Compensated Hydroxyl Radical Footprinting: A Flexible, Quantitative Probe of Protein Topography

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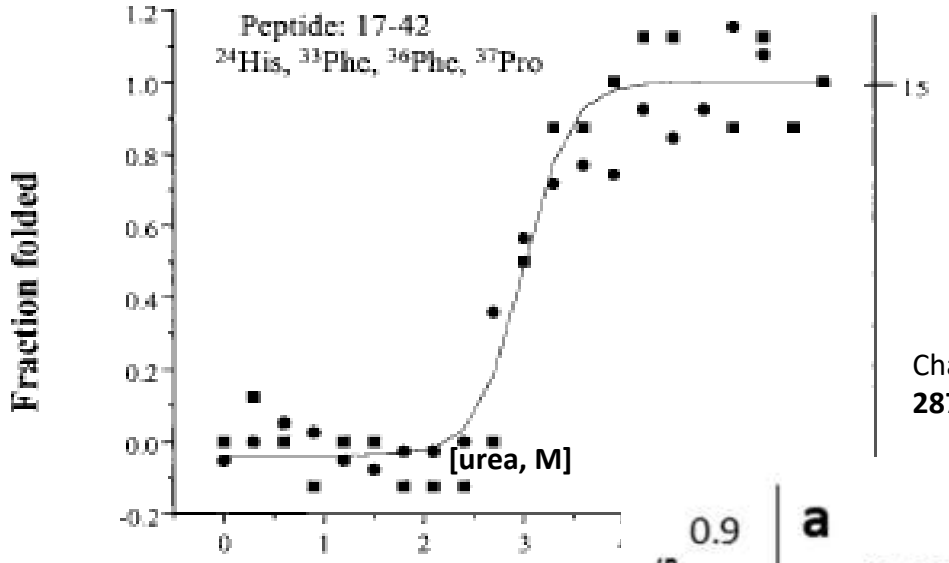
FCOI Statement: J.S.S. discloses a significant financial interest in GenNext Technologies, Inc., an early-stage company seeking to commercialize benchtop HRPF to support the pharmaceutical industry

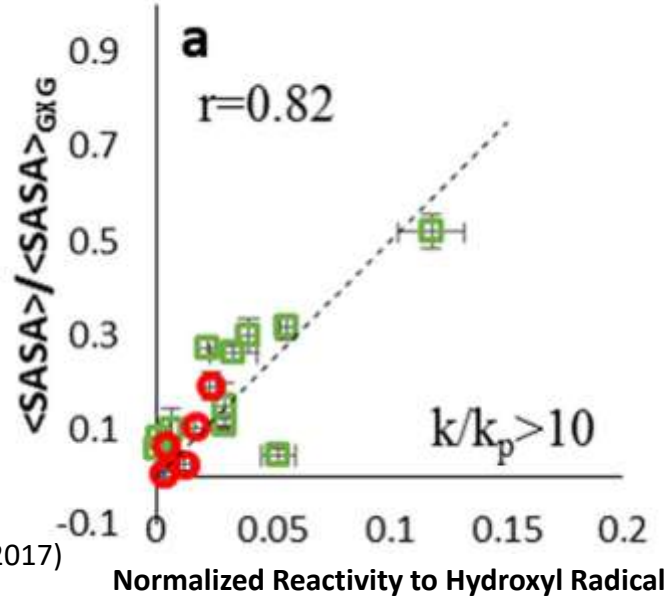
Foundation of Hydroxyl Radical Protein Footprinting

- Proteins in dilute aqueous solution exposed to diffusing hydroxyl radicals → oxidized amino acid side chains
- All amino acids reactive, but at widely differing rates
- Sequence context can influence inherent reactivity, especially for less reactive amino acids
- Rate of oxidation appears to primarily be a function of two factors
  - Chemical nature of oxidized residue
  - Average accessibility of oxidation target to the hydroxyl radical over the time of radical exposure



Chance, *Biochem Biophys Res Commun* **287,**614-621 (2001)

- **Changes in reaction rates in the same sequence reflects changes in average solvent accessibility**

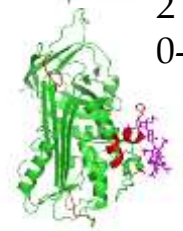


Xie *et al*, *Sci Rep* **7,**4552 (2017)

Flash Photochemical Oxidation of Proteins—How It Works

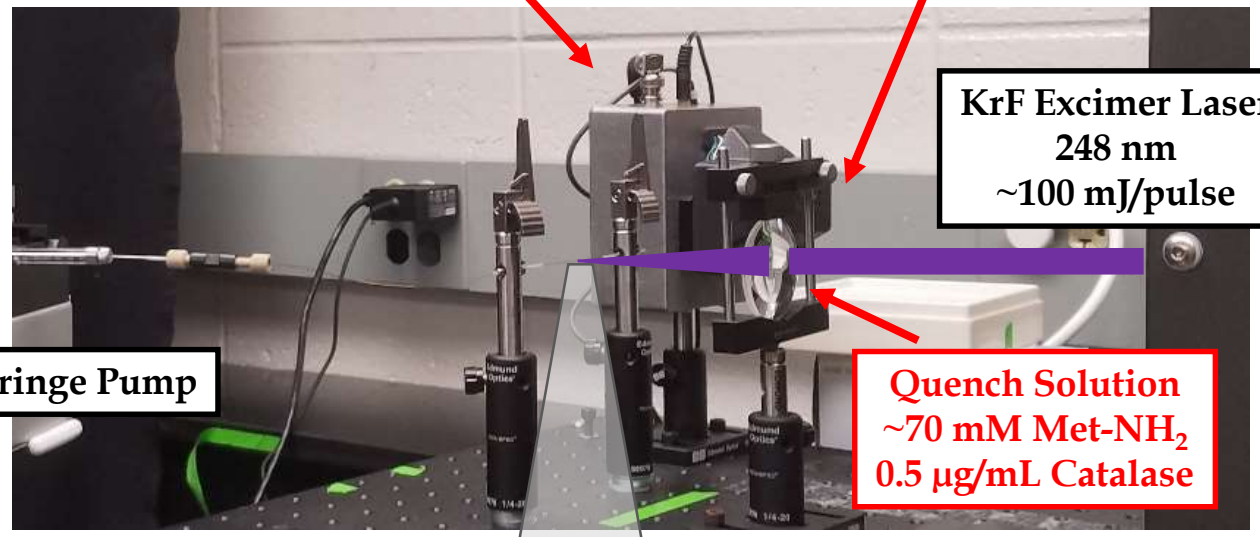
Condition 1

17 mM Glutamine
2 mM Adenine
0-100 mM H_2O_2



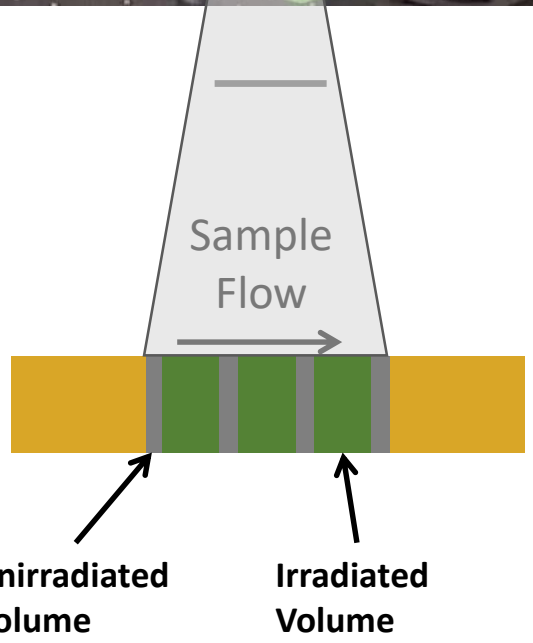
Condition 2

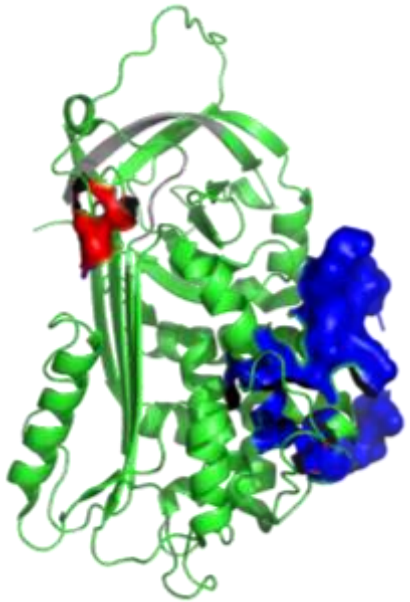
Syringe Pump

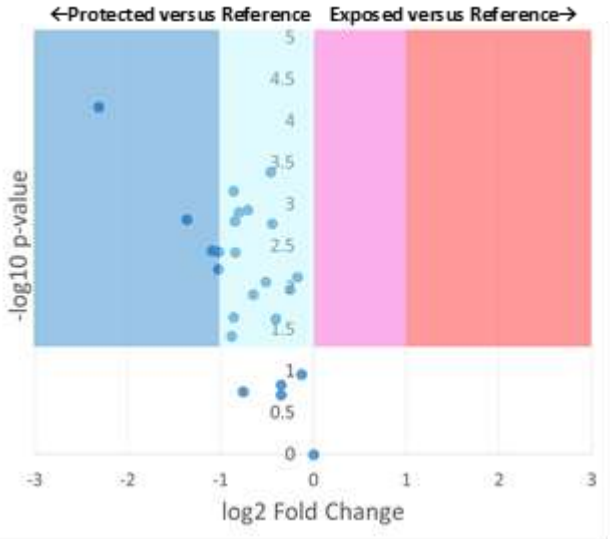


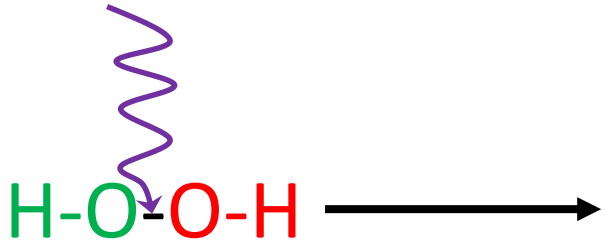
Sample Processing
—Reduction
—Proteolytic Digestion
—Deglycosylation
—Clean-Up

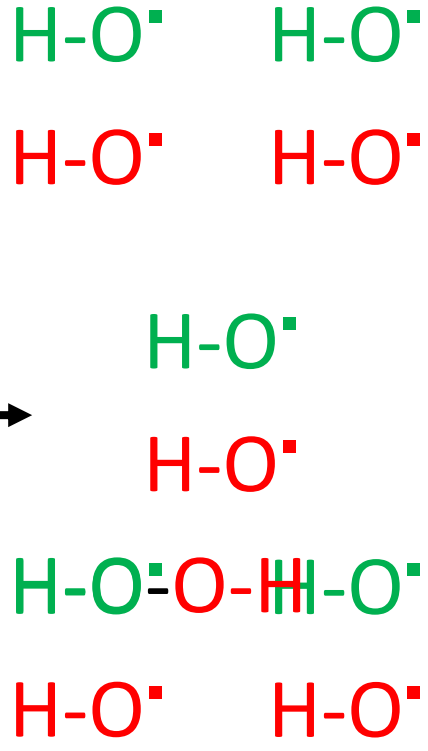
LC-MS

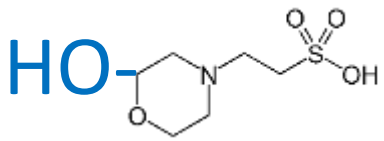


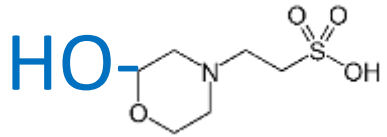


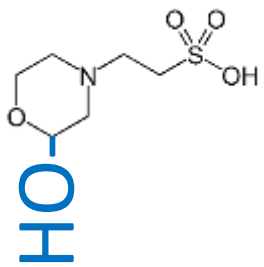


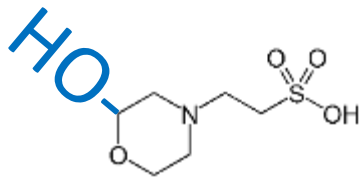


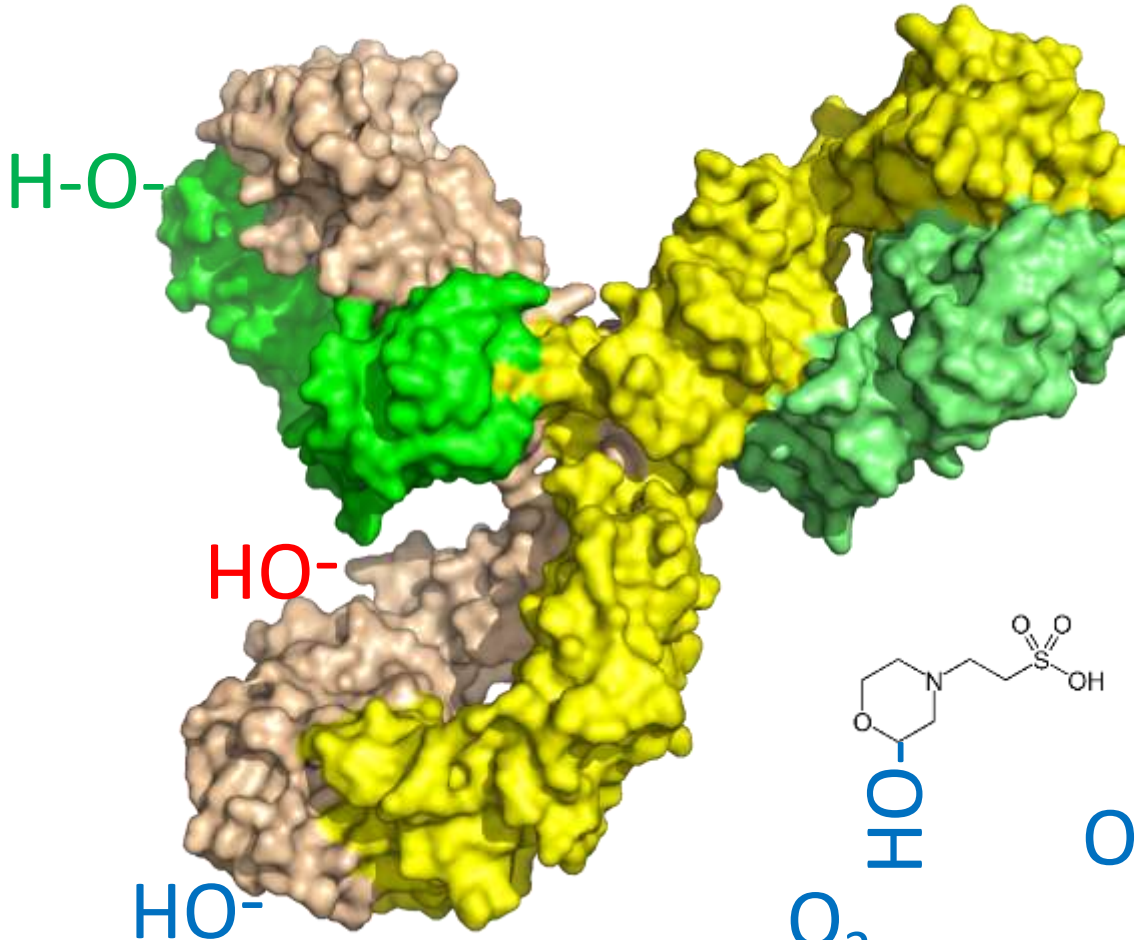






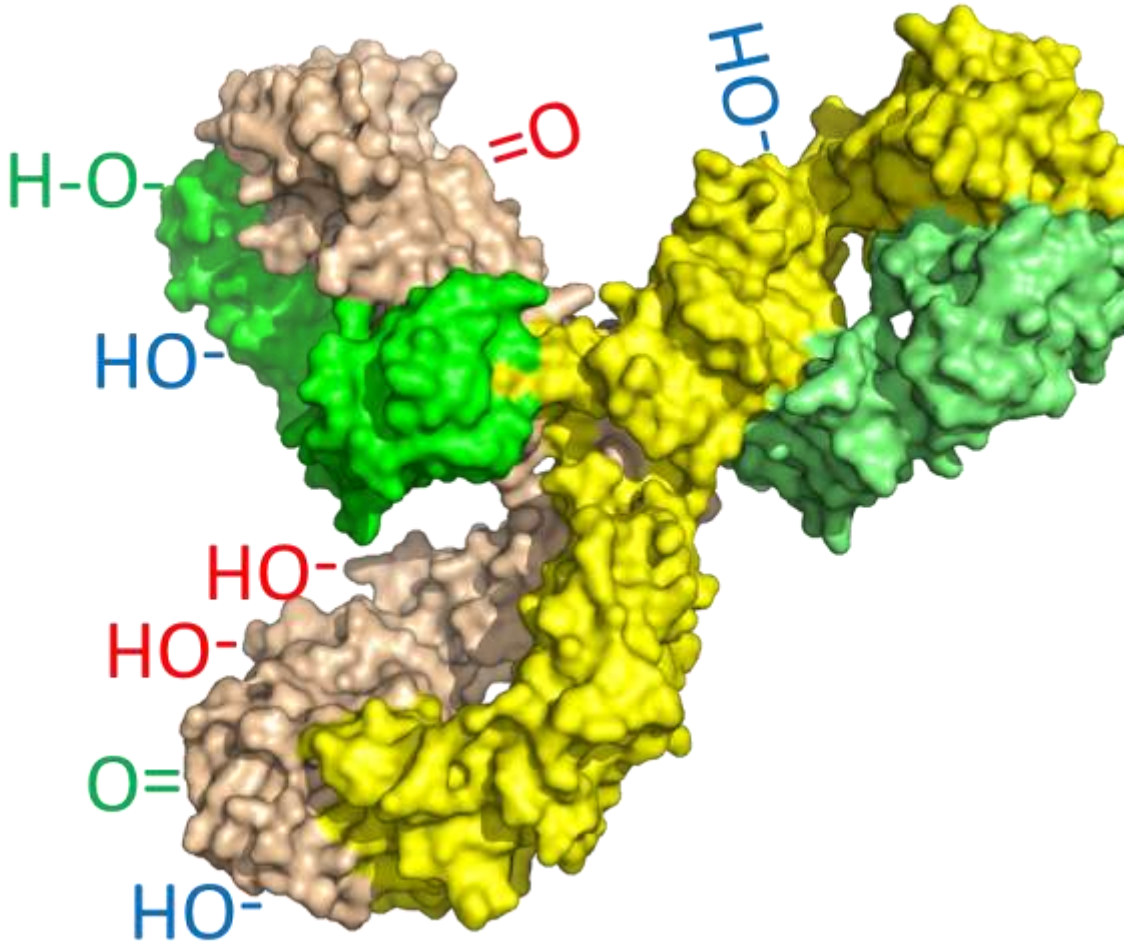




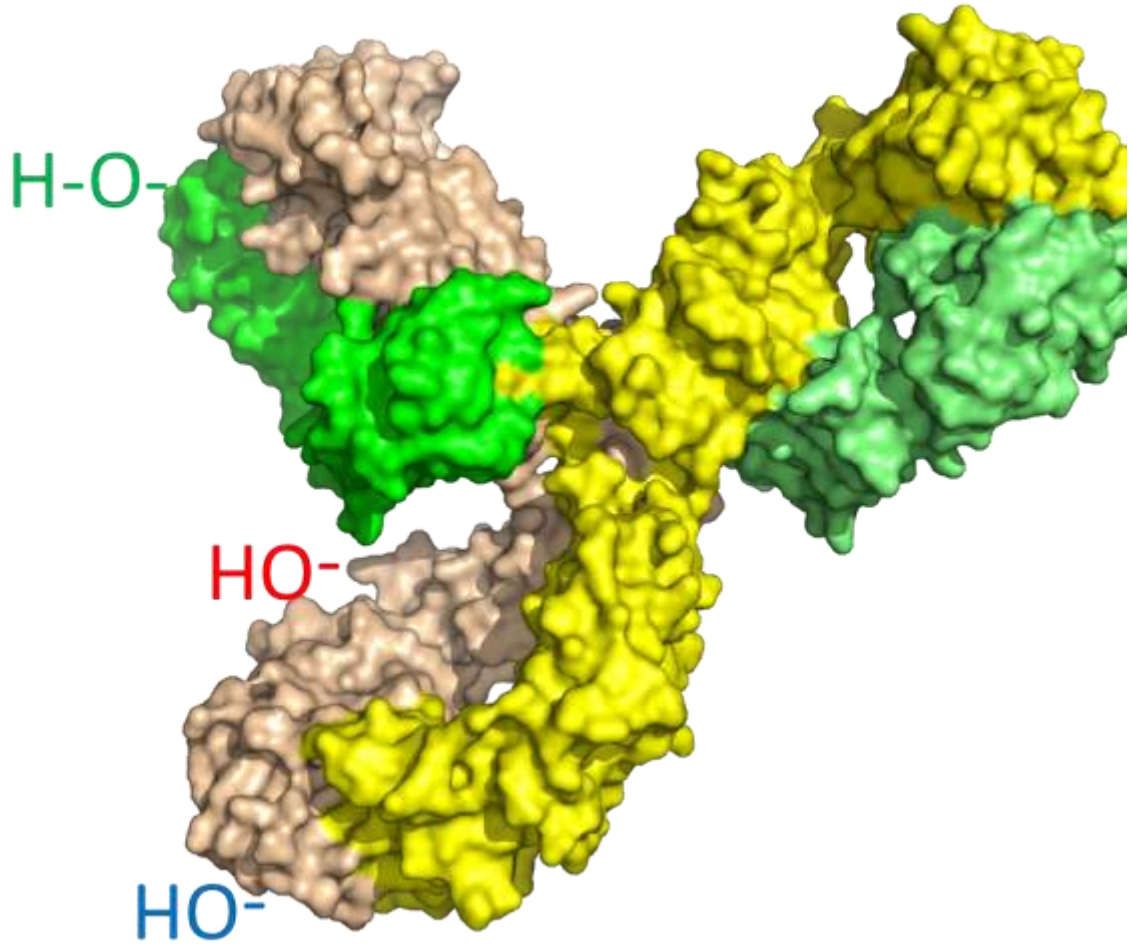


O_2 O_2 O_2 O_2 O_2 O_2 O_2

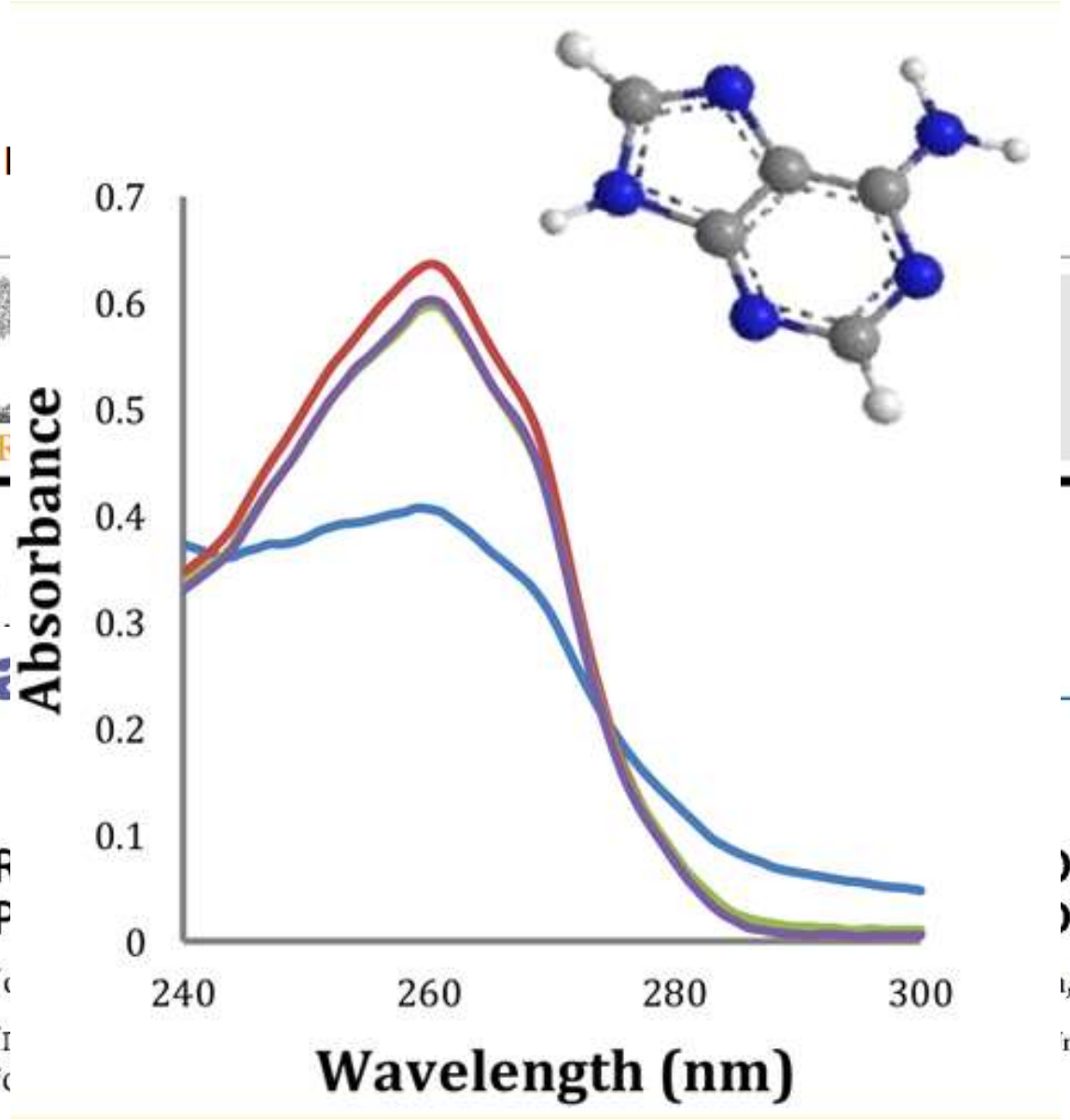
No Radical Scavenger



With Radical Scavenger



Adenine UV Absorbance



Technical Note

Hydroxyl Radical I
Footprint

Boer Xie and
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Anal. Chem., 20
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Publication Date
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Article

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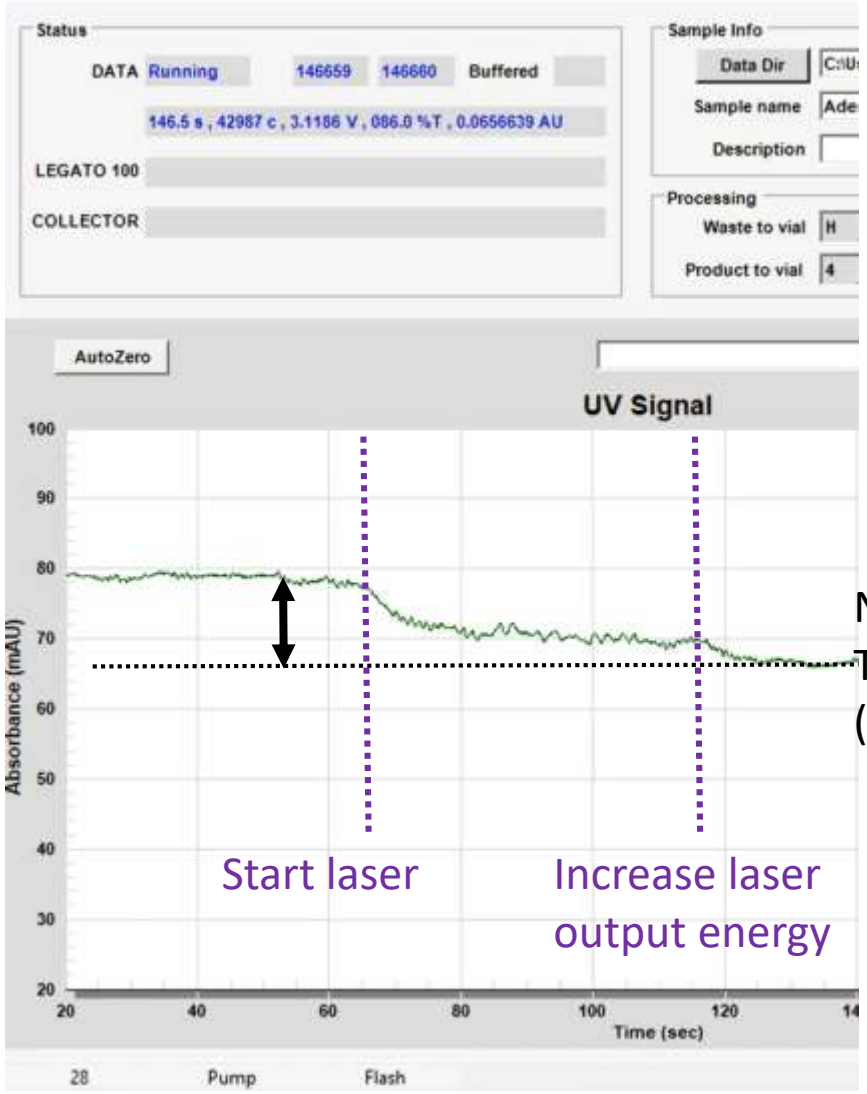
Oxidation of
Dosimetry

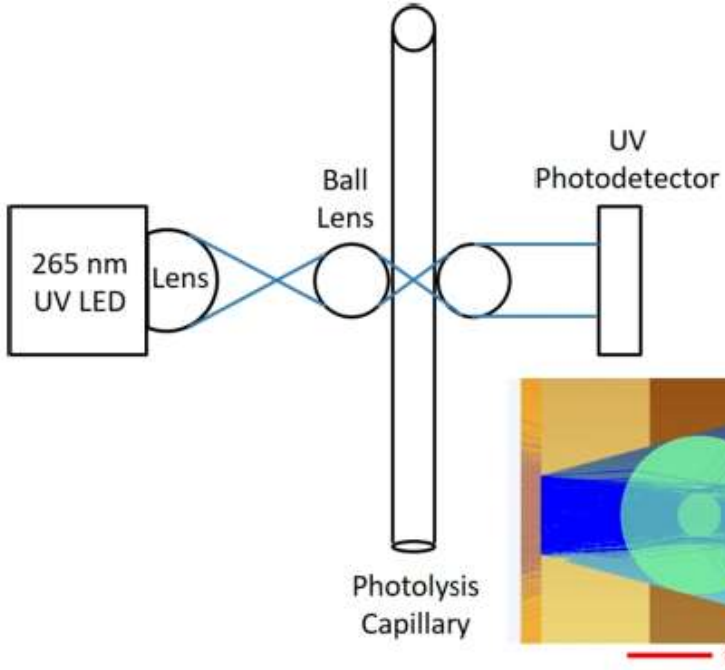
and Scot R. Weinberger

United States

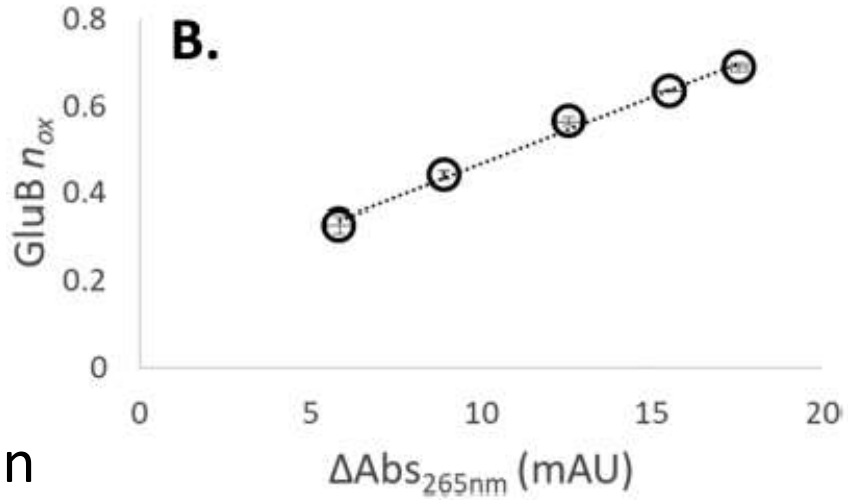
Real-Time Compensation of FPOP Experiment



Normalization Target (79 mAU→66mAU)



Capillary UV spectrophotometer optical bench



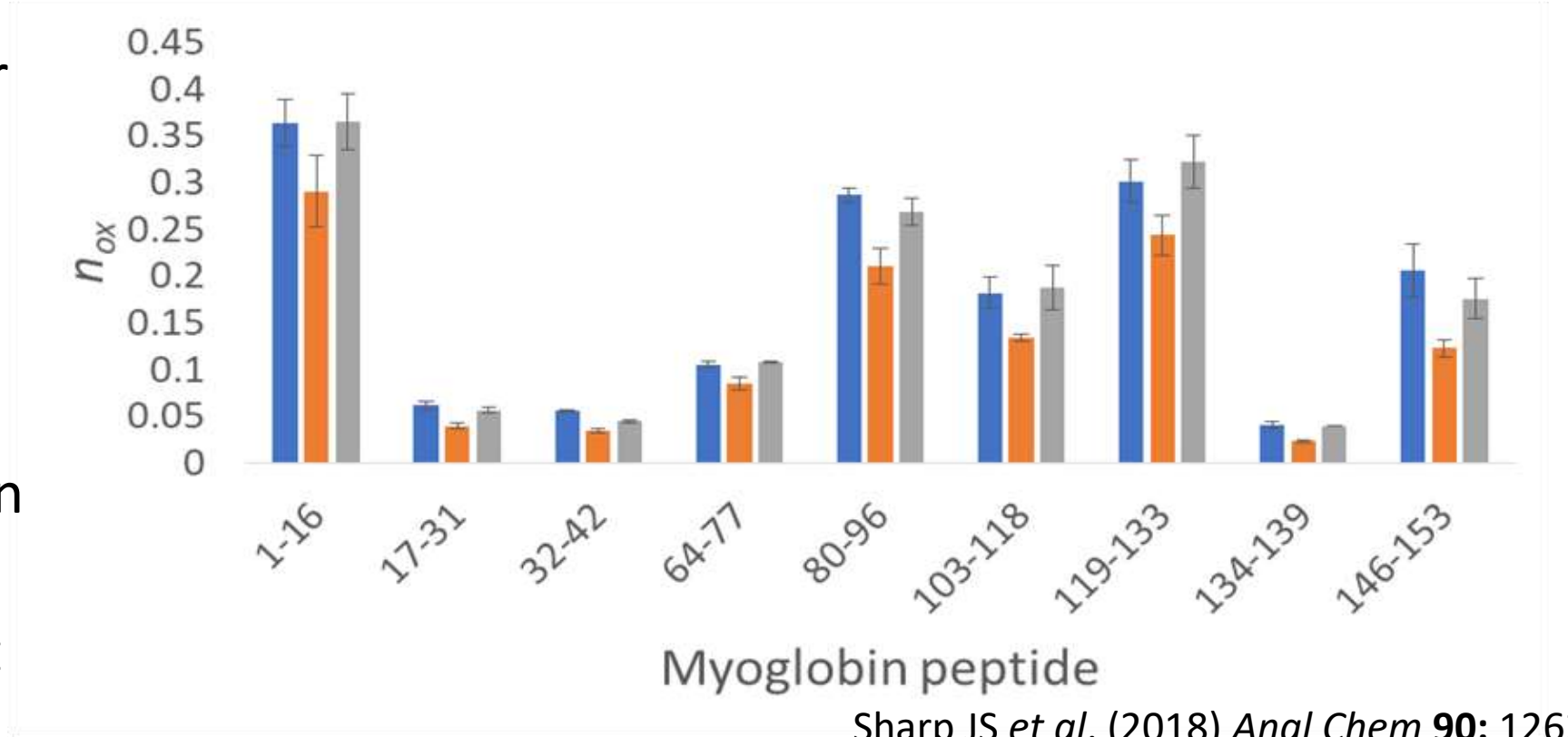
Dosimetry correlates with peptide oxidation

Sharp JS *et al*, (2018) *Anal Chem* **90:** 12625

Adenine Dosimetry-Based Normalization Effectively Compensates for Differential Scavenging

- Myoglobin
- 100 mM H_2O_2
- 15% Exclusion Volume

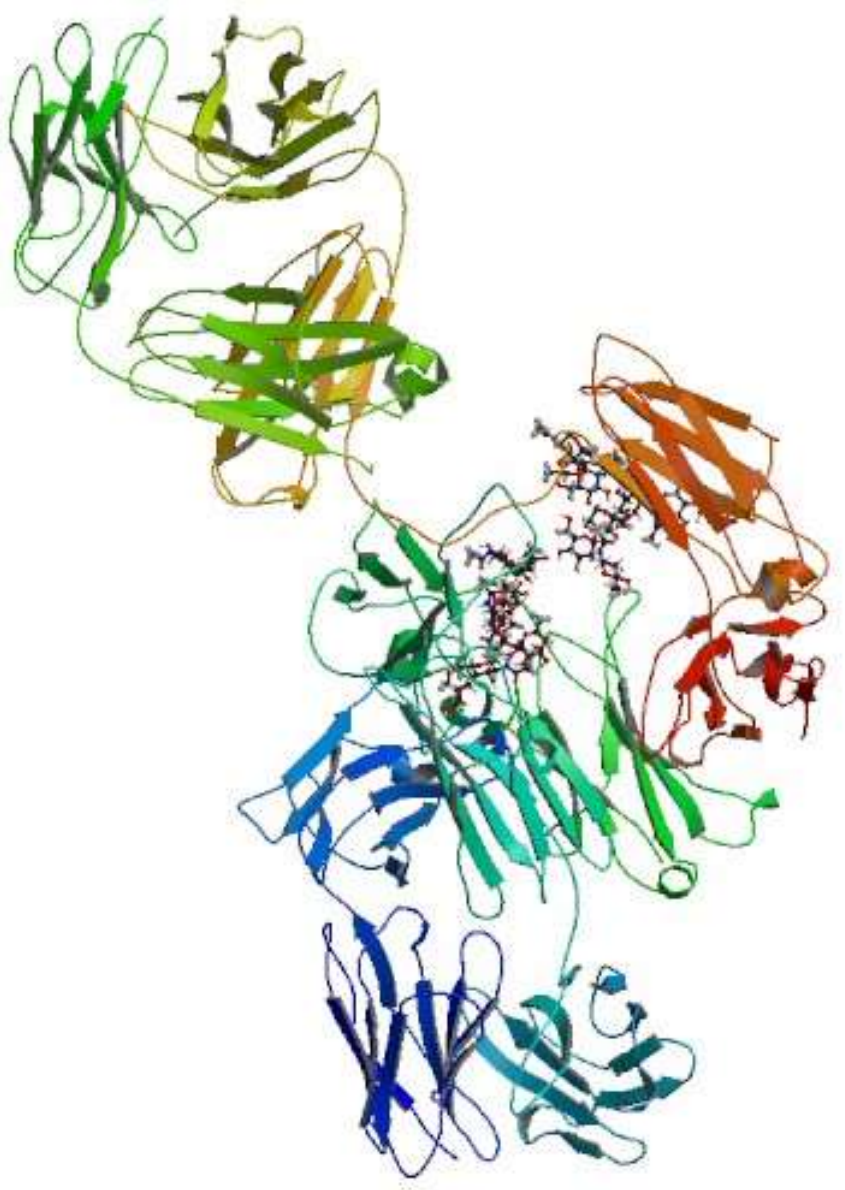
Blue: $NaPO_4$ buffer, 11.66 mJ/mm^2/pulse, $\Delta Abs_{(265nm)}$=19.13 ± 1.03
Orange: 10 mM MES buffer, 11.66 mJ/mm^2/pulse, $\Delta Abs_{(265nm)}$=7.07 ± 1.10
Grey: 10 mM MES buffer, 18.75 mJ/mm^2/pulse, $\Delta Abs_{(265nm)}$=18.17 ± 0.26



- No evidence that buffer changes protein structure/dynamics
- FPOP HRPF shows no significant differences after dosimeter-based compensation
- With compensation, can differentiate scavenger effect from structural effect in FPOP footprint

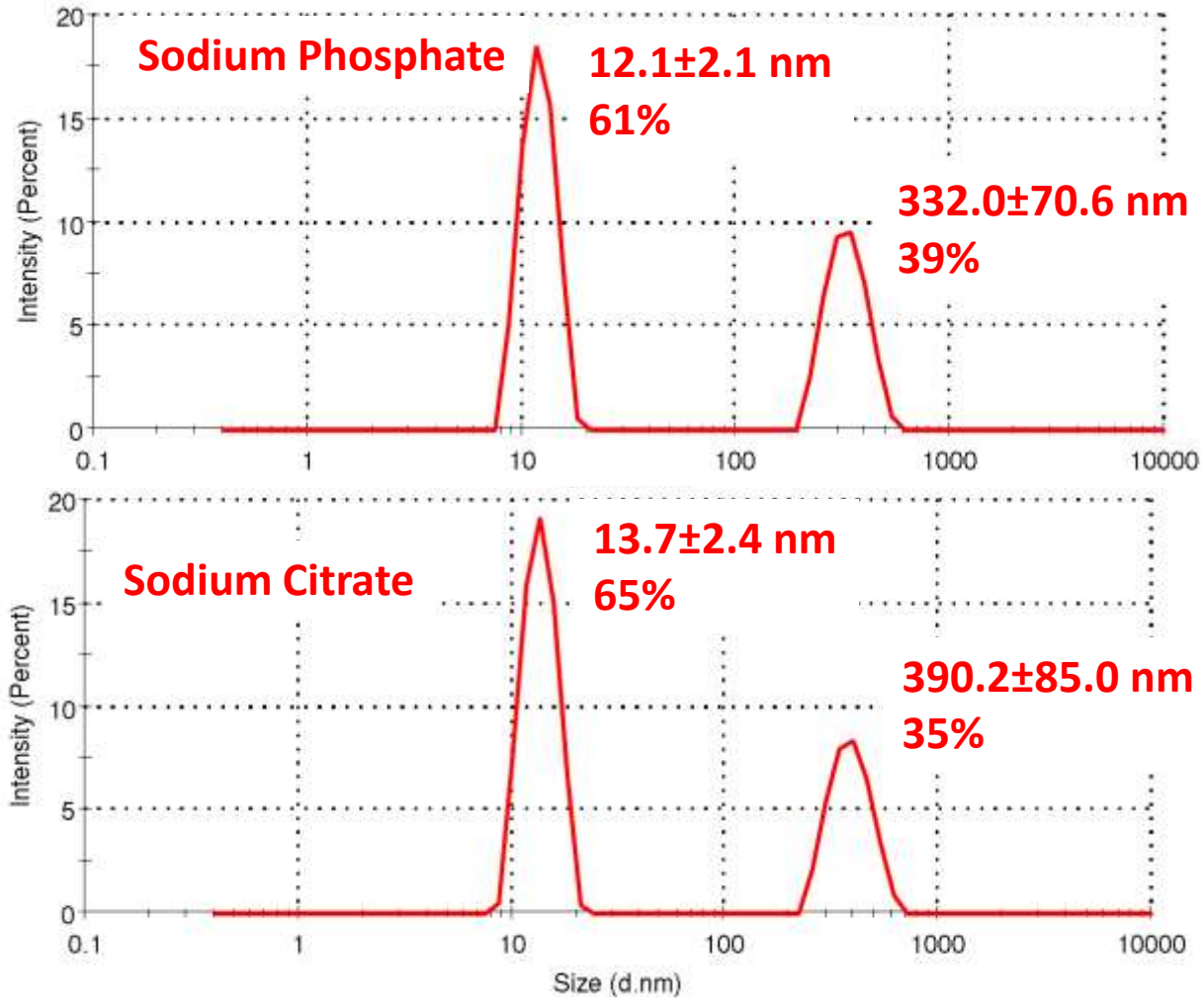
Sharp JS *et al*, (2018) *Anal Chem* **90:** 12625

Probing Formulations of an Adalimumab Biosimilar



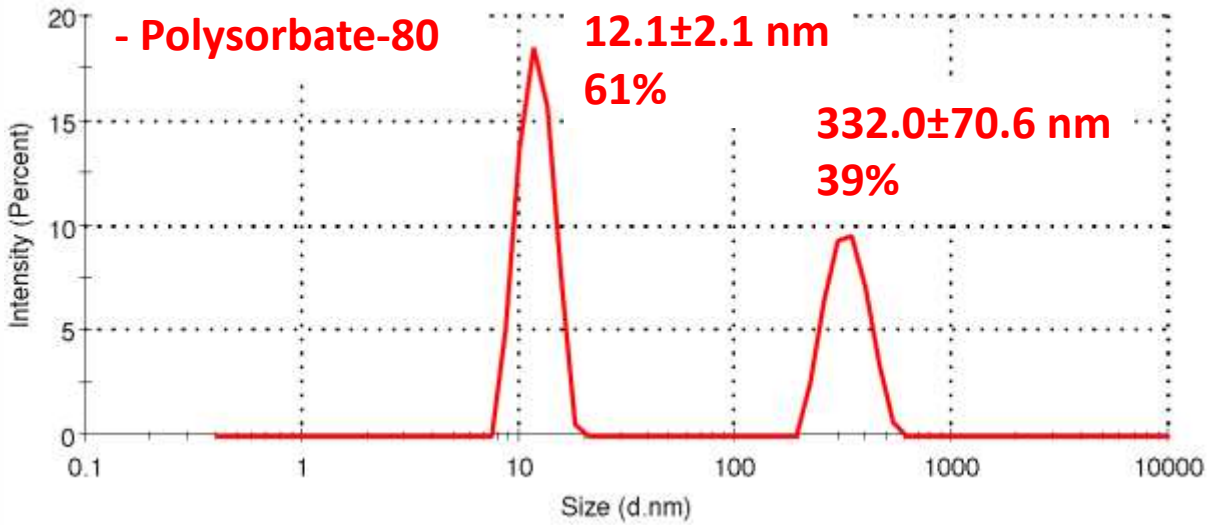
- Recombinant anti-TNFα IgG1
- Launched as Humira in 2003
- Active biosimilar market
- Interested in effect of different buffers and excipients on HOS
  - Native HOS
  - Ability to preserve HOS after temporary break in cold chain

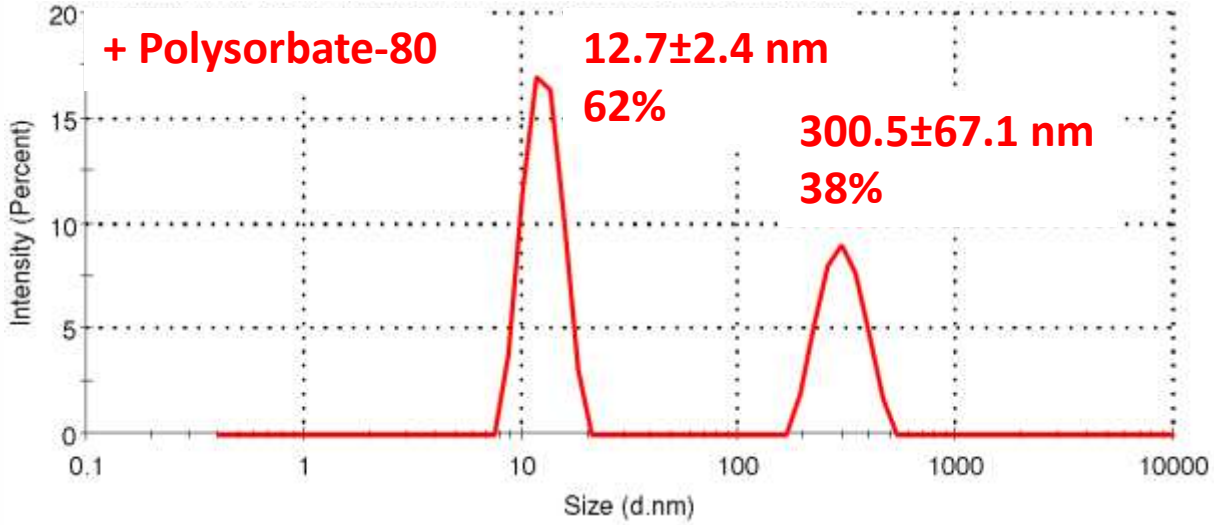
Effect of Buffer on Biosimilar HOS: Phosphate vs. Citrate (Compensated)



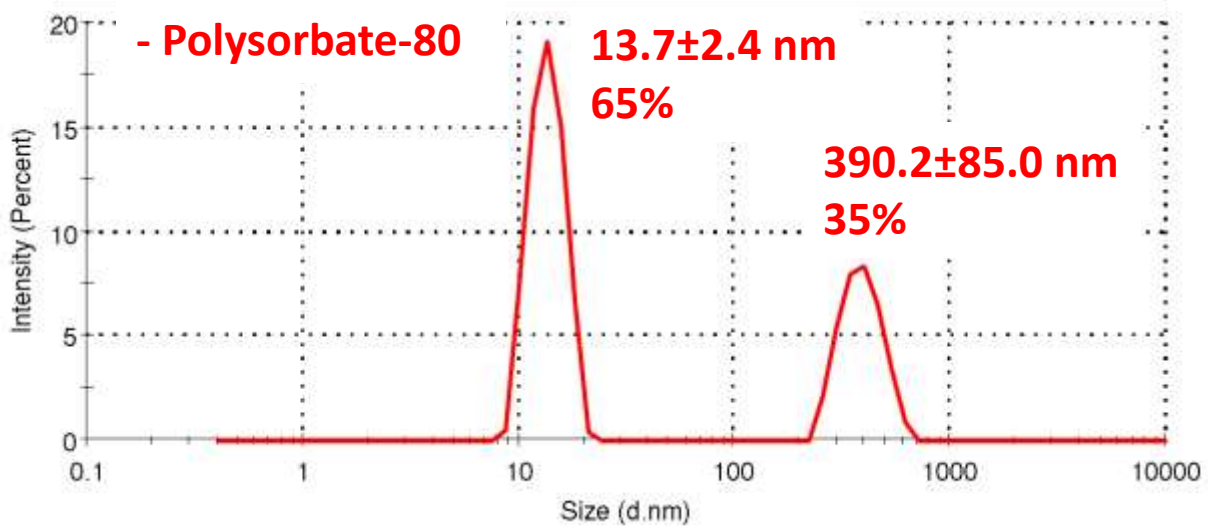
Effect of 0.1% Polysorbate-80 at Room Temperature (Compensated)

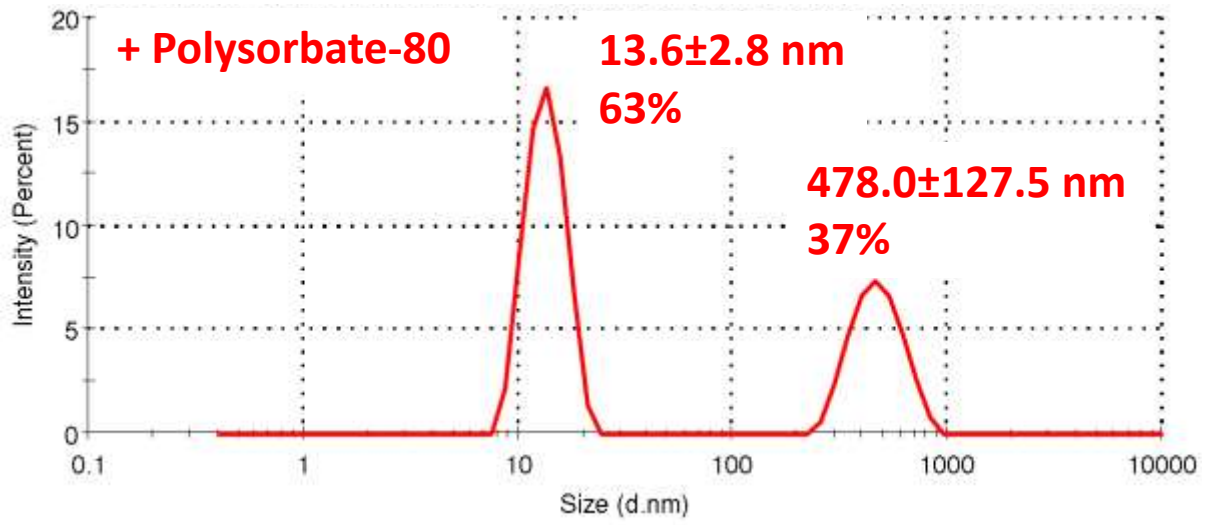
Sodium Phosphate ± 0.1% Polysorbate-80





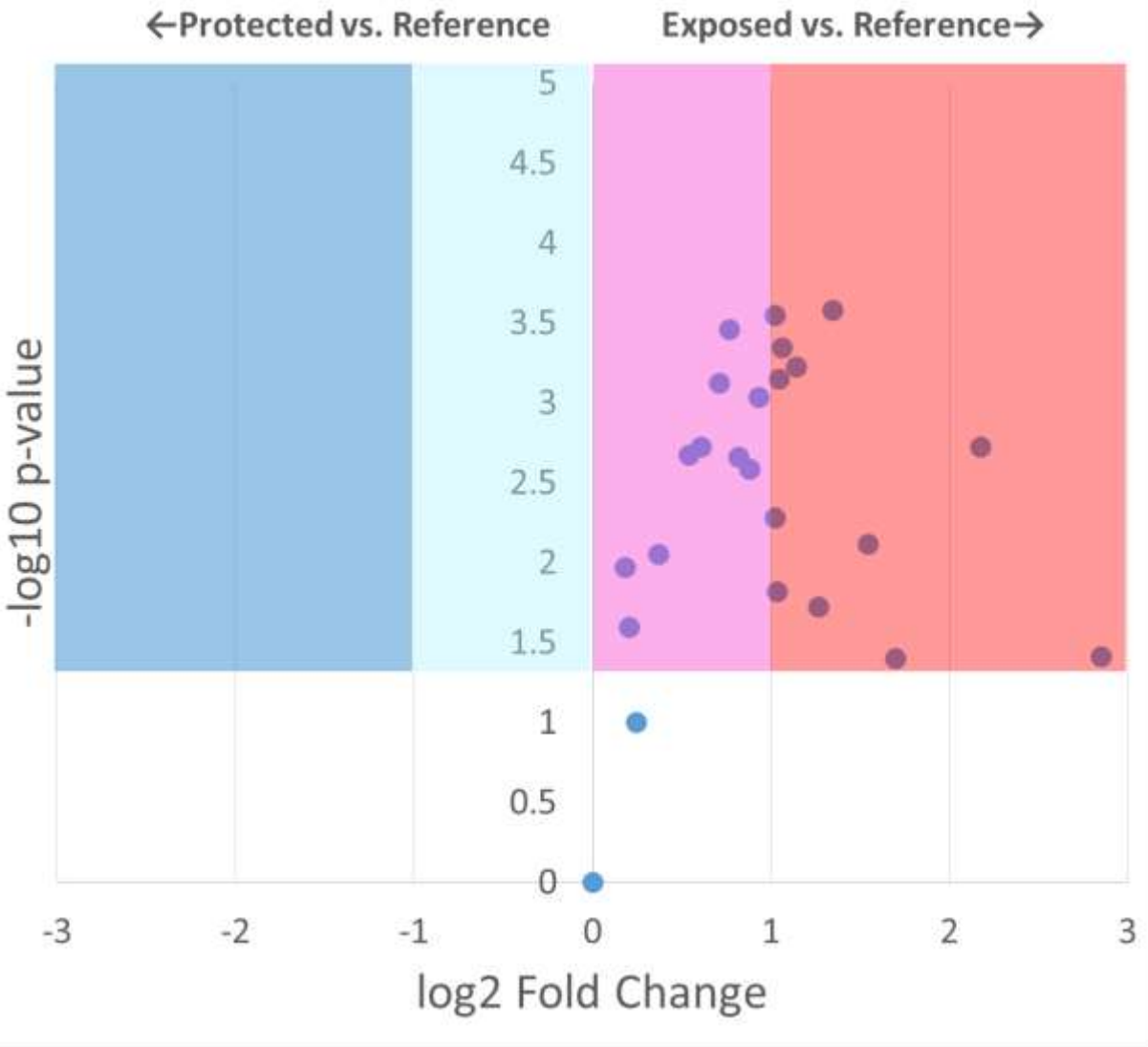
Sodium Citrate ± 0.1% Polysorbate-80

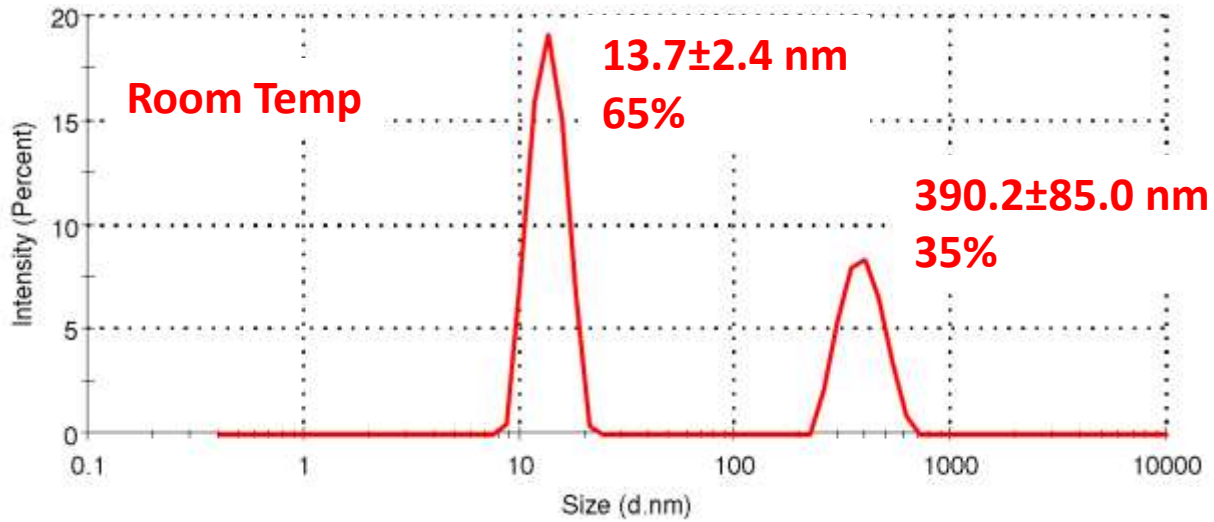


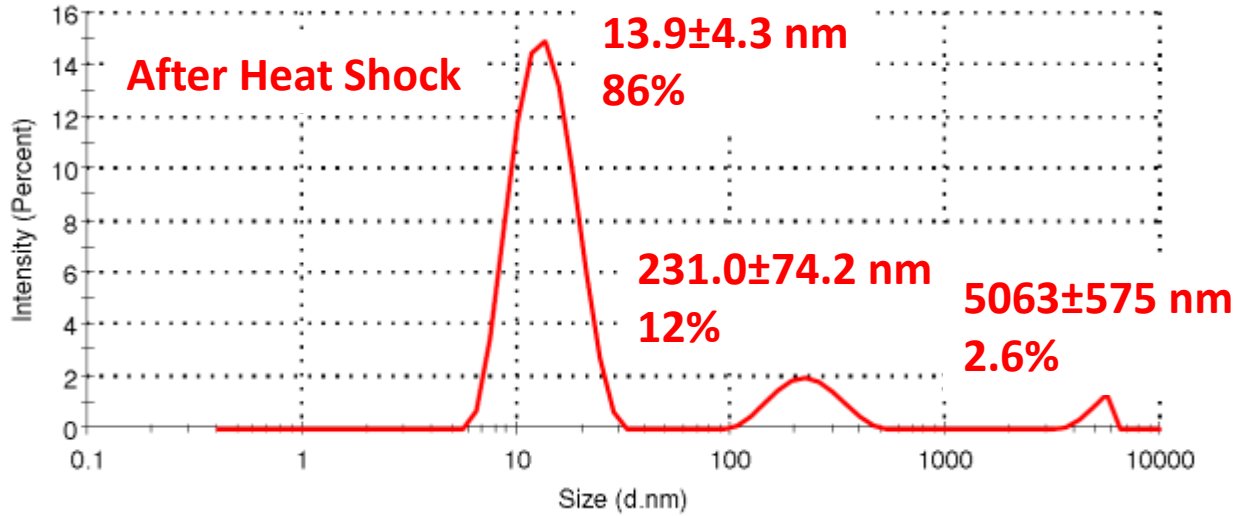


Heat Shock Stability: Sodium Citrate Buffer

55°C, 1 hour; then cooled to room temperature

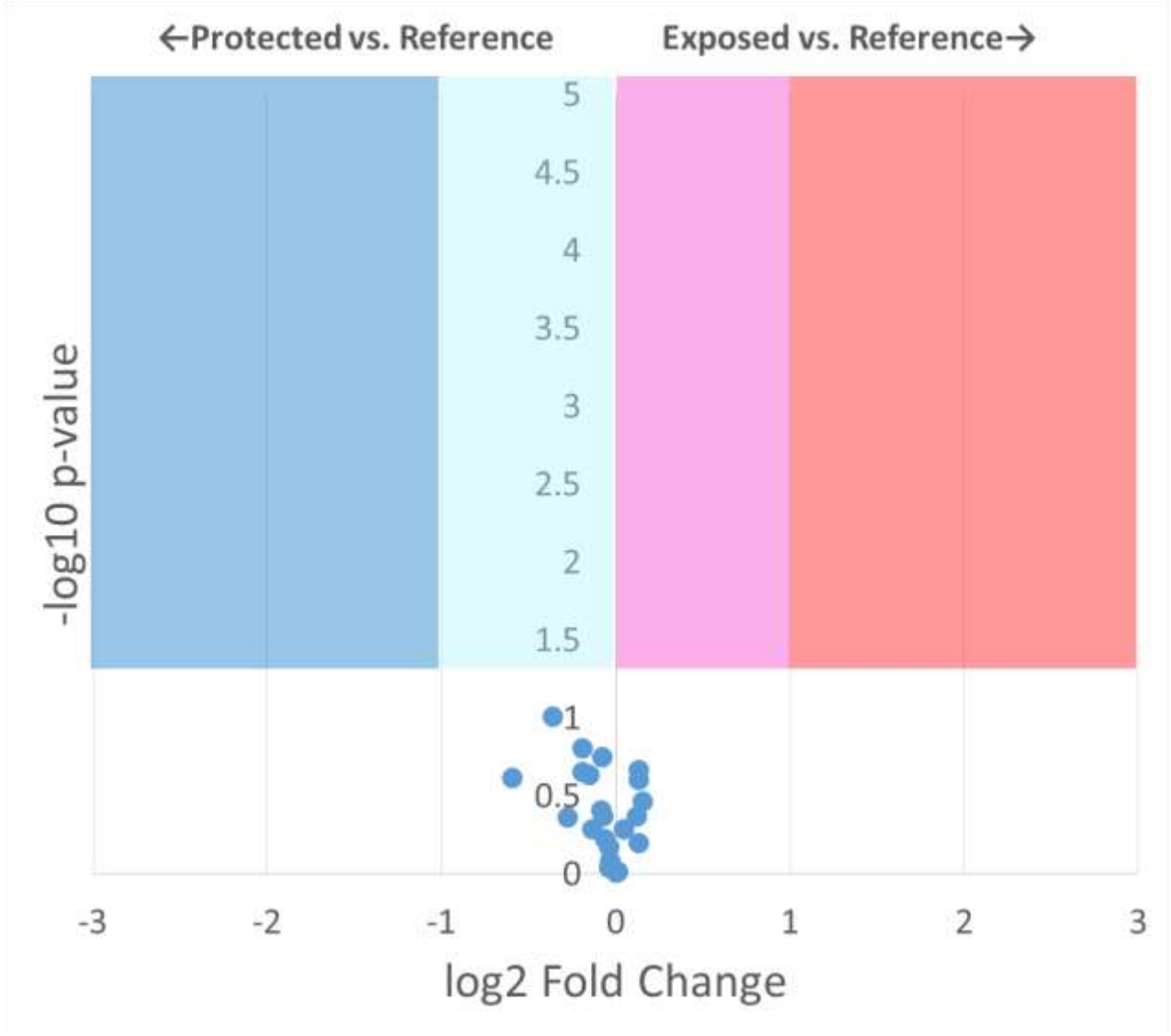


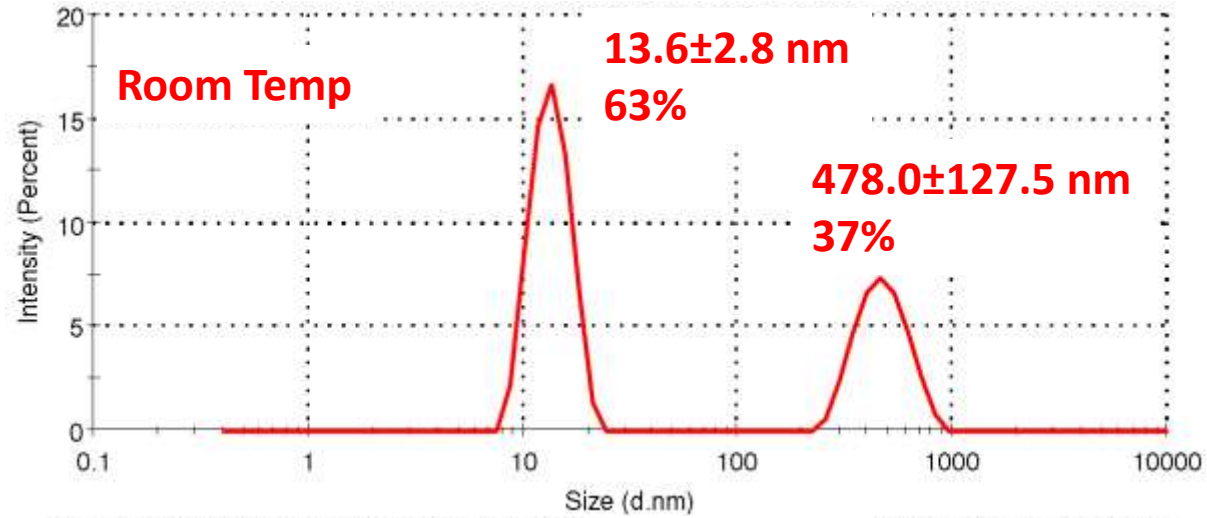


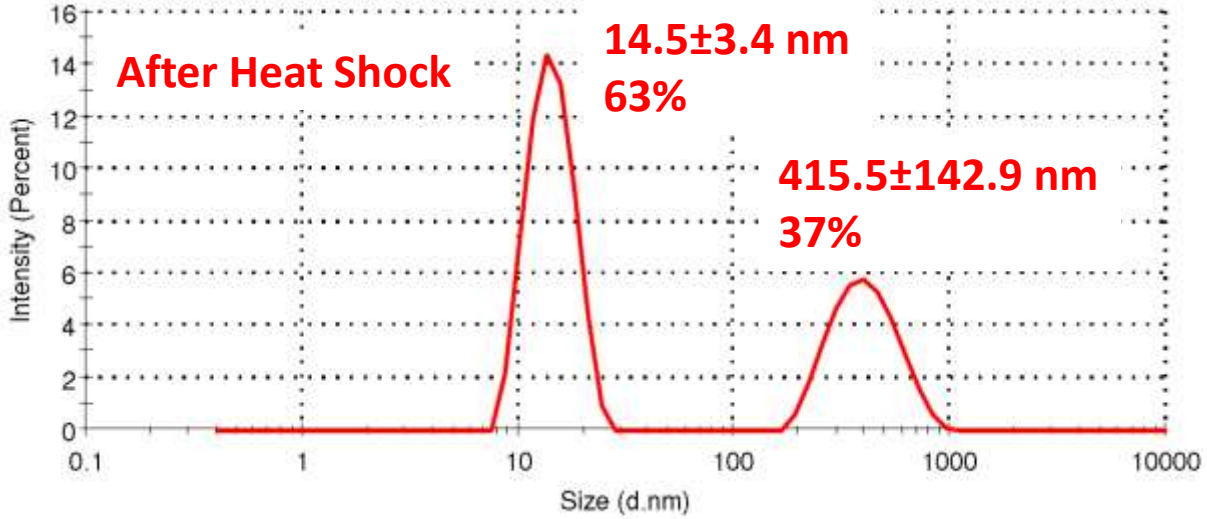


Heat Shock Stability: Sodium Citrate Buffer, 0.1% Polysorbate-80

55°C, 1 hour; then cooled to room temperature

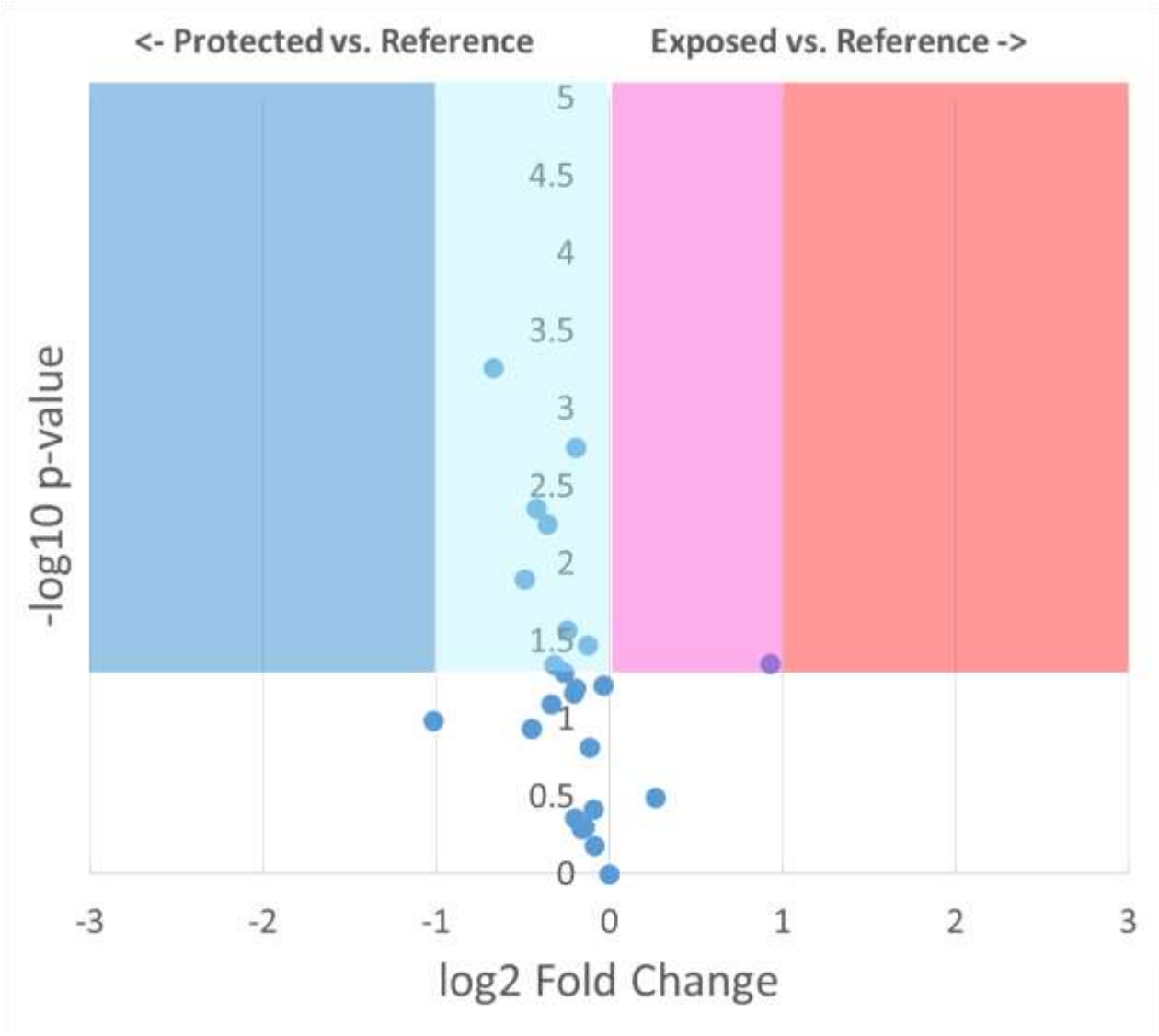


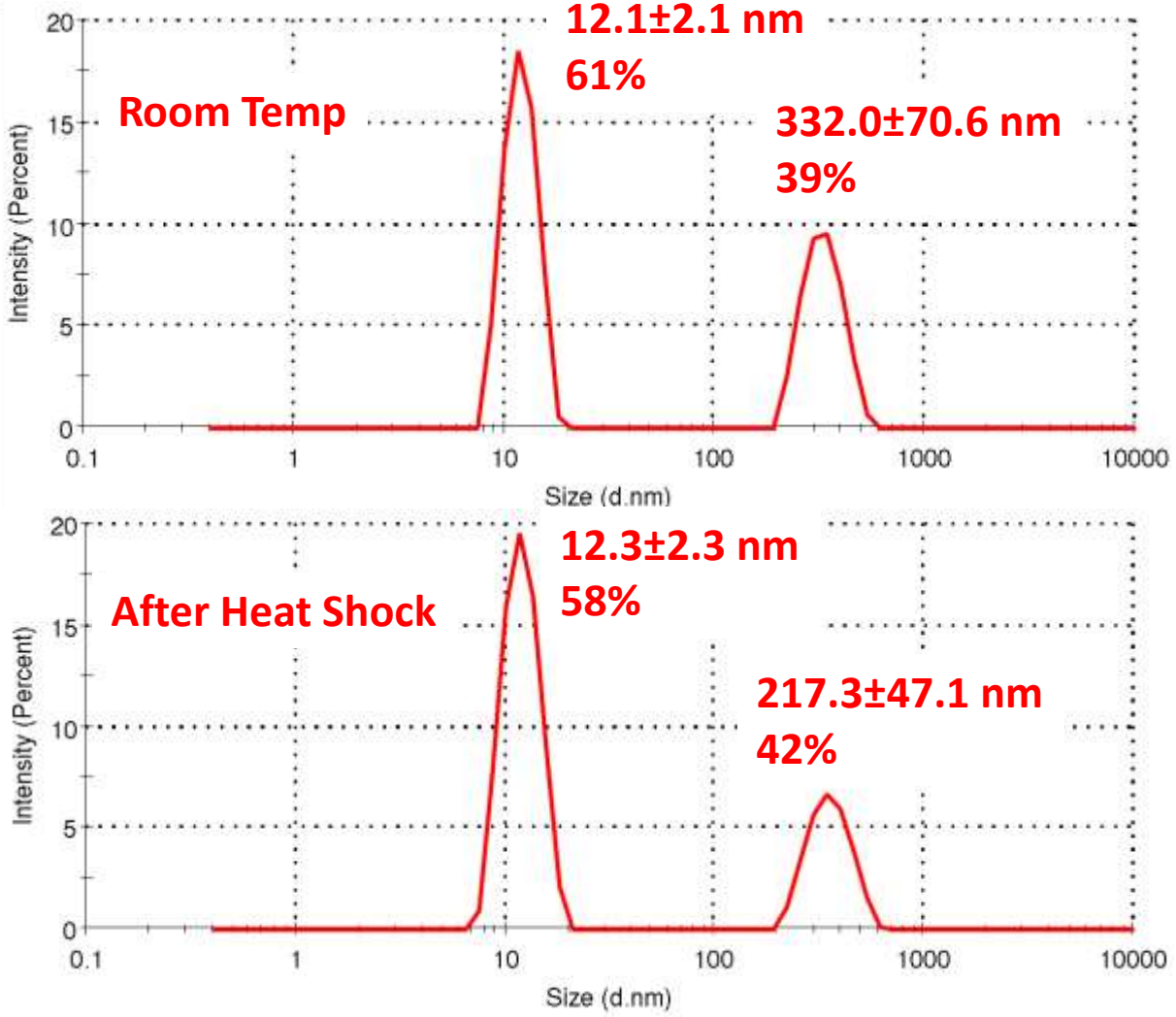




Heat Shock Stability: Sodium Phosphate Buffer

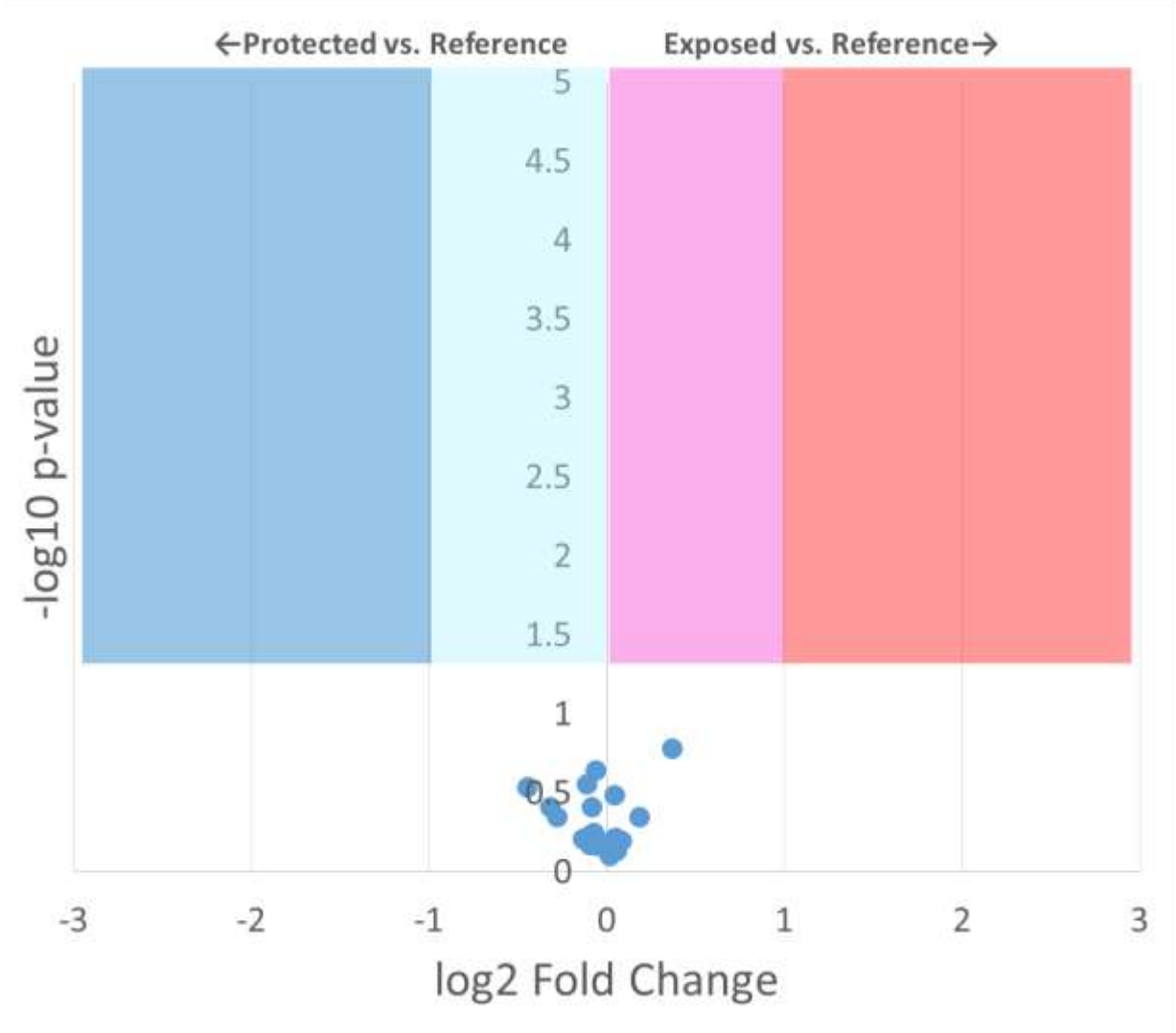
55°C, 1 hour; then cooled to room temperature

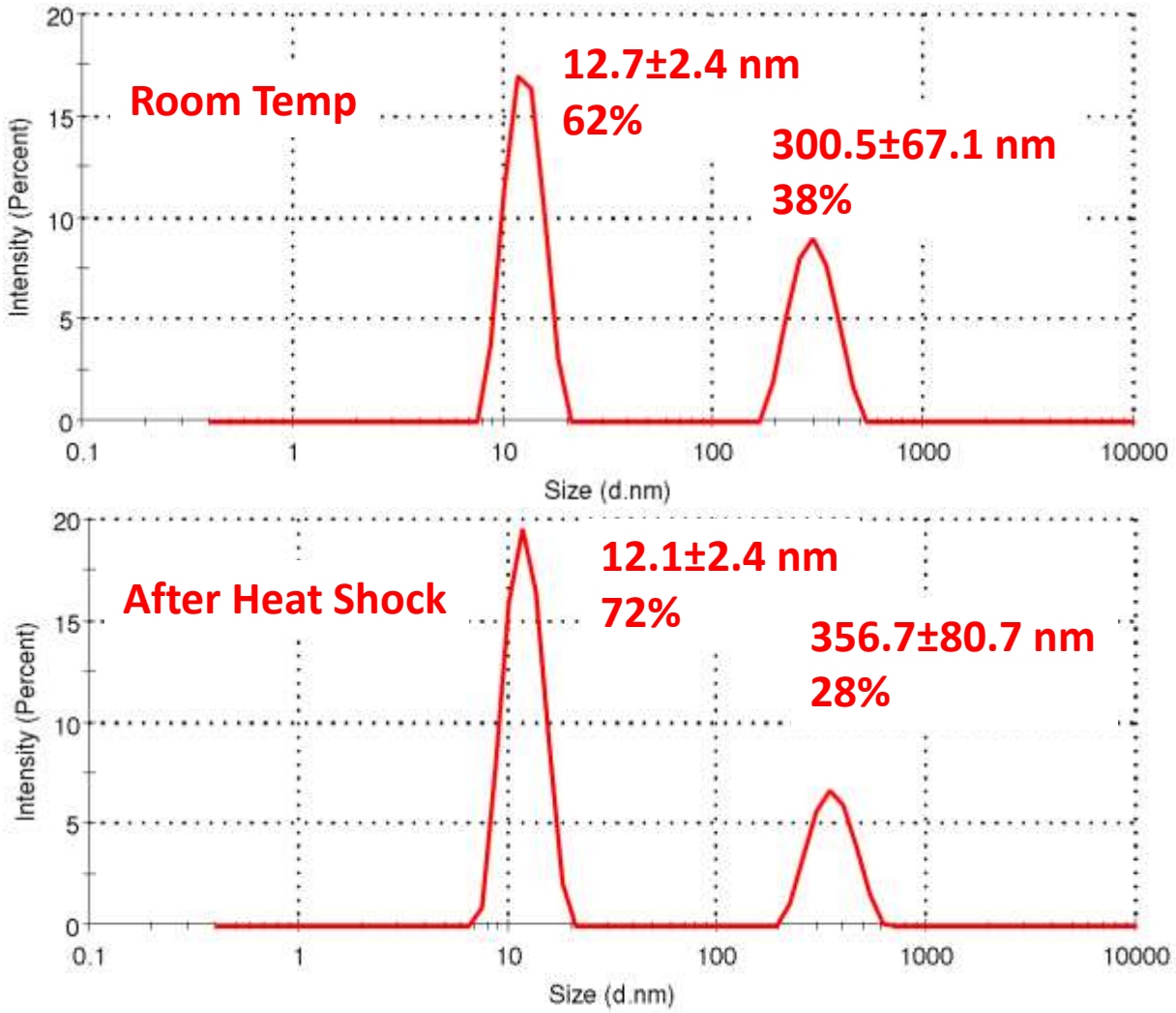




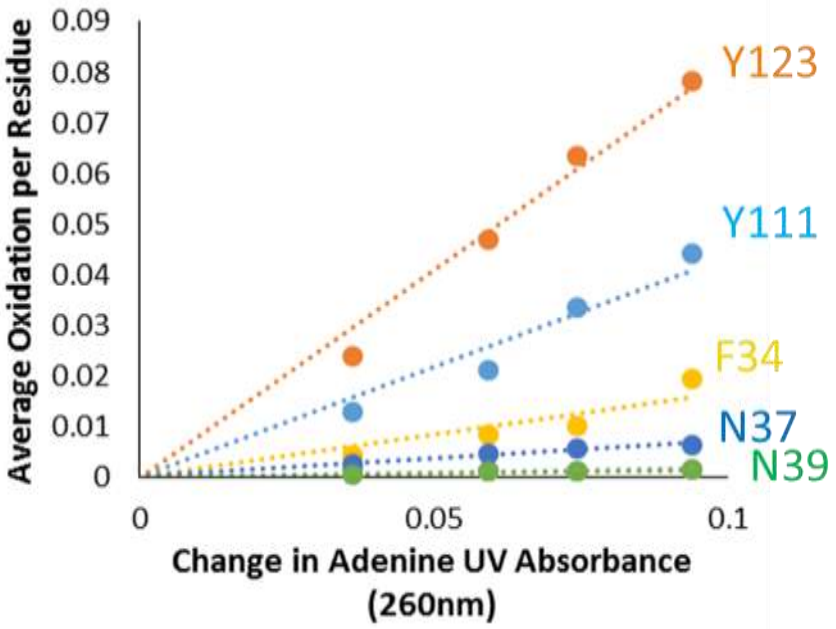
Heat Shock Stability: Sodium Phosphate Buffer, 0.1% Polysorbate-80

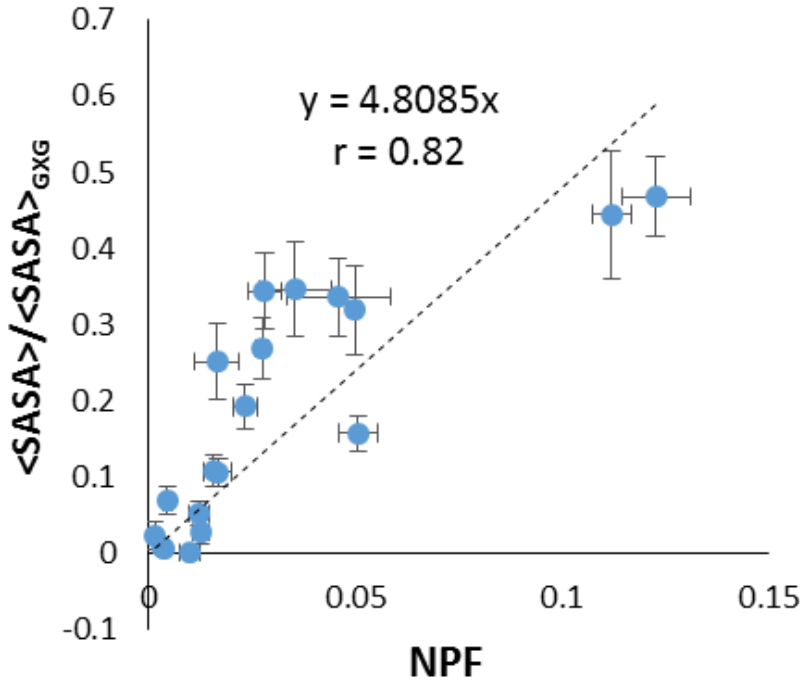
55°C, 1 hour; then cooled to room temperature





Radical Dosimetry Allows Quantitative Measurements of SASA





Normalized Protection Factor (NPF) $R_i = \dfrac{Slope\ R_i}{k_{R_i} / k_p}$

- With accurate radical dosimetry, we can measure the scavenger-independent reactivity of an amino acid or peptide
- Using this along with empirical models, we can convert scavenger-independent reactivities into fractional average solvent accessible surface area values

Xie B *et al*, (2017) *Sci Rep* **7:** 4552

HRPF is a Unique Tool in the Biophysical/Structural Toolkit

- Provides a stable covalent modification for a wide variety of amino acids
  - Oxidation of every amino acid but glycine has been reported in HRPF literature
  - Compatible with most post-labeling sample workup processes
- Probes protein topography at peptide or better resolution
  - Highly sensitive to changes in average topography including tertiary structure, aggregation, ligand binding, etc.
  - Provides **complimentary** data to many other popular biophysical methods (H/D exchange, CD spectroscopy, DLS, etc.)
- **With compensation**, is amenable to highly complex mixtures, very large proteins and complexes, wide variety of buffers and conditions
  - Real-time scavenging measurements and compensation now possible
  - Can measure HOS changes induced by buffer, excipients, pH, heat, etc.

Acknowledgements

Sharp Group

- Dr. Sandeep Misra
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- Joseph Mason
- Martin Biggs

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Collaborators

Online Dosimetry; IgG Biosimilar

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- Jeffrey Persoff
- Robert Egan