



# **IMMOBILIZATION**

**of**

# **BIOMOLECULES**

**Prof. Marco Mascini**

**Grenoble 2004**



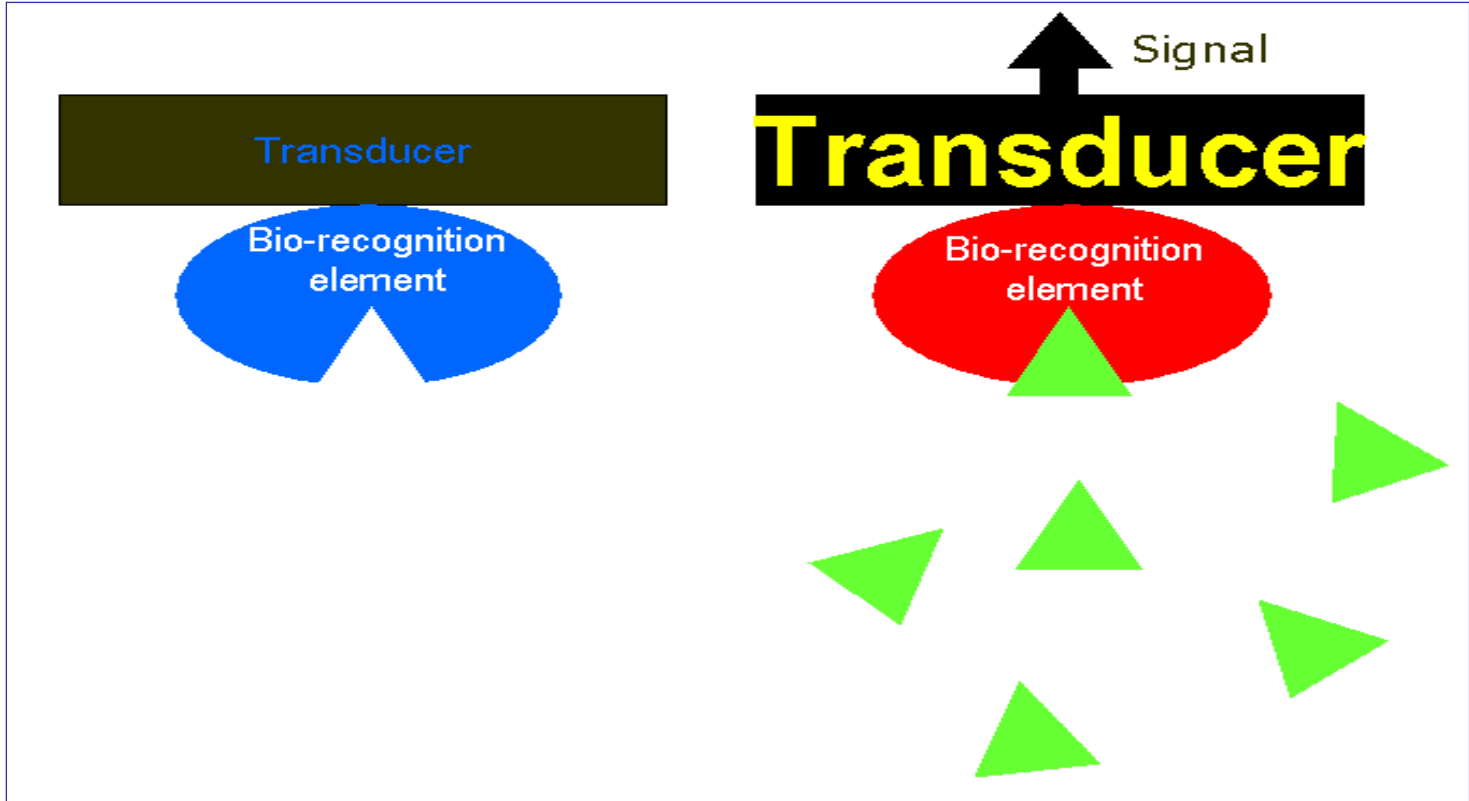
## What is a Biosensor?

Analytical devices incorporating a biological material or a biomimic (e.g. tissue, microorganisms, organelles, cell receptors, enzymes, antibodies, nucleic acids etc.), intimately associated with or integrated within a physicochemical transducer or transducing microsystem, which may be;

- optical,
- electrochemical,
- thermometric,
- magnetic or
- *piezoelectric*.



# What is a Biosensor?





## Where are Biosensors Being Used?

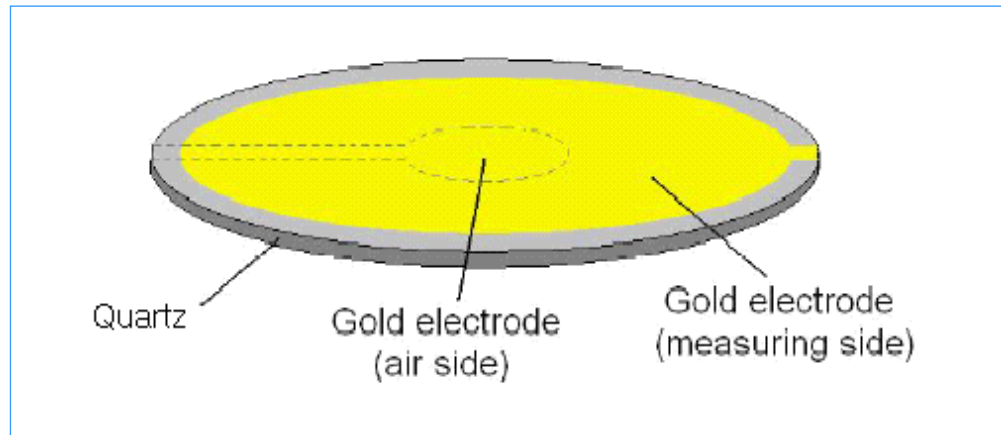
Biosensors are finding use in increasingly broader ranges of application. The following list describes some of the current applications.

- Clinical diagnosis and biomedicine
- Farm, garden and veterinary analysis
- Process control: fermentation control and analysis
- Food and drink production and analysis
- Microbiology: bacterial and viral analysis
- Pharmaceutical and drug analysis
- Industrial effluent control
- Pollution control and monitoring
- Mining, industrial and toxic gases
- Military applications



## Quartz Crystal Microbalance (QCM)

The quartz crystal microbalance (QCM, right) is an extremely sensitive mass sensor, capable of measuring mass changes in the nanogram range. QCM's are piezoelectric devices fabricated of a thin plate of quartz, with gold electrodes affixed to each side of the plate.





# APPLICATIONS OF QCM

## **Biotechnology**

- o Interactions of DNA and RNA with complementary strands
- o Specific recognition of protein ligands by immobilized receptors, immunological reactions
- o Detection of virus capsids, bacteria, mammalian cells
- o Adhesion of cells, liposomes and proteins
- o Biocompatibility of surfaces
- o Formation and prevention of formation of biofilms

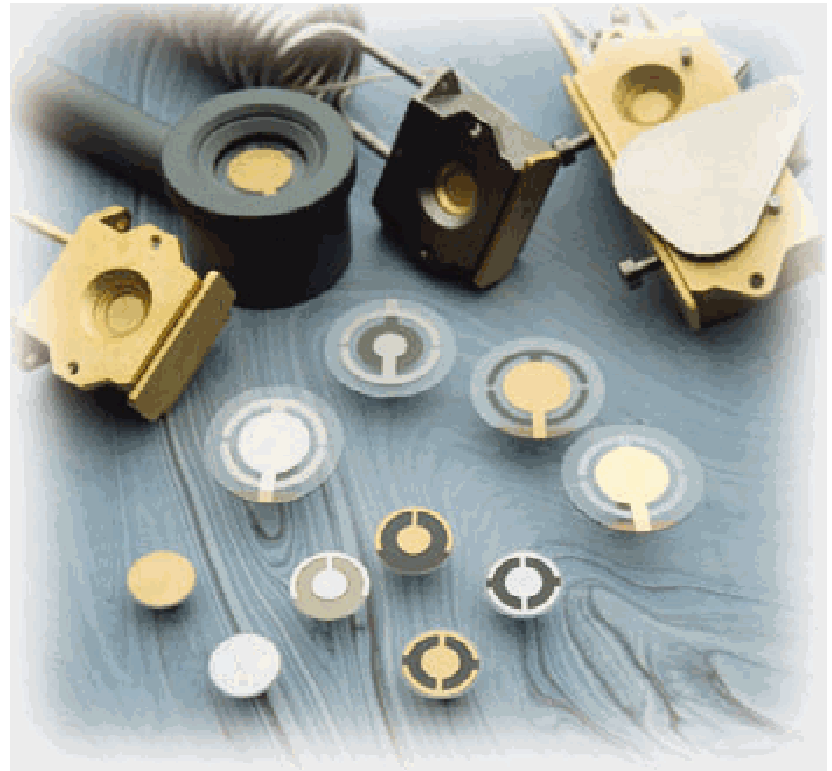
## **Drug Research**

- o Dissolution of polymer coatings
- o Molecular interaction of drugs
- o Cell response to pharmacological substances
- o Drug delivery



## Types of Electrodes used in QCM Studies

- Gold (Au)
- Platinum (Pt)
- Silver (Ag)





## Affinity Interactions used in QCM

### Ligand

Antibody

Inhibitor

Lectin

Receptor

Nucleic acid

Hormone, vitamin

### Counter ligand

Antigen, virus, cell

Enzyme (ligands are often substrate analogs or cofactor analogs)

Polysaccharide, glycoprotein, cell surface

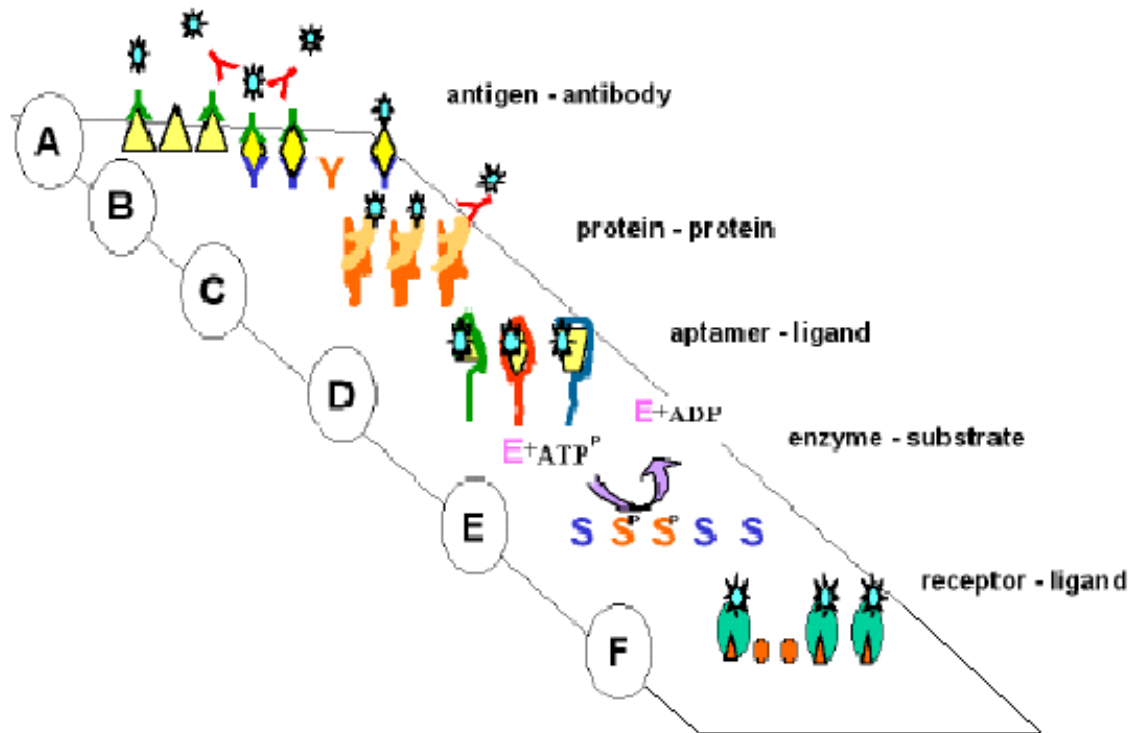
membrane protein, cell

Nucleic acid binding protein (enzyme or histone)

Receptor, carrier protein



# Affinity Interactions used in QCM





## Immobilization

Molecules may be immobilized either passively through;

- ❖ Hydrophobic
- ❖ Ionic interactions
- ❖ **Covalently** by attachment to activated surface groups.

Noncovalent surfaces are effective for many applications; however, passive adsorption fails in many cases.

Covalent immobilization is often necessary for binding of molecules that do not adsorb, adsorb very weakly, or adsorb with improper orientation and conformation to noncovalent surfaces.

Covalent immobilization may result in better biomolecule activity, reduced nonspecific adsorption, and greater stability.



## Immobilization

Immobilization reaction should have several characteristics;

Firstly, the reaction should occur rapidly and therefore allow the use of low concentrations of reagents for immobilization.

The chemistry should require little, if any, post-synthetic modification of ligands before immobilization to maximize the number of compounds that can be generated by solution or solid-phase synthesis and minimize the cost of these reagents.

Immobilized ligands must be in an oriented and homogeneous manner.



## Immobilization

The immobilization process should occur selectively in the presence of common functional groups, including amines, thiols, carboxylic acids, and alcohols.

**Amino-NH<sub>2</sub>,**

**Carboxy-COOH,**

**Aldehyde-CHO,**

**Thiol-SH,**

**Hydroxyl-OH**

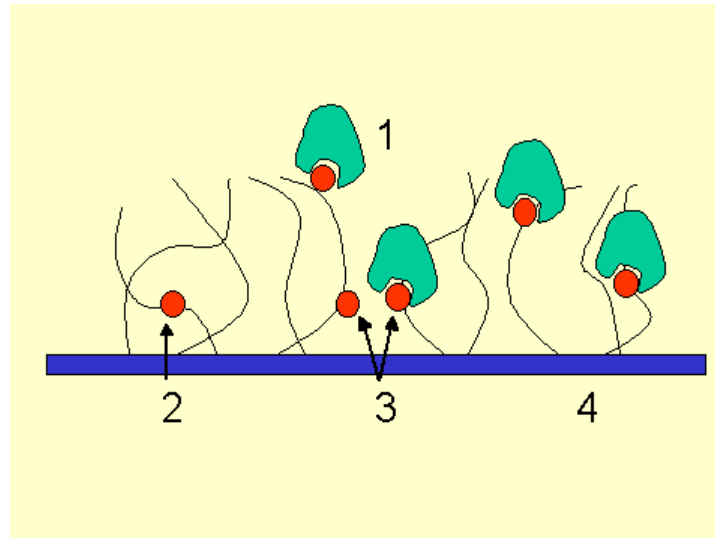


## Immobilization

Surface density of the ligand should be optimized.

Low density surface coverage will yield a correspondingly low frequency.

High surface densities may result steric interference between the covalently immobilized ligand molecules, impeding access to the target molecules.

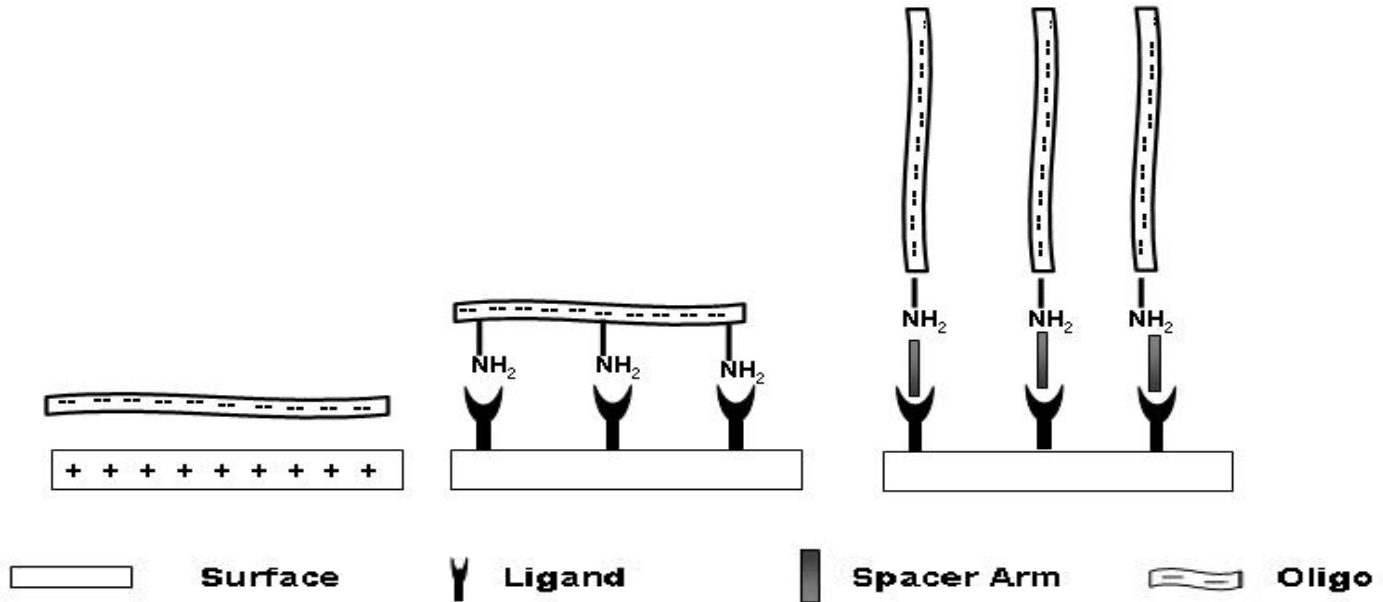


1) unhindered binding. 2) inaccessible binding site. 3) hindered binding site when adjacent site is occupied. 4) restricted access binding site.



# Immobilization

Correct orientation of the ligand molecules on the surface, and using a spacer arm are important and critical and makes the ligand available for the target.



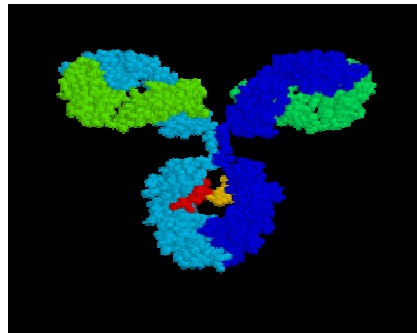


## Immobilization

Proteins are much more sensitive to their physiological environments and can easily be degraded or denatured by physical or chemical effects. Protein's 3-D confirmation must not change during immobilization procedure.

DNA molecules are much more stable then proteins.

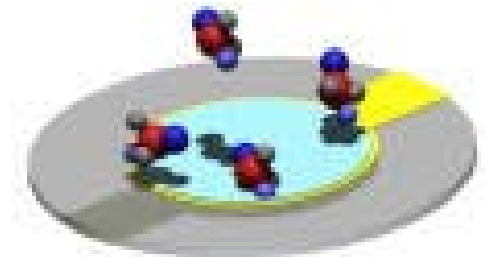
It is easier to immobilize DNA molecules.





## Preparation of Surface for Biomolecule Immobilization

- Modification of the gold electrode surface to create functional groups.
- Modification of biomolecules for covalent attachment to the surface.





# Preparation of Surface for Biomolecule Immobilization

- Cleaning the surface of electrode
- Modification of the surface to create functional groups by;

## **Polymer Coating**

Glow Discharge

Dipping

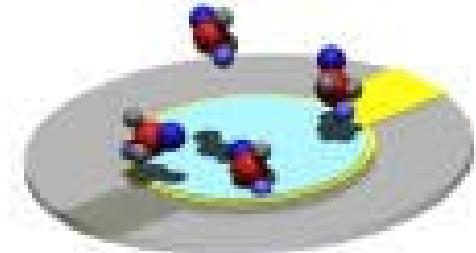
Spin Coating

Electrochemical deposition

## **Langmuir and Langmuir-Blodgett films**

## **Chemical Modificaitons**

Self-assembled monolayers (SAMs)





## Preparation of Surface for Biomolecule Immobilization

Proteins and nucleic acids must first be immobilized on the gold surface of the quartz crystal. In principle, proteins can be fixed noncovalently onto gold by physical adsorption. However, these surfaces are not stable during prolonged buffer rinses, especially if regeneration procedures are carried out. To sterically assist analyte binding, it is advantageous to couple the receptors to the gold surface via a linker molecule.

The linker layer serves as a functionalized structure for further modification of the surface, as well as creates a barrier to prevent proteins, DNA and other ligands from coming into contact with the metal.



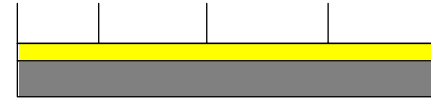
# General Route for Immobilization

Piranha Solution



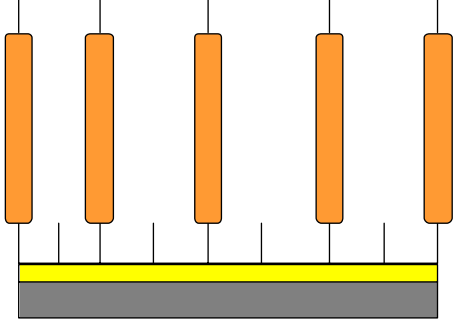
Cleaning the surface

OH NH<sub>2</sub> COOH COH SH

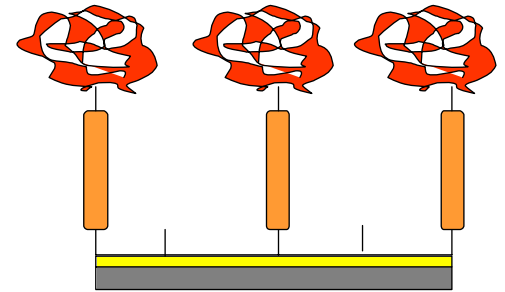


Modification of surface

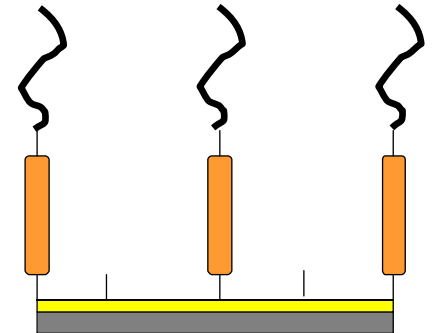
OH NH<sub>2</sub> COOH COH SH



Immobilization of Biomolecule



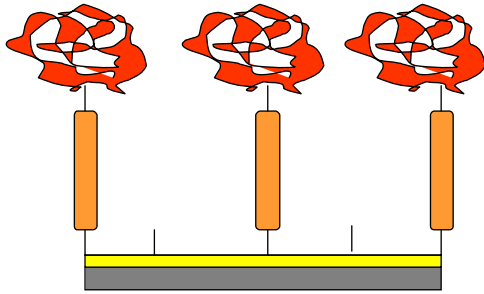
Proteins



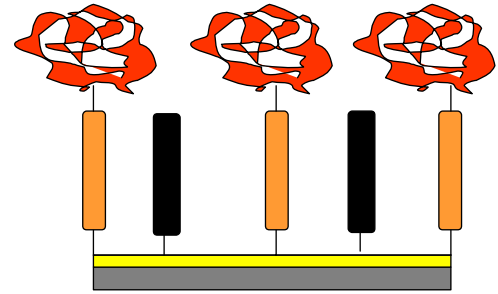
ss-DNA



# General Route for Immobilization



Proteins



Proteins

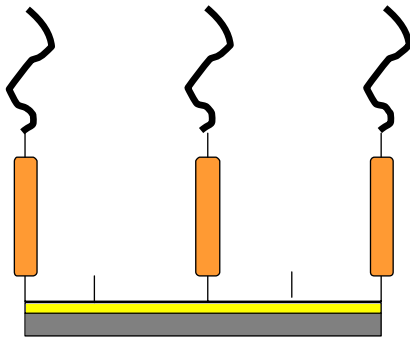


Addition of Blocking agents

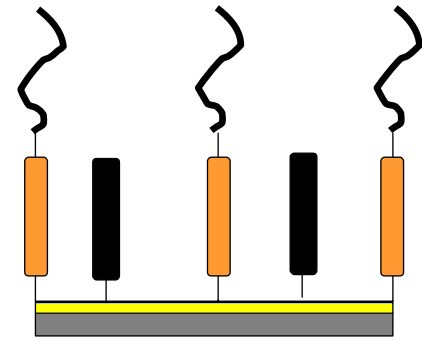
Glycine

6-mercapto-1-hexanol

Ethanolamine HCl



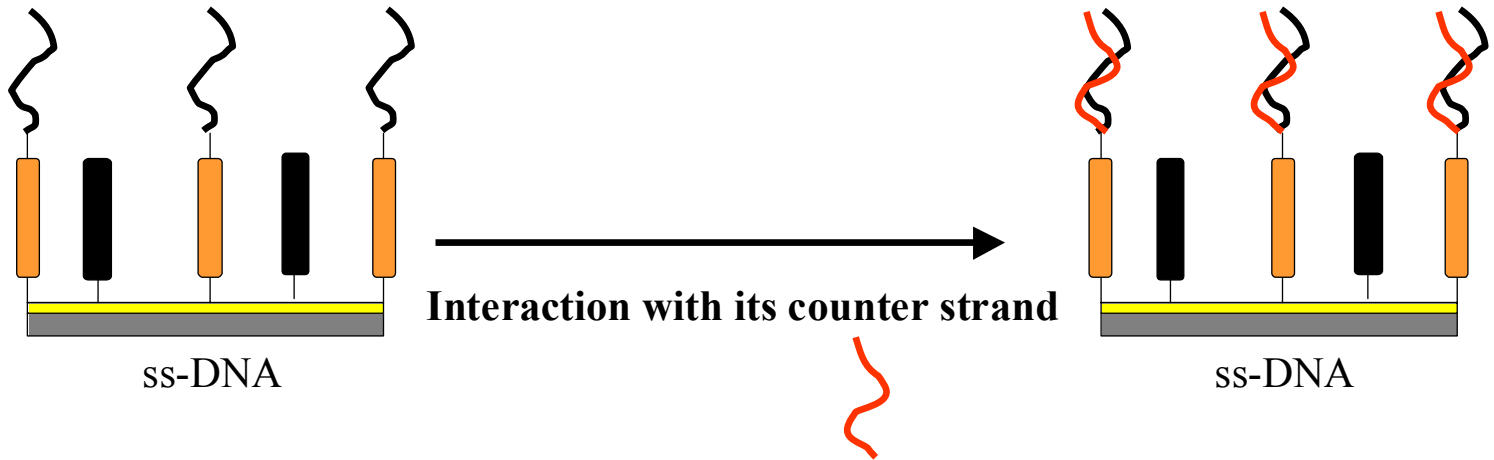
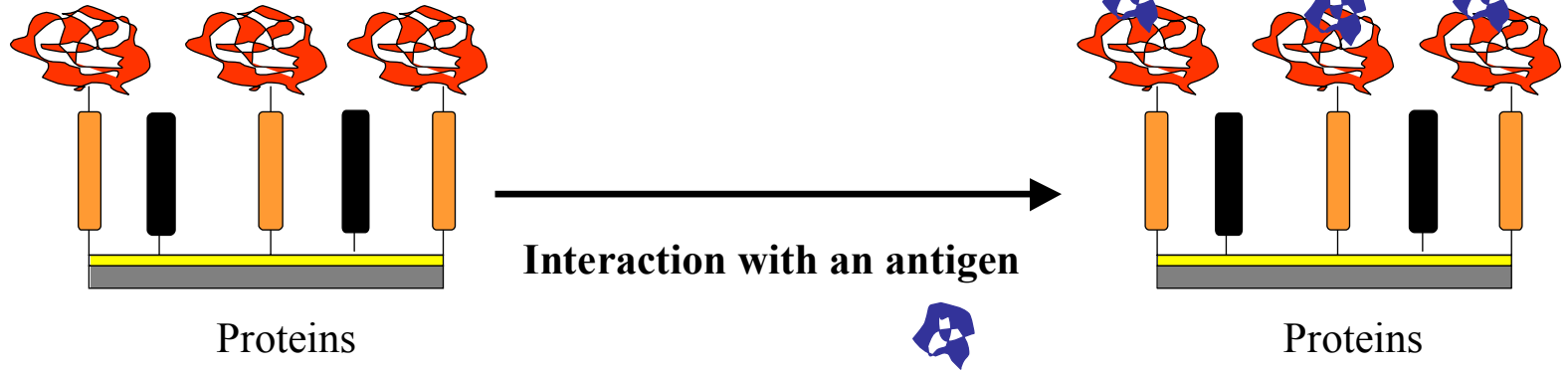
ss-DNA



ss-DNA



# General Route for Immobilization

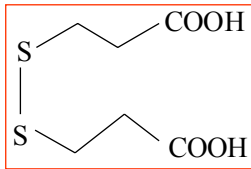




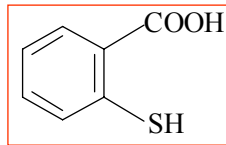
## Creating Functional Groups

Organosulfur compounds on gold was a widespread method for the preparation of self-assembled monolayers (SAMs).

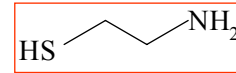
If the alkyl chain is long enough, these architectures are very stable and show a molecular orientation nearly perpendicular to the surface.



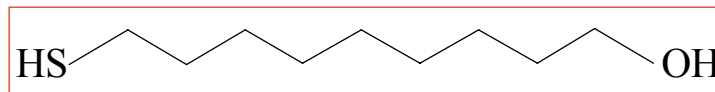
3,3'-dithiodipropionic acid



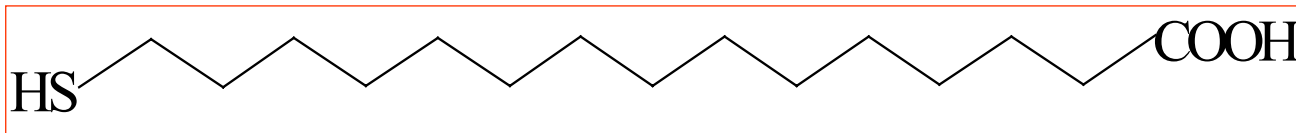
Thiosalicylic acid



Cysteamine



11-Mercaptoundecanol



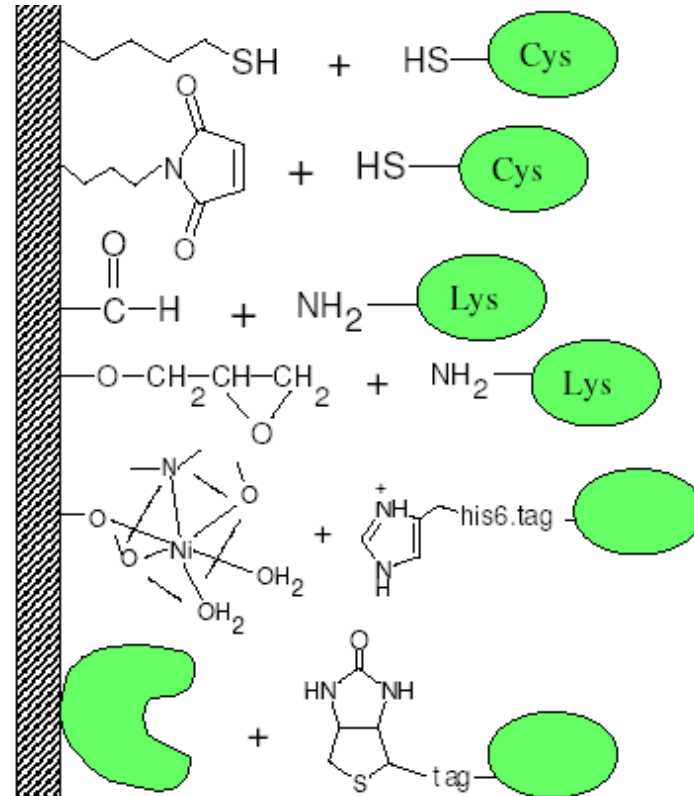
16-mercaptohexadecanoic acid



# Surface Chemistry

## Cross-linking Strategies for Protein Immobilization

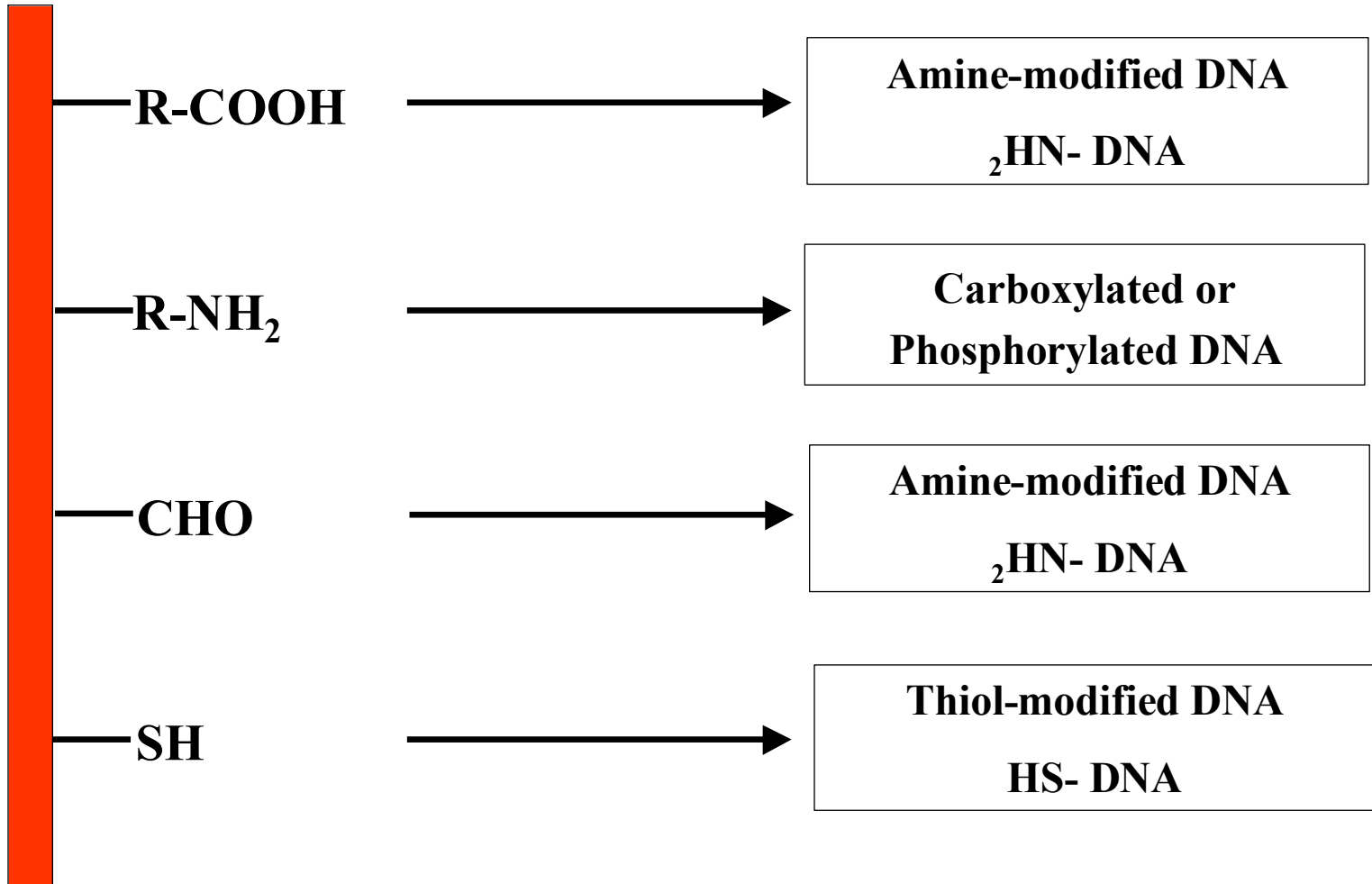
- Thiol surface
- Maleimide surface
- Aldehyde surface
- Epoxy surface
- Nickel Chelate surface
- Streptavidin surface
- Biotin surface





# Surface Chemistry

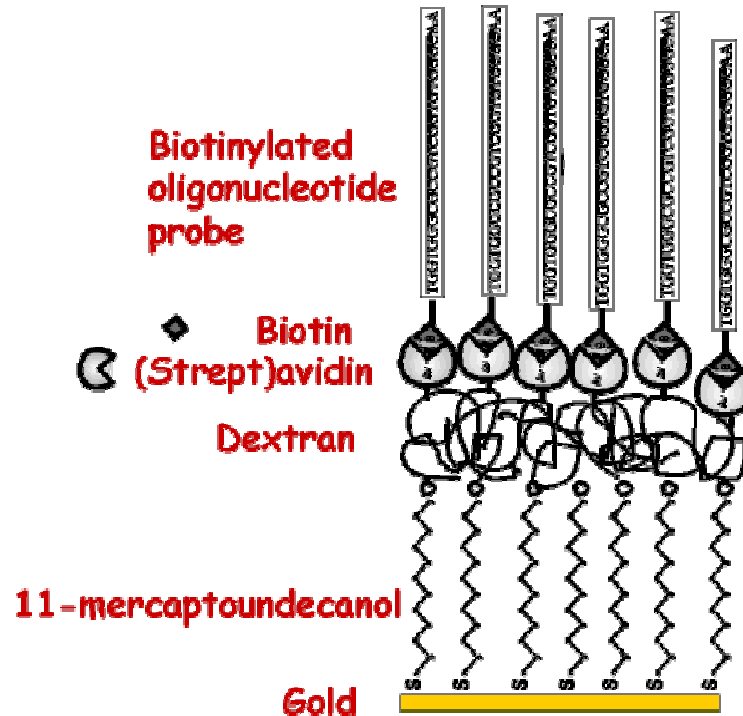
## Cross-linking Strategies for DNA Immobilization





# Surface Chemistry

## Cross-linking Strategies for DNA Immobilization



**Interaction or immobilized streptavidin carrying surfaces with Biotin modified-DNA**



## Examples from Literature

### Immobilization of Antibodies

A piezoelectric immunosensor was developed for rapid detection of *Escherichia coli* O157:H7. The immunosensor could detect the target bacteria in a range of 10<sup>3</sup>–10<sup>8</sup> CFU/ml within 30–50 min.

The development of new methods for rapid detection of microorganisms, particularly the infectious and toxigenic, remains a challenge to the scientists in various fields.

*Escherichia coli* O157:H7 is one of the most dangerous pathogens. First discovered in 1982, this bacterium has been implicated in hemorrhagic colitis and hemolytic uremic syndrome, both of which cause watery followed by bloody diarrhoea, bloody feces, kidney failure, and even death, especially in children.



# Examples from Literature

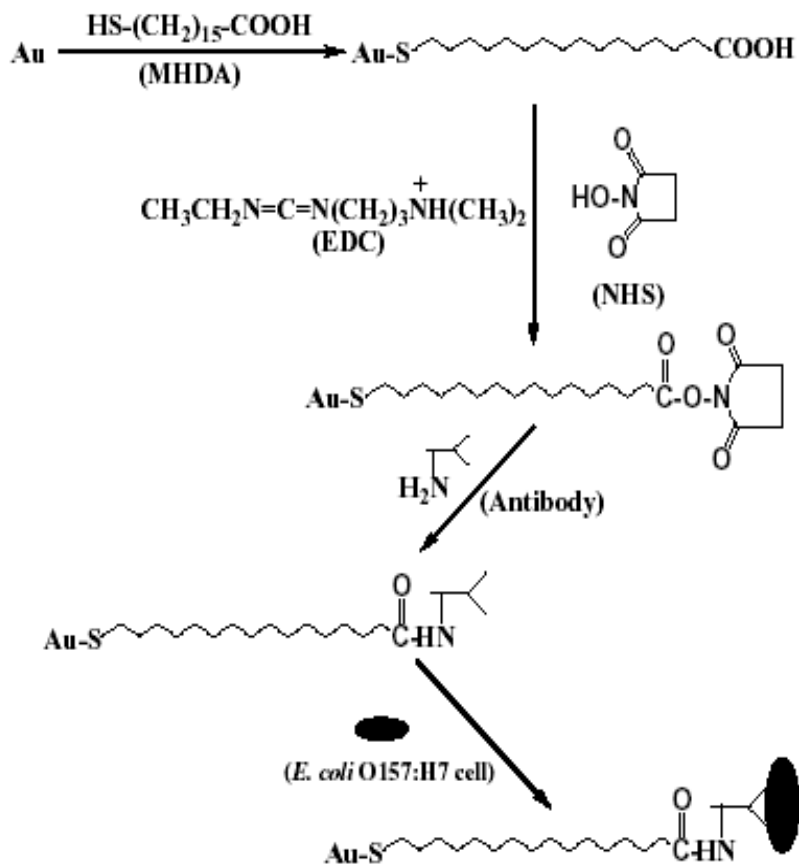
## Immobilization of Antibodies

The cleaned crystals were immersed in an ethanol solution of 16-mercaptohexadecanoic acid (MHDA).

MHDA-modified crystals were treated with EDC (Carbodiimide)-NHS (N-hydroxysuccinimide) to convert the terminal carboxylic group to an active NHS ester.

After rinsing with water and drying, anti-*E. coli* O157:H7 antibodies were added onto Au electrodes and stored at 4 C overnight (at least 15 h).

The excess antibodies were removed by rinsing with PBS.





## Examples from Literature

### Immobilization of Antibodies

Ferritins are a class of storage proteins widely distributed in vertebrates, invertebrates, plants, fungi, and bacteria. The major function of ferritin is to store and detoxify intracellular iron. At extremely elevated iron levels, ferritin can be disproportionately increased. The increased level of ferritin is known as a nonspecific marker of inflammatory processes and neoplasms.

It was demonstrated to be elevated in the sera of patients of a wide variety of tumors,

- human breast cancer,
- renal cell carcinoma,
- hepatocellular carcinoma,
- larynx cancer and
- malignant neoplasms of maxilla, etc.

or in significant diseases,

- like HIV infection,
- Still's disease,
- leukocytosis,
- reactive hemophagocytic syndrome.



# Examples from Literature

## Immobilization of Antibodies

A new human ferritin immunosensor was developed using anti-human ferritin antibodies (Abs) immobilized on the gold disc of a quartz crystal microbalance (QCM).

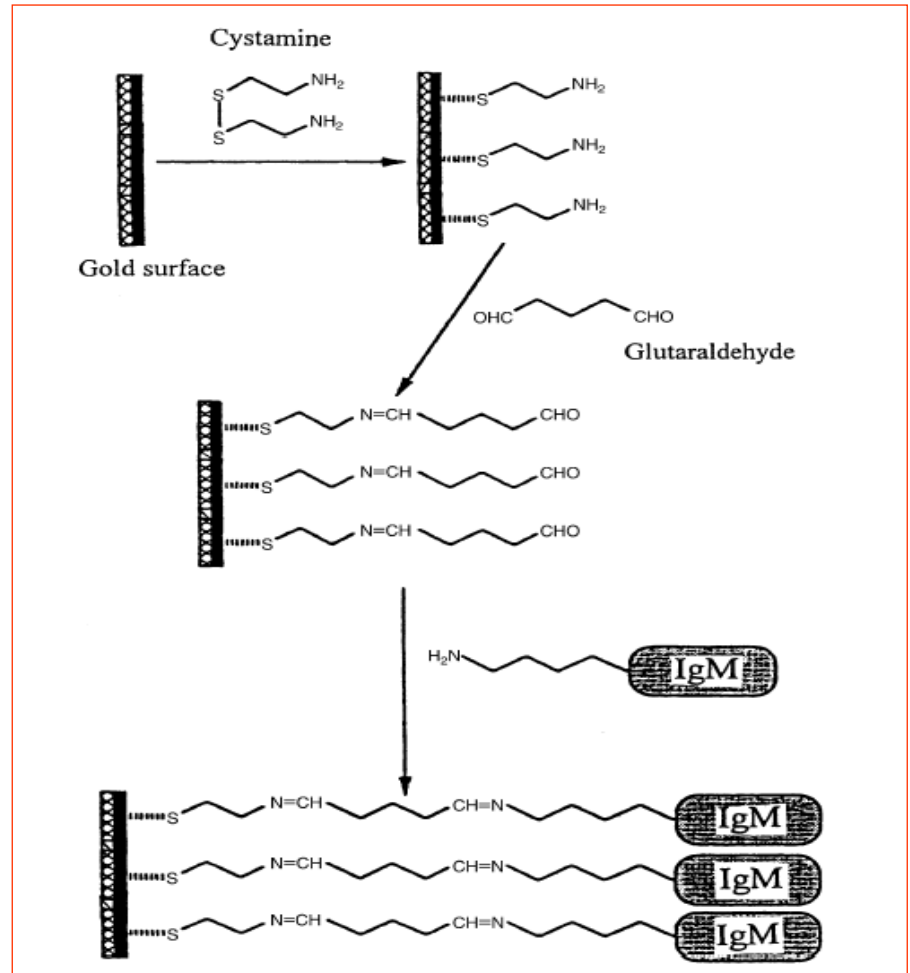
This human ferritin immunosensor had some advantages: high sensitivity, high specificity, low sample requirement, high reusability.

The crystal was immersed with a cystamine solution.

The crystal was dipped into aqueous glutaraldehyde solution.

Then, the crystal was immersed with a Ab solution (IgM).

The crystal was blocked with a glycine-PBS solution.





## Examples from Literature

### Immobilization of Antibodies

An immunosensor for the determination of okadaic acid (OA) using a quartz crystal microbalance (QCM) was developed and optimised in standard solutions. Several coupling techniques, protein A, protein G and polyethylenimine (PEI) with glutaraldehyde (GA) cross-linking, were investigated for the determination of okadaic acid and a very good result was obtained with PEI coupling.

The crystal surface was modified with polymer polyethylenimine and the free amino groups obtained were activated by glutaraldehyde. OA–BSA conjugate was then bound to the activated derivatives to form a cross-linked complex, which strongly attached to the gold surface of the crystal, resulting in good long-term storage properties.

The detection of okadaic acid, a key algal toxin in the environment.



## Examples from Literature

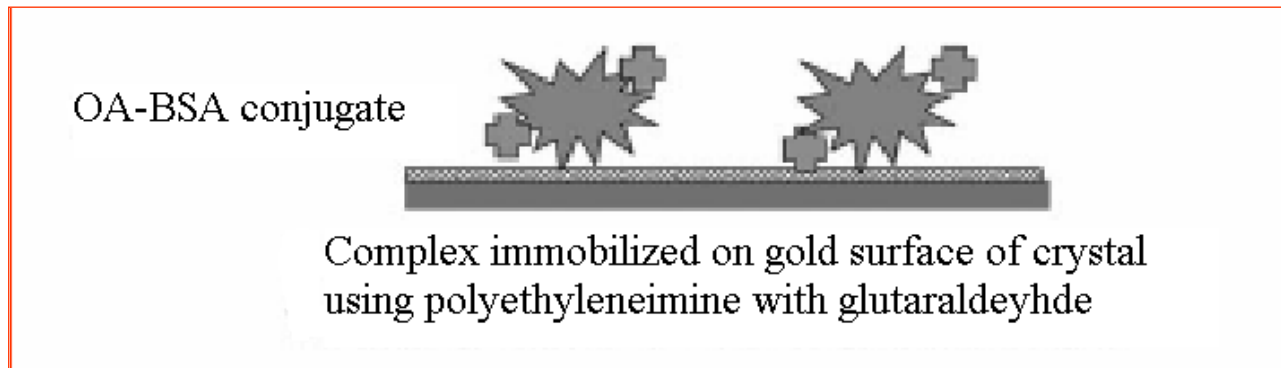
### Immobilization of Antibodies

The crystal surface was cleaned.

PEI (polyethyleneimine) was added onto the crystal surface.

The free amino group obtained on the crystal surface was activated using glutaraldehyde.

Okadaic acid-Bovine Serum Albumin (OA-BSA) conjugate was then bound to the activated derivatives to form a cross-linked complex to detect the amount of Anti-okadaic acid antibody (Anti-OA Ab).





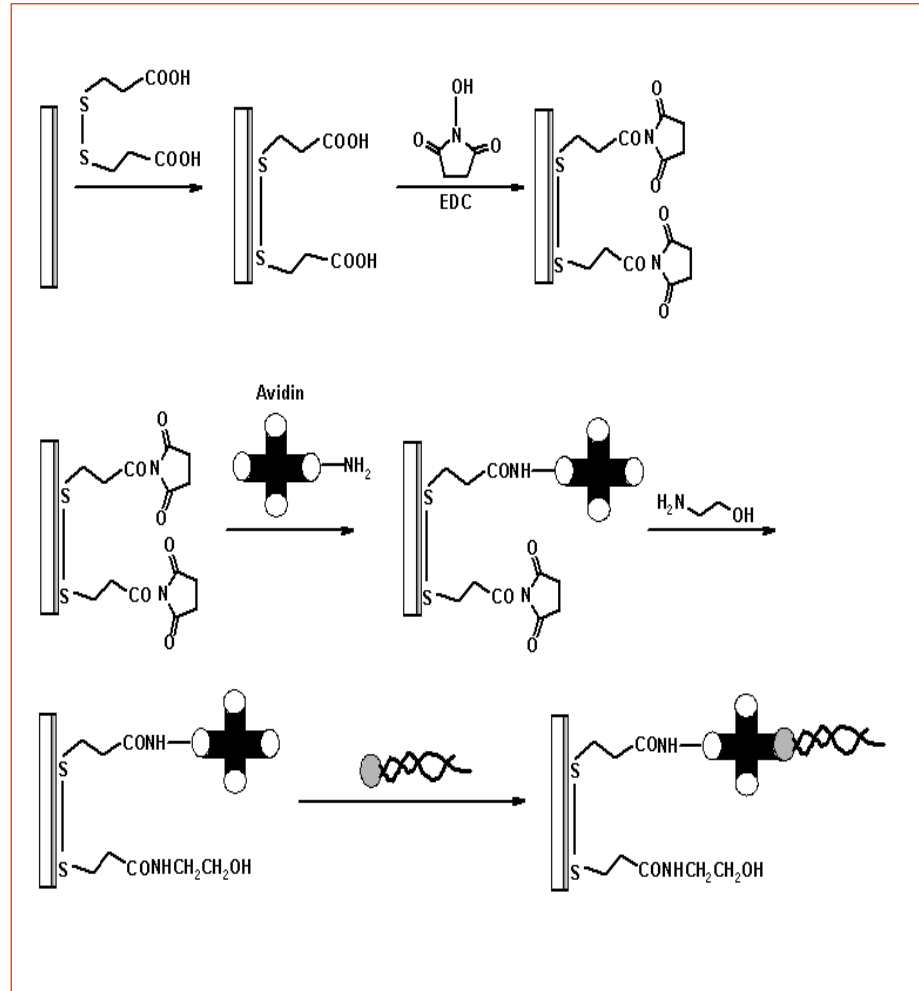
# Examples from Literature

## Immobilization of biotin modified-DNA

Dithiodipropionic acid is reacted with the gold surface,

Then by carbodiimide activation of the carboxylic acid groups, avidin (or streptavidin) is attached.

Finally biotin carrying DNA is immobilized specifically with the binding reaction of avidin with biotin.





## Examples from Literature

### Immobilization of biotin modified-DNA

A DNA piezoelectric sensor has been developed for the detection of genetically modified organisms (GMOs).

Two different probe immobilisation procedures were applied:

- (a) a thiol/dextran procedure and
- (b) a thiol-derivatised probe and blocking thiol procedure.

The results obtained showed that both immobilisation procedures enabled sensitive and specific detection of GMOs, providing a useful tool for screening analysis in food samples.

A genetically modified organism (GMO) is referred to as a living organism whose genome has been modified by the introduction of an exogenous gene able to express an additional protein that confers new characteristics, i.e. herbicide tolerance or resistance to virus, antibiotic and insect.



# Examples from Literature

## Immobilization of biotin modified-DNA

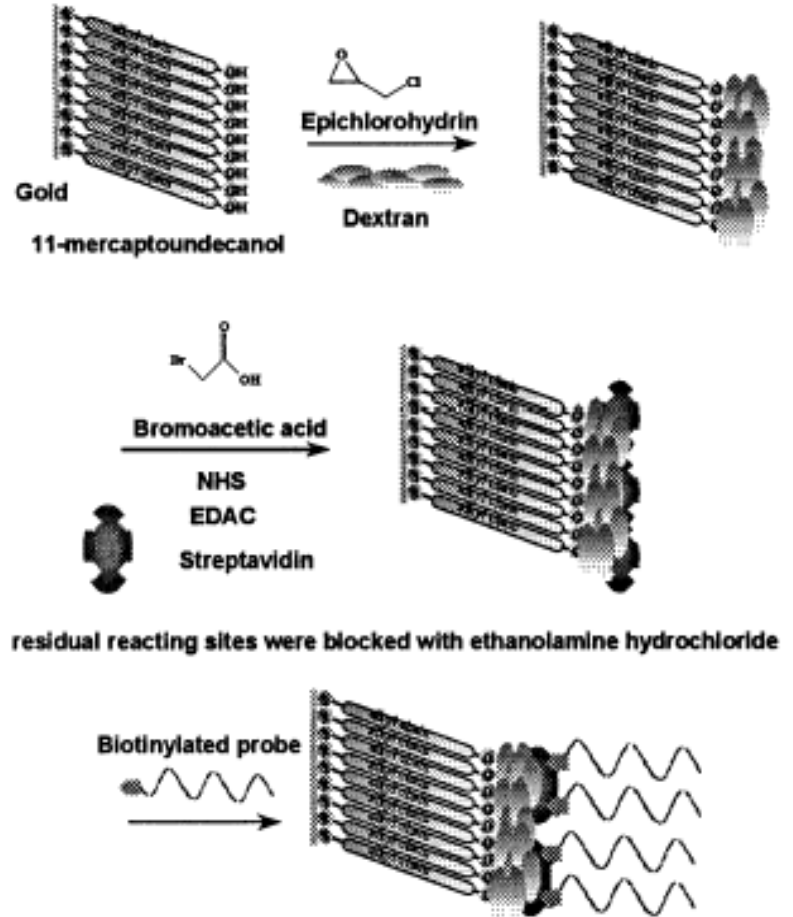
The gold sensor surface was modified with 11-mercaptoundecanol.

Activation of 11-mercaptoundecanol modified surface with epichlorohydrin for carboxylated dextran immobilization.

Immobilisation of streptavidin in the presence of carbodiimide and NHS.

Blocking of residual reactive sites with ethanolamine HCl.

Then the immobilisation of biotinylated oligonucleotide.



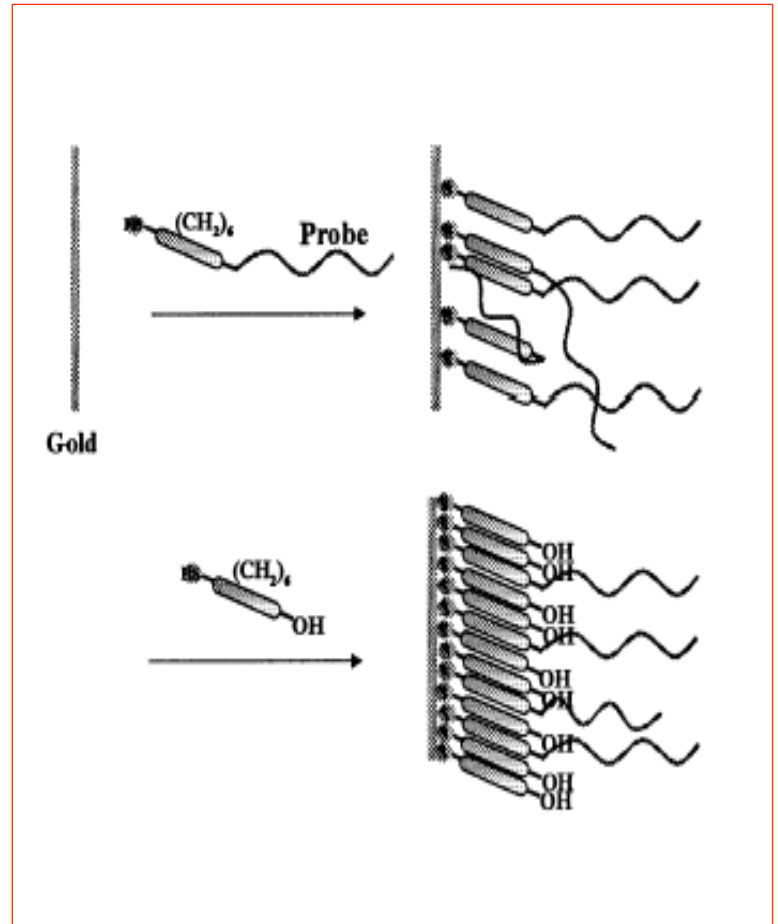


## Examples from Literature

### Immobilization of thiol modified-DNA

The gold sensor surface was modified with a thiol-derivatised probe (Oligo-C6-SH).

And then, with a thiol (6-mercapto-1-hexanol, MCH, for blocking of residual reactive sites.





## Examples from Literature

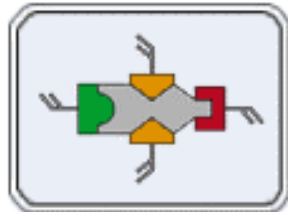
### Molecular Imprinting

Molecular imprinting is a process by which functional monomers are allowed to self-assemble around a template molecule and subsequently crosslinked into place. Under defined conditions, the template molecule can be removed, leaving behind a cavity complementary in shape and functionality, which will bind molecules identical to the template. The imprint functions like a lock that is only compatible with the correct key.

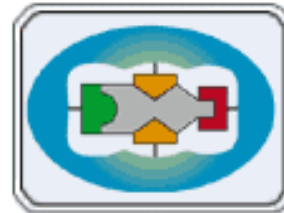
Selection



Self-Assembly



Polymerization



Extraction

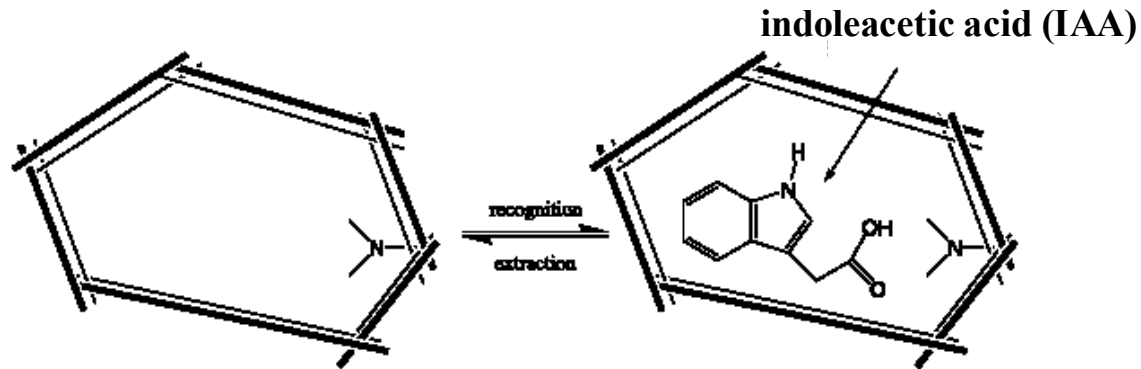




## Examples from Literature

### Imprinted polymer-coated QCM sensors

In this study, imprinted polymers for indoleacetic acid (IAA), which is a plant hormone and acts as a plant growth factor, were prepared with N,N-dimethylaminoethyl methacrylate as the functional monomer and the polymers showed high affinity and selectivity for IAA.





## Samples from Literature

### Imprinted polymer-coated QCM sensors

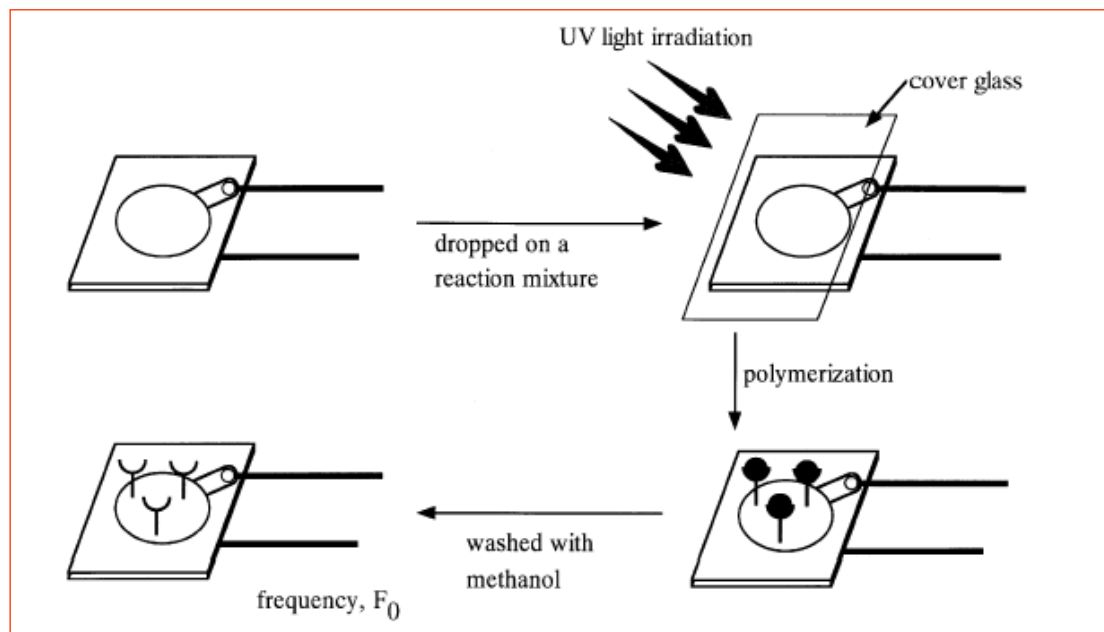
IAA (Indoleacetic acid), N,N-dimethylaminoethyl methacrylate, ethylene glycol dimethacrylate (EGDMA), azobisisobutyronitrile (AIBN) were dissolved in chloroform and the reaction mixture was degassed. The reaction mixture was dropped onto the QCM plate and held with a microcover glass coated with trimethylchlorosilane. The polymerization was initiated by UV light irradiation at room temperature under nitrogen atmosphere.



IAA imprinted polymer



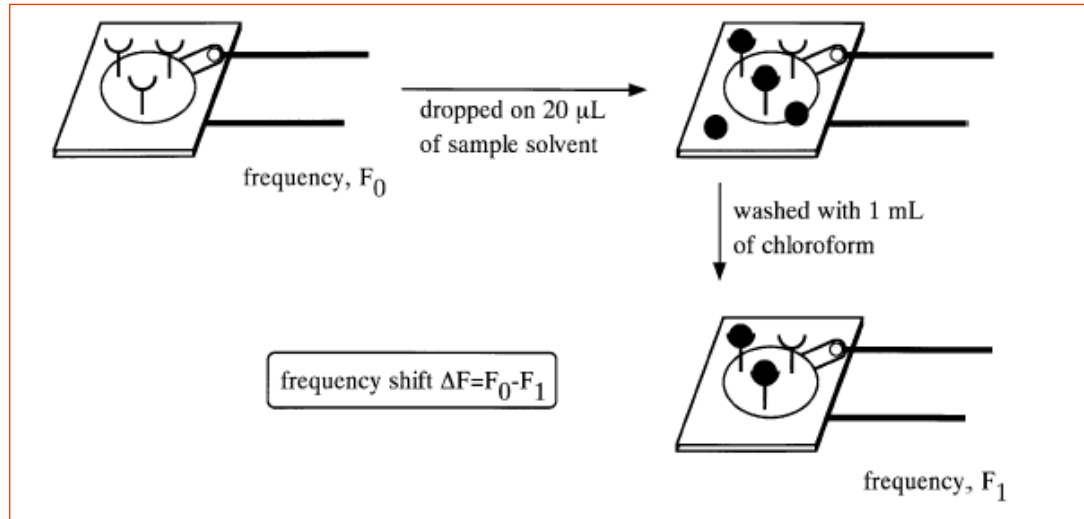
IAA





# Samples from Literature

## Imprinted polymer-coated QCM sensors



IAA imprinted polymer

● IAA

A. Kugimiya, T. Takeuchi, *Electroanalysis*, 1999, 11, No. 15, 1158-1160.