Group Epitope Mapping by Saturation Transfer Difference NMR To Identify Segments of a Ligand in Direct Contact with a Protein Receptor

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Aim

- Confirmation that GEM can be investigated by STD NMR studies
  - GEM for β-GalOMe
  - GEM for NA_2
  - Determining the effect of ligand excess on STD NMR
  - Determination of competitive binding

Group Epitope Mapping (GEM)

- Relevant for the understanding of binding systems:
  - Cellular recognition
  - Drug-receptor complexes
  - Signal transduction processes
  - Colloidal matrices

GEM by STD

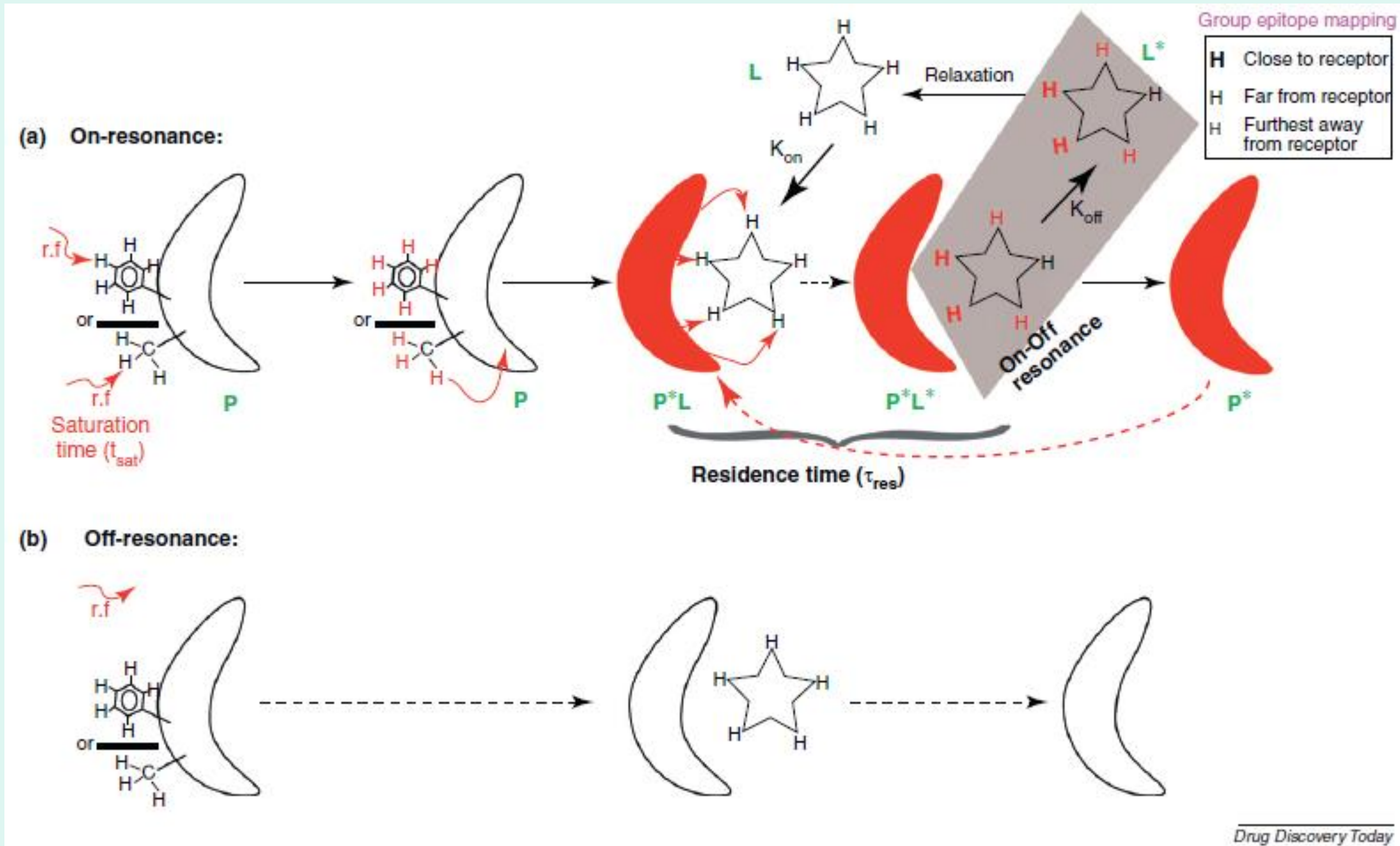


Fig. 1 The mechanism of STD experiments:

a) On-resonance, b) Off-resonance, grey) Difference spectrum

GEM is based on the proximity of ligand protons to the receptor core

GEM – Past and Present

- Historically achieved via X-Ray crystallography
- Before the widespread use of STD NMR
  - trNOE
  - SAR by NMR
  - These NMR techniques complement STD NMR

STD Advantages

- Direct identification of the binding component
- Identification from a mixture of compounds (K_D= 10^{-3}-10^{-8} M)
- Suitable for HTS
- Epitope mapping based on NMR signal Intensity.
- Highly sensitive, 1nmol limit for proteins >10KDa
- No isotopic labeling required (native states)
- Ligand choice relatively wide
  - Carbohydrates, peptides, glyopeptides, drug candidates
- Can be coupled with other spectroscopic techniques
  - TOCSY, HQSC, NOESY

Binding system studied

- Protein Receptor:
  - *Ricinus Communis* Agglutinin I, RCA_{120}

- Ligands:
  - Methyl β-D-galactopyranoside, β-GalOMe
  - Biantennary decasaccharide, NA_2

RCA_{120}

- *Ricinus Communis* Agglutinin I (RCA_{120})
  - Castor Bean
- Lectin family
  - Tetramer
  - 2 As-sB dimers
    - B-chain: lectin domain
- Affinity for terminal β-D-galactosyl residues
  - binding prevents attachment to carbohydrates in the cell membrane.

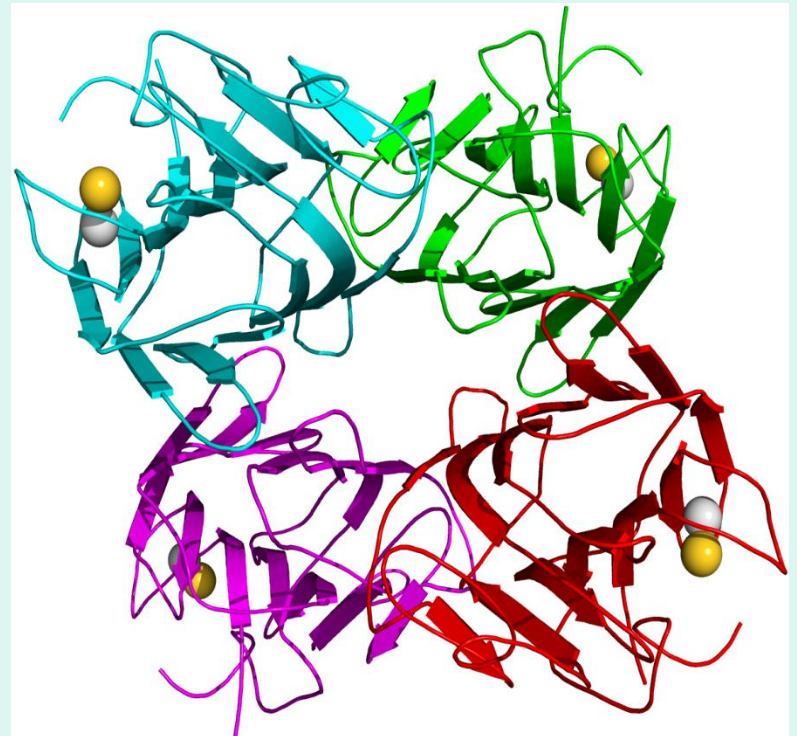
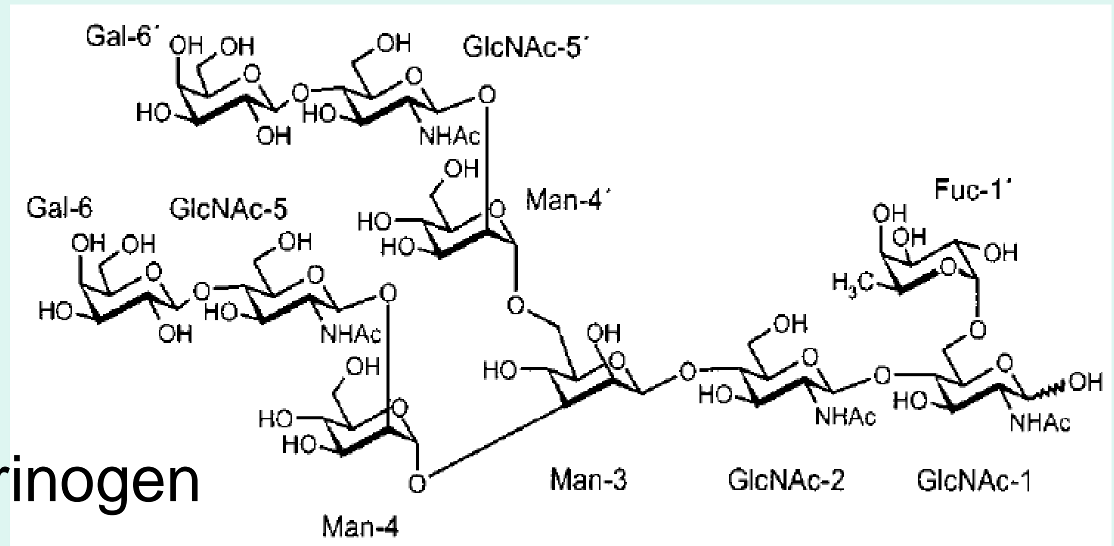


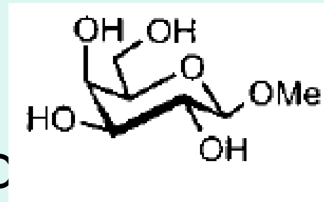
Fig. 2 A lectin, derived from Jack Bean

The ligands studied

- NA_2
  - 1836 Da
  - Isolated from fibrinogen



- β-GalOMe
  - 194 Da
  - Purchased, not isolated



Experimental conditions

- Measured on:
  - Bruker Avance DRX 500 MHz Spectrometer
- Sample preparation:
  - 500μL D_2O buffer
  - 20mM NaCl, 10mM Phosphate buffer pH 7, 0.04% NaN_3
- Protein conc.
  - Between 20 and 50 μM
  - (UV Abs 280nm, Beer-Lambert Law)
- Ligand added to Protein from stock solutions

STD pulse scheme

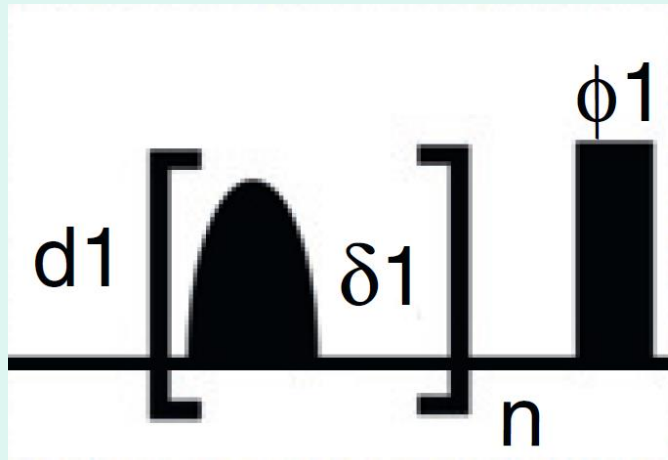


Fig. 3 Pulse sequence for a typical 1D STD NMR spectra recorded in D_2O

- Saturation period:
- selective pulse, 50 ms
  - (x 40)
  - Gaus
  - Strength 86Hz
- delay between pulses, 1ms
- saturation train, 2.04s
- subtraction (on and off-res) performed via phase cycling after every scan, δ
- On-res. irradiation, -0.4 ppm
- Off-res. irradiation, 30 ppm

STD pulse scheme

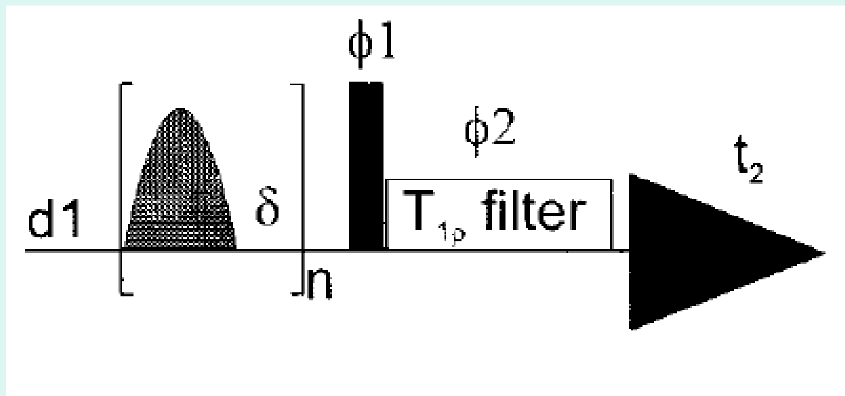


Fig. 4 Pulse sequence for the 1D STD NMR spectra recorded in D_2O with an additional $T_{1\rho}$ filter.

- Eliminating background Protein Resonances:
- $T_{1\rho}$ filter
  - 30-ms-spin-lock pulse
  - (after π/2 pulse)
  - Strength $(\gamma/2\pi)B_1$= 4960 Hz

Facilitates clearer analysis

- Reduces ligand STD signal intensity
- Solution: Ref. NMR spectra were also recorded with the same spin-lock pulse

STD pulse scheme

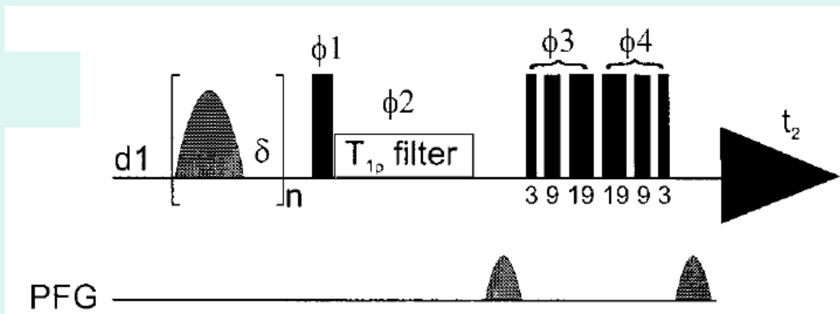
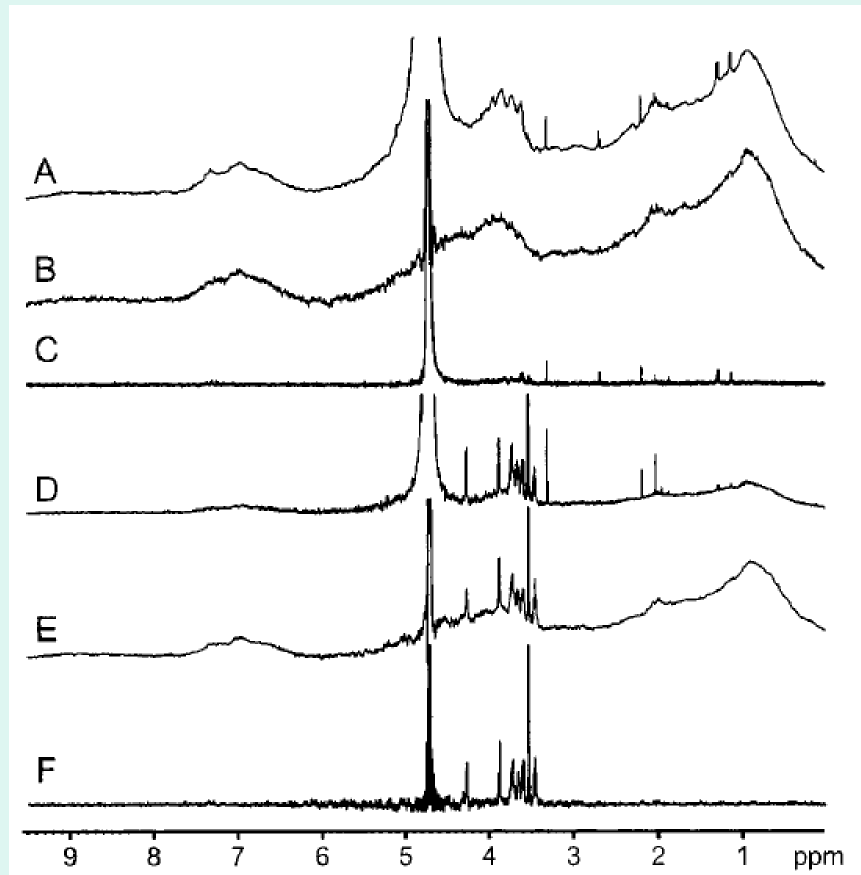


Fig. 5 Pulse sequence for the 1D STD NMR spectra recorded for H_2O samples with an additional $T_{1\rho}$ filter and WATERGATE.

- Suppression of residual HDO signal:
- WATERGATE
- Binomial 3-9-19 pulse sandwich
- 2 ms delay between pulses
- Strength $(\gamma/2\pi)B_1$= 6944 Hz

- Inverts all signals except the HDO signal at the carrier frequency

Steps involved in an STD NMR experiment



A. Ref 1D NMR of 120kDa RCA_{120} (50μM in binding sites)

B. Corresponding STD NMR spectrum

C. 1D NMR spectrum with a $T_{1\rho}$ filter

D. Ref 1D NMR of RCA_{120} and 1.2mM β-GalOMe without $T_{1\rho}$ filter

E. Corresponding STD NMR

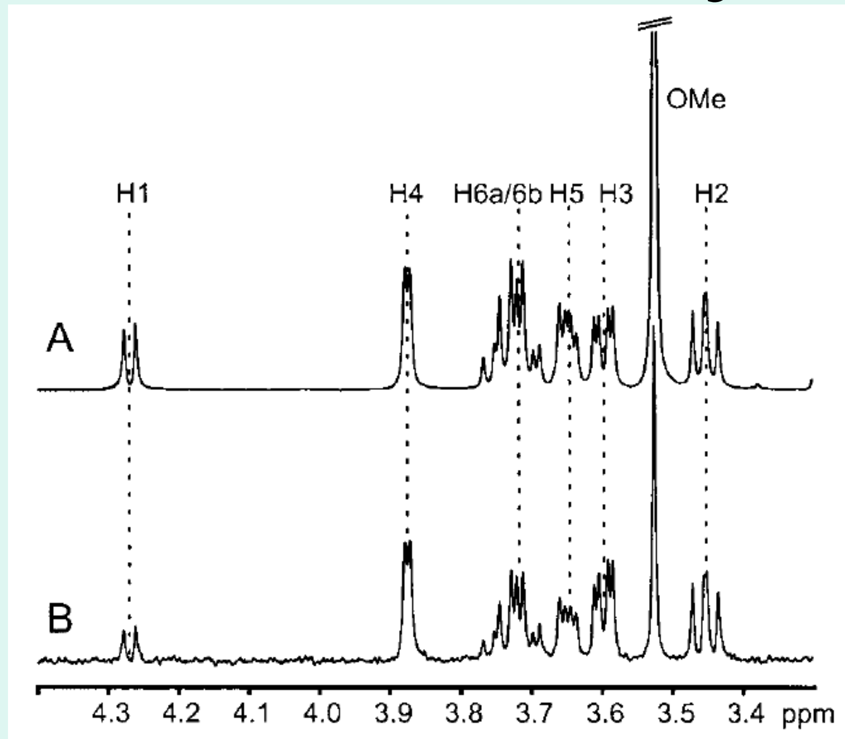
F. STD NMR spectrum as in E with $T_{1\rho}$ filter

Fig.6 Shows the steps involved in the STD NMR experiment.

Experiments:

- Analysis of β-GalOMe
  - GEM
  - Titration
  - Ligand excess
- Analysis of $NA_{2.}$
  - GEM
  - 1D STD Experiment
  - 2D STD TOCSY Experiment
  - Ligand excess
- Comparative analysis of NA_2 and β-GalOMe
  - Ligand excess
  - Competition Studies

GEM; Analysis of β-GalOMe



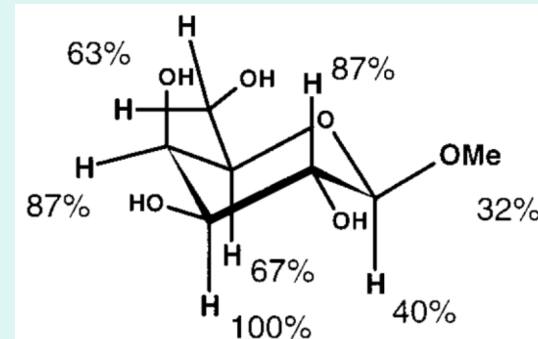


Fig. 7 **Left**: (A) Ref. WATERGATE NMR spectrum of a mixture of RCA120 (40 *μ*M binding sites) and β-GalOMe (4 mM) in a ratio of 1:100.
(B) WATERGATE STD NMR spectrum of the same sample. **Above Right**: Relative saturation of Protons in β-GalOMe

- STD spectrum proves binding
- Ligand protons nearest to the Protein identified
- Binding epitope characterised using relative integral STD signal intensities.
- H3 reference, set to 100%
- H2 and H4 87%
- H5, H6a, H6b approx. 63-67%
- H1 and OMe H, 40 and 32%

Titration; Analysis of β-GalOMe

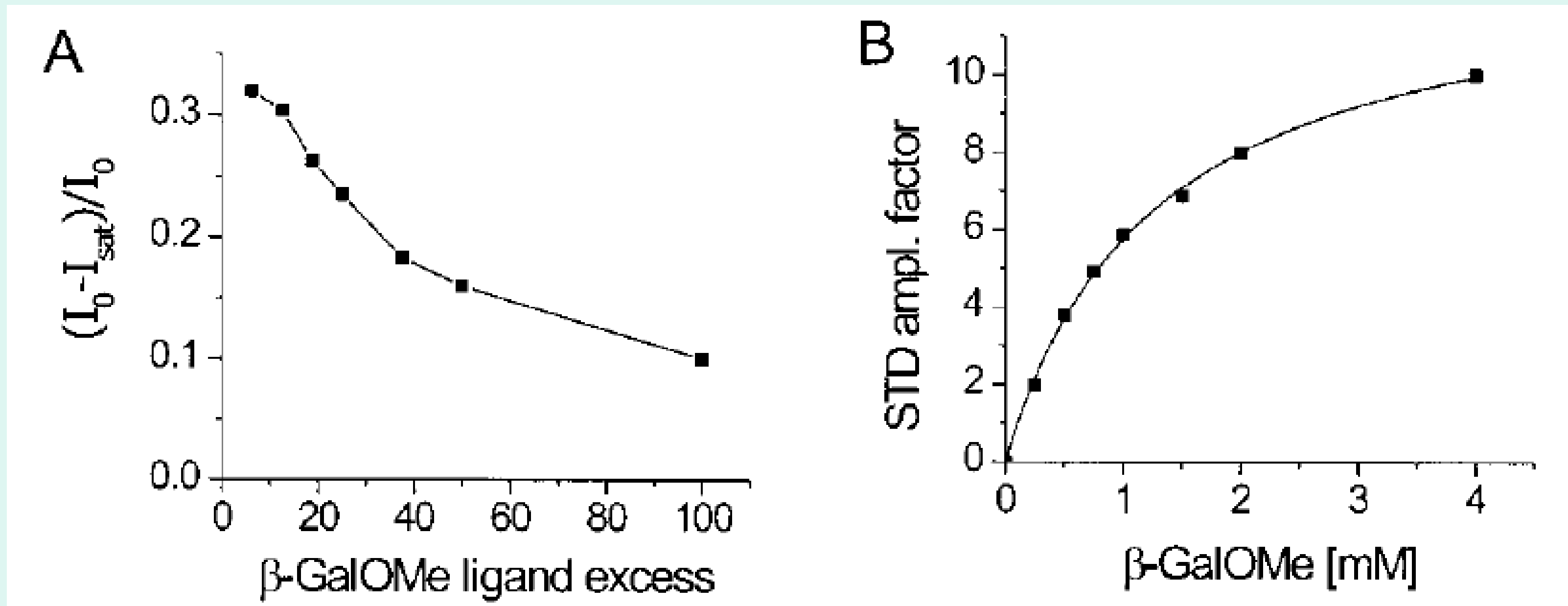


Fig. 8 (A) Diagram showing the fraction of the H4 signal of β-GalOMe which is saturated at a given ligand excess. The concentration of RCA120 was 40 *μ*M and the saturation time 2 s. (B) Display of the same data in terms of the STD amplification factor. This second plot shows that even though the fraction of ligand which is saturated decreases at a higher ligand excess, the absolute STD signal intensity increases in the form of a saturation curve.

STD effect :

$$\eta_{STD} = \frac{I_o - I_{sat}}{I_o} = \frac{I_{STD}}{I_o}$$

STD amplification factor :

$$A_{STD} = \frac{I_o - I_{sat}}{I_o} \times \text{ligand excess}$$

Ligand excess; Analysis of β-GalOMe

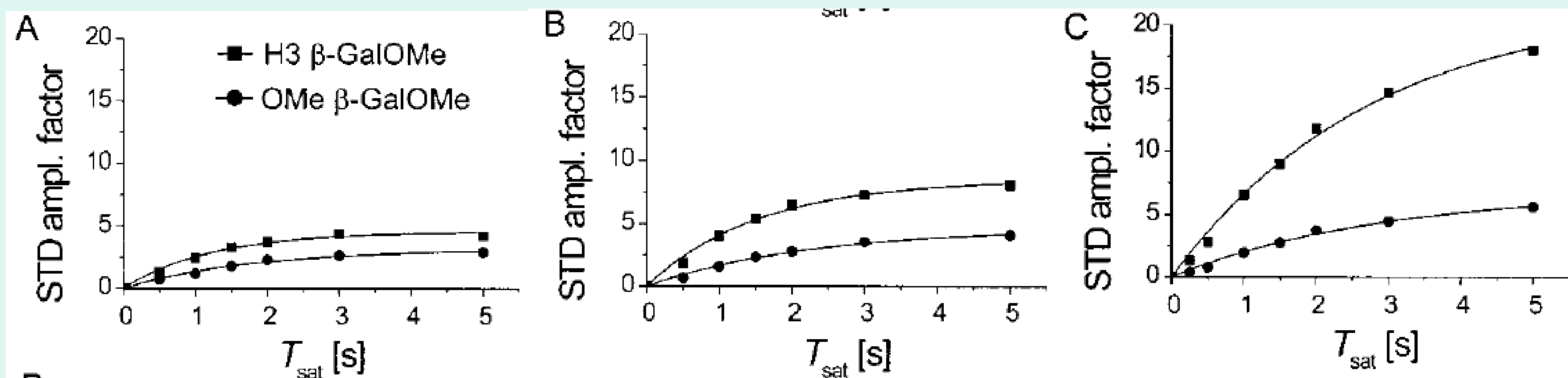
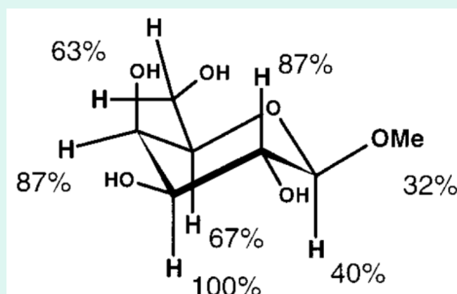
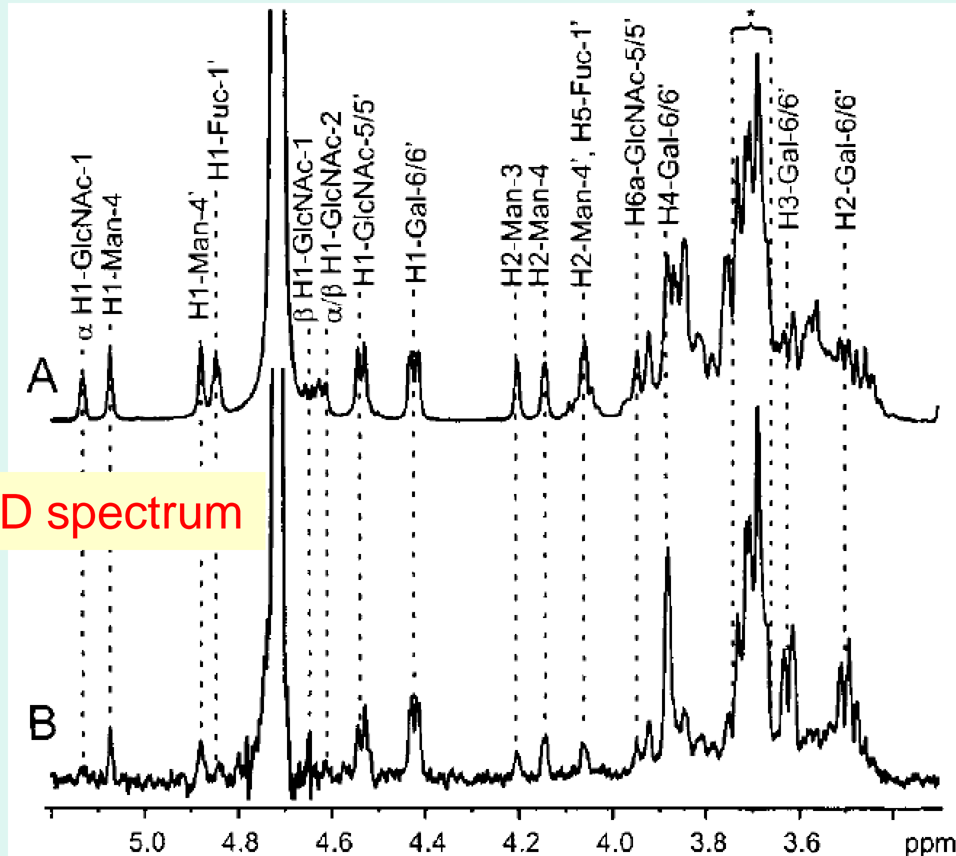


Fig. 9 Observed STD amplification factors of two resonances of β-GalOMe plotted against the saturation time *T*sat at three different ligand concentrations (9, H3 proton; b, OMe protons). STD amplification factors at concentrations of (A) 0.5 mM, (B) 1 mM, and (C) 4 mM β-GalOMe in the presence of 40 μM binding sites of RCA120. A large ligand excess yields larger STD intensities and better discrimination between strongly and weakly binding groups.



Relative saturation of Protons in β-GalOMe

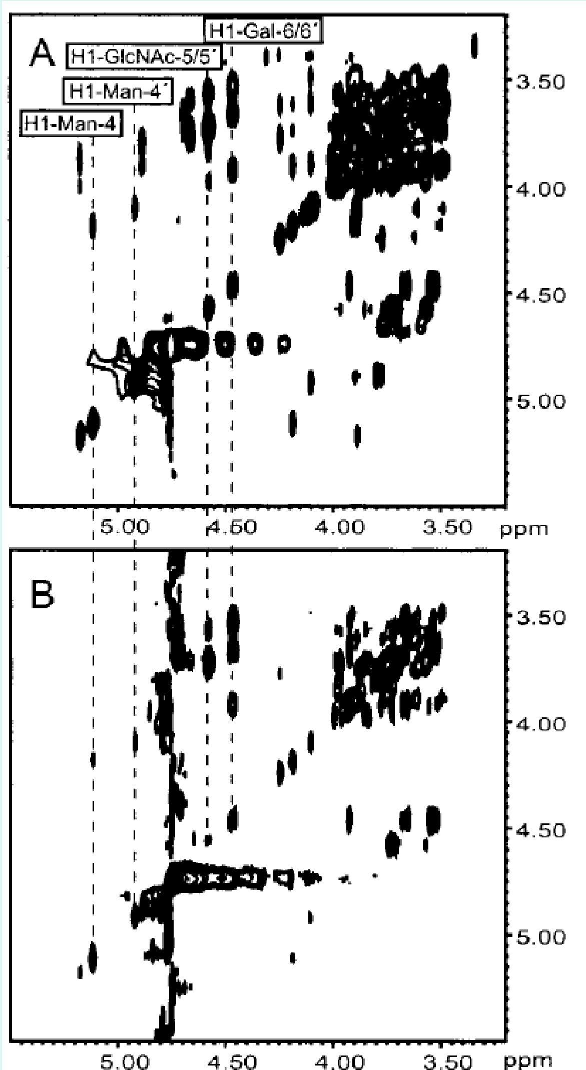
GEM, Analysis of $NA_{2.}$ 1D STD Experiment



- most intensive STD signals
  - Gal-6/6’
  - GlcNAc-5/5’
- The spectral region, 3.65 to 3.75 ppm strong STD signals
  - From H5 and H6a/6b of Gal 6/6’ and the H2, H3, and H4 of GlcNAc-5/5’ protons in equal parts
- almost no detectable STD signal int.
  - H1-Fuc-1’ and R-H1-GlcNAc-1

Fig. 10 (A) Section of a reference NMR spectrum of a mixture of RCA120 (50μM binding sites) and NA2 (0.55 mM) in a ratio of 1:11. (B) STD NMR spectrum revealing that the directly interacting residues of NA2 have the strongest signals.

GEM, Analysis of $NA_{2.}$ 2D STD TOCSY Experiment



- STD spectrum B:
- strong traces corresponding to:
  - H1-Gal-6/6’ and H1-GlcNAc-5/5’
- reduced intensities:
  - H1-Man-4 and H1-Man-4’
  - due to futher distance to the binding site of RCA120.
- cross-peaks absent:
  - GlcNAc-1/2 and Fuc-1’
  - proving that they have no interaction with the protein.

Fig. 11 (A) Reference or off-resonance TOCSY spectrum of NA2. (B) STD TOCSY spectrum obtained by subtraction of an on-resonance TOCSY spectrum from spectrum A.

Ligand excess; Analysis of $NA_{2.}$

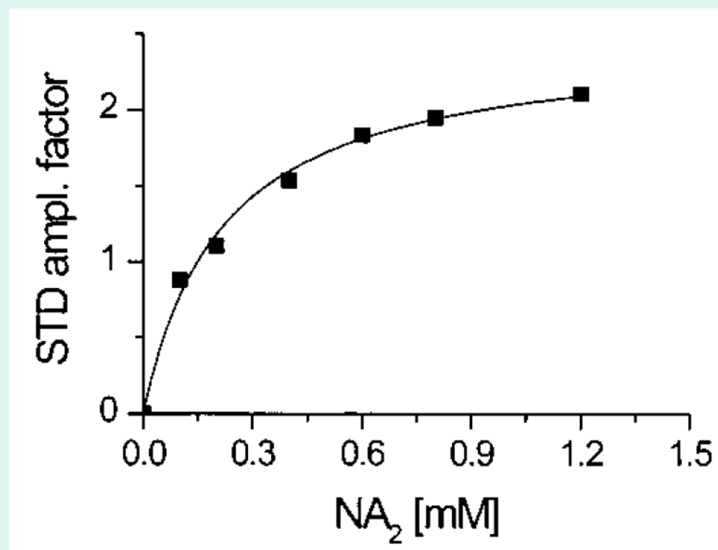


Fig. 12 Titration plot of NA2 to NMR sample containing RCA120 (20 M in binding sites), monitoring the increase of the STD amplification factor of the H4-Gal-6/6’ proton versus the ligand concentration (*T*sat) 2 s).

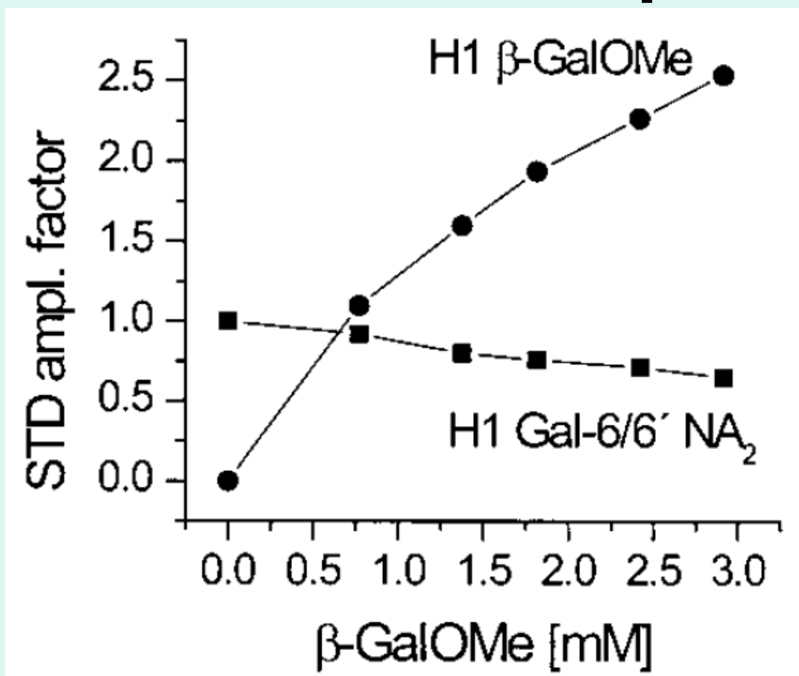
Ligand excess; Comparative analysis of NA_2 and β-GalOMe

Table 1. STD Signal Intensity of NA2 and β-GalOMe at a Ligand excess of 12.5- and 100-Fold, Respectively

	STD signal (%)		
NA_2 (β-GalOMe) resonance	11-fold excess NA_2	12.5-fold excess β-GalOMe	100-fold excess β-GalOMe
H4-Gal-6/6′ [b] (H4-β-GalOMe)	~100	90	87
H3-Gal-6/6′ [b] (H3-β-GalOMe)	~100	100	100
H2-Gal-6/6′ [b] (H2-β-GalOMe)	~100	95	87
H1-Gal-6/6′ (H1-β-GalOMe)	70	61	40
H5-Gal-6/6′ [b] (H5-β-GalOMe)	~80	74	67
H6a/6b-Gal-6/6′ [b] (H6a/6b-β-GalOMe)	~80	72	63
H2, H3, H4-GlcNAc-5/5′ [b]	~70		
H1-GlcNAc-5/5′	52		
H2-Man-4	44		
H2-Man-4′	42		
H1-Man-4	40		
H1-Man-4′	33		
H2-Man-3	28		
NHAc-5/5	37		
NHAc-2	17		
H6-Fuc-1′	2		

- Ligand excess on STD effects:
- Differentiation between direct and indirect contact could have been more pronounced at a higher excess for NA_2
- H1- β-GalOMe
  - 61% at 12.5 fold excess
  - 40% at 100% fold excess

Competition Studies



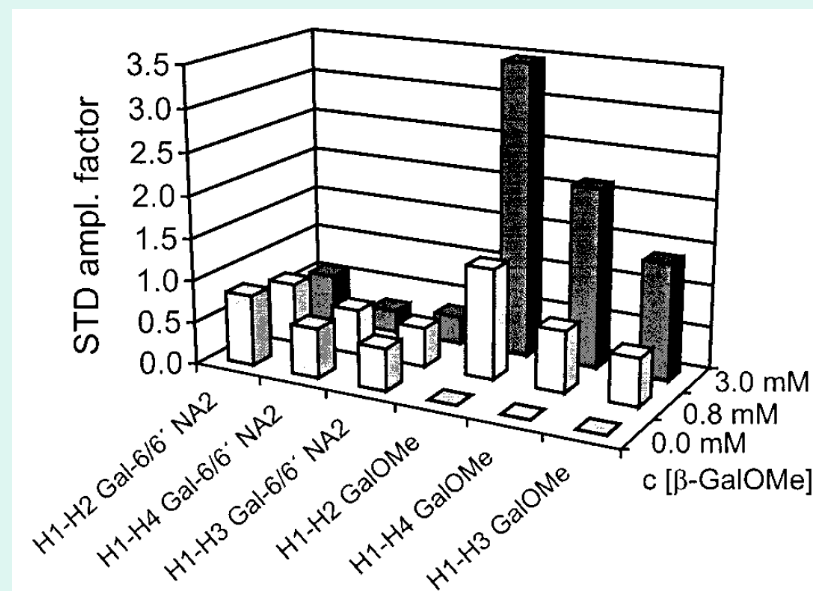


Fig. 12 **Left**:Diagram showing the STD amplification factors H1 β-GalOMe; H1 Gal-6/6‘ NA_2) determined from STD spectra on titration of β -GalOMe to a sample of RCA120 (50 *μ*M in binding sites) and NA2 (0.55 mM). The STD amplification factor of the signal corresponding to NA2 decreases from 1 to 0.66 with increasing concentration of β -GalOMe. This competition experiment gives evidence for the specificity of the RCA_{120} toward galactose-containing saccharides. The K_D of NA_2 can be calculated to be 27 *μ*M.

Right: The STD amplification factors of selected cross-peak intensities of NA2 and β-GalOMe determined from STD TOCSY spectra. The selected cross-peaks represent the F1 traces of the two H1 protons of the galactose residues. These values are consistent with those obtained from the 1D STD spectra therefore, even a few cross-peaks can be sufficient to perform titration experiments

Conclusions

- STD NMR spectroscopy:
  - analyzing binding processes
  - screening libraries
  - mapping of ligand epitopes

- The use of a high ligand excess is advantageous:
  - signal intensities are larger, making the STD experiment more sensitive
  - Differentiation between direct and indirect contact could have been more pronounced at a higher excess

- Determination binding epitope
  - integrals of the signals in 1D spectra
  - 2D cross-peak integrals

References

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Improvements and future outlooks

- Regarding cell-peptide interactions:
  - Second generation saturation transfer double difference (STDD)
    - Cell and membrane protein interactions can be studied
- Host-guest interactions:
  - Group selective STD (^{15}N GS STD NMR)
    - Reduces signal overlap
- Receptor-small molecule interactions:
  - Clean STD NMR
    - 3 x more sensitive