



Characterizing Insulin as a Biopharmaceutical Using Analytical Ultracentrifugation

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Abstract

Insulin is a well characterized drug used to treat diabetes. A large number of insulin formulations have been investigated in order to develop the optimum time delivery suited to patient needs. In addition, a number of insulin analogs such as Insulin Glargine and LysPro insulin (Humalog) have been developed and marketed as derivatives for immediate use at meal time and a more sustained release. With the regulatory release of a number of guidances on biosimilars, a number of biopharmaceutical companies are actively pursuing versions of these marketed products to address patient needs in this multibillion dollar market. The bioavailability of all these products is strongly dependent on not only the primary structure of the analog but also the formulation. Here, analytical ultracentrifugation (AUC) is investigated as a potential tool to analyze the higher-order structure of insulin under typical formulation conditions. AUC was used to probe the effect of insulin concentration, zinc addition, and the zinc chelating agent EDTA on monomer, dimer, and hexamer formation of USP Human Insulin Reference Standard. The zinc hexamer showed EDTA concentration dependence with increasing dimer and monomer species at higher EDTA concentrations. The methods outlined here highlight the use of the Beckman Coulter ProteomeLab XL-I for characterizing biopharmaceuticals in varying conditions for formulation development.

Introduction

In 2012, 29.1 million Americans, or 9.4% of the population, had either Type 1 or Type 2 Diabetes according to the American Diabetes Association and was the 7th leading cause of death in the United States in 2010 with 69,071 deaths¹. The primary treatment for Type 1 diabetes is daily insulin therapy since these patients are incapable of producing enough insulin on their own. Typically, Type 1 diabetes patients require administering several insulin injections every day at mealtime. However, even when properly administered, insulin injections do not replicate the natural time action profile of insulin, and thus analogs have been developed and are currently in development, to enhance effectiveness. Much of the existing research on the action profile of insulin relies upon the stoichiometry of the molecule in formulation conditions and under dilution conditions after injection into the human body.

Many methods are routinely used by biopharmaceutical companies to measure particle size and heterogeneity in a solution state. AUC functions by defining particle size, heterogeneity, and frictional ratio through manipulation of the Lamm equation which describes particles as they sediment through a liquid under force. AUC has previously been reported to measure the impact of the formulation on the sedimentation coefficient of insulin^{2,3} but never empirically to assess the impact of zinc and a chelating agent on higher order structure.

Materials

Dibasic sodium phosphate, zinc chloride, EDTA and glycerol were purchased from Fisher Scientific. Sodium hydroxide was purchased from Fluka and hydrochloric acid was purchased from BDH Chemicals. U.S. Pharmacopeia (USP) Human Insulin reference standard was purchased from Fisher Scientific.

ProteomeLab XL I, two-sector sedimentation velocity analytical cells, quartz windows, An 60 Ti rotor, An 50 Ti rotor, and torque stand were all purchased from Beckman Coulter, Inc..

Methods

Non formulated Insulin Concentration Titration: Insulin was resuspended to 50 mg/mL in 0.04 N HCl and diluted to respective concentrations. A reference buffer matching the sample solvent, void of Insulin, was also created. 420 μ l of both reference and sample were loaded into a 2 sector analytical cell, aligned with An 60 Ti rotor, and equilibrated at 20°C for greater than 1 hour. Samples were subsequently spun at 60,000 rpm, 20°C, 4 hours scanning at Abs_{280nm} in continuous mode.

Formulated Insulin Concentration Titration: Insulin was resuspended to 50 mg/mL in 0.04 N HCl and diluted to respective concentrations and formulated with 150 μ M ZnCl₂, 16 mg/mL glycerol, 1.9 mg/mL dibasic sodium phosphate, and 2.2 mM NaOH. A reference buffer matching the sample solvent, void of Insulin, was also created. 420 μ l of both reference and sample were loaded into a 2 sector analytical cell, aligned with An 60 Ti rotor, and equilibrated at 20°C for greater than 1 hour. Samples were subsequently spun at 60,000 rpm, 20°C, 4 hours scanning at Abs_{280nm} in continuous mode.

Zinc Concentration Titration: Insulin was resuspended to 50 mg/mL in 0.04 N HCl and diluted to 4 mg/mL formulated with varying concentrations of ZnCl₂, 16 mg/mL glycerol, 1.9 mg/mL dibasic sodium phosphate, and 2.2 mM NaOH. A reference buffer matching the sample solvent, void of Insulin, was also created. 420 μ l of both reference and sample were loaded into a 2 sector analytical cell, aligned with An 60 Ti rotor, and equilibrated at 20°C for greater than 1 hour. Samples were subsequently spun at 60,000 rpm, 20°C, 4 hours scanning at Abs_{280nm} in continuous mode. The rotor was stopped but left under vacuum at 20°C. 15 days later, the cells were removed from the rotor, shaken vigorously, and rerun under the same conditions.

EDTA Concentration Titration: Insulin was resuspended to 50 mg/mL in 0.04 N HCl and diluted to 2 mg/mL formulated with 150 μ M ZnCl₂, 16 mg/mL glycerol, 1.9 mg/mL dibasic sodium phosphate, and 2.2 mM NaOH. Varying concentrations of EDTA were added to the formulated product and incubated for 20 minutes at room temperature. A reference buffer matching the sample solvent, void of Insulin, was also created. 420 μ l of both reference and sample were loaded into a 2 sector analytical cell, aligned with An 50 Ti rotor, and equilibrated at 20°C for greater than 1 hour. Samples were subsequently spun at 50,000 rpm, 20°C, 5 hours scanning at Abs_{280nm} in continuous mode.

Data Analysis[†]: Data was extracted from the AUC controller and imported into SEDFIT 14.7 g⁴ (www.analyticalultracentrifugation.com). Absorbance data were analyzed in terms of a continuous c(s) distribution of sedimenting species using a maximum entropy regularization confidence interval of 0.68. In all cases excellent fits were observed with root mean square deviations ranging from 0.0016 to 0.0093 absorbance units. The partial specific volume of insulin was calculated based on the amino acid composition in SEDNTERP⁵ (<http://sednterp.unh.edu>); the density ρ and viscosity η for each formulation buffer were also calculated in SEDNTERP. A c(s) model was used in which the frictional ratio f/f_0 is floated and the partial specific volume refined. The data was extracted into GUSSI (<http://biophysics.swmed.edu/MBR/software.html>) and plotted for c(s) with an s value minimum constraint at 0.3S.

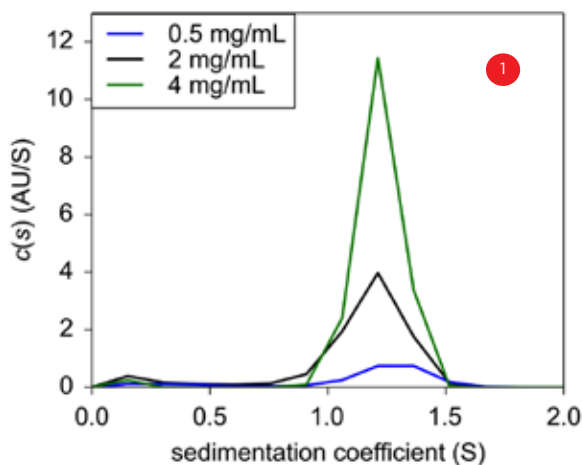


Figure 1. Sedimentation velocity c(s) of Insulin in 0.04N HCl only.

Results & Discussion

USP Human Insulin was diluted to three different concentrations in 0.04 N HCl and loaded into 2 sector sedimentation velocity AUC cells. Analysis revealed that all three concentrations were centered on 1.2 S through c(s) analysis by the popular software SEDFIT (Fig. 1). The c(s) values were left non normalized to show the change in signal with a decrease in concentration. The obtained value of 1.2 S is most likely representative of monomeric insulin, in agreement with Pohl, et.al.⁵ and consistent with monomer behavior^{6,7}.



Under typical formulation conditions⁵, insulin was again sedimented and analyzed for sedimentation coefficient. Here, insulin had a $s_{20,w}$ of between 2.95 – 3.1 S, but at 4.0 mg/mL, a minor species at 2.12 S emerged at a signal weight value of 8.3% of the overall sedimenting material (Fig. 2). This new peak is most likely representative of dimeric insulin. Interestingly, the protein dissociated at the highest concentration, suggesting that assembly is not concentration dependent; rather the dissociation is more likely from non-saturating conditions of one of the excipients.

To explore the effect, the concentration of zinc in the formulation buffer and the impact on sedimentation coefficient was probed. At a fixed concentration of insulin, addition of zinc pushed complex formation toward the hexamer around 3.0 S (Fig. 3A). Thus, zinc is critical to proper hexamer formulation, and at high concentration of insulin, the amount of zinc is no longer at saturated levels, leading to a smaller species most likely representative of insulin dimer. To determine if the effect was time dependent, the same formulations were left in the analytical cells for 15 days under vacuum at 20°C and sedimented for a second time. The results were the same for both spins, suggesting that insulin hexamer is stable for at least 15 days following formulation (Fig. 3B).

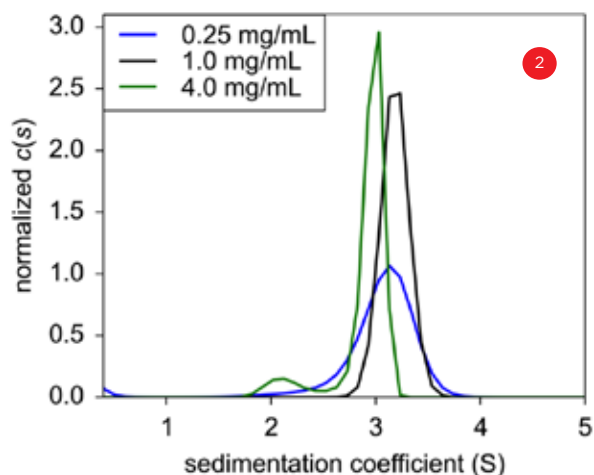


Figure 2. Sedimentation velocity normalized $c(s)$ of concentration titration of formulated Insulin. 0.25 mg/mL (black), 1.0 mg/mL (blue), and 4.0 mg/mL (green) were analyzed for sedimentation coefficient following formulation.

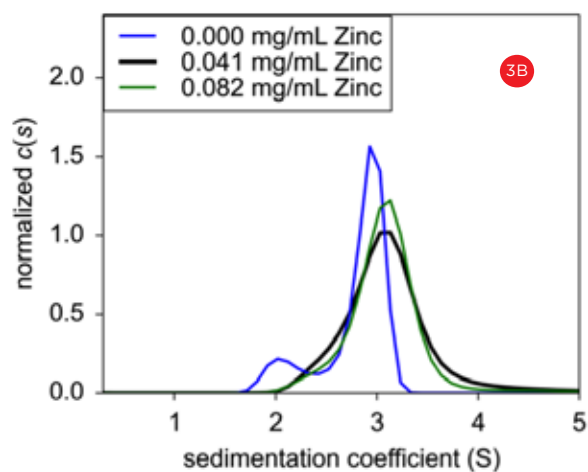
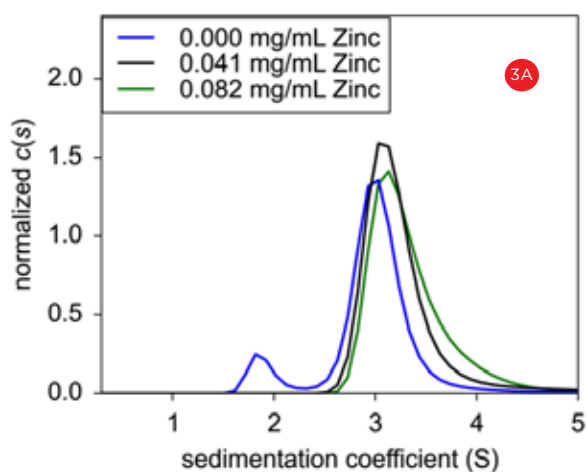


Figure 3. Sedimentation velocity normalized $c(s)$ of concentration titration of formulated Insulin. 0.25 mg/mL (black), 1.0 mg/mL (blue), and 4.0 mg/mL (green) were analyzed for sedimentation coefficient following formulation.

Finally, to confirm zinc's role in insulin hexamer formation, EDTA was titrated into the formulation to act as a chelating agent. Here, both insulin and zinc concentration were fixed to hexamer stable conditions, and EDTA was then added and incubated for 20 minutes. At increasing concentrations of EDTA, hexameric insulin was dissociated into monomer and dimer species (Fig.4). At 75 μM EDTA, approximately 11% of the hexameric complex had dissociated into monomer (Table 1). At 150 μM , an additional 15% had dissociated up to a total monomeric weight signal at 26.4%. At 300 μM , the hexameric species had completely dissolved into approximately 60% monomer and 40% dimer. Finally, at 600 μM , insulin existed at about 68% monomer and 32% dimer.

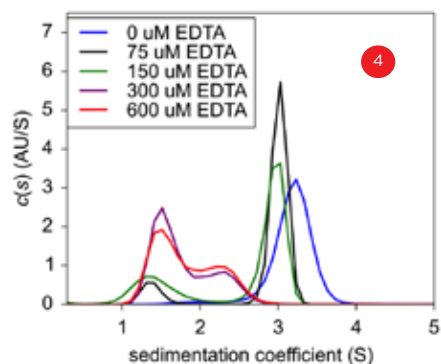


Figure 4. Sedimentation velocity $c(s)$ of EDTA titration with fixed Insulin concentration.

Zinc Titration	Hexamer	Dimer	Monomer
0 μM EDTA	100% (3.201 S)	N/A	N/A
75 μM EDTA	88.88% (3.015 S)	N/A	11.11% (1.373 S)
150 μM EDTA	73.59% (2.949 S)	N/A	26.41% (1.400 S)
300 μM EDTA	N/A	40.63% (2.258 S)	59.37% (1.52 S)
600 μM EDTA	N/A	31.99% (2.262 S)	68.01% (1.551 S)

Table 1. Weight signal average of insulin oligomeric species following EDTA treatment.

Conclusions:

Insulin is a well characterized drug created by many different manufacturers worldwide, and has been used to treat diabetes for more than 80 years. Today, large efforts are currently being undertaken by biopharmaceutical companies to create insulin derivatives and novel formulation to improve efficacy, administration methods, and time action profiles. In future development of insulin analogs, the effect of excipients such as zinc will need to be considered on the assembly state of the drug. These effects can be probed by a number of analytical techniques.

Insulin aggregation levels have been traditionally controlled using the Human Insulin monograph SEC method. This method is used to measure insulin covalent aggregates such as the covalent dimers as a result of the condensation reaction of free amines at the N-terminus and/or at free lysines. Addition covalent aggregation can occur through the disruption and reformation of the disulfide links. However, this method can not measure the relative levels of non-covalent aggregates such as insulin hexamer to monomer levels. These non-covalent aggregates are disrupted during the SEC sample preparation and/or analyses procedure. AUC as demonstrated in this application note, is shown to be useful in assessing the level of non-covalent aggregates such as the insulin hexamer. Here, we demonstrated a straight-forward approach to characterize the higher-order structure of a common biopharmaceutical with minimal development. Most higher-order methods, such as SEC, require adulterating the sample through large dilutions and/or column-matrix interactions that can further alter the aggregation state. Furthermore, although SEC is currently the preferred method for measuring covalent aggregates, the molar mass range of AUC is approximately from a few hundred to 10^9 daltons, a range that surpasses the typical capability of analytical SEC⁷. Finally, AUC can generate quantitative information around protein properties including molecular weight, shape, solvation properties⁸, post-translational modifications⁹, and conformational fluctuations¹⁰ at concentrations approaching 40-50 mg/mL¹¹, which positions the tool as an independent or orthogonal technique to the traditionally-used biopharmaceutical method of SEC to study biosimilarity, aggregation, lot-to-lot comparability, and formulation development⁷.

†Results generated from the softwares listed in this section are not guaranteed. Please see specific software tools for individual disclaimers.

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