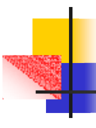
Welcome to Lecture 4

Plan for today

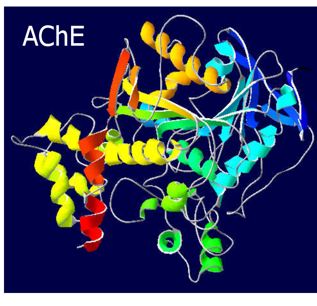
- A few words about homework
- Antibodies and AB-based sensors
- Chemical receptors and a "plastic antibody" technology

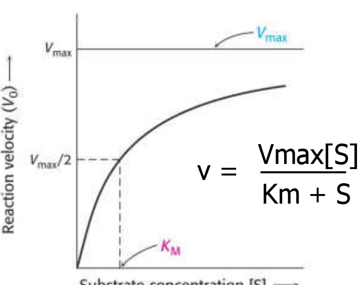
but first…



Last time

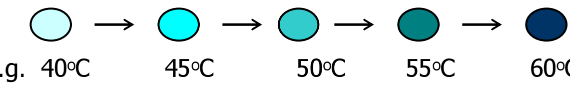
We discussed enzyme-based biosensors, touched upon protein structures and enzyme kinetics



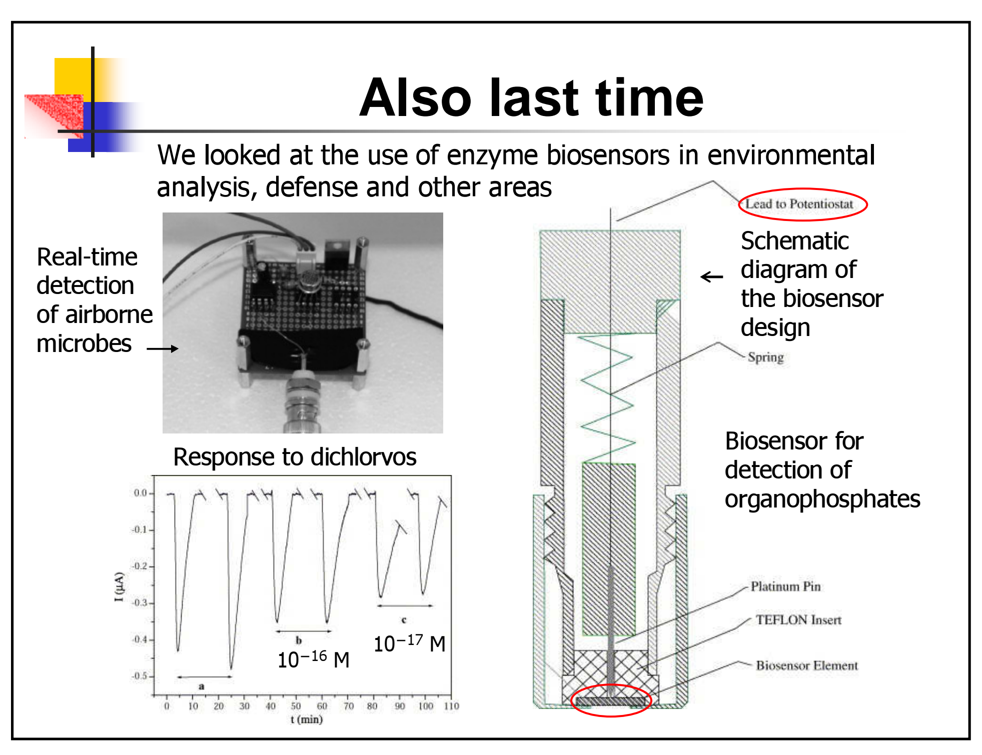


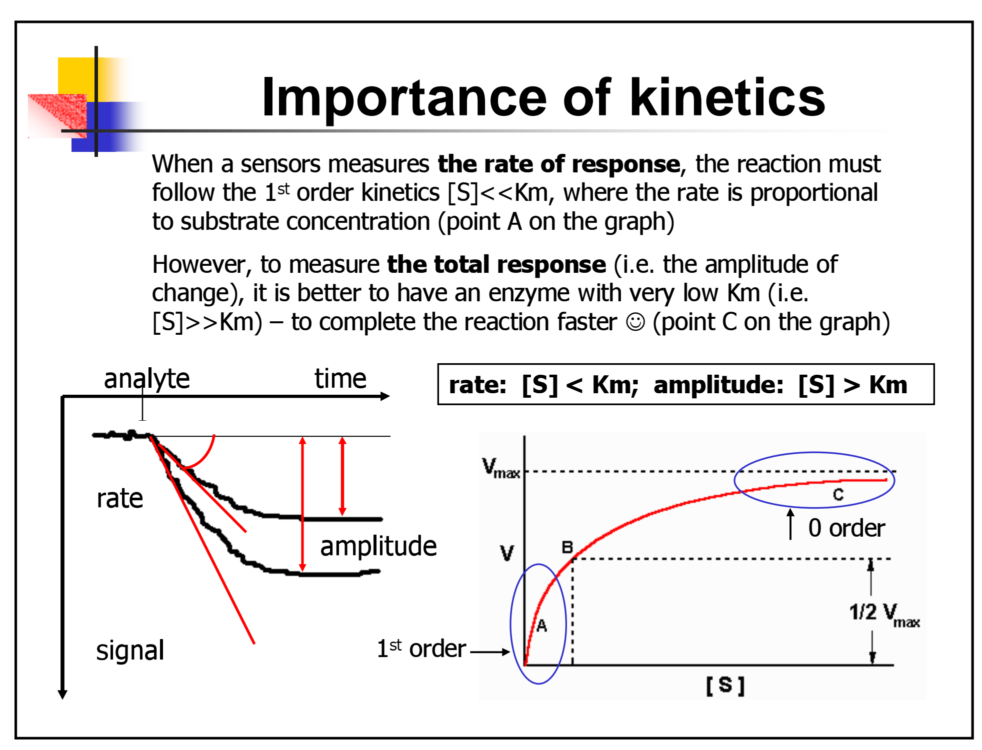
$$v = \frac{Vmax[S]}{Km + S}$$

We also talked about protein engineering, directed evolution



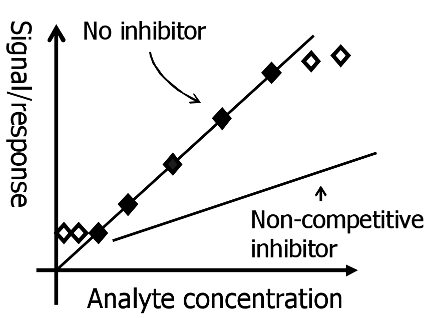
e.g. 40°C 45°C 50°C 55°C 60°C

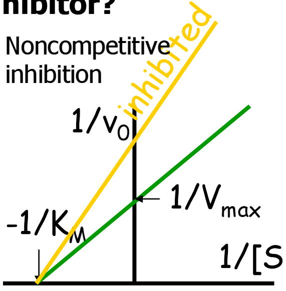

Also last time
We looked at the use of enzyme biosensors in environmental analysis, defense and other areas
Real-time detection of airborne microbes
Response to dichlorvos
10−16 M
10−17 M
t (min)
I (μA)
Lead to Potentiostat
Schematic diagram of the biosensor design
Spring
Biosensor for detection of organophosphates
Platinum Pin
TEFLON Insert
Biosensor Element


Importance of kinetics
When a sensors measures the rate of response, the reaction must follow the 1st order kinetics [S]<<Km, where the rate is proportional to substrate concentration (point A on the graph)
However, to measure the total response (i.e. the amplitude of change), it is better to have an enzyme with very low Km (i.e. [S]>>Km) – to complete the reaction faster ☺ (point C on the graph)
analyte
time
rate
amplitude
signal
rate: [S] < Km; amplitude: [S] > Km
Vmax
V
B
C
0 order
A
1/2 Vmax
1st order
[S]

HW: how do we get it back

if samples contain a NC inhibitor?

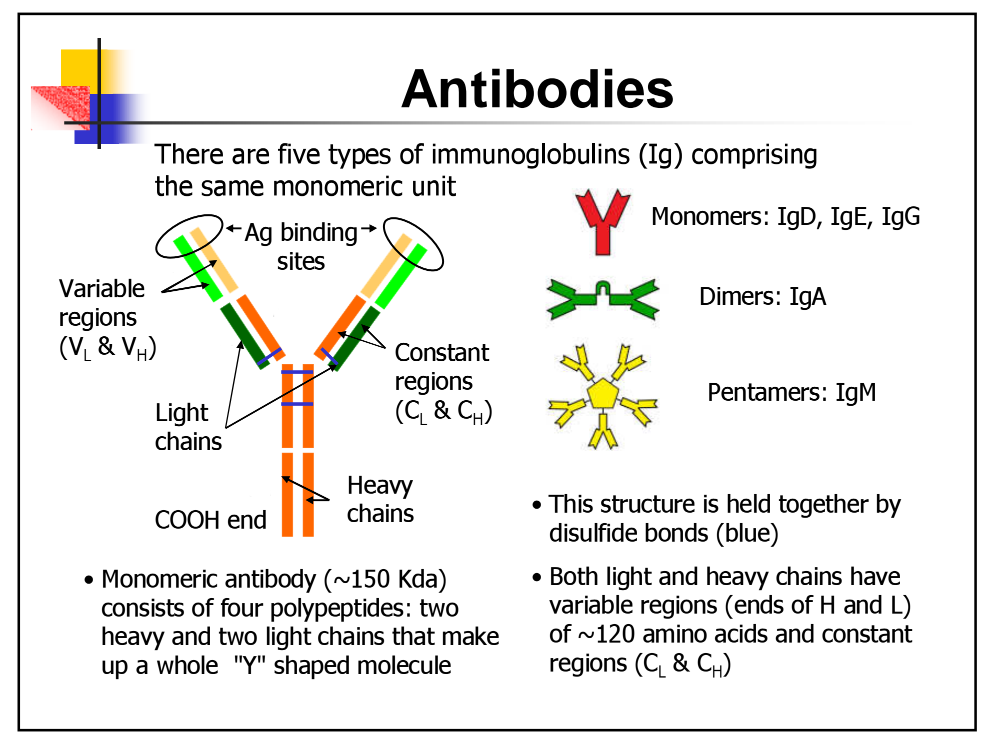


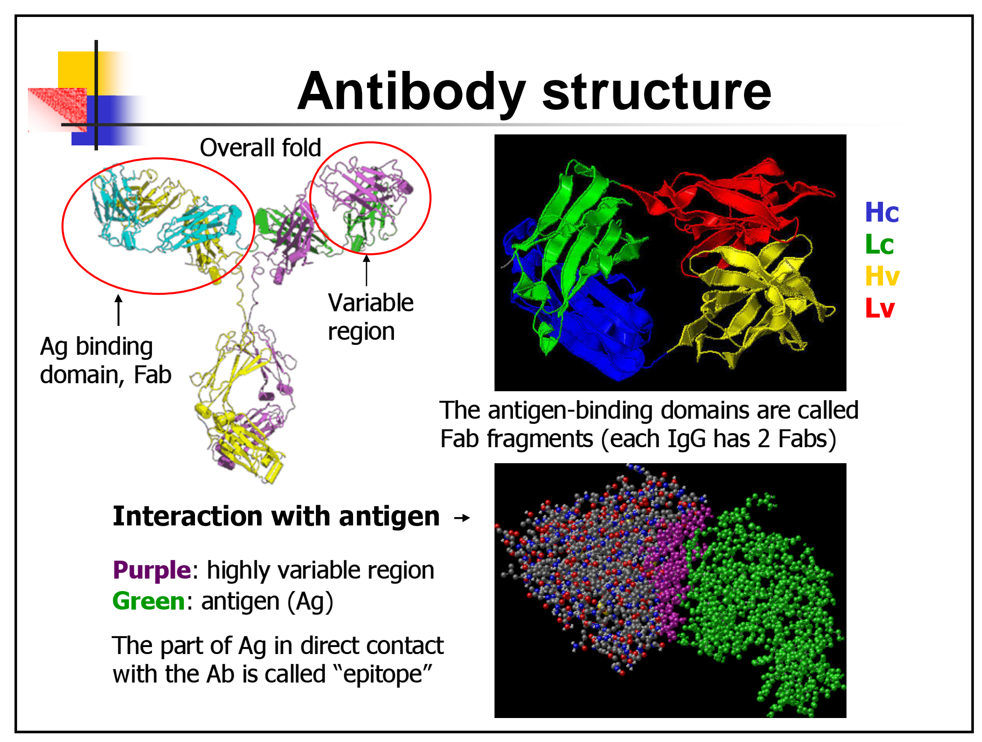


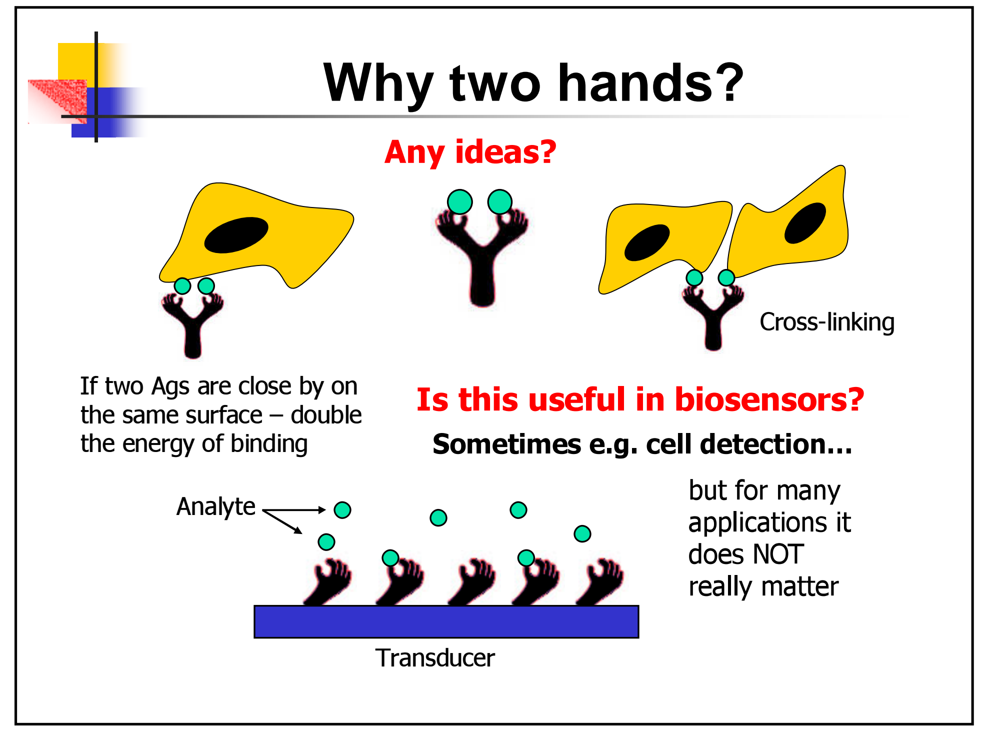
- Non-competitive inhibitors affect kcat: interact with both E and ES
- If kcat is 50% of what it was, v (rate) will be 50% too
- But there is a liner dependence between v and Eo

$$v = \frac{k_{cat}\, Eo\, S}{Km + S}$$

How about using more enzyme then? ☺



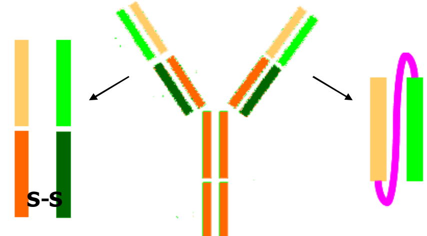

Antibody structure
Overall fold
Ag binding
domain, Fab
Variable
region
Hc
Lc
Hv
Lv
The antigen-binding domains are called
Fab fragments (each IgG has 2 Fabs)
Interaction with antigen →
Purple: highly variable region
Green: antigen (Ag)
The part of Ag in direct contact
with the Ab is called "epitope"


Why two hands?
Any ideas?
Cross-linking
If two Ags are close by on
the same surface – double
the energy of binding
Is this useful in biosensors?
Sometimes e.g. cell detection…
Analyte
but for many
applications it
does NOT
really matter
Transducer

Antibody formats

Fab fragment:

Can be obtained by protease (e.g. papain) cleavage of the parent Ab



Single chain Abs (**scFv**) -

the smallest structure that retains binding properties of the parent Ab

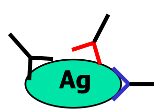
- scFv are engineered as a single polypeptide where VH and VL domains are joined by about 15 amino acids long linker
- The properties (e.g. stability and specificity) can be affected by the linker but various linker designs are available
- scFv are expressed in bacteria and, hence, can be very inexpensive to manufacture

Polyclonal vs monoclonal

Polyclonal antibodies: derived from different B cells i.e. a mixture if IgGs specific to a particular antigen

- Produced by immunization (e.g. rabbits, sheep) and purified from the serum; the bigger the animal, the better ☺

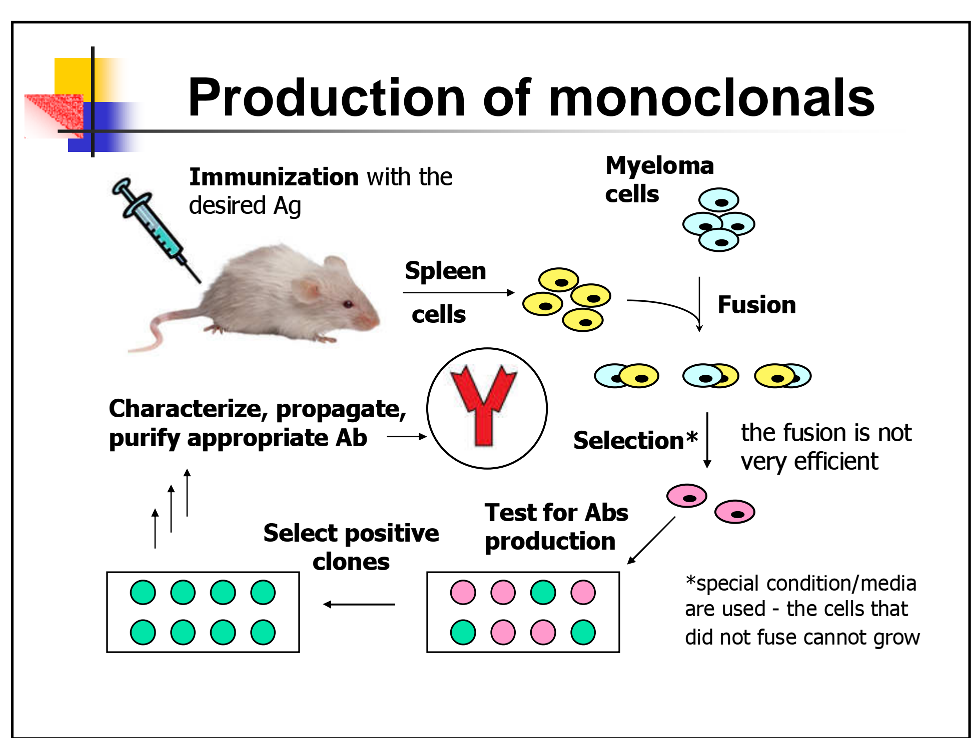
Problem:
bind to different epitopes

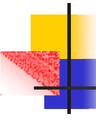


Selectivity and affinity (i.e. sensitivity) will vary from one Ab to another ☹

Monoclonal antibodies: derived from a single line of B cells i.e. all IgGs are identical – bind to a single epitope

- Produced using well established Hybridoma Cell Production Technology





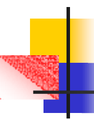
Phage display technology

- Powerful tool for identifying high affinity protein ligands to different target molecules
- A rapid directed evolution methodology for improving proteins' binding characteristics

Key feature: Direct linkage between a displayed peptide and its coding sequence enables rapid screening of vast libraries (e.g. 10^{12} clones) by a simple process of affinity-based selection

- Random oligopeptide libraries
- Custom-designed peptides or proteins
- Antibodies e.g. scFv fusion

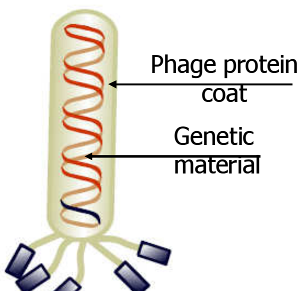
Smith GP (2005) Combinatorial display of peptides or proteins by fusion to phage coat proteins. Science 228:1335

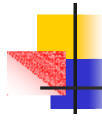


What is a phage?

- Bacteriophage is a virus that infects bacteria (it is not infectious to humans)
- Made of two main components – genetic material (inside) and protective protein coat (outside).
- The information for making all phage proteins and assembly is contained in the phage genome
- The phage multiplies by inserting its genome into bacteria and uses the cell machinery to make more of copies of itself

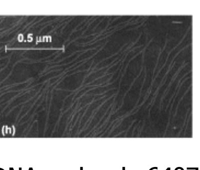
3 types of common phages used in phage display are the M13, F1, FD





M13 phage

Dimensions:
Diameter: 6.5nm in length: 930nm (wt)

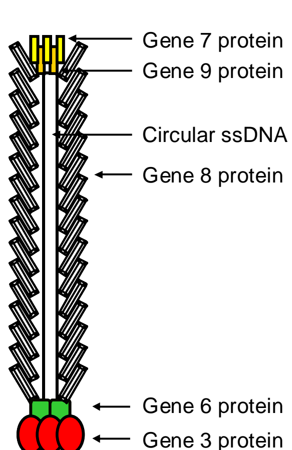


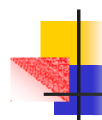
Genome:
Single stranded circular DNA molecule 6407 nucleotides long; enclosed in a flexible protein cylinder

Proteins:
The long phage cylinder comprises 2700 copies of the 50-amino-acid major coat protein p8

At one terminus:
- 5 copies of 33 AA residue p7
- 5 copies of 32 AA residue p9

At another terminus:
- 5 copies of 406 AA residue p3
- 5 copies of 112 AA residue p6





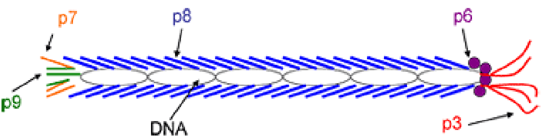
M13 phage display

p8 Peptide Display:

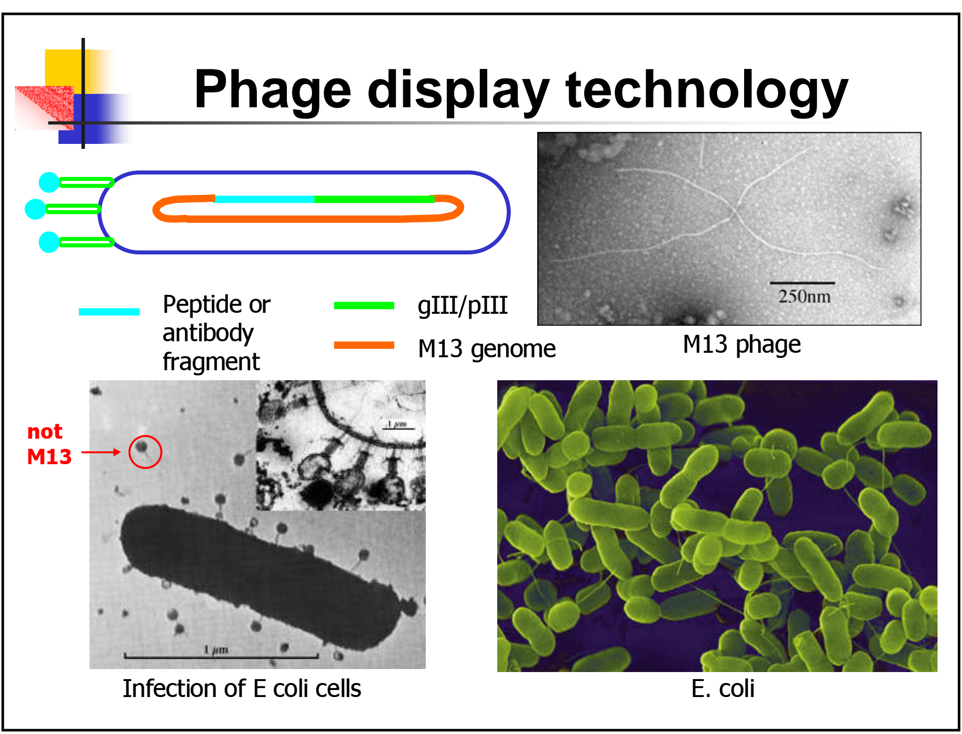
- Typically, peptides are displayed on every copy of p8 but the size is very limited to about 8 amino acids
- Larger peptides and proteins can be displayed on a hybrid phage in which 80% of p8 are wild-type

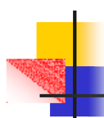
p3 Peptide Display:

- Only five molecules can be displayed per phage
- Large inserts can be used, although they reduce infectivity



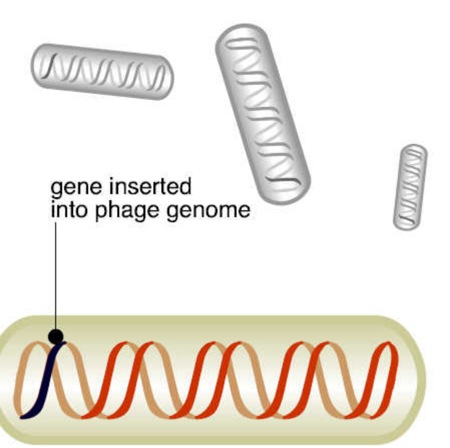
Numerous modified and improved systems are available



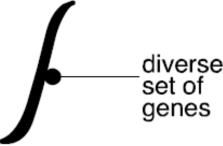


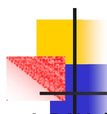
How does it work?

A diverse set of genes containing sequences for peptides/ proteins of interest are inserted into gene 3 or gene 8 to generate a library



Ideally, each phage receives a single unique protein or peptide, which it would express on the surface i.e. "display" to the world

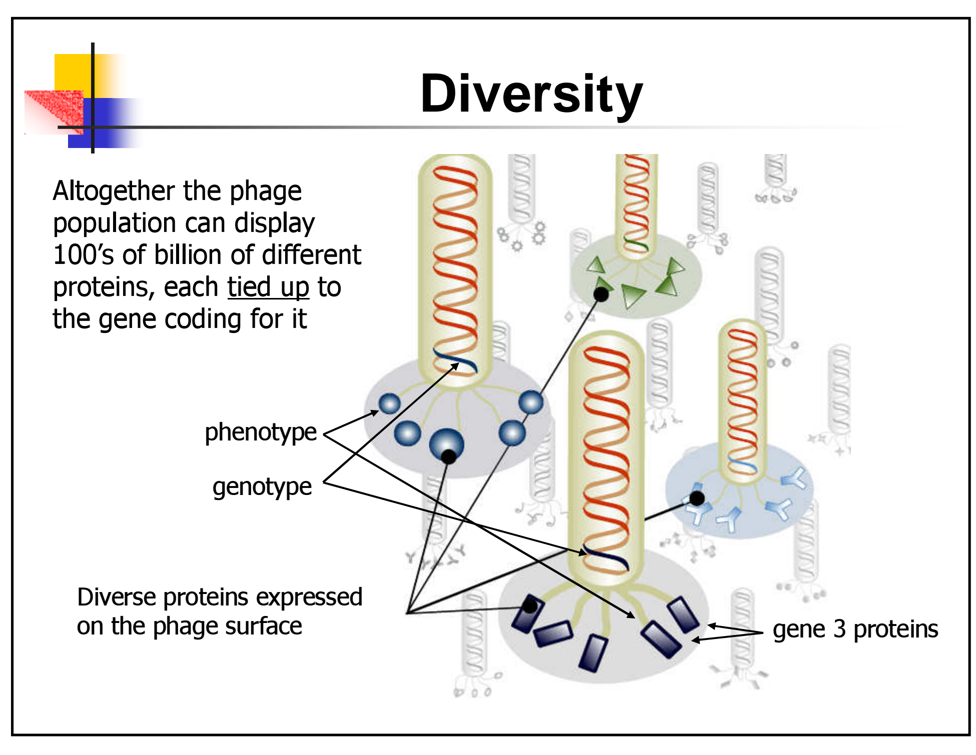


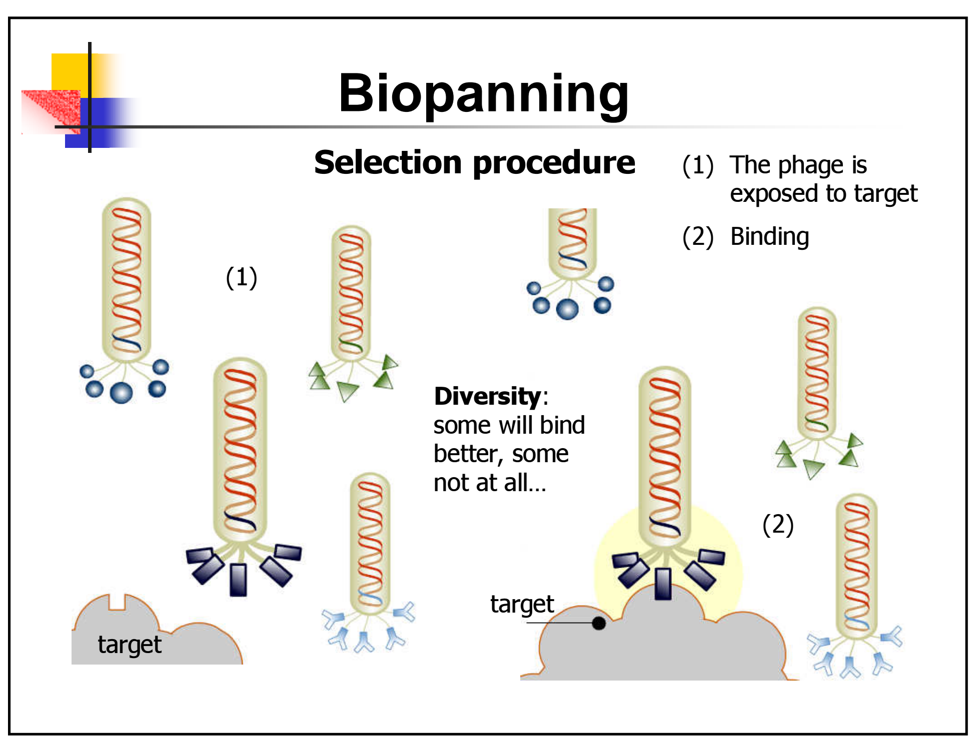


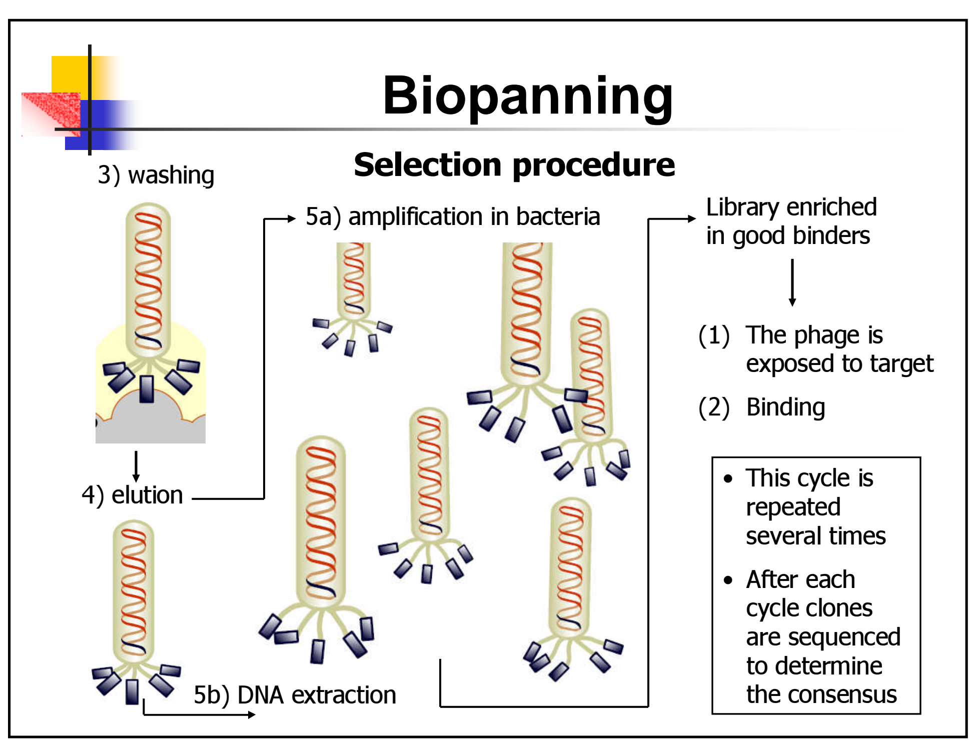
How it works: clarification

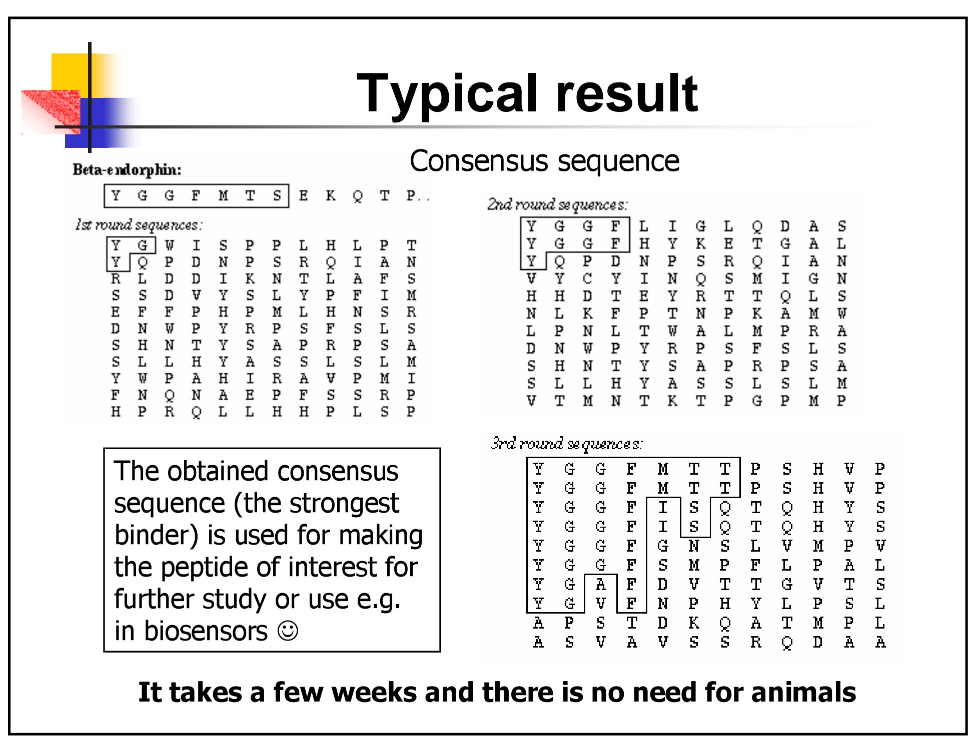
A DNA library coding for a variety of different oligopeptides/proteins is inserted into phage DNA (gene for P3 or P8). This can be a library coding for antibody fragments or library encoding polypeptides comprising 10 or 13 amino acids with each possible sequence present - huge diversity! Each phage carries a single DNA sequence and because this sequence is now a part of one of the coat proteins, the phage will make its coat with the additional oligopeptide/protein inserted and "display" it. Note that multiple copies of the same protein/peptide are normally displayed because there is more than one copy of each coat protein on the phage surface.

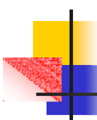
The cool part is that initially we don't even need to know which phage carries what sequence - there is no way we could checked them all anyway. We just let the phage to compete for binding to our target and pick up the best. After the first round we will multiply good binders by growing them in bacteria and do the second round (i.e. let them compete again). This will bring out the better out of the good ones and in the third round we will pick up the best among the better. Finally, when only the best binders are left, it is often the case that they have the same sequence with some variations ("consensus sequence"). Intermediate sequencing is also done as a "quality control" to see whether the sequences are converging or not. Although the selection is based on binding (phenotype), the sequence is actually read from the gene ("phenotype-genotype" I referred to).


Diversity
Altogether the phage population can display 100's of billion of different proteins, each tied up to the gene coding for it
phenotype
genotype
Diverse proteins expressed on the phage surface
gene 3 proteins


Biopanning
Selection procedure
(1) The phage is exposed to target
(2) Binding
(1)
Diversity: some will bind better, some not at all…
(2)
target
target


Biopanning
Selection procedure
3) washing
4) elution
5a) amplification in bacteria
5b) DNA extraction
Library enriched in good binders
(1) The phage is exposed to target
(2) Binding
This cycle is repeated several times
After each cycle clones are sequenced to determine the consensus


Typical result
Consensus sequence
Beta-endorphin:
Y G G F M T S E K Q T P..
1st round sequences:
Y G W I S P P L H L P T
Y Q P D N P S R Q I A N
R L D D I K N T L A F S
S S D V Y S L Y P F I M
E F F P H P M L H N S R
D N W P Y R P S F S L S
S H N T Y S A P R P S A
S L L H Y A S S L S L M
Y W P A H I R A V P M I
F N Q N A E P F S S R P
H P R Q L L H H P L S P
2nd round sequences:
Y G G F L I G L Q D A S
Y G G F H Y K E T G A L
Y Q P D N P S R Q I A N
V Y C Y I N Q S M I G N
H H D T E Y R T T Q L S
N L K F P T N P K A M W
L P N L T W A L M P R A
D N W P Y R P S F S L S
S H N T Y S A P R P S A
S L L H Y A S S L S L M
V T M N T K T P G P M P
3rd round sequences:
Y G G F M T T P S H V P
Y G G F M T T P S H V P
Y G G F I S Q T Q H Y S
Y G G F I S Q T Q H Y S
Y G G F G N S L V M P V
Y G G F S M P F L P A L
Y G A F D V T T G V T S
Y G V F N P H Y L P S L
A P S T D K Q A T M P L
A S V A V S S R Q D A A
The obtained consensus sequence (the strongest binder) is used for making the peptide of interest for further study or use e.g. in biosensors ☺
It takes a few weeks and there is no need for animals

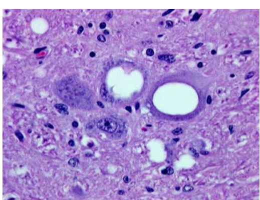


For example,

Directed Evolution of an Anti-prion Protein scFv Fragment to an Affinity of 1 pM and its Structural Interpretation

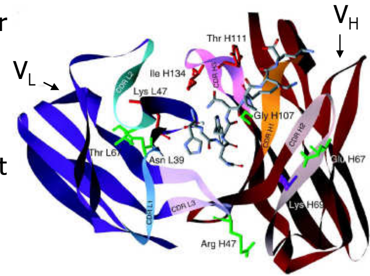
Luginbühl et al J. Mol. Biol. (2006) 363, 75–97

Prion protein is presumed to be a cause of bovine spongiform encephalo-pathy ("mad cow disease") and Creutzfeldt-Jakob disease (CJD)



spongiform change of the brain (vacuolation)

There is a need for highly sensitive, noninvasive diagnostic test which can detect even small amount of the disease-associated PrP conformer



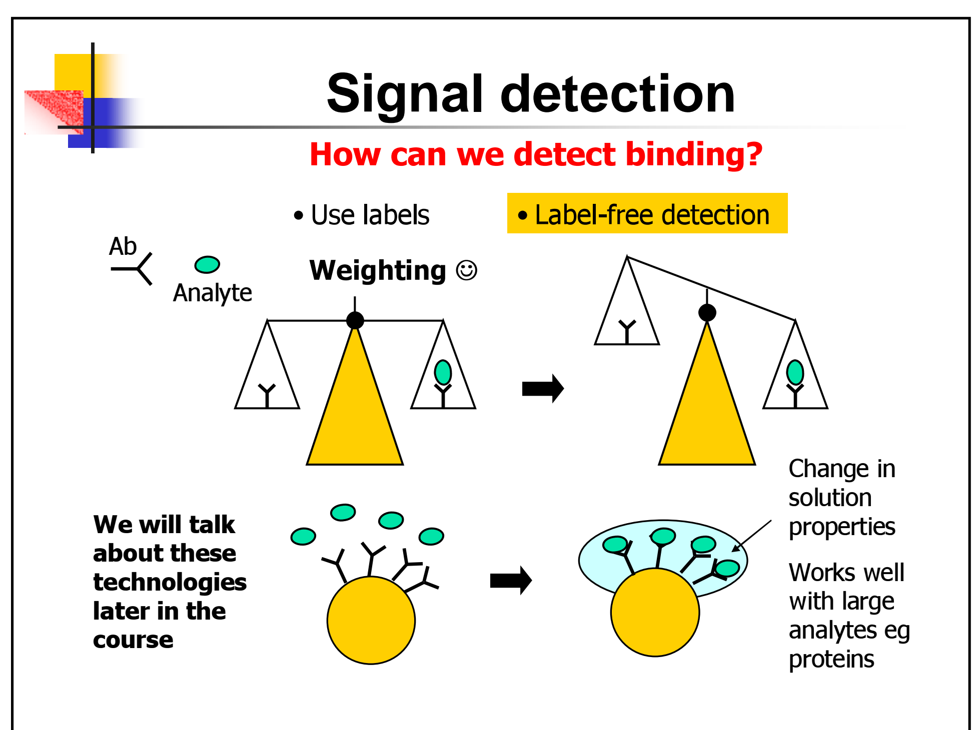
This is an **ultra-high affinity** Ab and the tightest peptide-binding antibody reported to date

Antibodies vs enzymes

Why use different directed evolution techniques for evolving/selecting enzymes and antibodies?

- To perform directed evolution a vast libraries are required
- To deal with this library an efficient screening is a must
- Enzymes excel at catalyzing reactions, while antibodies are great “binders”
- Screening enzymes for activity and Abs for affinity makes more sense…

Any questions?

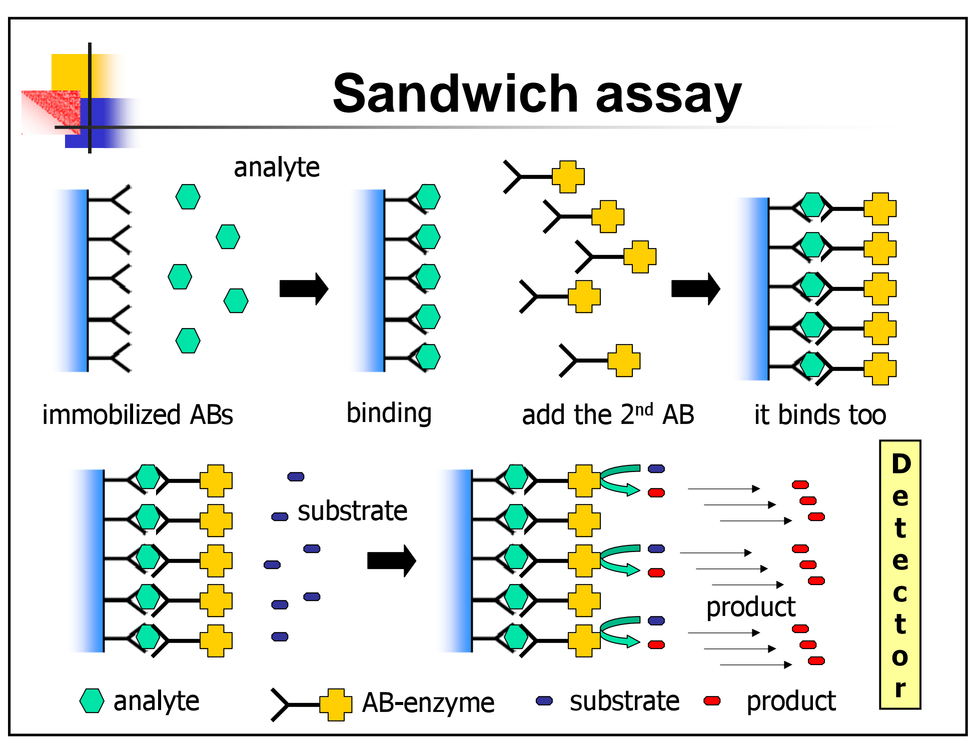


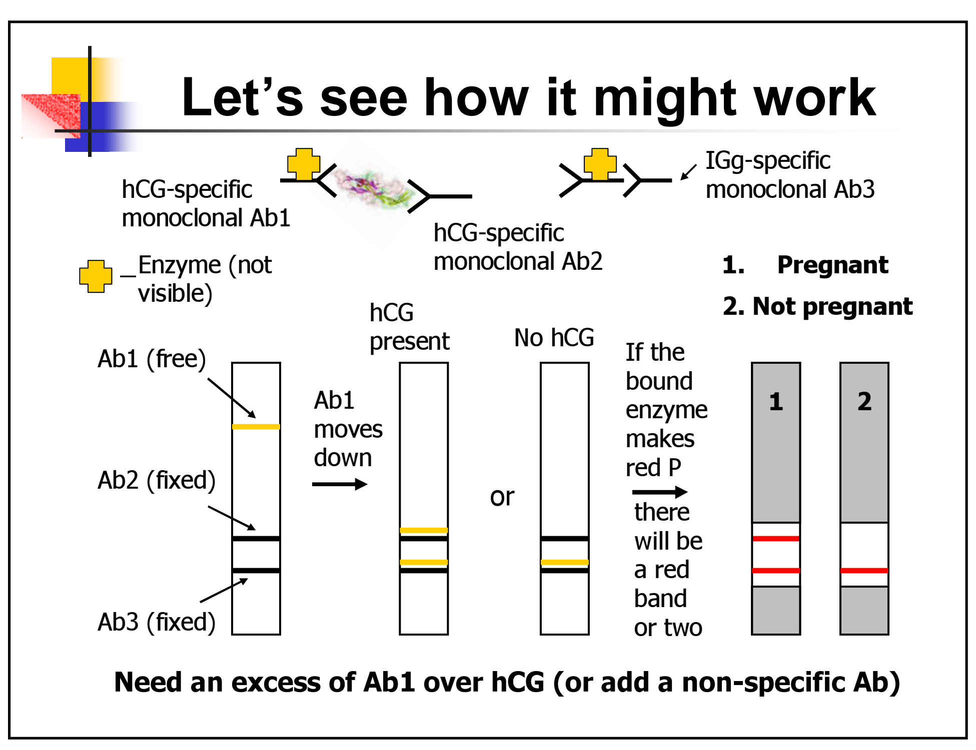
Amplification

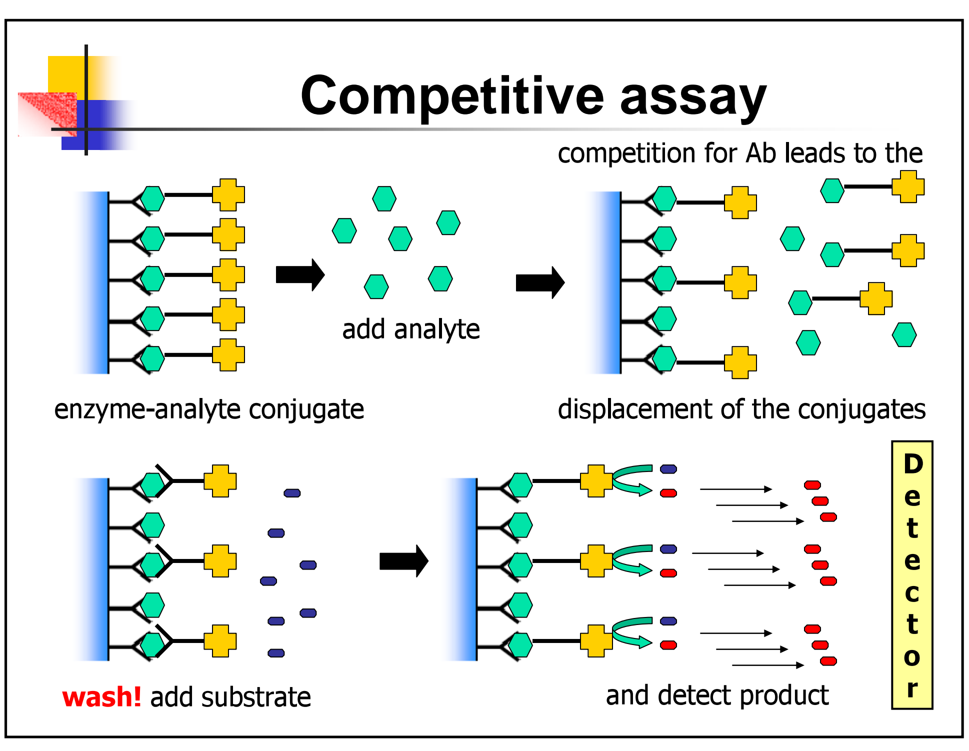
- Suppose we wish to build a sensitive Ab-based biosensor to detect a small MW analyte
- Detecting small targets at very low concentrations can be difficult and/or expensive
- To increase the sensitivity we can amplify the binding signal e.g. by generating a large number of molecules for every molecule bound by the antibody
- This would certainly make detection much easier…

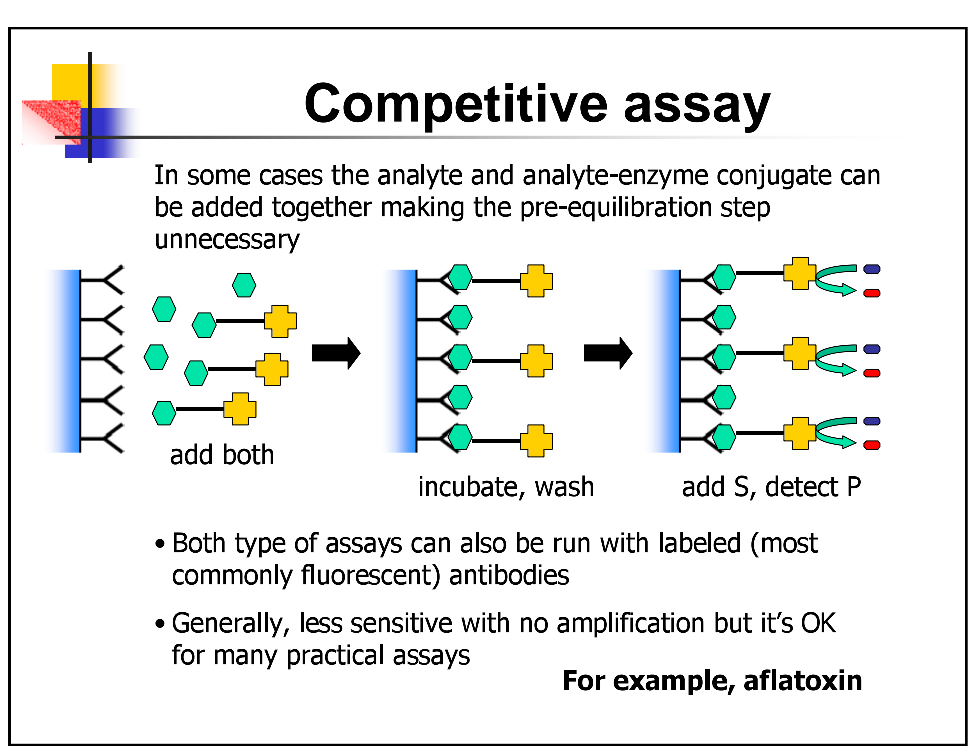
ELISA: Enzyme-Linked ImmunoSorbent Assay

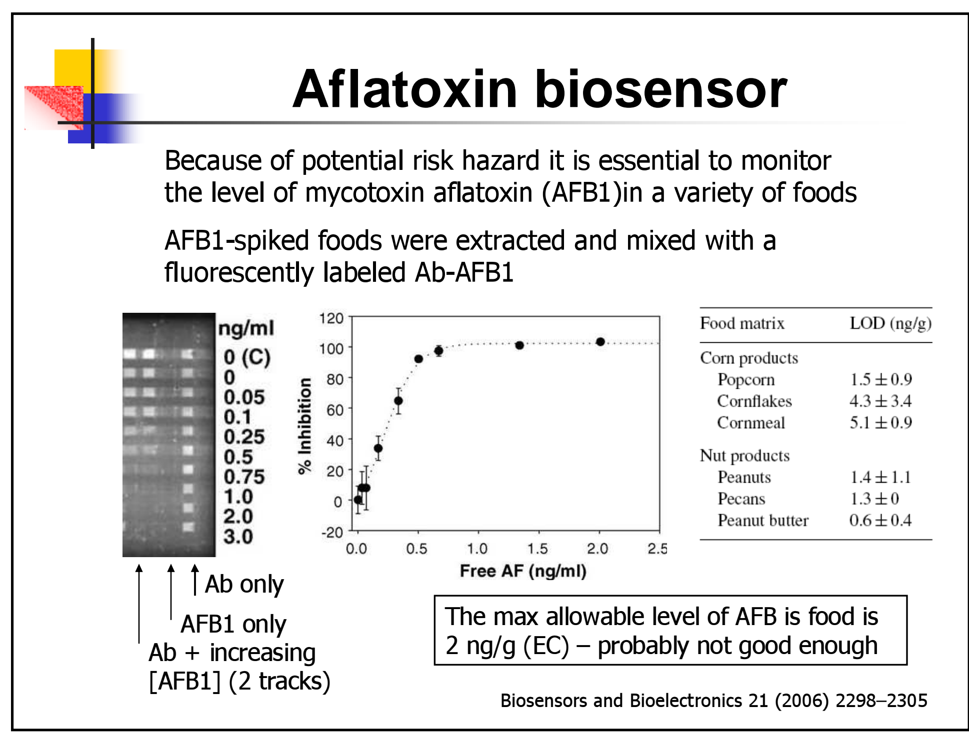
This is a technique for detecting target analytes with two antibodies, one of which is specific to the antigen and the second one is coupled to an enzyme (hence, the name). The enzyme linked to the second antibody catalyzes a reaction, the product of which is easily detected


Sandwich assay
analyte
immobilized ABs
binding
add the 2nd AB
it binds too
substrate
product
Detector
analyte
AB-enzyme
substrate
product

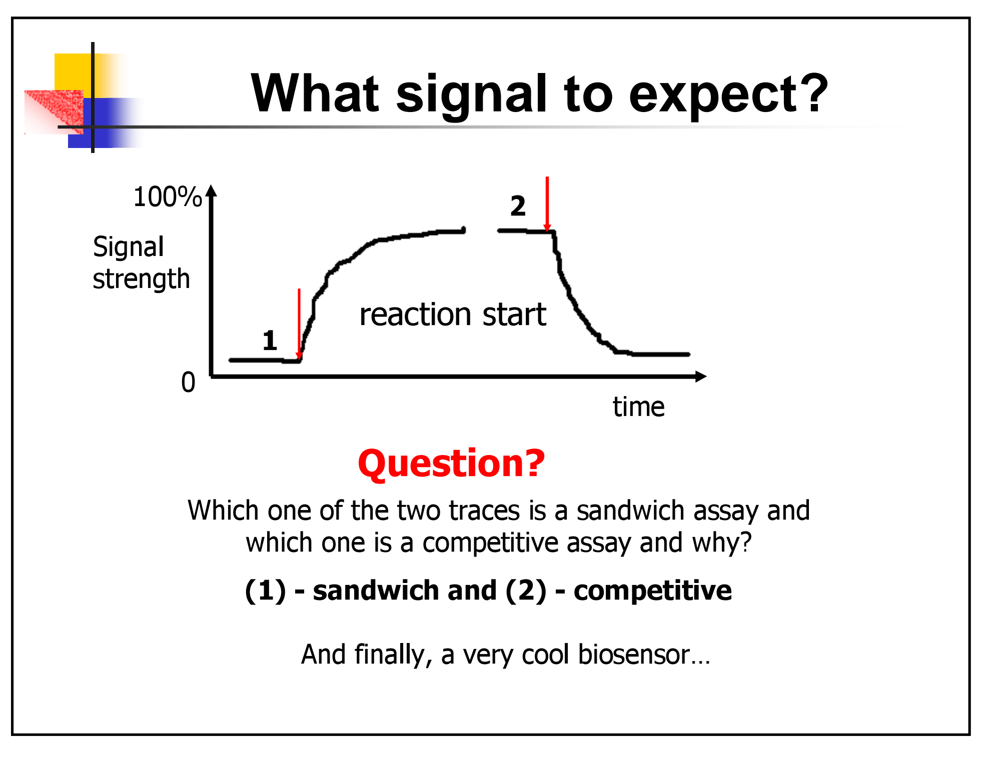

Let's see how it might work
hCG-specific monoclonal Ab1
IGg-specific monoclonal Ab3
hCG-specific monoclonal Ab2
Enzyme (not visible)
1. Pregnant
2. Not pregnant
Ab1 (free)
Ab2 (fixed)
Ab3 (fixed)
Ab1 moves down
hCG present
or
No hCG
If the bound enzyme makes red P
there will be a red band or two
1
2
Need an excess of Ab1 over hCG (or add a non-specific Ab)


Competitive assay
competition for Ab leads to the
add analyte
enzyme-analyte conjugate
displacement of the conjugates
wash! add substrate
and detect product
Detector

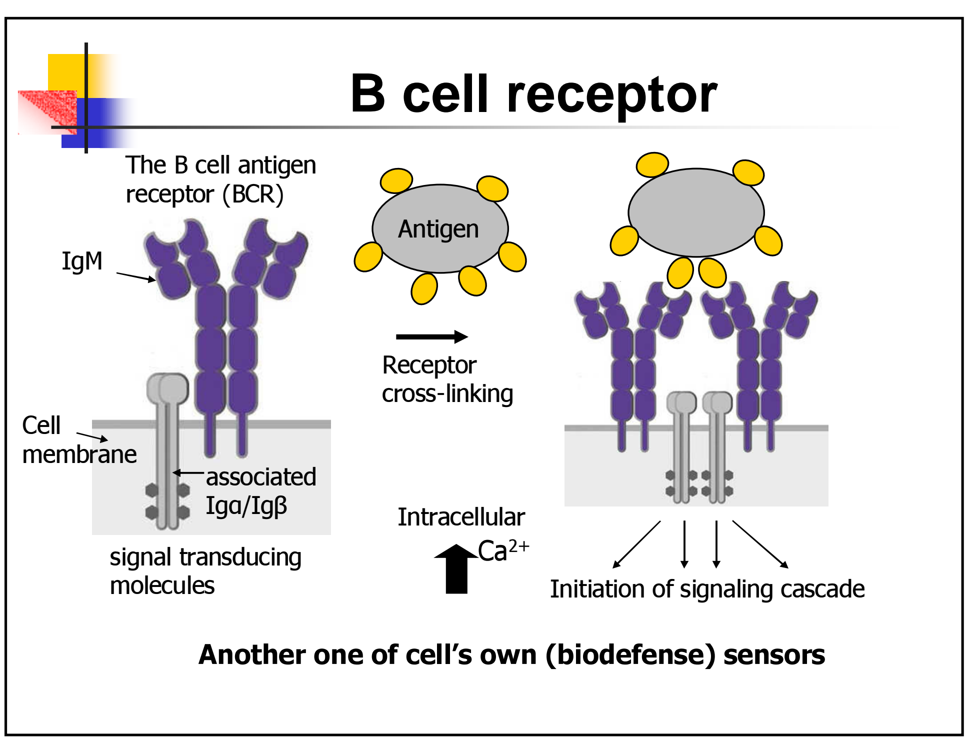

Competitive assay
In some cases the analyte and analyte-enzyme conjugate can be added together making the pre-equilibration step unnecessary
add both
incubate, wash
add S, detect P
• Both type of assays can also be run with labeled (most commonly fluorescent) antibodies
• Generally, less sensitive with no amplification but it's OK for many practical assays
For example, aflatoxin



Food matrix	LOD (ng/g)
Corn products	
Popcorn	1.5 ± 0.9
Cornflakes	4.3 ± 3.4
Cornmeal	5.1 ± 0.9
Nut products	
Peanuts	1.4 ± 1.1
Pecans	1.3 ± 0
Peanut butter	0.6 ± 0.4



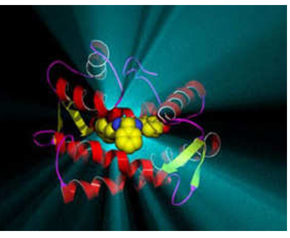
B cell receptor



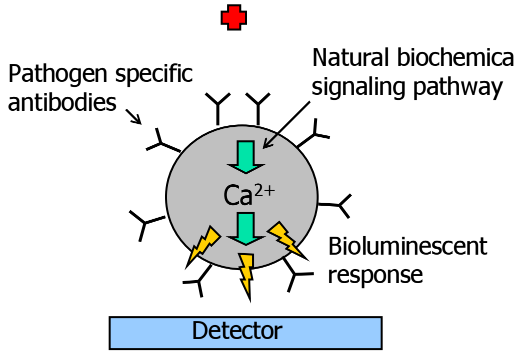
Another one of cell's own (biodefense) sensors

Hijacking BCR

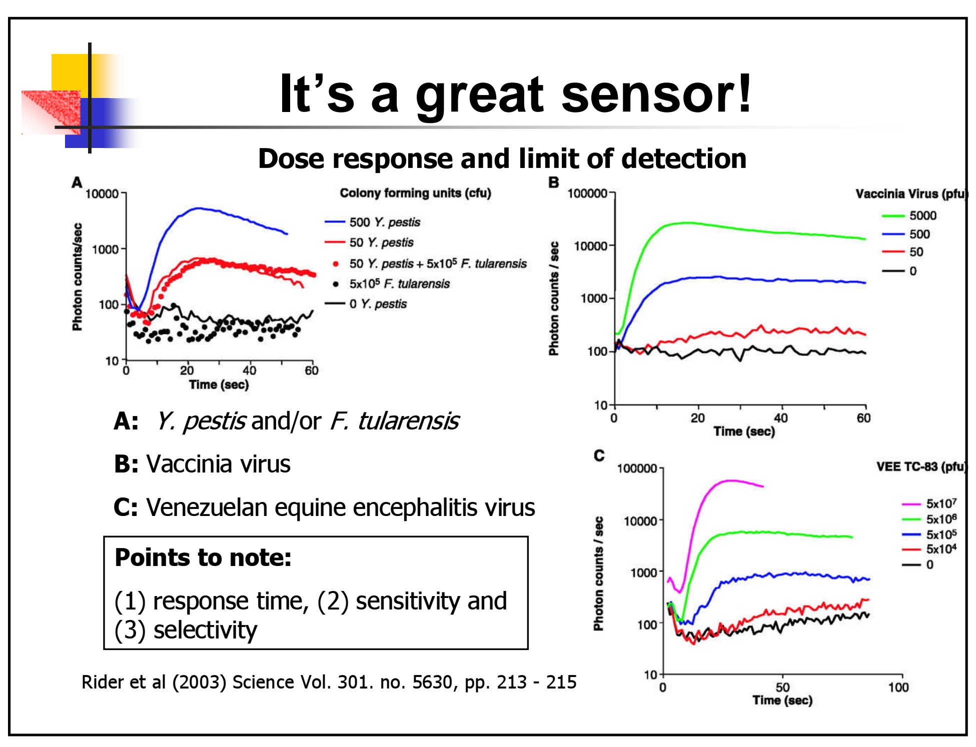
B cells were engineered to express aequorin, a Ca^{2+} sensitive bioluminescent protein from the Aequoria victoria jellyfish

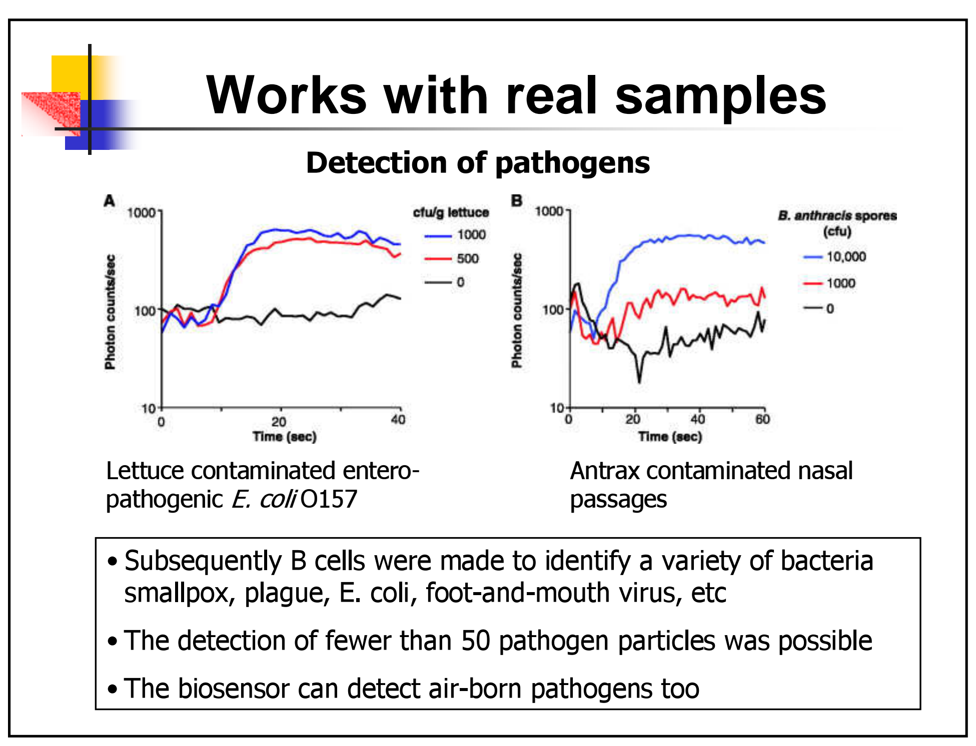


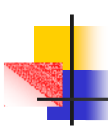
- 22 KDa protein from the luciferin family
- On Ca^{2+} binding aequorin undergoes a conformational change, and emits light on relaxation



The cells were also engineered to express membrane-bound antibodies specific for pathogens of interest

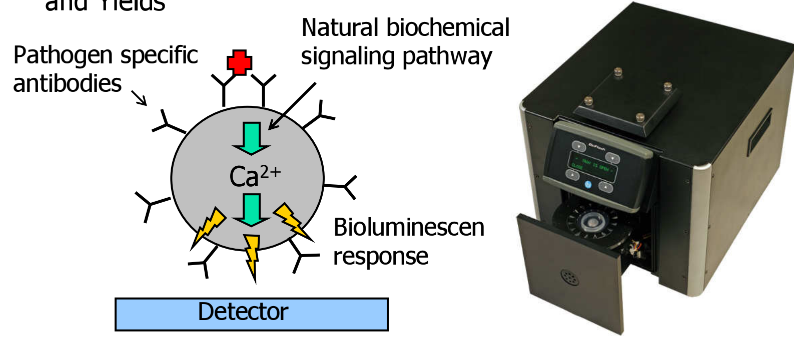

It's a great sensor!
Dose response and limit of detection
Colony forming units (cfu)
500 Y. pestis
50 Y. pestis
50 Y. pestis + 5x10^5 F. tularensis
5x10^5 F. tularensis
0 Y. pestis
Photon counts/sec
Time (sec)
Vaccinia Virus (pfu)
5000
500
50
0
Photon counts / sec
Time (sec)
VEE TC-83 (pfu)
5x10^7
5x10^6
5x10^5
5x10^4
0
A: Y. pestis and/or F. tularensis
B: Vaccinia virus
C: Venezuelan equine encephalitis virus
Points to note:
(1) response time, (2) sensitivity and (3) selectivity
Rider et al (2003) Science Vol. 301. no. 5630, pp. 213 - 215


Works with real samples
Detection of pathogens
cfu/g lettuce
1000
500
0
Photon counts/sec
Time (sec)
B. anthracis spores (cfu)
10,000
1000
0
Lettuce contaminated entero-pathogenic E. coli O157
Antrax contaminated nasal passages
• Subsequently B cells were made to identify a variety of bacteria smallpox, plague, E. coli, foot-and-mouth virus, etc
• The detection of fewer than 50 pathogen particles was possible
• The biosensor can detect air-born pathogens too



It is now commercial

CANARY® - Cellular Analysis and Notification of Antigen Risks and Yields



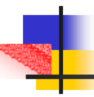
- CANARY has been developed at MIT who licensed the technology to Innovative Biosensors (Rockville, MD)
- Commercially available for biodefense use and a variety of non-military applications (http://www.innovativebiosensors.com/)

To summarize

- Antibody structure – needed for the exam
- Production and directed evolution of Abs
- Assay formats
- Some pretty sensors

and now let's take a

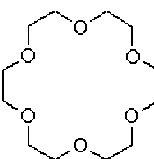
and the quiz ☹

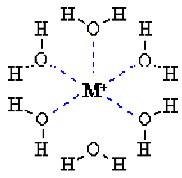


Biomimetic receptors

Can we make chemical receptors with selectivity comparable to biomolecules?

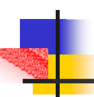
Crown ethers are heterocyclic compounds that, in their simplest form, are cyclic oligomers of ethylene oxide





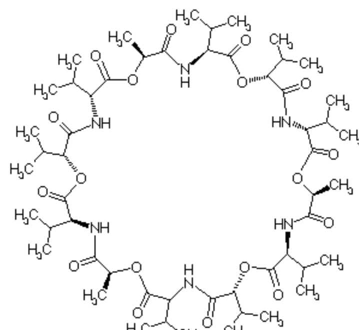
18-Crown-6 Me-complex hydration of a metal ion

The basic repeating unit of a simple crown ether is -CH2CH2O-; in 18-crown-6 it is repeated six times



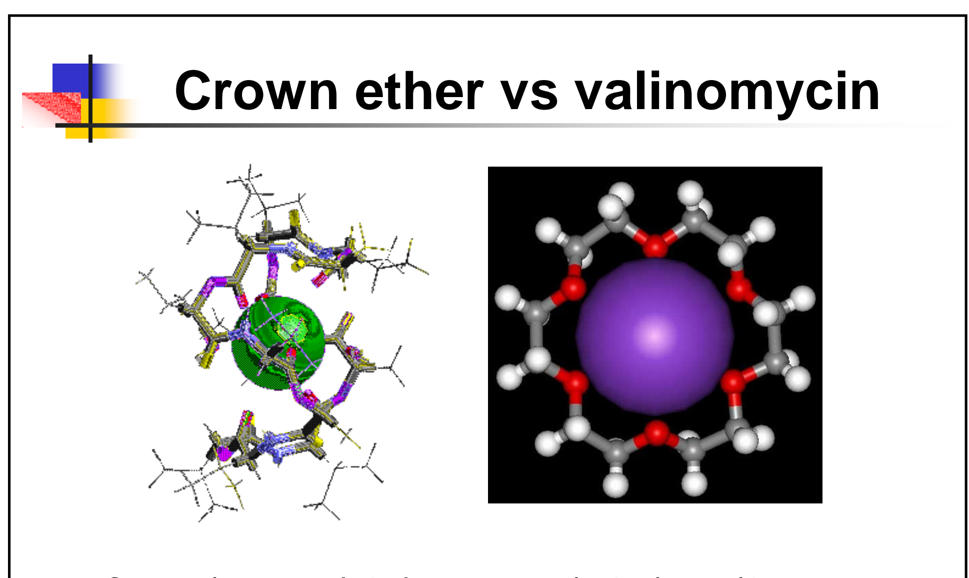
Biomimetic receptors

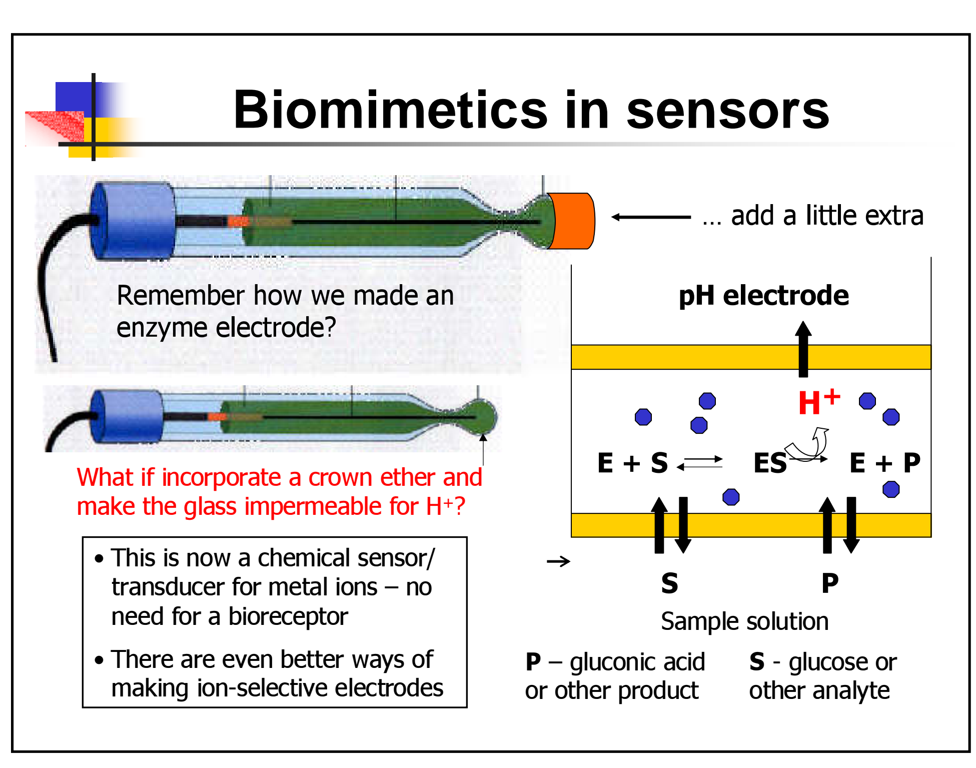
Crown ethers bind metal ions very strongly and with high selectivity, almost like valinomycin

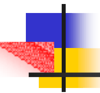


- Valinomycin is a cyclical poly-peptide
- It binds K^+ ~10,000 times stronger than Na^+

Difficult to compete with nature ☺

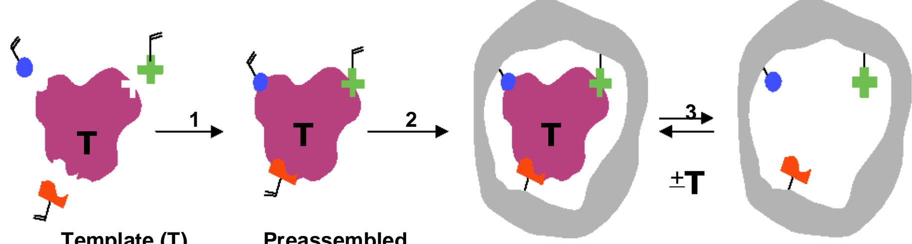

Crown ether vs valinomycin
Crown ethers are relatively easy to synthesize but making more complex artificial receptors/biomimetics typically requires a substantial synthetic effort


Biomimetics in sensors
… add a little extra
Remember how we made an enzyme electrode?
What if incorporate a crown ether and make the glass impermeable for H+?
This is now a chemical sensor/ transducer for metal ions – no need for a bioreceptor
There are even better ways of making ion-selective electrodes
pH electrode
H+
E + S
ES
E + P
S
P
Sample solution
P – gluconic acid or other product
S - glucose or other analyte



Plastic Antibodies

Molecular Imprinting is a technology for introducing highly selective recognition sites into cross-linked polymeric matrixes



Principle: To use the target molecule (e.g. analyte) as a template for creating its own recognition site

Imprinting Strategies: Covalent, non-covalent and "mixed"

Molecular Imprinting

Non-covalent approach

Dipeptide derivative

Template?

Phe

Functional monomer

EGDMA, polymerize

Trp

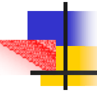
Non-covalent template-monomer complex

Imprint is spatially and functionally complementary to template

Advantages: simplicity, simplicity and simplicity

Disadvantages: heterogeneity and low binding capacity – many more sites with 1, 2, 3, etc COOH groups than with 5

K Mosbach and co-workers



Molecular Imprinting

Covalent approach

polymerize

- galactose

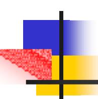
+ galactose

Easily/spontaneously formed covalent bonds

Advantages: low non-specific binding and much better control over polymerization conditions

Disadvantages: rather limited chemistry

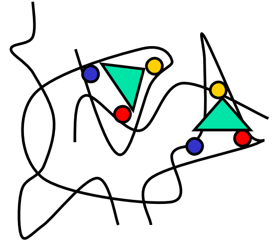
G. Wulff and co-workers

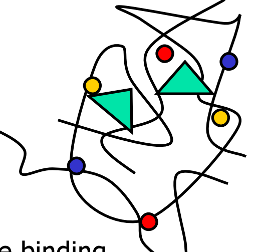


Very important point!

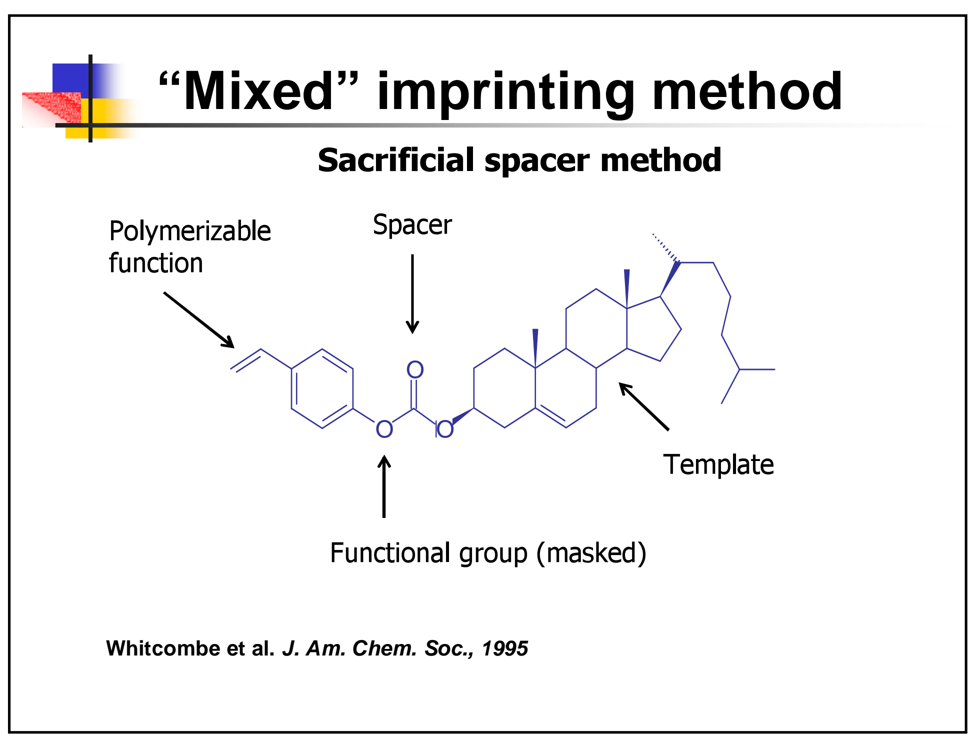
Imprinted and non-imprinted polymer have exactly the same chemical composition and functionality i.e. chemically identical

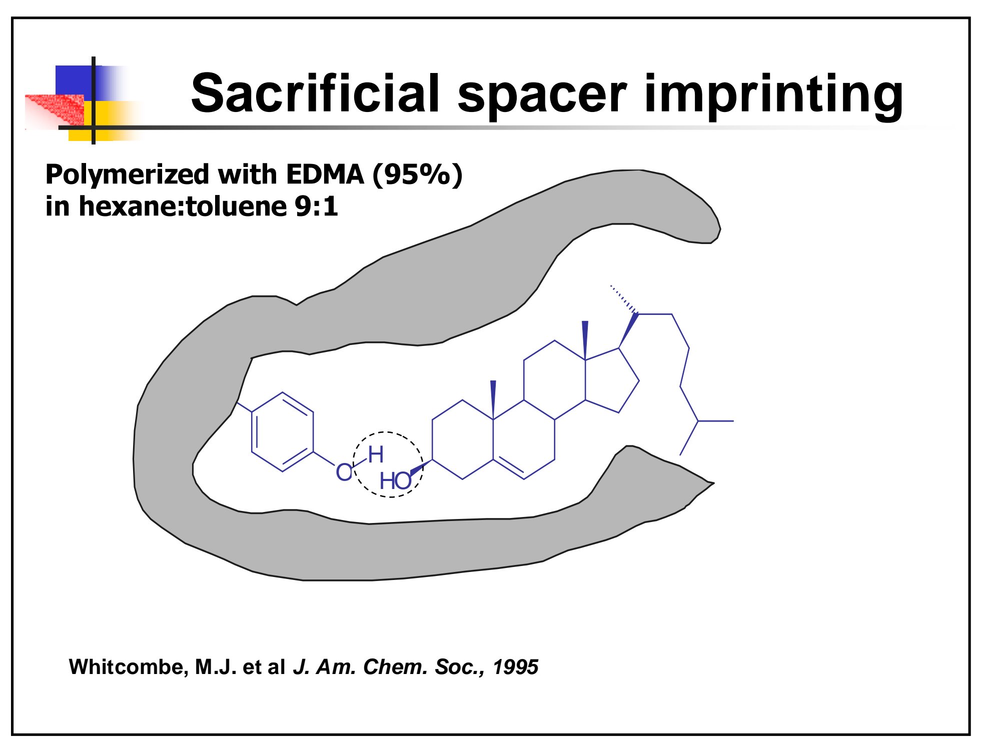
What is the difference?

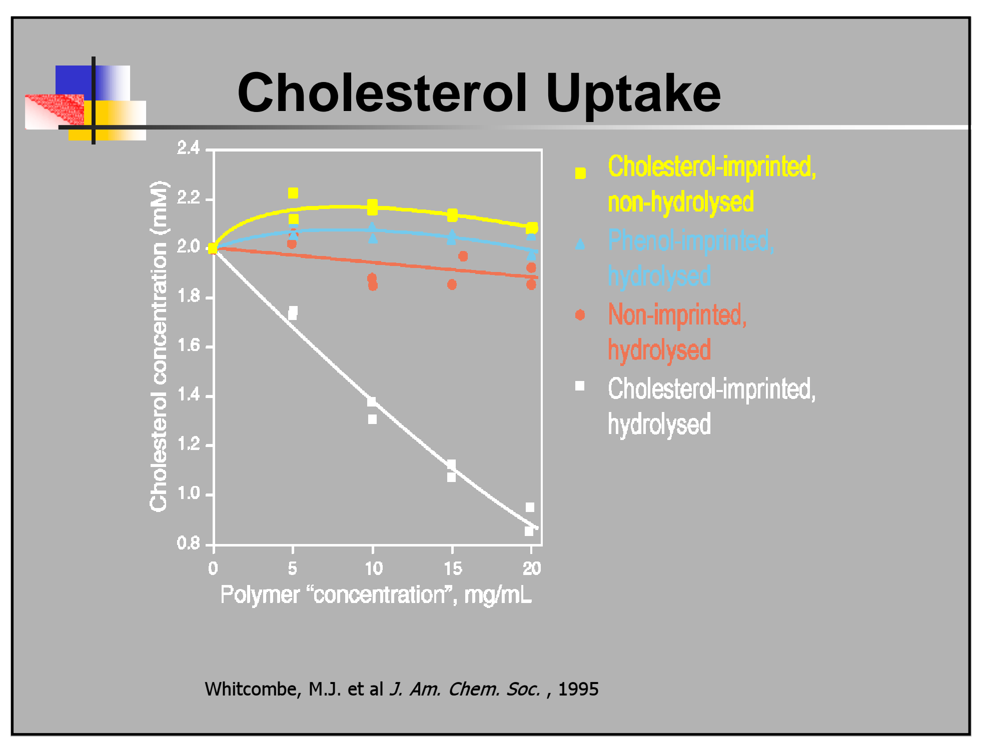


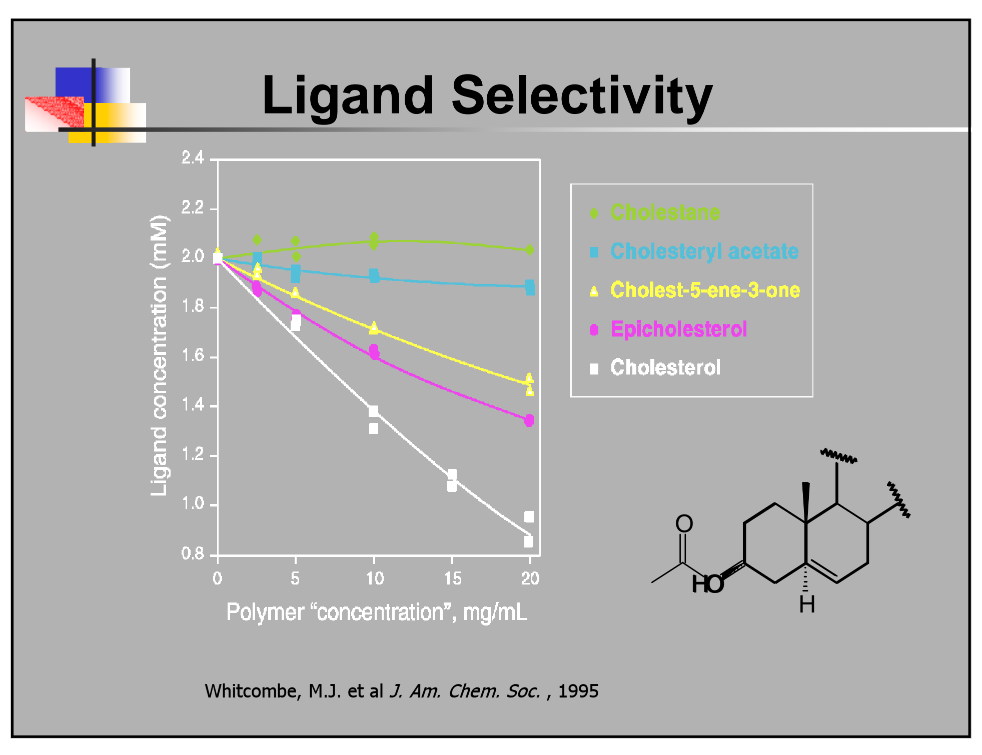


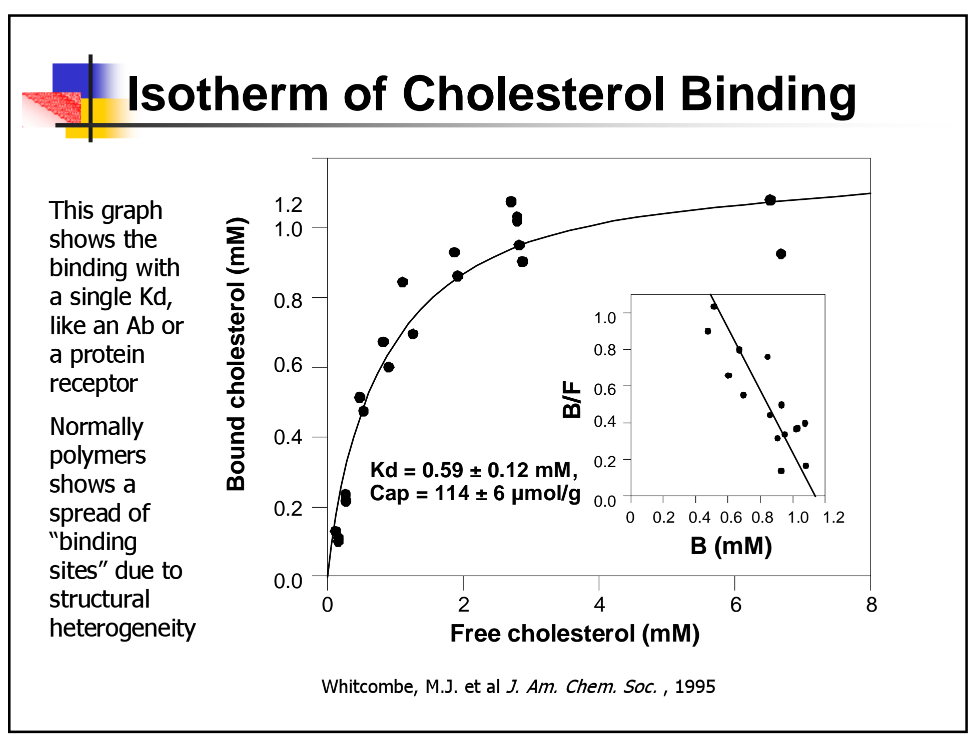
- The functional groups that make up the binding site are positions (or should be) in the precise spatial arrangement
- The size of the site (cavity) is more accurately defined

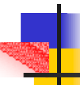

"Mixed" imprinting method
Sacrificial spacer method
Polymerizable function
Spacer
O
O
O
Template
Functional group (masked)
Whitcombe et al. J. Am. Chem. Soc., 1995


Sacrificial spacer imprinting
Polymerized with EDMA (95%)
in hexane:toluene 9:1
O
H
HO
Whitcombe, M.J. et al J. Am. Chem. Soc., 1995


Cholesterol Uptake
Cholesterol concentration (mM)
Polymer “concentration”, mg/mL
Cholesterol-imprinted, non-hydrolysed
Phenol-imprinted, hydrolysed
Non-imprinted, hydrolysed
Cholesterol-imprinted, hydrolysed
Whitcombe, M.J. et al J. Am. Chem. Soc. , 1995

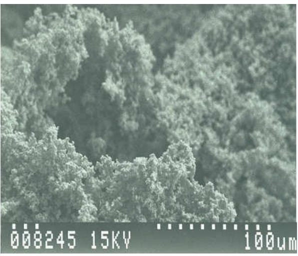

Ligand Selectivity
Ligand concentration (mM)
Polymer “concentration”, mg/mL
Cholestane
Cholesteryl acetate
Cholest-5-ene-3-one
Epicholesterol
Cholesterol
O
HO
H
Whitcombe, M.J. et al J. Am. Chem. Soc. , 1995

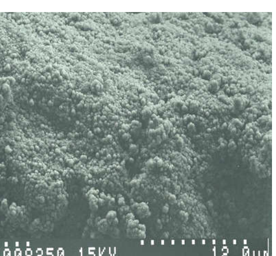


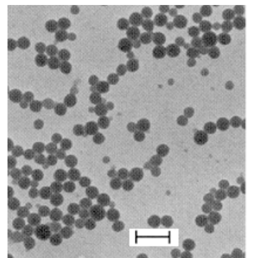


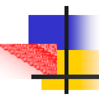
Sacrificial spacer: Summary

- Introduces functionality only into the intended recognitions site (covalent template – no free functional monomer) and binding is faster (the site relies on non-covalent interactions)
- More uniform binding constants (like Abs)
- Compatible with a wide range of polymerization conditions (bulk, emulsion, suspension polymerization, etc) – allows for polymer fine tuning in terms of performance, manufacturing flexibility









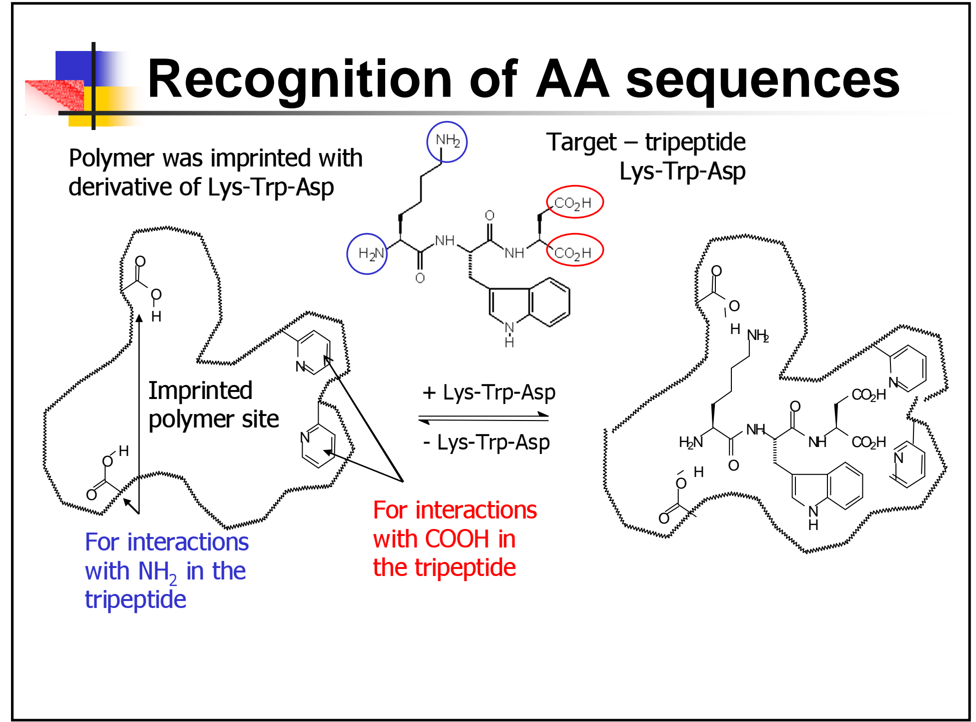
Imprinted polymers

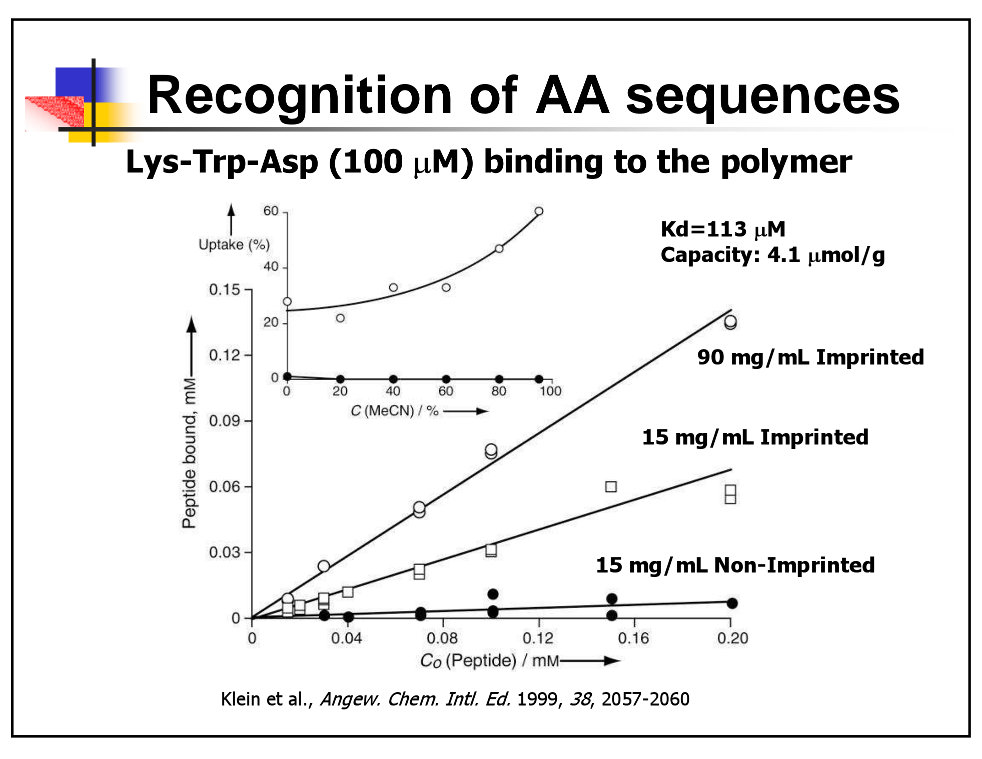
How specific is "specific"?

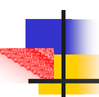
- Imprinted polymers have been shown to display selectivities similar to that of polyclonal antibodies (Vlatakis et al, Nature, 1993)
- The polymers can bind ligands with a single dissociation constant like a true biological receptor (Whitcombe et al, JACS, 1995)
- Can they also recognize specific amino acid or nucleotide sequences – a much more difficult test of the plastic antibodies' ability?

Is it possible to make a piece of plastic specific to an amino acid sequence?

Recognition of AA sequences





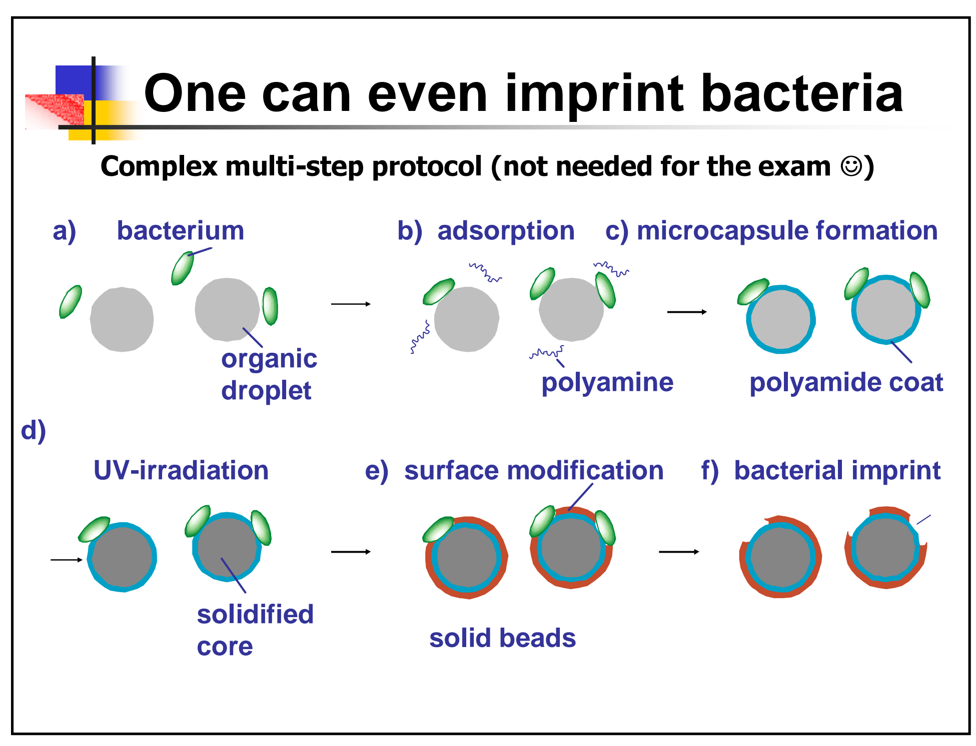


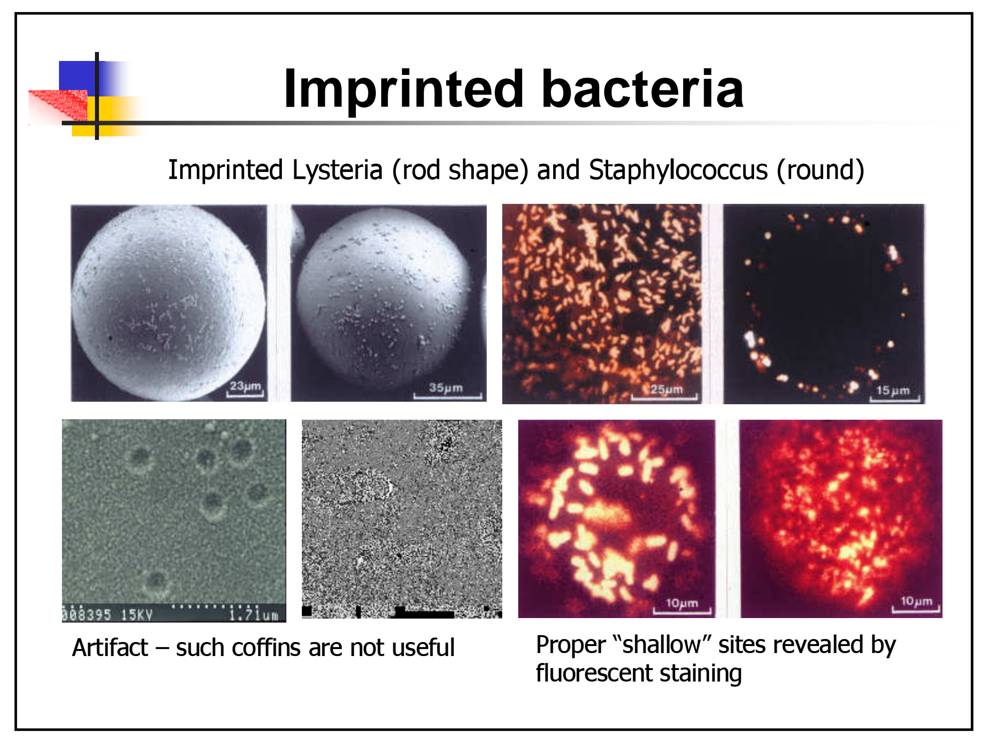
Specificity study:

Peptide binding

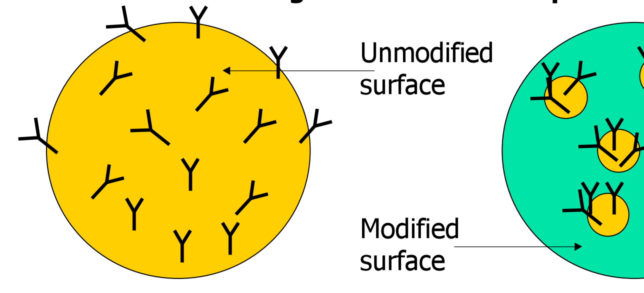
Sequence	Percentage bound	
	Imprinted polymer	Non-imprinted
Lys-Trp-Asp	43	11
Arg-Trp-Asp	24	9
Leu-Trp-Asp	<2	6
Gln-Trp-Asp	<2	<2
Lys-Phe-Asp	4	<2
Lys-Trp-Asp	5	3
Lys-Trp	35	17
Lys-Phe	9	5
Lys-Val	15	4

Klein et al., *Angew. Chem. Intl. Ed.* 1999, *38*, 2057-2060

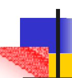

One can even imprint bacteria
Complex multi-step protocol (not needed for the exam ☺)
a) bacterium
organic droplet
b) adsorption
polyamine
c) microcapsule formation
polyamide coat
d)
UV-irradiation
solidified core
e) surface modification
solid beads
f) bacterial imprint


Imprinted bacteria
Imprinted Lysteria (rod shape) and Staphylococcus (round)
23μm
35μm
25μm
15μm
10μm
10μm
Artifact – such coffins are not useful
Proper "shallow" sites revealed by fluorescent staining

Why bother with bacteria/cells?



- Targeting to the right spot - less antibodies required
- Much lower non-specific binding to chemically modified "non-sticky" surface
- Higher specific binding due to higher concentration of Abs per unit of surface

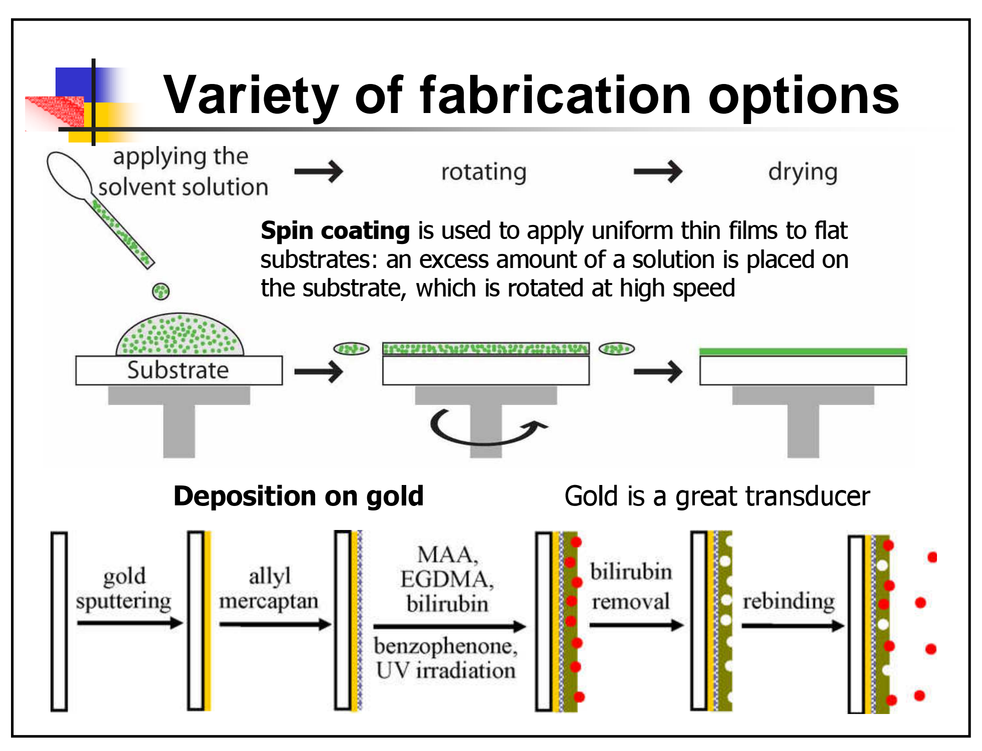


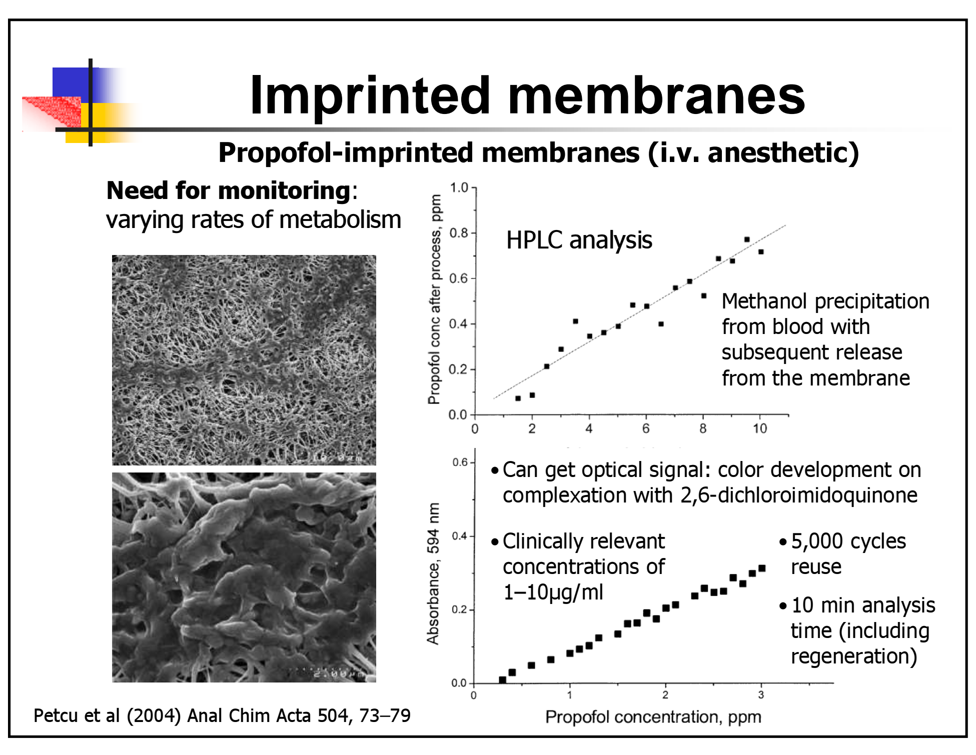
Applications of Imprinted polymers

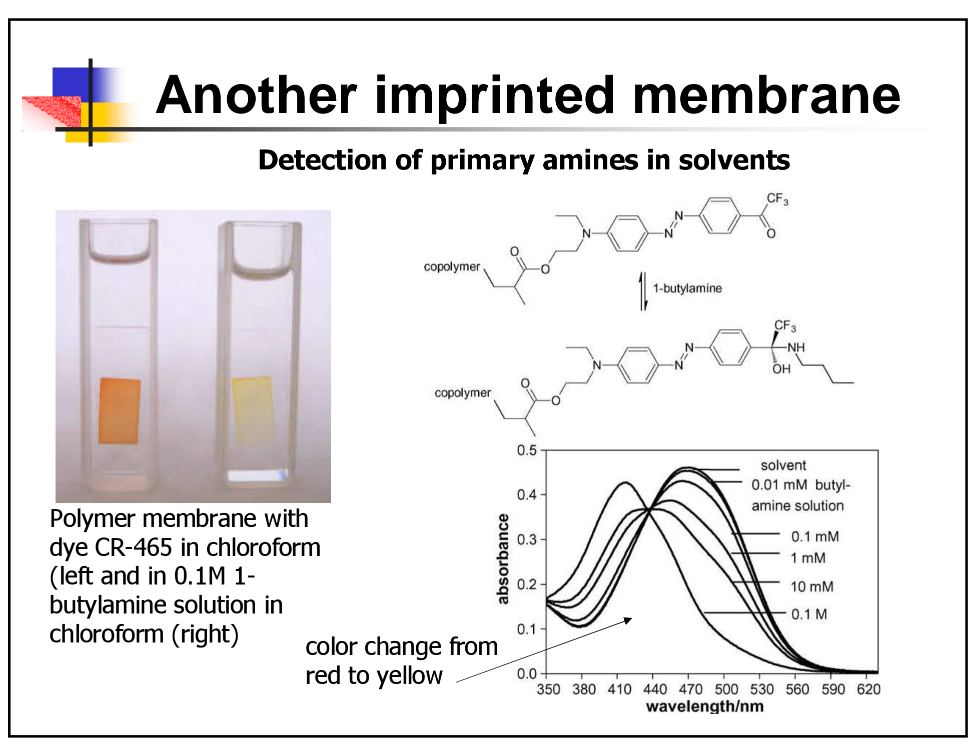
- Biomedical analysis and sensors
- Selective chromatographic materials
- Resolution of racemic mixtures
- Removal of undesirable components from complex mixtures/dilute fermentation streams
- **And in sensors!**

Generally, the selectivity of imprinted polymers is <u>much lower</u> than that of antibodies but they are by far cheaper and work well/better in some applications e.g. solvents

- **Antibodies cost:** **$1 per mg** (yet to be achieved)
- **Polymer cost:** **$1 per kg** (now!)


Variety of fabrication options
applying the solvent solution
rotating
drying
Spin coating is used to apply uniform thin films to flat substrates: an excess amount of a solution is placed on the substrate, which is rotated at high speed
Substrate
Deposition on gold
Gold is a great transducer
gold sputtering
allyl mercaptan
MAA, EGDMA, bilirubin
benzophenone, UV irradiation
bilirubin removal
rebinding


Imprinted membranes
Propofol-imprinted membranes (i.v. anesthetic)
Need for monitoring: varying rates of metabolism
HPLC analysis
Methanol precipitation from blood with subsequent release from the membrane
Propofol conc after process, ppm
Can get optical signal: color development on complexation with 2,6-dichloroimidoquinone
Clinically relevant concentrations of 1–10μg/ml
5,000 cycles reuse
10 min analysis time (including regeneration)
Absorbance, 594 nm
Propofol concentration, ppm
Petcu et al (2004) Anal Chim Acta 504, 73–79



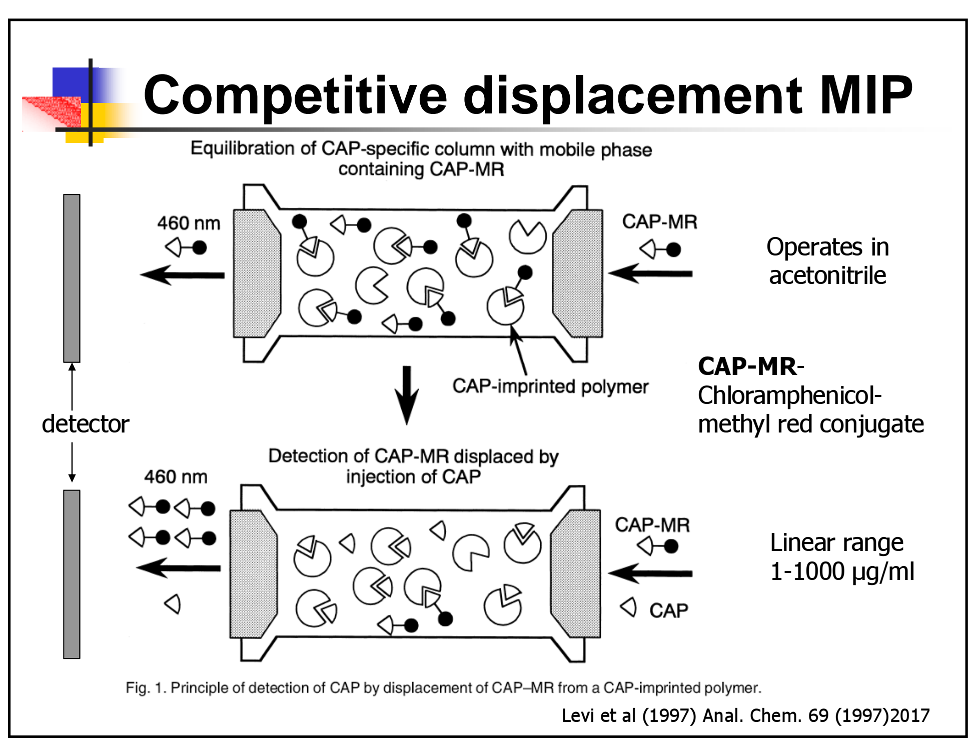
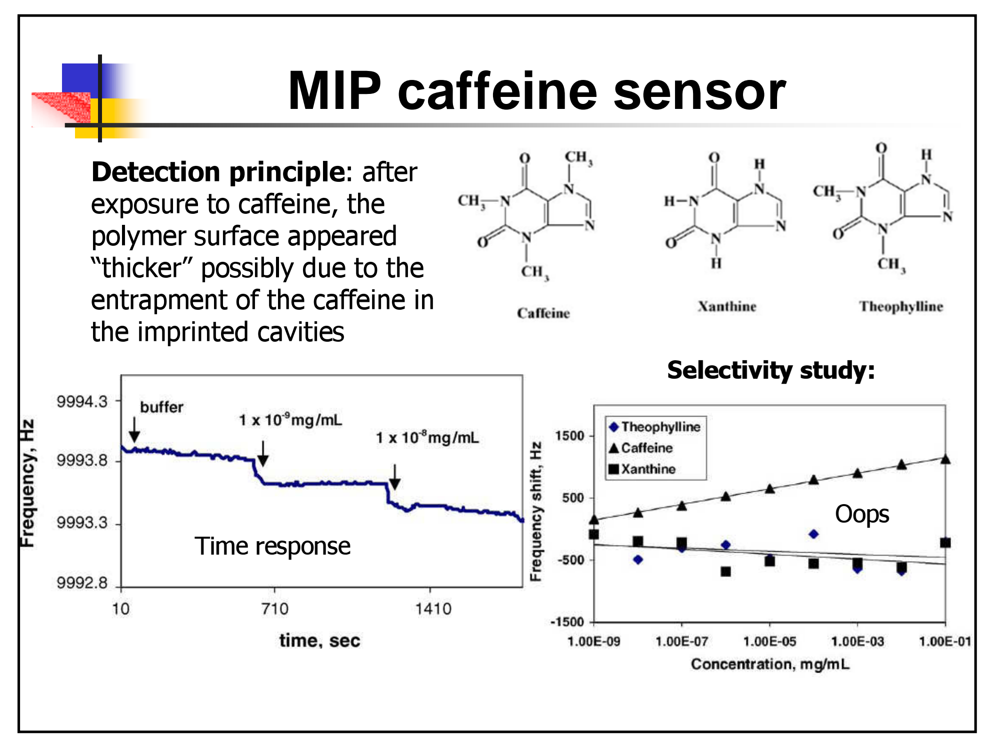


Fig. 1. Principle of detection of CAP by displacement of CAP–MR from a CAP-imprinted polymer.

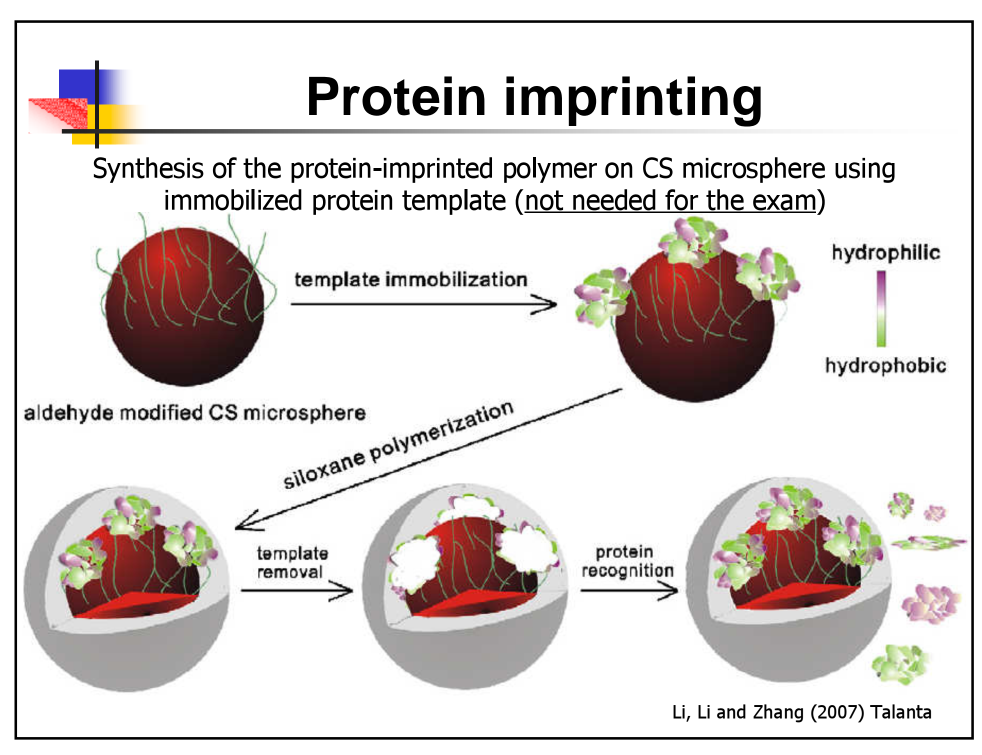


Imprinter polymers in sensors

Summary of sensor applications using molecularly imprinted polymers

Class	Typical analyte	Functional monomer	Detection range
Fluorimetry	Triazine	MAA	0.01–100 m*M*
	Sialic acid	Allylamine+TVPhB	0.5–10 µ*M*
	Dansyl-L-phenylalanine	MAA, 2VPy	0–30 µg/ml
	Pyrene	Aromatic polyurethane	0–40 µg/l
	cAMP	DMASVBP+HEMA	0.1–100 µ*M*
	NATA	HEAPTES+TES	Qualitative
Conductometry	Atrazine	DEAEM	0.01–0.5 mg/l
	Sialic acid	Allylamine+TVPhB	1–50 µ*M*
	Morphine	MAA	Qualitative
	L-Phenylalanine	DEAEM	0.05–0.4 m*M*
Spectrometry	Chloramphenicol	DEAEM	1–1000 µg/ml
	Chloramphenicol	DEAEM	3–30 µg/ml
	Testosterone	MAA	0.10-1.25 m*M*
Potentiometry	Phenylalanine anilide	MAA	33–3300 µg/ml
Capacitance	Phenylalanine anilide	MAA	Qualitative
Amperometry	Morphine	MAA	0.1–10 µg/ml
SAW, QMB	*o*-Xylene	Aromatic polyurethane	Qualitative
Luminescence	PMP	Eu^{III}+DVMB	0.125–150 000 µg/l
pH	Glucose	STACNCu	0–25 m*M*
SPR	Theophylline	MAA	0.4–6 mg/ml

Yano and Karube Trends Anal Chem (1999) 18, 1999



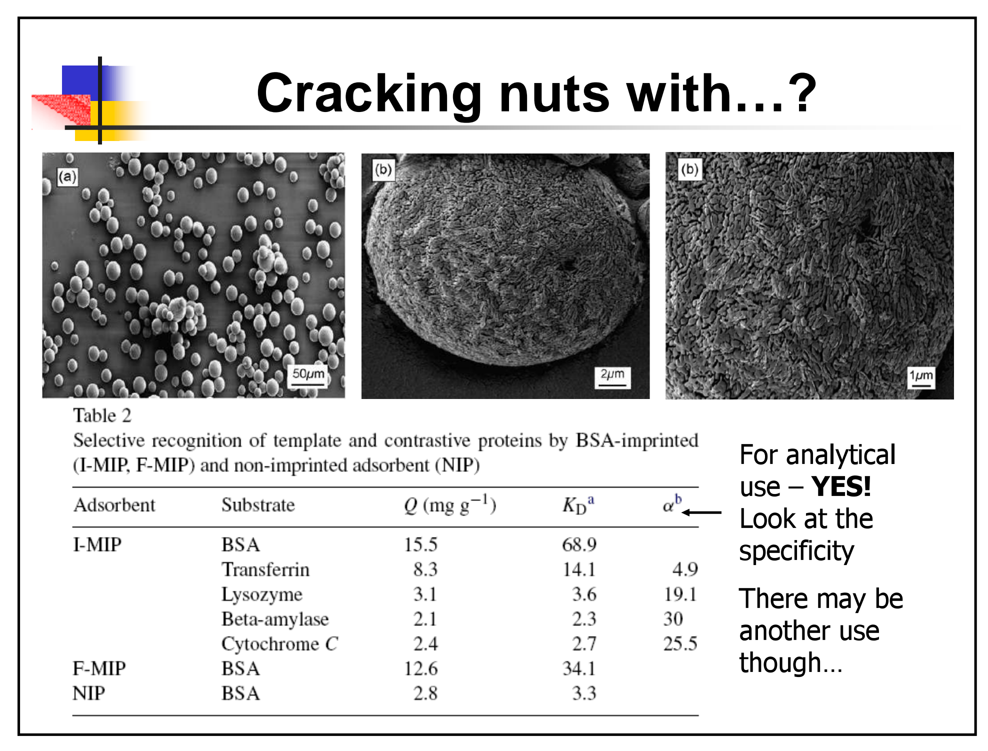
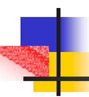


Table 2
Selective recognition of template and contrastive proteins by BSA-imprinted (I-MIP, F-MIP) and non-imprinted adsorbent (NIP)

Adsorbent	Substrate	Q (mg g^{-1})	K_D[a]	α[b]
I-MIP	BSA	15.5	68.9	
	Transferrin	8.3	14.1	4.9
	Lysozyme	3.1	3.6	19.1
	Beta-amylase	2.1	2.3	30
	Cytochrome *C*	2.4	2.7	25.5
F-MIP	BSA	12.6	34.1	
NIP	BSA	2.8	3.3	



In conclusion

- There is a fierce competition between technologies in the sensor's space – price vs quality
- Chemical sensors have upper hand in a number of applications (e.g. gases, ions), especially with simpler analytes and/or molecular mixtures in a sample
- Biosensors are generally superior in more complex, especially biomedical analysis, and when a very high selectivity/sensitivity is required, but this may change…
- **The bottom line**: never underestimate the competition

Have fun and
see you next week