Extraction and Reconstitution of Membrane Proteins into Lipid Nanodiscs Encased by Zwitterionic Styrene-Maleic Amide Copolymers

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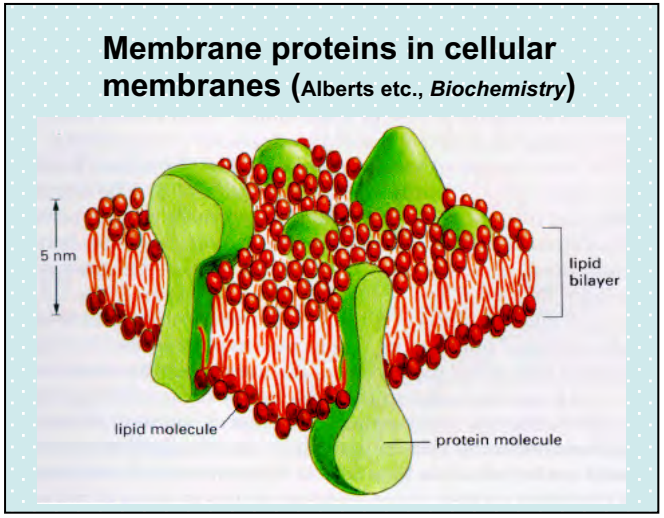
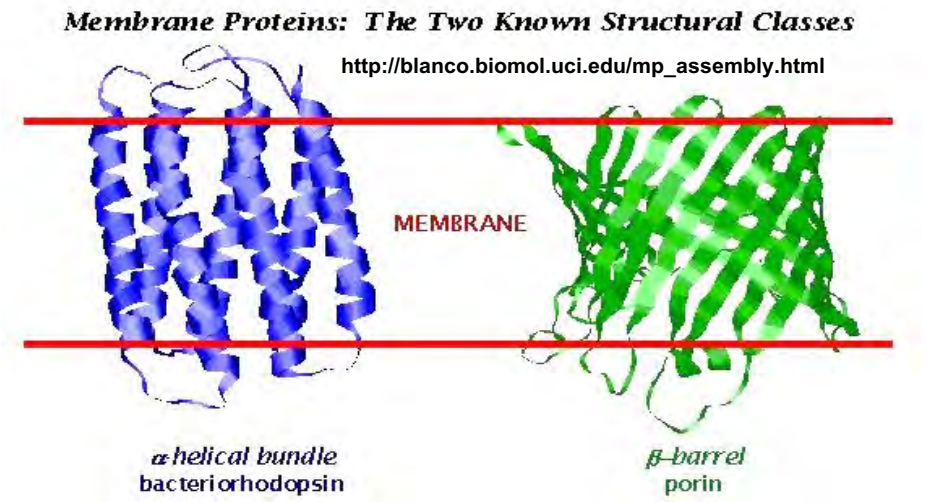
Understanding the Structure and Function of Membrane Proteins (MPs) Remains Challenging

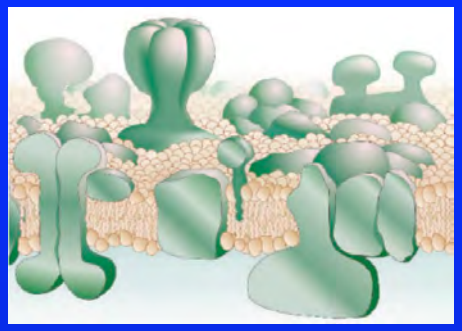




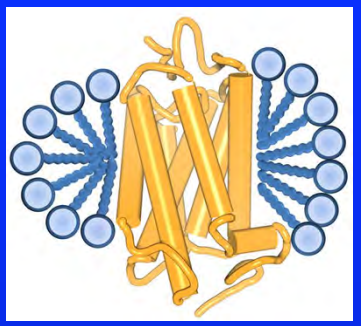
- **Represented by ~30% of the sequenced genomes**
- **Targeted by ~70% of all drugs in the market**
- **Due to the difficulty to stably extract and reconstitute MPs with physiologically relevant conformational states, the structure and function of many MPs remain elusive**

→ New tools needed to support MPs in their "native-like" states

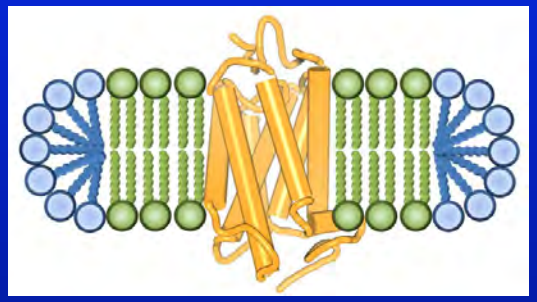
Some Common MP-Supporting Platforms



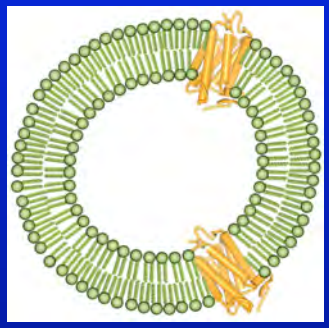
→ Whole cell or VLP suffers from low-abundancy of target MP and interference from irrelevant MPs



→ Detergent micelle may destabilize MPs and alter their native conformations



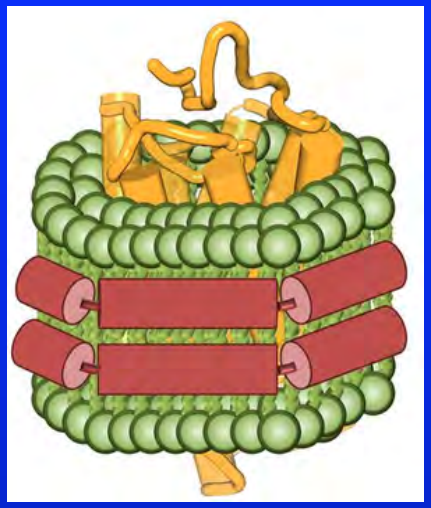
→ Bicelle is limited by specific lipid/amphiphile combinations, and the need to purify target MP by detergent



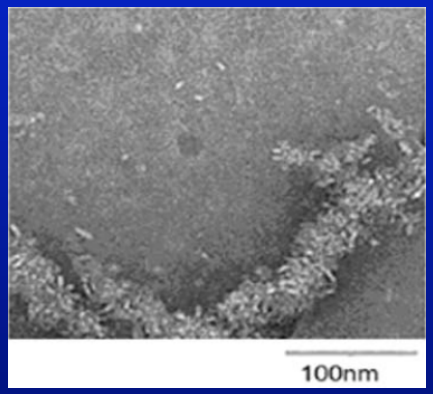
→ Proteoliposome is limited by the purification of MP target by detergent, the random orientation of reconstituted MPs, and undesirable light scattering for spectroscopy studies

Millic D and Veprintsev DB, *Front. Pharm.*, 6:66 (2015)

Supporting MPs with Lipid Nanodiscs (LNDs) Encased by Membrane Scaffold Proteins (MSPs)



Denisov IG and Sligar SG, *Chem. Rev*, 117:4669-4713 (2017)



He W et al, *Protein Sci.*, 22:1078-1086 (2013)

Pros

- Support individual MPs in a native-like membrane environment
- Display target MP in aqueous solution with well-accessible extracellular loops
- Allow tag or fluorescent label to sort or purify target MP

Cons

- Still needs detergents to release MPs from their native membranes before reconstituting into LNDs
- Stability problem − aggregation of LNDs into stacked "rouleaux" during storage or freeze-thaw cycles
- Interference from MSPs for some spectroscopic studies of target MPs, such as FT-IR, CD, Trp fluorescence etc.

MSPs Are Amphipathic Random Copolymers

MSP1: 200 AAs, 10 α-helices

MGHHHHHHIEGR
LKLLDNWDSV TSTFSKLREQ
LGPVTQEFWD NLEKETEGLR
QEMSKDLEEV KAKVQPYLDD
FQKKWQEEME LYRQKVEPLR
AELQEGARQK LHELQEKLSP
LGEEMRDRAR AHVDALRTHL
APYSDELRQR LAARLEALKE
NGGARLAEYH AKATEHLSTL
SEKAKPALED LRQGLLPVLE
SFKVSFLSAL EEYTKKLNTQ

Table 1. Labels and Amino Acid Sequences of Membrane Scaffold Proteins Used for Self-Assembly of Nanodiscs[a]

Abbreviation	Description	Amino Acid Sequence
H1	Helix 1	LKLLDNWDSVTSTFSKLREQLG
H1Δ(1–11)	Truncated Helix 1	STFSKLREQLG
H1Δ(1–17)	Truncated Helix 1	REQLG
H2	Helix 2	PVTQEFWDNLEKETEGLRQEMS
H3	Helix 3	KDLEEVKAKVQ
H4	Helix 4	PYLDDFQKKWQEEMELYRQKVE
H5	Helix 5	PLRAELQEGARQKLHELQEKLS
H6	Helix 6	PLGEEMRDRARAHVDALRTHLA
H7	Helix 7	PYSDELRQRLAARLEALKENGG
H8	Helix 8	ARLAEYHAKATEHLSTLSEKAK
H9	Helix 9	PALEDLRQGLL
H10	Helix 10	PVLESFKVSFLSALEEYTKKLNTQ
FX	Original N-terminus	MGHHHHHHIEGR
TEV	Modified N-terminus	MGHHHHHHH DYDIPTTENLYFQG

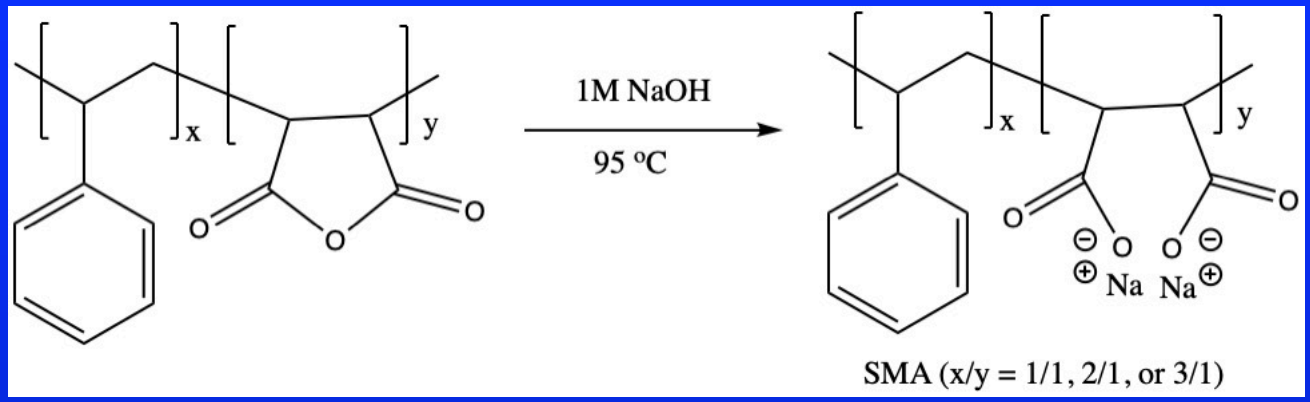
Helix 4, 5, and 6 are the 22mers that can be repeatedly added between H3 and H4 (i.e. Q55 and P56) to become MSP1E1 (…H4-H4…), MSP1E2 (…H4-H5-H4-H5…), and MSP1E3 (…H4-H5-H6-H4-H5-H6…) with longer length.

Denisov IG and Sligar SG, *Chem. Rev*, 117:4669-4713 (2017) Bayburt et al, *Nano Lett.*, 2(8):853-856 (2002)

SMALPs: Styrene-Maleic Acid/Lipid Particles

S

MA



S/MA=1/1 ...

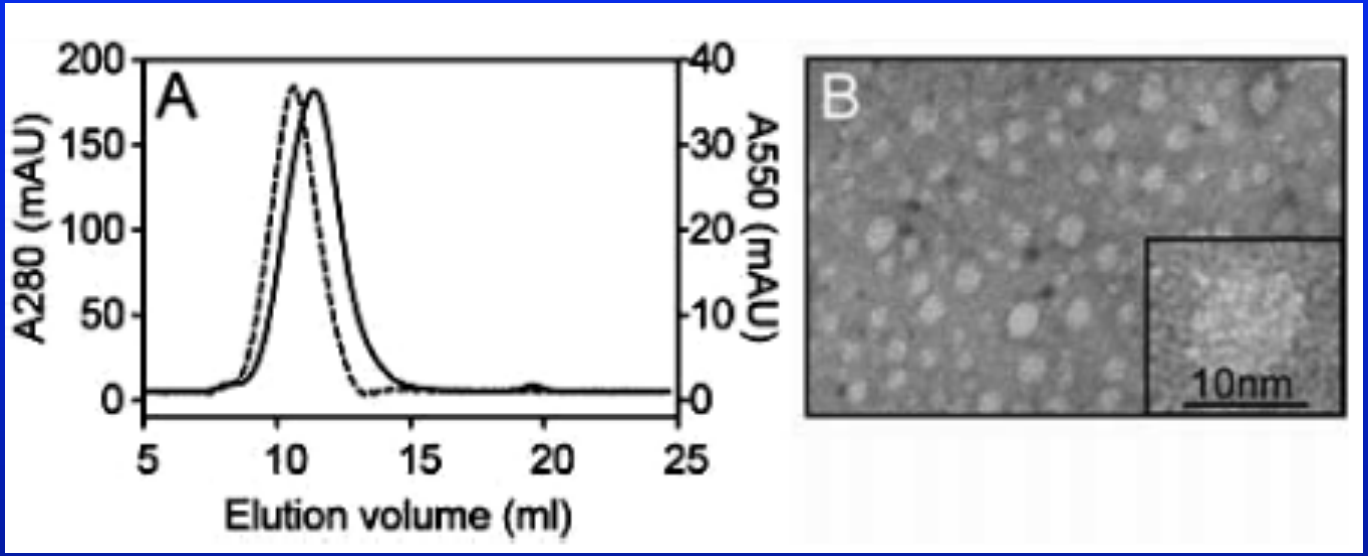
S/MA=2/1 ...

"The present invention … provide useful surfactants or solubilizing agents for certain substances, particularly drugs or other bioactive materials, and can be especially useful for producing aqueous solutions of substances that are lipid soluble but have poor aqueous solubility …" (Malvern Lipodisq™)

Tonge S and Tighe BJ, "Lipid-containing compositions and uses thereof", *WO1999009955 A2* (1999)

Polymer Encased Nanodiscs: SMALPs

SMA/lipid particle (SMALP): "monodispersed lipid disks" formed by treating liposomes or proteoliposomes with SMA copolymers. "The disks are ~11 nm in diameter and contain ~11 PC lipids and a single protein" as estimated by the phosphate assay and A_{280}.

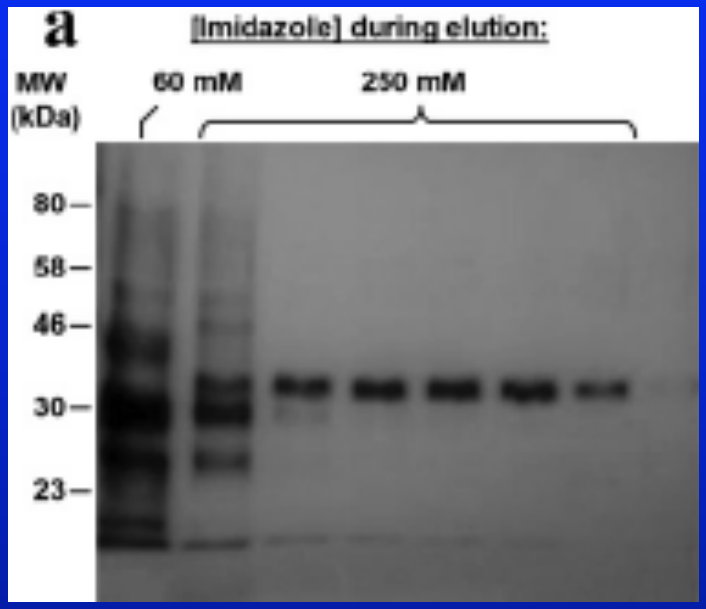


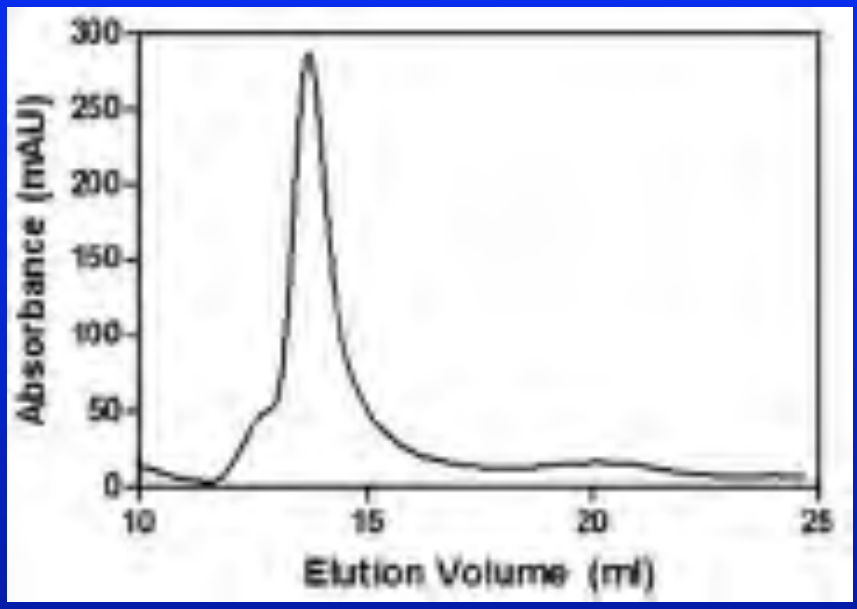
(A) SEC of PgP (solid line) and bR (dashed line) incorporated into SMALPs; (B) TEM of SMALPs

Knowles TJ, Finka R, Smith C, Lin YP, Dafforn T, and Overdurin M, *JACS*, 131:7484-7485 (2009)

Extract MPs from Native Membranes into SMALPs

"The first solubilization and purification of a functional GPCR [human adenosine A_{2A} receptor ($A_{2A}R$)], in the total absence of detergent at any stage" by forming SMALPs.

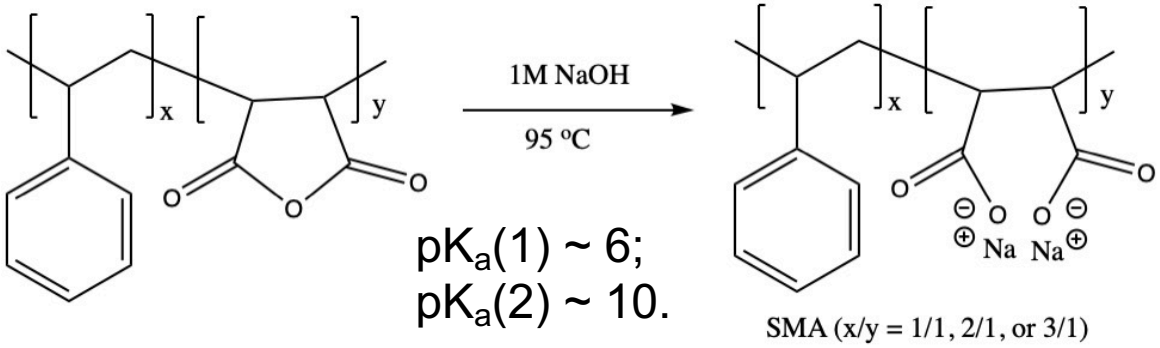




(A) Purification of SMALP-solubilized His-tagged $A_{2A}R$ from *P. pastoris*; (B) Analysis of $A_{2A}R$–SMALP from *P. pastoris* by SEC.

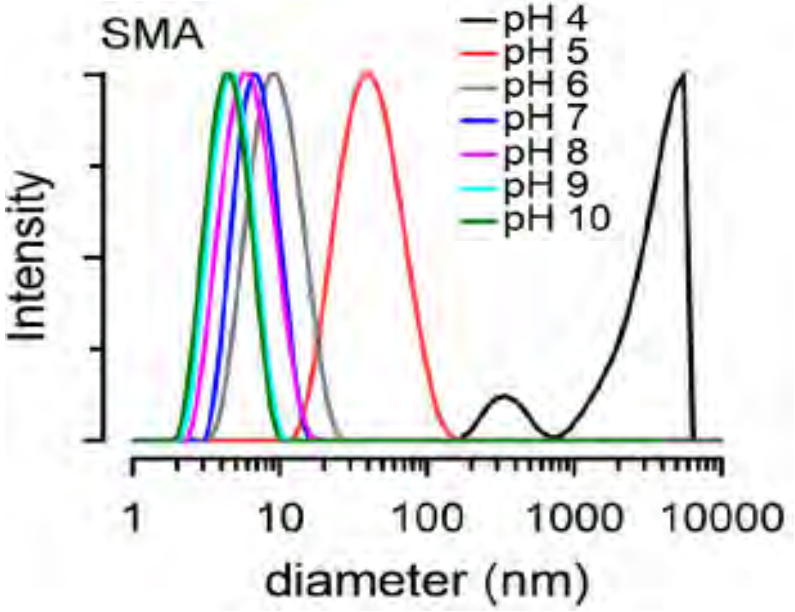
Jamshad M et al, *Biosci. Rep.*, 35:e00188 (2015)

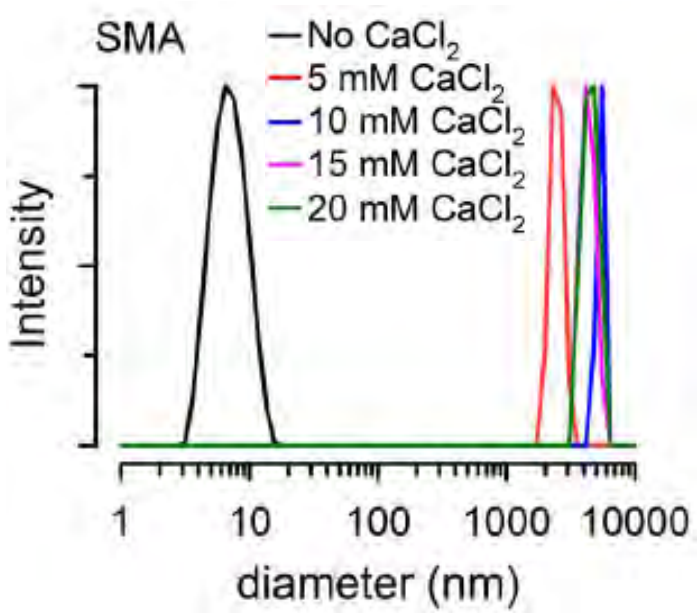
Limitations of SMAs: Buffer Incompatibility



$pK_a(1) \sim 6$;
$pK_a(2) \sim 10$.

SMA (x/y = 1/1, 2/1, or 3/1)

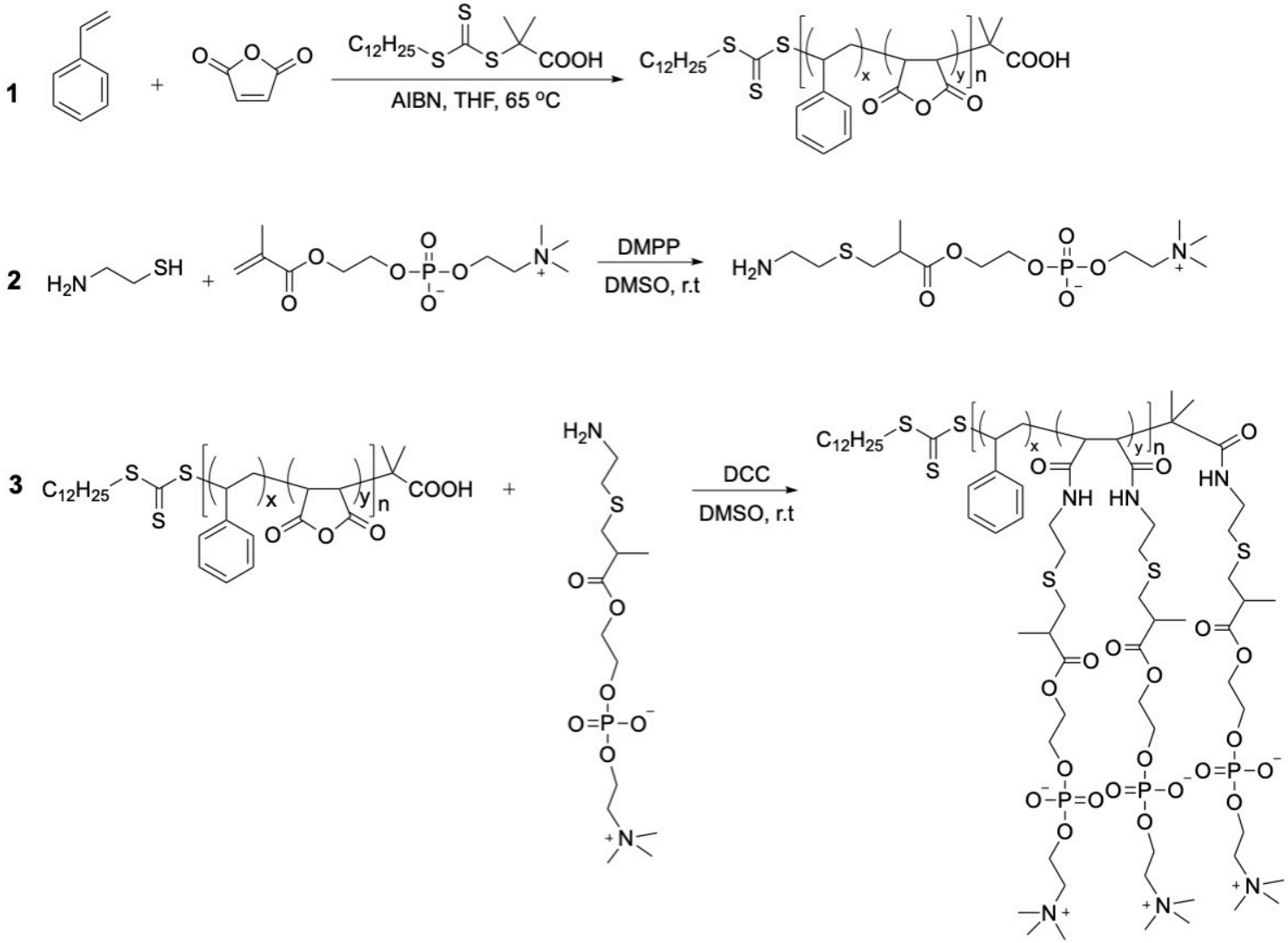




Fiori MC et al, *Sci. Rep.*, 7:7432 (2017)

→ SMALPs precipitate in buffers at low pH (i.e. pH<6) or in the presence of multivalent ions (Ca^{2+}, Mg^{2+}, etc.)

Zwitterionic Styrene-Maleic Amide Copolymers



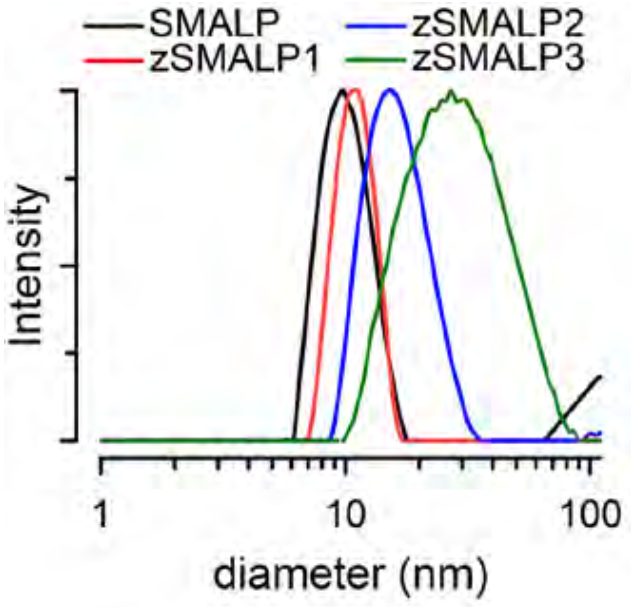
Altenberg GA and Liang HJ, "Polymer-encased nanodiscs with improved buffer compatibility", *USP application no. 62552605* (2017); *Licensed to Anatrace, Inc. (2019).*

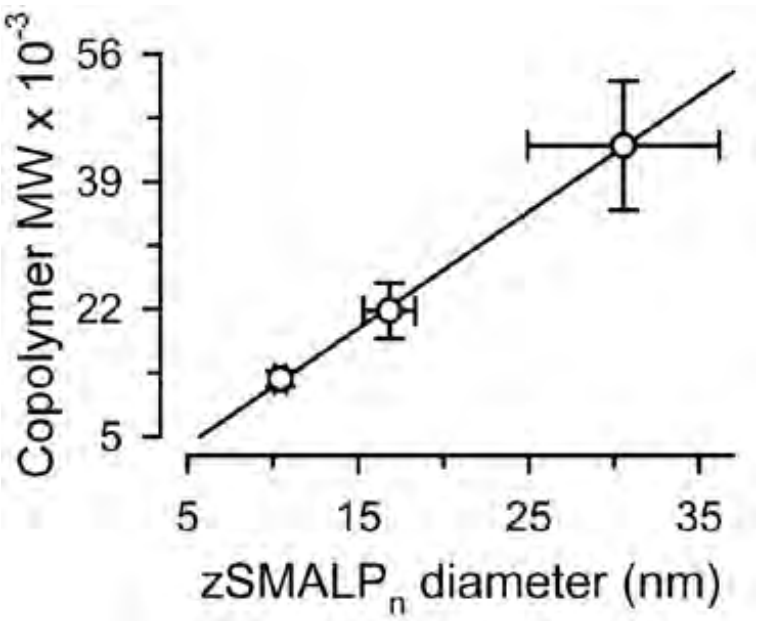
Polymer Encased Nanodiscs: zSMALPs

S/MA=1/1

zSMA1
zSMA2
zSMA3
SMA

Sample	MW from conversion	MW from NMR	GPC in DMF	
			Mn	PDI
P(S-*at*-MA)$_{59}$	12,451	12,675	NA	1.085
P(S-*at*-MA)$_{106}$	21,576	21,777	35,000	1.170
P(S-*at*-MA)$_{215}$	43,708	43,795	53,800	1.197
SMA (Xiran)	NA	NA	NA	1.341

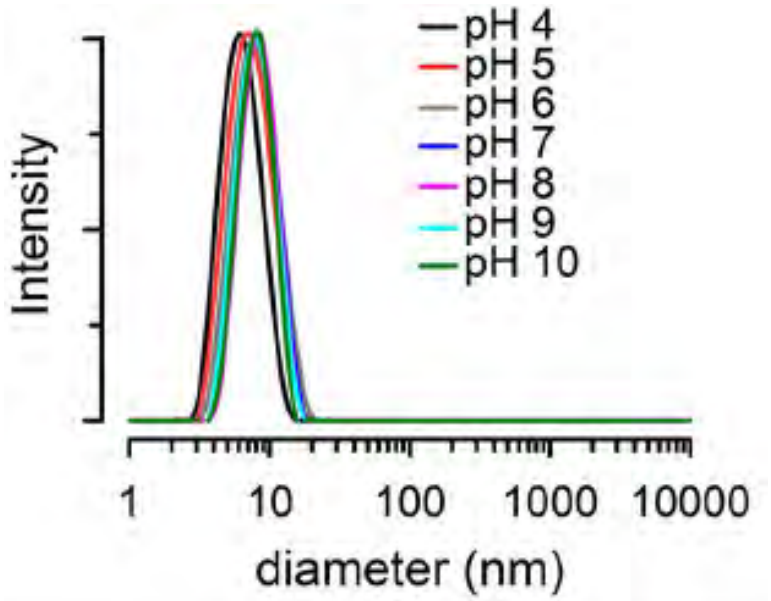


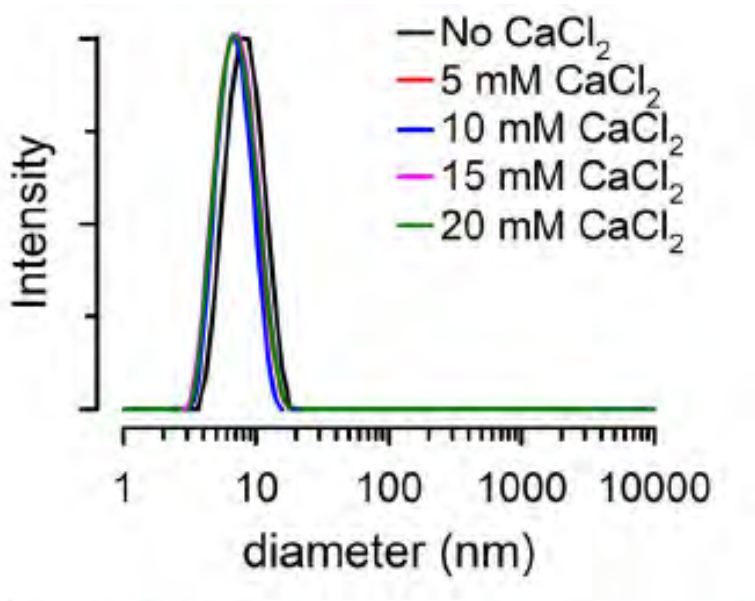


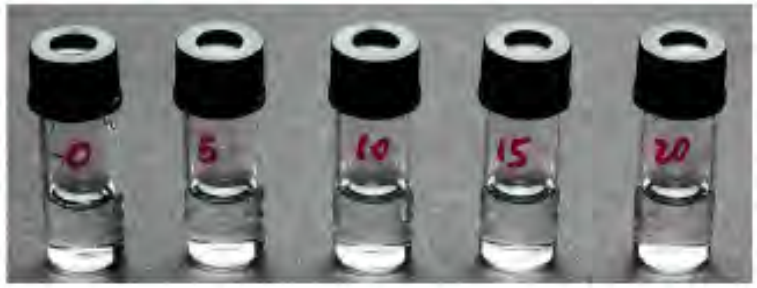
Fiori MC et al, *Sci. Rep.*, 7:7432 (2017)

→ Control the average size of zSMALPs by well-defined polymers

zSMALPs with Unlimited Buffer Compatibility





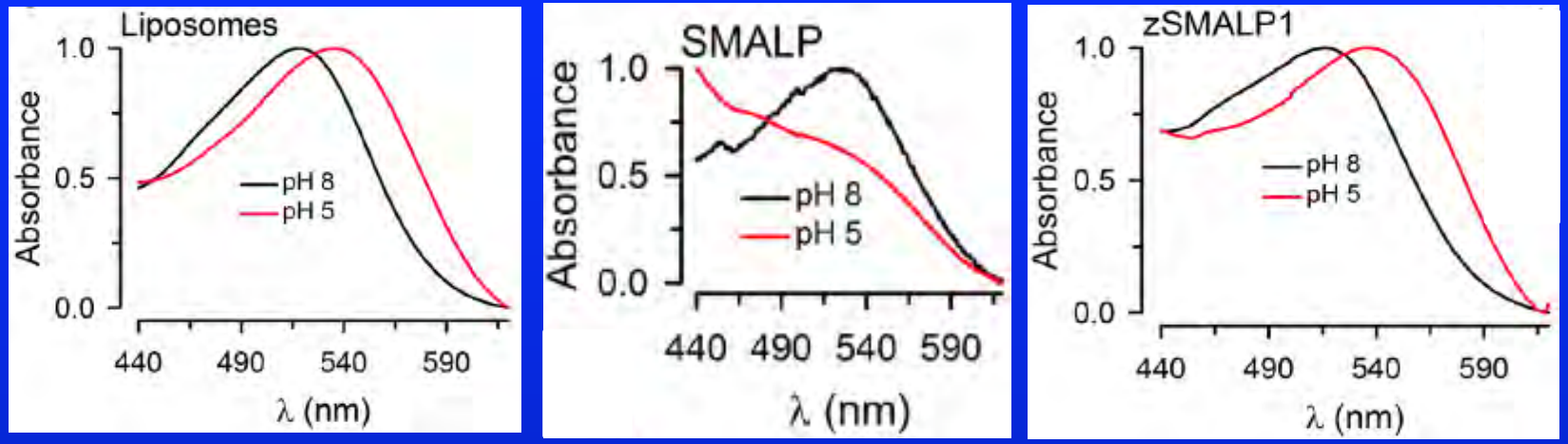


Fiori MC et al, *Sci. Rep.*, 7:7432 (2017)

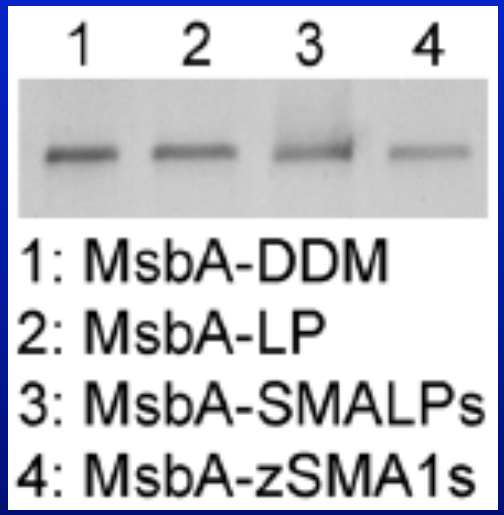
→ Unchanged size distribution of zSMALPs in buffers at low pH (i.e. pH<6) or in the presence of multivalent ions

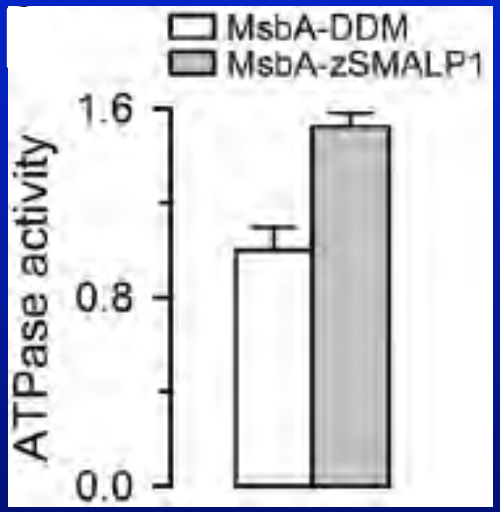
Functional Assays of Membrane Proteins in zSMALP

Proteorhodopsin functional assays at different pH



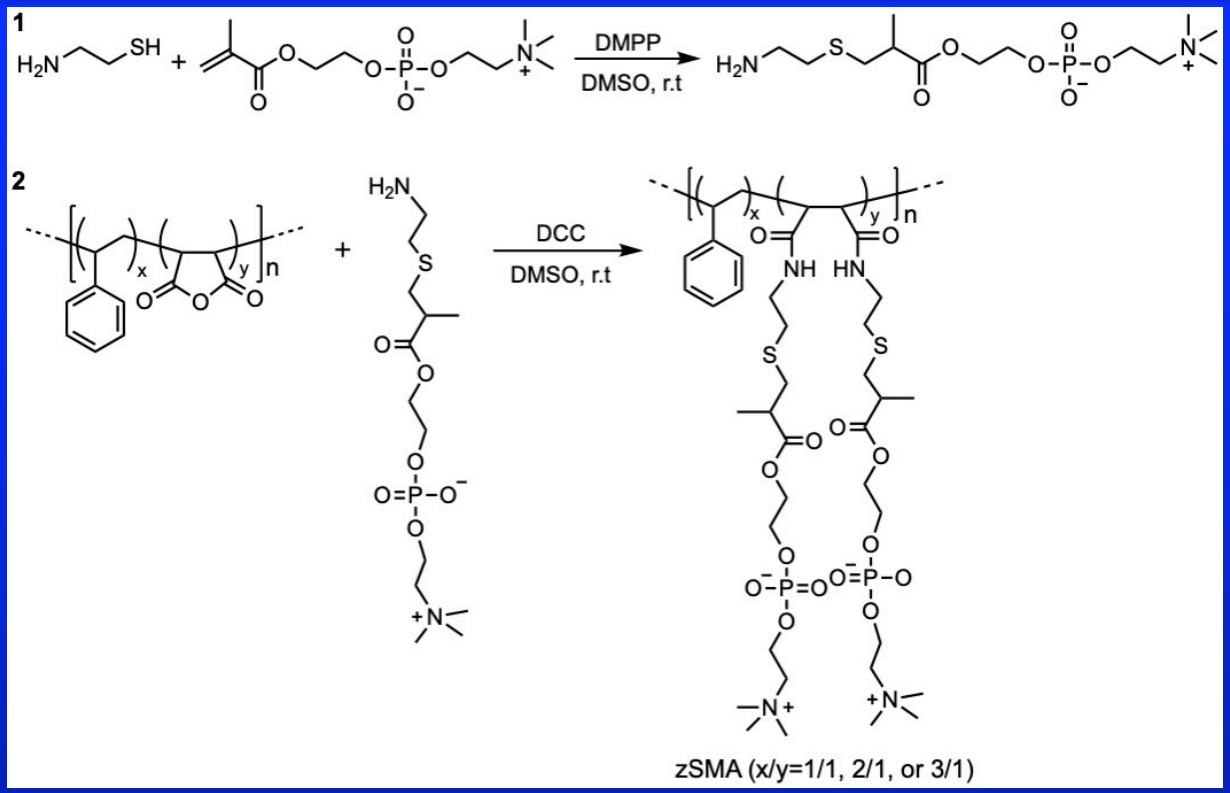
ATPase activity of MsbA in the presence of $MgCl_2$





Fiori MC et al, *Sci. Rep.*, 7:7432 (2017)

Compare zSMALPs Encased by Different zSMAs



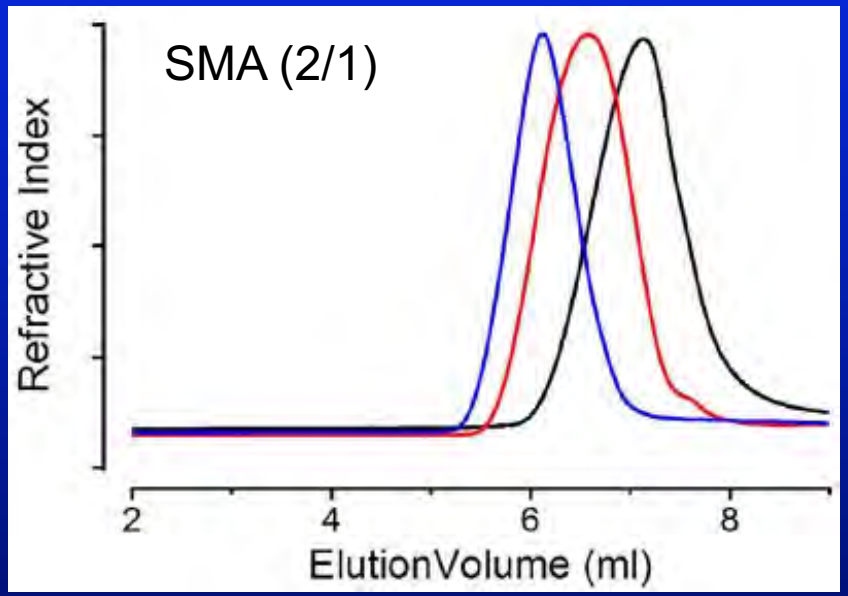
→Turning polydisperse Malvern Lipodisq™ SMA into zSMA ("M zSMA") and compare it with well-defined zSMA prepared via controlled/"living" polymerization;

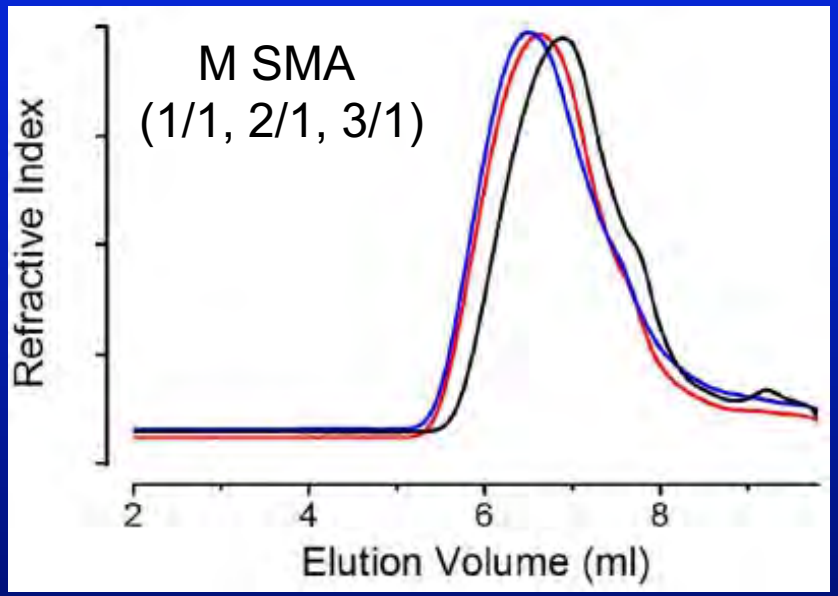
→ Compare the effect of buffer conditions, polymer-lipid interaction conditions, and polymer structure characteristics on solubilizing Halorhodopsin (HR) and MsbA into zSMALPs.

Compare zSMALPs Encased by Different zSMAs

S/MA	Sample Name	St:MA[c]	M.W. (kDa)[c]	M.W. (kDa)[d]	PDI[d]
1/1	P(St_{31}-*ran*-MA_{31})[a]	1.00/1.00	6.6	6.7	1.05
2/1	P(St_{18}-*ran*-MA_{9})[a]	2.00/1.00	3.1	3.1	1.05
2/1	P(St_{40}-*ran*-MA_{21})[a]	1.93/1.00	6.6	6.4	1.04
2/1	P(St_{76}-*ran*-MA_{39})[a]	1.95/1.00	12.6	12.0	1.13
1/1	P(St-*ran*-MA)[b]	0.95/1.00	/	4.6	1.22
2/1	P(St-*ran*-MA)[b]	2.00/1.00	/	5.0	1.52
3/1	P(St-*ran*-MA)[b]	2.86/1.00	/	5.7	1.59

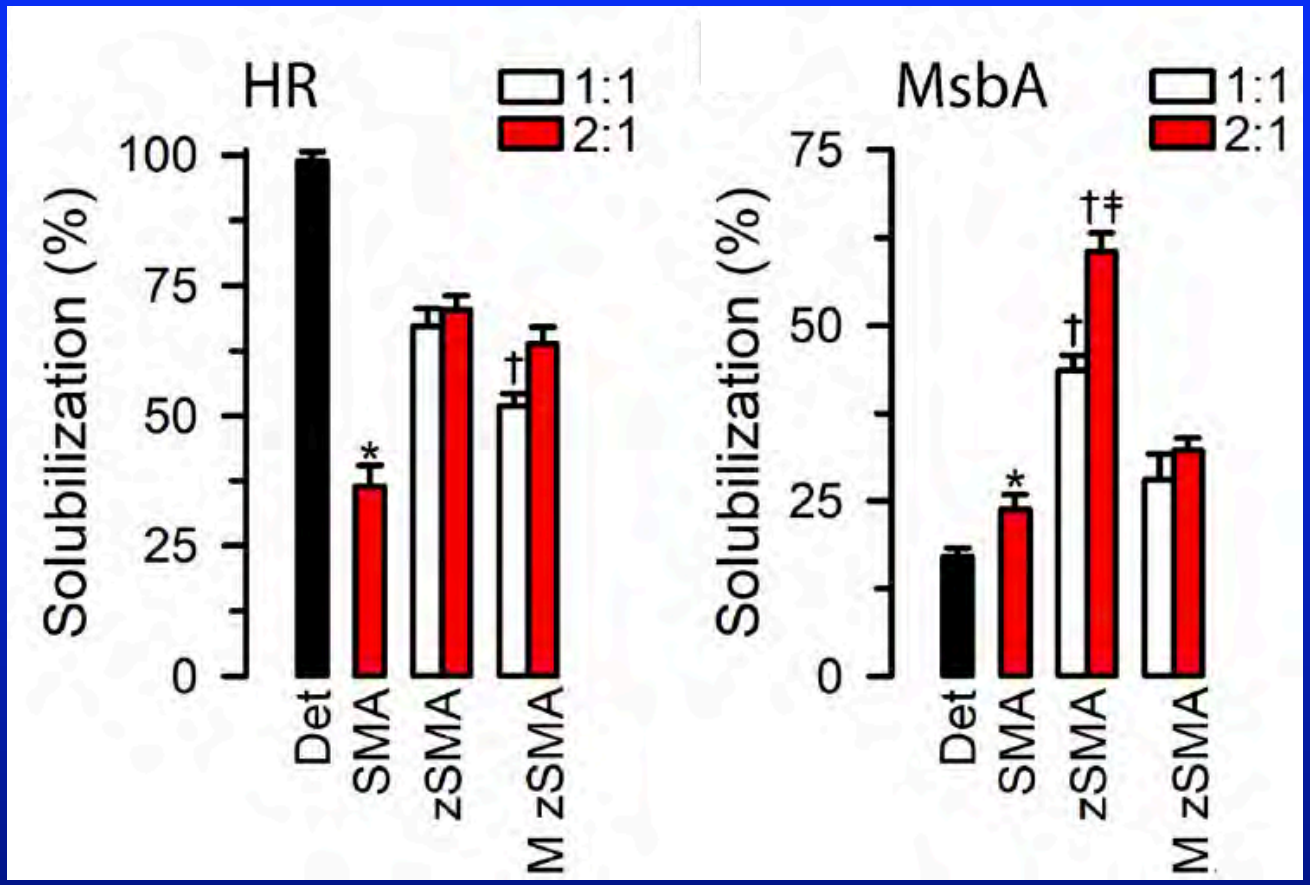
[a]Synthesized *via* RAFT polymerization; [b]Lipodisq® copolymers obtained from Malvern Cosmeceutics; [c]Obtained by NMR analysis; [d]Obtained by GPC analysis.





zSMALPs Encased by Different zSMAs: S/MA ratio

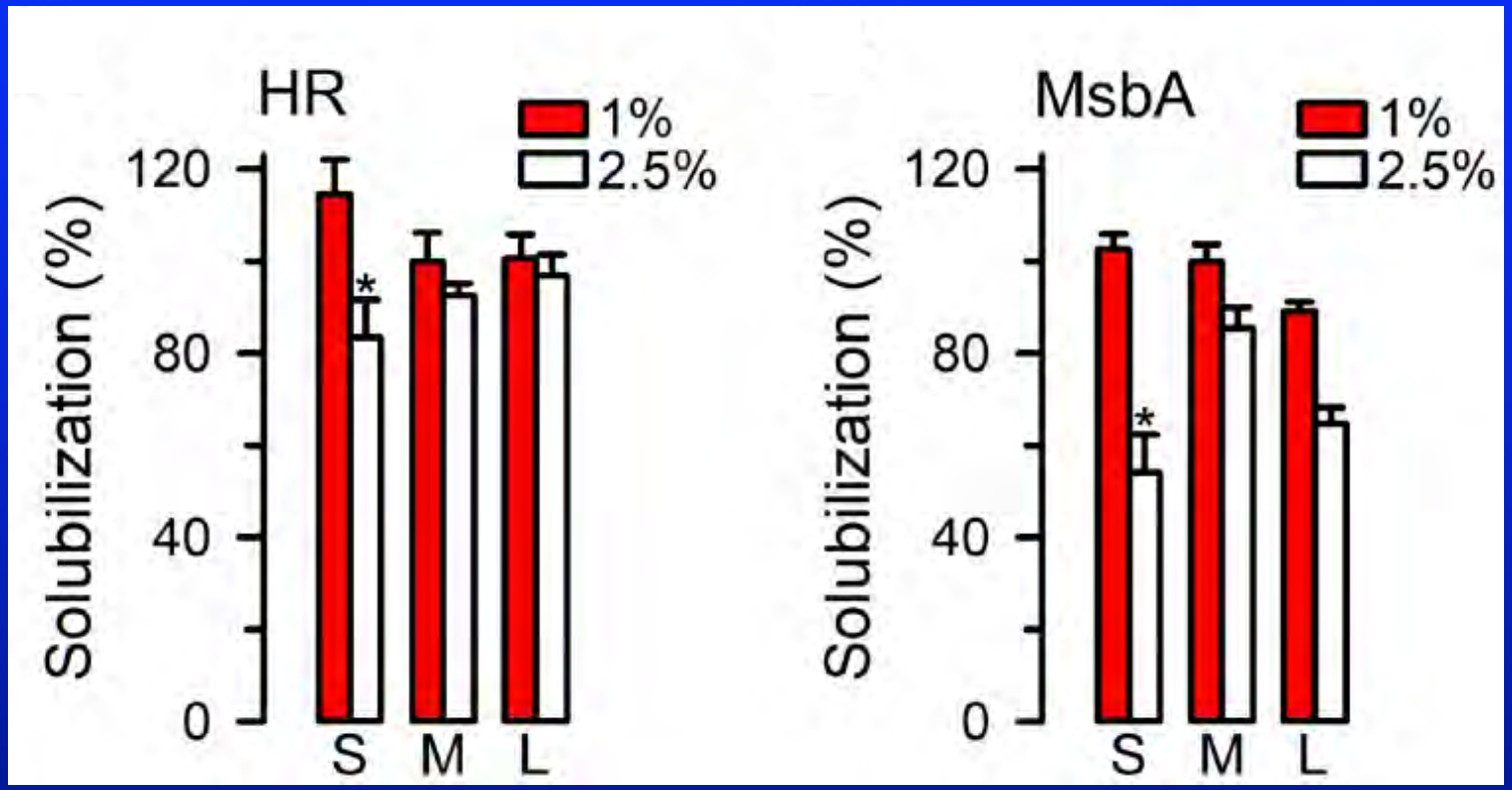
Crude membrane: B21 *E. coli*; [Copolymer] = 1%; [Salt] ~500 mM; pH 7.5 Tris; Copolymer M.W. (before converted to zSMA) ~ 5-6 kD; Det: 1.5% DDM for HR; 2% DDM/0.04% sodium cholate for MsbA; Incubation time: 2h; SMA (2/1) control



→ zSMA performs better than SMA or M zSMA for both MPs, and S/MA=2/1 zSMA works better than 1/1 zSMA for MsbA not HR

zSMALPs Encased by Different zSMAs: Chain Size

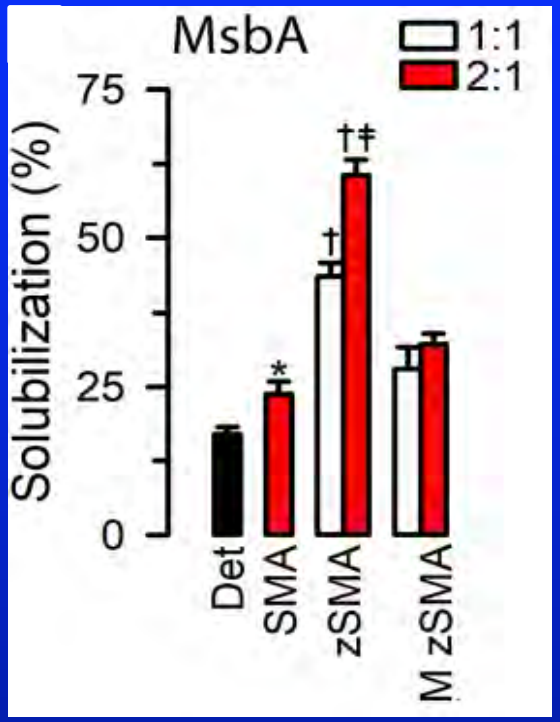
Crude membrane: B21 *E. coli*; [Salt] ~ 500 mM; pH 7.5 Tris buffer;
zSMA: S/MA=2/1; SMA M.W. (before conversion) ~ 3(S), 6(M), and 12(L) kD;
Det: 1.5% DDM for HR, 2% DDM/0.04% sodium cholate for MsbA; Time: 2h



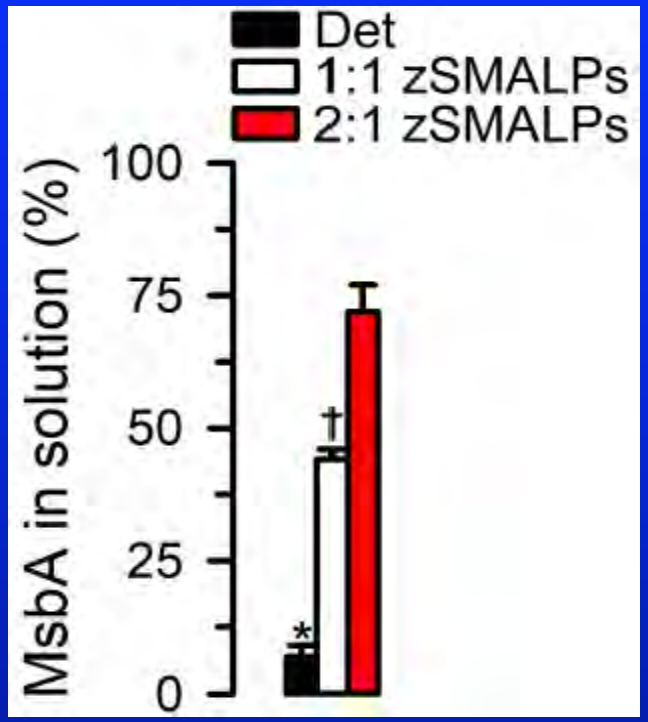
→ [zSMA]=1% solubilize crude membrane better than 2.5%;
→ At [zSMA]=1%, smaller zSMA solubilizes crude membrane slightly better than larger zSMAs

zSMALPs Encased by Different zSMAs: Stability & Function

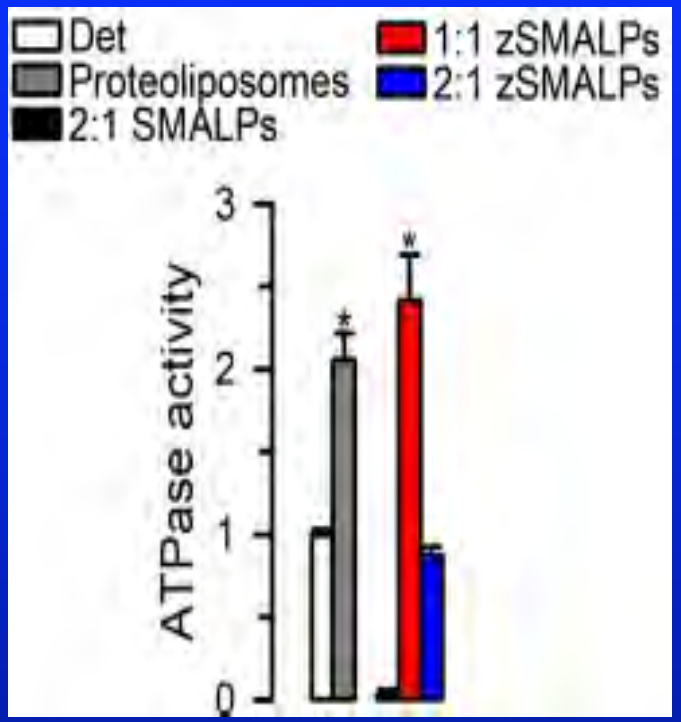
Crude membrane: B21 *E. coli*; pH 7.5 Tris buffer; [Salt]=500mM; M.W. (before conversion) ~ 5-6 kD; MP: MsbA; Det: 2% DDM/0.04% sodium cholate



Solubilization of crude membrane



Thermostability (heat shock@65ºC;15 min)



ATPase activity

→ S/MA=2/1 zSMA is more efficient than 1/1 in solubilizing MsbA from crude membrane to form zSMALPs with higher thermal stability, but the zSMALPs from 1/1 zSMA show better activity

Summary

- All zSMA shows higher efficiency than SMA (2/1) in extracting HR and MsbA from crude membranes into nanodiscs with no limitation on buffer conditions;
- Well-defined zSMA shows higher efficiency than polydisperse zSMA in extracting HR and MsbA from crude membranes, and can form nanodiscs with tunable diameters depending on the polymer chain size;
- A good starting point: 1% zSMA in regular buffers (pH 7-9) with intermediate ionic strength (150-500 mM NaCl) at RT or 37ºC with 2 h incubation time works well for both HR and MsbA;
- The high efficiency of SMA-like polymers to solubilize membrane not necessarily translates to high yield of nanodisc formation – needs test with individual MPs.

Acknowledgement

Group members

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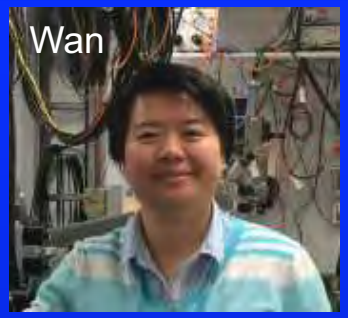
Miguel Anzaldua, Geuel Simiyu, Max Ahn



Collaborators

Guillermo Altenberg (TTUHSC)
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Steve Tonge (Malvern Cosmeceutics)

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NSF-DMR 1623241;
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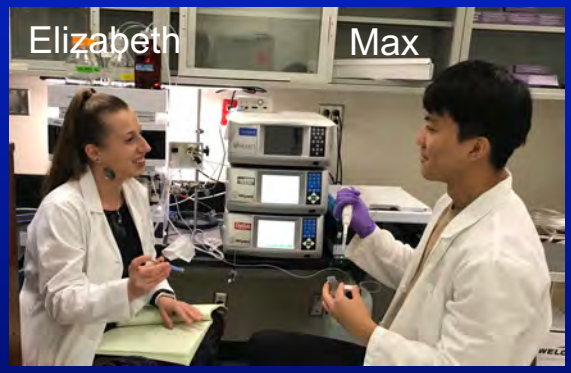

Thank You!