2017 Soft Matter Summer School on Membranes

Protein-Membrane Interaction Studies using NMR Spectroscopy

Jung Ho Lee

Department of Chemistry
Seoul National University, Korea
Motivation

- Compartmentalization by membranes is essential for life.
- There must be specific ways to overcome the boundaries of the compartment (membrane).
- Macromolecules exist to pass materials and information between a cell and its environment.
- **a**: Fluid Mosaic Model (Science 175, 720-731, 1972)
- **b**: High protein occupancy, variable patchiness, and thickness.

Course Outline

1. NMR spectroscopy
2. Membrane mimetics for NMR studies
3. Membrane proteins
4. Induction of Protein Structure upon Lipid Binding
5. Protein Motions on the Membrane
1. Nuclear Magnetic Resonance (NMR)

ethyl alcohol (1951)

Development of pulse sequence

Development of hardware

Increase in magnetic field

- 1952 Physics Bloch, Purcell NMR discovery
- 1991 Chemistry Ernst High-resolution FT NMR
- 2002 Chemistry Wüthrich 3D structure of biological macromolecules by NMR
- 2002 Medicine Mansfield, Lauterbur MRI
Applications of NMR

Structure of small molecules

Metabolomics
Identification and quantitative measurement of many metabolites in biological samples.

Reaction Kinetics

Applications of NMR

Structure of large biomolecules
- small proteins
- biomolecular complexes
- amyloid fibrils
- membrane proteins

Dynamics of large biomolecules
- Relaxation Dispersion
- $T_1$, $T_2$, hetNOE Modelfree
- Residual Dipolar Couplings
- Real-Time NMR
- Angular Dynamics of Protein Backbone Torsion Angles

Time scales:
- s
- ms
- μs
- ns
- ps
- fs

Global Motions
Local Flexibility
Applications of NMR

Dynamic Equilibrium

Slow exchange process

Spectroscopically invisible states

Four-state exchange


Ensemble of encounter complexes


Applications of NMR

Flexible Proteins and Regions

Conformation


Binding


MDa Proteins

~ MDa Proteins

670-kDa αββα 20S proteasome CP

Applications of NMR

In-Cell NMR

Protein conformation in live neuron cells


Protein phosphorylation in live oocyte cells


Protein structure in live E. coli cells

In-buffer

In-cell

Applications of NMR

MRI

Hyperpolarized Metabolite


Quantum Computation

Lithium-ion batteries

NMR Signal

Magnetic field

Boltzmann Distribution of Nuclear Spins

\( \beta \)-spins

\( \alpha \)-spins

NMR signal

http://mutuslab.cs.uwindsor.ca/
**T\(_1\) and T\(_2\) Relaxation**

Rate of relaxation: \(R_1 = 1/T_1\) and \(R_2 = 1/T_2\)

http://mri-q.com/
Dipolar Interactions between Nuclear Spins

\[ H_D = \frac{\hbar \gamma_I \gamma_S}{4\pi r_{IS}^3} \left[ 1 - 3 \cos^2 \theta \right] (3I_z S_z - I \cdot S) \]

Claridge TDW. High-Resolution NMR Techniques in Organic Chemistry 3rd Ed. Elsevier Science

Nuclear Overhauser Effect (NOE)

Saturation of S spin

The first protein structure determined by NMR

I-S dipolar interaction mediated NOE
Dipolar Relaxation through a Covalent Bond

- Ratio of global to local motion (order parameter) → local dynamics
- Timescale of local and global motions

- Local motions are often described as a bond wobbling in a cone
- Special motions lead to certain relaxation patterns.


Line Broadening by Chemical Exchange

\[ k_{ex} \]

\[ A + B \]

10⁶ s⁻¹

A  \rightleftharpoons  B

10³ s⁻¹

10² s⁻¹

A  B

10⁰ s⁻¹
2. Lipid Membrane Mimetics for NMR studies

Micelle

- The most popular and simplest way to prepare membrane proteins for NMR studies is to disperse them in lipid micelles.
- Denatured (e.g. by urea) membrane protein is added dropwise to a detergent solution to refold the protein → Micelle solution is added dropwise to the refolded protein while stirring → Buffer is exchanged to the micelle solution.

dodecylphosphorylcholine (DPC)

[Chemical structure diagram]

diheptanoyl / dihexanoyl phosphocholine (DHPC)

[Chemical structure diagrams]

*Front. Pharmacol. 6, 1-24 (2015)*
Bicelle

- Bicelles are mixture of bilayer-forming phospholipids (e.g. DMPC) and non-bilayer-forming phospholipids (e.g. DHPC).
- The ratio DMPC(long-chain)/DHPC(short-chain) determines the size of the bicelle, with higher ratio leading to larger and flatter bicelle.

DMPC

DHPC
Nanodiscs are small patches of lipid bilayer surrounded by segments of amphipathic helical proteins that stabilize the patches.

- Usually two copies of the membrane scaffold protein (MSP) are at their perimeter. MSPs are derived from apolipoprotein A-1.

- Nanodiscs are assembled by adding MSPs to cholate-solubilized phospholipids and detergent-solubilized membrane proteins. Detergents are removed afterwards.


Liposomes for Functional Studies

- **Small Unilamellar Vesicle (SUV)**: <100 nm
- **Large Unilamellar Vesicle (LUV)**: 100-1000 nm
- **Giant Unilamellar Vesicle (GUV)**: >1 μm
- **Multilamellar**

https://www.mirusbio.com/
Making Liposomes

Image: Avanti Lipids
Motivation for Studying Membrane Proteins by NMR
3. Characterization of Membrane Proteins by NMR

Membrane-Protein Function and Dynamics

Faster Dynamics in the ligand-free apo form

Ligand-induced conformational selection


Science 355, 1106-1110 (2012)
Val, Leu, Ile methyl-protonated and $^{15}\text{N}$-, $^{13}\text{C}$-, $^2\text{H}$-labeled membrane protein

$^{15}\text{N}$ ammonium chloride, U-$^{13}\text{C}/^2\text{H}$-labeled glucose, $^2\text{H}_2\text{O}$

CDLM-7317 $\alpha$-Ketoisovaleric Acid, Sodium Salt
(3-Methyl-$^{13}\text{C}$, 99%; 3,4,4,4-$^2\text{H}$, 98%)

CDLM-7318 $\alpha$-Ketobutyric Acid, Sodium Salt
(Methyl-$^{13}\text{C}$, 99%; 3,3-$^2\text{H}$, 98%)

$^1\text{H}$-$\delta$-Methyl-L-leucine

$^1\text{H}$-$\gamma$-Methyl-L-valine

$^1\text{H}$-$\delta$-Methyl-L-isoleucine

Proteins Affect Membranes in Many Ways

PROTEIN AGGREGATION PATHWAY

4. Structure Induction upon Membrane Binding

Alpha-Synuclein (αS) and Lipid-Membrane Interaction


J. Biol. Chem. 280, 9595-9603 (2005)
Alpha-Synuclein ($\alpha$S) Protein

$\alpha$S Primary Structure

$\alpha$S Function

- Helps SNARE-Complex Assembly
- Maintain the size of synaptic vesicle pools
- Impaired learning and memory in $\alpha$S knockout mice

$\alpha$S and Parkinson’s Disease (PD)

- $\alpha$S gene triplication and mutations are found in familial PD.
- $\alpha$S is the main component of Lewy bodies, which are aggregates of proteins in PD patient’s brain.
Challenges in Studying $\alpha$S-Membrane Interaction

Dynamic Equilibrium

$\alpha$S-Membrane Complex is too large for NMR

Heterogeneous Population

Bodner et al. *J. Mol. Biol.* (2009)

Liposome-αS Interaction

αS: 150 μM
Lipid: 400(black), 800(red) μM

- Signals from the N-terminal residues are significantly more attenuated than signals from the C-terminal residues upon addition of SUVs.
- No new or shifted resonance positions are observed.
- Minimal line-broadening is observed.
- Exchange is slow on the NMR timescale (<10 s⁻¹).

J. Mol. Biol. 390, 775-790 (2009)
Multiple Competing-Membrane-Binding-Modes of αS

- At low lipid/protein ratios (A), there exists a pool of protein, where only the first ~25 N-terminal residues are bound (SL1), and the second pool (SL2), where residues 1-97 are bound and invisible.
- Residues that are not directly bound to the lipids are dynamically disordered, even when other parts of the same protein molecule are immobilized.
- The relative degree of attenuation changes when the protein concentration is lowered (B), indicating competition between different binding modes. For example, SL1 is 20% and SL2 is 40% in (A).
- NMR data recorded at high lipid/αS ratio (C), suggests that more than two distinct states exist.

J. Mol. Biol. 390, 775-790 (2009)
**Kinetics of Binding**

- The observed $R_2^T$ equals the sum of the $R_2^T$ of the highly mobile $R_2^T_{\text{random-coil}}$ and the forward rate of the free-to-bound transition $k_{\text{on}}$.
- $k_{\text{on}} = 3-5 \text{ s}^{-1}$

![Graph of Transverse relaxation rate](image1)

- Selective saturation of the magnetization of phospholipid methylene resonances at 1.16 ppm.
- The fact that magnetization can be transferred from the lipid-bound state to the free state indicates that the timescale of bound-to-free transition is on the order of longitudinal relaxation rate of $\alpha$S amide protons. $k_{\text{off}} = \sim 1\text{ s}^{-1}$.

![Saturation transfer NMR](image2)

*J. Mol. Biol.* 390, 775-790 (2009)
Transferred NOE between $H^N$ and $H^N$

- (Free state $\rightarrow$ Bound state $\rightarrow$ Free state) during the NOE mixing time. Fast NOE transfer indicates that the bound complex is huge and $\alpha S$ is helical.
- Both SL1 and SL2 regions show $H^N$-$H^N$ connectivities to eight or more adjacent amide protons.

Transferred NOE between Leu-CH$_3$ and $H^N$

- NOE difference effect is large for Leu8 and Leu38 and extends over a significant number of residues.

$J. \text{ Mol. Biol.} \ 390, \ 775-790 \ (2009)$
Size of the $\alpha$S-Lipid Complex

Cryo-EM Images

600$\mu$M $\alpha$S + 0.03% SUV (400$\mu$M lipid)

NMR diffusion experiment

J. Mol. Biol. 390, 775-790 (2009)
Open Questions

- If $\alpha$S binds to the surface of SUVs with its 100 N-terminal residues in a contiguous $\alpha$-helical conformation, it would occupy a minimum of 1400 Å², approximately to the surface area of 28 phospholipid headgroups. (surface area of a single lipid headgroup in a bilayer is 50 Å²).

- With two leaflets per bilayer, the minimal stoichiometry for such a binding mode requires at least 56 lipids per $\alpha$S molecule, assuming that the surface of an SUV to be 100% covered by $\alpha$S.

- Even with $\alpha$S : lipid = 1 : 2.6, 40% of $\alpha$S is bound in SL2 mode.

- The N-terminally acetylated form of $\alpha$S binds to the lipid membranes even more tightly worsening the dilemma.

- Does a special stable, oligomeric, lipid-bound species of $\alpha$S exist (e.g. bundle of $\alpha$S with a modest number of phospholipids at its core)?

---

$J. \text{Mol. Biol.} \ 390, \ 775-790 \ (2009)$
Monitoring Acetylation Reactivity of Lysine Side Chains

Small Unilamellar Vesicles (SUV)
DOPE:DOPS:DOPC = 5:3:2

$\alpha$-Synuclein

High resolution NMR of acetylated lysine side chains

Add

Lipid removal by methanol precipitation

Lee JH et al. Biochemistry. 55, 4949 (2016)
NMR is a High Resolution Technique

Multidimensional NMR

High Magnetic Field


A Non-Destructive Technique
Assignment of Acetylated $\alpha$S Lysine Side Chains

15 Lysines
Lipid-Induced Protection of \( \alpha \)S Acetylation
Effect of SUV Chemical Composition on $\alpha$S Binding

**Same Helicity**

**Different Protection**

$\alpha$S may preferably bind to regions of lipid disorder and annealing defects OR lipid rafts.
5. Transient Membrane-Protein Interactions

- Ubiquitin
- Liposome

**Motional Model for the Bound State**

Lifetime = \(~10\mu s\)

Rotational correlation time = 1-100\(\mu s\)
Sequence Variation of $^{15}$N-$\Delta R_2$

Local and global motions of the NH bond

$^{15}$N Line-broadening

Rotation of Ubiquitin on the Surface of Liposome

local

intermediate

what kind?

global

$\alpha$ (degrees): the angle between N-H bond vector and the axis of rotation

$\text{d}=27\,\text{nm}$

$\text{d}=103\,\text{nm}$

$\text{SUV}$

$\text{LUV}$

$\text{J. Am. Chem. Soc.} \ 138, 5789-5792 \ (2016)$
Rotation of Ubiquitin on the Surface of Liposome

Ubiquitin rotates and wobbles on the surface of a liposome, upon encounter.
Probing the Surface of Interaction

Paramagnetic Relaxation Enhancement (PRE)

Ubiquitin in the presence of Gd$^{3+}$-tagged POPG LUVs

PRE mapping at the interface

Electrostatic potential

purple: high $^1$H$_N$ PRE
green: high methyl PRE
blue: positive, white: neutral, red: negative
Summary

Membrane Mimetics and Study of Membrane Proteins

αS-Membrane Interaction

Ubiquitin-Membrane Interaction

NMR Spectroscopy

PRE mapping
Further Reading


2) Engelman D.M. “Membranes are more mosaic than fluid”, Nature. 438, 578-580 (2005)

