Use of Chaperone Proteins and Octet Bio-Layer Interferometry To Detect Protein Folding /Unfolding Events in a High-Throughput Format.

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Outline – Chaperonin based interferometry detection platform

• Proof of concept – detecting partially folded proteins.
• detecting transient folds and screening for stabilizers (misfolding disease proteins)
• Detecting folding on the tips – reversible
• Assessing the Kinetic stability of the fold (Denaturant dip and read).
• Detection of unfolding/refolding toxin transitions – Anthrax pore translocon soluble to membrane insertion states using BLI systems.
Significance of protein misfolding diseases (a sampling).

Protein misfolding/folding diseases

Aggregation: *Sickle cell anemia, Light or Heavy Chain IgG amyloid

Neurodegenerative: *Alzheimer's, *Parkinson's, Huntington's, Prions

Cancers: Most Activated Oncogenic proteins are transiently stable

Bacterial Infections: *Anthrax, MRSA, Cholera, Diptheria

* Cystic Fibrosis, ALS(Lou Gehrig's), Type II diabetes

Rare diseases (afflicts 30M Americans): *Friedreich's Axtaxia, Phenylketouria, *Transthyretin associated neurocardiomyopathies, Gaucher’s disease

Prevalence: 30-50 % Human population afflicted with diseases that are due to Protein folding or aggregation defects.

* Protein misfolding reactions easily detected by our Chaperonin technology platform

CHAPRx: Confidential
Problem: Rapid identification of Potential Stabilizers or therapeutics for folding diseases is difficult because Disease Proteins are often transiently stable.

Disease/Protein misfolding/ Product degradation

Corrector (Drug)

Corrector stabilized mutant

Aggregation (common)
Current screening technologies:

1) Aggregation assays

Stabilizing ligand

A protein can remain misfolded* but not aggregate
Extremely lengthy assay time: Hours - days
2) Common High Throughput Screening technology to identify ligand stabilization of proteins: *Thermofluor*

Ligand binding shifts thermal melt curve (well behaved system).
Disadvantages of thermofluor

- Cannot be used for metastable proteins that readily aggregate/misfolding disease states
- Cannot be used for oligomeric proteins
- Cannot be used for membrane proteins as detergents and hydrophobic portions lead to noisy signal
- Dye binding itself may perturb the equilibrium between the folded and unfolded populations
- Ligand binding occurs under non-physiological conditions (high temperatures)
- Large quantities of protein needed (1 mg /96 well plate)
Missing element- monitoring the initial unfolding reaction
Rather than downstream events

Protein structure perturbant
(e.g. temp. denaturant etc.)
Chaperonin captures intermediates and **unfolded hydrophobic species** – Measurable parameter to identify stabilizers that correct the fold.

The solution: Use the High affinity ATP free chaperonin GroEL as a **kinetic trap**.

Disease

Unfolded (hydrophobic)

**- Large signal - Yes/no readout.**

YES

NO

Corrector (Drug)

Corrector stabilized mutant

Our IP:

- Proprietary large scale production of Extremely PURE Chaperonin
- One patent issued,
- Two patent pending,
- One provisional submitted.
The High Affinity Chaperonin GroEL (cpn60₁₄) can distinguish between native (N) and partially unfolded (I) proteins


How do we construct a high throughput screen using the chaperonin as a kinetic trap?

Important known properties.

• Chaperonin binding is promiscuous (binds **hydrophobic** regions/fragments/partially folded proteins/exposed transmembrane elements etc.

• Chaperonin is very large protein - 802 kDa (**large BLI signal**)
Automate the ASSAY – Label free technologies Bio-Layer Interferometry (BLI)*

- A layer of molecules attached to the tip of an optic fiber creates an interference pattern at the detector.
- Any change in the number of molecules bound causes a measured shift in the pattern
- Minimize mass transfer affects – Avoided with platform or tip stirring (single channel).

*Initially confirmed using BiaCore Surface Plasmon Resonance with Phil Gao and Na Zhang.
CHAPERONIN IN SOLUTION to detect Immobilized Transitioned Proteins

**PANEL A** In the absence of stabilizer - chaperonin binds – Large signal generated

**PANEL B** - presence of Stabilizer or corrector (C) – Low binding of chaperonin – Lower signal

**PANEL A**
- Folded protein
- Transitioned protein
- Spacer (stable chimeric protein inert to GroEL binding)

**PANEL B**
- Corrector stabilized mutant
- Transitioned protein
- Low or no binding Of Chaperonin onto Stabilized protein.

**BLI signal**

**Signal** vs **Time**
REVERSE CONFIGURATION:
Chaperonin bound to the tips also binds and therefore detects transitioned protein.

PANEL A: No stabilizer - Large signal generated when substrate protein binds.
PANEL B: + Stabilizer or corrector – Low binding- lower signal (potential HIT).

PANEL A:

- Immobilized GroEL binds mutant fold.
- Folded substrate protein.
- Misfolded mutant.

PANEL B:

- Stabilizer or correcter [C] stabilizes mutant - i.e. Low or NO BNDING.
Detected of Folded States

Immobilized Chaperonin on Biosensor tip Distinguishes between folded and partially folded α-lactalbumin.

![Graph of BLI Signal vs. Time](image)

- **Association**
- **Dissociation**

**Blind Signal (nm)**

- **Folded α-lactalbumin**
- **Partially unfolded α-lactalbumin**

**Time (Sec)**

0 100 200 300 400 500 600
Validation of Native Protein Stabilizers. DHFR as Example/(Control for Secondary screens)

DHFR + Chaperonin

Chaperonin + DHFR + 10 mM NADPH (Diminished binding).

Slow Dissociation $K_d = 140 \text{nM}$

Association of Dihydrofolate Reductase onto Chaperonin tip

+ DHFR Stabilizers (NADPH or Hydrofolate)

TIME (sec)

BLI Signal nm

0.25

0.20

0.15

0.10

0.05

0.00

0.00

0

100

200

300

400

500

600
### Native folds that exist in dynamic equilibria with partially folded conformers.

\[
N \text{ (native)} \leftrightarrow I \text{ (folding intermediate)}
\]

<table>
<thead>
<tr>
<th>Protein/Pathway</th>
<th>Associated Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malate dehydrogenase</td>
<td>Beta-2-microglobulin</td>
</tr>
<tr>
<td>Dihydrofolate reductase (human)</td>
<td>Dialysis related amyloidosis</td>
</tr>
<tr>
<td>Hemoglobin tetramer ↔ dimer (GroEL captures dimer)</td>
<td>Transthyretin A25T, L55P WT – No Binding</td>
</tr>
<tr>
<td>Cystic fibrosis Nucleotide binding domain fragment (NBD1).</td>
<td>Frataxin V122D and I154F</td>
</tr>
<tr>
<td>Alpha synuclein (WT)* Parkinson’s (trisomy)</td>
<td>Delta F508 (mouse model protein). Cystic fibrosis</td>
</tr>
<tr>
<td>IgG Antibody Partially folded monomers and dimers (?)</td>
<td>Alpha synuclein (A53T ) – aggregation prone.</td>
</tr>
<tr>
<td>Rhodanese (Bovine) (removal of oxygen prevents Cys oxidation driven unfolding)</td>
<td>Transitioned bacterial toxins (membrane insertable forms).</td>
</tr>
<tr>
<td></td>
<td>Maple syrup Urine disease (dimer) branched-chain alpha-keto acid dehydrogenase</td>
</tr>
</tbody>
</table>

* Alpha Synuclein is proposed to exist as a dynamic tetramer in equilibrium with disordered species.
Therapeutics: Cystic Fibrosis Example

*NIH designated orphan disease affecting 30k in the US*

- **Common Mutations in the CFTR gene (NBD1)**
- **Autosomal recessive**

**Genetic mutation**

**Malformed protein**
- Protein does not fold normally and is degraded by the cell
- Abnormal transport of chloride and sodium across the epithelium

**Disease**
- Lung & pancreatic disease
- Chronic infection
- Gastrointestinal problems
- Infertility

Chaperonin platform detects the moving target (misfolding) allowing us to identify of the Pharmacological chaperones that **DIRECTLY repair malfolded proteins** -- the cause of disease.

Current standard-of-care treats the symptoms – antibiotics, respiratory therapy, exercise/nutrition, organ transplant, etc.
Stabilizing a folding fragment—Relevance to Protein synthesis.

Idea here: Pharmacological Chaperone Stabilized Fragment may facilitate/rescue downstream folding.

Δ F508 at this position prominent mutant in Cystic Fibrosis)

Figure modified from “Protein folding on the Ribosome” Cabrita et al., 2012 Struc. Biol. 20, 1-13.
Using CFTR bound to immobilized CFTR antibodies to search for stabilizers.

Panel A: CFTR NBD1 without stabilizers

Panel B: Stabilized CFTR NBD1
GTP stabilization of CFTR NBD1 can be observed using BLI. GTP does not bind to GroEL. Association: Dipping into GroEL + Corrector. Dissociation: Dipping into Buffer + Corrector. GroEL + CFTR (no stabilizer). GroEL + CFTR + GTP (10mM). GTP conc used was 2000x the Kd.
Corrector Candidates Supplied by Cystic Fibrosis foundation used for screening

<table>
<thead>
<tr>
<th>C1</th>
<th>6-(1H-Benzimidazol-2-ylsulfanylmethyl)-2-(6-methoxy-4-methyl-quinazolin-2-ylamino)-pyrimidin-4-ol</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4</td>
<td>N-[2-(5-Chloro-2-methoxyphenylamino)-4'-methyl-[4,5']bithiazol-2'-yl]-benzamide</td>
</tr>
<tr>
<td>C6</td>
<td>N-(4-bromophenyl)-4-methylquinolin-2-amine</td>
</tr>
<tr>
<td>C7</td>
<td>2-(4-isopropoxypicolinoyl)-N-(4-pentlyphenyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide</td>
</tr>
<tr>
<td>C9</td>
<td>7-chloro-4-(4-(4-chlorophenylsulfonyl)piperazin-1-yl)quinoline</td>
</tr>
<tr>
<td>C12</td>
<td>N-(4-fluorophenyl)-4-p-tolylthiazol-2-amine</td>
</tr>
<tr>
<td>C13</td>
<td>N-(2-(3-acetylphenylamino)-4'-methyl-4,5'-bithiazol-2'-yl)benzamide</td>
</tr>
<tr>
<td>C15</td>
<td>N-phenyl-4-(4-vinylphenyl)thiazol-2-amine</td>
</tr>
<tr>
<td>C16</td>
<td>2-(6-methoxy-4-methylquinazolin-2-ylamino)-5,6-dimethylpyrimidin-4(1H)-one</td>
</tr>
<tr>
<td>C17</td>
<td>N-(2-(5-chloro-2-methoxyphenylamino)-4'-methyl-4,5'-bithiazol-2'-yl)pivalamide</td>
</tr>
</tbody>
</table>
Screening a sample set of CFTR corrector candidates

CFTR corrector compounds sourced from the CF foundation were screened at a conc of 200 uM. Compounds C1 (red) and C7 (yellow) showed up as potential hits (🌟).
Secondary screen and Ranking CFTR stabilizer Hits

Positive “Hit” signal decreases Chaperonin (GroEL) Binding.
Δ F508 CFTR mutant

• Δ F508 is the most common CF mutant (>90% clinical cases)

• Mouse F508 mutant CFTR NBD1 was used
Previously Identified Correctors for WT also show potential to Stabilize ΔF508 CFTR – (Most common Cystic Fibrosis Protein mutant)
Validation of hits using hDHFR partitioning (single channel BLI)

We rule out false positives using an alternative GroEL substrate, DHFR. If compounds interfere with DHFR partitioning, they may not be valid hits.
CFTR-NBD1 potential stabilizers

C15

N-phenyl-4-(4-vinylphenyl)thiazol-2-amine

C16

2-(6-methoxy-4-methylquinazolin-2-ylamino)-5,6-dimethylpyrimidin-4(1H)-one

Another study by Dr. Phil Thomas has also demonstrated that C16 prevents CFTR-NBD1 aggregation.

These compounds still have to be directly validated by direct binding analysis and/or by Denaturation dip procedure (under development).
CURRENT
TECHNOLOGIES

• Protein Aggregation Interference!
• Cumbersome
• Expensive
SLOW - Low Turnaround (Low throughput)
• Nonreusable (lose of target)

• Hard To Use

MOST WILL FAIL TO IDENTIFYING PROTEIN FOLDING DISEASE STABILIZERS DUE TO AGGREGATION!!

CHAPR\textsubscript{x} TECHNOLOGY

• Extremely Sensitive
• Real Time Measurement
• Detects transient misfolds of 11 out of 11 disease proteins tested so far.
• Higher Throughput with Octet System
• Dramatically Accelerates Detection
• Drugs Bind In Environment Closer To Reality
• User Friendly

• Detecting kinetic stabilization of the fold.
• Antibacterial/Antiviral ligand screen Development
Protein folding/unfolding on the tip – GroEL binding detects incomplete folding
His Tagged maltodextrin glucosidase (70 kDa) refolding on NiNTA tip

Denaturant dip
(4 M urea -3 min)

ATP binding
GroEL
Dissociation
(return to baseline)

GroEL
REBinding
Signal not as
Large ~
%50 Refolding
(15 min)

GroEL
Binding
Virtually NO LOSS OF
BOUND SUBSTRATE

3X ATP
Wash
~ 15 min 25C

GroEL
Binding
Virtually NO LOSS OF
BOUND SUBSTRATE

GroEL DOES NOT BIND TO NiNTA (No NBS)
• Since we can detect folding (indirectly through Loss of GroEL binding), can we use this approach to screen for stabilizers of stable folds?
Introduction to the Denaturant Dip and Read – Assessing the **Kinetic Stability** of Folded Proteins or Oligomers (slow dissociation).

Load - Brief wash - Dip into Denaturant - Brief wash - Chaperonin binding

Ni-NTA

Ni-NTA

Ni-NTA

Ni-NTA

Ni-NTA

(e.g. His-Protein Binding)

(KINetically Sensitive STEP - Time regime can Be controlled) + stabilizer

Read out.... Amplitude Increase due to GroEL binding.
Generating an Isothermal Kinetic Denaturation Profile using a Chaperonin-based Biolayer interferometry platform.

![Graph showing GroEL binding amplitude against increasing concentration of denaturant (urea or GnHCl). The graph illustrates how ligand/conditions can either stabilize or destabilize protein fold.](image-url)
Test Substrates Used. Large proteins that bind to GroEL

1) IgG – immobilized on biosensorTips

2) His tagged Maltodextrin glucosidase (MalZ) – 69 kDa

3) His Tagged Malate synthetase (MSG) – 82 kDa.
Complexation between GroEL and urea pulse denatured MalZ monitored through Biolayer Interferometry

Concentration of Urea (M)

GroEL binding amplitude (nm)
Complexation between GroEL and Urea pulse denatured MalZ in presence of 1M sucrose monitored by Biolayer Interferometry

<table>
<thead>
<tr>
<th>Urea Concentration</th>
<th>GroEL Binding (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6M urea + 1M sucrose</td>
<td>56.0</td>
</tr>
<tr>
<td>5M urea + 1M sucrose</td>
<td>52.0</td>
</tr>
<tr>
<td>4M urea + 1M sucrose</td>
<td>48.0</td>
</tr>
<tr>
<td>3M urea + 1M sucrose</td>
<td>44.0</td>
</tr>
<tr>
<td>2M urea + 1M sucrose</td>
<td>40.0</td>
</tr>
<tr>
<td>1M urea + 1M sucrose</td>
<td>36.0</td>
</tr>
<tr>
<td>0M urea + 1M sucrose</td>
<td>32.0</td>
</tr>
</tbody>
</table>
Kinetic denaturation isotherm profile (GroEL binding amplitude)
MalZ is stabilized by Glycerol and Sucrose during urea induced denaturation monitored by GroEL binding.

Stabilizing conditions identified in ~ 1 minute.
Applications?

• Probe stabilities of stable folds (i.e. equilibrium results in very small population of transient fold.)

• Oligomer stabilization. - Attach oligomers to tips and probe kinetic hydrophobic states with chaperonin (particularly useful in both accelerating dissociation).

• Could be a method to supplement thermofluor for oligomers and problem proteins.
Monitoring refolding transitions on BLI tips

- Real time measurements that follows the refolding the Protective Antigen Pore translocon formation

- Validation of transition
  - Micelle binding to exposed hydrophobic tip.
  - Visualization of Pore translocon that transitioned directly onto BLI biosensors.
The Anthrax toxin Protective antigen prepore to the pore transition involves massive unfolding refolding to create the extended Pore translocon during Endosome acidification.

Crystal structure of the prepore

85 Å

180 Å

Latest Model constructed from Low resolution EM structure of anthrax toxin pore *

Functional Pore Complex inserted Into lipid bilayer

Katayama et al., PNAS 2010. (lipid nanodiscs)

Modified from Heijne (2005) Science
LFn or Anthrax Prepore can be immobilized onto BLI or SPR surface via Thiol coupling.

**Thiol coupling:** an active disulfide moiety can be introduced either on the dextran matrix or on the ligand molecule. 2-(2-pyrdinyldithio) ethaneamine (PDEA) can be used to introduce the di-sulfide group for attachment exchange.
Immobilization and reaction sequence scheme for SPR (Panel A) or BLI (Panel B) detection of Membrane insertion transition (example given here Anthrax toxin prepore to pore transition).

**Panel A – SPR Scheme**

- Flow PA over LFn Immobilized on SPR surface
- Change Buffer to pH 5-6.5 Induce PA prepore to pore Conversion induced by pH change
- Monitor conformational change via change in Protein thickness / refractive index during The transition.

**Panel B – BLI Scheme**

- Immobilized LFn via sulphydryl linkage onto BLI tip
- Prepore binding in an orientation dependent manner onto BLI tip
- Immersion of BLI tip from pH 7.5 to In pH 7 or below induces transition that is detect by BLI
This conformational Extension of the Anthrax Translocon can be observed using label free methods (SPR).

Flow buffer changed to pH 6.5

Transition exclusively Occurs Away from surface (observable signal)

Return flow to pH 7.5

Transition remains

Flow Rate: 5ul/min

TIME (sec)

RU (Response)

! Time consuming 3-4 pH runs - two days!@!
Anthrax toxin pore affinity orientated so that Pore transitions away from the tip surface. Conformation changes detected at 7, 6.75 and 6.5 (2x)/pH.

Octet Bilayer interferometry (BLI) Detect same toxin prepore to pore transition

(MUCH MORE RAPID ANALYSIS! – 30 min vs 4 Days!)

Transition kinetics depends on pH jump

BLI Tip returned to pH 7.5

Transition remains

TIME (sec)
**Fortebio** Blitz system (Single channel.)

- **Drop. Read. Done.**
- **Time (sec):**
  - Run 1
  - Run 6

**Shift (nm):**
- pH 6.75
- Return to pH 7.5

**Time (min):**
- 0
- 5
- 10
Monitoring a Anthrax Pore translocon transition on the Biosensor (Before pH transition – Only Prepore is visible (TEM))

No Transition Control

Removed from tip With 1M DTT pH 8
Monitoring the insertion of Anthrax toxin Protective antigen (PA) into lipid nanodisc on immobilized solid (BLItz tip) surface after prepore to pore transition

Removal from solid surface (BLItz tip) indicating that PA pore aggregates are present, confirmed by TEM

Control experiment indicating that no insertion of lipid nanodisc without prepore to pore transition

pH 5.5, prepore to pore transition

Binding of MSP+Cholate+POPC mixture

Removal from solid surface (BLItz tip) indicating that insertion of PA in to lipid nanodisc, confirmed by TEM

NO transition

No binding of MSP+Cholate+POPC mixture
200 mM Imidazole knocks GroEL-Protein Substrate Complex off of the tip onto EM grid.

Rounded top GroEL indicative of substrate bound form. (large Dome- large substrate)

Free GroEL (square barrel)

GroEL-MalZ GroEL-GS
• Chaperonin readily detects kinetically-transient partially-folded states and burst populations. - REVERSIBLE with ATP addition.
• Large BLI signals are generated for both configurations (Chaperonin or Target Protein immobilized on BLI tip).
• The kinetics of partitioning can be controlled (e.g. modest temperature increases ~ 40-45°C = denaturant dip)
• Octet BLI system much more rapid, versatile, and robust compared to SPR systems. (will be particularly useful for slow protein partitioning reactions).
• Denaturant dip and read protocol allows one to evaluate the kinetic stability of the fold for native proteins and oligomers.
• As in SPR, Octet BLI system can also detect large unfolding/refolding conformational transitions. (85 to 170 Å extension of Anthrax toxin). In this case also, throughput is dramatically more rapid using an Octet BLI compared with SPR.
• Conversion to extended form confirmed by EM.
After the pH transition, TEM reveals that all anthrax Protective antigen removed from Biosensor tip has transitioned to the Pore translocon.