Application Note

Wyatt Technology Corporation

Self-Association of Insulin Quantified by CG-MALS

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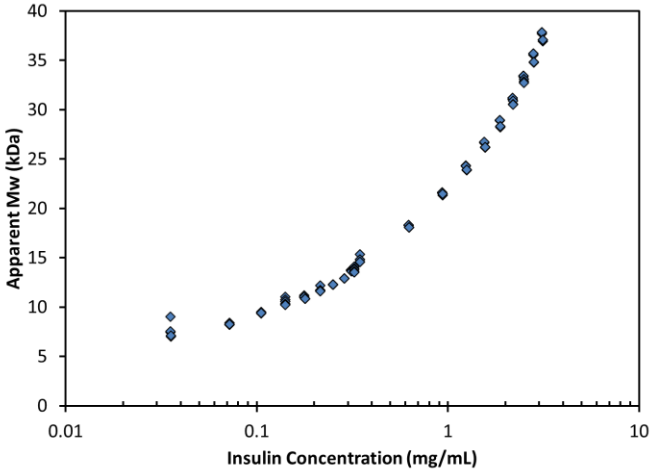
Summary

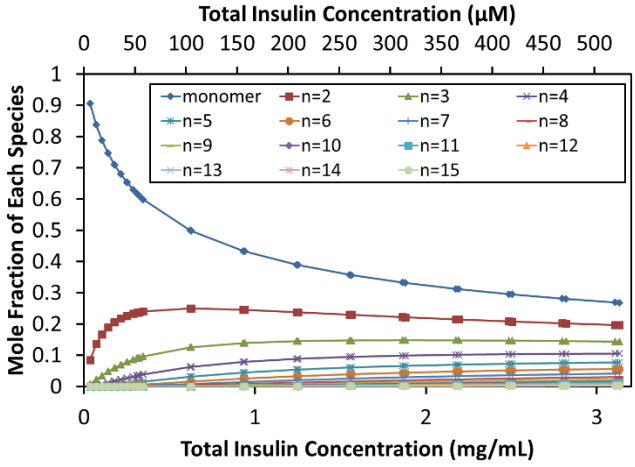
Therapeutic insulin analogs, engineered for specific states of self-association, have revolutionized the treatment of diabetes mellitus. Modifications that impact the self-association of these molecules in turn alter their pharmacokinetics and pharmacodynamics, resulting in fast-acting drugs best suited for prandial regimens and insulin pumps or extended-release versions for once daily dosage [1]. Hence the ability to quantify the affinity and stoichiometry of insulin self-association is central to developing efficacious analogs.

In this note, we quantify the self-association of insulin at neutral pH in the absence of Zn^{2+} using composition-gradient multi-angle light scattering (CG-MALS). CG-MALS enables rapid, reproducible, label-free quantification of biomolecular self-association. The increase in weight-average molar mass as a function of concentration is fit to an appropriate association model to yield the absolute stoichiometry and affinity of the interactions.

Under these conditions, insulin undergoes isodesmic self-association. Monomers self-associate to form dimers, trimers, and all higher order complexes. Each monomer adds to the growing cluster with equivalent affinity, in this case K_D = 52 µM. At the maximum concentration tested (3.2 mg/mL, 536 µM), an adequate description of the insulin solution must include oligomers >10-mer.



The weight-average molar mass of the insulin solution increases as a function of concentration, indicating the assembly of higher order oligomers.



The distribution of species can be determined from the CG-MALS data. In this case, oligomers with n>15 made up <0.5% mol/mol of the solution.

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WYATT TECHNOLOGY

I. Introduction

Composition-gradient multi-angle light scattering (CG-MALS) has been previously applied to quantify the self-association of macromolecules in solution, including insulin [2,3,4,5]. In the presence of Zn^{2+}, insulin forms native hexamers that further self-associate into higher order structures [2]. In the absence of Zn^{2+}, however, insulin monomers associate according to an isodesmic model, with each monomer adding to the growing clus-ter with equal affinity [3].

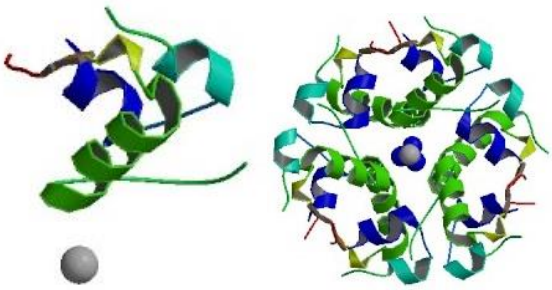

Figure 1: Crystal structure of insulin monomer (left) and hexamer (right) in the presence of zinc, PDB ID: 1TRZ

II. Materials and Methods

Human insulin samples were prepared in a buffer containing 20 mM sodium phosphate pH 7.2, 0.1 M NaCl, 1 mM EDTA and quantified using an extinction coefficient of 1.05 AU/(g/L*cm) at 276 nm. Insulin solutions and buffers were immediately filtered using Anotop 0.02 µm pore size syringe filters and degassed by centrifugation at 2500*g* for 15 minutes. Experiments were performed at 25°C in duplicate using two stock concentrations of either 3.2 mg/ml or 0.3 mg/ml.

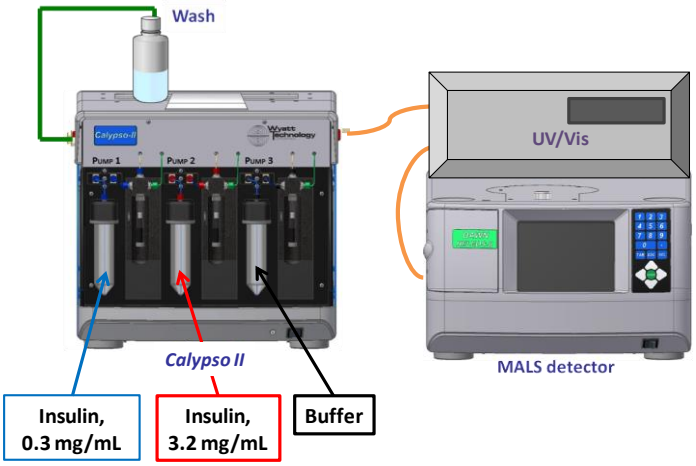

Figure 2: Calypso hardware setup

CG-MALS experiments were performed using a Calypso II to create a concentration gradient in line with a Shimadzu SPD-6AV UV/Vis spectrometer and a miniDAWN TREOS three-angle light scattering photometer (Figure 2). The light scattering and concentration data were fit to various self-association models using the CALYPSO software in order to determine the association scheme that best described the data.

III. Results and Discussion

As expected, the measured light scattering and concentration data for insulin in the absence of Zn^{2+} are characteristic of a self-associating molecule (Figure 3). Although the maximum measured M_w under these conditions (38 kDa) is approximately that of the hexamer (36 kDa), the increase in molar mass cannot be described by a simple model of monomer-hexamer equilibrium $6I \rightleftharpoons I_6$ with equilibrium association constant, $K_A = [I_6]/[I]^6$. As shown in Figure 4, this type of association underestimates the measured M_w by as much as 30% for concentrations less than ~0.2 mg/mL (34 µM) and overestimates the M_w by as much as 25% for concentrations between 0.2 and 2 mg/mL (34-340 µM).

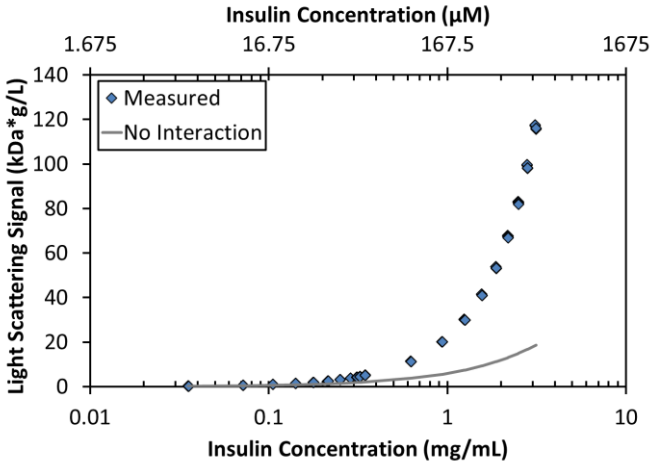

Figure 3: The measured LS signal as a function of concentration (blue diamonds) is significantly greater than the expected LS signal for a non-interacting monomer with M_w = 6 kDa (gray line). Data for duplicate experiments with stock concentrations of 0.3 mg/mL and 3.2 mg/mL, as shown in Figure 2.

Rather, the light scattering and concentration data are best fit by a model of isodesmic self-association. According to this mechanism, each insulin monomer adds to a growing insulin cluster with constant affinity as follows:

$$I + I \rightleftharpoons I_2 \qquad K_2 = \frac{[I_2]}{[I]^2}$$

$$I + I_2 \rightleftharpoons I_3 \qquad K_3 = \frac{[I_3]}{[I]^3} = K_2^2$$

$$\vdots \qquad \vdots$$

$$I + I_{n-1} \rightleftharpoons I_n \qquad K_n = \frac{[I_n]}{[I]^n} = K_2^{n-1}$$

The equilibrium association constant K_2 is related to the affinity per binding site, $K_D = 1/K_2$. For the data in Figure 3 and Figure 4, the best fit reveals isodesmic self-association with affinity K_D = 52 µM.

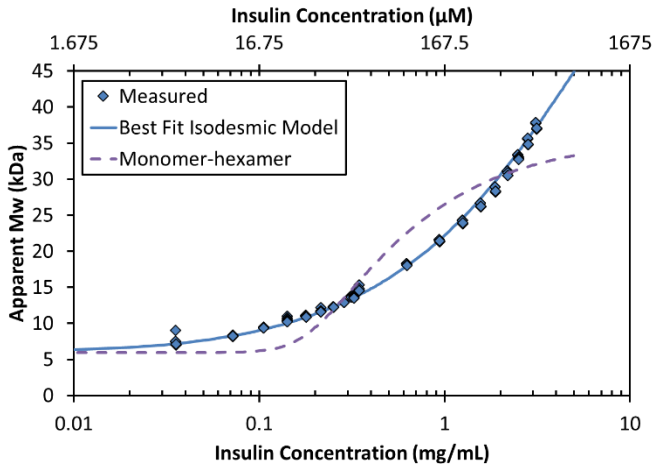

Figure 4: The increase in M_w as a function of concentration corresponds to an isodesmic self-association and is not characteristic of a simple model of monomer-hexamer equilibrium. Data for duplicate experiments with stock concentrations of 0.3 mg/mL and 3.2 mg/mL, as shown in Figure 2.

The equilibrium distribution of oligomers can be calculated from the self-association model (Figure 5). As the total concentration approaches the isodesmic binding site affinity (~50 µM or 0.3 mg/mL), insulin molecules self-associate, and the fraction of monomer in solution quickly decreases. At concentrations greater than ~0.6 mg/mL (~100 µM), even the contribution of dimer decreases as higher order oligomers form. At the maximum concentration (~500 µM), monomers make up only 27% mol/mol, with the remaining solution consisting of 20% mol/mol dimers and decreasing compositions of other oligomers. Figure 5 gives the molar compositions up to 15-mers, which comprise 0.3% mol/mol of the stock solution.

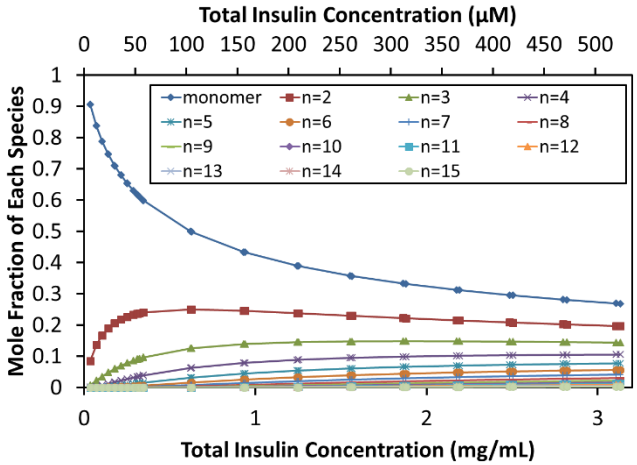

Figure 5: Equilibrium distribution of species

IV. Conclusions

Using CG-MALS, we measured the isodesmic self-association of human insulin in the absence of zinc. According to this model, insulin monomers form dimers, trimers, and higher order oligomers, with each insulin monomer adding to the growing cluster with equal affinity, K_D = 52 µM. Although the overall M_w only changed by ~6-fold over the concentrations studied (~0.3-3 mg/mL, ~5-500 µM), the change in M_w as a function of concentration is not described by a simple monomer-hexamer equilibrium. Rather, a true description of the complexes present at equilibrium must consider higher order insulin oligomerization, with species >10-mer present under these conditions.

V. References

1 Berenson DF, Weiss AR, Wan Zl, Weiss MA. Insulin analogs for the treatment of diabetes mellitus: therapeutic applications of protein engineering. Annals of the New York Academy of Sciences. 2012;1243(1):E40-E54.

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