

WATER NMR FOR PROTEIN AGGREGATION CHARACTERIZATION

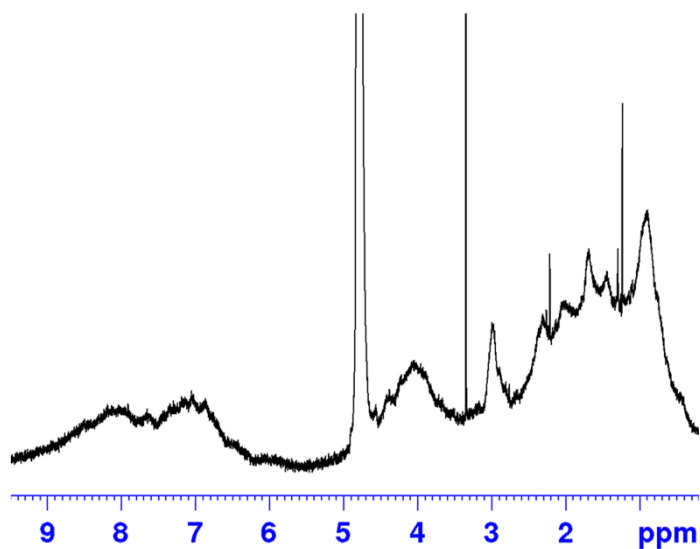
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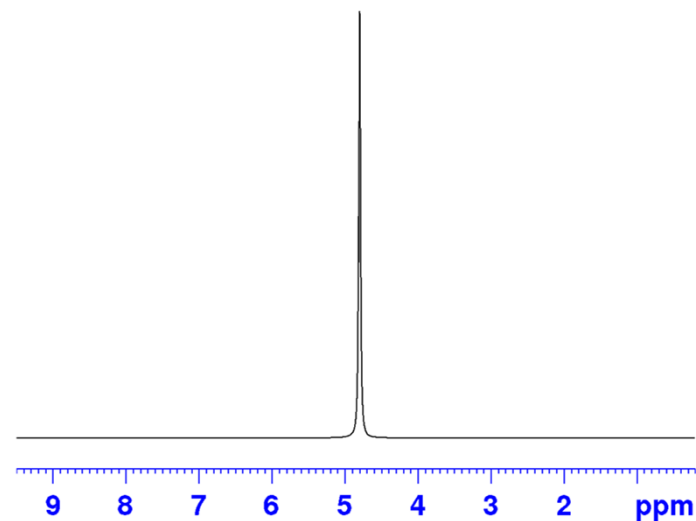
December 5, 2016, Baltimore
Protein Aggregation Measurement in Biotherapeutics: Established and Emerging
Techniques

WATER NMR—A NUISANCE OR A TOOL?

In aqueous solutions, solute resonances are considered the most important for NMR, and deuteration or suppression is used to remove interfering water signal

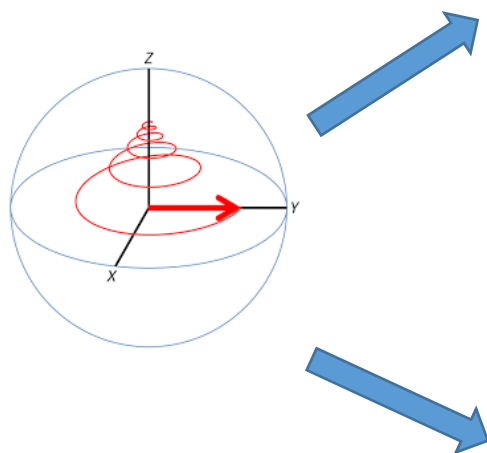


BSA (15 mg/mL in PBS buffer)
with water suppression (100
scans): bad resolution of
protein resonances.

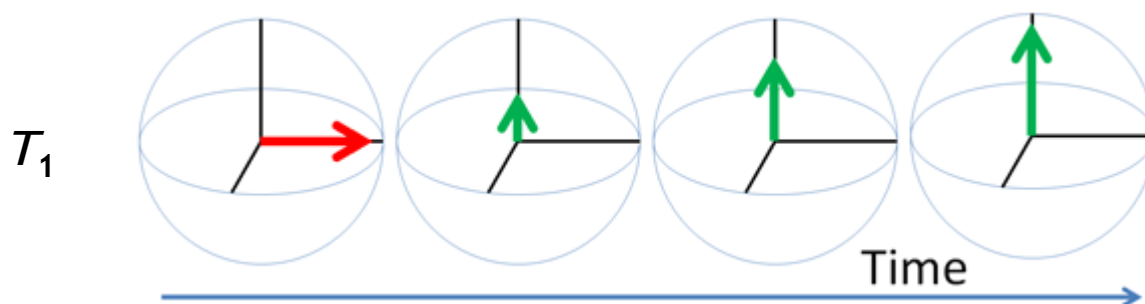


BSA (15 mg/mL in PBS buffer)
without water suppression (1
scan): high S/N for narrow
water signal, protein
resonances are invisible.

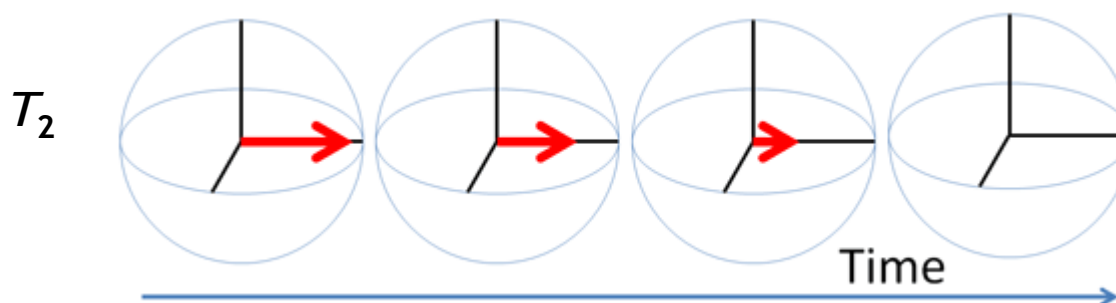
RELAXATION RATES IN NMR



Relaxation of the nucleus to its ground state (aligned with external magnetic field) is controlled by two mechanisms.



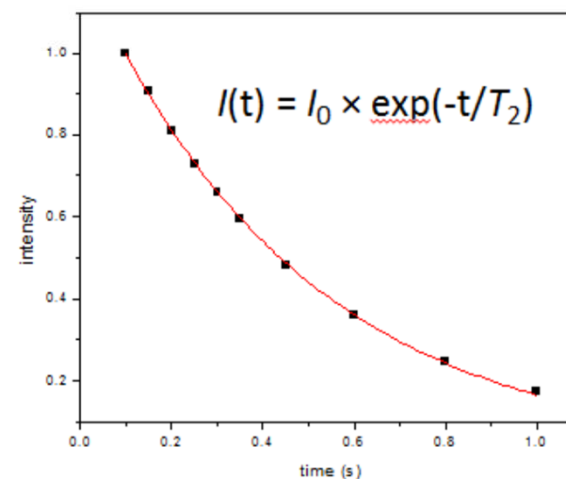
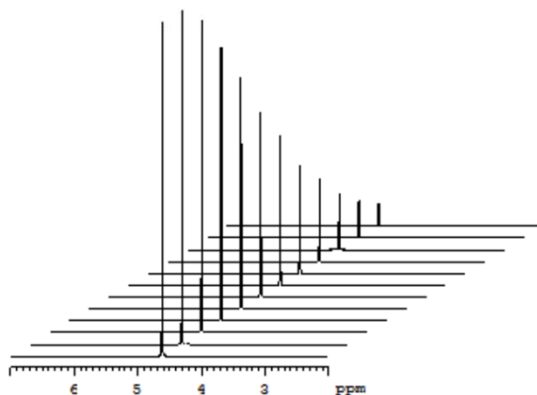
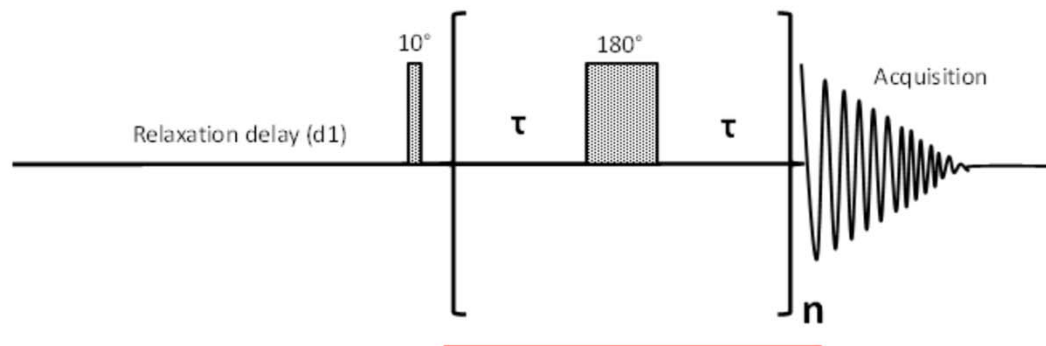
T_1 is spin-lattice or longitudinal relaxation, equal to time of energy transfer from excited to ground state along z-axis, often is defined by interaction between nucleus and media (solvent, diffusion).



T_2 is spin-spin or transverse relaxation, equal to time of energy transfer within the nucleus in the xy-plane due to dephasing, NMR line broadening down to the disappearance of the NMR signal, often defined by dipolar interactions, anisotropy of molecule, etc.

TRANSVERSE RELAXATION OF WATER CPMG PULSE SEQUENCE

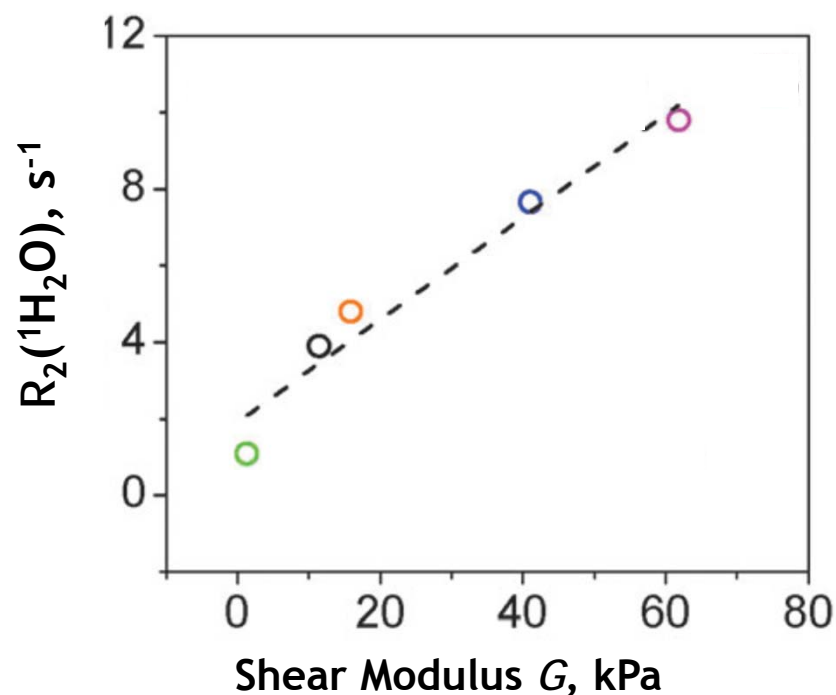
$$R_2(^1\text{H}_2\text{O}) = 1/T_2$$



T_2 is measured using classic CPMG pulse sequence that allows to monitor the drop in magnetization in xy -plane.

WATER NMR—A TOOL

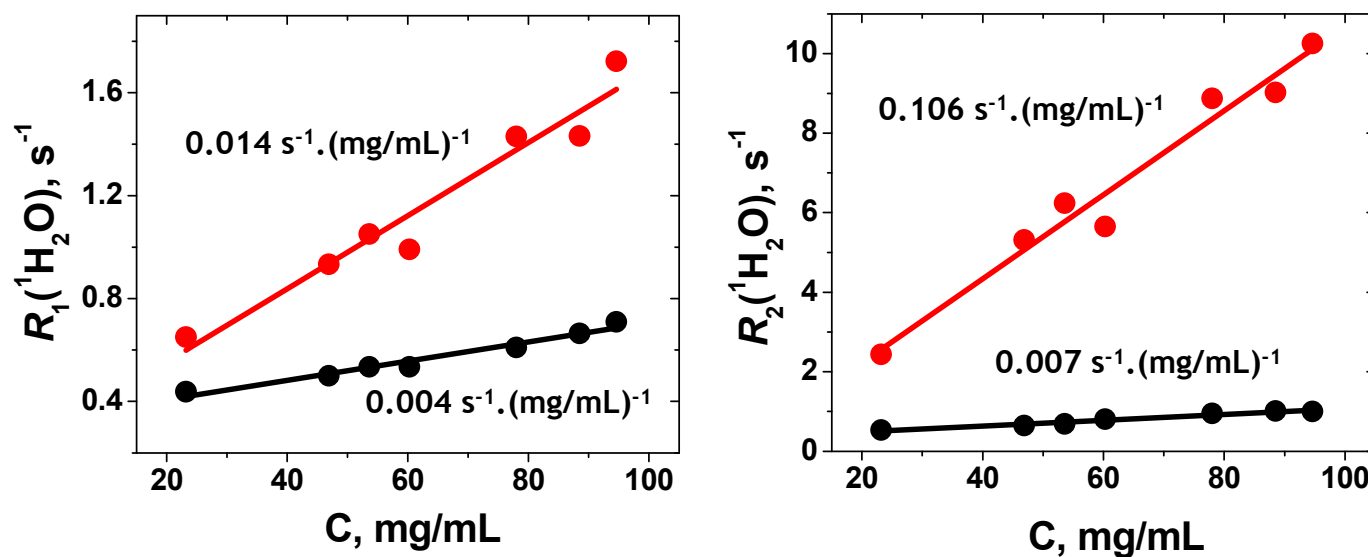
Water signal carries information on the global changes in the solute–water molecules interact with solute molecules and become sensitive to its changes, e.g., association



Water proton transverse relaxation rate, $R_2(^1\text{H}_2\text{O})$, could be used to measure the stiffness of peptide-based hydrogel. Gelation and aggregation both involve association, so would $R_2(^1\text{H}_2\text{O})$ also be sensitive to protein aggregation?

PRIOR ART–WATER RELAXATION IN HEAT-DENATURED PROTEINS

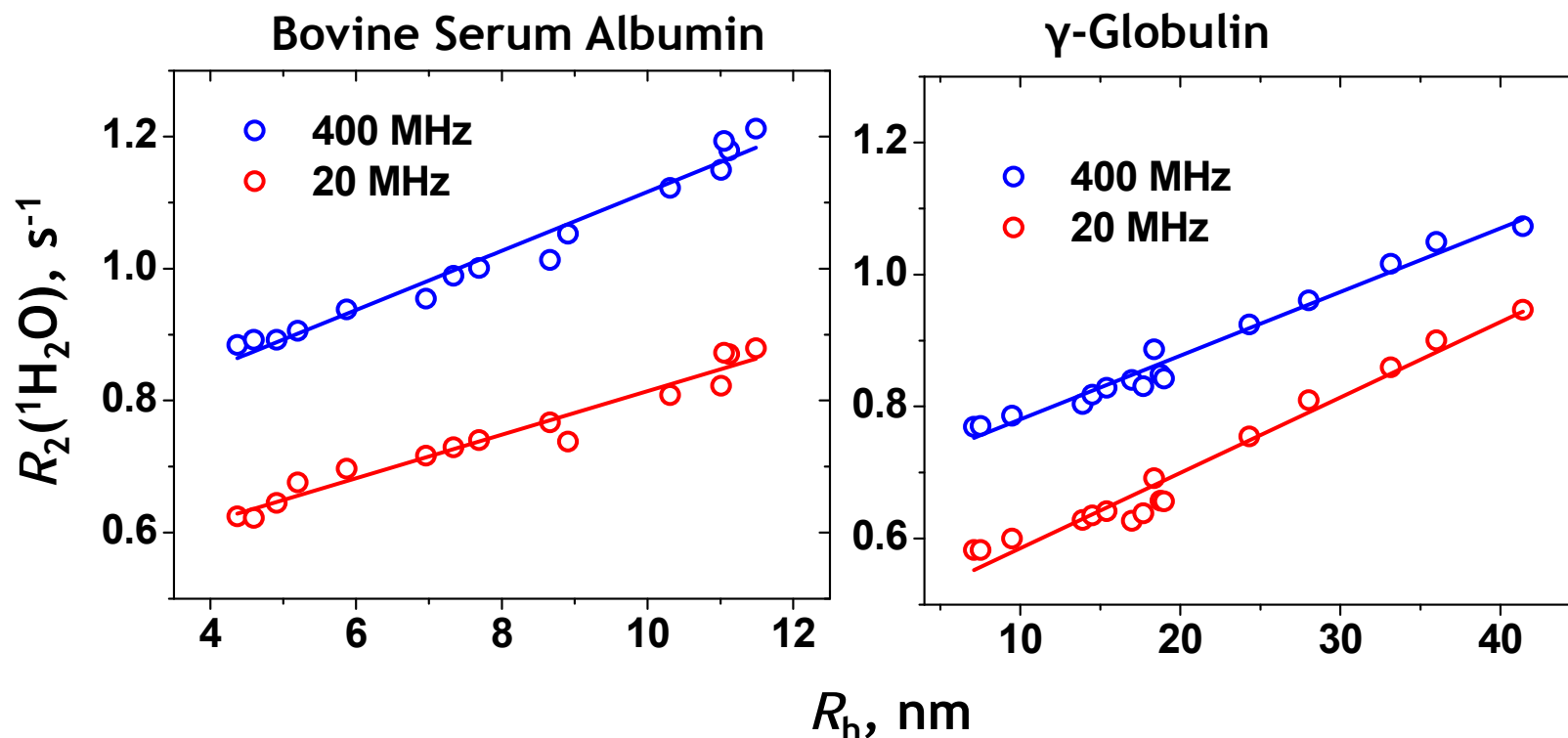
Ovalbumin • fresh; • heat denatured (0.33 Tesla, 14 MHz ^1H)



Does $R_2(^1\text{H}_2\text{O})$ correlate with aggregate size?

A PROBE FOR PROTEIN AGGREGATION

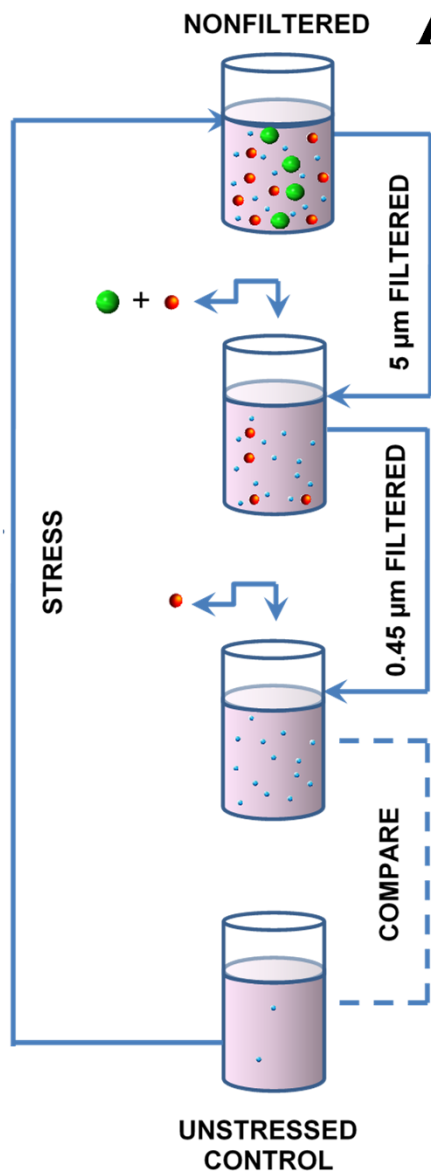
Water proton NMR is sensitive towards heat-induced aggregation of BSA and human γ -globulin, and could be used to quantify protein aggregation



$R_2(^1\text{H}_2\text{O})$ linearly increases with the growth of average hydrodynamic radius of protein aggregates.

Similar sensitivity observed in high (400 MHz) and low-field (20 MHz, BT NMR)

GENERATION OF MONOCLONAL ANTIBODY AGGREGATES OF VARIOUS SIZES



mAb has been stressed by:

Freeze-Thaw ($-40^{\circ}\text{C} \leftrightarrow 5^{\circ}\text{C}$, 16 cycles)

Heating at 50°C (36 h)

Agitation (24 h)

Aggregation was studied by

Conventional Techniques

Size-Exclusion Chromatography (SEC)

Dynamic Light Scattering (DLS)

Micro-Flow Imaging (MFI)

&

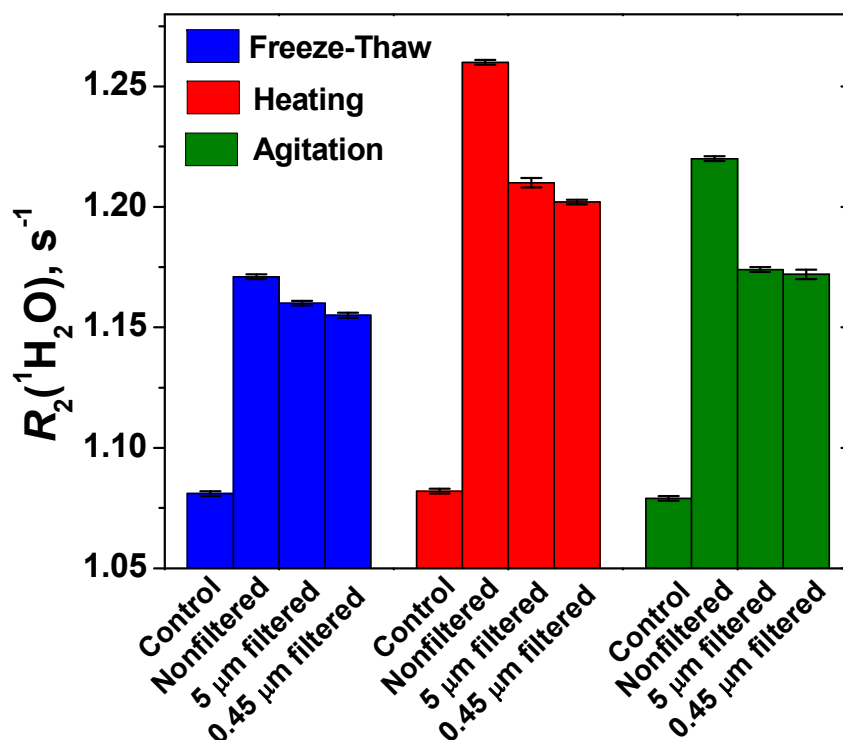
Water NMR (^1H NMR)

Transverse Relaxation Rate of Water

$R_2(^1\text{H}_2\text{O})$

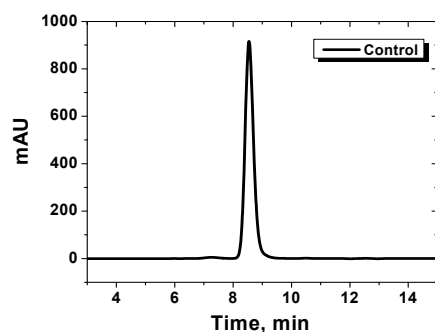
MEASUREMENT OF MAB AGGREGATION BY WNMR

$R_2(^1\text{H}_2\text{O})$ responded to aggregate formation under different stresses and differs from control after filtration

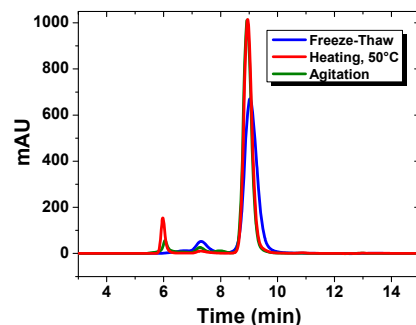


- $R_2(^1\text{H}_2\text{O})$ increased in each stressed sample compared to the unstressed control sample
- Filtration reduced the increase in $R_2(^1\text{H}_2\text{O})$ for all stresses
- $R_2(^1\text{H}_2\text{O})$ was still different after 0.45 micron filtration between stresses

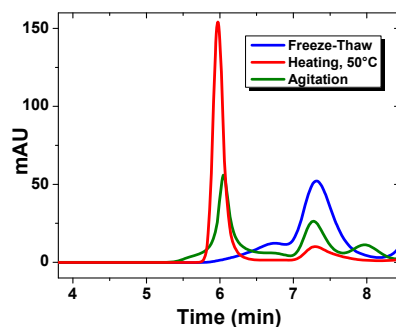
MEASUREMENT OF mAb AGGREGATION BY SEC



Control



Stressed samples



Aggregates

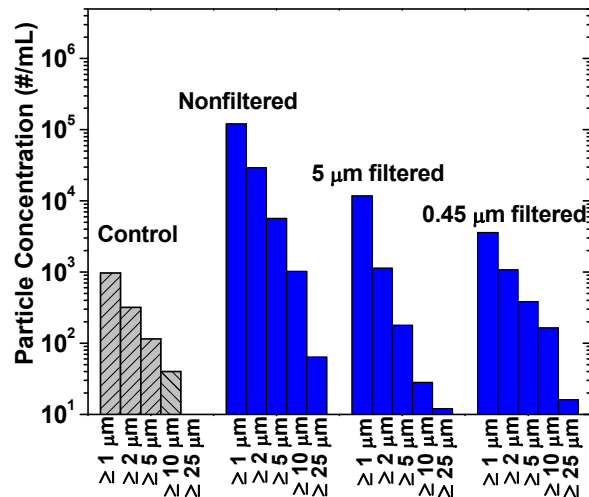
% Low (LMW) and % High Molecular Weight (HMW) and Total % soluble mAb aggregates for three stresses

	Aggregates	Control	Nonfiltered	5 μ m filtered	0.45 μ m filtered
Freeze-Thaw	% LMW	0.8	7.4	7.3	7.3
	% HMW	0.0	1.9	2.0	2.0
	Total % Aggr	0.8	9.3	9.3	9.3
Heating 50 °C	% LMW	0.8	1.4	1.4	1.4
	% HMW	0.0	6.5	6.4	6.4
	Total % Aggr	0.8	7.9	7.8	7.8
Agitation	% LMW	0.6	3.2	3.2	3.4
	% HMW	0.0	6.9	6.0	5.9
	Total % Aggr	0.6	10.1	9.2	9.3

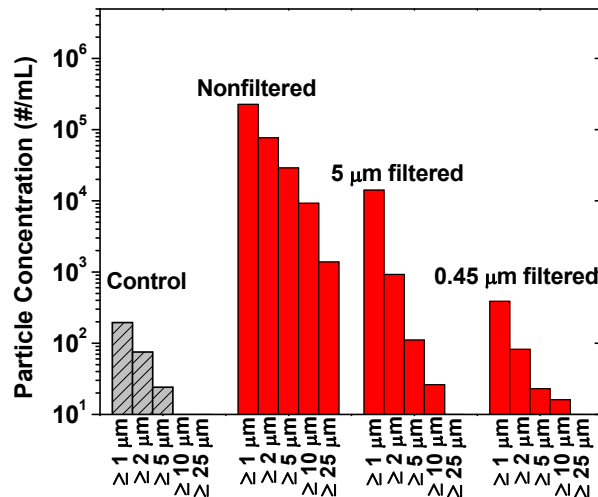
- Total percentage of aggregates were similar, but aggregate profile was different between each stress type
- 5 μ m & 0.45 μ m filtration did not change the ratio between LMW and HMW aggregates or total percentage of aggregates

MEASUREMENT OF MAB AGGREGATION BY MFI

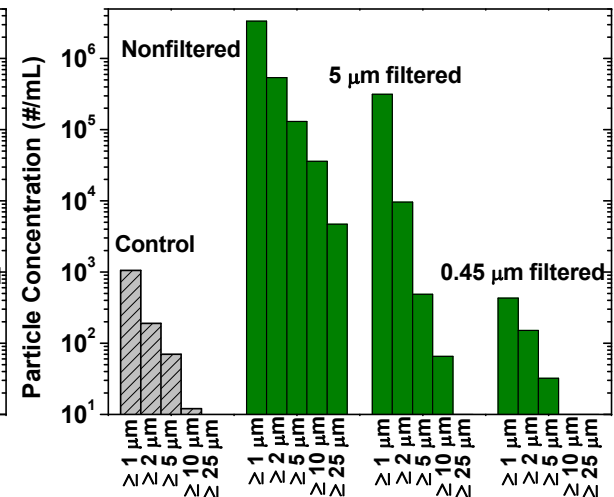
Decrease in particle counts during filtration from MFI



Freeze-Thaw



Heating, 50 °C

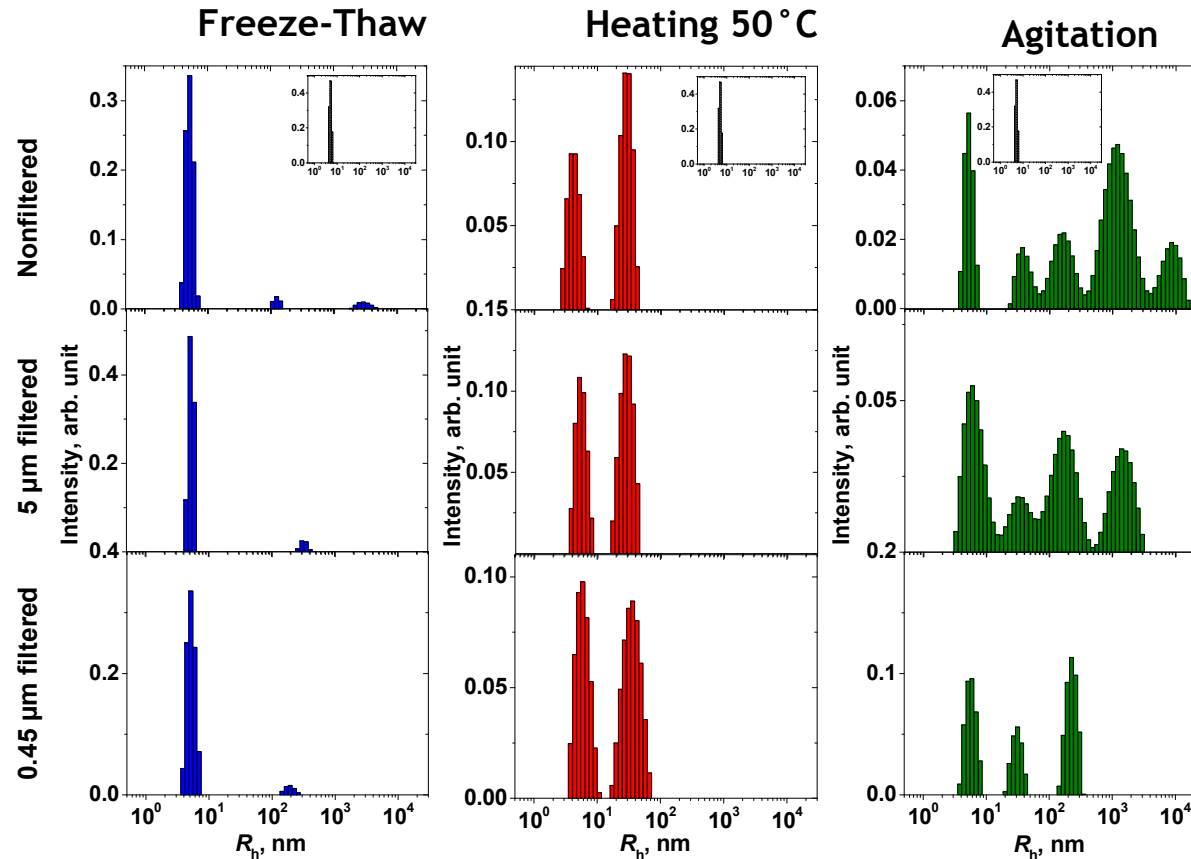


Agitation

- 5 μm filtration reduced particle counts from ≥ 1 μm to ≥ 25 μm (not only for ≥ 5 μm particles)
- After 0.45 μm filter, the samples for all three stresses are very close to the unstressed control

MEASUREMENT OF MAB AGGREGATION BY DLS

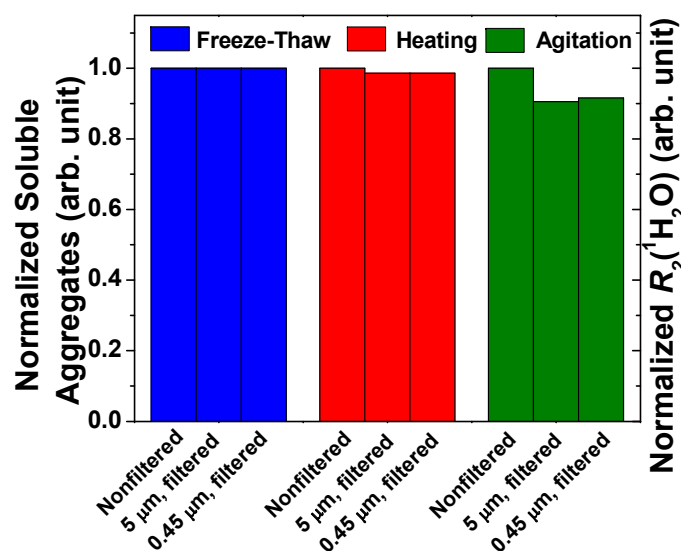
Each stress produced different particle size distributions (PSD) of aggregates



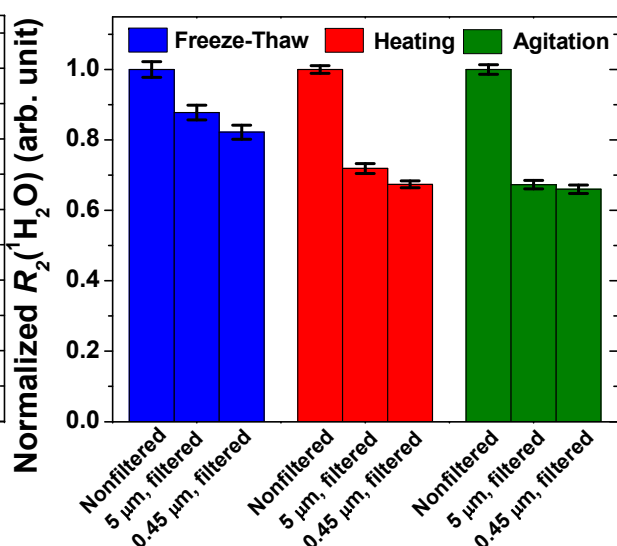
- 5 µm and 0.45 µm filters had minimal effect on PSD for freeze-thaw and heating
- Both filtration steps affected the PSD for agitation stress significantly

SENSITIVITY OF EACH TECHNIQUE TO SIZE AND NUMBER OF AGGREGATES

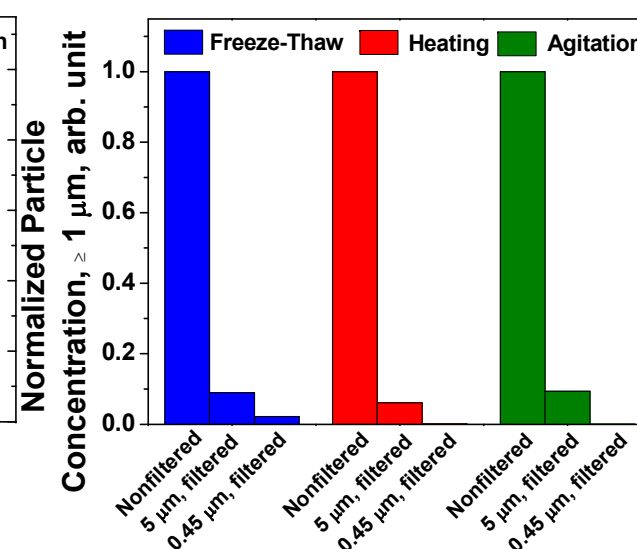
SEC



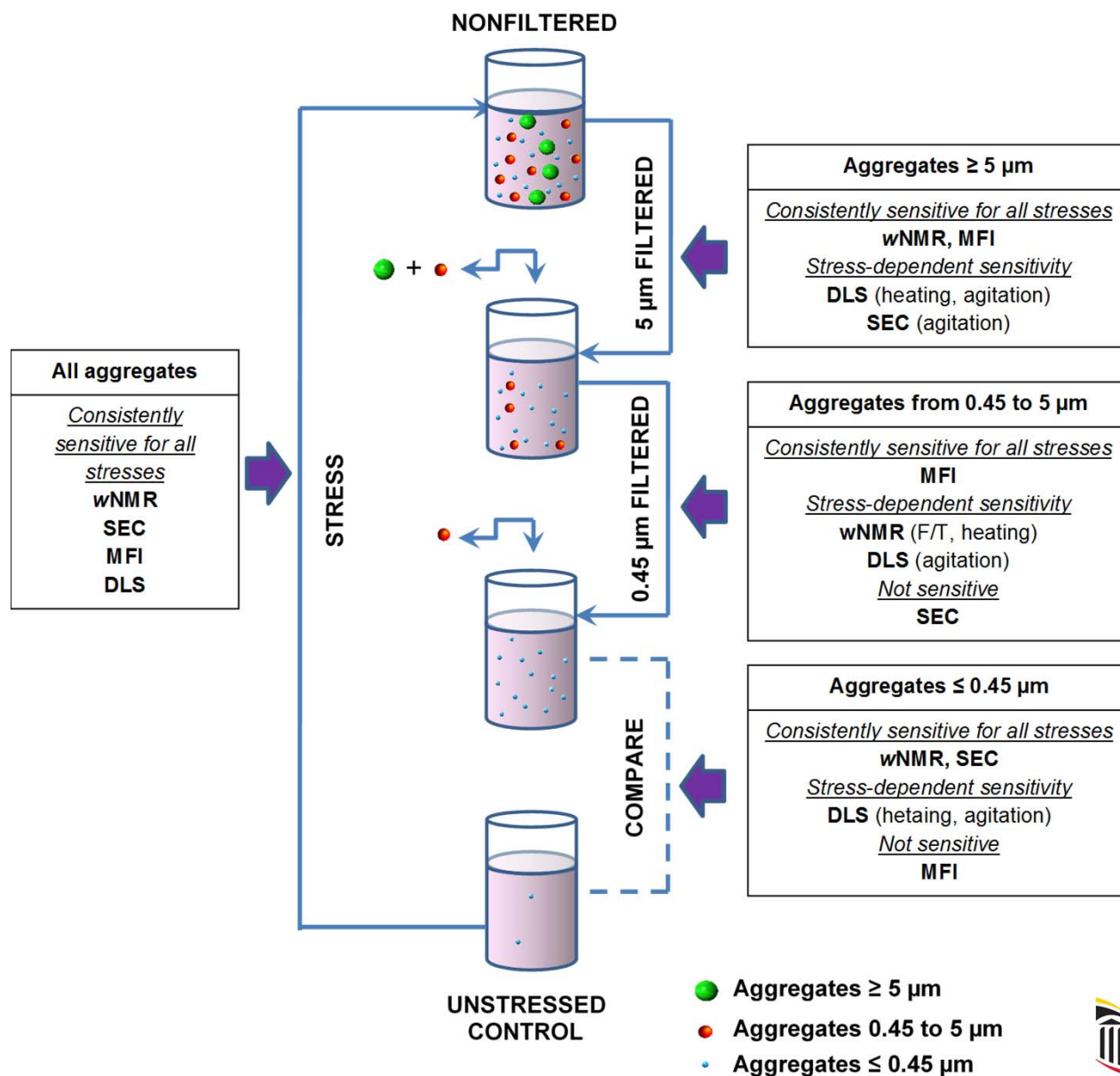
WNMR



MFI



SENSITIVITY RANGE OF EACH METHOD TO ANTIBODY AGGREGATES

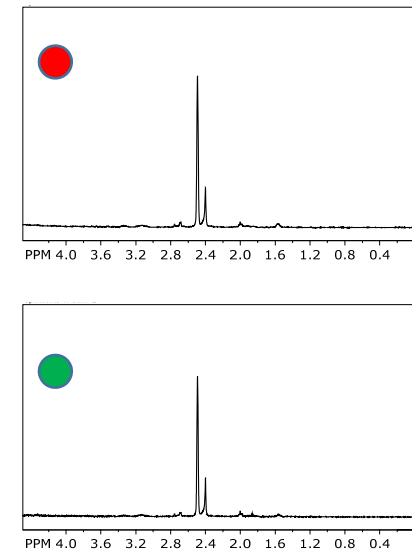
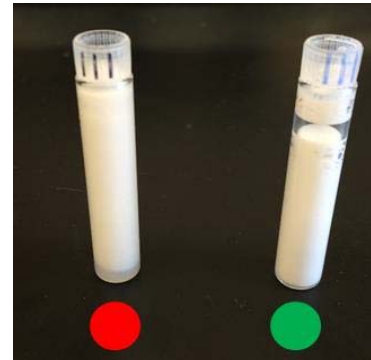
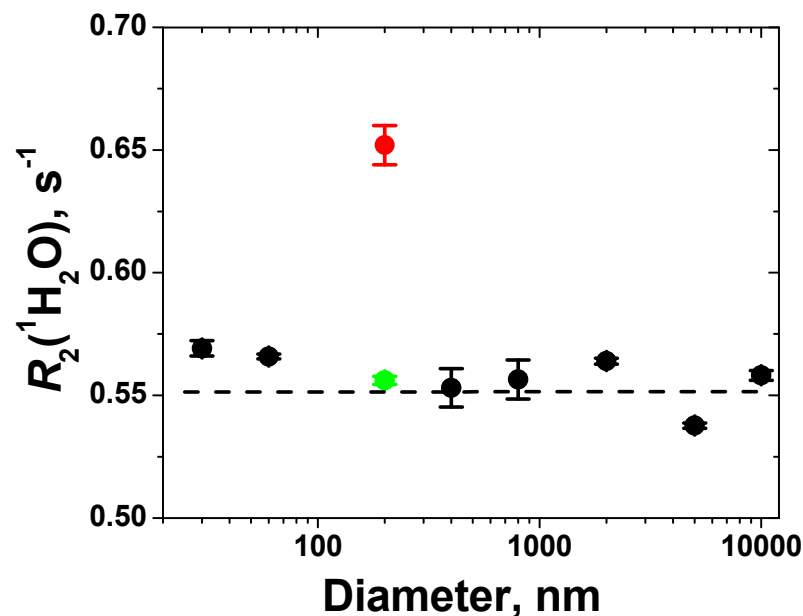


wNMR was most consistently sensitive to differences in sample quality across each stress type and after sample filtrations

POSSIBLE MECHANISMS OF SENSITIVITY OF PROTON NMR TO PROTEIN AGGREGATES

WATER NMR SENSES NANOPARTICLE CLUSTERING

- Two 200 nm polystyrene nanoparticle samples are visually indistinguishable
- ^1H NMR spectra show no difference in the signal intensities or chemical shifts

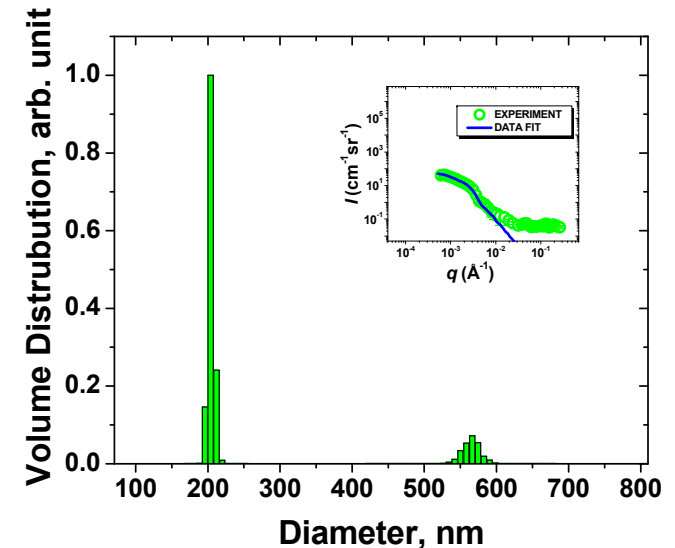
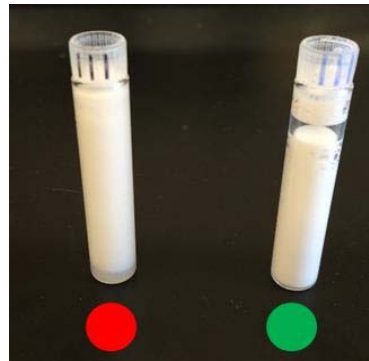
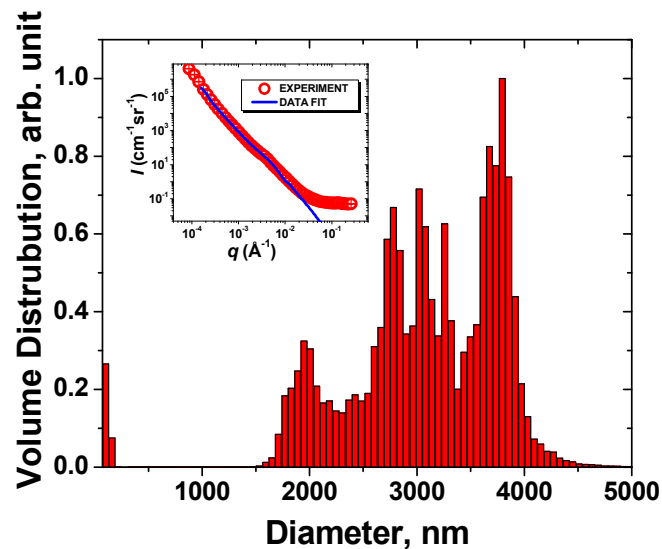


But one of them demonstrate **anomalously high water relaxation rate**

WHY?

WATER NMR SENSES NANOPARTICLE CLUSTERING

USAXS shows that anomalous sample contain mainly 2-4 μm particulates
While the good quality sample overwhelmingly contains 200 nm particles

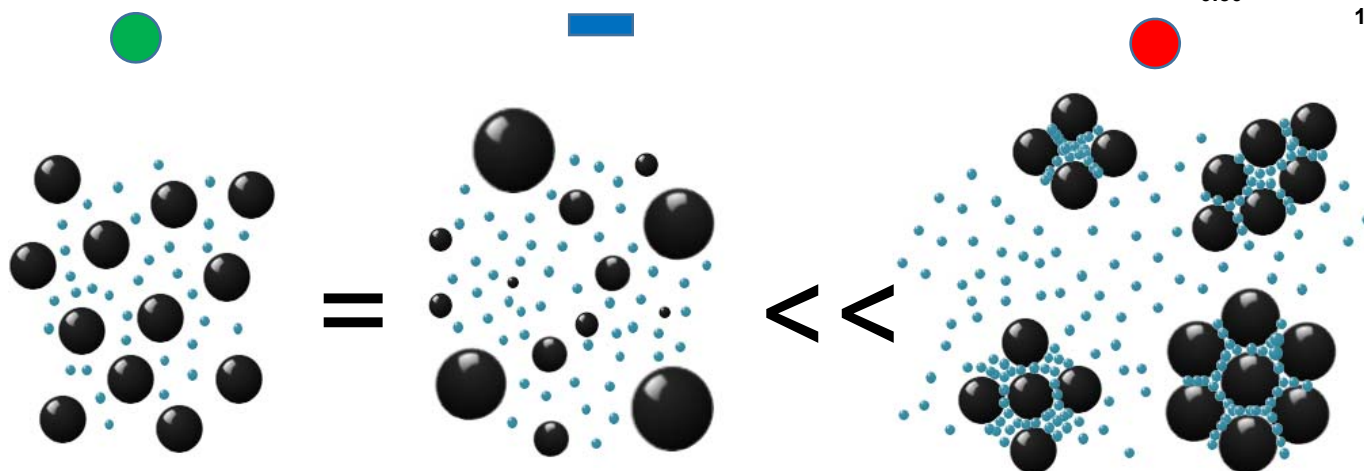
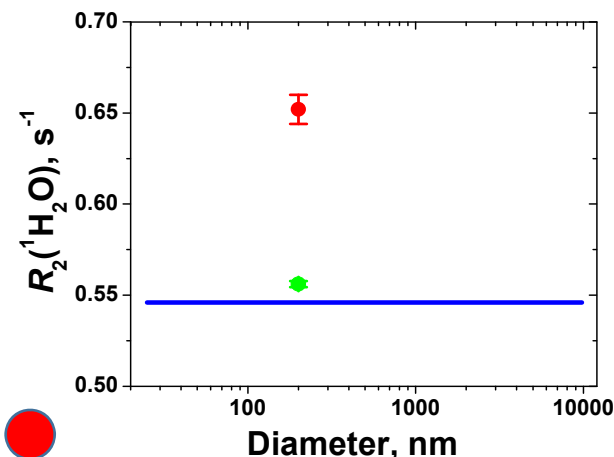


The anomalous sample had quality issues (confirmed by manufacturer), and nanoparticles in this sample are clustered and formed larger assemblies

USAXS = Ultra-small angle X-ray Scattering

WATER NMR SENSES NANOPARTICLE CLUSTERING

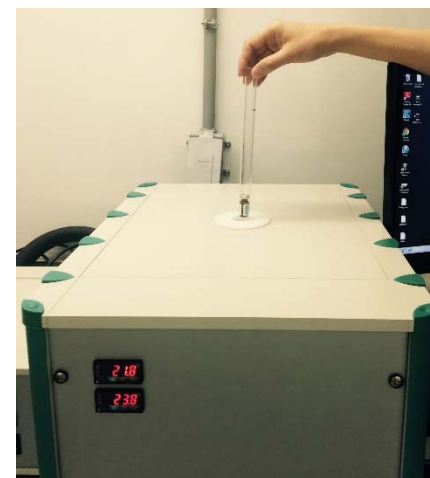
Sample polydispersity is not affecting $R_2(^1\text{H}_2\text{O})$ —mixture of different sizes is close to monodisperse sample/blank buffer (blue line).



Water molecules in the clustered compartments have different diffusive exchange and local magnetic field gradient resulting in anomalously high $R_2(^1\text{H}_2\text{O})$

CONCLUSIONS

- Water transverse relaxation rate $R_2(^1\text{H}_2\text{O})$ was a sensitive probe responding to changes in solute molecules: association, clustering, aggregation, etc.
- In protein aggregation, $R_2(^1\text{H}_2\text{O})$ was sensitive to the presence of insoluble particulates $\geq 5 \mu\text{m}$, from $\geq 1 \mu\text{m}$ to $5 \mu\text{m}$ as well as to soluble protein aggregates below $1 \mu\text{m}$.
- $R_2(^1\text{H}_2\text{O})$ can be monitored noninvasively using inexpensive benchtop low-field NMR spectrometers with wide bore capable to accommodate drug product vials without opening or sampling.



DON'T THROW THE BABY OUT WITH THE BATHWATER!

ACKNOWLEDGEMENTS

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