USZ Universitäts Spital Zürich

Universität Zürich[UZH]

FIDA: Flow-induced dispersion analysis

An immobilization-free technology used for characterization and quantification of biomolecular interaction and protein concentration under native conditions

Breuer Eva, LiverLab Prof. Clavien, 18.10.2022

Why use separation-based procedures for clinical bioanalysis?

- Clinical bioanalysis of therapeutic proteins and biomarkers
- Traditionally ligand-binding assays used: **ELISA**
  Surface Plasmon Resonance (SPR)
  Bio-Layer Interferometry (BLI)

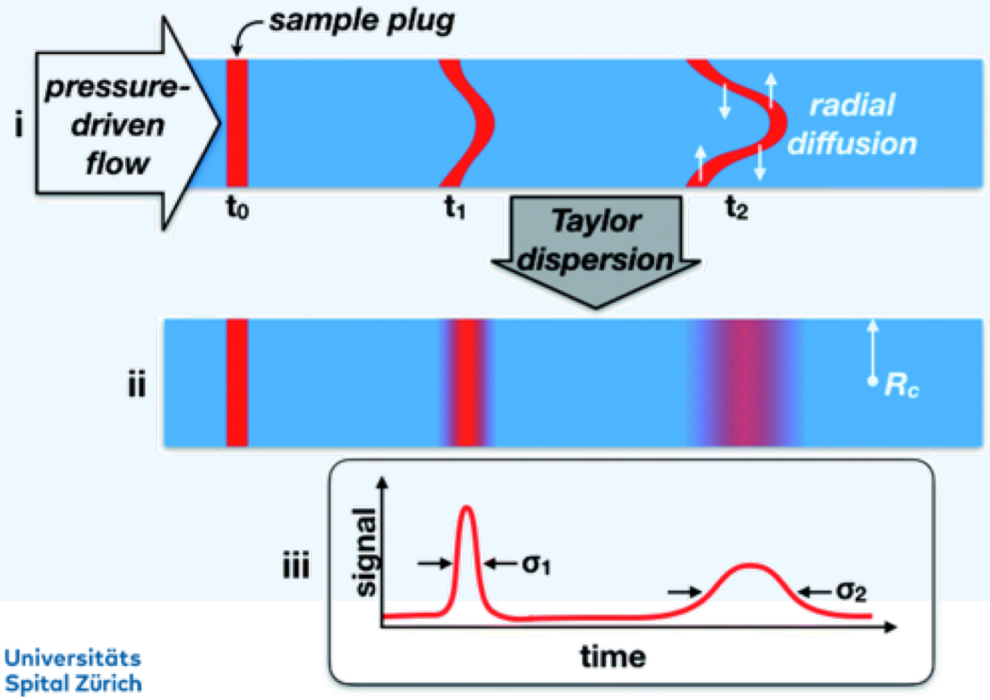
  → Downsides: Involve surface chemistries, multistep nature complicates development and validation, artifacts due to nonspecific surface adsorption in complex samples (e.g. plasma or serum)
- Separation-based procedures: **Capillary electrophoresis (CE)**
  Liquid chromatography (LC)

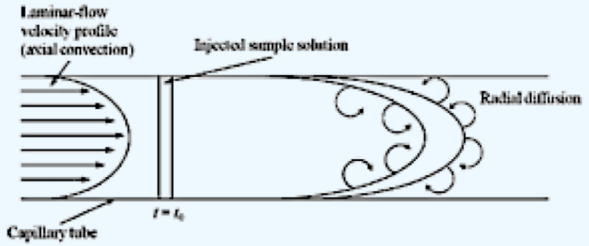
  → Sensitive and low cost optical detectors or mass spectrometry, development less cumbersome, detailed information on protein isoforms

  → Downsides: No functional information on binding activity → **FIDA**

Taylor Dispersion Analysis (TDA)

- Backbone for Flow-Induced Dispersion Analysis (FIDA)
- A sizing technique requiring no calibration and consumes only nL to pL sample volume
- Characterization of band broadening of an analyte plug under well-controlled laminar flow condition → Determination of diffusion coefficients via the Taylor-Aris equation





Contribution of longitudinal diffusion to band broadening negligible

Taylor-Aris equation

$$D = \frac{R_c^{\,2}\left(t_2 - t_1\right)}{24\left(\sigma_2^{\,2} - \sigma_1^{\,2}\right)}$$

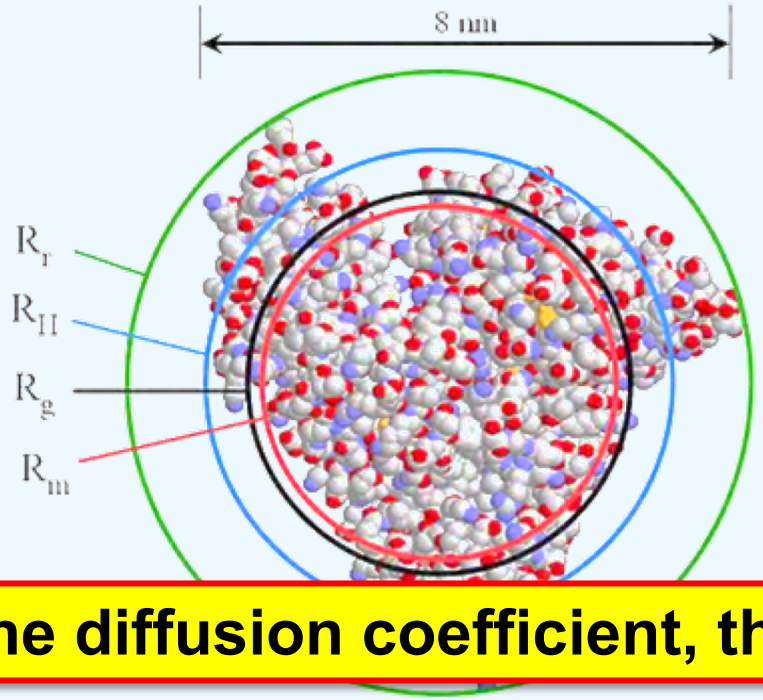
Taylor G et al.: Dispersion of soluble matter in (…). Proc R Soc A Math Phys Eng Sci 219:186–203, 1953
Moser M et al.: Taylor Dispersion Analysis in Fused Silica Capillaries. Anal. Methods, 2021, 13, 2357-2373.

Taylor Dispersion Analysis (TDA)

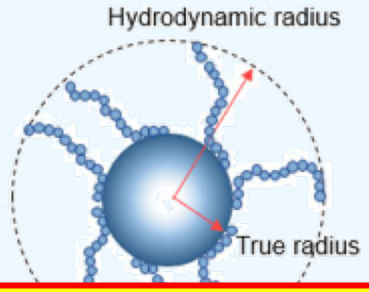
- The diffusion coefficient ***D*** can be used as a structural descriptor of the analyte when transformed to the hydrodynamic radius R_H via the Stokes-Einstein equation:

Radius of protein rotated about geometric center (R_r)

Hydrodynamic radius (R_H)



$$R_H = \frac{k_B T}{6\pi\eta D}$$



The lower the diffusion coefficient, the bigger the particle.

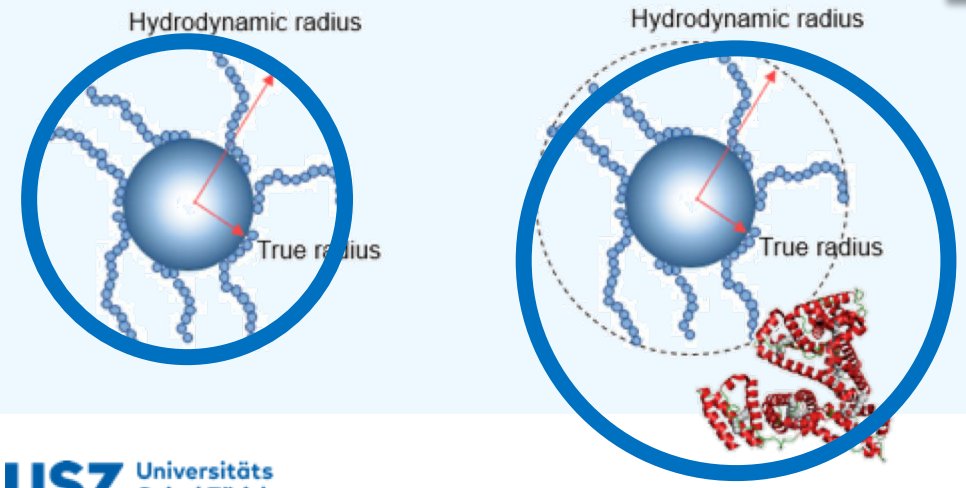
Taylor G et al.: Dispersion of soluble matter in (…). Proc R Soc A Math Phys Eng Sci 219:186–203, 1953
Moser M et al.: Taylor Dispersion Analysis in Fused Silica Capillaries. Anal. Methods, 2021, 13, 2357-2373.

Taylor Dispersion Analysis (TDA)

- Taylor Dispersion Analysis (TDA) provides an accurate measure of diffusivity and hydrodynamic radius (=size).

The binding of a ligand increases the hydrodynamic radius

R_H < R_H



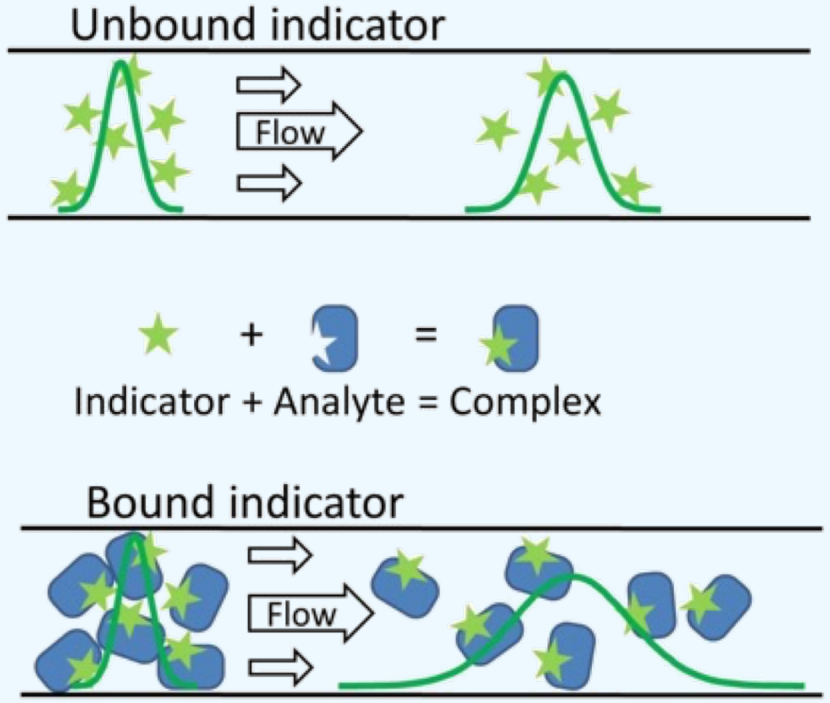
Lower diffusion coefficient (slower)

Band-broadening in TDA

Jensen H and Ostergaard J. Flow Induced Dispersion Analysis Quantifies Noncovalent Interactions in Nanoliter Samples. J. Am. Chem. Soc., 2010, 132, 12, 4070-4071.

Flow-Induced Dispersion Analysis (FIDA)

- In the assay, the hydrodynamic size of a **small ligand (indicator)** known to bind the **protein (analyte)** is measured.
- Then the **indicator** is bound by the **analyte** the apparent size increases and this change in size can be used to estimate the concentration of the protein in the sample.



→ Completed in minutes

→ Pico-nano molar sensitivity

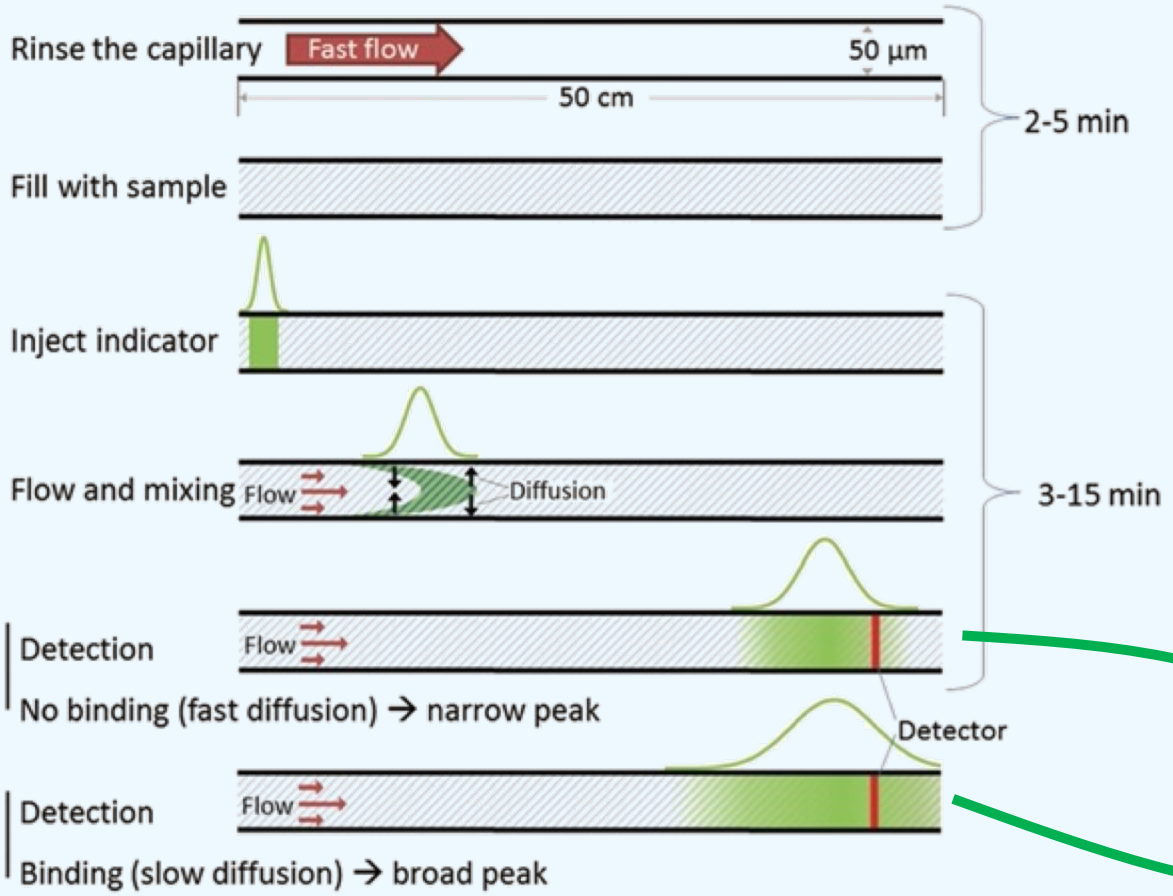
→ Fast assay development

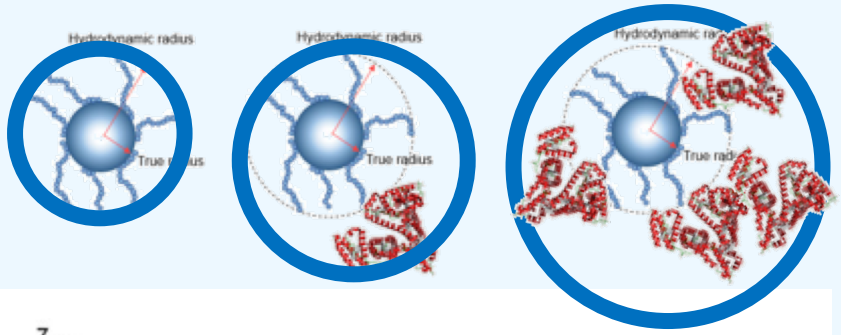
→ Selective (analysis of blood plasma)

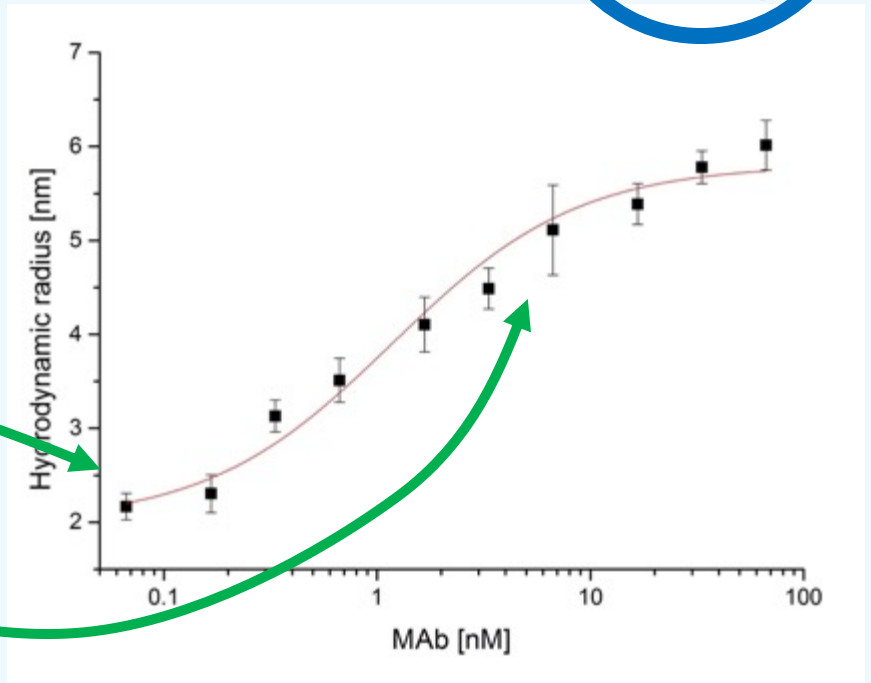
→ Performable on standard equipment (e.g. for capillary electrophoresis)

Poulsen N et al.: Flow Induced Dispersion Analysis for Probing non-Covalent Interactions and Quantifying large Biomolecules. 2022.

Flow-Induced Dispersion Analysis (FIDA)







Poulsen N et al.: Flow Induced Dispersion Analysis for Probing non-Covalent Interactions and Quantifying large Biomolecules. 2022.

CrossMark

Cite this: *Analyst*, 2015, **140**, 4365

Received 10th April 2015,
Accepted 27th May 2015

DOI: 10.1039/c5an00697j

www.rsc.org/analyst

Flow induced dispersion analysis rapidly quantifies proteins in human plasma samples†

Nicklas N. Poulsen, Nina Z. Andersen, Jesper Østergaard,‡ Guisheng Zhuang, Nickolaj J. Petersen and Henrik Jensen*‡

ROYAL SOCIETY OF CHEMISTRY

analytical chemistry

Technical Note

Cite This: *Anal. Chem.* 2019, 91, 4975−4979

pubs.acs.org/ac

Protein Characterization in 3D: Size, Folding, and Functional Assessment in a Unified Approach

Morten E. Pedersen,† Sarah I. Gad,† Jesper Østergaard,†,‡ and Henrik Jensen*,†,‡

OPEN

Size-based characterization of adalimumab and TNF-α interactions using flow induced dispersion analysis: assessment of avidity-stabilized multiple bound species

Morten E. Pedersen[1,2,3], Ragna M. S. Haegebaert[2,3], Jesper Østergaard[2] & Henrik Jensen[1,2]✉

scientific reports

Protein quantification in human plasma samples

- Can FIDA be used to reliably determine protein concentrations in human plasma samples?

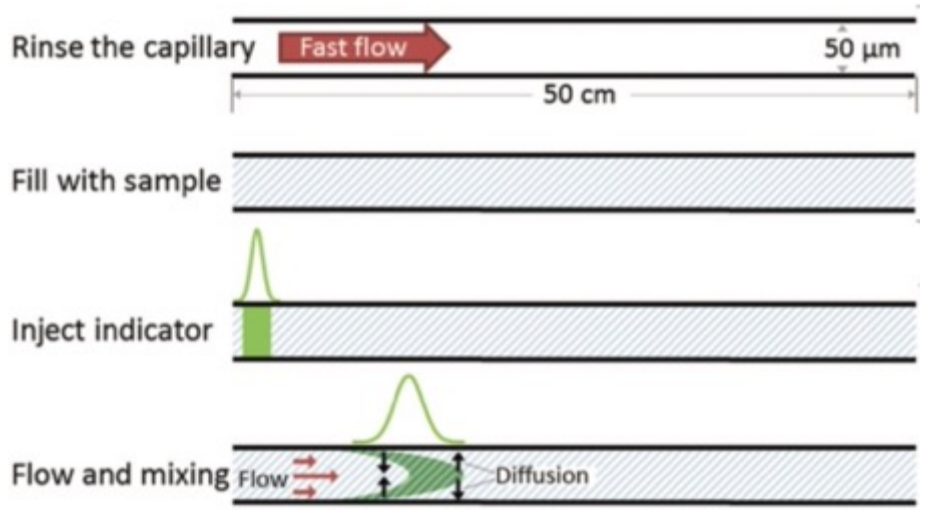
Standard fused silica capillary
Standard apparatus for capillary electrophoresis
488 nm laser for excitation – fluorescence detection

1. Fused silica capillary (50 µm inner diameter) is rinsed.

2. Capillary is filled with analyte (e.g. plasma)

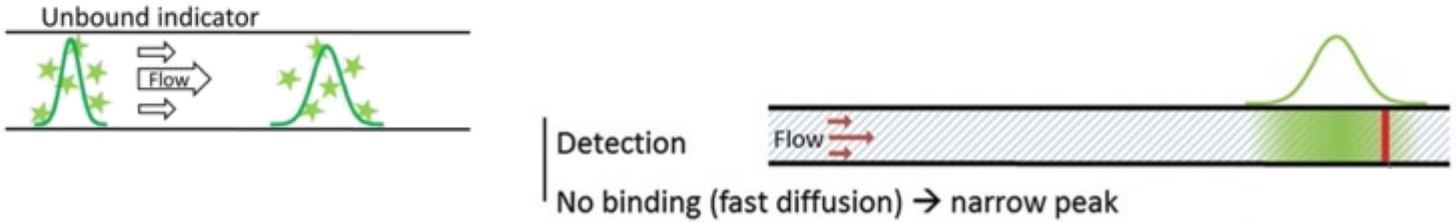
3. Small (10 nL) indicator zone is introduced.

4. Indicator is mobilized by pressure and mixes with the analyte. Binding of indicator and analyte occurs.



Protein quantification in human plasma samples

- Signal is recorded at the detection window located at 48 cm
- From the indicator peak shape, the apparent indicator diffusivity is obtained

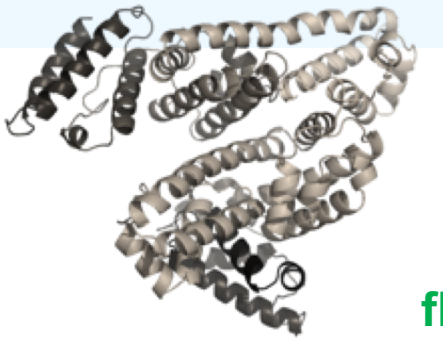


Taylor-Aris equation

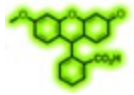
$$D = \frac{R_c^{\,2}\left(t_2 - t_1\right)}{24\left(\sigma_2^{\,2} - \sigma_1^{\,2}\right)}$$

Quantification of Human Serum Albumin (HSA) using FIDA

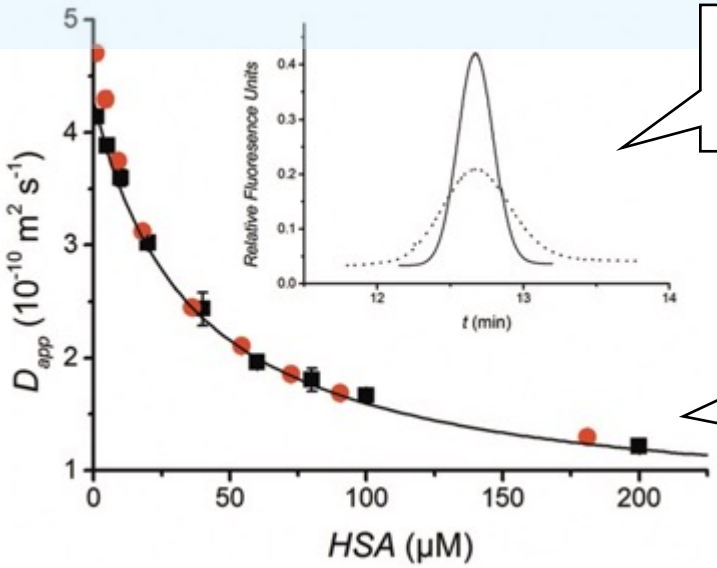
- Human Serum Albumin (HSA) was used as protein to detect in buffer/plasma
- Fluorescein is indicator:
  1. Known to bind to HSA
  2. Sensitive and selective fluorescence detection
  3. Small molecule (332 g/mol)
- Considerable change in hydrodynamic radius and therefore diffusivity is expected



HSA (analyte)



fluorescein (indicator)



Significant broadening of the fluorescein peak

The indicator apparent diffusivity as a function of the HSA concentration (in buffer)

Quantification of Human Serum Albumin (HSA) using FIDA

- Standard curve used to quantify HSA in diluted (2%) human plasma
- The colorimetric Bromo-Cresol Purple Assay (BCP) was used as comparative method to quantify HSA, as it is widely used to quantify HSA.
- Results from 3 different plasma samples → comparable results:

Table 1 The concentration of HSA in three different human plasma samples was determined by FIDA and BCP assays respectively

Plasma sample	HSA ($g\ l^{-1}$)	
	FIDA[a]	BCP[b]
1	33 (±0.6%)	32 (±0.2%)
2	30 (±5.8%)	27 (±0.5%)
3	32 (±6.8%)	32 (±0.2%)

Matrix effects possible, as fluorescein and BCP both can bind to other proteins. Very limited effect from plasma on FIDA assay.

Conclusion: POC of FIDA principle

- Increase in size of the indicator is an accurate approach for protein quantification
- Separation/isolation of an affinity complex is not required
- Not dependent on a secondary antibody interaction
- Fast (minutes compared to hours for e.g. ELISA)
- Easy to automate

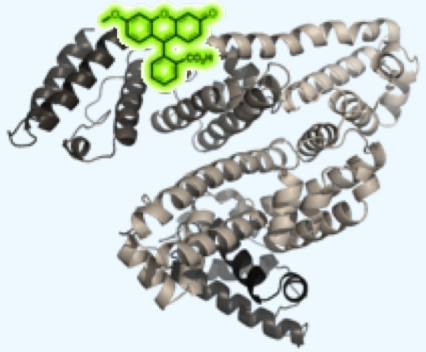
FIDA may be used to quantify any protein to which an indicator molecule (affinity ligand) exists, given high affinity and specificity.

- Indicator molecules available as antibodies

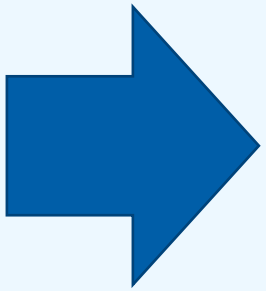
Antibody-based quantification?

- A strong specific binding between indicator and analyte is optimal
- Antibody-antigen interactions → high affinity and specificity
- Is FIDA suited for antibody based quantification?

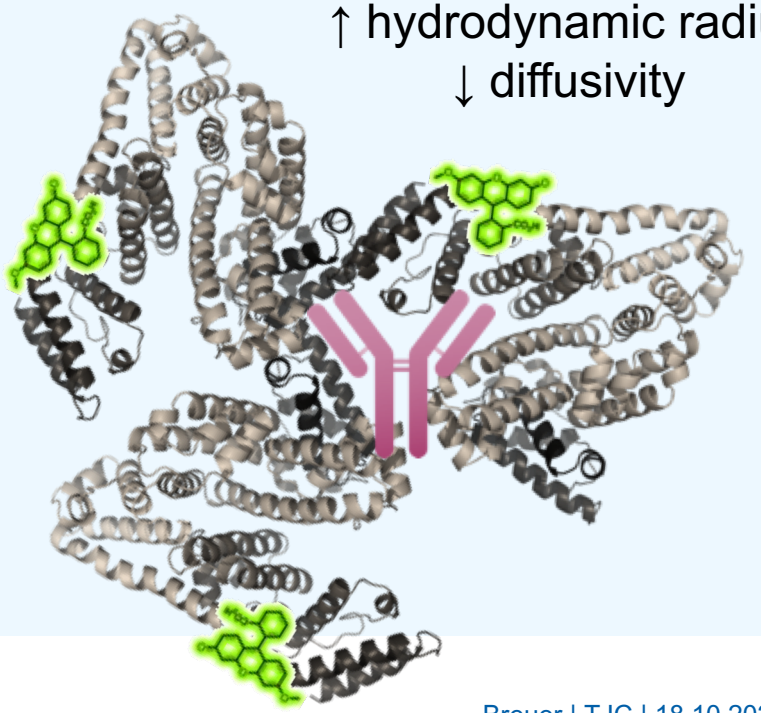
anti-HSA (analyte)



F-HSA
HSA tagged with ATTO488A (indicator)



complex formation
↑ hydrodynamic radius
↓ diffusivity



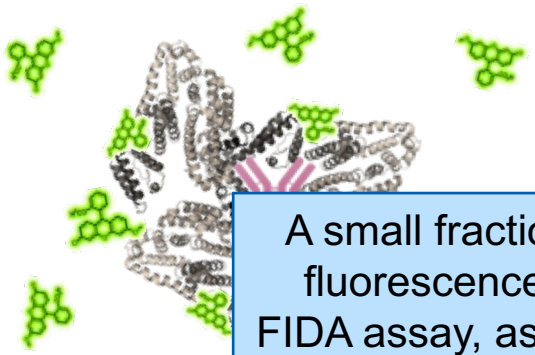
Not 100% convincing

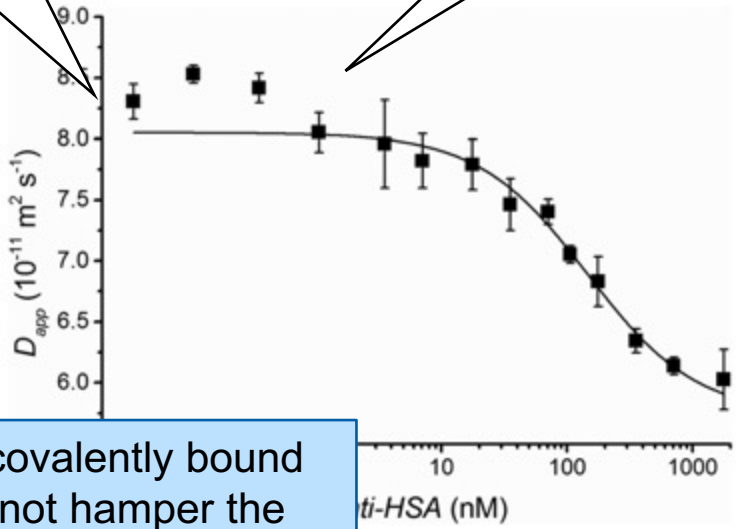
Diffusivity of HSA without indicator is higher compared to HSA alone (8.6 vs 6.4 10^{-11} m^2/s). → smaller than expected?

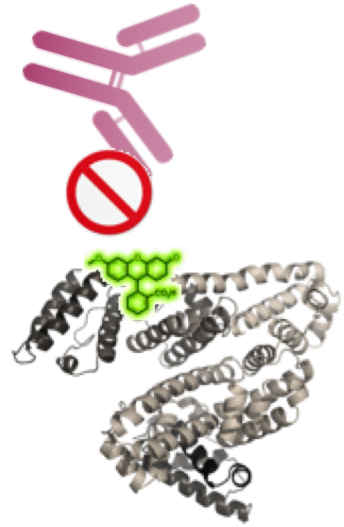
Heterogenic binding.
Main binding event occurs above 10 nm.

Possible fluorescence tags hampering the epitope recognition for part of the HSA molecule.

Unreacted fluorescent dye has not been completely removed from tagged F-HSA.







A small fraction of non-covalently bound fluorescence tag does not hamper the FIDA assay, as the whole curve is affected.

CrossMark

Cite this: *Analyst*, 2015, **140**, 4365

Received 10th April 2015,
Accepted 27th May 2015

DOI: 10.1039/c5an00697j

www.rsc.org/analyst

Flow induced dispersion analysis rapidly quantifies proteins in human plasma samples†

Nicklas N. Poulsen, Nina Z. Andersen, Jesper Østergaard,‡ Guisheng Zhuang, Nickolaj J. Petersen and Henrik Jensen*‡

ROYAL SOCIETY OF CHEMISTRY

analytical chemistry

Technical Note

Cite This: *Anal. Chem.* 2019, 91, 4975−4979

pubs.acs.org/ac

Protein Characterization in 3D: Size, Folding, and Functional Assessment in a Unified Approach

Morten E. Pedersen,† Sarah I. Gad,† Jesper Østergaard,†,‡ and Henrik Jensen*,†,‡

OPEN

Size-based characterization of adalimumab and TNF-α interactions using flow induced dispersion analysis: assessment of avidity-stabilized multiple bound species

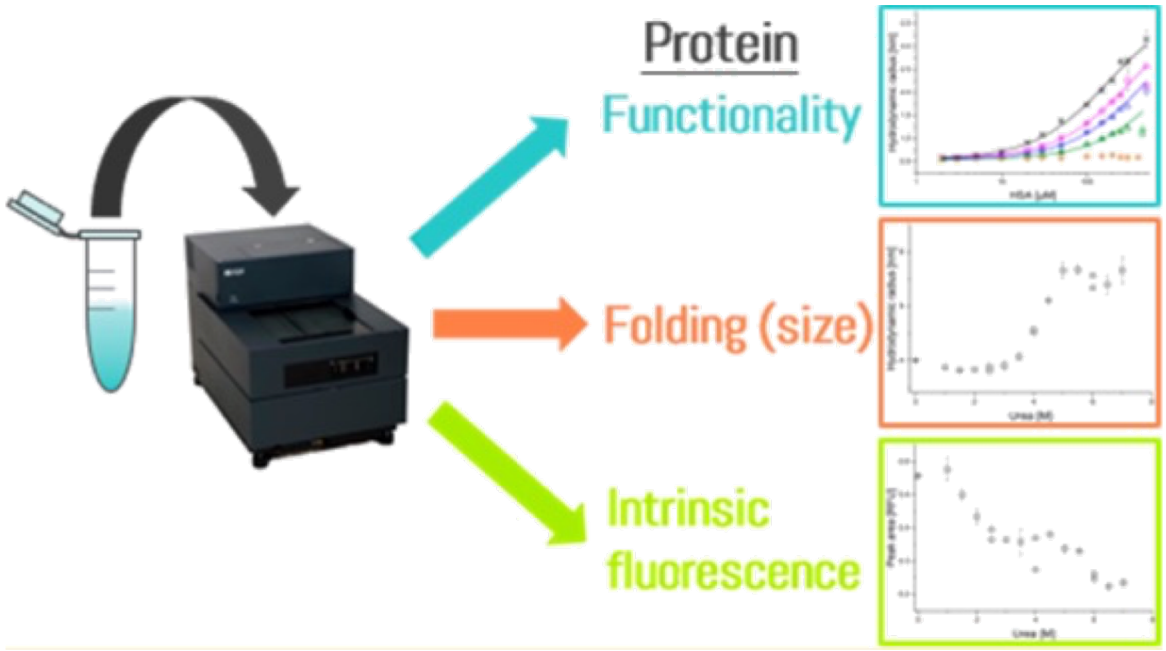
Morten E. Pedersen[1,2,3], Ragna M. S. Haegebaert[2,3], Jesper Østergaard[2] & Henrik Jensen[1,2]✉

scientific reports

Using TDA/FIDA to assess protein stability and unfolding

- Platform for detailed automated protein characterization: stabiity and unfolding

Human Serum Albumin + Urea → Denaturation/Unfolding of HSA

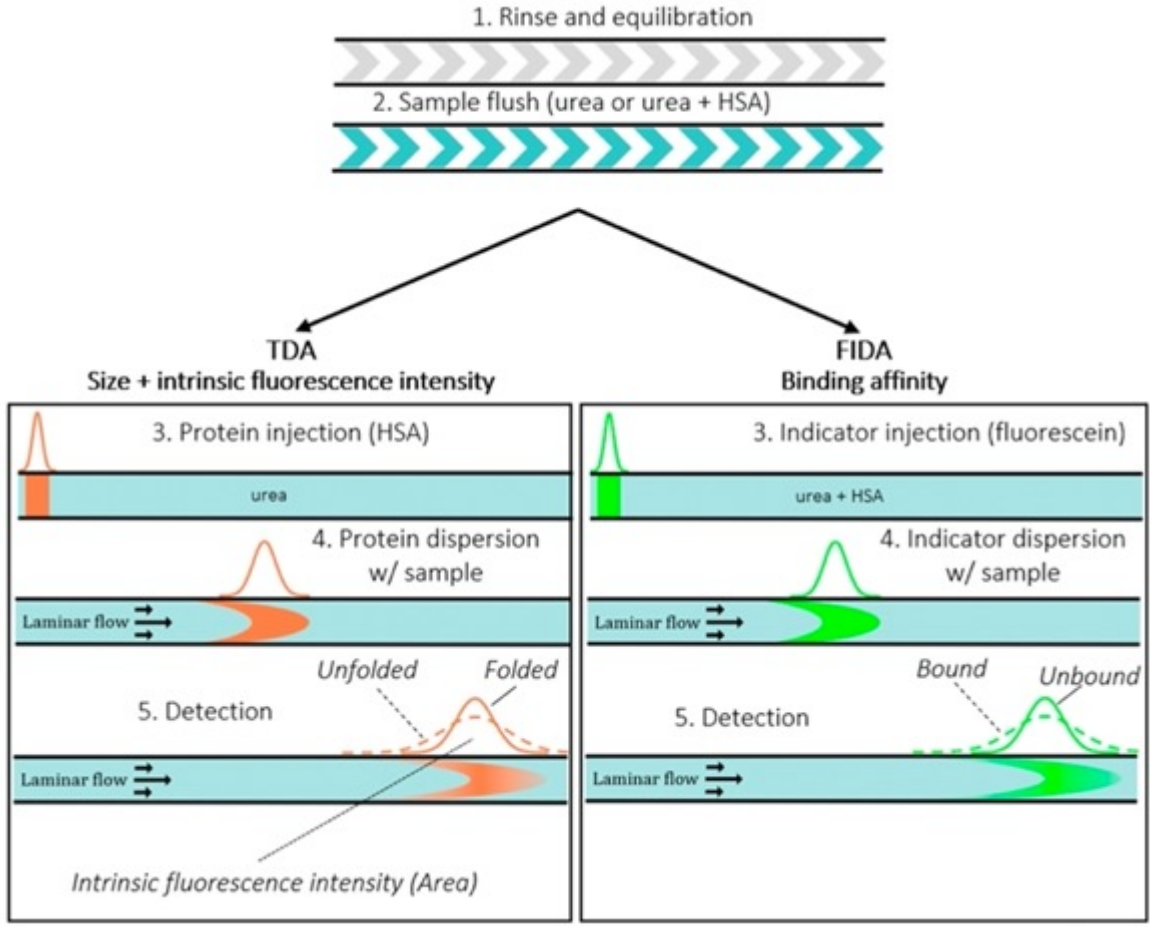


Loss of binding of a low-molecular weight ligand measured by FIDA during unfolding

Measuring increasing size by TDA during unfolding

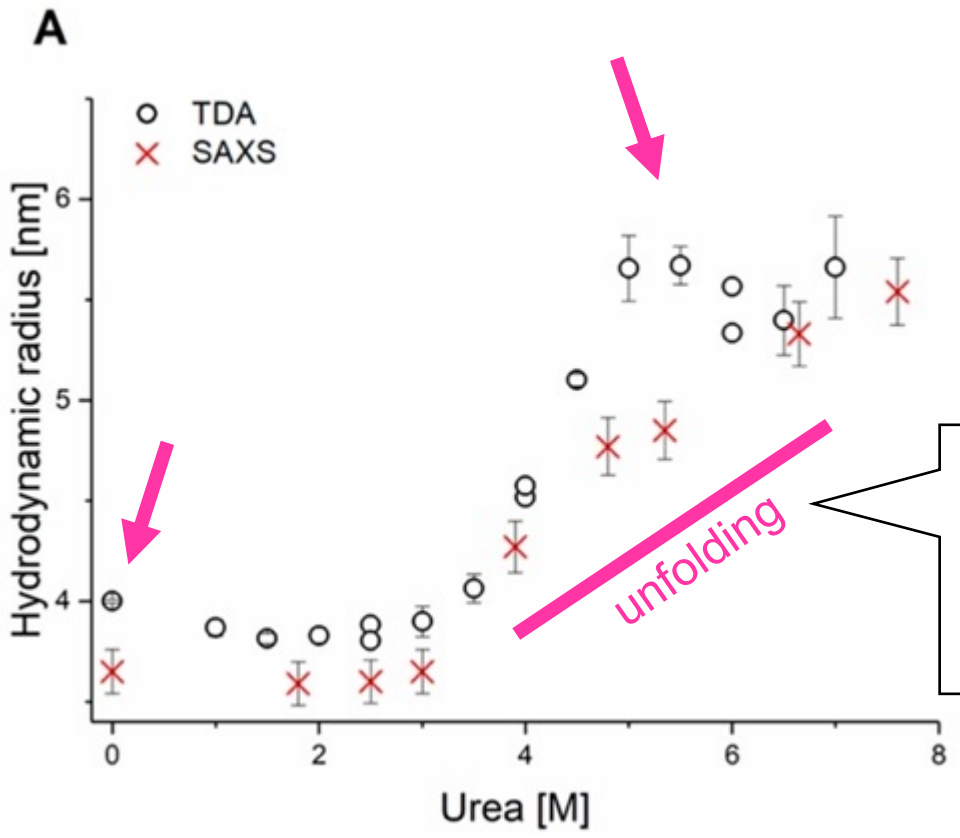
Intrinsic fluorescence of HSA changes when HSA is unfolded

Using TDA/FIDA to assess protein stability and unfolding



Urea-induced unfolding of HSA as measured by TDA

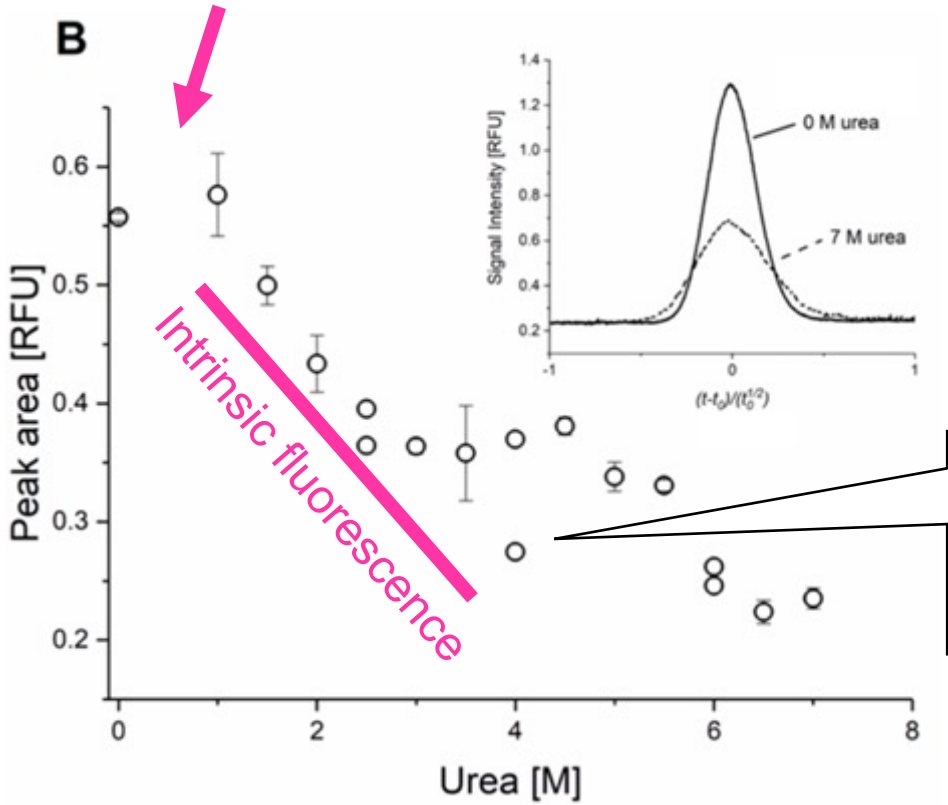
- The hydrodynamic radius of HSA was measured as a function of urea concentration
- SAXS (small-angle X-ray scattering) as comparison



Global HSA unfolding between 4 - 6 M urea, as measured in increased protein size

Urea-induced unfolding of HSA as measured by TDA

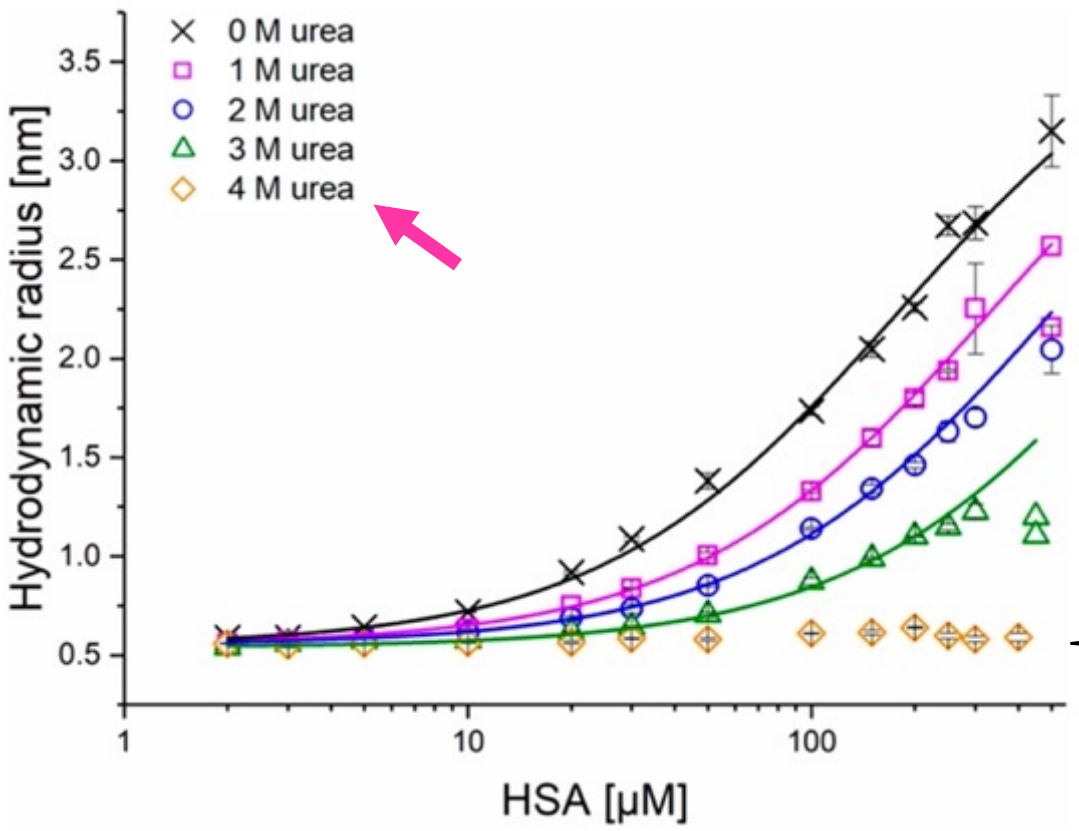
- Probing the intrinsic fluorescence intensity of HSA at increasing urea concentration



Structurally local changes below 4 M, prior to overall unfolding

Loss of HSA‘s binding affinity to fluorescein, determined by FIDA

- The normal interaction between HSA and fluorescein was investigated with increasing urea concentration using FIDA



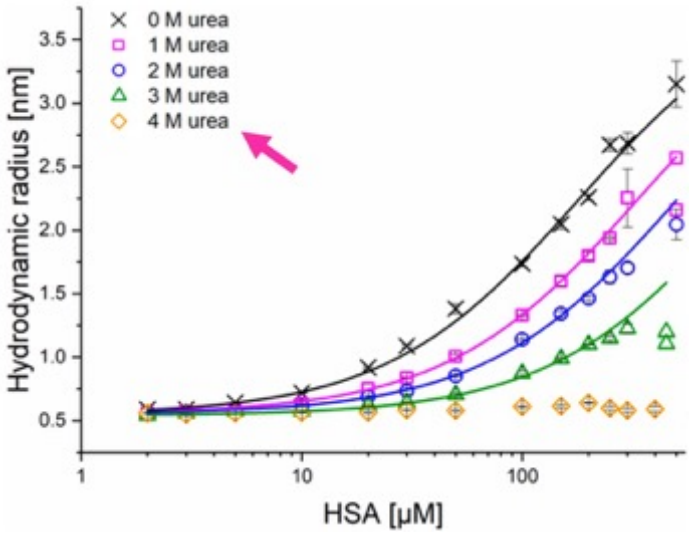
The more HSA, the more of the fluorescein is bound to HSA → higher hydrodynamic radius of the HSA-fluorescein complex (4 nm for HSA alone)

Gradual decrease in binding affinity from 1 to 3 M urea.

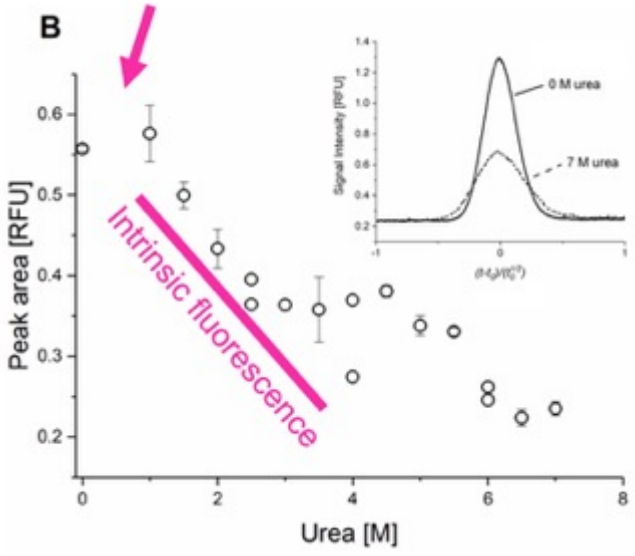
Completely lost binding at 4 M and higher urea conc.

Conclusion: FIDA to understand protein stability and function

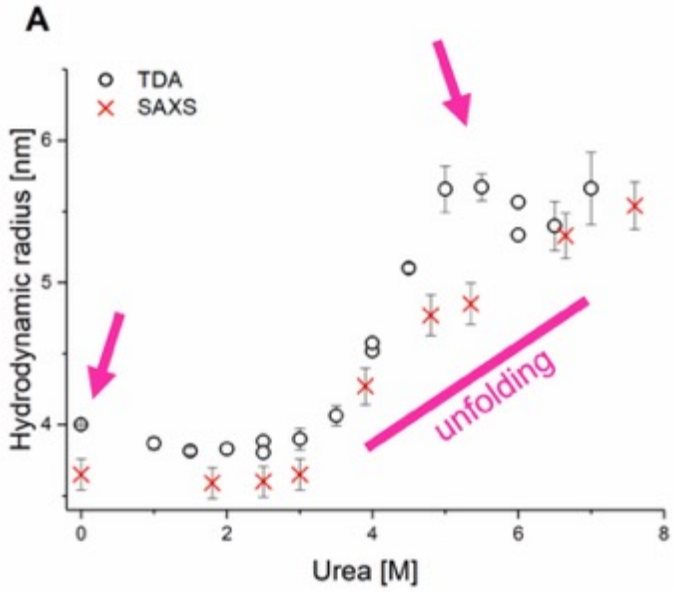
The presented approach was capable of a detailed characterization of the denaturation and unfolding process of HSA as induced by urea



decreased binding affinity to fluorescein at 1 M urea, indicating local changes at binding site (domain IIA)



Decreased intrinsic fluorescence at 1.5 M, indicating local changes near tryptophan and tyrosine residues



At urea 4 M increased hydrodynamic radius, indicating global unfolding

CrossMark

Cite this: *Analyst*, 2015, **140**, 4365

Received 10th April 2015,
Accepted 27th May 2015

DOI: 10.1039/c5an00697j

www.rsc.org/analyst

Flow induced dispersion analysis rapidly quantifies proteins in human plasma samples†

Nicklas N. Poulsen, Nina Z. Andersen, Jesper Østergaard,‡ Guisheng Zhuang, Nickolaj J. Petersen and Henrik Jensen*‡

ROYAL SOCIETY OF CHEMISTRY

analytical chemistry

Technical Note

Cite This: *Anal. Chem.* 2019, 91, 4975−4979

pubs.acs.org/ac

Protein Characterization in 3D: Size, Folding, and Functional Assessment in a Unified Approach

Morten E. Pedersen,† Sarah I. Gad,† Jesper Østergaard,†,‡ and Henrik Jensen*,†,‡

OPEN

Size-based characterization of adalimumab and TNF-α interactions using flow induced dispersion analysis: assessment of avidity-stabilized multiple bound species

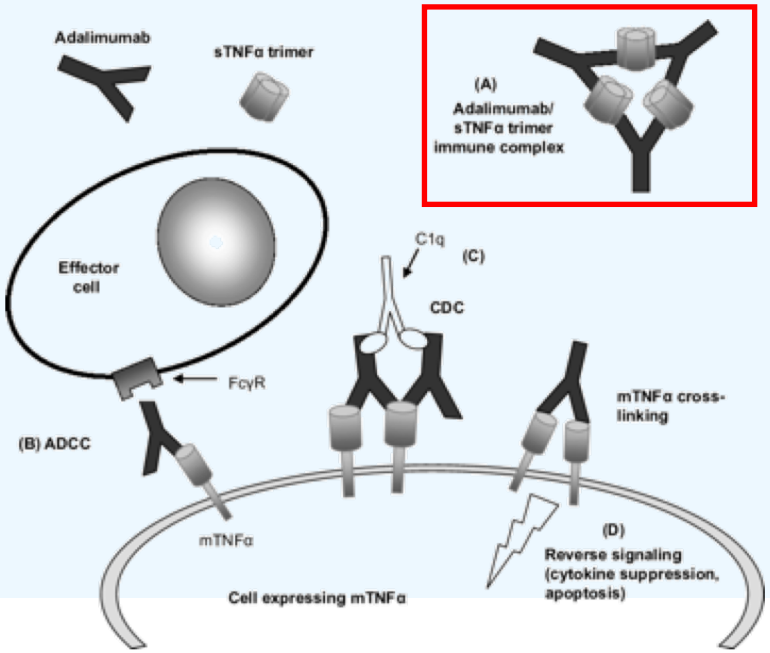
Morten E. Pedersen[1,2,3], Ragna M. S. Haegebaert[2,3], Jesper Østergaard[2] & Henrik Jensen[1,2]✉

scientific reports

Interaction of Adalimumab and TNF-α

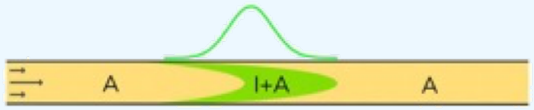
- The understanding and characterization of protein interactions is crucial, but often involves multiple binding, avidity, oligomerization and is dependent on local environment
- Current analytical methods fail…
- Flow-induced dispersion analysis (FIDA) for characterization of complex interactions under in vivo like conditions:

Size-based characterization of the interaction between TNF-α and adalimumab



Interaction of Adalimumab and TNF-α

- The apparent size of TNF-α increased with increasing adalimumab concentrations resulting in a binding curve. The increase in apparent size → formation of complexes

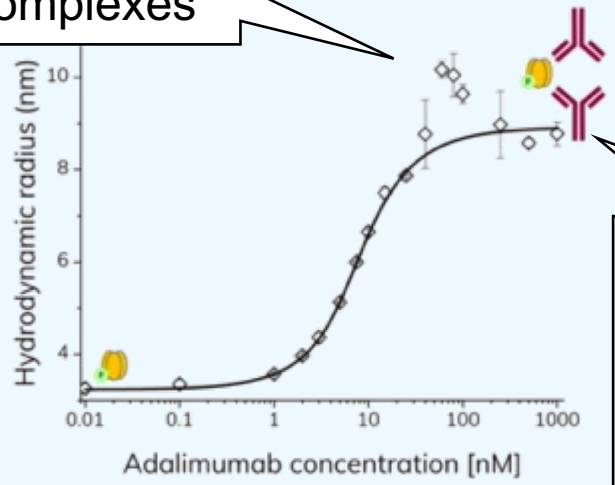


Pre-incubation of TNF-α-alexa 488 with adalimumab prior to the FIDA measurements

hook-shaped binding curve, indicates higher-order complexes

5.6 nm
(adalimumab alone)

3.2 nm
(unbound TNF-a-alexa488 trimer)

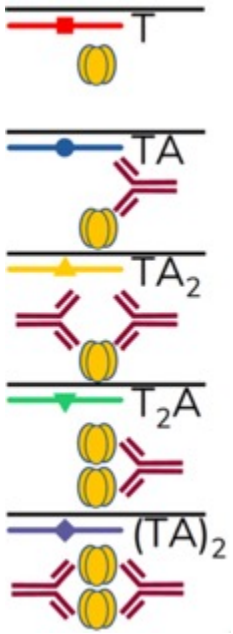


At excess adalimumab concentrations → shift towars smaller complexes

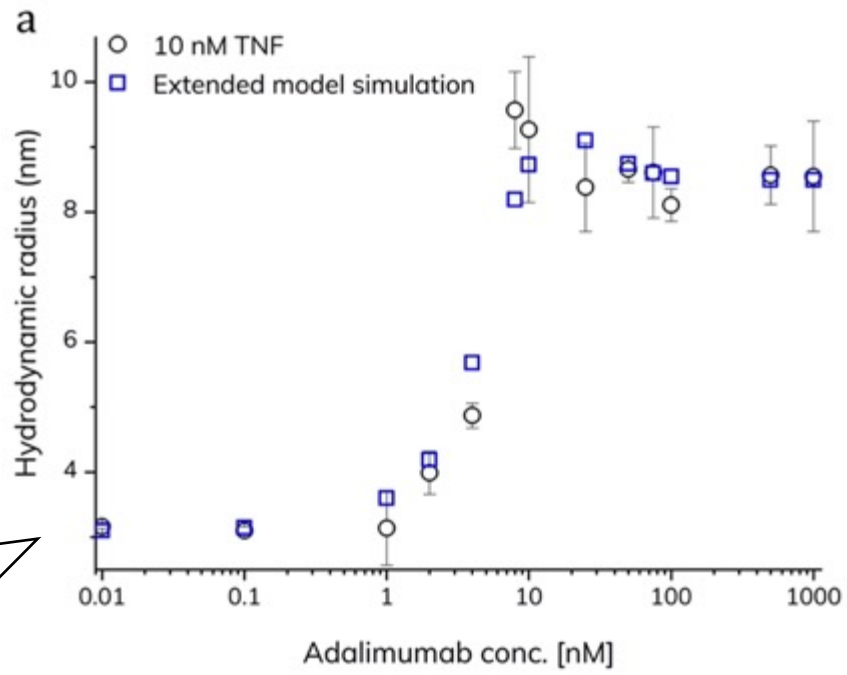
More advanced binding model is needed to further quantify the formation of higher-order complexes

Interaction of Adalimumab and TNF-α

- A range of TNF-α-adalimumab stoichiometries has been reported, e.g. 1:1, 1:2, 2:2, 3:2
- Creation of extended binding model to explain »hook shape«



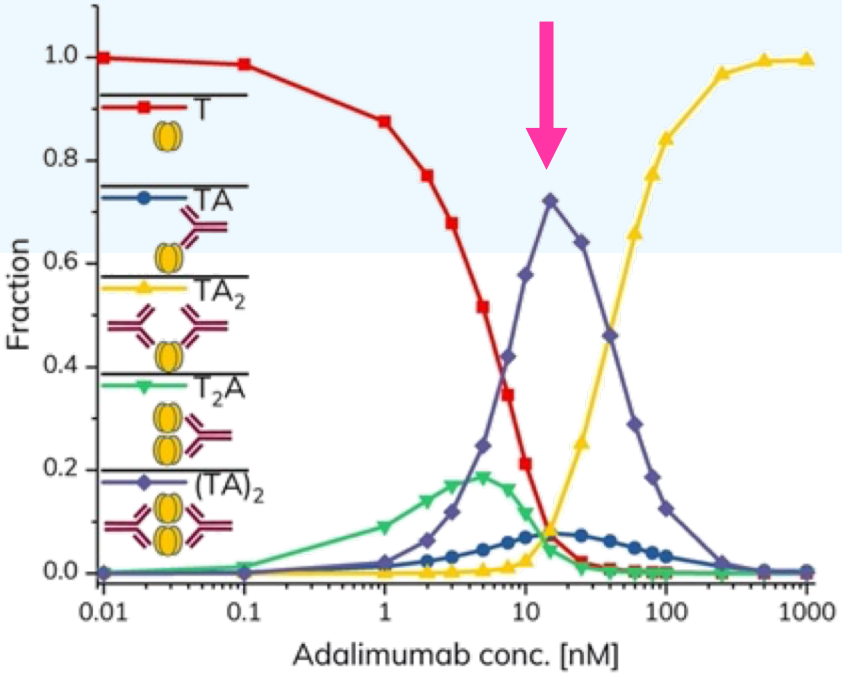
Complex size reported as hydrodynamic radius (nm)					Dissociation constant (nM)					
T	TA	TA_2	$(TA)_2$	T_2A	K_{d_1}	K_{d_2}	K_{d_3}	K_{d_4}	K_{d_5}	K_{d_6}
3.1	7.0	8.5	12	7.5	3	3	3	0.2	0.2	0.2



The model and selected simulation parameters explain and fit the experimental data well

Interaction of Adalimumab and TNF-α: Complex complexes

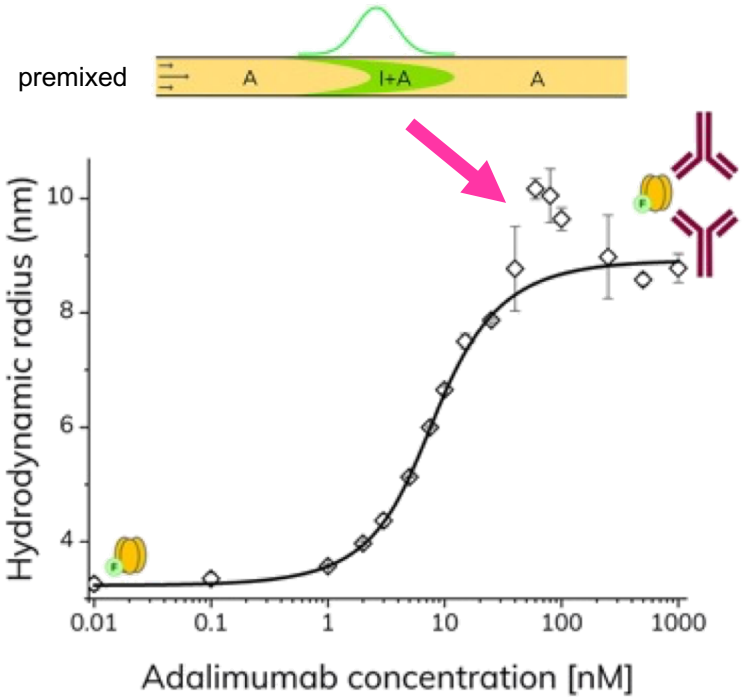
- Simulated fractions of the various complexes between TNF-α and adalimumab as a function of adalimumab concentration:
- The large $(TA)_2$ complex is the dominant species at intermediary adalimumab concentrations.

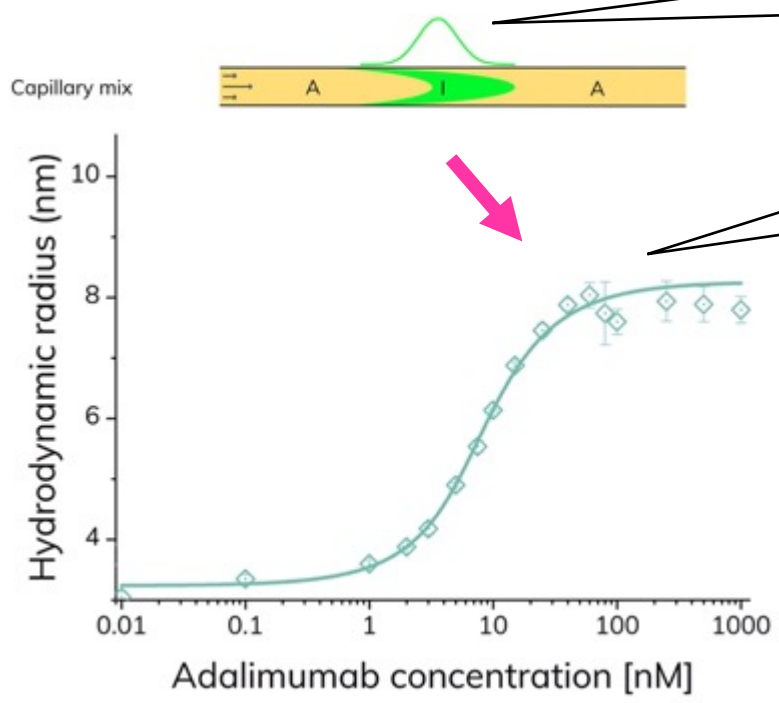


The amounts of the different species were heavily dependent on both the absolute and relative concentrations of TNF-α and adalimumab.

Assessment of binding kinetics

- Simulated fractions of the various complexes between TNF-α and adalimumab as a function of adalimumab concentration



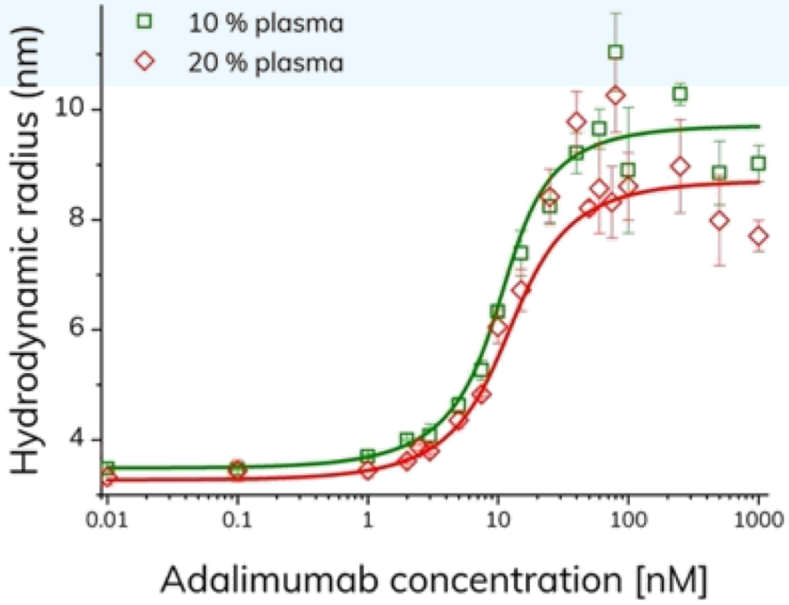


Samples not pre-mixed, short reaction time

No higher-order complexes were observed

Interaction of Adalimumab and TNF-α in human plasma

- Characterization of interaction in human plasma (physiological relevant conditions)
- The binding curves show an increased size of TNF-α with increasing concentrations of adalimumab as well as hook-shaped curves → similar to results in assay buffer



Plasma concentrations based on expected steady-state concentrations of adalimumab in patients receiving 40 mg every other week. Corresponding to 5.4 and 10.8 nM in 10 and 20% v/v plasma, respectively.

Minor differences in complex size as function of adalimumab concentration: Importance of physiological relevant conditions!

Conclusion: Interaction of Adalimumab and TNF-α

- Essential information on the TNF-α-adalimumab binding mechanism:

  Concentration-dependent complex sizes
  Binding affinities
  Kinetics
  Higher-order stoichiometries

- Confirmed the oligomeric state of TNF-α
- Larger and higher-order complexes require longer reaction time to form
- The nature of the complexes formed is not only of importance for characterizing the mode of action on a molecular level, but it may also provide information on **possible adverse effects** (e.g. some complexes prone to immunogenic responses).

CrossMark

Cite this: *Analyst*, 2015, **140**, 4365

Received 10th April 2015,
Accepted 27th May 2015

DOI: 10.1039/c5an00697j

www.rsc.org/analyst

Flow induced dispersion analysis rapidly quantifies proteins in human plasma samples†

Nicklas N. Poulsen, Nina Z. Andersen, Jesper Østergaard,‡ Guisheng Zhuang, Nickolaj J. Petersen and Henrik Jensen*‡

ROYAL SOCIETY OF CHEMISTRY

analytical chemistry

Technical Note

Cite This: *Anal. Chem.* 2019, 91, 4975−4979

pubs.acs.org/ac

Protein Characterization in 3D: Size, Folding, and Functional Assessment in a Unified Approach

Morten E. Pedersen,† Sarah I. Gad,† Jesper Østergaard,†,‡ and Henrik Jensen*,†,‡

OPEN

scientific reports

Size-based characterization of adalimumab and TNF-α interactions using flow induced dispersion analysis: assessment of avidity-stabilized multiple bound species

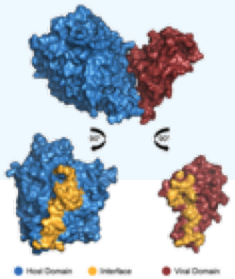
Morten E. Pedersen[1,2,3], Ragna M. S. Haegebaert[2,3], Jesper Østergaard[2] & Henrik Jensen[1,2]✉

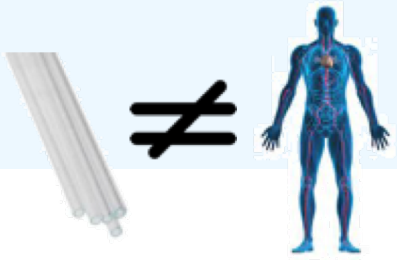
Summary

- Flow-induced dispersion analysis (FIDA) based on Taylor Dispersion Analysis (TDA)
- Applicable to measurement of both binding affinity and structural changes of biomolecular interactions under biorelevant conditions in a single assay format
- <u>Specific advantages</u>

  → Fast
  → Pico-nano molar sensitivity
  → Easier and faster assay development
  → Selective (analysis of blood plasma)
  → Performable on standard equipment
  → Functional information on binding activity

- <u>Downsides:</u>





Fidabio

Thank you for your attention