# Surface Plasmon Resonance (SPR) Protein interactions

Dr. S. Sadeghi

## SPR historical prospective

Brief History of Surface Plasmons

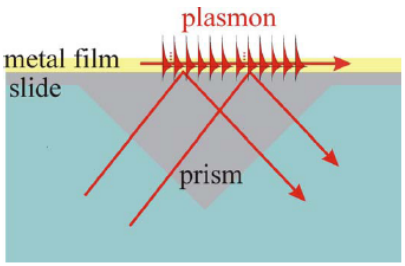
- first observed in 1902 by R. Wood as narrow dark bands in the spectrum of metal gratings
- observed in thin metal films as a drop in reflectivity by Thurbadar in 1958 and explained by Otto, Kretchmann and Raether in 1968.
- 1970s plasmons used to characterize metal films and study processes on the metal surfaces.
- 1990 first commercial SPR (Surface Plasmon Resonance sensor is launched by BIAcore AB.

# Definitions

- PLASMONS: Electromagnetic waves.
- SURFACE PLASMONS: Those plasmons that are confined to surfaces and that interact strongly with light.
- SURFACE PLASMON RESONANCE (SPR): The excitation of surface plasmons by light.
- RESONANCE UNITS (RU): SPR response values are expressed in resonance units (RU).
  - For most proteins, 1.0 RU is equivalent to a change in concentration of about 1 pg/mm$^2$ on the sensor surface.
- SENSORGRAM: A plot of the SPR Response, in RUs, as a function of time.
- ANALYTE: Interactant that is being flowed over the surface in the solution phase.
- LIGAND: Interactant that is immobilised onto the surface.



# SPR

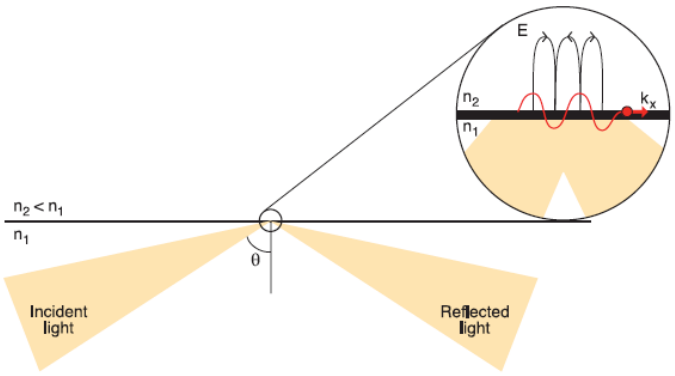



Surface Plasmon Resonance

- an optical method for measuring the refractive index of very thin layers of material adsorbed on a metal.
- In case of e.g. protein-adsorption the difference between the refractive index of the buffer (i.e. water) and the refractive index of the adsorbate can be easily converted into mass and thickness of the adsorbate
  - as all proteins have almost identical refractive indices.
- EVANESCENT-WAVE PHENOMENON
  Total internal reflection of light at a surface–solution interface produces an electromagnetic field, or evanescent wave, extends a short distance (~100–200 nm) into solution.
- ***SPR is an evanescent wave phenomenon that occurs at certain metallic surfaces***.

# Total internal reflection

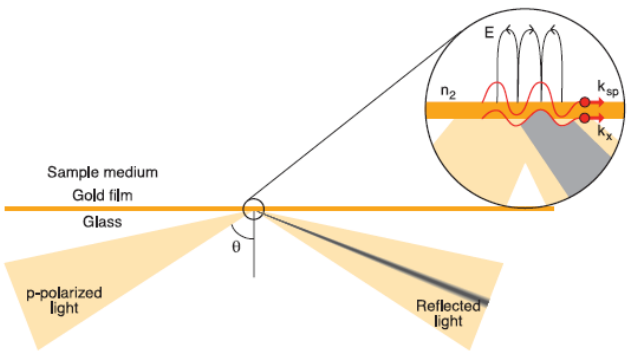
- total internal reflection (TIR) = occurs at an interface between non-absorbing media.
  - When a light beam propagating in a medium of higher refractive index meets an interface at a medium of lower refractive index at an angle of incidence above a critical angle the light is totally reflected at the interface and propagates back into the high refractive index medium



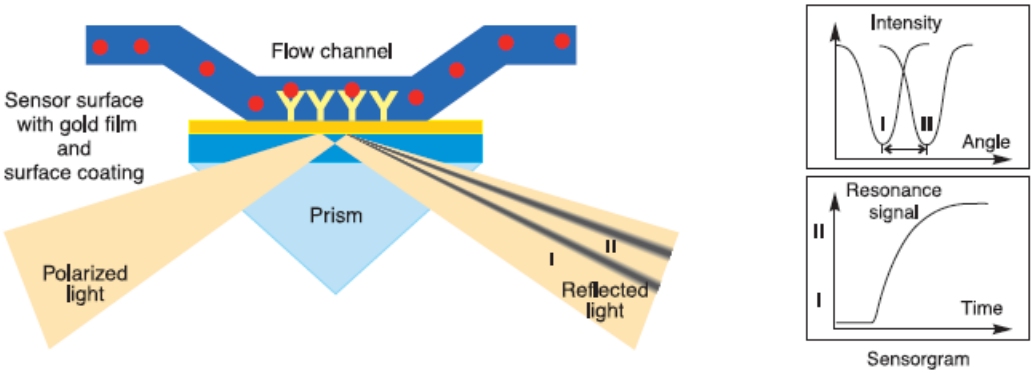

TIR for non-absorbing media.
Light propagating in a medium of refractive index n1 undergoing total internal reflection at the interface with the medium of a lower refractive index n2.
The evanescent field, E, is a non-transverse wave having components in all spatial orientations, decreasing in field intensity with penetration into medium of n2. θ is the angle of incidence.



- Although the fully reflected beam does not lose any net energy across the TIR interface, the light beam leaks an electrical field intensity called an evanescent field wave into the low refractive index medium.
- If the TIR-interface is coated with a layer of a conducting material (metal), of a suitable thickness the evanescent field wave, may penetrate the metal layer and excite electromagnetic surface plasmon waves propagating within the conductor surface that is in contact with the low refractive index medium.



- For a non-magnetic metal like gold, this surface plasmon wave will create an enhanced evanescent wave.
- Biomolecular interactions at the sensor surface change the solute conc + thus the refractive index within the evanescent wave penetration range.
- The angle of incidence required to create the SPR phenomenon (SPR angle) is therefore altered and measured as a response signal.

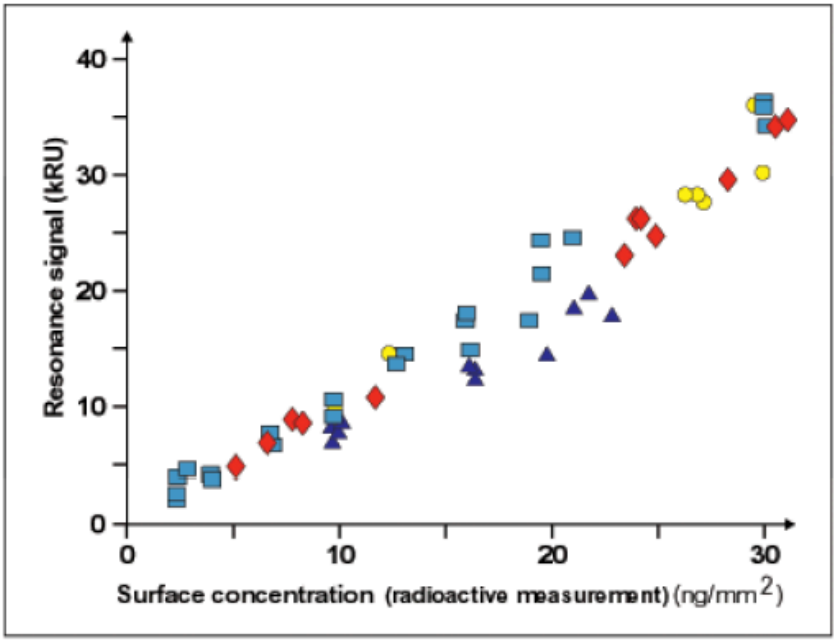





# SPR technique description

- The SPR-technique exploits the fact that at certain conditions, surface plasmons on metallic slabs can be excited by photons, thereby transforming a photon into a surface plasmon.
- The conditions depend on the refractive index of the adsorbate.The most common geometrical setup (*Kretschmann configuration*) is when the incoming light is located on the opposite side of the metalic slab than the adsorbate.
  - This is due to the fact that photons cannot excite surface plasmons on the surface being hit. The photons will however induce an evanescent light field into the metallic slab.
- Normally no transport of photons takes place through this field, but photons incident at a certain angle are able to tunnel through the field and to excite surface plasmons on the adsorbate side of the metallic slab.
- Whenever a plasmon is excited, one photon disappears, producing a dip in reflected light at that specific angle. The angle, which is dependent on refractive index of the adsorbate, is measured.

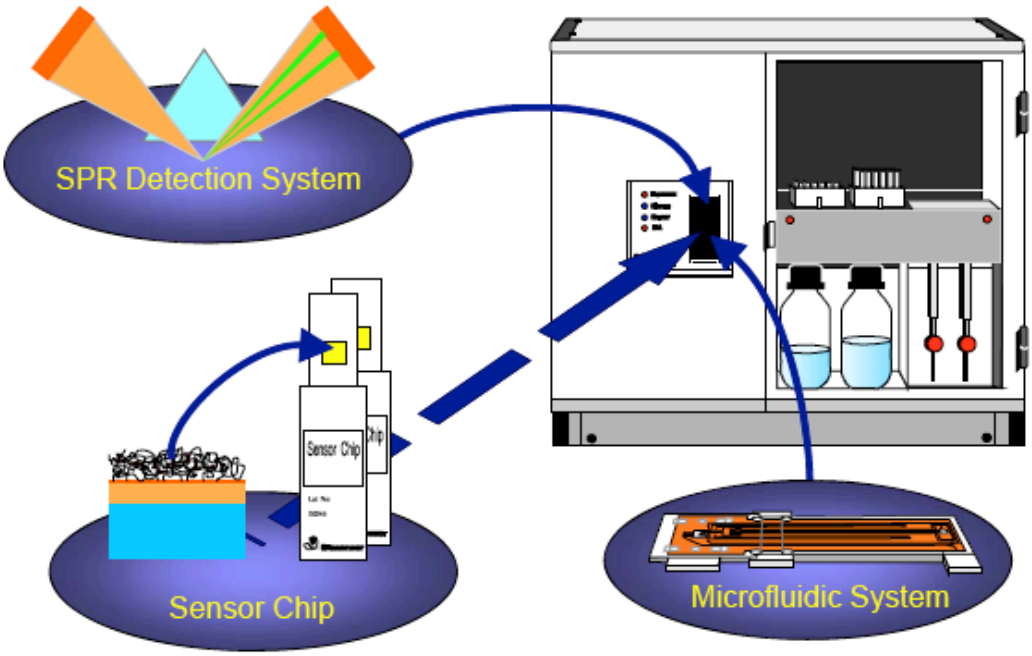
# There is a Linear Relationship Between the SPR Signal and the Mass on the Surface



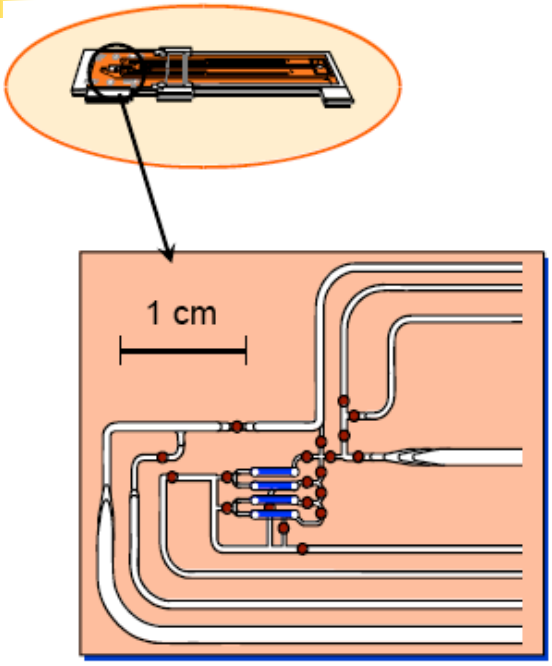

Signal proportional to mass
Same specific response for different proteins



# Typical commercial SPR



# 1. Microfluidic system

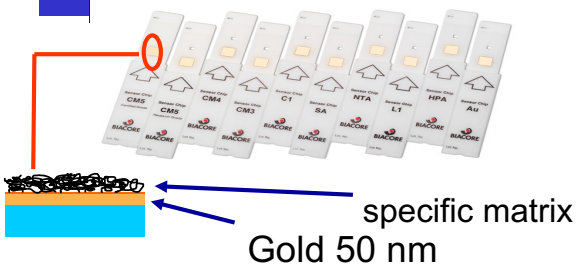


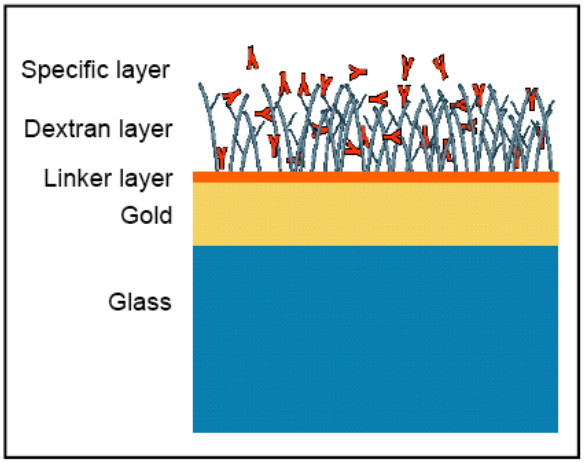

## Liquid handling

- Miniaturized system
- Integrated and automated
- Very small dead-volume
  - ↙Fast liquid exchange rates
  - ↙Low dispersion
  - ↙Excellent time control
- Temperature control



# 2. Sensor chip

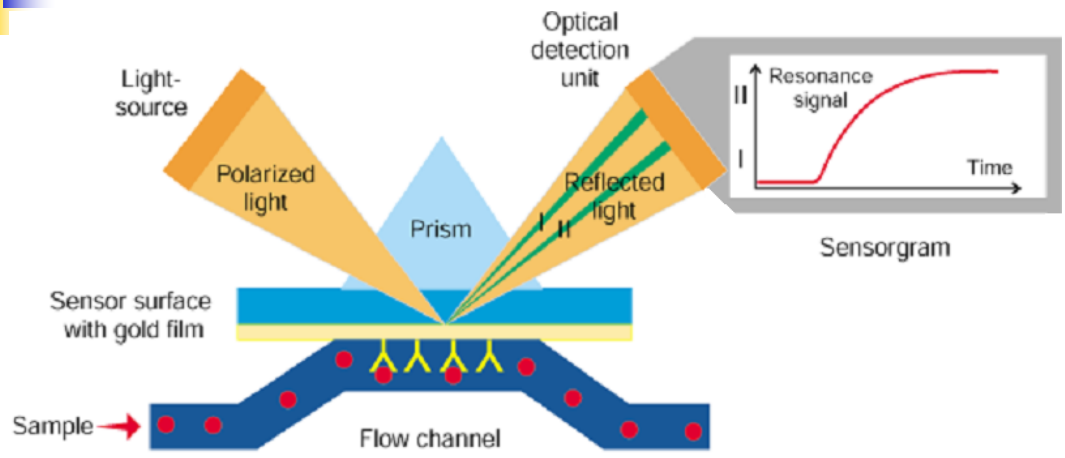



The dextran is a linear chain of 1,6-linked glucose units
Exhibits a **very low non-specific adsorption** of biomolecules.
Each glucose unit is modified with one carboxyl group.
Dextran hydrogel is the starting point to covalently bind a ligand to the sensor surface by amine-, thiol- or aldehyde chemistry.

- Dextran matrix covered with carboxyl groups (red circles)
- Captures ligands such as proteins, lipids, carbohydrates and nucleic acids (irreversible)
- Study of analytes ranging in size from small organic molecules, e.g. drug candidates, to large molecular assemblies or whole viruses.

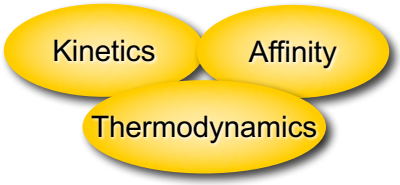
# 3. The SPR Detector




See youtube movies:
http://www.youtube.com/watch?v=o8d46ueAwXI
https://www.youtube.com/watch?v=sM-VI3alvAI



# What SPR Measures




How fast, strong & why…

Is the binding of a lead compound


How specific & selective...

Is this drug binding to its receptor?


How much...

Biologically active compound is in a production batch?

# Immobilisation on sensor

- Immobilisation of a receptor to the sensor surface is of central importance to the success.
- The coupling method must be:
  - efficient,
  - produce a highly stable association (to prevent signal drift)
  - allow control of the amount of receptor that is immobilised.
- **Amine coupling** (surface lysine on a protein) will lead to a heterogeneous population of receptors with random orientation on the surface.
- **sulphydryl couplings** can be used to produce a more homogeneous population of oriented receptors on the surface.



# Immobilisation on sensor

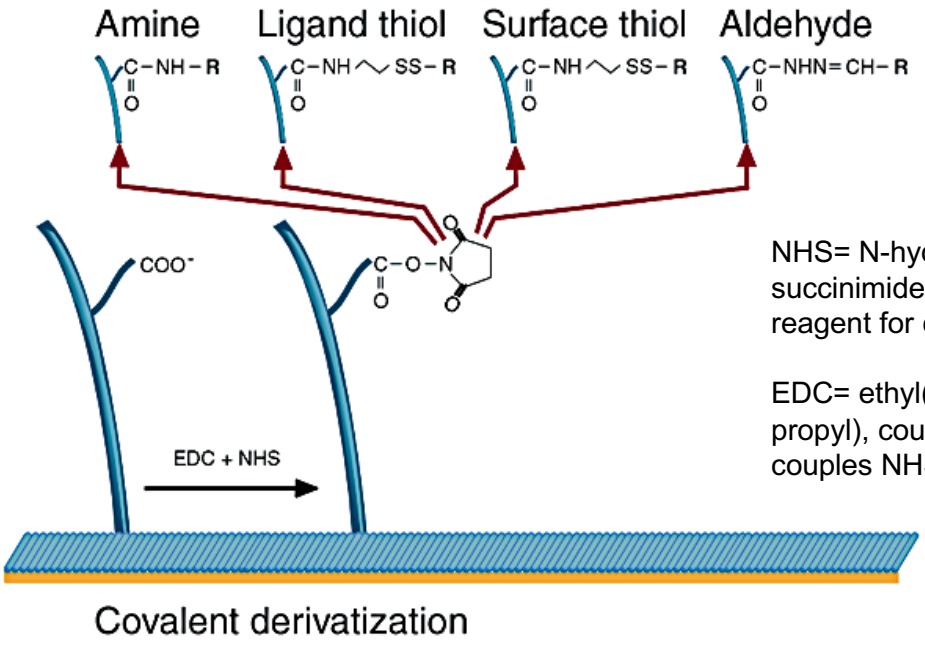
- Strategies that are commonly used to attach a receptor to a surface include:

- 1.Covalent attachment
  - *Water-soluble EDC-mediated activation of a carboxymethylated support, such as dextran.*

- 2. Non-covalent attachment
  - *Biotin- or streptavidin-presenting surfaces*
  - *Monoclonal antibodies*
  - *Metal-coordinating groups*

# 1. Covalent attachment

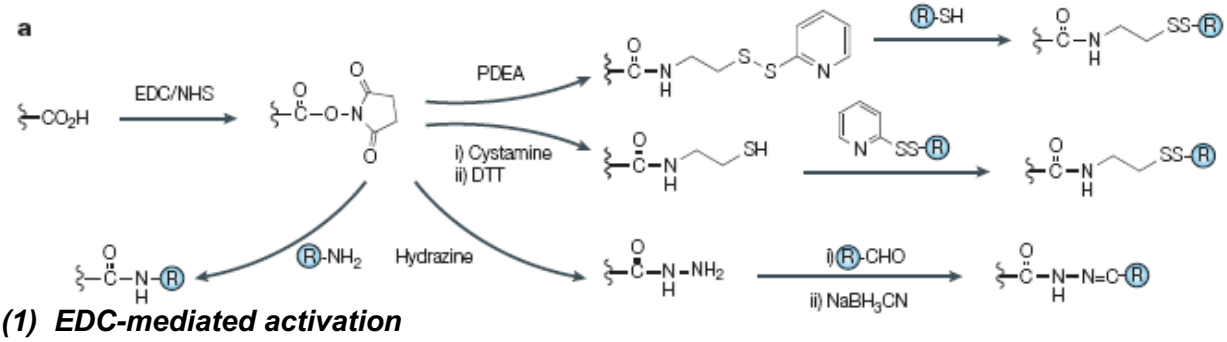
## Covalent immobilisation




NHS= N-hydroxy succinimide, activating reagent for carboxylic acids

EDC= ethyl(dimethyl amino propyl), coupling reagent, couples NHS to COO-

# Receptor immobilisation: covalent attachment




***(1) EDC-mediated activation***

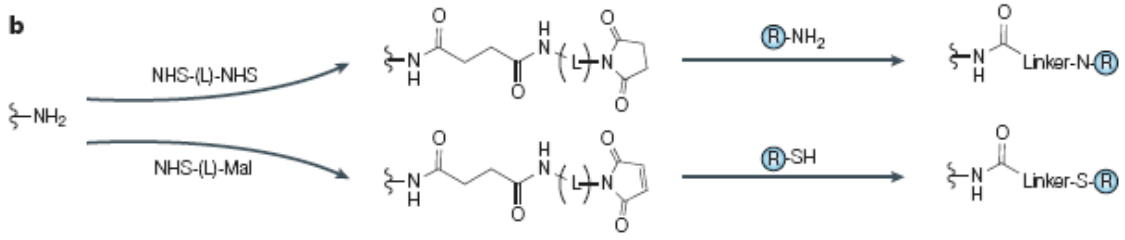
The resultant reactive NHS ester can be coupled to amino moieties of a receptor (R) to form a stable amide linkage.

Derivatisation with sulphydryl-reactive reagents (for example, PDEA or SPDP) allows reaction with free surface thiols (cysteine or methionine) to form a reversible disulfide linkage. In a similar manner, stable thioether bonds can be formed using maleimide coupling reagents, (sulpho-SMCC and GMBS). The surface can also be derivatised with cystamine to effect coupling with disulfide-activated receptors.

Treatment with hydrazine followed by a reductive amination allows coupling with aldheydes. The aldehyde groups could be native to the receptor or formed by mild oxidation of any cis-diols that are present



# Receptor immobilisation: covalent attachment

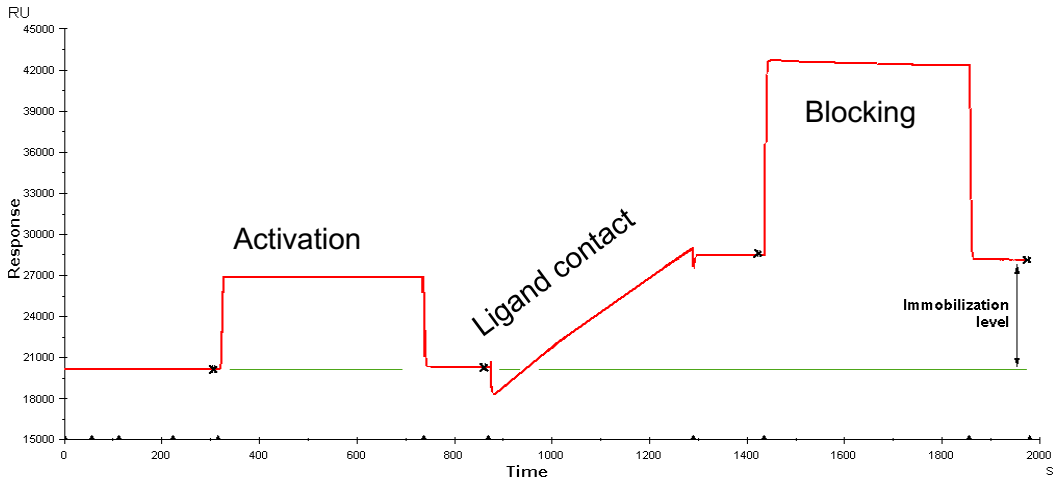



(2) *Amino-presenting surfaces* can be treated with commercially available bi-functional linking reagents to effect coupling with free amino or sulphydryl groups on the receptor (panel b).

L, linker; Mal, maleimide; NHS,N-hydroxysuccinamidyl

# Amine Coupling - Sensorgram

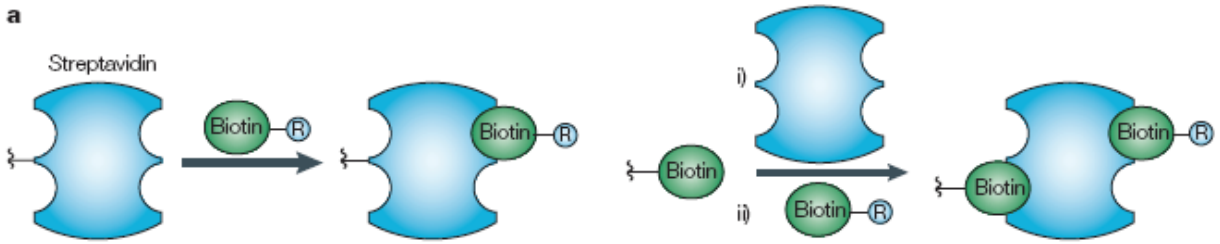
- Activation = EDC/NHS injection  surface esters
- Ligand contact = reaction with amine groups on ligand
- Blocking = deactivation of free esters with ethanolamine






# 2. Non-covalent attachment

# Receptor immobilisation: non-covalent




*(1) Biotin- or streptavidin-presenting surfaces*

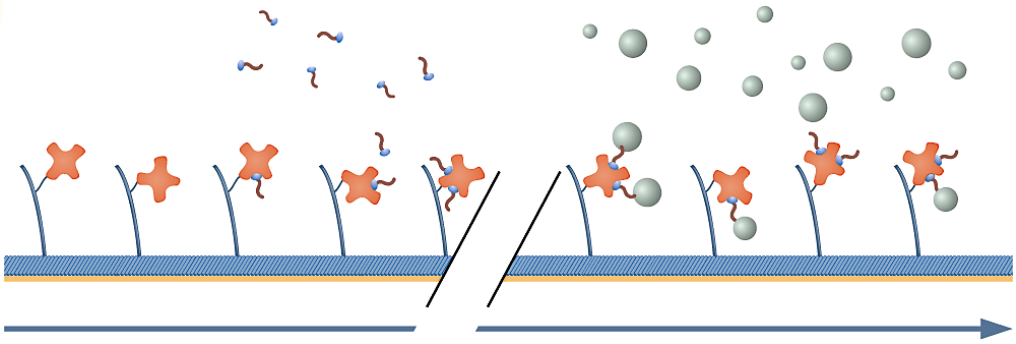
These can be used to capture biotinylated-receptors (panel a). The multiple BIOTIN-binding sites of streptavidin on each face of the molecule allow biotinylated ligands to be cross-linked by the streptavidin 'double adaptor'. This method is highly efficient and leads to stable complexes, but is effectively irreversible.



# Biotin/streptavidin background

- **Streptavidin** is a 60 kDa tetrameric protein purified from *Streptomyces avidinii*.
  - widely used due to its extraordinarily strong affinity for biotin (vitamin H);
    - the $K_d$ of the biotin-streptavidin complex is in the order of ~$10^{-15}$ M, ranking among one of the strongest known non-covalent interactions.

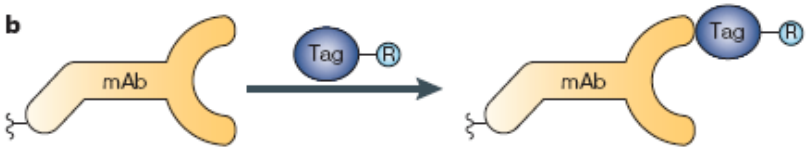
# Commercial Sensor Chip



- CM dextran matrix pre-immobilized with streptavidin
- Captures biotinylated ligands such as carbohydrates, peptides, proteins and DNA (irreversible)



# Receptor immobilisation: non-covalent




## (2) *Monoclonal antibodies*

These can be covalently attached to a solid support by means of amine coupling. Epitope-tagged or fusion proteins can then be directly and reversibly coupled to the surface through the antibody–antigen interaction.

Commonly used tags include,

- glutathione S-transferase (GST),
- herpes simplex virus glycoprotein D epiptope,
- FLAG epitope (polypeptide protein tag)
- Histidine tags (6 × His)

# Receptor immobilisation: non-covalent


(3) *Metal-coordinating groups*

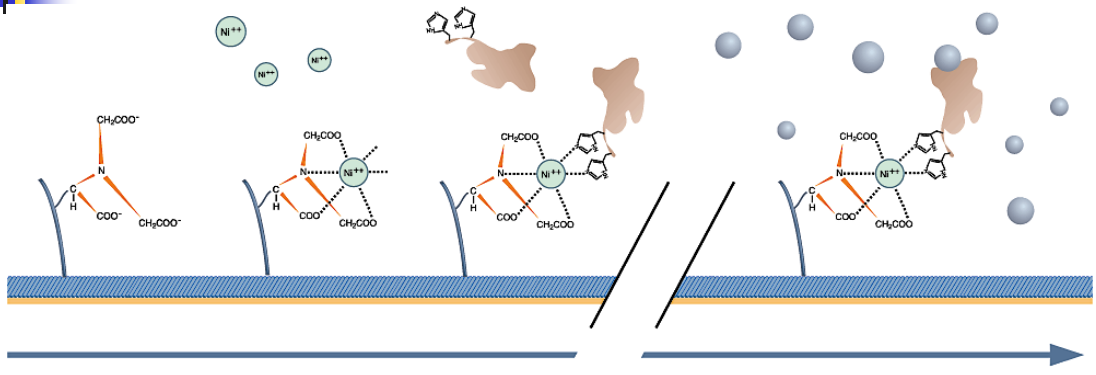
Groups such as iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) have been widely used for direct immobilisation of 6 × His- and 10 × His-tagged receptors.

The moderate affinity of the chelate–$Ni^{2+}$–histidine ternary interaction means that there is sometimes considerable decay in the level of immobilised receptor.

For this reason, anti-6 × His monoclonal antibodies are often used to enable stable, oriented immobilisation of His-tagged receptors

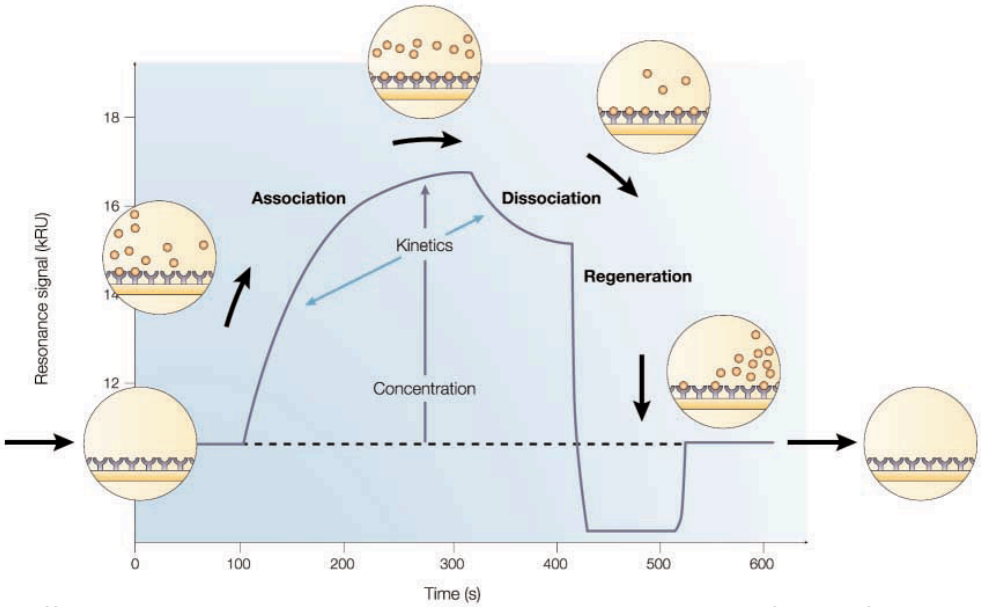


# Commercial Sensor Chip NTA



- CM dextran matrix pre-immobilized with nitrilotriacetic acid (NTA)
- Capture of His-tagged ligands via metal chelation
- Controlled steric orientation of ligand for optimal site exposure
- Regeneration by injection of EDTA to remove metal ions

# SPR experiment (sensorgram)




t = 0 s, buffer is contacted with the receptor through a microfluidic flow cell.
t = 100 s, a solution of analyte in the running buffer is passed over the receptor
t = 320 s, the analyte solution is replaced by buffer, receptor–analyte complex dissociate
t = 420 s disrupt binding and regenerate free receptor



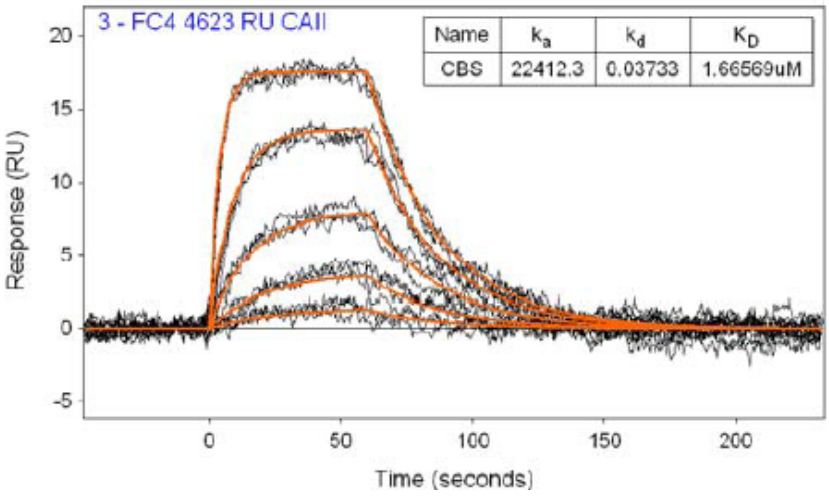
# Binding cycle

A typical binding cycle observed with an optical biosensor. A molecule is immobilised on the sensor surface with appropriate coupling chemistry.
At t = 0 s, buffer is contacted with the receptor through a microfluidic flow cell.
At t = 100 s, a solution of analyte in the running buffer is passed over the receptor. As the analyte binds to the surface, the refractive index of the medium adjacent to the sensor surface increases, which leads to an increase in the resonance signal. Analysis of this part of the binding curve gives the observed association rate (kobs). If the concentration of the analyte is known, then the association rate constant of the interaction (kass) can be determined. At equilibrium, by definition, the amount of analyte that is associating and dissociating with the receptor is equal. The response level at equilibrium is related to the concentration of active analyte in the sample.
At t = 320 s, the analyte solution is replaced by buffer, and the receptor–analyte complex is allowed to dissociate. Analysis of these data gives the dissociation rate constant (kdiss) for the interaction.
Many complexes in biology have considerable half-lives, so a pulse of a regeneration solution (for example, high salt or low pH) is used at t = 420 s to disrupt binding and regenerate the free receptor. The entire binding cycle is normally repeated several times at varying concentrations of analyte to generate a robust data set for global fitting to an appropriate binding algorithm. The affinity of the interaction can be calculated from the ratio of the rate constants (KD = 1/KA = kdiss/kass) or by a linear or nonlinear fitting of the response at equilibrium at varying concentrations of analyte.

# Examples of SPR

## 1.Kinetic Analysis of a Protein Binding to a Small Molecule

Ligand: Carbonic Anhydrase II immobilized on a CM5 chip by amine coupling
Analyte: 4-carboxybenzenesulfonamide (CBS) ( 0.12, 0.37, 1.11, 3.33, and 10
M )
Running Buffer:
PBS
Flow Rate: 50 ul/min

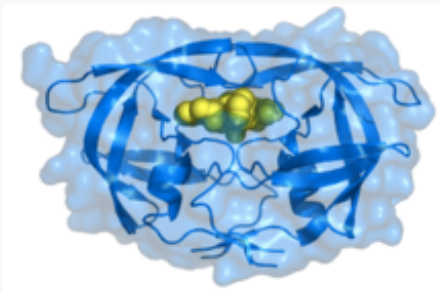



Carbonic anhydrase II is one of 14 forms of human α carbonic anhydrases.
catalyzes reversible hydration of carbon dioxide.
Defects in this enzyme are associated with osteopetrosis and renal tubular acidosis.

http://cmmi.uthscsa.edu/

# 2. HIV-1 protease

- **HIV-1 protease** is a retroviral aspartyl protease that is essential for the life-cycle of HIV, the retrovirus that causes AIDS.
- HIV protease cleaves newly synthesised polyproteins at specific sites to create the mature protein components of an infectious HIV.
- Without this protease, HIV virions remain uninfectious.
- Inhibition of its activity= anti-HIV drugs



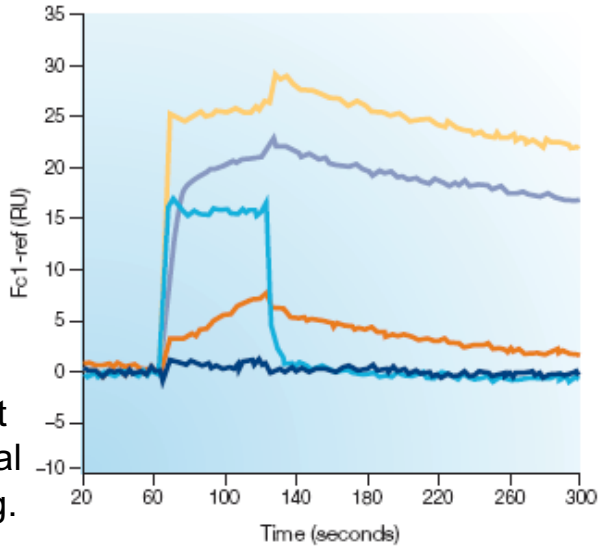
HIV-1 protease (blue) complexed with inhibitor (yellow) based on 1EBZ[1]



# 2. Protein/inhibitor interaction

## Hamalainen, M. D. *et al*. 2000

Traces from a screen in which (HIV)-1 protease was immobilised on a carboxymethyl-dextran surface, and compounds were passed over the receptor with automatic subtraction of data from a reference surface that contains no receptor. The reference surface is needed to correct for bulk refractive index changes and signal drift, and to control for non-specific binding.




Orange trace: lead with slow on- and slow off-rates; KD = 36 µm.
Turquoise trace: lead with high on- and high off-rates; KD = 1 µm.
Lilac trace: optimised lead with high on- and slow off-rates obtained by combining structural features of leads with orange and turquoise traces; KD = 27 nm.
Yellow trace: reference drug (ritanovir) with high on- and slow off-rates; KD = 22 nm.
Dark blue trace: negative control

## 3. Drug–Target Interactions: Direct Binding Assay for Interactions between Thrombin and Thrombin Inhibitors

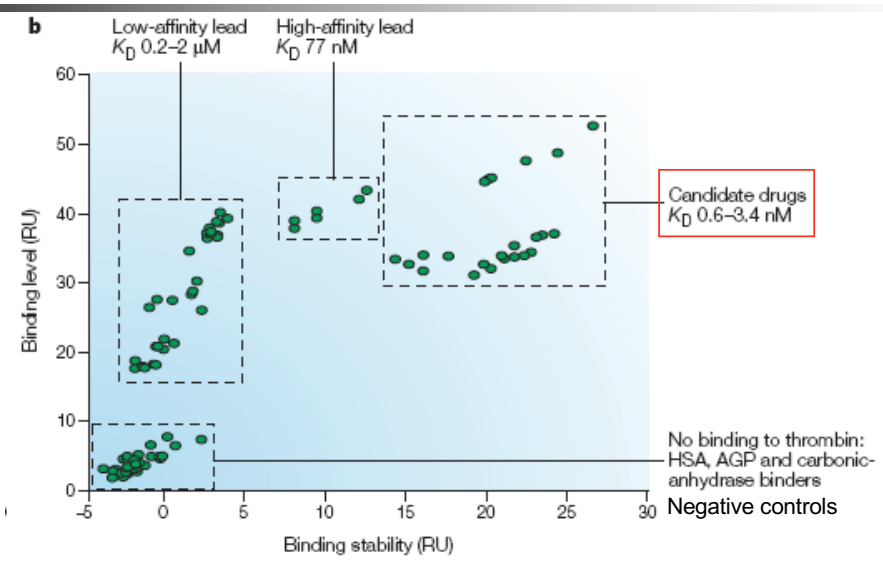
Karlsson, R. *et al*. 2000

- Thrombin inhibitors are a class of medication that act as **anticoagulants** (delaying blood clotting) by directly inhibiting the enzyme thrombin (factor II).
  - Some are in clinical use, while others are undergoing clinical development.
  - Several members of the class are expected to replace heparin (and derivatives) and warfarin
- In the direct binding assay, the target (thrombin) is immobilised to the sensor surface and compounds are passed over the surface. A positive signal indicates that the compound has bound to thrombin.
- A reliable direct binding assay for compounds binding to immobilised thrombin using a combination of two reference surfaces:
  - a dextran surface for subtraction and calibration of solvent effects and
  - a protein surface for identification of compounds that tend to bind proteins.
- 11 compounds with known binding specificity to thrombin and 159 additional compounds were investigated. All compounds with known binding specificity were identified at 1 and 10 µM concentration. One additional compound was scored as positive.



# Examples of SPR biosensors

Karlsson, R. *et al*. 2000




Thrombin was immobilised on a carboxymethyl-dextran surface and compounds assayed. The thrombin binding level attained at equilibrium during the association phase (a measure of binding affinity) was plotted against the level reached at a set time during the dissociation phase (a measure of binding stability).

# Membrane proteins and SPR



# Membrane proteins/receptors

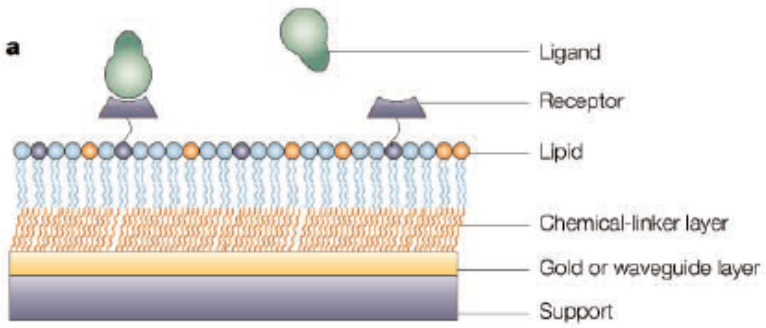
- Many interactions probed for drug discovery
  - Occur with membrane-bound receptors
    - e.g. ion channels, antibody receptors
    - Half of drugs on the market are targeted to membrane receptors
  - To understand interaction between drug and receptors:
    - Probe directly in vivo, OR
    - Model membrane systems
  - Usual techniques such as fluorescence, FRET, NMR etc are difficult to apply to membrane receptors
    - Therefore use immobilisation of membranes on a biosensor and probe interaction by SPR.

# Immobilisation of membranes on sensor surface

- (1) supported lipid monolayers
  - Simplest method for immobilisation of membranes on sensor surfaces is to adsorb the lipid onto a hydrophobic surface.
    - This results in the formation of a supported monolayer
      - Hydrophobic acyl chains of the lipids contact the hydrophobic surface
      - Polar lipid head groups face the solution
  - Disadvantage: cannot accommodate transmembrane proteins with large cytosolic domains



# Supported lipid monolayers



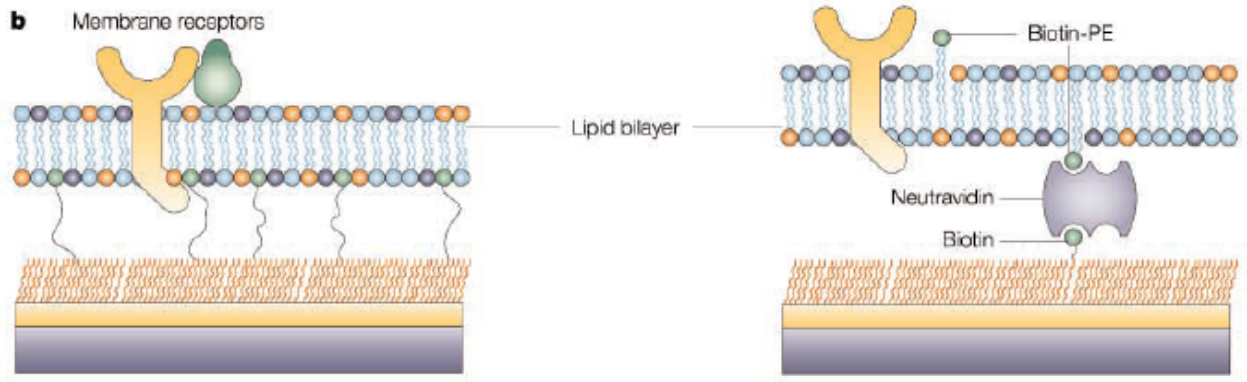

A supported lipid monolayer that has been formed on top of a hydrophobic, self-assembled monolayer on a gold surface.

# Immobilisation of membranes on sensor surface

- (2) Tethered lipid bilayers
  - Developed to accommodate larger transmembrane proteins.
  - Lipid bilayer placed at some distance away from the surface and attached in various ways to the sensor surface:
    - Triethyleneglycol spacer
    - Teflon spacer



# Tethered lipid bilayers



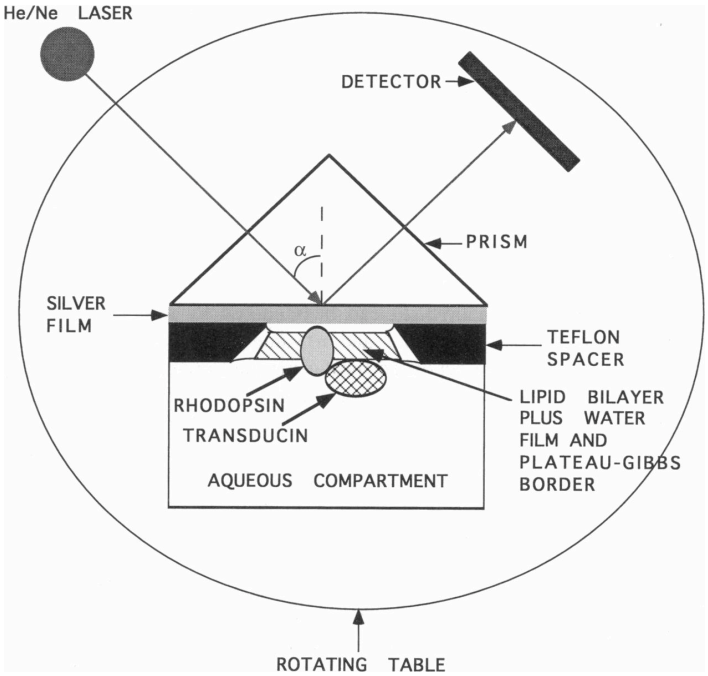

Examples of tethered lipid bilayers that contain an integral (transmembrane) receptor. The bilayer is either captured on the surface using synthetic phospholipids that are tethered to the support by flexible, hydrophilic linkers (left), or through immobilised neutravidin (deglycosylated avidin) in conjunction with biotinylated lipids or a biotinylated receptor.

PE, biotinyl-phosphoethanolamine-N-(biotinyl).

# Example of Tethered lipid bilayers (Salamon *et al*., 1996)

SPR to monitor interactions among the **proteins associated with signal transduction** in membrane-bound systems.

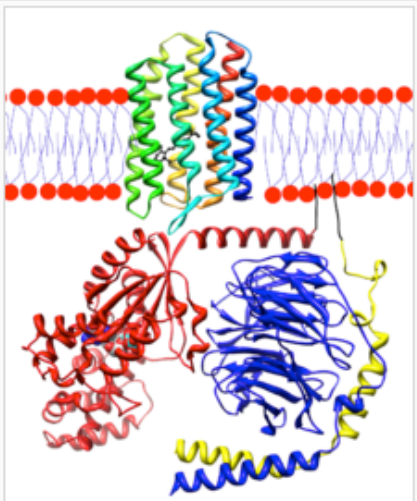
SPR for binding and activation of G-protein (transducin or G5) by bovine rhodopsin incorporated into an egg phosphatidylcholine bilayer deposited on a silver film.
Before photolysis, the SPR data show that Gt binds tightly (Kq- 60 nM) and with positive cooperativity to rhodopsin in the lipid layer to form a closely packed film.
calculated average thickness of about 57 A, in good agreement with the structure of Gt.
Upon visible light irradiation, characteristic changes occur in the SPR spectrum, which can be modeled by a 6 A increase in the average thickness of the lipid/protein film caused by formation of metarhodopsin 11 (MII). Upon subsequent addition of GTP, further SPR spectral changes are induced. These are interpreted as resulting from **dissociation of the a-subunit of Gt, formation of new MII-Gt complexes, and possible conformational changes of Gt as a consequence of complex formation.**






# Phototransduction cascade

- Transducin (also called $G_t$) is a heterotrimeric G protein that is naturally expressed in vertebrate retina rods and cones.
- Heterotrimeric Transducin (alpha-beta-gamma subunits) is activated by a conformational change in rhodopsin due to the absorption of photon by retinal (rhodopsin's active group).
- Activation causes GDP bound to alpha to be exachanged with GTP resulting in activated alpha, dissociating from other subunits.



Sensory rhodopsin II (rainbow colored) embedded in a lipid bilayer (heads red and tails blue) with Transducin below it. $G_t\alpha$ is colored red, $G_t\beta$ blue, and $G_t\gamma$ yellow. There is a bound GDP molecule in the $G_t\alpha$-subunit and a bound retinal (black) in the rhodopsin. The N-terminus terminus of rhodopsin is red and the C-terminus blue. Anchoring of transducin to the membrane has been drawn in black.

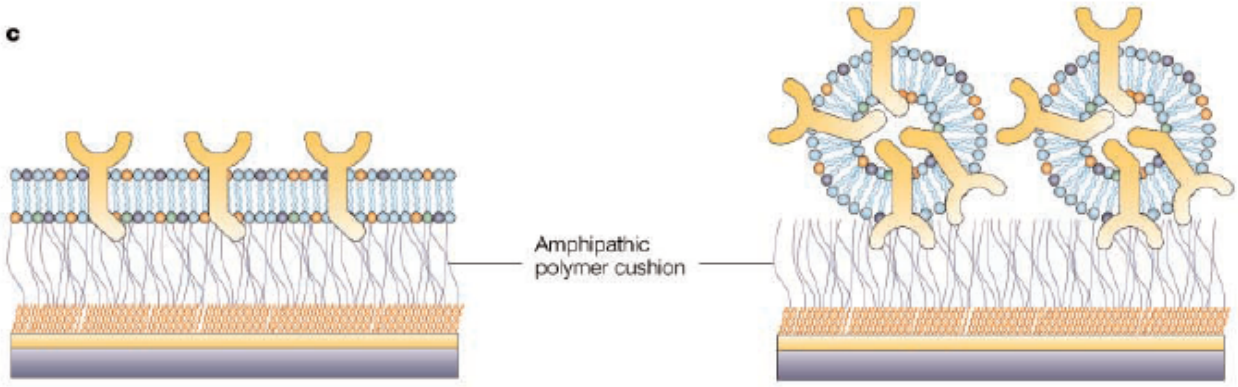
http://en.wikipedia.org/wiki/Transducin

# Immobilisation of membranes on sensor surface

- (3) polymer-supported bilayers
  - To overcome problems associated with roughness of the underlying surface, lipid bilayers can be bound to, but structurally de-coupled from the solid support by first coating with a flexible polymer.
  - These soft polymer cushions provide a hydrated lubricating layer between the surface + membrane that allows the "self-sealing" of surface defects.



# Polymer-supported bilayers




Flexible, amphipathic polymer cushions support membranes as either supported lipid bilayers or captured proteoliposome layers.

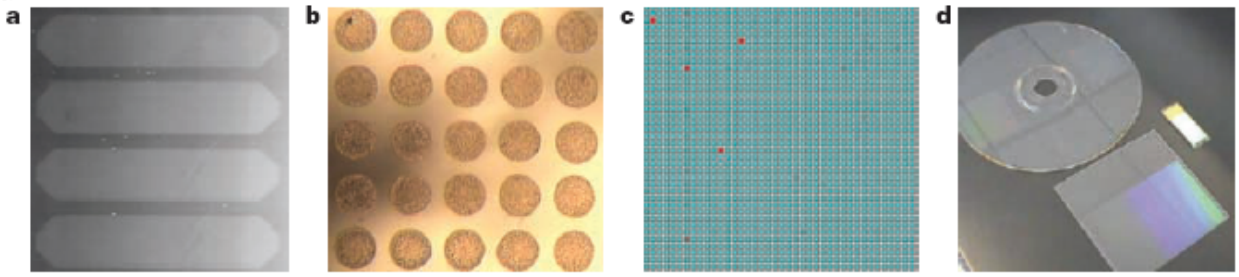
# Immobilisation of membranes on sensor surface

- (4) Microarrayed lipid layers
- With the numerous membrane-receptor targets cloned, there is considerable interest in methods to create spatially addressable membrane arrays.
- scientists use a process called micro-contact printing (μCP), in which a patterned stamp made from poly(dimethylsiloxane) (PDMS), is brought into contact with a planar supported lipid bilayer. This results in the displacement of adsorbed lipid at the areas of contact between the stamp and the slide, which can then be filled with a blocking molecule such as BSA.

- In an alternative approach, mixed SAMs of hydroxyl- and cholesterol terminating thiols have been formed to capture lipid bilayers, which can be micro-arrayed using micro-contact printing techniques.



# Micro-arrayed SPR




a | The four-channel flow cell that was pioneered by Biacore within an area of 2.4 mm × 2.6 mm.
b | Schematic representation of a Biacore functional array that is under development at present — 25 spots in a 5 mm × 5 mm area. The array will provide functional information on the protein-binding patterns and generate detailed kinetic data, in addition to concentration and affinity measurements.
c | An image taken with the Plasmon Imager from Graffinity Pharmaceuticals of part of an array of 4,089 spots in an area of 80 × 80 mm. The figure shows a schematic representation of the binding profile of a protein against 4,608 compounds, each immobilised on a different field of a sensor plate. The SPR wavelength shifts are detected after addition of 200 nM of protein. Larger wavelength shifts indicate stronger binding.
d | HTS Biosystems' plastic optical diffraction gratings, which could support a surface density of 10,000 spots in an area of 10 × 10 mm. The plastic chips are produced at low cost with the same methods that are used to manufacture CD-ROMs and DVDs, which allows for a great deal of flexibility in format design.

# Learning outcome

- From this lecture you should understand:
  - The basics of SPR
  - Some examples of different forms of immobilisation of biomolecules on sensor surface:
    - Non-covalent attachment
    - Covalent attachment
  - Examples of SPR use in biosensing with special reference to membrane bound receptors.



# References

M.A. Cooper. optical biosensors in drug discovery. *Nature Reviews*, vol. 1, 515-528 (2002).

Karlsson, R. *et al*. Biosensor analysis of drug target interactions: direct and competitive binding assays for investigation of interactions between thrombin and thrombin inhibitors. *Anal. Biochem*. 278, 1–13 (2000).

Hamalainen, M. D. *et al*. Characterisation of a set of HIV-1 protease inhibitors using binding kinetics data from a biosensor-based screen. *J. Biomol. Screen*. 5, 353–360 (2000).

Salamon, Z., Wang, Y., Souagales, J. L. & Tollin, G. SPR spectroscopy studies of membrane proteins: transducin binding and activation by rhodopsin monitored in thin membrane films. *Biophys. J.* 71, 283–294 (1996).

Hoaa X.D. *et al.* Towards integrated and sensitive surface plasmon resonance biosensors: A review of recent progress. *Biosensors and Bioelectronics* 23, 151–160 (2007).