# Immobilization

**IUPAC:** The technique used for the physical or chemical fixation of cells, organelles, enzymes, or other proteins (e.g. monoclonal antibodies ) onto a solid support, into a solid matrix or retained by a membrane, in order to increase their stability and make possible their repeated or continued use.

•Attachment of the biomolecules in or onto the surface of an insoluble support

- Immobilized enzymes have several advantages over the soluble enzyme:
  - **Convenience**
  - **Reusability**
  - **Stability**

# Immobilization criteria

- There are a number of requirements to achieve a successful immobilization:
  - The biological component must retain substantial biological activity after attachment
  - It must have a long-term stability
  - The sensitivity of the biomolecule must be preserved after attachment

# Importance of immobilization step

- Sensitivity decreases if immobilization causes enzyme denaturation or conformational changes or if the enzyme has been modified, especially on its active site.

- A better sensitivity is obtained with oriented immobilization of enzymes or by selecting the nature of the spacer arm between the enzyme and the support under covalent binding.

- Numerous immobilization techniques involve random distribution or poor orientation of enzyme molecules inducing a partial or a total loss of activity due to enzyme denaturation or blocking of the active site from substrate accessibility.

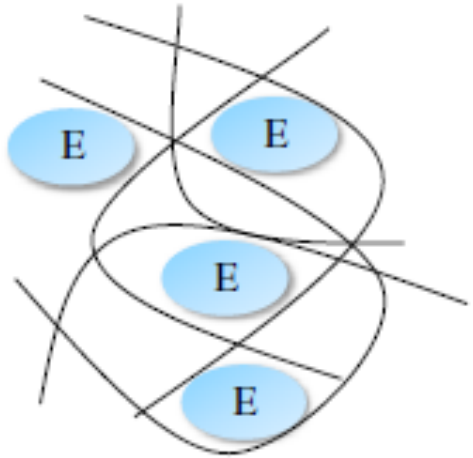
- Techniques based on affinity interactions in an ordered and site-specific manner are more useful in order to develop efficient biosensor.

- In the same way, self-assembled monolayer-based immobilization reduces the number of random orientations, generates uniform, reproducible and stable structures with high coverage.

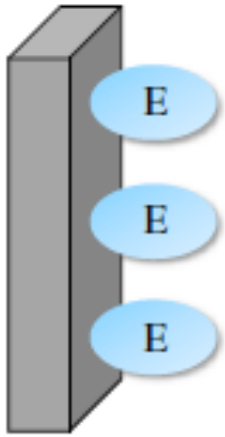
# Immobilization to nano-

- Biomolecules have also been immobilized with nanostructure materials
- Nanoparticles are interesting immobilization surfaces presenting a large surface area
- Moreover, direct adsorption of enzymes onto bulk metal surfaces frequently results in denaturation of the protein and loss of bioactivity which can be avoided if enzymes are first adsorbed onto metal nanoparticles before being electrodeposited on the electrode surface
- $SiO_2$ nanoparticles are also excellent matrices for enzyme immobilization due to their good biocompatibility and easy preparation

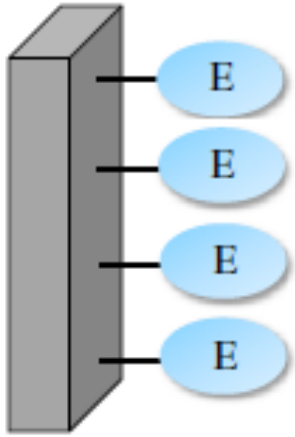
# Classical immobilization methods



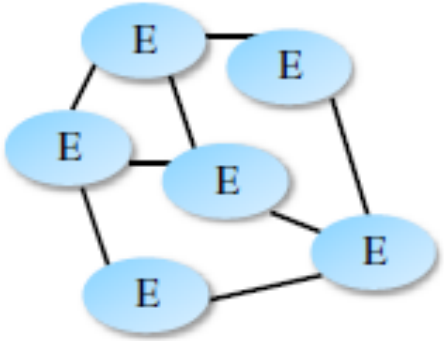

Entrapment

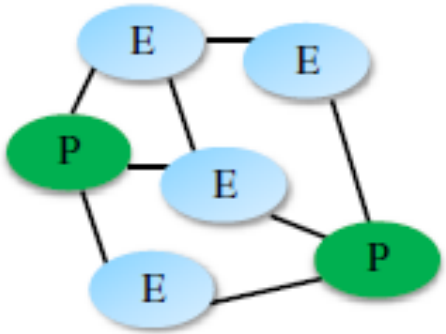



Adsorption

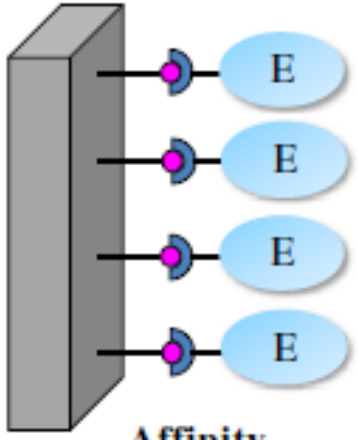



Covalence




Cross-linking



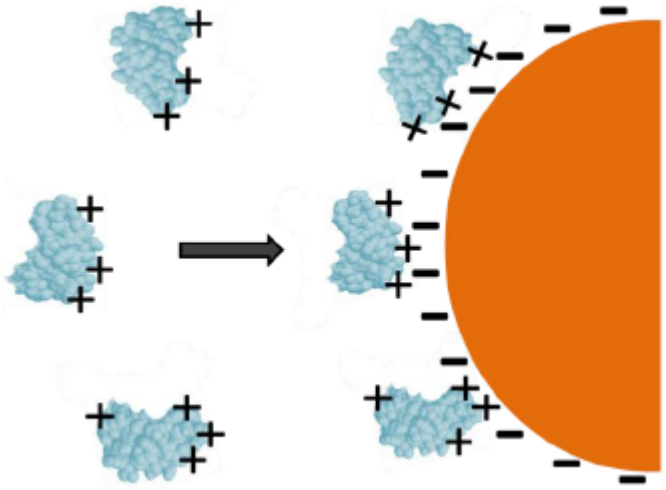

Affinity

# Adsorption

- ☺ Simplest immobilization method
  - Mix the enzyme and support in suitable conditions
- ☺ With a suitable charged matrix, ionic interactions may also be promoted
- ☺ This technique is technically undemanding and economically attractive
- ☺ Regeneration is easy
- ☹ Forces are weak (e.g. Van der Waal's forces and electrostatic and/or hydrophobic interactions) so leakage is generally a problem
- ☹ Biosensors based on adsorbed enzyme suffer from poor operational and storage stability.
- ☹ Non-specific adsorption of other proteins or substances.

# Adsorption: electrostatic interactions

- Enzymes can be electrostatically immobilized onto charged surfaces.
- If the isoelectric point of the enzyme is lower than the pH value of the solution, enzyme is negatively charged and thus can be bound to a positively charged support or vice versa



# For example: Isoelectric Point for an amino acid

- At a certain pH called the **isoelectric point (pI)**, the positive and negative charges on the amino acid are equal and the overall charge is zero.
  - In a solution that is more basic than pI, the -$NH_3^+$ group will loose $H^+$, and the overall charge will be negative.
  - In a solution that is more acidic than pI, the -$COO^-$ accepts an $H^+$, and the overall charge will be positive.

# Adsorption: electrostatic interactions

Immobilization to a nanoparticle:

- cover the gold nanoparticles (GNP) with a negatively charged ligand such as citrate.
- Positively charged amino acid residues allow enzymes in solution to be electrostatically adsorbed on the surface by merely dipping the modified electrode into the solution.
- Although this method has the benefit of speed and simplicity, unfavorable orientations and decreased functionality are likely

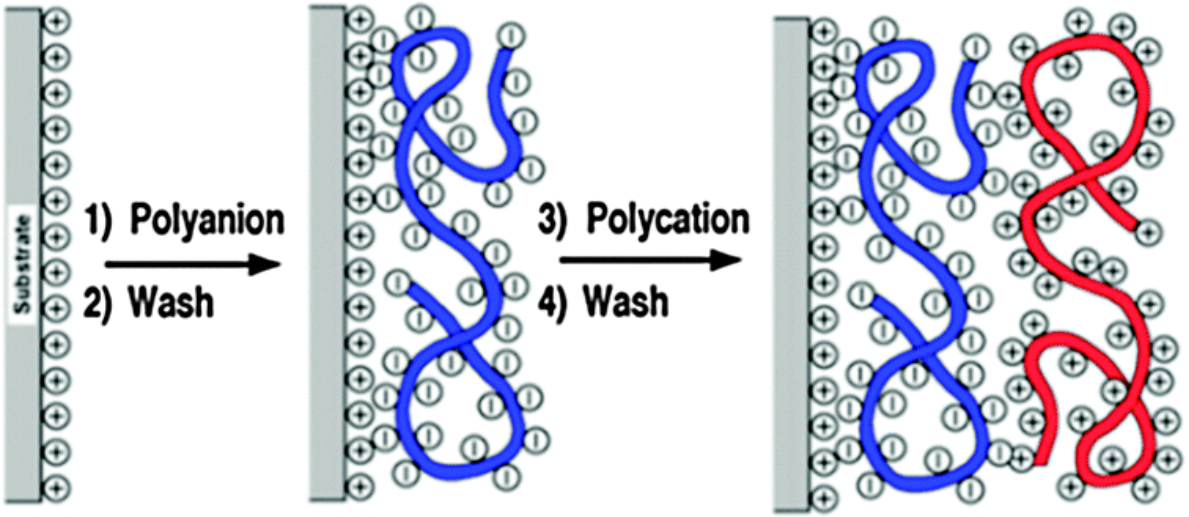
# Adsorption: electrostatic interactions

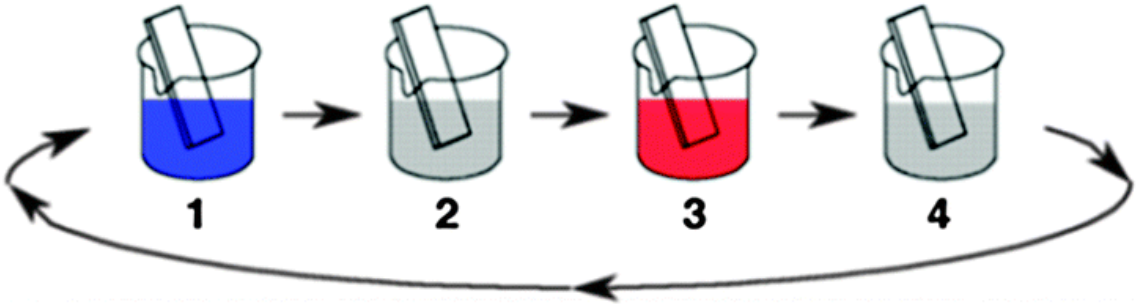
Layer-by-layer deposition

- Layer-by-layer (LbL) deposition was first described by Decher in 1991
- Based on alternate layers of polyelectrolyte and enzyme with opposite charges
  - Polycations: poly(L-lysine), poly(ethyleneimine), chitosan (or chitosan derivatives), etc.
  - Polyanions: poly(vinylsulfonate), poly(acrylic acid) and poly(methacrylic acid), etc
- Other materials like enzymes, DNA, nanoparticles, etc could also be introduced to the assembly

# Adsorption: electrostatic interactions

Layer-by-layer deposition




Polyanion/cation + Polycation/anion $\xrightarrow{\text{release of counterions}}$ Polyelectrolyte complex

# Entrapment in biosensor design

Enzymes can be immobilized in three-dimensional matrices such as electropolymerized films, amphiphilic networks composed of polydimethylsiloxane (PDMS), a photopolymer, a silica gel, a polysaccharide or a carbon paste

☺ Easy to perform

☺ Enzyme, mediators and additives can be simultaneously deposited in the same sensing layer.

☺ There is no modification of the biological element

☺ High operational and storage stability.

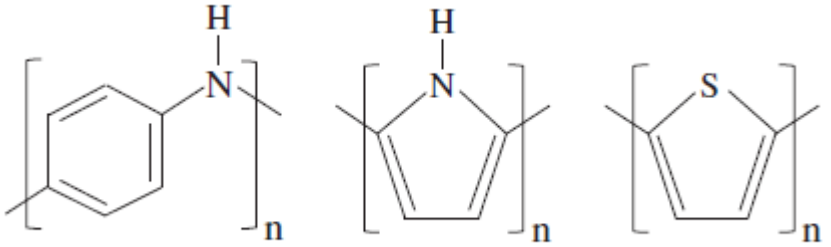
☹ Leaching of biocomponent

☹ Possible diffusion barriers

# Entrapment by Electropolymerization

Electrochemical polymerization (or electropolymerization)

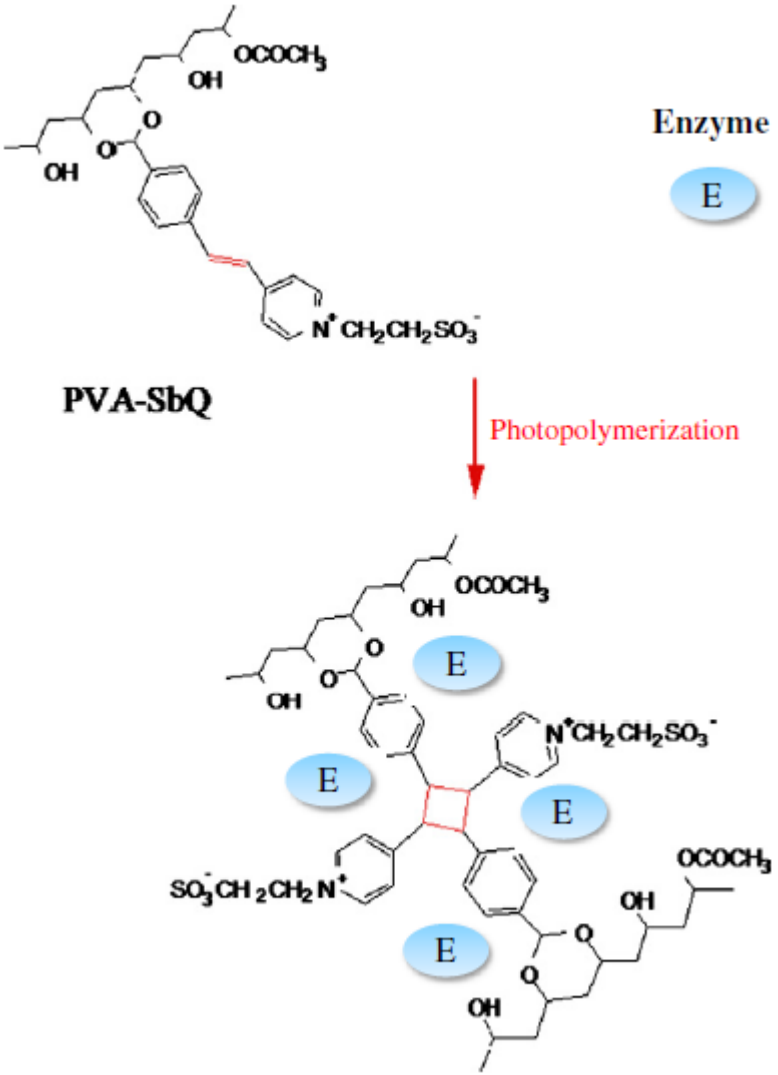
- One-step method consists in applying an appropriate potential or current to the transducer soaked in an aqueous solution containing both enzyme and monomer molecules
- Enzyme molecules in the immediate vicinity of the electrode surface are physically incorporated within the growing polymer network on the surface.
- Most of electropolymerized films used for biomolecule immobilization are conducting polymers




Polyaniline Polypyrrole Polythiophene

# Entrapment by photopolimerization

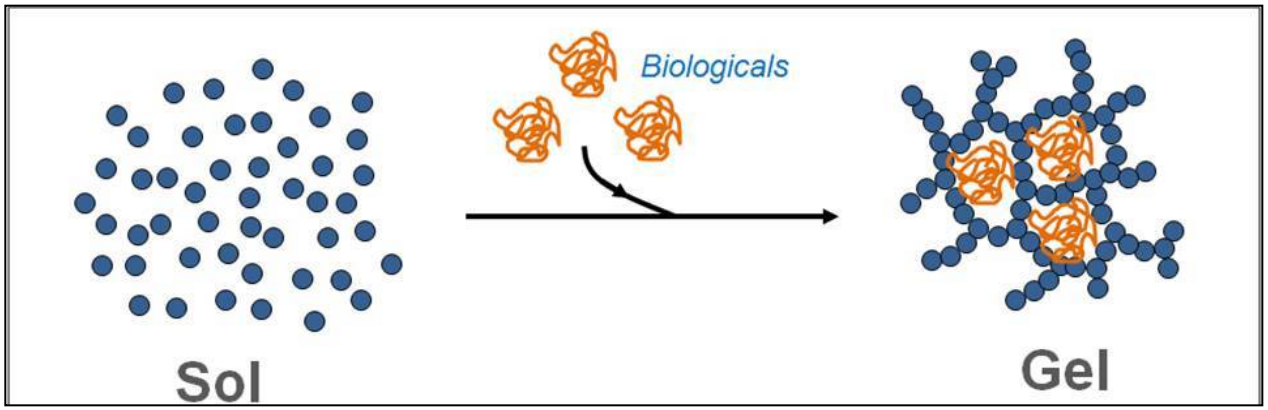
- The poly(vinyl alcohol)-bearing styrylpyridinium groups (PVASbQ) (photo-crosslinkable groups)
- An insoluble matrix, polymerization is initiated by light exposition



# Sol–gel entrapment process

- Sol–gel process involves hydrolysis of alkoxide precursors under acidic (or alkaline) conditions followed by condensation of the hydroxylated units, which leads to the formation of a porous gel
- Start with low-molecular weight metal alkoxide precursor (e.g. tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS)) with silanol (Si–OH) groups.
- Then condensation reaction between silanol moieties to form siloxane (Si–O–Si) polymers

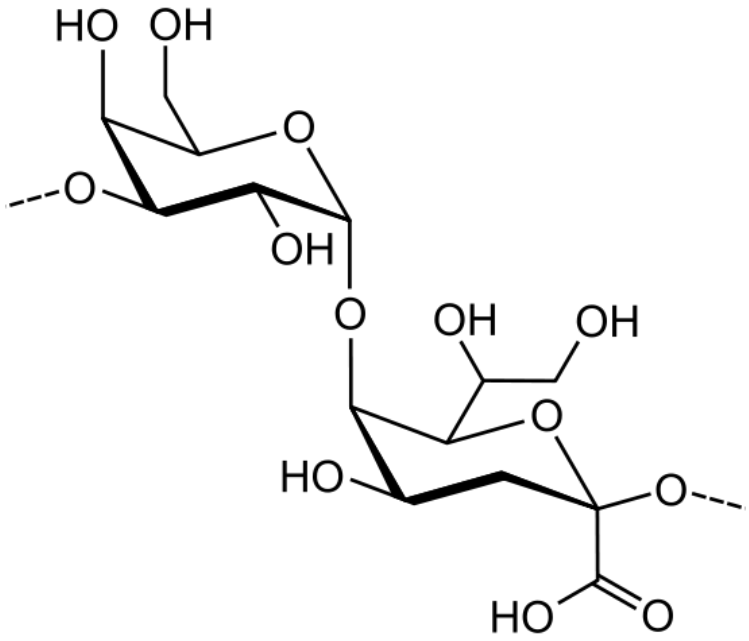
☺ Silica gels are highly porous, showing physical rigidity, chemical and biological inertness and thermal stability



# Entrapment in a polysaccharide-based gel

- Polysaccharides used (e.g. alginate, chitosan or agarose).
- Biocompatible, non toxic, provide natural microenvironment to the enzyme and also give sufficient accessibility to electrons to shuttle between the enzyme and the electrode.
- Alginate
  - derived from brown seaweed extracts
  - very porous and its stability is poor
- Chitosan

  a derivative of chitin which is found in the exoskeleton of crustaceans or insects and in fungal cell wall

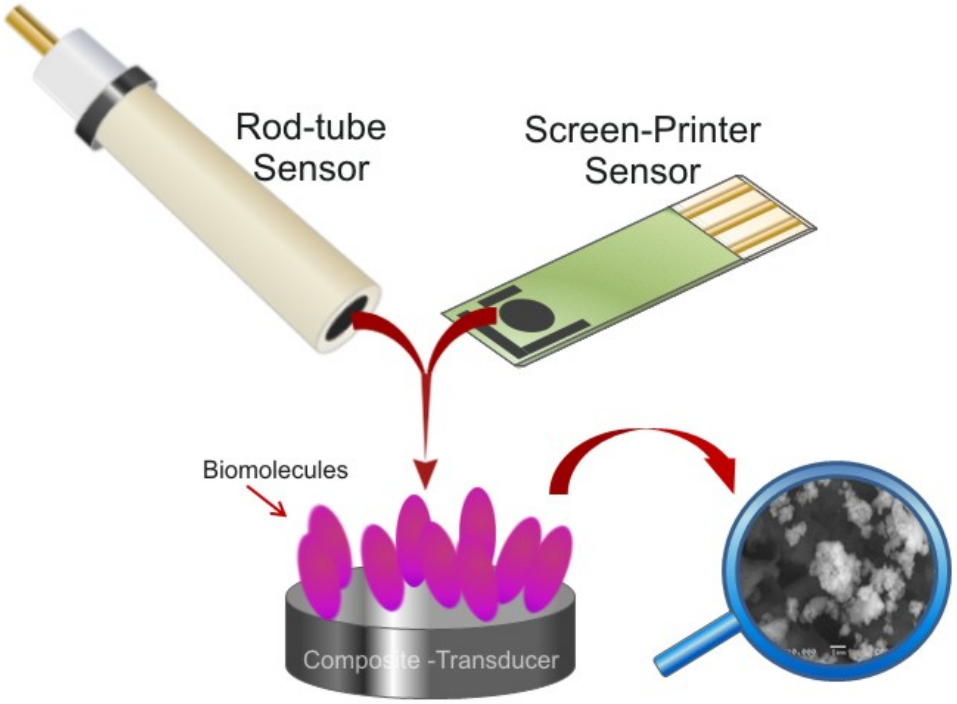


# Entrapment in a carbon paste

- A mixture of carbon (graphite) powder and a binder (pasting liquid) is a popular electrode material used for the preparation of various electrodes, sensors and detectors
- It allows an intimate contact between incorporated enzymes, mediator and sensing sites permitting a fast electron transfer.
- It is versatile, stable and the surface is easily renewed with good reproducibility.

# Entrapment in a carbon paste

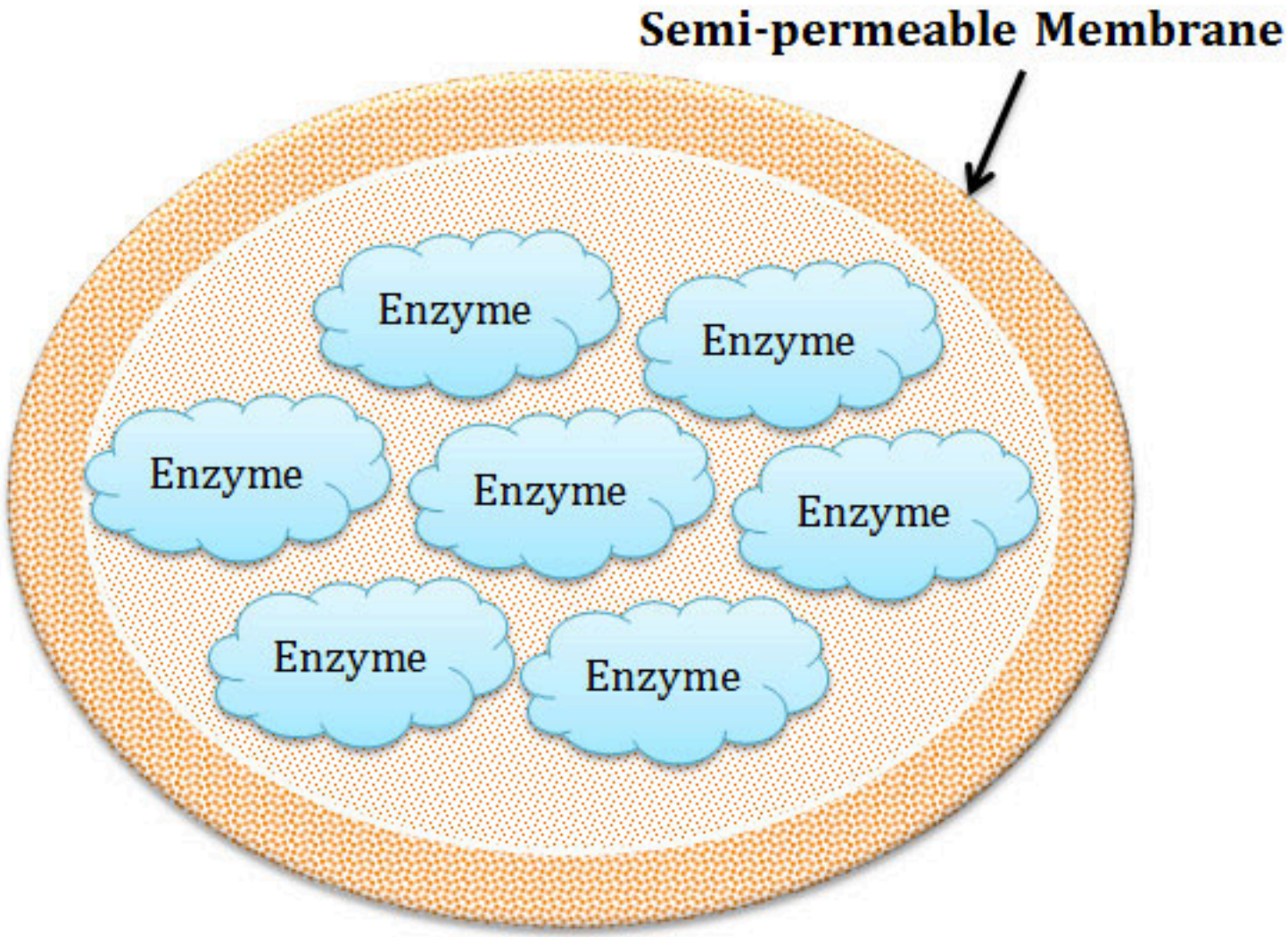
- First mix enzyme solution and graphite powder
- Then, mix with mineral oil (e.g. paraffin)
- The final paste is filled into a plastic cylindrical cartridge or used as screen-printed electrode



# Encapsulation

Biomolecule held in place behind an inert membrane – close contact between biomolecule and transducer

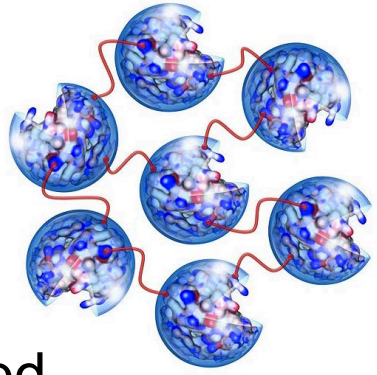
- cellulose acetate (dialysis membrane) – excludes proteins and slows transportation of interfering species
- polycarbonate (Nucleopore) – synthetic
- collagen – natural
- PTFE : polytetrafluoroethylene (Teflon) –synthetic- selectively permeable to gases such as oxygen
- Nafion
- Polyurethane



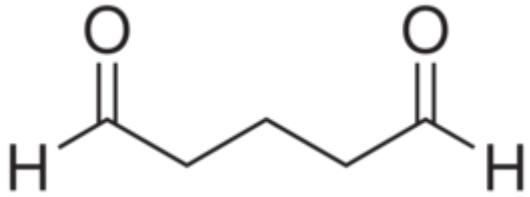

# Encapsulation

(Enzyme Immobilization)

# Crosslinking



- Glutaraldehyde or other bifunctional agents are used.




- The enzyme can be either cross-linked with each other or in the presence of a functionally inert protein such as bovine serum albumin.

☺ Simple and provides strong chemical binding

☹ Possibility of activity losses due to the distortion of the active enzyme conformation

☹ Possibility of chemical alterations of the active site

# Covalent immobilization

- The most widely used method for enzyme immobilization
  - It is technically more complex
  - It requires a variety of often expensive chemicals
  - It is time-consuming
  - ➢ But immobilized enzyme preparations are stable and leaching is minimal
- Enzymes are immobilized by a suitable group in the surface:
  - Hydroxyl groups in supports (e.g cellulose, dextran, agarose)
  - Amino, carboxyl and sulfhydryl groups in amino acids

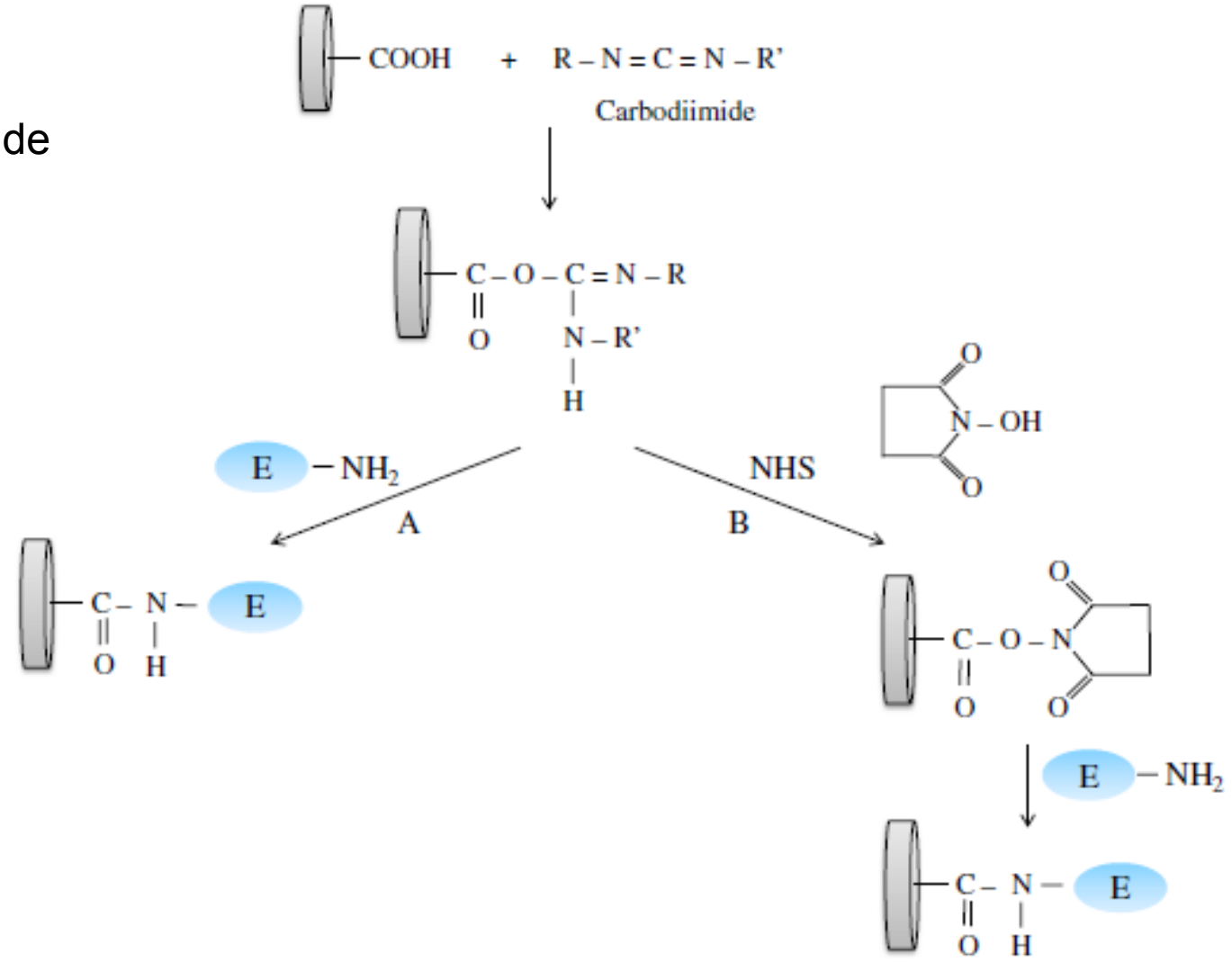
# Covalent immobilization

**Activation of carboxylic groups**

- Carbodiimides (e.g. EDC) allow the binding between the carboxyl groups of a support and the amino function of an enzyme
- N-hydroxysuccinimide (NHS) can be associated to carbodiimide in order to improve immobilization efficiency
- This procedure is widely used to develop enzymatic biosensors.

# Covalent immobilization

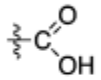
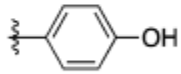
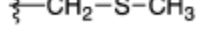
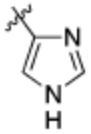
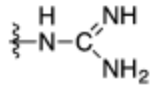
Carbodiimide coupling



# Covalent immobilization

- The amine groups of proteins are the most used moieties for covalent immobilization.
- Lysines are present in most proteins, and can make up 6-10% of the overall amino acid sequence and are frequently located on the surface of the protein.
- Lysines are very reactive toward electrophilic agents without the need to be activated and provide good stability

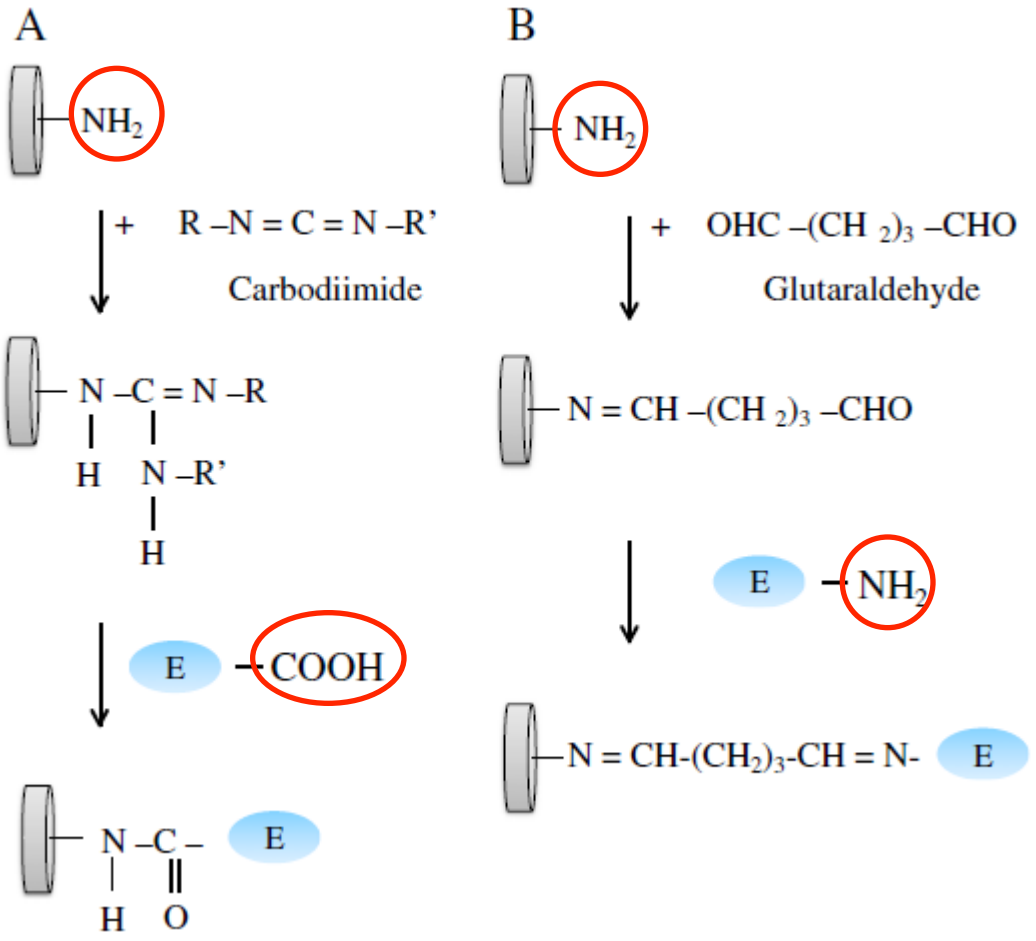
Table 1. Reactive Functional Groups in Naturally Occurring Amino Acids

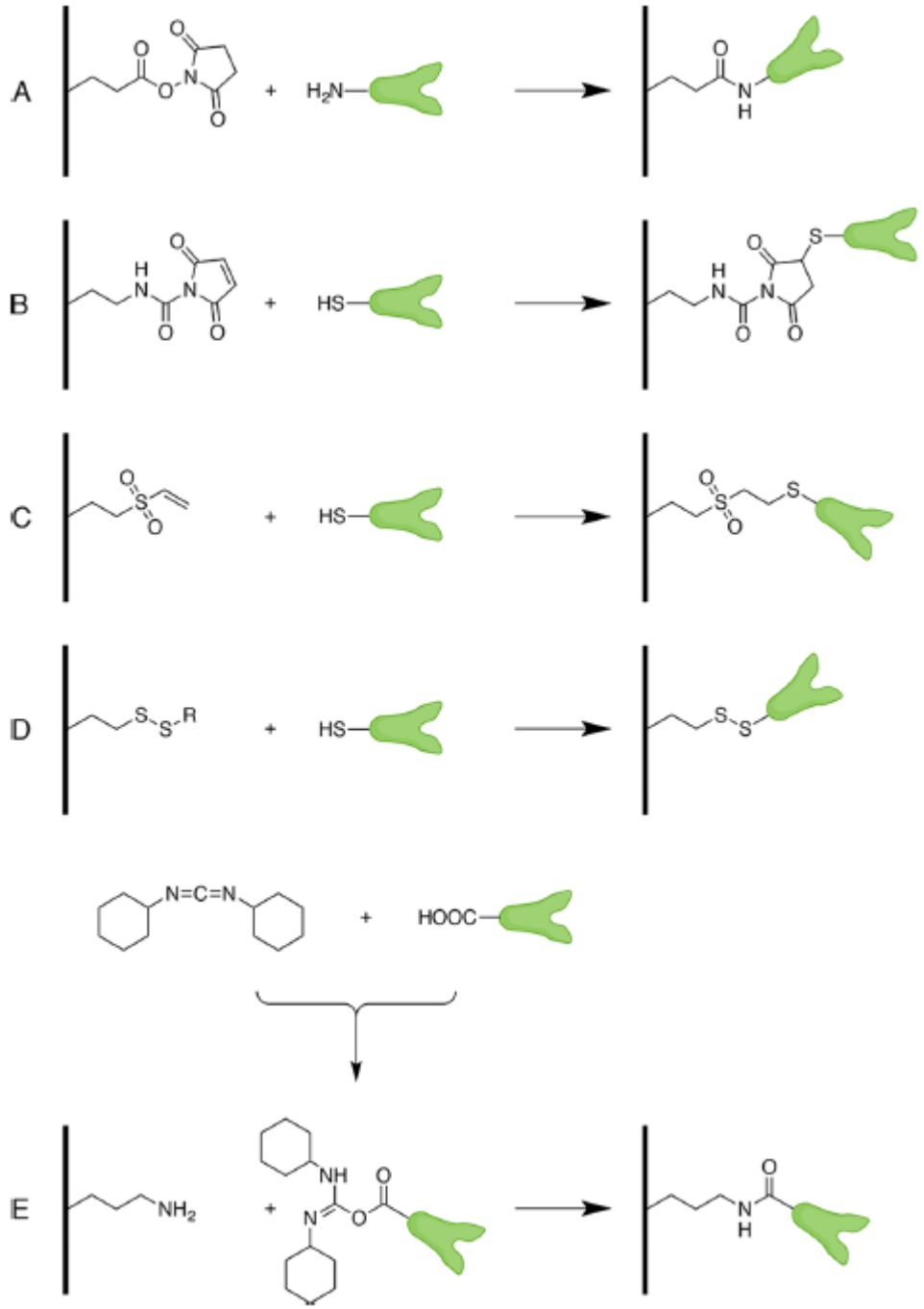
| Reactive group | | Amino acid |
|---|---|---|
|  | Primary amine | N-terminus<br>ε-amino group of Lysine |
|  | Carboxylic acid | C-terminus<br>Glutamic acid<br>Aspartic acid |
|  | Thiol | Cysteine |
|  | Hydroxyl | Serine<br>Threonine |
|  | Phenol | Tyrosine |
|  | Thioether | Methionine |
|  | Imidazole | Histidine |
|  | Guanidino | Arginine |

# Covalent immobilization

**Activation of amino groups**

- Carbodiimides
- Glutheraldehyde
- Etc






# Covalent Immobilization

A. EDC-NHS
B. Maleimide
C. Vinyl sulfone
D. Disulfide-modified surface
E. N,N dicyclohexyl carbodiimide (DCC)

# Chemisorption

Thiol (–SH) - metal bonds

- Strong adsorption properties of –SH and Au could be used for immobilization
- Thiol-containing enzymes can be directly immobilized on gold surface
- Simple and the strong and stable biomolecule attachment is achieved.
- The enzyme can be modified to contain thiol moieties

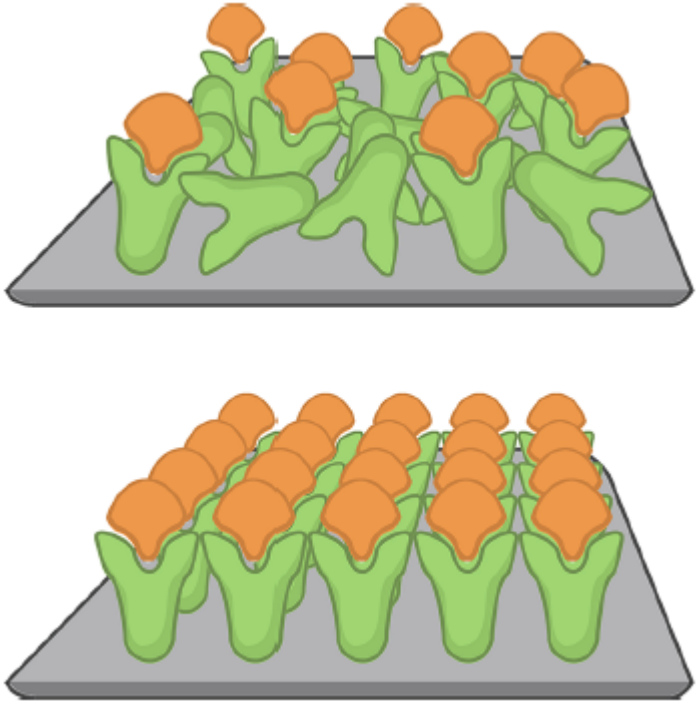
# Site-specific immobilization

Efforts have been achieved in order to develop biosensors based on oriented and site-specific immobilization of enzymes.

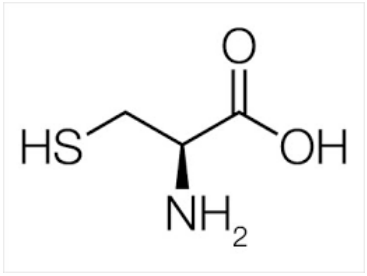
a) Gene fusion to incorporate a peptidic affinity tag. The enzymes are then attached from this affinity tag to anti-tag antibodies on membranes

b) Modification to incorporate a single biotin moiety on enzymes

c) Site-directed mutagenesis to introduce unique cysteines to enzymes. The enzymes are attached on thiol-reactive surfaces through the sulfhydryl group

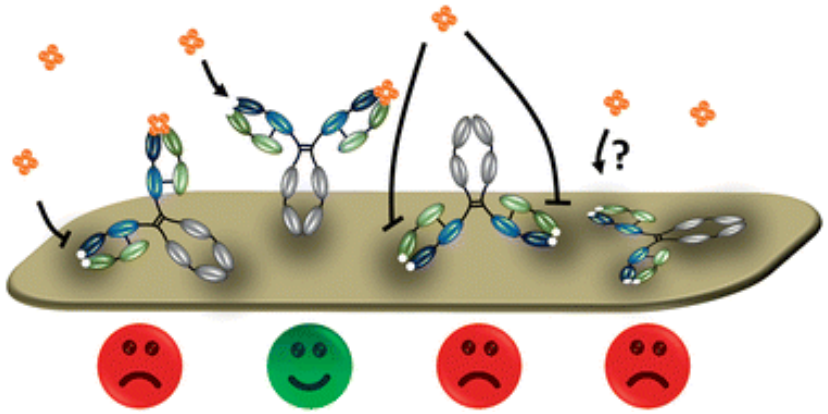


cysteine



# Affinity based
# Site-specific immobilization

- (bio)affinity bonds between an activated support and a specific group (a tag) of the protein sequence
- This method allows to control the biomolecule orientation in order to avoid enzyme deactivation and/or active site blocking.
  - (strept)avidin-biotin
  - lectin-carbohydrate
  - metal cation–chelator
  - DNA-mediated




*Trilling AK, 2013*

# Affinity based
# Site-specific immobilization

**Biotin-(strept)avidin**

- Biotinylation of proteins can be achieved through a covalent coupling of biotin to the protein by the use of biotin-ester reagents that preferentially modify lysine residues
- Enzymes can also be genetically biotinylated using a biotin acceptor peptide sequences fused to the C-terminus of enzyme