DNP: Practical aspects and applications in biomolecular applications

Joanna R. Long

DNP Workshop

August 27, 2025

NATIONAL MAGLAB

UF UNIVERSITY of FLORIDA

NATIONAL MAGLAB DNP User Program

600 MHz MAS DNP

- 90 – 300K range
- 3.2 mm/1.3 mm probes
- Triple resonance NMR
- >100x ^{13}C Enhancement
- User facility 2016

Applications:

Biosolids

Materials

Dissolution DNP

- 1 K, 5 T polarizer
- Interfaced with 7, 11.1, 14.1 T MRI/NMR systems
- >10^4x ^{13}C Enhancement
- User facility 2014

Applications:

Mechanistic studies

In vivo metabolic flux

600 MHz Overhauser DNP

- 293-373K range
- Organic/CO_2 solvent
- High resolution NMR
- Goal: 10-20x ^{1}H
- Development project

Applications:

Metabolomics

Natural products

NMR division
Rob Schurko (Tim Cross)
Fred Mentink-Vigier
Lucio Frydman
Zhehong Gan
Faith Scott
Sungsool Wi

AMRIS division
Joanna Long
Matt Merritt
James Collins
Luiza Caldas-Nogueira
Nhi Tran
Adam Smith

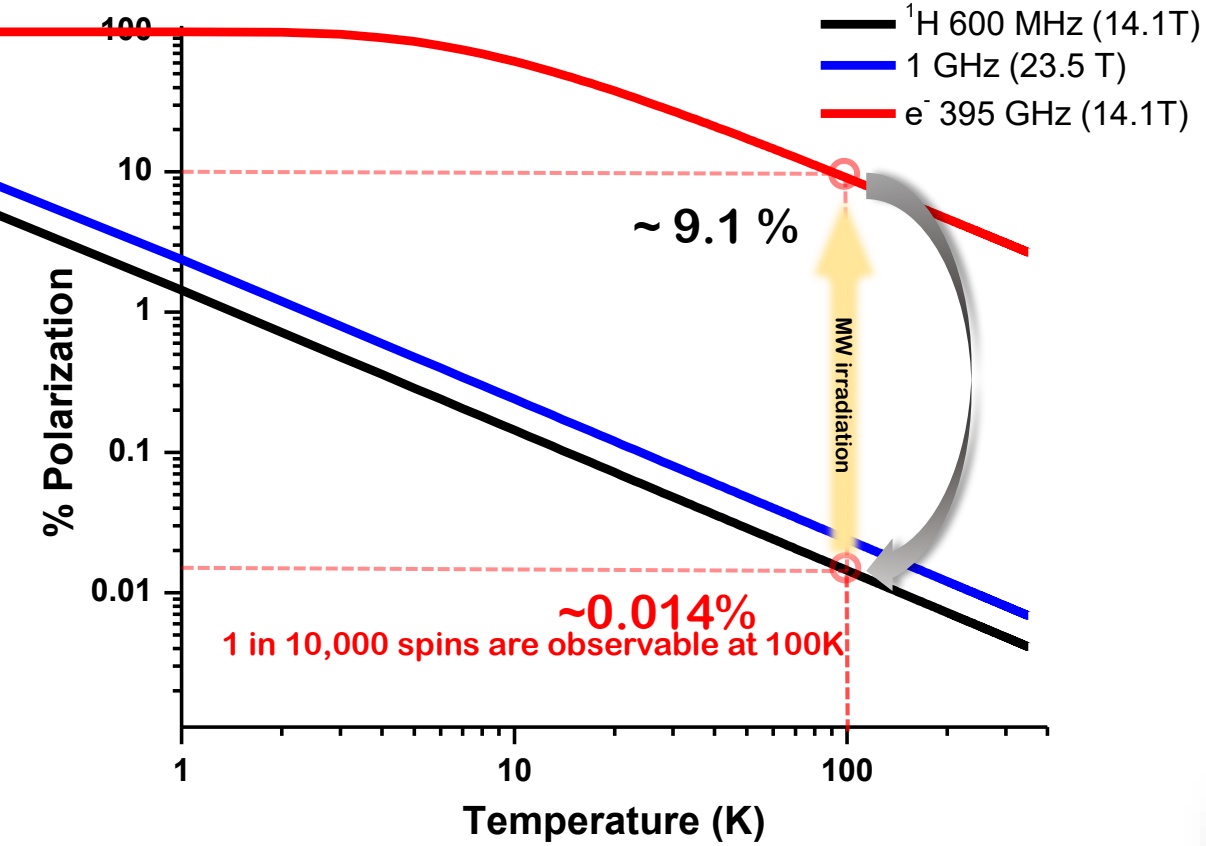
EMR division
Steve Hill
Thierry Dubroca
Hans van Tol
Bianca Trociewitz
Gail Fanucci

NIH S10 OD018519, P41 GM122698, RM1 GM148766

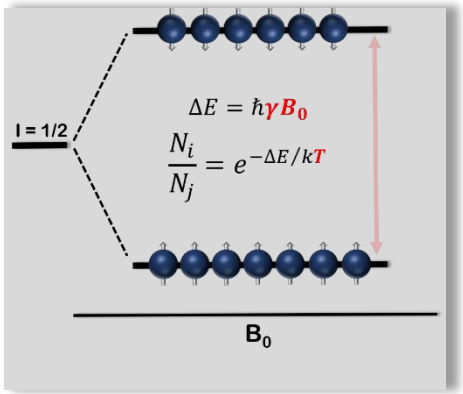
NSF MRI Grant CHE 1229170

The promise…..

Population difference between energy states ~ “polarization”



$$Polarization \propto \frac{N_i}{N_j} = e^{-\Delta E/kT}$$



Only 1 in ~8 million ^{1}H spins are NMR observable at RT!

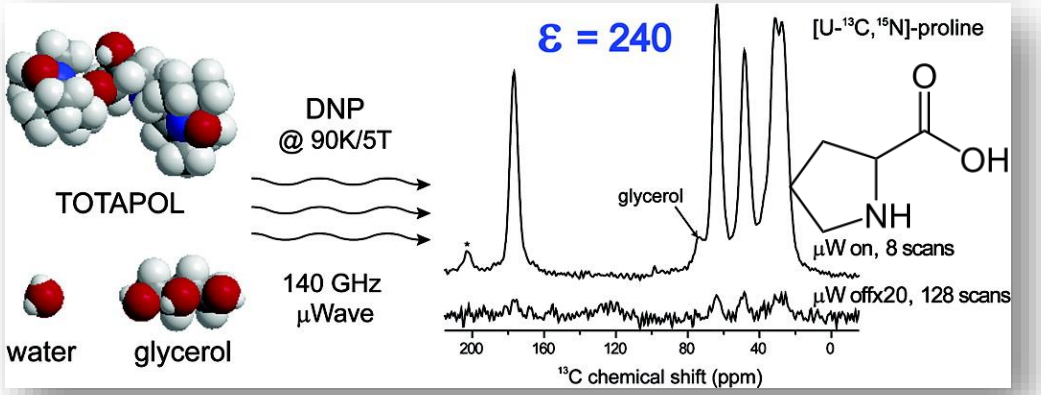
$$\varepsilon_{Theoretical\ max} = \frac{\gamma_e}{\gamma_H} \sim 660$$

Time savings $\propto \varepsilon^2$

ε = 240

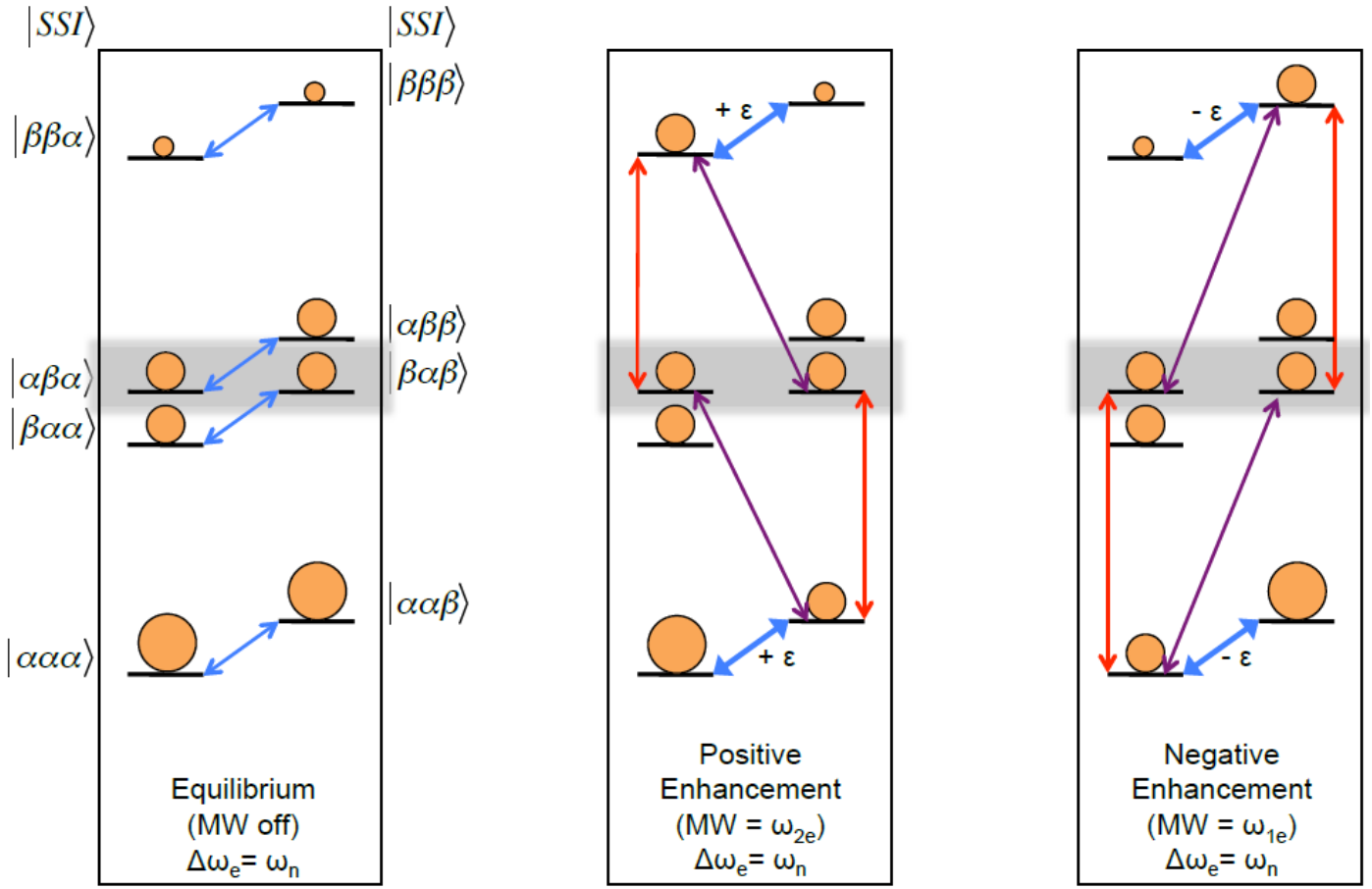
1 week signal averaging reduced to ~11 sec !

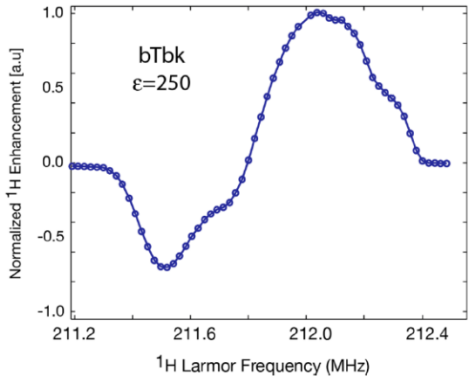
1 year signal averaging reduced to ~9 min!!!

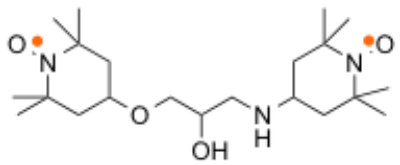


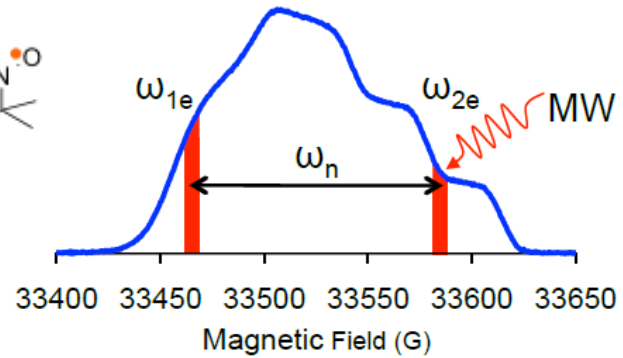
Changsik Song; Kan-Nian Hu; Chan-Gyu Joo; Timothy M. Swager; Robert G. Griffin; *J. Am. Chem. Soc.* **2006,** 128, 11385-11390.

DNP: the cross effect at ~100 K









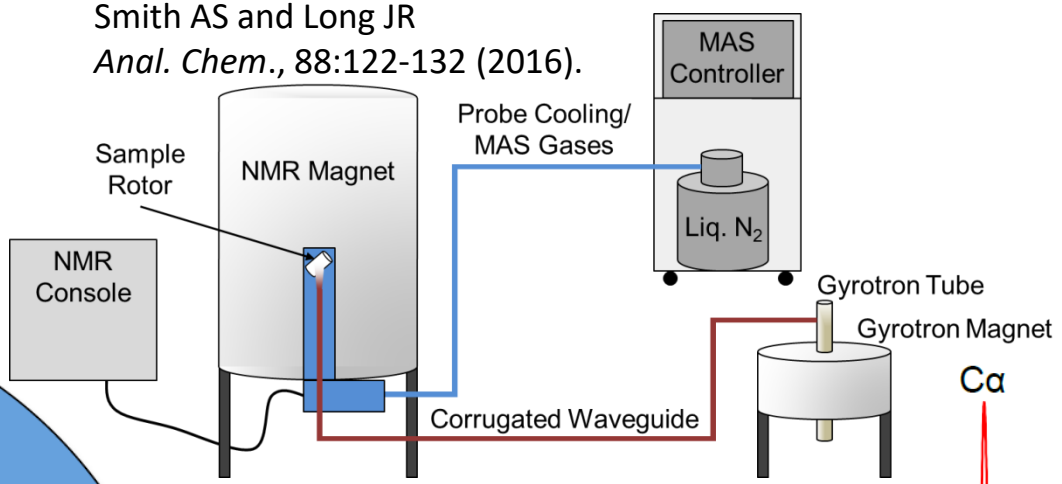
Smith AS and Long JR
Anal. Chem., 88:122-132 (2016).

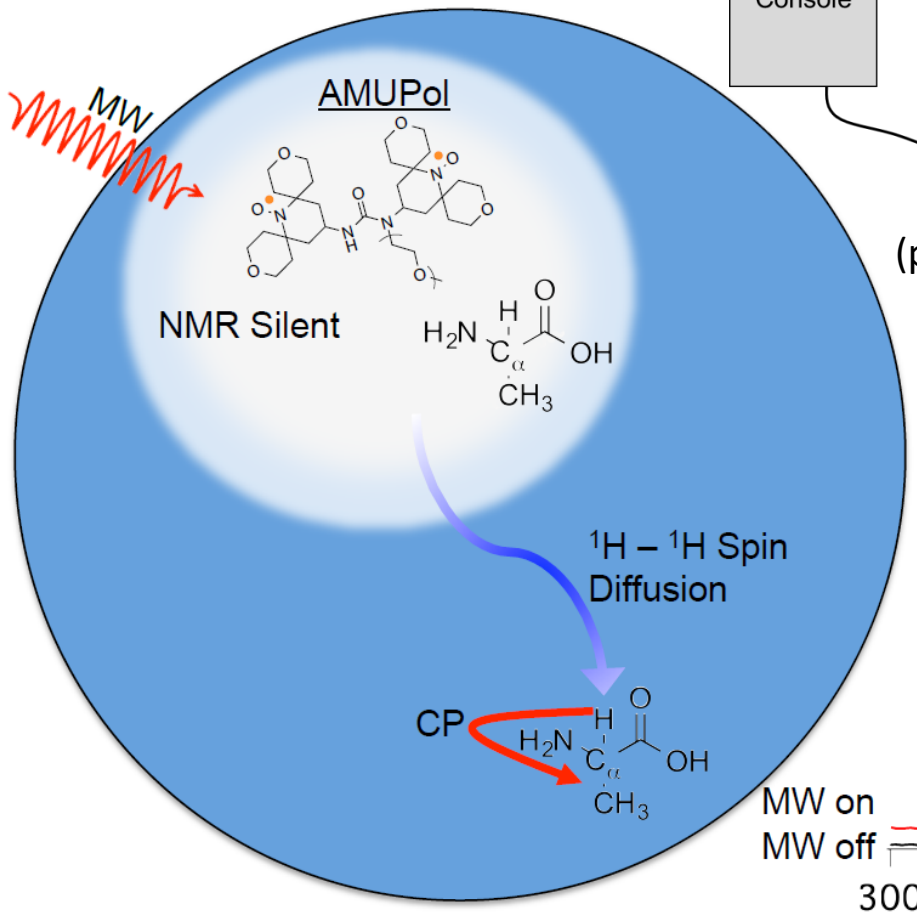
MAS DNP on a model system at 600 MHz

ε = 128 leads to a time savings of ≈16384

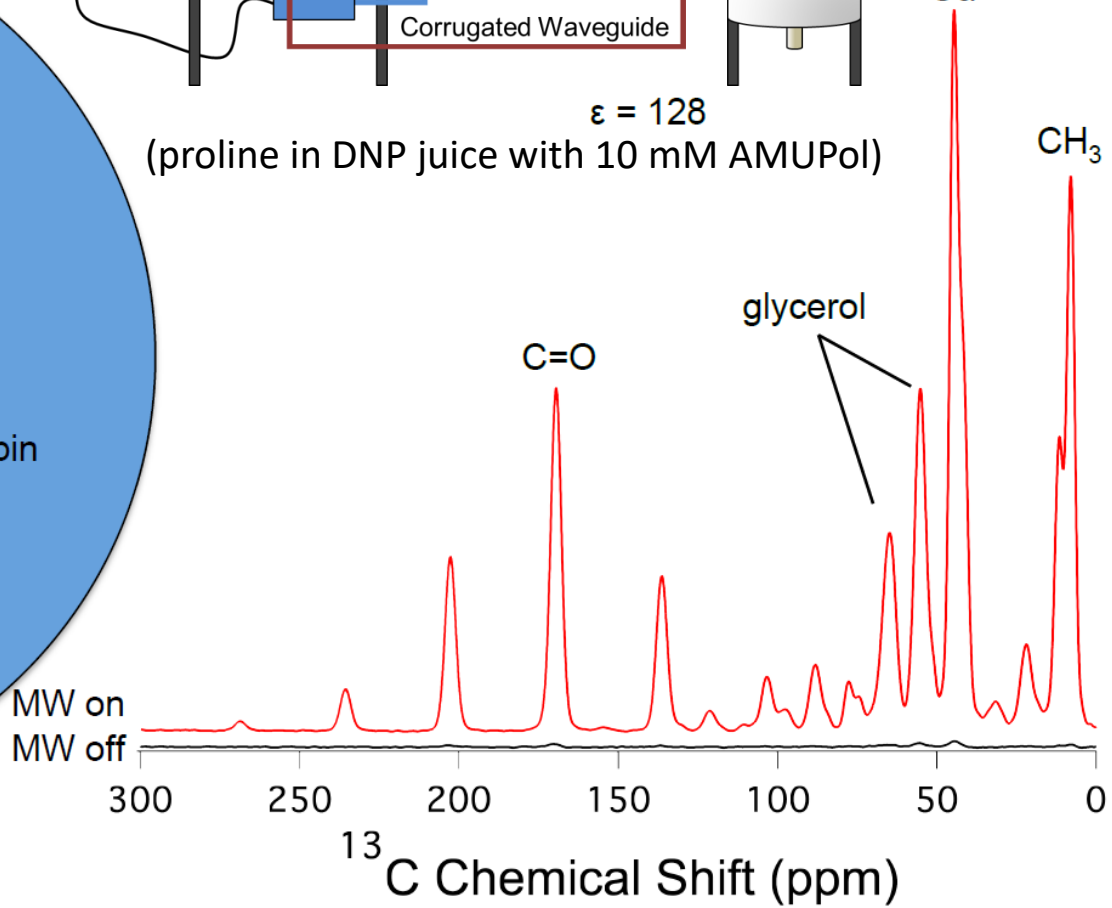
10-12 days ➔ 1 minute

Smith AS and Long JR
Anal. Chem., 88:122-132 (2016).





ε = 128
(proline in DNP juice with 10 mM AMUPol)



Biomolecular DNP MAS ssNMR

Biosystem	ε	Temp. (K)	Radical (mM)	B_0 (^{1}H freq.)
GNNQQNY	20	100	TOTAPOL	400 MHz
TTR105-115	12	100	TOTAPOL	400 MHz
PI3-SH3	30	100	TOTAPOL	400 MHz
Aβ1-40	20	96	TOTAPOL	400 MHz
Peptidoglycan	8.8	100	TOTAPOL	400 MHz
hΦ17W	18	100	bTbKa	400 MHz
M218-60	2.5	100	TOTAPOL	600 MHz
Arabidopsis cell wall	27	100	TOTAPOL	600 MHz
Whole cells	10	100	TOTAPOL	400 MHz
Cell envelopes	26	100	TOTAPOL	400 MHz
Ribosome	25	100	TOTAPOL	400 MHz
Cell wall	20	100	TOTAPOL	400 MHz
nAChR-bound NTII	26	100	TOTAPOL	400 MHz
bR	75	83	AMUPol	380 MHz

Su Y. et al, *Annu. Rev. Biochem.*, 2015

Using DNP to capture biomolecular structure and dynamics at atomic resolution

Insights / Understanding

Simulations incorporating multimodal data

Optimal experiments

Resolution, SNR, processing, quantitation

Trapping biological states

Physiologic conditions, sample freezing

DNP to enhance sensitivity

MW source, biradicals, DNP probe

Isotopically enriched samples

Synthesis / heterologous expression

Solid-State NMR

MAS, RF, pulse sequences

High Field Magnets

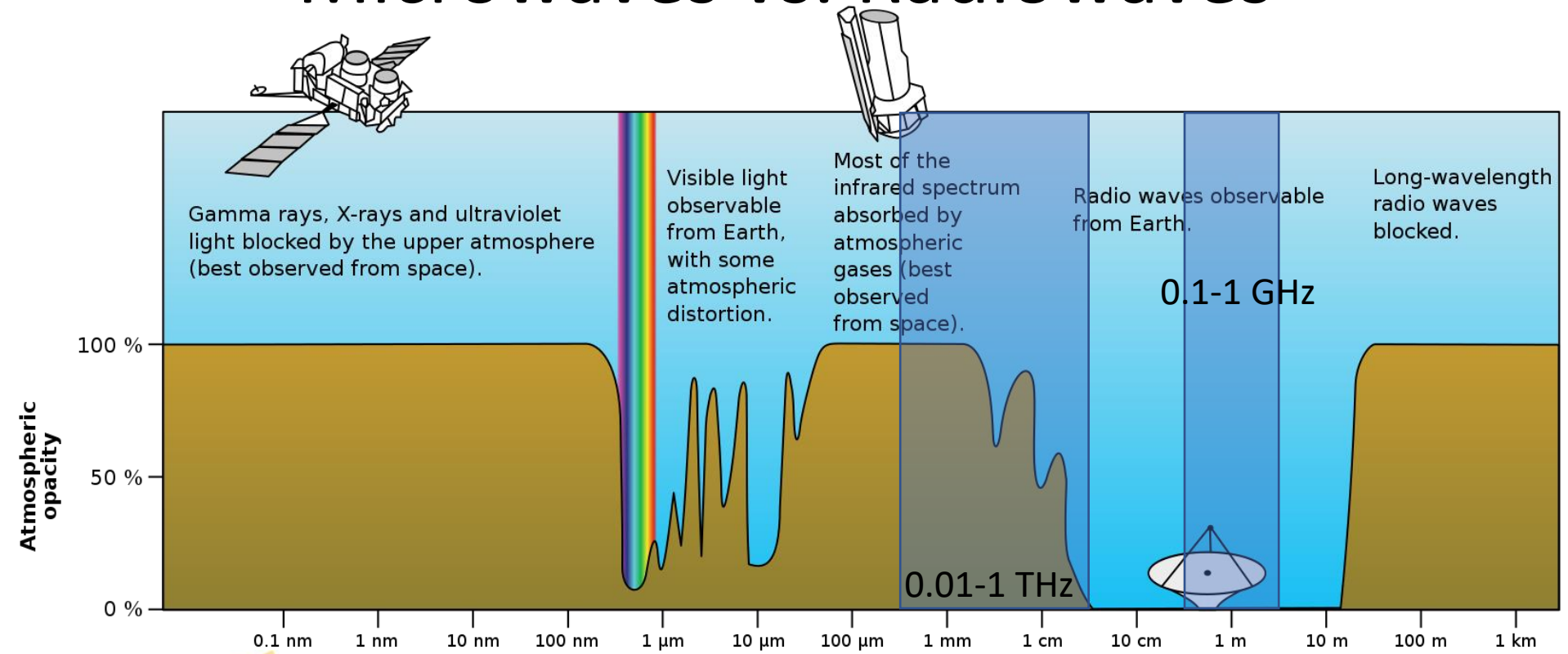
Superconductors/cryogenics

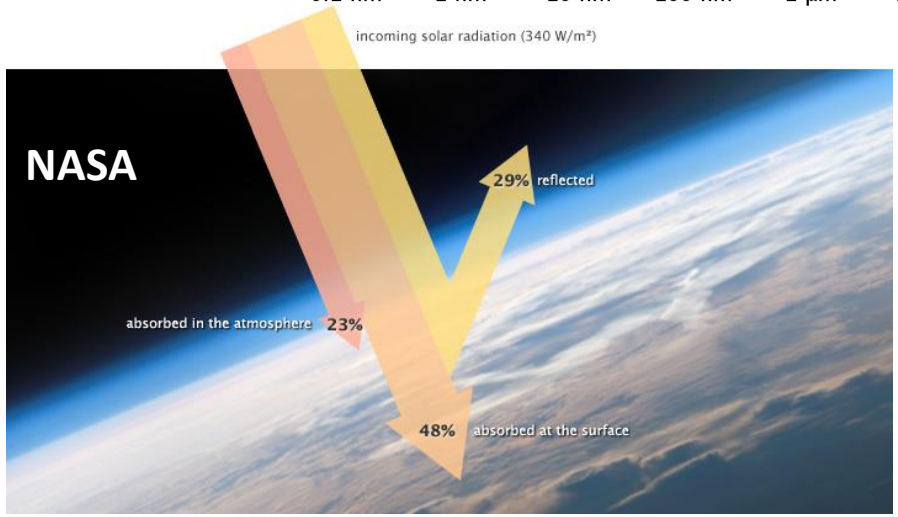
DNP: Practical aspects of irradiation

NATIONAL MAGLAB

UF UNIVERSITY of FLORIDA

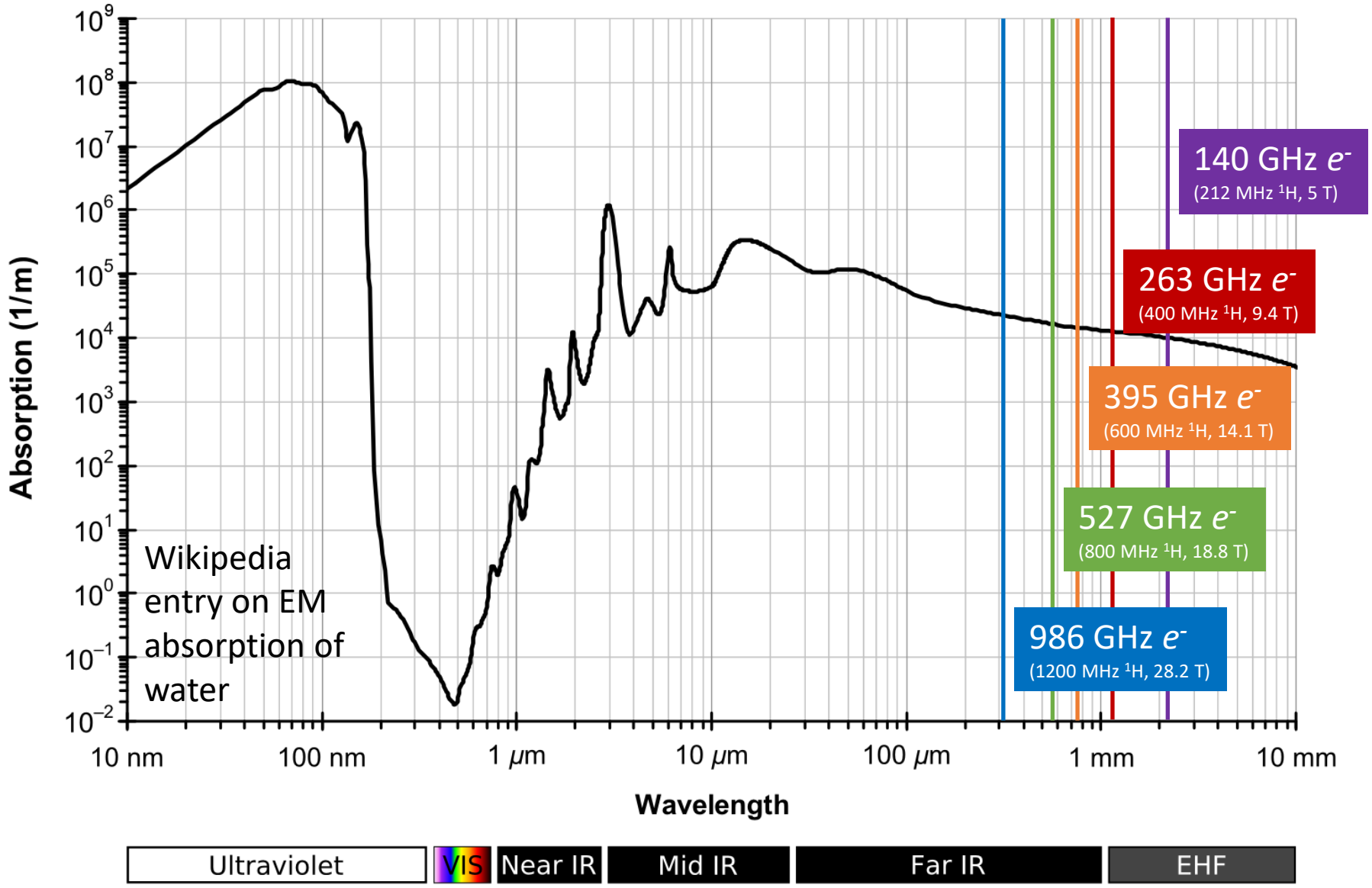
Microwaves vs. Radiowaves



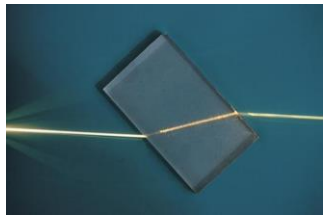


- ~29 percent of solar energy is reflected
- ~23 percent is absorbed by water vapor, dust, and ozone in the atmosphere
- ~48 percent passes through the atmosphere and is absorbed by the surface.

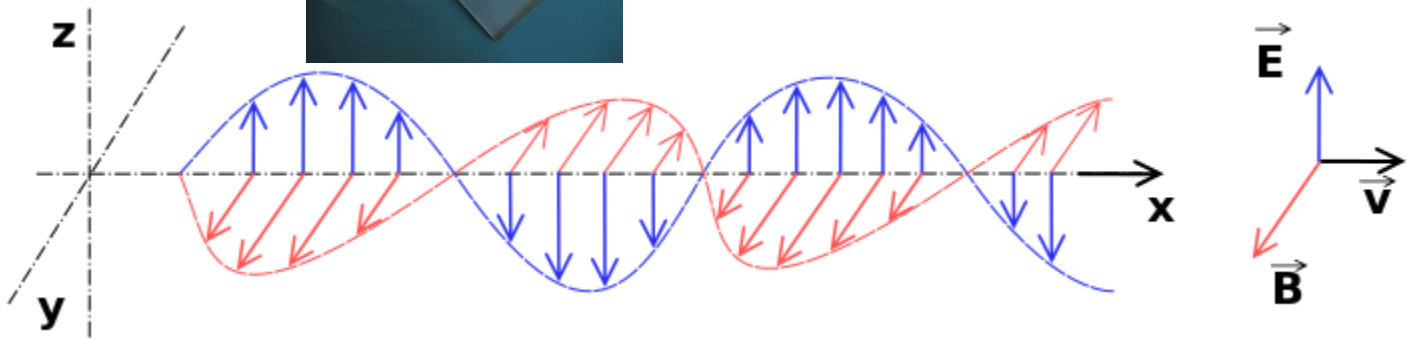
Microwaves and absorption by (liquid) water

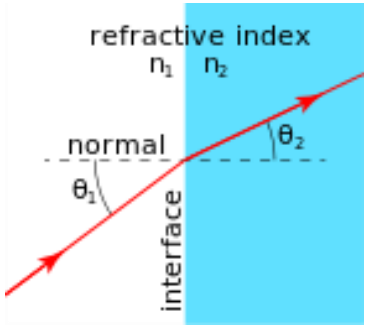


2-4 fold loss in efficiency over conventional NMR range



Minireview of electromagnetic propagation





$$n = \frac{c}{v}$$

$$\lambda = \frac{\lambda_0}{n}$$

$$f = \frac{v}{\lambda}$$

$$\alpha = \frac{4\pi\kappa}{\lambda_0}$$

α is the attenuation coefficient

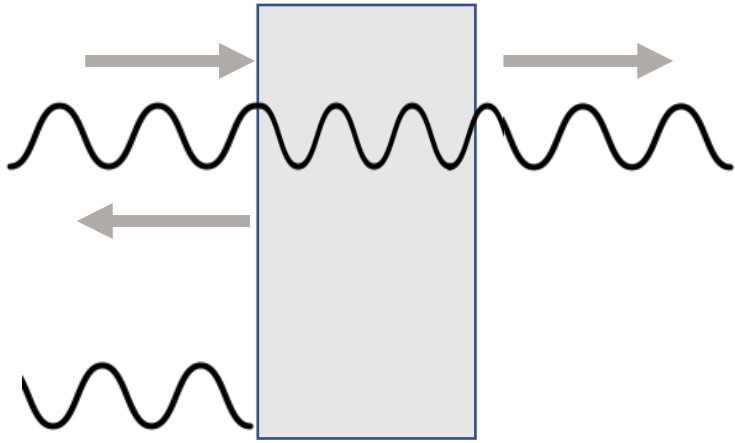
$$\delta_p = 1/\alpha$$

δ_p is the distance for attenuation by 1/e

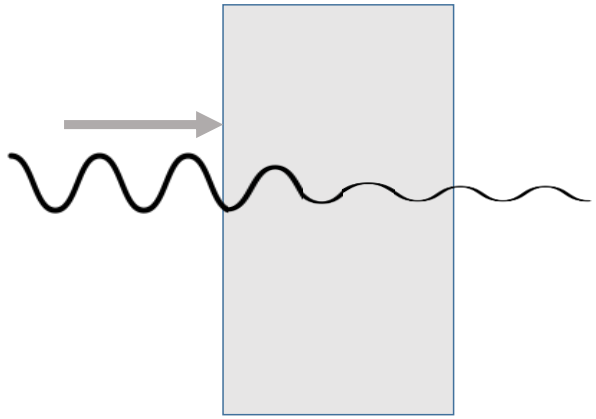
- n is dependent on the electric susceptibility of a material and is frequency dependent
- Interactions of the incoming light with electrons in the material lead to their spontaneous emission of light at varying angles relative to the incoming beam
- 90° out of phase→ $n > 1$ (refraction)
- 180° out of phase→interference (absorption)
- 270° out of phase→anomalous refraction ($n < 1$)
- 0° or in phase→amplification of light (lasers)
- For most materials at visible frequencies, a combination of refraction and absorption occurs

$$\underline{n} = n + i\kappa$$

λ in the sample — Extinction coefficient



n>1



High k

Ice vs. Water at wavelengths we care about

THE FLORIDA STATE UNIVERSITY

COLLEGE OF ARTS AND SCIENCES

COMPLEX INDICES OF REFRACTION FOR WATER AND ICE

FROM VISIBLE TO LONG WAVELENGTHS

By

MARK LEE MESENBRINK

A Thesis submitted to the
Department of Meteorology
in partial fulfillment of the
requirements for the degree of
Masters of Science

Degree Awarded:

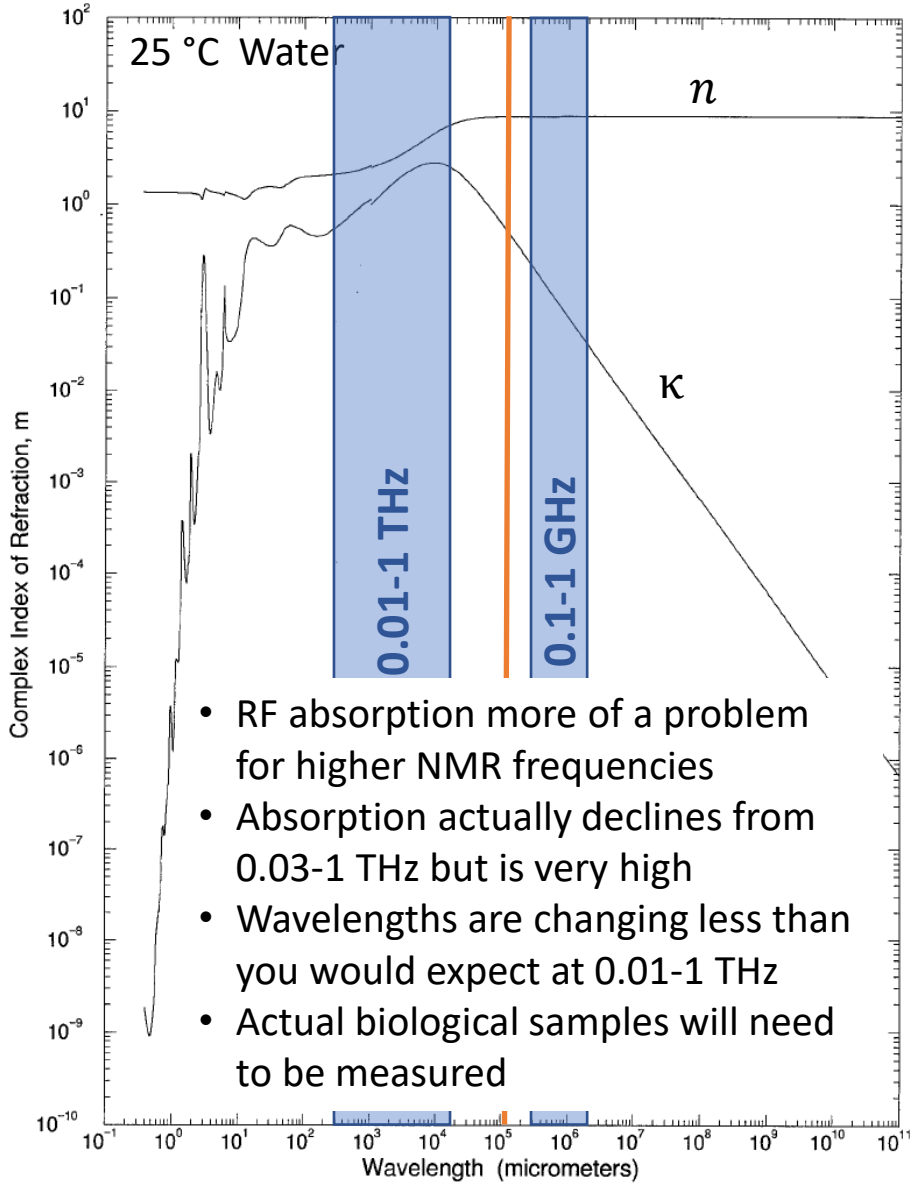
Spring Semester, 1996

ACKNOWLEDEMENTS

I would like to acknowledge the many people that have assisted and supported me with my research. My deepest and dearest thanks goes to Cyndi. Without her love, I would not have been able to keep focused. My Committee members, Dr. Peter Ray, Dr. Melody Owens and Dr. T. N. Krishnamurti, deserve many sincere thanks. Without their guidance and instruction, my research would never have been complete. I would like to also thank the three "behind the lines" people who made it all happen: Dr. Craig Bohren from Penn State University for his wisdom; Dr. Jesse Stephens for pointing me in the right directions; and Bret Whissel for his god-like knowledge of FORTRAN and countless other programs. Finally, I would like to thank the Air Force for allowing me the opportunity to further my education.

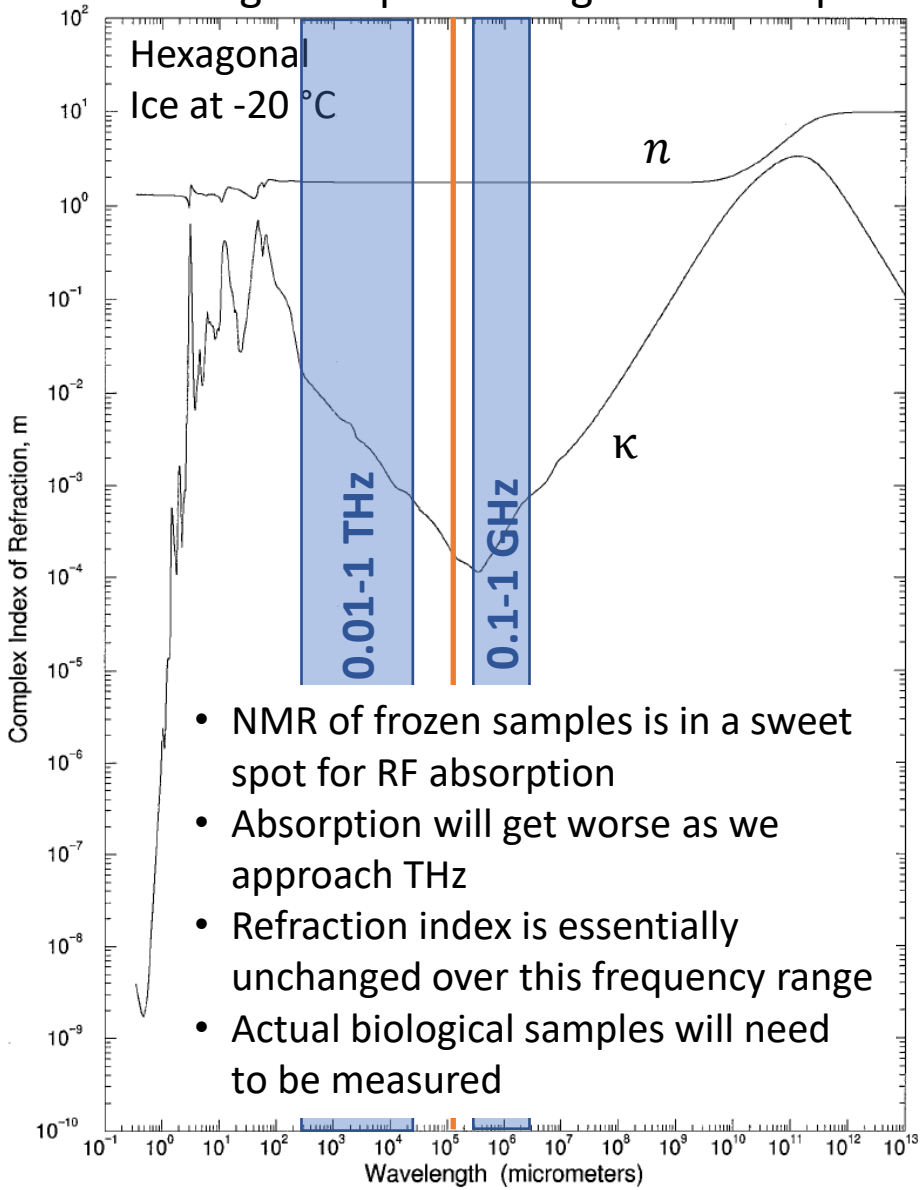
Ice vs. Water at wavelengths we care about

M. Mesenbrink, master's thesis, FSU, 1996



- RF absorption more of a problem for higher NMR frequencies
- Absorption actually declines from 0.03-1 THz but is very high
- Wavelengths are changing less than you would expect at 0.01-1 THz
- Actual biological samples will need to be measured

https://www.youtube.com/watch?v=HwREvdUWSKE
Finding The Speed of Light with Peeps and a Microwave



- NMR of frozen samples is in a sweet spot for RF absorption
- Absorption will get worse as we approach THz
- Refraction index is essentially unchanged over this frequency range
- Actual biological samples will need to be measured

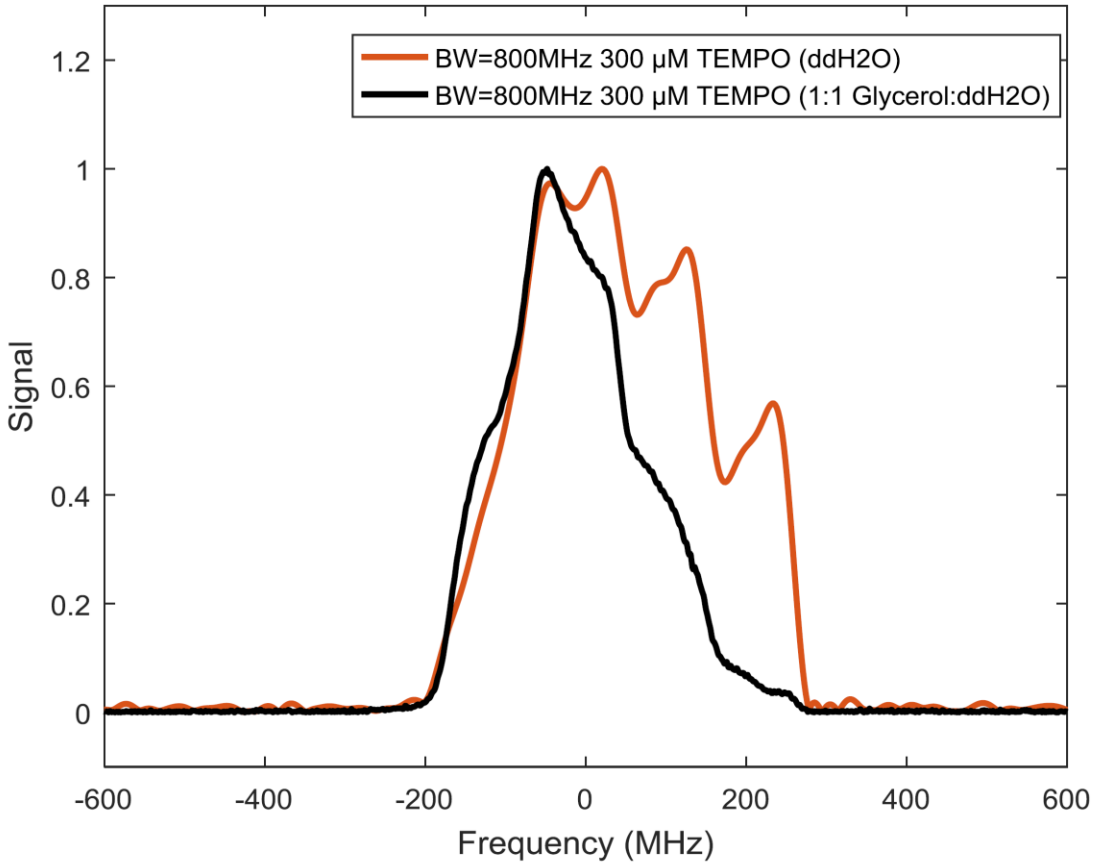
DNP: Practical aspects of freezing and temperature

NATIONAL MAGLAB

UF UNIVERSITY of FLORIDA

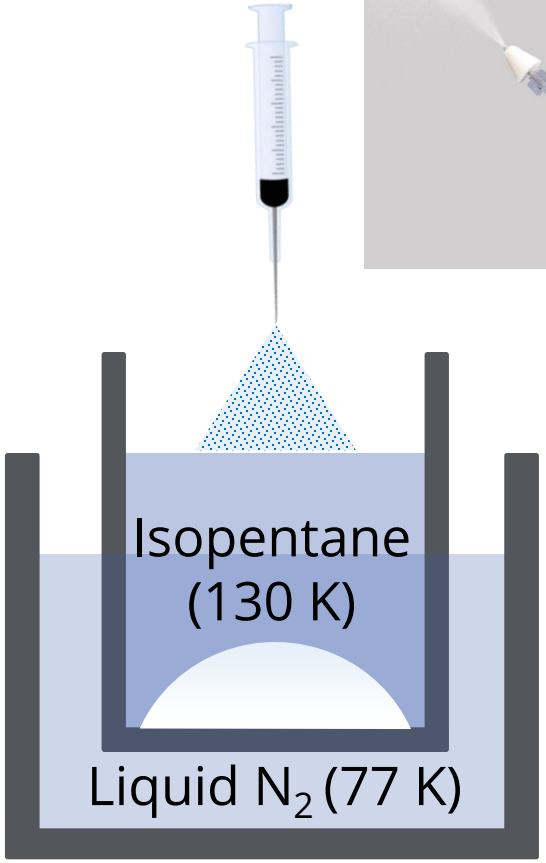
Sample freezing

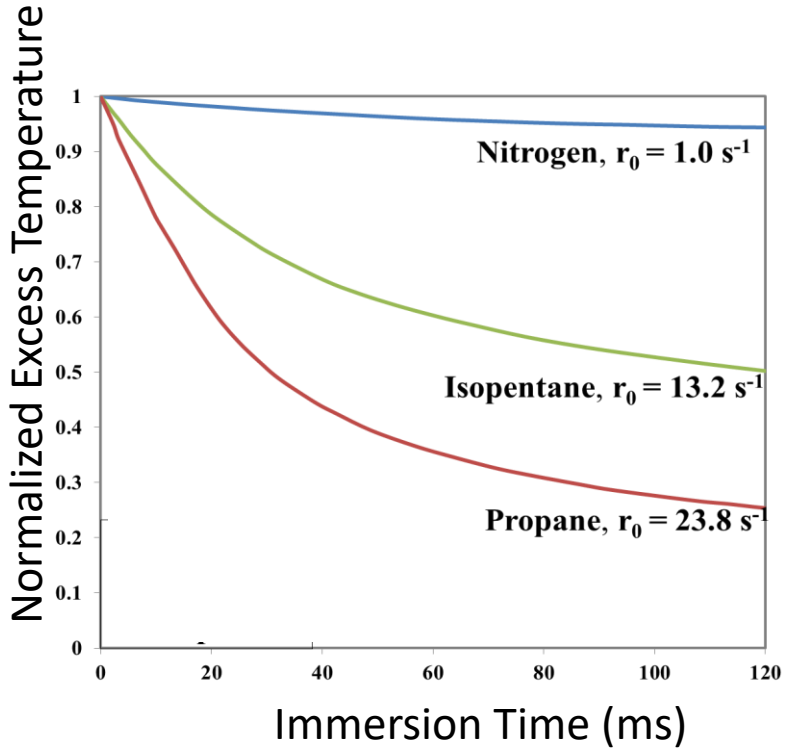
- Ice crystals can lead to biradical aggregation as well as degrade the sample
- MAS DNP typically relies on freezing the sample on insertion of the rotor into the probe (which takes a few seconds)
- Glassing agents can prevent crystal formation but can create other issues
- Alternatively faster freezing can be done outside of the rotor
- Freezing can also alter the pH of the sample
- The fields of cryoEM and cell biology have extensive literature on cryoprotectants and freezing protocols



Sample freezing

- An efficient freezing agent can remove the need for a glassing agent
- Vitrobots for cryoEM typically use liquid ethane or propane
- Enzyme kinetics people often use isopentane as it is a liquid at room temperature
- EPR and NMR labs have demonstrated freezing via spraying onto cold metal surfaces





Glassing agents

- An effective glassing agent allows you to slowly freeze in the probe
- A popular (though not ideal!) glass is DNP juice (60/40 glycerol/water)
- Cell biology typically uses DMSO, betaine, or trehelose as cryoprotectants which also work well for DNP glasses (with caveats to be shown)
- For biological solids, membranes and crystallation additives (like PEG) act as glassing agents too
- *Dehydration*—via high glassing agent concentration or removal of water through ultracentrifugation/drying/lyophilization will cause *inhomogeneous broadening*. This is widespread in the DNP literature.
- My lab likes 10% DMSO or 10% betaine as a glassing agent as they preserve structure/viability of the samples. We also like minimal ^{2}H and full hydration.

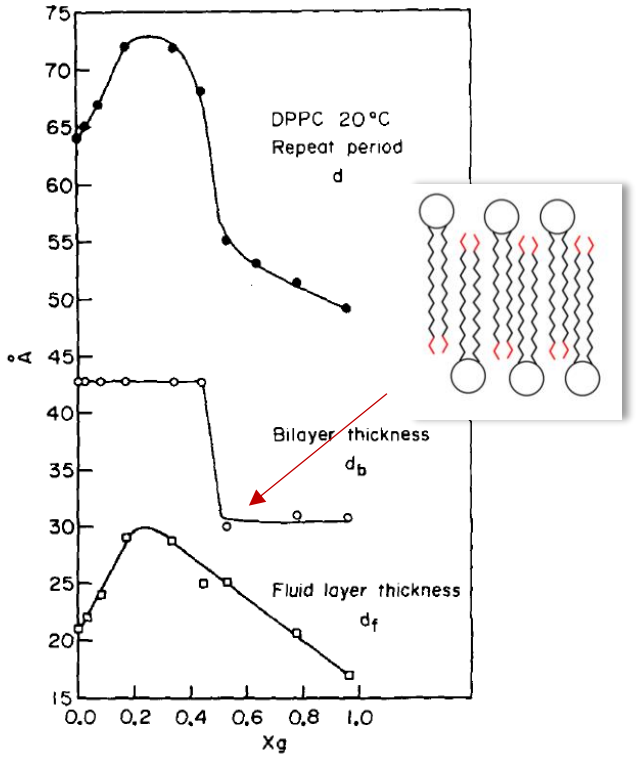


Fig. 4. X-ray parameters for DPPC as a function of mole fraction of glycerol (X_g). The solid circles (●) represent the repeat periods *d* and the open circles (○) and open squares (□) represent the bilayer widths and fluid layer thicknesses, respectively, as determined from electron density profiles (see Fig. 7).

R. V. McDaniel et al. , *Biochim Biophys Acta* .1983,731, 97-108

Classical DNP Matrix Considerations

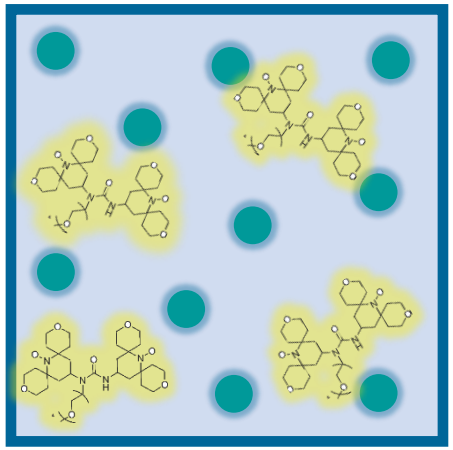
- ❑ Complete radical solubility
- ❑ Homogenous distribution of radical
- ❑ Dilute proton bath
- ❑ High protein concentration
- ❑ Preserve native environment of biological sample

~ 5-10 mM biradical

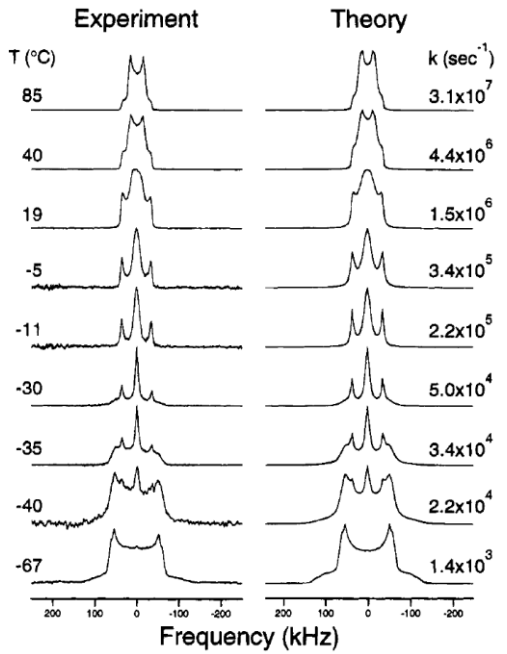
"DNP Juice"

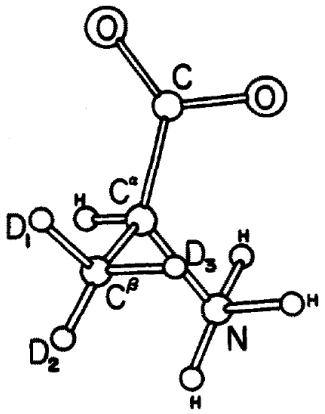
60/30/10 (v/v) glycerol-d_8 / D_2O / H_2O

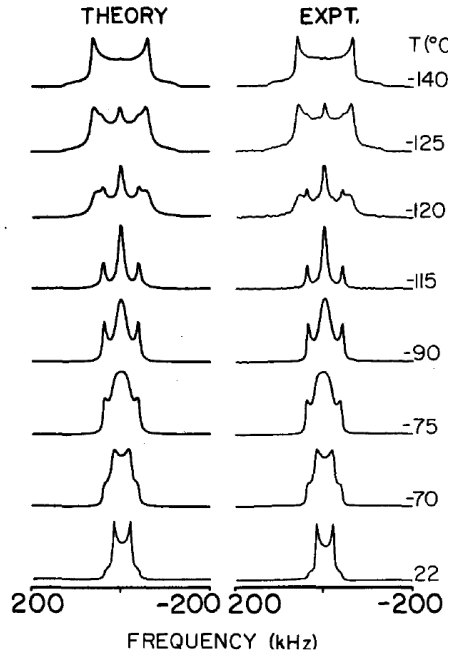
% glassing agent % deuteration



Biological dynamics also impact polarization



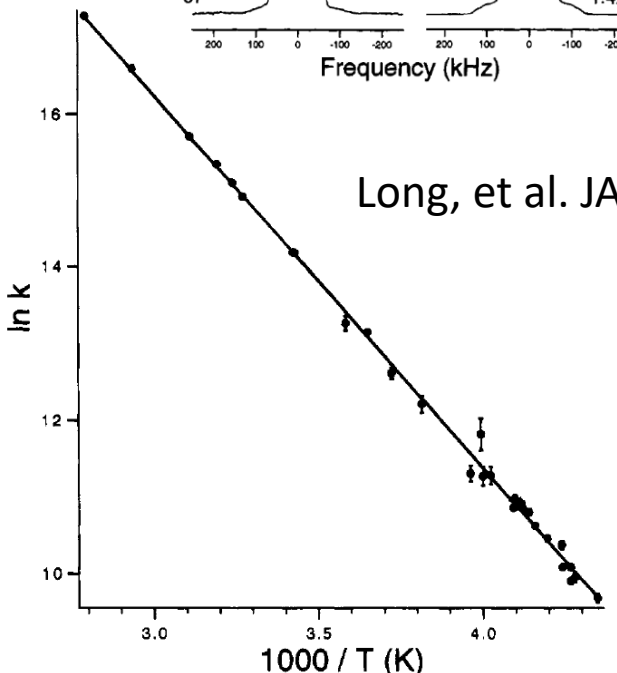




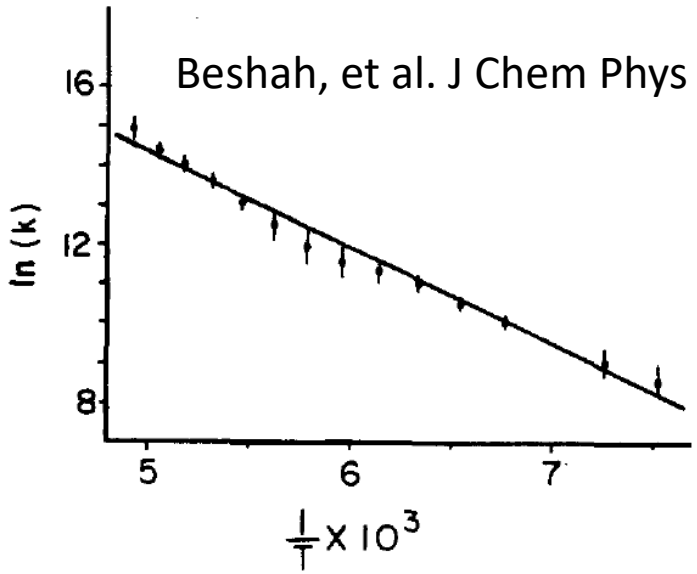
^{2}H NMR of Bacteriorhodopsin and Retinal Model Compounds

Table 1: Rates and Activation Energies for Methyl Three-Site Hop

T, K	18-C: 6-s-cis[a]	18-C: 6-s-trans[b]	18-C: bR[c]	16,17-C: 6-s-trans[d]	16,17-C: bR[e]
91	2.25×10^4				
95					8.65×10^5
98	5.06×10^4		$>5 \times 10^7$		
101				1.71×10^5	
108	3.84×10^5				
113	1.30×10^6				
116		2.1×10^{10}			
117				3.84×10^5	
118	1.94×10^6				
123			5×10^6		2.96×10^6
126				1.95×10^6	
128	4.38×10^6				
140		4.0×10^{10}			
139				2.96×10^6	
139				2.96×10^6	
158					$>5 \times 10^7$
179	2.0×10^8				
198			8×10^9		
228	2.6×10^9				
263	7.6×10^9				
297	1.5×10^{10}	1.2×10^{12}			
E(act), kJ/mol	14.5 ± 1	5 ± 1	9	14 ± 2	13 ± 2

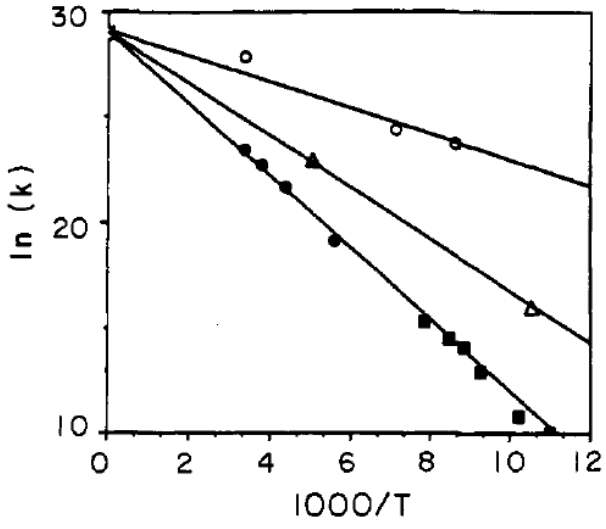


Long, et al. JACS 1994



Beshah, et al. J Chem Phys 1987

Copie, et al. Biochem 1994



DNP: Practical aspects of ssNMR hardware

NATIONAL MAGLAB

UF UNIVERSITY of FLORIDA

Sizes of Interactions: Solids

$$H_{total} = H_{external} + H_{internal}$$

$$H_{ext} = H_{Zeeman} + H_{Radio\ Frequency}$$

500 - 900 MHz 0.001 - 200 kHz

$$H_{Int} = H_{Dipole\text{-}Dipole} + H_{Chemical\ Shift} + H_{J\text{-}coupling}$$

0.05 - 40 kHz 20 - 40 kHz <200 Hz

Magic Angle Spinning

0.2 - 50 kHz

Challenges of biomolecular ssNMR

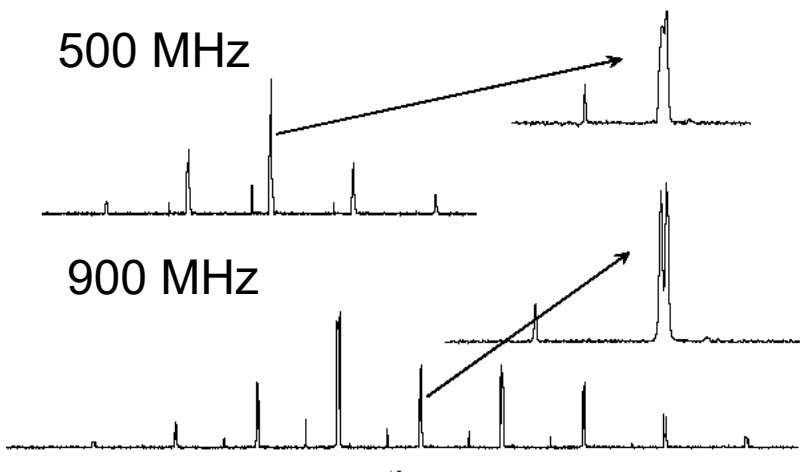
- Sensitivity→ Optimizing for proteins which are inherently dilute
- Temperature matters→Minimizing RF/MAS heating

Water is a major component of most samples

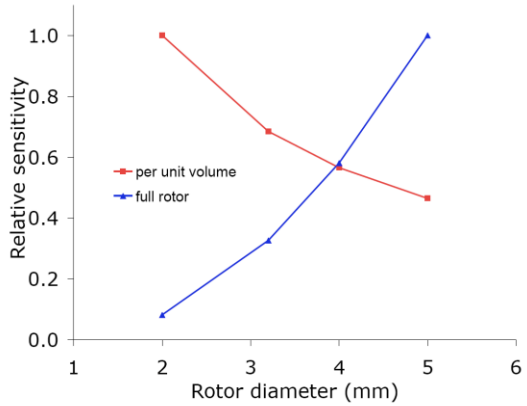
- Filtering out the signal of interest from a messy background→ Double quantum filtering techniques

Encyclopedia of Magnetic Resonance (2010)

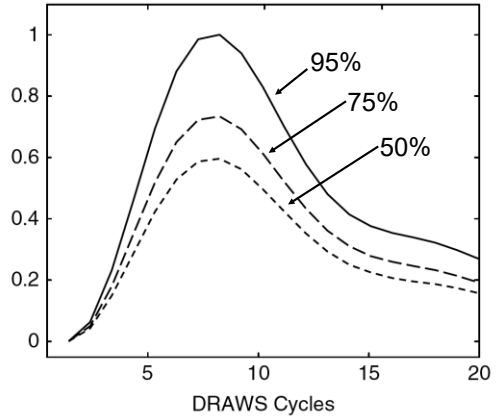
CSA field dependence



MAS vs sensitivity



RF homogeneity

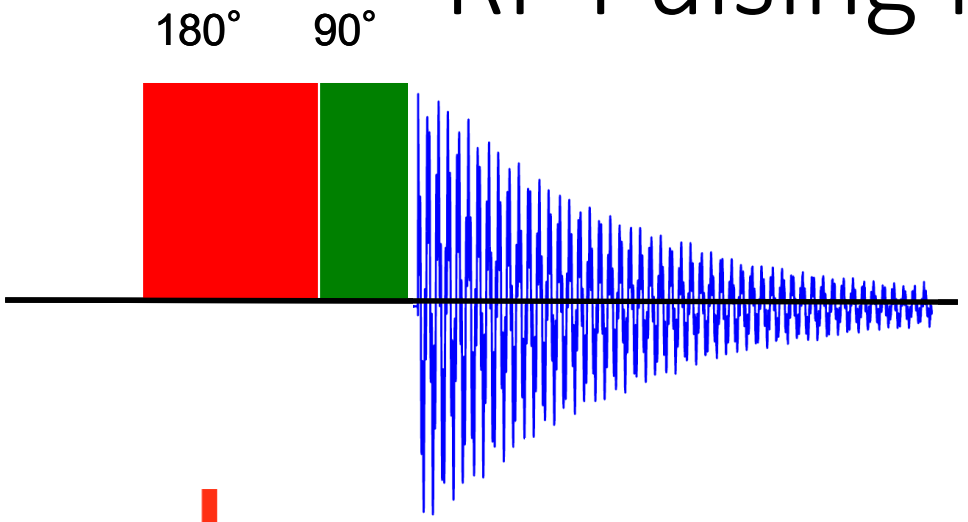


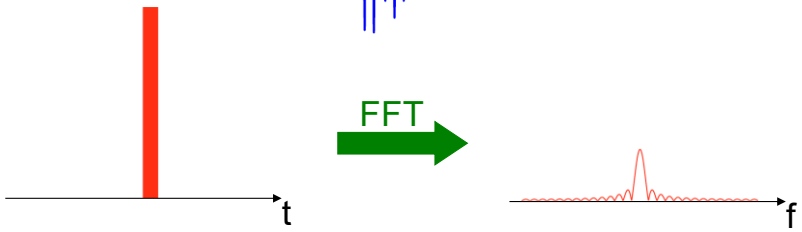
In dry air at 20 °C (68 °F), the speed of sound is 343 m/s (1235 km/h, or 770 mph, or 1129 ft/s, or approximately 5 seconds per mile)

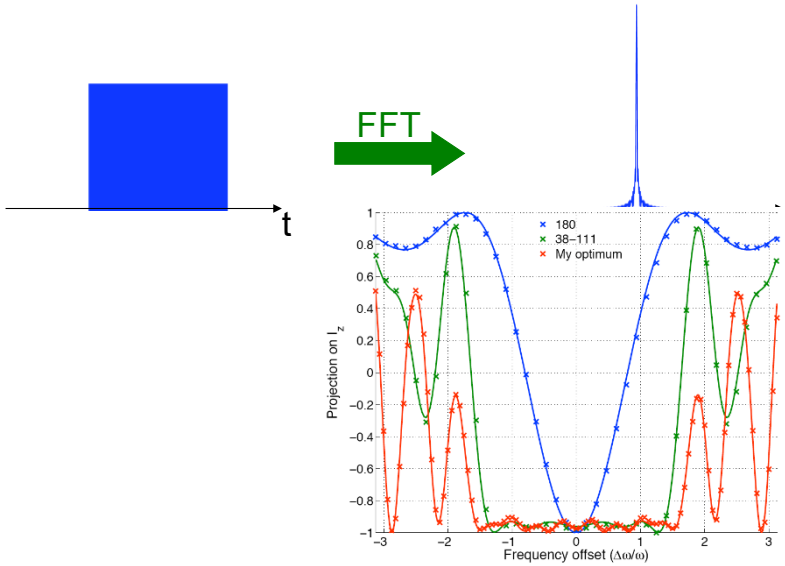
MAS rate	Rotor Dia (mm)	Outer Speed (m/s)	km/h	Outer Speed (mph)
16000	4	201	724	450
50000	2.5	393	1414	880
68000	1.3	278	1000	622
70000	1.3	286	1029	640

Magn. Reson. Chem. 2007; **45**: S209–S220

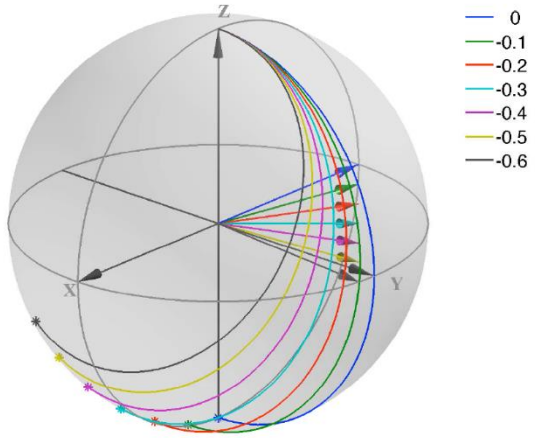
RF Pulsing in NMR

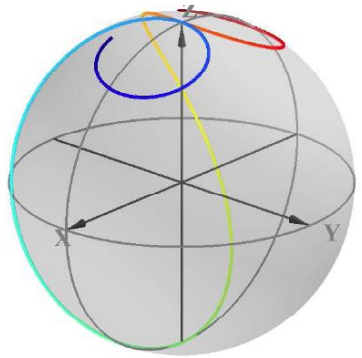


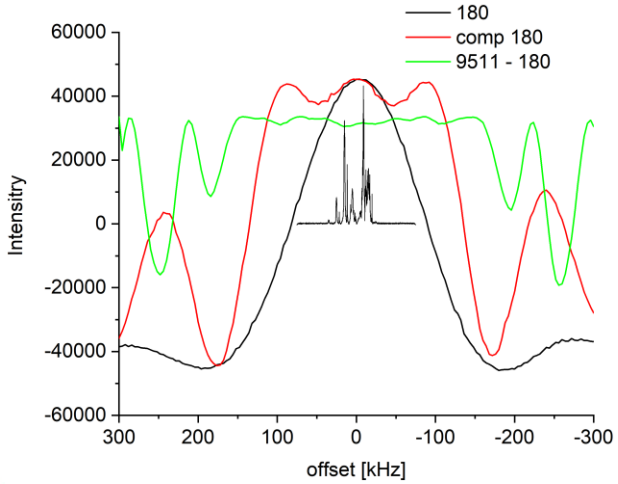


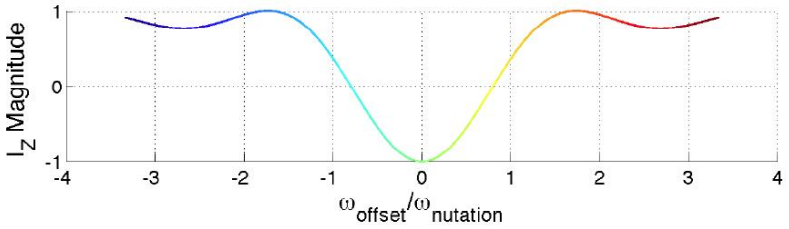


Inversion pulse: $\omega_{offset}/\omega_{nutation}$









Low-E MAS probes for dilute protein samples

RF Coil Design

Pencil rotors (longest)

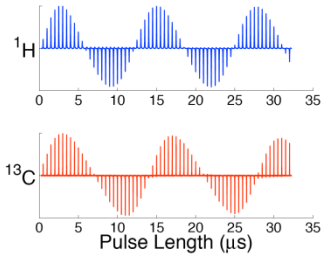
Stators custom-designed

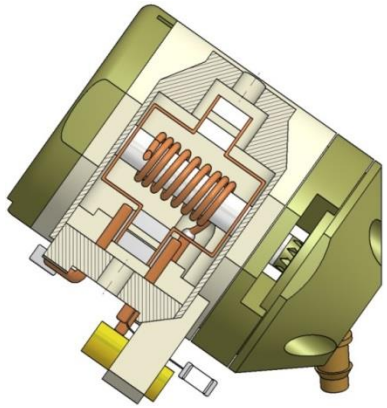
B_1 homogeneity over *full* 36uL rotor (900 MHz probe):

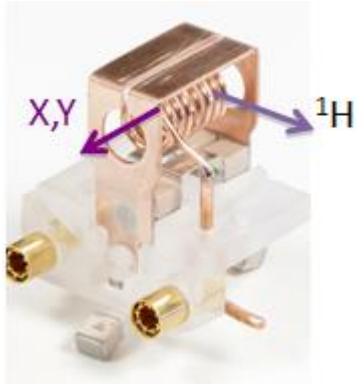
^{1}H 810°/90° = 97%

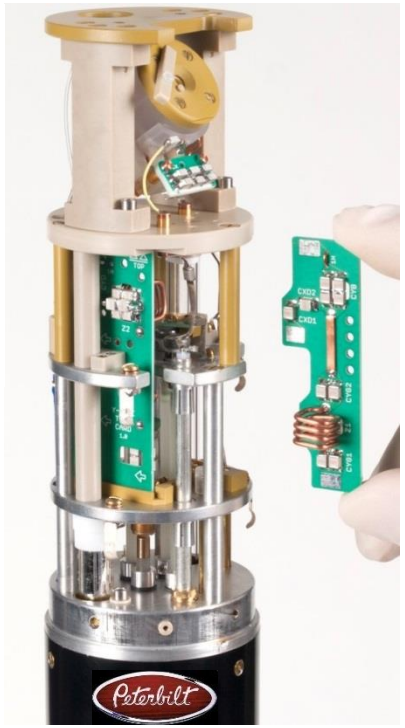
^{13}C 810°/90° = 88%

^{15}N 810°/90° = 82%





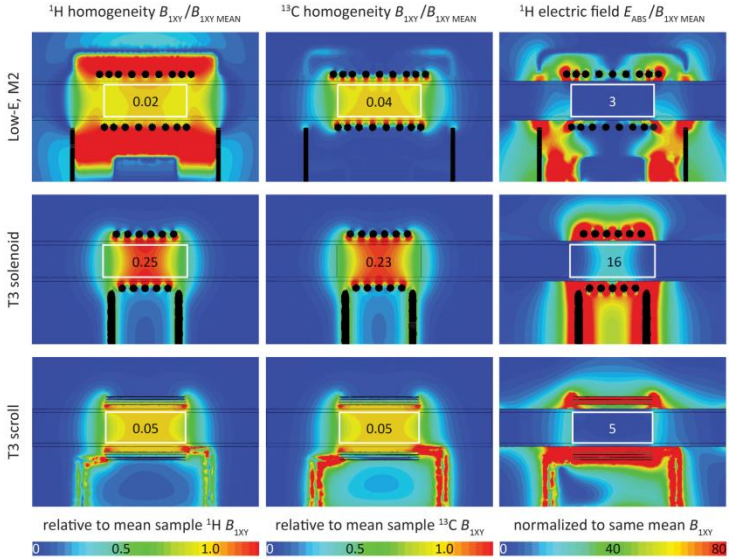




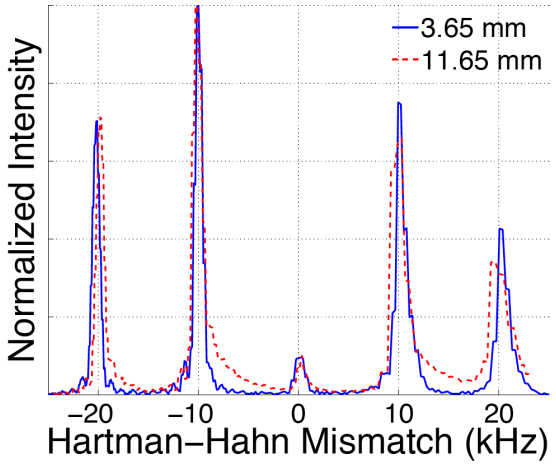
- High B_1 homogeneity
- Stable under high power
- Minimal RF heating of sample

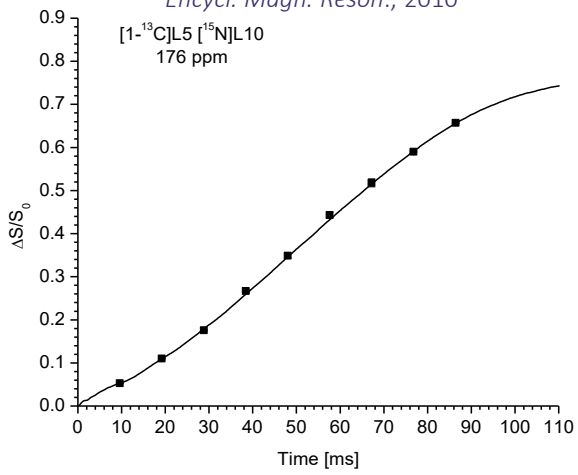
J Magn Reson., 2009, 197:135-144

Encycl. Magn. Reson., 2010



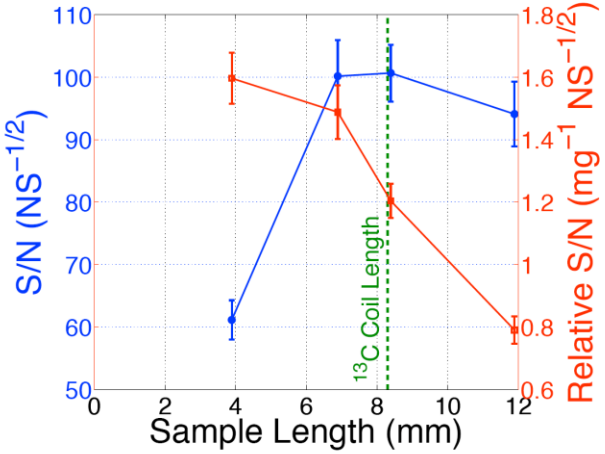
Standard deviations of B_1 field and sample-averaged values of E/B_1



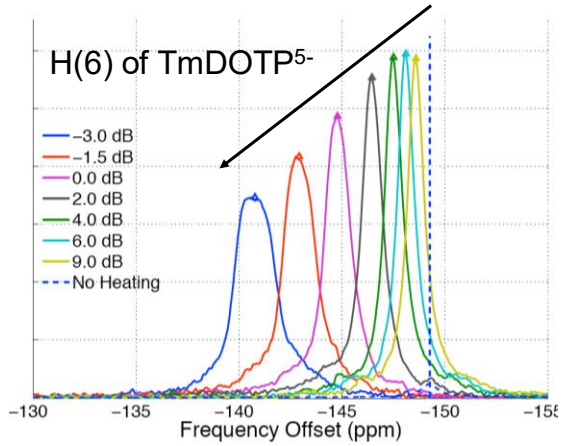


Characterizing a probe

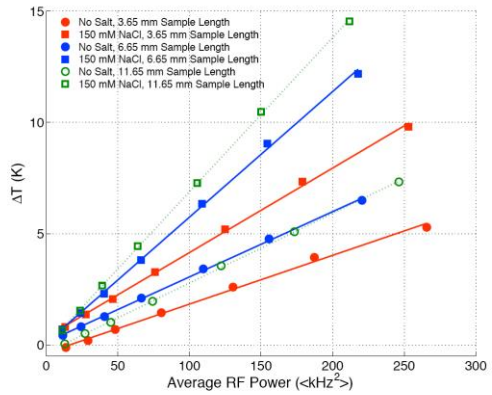
Rotor packing



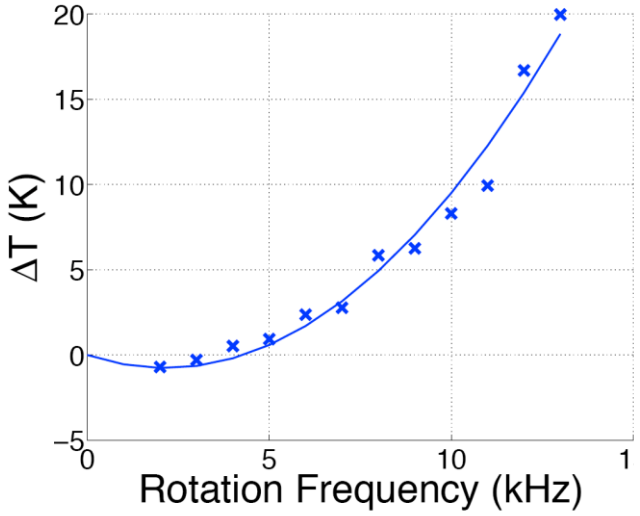
RF heating



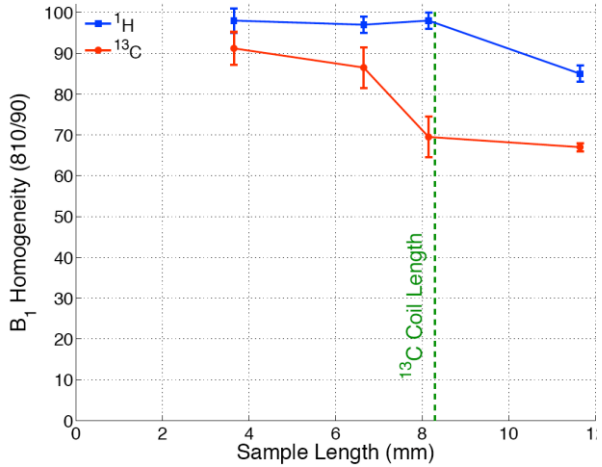
Dielectric effects



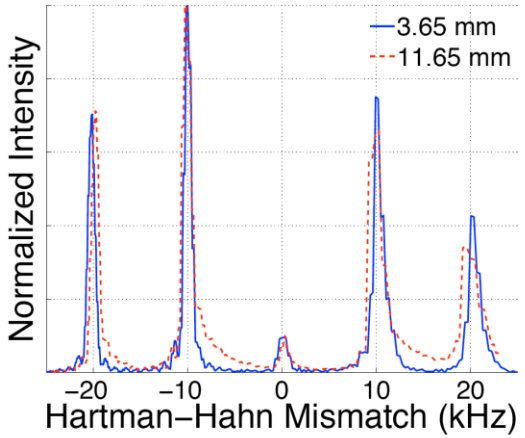
MAS heating



RF homogeneity



B1 field profiles



RF efficiency

	^{1}H kHz/W	^{13}C kHz/W
Low-E MAS	0.47	0.96
MAS Scroll	2.37	0.23
Balanced Solenoid MAS	1.10	0.71
Crossed Coil	0.63	0.12
Z-Coil	0.43	

J Magn Reson., 2009, 197:135-144
Encycl. Magn. Reson., 2010

Calibrating a probe:KBr

Setting the magic angle

J Magn Reson., 1982, 48:125-131

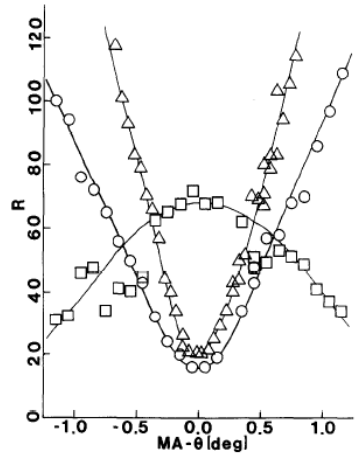


FIG. 3. Plot of peak height ratio vs deviation from magic angle. For KBr, R = (average he second-order spinning sidebands)/(height of central peak) with powdered (○) and crystalline (∠ ples. For HMB = (□), R = (height of aromatic peak)/(height of methyl peak).

TABLE 1

CANDIDATES FOR ANGLE REFERENCE MATERIALS

I = 1/2 Nuclide	Quadrupolar reference nuclide	$\lvert\gamma_I - \gamma_S\rvert / \lvert\gamma_I\rvert^a$
^{13}C	^{79}BR	0.004

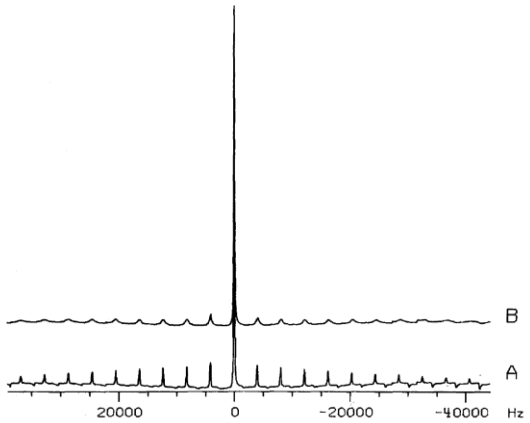
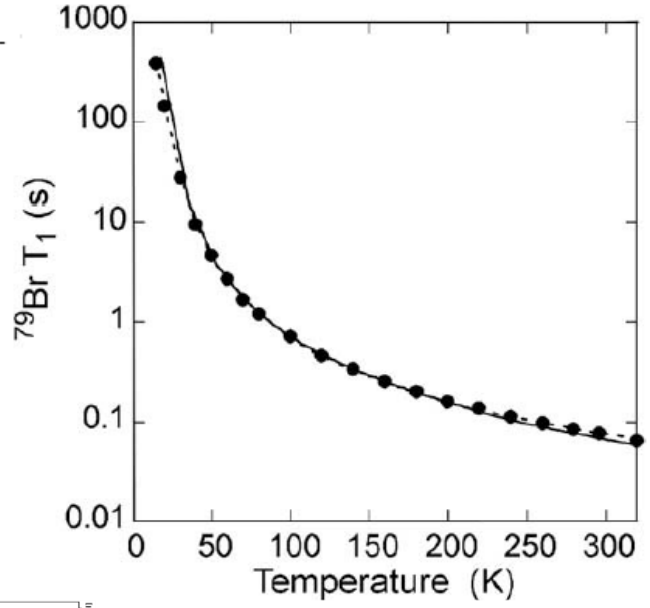
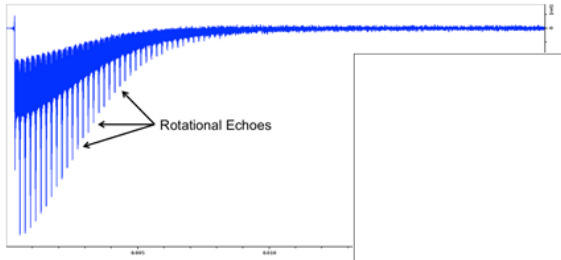


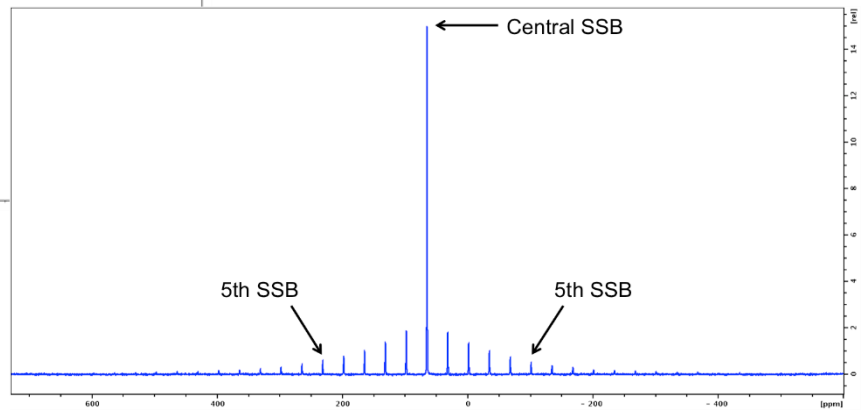
FIG. 2. FT/MAS ^{79}Br spectrum of KBr at the magic angle (A) and 0.5° off angle (B), at 37.6 MHz. 1000 3-μsec pulses were taken with a 70-msec repetition time. Extensive aliasing of high-order spinning sidebands is evident in A.

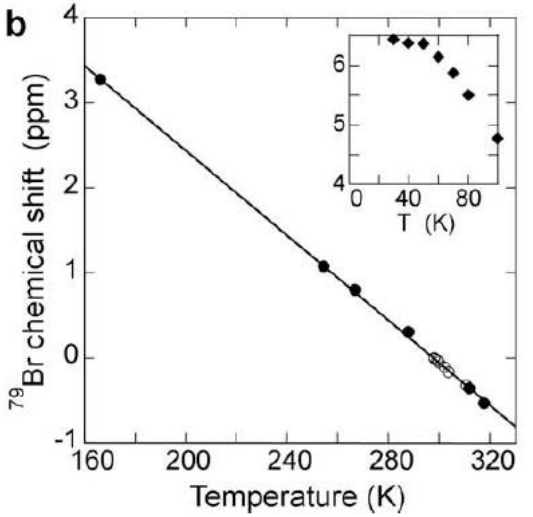
Calibrating temperature

J Magn Reson., 2009, 196:84-87









The FWHM of the central SSB should be ~ 120- 130 Hz and the 5th SSB FWHM should be within 10% of the measured FWHM for the central SSB.

Roughly calibrate the field: 60.15 ppm for KBr

Calibrating a probe:Adamantane

Shimming

J Magn Reson., 1997, 128:87-91

TABLE 3
Spherical Harmonic Shims in the Tilted Frame Expressed as Linear Combinations of Laboratory Frame Shims

First order

$$B_z^{tilt} = \frac{1}{\sqrt{3}} B_z^{lab} - \frac{\sqrt{2}}{\sqrt{3}} B_x^{lab}$$

$$B_x^{tilt} = \frac{1}{\sqrt{3}} B_x^{lab} + \frac{\sqrt{2}}{\sqrt{3}} B_z^{lab}$$

$$B_y^{tilt} = B_y^{lab}$$

Second order

$$B_{z^2}^{tilt} = B_{(x^2-y^2)}^{lab} - 2\sqrt{2} B_{xz}^{lab}$$

$$B_{xz}^{tilt} = -\frac{1}{3} B_{xz}^{lab} + \frac{\sqrt{2}}{6} B_{z^2}^{lab} - \frac{\sqrt{2}}{6} B_{(x^2-y^2)}^{lab}$$

$$B_{yz}^{tilt} = \frac{1}{\sqrt{3}} B_{yz}^{lab} - \frac{\sqrt{2}}{\sqrt{3}} B_{xy}^{lab}$$

$$B_{(x^2-y^2)}^{tilt} = \frac{2\sqrt{2}}{3} B_{xz}^{lab} + \frac{1}{3} B_{z^2}^{lab} + \frac{2}{3} B_{(x^2-y^2)}^{lab}$$

$$B_{xy}^{tilt} = \frac{1}{\sqrt{3}} B_{xy}^{lab} + \frac{\sqrt{2}}{\sqrt{3}} B_{yz}^{lab}$$

Third order

$$B_{z^3}^{tilt} = -\frac{2}{3\sqrt{3}} B_{z^3}^{lab} - \frac{1}{\sqrt{6}} B_{z^2x}^{lab} + \frac{5}{\sqrt{3}} B_{xz(x^2-y^2)}^{lab} - \frac{5}{3\sqrt{6}} B_{x^3}^{lab}$$

$$B_{z^2x}^{tilt} = \frac{\sqrt{2}}{3\sqrt{3}} B_{z^3}^{lab} - \frac{\sqrt{3}}{2} B_{z^2x}^{lab} + \frac{5}{6\sqrt{3}} B_{x^3}^{lab}$$

$$B_{z^2y}^{tilt} = -\frac{10\sqrt{2}}{3} B_{xyz}^{lab} + \frac{1}{6} B_{z^2y}^{lab} + \frac{5}{6} B_{y^3}^{lab}$$

$$B_{xz(x^2-y^2)}^{tilt} = -\frac{1}{3\sqrt{3}} B_{z^3}^{lab} - \frac{\sqrt{2}}{3\sqrt{3}} B_{x^3}^{lab}$$

$$B_{xyz}^{tilt} = -\frac{1}{3} B_{xyz}^{lab} + \frac{\sqrt{2}}{12} B_{z^2y}^{lab} - \frac{\sqrt{2}}{12} B_{y^3}^{lab}$$

$$B_{x^3}^{tilt} = \frac{\sqrt{2}}{3\sqrt{3}} B_{z^3}^{lab} + \frac{1}{2\sqrt{3}} B_{z^2x}^{lab} + \frac{2\sqrt{2}}{\sqrt{3}} B_{xz(x^2-y^2)}^{lab} + \frac{5}{6\sqrt{3}} B_{x^3}^{lab}$$

$$B_{y^3}^{tilt} = 2\sqrt{2} B_{xyz}^{lab} + \frac{1}{2} B_{z^2y}^{lab} + \frac{1}{2} B_{y^3}^{lab}$$

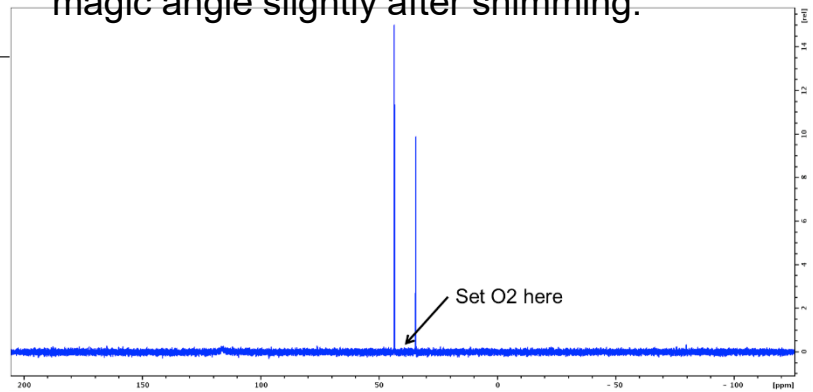
Fourth order

$$B_{z^4}^{tilt} = -\frac{7}{18} B_{z^4}^{lab} + \sum_{m=1}^{4} c_m T_{4m}$$

Fifth order

$$B_{z^5}^{tilt} = -\frac{1}{6\sqrt{5}} B_{z^5}^{lab} + \sum_{m=1}^{5} c_m T_{5m}$$

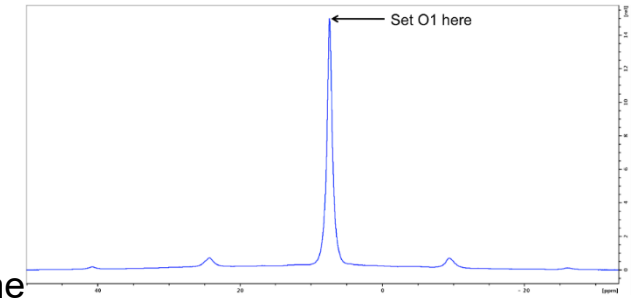
- Align magic angle axis with either x or y axis of shim set. Use simple pulse-acquire sequences
- Spin adamantane at 10 kHz, observe proton resonance (should be close to 2.3 ppm)
- Observe carbon with low power decouple on proton at 2.3 ppm (~50 mW), extend fid acquisition to 200-300 msec
- Set field for left peak at 38.48 ppm
- Shim using the X (or Y) shim and adjust; only the X OR Y shim should effect the shimming, NOT both; Z may help too
- Select the XZ (or YZ) shim and adjust
- Select the XZ^2 (or YZ^2) shim and adjust
- Adjust field as needed to keep peak at 38.48 ppm as decoupling needs to be on resonance since low power
- If shims are way off, you may need to readjust magic angle slightly after shimming.

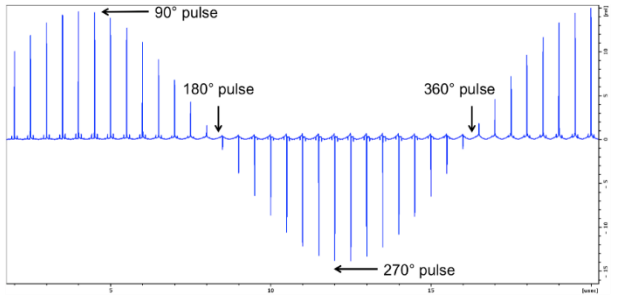


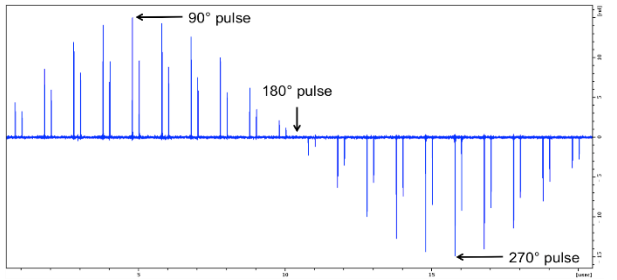
^{13}C spectrum of adamantane, with proper shimming (FWHM ~ 4 Hz)

Calibrating RF

J Magn Reson., 2009, 196:84-87



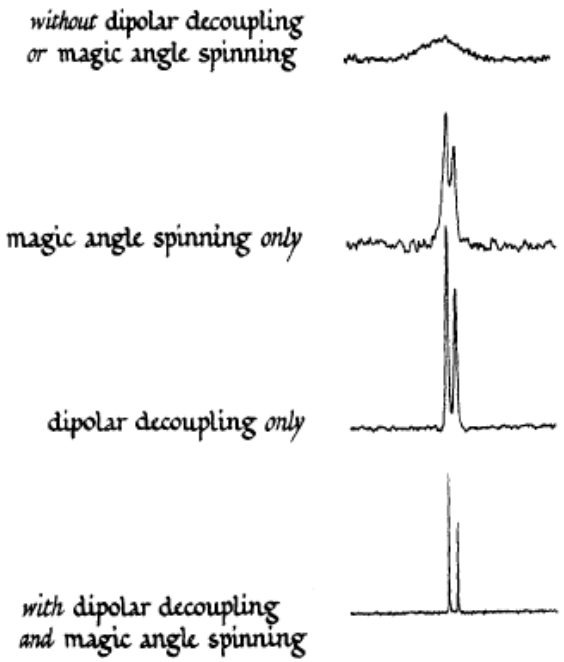


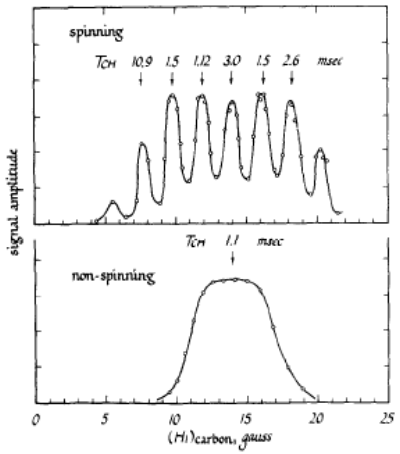


If console/amplifiers are linear, measuring 90 times at 50 W is sufficient to calculate power levels fairly closely for a range of experiments

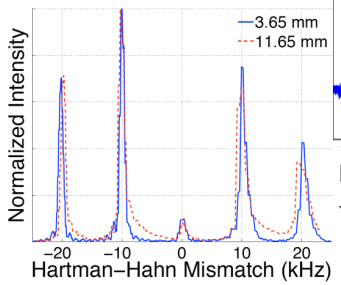
Calibrating a probe:CP and DCP

CP and magic angle spinning
J Magn Reson., 1977, 28:105-112

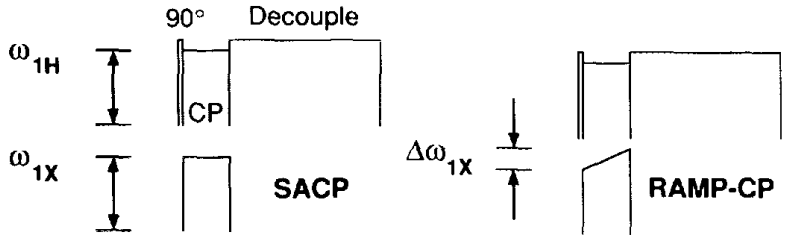


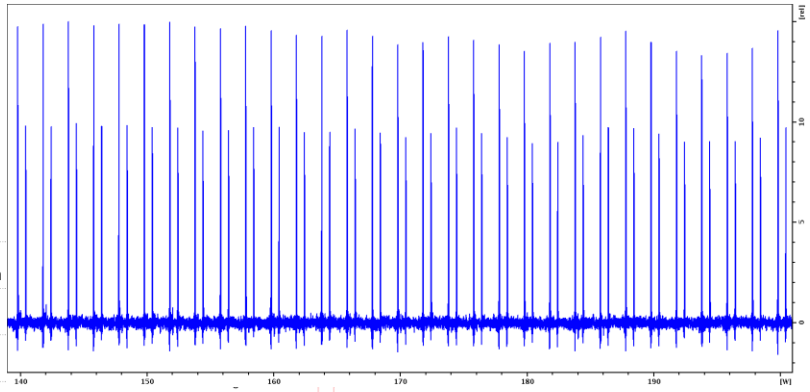


CP match on low-E probe
(10 kHz MAS adamantane)

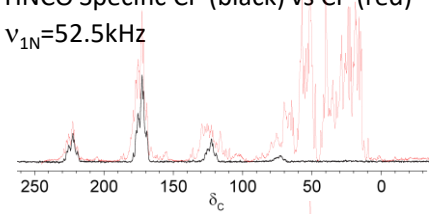


Ramped CP overcomes the MAS dependence (to some extent)
J Magn Reson., 1994, 110:219-227

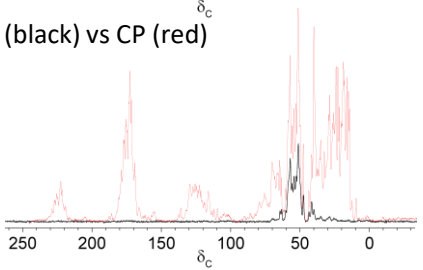




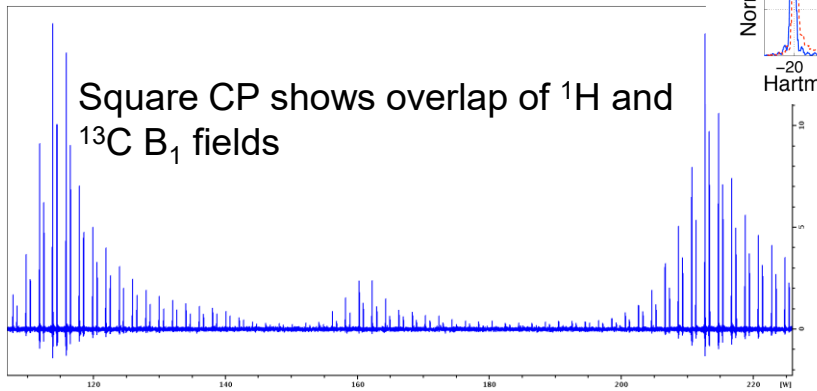
HNCO Specific CP (black) vs CP (red)
ν_{1N}=52.5kHz



HNCA Specific CP (black) vs CP (red)
ν_{1N}=54 kHz



Specificity and efficiency of specific CP (DCP) sequences are dependent on homogeneity of RF and B_1 strength for ^{13}C and ^{15}N



^{13}C power dependence at 10 kHz MAS for a commercial probe

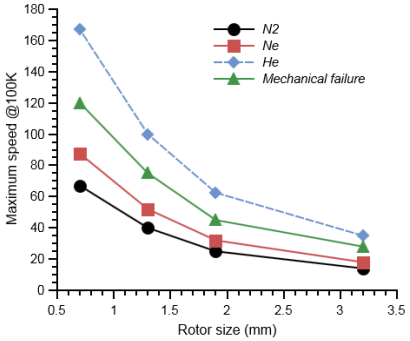
DNP MAS probe cryogenic considerations

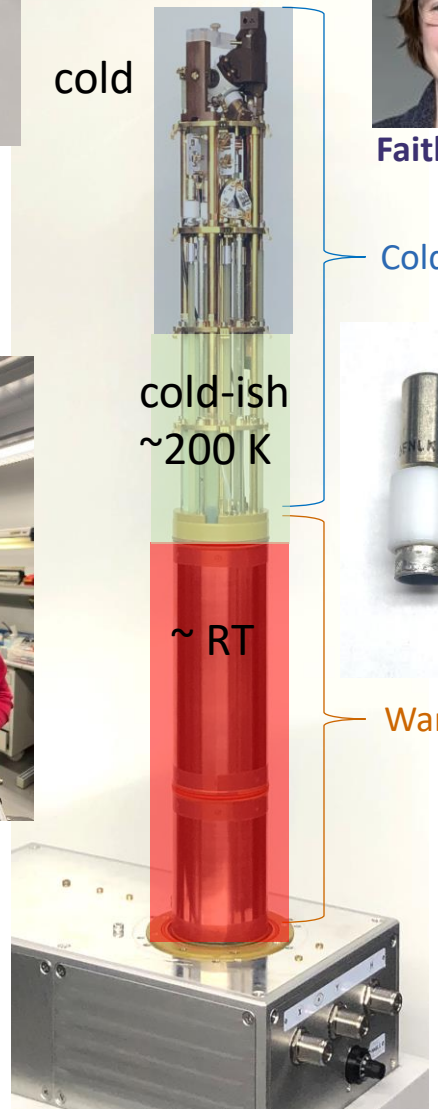
3.2 mm 600 MHz /395 GHz

Collaboration with Bruker

- ✓ Compatible with Bruker LT MAS cooling cabinet
- ✓ **^{1}HXY RF circuit cloned** from ^{1}H-detection probes
- ✓ Large choice of X-Y isotopes by change of tuning cards:

  ^{13}C, ^{15}N, ^{2}H, ^{17}O, ^{31}P, ^{14}N, metalloproteins, etc.
- ✓ Switches between 3- and 2-resonance circuits
- ✓ ^{1}H channel 2x more efficient
- ✓ Magic angle stable at 95K
- ✓ Internal cold space is half as small
  - ✓ <1 hour to warmup (vs. 2-3 hours)
  - ✓ <25” to cool down (vs. 45”)
- ✓ Stable, predictable tuning at 95K
- ✓ Weighs ~1/3 less



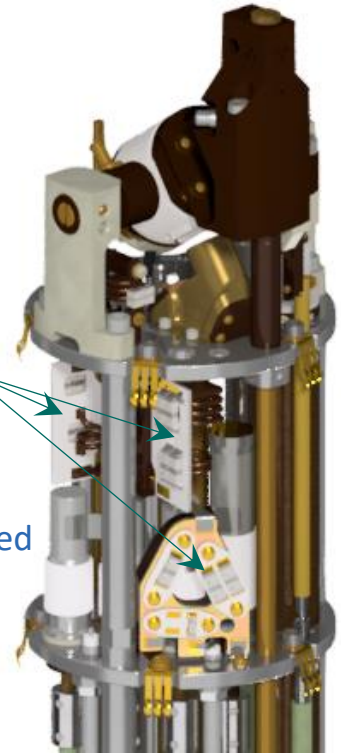


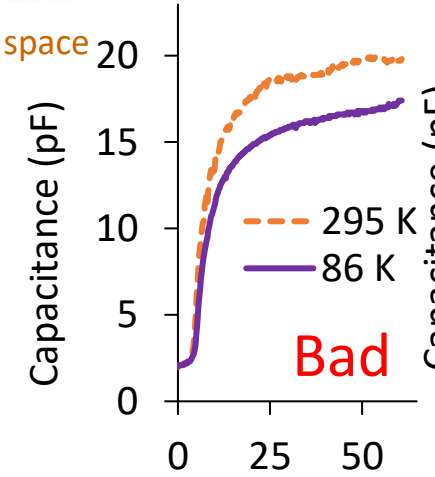
3.2 mm probe w/o outer dewar

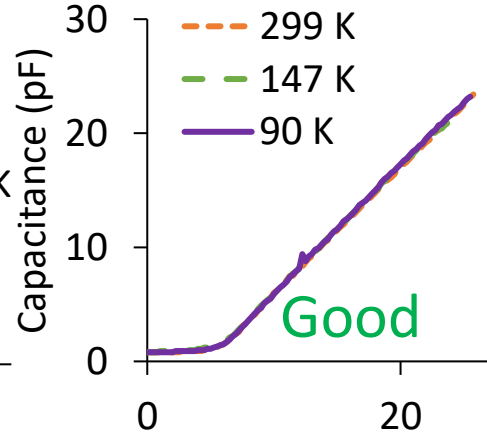
Faith Scott

Peter Gor’kov

Commercial cryogenic trimmers were modified for 95K operation





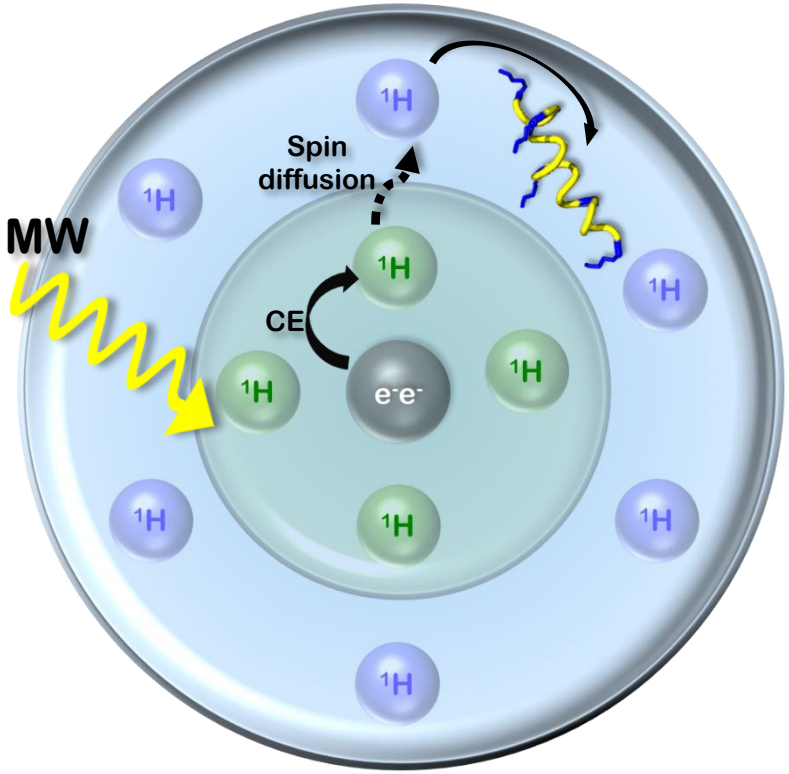


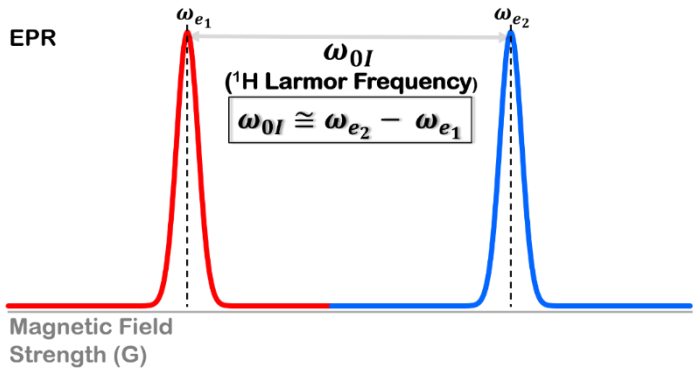
DNP: Practical aspects of determining optimal enhancements

NATIONAL MAGLAB

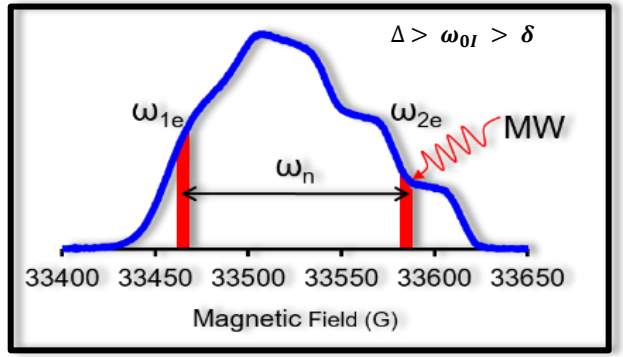
UF UNIVERSITY of FLORIDA

Optimizing enhancements at high field





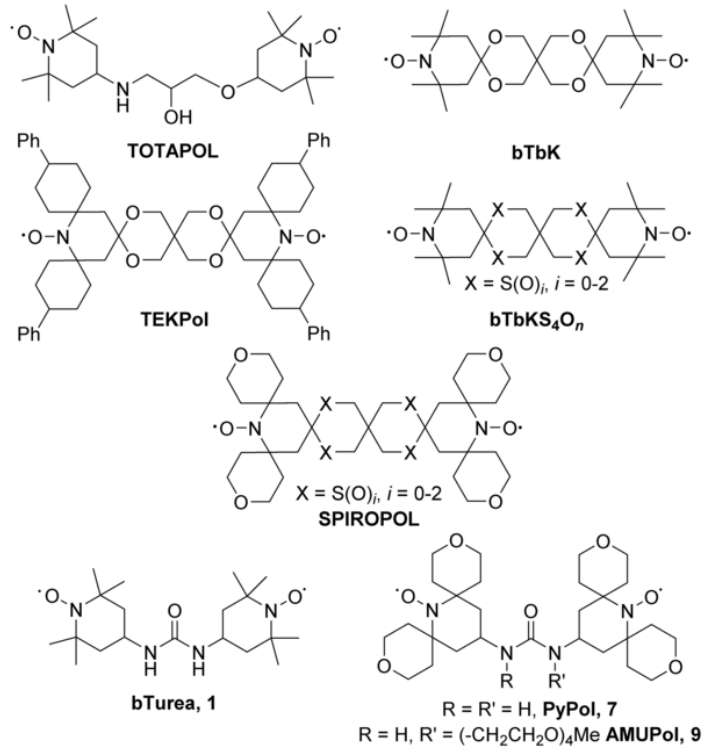
"Wide line radical"



Imhomogenous broadening

- g-anisotropy
- hyperfine interaction

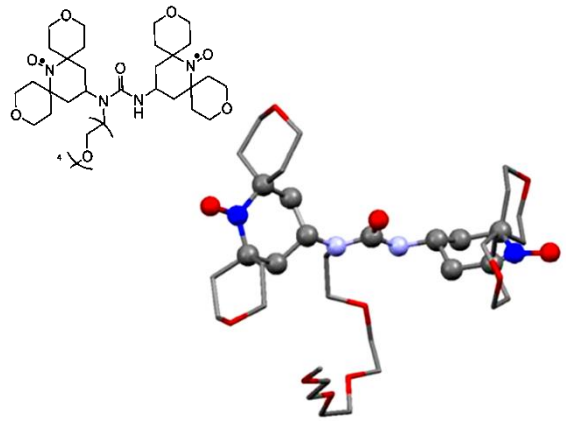
Developing Water Soluble Nitroxide Biradicals



Optimal DNP enhancements

- Perpendicular g-tensor orientation
- strong dipolar coupled e-
- rigid structure

AMUPol



A. Smith et al. *Anal. Chem.* 2016, 88, 122−132
C. Sauvée et al. Chem.Eur.J.2016, 22,5598 –5606
C. Sauvée et al. , *Angew. Chem. Int. Ed* .2013,52, 10858 –10861

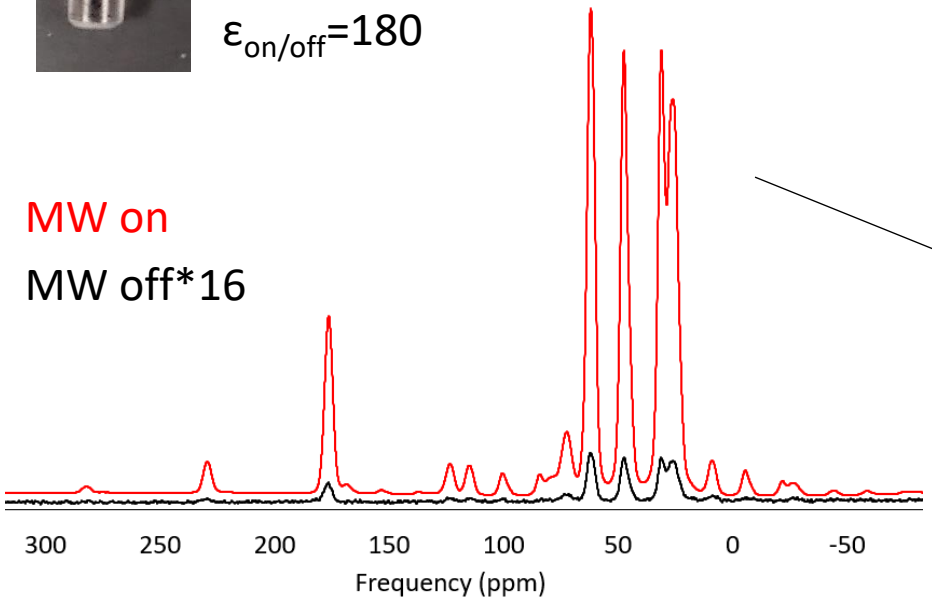
Proline enhancement of ~160 on our instrument (AMUPol) operating at ~95 K

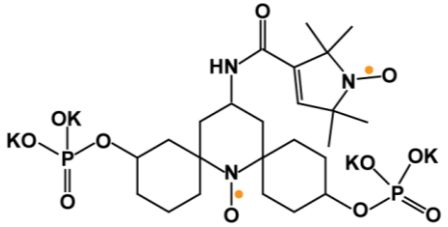
Optimizing enhancements at high field

150 mM ^{13}C-proline
10 mM Amupol
60% d_8-glycerol/30% D_2O/10% H_2O
Standard-wall sapphire rotor
$\varepsilon_{on/off}$=180

Sauvee, C., et al. (2013) *Angew.Chem.Int.*,41
D ~ 35 MHz
|J| ~ 15 MHz

MW on
MW off*16

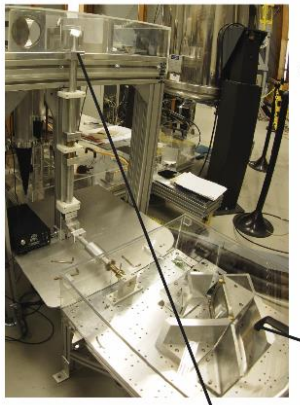


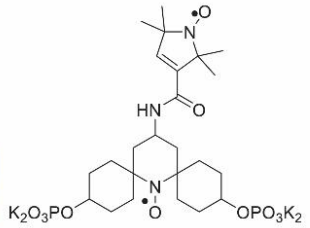


AsymPolPOK

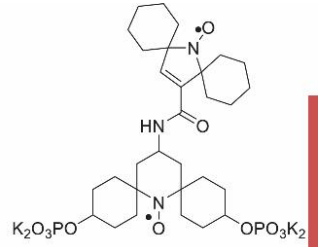
D ~ 56 MHz
|J| ~ 70 MHz

- Faster DNP buildup time
- Insensitive to protonation





AsymPol-POK



cAsymPol-POK

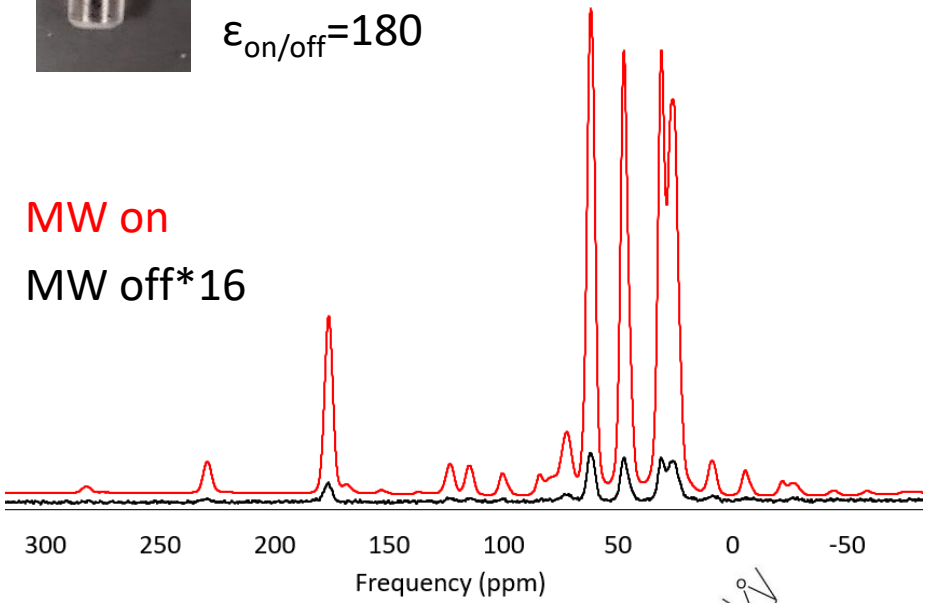
Mentink-Vigier, F., et al. (2018) *J.Am.Chem.Soc.*,141
Harrabi, R et al. (2022) *Ang. Chemie* 61 e202114103

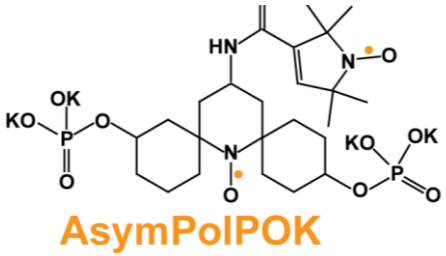
What should our standard be?

150 mM ^{13}C-proline
10 mM AMUpol
60% d_8-glycerol/30% D_2O/10% H_2O
Standard-wall sapphire rotor
$\varepsilon_{on/off}$=180

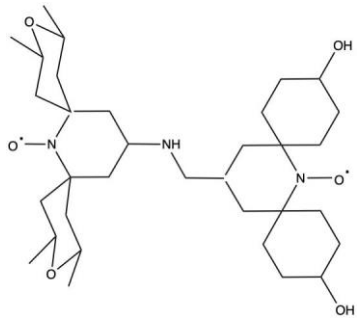
Sauvee, C., et al. (2013) *Angew.Chem.Int.*,41
D ~ 35 MHz
|J| ~ 15 MHz

MW on
MW off*16





AsymPolPOK



Mentink-Vigier, F., et al. (2018) *J.Am.Chem.Soc.*,141
Harrabi, R et al. (2022) *Ang. Chemie* 61 e202114103

Lund, A., et al. (2020) *Chem. Sci.*,11

Considerations:
- Biradical: AMUPol vs AsymPolPOK vs TinyPol
- MAS Temperature: 90 K vs 100 K and higher
- Glassing agent (MW absorption)
- O_2 content
- Sample integrity

Our observations:
- TinyPol and AsymPolPOK give roughly equivalent sensitivity
  - TinyPol wins for on/off; AsymPolPOK wins for buildup time
  - Both enable high levels of protonation
- DNP juice is unreliable
  - Very sensitive to temperature
  - Absorbs MW
  - Hard to control O_2 concentration
  - Denatures proteins/impacts lipid organization
- Other glassing agents work really well
  - Read the cryobiology literature
  - MW absorption is reduced relative to DNP juice
  - DNP buildup times are an order of magnitude faster
  - ***BUT*** don't expect your model compounds to look as good

Summary of DNP conditions (using proline)

$\varepsilon_{on/off}$: deuterate, use tinypol or asympolPOK

Biradical	^{2}H	Juice	DMSO	Betaine
none	yes	--	--	--
	no	--	--	--
10amupol	yes	86	61	39
	no	43	22	12
5asympolpok	yes	75	78	58
	no	75	84	79
10asympolpok	yes	62	***92***	82
	no	60	63	72
10tiny pol	yes	***157***	73	***118***
	no	141	61	50

Overall improvement: no ^{2}H needed!, asympolPOK

Biradical	^{2}H.	Juice	DMSO	Betaine
none	yes	1	2	4
	no	2	6	7
amupol	yes	151	43	74
	no	121	75	45
5asympolpok	yes	162	217	***191***
	no	197	238	168
10asympolpok	yes	279	395	93
	no	***426***	***484***	112
tiny pol	yes	230	135	128
	no	223	91	123

Luiza Caldas Nogueira

DNP Improvement: no ^{2}H, asympolPOK

Biradical	^{2}H.	Juice	DMSO	Betaine
none	yes	0.6	0.3	0.6
	no	1	1	1
amupol	yes	97	14	11
	no	78	8	7
5asympolpok	yes	103	39	14
	no	126	43	17
10asympolpok	yes	179	72	***28***
	no	***272***	***88***	25
tiny pol	yes	147	24	19
	no	143	17	18

Considerations:

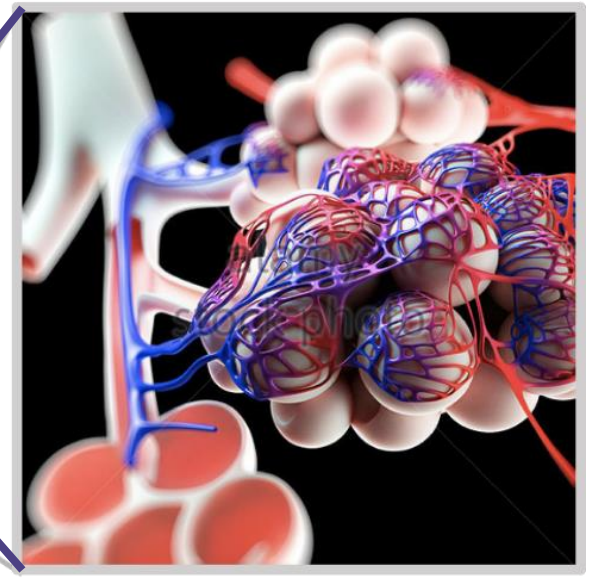
- Field: (we have a 600 MHz / 396 GHz instrument)
- MAS rate and Temperature (10.4 kHz and 90 K)
- Solvent/Glassing agent (MW absorption, Temp/phase behavior)
- O_2 content (solubility, degassing)
- Enhancement vs. Improvement--SNR/sqrt(Tb)
- Methyl-containing glassing agents degrade enhancement but improve SNR/sqrt(Tb) and likely reflect performance in biological samples
- ***Sample integrity***

DNP: What makes me want to add a gyrotron to all my magnets

NATIONAL MAGLAB

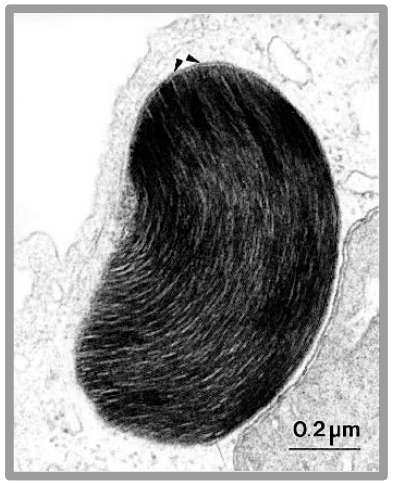
UF UNIVERSITY of FLORIDA

Pulmonary surfactant and lipid trafficking

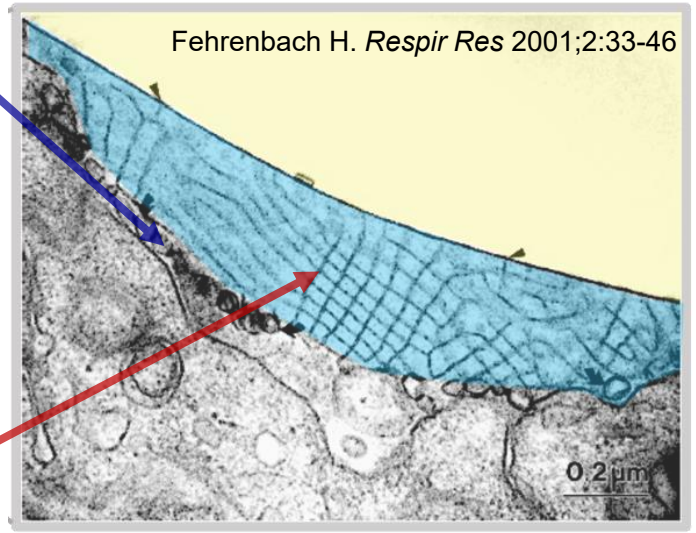


- Surface area ~300 cm^2 per cm^3 (~40x that of skin)
- Type II epithelial cells secrete pulmonary surfactant (PS) as lamellar bodies to lower surface tension
- Lamellar Body → Tubular Myelin → Air/Water interface (monolayer with reservoir beneath)
- PS is highly dynamic with lipid half lives of 5-10 hrs
- Surfactant protein B (SP-B) is absolutely required for lamellar body formation, trafficking into tubular myelin, and the recycling of PS

PS Lamellar Body

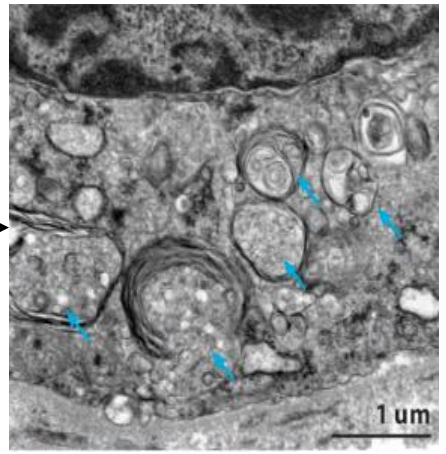


Tubular Myelin



Knockout of SP-B in mice is lethal

ΔSP-B



Whitsett JA. Annu. Rev. Med. 2010;61:105-119

SP-B: Protein critical to lipid trafficking

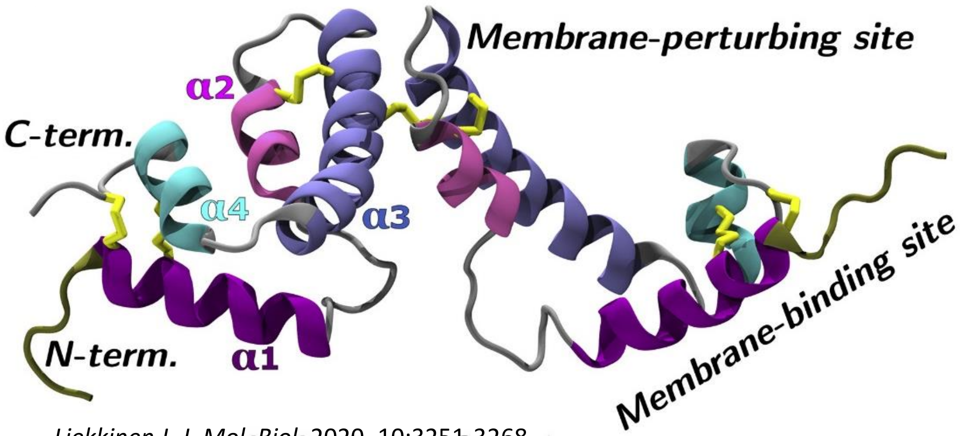
FPIPLPYCWLCRALIKRIQAMIPKGALAVAVAQVCRVVPLVAGGICQCLAERYSVILLDTLLGRMLPQLVCRLVLRCSMD

SP-B

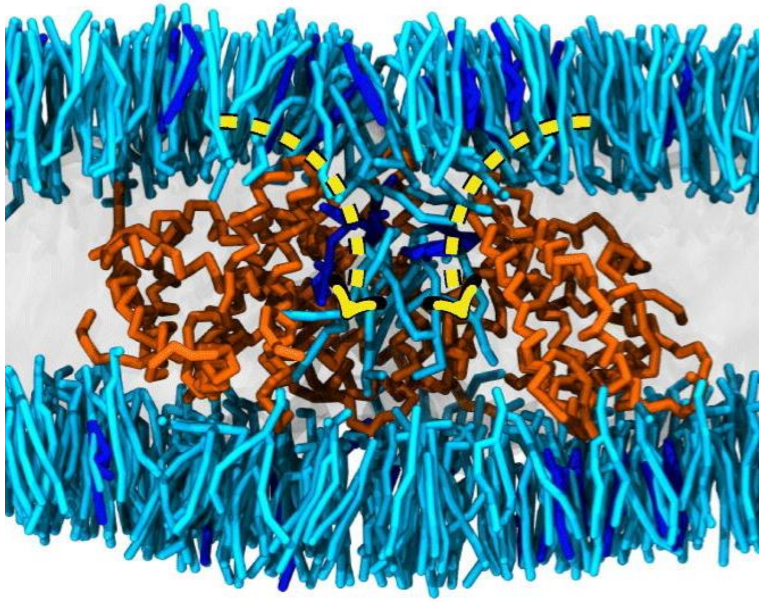
FDA approved ~1989

Most hydrophobic member of saposin family

Saposin B homology model



Liekkinen J. J. Mol. Biol. 2020, 10:3251-3268



$SP\text{-}B_{1-25}$
FPIPLPYCWLCRALIKRIQAMIPKG

$SP\text{-}B_{59-80}$
DTLLGRMLPQLVCRLVLRCSMD

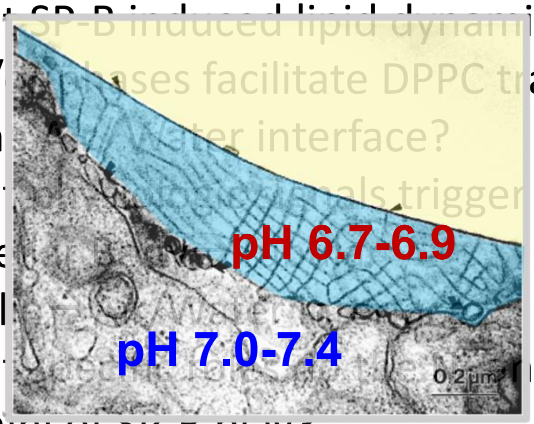
KL_4
KLLLLKLLLLKLLLLKLLLLK

Surfaxin
30 mg/mL phospholipids (22.5 mg DPPC + 7.5 mg POPG) with 4.05 mg palmitic acid and 0.862 mg KL_4 (~1 mol% relative to the phospholipids) suspended in tris buffer, pH 7.4

Surfaxin
(lucinactant)
Intratracheal Suspension

FDA approved 2012

- What SP-B induced lipid dynamics and/ ... phases facilitate DPPC transfer to th ... ater interface?
- What ... als trigger Lame ... Myel ...
- What ... nd C-termini of SP-B play?



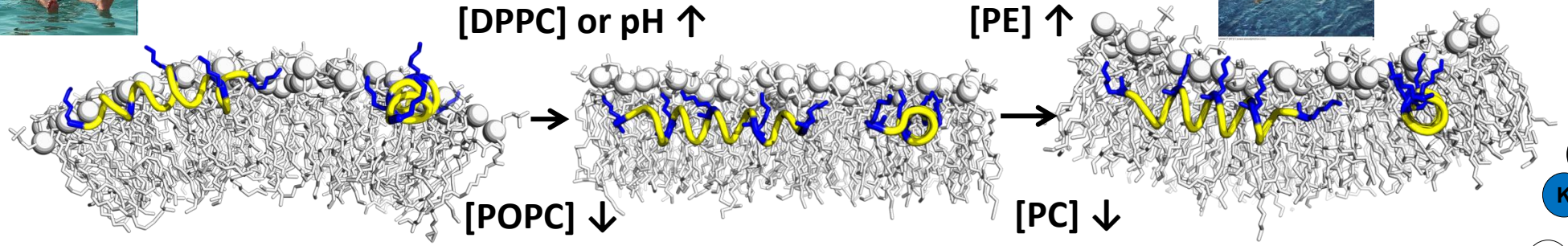
Partitioning, pH, and helicity of KL_4

KLLLLKLLLLKLLLLKLLLLK

Otonye Braide-Moncoeur

[DPPC] or pH ↑

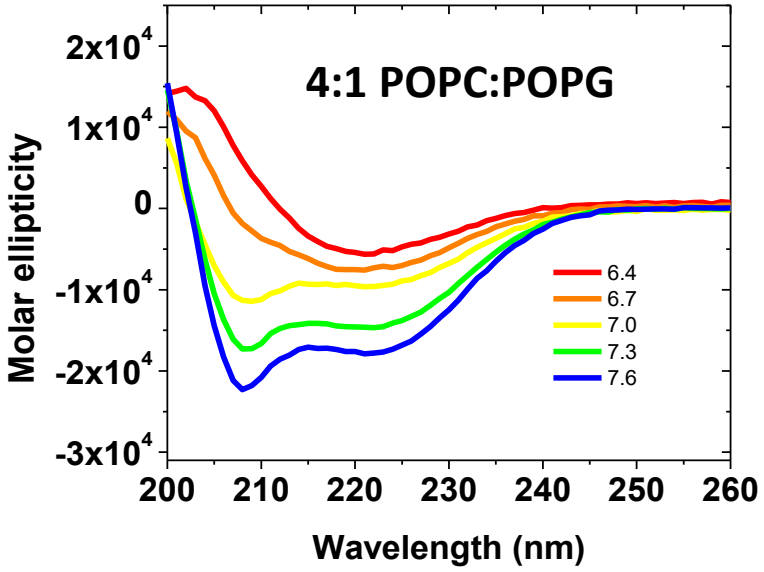
[PE] ↑



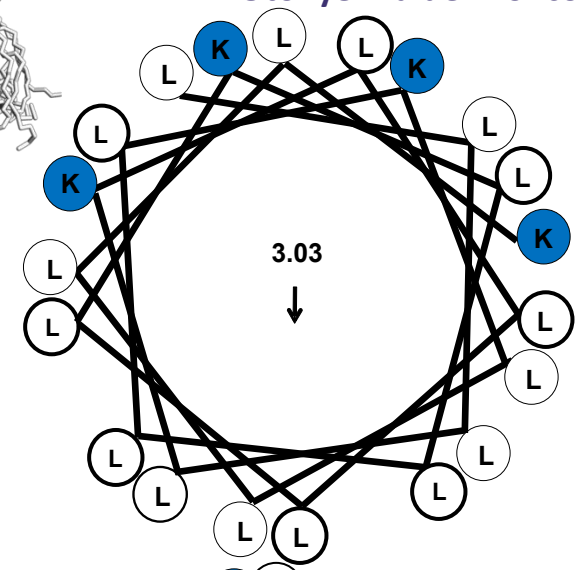
[POPC] ↓

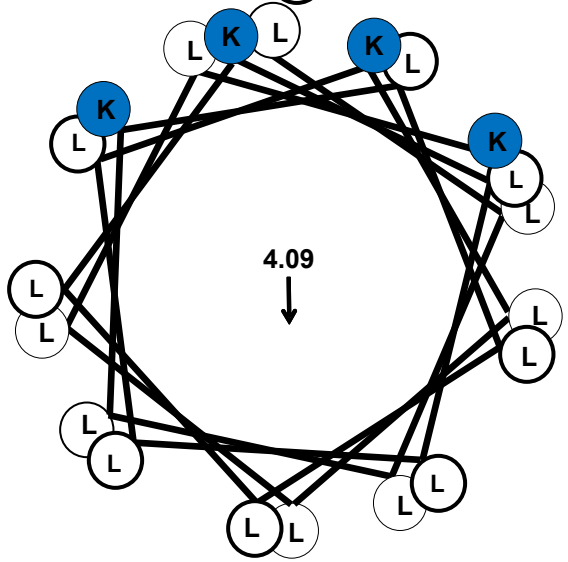
[PC] ↓

Current Opinion in Chemical Biology 32: 22-28 (2015)
BBA - Biomembranes 1838: 3212-3219 (2014)
BBA - Biomembranes 1798: 216-222 (2009)
Biophysical Journal 96: 4085-98 (2009)
Biochemistry. 47: 8292-8300 (2008)



- Deeper peptide partitioning in DPPC:POPG stabilizes a planar lipid assembly
- More shallow partitioning in POPC:POPG leads to positive curvature strain
- This enables differentiation of lipids for selective trafficking of DPPC to the air/water interface
- pH has marked effect on helicity





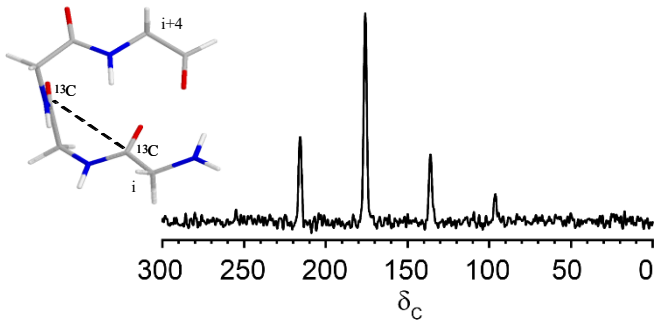
Optimal behavior (and clinically relevant) in lipids at 1:50 peptide:lipid ratio

pH-dependent characterization of KL_4

KL_4 1-^{13}C-L4,L5 in 4:1 POPC / POPG

CPMAS

DQ filtered spectrum



KL_4 in 4:1 POPC / POPG

L4,L5
pH 6.7

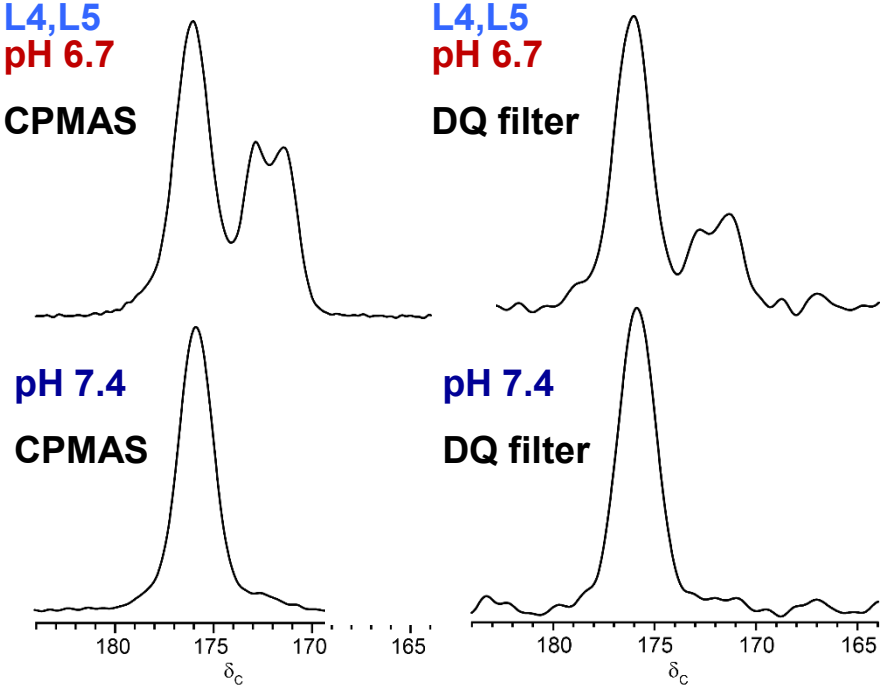
CPMAS

DQ filter

pH 7.4

CPMAS

DQ filter



KL_4 in 4:1 DPPC / POPG, pH 6.7

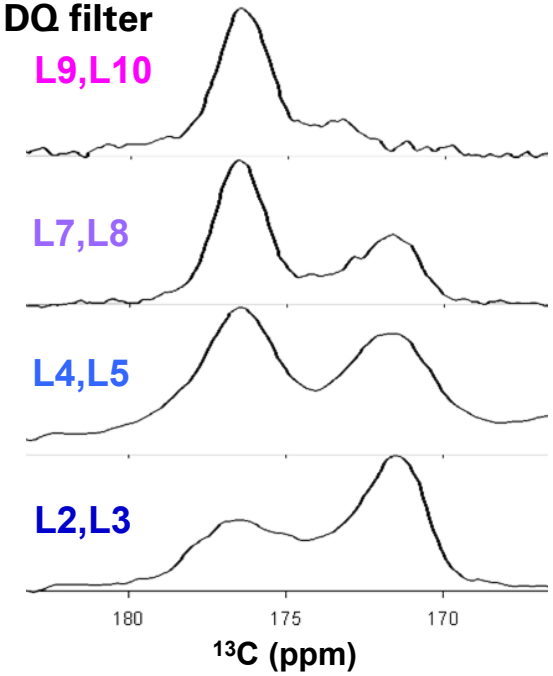
DQ filter

L9,L10

L7,L8

L4,L5

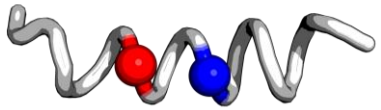
L2,L3



DNP CPMAS, ~95 K

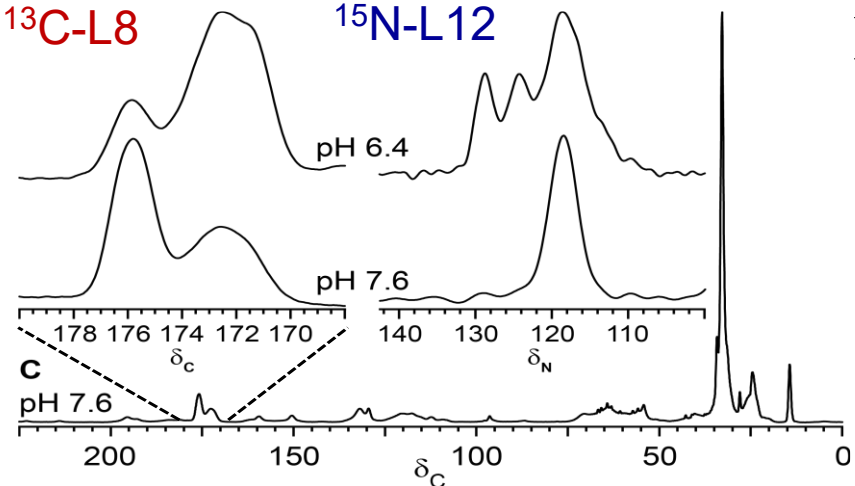
Sample frozen in rotor on insertion into probe

KL_4 1-^{13}C-L8, ^{15}N-L12



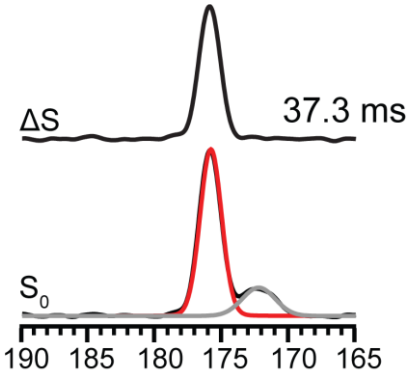
i,i+4

^{13}C-L8 ^{15}N-L12



KLLLLKLLLLKLLLLKLLLLK

REDOR, ~95 K



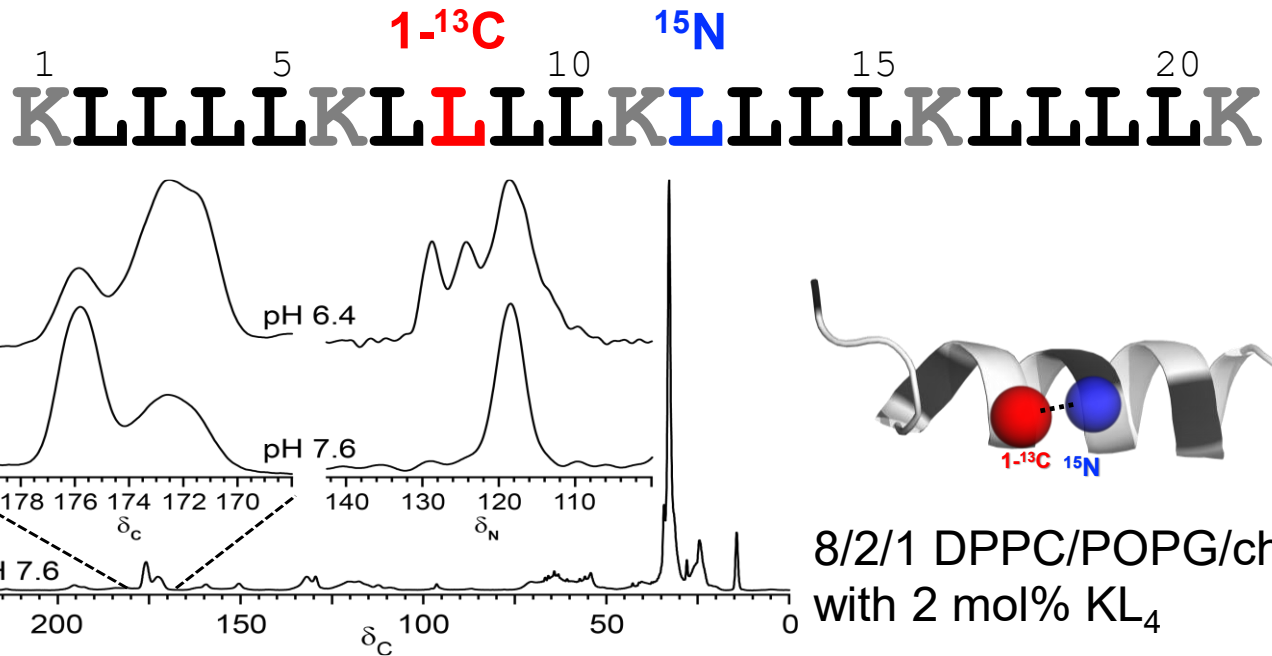
Buffers / pH are also important to consider

Biochem J.
167: 593-600 (1977)

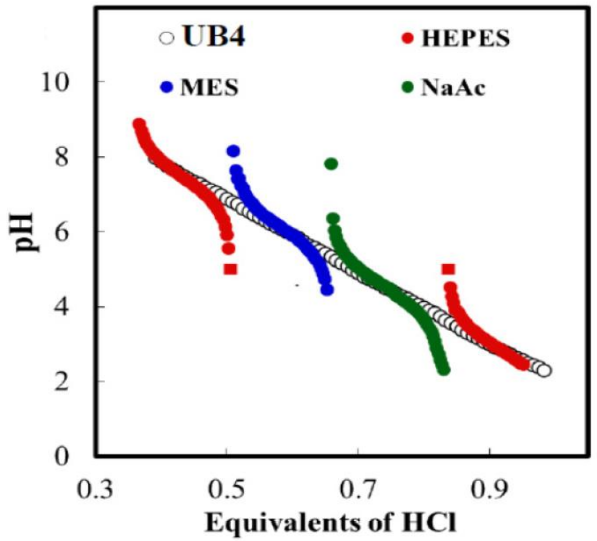
Buffer	$\Delta pH_{app.}$ for pH 8.1–8.2 at 20°C Manual freeze	$\Delta pH_{app.}$ for pH 6.9–7.4 at 20°C Rapid freeze	Manual freeze: Alone	Manual freeze: With albumin	Average pH change (without albumin)
Tris	+1.6±0.5	+2.1±0.3	+3.3±0.4*	+0.1±0.2	+2.3
Taps	+1.8±0.6	+2.0±0.5	+0.9±0.7	—	+1.6
Morpholine	+1.3±0.2	—	—	—	+1.3
Hepps	+0.6±0.2	—	—	—	+0.6
Bicine	+0.4±0.2	−0.4±0.2	−0.4±0.1*	0 ±0.1	−0.1
Tricine	—	0 ±0.2	−0.4±0.1	+0.1±0.2	−0.2
Hepes	—	—	−0.4±0.2	+0.4±0.1	−0.4
Potassium phosphate	−1.2±0.1	−1.4±0.7	−1.1±0.3*	+0.1±0.1	−1.2
Sodium phosphate	—	−1.8±0.2	−1.9±0.5*	−0.3±0.1	−1.8
Sodium pyrophosphate	−2.5±0.2	−2.9±0.3	−2.9±0.7*	−1.2±0.5	−2.8

AIMS Biophysics
2:336-342 (2015)

Buffer	pKa at 25°C	dpKa/°C at pH 7.0
Bis-Tris	6.46	N/A
HEPES	7.55	−0.014
MES	6.15	−0.011
NaAc	4.76	Negligible[1]
Tricine	8.05	−0.021
Tris	8.06	−0.028

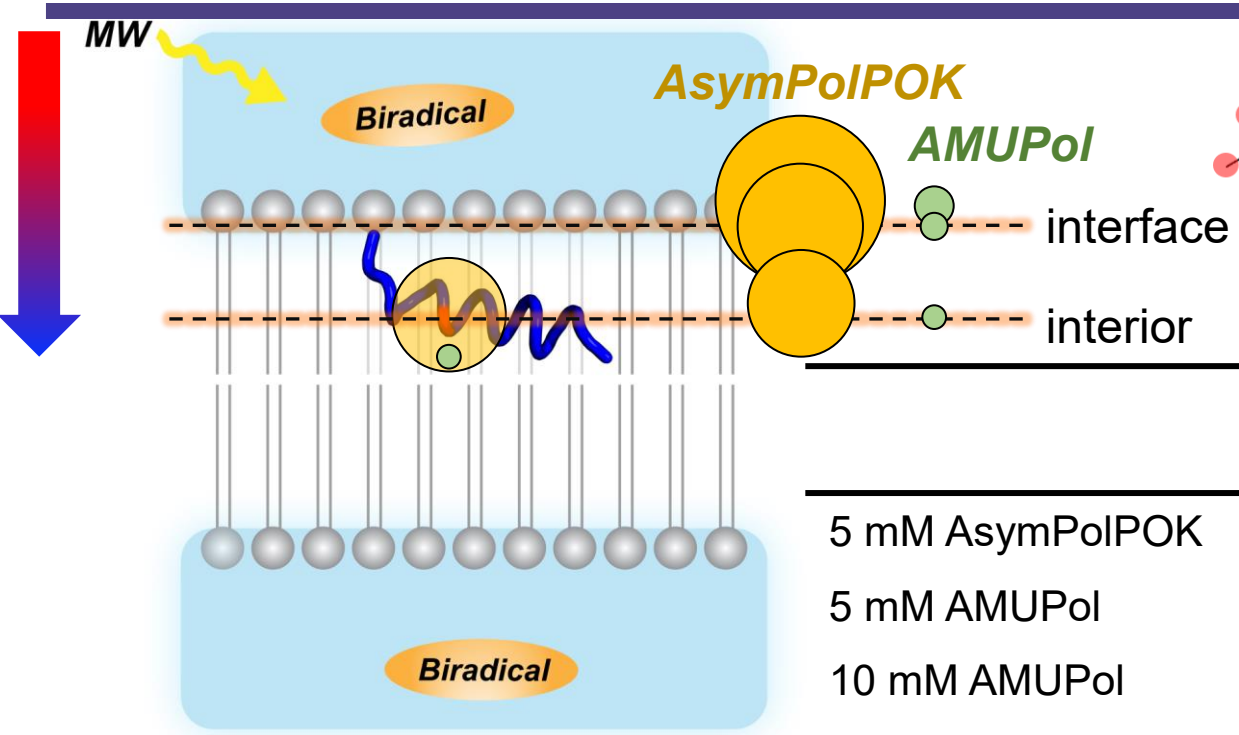


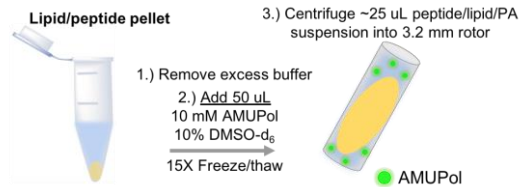
8/2/1 DPPC/POPG/cholesterol MLVs with 2 mol% KL_4

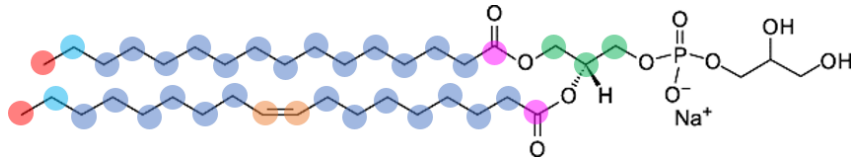


Archives Biochem. Biophysics **384**: 398-406 (2000)
Biotechnol. Prog. **26**:727-733 (2010)

KL_4 DNP enhancements



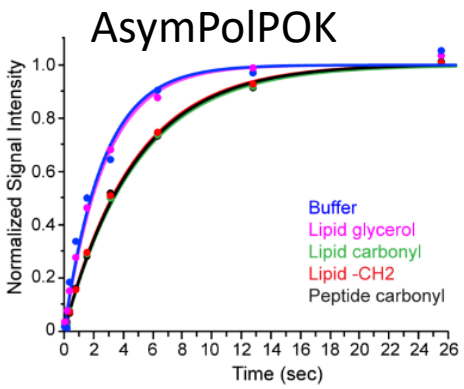


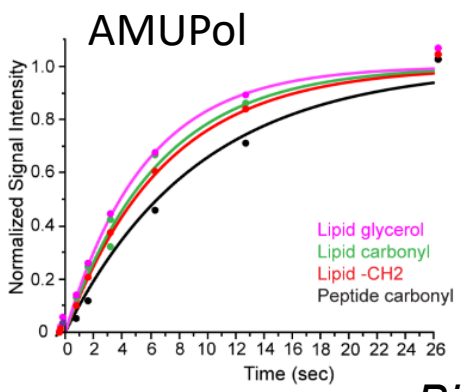


With AsymPolPOK,
only the water is deuterated!

	Buffer	Lipid glycerol	Lipid C=O	KL4 C=O	$-CH_2$
5 mM AsymPolPOK	$93^{\pm 27}$	$57^{\pm 2}$	$42^{\pm 6}$	$38^{\pm 3}$	$36^{\pm 0.5}$
5 mM AMUPol	$20^{\pm 10}$	$13^{\pm 0.5}$	$9^{\pm 1.2}$	$8^{\pm 1.2}$	$8^{\pm 0.1}$
10 mM AMUPol	50	24	13	14	14

Biomolecules. 2020, 10, 1246.



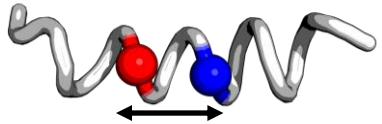


Nhi Tran

Biomolecules. 2020, 10, 1246.

$^{13}C\{^{15}N\}$ REDOR: Conventional ssNMR & DNP

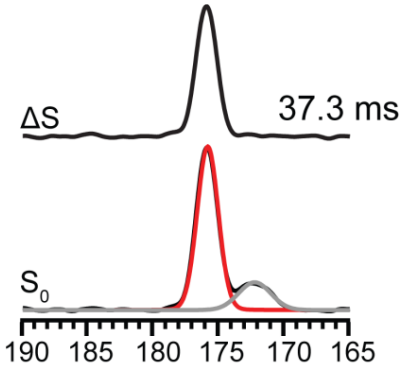
KL_4 1-^{13}C-Leu8, ^{15}N-Leu12, pH 7.4



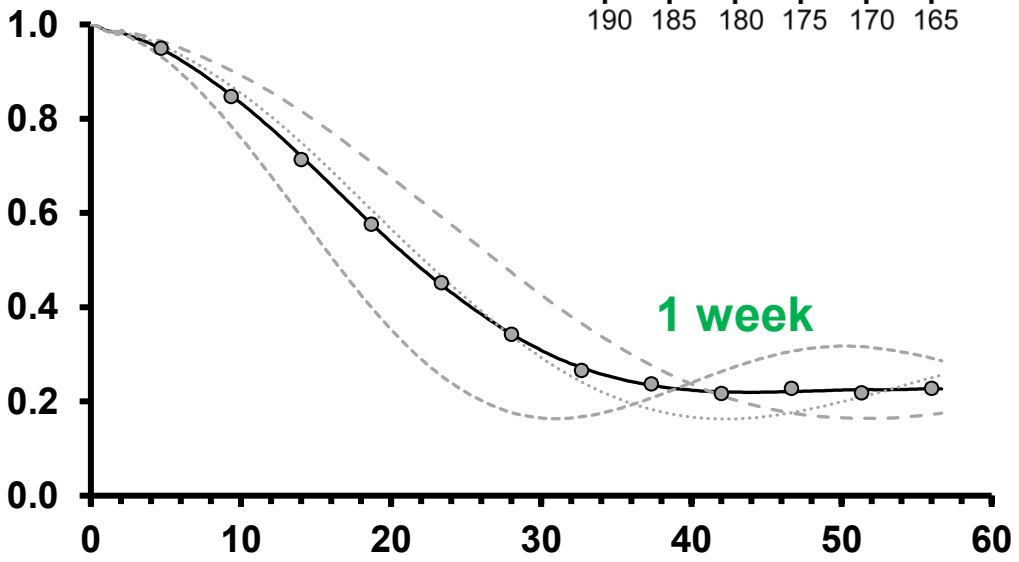
i,i+4

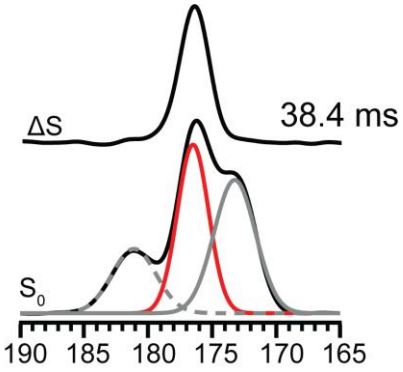
Luiza Caldas Nogueira

Diana Tymochko

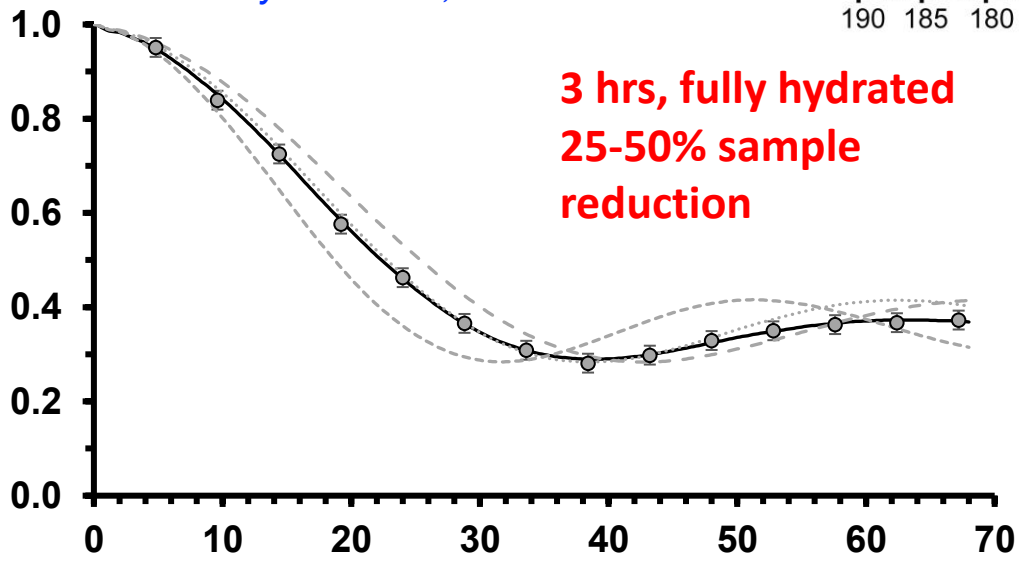


Lyophilized peptide/lipid
238K, 750 MHz, 12 kHz





Hydrated peptide/lipid
100K, 600 MHz/395 GHz,
5mM AsymPolPOK, 10 kHz



3 hrs, fully hydrated
25-50% sample reduction

Measuring multiple conformers

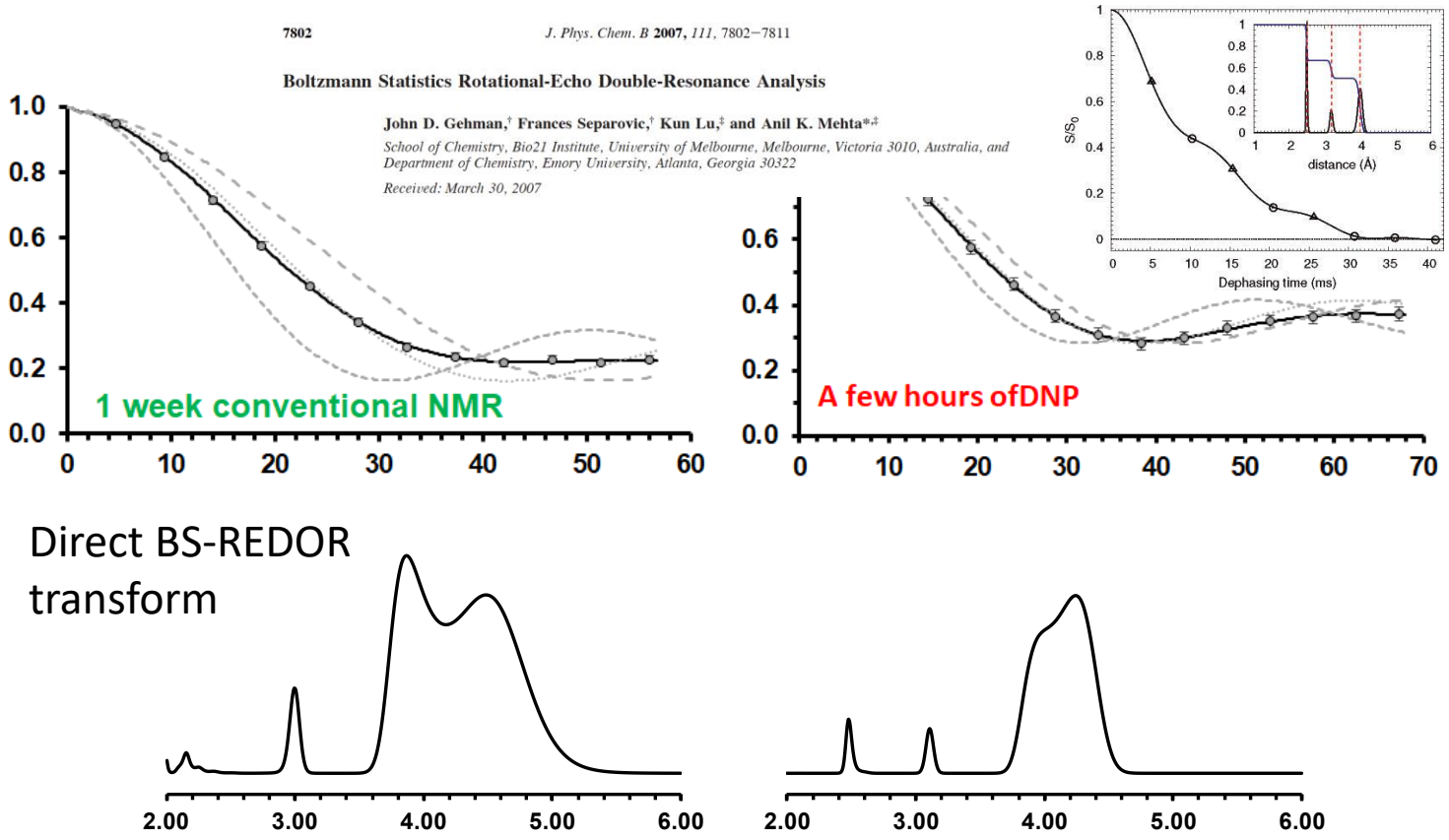


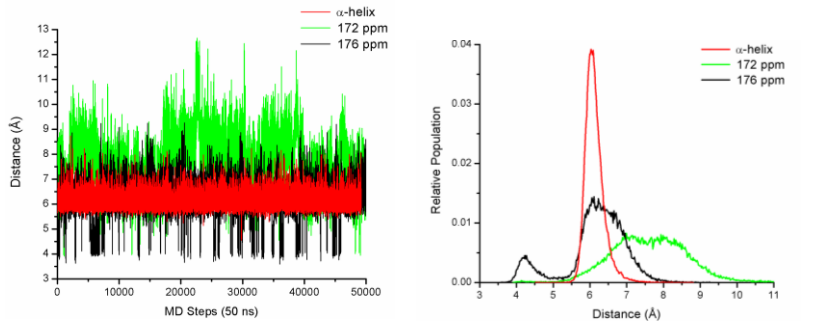
Table 1: Distances from ^{15}N for ideal and x-ray structures.

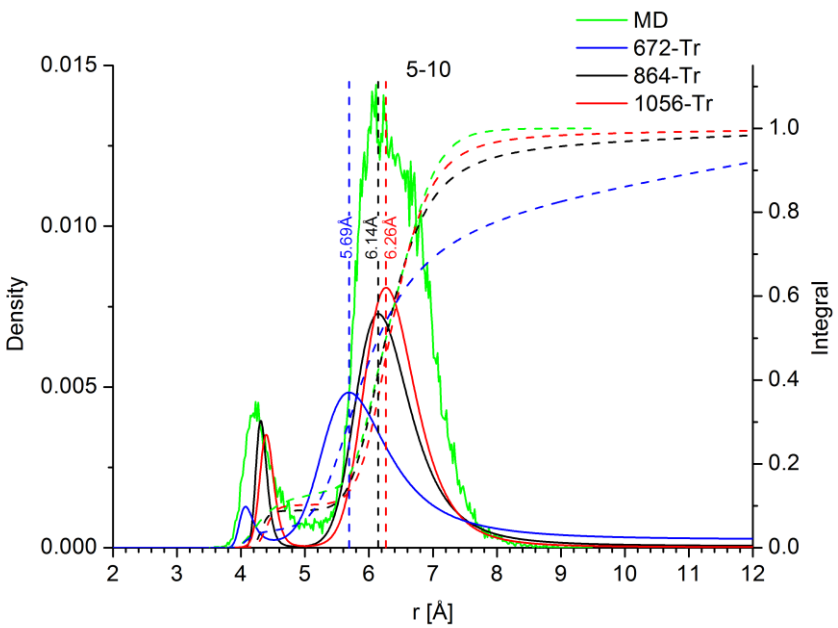
^{13}CO	α-helix -52°,-53°	α-helix GCN4 1CE9.pdb	π-helix -57°,-70°	2QD1 resid 342-353
i-1	1.38 Å	**1.33 Å**	1.38 Å	1.32 Å
i-2	3.30 Å	**3.29 Å**	3.59 Å	3.54 Å
i-3	3.64 Å	**3.71 Å**	4.21 Å	5.18 Å
i-4	**3.84 Å**	**4.19 Å**	**3.08 Å**	**4.18 Å**
i-5	5.73 Å	6.05 Å	3.06 Å	4.39 Å
i+1	2.5 Å	**2.5 Å**	2.5 Å	2.5 Å

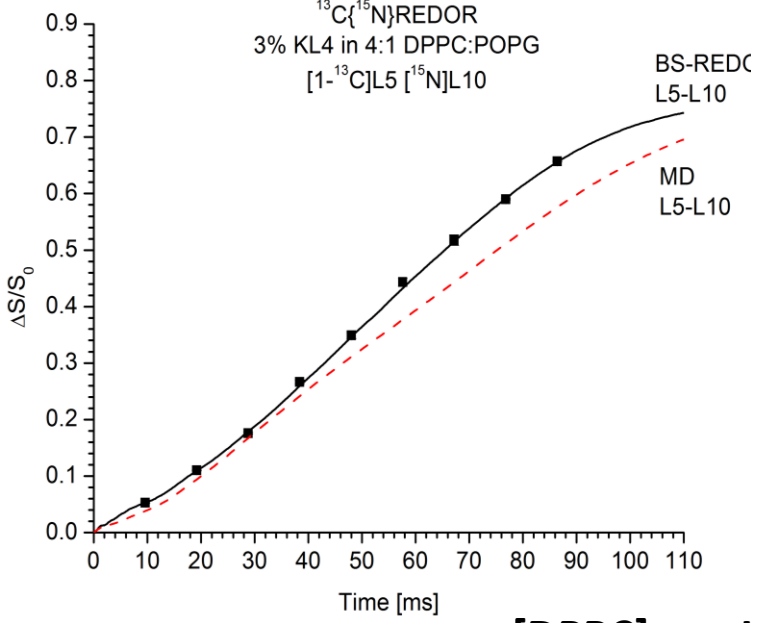
- RF inhomogeneity / natural abundance background corrected by L-curve

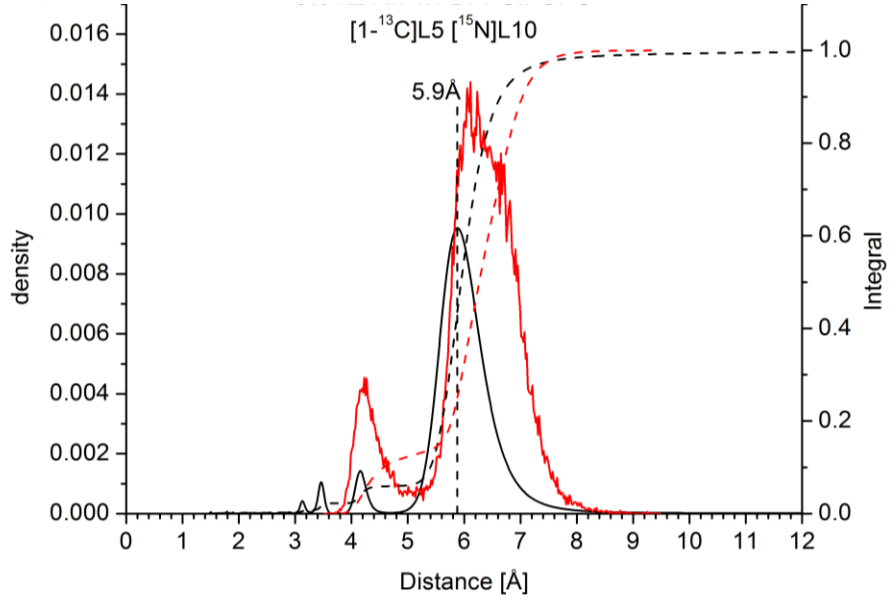
MD guidance of next experiments

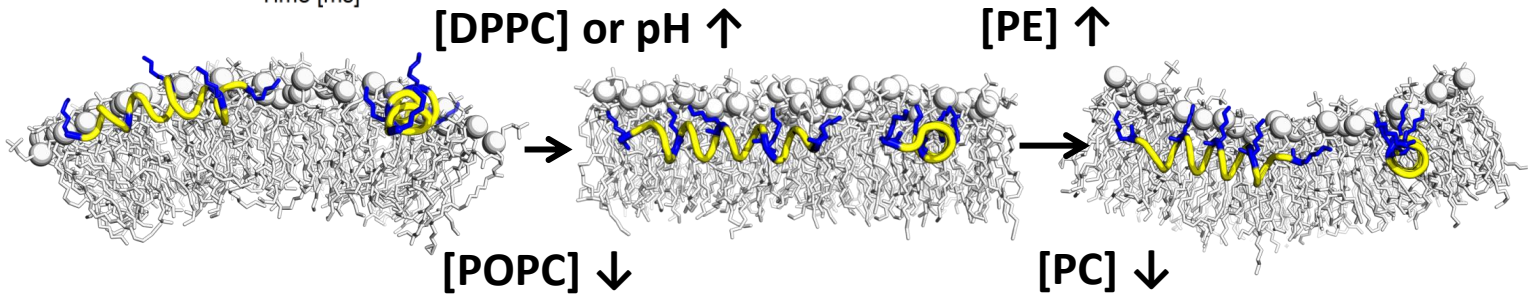
KL_4 1-^{13}C-Leu5, ^{15}N-Leu10, pH 7.4 in DPPC/POPG











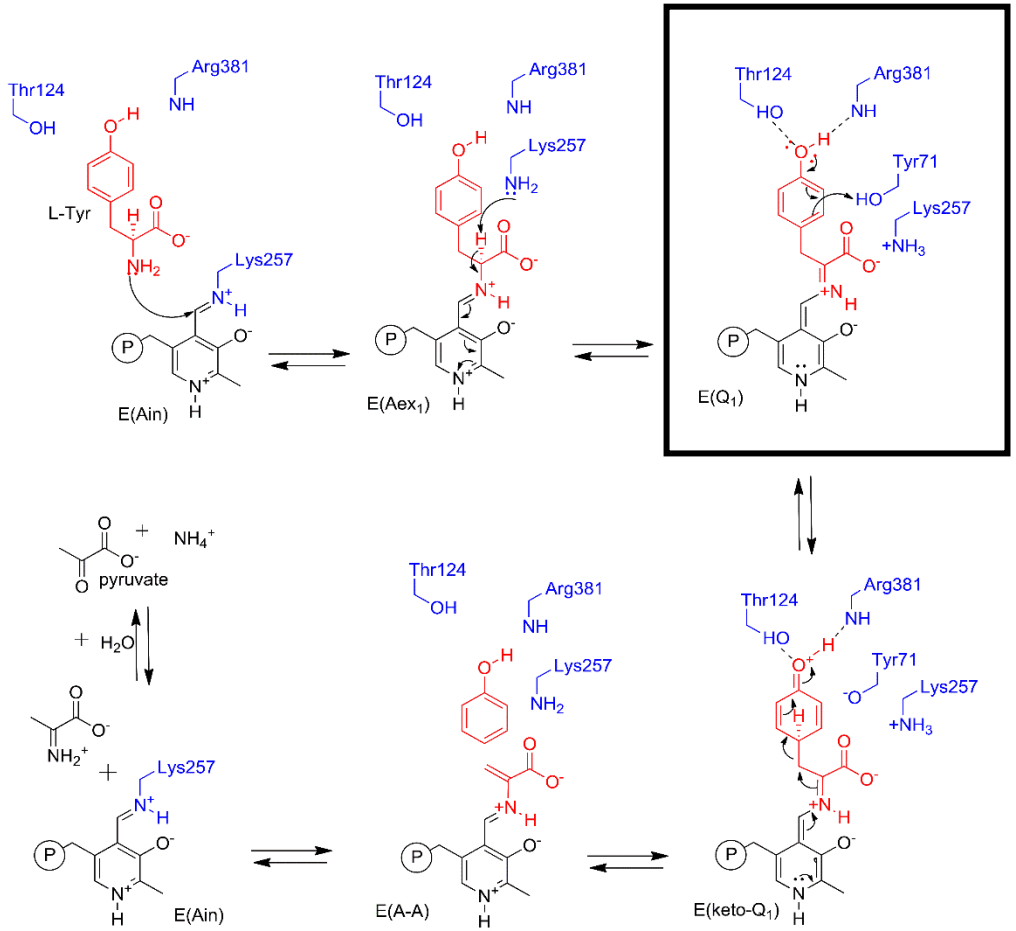
Measurements underway for six sample conditions,
~40 constraints per condition (~120 days with DNP under ideal operations)

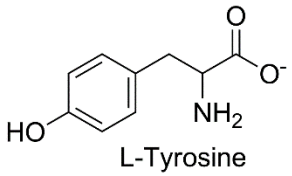
- MD predicts fluctuations in hydrogen bonding patterns with a bimodal distance distribution
- MD guides experimental REDOR measurements needed to complete our model of KL_4 coupling partitioning and helicity
- Differences between initial MD and experimental data now refining MD (iterative fitting)

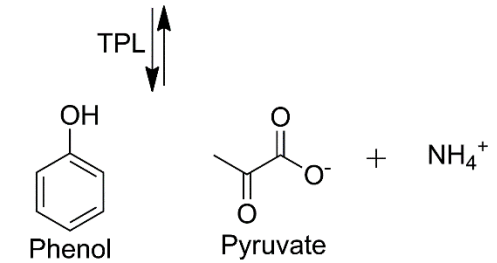
Tyrosine Phenol-Lyase (TPL)

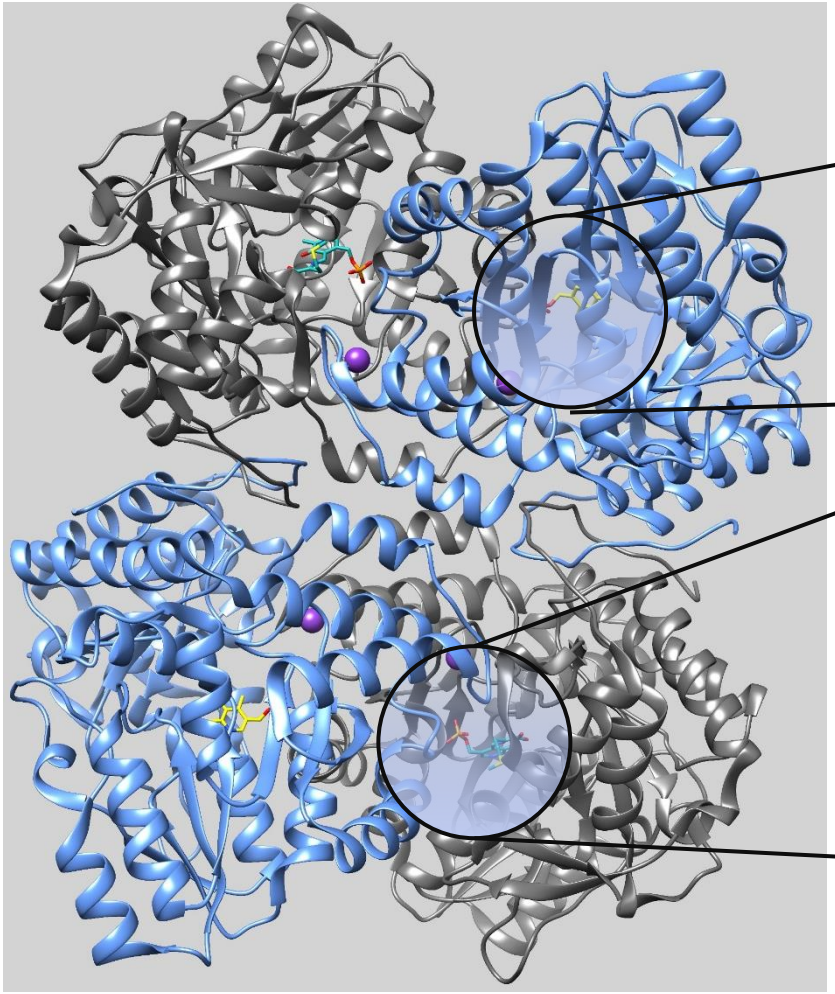
Tetramer complex

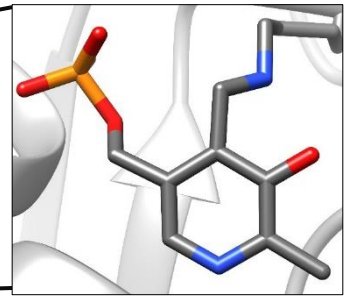
206 kDa effective asymmetric unit

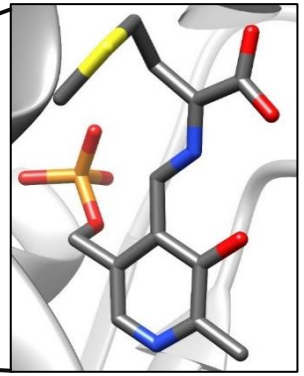








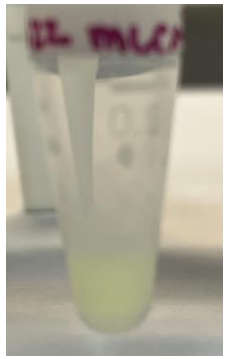




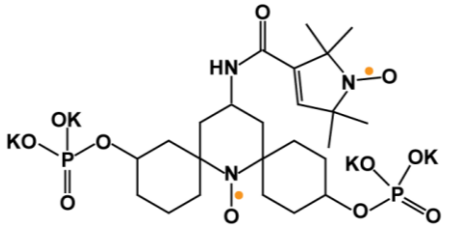
Phillips, R. S., Craig, S., Kovalevsky, A., Gerlits, O., Weiss, K., Iorgu, A. I., ... & Hay, S. (2019). Pressure and temperature effects on the formation of aminoacrylate intermediates of tyrosine phenol-lyase demonstrate reaction dynamics. *ACS Catalysis*, *10*(3), 1692-1703.

Milic, D., Demidkina, T. V., Faleev, N. G., Matkovic-Cˇalogovic, D., & Antson, A. A. (2008). Insights into the Catalytic Mechanism of Tyrosine Phenol-lyase from X-ray Structures of Quinonoid Intermediates⁎. *Journal of Biological Chemistry*, *283*(43), 29206-29214.

Sample preparation and evaluation: TPL example



10x solution in crystallization buffer

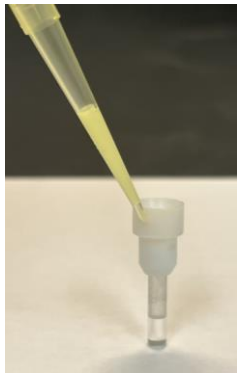


AsymPolPOK

Mentink-Vigier, F., et al. (2018) *J.Am.Chem.Soc.,*141

D ~ 56 MHz
|J| ~ 70 MHz

- 5 mM final concentration
- Faster DNP buildup time
- Insensitive to protonation



Protein microcrystals
10% DMSO as glassing agent
optimized crystallization conditions

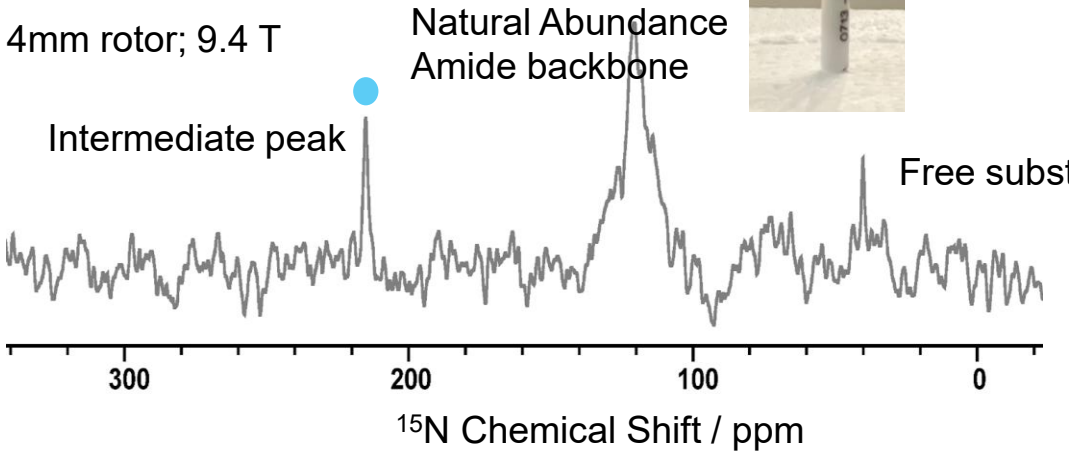
Rittik Ghosh

Luiza Caldas Nogueira

^{15}N, ^{13}C L-Methionine added to unlabeled protein

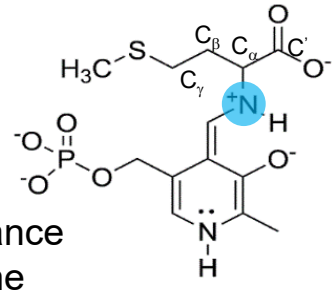
ssNMR ~40 hrs (~283 K)

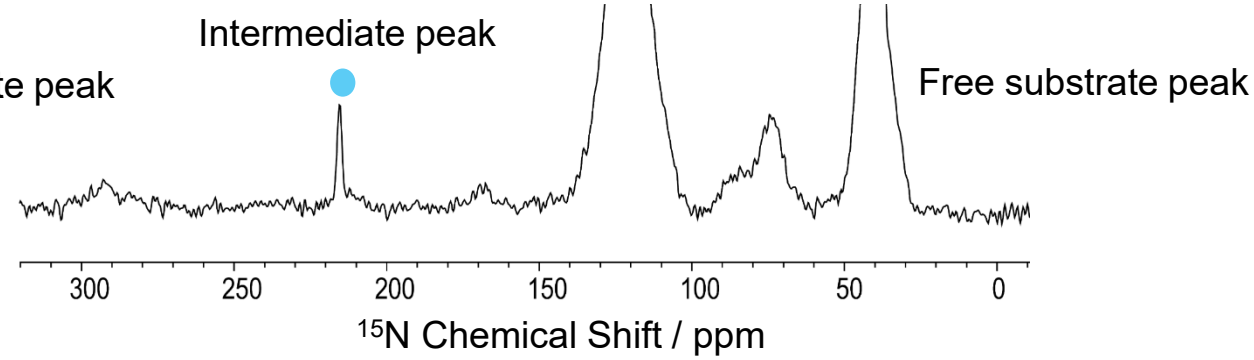
4mm rotor; 9.4 T



DNP <1 hr (~95 K)

~1.5 ppm LW



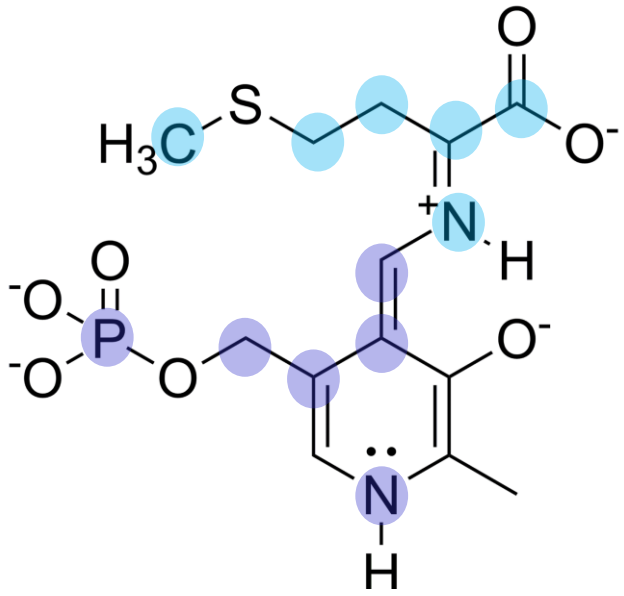


TPL quinonoid intermediate

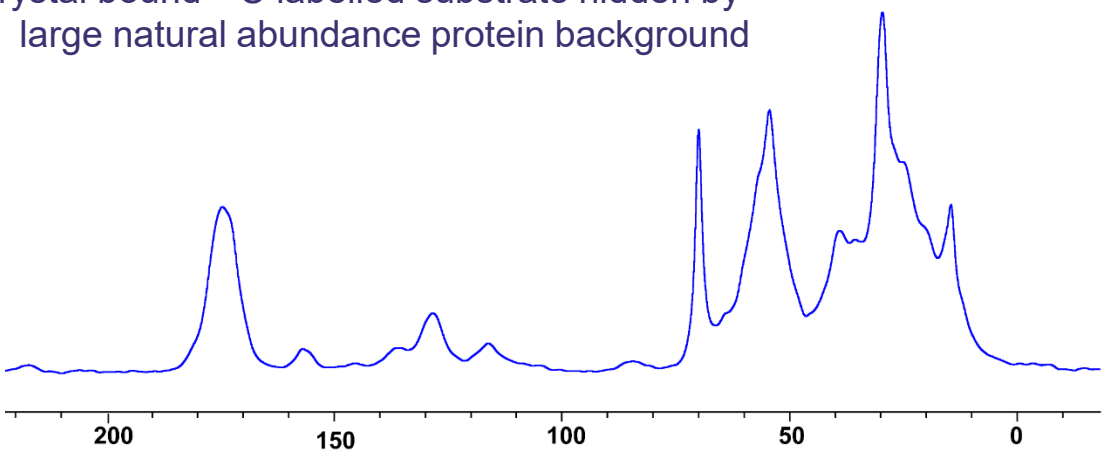
Tetramer complex

206 kDa effective asymmetric unit

- NA TPL in *either* protonated or deuterated crystallization buffer, 10% DMSO
- 5 mM AsymPolPOK, ε >50
- Buildup times shorter with ^{2}H, but ε constant
- ^{15}N, ^{13}C L-Methionine added (substrate analog)



Crystal bound ^{13}C labelled substrate hidden by large natural abundance protein background



Why doesn't the L-Met turnover ?

What can this tell us about the mechanism for the natural substrate L-Tyr ?

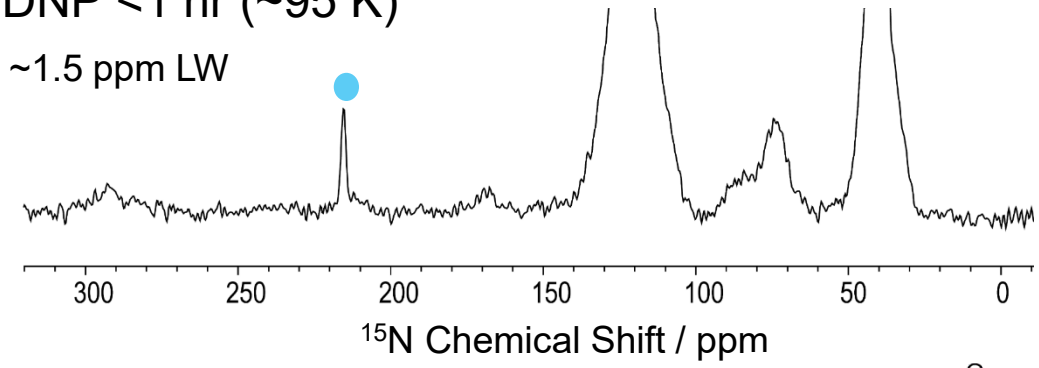
TPL quinonoid intermediate


Rittik Ghosh Luiza Caldas Nogueira

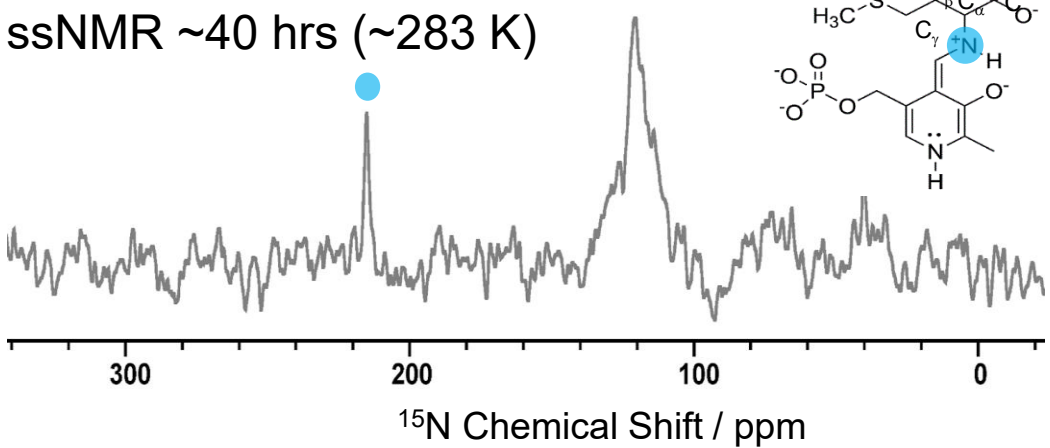
- NA TPL in *either* protonated or deuterated crystallization buffer, 10% DMSO
- 5 mM AsymPolPOK, ε >50 and DNP buildup < 1 sec
- Buildup times shorter with ^{2}H, but ε constant
- ^{15}N, ^{13}C L-Methionine added

DNP <1 hr (~95 K)

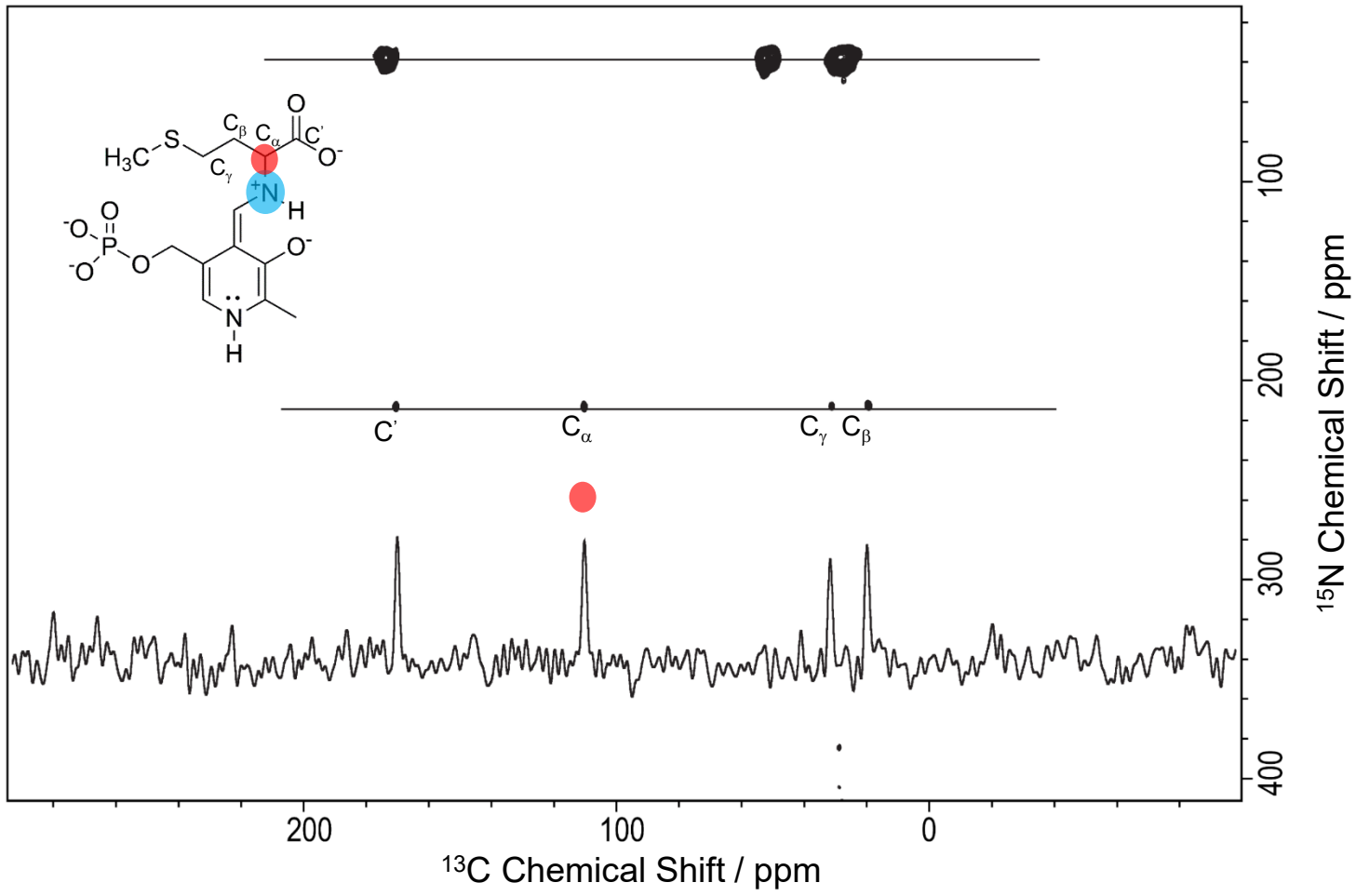
~1.5 ppm LW



ssNMR ~40 hrs (~283 K)



NCaCx 2D



Acknowledgements

Luiza Nogueira
Emily Peng
Mira Menon
James Collins
Faith Scott
Brooke Mangano
Adam Smith
Gwladys Riviere
Nhi Tran
Dan Downes
Bimala Lama
Lauren Schaffer

Len Mueller
Jacob Holmes
Rittik Ghosh
Viktoriia Liu
Bethany Caulkins
Yangyang Wang

NIH R01 GM97569

Funding

NSF, NIH

Melanie Rosay

Steve Hill
Rob Schurko
Hans van Tol
Fred Mentink-Vigier
Thierry Dubroca
Wenping Mao

Jonathan Mabrey
Peter Gor'kov
Jason Kitchen

DMR-1644779

P41 GM122698
S10 OD108519