

Ultra-Rapid Absorption of Recombinant Human Insulin Induced by Zinc Chelation and Surface Charge Masking

Roderike Pohl, Ph.D.,¹ Robert Hauser, Ph.D.,¹ Ming Li, Ph.D.,¹ Errol De Souza, Ph.D.,¹
Robert Feldstein, B.A.,¹ Richard Seibert, B.S.,¹ Koray Ozhan, M.S.,²
Nandini Kashyap, M.S.(Pharm.),³ and Solomon Steiner, Ph.D.⁴

Abstract

Background:

In order to enhance the absorption of insulin following subcutaneous injection, excipients were selected to hasten the dissociation rate of insulin hexamers and reduce their tendency to reassociate postinjection. A novel formulation of recombinant human insulin containing citrate and disodium ethylenediaminetetraacetic acid (EDTA) has been tested in clinic and has a very rapid onset of action in patients with diabetes. In order to understand the basis for the rapid insulin absorption, *in vitro* experiments using analytical ultracentrifugation, protein charge assessment, and light scattering have been performed with this novel human insulin formulation and compared with a commercially available insulin formulation [regular human insulin (RHI)].

Method:

Analytical ultracentrifugation and dynamic light scattering were used to infer the relative distributions of insulin monomers, dimers, and hexamers in the formulations. Electrical resistance of the insulin solutions characterized the overall net surface charge on the insulin complexes in solution.

Results:

The results of these experiments demonstrate that the zinc chelating (disodium EDTA) and charge-masking (citrate) excipients used in the formulation changed the properties of RHI in solution, making it dissociate more rapidly into smaller, charge-masked monomer/dimer units, which are twice as rapidly absorbed following subcutaneous injection than RHI (T_{max} 60 ± 43 versus 120 ± 70 min).

Conclusions:

The combination of rapid dissociation of insulin hexamers upon dilution due to the zinc chelating effects of disodium EDTA followed by the inhibition of insulin monomer/dimer reassociation due to the charge-masking effects of citrate provides the basis for the ultra-rapid absorption of this novel insulin formulation.

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Author Affiliations: ¹Biodel Inc., Danbury, Connecticut; ²Louisiana State University, Baton Rouge, Louisiana; ³Pii, Hunt Valley, Maryland; and ⁴Steiner Ventures, Mt. Kisco, New York

Abbreviations: (DLS) dynamic light scattering, (ECF) extracellular fluid, (EDTA) ethylenediaminetetraacetic acid, (RHI) regular human insulin, (RI2) recombinant human insulin at pH 2, (RI7) recombinant human insulin at pH 7

Keywords: analytical ultracentrifugation, dynamic light scattering, recombinant human insulin, ultra-rapid absorption

Corresponding Author: Roderike Pohl, Ph.D., Biodel Inc., 100 Saw Mill Rd., Danbury, CT 06810; email address rpohl@biodel.com

Introduction

Insulin solutions are composed of mixed populations of monomers, dimers, and hexamers that are in dynamic equilibrium. The amount of each species in solution is dependent on the concentration and pH of the solution.¹⁻⁵ At neutral pH and/or high concentration (>0.1 mM), this equilibrium favors the zinc-stabilized hexamer (molecular weight ~36 kDa), while diluted (10^{-5} to 10^{-8} M) or at low pH, the monomer is prevalent.⁶

The rate of subcutaneous insulin absorption is dependent on the population of monomers/dimers available for absorption. Postsubcutaneous injection, dilution with extracellular fluids (ECFs) shifts the dynamic equilibrium toward the more rapidly absorbed monomers (~5800 Da).^{7,8} Rapid-acting insulin analogs achieve rapid absorption by reengineering the insulin molecule to dissociate quickly by changing the amino acid sequence of the polypeptide chain.⁹⁻¹¹ It is the objective of the new ultra-rapid-acting human recombinant insulin formulation (BIOD-095) to enhance the rate of subcutaneous absorption by increasing the rate of monomer formation postinjection by using excipients that (1) enhance the dissociation of the hexamer through zinc chelation, (2) maintain the insulin in the form of monomers/dimers by reducing reassociation around ubiquitous divalent cations, and (3) neutralizing surface charge to hasten intracellular absorption.

BIOD-095 is a formulation of recombinant human insulin that uses a chelating agent, disodium ethylenediaminetetraacetic acid (EDTA), in conjunction with citric acid or its salt (sodium citrate) in order to sequester zinc, thereby destabilizing the insulin hexamer. After injection and dilution in the subcutaneous milieu, BIOD-095 rapidly disassembles into smaller monomeric/dimeric subunits. The citrate ions added to the formulation inhibit re-association of the monomers and reduce surface charge. The ultra-rapid onset of action of this formulation has been demonstrated in clinical studies.¹²⁻¹⁵

In order to elucidate the differences between BIOD-095 and regular human insulin (RHI), dynamic light scattering (DLS)¹⁶ and analytical ultracentrifugation¹⁷⁻²⁰ were used to characterize the dissociation of insulin hexamers following dilution in a buffer composed of the salts found in ECF, pH 7.4. In addition, the association of citrate with insulin was characterized using resistance measurements.

The goal of the present analysis was to simulate insulin dissociation *in vitro* to characterize the rate of appearance

of smaller monomeric/dimeric insulin subunits available for ultra-rapid absorption *in vivo*.

Research Design and Methods

The two primary test articles used in these studies were BIOD-095 (VIAject® 100 IU/ml, pH 4; 1.8 mg/ml citric acid, 1.8 mg/ml disodium EDTA, 22 mg/ml glycerin, 3 mg/ml m-cresol, 100 U/ml recombinant human insulin; Diosynth, Netherlands) and RHI (Humulin® R, Eli Lilly, Indianapolis, IN).²¹ Control solutions were made with pure recombinant RHI in water with pH adjusted to 2 (RI2) with HCl or 7 (RI7) with NaOH.

Resistance of Formulations

The association of citrate to insulin was analyzed by first measuring the resistance of water containing either insulin (MR_1), citrate (MR_2), or citrate and insulin (MR_t). Due to the limited solubility of insulin in pure water, MR_1 was extrapolated from measured resistance values of dilute insulin solutions. The total predicted resistance (PR_t) of the solution (insulin with trisodium citrate) was calculated using the equation $1/PR_t = 1/MR_1 + 1/MR_2$. The ratio of measured/predicted resistance was used as an indication of charge neutralization. A ratio equal to 1 would imply there is no association of the citrate to the insulin, while values greater than 1 demonstrate a reduction in the charge of the solution. The reduction in the charge of the solution (or increase in resistance) is most likely due to the association of insulin with citrate ions, reducing the overall conductivity of the solution.

Dynamic Light Scattering Particle Size Analysis

Particle size analysis was done using a Malvern Zetasizer Nano (Model Zen 1600, Malvern instruments, Malvern, UK). Following the initial full-strength analysis, a dilution series was performed from 1:2 to 1:16 in a buffer having similar pH and buffering capacity of ECF (0.7 mM magnesium chloride, 1.2 mM calcium chloride, 2 mM potassium chloride, 2 mM monopotassium phosphate, 0.5 mM sodium sulfate, 104 mM sodium chloride, 28.3 mM sodium bicarbonate; pH 7).²²

The procedure for size analysis was to filter 1 ml of each sample, using a low protein binding 0.2 μ m filter (Pall #4192, Pall Life Sciences, Ann Arbor, MI), into a square aperture glass cuvette. The cuvette was wiped dry and inserted into the sample holder. Instrument settings were protein (refractive index 1.45, absorption 0.001), dispersant = water

(refractive index 1.330, viscosity 0.8872 cp at 25 °C), temperature 25 °C, with 2 min equilibration and duration of measurement (automatic). A total of seven measurements were made per sample. Data processing was done using the general-purpose mode to determine a mean average volumetric size distribution (nm) of the insulin complexes in solution. The mean of at least two samples was used as a basis for comparison and was reported as mean \pm standard deviation in **Figure 1**.

Analytical Ultracentrifugation

A similar set of experiments was developed using analytical ultracentrifugation, which determines an estimate of the weight-averaged sedimentation coefficient [Svedbergs 20 °C, water S(20,w)], which is proportional to the buoyant effective molar weight. Sedimentation velocity analysis was conducted at 20 °C and 55,000 RPM using interference optics with a Beckman-Coulter XL-I analytical ultracentrifuge (University of Connecticut Analytical Ultracentrifugation Facility; Beckman Coulter, Brea, CA). Double sector synthetic boundary cells equipped with sapphire windows were used to match the sample and reference menisci. The rotor was equilibrated under vacuum at 20 °C, and after a period of ~1 h at 20 °C, the rotor was accelerated to 55,000 RPM. Interference scans were acquired at 50 s intervals for 4.5 h.

The interference optical system requires diluting the protein using a solution with the exact composition of the formulation, without the protein. The diluent for RHI was generated directly from the commercial preparations by filtering the formulation through a Centriprep filtration device with a molecular weight cutoff of 3 kDa (Centriprep YM-3, 3 kDa NMWL, Millipore, Billerica, MA). The filtrate was checked for protein content and confirmed insulin-free diluents were used to dilute the commercial product.

The diluent for BIOD-095 was prepared as a large batch and split into two portions, with 100 U/ml insulin added to one of the two portions. Included in the analysis were two insulin solutions composed of pure recombinant human insulin, made as a monomer control in 0.01 N HCl (RI2) or brought up to pH 7 with NaOH for a hexamer control (RI7). These first data sets were used to characterize the insulin as either a stable single species or one that changes from hexamer to dimer and/or monomer in its own diluents (**Figures 2, 3A and 4A**).

A second set of data was acquired to assess the behavior of the various insulin formulations in the presence of

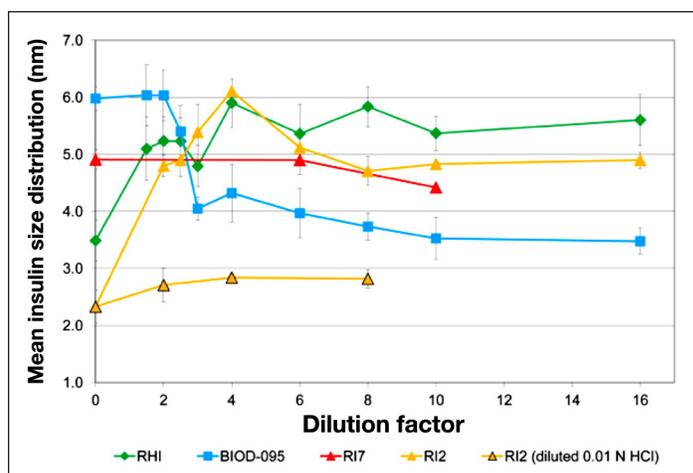


Figure 1. Insulin size distributions following dilution with ECF. BIOD-095 (blue square), RHI (green diamond), RI7 (red triangle), RI2 (orange triangle, ECF dilution; black-lined orange triangle, 0.01N HCl dilution). Data represent the mean \pm standard deviation.

ECF buffer. To accomplish this, an initial dilution was made with the undiluted commercial preparation and ECF. Then further dilutions were made with a blend of the diluents: ECF in the same ratio. These experiments were intended to mimic the postsubcutaneous injection environment (**Figures 3B and 4B**).

Experimental Design

The first set of dilution experiments was done using the exact diluents of each of the formulations at 1:2, 1:6, and 1:20. Formulations studied were RHI, BIOD-095, RI2, and RI7.

The second set of experiments was done using ECF buffer to simulate dilution postsubcutaneous injection. This experiment was specially adapted for BIOD-095, since a 1:2 dilution with ECF brought the pH to the isoelectric point, creating a precipitate. Therefore the first dilution was 1:4 (BIOD-095:ECF) to directly transition the formulation to physiological pH. Subsequent dilutions to ~1:8 and 1:20 were done with this 25:75 (BIOD-095 diluent:ECF) buffer. The first dilution for RHI (pH 7) was 1:2 using ECF. Subsequent dilutions were done with a diluent composed of 50:50 (RHI diluent:ECF) at 1:6 and 1:20.

Data Analysis

The first analysis was done using the program DcDt+ (version 2.1.0),^{23,24} which provides a model-independent, sedimentation coefficient distribution, $g(s^*)$, analysis using the time derivative of the concentration profile.²⁵ If there is no shift to higher sedimentation coefficient values with an increase in concentration, it is considered strong evidence that there are no reversible reactions occurring

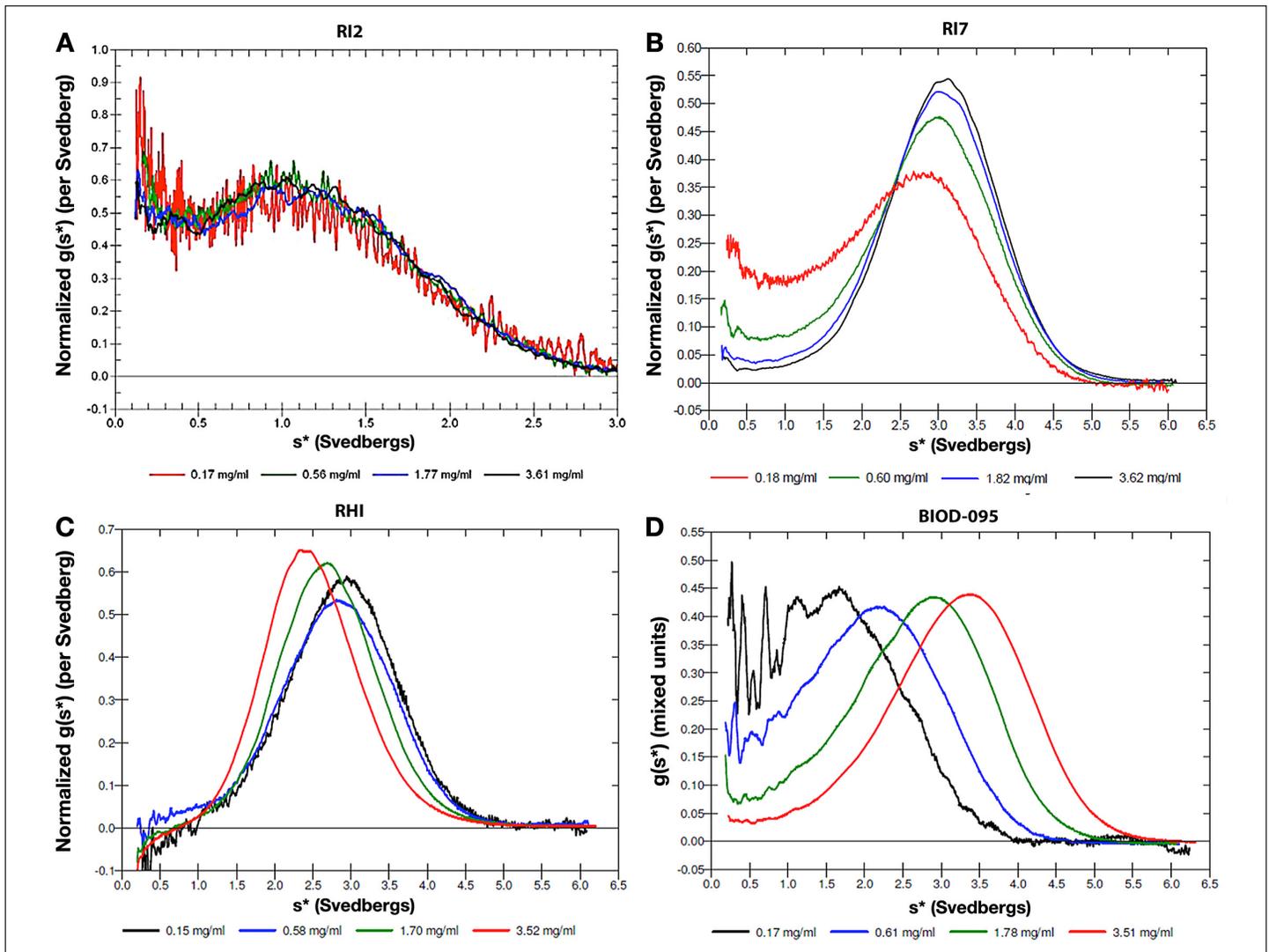


Figure 2. Comparison of the model-independent sedimentation coefficient distribution $[g(s^*)]$ analysis using the time derivative of the concentration profile for (A) RI2, (B) RI7, (C) RHI, and (D) BIOD-095. Concentrations of insulin solutions (in diluents) are ~ 3.5 mg/ml (undiluted), ~ 1.75 mg/ml (1:2), ~ 0.6 mg/ml (1:6), and ~ 0.17 (1:20).

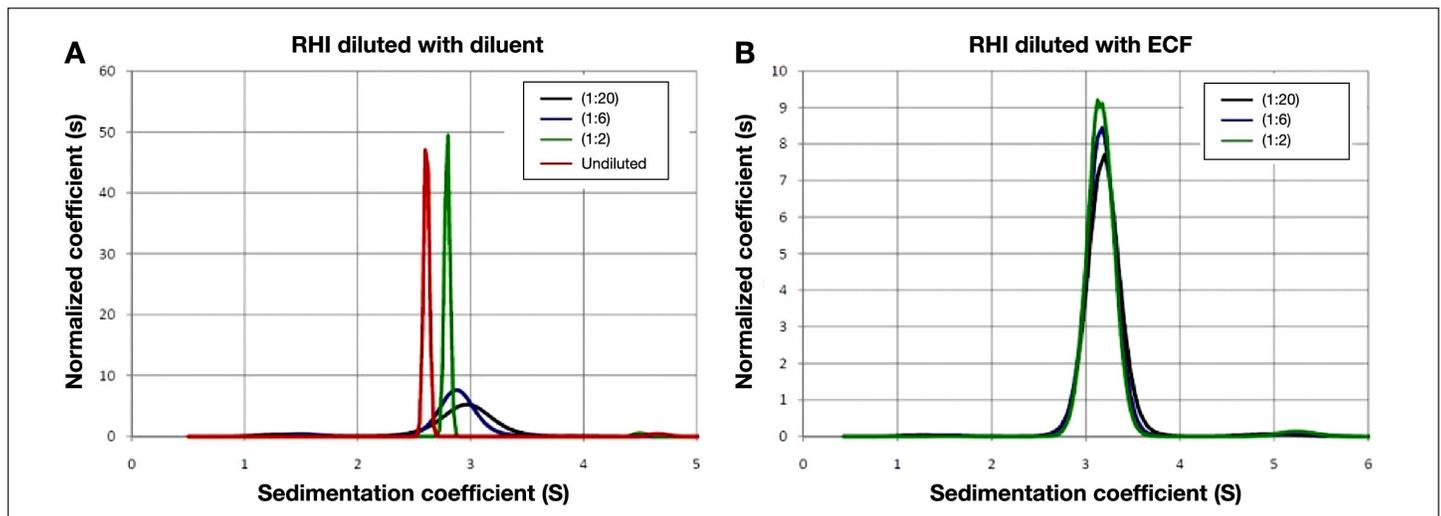


Figure 3. Graph of the normalized continuous sedimentation coefficient versus sedimentation coefficient (S) following dilution of RHI in (A) its own diluents and (B) ECF buffer. The dilutions shown are undiluted (red), 1:2 (green, 1.72 mg/ml), 1:6 (blue, 0.55 mg/ml), and 1:20 (black, 0.18 mg/ml).

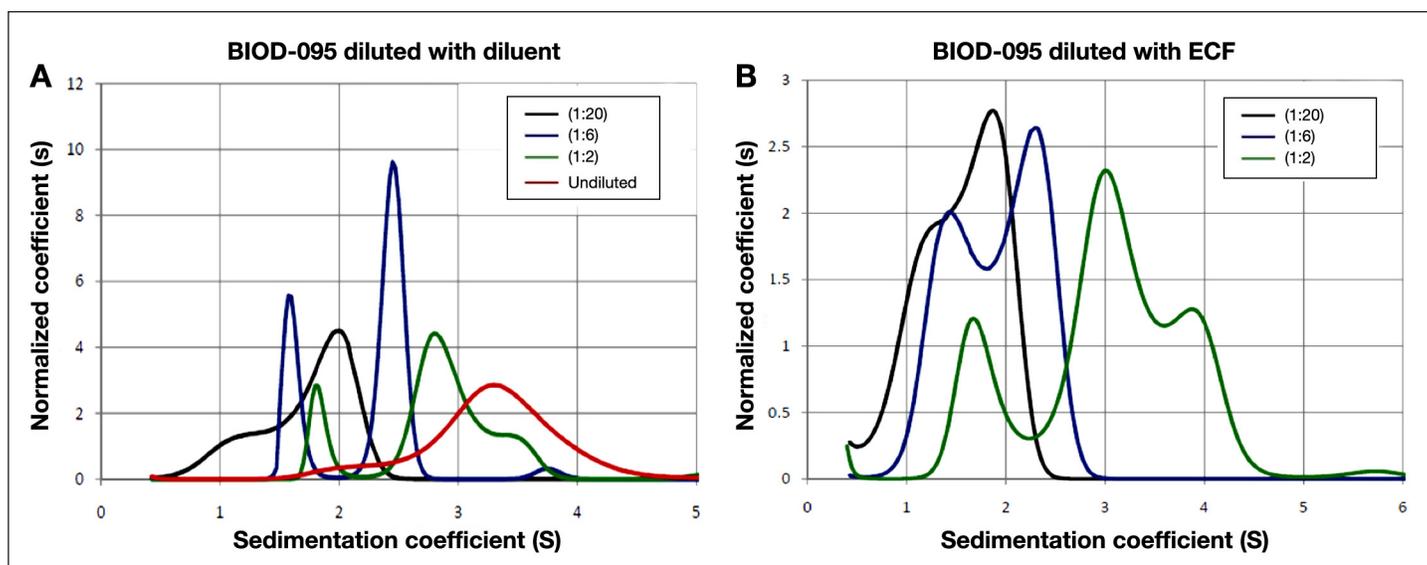


Figure 4. Plot of the continuous sedimentation coefficient versus sedimentation coefficient (S) following dilution of BIOD-095 in diluents and ECF buffer. **(A)** The dilutions are undiluted (red, 3.5 mg/ml), 1:2 (green, 1.78 mg/ml), 1:6 (blue, 0.61 mg/ml), and 1:20 (black, 0.17 mg/ml). **(B)** The dilutions are 1:4 (green, 0.94 mg/ml), ~1:8 (blue, 0.42 mg/ml), and 1:20 (black, 0.19 mg/ml).

(i.e., monomers and/or dimers forming hexamers).²⁶ Therefore, in the case of RHI, which is a stabilized hexamer throughout these dilutions, the molecular weight may be estimated. If the size and shape are changing on dilution (shifting from hexamer to dimer to monomer), it is not possible to determine an estimate of the molecular weight, but useful information can be obtained from the Sedfit program on the sedimentation coefficient $S(w)$ (Sedfit version 11.3b). Sedfit is a direct boundary modeling program for individual data sets using a model-based numerical solution to the Lamm equation.²⁷ It plots the continuous sedimentation coefficient $c(s)$ versus sedimentation coefficient (S) to produce curves that describe relative sizes of the sedimenting species. The $c(s)$ distribution plots are sharpened relative to other analysis methods because the broadening effects of diffusion are removed by use of an average value for the frictional coefficient. This model is only applicable to noninteracting mixtures, but in the case of interacting species such as insulin, it can yield an idea of what species (monomer, hexamers, dimer) are present in the solution.

Results

Resistance Values

The resistance of the individual excipients in solution permits the combined resistance of the mixture to be predicted if each of the excipients continues to act independently, unaffected by the presence of the other excipients. If there is an interaction, the total ion population available to contribute to the final resistance is reduced by weak charge binding. Therefore a combined measured resistance (MR_t) higher than predicted (PR_t) from the individual contributions from each excipient (MR_1 , MR_2) reveals a charge/charge interaction (**Table 1**).

The MR_t/PR_t ratio value of 1.8 indicates there was considerably more resistance in the solution than predicted by the sum of conductivity of the individual components. Therefore a significant binding affinity and charge-masking effect is present when the trisodium citrate and insulin are combined in solution. Because no irreversible changes were observed, binding was most

Table 1.
Comparison of Resistance of Excipient Components in Water^a

Trisodium citrate concentration (mg/ml)	Insulin concentration (IU/ml)	Insulin MR_1 (Ω)	Trisodium citrate MR_2 (Ω)	Insulin + trisodium citrate MR_t (Ω)	Insulin + trisodium citrate PR_t (Ω)	Ratio MR_t/PR_t
1.0	100	342.6	191.1	221.1	122.7	1.8

^a MR_1 , measured resistance insulin (extrapolated); MR_2 , measured resistance trisodium citrate; MR_t , total measured resistance; PR_t , total predicted resistance ($1/PR_t = 1/MR_1 + 1/MR_2$).

likely electrostatic, which masks or shields the local charge distribution on the insulin molecule.

Dynamic Light Scattering

The control insulin solutions at pH 2 and pH 7 were first analyzed undiluted to determine the average size of an insulin monomer (RI2) and hexamers (RI7). Dilution of acidic formulations in ECF buffer will shift the pH to 7, while pH 7 formulations will remain at physiological pH throughout the dilution series. The undiluted RI2 had a size of 2.3 ± 0.3 nm, consistent with the size of an insulin monomer. When diluted in ECF (pH 7), the size of RI2 rapidly increased to the hexameric size range, similar to RI7. However, RI2 dilution in 0.01N HCl did not further reduce the mean insulin size, confirming monomers were present at these dilutions (**Figure 1**). Undiluted RI7 had a size of 4.91 ± 0.17 nm, the approximate size of a hexamer.⁶ This “unstabilized” insulin solution at pH 7 starts to slightly decrease in size after a 1:10 dilution in ECF buffer, consistent with hexamers starting to dissociate into smaller subunits.

Undiluted BIOD-095 is larger than RI7, possibly indicating an association of citrate and/or disodium EDTA around the molecule. However, with a 1:3 dilution, the mean size of BIOD-095 was reduced by 2 nm, indicating rapid dissociation. Undiluted RHI appears smaller but grows in size to greater than 5 nm once diluted 1:2 and remains this size to a dilution of 1:16. Based on this data, it is hypothesized that 100 U/ml RHI is a stabilized compact hexamer, which, on dilution, becomes less compact.

Analytical Ultracentrifugation

The plots in **Figure 2** represent the sedimentation coefficient distribution profiles of RI2, RI7, RHI, and BIOD-095 in their own diluents. This provides an indication of whether there are interacting or noninteracting species in solution. The RI2 does not show a shift toward higher values of *S* with an increase in concentration, indicating there are no interacting species in this dilution range. This is consistent with the light-scattering results, confirming the smallest monomeric insulin subunit is present in undiluted RI2. The RI7 shows a shift to the left at the 1:20 dilution, while BIOD-095 has a left shift at all dilutions. The left shift indicates that there are interacting species in these solutions (monomers/dimers/hexamers).²⁸

In contrast, RHI does not show a shift toward higher values of *S* with an increase in concentration. This is strong evidence that there are no reversible reactions occurring over the concentration range covered by the samples. The peak shift upon dilution toward higher

values of *S* can be attributed to hydrodynamic nonideality, which arises from both the increased viscosity at higher concentrations of the protein and the solvent displacement by the sedimenting particles. Since RHI was a noninteracting species, it was possible to estimate its molecular weight of 34.75 kDa (DcDt+ software), which is in agreement with the molecular weight of insulin hexamers. Therefore the sedimentation coefficient value of ~ 3 S(w) corresponds to hexameric insulin species in solution.

The results from the c(s) analysis are graphically presented in **Figures 3** and **4**. The purpose of these analyses was to measure the effect of dilution with diluent and ECF buffer on sedimentation, using the direct boundary modeling program for individual data sets (Sedfit program). The model is useful in the case of interacting species (i.e., monomer, dimer, hexamer) and can provide an indication of which species are present in solution. The results from the analysis of RHI diluted with diluent and ECF are shown in **Figures 3A** and **3B**.

BIOD-095 was evaluated under similar experimental conditions, and the analyzed data are shown in **Figure 4**. The data show distributions consistent with smaller insulin forms: tetramers, dimers, and monomers at the 1:4 dilution in ECF. On further dilution, the insulin shifts to the smaller forms. Overall, the ultracentrifugation results are consistent with the size distributions determined from the light-scattering experiments (**Figures 1** and **4**).

The sedimentation coefficient values corresponding to the ECF dilution series are graphed in **Figure 5**. Regular human insulin essentially remains unchanged at ~ 3 S(w) across the dilution range. BIOD-095 shows a distinct drop in average S(w) at all dilutions, indicating a shift in the equilibrium of the interacting species toward the smaller monomer/dimers species. The RI2 (1.28 S(w)) and RI7 (3.1 S(w)) were not diluted with ECF but were provided here for reference. These observations are consistent with the light-scattering results.

Discussion

These analyses investigated the mechanism of rapid absorption of BIOD-095 (VIAject 100 U/ml, pH 4). This formulation has been shown in clinical studies to have a faster absorption rate than commercially available RHI and insulin lispro (Humalog[®]) following subcutaneous injection.¹³ The putative rate of absorption is enhanced by the chelation of zinc by disodium EDTA, creating a rapid dissociation of the insulin hexamers into more rapidly

absorbed insulin subunits. In addition, the association of citrate and possibly disodium EDTA with the insulin molecule reduces electrostatic binding forces, providing steric hindrance to hexamer reassociation postinjection.

To test these hypotheses, the molecular size of insulin was determined using DLS and analytical ultracentrifugation. Control solutions of pure recombinant human insulin under acidic conditions (monomer) and physiological pH (hexamer) were first measured without dilution to verify insulin monomer and hexamer size. According to Brange and coworkers,⁶ the approximate sizes of hexamers and monomers are $2.5 \times 2 \times 3$ nm (monomer) and 5×3.5 nm (hexamer). The measured volumetric size distributions of the control insulin solutions in our study were 2.3 nm (monomer) and 4.9 nm (hexamer), which are very close to reported estimates.

The measured sizes of undiluted BIOD-095 and RHI were not consistent with the insulin hexamer control. Undiluted BIOD-095 appeared larger than a hexamer (~6 nm), implying an association of the excipients (citric acid and disodium EDTA) with the insulin. On dilution with ECF, BIOD-095 quickly reduced in size to the dimer range, consistent with its rapid absorption *in vivo*. Regular human insulin, considered to be a stable hexamer, was measured at ~3.5 nm, noticeably smaller than the hexamer size of ~5 nm. The ECF dilution of RHI showed an increase in size to ~5 nm, consistent with the size of a hexamer, and did not decrease in size following dilution to 1:16 (Figure 1). The persistence of the hexamer following dilution is consistent with the slower subcutaneous absorption rate of RHI.

Analytical ultracentrifugation confirmed the DLS observations and discerned additional differences between the two formulations. This technique uses several dilutions of insulin to detect changes in the species (monomer, hexamer) using calculated Swedberg constants $S(w)$. Using this methodology, the control monomer solution (RI2) had a $S(w)$ of 1.28, which did not change on further dilution with 0.01 N HCl, consistent with monomer behavior.^{19,20} Undiluted RI7 was initially 3.1 $S(w)$, which reduced to ~2.4 $S(w)$ at a 1:20 dilution, consistent with a predominantly hexameric species beginning to shift its dynamic equilibrium toward smaller species (Figure 2). This slight shift with RI7 was also observed with DLS.

Continuous sediment coefficient ($c(s)$) versus sedimentation coefficient plots of RHI and BIOD-095 are shown in Figures 3 and 4. Regular human insulin maintains

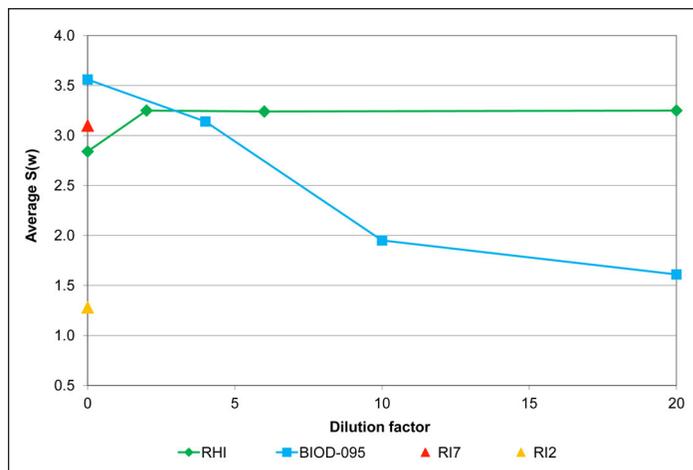


Figure 5. Graphic representation of the average sedimentation coefficients $S(w)$ following dilution with ECF versus dilution factor. BIOD-095 (blue square), RHI (green diamond), RI7 (red triangle), and RI2 (orange triangle). Control solutions (RI7 and RI2) were not diluted with ECF but are shown on graph for size reference.

its position at ~3 $S(w)$ throughout the dilution series, consistent with a hexameric population. Since there was no shift in $S(w)$, RHI is considered to be a noninteracting species at these dilutions. Therefore it was possible to calculate the molecular weight of RHI over this dilution range (34.75 kDa), which is consistent with the size of an insulin hexamer. This calculated molecular weight also applied to the undiluted insulin solution, which had a small size distribution by DLS (3.5 nm). Therefore, in this case, the small undiluted RHI insulin molecule is suggestive of a hexamer, perhaps in a very stable, compact state. The lack of dissociation of RHI into smaller insulin species is consistent with the literature, where it states that stabilized formulations of RHI require a dilution of 50- to 100-fold to achieve mostly dimeric units.^{6,29} Dilutions to this extent were not performed in this study, therefore dissociation to monomers was not observed.

In contrast, BIOD-095 shows a multimodal distribution in the first dilution, with a shift to the left on further dilution. This indicates interacting species, probably populations of hexamers, tetramers, dimers, and monomers (far left ~1.3 $S(w)$). Therefore DLS and analytical ultracentrifugation techniques confirm BIOD-095 rapidly dissociates into smaller subunits following dilution with ECF, which is consistent with its ultra-rapid absorption profile seen in clinic.

Lastly, we investigated why monomers created by BIOD-095 do not reassociate into hexamers postinjection, a problem documented to delay the absorption of

monomeric insulin formulations in the past.⁶ It was hypothesized that citrate ions in solution associate with the smaller monomeric insulin species created postinjection, neutralizing localized surface charges and thus reducing the tendency of the monomers to reassociate into hexamers. To confirm the association of the citrate ion to insulin, the resistance of the formulation was tested with individual excipients and as a complete solution. Assuming there was no association between the molecules in solution, the relationship $1/R_t = 1/R_1 + 1/R_2$ should apply. However, in the case of trisodium citrate and insulin, the relationship was not equal, instead showing a reduction of the total ions in solution by an increase of resistance of 1.8-fold. This evidence shows that there is an association of the citrate with insulin, reducing the total charge of the solution. This finding is consistent with the size distribution shown by light scattering, which showed that the size of the BIOD-095 hexamer was ~6 nm, slightly greater than the control solution of insulin without excipients.

Conclusions

BIOD-095 is an ultra-rapid-acting formulation of recombinant human insulin containing citrate and disodium EDTA. The beneficial effect of these excipients is rapid dissociation of insulin hexamers into smaller subunits (monomer/dimers) that are inhibited from reassociation postinjection. The rapid availability of monomers/dimers postinjection increases the rate of insulin absorption. Formulations of these excipients have been evaluated in multiple phase 1–3 clinical trials and have shown a 50% improvement in the timing of C_{max} (T_{max} 60 ± 43 versus 120 ± 70 min RHI), reduction in hypoglycemia, and improved postprandial glycemic control.^{12–15} This combination of rapid hexamer dissociation, inhibition of insulin monomer/dimer reassociation, and neutralization of surface charge result in an insulin formulation with an ultra-rapid absorption profile.

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Disclosure:

All authors are current or former employees of Bidel Inc.

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