Application Note

Wyatt Technology Corporation

Identification of Insulin Oligomeric States using SEC-MALS

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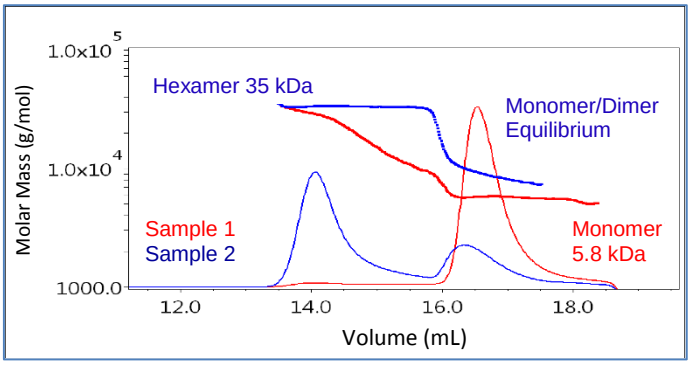
Summary

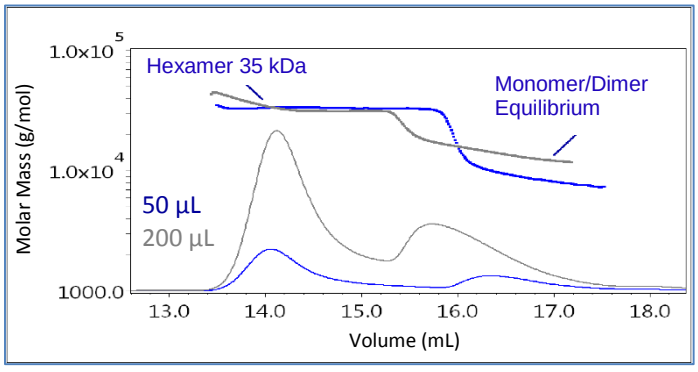
Insulin, one of the most important mammalian hormones, regulates a multitude of metabolic functions including the control of the blood glucose level in the body. Under healthy conditions, insulin is produced and stored in the islet tissues of the pancreas and released depending on the metabolic situation. In patients suffering from diabetes, insulin cannot be sufficiently produced by the body. It has to be administered as a pharmakon via oral or injection pathways. Large amounts of human insulin are nowadays produced industrially by genetically engineered microorganisms.

Under physiological conditions, insulin forms hexamer complexes in the presence of zinc ions. In the pancreas the hormone is also stored as zinc complexes. In pharmacological preparations therefore zinc is added to enable complex formation. To ensure safe and effective administration of insulin formulations, it is of vital interest to investigate, which oligomeric species are present in a specific preparation.

This application note shows how the combination of column chromatography (SEC/GPC) with Multi-Angle Light Scattering (MALS), Dynamic Light Scattering (DLS) and RI detection can be applied as a powerful tool to identify monomers, dimers, hexamers and higher aggregates of insulin. Using this approach, each preparation can be comprehensively characterized to determine optimal formulation, storage and administration conditions tor the patient's benefit.



Molar mass vs. time of insulin formulated in two different buffers, determined by SEC-MALS. Sample 1 shows a stable monomer with a small fraction of hexamer, while Sample 2 shows monomer-dimer equilibrium in conjunction with a stable hexamer.



Molar mass vs. time of sample 2, showing the concentration-dependent behavior of the monomer-dimer equilibrium assessed by SEC-MALS. Two different injection volumes lead to different overall different concentrations, resulting in different monomer-dimer fractions.

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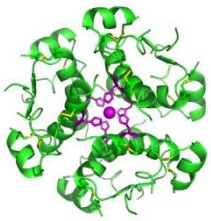
Introduction

In the analysis of proteins and peptides the question arises frequently whether the molecules under investigation are monomeric or form dimers or higher aggregates. The knowledge of this stoichiometric relation enables the investigator to estimate if the protein is present in its biologically active form.



In such determinations the first step is usually the separation of the molecular species and their characterization. To achieve this, size exclusion chromatography (SEC) is often used as the method of choice. Since this approach separates the molecules according to their volumes, it does not automatically allow the exact mass determination because molar mass is not necessarily a function of elution time but rather due to hydrodynamic properties.

A technology which overcomes this limitation is multi-angle light scattering (MALS). MALS measures molar mass in an absolute manner. This means no assumptions on the molecule's structure are made and no molar mass standards are needed. The determination relies on first principle measurements only and is therefore absolute. The MALS detection can be coupled to online DLS measurement to obtain hydrodynamic radii of the molecules. Moreover, CG-MALS measurements have been shown to be useful to detect the aggregation status of insulin (1,2).

The subject of the investigations presented here is the hormone insulin. Insulin plays a vital role in the regulation of mammalian blood glucose level and metabolism, cell growth and fat metabolism – only to name a few of its manifold functions. The discovery of insulin in 1921 represented a starting point for a revolution in the treatment of diabetes. Human insulin is a protein consisting of two peptide chains of 21 and 30 amino acids respectively, with total molar mass of 5.8 kDa. Human insulin self-associates and in the presence of zinc ions a hexameric complex is formed, 2 zinc ions bind per hexamer. Insulin is stored in the β–cells of the pancreas as zinc complexes. In pharmaceutical preparations insulin is normally formulated at concentrations where the self-association is pronounced and hence zinc is added to stabilize the hexamer and thereby a more stable formulation. Upon dilution after delivery the hexamer dissociates and ends up as monomers in the blood stream. Hence it is of interest to investigate the oligomerization both from a formulation as well as pharmacological perspective.

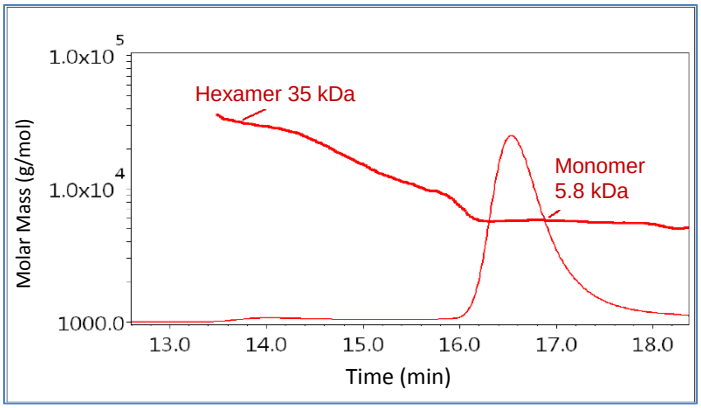


Figure 1. Plot of molar mass vs. time of sample 1. The UV signal @ 280 nm is plotted as an overlay. The molar masses were calculated using DRI and LS.

Materials and Methods

An Agilent 1260 HPLC system was used which included an auto sampler, an isocratic pump and a degasser. The separation column was a Superose 12 300/10 from GE Healthcare. For the detection we used a variable wavelength detector at 280 nm (Agilent), a DAWN® HELEOS® 18 angle light scattering detector equipped with a WyattQELS™ for online DLS detection, and an Optilab® rEX™ refractive index detector. The LS, DLS and dRI detection instruments, as well as ASTRA® software for SEC-MALS analysis, were from Wyatt Technology, Santa Barbara, CA, USA.

The mobile phase consisted of 10 mM Tris, 140 mM NaCl, 2 mM Phenol and 200 ppm NaN3 at pH = 7.7.

Three different insulin sample preparations all containing 0.6 mM insulin were analyzed:

Sample 1: insulin analogue without added zinc

Sample 2: human insulin with 0.1 mM zinc

Sample 3: human insulin with 0.3 mM zinc

Results and Discussion

Reversible protein assembly that increases with protein concentration, or self-association, is a well-known effect for e.g. monoclonal antibodies and insulin (3). In the case of insulin, the molecule forms dimers and other oligomers which can be separated by SEC and detected by light scattering techniques (4,5). Here we show the characterization of different insulin preparations by SEC-MALS.

Results of sample 1 are shown with molar masses indicated throughout the chromatogram (figure 1). The major fraction with molar mass corresponding to a

monomer is well separated from the very minor fraction corresponding to hexamer. Although not seen by specific peaks in UV it is observed by the LS signal that molar mass distribution covers the range from monomer to hexamer. It should be emphasized that the light scattering identified the hexamer easily even though the UV signal suggests that only a very small amount of this fraction is present. This is due to the extremely high sensitivity of the LS detection method for large molecules.

Further addition of zinc completely shifts the equilibrium towards the hexamer as seen in sample 3 (figure 3). In sample 3 another molecular species is detected which is identified as the dodecamer of the insulin molecule. It shows a molar mass of 70 kDa and, although present only in a small amount, generates a distinct signal clearly observed in by the LS detector.

SEC-MALS allows the determination of self-association tendencies. The effect of injection volumes on the sample was investigated, i.e. 50 and 200 µL of the same sample solution was injected to the SEC-MALS.

The increase of injected volume clearly affects the equilibrium of sample 2 (see figure 4). As a higher insulin concentration is achieved, the equilibrium of the monomer-dimer is shifted towards dimer. The smallest species observed when 50 µL is injected is monomer while the corresponding smallest species is dimer when 200 µL is injected. The hexamer fraction is unaffected and shows no tendency to form larger oligomers.

In the case of sample 3 there are basically no effect of varying the injection volume (see figure 5). In presence of 0.3 mM zinc the system is "locked" on hexamer and identical molar masses are detected at both concentrations (50 and 200 µL injected). Obviously, the self-association behavior of the hexamer is significantly different from that of the monomer.

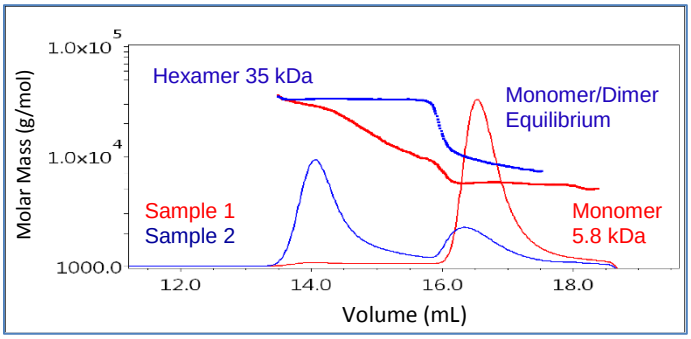


Figure 2: Plot of molar mass vs. time of sample 1 and 2 (monomer, dimer, hexamer). The UV signal @ 280 nm is plotted as an overlay. The molar masses were calculated using DRI and LS.

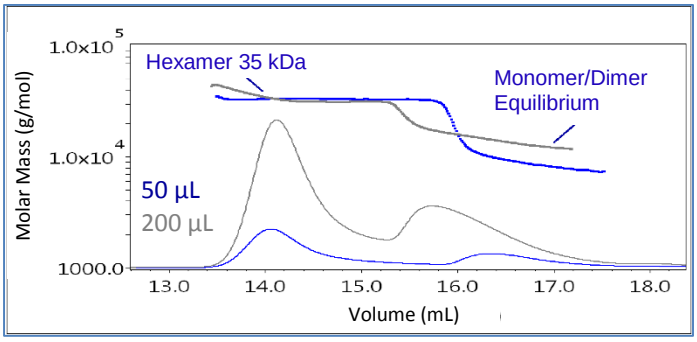


Figure 4: Plot of molar mass vs. time of sample 2. The UV signal @ 280 nm is plotted as an overlay. The molar masses were calculated using DRI and LS.

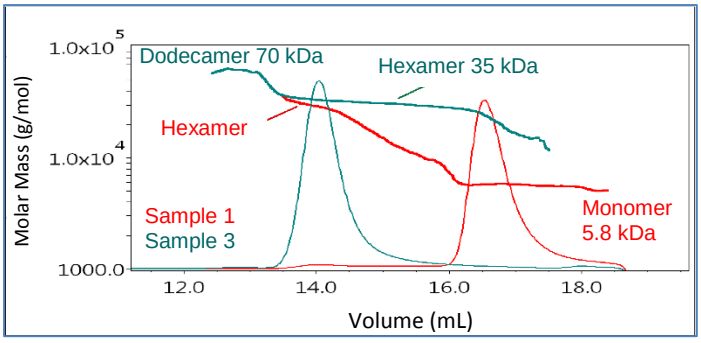


Figure 3: Plot of molar mass vs. time of sample 1 and 3. The UV signal @ 280 nm is plotted as an overlay. The molar masses were calculated using DRI and LS.

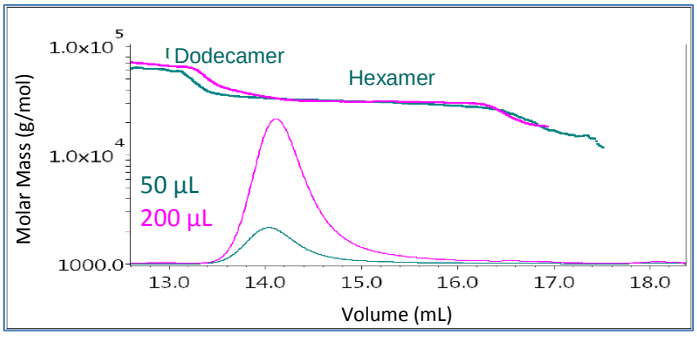


Figure 5: Plot of molar mass vs. time of sample 3. The DRI signal is plotted as an overlay. The UV signal is saturated due the high sample load. The molar masses were calculated using DRI and LS data.

Conclusions

Multi-angle light scattering proves to be the method of choice for the examination of association phenomena not only for large proteins such as antibodies, but also for peptide molecules with lower molecular masses. Since the use of proteins and peptides for therapeutic purposes is permanently increasing, the application of light scattering as a powerful method of molecular characterization will also become more and more important.

References

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