Protein and Peptide Release from in Situ Gelling Polymer

Michal Y. Krasko,[†] Neeraj Kumar,[‡] and Abraham J. Domb*[,†]

Department of Medicinal Chemistry and Natural Products, School of Pharmacy − Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem-91120, Israel, and Department of Pharmaceutics, National Institute of Pharmaceutical Education & Research (NIPER), Sector 67, S.A.S. Nagar (Mohali)-160 062, India

Received March 19, 2006
Revised Manuscript Received May 9, 2006

Introduction

Over the past decade, developments in the field of biotechnology have led to the cloning, characterization, and commercial availability of many clinically useful proteins and peptides. While the technology exists for the discovery and development of these molecules, several challenges need to be solved with regard to their delivery in convenient, controlled release, and targeted formulations. In contrast to conventional synthetic pharmaceuticals, proteins are large molecular weight polypeptides, which are susceptible to proteolysis, chemical modification, and denaturation during storage and administration.[1,2] Extensive investigations have been carried out on developing controlled-release systems for peptides and proteins.[3,4]

Degradable polymeric drug delivery systems have several advantages compared to conventional drug therapies. These include improved patient compliance, avoidance of the peaks and valley of drug plasma levels associated with conventional injections, localized delivery of the drug to a particular body compartment or cell type, thereby lowering the systemic drug level, protection of drugs that are rapidly degraded in the body, and improved drug efficacy. The obvious advantage of biodegradable polymers for drug delivery over nondegradable systems is that they are excreted from the body by natural processes and thus need not be removed. There are several challenges in the development of drug delivery systems with regard to maintaining the integrity and activity of incorporated proteins. First, in the process of preparing drug delivery systems, proteins may be exposed to extreme stresses. Necessary manufacturing steps may include excessive exposure of the protein to heat, shear forces, pH extremes, organic solvents, freezing, and drying, to name a few. Following manufacture or preparation, the drug delivery systems must be stored for some extended period of time prior to administration.[5]

There are many reports focused on poly(lactide-*co*-glycolide) (PLGA) as a choice of material for controlled drug release devices for various drugs, but there are limitations for peptides and proteins.[6] Unfortunately, bulk degradation of PLGA creates an inside acidic environment, which is not suitable for pH-sensitive peptides and proteins. Recently, a series of new poly(ester anhydride)s based on poly(sebacic acid) (PSA) and ricinoleic acid (RA) have been investigated as matrix for controlled drug delivery.[7,8] The main characteristics of these poly(sebacic-*co*-ricinoleic ester anhydride)s (p(SA-RA)s) are their simple one-pot preparation, biodegradability, and biocompatibility. p(SA-RA)s having 70% or more RA are pasty at room temperature and can be mixed with heat-sensitive proteins at room temperature and remain injectable. Heating of the protein−polymer system may not only cause a degradation of the protein, but also a reaction between functional groups of polymeric backbone (usually ester or anhydride) and protein. The incorporation of proteins in the polymer paste without the use of organic solvents or application of heat simplifies the incorporation and reduces possible deterioration of incorporated proteins.

The presence of water with pasty p(SA-RA) 30:70 w/w increases the viscosity of the polymer, which leads to a semisolid material. This pasty material may retain an incorporated drug for weeks while gradually releasing its content with time while being degraded.[9] The hydrophobicity of polymeric gel reduces the penetration of water and increases the degradation time of the polymer.

In this study, pasty p(SA-RA)s with 20:80 and 30:70 w/w ratios were prepared and investigated for controlled release of water-soluble macromolecules, like peptides and proteins. The peptides, leuprolide (1270 Da) and octreotide (1019 Da), and the proteins, bovine serum albumin (BSA) (68 000 Da), insulin (6860 Da), interleukin (53 000 Da), and interferon alpha-2a (IFN-alpha) (19 000 Da) were used.

Experimental Section

Molecular weights of the polymers were estimated on a gel permeation chromotography (GPC) system consisting of a Waters 1515 isocratic HPLC pump with a Waters 2410 refractive index (RI) detector and a Rheodyne (Coatati, CA) injection valve with 20-μL loop (Waters Ma). Samples were eluted with $CHCl_3$ through a linear Styragel HR1 column (Waters; 7.8 × 300 mm) at a flow rate of 1 mL/min. The molecular weights were determined relative to polystyrene standards (Polyscience, Warrington, PA) with a molecular weight range of 120−4500 using a Breeze computer program.

Octreotide, insulin, and leuprolide concentrations in buffer solutions were determined by an HPLC (Hewlett-Packard, Waldbronn, Germany) system composed of an HP 1100 pump, HP 1050 UV detector, and HP ChemStation data analysis program using a C18 reverse-phase column (LichroCart 250−4, Lichrospher 100, 5 μm).

A mixture of acetonitrile/phosphate buffer(pH 7.4, 20 mM) 40:60 v/v at a flow rate of 1 mL/min for 12 min was used as an eluent for the determination of octreotide with UV detection at 218 nm.

For insulin determination, the gradient was carried out using 0.13% trifluoracetic acid (TFA) in double distillated water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The gradient started at 0% of B (v/v) and was increased linearly to reach 85% of B in 9 min, then maintained for 3 min, and the volume percent of B was decreased linearly to reach 15% within 3 min, then maintained for another 2 min. The post time was set at 17 min. The flow rate was set at 1 mL/min, and detection wavelength was 214 nm.

A mixture of acetonitrile/tetraethylene ammonium phosphate buffer (0.01 M, pH 7.4) 30:70 v/v at a flow rate of 1 mL/min for 10 min was used as an eluent for the determination of leuprolide[10] with UV detection at 278 nm.

Interleukin and IFN-alpha concentrations in buffer solutions were determined using the Lowry assay method by UV spectrometer (Kontron Instruments Uvicon model 930 (Berlin, Germany)) at 270 nm.

* Correspondence author. Phone 972−2−6757573; Fax 972-2-6757629; E-mail adomb@md.huji.ac.il. Affiliated with the Devid R. Bloom Center for Pharmacy and the Alex Grass Center for Synthesis and Drug Design at The Hebrew University of Jerusalem, Jerusalem-91120, Israel.

[†] The Hebrew University of Jerusalem.

[‡] National Institute of Pharmaceutical Education & Research (NIPER).

10.1021/bm060255q CCC: $33.50
Published on Web 06/29/2006

Polymer Synthesis. Poly(SA-RA) 70:30 and 80:20 were synthesized as previously described.[7,11] Briefly, PSA ($M_w \geq 40\,000$ Da, 2 g) was mixed with 8 g of purified RA (98% pure)[12] at 120 °C for 3 h. The transesterification was stopped while the molecular weight reached its lowest constant value. Acetic anhydride was added to the reaction flask at 1:1 w/v ratio. The solution was refluxed at 140 °C for 30 min, filtered, cooled, and evaporated to dryness. The reaction flask was connected to a vacuum line. The polycondensation was performed at 170 °C, 0.1 mmHg for 2 h with continuous stirring. The polymerization was terminated when the molecular weight reached its highest constant value. The polymers had a typical weight average molecular weight of 5000 Da and a melting temperature of about 30 °C.

Drug Incorporation. The peptides and the proteins were ground separately in a mortar at room temperature to a fine powder. The drug powder was mixed with p(SA-RA) 20:80 or 30:70 until a homogeneous paste was achieved.

In Vitro Release and Hydrolytic Degradation of the Polymers. Formulations of p(SA-RA) 20:80 loaded with 10% w/w leuprolide and octreotide were prepared. These pasty formulations were injected as pasty droplets (20 mg each) into 15 mL of 0.1 M buffer phosphate solution (pH 7.4) followed by incubation at 37 °C with orbital shaking (100 rpm). Nonloaded polymer was also incubated under similar conditions and served as control. Samples of 2 mL were taken and analyzed by HPLC for controlled release of peptides. The degradation of these formulations under physiological conditions was followed by weight loss and GPC analysis for 40 days.

To study protein release from the polymers, formulations of p(SA-RA) 20:80 loaded with 10% w/w of BSA were prepared, injected as pasty droplets (50 mg each) in 20 mL of buffer, and incubated as described above. Samples of 0.2 mL were taken from the medium each 48 h, incubated with Folin-Ciocalteu's reagent according to the Lowry assay method,[12] and analyzed by UV spectrophotometer at 270 nm. Formulations of p(SA-RA) 30:70 loaded with 5% w/w of insulin were prepared, injected as pasty droplets (50 mg each) in 20 mL of buffer, and incubated as described above. Samples of 2 mL were taken and analyzed by Lowry assay method and HPLC.[14] Formulations of p(SA-RA) 30:70 loaded with 10% w/w of interleukin and IFN-alpha were prepared, injected as droplets (20 mg) in 15 mL of buffer, and incubated as described above. Samples of 0.2 mL were taken from the medium each 48 h, incubated with Folin-Ciocalteu's reagent according to the Lowry assay method and analyzed by UV spectrophotometer at 270 nm.

During the study, the samples did not crack and degraded from their surface, gradually decreasing their dimensions. The buffer solutions were replaced with fresh buffer every 48 h to avoid protein saturation and deterioration in solution. All experiments were carried out in triplicate.

Results

The polymers that were chosen for this study were p(SA-RA)s with 20:80 and 30:70 w/w ratios, having a molecular weight of about 5000 Da and melting point in the range of 30 °C.

The hydrolytic degradation of p(SA-RA) 20:80 loaded with 10% leuprolide and octreotide was studied by change in molecular weight (Figure 1) and weight loss (Figure 2). Both analyses were performed on the remaining polymer that was withdrawn from the degradation medium and dried prior to analysis. There was no significant difference in the molecular weight loss between blank and peptide-loaded polymers. The weight loss of polymer was slower for the peptide-loaded polymers, but after 20 days when about 70% of the peptide was released, the weight loss of blank and peptide-loaded polymers became close.

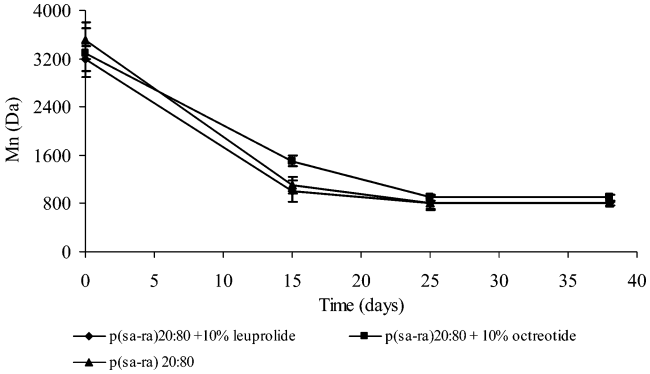

Figure 1. Hydrolysis of p(SA-RA) 20:80 w/w loaded with 10% leuprolide, 10% octreotide, and blank monitored by molecular weight (M_w) loss of the degraded polymer.

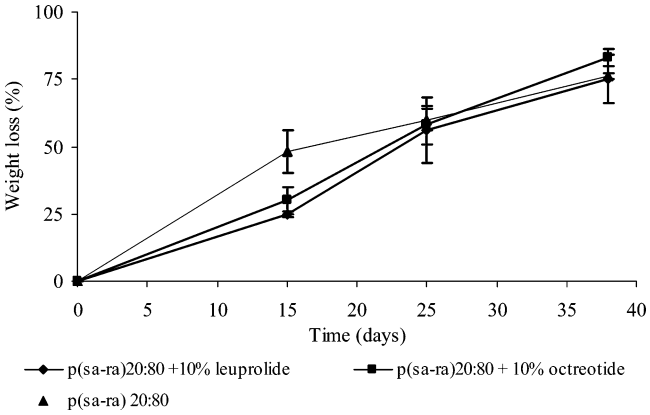

Figure 2. Hydrolysis of p(SA-RA) 20:80 w/w loaded with 10% leuprolide, 10% octreotide, and blank monitored by weight loss of the degraded polymer. Hydrolysis was conducted in 0.1 M phosphate buffer (pH 7.4) at 37 °C. At each time point, the remaining polymer was dried and weighed. Hydrolysis was conducted as described in Figure 1.

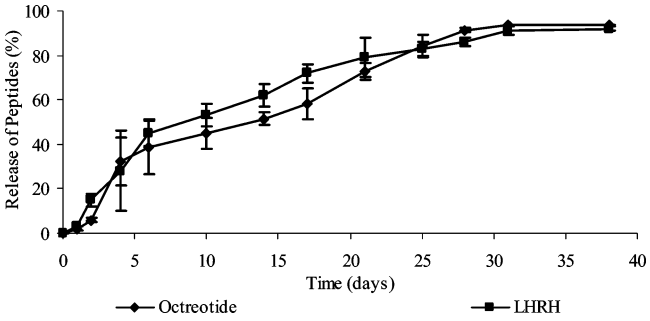

Figure 3. In vitro release of leuprolide and octreotide from p(SA-RA) 20:80 w/w. The release of peptides was conducted in 0.1 M phosphate buffer (pH 7.4) at 37 °C. The drug content in the releasing medium was determined by HPLC.

The leuprolide and octreotide release (Figure 3) was monitored by HPLC for 38 days. Both peptides having a similar molecular weight showed a similar release profiles.

The release of the proteins was monitored by the Lowry and HPLC methods for 25 days. Controlled release study of insulin showed that the more simple Lowry method can be used for detection of the protein in solution. The accuracy of the results was confirmed by HPLC analysis. The results are summarized in Figure 4. All proteins were released in a controlled manner similar to the peptides where a faster release is found during the first 5 days followed by a close to zero-order release for the next 20 days.

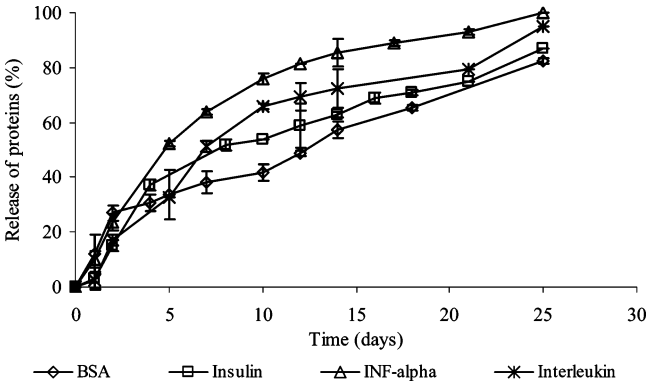

Figure 4. In vitro release of BSA, insulin, INF-alpha, and interleukin from p(SA-RA) 20:80 and p(SA-RA) 30:70 w/w. The release of peptides was conducted in 0.1 M phosphate buffer (pH 7.4) at 37 °C. The drug content in the releasing medium was determined by Lowry method assay and HPLC.

Discussion

Although there are many different types of biodegradable polymers that can potentially be used in the preparation of protein delivery systems,[15] the pasty in situ gelling hydrophobic polymers possess certain advantages over other polymeric delivery systems. The incorporation of sensitive proteins or peptides is done at room temperature by gently mixing the drug powder into the oily polymer with no solvents, additives, or water involved and without any mechanical forces. The polymer−protein paste can be injected via a common syringe into a desired site. Once the loaded polymer meets aqueous medium, it gels and form a hydrophobic protection for the entrapped protein. The hydrophobic gel degrades by hydrolysis mainly from the surface while releasing the entrapped protein. The common incorporation methods for proteins in polymeric delivery systems require mixing the protein in a hot polymeric melt, use of organic solvents and mixtures with water, or the use of additives such as surfactants and organic solvents, which deteriorate the proteins. For example, several systems were developed for controlled release of peptide analogues of luteinizing hormone-releasing hormone (LHRH) prepared from PLGA and include implants[16] and microsphere delivery systems. Preparation of such system involved heating[16] and use of organic solvents[10,17] and additives.[18]

It is also preferred over in situ cross-linking[19] and polymer precipitation[20] delivery systems that involve organic solvents such as *N*-vinyl pyrrolidinone, *N*-methyl pyrrolidinone, propylene glycol, acetone, DMSO, and THF or include free-radical reactions that promote generation of free radicals which can cause tissue injury or reaction with the protein. The polymer is hydrophobic and does not contain hydrophilic segments such as poly(ethylene glycol) in thermogelling systems;[21] the hydrophobic polymeric matrix protects peptides from the deterioration effects of aqueous medium and the interface of the water−organic medium. According to our previous study,[9] the hydrophobic p(SA-RA) can be compared with a hydrophobic organogel system, which is composed of water-insoluble amphiphilic lipids that swell in water and form various types of lyotropic liquid crystals.[22] However, the organogels suffer from the following disadvantages: purity of waxes and stability of oils—oils need a stabilizer, antioxidant, and preservative to increase their shelf life and stability. The hydrophobic pasty p(SA-RA) with or without the drug can be kept under refrigeration for months[7] without any stabilizers. Moreover, the difference between the melting points of waxes and oils makes this system susceptible to phase separation. In addition, the equilibrium water content of the organogel formed is typically approximately 35%, which therefore produces relatively short release duration for hydrophilic drugs. The proteins and peptides that were presented in this study were released from the p(SA-RA) in the time frame of one month. It can be compared to the release of some proteins such as lysozyme, trypsin, heparinase, ovalbumin, albumin, and immunoglobulin from polyanhydride microspheres that were prepared by a solvent evaporation technique method using a double emulsion.[23]

Incorporation of peptides into pasty polymer takes place at room temperature and with no solvent or additives involved. Peptide release from the pasty polymer was complete in 1 month with polymer elimination within 10 weeks postadministration. Comparing hydrophobic pasty polymer protein delivery to other injectable implant systems shows the advantages of using pasty polymer. Pasty p(SA-RA) possesses good syringe-ability at injection conditions and immediately forms semisolid droplets when injected.[9] It is a synthetic polymer made from natural fatty acids, which is preferred over natural, since it is presumed to be free of immunogenicity, and its physicochemical properties are more predictable, reproducible, and easy to modify.

Conclusions

This study demonstrates the usefulness of the pasty polymers for the constant release of peptides and proteins by simply mixing the drug powder into the polymer paste at room temperature. Leuprolide, octreotide, insulin, interleukin, interferon alpha-2a, and bovine serum albumin were incorporated into pasty poly(ester anhydride)s. They were released under physiological conditions for about one month.

References and Notes

(1) Manning, M. C.; Patel, K.; Borchardt, T. *Pharm. Res.* **1989**, *6*, 903.
(2) Wang, Y. J.; Hanson, A. *J. Parenter. Sci. Technol. Suppl.* **1988**, *42*, S3.
(3) Langer, R. *Chem. Eng. Commun.* **1980**, *6*, 1.
(4) Schwendeman, S. P.; Costantino, H. R.; Guppa, R. K.; Langer, R. *Controlled Drug Delivery, Challenges and Strategies*; American Chemical Society: Washington, DC, 1996; pp 229−67.
(5) Gombotz, R. W.; Pettit, D. K. *Bioconjugate Chem.* **1995**, *6*, 332.
(6) Couvreur, P.; Puisieux, F. *Adv. Drug Delivery Rev.* **1993**, *10*, 141.
(7) Krasko, M. Y.; Shikanov, A.; Ezra, A.; Domb, A. J. *J. Polym. Sci., Part A: Polym. Chem.* **2003**, *41*, 1059.
(8) Shikanov, A.; Vaisman, B.; Krasko, M. Y.; Nyska, A.; Domb, A. J. *J. Biomed. Mater. Res., A* **2004**, *69*, 47.
(9) Shikanov, A.; Domb, A. J. *Biomacromolecules* **2006**, *7*, 288.
(10) Slager, J.; Domb, A. J. *Biomacromolecules* **2003**, *4*, 1316.
(11) Krasko, M. Y.; Domb, A. J. *Biomacromolecules* **2005**, *6*, 1877.
(12) Borsotti, G.; Guglienlmetti, G.; Spera, S.; Battistel, E. *Tetrahedron* **2001**, *57*, 10219.
(13) Leger, G.; Mullerplatz, C. M.; Mentgeshettkamp, M.; Pflieger, G.; Julich, E. *Anal. Biochem.* **1985**, *150*, 278.
(14) Merisko-Liversidge, E.; McGurk, S. L.; Liversidge, G. G. *Pharm. Res.* **2004**, *21*, 1545.
(15) Mainardes, R. M.; Silva, L. P. *Curr. Drug Targets* **2004**, *5*, 449.
(16) Asano, M.; Yoshida, M.; Kaetsu, I.; Imai, K.; Mashimo, T.; Yuasa, H.; Yamanaka, H.; Suzuki, K.; Yamazaki, I. *Makromol. Chem., Rapid Commun.* **1995**, *6*, 509.
(17) Okada, H.; Doken, Y.; Ogawa, Y.; Toguchi, H. *Pharm. Res.* **1994**, *11*, 1143.
(18) Lu, W.; Park, T. G. *J. Pharm. Sci. Technol.* **1995**, *49*, 13.
(19) Nivasu, V. M.; Reddy, T. T.; Tammishetti, S. *Biomaterials* **2004**, *25*, 3283.
(20) Graham, P. D.; Brodbeck, K. J.; McHugh, A. J. *J. Controlled Release* **1999**, *58*, 233.
(21) Choi, S.; Kim, S. W. *Pharm. Res.* **2003**, *20*, 2008−2010.
(22) Engstrom, S.; Engstrom, L. *Int. J. Pharm.* **1992**, *79*, 113−122.
(23) Tabata, Y.; Gutta, S.; Langer, R. *Pharm. Res.* **1993**, *10*, 487.

BM060255Q