Efforts to Develop a High-Throughput Screening Tool Using Isoprenylcarboxyl Methyltransferase as a Membrane Protein-based Sensor

David H. Thompson, Purdue-Chemistry
Current Drug Discovery Limitations for Integral Membrane Protein (IMP) Targets

- ~60% of pharma targets are integral membrane proteins (IMP)
- fewer than 200 IMP structures are known to the < 3Å resolution necessary for rational drug design approaches (i.e., < 0.5% PDB)
  
  :: screening methods are still required to discover active agents for IMP targets

- issue is compounded by the fact that most IMP assays are:
  - slow
  - noisy
  - lack sensitivity

Need

- Platform technology for integral membrane protein-based sensors that are:
  - fast, reproducible & sensitive
  - compatible with high-throughput platforms (i.e., planar geometries, optical detection schemes)
  - capable of real-time read-out for kinetic & mechanistic studies
  - rugged
  - low false positive response rate
  - etc (patternable, controllable areal density of IMP, microfluidic/microelectronic interfacing….)

Bakheet & Doig, Bioinformatics 2009 25, 451

Iyengar et al., Mt. Sinai J Med 2007 74, 27
Supported Membrane Sensor Concept

- Bolalipid & tether synthesis
- Microfabrication
- Membrane-Support Gap
- Sensing method
- Bolalipid & tether design

APPLICATIONS
- Platform technology for membrane protein-based sensors
- High throughput screening for drug discovery
- Mechanistic studies of IMP
K-Ras Signaling Pathway & Mislocalization

Serial C-terminal PtM of eukaryotic CaaX proteins

GFP-K-Ras Localization in Mouse ES Cells

SAM: S-Adenosyl methionine
(substrate for Icmt)

SAH: S-Adenosyl homocysteine
(product of Icmt enzymatic reaction)

Hrycyna & Gibbs, *BOMCL 2006* 16, 4420-4423

Bergo, Leung, Ambroziak, Otto, Casey, Young, *J. Biol. Chem.* 2000, 275, 17605-17610
Isoprenylcysteine Methyltransferase (ICMT): A Target for Discovery of anti-K-Ras Agents

**Yeast ICMT** ✧ Ste14p, 26 kD
His<sub>10</sub>myc<sub>3</sub>-Ste14p, 37 kD

✎ mutations in the K-Ras oncogene are responsible for nearly 15% of all human cancers
✎ inhibition of Icmt results in the loss of transforming ability of K-Ras

∴ ICMT is a novel and attractive anti-cancer target
AFM Analysis of Ste14p in Supported Membranes

Dodecylmaltoside-Mediated His\textsubscript{10}-Ste14p Reconstitution Produces Liposomes with 80% of the Catalytic Domains on the Outer Surface

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Feature Height (nm)</th>
<th>Supported Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLPC</td>
<td>0.9-2.5</td>
<td>continuous bilayer</td>
</tr>
<tr>
<td>DMPC</td>
<td>0.6-1.0</td>
<td>continuous bilayer</td>
</tr>
<tr>
<td>POPC</td>
<td>0.7-1.8</td>
<td>continuous bilayer</td>
</tr>
<tr>
<td>DSPC</td>
<td>0.3-0.5</td>
<td>bilayer patches</td>
</tr>
</tbody>
</table>

Shan Zou, Akiko Murakawa, Linda Johnston
Strategies to Orient Immobilize Membrane Proteins Within a Supported Membrane on PEG Modified Glass

- Water gap thickness varies with PEG MW
- Water gap thickness varies with PEG surface concentration

I. Szleifer

### PEG3400-NTA on Si
- 16.9 nm ± 3.3 nm

### mPEG5000 on Si
- 13.7 nm ± 2.9 nm

### FLIC Microscopy
- POPC on bare Si: 1.83 nm ± 0.29 nm
- POPC on C18-PEG4000-Si(OEt)_3 modified Si: 16.9 nm ± 0.84 nm

Jong-Mok Kim/Elias Franses/Lukas Tamm
$K_d$ for His$_{10}$-GFP-Ste14p on Ni$^{2+}$:NTA-PEG Surfaces

$K_d = 52.7$ nM
Supported Membrane Sensor Fabrication

1. Si or Au Substrate
2. Liposome
3. Si or Au Substrate
Binding & Release Behavior of His$_{10}$-Ste14p on PEG-NTA Determined by SPR

Flow rate: 5 μl/min
Buffer(+DDM): 10 mM Tris-HCl, 150 mM NaCl, 0.2 mM DDM, pH 7.5

- 1 M Imidazole
- Buffer
- 200 nM Ste14p
- Buffer

PEG3400

A. Murakawa
Binding & Release of his-GFP-Ste14p on PEG Substrates

- **Binding of Ste14p to PEG-OMe**
  - OMe
  - 1000 nM
  - OMe

- **Binding of Ste14p to PEG-NTA**
  - NTA
  - 1000 nM

- **Release of Ste14p from PEG-NTA by Imidazole**
  - 1 M Imidazole
  - NTA
Preparation of his-Ste14p Supported Membrane by Surface-mediated Reconstitution

Buffer(+DDM): 10 mM Tris-HCl, 150 mM NaCl, 0.2 mM DDM, pH7.5
Buffer(-DDM): 10 mM Tris-HCl, 150 mM NaCl, pH7.5

Graph showing the kinetics of membrane reconstitution with Ste14p and POPC liposomes.
Natural Archae & Synthetic Bolalipids

Halophilic Bacteria and Methanogens

Thermophilic Bacterial Bolalipids

Bolalipid (membrane-spanning chain)

FFEM of Bilayer Membrane

FFEM of Bolalipid Membrane

C\textsubscript{20}BAS

C\textsubscript{32}PhytBAS

POPC

Bilayer Membrane delamination

200 nm

200 nm
C$_{20}$BAS Characterization

**T$_m$ = 17°C for C$_{20}$BAS Membranes**

31P NMR Chemical Shift Anisotropy for C$_{20}$BAS

C$_{20}$BAS Membranes are Highly Ordered

C$_{20}$BAS Lateral Diffusion Rate is Similar to Monopolar Lipids by FRAP & PFG-NMR

7:3 C$_{20}$BAS:Chol Vesicles Retain a Chemical Gradient

Cryo-TEM of 7:3 C$_{20}$BAS:Chol 200 nm

% Calcein Release

D$_{PFG-NMR}$ = 1.8 x 10$^{-8}$ cm$^2$/s

D$_{PFG-NMR}$ = 1.9 x 10$^{-8}$ cm$^2$/s

C$_{20}$BAS Lateral Diffusion Rate is Similar to Monopolar Lipids by FRAP & PFG-NMR

C$_{20}$BAS Membranes are Highly Ordered


Functional Assay of Ste14p in Bolalipid Membrane Vesicles of Varying Composition

(Bolalipid:E. Coli lipid mixtures)

Specific Activity of Ste14p (x 10^{-3} pmol/min/mg)

- Dashed line: C20BAS
- Solid line: C32-phyt

0:100 10:90 20:80 50:50 75:25 100:0

bolalipid:E. coli polar lipid

A. Patwardhan & D. H. Thompson, Org. Lett. 1999 1, 241-244

C_{20}BAS

C_{32}phytBAS

Conceptual Diagram of Interferometric Detection

SAH solution → anti-SAH-magnetic microbeads

SAH capture by anti-SAH-magnetic microbeads → Magnetic isolation and rinsing of microbeads

Laser diode

Preparation of Stamp

Master → PDMS-Stamp

Stamping

Inking biotinylated BSA → Stamping → Patterned Surface

Optical Microscopy of \( \mu \)CP Au Surfaces After Exposure to SAH-Immunocaptured Beads

Calibration Curve for S-Adenosyl Homocysteine Detection Using SAH-Immunocaptured Beads on μCP Au Surfaces

Controls

High-Throughput Platform for Interferometric Detection

Streptavidin-immobilized disc

Prime disc with biotinylated adenosine aptamer

Transfer magnetically-captured SAH produced in multiple assay reactions

Assay readout via interferometric detection device

SAH

biotin

www.quadraspec.com
Conclusions

• NTA-PEG-modified surfaces are capable of efficient his$_{10}$-Ste14p capture and his$_{10}$-Ste14p orientation during supported membrane formation

• C$_{20}$BAS bolalipid forms monolayer membranes that have similar permeability, melting transition, and lateral diffusion as conventional monopolar lipids

• C$_{32}$phytBAS bolalipid vesicles retains $\geq 75\%$ Ste14p activity, however, it is lost in C$_{20}$BAS vesicles that have significant hydrophobic mismatch

• The membrane-support gap can be controlled through appropriate choice of grafted PEG MW

• Interferometric sandwich assays provide an attractive method for SAH detection
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David Holland
Functional Assay of Ste14p in Bolalipid Membrane Vesicles of Varying Composition (Bolalipid:E. Coli Lipid Mixtures)

Specific Activity of Ste14p ($\times 10^{-3}$ pmol/min/mg)

- C20BAS
- C32-phy

bolalipid:E. coli polar lipid

0:100 10:90 20:80 50:50 75:25 100:0

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