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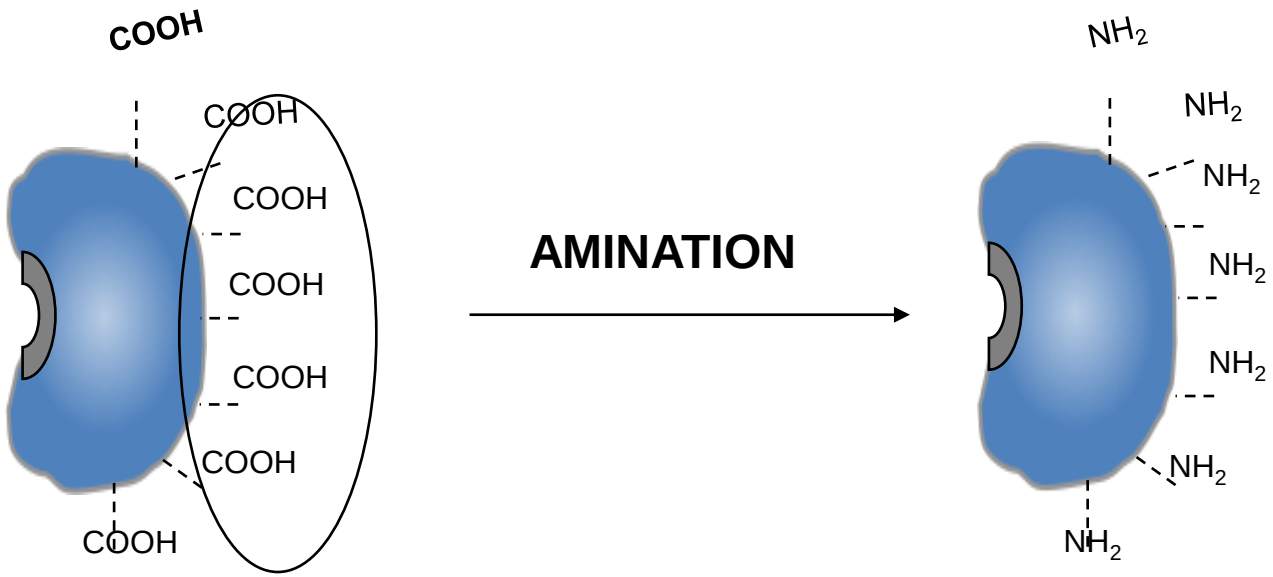

IMPOVED CATALYTIC FEATURES??
IMPROVED IMMOBILIZATION
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Amination of enzymes to improve biocatalyst performance: Coupling genetic modification and physicochemical tools

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Abstract

Improvement of the features of an enzyme is in many instances a pre-requisite for the industrial implementation of these exceedingly interesting biocatalysts. To reach this goal, the researcher may utilize different tools. For example, amination of the enzyme surface produces an alteration of the isoelectric point of the protein along with its chemical reactivity (primary amino groups are the most widely used to obtain the reaction of the enzyme with surfaces, chemical modifiers, etc) and even its "*in vivo*" behavior. This review will show some examples of chemical (mainly modifying the carboxylic groups using the carbodiimide route), physical (using polycationic polymers like polyethyleneimine) and genetic amination of the enzyme surface. Special emphasis will be put on cases where the amination is performed to improve subsequent protein modifications. Thus, amination has been used to increase the intensity of the enzyme/support multipoint covalent attachment, to improve the interaction with cation exchanger supports or polymers, or to promote the formation of crosslinkings (both intra-molecular and in the production of crosslinked enzyme aggregates). In other cases, amination has been used to directly modulate the enzyme properties (both in immobilized or free form). Amination of the enzyme surface may also pursue other goals not related with biocatalysis. For example, it has been used to improve the raising of antibodies against different compounds (both increasing the number of haptamers per enzyme and the immunogenicity of the composite) or the ability to penetrate cell membranes. Thus, amination may be a very powerful tool to improve the use of enzymes and proteins in many different areas and a great expansion of its usage may be expected in the next future.

Key words: enzyme chemical amination, enzyme genetic amination, polymer coating of enzymes, polyethylenimine, enzyme multipoint covalent attachment, crosslinking, enzyme stabilization, enzyme modulation.

Introduction

Enzyme features, such as specificity, selectivity and activity under mild conditions, have attracted the attention of researchers on these molecules as catalysts for industrially relevant reactions.[1] However, together with the positive properties, enzymes also have some features that are in opposition with their use as industrial catalysts: e.g., enzymes are soluble, unstable, inhibited by substrates, products and other compounds, and the good catalytic properties are only optimized towards the physiological substrate.[2] In nature enzymes are submitted to strict regulations in complex metabolic routes to give a rapid response to changes in the medium. However, now we intend to use the enzymes in an industrial reactor, where they are no longer required to have this regulative behavior.

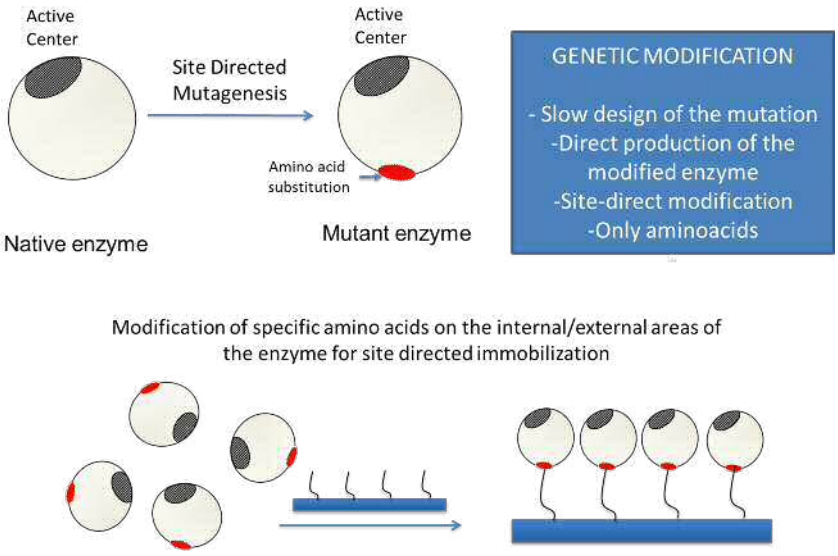


Figure 1. Site-directed mutagenesis in biocatalysts design

Genetic tools have permitted us to obtain more stable and efficient biocatalysts using site-directed mutagenesis or directed evolution.[3] This strategy may be more or less complex and time-consuming to produce the desired enzyme (Figure 1), but once the variant enzyme is ready, the large scale production will not be more expensive than using a native enzyme (it may actually become cheaper if enzyme overproduction is achieved).

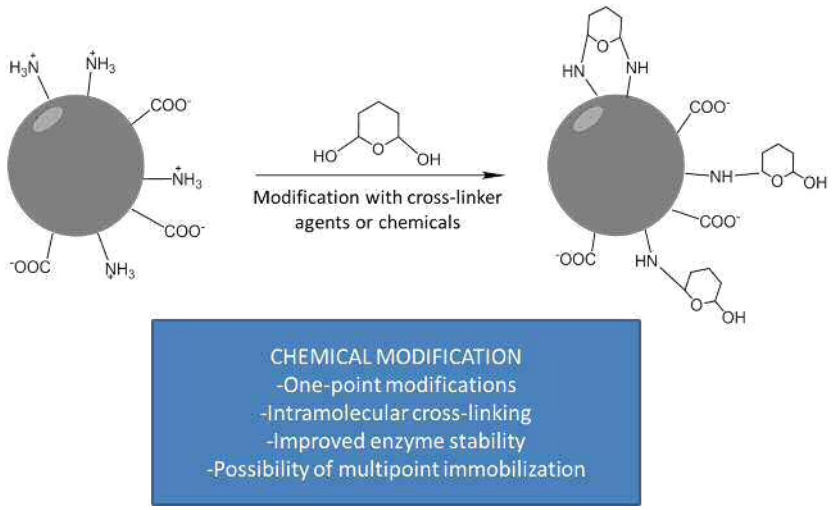


Figure 2.- Chemical modification of enzymes in biocatalysts design.

Another tool to improve enzyme properties is the chemical modification of enzymes.[4,5] (Figure 2) Chemical modification may pursue one-point modifications (the effect of the modification on the enzyme features may be hard to predict)[6,7] or the introduction of intramolecular crosslinkings to increase the enzyme rigidity and thus, enzyme stability may be enhanced.[8] On one hand, the modification may be performed quite rapidly, but the enzyme will need to be modified each time the biocatalyst is prepared. On the other hand, it is not necessary to be restricted to natural amino acids and it is not limited to enzymes with available genes.[5]

Immobilization also is used to improve enzyme properties.[9,10,11] This technique needs to be used to solve the water-soluble nature of enzymes.[12,13] (Figure 3) Immobilization consists in the confinement of the enzyme molecules in a limited space, and permits to have a heterogeneous catalyst, easy to separate from the reaction medium, and to reuse it, if the enzyme is stable enough. There are many immobilization techniques,[14] more or less adequate for each specific case depending on the enzyme and the process (e.g., substrate size).[15] However, as this immobilization step is almost compulsory in the preparation of an industrial biocatalyst, many authors are trying to solve other enzyme limitations during

immobilization.[9,10,11] Thus, immobilization inside porous structures avoids the interaction of the enzyme molecules with other enzyme molecules (preventing enzyme aggregation) or with interfaces such as gas bubbles, able to inactivate enzymes[11] (Figure 3). Rigidification of the enzyme three-dimensional structure may be achieved via multipoint covalent attachment[9], while the multisubunit immobilization of multimeric enzymes prevents their inactivation via dissociation (Figure 3).[16] In some cases, the generation of favorable environments may permit the stabilization of the enzyme under certain conditions.[17,18]

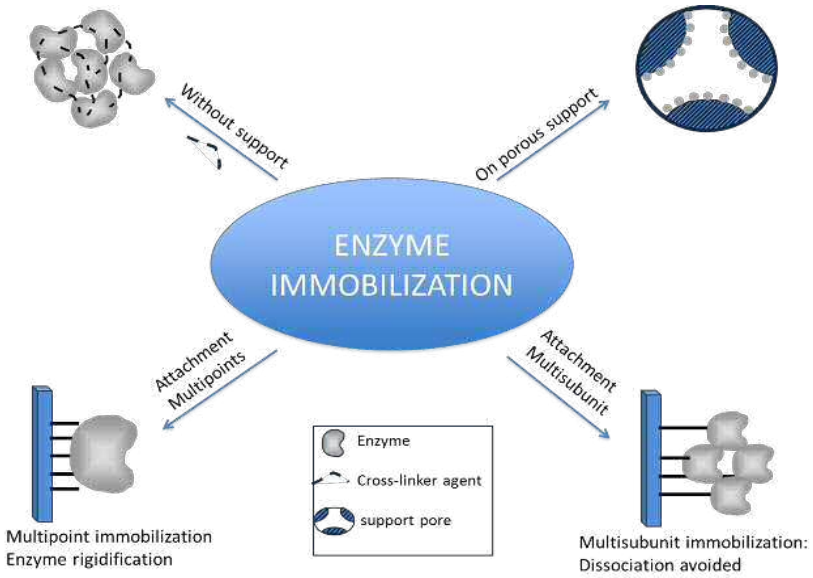


Figure 3. Strategies of enzyme immobilization in biocatalysts design.

With a handful of exceptions, these three tools are used in an individual way to design a biocatalyst, without considering that all of them may (or even must) be used simultaneously to have a biocatalyst with enhanced properties.[19-21] This becomes especially relevant considering, as previously discussed, that the enzymes must be finally used in an immobilized form.[12]

In this review, we will focus on the amination of the enzyme molecule surface, using physical, chemical or genetic strategies, to improve its properties, such as stability, but also activity or selectivity. Special emphasis will be paid to the coupled use of amination to improve the immobilization, chemical or physical modifications of the enzyme.

The amination of the surface of a protein may fulfill many different objectives (Figure 4). For example, it may alter the existing interactions between the groups in the enzyme surface to tune the enzyme properties.[19] This is easily obtained using chemical modification because chemical amination is usually based on the amidation of carboxylic acids (see section below).[22,23] This modification produces a clear alteration of the ionic interactions on the protein surface: ionic bridges may be broken and changed by repulsion forces. These changes may affect the conformation of the enzyme, and thus its stability, activity, specificity or selectivity.[24,25]

This alteration of the sign in the ionic character of areas of the protein surface may facilitate the use of cation exchangers to purify the enzyme that does not naturally have a tendency to become adsorbed on these supports (e.g., using poly-Lys tags).[26-28]

Amination may also increase the enzyme chemical reactivity versus a support used for covalent immobilization.[21] Most of the supports used to immobilize proteins are designed to involve the primary amino groups of the protein. That is because the amino group of the Lys is an nucleophile, relatively frequent in enzyme sequences, usually placed on the protein surface due to its hydrophilicity and can directly react with a broad diversity of groups that may be introduced in the support (epoxyde,[29] vinyl sulfone,[30] glutaraldehyde,[31,32] cyanogen bromide,[33] tosyl chloride,[34] tresyl chloride,[35] glyoxyl,[36] etc.). An enrichment of the enzyme surface in primary amino groups will produce an increase in the immobilization rate. Site-directed introduction of Lys residues may also permit the immobilization/purification of the

enzyme, using supports such as glyoxyl, which require immobilizing the enzyme via several enzyme/support attachments.[37,38]

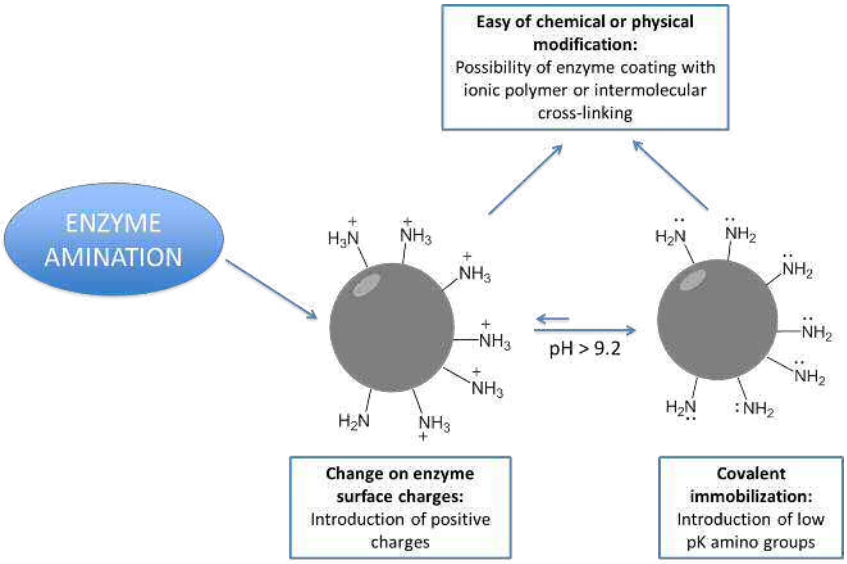


Figure 4. Chemical amination in biocatalysts design.

Amination increases the possibility of achieving a higher interaction between enzyme and activated supports,[19,21] that is, a higher number of covalent attachments that increase enzyme stabilization, or even controlling the immobilization area.[39,40]

If the amino groups are chemically introduced using ethylenediamine, the new amino groups present a lower pK value than that of the Lys (9.2 versus 10.7 without considering alterations caused by the local environment),[41] being thus more reactive and permitting both, immobilization and multipoint covalent attachment under milder conditions.[19] This may be very important when the enzyme is unstable at alkaline pH values.[42] However, this modification will be uncontrolled along the whole protein surface, while the site directed mutagenesis permits to introduce reactive groups just in the desired area of the protein, not altering the other areas of the protein.

The increase of amino groups on the enzyme surface may also facilitate some further chemical or physical modification of the enzyme. For example, it may simplify the coating of the enzyme with anion exchangers.[43,44] The increase on primary amino groups has also been

used in certain cases to improve the prospects of achieving intra (to stabilize enzymes)[45] or intermolecular covalent attachments (to prepare crosslinked enzyme aggregates, CLEAs).[46] The lower pK value of the chemically introduced amino groups using ethylenediamine has also permitted to have a more general chemical modification of protein surfaces with other molecules via modification of these amino groups under milder conditions than that required by the unmodified enzyme.[47]

The physical coating of the enzyme surface with poly-amine polymers, such as polyethylenimine or polyallylamine, may have many positive effects on enzyme properties, effects that are derived from the physical and chemical features of the polymer[48,49]. Among these, we can point out the partition effect, keeping away from the enzyme environment some deleterious hydrophobic compounds (oxygen,[50,51] hydrophobic organic cosolvents,[52,53]), the prevention of interaction with inactivating interfaces,[54] and stabilization of multimeric structures.[54,55]

However, in the context of this review, it be remarked that the coating with poly-amine polymers of the enzyme surface permits, in an indirect way, the enzyme ionic exchange on a cation exchanger, even though initially the enzyme had no tendency to become adsorbed to this cation exchanger.[54]

In the next sections of this review, we will present and discuss in a deeper way all these general ideas, supplying some of the available examples.

2. Chemical amination

2.1. Chemical amination of enzymes using the carbodiimide route

This is the most used strategy to achieve the chemical amination of protein surfaces. The use of water-soluble carbodiimides in conjunction with reactive nucleophilic species, as a technique for the modification of carboxyl groups in enzymes and other proteins, was

introduced many years ago.[56,57] Proteins have many reactive groups that can react with carbodiimides in the same fashion as with simple nucleophiles. [58-60]

Ethyl-di-methyl-amino-propyl carbodiimide (EDC) allows the modification of amino acid side chains thereby generating "new" enzymes via covalent modification of existing proteins. For this reason it has been used extensively for the chemical modification of proteins.[22,58,60]

Using carbodiimides and nucleophiles such as primary amines it is possible to modify carboxyl groups from different proteins. The nature of the current chemical reactions involved in carboxyl group modifications using water-soluble carbodiimides has been previously described.[57,61] This chemistry is summarized in Figure 5. In the first step of the reaction, the carboxyl group is added to the carbodiimide, forming a very labile O-acyl-iso-urea intermediate. As a result of the re-protonation at the site of the Schiff's base, the intermediate will change into a carbocation, followed by reaction with nucleophilic species such as ethylenediamine at high concentrations in order to give a stable amide bond (Figure 5, route 1).

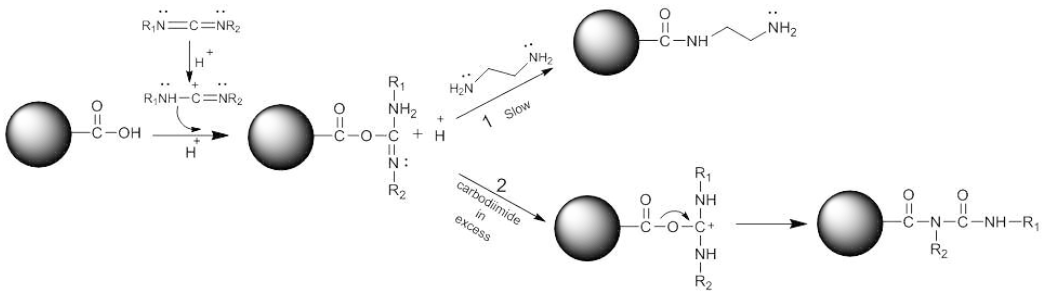
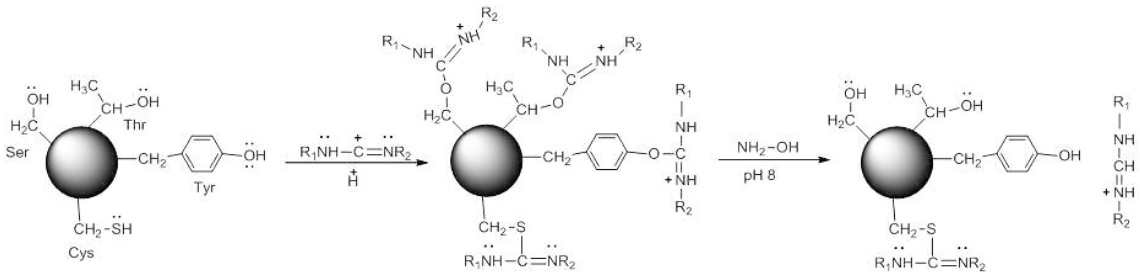


Figure 5. Reactions between carbodiimide and carboxylic groups of proteins.

On the other hand, the O-acyl-iso-urea intermediate can form N-acyl-urea via an intramolecular acyl transfer mechanism. If the nucleophile is water, the carboxyl group will be regenerated with the conversion of 1 molecule of carbodiimide into its corresponding urea (Figure 5, route 2).[57,61] However, kinetic studies on the modeling of carbodiimide-carboxyl-

196 nucleophile system have shown that the rearrangement can be slow compared to the
197 nucleophilic attack if the concentration of nucleophile is sufficiently high.[57] Therefore, the
198 coupling reaction of carboxyl and nucleophile groups can be driven essentially to completion
199 in the presence of excess of both carbodiimide and the nucleophilic reagent.

200 In aqueous solutions at acidic pHs, carbodiimides may react also with free sulfhydryl
201 groups from side chains of cysteine,[62] as well as accessible phenolic groups of tyrosines.[63]
202 Indeed, it has been reported that the carbodiimide activated O-acyl-iso-urea on one molecule
203 may undergo displacement by the slightly nucleophilic hydroxyl of tyrosine (Figure 6).[60,63,64]
204 Kinetic studies have shown that reaction rates of sulfhydryl and carboxyl groups with EDC are
205 approximately equal, while tyrosine reacts more slowly. Carraway and Koshland[63] have
206 shown that EDC converts accessible tyrosine residues in proteins to O-arylisourea derivatives,
207 which are resistant towards acid hydrolysis. However, they have also shown that
208 hydroxaminolysis of the modified protein quantitatively reverses this tyrosine modification.



209

210 **Figure 6. Side reactions during protein modifications with carbodiimide.**
211

212 The reaction of carbodiimides with the carboxyl group in proteins can lead to
213 inhibition; this can be caused by interaction of neighboring nucleophiles that could generate
214 intramolecular crosslinkings (Figure 7, route A). For example, erythrocyte membrane ATPase
215 is inhibited by the carbodiimide. The mechanism of the inhibition is thought to be via
216 formation of the O-acyl-iso-urea species followed by the attack of an adjacent nucleophile
217 causing the loss of urea, covalent binding of the nucleophile with the binding site to produce
218 crosslinking. Protection of the enzyme by using methyl glycinate only occurs when this

nucleophile is added simultaneously with the carbodiimide; subsequent addition to the nucleophile does not cause regeneration of the O-acyl-iso-urea.[58,65]

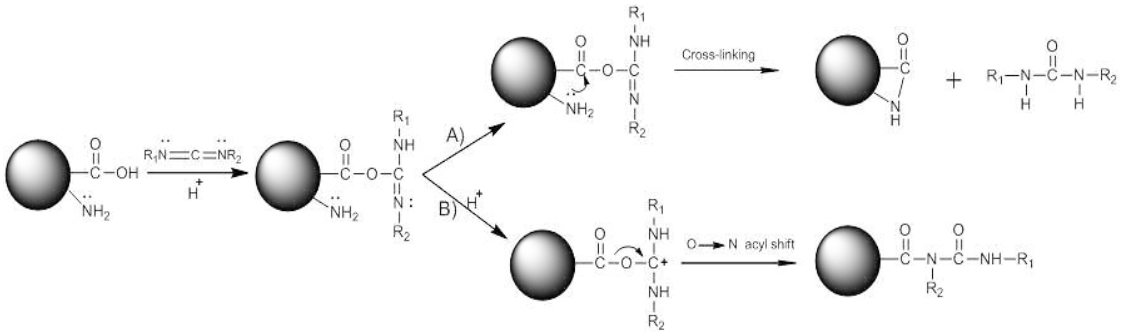


Figure 7. Rearrangement of proteins following treatment with carbodiimide. A) intramolecular crosslinkings. B) O-N-acyl shift rearrangements.

Furthermore, another cause of enzymatic inhibition by use of carbodiimides can be attributed to O-N-acyl shift rearrangements (Figure 7, route B). The O-acyl-iso-urea is relatively labile to hydrolysis, which causes regeneration of the active enzyme. However, residues partially shielded from solvolysis are susceptible to the stable N-acyl-urea rearrangement. Functionally important acid groups may frequently be found shielded in active sites and this type of chemical modification becomes now feasible.[58,64]

If properly performed, this route may permit the simple amination of the enzyme surface in a very controlled way.

2.1. Chemical amination of free enzymes

The first interest of , the amination of enzymes via the carbodiimide route was the modification of the carboxylic acids of the protein to discriminate the existence of essential carboxylic groups for the function of the proteins, and that was performed with diamines,[59] but also with just mono amine compounds[60,66] as the final goal was not the amination of the enzyme surface but the modification of the carboxylic residues.

Table 1. Effect of chemical amination on biochemical properties of free enzymes

Enzyme	Source	EC Number	Type of Modification	Effect of amination on enzyme properties	Reference
Alpha-Chymotrypsin	Bovine Pancreas	3.4.21.1	Succinylation of the enzyme followed by carbodimide activation and ethylenediamine cross-linking	Increase in thermostability from 3- to 21-fold	67
Glucoamylase	*Aspergillus niger*	3.2.1.4	Modification of three carboxyl groups available in the enzyme with carboddiimide and ethylenediamine activation	Increase on thermostability	68
Lysozyme	Hen egg white	3.2.1.17	Carbodiimide route activation of the enzyme followed by modification with athenolamine, ethylenediamine, methylamine, or 4(5)-(amino-methyl)-imidazole	Specific modification of Asp-101 decreases enzyme activity (83-52% of the native enzyme)	69
Beta-glucosidase	*A. niger* NIAB280	3.2.1.21	Modification of the enzyme with 1-ethyl-3(3-dimethylaminopropyl) carboddiimde in presence of glycinamide or ethylenediamine	Increase of half-lives at high temperatures (64 and 67 ºC), with better results using ethylenediamine	70
Carboxymethyl-cellulase	*A. niger*	3.2.1.4	Modification of the enzyme with 1-ethyl-3(3-dimethylaminopropyl) carboddiimde in presence of dimethylamine hydroghloride and ethylenediamine dihydrochloride as nucleophile	Improving catalytic efficiency (Vmax/Km) from 0.16 to 1	71
Glucoamylase	*Fusarium solani*	3.2.1.3	Chemical Amination of the enzyme using ethylenediamine	Increase on activity and stability depending on the modification degree improving catalytic efficiency from 136 to 225	72
Xylanase	*Scopulariosis*	3.2.1.8	Carbodiimide activation and	Decrease on catalytic efficiency and	

	sp.		ethylenediamine modification of the enzyme	obtaining of two optimal pHs	73
Serine protease	*Bacillus lentus*	3.4.21.-	Combined use of chemical modification and site-directed mutagenesis of the enzyme	Modification of enzyme selectivity allowing catalyzing coupling reactions of both L- and D-amino acid esters.	74
Invertase	NA	3.2.1.26		Increase of stability to temperature of the enzyme around 10 °C, to pH below 3.0 by 20% and denaturing compounds such as urea by 2 h.	75
Invertase	NA	3.2.1.26	Pectin was attached to ethylenediamine-activated carbohydrate moieties of the enzyme using Modification with 1-ethyl-3(3-dimethylaminopropyl) carbodiimide.	Increase of optimal temperature by 8 ºC and thermostability by 7.3 °C. Improving on half-life at 65 °C from 5 min to 2 days, enzyme stability at pH 2 by 33% and pH 12 by 27%.	76
Trypsin	Pancreas	3.4.21.4	Modification of the enzyme with polysaccharides derivatized with 1,4-diaminobutane through a transglutaminase-catalyzed reaction .	Shift of the optimal pH to alkaline values. Increase of thermostability around 22- to -48 fold in the range 50-60 °C. Increase of half-life time ranging from 9- to -68 fold in presence o 0.3%(w/v) sodium dodecylsulfate (SDS)	77

NA: Not Available

However, some examples may be found where the objective was to aminate the enzyme surface and check the effects of this modification on the enzyme performance. Table 1 shows a resume of the main examples[67-77]. The objectives could be enzyme crosslinking (analyzing the effect of the crosslinking size)[67], or just to check the effect of the general modifications. Stabilities or activities could be improved in some cases. In a quite sophisticated strategy, several polysaccharides were derivatized with 1,4-diaminobutane and covalently attached to bovine pancreatic trypsin through a transglutaminase-catalyzed reaction.[77]

Thus, amination of free enzymes, even although not very utilized, has been used in diverse examples with good results.

2.3. Chemical amination of enzymes to improve its immobilization

2.3.1. Increase of the number of the enzyme/support covalent attachments

One of the goals that may be pursued by amination of the enzyme surface is to increase the amount of reactive groups on the enzyme surface and thus improve the prospects of getting an intense multipoint covalent attachment during immobilization. This approach is effective if the support (e.g., glyoxyl-agarose,[36] epoxy,[31] etc) and immobilization protocol are chosen in a way that may permit to get this multipoint covalent attachment. Table 2 shows a resume of the main examples.

Initially, the enzymes were aminated in solution, requiring extensive dialysis to eliminate the excess of ethylendiamine[78-85]. Most results used glyoxyl-agarose supports. Results pointed that the aminated enzymes were more rapidly immobilized and permitted higher stabilization factors. Interestingly, immobilization could be performed now under milder pH conditions on glyoxyl supports, a key feature when the enzyme was unstable at pH 10[78]. Immobilization at different pH values permitted to alter the rea involved in the

Table 2. Chemical amination of enzymes to improve their immobilization

Enzyme	Source	EC Number	Type of modification and Immobilization	Effect of amination on enzyme properties	Reference
Penicillin acylase	*E. coli*	3.5.1.11	Aminated enzyme immobilized on glyoxyl agarose by multipoint covalent attachment	Improvement of enzyme immobilization at pH 9 by multipoint covalent attachment. Increase of thermostability by a 4 –fold factor compared to the unmodified enzyme	78, 79
Glutaryl acylase	NA	3.5.1.93	Aminated enzyme immobilized on glyoxyl agarose by multipoint covalent attachment	Improvement of enzyme immobilization at pH 9 by multipoint covalent attachment Increase of thermostability by a 20–fold factor compared to the unmodified enzyme	80
Glucoamylase	*A. niger*	3.2.1.3	Aminated enzyme immobilized on glyoxyl agarose by multipoint covalent attachment	Improvement of enzyme stability by 500-fold factor, keeping 50% of the initial activity of the immobilized enzyme	81
Laccase	*Trametes versicolor*	1.10.3.2	Aminated enzyme immobilized on glyoxyl agarose by multipoint covalent attachment	Stabilization of the enzyme 280-folds with a 60% of the initial activity. The biocatalyst can be used for 10 cycles in oxidation of phenyl compounds without detecting decrease in enzyme activity	82,83
Lipase	*Candida rugosa*	3.1.1.3	Aminated enzyme immobilized on electrochemically PANI activated with glutaraldehyde	Higher specific activity (52%) and thermal stability (3-times) after immobilization compared to immobilized unmodified enzyme. Increase of reuse of the enzyme at pH 10.	84
Invertase	Baker´s Yeast	3.2.1.26	Different aminated	Higher yields of immobilization, and	

			enzymes (periodate and ethanolamine-treated enzyme, periodate and ethylenediamine-treated enzyme and TNBS followed by periodate and ethylenedianzine-treated enzyme) were immobilized on Sepharose	improvements on thermal and storage stability of the enzyme	85
Lipase	*Bacillus thermocatenulatus*	3.1.1.3	Enzyme immobilized on octyl-agarose, aminated and then desorbed, and finally immobilized on Glyoxyl agarose	Stabilization of the enzyme around 1200-fold compared to enzyme immobilized on CNBr and further aminated	88
Lipase	*Thermomyces lanuginosus*	3.1.1.3	Enzyme immobilized on octyl-agarose, aminated and then desorbed, and finally immobilized on Glyoxyl agarose	Immobilization of the enzyme without inactivation can be performed at pH 9 or 10. Enzyme activity is kept at 70% and stability is improved 5-fold compared to the non-aminated enzyme	42
Lipase	*Rhizomucor miehei*	3.1.1.3	Enzyme immobilized on octyl-agarose, aminated and then desorbed, and finally immobilized on Glyoxyl agarose and CNBr	Improvement in enzyme immobilization rates at pH 9.1	95
Penicillin G acylase	*E. coli*	3.5.1.11	Amination of the enzyme by ethylenediamine and carbodiimide	Immobilization of the enzyme on carboxymethyl or dextran sulphate-coated supports is facilitated. Significant increase in enzyme stability to organic solvents are achieved	43
Invertase	*Saccharomyces*	3.2.1.26	Introduction of chitin	High yields and enzyme recovery	

	cerevisiae		enzyme structure	during immobilization of alginate-coated chitin supports. Optimal temperature is increased by 10 °C and thermostability enhanced around 9 °C (4 –fold more resistant to thermal treatment at 65 °C than native enzyme)	107
Invertase	*S. cerevisiae*	3.2.1.26	Modification of the enzyme on pectin-coated chitin support via polyelectrolyte complex formation	High enzyme recovery (97%) and immobilization yield (85%). Optimal temperature is increased by 10 °C and its thermostablity enhanced by about 10 °C after immobilization (4 –fold more resistant to thermal treatment at 65 °C than native enzyme)	108
Invertase	*S. cerevisiae*	3.2.1.26	Modification of the enzyme on hyaluronic-acid-modified chitin	Optimal temperature for sucrose hydrolysis is increased by 5 °C and thermostability enhanced by about 10 °C after immobilization (4 –fold more resistant to thermal treatment at 65 °C than native enzyme)	109

immobilization and different stabilizations could be obtained (after incubation at pH 10 of the already immobilized enzyme). Immobilization of lipase from *Candida rugosa* electrochemically synthesized PANI activated with glutaraldehyde was improved via chemical amination. [84] Aminated lipases exhibited higher specific activity (52%) and thermal stability (3-times) after immobilization, compared with the immobilized unmodified lipase. Also, reusability of the immobilized enzyme was significantly increased with amination, especially when immobilization was performed at pH 10, this biocatalyst retained 91% of activity after 15 reaction cycles.[84]

To solve the problem of elimination of the excess of ethylenediamina and also to beneficiate of the solid phase modification of proteins, the previous reversible immobilization of enzymes seems advantageous. Using lipases, this could be accomplished by reversibly immobilized on octyl-agarose,[86] a support that did not produce any cross-reaction with carbodiimide. These immobilized enzymes were aminated, washed in a very simple fashion to eliminate the residual ethylenediamine, desorbed from the octyl-agarose beads using a detergent, and immobilized on glyoxyl-agarose (Figure 8).[19] The presence of detergent during the covalent immobilization was useful to avoid the risk of lipase/lipase aggregation.[87] Results were similar to the described aminating free enzymes: higher immobilization rates, possibility of immobilization at lower pH values on glyoxyl agarose, higher stabilization factors[42,88,95].

The solid phase amination produces a clear simplification of the process, new methods for the reversible immobilization of enzymes on supports that did not interfere with the amination reaction may open the opportunity of extending this strategy to any other enzyme or protein.

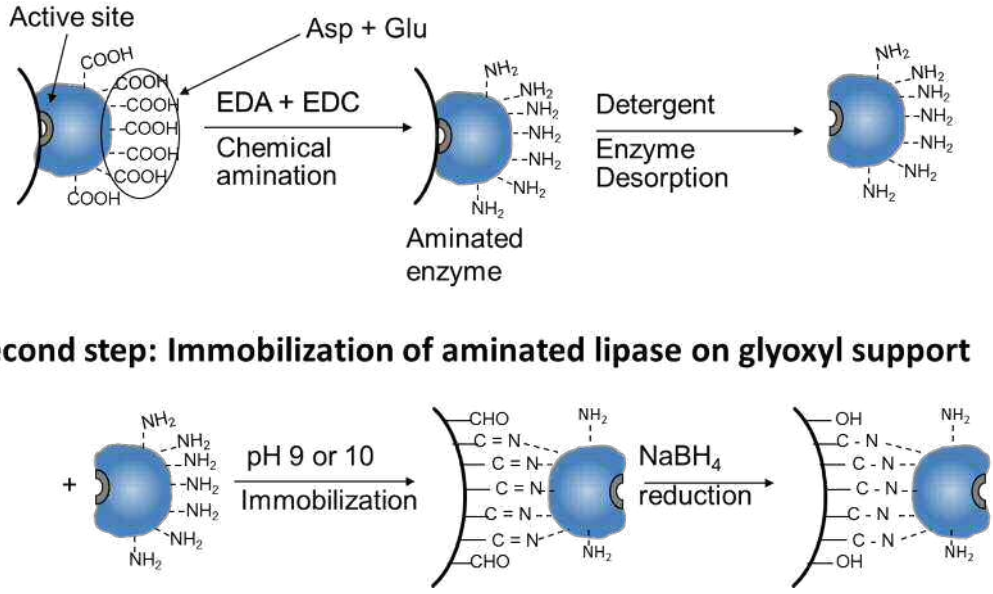
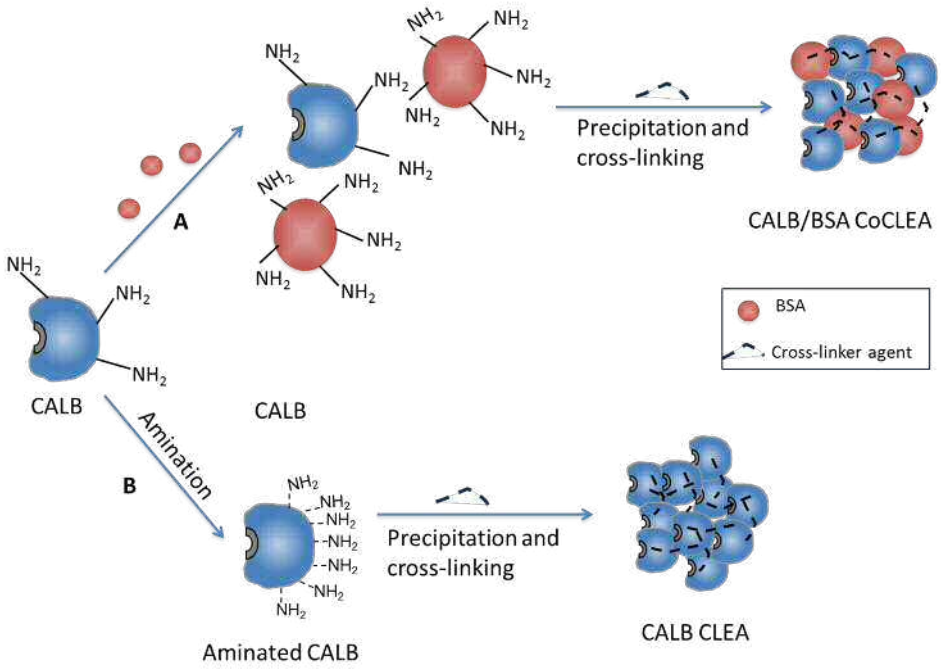


Figure 8. Solid-phase amination of lipases and its further immobilization on glyoxyl supports.

2.3.2. Improved production of crosslinked enzyme aggregates

The preparation of crosslinked enzyme aggregates (CLEAs) is a relatively recent enzyme immobilization technique developed in the group of Prof Roger Sheldon.[97] The strategy is relatively simple, consisting on the precipitation of the enzyme in an active form and the physical stabilization of the aggregate particles via chemical crosslinking to prevent re-dissolution when the aggregation reagent is eliminated.[98] However, in some instances, the crosslinking step of the enzyme may not be simple, e.g., if the enzyme has few reactive groups on its surface.[99] The amino groups tend to be the most utilized groups for the crosslinking step.[113, 114] Co-aggregation of the enzyme with other Lys rich proteins (Figure 9) is one of the possible solutions,[100] as well as the use of PEI (see section 4 of this review).[101,102] However, both strategies reduce the volumetric loading of the target protein on the final biocatalyst. The amination of the enzyme may be a simple solution to solve this problem. Lipase B from *Candida antarctica* presents a low amount of Lys on the surface.[103] Although the precipitation step is easy using different precipitants, the crosslinking step becomes a problem due to the low amount of Lys residues in this enzyme.[46] The enzyme surface was enriched in amino

306 groups by chemical amination of the enzyme using ethylenediamine and carbodiimide (Figure
307 9). Using this aminated enzyme, precipitation is also effective and the crosslinking step is no
308 longer a problem. Stability of this CLEA was higher in both thermal and cosolvent
309 inactivation experiments than that of the coCLEA produced by co-aggregation of BSA and
310 enzyme;[46] another alternative to produce a CLEA of this interesting enzyme.[104]



311

312 **Figure 9. Strategies to crosslink enzyme aggregates with glutaraldehyde when the**
313 **enzyme is poor on external amino groups.** A) Mixture with BSA. B) Chemical amination.
314

315 **2.3.3. Improved enzyme immobilization on cation exchangers**

316 Immobilization of proteins on ion exchangers requires the simultaneous
317 establishment of several enzyme-support interactions.[105,106] Most enzymes have an isoelectric
318 point ranking from 4 to 5, and this makes that most enzymes can hardly become adsorbed on
319 cation exchangers under a wide range of pH values. Table 2 shows examples where the
320 enzyme was aminated using ethylenimine [43] or cationic polymers like chitosan [107-109].
321 Immobilization via cation exchange could be only successfully employed using the modified
322 enzymes. The use of ionic polymers on the support and/or on the enzyme permitted to

improve the enzyme stability in the presence of organic solvents. Thus, amination seems to be a powerful tool to prevent one of the problems of immobilizing enzymes via ion exchange, the risk of enzyme desorption.

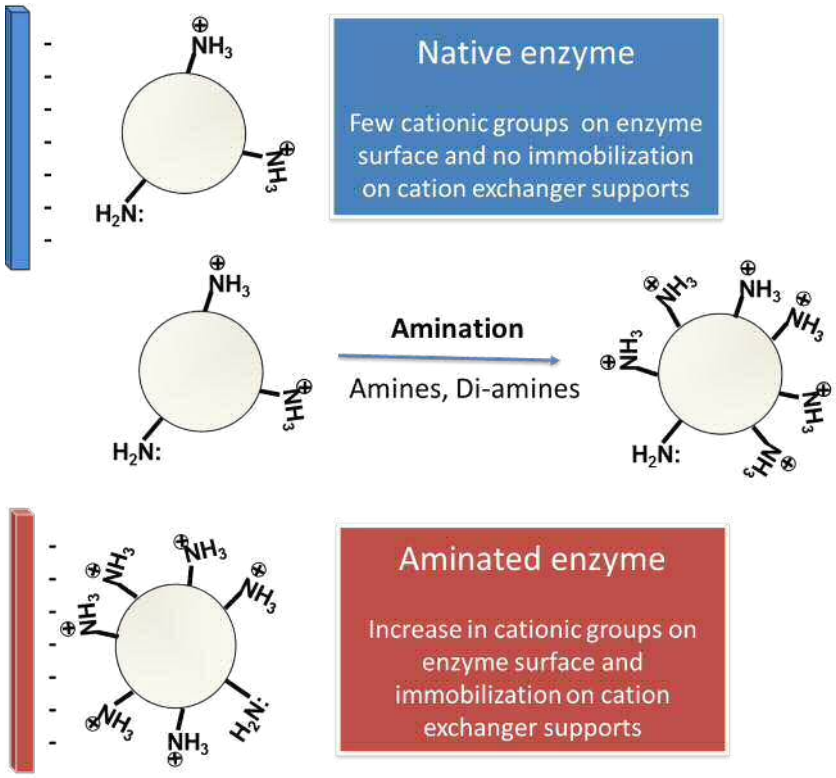


Figure 10. Improved ionic exchange of aminated proteins on anion exchangers.

2.4- Chemical amination of immobilized enzymes to improve their catalytic performance

The chemical modification of enzymes in the solid phase has many advantages[19]: prevention of aggregation, possibility of using stabilized enzymes, easy performance and control, etc. Most of the examples found using the chemical amination of the immobilized enzymes are quite recent. Table 3 summarizes the main results [24,24,110-114]. The amination of immobilized enzymes, mainly lipases, has permitted to improve their activity, stability, tuning selectivity and specificity. The results are not easy to predict, and depend on the immobilization protocol. However, due to the rapid way this modification may be

Table 3. Improvement of immobilized enzyme properties via chemical amination.

Enzyme	Source	EC Number	Type of Modification	Effect of amination on enzyme properties	Reference
Lipase	*Candida antarctica* (B), *T. lanuginosus* and *Pseudomonas fluorescens*	3.1.1.3	Modification with ethylenediamine via carbodiimide	Activity and enantioselectivity of the enzymes can be modulated, and it is possible to obtain high enantiomeric excess (ee) in the kinetic resolution of (±)-2-hydroxyphenylacetic acid methyl ester.	110
Lipase	(Novozyme 435) *C. antarctica* B in immobilized form	3.1.1.3	Modification by amino-ethyl-amidation of the enzyme	Improvement in enzyme activity against 3-phenylglutaric dimethyl diester (Two-fold)	111
Lipase	*T. lanuginosus*	3.1.1.3	Modification of the enzyme immobilized on octyl-agarose with ethylene-di-amine of carboxylic groups previously activated with carbodiimide at different extensions (10, 50 and 100%)	Improvement in enzyme activity against p-nitrophenyl-propionate (p-NPP). Fully aminated and hydroxylamine-treated enzyme exhibits higher thermostability (at pH 5 almost 30-fold factor compare to unmodified enzyme)	25
Lipase	*C. antarctica* B, *T. lanuginosus, R. miehei*	3.1.1.3	Amination of the enzyme immobilized on CNBr-activated Sepharose via a mild covalent immobilization or adsorbed onto octyl-Sepharose	Alteration of enzyme performance on the selective hydrolysis of sardine oil to produce both eicosapentanoic and docosahexaenoic acid.	112
Lipase	*C. antarctica* B	3.1.1.3	Modification with	Activity on p-nitrophenylbutyrate (p-	

			ethylenediamine (EDA) or 2,4,6-tri-nitro-benzen-sulfonic acid (TNBS) by different strategies (by single or sequential mode) of the enzyme covalently immobilized on CNBr-activated Sepharose or adsorbed onto octyl-Sepharose	NPB) is improved by 2-fold factor. Significant changes in activity/pH profiles and enzyme specificity are observed	24
Phospholipase (Lecitase Ultra)	*Artificial enzyme* (Novozymes)	3.1.1.4	Modification of the enzyme by different individual or cascade chemical modifications (amination, glutaraldehyde or 2,4,6-trinitrobenzensulfonic acid)	Most of the modifications presented a positive effect on some enzyme properties at least under certain conditions, and a negative effect under other conditions. For instance, glutaraldehyde modification of immobilized or aminated immobilized enzyme permitted to improve enzyme stability of both immobilized enzymes at pH 7 and 9 (around a 10-fold factor)	113
Lipase	*Geobacillus thermocatanulatus*	3.1.1.3	Modification of the enzyme by site directed amination by thiol-disulfide exchange with pyridyldisulfide poly-aminated-dextrans and then immobilized bycolvanet attachment in BrCN or Glyoxyl-agarose	Increase of enzyme activity on aliphatic carboxylic esters	114

accomplished, may be a simple way to increase the library of biocatalyst when looking for an optimal biocatalyst for a particular process.

2.5. Chemical amination to improve the crosslinking of immobilized enzymes

Chemical crosslinking of enzymes is a way to greatly increase their structure rigidity, and thus, their stability.[6,8,115,116]. Here we will focus on the crosslinking, using bi or multifunctional molecules, of previously immobilized enzymes. Intermolecular crosslinking is a quite complex process, as it must compete with the one-point modifications (if using homo-bifunctional reagents), and most important, only if there are reactive groups located on the appropriate distance (similar to the crosslinking reagent) the crosslinking will take place. This strategy is also valid to stabilize multimeric enzymes, if it involves all enzyme subunits.[16] The majority of the most widely used and effective crosslinkers are based on reaction with amino groups, as is the case of the glutaraldehyde.[32,117] Thus, amination of the enzyme surface could be a proper tool to achieve an intense intramolecular or intersubunit crosslinking (Figure 11).

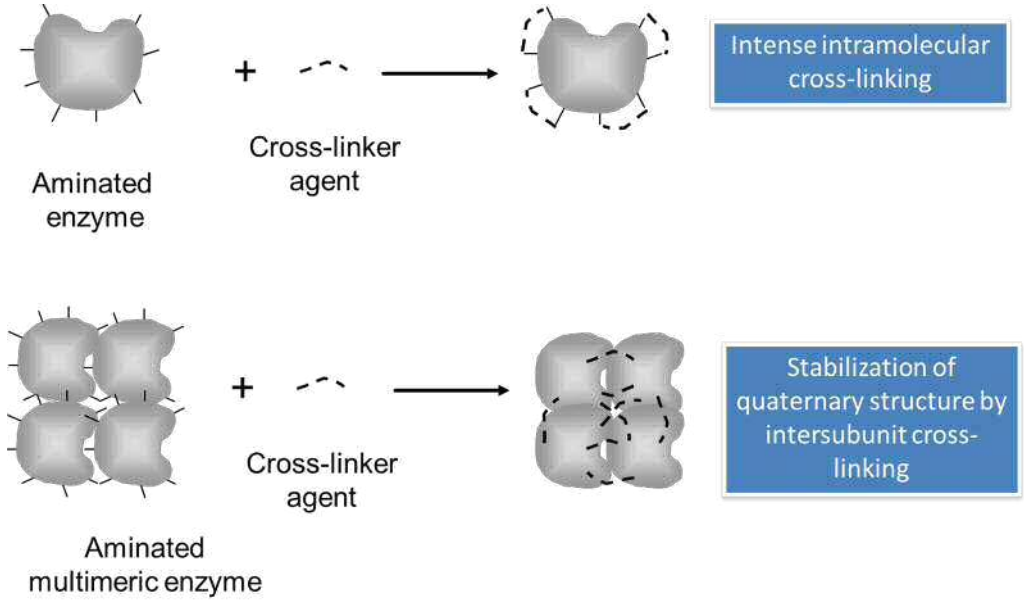


Figure 11. Increased prospects of crosslinking via amination.

However, although there are many reports on crosslinking of immobilized proteins,[19] we have been able to find just one example where the amination was performed on previously immobilized enzyme before the crosslinking. This example was on penicillin G acylase previously multipoint-immobilized on glyoxyl-agarose.[45] After amination, the enzyme was submitted to full modification with glutaraldehyde, the excess of reactive was eliminated by a simple washing, and the modified enzymes were long term incubated to permit an intense crosslinking (crosslinking is a quite slow process, as it requires the reaction between two groups attached to a rigid structure, a protein surface). Stabilization factors of more than 40 were achieved.[45] Using formaldehyde, stabilization did not take place, suggesting that this reactive may have a most complex crosslinking behavior.[45] However, leaving an excess of formaldehyde, similar stabilization factors were found,[118] indicating that formaldehyde required to form some multi-formaldehyde structures to give some crosslinking.[119]

2.6. Chemical amination to improve the physical coating with anionic polymers

The coating of enzymes with polymers has been reported as an efficient way to improve the enzyme stability versus interaction with interfaces, such as gas bubbles, subunit dissociation,[121] organic solvents by generating a certain partition, etc.[19] The use of ionic polymers may be a simpler solution than the covalent modification.

One requirement to use this strategy is that the polymer must coat the enzyme surface, and the enzyme-polymer interaction must be strong enough to enable the use of this composite under a wide range of pH values without breaking the composite. In fact, in some instances, this stabilization of the polymer-enzyme composite has been achieved by using a chemical crosslinker,[54] but in other cases this may not be possible, e.g., if the enzyme is inactivated by this treatment.[55]

Most of the examples dealing with coating enzymes with ionic polymers use polyethylenimine (see section 3 of this review) because most enzymes have too low isoelectric point to become coated using polyanionic polymers at neutral pH values. This coating with anionic polymers may be easily achieved using previously chemically aminated enzyme: the protein will have a cationic nature in pH values as high as 12 if total amination is achieved,[24] permitting to get a very stable enzyme-anionic polymer composite (Figure 12). Although this strategy should work, we have been unable to find an example where aminated enzymes are coated using poly-ionic polymers, the only examples we have found are related to immobilization of enzymes on anionic supports (see section 2.3.3).[43,107-109] However, as we thought that this application should work properly, we have decided to include this possibility in the present review.

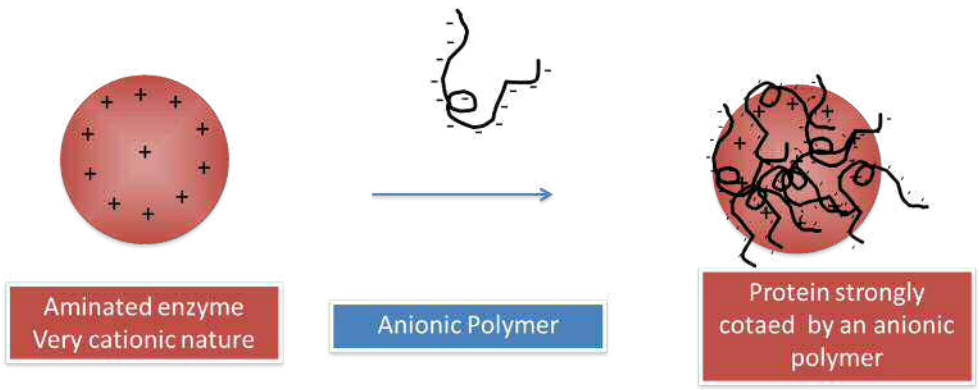


Figure 12. Physical coating of aminated enzymes with anionic polymers.

3.7. Chemical amination to improve their further modification with other compounds

In some instances, the researcher may intend to introduce some molecules on the enzyme surface to alter its physical properties, or alter its catalytic efficiency. The reaction with amino groups of the protein used to be one of the most applied strategies due to the good reactivity of these groups with many reagents.[122] However, if we really desire a massive modification of the protein surface, this may not be so simple, as the p*K* of the amino group in

the lateral chain of Lys is 10.5, and this p*K* will be quite similar on all residues exposed to the medium, that are the ones that we can modify. The terminal amino groups may have a lower p*K* value, but this group only permits a one-point modification. The massive modification of an enzyme surface with an amino-reactive compound was the goal of a recent paper.[47] While immobilized native lipase B from *Candida antarctica* cannot be massively modified with succinic polyethyleneglycol via the carbodiimide route, the aminated enzyme can be modified with 14-15 PEG molecules could be introduced per enzyme molecule. The effects on enzyme feature depended on the immobilization protocol.[47]

2.8. Chemical amination of proteins to improve their uses "*in vivo*"

Amination of enzymes has not only been used *in vitro*, but it has also been used to improve the enzyme and proteins performance *in vivo*. Covalently aminated enzymes, using polymers such as polyethylenimine or small amines attached to the carboxylic groups, have been used *in vivo* due to several advantages.

Regarding the preparation of antibodies versus small compounds, the use of aminated carrier proteins have two main advantages. First, the modified protein has usually a more potent immunogenecity that unmodified protein.[123,124] Second, and related to the point 2.6 of this review, the larger amount and higher reactivity of the aminated enzymes, may permit to introduce a higher number of antigen molecules per molecule of carrier protein.[125]

Regarding the use of proteins as medicament, the cationized protein is able to penetrate membranes in a more efficient way than the unmodified proteins.[126,127]

Now we will make a rapid overview on some examples of these *in vivo* uses of amination of proteins.

2.8.1. Use of aminated proteins to raise antibodies versus small molecules.

To raise antibodies versus small molecules, it is necessary to attach these small haptens to large proteins, because if the size is under 5000, the immunologic response is very low or inexistent.

In the late 1980s, it was shown that a cationized form of bovine serum albumin produced by substituting the anionic side chain carboxylic groups with aminoethylamide groups possesses unique immunologic properties.[124] It was possible to use 500-fold lower amount of cationized protein to reach the same immunogenic response. Moreover, antibodies were produced in response to the administration of cationized protein but not using unmodified enzyme unless an adjuvant was used. An inverse correlation between the degree of cationization and the amounts of antigen needed for optimal T cell reactivity was observed. The results suggested that native albumin enters the cell by fluid phase pinocytosis, whereas the aminated protein enters by a nonspecific adsorptive mechanism. [123] Ethylenediamine modified bovine albumin was modified with aflatoxin B1 using a Mannich-type protocol, and utilized to raise antibodies versus aflatoxin B1, achieving a quicker immunological response.[128] Later, a similar strategy was used to raise antibodies versus bisphenol A.[129] Compared with the non-aminated protein, the aminated bovine serum albumin improved the efficiency of coupling and enhanced the immune response against the target antigen.[129] In a third research, dichlorvos was coupled to cationized bovine serum albumin using also a method based on Mannich-type reaction, and utilized to produce a monoclonal antibody versus diclorvos.[130] In a nice report, it was shown that combining double-chemically modified carrier proteins and hetero-functional crosslinkers allowed preparing tailor-made hapten-protein carrier conjugates.[125] The protein was aminated and further modified by different crosslinkers (hyper-reactive proteins) at different conditions in order to control the conjugation ratio from 1 to > 12 molecules of hapten per carrier protein. Finally, this novel strategy has been successfully used to develop antibodies against a short specific peptide

corresponding to a one point mutation (D816V) of cKIT, which is a clinically relevant mutation related to mastocytosis and gastrointestinal stroma tumor.[125]

2.8.2. Improving the protein function *in vivo* via chemical amination

2.8.2.1. Improved function in vivo of aminated antibodies

Proteins and enzymes may be used as medicaments. In other cases, enzymes are used as a way to make some studies on their effect on cells. In most cases, the enzymes need to be inside the cells to be useful, or to penetrate complex barriers, such as the brain barrier. It has been demonstrated that proteins artificially cationized by chemical conjugation show efficient intracellular delivery via adsorptive-mediated endocytosis and then may exert their biological activity inside cells.[126] As the mammalian cell membrane possesses an abundance of negatively charged glycoproteins and glycosphingolipids, amination of proteins is a reasonable choice to endow them with the ability for intracellular delivery.[127]

One of the applications of the amination of proteins has been the improvement of antibody penetration on cells and different tissues. Owing to the poor transport of monoclonal antibodies across either capillary or cell membrane barriers, drug delivery strategies are needed to target monoclonal antibodies to intracellular sites where proteins function. Aminated antibodies may be therapeutic and allow for intracellular immunization because their better penetration in cells. For example, the improved issue uptake of aminated immunoglobulin G was shown after intravenous administration relative to the uptake of native protein.[131] Polyclonal antibodies directed against a 16-amino acid synthetic peptide corresponding to amino acids 35-50 of the 116-amino acid rev protein of human immunodeficiency virus type 1 were used as a model of the effect of the amination on protein cell uptake.[132] The study demonstrated that cationization resulted in enhanced endocytosis of

the antibody and enhanced inhibition of HIV-1 replication, consistent with intracellular immunization of the rev protein. The amination of a monoclonal antibody prepared against a synthetic peptide encoding the Asp_{13} point mutation of the ras proto-oncogenic p21 protein permitted to improve the uptake in vitro.[133] The *in vivo* pharmacokinetics and efficacy of cationized human immunoglobulins in the human-peripheral blood lymphocytes-severe combined immune deficiency mouse model were evaluated using the severe combined immunodeficient mouse transplanted with human lymphocytes and infected with human immunodeficiency virus (HIV)-1.[134] The aminated immunoglobulins have a markedly reduced mean residence time and a marked increase in organ uptake compared to the native immunoglobulins.[134] The amination of humanized 4D5 monoclonal antibody directed against the p185(HER2) oncogenic protein permitted to improve its cell uptake.[135] Native antibody was confined to the periplasma membrane space with minimal endocytosis into the cell. In contrast, robust internalization of the cationized 4D5 antibody by the SK-BR3 cells was demonstrated.[135] Aminated goat colchicine-specific polyclonal immunoglobulin G and antigen binding fragment decreased more rapidly in plasma than the non-modified counterparts.[136]

2.8.2.1. Improved function in vivo of aminated enzymes

In other instances, the objective of the amination was to achieve that exogenous enzyme may perform their function inside the cells to solve some problems, that is, to use the enzymes as medicaments. Table 4 shows a resume of the most significant examples. In general, aminated enzymes can penetrate better inside the cells, and exert inside the function, making that this strategy may be very useful to use enzymes to treat illness related to cell metabolic failure. Aminated Catalase is used in some these studies to prevent hydrogen peroxide-induced damage[137-138], aminated glucose oxidase is used for the treatment of metabolic deficiencies[137], but most examples are related to ribonucleases. Ribonucleases are

Table 4. Chemical amination of free enzymes to improve *in vivo* biological properties of enzymes

Enzyme	Source	EC Number	Type of modification and/or Immobilization	Effect of amination on enzyme properties	Reference
Catalase	NA	1.11.1.6	Enzyme is aminated with ethylendiamine	Significant protection against Fe(II)/H_2O_2 and ascorbic acid/copper ion-mediated damage is obtained	137
Glucose oxidase	NA	1.1.3.4	Enzyme is aminated with ethylendiamine	Treatment of pathological processes in the intestine is suggested	137
Catalase	NA	1.11.1.6	Enzyme is aminated with ethylenediamine or hexylenediamine	Aminated enzymes show increased binding capacities to HepG2 cells, and rapidly are taken up by the liver. Hydrogen peroxide induced cytotoxicity in HepG2 cells is significantly prevented by preincubation of the cells with aminated enzyme	138
RNAase	NA	3.1.27.-	Enzyme is aminated by ethylendiamine by the carbodiimide route	Improvement in ability to digest intra-cellular RNA, endocytosis and decreased affinity to the endogenous RNase inhibitors is achieved	140
RNAase	*Streptomyces aureofaciens*	3.1.1.27.-	Enzyme is aminated by ethylendiamine by the carbodiimide route	Toxic effects of the enzyme are enhanced	141
RNAase A	Bovine	3.1.27.5	Enzyme is modified by ethylendiamine by the carbodiimide route	Modified RNases are cytotoxic toward 3T3-SV-40 cells despite their decreasing on ribonucleolytic activity	142,143
RNAase 1	Human	3.1.27.3	Enzyme is modified by ethylendiamine by the carbodiimide route	Modified RNases are cytotoxic toward 3T3-SV-40 cells despite their decreasing on ribonucleolytic activity	142
RNAase	NA	3.1.27.-	Enzyme is modified with	Enzyme is efficiently uptaken and	147

			polyethyleneimine (PEI)	functioned into the cytosol	
Glutathione S-transferase	*Schistosoma japonicum*	2.5.1.18	Enzyme is fusioned with green fluorescent protein and cationized by forming a complex with a polycationic polyethylenimine-glutathione conjugate	Increase on both penetrability and enzyme delivery into CHO cells	148
RNAase	NA	3.1.27.5	Enzyme is biotinylated and mixed with PEI-streptavidin	Inhibition of cell growth of 3T3-SV-40 cell lines	150

potential anti-tumor drugs due to their potential cytotoxicity. A general model for the mechanism of the cytotoxic action of RNases includes the interaction of the enzyme with the cellular membrane, internalization, translocation to the cytosol, and degradation of ribonucleic acid.[139] The cytotoxic properties of naturally occurring or engineered RNases correlate well with their efficiency of cellular internalization and digestion level of cellular RNA. Aminated RNases are considered to be adsorbed on the anionic cellular surface by Coulombic interactions, and then become efficiently internalized into cells by an endocytosis-like pathway.[140-143] Although chemically modified cationized RNases showed decreased ribonucleolytic activity, improved endocytosis and decreased affinity to the endogenous RNase inhibitor improve their ability to digest intra-cellular RNA.

Another application in vivo of aminated proteins is their use as carrier proteins for different drugs or peptides towards target tissues. For example, rat albumin was cationized with hexamethylenediamine, and the isoelectric point of the protein was raised from 5.5 to approximately 8.[144] The aminated rat serum albumin was taken up by isolated rat or bovine brain microvessels, whereas native protein was not taken up by the capillaries in vitro. Therefore, cationized rat albumin may be used in future studies that use the repetitive administration of aminated rat albumin chimeric peptides for the evaluation of the transport of these substances through the blood-brain barrier *in vivo*.[144]

Bovine serum albumin was aminated with hexamethylenediamine or ethylenediamine to obtain cationized proteins and study the relation between physical properties and hepatic delivery.[145] Aminated albumins were rapidly taken up by liver, but the protein modified using hexylenediamine showed a faster uptake than when using ethylenediamine, with a similar number of free NH_2 groups, suggesting that the diamine

reagent with a longer carboxyl side chain results in more efficient hepatic targeting. A low degree of amination is sufficient for efficient hepatic targeting of proteins.[145]

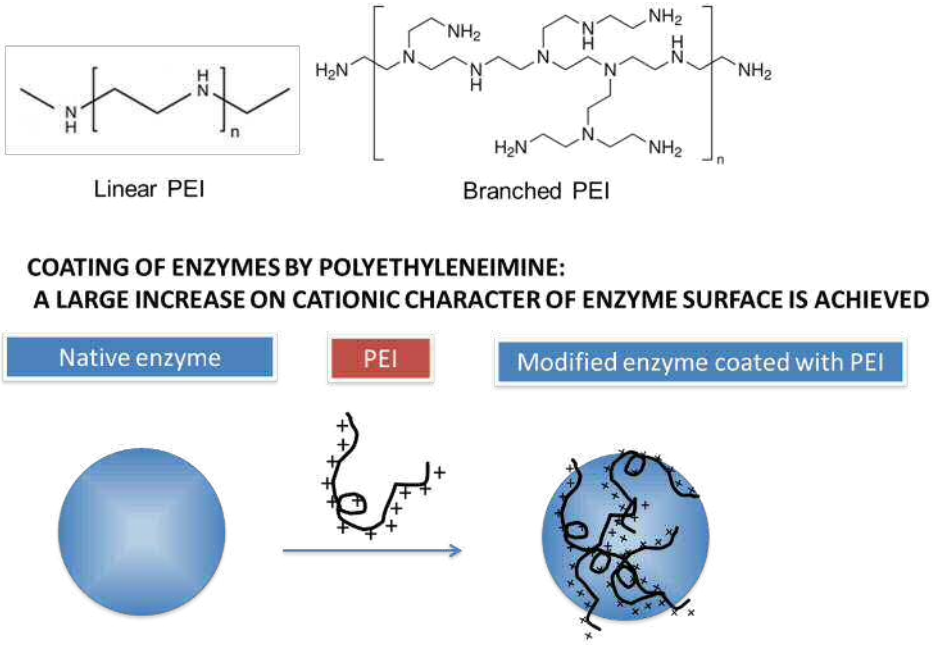
The use of aminated β-lactoglobulin (11 carboxylic groups were modified with ethylenediamine) as carrier protein improved the bioavailability for poorly absorbed bioactive compounds.[146]

In other cases, amination of enzymes and proteins has been used to facilitate the study of proteins in living cells. In the post-genomic era, there is interest for developing methodologies that permit protein manipulation to analyze functions of proteins in living cells. For this purpose, techniques to deliver functional proteins into living cells are of great relevance and protein amination seems to be an obvious option. Table 4 shows some of the most relevant examples. In some examples, the modification is performed using polymers like polyethylenimine.[147-149] An original approximation shows the indirect protein amination using non-covalent interaction using PEI-cationized avidin, streptavidin and protein G were used to deliver biotinylated proteins and antibodies into living cells.[150]

Finally, amination has been proposed to improve the activity recovery of proteins expressed as inclusion bodies opening a novel method to deliver a denatured protein into cells and simultaneously let it fold to express its function within cells. [151]

3. Physical amination of enzymes using aminated polymers

In the section 2, we have shown many examples where a protein was chemically attached to a poly-aminated polymer, usually chitosan or polyethyleneimine (PEI). This section will focus on the coating of the protein surface by polycationic polymers, but not in a covalent way, but simply by physical ionic exchange (Figure 13). The polymers may be quite large, even millions of Da, and that may facilitate the multipoint adsorption that is require to keep the polymer/enzyme interaction.[105,106]



549

550 **Figure 13. Physical coating of proteins with cationic polymer.**
551

552 PEI has been described to present some stabilizing effect on diverse proteins due to
553 diverse causes: prevention of enzyme aggregation, prevention of loss of secondary structure,
554 reduction of metal oxidation, prevention of multimeric enzyme dissociation, avoiding
555 inactivation by deleterious substrates, etc.[49,53,55,152] Some reports pointed that the stability-
556 effect of poly-ionic polymers did not really depend on their cationic or anionic nature of the
557 polymer, stating that perhaps a direct electrostatic enzyme/polymer interaction was not
558 required.[48] However, considering that most enzymes may be adsorbed under the same
559 conditions on PEI and dextran sulfate coated supports[153]; it is not clear that this electrostatic
560 interaction may be discarded.

561 Table 5 shows some of the most relevant examples. In some cases the effects of the
562 coatings were negative[154] , but usually some positive effects were described: stabilization
563 being the most usual,[155-156] but also improvement of detection limit ion biosensors may be
564 found[157].

Table 5. Effects of physical coating of enzymes with poly cationic polymers; effect on enzyme properties and immobilization performance

Enzyme	Source	EC Number	Type of modification (and immobilization)	Effect of amination on enzyme properties	Reference
Glyceraldehyde-3-phosphate dehydrogenase	NA	1.2.1.9	Enzyme is modified with quaternized polyamines (poly-N-alkyl-4-vinylpyridinium bromides)	Modification suppresses the thermoaggregation of glyceraldehyde-3-phosphate dehydrogenase but not thermodenaturation of the enzyme	154
Lactate deshydrogenase	NA	1.1.1.27	Enzyme is coated with polyethylenimine (PEI)	Protection of the enzyme against oxidative stress	155
Chloroperoxidase	*Caldariomyces fumago*	1.11.1.10	Enzyme is coated with polyethylenimine (PEI)	Improvement in the stability of the enzyme towards peroxide dependent inactivation	156
L-glutamate oxidase	NA	1.4.3.11	Enzyme is modified with polyethylenimine and o-phenylenediamine	Increase in detection limit of glucose	157
Glutamate dehydrogenase	*Thermus thermophilus*	1.4.1.2	Enzyme is coated with PEI and subsequently treated with glutaraldehyde	Subunit dissociation is prevented and adsorption on cationic exchangers facilitated	158
Formate dehydrogenase	*Pseudomonas sp.*	1.2.1.2	Enzyme is coated with PEI and subsequently treated with glutaraldehyde	Subunit dissociation is prevented and adsorption on cationic exchangers facilitated	158
Glucose oxidase	*A. niger*	1.1.3.4	Enzyme is microencapsulated in PEI before immobilization in paper substrates	Improvement in thermal stability at temperatures up to 60 ºC	159
Laccase	*Trametes versicolor*	1.10.3.2	Enzyme is microencapsulated in PEI	Improvement in activity retention at room temperature. Reduction in	159, 160

			before immobilization in paper substrates	thermal stability due to increased coordination between PEI and copper atoms present in the active site of the enzyme	
Lysozyme	NA	3.2.1.17	Enzyme is coated with PEI and then immobilized on layered titanates	High yields of immobilization and reuse of the composite	162
Lipase	*C. rugosa*	3.1.1.3	Enzyme is coated with PEI and then immobilized on layered titanates	High yields of immobilization and reuse of the composite	162

However, the most interesting examples are when the enzyme coating with the polymer is a simple step in the development of a more complex strategy to prepare an immobilized biocatalyst, as are some of the examples listed below.

3.1. Immobilization of enzymes coated with cationic polymers on cation exchangers

Modification of the enzyme using ionically exchanged poly-amines may permit to further immobilize the enzyme on a cation exchanger, when the free enzyme may have very low affinity by its anionic surface (in fact, the enzymes used in this strategy will be coated with a cationic polymer, that way the unmodified enzyme should have also a anionic surface).

Up to date, there is only one published paper on this strategy, using glutamate dehydrogenase from *Thermus thermophilus* and formate dehydrogenase from *Pseudomonas sp* (Table 5). Both enzymes were coated with a large PEI to prevent subunit dissociation, treated with glutaraldehyde to prevent enzyme/polymer dissociation at acidic pH value and adsorbed to carboxymethyl agarose.[54]

3.2. Protection of enzymes from undesired interactions with a immobilization matrix via coating with poly-amine polymers

In other cases, the enzyme coating with the polymers was just a first step in a longer immobilization strategy; the coating may increase the size of the enzyme, making their trapping easier, or preventing the interaction with deleterious interfaces (see Table 5). Trapping of enzymes in a paper matrix to be used in food packing is improved via physical coating with PEI. [159-160] Other material used to immobilize proteins after coating with PEI are surface anionic surface titanates.[161] The coating of enzymes with PEI permit the strong ion exchange in this material, together to the spontaneous flocculation of the material: the bio-molecules are incorporated within the interlayer space of layered structure. [162]

590

591 **3.3. Generation of artificial environments on immobilized enzymes**

592 Polyaminated polymers, like PEI, chitosan, polyalylmine, etc. are quite hydrophilic,
593 their cationic nature may permit to cover the immobilized enzyme molecules surface of a very
594 hydrophilic shell that can produce some partition of hydrophobic compounds, like gases,
595 organic solvents, etc, enabling the preparation of biocatalysts with improved stability in these
596 media. The strategy may be used for enzymes immobilized on preexisting supports, or
597 enzymes to be immobilized via the crosslinked enzyme aggregates (CLEA) technology. Table
598 6 resumes some of the examples. The very useful penicillin G acylase[163] is one of the
599 examples, whose uses are reduced due to the low stability in organic media[164-165] the strategy
600 permits to improve its stability versus organic solvents and use the enzyme in some interesting
601 reaction[18, 166-169] The biocatalyst prepared by co-aggregation of enzymes and PEI[170] was mosr
602 stable the much more thermostable glyoxyl-agarose biocatalyst.[171] This CoCLEAs permitted
603 also improve enzyme resistance to oxygen.[51]

604

605 **3.4. Improved preparation of CLEAs by co-aggregation with aminated polymers**

606 Polyaminated polymers have found several advantages in the preparation of
607 crosslinking enzyme aggregates (CLEAs). First, as commented in the point above, co-
608 aggregation with PEI (combined or not with sulfate dextran) is able to generate a hydrophilic
609 environment around the enzyme, producing partition of hydrophobic solvent or oxygen
610 molecules. In this point we will focus on the second advantage: it may be used to solve the
611 problems generated in the crosslinked step of proteins having just some few Lys superficial
612 residues, or it may just be used to have a more intensively crosslinked CLEA particle. Table 6
613 summarizes some relevant examples: the use of PEI permit the production of properly
614 crosslinked CLEAs of enzymes that did not give this result in unmodified form. [99, 101, 172-173]

Table 6. Physical coating of immobilized enzymes with ionic polymers to improve enzyme performance.

Enzyme	Source	EC Number	Type of modification	Effect of amination on enzyme properties	Reference
Penicillin G acylase	*Escherichia coli*	3.5.1.11	Enzyme is co-immobilized with PEI and submitted to successive modifications with aldehyde dextran and PEI, and finally with sulphate dextran.	High stability to organic co-solvents (up to 95%) such as tetraglyme in synthesis reactions.	18, 166
Penicillin G acylase	*Escherichia coli*	3.5.1.11	Enzyme is co-aggregated with PEI and dextran sulphate for synthesis of cross-linked enzyme co-aggregates (co-CLEAs)	Improvement in enzyme properties in presence of organic solvents	170, 171
Nitrilase	*Pseudomonas fluorescens*	3.5.5.1	Enzyme is modified and co-aggregated with PEI and cross-linked with glutaraldehyde for synthesis of co-CLEAs	Enzyme activity is retained upon exposition to oxygen for 40 h	51
Glutaryl acylase	*Pseudomonas sp*	3.5.1.93	Enzyme is co-aggregated with PEI and cross-linked with glutaraldehyde for synthesis of co-CLEAs	Enzyme maintains more than 60% of its initial activity after 72 h if incubation at 45 ºC.	101
Lipases	*Alcaligenes sp. Candida antarctica B*	3.1.1.3	Enzyme is co-aggregated with PEI or PEI-sulfate dextran mixtures and cross-linked with glutaraldehyde for synthesis of CLEAs (Co-CLEAs)	Alteration of enzyme activity, enantioselectivity and specificity	99

Lipase	*Serratia marcescens*	3.1.1.3	Enzyme is co-aggregated with PEI and cross-linked with glutaraldehyde for synthesis of CLEAs (Co-CLEAs)	Optimum temperature is increased from to 50 to 60 ºC. Thermal stability is also significantly improved.	102
Lipase	*Geotrichum sp.*	3.1.1.3	Enzyme is co-aggregated with PEI and cross-linked with glutaraldehyde for synthesis of CLEAs (Co-CLEAs)	Enzyme maintains more than 65% of relative hydrolysis degree after incubation in the range of 50-55 °C for 4 h and more than 85% of relative hydrolysis degree after being treated by acetone, tert-butyl alcohol and octane for 4 h	172
L-aminoacylase	*Aspergillus melleus*	3.5.1.14	Enzyme is co-aggregated with PEI and cross-linked with glutaraldehyde for synthesis of CLEAs (Co-CLEAs)	Enzyme shows 75% activity recovery and 81% aggregation yield. Improvement of enzyme stability and enantioselectivity of amino acid amides is obtained hydrolysis of N-acetyl amino acid amides.	173
Lipase	*Candida antarctica B* (immobilized Novozym 435)	3.1.1.3	Enzyme is coated with different ionic polymers	Improvement in enzyme activity is achieved (3 fold factor)	111
Lipase	*Candida rugosa*	3.1.1.3	Enzyme is immobilized and coated with PEI	Improvement in enantioselectivity is achieved (enantiomeric ratio is increased from 8 to 20 after PEI coating)	174
Phospholipase	*Lecitase Ultra, a artificial enzyme from Novo*	3.1.1.4	Immobilized enzyme is coated with poly ionic polymers	Increasing in enzyme activity (more than 3 fold) factor.	175
Phospholipase	*Lecitase Ultra, a artificial enzyme*	3.1.1.4	Immobilized enzyme is coated with poly ionic	Increasing in enzyme activity (more than 3 fold) factor in absence of	177

	from Novo		polymers in the presence of SDS	detergent	

615

616 **3.5. Tuning catalytic properties of immobilized enzymes by coating their**
617 **surfaces with poly-amine polymers**

618 Physical coating of enzymes with PEI has been used in some instances to improve
619 enzyme properties, mainly using lipases. The physical coating is far simpler than the chemical
620 modification, and in some instances may become as effective. Table 6 shows some of the most
621 relevant examples: enzyme activity or enantiospecificity versus certain substrates could be
622 improved[111,17] While the coating of Lecitase in aqueous media did not increase the rate of
623 irreversible inhibition, suggesting that the open form has no been stabilized,[176] the physical
624 coating of Lecitase Ultra in the presence of SDS has permitted to "freeze" the open structure
625 induced by the presence of the detergent.[177]

626

627 **4. Genetic amination of enzymes as a tool to improve their performance**

628

629 **4.1. Use of poly-Arg or poly-Lys tags to improve enzyme performance**

630 Protein fusion tags have been developed as indispensable tools for protein
631 expression, purification, and the design of functionalized surfaces or artificially bifunctional
632 proteins.[178] A recent review[179] has summarized how positively or negatively charged polyionic
633 fusion peptides with or without an additional cysteine can be used as protein tags for protein
634 expression and purification, for matrix-assisted refolding of aggregated protein, and for
635 coupling of proteins either to technologically relevant matrices or to other proteins.

636 Orientation of immobilized enzymes may play a critical role on the features of the
637 enzyme.[180] On one hand, this protein area will be the most involved one in the enzyme/support
638 interaction, being the most altered (improved/worsened) by the immobilization.[181] On the

other hand, this may define the access of large substrates or ligands to this active center,[182,183] or the communication between the active center of the enzyme and an electrode.[182,184,185]

Site directed mutagenesis is the most efficient tool to achieve this site directed immobilization, via introduction of specific groups on desired areas of the protein.[21] Usually, this orientation is achieved using a Cys inserted in the desired region, and immobilized on a support bearing disulfide groups.[181] Other popular strategy is the use of poly-His tags,[185,186] or generation of His pairs,[187] and immobilization the variant enzymes on immobilized metal chelates matrices.

In this review, we will try to focus on how this Poly-cationic tags may be used for protein immobilization.

5.1.1. Purification/immobilization of enzymes and proteins using cationic tags and cation exchangers

Most enzymes have an ionic surface nature that makes them unable to become adsorbed on cationic exchangers, and the adsorption of proteins on that matrices may be used as a way to purify proteins that can be adsorbed on this kind of ionic exchangers. This may be achieved by the introduction of cationic tags/domains on the target protein.[179,188]

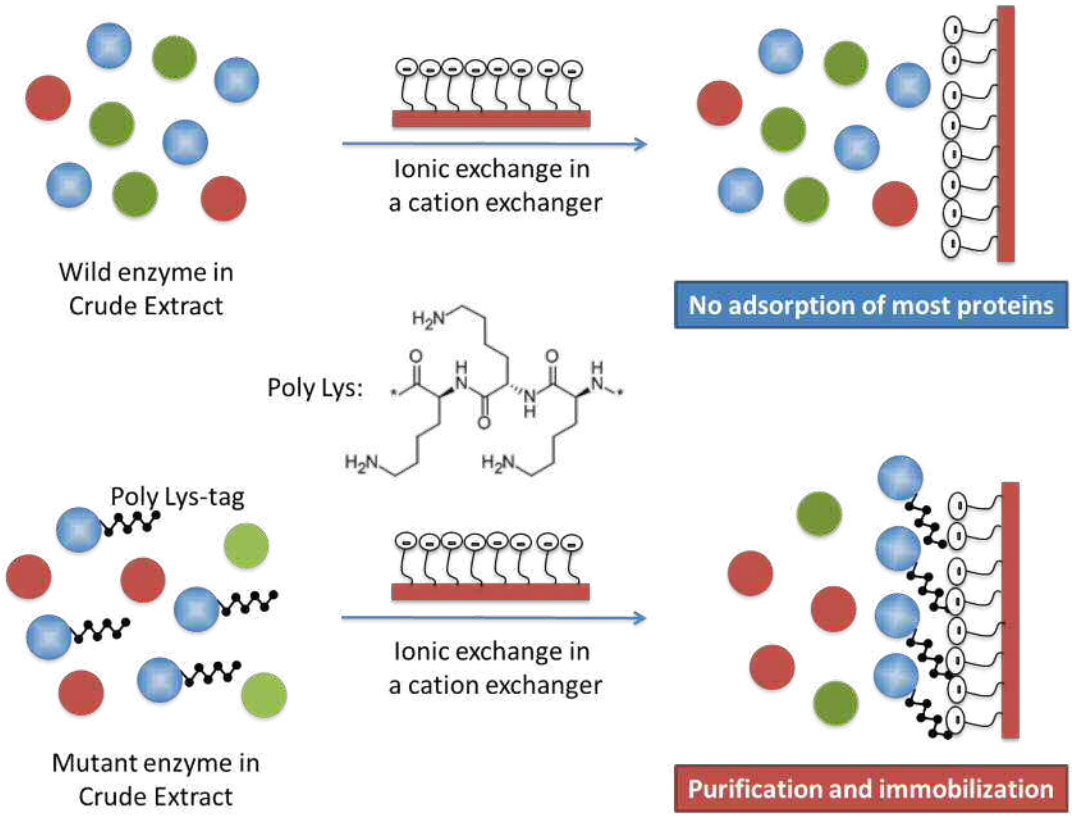


Figure 14. One step purification/immobilization of poly-Lys tagged proteins.

Thus, some examples on the usage of poly-Lys or poly-Arg may be found in the literature to obtain the one step purification and immobilization of enzymes on cationic exchangers (Figure 14). It has been shown that a poly-lysine tag facilitates protein purification and refolding processes. Table 7 resume some of the most relevant examples: immobilization of the poly-Lys or Arg tagged enzymes on cation exchangers is a specific way and used in diverse reactions[28,27-191-193]

4.1.2. Improving covalent immobilization via addition of poly cationic tags to the proteins

The addition of poly-Lys tags may be also advantageous to reach a further covalent immobilization of the peptide after ionic exchange. The idea would be similar to the use of heterofunctional supports: first the enzyme is adsorbed, second the covalent reaction takes place due to the very high apparent concentrations of reactive groups on both support and

Table 7. Effect of genetic amination to improve biocatalytical and biological properties of enzymes

Enzyme	Source	EC Number	Type of modification and/or Immobilization	Effect of amination on enzyme properties	Reference
Cyclodextrin glycosyltransferase	*Bacillus macerans*	2.4.1.19	Enzyme is tagged with poly-Lys (10 Lys residues) and immobilized on sulphopropyl–Sepharose	Enzyme activity is fully retained after reversible immobilization	28
Aminopeptidase II	*Bacillus stearothermophilus*	3.4.11.-	Enzyme is tagged C-terminally with either tri- or nona-lysines and immobilized in carboxylated iron oxide particles	Improvement in enzyme stability	190, 191
Protease	*Saccharomyces cerevisiae*	3.4.-.-	Enzyme is fused with a poly lysine tag containing 10 Lys residues at its C-terminus, purified, and immobilized on carboxyethyl chitosan magnetic nanoparticles	Enzyme is simply purified from cell extracts with very high purity in just one-step.	27
D-xylose isomerase	*Escherichia coli*	5.3.1.5	Enzyme is fused with a 10-Arg tag in its C-terminus, purified and immobilized by a single step of cation exchange chromatography	Enzyme is simply purified from cell extracts with very high purity in just one-step.	192
Penicillin G acylase	*Escherichia coli*	3.5.1.11	Enzyme is tagged with a poly-Lys and immobilized by directed	Enzyme keeps catalytic properties of the soluble enzyme on kinetically synthesis of cefamadole and cefonicid	11, 40, 196

			covalent immobilization		
Lipase	*Candida antarctica B*	3.4.19.12	Enzyme was fused with various polycationic amino acid tags	Solubility of the enzyme is increased by five- to nine fold during over expresion	199
Penicillin G acylase	*Escherichia coli*	3.5.1.11	Enzyme is site-directed mutagenized with Lys several residues and covalently immobilized on glyoxyl-agarose	Improvement on enzyme and organic co-solvent stability ranging from 4 to 11	39
Peroxidase	*Horseradish*	1.11.1.7	Enzyme is mutated replacing Arg by Lys and immobilized by oriented immobilization a polyethersulfone matrix modified with aldehyde residues	Excellent retention of catalytic activity is achieved, also stabilization is improved	201
Penicillin G acylase	*Escherichia coli*	3.5.1.11	One residue of Cys was introduced on different regions of the enzyme by site directed mutagenesis. A second round of site directed mutagenesis, for enrichment in 4 additional lysine residues in the zone of Cys380, and then immobilized on an epoxy support	Enzyme stabilization was increased 1500-fold in the second round of site directed mutagenesis regarding to variant one point immobilized through Cys380.	202
Penicillin G acylase	*Escherichia coli*	3.5.1.11	Enzyme is enriched with Lys residues in the opposite area of the active center and immobilized	Improvement in the behavior of the enzyme in kinetically controlled synthesis of semi-synthetic β-lactam antibiotics	203

			onto glyoxyl agarose		
Penicillin G acylase	*Escherichia coli*	3.5.1.11	Enzyme is modified by site directed mutagenesis increasing carboxylic groups on the enzyme surface.	Improvement in its immobilization on anion exchangers	44

NA: Not available

672 adsorbed protein.[31] Combination of cation exchangers and chemically reactive groups have
673 improved the immobilization of several enzymes, as shown in [194-195]
674
675 **4.1.3. Modulation of enzyme properties via site-directed covalent**
676 **immobilization using poly-Lys tags**
677 It has been shown on some papers and recent reviews how the control of the area of
678 the protein involved in the reaction with the support may produce different changes on the
679 enzyme structure (or prevent some changes that should occur), being this tool a very powerful
680 strategy for improving enzyme performance in different reactions whose yield depend on the
681 catalytic performance of the catalyst.[11,15,20]
682 The control of the immobilization of penicillin G acylase using a poly Lys tag is the
683 only example that we have been able to find regarding the use of poly Lys tag to reach this
684 goal (Table 7).[11,196] The poly-Lys tagged and site directed immobilized enzyme on glyoxyl
685 permitted to improve enzyme performance in kinetically controlled synthesis of several
686 antibiotics compared to the results obtained using the free enzyme[196-197]
687
688 **4.1.4. Other uses of chimeric poly-Lys tagged enzymes**
689 Poly-Lysine tags may have some other applications. For example, this strategy was
690 used for the efficient production of the intact glucagon-like peptide-1 using a recombinant E.
691 coli system, avoiding degradation.[198]
692 In other cases, poly-cationic tags have been used to improve the expression of a
693 hyper-expressed enzyme (Table 7).[199]
694
695 **5.2. Genetic amination of protein surface areas to improve enzyme multipoint covalent**
696 **attachment**

In other cases, the increase on Lys residues is not performed using a tag, but by selecting different regions to increase the density of Lys groups in the specific region on which we intend to use to immobilize the enzyme, or disperse along the protein surface, if we just intend to increase the cationic groups on the surface. Such modifications are expected to be more successful when based on a good quality 3D structure of the protein.

In immobilization, to take full advantage of this Lys enrichment, the immobilization should be based on multipoint processes, that way the factor directing the immobilization will be the density of reactive groups in one protein area and not the reactivity of a special residue or its global amount. Among the support for covalent immobilization, glyoxyl supports fulfill this requirement.[36,37] For reversible immobilization, most of the supports follow this multipoint interaction to fix the enzyme to the support.[15,106]

4.2.1. Improved covalent attachment via enrichment in Lys residues in specific areas of the enzyme surface

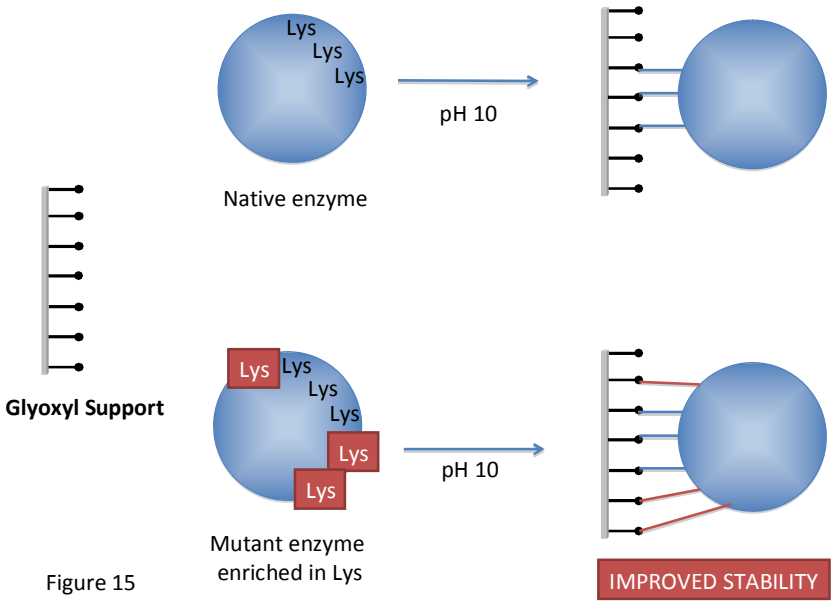


Figure 15

Figure 15. Increase number of enzyme-support covalent attachments on aminated proteins.

714

715 Main examples are resumed in Table 7: important additional stabilization regarding
716 the use of the native enzyme was found using glyoxyl agarose [39] or a modified
717 polyethersulfone matrix presenting aldehyde residues.[201]

718 A more directed strategy was later proposed. First, one Cys residue was introduced
719 on different regions of the enzyme penicillin G acylase, to find the area that was more
720 determinant for enzyme stability.[202] The immobilization was performed on an epoxy support,
721 because Cys was by far the most reactive amino group on a protein and that was enough to
722 direct the enzyme. The mutant enzyme where the Cys was in the position 380 of the β subunit
723 of the enzyme was the one that gave the highest PGA stabilization values. In a second round
724 of site-directed mutagenesis, that region was further enriched in 4 additional lysine residues,
725 and the resulting immobilized derivative was 1500-fold more stable than the same protein
726 variant uni-punctually immobilized through position β380.[202] It is expected that in the near
727 future, this strategy may be extended to more enzymes.

728 In other cases, the objective was more to have a fully oriented immobilized enzyme
729 than to improve the multipoint covalent attachment or the enzyme stability (Table 7). This was
730 the case of the immobilization of mutant penicillin G acylase enzymes enriched in Lys areas
731 in the area opposite to the active center, which permitted to improve the behavior of the
732 enzyme in kinetically controlled synthesis of semi-synthetic β-lactam antibiotics.[203-204]

733

734 **4.2.2. Improvement of immobilization in anionic exchangers via Lys enrichemnt**

735 We have not been able to find a example using genetic amination to improve
736 immobilization on anionic supports. In fact, and this may serve as a proof of concept, there is
737 one example where a genetics-based increase on carboxylic groups of the surface of penicillin
738 G acylase improved its immobilization on anion exchangers.[44]

739

740 **4.2.3. Improvement of intermolecular crosslinking via enrichment in Lys residues**

741 Again, we have not been able to find any papers concerning the use of enzymes with
742 enriched areas in Lys residues and the stabilization of this enzyme by using intermolecular
743 crosslinkers. However, in a similar way as that described when using chemical amination (see
744 section 2.5 of this review), this should permit to greatly improve the enzyme crosslinking by
745 increasing the prospects of having two residues of the protein at the right distance.[45] In fact,
746 this can be even more favorable than chemical amination, where it is only possible to get a
747 general enrichment on the enzyme surface of amino groups, using the carboxylic groups of the
748 enzyme. Now, using site-directed mutagenesis and if the enzyme has a well described
749 structure, it is possible to place the new Lys residues on the right position to permit the
750 enzyme crosslinking, a critical point to get an intramolecular crosslinking.[116]

751

752 **4.2.4. Improvement of coating with anionic polymers via enrichment on Lys**
753 **residues**

754 Again, we have not found examples where the enrichment in Lys residues of the
755 protein is used to facilitate the adsorption of cationic polymers on their surface. Using the
756 enzyme penicillin G acylase, there is, however, an example of enrichment on carboxy groups
757 of the enzyme surface to improve the adsorption of cationic polymers on the enzyme
758 surface,[205] and in section 2.6 the chemical amination to this goal is presented.[43] Perhaps,
759 although this coating may have very good effects on enzyme performance (see section 3 of
760 this review), it has been considered too sophisticated for the researchers to improve the
761 interaction via site-directed mutagenesis.

762

763 **4.2.5. Other uses of Lys enrichment of protein surface**

764 Ribonuclease Sa (pI = 3.5) from *Streptomyces aureofaciens* and its 3K (D1K, D17K,
765 E41K) (pI = 6.4) and 5K (3K + D25K, E74K) (pI = 10.2) variants were tested for
766 cytotoxicity.[206] The 5K mutant was cytotoxic to normal and v-ras-transformed NIH3T3 mouse
767 fibroblasts, while RNase Sa and 3K were not. The cytotoxic 5K mutant preferentially attacks
768 v-ras-NIH3T3 fibroblasts, suggesting that mammalian cells expressing the ras-oncogene are
769 potential targets for ribonuclease-based drugs.
770
771 **6. Conclusion and future trends**
772 This review has shown the high interest that the amination of enzymes and proteins has
773 with views towards improving their behavior *in vitro* as industrial biocatalysts, but also i*n vivo*
774 when using proteins as carriers or even as medicaments.
775 Amination has proved to be very useful to improve enzyme immobilization via
776 multipoint covalent attachment or cation exchange, to improve intramolecular crosslinking, to
777 improve enzyme stability, or to improve intermolecular crosslinking, which is a critical step in
778 the preparation of CLEAs. The amination also increases the immunogenicity and potential to
779 penetrate cell walls, enabling the use of some enzymes as biocides, improving the production
780 of antibodies, or just permitting to study the role of certain proteins *in vivo* after introduction
781 in the cell.
782 In some cases, amination may produce drastic changes in enzyme stability, activity or
783 selectivity/specificity. Considering the change of ionic interactions on the enzyme surface, a
784 negative effect should be expected. However in many instances the effect is positive.
785 Most examples cited in this review use chemical or physical amination. This may be
786 derived from the rapid preparation of the modified enzymes using these techniques, and the
787 relatively simple preparation of a collection of enzymes having different modification degrees,
788 mainly if a solid phase modification may be performed. Perhaps this may be the best solution

to alter enzyme properties such as selectivity of specificity, because the current knowledge on enzyme dynamics cannot give the exact groups to be modified to mimic the effects using site-directed modification. Moreover, this may be a first and rapid step to evaluate if the amination really permits to improve enzyme immobilization. However, these strategies in general will produce a general modification of the enzyme surface, and that may not be the best solution in some instances.

Site-directed mutagenesis is a slower technique, which requires expertise in fields different from those required for the researches involved in enzyme chemical modification or enzyme immobilization. However, together with the advantages derived from the fact that the modified enzymes will be always produced in this way (once the mutation has been introduced); this strategy may give some further possibilities. For example, only site directed genetic amination may permit to get a site-directed immobilization of enzymes on supports such as glyoxyl or cation exchangers, or to select the modified groups in a way that the introduction of an intramolecular crosslinker may be facilitated.

This relative complexity of the preparation of a collection of mutant enzymes may be an explanation of the relatively low amount of examples where genetic amination has been used, even though these examples have shown the very high improvement that this amination may have in the behavior of the final biocatalyst. In fact, it has never been used to improve the chemical reactivity versus crosslinking reagents, although chemical amination has proved that this may be a critical point to use this strategy.

Thus, we are before a clear example of the convenience of a close collaboration between experts in scientific areas apparently quite far in the design of biocatalysts. If this collaboration is achieved, it seems obvious that the genetic amination should be a future way of improving enzymes and proteins to be used as biocatalysts, but also as medicaments or protein carriers.

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References

1. M. T. Reetz, *J. Am. Chem. Soc.*, 2013, **135**, 12480-12496; C. M. Clouthier and J. N. Pelletier, *Chem. Soc. Rev.*, 2012, **41**, 1585-1605; G. W. Zheng and J. H. Xu, *Curr. Opin. Biotechnol.*, 2011, **22**, 784-792; R. N. Patel, *ACS Catalysis*, 2011, **1**, 1056-1074.
2. H. E. Schoemaker, D. Mink and M. G. WubboLts, *Science*, 2003, **299**, 1694-1697.
3. C. L. Windle, M. Müller, A. Nelson and A. Berry, *Curr. Opin. Chem. Biol.*, 2014, **19**, 25-33; A. Kumar and S. Singh, *Crit. Rev. Biotechnol.*, 2013, **33**, 365-378; T. Davids, M. Schmidt, D. Böttcher and U. T. Bornscheuer, *Curr. Opin. Chem. Biol.*, 2013, **17**, 215-220; M. B. Quin and C. Schmidt-Dannert, *ACS Catalysis*, 2011, **1**, 1017-1021.
4. G. DeSantis and J. B. Jones, *Curr. Opin. Biotechnol.*, 1999, **10**, 324-330; J. M. Chalker, G. J. L. Bernardes, Y. A. Lin and B. G. Davis, *Chem.-Asian J.*, 2009, **4**, 630-640; G. E. Means and R. E. Feeney, *Bioconjug. Chem.*, 1990, **1**, 2-12; E. Baslé, N. Joubert and M. Pucheault, *Chem. Biol.*, 2010, **17**, 213-227.
5. A. Díaz-Rodríguez and B. G. Davis, *Curr. Opin. Chem. Biol.*, 2011, **15**, 211-219; B. G. Davis, *Curr. Opin. Biotechnol.*, 2003, **14**, 379-386.
6. C. Ó'Fágáin, *Enzyme Microb. Technol.*, 2003, **33**, 137-149.
7. A. Marie O'Brien, A. T. Smith and C. O'Fágáin, *Biotechnol. Bioeng.*, 2003, **81**, 233-240.
8. S. S. Wong and L. J. C. Wong, *Enzyme Microb. Technol.*, 1992, **14**, 866-874.
9. A. S. Bommarius and M. F. Paye, *Chem. Soc. Rev.*, 2013, **42**, 6534-6565; C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2007, **40**, 1451-1463; P. V. Iyer and L. Ananthanarayan, *Process Biochem.*, 2008, **43**, 1019-1032.
10. D. Brady and J. Jordaan, *Biotechnol. Lett.*, 2009, **31**, 1639-1650; A. M. Klibanov, *Anal. Biochem.*, 1979, **93**, 1-25; L. Gianfreda and M. R. Scarfi, *Mol. Cell. Biochem.*, 1991, **100**, 97-128.
11. R. C. Rodrigues, C. Ortiz, A. Berenguer-Murcia, R. Torres and R. Fernandez-Lafuente, *Chem. Soc. Rev.*, 2013, **45**, 6290-6307.
12. R. A. Sheldon and S. Van Pelt, *Chem. Soc. Rev.*, 2013, **42**, 6223-6235.
13. W. Hartmeier, *Trends Biotechnol.*, 1985, **3**, 149-153; E. Katchalski-Katzir, *Trends Biotechnol.*, 1993, **11**, 471-478.
14. A. A. Homaei, R. Sariri, F. Vianello and R. Stevanato, *J. Chem. Biol.*, 2013, **6**, 185-205; B. M. Brena and F. Batista-Viera, in *Immobilization of Enzymes and Cells*, 2006, vol. 22, pp. 15-30.
15. C. Garcia-Galan, A. Berenguer-Murcia, R. Fernandez-Lafuente and R. C. Rodrigues, *Adv. Synth. Catal.*, 2011, **353**, 2885-2904.
16. R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2009, **45**, 405-418.
17. C. M. Moore, N. L. Akers, A. D. Hill, Z. C. Johnson and S. D. Minteer, *Biomacromolecules*, 2004, **5**, 1241-1247.
18. R. Fernandez-Lafuente, C. M. Rosell, L. Caanan-Haden, L. Rodes and J. M. Guisan, *Enzyme Microb. Technol.*, 1999, **24**, 96-103.
19. R. C. Rodrigues, Á. Berenguer-Murcia and R. Fernandez-Lafuente, *Adv. Synth. Catal.*, 2011, **353**, 2216-2238.
20. D. A. Cowan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2011, **49**, 326-346.
21. K. Hernandez and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2011, **48**, 107-122.
22. K. L. Carraway and D. E. Koshland Jr, *Methods Enzymol.*, 1972, **25**, 616-623.
23. K. L. Carraway, P. Spoerl and D. E. Koshland Jr, *J. Mol. Biol.*, 1969, **42**, 133-137.

24. O. Barbosa, M. Ruiz, C. Ortiz, M. Fernández, R. Torres and R. Fernandez-Lafuente, *Process Biochem.*, 2012, **47**, 867-876.
25. M. Galvis, O. Barbosa, R. Torres, C. Ortiz and R. Fernandez-Lafuente, *Process Biochem.*, 2012, **47**, 460-466.
26. J. Li, Y. Dong, Y. Zhang and Y. Yang, *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.*, 2013, **917-918**, 30-35.
27. J. Li, Y. Zhang, F. Shen and Y. Yang, *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.*, 2012, **907**, 159-162.
28. D. H. Kweon, S. G. Kim, N. S. Han, J. H. Lee, K. M. Chung and J. H. Seo, *Enzyme Microb. Technol.*, 2005, **36**, 571-578.
29. C. Mateo, V. Grazú, B. C. C. Pessela, T. Montes, J. M. Palomo, R. Torres, F. López-Gallego, R. Fernández-Lafuente and J. M. Guisán, *Biochem. Soc. Trans.*, 2007, **35**, 1593-1601; E. Katchalski-Katzir and D. M. Kraemer, *J. Mol. Catal. B: Enzym.*, 2000, **10**, 157-176; L. Hilterhaus, B. Minow, J. Müller, M. Berheide, H. Quitmann, M. Katzer, O. Thum, G. Antranikian, A. P. Zeng and A. Liese, *Bioprocess Biosyst. Eng.*, 2008, **31**, 163-171.
30. M. Ortega-Muñoz, J. Morales-Sanfrutos, A. Megia-Fernandez, F. J. Lopez-Jaramillo, F. Hernandez-Mateo and F. Santoyo-Gonzalez, *J. Mater. Chem.*, 2010, **20**, 7189-7196; J. Morales-Sanfrutos, J. Lopez-Jaramillo, M. Ortega-Muñoz, A. Megia-Fernandez, F. Perez-Balderas, F. Hernandez-Mateo and F. Santoyo-Gonzalez, *Org. Biomol. Chem.*, 2010, **8**, 667-675.
31. O. Barbosa, R. Torres, C. Ortiz, Á. Berenguer-Murcia, R. C. Rodrigues and R. Fernandez-Lafuente, *Biomacromolecules*, 2013, **40**, 2433-2462.
32. O. Barbosa, C. Ortiz, A. Berenguer-Murcia, R. Torres, R. C. Rodrigues and R. Fernandez-Lafuente, *RSC Advances*, 2014, **4**, 1583-1600.
33. J. Schnapp and Y. Shalitin, *Biochem. Biophys. Res. Commun.*, 1976, **70**, 8-14.
34. K. Nilsson and K. Mosbach, *Eur. J. Biochem.*, 1980, **112**, 397-402.
35. K. Nilsson and K. Mosbach, *Methods Enzymol.*, 1987, **135**, 65-78.
36. C. Mateo, J. M. Palomo, M. Fuentes, L. Betancor, V. Grazu, F. López-Gallego, B. C. C. Pessela, A. Hidalgo, G. Fernández-Lorente, R. Fernández-Lafuente and J. M. Guisán, *Enzyme Microb. Technol.*, 2006, **39**, 274-280.
37. C. Mateo, O. Abian, M. Bernedo, E. Cuenca, M. Fuentes, G. Fernandez-Lorente, J. M. Palomo, V. Grazu, B. C. C. Pessela, C. Giacomini, G. Irazoqui, A. Villarino, K. Ovsejevi, F. Batista-Viera, R. Fernandez-Lafuente and J. M. Guisán, *Enzyme Microb. Technol.*, 2005, **37**, 456-462.
38. V. Grazu, L. Betancor, T. Montes, F. Lopez-Gallego, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2006, **38**, 960-966.
39. O. Abian, V. Grazú, J. Hermoso, R. González, J. L. García, R. Fernández-Lafuente and J. M. Guisán, *Appl. Environ. Microbiol.*, 2004, **70**, 1249-1251.
40. F. Scaramozzino, I. Estruch, P. Rossolillo, M. Terreni and A. M. Albertini, *Appl. Environ. Microbiol.*, 2005, **71**, 8937-8940.
41. R. Fernandez-Lafuente, C. M. Rosell, V. Rodriguez, C. Santana, G. Soler, A. Bastida and J. M. Guisan, *Enzyme Microb. Technol.*, 1993, **15**, 546-550.
42. R. C. Rodrigues, C. A. Godoy, G. Volpato, M. A. Z. Ayub, R. Fernandez-Lafuente and J. M. Guisan, *Process Biochem.*, 2009, **44**, 963-968.
43. T. Montes, V. Grazu, F. López-Gallego, J. A. Hermoso, J. M. Guisán and R. Fernández-Lafuente, *Biomacromolecules*, 2006, **7**, 3052-3058.
44. T. Montes, V. Grazú, F. López-Gallego, J. A. Hermoso, J. L. García, I. Manso, B. Galán, R. González, R. Fernández-Lafuente and J. M. Guisán, *Appl. Environ. Microbiol.*, 2007, **73**, 312-319.

45. R. Fernandez-Lafuente, C. M. Rosell, V. Rodriguez and J. M. Guisan, *Enzyme Microb. Technol.*, 1995, **17**, 517-523.
46. M. Galvis, O. Barbosa, M. Ruiz, J. Cruz, C. Ortiz, R. Torres and R. Fernandez-Lafuente, *Process Biochem.*, 2012, **47**, 2373-2378.
47. M. Ruiz, M. Galvis, O. Barbosa, C. Ortiz, R. Torres and R. Fernandez-Lafuente, *J. Mol. Catal. B: Enzym.*, 2013, **87**, 75-82.
48. T. M. Foreman, M. Khalil, P. Meier, J. R. Brainard, L. A. Vanderberg and N. N. Sauer, *Biotechnol. Bioeng.*, 2001, **76**, 241-246.
49. J. Bryjak, *Bioprocess Eng.*, 1995, **13**, 177-181; M. M. Andersson and R. Hatti-Kaul, *J. Biotechnol.*, 1999, **72**, 21-31.
50. A. M. Klibanov, N. O. Kaplan and M. D. Kamen, *Proc. Natl. Acad. Sci. U. S. A.*, 1978, **75**, 3640-3643.
51. C. Mateo, B. Fernandes, F. Van Rantwijk, A. Stolz and R. A. Sheldon, *J. Mol. Catal. B: Enzym.*, 2006, **38**, 154-157.
52. L. Gianfreda, D. Pirozzi and G. Greco Jr, *Biotechnol. Bioeng.*, 1989, **33**, 1067-1071.
53. D. Costes, G. Rotčenkovs, E. Wehtje and P. Adlercreutz, *Biocatal. Biotransform.*, 2001, **19**, 119-130.
54. J. M. Bolivar, J. Rocha-Martin, C. Mateo, F. Cava, J. Berenguer, R. Fernandez-Lafuente and J. M. Guisan, *Biomacromolecules*, 2009, **10**, 742-747.
55. C. Garcia-Galan, O. Barbosa and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2013, **52**, 211-217.
56. J. P. Riehm and H. A. Scheraga, *Biochemistry*, 1966, **5**, 99-115.
57. D. G. Hoare and D. E. Koshland Jr, *J. Biol. Chem.*, 1967, **242**, 2447-2453.
58. A. Williams and I. T. Ibrahim, *Chem. Rev.*, 1981, **81**, 589-636.
59. L. F. Matyash, O. G. Ogloblina and V. M. Stepanov, *Eur. J. Biochem.*, 1973, **35**, 540-545.
60. R. B. Perfetti, C. D. Anderson and P. L. Hall, *Biochemistry*, 1976, **15**, 1735-1743.
61. N. Nakajima and Y. Ikada, *Bioconjug. Chem.*, 1995, **6**, 123-130.
62. K. L. Carraway and R. B. Triplett, *Biochim. Biophys. Acta, Protein Struct.*, 1970, **200**, 564-566.
63. K. L. Carraway and D. E. Koshland Jr, *Biochim. Biophys. Acta, Protein Struct.*, 1968, **160**, 272-274.
64. R. Timkovich, *Biochem. J.*, 1980, **185**, 47-57.
65. D. V. Godin and S. L. Schrier, *Biochemistry*, 1970, **9**, 4068-4077.
66. M. H. Rashid and K. S. Siddiqui, *Biotechnol. Appl. Biochem.*, 1998, **27**, 231-237.
67. V. P. Torchilin, A. V. Maksimenko, V. N. Smirnov, I. V. Berezin, A. M. Klibanov and K. Martinek, *Biochim. Biophys. Acta*, 1978, **522**, 277-283.
68. O. Munch and D. Tritsch, *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.*, 1990, **1041**, 111-116.
69. R. Kuroki, H. Yamada and T. Imoto, *J. Biochem. (Tokyo)*, 1986, **99**, 1493-1499; H. Yamada, T. Imoto, K. Fujita, K. Okazaki and M. Motomura, *Biochemistry*, 1981, **20**, 4836-4842.
70. M. H. Rashid and K. S. Siddiqui, *Process Biochem.*, 1998, **33**, 109-115.
71. K. S. Siddiqui, A. M. Shemsi, M. A. Anwar, M. H. Rashid and M. I. Rajoka, *Enzyme Microb. Technol.*, 1999, **24**, 599-608.
72. H. N. Bhatti, M. H. Rashid, M. Asgher, R. Nawaz, A. M. Khalid and R. Perveen, *Can. J. Microbiol.*, 2007, **53**, 177-185.
73. A. J. Afzal, S. A. Bokhari and K. S. Siddiqui, *Appl. Biochem. Biotechnol.*, 2007, **141**, 273-297; A. J. Afzal, S. A. Bokhari and K. S. Siddiqui, *Appl. Biochem. Biotechnol.*, 2008, **150**, 113.

74. K. Matsumoto, B. G. Davis and J. B. Jones, *Chem.-Eur. J.*, 2002, **8**, 4129-4137.
75. L. Gómez, H. L. Ramírez and R. Villalonga, *Biotechnol. Lett.*, 2000, **22**, 347-350.
76. L. Gómez and R. Villalonga, *Biotechnol. Lett.*, 2000, **22**, 1191-1195.
77. M. L. Villalonga, R. Villalonga, L. Mariniello, L. Gómez, P. Di Pierro and R. Porta, *World J. Microbiol. Biotechnol.*, 2006, **22**, 595-602.
78. F. López-Gallego, T. Montes, M. Fuentes, N. Alonso, V. Grazu, L. Betancor, J. M. Guisán and R. Fernández-Lafuente, *J. Biotechnol.*, 2005, **116**, 1-10.
79. C. E. McVey, M. A. Walsh, G. G. Dodson, K. S. Wilson and J. A. Brannigan, *J. Mol. Biol.*, 2001, **313**, 139-150.
80. J. K. Kim, I. S. Yang, S. Rhee, Z. Dauter, Y. S. Lee, S. S. Park and K. H. Kim, *Biochemistry*, 2003, **42**, 4084-4093.
81. P. W. Tardioli, M. F. Vieira, A. M. S. Vieira, G. M. Zanin, L. Betancor, C. Mateo, G. Fernández-Lorente and J. M. Guisán, *Process Biochem.*, 2011, **46**, 409-412.
82. V. Addorisio, F. Sannino, C. Mateo and J. M. Guisan, *Process Biochem.*, 2013, **48**, 1174-1180.
83. K. Piontek, M. Antorini and T. Choinowski, *J. Biol. Chem.*, 2002, **277**, 37663-37669.
84. D. Bezbradica, B. Jugovic, M. Gvozdenovic, S. Jakovetic and Z. Knezevic-Jugovic, *J. Mol. Catal. B: Enzym.*, 2011, **70**, 55-60.
85. S. Husain, F. Jafri and M. Saleemuddin, *Enzyme Microb. Technol.*, 1996, **18**, 275-280.
86. A. Bastida, P. Sabuquillo, P. Armisen, R. Fernández-Lafuente, J. Huguet and J. M. Guisán, *Biotechnol. Bioeng.*, 1998, **58**, 486-493.
87. G. Fernández-Lorente, J. M. Palomo, M. Fuentes, C. Mateo, J. M. Guisán and R. Fernández-Lafuente, *Biotechnol. Bioeng.*, 2003, **82**, 232-237; J. M. Palomo, M. Fuentes, G. Fernández-Lorente, C. Mateo, J. M. Guisan and R. Fernández-Lafuente, *Biomacromolecules*, 2003, **4**, 1-6.
88. G. Fernandez-Lorente, C. A. Godoy, A. A. Mendes, F. Lopez-Gallego, V. Grazu, B. de las Rivas, J. M. Palomo, J. Hermoso, R. Fernandez-Lafuente and J. M. Guisan, *Biomacromolecules*, 2008, **9**, 2553-2561.
89. C. Carrasco-López, C. Godoy, B. de las Rivas, G. Fernández-Lorente, J. M. Palomo, J. M. Guisán, R. Fernández-Lafuente, M. Martínez-Ripoll and J. A. Hermoso, *J. Biol. Chem.*, 2009, **284**, 4365-4372.
90. A. M. Brzozowski, H. Savage, C. S. Verma, J. P. Turkenburg, D. M. Lawson, A. Svendsen and S. Patkar, *Biochemistry*, 2000, **39**, 15071-15082.
91. R. C. Rodrigues and M. A. Z. Ayub, *Process Biochem.*, 2011, **46**, 682-688; R. C. Rodrigues, B. C. C. Pessela, G. Volpato, R. Fernandez-Lafuente, J. M. Guisan and M. A. Z. Ayub, *Process Biochem.*, 2010, **45**, 1268-1273.
92. M. Marciello, C. Mateo and J. M. Guisan, *Colloids Surf., B*, 2011, **84**, 556-560.
93. S. Moreno-Perez, M. Filice, J. M. Guisan and G. Fernandez-Lorente, *Chem. Phys. Lipids*, 2013, **174**, 48-54.
94. R. C. Rodrigues, J. M. Bolivar, A. Palau-Ors, G. Volpato, M. A. Z. Ayub, R. Fernandez-Lafuente and J. M. Guisan, *Enzyme Microb. Technol.*, 2009, **44**, 386-393.
95. Z. Habibi, M. Mohammadi and M. Yousefi, *Process Biochem.*, 2013, **48**, 669-676.
96. J. M. Bolivar, F. López-Gallego, C. Godoy, D. S. Rodrigues, R. C. Rodrigues, P. Batalla, J. Rocha-Martín, C. Mateo, R. L. C. Giordano and J. M. Guisán, *Enzyme Microb. Technol.*, 2009, **45**, 477-483.
97. R. A. Sheldon, *Appl. Microbiol. Biotechnol.*, 2011, **92**, 467-477; L. Cao, F. Van Rantwijk and R. A. Sheldon, *Org. Lett.*, 2000, **2**, 1361-1364.
98. S. Talekar, A. Joshi, G. Joshi, P. Kamat, R. Haripurkar and S. Kambale, *RSC Advances*, 2013, **3**, 12485-12511.

99. L. Wilson, G. Fernández-Lorente, R. Fernández-Lafuente, A. Illanes, J. M. Guisán and J. M. Palomo, *Enzyme Microb. Technol.*, 2006, **39**, 750-755.
100. S. Shah, A. Sharma and M. N. Gupta, *Anal. Biochem.*, 2006, **351**, 207-213; J. J. Karimpil, J. S. Melo and S. F. D'Souza, *J. Mol. Catal. B: Enzym.*, 2011, **71**, 113-118; T. Dong, L. Zhao, Y. Huang and X. Tan, *Bioresour. Technol.*, 2010, **101**, 6569-6571.
101. F. López-Gallego, L. Betancor, A. Hidalgo, N. Alonso, R. Fernández-Lafuente and J. M. Guisán, *Biomacromolecules*, 2005, **6**, 1839-1842.
102. J. Pan, X. D. Kong, C. X. Li, Q. Ye, J. H. Xu and T. Imanaka, *J. Mol. Catal. B: Enzym.*, 2011, **68**, 256-261.
103. J. Uppenberg, S. Patkar, T. Bergfors and T. A. Jones, *J. Mol. Biol.*, 1994, **235**, 790-792.
104. J. Cruz, O. Barbosa, R. C. Rodrigues, R. Fernandez-Lafuente, R. Torres and C. Ortiz, *J. Mol. Catal. B: Enzym.*, 2012, **80**, 7-14.
105. A. Kumar, I. Y. Galaev and B. Mattiasson, *J. Chromatogr. B Biomed. Sci. Appl.*, 2000, **741**, 103-113; B. C. C. Pessela, R. Munilla, L. Betancor, M. Fuentes, A. V. Carrascosa, A. Vian, R. Fernandez-Lafuente and J. M. Guisán, *J. Chromatogr. A*, 2004, **1034**, 155-159.
106. M. Fuentes, C. Mateo, B. C. C. Pessela, P. Batalla, R. Fernandez-Lafuente and J. M. Guisán, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2007, **849**, 243-250.
107. L. Gómez, H. L. Ramírez, M. L. Villalonga, J. Hernández and R. Villalonga, *Enzyme Microb. Technol.*, 2006, **38**, 22-27.
108. L. Gómez, H. L. Ramírez, A. Neira-Carrillo and R. Villalonga, *Bioprocess Biosyst. Eng.*, 2006, **28**, 387-395.
109. L. Gomez, H. L. Ramirez, G. Cabrera, B. K. Simpson and R. Villalonga, *J. Food Biochem.*, 2008, **32**, 264-277.
110. J. M. Palomo, G. Fernández-Lorente, J. M. Guisán and R. Fernández-Lafuente, *Adv. Synth. Catal.*, 2007, **349**, 1119-1127.
111. Z. Cabrera, G. Fernandez-Lorente, R. Fernandez-Lafuente, J. M. Palomo and J. M. Guisan, *Process Biochem.*, 2009, **44**, 226-231.
112. G. Fernández-Lorente, L. Betancor, A. V. Carrascosa, J. M. Palomo and J. M. Guisan, *J. Am. Oil Chem. Soc.*, 2012, **89**, 97-102.
113. C. Garcia-Galan, J. C. S. d. Santos, O. Barbosa, R. Torres, E. B. Pereira, V. C. Corberan, L. R. B. Gonçalves and R. Fernandez-Lafuente, *Process Biochem.*, 2014, **49**, 604-616.
114. C. A. Godoy, B. de las Rivas, M. Filice, G. Fernández-Lorente, J. M. Guisan and J. M. Palomo, *Process Biochem.*, 2010, **45**, 534-541.
115. K. Martinek and V. P. Torchilin, in *Methods Enzymol.*, 1988, vol. 137, pp. 615-626; R. Tyagi and M. N. Gupta, *Biochemistry (Mosc.)*, 1998, **63**, 334-344.
116. V. P. Torchilin, A. V. Maksimenko, V. N. Smirnov, I. V. Berezin and K. Martinek, *Biochim. Biophys. Acta, Enzymol.*, 1979, **568**, 1-10.
117. I. Migneault, C. Dartiguenave, M. J. Bertrand and K. C. Waldron, *Biotechniques*, 2004, **37**, 790-802.
118. R. Fernandez Lafuente, PhD Doctoral Thesis, UAM, 1992.
119. D. P. Kelly, M. K. Dewar, R. B. Johns, S. Wei-Let and J. F. Yates, in *Protein Crosslinking*, ed. M. Friedman, Springer US, 1977, vol. 86A, ch. 38, pp. 641-647.
120. L. Betancor, M. Fuentes, G. Dellamora-Ortiz, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, C. Mateo, J. M. Guisán and R. Fernández-Lafuente, *J. Mol. Catal. B: Enzym.*, 2005, **32**, 97-101; L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, M. Fuentes, R. Fernández-Lafuente and J. M. Guisán, *J. Biotechnol.*, 2004, **110**, 201-207.

121. M. Fuentes, R. L. Segura, O. Abian, L. Betancor, A. Hidalgo, C. Mateo, R. Fernandez-Lafuente and J. M. Guisan, *Proteomics*, 2004, **4**, 2602-2607.
122. P. Bailon and W. Berthold, *Pharm. Sci. Technol. To.*, 1998, **1**, 352-356; M. J. Hernáiz, J. M. Sánchez-Montero and J. V. Sinisterra, *Biotechnol. Bioeng.*, 1997, **55**, 252-260; M. J. Roberts, M. D. Bentley and J. M. Harris, *Adv. Drug Delivery Rev.*, 2002, **54**, 459-476; Y. Kodera, A. Matsushima, M. Hiroto, H. Nishimura, A. Ishii, T. Ueno and Y. Inada, *Progress in Polymer Science (Oxford)*, 1998, **23**, 1233-1271.
123. R. J. Apple, P. L. Domen, A. Muckerheide and J. G. Michael, *J. Immunol.*, 1988, **140**, 3290-3295.
124. A. Muckerheide, R. J. Apple, A. J. Pesce and J. G. Michael, *J. Immunol.*, 1987, **138**, 833-837.
125. R. Jara-Acevedo, M. Gonzalez-Gonzalez, M. Jara-Acevedo, J. Claros, A. Conde, R. López-Perez, A. Orfao and M. Fuentes, *J. Immunol. Methods*, 2012, **384**, 171-176.
126. J. Futami, M. Kitazoe, H. Murata and H. Yamada, *Expert Opin. Drug Discov.*, 2007, **2**, 261-269.
127. J. Futami, *Seikagaku*, 2013, **85**, 21-25.
128. Y. Zhou, J. Wu, W. Yu, Y. Xu, P. Wang, B. Xie and F. Chen, *J. Immunol. Methods*, 2007, **328**, 79-88.
129. Y. Feng, Y. Zhou, Q. Zou, J. Wang, F. Chen and Z. Gao, *J. Immunol. Methods*, 2009, **340**, 138-143.
130. Q. Feng, Y. Xu, Y. Zhou, L. Lu, F. Chen and X. Wang, *J. Mol. Struct.*, 2010, **977**, 100-105.
131. D. Triguero, J. L. Buciak and W. M. Pardridge, *J. Pharmacol. Exp. Ther.*, 1991, **258**, 186-192.
132. W. M. Pardridge, U. Bickel, J. Buciak, J. Yang and A. Diagne, *J. Infect. Dis.*, 1994, **169**, 55-61.
133. W. M. Pardridge, Y. S. Kang, J. Yang and J. L. Buciak, *J. Pharm. Sci.*, 1995, **84**, 943-948.
134. W. M. Pardridge, Y. S. Kang, A. Diagne and J. A. Zack, *J. Pharmacol. Exp. Ther.*, 1996, **276**, 246-252.
135. W. M. Pardridge, J. Buciak, J. Yang and D. Wu, *J. Pharmacol. Exp. Ther.*, 1998, **286**, 548-554.
136. G. Hong, M. I. Bazin-Redureau and J. M. G. Scherrmann, *J. Pharm. Sci.*, 1999, **88**, 147-153.
137. R. Kohen, A. Kakunda and A. Rubinstein, *J. Biol. Chem.*, 1992, **267**, 21349-21354.
138. S. F. Ma, M. Nishikawa, H. Katsumi, F. Yamashita and M. Hashida, *J. Control. Release*, 2006, **110**, 273-282.
139. U. Arnold and R. Ulbrich-Hofmann, *Biotechnol. Lett.*, 2006, **28**, 1615-1622.
140. J. Futami and H. Yamada, *Curr. Pharm. Biotechnol.*, 2008, **9**, 180-184.
141. O. N. Ilinskaya, A. Koschinski, V. A. Mitkevich, H. Repp, F. Dreyer, C. N. Pace and A. A. Makarov, *Biochem. Biophys. Res. Commun.*, 2004, **314**, 550-554.
142. J. Futami, T. Maeda, M. Kitazoe, E. Nukui, H. Tada, M. Seno, M. Kosaka and H. Yamada, *Biochemistry*, 2001, **40**, 7518-7524.
143. J. Futami, E. Nukui, T. Maeda, M. Kosaka, H. Tada, M. Seno and H. Yamada, *J. Biochem. (Tokyo)*, 2002, **132**, 223-228.
144. W. M. Pardridge, D. Triguero, J. Buciak and J. Yang, *J. Pharmacol. Exp. Ther.*, 1990, **255**, 893-899.
145. S. F. Ma, M. Nishikawa, H. Katsumi, F. Yamashita and M. Hashida, *J. Control. Release*, 2005, **102**, 583-594.

146. Z. Teng, Y. Li, Y. Luo, B. Zhang and Q. Wang, *Biomacromolecules*, 2013, **14**, 2848-2856.
147. J. Futami, M. Kitazoe, T. Maeda, E. Nukui, M. Sakaguchi, J. Kosaka, M. Miyazaki, M. Kosaka, H. Tada, M. Seno, J. Sasaki, N. H. Huh, M. Namba and H. Yamada, *J. Biosci. Bioeng.*, 2005, **99**, 95-103.
148. H. Murata, J. Futami, M. Kitazoe, T. Yonehara, H. Nakanishi, M. Kosaka, H. Tada, M. Sakaguchi, Y. Yagi, M. Seno, N. H. Huh and H. Yamada, *J. Biochem. (Tokyo)*, 2008, **144**, 447-455.
149. H. Murata, J. Futami, M. Kitazoe, M. Kosaka, H. Tada, M. Seno and H. Yamada, *J. Biosci. Bioeng.*, 2008, **105**, 34-38.
150. M. Kitazoe, H. Murata, J. Futami, T. Maeda, M. Sakaguchi, M. Miyazaki, M. Kosaka, H. Tada, M. Seno, N. H. Huh, M. Namba, M. Nishikawa, Y. Maeda and H. Yamada, *J. Biochem. (Tokyo)*, 2005, **137**, 693-701.
151. H. Murata, M. Sakaguchi, J. Futami, M. Kitazoe, T. Maeda, H. Doura, M. Kosaka, H. Tada, M. Seno, N. H. Huh and H. Yamada, *Biochemistry*, 2006, **45**, 6124-6132.
152. J. D. Breccia, M. M. Andersson and R. Hatti-Kaul, *Biochim. Biophys. Acta, Gen. Subj.*, 2002, **1570**, 165-173.
153. M. Fuentes, B. C. C. Pessela, J. V. Maquiese, C. Ortiz, R. L. Segura, J. M. Palomo, O. Abian, R. Torres, C. Mateo, R. Fernández-Lafuente and J. M. Guisán, *Biotechnol. Prog.*, 2004, **20**, 1134-1139.
154. I. N. Shalova, I. N. Naletova, L. Saso, V. I. Muronetz and V. A. Izumrudov, *Macromol. Biosci.*, 2007, **7**, 929-939.
155. L. Mazzaferro, J. D. Breccia, M. M. Andersson, B. Hitzmann and R. Hatti-Kaul, *Int. J. Biol. Macromol.*, 2010, **47**, 15-20.
156. M. Andersson, M. M. Andersson and P. Adlercreutz, *Biocatal. Biotransform.*, 2000, **18**, 457-469.
157. C. P. McMahon, G. Rocchitta, S. M. Kirwan, S. J. Killoran, P. A. Serra, J. P. Lowry and R. D. O'Neill, *Biosens. Bioelectron.*, 2007, **22**, 1466-1473.
158. J. M. Bolivar, F. Cava, C. Mateo, J. Rocha-Martín, J. M. Guisán, J. Berenguer and R. Fernandez-Lafuente, *Appl. Microbiol. Biotechnol.*, 2008, **80**, 49-58; J. M. Bolivar, L. Wilson, S. A. Ferrarotti, R. Fernandez-Lafuente, J. M. Guisan and C. Mateo, *Biomacromolecules*, 2006, **7**, 669-673.
159. Y. Zhang and D. Rochefort, *Process Biochem.*, 2011, **46**, 993-1000.
160. M. P. Guerrero, F. Bertrand and D. Rochefort, *Chem. Eng. Sci.*, 2011, **66**, 5313-5320.
161. Q. Wang, Q. Gao and J. Shi, *J. Am. Chem. Soc.*, 2004, **126**, 14346-14347; L. Zhang, Q. Zhang and J. Li, *Adv. Funct. Mater.*, 2007, **17**, 1958-1965.
162. Z. P. Han, J. Fu, P. Ye and X. P. Dong, *Enzyme Microb. Technol.*, 2013, **53**, 79-84.
163. A. K. Chandel, L. V. Rao, M. L. Narasu and O. V. Singh, *Enzyme Microb. Technol.*, 2008, **42**, 199-207.
164. R. Fernandez-Lafuente, C. M. Rosell and J. M. Guisan, *Enzyme Microb. Technol.*, 1991, **13**, 898-905.
165. G. Volpato, R. C. Rodrigues and R. Fernandez-Lafuente, *Curr. Med. Chem.*, 2010, **17**, 3855-3873.
166. O. Abian, L. Wilson, C. Mateo, G. Fernández-Lorente, J. M. Palomo, R. Fernández-Lafuente, J. M. Guisán, D. Re, A. Tam and M. Daminatti, *J. Mol. Catal. B: Enzym.*, 2002, **19-20**, 295-303; O. Abian, C. Mateo, G. Fernández-Lorente, J. M. Palomo, R. Fernández-Lafuente and J. M. Guisán, *Biocatal. Biotransform.*, 2001, **19**, 489-503.
167. O. Abian, C. Mateo, G. Fernández-Lorente, J. M. Guisán and R. Fernández-Lafuente, *Biotechnol. Prog.*, 2003, **19**, 1639-1642.

168. O. Abian, C. Mateo, J. M. Palomo, G. Fernández-Lorente, J. M. Guisán and R. Fernández-Lafuente, *Biotechnol. Prog.*, 2004, **20**, 984-988.
169. O. Abian, C. Mateo, G. Fernández-Lorente, J. M. Guisán and R. Fernández-Lafuente, *Biotechnol. Prog.*, 2004, **20**, 117-121.
170. L. Wilson, A. Illanes, O. Abián, B. C. C. Pessela, R. Fernández-Lafuente and J. M. Guisán, *Biomacromolecules*, 2004, **5**, 852-857.
171. L. Wilson, A. Illanes, O. Romero, J. Vergara and C. Mateo, *Enzyme Microb. Technol.*, 2008, **43**, 442-447.
172. J. Yan, X. Gui, G. Wang and Y. Yan, *Appl. Biochem. Biotechnol.*, 2012, **166**, 925-932.
173. B. K. Vaidya, S. S. Kuwar, S. B. Golegaonkar and S. N. Nene, *J. Mol. Catal. B: Enzym.*, 2012, **74**, 184-191.
174. Z. Cabrera, M. L. E. Gutarra, J. M. Guisan and J. M. Palomo, *Catal. Commun.*, 2010, **11**, 964-967.
175. J. C. S. d. Santos, C. Garcia-Galan, R. C. Rodrigues, H. B. d. Sant'Ana, L. R. B. Gonçalves and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2014, **60**, 1-8.
176. G. Fernández-Lorente, J. M. Palomo, C. Mateo, R. Munilla, C. Ortiz, Z. Cabrera, J. M. Guisán and R. Fernández-Lafuente, *Biomacromolecules*, 2006, **7**, 2610-2615.
177. J. C. S. d. Santos, C. Garcia-Galan, R. C. Rodrigues, H. B. d. Sant'Ana, L. R. B. Gonçalves and R. Fernandez-Lafuente, *Process Biochem.*, 2014, **accepeted**.
178. D. S. Waugh, *Trends Biotechnol.*, 2005, **23**, 316-320.
179. H. Lilie, S. Richter, S. Bergelt, S. Frost and F. Gehle, *Biol. Chem.*, 2013, **394**, 995-1004.
180. E. Steen Redeker, D. T. Ta, D. Cortens, B. Billen, W. Guedens and P. Adriaensens, *Bioconjug. Chem.*, 2013, **24**, 1761-1777.
181. A. Schellenberger and R. Ulbrich, *Biomed. Biochim. Acta*, 1989, **48**, 63-67; J. Mansfeld, G. Vriendl, O. R. Veltman, B. Van Den Burg, G. Venema, V. G. H. Eijsink and R. Utbrich-Hofmann, *FASEB J.*, 1997, **11**; R. Ulbrich-Hofmann, U. Arnold and J. Mansfeld, *J. Mol. Catal. B: Enzym.*, 1999, **7**, 125-131; J. Mansfeld, G. Vriend, B. Van Den Burg, V. G. H. Eijsink and R. Ulbrich-Hofmann, *Biochemistry*, 1999, **38**, 8240-8245.
182. C. Nowak, D. Schach, J. Gebert, M. Grosserueschkamp, R. B. Gennis, S. Ferguson-Miller, W. Knoll, D. Walz and R. L. C. Naumann, *J. Solid State Electrochem.*, 2011, **15**, 105-114.
183. M. L. Jeong, K. P. Hyun, Y. Jung, K. K. Jin, O. J. Sun and H. C. Bong, *Anal. Chem.*, 2007, **79**, 2680-2687; B. C. C. Pessela, R. Torres, M. Fuentes, C. Mateo, R. Fernandez-Lafuente and J. M. Guisán, *Biomacromolecules*, 2004, **5**, 2029-2033; J. Turková, *J. Chromatogr. B Biomed. Sci. Appl.*, 1999, **722**, 11-31; J. A. Camarero, *Biopolymers (Pept. Sci.)*, 2008, **90**, 450-458.
184. J. R. Siqueira Jr, L. Caseli, F. N. Crespilho, V. Zucolotto and O. N. Oliveira Jr, *Biosens. Bioelectron.*, 2010, **25**, 1254-1263; L. S. Wong, F. Khan and J. Micklefield, *Chem. Rev.*, 2009, **109**, 4025-4053; J. M. Pingarrón, P. Yáñez-Sedeño and A. González-Cortés, *Electrochim. Acta*, 2008, **53**, 5848-5866; Z. Naal, J. H. Park, S. Bernhard, J. P. Shapleigh, C. A. Batt and H. D. Abruña, *Anal. Chem.*, 2002, **74**, 140-148.
185. V. Balland, C. Hureau, A. M. Cusano, Y. Liu, T. Tron and B. Limoges, *Chem.-Eur. J.*, 2008, **14**, 7186-7192.
186. S. Andreescu, V. Magearu, A. Lougarre, D. Fournier and J. L. Marty, *Anal. Lett.*, 2001, **34**, 529-540.
187. J. Madoz-Gúrpide, J. M. Abad, J. Fernández-Recio, M. Vélez, L. Vázquez, C. Gómez-Moreno and V. M. Fernández, *J. Am. Chem. Soc.*, 2000, **122**, 9808-9817.

188. J. M. Bolivar and B. Nidetzky, *Langmuir*, 2012, **28**, 10040-10049; J. M. Bolivar and B. Nidetzky, *Biotechnol. Bioeng.*, 2012, **109**, 1490-1498; J. Wiesbauer, J. M. Bolivar, M. Mueller, M. Schiller and B. Nidetzky, *ChemCatChem*, 2011, **3**, 1299-1303; S. M. Fuchs and R. T. Raines, *Protein Sci.*, 2005, **14**, 1538-1544; T. Gräslund, G. Lundin, M. Uhlén, P.-Å. Nygren and S. Hober, *Protein Eng.*, 2000, **13**, 703-709; T. Ikeda and A. Kuroda, *Colloids Surf., B*, 2011, **86**, 359-363; M. Hedhammar, T. Gräslund and S. Hober, *Chem. Eng. Technol.*, 2005, **28**, 1315-1325.
189. C. S. Rha, D. H. Lee, S. G. Kim, W. K. Min, S. G. Byun, D. H. Kweon, S. H. Nam and J. H. Seo, *J. Mol. Catal. B: Enzym.*, 2005, **34**, 39-43.
190. C.-L. Huang, W.-C. Cheng, J.-C. Yang, M.-C. Chi, J.-H. Chen, H.-P. Lin and L.-L. Lin, *J. Ind. Microbiol. Biotechnol.*, 2010, **37**, 717-725.
191. C. L. Wu, Y. P. Chen, J. C. Yang, H. F. Lo and L. L. Lin, *J. Mol. Catal. B: Enzym.*, 2008, **54**, 83-89.
192. N. T. Hang, S. G. Kim and D. H. Kweon, *Korean J. Microbiol. Biotechnol.*, 2012, **40**, 163-167.
193. S. G. Kim, J. A. Kim, H. A. Yu, D. H. Lee, D. H. Kweon and J. H. Seo, *Enzyme Microb. Technol.*, 2006, **39**, 459-465.
194. C. Ladavière, T. Delair, A. Domard, A. Novelli-Rousseau, B. Mandrand and F. Mallet, *Bioconjug. Chem.*, 1998, **9**, 655-661.
195. C. Ladavière, C. Lorenzo, A. Elaïssari, B. Mandrand and T. Delair, *Bioconjug. Chem.*, 2000, **11**, 146-152.
196. V. Kasche, *Enzyme Microb. Technol.*, 1986, **8**, 4-16.
197. I. Serra, D. A. Ceechini, D. UbiaIi, E. M. Manazza, A. M. Albertini and M. Terreni, *Eur. J. Org. Chem.*, 2009, 1384-1389.
198. S. G. Kim, S. Y. Shin, Y. C. Park, C. S. Shin and J. H. Seo, *Protein Expr. Purif.*, 2011, **78**, 197-203.
199. H. J. Jung, S. K. Kim, W. K. Min, S. S. Lee, K. Park, Y. C. Park and J. H. Seo, *Bioprocess Biosyst. Eng.*, 2011, **34**, 833-839.
200. G. Alvaro, R. Fernandez-Lafuente, R. M. Blanco and J. M. Guisán, *Appl. Biochem. Biotechnol.*, 1990, **26**, 181-195.
201. B. J. Ryan and C. Ó'Fágáin, *BMC Biotechnol.*, 2007, **7**.
202. V. Grazu, F. López-Gallego and J. M. Guisán, *Process Biochem.*, 2012, **47**, 2538-2541.
203. D. A. Cecchini, I. Serra, D. Ubiali, M. Terreni and A. M. Albertini, *BMC Biotechnol.*, 2007, **7**.
204. I. Serra, D. Ubiali, D. A. Cecchini, E. Calleri, A. M. Albertini, M. Terreni and C. Temporini, *Anal. Bioanal. Chem.*, 2013, **405**, 745-753.
205. T. Montes, V. Grazú, I. Manso, B. Galán, F. López-Gallego, R. González, J. A. Hermoso, J. L. García, J. M. Guisán and R. Fernández-Lafuente, *Adv. Synth. Catal.*, 2007, **349**, 459-464.
206. O. N. Ilinskaya, F. Dreyer, V. A. Mitkevich, K. L. Shaw, C. Nick Pace and A. A. Makarov, *Protein Sci.*, 2002, **11**, 2522-2525.