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The encapsulation of ribozymes in biodegradable polymeric matrices

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Abstract

Ribozymes are catalytic RNA that bind and cleave specific regions of target RNA. Therefore, protein synthesis by the target RNA may be specifically inhibited by ribozymes. However, ribozymes are rapidly cleared from plasma so effective treatment of proliferative diseases may rely on the repeated administration of these agents to maintain therapeutic ribozyme concentrations. Therefore, the objective of this study was to encapsulate ribozymes in injectable polymeric paste and microsphere formulations to allow for the controlled release of these agents over extended periods of time. Ribozymes were effectively encapsulated in poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic) (PLGA) microspheres in various size ranges using a modified water-in-oil-in-water emulsion system and in poly(ε-caprolactone) (PCL) pastes by physical blending. These formulations released non-degraded ribozymes, in vitro, in a controlled manner. PLLA microspheres released the ribozymes rapidly whereas PLGA released drugs more slowly. The release rate of ribozymes from PCL pastes could be effectively controlled by altering the loading concentration of ribozymes in the paste. These polymeric injectable formulations of ribozymes may allow for the extended treatment of localized disease sites, such as cancer and arthritis, without the need for repeated dosing.

Keywords: Ribozymes; Polymeric drug delivery; Controlled release; Poly(lactic acid); Polycaprolactone; Microspheres; Polymeric pastes

1. Introduction

The concept of targeting a disease process by modulating the expression of specific genes has been known for many decades. Whereas more conventional drugs generally target the function of proteins, ribozymes and antisense oligonucle-

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otides (ASO's) target the RNA (or DNA) sequence that encodes proteins. These compounds function by binding to a complementary sequence on the RNA and preventing normal RNA function by blockade of nucleotide sites or activation of RNase H (for ASO's) or enzymatic cleavage of RNA (ribozymes). These site-specific agents may avoid the unwanted toxicities often associated with non-specific conventional small molecule drugs.

Ribozymes have demonstrated in vitro and in vivo activity in a variety of models. In vitro, ribozymes have been shown to effectively inhibit cancer cell growth (Shore et al., 1993; Snyder et al., 1993; Dorai et al., 1997; Parry et al., 1999) and to reverse multi drug resistance in cancer cells (Holm et al., 1994; Kiehntopf et al., 1994; Kobayashi et al., 1994). Local administration of anti-stromelysin ribozymes has been reported to inhibit arthritic processes in rabbit joints following intra-articular injection (Flory et al., 1996) and systemic administration of ribozymes targeting vascular endothelial growth factor receptors reduce solid tumor growth and metastasis in mice (Pavco et al., 2000).

However, ribozymes are rapidly cleared from plasma so effective treatment of chronic diseases may rely on the repeated administration of these agents to maintain therapeutic concentrations (Sandberg et al., 1999, 2000). Chemical modifications have greatly improved the stability of ribozymes (Beigelman et al., 1995), but have not completely eliminated susceptibility to in vivo degradation (Sandberg et al., 1999). An alternative delivery approach for local ribozyme administration may be to encapsulate the agents in polymeric compositions that can protect the nucleotide chain from nuclease attack. Site-directed, polymeric drug delivery might be appropriate for treatment of localized diseases such as tumors or inflammation by providing a continuous slow release of compound. In this laboratory, we have previously described polymeric drug delivery systems for use with anticancer drugs (Winternitz et al., 1996; Zhang et al., 1996; Dordunoo et al., 1997; Jackson et al., 1997; Liggins et al., 2000). Polymeric pastes based on poly(ε-caprolactone) (PCL) and microspheres prepared using poly(lactic acid) that release anti-cancer drugs in a controlled manner have been shown to effectively inhibit tumor growth in vivo (Winternitz et al., 1996; Jackson et al., 1997; Liggins et al., 2000).

The encapsulation and release of ASO's from biodegradable, biocompatible microspheres has been described by many workers (Lewis et al., 1995; Akhtar and Lewis, 1997; Cleek et al., 1997; Lewis et al., 1998; Putney et al., 1999). Indeed some of these formulations were shown to be effective in animal disease models (Putney et al., 1999). In Hudson et al. (1996), encapsulation and release of ribozymes from poly(L-lactic acid) (PLLA) implants were demonstrated indicating the potential use of biocompatible, biodegradable polymers for use with ribozymes. However, to our knowledge, there are no reports of the effective encapsulation and controlled release of ribozymes from injectable polymeric delivery systems such as microspheres or pastes. Although ribozymes and ASO's both bind their targeted RNA, there are important structural and mechanistic differences between these two types of compounds. Ribozymes currently in development are generally longer than most ASO's and have a different secondary structure. In addition, the chemical modifications used to stabilize both classes of compounds are different. In first-generation ASO's, all nucleotides contain phosphorothioate linkages to protect from cellular nuclease degradation. Nuclease stabilized-ribozymes like those used in this study contain 2′-*O*-methyl modified nucleotides at all but five nucleotides within the catalytic core. Additional modifications to increase stability include a 3′-3′ deoxyabasic nucleotide, a 2′-deoxy-2′-C-allyl-uridine, and typically four phosphorothioate linkages at the 5′ end of the molecule (Beigelman et al., 1995; Sandberg et al., 1999). The structural differences in these types of compounds could affect the ability to encapsulate and release ribozymes from polymeric formulations.

The objective of this study was to evaluate the encapsulation and release properties of ribozymes in injectable polymeric paste and microsphere formulations, as potential site-directed, controlled release drug delivery systems.

2. Materials and methods

2.1. Microsphere preparation

Microspheres were prepared using the solvent evaporation method from a water-in-oil-in-water emulsion system. Known weights of ribozymes (proprietary sequence anti-stromelysin ribozyme from Ribozyme Pharmaceutical Incorporated, Boulder, CO) were dissolved in 200 μl of distilled water. This solution was added to a 5 ml solution of a PLLA, (2K g/mol molecular weight from Polysciences, Warrington, PA) or poly(lactic-co-glycolic acid) (85:15) (PLGA, molecular weight given as inherent viscosity (i.v.) = 0.61 Birmingham Polymers, Birmingham, AL) in dichloromethane (DCM). The concentration of PLGA in DCM was 5% (w/v). The concentration of PLLA in DCM was variable. Eighty microlitres of Span 80 surfactant (Fisher Scientific) was added and this solution was homogenized for 10 s (Polytron homogenizer set on mark 1). The water-in-oil emulsion was immediately pipetted into 100 ml of a poly(vinyl alcohol) solution (PVA, 13–21K molecular weight, Aldrich Chemical Company, Oakville, Ontario, Canada) containing polysorbate 40 (Tween 40) surfactant (Fisher Scientific) at a concentration of 0.2% (v/v) with an overhead propeller stirring to form a water-in-oil-in-water emulsion. (The PVA concentration and the stir speed of the emulsion were varied in these experiments). The resulting emulsion was stirred for 2 h at room temperature and the resulting microspheres were washed three times in water with centrifugation at $400 \times g$ for 10 min between washes. Microspheres were then placed into preweighed vials and dried for 3 days under vacuum.

2.2. Microsphere particle size analysis

Microsphere size distributions were obtained by suspending microspheres at approximately 20 mg in 20 ml of water containing approximately 0.02% Tween 40. This suspension was placed in the glass cell of a laser diffraction particle size analyser (Coulter LS 100, Beckman Coulter Corporation, Hialeah, FL) and analyzed over 1 min using Fraunhofer algorimithic computing.

2.3. Encapsulation efficiency determinations

To determine the amount of ribozyme encapsulated in the microspheres, a known weight of microspheres was weighed into a 10 ml glass tube and 1 ml of dichloromethane was added to dissolve the microspheres. After 5 min, 2 ml of distilled water was added with vortexing to partition the free ribozymes into the aqueous phase. The ribozyme concentration in the aqueous phase was measured using UV/VIS absorbance at 260 nm. This assay had a linear calibration curve in the 1–100 μg/ml concentration range with a correlation coefficient of 0.998. The encapsulation efficiency was then determined as the percentage of the theoretical maximum loading based on the ratio of ribozyme to polymer used in the original emulsion method.

2.4. Preparation of polymeric pastes

Polymeric pastes were made from PCL (molecular weight 10 000 from Birmingham polymers, Birmingham, AL) with or without 20% (w/w) methoxypolyethylene glycol (MePEG—molecular weight 350 g/mol, from Union Carbide, Danbury, CT) as previously described (Winternitz et al., 1996). Ribozyme was incorporated into the pastes by melting a small known weight of paste on a microscope slide at 56 °C followed by spatula mixing of a known weight of the ribozyme into the paste.

2.5. Ribozyme release studies

To measure the release rate of ribozymes from microspheres or pastes, known weights of these formulations were placed in 2 ml plastic Eppendorf tubes. Two millilitres of phosphate buffered saline (10 mM) (pH 7.4) was added and the tubes were capped and tumbled end-over-end at 8 rpm. After a given time period, the tubes were centrifuged at 200 rpm and the supernatant was analyzed for ribozyme using UV/VIS absorbance at 260 nm as described above. In the early stages of these release experiments, the supernatant was discarded and replaced. As the ribozyme release rate decreased, the supernatant was placed back

in the tube for two or three time periods to allow for more accurate determination of the ribozyme concentration above the detection limit of the assay.

2.6. Stability of ribozymes encapsulated in polymers

Microsphere or paste samples remaining at the end of the release studies were placed in 1 ml of water for 2 days. The concentration of released ribozyme was analyzed by UV/VIS. Dry ribozyme was dissolved in water at the same concentration. These solutions were then analyzed by denaturing 20% polyacrylamide gel electrophoresis. The stability of the ribozyme that had been encapsulated in the polymeric matrices for 52 days and then released into the water was then qualitatively assessed by comparing the intensities to baseline and the released RNA bands. The gels were evaluated to determine the presence of other bands that might represent degraded fragments of RNA.

3. Results

3.1. PLGA microsphere preparation

At a given stir speed, the diameter of PLGA microspheres obtained using the water-in-oil-in-water emulsion method was inversely related to the concentration of PVA as shown in Fig. 1. The addition of a small amount of polysorbate 40 (0.2%) to the emulsion generally increased the mean diameter of microspheres compared to those produced in PVA alone. The effect of PVA concentration, in the presence or absence of Tween 40, on the mean diameters of microspheres is summarized in Table 1. The addition of Tween 40 improved the yield of microspheres obtained at each PVA concentration so that yields of more than 80% were always obtained when Tween 40 was added to the emulsion as shown in Table 1.

At a given PVA concentration, containing 0.2% Tween 40, the diameter of the PLGA microspheres decreased as the stir speed of the emulsion increased. This allowed for good control of microsphere diameter in the 30–100 μm size range

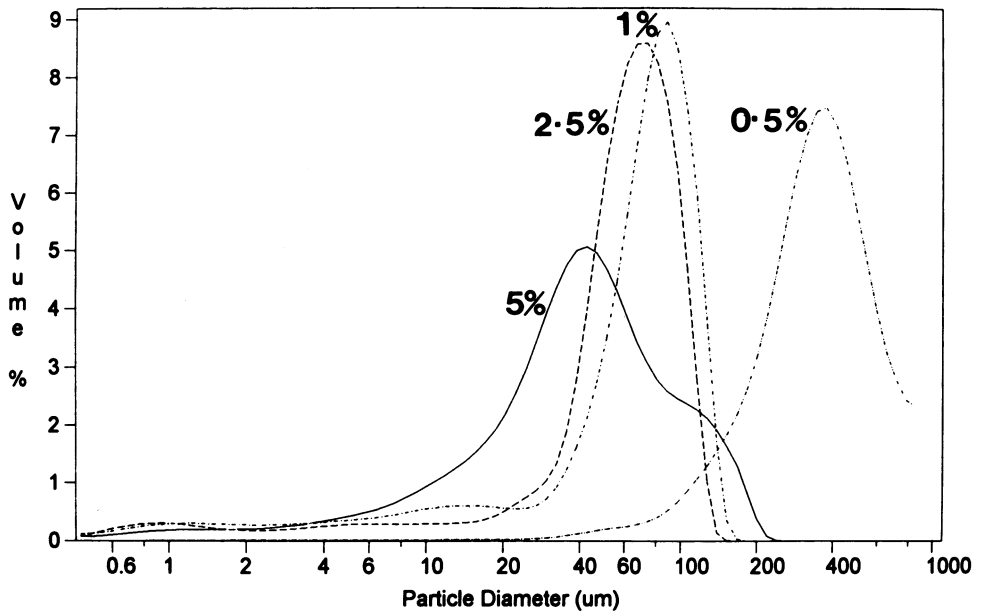


Fig. 1. The effect of varying PVA concentrations (0.5–5%) on the size distribution of PLGA (85:15, i.v. = 0.61 dl/g) microspheres. Microspheres were manufactured using a double emulsion method with a stir rate of 600 rpm. Polysorbate 40 at 0.2% was added to all PVA solutions.

Table 1
The effect of PVA concentration on the mean diameter and yields of PLGA (85:15, i.v. = 0.61 dl/g) microspheres manufactured at a stir rate of 600 rpm in the presence or absence of 0.2% polysorbate 40 in the PVA solution

PVA concentration (% w/v)	Mean diameter (μm)	Yield (%)	PVA concentration (%w/v) with 0.2% Tween 40	Mean diameter (μm)	Yield (%)
5.0	51	47	5.0	57	84
2.5	62	76	2.5	107	99
1.0	69	63	1.0	213	100
0.5	373	44	0.5	278	85

using stir speeds in the 650–1500 rpm range with a PVA concentration of 2.5%.

Microspheres were prepared in three size ranges, small (mean diameter 20 μm), medium (mean diameter 70 μm) and large (mean diameter 130 μm) using the following conditions of PVA concentration and stir speed (plus 0.2% polysorbate 40): small: 3.5% PVA, stir speed 1400 rpm, medium: 2.5% PVA, stir speed 700 rpm, large: 0.5% PVA, and a stir speed 500 rpm.

3.2. PLLA microsphere preparation

Preliminary studies with the low molecular weight PLLA (2K), using similar methods to those described for the PLGA polymer, produced microspheres in low yields and which were generally larger, with broad size distributions and low ribozyme encapsulation efficiencies. The 5% PLLA solution in DCM had a low viscosity and emulsified poorly in the PVA solution and therefore increased concentrations of PLLA in DCM were used in subsequent trials. Using a 25% PLLA solution, microspheres of more uniform sizes were produced in reasonable yields and increasing both stir speeds and PVA concentrations allowed for the manufacture of smaller size range microspheres.

3.3. Encapsulation of ribozymes

To determine the efficiency of encapsulation of ribozymes in PLGA microspheres the maximum theoretical loading was varied from 0.05 to 1% (initial weight of ribozyme mixed with PLGA expressed as % of PLGA) and microspheres were made in small, medium and large sizes with each loading. The encapsulation efficiencies of ribozyme loaded microspheres are shown in Table 2. Small size microspheres were produced with high yields (above 70%) and high encapsulation efficiencies for ribozymes (between 49 and 88%). Although the yields of large size microspheres was high (above 70%), the encapsulation efficiency of ribozymes was low (between 21 and 29%). Medium size microspheres were produced with lower yields and variable encapsulation efficiencies (between 19 and 74%).

The efficiency of encapsulation of ribozymes loaded in the PLLA microspheres was lower than for ribozyme-loaded PLGA microspheres. The encapsulation efficiency was dependent on the concentration of PLLA in the DCM solution. 'Medium' size (70 μm mean diameter) microspheres were prepared using a 2.5% PVA solution containing 0.2% polysorbate 40 and a stir speed of 600 rpm. At PLLA concentrations in DCM of 5, 10, 15 and 20%, the encapsulation efficiencies of ribozymes in PLLA microspheres were 4, 13, 35 and 50% respectively.

3.4. The release of ribozymes from microspheres

PLGA microspheres were prepared in three size ranges (small, medium and large) with ribozyme loadings between 0.05 and 2% (w/w). All microspheres released less than 20% of the total encapsulated ribozyme within 50 days with only small differences in the percent released within or between the size groups. Generally, all microsphere

samples released the ribozymes with a burst phase over about 1–3 days followed by a slower, sustained release over 50 days. Residual amounts of ribozymes in the microsphere samples showed that more than 70% of the encapsulated ribozymes remained in the microspheres at the end of the study in good agreement with the release studies reporting between 5 and 20% release.

Fig. 2 shows release profiles for small sized microspheres expressed as amount of ribozyme released and percent of loaded ribozyme released. Release profiles for medium and large sized microspheres are shown in Fig. 3A and B.

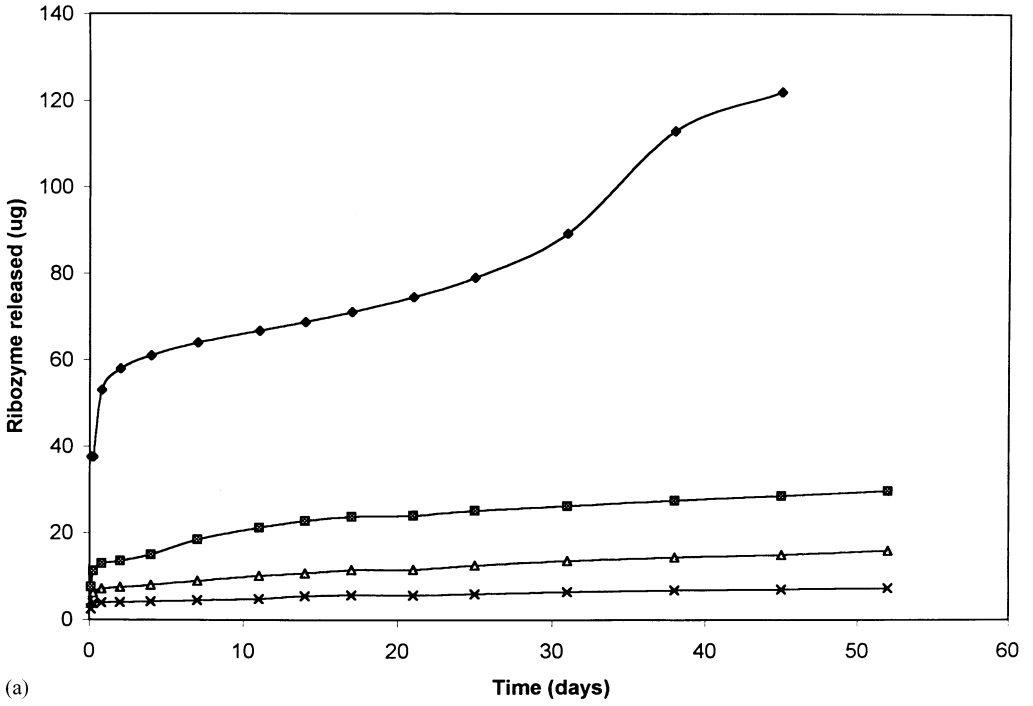
Fig. 4 shows release profiles for medium size (70 μm) and large size (130 μm) ribozyme loaded PLLA microspheres prepared using high concentration of PLLA in DCM to produce high ribozyme loading efficiencies. The PLLA microspheres released ribozymes more rapidly than the PLGA microspheres and between 50 and 100 μg (35–55%) of the encapsulated ribozyme was released within 11 days. Both medium and large microspheres gave a burst phase of ribozyme release of between 20 and 45% of loaded amount of ribozyme over 1 day followed by a much slower release over the next few days. It was not possible to continue the release experiment past 11 days as these low molecular weight PLLA microspheres began to disintegrate and it was not possible to pellet the small fragments of microspheres in the supernatant by centrifugation.

3.5. The release of ribozymes from PCL pastes

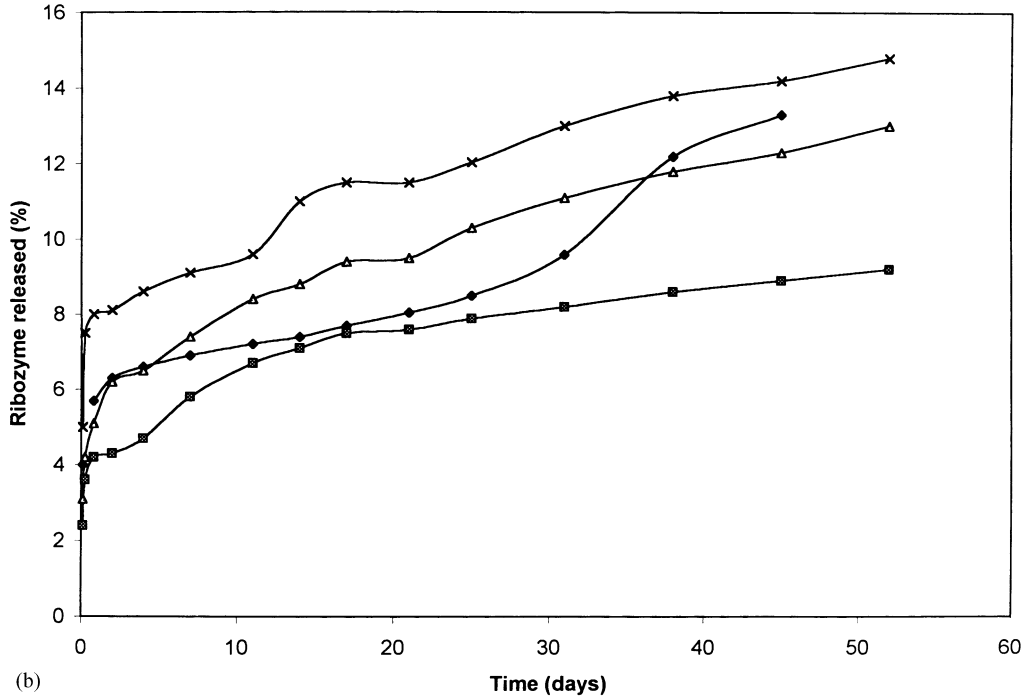
The dry ribozyme powder was readily and homogeneously incorporated into the PCL pastes in the presence or absence of 20% MePEG. Release profiles for PCL pastes loaded with 0.1, 0.5 or 1% ribozyme (w/w) are shown in Fig. 5. All release profiles were characterized by a rapid phase of release between about 1 and 5 days, followed by a slower sustained release over the next 50 days. The 1% ribozyme loaded PCL paste released a greater percentage of encapsulated ribozyme than either the 0.1 or 0.5% loaded pastes (Fig. 5). By day 52 almost 80% of the ribozymes were released from the 1% loaded paste as compared to approximately 50% from the 0.1 to 0.5% loaded pastes. The effect of the addition of 20% MePEG 350 to a 1% ribozyme loaded paste was to markedly increase the release rate of the ribozymes from the polymeric paste as shown in Fig. 5. By day 7 almost all (over 90%) of the encapsulated ribozymes had been released from the PCL–MePEG paste. At the end of the release study, the polymer pellets were analyzed for residual ribozyme. The residual values were in approximate agreement with the release curves so that the 1% ribozyme loaded PCL–MePEG and PCL alone paste contained 1.2% ($\pm$ 1%) and 7% ($\pm$ 2%) of the originally encapsulated ribozyme, respectively. The residual values for the 0.5 and 0.1% ribozyme loaded PCL pastes were 71% ($\pm$ 6%) and 45%

Table 2
The efficiency of encapsulation of ribozymes in PLGA (85: 15, i.v. = 0.61 dl/g) microspheres manufactured in three size ranges

Mean size (μm)	Theoretical loading (% w/w)	Actual loading (% w/w)	Encapsulation efficiency (%)
20 'small'	1.0	0.63	63
	0.5	0.24	49
	0.1	0.08	76
	0.05	0.04	88
70 'medium'	1.0	0.20	20
	0.5	0.095	19
	0.1	0.048	48
	0.05	0.036	74
130 'large'	1.0	0.22	22
	0.5	0.10	21
	0.1	0.027	27
	0.05	0.014	29

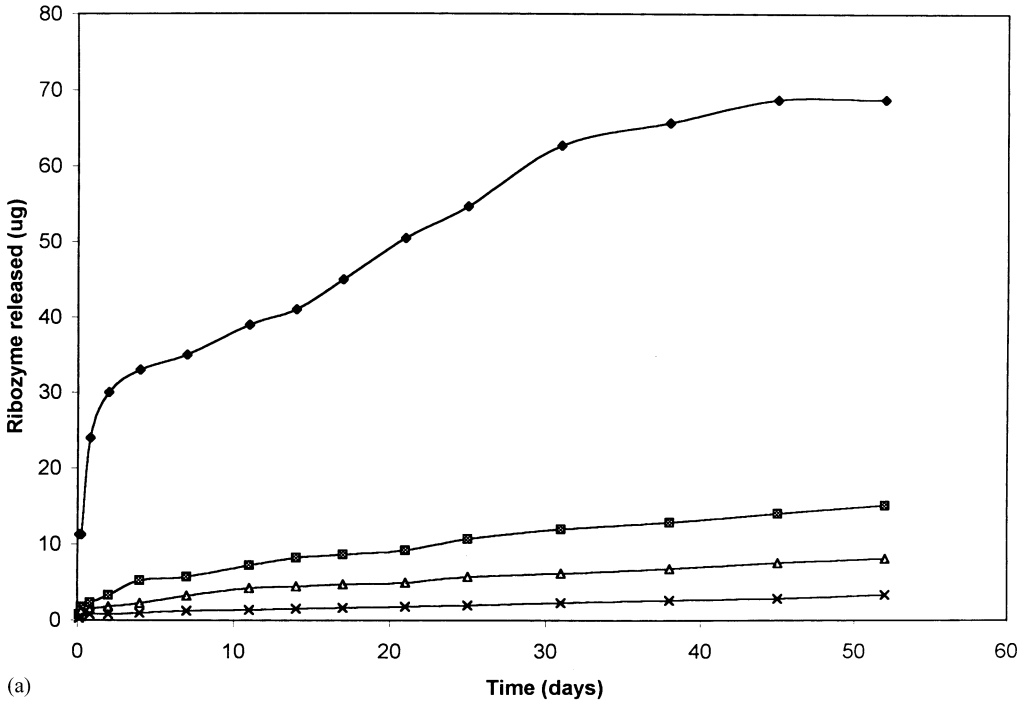


(a)

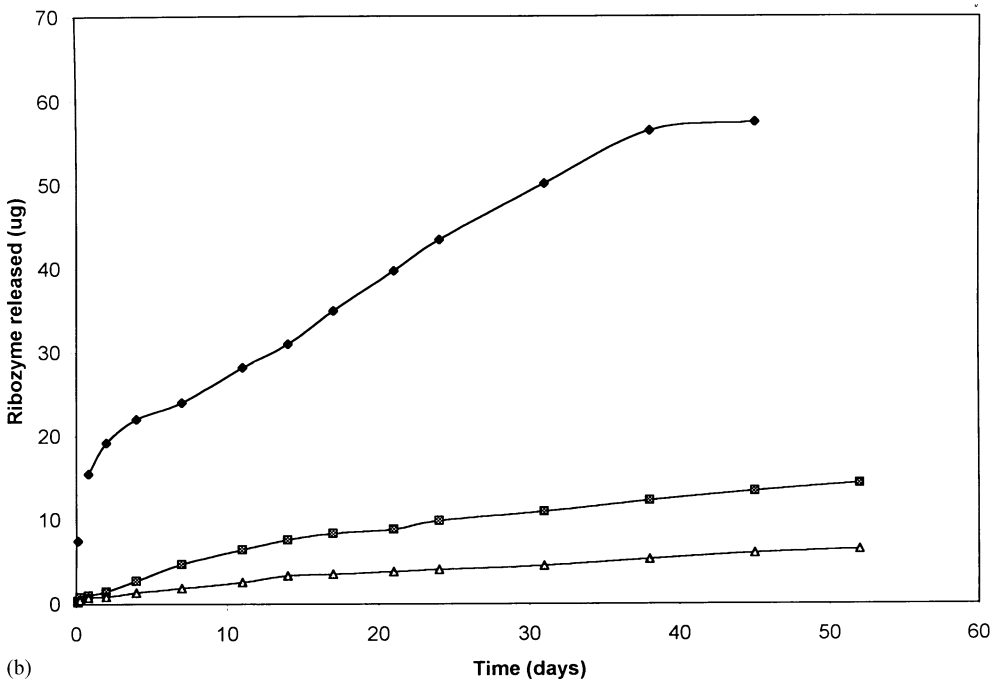


(b)

Fig. 2. The release of ribozymes from PLGA (85:15, i.v. = 0.61 dl/g)microspheres with a mean diameter of 20 μm (small) prepared with ribozyme loadings of: 1.85% (◆); 0.6% (■); 0.26% (△); and 0.1% (X). (A) The cumulative release of ribozymes in microgram as a function of time from 50 mg of microspheres. (B) The cumulative release of ribozymes as a percent of the loaded amount as a function of time from 50 mg of microspheres.



(a)



(b)

Fig. 3. The release of ribozymes from PLGA (85:15, i.v. = 0.6 dl/g) microspheres. (A) Mean diameter of 70 μm (medium) and ribozyme loadings of: 1.55% (◆); 0.2% (■); 0.1% (△); and 0.05% (X). (B) Mean diameter of 130 μm (large) and ribozyme loadings of 1.75% (◆); 0.22% (■); and 0.1% (△).

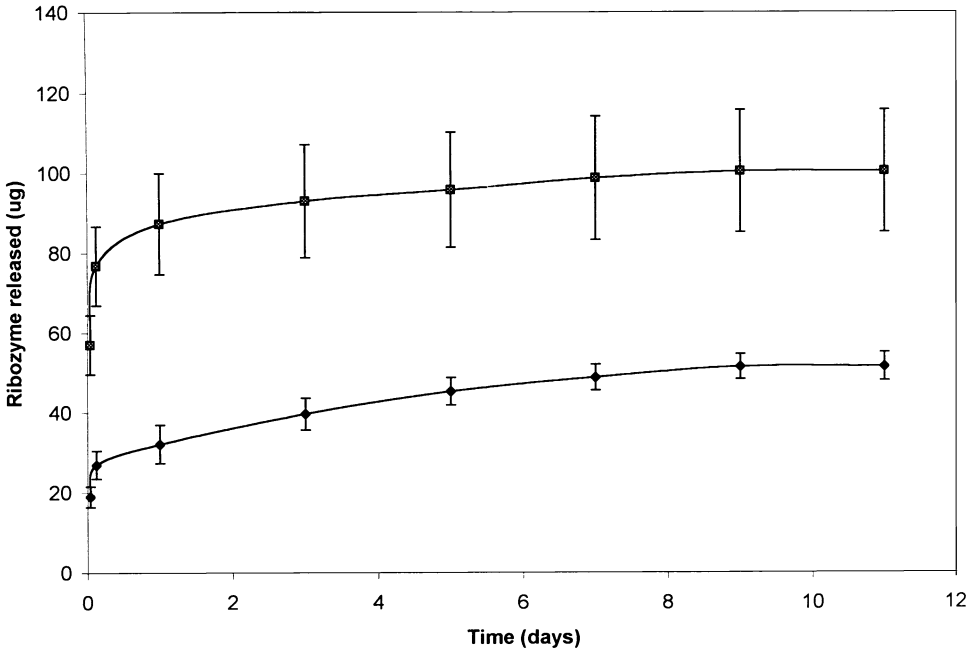


Fig. 4. The release of ribozymes from PLLA (2K g/mol) microspheres. (◆), large diameter (130 μm) with a 0.33% ribozyme loading and (■), medium diameter (70 μm) with a ribozyme loading of 0.45% (w/w).

(±9%). Although these mass balance values are not in close agreement with the release curves in Fig. 5, they do show that a significant amount of ribozyme remained in the pastes after 50 days in the release study.

3.6. Viability of ribozymes released from polymeric formulations

The critical aspect of viability of encapsulated ribozymes is that these agents should be released from the polymers in an intact, non-degraded, form. Therefore the supernatant from release studies was analyzed for intact ribozyme. Close to the end of the release study time course, the ribozyme released from the polymer was applied to a denaturing SDS PAGE gel and analyzed for molecular weight. Equivalent amounts of the original ribozyme (100% full-length) were then run on the same gels. Ribozymes released from medium sized PLGA microspheres at day 50 showed a single strong band at the same molecular weight position and approximate density as the as received ribozyme baseline sample (Fig. 6B). Ribozymes released from PCL paste at day 27 in the release study (both 1 and 5% ribozyme loaded) gave strong single bands on the gel at the same molecular weight and approximate density as the baseline sample (Fig. 6A). However, ribozymes released from the 1% ribozyme loaded paste containing 20% MePEG produced no bands on the SDS PAGE gel, probably due to extensive degradation of the ribozyme.

4. Discussion

Preliminary studies using the oil-in-water emulsion solvent evaporation method did not allow for the effective encapsulation of ribozymes in microspheres (data not shown) Since the water soluble ribozymes were suspended as solid material in the polymer phase, they likely dissolved out into the water phase during the microsphere preparation process. Several groups have described water-in-oil-in-water emulsion solvent evaporation methods that permit the effective encapsulation of water soluble compounds such as proteins and

nucleotide chains (Heya et al., 1991; Akhtar and Lewis, 1997; Cleek et al., 1997; Uchida et al., 1997; Leo et al., 1998). In order to avoid physical degradation of the ribozymes, the water- in-oil emulsion was initially vortexed before pipetting into the water phase (PVA solution). This method did not allow for efficient encapsulation of ribozymes. Therefore, a brief polytron homogenization step was included in the preparation method. A small amount of Span 80 was added to this water-in-oil emulsion to inhibit phase separation in the time between the initial production of this emulsion and pipetting into the PVA-polysorbate 40 solution. By varying the PVA concentration and the stir rate, this water-in-oil-in-water emulsion method permitted the preparation of PLGA microspheres in different size ranges in good yields (Fig. 1 and Tables 1 and 2). The addition of a small amount of polysorbate 40 to the PVA phase was found to increase the yield of microspheres in all size ranges probably by improving the stability of the water-in-oil-in-water emulsion.

Ribozymes were loaded at reasonable, although variable, encapsulation efficiencies in PLGA microspheres using these methods as shown in Table 2. Encapsulation efficiencies in the 20–80% range have been reported by other groups for water soluble compounds such as proteins or ASO's using water-in-oil-in water emulsion methods (Akhtar and Lewis, 1997; Uchida et al., 1997; Leo et al., 1998). Interestingly, the preparation of small sized microspheres allowed for the greatest efficiency of ribozyme encapsulation. This might be due to the more rapid evaporation of DCM from the polymer droplet and the correspondingly short solidification time of these microspheres, thus trapping ribozymes in the polymer core before they can migrate to the surface of the polymer droplet and partition into the aqueous phase.

Similar methods were used to manufacture low molecular weight (2K g/mole) PLLA microspheres. However, the use of a 5% PLLA in DCM solution did not allow for microsphere production in any size range. Other investigators have noted that low viscosity polymer solutions may result in

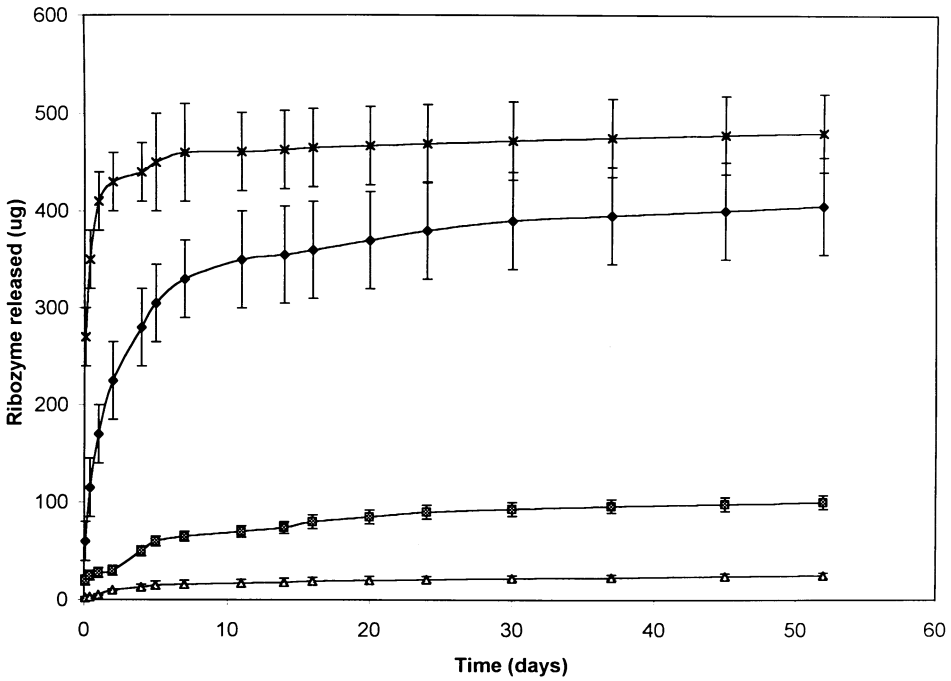


Fig. 5. The release of ribozymes from 50 mg PCL pastes manufactured with ribozyme loadings of: 1% (◆); 0.5% (■); 0.1% (△); and 1% (X) ribozyme in PCL paste containing 20% MePEG 350 (w/w).

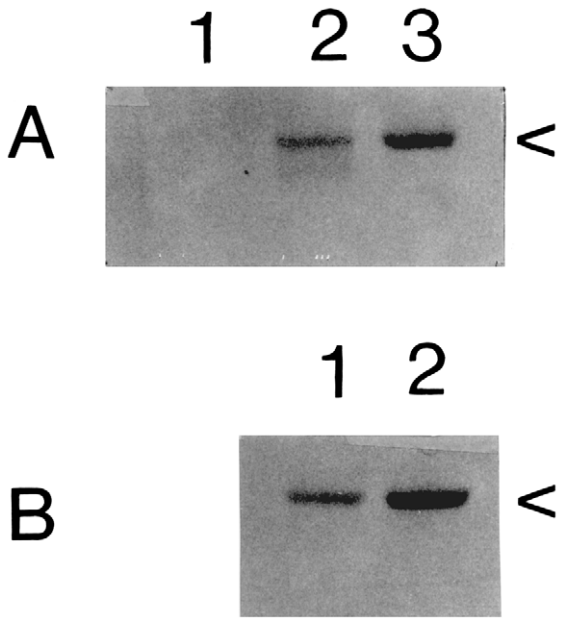


Fig. 6. SDS-PAGE of ribozymes residual remaining in (A) PCL paste after 27 days or (B) PLGA microspheres after 50 days. The upper gel (A) shows ribozymes released from; (lane 1), 1% loaded PCL–MePEG (20%) paste; (lane 2), 1% PCL paste and (lane 3), baseline sample. The lower gel (B) shows residual ribozymes released from; (lane 1), medium sized (70 μm) 0.5% ribozyme loaded PLGA microspheres and (lane 2), baseline sample.

destabilization of the polymer droplet and poor microsphere preparation and encapsulation of proteins (Leo et al., 1998). This effect was suggested to arise from the movement and coalescence of the water droplets within the low viscosity polymer solution. The use of higher concentrations and higher viscosity solutions of PLLA in DCM allowed for the controlled production of microspheres in various size ranges as shown in Table 3. The encapsulation efficiency of ribozymes in PLLA microspheres was also strongly dependent on the viscosity of the polymer solutions so that good ribozyme encapsulation could only be achieved at very high concentrations of PLLA in DCM.

Both PLGA and PLLA microspheres released ribozymes in a controlled manner (Figs. 2–4). The burst phase followed by a slower sustained phase of release is typical for the release of high molecular weight, water soluble compounds from such matrices and has been reported elsewhere (Akhtar and Lewis, 1997; Cleek et al., 1997; Uchida et al., 1997; Leo et al., 1998). Ribozymes were released more slowly from PLGA microspheres than from the low molecular weight PLLA microspheres. This was probably due to the faster degradation of the low molecular weight PLLA microspheres. The 85/15 PLGA higher molecular weight polymer used in this study typically degrades over a period of 3–6 months (Manufacturers data) whereas the 2K PLLA polymer degrades over a few weeks (Lin et al., 1999). In our release study with PLLA microspheres there was clear evidence of degradation since fragments of these microspheres were visible as suspended particles in the release media after 9 days. The faster release rate of proteins from microspheres manufactured from lower molecular weight PLGA polymers has been shown to result from faster degradation of the lower molecular weight polymers (Heya et al., 1991).

The ribozymes released from PLGA microspheres were shown to have the same molecular weight as the starting material. This demonstrated that the preparation processes involving the use of organic solvent (DCM) and rapid shear rates (polytron homogenization) did not compromise the structural integrity of the ribozymes. It has been previously reported that the use of organic solvents such as chloroform or DCM to prepare polymeric formulations containing ribozymes or ASO's did not cause drug degradation (Lewis et al., 1995; Hudson et al., 1996). Furthermore, the structural integrity of hormones was maintained when encapsulated in PLGA and PLLA micro-

Table 3
The effect of PVA concentration and stir rate on the mean particle size of PLLA (2K g/mol) microspheres prepared using a double emulsion system in the presence of 0.2% polysorbate 40

PVA concentration (%)	Stirring rate (rpm)	Mean particle size (μm)
4	1400	42.59
2.25	700	112.8
0.5	500	157.7

spheres prepared by a water-in-oil-in-water emulsion solvent evaporation method using polytron homogenization and DCM solvent (Heya et al., 1991; Uchida et al., 1997).

In summary, the emulsion method used in this study included the following modifications to increase both microsphere yield and ribozyme encapsulation. The water-in-oil emulsion was briefly homogenized using a polytron to reduce the droplet size of water in the oil phase. Span 80 was included in this emulsion to stabilize the small droplet size of the water in the oil. The concentration of the polymer in the organic phase was increased in order to increase the viscosity of the oil phase and hence to reduce the movement of the water droplets in the oil phase. Polysorbate 40 was included in the PVA solution to improve the stability of the oil in water emulsion and reduced aggregation of microspheres during precipitation of the polymer.

The use of injectable, biodegradable polymeric pastes for the controlled release of ribozymes has not previously been described. The particular advantage of such a system over microspheres may be that losses of ribozymes during encapsulation are negligible since the agent is simply blended directly into the paste. Ribozymes encapsulated readily in polycaprolactone pastes without any apparent aggregation of the solid ribozymes in the molten polymer. The amount of ribozyme released depended on the initial loading concentration of ribozymes in the polymer. The addition of the PCL-miscible and water soluble polymer MePEG 350 into the matrix resulted in the accelerated release of the ribozyme from the PCL–MePEG paste. This effect of adding MePEG has been previously reported for the release of a water soluble drug from PCL pastes and is due to the dissolution and partitioning of MePEG into the release buffer, thus allowing water to enter the matrix, and accelerated dissolution of the drug (Jackson et al., 1997). The structure integrity of the ribozymes was unaffected by encapsulation in the PCL paste since released ribozyme was shown to have the same molecular weight as the baseline sample (Fig. 6). However, the addition of MePEG to the PCL paste allowed the degradation of the ribozymes within the PCL matrix so that, although released ribozyme could be detected by UV/VIS, there was no evidence of intact ribozyme on SDS PAGE gels. This effect probably arose from the entry of water into the PCL matrix as the MePEG partitioned out so that hydrolytic degradation of the ribozymes occurred within the PCL matrix. The in vitro cleavage activity of released ribozymes were also assessed at the end of the release study. These qualitative determinations (data not included) showed that the ribozymes released from the microspheres and the polycaprolactone (alone) paste had activity comparable to those of the original (stock) ribozymes. However, the ribozymes released from the paste containing MePEG demonstrated reduced cleavage activity. Due to the very low concentrations of ribozymes released, it was not possible to quantitatively compare the activity of the released ribozymes with equivalent weights of stock ribozymes. However, these activity assessments support the degradation data obtained by SDS-PAGE.

This study demonstrates that ribozymes may be effectively encapsulated in injectable microspheres and paste. These formulations all allow for the controlled release of ribozymes over extended time periods and may be suitable for the site-directed treatment of localized diseases such as cancer and arthritis.

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