5.4, carbonic anhydrase II (bovine erythrocyte); 4.6, trypsin inhibitor (soybean); and 4.2, glucose oxidase (*Aspergillus niger*).

In-vitro release study

Microsphere samples (50 mg) were suspended in 2 mL phosphate-buffered saline (PBS) 0.01 M containing 0.15 M NaCl and 0.01% (w/v) of sodium azide (NaN_3) in three vials prepared for each time interval for one month. These vials were gently agitated at 37°C and 100 rev min^{-1}. The supernatant containing released protein was filtered and the protein content was measured using the BCA method.

The released protein profiles were normalized in percent compared with the initial amount of encapsulated protein in 50 mg microspheres. The release profiles were analysed by non-linear regression using Minim 3.0 software (Purves 1993) and the simple exponential equation described below:

$$C = C_0(1 - e^{-kt}) \quad (1)$$

where C is the released protein concentration, C_0 is the maximum concentration of released protein or initially loaded amount of protein, k is the release constant (day^{-1}), and t is the time in days.

Each parameter was compared for each microsphere preparation and differences were calculated using Student's *t*-test. $P < 0.005$ was considered significant.

Gel permeability chromatography (GPC)

Degraded microsphere samples (3.2 mg) were taken at each time point from the release studies above. Samples were dissolved in distilled tetrahydrofurane (4 mL) and filtered (0.45-μm filters) to eliminate precipitates and insoluble protein. The injection volume was 150 μL and the flow rate was 1 mL min^{-1}. The continual column system in a Waters 150-C ALC/GPC was set for different molecular weights: Jordi DVB for 10^5/100 Å, Ultrastyragel for 10^4/500 Å and the temperature was set at 37°C. A UV detector at 254 nm and a refractometer were used. Data acquisition was conducted using Millenium software provided by Waters. Samples were analysed in triplicate. The morphology of the degraded microspheres was observed using scanning electron microscopy.

Results

Characterization of modified proteins

The BCA method, which is independent of protein conformation and hydrophobicity, was used to measure native and modified protein content. This method depends on the number of peptide bonds, which does not change following the protein modification procedures reported here. The extent of protein modification was measured by counting the amino groups at 25°C before and after modification. The amino group content of the modified proteins was significantly decreased when compared with native RSA. The amino group content of native RSA is 48 mol mol^{-1} of RSA including the *N*-terminal (Jagodzinski et al 1981). Approximately 5% of the amino groups remained for RSA-heparin conjugate (2.4 ± 0.3 mol of amino group (mol RSA)$^{-1}$) and cross-

linked RSA (2.5 ± 0.3 mol of amino group (mol RSA)$^{-1}$). Reductive alkylation was slightly less complete compared with the other reactions as approximately 10% (4.8 ± 0.5 mol of amino group (mol RSA)$^{-1}$) of the amino groups remained following this reaction. Heparin conjugation was approximately 60 ± 5% of the amount of heparin added as determined using the metachromic assay.

SDS-PAGE and isoelectric focusing of native and modified RSA

Native RSA has a molecular weight of 66 kDa (Jagodzinski et al 1981). After SDS-PAGE every preparation showed a band corresponding to 66 kDa (Figure 1A), indicating that unreacted native RSA may have been present. In the case of propylated RSA, this band probably included propylated RSA, since this reaction resulted in only a slight increase in molecular weight (1.92 kDa) which was not detectable using SDS-PAGE (Figure 1A). The RSA-heparin conjugate had one additional band corresponding to 180 kDa, which may have been cross-linked RSA with or without attached heparin. The RSA samples cross-linked with glutaraldehyde contained three additional bands corresponding to 180, 186, and 203 kDa as determined by calibration with standard protein markers (Figure 1A).

Although the standard isoelectric point (pI) markers were correctly migrated (Figure 1B), the pI of native RSA was not stable during focusing as the band was widely distributed from pI 5.9 to 4.6. The clearest band occurred at pI 5.87, which was close to the literature value for RSA (Pardridge et al 1990). This band at pI 5.87 was observed in native RSA, cross-linked RSA and propylated RSA. However, there were no common bands between the RSA-heparin conjugate and the other proteins (Figure 1B). The attachment of heparin to RSA had the effect of decreasing the total pI of the RSA-heparin conjugate.

Size exclusion chromatography

There was no significant difference in size between the native RSA (eluent volume: 8.22 ± 0.10 mL) and the propylated RSA (8.11 ± 0.13 mL). The RSA-heparin conjugate and cross-linked RSA had significantly different eluent volumes of 6.18 ± 0.10 and 6.26 ± 0.10 mL, respectively, compared with native RSA. However, these peak shoulders, 25% and 40%, respectively, were at eluent volumes of 7.73 ± 0.15 and 7.65 ± 0.13 mL, respectively. This size corresponded to neither native RSA nor the main product of the conjugation or cross-linking reactions. Chemical modifications of RSA by heparin conjugation and by RSA cross-linking are heterogeneous reactions. This can be observed in the extra bands corresponding to greater than 180 kDa in the SDS-PAGE experiment.

Hydrophobicity determination

There was a significant difference in the retention times between native RSA and the modified RSAs. Native RSA was the most hydrophilic (26.7 ± 0.4 min), the cross-linked RSA and propylated RSA were the most hydrophobic (34.3 ± 0.7 min and 35.6 ± 0.7 min, respectively) and the RSA-heparin conjugate had an intermediate retention time (31.3 ± 0.6 min). The difference in the retention times between cross-linked RSA and propylated RSA was not significant. These results imply that chemical modification increased the effective hydrophobicity of RSA compared with native RSA, probably due to a decrease in the number of hydrophilic groups (-NH_2 and -COOH) through reactions with carbodiimide and glutaraldehyde.

Characterization of microspheres

The mean particle size of the microspheres was 15 ± 9 μm with a Gaussian distribution. Approximately 10000 particles were counted for each preparation. The encapsulation efficiency of RSA into microspheres was dependent on protein modification; native protein had the lowest encapsulation efficiency (4%), whereas the encapsulation efficiencies for the RSA-heparin conjugate (70 ± 10%), the cross-linked RSA (75 ± 10%) and the propylated RSA (75 ± 10%) were significantly improved. This may be a consequence of the increased hydrophobicities and molecular weights of the modified RSAs determined by HPLC and SDS-PAGE, respectively.

In-vitro release study

There was a relatively small initial burst release of protein from the microspheres followed by a period of slow release and then a period of rapid release. The initial burst phase was probably due to release from the surface of the microspheres. The rapid release phase began at approximately the tenth day of the study and was continuous through to the end of the study. For the purpose of modelling the release profiles, the data were divided into two phases; days 0–9 and days 10–30

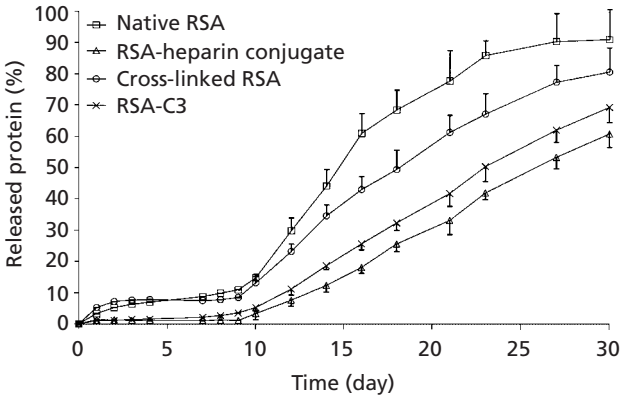

Figure 2 Normalized release profile of each modified protein from PLGA microspheres (37°C, PBS pH 7.4, stirring at 100 rev min^{-1}).

(Figure 2). The release profiles appeared to be first order for both phases and therefore the profiles were modelled using the same equation. The model using the proposed equation matched the data with relatively low variation (Table 1).

The RSA-heparin conjugate microspheres released the lowest quantity (2%), while the native RSA microspheres released the highest quantity (11%) during the burst release phase. The second phase (days 10–30) of the release study resulted in significantly different release rates for the different modified proteins. Native RSA, which is more hydrophilic than the others, had the most rapid release rate ($k = 11.63 \pm 1.06$ day^{-1}). The RSA conjugated with heparin had the slowest release rate ($k = 0.55 \pm 0.09$ day^{-1}). The cross-linked and propylated RSA had intermediate release rates (Table 1).

An exponential equation was used to model release from the microspheres. Each parameter (k) was compared, all values had minimum significant differences of $P < 0.005$ with the exception of the native and cross-linked RSA between the twenty-seventh and thirtieth day. This comparison revealed that all protein modifications influenced the release rate.

The second phase release profiles of the RSA-heparin conjugate and the propylated RSA from PLGA microspheres appeared to follow zero-order release, therefore these release profiles were modelled using linear regression to enable the results to be compared with results obtained by non-linear regression (Table 2). It appeared that the release profiles for the RSA-heparin conjugate and the propylated RSA were closer to zero than first order.

Degradation of PLGA microspheres

All the PLGA microsphere samples investigated conserved high molecular weight (50–53 kDa) until the ninth day of the release study (Figure 3). The gradual decrease in the molecular weight observed suggested that degradation was initiated from the ends of the polymer chains. Differences between the various modified RSAs were not significant up to this period of degradation.

The degradation rates accelerated from the ninth day of the release study. The degradation rates of the native and cross-linked RSA microspheres were significantly more rapid than those of the RSA-heparin conjugate and propylated RSA microspheres. Differences in the microsphere degradation rates (native RSA and cross-linked RSA microspheres vs RSA-heparin and propylated RSA microspheres) were even more evident from the sixteenth day of the release study. These two groups had a significant difference of $P < 0.001$ at the sixteenth day and of $P < 0.005$ at the twenty-first day of the release studies, respectively. The accelerated degradation at the ninth day of the release study was verified by significant percolation on the PLGA microsphere surfaces using scanning electron microscopy. These phenomena were not observed on the first day of the release study.

Table 1 Release of modified proteins from PLGA microspheres.

Parameters	Native RSA	RSA-heparin conjugate	Cross-linked RSA	Propylated RSA
Percent burst release from surface up to 9 days	11 ± 0.72	2 ± 0.03	9 ± 0.15	5 ± 2.34
Percent total amount released at 30 days	91.4 ± 9.7	61.1 ± 4.3	81.0 ± 7.8	69.6 ± 4.9
k (day^{-1}) $\times 10^{-2}$	11.63 ± 1.06	0.55 ± 0.09	6.02 ± 0.40	1.22 ± 0.08

Release conditions: 37°C in PBS at pH 7.4.

Table 2 Comparison between linear and non-linear regression for the slow release phase between days 10 and 30.

Per day	RSA-heparin conjugate	Propylated RSA
k (by non-linear regression) $\times 10^{-2}$	0.55 ± 0.09 (16%)*	1.22 ± 0.08 (7%)
k (by linear regression)	3.00 ± 0.07 (2%)	3.30 ± 0.06 (2%)

* The numbers in parentheses are the percent of error.

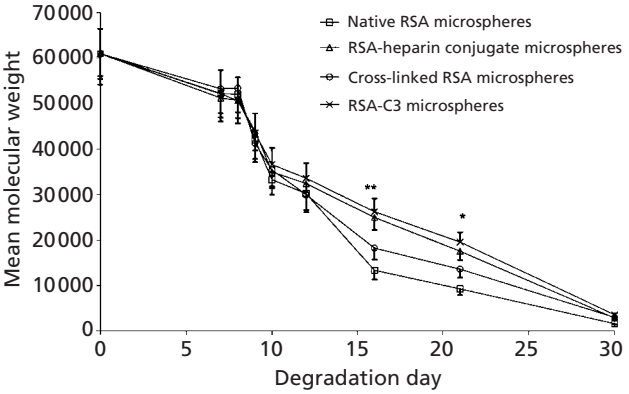

Figure 3 Degradation of microspheres measured by mean molecular weight using GPC at 37°C and tetrahydrofurane as the mobile phase. The error bars present the variation in the mean molecular weight for three injections. $^{**}P < 0.001$; $^{*}P < 0.005$. RSA-C3 MS, propylated RSA microspheres.

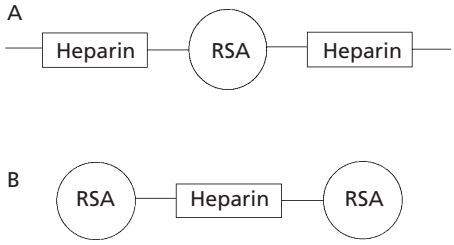

Figure 4 Illustration of the heterogeneous nature of the conjugation between RSA and heparin. A. Conjugation of one RSA with one or more heparin. B. Conjugation of one heparin molecule with more than one RSA.

Discussion

The RSA/heparin conjugation was heterogeneous since following activation of the heparin carboxyl groups several reactions may occur such as conjugation of one RSA with one or more heparin, and conjugation of one heparin with more than one RSA (Figure 4). The RSA/glutaraldehyde reaction could be heterogeneous and result in intra- and intermolecular cross-linking of RSA. The number of bands observed in the SDS-PAGE, and the shoulders observed on the peaks obtained using size exclusion chromatography confirmed the heterogeneous nature of these reactions. The reductive propylation reaction was relatively homogeneous as only one band was observed in SDS-PAGE and one peak in size exclusion chromatography.

The RSA-heparin conjugate and the cross-linked RSA had not only increased molecular weight, but also decreased hydrophilicity as a result of a reduction in the number of hydrophilic groups. The propylated RSA had increased hydrophobicity also. Consequently, these modified RSAs may have decreased partitioning into the aqueous phase during microsphere preparation, which may explain the high encapsulation efficiencies of modified RSA compared with native RSA. Since there was no difference in apparent molecular weight between native and propylated protein, the hydrophobicity appeared to have a dominant influence compared with molecular weight on the encapsulation efficiency. The encapsulation efficiency may also be improved as a result of interaction between the PLGA polymer and the modified proteins. For example, the propyl chain may interact with PLGA.

The slow release profiles of the modified proteins from PLGA microspheres correlated with the PLGA microsphere degradation rates as determined by GPC. Those microspheres containing the RSA-heparin conjugate and the propylated RSA had the slowest degradation rates. The RSA-heparin conjugate may have a buffering effect preventing significant pH reduction within the microspheres and therefore reducing acid catalysed PLGA hydrolysis and consequently reducing release rates. The propylated RSA was the most hydrophobic of the modified proteins, according to the HPLC data, and consequently water uptake into these microspheres was likely to be slower, which would reduce the PLGA degradation rate. This is supported by the increased association constant and decreased dissociation constant of alkylated protein from the lipid bilayer

coated optical waveguides (Michielin et al 1999). The release profile from the cross-linked RSA microspheres was similar to that of native RSA. A possible explanation for this is that the reduction in the buffer capacity of the cross-linked RSA, as a result of a reduction in the number of carboxyl and amino groups, may have the effect of offsetting the reduction in degradation rate expected as a consequence of the increased hydrophobicity of this molecule. The polydispersities of all the degraded PLGA microsphere samples were large compared with PLGA microspheres on the first day of the release studies. This may be due to auto-acceleration of PLGA degradation inside the microspheres by concentrated acid catalysed hydrolysis and conservation of PLGA molecular weight at the surface of the microspheres as a consequence of dilution of acid in the aqueous media.

In conclusion, it appears that chemical modification of RSA can result in a significant increase of encapsulation efficiency and in a reduction in release rates from PLGA microspheres. Modified RSA may be useful as a carrier protein to aid in the formulation of potent therapeutic proteins into PLGA microspheres.

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