Application Note
DEC 2022
AN-850-0130

Assessment of Protein Hydration, Surface Hydrophobicity, and Structure in One Single Automated Measurement

- Biosimilars
- mAbs
- ADCs
- AAVs
- Ligand Binding
- Protein/Peptide Analysis
- VLPs
- Nucleic Acid
- Fusion Proteins
- Enzyme Analysis

- Aggregation
- Quantitiation
- Structure
- Stability
- Similarity

Introduction

When dry proteins are exposed to water, they rapidly bind water molecules up to a maximum quantity. This interaction between a protein molecule and water molecules is oftentimes referred to as protein hydration and it plays an important role in protein folding, stability, and activity[1,2]. Figure 1(a) shows a protein with its functional domain highlighted in the black rectangle. Once the protein is dissolved in an aqueous solution, it is surrounded by water molecules, thanks to polarity. When the domain is open, water molecules get inside, and once the domain closes, water molecules are squeezed out. This shows that protein hydration is important for collective domain motions, which are general functional motions. In Figure 1(b), we have an unfolded protein on the left side and its folded state on the right, which have different interactions with water molecules, thus, different surface hydrophobicity. It illustrates how protein hydration plays an important role in defining the structure of the protein and affects its structural stability.

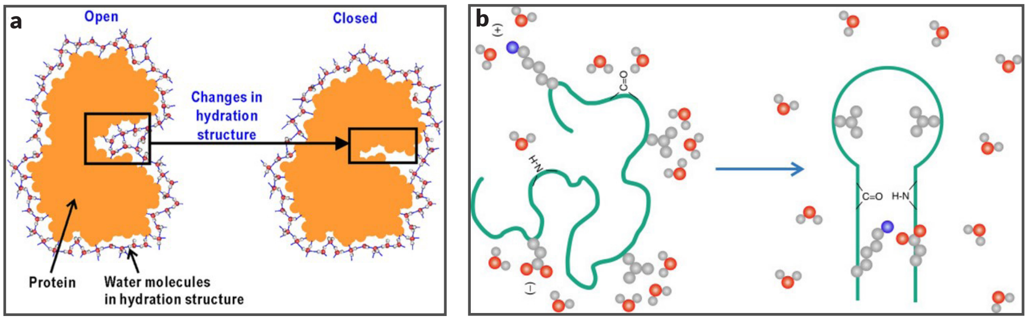

Figure 1. Schematic illustrations of (a) how water molecules play an important role in protein activity[2] ; and (b) how protein in its unfolded (left) and folded (right) state have different interactions with water molecules, thus, different surface hydrophobicity[3].

While protein structural stability assessment is critical in the drug development processes to meet regulatory requirements, e.g. ICH Q5E, protein hydration is usually overlooked due to the difficulty in experimental measurements[4 5 6]. One way to experimentally quantify protein hydration is through partial specific volume (PSV) (µL/mg)[7] , which is defined as the increase in volume (µL) upon the addition of 1 mg of dry protein to a solution[8]. The protein's PSV, indicates protein hydration, and can vary in different formulation buffers[9]. However, the experimental procedure to quantify the PSV in every formulation is labor-intensive and time-consuming.

Here, we provide a method to assess protein hydration together with protein structural analysis in a single automated measurement using the RedShiftBio Apollo powered by Microfluidic Modulation Spectroscopy (MMS)[10]. Our results show that there is a strong correlation between the level of protein hydration and the amount of sucrose added, suggesting a stabilization effect on the protein using sugar.

Methods

Hen Egg White Lysozyme (HEWL) (Sigma #L6876) was dissolved in water with 0, 0.5, 1, 2, 8, or 16% w/v sucrose at 2 mg/mL.

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Methods, continued

Samples were analyzed in triplicate at room temperature using Apollo at a modulation rate of 1 Hz, backing pressure of 5 psi, and all advanced parameters set as default. The secondary structure components of the prepared protein solutions were determined using the ***delta*** data analysis software.

MMS measurements are collected by modulating between protein sample and reference buffer and performing a real-time buffer subtraction. However, this over-subtracts the buffer absorbance because the protein itself takes up space in the buffer as shown in Figure 2A. Since the buffer that is displaced by protein has absorption in the IR range, there needs to be a correction factor for adding back in the displaced buffer, shown in Figure 2B. We call this parameter the "displacement factor" and this not only allows us to more precisely measure the protein structure, but also gives us an insight to the protein hydration.

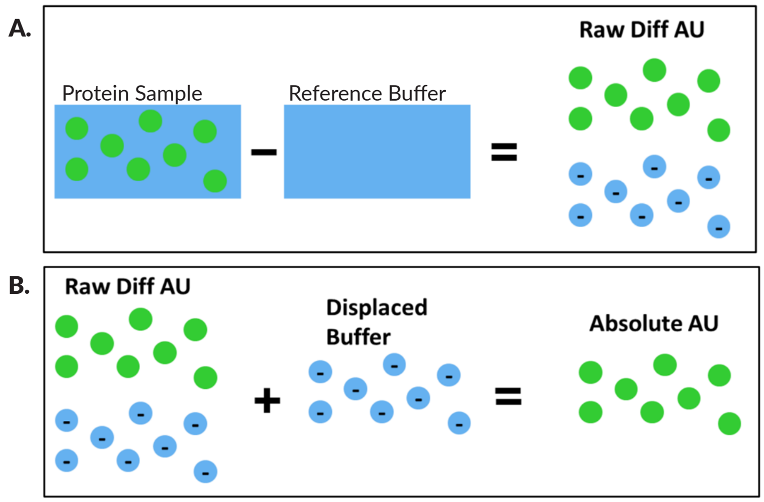

Figure 2. A. The subtraction of buffer absorbance from sample absorbance during MMS modulation is depicted above, where green circles indicate protein molecules and blue indicates buffer molecules. The blue circles with negative signs represent the over-subtraction of buffer molecules. B. Illustration of how absolute AU is generated by adding in the displaced buffer to the Raw Diff AU to account for the over-subtraction.

The amount of the displaced buffer is not directly measured but calculated as below:
(Displaced Buffer AU) = (Buffer AU) * (Displacement Factor) * (Protein Concentration)

If a model protein is chosen, the software will start with nominal displacement factor and nominal concentration to find the best mathematical fit. If no model protein is chosen, the absolute AU is calculated from given nominal displacement factor and concentration.

Results

The secondary structures of HEWL in water with 0, 0.5, 1, 2, 8, and 16% w/v of sucrose are characterized by MMS and shown in Figure 3. Figure 3 (A) shows the Absolute AU spectra, and the second derivatives are taken and shown in Figure 3 (B). Here we can see slight formulation-induced differences caused by the increasing amount of sucrose. Then in Figure 3 (C), we inverted and baseline-corrected the second derivative spectra for better visualization and quantification of the overall structural similarities. The quantification results are shown in Table 1 below – Similarity via Area of Overlap (AO).

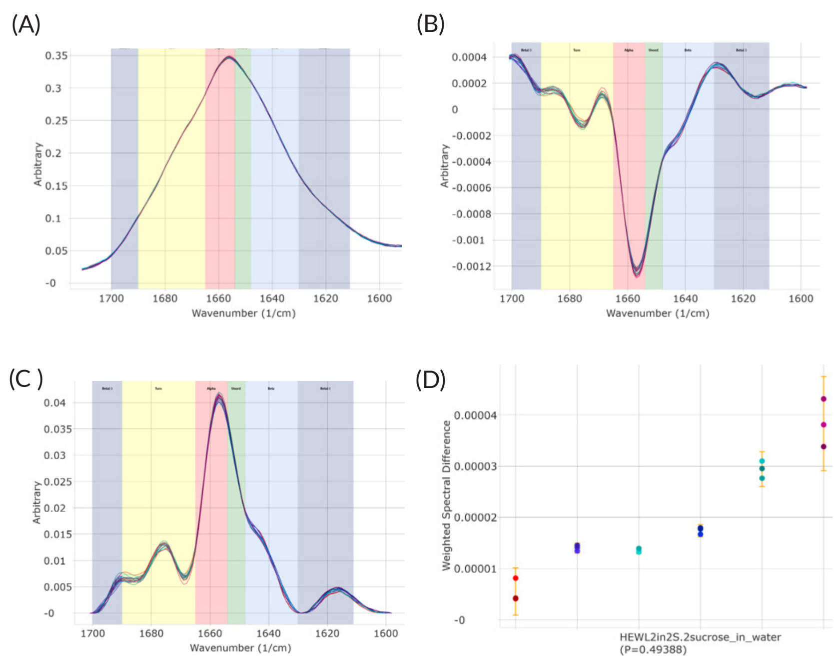

Figure 3. Structure characterization of HEWL in water with sucrose of 0, 0.5, 1, 2, 8, and 16% w/v by (A) Absolute AU spectra, (B) Second Derivatives of the Absolute AU spectra, (C) Similarity plots, and (D) Weighted Spectral Differences. Three replicates were taken for each condition. The colors in (A), (B) and (C) are coding wavenumber ranges for characteristic motifs. From left to right, they are beta(-) (or, intermolecular beta sheet which indicates structural aggregates), turn, alpha-helix, unordered, beta, and beta(-).

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Results, continued

The repeatability among replicates is all above 99%. On the contrary, the similarity of the structure in different amounts of sucrose compared to the sample in water decreases as the amount of sucrose increases. The comparison between repeatability and similarity shows that the structural differences, though subtle, are detectable and statistically significant. Figure 3 (D) uses another mathematical method – Weighted Spectral Differences (WSD) – to quantify the overall structural differences among samples. There is a clear trend of increasing structural differences with the increasing amount of sucrose in the formulation, the detailed data is summarized in Table 1. The HEWL WSDs in different amounts of sucrose as compared to no sucrose are one order of magnitude higher than the repeatability among replicates, indicating subtle but detectable structural differences induced by sucrose.

Table 1 summarizes the structural similarity via area of overlap (AO), Weighted Spectral Differences (WSD) and Hydration via displacement factor. While there is no significant difference between HEWL in 0.5% and 1% sucrose, the 2%, 8% and 16% samples cause consistently increasing sucrose-induced changes in both HEWL structure and hydration. Our results are consistent with previous studies using x-ray scattering to study the hydration effects of sugar on myoglobin[11].

	Similarity via AO		Weighted Spectral Differences (WSD)		Hydration
Sucrose conc. %w/v	Repeatability among Replicates %	Similarity Compared to Water %	Repeatability Among Replicates ($x10^{-6}$)	WSD Compared to Water ($x10^{-6}$)	Displacement Factor
0	99.35+/-0.3	100	5.5+/-2.3	0	0.59+/-0.01
0.5	99.35+/-0.2	98.34+/-0.07	6.17+/-2.5	14+/-0.6	0.58+/-0.00
1	99.23+/-0.3	97.70+/-0.2	6.1+/-1.2	13.5+/-0.4	0.58+/-0.00
2	99.46+/-0.2	97.55+/-0.1	4.6+/-1.0	17.4+/-0.6	0.38+/-0.00
8	99.36+/-0.3	97.71+/-0.3	5.43+/-1.1	29.4+/-1.7	0.29+/-0.01
16	99.47+/-0.1	97.41+/-0.08	6.8+/-1.2	38.3+/-4.7	0.13+/-0.00

Table 1. Summary of the structural similarity via area of overlap (AO), Weighted Spectral Differences (WSD) and Hydration via displacement factor. Three replicates were measured for each condition and show the average of the three replicates +/- standard deviation.

Conclusions

Literature has indicated that sugars can stabilize the native protein structure through the protective action of the protein hydration shell, experimentally verified via X-ray and neutron scattering methods[11]. However, the exact mechanism on how sugar stabilizes protein molecules is still under debate[12].

In this study, we show that protein surface hydration (or, hydrophilicity) increases with an increasing amount of sucrose in water, with subtle but detectable structural changes. Changes in the hydration were measured through the "Displacement factor," or how much water is displaced by the protein. This is directly related to partial specific volume[13], but not exactly the same because the former is mathematically fitted based on light absorption signals. Nevertheless, like the PSV (µL/mg), the displacement factor (µL/mg) also represents the increase in volume upon the addition of protein to a solution, which comprises the solvent (water for an aqueous solution), and a low-molecular-weight buffer or cosolvent (for example, sugar). Therefore, displacement factor comprises the information of protein hydration and protein-solvent interactions. It can be further related to the hydrodynamic size of the protein in the solution[14]. In summary, a typical trend is: surface hydration ↑ (surface hydrophilicity ↑), displacement factor ↓.

With a single automated run, MMS showed sucrose-induced increase in protein surface hydration and subtle but detectable structural changes. MMS provides a simple way of assessing protein hydration together with an ultra-sensitive secondary structural analysis. Knowing protein hydration and protein-solvent interactions will help us understand protein folding/unfolding, stability, and activity. This additional information on protein hydration together with the assessment of structural stability by comparing before and after stresses would provide valuable insights on drug formulation. Thus, MMS is an optimal tool for proteinaceous drug formulation development.

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