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Review

Control of protein immobilization: Coupling immobilization and site-directed mutagenesis to improve biocatalyst or biosensor performance

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ABSTRACT

Mutagenesis and immobilization are usually considered to be unrelated techniques with potential applications to improve protein properties. However, there are several reports showing that the use of site-directed mutagenesis to improve enzyme properties directly, but also how enzymes are immobilized on a support, can be a powerful tool to improve the properties of immobilized biomolecules for use as biosensors or biocatalysts. Standard immobilizations are not fully random processes, but the protein orientation may be difficult to alter. Initially, most efforts using this idea were addressed towards controlling the orientation of the enzyme on the immobilization support, in many cases to facilitate electron transfer from the support to the enzyme in redox biosensors. Usually, Cys residues are used to directly immobilize the protein on a support that contains disulfide groups or that is made from gold. There are also some examples using His in the target areas of the protein and using supports modified with immobilized metal chelates and other tags (e.g., using immobilized antibodies). Furthermore, site-directed mutagenesis to control immobilization is useful for improving the activity, the stability and even the selectivity of the immobilized protein, for example, via site-directed rigidification of selected areas of the protein. Initially, only Cys and disulfide supports were employed, but other supports with higher potential to give multipoint covalent attachment are being employed (e.g., glyoxyl or epoxy-disulfide supports). The advances in support design and the deeper knowledge of the mechanisms of enzyme–support interactions have permitted exploration of the possibilities of the coupled use of site-directed mutagenesis and immobilization in a new way. This paper intends to review some of the advances and possibilities that these coupled strategies permit.

Contents

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1. Introduction

Enzymes are biological catalysts with very good prospects for application in chemical industries due to their high activity under mild conditions, high selectivity and specificity [1–4]. However, enzymes do not fulfill all of the requirements of an industrial biocatalyst or biosensor [5,6]. They have been selected throughout natural evolution to perform their physiological functions under stress conditions and quite strict regulation. However, in industry, these biocatalysts should be heterogeneous, reasonably stable under conditions that may be quite far from their physiological environment [7–9] and retain their good activity and selectivity when acting with substrates that are in some instances quite different from their physiological ones.

Currently, substantial developments in many different scientific areas have been able to shortcut these enzyme "deficiencies" as industrial biocatalysts. These advances range from microbiology and molecular biology (e.g., using enzymes from thermophilic environments or improving enzyme properties via directed evolution) [10–14] to biochemical engineering (new bioreactors [15–18] or immobilization supports and protocols [19–23]). These developments have permitted an increase in the number of enzymes and proteins used as biosensors or biocatalysts at the industrial level in recent years.

Among these techniques, the immobilization of enzymes or proteins is almost compulsory for most of their practical applications. The immobilization permits heterogeneous adsorbents (e.g., antibodies) or biocatalysts (e.g., enzymes), as well as the ability to locate the enzyme in the defined area of the sensor where the transductor is placed [24]. Therefore, the enzyme can be reused, provided it is stable enough. When properly designed, the immobilization of enzymes and proteins has also been a very powerful tool to improve enzyme or protein stability, and in certain cases even their activity or selectivity [25–28]. On the other hand, site-directed mutagenesis has been a very useful instrument in improving enzyme features from activity to stability [27,29,30].

Site-directed mutagenesis and immobilization have been traditionally considered to be unrelated tools, even though the mutant enzyme (an improved enzyme) has in many cases been further immobilized for use in an industrial bioreactor [31–34].

This review will focus on the use of site-directed mutagenesis not to directly improve protein features but to obtain an immobilized protein with improved properties compared to the immobilized native enzyme.

The protein or enzyme orientation on the support surface may be critical if the enzyme active center needs to be in a proper connection with the support, which is the case for redox enzymes that should receive or transfer electrons from or to the support [35–40]. Another issue that makes protein orientation critical is that when the substrate is very large, the support surface may generate steric hindrance in its recognition by the immobilized protein [40–42]. Thus, in many examples, the coupled use of site-directed mutagenesis and immobilization was addressed to control the orientation of the protein on the support surface [43–45].

On the other hand, the immobilization of an enzyme involving different areas of the enzyme may affect all of its properties (from activity to selectivity or stability) [26]; there are some examples where site-directed mutagenesis has been used to improve some specific catalytic properties of the immobilized enzyme. In some cases, just by orienting the enzyme in a particular way or by improving the intensity of the enzyme–support reaction, the protein stability can be improved [26].

2. Conventional immobilization of enzymes and proteins

Immobilization of enzymes and proteins using standard protocols is usually called "random immobilization." However, in most cases, the controlled immobilization of a protein on a support is not a completely random phenomenon but results in a determined protein orientation.

When an enzyme is immobilized using a particular immobilization protocol, the enzyme can interact with some antibodies and not with others, suggesting that all of the immobilized protein molecules have a specific inaccessible surface area [46]. The use of different immobilization protocols changes the protected area of the same protein [46]. Moreover, if conventional immobilization produced a randomly immobilized enzyme, then the dramatic changes in enzyme properties caused by the use of different immobilization protocols, even inversion of enzyme specificity [26,47–54], would be hard to explain. A recent paper [55] shows that the tryptic hydrolysis of penicillin G acylase attached to Eupergit (via a first hydrophobic adsorption) [56,57] or glyoxyl-agarose (via the area richest in Lys residues) [58,59] identifies some Lys residues that are involved in the immobilization while others remained fully unmodified, showing differences in the protein orientation in the two preparations [55].

Thus, in many cases, a particular controlled immobilization protocol yields an immobilized enzyme preparation in which most of the immobilized molecules have a very similar orientation. In fact, truly random immobilization of a protein on a support may be quite difficult to achieve, and some authors propose that when this is required, the best option may be to use different immobilization protocols and use all these immobilized preparations in a coupled way [46].

An explanation for this oriented immobilization may be found in the mechanism of immobilization that follows using different supports.

First, supports able to covalently immobilize proteins at just one point (e.g., a primary amino group) will be analyzed. Many authors consider that the first immobilization of the protein could be produced mainly via any ε-amino group of the most abundant primary amino groups in the protein, which are the Lys amino acid, leading to a randomly immobilized enzyme preparation. However, it should be considered that only non-ionized amino groups will be good nucleophiles and will take part in the reaction. At pH 7, the reactivity of an external Lys (with a pK_a over 10.5) will be significantly reduced; less than $10^{-3.5}$ of the groups will be reactive. However, the terminal amino group of the protein will present a pK value between 7 and 8. That means then even in the least positive case, the reactivity of this group will be over $10^{2.5}$ higher than that of a Lys and much higher than the overall Lys groups present in a standard protein surface. Thus, when using supports activated with tosyl chloride [60,61], cyanogen bromide [62,63] or glutaraldehyde (at high ionic strength or using lowly activated supports) [64] at a neutral pH value, the proteins will be first immobilized via this terminal amino group (if it is exposed to the surface) (Fig. 1). After the first immobilization, it may be possible that the ε-amino group of the Lys groups, in a "pseudo-intermolecular" way, can react with the support groups to give a certain multipoint covalent attachment. Of course, in some cases, a protein may have several terminal amino groups that might have similar pK values and exposure to the medium, being located in different areas of the protein, which would produce a mixture of protein orientations. In other cases, this group may not be exposed to the medium, leaving the Lys residues as the only reactive groups. However, these cases should be considered to be an exception rather than a rule.

Another situation arises when the enzyme needs to produce several simultaneous "weak" interactions with the support to

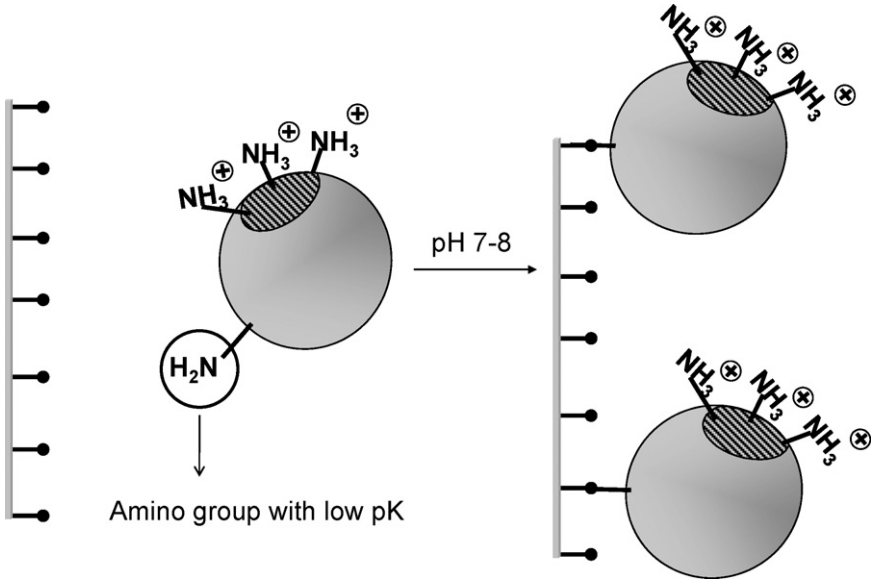

Fig. 1. One-point immobilization by the most reactive amino group of the protein.

become immobilized (Fig. 2). This situation occurs in most physical adsorptions of proteins in supports, such as ionic exchange, hydrophobic adsorptions or interactions with immobilized metal chelates (an exception is when the enzyme has an engineered His-tag or two wild-type vicinal His residues) [65–68]. In covalent attachment, proteins become immobilized on glyoxyl supports in this way in the absence of reagents able to stabilizing imino bond formed between the enzyme and the support [58,69]. In all of these cases, the immobilization rate depends exponentially on the surface density of groups on the support and, obviously, on the protein surface [58]. Moreover, the enzyme–support multi-interaction may be facilitated if the distance between several reactive groups of the protein corresponds to the distance between the reactive groups on the support. Thus, proteins will become mainly immobilized by the area where the highest possibility of giving this simultaneous multipoint interaction between the enzyme and support exists, and immobilization will also be quite far from being random, resulting in a main orientation of the protein molecules. The orientation of the protein on the support surface can only be changed by altering the event that produces the first immobilization [70].

Another aspect of immobilization of a protein on a support is the possibility of establishing multiple enzyme–support interactions. If these interactions are controlled and occur during the immobilization step, the effects should be mainly beneficial for the final properties of the biocatalysts [26,71–73]. For example, using a covalent immobilization, the effects of this controlled multipoint covalent attachment are a higher stability with a moderate (or even nonexistent) decrease in enzyme activity [26]. This is the case for the immobilization of enzymes on glyoxyl agarose. After reduction, the support is fully inert and hydrophilic. However, reactive glyoxyl supports are able to produce an intense multipoint covalent attachment [59]. Using some epoxy supports, the remaining

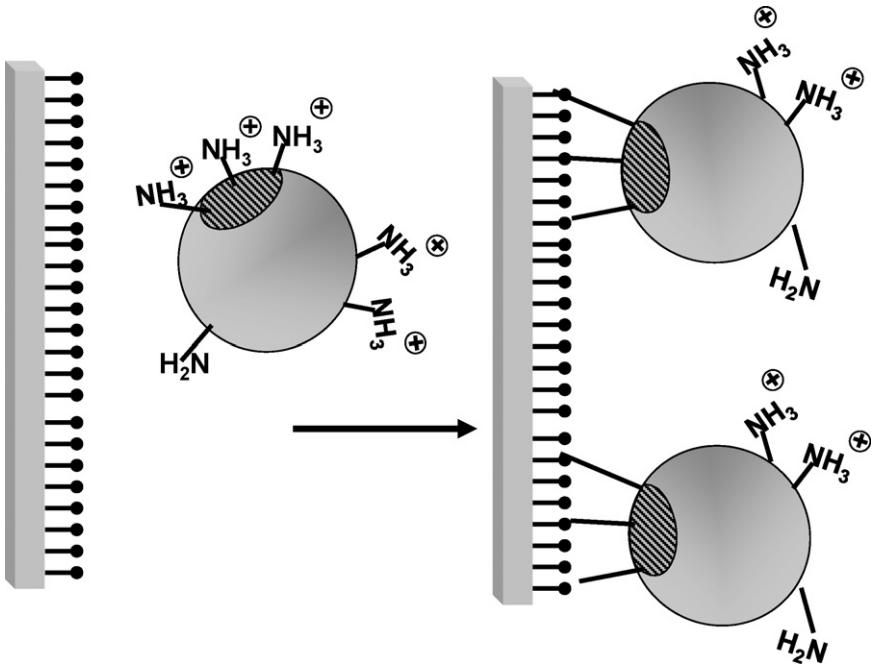

Fig. 2. Multipoint immobilization by the area with the highest density of reactive groups.

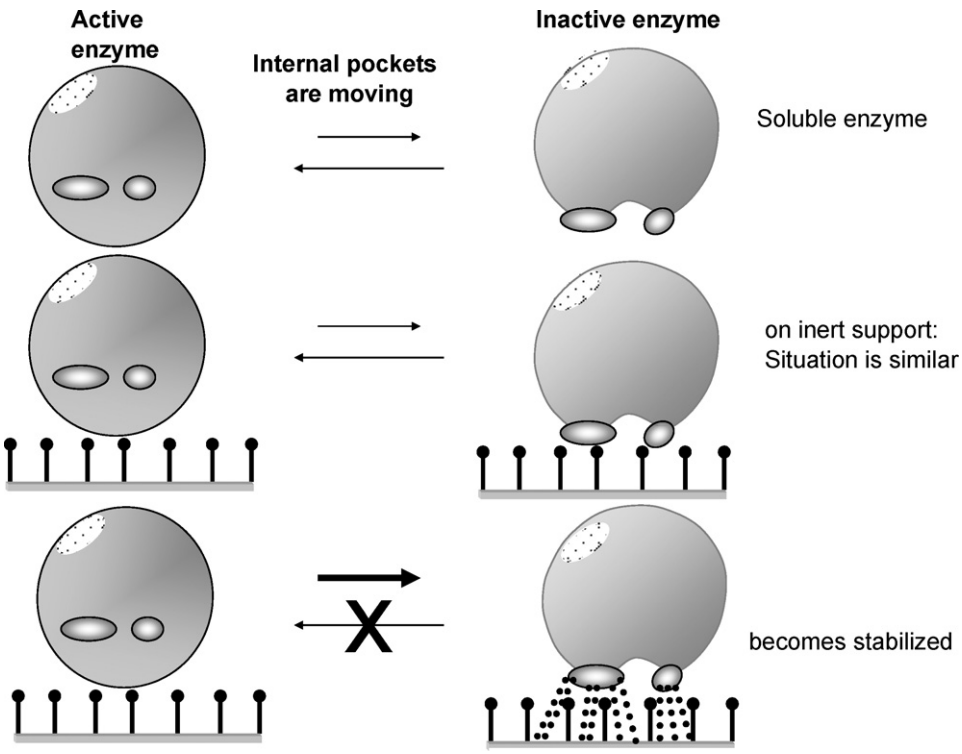

Fig. 3. Inert versus non-inert supports.

epoxy groups can be blocked with hydrophilic compounds to avoid covalent reactivity and to enhance support inertness [74]. Both supports have been successfully used to prepare biocatalysts with high stabilization values and high activity recoveries [59,75].

An entirely different matter is when the support cannot be transformed into an inert one after enzyme immobilization and is able to establish uncontrolled interactions with the immobilized protein during storage or application. These uncontrolled enzyme–support interactions are undesired and in many instances may lead to enzyme inactivation (Fig. 3). These uncontrolled interactions are not avoided by using site-directed immobilization of the protein. Even if the reactivity of the groups in the support is not enough to significantly immobilize the protein on the support, after immobilization, the surface of the support will be very near to the surface of the enzyme [76]. That way, the huge increase of the apparent concentration of the groups in the protein and the poorly reactive groups in the support facilitates the establishment of these unwanted interactions. Therefore, the design of the support should always consider that having a final surface as inert as possible is of interest, even if a first site-directed immobilization is produced.

In some cases, immobilization of enzymes or proteins in some determined protein orientations may produce severe activity drops because immobilization may distort critical areas of the protein or block the active center of the enzyme, especially when using large substrates. For example, β-galactosidase from *Aspergillus oryzae* remained fully active when immobilized on amino-epoxy supports but lost all of its activity when immobilized on hydrophobic-epoxy supports [77]. The case of the function of immobilized antibodies or protein A is another example where immobilization may greatly alter the final properties of the immobilized biomacromolecule [78–83].

However, protein immobilization via areas near the active site may be beneficial in some cases. The interfacial activation of lipases *versus* hydrophobic supports produces hyper-activation of the enzyme using hydrophobic substrates [26,48,84].

Thus, a controlled immobilization of a protein on a support is not site-directed control but yields a quite homogenous population of immobilized protein molecules that may serve many applications. However, this orientation may be hard to predict and even harder to alter using a determined immobilization protocol, if it is not the desired orientation. For this reason, site-directed and completely controlled immobilization should be very advantageous in many instances because it ensures obtaining the desired orientation of the enzyme on the support.

3. Controlling the immobilized enzyme orientation using site-directed mutagenesis to maintain protein function

As previously stated, the enzyme orientation may be a key point in certain cases. In some instances, the control of the orientation may be performed by controlling the support activation. For example, the use of a lowly activated amino support may direct the immobilization of multimeric enzymes to the area that involves more enzyme subunits [85–86]. The control of the immobilization conditions, for instance, by employing high ionic strength using ionic exchange matrices, may also give a specific protein orientation [87,88]. The most obvious way of getting a site-directed immobilization is to select a specific group on the protein, such as the sugar chains [42]. However, this method may give a specific orientation that in certain cases may not be the desired one, or make the use of a determined support and immobilization protocol compulsory, which may not be adequate for the specific enzyme or application under study.

Therefore, site-directed mutagenesis appears to be an elegant and versatile way of controlling protein orientation, permitting the immobilization of a protein via different orientations on the same support and conditions as well as determining the first area that interacts with the support. Compared to the “controlled” immobilization in the previous point, some additional requisites are necessary: the structure of the protein (or some analogous one) needs to be resolved, and the enzyme needs to be cloned

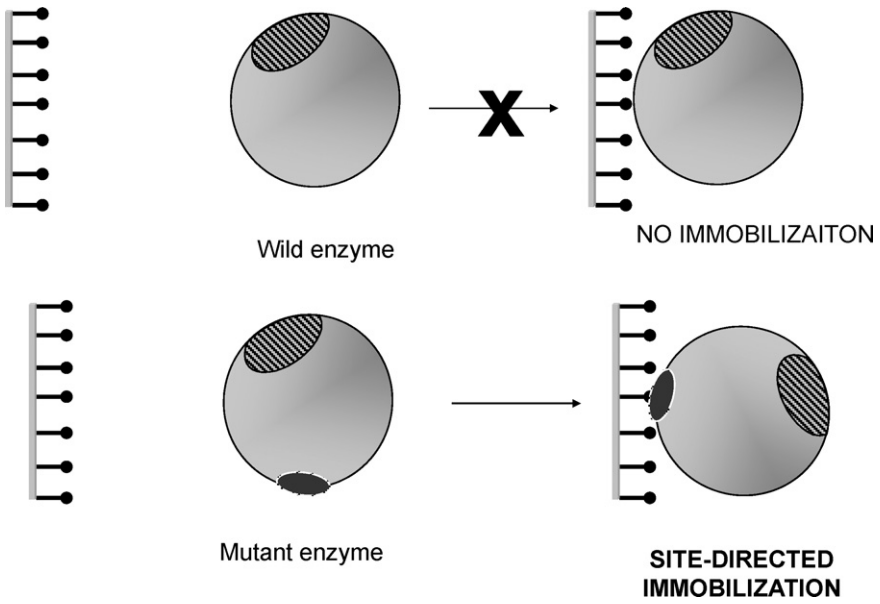

Fig. 4. Site-directed mutagenesis for a site-directed immobilization.

in a microorganism where genetic manipulation tools are available. Although the use of genetic tools requires some additional expenses in biocatalyst development, these tools are already used to improve enzyme properties, in some cases at the industrial level. Once the optimal protein is designed, the production cost will be similar to the native one. If stability, activity or selectivity of the enzyme is increased in a significant enough way, the economical balance of the process should be positive, although a specific economic study may be necessary in each case.

To this goal, the research needs to introduce changes in the sequence of the gene of the target protein that, after its expression, permits the immobilization of the protein using an immobilization protocol that cannot immobilize the native enzyme (Fig. 4).

Butterfield et al. revised these developments to get a site-directed immobilization of proteins and enzymes to form biofunctional membranes to be used in different areas [89]. In addition, Campàs et al. have recently published a review on the use of genetically engineered enzymes in electrochemical biosensors to improve the sensor sensitivity [45]. The authors state that immobilization of biomolecules on electrodes is a key issue in the development of electrochemical biosensors. Immobilized proteins should retain their function and keep their active sites accessible. Moreover, a close proximity between the bio-recognition molecule and the transducer is also desired to obtain an efficient electron transfer. Thus, together with the improvements in enzyme activity and specificity using mutagenesis, enzyme orientation control on the support surface is stated in these reviews as one of the main targets to create an improved sensor by improving the electron transference or by improving the substrate accessibility [45].

This joint use of both techniques has been carried out using different strategies that may permit achieving site-directed immobilization in different situations [45,89].

3.1. Use of tagged proteins and antibodies

One of these strategies is somewhat complex, but quite elegant. It involves the production of antibodies versus a specific tag, the immobilization of the antibody and the gene fusion of the target protein to incorporate the peptidic tag at the N- or C-terminus of the enzyme. The tagged enzymes are then attached using the affinity tag to the immobilized anti-tag antibodies (Fig. 5). For example, this strategy was employed for the site-specific immobilization of an alkaline phosphatase [90]. First, a fusion protein of alkaline phosphatase and a polypeptide tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) was produced. The fusion protein was incubated in the presence of a monoclonal antibody directed against the tag. Finally, a membrane containing immobilized protein A was used to adsorb the protein–antibody complex. This process resulted in the site-specific immobilization of the enzyme, which permitted the recovery of over 85% of the enzyme activity [90]. This gene fusion/affinity immobilization approach is appropriate when either the N- or C-terminus is exposed and permits the orientation that the researcher requires. However, it is only valid for orientations via these two positions.

3.2. Use of enzymes to immobilize proteins via specific tags

Another similar strategy proposes the use of a poly-peptide tag with transglutaminase-reactive Lys, and the use of transglutaminase to achieve site-specific immobilization of the enzyme via this tag (Fig. 6). The use of transglutaminase to immobilize and cross-link proteins is a method of immobilization used in some examples [91–93].

Using alkaline phosphatase from *E. coli*, two designed specific peptide tags containing a reactive lysine residue with Gly-Ser linkers of different lengths for microbial transglutaminase were genetically attached to the N- or C-terminus of the target enzyme [94]. β-Casein was immobilized on agarose beads to obtain reactive Gln residues on the support surface. Immobilization was found to depend on both the position and the length of the peptide tags incorporated into the enzyme, suggesting steric constraint upon enzymatic immobilization. This immobilized alkaline phosphatase showed comparable activity with the soluble counterpart and comparable operational stability with the chemically immobilized enzyme [94].

A similar method was used for the site-specific immobilization of this enzyme on a plastic surface. The researchers used alkaline phosphatase from *E. coli* with a specific peptide tag (MKHKGS) genetically incorporated at the N-terminus, microbial transglutaminase and a 96-well polystyrene microtiter plate coated with casein to produce site-directed immobilization [93]. There are more examples of the use of this technique to achieve the site-directed

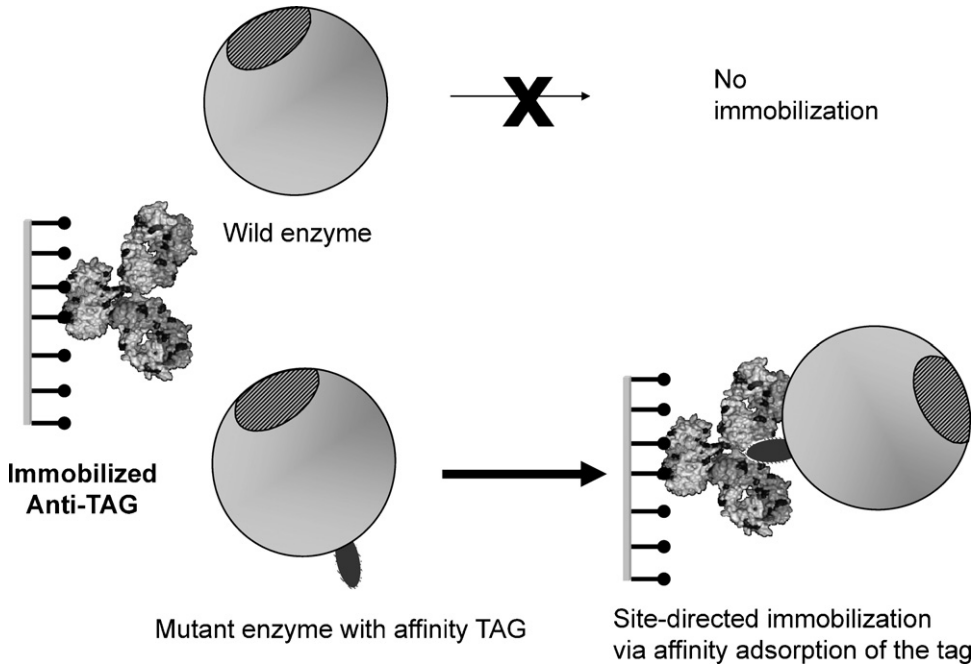

Fig. 5. Use of chimerical proteins with inserted tags and immobilized anti-tags to achieve site-directed immobilization of the target protein.

immobilization of different enzymes [95,96]. It is a very mild way to site-directly immobilize an enzyme, but hardly any significant stabilization may be expected after enzyme immobilization.

3.3. Use of biotin/avidin site-modified proteins

Another elegant but complex strategy is the use of molecular biology to add a single biotin addition in a site-specific manner to the structure of the target protein [97]. This biotinylated protein may be attached to a support containing avidin through the strong interaction between both molecules. This method allows the use of an avidin spacer between the enzyme and the immobilization surface, decreasing steric hindrance, but also reducing the possibility of a controlled multipoint covalent attachment between immobilized enzyme and support; therefore, the stabilization resulting from the immobilization process should be low. The

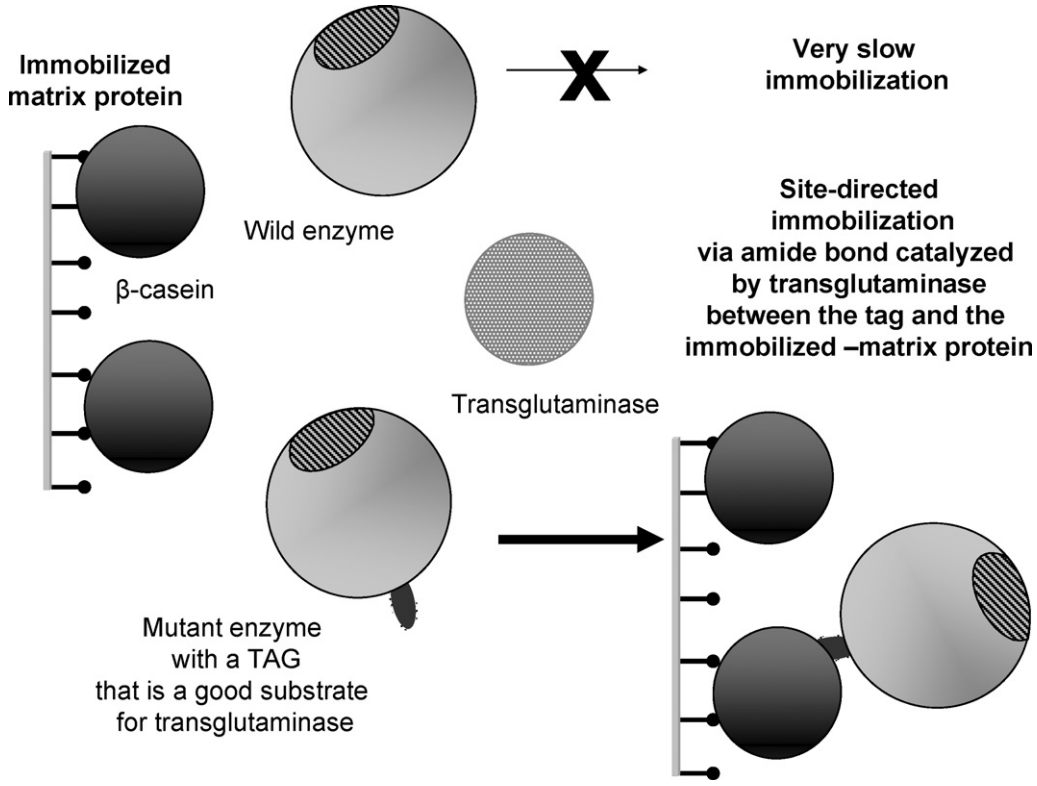

Fig. 6. Use of transglutaminase and chimerical proteins having tags with selected sequences to get site-directed immobilization of the target protein.

tag will again be in two possible positions on the protein (C or N terminal); therefore, this method is again only valid for certain applications. A nice example of this strategy was the immobilization of a β-galactosidase (β-Gal). The enzyme was biotinylated at a single site following expression of a plasmid in *E. coli* that encodes a fusion protein of β-Gal and a polypeptide tag at its N-terminus. When this fusion product is expressed in *E. coli*, one lysine residue on the polypeptide tag is specifically biotinylated by biotin ligase (a bacteria-resident enzyme) during the post-translational modification process. The incubation of this biotinylated enzyme in the presence of poly(ether sulfone) membranes onto which avidin was already immobilized permitted the site-directed immobilization of the β-Gal. A two-fold improvement in immobilized enzyme activity was observed relative to that of the fully biotinylated enzyme (that was immobilized in a random way) [97].

In other examples, protein–streptavidin fusion proteins were prepared and immobilized on a biotinylated matrix [98]. For example, extracellular production of a keratinase–streptavidin fusion protein was accomplished by cloning in *Bacillus subtilis* a transforming plasmid carrying the keratinase–streptavidin fusion gene. The fusion protein was immobilized onto a biotinylated solid matrix by mixing in the culture medium. The heat stability and pH tolerance of the enzyme were greatly improved by its immobilization, but its catalytic efficiency (k_{cat}/K_m) was reduced eight-fold, perhaps due to diffusion problems [98].

3.4. Use of site-directed exchanged His residues or His tags

The use of His to immobilize proteins on immobilized metal chelate (IMAC) or gold supports has also been used in many instances. In these cases, the support is activated with immobilized transition metal chelates (IMAC) (Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+} or Fe^{3+}) [99]. This immobilization method is based on the reversible interaction between the transition metal ions and electron donating groups, mainly His residues (although Cys and Trp may also participate) [100,101]. Wild-type proteins are immobilized on IMAC supports only via the interaction between several chelate groups in the support and several groups dispersed on the protein surface. However, poly-His tagged proteins may be fixed to the support by the interaction of two His groups of the tag with a single group in the support [66]. This adsorption via just one chelate also occurs when using wild-type (or mutated) proteins having two proximal His residues. The immobilization is reversible upon addition of a competitive ligand (e.g., imidazole or His) or removal of the metal ion via EDTA complexation [68,100,101], a reversibility that has been exploited both in enzyme purification and in the reuse of supports of expensive biosensors or biocatalysts after enzyme inactivation. This methodology is more versatile than the previous ones because the introduction of His pairs in the middle of the protein structure does not limit the likely orientations of the protein to those achieved when immobilizing it by the terminal amino acids, although the introduction of two His groups may have some impact on the protein properties. However, in some cases, one His may be presented on the desired area of the wild-type protein, so that only one additional His needs to be added.

For example, a biosensor for organophosphorus insecticides has been developed, based on the immobilization of a hexa-His-tagged acetylcholinesterase on a nitrilotriacetic acid metal ion electrode [102]. The hexa-His tail was incorporated at the position where the wild-type enzyme presents a glycolipid anchor that *in vivo* links the enzyme to the cytoplasmic membrane to preserve the enzyme conformation and activity. The biosensor showed a sensitivity, linear range and response time not significantly different from those based on trapping into polymers, but showed a lower detection limit, probably due to the absence of diffusion barriers. Although the metal affinity-based biosensor presented shorter storage stability, it could be restored by desorption of inactivated enzyme and immobilization of fresh enzyme solution on the electrode. The sensor was tested for the detection of paraoxon, dichlorvos and chlorpyrifos ethyl oxon, with the detection levels being 4.1, 0.5 and 0.1 ppb, respectively [103].

In another example, the hexa-His-tagged acetylcholine esterase was conjugated to Ni^{2+}-iminodiacetic acid modified magnetic beads for their subsequent immobilization on magnetized supports [104]. The use of magnetic beads enabled a decrease in the inhibitor incubation time and provided a 10-fold lower limit of detection compared to immobilization of the enzyme by entrapment.

Horseradish peroxidase is another enzyme frequently used in biosensors. The direct electrochemical reduction of wild-type horseradish peroxidase adsorbed on electrodes is not very efficient. This finding may be due to the long distance between the active site of the glycosylated native enzyme and/or an unfavorable orientation of the enzyme [105,106]. The use of a non-glycosylated recombinant horseradish peroxidase containing a hexa-His tag accelerated the electron transfer kinetics, improving the sensitivity of the gold electrodes towards hydrogen peroxide, and provided more stable biosensors due to the higher binding strength of the enzyme with gold [107,108]. On the one hand, the deglycosylation of horseradish peroxidase decreases the electron transfer distance between the active site and the electrode. On the other hand, the immobilization via the hexa-His tag may be orientating the enzyme in an optimal configuration for the electron transfer.

The importance of the orientation of the enzyme in the surface-enzyme electron transfer was also demonstrated using ferredoxin:nicotinamide adenine dinucleotide phosphate reductase [109]. The researchers introduced a His pair at two different positions of the protein surface. The two electrode-bound mutant enzymes manifested differences in the amount and distribution of bound molecules, in the kinetic constants of their redox catalytic steps, and most interestingly, in their ability to transfer electrons to a redox mediator covalently attached to the self-assembled monolayer. The authors concluded that the position of the His-pair determined the orientation of the protein with respect to the electrode surface and its competence to establish direct electrical communication with the electrode. Atomic force microscopy confirmed the different orientation of both immobilized mutant proteins [109]. There are many other examples of this strategy [110,111], e.g., human neuroglobin [112], laccase [37], cellulase, [113], firefly luciferase, [114], RNA polymerase [115] and D-xylose isomerase [116].

3.5. Use of site-directed exchanged Cys residues

The most simple and versatile way of controlling enzyme orientation is the site-directed mutagenesis of the enzyme to introduce unique Cys residues into the enzyme surface (Fig. 7). The enzymes are attached on thiol-reactive surfaces (gold, or disulfide activated supports) through the thiol group on the side chain of the introduced Cys [44,45,89,117,118]. If the wild-type enzyme presents some surface Cys, a previous change of this (these) residue(s) by a serine or threonine will be necessary.

Using gold as an immobilization surface, the immobilization is produced by the binding between thiol groups (although halogen, hydroxyl or aldehyde functionalities may also be involved) and gold surfaces [44,45,89]. The main advantages are the simplicity and mildness of the protocol and the strong and stable biomolecule attachment. Using disulfide activated supports, immobilization is produced by a thiol-disulfide interchange, and only the SH in the Cys will be involved [117,118].

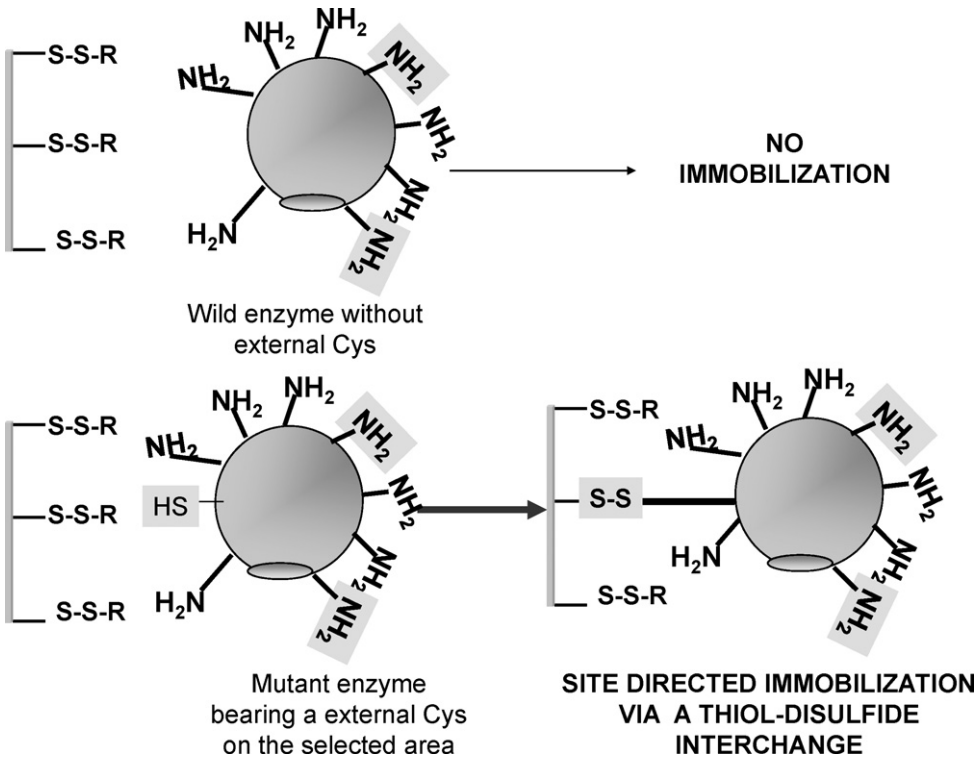

Fig. 7. Introduction of Cys for site-directed immobilization of proteins via thiol-disulfide interchange.

For example, site-directed mutagenesis was used to introduce a Cys into subtilisin, a protein that does not contain Cys [119]. The researchers intended in this case to immobilize the enzyme as far as possible from the active center. To this purpose, Ser residues located on the distal side of the protein surface away from the active site Ser were changed to Cys (one at a time). The changes of Ser to Cys were innocuous to the structure and function of subtilisin. Employing the unique Cys located away from the active site, the SH group was reacted with a thiol-reactive membrane. Using this methodology, an increase in recovered activity from 48 to 83% was observed for conventionally immobilized and site-specifically immobilized subtilisin on PVC-silica membranes [120].

The use of recombinant horseradish peroxidases that, in addition to the hexa-His tag, contained a Cys residue at different positions and were immobilized on gold electrodes demonstrated that the electron transfer efficiency depended on the orientation of the HRP at the electrode surface that, in turn, depended on the position of the mutations [121]. This enzyme has been immobilized using this strategy in other instances [122,123].

Cys residues have been introduced into protein sequences for the immobilization of enzymes by the formation of self-assembled monolayers on gold surfaces. Six mutants of recombinant monomeric superoxide dismutase were engineered to contain one or two additional Cys residues for self-assembly on gold electrodes [124]. The one-step immobilization protocol, based on the incubation of the gold electrode into an enzyme solution, not only simplified the construction of the biosensor but also provided more reproducible results. The biosensor showed a higher sensitivity towards superoxide radicals than that obtained by conventional cytochrome C monolayer electrodes, which are also used for this purpose [125].

A high number of Cys residues involved in the protein interaction with the support may permit a stronger immobilization, reducing enzyme leakage. On the other hand, it can compromise the function of the immobilized biomolecule. Thus, stripping voltammograms of an immobilized dimeric nitroreductase containing different numbers of Cys residues per monomer (adjacent to His tags used for enzyme purification) [126] showed optimal results at Cys 12 (two Cys6-modified monomers). Because the activity of all modified enzymes in solution was essentially the same, the observed effect was likely due to a minimal loss of the native conformation upon immobilization with the Cys12-modified enzyme.

In another example, a nitroreductase containing a sequence of six Cys was genetically engineered. This protein enabled the establishment of strong thiolate bonds between the enzyme and gold supports. This enzyme was adsorbed on a gold electrode surface without the need for pre-treatment of the surface and without the loss of enzyme activity. The enzyme was directly immobilized on a gold electrode. This technique was used to develop an amperometric biosensor for the detection of explosives containing nitroaromatic compounds, with detection levels down to 100 parts per trillion [126].

There are many other examples of using genetically introduced Cys to get site-directed immobilization enzymes, including protein receptor [127], plastocyanin [128], dihydrofolate reductase [129,130], dehydrogenases [131,132] and immunoglobulin binding receptors [133].

In general, the replacement of one amino acid on the surface of the proteins by a Cys presented a negligible effect on the enzyme features, could be introduced in any region of the enzyme, and permitted a complete site-directed immobilization of the proteins, mainly when using disulfide-thiol interchange chemistry. Thus, this seems to be the most promising technique to have a site-directed orientation at any position of the protein.

3.6. Immobilization of proteins via a cellulose binding domain on cellulose supports

There are many peptide domains that permit the one-step purification and immobilization of proteins on different supports, and an exhaustive revision of all of these technologies may be beyond the scope of this review because in many cases directed

immobilization is not the final target. Nevertheless, we will use the cellulose-binding domain (CBD) as an example of the usefulness of these domains because the CBD has found diverse applications in protein immobilization on carbohydrate supports, particularly cellulose. Such carbohydrate-binding modules may be artificial as well as of natural origin. They are well known and extensively studied in many prokaryotes as well as eukaryotes, so their genetic manipulation is relatively straightforward.

One of the simplest applications of this tag is the immobilization of protein A or antibodies to achieve a proper orientation of these biomacromolecules so that they can be used as biosensors or in protein purification [134–136]. For example, a novel method for immobilizing antibodies onto magnetic cellulose microspheres (MCMS) using a cellulose-binding domain-protein A (CBD-ProA) linkage was reported [137]. Biospecific connection between antibodies and MCMS exhibited significant advantages compared to chemical coupling, including convenient and simple preparation, elimination of toxic compounds, and highly efficient antibody utilization. To evaluate the application of this method, interferon α-2b (IFN α-2b) was chosen as a model target. After optimization and characterization, IFN α-2b was successfully purified from crude cell lysate in a single step by cross-linked anti-IFN α-2b IgG protein A-CBD-MCMS, purifying 106.1 μg IFN α-2b/mL matrix, corresponding to a 13-fold increase over the chemical coupling method [137].

There are also some examples where CBDs have been used to purify and immobilize enzymes [138–140]. For example, the *Zymomonas mobilis* gene invB, encoding an extracellular invertase, was translationally fused with the cellulose-binding domain Cex (CBD_{Cex}) from *Cellulomonas fimi* and expressed in *E. coli*. The fusion protein obtained, termed INVB-CBD, was easily purified and immobilized to Avicel (crystalline cellulose) in a single step [141]. In another example, organophosphorus hydrolase (OPH) was used. OPH is an enzyme that catalyzes the hydrolysis of neurotoxic organophosphate nerve agents. To simplify the production of the biocatalysts, a fusion between OPH and a CBD was generated to allow one-step purification and immobilization of OPH on cellulose, resulting in a preparation useful for degrading coumaphos [142]. Later, the system was applied for the biodetoxification of paraoxon in a continuous flow mode with good results [143].

Finally, this domain has found two specific and elegant applications. Nahalka and Nidetzky used a novel concept of controlled precipitation *in vivo* [144]. To this goal, the target enzyme is fused with a CBD of *Clostridium cellulovorans*, expressed in *Escherichia coli*, and produced under conditions that induce selective pull-down of the folded fusion protein via intermolecular self-aggregation of the CBD. Aggregates of D-amino acid oxidase from *Trigonopsis variabilis* were produced using this technique with good results. The aggregated CBD retained the ability to bind microcrystalline cellulose and flocculated polysaccharide particles upon attachment to them. The cellulose-bound oxidase was about 36-fold more stable than the soluble enzyme [144]. A second specific application is the use of this domain to generate a thermoswitched immobilization, proposed by Nahálka and Geneminer for thermophilic enzymes in an interesting paper [145]. They fused the mesophilic carbohydrate-binding domain from *Clostridium cellulovorans* with thermophilic enzymes from *Pyrococcus furiosus*. Then, they showed that the fusion enzyme can be reversibly immobilized/released just by thermal denaturation and renaturation of the fused protein. Modular recombinant enzymes are active and free in the reaction mixture at 80–90 °C and deactivated and immobilized by affinity adsorption on cellulose at 40–30 °C. The temperature transition between both the modes is rather sharp and occurs within the range of 40–50 °C. After the reaction, the enzymes are quickly deactivated, adsorbed on the affinity matrix, removed from the reaction mixture and are ready to be used in another reaction cycle [145].

4. Site-directed mutagenesis to improve the enzyme properties of immobilized enzymes

A deeper knowledge of the protein structure and of the mechanism of immobilization on different supports has permitted the use of site-directed mutagenesis not only to preserve the enzyme function after immobilization but also to improve it.

The genetic increase in the number of the desired ionized amino acids on proteins that are unable to become adsorbed on ionic exchangers has permitted the immobilization of these enzymes on those supports. This was exemplified using penicillin G acylase from *E. coli*, where eight Glu residues were introduced on the enzyme surface by site-directed mutagenesis, decreasing the Ip of the protein from 6.4 to 4.3 [146]. Thus, from a wild-type enzyme that cannot be adsorbed on anionic exchangers, it was possible to prepare an enzyme able to become adsorbed on DEAE or polyethyleneimine-coated supports. Considering that this is a very simple immobilization technique and may produce stabilization versus organic solvent, mainly if using polymeric beads [147], this is a simple way of improving the enzyme immobilization properties via site-directed mutagenesis.

An original way of using genetic tools to improve the immobilization was the research developed by Ansorge-Schumacher et al. to improve the activity recovery of formate dehydrogenase from *Candida boidinii* after entrapment of the enzyme in polyacrylamide gels [148]. It is not really site-directed mutagenesis to get a directed immobilization but rather the use of a potent genetic tool to improve the results of an immobilization protocol.

Enzymes exposed to acrylamide and tetramethylethylenediamine may become inactivated due to chemical modifications of Lys or the hydrophobicity of the medium [149–152]. Stabilization resulted from an exchange of distinct amino acids after two cycles of an error-prone PCR process. Lys 35 was exchanged for Arg and Glu 53 for Val in the first round. Cys (position 23 exchanged for Ser) was found in the third round. All residues were remote from the active site and did not significantly affect the kinetic parameters of the enzyme. Thermal stability of the mutant enzymes significantly increased at the exchange of lysine and glutamic acid but decreased due to the additional exchange of cysteine. This result agreed with previous findings [153], where an 80% decrease in the half-life of mutant formate dehydrogenase-C23S at 50 °C was found, and confirms the special relevance of C23 for enzyme stability. The better thermal stability of the new formate dehydrogenase variant compared with that of formate dehydrogenase-C23S was obviously a result of the stabilizing effects of the additional mutations that were introduced during the first step of evolution [158].

The formate dehydrogenase variants of both generations retained clearly higher residual activity after entrapment in polyacrylamide gels than the wild-type enzyme. The results found during immobilization were less clear than during screening, where soluble acrylamide was used, but even with that being the case, the activity increased by 4.4-fold in the enzyme with three mutations. This lower impact was explained by diverse causes; for example, the polymerization process itself affects the enzyme variants in different ways, depending on their respective mutations. In any case, screening with a mixture of acrylamide and TEMED turned out to be well-suited for resembling the deactivating forces of polyacrylamide gels PAA formation on the enzyme and thus enabling the identification of variants with considerably improved activity recovery after immobilization. Thus, two apparently unrelated strategies, such as immobilization and directed evolution, may be used in a coupled way to get a final optimized catalyst. The coupled use of both strategies should become beneficial if high-throughput screening is utilized to study the immobilization, as recently described by Brant and co-workers using microtiter plates and an esterase as a model [154].

4.1. Enrichment in Lys residues on the desired region

The first immobilization of enzymes on glyoxyl agarose is via a multipoint covalent attachment [58]. This attachment causes enzymes to become immobilized on this support at the areas where most of their reactive groups are located [58,59]. On the other hand, it has been shown that the enrichment of reactive groups on the protein surface (using chemical modification) increases the possibility of getting a more intense stabilization by multipoint covalent attachment on these supports [155,156]. Thus, if a region on the protein surface was created that was very rich in Lys residues, it could be expected that the enzyme was mainly immobilized on glyoxyl-agarose via this area and could be stabilized in a more intense way (Fig. 8).

Following this idea, Guisan's group used the enzyme penicillin G acylase from *E. coli* and genetically introduced three Lys residues on the enzyme surface in a region that was already rich in Lys groups [157]. These mutations did not alter the enzyme stability or the kinetic properties of the soluble enzyme. However, the rate of immobilization on glyoxyl-agarose was increased more than 10-fold when compared to the wild-type enzyme, even though the number of overall external Lys was not significantly altered (around 10%). This finding confirmed that the immobilization was mainly performed via the region where the 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 the native enzyme immobilized on glyoxyl-agarose following the same protocol [158]. Considering the stabilization obtained by the immobilization of the wild-type enzyme (near to 10,000) [158], the final stabilization factors achieved with this strategy were impressive.

Terreni and co-workers used a similar strategy to direct the immobilization of penicillin G acylase, focusing their efforts on improving the catalytic behavior of the enzyme in the kinetically controlled synthesis of antibiotics. For this purpose, they designed a mutant protein with a tag of three lysines alternating with three glycines on the C-terminal end of the β chain of the enzyme. This modification improved the immobilization efficiency of PGA on glyoxyl agarose and the catalytic properties of the immobilized derivative on the kinetic synthesis of antibiotics [159,160]. They evaluated other Lys enriched mutant enzymes on different regions of the protein, obtaining different catalytic behaviors of the different mutant enzymes after immobilization on glyoxyl agarose [161].

Using a similar strategy, but changing glyoxyl agarose to a modified polyethersulfone matrix presenting aldehyde residues, Ryan and Ó'Fágáin improved the immobilization of horseradish peroxidase [162]. The wild-type enzyme presented only two exposed Lys residues. One triple mutant and one pentuple mutant were generated by substitution of external arginines on the "back" of the polypeptide (R118, R159 and R283) and of residues known to influence enzyme stability (K232 and K241). The immobilized protein was forced to orientate its active site away from the membrane and towards the bulk solution phase. Mutations presented a negative effect on enzyme stability that was not regained by immobilization, perhaps due to the unsuitability of the support for giving an intense multipoint covalent attachment, and was partially solved by reverting one of the mutations. Optimal values were achieved with the mutant with R118K, R159K, K232F, K241N and R283K. Thus, directional, oriented immobilization of horseradish peroxidase mutants onto an aldehyde-activated polyethersulfone membrane was achieved with excellent retention of catalytic activity; however, stability was not good enough due to the negative effects of the mutations and further studies are necessary [162].

Thus, the incremental addition of Lys residues may permit site-directed immobilization, as well as bring forth an improved stability and properties of the final biocatalyst. However, this strategy needs several amino acid replacements on a determined area. Moreover, this strategy may not be not useful for immobilizing proteins via surface areas lacking in Lys residues because it will be

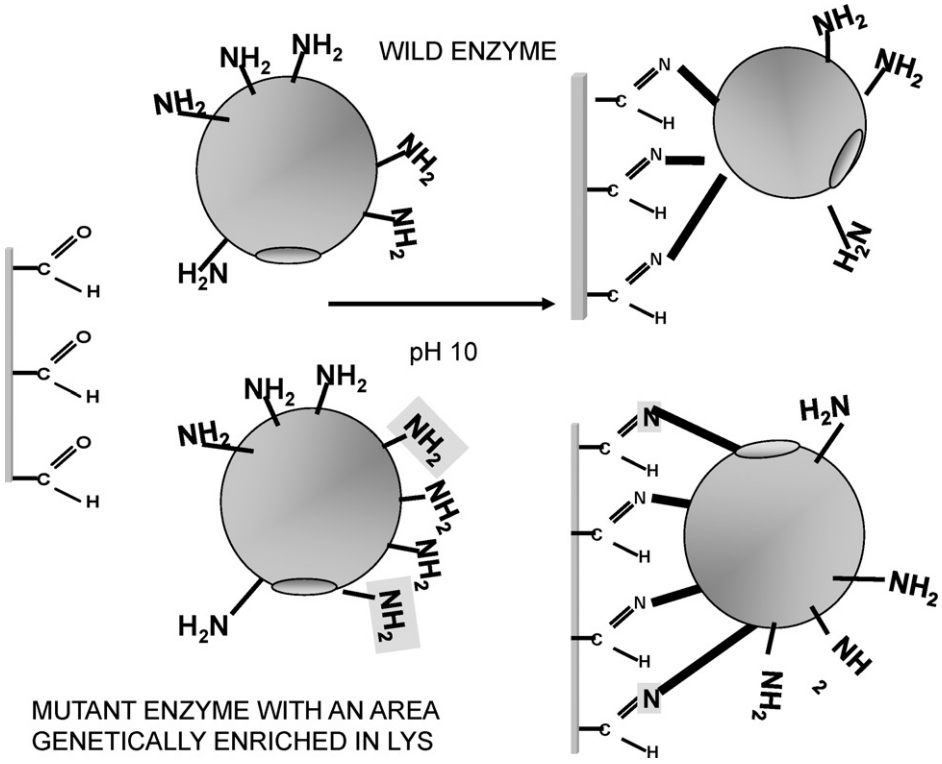

Fig. 8. Site-directed and improved multipoint attachment of enzymes with an area genetically enriched in Lys residues using glyoxyl supports.

necessary to reduce the Lys number of other Lys-rich areas and introduce a high number of Lys on the desired area. Thus, although very useful, it did not appear to be a general and simple solution.

4.2. Use of thiol/disulfide supports to get more stabilized enzyme preparations

The pioneers of this strategy were Ulbrich, Mansfeld and co-workers. In a first approximation, studies on the thermal inactivation of immobilized enzymes made the authors propose a novel conception of protein stabilization [163]. The hypothesis considered that the native protein molecule is characterized by a specific structural region where the unfolding process starts. Accordingly, enzyme stabilization by immobilization could be obtained if this unfolding nucleus was blocked. Some years later, the researchers published results that reinforced that idea [164–167]. The introduction of Cys residues (just one per mutant) on different areas of the surface of a cysteine-free mutant of a thermolysin-like protease from *Bacillus stearothermophilus* by site-directed mutagenesis enabled the site-directed immobilization of this protease via a single thiol group onto thiol-Sepharose. With some exceptions, introduction of this surface Cys did not present a significant effect on the thermal stability of the free mutant enzymes. However, the position of the introduced single Cys residues determined the thermal stability of the protease after immobilization. The resulting stabilization effects were higher if the attachment of the enzyme to the carrier was within the residues 56–69, an area that was known to be critical for enzyme stability [167]. Thus, two immobilized mutants (T56C and S65C) having their Cys in the proposed unfolding region of the enzyme showed a 9- and 12-fold increase in half-lives at 75 °C compared to the wild-type enzyme. The stabilization by immobilization was even larger (33-fold) for the T56C/S65C double mutant enzyme, where a two-point immobilization could be obtained [168]. In contrast, mutants containing Cys in other parts of the enzyme showed only small increases in half-lives due to immobilization (a maximum of 2.5-fold). This finding suggested that the previous hypothesis could be correct: every protein is characterized by a specific structural region where the unfolding process starts, and when this unfolding region is bound by covalent or non-covalent bonds to a support, it produces a high stabilization. Considering the comparison between the different immobilized mutant enzymes, where proteolysis is not possible, the reasons for the stabilization after just one-point immobilization of the enzyme may not easily be described. It might be founded on the promotion of some steric hindrance to the movements of the enzyme chains: the near proximity of the support surface can reduce the mobility of these chains and thus produce certain stabilization. However, the fact that a multipoint covalent attachment (in this case a two-point covalent attachment) on the critical area could greatly improve the results was a clear message from this work. Using Cys and disulfide supports, a higher multipoint attachment could be hard to achieve because it is not simple to have a protein with many Cys residues concentrated in a reduced area of the protein surface.

More than 20 years elapsed before a solution was proposed for coupling thiol-directed immobilization and multipoint covalent attachment. As a first step to reach the site-directed enzyme rigidification via immobilization, a thiol-epoxy support was designed [168]. Epoxy groups are very poorly reactive for soluble proteins [57], so this new support permitted the immobilization of proteins via thiol residues and produced a certain multipoint covalent attachment between the enzyme and the support by involving other nucleophiles of the protein surface, mainly the ε-amino group of the Lys residues [169] (Fig. 9). Penicillin G acylase from *E. coli*

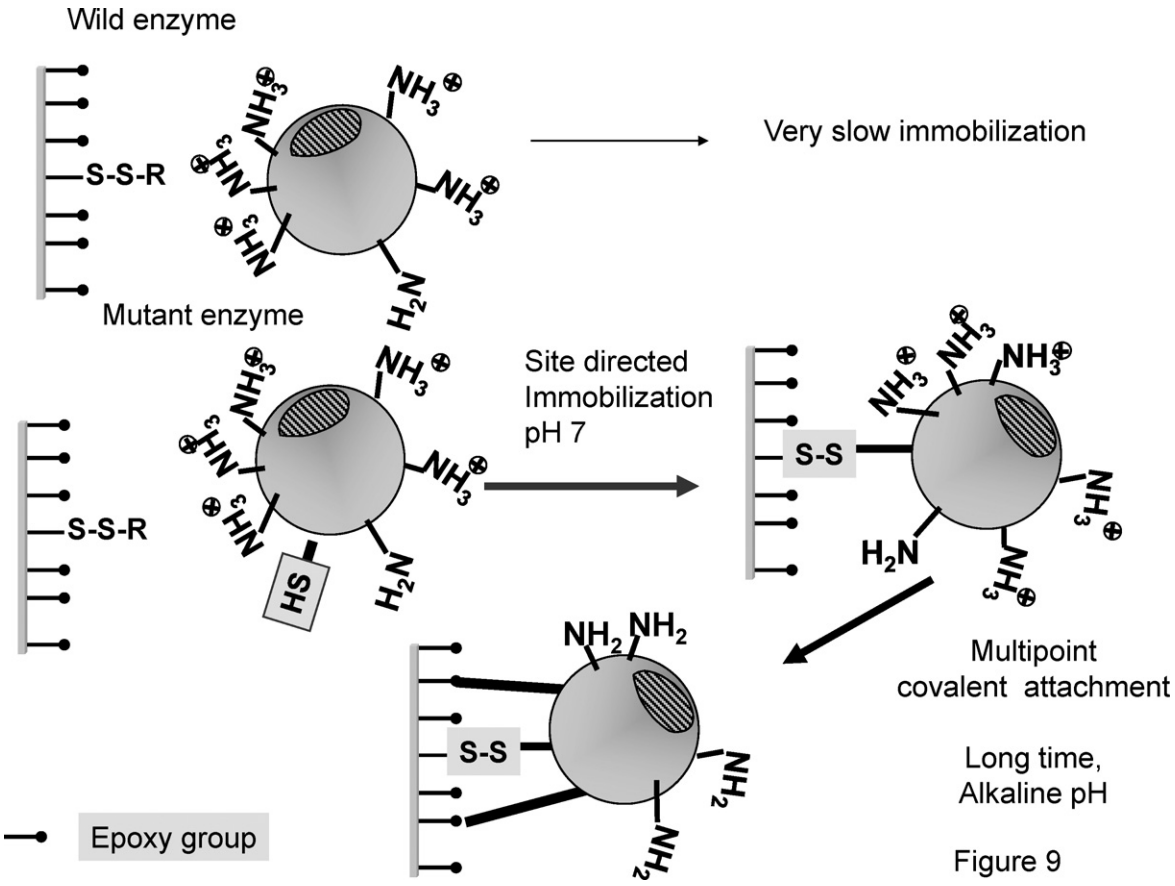

Fig. 9. Site-directed rigidification using thiol-epoxy supports and a protein with a Cys inserted in the desired area.

was chosen as a model enzyme. There is no clear information on the most critical regions of this enzyme for its inactivation, and researchers have proposed that, contrary to the preliminary papers, multipoint immobilization via different regions could give information on the regions critical for the stability of the immobilized enzyme, and the enzyme stability could be analyzed under different inactivation conditions. Thus, six different variants of penicillin G acylase from *E. coli* (which lacks in Cys residues) were designed, introducing a unique Cys residue via site-directed mutagenesis in six different enzyme regions [170]. As the objective was to give an intense multipoint attachment around each Cys, areas that were rich in Lys residues were chosen. All soluble variants exhibited a similar activity and stability compared to those of the native enzyme, and contrary to the previous reports presented above, all of the one-point thiol-immobilized enzyme preparations presented exactly the same stability. However, when the enzymes were immobilized on supports having a low concentration of reactive disulfide moieties and a high concentration of poorly reactive epoxy groups and incubated under conditions where multipoint attachment between epoxy and protein groups was favored [56,169], the different derivatives exhibited diverse stabilities against several distorting agents. The immobilized derivative of the mutant enzyme obtained by replacement of GlnB380 by Cys (a group near the active center of the enzyme) was the most stable biocatalyst against heat and organic cosolvents: it preserved 90% of the initial activity after multipoint covalent attachment and was 30-fold more stable than the soluble enzyme in the presence of dioxane [170]. This derivative also exhibited an improved enantioselectivity in the hydrolysis of chiral esters (E was improved from 8 to 16) and in the kinetically controlled synthesis of amides (synthetic yields were increased from 31 to 49%) [170]. Thus, the site-directed rigidification not only permitted an improvement in the enzyme stability but also in other features of the enzyme, as could be expected after enzyme modulation using different immobilization protocols [26,37–54]

However, the enzyme immobilized using this protocol did not result as highly stabilized as expected. The reported 30-fold factor cannot compare with the stabilization obtained on standard Eupergit C reported by the same research group [56], as the authors recognize in the paper. A likely reason for these less than satisfactory results could be the generation of steric hindrance by the disulfides introduced on the support. They were over the epoxy groups layer, which could reduce the possibilities of getting an intense multipoint covalent attachment. One likely solution to this problem may be to place the disulfide groups under the layer of epoxy groups. While the one-point interaction is hardly limited in the immobilization because the enzyme is in soluble form and its mobility is complete, after immobilization, any steric hindrance may result in a very difficult enzyme support multipoint reaction. Nevertheless, this technique seems to be valid for detecting the areas of the enzyme where a higher stabilization may be achieved if involved in a multipoint immobilization process, and these critical areas can be stabilized by other strategies (e.g., protein engineering). The authors also propose some ways of improving the stabilization results by this immobilization-stabilization technique, as the enrichment of this key region with more Lys residues could achieve a more intense multipoint covalent attachment, as was previously shown [157] (Fig. 10).

At first glance, one likely problem with this strategy is that one point did not fully determine the enzyme orientation, However, it should be considered that the reactivity of the groups in the support with the nucleophiles on the protein surface surrounding this Cys should be very different: exposition, reactivity and (perhaps most importantly) distance may be quite different. Thus, an almost fully controlled orientation of the enzyme on the support (as is suggested by the differences in the enzyme properties of penicillin G acylases with very close inserted Cys residues) can be expected. To be completely sure of that orientation, Cys2 mutants could be used, although these may generate some problems due to the necessary proximity of both groups.

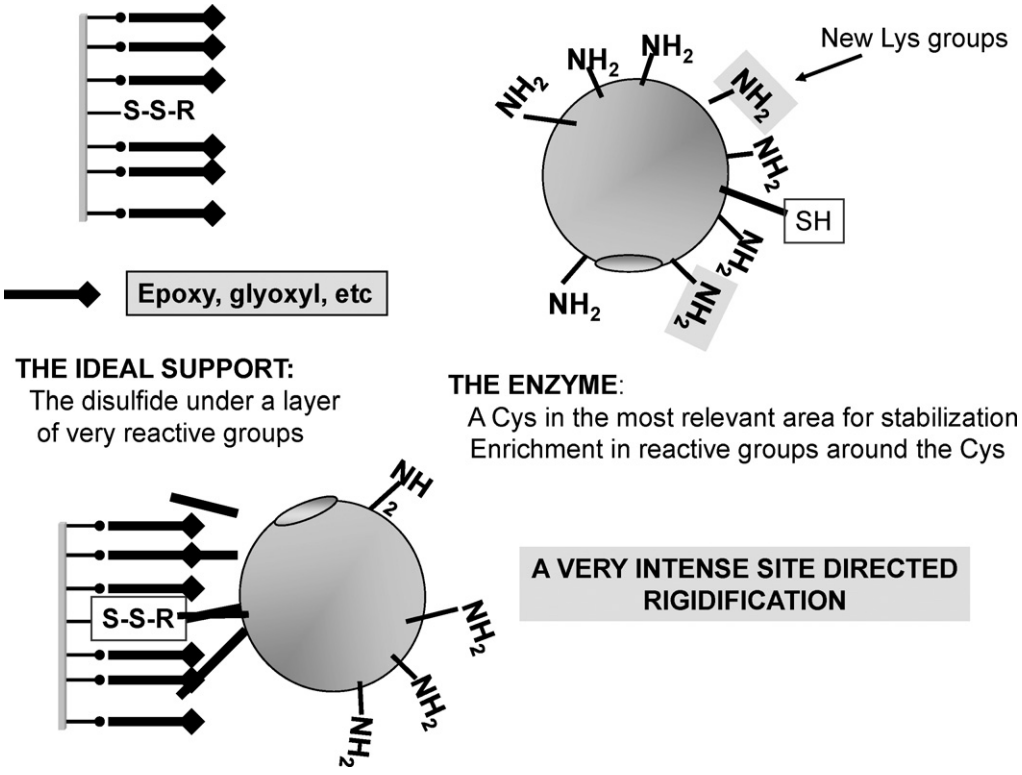

Fig. 10. Optimal site-directed rigidification by improving the protein–support complementarities support: site-directed immobilization followed by a very intense multipoint covalent attachment.

5. Future prospects

The results presented in this review are the first steps on a path that may lead to almost complete control of the immobilization of an enzyme, such that the orientation and the intensity of the enzyme–support interaction can be chosen. Some years ago, researchers could only try to optimize the support to achieve more complementary to the enzyme surface. Currently, site-directed mutagenesis of the protein may be used to improve this complementarity of enzyme and support surface in a more complete way. Therefore, perfectly oriented and very rigid enzyme molecules could be prepared.

At first glance, the use of site-directed exchanged Cys on the protein surface to direct the enzyme immobilization on disulfide supports is the best way to get an oriented immobilization [170]. In the second step, the further site-directed rigidification should be reinforced by adding nucleophiles on the enzyme surface (e.g., Lys groups), as this has proven to yield very good results [157].

However, there is still a necessity for improved supports. Disulfide-epoxy-Eupergit C supports have proven to be not suitable for intense multipoint covalent attachment. This result can be based on the existence of steric hindrance for the enzyme–support reaction caused by the disulfides being over the layer of epoxides, the unsuitability of epoxy groups for giving a very intense and rapid multipoint covalent attachment [171] or the inadequate internal geometry of the support. Eupergit C offers thin fibers where only a small percentage of the protein surface can interact. Regarding this last point, immobilization of proteins on two different epoxy-activated supports, such as Eupergit C and Sepabeads, has been reported to give very different enzyme stabilizations, even when using similar immobilization protocols [74]. Sepabeads have been described as a support that offers a much better internal geometry than Eupergit to give a good geometrical congruence between the protein and its internal surfaces.

Thus, new supports need to be prepared to take full advantage of these methodologies and achieve site-directed rigidification (Fig. 10). The new supports should reduce the steric hindrance of the further multipoint attachment, for example, by presenting the disulfide groups under the layer of the other reactive groups of the support. Moreover, the support should offer good geometrical congruence for the enzyme–support reaction (internal flat surfaces). It is also necessary to develop a protocol that will produce fully inert surfaces after enzyme immobilization. Finally, the reactive groups on the support should have a high reactivity with the enzyme groups [171], perhaps using glyoxyl groups but obviously, only after the first site-directed immobilization.

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