Controlled release of polypeptides from polyanhydrides

(bovine growth hormone/drug delivery systems/controlled release/degradable polymers)

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ABSTRACT The effects of both polymer hydrophobicity and addition of stabilizers on the release and integrity of polymer-encapsulated proteins were studied. By using very hydrophobic poly[1,3-bis(*p*-carboxyhydroxy)hexane anhydride] with sucrose as an excipient, both recombinant bovine somatotropin and zinc insulin were released intact over 3 weeks. The released proteins appeared to maintain their integrity as judged by acidic reverse-phase HPLC, size-exclusion HPLC, radioimmunoassay, and conformation-sensitive immunoassays. Our results also suggest how polymer hydrophobicity can be used to enhance protein stability.

Most peptidic and proteinic drugs are susceptible to degradation at the site of administration, whether administered by subcutaneous, intramuscular, intestinal, buccal, rectal, nasal, or vaginal routes (1, 2). In addition, some proteins have very short *in vivo* half-lives and, as a consequence, multiple injections are required to achieve desirable therapy. One way to increase the therapeutic efficacy of these proteins is to incorporate them in a controlled release system that releases the proteins continuously. Several controlled release systems have been tested for their ability to sustain the release of peptidic drugs such as insulin (3), epidermal growth factor (4), interferon (5), and luteinizing hormone-releasing hormone and its analogs (6–9). It is also possible that, in addition to providing sustained release, polymers may help to protect the polypeptides from denaturing conditions, water, and proteolytic enzymes. Furthermore, the use of bioerodible polymers does not require posttreatment removal of the device. However, the polymers used for such delivery are limited to those that are inert toward the incorporated proteins—i.e., those polymers that neither bind to irreversibly nor promote decomposition of the proteinaceous therapeutic agent. Polyanhydrides have been investigated in our and other laboratories as biodegradable implants (10–12). Furthermore, polyanhydrides are one of the few synthetic biodegradable polymers being studied clinically as injectable or as implantable matrices. We used several polyanhydrides with different hydrophobicities to explore the effect of hydrophobicity and excipients on the release and activity of two proteins. We also studied the implications of possible interactions between the polymer, its degradation products, and the incorporated proteins.

We chose recombinant bovine somatotropin (rb-STH) and insulin as model proteins. b-STH is a single-chain protein hormone consisting of 191 amino acids. The native structure of rb-STH consists of a four-helix bundle motif with two intrachain disulfide bridges at positions 153/164 and 181/189 (13, 14). Physical instability due to perturbation of the tertiary structure results in partially unfolded intermediates and potential formation of insoluble aggregates (15). Potentially

more important is the chemical instability of rb-STH in aqueous environments leading to deamidated, chain-cleaved, and covalently bonded oligomeric products (16). This instability presents an intriguing problem to attempt to overcome; solving it will demonstrate the potential of using hydrophobic polymers to reduce aggregation and to control the release of unstable proteins. Insulin was used to study the extent to which *in vitro* release and activity would compare with *in vivo* activity. The diabetic rat model is well documented in the literature and the successful use of our final formulation for delivery of intact protein would be a good affirmation.

EXPERIMENTAL PROCEDURES

Materials. Sebacic acid, 4-hydroxybenzoic acid, 1,3-dibromopropane, and 1,6-dibromohexane were all from Aldrich. All solvents were reagent grade (J. T. Baker). Chloroform-*d*₁ (gold label with 0.01% tetramethylsilane) was from Aldrich. rb-STH was a gift from Upjohn; zinc insulin (Sigma; I5500) and sucrose were from Sigma.

Instrumentation. Infrared spectroscopy was performed on a Perkin–Elmer 1430 spectrophotometer. Polymeric samples were film cast onto NaCl plates from a solution of the polymer in chloroform. Formulated samples were either pressed into KBr pellets or dispersed in nujol onto NaCl plates. Viscometry of the polymers in chloroform (J. T. Baker) was measured in a Cannon 75 Ubbelohde dilution viscometer. Afflux times were measured at four concentrations at 23°C, and the data were analyzed by standard methods (17). The molecular weights of the polymers were estimated on a Perkin–Elmer gel-permeation chromatography system consisting of the series 10 pump and the 3600 data station with the LKB 214 rapid spectral detector (wavelength, 254 nm). Samples were eluted in chloroform (alcohol free) through a 5- μ m mixed column gel (Polymer Laboratories, Amherst, MA) at a flow rate of 0.9 ml/min at 23°C. Molecular weights of polymers were determined relative to polystyrene standards (Polysciences; *M*_r 500–160,000) using CHROM2 and GPC4 computer programs (Perkin–Elmer). ¹H NMR spectra were obtained on a Varian 270 MHz spectrophotometer using chloroform-*d*₁ as a solvent and tetramethylsilane as an internal reference.

Proteins were analyzed by a Waters 840 HPLC system consisting of two 510 pumps, a Wisp 712 automatic injector, and a 490 multiwavelength detector all controlled by a DEC

Abbreviations: b-STH, bovine somatotropin; rb-STH, recombinant b-STH; poly(CPP-SA), poly[1,3-bis(*p*-carboxyphenoxy)propane-co-sebacic anhydride]; poly(CPH), poly[1,3-bis(*p*-carboxyphenoxy)-hexane]; CSI, conformation-sensitive immunoassay; SEC, size-exclusion chromatography.

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350 data station. Acidic reverse-phase HPLC runs were performed on either a C₁₈ column (μ Bondapak 150 $\times$ 4.6; Waters) for insulin or a C₄ column (Scout C₄ 50 $\times$ 4.6 or WidePore C₄ 250 $\times$ 4.6; both from J. T. Baker) for b-STH. The mobile phase consisted of a 0.1% trifluoroacetic acid (A)/acetonitrile (B) gradient (50–60% B in 20 min for rb-STH; 0–90% B in 25 min for insulin). Size-exclusion chromatography (SEC) was performed on a TSK-125 column (300 $\times$ 7.5; Bio-Rad) using sodium sulfate (0.05 M)/sodium phosphate (0.02 M) buffer, pH 6.8, with 10% acetonitrile as the isocratic mobile phase. Reduced SDS gel electrophoresis and isoelectric focusing were performed on a PhastSystem using the appropriate PhastGels (Pharmacia).

Polymers. Poly[1,3-bis(*p*-carboxyphenoxy)propane-*co*-sebacic anhydride] [poly(CPP-SA)], and poly[1,3-bis(*p*-carboxyphenoxy)hexane] [poly(CPH)], were prepared by melt polycondensation of mixed anhydrides of diacids and acetic acid as described (18). The polyanhydrides after purification (12) were frozen in liquid nitrogen, micronized in a Technilab micromill (Baxter Scientific Products, McGaw, IL), and sieved to 130–150 μ m.

All the polyanhydrides were analyzed by gel-permeation chromatography and had an average M_r between 20,000 and 120,000. Copolymer composition was verified by ¹H NMR as described (19).

Device Preparation and Release. Proteins were dissolved in Milli-Q water (0.05–0.2%) with or without excipients and lyophilized. The resulting dry powder was sieved through a 150- μ m sieve (Thomas) before use. Final moisture content of the preparation was between 1.1% and 3.6% as determined by (29) Karl Fischer titration.

Proteins (with and without excipients) were mechanically mixed (Vortex) with polymer in a test tube. A 10% loading was achieved by using the weight of protein and excipient divided by the weight of polymer. Discs of drug-impregnated polyanhydrides were fabricated by using a Carver Laboratory (Menomonee Falls, WI) press. Discs (200 mg; diameter, 1.4 cm; width, 1.4 mm) were placed in periodically changed 0.1 M potassium phosphate buffer (pH 7.4) (10 ml). Throughout the release period, sink conditions were maintained in which the concentration of released drug in solution was less than 5–10% of saturation. The decanted buffer solutions were monitored by both reverse-phase and size-exclusion HPLC. Additional release quantitation was done by RIA for both rb-STH and insulin and by conformational analysis (CSI; conformation-sensitive immunoassay) for rb-STH samples.

When the released proteins were undetectable by HPLC, the discs were washed with a few portions of Milli-Q water and lyophilized. The remaining powder was sent for elemental analysis (Galbraith Laboratories, Knoxville, TN).

Protein Quantitative Assays. *Immunoassays.* b-STH was quantified, in addition to the HPLC methods, by a double-antibody RIA (20) and by a CSI (21, 22). The latter two assays allow examination of the native state of the protein in solution and identification of alterations in protein conformation.

RIA. The RIA was used for measuring concentrations of rb-STH. The competitive assay utilizes a guinea pig polyclonal anti-b-STH antibody (Upjohn), a goat anti-guinea pig immunoglobulin (Antibodies, Inc.), and iodinated b-STH.

Insulin concentrations (by HPLC and reverse-phase HPLC) were verified by RIA (ICI).

CSI. The CSI was used on selected samples both to confirm conformational integrity and for quantitation purposes. The CSI utilized two anti-b-STH monoclonal antibodies (Upjohn) with apparent conformational specificity. The assay was based on particle concentration fluorescence immunoassay technology with a Pendex screen machine (Pendex Division, Travenol Labs, Mundelein, IL). The CSI consists of sandwiching b-STH with antigens that are cova-

lently coupled to a hydrophilic membrane support and probed with monoclonal antibodies that recognize a specific epitope.

In Vivo. Female Sprague-Dawley rats (190 $\pm$ 10 g) were obtained from Charles River Breeding Laboratories. Diabetes was induced by tail vein injection of streptozotocin (Upjohn) at a dose of 65 mg per kg of body weight in 100 mM sodium citrate/150 mM NaCl buffer, pH 4.5. All rats were placed in metabolic cages and were fed laboratory rat chow (Purina) and water ad libitum.

Discs (diameter, 1.4 cm; width, 1.4 mm) were surface-sterilized under UV light (240 nm) for 40 min on each side. Implantation was conducted 9 days after induction of diabetes. The rats were anesthetized with methoxyflurane (Mettaphen; Pittman-Moore, Mundelein, IL). The implantation was made in the lower abdominal area (3). Both plasma and urinary glucose levels were monitored to assess the effects of *in vivo* insulin release. Plasma glucose levels were determined with a Diascan blood glucose analyzer (model 1200; Home Diagnostics, Eatontown, NJ). Urinary glucose levels were determined with Chemstrip μ G (Bio-Dynamics, Indianapolis). Blood and urinary glucose samples were normally performed between 1100 and 1300 h.

RESULTS AND DISCUSSION

There was a concern that rb-STH could not be released over an extended period due to its decomposition/aggregation in the presence of moisture (15, 16). In addition, it was reported (11) that incorporating different amine-containing molecules into polyanhydrides by injection molding (120°C) resulted in the appearance of new amide bonds as observed by IR spectroscopy. This phenomena was explained by interaction between the incorporated amine-containing molecules and the carboxylic group of degraded polyanhydrides. Therefore, we wanted to examine whether the polymer may interact with the incorporated rb-STH. Both covalent oligomerization and polymer–rb-STH interaction should give rise to the appearance of additional signals during analytical analysis. On the other hand, a single signal supports the notion that no interactions or cross-linking had taken place.

We used compression-molded discs in our study as opposed to solvent casting/evaporation to avoid possible organic solvent effects on the protein (e.g., denaturation). Furthermore, we fabricated the devices at 25°C to achieve gentler conditions than any other available method. No new amide bond formation was observed by IR spectroscopic analysis (data not shown).

The discs of b-STH- and insulin-impregnated polyanhydrides were placed in periodically changed phosphate buffer. There was no difference between the SEC-HPLC chromatogram peak shape of the rb-STH prior to incorporation and the released protein, even after 288 h (Fig. 1 *Left*). No additional peaks were observed. Periodically, buffer samples were concentrated, dialyzed, and analyzed by isoelectric focusing and SDS gel electrophoresis. Preliminary analysis (data not shown) of these buffer samples did not reveal any change in the pI pattern and the molecular weight (20,000 by SDS/PAGE) of the released protein, suggesting no alteration due to interaction with the polymer. b-STH release determined by RIA closely matched the results obtained by SEC-HPLC (Table 1). Similarly, in a second study, a significant correlation of rb-STH release, as determined by SEC-HPLC, RIA, and CSI, was achieved (Table 2). Correlation between SEC-HPLC, RIA, and CSI supports the release of rb-STH with maintained conformational integrity.

The postrelease devices were washed and lyophilized. The remaining powder was analyzed for both sulfur and nitrogen content. Since the polymer structure contains neither nitrogen nor sulfur, postrelease analysis of the two elements is related to the unreleased protein content. A mass balance

FIG. 1. (Left) SEC of rb-STH before fabrication (A), after 6 h (B), and after 288 h (C). (Right) SEC of zinc insulin before fabrication (A), after 12 h (B), and after 196 h (C).

calculation enabled quantitative determination of the rb-STH remaining within the device and direct comparison to the released protein. Incorporated rb-STH released as determined by SEC-HPLC and RIA and confirmed by postrelease elemental analysis was 92.6%, 88.8%, and 93.5%, respectively.

We also wanted to examine whether rb-STH would decompose within the device. Generally, the release of a protein from a matrix could be viewed as surface protein dissolution followed by polymer erosion/protein release. Therefore, reducing the device thickness should reduce the relative amount of the core "entrapped" protein. As predicted, the thickness of the device affected total protein release. Matrices with different widths were fabricated by using the same die and the same protein concentration (wt/wt). By loading the same die with different amounts of polymer/protein mixture (50, 100, and 200 mg) matrices with different widths were formed. All the protein was released from a 0.4 mm (50 mg) poly(CPP-SA) 20:80 device (CPP-SA, 20:80), while a lower percentage of protein release was observed with an increase in the thickness of the slab (Fig. 2A). This phenomenon could be explained by diffusion of the protein from the device. The thinner device had a greater percentage of the incorporated protein molecules bordering the matrix surface and therefore the surface protein's ability to dissolve increased.

An effect similar to changing the device thickness could be achieved by varying the device hydrophobicity. Increasing polymer hydrophobicity should enhance the surface-eroding ability of the polymeric matrix and limits bulk erosion. A surface-eroding polymeric matrix should reduce water pen-

etration into its core and therefore help protect a water-labile protein from decomposition. As expected, changing the polyanhydride hydrophobicity affected the rb-STH cumulative release. We have previously shown (23) that 1-mm-thick disks of poly(CPP-SA) 20:80 erode completely *in vitro* within <2 weeks and poly(CPP-SA) 50:50 (same thickness) erodes completely in >3 months, while the more hydrophobic poly(CPH) erodes completely in several years. We correlated these different erosion times to the increased hydrophobicity of the monomers: SA < CPP < CPH. For 1.5-mm-thick devices of less-hydrophobic poly(CPP-SA) 50:50, <50% of the total protein was released, while for the very hydrophobic poly(CPH), ~90% of the total protein was released (Fig. 2B). Both SDS gels and guanidine hydrochloride treatment of postreleased devices revealed intact undelivered protein. The appearance of intact rb-STH after reducing and denaturing treatment of the postreleased devices may indicate either a reversible noncovalent aggregation of the protein in the devices due to the presence of moisture and/or hydrophobic polymer or reversible adsorption of the protein to the polymer.

To minimize the possibility of rb-STH aggregation in the device, additives were incorporated. As a model stabilizer we chose sucrose. Several reports have supported the idea that sucrose may stabilize the structure and inhibit the denaturation of proteins (24, 25). Indeed, sucrose doubled the release duration and increased the total amount of rb-STH that was released by 50% (Fig. 2C).

We combined these two parameters—high polymer hydrophobicity and a stabilizer—and incorporated rb-STH with sucrose in poly(CPH) by compression molding. This protein was slowly released for >3 weeks (Fig. 2D). Although there have been reports of active b-STH being released from waxes (26), we report here that active, chemically unaltered, rb-STH was still released intact, as determined by both RIA and conformational immunoassay, from a synthetic polymeric

Table 1. rb-STH released from poly(CPH): Comparison between SEC-HPLC and RIA

Time, h	SEC-HPLC, $\mu\text{g/ml}$	RIA, $\mu\text{g/ml}$
5	152.3	148.5
12	58.2	72.3
48	56.0	42.0
60	23.9	18.5
72	45.3	41.1
144	31.2	24.2
216	24.2	12.6
264	9.7	3.7
288	6.7	3.5
336	10.0	5.8

Table 2. Release of b-STH from poly(CPH): Comparative results of SEC-HPLC, RIA, and CSI

Time, h	SEC-HPLC, $\mu\text{g/ml}$	RIA, $\mu\text{g/ml}$	CSI, $\mu\text{g/ml}$
60	23.9	18.5	23.8
96	32.1	16.5	23.1
264	9.7	3.7	5.9
288	5.4	2.8	2.6

FIG. 2. (A) Effect of increased thickness of the device on cumulative protein release. (B) Effect of device hydrophobicity on cumulative protein release. (C) Effect of excipient (sucrose) on cumulative protein release. (D) Combined effect of high polymer hydrophobicity and sucrose, as an additive, on the release of both rb-STH and insulin.

erodible device after exposure to aqueous media for >2 weeks.

To further investigate the ability of sucrose and hydrophobic polymer to stabilize proteins, we analyzed insulin. SEC of insulin displayed a single peak before device fabrication and for up to 196 h of release (Fig. 1 *Right*). A small peak (<10%) indicative of oligomers was observed after 196 h of release. Using reverse-phase HPLC, insulin prior to device fabrication had a retention time of 6.8 min. The placebo poly(CPP-SA) 20:80 displayed, at first, hydrophobic degradation products at a retention time of 7.9 min and additional hydrophilic degradation products at 4.8 min. The analysis of the release of the insulin from the polymeric matrix did not reveal any peaks in addition to the peaks of intact insulin and the polymer degradation products (Fig. 3). Analysis of released insulin by RIA and SEC-HPLC reveals an excellent

correlation between these two methods (Fig. 4A). Mass balance analysis using elemental analysis supported the HPLC results. Again, the data suggested that there was minimal or no interaction between insulin and polyanhydrides; furthermore, the data suggest conservation of integrity of the conformation of insulin for >198 h of release.

FIG. 3. Reverse-phase HPLC of released insulin before fabrication (A) and of insulin released after 1 (B) and 3 h.

FIG. 4. (A) Comparison of released insulin by RIA and reverse-phase HPLC. (B) Glucose control in urine of diabetic rats by insulin released from poly(CPP-SA) 20:80 and poly(CPH).

As before, we combined these two parameters: high polymer hydrophobicity [poly(CPH)] and a model stabilizer (sucrose). The release of insulin was still noted after 1.5 weeks, with >90% of the insulin originally incorporated in the device being released (Fig. 2D).

To further confirm the results described above, the effect of hydrophobicity of matrices made of biodegradable polymers as carriers for the controlled release of insulin in treating diabetes mellitus was assessed in streptozotocin-induced diabetic rats. The rats were monitored for both blood and urine glucose levels. The high level of glucose was lowered to a normal level with the administration of insulin-impregnated polyanhydrides. Discs made of poly(CPH) containing insulin demonstrated almost 2 weeks of urine and blood glucose control in diabetic rats. On the other hand, devices made of poly(CPP-SA) 50:50, the less hydrophobic polymer, with the same insulin content, demonstrated less than a week of urine and blood glucose control in diabetic rats (Fig. 4B).

At the end of study, the rats were sacrificed and analyzed histologically. The histology was characterized by a small fibrous connective tissue capsule surrounding the implant that was infiltrated with lymphocytes and macrophages. This was a normal mild foreign body reaction as seen previously for similar polyanhydrides and other biocompatible implants (27).

Other methods for protein integrity analysis may be used (e.g., circular dichroism, basic reverse-phase HPLC, quasi-elastic light scattering, nonreduced SDS gel electrophoresis) to extend analysis of the integrity of the incorporated protein.

The release of hydrophilic proteins from polyanhydrides, formulated as described, may possibly be explained by a multiple-mechanism procedure. First, dissolution and diffusion of the surface protein occurs. Second, proteins are released by a combined mechanism of polymer erosion and drug diffusion. Nonetheless, the surface proteins diffuse faster than the rate of polymer degradation. The proteins leave channels at the device's surface and water penetrates through these channels (28). The trapped proteins at the core of the device, which contact water but cannot diffuse through the narrow channels, eventually aggregate. Thus, more hydrophobic devices that prevent water entry and/or thin devices with a greater percentage of protein near the surface of the matrix should minimize aggregation and promote a greater percentage of the incorporated proteins to be released.

CONCLUSIONS

We successfully used the polyanhydrides' changeable hydrophobicity to explore the effect of hydrophobicity on release and activity of two proteins. We established that higher hydrophobicity is advantageous for moisture-labile proteins. This higher hydrophobicity could be achieved by using a very hydrophobic biodegradable poly(CPH). We also demonstrated that different excipients/stabilizers can be an important part of the formulation and can be essential to support and extend the life of sensitive proteins. Finally, the inertness of both polyanhydrides and their degradation products toward specific proteins was established.

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