GLYCOBIOLOGY GROWTH IN THE BIOANALYTICAL SPACE:

THE DEVELOPMENT OF AN LC-MS ASSAY FOR QUANTITATION OF GLYCOSAMINOGLYCANS

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GLYCOBIOLOGY

What is it and why is it important?

- Glycobiology = study of the structure, biosynthesis and biological functions of carbohydrates

- Simple Sugar Monosaccaharides
  → glycans and glycosaminoglycans (GAGs)

  Diverse Structures = Diverse Functions

Image: Examples of glycans and GAGs

- Participate in human disease

- Unique therapeutic opportunities

- Glycobiology market predicted to double in next five years from $1.8 to $3.7 billion

GLYCOSAMINOGLYCANS (GAGS) ASSAY REQUEST

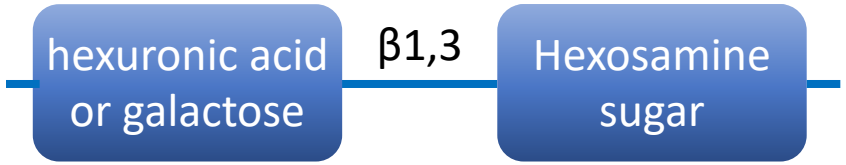
Aim:

- **Develop an assay to monitor GAG levels in rat spinal cord tissue**
    - Therapeutic drug designed to impact GAG levels
    - Assay required to support early-stage discovery studies

GLYCOSAMINOGLYCANS (GAGS)

Structure and Function

- Long linear polysaccharides
- repeating disaccharide units



- Chondroitin sulfate (CS) is involved in cartilage health and related pathologies.

Image (5 classes of GAGs)

hyaluronic acid (HA)

dermatan sulphate (DS

chondroitin sulphate (CS)

heparin sulphate (HS)

keratan sulphate (KS)

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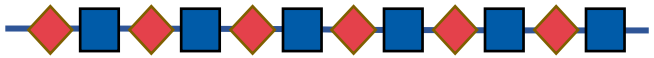
APPROACHES TO MEASURE GAGS

- Conventional approaches based on binding assays:
    - Dimethylmethylene blue dye ,fluorescence-based assays and ELISA.
    - Can be limited by sensitivity or selectivity
- Mass spectrometry approaches:
    - High sensitivity and specificity
    - Selective detection of specific GAG classes
    - Can distinguish between sulphated and non-sulphated GAG forms

MASS SPEC OF GAGS

Top Down



M.W.
~10k–100kDa

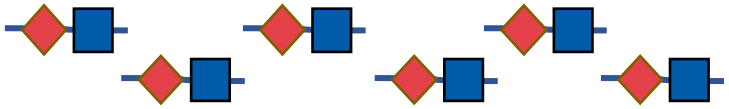
Glycosaminoglycan chain

Mass Spec

depolymerisation

Bottom Up

M.W.
400-700 Da



disaccharides

Mass Spec

Measure specific surrogate dissacahrides to represent total GAGs

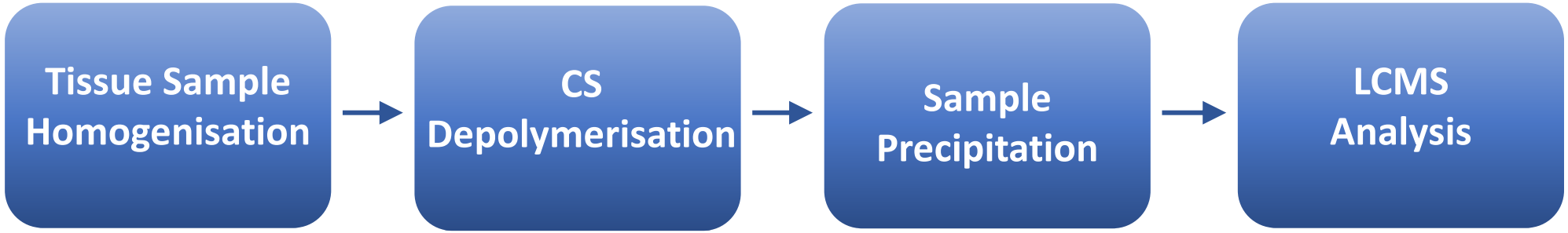
ASSAY AIM

Aim:

- **Develop an assay to monitor GAG levels in rat spinal cord tissue**

- **Develop an LC-MS assay for the indirect quantification of Chondroitin Sulphate - using a surrogate disaccharide approach in rat spinal cord tissue**

ASSAY APPROACH



Assay Considerations and Challenges:

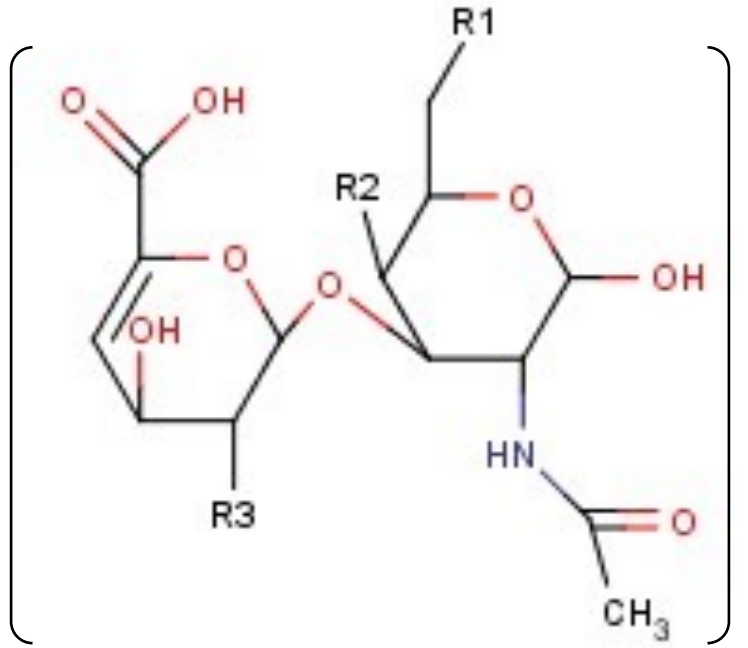
1. Selection of suitable surrogate disaccharides and proxy matrix
2. Specific degradation of CS GAGs
3. Development of selective LCMS conditions for analysis

SELECTION OF SURROGATE DISACCHARIDES TO REPRESENT CS

Sulphation State			
Non	Mono	Di	Tri
Δ0S-CS	Δ2S-CS	Δ2S,4S-CS	ΔtriS-CS
	Δ4S-CS	Δ2S,6S-CS	
	Δ6S-CS	Δ4S,6S-CS	

- Calibration standards/QCs prepared
  - with pure disaccharides
  - proxy homogenate
  - additional QCs in rat spinal cord homogenate matrix
  - Internal Standard – synthetic disaccharide

GlcA **GalNAc**



R = H or SO_3^-

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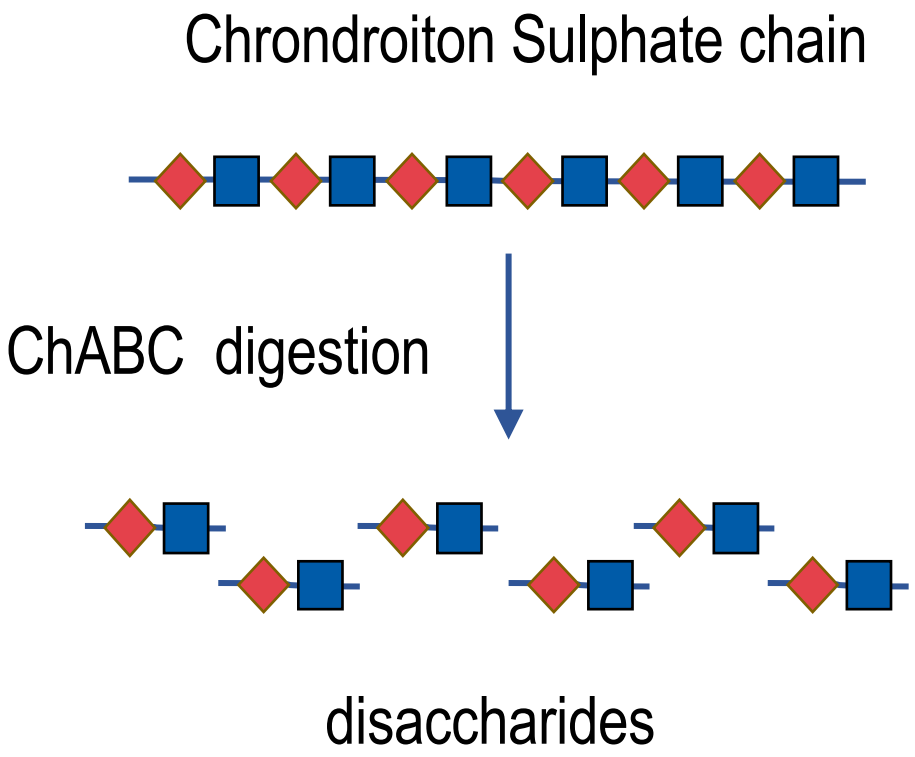
SPECIFIC DEGRADATION OF CHONDROITIN SULPHATE



- Chondriotinase ABC (ChABC) cleaves CS into its 8 different disaccharide's

- ChABC also cleaves:
  - DS - no impact on assay specificity
  - HA - additional selectivity challenge of a disaccharide isomeric to CS

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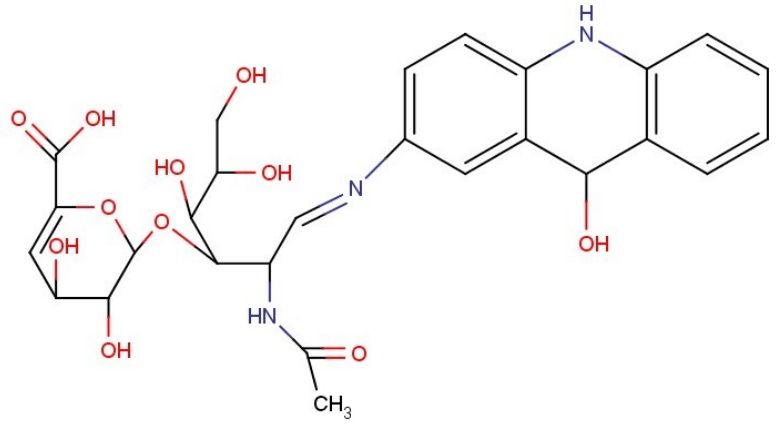
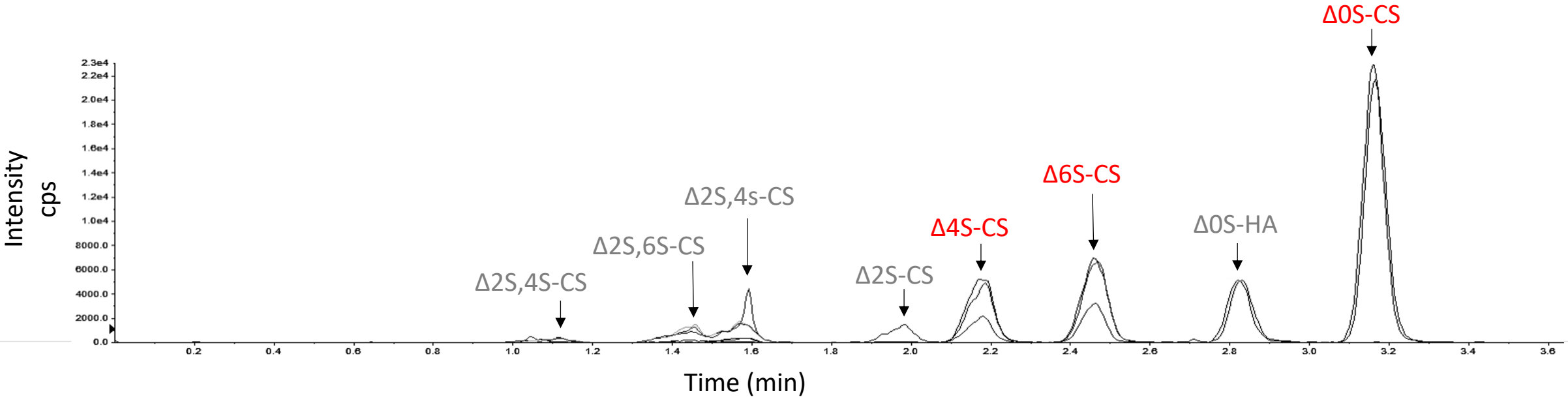
DEVELOPMENT OF SELECTIVE LC-MS

1. Disaccharides are highly polar – challenging to retain on LC
   → derivatisation required

2. Multiple and isomeric disaccharides present
   = assay selectivity challenge





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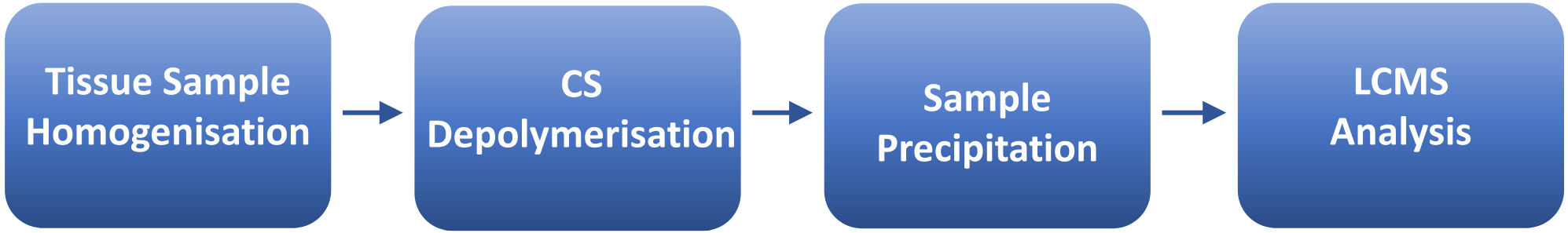
ASSAY APPROACH



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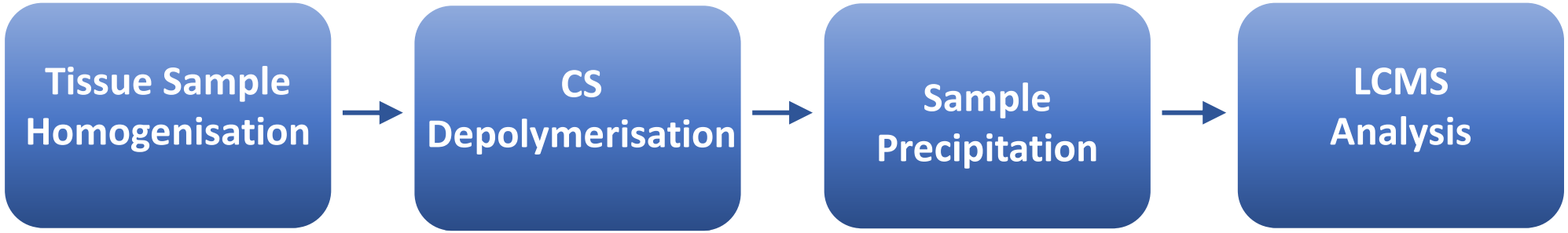
2. Selective degradation of CS GAGs

3. Development of selective LCMS conditions for analysis

FINAL ASSAY APPROACH



Assay Considerations and Challenges:

1. Selection of suitable surrogate disaccharides and proxy matrix

   → Three surrogate disaccharides, proxy homogenate selected

2. Selective degradation of CS GAGs

   → ChABC digestion

3. Development of selective LCMS conditions for analysis

   → Dervisitation and chromatographic separation

ASSAY PERFORMANCE

- Acceptable linearity, accuracy and precision
  - Δ0S-CS $1/X^2$ r=0.9975
  - Δ4S-CS $1/X^2$ r=0.9925
  - Δ6S-CS $1/X^2$ r=0.9963
- fit-for-purpose acceptance criteria of 30%.
- Carryover acceptable
- Analyte selectivity acceptable

		Proxy QCs				Spinal Cord QCs	
		LQC	MQC	Mid-HQC	HQC	Mid-HQC	HQC
ΔCS-0S	**Nominal**	0.120	3.60	12.0	32.0	16.2	36.2
	Measured	0.128	3.46	11.6	31.1	13.5	27.4
	RE%	6.7	-3.9	-3.3	-2.8	-16.6	-24.3
	CV%	9.3	5.0	3.7	4.7	10.8	6.5
ΔCS-4S	**Nominal**	0.480	14.4	48.0	128	90.4	170
	Measured	0.499	13.0	45.4	126	83.2	190
	RE%	4.0	-9.7	-5.4	-1.6	-7.9	11.5
	CV%	12.0	5.0	4.6	11.4	6.7	5.3
ΔCS-6S	**Nominal**	0.360	10.8	36.0	96.0	43.8	104
	Measured	0.350	10.5	35.2	94.0	37.9	84.7
	RE%	-2.8	-2.8	-2.2	-2.1	-13.5	-18.4
	CV%	6.6	5.6	3.1	6.3	9.7	6.0

ASSAY PERFORMANCE

- Initial assay range = 3 order of magnitude

  → LLOQ was raised 2-fold to maintain linearity of Δ4S-CS

Analyte	Range µg/g	New Range µg/g
Δ0S-CS	0.0400-40.0	0.0800-40.0
Δ4S-CS	0.160-160	0.320-160
Δ6S-CS	0.120-120	0.240-120

ASSAY PERFORMANCE

- Initial assay range = 3 orders of magnitude

  → LLOQ was raised 2-fold to maintain linearity of Δ4S-CS

- Sensitivity and ranges allow sponsor to monitor 10-fold reduction in endogenous CS concentration

Analyte	Range µg/g	New Range µg/g	Endogenous Conc µg/g
Δ0S-CS	0.0400-40.0	0.0800-40.0	3.28
Δ4S-CS	0.160-160	0.320-160	37.1
Δ6S-CS	0.120-120	0.240-120	4.29

SUMMARY AND CONCLUSIONS

- Glycobiology important in fundamental aspects of cell biology

  → Huge potential in the future drug discovery market

- At Charles River we are now seeing increased requests for assays with a glycobiology focus.
  - More challenging than typical small molecules

- Successfully developed an LCMS assay for quantification of chondroitin sulphate
  - Currently being employed for sample analysis

ACKNOWLEDGEMENTS

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