Presentation III: Sample preparation.

Stanley J. Opella
Department of Chemistry and Biochemistry
University of California, San Diego
La Jolla, California USA
Relative effort devoted to the four pillars.

80% Sample preparation
Expression and purification of membrane proteins I.

- Identify target protein.
- Select polypeptide sequence.
  - Wild-type.
  - Domain.
  - Truncation.
  - Chimera.
  - Mutations.
- Select fusion partner.
  - KSI.
  - ΔTrp.
  - GST.
  - Bcl-XL.
  - MBP.
- Select cleavage method.
  - Protease.
  - Cyanogen bromide.
- Obtain codon-optimized gene.
Codon optimization. DNA 2.0 (www.dna20.com)
Expression and purification II.

- Assemble plasmid.
- Optimize expression levels in various media.
- Optimize expression levels in E. coli strains.
  - BL21 – tightest control of uninduced expression
  - BL21 (DE3) – high level expression
  - C41 (DE3) – resistant to membrane protein toxicity
- Protein isolation.
  - Separate/solubilize inclusion bodies.
  - Nickel column chromatography.
- Cleavage.
- Protein purification.
  - HPLC.
  - FPLC.
Expression and purification III: media for isotopic labeling.

- Unlabeled samples.
  - LB
- 100% uniform $^{15}$N.
  - M9 with $^{15}$N ammonium sulfate.
- 100% uniform $^{13}$C and $^{15}$N.
  - M9 with $^{13}$C glucose and $^{15}$N ammonium sulfate.
  - CIL BioExpress.
- Tailored 13C labeling.
  - CIL BioExpress with 15% - 45% uniform $^{13}$C and 100% uniform $^{15}$N.
  - Metabolic labeling.
    - M9 with [2-$^{13}$C]-glycerol.
    - M9 with [1,3-$^{13}$C]-glycerol.
    - M9 with 2-$^{13}$C glucose.
- Selective (by residue type) $^{15}$N and/or $^{13}$C amino acids.
  - M9 with added amino acids.
  - NP with added amino acids.
NP media for selective (by residue type) labeling.

Media Comparison Test (OD vs Time)

OD @ 600nm vs Time (hours)

- LB
- M9
- NP
Selective labeling (by residue type) with NP media: ~ 3X higher OD and protein yield than M9.

NP contains salts, amino acids, nucleotides, glucose and other carbon sources
Example: *p7 of Hepatitis C Virus (HCV).*

- 63 residues.
- Two trans-membrane helices.
- Oligomers with ion-channel activity.
- Channel blockers have anti-viral activity.
- Viroporins.
  - M2 of influenza.
  - p7 of BVDV.
  - Vpu of HIV-1 (not HIV-2 or SIV).
  - K of Semliki Forest virus.
  - p10 of avian and Nelson Bay reoviruses.
  - 2B of picornavirus
  - NS3 of bluetongue virus.
  - E of MHV (murine hepatitis virus).
p7 polypeptide constructs: full-length, C-terminal truncated, and N-terminal truncated.

Codon-optimized sequence of full-length p7.

HindIII

MALENLVLNAAASVAGAHGILSVLVFFSAAWYIKGRLAPGAAAYAFFGVWPPLLLLLLALPPRAYA

AAWYIKGRLAPGAAAYAFFGVWPPLLLLLLALPPRAYA

G V W P L L L L L L L A L P P R A Y A BamHI

GGT GGT TGG CGG CTG CTG CTG CTG CTG CTG CTG CTG CTG CTG CTG CTG CTG CTG GGT TGC GCT TAA TAA GGA TCC -3'
CCA CAA ACC GGC GAC GAC GAC GAC GAC GAC GAC GAC GAC GAC GAC GAC GAC GAC ATG CAA ATT CCT AGG -5'
The DNA and protein sequence of the full-length protein are shown above the vector map. The pHLV plasmid containing the His tagged TrpDLE fusion partner was used by inserting the p7 DNA sequence into the vector between the HinDIII and BamHI restriction sites. The DNA insert included an N-terminal methionine for cleavage and two C-terminal stop codons.

**Plasmid.**

Agarose gel of PCR products for p7 construct insert DNA. The 1.5% gel shows the amplification of the sequences for (A) p7, (B) p7TM1 and (C) p7TM2. Lane 1 for each of the gels is the base pair size marker 100 Ladder (New England Biolabs).
Expression and purification of p7 polypeptide constructs.

Growth of BL21 (DE3) pLysS cells and overexpression

Lysis and isolation of the protein-containing inclusion bodies

Purification by Immobilized metal ion affinity chromatography

Removal of the TrpLE fusion protein partner by Cyanogen Bromide Cleavage

Purification by FPLC/HPLC
Increased cell density (and yield of protein) using a fermentor.
Solution NMR and solid-state NMR spectra of uniformly $^{15}\text{N}$ labeled p7 constructs.

Q=0 DHPC micelles

q=3.2 DMPC:DHPC bicelles
Sample preparation in lipids.
Phospholipid bilayer assemblies are characterized by $q$, the molar ratio of the long chain lipid to the short chain lipid.

- **Micelles**
  - $q = 0$
  - $T = 50^\circ C$
  - Isotropic
  - Weakly aligned

- **Isotropic bicelles**
  - $q < 1.0$
  - $T = 50^\circ C$
  - Isotropic
  - Weakly aligned

- **Magnetically aligned bilayers**
  - $q = 2.5 - 10$
  - $T = 15^\circ C - 65^\circ C$
  - Completely aligned
  - Parallel or perpendicular

- **Unoriented bilayers.**
NMR of lipid membranes is a well-established technique.

$^{31}$P NMR demonstrates that identical phospholipids are affected differently by different proteins.
$^{31}$P NMR spectra consistent with specific modes of interactions between proteins and phospholipids.
Alignment of protein-containing bicelles is sensitive to lipid composition and temperature.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>DMPC/POPC/DHPC Composition</th>
<th>q Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>23°C</td>
<td>24/6/5.5 (w/w), q=3.58</td>
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</tr>
<tr>
<td>30°C</td>
<td>24/6/6.0 (w/w), q=3.28</td>
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</tr>
<tr>
<td>40°C</td>
<td>24/6/6.5 (w/w), q=3.03</td>
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</tbody>
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Samples with bilayer normals parallel to the field.

glass plates

TBBPC bicelles

‘flipped’ bicelles with lanthanide
Resource for NMR Molecular Imaging of Proteins at UCSD.

Research group
Lauren Albrecht (GPCR)
Eugene Lin (triple-resonance NMR)
Hua Zhang (Vpu)
Sang Ho Park
Gabriel Cook
Albert Wu
Leah Cho
Christopher Grant
Woo Sung Son
Yan Wang
George Lu
Aubrey Davis
Yanwen Mai
Megan Chu

Supported by the National Institutes of Health