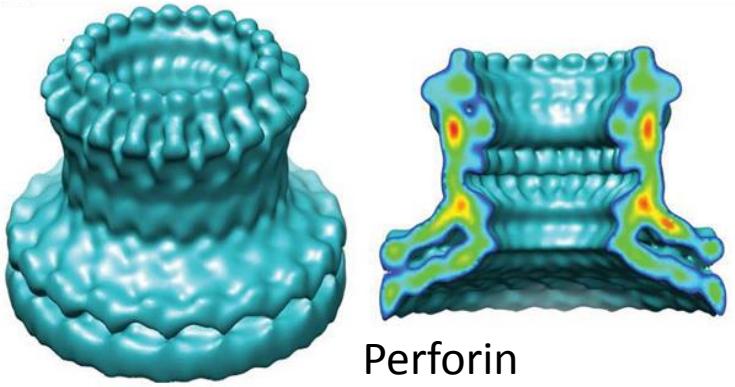
Understanding protein-membrane interactions using Cytolysin A as a model

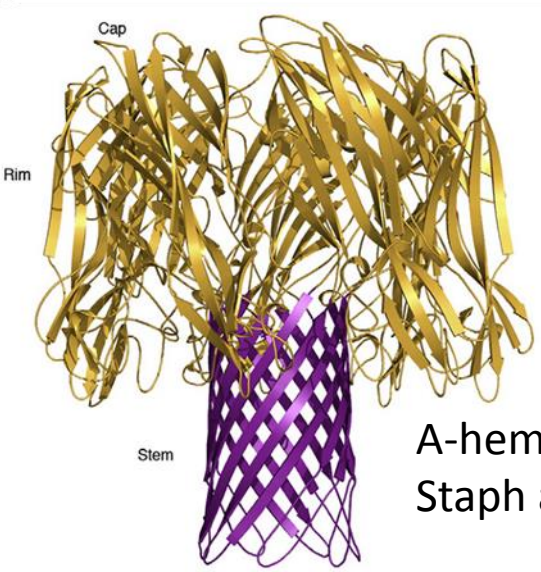
- Omkar Banare

IIT Kanpur

Pore forming toxins



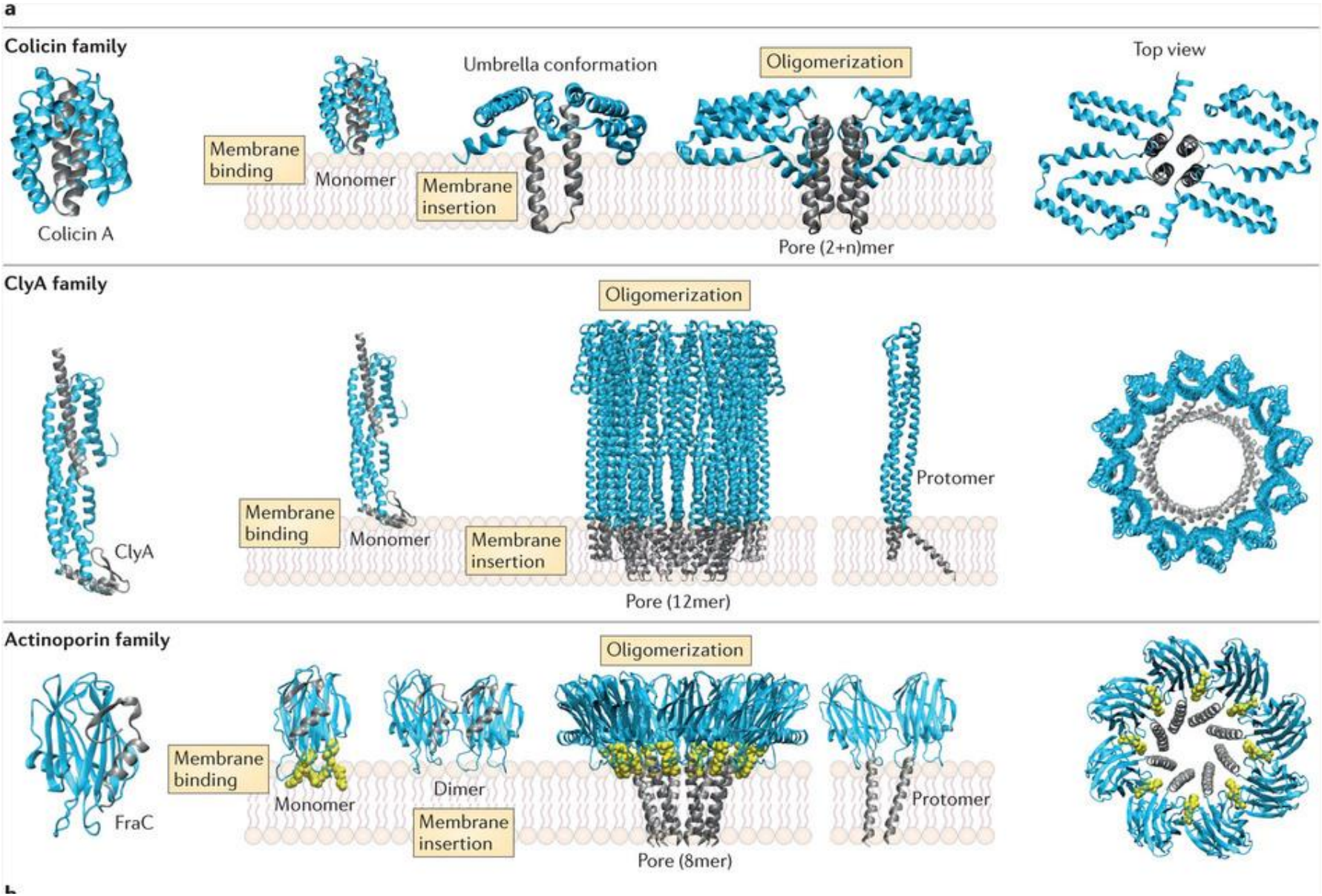
Perforin



A-hemolysin from Staph aureus

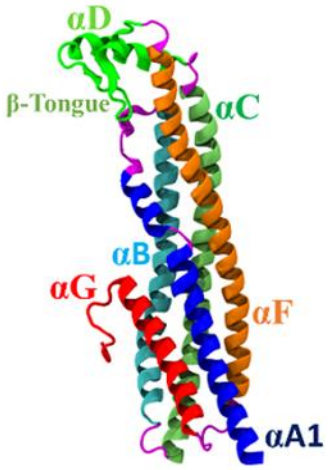
- Pore-forming toxins (PFTs) are the largest class of bacterial protein toxins
- Anemones [equinatoxin], mushrooms [flammutoxin] and humans [perforin] also produce PFT like proteins
- Pores cause cellular ion imbalance leading to cell lysis and death
- But are also used for other cellular purposes, such as immunoregulation by perforin in humans
- Characteristic feature of PFTs : Exist in bistable states
- Great to learn about protein structure!
- Can be classified broadly into alpha and beta toxins based on the secondary structure elements that span the membrane

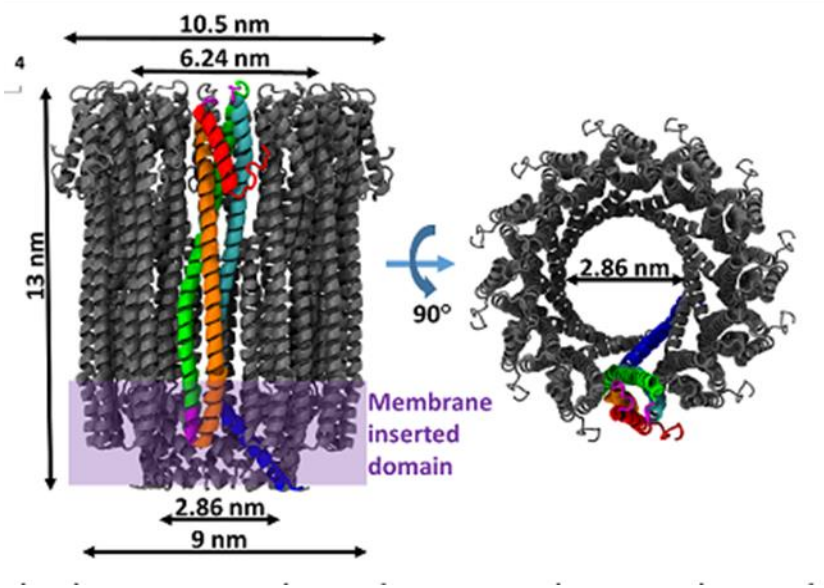
Dal Peraro, Matteo, and F. Gisou Van Der Goot. "Pore-forming toxins: ancient, but never really out of fashion." Nature Reviews Microbiology 14.2 (2016): 77-92.


a
Colicin family
Colicin A
Membrane binding
Monomer
Umbrella conformation
Membrane insertion
Oligomerization
Pore (2+n)mer
Top view
ClyA family
ClyA
Membrane binding
Monomer
Membrane insertion
Oligomerization
Pore (12mer)
Protomer
Actinoporin family
FraC
Membrane binding
Monomer
Dimer
Membrane insertion
Oligomerization
Pore (8mer)
Protomer
b

E. coli Cytolysin A

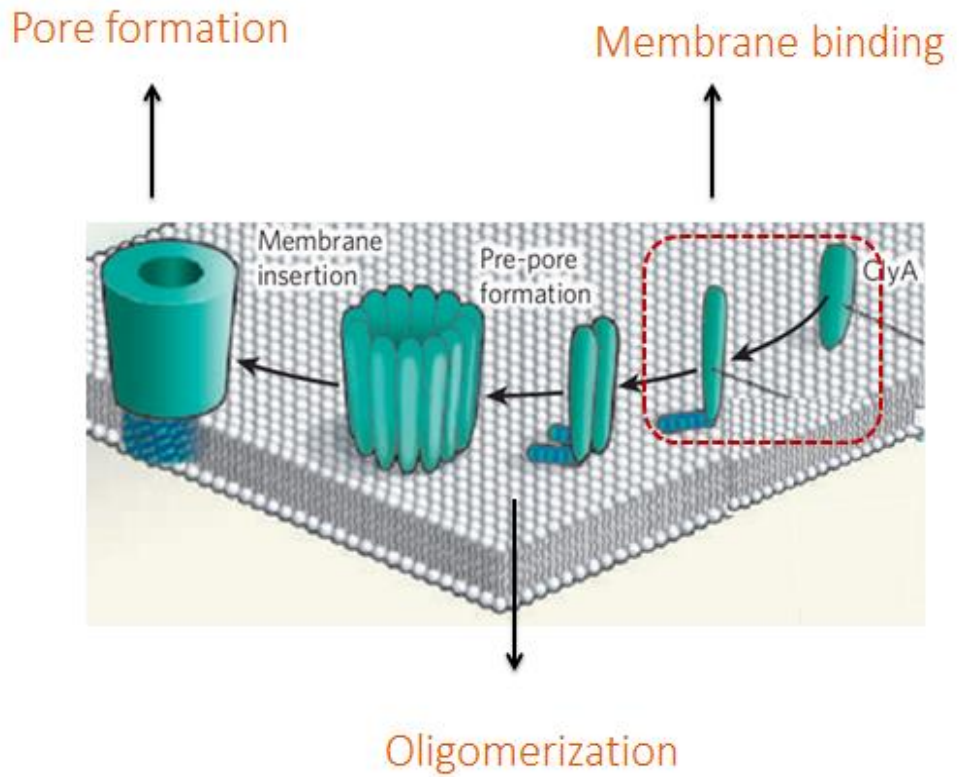
- Cytolysin A (ClyA, also known as HlyE), a α-PFT, is a cytolytic α-helical toxin responsible for the hemolytic phenotype of several *Escherichia coli* and *Salmonella typhi* strains
- Assembles as a dodecameric pore with a 2.8 nm diameter
- Transition from monomer to protomer involves structural rearrangement of ~55% of residues
- While studies on crystal structure elucidation for both the monomer and the pore are available, kinetic studies for the same have not been done so far.





Mueller, Marcus, et al. "The structure of a cytolytic α-helical toxin pore reveals its assembly mechanism." Nature 459.7247 (2009): 726-730.

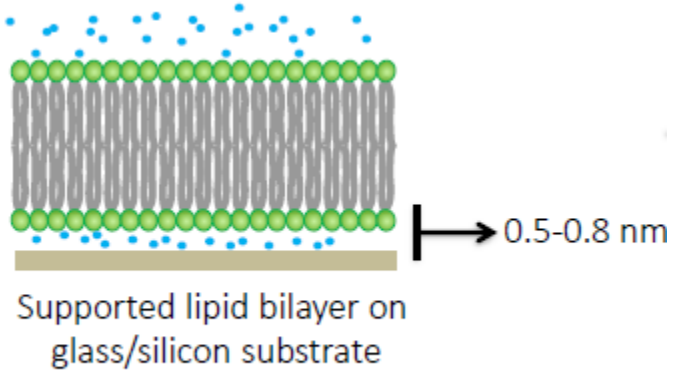
E. coli Cytolysin A

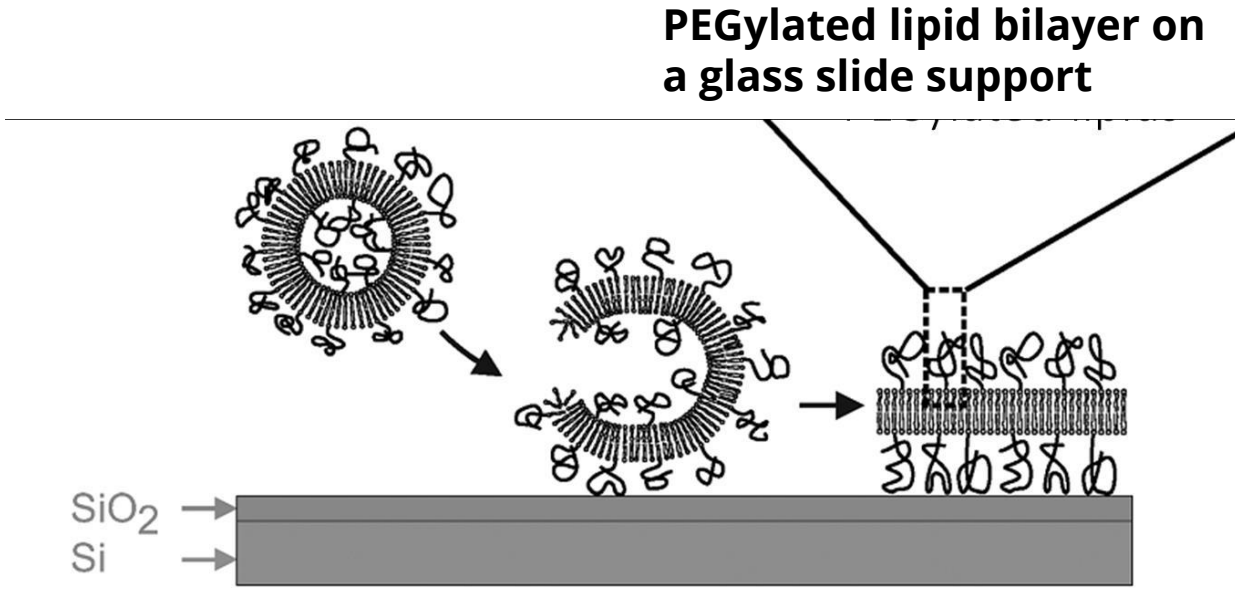


- Reports propose a sequential assembly mechanism of the ClyA monomer, thought to occur through three main stages – membrane binding, conformation change and oligomerisation, and pore formation and insertion

Benke, Stephan, et al. "The assembly dynamics of the cytolytic pore toxin ClyA." *Nature communications* 6 (2015).

Single molecule experiments





PEGylated lipid bilayer on a glass slide/silicon support. Vesicles with PEG are formed, broken and eased onto a silicon substrate for single molecule experiments on ClyA. The PEGylation was done in the first place to lift the lipid bilayer over the substrate to provide room for ClyA to insert into the membrane completely,

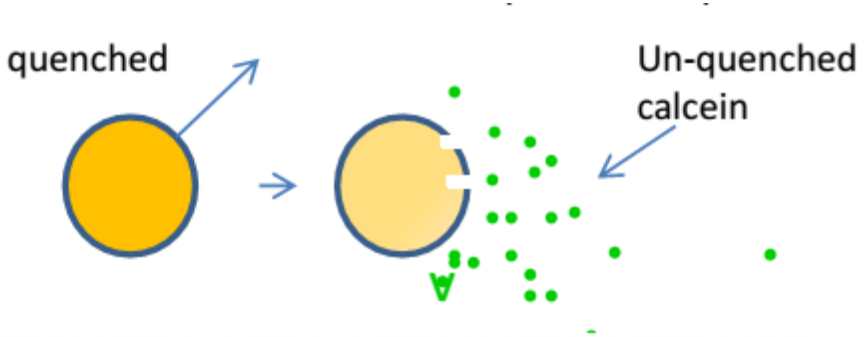
Questions

- Does the C-terminal loop play any role in activity?
- Kinetics studies on the lytic behavior at various concentrations of ClyA Q56C.
- Does PEGylation of vesicles change the activity of ClyA?

Methods

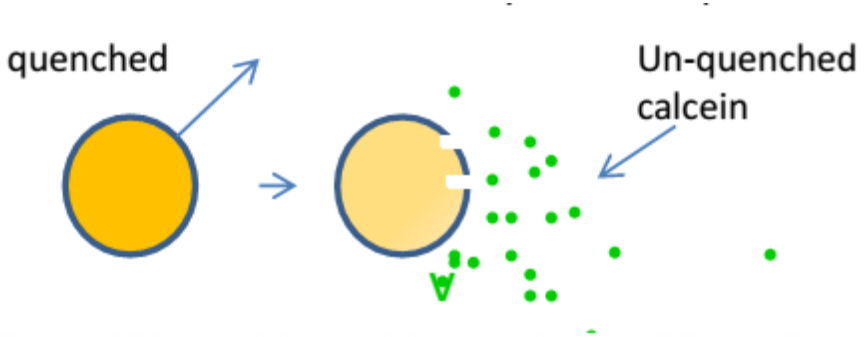
- Expressed, purified and verified (using SDS-PAGE) ClyA Q56C and ClyA Q56C Δ292-303.
- Hemolysis assays using diluted rabbit erythrocytes along with varying concentrations of the wild type and the deletion mutant.
- Hemolysis assay using a fixed amount of the wild type, along with varying concentrations of the deletion mutant added to it.
- Calcein leakage experiments (next slide)

Calcein leakage experiments



- Calcein is present at a self-quenching concentration (80mM) in the vesicles.
- As calcein leaks, the self-quenching property is lost, leading to increase in fluorescence.
- So as the no. of pores formed increase, the fluorometer reading goes up.
- Detergent completely solubilizes the membrane and releases all the calcein, giving the fluorescence reading for complete lysis.
- Vesicles of POPC: Chol (50% molarity, 50% molarity respectively, 5mM) and POPC: Chol: DOPE-PEG (47.5% molarity, 47.5% molarity, 5% molarity respectively, 5mM were made

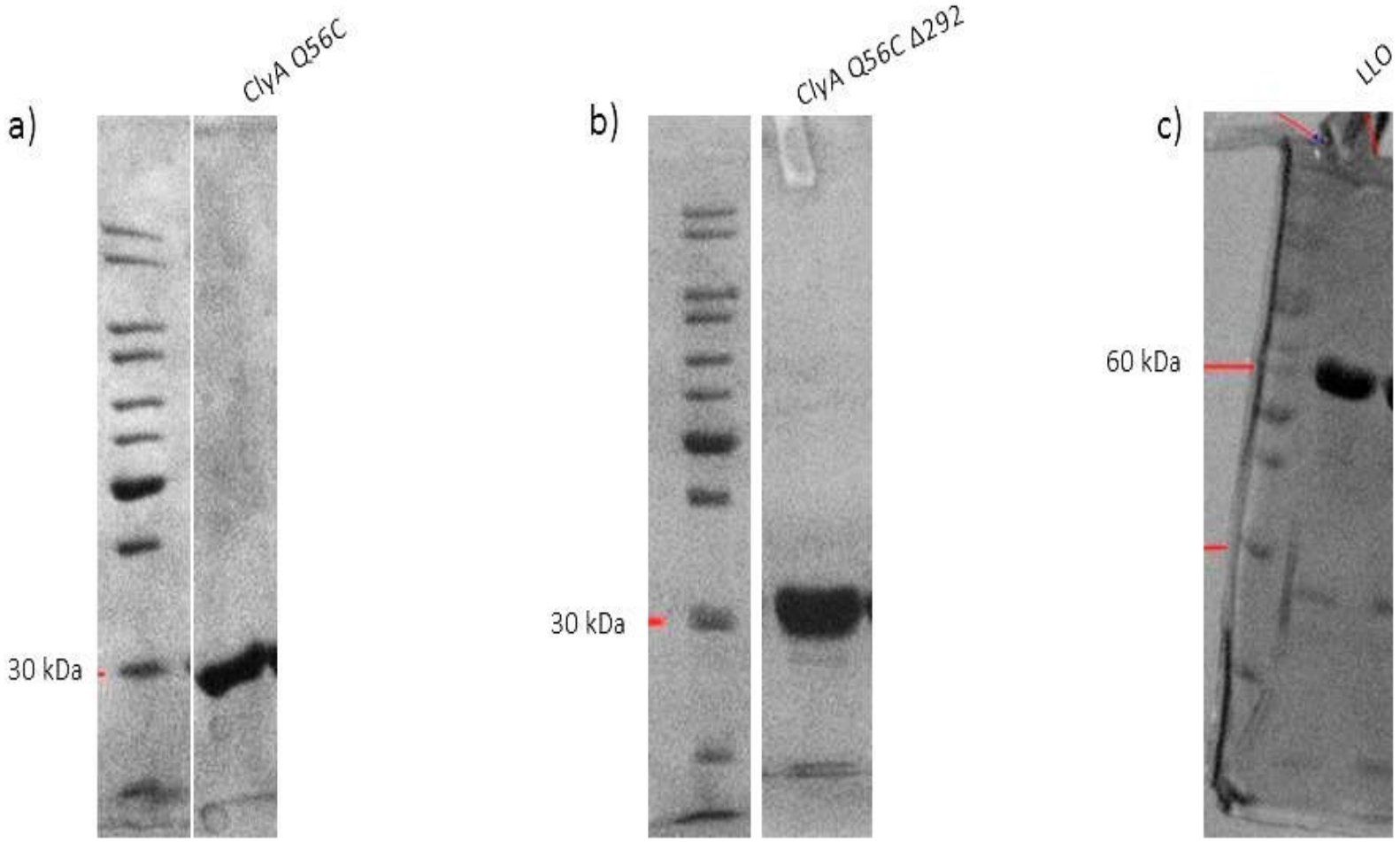
Calcein leakage experiments



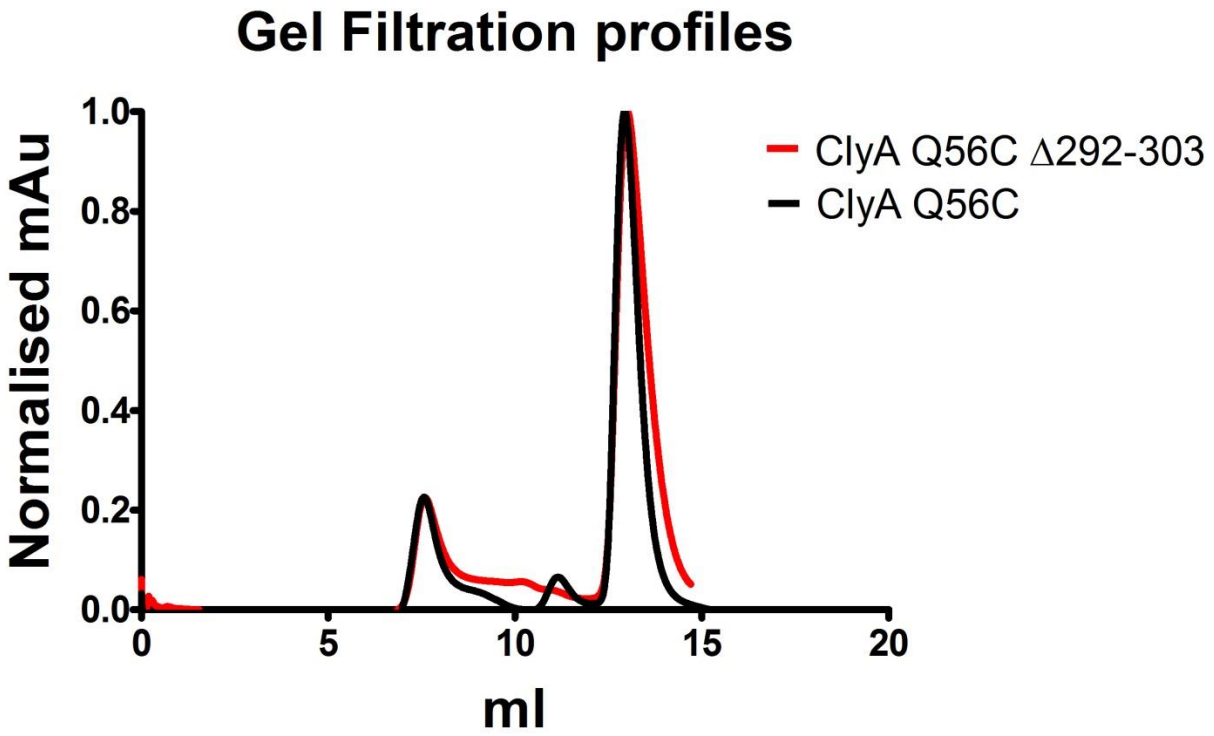
- Varying concentrations of ClyA Q56C were added to POPC: Chol vesicles to look at the dose response and generate kinetics
- Equimolar concentrations of ClyA Q56C were added to both POPC:Chol and POPC:Chol:PEG

Results

SDS-PAGE for the proteins purified

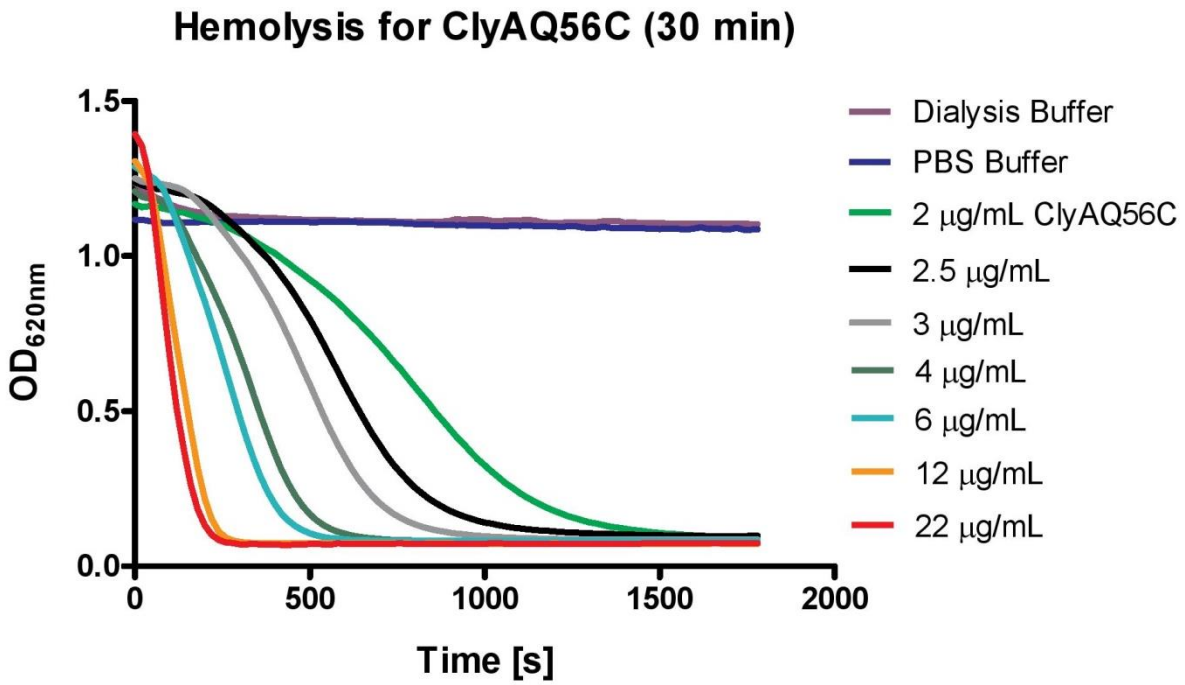


Gel Filtration profile



- **Gel Filtration profiles for both the wild type and the mutant protein** show a single broad monomeric peak at around 14 seconds, indicating no aberrant aggregated proteins formed due to the mutations. The curves were normalized with the maximum mAu of the individual curves obtained.

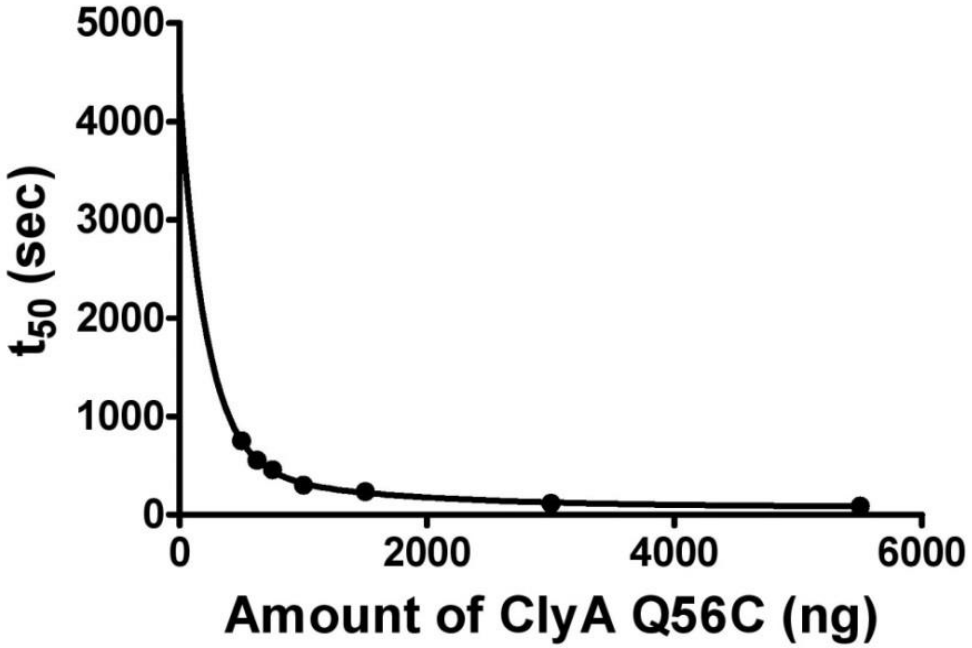
Results of the hemolysis assays



	2 μg/mL	2.5 μg/mL	3 μg/mL	4 μg/mL	6 μg/mL	12 μg/mL	22 ng/μL
t_{50}(sec)	752.8	555.4	458.6	302.8	236.9	119	88.42
Slope	-202.6	-135.9	-115.6	-84.63	-75.94	-39.91	-36.34

- Lysis activity increases with increase in concentration of toxin present

Results of the hemolysis assays

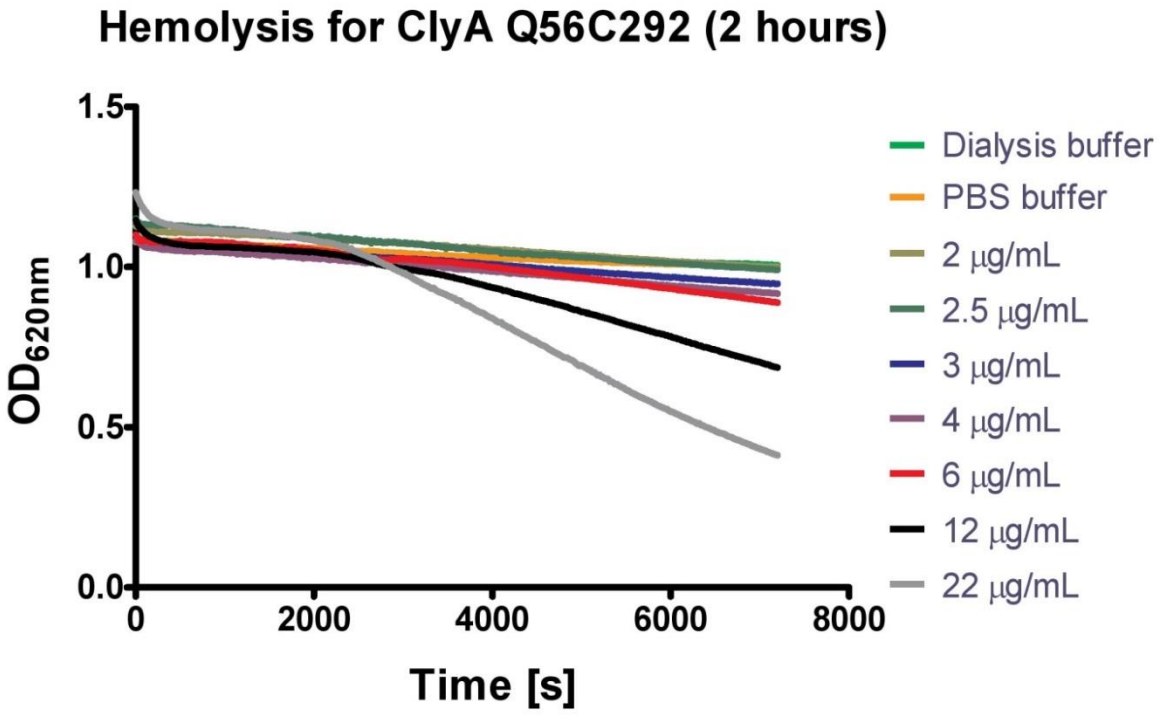


$$t_{50} = 424.4e^{-0.00073x} + 3790e^{-0.0046x} + 78.2$$

where,

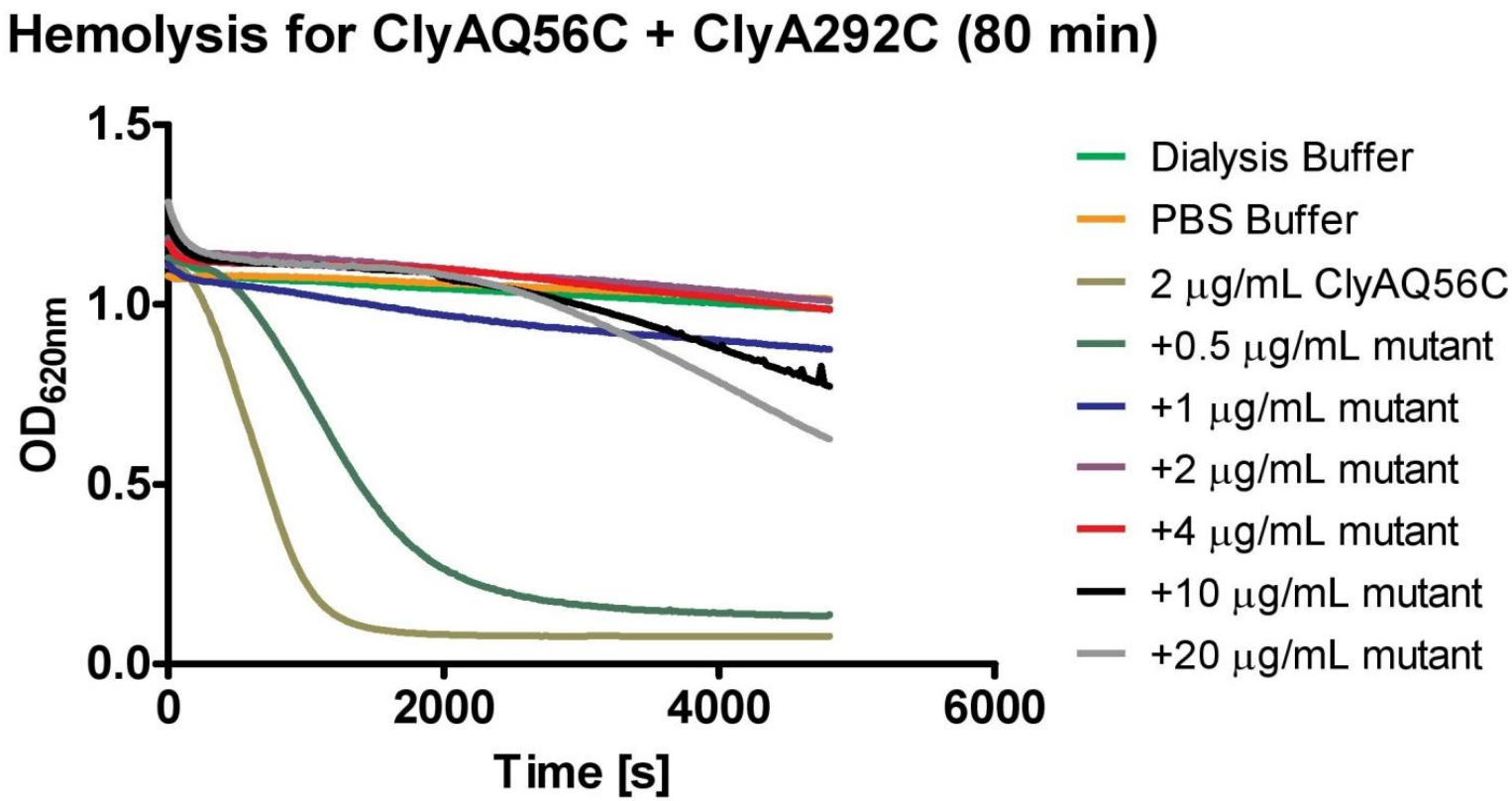
x = Amount of ClyA Q56C added

Results of the hemolysis assays



- Deletion mutant shows loss of lytic activity, even at high concentrations of the toxin
- As expected, lysis rates increased with increasing concentration of ClyA Q56C Δ292-303.

Results of the hemolysis assays

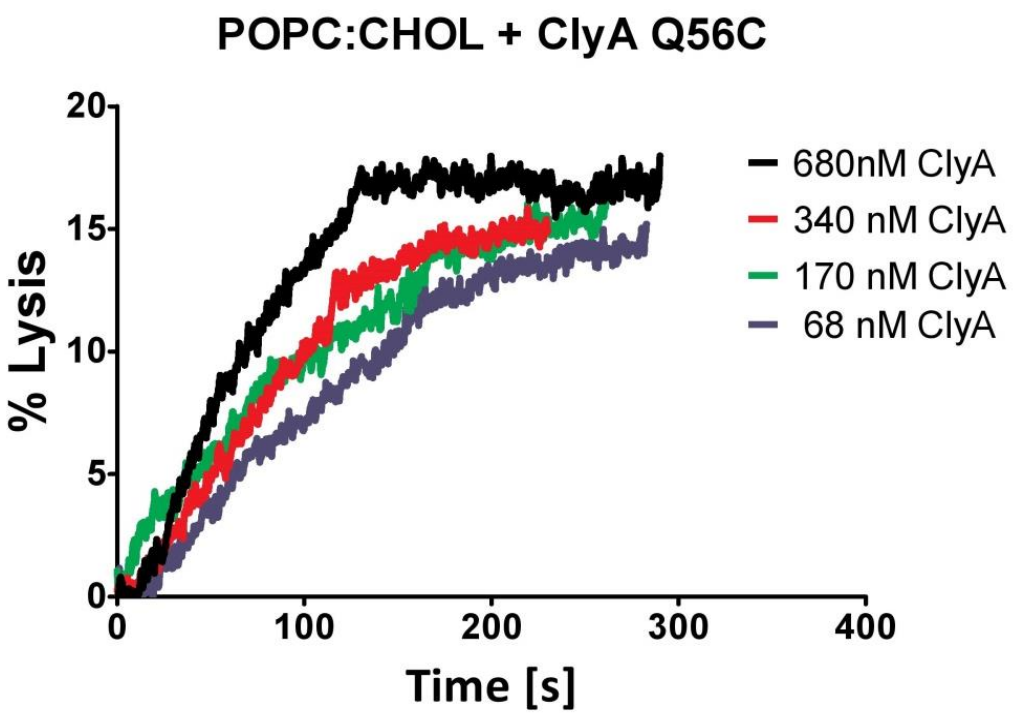


- Increasing the amount of the deletion mutant gradually decreases the rate of lysis until no lytic activity can be observed
- Equimolar concentrations of the wild type and mutant show no lysis activity, suggesting hetero-oligomerisation
- Lytic activity observed for 10 µg/mL and 20 µg/mL of truncated toxin added to 2 µg/mL of ClyA Q56C is due to oligomerisation of the truncated toxin itself.

Questions

- ✓ Does the C-terminal loop play any role in activity?
- ✓ Kinetics studies on the lytic behavior at various concentrations of ClyA Q56C.
- Does PEGylation of vesicles change the activity of ClyA?

Results from calcein leakage



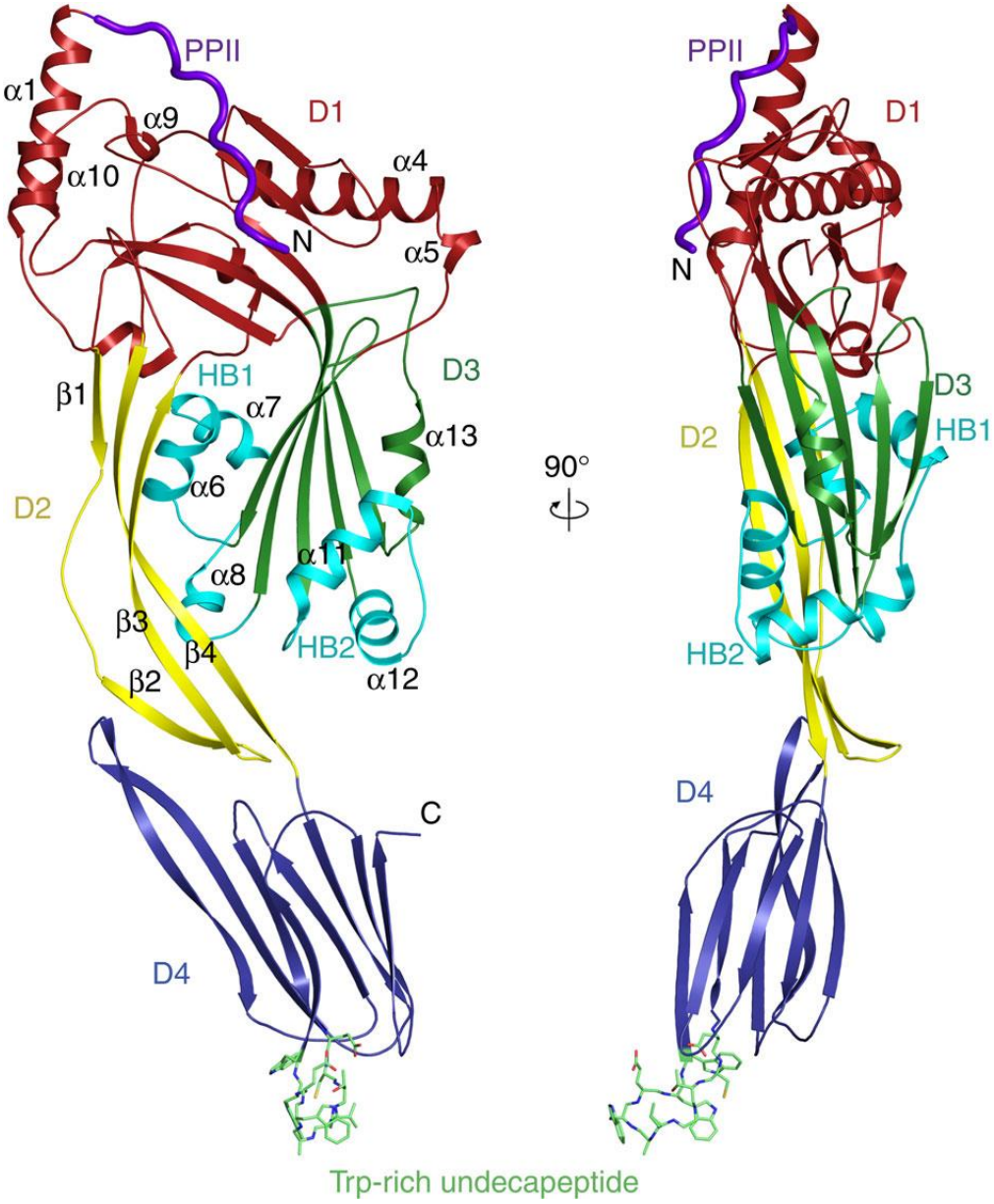
	680 nM	340 nM	170 nM	68 nM
K	0.01566	0.008568	0.007054	0.005889
Tau	63.87	116.7	141.8	169.8
Half-time	44.27	80.9	98.27	117.7

- ClyA is not able to lyse above 16-17% of the vesicles, indicating its poor potency.
- The highest concentration added (680 nM) lyses 17% of the vesicles in 100 seconds, and halts further lytic activity
- Similarly all other concentrations saturate to 16%
- Increasing the concentration increase the rate of lysis (but not the total lysis)

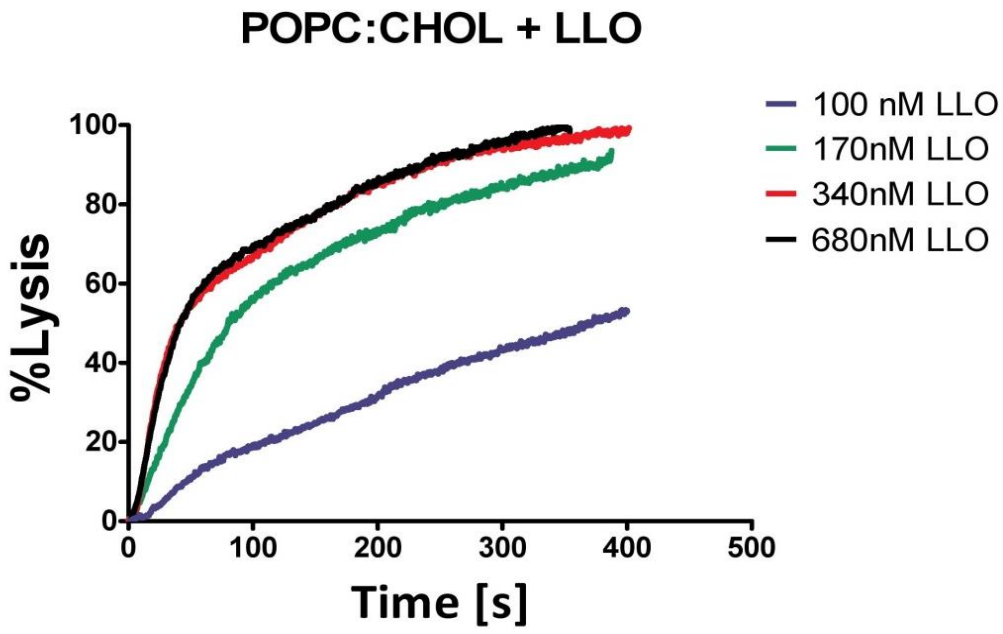
Why does ClyA lyse only 16% of the vesicles during calcein leakage?
Are vesicles the problem?

Listeriolysin O

- Cholesterol-dependent PFT of the hemolysin family produced by the bacterium *Listeria monocytogenes*, the pathogen responsible for causing listeriosis.
- Potent toxin with high lytic activity



Results from calcein leakage



	100nM LLO	170nM LLO	340nM LLO	680nM LLO
K	0.002295	0.008968	0.01125	0.01227
Tau	435.7	111.5	88.9	81.51
Half-time	302	77.3	61.62	56.5

- Lysis observed is almost complete (~95%), showing high potency of LLO in artificial lipid vesicles
- Even a low concentration of LLO is able to lyse upto half the vesicles as compared to ClyA which lyses just 16% of vesicles regardless of concentration.
- Saturation of lysis is seen to be dose dependent, as 680nM of LLO lyses 98% of the vesicles, 340nM lyses 95%, 170nM lyses 80% and 100nM lyses just about 50% at the end of 400 seconds.
- But with ClyA, saturation of lysis activity is not seen to be dose dependent, all concentrations saturate at 16-17% of lysis.
- The cause for such difference in potency is, however, not known.

Why does ClyA lyse only 16% of the vesicles during calcein leakage?
Are vesicles the problem?

No!

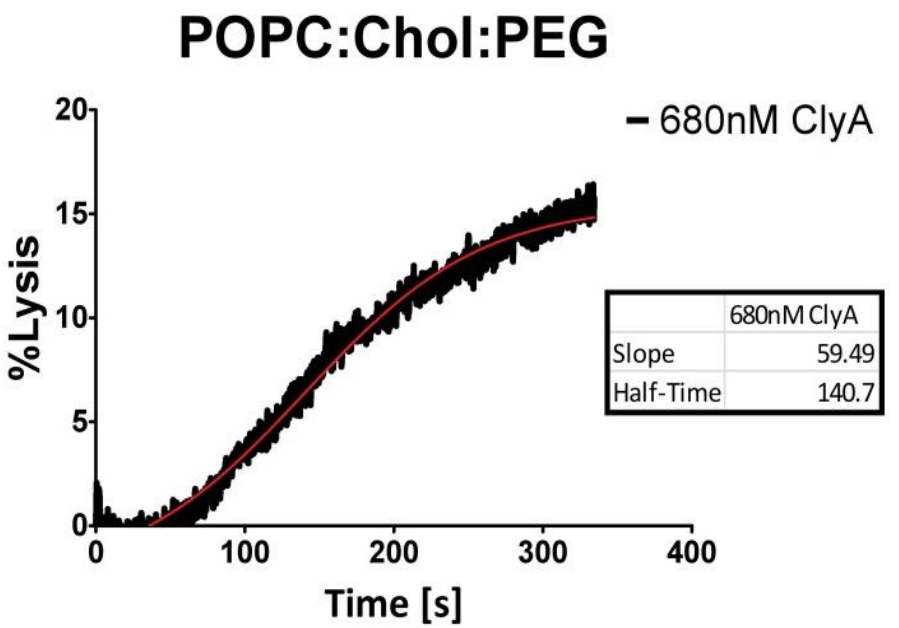
Results indicate the high potency of LLO on vesicles, as well the low potency of ClyA Q56C on the same vesicles

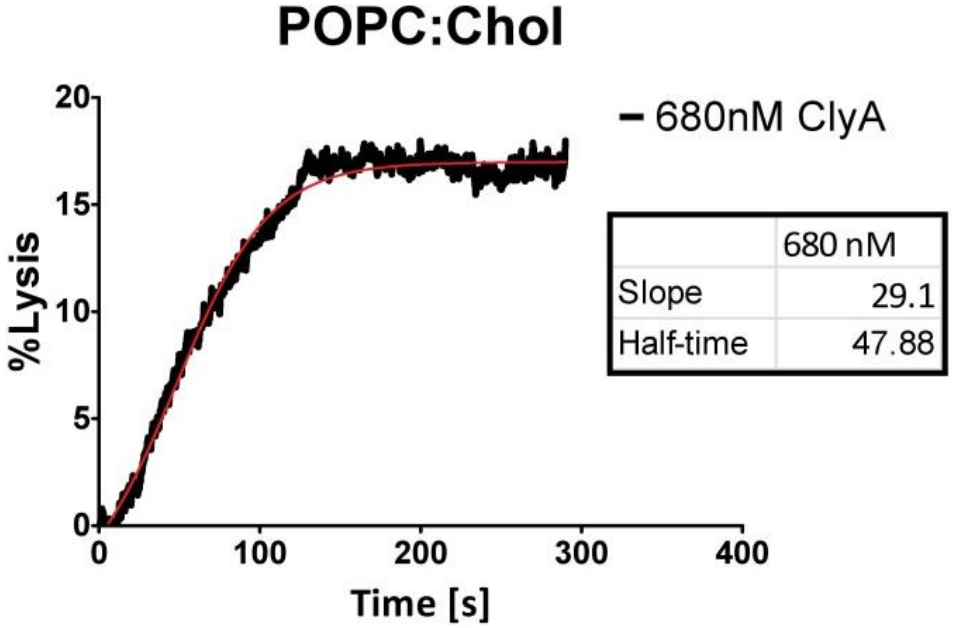
Questions

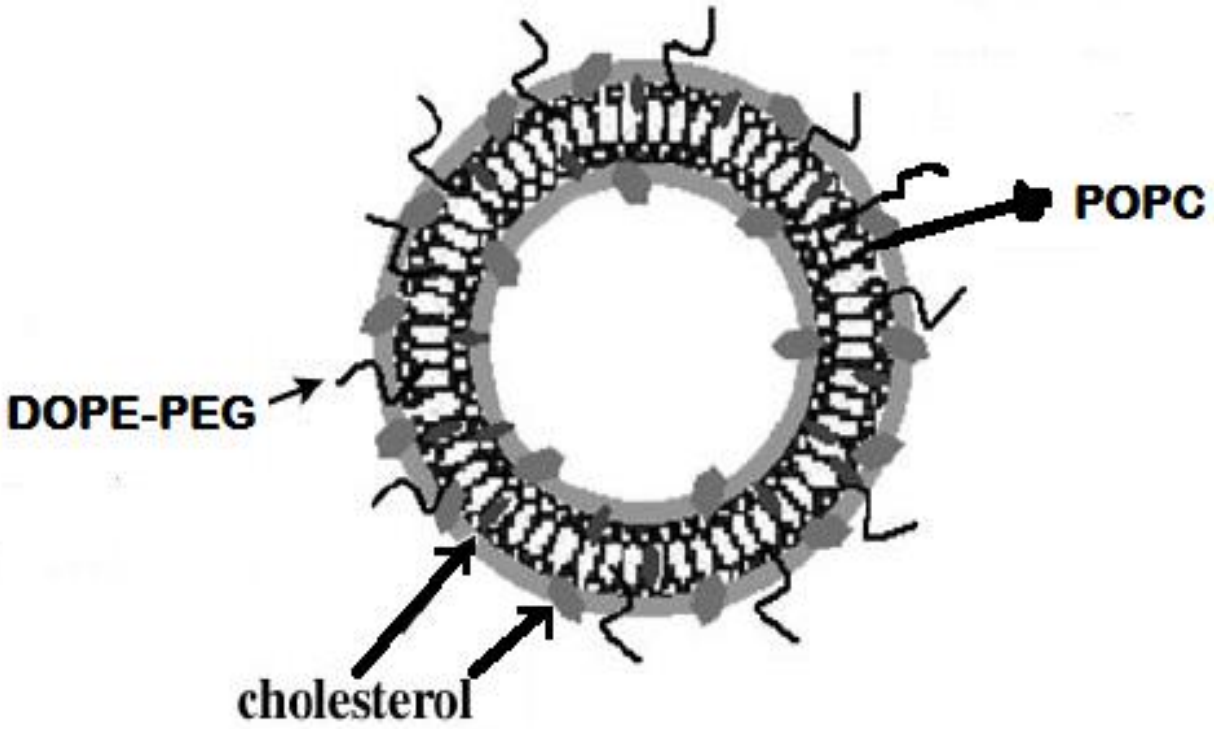
- ✓ Does the C-terminal loop play any role in activity?
- ✓ Kinetics studies on the lytic behavior at various concentrations of ClyA Q56C.
- Does PEGylation of vesicles change the activity of ClyA?

Results from calcein leakage

- While the rate constant (inverse of slope) for lysis was around 0.017 for PEGylated vesicles, lysis with the normal vesicles has a rate constant of 0.034, showing a slight decrease in the rate of lysis in the case of PEGylated vesicles.
- More importantly, the difference in the half-life of the vesicles is just less than 100 seconds indicating the sustained lysis activity in the case of PEGylated vesicles.
- However, this sustained lysis does not decrease the amount of lysis occurring, as it is still seen to saturate at 16%.
- These observations point out towards the possible steric hindrance being offered by PEG while oligomerisation of the toxin.






POPC
DOPE-PEG
cholesterol

Questions

- ✓ Does the C-terminal loop play any role in activity?
- ✓ Kinetics studies on the lytic behavior at various concentrations of ClyA Q56C.
- ✓ Does PEGylation of vesicles change the activity of ClyA?

Conclusions

- C-terminal loop plays an important role in regulating lytic activity of the toxin.
- Kinetics data for lysis rates are now available for different concentrations of ClyA for both natural membranes (using rabbit RBCs as a model) and artificial ones (using POPC: Cholesterol vesicles as a model).
- PEGylated vesicles do not compromise the activity of Cytolysin A; however a gradual shift in lysis profile is observed implying sustained lysis of the vesicles when treated with ClyA.
- This is likely due to PEG chains sticking out over the liposome surface, providing steric hindrance to pore formation.
- Potency of Cytolysin A is quite poor in synthetic vesicles , as compared to LLO which is quite potent in activity on the vesicles.
- Hetero-oligomerisation between the wild type and the deletion mutant takes place when co-incubated together, effectively reducing the lytic activity. Hence the deletion mutant can act as a suitable blocker for reducing lytic activity of the toxin.

rekt.