Amination of enzymes to improve biocatalyst performance.

Coupling genetic modification and physicochemical tools

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Abstract

Improvement of enzyme features 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) 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 exchanges 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 to improve the use of enzymes and proteins in many different areas and may be expected a great expansion of its usage in the next future.

Key words: enzyme chemical amination, enzyme genetic amination, polymer coating of enzymes, 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 theses molecules as catalysts of industrially relevant reactions since the middle of the last century.\(^1\sim^4\) 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.\(^5\) Many of these limitations are based on their biological origin. In nature enzymes are submitted to strict regulations in complex metabolic routes to give a rapid answer 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.

Genetic tools have permitted to obtain more stable and efficient biocatalysts by diverse tools, such as site-directed mutagenesis or directed evolution.\(^6\sim^9\) This strategy may be more or less complex and time-consuming to produce the desired enzyme, but once the mutant enzyme is ready, the large scale production will not be more expensive than using a native enzyme (it may become cheaper if enzyme overproduction is achieved. (Figure 1)

Another useful tool to improve enzyme properties is the chemical modification of enzymes.\(^10\sim^{15}\) (Figure 2) Chemical modification may pursue producing a one-point modification (and although the effect of the modification on the enzyme features may be hard to predict, in some cases enzyme performance improves)\(^16\), \(^17\) or the introduction of intramolecular crosslinkings to increase the enzyme rigidity and thus, enzyme stability may be enhanced.\(^18\) 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, as an additional advantage to genetic modifications, the only limit to the nature of the introduced
groups will be the imagination of the researcher, and it is not limited to enzymes with available genes.14,15

Another tool to improve enzyme performance is the immobilization.19-25 This technique needs to be used to solve the first of the protein problems as industrial biocatalyst: the water-soluble nature of enzymes.26-28 (Figure 3). This 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,29,30 more or less adequate for each specific case depending on the enzyme and the process (e.g., substrate size).31 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.19-25 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 enzymes25 (Figure 3). Rigidification of the enzyme tridimensional structure may be achieved via multipoint covalent attachment19-21, while the multisubunit immobilization of multimeric enzymes prevents their inactivation via dissociation.32 In some cases, the generation of favorable environments may permit the stabilization of the enzyme under certain conditions.33,34 (Figure 3).

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 (and even must) be used simultaneously to have a biocatalyst with enhanced properties.35-37 This is even more stressed considering, as stated above, that the enzymes must be finally used in an immobilized form.26 For example, the more stable the free enzyme is, the higher the range of conditions that may be used to submit the enzyme to immobilization or chemical modification processes.36
In fact, the relevant point is the final stability of the immobilized enzyme, and not the stability of the free enzyme (Figure 4).

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.

2. Importance of the amination of enzyme surface

The amination of the surface of a protein may fulfill many different objectives (Figure 5). For example, it may alter the existing interactions between the groups in the enzyme support to tune the enzyme properties. This is easily obtained using chemical modification because chemical amination is based on the amidation of carboxylic acids (see section below). 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.

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 tendency to become adsorbed on these supports (e.g., using poly-Lys tags).

Another likely objective to be achieved via amination of the enzyme surface is to increase the enzyme chemical reactivity versus a support used for covalent immobilization. Most of the supports used to immobilize proteins are designed to involve the primary amino groups of the protein (terminal amino group and ε amino group of Lys). That is because Lys is an ionic nucleophilic group, relatively frequent in the enzyme sequence, usually placed on the protein surface due to its hydrophilicity and its reactivity with a broad diversity of groups that
may be introduced in the support (epoxyde, vinyl sulfone, glutaraldehyde, cyanogen bromide, tosyl chloride, tresyl chloride, glyoxyl, etc.) without requiring any activation step. As a first obvious effect, an enrichment of the enzyme surface in primary amino groups will produce an increase on the immobilization rate of all these supports. Introduction of Lys residues may also permit the immobilization/purification of the enzyme, using supports such as glyoxyl ones that require immobilizing the enzyme via some enzyme/support attachments.

However, as it will be discussed in a following section, the main interest of this modification is the possibility of achieving a more intense enzyme/support reaction, that is, a more intense multipoint covalent attachment that can drive to higher enzyme stabilization, or controlling the immobilization area (in this case, just using site-directed mutagenesis).

If the amino groups are introduced chemically using ethylendiamine, the new amino groups present a pK value that is lower than that of the Lys (9.2 versus 10.7), being thus more reactive and permitting both, immobilization and multipoint covalent attachment under milder conditions. This may be very important when the enzyme is unstable at alkaline pH values. 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, leaving the other areas of the protein unmodified.

The increase of amino groups in the enzyme surface may also be a tool to facilitate some further chemical or physical modification of the enzyme. For example, it may facilitate the coating of the enzyme with anion exchangers. The increase on primary amino groups has been also used in certain cases to improve the prospects of achieving intra (to stabilize enzymes) or intermolecular (to prepare crosslinked enzyme aggregates, CLEAs). The lower pK value of the chemically introduced amino groups using ethylendiamine has also permitted to have a more general chemical modification of protein surfaces with other
molecules via modification of these amino groups under mild conditions that requires using the unmodified enzyme.\textsuperscript{66}

The physical coating of the enzyme surface with a poly-amine polymer, like polyethylenimine or poly allyl amine, will have many positive effects on enzyme properties, effects that are derived from the physical and chemical features of the polymer\textsuperscript{67-69}. Among these, we can remark out the partition away from the enzyme environment of deleterious hydrophobic compounds (oxygen,\textsuperscript{70, 71} hydrophobic organic cosolvents,\textsuperscript{72, 73} prevention of interaction with inactivating interfaces,\textsuperscript{74} stabilization of multimeric structures,\textsuperscript{74, 75} etc).

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.\textsuperscript{74}

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

3. Chemical amination

3.1. Chemistry of the chemical amination of enzymes using the carbodiimide route

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 several years ago.\textsuperscript{76, 77} Proteins have many reactive groups that can react largely with carbodiimides in the same fashion as with simple nucleophiles.\textsuperscript{78} Versatility and usefulness of carbodiimides as chemical modifying agents has been widely demonstrated.\textsuperscript{41, 61, 78-80}

Ethyl-di-methyl-amino-propyl Carbodiimide (EDC) is often used in the chemical modification of biocatalysts, such as proteases, ribonuclease and glucose oxidase, among
others, and allows the alteration 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.\textsuperscript{38, 78, 80}

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.\textsuperscript{77, 81} This chemistry is summarized in Figure xx. 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 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 XX, route 1).

On the other hand, if carbodiimide is added in excess, the O-acyl-iso-urea intermediate can be rearranged to form N-acyl-urea as byproduct via an intramolecular acyl transfer mechanism. In the special case, where the nucleophile is water, the carboxyl group will be regenerated with the conversion of 1 molecule of carbodiimide into its corresponding urea (Figure XX, route 2).\textsuperscript{77, 81} However, kinetic studies on the modeling of carbodiimide-carboxyl-nucleophile system have shown that the rearrangement can be slowly compared to the nucleophilic attack if the concentration of nucleophile is sufficiently high.\textsuperscript{77} Therefore, the coupling reaction of carboxyl and nucleophile can be driven essentially to completion in the presence of excess of both carbodiimide and the nucleophilic reagent.

Carbodiimides are not only specific for carboxyl groups. In aqueous solutions at acidic pHs, carbodiimides would react also with free sulfhydryl groups as for example the thiol group from side chains of cysteine,\textsuperscript{82} as well as accessible phenolic hydroxyl groups and tyrosines.\textsuperscript{83} Indeed, it has been reported that the carbodiimide activated O-acyl-iso-ureas on
one molecule may undergo displacement by the slightly nucleophilic hydroxyl of tyrosine (Figure 3). In fact, kinetic studies have shown that reaction rates of sulfhydryl and carboxyl groups with EDC are approximately equal, while tyrosine reacts more slowly. Carraway and Koshland have shown that EDC converts accessible tyrosine residues in proteins to O-arylisourea derivatives, which are resistant towards acid hydrolysis. However, they have also shown that hydroxaminolysis of the modified protein quantitatively reverses this tyrosine modification.

The reaction of carbodiimides with the carboxyl group in proteins can lead to inhibition; this can be caused by interaction of neighboring nucleophiles that could generate intramolecular cross-linkings (Figure xxa). For example, ATPase is inhibited by the carbodiimide. The mechanism of the inhibition is thought to be via formation of the O-acyl-iso-urea species followed by the attack of an adjacent nucleophile causing the loss of urea, covalent binding of the nucleophile with the binding site to produce cross-linking, and no loss of inhibition. Protection of the enzyme by 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.

Furthermore, another cause of enzymatic inhibition by use of carbodiimides can be attributed to O-N-acyl shift re-arrangements (figure xxxb). If the external nucleophile is water, the enzyme is then regenerated. The O-acylisourea 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.

3.2. Chemical amination of free enzymes
The amination of enzymes via the carbodiimide route is a very old technology. The first interest of the modifications is usually 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, but also with just mono amine compounds as the final goal was not the amination of the enzyme surface but the modification of the carboxylic residues.

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.

One of the first approaches in using diamines and carbodiimide to improve enzyme properties was the test of using modification to introduce intramolecular cross-linkages. The effect of the length of the diamine chain on the thermostability of α-chymotrypsin has been studied. To increase the prospects of having an intense crosslinking, α-chymotrypsin was succinylated. For succinylated α-chymotrypsin, the dependence of the rate constant of monomolecular thermoinactivation of the enzyme on the length of the cross-linking agent has a minimum for a shorter bifunctional reagent, ethylenediamine. The maximum stabilizing effect (compared to the native enzyme) increased (from 3- to 21-fold) when α-chymotrypsin was modified with tetramethylenediamine or succinylated α-chymotrypsin modified with ethylenediamine is used. However, they did not check if the amination degree was similar using the different diamines (and very likely it was not, due to the different pK of the amino groups), neither checked the likely existence of enzyme aggregates.

In a further research, the modification of 3 carboxyl groups of the glucoamylase from *Aspergillus niger* by ethylenediamine increased the thermostability of the enzyme for temperatures above the temperature of compensation, which is 60 °C. However, in some examples, a specific modification of a target carboxylic residue could be achieved if the carbodiimide presented some affinity towards those groups. This was the case
of the specific modification of Asp-101 of hen egg white lysozyme, via the carbodiimide
route, and using nucleophiles as different as ethanolamine, ethylenediamine, methylamine, or
4(5)-(aminomethyl)imidazole. The specific modificaion could be attained using a small
excess of carbodiimide, and that was explained by the specific binding of EDC to the substrate
binding site close to Asp-101. With histamine or D-glucosamine, the selectivity of the
modification towards Asp-101 was somewhat lower. This may be due to the specific binding
of these amines to lysozyme in competition with the carbodiimide. Depending on the amine
employed, the modified lysozyme exhibited a decreased activity (83-52% of native enzyme),
suggesting that the modification of Asp-101 weakened substrate binding.

In another example, the carboxyl groups of β-glucosidase from *Aspergillus niger*
NIAB280 were modified by water soluble 1-ethyl-3(3-dimethylaminopropyl) carbodiimide in
the presence of glycinamide or ethylenediamine. The half-lives of both modified enzymes at
low temperatures (55 and 60°C) were reduced, whereas at higher temperatures (64 and 67°C)
half-lives were enhanced. At 70°C the half-life of the enzyme modified with glycinamide
became equal to the native whereas that of the EDA modified enzyme was increased.

Chemical amination may produce very different effects when changing the inactivation
conditions.

In some instances, amination was not the target reactions but a reference composite.
Carboxymethylcellulase from *Aspergillus niger* was modified by 1-ethyl-3(3-
dimethylaminopropyl) carbodiimide in the presence of dimethylamine hydrochloride and
ethylenediamine dihydrochloride as nucleophiles. The amino groups of the enzyme modified
with dimethylamine hydrochloride were further modified by acetic anhydride for the complete
elimination of surface charges. In all cases the specificity constants (V(max)/K(m)) was
improved from 0.16 to around 1. Gibbs activation free energies of denaturation of native and
aminated enzyme at 80°C and pH 5.2 were 110 and 107 kJ mol⁻¹, whereas enthalpy of
denaturation were 143 and 144 kJ mol\(^{-1}\), and the entropies of denaturation were 91 and 105 kJ mol\(^{-1}\) K\(^{-1}\), respectively, indicating highly disordered conformations of all the transition states of modified enzyme. However, the authors focused on the stabilization of the double modified enzyme in the presence of solvents.\(^92\)

Chemical amination using ethylenediamine of a glucoamylase from *Fusarium solani* permitted to increase activity and stability of the enzyme, being the effect depended on the exact modification degree.\(^93\) Temperature and pH optima of modified glucoamylase increased after modification while the specificity constant (\(k_{\text{cat}}/K_m\)) of unmodified and optimal modified enzyme went from 136 to 225. Thus, the chemical amination of this enzyme offered very interesting enhances of the enzyme performance.

Three to four carboxyl groups of a xylanase from *Scopulariopsis sp.* were chemically modified using ethylenediamine and carbodiimide.\(^94, \, 95\) There were no differences in pH optima between the native and modified enzyme, but there was a double pH optimum for the modified enzyme. The \(V_{\text{max}}/K_m\) decreased relative to the non-modified enzyme.

In a very interesting paper, Matsumoto and co-workers showed the combined use of chemical modification and site-directed mutagenesis to get an optimized enzyme. The target enzyme was serine protease subtilisin Bacillus lentus A significant enhancement of the applicability of this enzyme in peptide synthesis was achieved by using the strategy of combined site-directed mutagenesis and chemical modification to create chemically modified mutant enzymes.\(^96\) The introduction of polar and/or homochiral auxiliary substituents, such as \(X = \text{oxazolidinones, alkylammonium groups, and carbohydrates at position 166 at the base of the primary specificity } S_1 \text{ pocket created an enzyme with strikingly broad structural substrate specificities. These modified mutante enzymes are capable of catalyzing the coupling reactions of not only L-amino acid esters but also D-amino acid esters as acyl donors with glycaminamide to give the corresponding dipeptides in good yields. These powerful enzymes are}
also applicable to the coupling of L-amino acid acyl donors with L-alaninamide. Typical increases in isolated yields of dipeptides of 60-80% over the wild type enzyme (e.g. 0% yield of Z-D-Glu-GlyNH\textsubscript{2} using wild type enzyme versus 74% using S166C-S-(CH\textsubscript{2})\textsubscript{2} NMe\textsubscript{3}+) demonstrate the remarkable synthetic utility of this "polar patch" strategy. Such wide-ranging systems displaying broadened and therefore similarly high, balanced yields of products (e.g. 91% Z-L-Ala-GlyNH\textsubscript{2} and 86% yield of Z-D-Ala-GlyNH\textsubscript{2} using S166C-S-(3R,4S)-indenooxazolidinone) was proposed as a tool to allow the use of biocatalysts in parallel library synthesis.\textsuperscript{96}

In another cases, the covalent modification of the enzyme was carried out using polymers. For example, chitosan was linked to invertase by covalent conjugation to periodate-activated carbohydrate moieties of the enzyme.\textsuperscript{97} The thermostability of the modified enzyme was enhanced by about 10 °C. The half-life at 65 °C was increased from 5 min to 5 h. The enzyme stability was enhanced by 20% at pH below 3.0. The half-life of denaturation by 6 M urea was increased by 2 h.

In another instance, the sugar chain of glycosilated portion was aminated before a further modification. For example, pectin was attached to ethylenediamine-activated carbohydrate moieties of invertase using 1-ethyl-3-(3-dimethylaminopropyl)carboimide as coupling agent.\textsuperscript{98} The modified enzyme retained 57% of the original activity and contained 2.7 mol polymer per mol of holoenzyme. Its optimum temperature was increased by 8 °C and its thermostability by 7.3 °C. The half-life at 65 °C was increased from 5 min to 2 days. The enzyme stability was enhanced by 33 % at pH 2.0, and also by 27 % at pH 12.0. The conjugate retained about 96 % of its initial activity after 3 h incubation in 6 M urea.

A more sophisticated strategy involves the use of an enzyme to produce the chemical modification of the target enzyme with the aminated polymer. Several polysacharides were derivatized with 1,4-diaminobutane and covalently attached to bovine pancreatic trypsin
through a transglutaminase-catalysed reaction. The conjugates retained about 61–82% of the original esterolytic activity of trypsin, while the optimum pH was shifted to alkaline values. The prepared conjugates were also more stable against thermal incubation at different temperatures ranging from 50°C to 60°C, and were about 22- to 48-fold more resistant to autolytic degradation at pH 9.0. Transglutaminase-catalysed glycosidation also protected trypsin against denaturation in surfactant media, with 9- to 68–fold increased half-life times in the presence of 0.3% (w/v) sodium dodecylsulfate.

3.3. Chemical amination to improve the immobilization of the enzyme

3.3.1. Increase of the intensity of the enzyme/support multipoint covalent attachment

As stated above, 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. It is possible to find diverse examples of this in the literature. However, this strategy may be effective only if the support and protocol are chosen in a way that may permit to get this multipoint covalent attachment (e.g., glyoxyl-agarose, epoxy, etc).

The first example was the amination of the enzymes glutarayl acylase and penicillin G acylase to improve their multipoint covalent immobilization on glyoxyl-agarose. Both enzymes were quite different regarding the density of Lys residues on the surface. While penicillin G acylase presented 41 superficial Lys, glutarayl acylase presented just 9 groups. In fact, penicillin G acylase could be greatly stabilized via immobilization on glyoxyl agarose, while glutarayl acylase immobilized very slowly in this support and the stabilization obtained was reduced. After full chemical amination of the exposed carboxylic groups (following the carbodiimide route described above), it was found that the aminated
penicillin acylase almost did not reduce its activity, but severely reduced enzyme stability. For this reason, only 50% modification was utilized. In the case of glutarayl acylase the lack of stability at pH 4.75 forced to use pH 6 in the modification and after this the activity decreased by 20%, but its stability remained almost unaltered. This shows the heterogeneity of the effects of the chemical modification on enzyme properties, as it has been shown above. Moreover, it also suggests that the chemical modification may be at a disadvantage regarding the genetic modification, where only the desired groups will be modified.

As a further advantage, both enzymes could be now immobilized at pH 9 (while the non aminated enzyme required a pH value near 10). This permitted to alter the orientation of the enzymes on the enzyme support and after immobilization at pH 9, the pH was increased to 10 to favor the multipoint covalent attachment. For glutarayl acylase, results were similar to the direct immobilization at pH 10, but for penicillin G acylase, the stability increased by a 2-fold factor compared to the enzyme directly immobilized at pH 10. Thus, after immobilization of the partially animated enzymes, the comparison of the unmodified/ modified enzymes immobilized on glyoxyl support permitted to get a stabilization of a four-fold factor in the case of penicillin G acylase and a 20-fold factor in the case of glutarayl acylase, showing the potential of the strategy.

Glucoamylase immobilized very slowly on glyoxyl-agarose, stabilizing the enzyme only by a 6-fold factor. After amination, enzyme stability was maintained, but now the immobilization rate was higher and the final stabilization factor was 500, maintaining a 50% of the initial activity after the whole protocol.

Laccase from *Trametes versicolor* was aminated and immobilized on glyoxyl supports, enabling a stabilization of 280 folds while maintaining a 60% of the activity. Without the amination step, the immobilization of the enzyme on the support results negligible, due to the poor density of Lys residues on the enzyme surface (just 8 Lys). This
biocatalyst could be used 10 cycles in oxidation of phenyl compounds without detecting a
decrease in enzyme activity.

Immobilization of lipase from *Candida rugosa* on electrochemically synthesized
PANI activated with glutaraldehyde could be improved after chemical amination of the
enzyme.\(^{106}\) Aminated lipases exhibited higher specific activity (52\%) and thermal stability (3
times) after immobilization, compared with the unmodified lipase. Also, reusability of the
immobilized enzyme was significantly increased with amination, especially if immobilization
was performed at pH 10, this biocatalyst retained 91\% of activity after 15 reaction cycles.

The effect of different chemical modifications, before or after immobilization, on the
properties of immobilized invertase from baker's yeast immobilized was studied.\(^{107}\) The
immobilized preparations obtained were Sp-INV by direct coupling of invertase to Sepharose,
Sp-PEA-INV by coupling of periodate and ethanolamine-treated invertase to Sepharose, Sp-
PEDA-INV by coupling of periodate and ethylenediamine-treated invertase to Sepharose, and
Sp-PEDA-2-4-6-trinitrobenzene sulfonic acid (TNBS)-INV by coupling of TNBS followed by
periodate and ethylenediamine-treated invertase to Sepharose. All of the immobilized
preparations exhibited higher stability against heat and urea-induced inactivation as compared
to native invertase. Among the procedures employed for immobilization of invertase, the Sp-
PEDA-INV preparation exhibited highest yield of immobilization, and thermal and storage
stability.

However, this strategy was complicated for industrial implementation, as it requires
the complete elimination of the remaining ethylenediamine, a competitor for the glyoxyl
groups that could reduce the prospects of getting an intense multipoint attachment. The use of
free enzyme makes the use of more or less complex techniques (e.g., ultrafiltration) necessary
to eliminate this reagent. This was solved in a new evolution of the strategy. The target
enzymes were lipases, which could be reversibly immobilized on octyl-agarose,\(^{108}\) a support
that did not produce any cross-reaction. These immobilized enzymes were aminated in solid phase, 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. The presence of detergent was useful to avoid the risk of lipase/lipase aggregation during covalent immobilization.109, 110

In a first example, the lipase from *Bacillus thermocatenulatus* was used as model.111 The enzyme is not very rich in external Lys residues.112 The chemical amination did not present a significant effect on the enzyme activity and only reduced the enzyme half-life by a 3-4-fold factor in inactivations promoted by heat or organic solvents. The optimal stabilization protocol was the immobilization of aminated BTL2 at pH 9 and the further incubation for 24 h at 25 °C and pH 10. This preparation was 5-fold more stable than the optimal BTL2 immobilized on glyoxyl agarose and around 1200-fold more stable than the enzyme immobilized on CNBr and further aminated.

In a further example, the lipase from *Thermomyces lanuginosus* was submitted to a similar treatment.61 The enzyme presented few external Lys groups,113 offering low prospects to get multipoint covalent attachment on glyoxyl supports. Even immobilization was quite slow. This case was even more complex, as the free enzyme at pH 10 was inactivated making its immobilization on glyoxyl agarose very complex, while at pH under 10 the enzyme was not immobilized. However, after amination, the enzyme could be rapidly immobilized at pH 9 or 10, avoiding enzyme inactivation. This permitted to maintain 70 % of the enzyme activity with a 5-fold improved stability compared to the immobilized non-aminated enzyme (that also presented very low activity recovery). This stabilized enzyme showed its good performance in some reactions such as the production of biodiesel,114, 115 hydrolysis of sucrose laurate,116 and synthesis of ascorbyl oleate by transesterification of olive oil with ascorbic acid in polar organic media.117 It was also shown that the aminated and multipoint covalently attached
 enzyme could be unfolded and refolded even in a more efficient fashion than the unmodified-
one point immobilized enzyme.\textsuperscript{118}

In another research, octyl-agarose immobilized lipase from \textit{Rhizomucor miehei} was
aminated and immobilized on glyoxyl-agarose and cyanogen bromide-agarose.\textsuperscript{119} Results in
stability were not analyzed, but the immobilization rate was higher in glyoxyl agarose (even
using pH 9.1 for the aminated enzyme). However, using the cyanogen bromide-agarose
immobilization rate was slower for the aminated enzyme that was not explained by the
authors. Using diothitritol (to stabilize the one-point imino bonds with the support) the
aminated enzyme could be immobilized even at pH 8.\textsuperscript{120}

\textbf{3.3.2. Improved production of crosslinked enzyme aggregates}

Crosslinked enzyme aggregates (CLEAs) is a relatively recent immobilization
technique developed the group of Prof Roger Sheldon.\textsuperscript{121, 122} The strategy is relatively simple,
consisting on the precipitation of the enzyme in an active form and the physical stabilization
of the aggregate articles via chemical crosslinking to prevent re-dissolution when the
aggregation reagent is eliminated.\textsuperscript{123} 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.\textsuperscript{124} The
amino groups tend to be the most utilized groups for crosslinking.\textsuperscript{113, 114} Co-aggregation of the
enzyme with other Lys rich proteins is one of the possible solutions,\textsuperscript{125-127} as well as the use of
PEI (see section 4 of this review).\textsuperscript{128, 129} However, both strategies reduce the volume loading
of the target protein on the final biocatalyst. The amination of the enzyme may be a simple
solution to solve this problem.

This has been used, to date, in a single paper.\textsuperscript{65} Lipase B from \textit{Candida antarctica} is
not very adequate to prepare CLEAS due to the low amount of surface Lys.\textsuperscript{130} Although the
precipitation step is easy using different precipitants, the cross-linking step becomes a
problem due to the low amount of Lys residues in this enzyme. The enzyme surface was enriched in amino groups by chemical amination of the enzyme using ethylenediamine and carbodiimide. Using this aminated enzyme, precipitation is also effective and the crosslinking step is no longer a problem. Stability of this CLEA was higher in both thermal and cosolvent inactivation experiments than that of the coCLEA produced by co-aggregation of BSA and enzyme, another alternative to produce a CLEA of this interesting enzyme.

3.3.3. Improved immobilization on cation exchangers

Immobilization of proteins on ion exchangers requires the simultaneous establishment of several enzyme-support interactions. Most enzymes have an isoelectric point ranking from 4 to 5, and this makes that the enzymes can hardly become adsorbed on cation exchangers under a wide range of pH values. This may be facilitated if the carboxylic groups of the enzyme are modified to amino groups via chemical amination, as the number of cationic groups may be greatly increased and thus, the enzyme may become easily exchanged in anionic supports in a wide range of conditions. However, there are few examples of this strategy. The enzyme penicillin G acylase is not adsorbed at pH 7 on carboxymethyl or dextran sulfate-coated supports. The chemical amination of the protein surface permitted the immobilization of the enzyme on both anionic supports. Immobilization was very strong on these supports, mainly in the polymeric ones, and dependent on the degree of modification, although the enzymes can still become desorbed after inactivation by incubation under drastic conditions. Moreover, the immobilization on ionic polymeric beds allowed a significant increase in enzyme stability against the inactivation and inhibitory effects of organic solvents, very likely by the promotion of a certain partition of the organic solvent out of the enzyme environment.
The chemical introduction of aminated polymers has also been used to improve the ionic exchange of proteins on ion exchangers. Invertase from *Saccharomyces cerevisiae* was chemically modified with chitosan and further immobilized on sodium alginate-coated chitin support. The positive charges of the chitosan permitted to keep the enzyme retained by interactions with the anionic alginate. The yield of immobilized protein was determined as 85% and the enzyme retained 97% of the initial chitosan-invertase activity. The optimum temperature for invertase was increased by 10 °C and its thermostability was enhanced by about 9 °C after immobilization. The immobilized enzyme was stable against incubation in high ionic strength solutions and was four-fold more resistant to thermal treatment at 65 °C than the native counterpart. The biocatalyst prepared retained 80% of the original catalytic activity after 50 h under continuous operational regime in a packed bed reactor. The strategy was further extended to the immobilization of the modified enzyme on pectin-coated chitin support via polyelectrolyte complex formation. The yield of immobilized enzyme protein was determined as 85% and the immobilized biocatalyst retained 97% of the initial chitosan-invertase activity. The optimum temperature for invertase was increased by 10°C and its thermostability was enhanced by about 10°C after immobilization. The immobilized enzyme was stable against incubation in high ionic strength solutions and was 4-fold more resistant to thermal treatment at 65 °C than the native counterpart. The biocatalyst prepared retained 96 and 95 % of the original catalytic activity after 10 cycles of reuse and 74 h of continuous operational regime in a packed bed reactor, respectively.

The same chemical modification strategy was used to immobilize this enzyme on hyaluronic-acid-modified chitin. The immobilized enzyme retained 80 % of the initial invertase activity. The optimum temperature for sucrose hydrolysis was increased by 5 °C, and its thermostability was enhanced by about 10 °C after immobilization. The immobilized enzyme was stable against incubation in high-ionic-strength solutions, and was six-fold more...
resistant to thermal treatment at 65 °C than the native counterpart. The biocatalyst prepared
retained 100 % of the inicial activity after 10 cycles of reuse as well as after 74 h of
continuous sucrose hydrolysis in a packed bed reactor, respectively.

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

As previously discussed (point 3.2), the chemical amination of enzymes may be a
potent tool to improve enzyme performance. As stated in point 3.3.1. of this review and
discussed in, the chemical modification of enzymes in the solid phase has many advantages:
prevention of aggregation, possibility of using stabilized enzymes, easy performance and
control, etc.

Therefore, if the modification is performed to alter enzyme properties of an enzyme
that is going to be used in an immobilized way, it makes sense to perform the modification on
an already immobilized enzyme,

Most of the examples found using the chemical amination of the immobilized
enzymes are quite recent. In one of the first examples, three different immobilized lipases
[those from Candida antarctica (form B), Thermomyces lanuginosus and Pseudomonas
fluorescens] were modified with ethylenediamine. In some cases, the activity of the lipases
increased after the chemical modification while in other cases the activity was strongly
reduced. The enantioselectivity of the enzymes in the hydrolysis of different mandelic acid
derivatives was also highly modulated. For example, amination of the CNBr-CAL-B
preparation greatly increased the enantioselectivity of the enzyme in the hydrolysis of (±)-2-
hydroxyphenylacetic acid methyl ester, from an E value of 2 without modification up to
E>100, affording (R)-mandelic acid in high purity (ee>99% at 50% conversion) at pH 7 and
4°C.
Novozym 435 (a commercial immobilized preparation of lipase B from *C. antarctica*) was modified via aminoethylamidation among other compounds.\(^{139}\) The modified enzyme improved the activity versus 3-phenylglutaric dimethyl diester by around a two fold factor, while decreased the activity versus mandelic acid methyl ester or 2-\(O\)-butyryl-2-phenylacetic acid. However, the enantiospecificity of the enzyme in the hydrolysis of racemic mandelic acid methyl ester improved while the enantioselectivity in the hydrolysis of 3-phenylglutaric dimethyl diester.

The lipase from *Thermomyces lanuginosus* was immobilized on octyl Sepharose and further modified with ethylenediamine after activation of the carboxylic groups with carbodiimide.\(^{41}\) Different degrees of modification of the carboxyl groups were carried out by controlling the concentration of carbodiimide (10\%, 50\% or 100\%). Interestingly, the chemical modification of the immobilized lipase produced an improvement in its activity versus p-nitrophylpropionate, and it increased with the modification degree. This increase in activity was much more significant at pH 10, where the fully modified preparation increased the activity by a factor of 10 as compared to the unmodified preparation. Moreover, the incubation of the chemically aminated preparations in a hydroxylamine solution (to recover modified Tyr residues) improved the activity by an additional factor of 1.2. The fully aminated and incubated enzyme in hydroxylamine preparation exhibited a higher thermostability than that of the unmodified preparation, mainly at pH 5 (almost a 30 fold factor). In the presence of tetrahydrofuran, some stabilization was observed at pH 7, while at pH 9 the stability of all modified enzymes decreased.\(^{41}\)

In another example, three different lipases (from *Candida antarctica* fraction B, *Thermomyces lanuginosa*, and *Rhizomucor miehei*) were immobilized on CNBr-activated Sepharose via a mild covalent immobilization or adsorbed onto octyl-Sepharose and submitted to amination among other modifications, altering (and in some cases improving) the enzyme
performance in the selective hydrolysis of sardine oil to produce eicosapentaenoic acid and
docosahexaenoic acid, being the lipase from *Candida antarctica* fraction B the lipase with a
lower change in its properties in this reaction.\textsuperscript{140}

In a further extension of the strategy, the fact of the increase in amino groups in the
surface of the protein was not the only target. As a second target, the fact that, now, the
enzyme surface is enriched in amino groups was utilized to achieve a larger modification of
the protein surface with a second amine-modifying reagent. Together to the potential to
modulate enzyme properties of the chemical modifications, the research was also focused on
the decisive effect that the immobilization protocol has on the effects of the chemical
modifications. In a first example, *Candida antarctica* fraction B adsorbed on octyl-agarose or
covaantly immobilized on cyanogen bromide agarose was modified with ethylenediamine
(EDA) or 2,4,6-trinitrobenzensulfonic acid (TNBS) using one reagent or using several
modifications in a sequential way (the most complex preparation was CALB-TNBS-EDA-
TNBS).\textsuperscript{40} The covalently immobilized enzyme decreased the activity by 40-60% after
chemical modifications, while the adsorbed enzyme improved the activity on p-
nitrophenylbutyrate (pNPB) by EDA modification (even by a 2-fold factor). Moreover,
significant changes in the activity/pH profile and in the enzyme specificity by the chemical
modification were observed. In a second research effort, the utilized enzyme was a
commercial quimeric fosfolipase commercialized by Novozymes), Lecitase Ultra,
immobilized in the same supports. Both immobilized preparations have been submitted to
different individual or cascade chemical modifications (amination, glutaraldehyde or 2,4,6-
trinitrobenzensulfonic acid (TNBS) modification) in order to check the effect of these
modifications on the catalytic features of the immobilized enzymes (including stability and
substrate specificity under different conditions).\textsuperscript{141} As in the previously presented case, the
effects of the chemical modifications strongly depend on the immobilization strategy used.
For example, using one immobilization protocol a modification improves activity, while for the other immobilized enzyme it is even negative. 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 example, glutaraldehyde modification of immobilized or modified and aminated enzyme permitted to improve enzyme stability of both immobilized enzymes at pH 7 and 9 (around a 10-fold), but only the adsorbed aminated enzyme improved the enzyme stability at pH 5 by glutaraldehyde treatment. This occurred even though some intermolecular crosslinking could be detected via SDS-PAGE. Amination improved the stability of octyl-Lecitase, while it reduced the stability of the covalent preparation.\textsuperscript{141}

Following a different amination strategy using an aminated polymer a nice proposal is described in a previous work.\textsuperscript{142} A poly-aminated dextran was site-specifically introduced on a lipase from \textit{Geobacillus thermocatenulatus} (BTL2). The chosen site was Cys64, it is placed in the proximity of the region where the lid is allocated when the lipase exhibits its open and active form,\textsuperscript{112} and the modification was performed on two differently immobilized lipase preparations. This position of the enzyme was specifically modified by thiol-disulfide exchange with pyridyldisulfide poly-aminated-dextrans. If the enzyme was immobilized on cyanogen bromide agarose, the modification increased the activity by around a 2 fold factor versus aliphatic carboxylic esters, but if the substrate contained an aromatic carboxylic group the activity remained unchanged.\textsuperscript{142} If the enzyme was attached to glyoxyl-agarose (multipoint covalent attachment), a significant increase in activity was only observed using p-nitrophenyl butyrate. The stabilization of the open form of the lipase induced by the modification was shown by irreversible inhibition experiments.

\textbf{3.5. 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.\textsuperscript{16, 18, 143-145} From a very wide perspective, multipoint covalent attachment on a support may be considered a very intense crosslinking process, where the support is the crosslinker reagent.\textsuperscript{31} This was treated in section 3.3.1. Here we will focus on the crosslinking using bi or multifunctional molecules of already immobilized enzymes. Intermolecular crosslinking is a quite complex process, as it must make a competition with one-point modification (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.\textsuperscript{32} It seems obvious that an increase in the amount of reactive groups on the protein surface may be advantageous for both objectives. Moreover, most of the most used and effective crosslinkers are based on reaction between amino groups, as is the case of the glutaraldehyde.\textsuperscript{51, 146} Thus, amination of the enzyme surface could be a proper tool to achieve an intense intramolecular or intersubunit crosslinking.

However, although there are many reports on cross-linking of immobilized proteins,\textsuperscript{35} we have been able to find just one example where the amination was performed on previously aminated enzyme. This example was on penicillin G acylase previously multipoint-immobilized on glyoxyl-agarose.\textsuperscript{64} After amination, the enzyme was submitted to full amino-modification with one molecule or two molecules of glutaraldehyde per amino group, the excess of reactive was eliminated and both preparations 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). After 20 h of incubation, stabilization factors of more than 40 were found when using one glutaraldehyde molecule per amino group, while results were poorer using two glutaraldehyde molecules.\textsuperscript{64} The incubation pH value, 7 or 9, presented a marginal effect, suggesting the high reactivity of the amino-
glutaraldehyde groups with another glutaraldehyde amino groups in a wide range of pH values. Using formaldehyde, stabilization did not take place, suggesting that this reactive may have a most complex crosslinking behavior. Using an excess of formaldehyde, similar stabilization factors were found, suggesting that formaldehyde require to form some multiformaldehyde structures to give some crosslinking.

3.6. 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 some inactivating causes. For example, the enzyme may become stabilized versus interaction with interfaces, such gas bubbles gas produced by stirring (e.g., if adjusting the pH value is necessary) or gas bubbling (e.g., if oxygen needs to be supplied). It may also be used to prevent multimeric enzyme inactivation by subunit dissociation, to increase enzyme stability versus organic solvents by generating a certain partition, etc. Previous examples use chemical modifications, for example using aldehyde dextran, but this modification may be somehow complex, and may affect enzyme activity (chemical reaction, reduction step, etc). In this sense, the use of ionic polymers may be a simpler solution.

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

Most of the examples dealing with coating enzymes with ionic polymers use polyethylenimine (see section 4 of this review) because most enzymes have an Ip too low to
become coated using polyanionic polymers under neutral pH values. Ionic exchange, as it has
been previously stated, requires a multipoint ion exchange.\textsuperscript{132, 133} In this case, we intend that
the full protein surface may be coated by the polymer. This may be harder than just the
immobilization, which only involves a determined enzyme area.

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,\textsuperscript{40} permitting to have a very stable enzyme-anionic polymer
composite. 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 3.3.3).\textsuperscript{62, 135-}
137 However, as we thought that this application should work properly, we have decided to
include this possibility in the present review.

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 their catalytic efficiency. The reaction
with amino groups of the protein used to be one of the most used ones due to the good
reactivity of amino groups with many reactive.\textsuperscript{152-155} However, if we really desire a massive
modification of the protein surface, this may not be so simple, as the pK of the amino group in
the lateral chain of Lys is 10.5, and this pK will be quite similar on medium exposed residues.
The terminal amino groups may have a far lower pK value, but this group may only permit a
one-point modification. This was the goal of a recent paper.\textsuperscript{66} The researches intended to
modify the surface of the lipase B from \textit{Candida antarctica} with succinic polyethyleneglycol
via the carbodiimide route. Immobilized enzyme (on octyl Sepharose or Eupergit C) were
used, to analyze the effect of the immobilization protocol. Modification of the native amino
groups of the enzyme did not produce a significant alteration in the amount of the amino
groups of the enzyme (just around 1 group per enzyme molecule could be modified).
However, if the enzyme was previously aminated, around 14-15 PEG molecules could be
introduced per enzyme molecule. As in other examples commented in other sections, it has
been found that the effect of this modification depends on the immobilization protocol. For
example, activity versus pNPP increased using CALB-octyl Sepharose while it decreased
when using Eupergit C following amination and PEGylation. In hydrolysis of R/S methyl
mandelate, enantioselectivity in this hydrolysis significantly improved after modification
using the covalent preparation (from 7.5 to 20), while using octyl Sepharose almost had no
effect.66

3.8. Chemical amination of proteins to improve their usefulness “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
proteins have two main advantages. First, the modified protein has a different, usually more
potent immunogenicity that unmodified protein.156, 157 Second, and related to the point 3.6 of
this review, the larger amount and higher reactivity of the aminated enzymes, may permit to
introduce a higher number of antigen molecules per carrier protein.158
Regarding the use of proteins as a medicament the cationized protein is able to penetrate
membranes in a more efficient way than the unmodified proteins.159, 160

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

3.8.1. Use of aminated proteins to raise antibodies versus small molecules.
To raise antibodies versus small molecules, it is necessary to attach this small haptamers 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. 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 administration of cationized protein but not using unmodified enzyme unless an adjuvant was used. It was speculated that the aminated protein may have a greater affinity for antigen-presenting cells or for the T cell receptor, or that the altered structure may enhance recognition of the molecule by APC and/or helper T cells. The authors tried to explain these results investigating the uptake of unmodified and cationized serum albumin by splenic APC. Amination was performed at different degrees of carboxylic modification. 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 aminated BSA enters by a nonspecific adsorptive mechanism. The different modes of cellular entry for the two molecules, nBSA and cBSA, resulting in a rapid uptake of aminated BSA. This was proposed to have important ramifications on T cell activation and immunoregulation.

In another paper, 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 and a similar sensitivity of antisera against AFB1 were observed, compared with immunization by AFB1-oxime-albumin. Later, a similar strategy was used to raise antibodies versus bisphenol A. Compared with non-aminated protein, the aminated bovine serum albumin improved the efficiency of coupling and
enhanced the immune response against the target antigen. The sensitivity of antisera against bisphenol A was similar to the sera obtained using non-aminated protein. In a third research, dichlorvos was coupled with cationized bovine serum albumin using also using a method based on Mannich-type reaction, and utilized to produce a monoclonal antibody versus diclorvos.

In a nice report, it was shown that combining double-chemically modified carrier proteins and hetero-functional cross-linkers allows preparing tailor-made hapten-protein carrier conjugates. The protein was aminated and further modified by different cross-linkers (hyper-activated 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.

3.8.2. Improving the enzyme function in vivo

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 of these 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 can exert their biological activity in cells. As the mammalian cell membrane possesses an abundance of negatively charged glycoproteins and glycosphingolipids, cationization of proteins is a reasonable choice to endow them with the ability for intracellular delivery.
One of the applications of 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. There are many examples of this strategy in literature.

The improved issue uptake of cationized immunoglobulin G was shown after intravenous administration relative to the uptake of native protein. The studies demonstrate that cationization of immunoglobulin greatly increases organ uptake of the plasma protein compared to native immunoglobulins, and suggests that cationization of monoclonal antibodies may represent a potential new strategy for enhancing the intracellular delivery of these proteins. The ratio of the volume of distribution of the $^3$H-cationized IgG compared to $^3$H-labeled native albumin ranged from 0.9 (testis) to 15.7 (spleen) in the rat and in primates.

In another study, 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. The study demonstrated that cationization results in enhanced endocytosis of the antibody and enhanced inhibition of HIV-1 replication, consistent with intracellular immunization of the rev protein.

In another paper, the cationization 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. While the $^{125}$I-labeled native D146 antibody uptake by MDA-MB231 human carcinoma cells was negligible, there was a marked increase in the endocytosis of the antibody following cationization. The in vivo organ uptake of the
cationized monoclonal antibody was increased relative to the native antibody; there was a 6-
fold increase in the systemic volume of distribution, a 58- fold increase in the systemic
clearance of the cationized antibody from the plasma compartment, and a 9-fold reduction in
the mean residence time of the cationized antibody as compared to the native D146 antibody.

The in vivo pharmacokinetics and efficacy of cationized human immunoglobulins in
the human-peripheral blood lymphocytes-severe combined immune deficiency mouse model
were evaluated in another study using the severe combined immunodeficient mouse
transplanted with human lymphocytes and infected with human immunodeficiency virus
(HIV)-1.\textsuperscript{167} Immunoglobulins from noninfected humans and from HIV-infected individuals
were cationized. The pharmacokinetic analysis showed that the cationized immunoglobulins
have a markedly reduced mean residence time and a marked increase in organ uptake
compared to the native immunoglobulins. Treatment of HIV-infected severe combined
immune deficiency mice that were transplanted with human lymphocytes demonstrated
therapeutic efficacy for a 2-week treatment at a dose of 5 mg/kg cationized HIV immune
globulin.\textsuperscript{167}

In another study, the feasibility of cationizing the humanized 4D5 monoclonal
antibody directed against the p185(HER2) oncogenic protein was analyzed to analyze its cell
uptake.\textsuperscript{168} 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. The systemic volume of distribution of the cationized
4D5 antibody was 11-fold greater than that of the native antibody

In another example, it was found that aminated goat colchicine-specific polyclonal
immunoglobulin G and antigen binding fragment in plasma decreased more rapidly than the
non-modified counterparts.\textsuperscript{169} In addition, there was a 74-fold increase in the volume of
distribution and a 114-fold increase in the systemic clearance of aminated antibody with the
native one. Amination of colchicine-specific antibibidy or their fragments increased the
organ distribution and greatly altered their pharmacokinetics.\(^{169}\)

In other cases, the amination has as objective to achieve the function of enzymes
inside the cells to solve some problems, that is, use the enzymes as medicaments. For
example, the successful prevention of hydrogen peroxide-induced damage to the rat jejunal
mucosa by cationized catalase and compared to the protection achieved using unmodified
enzyme.\(^{170}\) It was found that in all cases the cationized enzymes were superior to the native
catalase in their shielding capability. A significant protection against Fe(II)/H\(_2\)O\(_2\) and ascorbic
acid/copper ion-mediated damage was obtained when the cationized enzymes were used. In
the presence of glucose, native glucose oxidase failed to cause damage in the rat jejunal
mucosa; however, the cationized enzyme caused profound tissue injury. These findings
indicate the potential therapeutic merit of cationized enzymes for the treatment of pathological
processes in the intestine, whenever oxidative stress is involved.\(^{170}\)

In another research, the objective was to achieve hepatic delivery of catalase for the
prevention of CCl\(_4\)-induced acute liver failure in mice, two types of cationized catalase
composites were developed using ethylendiamine (13.6 amino groups/molecule could be
introduced) or hexylendiamine (introduction of 3.1/molecule).\(^{171}\) Aminated enzyme showed
an increased binding to HepG2 cells, and were rapidly taken up by the liver. Hydrogen
peroxide induced cytotoxicity in HepG2 cells was significantly prevented by preincubation of
the cells with aminated enzyme.

Perhaps ribonucleases (RNases) are the most studied enzymes as therapeutics.
Ribonucleases are potential anti-tumor drugs due to their 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.\(^{172}\) The cytotoxic properties of naturally occurring or engineered RNases correlate well
with their efficiency of cellular internalization and digestion level of cellular RNA. Cationized RNases are considered to adsorb to the anionic cellular surface by Coulombic interactions, and then become efficiently internalized into cells by an endocytosis-like pathway. Although chemically modified cationized RNases showed decreased ribonucleolytic activity, improved endocytosis and decreased affinity to the endogenous RNase inhibitor improve their ability to digest cellular RNA.

Toxic effects of aminated *Streptomyces aureofaciens* RNases Sa, Sa2, Sa3, are enhanced, indicating the major role of a cationic nature on the enzyme surface. Another study shows how carboxyl groups of bovine RNase A and human RNase 1 were modified with ethylenediamine by the carbodiimide route. The modified RNases were cytotoxic toward 3T3-SV-40 cells despite their decreased in ribonucleolytic activity. RNase inhibitor R1 cannot eliminate their enzymatic activity, while native enzymes were completely inactivated by RI. The cytotoxicity correlated well with the net cationic residues. Cationic RNases were more efficiently adsorbed by the cells. In a more detailed study, they found that if modifying 5 to 7 out of 11 carboxyl groups in RNase A, a maximum on cytotoxicity toward MCF-7 and 3T3-SV-40 cells were found.

Another application of aminated proteins is their use as carrier proteins for different drugs or peptides. For example, rat albumin was cationized with hexamethylenediamine, and the isoelectric point of the protein was raised from 5.5 to approximately 8. 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. The brain volume of distribution of the $^3$H-cationized rat serum albumin increased linearly over a 5-hr period after an intravenous injection of the isotope and reached a value of $46 \pm 3 \mu l/g$ (mean $\pm$ S.E.) by 5 hr, whereas the brain volume of distribution of the $^{125}$I-native rat serum albumin was constant during the 5-hr time period ($9.3 \pm 0.7 \mu l/g$, which is equal to the brain blood volume). Therefore, cationized
rat albumin may be used in future studies that use the repetitive administration of cationized rat albumin chimeric peptides for the evaluation of the transport of these substances through the blood-brain barrier in vivo.\textsuperscript{177} In another example, bovine serum albumin was aminated with hexamethylenediamine or ethylenediamine to obtain cationized proteins and study the relation between physical properties and hepatic delivery.\textsuperscript{178} Aminated albumins were rapidly taken up by liver, but the protein modified using hexylenediamine showed a faster uptake than is using ethylenediamine, with a similar number of free NH\textsubscript{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.\textsuperscript{178}

Another research used the cationic β-lactoglobulin as carrier. This protein was assayed as a bioavailability enhancer for poorly absorbed bioactive compounds.\textsuperscript{179} At most 11 anionic amino acid residues of β-lactoglobulin were substituted by ethylenediamine, resulting in a highly cationic surface and significantly increased surface hydrophobicity. These changes improved also improved mucoadhesion.\textsuperscript{179}

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 efficient strategy. A method for efficient protein transduction into living cells in which a protein is simply cationized with PEI by limited chemical conjugation was described in an interesting paper.\textsuperscript{180} PEI-cationized proteins appeared to adhere to the cell surface by ionic charge interaction and then internalize into cells in a receptor- and transporter-independent fashion. Since PEI is an organic macromolecule with a high cationic-charge density, limited coupling with PEI results in endowment of
sufficient cationic charge to proteins without causing serious decline in their fundamental functions. A number of PEI-cationized proteins, such as ribonuclease (RNase), green fluorescent protein (GFP) and immunoglobulin (IgG), efficiently entered cells and functioned in the cytosol. The glutathione S-transferase-fused protein expression system has been extensively used to generate a large quantity of proteins and has served for functional analysis in vitro. A novel approach for the efficient intracellular delivery of GST-fused proteins into living cells to expand their usefulness up to in vivo use has been intended using the amination of the enzyme to improve the enzyme penetrability. The glutathione S-transferase fused proteins were cationized by forming a complex with a polycationic polyethylenimine-glutathione conjugate. On screening of protein transduction, optimized PEI-glutathione conjugate for protein transduction was characterized by a partly oligomerized mixture of PEI with average molecular masses of 600 (PEI600) modified with multiple glutathiones, which could have sufficient avidity for glutathione S-transferase. These PEI-glutathione conjugates seem to be convenient molecular tools for protein transduction of widely used glutathione S-transferase-fused proteins in in vitro studies.

Another example is the artificial regulation of cell proliferation by protein transduction of the N-terminal domain (1-132 amino acids) of the simian virus 40 large T-antigen, which inactivates retinoblastoma family proteins but no p53 has been intended by PEI modification of this protein. To deliver proteins into cells, an indirect cationization method was used by forming a complex of biotynylated protein through disulfide bonds and PEI-cationized avidin. Using this complex, the virus was transduced into the nucleus of confluent and quiescent Balb/c 3T3 cells and was found to be complexed with a cellular target protein, pRb. Furthermore, this viral protein produced transduction induced cell proliferation in spite
of confluent conditions. These results suggest that oncogene protein transduction technology has great potential for in vitro regulation of cell proliferation.\textsuperscript{182}

In another original approximation, indirect protein amination using non-covalent interaction was evaluated for the transduction of proteins into living cells and for the expression of their functions in the cytosol. PEI-cationized avidin, streptavidin and protein G were prepared, and examined whether they could deliver biotinylated proteins and antibodies into living cells.\textsuperscript{183} PEI-avidin (and/or PEI-streptavidin) carried biotinylated GFPs into various mammalian cells very efficiently. A GFP variant containing a nuclear localization signal was found even in the cell nucleus. The addition of a biotinylated RNase A derivative mixed with PEI-streptavidin to a culture medium of 3T3-SV-40 cells resulted in remarkable cell growth inhibition, suggesting that the biotinylated RNase A derivative entered cells and digested intracellular RNA molecules. Furthermore, the addition of a fluorescein-labeled anti-S100C (beta-actin binding protein) antibody mixed with PEI-protein G to human fibroblasts resulted in the appearance of a fluorescence image of actin-like filamentous structures in the cells.\textsuperscript{183}

Finally, amination has been proposed to recover the activity of proteins expressed as inclusion bodies. In a different approach, a reversibly aminated denatured protein through disulfide bonds is not only soluble in water but also able to fold to the native conformation in vitro.\textsuperscript{184} Taken together this and the easy penetration of aminated protein in cells, a novel method to deliver a denatured protein into cells and simultaneously let it fold to express its function within cells was presented. This "in-cell folding" method enhances the utility of recombinant proteins expressed in \textit{Escherichia coli} as inclusion bodies. The strategy includes several steps: the recombinant proteins in inclusion bodies are solubilized by reversible cationization through cysteine residues by disulfide bonds with aminopropyl methanethiosulfonate or pyridyldithiopropionylpolyethylenimine and then incubated with cells without an in vitro folding procedure. This was shown using human tumor-suppressor
p53. Treatment of p53-null Saos-2 cells with reversibly cationized p53 revealed that all events examined as indications of the activation of p53 in cells, such as reduction of disulfide bonds followed by tetramer formation, localization into the nucleus, induction of p53 target genes, and induction of apoptosis of cells, occurred.184

4. Physical amination of enzymes using aminated polymers

In the previous section, 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. The polymers may be quite large, even million of kDa, and that may facilitate the multipoint adsorption that is require to keep the polymer/enzyme interaction.132-134

PEI has been described to present some stabilizing effect on diverse proteins due to diverse causes: prevention of enzyme aggregation, prevention of lost of secondary structure, reduction of metal oxidation, prevention of multimeric enzyme dissociation, inactivation by deleterious substrates, etc.68, 69, 73, 75, 185 Some reports pointed that the stability-effect of poly-ionic polymers did not really depend on their cationic or anionic nature of the polymer was not critical to get the stabilization, effect, stating that perhaps a direct electrostatic enzyme/polymer interaction was not required.67 However, considering that most enzymes may be adsorbed under the same conditions on PEI and dextran sulfate coated supports; it is not clear that this electrostatic interaction may be discarded.186

The effects of the polyamine polymer were not always positive on enzyme features. Quaternized polyamines (poly-N-alkyl-4-vinylpyridinium bromides suppress the thermoaggregation of glyceraldehyde-3-phosphate dehydrogenase but not thermodenaturation
of the enzyme. The adverse effect was reduced by the addition of sodium chloride, which destroyed the enzyme-polymer complex and resulted in a noticeable reactivation.

In another example, PEI was found to have not effect on the melting temperature of basic proteins while for the acidic ones there was a shift in the melting temperature towards lower temperatures. The secondary structures of the basic proteins were essentially the same in presence of the polymer, with none or a slight increase in the CD spectra. In the case of acidic proteins, the CD spectra were diminished mostly due to phase separation. Despite lowering the thermal stability of acidic proteins, PEI protected heart lactate dehydrogenase at an increasing oxidative stress. In another example, the addition of polyethyleneimine to chloroperoxidase from *Caldariomyces fumago* dramatically improved the stability of the enzyme towards peroxide dependent inactivation.

Biosensors were fabricated at neutral pH by sequentially depositing the polycation polyethyleneimine (PEI), the stereoselective enzyme l-glutamate oxidase (GluOx) and poly-ortho-phenylenediamine onto 125-μm diameter Pt wire electrodes. The presence of PEI produced a 10-fold enhancement in the detection limit for Glu (compared with the corresponding PEI-free configurations, without undermining the response time. Most remarkable was the finding that, although some designs of PEI-containing biosensors showed a 10-fold increase in linear region sensitivity to Glu, their oxygen dependence remained low.

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

### 4.1. Immobilization 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 they should have also a anionic surface).

This has been exemplified by a single paper to date. Glutamate dehydrogenase from *Thermus thermophilus* and formate dehydrogenase from *Pseudomonas sp.* were coated with large PEI to prevent subunit dissociation. Both enzymes are very unstable at acidic pH values due to the rapid dissociation of their subunits (half-life of diluted preparations is few minutes at pH 4 and 25 °C). The enzyme-PEI composites exhibited full activity after preparation. The enzyme-polymer composites were treated with glutaraldehyde to prevent enzyme/polymer dissociation at acidic pH value, that was the pH values range of higher interest in these enzymes. This step was performed by previously immobilizing the composite onto a weak cationic exchanger to prevent enzyme covalent aggregation. The composite could be very strongly, but reversibly, adsorbed on cationic exchangers.

4.2. Coating with poly-amine polymers before immobilization to prevent undesired interactions with the matrix

In other cases, the enzyme coating was just a first step in a longer immobilization strategy; the coating may increase the size of the enzyme, making their trapping easy, or preventing the interaction with deleterious interfaces.

Examples to improve the enzyme trapping in paper to be used in food packing may be found in the literature. To this goal, the microencapsulation of glucose oxidase from *Aspergillus niger* and laccase from *Trametes versicolor* in PEI with the goal of immobilizing these enzymes in paper substrates to develop biosensors and bioreactors. The technique caused a severe decrease (up to 65%) in the specific activities of both enzymes once microencapsulated. Microencapsulation improved the thermal stability of glucose oxidase at temperatures up to 60 °C due to stabilization of its active conformation but reduced the
thermal stability of laccase because of the increased coordination between PEI and copper atoms in the enzyme's active site. Glucose oxidase bioactive paper was fabricated, which could be potentially used as food packaging paper. In a further optimization, results using the laccase was improved, using a starch-based coating suspension. The use of microencapsulation allows for better activity retention in papers over time at room temperature (50% loss after 28 days) compared to papers modified with free laccase (50% loss after 4 days). Microcapsules also decrease the inhibition of laccase by azide.

Another example is an interesting immobilization of enzymes using a “sandwich” strategy. Layered titanates have been employed to support active proteins, which have been widely used in biocatalysis and bioelectrochemistry. Their interest lay on their good biocompatibility, nontoxicity, relatively high conductivity, and chemical and thermal stability. The titanate nanosheets are negatively charged, and stable in aqueous solution. They can easily immobilize positively charged protein molecules, where a spontaneous flocculation occurs and biomolecules are incorporated within the interlayer space of layered structure. However, this did not occur if the enzyme has an anionic surface, that is, with most of the proteins. This paper shows how the preliminary coating of the enzyme with PEI can be used to immobilized the enzyme on Layered titanates. The native structures of proteins were retained after immobilizing although a significant difference in microstructures was observed among these composites. The amounts of immobilized proteins depend on the enzyme, were up to ~70% wt. for lysozyme, 37% wt. for bovine serum albumin and 21.5% wt. for lipase from Candida rugosa. These composites were stable under neutral and weakly acidic condition, and only releases <10% proteins at pH under 4. These composites are reusable, and the residual activities of immobilized enzymes are 68% for lysozyme and 61% for lipase after 10 recycles.
4.3. Generation of artificial environments on immobilized enzymes

Polyaminated polymers, like PEI, chitosan, polyalylmine, etc are quite hydrophilic, their cationic nature may permit to recover the immobilized enzyme molecules of a very hydrophilic shell that can produce some partition of hydrophobic compounds, like gases, organic solvents, etc, enabling the preparation of biocatalysts with improved stability in this media.

The strategy may be used for enzymes immobilized on preexisting supports, or enzymes to be immobilized via the CLEA technology.

One of the enzymes that has been subject to more studies using this stabilization strategy is penicillin G acylase. This enzyme has many potential uses, hydrolysis of antibiotics, resolution of racemic mixtures or synthesis of antibiotics. In many instances, the enzyme needs to be used in organic medium, and the enzyme is not very stable under these conditions. Even much stabilized immobilized enzymes via multipoint covalent attachment have reduced application on some of these reactions. Thus, the stabilization of this enzyme versus the deleterious effects of organic solvents is a key point for their applicability. In a first approach, this stabilized enzyme was co-immobilized with PEI, submitted to successive modification with aldehyde dextran and PEI. In an effort to further improve the enzyme stability, sulfate dextran was also used, to generate a thick shell of “poly-ammonium sulfate” that were able to stand even 95% of organic solvents like tetraglyme when the original immobilized enzyme only can be used at a maximum of 60%, and with a lower operational stability. Even more interestingly, this derivatives presented a higher activity, confirming that the random coil structure of the polymers avoid the promotion of diffusion barriers. These preparations permitted to perform some reactions under conditions where the untreated immobilized preparations exhibited a low stability, like hydrolysis of penicillin G.
acylase in the presence of organic solvent,\textsuperscript{203} enantioselective synthesis of phenylacetamides,\textsuperscript{204} or the synthesis of amides of high pK amines.\textsuperscript{205}

The co-aggregation of penicillin acylase, PEI and dextran sulfate permitted to prepare crosslinked enzyme aggregates with also significantly improved properties in the presence of organic solvents.\textsuperscript{206} This biocatalyst presented better behavior in organic solvents than the more thermostable glyoxyl-agarose biocatalyst.\textsuperscript{207}

Stabilization of oxygen labile enzymes has been also achieved by the salting out effect, using coCLEAs of PEI and enzyme. Oxygen labile nitrilases have been stabilized this way versus oxygen inactivation.\textsuperscript{71} While the nitrilases lost 50-100\% of their activity upon exposure to oxygen for 40 h, the PEI co-aggregates of the nitrilases were much more oxygen-tolerant. The nitrilase from \textit{Pseudomonas fluorescens} EBC 191, in particular, retained its full activity upon exposure to oxygen for 40 h.

4.4. Improved preparation of CLEAs

Polyaminated polymers have found several advantages in the preparation of crosslinking enzyme aggregates (CLEAs). First, as commented in the point above, co-aggregation with PEI (combined or not with sulfate dextran) is able to generate a hydrophilic environment around the enzyme, producing partition of solvent or oxygen. In this point we will focus on the second advantage: it may be used to solve the problems generated in the crosslinked step of proteins having just some few Lys superficial residues, or it may just be used to have a more intensively crosslinked CLEA particle.

The strategy was first established using the enzyme glutaryl acylase from \textit{Pseudomonas sp.}, enzyme that as previously described in this review, is quite poor in superficial Lys residues.\textsuperscript{102} Glutaryl acylase may be precipitated using polyethylene glycol, but the further treatment of the aggregate particles with glutaraldehyde did not permit to
Co-precipitating the enzyme and PEI, the cross-linking between the very reactive and abundant primary amino groups of the PEI and the few primary amino groups on the enzyme surface is favored, and the aggregates remain insolubilized in the absence of any precipitant. The enzyme /PEI CLEA maintained more than 60% of its initial activity after 72 h of incubation at 45 °C, whereas the soluble enzyme was fully inactivated in only 2.5 h of incubation under the same conditions.

A second example was using lipases. Standard CLEAs preparation using commercial preparations of lipases from *Alcaligenes sp.* and *Candida antarctica* (fraction B) is not fully effective, some leakage of enzyme from the CLEA can be observed, and the SDS-PAGE from those preparations reveals that many enzyme molecules have not cross-linked properly. The co-precipitation of the lipases with poly-ethyleneimine or PEI-sulfate dextran mixtures permitted to get fully physically stable CLEAs, with higher stability in the presence of organic solvents. Very interestingly, the conditions of precipitation and the nature of the polymers permitted to significantly alter the lipases activity, enantio-selectivity and specificity.

Lipases were also the subject of other studies. The lipase from *Serratia marcescens* was co-aggregated with PEI. Optimum temperature was increased from 50 °C to 60 °C after immobilization, and its thermal stability was also significantly improved. This coCLEA showed excellent operational stability in its repeated use in aqueous-toluene biphasic system for asymmetric hydrolysis of trans-3-(4′-methoxyphenyl) glycidic acid methyl ester (MPGM), without significant inactivation after 10 rounds of repeated use.

Another lipase immobilized using coCLEAs with PEI was the enzyme from recombinant *Geotrichum* sp. These coCLEAs maintained more than 65% of relative hydrolysis degree after incubation in the range of 50-55 °C for 4 h and maintain more than 85% of relative hydrolysis degree after being treated by acetone, tert-butyl alcohol and octane.
for 4 h. They were applied to hydrolyze fish oil for enrichment of polyunsaturated fatty acids successfully and increased hydrolysis degree to 42% from 12% by free lipase. After five batch reactions, PEI-CLEAs still maintained 72% of relative hydrolysis degree.

Not only lipases have been immobilized following this coCLEA strategy. L-Aminoacylase from *Aspergillus melleus* was co-aggregated with polyethyleneimine and subsequently cross-linked with glutaraldehyde to obtain aminoacylase-polyethyleneimine cross-linked enzyme aggregates. This biocatalyst expressed 75% activity recovery and 81% aggregation yield, and improved enzyme stability. Its enantioselectivity was the highest for hydrolysis of amino acid amides; was moderate for hydrolysis of N-acetyl amino acids and was the worse for hydrolysis of amino acid esters. It retained more than 92% of the initial activity after five consecutive batches of (RS)-homophenylalanine hydrolysis suggesting an adequate operational stability of the biocatalyst.

**4.5. Tuning catalytic properties enzymes by coating their surfaces with poly-amine polymer coated**

Physical coating of enzymes with PEI has been used in some instances to improve enzyme properties, mainly lipases due to the facility to modulate their properties. The physical coating is far simpler than the chemical modification, and in some instances may become as effective (and even more in some instances).

In a first report, the properties of the most popular commercial biocatalyst lipase of lipase, Nozovym 435, were modulated by coating with different ionic polymers. PEI coated Novozym 435 improved its activity versus 3-phenylglutaric dimethyl diester by a 3-fold factor. Later, using a covalently immobilized preparation of the same lipase instead of the commercial one, it was shown that PEI modification on immobilized lipases greatly enhanced
the enantioselectivity of the immobilized enzyme in the kinetic resolution of (±)-2-hydroxyphenylacetic acid methyl ester. The enantiomeric ratio went from E=1.5 (without coating) to E>100 (ee>99%). Using a covalently immobilized lipase from *Candida rugosa*, the E went from 8 (without coating) to 20 after PEI coating. Moreover, this coating strategy improved the activity in some instances, the stability at high temperatures or in the presence of high co-solvent.

Immobilized Lecitase Ultra (a chimeric fosfolipase commercialized by Novozymes), has also been coated with different poly ionic polymers. The effect of the coating depended on the immobilization protocol, however, the PEI coating generally produced a significant increase in enzyme activity, in some cases even by more than a 30-fold factor (using the octyl-Lecitase at pH 5 in the hydrolysis of methyl phenyl acetate). The rate of irreversible inhibition of the covalent preparation using diethyl p-nitrophenylphosphate did not increase after PEI coating suggesting that the increase in Lecitase activity is not a consequence of the stabilization of the open form of Lecitase.

In a further development, PEI was not used to just coat the enzyme surface, but to freeze the open conformation of Lecitase induced by the presence of a detergent (SDS). Coating the immobilized enzyme with polyethyleneimine in aqueous buffer (PEI) produced a 3-fold increase in enzyme activity. However, in the presence of 0.1 % SDS (v/v), this coating produced a 50-fold increase in enzyme activity. Using irreversible inhibitors, it could be shown that the PEI/SDS-covalent immobilized -Lecitase preparation presented its catalytic Ser more exposed to the reaction medium than the unmodified CNBr-Lecitase, suggesting that the enzyme open form was somehow stabilized.

5. Genetic amination

5.1. Use of poly-Arg or poly-Lys tags
Protein fusion tags have been developed as indispensable tools for protein expression, purification, and the design of functionalized surfaces or artificially bifunctional proteins. A recent review has summarizes how positively or negatively charged polyionic fusion peptides with or without an additional cysteine can be used as protein tags for protein expression and purification, for matrix-assisted refolding of aggregated protein, and for coupling of proteins either to technologically relevant matrices or to other proteins.

Immobilized enzyme orientation may play a critical role on the features of the enzyme. By one side, this protein area will be the most involved one in the enzyme/support interaction, being the most improved/worsened by the immobilization. By other side, this may define the access of large substrates or ligands to this active center, or the communication between the active center of the enzyme and an electrode.

Site directed mutagenesis is the most efficient tools to achieve this site directed immobilization, via introduction of specific groups on desired areas of the protein. Usually, this orientation is achieved using a Cys inserted in the desired region, and immobilized on a support bearing a disulfide groups. Other popular strategy is the use of poly-His tags, or generation of His pairs, and immobilization on immobilized metal chelates matrices. Other tools have been also utilized to get this oriented fixation of enzymes on supports.

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 using cationic tags,

Most enzymes have an ionic surface nature that makes them unable to become adsorbed on cationic exchangers, and that 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.
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 cation exchangers. It has been shown that a poly-lysine tag facilitates protein purification and refolding processes.

For example, a poly-lysine (10 lysine residues) tagged cyclodextrin glycosyltransferase from *Bacillus macerans* and used to immobilize the enzyme on Sulphopropyl–Sepharose a cation-exchange resin. Enzyme activity was fully retained after immobilization. Though the poly-lysine-mediated immobilization is reversible, the binding force is strong enough to block protein leakage from the solid support at neutral and basic pH.

The authors pointed out that the method needs improvements since the enzyme was released at acidic pH values. Perhaps the use of polyanionic polymers could be a likely solution for this problem. This biocatalyst was used to produce α-cyclodextrin from soluble starch. Destabilization of CGTase by poly-lysine fusion and immobilization onto a cation exchanger was detected. However, α-cyclodextrin productivity of 539.4 g l-1 h-1 was obtained with 2% soluble starch and the operational half-life of the packed-bed enzyme reactor was estimated 12 days at 25°C and pH 6.0.

In an interesting paper, surface-modified iron oxide particles were used for the simultaneous purification and immobilization of *Bacillus stearothermophilus* aminopeptidase II (BsAPII) tagged C-terminally with either tri- or nona-lysines. The adsorption strength depended on the size of the tag. Three Lys permitted purification to near homogeneity by the carboxylated magnetic particles, but it was not easy to elute the adsorbed Lys9 protein from the matrix. Immobilization improved the stability of the enzyme. That way, the Lys 9 tag-aminopeptidase could be recycled ten times without a significant loss of enzyme activity.

In another example, carboxyethyl chitosan magnetic nanoparticles were used to purify small ubiquitin-like modifier, a protease derived from *Saccharomyces cerevisiae*. The
enzyme was fused with a poly lysine tag containing 10 lysine residues at its C-terminus.43 The lysine-tagged protease can be simply purified by magnetite nanoparticles from cell extracts with very high purity in just one-step. A poly-Arg tag has been used in other cases. For example, D-xylose isomerase from *Escherichia coli* was fused with a 10-arginine tag at its C-terminus.243 The fusion protein XIR10 was purified to a high purity and immobilized by a single step of cation exchange chromatography. The immobilization to the cation exchanger has a small effect on the enzymatic function.

In another research, a minichaperone polypeptide was fused with a poly-arginine (10 residues).244 This chimeric peptide was purified through a single step of cation exchange chromatography with high purity. The purified chaperone was efficiently immobilized on the cation exchanger and applied to the refolding of *Bacillus macerans* cyclodextrin glycosyltransferase, which was expressed as inclusion body in recombinant E. coli.

5.1.2. Improving covalent immobilization via poly cationic tags

The addition of a Poly-Lys tag 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 adsorbed protein.50 Using this strategy, it was found that the covalent immobilization of a protein onto the maleic anhydride-alt-methyl vinyl ether copolymers, via the formation of amide bonds, occurred in moderate yields under aqueous conditions. This was exemplified using two genetically modified HIV-1 capsid p24 proteins, RH24 and RH24K.245 The addition of a six lysine unit tag at the COOH-terminus of RH24K greatly improved the grafting reaction which could take place under many different experimental conditions. The course of the reaction was controlled by electrostatic attractive forces between the protein and the
negatively charged polymer, as the chemical binding was more efficient at low ionic strength. This explanation was later confirmed using a peptide. The grafting reaction was improved by adding a sequence of three positively charged amino acids (lysine or arginine) at the amino terminus of the peptide. The arginine tag was more efficient than the lysine tag for enhancing the immobilization reaction, proving that the effect was due to an electrostatic driving force.

5.1.3. Using of poly-Lys to direct the covalent immobilization of proteins to modulate its catalytic behavior

It has been shown on some papers and recent reviews how the control of the area of the protein involved in the reaction with the support may produce different changes (or prevent some changes that should occur), being this tool a very powerful strategy for improving enzyme performance in different reactions whose yield depend on the catalytic performance of the catalyst.

The control of the immobilization of penicillin G acylase using a poly Lys tag is the only example that we have been able to find regarding the use of poly Lys tag to reach this goal. This biocatalyst was used for the kinetically controlled synthesis of different beta-lactamic antibodies. In this reaction strategy, the use of an activated acyl donor permits to reach transient maximum yields, and this yields are determined by the rate of synthesis and rates of activated acyl donor and product hydrolysis.

To achieve this, a tag of three lysines alternating with three glycines was added to the C-terminal end of the β chain of penicillin G acylase. This enzyme was then immobilized to glyoxyl agarose. As glyoxyl agarose only immobilized enzymes via several points, this new very rich area in Lys drives the immobilization by this area, even though the increment on total amino groups was under 10%, even permitting to immobilize the enzyme at
pH 9.59 The immobilization of this enzyme the catalytic properties of the immobilized
derivative on kinetically synthesis of cefamandole and cefonicid.248

5.1.4. Other uses of chimeric enzymes/poly-Lys tags

Poly-Lysine tags may have some other applications. For example, this strategy was
used to the efficient production of the intact glucagon-like peptide-1 using a recombinant E.
coli system, avoiding degradation.249 The peptide was fused to a 6-lysine tag, ubiquitin and the
peptide in a row. Solid-phase refolding of chimeric protein inclusion body using a cation
exchanger led to a refolding yield over 90%. Finally, the cleavage of the refolded protein with
ubiquitin-specific protease 1 gave an authentic form of the desired peptide.

In other cases, poly-caitonic tags have been used to improve the expression of a
hyper-expressd enzyme. They can favor the solubility of these hyper-expressed proteins. For
example, Lipase B from Candida antarctica was fused with various polycationic amino acid
tags and expressed in E. coli in order to increase a soluble expression level.250 The 10-arginine
and 10-lysine tags fused at the C-terminal of CalB significantly increased the solubility of the
lipase by five- to ninefold, relative to the case of the native enzyme expressed in a
recombinant E. coli.

5.2. Modification of the protein surface

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.

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.\textsuperscript{55, 56} For reversible immobilization, most of the supports follow this multipoint interaction to fix the enzyme to the support.\textsuperscript{31, 134}

### 5.2.1. Improvement of the multipoint covalent attachment

The strategy of improving the multipoint covalent attachment on glyoxyl supports have been developed using Penicillin G acylase from \textit{E. coli} as a model enzyme. The researchers chose a region of the enzyme that was already very rich in Lys resides and introduced there additional Lys residues.\textsuperscript{58} The immobilization rate was increased by more than a 10 fold factor when compared to the wild enzyme, even though the number of overall external Lys was increased by less than 10%. This confirmed that the immobilization was mainly performed via the region where the new Lys residues had been introduced. The immobilized mutant enzyme showed improved stability on thermal or cosolvent induced inactivations with stabilization factors ranging from 4 to 11 compared to that of the native enzyme immobilized on glyoxyl-agarose following the same protocol.\textsuperscript{58} Considering the stabilization obtained by the immobilization of the wild type enzyme (near to 10,000),\textsuperscript{251} the final stabilization factors achieved with this strategy were impressive.

In another research, the enzyme (horseradish peroxidase) and the support (a modified polyethersulfone matrix presenting aldehyde residues) were changed.\textsuperscript{252} The researchers replaced arginine residues on the face of glycan-free recombinant horseradish peroxidase opposite to the active site by lysines. These conservative Arg-to-Lys substitutions provide a means of multipoint covalent immobilization such that the active site will always face away from the immobilization matrix. One triple and one pentuple mutant were generated by substitution of solvent-exposed arginines on the "back" of the polypeptide (R118, R159...
and R283) and of residues known to influence stability (K232 and K241). Oriented a modified polyethersulfone matrix presenting aldehyde residues immobilization was demonstrated using the modified polyethersulfone membrane; the protein was forced to orientate its active site away from the membrane and towards the bulk solution phase. The reversion of K283R mutation permitted to improve enzyme stability, the quadruple mutant regained some stability over its mutant counterparts. A moderate improvement on the immobilization rate of the mutant enzymes on CNBr-activated Sepharose™ was noted with increased lysine content. This support was able to fix the enzyme via just one point, usually involving the most reactive group on the protein. However, only marginal gains in solvent stability resulted from immobilization on this latter matrix. The authors conclude that a directional and oriented immobilization of horseradish peroxidase mutants onto polyethersulfone membrane has been achieved with excellent retention of catalytic activity.

A more directed strategy was later proposed. First, one Cys residue was introduced on different regions of the enzyme penicillin G acylase, to find the area that was more determinant for enzyme stability. The immobilization was performed on an epoxy support, because Cys was by far the most reactive amino group on a protein and that was enough to direct the enzyme. The mutant enzyme where the Cys was in the position β380 was the one that gave the highest PGA stabilization values. In a second round of site-directed mutagenesis, that region was further enriched in 4 additional lysine residues, and the resulting immobilized derivative was 1500-fold more stable than the same protein variant uni-punctually immobilized through position β380.

It is expected that in the near future, this strategy may be extended to more enzymes.

5.2.2. Site directed immobilization: controlling enzyme catalytic features
In other cases, the objective was more to have a fully oriented immobilized enzyme than to improve the multipoint covalent attachment or the enzyme stability. This was the case of the immobilization of mutant penicillin G acylase enzymes enriched in Lys areas in the area opposite to the active center.\textsuperscript{254} The objective was to improve the behavior of the enzyme in kinetically controlled synthesis of semi-synthetic β-lactam antibiotics. Native enzyme immobilized mainly near to the active center, and that seemed to generate some steric hindrances to the entry of the nucleophile producing a severe worsening in its properties. Different mutants with an increasing number of Lys were designed and immobilized onto glyoxyl agarose. These immobilized Lys enriched mutants have similar performances to the free enzyme. Later, they show this differential immobilization of the enzyme using tryptic digestion of the immobilized enzymes followed by liquid chromatography-tandem mass spectrometry.\textsuperscript{255}

5.2.3. Improvement of immobilization in anionic exchangers

While using chemical amination there is at least one example of the use of amination to improve the immobilization on cation exchangers (see section 3.3.3), we have not been able to find a similar example using genetic amination. In fact, and this may serve as a proof of concept, there is one example where genetic increment on carboxylic groups of the surface of penicillin G acylase improves its immobilization on anion exchangers.\textsuperscript{63}

5.2.4. Improvement of intermolecular crosslinking

We have not been able to find any papers concerning the use of enzymes with enriched areas in Lys residues and the stabilization of this enzyme by using intermolecular crosslinkers. However, in a similar way as when using chemical amination (see section 3.5 of this review), this should permit to greatly improve the enzyme crosslinking by increasing the prospects of having two residues of the protein at the right distance.\textsuperscript{64} In fact, this can be even
more favorable than chemical amination, where it is only possible to get a general enrichment on the enzyme surface of amino groups, using the carboxylic groups of the enzyme. Now, using site-directed mutagenesis and if the enzyme has a well described structure, it is possible to place the new Lys residues on the right position to permit the enzyme crosslinking, a critical point to get an intramolecular crosslinking.

5.2.5. Improvement of coating with anionic polymers

Again, we have not found examples where the enrichment in Lys residues of the protein is used to facilitate the adsorption of cationic polymers on their surface. Using the enzyme penicillin G acylase, there is, however, an example of enrichment on carboxy groups of the enzyme surface to improve the adsorption of cationic polymers on the enzyme surface, and in section 3.6 the chemical amination to this goal is presented. Perhaps, although this coating may have very good effects on enzyme performance (see section 4 of this review), it is considered too sophisticated to improve the interaction via site-directed mutagenesis.

5.2.6. Other uses

As discussed in section 3, cationized enzymes have a higher potential to penetrate cell membranes and system barriers. Together with the previously presented chemical modifications, this increase in surface cations may be also achieved via site-directed modification. For example, Ribonuclease Sa (pI = 3.5) from *Streptomyces aureofaciens* and its 3K (D1K, D17K, E41K) (pI = 6.4) and 5K (3K + D25K, E74K) (pI = 10.2) mutants were tested for cytotoxicity. The 5K mutant was cytotoxic to normal and v-ras-transformed NIH3T3 mouse fibroblasts, while RNase Sa and 3K were not. The cytotoxic 5K mutant
preferentially attacks v-ras-NIH3T3 fibroblasts, suggesting that mammalian cells expressing
the ras-oncogene are potential targets for ribonuclease-based drugs.

Conclusion and future trends

This review has shown the high interest that the amination of enzymes and proteins has
with views towards improving their behavior \textit{in vitro} as industrial biocatalysts, but also \textit{in vivo}
when using proteins as carriers or as medicaments.

Amination has proved to be very useful to improve enzyme immobilization via
multipoint covalent attachment or cation exchange, to improve intramolecular crosslinking, to
improve enzyme stability, or to improve intermolecular crosslinking which is a critical step in
the preparation of CLEAs. The amination also increases the immunogenicity and potential to
penetrate cell walls, enabling the use of some enzymes as biocides, improving the production
of antibodies, or just permitting to study the role of certain proteins \textit{in vivo} after introduction
in the cell.

In some cases, amination may produce drastic changes in enzyme stability, activity or
selectivity/specifictiy. Considering the change of ionic interactions on the enzyme surface, a
negative effect should be expected. However in many instances the effect is positive..

Most examples cited in this review use chemical or physical amination. This may be
derived from the rapid preparation of the modified enzymes using these techniques, and the
relatively simple preparation of a collection of enzymes having different modification degrees,
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 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 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 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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