Two-Dimensional Liquid Chromatography Isolation and Quantitation of IgG and Exosomes from Cell Culture Media

Chris Topper and R. Kenneth Marcus
Department of Chemistry, Biosystems Research Complex
Clemson University, Clemson, SC 29634

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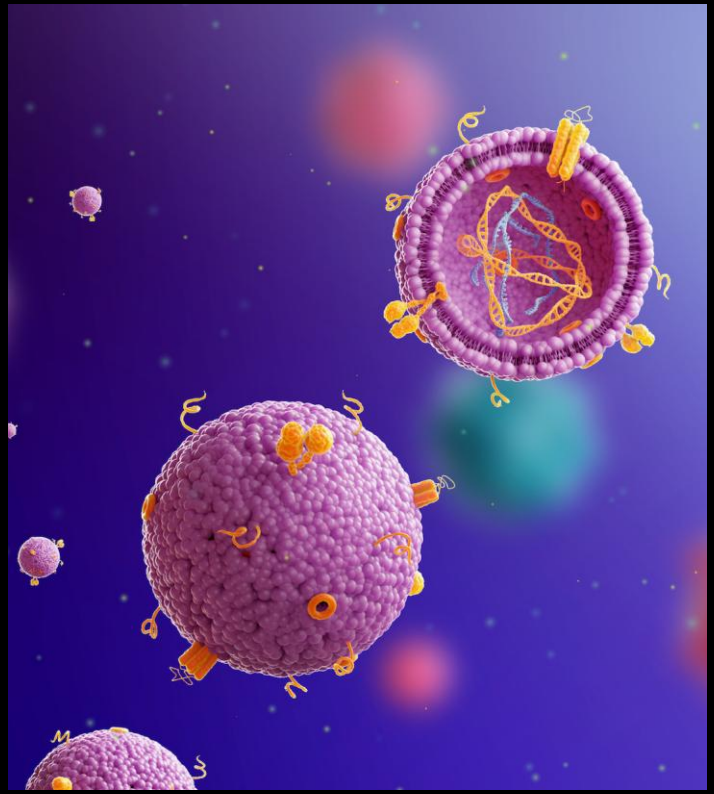
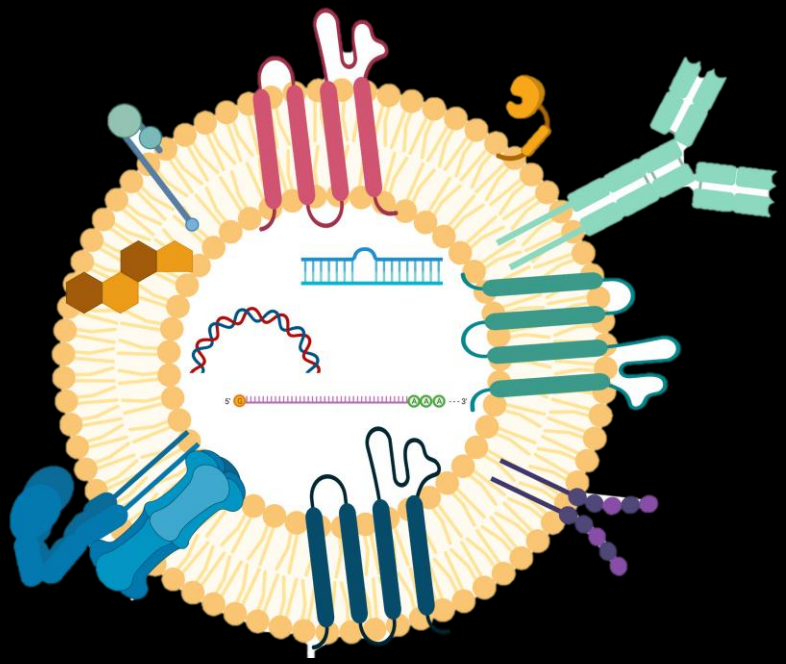
What are Exosomes?



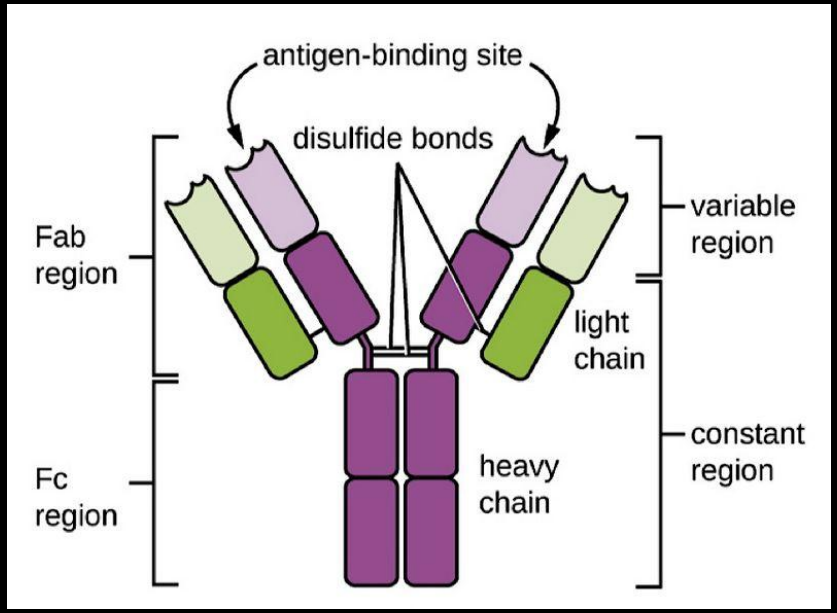
- Subset of extracellular vesicles (EVs)
  - 30 to 150 nm diameter
  - Phospholipid bilayer membrane enriched with tetraspanin proteins (CD9, CD63, CD81)
- Released by ALL cell types
  - Essential for intercellular communication
  - Homeostasis, tissue regeneration, immune system modulation
- Contain DNA, RNA, and proteins reflective of the cell of origin
  - Cargo reflects the biological state of a cell
  - Useful tool for disease diagnostics

Exosomes as Vectors



- Emerging use as vectors for targeted drug delivery to diseased cells
  - Low immunogenicity compared to LNPs, AAVs
  - Can be used to deliver DNA, RNA, proteins, small molecules, and other biotherapeutics
- Phase III clinical trials underway

Monoclonal Antibodies (mAbs)

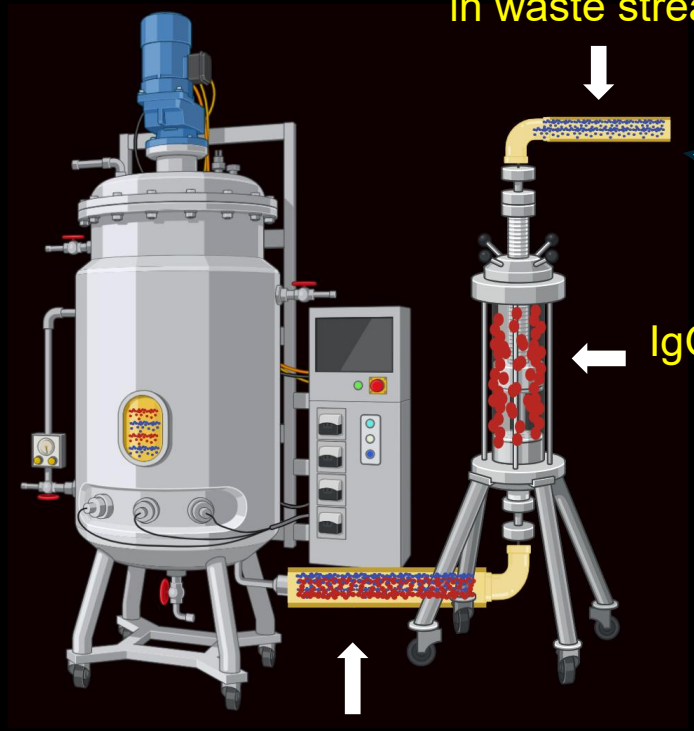


- Manufactured proteins designed to recognize and bind to a specific target (antigen)
  - Precision medicine
- Immunoglobulin G (IgG) is the primary isotype used to make therapeutic mAbs
  - Modifications to antigen-binding site
- IgG used for most Antibody-Drug Conjugates
  - Targeted delivery of cytotoxic payloads

Production of mAbs

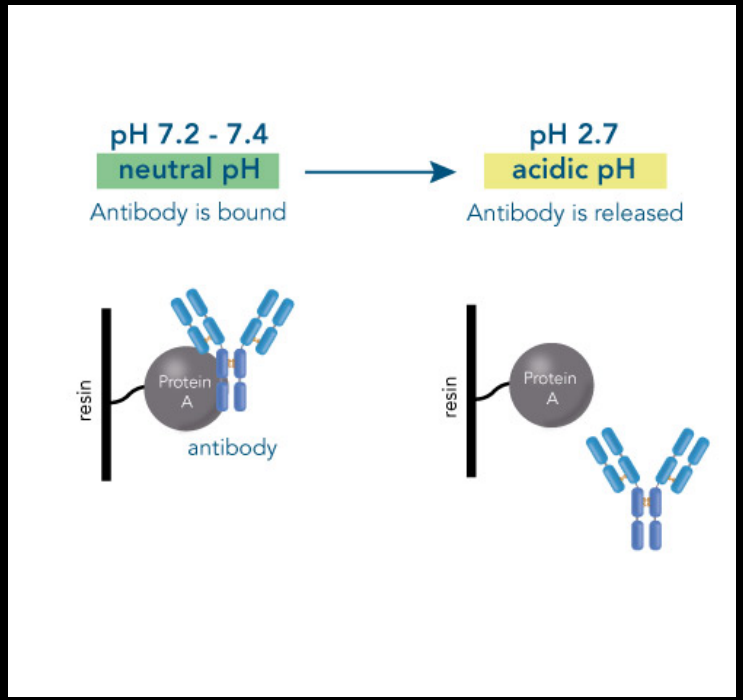
- Chinese hamster ovary (CHO) cells
  - Cultured in bioreactors
  - Engineered to express IgG
  - IgG purified from culture media
- Affinity chromatography using protein A (ProA) for initial purification
  - High specificity
  - pH based capture and elute mechanism
- ProA effluent discarded as waste
  - Rich in exosomes

Opportunity for by-product valorization!



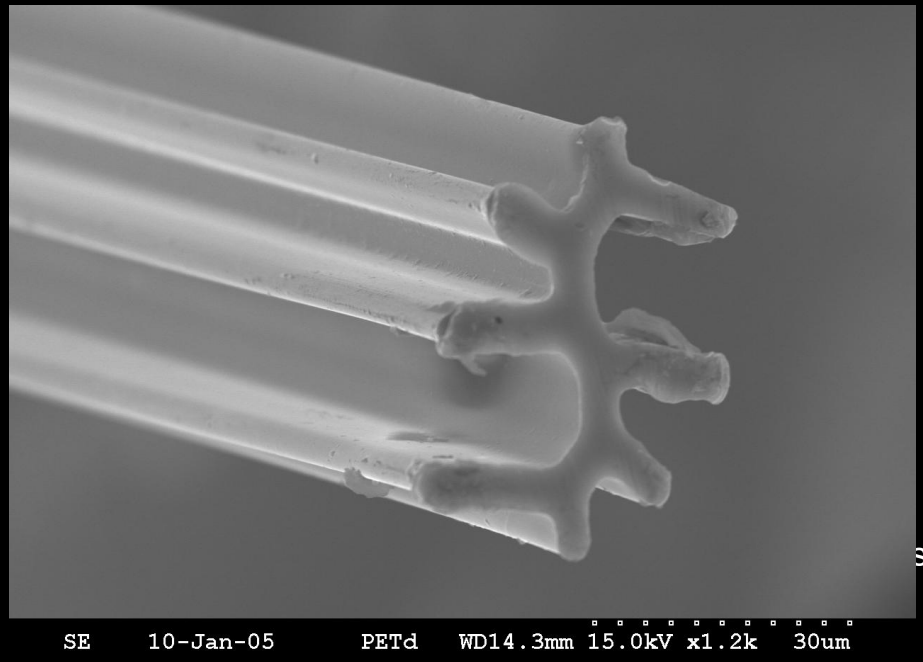
Protein Affinity Chromatography

- Binding – occurs at near neutral (physiological) pH
  - Protein A exhibits high specificity towards the Fc domain of mAbs
  - Interaction is driven predominantly by hydrophobic effects with some electrostatics involved
- Elution – decrease in pH causes conformational change in ProA
  - Partial unfolding due to altered electrostatic interactions
  - Causes mAbs to dissociate



Capillary-Channeled Polymer Fibers (C-CP)

- Commodity polymer fibers are melt-extruded with 8-pronged shape
  - Non-porous to large biomolecules
  - Excellent mass transfer properties in viscous fluids
  - < $5 per column
  - Column chemistries modified on-demand
- Polypropylene (PP)
  - Possess significant hydrophobicity
  - Recombinant protein A (rSPA) adsorbed onto fibers
  - 2 IgG molecules per protein A
- Polyester (PET)
  - Moderately hydrophobic
  - HIC, RP, desalting columns, capture loops

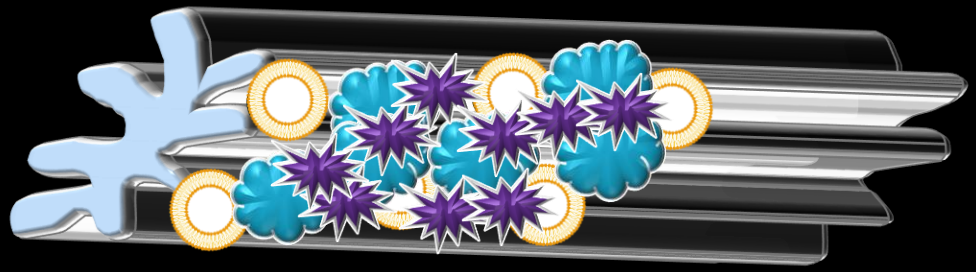


HIC Separation of Exosomes from Biofluids

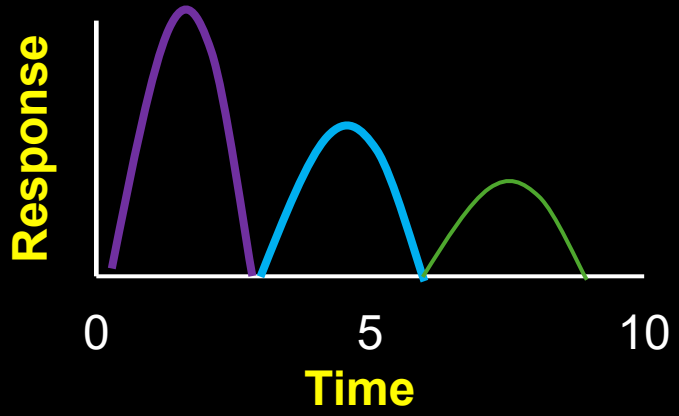
Matrix species

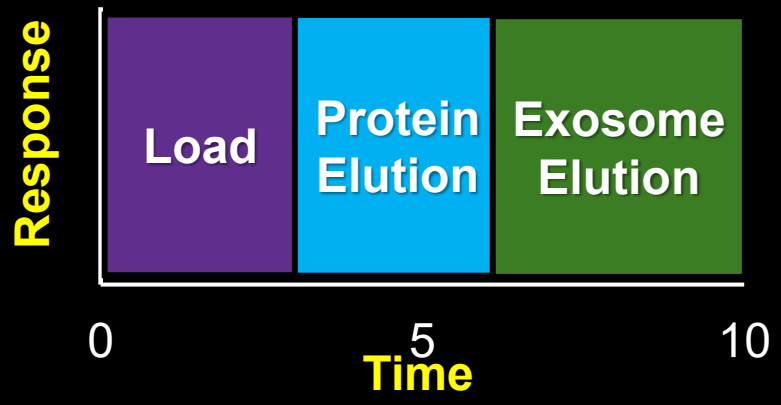
Proteins

Exosomes



C-CP Fibers





ProA-HIC Separation

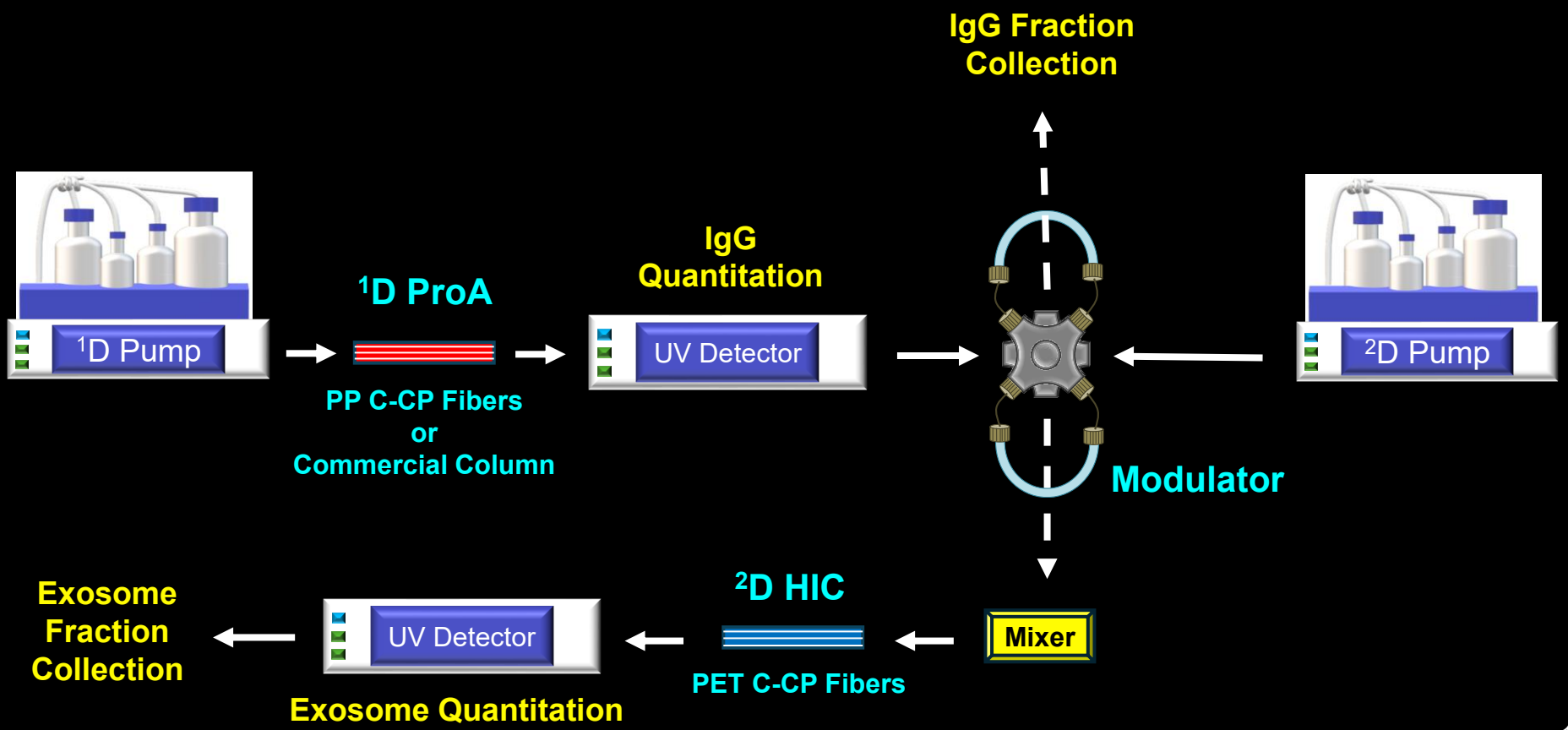
Goal

- Employ HIC in ^{2}D to isolate exosomes from ^{1}D-ProA waste streams
  - Downstream of ProA minimizes GMP regulatory burden

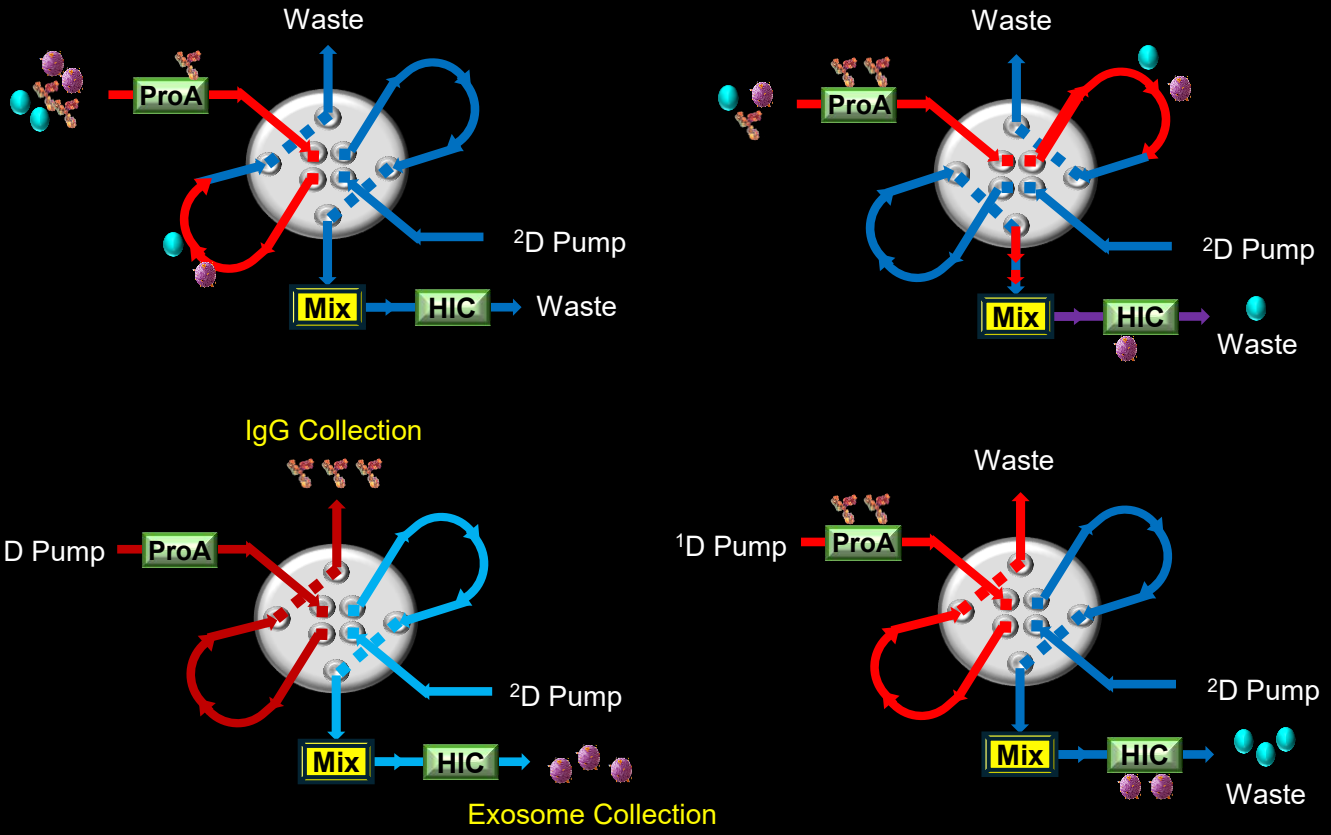
Challenge

- ^{1}D effluent results in exosome breakthrough in second dimension
  - Need sufficient salt concentration to get exosomes to “stick”
  - At-column “dilution” = increase salt concentration

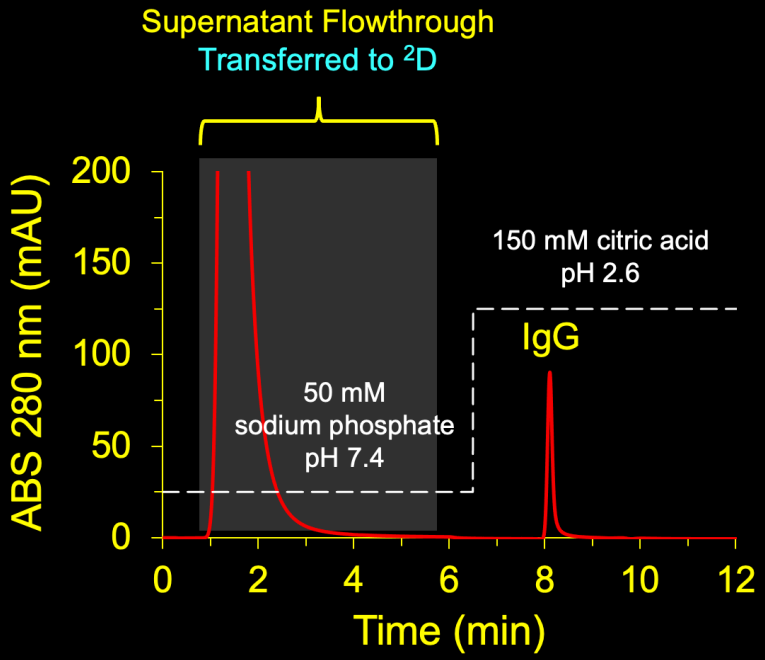
Instrument Configuration



Modulation Strategy



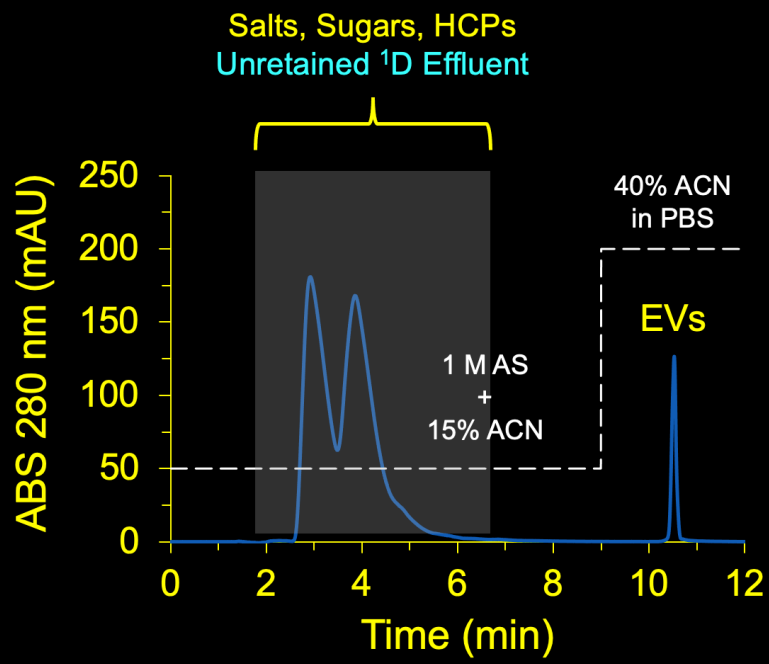
^{1}D-ProA



^{1}D – Affinity Chromatography

PP fibers modified with Protein A
pH gradient elution
IgG isolated from CHO supernatant

^{2}D-HIC

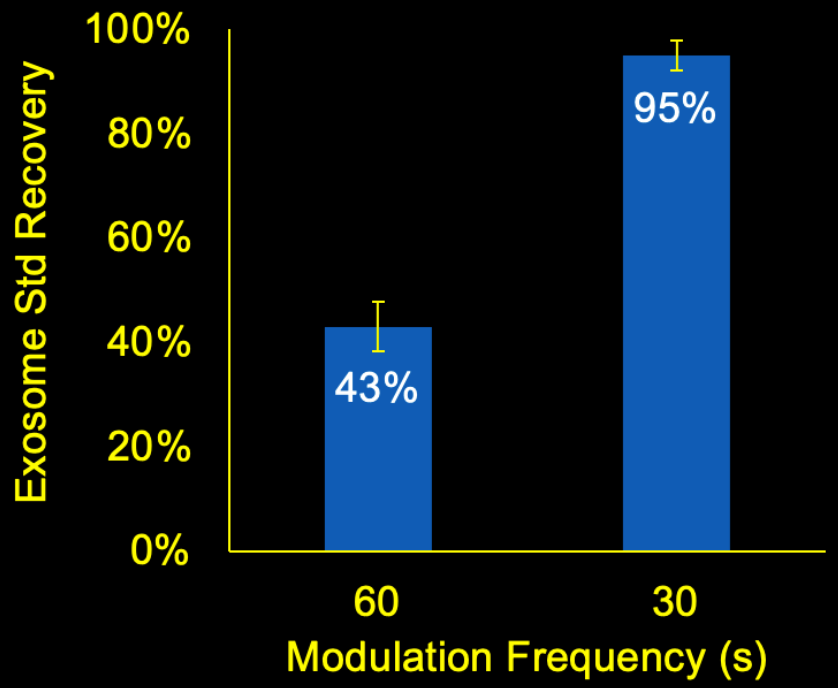


^{2}D – Hydrophobic Interaction Chromatography

PET fibers
Inverse salt gradient + organic modifier
Exosomes isolated and HCPs unretained

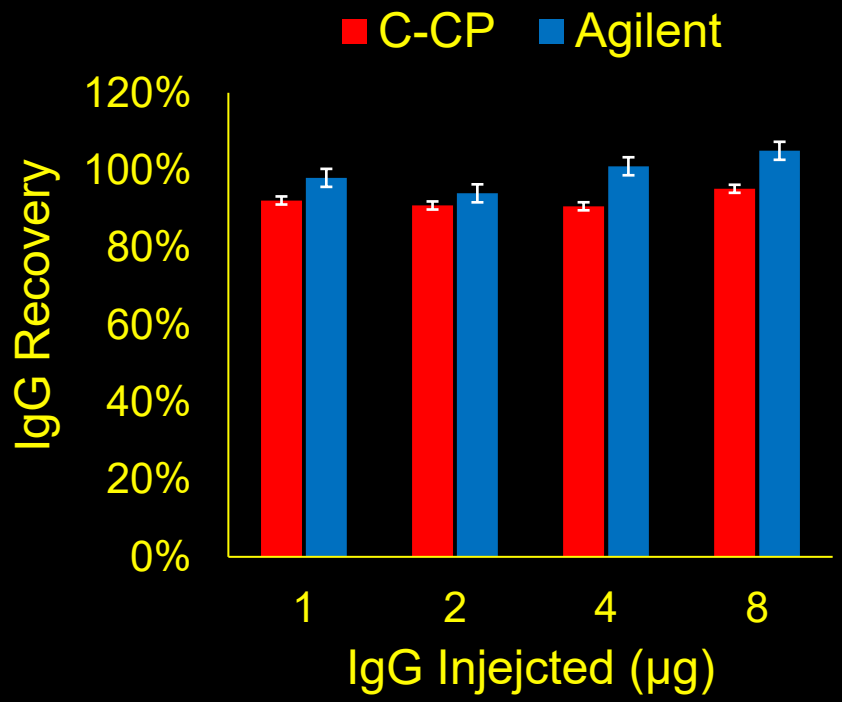
Modulation Efficiency

- ^{1}D-HIC
  - Peak area of exosome standard measured in absence of modulation activity
- ^{2}D-HIC
  - Exosome peak area assessed with modulations every 60 or 30 seconds
    - ^{1}D effluent occupies 50 and 25% of the transfer loop volume respectively



IgG – Recovery

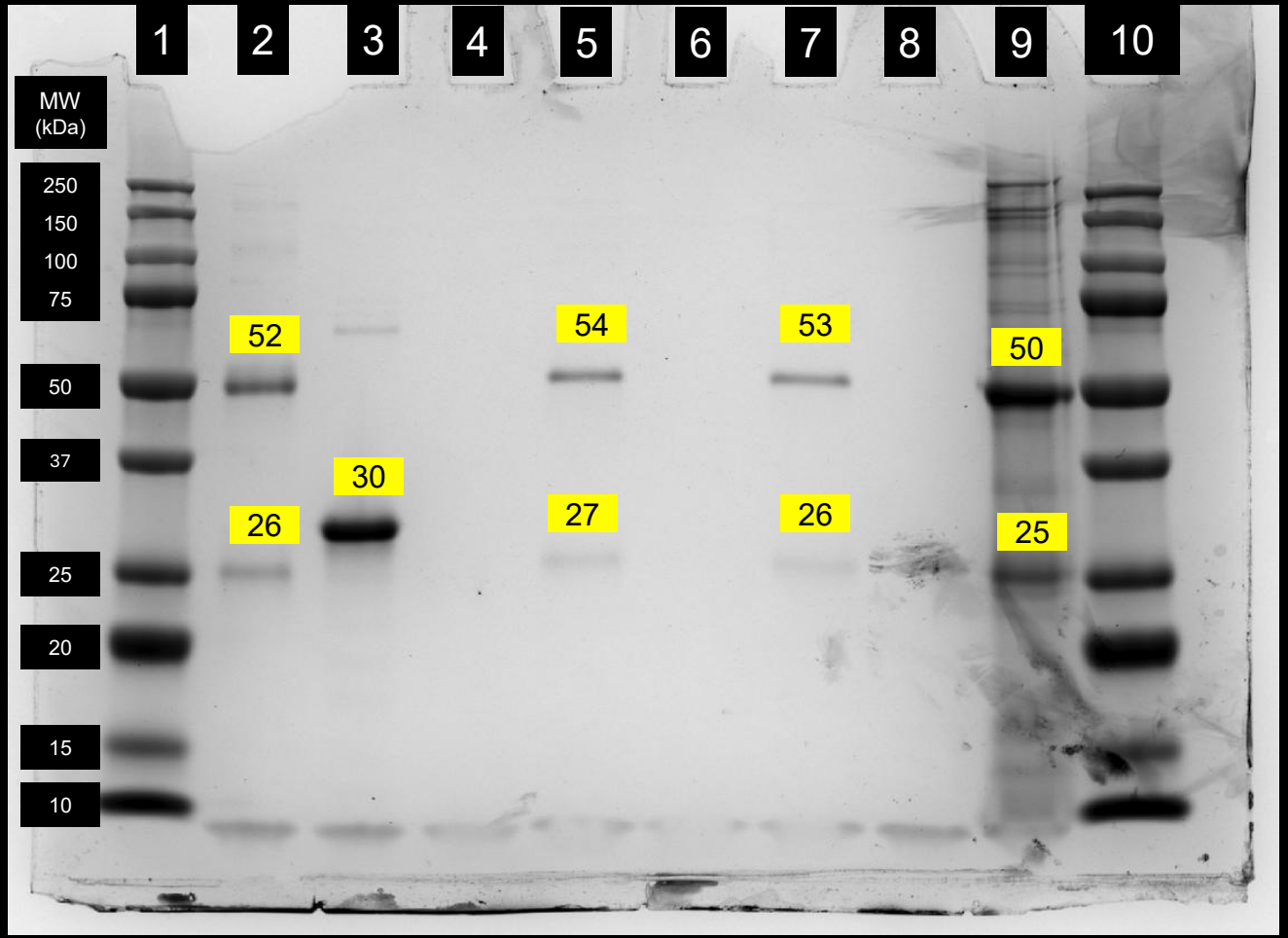
- CHO supernatant spiked with various concentrations of IgG
  - Excellent response linearity
  - R^2= 0.99
- Quantitative recoveries with RSD <5%
  - Comparable to Agilent commercial column



IgG – Purity

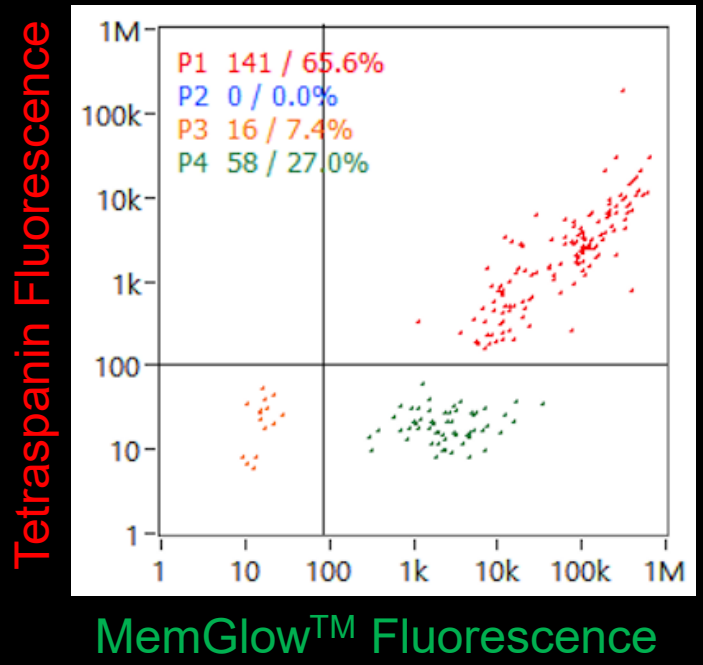
SDS-PAGE

Lane #	Sample
1	MW Ladder
2	IgG Standard
3	rSPA
4	Blanks (C-CP)
5	IgG (C-CP)
6	Blanks (Agilent)
7	IgG (Agilent)
8	Empty
9	CHO Supernatant
10	MW Ladder



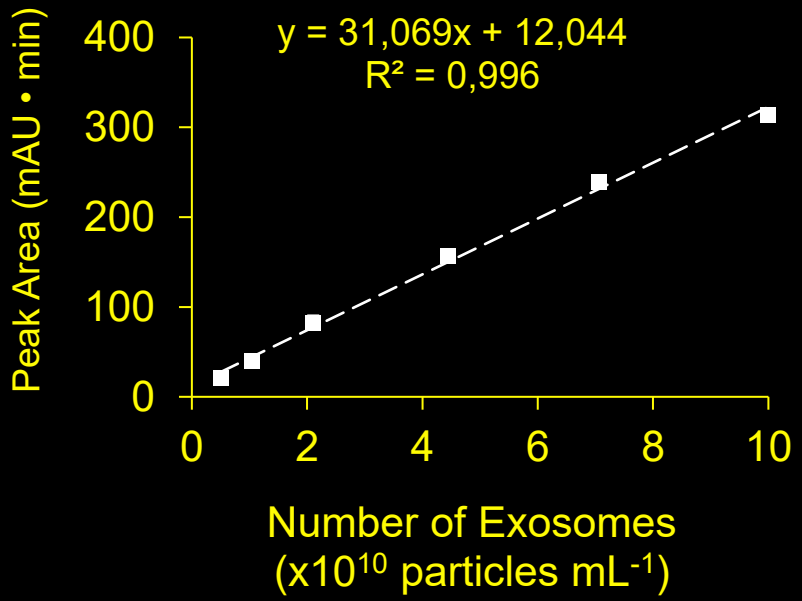
Exosome Characterization

- Particle-size distribution characterized using nano-flow cytometry (nanoFCM)
  - Mean diameter 83.2 ± 22.6 nm
- Immunolabeling of exosome tetraspanins using FITC anti-CD9, CD63, CD81 antibodies
  - ~ 66% of particles
- Phospholipid membranes labeled using lipophilic MemGlow™
  - ~ 93% of particles



Exosome Recovery and Purity

- Exosome recovery
  - 1.76 x 10^{11} particles mL^{-1}
  - < 7% RSD
- > 99% reduction of supernatant proteins
  - CHO supernatant 4 mg mL^{-1}
  - Exosome fraction 3 µg mL^{-1}
- Exosome purity
  - 5.86 x 10^{10} particles $µg^{-1}$ protein



Meets NIH guidelines for high purity exosomes (3 x 10^{10} particles $µg^{-1}$ protein)

Future Directions

- Characterize performance of 2.1 mm i.d. analytical C-CP columns
- Yb labeling of exosomes for quantitation using ICP-MS
- Cellular uptake studies of HIC isolated exosomes

- AEX separations using polyethyleneimine modified PET fibers
- Other novel fiber modifications to open additional separation modalities
- Separations of liposomes and AAVs

Summary

- Successfully demonstrated the use of 2D-LC to isolate and quantify both IgG and exosomes from CHO supernatant
  - Extract value from current waste streams
  - Expand Process Analytical Technology to monitor cell culture health and improve QA/QC
- Low-cost ProA C-CP achieved product quality comparable to leading commercial columns
  - No IgG carry-over or ProA leaching observed
- Successfully isolated high purity exosomes downstream of ProA separation
  - >99% reduction of host cell proteins from CHO supernatant
  - > 5 x 10^{10} particles μg^{-1} protein

Acknowledgments

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Thank You!

Questions?