

AN3004: Self-association of insulin quantified by CG-MALS

Vijay Pandyarajan, M.D., Ph.D.¹, Nelson B. Phillips, Ph.D.¹, Sophia Kenrick, Ph.D.², Michael A. Weiss, Ph.D.¹

¹Case Western Reserve University ²Wyatt Technology Corp.

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 dosage¹. 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 Zn²⁺ 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 $K_d = 52 \mu\text{M}$. At the maximum concentration tested (3.2 mg/mL, 536 μM), an adequate description of the insulin solution must include oligomers >10-mer.

Introduction

Composition-gradient multi-angle light scattering (CG-MALS) has been previously applied to quantify the self-association of macromolecules in solution, including insulin¹⁻⁵. In the presence of Zn²⁺, insulin forms native hexamers that further self-associate into higher order struc-

tures². In the absence of Zn²⁺, however, insulin monomers associate according to an isodesmic model, with each monomer adding to the growing cluster with equal affinity³.

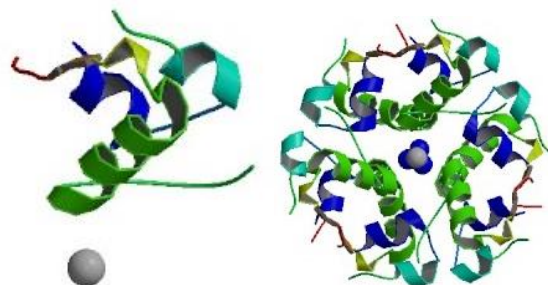


Figure 1: Crystal structure of insulin monomer (left) and hexamer (right) in the presence of zinc, PDB ID: 1TRZ

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. CG-MALS experiments were performed using a Calypso[®] concentration-gradient system to create a concentration gradient in line with a Shimadzu SPD-6AV UV/Vis spectrometer and a miniDAWN[®] 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.

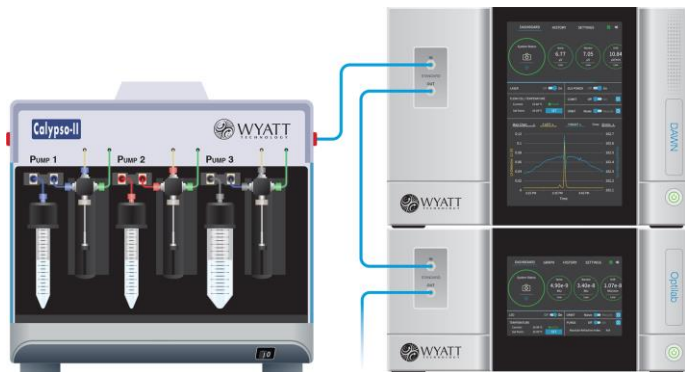


Figure 2: Calypso hardware setup

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 μ M) and overestimates the M_w by as much as 25% for concentrations between 0.2 and 2 mg/mL (34-340 μ M).

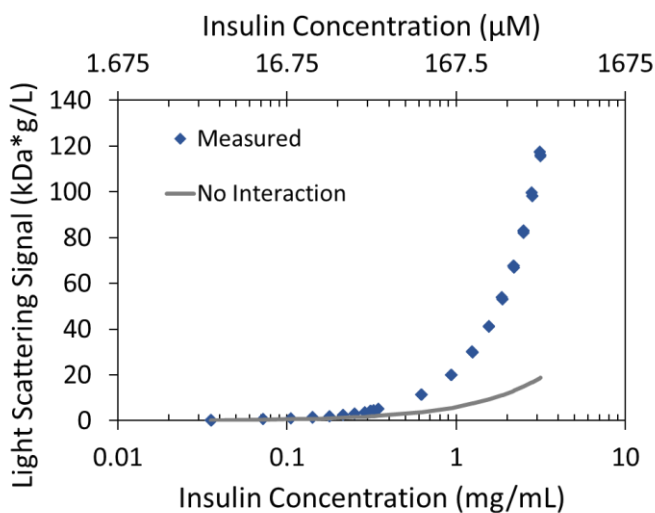
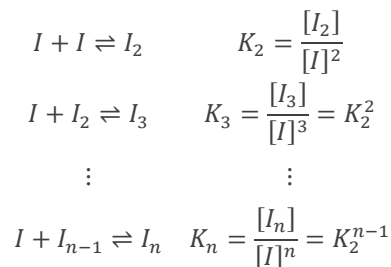


Figure 3: The measured LS signal as a function of concentration (blue diamonds) is significantly greater than the expected LS signal for a non-interacting monomer with $M_w = 6$ kDa (gray line).

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:



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 $K_d = 52$ μ M.

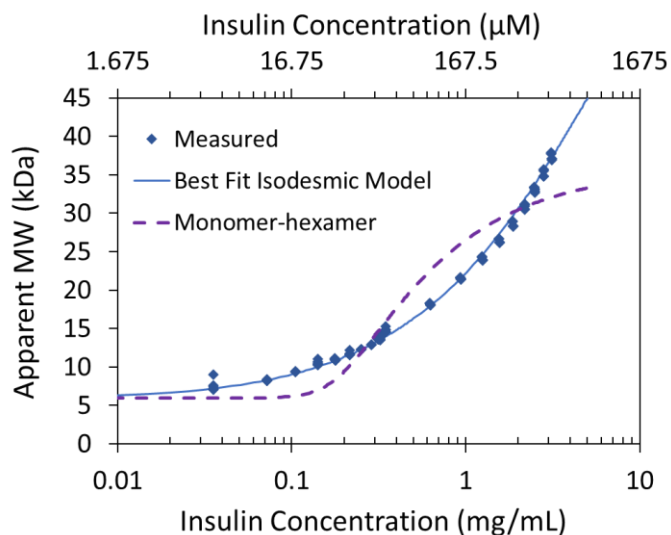


Figure 4: The increase in M_w as a function of concentration corresponds to an isodesmic self-association and is not characteristic of a simple model of monomer-hexamer equilibrium.

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 μ M 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 μ M), even the contribution of dimer decreases as higher order oligomers form. At the maximum concentration (~ 500 μ M), 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.

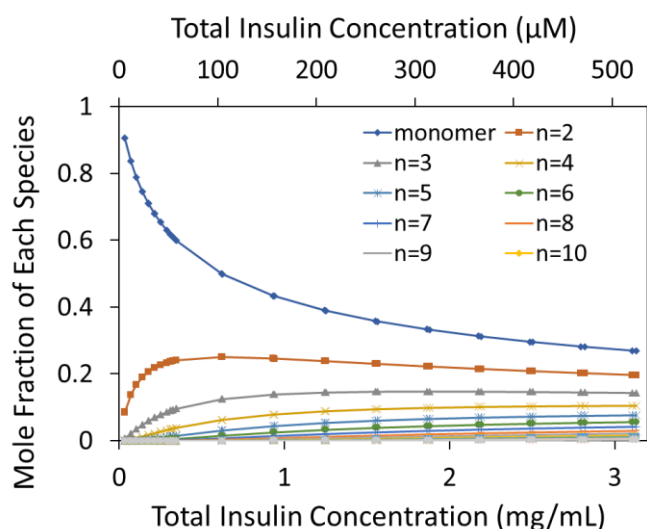


Figure 5: Equilibrium distribution of species

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, $K_d = 52 \mu\text{M}$. Although the overall M_w only changed by ~ 6 -fold over the concentrations studied (~ 0.3 - 3 mg/mL , ~ 5 - $500 \mu\text{M}$), 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.

References

1. Berenson D.F., Weiss A.R., Wan Z.I., Weiss M.A. Insulin analogs for the treatment of diabetes mellitus: therapeutic applications of protein engineering. *Annals of the New York Academy of Sciences* **1243**(1), E40-E54 (2012).
2. Attri A.K., Fernández C., Minton A.P. Self-association of Zn-insulin at neutral pH; Investigation by concentration-gradient static and dynamic light scattering. *Biophys. Chem.* **148**, 23-27 (2010).
3. Attri A.K., Fernández C., Minton A.P. pH-dependent self-association of zinc-free insulin characterized by concentration-gradient static light scattering. *Biophys. Chem.* **148**, 28-33 (2010).
4. Some D., Kenrick S. Characterization of Protein-Protein Interactions via Static and Dynamic Light Scattering in *Protein Interactions* ed. Cai, J. and Wang, R.E. 401-426 (InTech, 2012).
5. Some D. Light-scattering-based analysis of biomolecular interactions. *Biophys. Rev.* **5**(2), 147-158 (2013).



© Wyatt Technology Corporation. All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form by any means, electronic, mechanical, photocopying, recording, or otherwise, without the prior written permission of Wyatt Technology Corporation.

One or more of Wyatt Technology Corporation's trademarks or service marks may appear in this publication. For a list of Wyatt Technology Corporation's trademarks and service marks, please see <https://www.wyatt.com/about/trademarks>.