

**UNIVERSIDADE FEDERAL DO RIO DE JANEIRO**

**EQB738 - ENGENHARIA ENZIMÁTICA**

# **Imobilização de Enzimas**

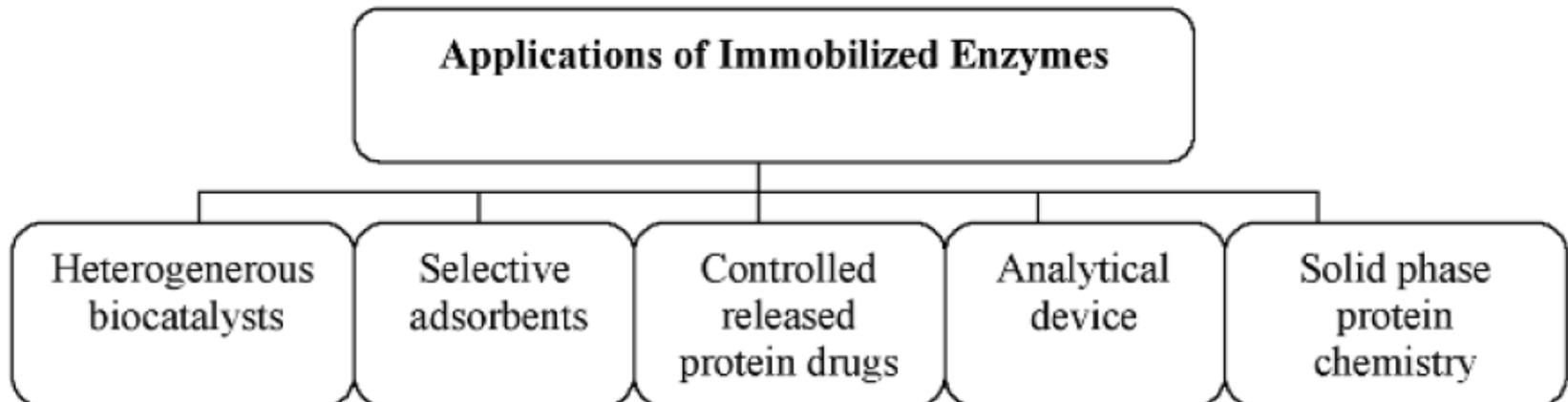
**Prof Bernardo Dias**

# Introdução

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## *Enzima imobilizada*

***Aquela física ou quimicamente associada a um suporte ou matriz, usualmente sólida, insolúvel em água e inerte, com retenção de sua atividade catalítica, e permitindo seu reuso de forma contínua.***



# Introdução

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<b>Vantagens</b>	<b>Desvantagens</b>
<b>Desenvolvimento de sistemas contínuos</b>	<b>Custo adicional de suportes, reagentes e da operação de imobilização</b>
<b>Maior estabilidade enzimática</b>	<b>Perdas de atividade durante a imobilização</b>
<b>Uso mais eficiente do catalisador através de reutilizações</b>	<b>Possíveis exigências adicionais de purificação do catalisador</b>
<b>Flexibilidade no projeto de reatores</b>	<b>Técnica pouco adequada a substratos insolúveis ou de alto peso molecular</b>
<b>Efluentes livres de catalisadores</b>	<b>Maiores riscos de contaminação na operação contínua dos reatores</b>
<b>Maior versatilidade na etapa de separação</b>	<b>Restrições difusionais e impedimento estéreo</b>
<b>Menor custo de mão de obra</b>	
<b>Facilidade de Automação e controle</b>	
<b>Possibilidade de utilização das enzimas “<i>in-natura</i>”</b>	

# Histórico

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**1916 → 1ª utilização de enzima imobilizada (*invertase* em carvão vegetal e alumina) por Nelson e Griffin.**

**1960 → Katchalski introduziu os primeiros suportes úteis.**

**1978 → Utilização industrial: *aminoacilase* imobilizada em DEAE–Sephadex para produção L-aminoácidos.**

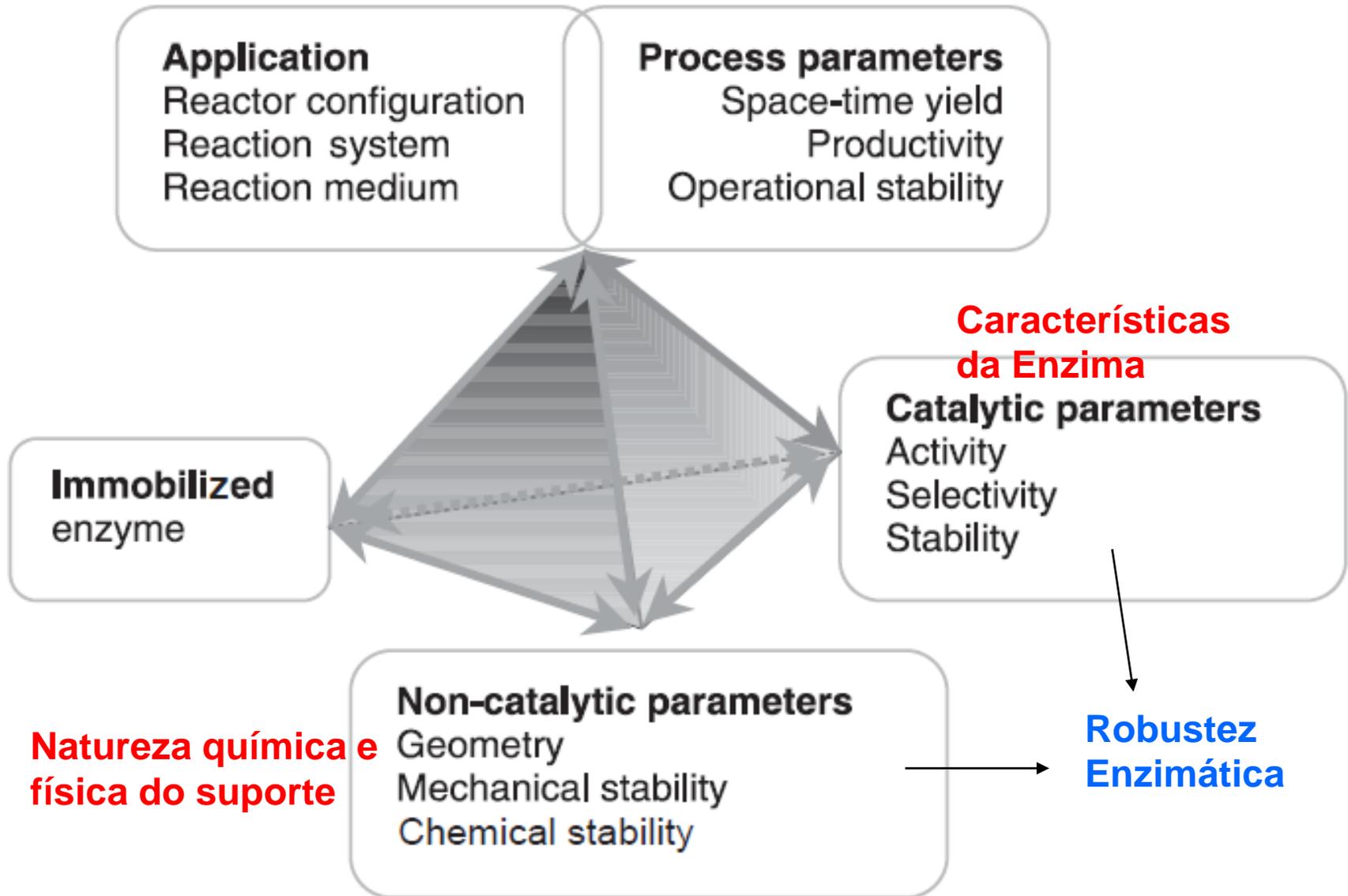
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**Table 2** Large scale industrial processes utilizing immobilized biocatalysts

Enzyme	Form <sup>a</sup>	Process	Product scale (ton per year)
Glucose isomerase	CWC, IME, CIE	High fructose corn syrup from corn syrup	10 <sup>7</sup>
Nitrile hydratase	CWC	Acrylamide from acrylonitrile	10 <sup>5</sup>
Lipase	IME	Transesterification of food oils	10 <sup>5</sup>
Lactase	IME	Lactose hydrolysis, GOS synthesis	10 <sup>5</sup>
Lipase	IME	Biodiesel from triglycerides	10 <sup>4</sup>
Penicillin G acylase	CIE	Antibiotic modification	10 <sup>4</sup>
Aspartase	CWC, IME	L-Aspartic acid from Fumaric acid	10 <sup>4</sup>
Thermolysin	IME	Aspartame synthesis	10 <sup>4</sup>
Lipase	IME, CIE	Chiral resolution of alcohols and amines	10 <sup>3</sup>

<sup>a</sup> CWC = cross-linked whole cell; IME = immobilized enzyme; CIE = covalently immobilized enzyme.

# Critérios para imobilização



# **Critérios para imobilização**

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- **Ausência de reagentes tóxicos durante e após a imobilização da enzima**
- **Uso de suporte muito estáveis**
- **Possibilidade de associar imobilização com a melhoria de propriedades funcionais (Atividade, Estabilidade e Especificidade)**
- **Obtenção de biocatalisadores que possam ser usados em diferentes reações, diferentes sistemas reacionais e reatores**
- **Simplicidade do processo**
- **Custo**
- **Métodos de imobilização**

# Exemplos

**Table 8** Immobilized commercial lipases and their characteristics (reprinted from ref. 229 with permission from the American Chemical Society)

Brand	Lipase species	Carrier	Specificity	Water content (wt%)	Porosity
Lipozyme TL IM	<i>Thermomyces lanuginosus</i>	Silica granules	<i>sn</i> -1,3-Specific	6.0	0.77
TL-lab-immobilized	<i>Thermomyces lanuginosus</i>	Accurel EP 100	<i>sn</i> -1,3-Specific	5.8	
Lipozyme RM IM	<i>Rhizomucor miehei</i>	Macroporous resin	<i>sn</i> -1,3-Specific	3.2	0.45
Novozym 435	<i>Candida antarctica</i> lipase B	Macroporous polymer based on methyl and butyl methacrylic esters	Non-specific	4.1	0.65
Lipase PS-C-I	<i>Burkholderia cepacia</i>	Ceramic particles	Non-specific <sup>a</sup>	4.3	
Lipase PS-D-I	<i>Burkholderia cepacia</i>	Diatomaceous earth	Non-specific <sup>a</sup>	3.7	

<sup>a</sup> No clear claim.

# Exemplos

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## Atividade da esterase de *Bacillus subtilis* imobilizada em vários suportes e por diferentes métodos

SUPORTE	MÉTODO	POROS (°A)	UI/g
Acetato de celulose	Microencapsulamento	0,02	45
Poliacrilamida	Microencapsulamento	2,5	50
Pérolas de vidro	Ligação covalente	0,2-1,0	200
DEAE-Sephadex A25	Adsorção	0,1	650
Dowex 1X1	Adsorção	0,4	820
DEAE- Celulose	Adsorção	0,06	4200

# Suportes de Imobilização



## Tipos

	Orgânicos	Inorgânicos
Naturais	Polissacarídeos: celulose, dextrana (Sephadex), agarose (Sepharose), alginato, quitina; Proteínas: albumina, colágeno; Carvão	Bentonita, Celite, sílica (Aerolyst)
Sintéticos ou Processados	Poliestireno, poliamidas, polipropileno (Accurel), polímeros vinílicos e acrílicos (Sepabeads, Eupergit)	Metais, óxidos, vidros



Vidro



sílica

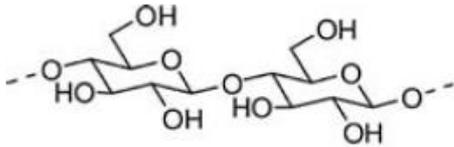


tela de nylon



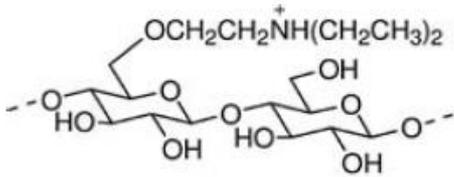
Alumina

# Suportes de Imobilização

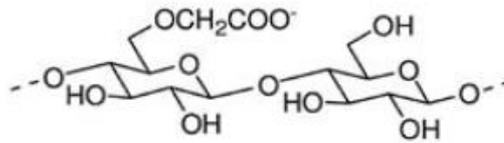


Cellulose

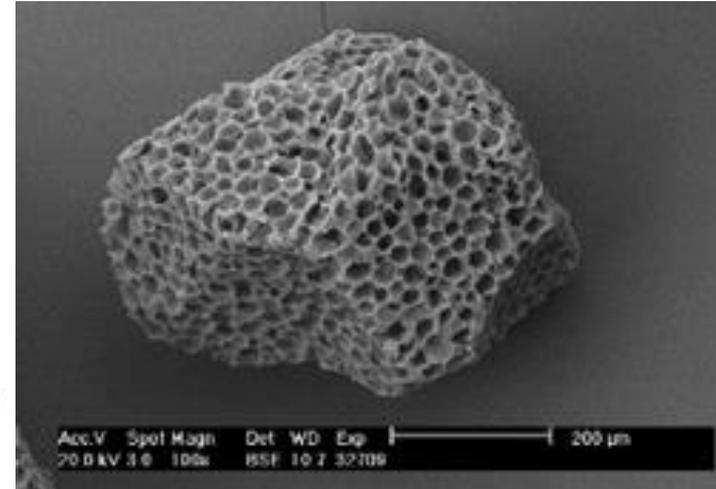
DEAE (diethylaminoethyl)  
CM (carboxymethyl)



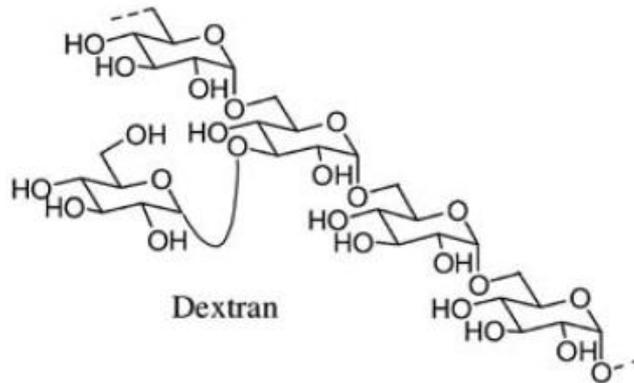
DEAE-cellulose



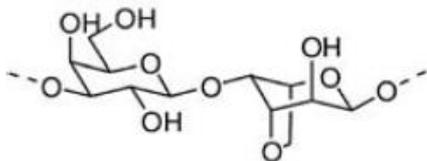
CM-cellulose



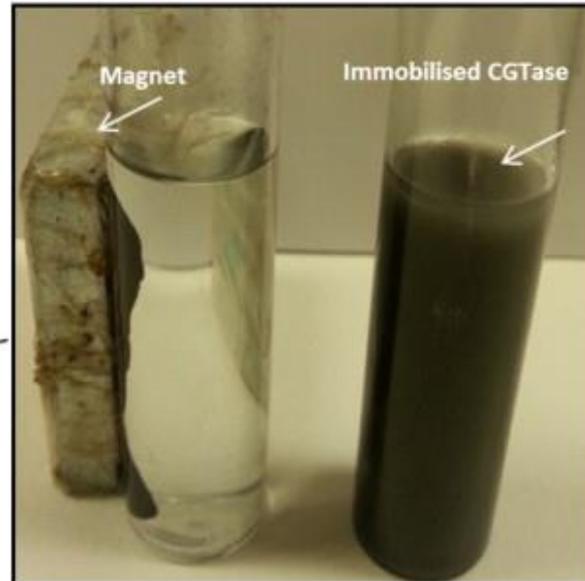
Accurel® MP1000 - microporoso



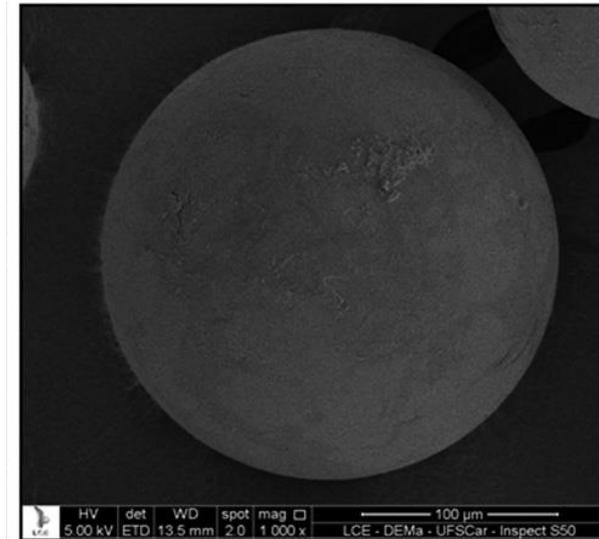
Dextran



Agarose



Nanopartículas magnéticas



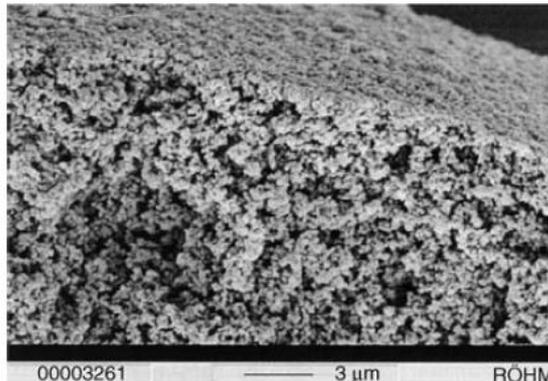
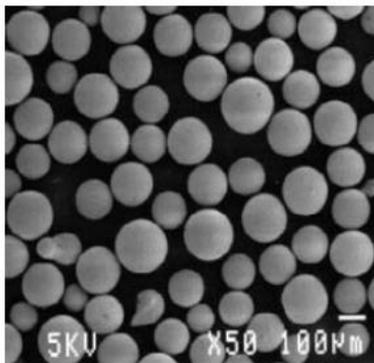
Sepabeads

# Suportes de Imobilização

- Alguns suportes necessitam de mudanças ou inclusão de grupos reativos – **ativação do suporte**.

## Classificação dos suportes utilizados na Imobilização de Enzimas

TIPO DE SUPORTE	MATERIAIS	POROSIDADE	ESTABILIDADE
Não poroso	Vidro, sílica, aço	-	Alta
Microencapsulado	Triacetato de celulose	35 °A	Moderada
Entrelaçado	Poliacrilamida, PVP	Variada	Baixa
Macroporoso	Alumina, sílica	200-1000 °A	alta



Synthetic polymer carrier; macroporous particle structure. Scanning electron microscopy (SEM) pictures of Eupergit<sup>®</sup> C 250L.

# Suportes de Imobilização

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## Geometria

Subclass	Remark
Bead	Beads with defined size and various functional groups and internal structures are widely used as adsorbents for various separation procedures/enzyme immobilization
Film	Often, the films used for enzyme immobilization are supported. In other words, the films are often a polymer matrix coating upon which the enzyme can be immobilized via normal enzyme immobilization techniques
Membrane	Which can combine the catalytic action and separation in one embodiment
Irregular	Not frequently used
Fibrous	Fibre-based carriers have the advantages of high volume activity, presumably because of the high surface available for the enzyme immobilization
Foam	Mainly used for enzyme entrapment. In special cases pre-designed foam can be used for enzyme adsorption

# Suportes de Imobilização

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## Estrutura

Microporous	Microporous carriers are carriers which generally have pores in the range 0.1–10 $\mu\text{m}$
Mesoporous	Mesoporous materials have pore size in the range 3–10 nm. The pore sizes are, therefore, usually in the same range as the enzymes to be immobilized
Macroporous	Macroporous carriers refer to the materials with permanent pores in the range 8–1000 nm and specific areas in the range of 25–100 $\text{m}^2 \text{g}^{-1}$
Non-porous	Less diffusion limitation might be expected, but enzyme loading might be lower because the available specific surface area is 2–3 order of magnitude less than for porous carriers
Gel	In a strict sense gel materials are generally regarded as non-porous materials, because they do not have permanent pores and the pores only exist when they swell in a solvent
Tentacle carrier	Preparation of tentacle carriers can be realized by grafting, coating, and covalent attachment

# Enzima-Suporte: Critérios

Properties	Desired	
<p>Chemical</p> <ul style="list-style-type: none"> <li>• Hydrophilicity/hydrophobicity</li> <li>• Swelling properties</li> <li>• Chemical stability</li> <li>• Microbial stability</li> </ul> <p>Morphological</p> <ul style="list-style-type: none"> <li>• Particle diameter, particle distribution</li> <li>• Pore size<sup>a)</sup></li> <li>• (Inner) surface for adsorption/binding</li> </ul> <p>Mechanical</p> <ul style="list-style-type: none"> <li>• Resistance to pressure/compressibility</li> <li>• Elasticity</li> </ul> <p>General</p>	<p>Low</p> <p>High Inerte, sem promover desnaturação enzimática</p> <p>Good</p> <p>0.2–1 mm/narrow distribution</p> <p>30–60 nm</p> <p>Large</p> <p>Good/low</p> <p>Sufficient (no abrasion by stirrer)</p> <p>Food grade (for application in food manufacture)</p> <p>Low cost</p> <p><i>Estabilidade de fixação</i> → a enzima deve estar bem fixa ao suporte.</p>	
	<p>Lower limit</p> <p>Upper limit</p>	
Pore diameter	20–40 nm	80–160 nm
Inner surface (corresponding)	250–125 m <sup>2</sup> g <sup>-1</sup>	45–25 m <sup>2</sup> g <sup>-1</sup>

a) Pore diameter and corresponding inner surface (example: porous glass):  
 Trypsin with a molar mass of 23 800 Da requires a surface of about 15 nm<sup>2</sup>.

# Suportes de Imobilização

**Capacidade de carga** → o suporte deve fixar um número elevado de unidades enzimáticas por unidade de área - um pequeno reator uma grande capacidade catalítica.

Morfologia do suporte	Porosa	Não Porosa
Área superficial por unidade de massa	Elevada	Reduzida
Restrições difusionais	Muito significativas	Pouco significativas
Carga máxima do biocatalisador	Elevada	Reduzida
Proteção do biocatalisador frente a ambiente hostil	Razoável	Reduzida
Compatibilidade com substratos macromoleculares	Reduzida	Elevada
Custo	Elevado (poro controlado) Reduzido (poro não controlado)	Reduzido

# Suportes de Imobilização

## Exemplo comercial

### Sephadex G-25 Medium, 100 g

BioProcess resin	Yes
Matrix	Cross-linked dextran
Particle size distribution, wet	38–235 $\mu\text{m}$
Partical Size Dry	>50 $\mu\text{m}$
pH stability, operational	2–13 <sup>1)</sup>
pH stability, CIP	2–13 <sup>2)</sup>
Pressure/Flow Specification	>100 cm/h, pressure drop cm H <sub>2</sub> O/bed height=2, bed height 30 cm, 2.6 cm i.d.
Fractionation range [Mr], Globular proteins	$1 \times 10^3$ – $5 \times 10^3$
Fractionation [Mr] Dextrans	$1 \times 10^2$ – $1 \times 10^2$
Exclusion Limit [Mr] [Globular Proteins]	$> 5 \times 10^3$
Exclusion Limit	$M_r$ 5000
Storage	4 to 30°C, dry resins. Used resins 4 to 8 °C in 20% Ethanol or 0.01 M NaOH
Storage Conditions	10 ROOM TEMPERATURE
Chemical Stability	Stable in commonly used buffers, 0.2 M NaOH, 0.2 M HCl, 1 M acetic acid, 8 M urea, 6 M guanidine HCl, 1 % SDS, 24 % ethanol, 30 % porpanol, 30 % acetonitrile
Certificate of Analysis	Yes

<sup>1)</sup>pH range where resin can be operated without significant change in function.

<sup>2)</sup>pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.

# Suportes de Imobilização

*Exemplo comercial*

## SEPABEADS™ EC-EP Porous hydrophilic enzyme carriers



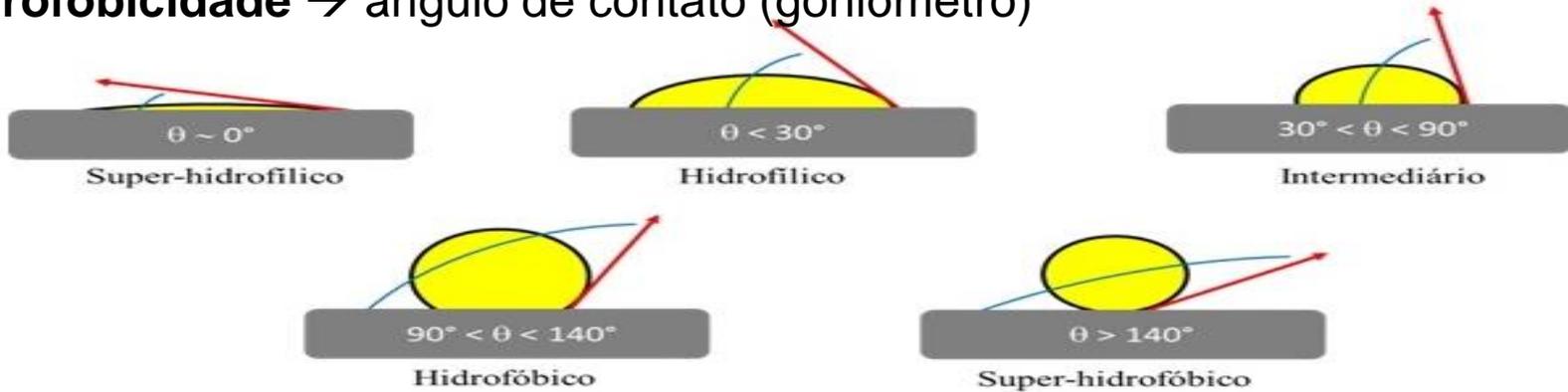
### Product Features

Matrix	polymethacrylate
Appearance	perfect spherical white opaque beads
Functional group	epoxide
Oxirane content	min. 100 $\mu\text{mol/g}$ wet
Median pore diameter	10 - 20 nm
True density	> 1.1 g/ml
Water retention	55 - 65 %
Temperature stability range	0 - 10 °C
pH stability range	5 - 8
Recommended storage temperature	4 - 6 °C
Storage time limit	6 months

<b>Available grades</b>	<b>S</b>	<b>M</b>
<b>Particle size range</b>	<b>100 - 300 <math>\mu\text{m}</math></b>	<b>200 - 500 <math>\mu\text{m}</math></b>

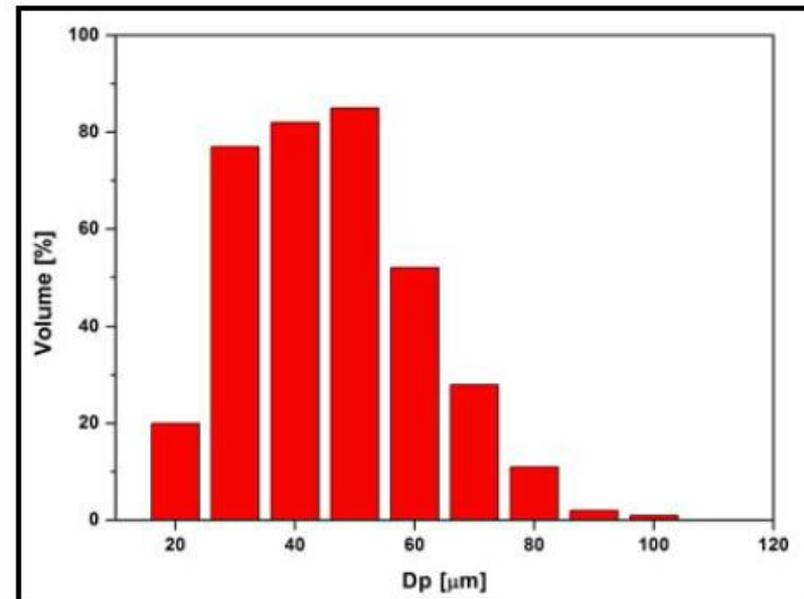
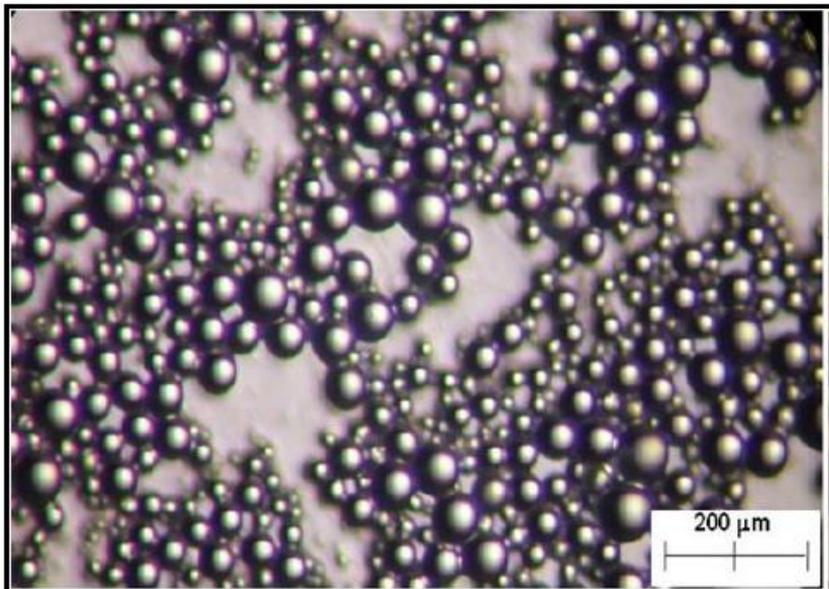
# Avaliação dos Suportes

Hidrofbicidade → ângulo de contato (goniômetro)



Morfologia das partículas → microscopia optica e eletronica de varredura

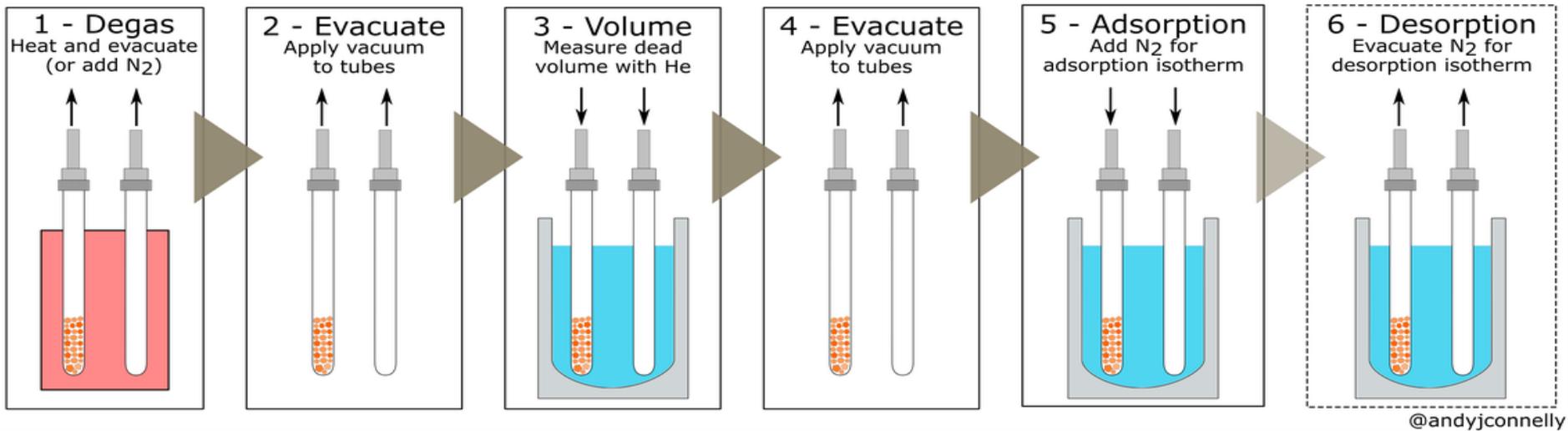
Diametro e distribuição de partículas → Espalhamento de luz (Malvern)



# Avaliação dos Suportes

Tamanho de poro e área superficial → isothermas de adsorção (BET)

## Measuring surface area with BET

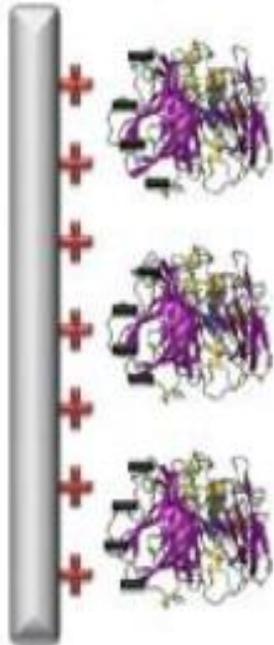


Measurement	Calculation methods	Notes
Surface area	BET, Langmuir, Temkin, Freundlich	Can be calculated from section of isotherm (generally $P/P_0=0.05-0.35$ )
Total Pore Volume	Kelvin equation	Generally carried out at $P/P_0 = 0.99 - 0.998$ although theoretically all pores should be full at $P/P_0=0.995$
Mesopore volume, area, and distribution	BJH, Dollimore-Heal	Requires full adsorption and desorption isotherms
Micropore distribution	Dubinin-Radushkevich and Astakhov, Horvath-Kawazoe, Saito-Foley, Cheng-Yang, MP method	Requires full adsorption isotherm
Pore size modeling	Density Functional Theory	Requires full adsorption isotherm
Surface energy	Density Functional Theory	Requires full adsorption isotherm

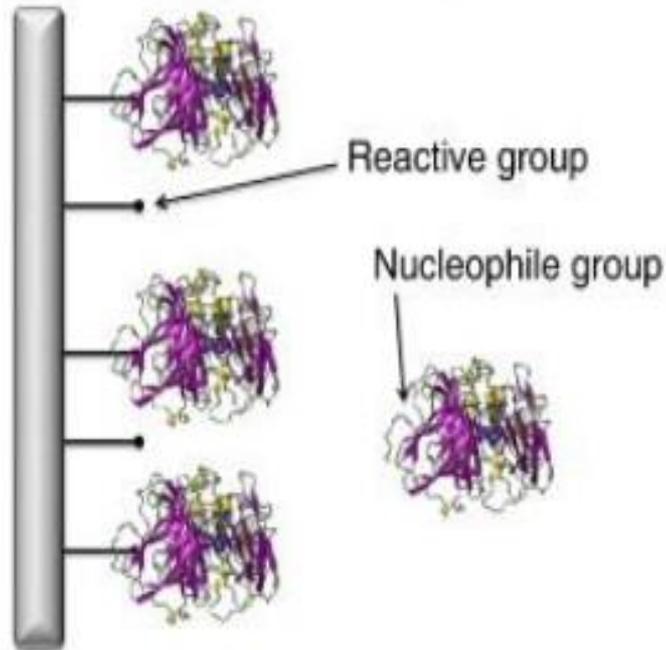
# Métodos de Imobilização

- Adsorção/Ligação covalente/CLAE:

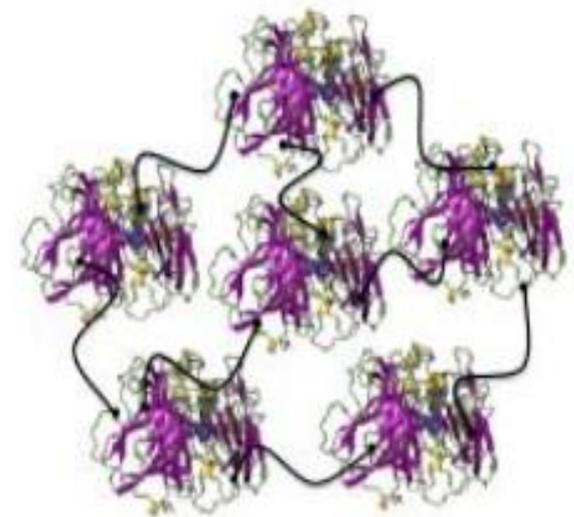
a) Adsorption



b) Covalent binding



c) CLAE



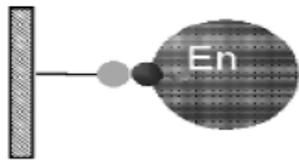
# Métodos de Imobilização

## Adsorção

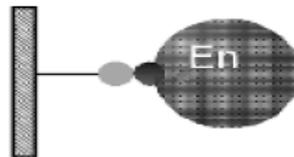
### Vantagens:

- \* Reversibilidade → purificação de proteínas e reuso de suporte;
- \* Simplicidade → processo em condições suaves;
- \* Possível alta retenção de atividade, por não ter modificação química.

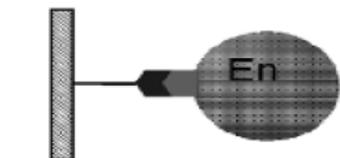
**Desvantagem:** tendência a dessorção frente a alterações no pH e altas forças iônicas → fraca interação entre enzima e suporte



Ionic adsorption (C-(+)---(-)-En



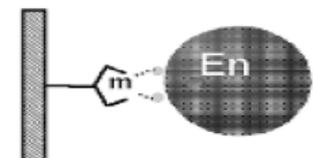
Ionic adsorption (C-(-)---(+)-En



Biospecific



Non-specific



Coordination

# Métodos de Imobilização

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## Tipos de Adsorção

- **Física não-específica** → ocorre via forças de Van der Waals, ligações hidrogênio e interação hidrofílica;

Durante o processo de imobilização por adsorção, o microambiente da enzima se altera, ocorrendo dessolvatação de água, e assim, uma mudança na sua conformação → altera propriedades catalíticas

Para evitar este problema:

- **Monocamadas:** as enzimas tendem a maximizar o contato com o suporte com baixas cargas enzimáticas, formam uma camada de 2 a 3 mg proteína por  $m^2$  → varia com a enzima e o suporte.

# Métodos de Imobilização

Table 2.2 Adsorption of enzyme via non-specific interaction

Polymer	Nature of adsorption	Comments	Enzyme
PET	Hydrophobic	Retention of activity was higher than when covalently immobilized on CNBr-activated Sepharose	Pectin esterases
NaY Zeolite/Accurel PA6	Hydrophobic	The payload is 0.025; activity retention is 85%. IME is more stable than on Accurel PA6 (no activity loss was observed after 45 days). Enzyme is desorbed more easily from Accurel PA6 than from NaY zeolite	Cutinase
Controlled-pore glass (CPG)/ A microporous ceramic	Hydrophilic	Better than conventional silica glass carriers, with regard to lower leakage of enzyme; the carrier is more stable than CPG	$\beta$ -Glucosidase
MCM-41 (4 nm)	Hydrophilic	Enzyme loading is inversely proportional to the size of the enzyme molecules. The payload for all the enzymes tested was below 0.01	Cytochrome C Papain Trypsin
Mesoporous silica matrixes	Adsorption	Enzyme properties depend on template content and on sol-gel matrix pore dimensions	HRP
Cross-linked starch entrapped in alginate	Adsorption	Fewer mass-transfer constraints than for beads with un-cross-linked starch	$\alpha$ -Amylase
ZrO <sub>2</sub> -coated glass carriers	Coordination	A metal whose oxide is more water-durable than is the carrier	Glucose isomerase and glucoamylase
Pumice-metal (TiCl <sub>4</sub> ) complex (coating)	Coordination	50.7% of the original enzyme activity was obtained	$\alpha$ -Amylase

\* Payload is defined as ratio of the mass of the immobilized enzyme to mass of carrier used

# Métodos de Imobilização

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## Tipos de Adsorção

- **Interação eletrostática ou iônica** → interação entre grupos carregados positivos e/ou negativos do suporte com os grupos amino da lisina e carboxila dos ácidos glutâmico e aspártico das enzimas;

### Suportes:

- \* Sintéticos como gels copolímeros de acrilamida/ácido maleico ou itaconico, e outros polímeros preparados por condensação de fenol e formaldeído. Ex.: Duolite.
- Derivados de polímeros sintéticos, como poly(styrene-co-divinylbenzene) resultando em resinas Dowex (Dowex SBR-P, Amberlite IRA-904, Amberlite IRC 50, Amberlite CG-50, Diaion CR-20); ou de poliacrilamida (Duolite A-7, Duolite S-761, porous resin Duolite ES 762);
- Derivados de cross-linked polysaccharides como DEAE-cellulose, QAE-cellulose, SP-cellulose, DEAE-Sephadex, QAE-Sephadex, CM-Sephadex, DEAE-dextran, CH-Sepharose 4B, AH-Sepharose 4B, Q-Sepharose, S-Sepharose, CM-Sepharose.

This ionic binding functionality can be prepared in situ during polymerization or post-synthesis by derivatizing ready-made polymers such as functionalized poly(styrene-divinylbenzene), e.g. Dowex, and cross-linked polysaccharide carriers.

# Métodos de Imobilização

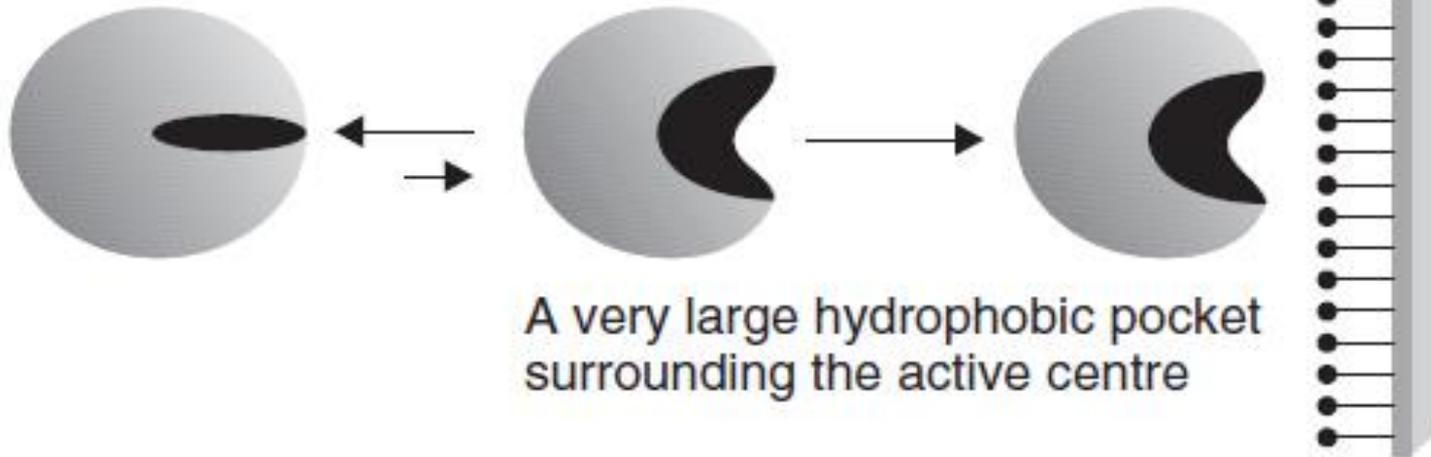
## Tipos de Adsorção

- **Interação hidrofóbica** → interação entre regiões hidrofóbicas de suporte e enzima.

Polymeric carrier	Hydrophobic tail	Comments	Enzyme
Sepharose gels	Butyl, hexyl, and octyl	Nearly completely adsorbed by the last two gels. Inhibitor and activator both influenced the adsorption behaviour of the enzymes, because of the conformational change	<i>Escherichia coli</i> phosphoenolpyruvate carboxylase
Sepharose 4B	<i>N</i> -Butylamine (A-C <sub>4</sub> ) or <i>n</i> -octylamine (A-C <sub>8</sub> )	Acidic enzymes can be virtually irreversibly immobilized on the carrier with A-C8	Xanthine oxidase, LDH DNase I, AKP, and urease
<i>N</i> -alkyl or aryl amino-agar beads	<i>n</i> -Alkyl or aryl amino	The payload is 0.020–0.035 with 80% retention of activity and increased thermostability	MPS
Triton X-100-substituted Sepharose 4B	Triton X-100	Specific interactions involved the hydrophobic region of Triton X-100 and apolar regions on the protein surface	BSA, Hb, GLD, Cyt C, pepsin
Palmityl-substituted Sepharose 4B	Palmityl	Immobilization conditions for individual enzymes are determined by the properties of the enzyme itself	Lysozyme, trypsin, α-CT, etc.
Remazol blue (RB)-cellulose beads	Remazol blue (RB)	Instead of the expected bio-specificity in the interaction of LDH with RB-cellulose beads, the predominant interaction is hydrophobic	LDH
Cellulose esters of both alkyl and aryl carboxylic acids	Alkyl and aryl carboxylic acids	The bound enzymes retained their catalytic activity almost completely	Ten of the enzymes tested
Adipic acid dihydrazido-Sepharose 4B	Sialic acids and sialyl-glycoconjugates	Immobilization is via its CO <sub>2</sub> H group, C-7 to C-9 side chain, or its NH <sub>2</sub> function as <i>d</i> -neuraminic acid-β-me glycoside or 2-deoxy-2,3-didehydro-neuraminic acid; hydrophobic interaction is involved	<i>Clostridium perfringens</i> sialidase
Different esters of cross-linked poly(vinyl alcohol) (PVA)	Alkyl carboxylic acids	Lipase CRL showed greater affinity for carbon chain length ranging from 8 to 12. The result was better than with Celite 545	Lipase of <i>Candida rugosa</i>
Phenylbutylamine-Eupergit/Eupergit 250L	Phenylbutylamine	The pore size of the support had a strong effect on the activity but did not influence stability	<i>Staphylococcus carnosus</i> lipase

## Purification and hyperactivation of lipases

Interfacial activation of lipases on artificial hydrophobic surfaces at low ionic strength



A very large hydrophobic pocket surrounding the active centre



The majority of proteins only have a few small hydrophobic pockets on their surface. They only adsorb on hydrophobic supports at very high ionic strength.

**Figure 54.6.** Interaction of soluble lipases on hydrophobic surfaces resembling drops of lipids.

## Hyperactivation of microbial lipases by adsorption on octyl-agarose

Microbial source	Hyperactivation
<i>C. antarctica</i> - fracción B	200
<i>T. lanuginosa</i>	2000
<i>M. javanicus</i>	300
<i>R. miehei</i>	700
<i>P. fluorescens</i>	150
<i>R. niveus</i>	600

Activity of derivatives is compared with soluble enzymes and it is measured in a fully aqueous medium, at pH 7.0 and 25°C, by using p-nitrophenil propionate as substrate.

**Figure 54.7.** Hyperactivation (regarding to soluble enzymes) of some microbial lipases after adsorption on hydrophobic supports.

# Métodos de Imobilização

## Tipos de Adsorção

- **Bio-específica** → utiliza anticorpos, substratos análogos e/ou inibidores como ligantes para adsorção. Ex.: concanavalina A (lectina)

Polymeric carrier	Ligand/binding site	Comments	Enzyme
Triazine bead celluloses MT-100	Concanavalin A/ mannan	The specific activity and storage and operational stability of the penicillin G acylase immobilized on Con A-cellulose is even better than that covalently immobilized on Eupergit C	Synthetic glycolated penicillin G acylase
Con A-Sepharose	Con A/sugar moiety	Compared with the native enzyme, there is no significant change in the enzyme kinetics, suggesting that the active site is far from the binding site	Laccase ( <i>p</i> -diphenol: oxygen oxidoreductase)
Chitosan	Tannin	Highest retention of activity, reduced pH optimum, and enhanced optimum temperature	<i>Aspergillus niger</i> NRC 107 xylanase and ( <i>R</i> )-xylosidase
Sepharose	Anti-CHT-IgG I	Biospecific immobilization offered great advantages over the covalent immobilized counterpart, because of the oriented immobilization	CHT
Cellulose beads Sepharose	CCBMPB Monoclonal anti- $\beta$ -galactosidase	90.12% retention of activity; reversibility of the binding > lowering pH The antibody immobilized rates of adsorption and desorption of the enzyme from the immobilized antibody and the specific activity of immunosorbents were affected	HRP $\beta$ -Galactosidase
Sepharose	Monoclonal antibody pH 8	The IME retained kinetic properties similar to those of the native enzyme and responded to activators and inhibitor in the same way as the native enzyme	Tryptophan hydroxylase
Porous glass	Biotin	Neither refolding of the fusion protein nor interaction of the streptavidin domain with immobilized biotin altered the structure of the substrate binding site	Streptavidin- $\beta$ -galactosidase

CCBMPB: catechol[2-(diethylamino)carbonyl-4-bromomethyl phenylboronate

# Métodos de Imobilização

## Tipos de Adsorção

- **Bio-específica** → utiliza anticorpos, substratos análogos e/ou inibidores como ligantes para adsorção. Ex.: concanavalina A (lectina)

Polymeric carrier	Ligand/binding site	Comments	Enzyme
Poly(2-HEMA-co-EDMA)	Anti-chymotrypsin antibodies	Immunsorbent adsorbed 166.7 $\mu\text{g}$ chymotrypsin $\text{g}^{-1}$ dry carrier with 100% retention of activity	Chymotrypsin
Sub-micron ferrite	Soybean trypsin inhibitor	Selective recovery of trypsin was obtained by adsorption with immobilized inhibitor	Trypsin
Sepharose	N6-(6-aminoethyl)-5'-AMP	Linear temperature gradients were successfully employed for elution of enzymes from affinity adsorbents	Glycerokinase and yeast alcohol dehydrogenase
Amine-derivatized agarose	Triazine scaffold bis-substituted with 5-aminoindan	The ligand, a triazine scaffold bis-substituted with 5-aminoindan, which mimics natural protein-carbohydrate interactions, can adsorb glycosylated enzyme	Glucose oxidase
Agarose-AA	Aminoethyl	Glycine enzyme can be desorbed with 0.5 M NaCl and 0.4 M phosphate or 0.14 M HAD; enhanced enzyme activity was obtained	Prephenate dehydratase
CNBr-activated-Sepharose 4B	Aminolevulinate	Ionic strength influenced the binding behaviour	Bovine liver I dehydratase
Sepharose	Divinylsulphone-activated methicillin	Carrier bearing substrate analogues were used to demonstrate reversible adsorption of enzyme	Penicillinase
Affinity-ligand-modified liposomes	<i>p</i> -Aminobenz-amidine (PAB) as the affinity ligand for trypsin	Recovery yield from the crude mixture was 68%; trypsin purity was 98%	Trypsin
Sepharose	Histamine/carboxyl-histidyl	The dissociation constant depends on the nature of the ligands, suggesting that the nature of interaction involved is different	Catechol-2,3-dioxygenase

# Métodos de Imobilização

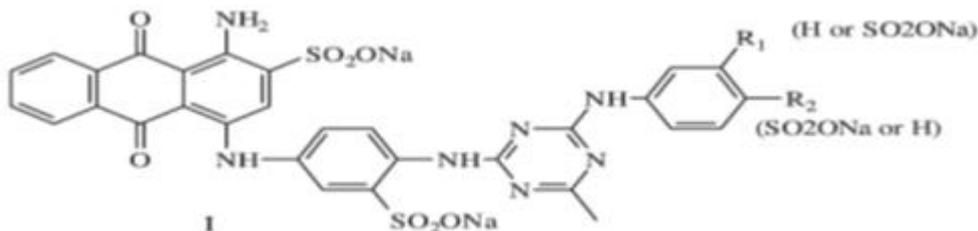
## Tipos de Adsorção

- **Afinidade** → utiliza corantes imobilizados; Ex.: *p*-(*N*-acetyl-L-tyrosine azo) benzamidoethyl-CL-Sepharose 4B para lactase

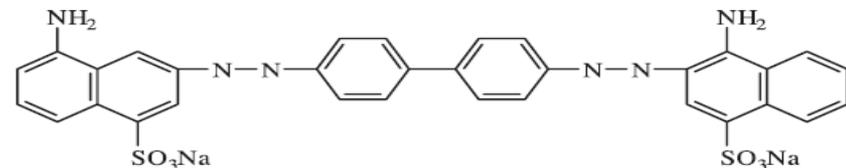
Exemplos de corantes triazinicos:, Procion red HE-3B, Pricon green, Pricon yellow, Pricon brown, Congo red.

It is worth mentioning that some transition metal ions, for example as Fe(III), Ni(II), and Cu(II) can be chelated to the immobilized dyes, leading to the formation of new adsorbents which have affinity behaviour analogous to that of the mixed ligands. In these cases, the binding capacity of the affinity adsorbent is usually enhanced compared with the immobilized dyes without the transition metal ions.

Cibacron blue F3G-A



Congo red



# Métodos de Imobilização

## Tipos de Adsorção

- **Afinidade** → utiliza corantes imobilizados; Ex.: *p*-(*N*-acetyl-L-tyrosine azo) benzamidoethyl-CL-Sepharose 4B para lactase

Polymeric carrier	Dye (ligand)	Comments	Enzyme
Poly(HEMA-co-MMA)-MS-CB-F3GA/or Cu(II)	I	Payload on poly(HEMA-MMA) microspheres was 0.0036, whereas on Cibacron blue F3G-A or Cu(II) the payload was increased to 0.248 or 0.319, respectively	Lysozyme
(pHEMA)-cb/(pHEMA)-CB-Fe(III)	I	30% increase in enzyme loading was achieved with Fe(III), compared with poly(HEMA)-CB. Slightly higher $K_m$ relative to the native enzyme; increased stability with immobilization, especially in the presence of Fe(III)	Catalase
Procion green H-E4BD-attached pHEMA	VIII	The enzyme could be repeatedly adsorbed and desorbed from the dye-attached pHEMA film without any significant loss in adsorption capacity	Lysozyme
Non-porous monodisperse silicas/triazine dyes	I	Procion red HE3B, Procion red MX5B, and Cibacron blue F3G-A	LDH, and MDH, ARD
Poly(HEMA) membranes/CB-F3G-A	I	The storage stability of the enzyme was found to increase upon immobilization	$\beta$ -Galactosidase
HMF (polyamide)-procion brown Mx-5 BR-Ni(II)	VII	The payload is 0.078, activity retention is 37%, and affinity immobilization enhanced the thermostability 3–10 times, depending on the temperature	Urease
Supported alkanethiol SAM-CB-F3G-A	I	NAD <sup>+</sup> -binding pocket is not involved in the binding of the dyes	LDH
Agarose-Cb	I	Affinity adsorbent	Sulphurtransferase
Activated silica/reactive triazine dyes		LDH, hexokinase, AKP, carboxypeptidase, tryptophanyl-tRNA synthetase	
Cibacron blue F3G-A	I	Procion red H-8BN, Procion yellow H-A and Cibacron blue F3G-A	Carboxypeptidase G2, hexokinase
Poly(HEMA-co-MMA)-Congo red/or CR-Fe <sup>3+</sup>	VI	The payloads are 0.126 and 0.165 for P-CR-GOD and P-CR-Fe <sup>3+</sup> , respectively	GOD

# Métodos de Imobilização

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## Tipos de Adsorção

- **Coordenação** → utiliza metais imobilizados como Cu(II), Ni(II), Co(II), Co(III), Fe(II), Fe(III), Zn(II), Ca(II), Al(III), que se ligam a resíduos de aminoácidos contendo -SH, -COOH, -histidyl, na superfície enzimática; possibilita uma carga enzimática alta; Também pode ser um método de purificação

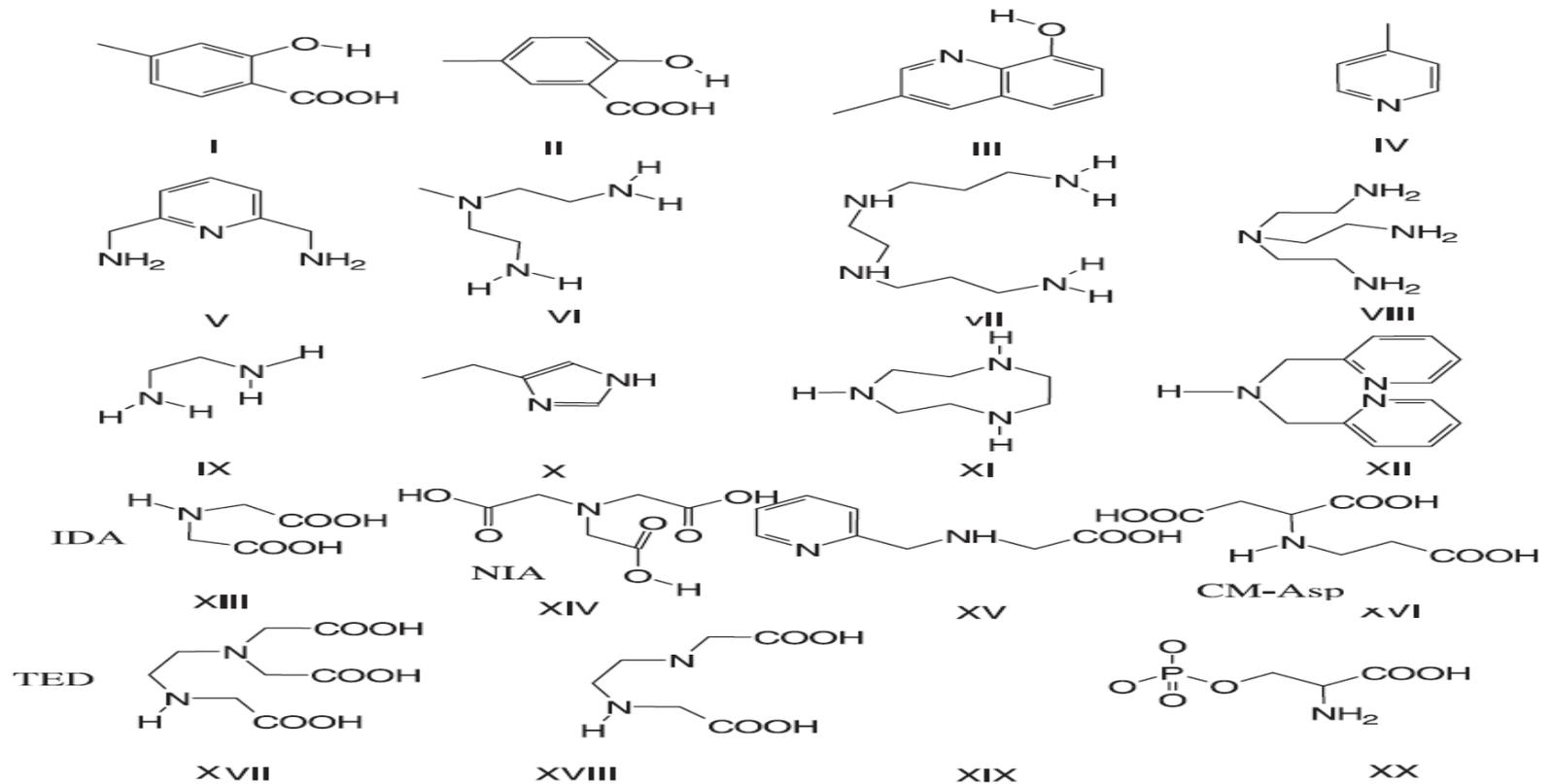
Exemplos de quelatos:

- salicylaldehyde-Cu(II),
- 8-hydroxyquinoline-Al(III)/Fe(II)/Yb(III)/Ca(II),
- iminodiacetic acid (IDA)-Cu(II)/Zn(II)/Ni(II)/Co(II),
- dipicolylamine (DPA)-Zn(II)/Ni(II),
- orthophosphoric (OPS) Fe(III)/Al(III),
- *N*-(2-pyridylmethylaminoacetate)-Cu(II),
- 2,6-diaminomethylpyridine-Cu(II),
- nitrilotriacetic acid (NTA)-Ni(II),
- carboxymethylated aspartic acid (CM-Asp)-Ca(II)/Co(II),
- *N,N,N'*-tris(carboxymethylethylenediamine) (TED)-Cu(II)/Zn(II),
- EDDA (ethylenediamine-*N,N'*-diacetic acid)/Fe(II)/Cu(II)/Ni(II)/Zn(II),
- cystine-Fe(III); tris(2-aminoethyl)amine (TREN)-Cu(II),
- imidazole-Cu(II); 1,4,7-triazacyclononane (TACN)-Cu(II)/Ni(II)/Zn(II).

# Métodos de Imobilização



A=



**B** = Histidine, Cysteine, Aspartic acid, Glutamic acid, Lysine, Arginine, Tryptophan, Tyrosine, Phenylalanine, N-Terminus

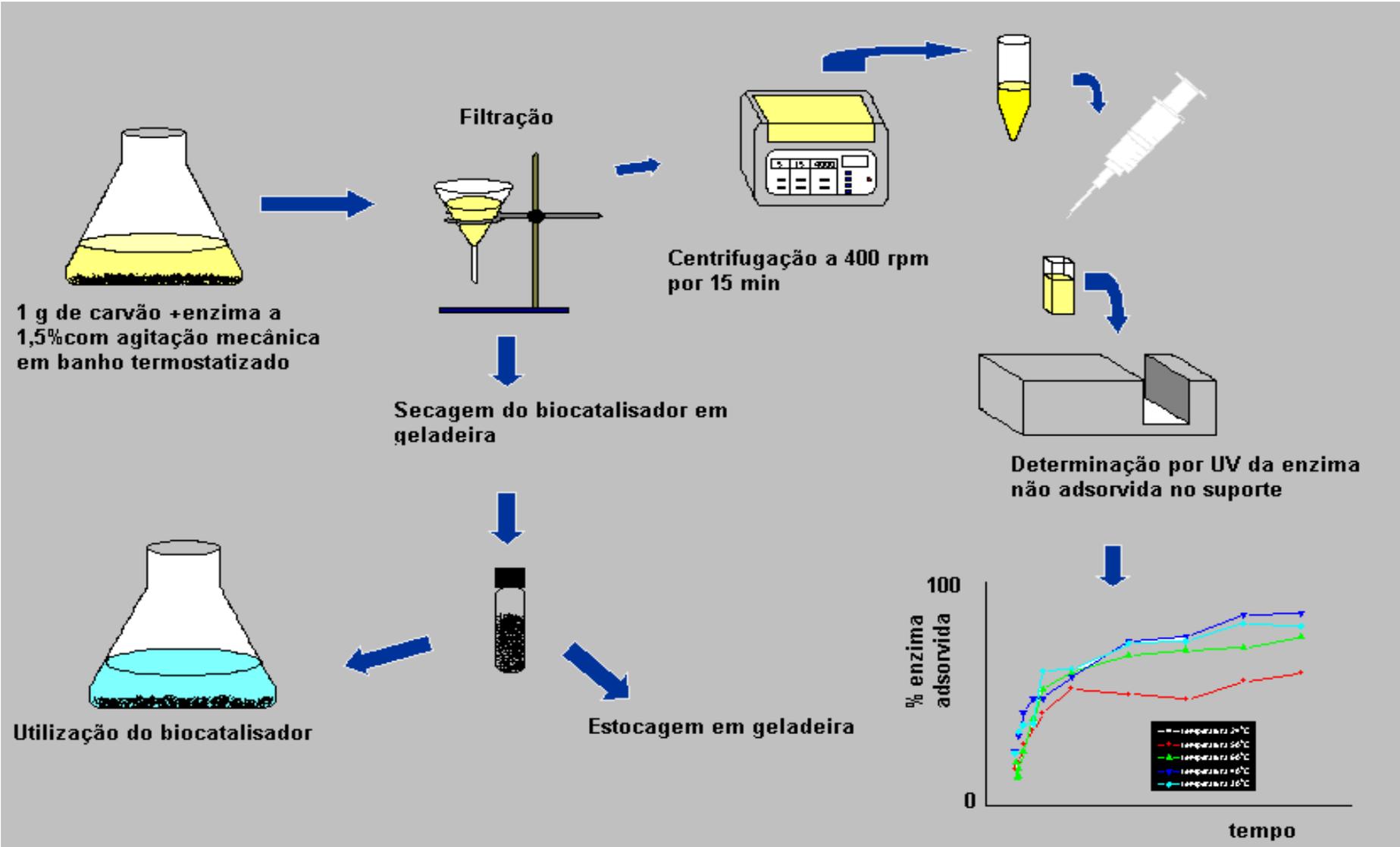
**M** = Cu(II), Ni(II), Co(II), Co(III), Fe(II), Fe(III), Zn(II), Ca(II),

# Métodos de Imobilização

Polymer	Ligand	Comments	Enzyme
Sepharose 4B, silica	IDA/Cu(II)	Regeneration of carrier is possible. Introduction of suitable spacer between the ligand and the carrier is essential for the adsorption	PGA
Sepharose	Activated by Cu <sup>2+</sup> ions	Activity retention is around 80%. The carrier can be repeatedly used without significant reduction of the capacity of adsorption	Papain
A chelating gel	Fe <sup>3+</sup> ions	The capacities were approximately 5 mg protein mL <sup>-1</sup> adsorbent	Phosphorylase and LDH
Agarose resin	Ni <sup>2+</sup>	The IME can withstand repeated buffer changes without substantial activity loss	His6-tags-RNA
Polystyrene supports	EDA/DET/TET chelated with Co(II), Ni(II), Cu(II), Fe(III), or Co(III)	IME has slightly low $K_m$ and $V_{max}$ , reduced by a factor of 2. High thermal stability was obtained by oxidation of Co(II) to Co(III) of IME; high stability was also found for Fe(III) bearing IME	Penicillin amidohydrolase
Polystyrene or silicrome supports	Co(II)-EDA, Cu(II)-EDA	Co(II)-EDA and Cu(II)-EDA-polystyrene and all metal complexes of DEA-polystyrene had the highest activity. Enzyme immobilized on Co(II) and Cu(II) complexes of EDA-polystyrene has the highest activity retention	Penicillin amidohydrolase
Poly(vinyl alcohol)	Glutamic acid resin/Cu(II)	The optimum pH for IME was 5.59 and 6.64, and $K_m$ was $1.70 \times 10^{-2}$ mol L <sup>-1</sup> , which are similar to those for free PPO. The IME can be recycled five times with 20% retention of activity	Polyphenol oxidase
Porous silica matrixes	Alkylamine derivatives of Ti(IV)	The $K_m$ of IME was higher, because of diffusion resistance and the pH optimum was slightly lower than for the free enzyme. The optimum temperature was reduced to 60 °C after immobilization	Glucoamylase
An agarose gel	$\alpha$ -Amino acid residue-Cu(II)	The method is useful not only for immobilization but also for cross-linking of proteins. It is characterized as being site-specific	Salicylaldehyde modified chymotrypsin
Sepharose 4B	Epibromohydrin-IDA/Cu <sup>2+</sup> , Zn <sup>2+</sup>	Sepharose 4B-Epi-IDA-Cu <sup>2+</sup> was the best carrier with regard to activity retention. The technique is advantageous in that costs and disposal problems can be reduced because of carrier re-use	Laccase
Silica	IDA/Cu(II)	Enzyme leakage can be avoided by post-immobilization cross-linking with glutaraldehyde	Poly(his) tagged hydantoinase

EDA, ethylenediamine, DET, diethylenetriamine, or TET, triethylenetetramine, LDH: lactate dehydrogenase

# Imobilização de $\alpha$ -amilase por adsorção física em carvão de casca de coco



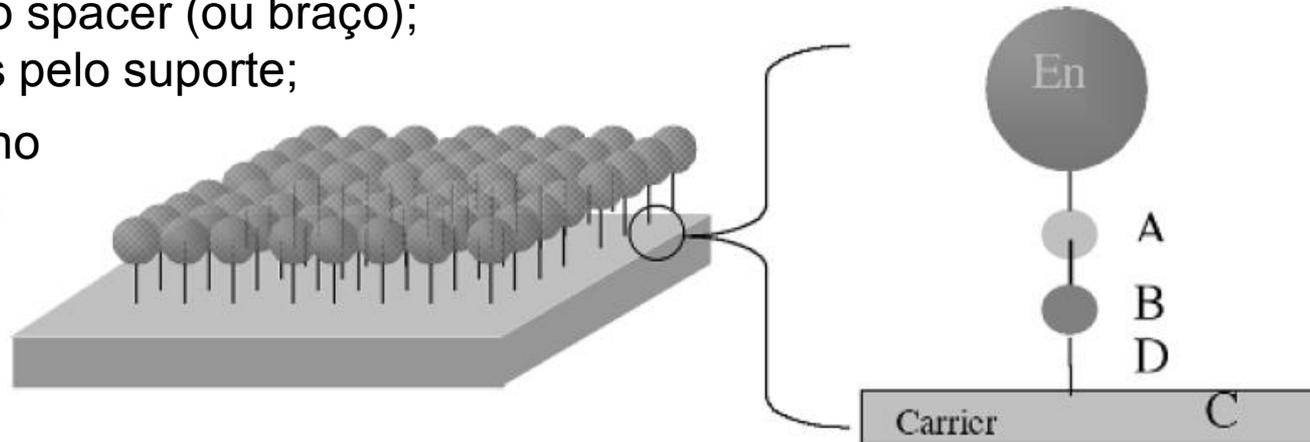
# Métodos de Imobilização

**Ligação Covalente** → ligações fortes e irreversíveis entre enzima e suporte, evitando perdas, fixando a conformação estrutural.

Ocorre pela reação entre resíduos ativos de aminoácidos na superfície enzimática e funcionalidades ativadas no suporte.

Além das propriedades físicas e químicas do suporte, outros fatores podem afetar este método:

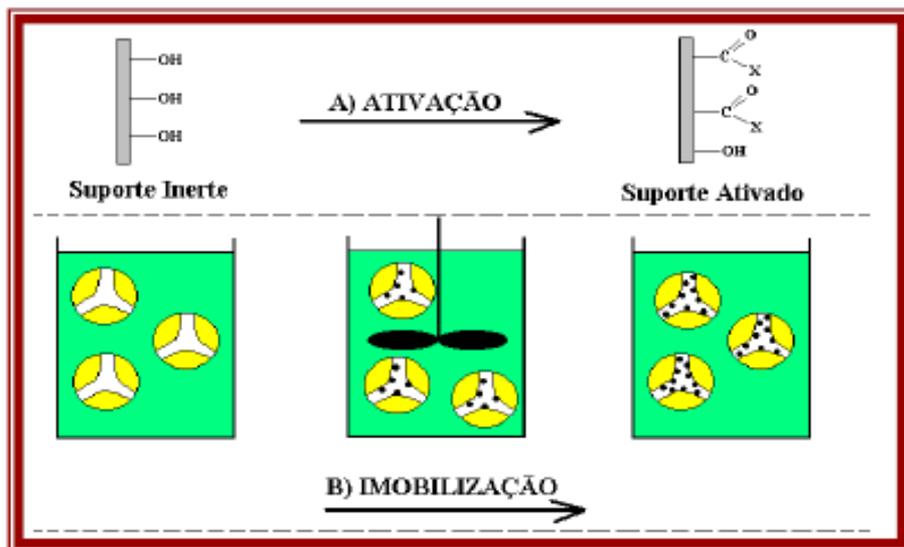
- a natureza da ligação química;
- a conformação e orientação da enzima durante ou depois da imobilização;
- a natureza e tamanho do spacer (ou braço);
- distribuição das enzimas pelo suporte;
- as condições utilizadas no processo de imobilização;
- o número de ligações formadas entre enzima e suporte;



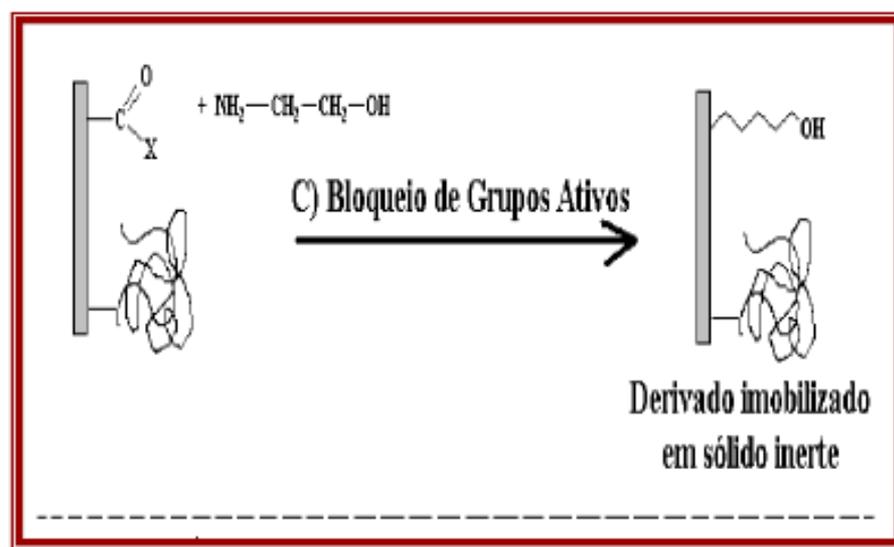
**Scheme 3.1** Covalent immobilization of enzyme on the carrier: (A) active amino acid residue; (B) binding functionality of the carrier; (C) carrier; (D) spacer.

# Etapas da imobilização covalente:

## Ativação/imobilização



## Bloqueio de grupos ativos



# Métodos de Imobilização

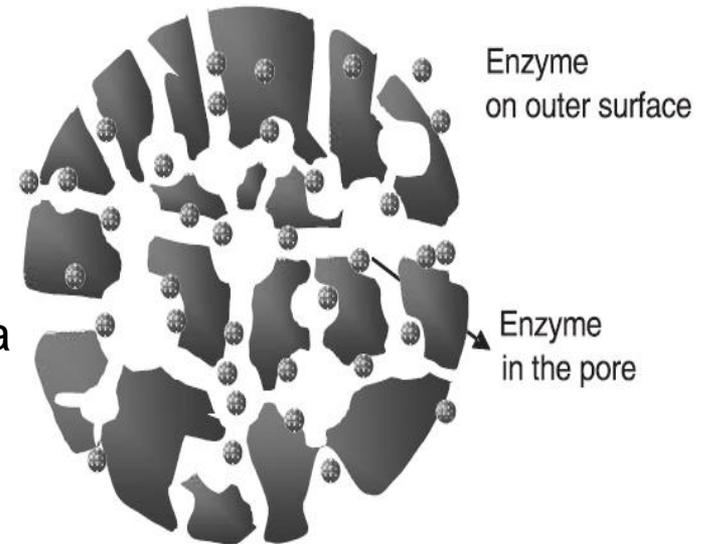
## Características Físicas do Suporte

**1) Superfície do suporte → ↑ superfície interna (poros) acessível favorece ↑ carga enzimática**

Superfície acessível → superfície externa e locais onde a enzima consegue se difundir

Superfície inacessível → poros de tamanho pequeno ou fechados

Superfície interna  $\approx 100 - 1000 \times$  Superfície Externa em um suporte macroporoso. Então, nesse caso, a carga enzimática na area externa é desprezível



Scheme 3.2 Internal and external surfaces of a porous carrier.

**2) Tamanho do poro → tem maior influencia na retenção de atividade** ( $E = \frac{[\text{Atividade medida}]}{[\text{Atividade ligada ao suporte}]}$  ou  $\frac{[\text{Atividade especifica da enzima imobilizada}]}{[\text{atividade especifica da enzima nativa}]}$ ) e na carga útil (payload = massa proteína imobilizada/massa suporte)

Devem ser  $3 - 9 \times$  o tamanho da enzima

↑ tamanho do poro, ↓ superfície acessível → ↓ carga enzimática, ↓ atividade especifica (sendo minima com poro de 60 nm, mas aumenta depois deste tamanho)

# Métodos de Imobilização

## **3) Distribuição de Tamanho dos Poros > Tamanho da enzima → ↑ carga enzimática, ↓ restrições difusionais, controle da distribuição de enzimas**

A distancia da camada de enzima ligada a parede do poro no suporte e o centro do poro deve ser maior que 20 nm, justamente para evitar restrições difusionais. Assim o valor minimo do tamanho do poro =  $2x(\text{tamanho da enzima} + 20)$  nm

Ex. Se o tamanho minimo da enzima for 5 nm, o tamanho minimo do poro é 50 nm

## **4) Porosidade → razão de volume interno/volume externo da partícula**

Se for zero é um suporte não poroso; a maioria dos suportes porosos tem valores entre 0,5 e 0,8.

Também influenciam a retenção de atividade a carga enzimática e o microambiente superficial.

## **5) Tamanho da Particula**

Para suporte não-porosos, ↓ tamanho da partícula, ↑ superficie acessivel → ↑ carga enzimática; mas o limite minimo para qualquer suporte seria 50 µm.

No caso de suportes porosos, ↑ carga enzimática → ↓ atividade, devido a maiores restrições difusionais

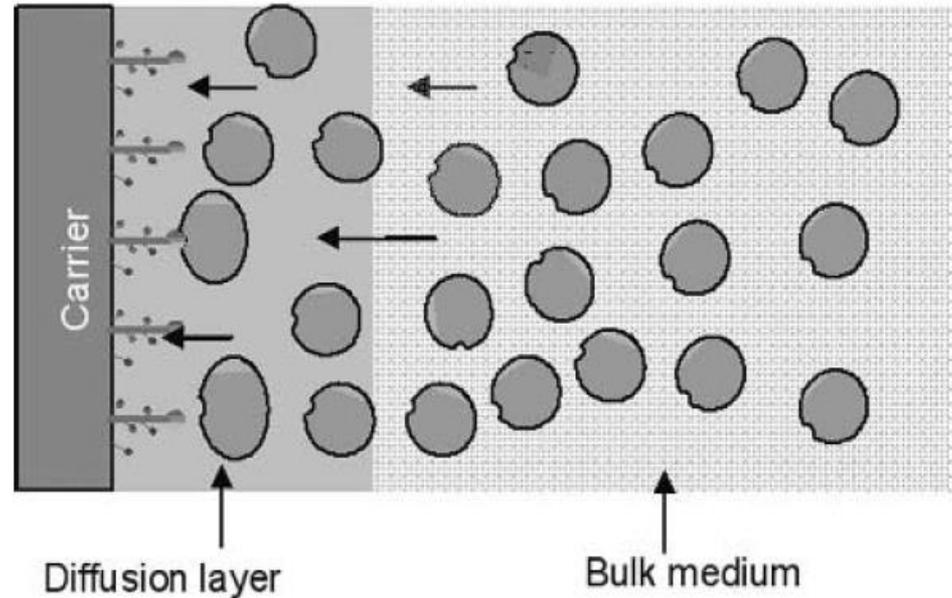
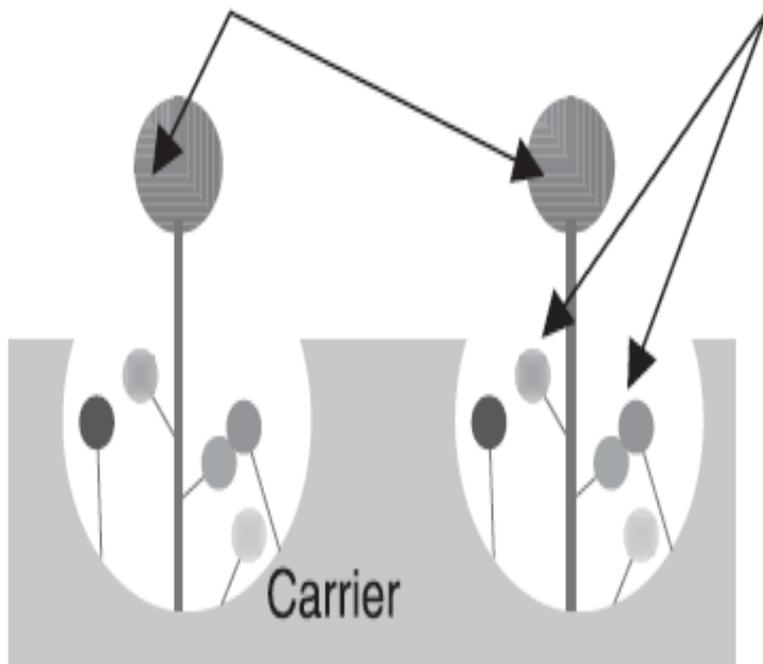
Para sistemas onde susbtratos ou produtos são sólidos, o tamanho das particulas das enzimas imobilizadas devem ser maiores que os mesmos, em torno de ou >100 µm, facilitando sua separação.

# Métodos de Imobilização

## Características Químicas do Suporte

Active functionality

Inert functionality

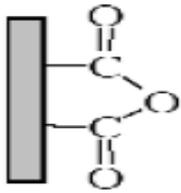


Scheme 3.11 Conformation change of enzyme approaching the carrier - effect of the chemical nature of the carrier on the enzyme molecules.

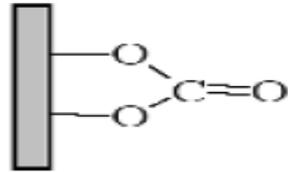
# Métodos de Imobilização

## Características Químicas do Suporte

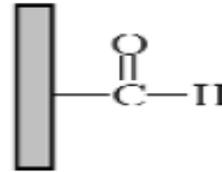
### Grupos Ativos ligados ao suporte



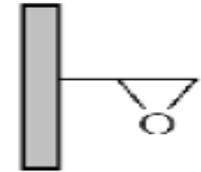
(A)  
polianidrido



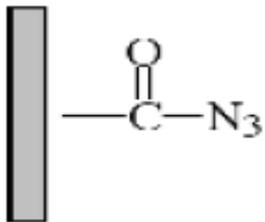
(B)  
policarbonato



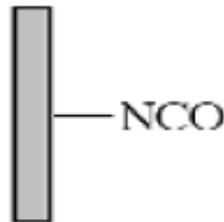
(C)  
polialdeído



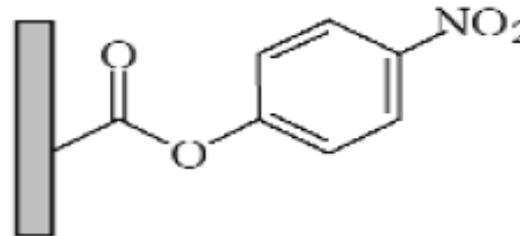
(D)  
poliepoxido



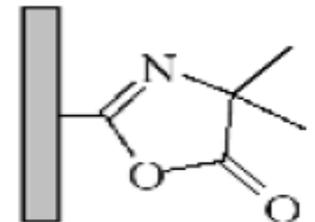
(E)  
poliacilazida



(F)  
poliisocianato



(G)  
policarboxílico ácido  
fenil éster



(H)  
poliazlactona

Mais comuns: grupos epoxido,  
carbonila, e amino

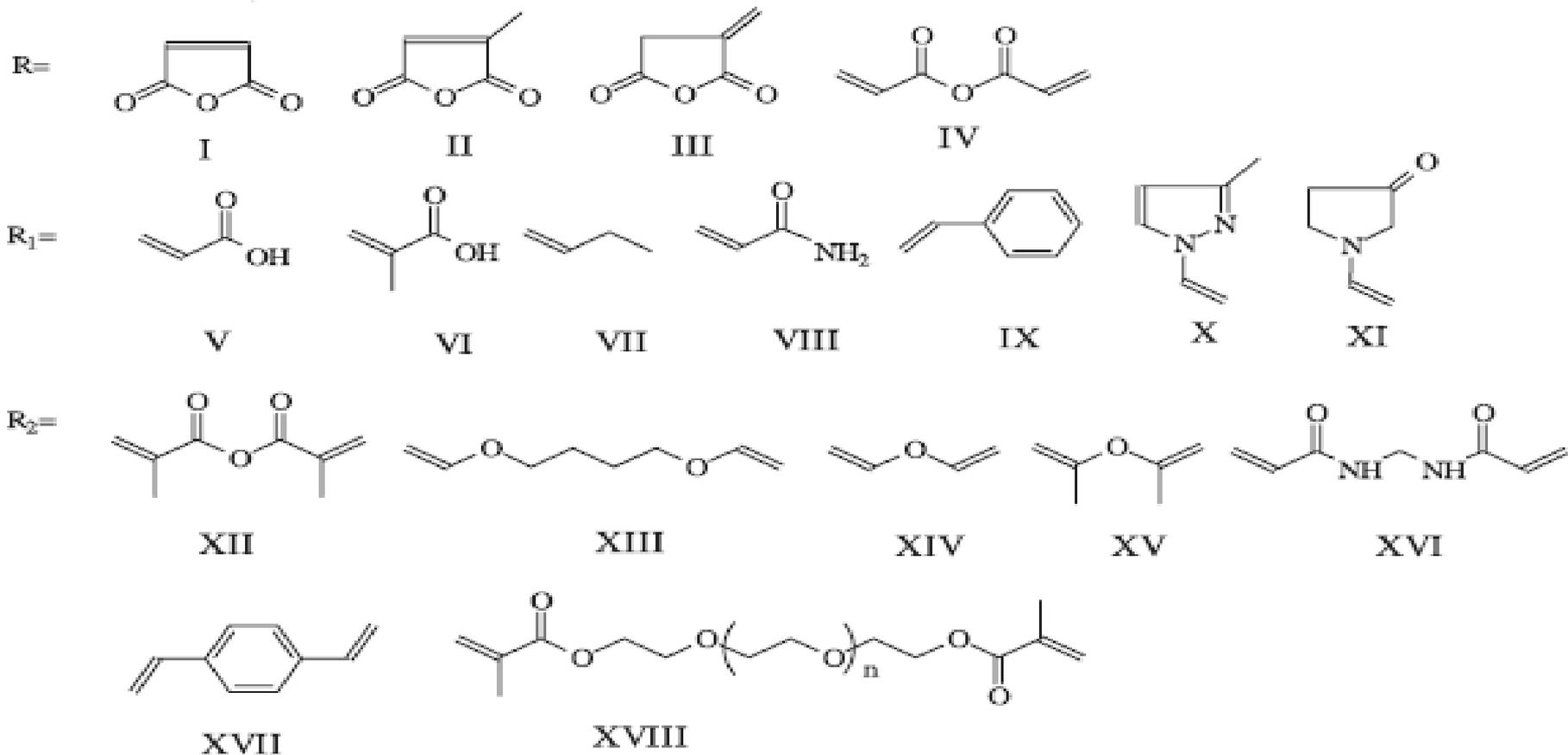
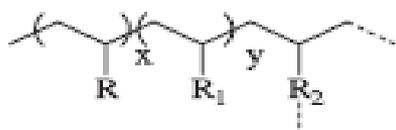
Ex.: Sepabeads (grupos epoxi),  
oxidized polysaccharide  
(aldehyde groups)

# Métodos de Imobilização

## **Preparação de Suportes Ativos**

- Polimerização direta (Sistema em suspensão bifásico) de monômeros apresentando a funcionalidade ativa;
- Polimerização de monômeros apresentando funcionalidade inerte (grupos hidroxila, carboxila, amino e nitrila), seguido de ativação antes da imobilização → pré-suportes inertes
- Recobrimento de pré-polímeros;
- Modificação de suportes prontos;
- Formação de ligações cruzadas de polímeros solúveis;
- Graftização de suportes prontos;
- Formação de compósitos com mais de dois polímeros distintos.

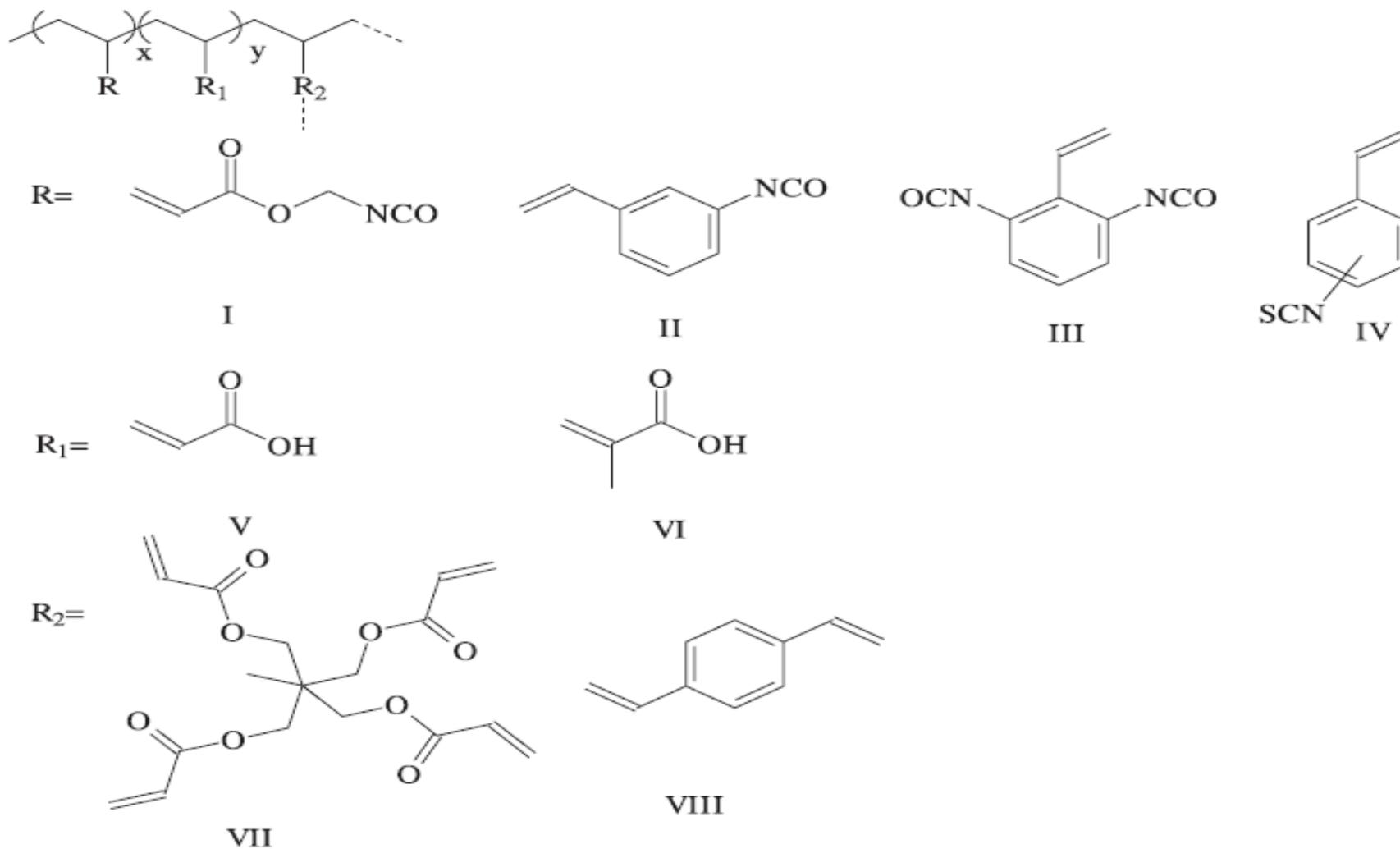
# Métodos de Imobilização



**Scheme 3.57** Monomers used for preparation of polymeric anhydrides for enzyme immobilization.

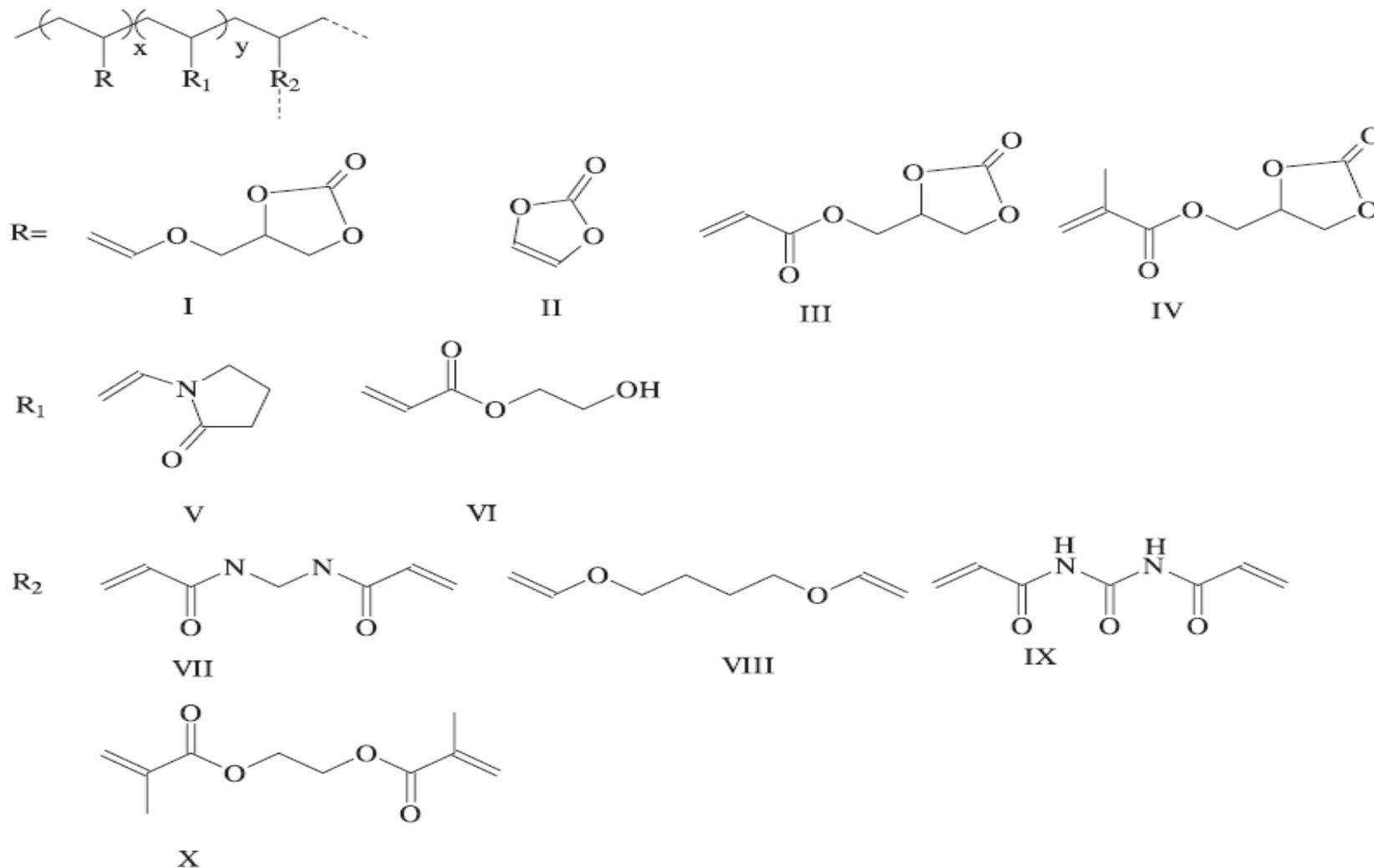


# Métodos de Imobilização



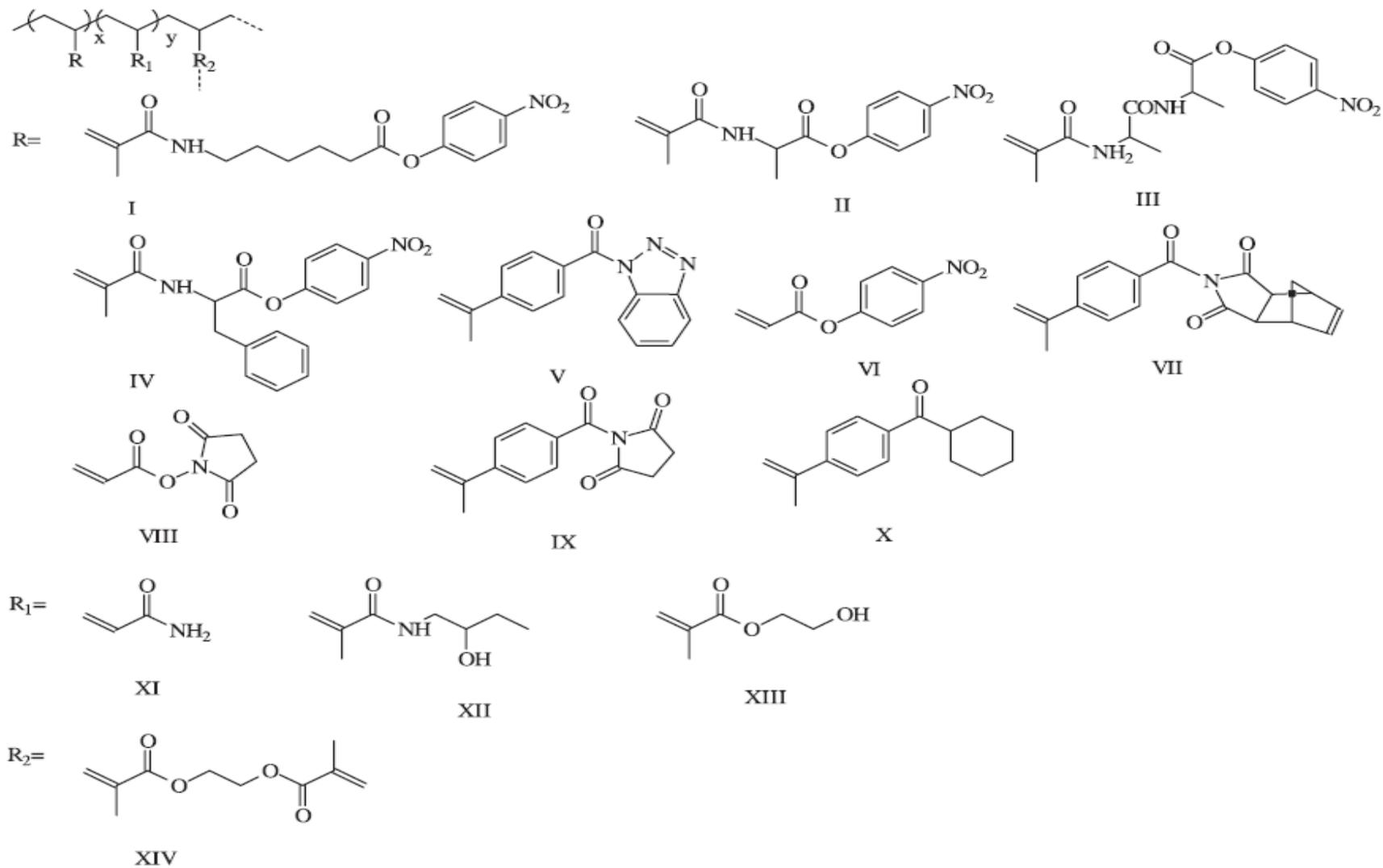
**Scheme 3.61** Monomers used for preparation of synthetic polymers bearing isocyanate groups.

# Métodos de Imobilização



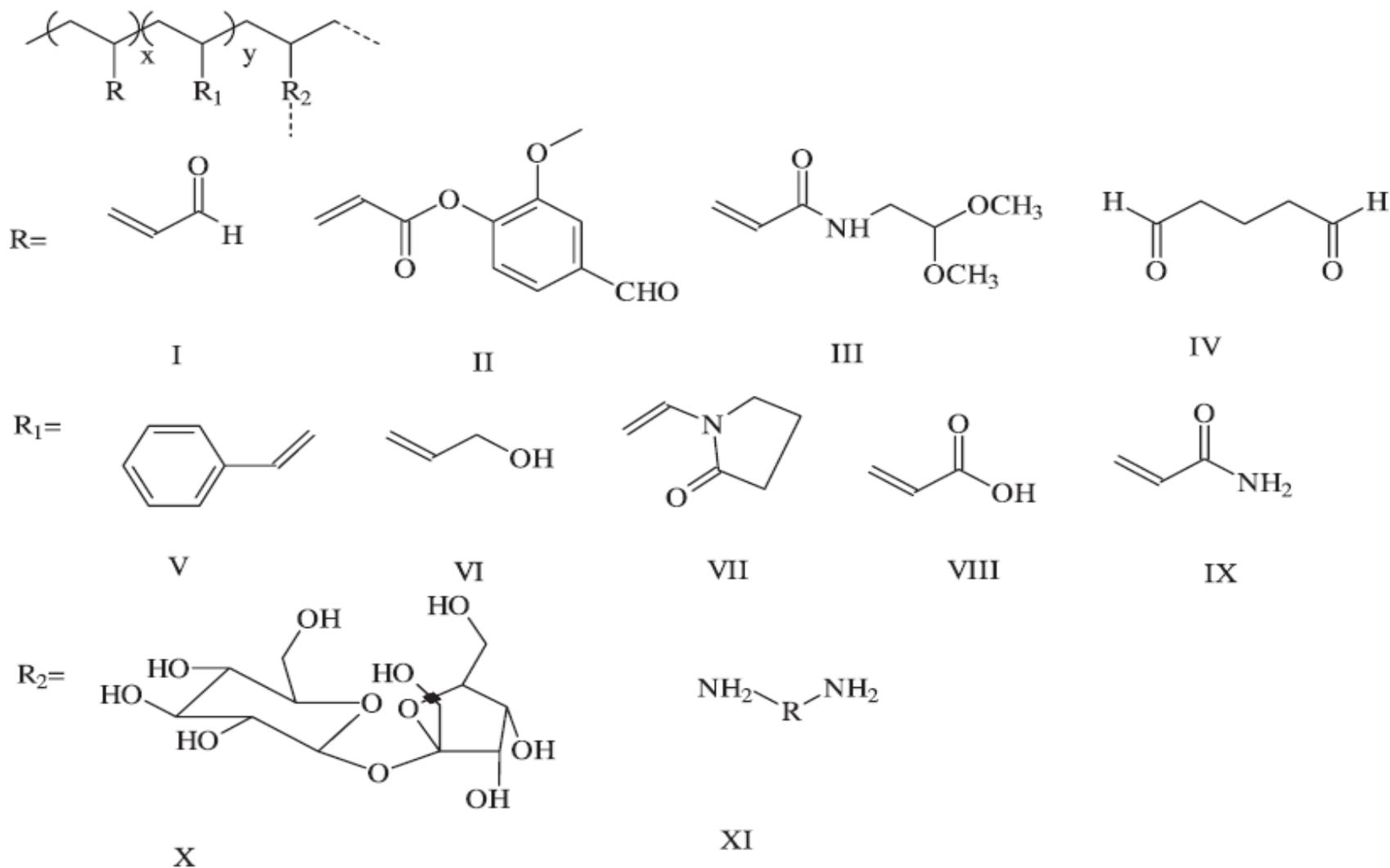
**Scheme 3.62** Monomers used for preparation of polymeric carriers with carbonate groups.

# Métodos de Imobilização



Scheme 3.63 Activated polycarbonyl polymers.

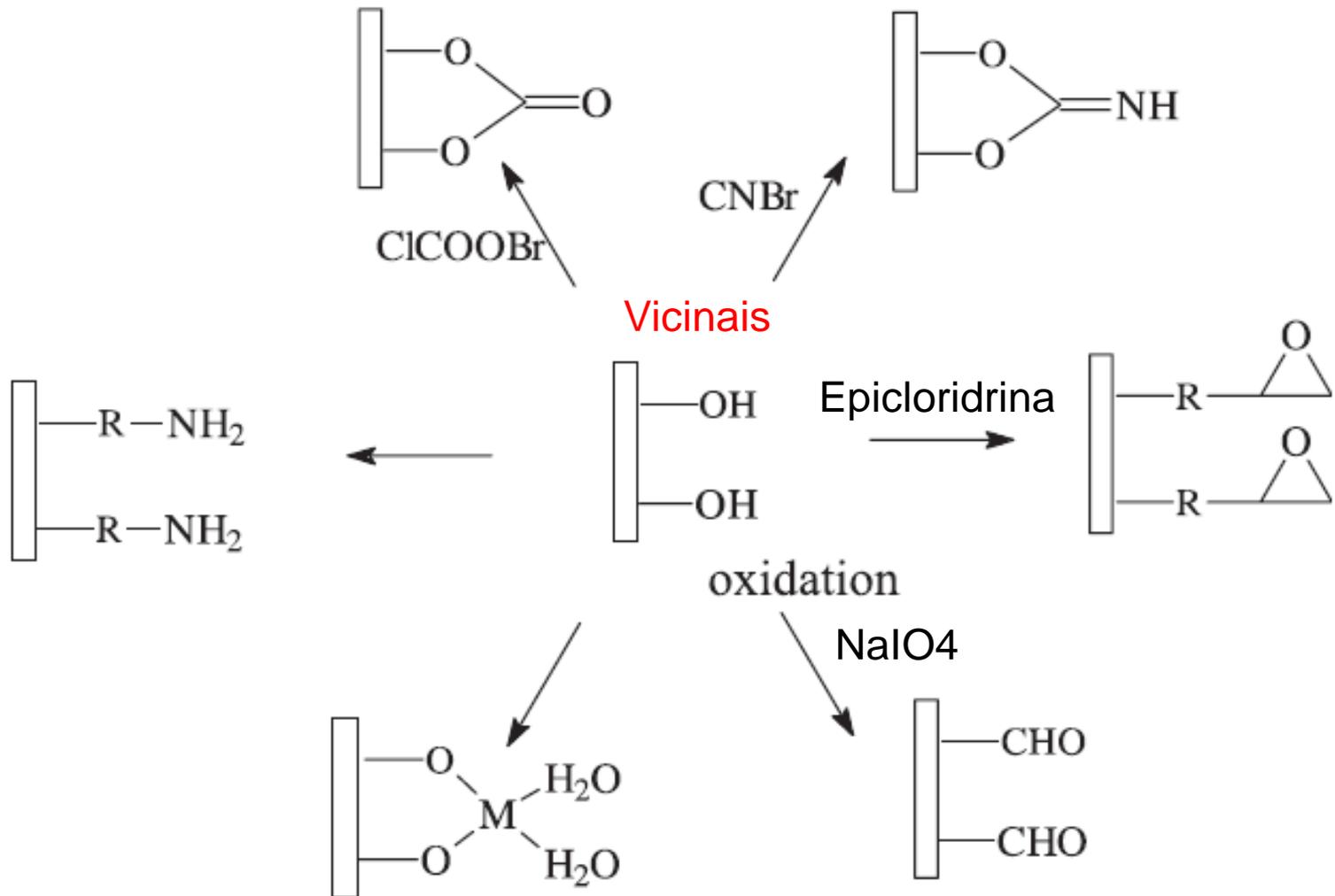
# Métodos de Imobilização



Scheme 3.65 Monomers used for preparation of polymers bearing aldehyde groups.

# Métodos de Imobilização

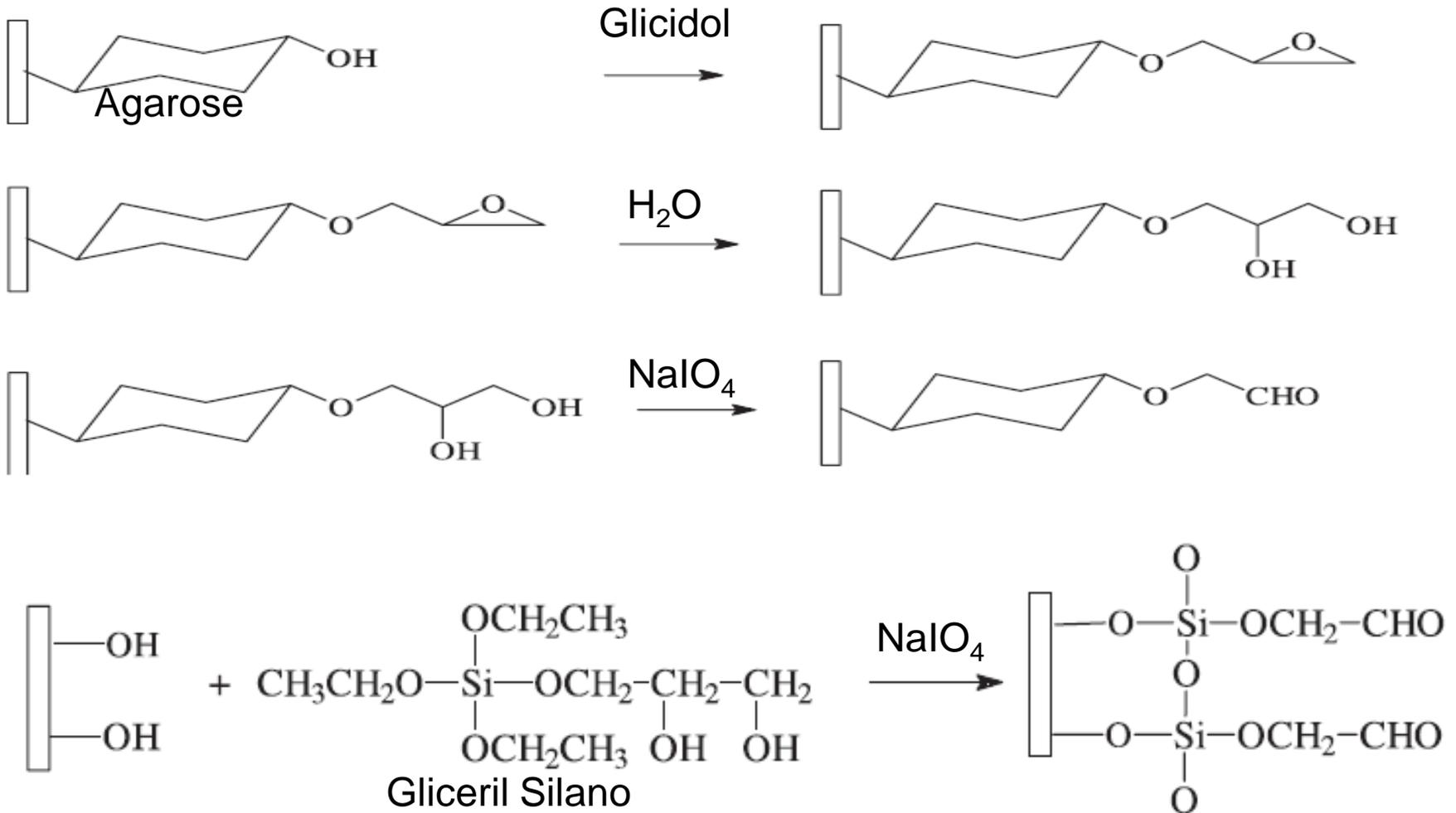
Ativação de Pré-suportes Inertes: Grupos Hidroxila Ex.: Sepharose e Sephadex



# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Hidroxila

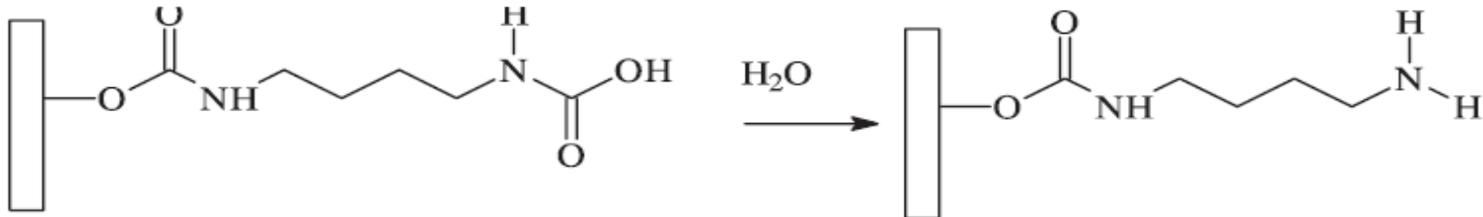
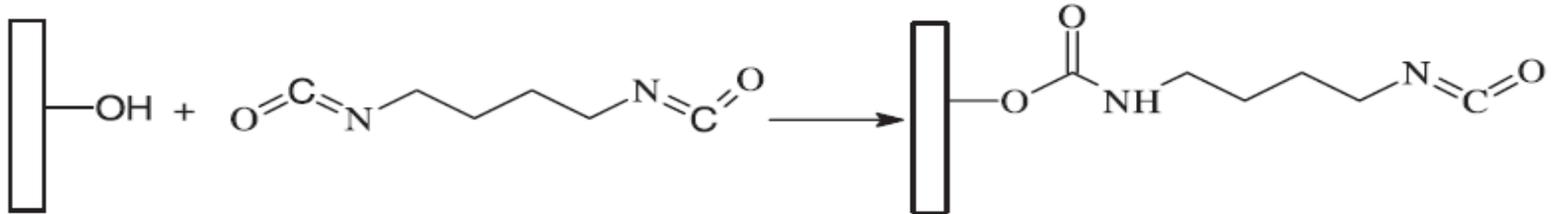
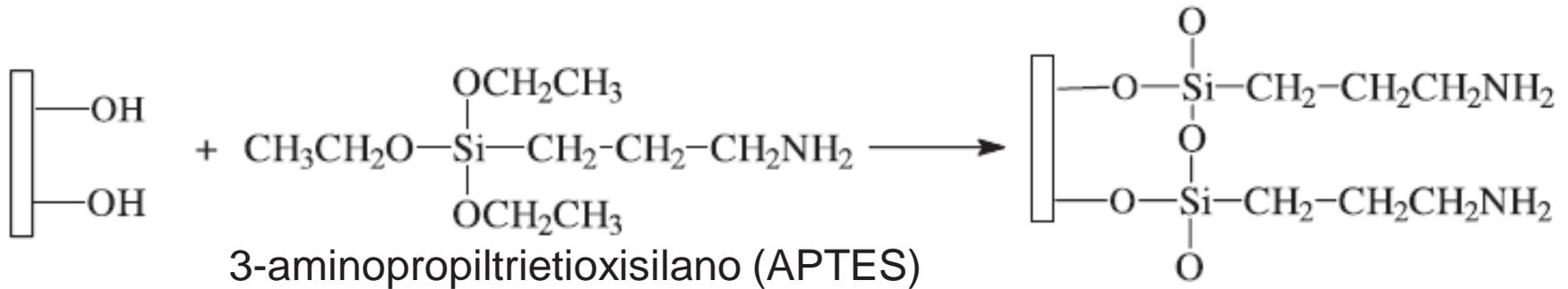
### Oxidação



# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Hidroxila

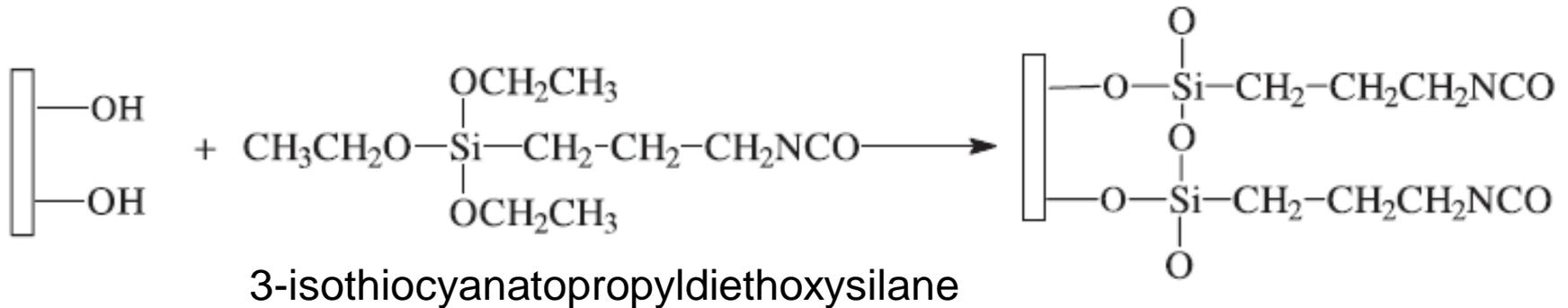
### Conversão a Grupos Amino



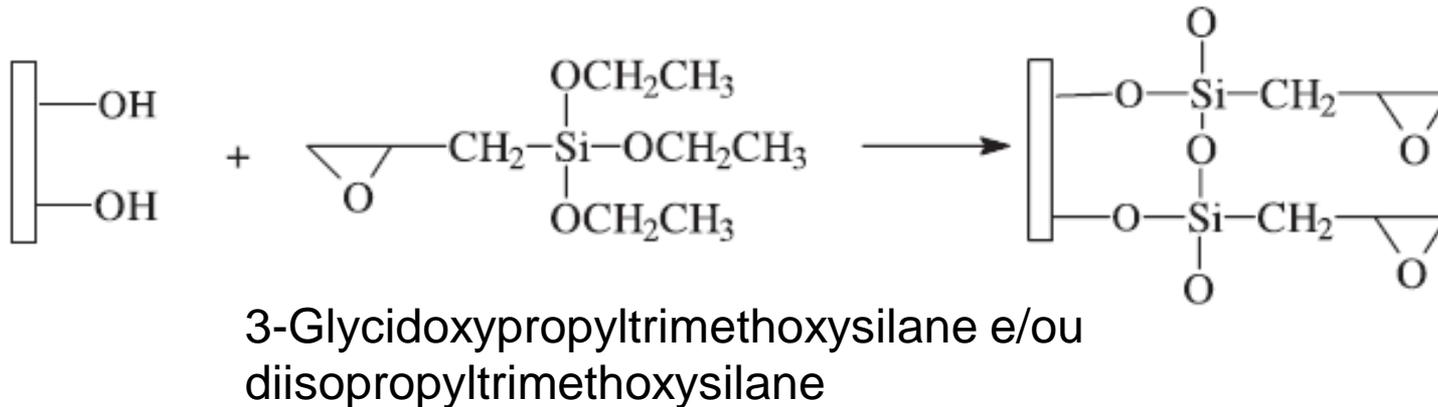
# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Hidroxila

### Conversão a Isocianato



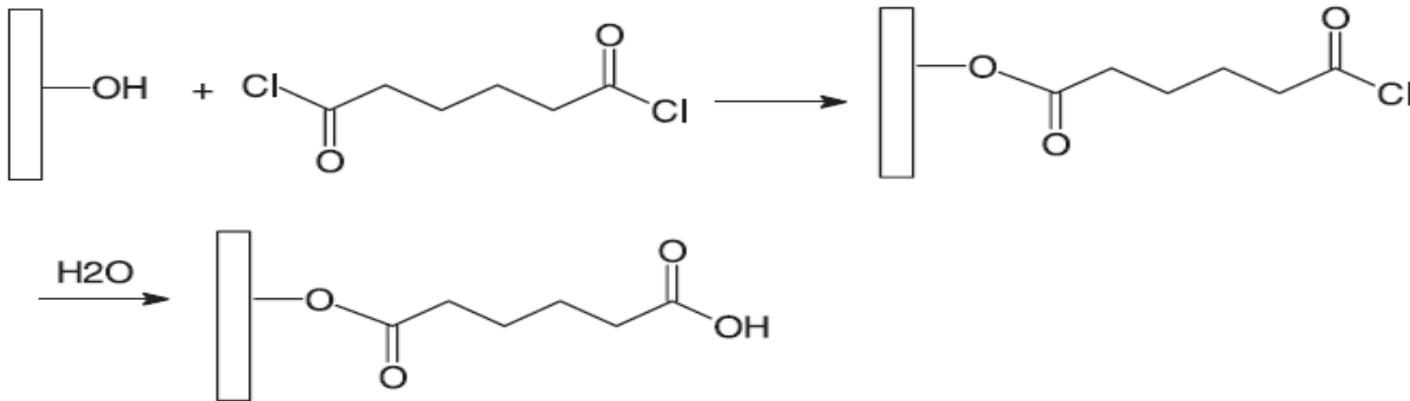
### Conversão a Grupos Oxirânicos



# Métodos de Imobilização

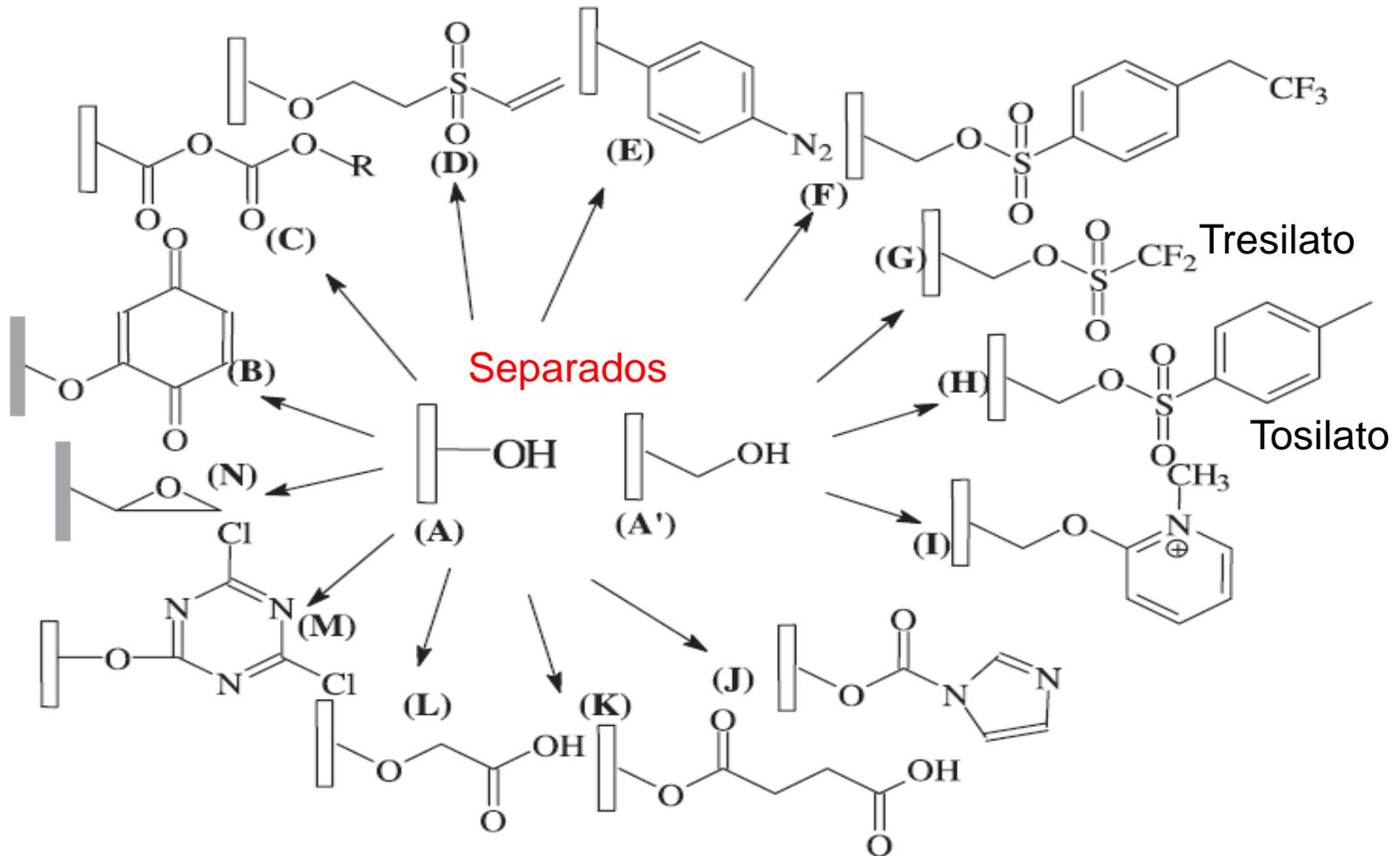
## Ativação de Pré-suportes Inertes: Grupos Hidroxila

### Conversão a Grupos Carboxílicos



# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Hidroxila

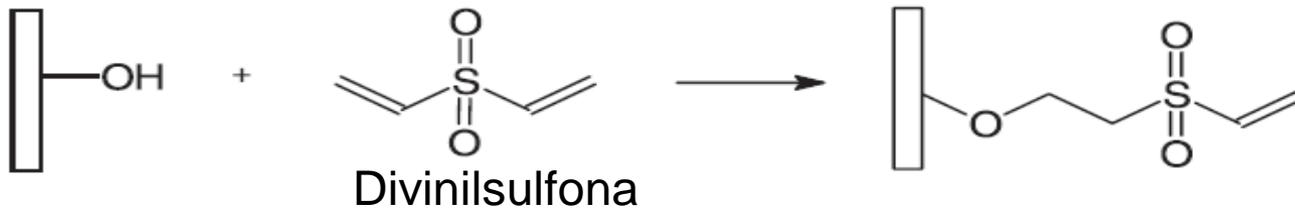
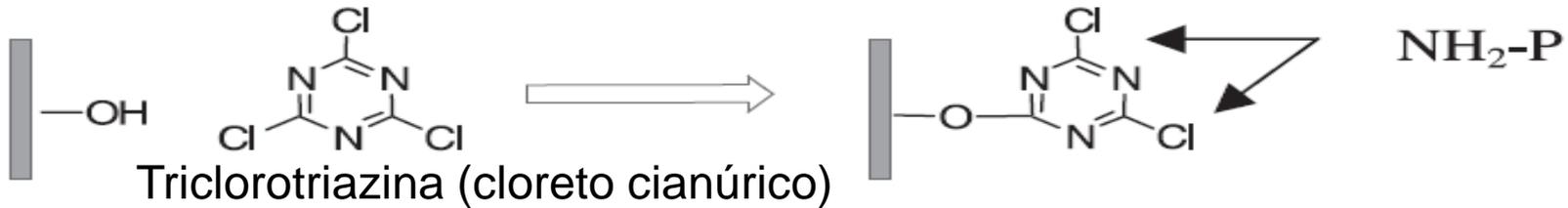


# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Hidroxila

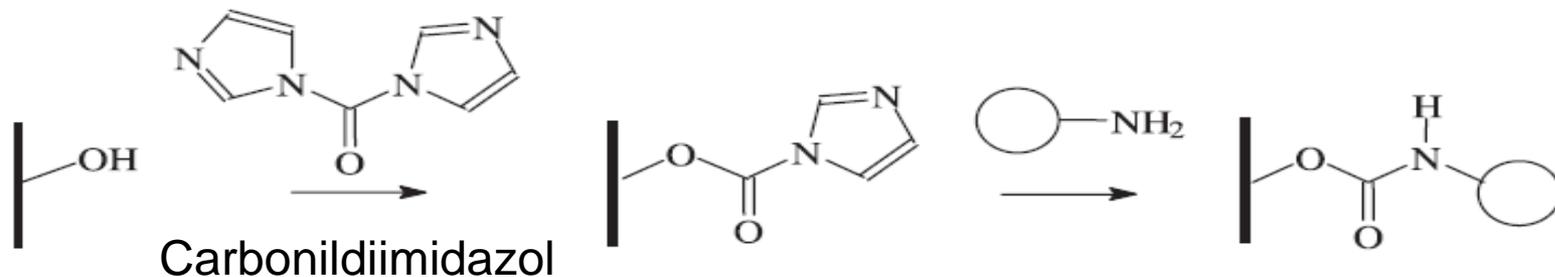
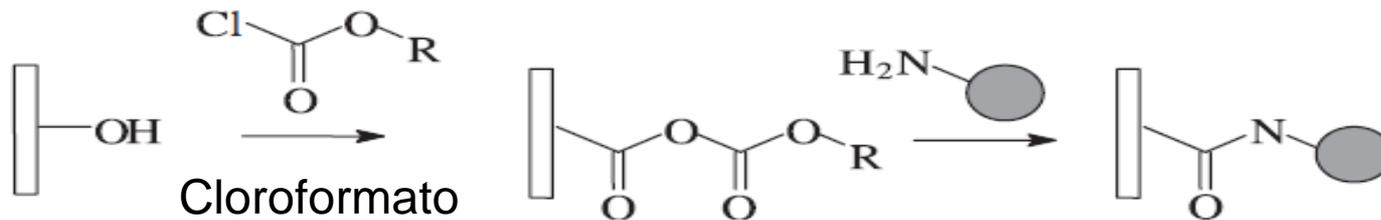
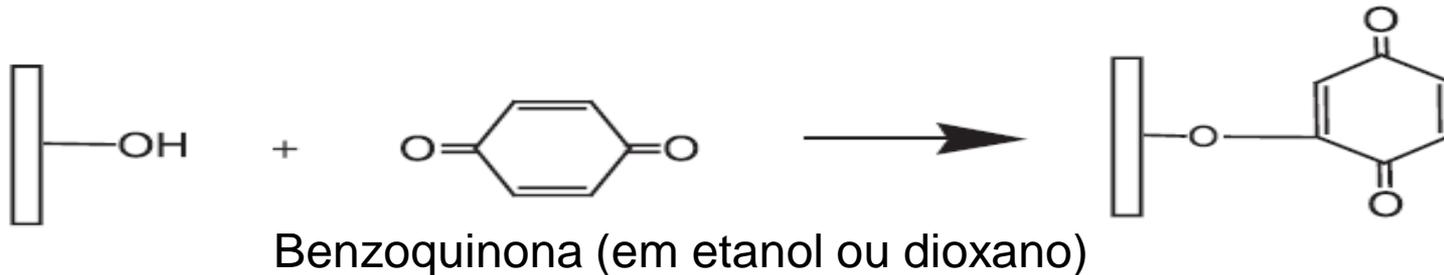


R =  $\text{CH}_2\text{CF}_2$  (tresylate),  $\text{C}_6\text{H}_4\text{CH}_3$  (tosylates)



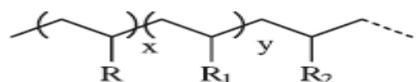
# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Hidroxila

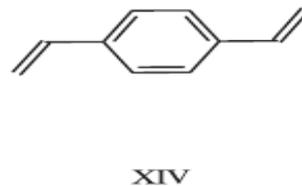
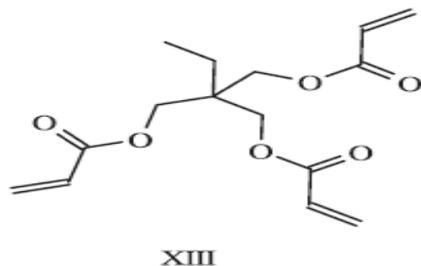
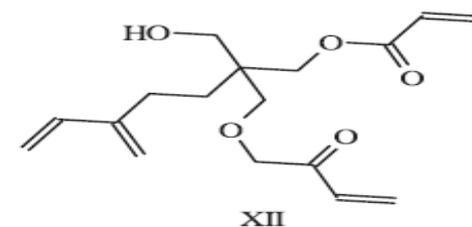
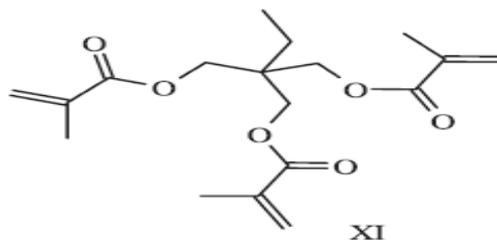
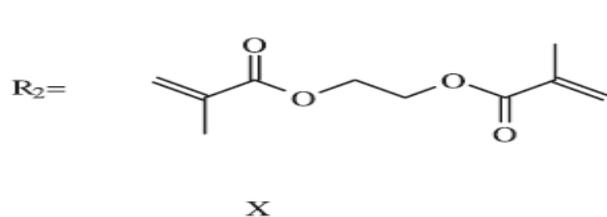
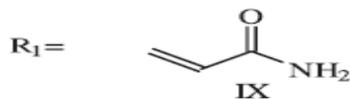
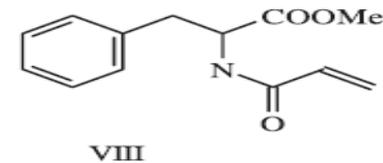
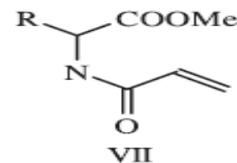
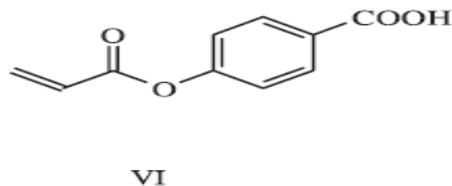
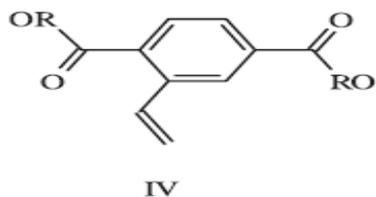
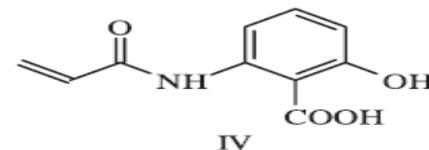
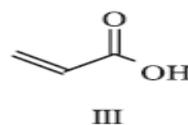
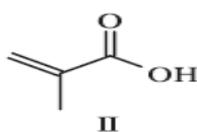
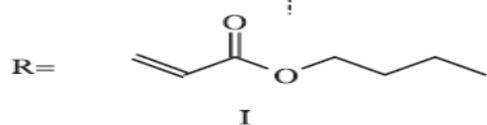


# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Carboxila



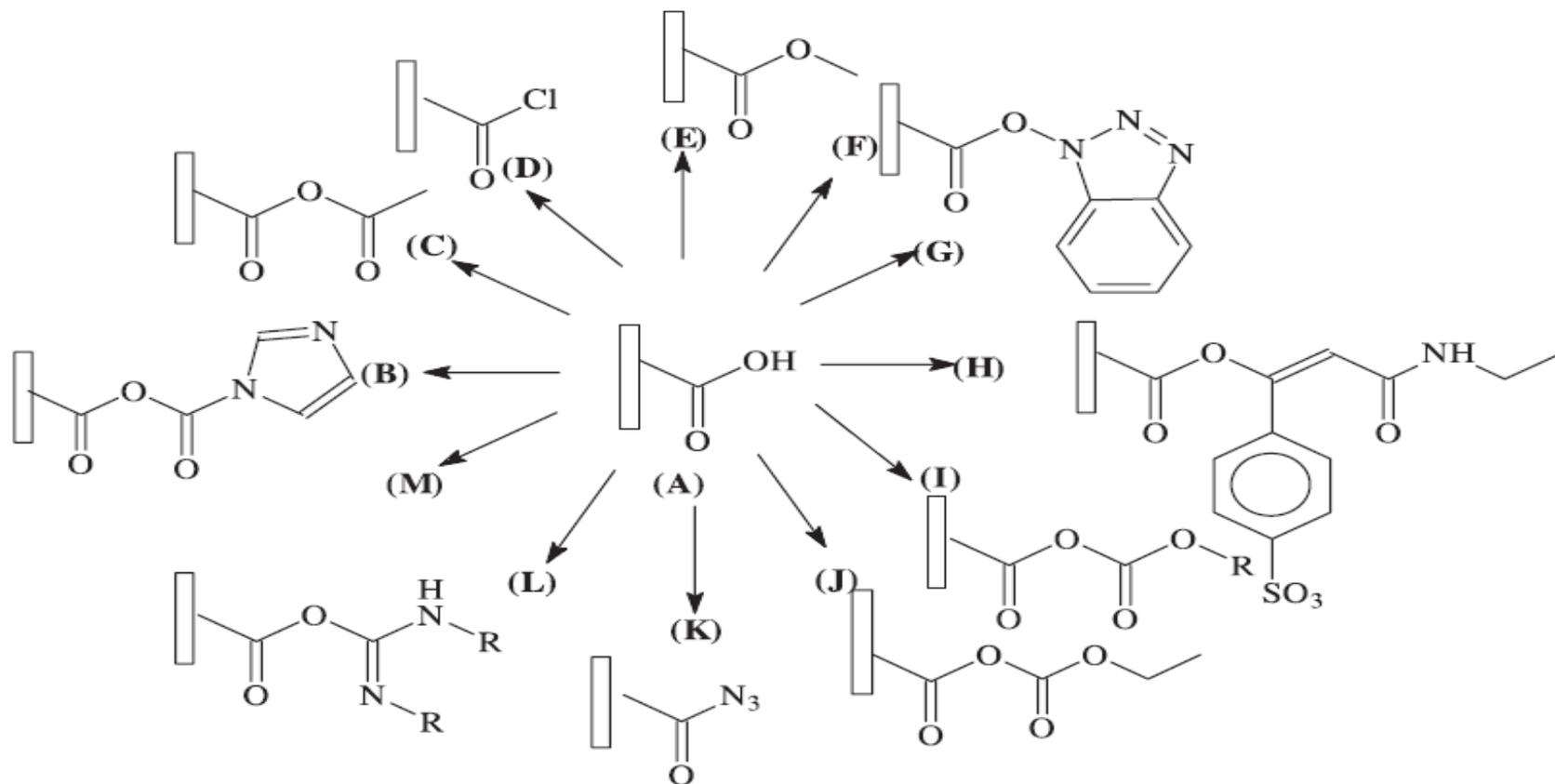
### Insoluble Polyacrylic Acid or Derivatives



Scheme 3.71 Monomers bearing carboxyl groups.

# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Carboxila

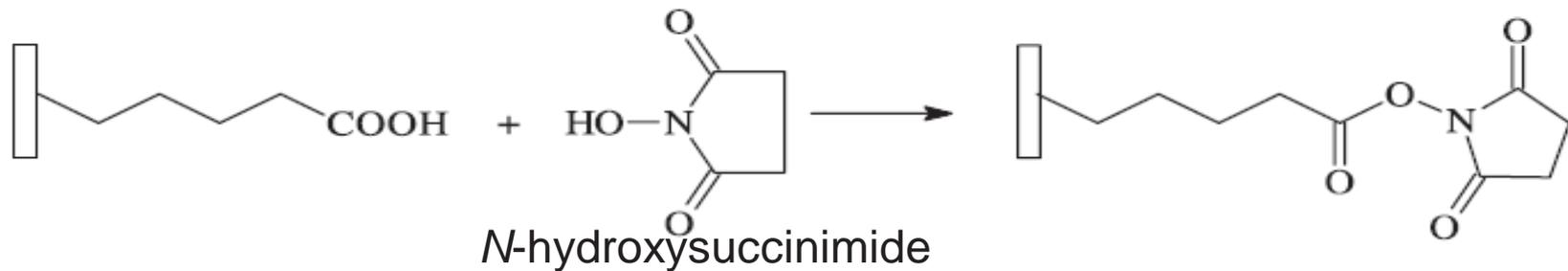


**Scheme 3.98** Activation of carriers containing carboxyl groups: (AB) activation with carbonyldiimidazole, (AC) mixed anhydride, (AD) thionyl chloride activation, (AE) esterification, (AF) activation with N-hydroxybenzotriazole, (AH) activation with Woodward reagent

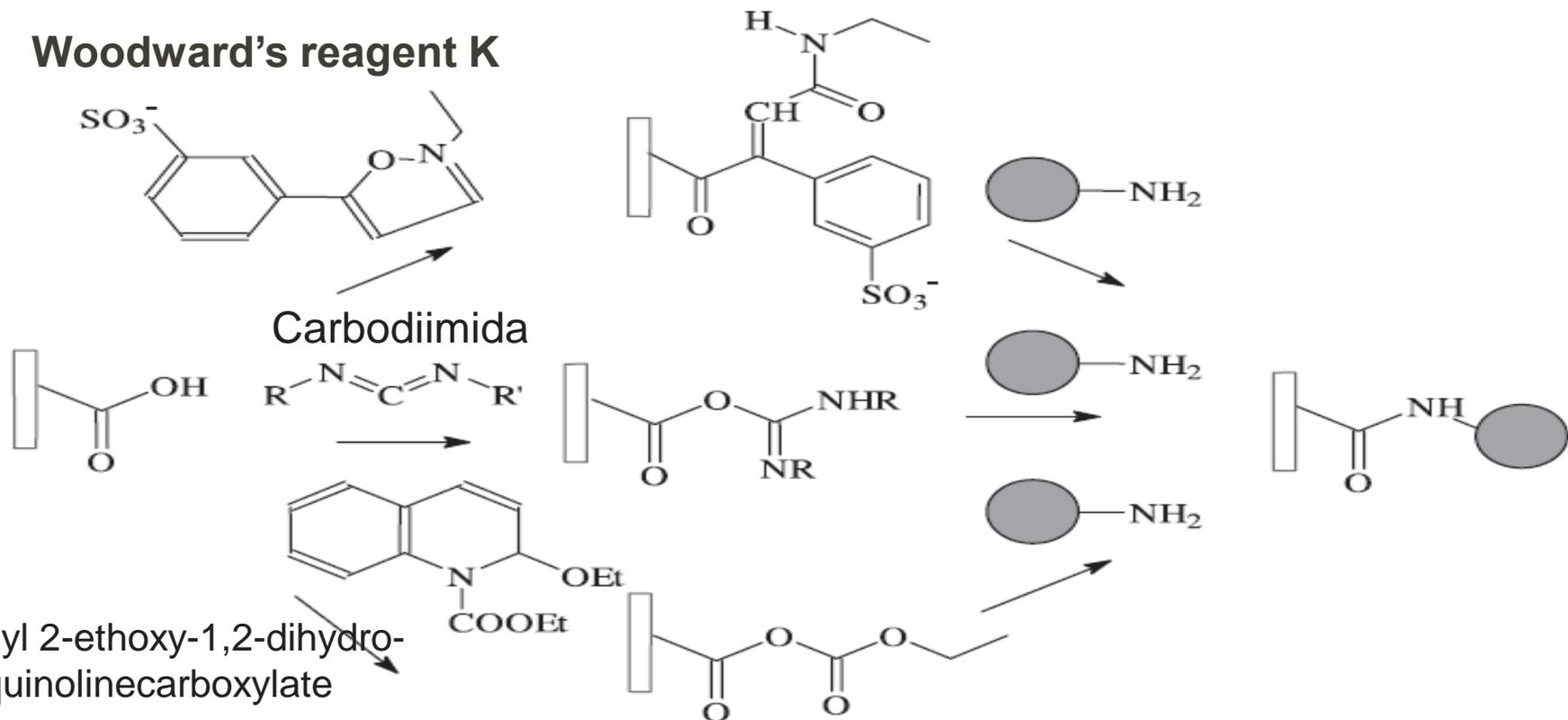
K, (AI) activation with ethyl 2-ethoxy-1,2-dihydro-1-quinolinecarboxylate, (AJ) activation with chlorocarbonate, (AK) acyl azide via three steps, i.e. esterification and further reaction with hydrazine and nitric acid, (AL) activation with various carbodiimides.

# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Carboxila

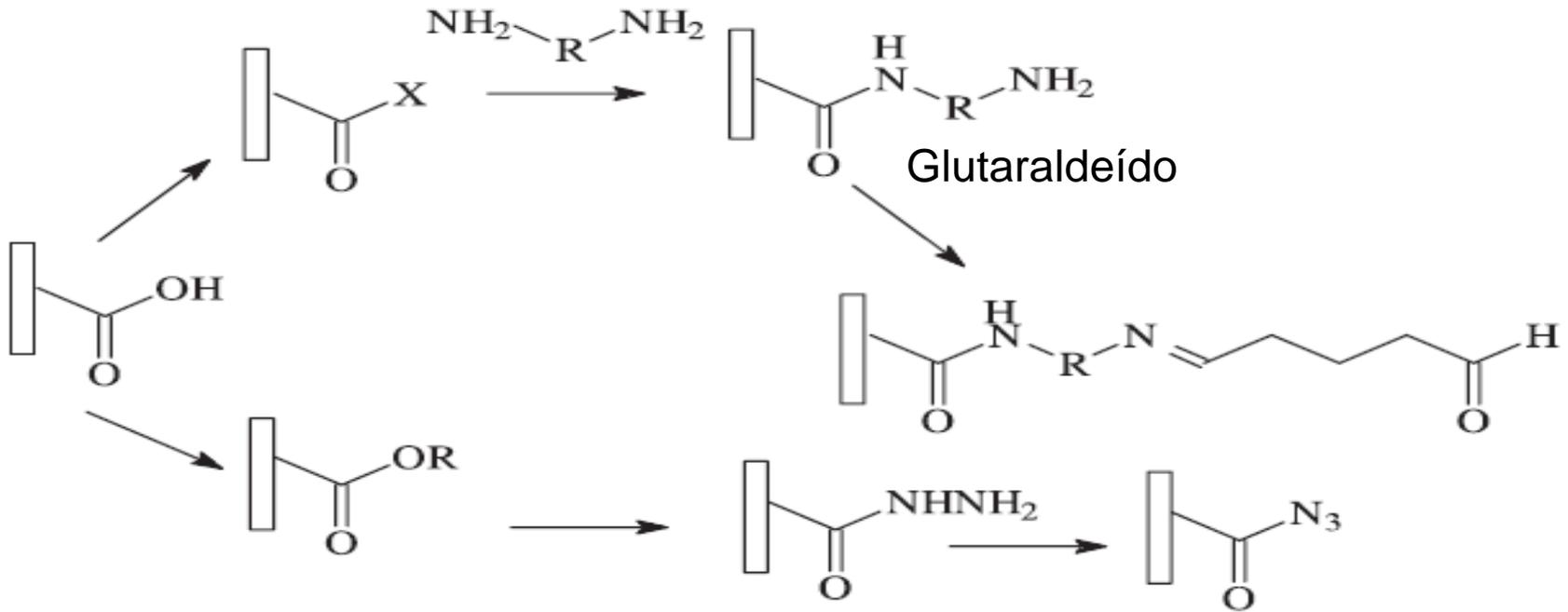


### Woodward's reagent K



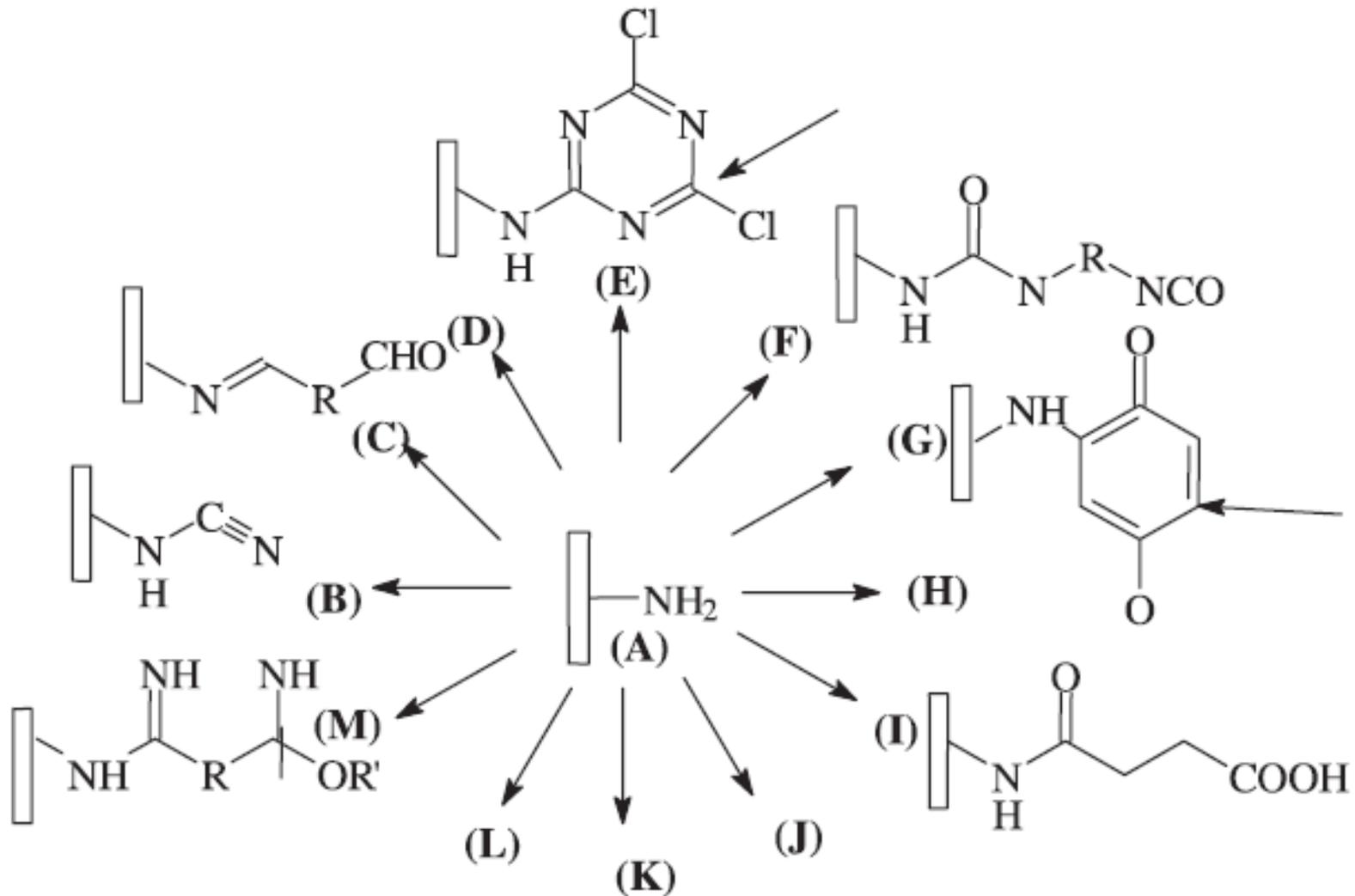
# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Carboxila



# Métodos de Imobilização

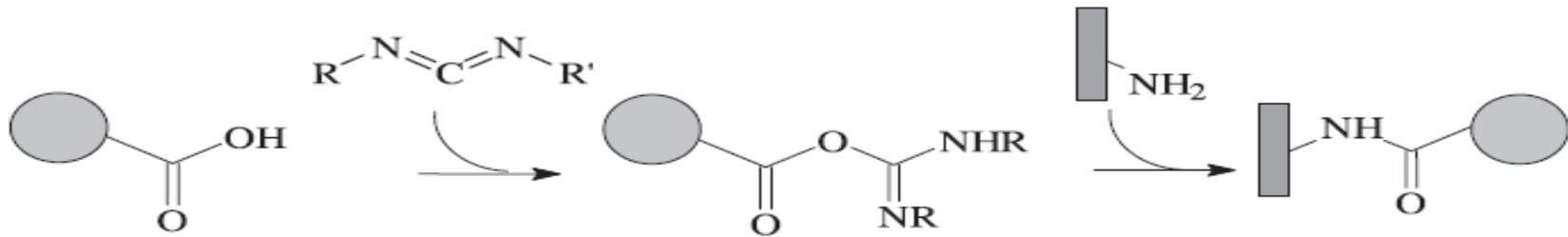
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# Métodos de Imobilização

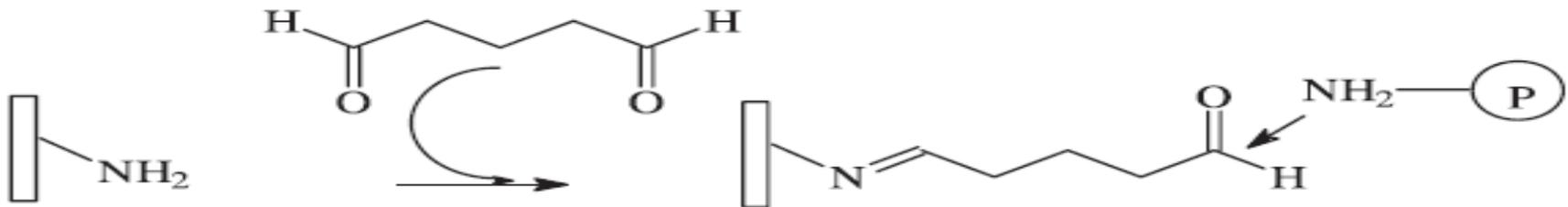
## Ativação de Pré-suportes Inertes: Grupos Amino

### Ativação da enzima com carbodiimida



Scheme 3.103 Zero coupling of protein with carriers.

### Reação com aldeído

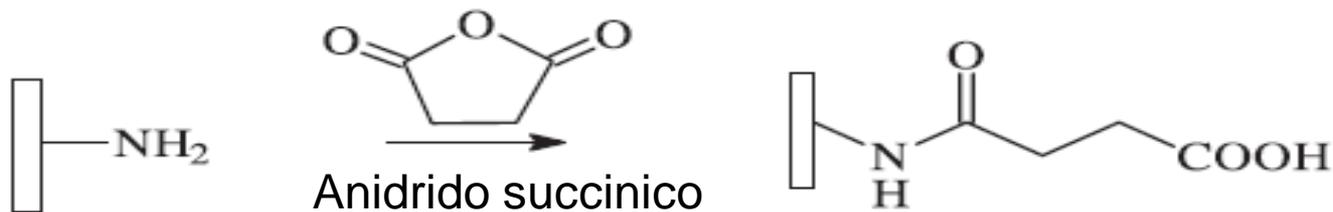
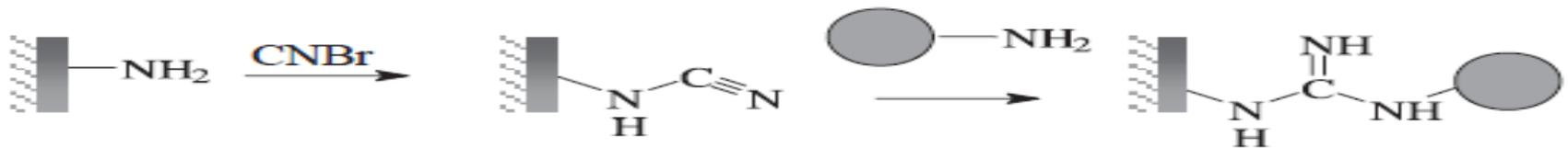
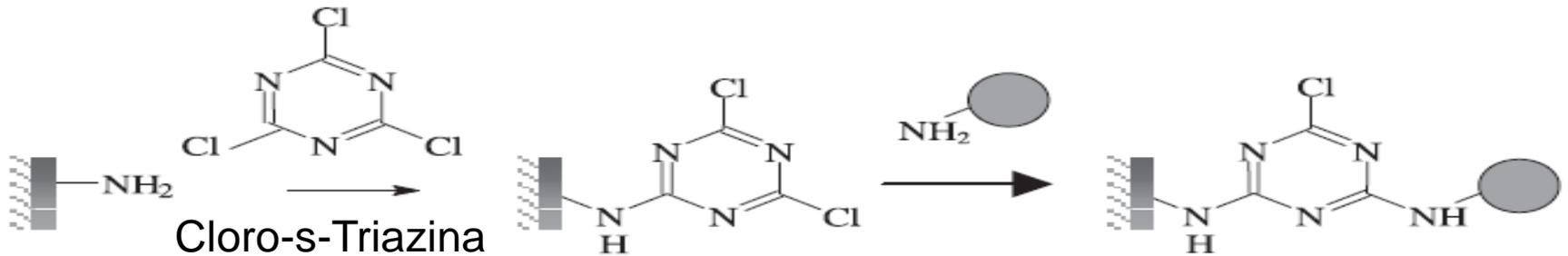


### Reação com diisocianato



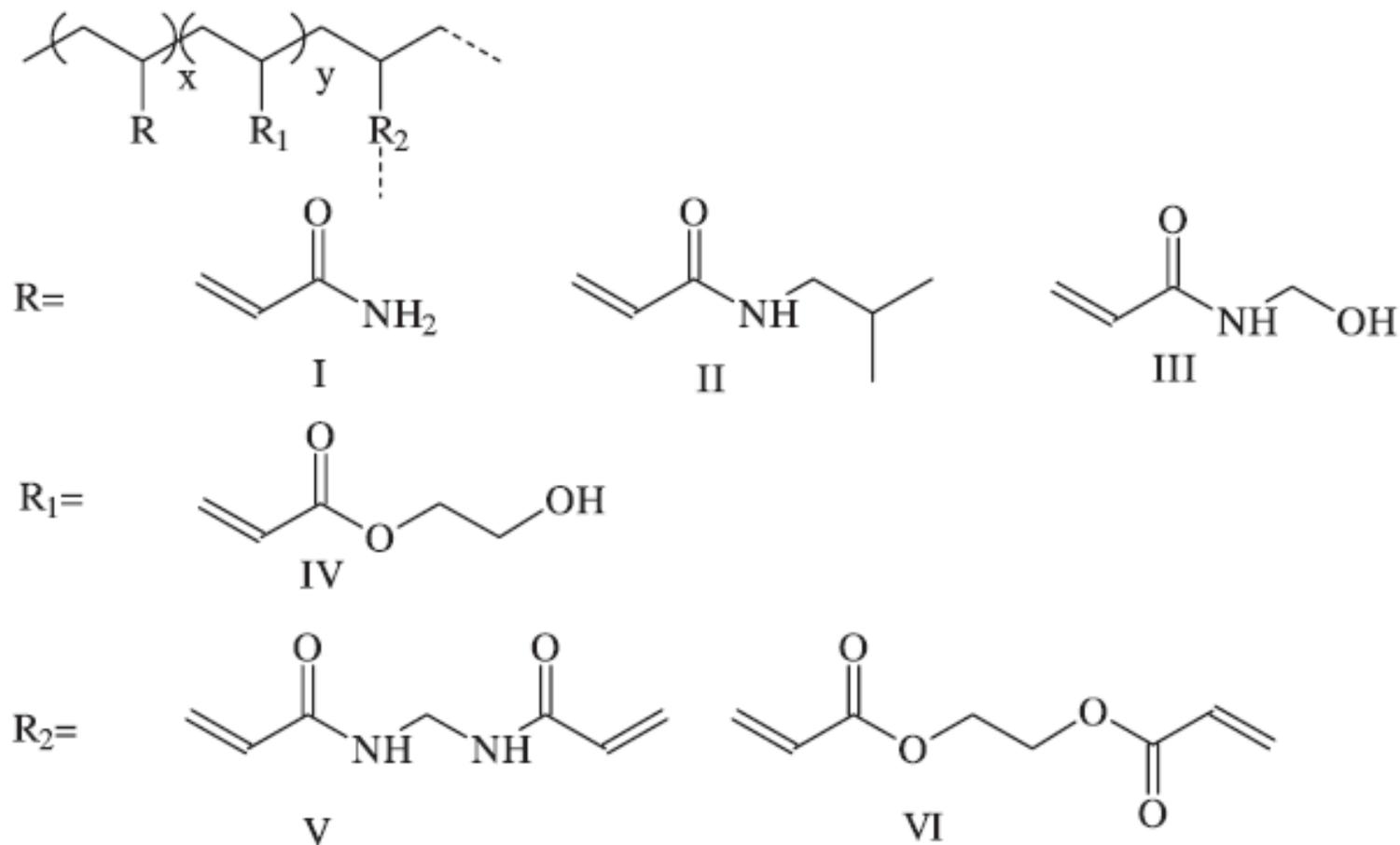
# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Amino



# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Amida

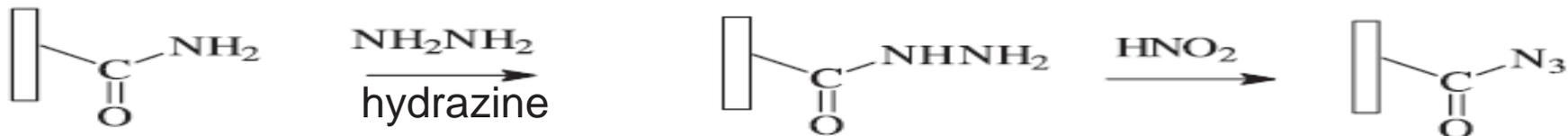


Scheme 3.70 Polyacrylamide.

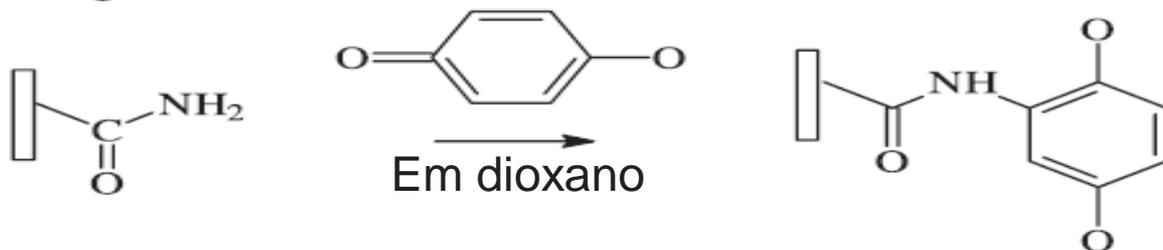
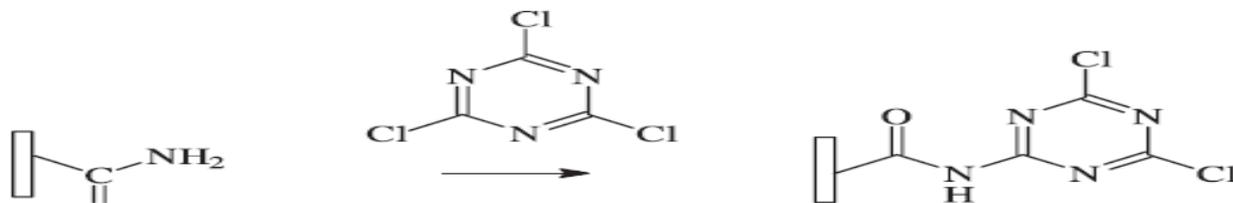
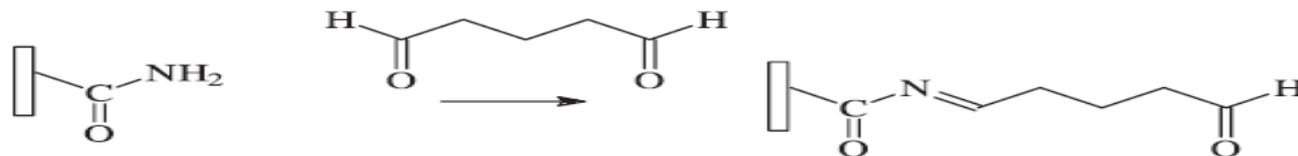


# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Amida

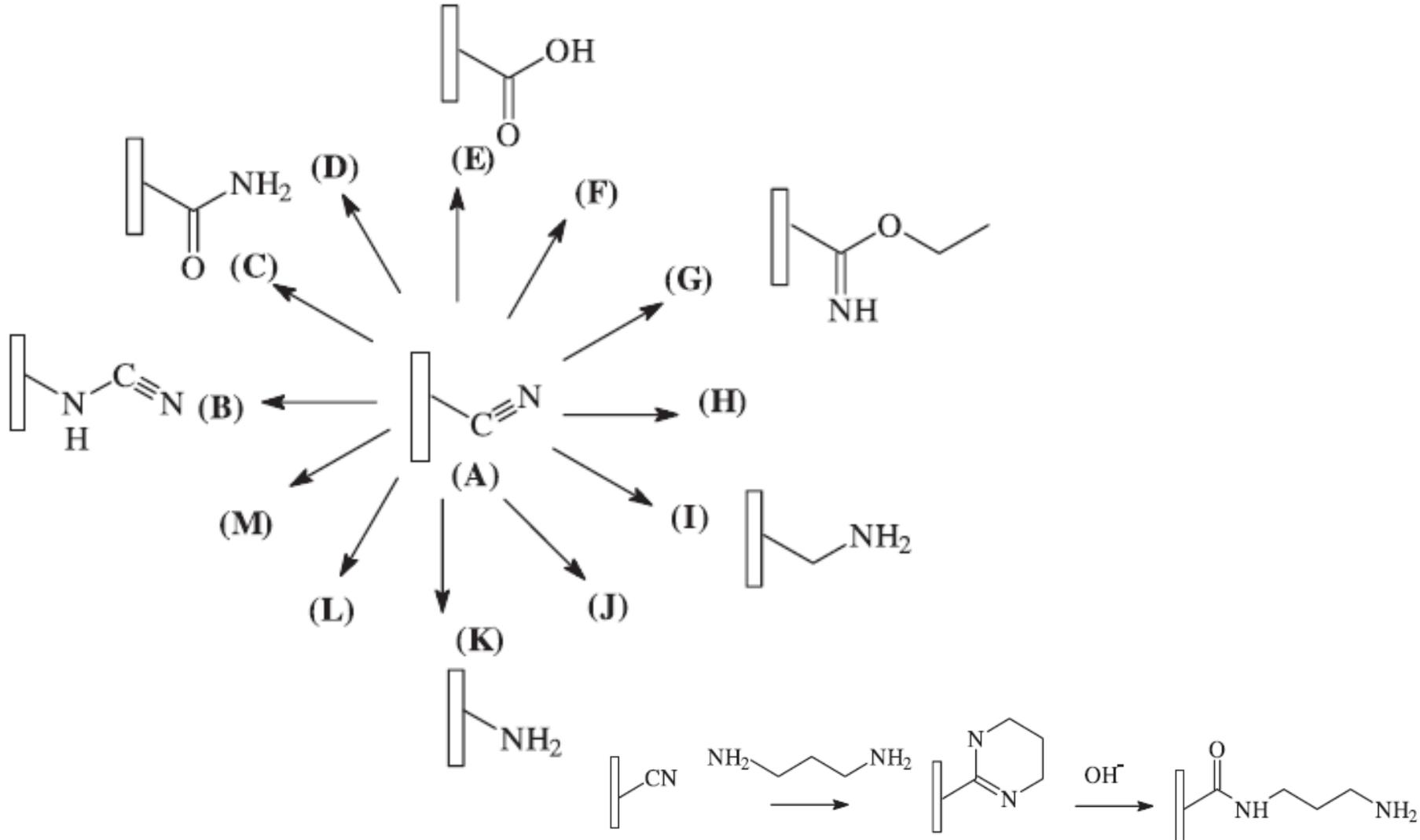


Scheme 3.112 Activation of polyacrylamide to polyacylazide.



# Métodos de Imobilização

## Ativação de Pré-suportes Inertes: Grupos Nitrila



# Métodos de Imobilização

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## Características Químicas do Suporte

### Grupos Inertes ligados ao suporte

Estão diretamente ligadas a estrutura polimérica do suporte via ligação covalente; ou ligadas ao spacer, que liga os grupos ativos a estrutura do suporte; ou como grupos de saída ligados aos grupos ativos, sendo liberados depois da formação da ligação covalente com a enzima

Embora não participem da ligação covalente, estes podem influenciar a imobilização em ligações não-covalentes, modificando o microambiente dos suportes

Tipos:

- carregados: possuindo grupos  $-NH_2$  ou  $-COOH$ ;
- hidrofóbicos, pode ser introduzido por monômeros hidrofóbicos ou cross-linkers;
- hidrofílicos, introduzidos por monômeros hidrofílicos;
- bioespecíficos, ideia similar ao processo de adsorção bioespecífico

# Métodos de Imobilização

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## Spacer-Arm

São necessários porque evitam o impedimento estérico e permitem a interação entre grupo ligante e os resíduos de aminoácidos da enzima, além de proporcionar certa flexibilidade da ligação com o suporte.

Tipos:

- compostos bifuncionais, como glutaraldeído;
- polímeros lineares, como PEG-diamine or PEO acid, dextran and PEI;
- polissacarídeos funcionalizados, como aldehyde dextran, amino dextran, e proteínas, como bovine serum albumin (BSA);
- poliéteres.

Uma imobilização direta, quer dizer, sem um spacer pode levar a uma baixa eficiência de imobilização e até alterações na estabilidade enzimática, devido a:

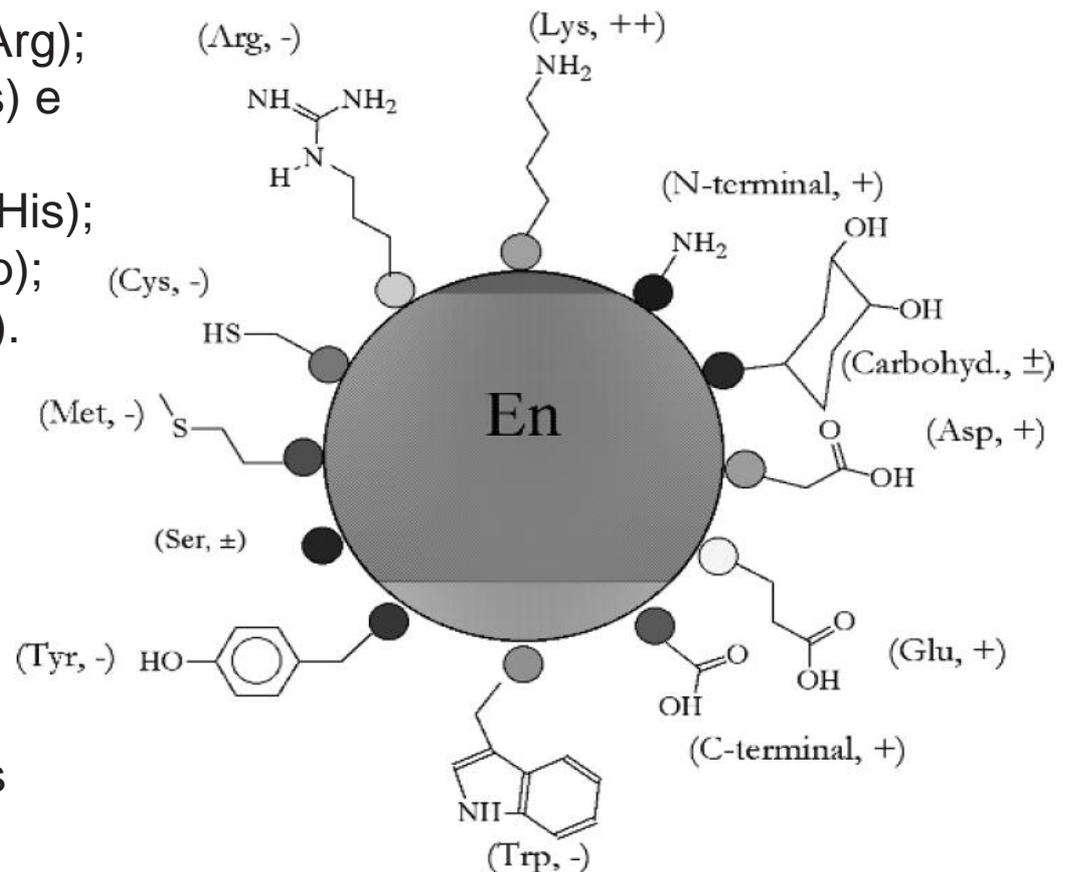
- impedimento estérico;
- efeitos ambientais na superfície (superfície hidrofóbicas promovem inativação da enzima);
- interação desfavorável da enzima com o suporte;
- orientação errada da enzima nos suportes, envolvendo ligação do sítio ativo com o suporte
- perda de flexibilidade conformacional devido ao excesso ligações multipontuais

# Métodos de Imobilização

## Resíduos de Aminoácidos disponíveis na Enzima

- grupos amino de N-terminal amino acids (NAA) e ( $\epsilon$ )-amino groups de lisina (Lys);
- $\gamma$  e  $\beta$  grupos carboxílicos de ácido glutâmico (Glu) e aspártico (Asp), e C-terminal carboxyl groups;
- grupo guanidínica da arginina (Arg);
- grupo sulfidrílica da cisteína (Cys) e tioéter da metionina (Met);
- grupo imidazólico da histidina (His);
- grupo indólico do triptofano (Trp);
- grupos fenólicos da tirosina (Tyr).

Além disso, alguns oligossacarídeos em enzimas glicosiladas podem ser convertidos a grupos aldeídicos por oxidação de hidroxilas vicinais e também serem núcleos de ligação covalente



Scheme 3.16 Reactive amino acid residues: + frequently used in enzyme immobilization; - not used; ± not frequently used; ++ very frequently used.

# Enzimas: reatividade e quantidade de resíduos

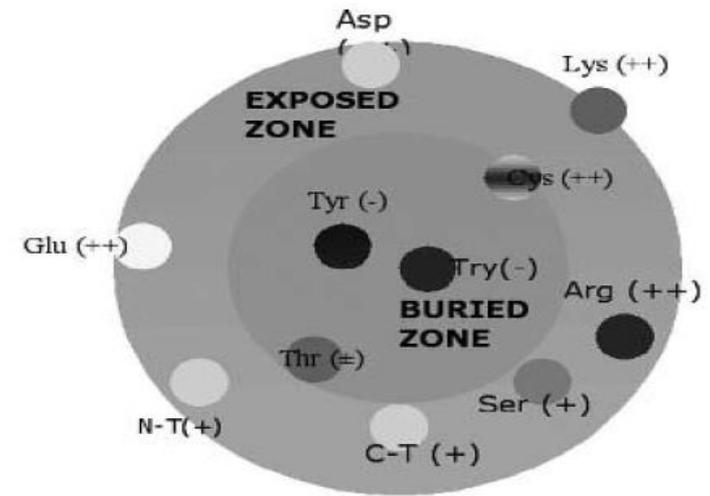
Resíduo	Porcentagem	Número de reações
L- Serina	7,8	0
L-Lisina	7,0	27
L-Treonina	6,5	0
Ácido L-Aspático	4,8	4
Ácido L-Glutâmico	4,8	4
L-Arginina	3,8	6
L-Tirosina	3,4	16
L-Cisteína	3,4	31
L-Histidina	2,2	13
L-Metionina	1,6	7
L-Triptofano	1,2	7

# Métodos de Imobilização

**Reatividade dos Resíduos → natureza química, microambiente, pH, força iônica, composição do meio de imobilização (normalmente aquoso)**

Reações químicas possíveis de ocorrer:

- Formação de peptideos → Lys, Asp, Glu;
- Diazotização → Arg, Cys, His, Lys, Tyr, Try;
- Alquilação; arilação e amidação → Lys
- reação de Ugi → Lys, Asp, Glu;
- Tiodissulfeto → Met;
- formação de base de Schiff → Lys, His, Cys, Tyr

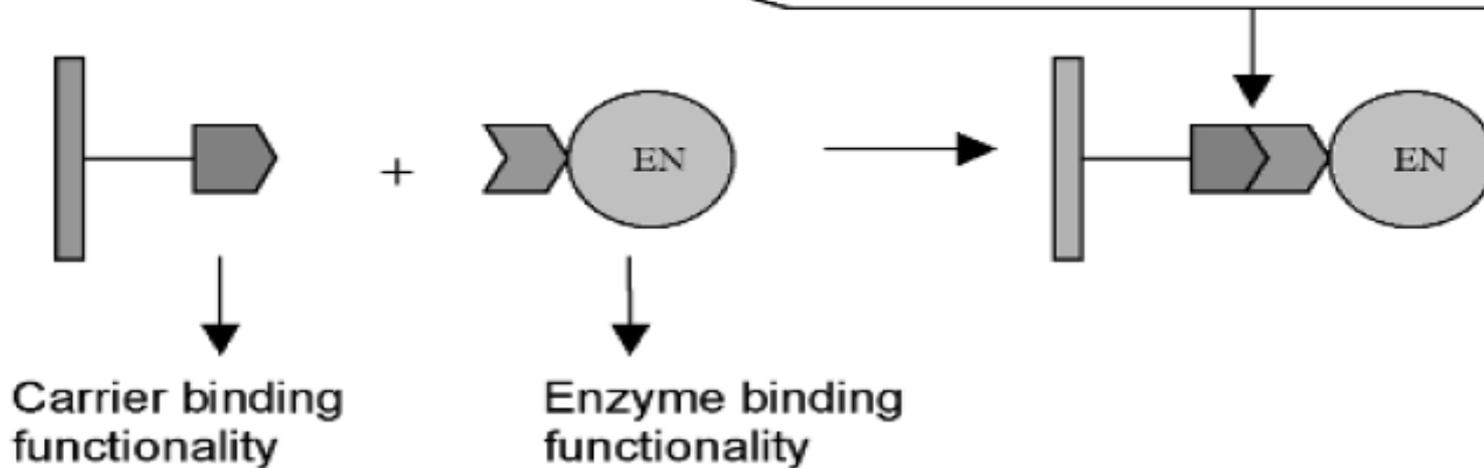


**Position of Active Amino Acids → normalmente os resíduos hidrofóbicos (Thr, Tyr e Trp) se mantem no interior da proteína e os hidrofílicos (Lys, Glu, Asp) na superfície, formamndo ligações hidrogênio com moléculas de água**

Em algumas situações isso pode ser alterado. Ex.: ↑ força iônica, ou adição de solvents orgânicos expõe os aminoácidos hidrofóbicos

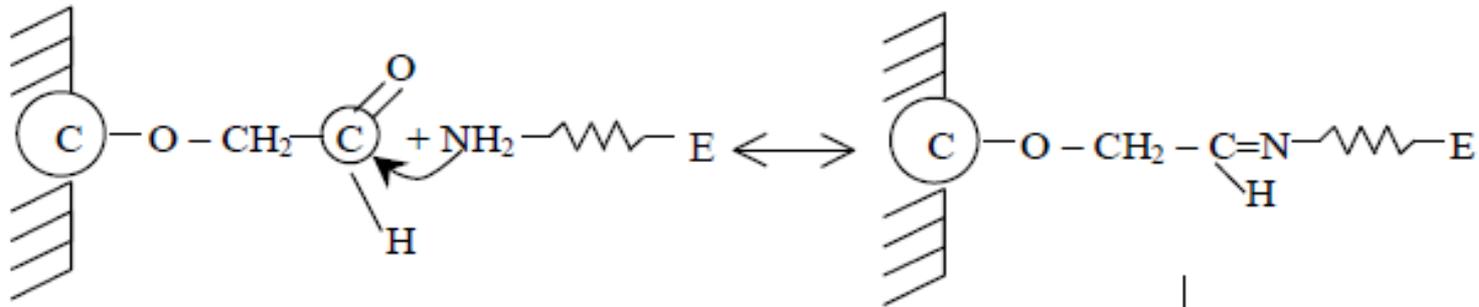
# Métodos de Imobilização

1. Diazotization
2. Peptide bond
3. Alkylation and Arylation
4. Schiff base formation
5. Ugi reaction
6. Amidination
7. Thiol-disulfide exchange reaction
8. Mercury-enzyme interaction
9. Oxidation

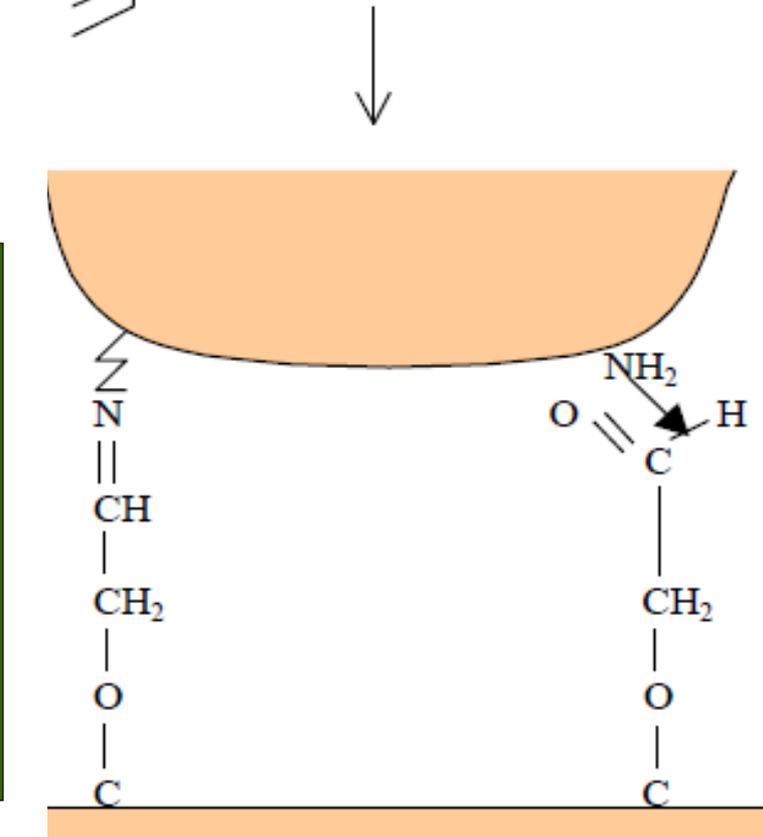
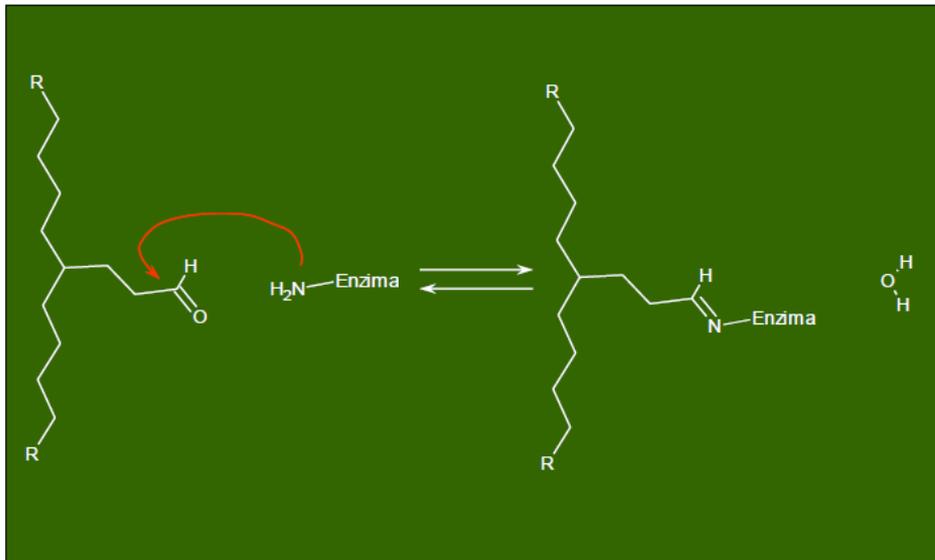


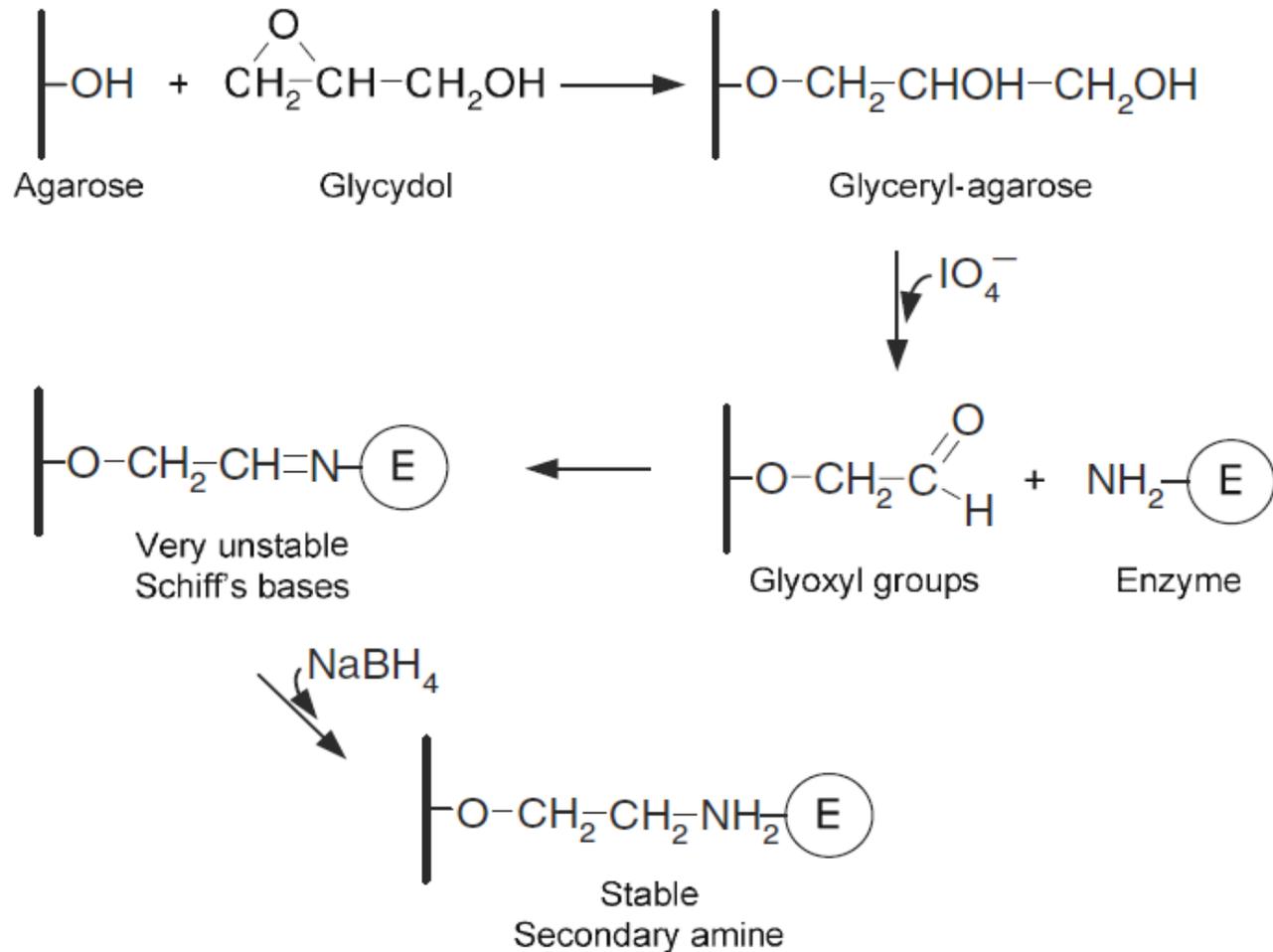
Scheme 3.14 Binding modes between carrier and enzyme.

# Multi-interação enzima-suporte



## Formação das bases de Schiffs

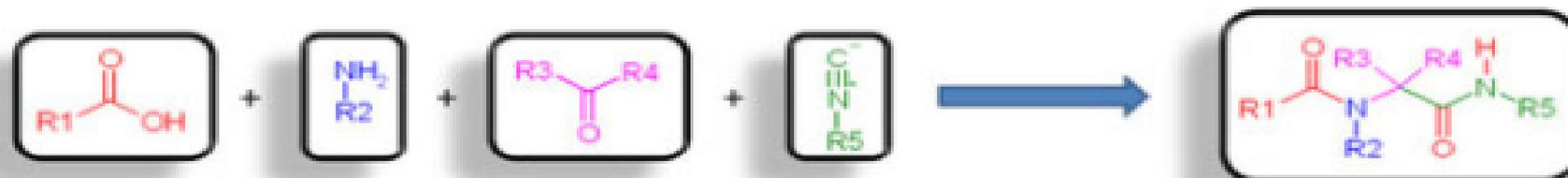




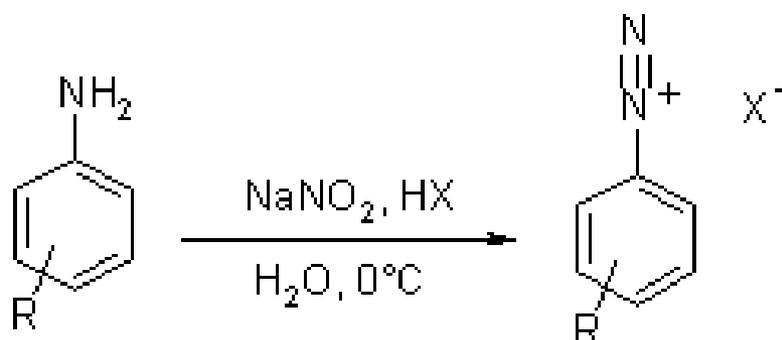
**Fig. 4.2** Scheme for enzyme immobilization in glyoxyl-agarose

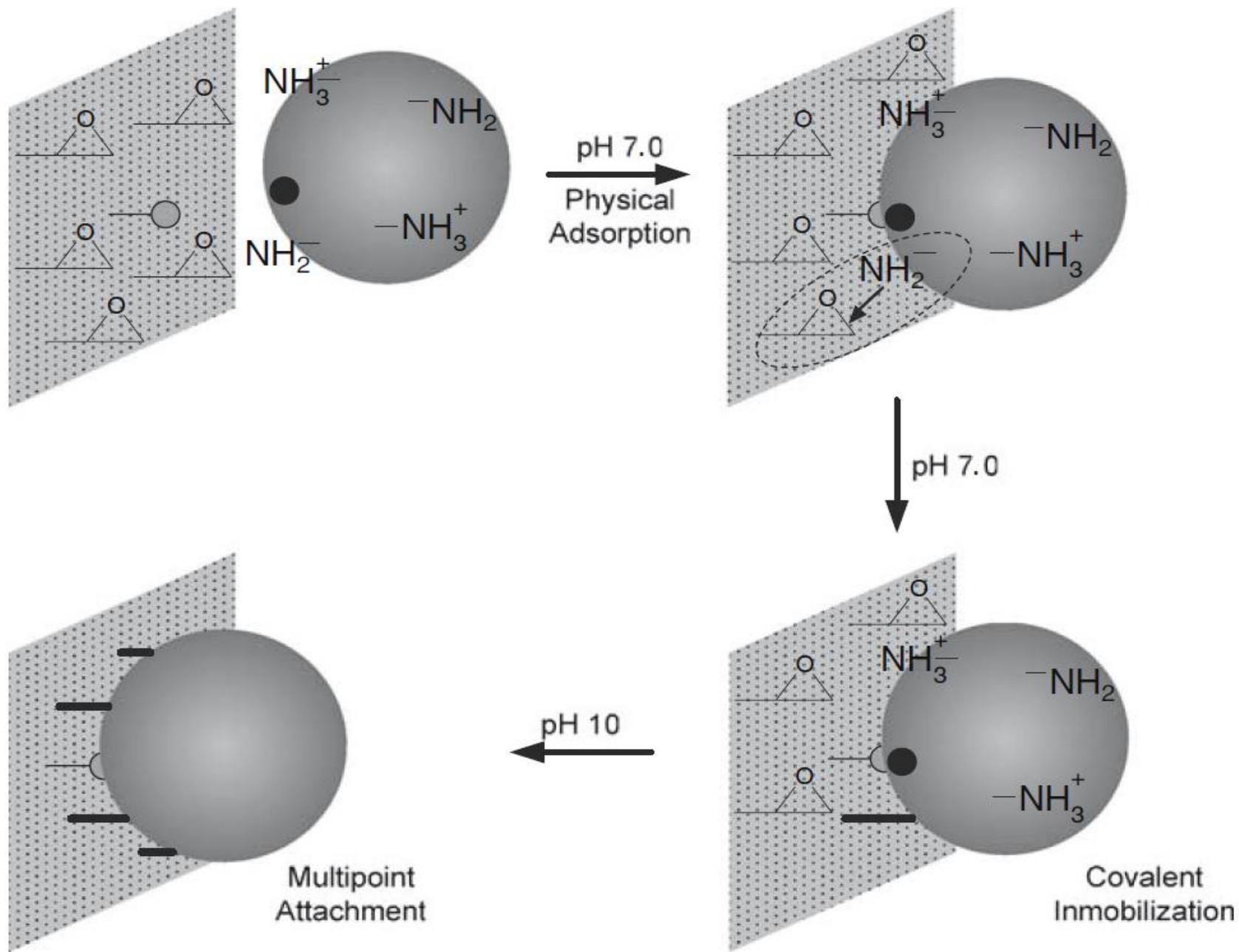
# Ugi reaction

The **Ugi reaction** is a multi-component reaction in organic chemistry involving a ketone or aldehyde, an amine, an isocyanide and a carboxylic acid to form a bis-amide. The reaction is named after Ivar Karl Ugi, who first published this reaction in 1962.



## Diazotisation





**Fig. 4.3** Immobilization of enzymes on heterofunctional epoxy supports

Some enzymes highly stabilized by  
multipoint covalent attachment on glyoxyl-agarose

Enzyme	Activity(%)	Stabilization
Trypsin	75%	10.000
Chymotrypsin	70%	60.000
Penicillin G acylase from <i>E. Coli</i>	70%	8.000
Penicillin G acylase from <i>K. citrophila</i>	70%	7.000
Ferredoxin NADP reductase from <i>Anabaena</i>	60%	1.000
Lipase from <i>C. rugosa</i>	50%	150
Glutamate racemase	70%	1.000
Esterase from <i>B. stearothermophilus</i>	70%	1.000
Thermolysin from <i>B. thermoproteolyticus</i>	100%	100

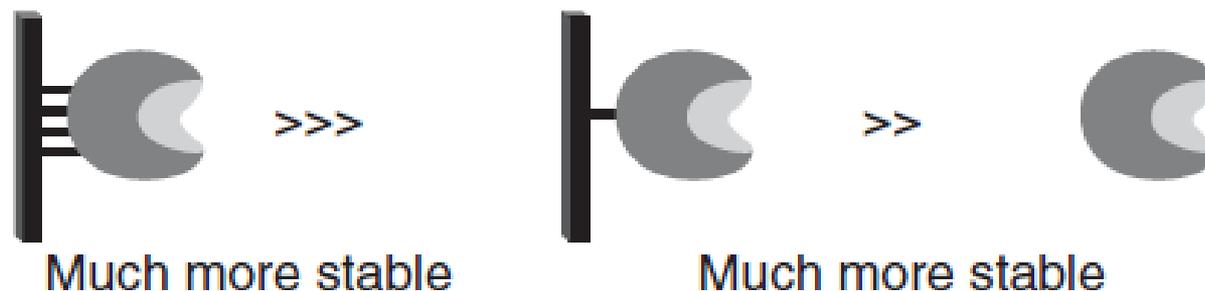


Figure 54.16. Some example of enzymes highly rigidified by multipoint covalent immobilization.

# Métodos de Imobilização

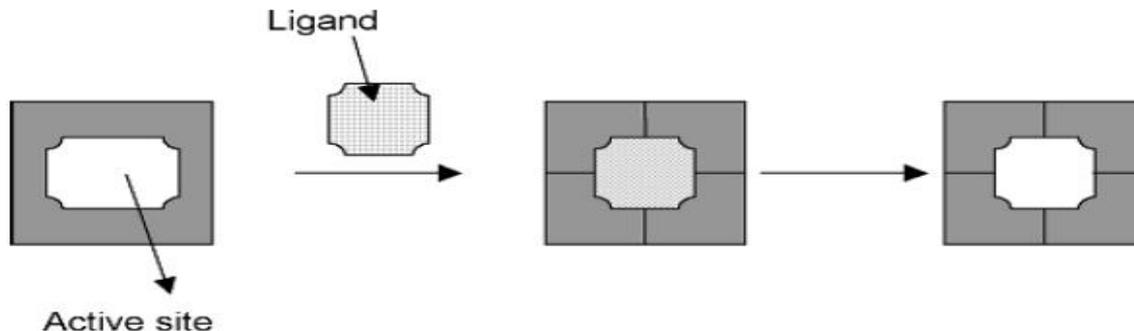
## Reticulação ou Ligação Cruzada

Método que envolve o uso de agentes bifuncionais ou multifuncionais, em geral diaminas alifáticas (di-ísoocianato), benzoquinona ou aldeídos (glutaraldeído, dextrana polialdeído), que induzem a auto-reticulação das enzimas, resultando assim na formação de uma rede tridimensional de moléculas de enzima.

Para facilitar o processo de reticulação, as enzimas são precipitadas na forma de cristais puros (CLEC) ou na de agregados (CLEAS).

A agregação é causada por sais (sulfato de amônio, sulfato de sódio), solventes (etanol, propanol, acetone, t-butanol) e outros (PEG, SDS, triton, crown ether).

**As condições de cristalização são afetadas por força iônica e pH. A presença de substratos, análogos, inibidores ou surfactantes no processo induz uma conformação correspondente no cristal formado → impressão molecular**



Scheme 6.20 Illustration of imprinting enzyme immobilization

# Métodos de Imobilização

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## Reticulação ou Ligação Cruzada

CLECs → enhanced thermostability, mechanical stability, stability against organic solvents and broad pH stability.

→ limited by the requirement for a successful crystallization of the purified enzyme. applied to a limited number of enzymes including ribonuclease A, subtilisin, carboxypeptidase B, alcohol dehydrogenase and some lipases.

CLEAs → more stable to denaturation by heat, organic solvents and proteolysis than the corresponding soluble enzyme. superior operational stability, volumetric productivities and recoverability.

→ fragile for many industrial applications in almost any kind of reactor configuration (basket reactors (BRs) may be an exception) and it is difficult to handle and fully recover the CLEA particle, the internal mass-transfer limitations of CLEAs brings about special accessibility problems for macromolecular substrates.

applied to an increasingly wide selection of hydrolases, oxidoreductases and lyases.

# Métodos de Imobilização

## Reticulação ou Ligação Cruzada

### CLEA

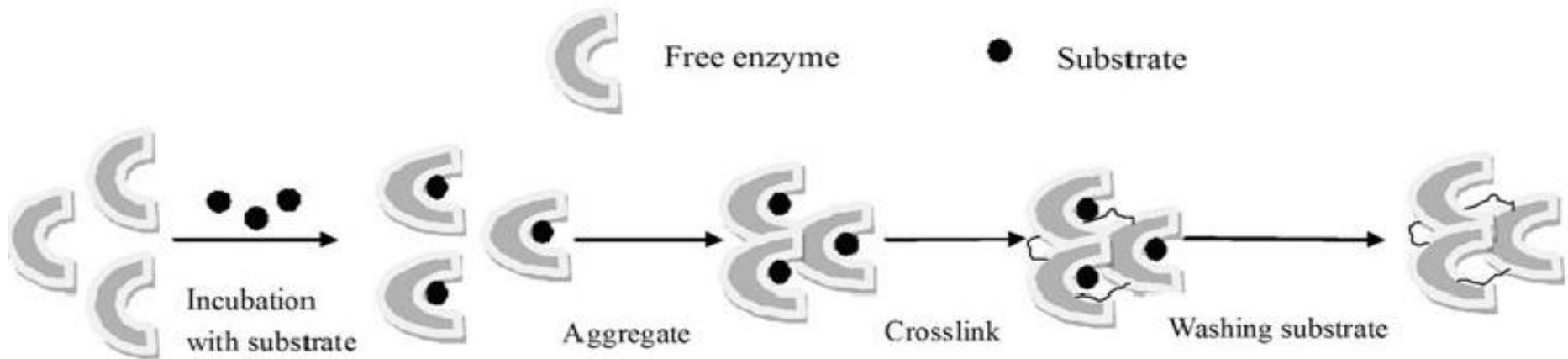


Figure 3. General scheme for imprinted CLEAs preparation.

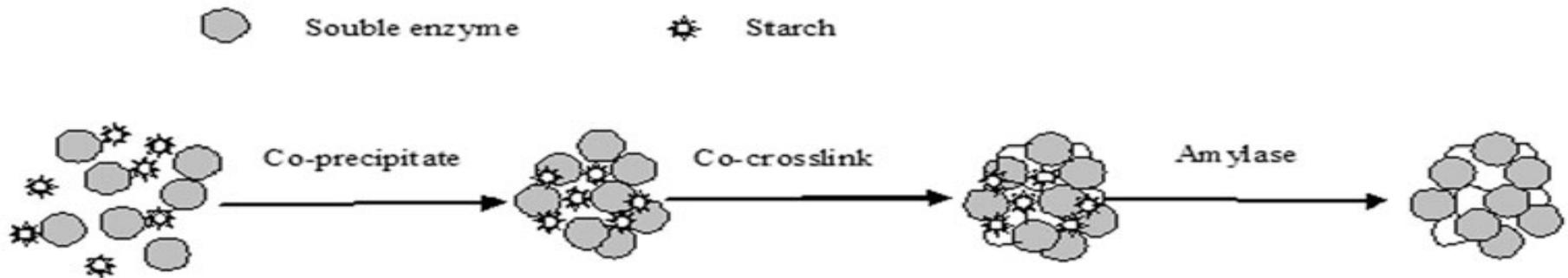
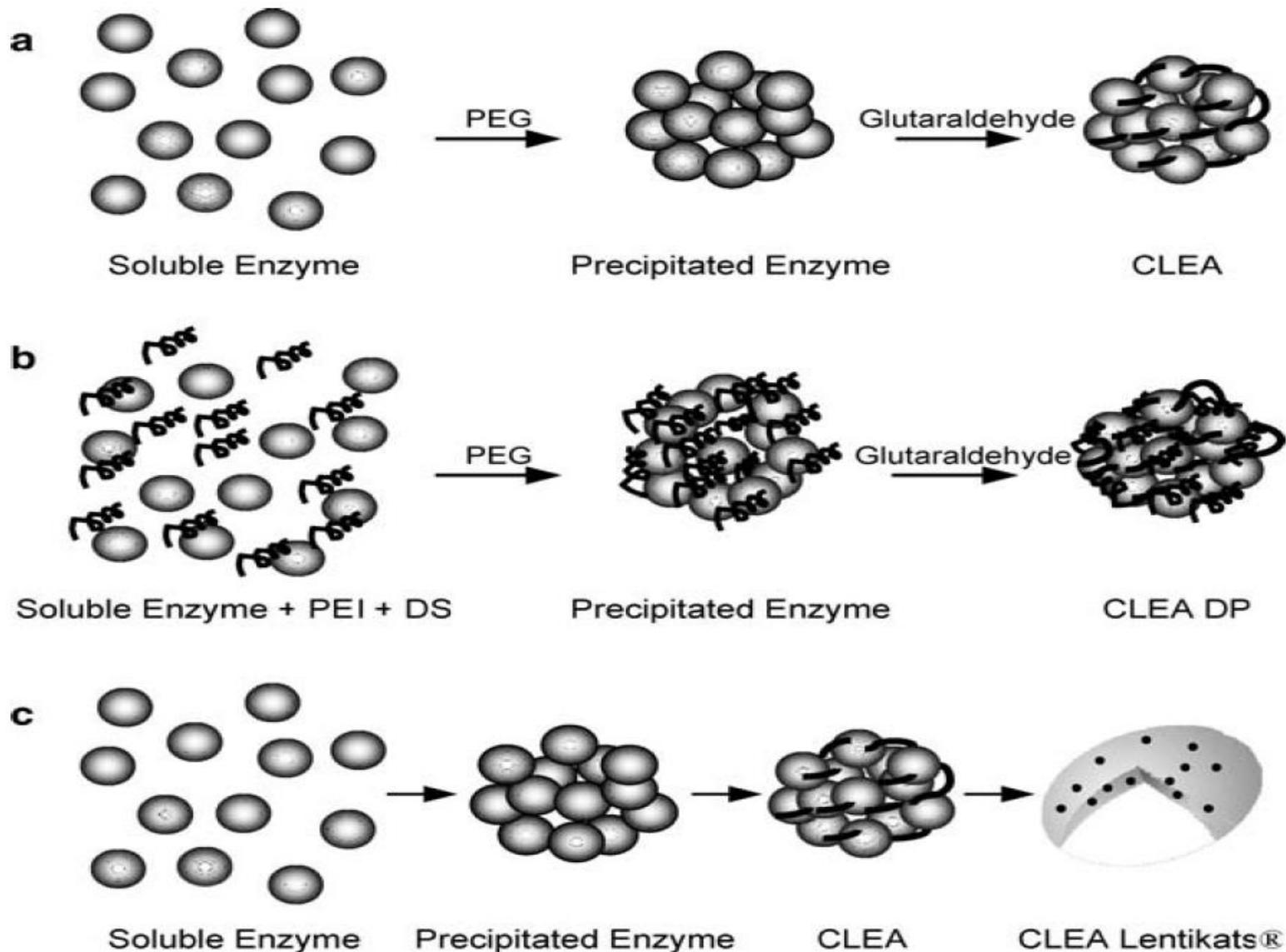


Figure 5. General scheme for porous-CLEAs preparation.



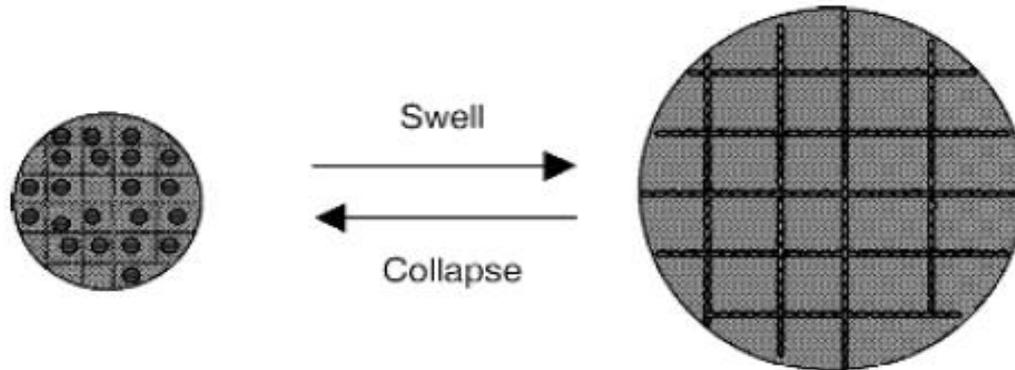
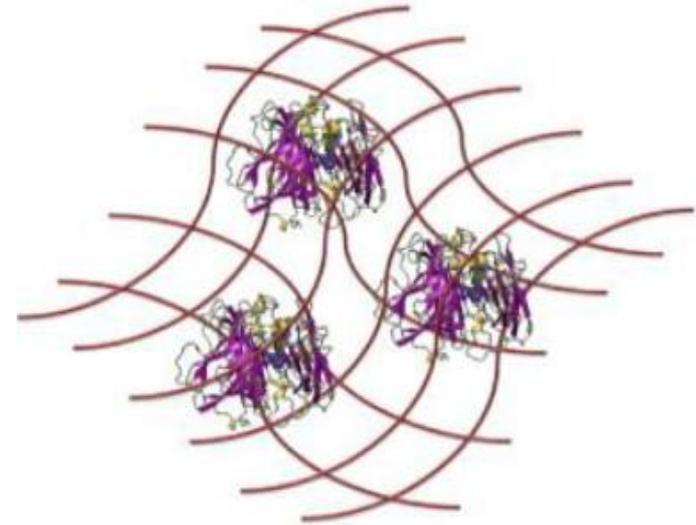
**Fig. 4.4** Schematic representation of the preparation of CLEAs: a) using polyethylene glycol (PEG) as precipitating agent and glutaraldehyde as cross-linking agent; b) using highly hydrophilic microenvironment composed by polyethyleneimine (PEI) and dextran sulfate (DS), (PEG) as precipitating agent and glutaraldehyde as cross-linking agent; c) using polyethylene glycol (PEG) as precipitating agent and glutaraldehyde as cross-linking agent and encapsulation into polyvinyl alcohol lens-shaped gel particles (LentiKats)

# Métodos de Imobilização

## Confinamento

A enzima é confinada (ou aprisionada) em uma matriz, permeável aos substratos, formada pela solução polimérica, utilizando métodos físicos ou químicos para processo de solidificação.

- A matriz pode ter forma de beads, membranas, filmes, discos e fibras.
- O processo de preparação dos suportes não pode ser separado do processo de imobilização.
- Quando a matriz fica turgida, esta pode ser considerada porosa



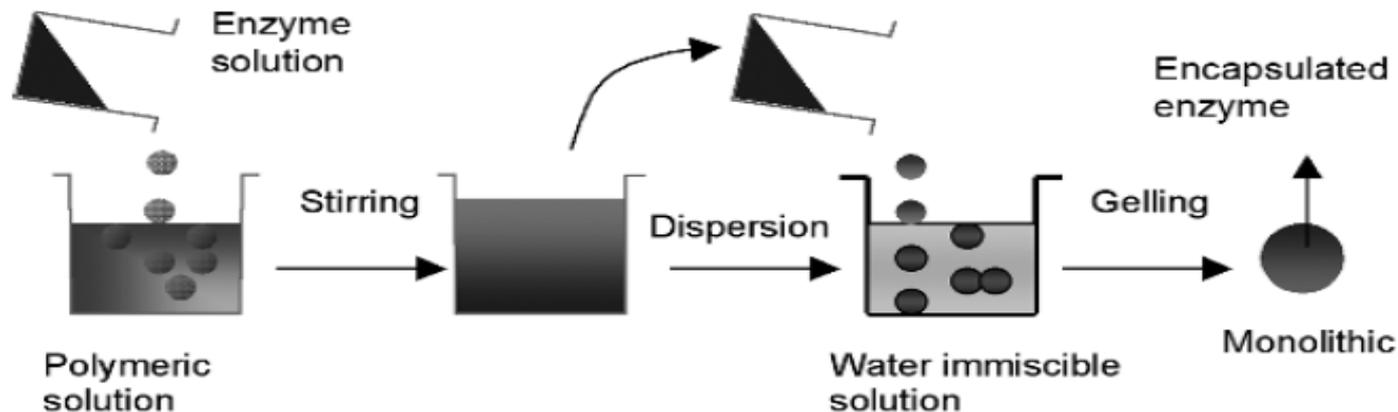
Scheme 4.2 Enzyme molecules immobilized in a gel matrix.

# Métodos de Imobilização

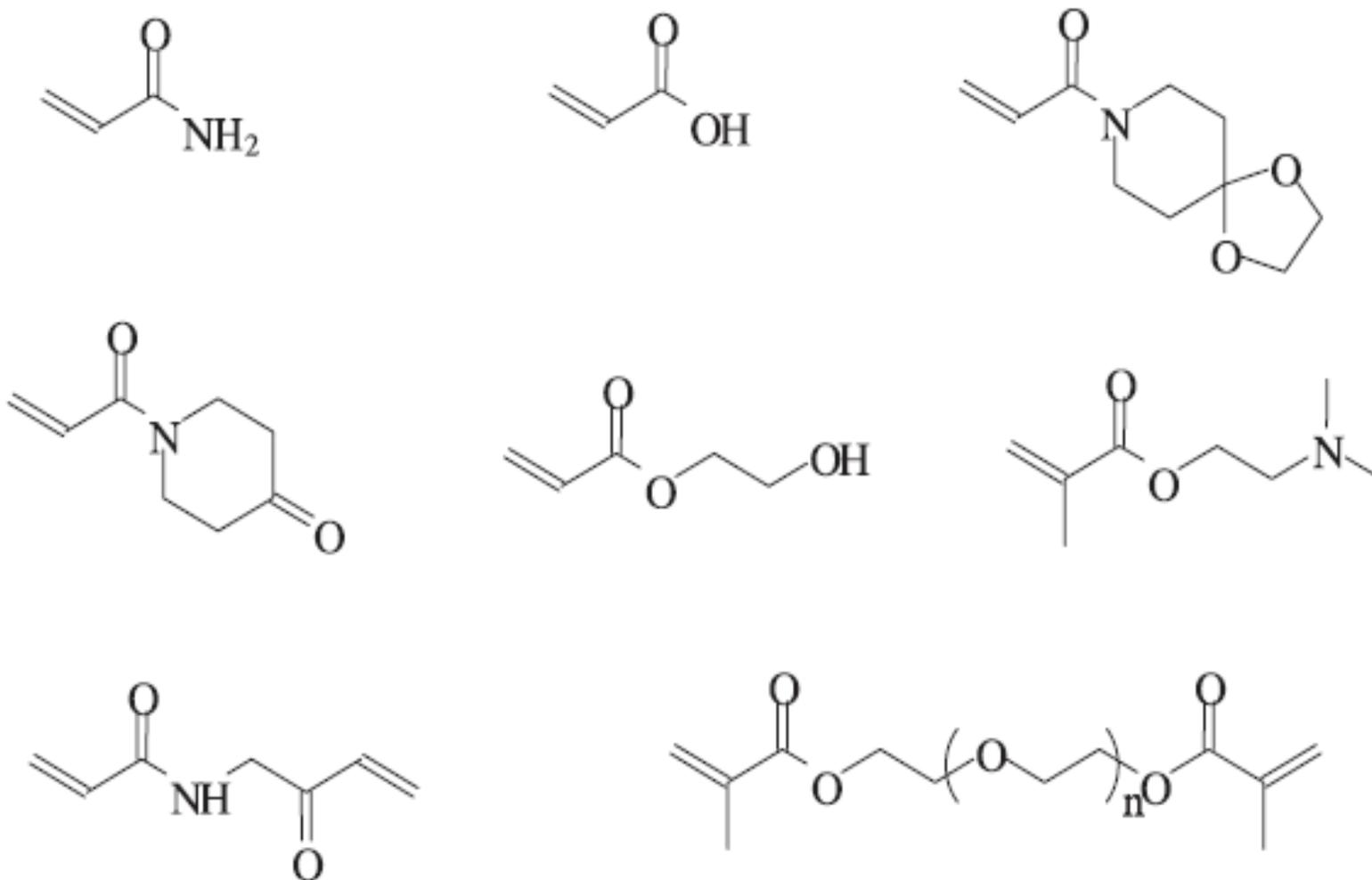
## Confinamento por Polimerização

Monômeros insaturados (ex. acrilamida, ácido acrílico com glicidilacrilato, 2-hidroxietilmetilacrilato, *N*-vinilpirrolidona, 2-hidroxipropilamida, 2-hidroxipropilacrilato, poli(etilenoglicol)metilacrilato, butanodiolacrilato, etilenoglicoldiacrilato) são utilizados como cross-linker, e a reação pode ser iniciada por irradiação, luz ou quimicamente. Controle do tamanho da matriz e da atividade enzimática estão relacionados com a concentração de monômeros e cross-linkers. Além disso o desempenho da enzima também dependerá da natureza dos monômeros e da concentração do iniciador (ou radical).

Ocasionalmente surfactantes, PVA e PEG podem ser utilizados para evitar a inibição da enzima e aumentando a porosidade da matriz

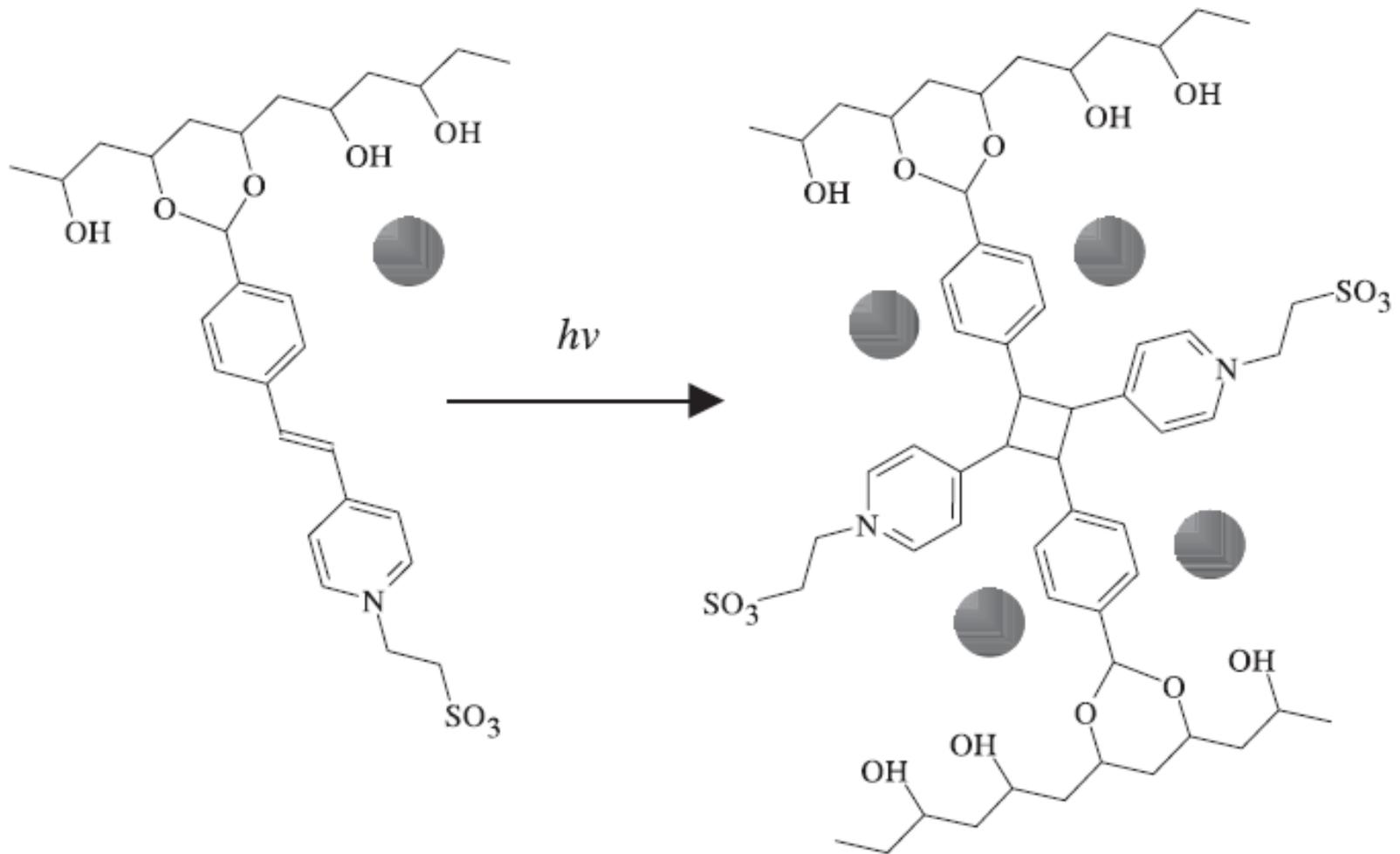


# Métodos de Imobilização



Scheme 4.7 Monomers used for polymerization entrapment.

# Métodos de Imobilização



Scheme 4.8 Entrapment of enzyme in a matrix formed by photo-initiated polymerization.



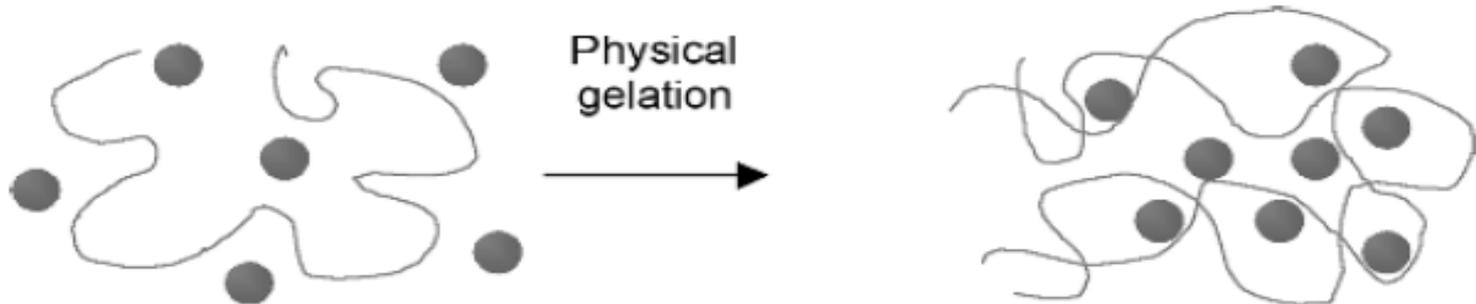
# Métodos de Imobilização

## Confinamento por Gelificação Física

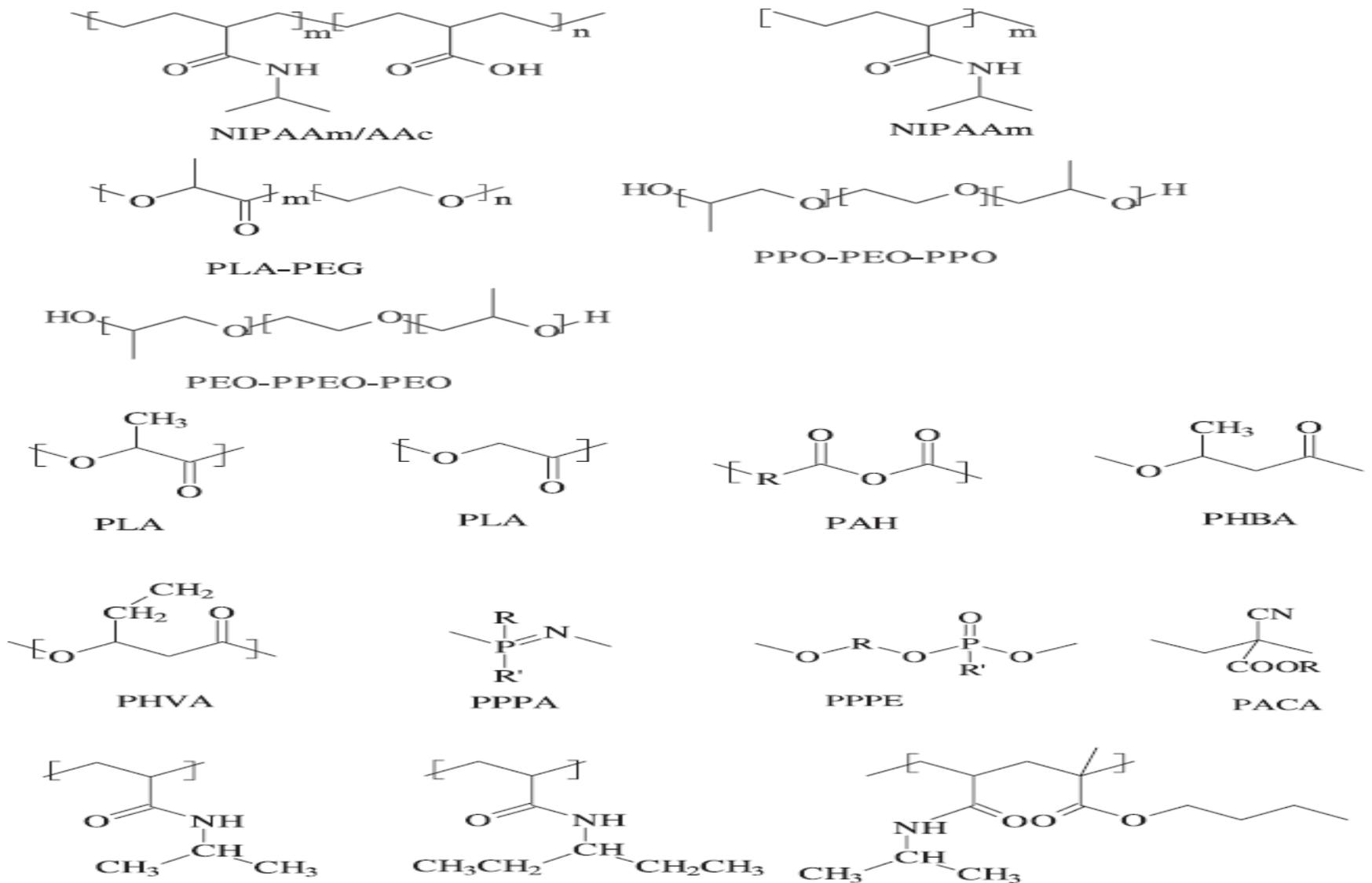
Enzima e a solução polimerica dissolvida confinadas a partir de alterações de condições ambientais como temperatura, pH, solventes ou força iônica. Ex: PVA em baixa temperatura; alginate e cálcio; inversão de fases por remoção de solventes.

Outros polímeros: proteínas (albumina, gelatina, colágeno, caseína), polissacarídeos (quitosana, agarose, amida, carragenana, cellulose, pectin, galactomananas, xantana) e derivados (etilcelulose, propilalginato); PEI, PAA; geis sintéticos (PLA-PEG, poly(*N*-vinylcaprolactam, *N*-alkylated polyacrylamides, poly(methyl vinyl ether), poly(*N*-isopropylacrylamide-coacrylic acid, PEO-PPO-PEO – Pluronic, polyoxamers)

Interação da enzima com a matriz por ligações de hidrogênio, pontes salinas e interações hidrofóbicas, além da restrição de mobilidade auxiliarem no aumento de termoestabilidade e também nas restrições difusionais



# Métodos de Imobilização



Scheme 4.10 Polymeric smart polymers for entrapment of enzymes.

# Métodos de Imobilização

Table 4.6 Enzyme entrapment by physical gelation effect of temperature

Gel/precursors	Method of gelation	Comments	Enzyme
Agarose	Cold-set gelation	Confinement increases enzyme thermostability by increasing its molecular rigidity	Mesophilic $\beta$ -galactosidase from <i>Aspergillus oryzae</i>
Gelatin	Cold-set gelation	The cells were immobilized in an open-pore gelatin matrix which was prepared by selectively leaching alginate/dopant	<i>K. marxianus</i> cells with inulinase
Agarose	Cold-set gelation	Among the methods tested (e.g. adsorption, cross-linking, and entrapment), entrapment proved to be best method of immobilization	P 450 monooxygenase containing chloroplasts and yeast microsomes
Agarose	Cold-set gelation	10% retention of activity	Cells of pseudomonas species
Agar	Cold-set gelation	Compared with adsorption on Dowex 1, the activity with entrapment is eight times higher, with improved stability	Rhodococcus AJ270 containing amidase activity
Agar	Cold-set gelation		Whole cell lactase of <i>E. coli</i>
Gelatin	Cold-set gelation	Retention of activity is approximately 88%, which is higher than in Cu alginate and PAAm gel (67 and 57%). Enzyme stability is also higher	Tyrosinase
	Iterative freezing-thawing	The presence of Con A facilitates the diffusion of the substrates through the pores	Glucoamylase
PVA	Freezing/thawing	The PVA concentration did not affect the kinetic behaviour of the immobilized cells. The pH of optimum enzyme activity was not changed by immobilization, although the activity profile was broader than that of the free cells	<i>E. coli</i> whole cells with $\beta$ -galactosidase
Agarose	Cold-set gelation	The stability of the sensors and their linear response range was strikingly improved by casting a cellulose acetate membrane on top of the agarose-enzyme gel	Glucose oxidase and lactate oxidase
Poly(vinyl Me ether) gel	Thermally induced reversible swelling-collapsing	The hydrolysis of maltose proceeds at temperatures below 37°C and stops above this temperature; the initiation and termination of this enzymatic process can be repeated by a rapid temperature switching, e.g. from 32 to 42°C and back to 32°C	<i>exo</i> -1,4- $\beta$ -D-Glucosidase
PEO-PPO-PEO	Heat-set gels	Temperature-sensitive polyurethanes are obtained by reacting trifunctional isocyanate with poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (Pluronic L 122)	Proteins

# Métodos de Imobilização

Table 4.7 Enzyme entrapment by physical gelation (pH-sensitive gel)

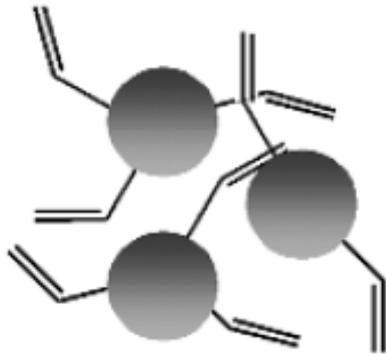
Gel/precursors	Method of gelation	Comments	Enzyme
Hydroxypropyl methyl-cellulose acetate succinate (AS)	Physical gelation	A reversibly soluble-insoluble transition occurred at pH 4.0; a sharp response of solubility to slight changes of pH without decrease in enzyme activity	Amylase
pH Responsive poly(MAAc-MA-MMA)	Physical gelation	High retention of activity; enhanced thermostability and stability in water-miscible organic solvent	Papain, chymotrypsin
Poly(ACr-co-AAc)	Physical gelation	Easily precipitated at pH 4–4.5 and re-dissolved at pH 7.0 with complete retention of activity	Trypsin
Polyethylenimine (PEI)	Physical gelation	Broad pH optimum in alkaline region	Invertase
Polyacrylic acid (PAAc)	Physical gelation	Broad pH optimum in acidic region	Invertase
PEI/Eudragit	Physical gelation	96 % retention of activity on Eudragit and more than 100 % activity was found with PEI; the payload is approximately 0.02–0.05	$\alpha$ -Amylase
AA poly(ethyleneimine) (CEPEI)	Physical gelation	Re-dissolved in organic solvent with full restoration of catalytic activity and remarkably high storage stability in the dry state	Chymotrypsin laccase
PVNEPB-PAAc (1 : 3)	Physical gelation	Slight change in pH and ionic strength can reverse the solubility	Penicillin G acylase
Eudragit L	Physical gelation	Reversibly soluble–insoluble, depending on pH	TV cellulase
Poly( <i>N</i> -vinyl-caprolactam)	Physical gelation	Increasing the NaCl concentration from 0.01 to 1.0 m shifted the half-precipitation temperature maximum from 34.5 to 24.5 °C	Penicillin amidase chymotrypsin
Eudragit L100-55	Physical gelation	Reversibly soluble–insoluble, depending on pH; similar performance to NE; and enhanced stability at 25–45 °C	<i>endo</i> -Pectinlyase
Eudragit S-100	Physical gelation	Retention of activity depends on the pH of the medium	AP, GL, GA, Trypsin xylanase
Eudragit S-100	Physical gelation	64 % retention of activity; 30 % reduced $K_m$	Trypsin

# Métodos de Imobilização

## Confinamento Covalente

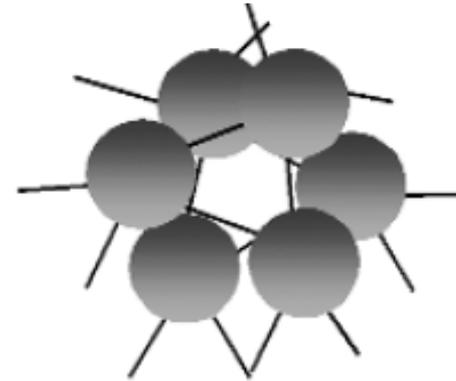
### - Polimerização de Enzimas Insaturadas

Inserção de grupos funcionais polimerizáveis na enzima e reação com cross-linkers



Modified enzyme with unsaturated bond

Polymerization



Chemically crosslinked enzyme

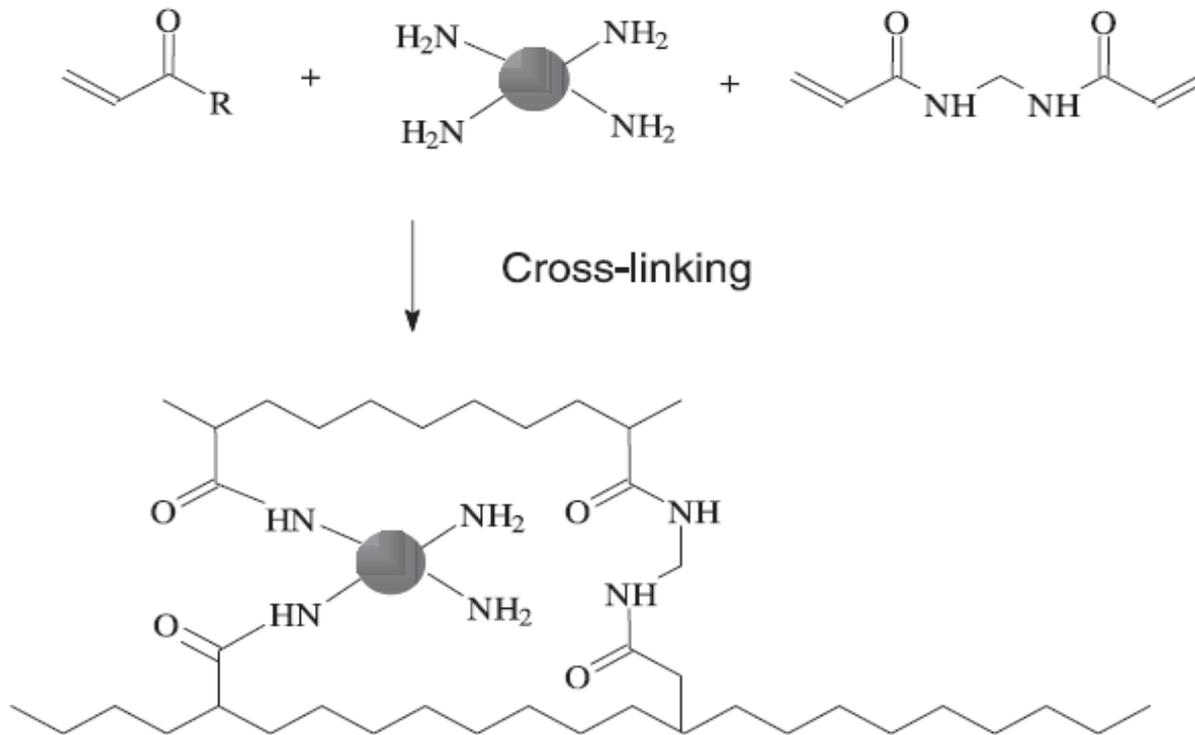
Scheme 4.12 Copolymerization of enzyme modified with unsaturated compounds.

# Métodos de Imobilização

## Confinamento Covalente

### - Híbrido Confinamento/Ligação Covalente in Situ

Enzimas podem se ligar a matriz durante ou depois da sua formação por polimerização, utilizando monômeros ativos e inertes



Scheme 4.13 Entrapment of an enzyme in a matrix by covalent bonding.

# Métodos de Imobilização

Table 4.10 Covalent entrapment

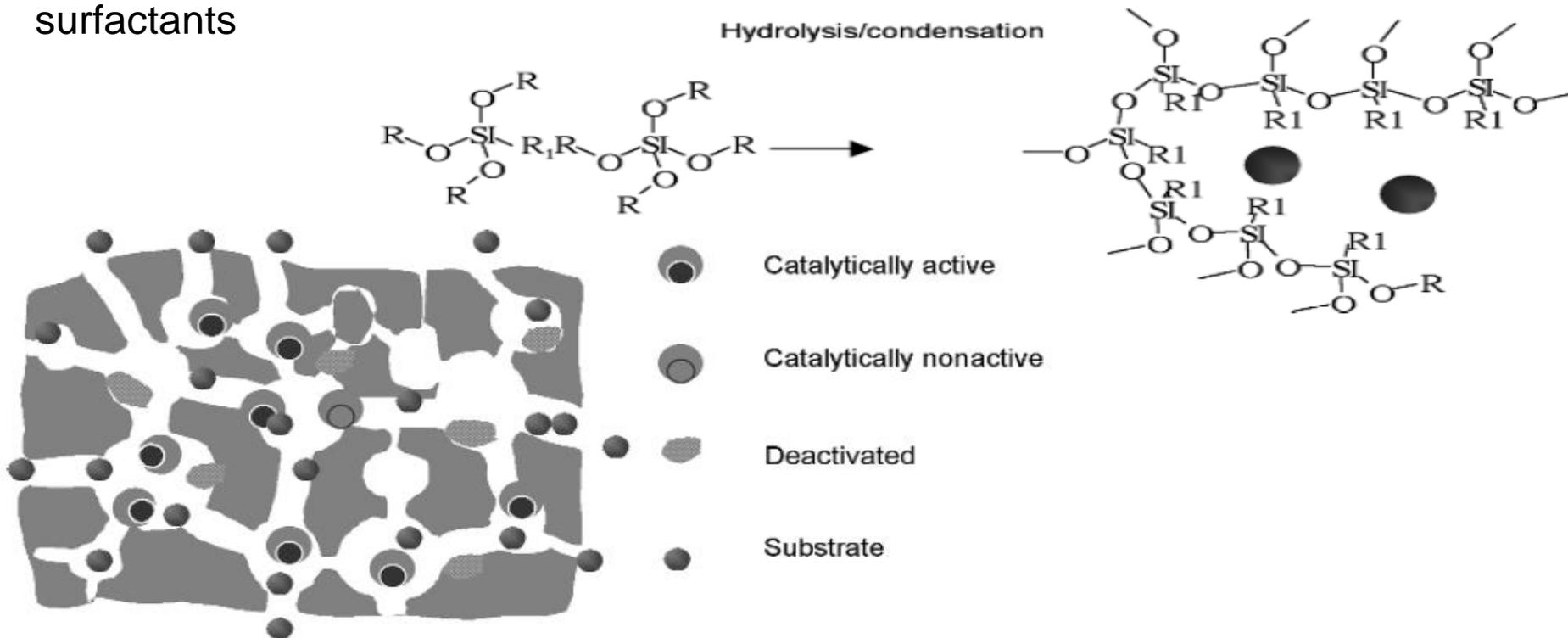
Matrix/monomers	Method	Comments	Enzyme
Poly(AAm-co-NAS-co-BAAm)	Entrapment	Covalently in good yields (20–90%)	ADK, ACK, HRP
A mixture of poly(acrylamide-co-N-acryloxysuccinimide) and triethylenetetraamine	Entrapment	A variety of enzymes were covalently immobilized in high (20–80%) yield. The resulting insoluble enzyme-containing gels have adequate physical properties for use in organic synthetic procedures	–
PAN, poly(acrylamide-N-acryloxysuccinimide/cystamine)	Entrapment	The gels are too soft to be used alone in columns, but by mixing them with filter aides or by forming them on glass beads, columns with excellent flow characteristics can be assembled	Enzymes
AAM/BisAAM/active monomer	Entrapment	The heat stability of immobilized glucoamylase gels is improved?	$\alpha$ -Amylases and glucoamylase
AAM/BisAAM/active monomer	Entrapment	Complimentary multipoint attachment	Enzymes
Glycidyl methacrylate/methylene bisacrylamide	Entrapment	Ca 15% activity retained; the immobilized enzyme obtained is more stable than the native enzyme	AMP deaminase
Polyacrylamide/AAM	Entrapment	The stabilizing effect increased with increasing the number of bonds formed	Chymotrypsin
AAM/BisAAM	Entrapment	The immobilized enzymes can be used repeatedly in the form of suspensions or columns without appreciable loss of activity	Modified penicillin G acylase and other enzymes
AAM/BisAAM	Entrapment	–	Maleic anhydride-modified penicillin G acylase

# Métodos de Imobilização

## Confinamento Sol-Gel

Enzima é adicionada a uma solução sol-gel de ácido silícico, seguido por um processo de gelificação sob influência de pH e aging process, formando materiais mesoporosos (2-20 nm), podendo ocorrer restrições difusionais

Para modular o tamanho dos poros, são utilizados PEG, polissacarídeos ou surfactants



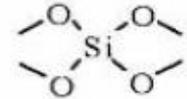
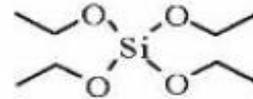
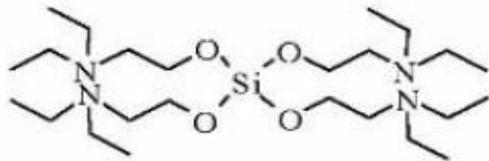
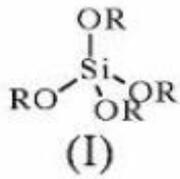
Scheme 4.16 Molecular accessibility of an enzyme entrapped in a gel matrix

# Métodos de Imobilização

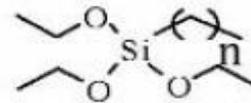
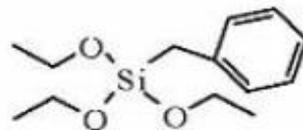
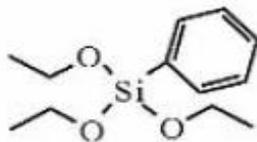
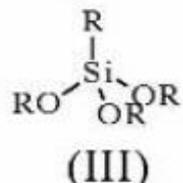
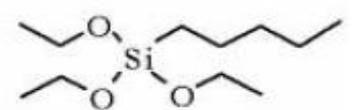
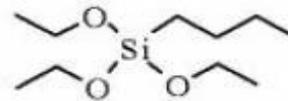
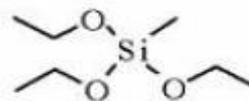
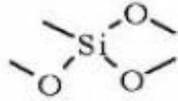
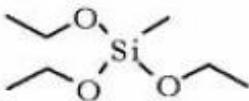
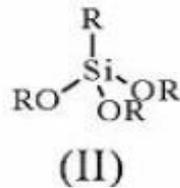
## Confinamento Sol-Gel

**Precursores:**  $(RO)_4Si$  (ex. tetrametoxisilano, TMOS, ou tetraetoxisilano, TEOS) e siloxanos modificados  $R''(R'O)_3Si$ .

simple tetraalkoxysilanes.



functional trialkoxysilanes; simple alkyl (methyl, ethyl, propyl, butyl, pentyl, cyclohexyl).



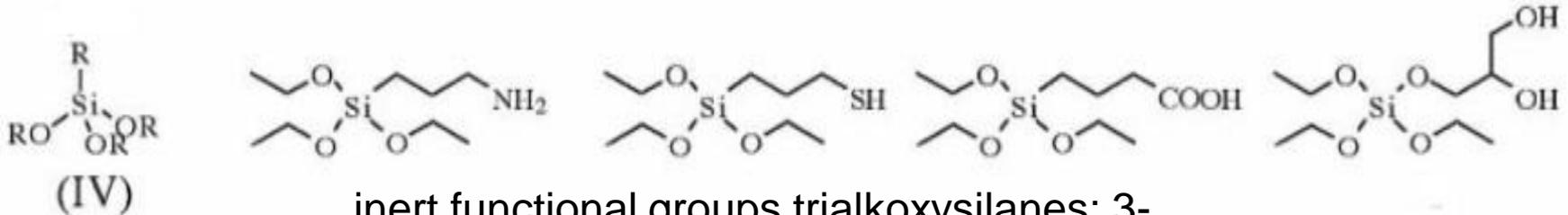
(n=6, 8, 10, 12, 14, 16, 18)

functional trialkoxysilanes; aryl (phenyl, benzyl, phenethyl) and long alkyl chains ( $R''=(C_nH_{2n+1}Si(OMe)_3)$ );

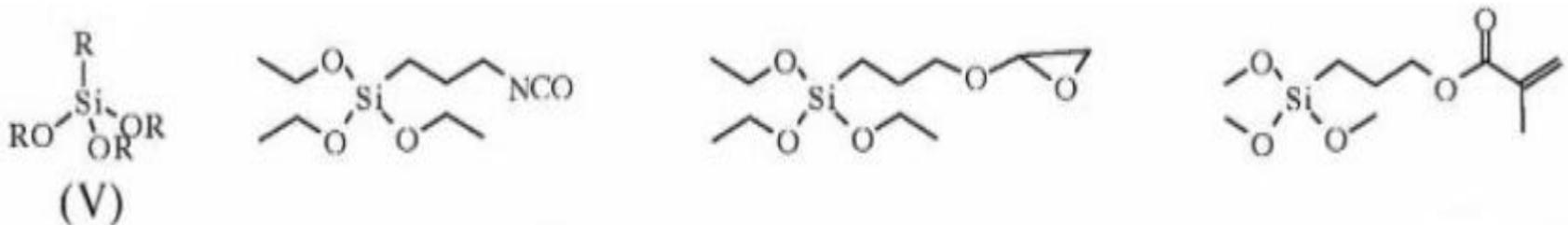
# Métodos de Imobilização

## Confinamento Sol-Gel

**Precursores:**  $(RO)_4Si$  (ex. tetrametoxisilano, TMOS, ou tetraetoxisilano, TEOS) e siloxanos modificados  $R''(R'O)_3Si$ .



inert functional groups trialkoxysilanes; 3-aminopropyl, 3-carboxylpropyl, 3-mercaptopropyl

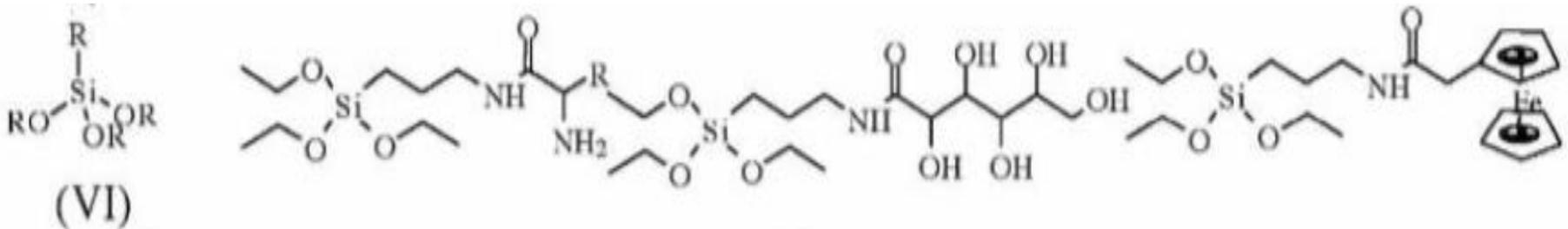


functional trialkoxysilanes; isocyanato, epoxy, photoactive double bonds.

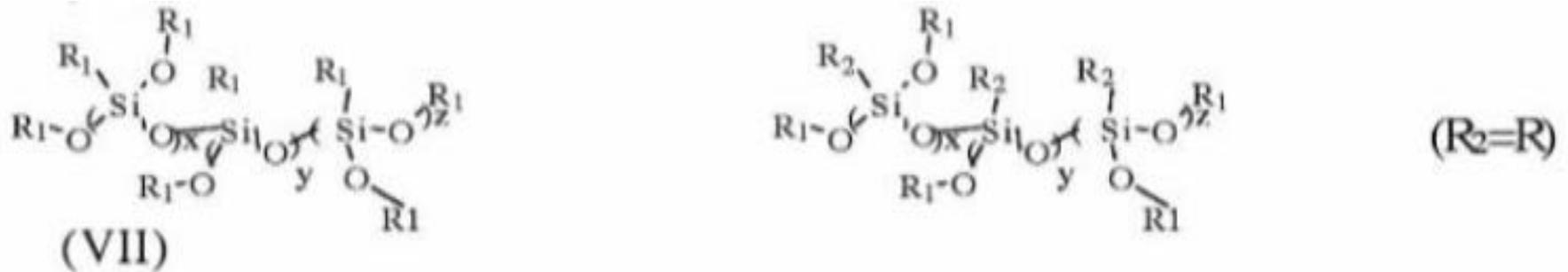
# Métodos de Imobilização

## Confinamento Sol-Gel

**Precursores:**  $(RO)_4Si$  (ex. tetrametoxisilano, TMOS, ou tetraetoxisilano, TEOS) e siloxanos modificados  $R''(R'O)_3Si$ .



inert functional groups trialkoxysilanes; which are usually used to modulate the microenvironment of the gel matrix.



poly(alkoxysilanes)

# Métodos de Imobilização

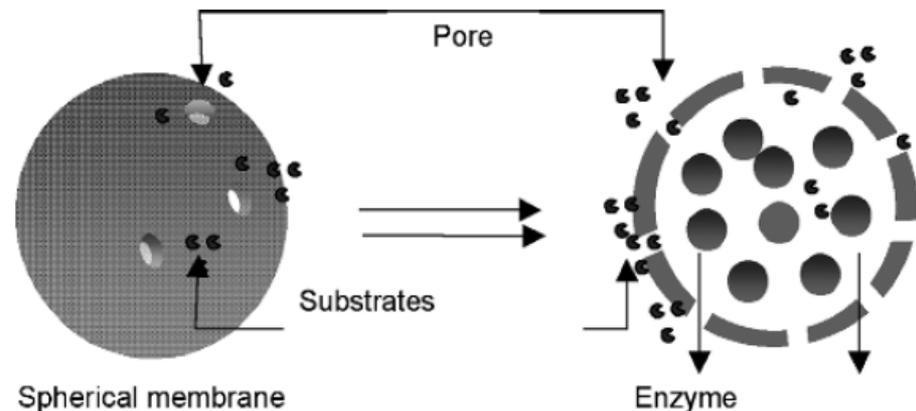
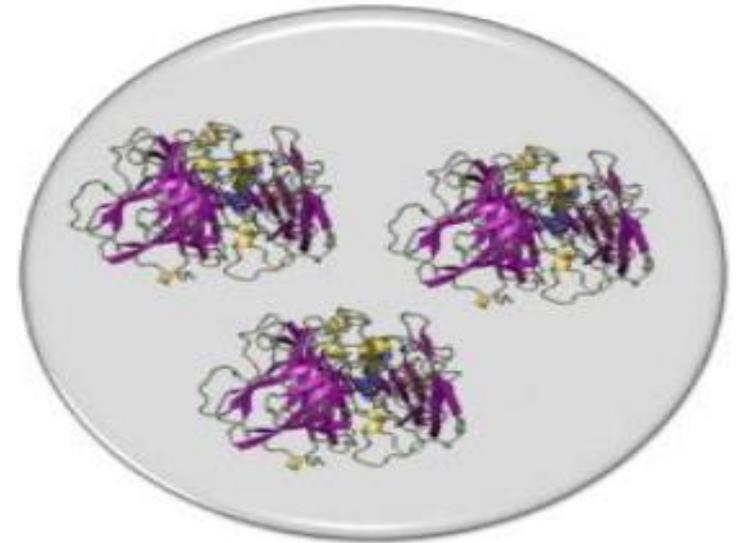
## Encapsulamento

Formação de uma barreira física em formato de membranas poliméricas semi-permeáveis ao redor da preparação enzimática, com diâmetros em torno de 1 a 100  $\mu\text{m}$ .

Há problemas com restrições difusionais, mas por outro lado permite a imobilização de várias enzimas diferentes juntas e reações sequenciais.

A natureza do monomer hidrofílico, a espessura da membrane, o processo de imobilização e o tamanho do poro podem afetar a atividade enzimática

A retenção de atividade varia de 20 a 90%



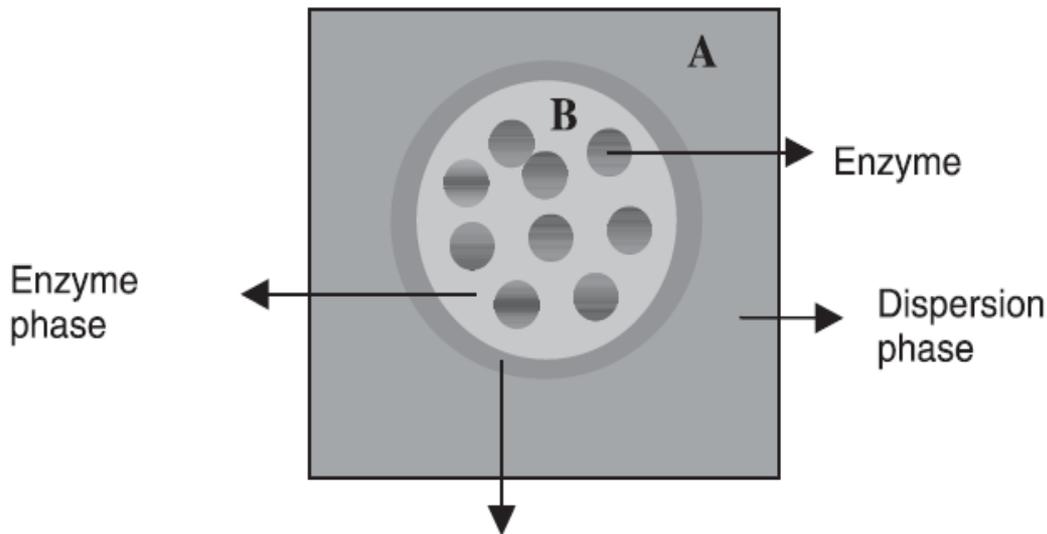
Scheme 5.2 Micro-encapsulation of enzymes in microcapsules.

# Métodos de Imobilização

## Encapsulamento: Métodos de preparo

### - Método Convencional: Processos interfaciais

- 1) Polimerização interfacial: Polimerizando os monômeros localizados na interface entre solução aquosa e solução orgânica imiscível
- 2) Deposição interfacial: Deposição do polímero na interface
- 3) Complexação interfacial: Formação de complexo insolúvel (A–B) na interface de duas soluções aquosas como na formação de um simplex (complexo entre polieletrólitos) Ex. Quitosana e alginato



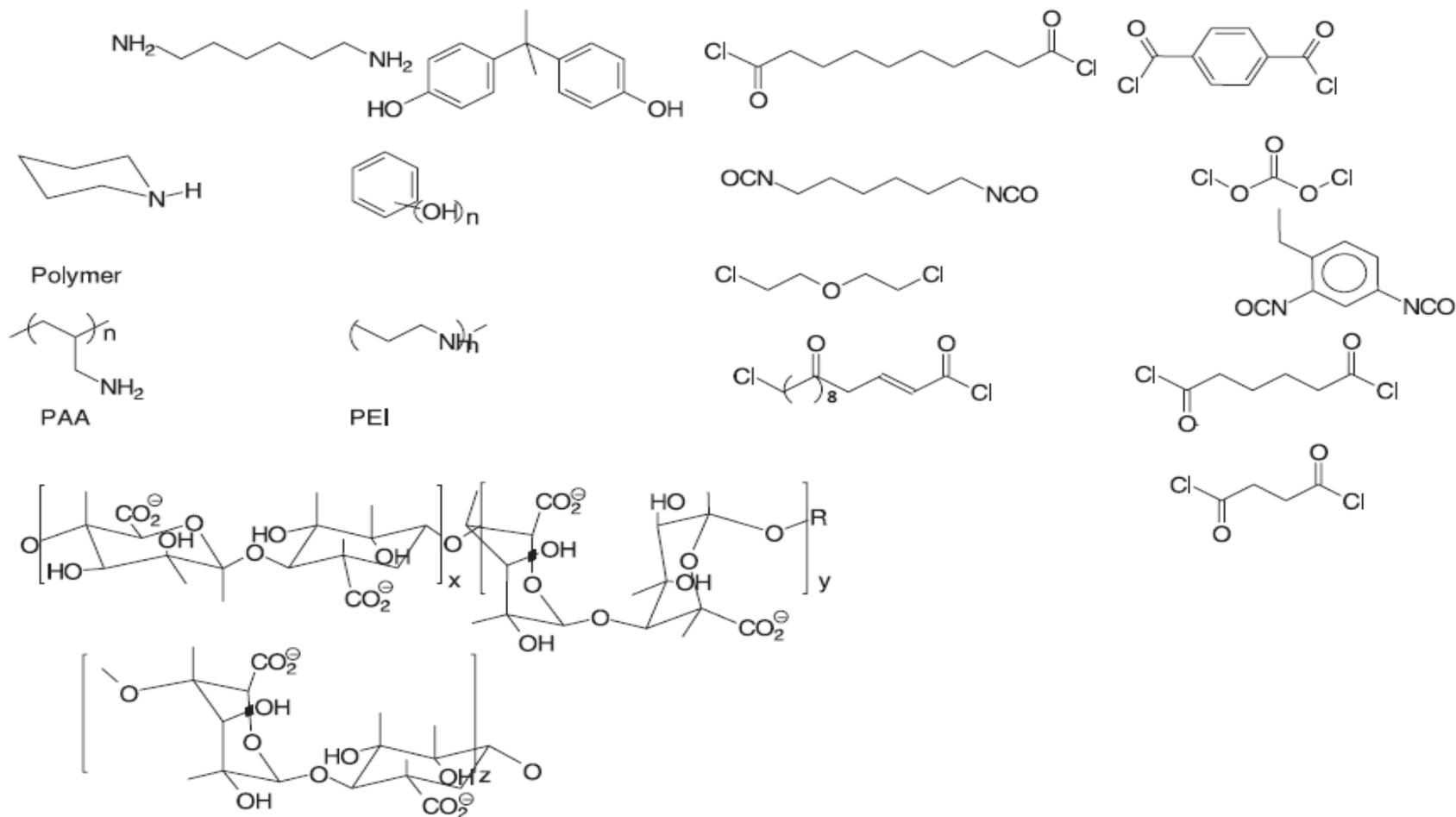
### Outros polieletrólitos

- Naturais: celulose sulfato, dextrana sulfato, pectina, carragenana e Xantana;
- Sintéticos: poli(l-lisina), PEI, gama-PGA, polidimetildialil amônio cloreto, poli(l-ácido aspártico), poly(methylene co-guanidine) (PMG), PLGA (poly(l-glutamic acid), poly(propylenimine), K poly(vinyl alcohol) sulphate (KPVS),

# Métodos de Imobilização

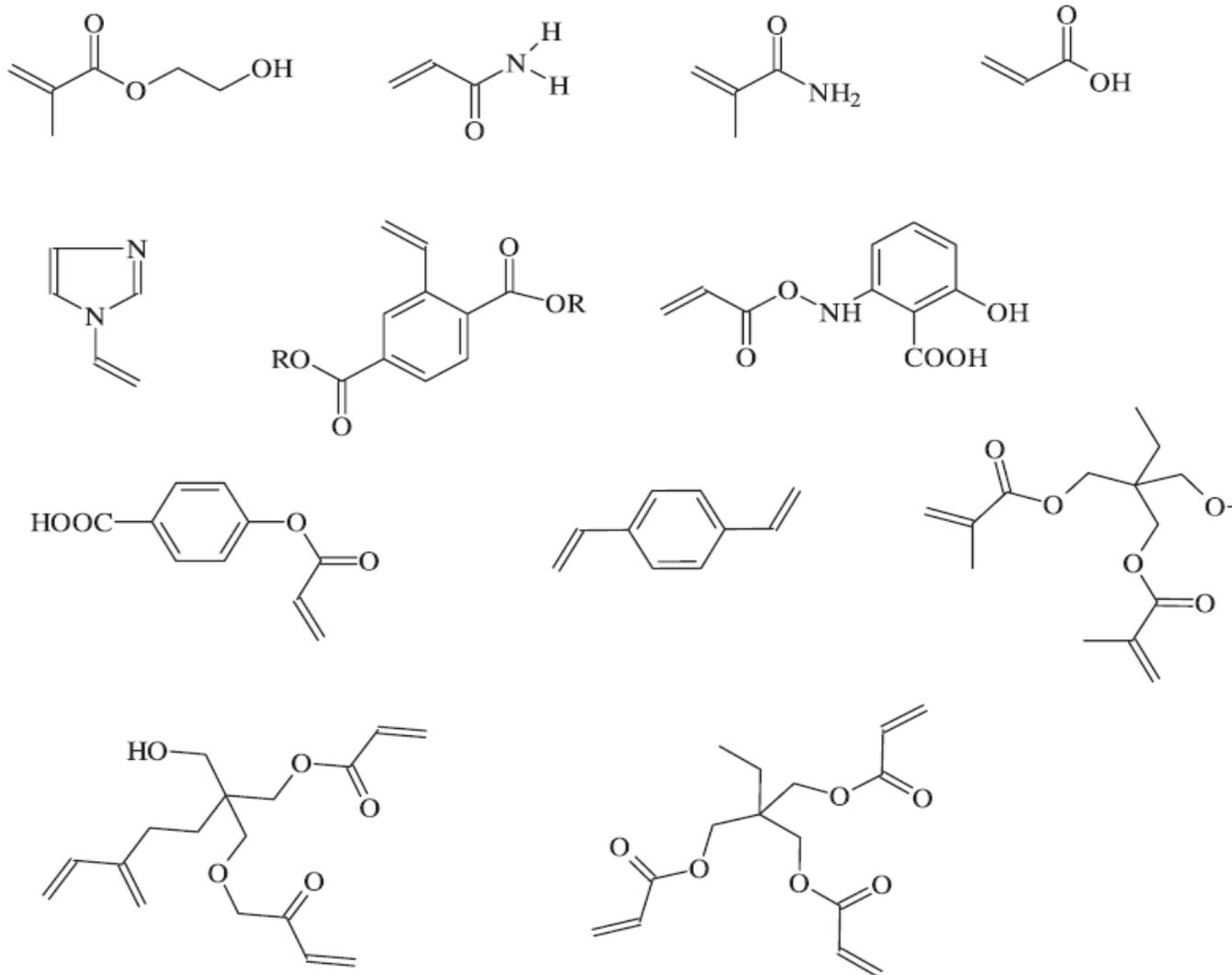
Water-soluble (B)  
Monomer

Water insoluble



**Scheme 5.9** Monomers and polymers for hollow sphere membrane (HSM) by interfacial cross-linking.

# Métodos de Imobilização



Scheme 5.10 Monomers for interfacial polymerization.

# Métodos de Imobilização

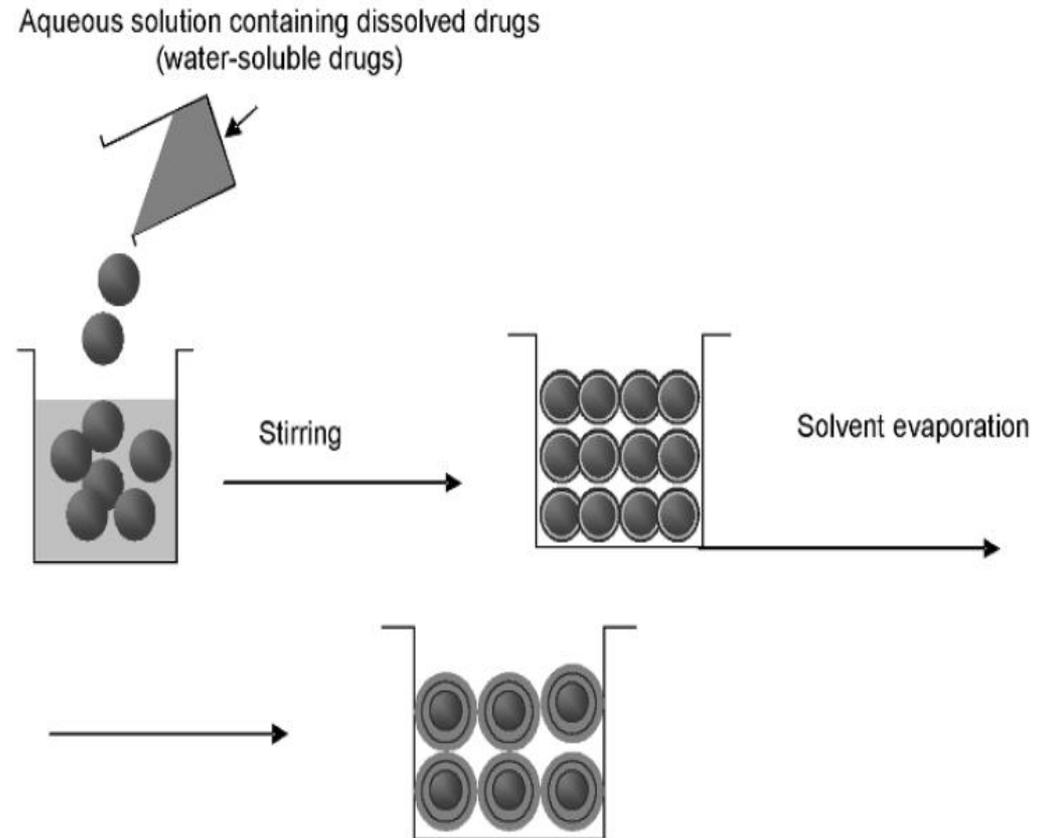
## Encapsulamento: Métodos de preparo

### - Método de Inversão de Fases:

A fase líquida em volta da enzima é solidificada devido a alteração de solubilidade estimulada por difusão ou evaporação dos solventes dos componentes a serem integrados a membrana.

Desvantagens: materiais custosos; inativação das enzimas; baixa eficiência de encapsulação, além de perdas por lixiviação

1) Coacervação: a solução aquosa da enzima é dispersa no solventes imiscível em água contendo o polímero, e depois a evaporação deste leva a deposição dos polímeros



Scheme 5.16 Preparation of monolithic microspheres.  
(This method is not suitable for entrapment of highly water-soluble drugs.)

# Métodos de Imobilização

Table 5.7 Coacervation process for preparation of HSM

Material	Method	Remarks	Enzymes
Cellulose nitrate?	Interfacial coacervation	Haemoglobin preserves the physical stability of the enzyme during microcapsule manufacture and also enhances the structural integrity of the microcapsules	–
Cellulose nitrate	Interfacial coacervation	Enzyme was immobilized in semipermeable microcapsules which retain the enzyme and allow asparaginase to diffuse in	Asparaginase
Cellulose nitrate	Interfacial coacervation		Asparaginase
Cellulose nitrate (collodion artificial cells)	Interfacial coacervation	Microencapsulated PAL reportedly loses 80 % of its activity. $V_{\max}$ is about 20 % of the free enzyme	Phenylalanine ammonia-lyase
Cellulose nitrate	Interfacial coacervation	Lower retention of activity was obtained because of deactivation of the enzyme during encapsulation. 70 % encapsulation efficiency of which 21 % is active	Phenylalanine ammonia-lyase
–	Interfacial coacervation	–	Phenylalanine ammonia-lyase
Collodion artificial cells	Interfacial coacervation	20 % activity retention was obtained	Phenylalanine ammonia-lyase
Collodion artificial cells	Interfacial coacervation	70 % entrapment efficiency and 20 % activity retention were obtained, because of deactivation of the enzyme by the organic solvents	Phenylalanine ammonia-lyase
Cellulose nitrate	Interfacial coacervation	Lost 88 % of its activity because of denaturation of the enzyme after contact with organic solvent	Arginase
Cellulose nitrate	Interfacial coacervation	$K_m$ was not changed but $V_{\max}$ decreased	Catalase
Cellulose nitrate	Interfacial coacervation	$K_m$ was not changed but $V_{\max}$ decreased	Histidase
Cellulose nitrate	Interfacial coacervation	–	Lactase
NH <sub>4</sub> polyacrylate-enzyme/ liquid paraffin	Interfacial coacervation	–	Alkaline protease
Cellulose nitrate	Interfacial coacervation	The artificial cells can be used to remove intestinal amino acids in diseases where they accumulate	Asparaginase, glutaminase, and tyrosinase

# Métodos de Imobilização

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## Encapsulamento: Métodos de preparo

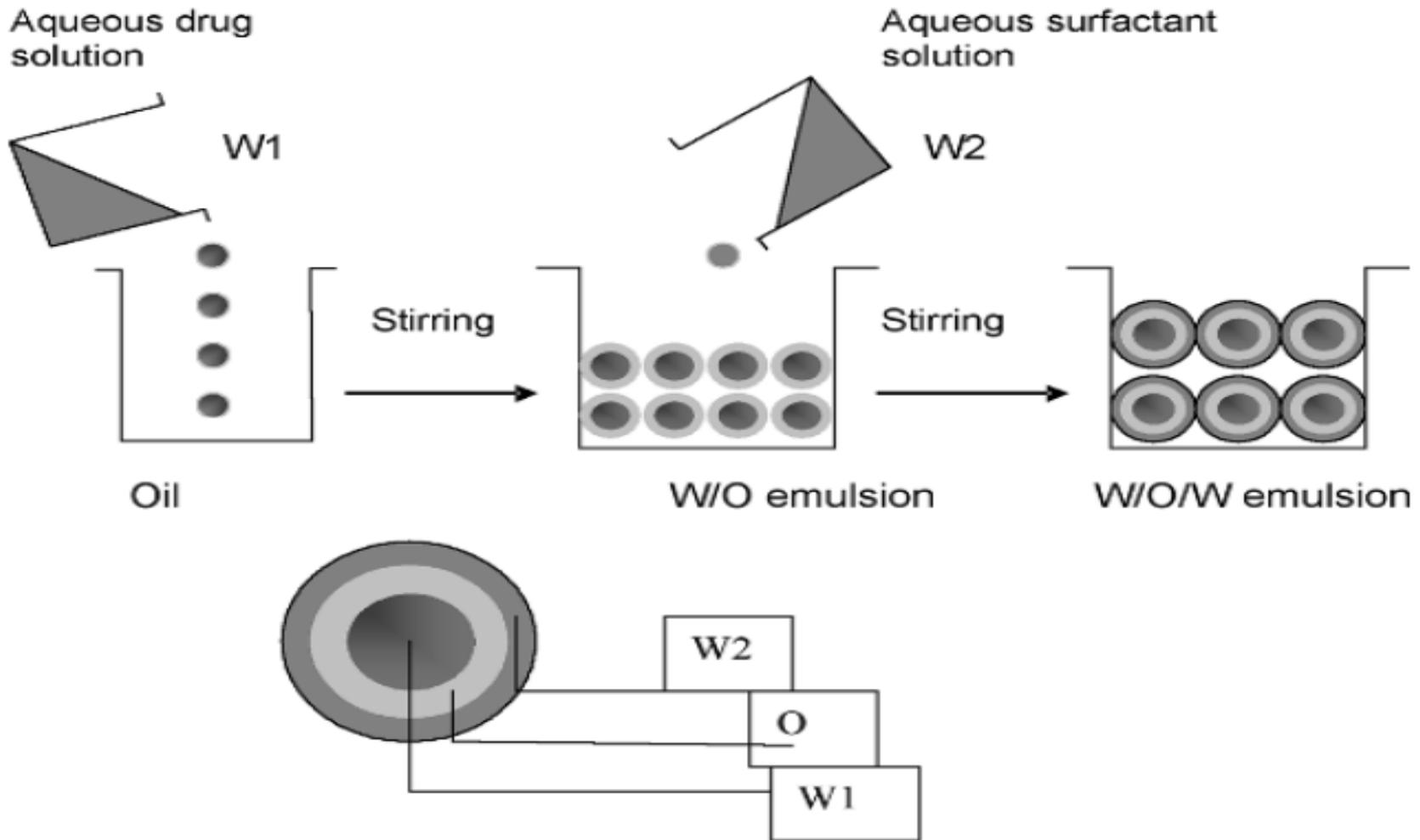
### - Método de Inversão de Fases:

2) Emulsões múltiplas, onde A é um polímero hidrofílico e B, um polímero hidrofóbico dissolvido em uma fase orgânica. O método de dupla emulsão W/O/W é o mais comum, mas existe: S/O/W (solid/oil/water), W/O/O (water/oil/oil), O/W<sub>1</sub>/W<sub>2</sub> (oil/water/water), O/W<sub>1</sub>/O (for encapsulation of oil).

Normalmente, a fase aquosa com a enzima é emulsificada com fase orgânica com polímero (PMM, PPP, Eudragit S, PLGA, poly(l-lactide-co-glycolide)-PEO), e depois a emulsão é reemulsificada com grande volume de fase aquosa contendo outro polímero (ex. PVA). O solvente orgânico evapora rápido e a precipitação ocorre na interface.

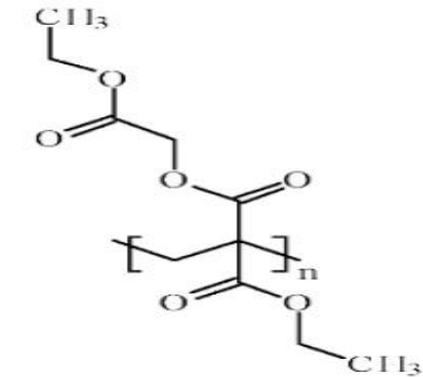
Na reemulsificação, é utilizado um polímero, surfactante ou glicerol para atuar como estabilizante

# Métodos de Imobilização

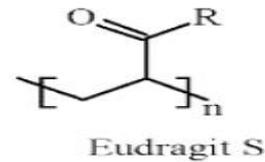


**Scheme 5.17** W/O/W emulsion for preparation of microspheres with in-situ encapsulation of drugs.

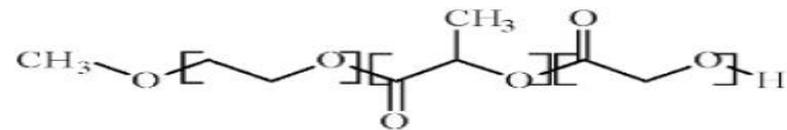
# Métodos de Imobilização



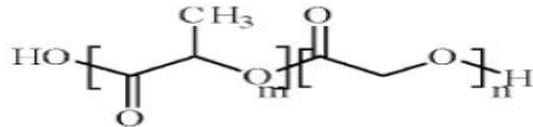
PMM (poly (methylidene malonate))



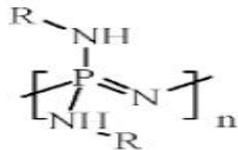
Eudragit S



PEG-PLA copolymer

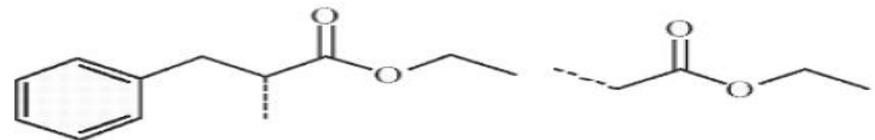


PLA (poly(lactide-co-glycolide))

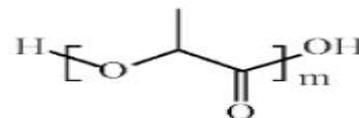


PPP (polyphosphazenes)

R =



Poly(glycolic acid) (PGA)



Poly(lactic acid)

Scheme 5.18 Polymers used for preparation of polymeric capsules for entrapment of drugs.

# Métodos de Imobilização

## Encapsulamento: Métodos de preparo

### - Método Post-loading

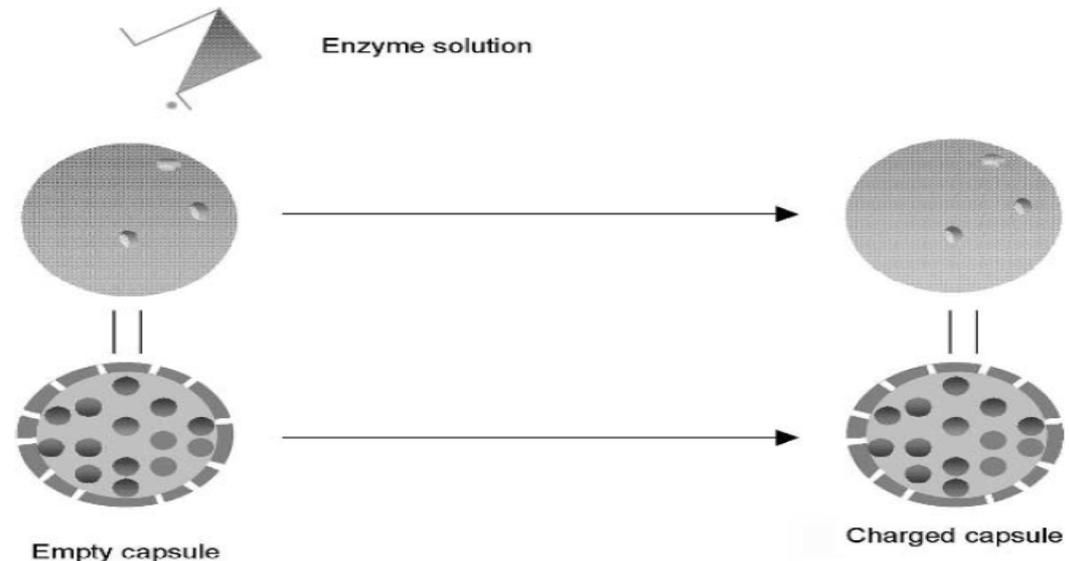
Encapsulamento da enzima em capsulas já prontas, tumescíveis ou não. Ex.

Polipirrois

Para não-tumescíveis, a carga enzimática = volume vazio da capsula x concentração da enzima. Para as tumescíveis, a carga enzimática = fator de inchamento (swelling factor) x carga enz das não-tumescíveis

O tamanho do poro e volume interno das microesferas tumescíveis podem ser controlados pela alteração de pH, temperatura, ou grau de hidratação.

Outra opção, casca-núcleo: suporte com núcleo insolúvel (melamine formaldehyde ou poliestireno) com camadas estruturadas de outros polímeros (poly(allylamine) (PAH) e poly(styrenesulphonate) (PSS)).

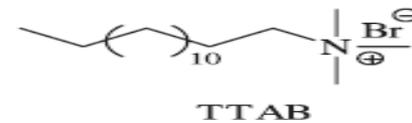
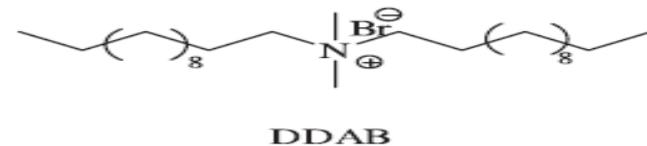
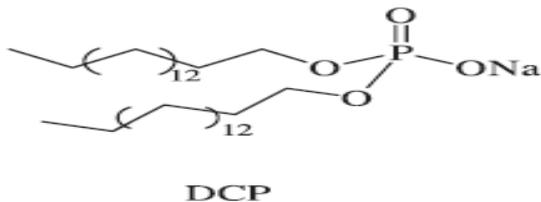
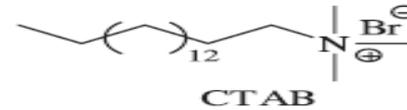
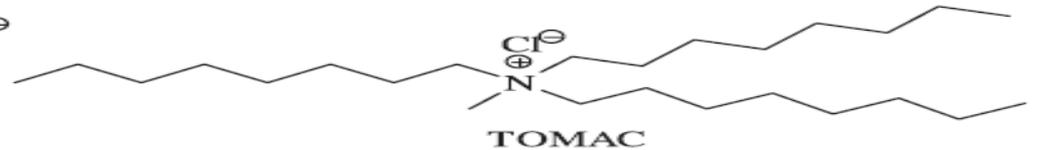
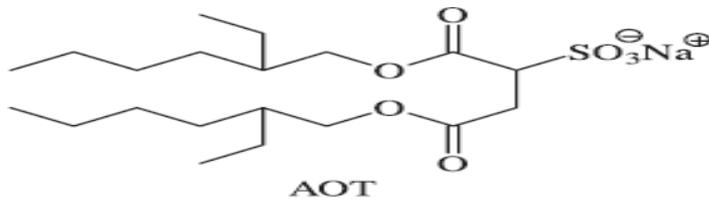
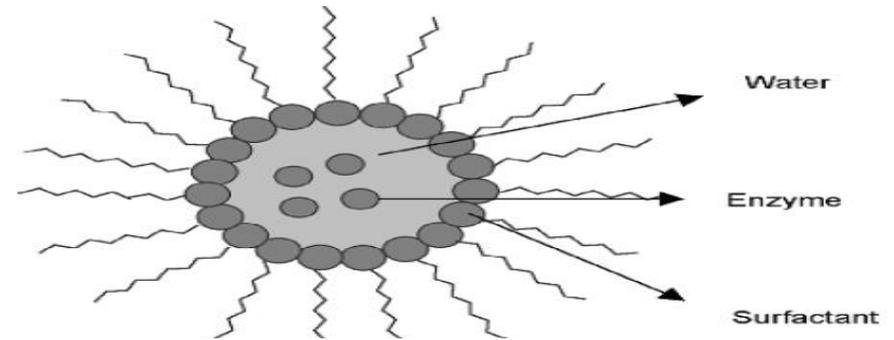


# Métodos de Imobilização

## Encapsulamento: Métodos de preparo

### - Método Microemulsão

A enzima é encapsulada em membrana esféricas ocas com núcleos líquidos em sistemas de microemulsão utilizando surfactants iônicos e não-iônicos



# Métodos de Imobilização

Table 5.3 Micelle-aided encapsulation of enzymes

Capsule	Method	Remarks	Enzyme
Detergentless microemulsion	Micelle	Alterations of the catalytic properties of polyphenol-oxidase in detergent-less microemulsion and ternary water–organic solvent mixtures	Polyphenol oxidase
Micelles of AOT/isooctane	Micelle	The enzyme-containing micelle was used in a dialysis stirred cell to integrate reaction and product recovery. The resistance of the dialysis membrane to reversed micelles was controlled by the water content	<i>Candida rugosa</i> lipase
Phosphatidylcholine/isooctane	Micelle	Esterification of butyric acid was performed in organic solvent	Chintase
Bis(2-ethylhexyl) sodium sulphosuccinate in isooctane	Micelle	The intermicellar diffusion and the intramicellar diffusion were used to describe the distribution of the micelle system	$\alpha$ -Chymotrypsin
–	Micelle	100 to 1000-fold increase in $K_m$ and simultaneous decrease in $k_{cat}$ by a factor of 2–5 was observed	$\alpha$ -Chymotrypsin
Lecithin reverse micelles	Micelle	The water activity in the micelle was controlled	Trypsin and $\alpha$ -protease
	Micelle		Hydrogenase
Detergentless microemulsion	Micelle	The emulsions are obtained with the use of hydrocarbon/alcohol/water, in defined zones of the phase diagram.	Laccase
Liquid membrane emulsions	Micelle		Cellulase
Lecithin reverse micelles	Adsorption–encapsulation	A 2.8-fold increase in the productivity of the interesterification when compared with shake-flask experiments	Celite– <i>Rhizopus arrhizus</i> lipase
Detergentless microemulsion	Micelle	The emulsions are obtained with the use of hydrocarbon/alcohol/water, in defined zones of the phase diagram	$\alpha$ -Chymotrypsin
Reversed micelles of sodium lauryl sulphate and sodium tauroglycolate	Micelle	–	Invertase

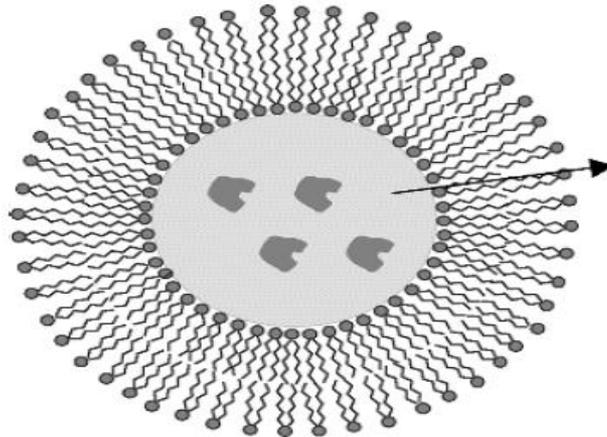
# Métodos de Imobilização

## Encapsulamento: Métodos de preparo

### - Método Microemulsão

Há também a opção de se utilizar surfactantes poliméricos, onde a membrana emulsionada formada por monômeros surfactants possa ser polimerizada e formar a capsula. Estas podem ser recobertas por outro polímero, sendo depois removida a micela e formando a nanocapsula polimérica

\*\* Lipossomos: vesículas lipídicas, formadas pela dispersão de bicamada anfifílica em solução aquosa. Seu tamanho varia de 20 a 1000 nm. As enzimas podem ser encapsuladas ou adsorvidas na superfície. Ex. lecitina



Scheme 5.14 Liposome capsule for enzyme encapsulation.

# Métodos de Imobilização

Table 5.4 Polymer-stabilized liposome-entrapped enzymes

Capsule	Method	Remarks	Enzyme
Surfactant nanocapsules in cyclohexane	The reversed hydrated micelles from <i>N,N</i> -diallyl- <i>N,N</i> -didodecyl ammonium bromide (DDAB)	Cross-linking enhanced the thermostability of the enzyme molecules	$\alpha$ -Chymotrypsin
Polymer-coated liposome	Poly(1,4-pyridinium diethylene salt) was coated on the liposome	Liposome of dicetyl phosphate (DCP), or of a 7:2:1 (mole ratio) mixture of phosphatidylcholine, DCP and cholesterol	Glucose oxidase
Polymeric micelles	Polymeric reversed micelles were based on modified polyethyleneimine	Successive alkylation of polyethyleneimine with cetyl bromide and ethyl bromide, thus being able to solubilize considerable amounts of water in the benzene/BuOH mixture	$\alpha$ -Chymotrypsin and laccase
Surface-modified polymeric nanogranules (SMPN)	Polymerization of an AAm/ <i>N,N'</i> -MBAm mixture in a mixed reversed of AOT and surfactant Pluronic F-108	Chromatographic removal of the auxiliary surfactant and AOT was necessary	$\alpha$ -Chymotrypsin
Surface-modified polymeric nanogranules (SMPN)	One of the surfactants bearing double bonds such as Pluronic F-108 was polymerized	Activity and stability of $\alpha$ -chymotrypsin entrapped in SMPN strongly depended on conditions of preparation of SMPN	$\alpha$ -Chymotrypsin
Polymeric surfactant	PEI modified with cetyl bromide and ethyl bromide	Polymeric reverse micelles of 20–50 nm were capable of solubilizing enzymes in non-polar solvents with retention of catalytic activity	$\alpha$ -Chymotrypsin and laccas
VET200	Polymeric liposome	Enzyme is entrapped inside polymerized PMOXA-PDMS–PMOXA-triblock copolymer vesicles mean diameter 250 nm; the membrane was 10 nm thick	Lactamase
AOT reverse micelles/gel	Gel-entrapped enzymes in reversed micelles	The enzymes are first gel-entrapped then “equilibrated” with a reversed micellar solution	$\alpha$ -Chymotrypsin
Poly(MA-co-MVE), poly(MA-co-St)-coated emulsion	Depositing the polymer around emulsified aqueous droplets	The permeability of the former responded to pH changes in the range pH 5–60	Invertase
FAT-VET200	POPC/polymer-stabilized POPC-based vesicles	Enzyme is entrapped inside POPC or polymer-stabilized POPC-based vesicles (FAT-VET200)	Lactamase

# Métodos de Imobilização

Table 5.5 Liposome-based capsules

Method of preparation of liposome	Type of liposome	Remark	Enzyme
DRV	–	AChE can be stabilized in porin-functionalized liposome against denaturation by proteolysis	AChE
FAT-VET100	POPC vesicles	No activity against the larger substrate Suc-Ala-Ala-Pro-Phe-pNA or casein. Inhibition of externally present enzyme by an inhibitor protein	$\alpha$ -Chymotrypsin
FAT-VET100	POPC vesicles	87 enzyme molecules per vesicle with a diameter of 125 nm was obtained in FAT-VET100	$\alpha$ -Chymotrypsin
DDV	DPPC–cholesterol–stearylamine 6:3:1 or 5:3:2	Liposome is around $61 \pm 18$ nm and $29 \pm 11$ nm, respectively. The enzyme was partially localized on the external vesicle surface, particularly with ratio of DPPC–cholesterol–stearylamine 6:3:1	Carbonic anhydrase
REV	Egg PG–egg PC–cholesterol, 1:4:5	Vesicles are characterized by a relatively large internal aqueous space (0.2–1 $\mu$ m)	Alkaline
DDV	Egg PC–lyso PC: SM–PE, 72.5:4.6:3.8:19.1	The liposome is ~30–125 nm and enabled the permeation of ( <i>N</i> -benzoyl- <i>dl</i> -arginine-4-nitroanilide) the interior of the vesicles	Trypsin
DDV	PC–cholesterol (2:1)	Entrapment in vesicles yielded about 150 enzyme molecules per vesicle	Alkaline phosphatase
VET/REV	Dipalmitoylphosphatidylcholine (DPPC)/ phosphatidylinositol	~20% encapsulation efficiency with the use of 1 mg mL <sup>-1</sup> enzyme content	Chloroperoxidase/ lactoperoxidase/ glucose oxidase
VET/REV	Dipalmitoylphosphatidylcholine (DPPC) and phosphatidylinositol (PI)	The antibacterial activity of these “reactive” liposomes arising from hydrogen peroxide and oxyacids in the presence of the substrates glucose and iodide ions	Glucose oxidase (GO) and GO in combination with horseradish peroxidase (HRP)
Dry-rehydration with enzyme–buffer solution/ extrusion	(SL) Palmitoyloleoylphosphatidylcholine (POPC) and dipalmitoyl phosphatidylethanolamine- <i>N</i> -PEG 2000/ cholesterol	There is no significant difference in $K_m$ values between free and encapsulated phosphotriesterase; therefore paraoxon readily penetrates the membrane of the carrier cells	A recombinant phosphotriesterase

# Métodos de Imobilização

Table 5.5 Continued

Method of preparation of liposome	Type of liposome	Remark	Enzyme	Ref.
VET	DMPC and CS-incorporating dimethyldioctadecylammonium bromide and DMPC incorporating PTI	On addition of glucose, H <sub>2</sub> O <sub>2</sub> is produced, which is toxic to the bacterial	Glucose oxidase/horseradish peroxidase (HRP)/lactoperoxidase (LPO)	118
Rehydration	PC liposome	An increase in the number of stearyl residues attached to the enzyme results in a dramatic decrease of ATEE binding to the active centre ( <i>K<sub>m</sub></i> increase)	Hydrophobized α-chymotrypsin derivatives	119
SUV, “Millipore-filtered MLV”, and “ether-injection method”	Egg PC-egg PA, 15:2:12	Entrapment experiments indicated that at least part of the enzyme is localized inside the vesicles	Aminolevulinate dehydratase	120
MLV	PC-cholesteroldicetylphosphate, 5:5:1	The enzyme-containing vesicles obtained was 2–3.5, depending on the lipid	Trypsin	12
FAT-VET400	POPC vesicles	The diameter is ~150 nm and addition of cholate enabled the passage of substrate across the membrane	Phosphorylase	122
REV or DRV	Soybean phosphatidylcholine-cholesterol vesicles	Enzyme entrapment in soybean phosphatidylcholine-cholesterol vesicles (REV or DRV) decreased with increasing cholesterol content. The enzyme was localized both inside and on the surface of the vesicles	β-Galactosidase	123
MLS	Egg-phosphatidylcholine lipids (eggPC), 1-palmitoyl-2-oleoyl- <i>sn</i> -glycero-3-[phosphoserine] (sodium salt) (POPS) etc.	Liposomes insert a barrier between the enzyme and the external environment and protects the enzyme	Acetylcholinesterase	124

*Abbreviations:* DDV, vesicles prepared by the detergent dialysis method; SUV, small (sonicated) unilamellar vesicles; FAT-VET200, vesicles prepared by means of freeze-thaw cycles then repeated extrusions; MLV, multilamellar vesicles; VET100, vesicles prepared by the extrusion method (without freezing-thawing cycles); REV, vesicles prepared by the reverse-phase evaporation; DRV, vesicles prepared by the dehydration-rehydration method; MLS, multilamellar spherulites prepared by shearing a lamellar phase

**Table 1. Preparation and Characteristics of Immobilized Enzyme**

Characteristic	Carrier-binding method			Cross-linking method	Entrapping method
	Physical adsorption	Ionic binding	Covalent binding		
Preparation	Easy	Easy	Difficult	Difficult	Difficult
Enzyme activity	Low	High	High	Moderate	High
Substrate specificity	Unchangeable	Unchangeable	Changeable	Changeable	Unchangeable
Binding force	Weak	Moderate	Strong	Strong	Strong
Regeneration	Possible	Possible	Impossible	Impossible	Impossible
General applicability	Low	Moderate	Moderate	Low	High
Cost of immobilization	Low	Low	High	Moderate	Low

# Parâmetros de Imobilização

## Rendimento da Imobilização Enzimática ( $Y_E$ )

$$Y_E = \frac{E_I}{E_C} = \frac{E_I}{E_I + E_R + E_L}$$

$E_I$  → Atividade de enzima imobilizada

$E_R$  → Atividade remanescente de enzima em solução depois da imobilização

$E_L$  → Atividade perdida de enzima durante a imobilização

$E_L$  pode ser atribuído a inativação da enzima imobilizada ou livre, ou a limitações de transferência de massa, ou por impedimentos estéricos

Obs.:  $Y_E > 1$  → hiperativação

## Rendimento da Imobilização Proteica ( $Y_P$ )

$$Y_P = \frac{P_I}{P_C} = \frac{P_C - P_R}{P_C}$$

$P_C$  → Massa de proteína contactada (total)

$P_R$  → Massa de proteína remanescente em solução depois da imobilização

Obs.:  $Y_P \gg Y_p$  → limitações de transferência de massa e impedimentos estéricos são significantes

## Atividade específica do catalisador

$$a_{sp} = \frac{E_I}{M_{cat}}$$

### Variáveis do Processo de Imobilização

Temperatura, pH, tempo de contato, proporção enzima/suporte (carga enzimática), condições de ativação do suporte (se necessário). Custo do suporte?

### Exercício 1

A  $\beta$ -galactosidase is immobilized in a heterofunctional agarose support. The immobilization yield in terms of protein ( $Y_P$ ) is 95% and in terms of expressed activity ( $Y_E$ ) is 85%. The immobilization procedure consists in contacting 10 g of support (density  $0.7 \text{ g} \cdot \text{mL}^{-1}$ ) with 9 mL of soluble  $\beta$ -galactosidase preparation and 93 mL of bicarbonate buffer under stirring for 3 hours. At the end of the process, the expressed activity in the immobilized catalyst is  $286 \text{ IU} \cdot \text{g}^{-1}$ , the remaining activity in solution is  $2.6 \text{ IU} \cdot \text{mL}^{-1}$  and the remaining protein in solution is  $0.18 \text{ mg} \cdot \text{mL}^{-1}$ .

Determine: the activity lost during immobilization, the protein load in the support (mass of protein immobilized per unit mass of support) and the specific activity of the soluble  $\beta$ -galactosidase preparation in  $\text{IU} \cdot \text{mg}_{\text{protein}}^{-1}$ .

## Solução

**Resolution:** Volume of remaining solution =  $93 + 9 = 102$  mL

$$E_R = 2.6(\text{IU} \cdot \text{mL}^{-1}) \cdot 102(\text{mL}) = 265.2(\text{IU})$$

$$E_I = 286(\text{IU} \cdot \text{g}^{-1}) \cdot 10(\text{g})^{-1} = 2860(\text{IU})$$

From Equation 3.1 and  $Y_E = 0.85$ :

$$E_L = 239.5(\text{IU})$$

$$E_c = E_I + E_R + E_L = 2860 + 265.2 + 239.5 = 3364.7(\text{IU})$$

$$P_R = 0.18(\text{mg} \cdot \text{mL}^{-1}) \cdot 102(\text{mL}) = 18.36(\text{mg})$$

From Equation 3.2 and  $Y_P = 0.95$ :

$$P_C = 367.2(\text{mg})$$

$$P_I = P_C - P_R = 348.84(\text{mg})$$

**Answer:**

$$\text{Activity lost} = 239.5(\text{IU})$$

$$\text{Support load} = \frac{348.84}{10} = 34.8(\text{mg} \cdot \text{g}^{-1})$$

$$\text{Specific activity of enzyme preparation} = \frac{3364.7}{367.2} = 9.16(\text{IU} \cdot \text{mg}^{-1})$$