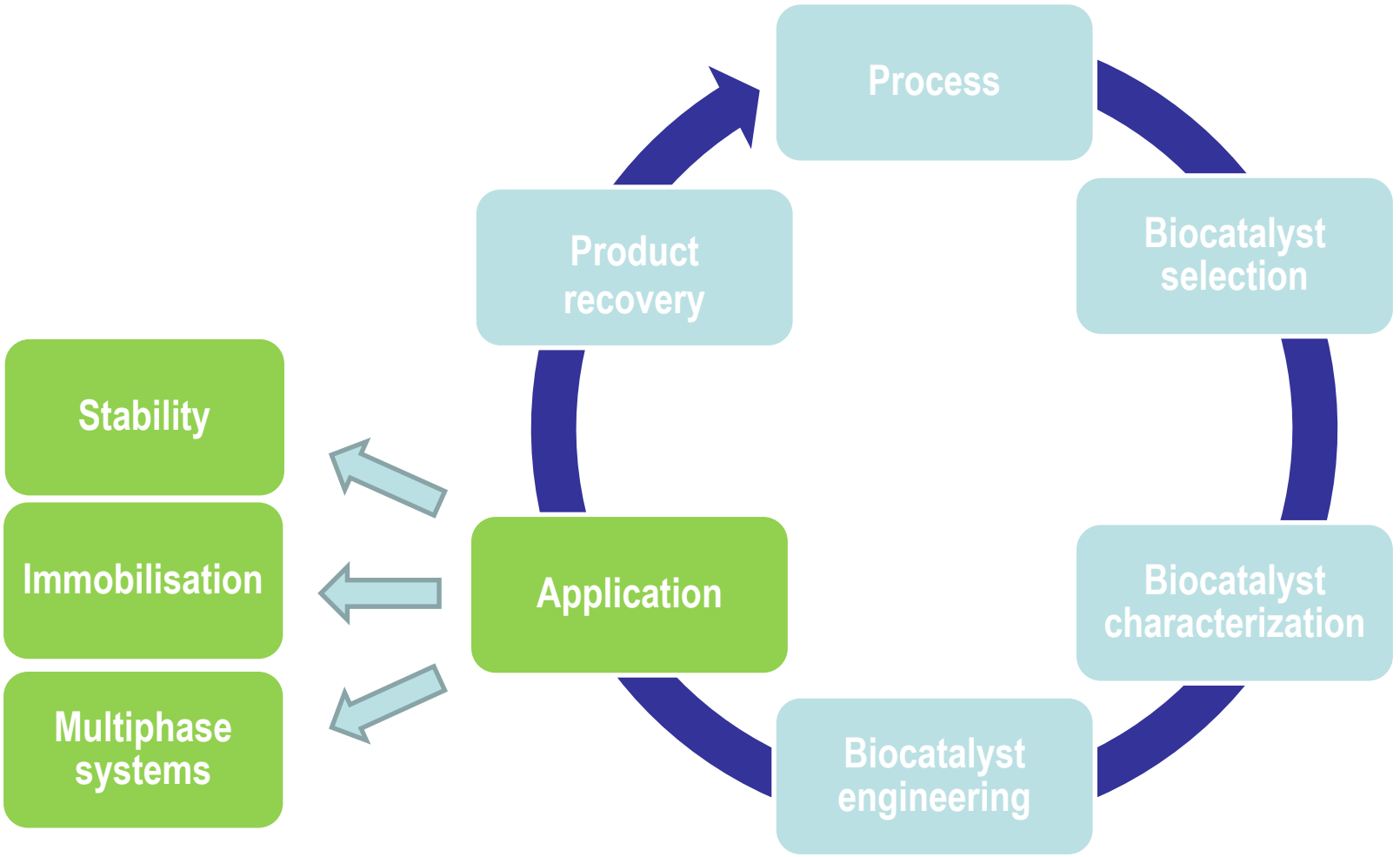
Formulation, stabilization and immobilization of enzymes for biotransformations

Biocatalysis for industrial synthesis



Trasform a good enzyme
into an efficient industrial biocatalyst

Chem Soc Rev

RSC Publishing

TUTORIAL REVIEW

View Article Online
View Journal

Cite this: DOI: 10.1039/c3cs35464d

Efficient immobilisation of industrial biocatalysts: criteria and constraints for the selection of organic polymeric carriers and immobilisation methods†

Sara Cantone,[a] Valerio Ferrario,[b] Livia Corici,[a] Cynthia Ebert,[b] Diana Fattor,[a] Patrizia Spizzo[a] and Lucia Gardossi*[b]

Adv. Synth. Catal. **2007**, *349*, 1289–1307

DOI: 10.1002/adsc.200700082

Enzyme Immobilization: The Quest for Optimum Performance

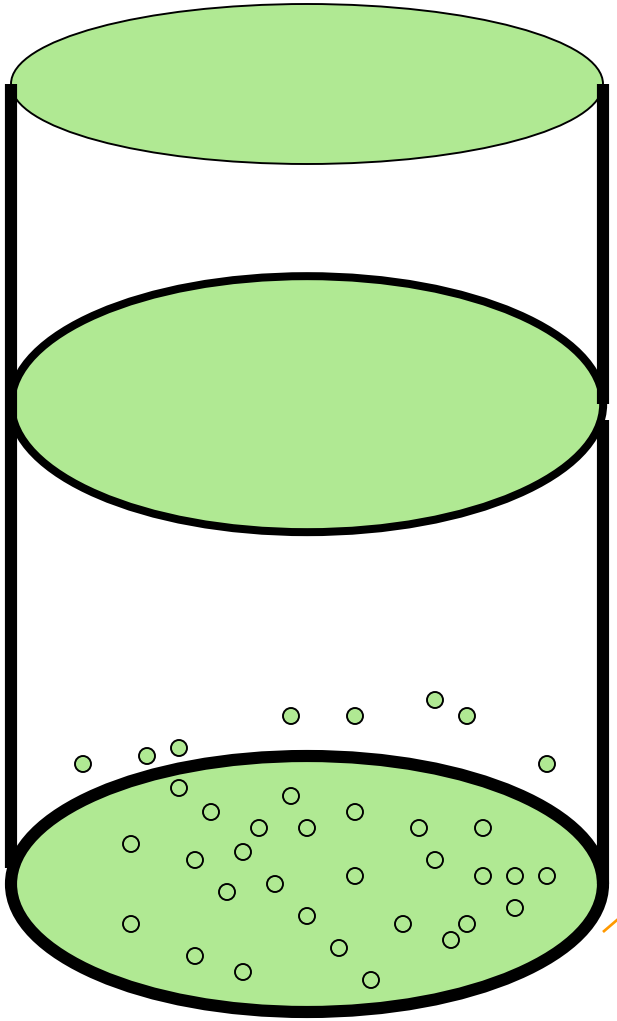
Roger A. Sheldon[a,*]

Understanding enzyme immobilisation

Ulf Hanefeld,*[a] Lucia Gardossi[b] and Edmond Magner[c]

Chem. Soc. Rev., 2009, **38**, 453–468

Immobilized enzymes: heterogeneous systems



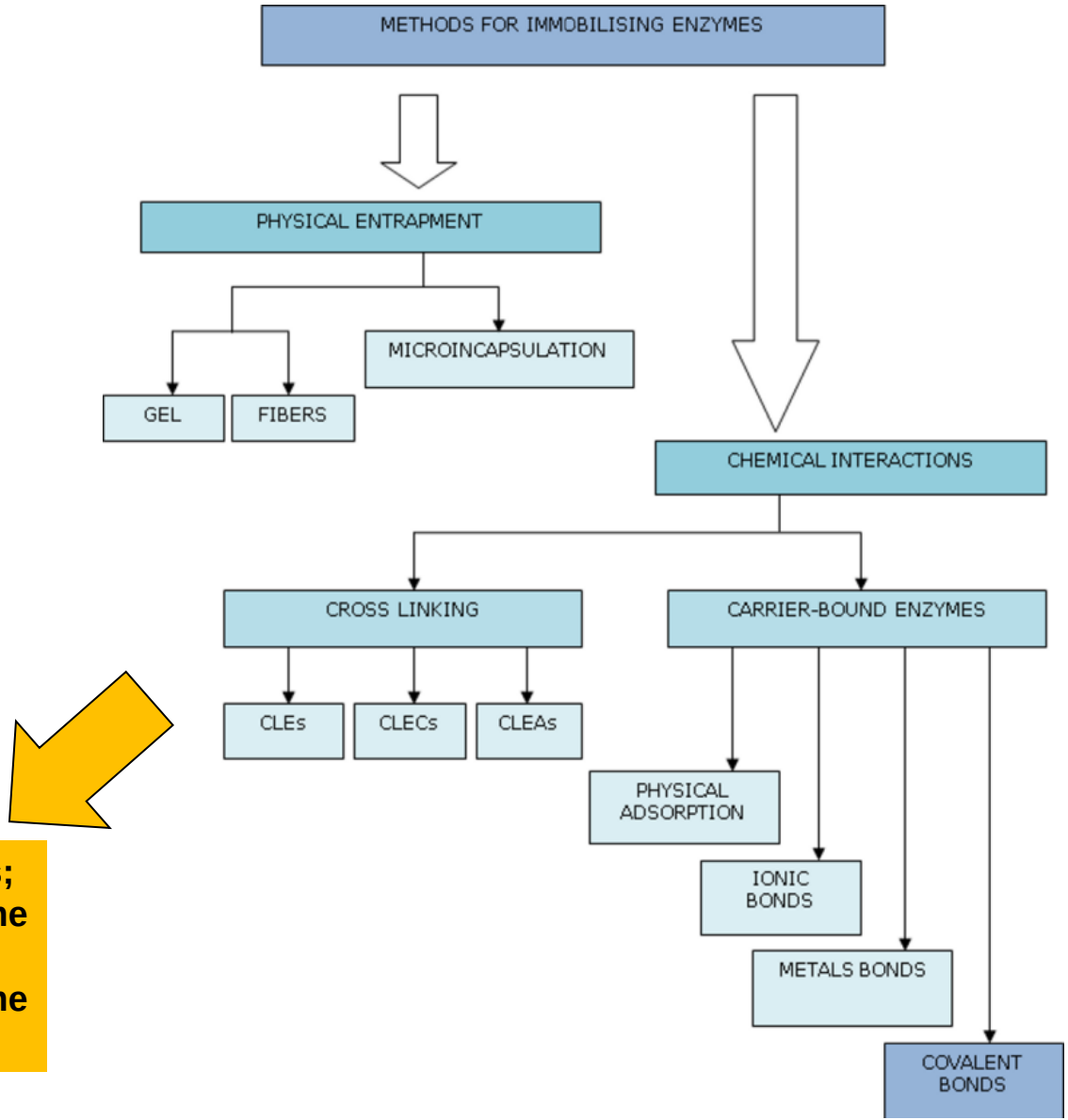
Immobilized enzymes: potential advantages

- enhanced stability,
- repeated or continuous use,
- easy separation from the reaction mixture,
- possible modulation of the catalytic properties,
- prevention of protein contamination in the product,
- easier prevention of microbial contaminations.

↓

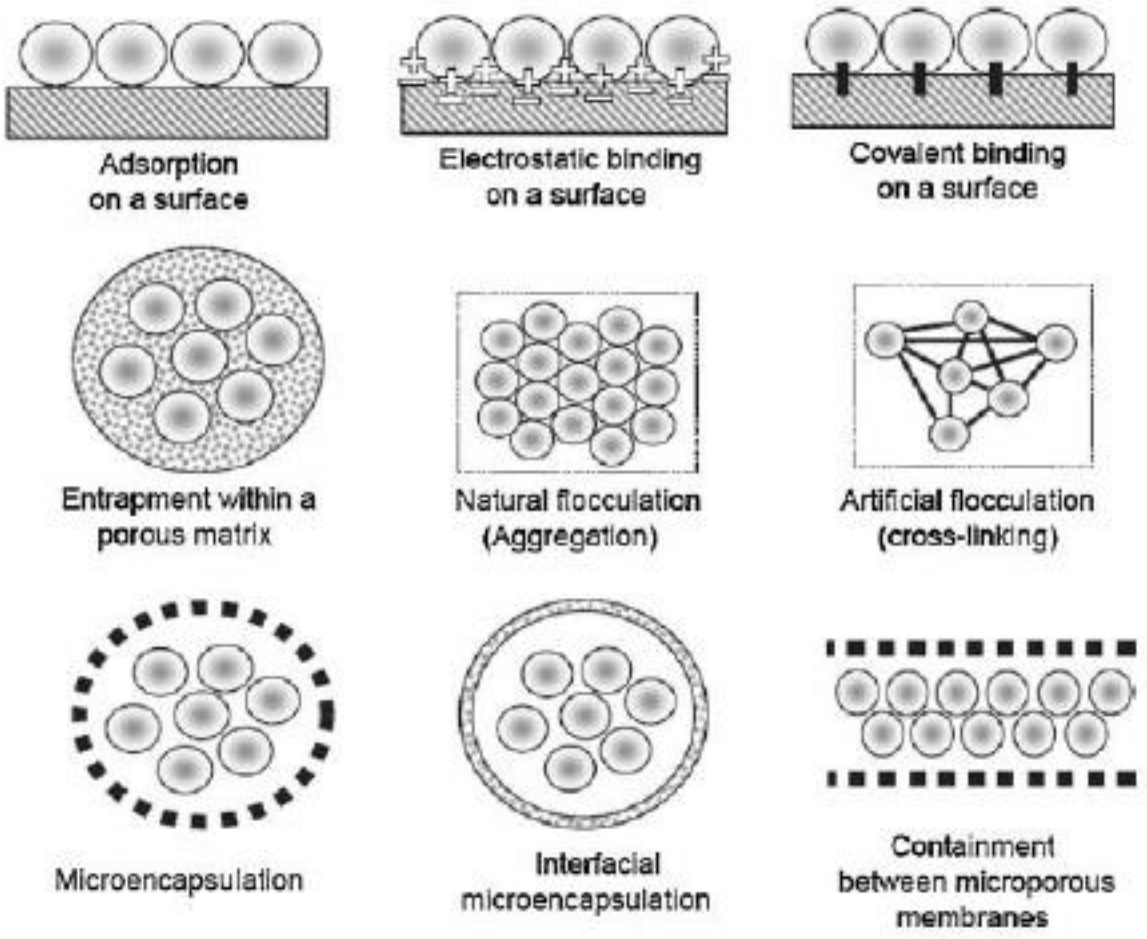
Most often a compromise is reached between stabilization and % of retained activity

Enzyme immobilization: selecting the method



CLEs:Cross-Linked Enzymes;
CLECs:Cross-Linked Enzyme Crystals;
CLEAs:Cross-Linked Enzyme Aggregates.

Most common immobilization methods



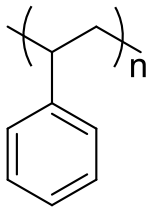
Immobilization on solid carriers

Dott. Simone Lotteria, Thesis 2016

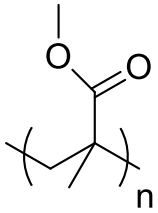
Carrier

- Either organic or inorganic (e.g. silicates)
- Must be chemically and mechanically stable under operational conditions
- When immobilization occurs via covalent linking the carrier must present suitablefunctional groups on the surface

Synthetic polymers

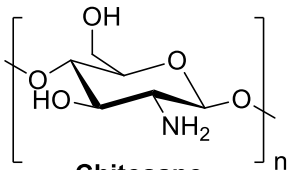


Polistirene

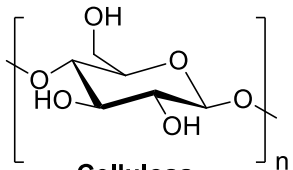


Poliacrilato

Biopolymers



Chitosano



Cellulosa

Inorganic

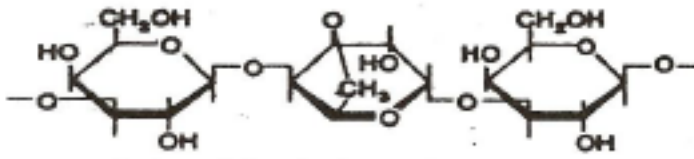
Silicates, celite

Organic polymers used as carriers

Polysaccharides:	Cellulose
	Agar/agarose
	Chitosan
	Dextran
	Carrageenan
	Alginate
	Pectate
	Xanthan gum
Proteins:	Collagen
	Gelatin
	Albumin
	Fibrin
Synthetic Polymers:	Polyacrylamide
	Methacrylate
	Polyurethane
	Epoxy resin
	Polystyrene
	Polyester
	Polypropylene
	Polyphenylene oxide
	Polyvinyl alcohol
	Polyvinyl chloride

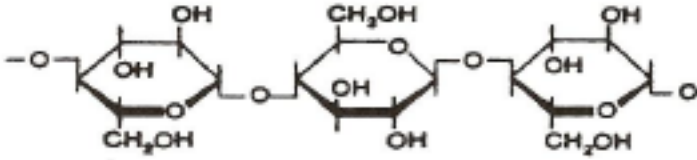
Polysaccharides and polyamides frequently serve as matrices

Agarose

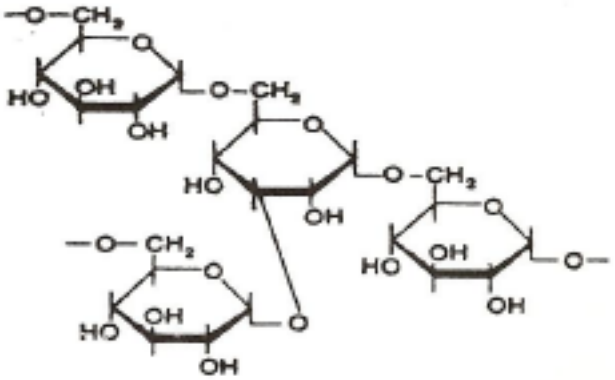


—o-galactose-3, 6-anhydro-L-galactose-o-galactose—

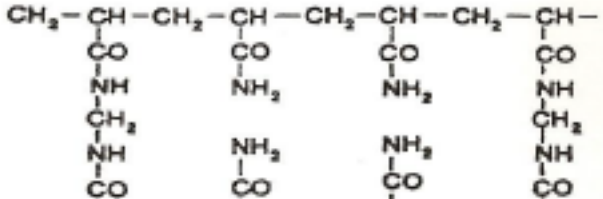
Cellulose



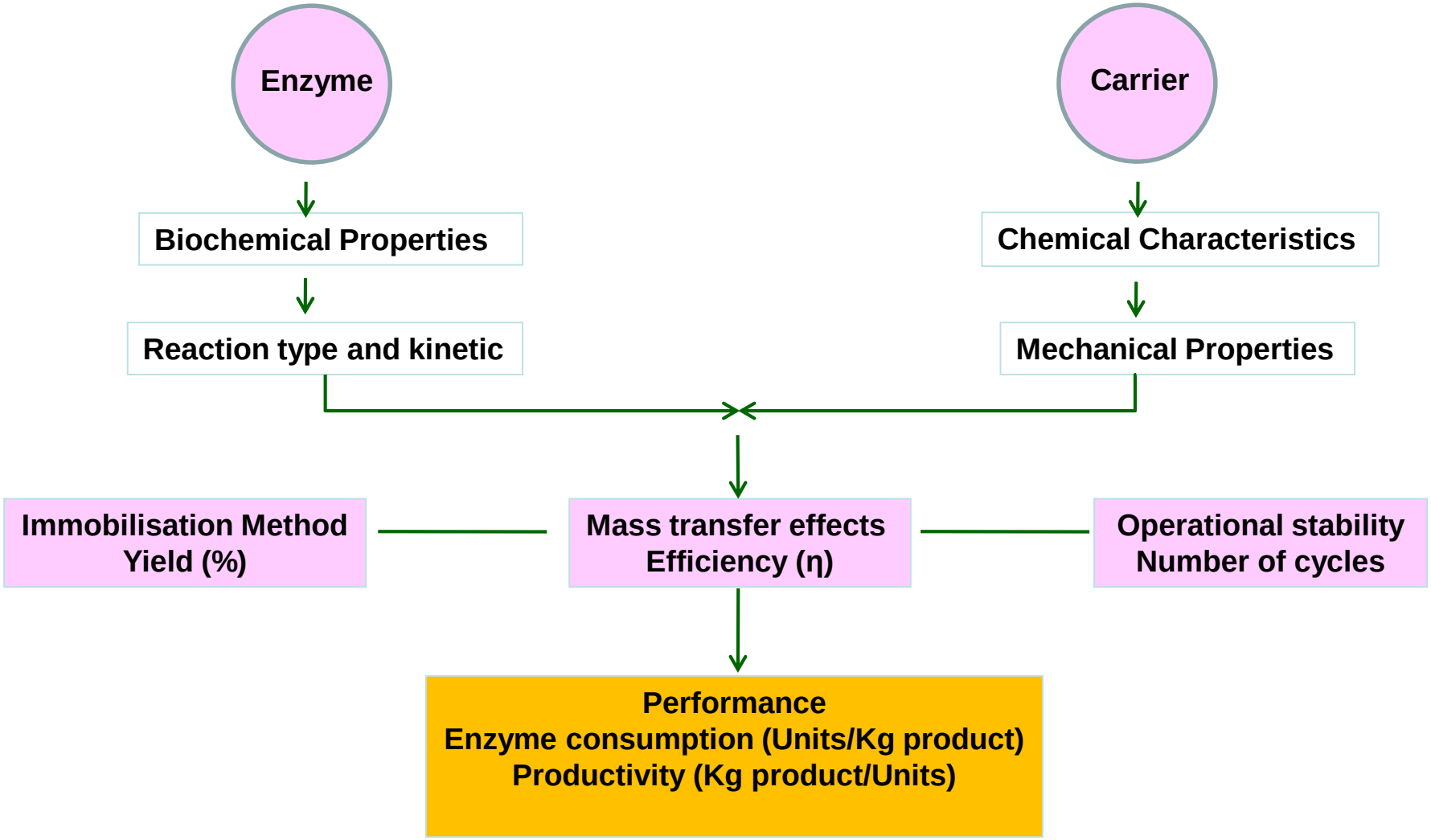
Crosslinked dextran (Sephadex)



Crosslinked polyacrylamide

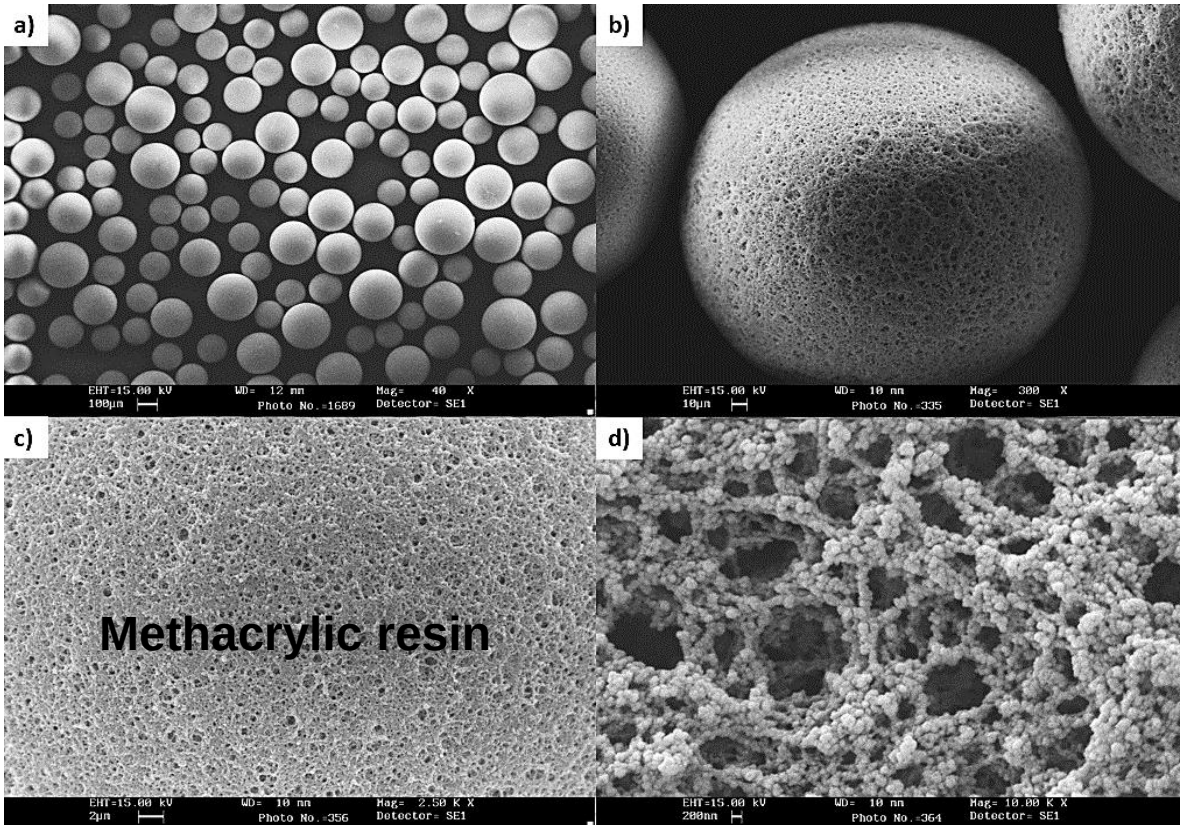


Immobilization on solid carriers



The physical characteristics of the supports (such as **particle diameter**, **swelling behavior, mechanical stability, and compression** behavior) are of paramount importance for the performance of the immobilised systems and will determine the type of reactor used under technical conditions.

In particular, **pore volume, pore diameter** and particle size determine the total surface area and thus critically affect the **loading capacity** of the resin.



In general, an essential requirement for any carrier is to have a **large surface area** (>100 m^2 g^{-1}) to promote the contact between the enzyme and the substrate.

The **pore size** of the selected carriers should meet three requirements: (i) suitable to enable the adsorption of the enzyme molecule in the interior of the carriers; (ii) larger than the size of the enzyme molecules thus preventing the decrease of enzyme-conformation mobility; and (iii) diffusion constraints should be mitigated to ensure the accessibility of the substrate to the catalytic site of the enzyme.

Very large substrates might require lower porosity to avoid the immobilisation of the enzyme in the inner pores that would be hardly accessible by bulky substrates.

Maintaining the catalyst on the external layer of the support can be preferable when scarcely soluble substrates are used, which might precipitate inside narrow pores.

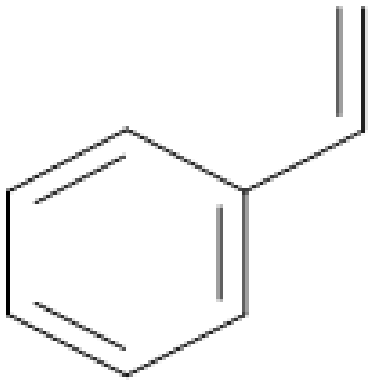
Organic synthetic polymers and resins

A range of **hydrophobic carriers**, such as polypropylene, acrylic or styrene, with different degrees of hydrophobicity and porosity are available on the market.

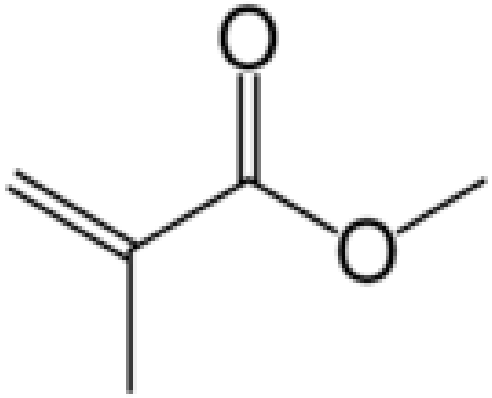
They are synthetized via radicalic polymerization

Indeed, **styrenic polymers** are widely used in refining of pharmaceuticals and natural extracts, since these are suitable for adsorbing large molecules because of their relatively large pore sizes and adsorption–desorption capacity.

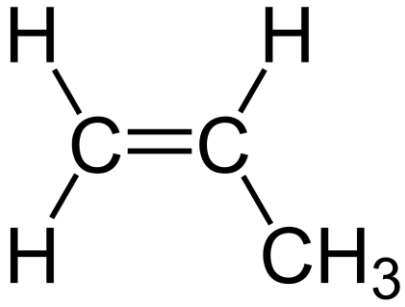
As a consequence, a considerable number of **acrylic or styrenic resins**, with different degrees of hydrophobicity, are available



Styrene

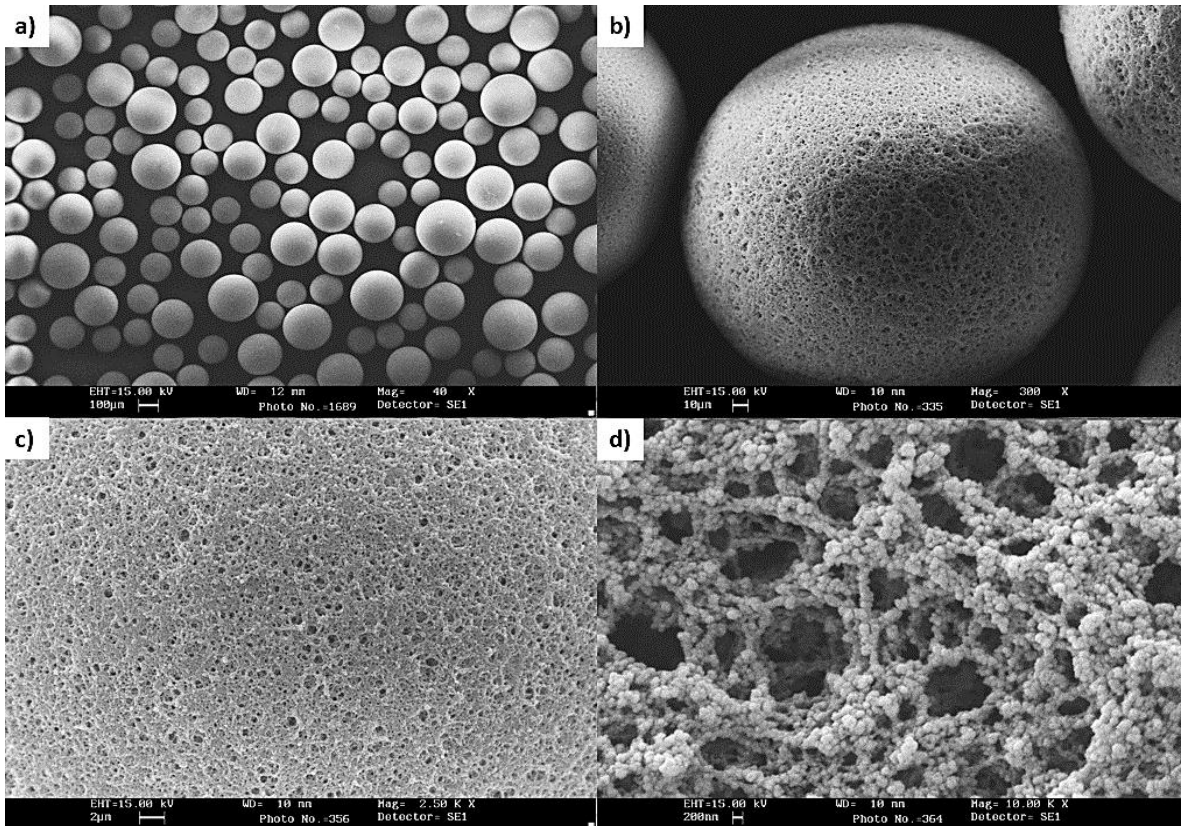


methyl acrylate



propilene

acrylic or styrenic resins,
with different degrees of hydrophobicity, are available and they usually have a surface area **>40 m^2 g^{-1}**

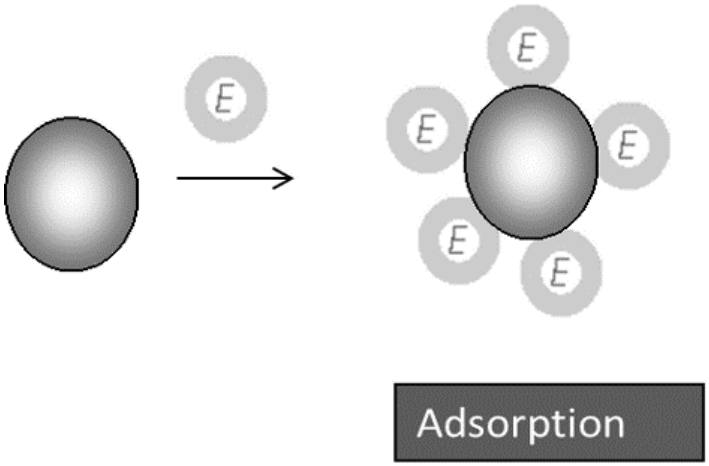


Porosity :
several materials used for enzyme adsorption can be considered **macroporous,** since pore diameters are higher than 50 nm.

Macroporous greater than 50 nm;
Mesoporous diameters between 2 and 50 nm.
Microporous less than 2 nm

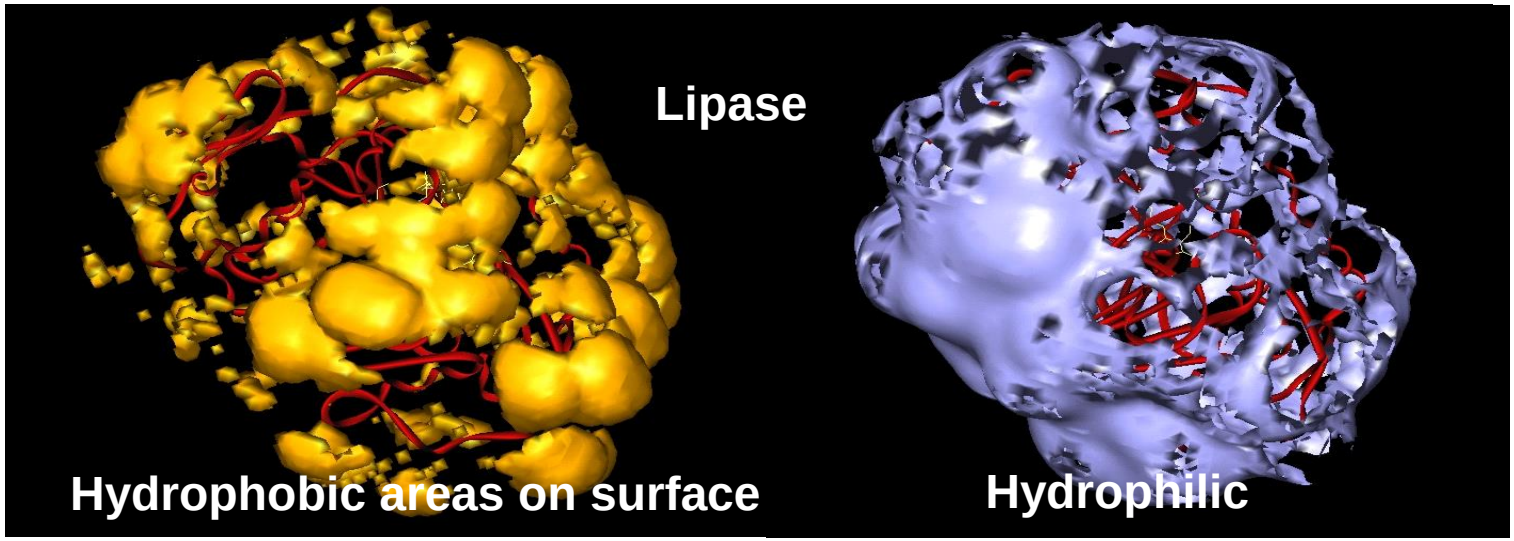
Enzyme immobilization on solid carriers: adsorption

Support binding can simply exploit weak interactions



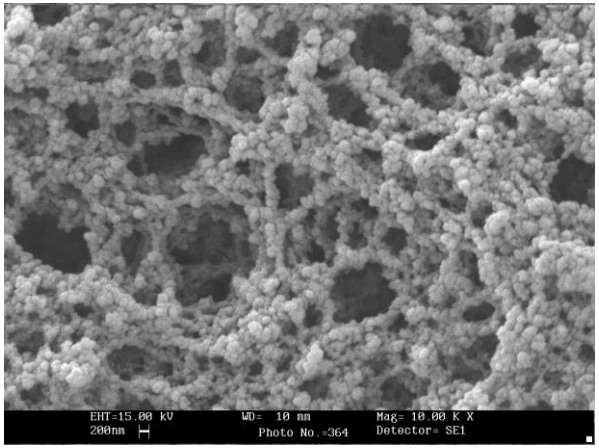
One of the most successful examples of lipase adsorption on organic resins is the widely used enzyme Candida antarctica lipase B commercially available in the immobilised form as Novozym 435$_s$ which consists of the enzyme adsorbed on a macroporous polymethyl/butylmethacrylate-divinylbenzene) resin.

Hydrophobic, van der Waals interactions



Methacrylic and styrenic porous polymers

High porosity and large internal surface

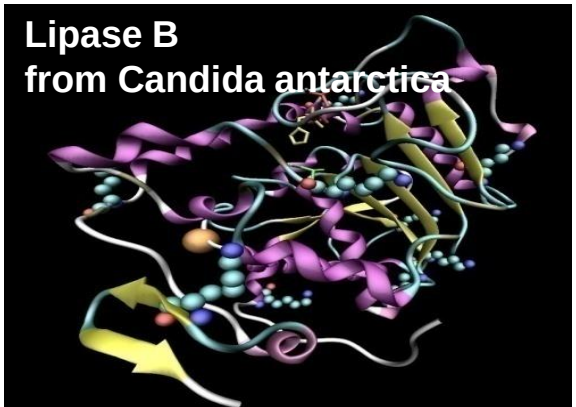


→

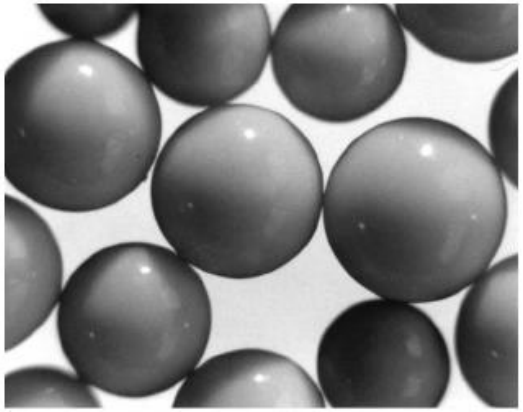
Increasing the ionic stenght for forcing the partition of enzyme onto the polymer

Important!!!
Commercial enzymes contain impurities and additives

Product	Appearance	Protein content (from the manufacturer)	Specific hydrolytic activity
Lipozyme CALB L	Viscous brown-yellowish liquid Small proteic impurities ì	6% Declared content for 1g solution: 440 mg water 250 mg sorbitol 250 mg glycerol 60 mg protein 2 mg sodium benzoate 1 mg potassium sorbate	4800-5200 U/ml
Chyrazyme L-2 Roche	White powder. Lyophilized	43%	60-70 U/mg_{prep}

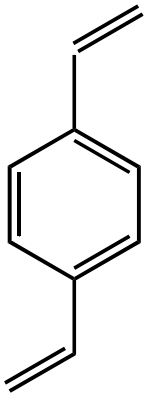


Acrylic + DVB



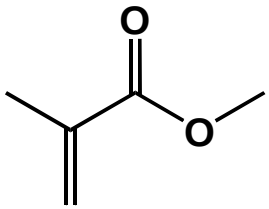
Novozym® 435

CAL-B immobilised (adsorption) on a hydrophobic polymer



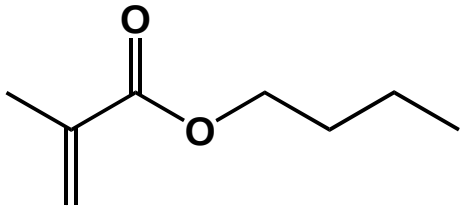
DVB (Divinylbenzene)

+



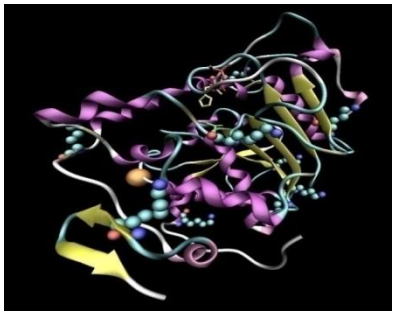
MA (Methylacrylate)

+

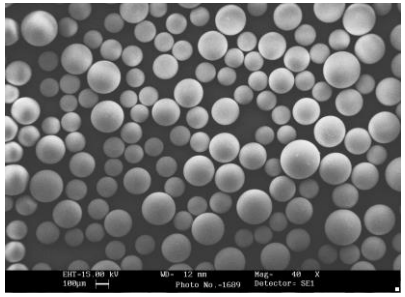


BA (Buthylacrylate)

Lipase B from *C. antarctica*



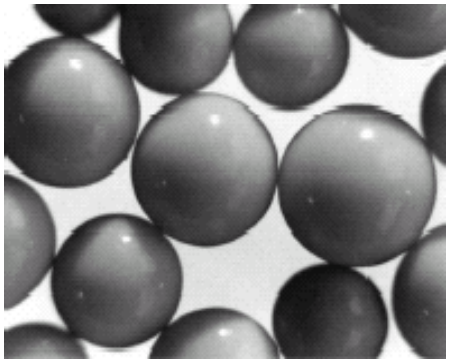
Enzyme



Solid support

Immobilised bio-catalyst

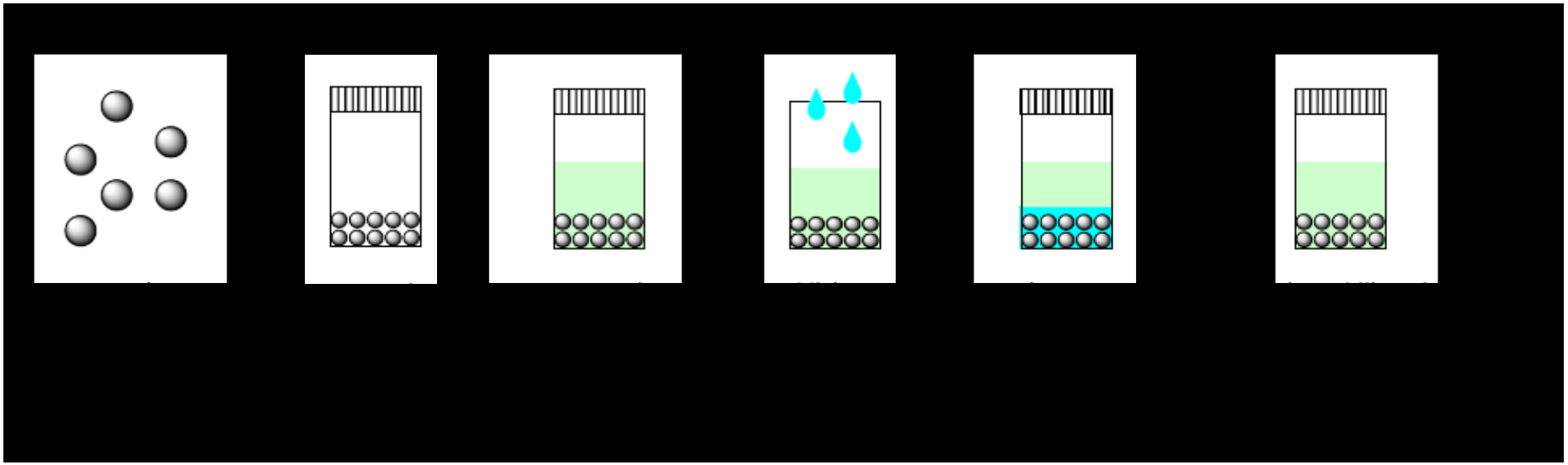
Adsorption **Immobilisation**



Novozyme 435

Lipase B from Candida antarctica, adsorbed on acrylic resin

Enzyme immobilization: enzyme solubilized in buffer is adsorbed on solid supports



Support binding can simply exploit weak interactions

Inorganic Supports

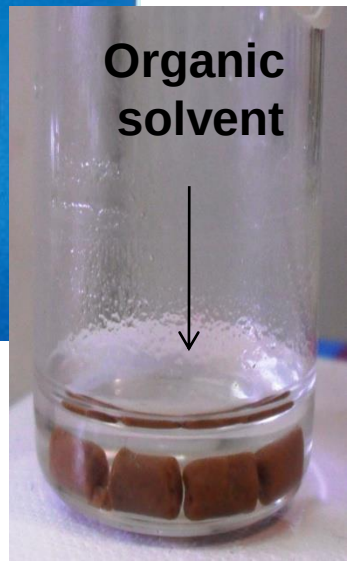
A variety of inorganic solids can be used for the immobilization of enzymes, e.g., alumina, silica, zeolites and mesoporous silicas.

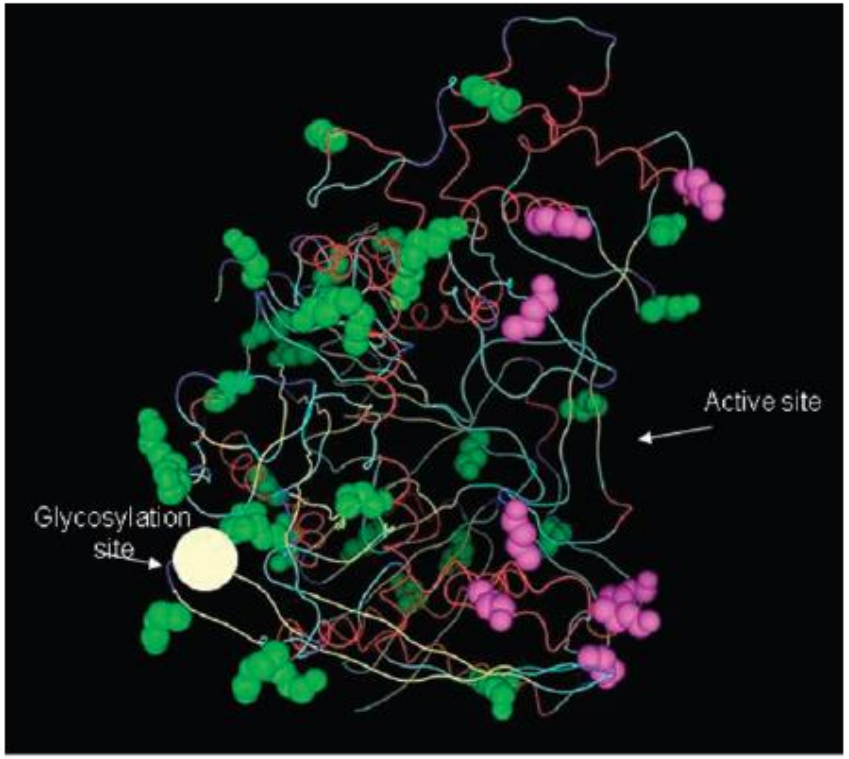
Silicates

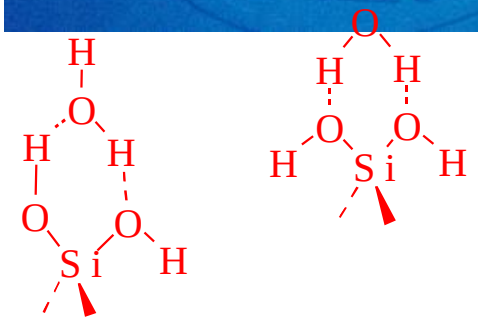
- Chemically inert, stable shape, do not swell

Celite derives from Diatomaceous earth (powder)

Immobilization on Celite: Hydrogen bonds and hydrophilic interactions. Suitable for applications in non aqueous media







In most cases hydrophilic amino acid residues prevail on the surface of enzymes.

In addition, enzymes may be glycosylated, further increasing the hydrophilicity of the protein. Therefore they can easily form hydrogen bonds and thus can be immobilised on hydrophilic carriers (cellulose, Celite, porous glass, clay, silica gel). A particularly popular carrier is Celite (diatomaceous earth), the silicate skeletons of diatoms. Powder are calcinated at high temperature.

Inorganic Supports

One of the simplest and most inexpensive methods to immobilize an enzyme is by **silica Granulation**.

It is used, for example, to formulate enzymes for detergent powders which release the enzyme into the washing liquid during washing. Granulation technology was used to immobilize lipase on silica granules, by first adsorbing the lipase on silica powder followed by agglomeration.

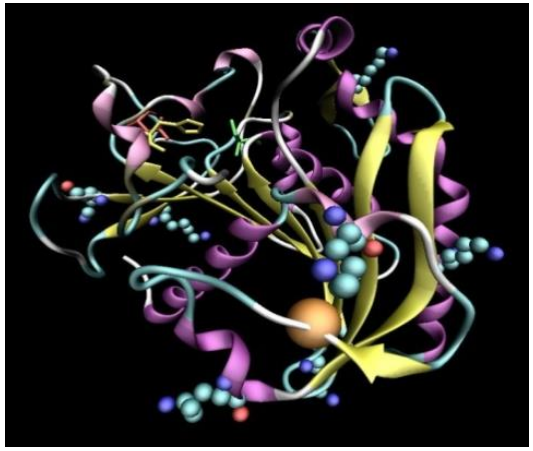
Owing to the composition of the granulates, they are intended for use only in organic media. In an aqueous medium the lipase is desorbed and the particle slowly disintegrates.

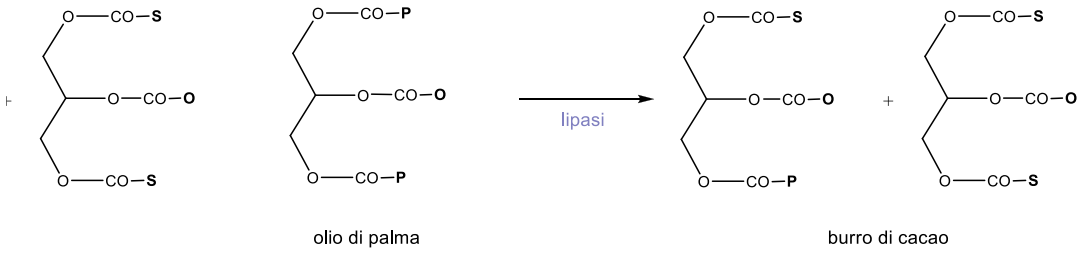
Granulation on silica: industrial scale

Lipozyme TL IM is a kosher- and halal-certified, food-grade lipase from *Termomyces lanuginosa*. In its non-immobilised form, it is a 1,3-specific lipase. As an immobilised enzyme it preferentially rearranges the fatty acids in the 1- and 3-positions on the fats (the 2-position is partly preserved).

The lipase is immobilized onto porous silica granulates which are insoluble in oil. Lipozyme TL IM is intended for use with interesterification of bulk fats for frying fats, shortenings & margarine hardstock.

This is done by a catalyst operating at about 100°C and under vacuum.





The enzyme and a liquid binder (gum, PVA,…) are sprayed by atomization onto a silica carrier with a particle size below 100 μm.
During the granulation, the silica particles become agglomerated into larger, porous particles with the enzyme distributed evenly over the whole surface area of the silica. The mean diameter of the particles is around 600 μm and the surface area is around 50 m^2 per gram. This gives a large area where the substrate can come into contact with the enzyme. Even though the silica granules are porous, they are mechanically stable both for batch and fixed bed column operation.
Furthermore, all the granulation components are of food-grade quality,

The immobilized lipase Lipozyme® TL IM viewed through a light microscope. The enzyme is bound to a silica carrier

Immobilization on functionalized solid carriers

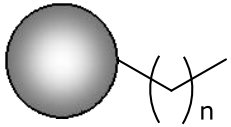
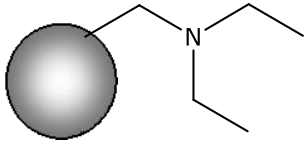
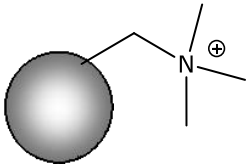
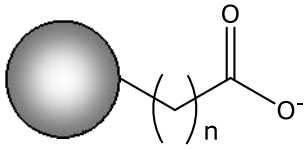
It is related to the presence of **specific chemical functionalities** on the surface of the carriers.

These functional groups can be part of the inherent **structure of the monomers comprising the carrier** (e.g. –OH groups of carbohydrates) or can be deliberately **introduced during the polymerization process** (e.g. a certain percentage of the glycidyl methacrylate monomer was added in the synthesis of methacrylic polymers).

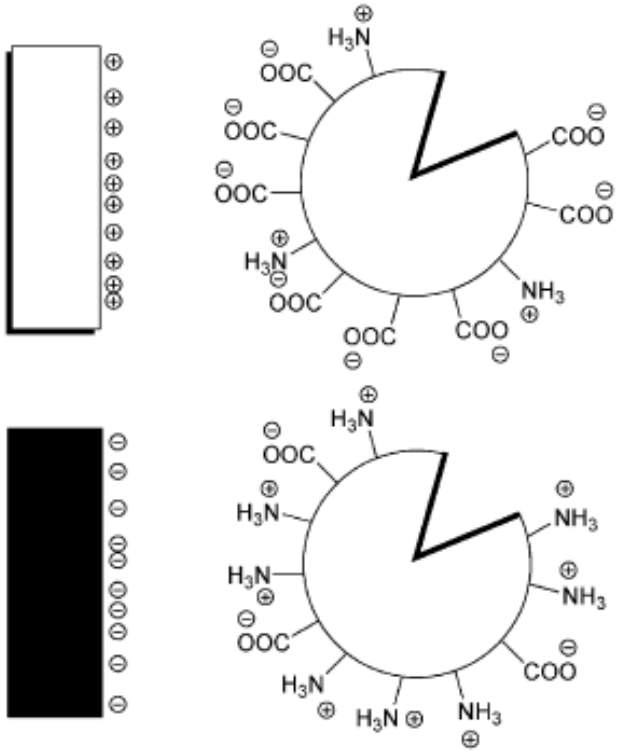
Finally, they can also be **introduced by chemical modification** of the surface of the carrier through a "pre-activation" treatment.

polymer–enzyme spacers:
Longer spacers are expected to allow a wider conformational flexibility to the protein.
Shorter spacers can confer higher thermal stability since they restrict the enzyme mobility and prevent unfolding. They can also cause steric hindrance and lower accessibility to the active site.

Organic polymeric resinswith functional groups: different types of interactions

Method of immobilisation	Functional group	Structure	Binding	Reactive group on enzyme
van der Waals and hydrophobic interactions	alkyl		maximizes hydrophobic interactions (adsorption)	Hydrophobic areas on surface of lipases
Ionic interactions	Trialkyl ammine		Ionic adsorption	Negatively charged a.a.
	Tetra alkyl ammonium		Ionic adsorption	Negatively charged a.a.
	Carboxylate		Ionic adsorption	Positively charged a.a.

Immobilization *via* ionic interactions



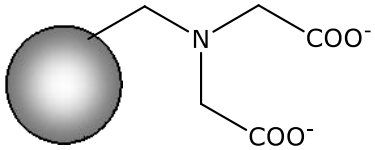
Depending on the pH of the solution and the isoelectric point the surface of the enzyme may bear charges. Using widely available modelling systems, the surface charge and charge distribution of an enzyme can be readily calculated and displayed. Essentially any ion exchanger can act as carrier in immobilisation via ionic and strongly polar interactions. Depending on the predominant charge on the enzyme, the ion exchanger needs to be negatively (for instance carboxylate) or positively charged (for instance protonated amino groups).

Chem. Soc. Rev., 2009, 38, 453–468

Table S2: A number of synthetic organic polymers employed for ionic immobilisation available on the market. Legend: a) Resindion S.r.l. (Mitsubishi Chemical Corporation); b) ChiralVision

Product Name	Chemical Matrix	Functional group	Pore diameter (Å)
[a]Sepabeads EC-EA	poly(methacrylate)	ethylamino	100-200
[a]Sepabeads EC-HA	poly(methacrylate)	hexamethylamino	100-200
[b]IB-D152	polyacrylic	carboxylic acid	N/A
[b]IB-C435	polyacrylic	carboxylic acid	N/A
[b]IB-A161	polystyrene	quaternary ammonium	N/A
[b]IB-A171	polystyrene	quaternary ammonium	N/A
[b]IB-A369	polystyrene	quaternary ammonium	N/A

Organic polymeric resins fo metal binding

Method of immobilisation	Functional group	Structure	Binding	Reactive group on enzyme
Metal affinity	Iminodiacetic		Loading metals such as Ni^{2+}, Zn^{2+}, Cu^{2+}	His-tag

Metal binding: used for protein purification but rarely for enzyme immobilization.

Metal chelated supports are used extensively in protein chromatography (IMA – **Immobilised Metal-Ion Affinity**).

The **DNA sequence specifying a string of six to nine histidine** residues is frequently used in vectors for production of recombinant proteins. The result is expression of a recombinant protein with a 6x His or poly-His tag fused to its N- or C-terminus. Expressed His-tagged proteins can be purified and detected easily because the string **of histidine residues binds to several types of immobilised metal** ions, including nickel, cobalt and copper, under specific buffer conditions.

Chelator ligands can be immobilised on the solid supports by means of stable covalent bonds and the metal ions are then bound by coordination. The chelators most commonly used as ligands for IMAC are **nitrilotriacetic acid (NTA)** and **iminodiacetic acid (IDA).**

The stable complexes formed can be used for the retention of proteins. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing pH. The support is **subsequently regenerated** by washing with a stronger chelator such as ethylene diamine tetraacetic acid (**EDTA**) when desired.

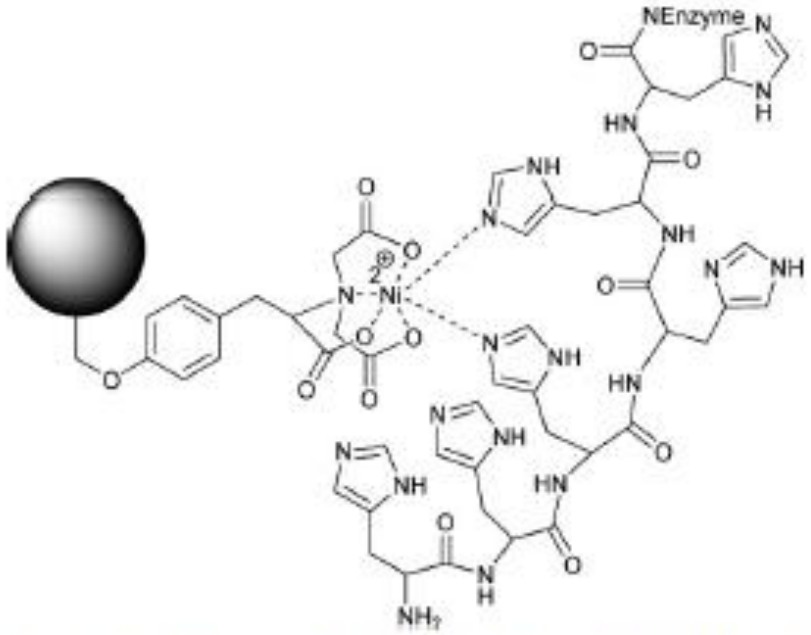
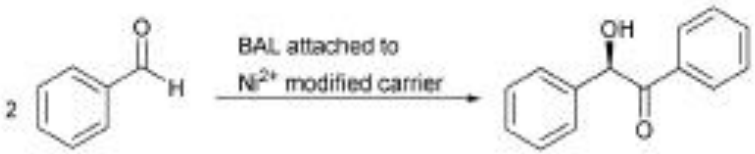


Fig. 5 Ni^{2+} attached to a carrier anchors an enzyme with a His tag to the carrier.



The His tag has little influence on the catalytic performance of the enzymes. **Benzaldehyde lyase (BAL)** immobilised via imidazole complexation of Ni2+ attached to a polyvinylpyrrolidinonebased matrix, could be reused several times for the formation of benzoin.

Covalent immobilization of enzymes

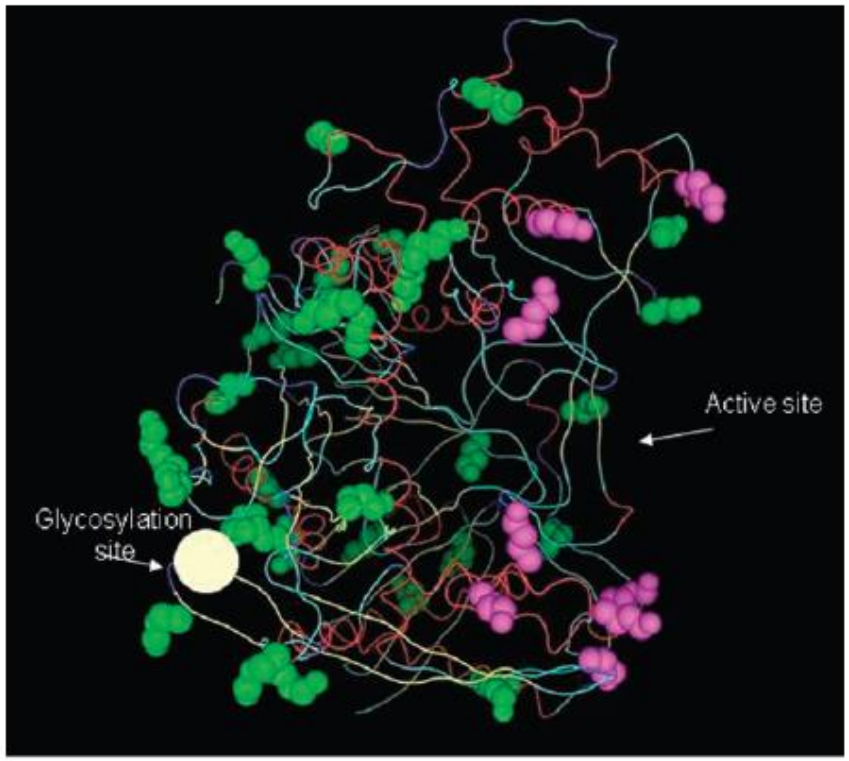
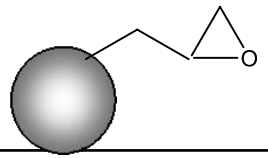
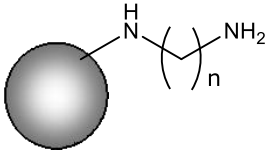
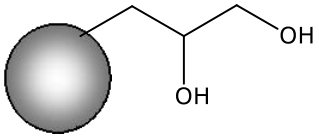


Fig. 13 Model of the structure of the PGA from *E. coli*. The residues of lysine are pointed out in the space-filling modality. The violet residues correspond to those lysines closer to the opening of the active site. On the opposite side there is the glycosylation site (yellow).

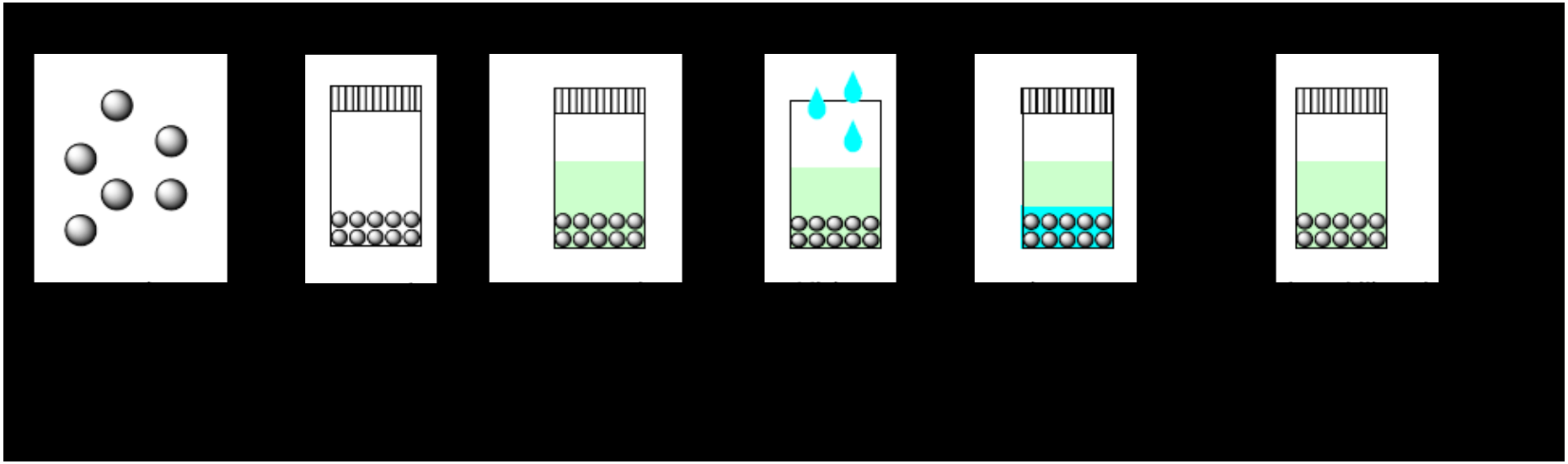
Most often protocols exploit the nucleophilic reactivity of amino groups on Lys side chain on the surface of enzyme

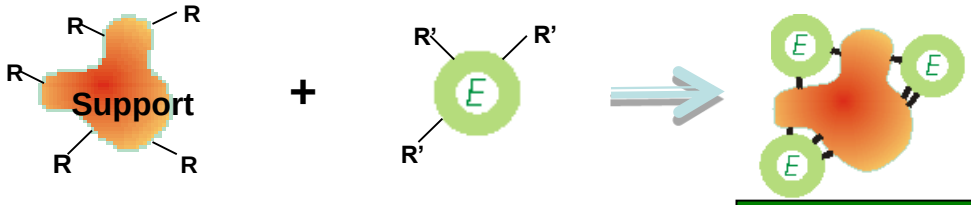
Covalent immobilization of enzymes on commercially available organic resins with functionalized surface

Organic polymeric resins: functional groups for covalent immobilization

Method of immobilisation	Functional group	Structure	Binding	Reactive group on enzyme
Covalent bonds	Epoxy		Formation of covalent bonds via nucleophilic attack and opening of epoxy ring	Nucleophilic groups (mainly – NH_2 and -SH)
	Amino		Pre-activation with glutaraldehyde and formation of imino bond with a primary amine	Primary amines (terminal amine and Lys side chains)
	Diol		Activation with BrCN to imido-carbonate. Oxidation of adjacent cis-diols with $NaIO_4$ to give dialdehydes.	Primary amines (terminal amine and Lys side chains)

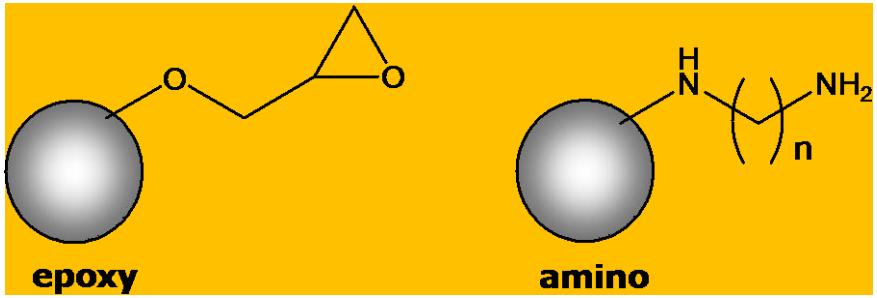
Enzyme immobilization: covalent binding on organic polymeric resins



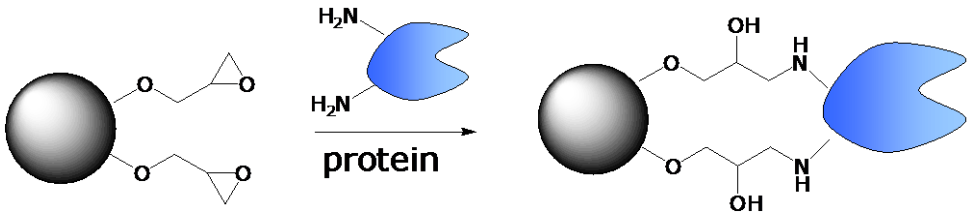


Covalent anchorage on support

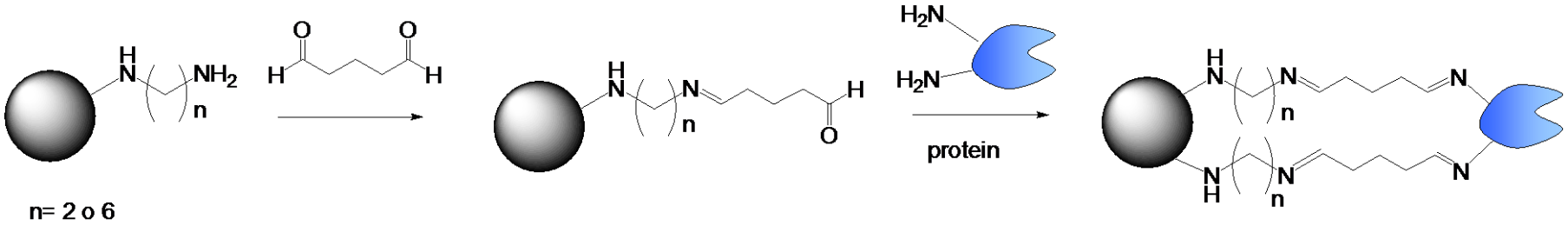
Covalent immoblization on functionalized supports



epoxy support



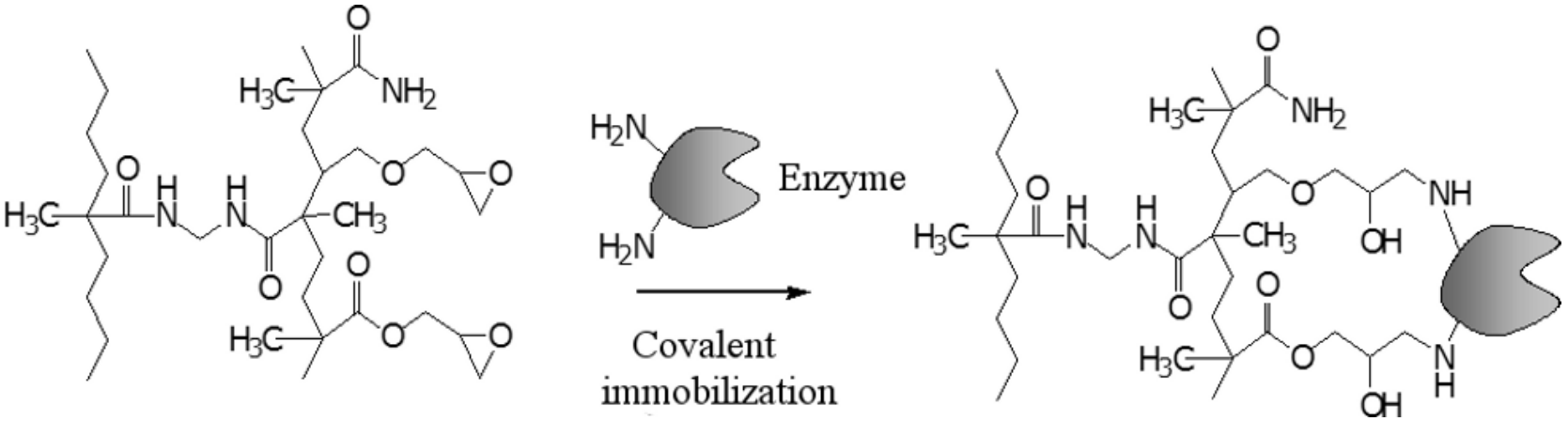
amino support



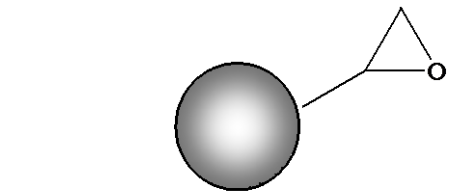
n= 2 o 6

Serenovic et al., Biotechnol. Bioeng. 2006.
Basso et al, Adv. Synth. Catal. 2007.

Covalent immoblization on methacrylic epoxy supports



Enzyme immobilisation on epoxy and amino carriers

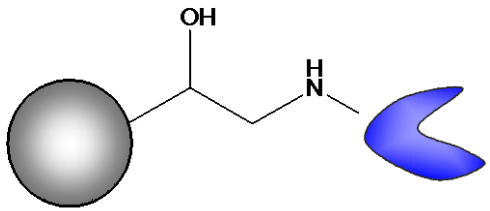


EC-EP

Kpi buffer

(pH 8.0, > 1M)

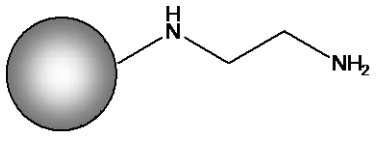
Enzyme



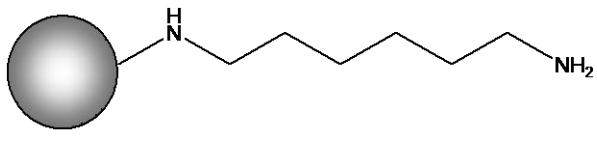
EC-EP

Covalent immobilisation

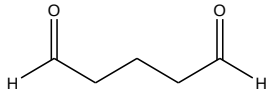
and post-blocking (Gly)



EC-EA

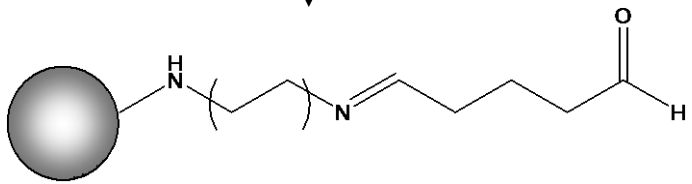


EC-HA



Support pre-activation

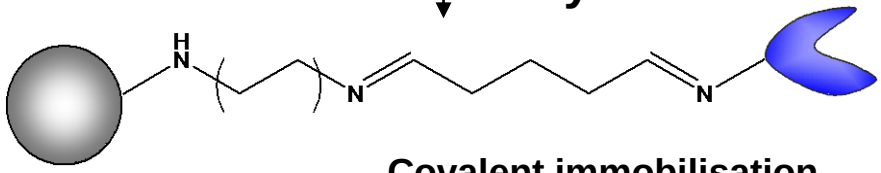
(2% glutaraldehyde)



Kpi buffer

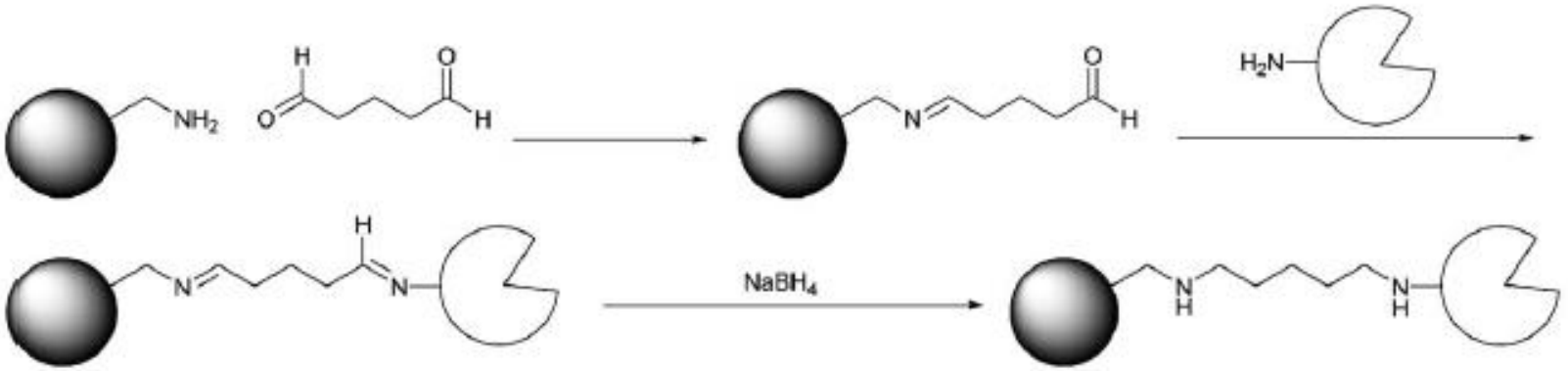
(pH 8.0, 0.02M)

Enzyme



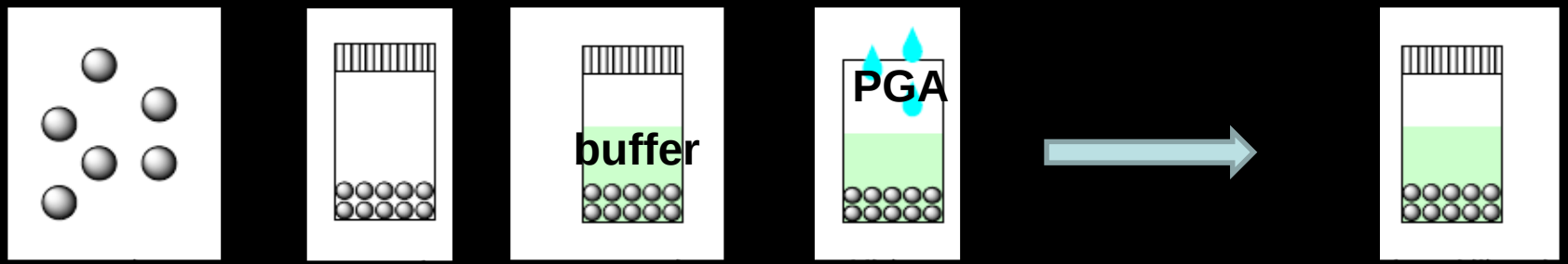
Covalent immobilisation

(Shiff base formation)

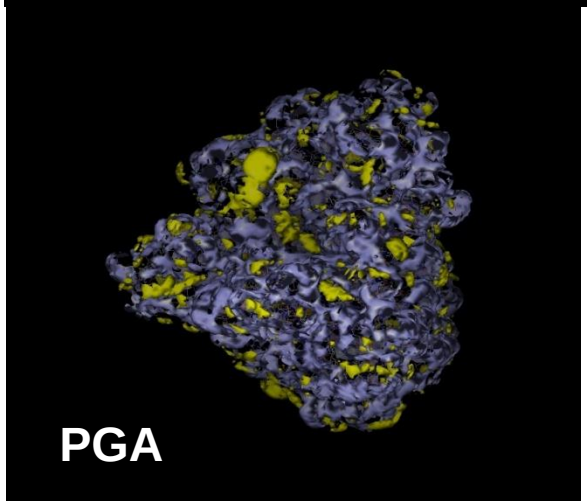


Imine bond is unstable at low pH: reduction with NaBH4 makes the anchorage more stable but enzyme activity can be lost

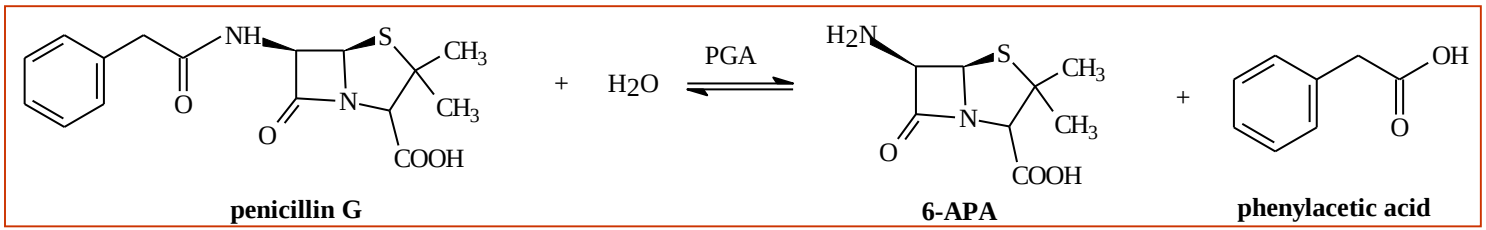
No general protocol for enzyme immobilization



Enzyme immobilization on carriers in aqueous buffer

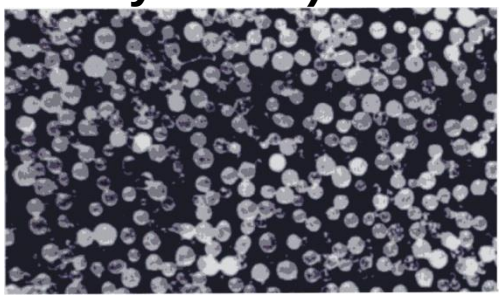


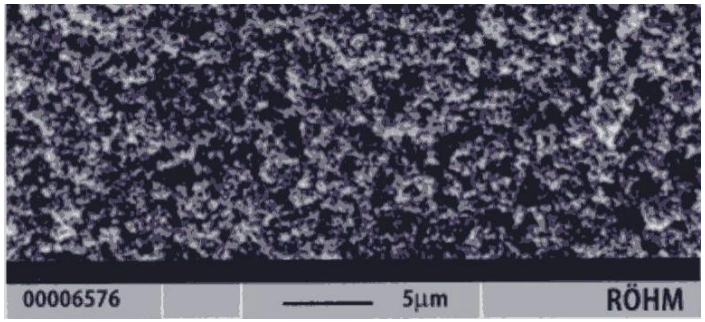
Tons of immobilized PGA consumed per year
At industrial level used for >600 cycles

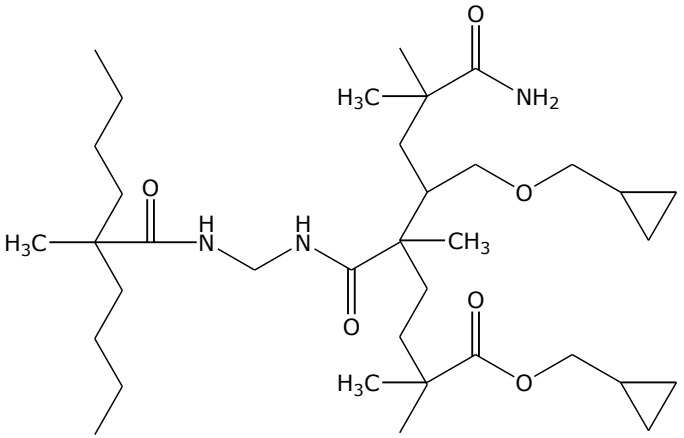


Acrylic polymers - Eupergit

Eupergit C is a macroporous copolymer of methacrylamide, glycidyl methacrylate and allyl glycidyl ether, cross-linked with N,N'-methylene-bis(methacrylamide)



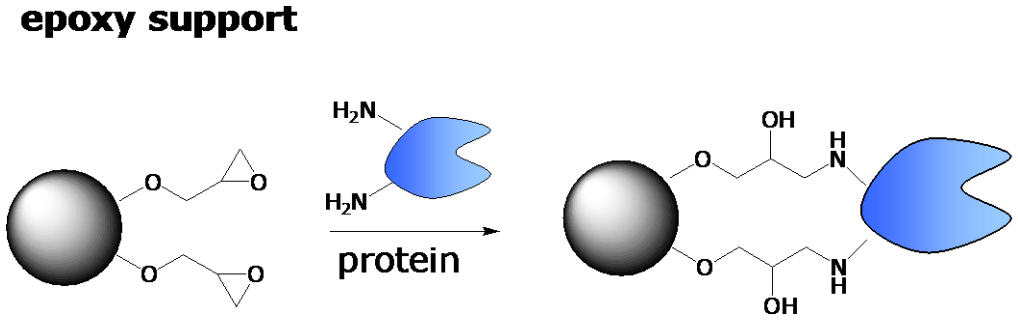




particle size 170 m and pore diameter 25 nm

T. Boller, C. Meier, S. Menzler, "Eupergit Oxirane Acrylic Beads: How to make enzymes fit for Biocatalysis", Org. Proc. Devel. 2002, 6, 509-519

Acrylic polymers - Eupergit



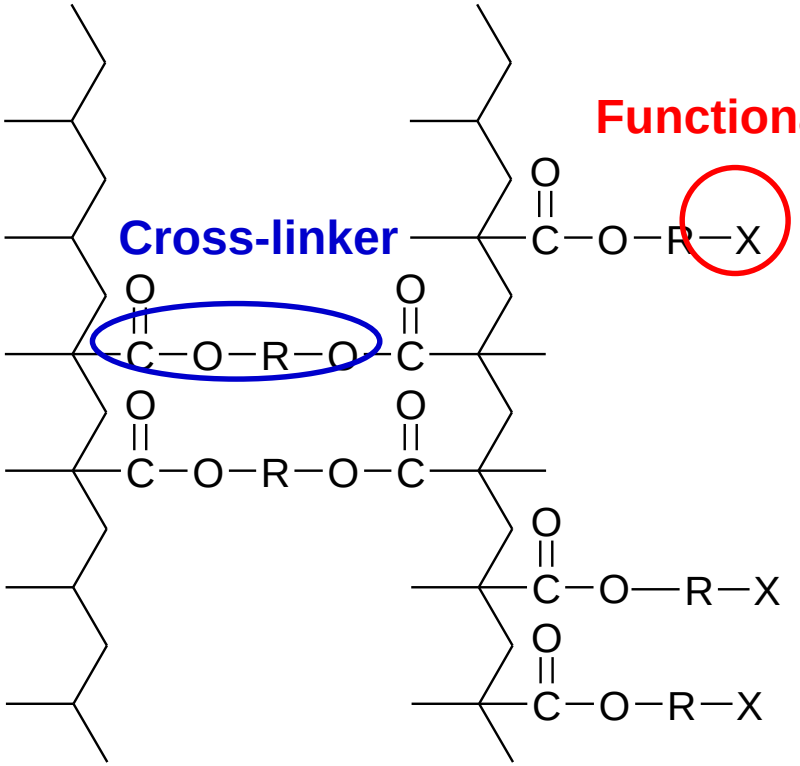
Beads dimension: 100 - 250µm

Multipoint attachment

1. oxidoreductases	
alcohol dehydrogenase	E.C. 1.1.1.2
lactate dehydrogenase	E.C. 1.1.1.27
α-hydroxysteroid dehydrogenase	E.C. 1.1.1.50
β-hydroxysteroid dehydrogenase	E.C. 1.1.1.51
pyranose oxidase	E.C. 1.1.3.10
nucleoside oxidase	E.C. 1.1.3.28
phenylalanine dehydrogenase	E.C. 1.4.1.20
D-amino acid oxidase	E.C. 1.4.3.3
formate dehydrogenase	E.C. 1.2.1.2
2. transferases	
transketolase	E.C. 2.2.1.1
3. hydrolases	
carboxylesterase	E.C. 3.1.1.1
triacylglycerol lipase	E.C. 3.1.1.3
β-glucosidase	E.C. 3.2.1.21
β-galactosidase	E.C. 3.2.1.23
trypsin	E.C. 3.4.21.4
thermolysin	E.C. 3.4.24.27
glutaryl-7-ACA acylase	E.C. 3.5.1.4
penicillin amidase	E.C. 3.5.1.11
aminoacylase	E.C. 3.5.1.14
cytidine deaminase	E.C. 3.5.4.5
2-haloacid dehalogenase	E.C. 3.8.1.2
4. lyases	
oxynitrilase	E.C. 4.1.2.10
Neu5ac aldolase	E.C. 4.1.3.3

Resindion S.R.L.
Mitsubishi Chemical Corporation

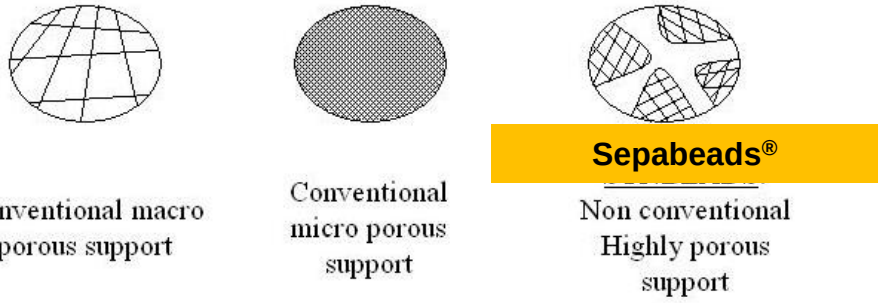
Acrylic polymers - Sepabeads



X = Active Functional Group

Sepabeads®

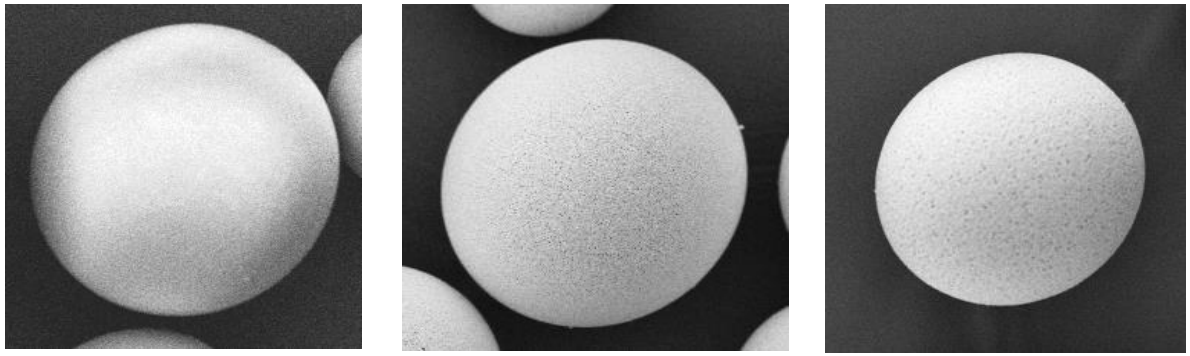
- methacrylic copolymers
- prepared through suspension polymerisation
- high mechanical stability



Mateo C, Abian O, Fernandez-Lorente G, Pedroche J, Fernandez-Lafuente R, Guisan JM, Tam A, Daminati M. Biotechnol Prog. 2002, 18, 629-634.

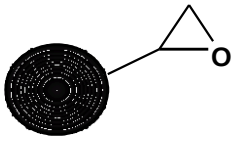
Carrier: criteria for the selection

Particle diameter (μm):
150-300
200-600

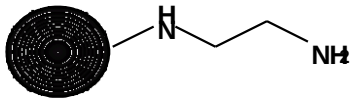


Porosity (Ø)

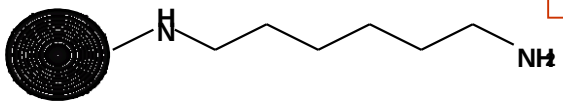
300 Å → **> 2000 Å**



EC-EP

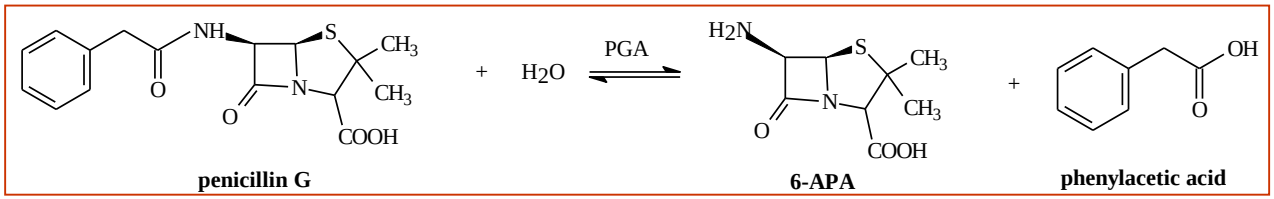


EC-EA

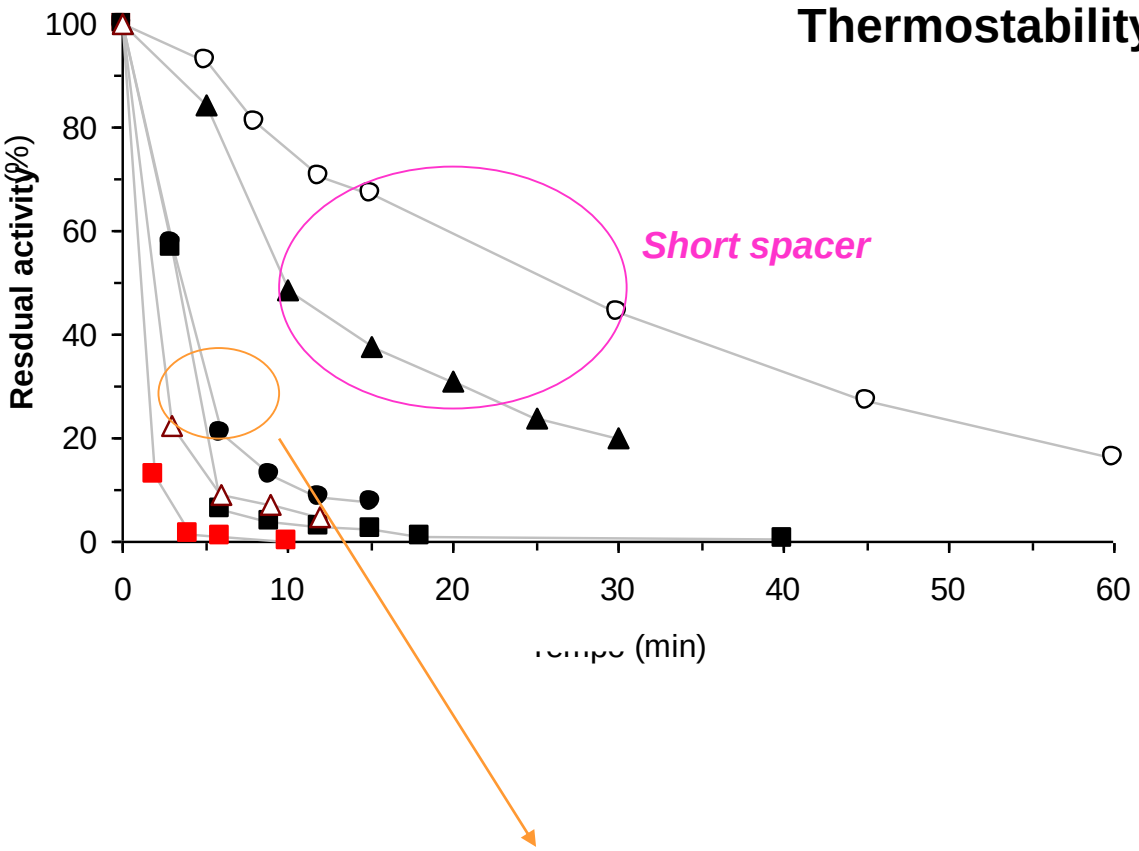


EC-HA

Penicillin G amidase from *E. coli*: Hydrolysis of pen-G in aqueous buffer



Penicillin G amidase: higher stabilization on supports having short spacers



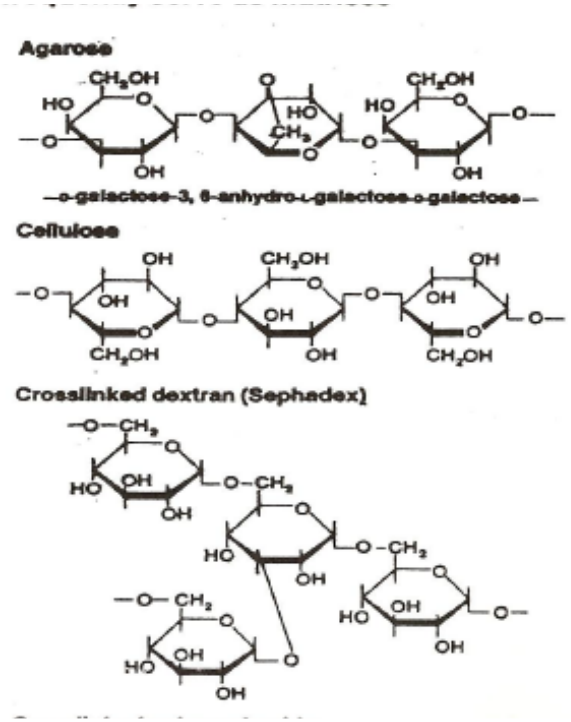
Long spacers:
higher conformational freedom → lower stability

BIOPOLYMERS AS CARRIERS

Polysaccharides:	Cellulose
	Agar/agarose
	Chitosan
	Dextran
	Carrageenan
	Alginate
	Pectate
	Xanthan gum
Proteins:	Collagen
	Gelatin
	Albumin
	Fibrin

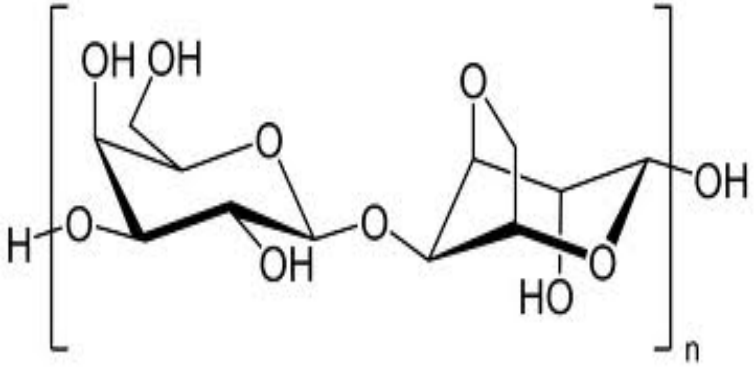
Natural biopolymers may represent an attractive alternative from the economic point of view. Water-insoluble **carbohydrates** such as **cellulose**, starch, dextran, **agarose** and **chitosan** and proteins such as albumin and gelatin have been widely used as supports for immobilising enzymes.

From this group, polysaccharides are of special interest, since they do not suffer from biological safety aspects like protein matrices isolated from animal sources and they are highly hydrophilic, which provides a desirable microenvironment for many enzymes.



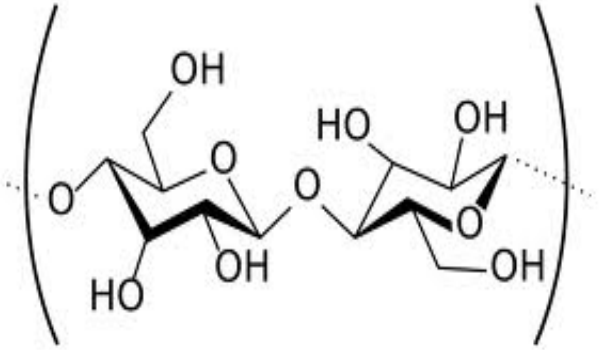
Sugar based bio-polymers as carriers.
The hydroxyl groups of polysaccharides can be exploited for covalent immobilisation of proteins after **ACTIVATION**.

- **Agarose**

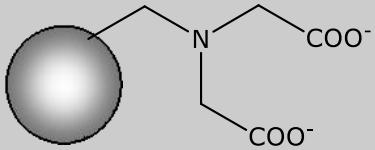
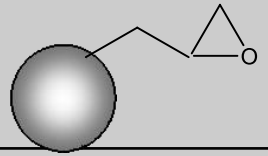
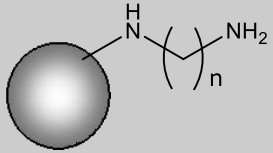
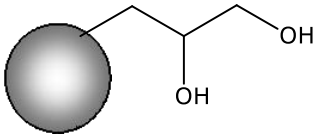


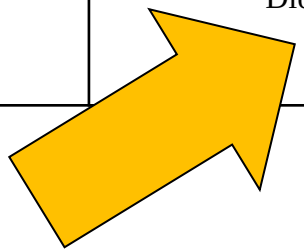
Chemically it is a (poly-{β-1,3-$_D$-galactose-α-1,4-(3,6-anhydro)-$_L$-galactose}) gel.

- **Cellulose**



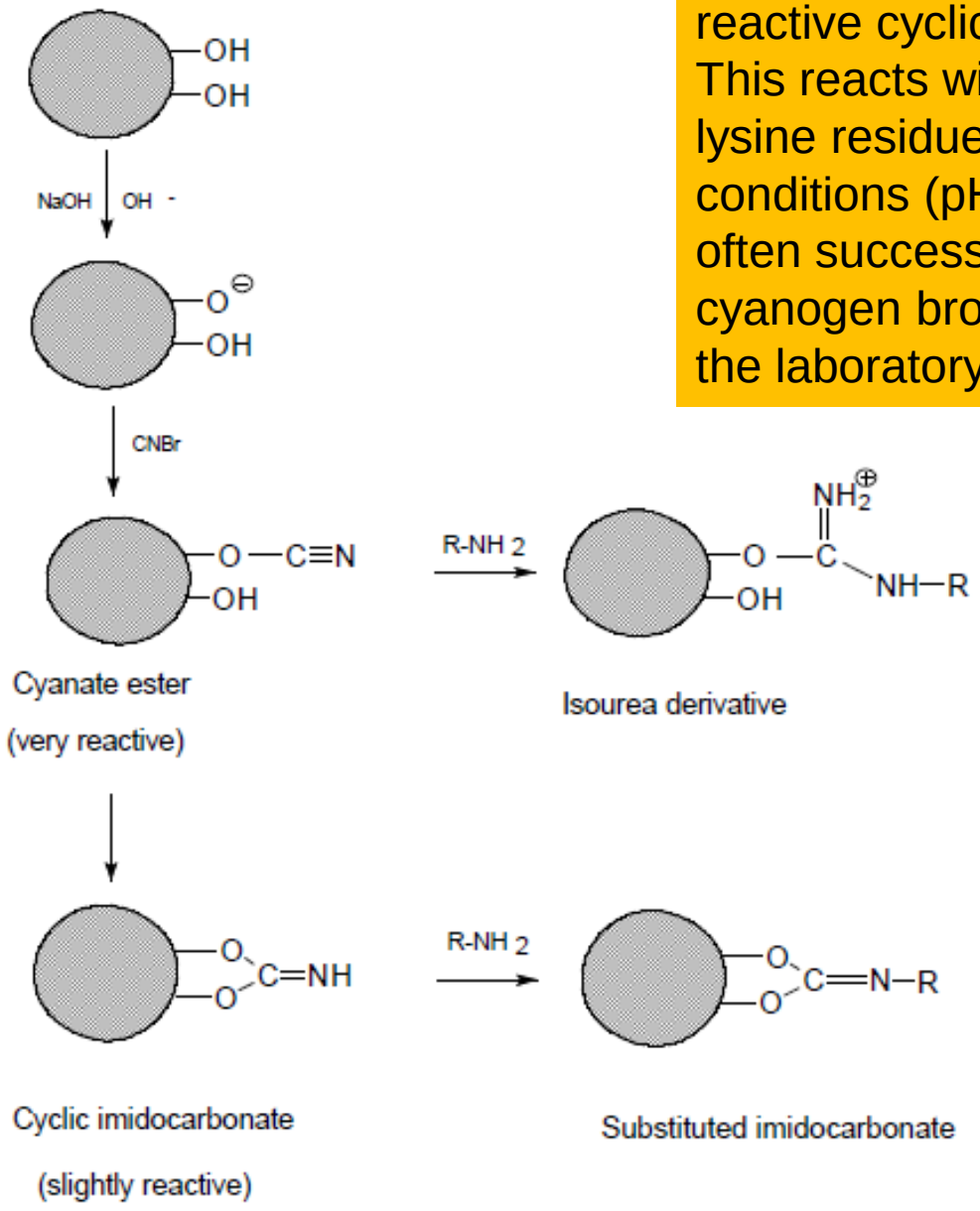
functional groups of carriers

Method of immobilisation	Functional group	Structure	Binding	Reactive group on enzyme
Metal affinity	Iminodiacetic		Loading metals such as Ni^{2+}, Zn^{2+}, Cu^{2+}	His-tag
Covalent bonds	Epoxy		Formation of covalent bonds via nucleophilic attack and opening of epoxy ring	Nucleophilic groups (mainly – NH_2 and -SH)
	Amino		Pre-activation with glutaraldehyde and formation of imino bond with a primary amine	Primary amines (terminal amine and Lys side chains)
	Diol		Activation with BrCN to imido-carbonate. Oxidation of adjacent cis-diols with $NaIO_4$ to give dialdehydes.	Primary amines (terminal amine and Lys side chains)

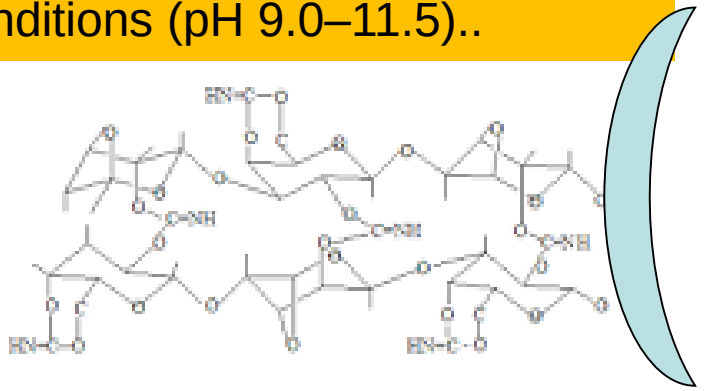


Activation of agarose

The hydroxyl groups of this polysaccharide combine with **cyanogen bromide** to give the reactive cyclic imido-carbonate.
This reacts with primary amino groups (i.e. mainly lysine residues) on the enzyme under mildly basic conditions (pH 9–11.5). This is a simple, mild and often successful method but the high toxicity of cyanogen bromide confined its use to the laboratory scale.



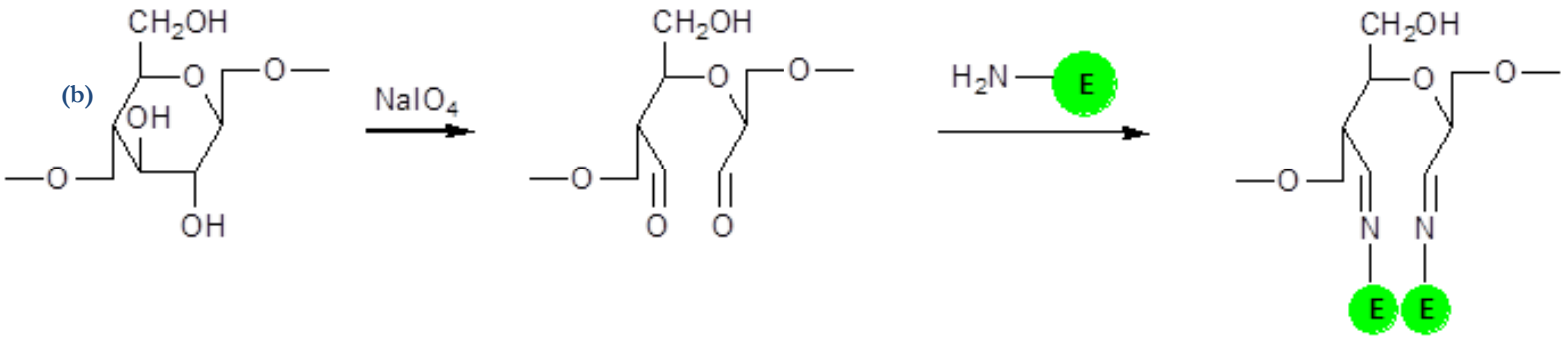
Cyanogen bromide reacts with vicinal diols to give the reactive cyclic imido-carbonate. This reacts with primary amino groups (i.e. mainly lysine residues) on the protein under mildly basic conditions (pH 9.0–11.5)..



Activated agarose

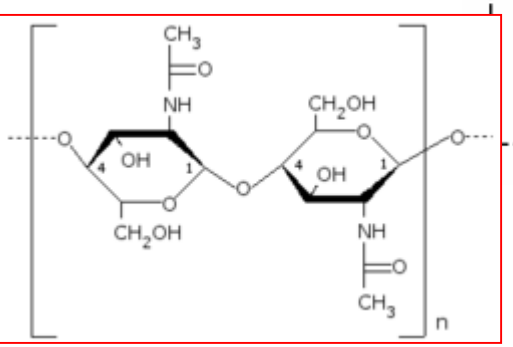
Immobilization by covalent coupling of enzyme on oxidized CELLULOSE support;

The carrier is activated by a process involving oxidation of cellulose to provide aldehyde groups, and covalent binding of enzyme molecules on aldehyde activated support.



The binding capacity for enzymes is generally lower as compared to agarose but it is inexpensive and commercially available in fibrous and granular forms. Some drawbacks are the low particle sizes, which affect their use in high pressure processes.

Il chitosano è un un polisaccaride di orgine animale ottenuto dalla deacetilazione della chitina, componente principale degli esoscheletri dei crostacei; è costituito da unità monometriche di 2-amino-2-deossi-D-glucopiranosio legate con legami (1-4)β, é strutturalmente simile alla cellulosa da cui però si discosta in quanto gli idrossili presenti sul C2 sono sostituiti con gruppi amminici come si può osservare dalle formula di struttura riportata.
Successivamente, attraverso il processo di deacetilazione, vengono rimossi gli acetili dalla molecola e viene ottenuto il chitosano (a seconda dell'efficienza del processo si può ottenere un differente grado di deacetilazione). Il chitosano è insolubile in H2O, ma è relativamente solubile in soluzioni diluite di acidi.



chitina

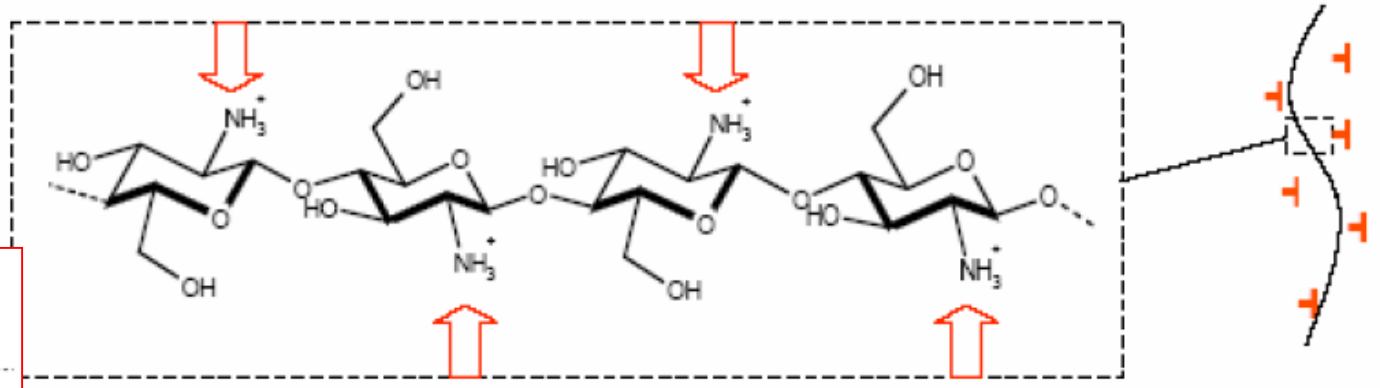
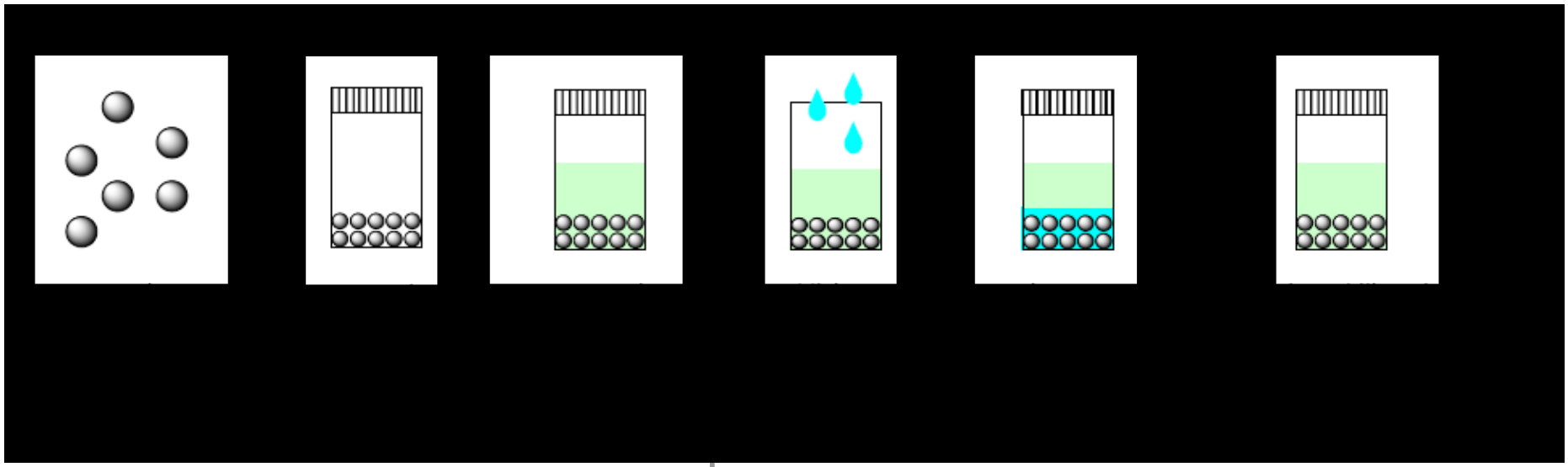
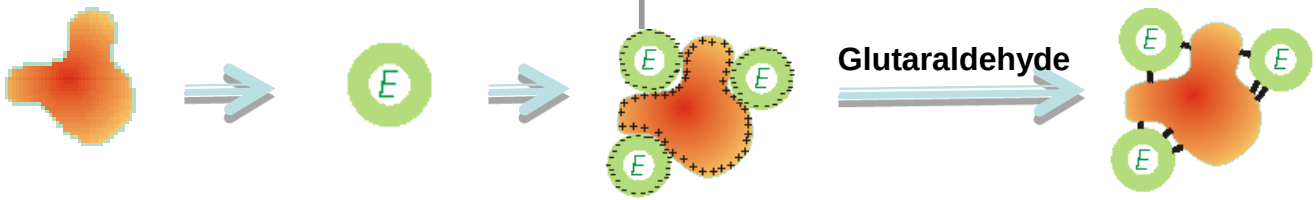


Fig1 dettaglio di una catena di chitosano

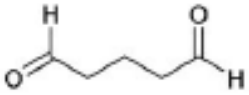
Covalent Enzyme immobilization via adsorption + crossInking





Adsorption

Adsorption + cross-linking



Use of a difunctional chemica reagent: imino group formation

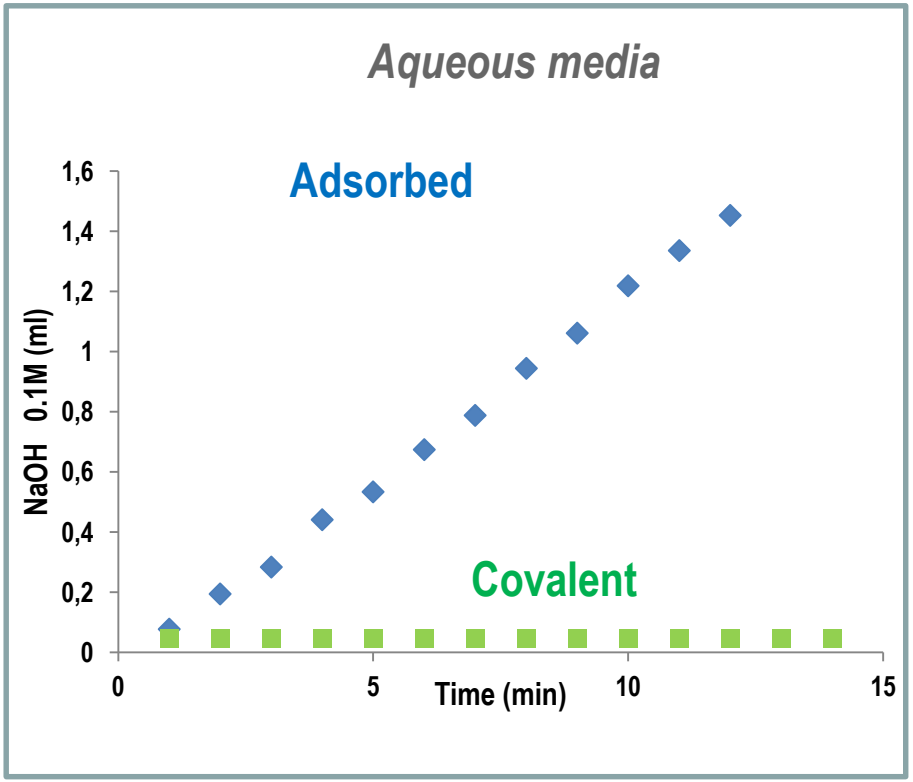
Criteria for selecting immobilization methods

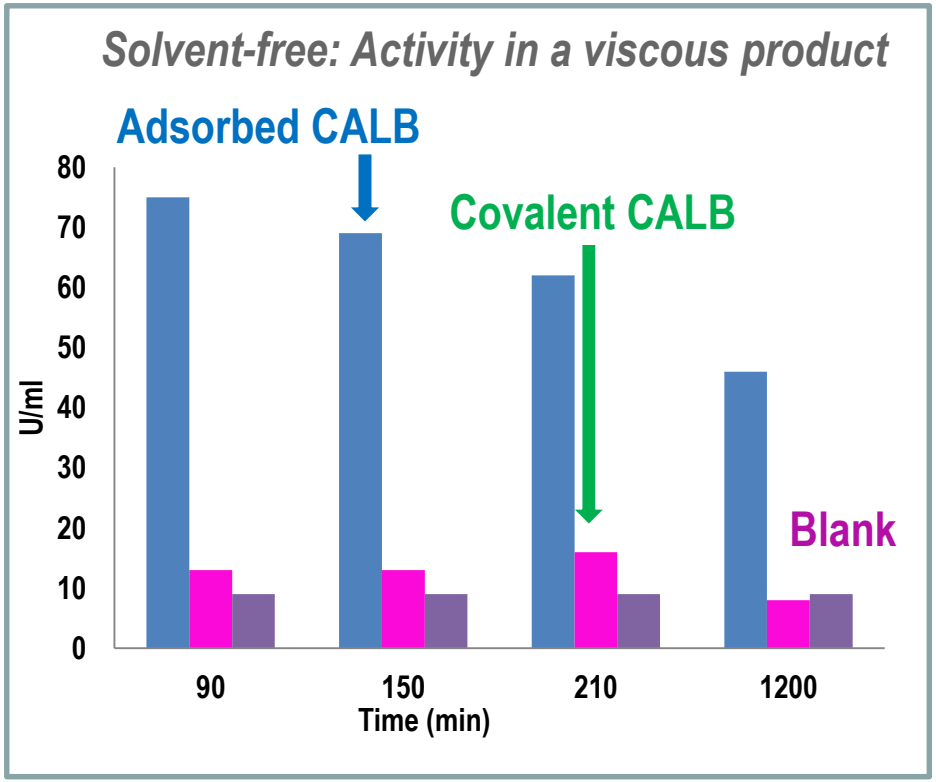
A different immobilization technique for each system

	Aqueous	Aqueous/solvent	Highly viscous	Hydrophobic solvent
Covalent	X	X	X	X
Adsorption				X
Adsorption + cross-linking	X	X	X	X

Comparison between adsorbed and covalently linked enzymes

Residual hydrolytic activity detected in the final product after filtration of the biocatalyst



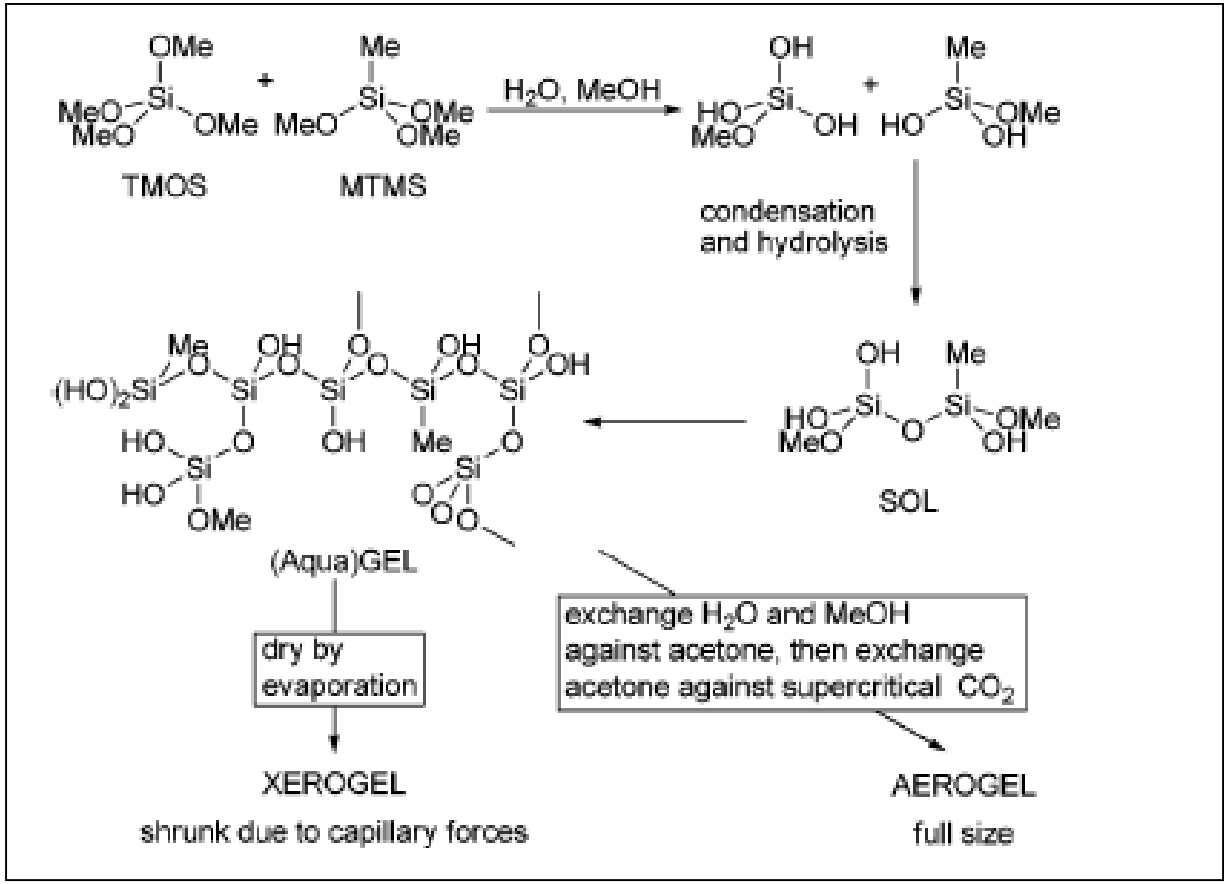


Leaching phenomena affect kinetic studies when adsorbed preparations are employed

Immobilization by entrapment:

Enzymes can be also entrapped in polymers network such as an organic polymer or a silica sol-gel, or a membrane device such as a hollow fiber or a microcapsule.

Synthesis of sol-gels

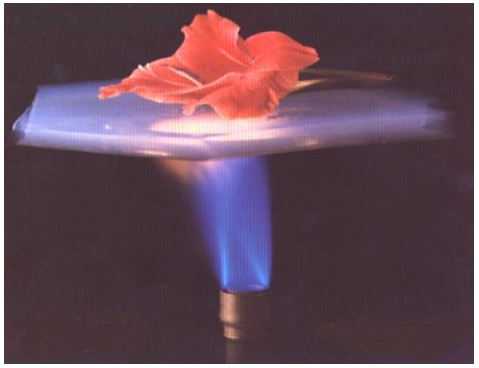


Chem. Soc. Rev., 2009, 38, 453–468

AEROGELs and XEROGELs

DRYING

GEL → SOLID



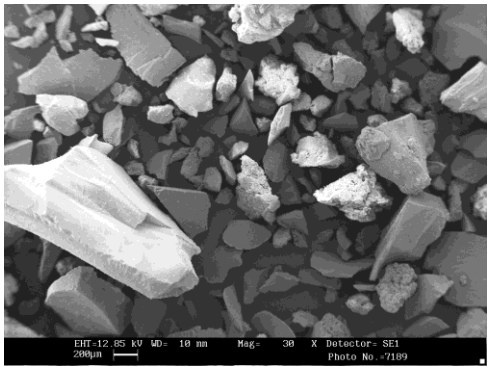
Aerogel

Low thermal conductivity

Transparency

Porosity

High surface area



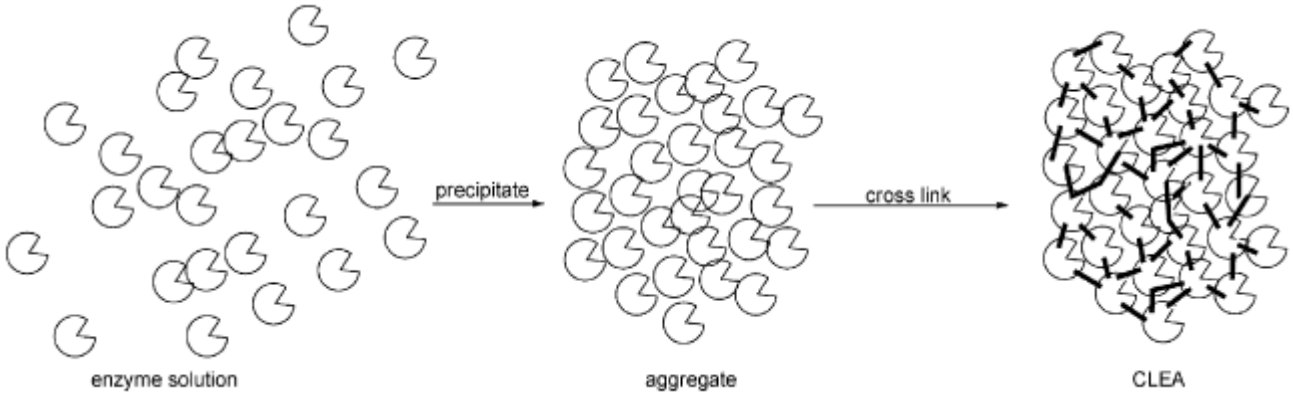
Xerogel

Obtained by means of supercritical CO2 drying

Obtained by classical drying

Carrier-free immobilised enzymes

Carrier-free immobilized enzymes are prepared by the cross-linking of enzyme aggregates or crystals, using a bifunctional reagent. This procedures lead to macroparticles, such as cross-linked enzyme crystals (CLECs) and cross-linked enzyme aggregates (CLEAs). This approach offers the advantage of highly concentrated enzyme activity in the catalyst and low production costs owing to the exclusion of an additional carrier. However, their mechanical stability in some cases may not match industrial requests.



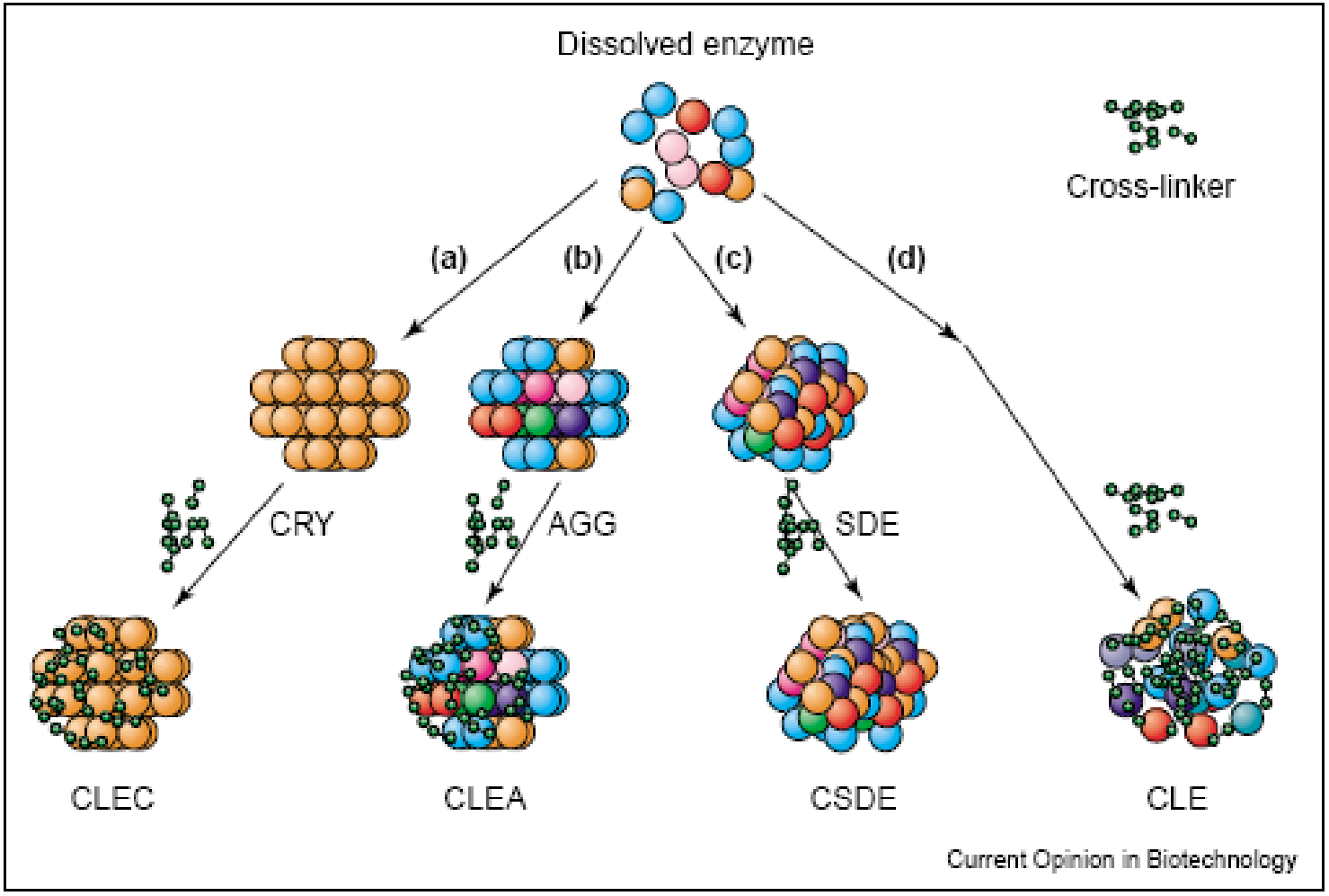
Chem. Soc. Rev., 2009, 38, 453–468

CLEs

- CLEs Cross-linked enzyme
- CLECs Cross-linked enzyme crystals
- CLEAs Cross-linked enzyme aggregates
- CLSDs Cross-linked spray-dried

Amotz S: Method for production of an immobilized enzyme preparation by means of a crosslinking agent. (Novo Industri A/S) 1987; US 4,665,028.

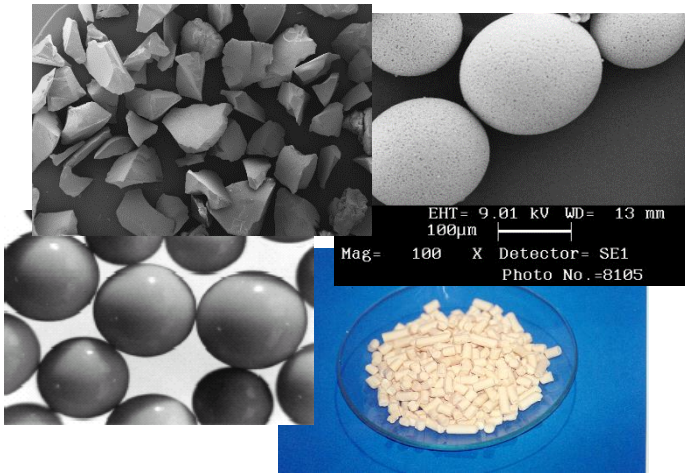
Carrier-free immobilised enzymes



Formulating active and stable immobilized enzymes for industry

The support

- Hydrophobic/hydrophylic
- Porosity
- Chemical functionality
- Polymer-enzyme spacer
- Parcicle size



The enzyme

- Additives in the enzyme formulation?
- Covalent linking possible?
- Conformational flexibility required?
- Hydration required?
- Enzyme glycosylated?

The process

- Reaction medium?
- Heterogeneous system?
- Diffusion limitations?
- Solutes adsorbtion/partition?
- Thermodynamics to be controlled?

COST?

When industry uses immobilized enzymes

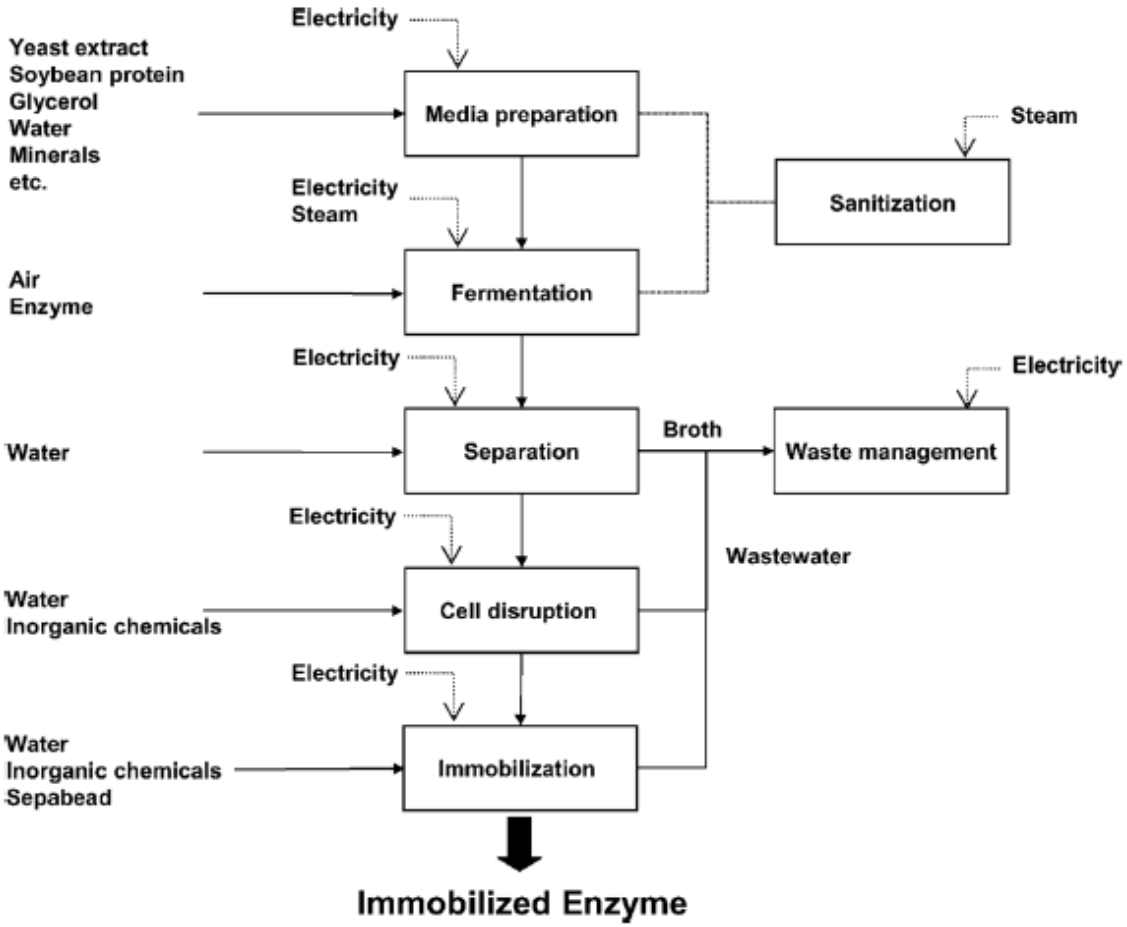
Table 1 Attributes of immobilized biocatalysts

Advantages	Disadvantages
Amenable to continuous and batch formats	Loss of enzyme activity upon immobilization
Reuse over multiple cycles possible	Unfavorable alterations in kinetic properties
Improved stability over soluble enzyme forms	Cost of carrier and fixing agents
Favorable alterations in pH and temperature optima	Cost of immobilization process
Sequester enzyme from product stream	Mass transfer limitations
Co-immobilization with other enzymes possible	Subject to fouling

In reality the cost of most industrial enzymes is in the $50 to $500 per kg enzyme protein range, and they are often only a minor component in overall process economics.

The **first full scale industrial use of an immobilised enzyme** was the production of L-amino acids by resolution of racemic acylamino acids using an aminoacylase from *Aspergillus oryzae* immobilised by adsorption on **DEAE-Sephadex**, which consists of **cross-linked dextran** functionalized with diethylaminoethyl groups. The process was performed in continuous operation in a fixed-bed reactor (Tanabe process – 1960)

Life Cycle Analysis: how sustainable are immobilized enzymes?



Chem Soc Rev

RSC Publishing

REVIEW ARTICLE

View Article Online
View Journal | View Issue

Industrial use of immobilized enzymes

Cite this: *Chem. Soc. Rev.*, 2013, **42**, 6437

Robert DiCosimo,*[a] Joseph McAuliffe,[b] Ayrookaran J. Poulose[b] and Gregory Bohlmann[b]

Received 10th December 2012

DOI: 10.1039/c3cs35506c

www.rsc.org/csr

Although many methods for enzyme immobilization have been described in patents and publications, relatively few processes employing immobilized enzymes have been successfully commercialized. The cost of most industrial enzymes is often only a minor component in overall process economics, and in these instances, the additional costs associated with enzyme immobilization are often not justified. More commonly the benefit realized from enzyme immobilization relates to the process advantages that an immobilized catalyst offers, for example, enabling continuous production, improved stability and the absence of the biocatalyst in the product stream. The development and attributes of several established and emerging industrial applications for immobilized enzymes, including high-fructose corn syrup production, pectin hydrolysis, debittering of fruit juices, interesterification of food fats and oils, biodiesel production, and carbon dioxide capture are reviewed herein, highlighting factors that define the advantages of enzyme immobilization.

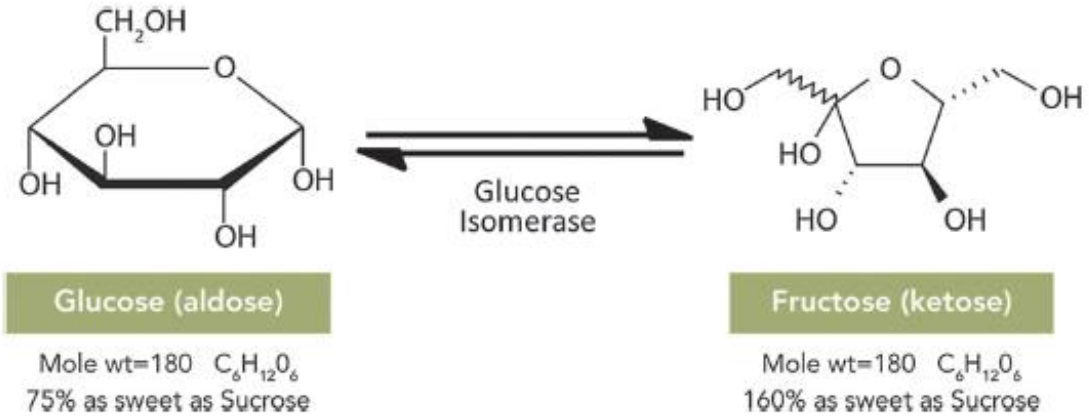
The cost contribution from an immobilized enzyme is dependent on the number of times the enzyme is reused, an indirect measure of total productivity on a kg product per kg biocatalyst basis. This amount varies between a few hundred $ per kg for specialty chemicals, down to a few cents per kg for bulk chemicals, and is often in the range of $0.1 to $10 per kg

Table 2 Large scale industrial processes utilizing immobilized biocatalysts

Enzyme	Form[a]	Process	Product scale (ton per year)	Ref.
Glucose isomerase	CWC, IME, CIE	High fructose corn syrup from corn syrup	10^7	7,19,21–23
Nitrile hydratase	CWC	Acrylamide from acrylonitrile	10^5	334–336
Lipase	IME	Transesterification of food oils	10^5	205–209
Lactase	IME	Lactose hydrolysis, GOS synthesis	10^5	337–339
Lipase	IME	Biodiesel from triglycerides	10^4	269,271–277
Penicillin G acylase	CIE	Antibiotic modification	10^4	340–342
Aspartase	CWC, IME	L-Aspartic acid from Fumaric acid	10^4	343–345
Thermolysin	IME	Aspartame synthesis	10^4	346–348
Lipase	IME, CIE	Chiral resolution of alcohols and amines	10^3	349–351

[a] CWC = cross-linked whole cell; IME = immobilized enzyme; CIE = covalently immobilized enzyme.

Glucose isomerase (GI), also known as xylose isomerase (D-xylose ketol isomerase; EC 5.3.1.5), is one of themost important industrial enzymes in commerce today, driven primarily by the rise of D-fructose as a sweetener for beverages and foodstuffs. Although D-xylose is the native substrate, the enzyme has broad substrate specificity and efficiently converts D-glucose to D-fructose (Scheme 1).



Scheme 1 Isomerization of D-glucose to D-fructose.

Table 3 Examples of commercial immobilized glucose isomerase products[22,23,44]

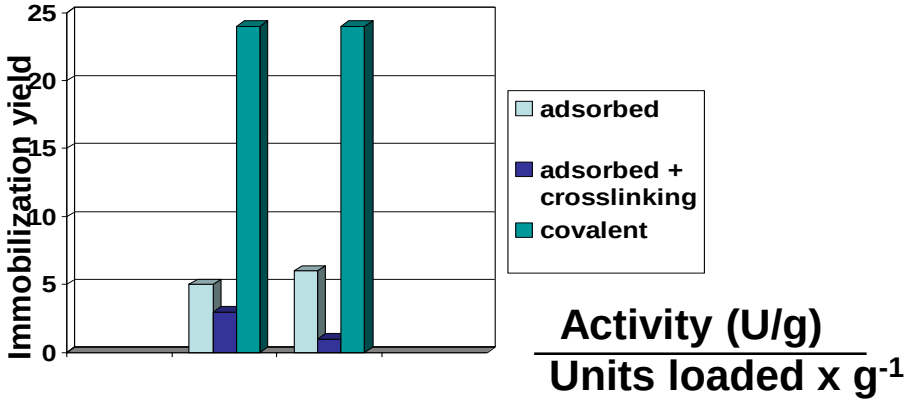
Product	Producer	GI source	Description	Currently sold?
Optisweet® 22	Miles-Kali/Solvay	*S. rubiginosus*	Adsorption of GI on to SiO_2 followed by crosslinking with glutaraldehyde	N
TakaSweet®	Miles Labs/Solvay	*Flavobacterium arborescens*	Polyamine/glutaraldehyde crosslinked cells extruded and spheronized	N
Maxazyme® GI	Gist-Brocades	*A. missouriensis*	Crosslinked cells entrapped within gelatin beads	N
Ketomax GI-100	UOP	*S. olivochromogenes*	Glutaraldehyde crosslinked GI adsorbed to PEI-treated alumina	N
Spezyme®	Genencor	*S. rubiginosis*	Crystallized crosslinked GI adsorbed to granular DEAE-cellulose	N
Sweetase®	Denki Kagku-Nagase	*S. phaeochromogenes*	Heat-treated cells entrapped within polymer beads	N
Sweetzyme® T	Novozymes A/S	*B. coagulans* *S. murinus*	Gluteraldehyde crosslinked whole cell homogenate containing inorganic carrier	Y
GENSWEET®SGI	Genencor/DuPont	*S. rubiginosis*	Soluble GI product for adsorption to DEAE-cellulose anionic resin	Y
GENSWEET® IGI	Genencor/DuPont	*S. rubiginosis*	PEI/glutaraldehyde crosslinked cells, mixed with inorganics (clay, DE)	Y

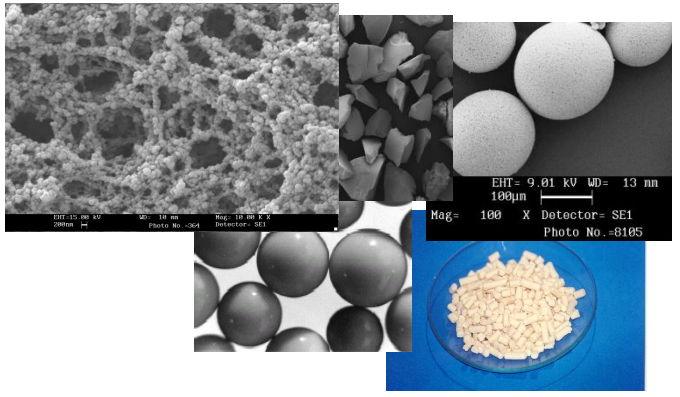
How to describe a biocatalysed process and report a biocatalysed experiment

Immobilized biocatalysts: data to be reported in protocols

Clear protocol of immobilization with characterization:

- **Amount of support and enzyme / cells**
- **amount immobilised, (e.g. difference method)**
- **activity of immobilised preparation**
- **residual water content**
- **data on support (when available)**
- **distribution within particles (when feasible)**

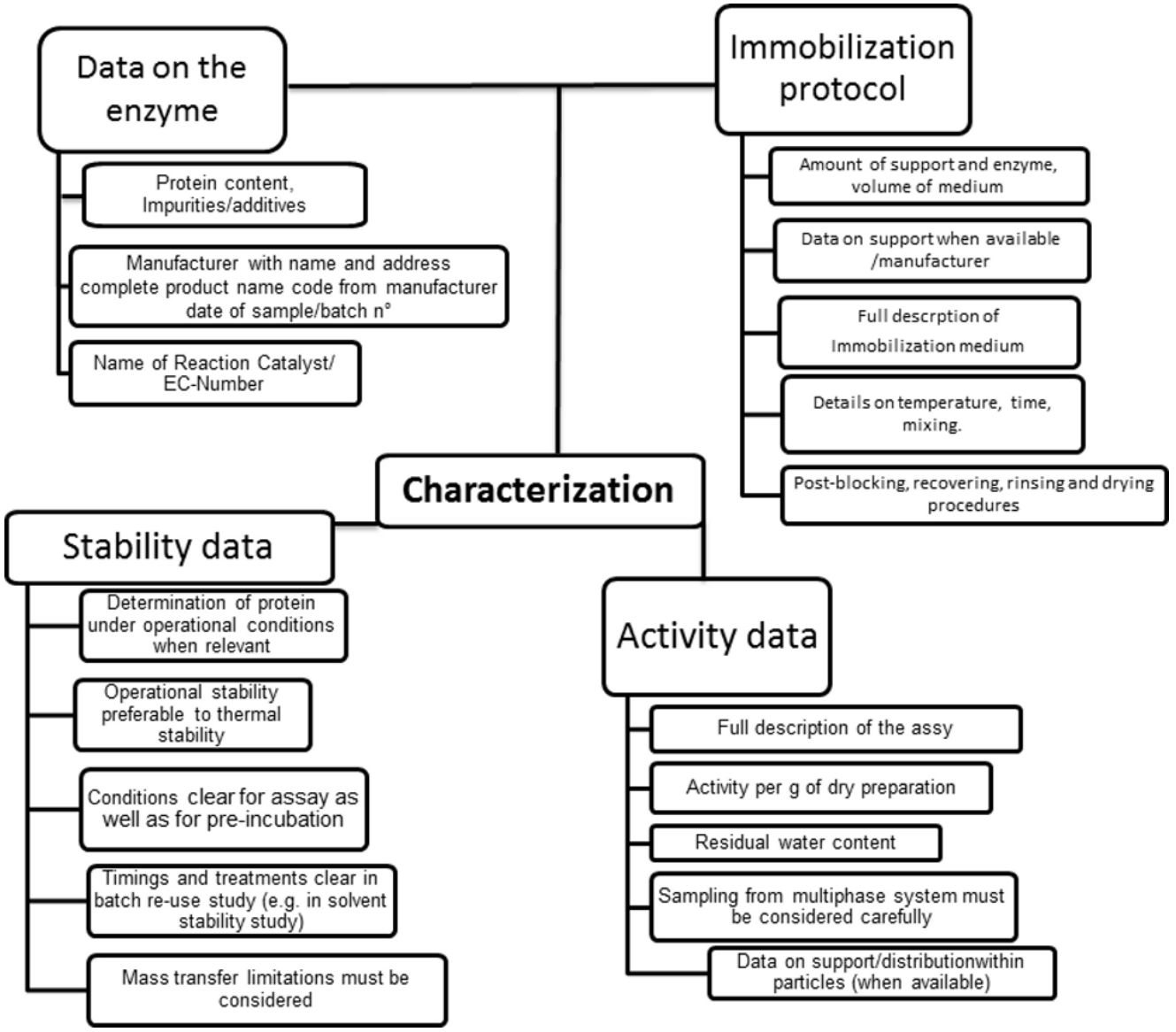




Proteomic Methods Applied to The Analysis of Immobilized Biocatalysts, I. Petry, et al. Biotechnol. Bioeng., 95, 984, 2006

ESAB Section on Applied Biocatalysis

- Rigorous experimental planning
- Detailed reporting of experimental conditions



Factors to be considered when planning enzyme immobilization

Enzyme
Size of the enzyme
Conformational flexibility required by the mechanism
Isoelectric point
Surface functional groups/charge density
Glycosylation
Stability under immobilisation conditions
Presence of hydrophobic regions
Presence of hydrophilic regions
Additives in the enzymatic preparation

Carrier
Organic or inorganic
Hydrophobic or hydrophilic
Surface charges
Surface functionalisation
Chemical and mechanical stability
Surface area
Porosity
Particle size

Specific factors related to the reaction system
Reaction medium
Diffusion limitations
Enzyme inhibition
Precipitation of products
Viscosity of the mixture
Reaction thermodynamics
Non-specific solute-support interactions

Method of immobilization	Relevant Factors
General	Additives in the enzyme preparation that might interfere Stability of the enzyme under immobilisation conditions Stability of the carrier under operative conditions Protein leaching under operative conditions Non-specific carrier-substrates interactions Cost and availability of the carrier
Adsorption/deposition	
Hydrophobic organic carrier	Presence of hydrophobic regions on enzyme Ionic strength of the immobilisation buffer to favour protein adsorption
Hydrophilic organic carrier	Presence of hydrophilic regions on enzyme/glycosylation

Chem. Soc. Rev., 2009, 38, 453–468

Method of immobilization	Relevant Factors
Ionic interactions	pI of the enzyme Charged residues (type and density) on the enzyme surface pH and ionic strength of immobilisation buffer
Covalent binding/crosslinking	Location of the residues necessary for linking pH of immobilisation suitable for nucleophilic attack Conformational flexibility required by the catalytic mechanism
Encapsulation	Size of the enzyme Synthesis conditions for the polymer

Chem. Soc. Rev., 2009, 38, 453–468

Reaction system	Method of immobilisation
Dilute aqueous solution	Covalent Crosslinking Encapsulation
Dilute organic solution	Any
Concentrated, viscous organic/ inorganic mixtures	Covalent, crosslinking