Investigation of membrane proteins using surface sensitive techniques: how to exploit amphipols?

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Outline

Immobilization of proteins: why?

Supports and techniques taking advantage of surfaces

Immobilization of proteins: how?

Immobilization of membrane proteins – a brief review

Amphipols for membrane proteins immobilization

Immobilization of proteins: Why?

Functional information:

How does the protein of interest interact with other molecules?

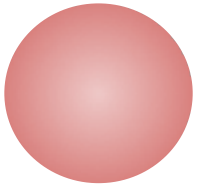
Molecules: ligand, interacting proteins

- Thermodynamics at equilibrium: Kd, Ki
- Kinetics of interactions: kon, koff, residence time of molecule

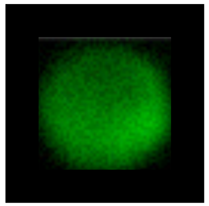
Requires:

- High-sensitivity of detection
- Monitoring kinetics of interactions

Taking advantage of the surface



- a solid support:
  - easy separation of free / bound molecules
    - magnetic particles
    - flow cytometry
    - fluorescence microscopy



Also important

- Upconcentration of the protein
  - Increase Signal / Noise
  - Reduced quantities of material

- Additional purification step (in case of specific immobilization)

Taking advantage of the surface

Control of the biological system (for specific immobilization)

- Orientation
- Stoichiometry
- Density

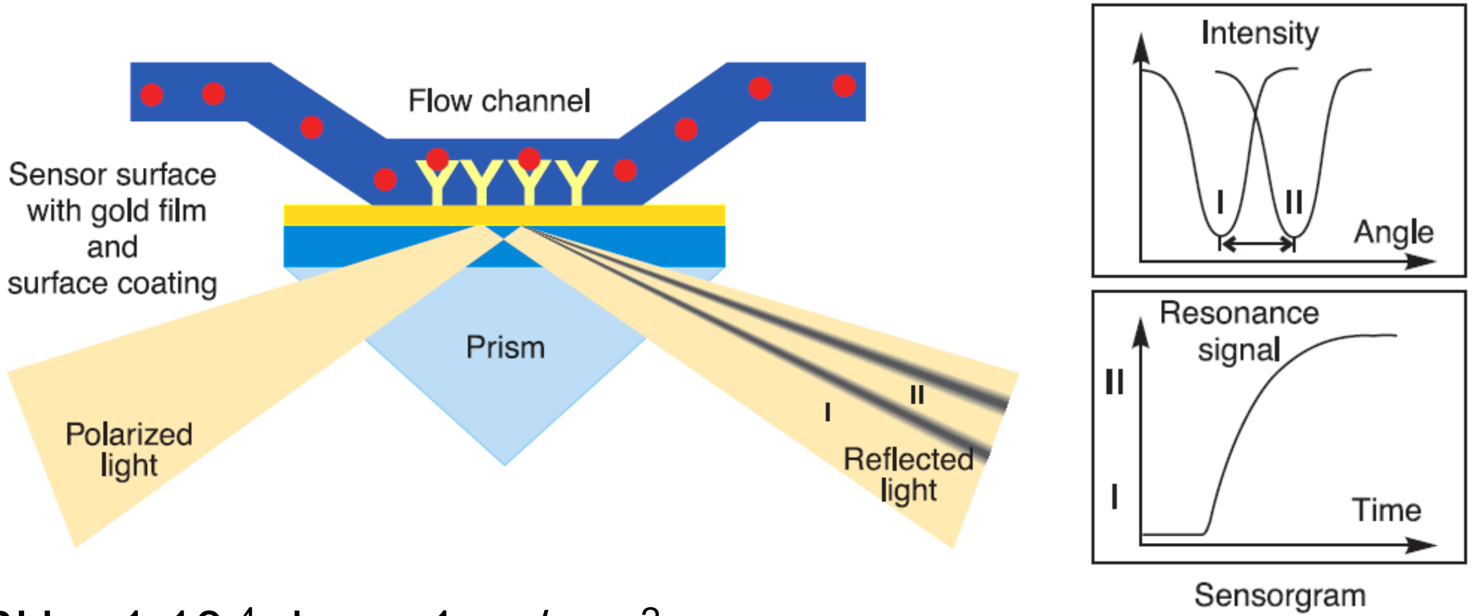
- 2D organization: can be important for membrane proteins

Detection methods

- Fluorescence
  - fluorescence microscopy, FACS
  - total internal reflection fluorescence
- Radioactivity
  - Scintillation Proximity Assay
- Label-free techniques
  - Surface Plasmon Resonance
    (change of refractive index, i.e. mass)
  - Quartz Crystal Microbalance
    (change of mass)

Biacore’s Kretschmann setup

- Wedge of light, SPR angle is transformed into resonance signal



- 1 RU ≡ $1 \cdot 10^{-4}$ deg ≈ 1 pg/mm^2
- 1 RU = RI change of 10^{-6}

Biacore AB, Technology Note 1
S. Lofas *et al.*, *Sens. Actuators, B* 1991
E. Stenberg *et al.*, *J. Colloid Interface Sci.* 1991

How to immobilize a protein?

- Various surfaces and their modifications

- Covalent / non covalent

- Specific / Non specific

- Important criteria
  - protein function
  - orientation
  - accessibility
  - non specific binding

How do proteins interact with surfaces?

- Proteins interact via:
  - Ionic bonds
  - Hydrophobic interactions
  - Polar interactions
- Influence of each parameters depends:
  - the protein
  - the surface
- There is no general rule

Consequences:
- proteins of interest randomly immobilized
- non-specific immobilization of proteins

Determines:
- the Signal / Noise of the assay
- the sensitivity of the assay
- the limit of detection

Which surface?

- The choice of the surface depends on the technique of detection
- Optical detection (glass, silica, polymer, 3D matrixes)
  - Substrate transparent
  - Substrate with low background signal
- Surface Plasmon Resonance
  - Gold
- All the surfaces can be chemically modified and grafted with some functional groups

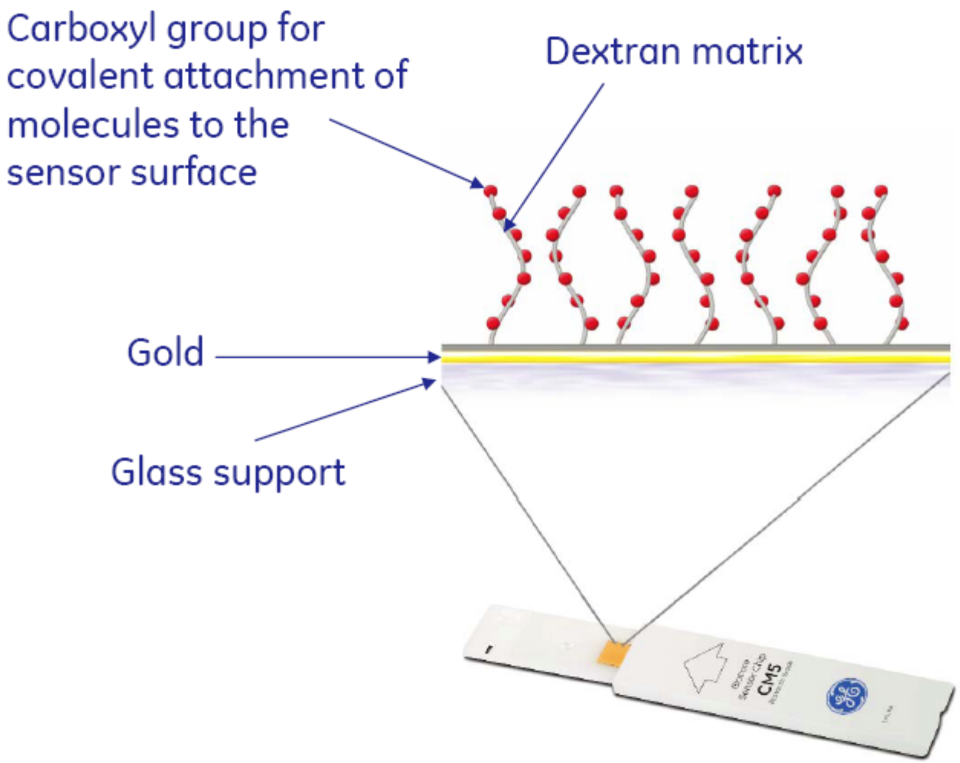
Non specific binding: how to reduce it?

- Traditional approaches:
  - “sticky proteins”:
    - – BSA (bovine serum albumin)
    - – WGA (weat germ agglutinin)
    - – milk powder
  - Low concentration of detergent:
    - – Tween 20
    - – SDS

- Coating with polymers:
  - – Poly(ethylene)glycol: PEG
  - – Poly(Lysine)-Poly(ethylene)glycol: PLL-PEG

Biacore: Sensor chips with dextran matrix

- Dextran
- Flexible
- Decrease NSB
- Increase density
- ~50% use CM5 chip
  (~100 nm dextran matrix)
- Few chips do not have dextran matrix (C1, HPA)



S. Lofas & B. Johnsson, *J. Chem. Soc., Chem. Commun.* 1990

Immobilization of the protein

2 options:

- Random immobilization
  - Adsorption
  - Covalent cross-linking

- Controlled (and oriented?) immobilization
  - Use of affinity tags
  - Reversible or not

Protein Immobilization : protein adsorption

Adsorption of proteins to surfaces

1- Direct interaction of the protein with the surface

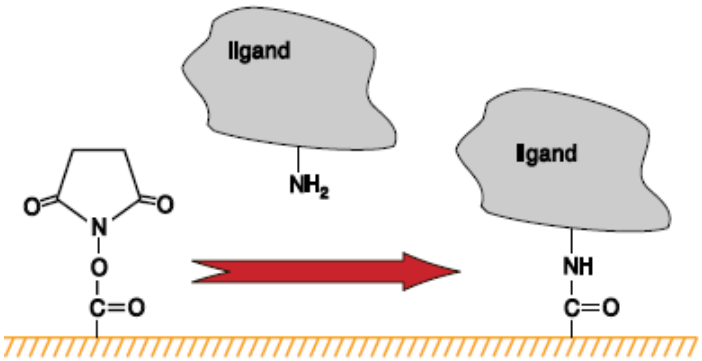
Structurally stable proteins: hydrophobic and electrostatic interactions
Poorly structurally stable proteins : adsorb on everything due to gain in conformational entropy

2- Random orientation of the proteins

+ Very easy – no modifications
- Denaturation - largest conformational change for soft proteins
  Alteration of the accessibility and the function

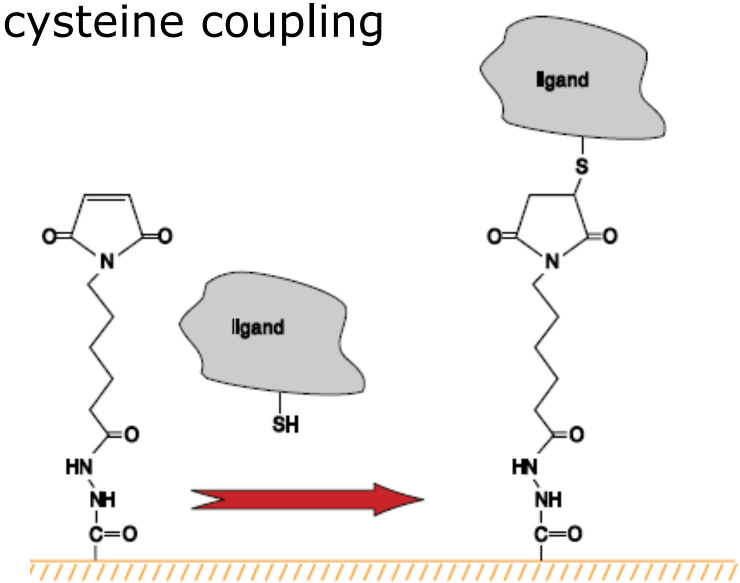
Protein Immobilization :"Random" linking

amine coupling



+ Easy – only surface modification
  Stability of the protein content

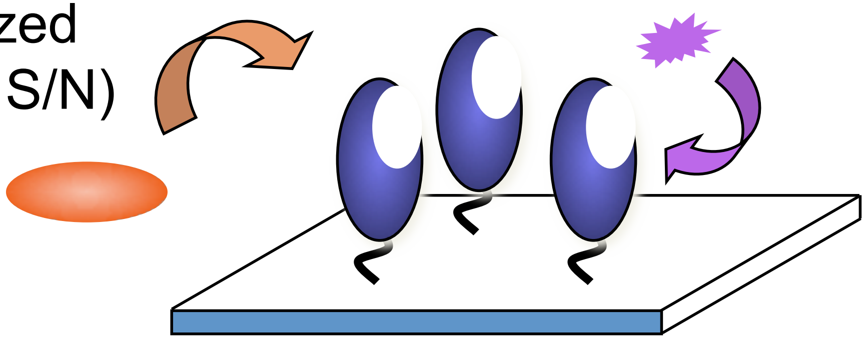
cysteine coupling



- Risk of denaturation from multiple linking sites
  Heterogeneous population
  Steric hindrance

Controlled immobilization via affinity tags

- Controlled orientation
  - Maintained protein function
  - Specificity of the protein immobilized
  - Homogeneous population (better S/N)



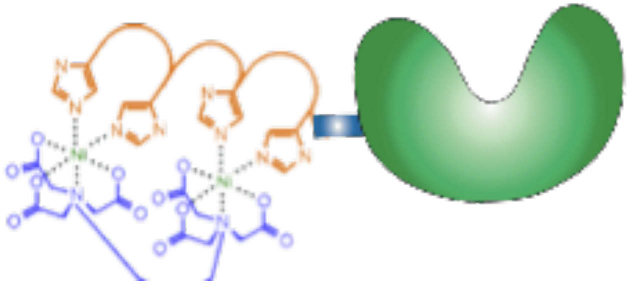
- Controlled density on surfaces
  - Better control of the Signal
  - Reversible / irreversible surface
  - Regeneration of the sensor

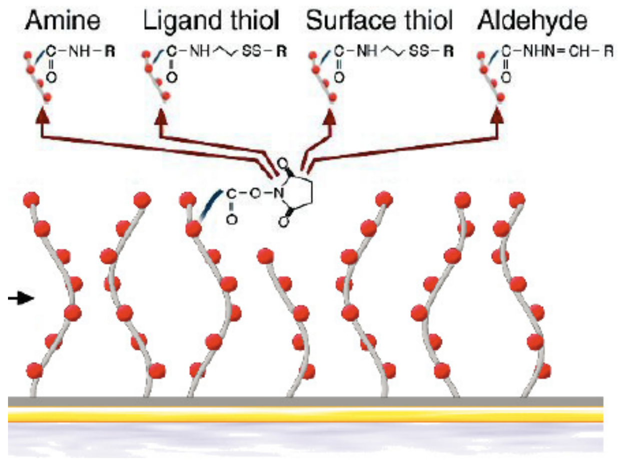
4 main aspects:

- Modification of the protein required
- Non invasive immobilization strategy
- Purification & immobilization in 1 step
- Possibility to tune the density i.e. signal

Immobilization Techniques

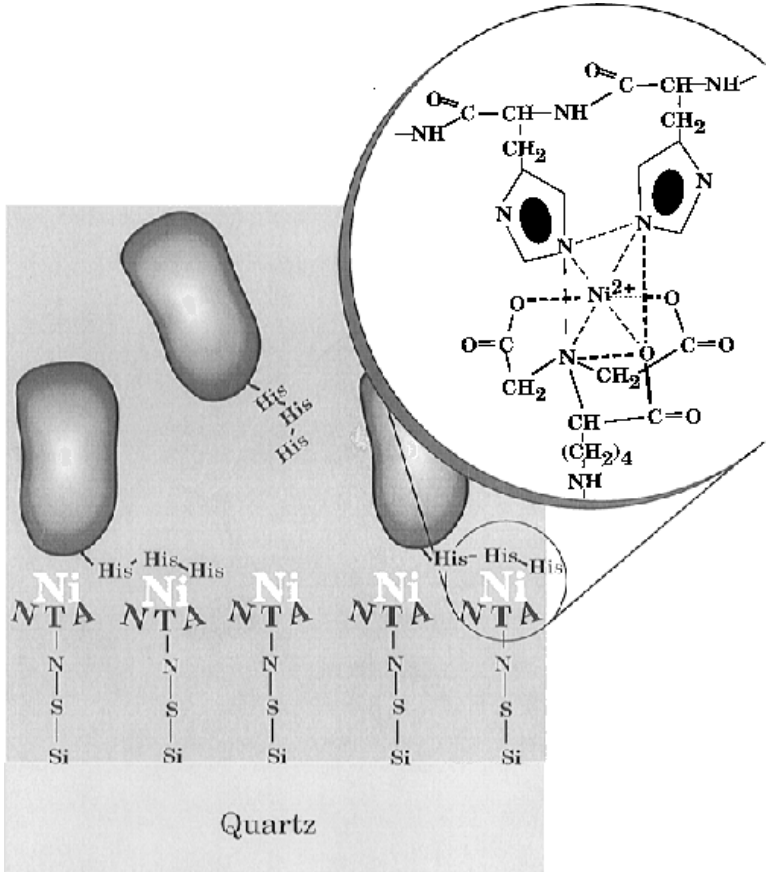
µM	• His-tag - NTA
nM	• Antibody - antigen
pM	• Streptavidin - biotin
irreversible	• Covalent bond





His tag / Ni:NTA

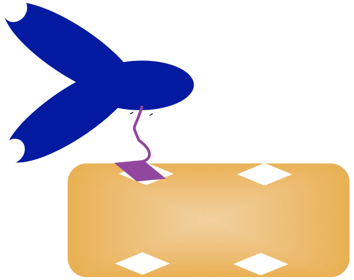
- How to tune the interaction?
  - Reversible
    - Imidazole
    - EDTA
  - Density of Ni:NTA motifs at the surface
  - Length of the His tag.



+ reversibility
  control of the orientation
  quasi universal
- Stability of the surface

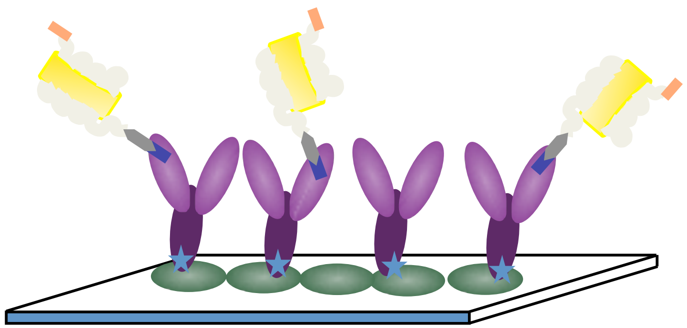
Antigen / Antibody

- Strong interactions
    - $10^{-9} – 10^{-10}$ M
    - Specific
    - Reversible



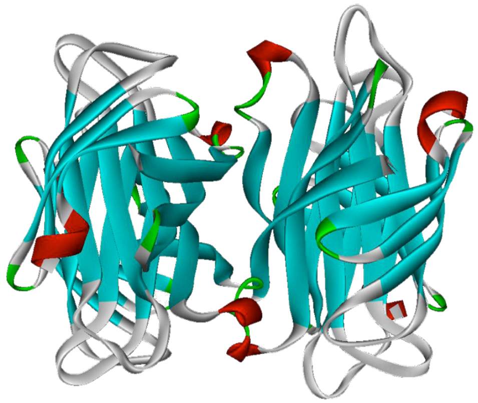
Orientation can be controlled?
- Protein A & protein G
  interact specifically with Fc fragments of IgG Ab
- biotinylated Ab

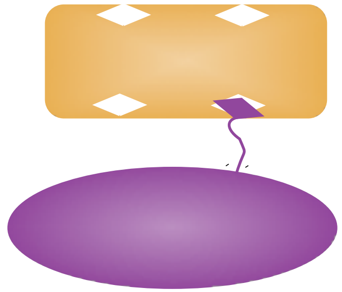
+ reversibility
  control of the orientation?
- control of the orientation?
  Stability of the surface
  complex surface engineering



Biotin / Streptavidin

- Streptavidin:
  4 identical subunits
  symmetrical protein
  60kDa





- Several analogs:
  - Streptavidin: pI = 5
  - Avidin: pI = 10
  - Neutravidin: pI = 7

+ Very stable surface
  Geometry ideal for self-assembly
- How to reverse the system?

How do surfaces influence protein function?

It might influence the protein function

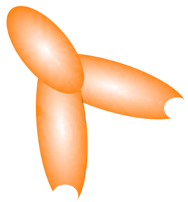
It alters binding capability of the protein modified surfaces

It might contribute to the low specificity of the signal
i.e. sensitivity of the assay

How do surfaces influence protein function?

Antibodies:

- usually adsorbed randomly
- specific orientation:
  - increases up to 10 times the binding capacity
  - increases the % of Ab active



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Optimizing antibody immobilization strategies for the construction of protein microarrays

Paul Peluso,[1] David S. Wilson,[1] Duc Do, Huu Tran, Maanasa Venkatasubbaiah, David Quincy, Bettina Heidecker, Kelli Poindexter, Neil Tolani, Michael Phelan, Krista Witte, Linda S. Jung, Peter Wagner, and Steffen Nock*

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Received 20 May 2002

Abstract

Antibody microarrays have the potential to revolutionize protein expression profiling. The intensity of specific signal produced on a feature of such an array is related to the amount of analyte that is captured from the biological mixture by the immobilized antibody (the "capture agent"). This in turn is a function of the surface density and fractional activity of the capture agents. Here we investigate how these two factors are affected by the orientation of the capture agents on the surface. We compare randomly versus specifically oriented capture agents based on both full-sized antibodies and Fab′ fragments. Each comparison was performed using three different antibodies and two types of streptavidin-coated monolayer surfaces. The specific orientation of capture agents consistently increases the analyte-binding capacity of the surfaces, with up to 10-fold improvements over surfaces with randomly oriented capture agents. Surface plasmon resonance revealed a dense monolayer of Fab′ fragments that are on average 90% active when specifically oriented. Randomly attached Fab's could not be packed at such a high density and generally also had a lower specific activity. These results emphasize the importance of attaching proteins to surfaces such that their binding sites are oriented toward the solution phase.

Keywords: Antibody immobilization; Oriented binding; Fab; Protein array; Streptavidin

Peluso 2002 Analytical Biochemistry

How do surfaces influence protein function?

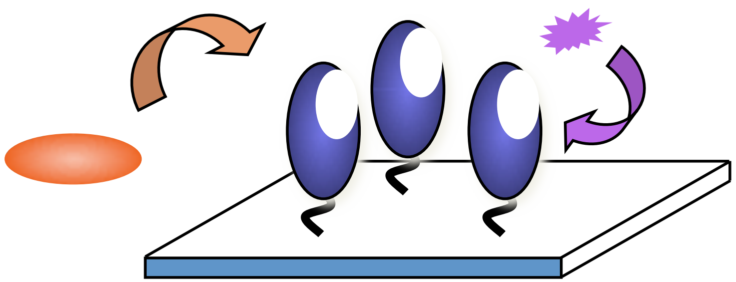
Enzymes:

- Immobilization of enzyme via biotin tag:
  - 65% increase of antibody accessibility
  - 24 times increase of the catalytic activity

  Holland-Nell et al. 2007 ChemBiochem

- Immobilization of enzyme via His tag:
  - 70% increase of antibody accessibility
  - 5 times increase of the catalytic activity

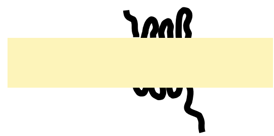
  Cha et al. 2005 Proteomics



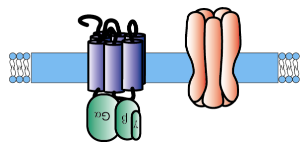
Immobilization of membrane proteins

Various environments for various level of complexity

Isolated domain

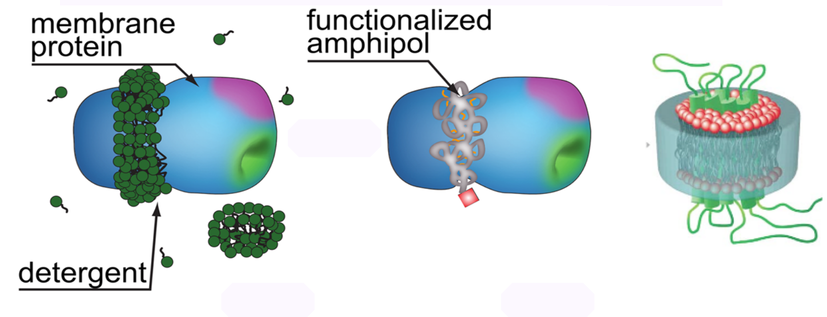


artIficial lipid bilayer



Supported-Cell Membrane Sheets

Isolated functional full length membrane protein



Bottlenecks:

- Overexpression of MPs
- Preserved function
- Accessibility

Modification of the protein or its surrounding

How are MP immobilized?

- Non specific immobilization
  - Spotted membranes
  - Supported Cell Membrane Sheets
- Via lipids
  - Planar membranes
  - Vesicles
- Via tags
  - In native membranes
  - In detergent
  - In Nanodiscs
  - In apols

Native membranes

Non specifically adsorbed membranes

Oriented immobilization of the membranes

- Supported Cell-Membrane Sheets
- Lipid tethered membranes
- Tagged membrane proteins

Protein arrays for GPCRs

- Technique:
  - Spotted membranes
  - Modified glass slides with aminopropylsilane / Porous glass slides
- Monitoring of ligand binding and G protein interactions

Fang *et al.* 2002 JACS
Hong *et al.* 2005 JACS

J|A|C|S COMMUNICATIONS
Published on Web 02/26/2002

Membrane Protein Microarrays

Ye Fang, Anthony G. Frutos, and Joydeep Lahiri*
Biochemical Technologies, Science and Technology Division, Corning Incorporated, Corning, New York 14831
Received October 22, 2001

This paper describes the fabrication of arrays of G protein-coupled receptors (GPCRs)[1] and assays for screening of ligands on these arrays. The attractiveness of obtaining large amounts of bioinformation[2] using extremely small volumes of samples has inspired the extension of DNA microarray technology to proteins.[3] There are two important reasons for the complementary development of protein microarrays: (i) analysis of protein expression from mRNA levels using DNA microarrays is prone to artifacts and does not provide information regarding posttranslational modifications; (ii) proteins are the molecular entities that bind drugs; hence, the analysis of protein−drug interactions provides direct information about compound design and selectivity. Although there have been several reports on protein microarrays,[4] there are no reports describing membrane protein arrays and their use for ligand screening.[5] Membrane-bound proteins represent the single most important class of drug targets—approximately 50% of current molecular targets are membrane-bound.[6] Therefore, the lack of microarray methods for membrane proteins is viewed as a fundamental limitation of protein microchip technology.

Arraying membrane proteins requires printing mixtures of the protein and associated lipids, which in turn warrants appropriate surface chemistry for the immobilization of lipids. We investigated the structure and properties of supported lipids on several surfaces and found that surfaces modified with γ-aminopropylsilane (GAPS)[7]

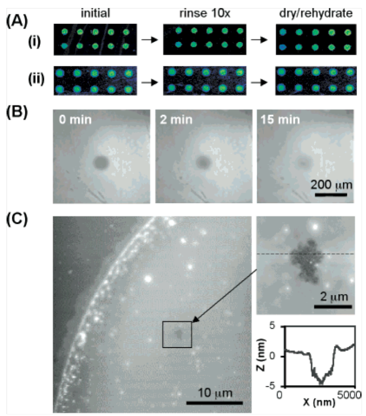
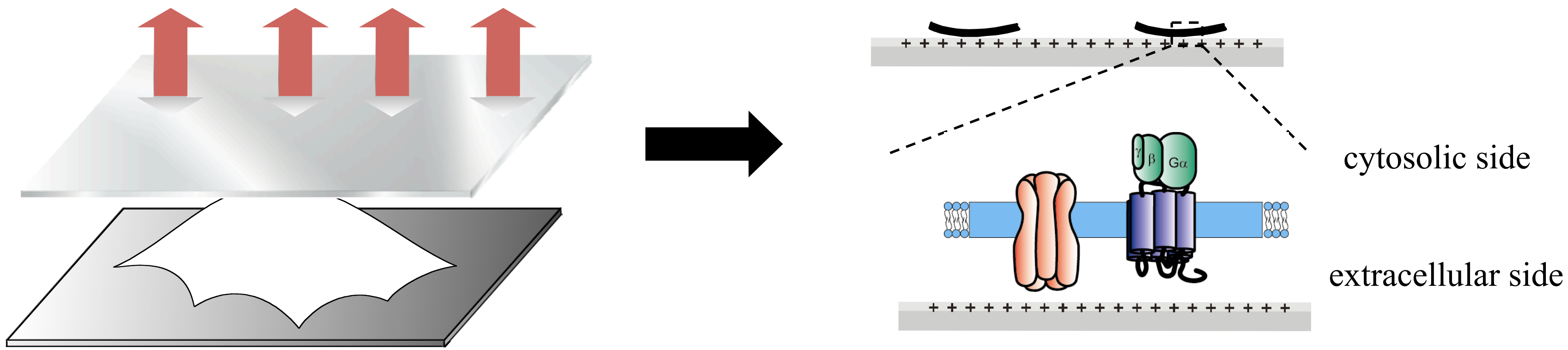


Figure 1. Characterization of model lipid arrays. (A) In situ fluorescence images of microarrays of (i) DPPC/DMPC (4:1 mol ratio) lipids and (ii) egg-yolk PC lipids, doped with TR-DHPE (1%, mol %) on GAPS slides.

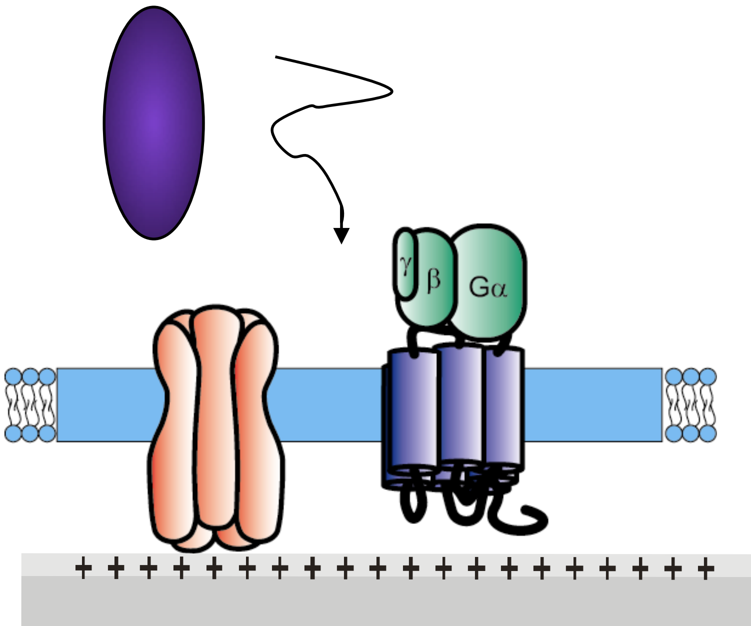
Native membranes: SCMS

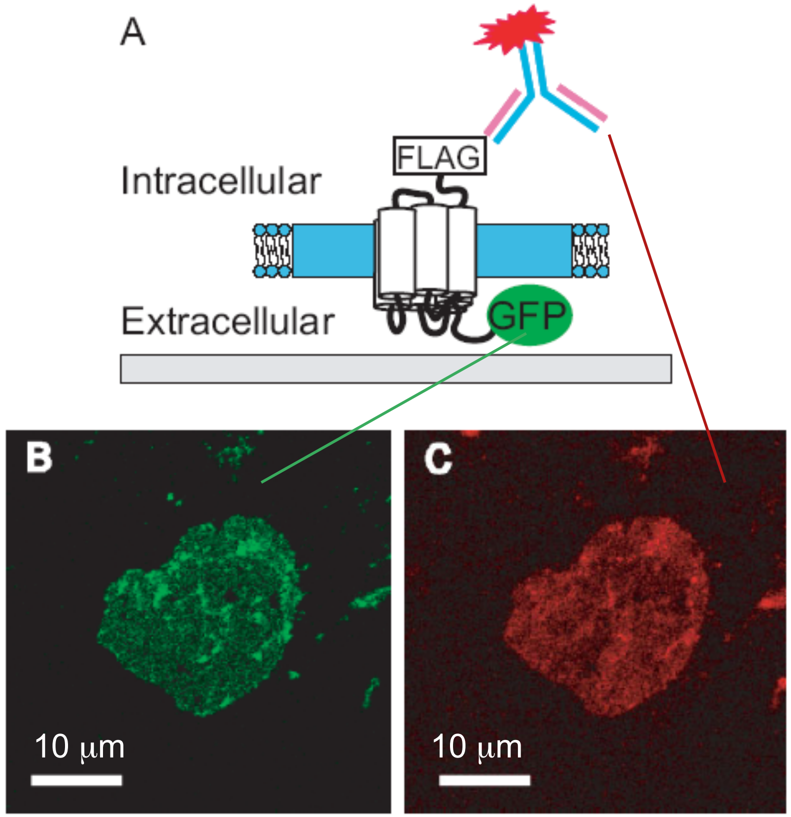
Supported cell-membrane Sheets
- intact hydrophobic environment
- Native protein composition



J.-B. Perez et al., *Adv. Func. Mat.,* 2006,16 (2): 306-312

Orientation : cytoplasmic leaflet accessible



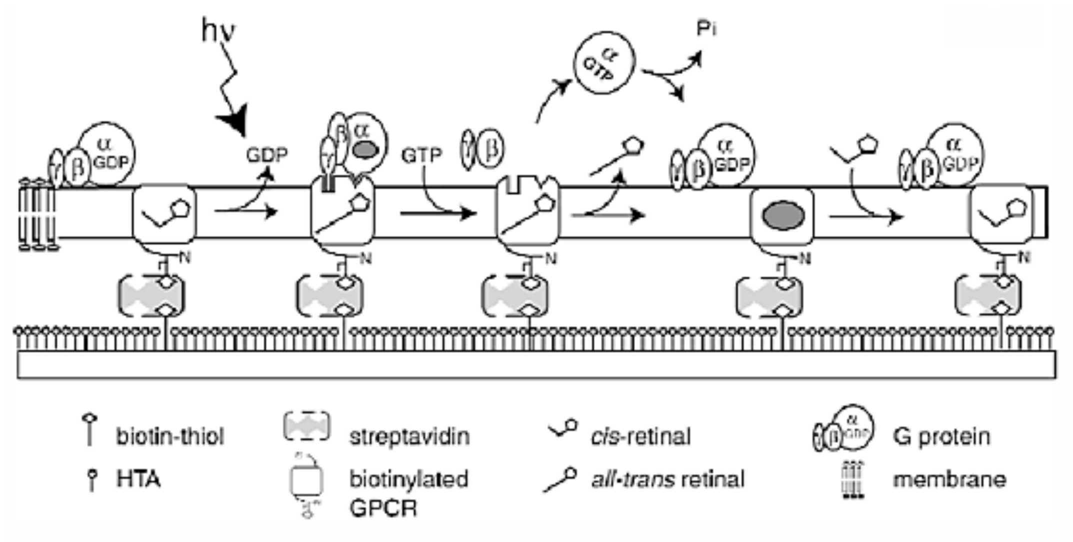


Accessibility to the inner leaflet, single bilayer

Controled orientation of the membrane

J.-B. Perez et al. (2006) *Adv. Func. Mat.* 16 (2): 306

Monitoring of rhodopsin by SPR

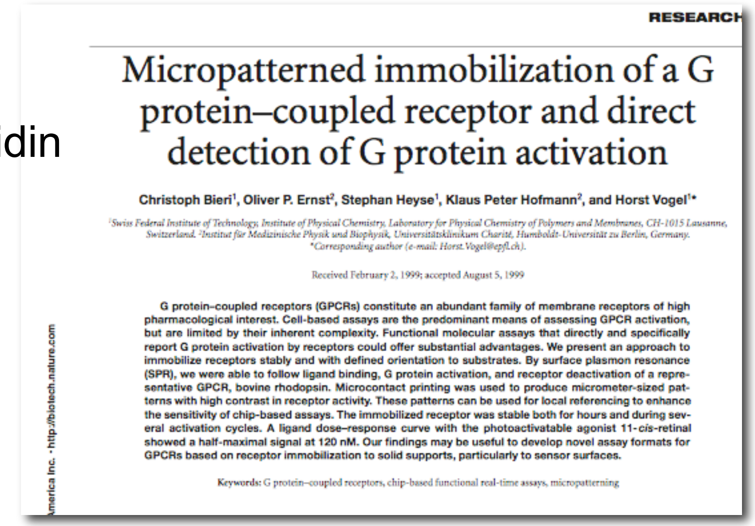


Chemical modification of the Rhodopsin

Native membranes

Patterns of streptavidin using μcontact printing of streptavidin

Bieri et al. Nature Biotech 1999



RESEARCH

Micropatterned immobilization of a G protein–coupled receptor and direct detection of G protein activation

Christoph Bieri[1], Oliver P. Ernst[2], Stephan Heyse[1], Klaus Peter Hofmann[2], and Horst Vogel[1*]

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G protein–coupled receptors (GPCRs) constitute an abundant family of membrane receptors of high pharmacological interest. Cell-based assays are the predominant means of assessing GPCR activation, but are limited by their inherent complexity. Functional molecular assays that directly and specifically report G protein activation by receptors could offer substantial advantages. We present an approach to immobilize receptors stably and with defined orientation to substrates. By surface plasmon resonance (SPR), we were able to follow ligand binding, G protein activation, and receptor deactivation of a representative GPCR, bovine rhodopsin. Microcontact printing was used to produce micrometer-sized patterns with high contrast in receptor activity. These patterns can be used for local referencing to enhance the sensitivity of chip-based assays. The immobilized receptor was stable both for hours and during several activation cycles. A ligand dose–response curve with the photoactivatable agonist 11-*cis*-retinal showed a half-maximal signal at 120 nM. Our findings may be useful to develop novel assay formats for GPCRs based on receptor immobilization to solid supports, particularly to sensor surfaces.

Keywords: G protein–coupled receptors, chip-based functional real-time assays, micropatterning

Native membranes: advantages & inconvenients

Advantages

- natural environment
- native stoichiometry of proteins
- sample preparation
- no need to modify the protein of interest

Inconvenients

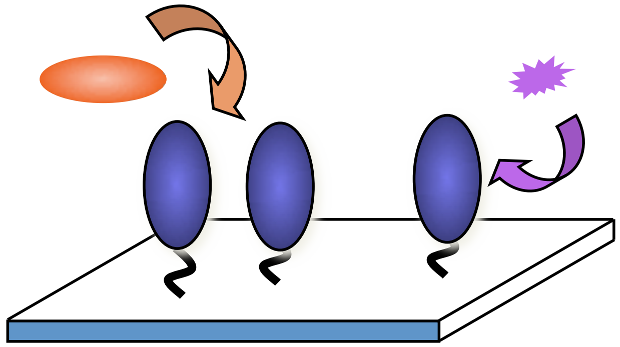
- function?
- accessibility to both sides of the membranes?
- quantity of membrane protein of interest?

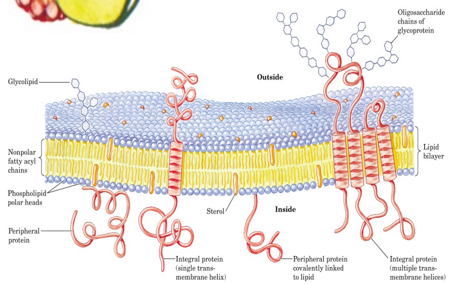
  Limited sensitivity & specificity of the signal…

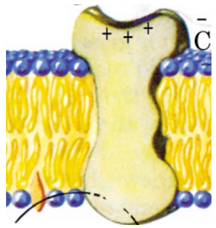
Immobilization of membrane proteins

Handling membrane proteins on surfaces

- Native lipid membrane
- Detergent
- Reconstituted artificial lipids
    - lipid vesicles
    - planar membranes
    - Nanodiscs
- amphipols





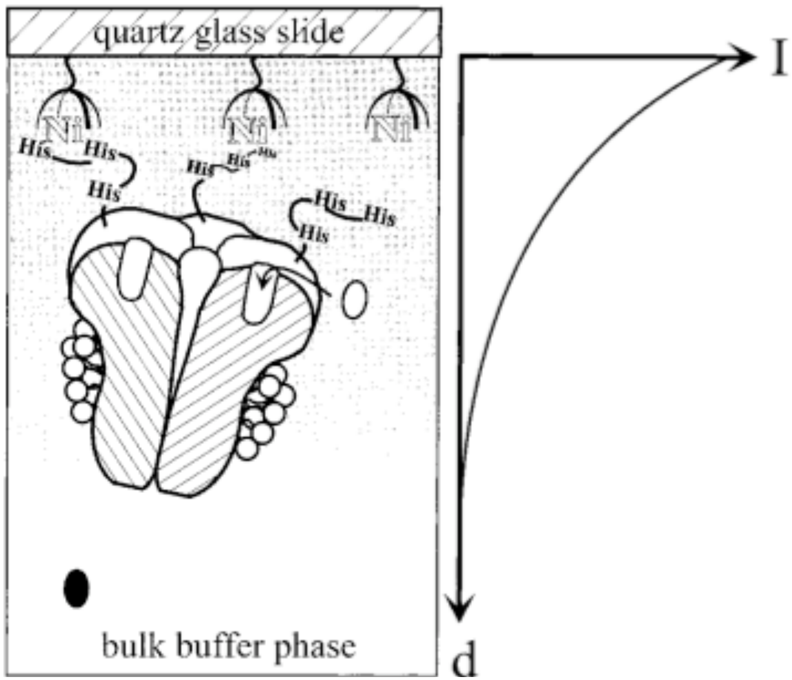


Modification of the protein or its surrounding

Detergent solubilized membrane protein

5HT3R – ligand gated ion channel

- Immobilization on glass surfaces via Ni:NTA
- Total internal reflection fluorescence
- Monitoring of ligand binding

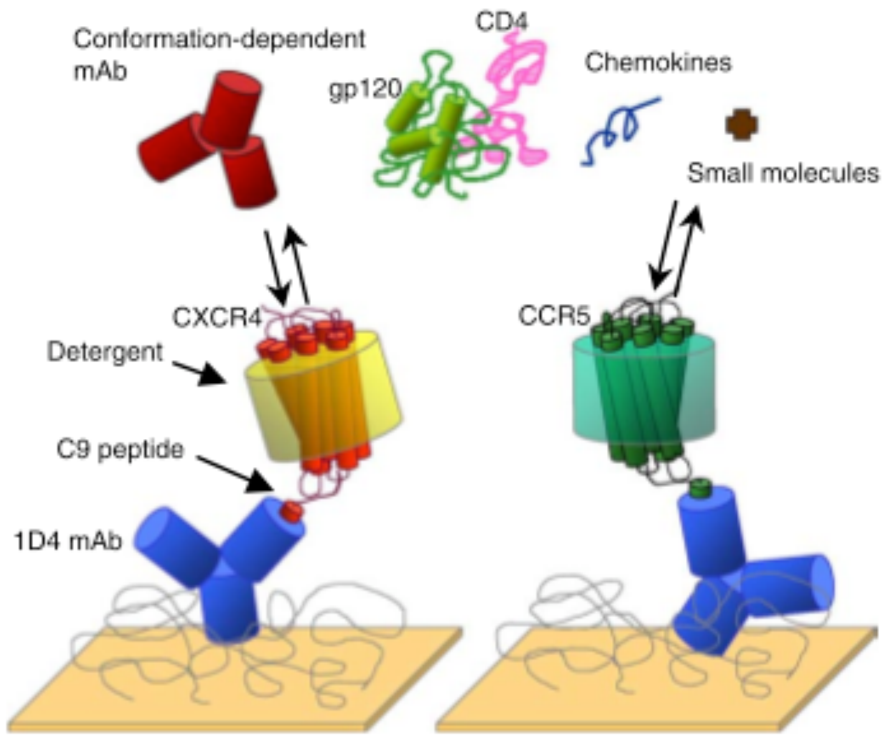


Schmid et al. 1998 Analytical Chemistry

Detergent solubilized membrane protein

GPCR

- Immobilization on SPR chip via antibodies
- Check conformation using antibodies
- Screen solubilization conditions
- Monitoring of ligand binding



Navratilova *et al.* 2006 Analytical Biochemistry

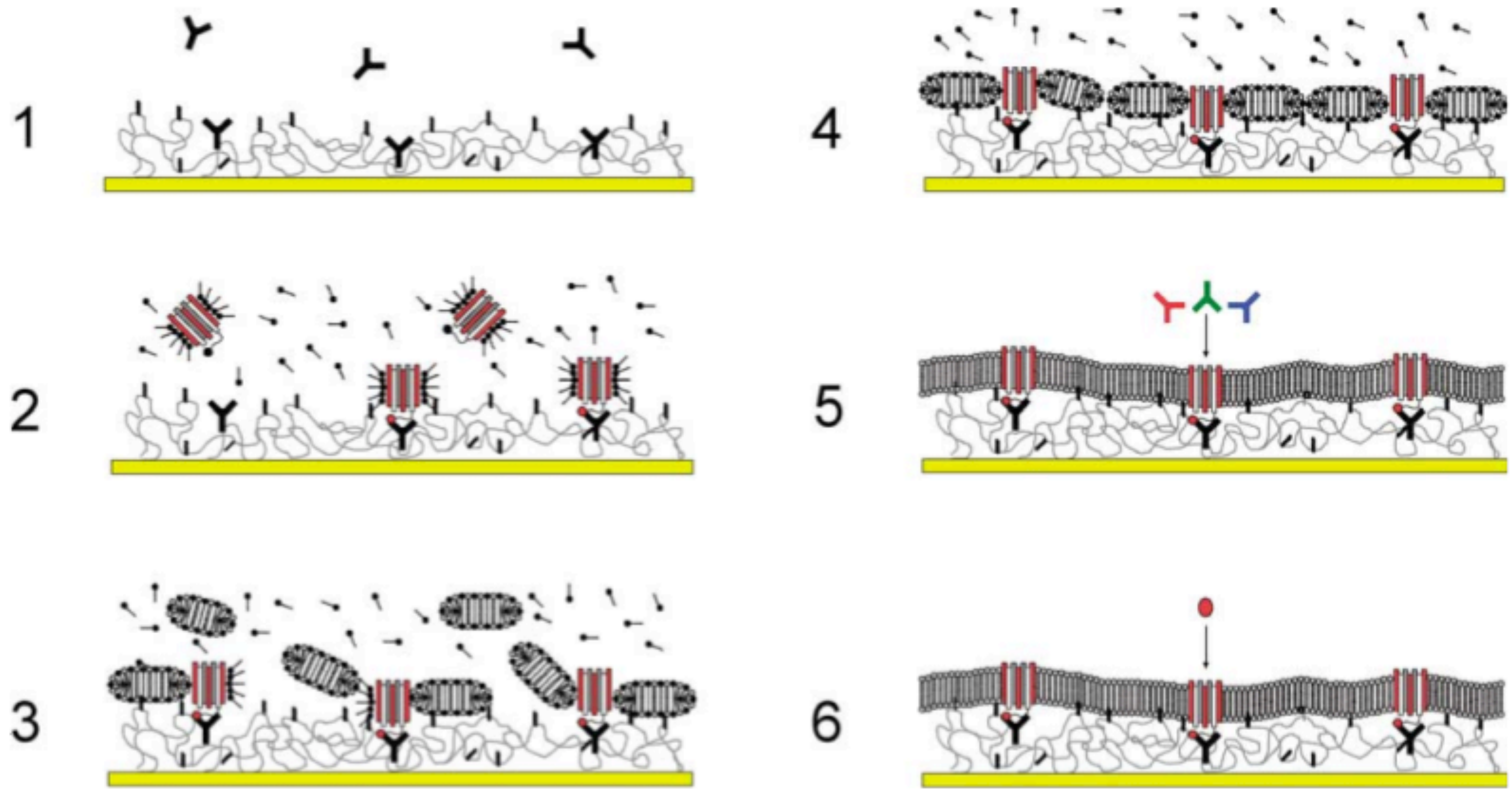
Membrane proteins in detergent

- Advantages
  - Control of the quantity of protein (better sensitivity)
  - Can be immobilized on the surface or interact with an immobilized ligand
- Inconvenients
  - Modification of the protein required
  - Function of the protein in detergent?

Reconstitution solubilized membrane protein

GPCR

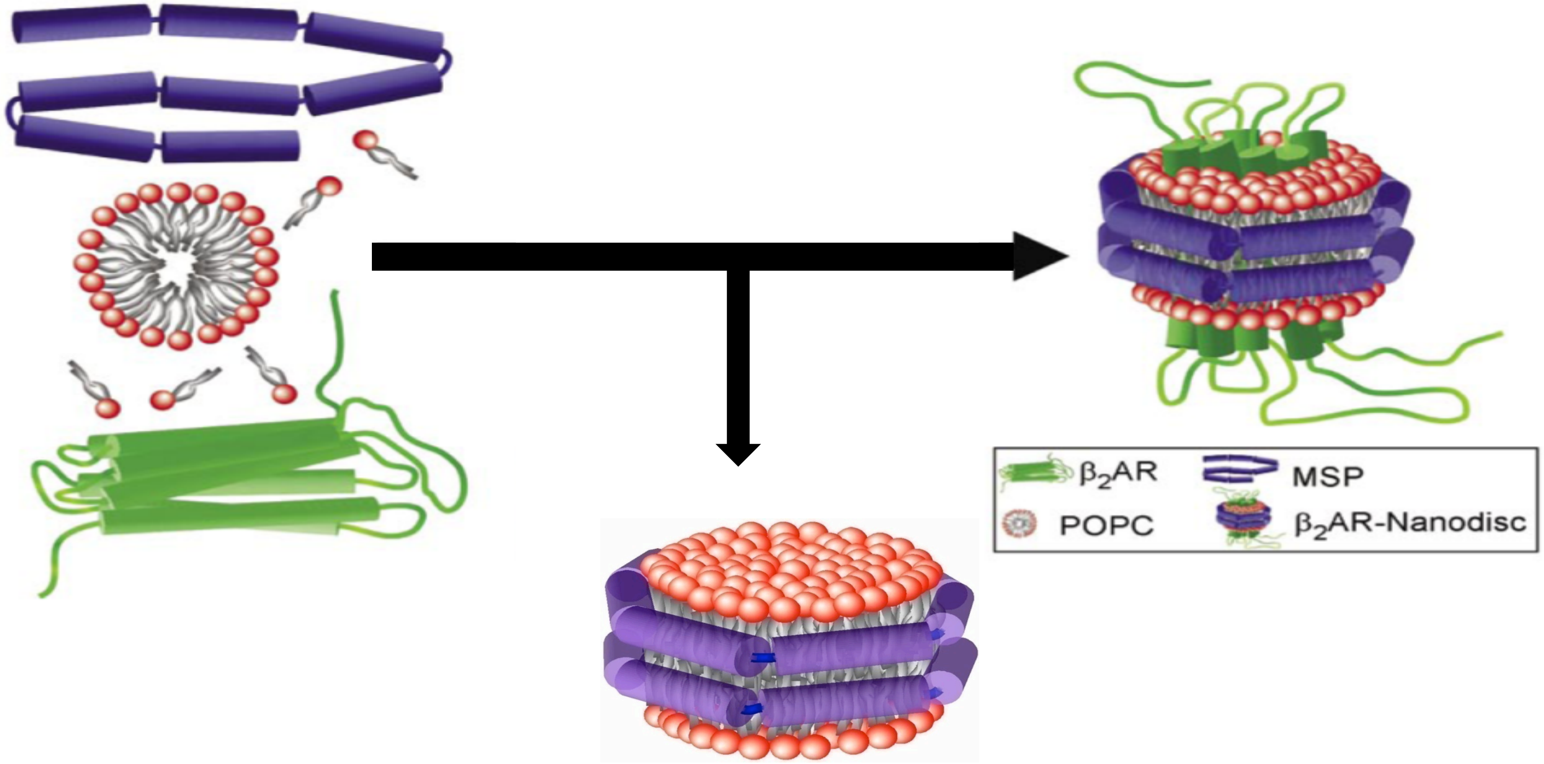
- Immobilization on SPR chip via antibodies



Stenlund *et al.* 2003 Analytical Biochemistry

Membrane proteins in nanodiscs

- Self-assembled soluble discoidal phospholipids bilayers
- Formed by an amphipatic protein belt

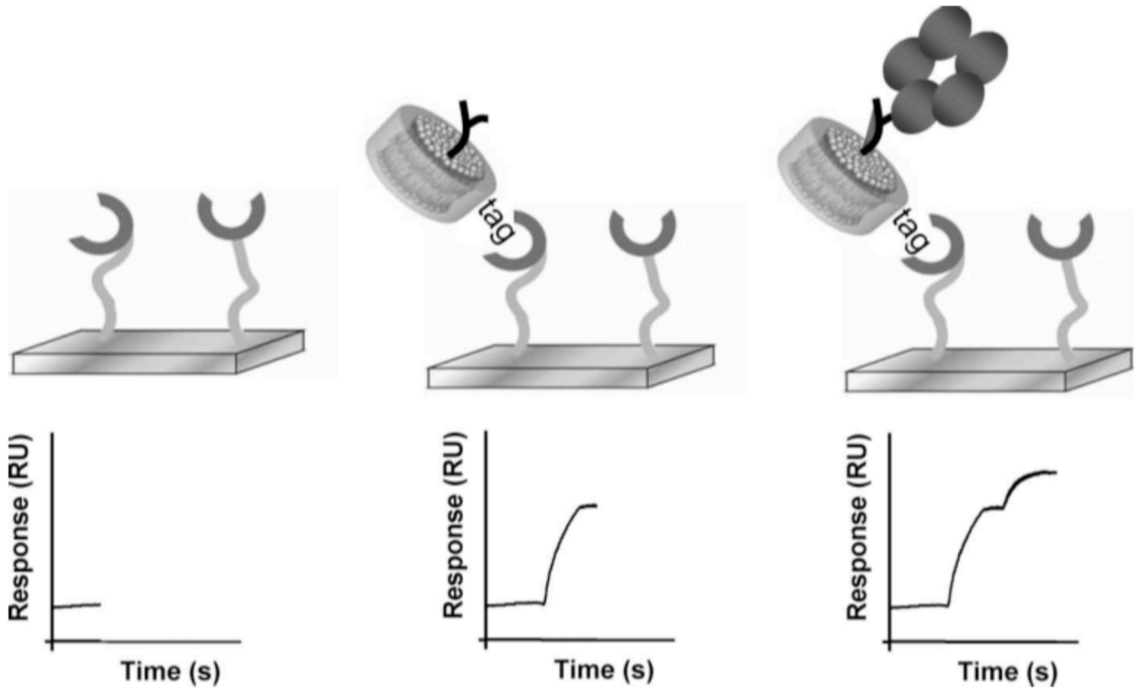


Nath et al. Biochemistry 2007 for review

Membrane proteins in nanodiscs

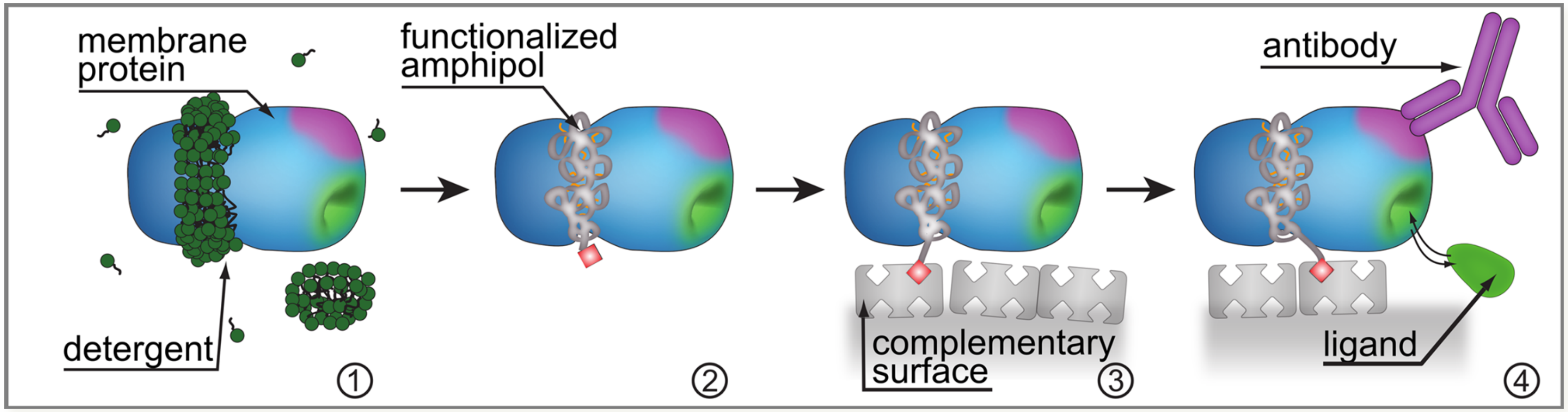
SPR investigations using:

- Ni-NTA immobilization of Histag MSP
- Antibody immobilization of Histag MSP
- Antibody immobilization of FLAG tag MSP



Borch et al. Analytical chemistry 2008

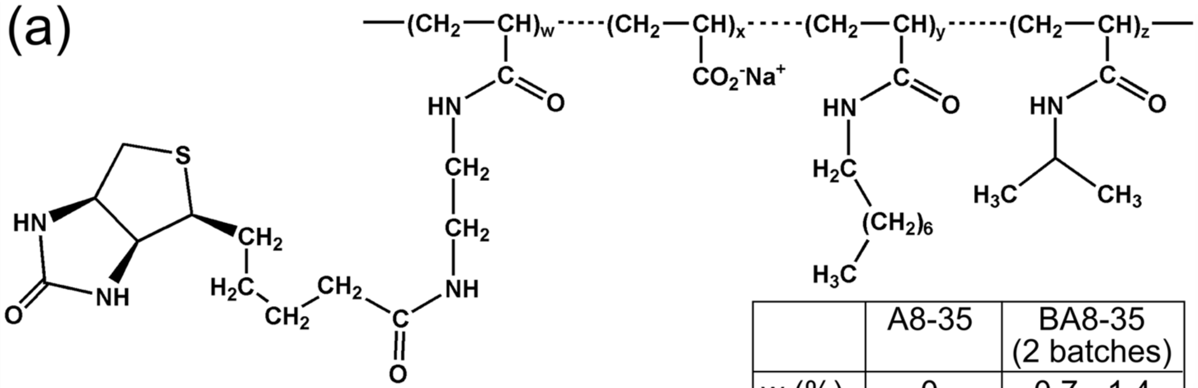
Immobilization of nAChR using biotinylated amphipols



biotinylated A8-35
("BAPol")

~0.5 or ~1 biotin/A8-35 molecule

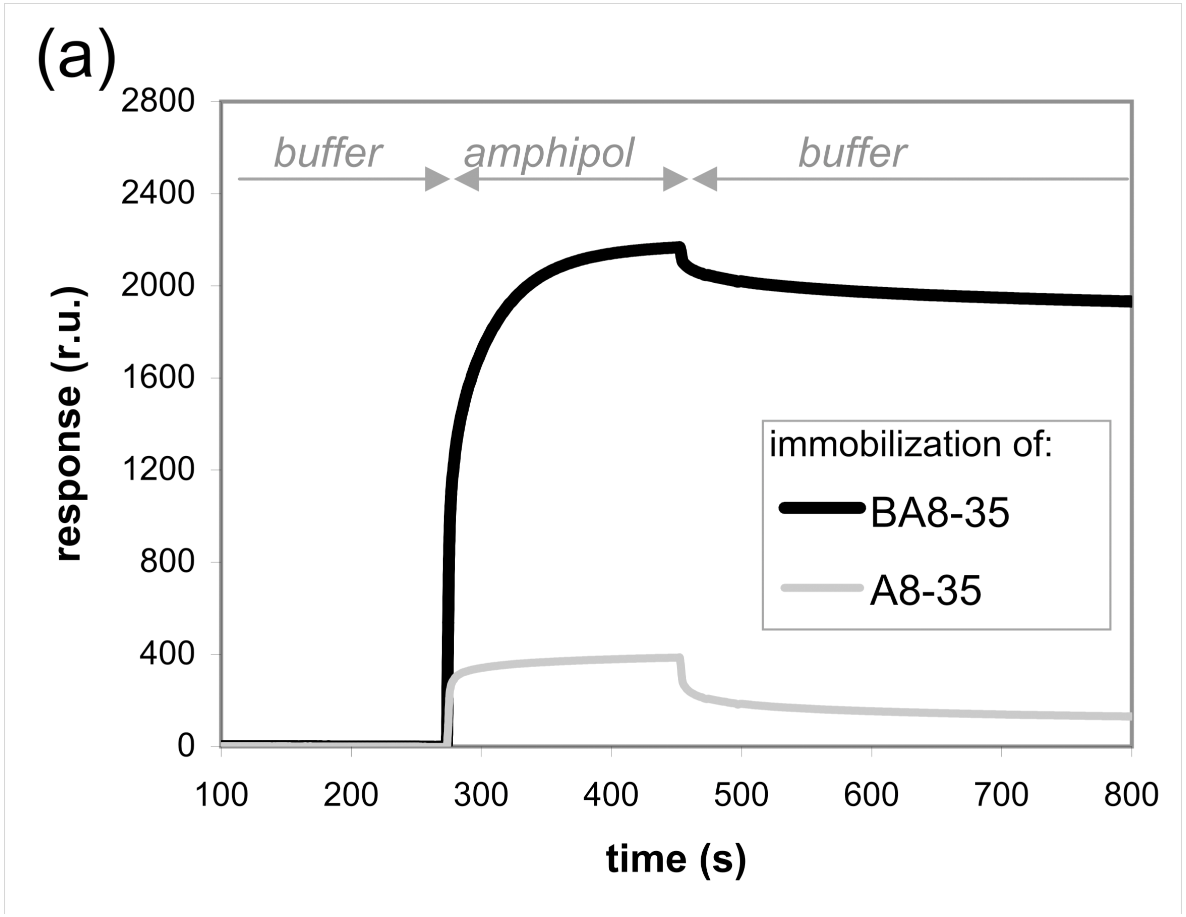
(a)



	A8-35	BA8-35 (2 batches)
w (%)	0	0.7 - 1.4
x (%)	37	30 - 35
y (%)	25	21 - 25
z (%)	36	36 - 41

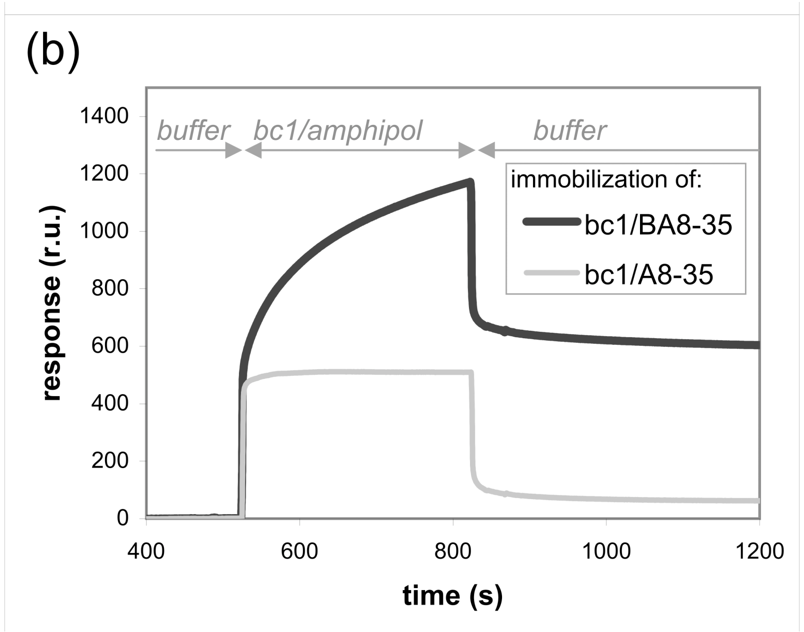
Coll. Jean-Luc Popot, CNRS, FR

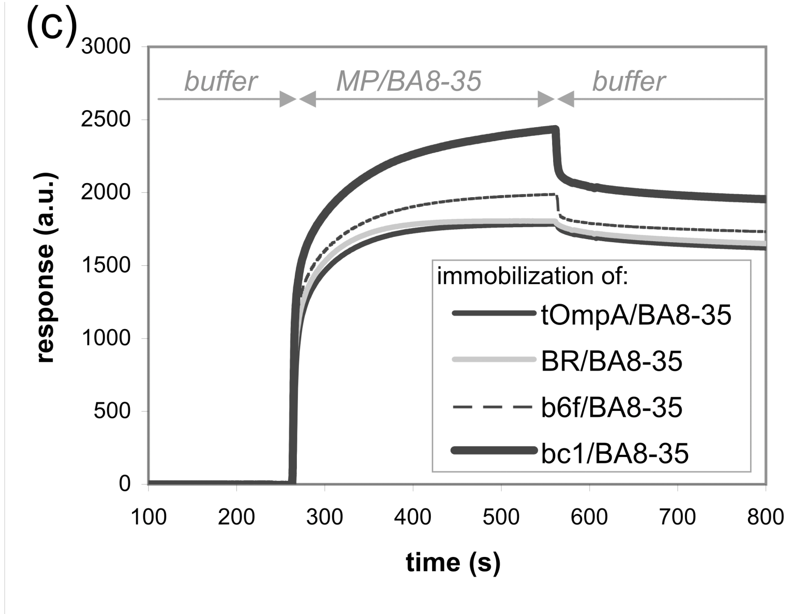
Specific interaction of the BAPols



Charvolin, Perez et al., PNAS 2009

Specific immobilization of membrane proteins





Charvolin, Perez et al., PNAS 2009

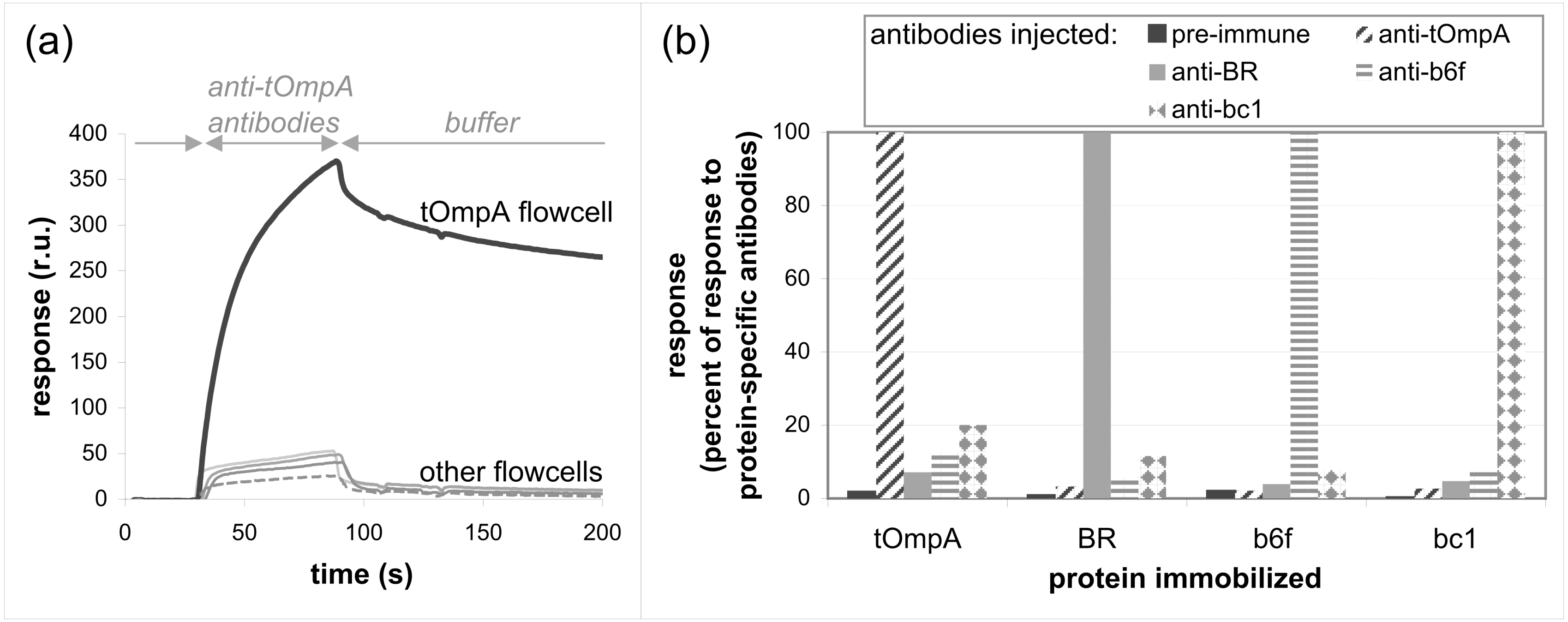
Applications for diagnostics?

- **Targets:** four distinct membrane proteins:

Protein	*M*	Origin	Composition	Structure
tOmpA	19 kDa	eubacterium	1 subunit	β-barrel
bacteriorhodopsin (BR)	27 kDa	archaebacterium	1 subunit	α-helix bundle
cytochrome b_6f	228 kDa	eukaryotic alga	16 subunits	α-helix bundle
cytochrome bc_1	490 kDa	beef mitochondria	22 subunits	α-helix bundle

Charvolin, Perez et al., PNAS 2009

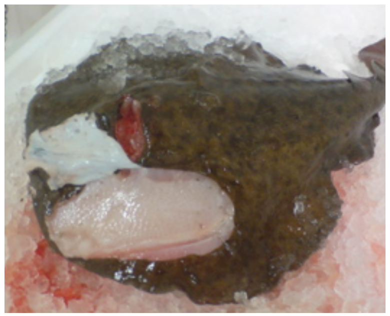
Applications for diagnostics?

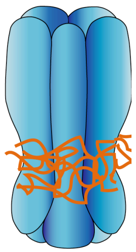


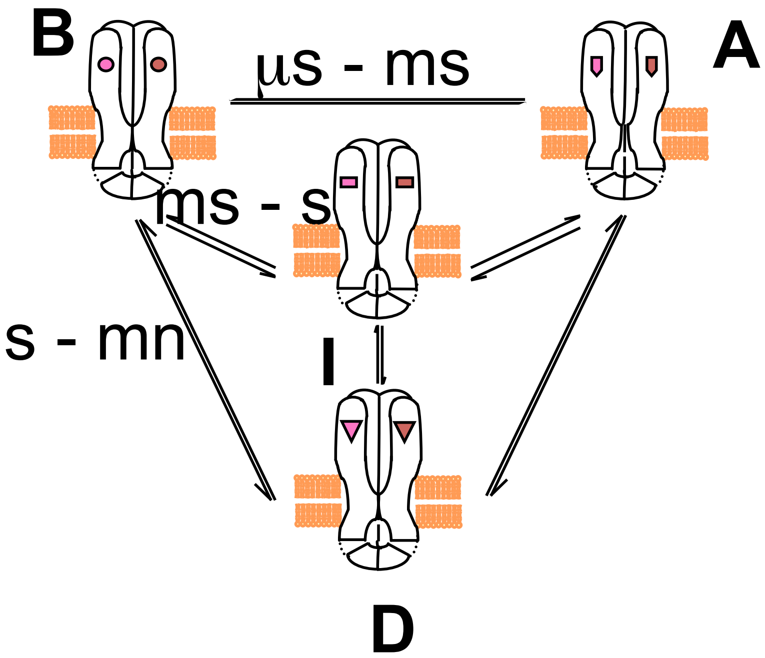
Charvolin, Perez et al., PNAS 2009

Stabilization the nAChR in amphipols

- Protein extracted from *Torpedo Marmorata* electric organs

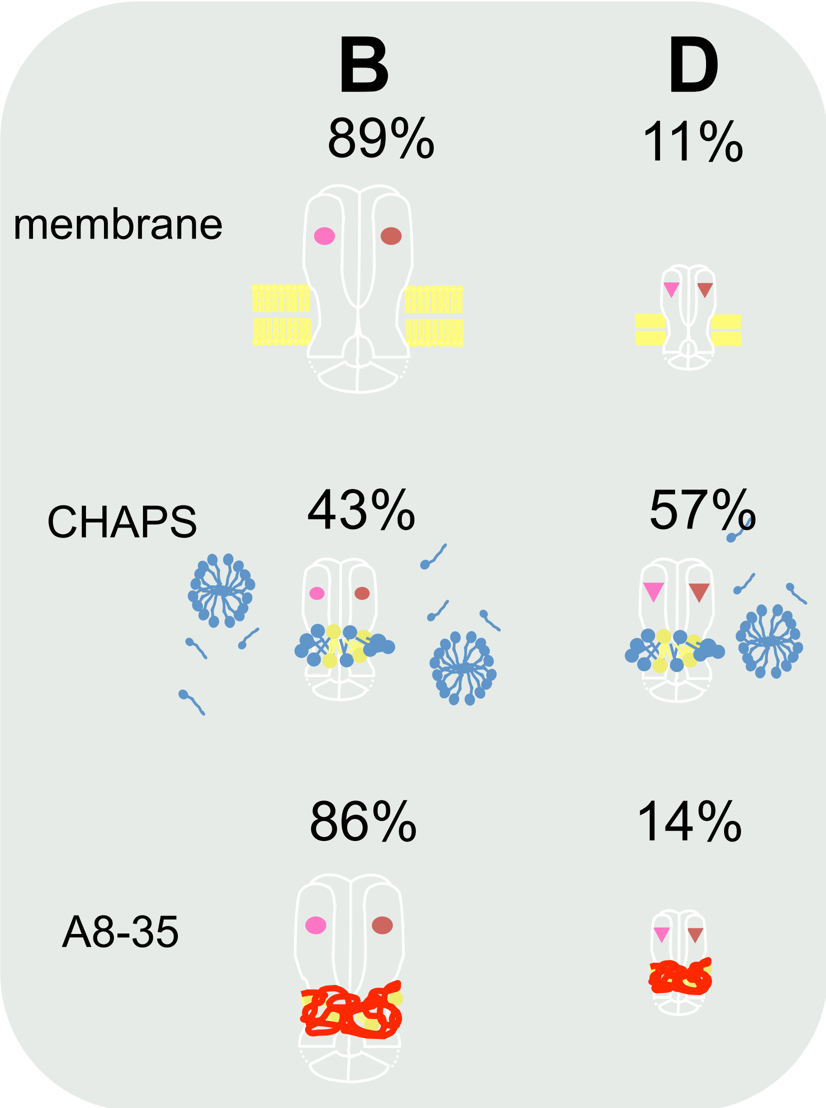


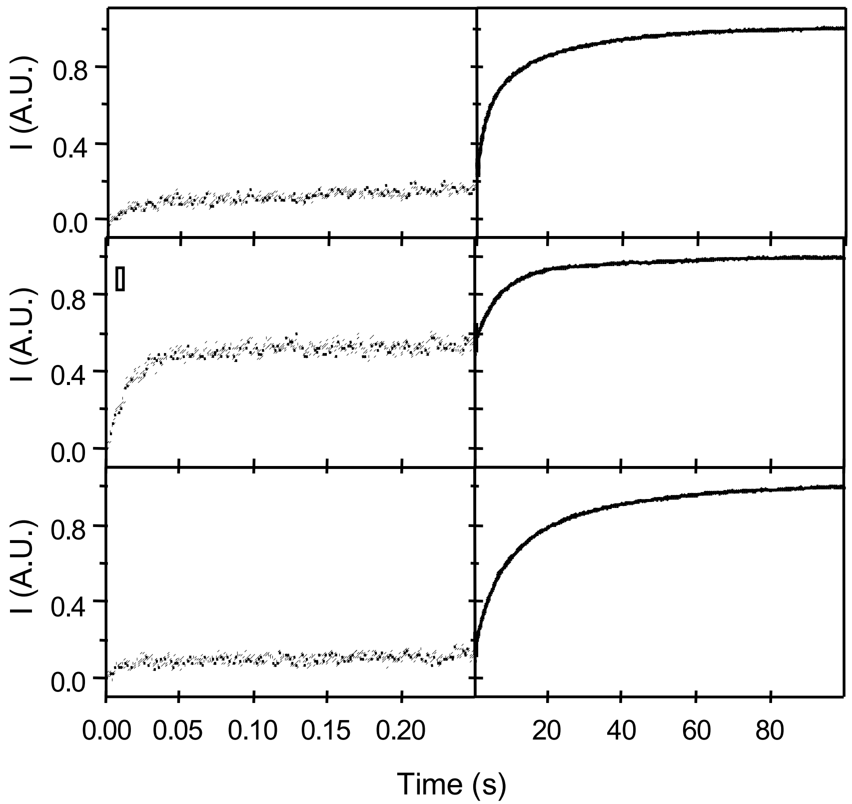




Coll. Jean-Pierre Changeux, Pasteur Institute, FR

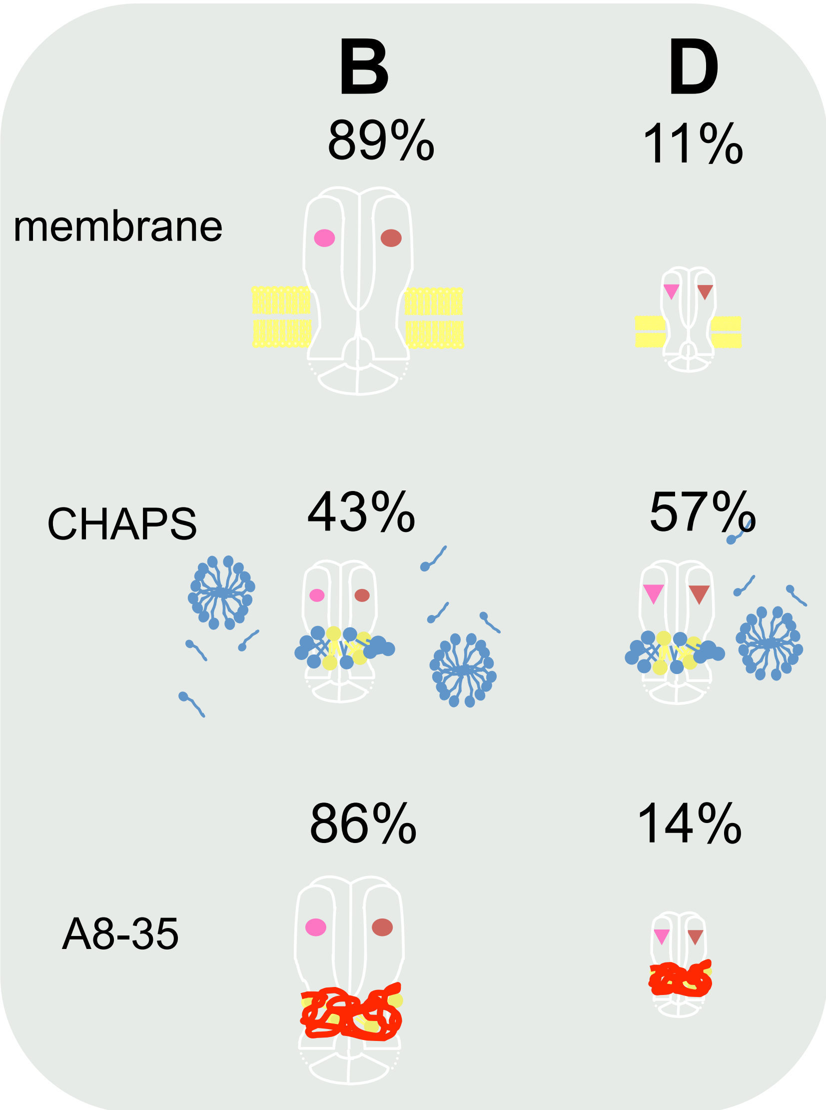
nAChR allosteric transitions maintained in amphipols

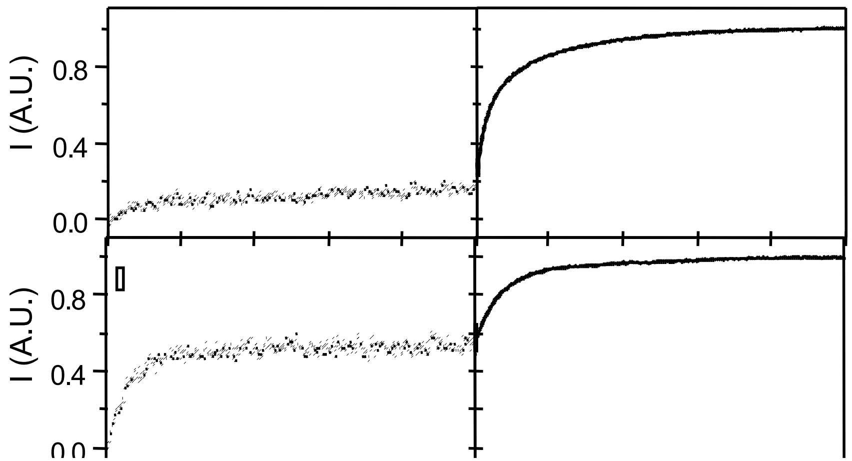




Martinez *et al.* (2002), *FEBS Lett.* **528**:251-256.

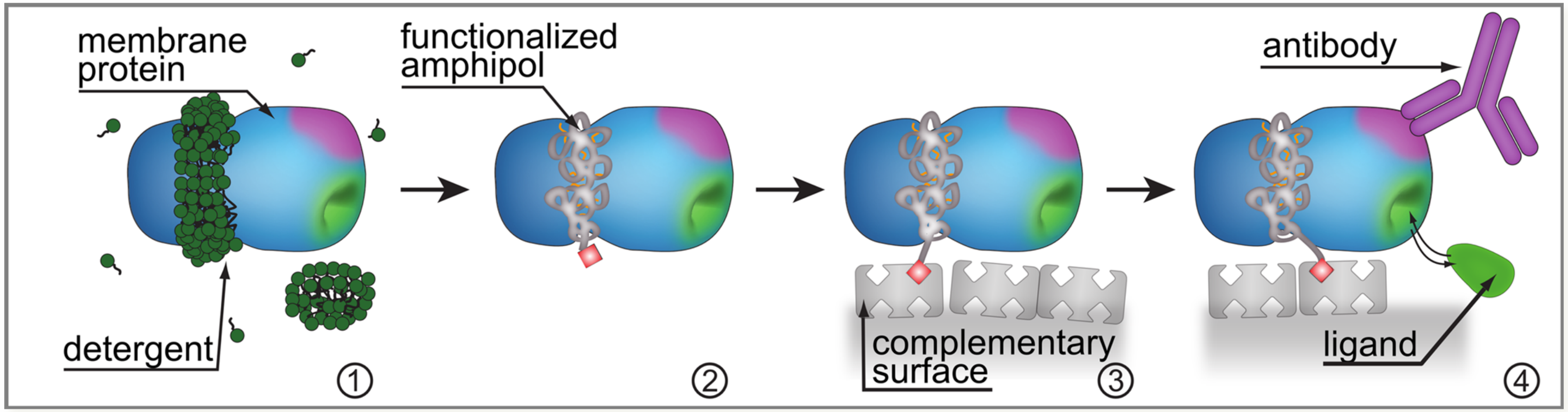
nAChR allosteric transitions maintained in amphipols





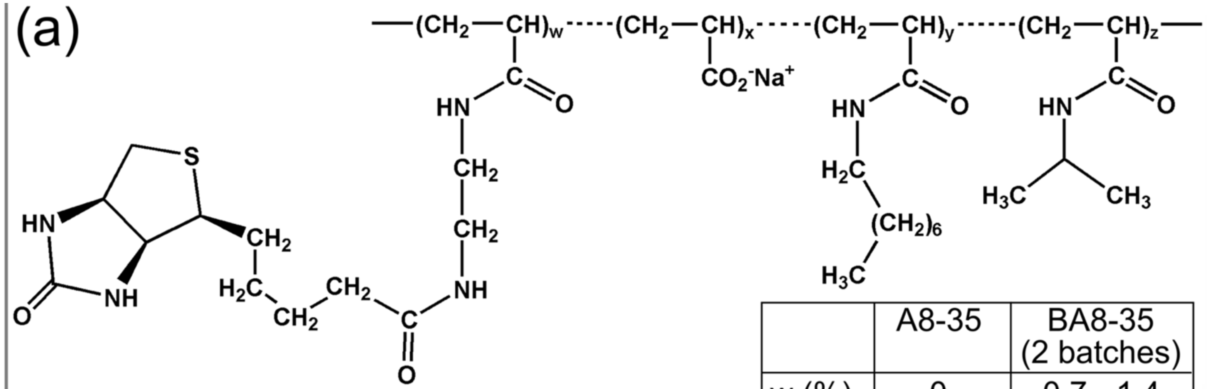
- Functional after trapping in amphipols:
  - binds ligands
  - undergoes conformational changes

Immobilization of nAChR using biotinylated amphipols



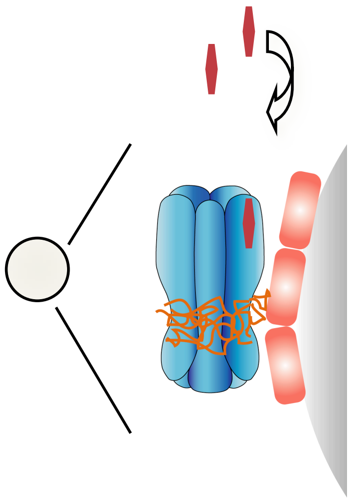
biotinylated A8-35 ("BAPol")

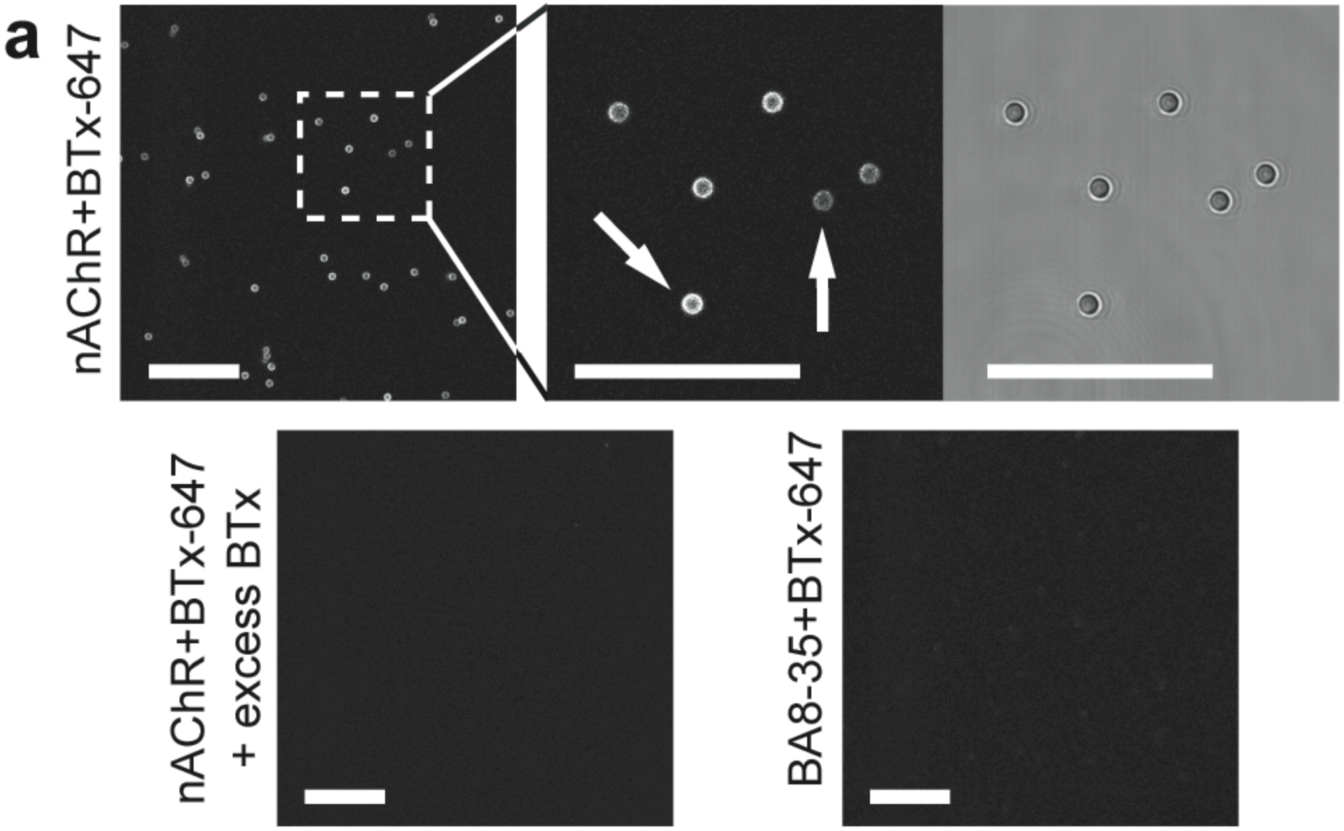
~0.5 or ~1 biotin/A8-35 molecule



	A8-35	BA8-35 (2 batches)
w (%)	0	0.7 - 1.4
x (%)	37	30 - 35
y (%)	25	21 - 25
z (%)	36	36 - 41

Binding kinetics of the toxin to the immobilized nAChR





25 μm

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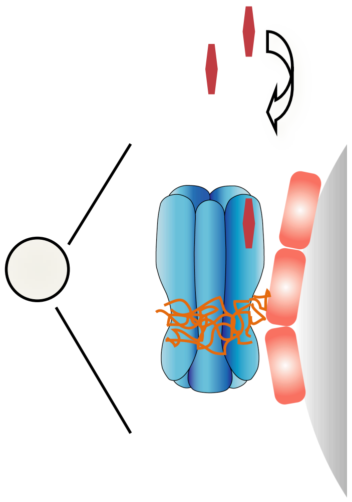
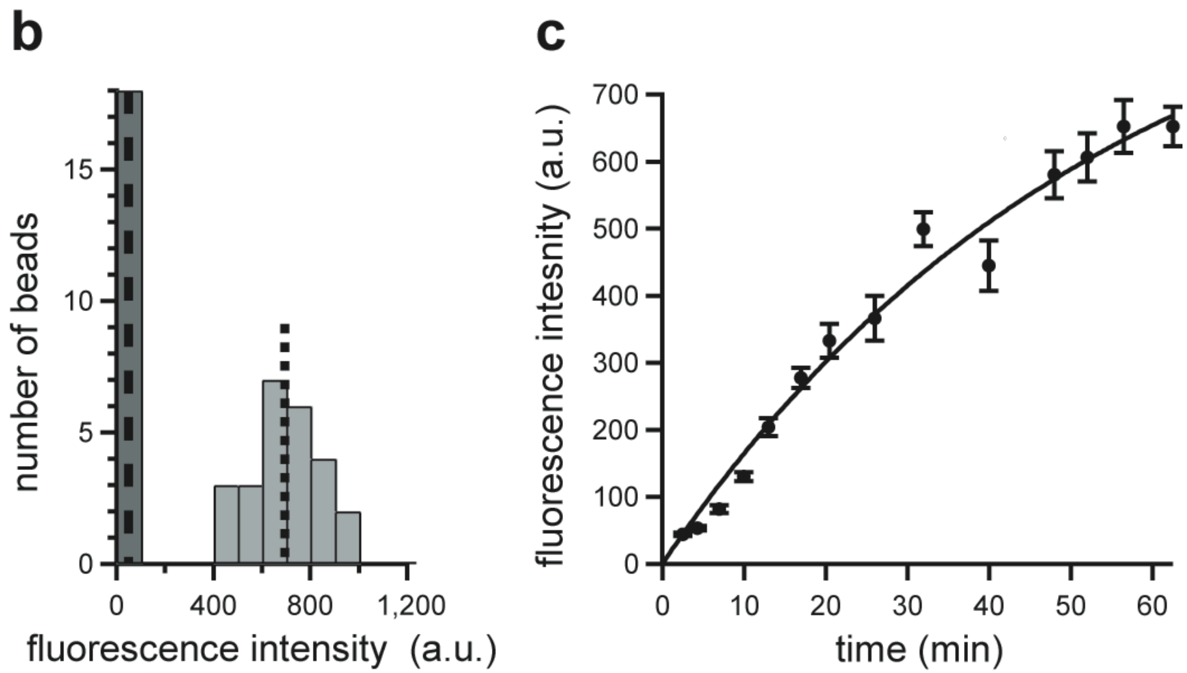
Binding kinetics of the toxin to the immobilized nAChR





Charvolin, Perez et al., PNAS 2009

Conclusion

- isolated and purified protein
- functional proteins
- All of the extramembrane surface is accessible
- Ligand binding measured in aqueous, detergent-free solutions

- Use of specific immobilization
    - biotin tag

- Tag engineered on the apols
    - No need of modification of the protein
    - access to natural proteins

- Tag can be engineered on the protein