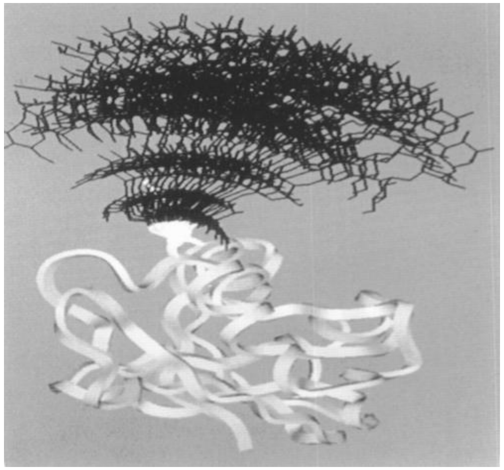
UNDERSTANDING THE EFFECTS OF PROCESS CONDITIONS ON PROTEIN GLYCOSYLATION AND PROTEOGLYCAN SYNTHESIS

Susan Sharfstein
Associate Professor of Nanobioscience
ssharfstein@albany.edu

GLYCOSYLATION IS A CRITICAL QUALITY ATTRIBUTE

Affects clearance, immunogenicity, stability, folding, secretion, and activity

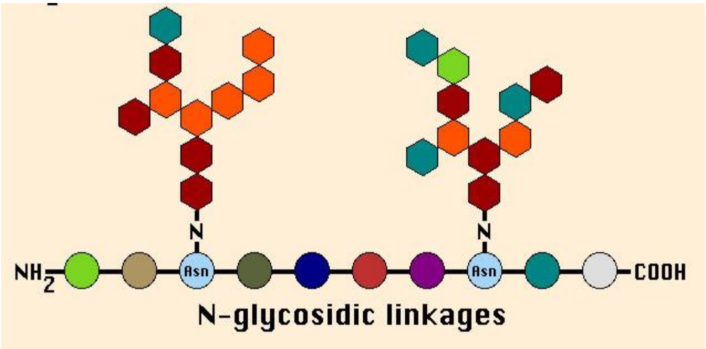
Non-templeted process so glycans exhibit macro and micro-heterogeneity



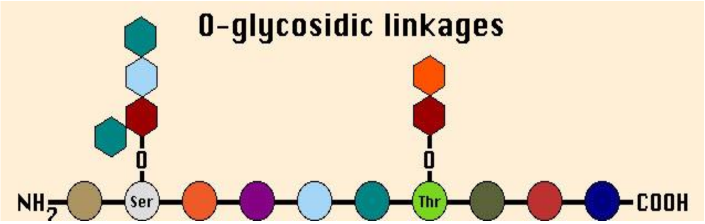
RNase B,(Dwek Chem. Rev., ***1996****, 96,683-720)*

Types of oligosaccharides & putative glycosylation sites

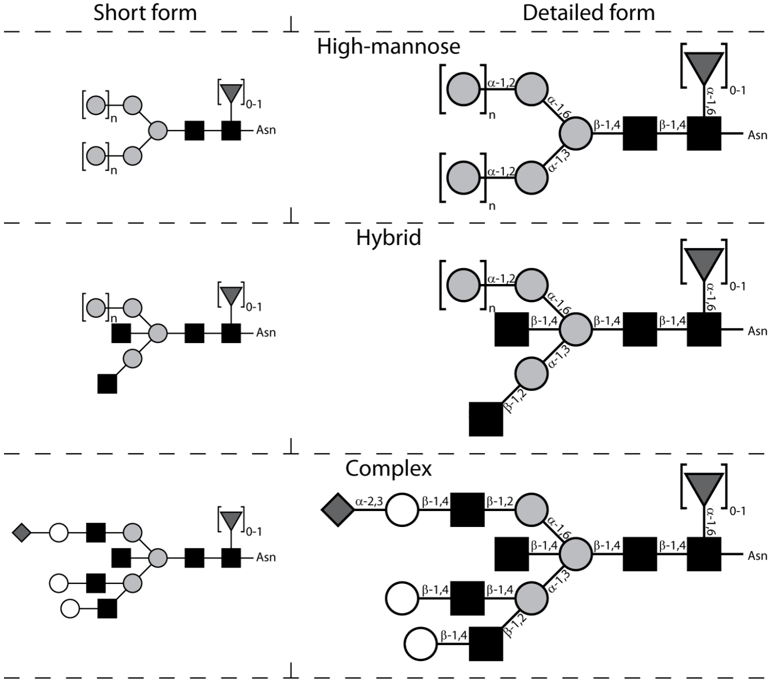
- N-linked site : Asn-X-Ser/Thr
(X: any amino acid except proline)



- O-linked site : Ser/Thr



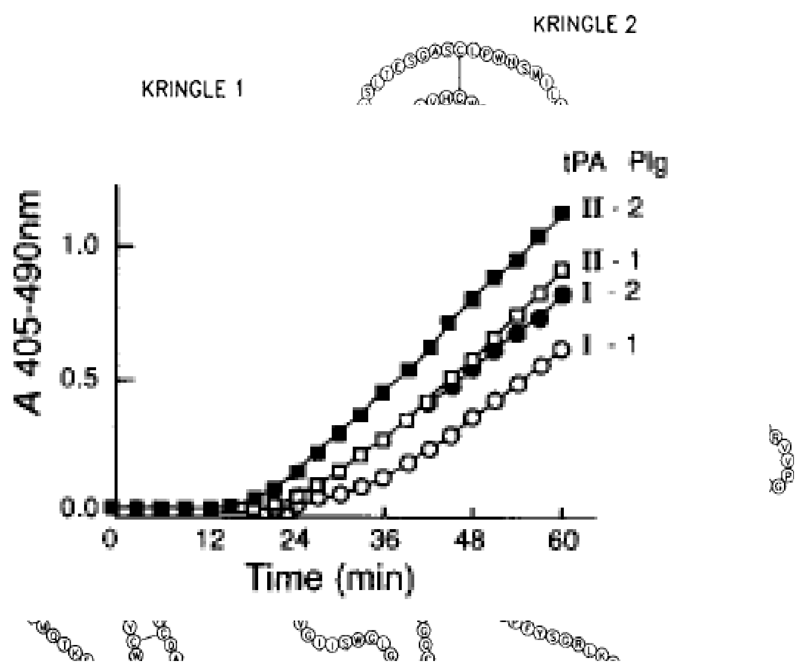
- N-linked type: Complex/High mannose/Hybrid type)



- O-linked type: No universal categorized types

Tissue Plasminogen Activator (tPA)

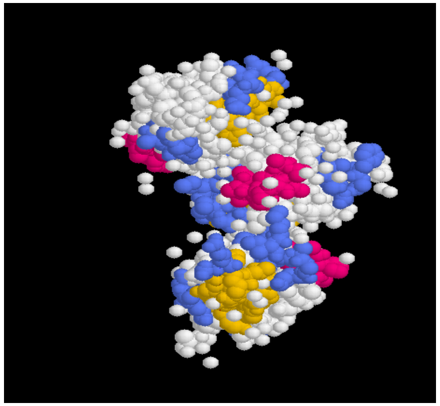
Type I Type II



Activity is affected by t-PA and plasminogen variants

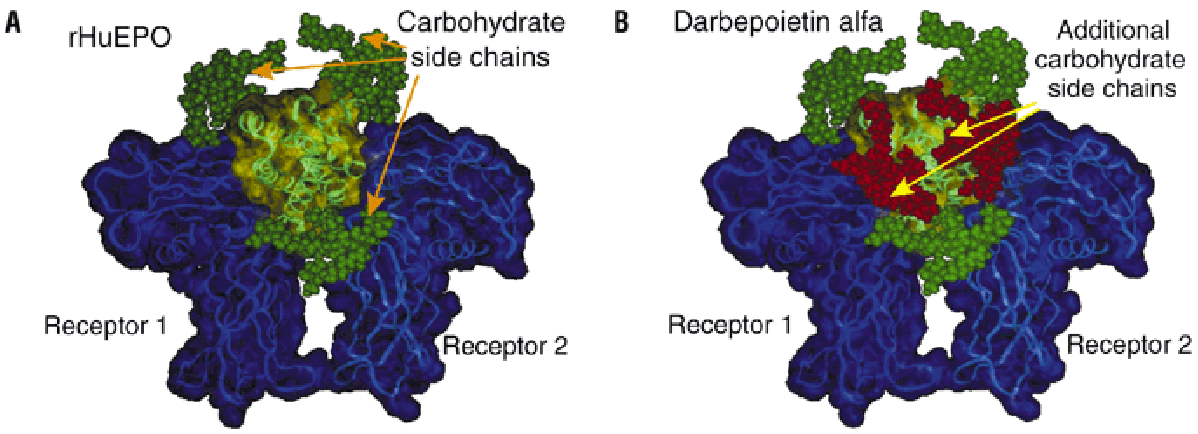
Rudd et al., Biochimica Biophysica Acta 1248 (1995) 1-10

(Rouf et al. , *Biotechnology Advances*, **1996**, 14(3), 239–266)

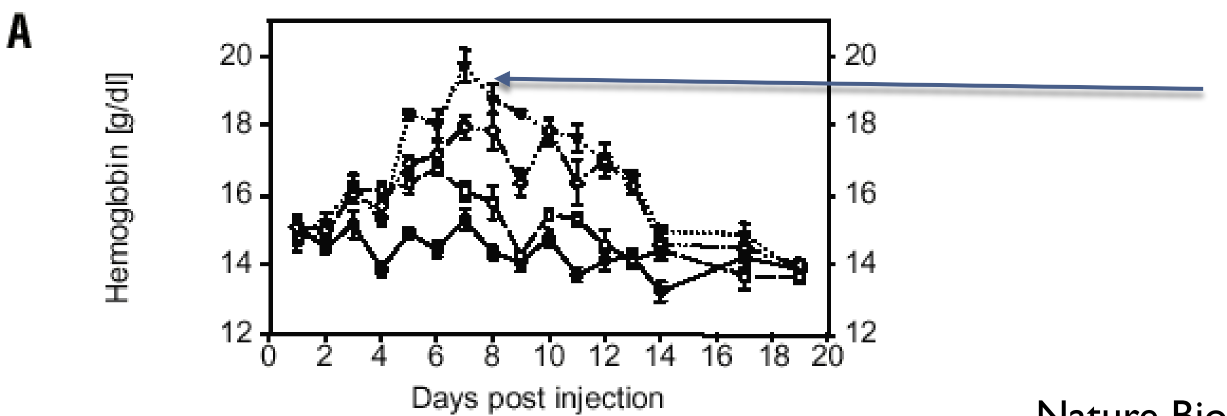


Properties of t-PA	
# of A.A.	527
M.W.	~ 70kD with glycans
# of disulfide bonds	17
# of putative N-glycosylation sites	4 (Asn-117,184,218 & 448)
Types	One-chain/two-chain Type I/Type II

Erythropoietin (EPO) vs. Darbepoietin



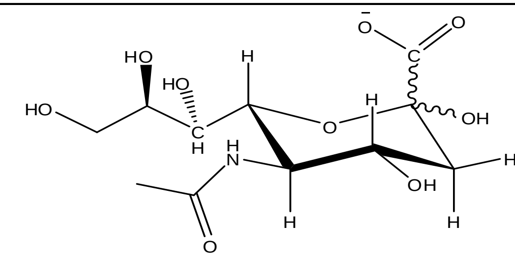
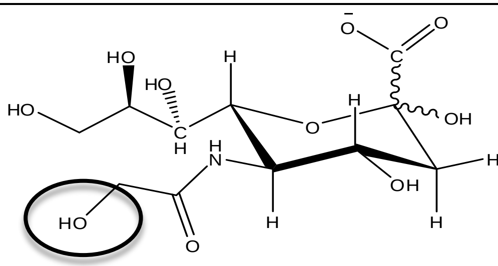
Three additional N-linked glycosylation sites were engineered into Darbepoietin



Darbepoietin showed higher activity, longer duration and lower dosing than EPO

Nature Biotechnology 21, 414 - 421 (2003)

Sialic acid immunogenicity

Sialic acid	N-acetylneuraminic acid	Neu5Ac or Sia		
	N-glycolylneuraminic acid	N[illegible]5[illegible]		

Avoid

Two examples

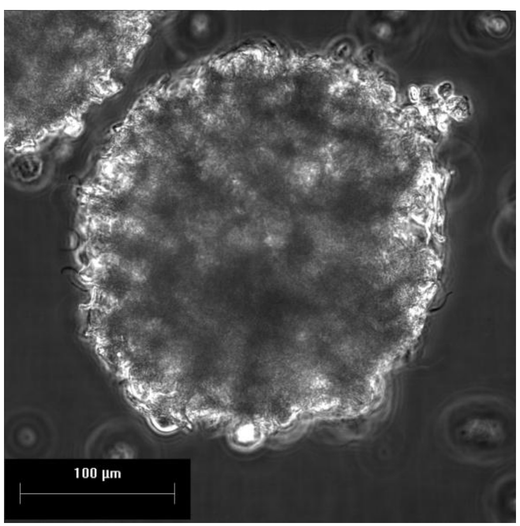
- Effects of process conditions on N-linked glycan structures
- Metabolic engineering of CHO cells to produce a bioengineered heparin

Culture conditions

- All cultures performed in a stirred bioreactor with pH and DO control, in fed-batch to maintain glucose and glutamine at target levels, HyQ SFM4CHO-Utility (protein-free)
- Four culture conditions
  - Suspension
  - Cytodex 3 microcarriers
  - Cytopore 1 microcarriers
  - Suspension with a temperature reduction from 37° C to 33° C at onset of stationary phase

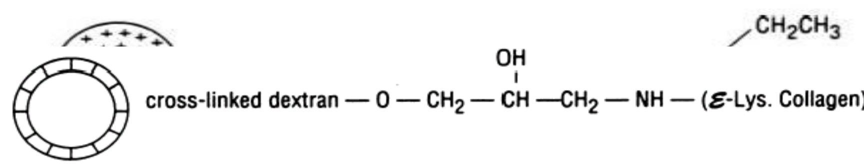
Model systems

- Chinese hamster ovary (CHO) cell line
  - Secreted alkaline phosphatase (SEAP) producing CHO cell line (TR2-255, a generous gift from Dr. Ermonval, Institute Andre Lwoff)
- Microcarriers

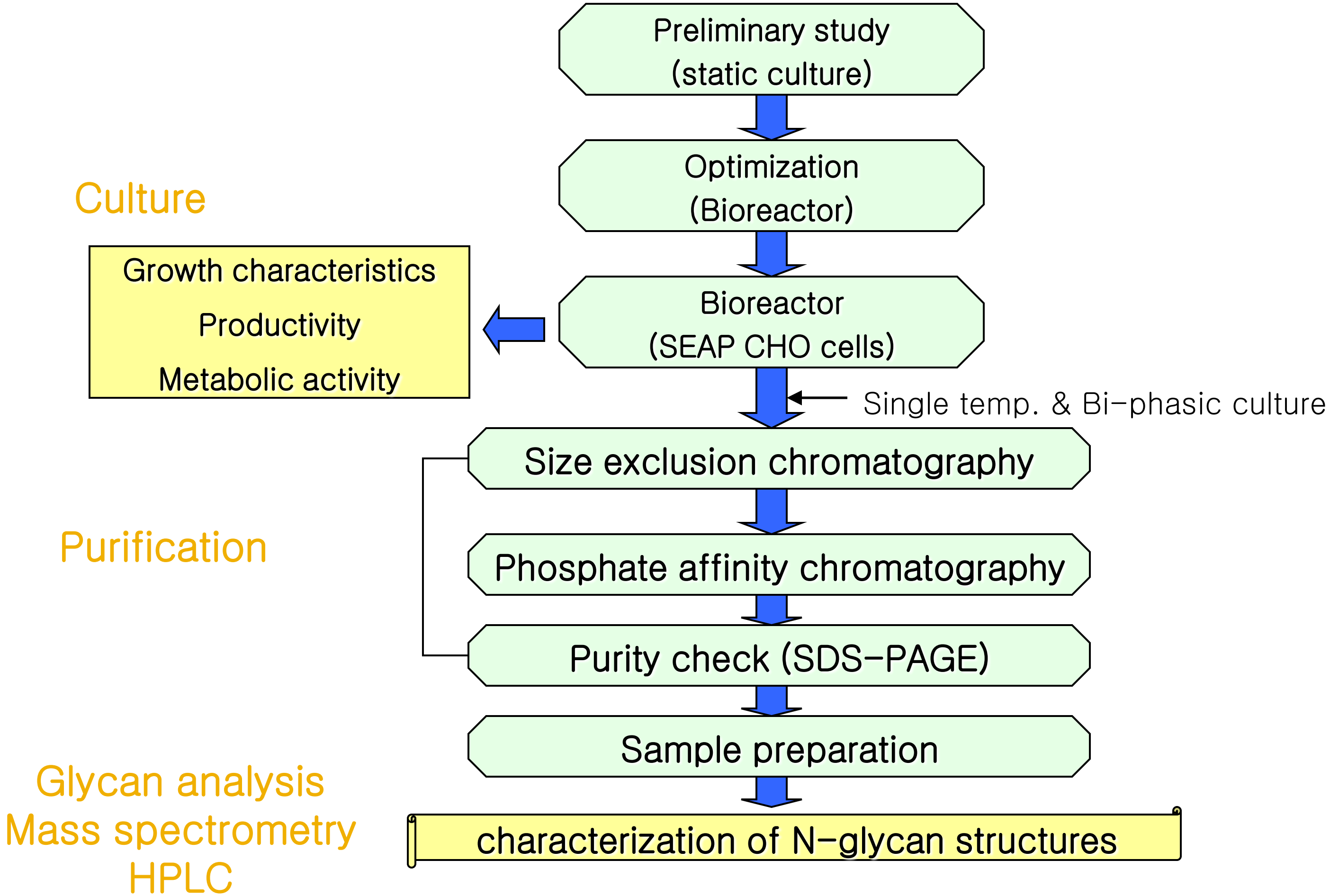


Type	Material	Size (µm)
Cytodex 3 (Solid)	Cross-linked dextran, collagen-coated	141-211 (175)
Cytopore 1 (Porous)	Macroporous, cross-linked cellulose, positively-charged	200-280 (240)

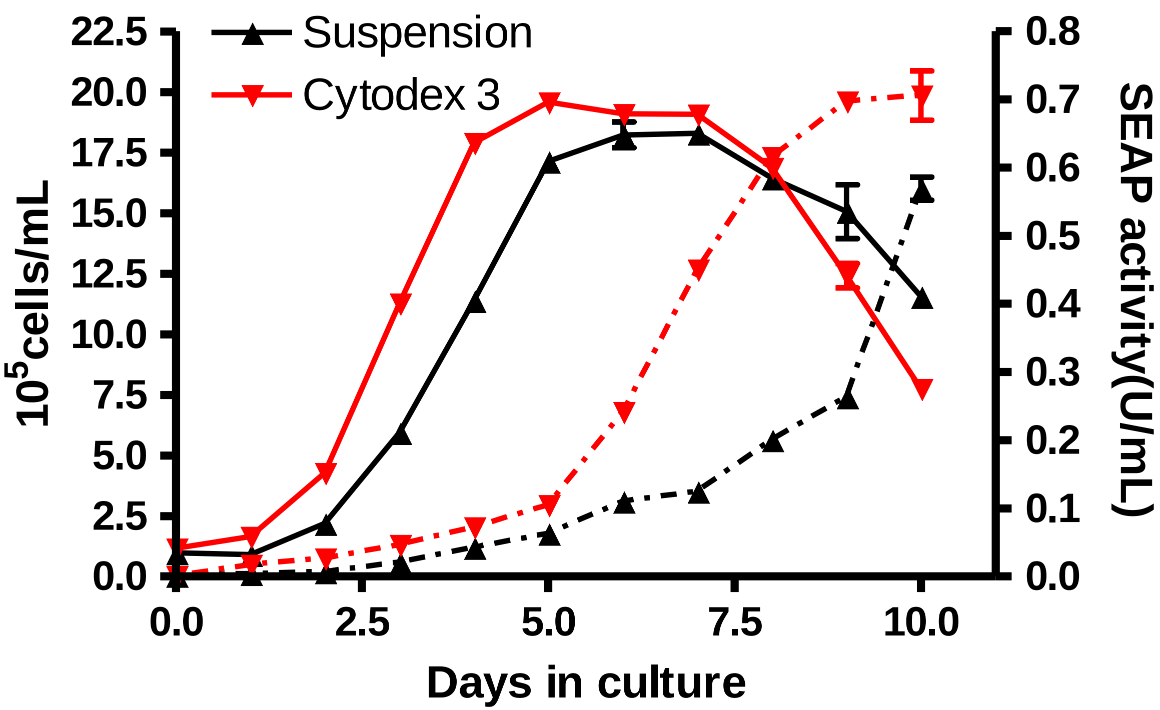
Cytodex 3
collagen layer coupled to surface



Overall experimental flowchart

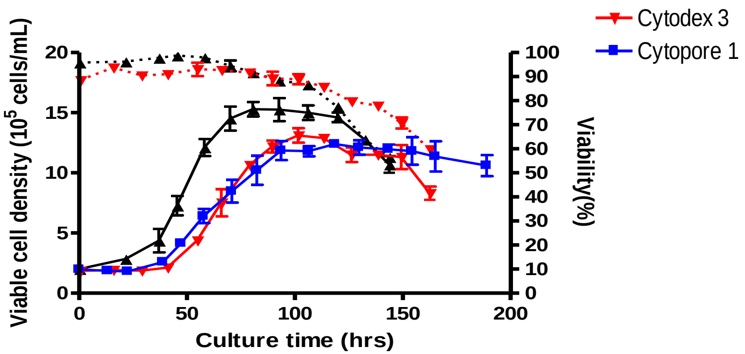


In static culture, microcarriers outperform suspension cells

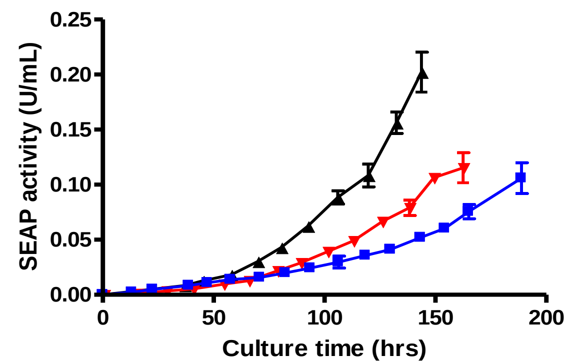


In bioreactor cultures, suspension cells show better growth and productivity

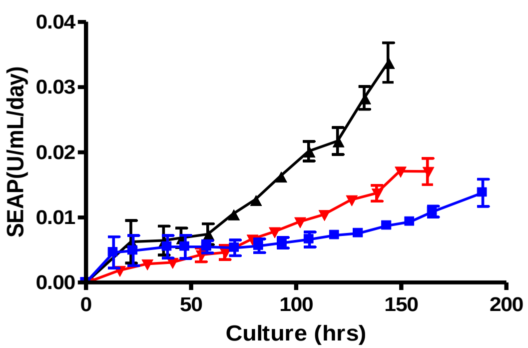
Growth curve



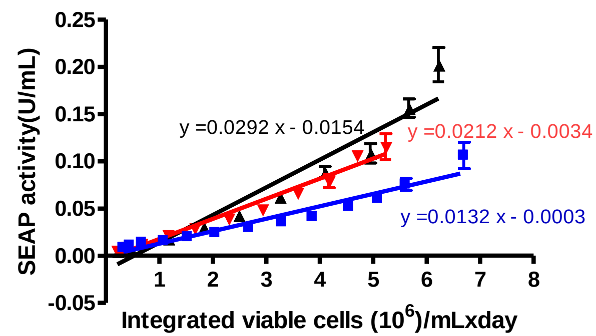
Accumulated activity of SEAP



Volumetric productivity of SEAP



Specific productivity of SEAP

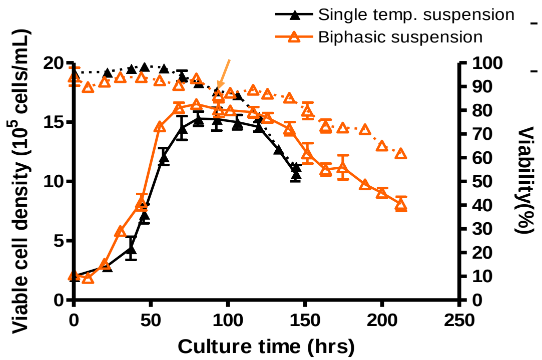


Reducing the temperature extends growth and improves productivity

Greatest benefits seen in suspension culture

Growth curve

Suspension



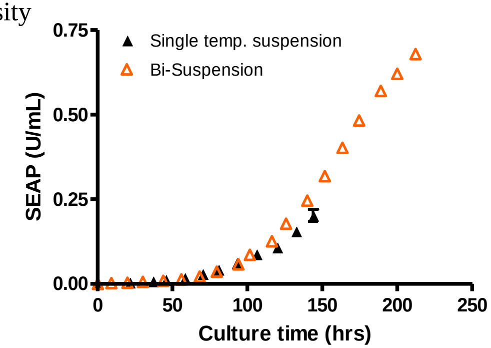
Arrows : temp. switch

- Solid line : viable cell density

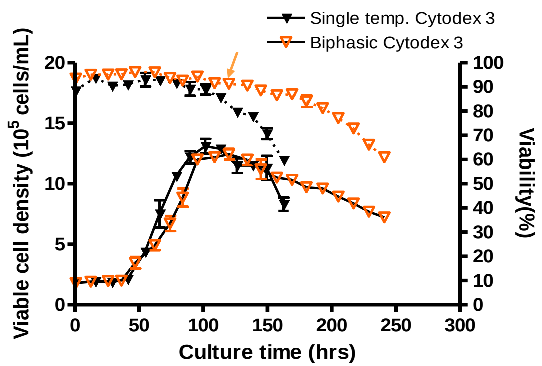
- Dashed line: viability

Accumulated activity of SEAP

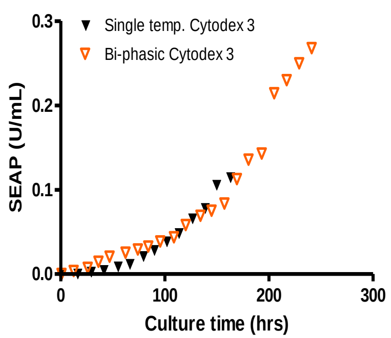
Suspension



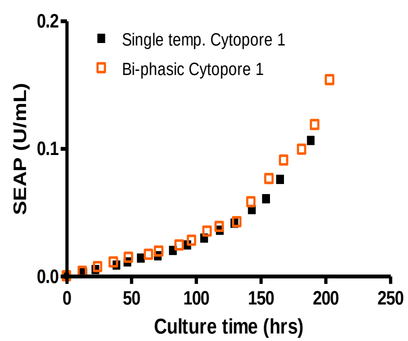
Cytodex 3



Cytodex 3



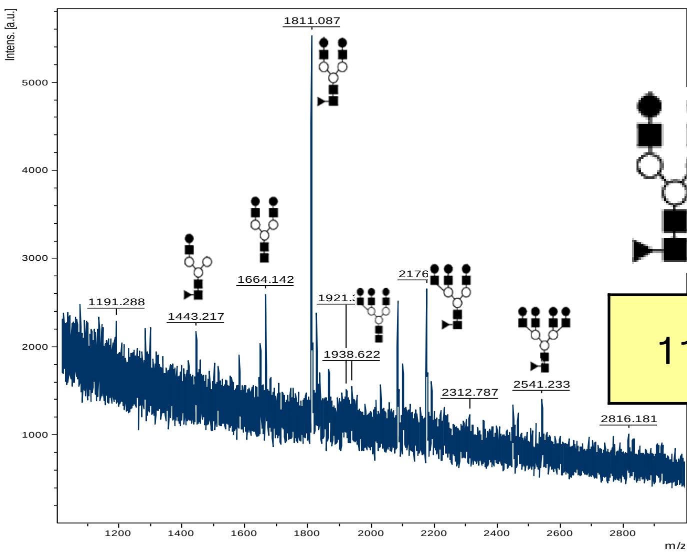
Cytopore 1



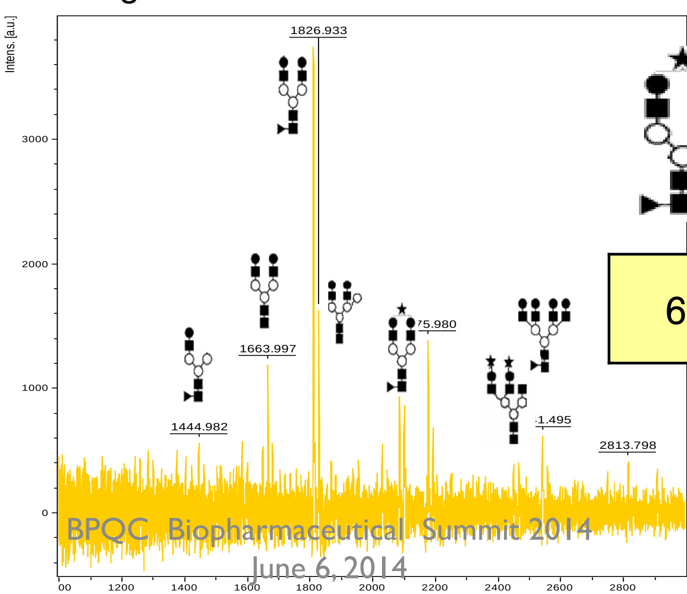
MALDI-TOF mass spectrometry is used to characterize N-linked glycans

Suspension

Positive-ion mode



Negative-ion mode



Composition/Sequence of N-linked glycan pool

No.	Positive-ion mode			
	Observed m/z * [M+Na]+	Calculated m/z * [M+Na]+	Composition/Sequence	Abbreviation
1	1136.9	1136.4	(HexNAc)1+(Man)3(GlcNAc)2	A1
2	1283.5	1282.5	(HexNAc)1(Deoxyhexose)1+(Man)3(GlcNAc)2	A1F1
3	1299.4	1298.4	(Hex)1(HexNAc)1+(Man)3(GlcNAc)2	A1G1
4	1420.4	1419.5	(Hex)3+(Man)3(GlcNAc)2	A0M3
5	1443.2	1444.5	(Hex)1(HexNAc)1(Deoxyhexose)1+(Man)3(GlcNAc)2	A1G1F1
6	1459.1	1460.5	(Hex)2(HexNAc)1+(Man)3(GlcNAc)2	A1M1G1
7	1502.3	1501.5	(Hex)1(HexNAc)2+(Man)3(GlcNAc)2	A2G1
11	1811.1	1809.6	(Hex)2(HexNAc)2(Deoxyhexose)1+(Man)3(GlcNAc)2	A2G2F1
12	1826.1	1825.6	(Hex)3(HexNAc)2+(Man)3(GlcNAc)2	A2M1G2
13	1866.0	1866.7	(Hex)2(HexNAc)3+(Man)3(GlcNAc)2	A3G2
14	1907.5	1907.7	(Hex)1(HexNAc)4+(Man)3(GlcNAc)2	A4G3
15	2029.7	2028.7	(Hex)3(HexNAc)3+(Man)3(GlcNAc)2	A3G3
16	2176.0	2174.8	(Hex)3(HexNAc)3(Deoxyhexose)1+(Man)3(GlcNAc)2	A3G3F1
17	2232.0	2231.8	(Hex)3(HexNAc)4+(Man)3(GlcNAc)2	A4G3
18	2541.2	2539.9	(Hex)4(HexNAc)4(Deoxyhexose)1+(Man)3(GlcNAc)2	A4G4F1

11	(Hex)2(HexNAc)2(Deoxyhexose)1+(Man)3(GlcNAc)2	A2G2F1

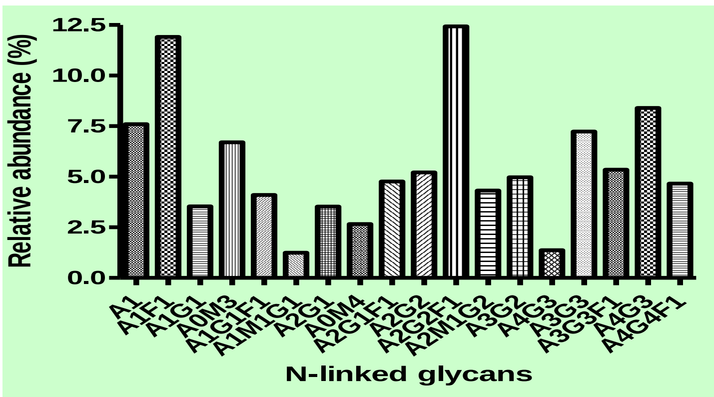
No.	Negative-ion mode			
	Observed m/z * [M+Na-H]-	Calculated m/z * [M+Na-H]-	Composition/Sequence	Abbreviation
1	1281.6	1281.5	(HexNAc)1(Deoxyhexose)1+(Man)3(GlcNAc)2	A1F1
5	1826.1	1824.6	(Hex)3(HexNAc)2+(Man)3(GlcNAc)2	A2M1G2
6	2098.9	2099.7	(Hex)2 (HexNAc)2(Deoxyhexose)1(NeuAc)1 +(Man)3(GlcNAc)2	A2G2F1S1
7	2449.0	2450.0	(Hex)2 (HexNAc)3(NeuAc)2+(Man)3(GlcNAc)2	A3G2S2
8	2465.1	2464.9	(Hex)3(HexNAc)3(Deoxyhexose)1(NeuAc)1 +(Man)3(GlcNAc)2	A3G3F1S1
9	2539.9	2538.9	(Hex)4(HexNAc)4(Deoxyhexose)1+(Man)3(GlcNAc)2	A4G4F1

6	(Hex)2(HexNAc)2(Deoxyhexose)1(NeuAc)1+(Man)3(GlcNAc)2	A2G2F1 S1

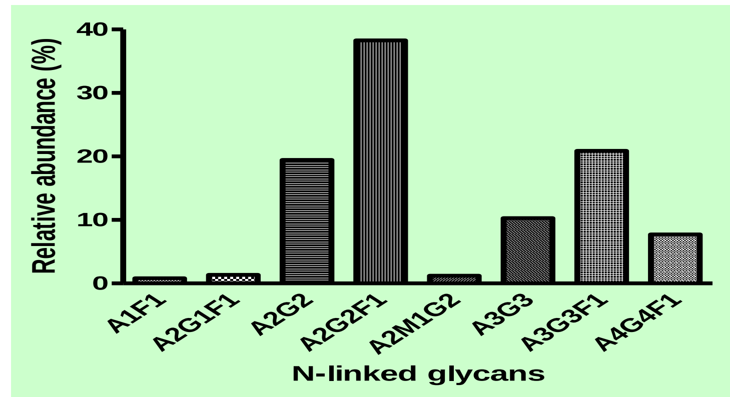
Relative abundance of N-linked glycans

(Based on Harvey, Rapid communications in Mass spectrometry, 1993, 7: 614-619)

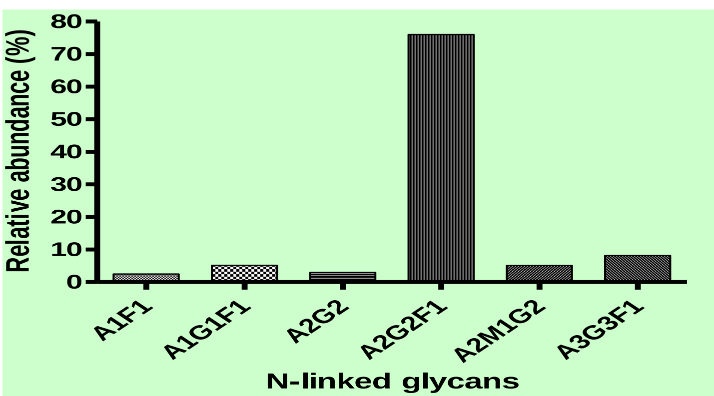
Suspension culture



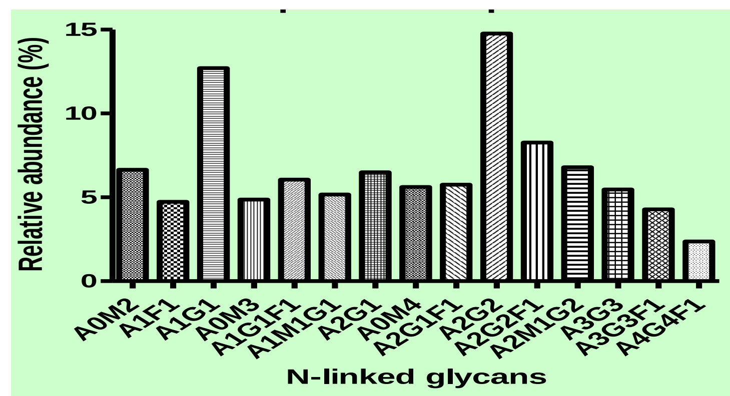
Cytodex 3 culture



Cytopore 1 culture

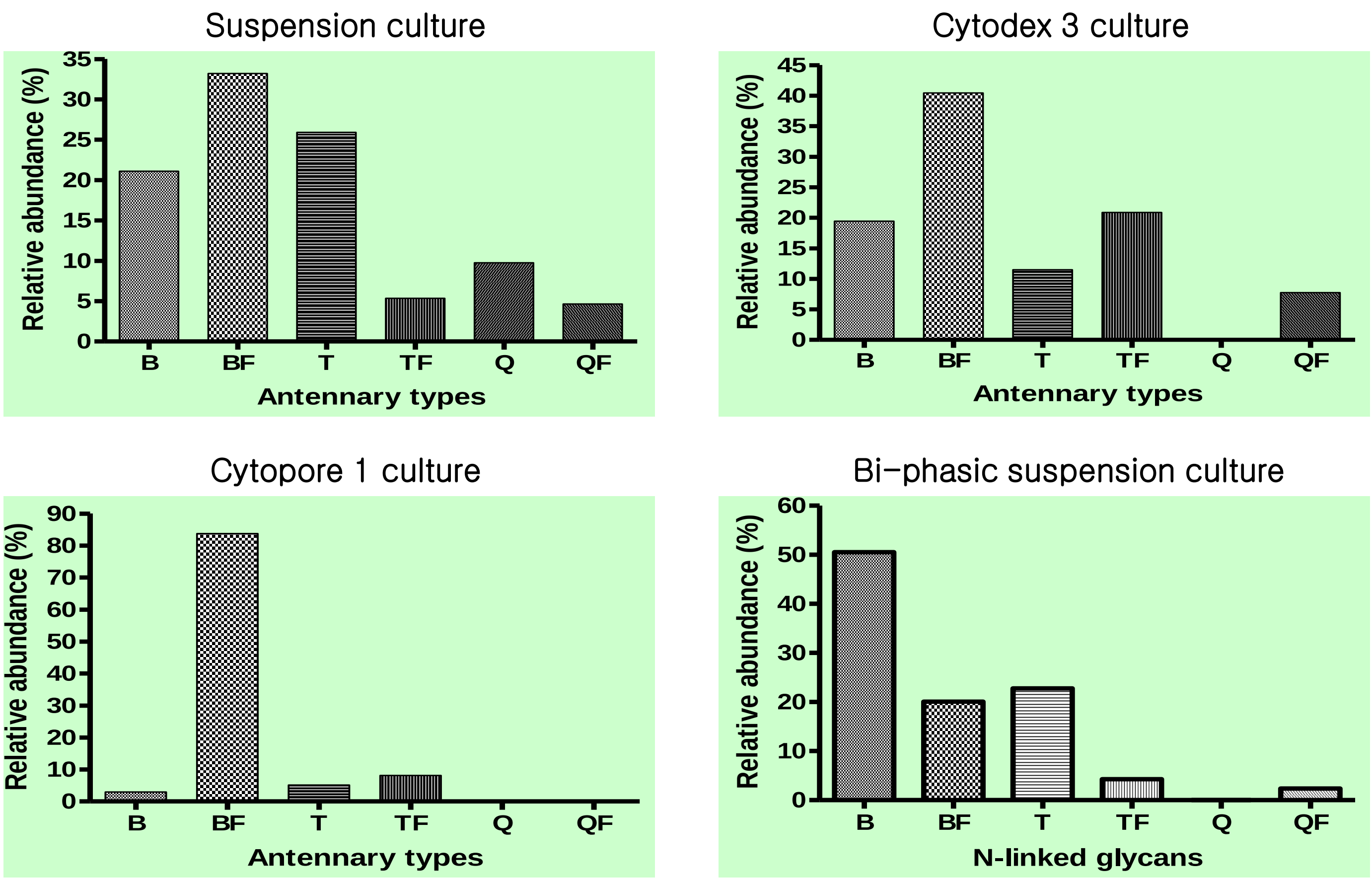


Bi-phasic suspension culture



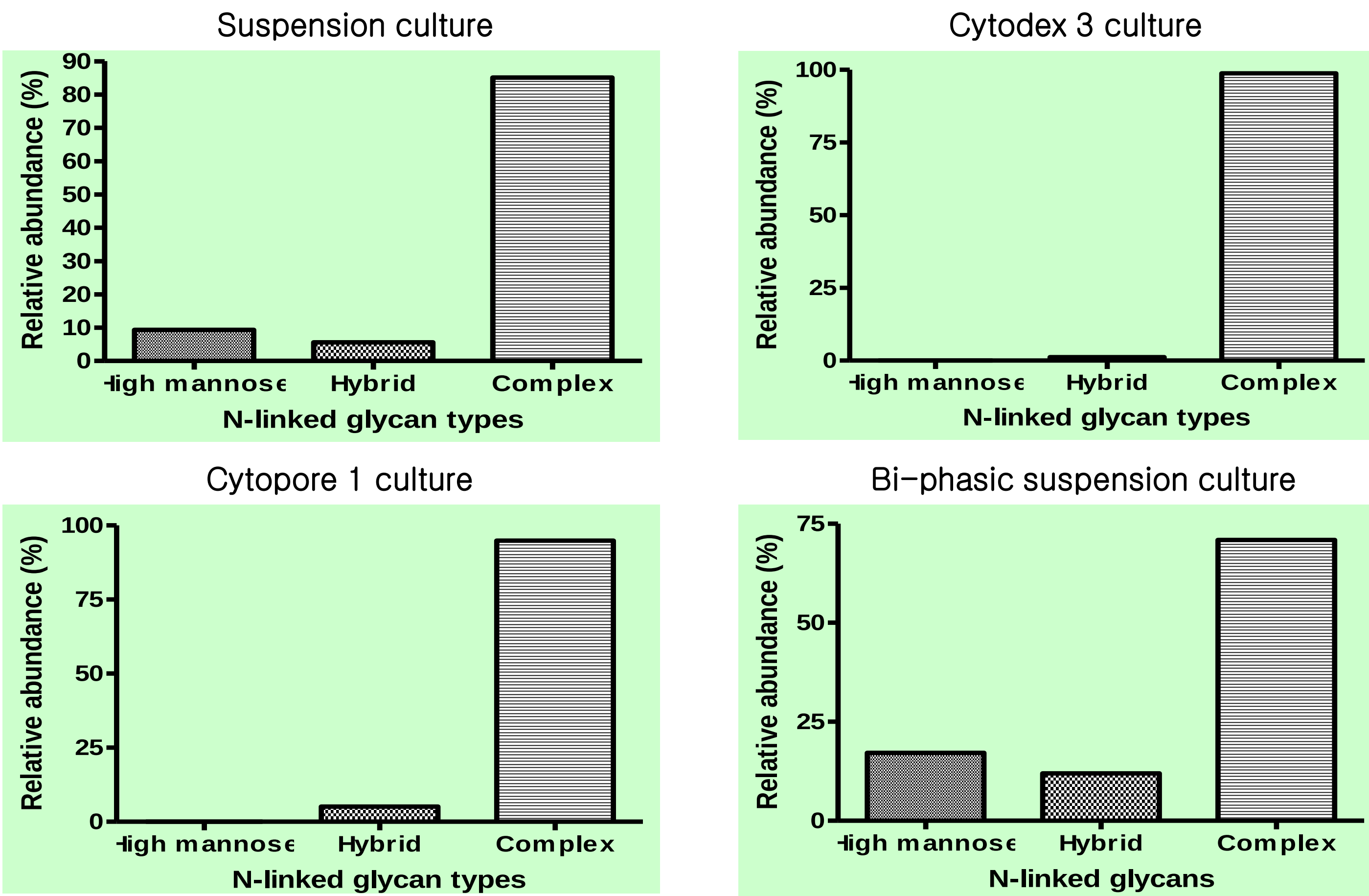
(A: GlcNAc, F: Fucose, G: Galactose, M: Mannose) N-glycan abbreviation based on Butler's paper, 2003, vol. 13, p601-622.

Culture conditions affect antennary distribution and fucosylation



(B: biantennary, T: triantennary, Q: tetrantennary, F: fucosyl residue)

Complex glycans are the predominant form

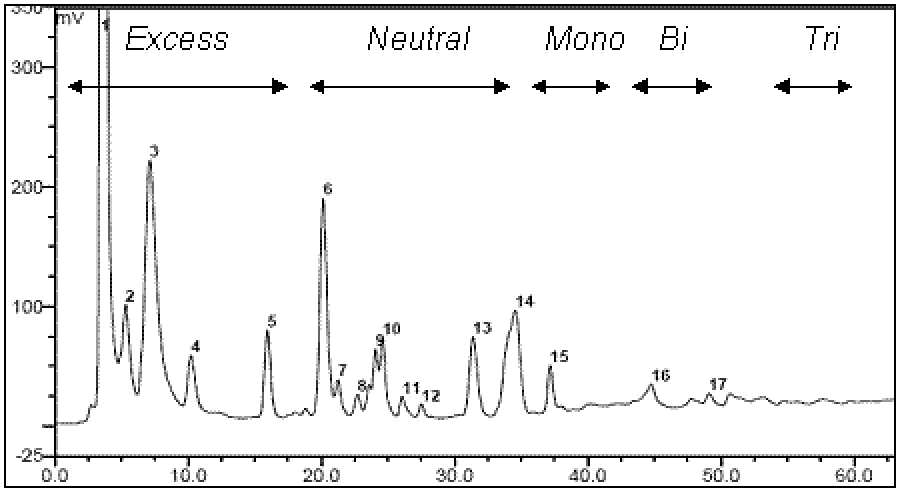


SUNY College of Nanoscale Science and Engineering

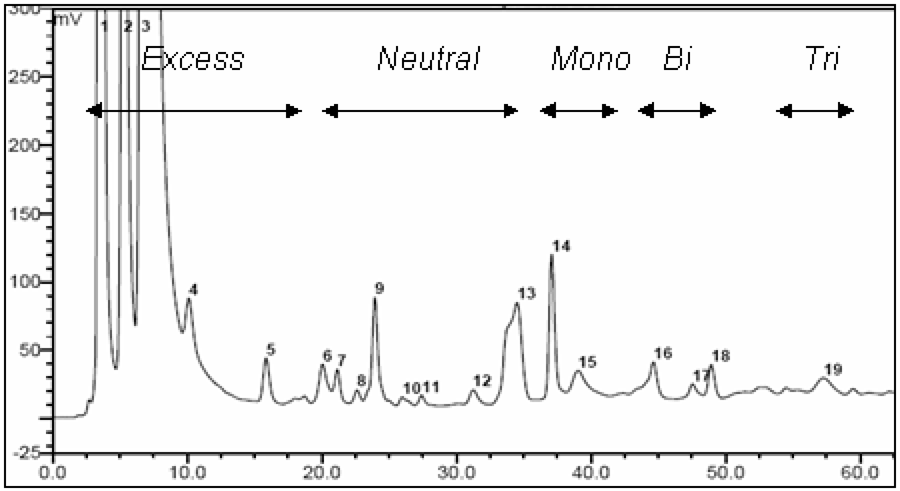
HPLC is used to quantitate glycans

Culture conditions have subtle, but potentially important effects on glycosylation

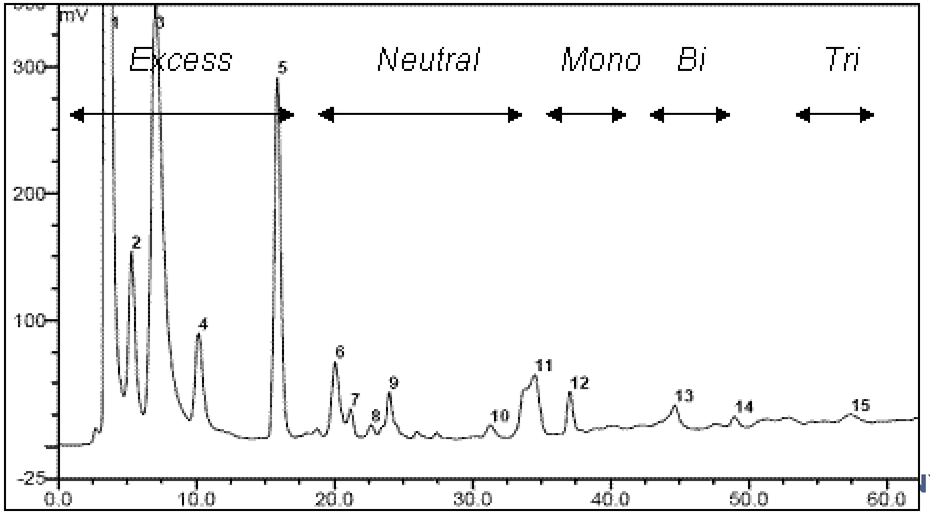
Suspension



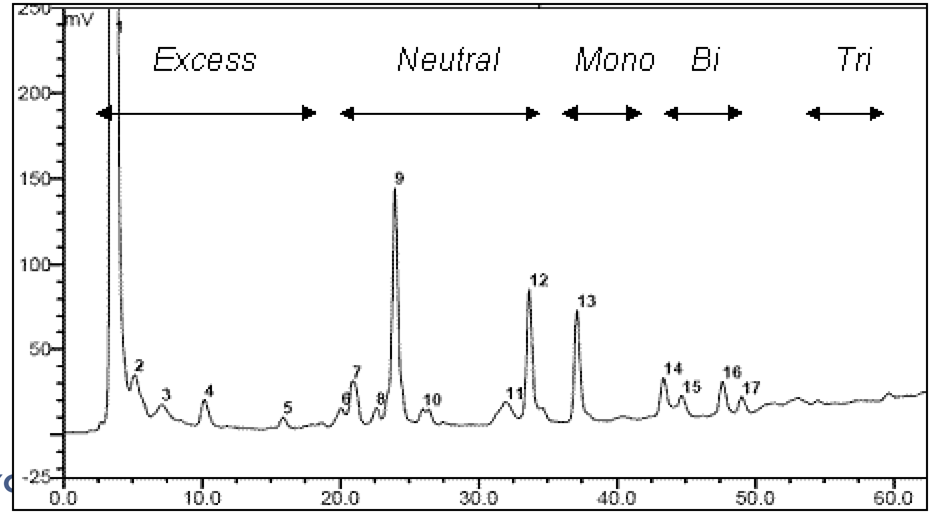
Cytodex 3



Cytopore 1



Biphasic

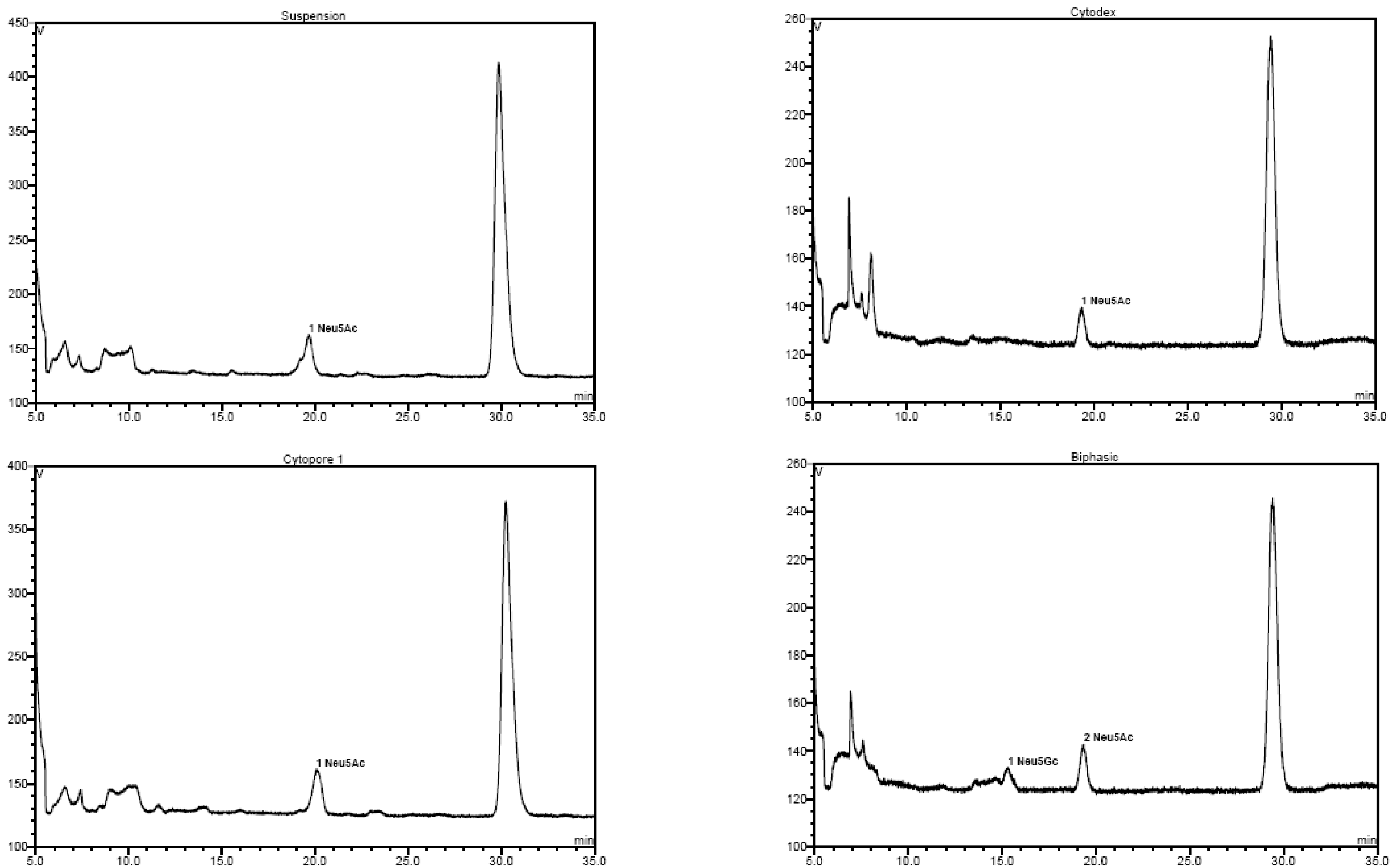


Culture conditions affect sialylation and fucosylation

Culture Condition	Neutral N-linked glycans (%)		Sialylated N-linked glycans (%)							
	fucosylated	non-fucosylated	Mono-sialylated		Di-sialylated		Tri-sialylated		Total sialylated	Total fucosylated
			fucosylated	non-fucosylated	fucosylated	non-fucosylated	fucosylated	non-fucosylated		
Suspension	35.1	20.0	11.3	30.1	0	3.5	0	0	44.9	46.4
Cytodex 3	11.3	14.4	2.2	55.1	6.7	6.1	0	4.2	74.3	20.2
Cytopore 1	27.0	13.1	3.3	42.2	0	10.4	0	4.1	59.9	30.3
Biphasic suspension	11.9	34.3	6.1	32.5	8.9	6.2	0	0	53.8	27.0

SUNY College of Nanoscale Science and Engineering

Reduced temperature cultures also show different sialic acids

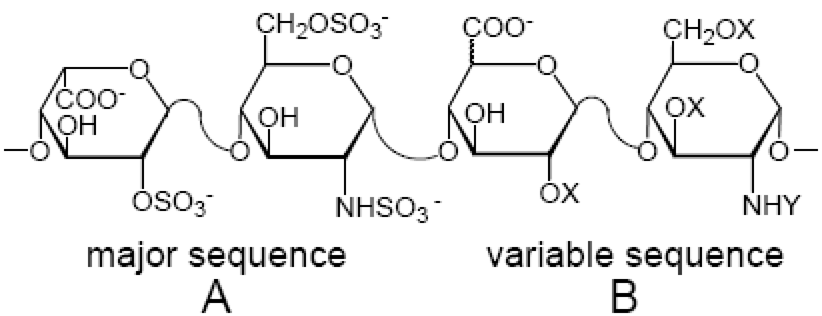


Conclusions

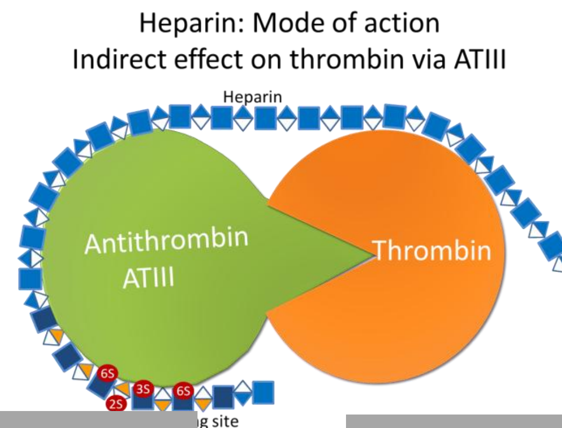
- Biphasic temperature reduction improves productivity for some proteins (Nam, et al, Cytotech., 59: 81-91 (2009)
- Microcarrier cultures do not necessarily improve cell density or productivity (Nam, et al. Biotech. Prog., 23: 652 -660, 2007)
- Sialic acid and fucosylation are affected by temperature profiles and microcarrier culture (Nam, et al., Biotech. Bioeng. 100: 1178–1192, 2008)

Heparin is a linear highly-sulfated polysaccharide (glycosaminoglycan)

Heparin is the most widely used anticoagulant drug in the world

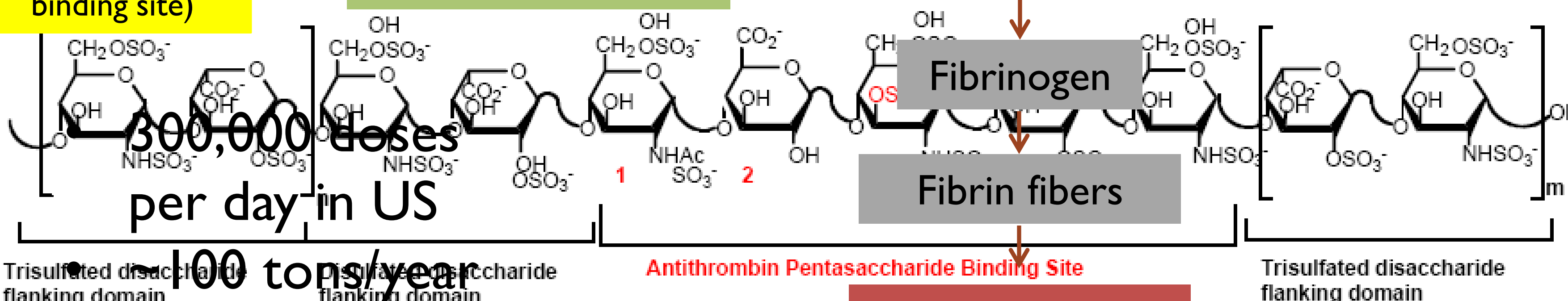


The structu... ...charide
units altern... ...and
N-acetyl gl...



Heparin (Antithrombin III binding site) + Antithrombin III ⊣ Thrombin ← Prothrombin

Thrombin → Fibrinogen → Fibrin fibers → Wound clotting



- 300,000 doses per day in US
- ~100 tons/year
- $7 billion/year

Loganathan D. et al., *Biochemistry*, 1990
Pervin A. et al., *Glycobiology*, 1995

Animal tissues are the current source of heparin

Source

- Primarily produced in mast cells
- Extracted from animal tissues (porcine intestine, bovine lung)

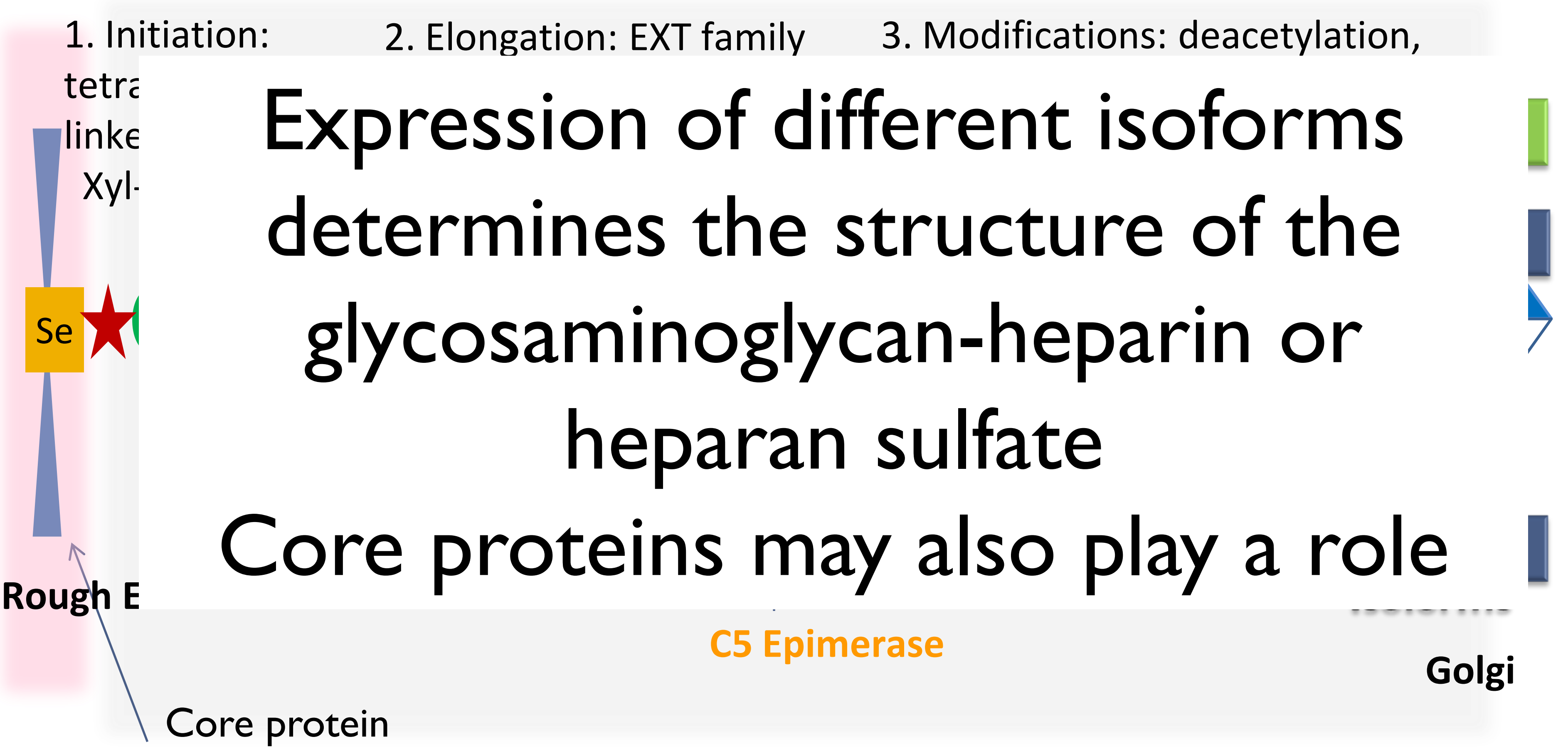
Problems

- Animal subspecies, feed and environment effect heparin quality
- Animal number (1700 million pigs annually)
- Intestines of 3 pigs are needed to provide 1 gram of heparin
- Contamination crisis of 2008: oversulfated chondroitin sulfate led to the death of over 100 people in US alone

Objective: Use metabolic engineering to take biopharmaceutical development to the next step by producing polysaccharide drugs in culture under GMP conditions

Heparin/Heparan Sulfate Biosynthesis

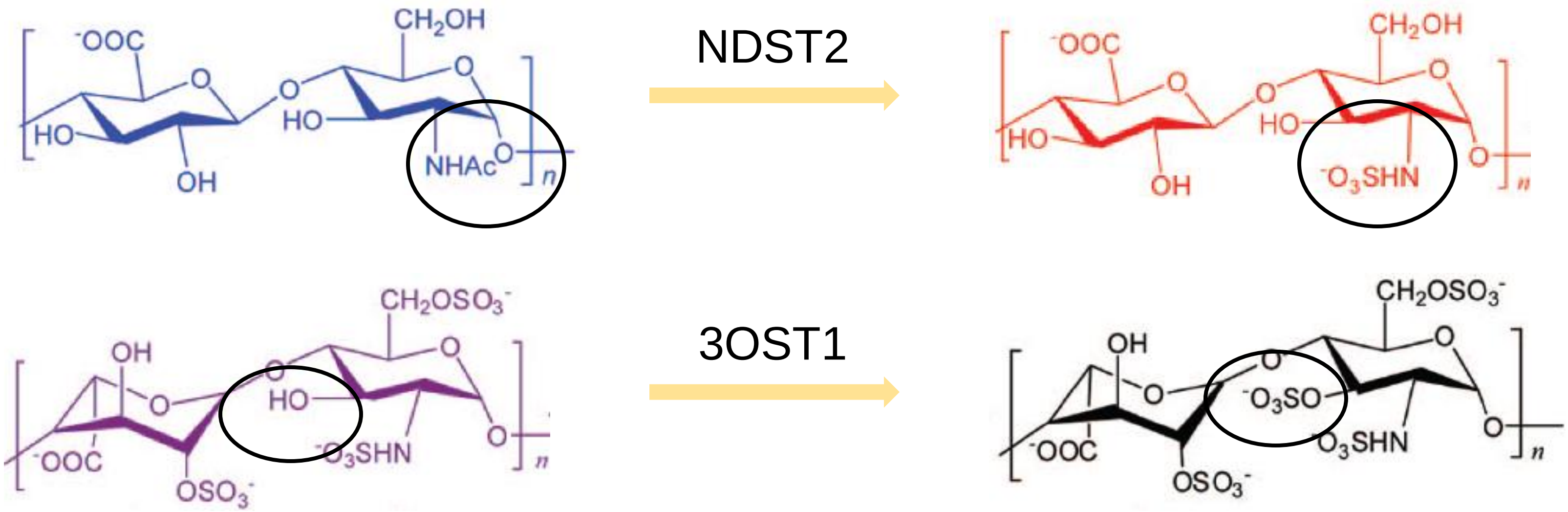
Heparin & heparan sulfate share a similar biosynthetic pathway

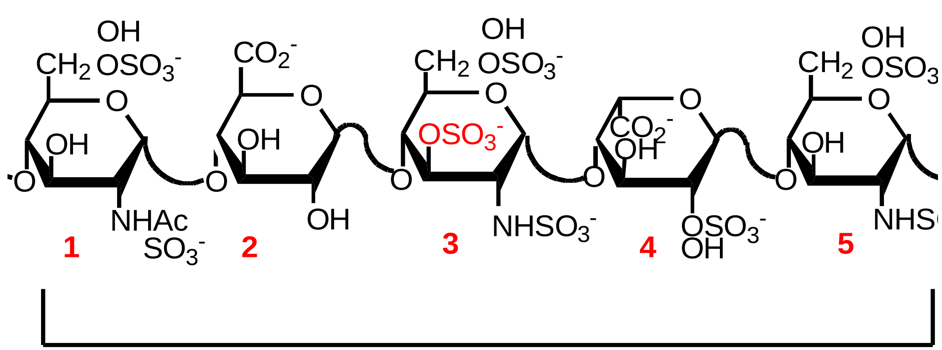


Expression of different isoforms determines the structure of the glycosaminoglycan-heparin or heparan sulfate

Core proteins may also play a role

NDST2 and 3OST1 are critical for heparin synthesis



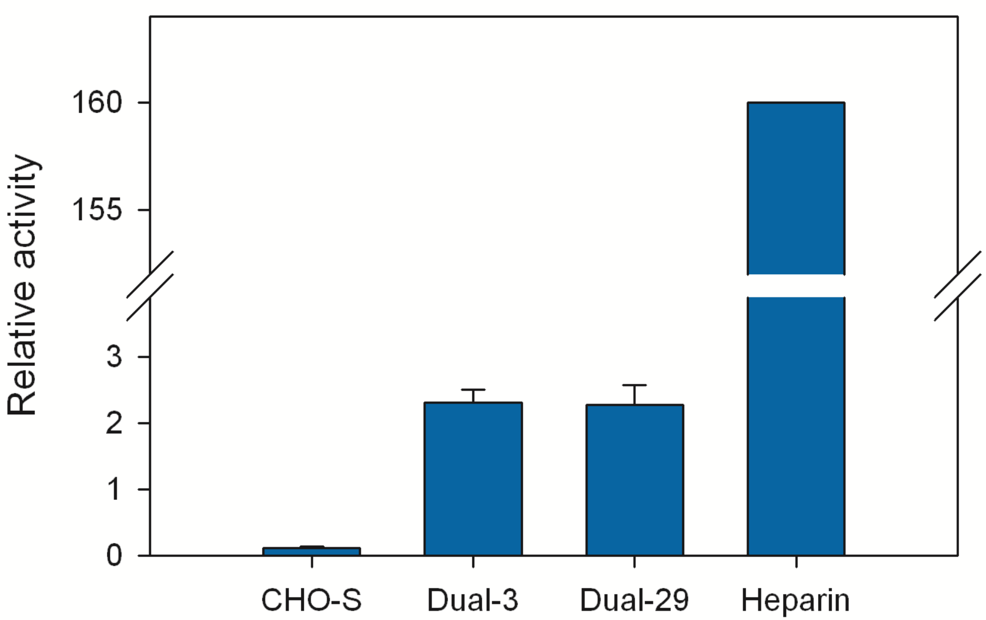


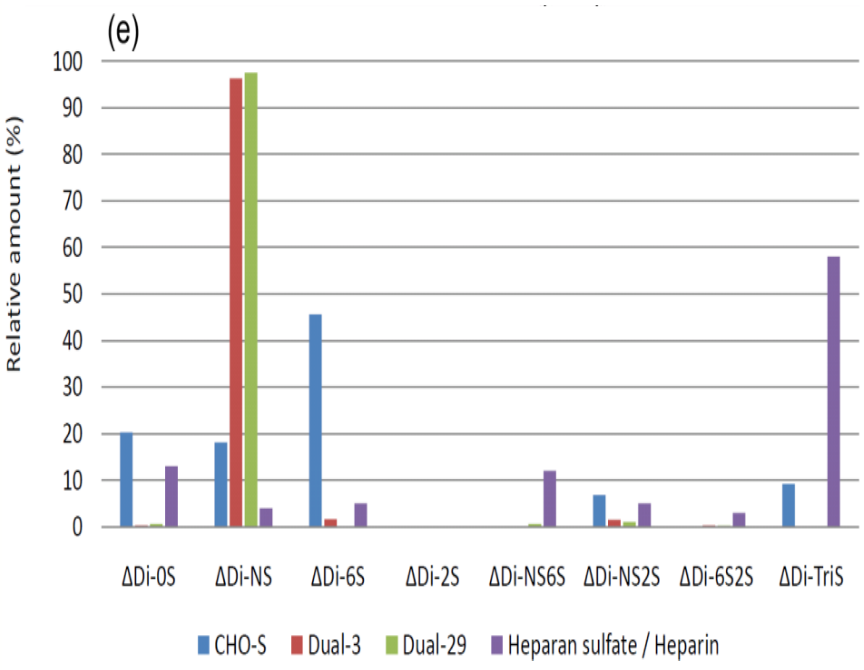
Antithrombin Pentasaccharide Binding Site

Anti-coagulant activity is increased, but not enough

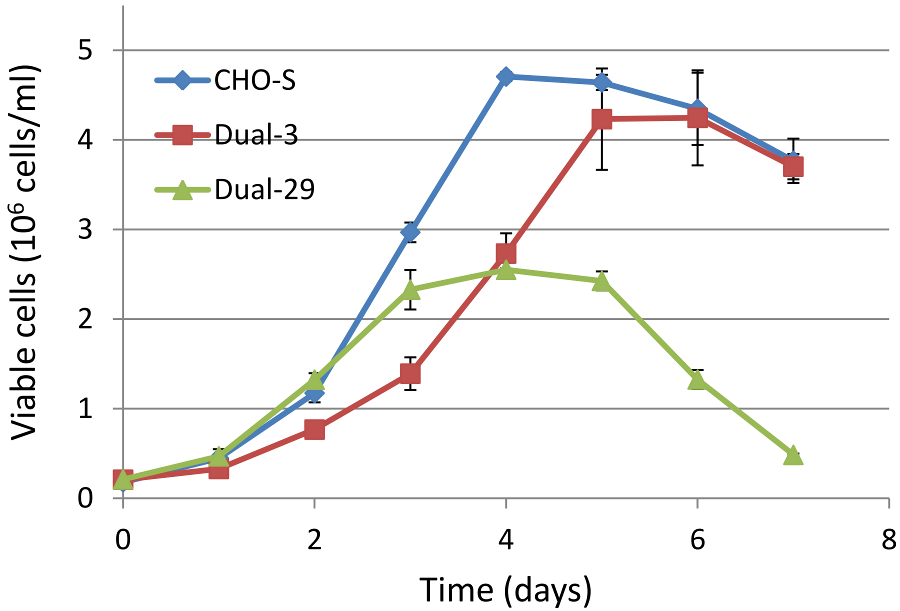
	CHO-S media	D3 media	D29 media
GAGs (μg)	19.3	85.2	173.2
HS/HP (μg)	**18.5**	**85.2**	173.2

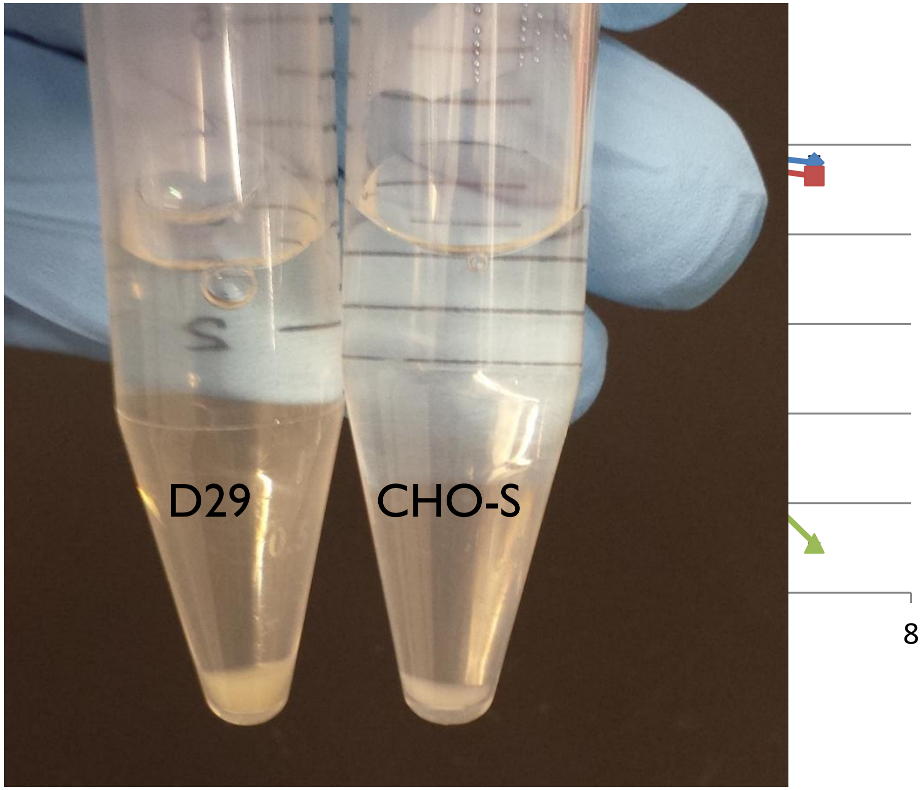
Baik et al. *Metabolic engineering of Chinese hamster ovary cells: Towards a bioengineered heparin*, Metabolic Engineering, 14: 81–90. (2012). doi.org/10.1016/j.ymben.2012.01.008





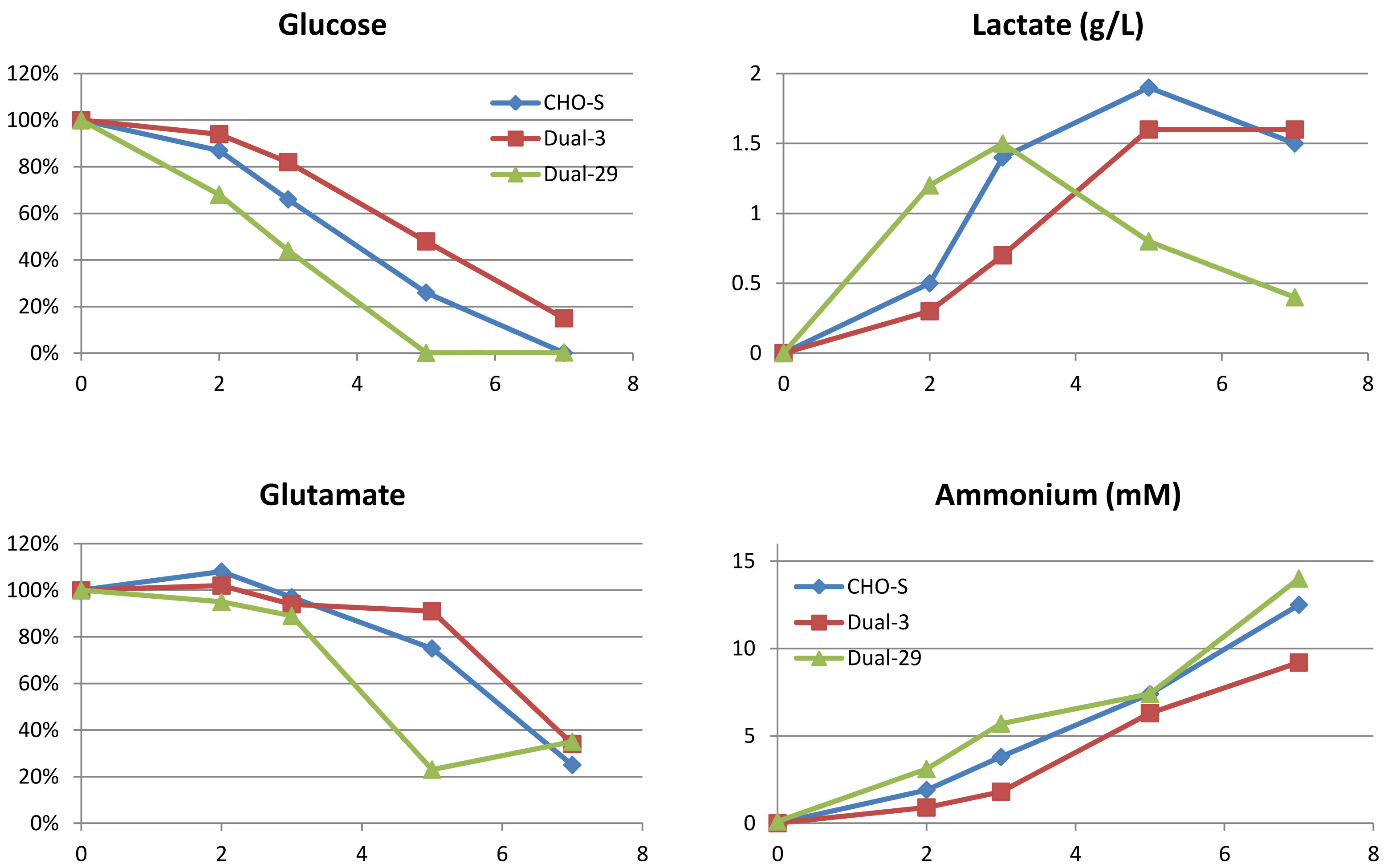
Can bioprocess manipulations improve productivity, activity, and structure?





Batch shake flask studies in CD-CHO supplemented with 8 mM Glutamax and HT

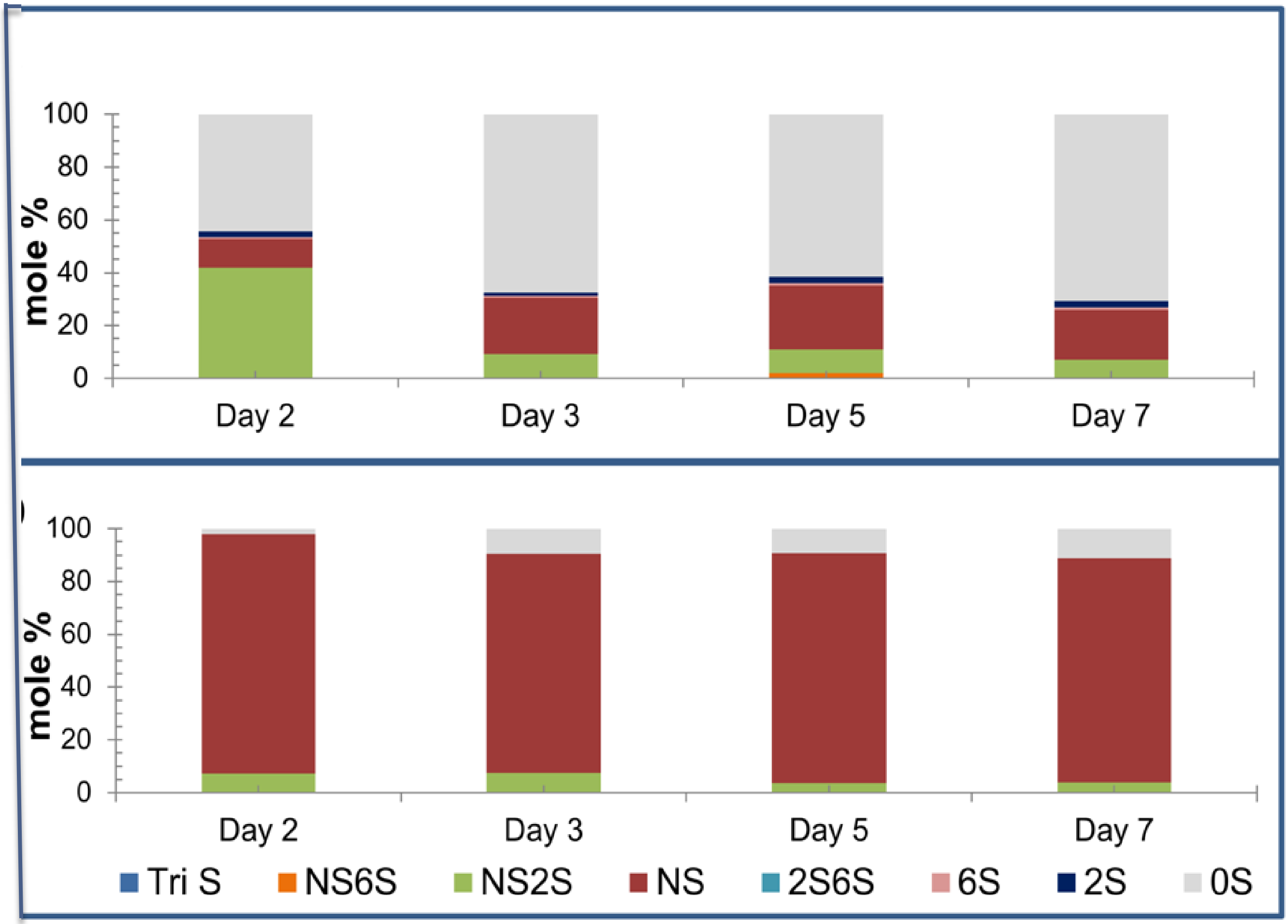
Glucose is rapidly depleted in D29 cultures



Sulfation levels decrease during batch culture

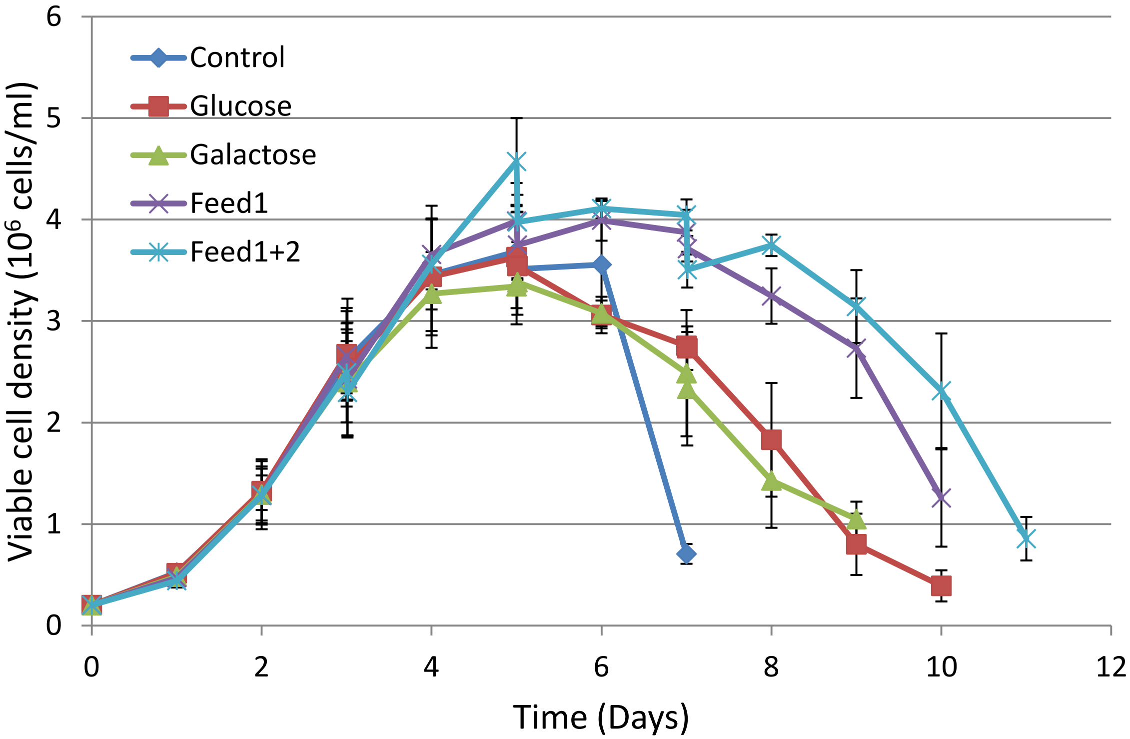
CHO-S®

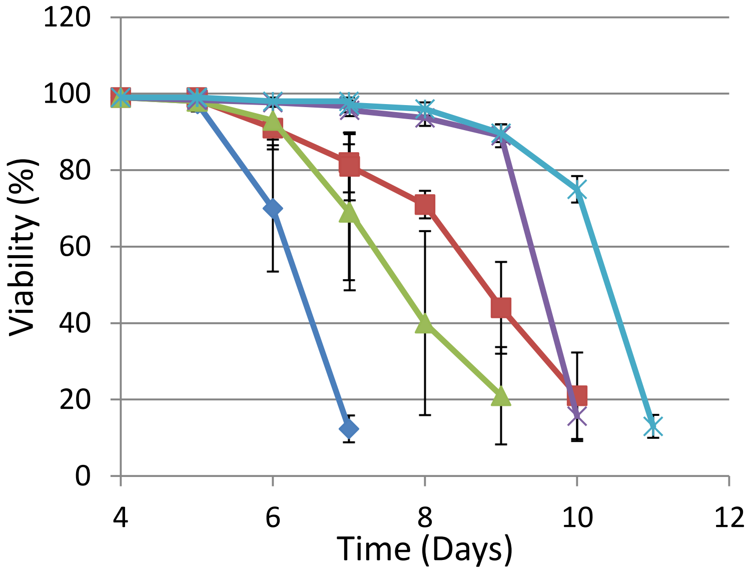
D29



Fed-batch experiments to improve IVCD and productivity

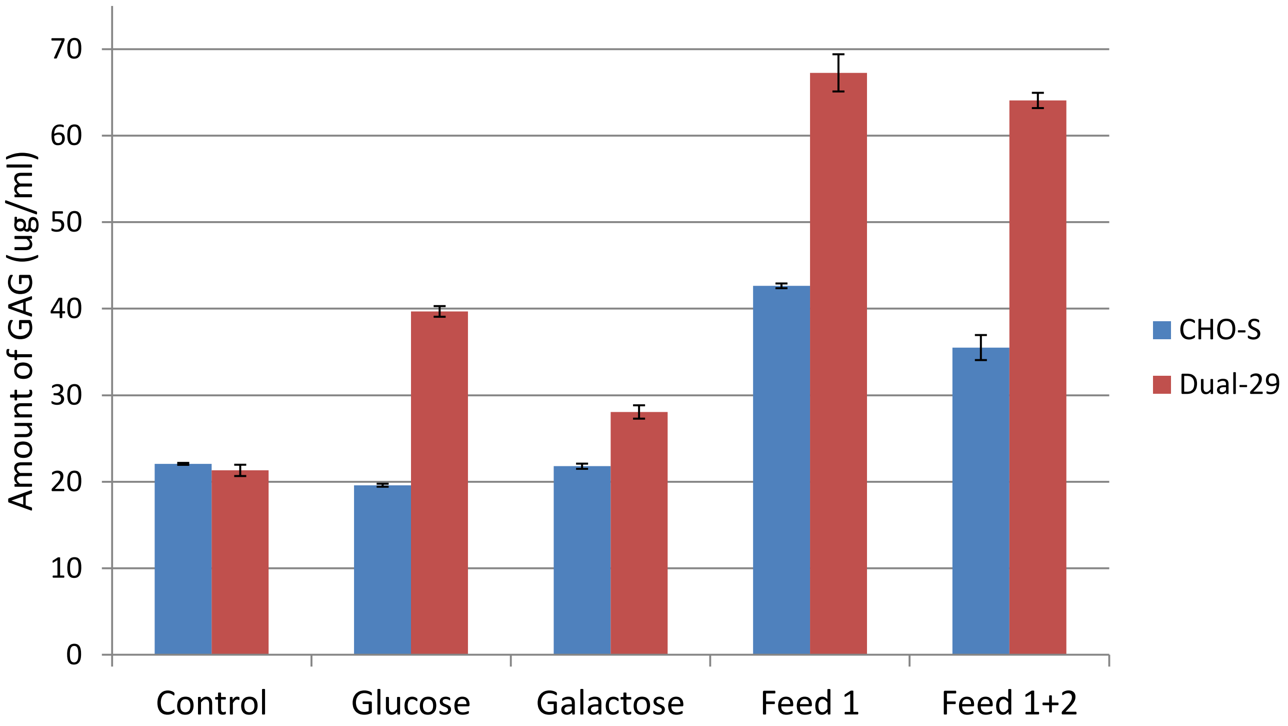
Growth profile of Dual-29 cell line



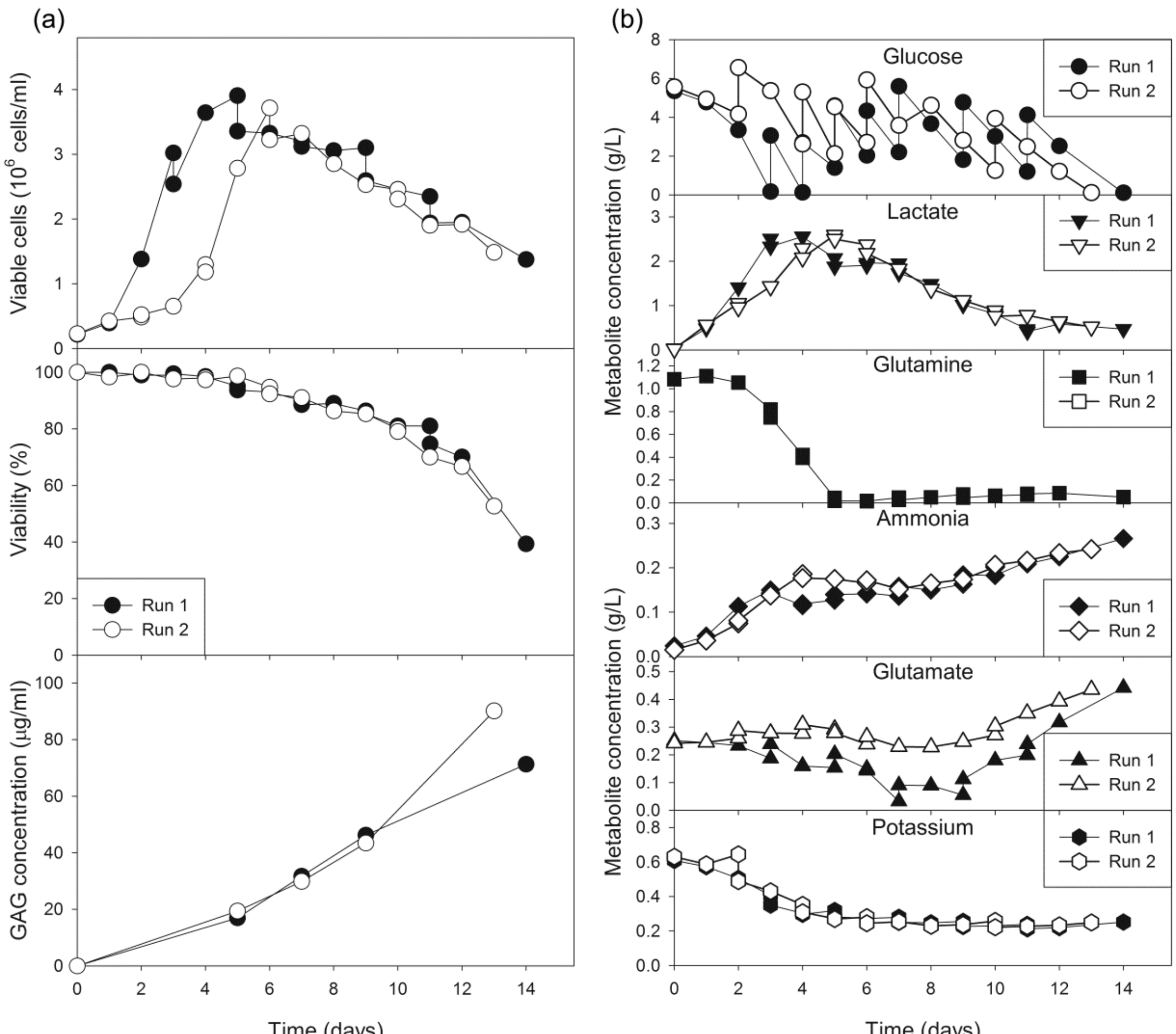


All feeds improved maximum cell density and IVCD. Feed 1(CD EfficientFeed™ B) and a combination of Feeds 1&2 (FunctionMax™ Titer Enhancer) increased maximum cell density and culture longevity

GAG production is increased upon feeding. D29 cells are most improved.



Fed-batch bioreactors show nearly 100 μg/mL



EfficientFeed™ B and glucose fed on alternate days

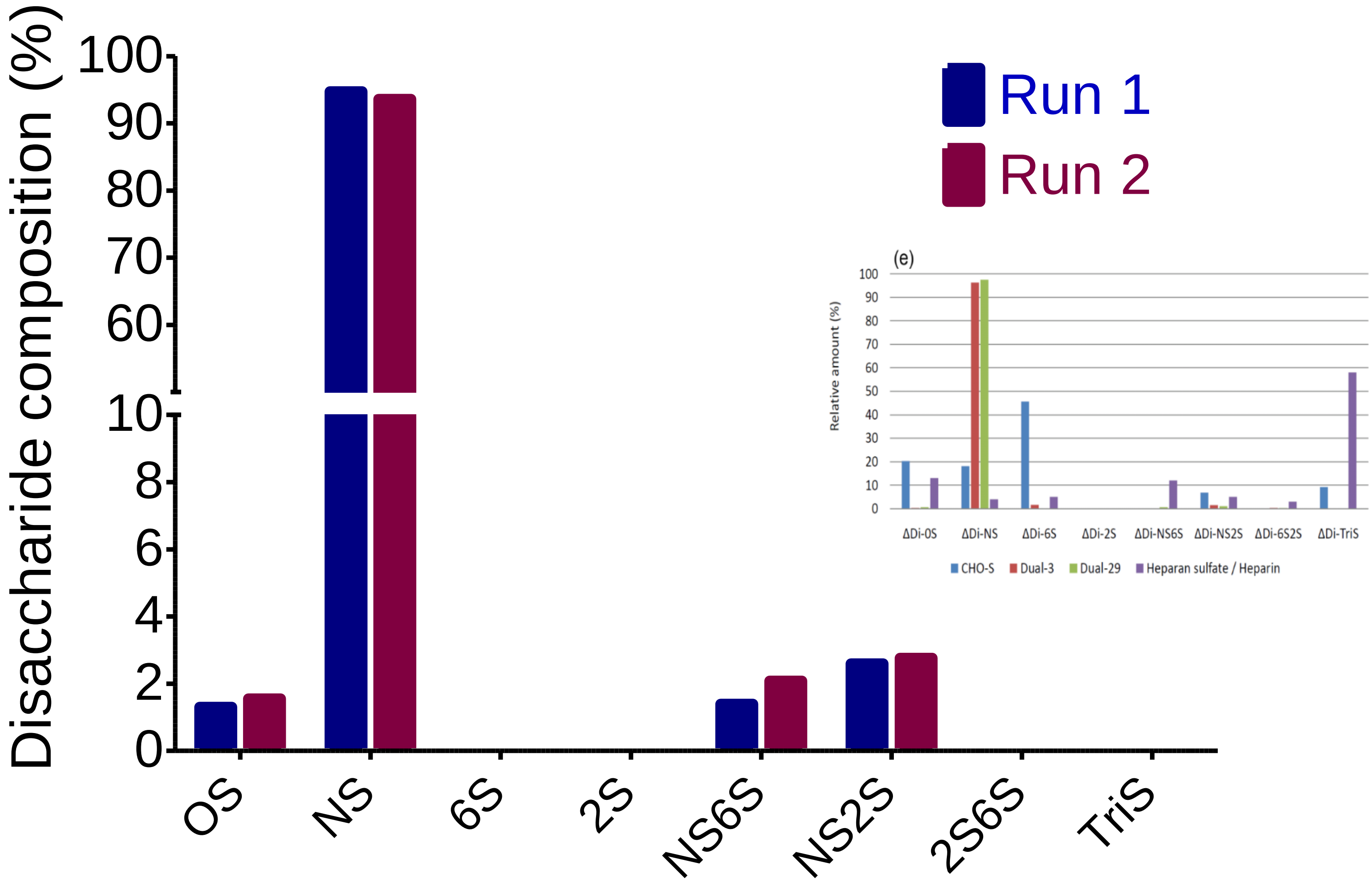
Cultures exhibit very high oxygen demand

Amino acid analysis shows cystine depletion

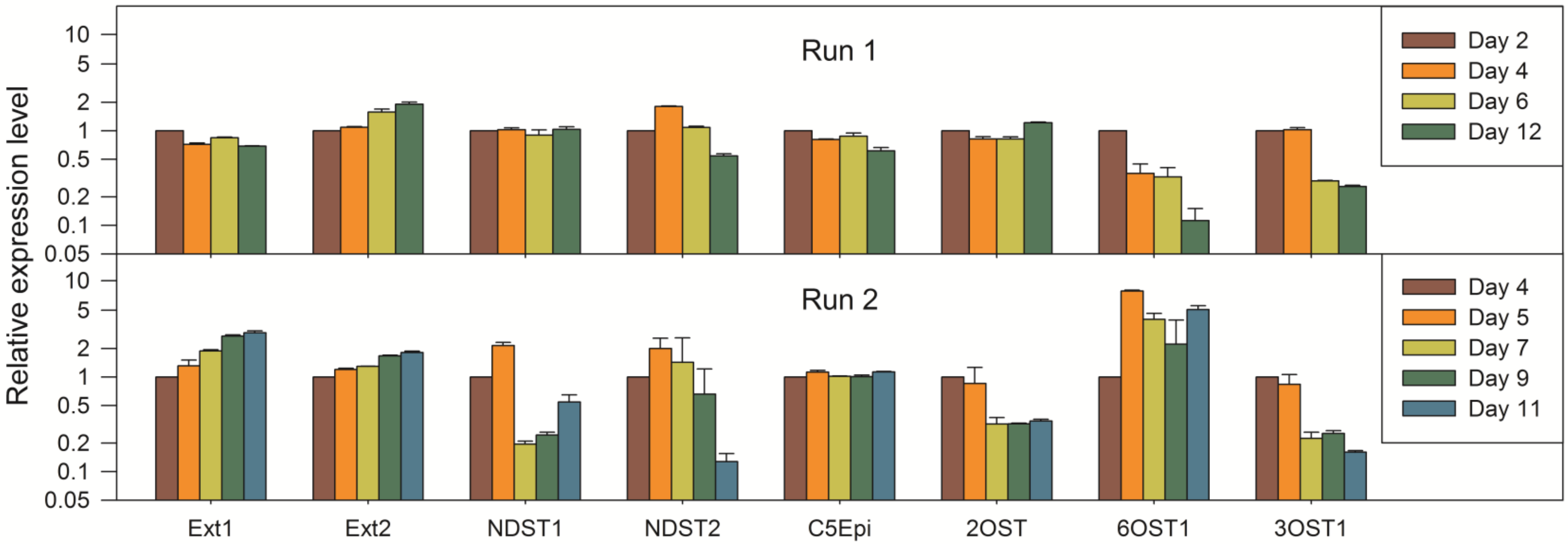
Run 1	Relative Concentrations					
COMPONENT	D0	D3	D5	D7	D9	D11
L-ALANINE	100%	3205%	11257%	11107%	11834%	7927%
L-ARGININE	100%	59%	72%	47%	55%	86%
L-ASPARAGINE	100%	8%	ND	ND	ND	ND
L-ASPARTIC ACID	100%	88%	8%	27%	NQ	36%
L- CYSTINE	100%	NQ	ND	ND	ND	ND
ETHANOLAMINE	100%	NQ	34%	NQ	16%	61%
L-GLUTAMINE (mM)	-	NQ	NQ	NQ	NQ	0.5
L-GLUTAMIC ACID	100%	74%	5%	13%	3%	17%
GLYCINE (mM)	NQ	0.9	3.3	2.0	2.6	3.7
L-HISTIDINE	100%	68%	86%	63%	71%	99%
HYDROXY-L-PROLINE	100%	99%	130%	101%	115%	139%
L-ISOLEUCINE	100%	64%	43%	42%	37%	53%
L-LEUCINE	100%	60%	25%	35%	23%	32%
L-LYSINE	100%	60%	78%	50%	59%	95%
L-METHIONINE	100%	53%	96%	53%	69%	120%
AMMONIA	0.1	4.8	6.3	4.2	4.5	9.7
L-PHENYLALANINE	100%	56%	62%	45%	48%	78%
L-PROLINE	100%	79%	100%	76%	86%	108%
L-SERINE	100%	38%	21%	18%	17%	26%
L-THREONINE	100%	73%	86%	65%	72%	97%
L-TRYPTOPHAN	100%	79%	111%	79%	90%	131%
L-TYROSINE	100%	54%	23%	30%	21%	29%
L-VALINE	100%	64%	47%	48%	43%	55%
B-12	100%	80%	1282%	371%	799%	1341%
FOLIC ACID	100%	98%	459%	190%	350%	583%
NIACINAMIDE	100%	53%	744%	266%	477%	1001%
RIBOFLAVIN	100%	32%	60%	38%	57%	72%
THIAMINE	100%	61%	31%	NQ	20%	70%

Run 2	Relative Concentrations					
COMPONENT	D0	D2	D4	D6	D10	
L-ALANINE	100%	1586%	6620%	10993%	10318%	
L-ARGININE	100%	97%	79%	65%	92%	
L-ASPARAGINE	100%	73%	15%	2%	ND	
L-ASPARTIC ACID	100%	118%	150%	90%	57%	
L- CYSTINE	100%	49%	NQ	ND	ND	
ETHANOLAMINE	100%	63%	NQ	9%	48%	
L-GLUTAMINE (mM)	-	NQ	NQ	NQ	NQ	
L-GLUTAMIC ACID	100%	95%	48%	20%	24%	
GLYCINE (mM)	NQ	0.9	1.5	2.5	3.7	
L-HISTIDINE	100%	98%	88%	81%	103%	
HYDROXY-L-PROLINE	100%	107%	112%	118%	135%	
L-ISOLEUCINE	100%	96%	87%	64%	68%	
L-LEUCINE	100%	96%	81%	52%	47%	
L-LYSINE	100%	97%	83%	68%	99%	
L-METHIONINE	100%	92%	85%	77%	122%	
AMMONIA (mM)	NQ	2.8	7.0	6.0	7.9	
L-PHENYLALANINE	100%	94%	76%	58%	83%	
L-PROLINE	100%	97%	95%	92%	109%	
L-SERINE	100%	80%	54%	23%	29%	
L-THREONINE	100%	101%	90%	81%	102%	
L-TRYPTOPHAN	100%	98%	105%	101%	130%	
L-TYROSINE	100%	92%	63%	33%	39%	
L-VALINE	100%	98%	83%	64%	70%	
B-12	100%	81%	531%	714%	1325%	
FOLIC ACID	100%	83%	265%	372%	496%	
NIACINAMIDE	100%	87%	342%	562%	1003%	
RIBOFLAVIN	100%	60%	57%	71%	86%	
THIAMINE	100%	108%	98%	60%	85%	

Disaccharide analysis shows little difference from shaker studies



qPCR shows variation in expression of endogenous and transgenes in heparin/heparan sulfate pathway



Summary

- We have successfully metabolically engineered CHO cells to produce a more heparin-like GAG
- Sulfation pattern does not match pharmaceutical heparin
- Engineered cells exhibit distinctly different metabolic behavior
  - Significant increases in glucose and oxygen uptake
  - Substantially larger cells
- Sulfur content in medium (as cysteine/cystine) appears to be insufficient

Fundamental Questions

- What is the relationship between enzymes activities and glycan structures?
- What is the relationship between culture conditions and media formulations and glycan structures?
- Can we rationally engineer cells and processes to produce the desired glycans?
- Can we use combinatorial approaches of knockin/knockout/process optimization to a produce desired glycans?
- What impact does this have on biosimilars?

Acknowledgements

- Professor Robert Linhardt
- Professor Fuming Zhang

GE Healthcare
National Institutes of Health
1R01GM090127
National Science Foundation
IIP-1321432
CBET-0083203

- Dr. Jong Hyun Nam
- Dr. Jong Youn Baik
- Dr. Leyla Gasimli
- Dr. Payel Datta
- Dr. Bo Yang
- Dr. Trent Gemmill
- Dr. Hussain Dahodwala