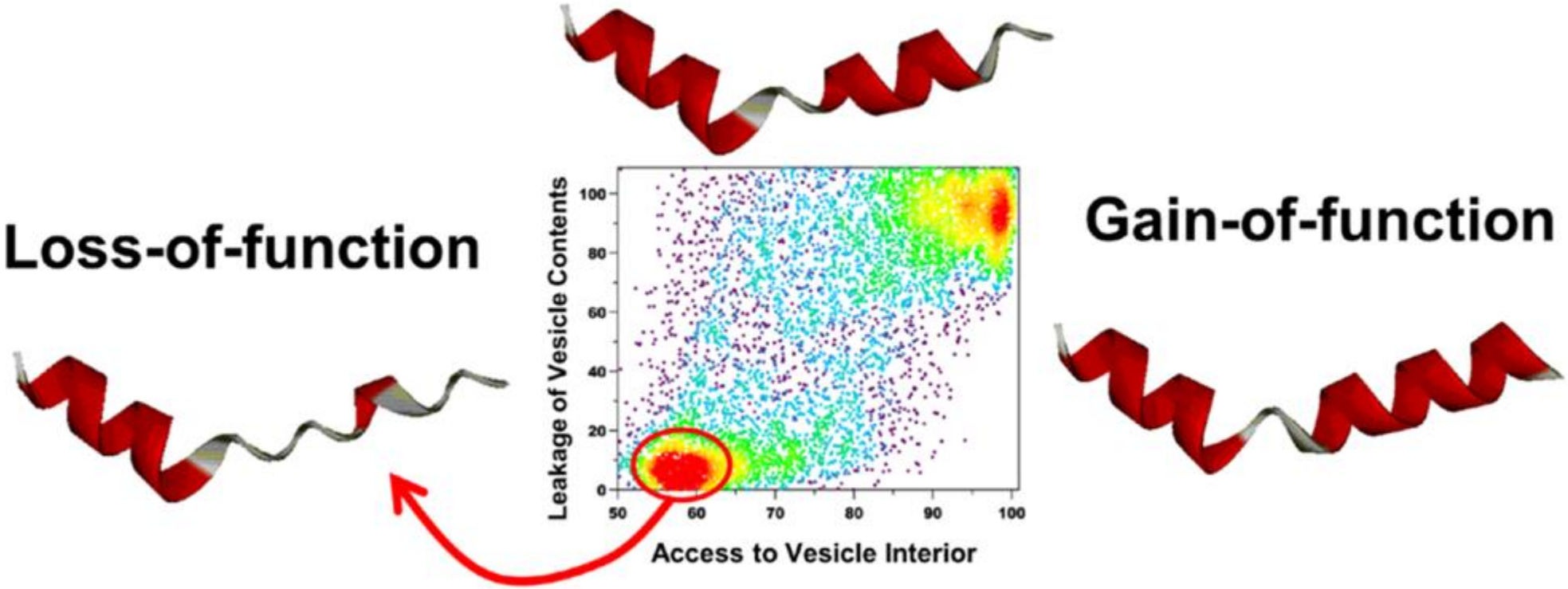
Discovery of peptides with selective cytotoxicity by screening a library for *loss-of-function* variants

2016/2/5 Kotaro Tokumoto



Krauson, A. J. *et. al. J. Am. Chem. Soc.* **2015**, *137*, 16144.

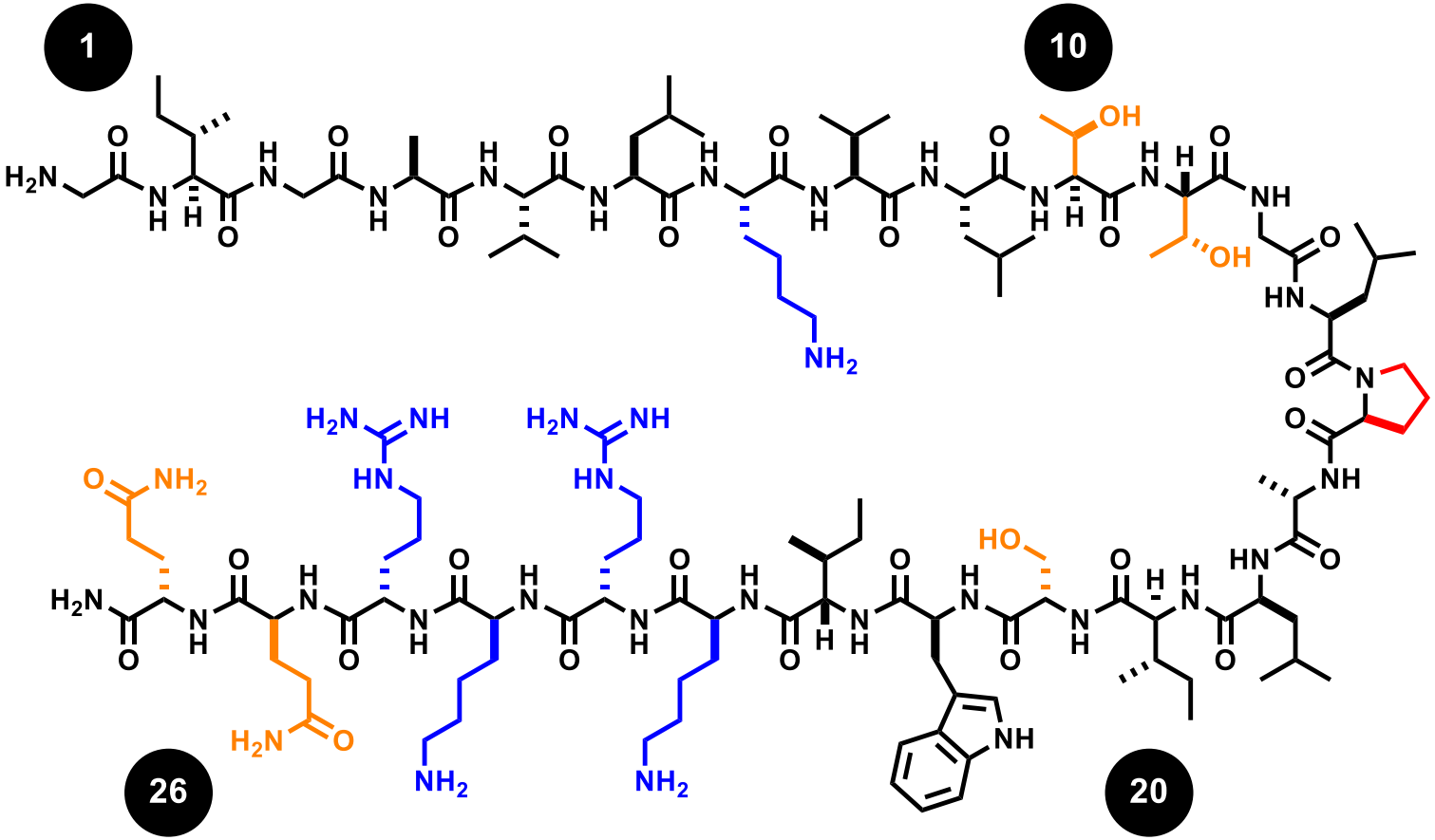
Outline

1. Introduction

   Melittin and its toxicity

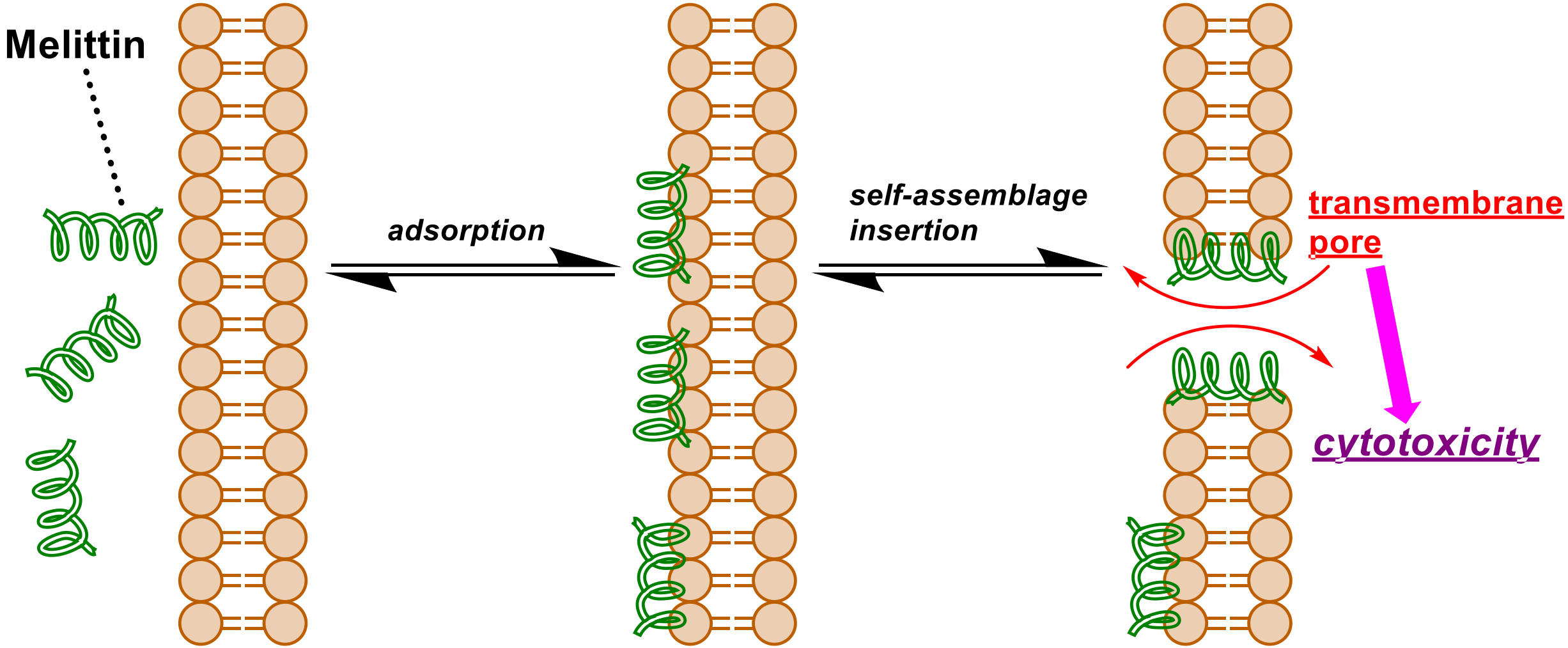
2. Screening for *gain-of-function* variants
   i. Split-pool method
   ii. High-throughput screening (Two-step assay)
   iii. Further study on *gain-of-function* variants
3. Screening for *loss-of-function* variants (main paper)

1-1. Melittin



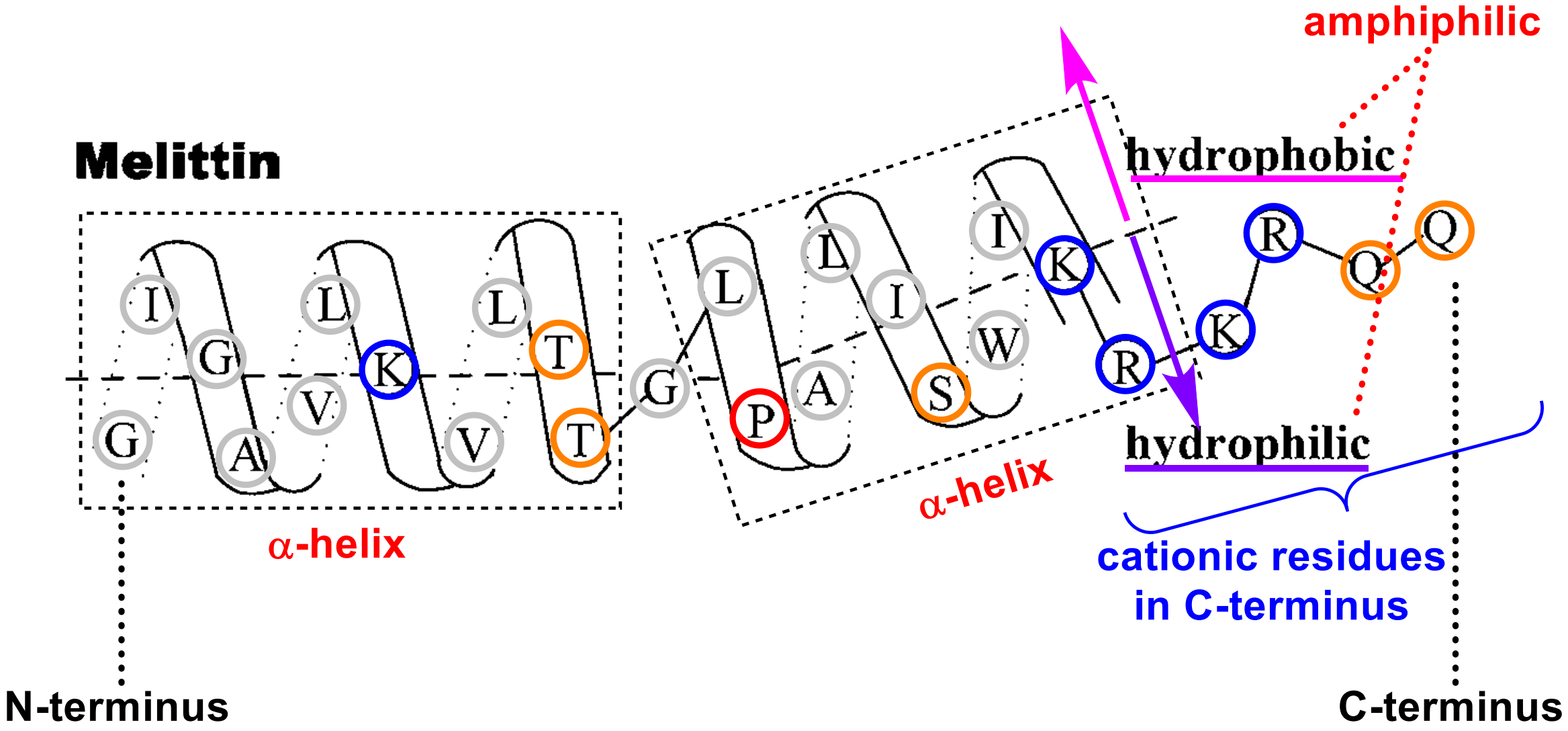
- Main component of European Honey Bee venom
- 26 residue α-helical peptide toxin (Only L-amino acids)

1-2. Pore formation



Sengupta, *D.* et. al. *Biochim. Biophys. Acta* **2008**, *1778*, 2308.

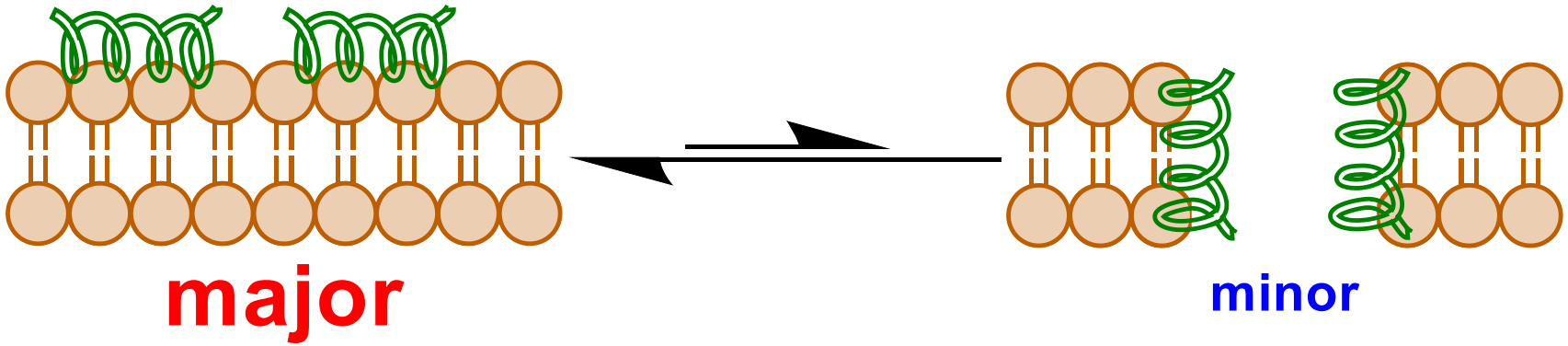
1-3. Melittin molecule in membrane



Lee, V. S. Y. et. al. *Insect Mol. Biol.* **2007**, *16*, 231.

1-4. Difficulty in detailed study and applications

- "Pore" is sensitive to various properties:
  - Peptide concentration
  - Lipid composition
  - pH
  - Ionic strength
  - Temperature
- Transmembrane pore state is usually a minor component of the total peptide population in equilibrium



Hristova, K. et. al. *Biophys. J.* **2001**, *80*, 801.

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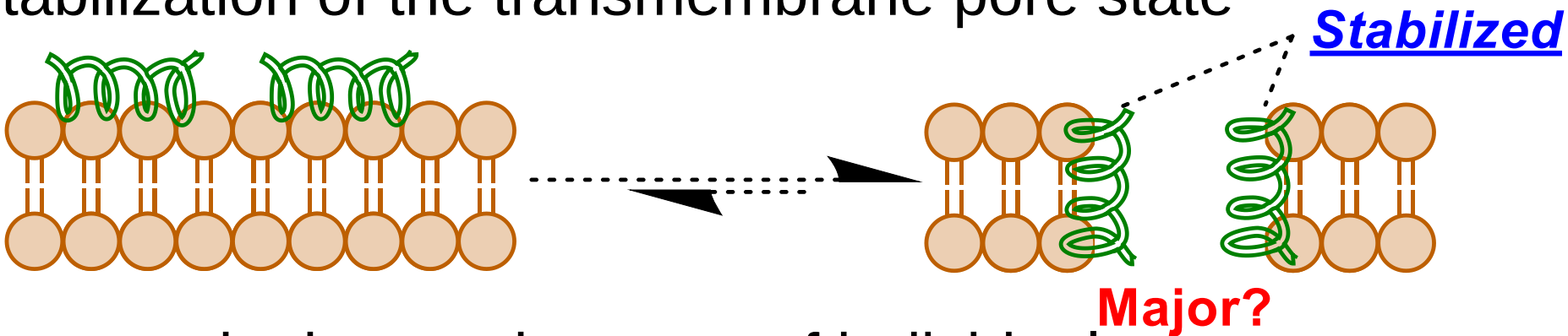
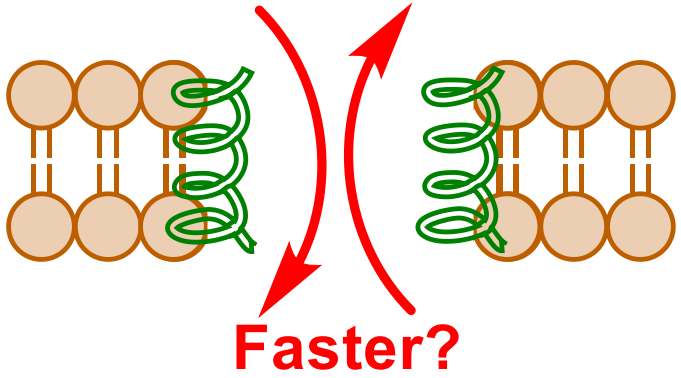
2-1. Strategy toward *gain-of-function* variants

1. Stabilization of the transmembrane pore state



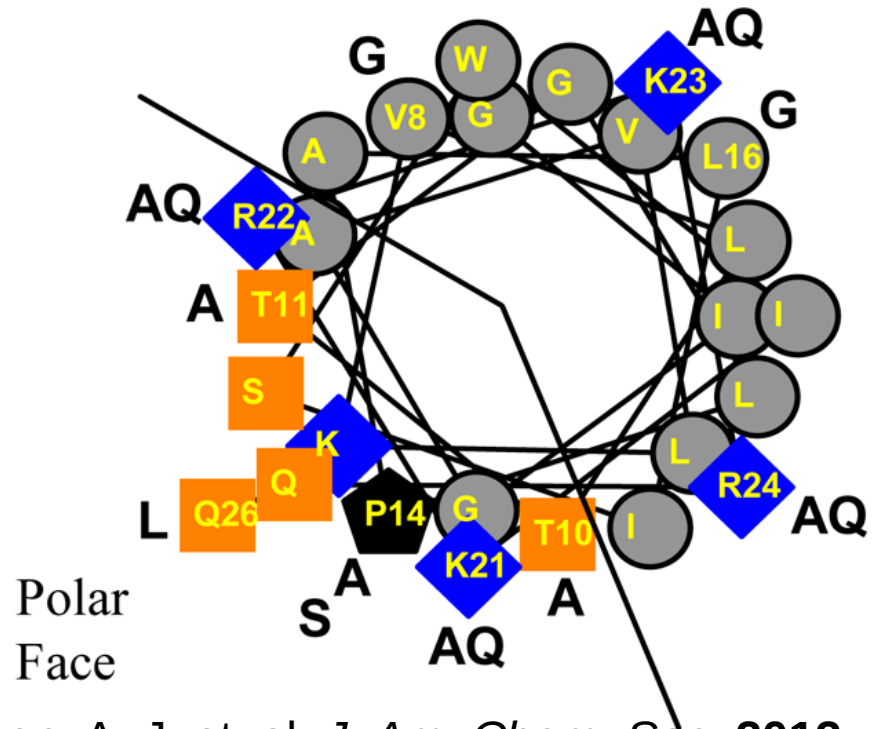
2. Increase in the conductance of individual pores



Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2012**, *134*, 12732.

2-2. Design of the melittin library

→ 2 × 2 × 2 × 3 × 2 × 3 × 3 × 3 × 3 × 2
= ***7776*** variants



V_8 and **L_{16}** → **G** : “glycine zipper” (GXXXG)?

T_{10} and **T_{11}** → **A** or **L** : change in amphipathicity and polar angle?

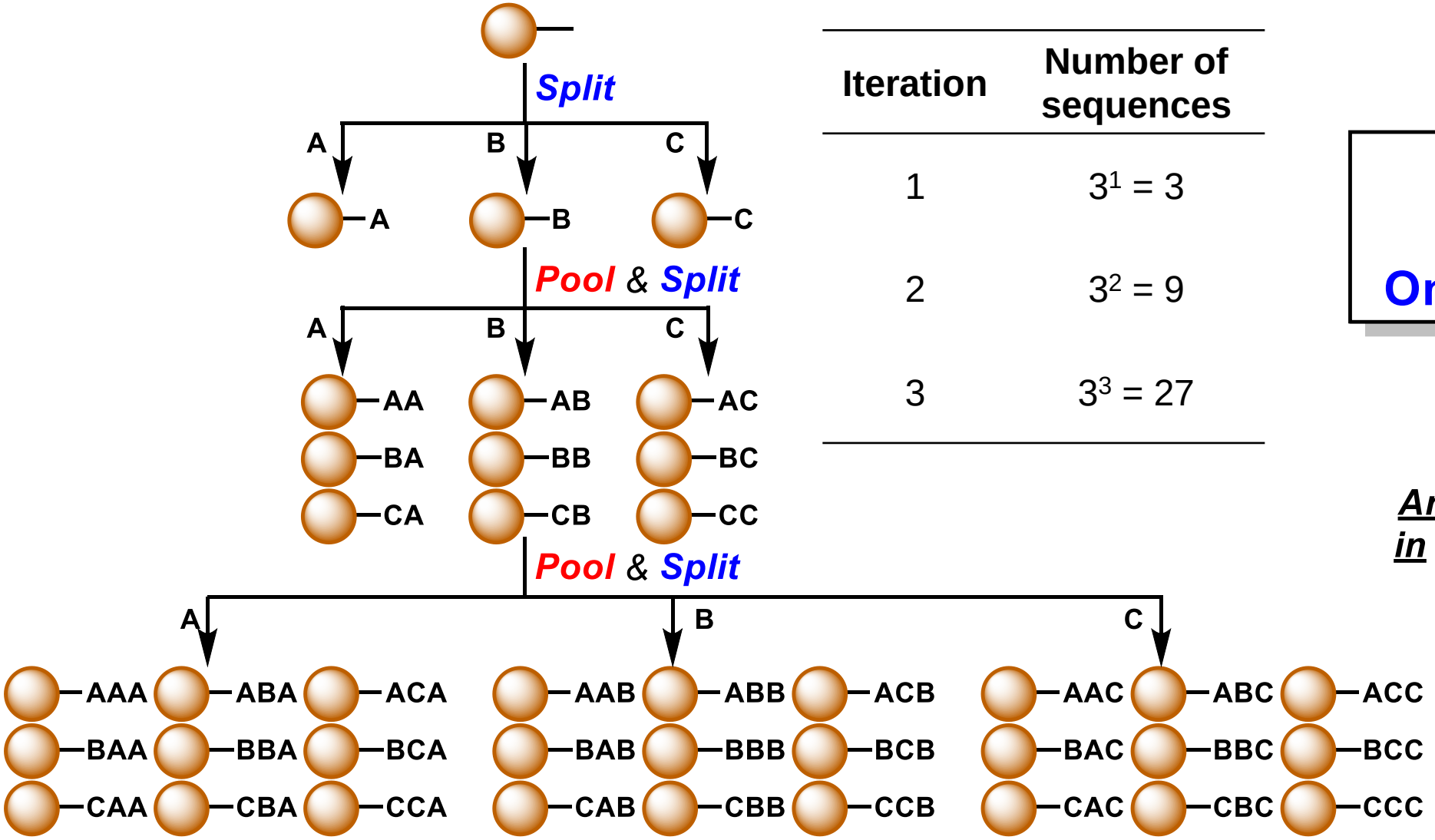
P_{14} → **S** or **A** : linear α-helix?

K_{23} and **R_{24}** → **A** or **Q** : increase in amphipathicity?

K_{21}, **R_{22}**, and **Q_{26}** → **A**, **Q** or **L** : extra helical conformation at C-terminus?

Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2012**, *134*, 12732.

2-3. Split-pool method (in case of SPPS)



Iteration	Number of sequences
1	$3^1 = 3$
2	$3^2 = 9$
3	$3^3 = 27$

One bead

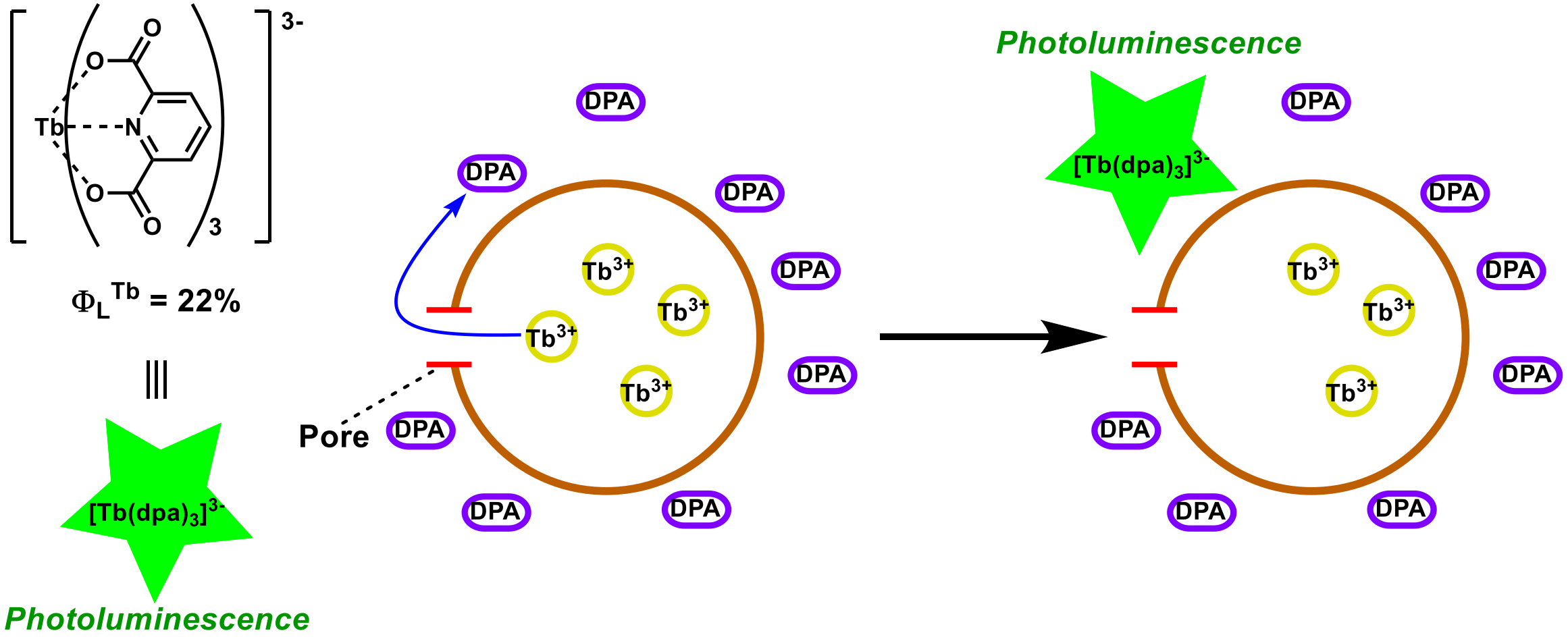
×

One sequence

Analytical method in very small scale (<1 nmol) is necessary

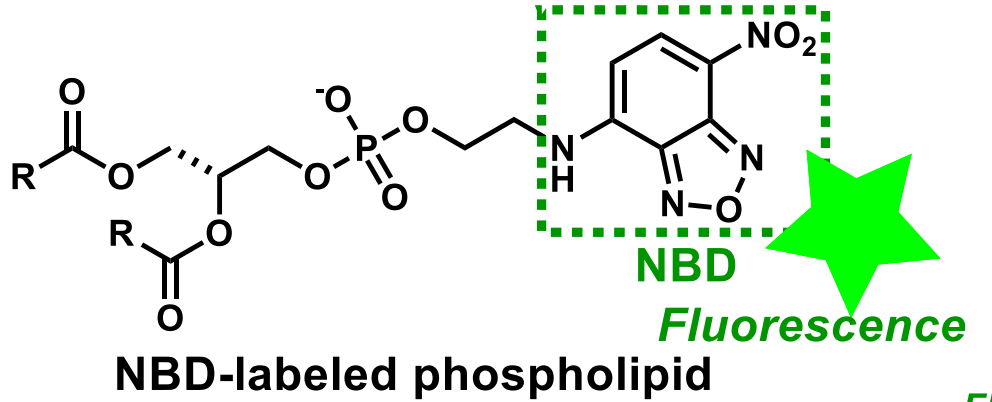
Lam, K. S. et. al. *Nature* **1991**, *354*, 82.

2-4. Tb^{3+} leakage



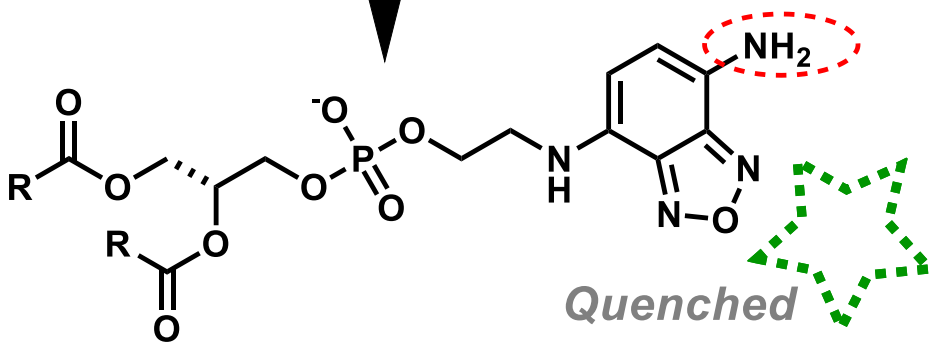
Andres, J. et. al. *Adv. Funct. Mater.* **2014**, *24*, 5029.

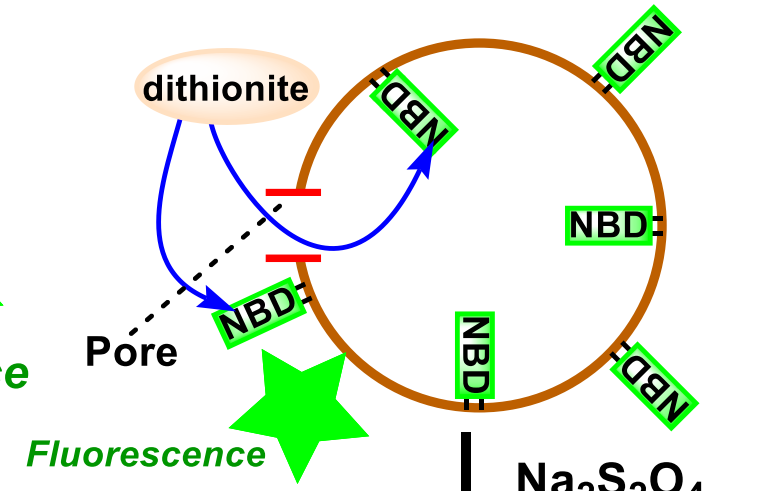
2-5. NBD quenching



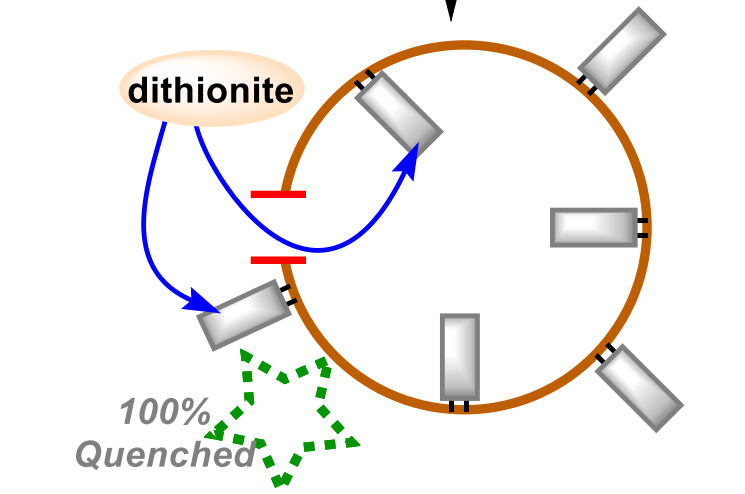
NBD-labeled phospholipid

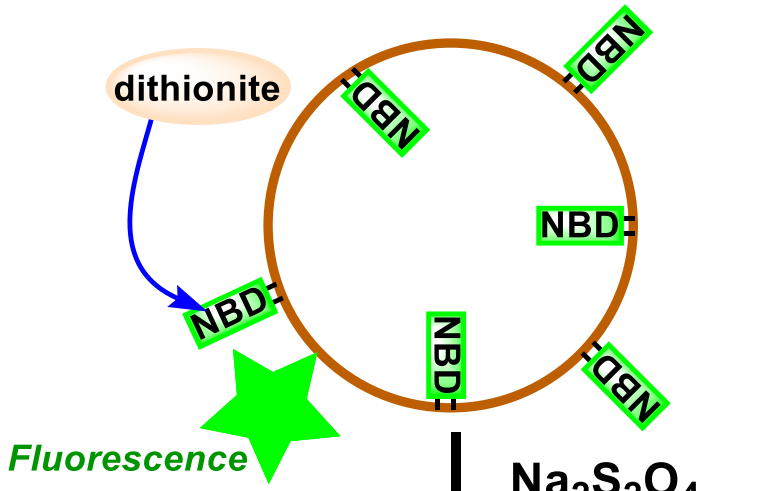
$Na_2S_2O_4$ (dithionite)



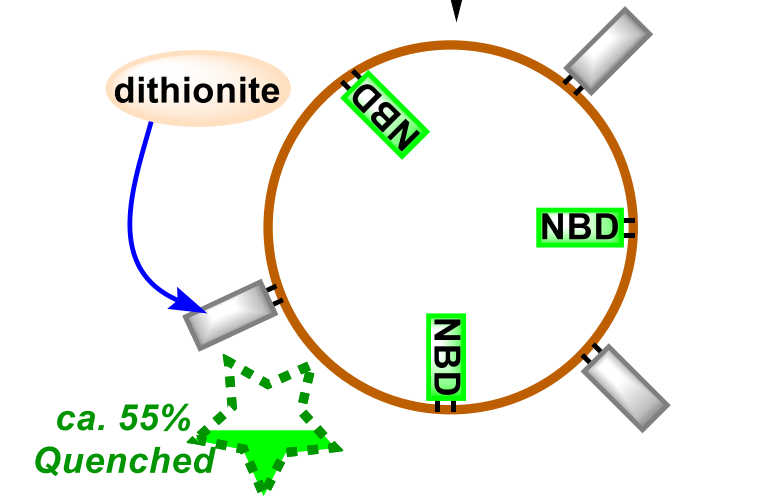


$Na_2S_2O_4$ (dithionite)



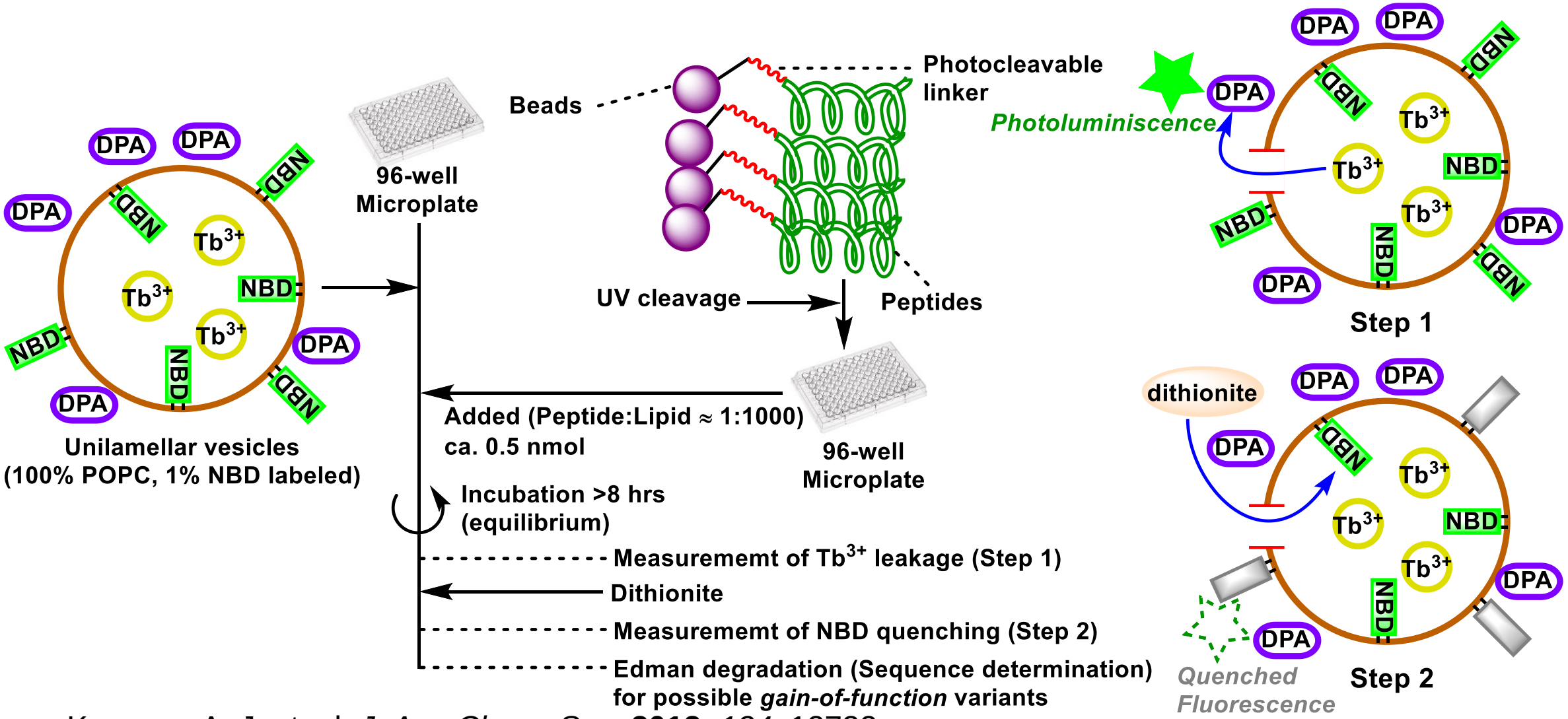


$Na_2S_2O_4$ (dithionite)



Moss, R. A.; Bhattacharya, S. *J. Am. Chem. Soc.* **1995**, *117*, 8688.

2-6. 2-step assay for *gain-of-function* variants



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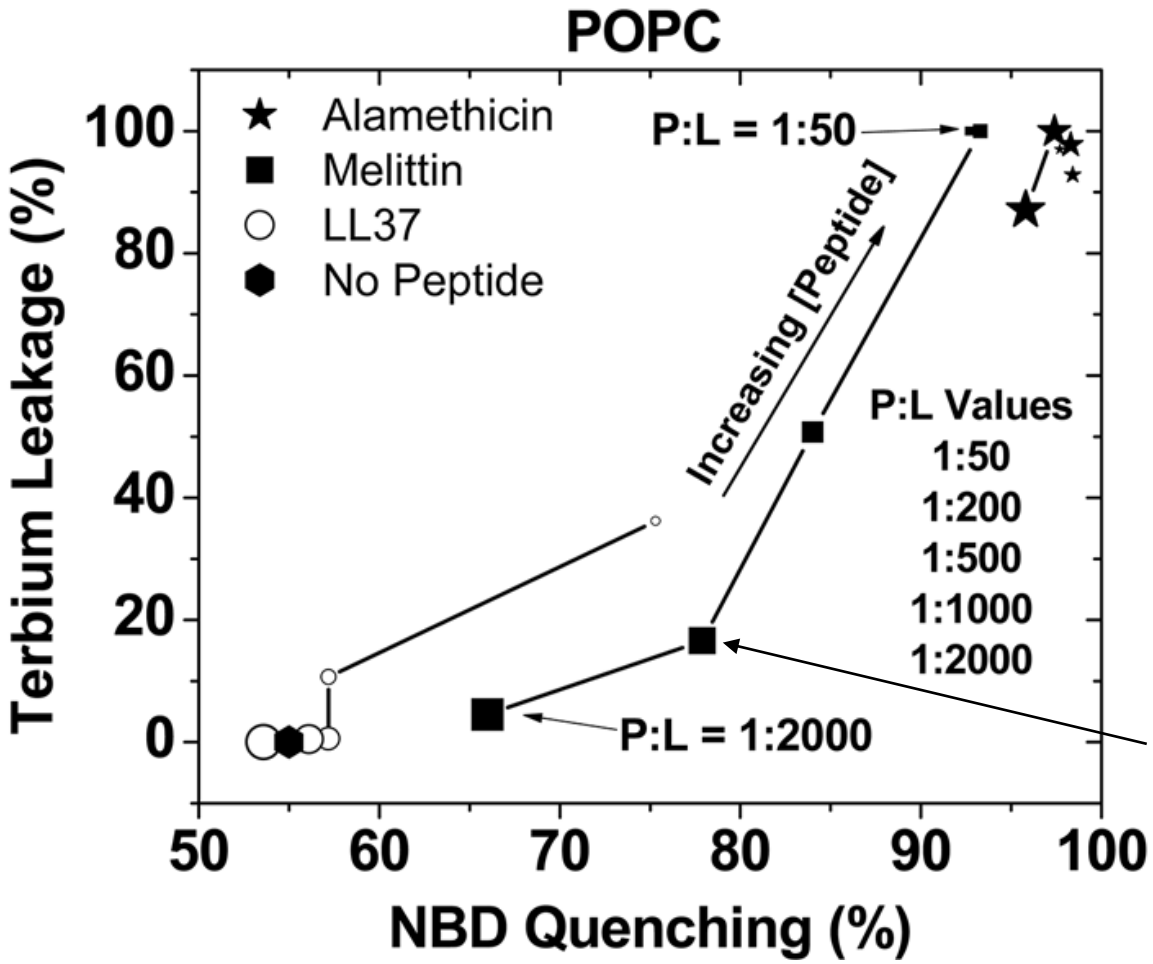
2-7. Control experiment



In order to find *gain-of-function* variants, this experiment was conducted at ***P:L = 1:1000*** where melittin has low activity.

P:L = 1:1000

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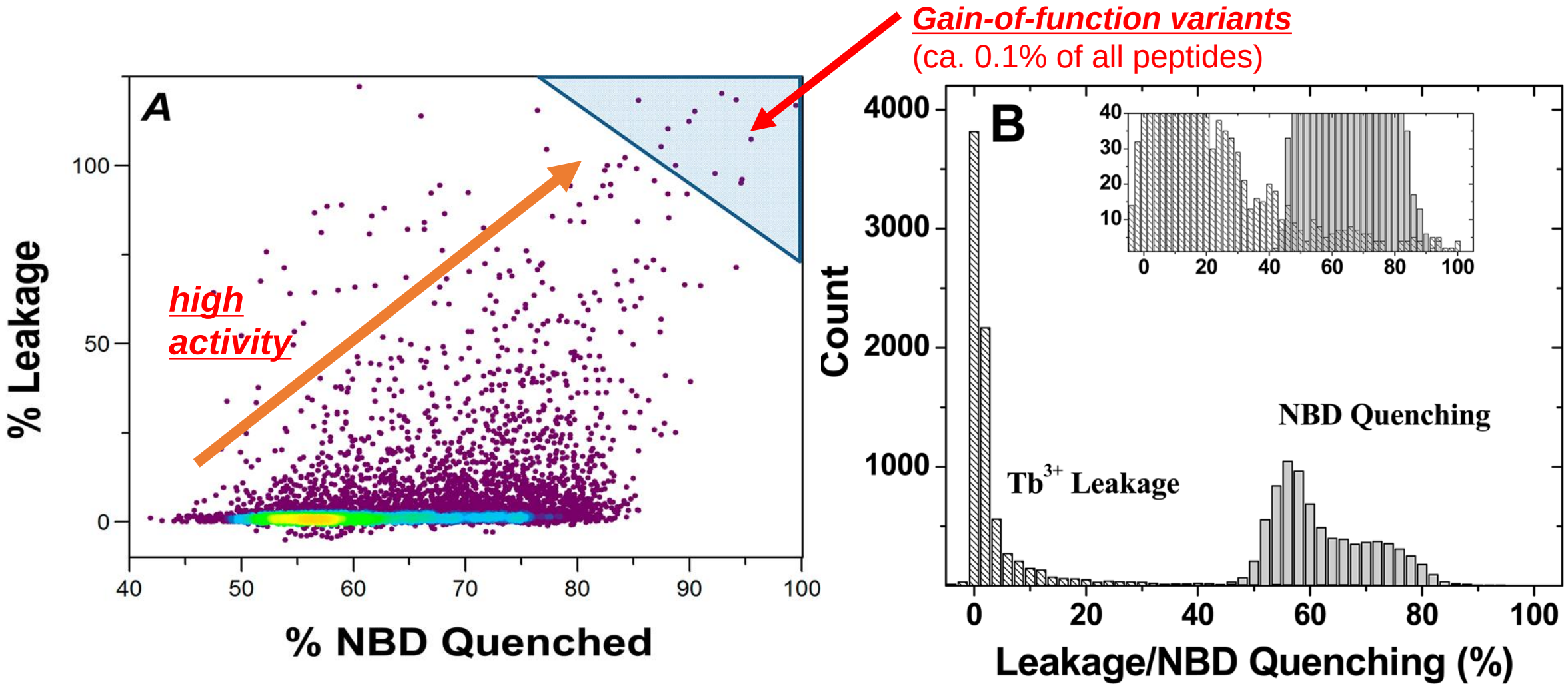
2-8. HTL results



Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2012**, *134*, 12732.

2-9. Highly active peptides found in HTS

G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	Q	Native Sequence	
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Residue #	
Residues present in library							VG		TA	TL			PSA		LG					KAQ	RAQ	KAQ	RAQ		QL	Two step screen values	
Screening Results																										Tb^{3+}	NBD
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	K	R	A	Q	Q	L	105	87
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	A	A	Q	Q	L	96	94
G	I	G	A	V	L	K	G	L	A	T	G	L	P	A	L	I	S	W	I	K	Q	A	Q	Q	Q	93	89
G	I	G	A	V	L	K	G	L	T	T	G	L	P	A	L	I	S	W	I	K	A	A	R	Q	L	100	94
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	K	A	A	Q	Q	L	92	90
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	R	A	R	Q	Q	107	95
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	Q	A	Q	Q	L	100	99
G	I	G	A	V	L	K	G	L	A	T	G	L	P	A	L	I	S	W	I	Q	R	A	Q	Q	Q	98	92
G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	Q	A	R	Q	Q	102	84
G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	A	Q	Q	Q	100	90
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	R	K	Q	Q	Q	96	87
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	G	I	S	W	I	Q	R	A	R	Q	L	92	83
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	RQ	X	R	Q	Q	95	83
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	A	Q	Q	Q	L	100	93
Strongly Excluded										L			SA		G					A		KQ	A				

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2-10. Highly active peptides found in HTS

G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	Q	Native Sequence	
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	Residue #	
Residues present in library							VG		TA	TL			PSA		LG					KAQ	RAQ	KAQ	RAQ		QL	Two step screen values	
Screening Results																										Tb^{3+}	NBD
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	K	R	A	Q	Q	L	105	87
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	A	A	Q	Q	L	96	94
G	I	G	A	V	L	K	G	L	A	T	G	L	P	A	L	I	S	W	I	K	Q	A	Q	Q	Q	93	89
G	I	G	A	V	L	K	G	L	T	T	G	L	P	A	L	I	S	W	I	K	A	A	R	Q	L	100	94
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	K	A	A	Q	Q	L	92	90
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	R	A	R	Q	Q	107	95
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	Q	A	Q	Q	L	100	99
G	I	G	A	V	L	K	G	L	A	T	G	L	P	A	L	I	S	W	I	Q	R	A	Q	Q	Q	98	92
G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	Q	A	R	Q	Q	102	84
G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	A	Q	Q	Q	100	90
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	R	K	Q	Q	Q	96	87
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	G	I	S	W	I	Q	R	A	R	Q	L	92	83
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	R/Q	X	R	Q	Q	95	83
G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	Q	A	Q	Q	Q	L	100	93
Strongly Excluded										L			S/A		G					A		K/Q	A				

almost conserved

frequently changed

Charge of C-terminus
average: +1.1
(melittin: +4)

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2-11. Selected sequences for further study

Engineered sequences
(Effects of T10 → A and K23 to A?)

***Gain-of-function* sequences found in HTL**
(Effects of the C-terminal charge?)

Sequences observed in HTL (not very strong)
(Effects of change in V8, T10 and C-terminal?)

Peptide	Sequence (8 10 21 24 26)	Δ	+
Melittin	G I G A V L **K** V L T T G L P A L I S W I **K R K R** Q Q	0	6
Mel-P1	G I G A V L **K** V L **A** T G L P A L I S W I **K R K R** Q Q	1	6
Mel-P2	G I G A V L **K** V L T T G L P A L I S W I **K R A R** Q Q	1	5
Mel-P3	G I G A V L **K** V L **A** T G L P A L I S W I **K R A R** Q Q	2	5
Mel-P4	G I G A V L **K** V L **A** T G L P A L I S W I **Q A A Q** Q **L**	6	2
Mel-P5	G I G A V L **K** V L **A** T G L P A L I S W I **K A A Q** Q **L**	5	3
Mel-P6	G I G A V L **K** V L **A** T G L P A L I S W I **K R A Q** Q **L**	4	4
Mel-P7	G I G A V L **K** V L T T G L P A L I S W I **K R A Q** Q Q	2	4
Mel-P8	G I G A V L **K** **G** L **A** T G L P A L I S W I **Q R A Q** Q Q	4	3
Mel-P9	G I G A V L **K** **G** L T T G L P A L I S W I **K A A Q** Q **L**	4	3

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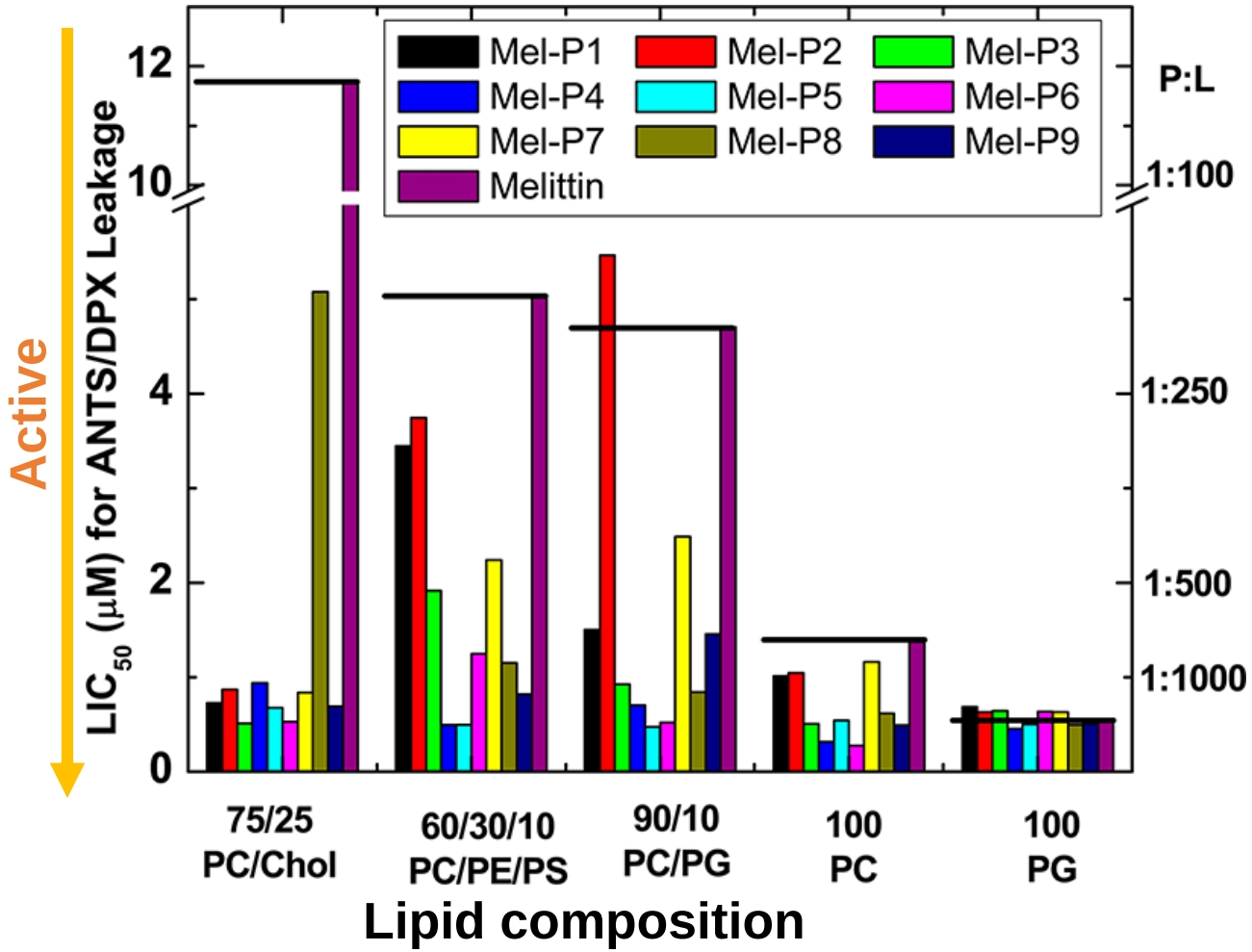
2-12. LIC_{50} values measured in various lipid composition



- **Mel-P4 and Mel-P5 exhibited high activity in all lipid compositions.**
- **Activity of other peptided varies according to lipid compositions.**

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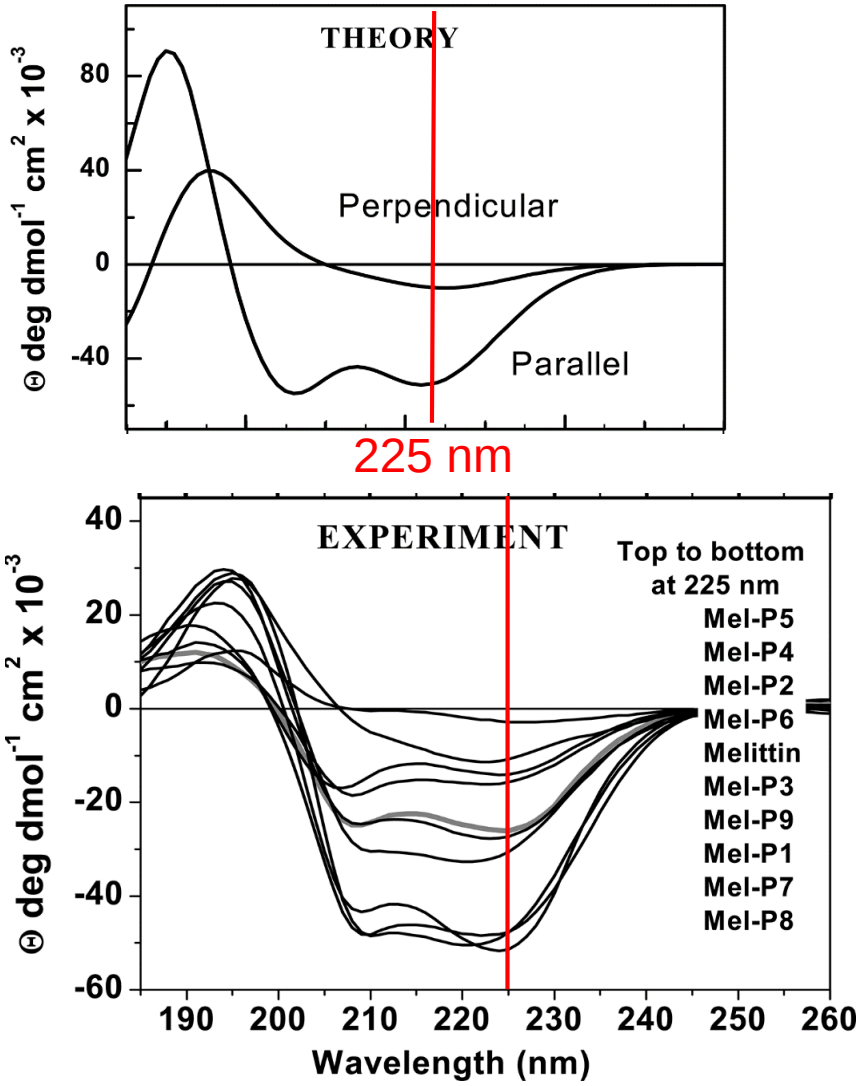
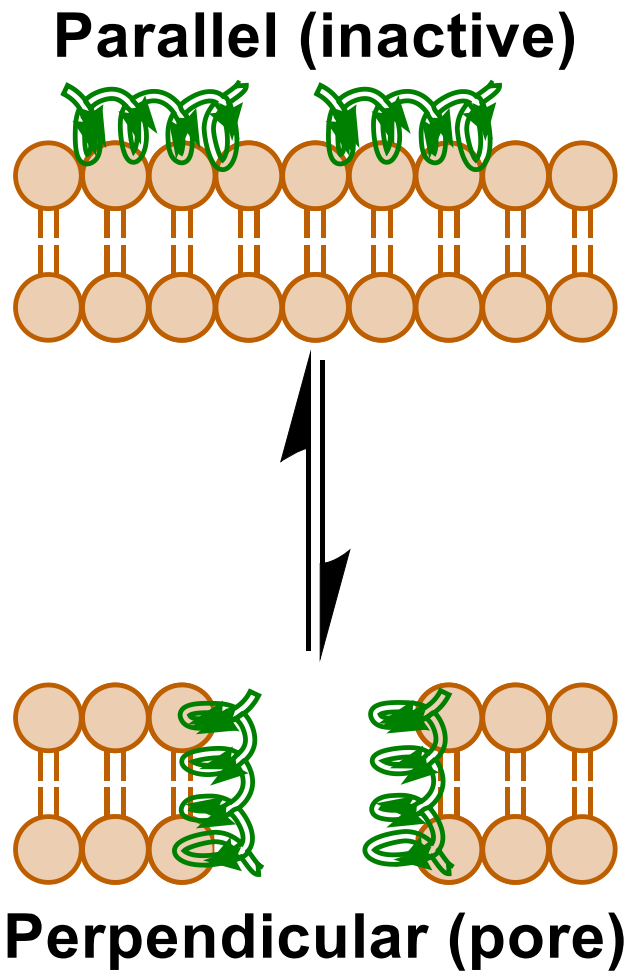
2-13. Membrane orientation of *gain-of-function* variants



Top to Bottom at 225 nm	
Mel-P5	**Perpendicular (active)**
Mel-P4	
Mel-P2	
Mel-P6	
Mellittin	
Mel-P3	**Parallel (inactive)**
Mel-P9	
Mel-P1	
Mel-P7	
Mel-P8	



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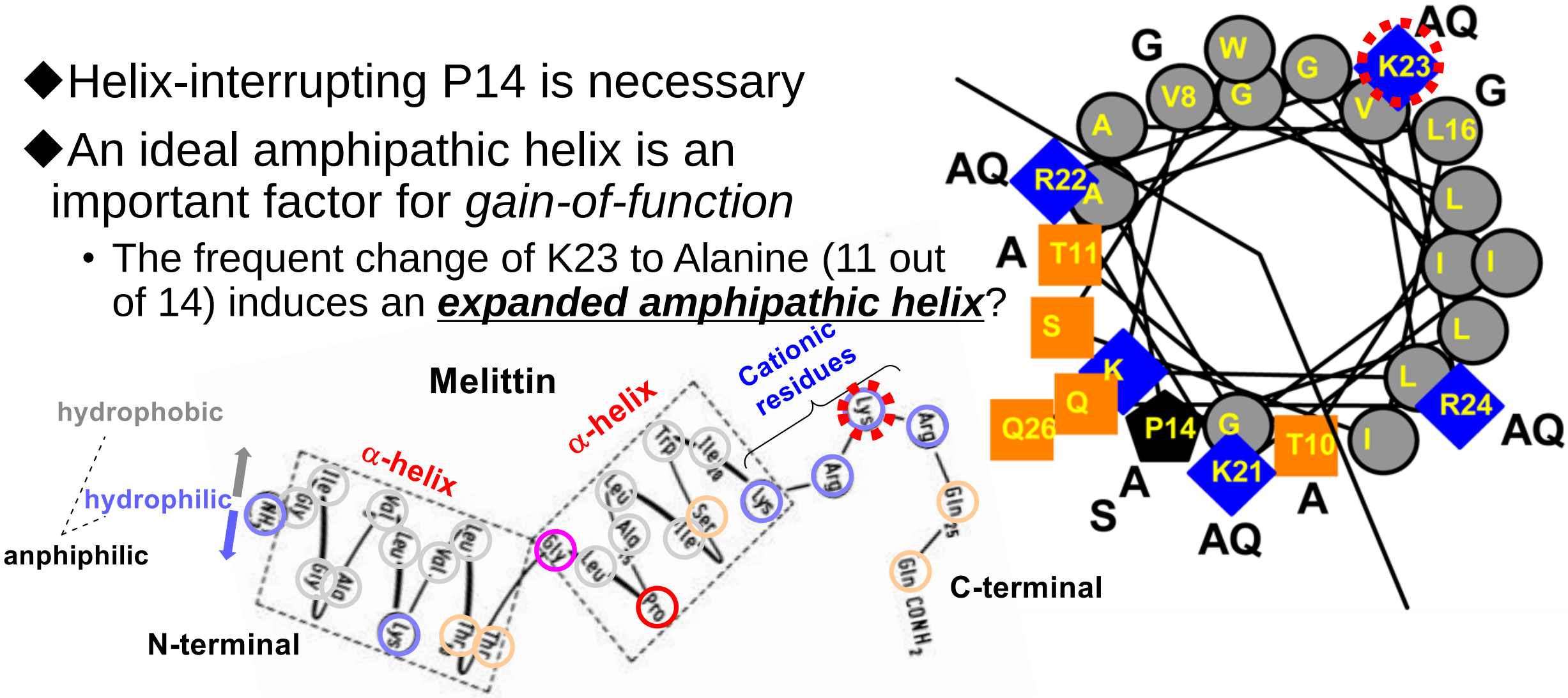
2-14. Structural implification

- Helix-interrupting P14 is necessary
- An ideal amphipathic helix is an important factor for *gain-of-function*
  - The frequent change of K23 to Alanine (11 out of 14) induces an ***expanded amphipathic helix***?



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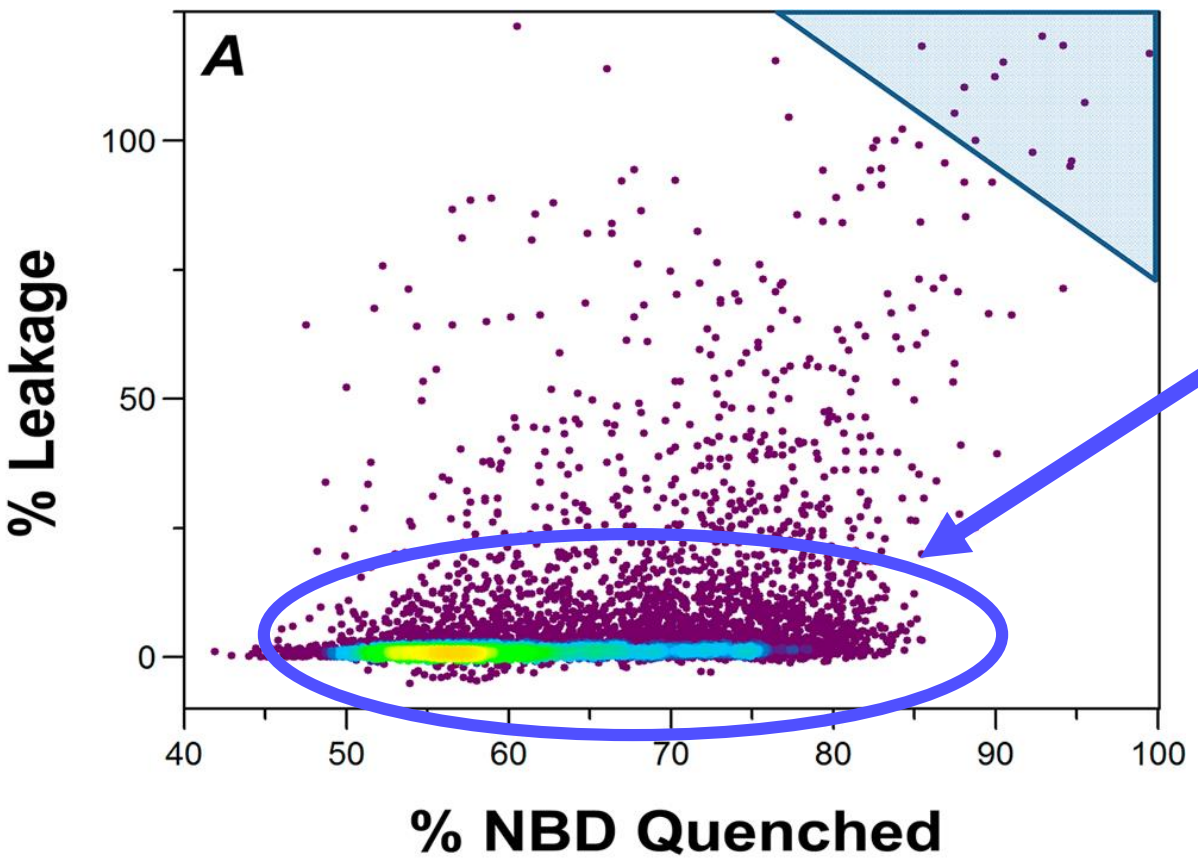
3. Screening for *loss-of-function* variants (main paper)

3-1. Design of screening for *loss-of-function* variants



***"loss-of-function"* variants**
··· important in investigating selectively toxic analogues

Study on sequences in this area is needed

	Peptide Concentration	Lipid Composition
Gain-of-function	**Pepeide:Lipid ≈ 1:1000**	**POPC 100%**
Loss-of-function	**Peptide:Lipid ≈ 1:20**	**POPC 90% POPG 10%**

* Melittin: becomes active at P:L ≈ 1:200

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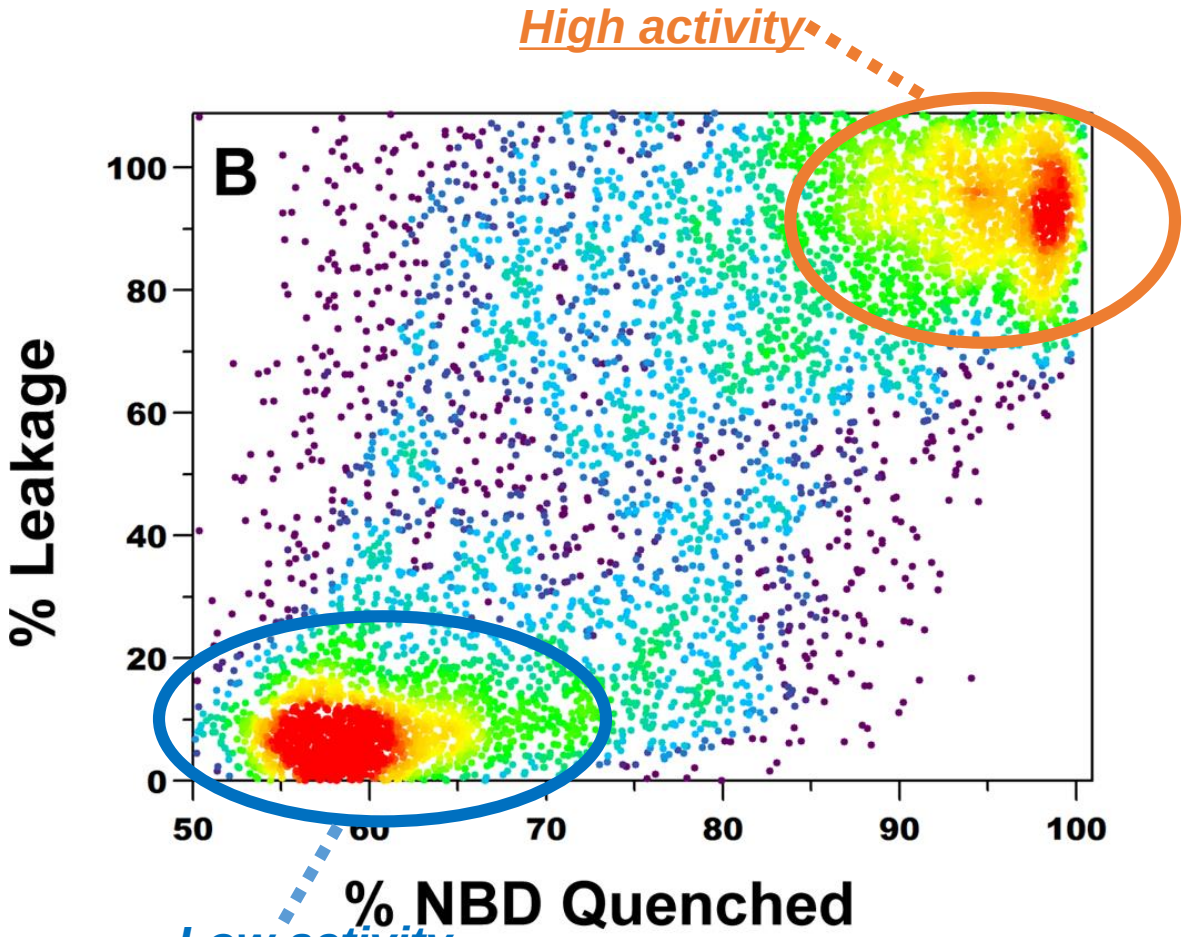
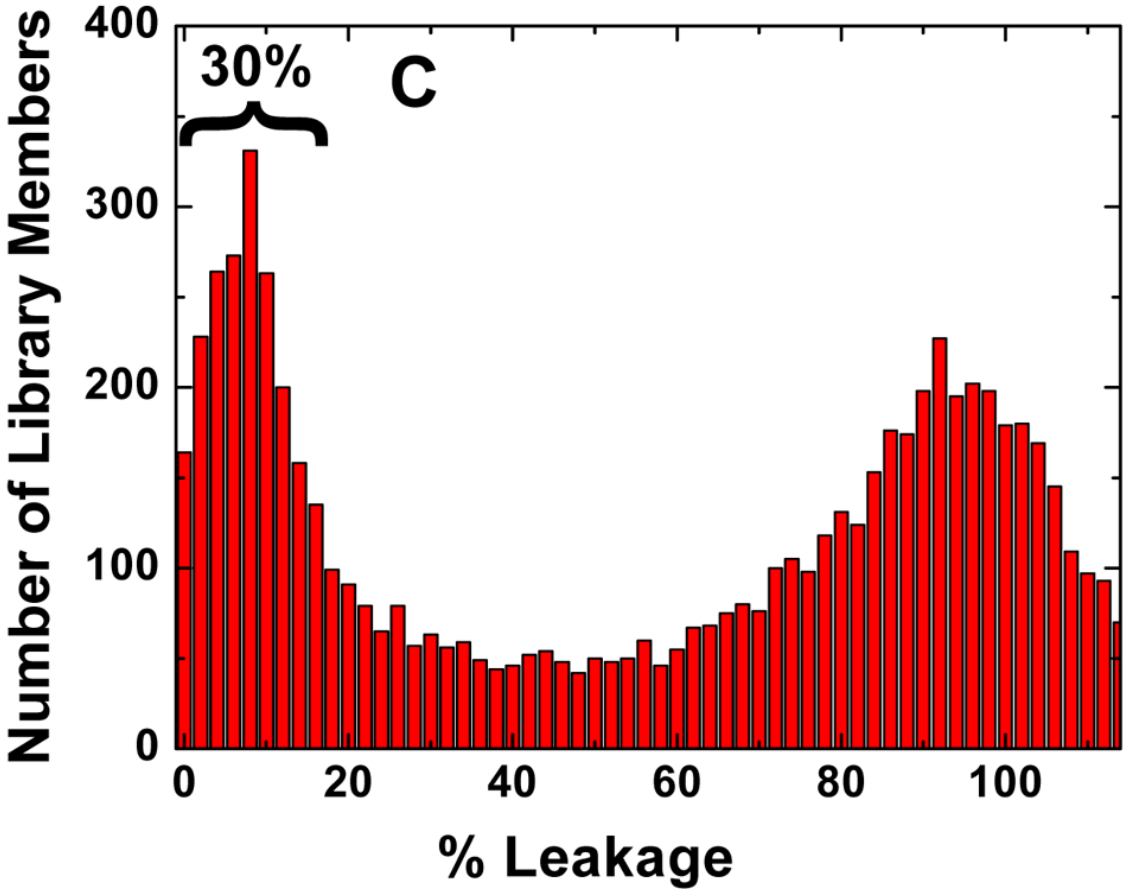
3-2. HTL results (high peptide conc.)

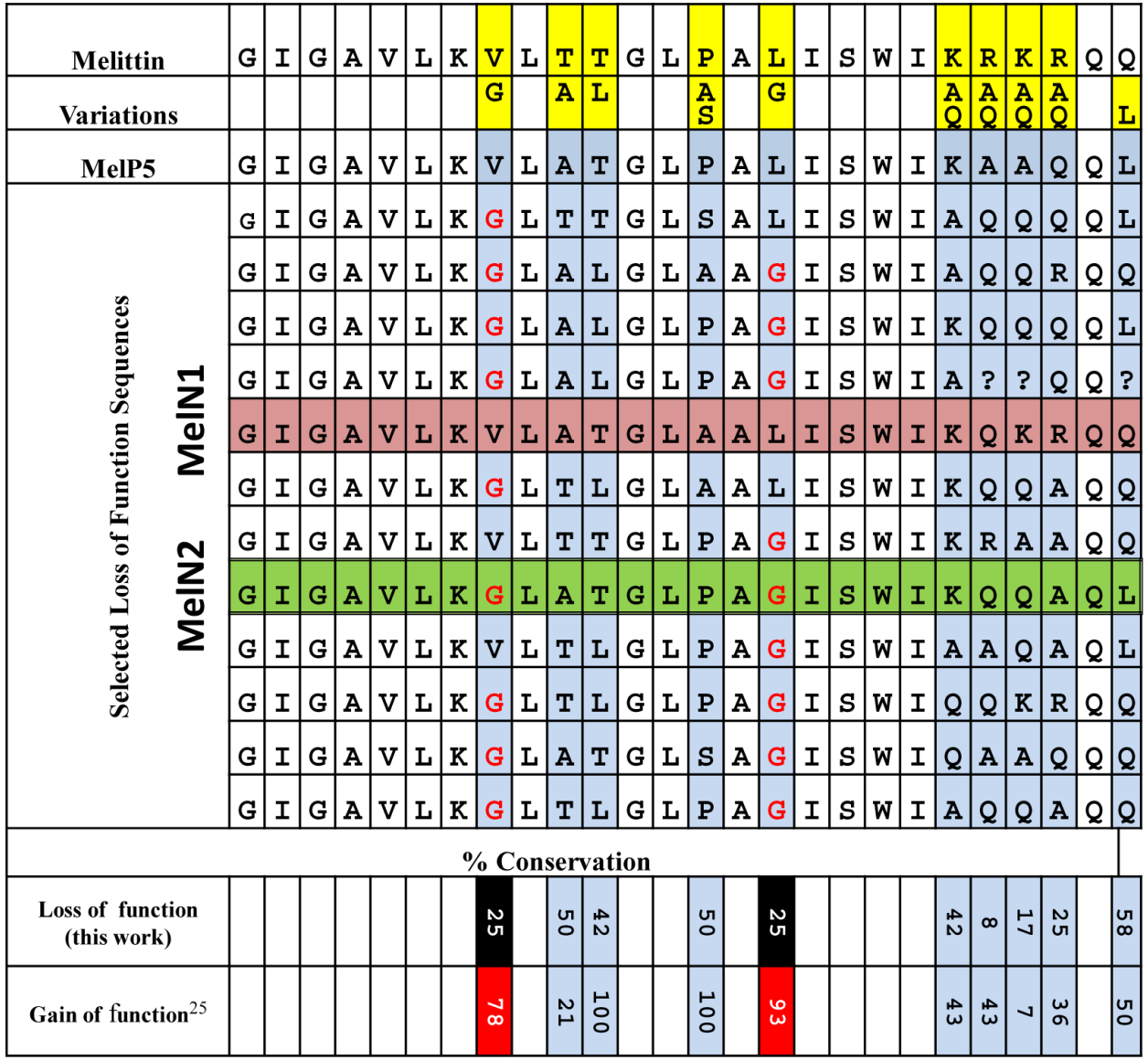




* Up to 1 or 2% of beads do not release sufficient peptide and they seems to be included in *loss-of-function* variants.

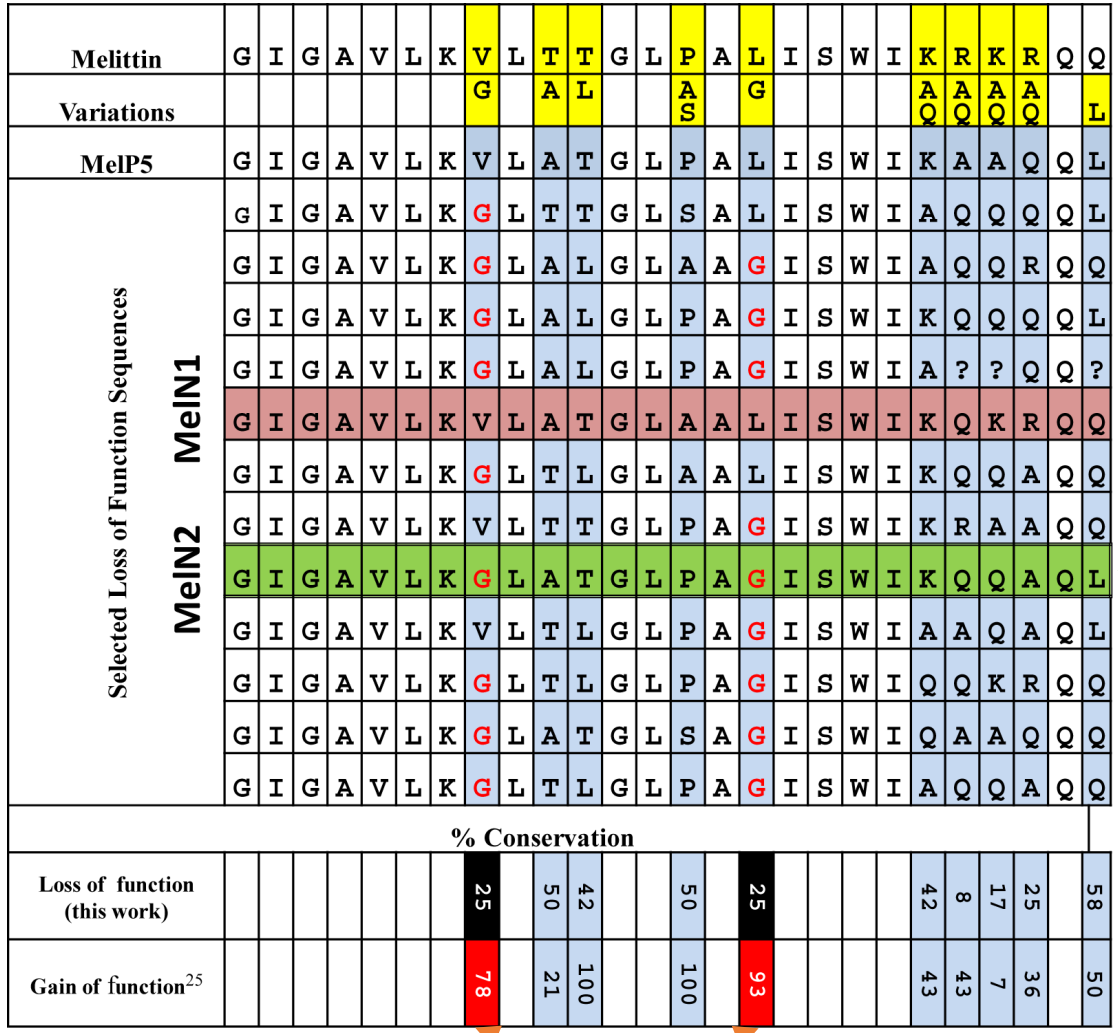
Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-3. Selected *loss-of-function* sequences



Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-4. Selected *loss-of-function* sequences



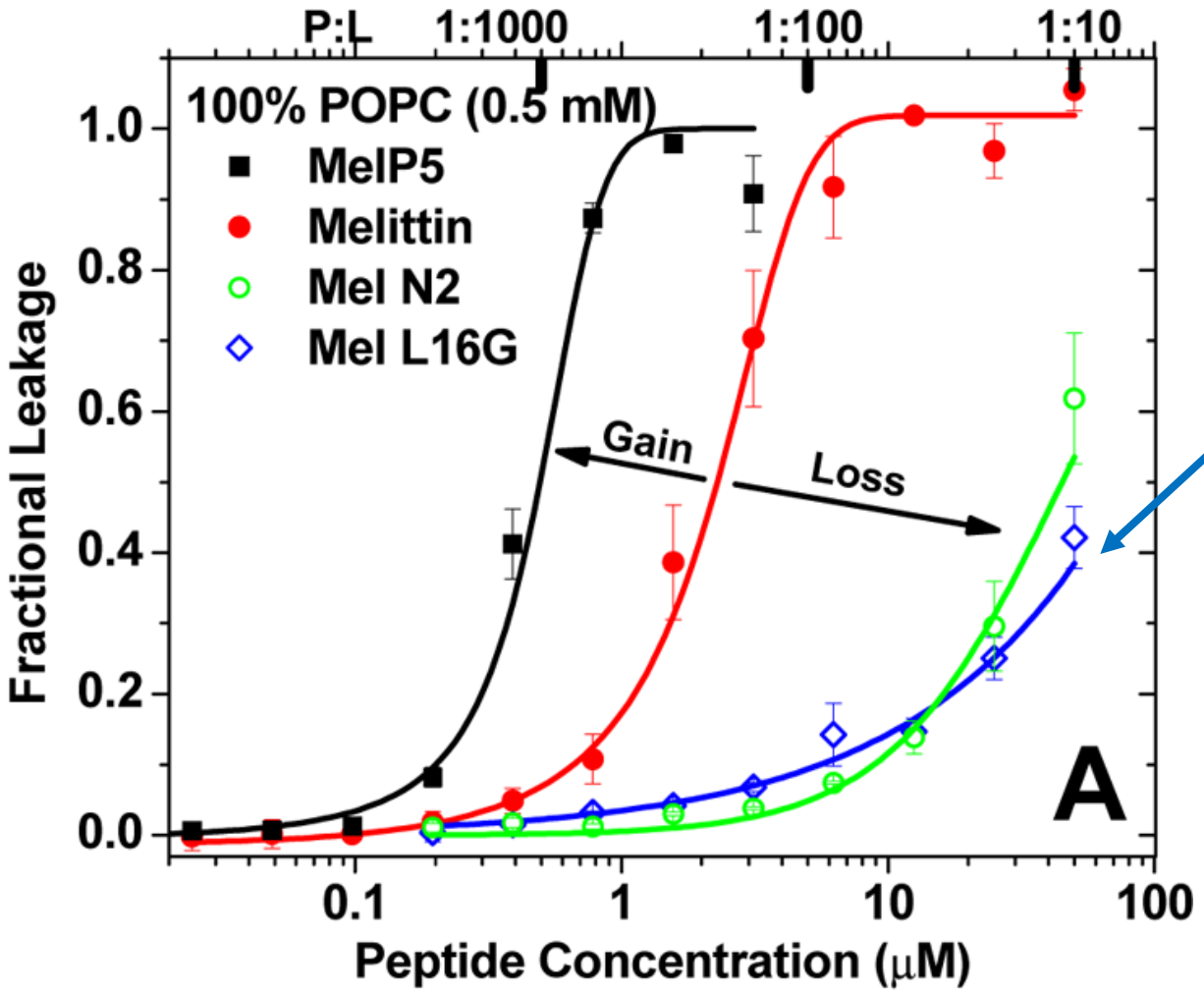
Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-5. Sequences used for further study

Residue #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
Melittin	G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	L	I	S	W	I	K	R	K	R	Q	Q	
MelP5	G	I	G	A	V	L	K	V	L	A	T	G	L	P	A	L	I	S	W	I	L	A	A	Q	Q	L	A ***gain-of-function*** sequence
MelN2	G	I	G	A	V	L	K	G	L	A	T	G	L	P	A	G	I	S	W	I	K	Q	Q	A	Q	L	A ***loss-of-function*** sequence
Mel L16G	G	I	G	A	V	L	K	V	L	T	T	G	L	P	A	G	I	S	W	I	K	R	K	R	Q	Q	An engineered sequence

Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-6. Vesicle leakage from POPC vesicles

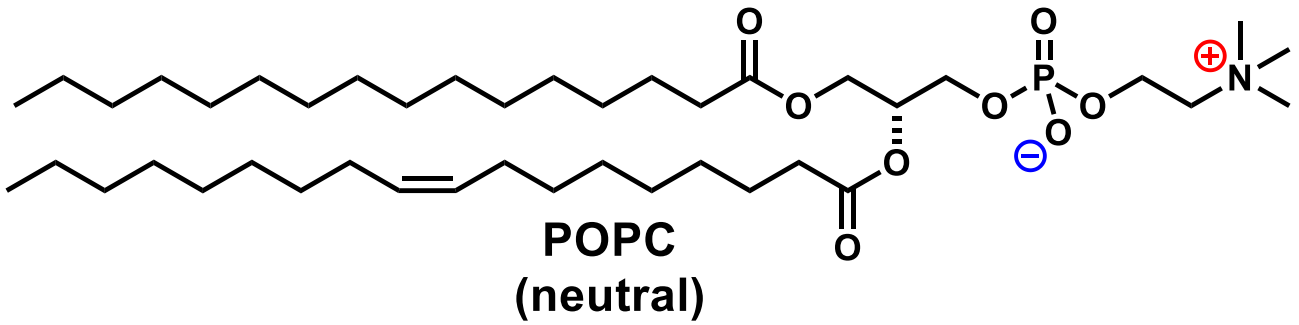


Single-residue change (L16G) caused a remarkable loss of function.

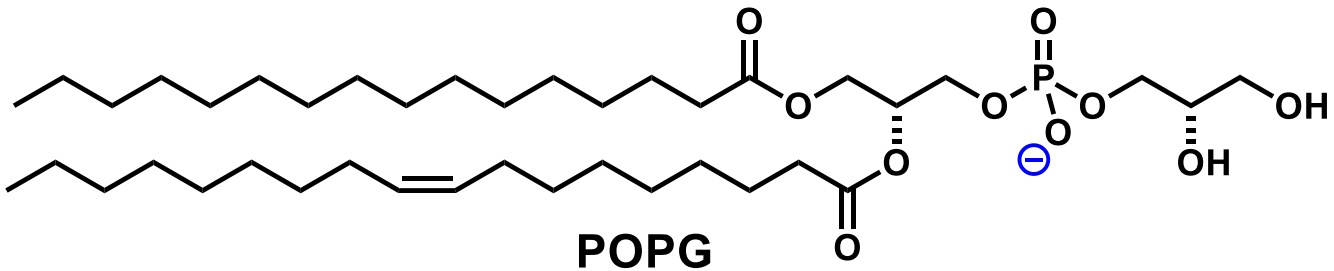
"Helix-breaking" glycine prevented Mel L16G from attaining helical active conformation?

Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-7. Anionic lipids and cationic peptides



POPC
(neutral)

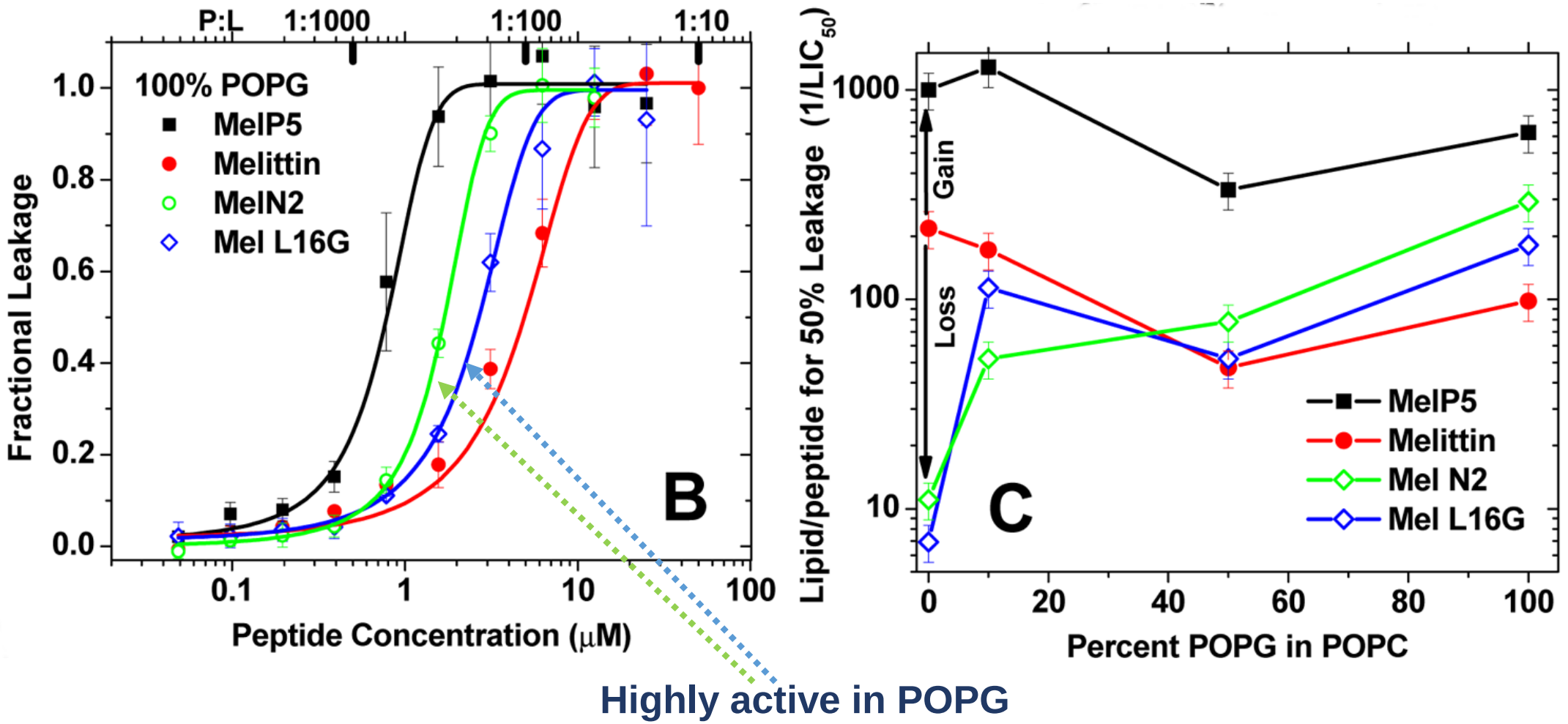


POPG
(anionic)

Sequence	Charge
Mellitin	+6
MelP5	+3
MelN2	+3
Mel L16G	+6

Stronger interaction?

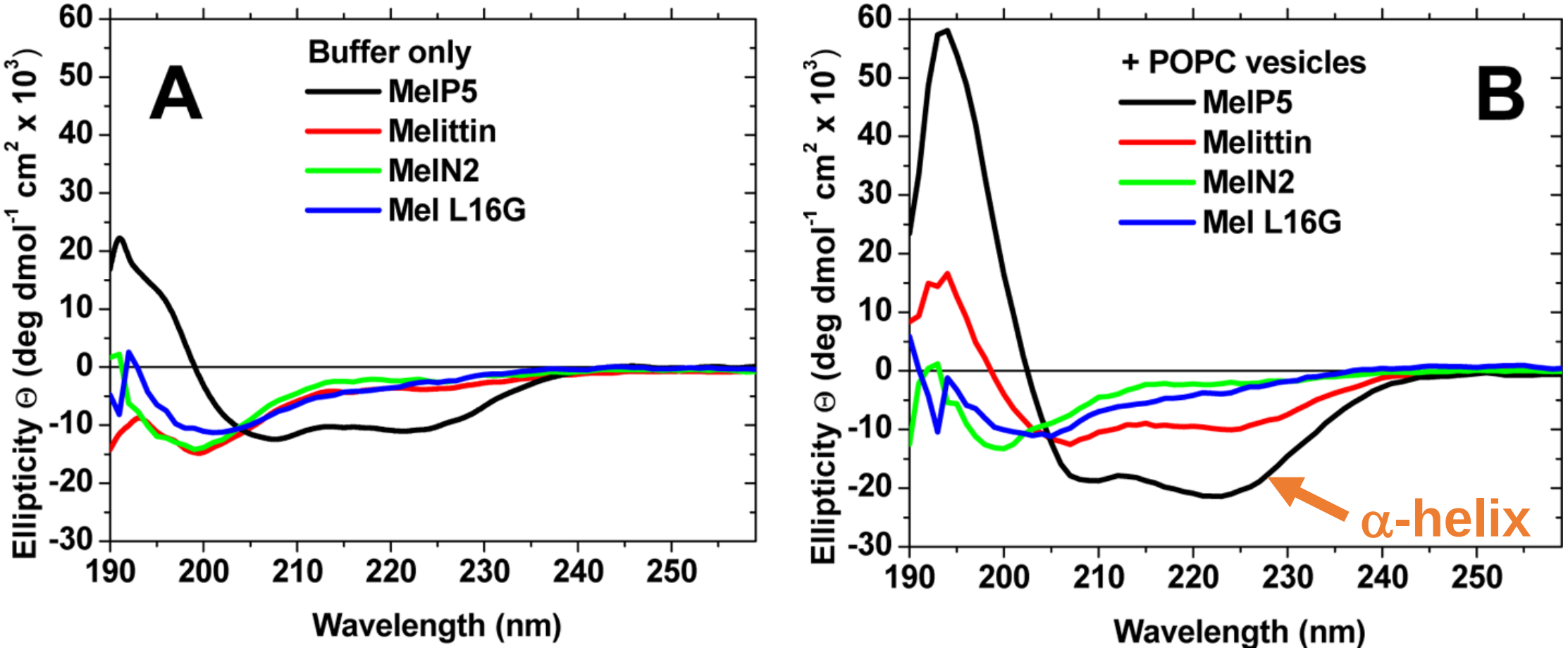
3-8. Leakage from POPG vesicles



Highly active in POPG

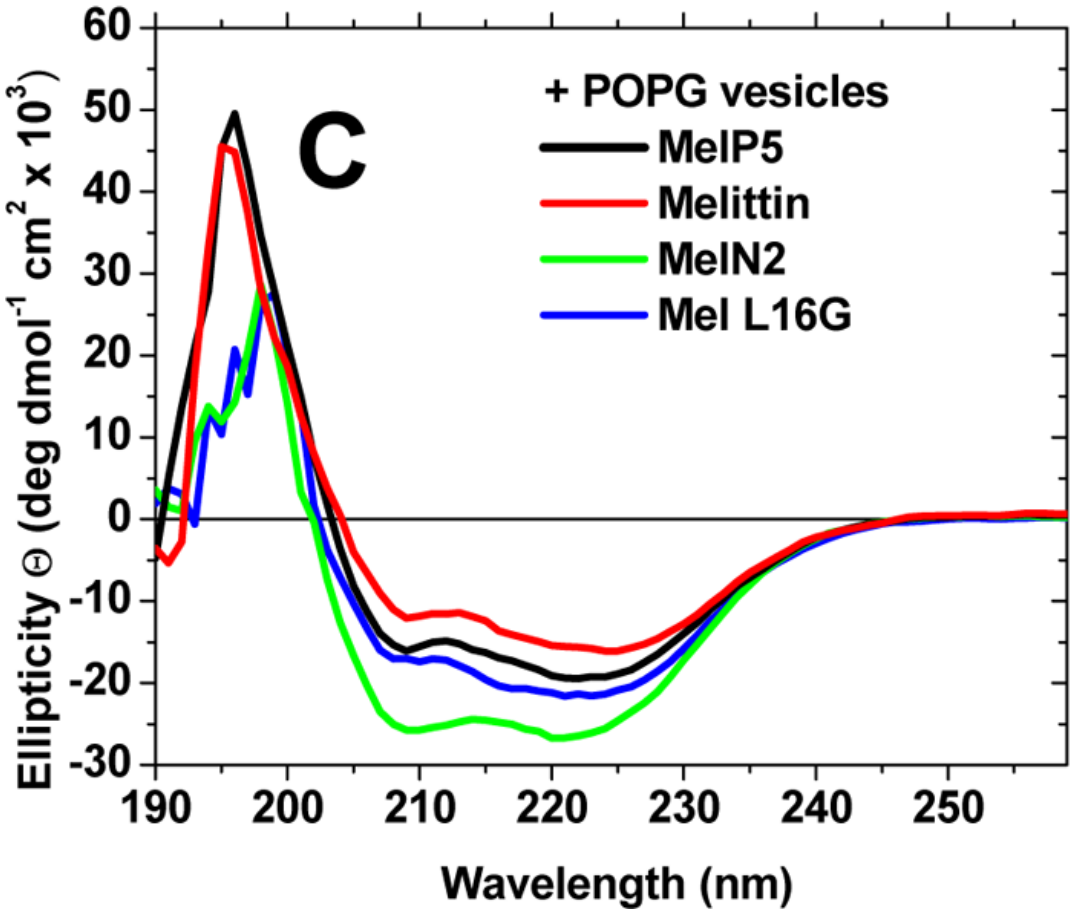
Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-9. Secondary structures-1



Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

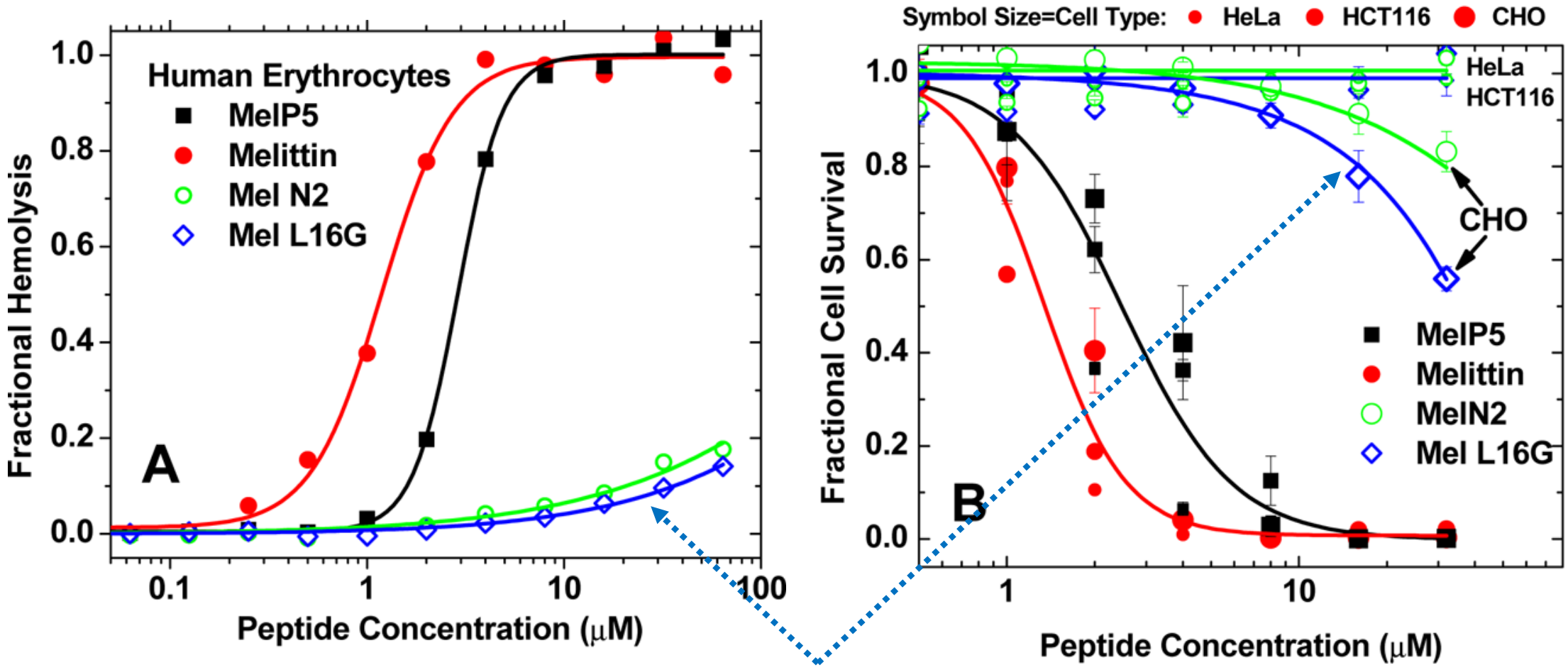
3-10. Secondary structures-2



	Buffer only	+POPC vesicles	+POPG vesicles
Melittin	random coil	partially α-helical	α-helix
MelP5	partially α-helical	α-helix	α-helix
MelN2	random coil	random coil	α-helix
Mel L16G	random coil	random coil	α-helix

Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-11. Lysis of mammalian cells



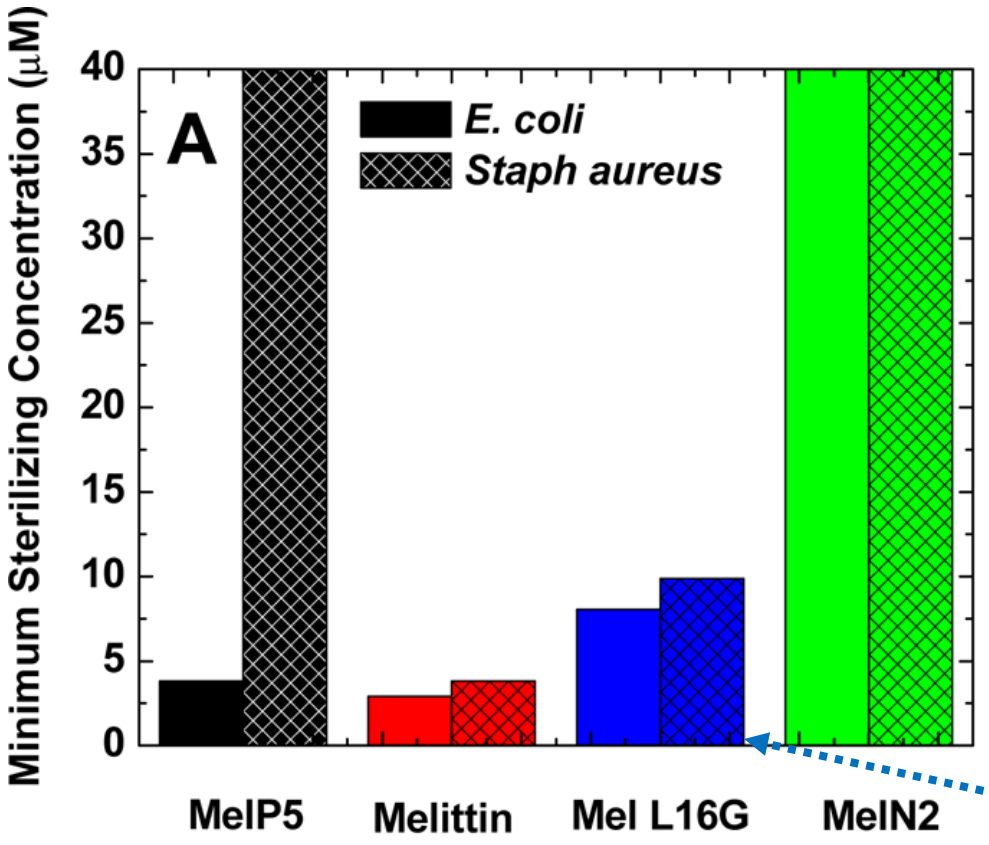
Low toxicity to mammalian cells

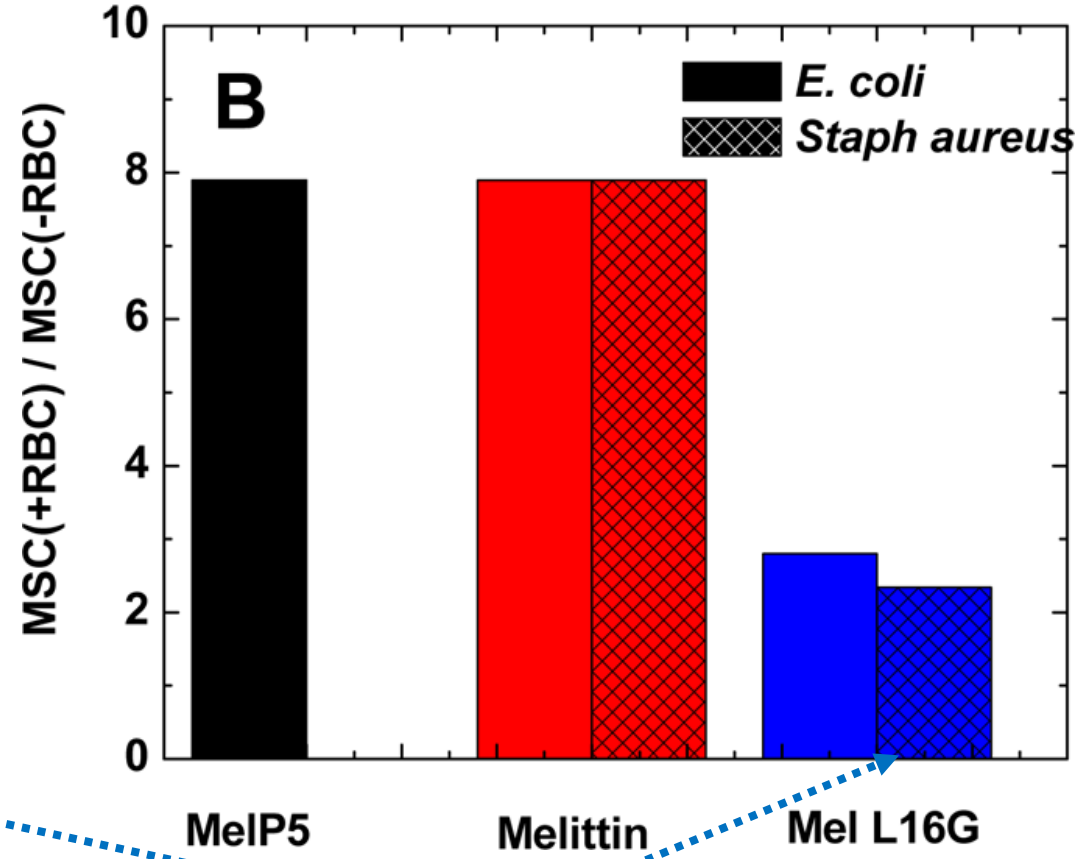
Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-12. Antibacterial activity

***E. coli*: gram-negative**

***Staph. aureus*: gram-positive**

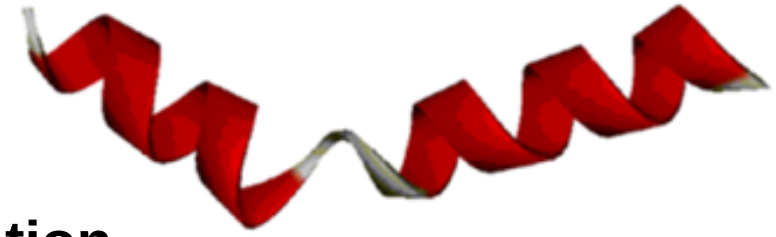




high antibacterial activity

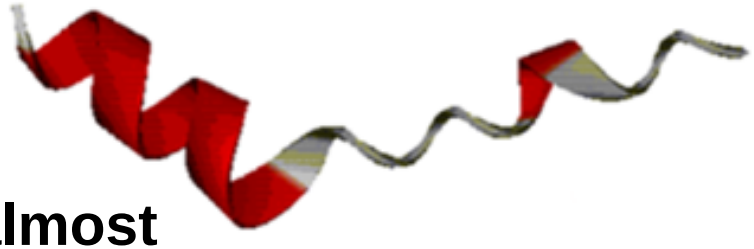
Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-13. Proposed comformation



◆***MelP5***

High tendency to helical conformation



◆***Mel L16G***

Low tendency to helical conformation

→ unable to induce lysis of human membranes (almost neutral)

High cationic charge at C-terminus

→ strong binding to bacterial membranes (anionic)

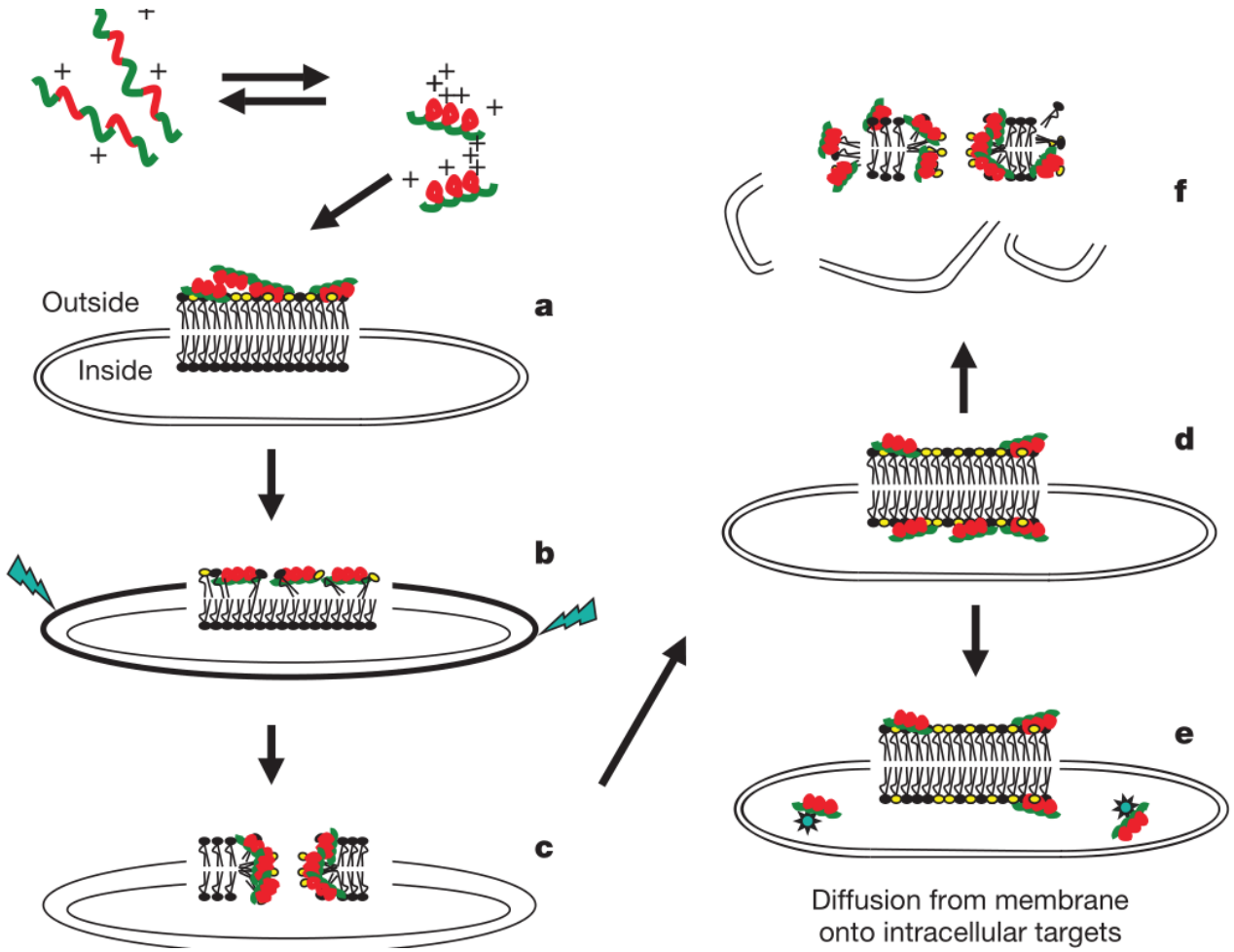
Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2015**, *137*, 16144.

3-14. Conclusion

- **A 7776-member peptide library** based on melittin was screened for ***loss-of-function*** **variants**.
- Mel L16G was prepared because large proportion of *loss-of-function* variants had L16 changed to glycine.
- Mel L16G did not induce lysis of POPC(neutral) vesicles but POPG(anionic) vesicles.
- Mel L16G exhibited potent **selective cytotoxicity** to both of **Gram-positive and negative bacterium**.

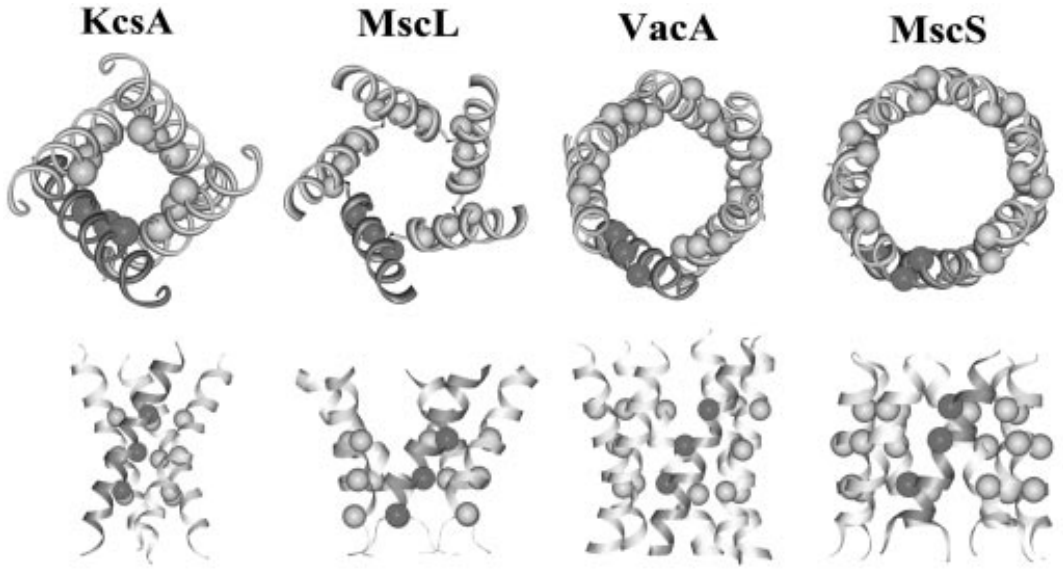
Appendix

A-1. Detailed mechanism of action of antimicrobial peptides



Zasloff, M. Nature **2002**, *415*, 389.

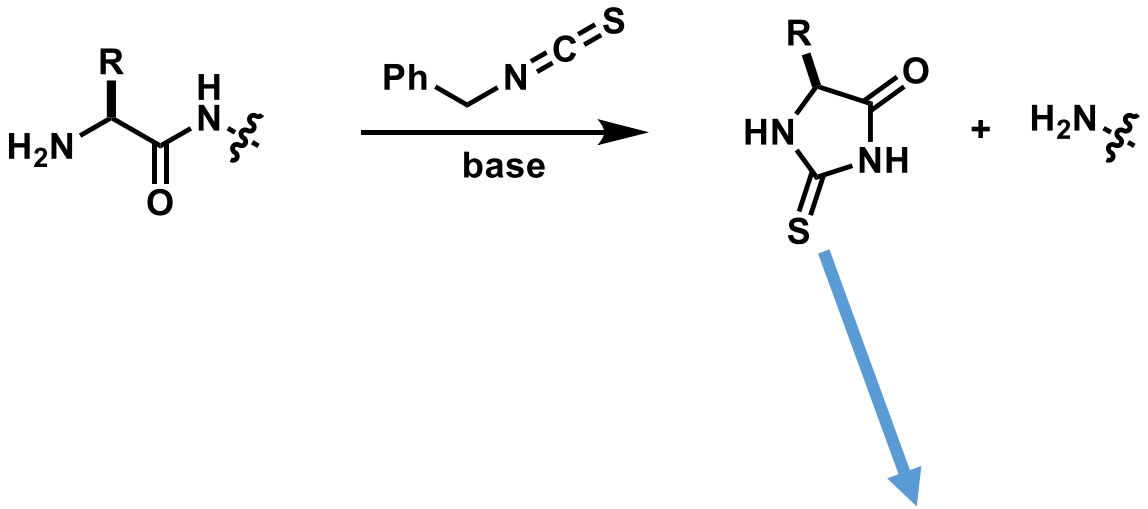
A-2. An example of “glycine zipper”



MscS	*E. coli*	95	SVIAVL**G**AA**G**LAV**G**LALQGSLS	116	heptamer
	C. tepidum	98	SLTVLS**G**TI**G**LGI**G**FGLQNIAD	119	
	S. enterica	95	SVIAVL**G**AA**G**LAV**G**LALQGSLS	116	
VacA	*H. pylori*	9	PAIVG**G**IAT**G**TAV**G**TVSGLLGW	30	hexamer
MscL	*M. tuberculosis*	15	VDLAV**A**VVI**G**TAF**T**ALVTKFTD	36	pentamer
	B. subtilis	15	VDLAI**G**VVI**G**GAF**G**KIVTSLVN	36	
	E. coli	17	VDLAV**G**VII**G**AAF**G**KIVSSLVA	36	
KcsA	*S. lividans*	95	VMVAGITSF**G**LVT**A**ALA**T**WFVG	116	tetramer
	T. volcanium	85	IMVSGIGLL**G**TLT**A**TIS**A**YLFQ	106	
	S. coelicolor	213	LMLSGIALL**G**VVT**A**NIA**A**WFIS	234	

Kim, S. et. al. *Proc. Natl. Acad. Sci.* **2005**, *102*, 14278.

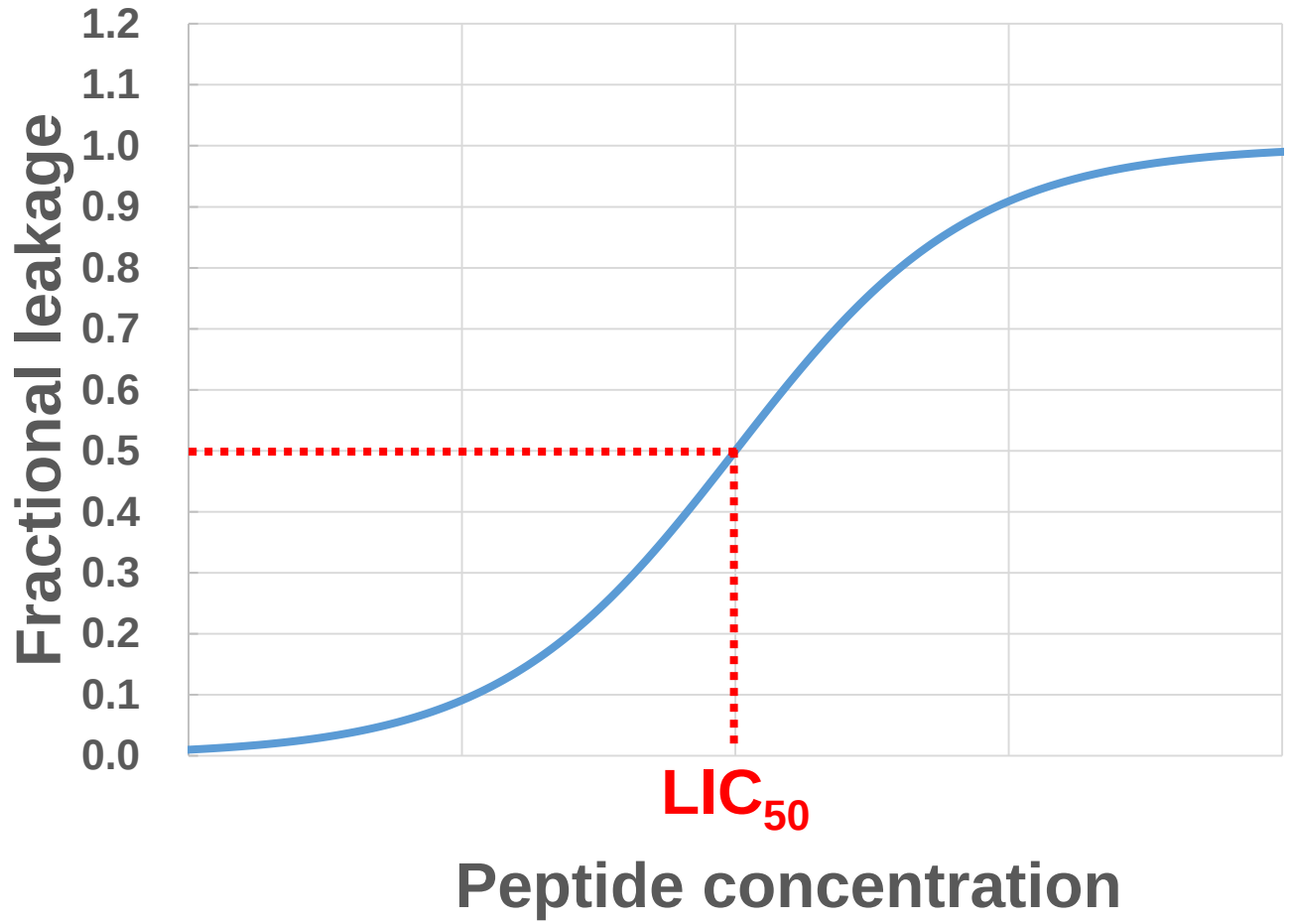
A-3. Edman degradation



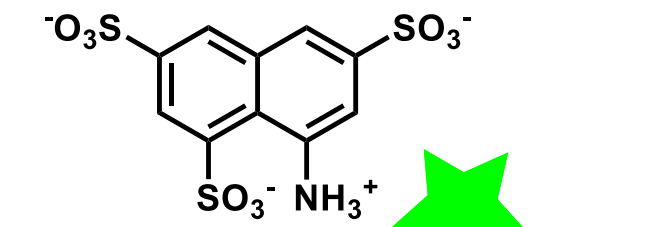
Determination of single amino acid

Edman, P. et. al. *Acta Chem. Scand.* **1950**, *4*, 283.

A-4. Definition of LIC_{50}

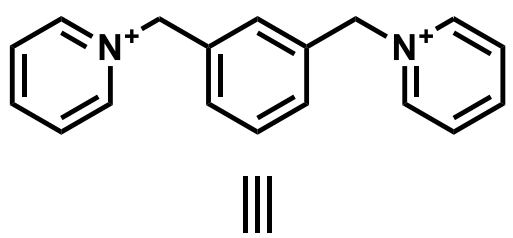


A-5. ANTS/DPX leakage



Fluorescence

ANTS

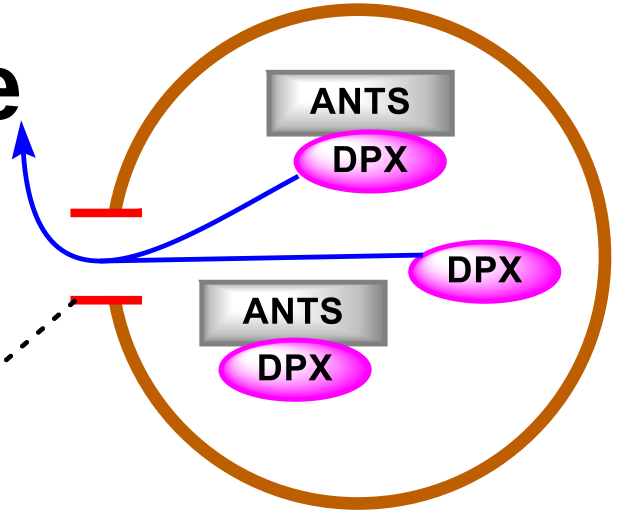


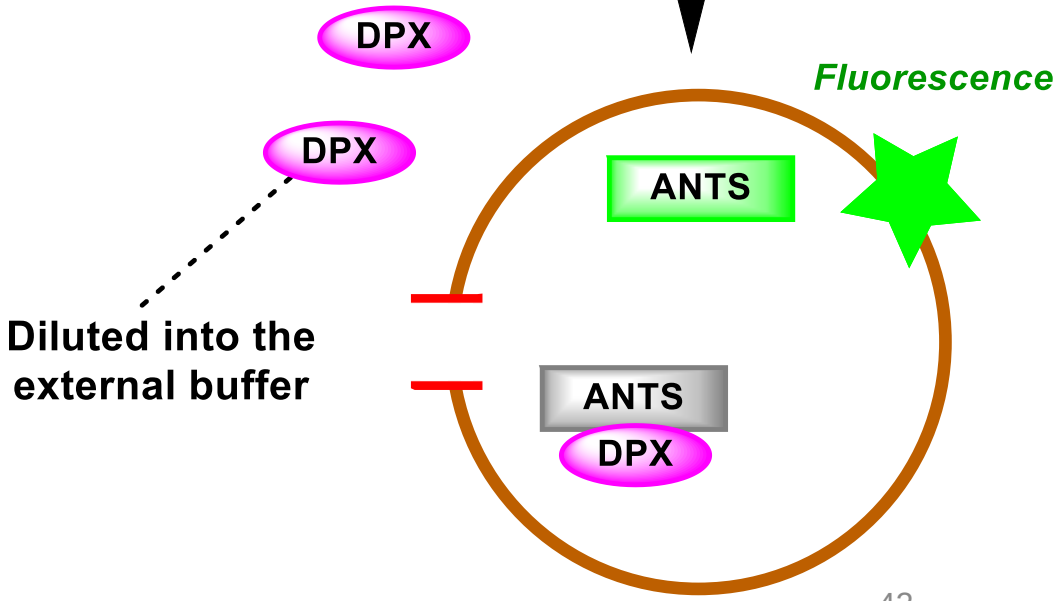
DPX

Quencher

ANTS
DPX

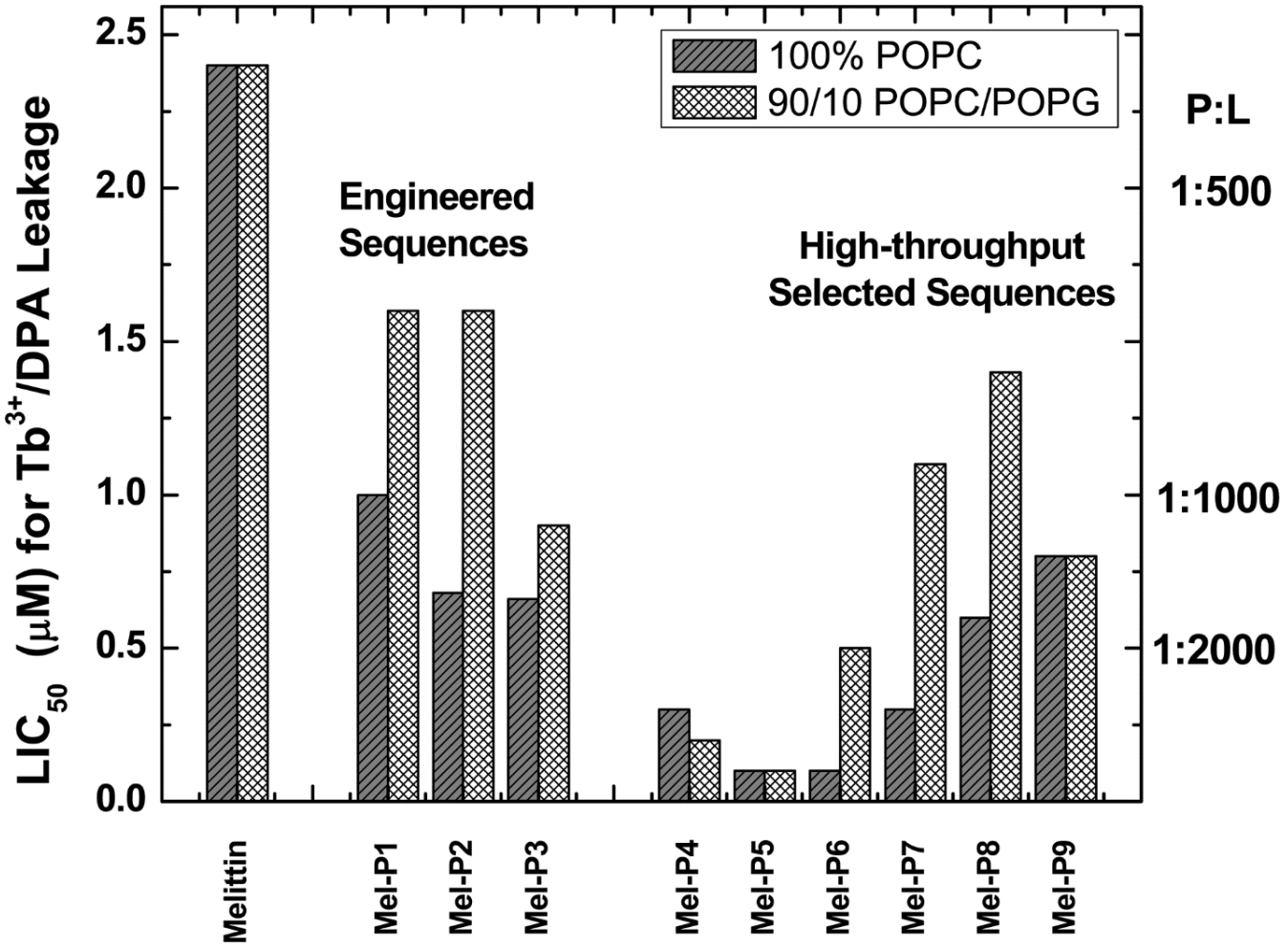
Quenched





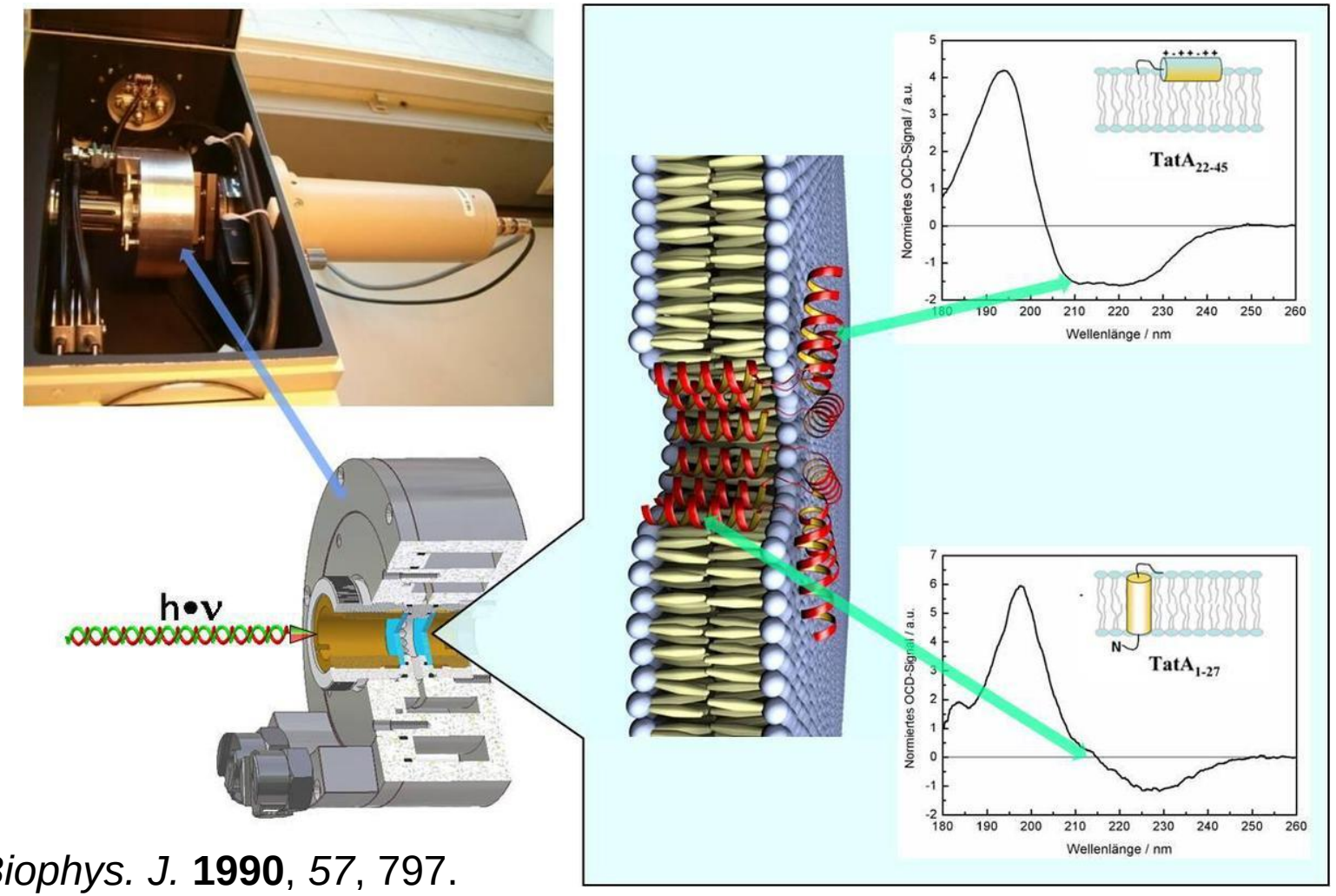
Ellens, H. et. al. *Biochemistry* **1985**, *24*, 3099.

A-6. Measurement of LIC_{50} for selected peptides by Tb^{3+} leakage (section 2)



Krauson, A. J. et. al. *J. Am. Chem. Soc.* **2012**, *134*, 12732.

A-7. Oriented Circular Dichroism



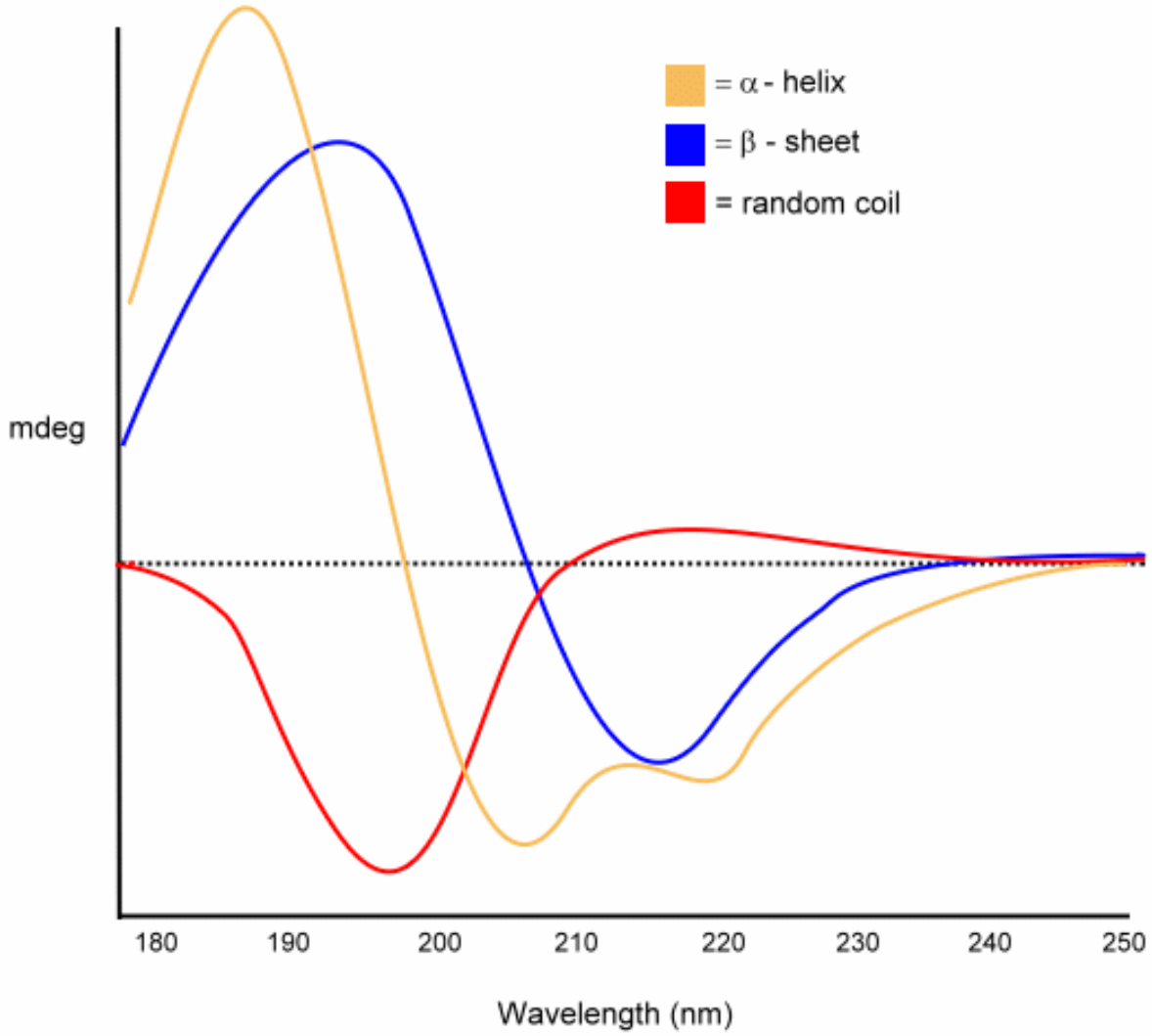
Wu, Y. et. al. *Biophys. J.* **1990**, *57*, 797.
http://www.ibg.kit.edu/nmr/48.php (last visited 2016/2/2)

A-8. Helix-breaking effect of Gly and Pro (relative helix propensities of amino acids)

Amino acid	Helix propensity	Amino acid	Helix propensity
Ala	0.00	Ile	0.41
Arg^+	0.21	Leu	0.21
Asn	0.65	Lys^+	0.26
Asp	0.43	Met	0.24
Asp^-	0.69	Phe	0.54
Cys	0.68	**Pro**	**3.16**
Gln	0.39	Ser	0.50
Glu	0.16	Thr	0.66
Glu^-	0.40	Trp	0.49
Gly	**1.00**	Tyr	0.53
His	0.56	Val	0.61
His^+	0.66		

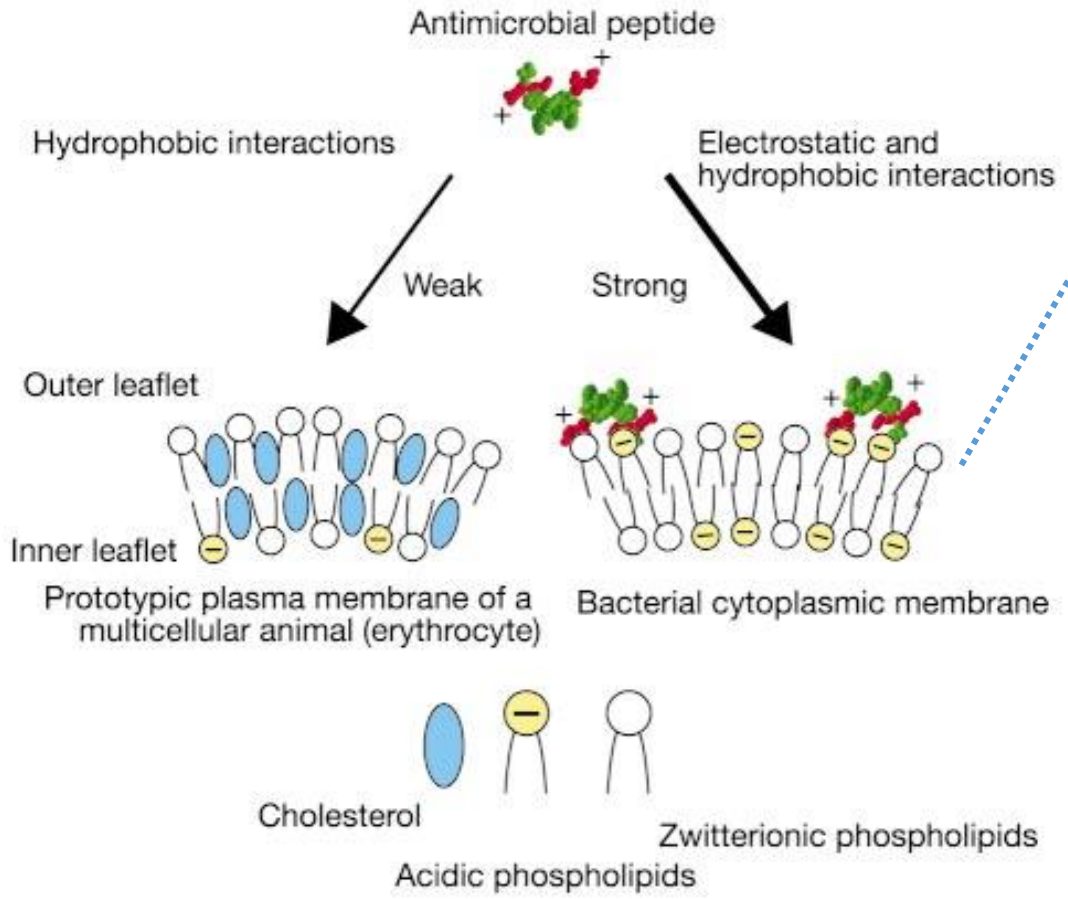
Nick Pace, C.; Martin Scholtz, J. *Biophys. J.* **1998**, *75*, 422.

A-9. Typical CD spectrum for α-helix and random coil



http://www.proteinchemist.com/cd/cdspec.html (last visited 2016/2/4)

A-10. Anionic nature of bacterial membrane



◆ Origin of negative charge

- **Gram-positive bacteria**:

  wall associated teichoic acid

- **Gram-negative bacteria**:

  acidic polymers

  (e.g. lipopolysaccharide)

Zasloff, M. *Nature* **2002**, *415*, 389.
Hancock, R. E. W.; Sahl, H.-G. *Nat. Biotechnol.* **2006**, *24*, 1551.