Welcome to Lecture 8

Fluorescence
Excitation: $S_0 + \text{hv} \rightarrow S_1$
Relaxation: $S_1 + \text{hv} \rightarrow S_0$

Last time:
Fluorimeters
Light source (can be a laser)

Stokes shift and filters:

Quantum Yield: $\Phi = \frac{N \text{ photons emitted}}{N \text{ photons adsorbed}}$

At the last lecture

Excitation
Quenching
More heat

Quencher
Less fluorescence

Photobleaching

Return to So
Cool sensors and materials

Antrax immunosensor

Melanophores on fluorescent beads

LMW dyes:

2.3nm → 5.5nm

UV-excited CdSe quantum dots

Conduction band

QDs: depends on size

Valency band

QDs in sensor and imaging

Specific marker at cell surface

Direct targeting

Active tumor targeting

Normal blood vessel

Tumor angiogenic vessel

Ab-QD conjugates in cancer detection

Imaging
How colors work

It’s all in our minds

How CCDs detect colors

Bayer filters

Or you have to do it three times

3 CCDs
Animal vision

**BIRDS**: the inner segment contains a colored oil droplet at the base of the outer segment – natural filter through which light must pass before reaching rhodopsin

- The oil droplets are of several colors, due to the presence of different carotenoids
- Also, birds have 4 chromatic channels (some 5) i.e. they are tetra- or penta-chromatic

**BEES and BUTTERFLIES**: The range of vision extends well into UV, presumably because many flowers they pollinate have special ultraviolet patterns that guide the insects deep into the flower

In dogs the central portion of retina is primarily composed of rods, they see well in the dark and probably detect motion and flickering much better. Also dogs have dichromatic vision - they only have two types of cones. Dogs are red-green color blind; occurs in ~4% of humans too

Human vision

We are also different from machines

Suppose a human and a robot look at the same colored picture

What would a robot like the Terminator “see” when looking at a colored object?
**Human vs Terminator**

**Difference in signal detection:**

<table>
<thead>
<tr>
<th></th>
<th>A nm</th>
<th>B nm</th>
<th>C nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>X nm</td>
<td>255</td>
<td>204</td>
<td>153</td>
</tr>
<tr>
<td>Y nm</td>
<td>102</td>
<td>51</td>
<td>0</td>
</tr>
</tbody>
</table>

**The Terminator**

R + G = Y  R + B = M  G + B = C  R + G + B = W

---

**Human vs Terminator**

- Do robots appreciate paintings? 😊

**Immobilization**

**Goals:**

- To bring the recognition (sensing) biomolecules in close contact with transducer to facilitate signal transduction
- To stabilize biomolecules with the aim of increasing reusability and shelf-life of the biosensor device

**Bioreceptor groups:**

- Cells (including bacteria) and tissues
- Proteins (e.g., enzymes, antibodies, receptors)
- Nucleic acids (DNA and RNA)

**Each group has its own requirements** such as acceptable matrix, chemical treatment, etc
Creating recognition interface

Means localization or attachment of a receptor on (or near to) the transducer surface

**Many approaches:**

- Physical entrapment near the surface e.g. the use of membranes or polymer gels
- Direct physical adsorption to the surface
- Covalent chemical coupling directly to the transducer or to an intermediate (e.g. polymer or a monolayer) on the transducer surface
- Non-covalent “capture” of the receptor, primarily using a highly specific bio-recognition system

**Whatever it is the biological activity must be preserved**

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Enzyme electrode

To do so, we’ll take a very conventional pH electrode and...

... add a little extra

Biocatalytic layer (sensor) is located between two membranes & contains free or immobilized enzyme

**glucose oxidase or other enzyme (E)**

Membrane permeable to S and P but not to E

**S - glucose or other analyte**

**P – gluconic acid or other product**

**Sample solution**

**pH electrode**

$E + S \rightarrow ES \rightarrow E + P$

$S \text{ glucose or other analyte}$

$P \text{ – gluconic acid or other product}$
Membrane entrapment

Entrapment behind semi-permeable membranes is a simple method of retaining bioreceptors in a compartment adjacent to the transducer.

Plenty of inexpensive commercially available membranes:
e.g. cellulose dialysis tubing – MW cut-off ~5-10KDa would keep most proteins in and allow small analytes diffuse in and out.

Glycerol biosensors

Integrated multi-enzyme electrochemical biosensors for the determination of glycerol in wines:

- Glycerol normally formed in fermentation (~1:10 ratio to EtOH); determination of glycerol is important for industrial quality control e.g. fermentation indicator, detection of possible adulteration
- Design: Enz immobilization of gold electrode + dialysis membrane

Two amperometric sensors:
(i) glycerol dehydrogenase and (ii) glycerol kinase, both coupled to other enzymes to get a signal

Comparison of the two biosensors with conventional enzyme-based assay (white bars)

Wine samples

Analytica Chimica Acta 609 (2008) 201–209
**Optical biosensor**

**Electrochemical sensor**

- Electrode
- \[ E + S \rightleftharpoons ES \rightleftharpoons E + P \]
- \( S \rightarrow P \)

**Optical biosensor**

- Optical transducer or fiber optic probe
- \[ AH \rightleftharpoons A^- + H^+ \]
- \[ H^+ \rightarrow \]

A: can be a highly colored substance (e.g. pH indicator) or a fluorescent dye, to measure absorbance (OD) or fluorescence, respectively.

**And another way**

**Schematic diagram of chemomechanical glucose sensors**

- Membrane stuck can be used too
- Requires covalent immobilization.

Membrane entrapment

Advantages:
- Very simple and broadly applicable system for a wide range of receptors
- Mild conditions used – no chemicals, no procedures that can cause damage: the loss of activity or viability
- The bioreceptor can be maintained hydrated at suitable conditions (temp, pH) throughout
- The amount of volume is physically defined; hence control over size; excess of bioreceptor, if required

Disadvantages:
- Limited to certain type of transducers (e.g. optical, electrochemical) but no go with others (e.g. SPR)
- Often difficult to transfer to mass production (e.g. sealing wet membranes over devices)

Physical adsorption

Proteins and nucleic acids can be attached to a variety of surfaces by simple physical adsorption
- Generally proteins adsorb strongly to hydrophobic surfaces
- This adsorption is often followed by slow unfolding of the protein structure
- The process is thermodynamically driven to maximize the interaction between the protein core and the surface

Advantage: simple!

Disadvantages:
- Possible loss of activity due to denaturation
- Non-specific binding: any protein will stick to the surface too 😊
Physical adsorption

The surface does not have to be hydrophobic:
Proteins and nucleic acids typically carry a net charge at neural pH and can be attached to a surface with opposite charge

**Does it solve the problem? Not, entirely**

- Denaturation may no longer be an issue, but non-specific binding (surface “fouling”) can still be...
- Nevertheless physical adsorption can be used advantageously in some circumstances e.g.
  - (i) Adsorption of enzymes on carbon paste - electrode
  - (ii) Adsortion of DNA to positively charged membranes

**Bottom line:**
Physical adsorption often works best for single use applications

Immuno-strip biosensor

Enzyme-linked immuno-strip biosensor to detect *Escherichia coli* O157:H7

- About 90% of food-borne illnesses are caused by pathogenic microorganisms with *Escherichia coli* O157 being one of the most harmful - estimated 73,000 infection and 61 deaths in the U.S. each year.
- ELISA is the most widely used method to detect and quantify bacteria but it takes relatively long time and appropriate equipment is required.
- A membrane strip is a simple tool for rapid, on-site analysis - colorimetric immuno-sensor based on sandwich ELISA.
- Colorimetric signal can be easily quantified, if required, by using a digital camera and simple image analysis software.
Cross-flow strip design

1. A prepared sensor strip is placed into a sample (100 µL) for 15 min to absorb the solution into the strip in the vertical direction.

2. After immune reaction two pads – substrate application pad and adsorption pad - are arranged on each side of the signal generation pad to supply substrate.

3. Five min after the substrate addition, the color signal is captured by a digital camera.

The analytical procedure: (A) immuno-reaction in vertical direction and (B) enzymatic reaction in horizontal direction.

Adsorption on membranes

Four different membranes were used for preparation.

In every case physical adsorption to the membrane.

After applying all the solutions the strip is dried at 37°C for 1h.

**Biosensor performance**

Colorimetric signal produced by Horse Reddish Peroxidase at two specific positions at the signal generation pad

E. coli O157:H7 (1.8XCFU/mL)

Upper: Control signal
Lower: E coli 1057 signal

SEM images of the nitrocellulose membrane (signal detection pad) with E. coli O157 (white arrows) bound to immobilized mAb

**Entrapment in hydrogels**

A simple alternative to membranes

Dissolve polymer at elevated temperature
Add enzyme and start cooling down
A gel forms

- Polymer hydrogels can be dried into a film to better preserve bioreceptors on storage or between usage
- More important, hydrogels can by covalently cross-linked to improve stability of receptors
Hydrogels

Water swollen cross-linked polymers

**Cross-linking is achieved by:**

- Non-covalent interactions e.g. hydrogen bonds, charge, van der Waals forces
- Covalent chemical reaction in the presence of a suitable cross-linking agent:
  - Small MW agents that links two chains together by reacting with its functional groups, e.g. cross-linking natural polysaccharides with bi-functional cross-linkers that react with -OH, -NH₂ or -COOH groups present
  - Copolymerization-crosslinking reactions between the monomers and a multifunctional monomer that is present in small quantities e.g. synthesis of polyacrylamide gels

Don't confuse with Abs!

Natural hydrogels

A variety of materials are available

**Natural:** e.g. Agarose, Alginate, Carrageenan

- Complex oligosaccharides derived from natural sources
- Gelation can be triggered by various factors e.g. temp (agarose), or addition of Ca ions (alginate); low melting points variants e.g. agarose gel at 40°C

Hyaluronan (aka hyaluronic acid)  
Carrageenan: ~25KDa MW sulfated galactose derivatives

Obtained from a seaweed

Proteins e.g. collagen is used too
**Lymphocytes sense toxins**

This setup allows multiple parallels assays

Exposure to hazardous bacteria/toxins

- Detects whether a toxin or bacteria have "broken" the lymphocytes
- If damaged, lymphocytes release alkaline phosphatase, which can be detected by a color reaction

Quantitative values calculated by ALP release from damaged cells

Arrows: Non-pathogenic

Lin: *L. innocua*; Bs: *B. subtilis

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**Natural hydrogels in sensors**

A novel and simple cell-based detection system with a collagen-encapsulated B-lymphocyte cell line as a biosensor for rapid detection of pathogens and toxins

Pratik Banerjee¹, Dominik Lenz², Joseph Paul Robinson², Jenna L. Rickus¹ and Anum K Bhuria³

Strategy for cell-based biosensing using collagen-encapsulated cells and the assay procedure

Viability of sensor cells in collagen microenvironment

How do you access cells viability in a gel?

Laboratory Investigation (2008) 88, 196–206
Fluorescence

Laser scanning cytometry of the collagen matrix with entrapped cells

The images (a–f) are shown as merged and virtually colored images of blue (Hoechst 33342, 405 excitation line) and red (Propidium iodide, 488 excitation line) as obtained by the photomultiplier of the iCys LSC.

The blue cells were counted as "live", the red cells - as "dead" (a) Control, uninfected cells; (b) and (c) infected with L. monocytogene and toxin from B. cereus

Can be quantified too

Image analysis of the data as in the previous slide

AP detection is simpler and cheaper
**Synthetic hydrogels**

**Synthetic:** e.g. poly(vinyl) alcohol (PVA) makes excellent dry film and polyacrylamide allows for well controlled cross-linking (important to minimize leaching out!)

**Entrapment within conducting polymers:** Many redox enzymes can be directly “wired” into electrodes and the polymer can play a key role in the signal transduction mechanism. Pyrrole is a typical example. Polymerization can be carried out under mild conditions.

**Entrapment within sol-gel glasses:** Porous silicate network is formed under mild aqueous conditions. Sol gels are optically transparent (hence, a great choice for optical sensors) and provide excellent stabilization to bioreceptors. However, small pore size makes it difficult to use sol-gels with large analyte targets e.g. proteins.

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**GOD-PVA with O₂ electrode**

**Reusability, reproducibility and stability**

- **Oxygen electrode**
- **GOD-PVA membrane** (not to scale)

**Stability**
- Retained 90% activity on storage at 4°C for 30d; then decreased.
- Storage, d

**Reusability**
- 90 mg/dl
- 22.5 mg/dl

**Correlation with standard hospital analysis**

*Kumar and D'Souza (2008) Talanta 75, 183–188*
**X-linked polymeric networks**

Potentiometric Hepatitis B surface antigen (HBsAg) immunosensor

- Silica nanoparticles (d~100nm) are suspended in solution of chitosan and deposited on the electrode surface
- Treated with glutaraldehyde to obtain a 3D network (X-linking) and create extra CHO
- Silica particles removed by dissolution is aq HF
- Excess of aldehyde groups used to covalently immobilized Hepatitis B Abs (HBsA)

The signal is due to the increase of the electric charge density on the electrode surface after the antigen binding to the Ab

*SEM of the resulting porous chitosan film*

**Extensive optimization**

1) Effect of the amount of silica nanoparticles in chitosan solution on potentiometric response in the presence of 100 ngmL$^{-1}$ HBsAg

2) Effect of the amount of immobilized HBsAb on potentiometric response in the presence of 100 ngmL$^{-1}$ HBsAg

3) Further biochemical and electrochemical optimization too...

**Detection limit:** 3.89 ngmL$^{-1}$ HBsAg and linear range: 7-700 ngmL$^{-1}$

*J Colloid Interface Sci 320 (2008) 125–131*
Sensor performance

- Hepatitis B surface antigen (HBsAg) is a protein antigen produced by HBV
- HBsAg is the earliest indicator of acute hepatitis B infection
- Often it can be detected before symptoms appear; hence used for early diagnostics

<table>
<thead>
<tr>
<th>Sample number</th>
<th>ΔE (mV)</th>
<th>This method (ng mL⁻¹)</th>
<th>ELISA (ng mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.2</td>
<td>15.3</td>
<td>14.9</td>
</tr>
<tr>
<td>2</td>
<td>38.8</td>
<td>163.4</td>
<td>162.3</td>
</tr>
<tr>
<td>3</td>
<td>43.3</td>
<td>270.6</td>
<td>270.9</td>
</tr>
<tr>
<td>4</td>
<td>45.6</td>
<td>355.4</td>
<td>354.7</td>
</tr>
<tr>
<td>5</td>
<td>48.7</td>
<td>561.2</td>
<td>561.8</td>
</tr>
</tbody>
</table>

Sol-gel chemistry

Organotrialkoxysilanes, RSi(OR')₃ are sol-gel monomers and coupling agents. They are commonly used to modify surfaces and to prepare coatings for microelectronics and photonics applications.

- Sol-gels are frequently used for entrapment of biomolecules
- In the absence of "interfering" molecules a very regular polymer network is obtained
**Immobilization in sol-gels**

Suitable for entrapment of a wide range of proteins

Drawbacks: accessibility, some inactivation, mass transfer

- Sol gels are optically transparent and provide excellent stabilization to biomolecules
- This chemistry is widely used for surface modification of certain type of transducers: coating thickness typically <5μm and often < 1μm

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**A sensor we discussed**

SEM of AChE/Al2O3 sol-gel-carbon electrode - granular (porous) structure due to presence of AChE

**Table 1**

Comparison between the three pesticides, 0.2 M phosphate buffer, pH 7.0, applied potential: 210 mV, 1 mM acetylcholine

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Limit of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraoxon (M)</td>
<td>7.5 ± 10^{-8}</td>
</tr>
<tr>
<td>Dichlorvos (M)</td>
<td>5.0 ± 10^{-9}</td>
</tr>
<tr>
<td>Chlorpyrifos-ethyl-ami (M)</td>
<td>2.5 ± 10^{-10}</td>
</tr>
</tbody>
</table>

*Zejli et al Talanta 77 (2008) 217–221*

**Note:**

Immiscibility can change pH dependence
Membranes vs gels

<table>
<thead>
<tr>
<th>Entrapment</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane</td>
<td>• Simple and universal&lt;br&gt;• Very mild conditions&lt;br&gt;• Often long shelf life</td>
<td>• Diffusion limitations can be significant&lt;br&gt;• Difficult to mass produce</td>
</tr>
<tr>
<td>Polymer gels</td>
<td>• Much easier to mass produce&lt;br&gt;• Highly efficient solution for many particular applications</td>
<td>• Higher possibility of denaturation&lt;br&gt;• Special handling may be required</td>
</tr>
</tbody>
</table>

**The rule of thumb**: typically mild conditions e.g. temperature- (agarose) or ion-induced x-linking (alginate) are used for immobilizing cells and very labile macromolecules, while chemical x-linking is preferred for many enzymes and ABs (more tolerant to conditions)

Let’s summarize

So far we looked at several methods:

- adsorption
- membrane entrapment
- gel entrapment and X-linking

**The common feature**: the biosensing layer is created by physical attachment to the transducer. **Drawbacks** – leaching, desorption

**Covalent attachment**: Biomolecules cannot escape but suitably reactive surface is required – **appropriate functionality is everything**

**Functional groups** are specific groups of atoms within molecules responsible for the characteristic chemical reactions of these molecules. Typically, the same functional group will undergo the same or similar chemical reaction(s) regardless of the size of the molecule it is a part of
### Coupling chemistry

![Chemical structures and functional groups](image)

- COOH → Functional groups on the receptor (proteins)
- NH₂ → Functional groups on the surface
- SH → Functional groups on the surface

- Other functional groups e.g. OH are used too but less frequently e.g. Serine is less reactive; Tyrosine is often in the protein core

### Covalent coupling

#### Relative usefulness of commonly used AA residues

<table>
<thead>
<tr>
<th>Residue</th>
<th>Content</th>
<th>Exposure</th>
<th>Reactivity</th>
<th>Stability</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Arginine</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Cysteine</td>
<td>-</td>
<td>±</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutamate</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Histidine</td>
<td>±</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Lysine</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Serine</td>
<td>++</td>
<td>+</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Threonine</td>
<td>++</td>
<td>±</td>
<td>±</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>C terminus</td>
<td>-</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N terminus</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
The good news

I am not going to discuss available reagents, reaction conditions and mechanisms – this chemistry is well developed and widely used.

The bad news: There is a book chapter on immobilization methods that you have to read (required). Pick up a hard copy when you hand in the quiz.

Quiz time

4) What color apple will you see?

Blue light source

Typical surfaces

A very wide range of different materials is used

Some important types:
- Nobel metals
- Glass and silica
- Metal oxides
- POLYMERS (vast range)
- Carbon

Chemical attachment can be accomplished by covalent linkage, non-covalent interactions or a combination of the two.

Either way functional groups (“handles”) must be introduced.
Gold

In biosensor research gold is the most widely used noble metal: electrochemical and acoustic, most SPR devices

Key features:

- Inert (e.g. does not oxidize in air)
- Compatible with many semi-conductor manufacturing processes (e.g. evaporation coating)
- Thin films maintain a degree of transparency

For biosensors research – a great method for controlled immobilization of bioreceptors:

SAMs – Self-Assembled Monolayers

Thiol SAMs on gold

Thiols spontaneously form monolayer on gold surface

$$R\text{-SH} + \text{Au} \rightarrow R\text{-S-Au}$$

Thiol solution

<table>
<thead>
<tr>
<th>Adsorption</th>
</tr>
</thead>
</table>

Gold

Long chain thiols ($n>10$) assemble into dense crystalline monolayers

Chains pack with a tilt to the surface normal (not shown on scheme below)
Thiols on gold

Important characteristics:
- Bond are very strong and show little tendency for dissociation or lateral migration under normal conditions.
- Adsorption is random and statistical with the composition of the monolayer reflecting the concentration of different thiols in solution.
- This means that mixed monolayers can be formed with chains of different length or different end functionality.

AFM: Molecules are "packed" together like in a crystal.

And now the exciting part

If we dilute simple alkyl thiols with suitably end-functionalized ones and self-assemble the mixture.

suitable functional group eg COOH, NH₂
One more touch…

Remember the discussion of hydrophobicity and stickiness?

Hence, we REALLY want this arrangement on the surface

This is very hydrophobic surface

but this one is hydrophilic!

Making surface hydrophilic

Poly(ethylene glycol) is polymerized ethylene oxide

PEG is the most widely used polymer for surface modification

The helical conformation of PEG chains in aqueous solution in such that water molecules fit perfectly into the "grove"
Glass silica and metal oxides

Main features:
- Contain a mixture of oxygen bridged atoms (Si and Me) and OH groups
- Provide a surface which is relatively easy to further modify
- Glass and silica are ideal for optical applications (transparency)
- Silicon dioxide is used in field effect transducers; other metal oxides are also used/compatible with semi-conductor manufacturing process

Siloxane monolayers

Same principle – different chemistry

Formation of alkyl-siloxane monolayers
Protein immobilized on silica support
Different SAMs

Siloxane chemistry vs thiols on gold

What is the difference?
Thiol on gold mono-layers self-assemble
Siloxane monolayers - chemistry

Which one is better 😊?

Functionalization of glass

Glass (or silica) is subjected to harsh hydrolysis to maximize the number of OH-groups on the surface

Basically, the same chemistry

Treatment with 3-Aminopropyltriethoxysilane (APTES)
• Other reagents are available for introducing other surface functionality e.g. COOH-groups, etc
• Once we got the functionality – we can do chemistry
Changing surface functionality

<table>
<thead>
<tr>
<th>Surface 1</th>
<th>Reagent</th>
<th>Surface 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>R−NH₂</td>
<td><img src="image1.png" alt="Reagent 1" /></td>
<td>R−COO⁻</td>
</tr>
<tr>
<td>R−OH</td>
<td><img src="image2.png" alt="Reagent 2" /></td>
<td>R−OH</td>
</tr>
<tr>
<td>R−SH</td>
<td><img src="image3.png" alt="Reagent 3" /></td>
<td>R−COO⁻</td>
</tr>
</tbody>
</table>

Points to note:
- Commercial reagents and conditions are available to switch from surface with ease.
- Functional groups used are often the same as present in proteins e.g. NH₂, OH, COOH, SH.
- Often (but not always!) the same reagents are used for surface modification and for protein coupling to the surface, albeit under different conditions.

Surface modification of QDs

QDs (left) and micelles PL-QD micelles (right)

Carbon

Hydrogen terminated NCD

\[ \text{CH CH CH CH CH} \]

10-undecenoic acid

\[ \text{COOH} \]

\[ (\text{CH}_2)_n \]

\[ \text{CH CH CH CH} \]

Immmobilization of DNA on chemical vapor deposited nano-crystalline diamond (NCD)

\[ \text{NH}_2\text{-modified FITS labeled DNA} \]

Polymers

The easiest proposition in terms of chemistry

- Modern polymer technology enables the synthesis of polymers with practically any functionality
- Cheap and versatile, but physical properties (conductivity, transparency, stability of very thin films) are often inferior

We have looked at several methods of introducing chemical functionality on or near the surface of the transducer with the aim of attaching biomolecules. However, sometimes there is a problem – just chemistry may not be good enough....
Non-covalent capture

A number of methods are available for immobilizing bioreceptors in non-random conformation.

Not all the Abs are active but more importantly some sites are hindered – hence, different binding characteristics e.g. lower sensitivity.

Immobilized Abs + analyte

Binding to “productive” sites

Immobilize Ab to Ab first

Other agents e.g. protein A

Streptavidin - biotin

Streptavidin is a tetrameric protein which binds vitamin B7 biotin with Kd about $10^{-14}$.

• Binding sites are on the opposite side of the globule.
**Biotynylated surface**

Antibody capture on the biotynylated surface of a transducer

**Histidine tags**

Approach used for the purification of proteins and their attachment and orientation on the surface. It relies on the ability of histidine to complex metals.

Nitriloacetic acid, its complex with Ni$^{2+}$ and a text complex with His.
The use of histidine tags

A (poly)histidine-tag is an amino acid sequence of at least 6 histidine residues, typically attached to the N- or C-terminus of proteins.

Purification of the target protein by Metal Chelate Affinity Chromatography

Simple and widely used technology

Surface capture

His-tag AChE on magnetic beads

Analysis of organophosphorus insecticides

The biosensor was fabricated by placing 1 µL of the enzyme functionalised beads suspension on the surface of the working electrode, fitted with a 4 mm-diameter magnet on the back of the electrode.

Biosensors and Bioelectronics 23 (2007) 506–512
Photonic activation

Light induced opening of a specific disulphide bridge which is a close proximity of aromatic amino acids in the protein structure.

The reduced disulphides leave free thiols that can anchor covalently to a thiol coated surface.

Advantages: (i) avoids chemicals in the reduction and (ii) modern laser technology allows focal spots with dimensions of <1μm – great for miniaturization and making arrays.

Was used with Abs

Precisely oriented antibodies on a biosensor surface with a spatial resolution of ~1μM.

Very cool!

Figure 1. UV light-induced immobilisation illustrated with an immunoglobulin Fab fragment onto a thiol-derivatised surface. The disulphide bridge (red) located near a Trp residue (blue) is located far away from the antigen binding site.
**Covalent vs non-covalent**

**Covalent immobilization:**
- Attached proteins do not leach, important for applications that require extensive washing and for continuous flow analysis
- Analytes can be stripped, allowing for further analysis
- Proteins are attached to surface directly and often non-specifically; often results in higher density

**Non-covalent (capture) immobilization:**
- No chemical inactivation due to the nature of chemical reagents/reaction conditions used
- No loss of biological activity due to the lack of orientation on the surface and steric hindrance

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**Membrane receptors**

**Phospholipid membrane sensor platforms**

![Diagram of membrane receptors](image)

Typically a semiconductor or oxide substrate is used for direct assembly of a supported lipid bilayer on the surface, acting as a working electrode (WE) for electrochemical measurements.

**Drawback:** little space between the lipid membrane and the solid support to accommodate hydrophilic domains of the integrated protein.

A supported lipid bilayer with a linker (often derived from lipids) to tether the lipid membrane to a gold surface.

The increased space of a few nanometers allows integration of membrane proteins without undesired surface interaction.
Nanoscale array

(a) Native membrane fragments are transferred onto the nanopore arrays by steps (i)–(iii)

(b) Scanning electron micrographs of 5 mm lattice array (top) of 500 nm holes fabricated in a 500 nm thick silicon nitride film (bottom). The inset shows a scheme of the 3D structure of the silicon chip

Why it is interesting

About 50% of drug target are extracellular membrane proteins

Membrane proteins e.g. GPCRs can be used with the native cell membrane, or partially purified and incorporated into an artificial phospholipid membrane

**Drawbacks:** Stability, stability and stability
The choice of method

depends on many parameters:

• Type of sensor is being produced
• Requirements for long-term stability e.g. will it be regenerated and re-used or is it a single use sensor that will be disposed
• The nature of the receptor e.g. how sensitive it is to handling (cells must be kept alive, enzymes active, etc)
• The nature of the transducer surface and the mechanism of signal generation (e.g. it’s pointless to have a thick biolayer, if the transducer only “sees” a small fraction of it)
• Research tool vs commercial product (issues such cost per unit, feasibility of mass production, safety concerns)

There are no hard rules – arriving to the right decision is often a matter of experience (and trial & error 😃)

In conclusion

We have looked at immobilization of biomolecules at the surface

What you need to clear about:

• Physical vs chemical attachment
• Monolayers vs multiple layers/pads
• Covalent vs non-covalent
• Orientation vs random