Winter School, Stowe, Vermont
January 2018

The Oriented Sample Approach to NMR Spectroscopy of Membrane Proteins

Stanley J. Opella
Department of Chemistry and Biochemistry
University of California, San Diego
Life.
Diverse macroscopic organisms are chemically similar.

Amazon tropical rainforest.  

Sea life off Palma de Mallorca.
Life on Earth.

- Life existed 3.5 billion years ago.
- Humans on earth for 200,000 years.
- Our understanding of life is very recent (100 years).
Abiogenesis describes how life arose from inorganic matter under the conditions of the early Earth.

- Protobionts are precursor organic molecules for amino acids, nucleotides, and saccharides formed in the chemical environment on early Earth.
- A membrane-like barrier formed by pre-biotic amphiphiles was essential to keep key molecules in close proximity.
- Single-cell prokaryotes left fossil evidence 3.5 billion years ago.

Stromatolites are pre-Cambrian petrified biofilm in a rock formation.
First observation of single cell organisms by Anton van Leeuwenhoek in 1676.

Bacteria from the human mouth.
Contents of a bacterium (prokaryote) is enclosed by its plasma (cell) membrane.
Membranes also define the boundaries of compartments within human eukaryotic cells.

- Nucleus.
- Mitochondria.
- Lysosomes.
- Vacuoles.
Membranes.
Phospholipids have polar “head groups” and long hydrophobic “tails”. Self-assemble into bilayers in water.
The Fluid Mosaic Model of the Structure of Cell Membranes

Cell membranes are viewed as two-dimensional solutions of oriented globular proteins and lipids.

S. J. Singer and Garth L. Nicolson

- By weight, biological membranes are 50% protein.
- 30% of all proteins encoded in the human genome are membrane proteins.
Proteins are immobilized (on NMR timescales) by interactions with membrane bilayers.

IL-8 bound to CXCR1 in phospholipid bilayers

IL-8 in aqueous solution

$^1$H solution NMR spectra of monomeric IL-8 (66 residues): free vs. bound.
Solid-state NMR.
The oriented sample solid-state NMR approach to structure determination of membrane proteins.

- Solve the correlation time problem.
  - High sensitivity.
  - High resolution.

- Determine protein structures.
  - Resolve resonances.
  - Assign resonances.
  - Measure frequencies.
  - Translate frequencies to bond angles relative to the axis of alignment.
  - Measure inter- and intra- molecular distances.

- Calculate structures.
  - Orientational constraints.
  - Distance constraints.
  - Comparisons to data bases.
APPROACH TO HIGH-RESOLUTION nmr IN SOLIDS*

J. S. Waugh, L. M. Huber, and U. Haeberlen†
Department of Chemistry and Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

1968

A. PINES, M. G. GIBBY,† AND J. S. WAUGH

Department of Chemistry and Research Laboratory of Electronics, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
in a 1 cm³ sample. One can thus contemplate high resolution NMR of very rare spins and/or very small samples. Studies of chemical shift anisotropy of rare species (e.g., metals bound to proteins) or the dipolar structure of rare spin groups (e.g., $^{31}$P in polyphosphate moieties) could be of value in structural studies.
Separated Local Field Spectra in NMR: Determination of Structure of Solids*

R. K. Hester, J. L. Ackerman, B. L. Neff, and J. S. Waugh

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
(Received 15 March 1976)

Separation by:
1) chemical shifts of the dilute spins
2) homonuclear decoupling of the abundant spins
High-Resolution Heteronuclear Dipolar Solid-State NMR Spectroscopy

C. H. Wu, A. Ramamoorthy, and S. J. Opella

Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19104
Instrumentation and experimental developments following the solutions to the correlation time problem.

- High magnetic fields.
- Multidimensional NMR experiments.
- “Low-E” probes.
- Fast Magic Angle Spinning.
- High field Dynamic Nuclear Polarization.
- Nonlinear sampling.
- Uniform isotopic labeling.
Molecular alignment.
Molecular alignment in solid-state NMR samples.

- No alignment.
  - Powders.
  - Glasses.

- Three-dimensional alignment.
  - Single crystals.

- Uniaxial alignment.
  - Mechanical alignment.
    - Drawn polymers.
    - Phospholipid bilayers* between glass plates.
  - Magnetic alignment.
    - Liquid crystals*.
    - Phospholipid bilayers* in bicelles and macrodiscs.

- Rotational alignment.
  - Unaligned phospholipid bilayers*.

*Can host other molecules.
Nuclear Resonance Absorption in Hydrated Crystals:
Fine Structure of the Proton Line

G. E. Pake*

Powder pattern.

Single crystal rotation pattern.
The chemical shift interaction is anisotropic. $^{13}$C is “dilute” by isotopic composition. There are no $^1$H.
Carbon-13 chemical shielding tensors in single-crystal durene*

S. Pausak, A. Pines†, and J. S. Waugh

Department of Chemistry and Research Laboratory of Electronics,
Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

*1973
Uniaxial alignment.
Two-dimensional $^{13}$C NMR of highly oriented polyethylene

Stanley J. Opella$^+$ and J. S. Waugh

*Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*
NMR SPECTROSCOPY OF MOLECULES DISSOLVED IN LIQUID CRYSTAL SOLVENTS

C. L. Khetrapal
(Raman Research Institute, Bangalore 560006, India)

$^1$H NMR spectrum
simulation
Study of the isotropic-nematic-solid transitions in a liquid crystal by carbon-13–proton double resonance

A. Pines and J. J. Chang
Department of Chemistry, University of California, and Inorganic Materials Division, Lawrence Berkeley Laboratory, Berkeley, California 94720

MBBA

<table>
<thead>
<tr>
<th>Orientation</th>
<th>State</th>
</tr>
</thead>
<tbody>
<tr>
<td>isotropic</td>
<td>46°</td>
</tr>
<tr>
<td>nematic</td>
<td>40.6°</td>
</tr>
<tr>
<td>solid</td>
<td>8°</td>
</tr>
<tr>
<td></td>
<td>14°</td>
</tr>
<tr>
<td></td>
<td>-6°</td>
</tr>
</tbody>
</table>
High-Resolution NMR Spectroscopy of Membrane Proteins in Aligned Bicelles

Anna A. De Angelis, Alexander A. Nevzorov, Sang Ho Park, Stanley C. Howell, Anthony A. Mrse, and Stanley J. Opella*

2004
Trans-membrane domain of channel-forming viral membrane protein Vpu.

Flipped bicelles

Aligned bicelles

uniformly $^{15}$N labeled Vpu trans-membrane domain in DMPC bilayers
Structure Determination of a Membrane Protein with Two Trans-membrane Helices in Aligned Phospholipid Bicelles by Solid-State NMR Spectroscopy

Anna A. De Angelis, Stanley C. Howell, Alexander A. Nevzorov, and Stanley J. Opella*
Orientationally-dependent frequencies provide high resolution and angular constraints in aligned bilayer samples.

uniformly $^{15}$N labeled membrane proteins in q = 3.2 DMPC:DHPC bicelles
Resolution and measurements from three-dimensional spectra.

Each signal has three orientationally dependent frequencies:
- $^1\text{H}$ Shift: 2.9 ppm
- $^{15}\text{N}$ Shift: 94.0 ppm
- $^1\text{H}$$^{-15}\text{N}$ Coupling: 2.2 kHz

- $^1\text{H}$ shift = 2.9 ppm
- $^1\text{H}$ shift = 13.5 ppm
- $^1\text{H}$ shift = 14.3 ppm (14.3 ppm)
Nanodiscs versus Macrodiscs for NMR of Membrane Proteins

Sang Ho Park, Sabrina Berkamp, Gabriel A. Cook, Michelle K. Chan, Hector Viadiu, and Stanley J. Opella*

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0307, United States
Preparation of macrodiscs using styrene-maleic acid (SMA) polymer.

- Protein-containing liposomes + SMA
- heat – vortex – chill cycles
- spin down protein-containing macrodiscs

- No detergents.
- Wide temperature range.
- Variety of lipids.
- Higher order parameter.
- Higher concentrations of protein and lipids in NMR samples.
Improved alignment yields narrower linewidths.

Bilayers on glass plates

1991

(Shon, Kim, Colnago, Opella, Science 252, 1303, 1991)

SMALP Macrodiscs

2017

(Park, Radoicic, Opella, unpublished, 2017)

$^{15}$N Chemical Shift

uniformly $^{15}$N labeled Pf1 coat proteins in aligned bilayers
900 MHz PISEMA spectrum of aligned SMALP macrodiscs.

2 mg uniformly $^{15}$N labeled Pf1 coat proteins in DMPC/DMPG SMALP macrodiscs

100:1 lipid:protein molar ratio
“Rotational” alignment of membrane proteins in unoriented phospholipid bilayers.
The sign of axial symmetry and reduction in frequency span are determined by the angle of rotation.

**dipole-dipole coupling**


**chemical shift anisotropy**

Membrane proteins undergo fast lateral and rotational motions in phospholipid bilayers.
‘Spinnin’ of membrane proteins.

“And you know that tilt-a-whirl down on the south beach drag
I got on it last night and my shirt got caught
And that joey kept me spinnin’ I didn’t think I ‘d ever get off"

Springsteen, B. (1973) “4th of July, Asbury Park / Sandy”
Motional averaging in unoriented bilayers.

NMR Structural Analysis of a Membrane Protein: Bacteriorhodopsin Peptide Backbone Orientation and Motion†

B. A. Lewis,‡§ G. S. Harbison,‖ J. Herzfeld,‖ and R. G. Griffin*†

Francis Bitter National Magnet Laboratory, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115

Received January 7, 1985

Unoriented DMPC

30°C gel phase

30°C liq crys phase

“...line shape can be interpreted in terms of the orientation of the groups with respect to the diffusion axis...similar to that obtained from oriented samples...”
‘Rotational alignment’ of phospholipids and proteins.

APPLICATION OF $^{31}$P NMR TO MODEL AND BIOLOGICAL MEMBRANE SYSTEMS

Biochemistry Department, Oxford University, Oxford, England

1971

Rotational diffusion of membrane proteins in aligned phospholipid bilayers by solid-state NMR spectroscopy

Sang Ho Park, Anthony A. Mrse, Alexander A. Nevzorov, Anna A. De Angelis, Stanley J. Opella

Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92037-0307, USA

2006
Orientation from rotational diffusion in unoriented bilayers.

Determining the Orientation of Uniaxially Rotating Membrane Proteins Using Unoriented Samples: A $^2$H, $^{13}$C, and $^{15}$N Solid-State NMR Investigation of the Dynamics and Orientation of a Transmembrane Helical Bundle

Sarah D. Cady,† Catherine Goodman,‡ Chad D. Tatko,‡ William F. DeGrado,‡ and Mei Hong*†

Figures 3 and 4: N-H dipolar couplings of $^{13}$C-$^1$N-labeled membrane-bound MDM2 in DLPC bilayers at 313 K. (a) $^1$D $^1$N CP-MAS spectrum. Two of the four $^1$N labels overlap completely. NS = 5336. (b) $^1$N MAS spectrum after 1.42 ms $^1$C-$^15$N REDOR time. All four $^1$C labels are resolved. NS = 77,696. (c) $^1$D-detected N-H dipolar relaxation times for $^1$C-labeled resonances. The dipolar couplings indicated are the true values after dividing the fit values by 1.5 ( $^1$D $^1$N for $^1$N-detection) and the doubling factor of 2. The experiment was conducted under 7 kHz MAS.
Mechanically, Magnetically, and “Rotationally Aligned” Membrane Proteins in Phospholipid Bilayers Give Equivalent Angular Constraints for NMR Structure Determination

Sang Ho Park, Bibhuti B. Das, Anna A. De Angelis, Mario Scrima, and Stanley J. Opella*
Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0307, United States

Vpu TM

One Site

Two Sites

unoriented

mechanically aligned parallel

* magnetically aligned parallel

5 °C

25 °C

unoriented

mechanically aligned parallel

fd coat protein

* scaled for order parameter
Membrane proteins undergo fast rotational diffusion about the bilayer normal in liquid crystalline phospholipid bilayers.
Unoriented proteoliposomes.
Unoriented proteoliposomes.
Magic angle sample spinning and rotational diffusion of proteins.
**G-protein coupled receptors (GPCRs).**

- Biological mechanism.
  - Activated by specific signals (hormone, drug, etc.).
  - Triggers activation of G-proteins and signaling.

- GPCRs as structure targets.
  - 800 GPCRs in the human genome.
  - Receptors for >40% of drugs.

- GPCRs as proteins.
  - 7 trans-membrane helices.
  - > 350 residues.
The innate immune system is the first line of defense against pathogens.

- Neutrophils directed to the site of inflammation by IL-8 interacting with CXCR1. (Chemotaxis).

- Neutrophils kill bacteria.
  - Phagocytosis.
  - Degranulation results in secretion of anti-microbials.
  - Respiratory burst (release of reactive oxygen compounds).
Chemokines.

- **1987** IL-8 the first chemokine.
- IL-8 is Specific for neutrophils.
- 50 different chemokines.
- IL-8 has 72 residues and is a homodimer at high concentrations.
- Monomer binds to CXCR1.
- **1990** structure determination by solution NMR and X-ray crystallography.
CXCR1.

- **1991** CXCR1 and CXCR2 the first chemokine receptors.
- 20 different chemokine receptors.
- IL-8 binds to CXCR1.
- **2012** CXCR1 structure determined by solid-state NMR.
Chemokine-mediated GPCR signaling pathway.
Representative data from three regions of CXCR1 show that it has a single conformation.
Angles between H-Cα bonds and bilayer normal are determined from rotationally-averaged $^1H-^{13}C_\alpha$ Pake doublets.
Calculate the protein structure.

1.) Select lowest energy decoy from Rosetta.
2.) Final refined structure from XPLOR-NIH.

Generate initial structure from primary sequence in Rosetta

Identify transmembrane helical domains from MAS measured dipolar couplings.

Prepare dihedral angle constraints for TM domains
Prepare $^{15}$N dipolar coupling & csa constraints

Low temperature refinement of the de novo structure in xplor-NIH.
Biological membranes at UCSD 1972 – 2012: “The Fluid Mosaic Model of a Membrane” to the “Atomic Resolution Structure of a GPCR”.

PDB: 2LNL
Interactions between IL-8 and CXCR1.
Current model of IL-8 - CXCR1 complex.


- Insights from other chemokine receptors.
  - Stevens and coworkers, Science 347, 1117 (2015). Crystallography of CXCR4-vMIP-II.

- IL-8 interacts with CXCR1.
  - Site I: N-loop region (residues 10-22).
  - Site II: N-terminal ELR (residues 4-6).

- CXCR1 interacts with IL-8.
  - Site I: N-terminal domain of receptor.
  - Site II: extracellular loops, especially E3.
CXCR1 constructs enable separation of the two IL-8 binding sites.

Solution NMR

Site I
- IL-8 (N-Domain: residues 1-38)
- 1TM-Domain (residues 1-72)

Site II
- IL-8 (ΔN-CXCR1: residues 39-350)

Solid-state NMR

Sites I+II
- IL-8 (CXCR1: residues 1-350)
$^1$H-detected MAS NMR of $^1$H back-exchanged perdeuterated $^{15}$N-labeled IL-8 and unlabeled CXCR1 in phospholipid bilayers.

[Diagram showing NMR spectroscopy results for IL-8 alone and in complexes with CXCR1.]
IL-8 is not sedimented by 60 kHz MAS. Signals from fast exchanging amide sites missing due to water suppression method.

IL-8 alone

Exchanging amide sites in blue

Red: INEPT-HSQC of IL-8 alone at 60 kHz MAS
Blue: Solution NMR HSQC of IL-8 alone
$^1$H-detected MAS NMR of IL-8 bound to ΔN-CXCR1: Site II. Cross-polarization of immobile amide sites.

IL-8: ΔN-CXCR1

Red: CP-HSQC of IL-8 bound to ΔN-CXCR1 at 60 kHz MAS
Blue: Solution NMR HSQC of IL-8 alone
$^1$H-detected MAS NMR of IL-8 bound to $\Delta N$-CXCR1: Site II. INEPT of mobile amide sites.

**IL-8: $\Delta N$-CXCR1**

**INEPT**

*Green: INEPT-HSQC of IL-8 bound to $\Delta N$-CXCR1 at 60 kHz MAS*

*Blue: Solution NMR HSQC of IL-8 alone*
$^1$H-detected MAS NMR of IL-8 bound to CXCR1: Sites I + II. Cross-polarization of immobile amide sites.

**IL-8:CXCR1**

Red: CP-HSQC of IL-8 bound to CXCR1 at 60 kHz MAS
Blue: Solution NMR HSQC of IL-8 alone
$^1$H-detected MAS NMR of IL-8 bound to CXCR1: Sites I + II. INEPT of mobile amide sites.

Green: INEPT-HSQC of IL-8 bound to CXCR1 at 60 kHz MAS
Blue: Solution NMR HSQC of IL-8 alone

Mobile amide sites in green
$^1$H-detected MAS NMR of IL-8 bound to CXCR1 constructs in bilayers. Intermolecular PREs between IL-8 and Mn$^{2+}$ in CXCR1.

$^1$H solid-state NMR spectra of $^{15}$N labeled IL-8 obtained with CP

IL-8:W10HQA 1TM-Domain  IL-8:W10HQA CXCR1  IL-8:CXCR1  IL-8:ΔN-CXCR1

- Mn$^{2+}$

B

+ Mn$^{2+}$

D

12 10 8 6

$^1$H shift (ppm)
Current status of IL-8 - CXCR1 complex.

**Dynamics**
- Immobile sites
- Mobile sites

**Perturbations**
- Interaction site I
- Interaction site II