Assessment of protein stability and functionality by Flow Induced Dispersion Analysis

Key Fidabio Benefits

- Rapid, HTP and efficient characterization of chemically induced unfolding of Human Serum Albumin
- Assessment of local and global structural changes in protein unfolding events
- Data in good agreement with SAXS
- Simultaneous assessment of in-solution binding affinity, protein stability and absolute size
- Native conditions and low sample volume
- Built-in quality control

Link to Tech-notes/App-notes

VERSION 01

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Introduction

The present application note is based on a peer reviewed paper of 2019 (1).
Protein-based pharmaceuticals represent a rapidly growing class of drug compounds. However, development of protein-based drugs is associated with significant challenges as these complex molecules are structurally labile. Further, the drug molecule, vehicle or degradation products may cause immunogenic responses, thereby leading to loss of therapeutic effect, toxicity or even anaphylaxis. Current methodologies cannot address these risks, as they typically are unable to probe stability under native conditions and require large amounts of sample. In this work, it is shown that Flow Induced Dispersion Analysis (FIDA) is used to measure the unfolding of Human Serum Albumin (HSA) and the loss of binding affinity to Fluorescein. The assay is performed under native conditions with minimal sample consumption.
FIDA is a new capillary-based technology for measuring binding affinity and assessing protein stability in-solution under native conditions. FIDA utilizes Taylor dispersion for accurate size determinations of analytes in a pressure driven flow system. The change in apparent size forms the basis for an accurate measure of binding affinity and protein stability.

Figure 1. Characterization of activity, stability and structural changes of HSA using FIDA.

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Material & Methods

Fida 1 instrument with 266 nm and 480 nm LIF and LED fluorescence detection for unfolding and binding experiments respectively (FIDA-Tech ApS). FIDA standard capillary (i.d.: 75 µm, LT: 100 cm, Leff: 90 cm). 67 mM phosphate buffer pH 7.4 was used as working buffer. HSA (0.5 mg/mL) as indicator in 0-7 M urea for unfolding experiment. Fluorescein as indicator (10 nM), HSA as analyte (0-500 µM) in 0-4 M urea solutions for binding experiments. Sample analysis was performed by filling the capillary with analyte, followed by injection of 39 nL indicator, which was mobilized towards the detector with analyte at 400 mbar.

Results

Urea-induced unfolding of HSA

As FIDA technology provides absolute measurements of hydrodynamic radius, it was used to address global changes upon HSA unfolding. Additionally, the intrinsic fluorescence of HSA and its binding to a low molecular weight fluorescent ligand, Fluorescein, were probed to detect local structural changes and associated functionality of the protein in increasing urea concentration.

The changes in the size (hydrodynamic radius) of HSA was plotted as a function of increasing urea concentration (0-7 M) at 25˚C as shown in Figure 2A. An increase in urea concentration around 4.0 M led to unfolding of HSA, observed as increase in size from 3.5 nm to 6.2 nm. The results are in line with a similar study using Small Angle X-ray Scattering (SAXS).

The peak areas of FIDA taylorgrams were exploited for simultaneously probing the intrinsic fluorescence intensity of HSA at increasing urea concentration.

Intrinsic fluorescence of HSA was affected by urea at 1.5 M indicating local structural changes prior to the overall unfolding (Figure 2B). This showcases the unique ability of FIDA to measure both local and global structural changes in a single measurement.

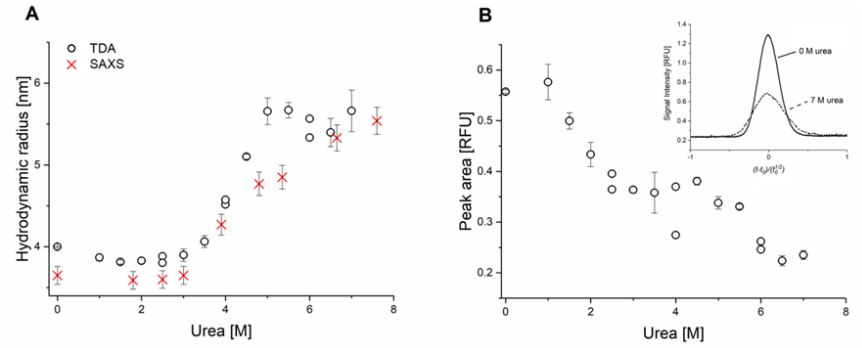

Figure 2. (A) Unfolding curve of HSA in 0-7 M urea. Hydrodynamic radius of HSA as a function of urea concentration determined by FIDA at 25˚C and compared with SAXS, **(B)** Intrinsic fluorescence area of 15 µM HSA as a function of urea concentration.

Loss of HSA binding to Fluorescein

Binding curves of the interaction between Fluorescein and HSA in 0-4 M urea were generated (Figure 3) and the Kd values were obtained as shown in Table 1. The binding affinity of the complex declines with increasing urea concentration; therefore, increased concentration of urea is associated with HSA unfolding and loss of functionality.

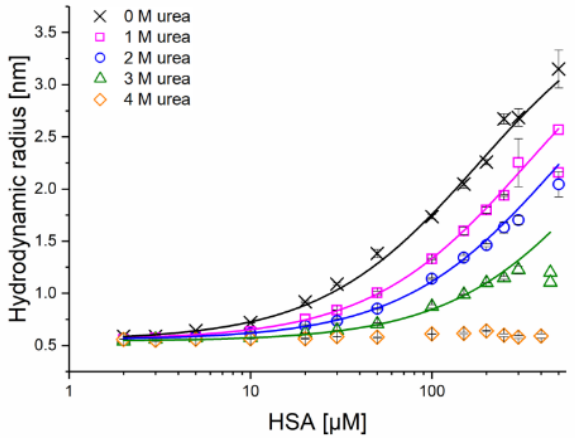

Figure 3. Binding curves of the interaction between HSA and Fluorescein in 0-4 M urea.

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Table 1. Dissociation constants (Kd) obtained for the binding of HSA to Fluorescein at varying urea concentrations at pH 7.4 and 25 ^{0}C.

Urea (M)	Kd (uM)	R^2 for binding isotherm fit
0	25,1	0,99
1	47,1	0,99
2	71,1	0,99
3	139	0,99

Conclusion

A combination of different techniques is required for a complete understanding of protein stability and function. The FIDA methodology is a multi-tiered approach capable of detailed characterization of the denaturation and unfolding process as depicted by HSA unfolding induced by urea.

With minimal sample consumption and using one instrument, FIDA allows in-depth assessment of protein activity combined with local and global structural changes by measuring the overall hydrodynamic radius of the protein, upon protein unfolding.

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