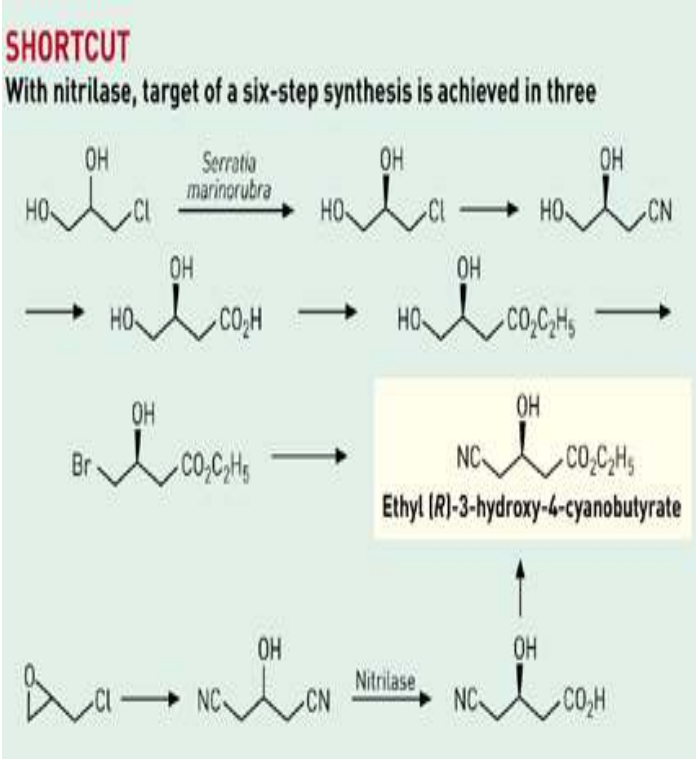
Enzyme Engineering

5. Immobilized Enzymes

Why Enzyme?

- Selectivity and specificity
- Reaction under mild conditions
- Environmental aspects
- Shortcut steps



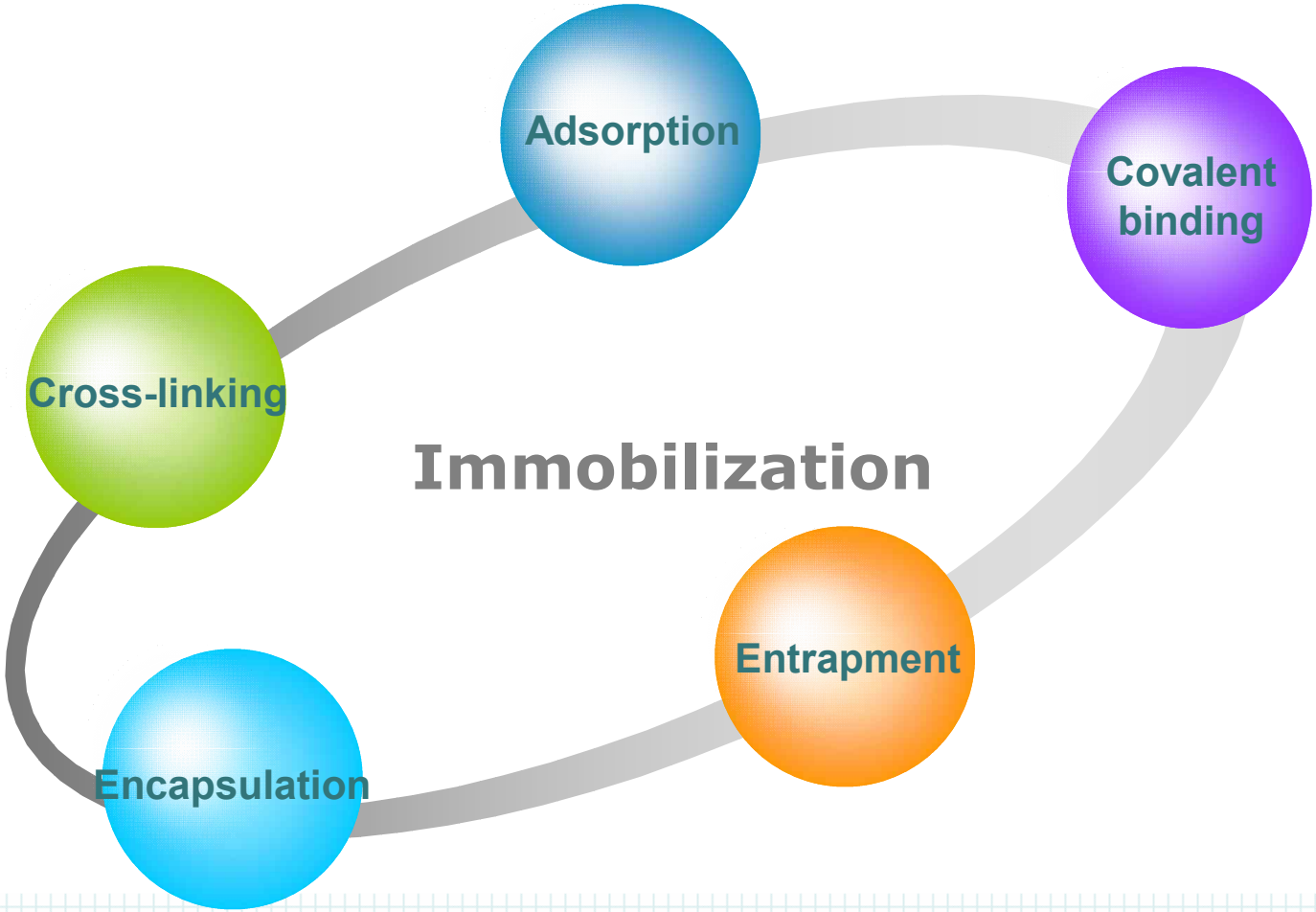
Ref.) C&EN, 80, 7, pp86-87, 2002

Why immobilized enzymes?

Definition : Immobilization means that the biocatalysts are limited in moving due to chemically or physically treatment

Reasons	**Limitations**
- Reuse of enzyme(reducing cost)	-Cost of carriers and immobilization
- Easy product separation	-Changes in properties(selectivity)
- Continuous processing	-Mass transfer limitations
- Stabilization by immobilization	-Activity loss during immobilization

Conventional Immobilization Methods



Enzyme Immobilization ; Adsorption

- DEAE-cellulose, activated carbon, etc.
- reversible electrostatic surface interaction
  -Van der Waals, Hydrogen interaction

Advantage	● Little damage to enzyme ● Simple / cheap / quick ● No chemical change to support / enzyme ● Reversible to allow regeneration
Disadvantage	● Leakage of enzyme ● Non-specific binding ● Over-loading on the support

Enzyme Immobilization ; Covalent binding

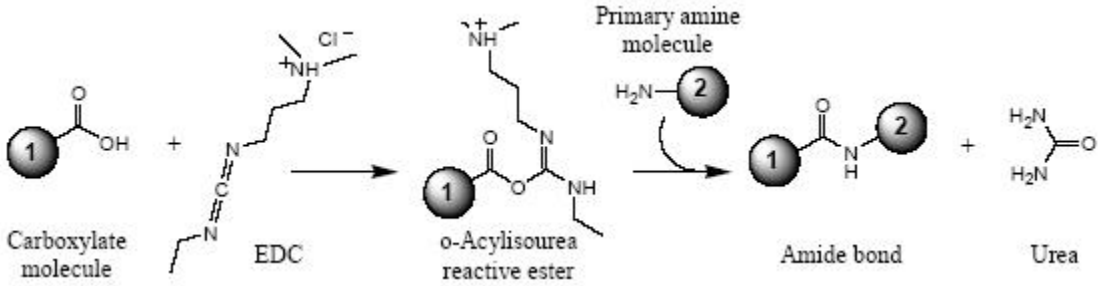
- Formation of covalent bond between enzyme and support

- -NH_2 of lysine or arginine
  -COOH of aspartic acid or glutamic acid
  -OH of serine or threonine
  -SH of cystein

- Hydrophilicity is the most important factor for maintaining enzyme activity in a support environment.
  → polysaccharide polymers (-OH)

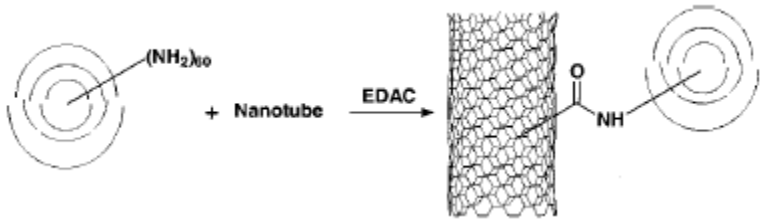
Enzyme Immobilization ; Covalent binding

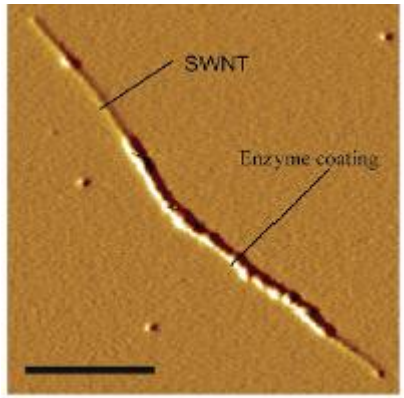
- EDC (1-Ethyl-3-(3dimythylaminopropyl)) activation



Ref.) http://piercenet.com/files/0475as4.pdf

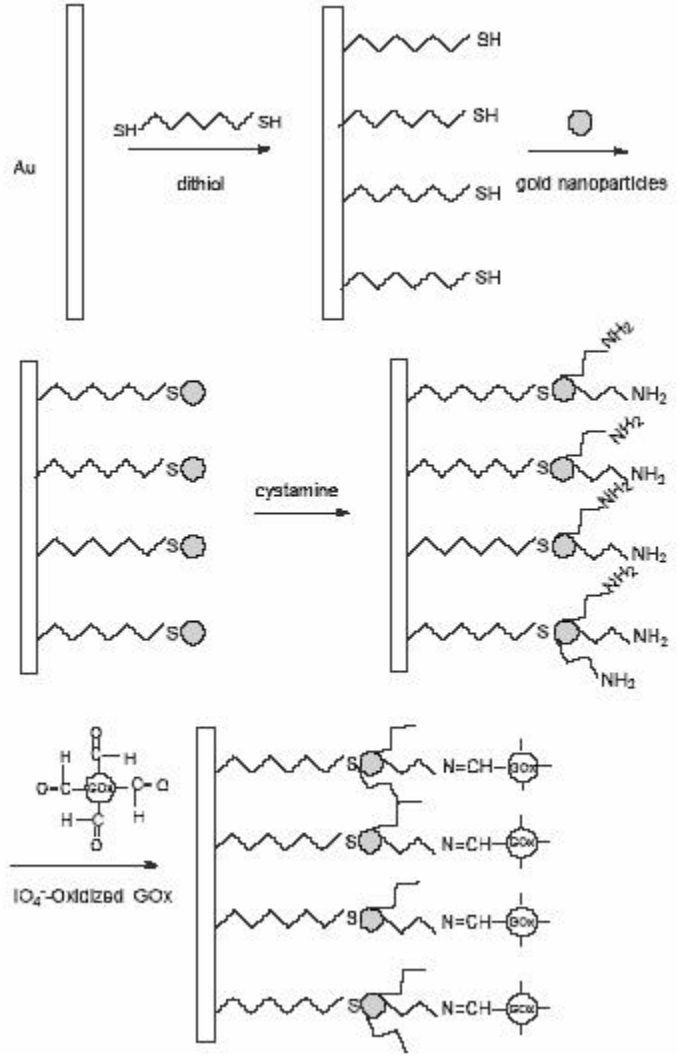
- Nanotube – protein conjugate



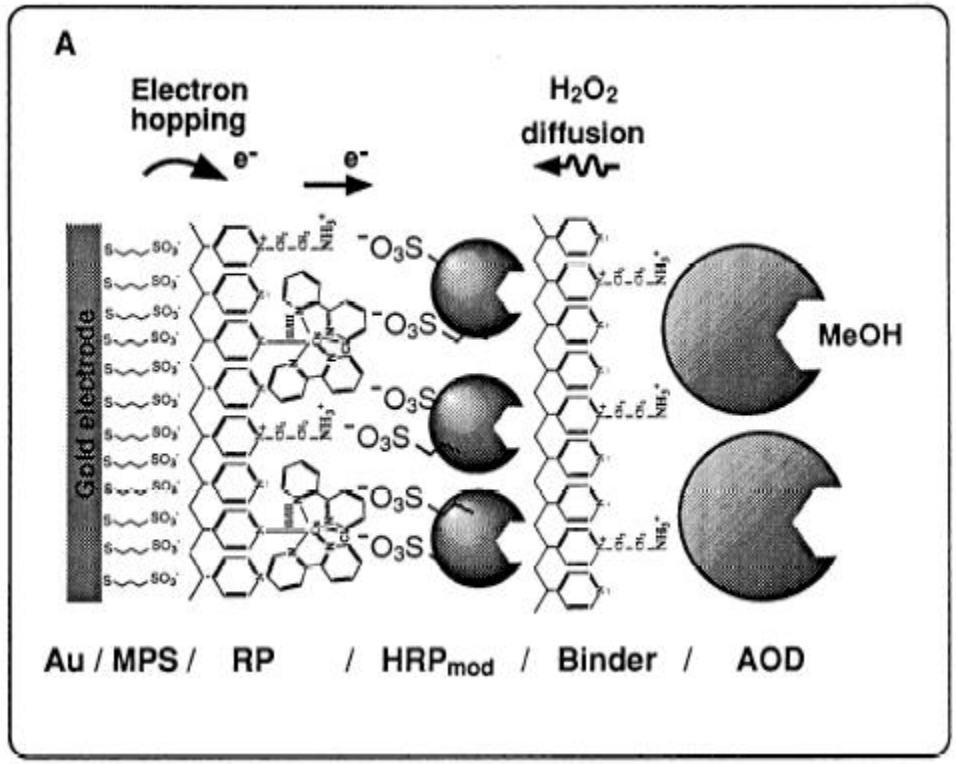


Ref.) JACS, 124 (2002) 12664 - 12665

Enzyme Immobilization ; Covalent binding



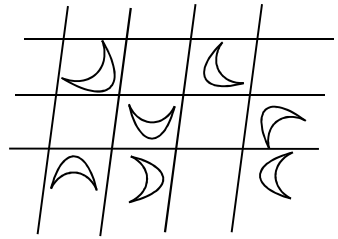
Ref.)
Bioelectrochemistry, 67, 1, 15-22 (2005)



Ref.) Biosensors and Bioelectronics 15 (2000), 43-52

Enzyme Immobilization ; Entrapment

- Entrapped enzymes into lattice by polymer structure
  - synthetic polymer ; polyacrylamide, polyvinyl alcohol
  - natural polymer ; *k*-carageenan, Ca-alginate



- Restricted in movement by lattice structure of gel

- The porosity of gel lattice is tight enough to prevent leakage of enzyme, yet the same time allow free movement of substrate and product.

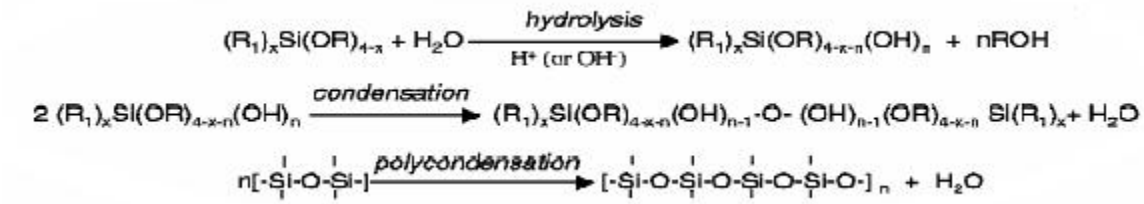
(Ex) Sol-gel Immobilization Method

- One of entrapment method that stabilizes the enzyme by silica structure

Additives
polymers, surfactant, protein stabilizer

Biomolecules
Proteins, DNA, cells

Precursor sol



Gelation
Sol-gel formation with entrapped biomolecules

Aging
Polycondensation by solvent evaporation and sol-gel shrinkage

Sol-gel Immobilization Method

- Colloidal suspension of silica particles that is gelled to form a solid

- Prevent the enzymes to leak out and easily mass transfer because of porous structure.

- Advantages

; Entrapped enzymes with beneficial microenvironments

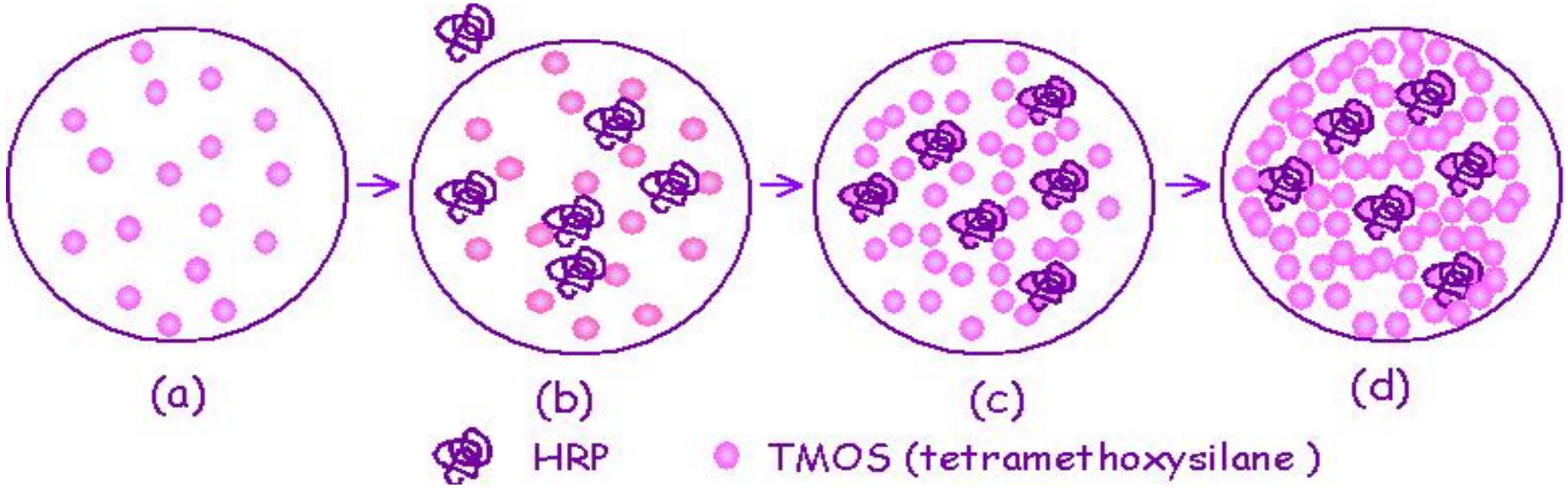
High enzyme activity

Easy mass transfer because of the porous structure

Optically transparent silicate materials

Ideal for the development of chemical / biochemical sensors

Sol-gel Immobilization Process



(a) precursor solution (TMOS, TEOS), sol phase

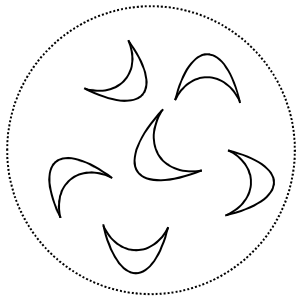
(b) addition of proteins solution

(c) networking

(d) immobilized proteins in porous silica gel

Enzyme Immobilization ; Encapsulation

- Enveloping the enzymes within various form of semi-permeable membranes such as nylon and cellulose nitrate



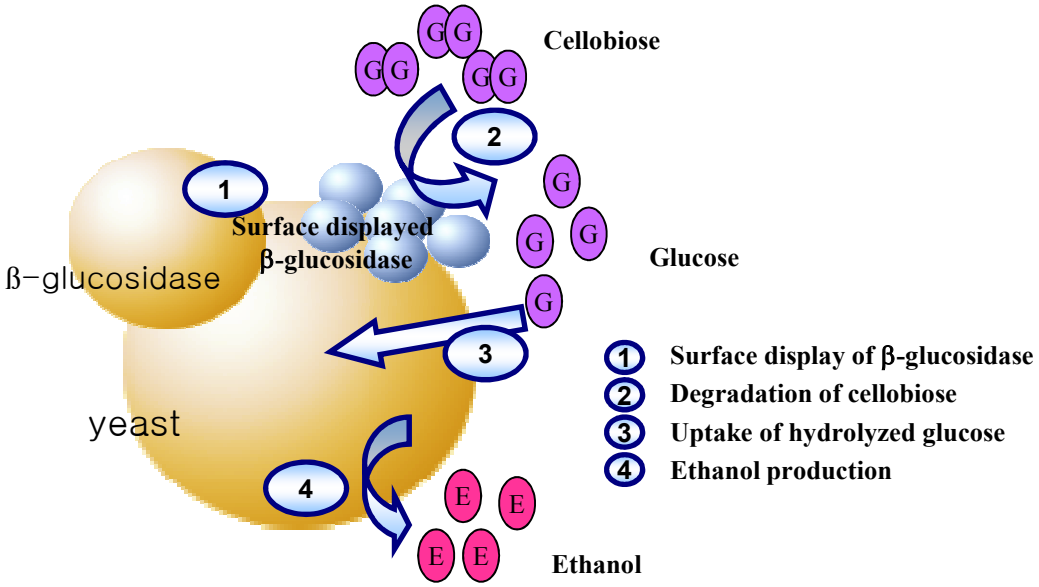
- Microcapsules varying from 10-100μm in diameter

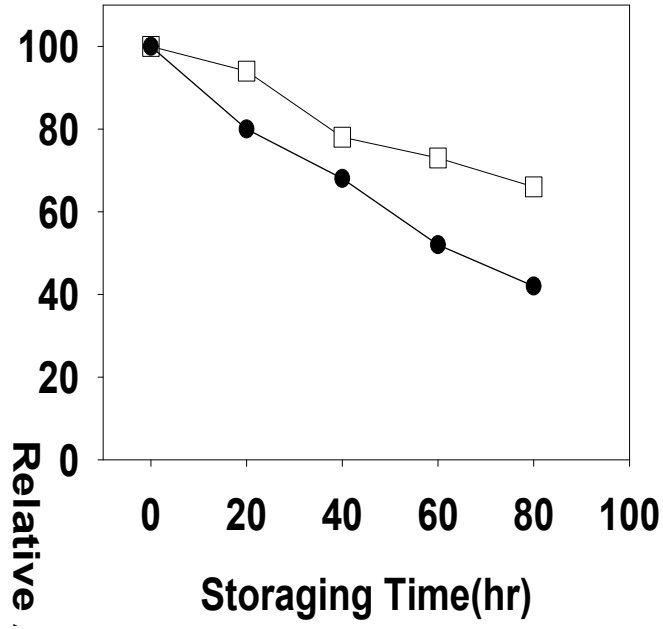
- Large enzymes cannot pass out or into the capsule, but small substrates and products can pass freely across the semi-permeable membrane.

Enzyme Immobilization ; Cross-linking

- Covalent bond formation between enzymes by means of a bi- or multi-functional reagent
- Glutaraldehyde, toluene diisocyanate
- Form a large, three-dimensional complex structure
- Toxicity of reagent or limiting factor.
- Multiple- binding of enzymes

Surface Display





□ : surface displayed β-glucosidase

● : β-glucosidase

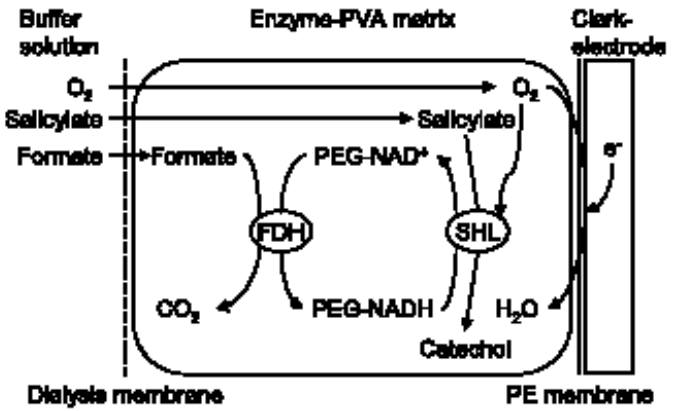
(Ex) Application to Protein-Chip

- Ag - Ab reaction
- Protein – protein interaction / Protein – DNA interaction
- Analysis of protein function
- Proteomics
- Medical diagnosis / drug discovery

Immobilization type	Adsorption / Absorption	Covalent crosslinking	Affinity attachment
Advantage	•Generally simple • No manipulation of the protein sample	• Reproducibility and stability of protein layer • The possibility of controlling the density and environment of the immobilized species	• Identical orientation by site specific immobilization • Direct immobilization by high affinity • Easy protein purification and array fabrication • Reproducibility and stability of protein layer • The possibility of controlling the density and environment of the immobilized species
Disadvantage	• Some protein denature and inactive • Unstable binding Non-specific random and multi-oriented protein immobilization: activity decreased • Irreproducibility of results	• Non-specific random orientation: activity decreased • Some protein denature and inactive • Additional chemical reaction for modification in vitro	• Difficult application in multi-subunit proteins • The possibility of elusion of some protein

Immobilization of Coenzyme ; Entrapment in Membrane Module

- NAD^+ immobilized to macromolecular carriers like polyethylene glycol
- The coenzyme is continuously available for the enzyme activity
- Some biosensors based on the co-entrapment of NAD(P)+-dependent dehydrogenase and NAD+-dextran of PEG-NAD+ have been reported.



[Schematic representation of the PEG-NAD= regeneration system. (FDH, formate dehydrotenase; SHL, salicylate hydroxylase)]

Immobilization of Coenzyme ; Entrapment in Membrane Module

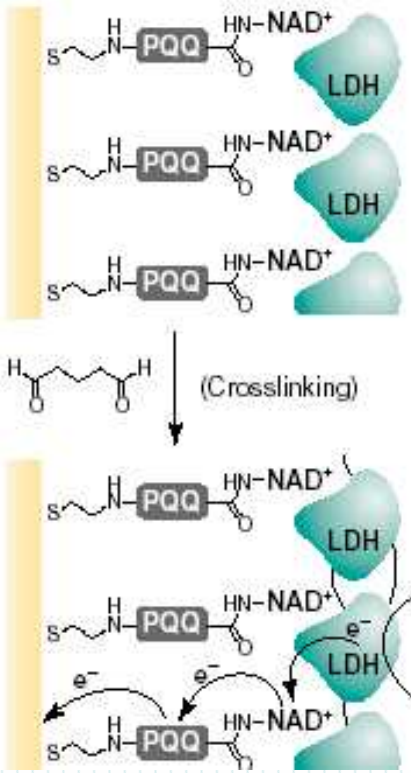
- Recent developments demonstrate the use of nanofiltration membranes for coenzyme retention in continuous enzyme synthesis.
- In this method, a native coenzyme could be used instead of a chemically modified coenzyme.

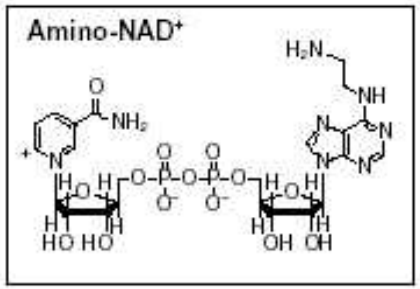
Parameter	Ultrafiltration membrane	Nanofiltration membrane
Trimethylpyruvate[a] (feed)	500 mM	500 mM
NAD+	0.2 mM	0.2 mM
Residence time	4 h	4 h
Mean conversion	93%	95%
Yield	366 g l^{-1} d^{-1}	373 g l^{-1} d^{-1}
Total turnover number	2325	7920

[Comparison of the production of L-tert-leucine in continuous working enzyme membrane reactors either equipped with an ultrafiltration or a nanofiltration membrane]

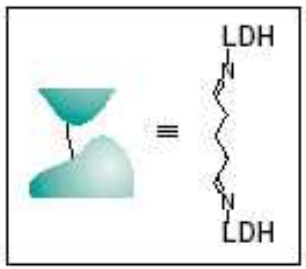
Immobilization of Coenzyme ; Entrapment in Membrane Module

- The N^6-(2-aminomethyl) –functionalized NAD+, was covalently linked to a pyrroloquinoline quinone monolayer associated with a gold electrode.





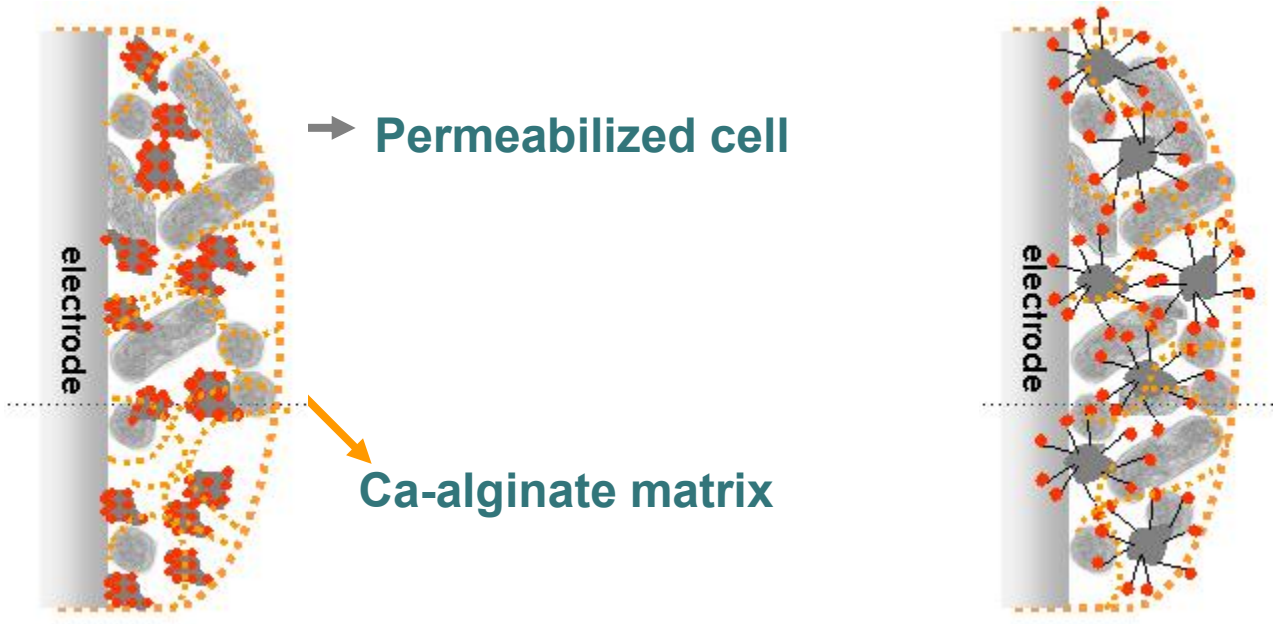
* The enzyme-electrode stimulates the biocatalytic oxidation of lactate and act as an amperometric biosensor for lactate.



[The assembly of an integrated lactate dehydrogenase monolayer-electrode by the crosslinking of an affinity complex formed between the enzyme and a PQQ_NAD+ monolayer-functionalized gold electrode.]

PQQ : Pyrroquinolinequinone

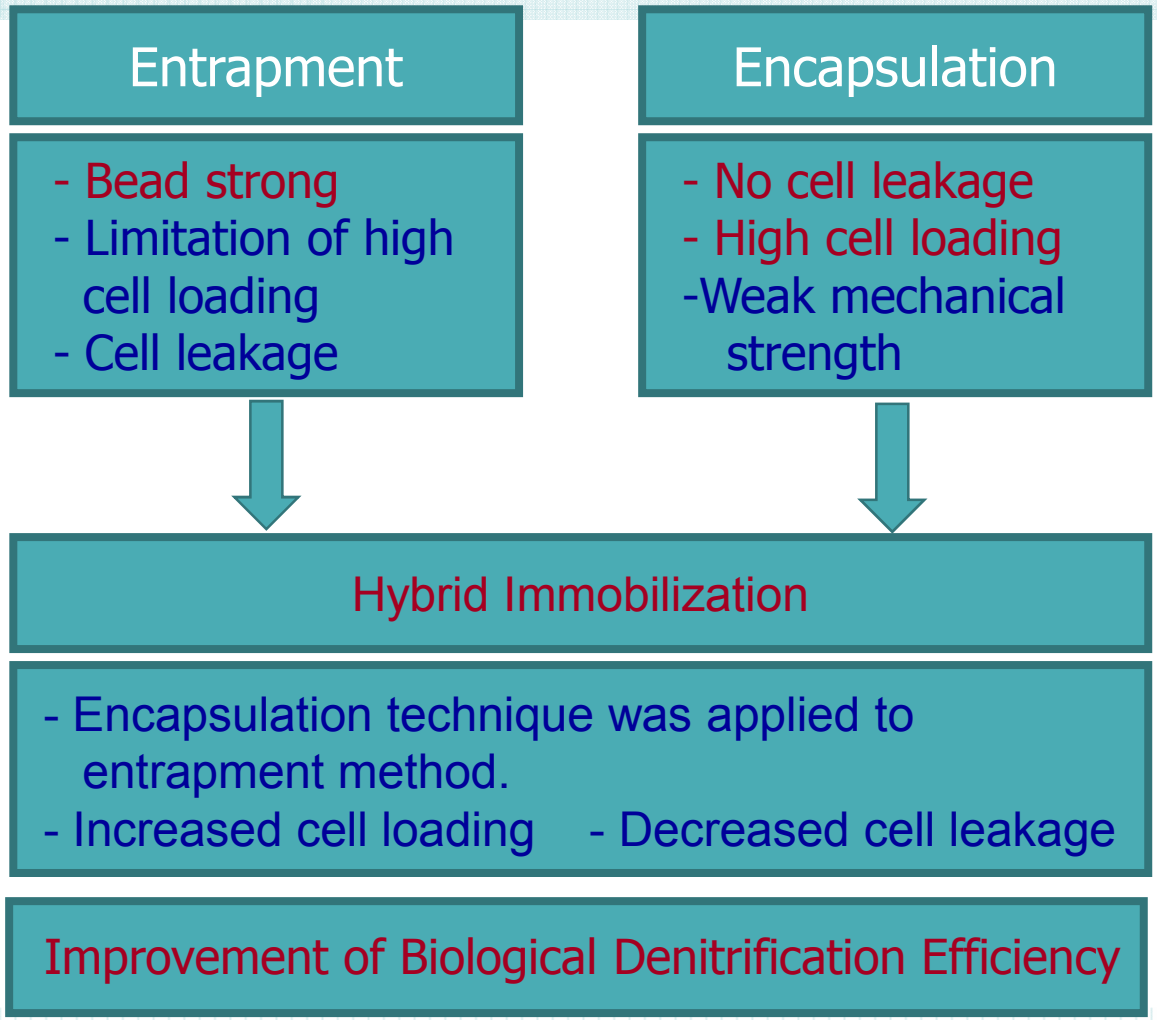
(Ex) Immobilization of Whole Cells



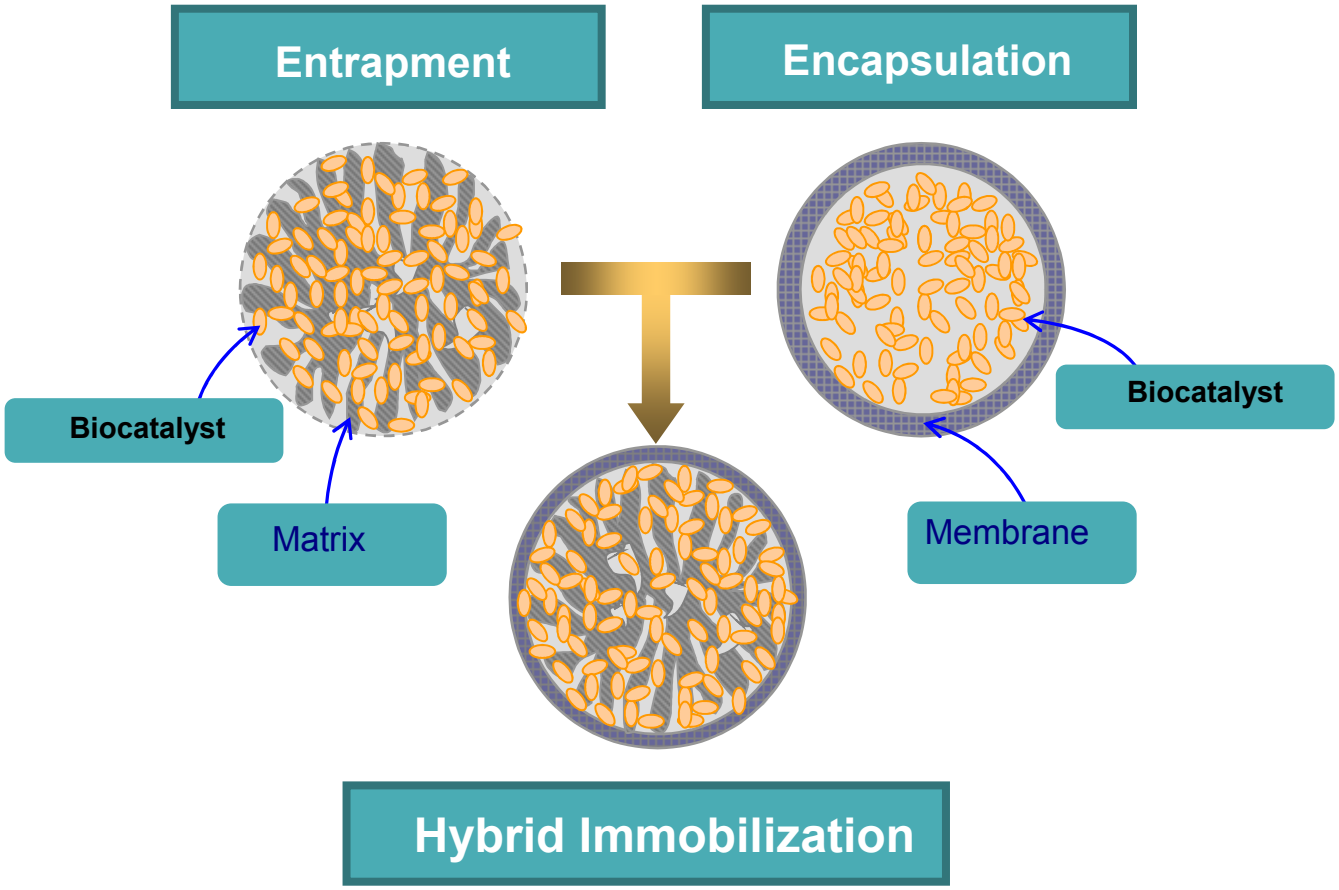
Carbon nanopowder-Neutral red

Carbon nanopowder-linker-Neutral red

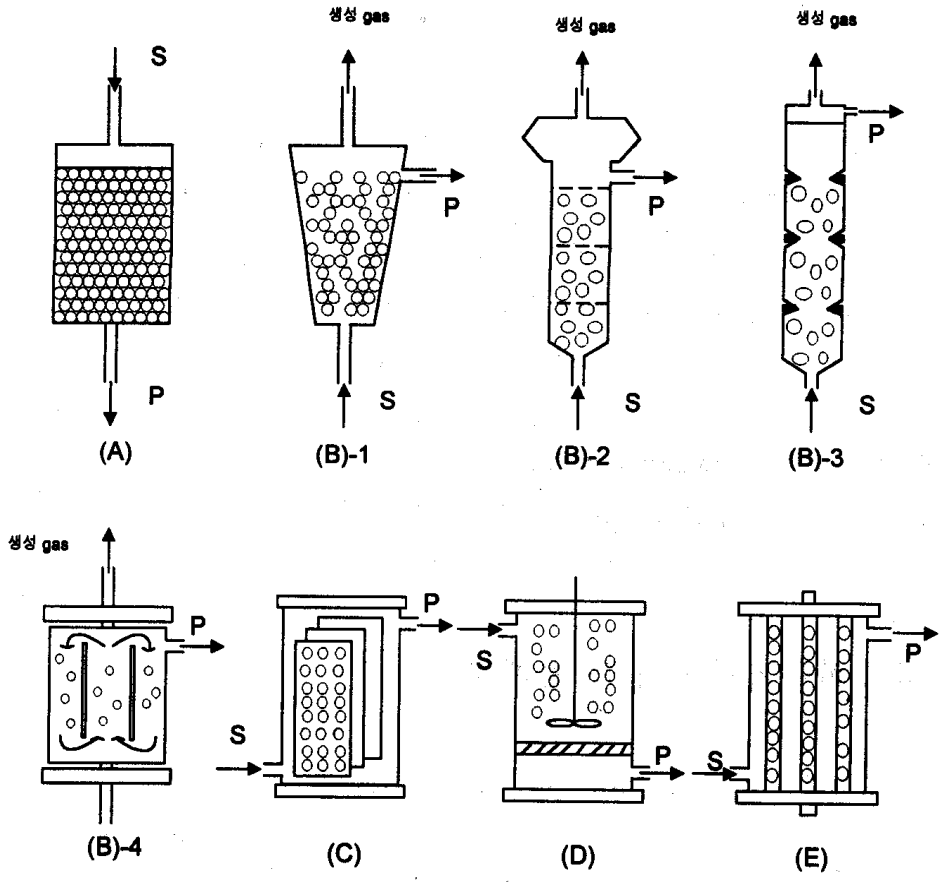
(Ex) Hybrid Immobilization



Hybrid Immobilization



Reactors for Immobilized Enzymes



(A) Packed-bed reactor

(B) Suspended reactor

(C) Panel type reactor

(D) Filter type reactor

(E) Membrane reactor

Characteristics of Bioreactors

Immobilized Bioreactor

Reactor	Characteristics
Batch	Separation is required Small amounts of fine chemicals
CSTR	Ease of control, operation Shear effect Substrate inhibition – favorable
Packed-bed	Most widely used reactor type Conversion – length of the reactor Product inhibition – favorable
Fluidized-bed	Packed-bed< < CSTR Hinimal pressure drop Ease of control – pll, temp. Oxygen supply, gas removal - better
Hollow-fiber	Economy of immobilization Lack of proper control of cell loading

Engineering Considerations

Biocatalysts : Engineering Consideration

1. Critical fluid velocity
   - shear rate
   - tensile strength (CO_2)
2. External and internal mass transfer
3. Operational stability (half-life)
4. Compression behavior, pressure drop

Considerations for commercialization

- Immobilization yield (Ex: Eupergit)
- Long-term stability
- Cost
- Contamination by microorganisms
- Impurity treatment in raw materials

  (ex) Production of high fructose corn syrup