Novel platforms for the expression of membrane proteins

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Genomics Technologies
Molecular Biology Reagents

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Membrane Proteins- why are they important?

1. Membrane proteins are critical components of cellular communication and transport machinery.

2. Membrane proteins account for more than 30% of the human genome (more than 10000 proteins)

3. More than 50% of the drug targets are membrane proteins

4. Less than 1% of the membrane proteins have been studied at the structural level

Challenging in every aspect:

- Expression
- Extraction
- Purification
- Functional analysis
- Structural analysis
Two Novel Solutions to Membrane Protein Expression

**In Vitro Approach:**

NLPs – Nanolipoprotein particles (Nanodiscs)

**In Vivo Approach:**

VLPs – Virus-like particles
From HDL to Nanodisc/NLP


Structural Studies of Apolipoprotein AI/Phosphatidylcholine Recombinants by High-Field Proton NMR, Nondenaturing Gradient Gel Electrophoresis, and Electron Microscopy
Christie G. Brouillette, James L. Jones, Thien C. Ng, Henri Kercret, B. Hong Chung, and Jere P. Segrest*
Biochemistry 1984, 23, 359-367

Self-Assembly of Discoidal Phospholipid Bilayer Nanoparticles with Membrane Scaffold Proteins
Timothy H. Bayburt,† Yelena V. Grinkova,† and Stephen G. Sligar*, Department of Biochemistry, Department of Chemistry, University of Illinois, Urbana, Illinois 61801
NANO LETTERS 2002 Vol. 2, No. 8 853-856

Nascent HDL (high density lipoproteins) in plasma are composed of ApoA1 and cholesterol

Nanodisc - Planar Phospholipid bilayer surrounded by a protein coat

Membrane scaffold protein + Membrane protein + Phospholipids + Detergent → Dialysis → NLP/membrane protein complex
NLP: Planar Phospholipid bilayer surrounded by a protein coat

Advantages

1. Planar
2. MP accessible from both sides of the membrane
3. Protein belt constrains dimensions
4. Monodisperse within preparations
5. Consistence between preparations
6. Very stable
7. No compartmentalization
8. Defined component stoichiometry and NLP composition determined by free energy of assembly
Solution: Cell-free protein expression combined with NLPs

Advantages of cell-free approach

- Open environment, easy to control
- Fast results (hours)
- Yields up to mg/ml
- Use of multiple templates (plasmid/PCR fragments)
- Expression of a variety of otherwise toxic products
- Easily scalable
- Fast purification and analysis
- Uniform/selective labeling
Atomic Force and Electron Microscopy of NLPs

NLPs-EmrE complex formation and purification

ApoA1 His-tag removal
(TEV co-migrates with uncleaved ApoA1)
Enhanced solubility for membrane proteins

Solid bars: + NLPs
Empty bars: - NLPs

NLPs

2 TMSs
NLPs

3 TMSs
NLPs

4 TMSs
NLPs

7 TMSs
NLPs

Empty Disc BR Disc
4.2nm

Invitrogen
### Expression of soluble GPCRs

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>MW (kDa)</th>
<th># aas</th>
<th>TMS</th>
<th>Description</th>
<th>Role/Family</th>
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</thead>
<tbody>
<tr>
<td>bR</td>
<td>28.2</td>
<td>262</td>
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<td>Halobacterium salinarum, bacteriorhodopsin</td>
<td>Proton pump</td>
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<td>IOH14234</td>
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<td>IOH28351</td>
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<td>7</td>
<td>Homo sapiens, cholinergic receptor, muscarinic 2 (CHRM2), transcript</td>
<td>GPCR</td>
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<td>GPCR</td>
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<tr>
<td>IOH29556</td>
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<td>Homo sapiens, dopamine receptor D1 (DRD1), mRNA</td>
<td>GPCR</td>
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<td>IOH29738</td>
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<td>Homo sapiens, melanocortin 5 receptor (MC5R), mRNA</td>
<td>GPCR</td>
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<tr>
<td>IOH39398</td>
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<td>7</td>
<td>Homo sapiens, corticotropin releasing hormone receptor 1</td>
<td>GPCR</td>
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<tr>
<td>IOH46452</td>
<td>46.1</td>
<td>422</td>
<td>7</td>
<td>Homo sapiens, 5-hydroxytryptamine (serotonin) receptor 1A (HTR1A)</td>
<td>GPCR</td>
</tr>
<tr>
<td>IOH56940</td>
<td>51.4</td>
<td>460</td>
<td>7</td>
<td>Homo sapiens, cholinergic receptor, muscarinic 1 (CHRM1), mRNA</td>
<td>GPCR</td>
</tr>
</tbody>
</table>
Model Proteins Demonstrate Functional Activity

**Bacteriorhodopsin**

- **bR photocycle**

FTIR measurement of trapped M intermediate

**EmrE Saturation Binding**

Kd = 19.17

[K^3H]TPP^+ (nM) vs CPM Counts

bR-NLP function is similar to wild type bR
Functional reconstitution of β 2-adrenergic receptors utilizing self-assembling Nanodisc technology

Andrew J. Leitz 1, Timothy H. Bayburt 1, Alexander N. Barnakov 2, Barry A. Springer 2, Stephen G. Sligar 1

Assembly of β 2-adrenergic receptor (β 2 AR)-Nanodiscs. β 2AR-Nanodisc self-assembly showing membrane scaffold protein (MSP), lipid 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), and the target β 2AR. Self-assembly is initiated by removal of the detergents solubilizing the components and results in the efficient formation of 10-nm nanobilayer assemblies.


β2-adrenergic –antagonist binding

- Proteins were synthesized using E Coli lipid/NLP by IVTT
- 5HT1a and b2 were purchased from Perkin Elmer as positive controls
- Microspin G-50 Columns were used to separate free ligand
Fluorescence correlation spectroscopy (FCS) measures molecular diffusion times, and association/dissociation rates.

Membrane protein

NLP: scaffold protein + phospholipid

10-30nm in diameter

FCS provides normalized Cross Correlation Curves

Ting Gao, Thomas Huser, John Voss, Paul Henderson – UC Davis/ Matt Coleman LLNL
Fluorescence correlation spectroscopy (FCS) measurements

FCS identifies Nuerokinin-GFP as associated with NLP

Beta-2 Adenergic receptor ligand binding

Signal mixture of all protein species. Cross-correlation of GFP/Texas red.

Saturation binding studies are in progress

Ting Gao, Thomas Huser, John Voss, Paul Henderson – UC Davis/ Matt Coleman LLNL
Crystals diffract at 0.6 nm

Large Single Crystals of B. Mori LipolIII NLPs Form in a Variety of Crystallization Conditions, Including a Number Compatible with Cryo-Preservation

Initial Diffraction Studies Reveal a Combination of Discrete Diffraction and Fiber Diffraction to ~6Å, Considerably Higher Resolution than Other Reports

Nicholas Fischer and Brent Segelke, LLNL
To date, we know we can...

1. Make stable, monodisperse, and functional NLPs
2. Solubilize membrane proteins with pre-formed and in-situ formed NLPs using...
3. ...a variety of cell-free protein expression platforms
4. Form and isolate NLP-MP complexes
5. Get a high yield of soluble membrane proteins
6. Make functional membrane proteins in solution
7. Crystallize NLPs and...
8. ... create arrays
9. Simple HIS tag purification of native membrane protein
What we don’t know is…

1. The mechanism of MP insertion into NLP. Is the insertion co-translational or post-translational?

2. Correlation between solubility and folding.

3. Impact of NLP lipid/scaffold composition on MP functionality

4. Can the multi-subunit MP complexes be formed and inserted into NLPs? (i.e. using Multi-Site Gateway approach)

5. GPCR/mammalian MP functionality as measured in standard format HTP ligand binding assay.

6. Can the NMR spectra be generated for NLP:MP complexes?

7. Can we use NLP approach to generate GPCR antibodies?
Potential for membrane-protein-specific NLP environment

<table>
<thead>
<tr>
<th>Scaffold Proteins</th>
<th>Lipid Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoA-1</td>
<td>DMPC</td>
</tr>
<tr>
<td>MSP1T2</td>
<td>POPC</td>
</tr>
<tr>
<td>Human ApoA-1</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>ApoE422K</td>
<td>Sphingolipids</td>
</tr>
<tr>
<td>ApoLp-III</td>
<td>• Sphingosine and Derivatives</td>
</tr>
<tr>
<td></td>
<td>• Gangliosides</td>
</tr>
<tr>
<td></td>
<td>Lipid Mixtures</td>
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<tr>
<td></td>
<td>Glycerol Based</td>
</tr>
<tr>
<td></td>
<td>• Lipid Tissue Extracts</td>
</tr>
<tr>
<td></td>
<td>Brain &amp; Egg</td>
</tr>
<tr>
<td></td>
<td>Escherichia Coli &amp; Heart</td>
</tr>
<tr>
<td></td>
<td>Liver &amp; Soy</td>
</tr>
<tr>
<td></td>
<td>• Choline</td>
</tr>
<tr>
<td></td>
<td>• Ethanolamine</td>
</tr>
<tr>
<td></td>
<td>• Inositol</td>
</tr>
<tr>
<td></td>
<td>Fluorescent Lipids</td>
</tr>
</tbody>
</table>
Publications and Collaborations


   Federico Katzen, Rob Bennett and Wieslaw Kudlicki (eds.)


4. Molecular and Cellular Proteomics, 2008. Cell-free co-expression of soluble nanolipoprotein particles supporting functional membrane protein. Jenny A. Cappuccio1†, Craig D. Blanchette1†, Todd Sulchek1, Erin S. Arroyo1, Angela K. Hinz1, Edward A. Kuhn1, Brett A. Chromy1, Julia Fletcher2, Federico Katzen2, Todd Peterson2, Wieslaw A. Kudlicki, Graham Bench1, Paul D. Hoeprich1* and Matthew A. Coleman1*


2008/2009 UC Discovery Grant- UC Davis/LLNL
Industrial collaborations on functionally expression and crystallization of membrane proteins
Second solution to membrane protein expression: VLPs (in development)

**Lentiviral Assembly**

**VLP Assembly Optimizations**

**Expression System**
- pLenti6.3/V5-GW/哌替啶 10822 bp
- pLenti6.3/V5-GW/哌替啶 10822 bp
- pcDNA3.3-TOPO 5.4 kb
- pcDNA3.3-TOPO 5.4 kb

**Packaging System**
- Env Gag Pol
- Gag
- Env Gag Pol
- Gag

**GPCR Activity**
- +
- ?
- ?
- ?

invitrogen®
NLP vs. VLP

- Planar
- MP accessible from both sides of the membrane
- Protein belt constrains dimensions
- Monodisperse within preparations
- Consistence between preparations
- Very stable
  (Do not delimit compartments)

Concept: Viral Gag (coat) protein buds from cell surface capturing overexpressed GPCRs

- Preservation of native MP activities
- Enriched sampling of GPCRs and other receptors
- MPs captured in their native environment
- Soluble 150nm particles
- Elimination of most cellular protein contamination
- Amenable to small-scale analytical to industrial scale
- HTP ligand screening.
**VLPs displaying active GPCRs, Initial Data**

### A. 5HT1a Receptor

- **Muscarinic M1 Receptor**
  - 

### B. Serotonin Ligand Binding to Lenti PVPs
- ![Graph showing CPM counts for serotonin ligand binding with and without metergoline for Neg VLPs and 5HT1a VLPs.]

### Muscarinic M1 Ligand Binding to Membranes
- ![Graph showing CPM counts for muscarinic M1 ligand binding with and without Atropine for Neg VLPs and M1 VLPs.]

### 5HT1a Receptor
- **Kd = 0.68 nM**

### Muscarinic M1 Receptor
- **Kd = 0.6 nM**

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*Invitrogen*
Isolated VLP/MP Complexes are Active and Highly Pure
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NLPs

- High Yield
- Structural Applications
- Purification
- Immobilization Protoarrays
- Functional ligand binding assays
- Native Environment Associated Factors
- In vivo Delivery
- Vaccines

VLPs
The team

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John Voss

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