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Review

Improvement of enzyme activity, stability and selectivity via immobilization techniques

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Abstract

In spite of their excellent catalytic properties, enzyme properties have to be usually improved before their implementation at industrial scale (where many cycles of high yield processes are desired). Generally, soluble enzymes have to be immobilized to be reused for long times in industrial reactors and, in addition to that, some other critical enzyme properties have to be improved like stability, activity, inhibition by reaction products, selectivity towards non-natural substrates. Some strategies to improve these enzyme properties during the performance of tailor-made enzyme immobilization protocols are here reviewed. In this way, immobilized enzymes may also exhibit much better functional properties than the corresponding soluble enzymes by very simple immobilization protocols. For example, multipoint and multisubunit covalent immobilization improve the stability of monomeric or multimeric enzymes. Moreover, enantioselectivity of different enzymes (e.g., lipases) may be also dramatically improved (from $E = 1$ to >100) by performing different immobilization protocols of the same enzyme. In all cases, enzyme engineering via immobilization techniques is perfectly compatible with other chemical or biological approaches to improve enzyme functions and the final success depend on the availability of a wide battery of immobilization protocols.

Keywords: Immobilization; Stabilization; Improved selectivity; Improved specificity; Reduced inhibition

Contents

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1. Advantages and limitations of enzymes as industrial catalysts

Enzymes are catalysts bearing some excellent properties (high activity, selectivity and specificity) that may permit to perform the most complex chemical processes under the most benign experimental and environmental conditions [1,2]. Thus, the engineering of enzymes from biological entities to industrial reactors is a very exciting goal. Fortunately, there are many techniques available that may permit to improve the enzyme features, involving many areas of science that have suffered impressive developments in the last years: microbiology, protein engineering, chemistry of proteins, etc. However, some apparently older fashioned techniques, as immobilization, have been revealed in the last times as a very powerful tool to improve almost all enzyme properties, if properly designed: e.g., stability, activity, specificity and selectivity, reduction of inhibition. In this review, we will try to make a brief summary of the most interesting and recent advances in these topics. Thus, more than a description of new immobilization protocols, we will focus on how immobilization may help to solve some of the problems of enzymes as industrial biocatalysts: enzyme recovery, enzyme stability, enzyme selectivity, reduction of inhibition by the medium or products. In all these cases, the use of a battery of immobilization solutions that permits to control the support–enzyme interaction, to immobilize the enzyme via different orientations or in different conditions, is the key point that greatly increases the possibilities of success.

2. Immobilization of enzymes

The use of a relatively expensive catalyst as an enzyme requires, in many instances, its recovery and reuse to make an economically feasible process. Moreover, the use of an immobilized enzyme permits to greatly simplify the design of the reactor and the control of the reaction [3–7]: the simple filtering of the enzyme stops the reaction; it is possible to use any kind of reactor, etc. Thus, immobilization is usually a requirement to the use of an enzyme as an industrial biocatalyst, and is the simplest solution to the solubility problem of these interesting biocatalysts.

However, the idea of enzyme reuse implicitly means that the stability of the final enzyme preparation should be high enough to permit this reuse. Therefore, the enzyme needs to be very stable or to become highly stabilized during the immobilization process to be a suitable process.

Thus, although there are hundreds of immobilization protocols [3–7], the design of new protocols that may permit to improve the enzyme properties during immobilization is still a exciting goal.

Moreover, bearing in mind that the final use will be as industrial catalyst, the ideal immobilization processes should limit the use of toxic or highly unstable reagents, be very simple, robust, etc.

3. Stabilization of enzymes via immobilization

One of the properties that have been generally considered to be improved via immobilization is enzyme stability [8–15]. However, this may be not fully exact if immobilization is not properly designed, and even the explanation for the observed stabilizations may be diverse.

Random immobilization may not really improve enzyme rigidity, even in some cases the enzyme stability may decrease after immobilization [8–18], e.g., if the support is able to establish undesired interactions with the enzyme.

Next, we will show the different "levels" of enzyme stabilization that immobilization may provide.

3.1. Operational stabilization of enzymes by immobilization on porous supports

Immobilization of enzymes inside the porous structure of a solid may permit to have the enzyme molecules fully dispersed and without the possibility of interacting with any external interface. Thus, this immobilization will stabilize the enzyme against interaction with molecules from the enzymatic extract, preventing aggregation, autolysis or proteolysis by proteases from the extract (that will be also dispersed and immobilized). Moreover, the immobilized enzyme molecules will not be in contact with any external hydrophobic interface, such as air bubbles originated by supplying some required gases or promoted by strong stirring, necessary to control pH. These gas bubbles may produce enzyme inactivation of soluble proteins [19–22], but cannot inactivate the enzymes immobilized on a porous solid [23,24] (Fig. 1).

In the presence of an organic solvent phase, the immobilized enzymes may be in contact with the molecules of solvent which

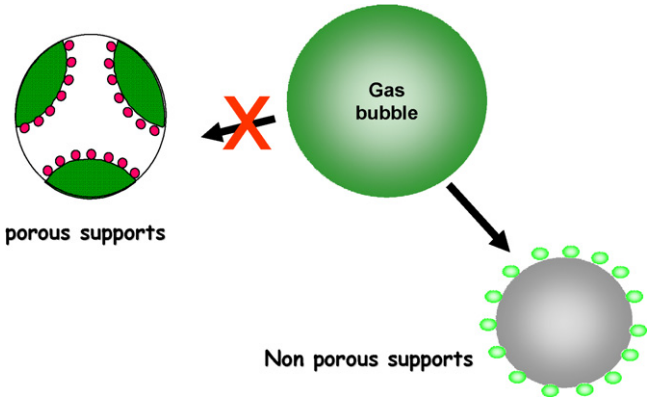


Fig. 1. Effect of the type of support on the immobilized enzyme stability against inactivation by gas bubbles.

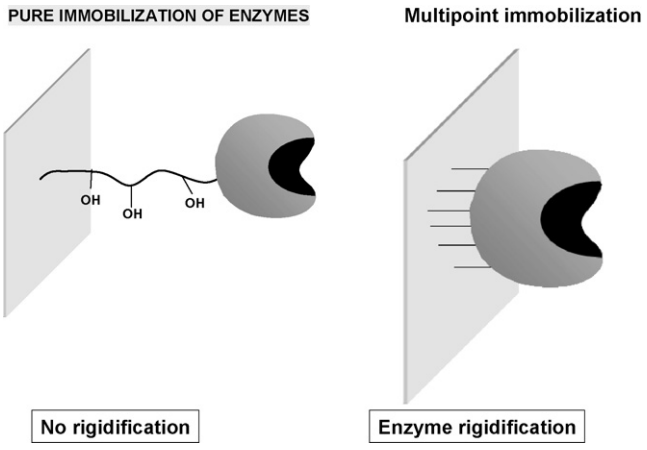


Fig. 2. Effect of immobilization on enzyme stability.

are soluble in the aqueous phase, but not with the organic phase-interface, preventing this inactivation cause again.

Thus, any immobilization protocol of an enzyme which yields the enzyme immobilized inside a porous solid as the final product may permit an "operational stabilization" of the enzyme, even without really affecting the structural stability of the enzyme, simply by the mechanisms described above.

However, this stabilization is not universally associated to the immobilization. For example, the increasingly popular use of non-porous nano-particles (mainly magnetic nano-particles) to immobilize enzymes [25–27] needs to consider that all the mechanisms of enzyme stabilization described above are lost in this case: enzymes immobilized on nano-particles are now able to interact with external interfaces, or even with enzyme molecules immobilized on other particles. This may be solved by using proper strategies (e.g., covering the immobilized proteins with polymers) [28] (Fig. 1).

3.2. Rigidification of the enzyme structure by multipoint covalent immobilization

Multipoint covalent attachment of enzymes on highly activated pre-existing supports via short spacer arms and involving many residues placed on the enzyme surface promotes a rigidification of the enzyme structure of the immobilized enzyme [8–15] (Fig. 2). Now, the relative distances among all residues involved in the multipoint immobilization have to be maintained unaltered during any conformational change induced by any distorting agent (heat, organic solvents, extreme pH values). This should reduce any conformational change involved in enzyme inactivation and greatly increase the enzyme stability.

However, to achieve these intense multipoint covalent attachments between the enzyme and the support – two dissimilar and rigid structures – is not a trivial problem. It becomes necessary to choose suitable immobilization systems to take advantages of the prospects of these techniques. Some authors had even discarded the possibility of getting a significant stabilization by this technique, due to the only moderate geometric congruence between the enzyme and the support [11,13,14].

Thus, the characteristics of the support, reactive groups and immobilization conditions need to be carefully selected to be able to involve the maximum number of enzyme groups in the immobilization. In fact, it has been possible to correlate the enzyme stabilization reached with the number of enzyme–support linkages [29].

3.2.1. Selection of proper immobilization supports

A support suitable for protein multipoint immobilization requires fulfilling some characteristics:

- The support should present large internal surface to have good geometrical congruence with the enzyme surface. If the support is formed by very thin fibers, e.g., smaller than the protein, it will hardly be possible to get an intense interaction between the enzyme and the support.
- The support should present a high superficial density of reactive groups. Only if there many reactive groups in the support under the protein surface, an intense multipoint covalent attachment may be achieved.
- The reactive groups in the protein and the support should present minimal steric hindrances in the reaction, because after the initial immobilization, multipoint covalent attachment requires the contact between groups bound to rigid structures.
- The reactive groups in the support should react with groups frequently placed in the enzyme surface.
- The reactive groups involved in the immobilization should be stable enough to permit long enzyme–support reaction periods.
- It should be easy to get a final inert surface in the support after immobilization, by destroying or blocking the remaining reactive groups in the support without affecting the enzyme.

Among the immobilization protocols described in literature, there are some that may fulfill most of these requirements. Supports like agarose beads, zeolites, porous glass, epoxy resins like Sepabeads, offer large areas for enzyme–support interactions.

Among the reactive groups, epoxy or glyoxyl groups may be considered very adequate [30,31]. Glyoxyl agarose has the additional advantage that directs the immobilization via the richest area(s) in reactive residues of the protein, enabling intense multipoint enzyme–support reaction [32,33]. Glutaraldehyde chemistry is other popular technique to immobilize enzymes. It has given some good stabilization factors in many instances [34–36]. The glutaraldehyde technique is very versatile and may be used in very different fashions [37–39]. However, in terms of stabilization, the treatment with glutaraldehyde of proteins previously adsorbed in supports bearing primary amino groups offers in many cases very good results, because permit the crosslink between glutaraldehyde molecules bound to the enzyme and glutaraldehyde molecules bound to the support. However, it implies the chemical modification of the whole enzyme surface [40].

3.2.2. Selection of suitable immobilization conditions

Although some multipoint covalent attachment may be expected using a correct support activated with a proper group, the selection of suitable immobilization conditions is critical to maximize the multipoint covalent attachment. Immobilization conditions should favor the enzyme–support reaction [30,41]. Some of these critical variables are:

- *Reaction time*. Although immobilization may be very rapid, multipoint interaction between the non-complementary enzyme and support surfaces is a slow and time-dependent process: it requires the correct alignment of groups located in the already immobilized, and partially rigidified enzyme, and the rigid surface of the support.
- *pH value*. Although immobilization may be performed at neutral pH value in many cases, incubation at alkaline pH values, where the reactivity of the nucleophiles of the protein (usually Lys) may be improved, is convenient to reach a high enzyme–support reaction. It should be kept in mind that while terminal amino groups may have a p*K* between 7 and 8, exposed Lys groups will present a p*K* over 10.5.
- *Temperature*. Moderately high temperature may favor the vibration of enzyme and support, increasing the possibilities of getting more enzyme–support linkages.
- *Buffers*. It should be chosen buffers that do not interfere in the reaction. For example borate may interfere in the aldehyde–amine reaction; amino compounds (Tris, ethanol amine) may modify epoxy supports or compete with the Lys by aldehyde groups [30].
- *Inhibitors or other protein protectors*. Multipoint immobilization, or the immobilization conditions, may reduce the enzyme activity. The presence of inhibitors and other compounds may reduce this lost in activity [30].

3.2.3. Enzyme stabilization by multipoint covalent attachment

Thus, a proper immobilization protocol designed to stabilize a protein should consider many different facts. However, if properly designed, results achieved via immobilization techniques may be really impressive, perfectly comparable to any other stabilization technique. For example, Table 1 shows some of the results obtained after immobilizing many different proteins on glyoxyl agarose under optimal conditions (see Refs. [23,24,29,30,33,41–70]). The stabilization factors are in many cases extremely high (1000–10,000-fold factors) with activity recoveries usually over 60%.

The use of epoxy-activated supports also may give very good results, perhaps lower that glyoxyl agarose but still very significant (see Table 2) [31,71–86]. In some cases, both supports have given similar results (e.g., penicillin acylase).

Moreover, it should be considered that any other technique employed to get a stable enzyme (protein engineering, screening, etc.) may be compatible with the stabilization of the enzyme by multipoint covalent attachment, once immobilization will be a almost necessary step for the preparation of an industrial biocatalyst. Thus, enzymes from extremophiles have been also stabilized by multipoint covalent attachment (see for example Refs. [49,50,56,64,86]).

3.3. Stabilization of multimeric enzymes by multisubunit immobilization

One specific problem in the design of a biocatalyst is if the enzyme is a multimeric one. The inactivation of these enzymes starts, in many instances, by the dissociation of the enzyme in its individual subunits [87]. Even if this is not relevant for enzyme stability, the release of enzyme subunits may contaminate the final product (e.g., a food). Stabilization of multimeric enzymes

Table 1
Recovered activities and stabilization factors by immobilization on glyoxyl agarose of some enzymes

Enzyme	Recovered activity (%)	Stabilization factor[a]	Ref.
Trypsin	75	10,000[a]	[41]
Chymotrypsin	70	60,000[a]	[45]
Penicilin G acylase (*E. coli*)	70	8,000[a]	[42]
Penicilin G acylase (*K. citrophila*)	70	7,000[a]	[43]
Ferrodoxin NADP reductase (*Anabaena*)	60	1,000[a]	[23]
Lipase (*C. rugosa*)	50	150[a]	[54]
Esterase (*B. stearothermophilus*)	70	1,000[a]	[49]
Thermolysin (*B. thermoproteolyticus*)	100	100[a]	[50]
Glutamate racemase	70	1,000[a]	[33]
Formate dehydrogenase (*Pseudomonas* sp. 101)	50	>5,000[a]	[24]
Formate dehydrogenase (*C. boidini*)	15	150[a]	[70]
Alcohol dehydrogenase (H. Liver)	90	>3,000	[68]
Cyclomaltodextrin glucanotransferase (*Thermoanaerobacter*)	30	>100	[64]
Cyclodextrin glycosyltransferase (*B. circulans*)	70	>100	[69]
Pullulanase (*K. pneumoniae*)	10	>50	[59]
Angiotensin-converting enzyme	nd	60	[65]
Alcalase	54	500	[46]
Urokinase	80	10	[55]

Stabilization factors are defined as the ratio between half-lives.
[a] Compared with one-point immobilized enzymes.

Table 2
Immobilization-stabilization of different enzymes on epoxy-activated supports

Epoxy support	Enzyme	Immobilization yield[a]	Stabilization factor[b] (*T*)	Ref.
Eupergit® C	Phosphodiesterase	92	nd	[76]
Eupergit® C	Trypsin (BAEE)	100	nd	[31]
Eupergit® C	Lipase (*Ps. fluorescens*)	83	nd	[31]
Eupergit® C	Glucose-oxidase	61	nd	[77]
Eupergit® C 250L	Glycolate oxidase	17	nd	[77]
Eupergit® C	Phospholipase-C (*B. cereus*)	25	nd	[74]
Sepabeads	Phospholipase-C (*B. cereus*)	75	nd	[74]
pHEMA-GMA	Cholesterol oxidase	67	2.5 (50 °C)	[72]
Poly(HEMA-GMA-2)	Alpha-amylase (*B. licheniformis*)	73	2 (70 °C)	[71]
pHEMA-GMA	Invertase	60	2 (70 °C)	[78]
Eupergit® C	Dextransucrase (*L. mesenteriodes*)	21	30 (30 °C)	[75]
Eupergit®C 250L	Dextransucrase (*L. mesenteriodes*)	22	40 (30 °C)	[75]
Curdlan matrix	*Pancreatic porcine* lipase	58.74	nd	[79]
Eupergit® C	Hydantoinase	15	nd	[80]
Eupergit® 250L	L-*N*-Carbamoylase	54	nd	[80]
Sepabeads	Uridine phosphorylase	60–70	nd	[81]
Sepabeads	Purine nucleoside phosphorylase	60–70	nd	[81]
Eupergit® C	Cyclodextrin glucanotransferase (*Thermoanaerobacter* sp.)	11–15%	5	[83]
Eupergit® C	Lacasse (*P. ostreatus*)	300%	nd	[57]

[a] Yields based on percentage of immobilized protein that retains enzymatic activity.
[b] Stabilization factor respect to the soluble enzyme.

by genetic engineering may be too complex, leaving the immobilization or the chemical modification as the simplest strategies to solve the dissociation problem of oligomeric proteins. However, in this case the immobilization–stabilization strategy should consider this multimeric nature of the enzyme, pursuing not only the multipoint attachment of the protein, but also the multisubunit attachment. The ideas behind will be very similar to the previously described for the multipoint covalent attachment. Thus, the multimeric structure of dimeric enzymes may be easily stabilized by immobilization on highly activated supports [88]. Some successful examples of this strategy obtained in our laboratory are: D-amino acid oxidases from *Trigonopsis variabilis* and *Rodotorula gracilis* [35,51], formate dehydrogenases from *Pseudomas* sp. and *Candida boidini* [24,70], alcohol dehydrogenase from horse liver [68] (Fig. 3).

However, if the enzyme structure is more complex, it is quite likely that not all the subunits of the enzyme may be attached to a plane surface. In these, cases multisubunit immobilization on pre-existing solid supports may be complemented by chemical crosslinking with polyfunctional-polymers of the already immobilized enzyme [88] (Fig. 4). In our laboratory we have been able to stabilize many complex multimeric enzymes following this two-step strategy, e.g., catalase from bovine liver or *Thermus* sp. [52,53] ampicillin acylase from *Acetobacter turbidans* [89], Asparaginase [90], beta-galactosidase from *Thermus* sp. [86], alcohol oxidases from *P. pastoris* and *Hansenula* sp. [91].

If the immobilization is produced by reaction between enzyme molecules like crosslinked crystal enzyme (CLECs) [92], crosslinked enzyme aggregates [93] (CLEAs), etc., they may be a good option to get immobilized enzymes with a stabilized multimeric structure, although there are not many examples on this [94].

4. Improved activity of lipases

A good immobilization protocol should keep high catalytic activity after enzyme immobilization. However, in some cases,

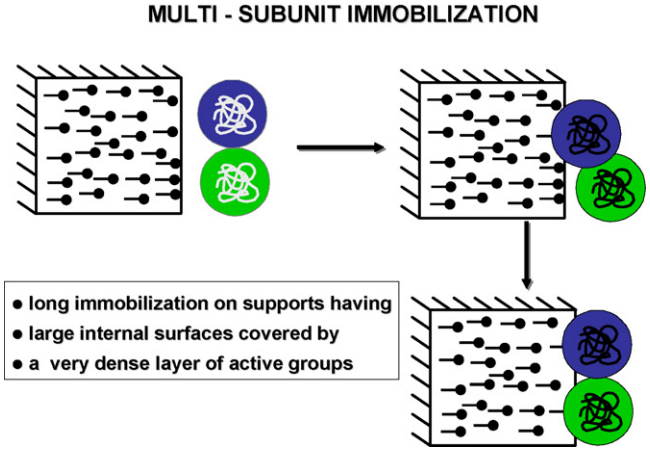


Fig. 3. Stabilization of dimeric enzymes by multisubunit stabilization.

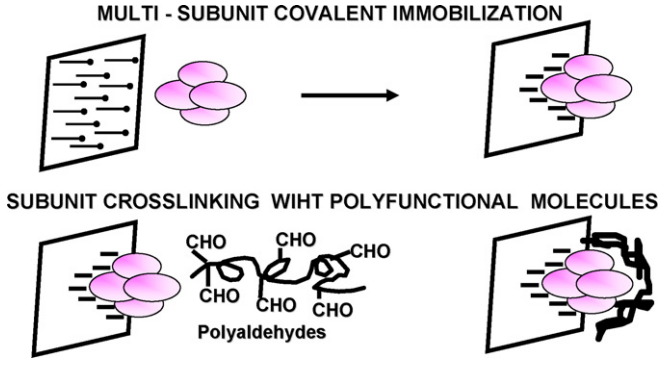


Fig. 4. Stabilization of complex multimeric enzymes by multisubunit stabilization plus crosslinking with polyfunctional-polymers.

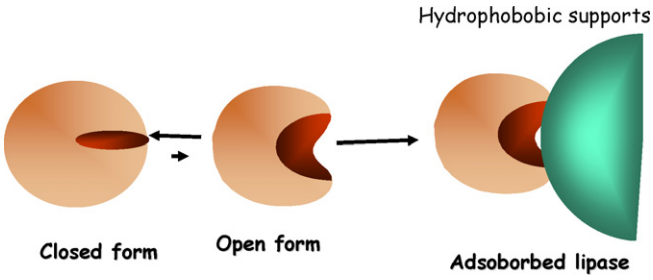


Fig. 5. Interfacial activation of lipases on hydrophobic supports at low ionic strength.

the enzyme may have two forms with very different activity, and if we were able to fix the enzyme form with higher activity, the final immobilized preparation may be more active than the native one. One of the clearest examples of this is the case of lipases. Lipases show two different conformations. The closed form, considered inactive, where the active site is isolated from the reaction medium by a polypeptide chain called lid, and the open form, where this lid is displaced and the active site is fully exposed to the reaction medium [95–98]. Both forms of lipases are in an equilibrium affected by the experimental conditions. In the presence of hydrophobic drops of substrate, lipases may become strongly adsorbed on the interface of these drops and the conformational equilibrium is shifted towards the open form of the lipases.

From these features, two strategies to get immobilized lipases molecules with an improved activity have been developed, both trying to fix the open form of the lipase.

4.1. Lipase adsorption on hydrophobic supports

It has been shown in many examples that lipases may become adsorbed on hydrophobic supports at low ionic strength (e.g., 5–25 mM sodium phosphate), conditions where other enzymes did not become immobilized, involving in the adsorption the hydrophobic areas surrounding the active centre and leaving stabilized the open form of the lipase [99] (Fig. 5). This permits to get immobilized preparations highly hyperactivated, mainly against small and hydrophobic substrates (even by a 20-fold factor) [99]. Reetz also have shown that immobilization of lipases on hydrophobic sol–gels yields a higher activity recovery [100]. However, if the substrate is very large or hydrophilic, the near presence of the hydrophobic support surface may generate some steric hindrances, reducing the activity of the lipase.

4.2. Lipase immobilization in the presence of detergents

Detergents have been described to permit the hyperactivation of lipases, very likely by stabilizing their open forms. Thus, Braco and co-workers [101] described the improved activity of lipases precipitated in the presence of detergents and used in organic medium. This idea has been used by many authors [102–104]. Unfortunately, the lack of chemical stabilization of these aggregates made them only useful in organic medium, being re-dissolved in aqueous medium. Recently, Sheldon and co-workers showed that the preparation of CLEAs of lipases in

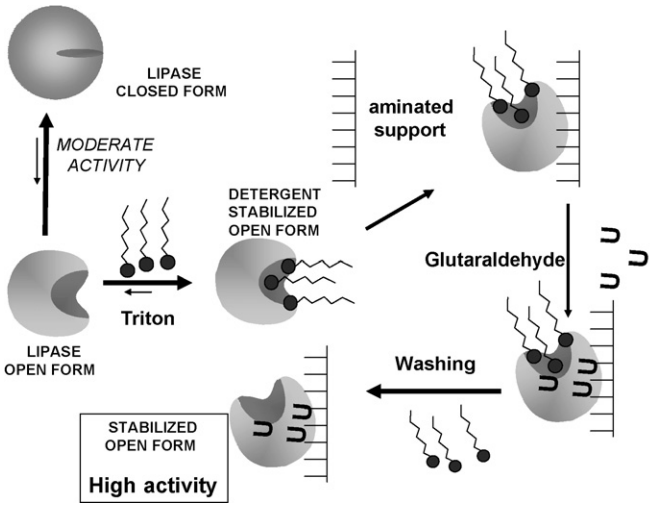


Fig. 6. Stabilization of the open forms of lipases induced by detergent by glutaraldehyde crosslinking.

the presence of detergents may permit to get improved lipase activity, very likely by fixing the open form of lipases during crosslinking [105]. These CLEAs, chemically crosslinked, could be already used in any reaction media without risking re-dissolution.

Recently, the stabilization of the open forms of lipases adsorbed on aminated supports has been shown. To this goal, immobilized lipases from different source were incubated in the presence of detergents and then, the crosslinking with glutaraldehyde of the amino groups placed in the support and in the enzyme was attempted. Results suggested that this is a good option to get lipases exhibiting a higher catalytic activity [106] (Fig. 6). To this purpose it was necessary to achieve an enough rigidity of the enzyme structure, enough to prevent the movement of the lid.

Thus, these results suggest that other enzymes, having different forms with dissimilar activity, could be immobilized by fixing their "most active form", getting hyperactivated biocatalysts in absence of the "hyperactivating" agent.

The key point will be that the immobilization may be able to "fix" the changes induced by the activator.

5. Modulation of the selectivity of enzymes by immobilization techniques

As commented in Section 1, the enzyme specificity should be very high against the natural substrate, but this value may be significantly reduced when the enzyme is intended to be used against compounds that are far from that natural substrates. In this case, the improvement of the enantioselectivity may be a critical requirement to get an industrially relevant process. Among the many tools to improve the enzyme selectivity (e.g., protein engineering or directed evolution [107–110]), again it is possible to find the protein immobilization as a powerful technique.

Immobilization of a protein may produce some distortion on the active site, reducing the overall mobility of the protein

Table 3
Effect of the immobilization strategy in the enantioselectivity of different lipases catalyzing the hydrolysis of several racemic compounds (see Scheme 1)

Substrate	Lipase	Support	Conditions	Product	ee_p [a] (%)	ee_s [b] (%)	*E*	Ref.
(±)-**1**	25L	GLUT-agarose	pH 7, 25 °C, 10% dioxane (v/v)	(*R*/*S*)-**5**	nd	0	1	[111]
		DEAE-agarose		(*R*)-**5**	nd	>99	>100	
	ROL	Octyl-agarose	pH 7, 25 °C	(*S*)-**5**	nd	25	2	[112]
		DS-agarose		(*S*)-**5**	nd	89	51	
(±)-**2**	CAL-B	CNBr-agarose	pH 7, 25 °C	(*R*)-**6**	73	nd	7.4	[113]
		PEI-agarose		(*R*)-**6**	>95	nd	67	
(±)-**3**	CRL	PEI-agarose	pH 5, 25 °C	(*S*)-**7**	12	nd	1.2	[114]
		Octyl-agarose		(*S*)-**7**	>95	nd	85	
		GLUT-agarose		(*R*)-**7**	>99	nd	>100	
	BTL	Octadecyl-Sepabeads	pH 9, 4 °C	(*R*)-**7**	70	nd	6	[115]
		Glyoxyl agarose		(*R*)-**7**	>99	nd	>100	
(±)-**4**	CAL-B	Octadecyl-Sepabeads	pH 7, 4 °C, 20%	(3*S*,4*R*)-**3**/(3*R*,4*S*)-**3**	nd	0	1	[116]
		Glyoxyl agarose	diglyme/dioxane 3:1 (v/v)	(3*S*,4*R*)-**3**	nd	>99	>100	

Abbreviations—25L: lipase of 25 kDa from the PPL comercial extract; ROL: lipase from *R. oryzae*; CAL-B: lipase from *C. antarctica* B; CRL: lipase from *C. rugosa*; BTL: lipase from *B. thermocatenulatus*; GLUT: glutaraldehyde; PEI: polyethylenimine; DS: dextran sulphate; **1**: glycidyl butyrate; **2**: methyl mandelate; **3**: 2-*O*-butyryl-2-phenylacetic acid; **4**: *trans*-4-(4′-fluorophenyl)-6-oxopiperidin-3-ethyl carboxylate.
[a] ee of product was calculated at conversion between 10 and 20%.
[b] ee of substrate was calculated at 50% conversion.

groups. Moreover, enzymes that suffer great conformational changes during catalysis could have these changes distorted when immobilized, producing immobilized enzymes with fully altered catalytic properties.

The requirement to utilize the possibilities of this distortion is to be able to access to a large battery of supports to immobilize proteins and of immobilization protocols that may permit to control the immobilization. This may permit the immobilization of a protein via different regions, conferring different rigidity and distorting the enzyme in very different ways, it may be even possible to generate different micro-environments around the enzyme bearing very different physical properties. That way, this may permit to achieve a large library of immobilized enzymes, perhaps with very different properties.

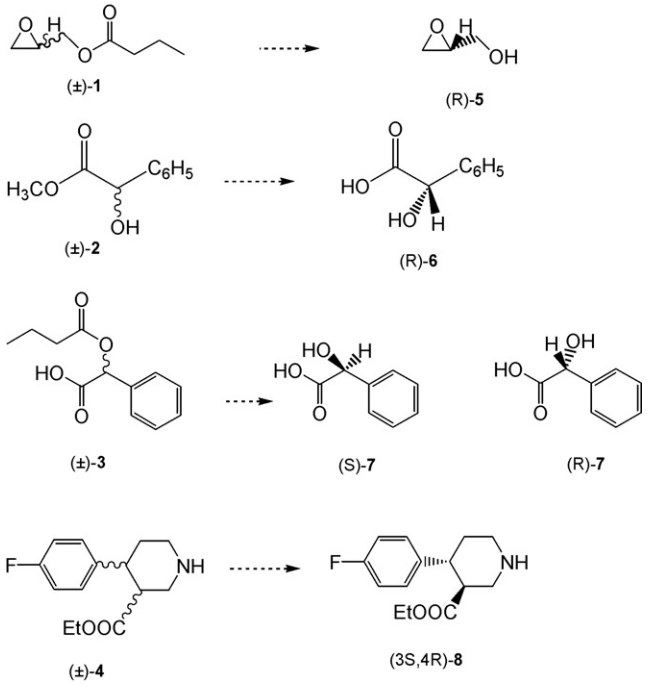
As stated above, lipases suffer very large conformational changes during catalysis [95–98]. Thus, these enzymes were chosen to develop this idea. In fact, the strategy has been very successful in the modulation of the enantiopreference of many lipases [111–129]. It has been reported that the same lipase immobilized on different supports may exhibit very different enantioselectivity (in some cases even an inversion being detected) in the same experimental conditions.

In some cases, the change in the experimental conditions can present an effect fully opposite on different immobilized preparations [110–115].

Table 3 shows some of the most relevant results obtained in our laboratory with some of the most used lipases, in the alteration of the lipase enantiopreference by using different immobilization techniques, in the hydrolysis of several racemic compounds (Scheme 1). Enantiomeric ratio may be changed from 1 to almost 100 just by using different immobilized preparations in some cases.

In some instances (e.g., methyl mandelate and CRL [114]) it was even possible to obtain both pure isomers by using different immobilized preparations.

A further step was showing that the *same* lipase immobilized on the *same* support and used under the *same* conditions, may behave in a very different fashion depending on the immobilization conditions. This required the immobilization protocol to be able to immobilize proteins in a wide range of conditions and, in certain extent, to fix the different structure of the lipase during immobilization. This was exemplified using lipase from *Candida antarctica* (form B) and its adsorption on a support coated with polyethylenimine, at different temperatures from pH 5 to 9 [130]. The use of PEI-coated support permitted



Scheme 1. Enantioselective hydrolytic resolution of different racemic compounds catalyzed by immobilized lipases.

to immobilize the enzyme in all these range of pH value. The interactions with the polymer of the different forms of the lipases, permitted to keep, at least partially, the different forms of the lipase molecule. Thus, enantioselectivity was varied from 3.5 to 25 just by adsorbing the enzyme under different pH and *T* values [130].

Other examples of modulation of the enzyme catalytic properties via immobilization techniques may be found. Penicillin G acylase is another enzyme that suffers a relatively large conformational change during catalysis, an acyl-induced exposition of the reactive serine [131]. The properties of this enzyme have been also modulated by immobilization, both in synthesis of antibiotics [132,133] and in resolution of racemic mixtures [134]. Similarly, the selectivity of multimeric enzymes, where the subunit assembly may play a critical role in the final properties, has been altered by immobilization techniques: beta-galactosidase from *E. coli* [135] or ampicillin acylase from *A. turbidans* [89], getting preparations with different features.

However, these results show another aspect: this kind of enzymes will alter its properties during immobilization even if it is not desired, and the use of just one random immobilization technique may produce an immobilized form with similar, worse or better properties than the soluble enzyme. If the enzyme naturally presents the desired properties, it is necessary to keep them unaltered during the immobilization. For these cases, it is necessary to use extremely mild immobilization protocols, preventing any undesired restriction to the enzyme mobility or distortion of the active center. In this regard, immobilization on supports bearing lowly activated aldehyde dextran may be a very good alternative [136]: this long and hydrophilic spacer arm produces a minimal modification on the enzyme.

We should consider that most of the immobilization strategies available will immobilize proteins by the areas that were the richest one in one kind of group, but a protein may have several areas very rich in a determinate group (Lys, in negative charges, in positive charges). Therefore, in many cases we can have a main orientation of the enzyme, but we can hardly assure that we have got just a unique orientation of the enzyme. However, even using this imperfect strategy, we have observed an extremely strong modulation of the enzyme properties when comparing enzymes immobilized on different supports. Thus, if it was possible to have all the protein molecules immobilized following exactly the same orientation, results may be expected even to be more significant. Therefore, the development of methodologies that could permit to immobilize enzymes in a fully controlled fashion, exactly via a particular area and conferring a desired rigidity, will greatly improve the potential of this technique to modulate the enzyme properties. This way, there is still a hard and exciting way ahead before exploring the possibilities of these methodologies.

Therefore, controlled immobilization of enzymes may be a very powerful and simple tool to modulate enzyme properties, with results such as those obtained using other techniques (e.g., directed evolution). In any case, all the technologies should be compatible, and this "combinatorial" preparation of biocatalyst may be performed on engineered proteins. There is a recent report where a possible robotization of the system to coupling side directed evolution and immobilization modulation is proposed by immobilizing enzymes in microtiter plate scale [137].

This strategy, although empiric, may permit in a very rapid fashion, using a collection of enzymes immobilized following a battery of supports under different conditions, to find a biocatalyst able to perform a particular resolution of a chiral compound, with quite high possibilities.

6. Decrease of inhibitions by immobilization techniques

The last possible problem of the enzymes when used as industrial catalyst that we are going to consider in this paper is the inhibition of the enzymes by the substrates, reaction products or components of the bulk medium [138,139]. In some cases, that may produce that the time to reach the desired yield was too long. In some extreme cases, from a practical point of view, the reaction may be stopped far from the thermodynamic yield.

Once again, among the techniques to solve these problems, immobilization has proven to be a very useful tool. The immobilization may permit to reduce the inhibition problems by different mechanism.

6.1. Exclusion of the inhibitor from the enzyme environment

It is possible to generate an artificial nano-environment fully covering the enzyme using polymers. This artificial nano-environment can promote the partition of hydrophobic or hydrophilic substances from the enzyme environment.

Covalently immobilized penicillin G acylase was covered by layers of hydrophilic polymers, greatly reducing the inhibition produced by organic solvents [140]. Other successful strategies were the enzyme co-aggregation with hydrophilic polymers [141] or the trapping in hydrophilic beads (Lentikats) [142]. In all cases, together with a higher stability in the presence of organic solvents, inhibition by organic solvents was greatly reduced.

6.2. Decrease of the affinity of the recognition places of the enzyme by the inhibitor

Specific inhibitors need to interact with specific places of the enzyme structure to produce the inhibition. Thus, immobilization may reduce the inhibition in two different situations.

The first one is when the inhibitor acts as an allosteric one, interacting with the protein in a place different from the active center. In this case it may be possible that the immobilization of the enzyme through that region may distort or block this place, greatly reducing the inhibition caused by this agent.

The second one is when the inhibitor interacts with the active site. In this case, if the immobilization slightly distorts the enzyme active center, in some cases may be achieved a higher distortion of the enzyme in the inhibitor place than in the substrate place. This may be more likely if the substrate is larger than the inhibitor and interacts with a larger number of groups in the enzyme.

Again, to get good results it is convenient to use a battery of immobilization methods, being able to immobilize the enzyme via different areas, with controlled intensity, etc. That way, it may be possible to find a support and protocol that can produce the desired effect.

As examples of the potential of these techniques, the hydrolysis of lactose by two different lactases will be discussed. Lactase from *Kluyveromyces lactis* presents competitive inhibition by galactose (χ was 45 mM) and non-competitive by glucose (χ was 750 mM) [143]. Using soluble enzyme, this inhibition constants were enough to stop the reaction of hydrolysis of 5% lactose (similar to the content in milk) after an 80% hydrolysis [143]. After immobilizing the enzyme on different supports, a immobilized preparation with a much higher inhibition constant by galactose (χ was over 40 M) was obtained and this preparation permitted the full hydrolysis of 5% lactose.

Lactase from *Thermus* sp. presented even a stronger competitive inhibition by galactose (χ 3.1 mM) and non-competitive inhibition by glucose (χ was 50 mM), the reaction of hydrolyses of 5% lactose was apparently stopped just at 60% of conversion [144]. The immobilization on different supports permitted to find one preparation where the χ by glucose were increased by a two-fold factor, while the competitive χ by galactose increased by a four-fold factor. These changes in the kinetic constants permitted, also in this case, the full hydrolysis of 5% lactose [144].

Thus, although this problem may be also reduced using genetic tools [145] immobilization may also solve or reduce it with a similar or even higher efficiency.

7. Integration of different techniques to improve the features of the immobilized enzyme

There are many papers showing that the enzyme properties may be directly improved by chemical or genetic modification of enzymes, screening of the most suitable enzyme, etc. [107–110,145–153].

In this revision we have shown some examples where the chemical or physical modification of immobilized enzymes permitted to solve some problems, e.g. (stabilization of multimeric enzymes [88], generation of artificial micro-environments [140]). Moreover, the immobilization–stabilization of enzymes from thermophiles has permitted to increase its stability against any distorting condition [49,50].

Recently, there are some new promising proposals that show the opportunities of the use of chemical or genetic modification not to directly improve the enzyme functional properties, but to improve the enzyme performance after immobilization. This may simplify the design of the mutations, because now we only intend to increase the reactivity of the enzyme surface, not to directly alter its stability. The chemical and genetic amination of the surface of some enzymes (e.g., glutaryl acylase, penicillin G acylase) have permitted to greatly improve the multipoint covalent attachment of the enzyme, increasing the stability of the immobilized enzyme when compared to the immobilized native one [154–155].

In a similar way, chemical and genetic modification of the protein surface has improved the reversible adsorption of penicillin G acylase on both, cationic and anionic exchangers [156–157].

A final nice approach that we would like to comment on has been the use of directed evolution to improve the activity recovery and stability of the enzyme after immobilization. In two cycles of an error-prone PCR process, variants of formate dehydrogenase from *Candida boidinii* were created which preserved 4.4-fold higher activity after entrapment in polyacrylamide gels than the wild-type enzyme [158].

Therefore, the use of site-directed mutagenesis of protein surfaces seems to be a powerful tool to greatly improve the control of the immobilization and the properties of the final immobilized biocatalyst, and more effort may be expected in the next years in this regard.

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