Methods for enzyme and protein immobilization

Metal ions in play

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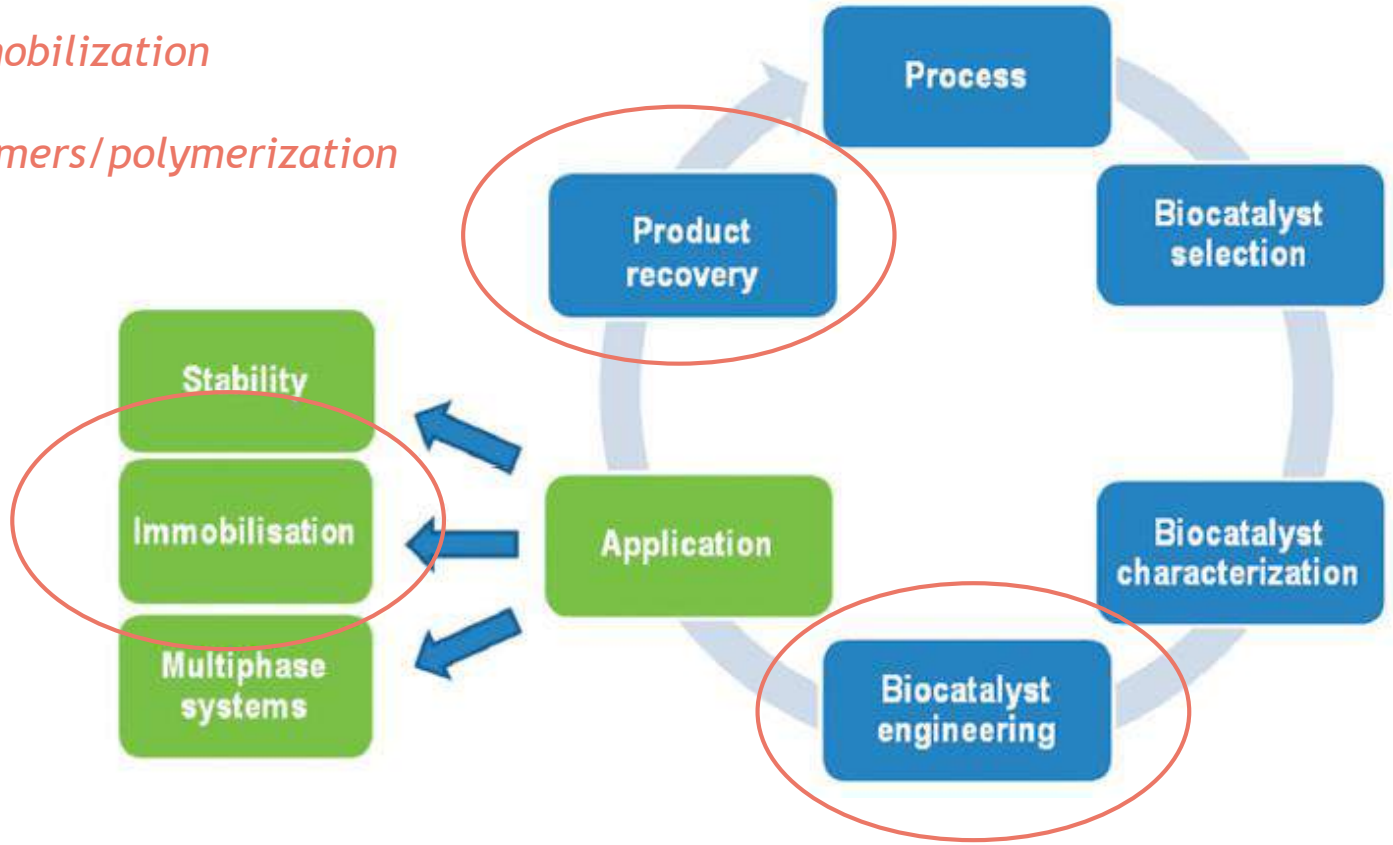


SynBiocat LLC, Budapest, Hungary

Steps of development of a biocatalyzed process

Biocatalysis: A *biocatalyst* can transform a well-defined substrate to well defined product(s) in a process called *biotransformation*

- *Enzyme immobilization*
- *Role of polymers/polymerization*

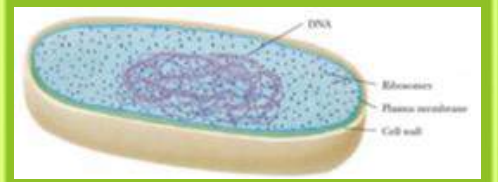


Cantone S. et al, *Chem. Soc. Rev.*, **2013**, *42*, 6262-6276

Biocatalysts

Different forms from whole cell to pure enzyme

Microorganisms, whole-cell systems

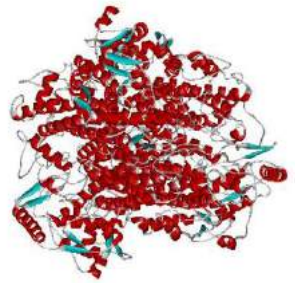


Non-living cells

Disrupted cells, crude enzyme mixture

Partially purified enzymes

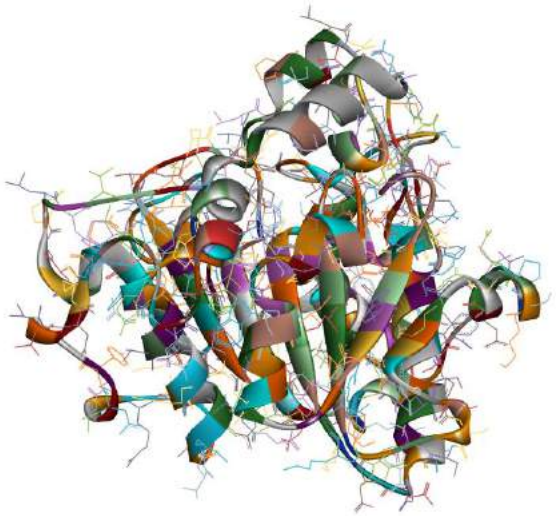
Homogenous enzyme



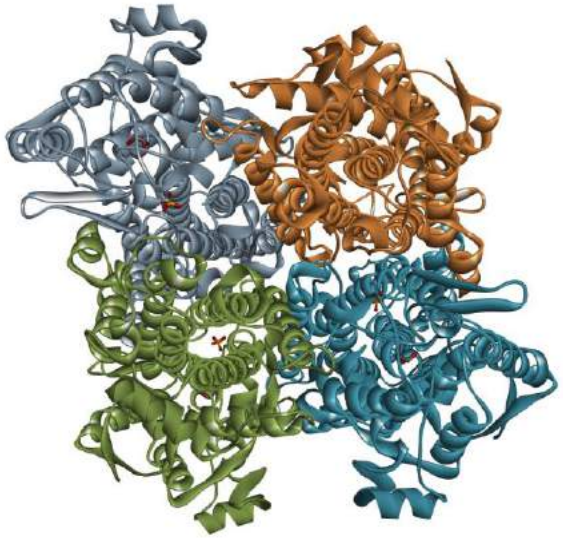
- Any of the above forms may be useful as biocatalyst for various applications
- The cost of of biocatalyst increases from whole cell to homogenous enzyme
- Any of the above forms can be immobilized

Enzyme are biopolymers

- Enzymes are proteins being ***copolymers of 20 kinds of amino acids*** (19 out of the 20 natural AAs are chiral)

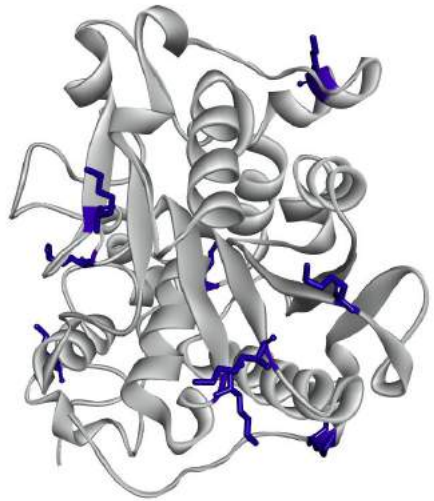


- Enzymes may be composed of ***more than one protein chains*** (a homotetrameric enzyme is shown)

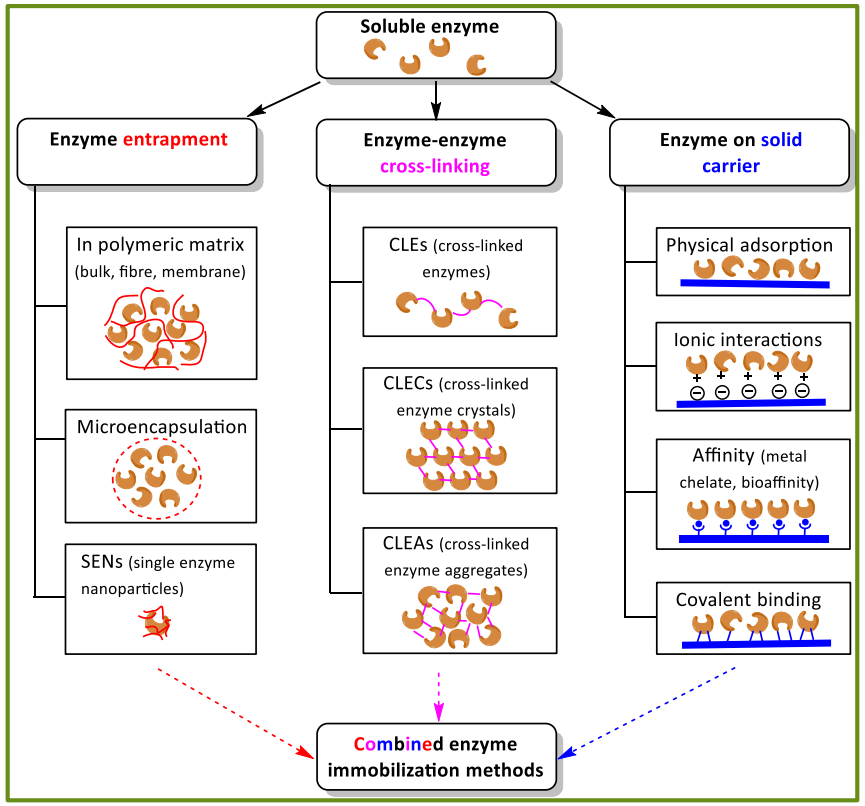


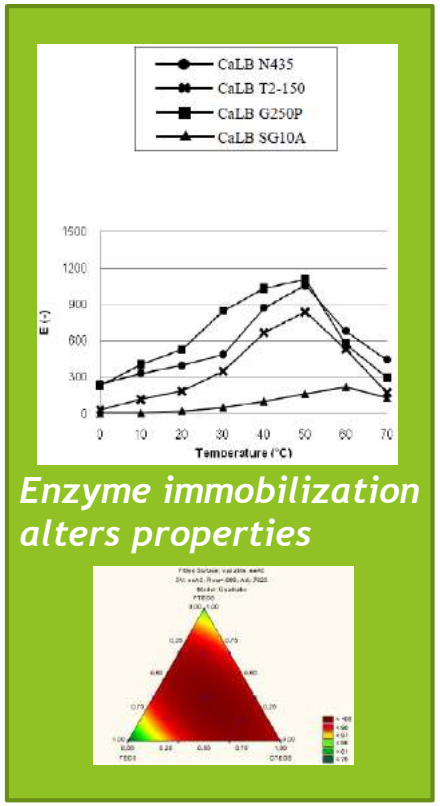
- Enzymes have ***reactive residues at their surface*** (e.g. surface exposed **Lysin** residues)

  => enzyme molecules may be ***a unit*** in a co-polymer forming a composit biopolymer



Enzyme immobilization





Enzyme immobilization alters properties

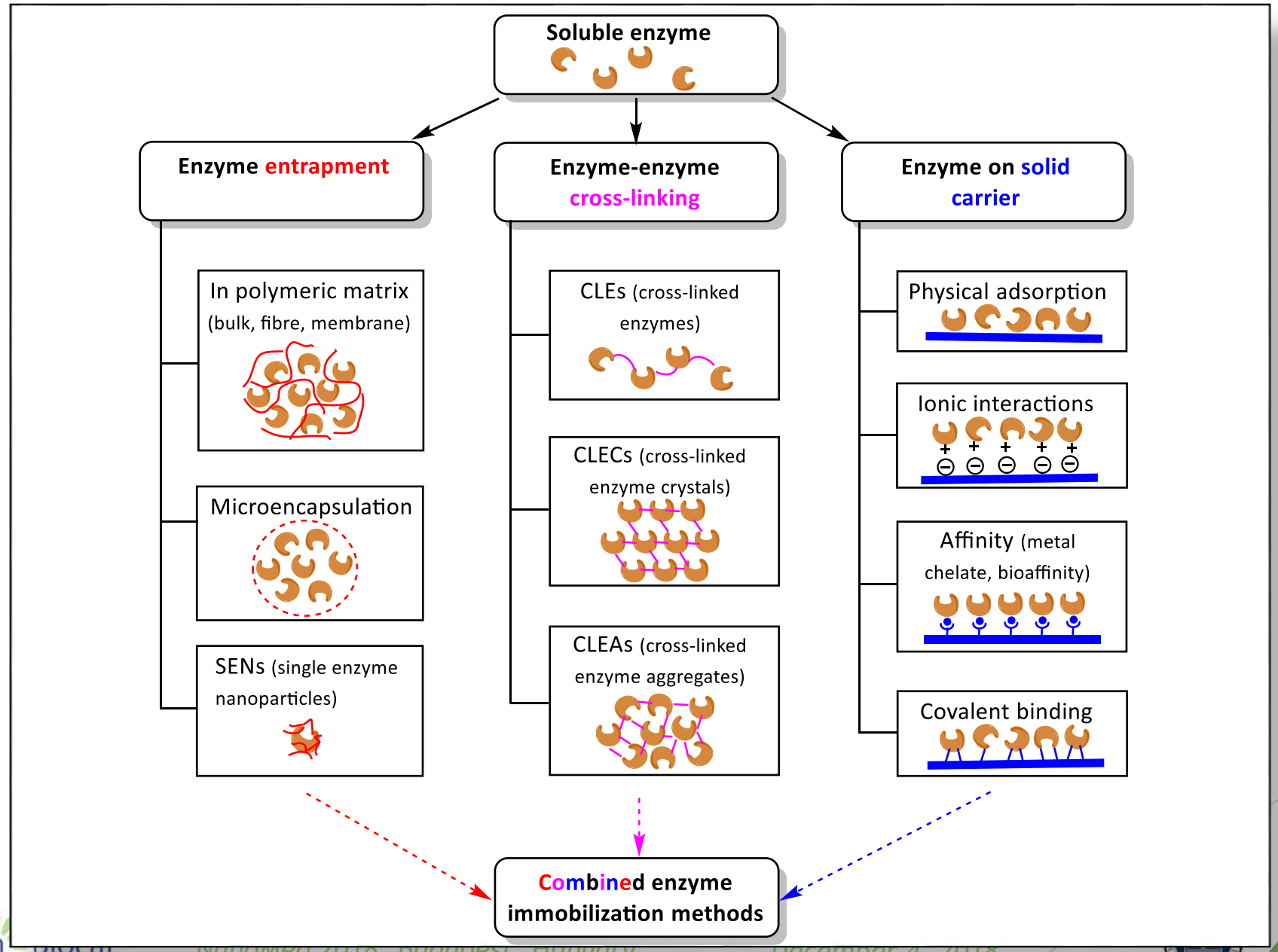
Immobilized enzyme (IE) libraries - different IE for each application





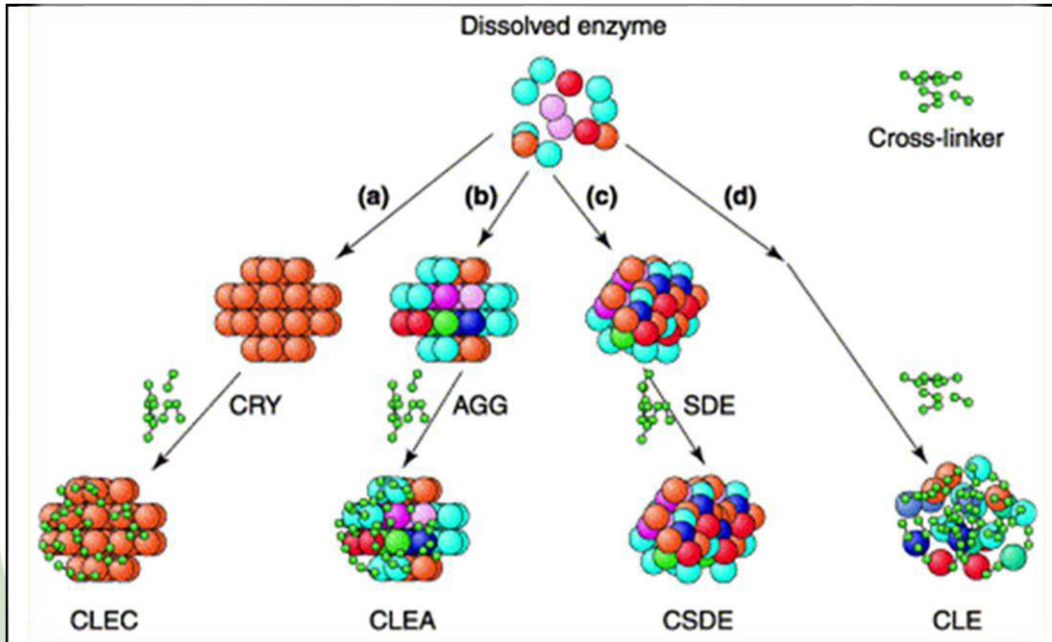
- There is no golden rule for enzyme immobilization.
- Although there are generalizable considerations, the **optimal way of immobilization may vary application by application**.
- **Polymers / polymerization are essential** in many types of immobilizations

Enzyme immobilization



Enzyme cross-linking

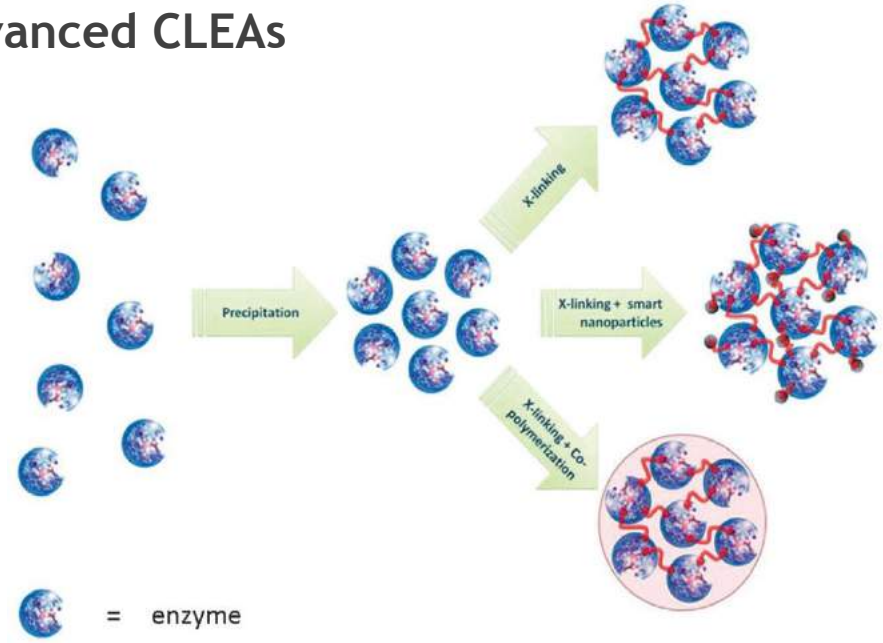
Types of enzyme cross-linked biocatalysts



CLE Cross-linked dissolved enzyme
CSDE Cross-linked spray-dryed enzyme
CLEA Cross-linked enzyme aggregate
CLEC Cross-linked enzyme crystal

Cao L. et al *Curr. Opin Biotech.* **2003**, *14*, 387-394

Advanced CLEAs

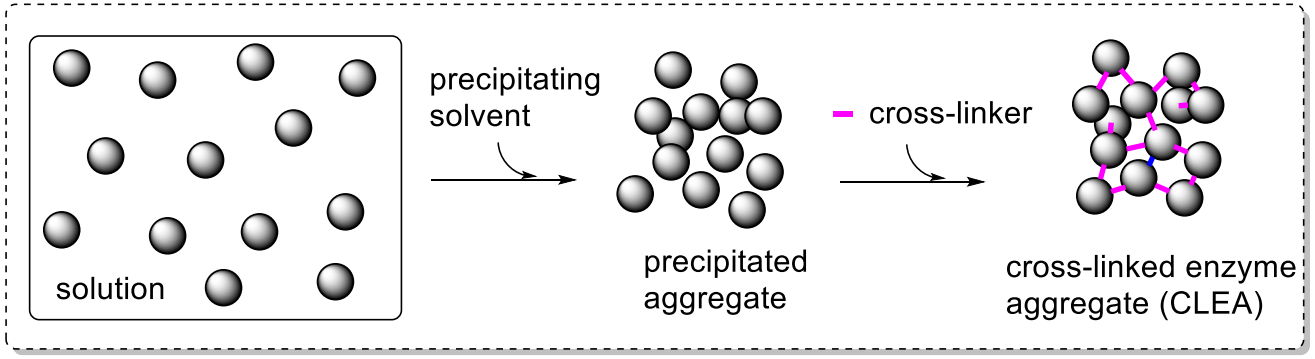


General features of CLEA-type immobilized enzymes:
✓ Easy and cheap preparation
✓ High enzyme loading
✓ Enhanced stability of enzyme
∅ Non-ideal mechanic properties
∅ High degree of diffusional limitation

Sheldon R.A., Pelt S. *Chem. Soc. Rev.*, **2013**, *42*, 6223-6235

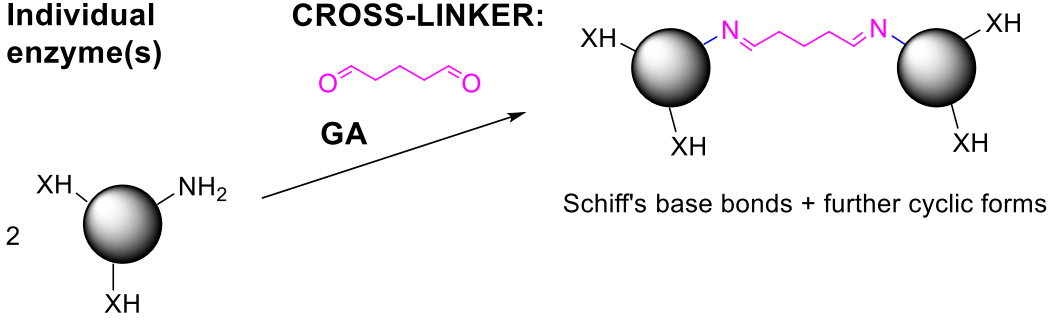
Enzyme cross-linking

Principle of CLEAs and role of cross-linking agent



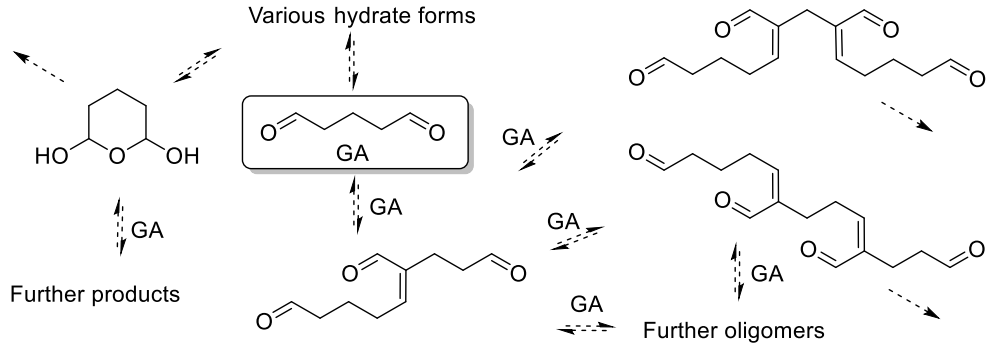
Individual enzyme(s)

CROSS-LINKER:



X= NH, S, C

Glutaraldehyde (**GA**) is the most widely used cross-linker but



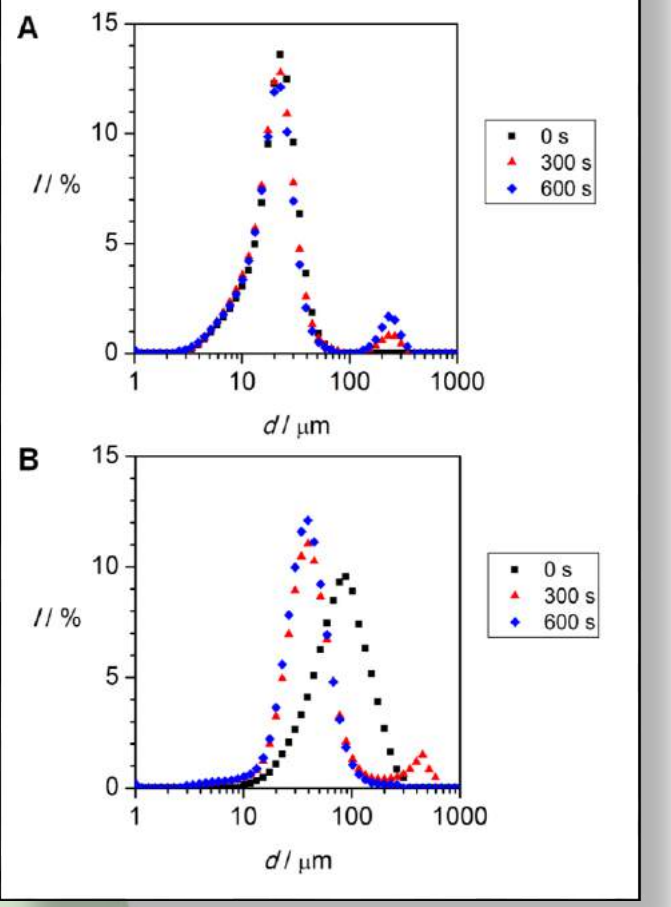
Our study indicated the usefulness of a bisepoxyde (**GDE**) as cross-linker

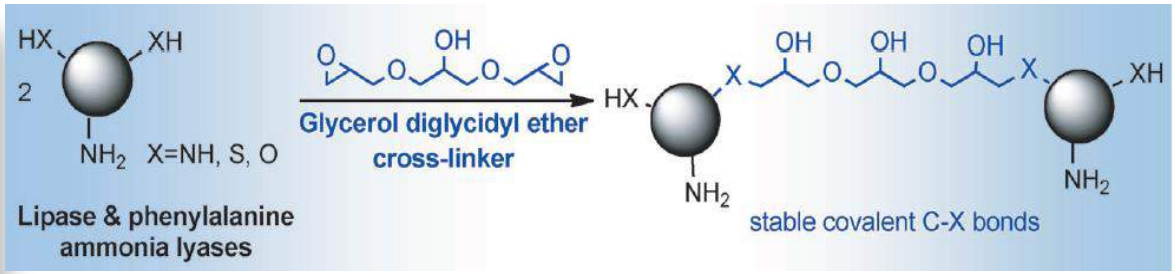
Weiser D. et al *ChemCatChem* **2014**, *6*, 1463-1469.

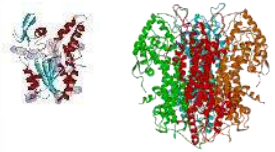
Novel enzyme cross-linking

Bisepoxide cross-linkers for stable CLEAs

Mechanical stability
(*Pc*PAL: PS after ultrasonication)



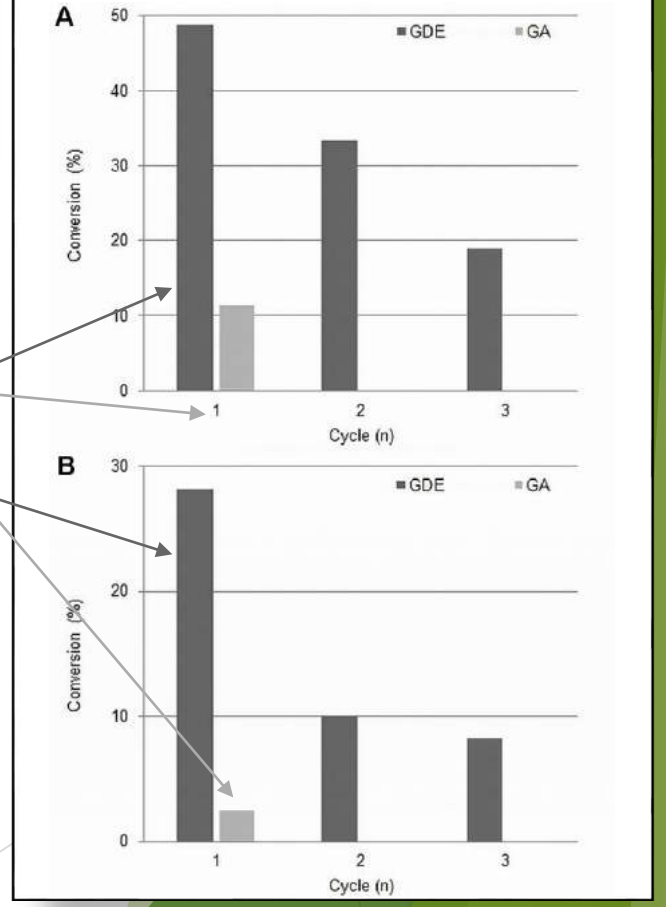




CLEAs of *Pc*PAL and *Rt*PAL crosslinked with GA or bisepoxides (**GDE**) were prepared. The GA or GDE based CLEAs prepared in the presence of starch/BSA were active **even in the ammonia addition reaction performed in 6 M NH_3 / $(NH_4)_2CO_3$**.

The **GDE-starch/BSA-CLEA of *Pc*PAL retained** most of the **initial activity after 3 times recycling** (3 days reaction at RT, ~20% conversion to L-Phe).

Recycling
(*Pc*PAL: *c* (%) after reuse)

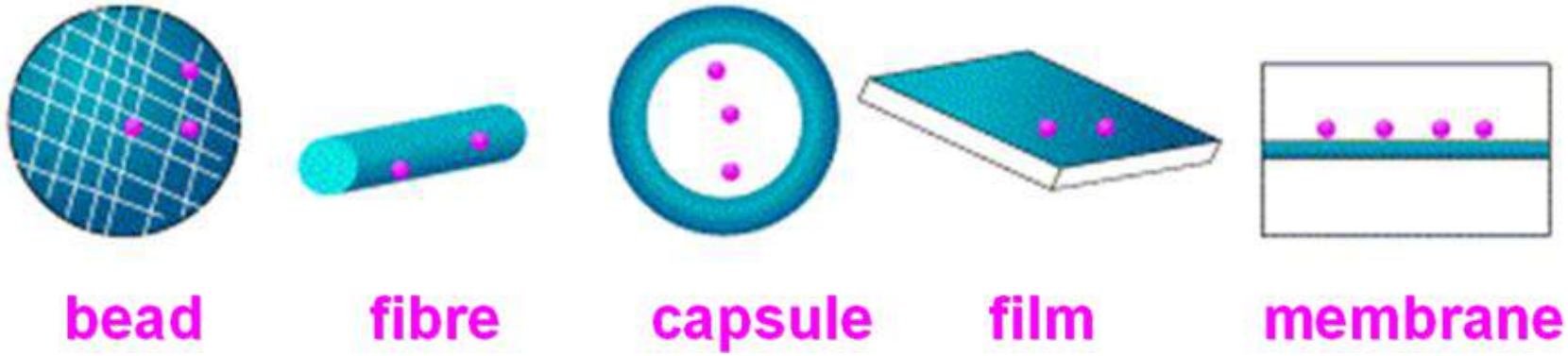


Weiser D. et al *ChemCatChem* **2014**, *6*, 1463-1469.

Enzymes on solid carrier

Most common types of carriers

Cao L. et al *Curr. Opin Biotech.* **2003**, *14*, 387-394



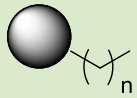
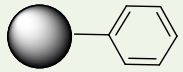
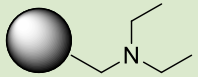
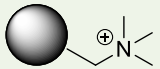
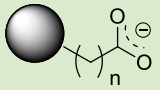
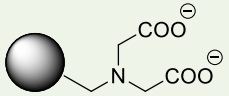
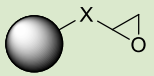
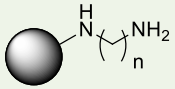
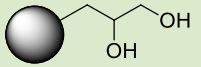
- Any of the above forms may be useful as **carrier to immobilize enzymes** for various applications
- Most common polymers for the above types of carriers:

Inorganic: e.g. **silica gel** (+ further metal oxydes)

Organic: e.g. various **acrylate** polymers, **styrene-divinylbenzene** polymers, etc.

Enzymes on solid carrier

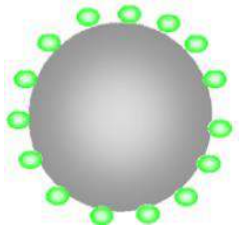
Most common interactions and functions on solid carriers

Mode of immobilization	*Functional group*	*Structure*	*Binding*	*Interacting groups on enzyme*
Adsorption (van der Waals and hydrophobic)	Alkyl		The long alkyl chain enhances the hydrophobic nature of the carrier and maximizes hydrophobic interactions for physical immobilisation (adsorption)	Hydrophobic areas on the surface of lipases
	Phenyl		The phenyl groups enable hydrophobic interactions for physical immobilisation (adsorption)	Hydrophobic areas on the surface of enzymes
Ionic interactions	Trialkyl amine		Ionic adsorption	Negatively charged a.a.
	Tetraalkyl ammonium		Ionic adsorption	Negatively charged a.a.
	Carboxylate		Ionic adsorption	Positively charged a.a.
Metal affinity	Iminodiacetic (Further chelating groups)		Chelates with metal ions such as Ni^{2+}, Co^{2+}, Zn^{2+} ...; Further chelating groups	His-tag
Covalent bonds	Epoxy		Formation of covalent bonds *via* nucleophilic attack and opening of the epoxy ring	Nucleophilic groups (mainly $-NH_2$ and $-SH$)
	Amino		Pre-activation with glutaraldehyde to introduce an aldehyde group that forms an imino bond via nucleophilic attack by a primary amine	Primary amines (terminal amines and Lys side chains)
	Diol		Activation with BrCN to give a reactive cyclic imido-carbonate. Oxidation of adjacent cis-diols with $NaIO_4$ to give dialdehydes	Primary amines (terminal amines and Lys side chains)

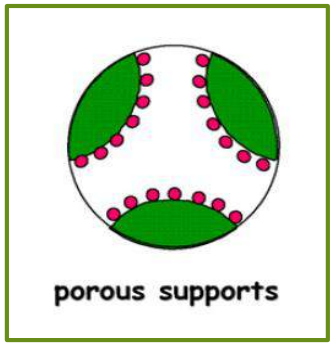
Cantone S. et al, *Chem. Soc. Rev.*, **2013**, *42*, 6262-6276

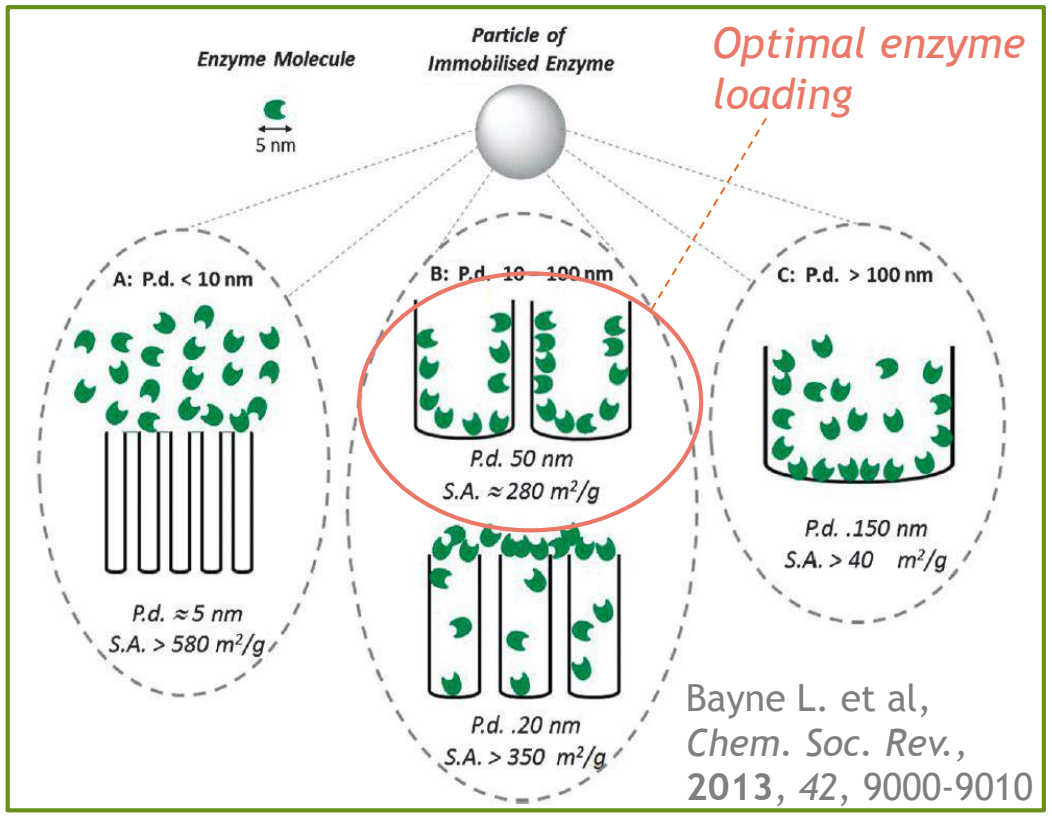
Enzymes on solid carrier

Effect of the pore diameter on enzyme loading



Non porous supports



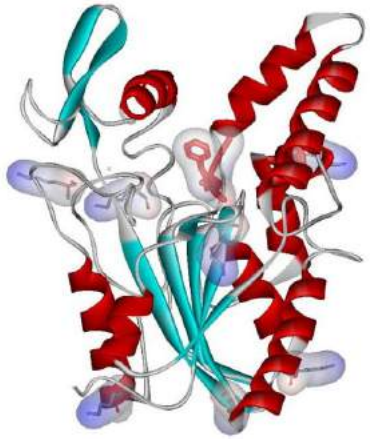


Bayne L. et al, *Chem. Soc. Rev.*, **2013**, *42*, 9000-9010

- (A) shows pore diameter (**P.d.**) approx. equal to the diameter of enzyme molecule and hence no access to the large surface area (**S.A.**) available.
- (B) shows various mesopores at **P.d.** between 10–100 nm. At 50 nm **P.d.** we see high protein loading. At **P.d.** of 20 nm we see reduced protein loading despite an increase **S.A.** This may be explained by protein-protein interaction at the pore entrance inhibiting further enzyme immobilization.
- (C) shows the reduced available area **A.A.** noted at larger P.D. > 100 nm, hence reduced protein loading.

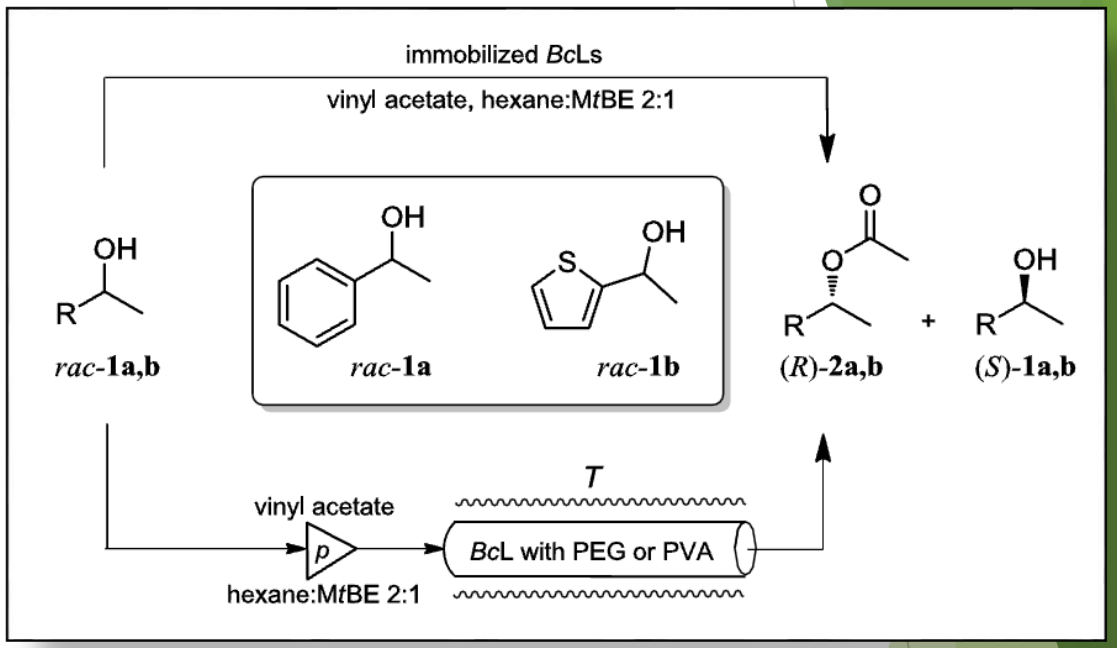
Enzyme on solid carrier

Adsorption: Effect on embedding polymers on thermostability

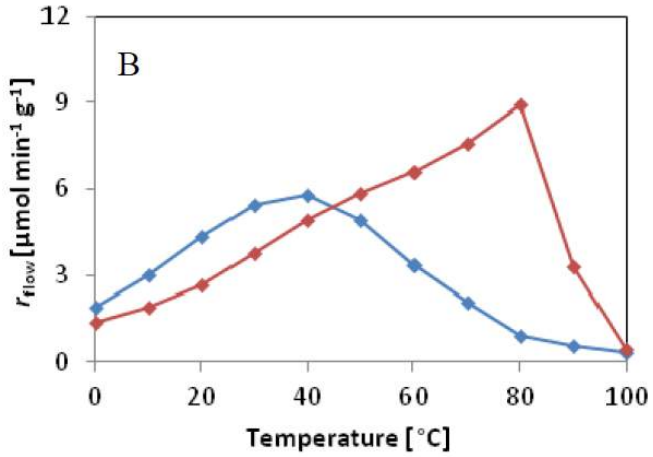


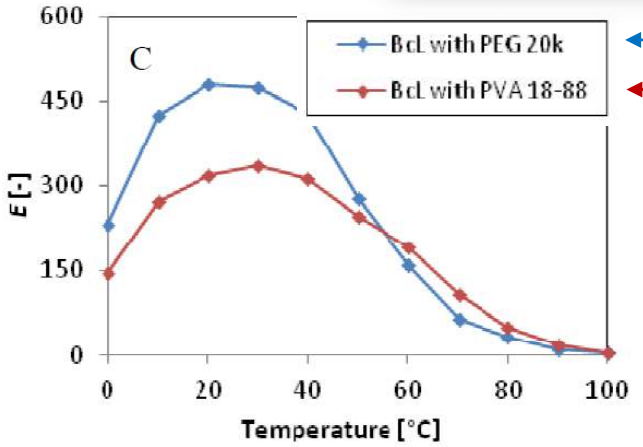
Abaházi E. et al. *Molecules* **2014**, *19*, 9816-9837.

BcL: Lipase from *Burkholderia cepacia*



Carrier: mesoporous silica (P.d.: 25 nm)
Additives: surfactants and polymers
(e.g. PEG 20 000 and PVA 18-88)





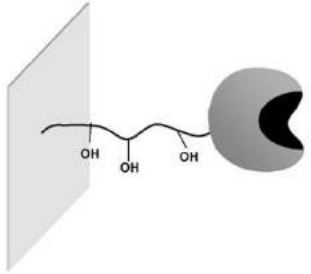
← soluble in media
← insoluble in media
ides rigidification

⇩

50 °C shift in thermal stability

Enzyme on solid carrier

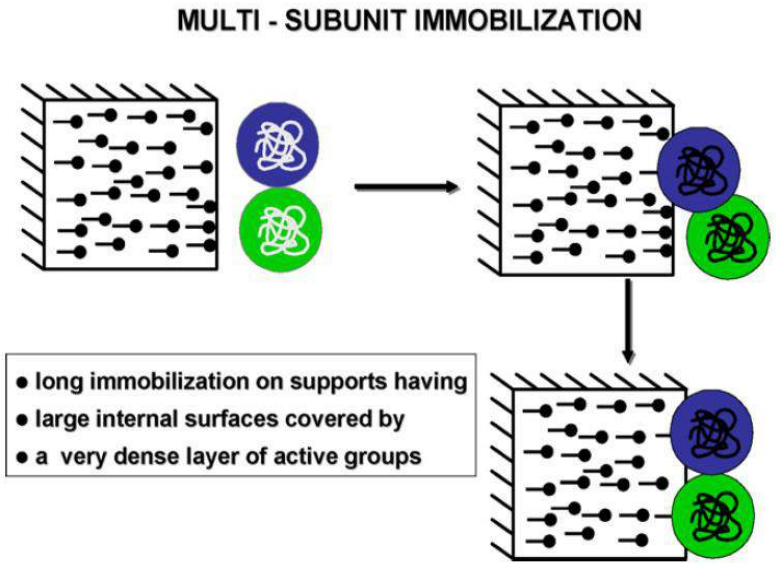
Covalent binding: Multipoint attachment and rigidification

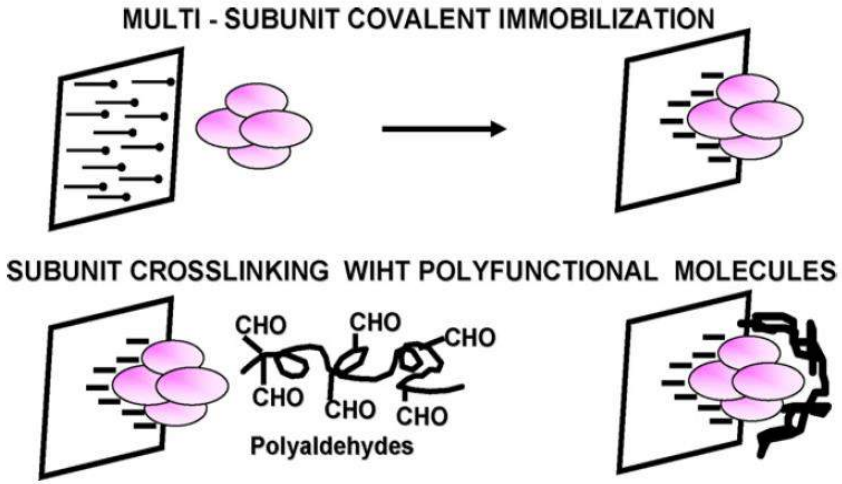


No rigidification



Enzyme rigidification

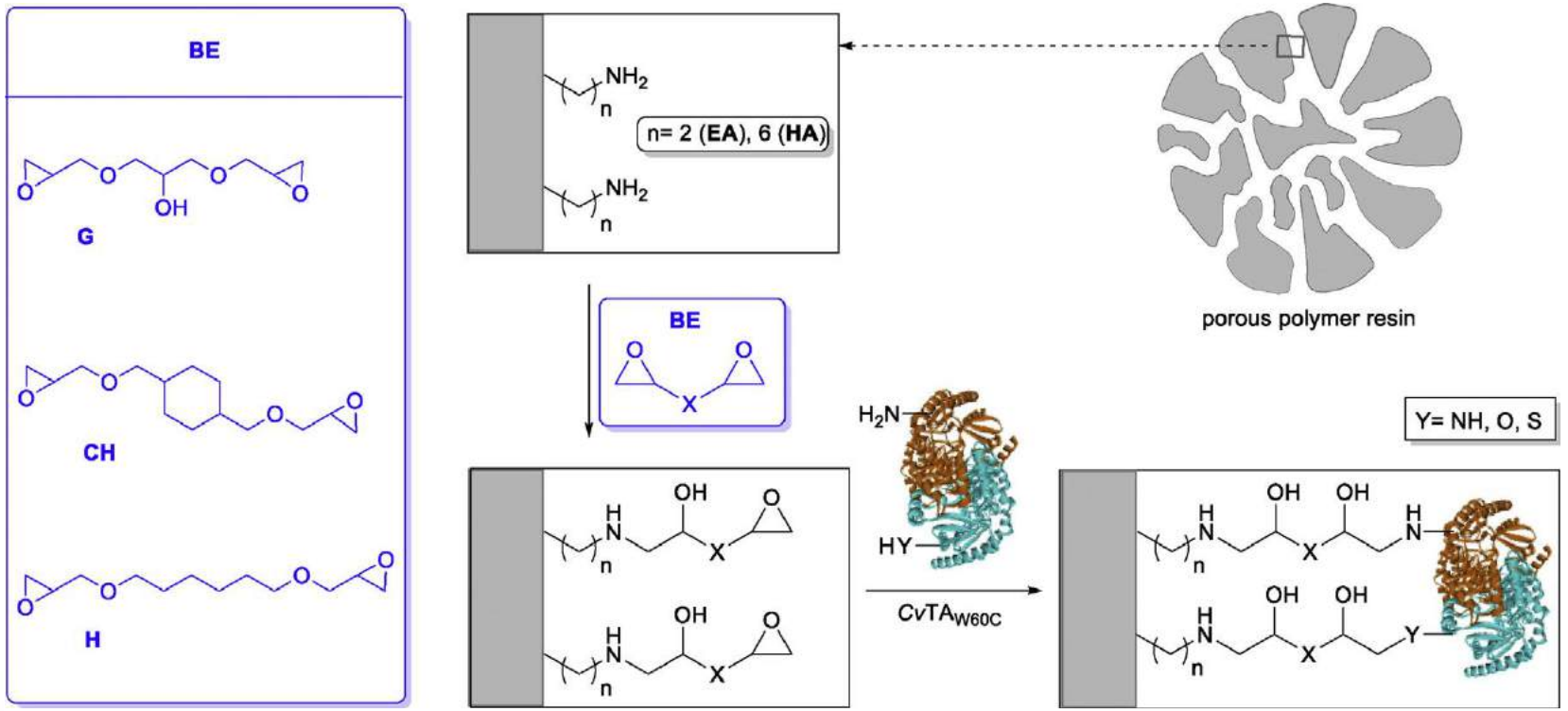


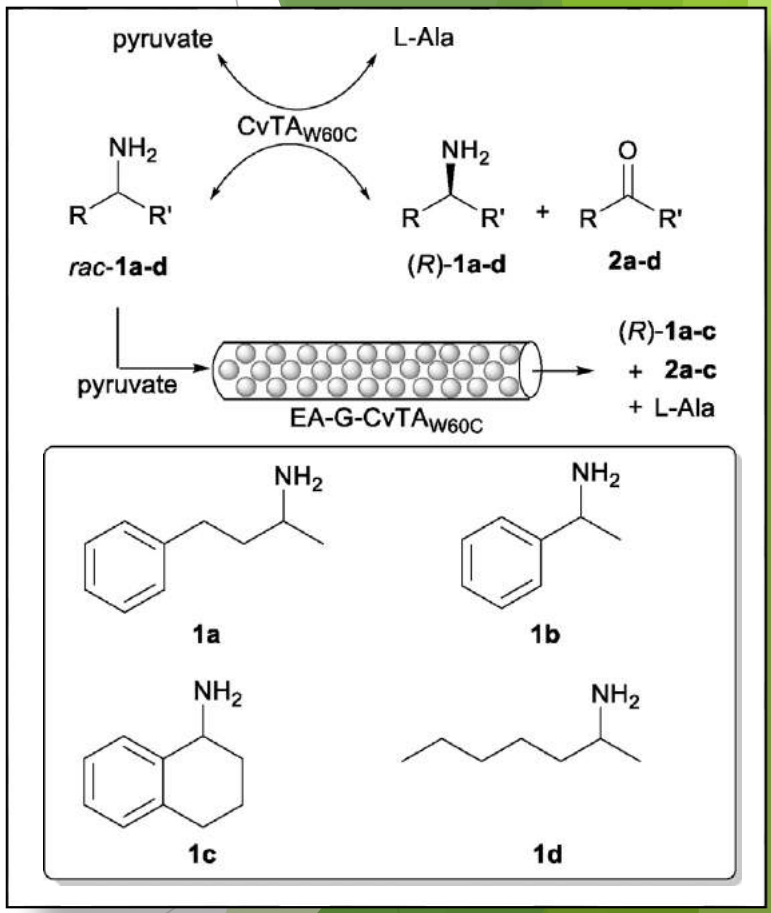


Mateo C. et al. *Enzyme Microb. Technol.* **2007**, *40*, 1451–1463.

ω-Transaminase on solid carrier

Covalent binding: Multipoint attachment with bisepoxides



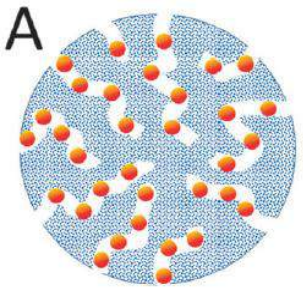


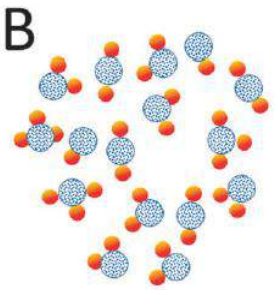
- Covalent immobilization of an engineered ω-transaminase mutant Trp60Cys from *Chromobacterium violaceum* ($CvTA_{W60C}$) was performed on bisepoxide-activated aminoalkylated acrylic resins.
- DMSO tolerance and thermotolerance was enhanced in continuous-flow mode applications.

Abaházi E, et al. *Biochem Eng J.*, **2018**, *42*, 6236-6249.

Enzyme on solid carrier

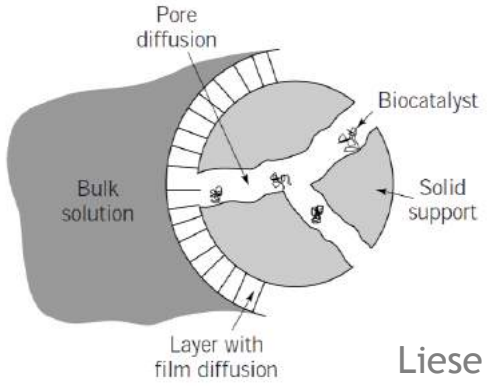
Effect of the pore length and particle diameter of beads





A mesoporous carrier:

- ✓ Easy to handle and recover
- ✓ Relative large surface area
- ∅ Long pores cause **diffusion limitations**
- ▶ **Smaller diameter** shorten the pores and thus **reduces diffusion limitation**

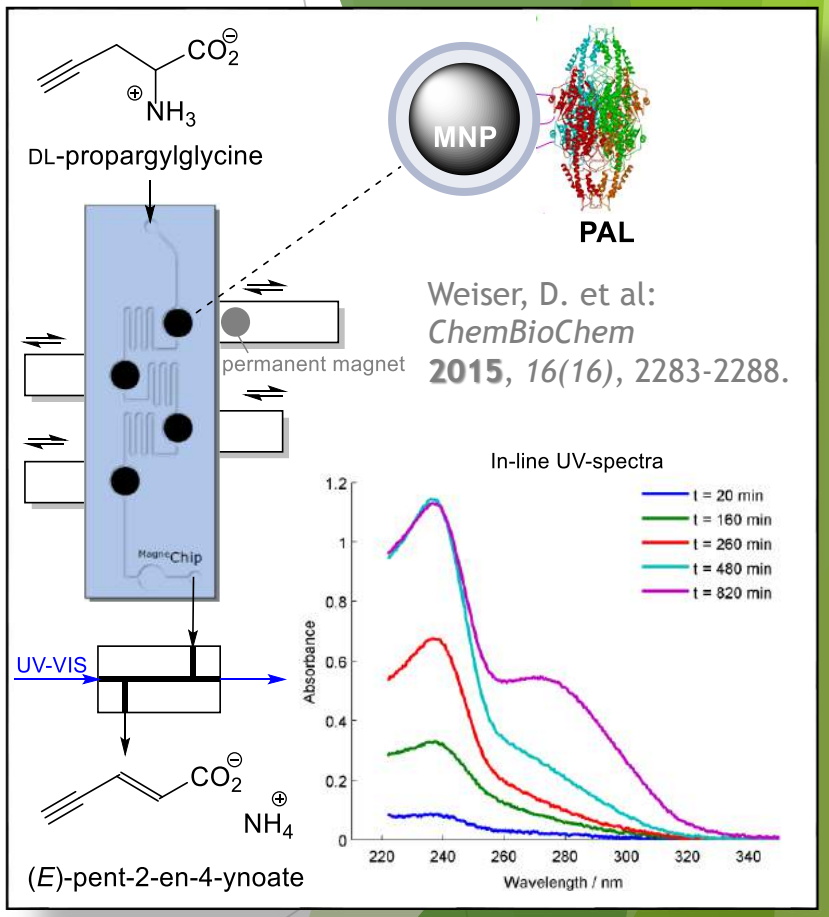


Liese A., Hilterhaus L.
Chem. Soc. Rev., **2013**, *42*, 6236-6249.

B nanoparticles:

- ✓ **No diffusion limitations**
- ▶ **The smaller the diameter the larger the surface area/volume ratio**
- ✓ Relative large surface area
- ✓ Can form **stable emulsion** behaving like homogenous solution
- ∅ More difficult to handle and recover
- ✓ **Magnetic nanoparticles** enable easy recovery

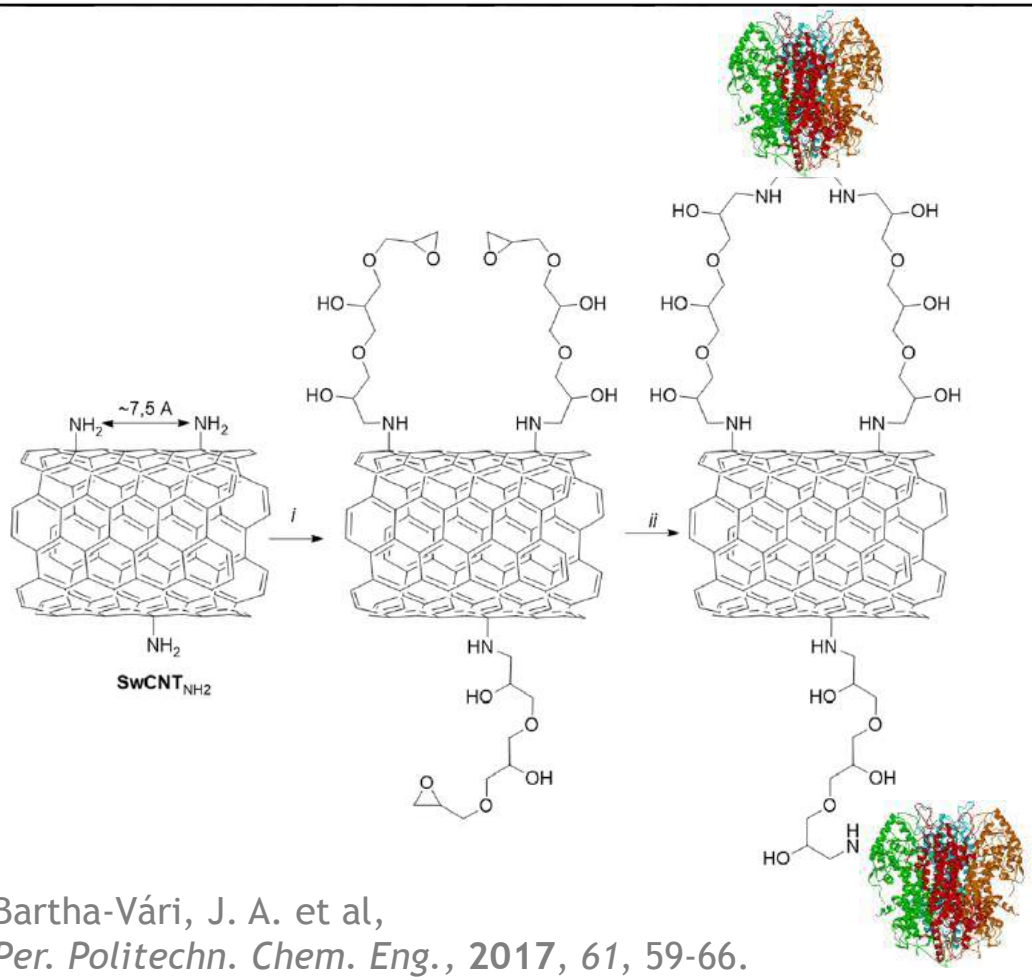




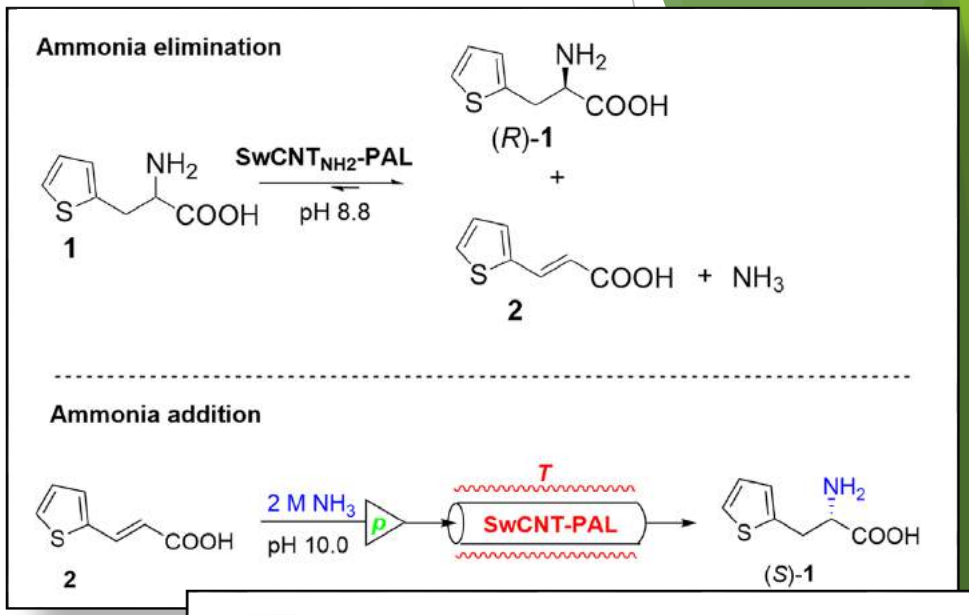
Weiser, D. et al:
ChemBioChem
2015, *16(16)*, 2283-2288.

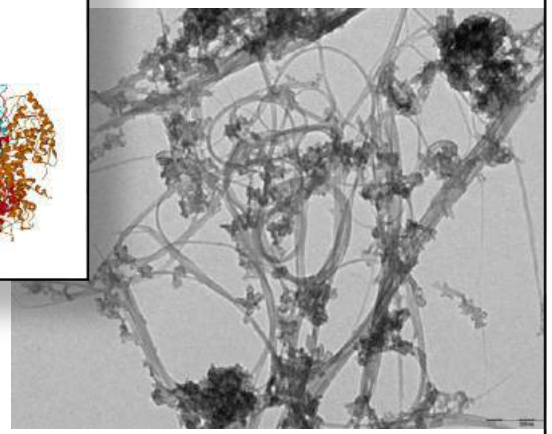
Covalent immobilization of PAL

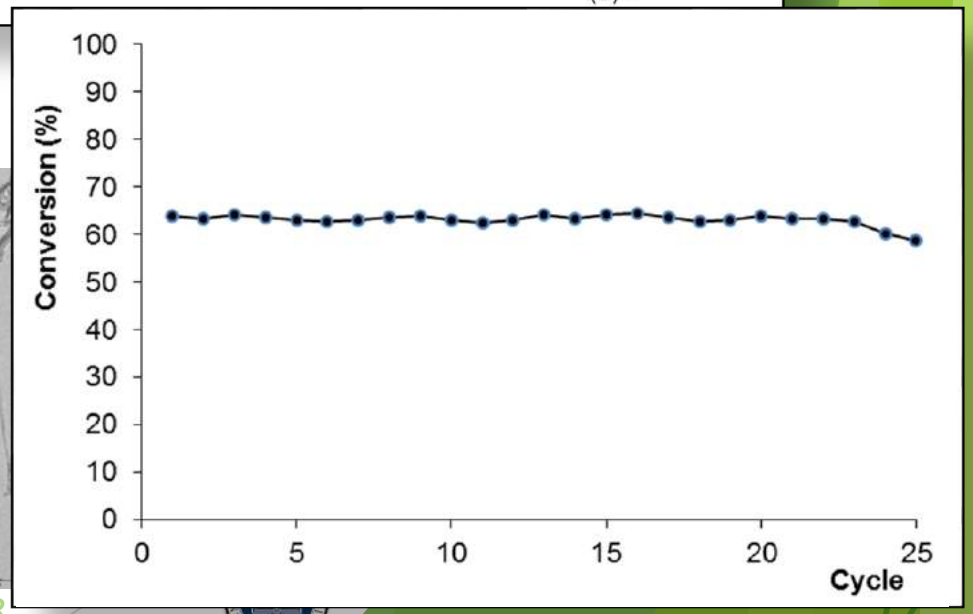
Covalent binding by bisepoxides onto single walled carbon nanotubes



Bartha-Vári, J. A. et al,
Per. Politechn. Chem. Eng., **2017**, *61*, 59-66.

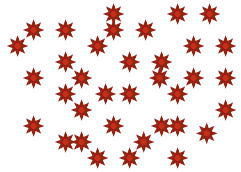






Enzyme entrapment

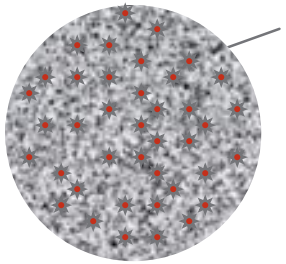
Methods for entrapment or encapsulation



Non-immobilized biocatalyst

Matrix encapsulation

Enzyme within the interstitial spaces of cross-linked, water-insoluble polymer gels.

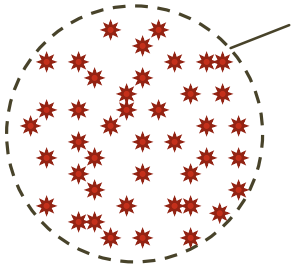


Porous polymeric matrix

- Synthetic polymers (e.g. acrylates)
- Natural polysaccharides (e.g. alginate, κ-carrageenan)

Microencapsulation

Enzyme is enclosed within spherical, semi-permeable membranes of 1-100 μm diameter.



Semipermeable membrane

- Interfacial polymerization (e.g. nylon or chitosan)
- Stable microemulsion (e.g. liposomes)

✓ Advantages of entrapment

- Enzymes are immobilized without a chemical or structural modification.
- A very general technique.

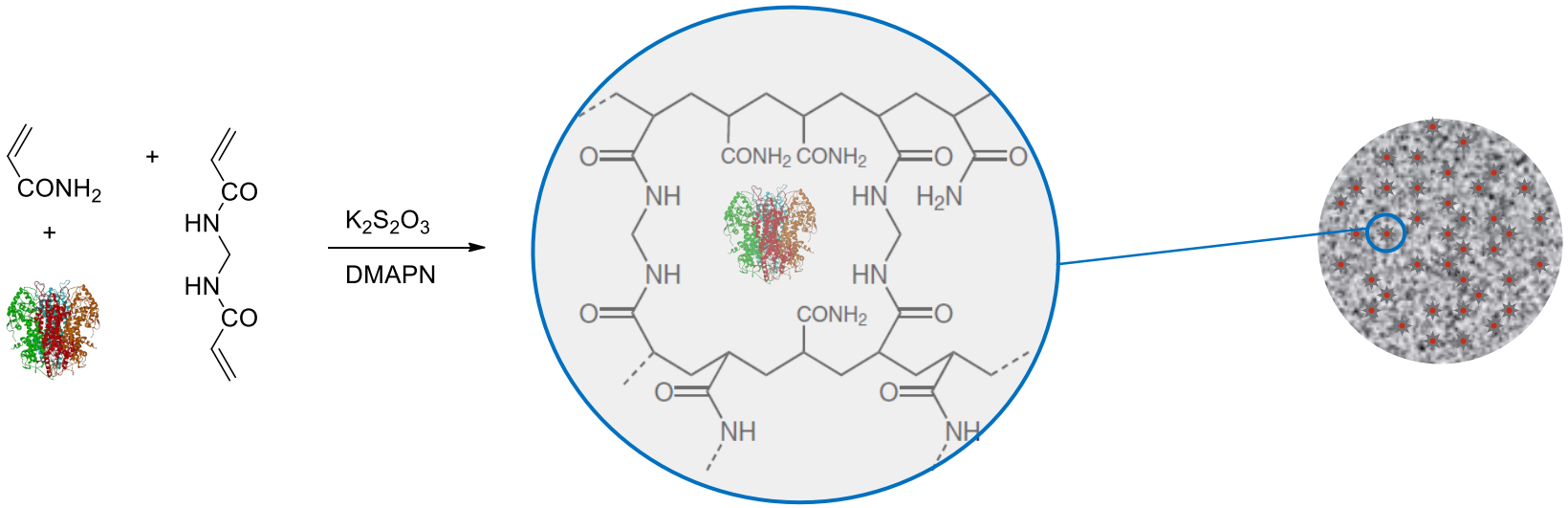
∅ Disadvantage of entrapment

- High molecular weight substrates have limited diffusivity, and cannot be treated with entrapped enzymes.

Enzyme entrapment

Entrapment within synthetic polymer gels

Polyacrylamide gel encapsulation

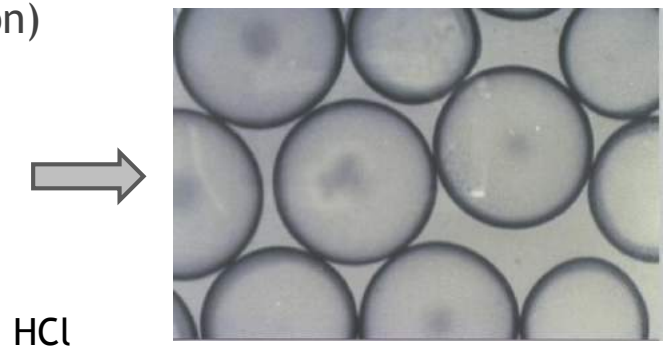


Variations of pore size result in enzyme leakage, even after washing. The effect of initiator used in polyacrylamide gels can be problematic.

Enzyme entrapment
Microencapsulation

- **Interfacial polymerization reaction** (e.g. nylon)

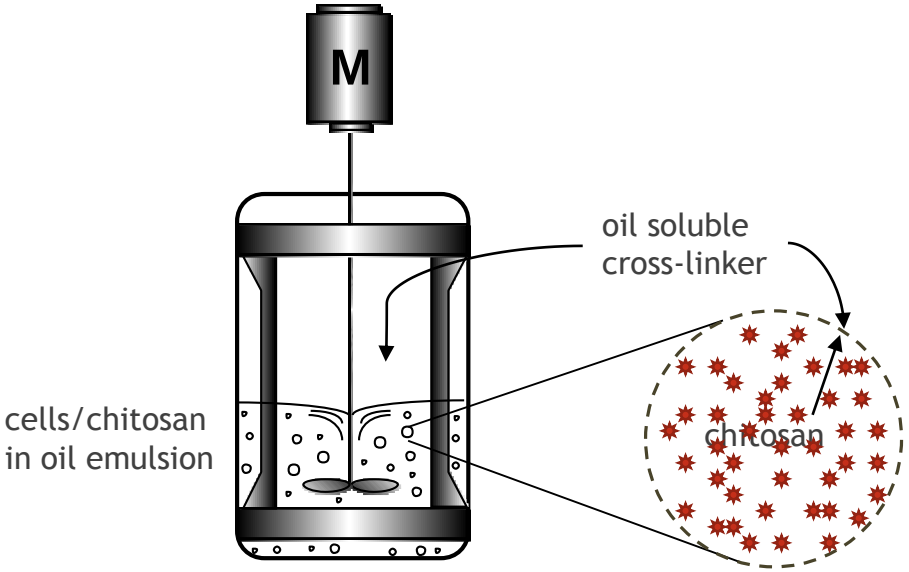
nylon 6-10 polyamide:

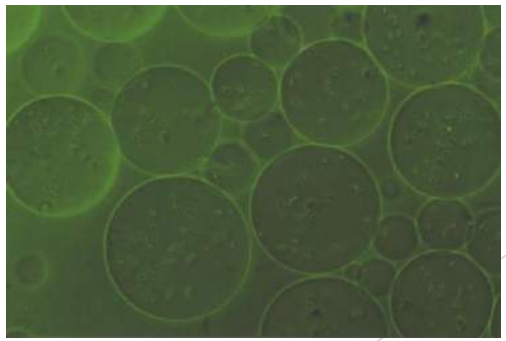


$NH_2(CH_2)_6NH_2 + ClCO(CH_2)_8COCl \longrightarrow$

$NH_2(CH_2)_6NH\text{-}CO(CH_2)_8CONH(CH_2)_6NH\text{-}CO(CH_2)_8CO\text{-} + HCl$

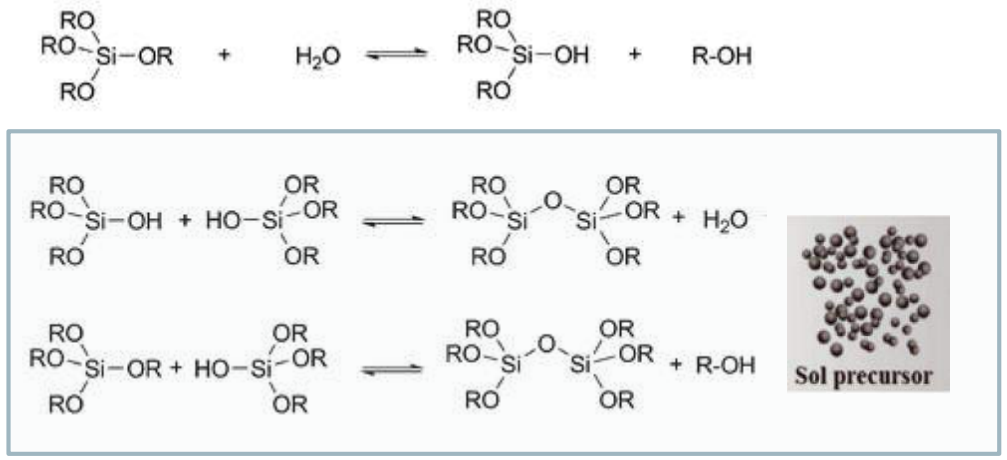
- **Microencapsulation protocol** (e.g. chitosan)



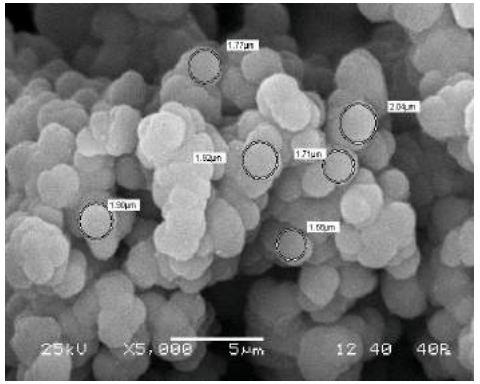


Enzyme entrapment

Entrapment in sol-gel matrices

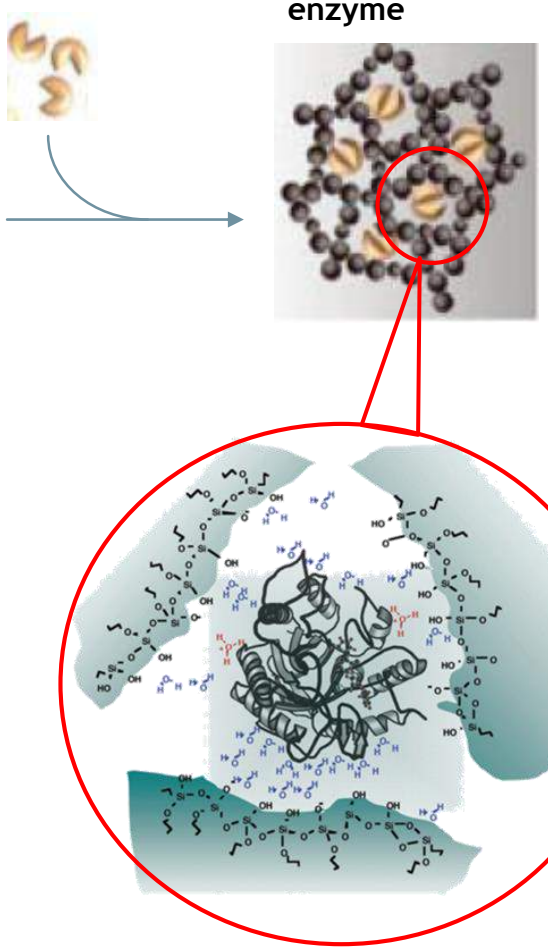






free enzyme

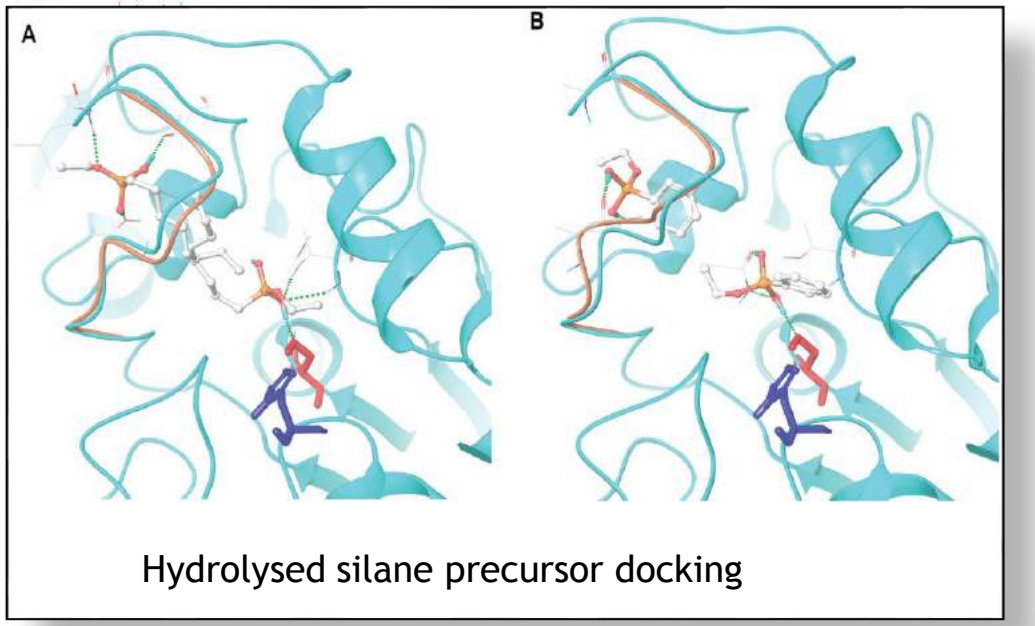
sol-gel entrapped enzyme



Complementary multipoint fixation of the enzyme in the mechanically stable sol-gel matrix can radically **enhance thermal stability and solvent tolerance**

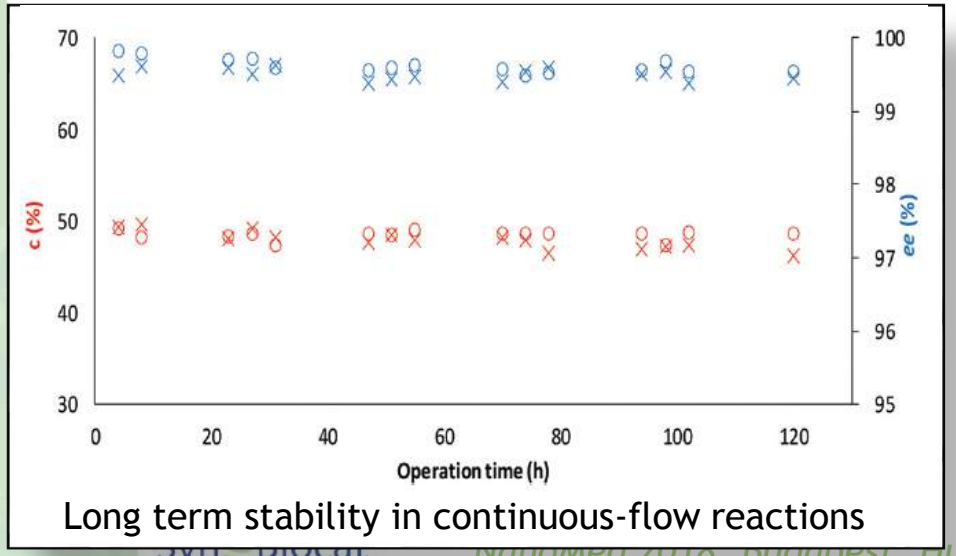
Enzyme entrapment

Fine tuning sol-gel matrices

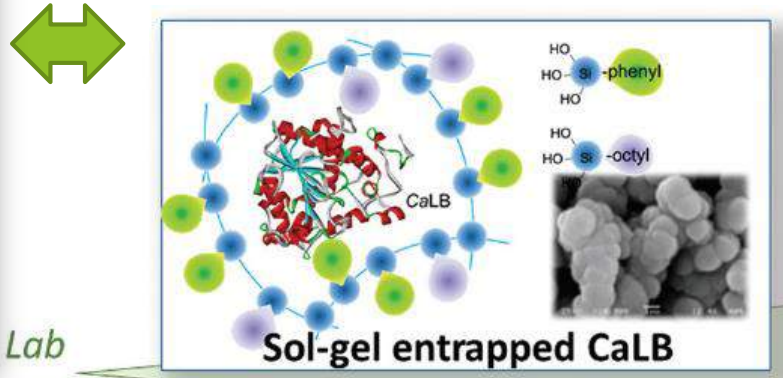


Hydrolysed silane precursor docking

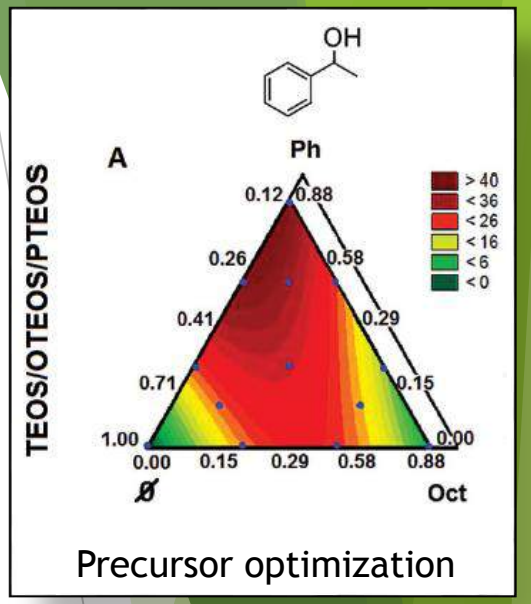
Weiser, D. et al,
Green Chem., **2017**, *19*, 3927.



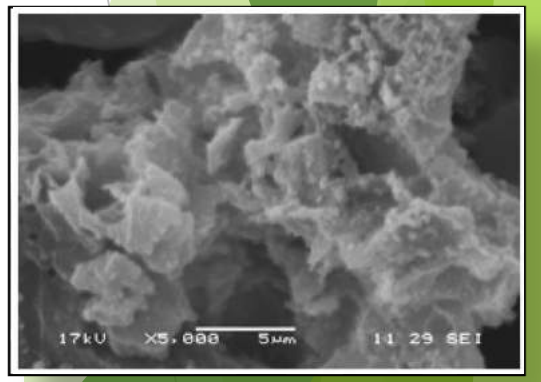
Long term stability in continuous-flow reactions

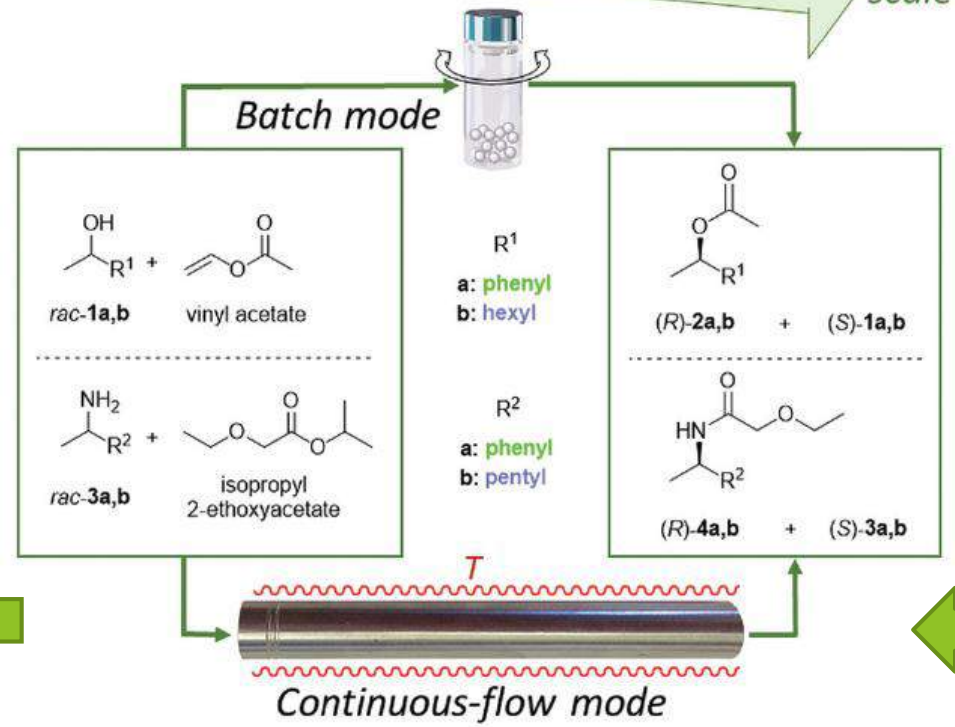


Lab scale → Pilot scale



Precursor optimization

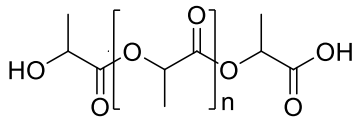


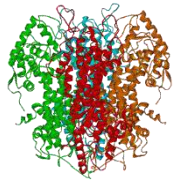


Entrapment of RxPAL by electrospinning

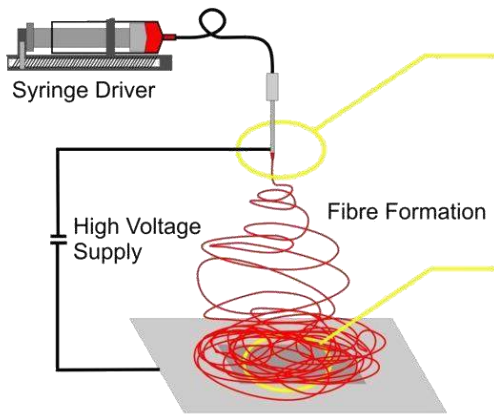
Electrospinning as a potent tool for biocatalyst **entrapment**

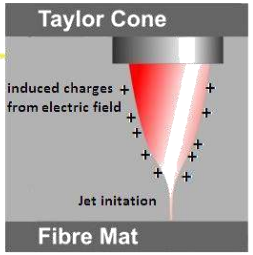
Polylactic acid (PLA) solution in organic solvent

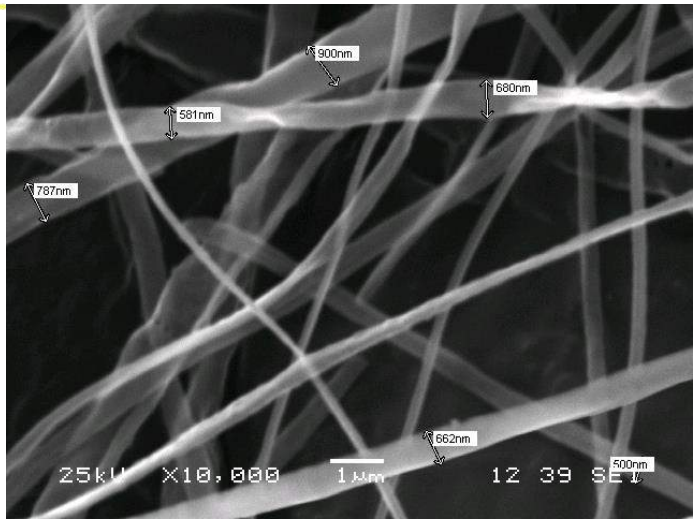




*Rx*PAL in buffer





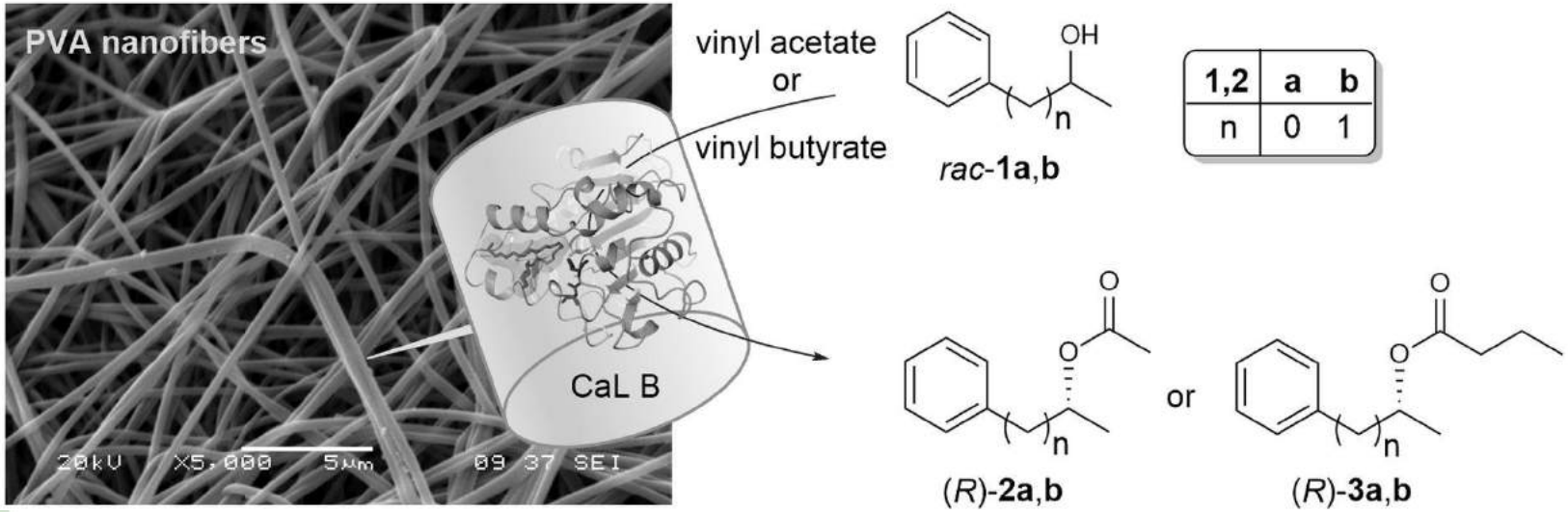


Electrospun PLA nanofibres with entrapped ***Rx*PAL** were unsoluble in aqueous media and active in the **L-Phe elimination reaction** (reaction at RT, full conversion to (*E*)-cinnamate).

Balogh-Weiser, D. et al, *unpublished results*, **2018**.

Entrapment of CaLB by electrospinning

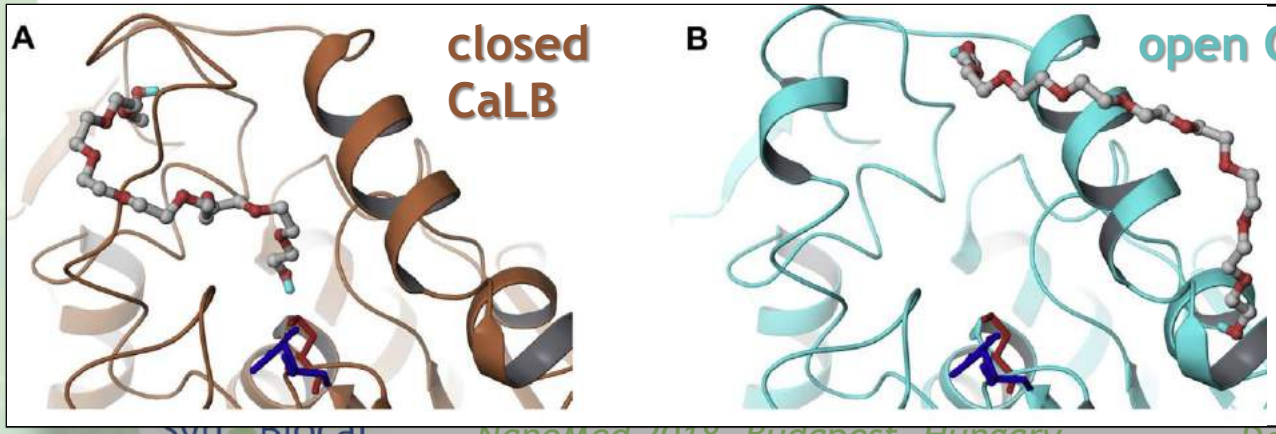
Electrospinning: entrapment matrix for bioimprinting



Minor amounts of well selected additives caused significant activity enhancement of the entrapped lipase by three factors:

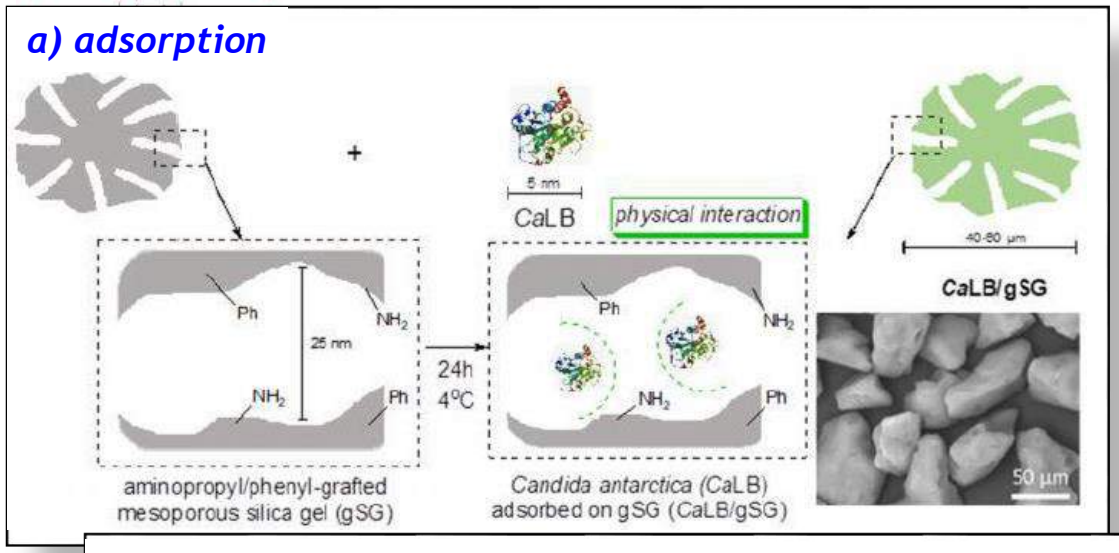
- by stabilizing the active conformation of the lipases;
- by providing extra room for the mobile active site-covering lids after their removal, thus enhancing the turnover frequency of the reaction;
- by slackening the polymeric chain structure of the entrapment matrix, thus decreasing mass transfer limitations.

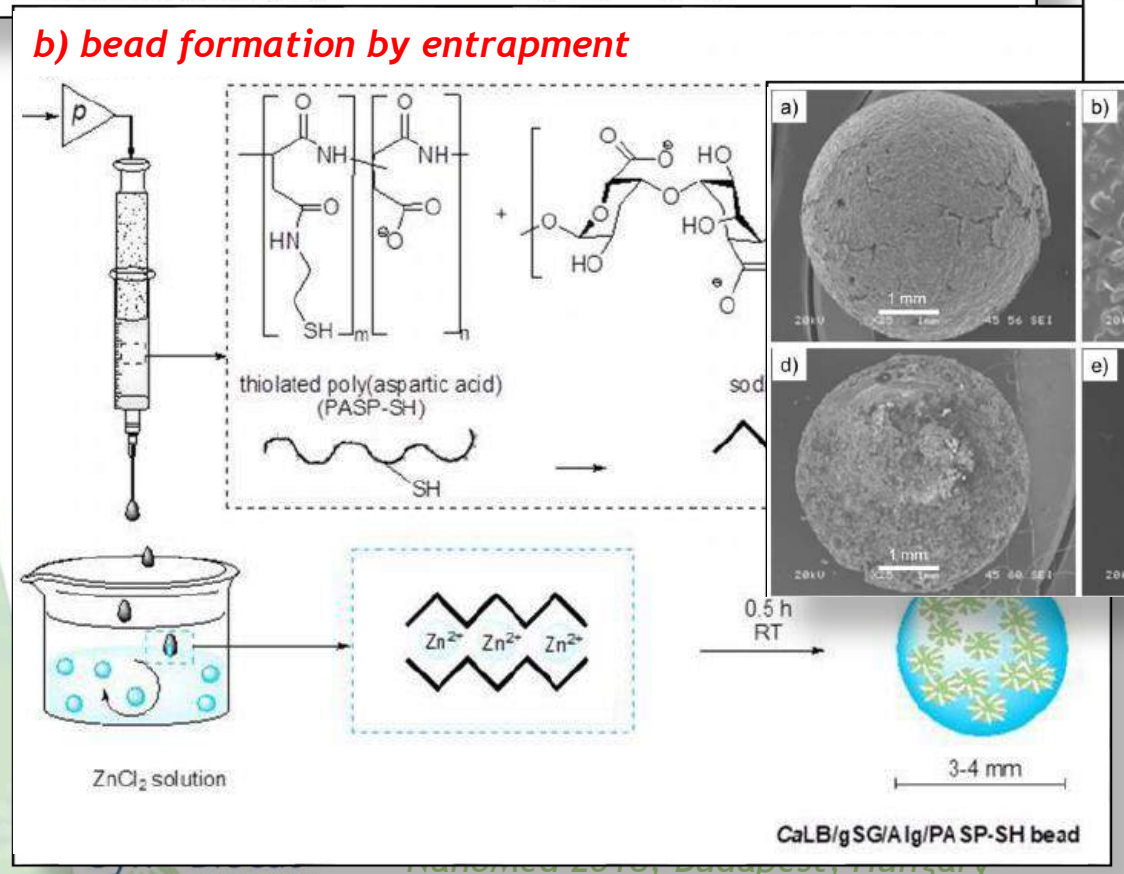
Weiser, D. et al, *Tetrahedron*, **2017**, *72*, 7335-7342.

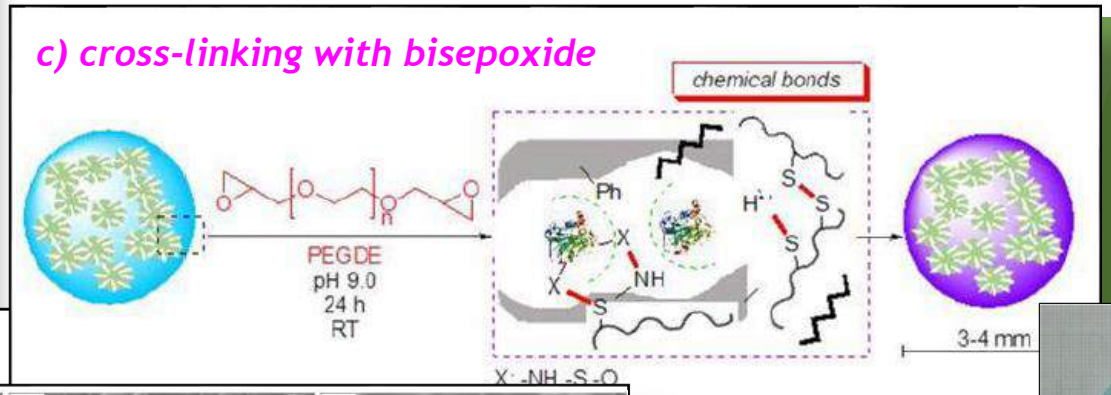


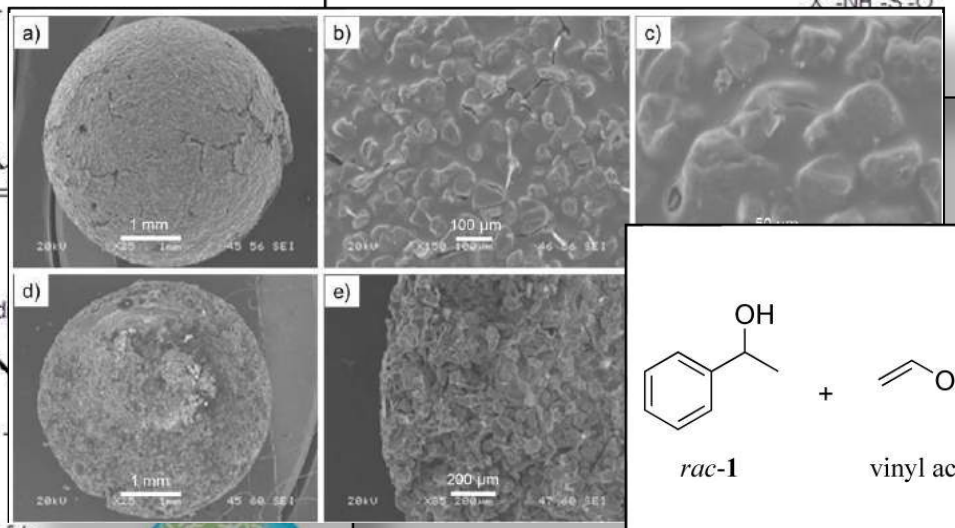
We could show that in case of **PEG 400** being the most effective additive for lipase immobilization by entrapment in PVA, all the above mentioned three factors play a synergistic role.

Combined enzyme immobilization methods

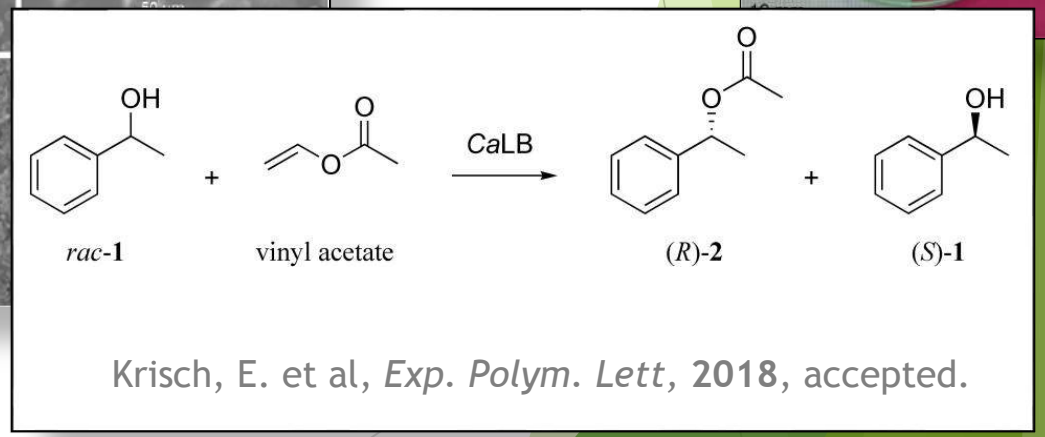










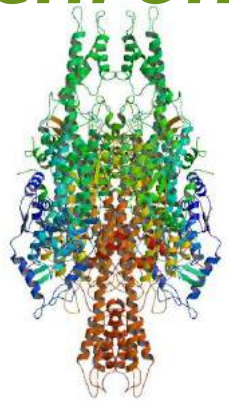


Krisch, E. et al, *Exp. Polym. Lett*, **2018**, accepted.

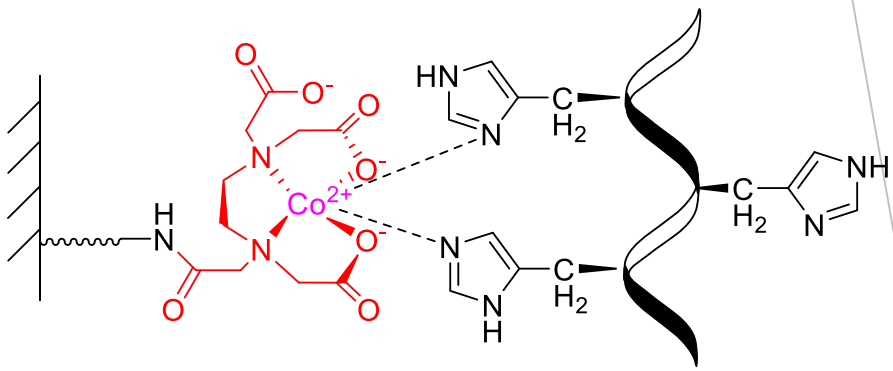
IMAC (immobilised metal ion affinity chromatography)

Petroselinum crispum
parsley
Phenylalanine ammonia-liase

PcPAL







6 solid carriers

Material	Type
PMMA polymer	Resindion EA 403/S Resindion HA 403/S ChiralVision T2-150
silica	Materium Innovations MAT 540 Geduran 60
magnetic nanoparticle	Own prepared

IMAC

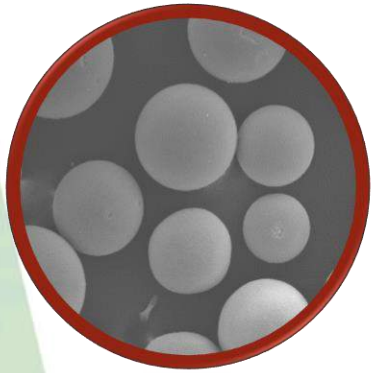
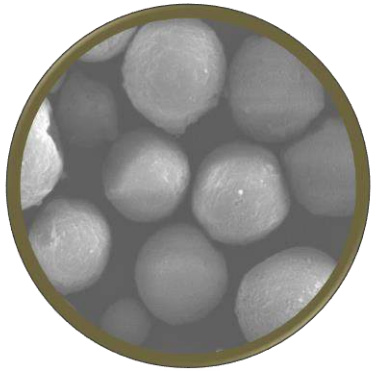
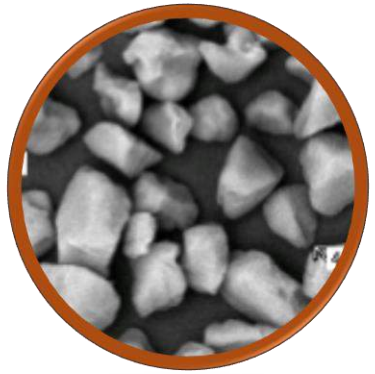
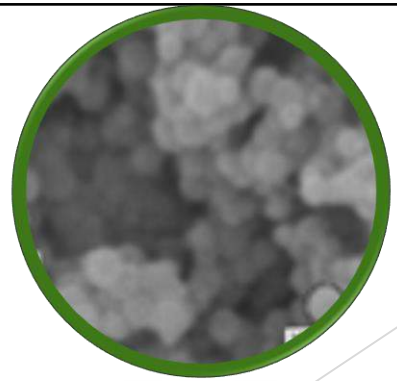
- Protein purification
- Solid Carrier + Tag on protein
- Useful for immobilization → Biocatalyst
- Benefit: regeneration

PAL enzyme

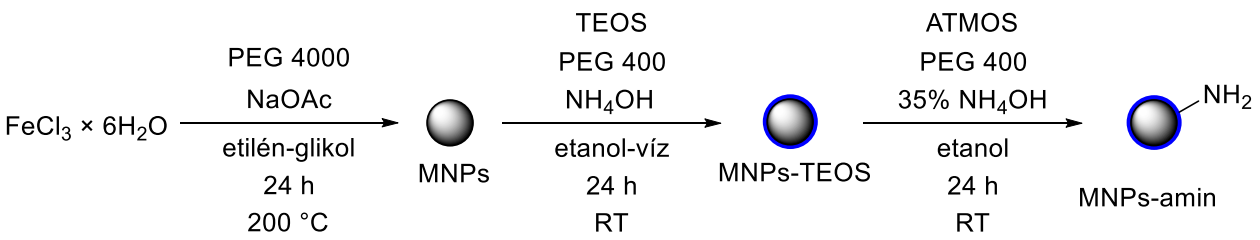
- PKU diagnostics + treatment
- L-Phe gyártás

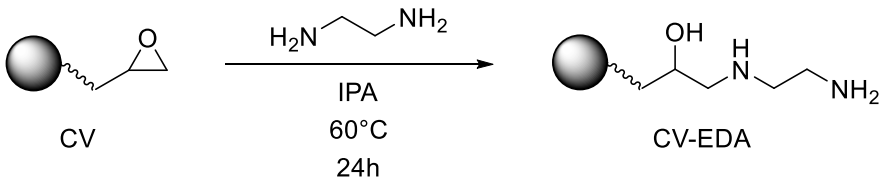
Carriers applied

Material	Trade mark	Size (OD, pore)	Shape
PMMA polymer	Resindion EA 403/S	100-300 µm, 40-60 nm	spherical
	Resindion HA 403/S	100-300 µm, 40-60 nm	spherical
	ChiralVision T2-150	250-500 µm, 200 nm	spherical
silica	Materium Innovations MAT 540	10 µm, 15-30 nm	Hollow globe
	Geduran 60	40-60 µm, 25 nm	irregular
magnetic nanoparticle	In house	300 nm, -	spherical

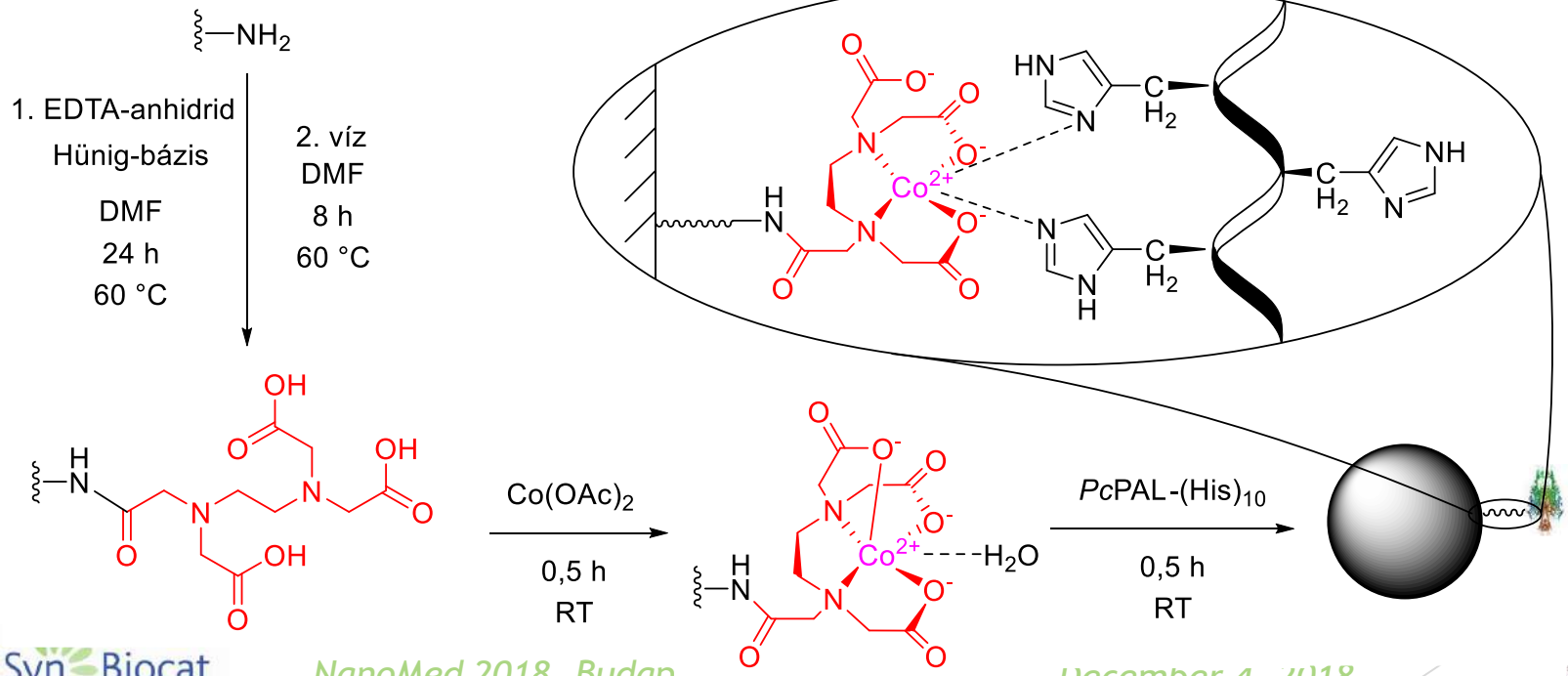





Surface modification, chelation





- TEOS: tetraethoxysilane
- ATMOS: (3-aminopropyl)trimethoxysilane



Results

- Catalytic activity
- Thermal stability and residual activity
- Recyclability

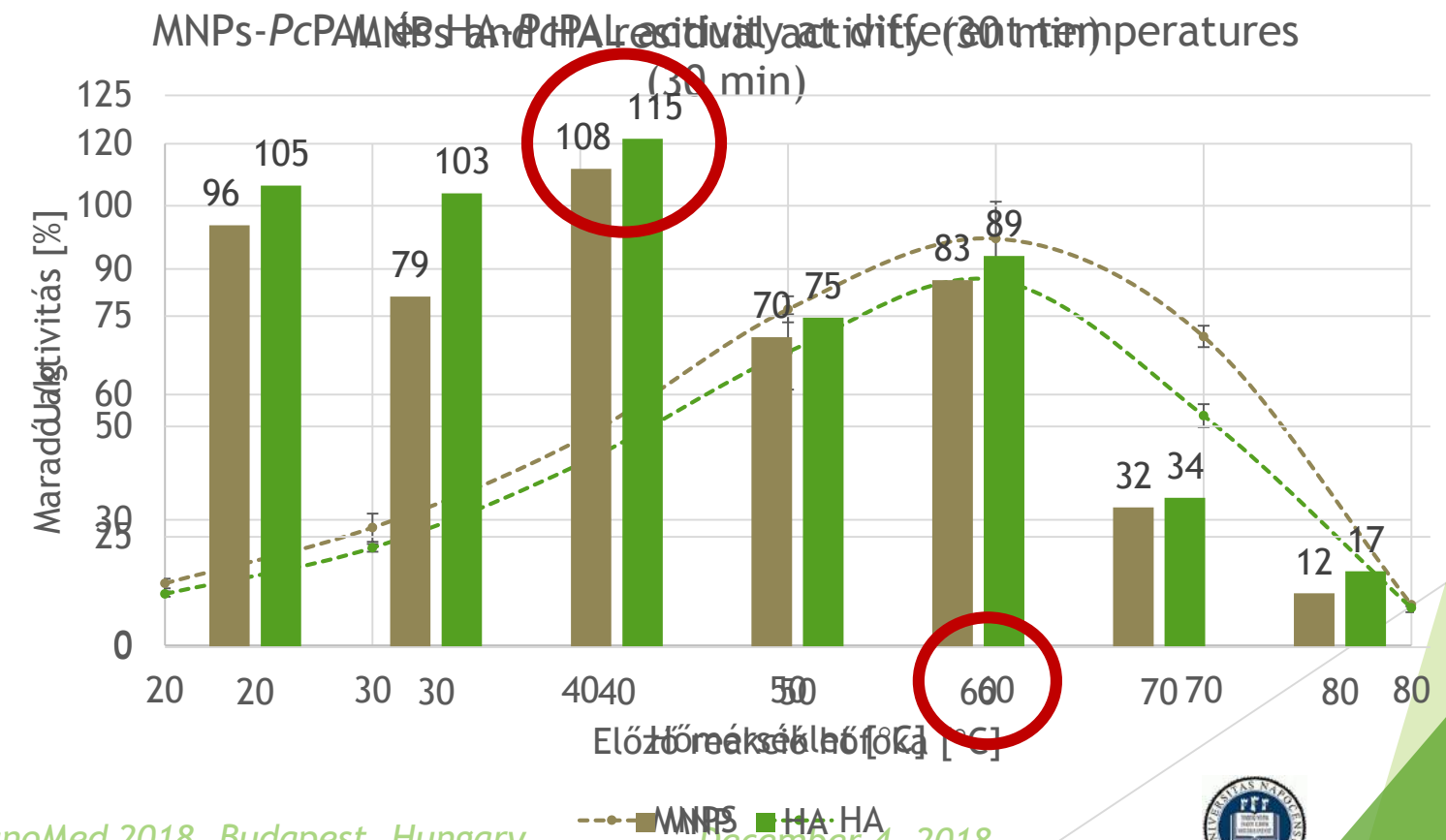
$$U/g = \frac{\mu mol}{min \cdot g}$$

	MNPs		HA (Polymer)	
	Activity [U/g]	SD [%]	Activity [U/g]	SD [%]
1	27,1	6,6	26,3	4,1
2	25,6	2,2	24,2	3,6
3	29,8	10,6	25,7	8,4
Mean	27,5	6,5	25,4	5,3

Results

- Catalytic activityitás
- Thermal stability and residual activity
- Recyclability

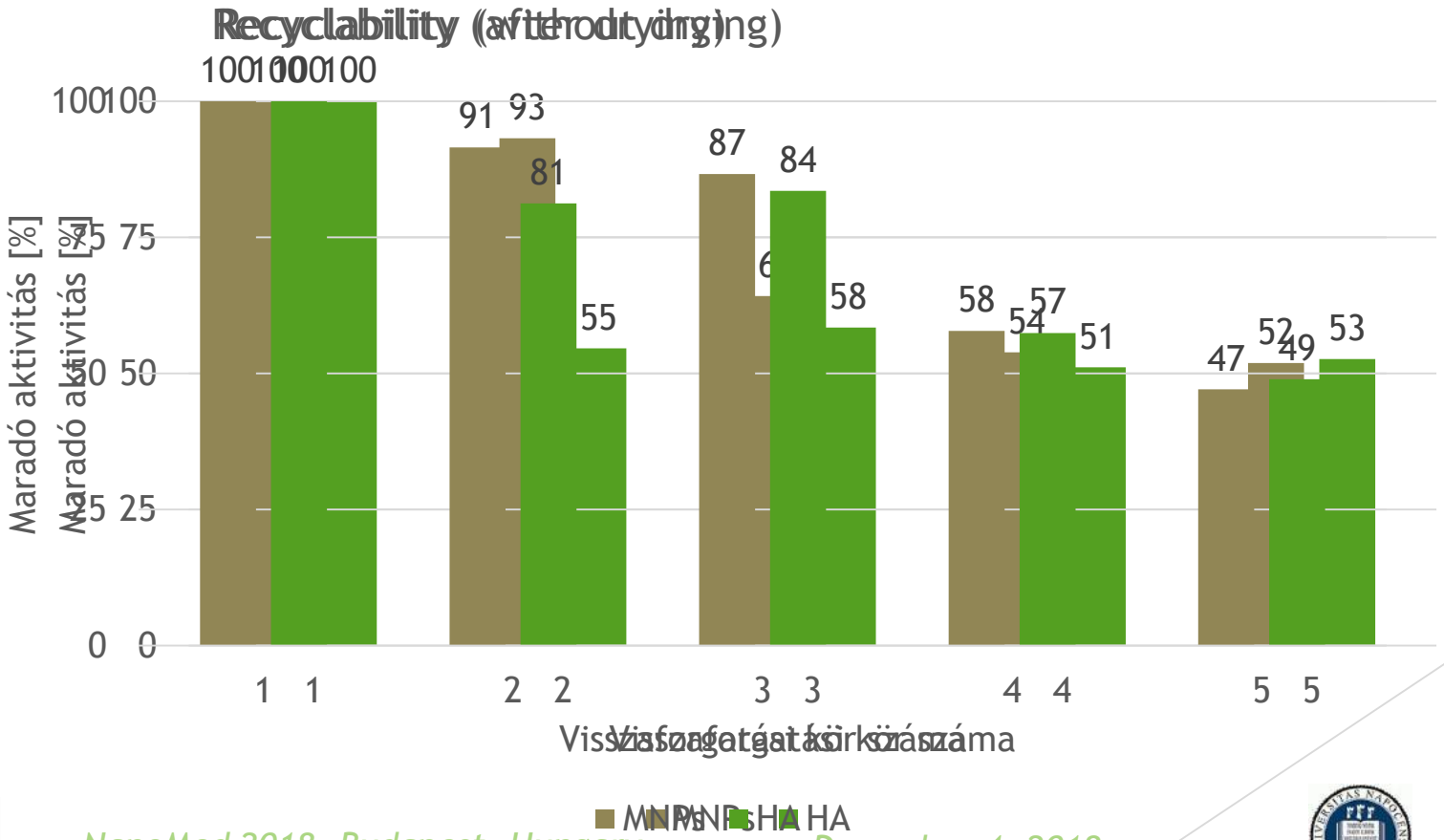
$$U/g = \frac{\mu mol}{min \cdot g}$$



Results

- Catalytic activity
- Thermal stability and residual activity
- Recyclability

SDS PAGE –
No protein leaching



Mode of enzyme immobilization

Conclusions

No single method can be regarded as the universal mode of immobilization for all applications or all enzymes. Consider,

- widely different chemical characteristics of enzymes
- different properties of substrates and products
- range of potential processes employed

Characteristic	*On carrier* *Phys. adsorption*	*Ionic binding*	*Chelation*	*Covalent binding*	*Cross-linking*	*Entrapment*
Preparation	Simple	Simple	Simple	Simple to Difficult	Intermediate	Intermediate/ Difficult
Binding force	Weak	Intermediate	Intermediate	Strong	Strong	Intermediate
Enzyme activity	High/ Intermediate	High	High	High/ Intermediate	Low	Low
Regeneration of carrier	Possible	Possible	Possible	Rare	Impossible	Impossible
Cost of immobilization	Low	Low	Intermediate	High/ Intermediate	Intermediate	Intermediate
General applicability	Yes	Yes	Yes	No	No	Yes
Mechanical stability	High/ Intermediate	High/ Intermediate	High/ Intermediate	High/ Intermediate	Low	High
Thermostability	Low	Low	Intermediate	High/ Intermediate	High	High
Protection from microbial attack	No	No	No	Possible	Possible	Yes

Thank you for your attention





FIKP-2018 BIO / Hungary

NEMSyB, ID P37_273, Cod MySMIS 103413 / Romania