Rapid Incorporation of Heterologously Expressed GPCRs in Nanoscale Apolipoprotein Bound Bilayers (NABBs)

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ABSTRACT

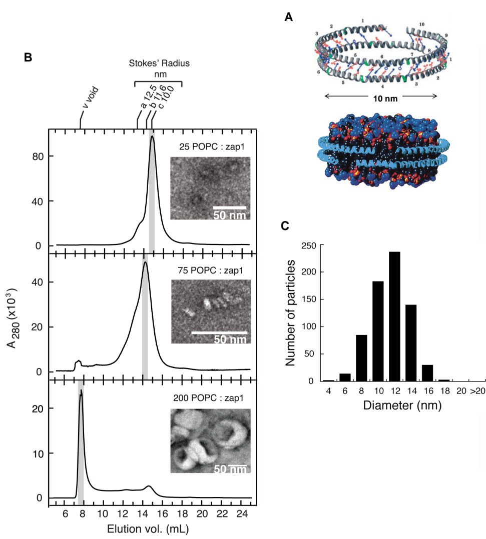
Nanoscale apolipoprotein bound bilayers (NABBs) are stable discoidal structures that allow access to both topological surfaces of transmembrane receptors. We showed earlier that the prototypical G protein-coupled receptor (GPCR), rhodopsin, is stable and functional when reconstituted into NABBs. [1] Here we report the incorporation into NABBs of an engineered C-C chemokine receptor 5 (CCR5) – a rhodopsin-like GPCR involved in the immune response and used as the primary coreceptor for HIV-1. Recombinant CCR5 was immunoaffinity purified from detergent extracts of a mammalian cell line. CCR5-NABBs were prepared by mixing phospholipids, zap1 and purified CCR5 followed by hydrophobic affinity chromatography to remove detergent. The resulting crude NABBs were purified by size exclusion chromatography. We also show that NABBs may be used to conveniently purify GPCRs produced using an *in-vitro* transcription and translation system, using opsin as an example. NABBs appear to be a flexible tool for a variety of biophysical studies of engineered GPCRs in a detergent-free, native-like environment.

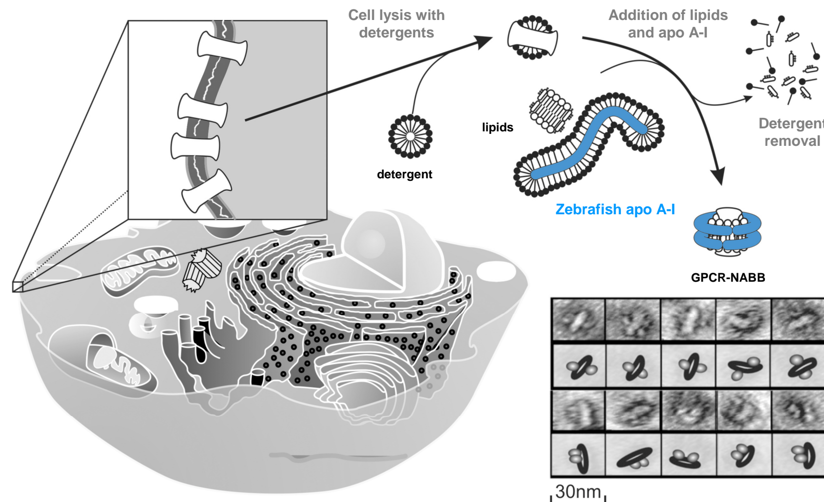
NABBs

(A) Apolipoprotein A-I is an amphipathic alpha-helical protein which forms discoidal lipoprotein particles. Two apo A-I molecules form one discoidal particle, with an anti-parallel orientation relative to each other.[2]

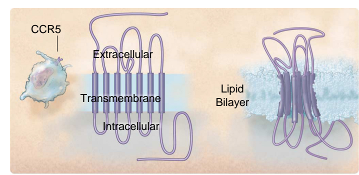
(B) Engineered zebrafish apo A-I (Zap1) forms homogeneous discoidal lipoprotein particles with POPC lipid, at an optimal molar ratio of 75 POPC : 1 Zap1, determined by gel-filtration chromatography. [1]

(C) Measurement of NABB diameters from EM images of 75 POPC : 1 Zap1 sample, shows a monodisperse population with diameter between 10 – 12 nm.

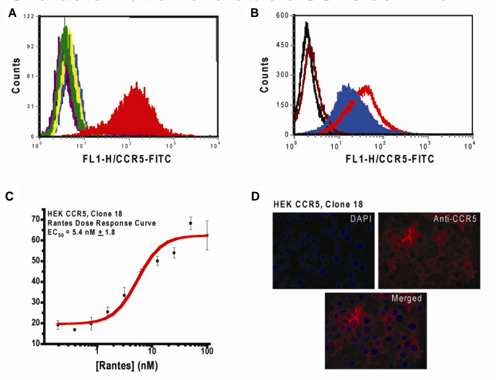




CCR5 [3]

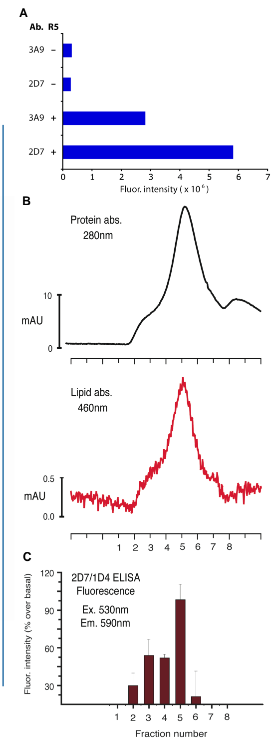


Characterization of a stable CCR5 cell line



(A) Histogram of HEK CCR5 clone 18 cells stained with a conformationally sensitive anti-CCR5 mAb 2D7 conjugated to FITC (red) analyzed by FACS. **(B)** CCR5 cells were incubated in the presence (blue) or absence (red) of RANTES (100 nM) before staining with anti-CCR5 2D7 mAb. **(C)** Intracellular Ca flux assay of CCR5 clone 18 cells demonstrates RANTES stimulation of CCR5 leads to an increase in intracellular calcium. **(D)** CCR5 clone 18 cells stained with anti-CCR5 2D7 mAb show a uniform membrane staining profile for CCR5.

2D7 binding to CCR5



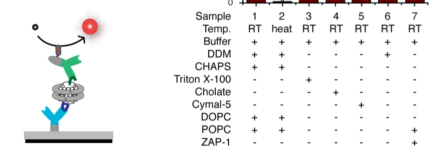
(A) Binding of Atto-655 linked CCR5 N-terminal antibodies 3A9 and 2D7 to detergent solubilized CCR5 purified on 1D4 (C-term. mAb)- sepharose beads. Efficient recognition by the conformationally sensitive 2D7 mAb indicates that CCR5 retains its correctly folded extracellular conformation upon solubilization in a buffer containing lipids and detergent.

(B) Detergent solubilized, 1D4 purified CCR5 was reconstituted into NABBs using Extracti Gel D. [1] 1 % (w/w) NBD-DOPE was added to the POPC mixture before starting the NABB reconstitution. The product was analyzed by gel-filtration chromatography on a Superose 6 PC 3.2/30 column, and the absorbance at 280 nm and 460 nm was recorded.

(C) The fractions from the gel-filtration column were analyzed by a sandwich ELISA protocol, using Amplex Red substrate. Equal protein concentrations were captured using 2D7 and probed using HRP-linked 1D4 antibody.

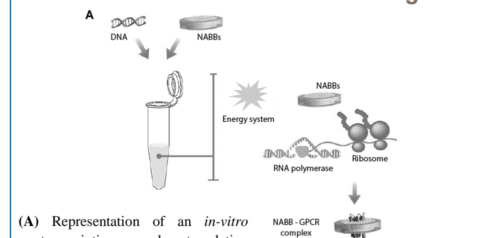
Stability of CCR5 in NABBs

CCR5 was incubated at room temp. for 15 min in different detergents and/or lipids, and in NABBs, followed by analysis using sandwich ELISA. CCR5 containing samples were captured with 2D7 and probed with HRP-1D4 using Amplex Red substrate.



Sample	1	2	3	4	5	6	7
Temp.	RT	heat	RT	RT	RT	RT	RT
Buffer	+	+	+	+	+	+	+
DDM	+	+	-	-	-	+	-
CHAPS	+	+	-	-	-	-	-
Triton X-100	-	-	+	-	-	-	-
Cholate	-	-	-	+	-	-	-
Cymal-5	-	-	-	-	+	-	-
DOPC	+	+	-	-	-	-	-
POPC	+	+	-	-	-	-	+
ZAP-1	-	-	-	-	-	-	+

In-vitro translation using NABBs



(A) Representation of an *in-vitro* transcription and translation system using nano lipoprotein particles. [4]

(B) 1D4 immunoblot of Ni affinity purified NABBs, after *in-vitro* translation of an opsin gene containing a 1D4 mAb recognition sequence at the C-terminus. Wheat Germ Extract and SP6 RNA polymerase was used for the reaction. The NABBs have a 6xHis tag on zap1 which was used for affinity purification on Ni-NTA beads.

CONCLUSIONS

1. NABBs may be used to isolate and purify heterologously expressed membrane proteins in a lipid environment, with high yields.
2. CCR5 reconstituted into NABBs retains the correct conformation of its extracellular side.
3. Both sides of CCR5 in a NABB are biochemically accessible, making the system ideal for high-throughput screening applications.
4. GPCRs may be expressed in a cell-free system and purified conveniently using affinity tags on NABBs.

REFERENCES

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2. Segrest JP *et al.* (2000), *Curr. Op. Lipidol.* **11**: 105 – 15.
3. Lederman MM *et al.* (2006), *JAMA* **296**: 815 – 26.
4. Katzen F *et al.* (2008), *J. Proteome Res.* **7**: 3535 – 42.