A Novel *in Vitro* Delivery System for Assessing the Biological Integrity of Protein upon Release from PLGA Microspheres

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Received November 28, 2001; revised February 28, 2002; accepted April 1, 2002

Purpose. The development of a novel *in vitro* system is required to assess the stability and release kinetics of a protein microsphere formulation used for drug delivery to the brain.

Methods. Microspheres containing lysozyme as model protein were prepared using a (w/o/w) emulsion-solvent evaporation process. Both the active and total (active + inactive) encapsulation efficiencies and release profiles were determined. The biologic activity of lysozyme was measured using bacterial cell lysis; total protein content was measured using a ^{125}I-radiolabel. A novel *in vitro* apparatus was developed to determine kinetics over a sustained time period (>30 days).

Results. The microencapsulation technique allowed an entrapment of active lysozyme at 80 ± 4% and a sustained (>42 days) *in vitro* release. The kinetics study showed that the novel *in vitro* system was able to detect the release of low amounts (ng) of protein. To improve the stability of the protein within microspheres and allow the release of biologically active lysozyme, a basic additive ($Mg(OH)_2$) was successfully encapsulated.

Conclusions. This novel *in vitro* system was appropriate to study protein microsphere release kinetics. In addition, the model is cost-effective and mimes brain physiological conditions more closely than previous models.

KEY WORDS: protein release; lysozyme; microspheres; poly(lactic-co-glycolic acid); stabilization.

INTRODUCTION

While the use of biologically-derived drugs (antigens, peptides, proteins) seems set to increase in the coming years, their application *in vivo* remains limited by issues of stability and delivery. For example, a number of growth factors have been identified as potential drug candidates for the treatment of various neurodegenerative disorders. However, the *in vivo* behavior of growth factors is characterized by inherently short biologic half-lives and a limited ability to cross the blood-brain barrier. In addition, they display a multi-specific activity in the central nervous system and in non–neuronal tissues due to a wide receptor distribution (1). Clearly, there is a need to develop novel, site-specific delivery systems.

Protein microencapsulation within biodegradable polymer matrices is a possible means of overcoming such obstacles. Poly(lactide-co-glycolide) (PLGA) microspheres for instance, are well known to exhibit continuous controlled drug release profiles over a period of weeks or months and may protect the protein from *in vivo* degradation (2). To date however, the success of protein microsphere formulations as drug delivery systems has been limited (3). During microsphere preparation, the formation of interfaces is a common destabilizing factor for proteins (4). Furthermore, PLGA hydrolysis leads to a decrease in pH within the polymer matrix and release medium, which is prejudicial to protein stability (5,6).

Protein loaded microspheres were characterized by their morphology, their size, their encapsulation yield and their *in vitro* release profile. The *in vitro* release study of protein is required to assess the retention of biologic activity and the performance of the microsphere formulation with regard to sustained release. *In vitro* release studies of protein from microspheres can not be realized in classical USP apparatus. *Inter alia*, large volumes are not compatible with the low protein quantities involved. The traditionally used *in vitro* systems are vials containing buffer in which incubated microspheres are shaken (7,8,9). At appropriate intervals, release medium is collected by centrifugation and replaced with fresh buffer. The advantages of these systems are their simplicity and their low cost. Their drawbacks are their adsorption risks, their need for agitation and centrifugation, and their increased risk of protein degradation in the release medium prior to sampling. In such instances, protein stability studies are frequently limited to a maximum of 24 h (10,11). One alternative is to measure the amount of drug remaining in the microspheres. However, this is cumbersome and requires large quantities of sample (12,13). Another strategy, particularly relevant to *in vivo* conditions, is to use a low continuous flow system.

The objective of our group is to encapsulate GDNF, (Glial cell line-Derived Neurotrophic Factor), a potential therapeutic agent for the treatment of neurodegenerative disorders such as Parkinson's disease (14). The first part of this study was to develop a novel *in vitro* model system with a view towards minimizing possible degradation of protein prior to analysis. It was required to monitor the release kinetics of the microsphere formulation over an extended time period (>30 days), under flow conditions approaching those found *in vivo*.

For development of the system a model protein, lysozyme, was selected as the model of choice. The physicochemical properties of lysozyme approach those of GDNF. GDNF is a homodimer with an apparent $\overline{M}w$ for the monomer of 16,000 Da (14); lysozyme is a monomeric globular protein with a $\overline{M}w$ = 14,300 Da. Both proteins are basic at pH 7.4. In addition, lysozyme is a well known characterized protein.

MATERIALS AND METHODS

Materials

Uncapped PLGA (free carboxylic acid group at the terminal end) was obtained from Phusis (PLGA 37.5/25) (Saint

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ABBREVIATIONS: GDNF, Glial cell line-derived neurotrophic factor; LYS-ms, lysozyme microspheres; LYS-$Mg(OH)_2$-ms, lysozyme microspheres containing $Mg(OH)_2$; NGF, nerve growth factor; PBS, phosphate buffer saline; PLGA, poly(lactic-co-glycolic acid); TRIS, Tris(hydroxymethyl)aminomethane.

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Ismier, France). The composition of the chains was 37.5% D-lactic units, 37.5% L-lactic units and 25% glycolic units. The mean molecular weight ($\overline{M}w$) was 21,000 Da ($I = 1.7$) as determined by steric exclusion chromatography. Lysozyme is a monomeric globular protein with 129 amino acids and an isoelectric point (pI) 10.7. Lysozyme from chicken egg white (95%; 3 × crystallized, dialyzed and lyophilized) and its substrate, *Micrococcus lysodeikticus*, HSA (fraction V 96 × 99% albumin), all AR grade chemicals and buffer components were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France). Polyethylene glycol (PEG 400) was purchased from Cooper (Melun, France) and poly(vinyl alcohol) (PVA; 88% hydrolyzed, Rhodoviol® 4/125) from Merck Eurolab (Paris, France).

Protein Assay

The biologic activity of lysozyme was determined by measurement of the turbidity change in a *Micrococcus lysodeikticus* bacterial cell suspension. The reported method by Mc Kenzie and White (15) was modified. Lysozyme was incubated in a 0.015% w/v *Micrococcus lysodeikticus* suspension in TRIS (0.084 M) buffer solution (37°C, 4h). The absorbance of the suspension was then measured at 450 nm (Uvikon 922 Spectrophotometer, Bio-Tek Kontron Instruments, Saint Quentin Yvelines, France). The comparison with a suspension without protein gives the lytic activity. The increase in transmittance was a second order polynomial function of lysozyme mass from 2 ng to 30 ng. Within and between day (3 days) coefficients of variation (CV) at 5 (low), 15 (mid) and 25 ng (high) were <8% in all cases.

Lysozyme Stability Study

The biologic activity of lysozyme upon storage was determined in a variety of TRIS buffer formulations. Different additives (BSA, dextrose, NaCl), which are frequently used as stabilizers in the literature (16,17), were tested. The % of remaining activity was measured at –20°C, 6°C and 37°C over time. Lysozyme solutions were prepared (100 ng.mL^{-1}, $n = 2$) in silanized glass vials and tested for lytic activity at 1, 5, 20 and 40 days.

^{125}I-labeled Lysozyme

Lysozyme was labeled by a chemical method using a fixed oxidant: Iodogen® (1,3,4,6-tetrachloro-3α–6α–diphenylglycouril) (18). Lysozyme (1 mg.mL^{-1}) was dissolved in buffer (NaH_2PO_4, pH 6). Na-^{125}I (74mBq) was added and gently shaken for two hours. The purification was made on a Dowex 1 × 4-200 column, Sigma-Aldrich (Saint Quentin Fallavier, France). First, the column was saturated with HSA and washed with buffer (NaH_2PO_4, pH 7). The labeled protein was subsequently purified by dialysis (Spectra/por, $\overline{M}$wCO: 3,500, Merck Eurolab, Paris, France) and lyophilized (RP2V Serail SGD, Argenteuil, France).

Microsphere Preparation

Microspheres containing lysozyme were prepared using a w/o/w emulsion solvent evaporation process previously developed in our laboratory (10,19,20).

Briefly, a 0.06 mL internal aqueous phase (16 mM citrate buffer, 0.1% w/v HSA, pH 6.0) containing 2.5 mg of additional HSA (5% w/w with respect to the amount of PLGA), 250 μg lysozyme (0.5%) and a fraction of ^{125}I-labeled lysozyme, was mixed with 0.09 mL of liquid PEG 400. The mixture was emulsified in an organic solution (2 mL; 3:1 methylene chloride:acetone). This organic phase contained 50 mg PLGA with or without 3% w/w $Mg(OH)_2$ (respectively LYS-$Mg(OH)_2$-ms and LYS-ms). Emulsification was performed in a polytetrafluoroethylene (PTFE) tube using sonication (15s; Microson XL2007, 100 W, 23KHz, with a 3.2 mm microprobe, Microsonix, Servilab, Le Mans, France). This w/o emulsion was subsequently poured into an external aqueous solution of PVA (30 mL, 5% w/v) and mechanically stirred at 500 rpm for 1 min (Heidolph RGH 500, Merck Eurolab, Paris, France). The resulting w/o/w emulsion was added to deionized water (400 mL) and magnetically stirred for a further 25 min to extract the organic solvent. Finally, the formed microparticles were filtered on a 0.45 μm filter (HVLP type, Millipore SA, Saint Quentin Yvelines, France), washed five times with 100 mL of deionized water and freeze-dried to obtain a free flowing powder. The dried particles were stored at +4°C until further required.

Following preparation particles were viewed by a light microscope (BH2, Olympus, Tokyo, Japan) and the particle size determined using a Coulter Multisizer (Coultronics, Margency, France).

Entrapment Efficiency

The reported method by Ghaderi and Carlfors (21) was modified. PLGA microspheres (10 mg) were dissolved in 0.9 mL DMSO in a silanized glass tube. After 1h, 2.4 mL 0.001 M HCl was added. The solutions were left to stand for a further 1 h, then diluted and incubated with *Micrococcus lysodeikticus* suspension (0.015% w/v) for 4h at 37°C, as previously described. Encapsulation yields were determined considering both total protein (active + inactive) (Yt) and biologically active protein (Yb).

$$Y_t(\%) = \frac{\text{recovered cpm in microparticles}}{\text{cpm introduced in the process}} \times \frac{\text{mg (PLGA + drug + additives)}}{\text{mg microparticles}} \times 100$$

$$Y_b(\%) = \frac{\text{mg drug biologically active recovered in microparticles}}{\text{mg drug introduced in the process}} \times \frac{\text{mg (PLGA + drug + additives)}}{\text{mg microparticles}} \times 100$$

In Vitro Release: Study in a Continuous Flow System

In vitro release of lysozyme from PLGA microspheres was determined using a novel *in vitro* apparatus (Fig. 1) . Microspheres (20 mg, n = 3) were placed in an unpacked Omega® HPLC tube (4.6 mm i.d. × 5 cm) fitted with 0.5 μm frits and connected with 1.6 mm o.d. HPLC tubing at each end. The column assembly, frits and tubing consisted of PEEK® polymer and were supplied by Upchurch Scientific (Oak Harbor, Washington). For the duration of the study, eluent (TRIS 0.084 M buffer pH 7.4, containing 0.1% w/v BSA and 0.09% w/v NaCl) was supplied to the column inlet at 5 μL.min^{-1} by syringe pump (PhD 2000, Harvard Appara-

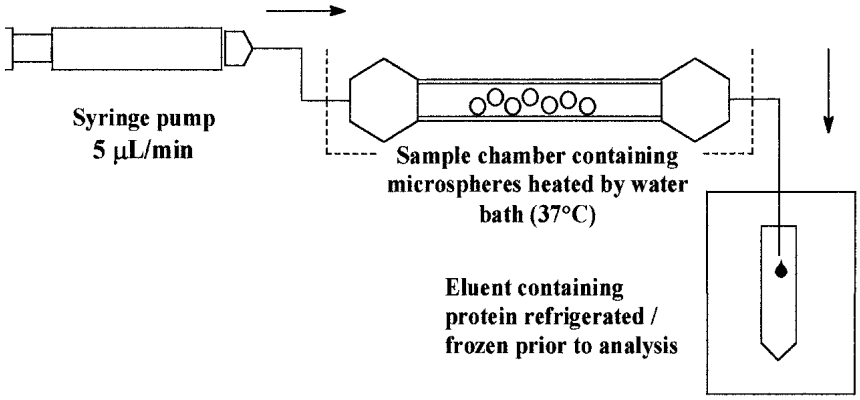

Fig. 1. Illustration of the system used for the *in vitro* release of protein from microspheres.

tus, les Ulis, France). Heating (37°C) of the chamber was achieved by immersion of the column assembly in a water bath. Tubing from the column outlet led directly into a refrigerated chamber (4°C), allowing the collection of fractionated eluent. Eluent containing the released protein was collected in fractions over 1, 3 or 7 day intervals in silanized glass vials and subsequently frozen (–20°C) until further required.

The released protein amount was determined by counting radioactivity (cpm) with a gamma counter Minaxi Auto-Gamma® 5000 Packard (Canberra, Australia). The radioactive decay was considered and corrected. The percentage of released lysozyme was expressed in relation to the active encapsulated lysozyme (y_b).

RESULTS AND DISCUSSION

Lysozyme Stability Study

This preliminary study was undertaken to identify an optimal buffer formulation for lysozyme stability. Importantly, the structural differences among different proteins are so significant that generalization of universal stabilization strategies has not been successful. In the literature, no particular lysozyme stability study has been undertaken. But generally, for microsphere *in vitro* release studies involving lysozyme, researchers have used PBS pH 7 at 37°C. The results are conflicting: Bezemer *et al.* (22) found no decrease in activity during a 50 day release period, whilst Park *et al.* (23) observed near complete loss of catalytic activity after 27 days. In our case, the eluent initially chosen was TRIS 0.084 M + 0.1% BSA pH 7.4, which permitted 80% of the lytic activity of lysozyme to remain after 40 days at 37°C. To improve this stability in solution further, several additives were added to the TRIS buffer and tested. An optimal solution consisting of 0.084 M TRIS, 0.1% BSA and 0.09% NaCl pH 7.4, allowed complete retention of lysozyme activity after 40 days at 37°C (Fig. 2). In this case, NaCl decreases reversible denaturation via non specific binding to the protein and the serum albumin prevents protein surface adsorption (24). In addition, excellent stability was observed at –20 and 6°C. This optimal buffer was used as the eluent for the *in vitro* release studies.

Characterization of Microspheres

Microspheres containing lysozyme were smooth and spherical with a mean (±SD) particle size of 24 ± 10 µm (volume weighted) for LYS-ms. The encapsulation efficiency was 84 ± 4% as determined by radioactivity and 80 ± 4% by biologic assay (Table I). Thus, the lysozyme was successfully encapsulated without significant loss in biologic activity (<5%).

The characteristics of the LYS-$Mg(OH)_2$-ms are similar to those of LYS-ms (Table I). The addition of $Mg(OH)_2$ did not unduly affect particle size or the encapsulation efficiency.

In Vitro Release: Study in a Continuous Flow System

Prior to commencing the release study, 100 µg of lysozyme in solution + 20 mg of blank microspheres was placed into the release column (n = 3). A complete recovery of

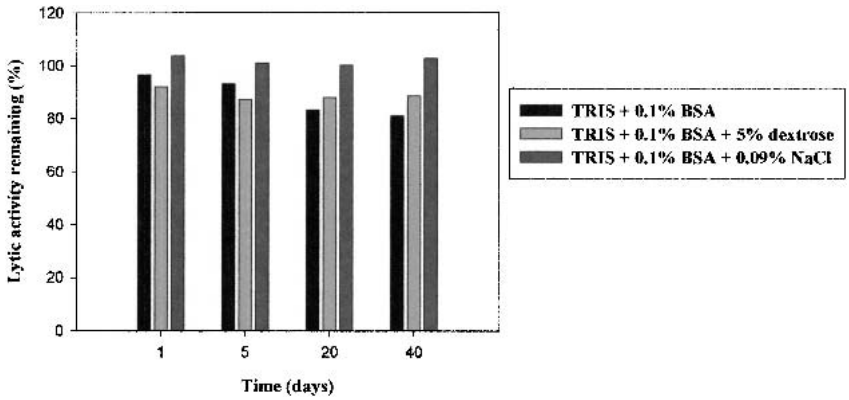

Fig. 2. Lytic activity of lysozyme (100 ng.mL^{-1}) remaining upon storage at 37°C (mean, $n = 2$).

Table I. Microsphere Characteristics

	LYS-ms		LYS-$Mg(OH)_2$-ms	
Particle size ± SD (μm)	24 ± 10 μm		23 ± 11 μm	
Encapsulated protein				
- total (Y_t)	84 ± 4%		89 ± 3%	
- active (Y_b)	80 ± 4%		83 ± 7%	
- ratio	95%		93%	
Release characteristics	D_1 (days)	D_{42} (days)	D_1 (days)	D_{42} (days)
- total	13 ± 2%	20 ± 3%	7 ± 1%	19 ± 3%
- active	4 ± 1%	13 ± 3%	7 ± 1%	22 ± 2%
- ratio	31%	65%	100%	100%
Lysozyme remaining in microspheres		D_{42} (days)		D_{42} (days)
- total		82 ± 4%		83 ± 1%
- active		41 ± 4%		65 ± 1%
- ratio		50%		78%

biologically active lysozyme was achieved within 48h, indicating negligible adsorption and degradation of the lysozyme in the system at 37°C in the TRIS/BSA/NaCl buffer pH 7.4.

The *in vitro* release apparatus has a number of advantages. It permits the observation of the release profile for several weeks. Moreover, the encapsulation of radiolabeled lysozyme, combined with the cell lysis assay, allowed the proportion of the total and biologically active protein released to be determined.

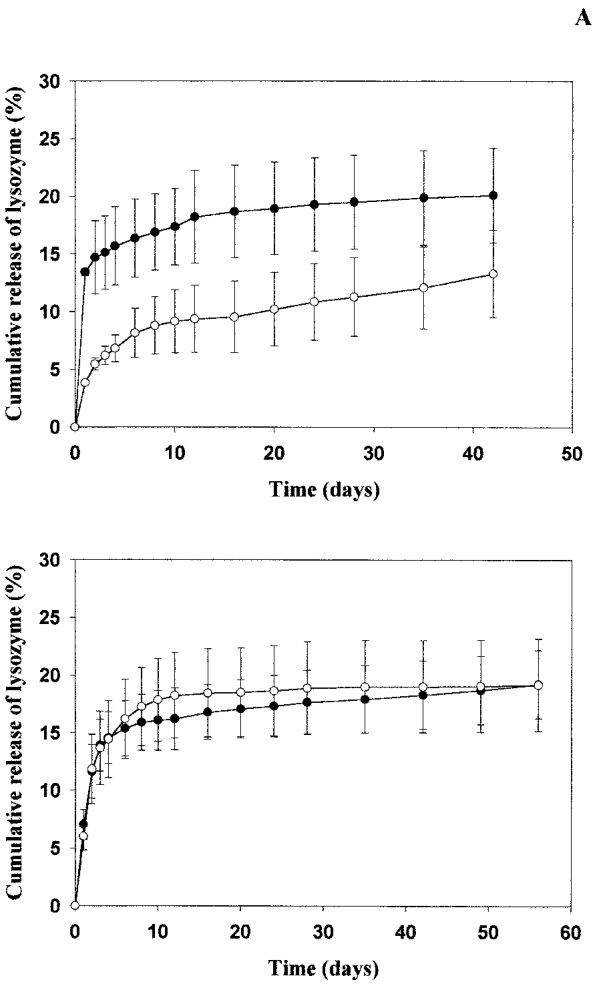

Fig. 3. *In vitro* release kinetics of lysozyme from PLGA microspheres (mean ± SD, $n = 3$). LYZ-ms, (A). LYZ-$Mg(OH)_2$-ms, (B). ○ active protein; ● total protein (active + inactive).

The continuous flow system is the most appropriate for such purposes. Previous researchers have used a semi-continuous flow analytical device at 15 μL/min (25,26). However, the flow rate should be as low as possible, to approach the conditions found within the brain parenchyma. Additionally, the system must prevent protein degradation prior to analysis. With this in mind, we used an adsorption resistant material (PEEK®), a refrigerator to conserve the collected samples and an eluent known to allow the retention of protein activity under temperatures ranging from −6 to 37°C.

For the LYS-ms, the release profile was characterized by a marked initial protein release (13% total protein within 24h, with 31% of this being active) followed by a low continuous release (Fig. 3). However, the microspheres exhibited an incomplete release with only 20% total lysozyme release after 42 days. This incomplete release is frequently observed with proteins (17).

After 42 days, the remaining microspheres were analyzed. Radioactivity measurements indicated 82% of the ^{125}I-lysozyme remained within the microspheres; 50% of remaining lysozyme was active protein.

The difference between active and total protein content is attributed to protein degradation within the polymer matrix over time. To prevent the pH drop during polymer degradation, a basic additive, $Mg(OH)_2$ was encapsulated as previously described in the literature (27).

For LYS-$Mg(OH)_2$-ms, the release profile of total protein was unchanged (7% total protein within 24h; 19% at day 42). However, the active protein release profile was dramatically increased: 100% of the released lysozyme was biologically active. In addition, after 8 weeks, 78% of the lysozyme remaining in the microspheres was active (*c.f.* 50% in the absence of the basic additive).

The co-encapsulation of $Mg(OH)_2$ improved the stability of the protein, in part, through inhibiting the decrease in pH associated with the polymer degradation. In addition, in the early stage of the *in vitro* release, other mechanisms of stabilization may be involved which are yet to be elucidated. In-

terestingly, the presence of the basic additive had no effect on the total protein release after 42 days (19%).

Incomplete release is a commonly encountered problem in protein-PLGA formulations. Park *et al.* (23) have shown that incomplete release was caused by non-covalent aggregation and hydrophobic PLGA-protein interactions. It is particularly acute for therapeutically potent proteins that have small loads within PLGA microspheres. Different strategies have been proposed to overcome this effect. First, the co-encapsulation of diluent proteins such as HSA. HSA enhances the microsphere porosity and facilitates faster release of the therapeutic protein (28). A second strategy is the co-encapsulation of urea. This compound is known to unfold proteins and allow their outward diffusion from the microsphere matrix. Upon reaching the release medium, the protein may then refold back to recover its native conformation (29). Finally, the co-encapsulation of an antacid may also improve protein release. As stated, such agents neutralize the acidic microenvironment within microspheres that develops in the course of the polymer degradation, and consequently prevents non-covalent aggregation. This effect has been observed using BSA (30). In our case, the use of an antacid will be further optimized, being chosen for its biocompatibility with a brain implantation.

Thus, we have developed a new, reliable tool, which permits the evaluation of protein-microsphere formulations. By minimizing protein degradation prior to analysis and distinguishing between active and total protein content, the system allows an assessment of the origins of the protein degradation.

To test this new system, we have worked with lysozyme microspheres loaded at 250 μg protein/ 50 mg polymer. Microspheres with lower lysozyme loads (15 to 100 μg protein/50 mg polymer) will now be considered. Such loadings are more relevant to the clinical setting, being closer to the concentrations used for the encapsulation of GDNF. The encapsulation of such powerful growth factors, like GDNF, necessitates the use of lower loads of the active compound and a high sensitivity for any *in vitro* analytical system.

CONCLUSION

The novel *in vitro* system proposed in this work seems to be appropriate to study protein microsphere release kinetics for several weeks, allowing an improvement in the protein formulation process (the co-encapsulation of a basic additive to reduce protein degradation). Whilst the system enabled us to successfully determine the release characteristics of a lysozyme-microsphere formulation, the technique may be applied to a variety of protein-polymer devices. It is reasonable to expect that an appropriate *in vitro* characterization of GDNF-microspheres and valid *in vitro/in vivo* correlations are now possible.

ACKNOWLEDGMENTS

The authors thank Myriam Moreau for her technical assistance. We are also grateful to "Conseil Général de Maine et Loire" for its financial support.

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