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Research Paper

Novel Long-Acting Crystal Formulation of Human Growth Hormone

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Purpose. The aim of the study is to solve a significant challenge of extending the half-life of therapeutic proteins using crystalline biopharmaceuticals and without redesigning the molecules.
Methods. Crystals of recombinant human growth hormone were coated with a monomolecular layer of positively charged poly(arginine). The pharmacokinetics and pharmacodynamics of this poly(arginine)-coated human growth hormone crystalline formulation were determined in hypophysectomized rats and monkeys.
Results. Here we have demonstrated for the first time that crystals of human growth hormone coated with positively charged poly(arginine) allowed for *in vivo* pharmacokinetic release profiles of over several days in animal models. The efficacy of this crystalline formulation injected subcutaneously once a week was found to be equivalent to seven daily soluble injections in the standard weight gain assay using the hypophysectomized rat model and in measurement of serum insulin-like growth factor in monkeys. The nonviscous nature of the suspension facilitated easy administration through a fine, 30-gauge needle and should provide for improved patient convenience and compliance.
Conclusions. The approach described here offers an exciting possibility of being broadly applicable to other therapeutic proteins.

KEY WORDS: crystalline hGH formulation; human growth hormone; insulin-like growth factor-1; poly(arginine) adsorption; sustained release; syringeability.

INTRODUCTION

The significant interest in extended release therapeutic proteins is well justified. Extended release formulations can provide greater safety and efficacy and improve patient compliance because of less frequent administration. Techniques used for this purpose include protein engineering, pegylation, glycoengineering, attachment of fatty acids, and fusion with other proteins (1). All these techniques are based on chemical modification and redesign of the molecule and thus create a new molecular entity. As far as regulatory agencies are concerned, this approach, while successful, is not much different from creating a molecule *de novo*.

The other approach, widely popular with small molecules and peptides, employs encapsulation with biodegradable polymers and is very attractive because it does not require the chemical modification of the underlying molecule (2–5). However, the manufacturing processes that are used for encapsulation of proteins are complicated and expensive and result in relatively low loadings of the active drug substance (6). Indeed, the only approved encapsulated protein [recombinant human growth hormone (rhGH)] (7) has recently been withdrawn from the market primarily because of the cost of manufacturing. Because polymeric encapsulation-based approaches rely to a large extent on diffusion control in addition to polymer degradation for slower drug release, the particle sizes tend to be large (about 50 μm) (8) and, as a result, require larger gauge needles for dose administration. For example, Nutropin Depot® (PLGA-encapsulated rhGH) required delivery through a specially designed thin-walled 21-gauge needle (Nutropin Depot® package insert). For patient comfort, compliance, and thus market acceptance, good syringeability and injectability of the drug product through markedly thinner (29–31 gauge) needle is much preferred.

Clearly, technology that will allow for extended release formulations, without the need for redesigning the underlying molecule and using expensive manufacturing processes, would be of great value. We believe that these challenges can be met by employing protein crystallization technology (9–13). Surprisingly, despite many promises and apparent advantages of crystallization, there is only one protein product, insulin, which is manufactured and delivered

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ABBREVIATIONS: μ_E, electrophoretic mobility; ζ, Zeta potential; σ, surface charge density; C, Coulomb; SC, subcutaneous; PK, pharmacokinetic; PD, pharmacodynamic; IGF-1, insulin-like growth factor 1.

in the crystalline form. Although the crystalline suspensions of insulin revolutionized the diabetes therapy by providing sustained release for up to 24 h, protein crystallization has yet to deliver additional extended release products. In general, the high intrinsic solubility of proteins makes crystallization-based long-acting biopharmaceuticals seem counterintuitive. Even co-crystallization of insulin with lipophilically modified insulin derivatives did not extend insulin release beyond 1 day (14). Yet several therapeutic proteins would benefit from much longer release profile. One of these molecules is rhGH, which is approved for treatment of pediatric and adult growth hormone deficiency, Turner syndrome, cachexia in AIDS patients, and others. All current products are administered for the most part by daily injections over several years.

Here we present a crystallization-based approach that allows for delivery of rhGH over a period of several days. This result is achieved by means of crystallization and complexation of polyelectrolytes to rhGH crystals to form polyelectrolyte coated crystals. The molecular coating offers additional control over dissolution of the protein (15) and may become a robust platform for additional molecules.

MATERIALS AND METHODS

Crystallization of Recombinant Human Growth Hormone

Screening

Different crystal morphologies were obtained by hanging drop vapor diffusion method (16). Protein solutions containing 13 mg/mL rhGH in 3.2 mM Tris pH 7.5 were mixed with equal volumes of crystallization reagents and divided into several drops. The screening plates were incubated at 25°C for >1 day. The crystallization reagents were formulated as (A) 8 wt.% PEG-4000, 0.08 M sodium citrate pH 5.6, 8 vol.% 2-propanol, 20 wt.% *n*-dodecyl-β-D-maltoside; (B) 10 wt.% PEG-4000, 0.1 M sodium citrate pH 5.6, 10 vol.% 2-propanol; (C) 20 vol.% ethanol.

Batch Scale-Up

From the initial crystal screening, three rhGH crystallization conditions were identified and successfully scaled up to batch crystallization mode (with modifications).

Crystallization with sodium containing mother liquor. Protein solution containing 35 mg/mL rhGH in 10 mM Tris pH 8.0 was mixed with concentrated mother liquor and deionized water to achieve the following final concentrations: 15 mg/mL rhGH, 100 mM Tris–HCl, pH 8.6, 6% PEG-6000, and 500 mM sodium acetate. The resulting solution was incubated at 33°C for 12–16 h. This condition produces acicular crystals with a yield of over 90%.

Crystallization with zinc containing mother liquor. Protein solution containing 15 mg/mL rhGH in 10 mM phosphate pH 6.1 was mixed with concentrated mother liquor and deionized water to achieve the following final concentrations: 6 mg/mL rhGH, 10 mM phosphate buffer pH 6.1, 10% (v/v) acetone, and 0.08 mg/mL zinc acetate. The resulting solution was incubated at 15°C for 21–24 h. This condition produces hexagonal crystals with a yield of over 50%.

Complexation of Polyelectrolyte to Human Growth Hormone Crystal Surfaces to Form Coated Crystals

Polyelectrolytes were complexed to crystalline human growth hormone (hGH) surfaces to form coated hGH crystals used in this study. To accomplish this, the crystalline suspension from the sodium containing mother liquor was pelleted by centrifugation (10,000 rpm, 2 min at ambient temperature) to remove soluble hGH. This step was performed to prevent complexation of the polyelectrolyte to any soluble, noncrystalline form of hGH remaining in the crystalline suspension. The hGH pellet was then resuspended in complexation buffer (150 mM NaOAc, 25 mM Tris–HCl, pH 8.6, 6% PEG-6000) to a final hGH concentration of 21 mg/mL.

A polyelectrolyte-containing solution in complexation buffer [poly(arginine) (weight-average molecular weight $\{M_w\}$ = 7,500, 4.2 mg/mL) or protamine sulfate (M_w = 4,500, 7 mg/mL)] was added to resuspend the pellet. The weight ratio of protein/polyelectrolyte is 5:1 for poly(arginine) and 3:1 for protamine, which correspond to molar ratios of 1:0.59 for poly(arginine) and 1:1.72 for protamine. The rhGH–polyelectrolyte mixture was then incubated overnight at 4°C. The polyelectrolyte-coated crystals were harvested, and excess polyelectrolytes were removed by washing with 100 mM NaOAc, 25 mM Tris–HCl, pH 7.5, 5% PEG 6000.

Dissolution Testing

An *in vitro* dissolution test was developed to evaluate the ability to extend protein release of both bare and polyelectrolyte-coated rhGH crystals. A 200-μL suspension of 2 mg/mL rhGH crystal was centrifuged at 10,000 rpm for 2.0 min. The supernatant was then removed and subjected to reversed-phase high-performance liquid chromatography (RP-HPLC) analysis to determine the amount of dissolved protein. Dissolution buffer (200 μl; 50 mM HEPES pH 7.2, 140 mM NaCl, 10 mM KCl, maintained at 37°C) is added to resuspend the pellet. The test tube was vortexed until a homogenous suspension is obtained. The centrifugation and resuspension steps were repeated until no visible pellet is formed following centrifugation. Cumulative percent rhGH dissolved was quantitated by RP-HPLC and plotted against the number of washes.

Peptide Mapping of Recombinant Human Growth Hormone

Recombinant rhGH (0.5 mg) was reduced in a solution of dithiothreitol (8 mM, 0.5 mL) for 30 min at 37°C. The cysteine residues were alkylated by treatment with 4-vinyl pyridine (3 μL) for 2 h at room temperature. The sample was dialyzed overnight using a dialysis cartridge (3.5 kDa), against ammonium bicarbonate (0.1 M) and calcium chloride (2 mM). The sample solution was mixed with an equal volume of a solution of 0.1 M ammonium bicarbonate and 4 M urea. Trypsin (~25 μg) was added, and the reaction mixture was incubated at 37°C for 24 h. The digest mixture was analyzed by reverse-phase HPLC using a C8 column (10 cm × 2 mm I.D.; 37°C column temperature, 0.5 mL/min flow). The gradient program was as follows: 95% buffer A [0.1% formic acid, 0.05% trifluoroacetic acid (TFA)] and 5% buffer B

(0.1% formic acid, 0.045% trifluoroacetic acid in acetonitrile) for 5 min; linear gradient to 60% buffer B at 40 min; linear gradient to 95% buffer B at 45 min. Peak detection was at 214 nm.

Zeta Potential

Electrokinetic properties of rhGH crystals were measured using a Malvern ZetaSizer NanoZ instrument. Sample cell type used was DTS1060-Disposable Zeta Cell; samples were run at a temperature of 25°C. Apparent zeta potentials (ζ) were calculated using the Smoluchowski model.

Adsorption Isotherms

Poly(arginine) surface adsorption on rhGH crystals was measured using reverse-phase HPLC. The initial and solution concentrations of poly(arginine) in the slurry of rhGH crystals were quantitated from aliquots of the supernatant after ~16 h using reverse-phase HPLC [Supelco BioWide C5, 50 × 4.6 mm I.D., 30-nm pore diameter, 3-μm particle size; buffer A, 0.1 vol.% trifluoroacetic acid in H_2O; buffer B, 0.1 vol.% trifluoroacetic acid in acetonitrile; gradient, 0–2.5 min (5% buffer B), 2.5–15.5 min (linear gradient to 90% buffer B), 15.5–17 min (90% buffer B); 1 mL/min flow rate; UV detection at 214 nm]. The decrease in solution concentration of poly(arginine) is because of adsorption onto the hGH crystal surface. These numbers were confirmed by subsequent reverse-phase HPLC analyses of the poly(arginine) released upon dissolution of the poly(arginine)-coated hGH crystals. The hGH content was determined by UV absorbance at 280 nm using a specific absorbance coefficient of 0.75 mL mg^{-1} cm^{-1}.

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry

Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) analyses were obtained using an Applied Biosystems Voyager DE PRO Biospectrometry Workstation. Samples were diluted to 10 pmol/μL and then mixed with sinapinic acid matrix (prepared by dissolving 10 mg of sinapinic acid in 1 mL of 50:50 mixtures of 0.1% TFA and 100% MeCN) at 1:1 ratio. Matrix-sample solutions (1 μL) were spotted onto the MALDI plate. Mass spectral calibration used external standards.

Secondary Structure Determinations

Circular dichroism spectropolarimetry was performed on a Jasco Model J810-1505 spectropolarimeter. Fourier transform infrared (FTIR) spectra were measured using a Bruker Tensor 27 spectrometer equipped with a thermostatted Harrick BioATR Cell II micro-ATR accessory. Secondary structure calculations were performed using software supplied with the respective spectrometers.

Electron Microscopy

Scanning electron micrographs (SEM) were measured with Topcon ABT-150F SEM. Samples were coated with either Cr or Pt (~2 nm) using a Cressington Sputter coater, Model 208HR. Scanning electron micrographs were calibrated with a Geller MRS-3 calibration standard (second generation NIST-traceable). Transmission electron micrographs (TEM) were obtained using a JEOL 1210 LaB6 TEM. The rhGH needles were negatively stained with Os using standard techniques. Image analyses were performed using Caldris software.

Animal Studies

All animal studies adhered to the guidelines outlined in the *Principles of Laboratory Animal Care* (NIH publication #85-23, revised 1985).

In Vivo Pharmacokinetic Release Profile in Rats

Hypophysectomized (hypox) rats were used for the *in vivo* studies to investigate the pharmacokinetic (PK) profiles of the coated and uncoated crystals. The test articles were given by subcutaneous injection at the nape of the neck with a 27-gauge needle. Nine rats were tested for each formulation evaluated. The study design included controls for daily soluble administered over a week and vehicle controls for crystalline groups. Blood samples were collected by orbital bleeds at 1, 3, 5, 7, 10, 24, 36, 48, 72, 96, 120, 168, and 240 h. Each serum sample was allowed to clot on ice for a minimum of 30 min and centrifuged at 12,000 rpm at 4°C for 10 min. Serum was decanted onto sterile-labeled cryotubes and then rapidly frozen in liquid nitrogen. The studies were performed in compliance with U.S. Food and Drug Administration Good Laboratory Practices. Female Sprague–Dawley rats, ~8 weeks old and weighing ~120 g, were purchased from Charles River Labs, Wilmington, MA, USA. Rats were housed three per cage and provided water and standard rodent chow *ad libitum* in accordance with standards established by the National Research Council and local Institutional Animal Care and Use Committee (IUCAC) regulations. The rats were allowed to habituate for a period of 15 days prior to entering the study. Serum rhGH was measured by ELISA with commercial kits purchased from Diagnostic Systems Laboratories Inc. (Webster, TX, USA).

Efficacy Testing in Hypophysectomized Rats

Efficacy of rhGH crystalline formulation was assessed by weight gain in a hypophysectomized rat model. Male Wistar rats, 11–13 rats/group, were used for this study. All rats except one group were hypophysectomized (pituitary gland was surgically removed). Nonhypophysectomized group had sham hypophysectomy (surgery was performed, but pituitary gland was not removed). Starting weight of hypophysectomized rats = 89 ± 6.5 g (average ± standard deviation). Subcutaneous (SC) injection was performed with 30-G, 5/16-in. (8 mm) needle. Dosing: seven daily SC injections of soluble growth hormone at 0.8 mg/kg (5.6 mg/kg/week cumulative dose of rhGH); single SC injection of crystalline rhGH suspension or bolus soluble rhGH at 5.6 mg/kg; vehicle controls for crystalline rhGH suspension and soluble rhGH were also administered. A single weekly crystalline rhGH suspension injection induces growth equivalent to seven daily

soluble injections. Each animal's predose body weight was used for calculating percent body weight gain. Error bars are standard error of mean values.

Pharmacokinetic/Pharmacodynamic Analysis in Monkeys

Time action profile of soluble and crystalline rhGH formulations was also assessed in female cynomolgus monkey model. Four monkeys, 4–7 years old, per group were used in this study. Dosing—soluble group: seven daily SC injections of soluble growth hormone at 0.8 mg/kg/day (cumulative dose of 5.6 mg/kg/week); crystalline group: single SC injection of crystalline growth hormone suspension at 5.6 mg/kg/week. Multiple blood samples on the first day and then daily blood samples each morning for 2 weeks were taken. Daily samples were taken prior to daily administrations for soluble group. Bioanalysis of serum GH concentrations and serum insulin-like growth factor 1 (IGF-1) concentrations were performed using commercial ELISA kits from Diagnostic Systems Laboratories Inc. Pharmacokinetic (serum GH concentrations) profile in monkeys of soluble and crystalline formulations: each animal's predose (time 0) GH level was used for calculating elevation above baseline. Error bars are standard error of mean values. Pharmacodynamic (serum IGF-1 concentrations) profile in monkeys of a soluble and crystalline formulations: each animal's predose (time 0) IGF-1 level was used for calculating elevation above baseline. Error bars are standard error of mean values.

RESULTS

Crystallization of Recombinant Human Growth Hormone

The results of rhGH crystallization experiments are shown in Fig. 1. The initial screening of crystallization conditions yielded several crystal forms. The crystallization of the rectangular needle-shaped crystals (Fig. 1a) of approximate dimensions (5–20) $\times$ 0.4 $\times$ 0.4 μm (Fig. 1b) was further scaled up to a gram level. Analytical characterization of crystalline suspensions upon dissolution at 25°C was performed by using sodium dodecyl sulfate/polyacrylamide gel electrophoresis, isoelectric focusing, static light scattering, low-angle laser light scattering (laser diffraction), MALDI-TOF, peptide mapping, FTIR, circular dichroism, reversed-phase HPLC, and size-exclusion HPLC (Fig. 2). These data show no differences in purity and structural integrity between the soluble starting protein used for crystallization and the protein obtained after the dissolution of naked or polyelectrolyte-coated crystals.

It is known that the addition of certain bivalent cations such as Zn^{2+} reduces solubility of both insulin crystals (17) and amorphous precipitates of rhGH (7). Interestingly, the use of Zn^{2+} in conjunction with rhGH crystallization did not produce any significant decreases in dissolution rates of rhGH crystals *in vitro* compared to highly soluble Na^{+} crystals (Fig. 3a). The same effect was confirmed by the pharmacokinetics (PK) studies in rats (Fig. 3b). Therefore,

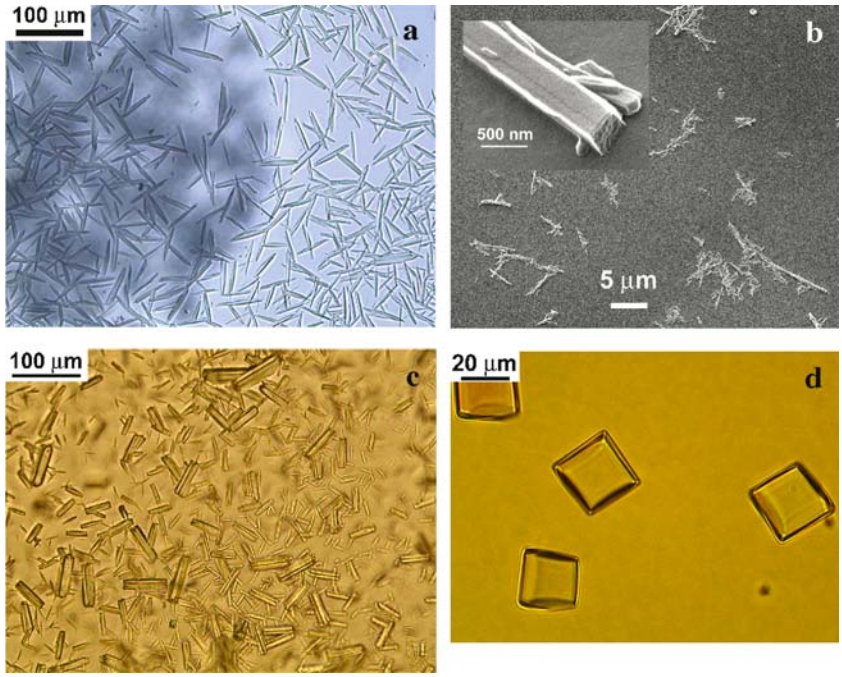


Fig. 1. Different crystal morphologies were obtained by hanging drop vapor diffusion method [light micrographs except (b)]. Protein solutions containing 13 mg/mL rhGH in 3.2 mM Tris pH 7.5 were mixed with equal volume of crystallization reagents and divided into several drops. The screening plates were incubated at 25°C for at lease 1 day. The crystallization reagents were formulated as (a) 8% PEG 4000, 0.08 M Na citrate pH 5.6, 8% IPA, 20% *n*-dodecyl-*b*-D-maltoside; (b) 6% PEG 6000, 0.5 M NaOAc, 0.1 M Tris, pH 8.6 (33°c), scale-up lot (SEM); (c) 10% PEG 4000, 0.1 M Na Citrate pH 5.6, 10% IPA; (d) 20% ethanol. Due to ready scale-up, acicular crystals (panel b) were used throughout this study.

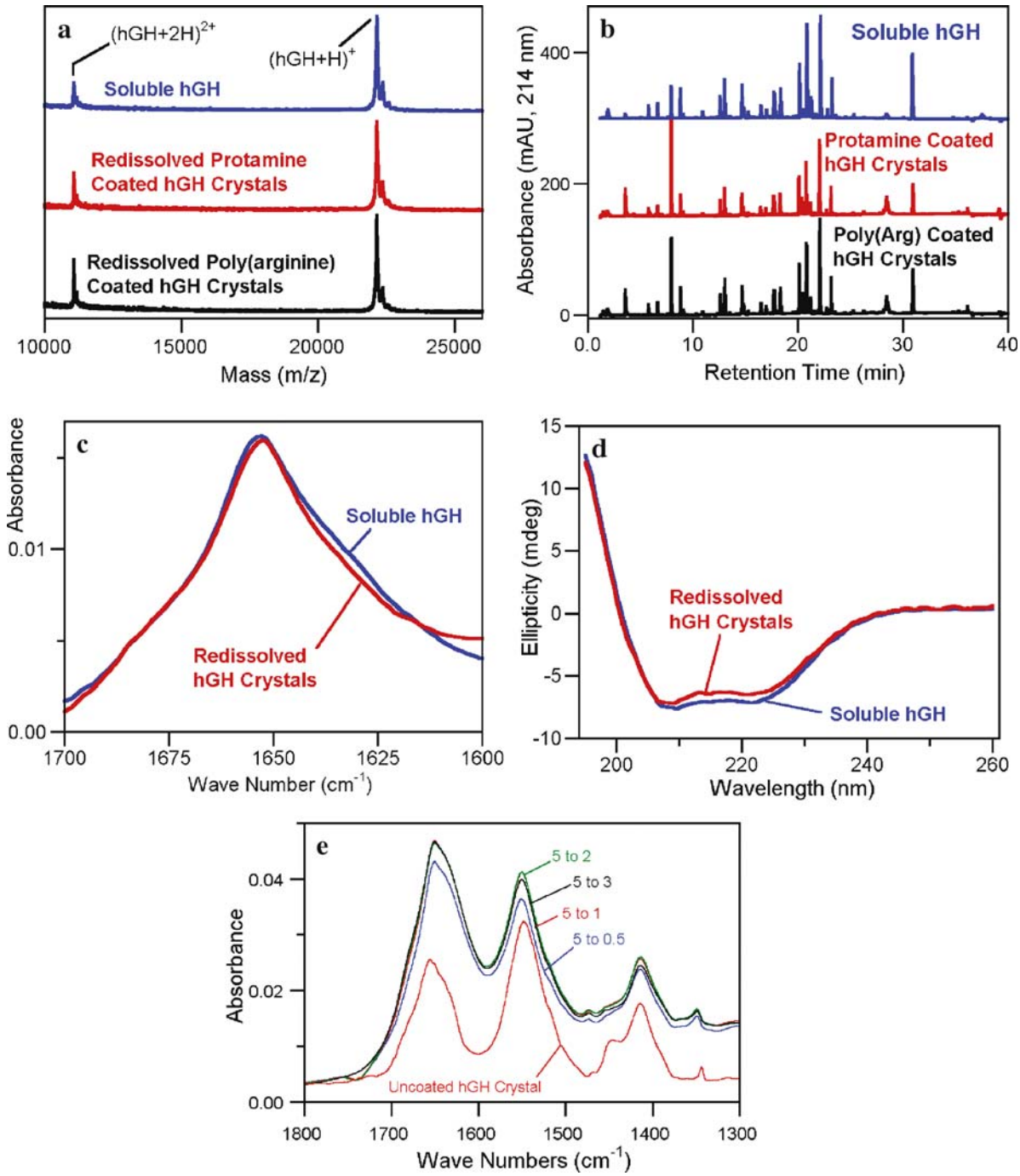


Fig. 2. No changes in primary structure (top row) or secondary structure (bottom row) were discernible upon polycation complexation of rhGH crystals and/or crystallization. (a) MALDI-TOF mass spectrometry; (b) peptide map reverse-phase HPLC; (c) micro-ATR FTIR (amide I region); (d) circular dichroism spectropolarimetry; (e) micro-ATR FTIR spectra showing adsorption of poly-L-arginine onto rhGH crystal surfaces. Buffer spectra subtracted from all spectra. Data confirm zeta potential and poly-L-arginine adsorption data showing saturation of the surface with poly-L-arginine at rhGH to total poly-L-arginine ratio of ca. 5:1 (w/w).

additional means of controlling protein crystal dissolution were needed.

Coating of Recombinant Human Growth Hormone Crystals

We hypothesized that a further reduction in the dissolution rate of rhGH crystals could be achieved by the adsorption of polycations on the negatively charged surface of the rhGH crystals (Fig. 4a). To this end, several polycations, such as poly(lysine), protamine, and poly(arginine), were investigated. Both protamine- and poly(arginine)-coated rhGH crystals exhibited significantly longer dissolution profile both *in vitro* and *in vivo* (Fig. 3). Further studies in several animal models including primates (see

below) demonstrated superior pharmacokinetic (PK) and pharmacodynamic (PD) properties of poly(arginine)-coated rhGH crystals. For this reason, poly(arginine)-coated rhGH crystals were chosen for further investigation.

Analysis of Poly(arginine)-Coated Recombinant Human Growth Hormone Crystals

The process of coating of rhGH crystals was studied by several techniques, such as HPLC, zeta potential (ζ) measurements, FTIR, as well as scanning and transmission electron microscopies. The adsorption of poly(arginine), from solution, onto rhGH crystal surfaces follows classic adsorption isotherms (18,19) and is shown in Fig. 5a. The shape of this adsorption isotherm is consistent with strong adsorption of the poly(arginine) onto the rhGH crystal surface. Electrostatically driven adsorption of the cationic polyelectrolyte onto the negative rhGH crystal surface is fast (completion in less than 10 min), which is consistent with the literature (20), and results in surface charge reversal as shown by electrophoretic mobility (μ_E) or zeta potential (ζ) measurements (Fig. 5b). The saturation is reached at about 5:1 weight ratio of rhGH crystal to total added poly(arginine). The maximum electrophoretic mobility of the coated crystals is ca. $+4 \times 10^{-6}$ $m^2 \cdot V^{-1}$ s^{-1} (apparent zeta potential ζ = +30 mV) and corresponds to an average net surface charge density (σ)[1] at the hydrodynamic shear plane of 4.0×10^{-3} C/m^2 or 0.025 +e/nm^2. These values are comparable to those calculated for the cationic polyelectrolyte adsorption onto *Escherichia coli* cell surfaces (21) and are much less than the expected σ = ~8.5 C/m^2 (~50 +e/nm^2) if all the adsorbed poly(arginine) molecules were fully charged and unscreened by surface counterions.

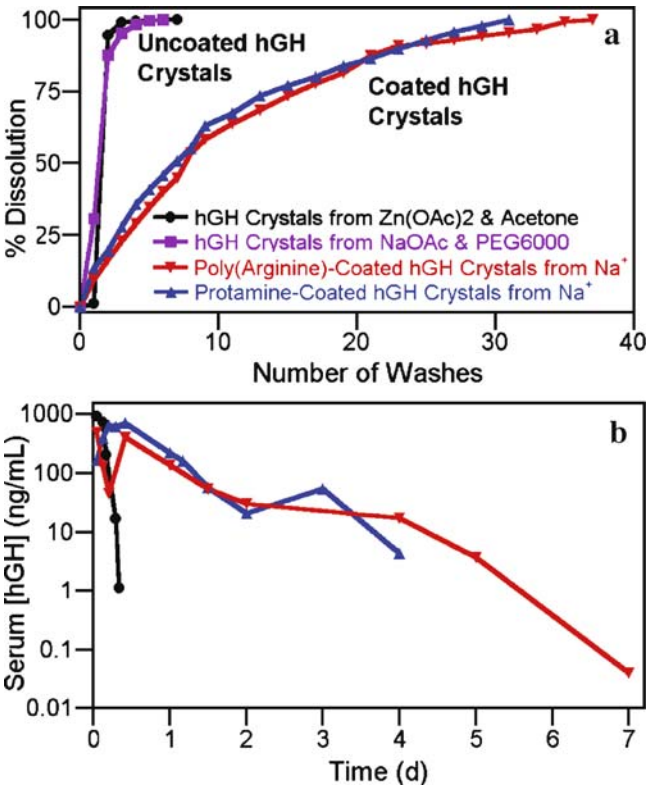


Fig. 3. Dissolution rates of uncoated and coated acicular rhGH crystals using (a) *in vitro* dissolution assays and (b) pharmacokinetics study in rats (see Materials and Method section).

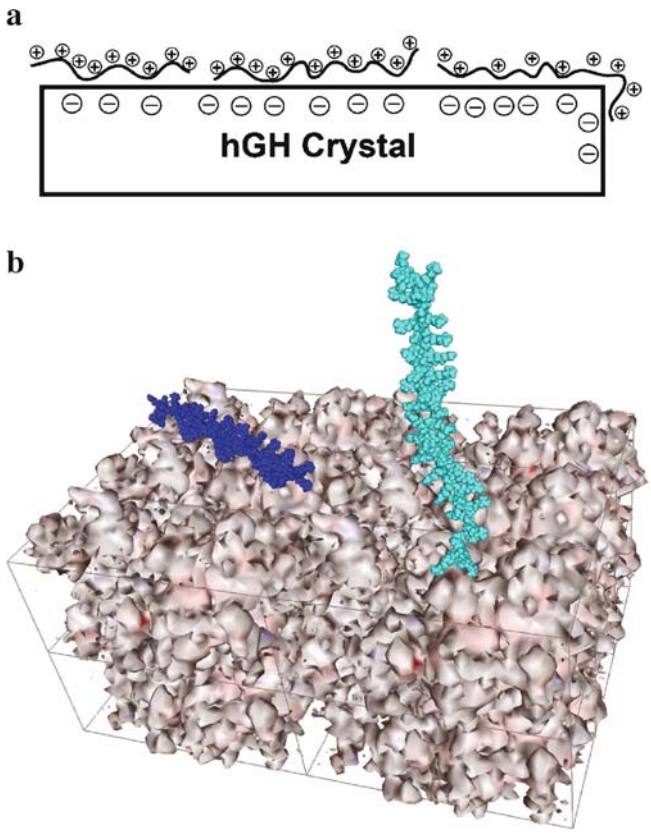


Fig. 4. (a) Schematic diagram of poly-L-arginine adsorption on rhGH crystal surface. (b) Artist rendition of two extreme conformations with poly-L-arginine lying flat (dark blue) or on end (brush phase; turquoise). Zeta potential data support the flat conformation.

The average surface coverages of poly(arginine) in these samples are in the range 1–3 arginine/nm^2 based on reverse-phase HPLC quantitation (Fig. 5a). These data suggest that the poly(arginine) charges are effectively screened by solution counterions. The length of the poly(arginine) used in this work is ~20 nm in the α-helical conformation, which only forms at high pH (>12) where the guanidinium groups are not charged (22).

However, under the solution conditions (pH 7.4), the Debye screening length is ≈2 nm, and these data suggest that the poly(arginine) adopted a two-dimensional flat conformation within this distance from the rhGH crystal

[1] Poly(arginine) with a weight-average molecular weight of ~7,500 corresponds to ca. +50 charges/molecule if all guanidinium groups were protonated. Calculations based on average hGH crystal size of 6 × 0.4 × 0.4 μm determined from light and scanning electron microscopies and laser diffraction particle sizing. Traditional BET surface area measurements were not performed due to expected mechanical destruction of the hGH crystals during the repeated *in vacuo* freeze/thaw cycles at liquid nitrogen temperatures. Even if possible, a BET surface area would not be relevant to these studies as this technique uses very small probes of the surface (e.g., N_2 molecule) and would not yield the area accessible to a linear macromolecule. Coulomb unit ≡ C.

surface (dark-blue, "flat" adsorbed conformation in Fig. 4b). This flat conformation is generally favored under conditions of low solution ionic strength (23). Comparable adsorbed polyelectrolyte two-dimensional layer thicknesses of 1.7 (24) and 0.65–2.5 nm (25) have been reported from small-angle neutron scattering and ellipsometry studies, respectively. Polymer layer thicknesses of ca. >10 nm have been measured with comparable length polymers (26,27).

The saturation of the rhGH crystal surface with poly(arginine) is also confirmed by the electrophoretic properties of the coated rhGH crystals and their FTIR spectra (Fig. 2e). The FTIR spectra are consistent with the adsorption isotherm and ζ studies showing a plateau of the amount of poly(arginine) surface coverage at the ratio of rhGH to poly(arginine) of 5:1. Thus, the data from adsorption, zeta potential, and FTIR experiments are consistent with a monomolecular layer of surface-adsorbed poly(arginine). This surface adsorption of poly(arginine) is fully reversible and does not change the integrity of rhGH molecule (Fig. 2) as well as the two-dimensional crystal lattice (Fig. 5c). The lattice planes are clearly seen in the negatively stained TEM (Fig. 5c) before and after coating with the poly(arginine) (TEM of uncoated rhGH not shown). These thin polyelectrolyte monomolecular coatings[1] (21,28) are below the detection limits of routine high-resolution electron microscopic techniques (ca. 5-nm thickness, Fig. 5c).

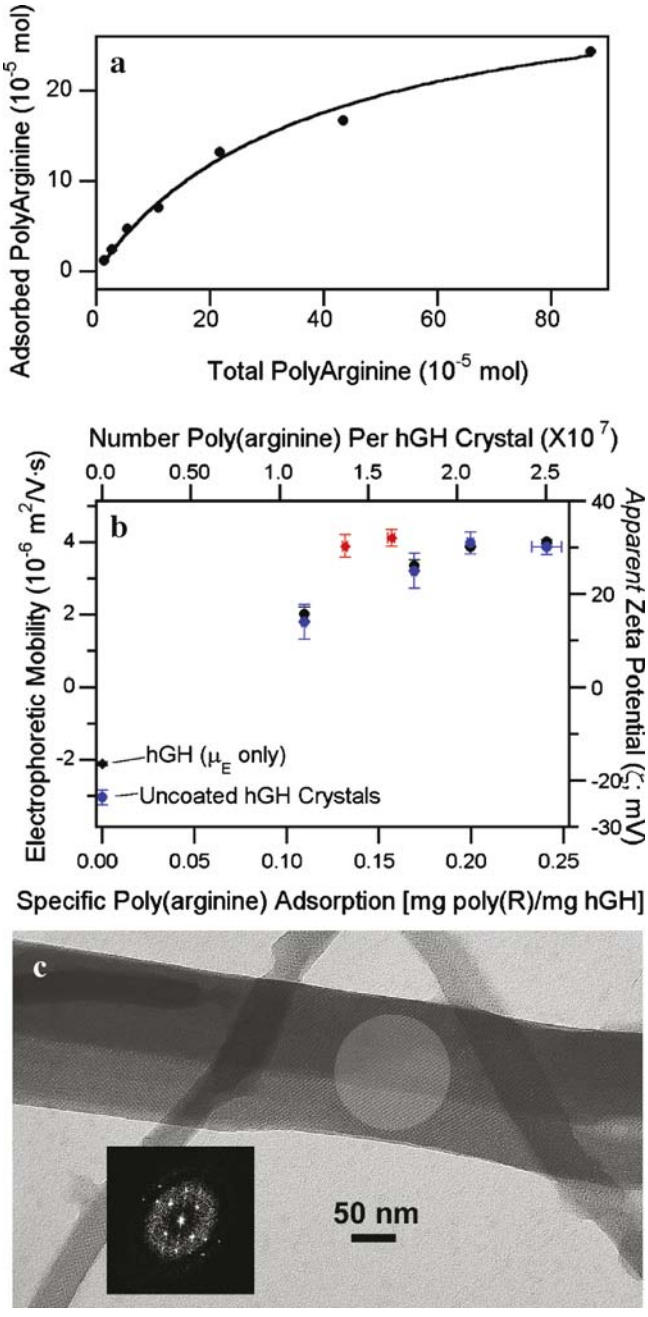


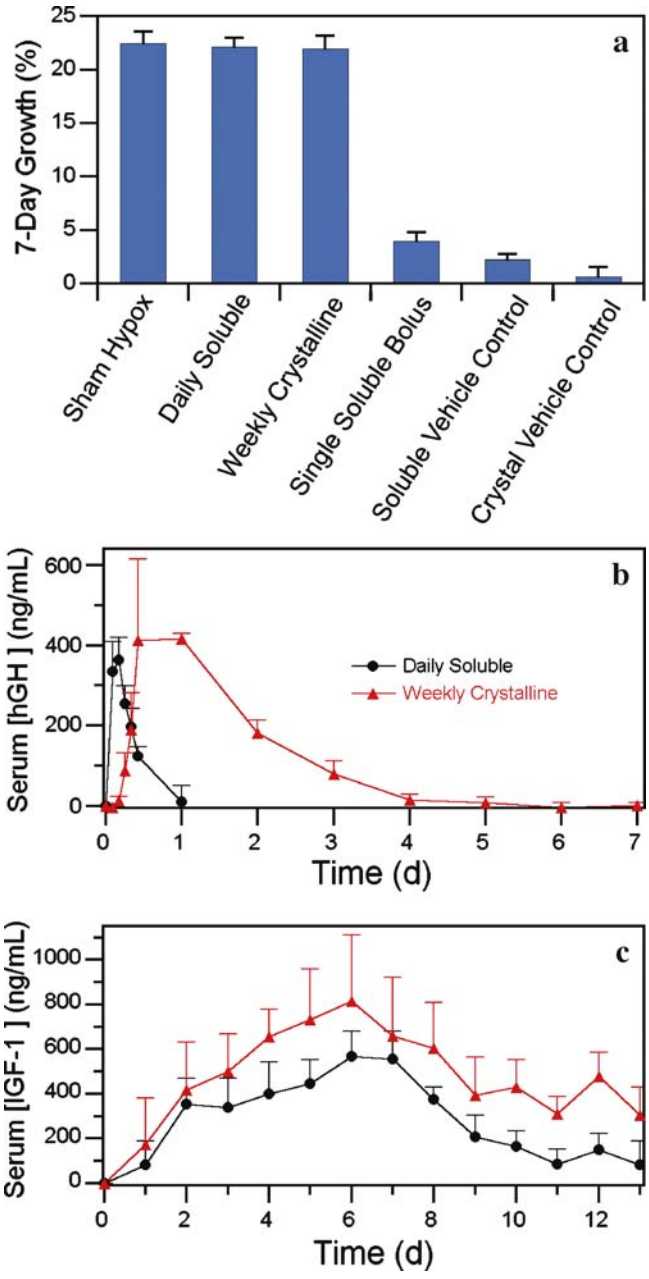
Fig. 5. Physical characterization of poly(arginine) adsorption on rhGH crystal surfaces. (a) Poly(arginine) adsorption isotherm on rhGH acicular crystals (ca. 6 × 0.4 × 0.4 μm). Plateau region is characteristic of saturation of the crystal surface by poly(arginine). (b) Electrophoretic mobility (μ_E) of poly(arginine)-coated rhGH crystals at pH 7.8 in 20 mM Tris–HCl. The apparent zeta potentials (ζ) were based on the Smoluchowski model. Note the negative ζ of uncoated rhGH crystals (rhGH has an isoelectric point pI = 5.4 and $\mu_E = -2.1 \times 10^{-6}$ m^2 V^{-1} s^{-1}). The theoretical numbers of adsorbed poly(arginine) molecules per crystal were calculated based on the above average crystal sizes from low-angle light scattering measurements, microscopy, and SEM. (c) High-resolution transmission electron micrograph monomolecular poly(arginine)-coated rhGH needles (>85 wt.% rhGH) shown with its fast fourier transform (FFT) optical diffraction pattern (circled area; found 2-D unit cell spacings: $a = b$ = 34.4 Å, γ = 90.0°). Similar results were obtained for rhGH crystals prior to poly(arginine) adsorption (not shown). The poly(arginine) coating was not seen in these electron micrographs (resolution limit <2nm) and is consistent with a monomolecular coating. These data also indicate that the polycation adsorption did not involve any changes in crystalline structure.

Pharmacodynamics Study in Hypophysectomized Rat Model

Encouraged by the *in vitro* dissolution (Fig. 3a) and initial PK studies in rats (Fig. 3b), we decided to test the efficacy of coated rhGH crystals in the well-established PD rat model (29). We compared the efficacy of formulations of rhGH when administered subcutaneously either once or daily for seven consecutive days to hypophysectomized male Wistar rats. Soluble rhGH formulations were administered as positive controls, and formulation vehicles were administered as negative controls. Sham hypophysectomized rats served as a control to establish normal rat growth in the absence of hypophysectomy. Hypophysectomized male Wistar and sham surgery male Wistar rats were subcutaneously injected with various formulations either daily for 7 days or given as a single dose. Body weights were recorded for all animals once prior to surgery and shipment, twice during week 2, and daily from day 7 prior to dosing until the end of the observation period on day 14. No mortality, adverse clinical signs, and macroscopic or microscopic findings were noted following the administration of any of the test article formulations (data not shown).

Soluble formulations were administered at a cumulative dose of 5.6 mg/kg. This dose was administered either once on the first day of rhGH administration or daily for seven consecutive days at a dose level of 0.8 mg/kg/day. Poly (arginine)-coated crystals were administered at a single rhGH dose of 5.6 mg/kg.

The poly(arginine)-coated crystal formulation induced growth equivalent to that of seven daily soluble rhGH injections at a dose of 5.6 mg/kg (0.8 mg/kg/day). The comparison of a weekly crystalline (bar 3; Fig. 6a) and a single bolus of soluble μrhGH (bar 4; Fig. 6a) at the same dose of 5.6 mg/kg is especially instructive. Although the crystalline rhGH induced growth equivalent to that measured in sham hypophysectomized rats, the same dose of a soluble rhGH induced growth only slightly higher than that of a control group (Fig. 6a). This observation is consistent with rapid elimination of soluble rhGH from circulation and therefore its inability to contribute toward prolonged efficacy. In contrast, rhGH from crystal formulation is apparently slowly dissolved in the subcutaneous (SC) space and therefore slowly absorbed and provide long time-action profile.



Pharmacokinetic/Pharmacodynamic Analysis in Monkeys

The final step of the investigation was to compare the efficacy of the crystalline rhGH formulation with daily injections of soluble rhGH in a nonhuman primate model. To this end, we conducted a PK and PD study in cynomolgus monkeys. In these studies, juvenile female cynomolgus monkeys age 4–7 years were subcutaneously administered with either soluble rhGH (seven daily injections at 0.8 mg/kg/day dose) or poly(arginine)-coated crystalline rhGH (single administration of 5.6 mg/kg on the first day of dosing). The data obtained during this study have been analyzed by a combination of simple descriptive statistics, compartmental analysis, and noncompartmental analysis to assess the PK performance of the crystalline rhGH formulations. The coated crystalline formulation provided a significantly prolonged PK profile compared to the daily soluble rhGH (Fig. 6b). The C_{max} from the single administration of long-acting crystalline hGH suspension is only 10% higher (C_{max} of 372 ng/mL for daily soluble and 413 ng/mL for crystalline hGH) than C_{max} of the daily soluble hGH administration (i.e., 1/7th of the dose). This low burst release from crystalline hGH avoids potentially problematic serum levels and thus provides safety (comparable C_{max} to daily injection) and efficacy (higher serum GH levels on later days). The coated crystalline formulation has an average t_{max} of about 17 h and a relative bioavailability based on serum rhGH levels of about 85%. The IGF-1 serum level from single weekly poly(arginine)-coated crystalline formulation and seven daily soluble injection was similar (Fig. 6c). This observation suggests that the growth levels that could be

Fig. 6. (a) Efficacy and pharmacokinetics of crystalline suspension formulation of recombinant human growth hormone (rhGH). Panel a: Efficacy of rhGH crystalline formulation assessed by weight gain in a hypophysectomized rat model. Male Wistar rats, 11–13 rats/group, were used for this study. Starting weight of hypophysectomized rats = 89 ± 6.5 g (average ± standard deviation). Subcutaneous (SC) injection performed with 30-G, 5/16-in. (8 mm) needle. Dosing: seven daily SC injections of soluble growth hormone at 0.8 mg/kg (5.6 mg/kg/week cumulative dose of rhGH); single SC injection of crystalline rhGH suspension or bolus soluble rhGH at 5.6 mg/kg; vehicle controls for crystalline rhGH suspension and soluble rhGH were also administered. Each animal's predose body weight was used for calculating percent body weight gain. Error bars are standard error of mean values. (b) Efficacy and pharmacokinetics of crystalline suspension formulation of rhGH. Panels b and c: Time action profile of soluble and crystalline rhGH formulations in female cynomolgus monkey model. Four monkeys, 4–7 years old, per group were used in this study. Dosing—soluble group: seven daily SC injections of soluble growth hormone at 0.8 mg/kg/day (cumulative dose of 5.6 mg/kg/week); crystalline group: single SC injection of crystalline growth hormone suspension at 5.6 mg/kg/week. Panel B: Pharmacokinetic (serum GH concentrations) profile in monkeys of a soluble and crystalline formulations. Each animal's predose (time 0) GH level was used for calculating elevation above baseline. Error bars are standard error of mean values. (c) Efficacy and pharmacodynamics of crystalline suspension formulation of rhGH. Panel c: Pharmacodynamic (serum IGF-1 concentrations) profile in monkeys of soluble and crystalline formulations. Each animal's predose (time 0) IGF-1 level was used for calculating elevation above baseline. Error bars are standard error of mean values.

obtained in this primate system would be comparable between the daily soluble and crystalline formulations.

DISCUSSION

Extending the half-life of the therapeutic proteins without redesigning the molecule is a significant challenge that potentially can be solved by protein crystallization. In general, crystalline proteins provide many potential advantages, such as improved stability during manufacturing, storage and delivery, ease of handling, and varied formulation and delivery options (11). For example, high-dose, low-viscosity formulations of crystalline antibodies have recently been reported (12). However, there has not been much use made of protein crystals for extended release formulations. In fact, the only known commercially available formulation, long-acting insulin, was discovered more than 70 years ago. The high solubility of protein crystals in the interstitial fluid remains the biggest unresolved problem. Several proposed approaches, such as co-crystallization with lipophilically derivatized molecules or specific ligands and complexation with bivalent cations, are specific to insulin and are difficult to transfer to the other molecules (30). We hypothesized that a significant reduction in dissolution rate of rhGH crystals and thus an extended release profile could be achieved by coating the negatively charged rhGH crystals with positively charged polyelectrolytes. This, indeed, turned out to be the case with poly(arginine) molecules. We have demonstrated that rhGH crystals coated with a thin monolayer of poly(arginine) provide extended release of rhGH in animal models. Moreover, one weekly injection of crystalline rhGH seems to be as effective (similar IGF-1 serum levels) as seven daily injections of the soluble formulation at a matched dose.

The proposed approach is readily implemented. It consists of crystallization of rhGH followed by adsorption of poly(arginine) onto the crystal surface. Both steps are conducted in aqueous solutions, are fully reversible, and do not change the integrity and structure of the underlying molecule. The proposed formulations of poly(arginine)-coated rhGH crystals contain >80 wt.% of rhGH, have low viscosity (5 cP), and were easily injected through fine needles (29–31 gauge). Preclinical studies in rats and monkeys have shown that these injections are well tolerated. In contrast, protein microencapsulation technology generally uses a vast molecular excess of polymers and requires organic solvents, low temperature, and sophisticated engineering scale-up (6). Moreover, the low loading of rhGH in microspheres and relatively large particle size leads to high viscosity of the formulation and necessitates administration through a large diameter needle. This, in turn, results in local irritation and pain (31) and may lead to poor patient compliance.

CONCLUSION

The crystal coating process proposed here is based on electrostatic interactions between opposite charges of a crystal surface and an appropriate polyelectrolyte. We believe that this approach is generally applicable to a variety of therapeutic proteins (32). The work on other targets is underway in our laboratories.

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