HETEROFUNCTIONAL SUPPORTS IN ENZYME IMMOBILIZATION: FROM TRADITIONAL IMMOBILIZATION PROTOCOLS TO OPPORTUNITIES IN TUNING ENZYME PROPERTIES

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Abstract

A heterofunctional support for enzyme immobilization may be defined as that which possesses several distinct functionalities on its surface able to interact with a protein. We will focus on those supports in which a final covalent attachment between the enzyme and the support is achieved. Heterofunctionality sometimes has been featured in very old immobilization techniques, even though in many instances it has been overlooked, giving rise to some misunderstandings. In this respect, glutaraldehyde activated supports are the oldest multifunctional supports. Their matrix has primary amino groups, the hydrophobic glutaraldehyde chain, and can covalently react with the primary amino groups of the enzyme. Thus, immobilization may start (first event of the immobilization) via different causes and may involve different positions of the enzyme surface depending on the activation degree and immobilization conditions. Other “classical” heterofunctional supports are epoxy commercial supports consisting of reactive covalent epoxy groups on a hydrophobic matrix. Immobilization is performed at high ionic strength to permit protein adsorption, so that covalent attachment may take place at a later stage. Starting from these old immobilization techniques, tailor-made heterofunctional supports have been designed to permit a stricter control of the enzyme immobilization process. The requirement is to find conditions where the main covalent reactive moieties may have very low reactivity towards the enzyme. In this review we will discuss the suitable properties of the groups able to give the covalent attachment (intending a multipoint covalent attachment), and the groups able to produce the first enzyme adsorption on the support. Prospects, limitations and likely pathways for the evolution (e.g., coupling of site-directed mutagenesis and thiol heterofunctional supports of enzyme immobilization on heterofunctional supports) will be discussed in this review.

Keywords: heterofunctional supports; enzyme immobilization; enzyme stabilization; multipoint covalent attachment;

1. **INTRODUCTION**

Immobilization is in most instances a requisite for using enzymes in industry.[1-3] Immobilized enzymes may be utilized in different reactor configurations, permitting an easy control of the reaction, avoiding contamination of the product by the enzyme (this is highly relevant in food technology) and permitting their reuse over many reaction cycles.[1-3] Thus, a proper immobilization system should give a strong enough immobilization in order to avoid enzyme release that may contaminate the product and result in loss of enzyme (and catalytic activity).[1-3] Moreover, immobilization and stability are closely related terms, as only stable enough biocatalysts could be reused.[4, 5] However, the term "immobilization" does not necessarily imply stabilization of an enzyme. In fact, if the immobilization protocol is not well designed, for example permitting uncontrolled enzyme-support interactions, immobilized enzymes may be even less stable than free enzymes.[6-13]

Considering this requirement as an opportunity, many researchers have tried to understand and control the immobilization of enzymes to use this process as a powerful tool to improve enzyme properties, such as stability, activity, selectivity, reduce inhibitions, etc.[7, 14-16]

The tuning of enzyme features by immobilization[16] should not be considered as an alternative to other tools to improve enzyme features, but as a tool that is compatible with any other enzyme tuning strategy (remember that in most cases, the enzyme will be finally used in an immobilized form). In fact, it has been recently reviewed how the joint use of microbiological tools (use of thermopilic mircoorganisms),[17] genetic tools[18] or chemical modification of enzymes[19] to achieve an improved immobilization has opened the door to new strategies for enzyme properties enhancement.

It is true that any immobilization protocol (at least, if the enzyme is placed inside porous particles) avoids some inactivation causes: aggregation, proteolysis (due to autolysis if the enzyme is a protease or due to the action of some contaminant protease) or interaction with external interfaces will no longer be possible (just a small percentage of enzymes placed on the

outer surface of the particle may suffer these detrimental phenomena).[6] Operational stabilization may be also achieved by decreasing the inactivation cause, as it occurs if an enzyme microenvironment is generated where the inactivating agent is partitioned away from the enzyme environment (e.g., a hydrophobic environment in the presence of hydrogen peroxide[20] or a hydrophilic environment in the presence of organic solvents or oxygen[14, 21-23]).

However, a true improvement on the enzyme rigidity may be achieved if an intense multipoint covalent attachment between the enzyme and a rigid support by short spacer arms is obtained.[3, 6, 7, 14, 15, 17, 18] All the enzyme moieties involved in the immobilization process must maintain their relative positions under any condition that may produce a conformational change. The support could be considered as a multifunctional crosslinker reagent, where the crosslinking will involve many groups in the enzyme structure. This rigidification of the enzyme structure should produce a stabilization of the enzyme in all cases where the inactivation cause was due to conformational changes. Stabilization may not be observed if the main cause for enzyme inactivation is a chemical modification of an exposed group[6] or if it involves the dissociation of enzyme subunits[24] or of some ion or cofactor.[6, 25]

In the case of multimeric enzymes, in many instances, enzyme inactivation starts via subunit dissociation.[24] If this is the case, prevention of this dissociation may be achieved also by multi-subunit immobilization among other strategies.[24]

Immobilization produces some alterations on the enzyme structure and its overall mobility in most cases, and that may lead to an alteration of enzyme properties, such as activity, selectivity or specificity.[7, 16, 26]. These effects may be unpredictable and related to the area of the enzyme involved in the immobilization, the intensity of the covalent attachment, etc.[18]

Moreover, it has been clearly established that the effect of a multipoint covalent attachment depends on the involved protein areas (e.g., enzyme stability). There are enzyme areas that are more relevant than others,[18, 27, 28] even though multipoint covalent attachment

produces a global rigidification of the enzyme structure, and the effects are more relevant when they directly involve the area of the enzyme structure where the inactivation starts.[18, 27, 28]

Using just one kind of support, changing the orientation of the enzyme on the support surface is a complex (although not impossible) problem.[18] It should be considered that most immobilization supports are able to immobilize enzymes and proteins with a quite well defined orientation, although in some instances it may be difficult to guess the exact area of the protein involved, even when using the enzyme structure and molecular dynamic programs.[18] Those immobilization methods, which directly provide a covalent attachment and are used at neutral pH values, immobilize enzymes mainly by the most reactive amine in the protein surface (usually the terminal amino group).[18, 29, 30] Ionic exchange, adsorption of enzymes on immobilized metal chelates or hydrophobic supports are multipoint processes, therefore they mainly involve the areas of the enzyme surface where there is a higher concentration of the target groups.[18, 31-40] Other supports, due to the reversibility of each individual bond, only immobilize the enzyme on their surface after several covalent linkages, therefore directing the immobilization by the area of the enzyme surface richest in support-reactive groups (that is the case of glyoxyl supports).[29, 30] Thus, to have proteins immobilized in different orientations (e.g., interesting for proteomics), the most effective solution is to use different immobilization protocols.[18, 41]

Immobilization of enzymes on a support may be more versatile if we use multifunctional supports. We will apply the term “heterofunctional support” to that which has several functionalities on its surface (introduced either by accident or by design) that are able to interact with groups of an enzyme under different circumstances. In this review, we will focus on those supports that yield covalent bonds as a final result. We will present the advantages and problems raised by the use of heterofunctional supports, as well as the most likely evolution of these systems.

2. STANDARD MULTIFUNCTIONAL SUPPORTS

Some of the oldest covalent immobilization strategies are based on the use of the multifunctional features of the support. These features are sometimes ignored during the use of the supports, making the understanding of the final results complex.

2.1. Glutaraldehyde activated supports: an old heterofunctional support

Supports activated with glutaraldehyde are expected to react mainly with non-ionized primary amino groups.[29, 42-44] Due to the relatively low stability of the glutaraldehyde groups at alkaline pH value, immobilization employing these supports is usually performed at neutral pH values. At these pH values, the most reactive amino group in the protein tends to be the terminal amino group (p*K* ranging from 7 to 8, much lower than the p*K* of the exposed Lys residues that is 10.7). However, after the first immobilization, if some nucleophiles of the protein are in the area exposed to the support, the high apparent concentration of the different groups may permit the establishment of some new covalent enzyme-support bonds.[45] Thus, it may be possible to reach some multipoint covalent attachment by using highly activated glutaraldehyde supports.[46-49]

However, the current scenario using glutaraldehyde-activated supports is far more complex, as these supports are really multifunctional ones. The multifunctionality of some supports is a direct consequence of the way they are prepared and this is the case for glutaraldehyde activated supports (figure 1). Their preparation begins with the modification of supports bearing primary amino groups (they are, in short, anion exchangers) with glutaraldehyde.[42-44] Following the procedures described in the literature, it is hard to give the exact structure of the groups formed by the glutaraldehyde, but the existence of some stable cycles instead of standard imino bonds seems to be certain (no linear aldehyde molecules should be expected).[44, 50, 51]

Moreover, for a long time, it has been established that the modification of each primary amino group on the support with one or two glutaraldehyde molecules may be achieved in a relatively simple way by controlling the time, the pH and the concentration of glutaraldehyde during support activation.[42] The most reactive species with amino groups are those obtained when two glutaraldehyde molecules per amino group are present.[49] Furthermore, the amino-glutaraldehyde-glutaraldehyde groups have low reactivity with other similar groups,[52] decreasing the risks of crosslinking that should lead to the loss of reactive moieties on the support. Whatever the exact structure of the glutaraldehyde on the support, the final result is a support having spacer arms bearing one or two amino groups (cationic groups that may function as anion exchangers), a fairly hydrophobic moiety formed by the glutaraldehyde chain and the covalent reactive group. That is, the support may give three different kinds of interactions with an enzyme: hydrophobic, anionic exchange and covalent (Figure 1).[53] Using highly activated supports, all of them will be able to immobilize the enzyme, each one being dominant under certain experimental conditions.[34]

This fact raises some problems when using this old fashioned multifunctional support, especially if the researcher ignores its multifunctional nature in the design of the experimental protocol. The first one is that although the support has moieties able to covalently react with the enzyme, the fact that all enzyme molecules are immobilized in a very rapid fashion is not a guarantee that covalent immobilization of the enzyme on the support will take place. Thus, the researcher should try to release the adsorbed enzyme molecules from the support (e.g., using cationic detergents) to verify the establishment of covalent attachments between enzyme and support.[53] A second problem is that it is not possible to obtain a fully inert surface after enzyme immobilization. In general, these finally inert surfaces will be always preferred in an immobilization protocol, with view towards permitting a fine control of the support-enzyme interactions.[18] Reduction of the support with sodium borohydride may eliminate the chemical reactivity of the glutaraldehyde group, but we still have a layer of hydrophobic groups over a

layer of cationic groups, that can produce uncontrolled enzyme-support interactions during storage or use. These interactions may in some cases have positive effects on enzyme performance, while in other cases the effects will be negative, but these will be solely uncovered after studying the biocatalyst properties.

These interactions will only have a real impact on enzyme performance when using supports bearing several amino-(glutaraldehyde)$_n$ moieties under each enzyme molecule.[34, 36, 38-41] Biomacromolecules are only immobilized on supports via ionic exchange or hydrophobic interactions when several enzyme-support interactions may be established. If there is a very small amount of groups on the support, (e.g. just one spacer arm per projected area of the enzyme), this multi-interaction will no longer be possible.[29, 34, 36, 38-41] Thus, using very lowly activated amino supports, immobilization using glutaraldehyde will be directly performed by a covalent reaction by the most reactive amino group on the enzyme (Figure 2). However, immobilization will be very slow due to the low activation of the support, and will offer no chance of reaching an intense multipoint covalent attachment.[7]

Using highly activated supports, it has been shown that in most cases an ionic exchange with the amino groups in the support is the first step in the immobilization of most enzymes on highly activated glutaraldehyde supports.[18, 46, 49, 53] Using lipases, interfacial activation on the hydrophobic surfaces formed by glutaraldehyde may give similar adsorption rates to those found for ionic exchange, making the final picture even more complex.[53] Both immobilization mechanisms are far more rapid that the direct covalent attachment via glutaraldehyde-enzyme covalent reaction.

One effect of this first ionic adsorption is that, even though glutaraldehyde is able to immobilize enzymes via just one attachment due to the stability of the bond formed, the activation degree of the support has an exponential effect on the immobilization reaction rate (figure 3). This is because the researcher is measuring the rate of ionic exchange of the enzyme on the support, which requires the establishment of several enzyme-support interactions, and

thus it is exponentially dependent on the surface density of amino groups on the support although the covalent reaction should be of order 1.[34, 36, 38-41, 49]

This multifunctionality may be (and actually is) an advantage in certain cases. The rapid ionic exchange of the enzyme on the support prevents the enzyme from fulfilling the requirement of staying in a soluble state for a long time before being immobilized.[7, 14] That way, inactivation of soluble enzymes via precipitation or proteolysis is reduced, and if enzyme adsorption has a positive effect on enzyme stability, enzyme inactivation by distortion will also be prevented.

However, the main advantage of the multifunctionality of glutaraldehyde is that we can alter the enzyme orientation on the support by changing the immobilization conditions, favoring one mechanism or another as the first immobilization cause. Thus, it has been shown that using lipases, it is at least possible to immobilize the enzyme via 4 different mechanisms.[53] In fact, there will be five different forms of having a biocatalyst from a given lipase using glutaraldehyde chemistry, if the ionic exchange of the enzyme on aminated supports and further modification with glutaraldehyde of the adsorbed enzyme and supports is included.[53]

If the researcher wishes to have a first hydrophobic adsorption, this can be achieved using a high enough ionic strength (figure 4). Using very high ionic strength, the areas of the protein with high concentration of external hydrophobic groups (Figure 5) may be involved in the first enzyme adsorption and delimit the area where the reactive groups of the enzyme which will react with the support should be located. After enzyme hydrophobic adsorption, the reactive groups of the enzyme near the support surface may produce some covalent reactions. However, this will be produced *after* enzyme immobilization, and there is no guarantee that the enzyme will finally have any covalent attachment with the support.

The second possibility is to permit ionic exchange of the enzyme prior to covalent immobilization. Using most water soluble enzymes, the use of low ionic strength is enough to reach this situation (an ionic strength that permits ionic exchange of the proteins on the non-

activated glutaraldehyde amino support).[53] (Figure 4). In this case, the enzyme will be first immobilized on the support by ionic exchange and this area will be the one where nucleophiles capable of reacting with the glutaraldehyde moieties should be located.

Using lipases, the situation is more complex. Due to the tendency of the open form of the lipases to become adsorbed versus hydrophobic interfaces,[54-57] if immobilization is just performed at low ionic strength, the enzyme will be immobilized by both immobilization mechanisms: interfacial activation and ionic exchange (Figure 5). Thus, depending on the enzyme, the support and the immobilization conditions, one or the other immobilization cause may be predominant.[53] This is the usual situation that we may find in the literature, and this may lead to a mixture of different immobilized forms of the lipases, making it difficult to understand the results. This situation may be avoided by using non-ionic detergents, which prevents the interfacial activation of the lipase versus a hydrophobic support.[55] Performing the immobilization in the presence of Triton X-100, lipases are mainly immobilized on the support via ionic exchange as first reason of immobilization.[53] (Figure 5)

Ionic exchange may also involve different enzyme regions depending on the experimental conditions and activation degree of the support. Ionic exchange at different pH values may in certain enzymes change the area where the highest concentration of available anionic charged groups may be found. Furthermore, the ionic strength may determine the area involved because the higher the ionic strength, the more restrictive the immobilization becomes (requiring more enzyme support-interactions).[31, 32]

Finally, it is possible to immobilize the enzyme via a direct first covalent attachment, involving the most reactive exposed group of the enzyme (usually the terminal amino group).[7] Using most water soluble enzymes, the moderate ionic strength used to prevent ionic exchange (100-250 mM of NaCl) is not enough to force the hydrophobic adsorption of the protein on the support and a direct covalent immobilization may be the first cause for the enzyme immobilization.[53] In most enzymes, the use of an ionic strength which is sufficient to prevent

ionic adsorption is enough (figure 4).[49] Using lipases, the situation is once again different. As ionic exchange is avoided, lipases become immobilized on the support first via a rapid interfacial activation on the support, which is still much faster than the direct covalent attachment.[53] (figure 5). Thus, in the case of lipases, the use of ionic strength and detergents, or ionic detergents, may be the only way to ensure a first covalent immobilization.[53] (Figure 5)

If we have a situation where the first phenomenon is covalent immobilization, the surface density of groups in the support will have a first order effect on the rate of enzyme immobilization.[29] (figure 3) Thus, we can ensure that the first step of the enzyme immobilization process is the chemical reaction between enzyme and support.

These are different ways of immobilizing any enzyme on glutaraldehyde activated supports, which may lead to different orientations of the enzyme on the support. The different immobilized enzyme preparations obtained have been shown to present different stabilities,[49] and in the case of lipases, they also exhibited different catalytic behavior (e.g., selectivity was altered).[53] This way, it is possible to have, using the same immobilization support, enzymes immobilized by different areas, with different numbers of enzyme molecule-support covalent bonds and different enzyme-support unspecific interactions. We should bear in mind that, due to the proximity between the groups of the support and of the enzyme, interactions between immobilized enzyme and support will be produced even though they may not be enough to be the only cause for immobilization.[45]

Thus, multifunctionality of glutaraldehyde supports may be in some instances a problem, mainly to understand the results as the cause for enzyme immobilization may be unclear. However, it may be an advantage if properly used, by giving a higher versatility to these supports.

2.2 Standard epoxy supports

Epoxy supports are another example of old and very popular protein immobilization matrices.[58-60] Epoxy groups may react with different protein moieties, including thiols (from Cys residues), primary amino groups (terminal amino aminoacid and the amino group of the lateral chain of Lys), hydroxyl (mainly from the phenol chain of Tyr), imidazol (from His), and also with carboxylic acids (lateral chain of Asp and Glu, carboxyl terminal groups of the enzyme) among other groups.[59] Most of the final bonds are very stable, such as thioethers, ethers or secondary amino bonds. In this respect the weakest bond is that formed after reaction with carboxylic acid (i.e. formation of an ester).[59] These supports are directly supplied as activated supports; therefore they do not require any further treatment to immobilize the enzyme. In dry form and at low temperatures, they can be stored for months without altering their reactivity, and the epoxy groups are also stable for weeks at neutral pH in wet conditions and at room temperature. At alkaline pH values, the epoxy groups are less stable but still the half-life may be measured in weeks at pH 10, enabling the incubation of the enzyme and support for long periods of time, a requisite for increasing the prospects of an intense enzyme-support reaction.[61, 62]

Thus, at first glance, these supports are very adequate not only to immobilize enzymes, but also to improve their stability via multipoint covalent attachment, at both laboratory and industrial scale.

However, current epoxy activated supports exhibit a moderate to very low reactivity versus the different reactive groups of a protein. Only thiol groups of Cys seem to be able to provide a significant immobilization rate on epoxy supports and this only takes place using a very high concentration of support, but even this is quite slow.[58, 61-64] Moreover, in most cases, exposed Cys residues in proteins will have an oxidized thiol group. Thus, this group will usually require to be submitted to a reduction treatment before being able to react with the epoxy groups.[65, 66]

Nevertheless, the fact is that epoxy supports have been available for immobilizing industrial enzymes for a long time, and they have proven their efficiency in certain cases.[58, 61-64]

This is possible because the protocol recommended by the suppliers involves the use of high concentrations of buffers (1 M sodium phosphate), and the commercially available supports have a hydrophobic nature (e.g., Eupergit supports, commercialized by Rhon Hass or Sepabeads, commercialized by Resindion). Thus, these supports are actually multifunctional, even though they only present one short spacer arm having chemical reactive moieties, because below the epoxy groups they have a fairly hydrophobic surface formed by the support matrix (Figure 6).[7, 62] The use of high ionic strength produces the hydrophobic adsorption of the enzyme on the support as the first step in the immobilization of enzymes on these standard epoxy supports.[58, 67] Then, the very high effective concentration of epoxy groups and nucleophile achieved by the proximity of enzyme and support allows the acceleration of the covalent reaction between enzyme and support in a second step.[61, 62] (Figure7) In fact, although agarose-epoxy beads are available, they are not recommended to immobilize enzymes. This is due to the high hydrophilicity of agarose.

It has been shown that after enzyme immobilization, which may be performed at neutral pH values and low temperatures (just after the hydrophobic adsorption or after a first covalent attachment), the increase in the pH value (e.g., to pH 9 or 10) may permit to increase the enzyme-support reactivity, yielding a relatively intense multipoint covalent attachment and permitting to get a high stabilization of the enzymes via this immobilization technique.[61, 62] (figure 7) However, this is only possible if there is a high number of groups that can react with the epoxy support in the most hydrophobic area of the protein (that involved in the immobilization).[67]

The versatility of these supports is not as high as in the case of the glutaraldehyde activated supports. Now, the direct covalent attachment is not possible at industrial scale due to its slow rate of proteins on epoxy-supports, and thus only hydrophobic adsorption is possible.

An exception may be once again found in lipases. Due to the tendency of these enzymes to become adsorbed via interfacial activation on hydrophobic surfaces, they can also become

adsorbed on this fairly hydrophobic supports.[53, 55, 68] Thus, using low ionic strength during immobilization, the lipases will become immobilized via interfacial activation on the hydrophobic support in a quite rapid fashion (figure 8).[55] Using high ionic strength, the lipase molecules tend to be in the closed form, due to the highly unstable large hydrophobic pocket that becomes exposed to the medium in the open form. In fact, it has been reported that lipases can be immobilized in a slower way on octyl-agarose when the ionic strength in increased.[68] Thus, under these conditions, adsorption via hydrophobic external residues of lipases may be favored versus interfacial activation. This has been exemplified using the lipase B from *Candida antarctica*, that was immobilized at low and high ionic strength on standard Eupergit. The resulting enzyme preparations showed different features (stability, activity and selectivity).[69]

The blocking step is recommended to prevent unwanted covalent reactions between enzyme and support, and it has also been used to solve the hydrophobicity problem of these supports (negative for enzyme stability) (Figure 7).[62] The problem is more relevant when the geometrical congruence between enzyme and support is high, but on the other hand only when this good geometrical congruence occurs should we expect a very high stabilization of the enzyme.[6, 7] In most cases, the hydrophobicity of the supports will be detrimental for enzyme stability as it may stabilize some inadequate conformations of the enzyme, as if it was a gas bubble.[6] This negative effect may even mask the enzyme rigidification obtained via multipoint covalent attachment.[62] This has been partially overcome using hydrophilic molecules to block the remaining epoxy supports after enzyme immobilization, such as amino acids.[62] This way, a very high enzyme stabilization has been achieved using these supports in some instances.[62]

2.3. Other multifunctional supports

From the aforementioned examples, it is evident that many immobilization supports may be in fact multifunctional ones. In some instances the multifunctionality may derive from the intrinsic properties of the matrix itself: in some cases it may be ionic (chitosan),[70] in others

hydrophobic.[58-60] In some specific cases, the spacer arm that we introduce having the covalent functionality may be enough to provide this multifunctionality, since many of these groups are not physically inert.[49, 53] For example, long spacer arms composed of just CH_2 chains will have a hydrophobic character. Similarly tosyl chloride may be considered hydrophobic.[71, 72]

In other cases, the new functionality may be directly derived from the inactivation of the active group of the enzyme during enzyme immobilization. For example, oxidation of aldehydes may produce acids able to immobilize proteins via cationic exchange. Thus, the researcher must identify the capability of the support to interact with the enzyme through different mechanisms, and design the experiments to take advantages of this multifunctionality, or discard the support if the unspecific functionalities produce a negative effect on enzyme features.

3. New tailor-made heterofunctional supports

The aforementioned examples have shown supports whose heterofunctionality was a property inherent to the support preparation or nature of the matrix, not produced by design. However, the case of commercial hydrophobic epoxy supports was the source of the starting hypothesis that finally originated tailor-made heterofunctional supports.[63, 73-75] They were designed so as to fulfill the requirements for the specific use that they were produced for.[67] The idea was to have a support surface as full as possible of groups able to produce a covalent reaction with the enzyme (the objective will be to have an intense multipoint enzyme-support covalent attachment), and other moieties able to produce a first immobilization of the enzyme (Figure 9).[67] The key point was to find conditions where the rate of enzyme immobilization produced by the main chemical group of the support was negligible compared to the immobilization rate produced by the groups responsible for the first protein immobilization.[58]

Now, we will discuss the ideal properties of the main group in the support and how some of the different existing supports may be near these requirements. Next, the ideal and actual

387 groups that result in adsorption will be revised. Finally, some specific uses of these new tailor-
388 made requirements will be presented.
389
390 **3.1. Designing an ideal heterofunctional support to obtain an intense multipoint covalent**
391 **attachment**
392 In order to have an intense multipoint covalent attachment, whatever the support
393 groups used, it is necessary to use supports offering large internal surfaces (Figure 10) .[6] Only if
394 these supports offer a high enough geometrical congruence with the enzyme, the enzyme–
395 support interaction may involve many groups of the support and the enzyme, and thus produce
396 an intense multipoint covalent attachment.[7] Thus, supports formed by thin chains, of a diameter
397 similar or smaller to that of the protein, can hardly yield many enzyme-support bonds, while
398 supports having large internal surfaces, like very thick cylindrical chains (agarose),[30] or pores in
399 solid materials (porous glass, Sepabeads) may permit intense enzyme support interactions.[14, 62]
400 Another requisite is that many reactive groups of the support should be under the surface
401 of each protein molecule.[7] Only if there are many reactive groups of the support under the
402 enzyme surface, the involvement of most of the available enzyme groups on the enzyme-support
403 multipoint covalent attachment can be expected (Figure 10). Thus, only supports having very
404 high surface densities of reactive groups will be useful to produce an intense multipoint covalent
405 attachment.[6, 7]
406 However, even using an adequate support, the heterofunctional support may only give an
407 intense multipoint covalent attachment if the reactive groups fulfill some features, as discussed
408 below.
409
410 **3.1. The main chemical group**
411 **3.1.1. The ideal group**

As stated above, the first requirement of a suitable group to be used to obtain an intense multipoint covalent attachment on a tailor-made heterofunctional support is that it must be unable to immobilize by itself the protein under the conditions used in the immobilization, or do this at a negligible rate.[58]

However, after enzyme immobilization controlled by the secondary group, the support should be able to give an intense multipoint attachment.[7] Thus, it appears convenient that, once the enzyme is immobilized, the main groups in the support should be able to react with lateral groups of amino acids that are abundant on the protein surface, without any kind of previous protein activation step that could produce some deleterious effect in the enzyme activity or increse the complexity of the process.[7] Primary amino groups (of the Lys chain and terminal amino groups) may be the most interesting ones. They will be mainly placed on the enzyme surface, exposed to the medium, and its non-ionized form will be reactive without any activation step.[7, 30] Carboxylic groups may be the most abundant in most enzyme surfaces, but they will usually require some activation step to react with the supports.[76-78] Other protein groups such as hydroxyl groups (Ser), phenol (Tyr), imidazol (His) or thiol (Cys) may be also reactive with certain groups but will not be so abundant on the enzyme surface. The other enzyme groups (aliphatic chains, amides) will be neither very reactive nor abundant on the enzyme surface.

The main properties of a support group to give an intense multipoint covalent attachment have been summarized in different papers.[7, 29, 30, 63, 79, 80] Here we point out the most relevant ones:

- The steric hindrances for the reaction between the enzyme and the support groups should be as low as possible, as the reaction between an already immobilized enzyme and a support, both rigid and non-complementary structures, may be complex enough to give good results even when adding additional problems. (Figure 11)

- The stability of the groups should be high under conditions where the enzyme reactivity with the support may be adequate. The maximization of the enzyme-support reaction takes a

longer time than the first immobilization, as it requires the correct alignment of groups placed on rigid and non-complementary structures. (Figure 12)

- The spacer arm should be long enough to avoid the support surface from generating steric hindrances for the reaction with the enzyme, and short enough to transmit the rigidity of the support to the enzyme. Longer spacer arms may in principle permit the production of more enzyme-support bonds (they have more mobility and may even involve protein regions far from the support surface), but the mobility of the spacer arm will generate a lower enzyme rigidification (Figure 13).

- A reaction end point that can generate a chemically and physically inert support. As stated before, any uncontrolled enzyme-support interaction may generate problems during storage or use of the immobilized enzymes.

Obviously, it is not simple to find activated supports that simultaneously show all these requirements. Next, we will show the two supports that are nearest to the whole set of requirements.

3.1.2. Epoxy supports

As previously discussed in this review, epoxy activated supports may react with many different groups present on a protein.[58, 59, 62, 63, 67] In fact, they are an exception concerning their reactivity with the groups of a protein; they can react with amino and carboxylic groups without the enzyme undergoing any treatment.[59] They can also react with phenol, hydroxyl or thiol groups.[59] Epoxy groups are also stable, have low steric hindrances for the reaction with the protein, have short spacer arms, and are usually highly activated (there are available supports bearing between 15 and 20 epoxy groups / 1000Å^2).[58, 62, 63, 67] Moreover, they can be blocked after enzyme-support reaction, using different compounds to have a final inert support.[62, 67] Most importantly, they immobilize proteins at any pH value in a very slow fashion, making it easy to find first immobilization causes far more rapid than the epoxy covalent reaction.[58]

Apparently, they seem to be ideal groups to give intense multipoint attachments, and in fact they have permitted to reach very good stabilization factors in some cases.[33, 62, 67, 81] Thus, epoxy heterofunctional supports were the first approximation to build tailor-made heterofunctional supports, and as we will show later, most reported examples are based on these supports.

Carboxylic groups react very slowly with epoxy groups, and even this low reactivity requires that the enzyme-support is incubated at acidic pH,[59] while amino groups react better at alkaline pH[61, 62] (a first incubation at acidic or basic pH followed by incubation at basic or acidic pH value may be a good strategy to involve both groups). Nevertheless, the fact is that the reactivity of the epoxy supports with the groups of a protein is so low that even after the first enzyme immobilization, when the concentration of reactive groups of both protein and support is very high, the covalent reaction takes a long time even using appropriate pH values, and enzyme stability continues to increase even after 10 days of immobilized enzyme-support reaction.[67] Thus, even though the epoxy groups have offered in some cases good results, and fulfill many of the aforementioned requirements, the use of a more reactive group seems to be necessary to reach very high enzyme-support reaction.

3.1.3. Glyoxyl supports

Glyoxyl supports have been described as very suitable supports to give an intense enzyme-support multipoint covalent attachment.[7, 29, 30, 80]

From the requirements described above, they fulfill most of them: very high stability, low steric hindrances for the reaction with a protein, short spacer arm, etc. One limitation is that they can only react with non-ionized primary amine groups of a protein, reducing the "theoretical maximum" number of enzyme-support bonds when compared to epoxy supports.[30, 59] Another problem is that the end point of the reaction must be a reduction step, and this is necessary to have an inert support as well as to transform the labile imine bonds in strong

secondary bonds.[30] For some companies, this reduction step using sodium borohydride may become a problem.[30]

The reversibility and weakness of the Schiff´s base formed by glyoxyl and amino groups is the point that has made these supports almost ideal to get an intense multipoint covalent attachment when used as monofunctional supports.[29] The reason is that this reversibility means that the enzyme only becomes immobilized on the support when there are several enzyme-support bonds (Figure 14).[29] Thus, using monofunctional glyoxyl supports, the enzyme is immobilized by the area where it is easiest to directly yield several enzyme support attachments simultaneously. Furthermore, that area is the one where the density of amino groups is higher and where the most intense multipoint covalent attachment may be expected.[7, 29, 30, 79]

As a second consequence, a glyoxyl-support can only immobilize a protein under conditions where the enzyme presents several non-ionized amino groups.[30] That means that the support can only immobilize most proteins at alkaline pH values.[29] A glyoxyl support at pH 7 should be unable to immobilize most proteins. Thus, we can control the immobilization of the protein by the secondary groups of the support, as it is our objective (Figure 9).

As an exception, proteins formed by several peptide chains (multimeric or proteolyzed proteins), that may have several terminal amino bonds, could become immobilized at neutral pH values on glyoxyl supports (Figure 14).[79] In fact, this has been used to immobilize, purify and stabilize multimeric enzymes,[24, 82] but now it may be considered a problem in the design of heterofunctional supports. The use of pH 5 during the first immobilization could solve this problem, because at this pH value even the terminal amino groups of the enzyme will be scarcely reactive.[29] If that decrease in the immobilization pH value is not convenient for any reason (e.g., enzyme stability, lack of adsorption of the protein via the secondary group), there are other solutions to prevent the first covalent immobilization of the protein on glyoxyl supports. Borate buffer reduces the reactivity of the glyoxyl groups, while small aminated compounds such as Tris buffer, ethanol amine, etc., may act as competitors for enzyme

immobilization.[29, 30] In some cases, by just using 10 mM Tris buffer, enzyme immobilization was fully avoided on highly activated glyoxyl agarose even at pH 10.[83] Any compound able to stabilize the created Schiff´s base should be avoided during the immobilization process to prevent a direct covalent enzyme-support reaction, like thiolated compounds[84] or cyanoborohydride.[85]

Thus, it is possible to find conditions where any enzyme cannot become covalently immobilized on glyoxyl supports. However, after enzyme immobilization via the secondary groups, the increase in pH and the elimination of any inhibitor to the aldehyde-amine reaction will permit the reaction between the glyoxyl groups and the non-ionized amine groups of the protein.[29, 30, 80] And glyoxyl supports have showed to be able to give impressive stabilization factors.[30]

Although glyoxyl groups can only react with primary amino groups, it is possible to develop relatively simple strategies to increase the reactivity of the protein with the support.

For example, the chemical amination of the enzyme, for instance using ethylenediamine and activating the carboxylic groups with carbodiimide, has been employed with good results in many examples to increase the number of enzyme-support bonds (Figure 15).[19, 76-78] Using heterofunctional supports, this strategy requires great care, as the new amino groups will have a lower pK and may have some reactivity with glyoxyl supports even at pH 7.[76-78]

Moreover, enrichment of Lys residues on the target area via genetic manipulation has been utilized in some other examples (Figure 15).[18, 86-90] In this case, we can focus on the area where we intend to immobilize the enzyme, leaving the other areas of the protein unaltered.

In any case, it has been recently shown that the higher reactivity at alkaline pH values of glyoxyl groups, when compared to epoxy supports, causes these supports to give a higher number of enzyme-support linkages and, therefore, higher enzyme stabilization.[91]

3.2. The secondary group

This group is the one that should cause the first immobilization of the enzyme on the heterofunctional support. That is, it should be the one that produces the orientation of the enzyme on the support. The nature and concentration of this group will depend on the final objective pursued for the heterofunctional support (see section 4). A support having the same main group may still present different secondary groups. Thus, it may be possible to attach the same enzyme with different orientations regarding the support surface via the same chemistry.

3.2.1. The ideal secondary groups

In this case it is hard to give general rules, as the secondary group should become adapted to the final objective of the heterofunctional support (see below). Apart from the capacity of generating a moderately rapid enzyme immobilization on the support, a general characteristic should be that the secondary group should produce the lowest steric hindrances possible to the subsequent multipoint covalent attachment with the primary group. Thus, bulky groups over the layer of chemically reactive groups of the support may not be very convenient.[6, 18]

3.2.2. Groups able to immobilize proteins via general interactions between enzyme and support

Almost any group able to adsorb proteins may be used. The larger the battery of secondary groups, the higher the possibility of altering the area of the protein that is going to be rigidified via multipoint covalent attachment and, the larger the final library of biocatalysts that will be obtained. We will give a rapid summary of the main groups used to this goal.

Physical adsorption of proteins is a quite rapid phenomenon. As stated above, the original epoxy supports already use the concept of heterofunctional supports by using hydrophobic adsorption of the protein on their hydrophobic matrix.[62, 63, 67, 73-75]

Cationic or anionic groups may produce enzyme adsorption via ionic exchange.[32, 58, 92-95] As explained for the glutaraldehyde supports, ionic exchange requires the involvement of several groups of the enzyme and the support to fix the enzyme to the support.[34, 49]

Metallic chelates are other groups able to absorb proteins by interactions with different groups of the proteins, the imidazol groups of His give the stronger interactions, but also Cys or Tyr may be involved in the adsorption process.[35-40, 58, 96-99] Among the transition metals used in this adsorption, the one that produces a stronger adsorption of the enzyme on the support is Cu^{2+}, while others like Zn^{2+} or Co^{2+} produce weaker adsorption.[40] This should be considered depending on the objectives. The immobilization of proteins requires the interaction of two His groups with the supports. Usually, this involves two different immobilized metal chelate groups, but if the enzyme has several His groups in its vicinity, this phenomenon may be produced in just one metal chelate group (this is the case of the poly-His tagged proteins).[81, 100, 101]

Immobilized phenyl boronic acid has also been used as secondary group.[58, 102-106] Although they form bonds with sugars and cis-alcohols,[107-112] it has been shown that they can immobilize most of the protein of a crude extract of *E. coli*.[113] As these proteins are not glycosylated, other mechanisms seem to be involved in the adsorption of the protein on boronic activated supports.[114]

Dyes may be also used to adsorb proteins, with a higher or lower affinity for a certain kind of proteins,[115-117] and later yield a covalent reaction. However, they are bulky and may promote severe difficulties to give an intense multipoint covalent attachment; thus, these are not recommended for this application. Nevertheless, if a further rigidification of the enzyme is not pursued and only some covalent linkages are intended, these dyes may be a complement to the other more general secondary groups.

Initially, the groups were introduced by modification of the support main group (e.g. epoxy groups).[58] A preliminary optimization of the support modification degree was necessary: the higher the modification, the faster the protein adsorption.[58, 69] However, the covalent

593 immobilization rate started to decrease when less available groups able to give covalent reaction
594 with the enzyme were left in the support (Figure 16). Thus, a compromise solution in this
595 support modification is necessary to achieve both high adsorption and high covalent reaction
596 rates.[67] Furthermore, this dependence on the first covalent reaction between enzyme and support
597 on the modification of the epoxides on the support advanced the likely effect of this
598 modification on the more complex multipoint covalent attachment.

599 However, as stated above, in most cases several adsorbing groups should be under the
600 protein surface to produce the first immobilization. This is necessary to permit the essential first
601 multipoint adsorption, and also to have this phenomenon at a reasonable rate. This layer of
602 adsorbing groups has a double negative effect on multipoint covalent attachment: they decrease
603 the amount of reactive groups and, even more importantly, they can generate some steric
604 hindrances to the reaction between enzyme and support (Figure 17).[18] As stated above, steric
605 hindrances for the enzyme-support reaction may become a serious problem when an intense
606 multipoint covalent attachment is pursued, even if very small groups are used.

607

608 **3.2.2.1. Second generation of supports**

609 Due to the problems to yield a very intense multipoint covalent attachment of enzymes
610 on the first generation of heterofunctional supports, these supports were mainly used to alter the
611 enzyme orientation, but could hardly highly rigidify the target areas of the proteins.[18, 58, 67] These
612 problems are not present when using the standard epoxy hydrophobic supports, where the
613 support matrix is the adsorbent and the epoxy groups are over it.[61, 62]

614 The coupling of both ideas permitted to design a new generation of heterofunctional
615 supports[118] that overcame the limitations of the first generation of heterofunctional supports.[58] In
616 this second generation of heterofunctional supports, the adsorbent groups were in the same
617 spacer arm as the epoxy groups, and nearer to the support surface (Figure 18).[118] This idea
618 reduced the problems of the first generation of heterofunctional supports.[58] First, as there is no

competition between secondary and primary groups in the support, the adsorption rate and covalent reaction may be maximized. Second, the support-enzyme reaction does not have any steric hindrances generated by the adsorbing groups.[67]

However, this is not a fully ideal solution. The first problem is that it is not so easy to design spacer arms having protein adsorbents and chemically reactive groups. Now, the company Resindion (Milan, Italy) has commercialized amino-epoxy supports (Figure 19).[118] The idea is based on the modification of a reactive support with a bifunctional reagent. One group reacts with the support; the other is used to react with epiclorhydrin or other similar compound to obtain a group reactive with proteins. To get amino-epoxy supports, epoxy supports have been used, and ethylenediamine has been utilized as the first modifying compound.[118] Using other heterofunctional molecules to modify other activated supports may be feasible to produce other kind of heterofunctional supports, although we have not found any examples in the literature. Even when using just amino-epoxy supports, as stated above, the anionic exchange may involve different regions of the enzyme depending on the pH or ionic strength; therefore it may be possible to get different enzyme orientations on the support.

This strategy of building the heterofunctionality generates a second problem; the spacer arm is longer than using the original epoxy support, and as commented before, this may produce more enzyme-support bonds but with a lower stabilization effect (Figure 13). Moreover, if using epoxy groups, the secondary amino bonds may be also attacked by the epoxy groups, finally reducing the reactivity stability of the activated support.[118]

The last problem is that the support will not be fully inert after enzyme multipoint covalent immobilization. This may be partially solved using the adequate blocking reagents if epoxy groups are used as main groups, but in the best situation a mixed ionic exchanger will be generated, and even if the net charge of the support surface is null, they are still able to produce ionic exchangers with immobilized proteins.[119] This is even more relevant considering that the protein is very near to the support surface.

In any case, the results reported using the first generation of supports bearing epoxy and amino groups are worse than the results obtained using amino-epoxy supports, suggesting that the advantages of these new supports are more important than their drawbacks.[58, 67, 118, 120]

A solution closer to the optimal one may be if the final system is near to the current standard epoxy supports, that is, if the support matrix has an ionic nature and may be activated with epoxy groups (e.g., chitosan) (Figure 20).[48] The main risk here is the crosslinking of the support during activation, which will reduce the number of active groups.

Thus, even though the results are promising, more efforts are necessary to get heterofunctional supports that are nearer to fulfilling the whole set of requirements.[18]

3.2.3. Site-directed immobilization/rigidification of enzymes

The adsorbent groups of previous heterofunctional supports are based on the general mechanisms of adsorption of proteins, which present as main usefulness the ability to immobilize the same enzyme without any treatment via different adsorption events (and for this reason, very likely by different protein areas) and finally rigidify the area of the enzyme involved in the adsorption process. Although that area is quite well defined when immobilizing an enzyme in a particular support (given the multipoint nature of most adsorption processes), the exact area of the protein that participates in the immobilization may not be easily guessed even in those cases where the protein structure is available.[18, 41] The distance between enzyme groups (that should match that of the support adsorbent groups), disposition to interact with the support, and/or susceptibility towards the interaction, in many cases, may seem to point to several regions of the protein, even though actually only one will be the predominant.

The next step would seem obvious, and may fulfill the dream of an enzyme technologist. Through the available tools, it may be easy to locate exposed residues on target areas of the protein and then to introduce mutations on these amino acids placed on the enzyme surface.[18] If this site-directed mutagenesis is coupled to the design of tailor made heterofunctional supports,

671 this may permit to immobilize different mutants of the same enzyme, using the same support,
672 involving very different areas of the protein (Figure 21).[18] This is a difference with the use of
673 the previously discussed heterofunctional supports.[58, 67] While using the aforementioned
674 supports the versatility of the immobilization arose from changing the adsorbent group in the
675 support, without modifying the enzyme, now the versatility of the immobilization came from a
676 change on the enzyme surface, while maintaining the support unaltered. Nevertheless, it is
677 compulsory to know the structure of the enzyme (or that of an analogous protein) and a plasmid
678 with the gene that codifies the protein in a suitable host to produce a battery of mutant enzymes
679 that will be immobilized on the same support. We can choose any area of the protein to interact
680 with the support and be completely sure of the first group involved in the immobilization
681 process (Figure 21). In contrast, using the initial heterofunctional supports,[58] a battery of
682 different supports was required, but the gen and structure of the enzyme was not necessary.
683 However, this previous strategy did not permit a full site-directed control of the enzyme
684 immobilization.[18]

685 The coupling of site-directed mutagenesis and immobilization has been recently
686 revised;[18] here we will call the attention upon the main features that the heterofunctional support
687 should present. In general, a single mutation on a protein surface may be expected to produce
688 small alterations on the overall enzyme properties. In any case, the objective here is not to
689 improve enzyme properties, only to direct the enzyme on the immobilization.[18]

690 The group in the protein used to orientate the enzyme on the support should be one
691 with very scarce presence on the enzyme surface. Two have been the most widely used groups
692 to orientate proteins. The first one is the imidazol groups of His, using a support containing
693 immobilized metal chelates,[33, 35, 37-40, 81, 97, 100, 101] and epoxy[67] or glyoxyl residues (Figure 22).[30]
694 Histidine residues are not very frequent on the enzyme surface, but as it has been discussed
695 above, proteins only become adsorbed on an IMAC support if several enzyme-support
696 interactions are established.[33, 35, 97, 98, 121] Usually, this is produced between several His residues

in the enzyme surface and several immobilized metal chelates in the support.[33, 35, 37-40, 81, 97, 100, 101] But if a couple of His residues are sufficiently close, they can directly adsorb the protein via interaction with just one chelate.[81, 100, 101] A poly-His tag may be used, but this leaves only two likely orientations for the protein, the amino and the carboxyl terminal positions (Figure 22).[18, 33, 81, 100] It is a better solution to introduce new His residues near other present His groups (Figure 22).[122, 123]

If in an area there are no His residues, it is possible to introduce a couple of His placed in an adjacent position on the enzyme surface.[122] Some examples of oriented immobilization of proteins directly on IMAC supports may be found in the literature,[124, 125] but not using heterofunctional supports (although some poly-His tagged proteins have been immobilized on IMAC-epoxy supports, the objective was other, as discussed below).

The other group used to attain an oriented immobilization of enzymes is the thiol group of Cys, immobilizing the enzyme via thiol/disulfide exchange, a very specific reaction that cannot be produced by any other group on the enzyme (Figure 23).[65, 126-133] Cysteine groups are quite scarce on the protein surface, and when needed, if the native enzyme has some external Cys, it may be transformed into Ser via site-directed mutagenesis, as the physical properties of both lateral chains are somehow similar. To achieve an immobilization fully directed by the Cys location, there are two possibilities: to use thiol reactive disulfide groups on the support[134, 135] or to generate it on the enzyme (e.g., by modification of the exposed Cys of the enzyme with 2,2-dipyridyl disulfide) (Figure 24).[136, 137]

The strategy is to introduce site-directed mutations on the enzyme surface that permit to have enzymes with just one Cys on the target position.[136, 137] We can produce as many mutant enzymes as desired, involving many different enzyme regions. The use of supports bearing some thiol reactive groups and a dense layer of glyoxyl[138] or epoxy[136, 137] groups may permit to rigidify the selected regions (Figure 23). Epoxy groups are able to immobilize enzymes directly via a thiol group, but at a much lower rate than the disulfide exchange; in fact immobilization

723 may take hours even when using a high concentration of support.[64] Thus, immobilization using
724 epoxy –thiol reactive supports is necessary to have adequate immobilization rates.

725 The tendency of medium exposed Cys to become oxidized creates the necessity for the
726 enzymes to be reduced just before the immobilization process, and even if the immobilization is
727 slow, some Cys may become oxidized again before immobilization, reducing the overall yield.[65,
728 66]

729 Current epoxy/thiol supports have been prepared using SH that reacts with a
730 percentage of the epoxide groups in the support. This is a quite small group; therefore it should
731 generate very low steric hindrances for the enzyme-support multipoint reaction that should be
732 the final objective after the directed immobilization.[18, 136, 137] However, in the few reported trials,
733 the support is activated as disulfide, not the enzyme, and in that case the steric hindrances for the
734 enzyme-support reaction are higher. In fact, reported stabilization factors are quite poor and that
735 has been attributed to these steric hindrances.[18, 134-137]

736 One further question remains. At first glance, just one Cys group may not fully
737 determine the area of the protein involved in the immobilization; a point does not determine a
738 planar surface. The use of a couple of near Cys residues, that should produce a fully controlled
739 orientation, is also risky. The support should present many thiol (or thiol reactive) groups to
740 involve both Cys residues in the immobilization, or this second Cys group will only increase the
741 indetermination of the enzyme orientation as the enzyme could be immobilized by one or the
742 other Cys. Moreover, a high number of thiol groups under the enzyme molecules should
743 produce a poor multipoint covalent attachment between the other nucleophile groups of the
744 enzyme and the epoxy or glyoxyl groups placed on the support surface.

745 After these appreciations, we would like to clarify that the actual situation is much
746 simpler. Considering the importance of the group reactivity and distance of the groups of the
747 protein to give the first enzyme-main group in the support reaction, we can be quite sure that in

most cases the final area of the protein involved in the immobilization will be almost fully determined by the Cys position.

As in the previous heterofunctional supports, a strategy that can permit to have the epoxy or glyoxyl groups *over* the thiol reactive groups may be a solution to really take full advantage of this strategy to get an intense and full site-directed rigidification of the enzyme.[18] Thus, even though these strategies are near to achieving full control over enzyme immobilization, more efforts seem to be necessary to optimize and take full advantage of them.

4.- Uses of heterofunctional supports

The multifunctionality of a support has at first glance an interesting effect; it gives some versatility to the immobilization of the protein. This means that different areas of the enzyme may become involved in the enzyme-support interaction, and that may be related to the activity and stability of the final immobilized enzymes.[18, 58, 137] However, tailor-made heterofunctional supports may permit to take advantage of their multifunctionality to solve some of the problems on the use of enzymes as industrial biocatalyst, like the purification of the proteins, the prevention of subunit dissociation (this may have a negative effect on enzyme stability and in any case will produce the contamination of the reaction medium and product),[24] etc. Next, we will show some examples and prospects of the uses of tailor-made heterofunctional supports.

4.1. Immobilization/purification of enzymes by using tailor-made heterofunctional supports.

One of the problems of the use of enzymes as industrial biocatalysts is the interest of using them with a reasonable degree of purity. This is important to maximize the volumetric activity, and even more, to avoid other enzymes present in the preparation producing some modification on substrates or products, thus decreasing the selectivity or specificity of the final biocatalyst.[139] On the other hand, purification is a time-consuming and expensive process.[116, 140,

[141] However, as enzyme immobilization is in most cases another necessary process to build an industrial biocatalyst, any strategy that may be used to simultaneously purify, immobilize and stabilize the enzyme, should be considered an important advance in enzyme technology.[6]

This has been obtained in certain cases just using monofunctional supports. In general, any strategy that permits a preferential adsorption of the target protein on a support may give a significant purification.[29, 32, 68, 94, 142, 143] However, if the forces that keep the enzyme on the support were just strong enough to maintain the enzyme in its immobilized form during use, this may be considered an immobilized biocatalyst. Furthermore, most of the described selective adsorptions are based on a low activation of the support to prevent uncontrolled multipoint-enzyme interactions and that produce mild adsorptions, very positive in purification, but not so much in immobilization.[36, 94, 142, 144]

However, there are some cases where monofunctional supports have reached a reasonable success in the one-step purification and immobilization of some enzymes. The purification-immobilization-stabilization of lipases on fairly hydrophobic supports *via* adsorption of the open form of the lipase (interfacial activation) is one of the most successful examples.[55, 68, 143, 145] This immobilization results in a strong adsorption, although there are some risks of desorption in the presence of detergents or organic cosolvents, solved by chemical crosslinking of the immobilized enzyme molecules.[146, 147]

In other examples, poly-His tagged enzymes have been purified-immobilized using IMAC matrices.[148-150] This has some more risks of enzyme desorption, as the metal chelate may become desorbed from the support and release the enzyme (and also contaminate the products). Another possibility is the use of immobilized antibodies,[151, 152] by the use of which purification during immobilization will be almost guaranteed, but stabilization may be very short and the matrix may be far more expensive than the enzyme we want to immobilize.[153-155]

The use of tailor-made heterofunctional supports has been a solution, as we can now design as weak an enzyme adsorption as desired, because finally the enzyme will covalently

react with the support. Next, we will show some examples where this idea has been fruitfully employed.

4.1.1 One step purification-immobilization-stabilization of multimeric enzymes

As it has been discussed in this review, ionic exchange of proteins on anionic exchangers or adsorption of proteins on IMAC supports is generally performed via multipoint adsorption.[18, 34, 36]

Focusing on ionic exchange, it is necessary that several counter-ions that will be interacting with the ionic groups on the support may be exchanged by several ionic groups on the enzyme (that will have also their corresponding counter-ions) to fix the protein on the support.[156, 157] The number of interactions that needs to be established between enzyme and support will depend on the ionic strength (as they can act as competitors in the exchange) and the pH value (that will control the ionization of the enzyme and support groups).[32, 157] In opposition with some extended ideas, a protein may become adsorbed on both, anionic and cationic exchangers even at the same pH value, mainly using immobilized ionic polymeric beds.[92] Besides, it has been shown that a high percentage of the proteins contained in a crude protein extract becomes adsorbed on supports having the same amount of cations and anions.[119] More importantly, some proteins that cannot become adsorbed on equivalent cationic or anionic exchangers, may become adsorbed on this mixed ionic exchanger supports.[119] The critical point is the possibility of establishing several enzyme-support ionic interactions.

Once this multipoint nature of ionic exchange is established, it seems obvious that a large protein may establish interactions at a longer distance that small proteins.[94] It was shown that using supports having a very low density of cationic groups on the support surface (around 2 residues / 1000Å^2), only large multimeric proteins could become adsorbed on the support, even though they can become desorbed at very low ionic exchange levels.[94] The next step was the development of heterofunctional amino and epoxy supports first and amino and glyoxyl

supports later.[45, 91, 158] The idea was to progressively decrease the number of amino groups on the support and use the lower activation on the support which could produce the adsorption of the target protein. This strategy not only permitted to immobilize large proteins selectively, but also to cause the enzyme to become immobilized by the largest area of the enzyme, that will be that where longer distances may be covered in the interaction with the support (Figure 25).[91, 158] This area of the multimeric proteins is that where more enzyme subunits area present. This multi-subunit immobilization produces a full prevention of the possibilities of subunit desorption or dissociation of the subunits involved in the immobilization, and also the increase in the rigidity of the maximum number of monomers.[91, 158] Thus, this immobilization strategy produces enzyme stabilization by both factors, stabilization of the tridimensional structure of the enzyme by multipoint covalent attachment and stabilization of the quaternary structure of the enzyme via multisubunit immobilization (Figure 25). Enzymes become purified from smaller proteins and from those unable to become adsorbed on the less activated cationic exchangers under those conditions. This may permit to reactivate the immobilized multimeric enzymes by unfolding-refolding strategies.[159] This will not be possible unless all enzyme subunits are immobilized.

A further step was to find situations where only one large multimeric protein is presented in a protein preparation. Extracts from mesophilic microorganisms hosting a multimeric thermophilic enzyme was one of these situations: a thermal shock produces the destruction of all mesophilic multimeric enzymes that precipitate.[36, 94] The supernatant contains just small proteins together with the large multimeric and thermophilic enzyme that may be purified (almost to homogeneity) and stabilized via immobilization on tailor made amino-epoxy or amino-glyoxyl supports.[45, 91, 158, 159]

IMAC supports having a low activation degree have been shown to be able to only immobilize very large proteins: the lower the activation on the support, the larger the proteins adsorbed on it.[36] This adsorption is quite weak, which becomes positive if just purification is

intended, but it is not useful if an immobilized biocatalyst is the main goal. However, this interesting idea has not been further developed in heterofunctional supports, where a combination of immobilized metal chelate and epoxy or glyoxyl supports may permit similar results to those obtained using ionic exchangers. Perhaps, the main reason is that IMAC-heterofunctional supports have been used for one specific case, the poly-His tagged proteins, as we will show below.

4.1.2. One step purification-immobilization-stabilization of poly-His tagged proteins.

Poly-His tagged proteins may become adsorbed via interactions between several His in the tag and just one immobilized metal chelate in the support, while native proteins having His on the surface require the interaction of several His residues with different immobilized metal chelates in the support (except if a pair of His are near enough to interact with one metal chelate).[35-40, 58, 96-99] Thus, poly-His tagged enzymes have been usually purified by using very low activated IMAC supports, having metals with low affinity, and using short spacer arms, conditions where one-point interactions have preference to multipoint interactions.[100] This permits very high purification factors for the enzymes, but the immobilization is relatively weak.[100]

The use of a heterofunctional support for enzyme immobilization seems to be an answer to solve this problem and to reach all the objectives. In fact, the immobilization of poly-His tagged proteins on heterofunctional epoxy-immobilized metal chelates (Figure 26) was the first instance of one step purification and stabilization via immobilization on heterofunctional supports, with very positive results enabling almost full purification of a glutarayl acylase[81] and later of a β-galactatosidase from *Thermus thermophilus*,[33] obtaining very high stabilization factors. Thus, the potential use of this kind of supports has been clearly established. Examples using IMAC-glyoxyl supports for this goal has not been reported to date, but at first glance, results should be similar to that described using epoxides, and owing to the greater potential to

878 stabilize enzymes of glyoxyl groups,[91] results may be expected to be even better than the
879 reported using epoxy supports.
880
881 **4.2. Rigidification of different areas of the enzyme**
882 The use of heterofunctional supports to immobilize enzymes may permit to alter enzyme
883 orientation on the support surface, involving different regions of the enzyme on the
884 immobilization process (Figures 9 and 21).[18, 58, 67] This means that different areas of the enzyme
885 may be protected or blocked by the support surface while other areas of the protein will be
886 oriented towards the reaction medium.[18] The protein area in contact with the support is the one
887 that may increase the rigidity via multipoint covalent attachment (rigidification that will be
888 transmitted to the whole protein structure), and also the most affected by the reaction with the
889 support groups. Orientation of the enzyme on the support may produce changes in enzyme
890 activity, stability, but also on the selectivity or specificity, as different regions of the enzyme
891 will suffer different distortions.[18]
892
893 **4.2.1. Effect on enzyme activity**
894 The orientation of the enzyme is a key point when Redox enzymes are involved and
895 the current of electrons must go via the support. This may work only if the active center is
896 properly oriented. The review from Hernandez and Fernandez-Lafuente[18] shows many examples
897 of this effect. However, they are mainly related to the use monofunctional supports to modify
898 enzymes, not to the use of heterofunctional supports. Nevertheless, enzyme orientation may
899 affect enzyme activity in many other cases.[108, 160]
900 The effect of orientation on enzyme activity is quite evident if the substrate is very large:
901 if the active center is not oriented towards the reaction medium, and depending on the protein
902 loading of the support, the expressed activity may be quite different (Figure 27).[18] If the
903 substrate is small, it is very likely that even if the active center is facing the support surface, the

substrate may reach the active center (Figure 27).[16] A clear example of this is the immobilization of lipases by interfacial activation on hydrophobic supports, whose activity, far from decreasing, even increases in this situation.[55, 68, 143, 145]

Involvement of key groups of the catalysis of the enzyme in the immobilization is not simple, as these groups will be mainly located in internal pockets, and therefore their access to the support surface will be minimal.

However, the situation is different considering the distortion generated by the enzyme-support reaction that may produce enzyme inactivation if the distortion is large enough.[18] If the distortion involves different areas of the protein, the effects of the immobilization may be quite diverse. Thus, using heterofunctional supports under identical immobilization conditions and, via the same chemistry, it may produce very different effects on enzyme activity by involving different regions with different relevance for the enzyme activity (Figures 9 and 21).[58, 67, 118, 161] One of the most extreme cases is the immobilization of the β-galactosidase from *Aspergillus niger* on epoxy supports,[120] that produces an almost inactive preparation using hydrophobic adsorption and retains almost 100% of the activity if using cationic exchange as first immobilization cause.

4.2.2 Effect on enzyme stability

As previously commented in this review, one of the most important goals of enzyme immobilization is the improvement of enzyme stability.[4, 5] The low stability of enzymes under operational conditions is one of the most relevant drawbacks that limit their industrial implementation.[7, 14] Multipoint and multisubunit immobilizations have revealed themselves as one of the most powerful tools to solve this limitation.[7, 29, 30]

Orientation of the enzyme on the support has two main effects on the final enzyme stabilization that may be achieved by immobilization.[18] The first one is due to the fact that not all enzyme areas will have the same density of groups able to react with the support. This way,

the first immobilization involving one or other enzyme area will determine the maximum degree of multipoint covalent attachment that may be achieved under ideal conditions. The second one is related to the fact that not all enzyme regions have the same relevance for enzyme stability.[27, 28, 162, 163]

There are regions more labile and relevant for enzyme activity and others more rigid or less related to enzyme activity.[27, 28, 162, 163] Thus, even though an intense multipoint covalent attachment may have very significant effect on overall enzyme stability,[7] the ideal situation will occur where the multipoint covalent attachment involves the most relevant region for the enzyme stability and produces the maximum number of enzyme-support attachments.

Immobilization of enzymes on different heterofunctional epoxy supports under the same conditions generally produces quite different enzyme stabilities, as expected from the points raised above (Figures 9 and 21).[58, 67, 118, 164] However, using standard heterofunctional supports, it may be hard to fully identify the area involved in the immobilization in some instances, even when using advanced molecular dynamics programs and when the enzyme structure is available. In other instances, it may be simpler to identify the area of the protein involved in the immobilization, and this can help to identify the most relevant areas for enzyme stability and permit to further improve the enzyme immobilization, e.g., increasing the number of nucleophiles in this enzyme area (Figure 15).[86]

Using thiol-epoxy or thiol-glyoxyl supports, it has been shown how the immobilization by different regions of the enzyme penicillin G acylase may have different impact on enzyme stability depending on the enzyme area where the Cys was located and on the inactivating conditions.[137] Even though the stabilization factors reported in this paper were not as high as those obtained using standard monofunctional supports[61, 62] (due to the steric hindrances generated by the groups over the epoxy layer),[18] they have permitted to identify the more relevant areas of the protein for enzyme stabilization under different inactivating conditions, and that way the researchers could focus all efforts on improving the reactivity of this area of the

enzyme with the support (adding some Lys via site-directed mutagenesis).[137] In fact, the final engineered enzyme was directly immobilized on monofunctional glyoxyl supports:[137] the enzyme immobilization proceeds via the area of the protein where the density of Lys residues had been increased, with stabilization factors increased by several orders of magnitude after enzyme immobilization.[30] A second enzyme, a lipase from *Bacillus thermocatenolatus*, was also immobilized on thiol-glyoxyl and thiol-epoxy via different regions, with similarly different results in terms of stabilization.[138]

Thus, thiol reactive heterofunctional supports showed a great potential to identify the regions that may have more or less relevance in the enzyme inactivation under different conditions, and this information can hardly be obtained from the current level of the tools used in modeling and molecular dynamics.

To really obtain an optimal stabilization using heterofunctional supports, it is still necessary to design a support where there are no obstacles for the reactions between enzyme and support.[18] An ideal support should be able to rapidly react with the thiol group of the Cys under conditions where the other nucleophiles of the protein were not reactive at all, and then, upon changing the conditions, achieve a good general enzyme-support reactivity (epoxy supports may be near to this situation, but reactivity is too low to have real industrial applicability). As an ideal heterofunctional support, the thiol reactive group on the support should be below a dense layer of reactive groups (Figure 28).[18]

4.2.3. Effect on enzyme specificity and/or selectivity

Immobilization has been shown as a very potent tool to modulate enzyme specificity and selectivity, mainly when the enzymes have a flexible active center (subject to drastic conformational changes, like lipases or penicillin G acylase from *E. coli*) or multimeric enzymes.[7, 16, 26, 161, 165, 166] The immobilization will reduce the mobility of some areas of the protein, distorting others.[7, 26] The final result is a protein that cannot adopt the original active structure. This has been show using completely different immobilization techniques, in some

cases even inversion on the enantiopreference was obtained and, the same enzyme immobilized on different supports offered very different catalytic behavior and even different answers to changes in the medium condition (temperature, pH, etc).[7, 26, 89, 166-172]

This tuning of enzyme properties via immobilization may benefit from the use of a battery of heterofunctional supports, where the orientation of the enzyme on the support is different but the chemistry of the immobilization is the same (Figure 9). In fact, the modulation of the enantiospecificity of the lipase from *Mucor miehei* on hydrolytic reactions via immobilization on different heterofunctional epoxy supports is among the first examples of lipase properties tuning via immobilization.[168] Recently, it has also been demonstrated using the lipase B from *Candida antarctica* on transesterification reactions in organic media.[69]

The next step was to study enzyme modulation using thiol reactive heterofunctional supports to get a (almost) fully controlled site-directed rigidification of different enzyme areas (using a battery of Cys mutant enzymes with the Cys placed in different regions of the enzyme surface) (Figure 21).

In a first example, the enzyme penicillin G acylase from *E. coli* was submitted to site-directed mutagenesis and each mutant immobilized-stabilized via site-directed immobilization.[137] The enzyme was used in a kinetic resolution of chiral esters by hydrolysis. Using monofunctional thiol reactive supports, where no rigidification was observed, all the immobilized mutant enzymes exhibited the same specificity. Using thiol-epoxy or thiol-glyoxyl supports, most enzymes remained unaltered in its enantiospecificity, but one mutant doubled the value.[137] This result pointed out two important facts:

First, only enzyme immobilization via one point (the thiol exchange) has no effect on enzyme mobility or conformation and, therefore, maintains the enzyme features, even when altering the position of the enzyme regarding the support surface, in the case where the support did not promote any uncontrolled interaction with the enzyme.

Second, site-directed rigidification of an enzyme may permit to modulate the enzyme properties and identify the most relevant areas for the process.[137]

The same battery of immobilized Cys mutant enzymes was used in a more sophisticated reaction, a kinetically controlled synthesis.[137] This process involves the use of an activated acyl donor (in this case, as an ester), and the yields came from the balance between three reactions: the synthesis of the target product, the hydrolysis of the ester substrate and the hydrolysis of the product. The yields reach a maximum and then, they decrease as the medium may be even fully aqueous and the thermodynamic constant of the process may offer very low yield at equilibrium.[16, 173] Therefore, the yields are strictly determined by the kinetic properties of the enzyme (affinity by the nucleophile, ester and product, activity in the 3 likely substrates). Again, while all the one-point attached Cys mutant enzymes remained with almost identical behavior, one of the site-directed immobilized-rigidified enzymes preparations permitted a significant increase in the yields.[137] This mutant is the same that permits to increase the enantioselectivity and it holds the same position that produces a higher stabilization; the new Cys was introduced in the position 380 of the B chain of penicillin G acylase.[137]

Similar studies were performed using the lipase from *Bacillus thermocatenolatus* (BTL2).[138, 174] In this case, the immobilization via one-point permitted to improve enzyme features in some instances.[134] This may be based on the drastic conformational changes of this lipase during catalysis, the enzyme has a double lid and any hindrance to the movement of this complex structure may alter the enzyme properties.[175]

However, if thiol-glyoxyl supports were used, permitting a certain rigidification of the areas involved in the immobilization, the changes were more significant.[138] For example, the simple orientated immobilization by the BTL2-S334C on monofunctional disulfide supports gave ee > 99% in the asymmetric hydrolysis of phenylglutaric acid dimethyl diester but not in the kinetic resolution of *rac*-2-*O*-butyryl-2-phenylacetic acid (ee = 27%). On the contrary, the

site-directed rigidification of the BTL2-S334C variant on disulfide-aldehyde supports generated a fully enantioselective biocatalyst in both processes (ee > 99%).[138]

4.2.4. Co-immobilization of enzymes

This is the last example of the advantages of heterofunctionality of supports that we will include in this review. Co-immobilization of enzymes, working in cascade reactions, has advantages and drawbacks.[6, 176, 177] From a kinetic point of view, the second enzyme will be working using higher concentrations of product from the first enzyme, increasing the global reaction course (Figure 29).[178-185] However, co-immobilization of two enzymes causes the life of the biocatalyst to be determined by the stability of the weaker component.[6] Moreover, co-immobilization results in both enzymes needing to be immobilized on the same support, and in some cases optimal immobilization conditions for an enzyme may be quite far from the optimal immobilization conditions and support for the other enzyme.[6]

The use of a bifunctional or even a multifunctional support may be a very suitable alternative to immobilize two enzymes whose immobilization on the same monofunctional support may be complex. In this case, we do not intend that one of the groups on the support makes a first immobilization and then the other produces a covalent reaction. In this case, we intend that the support may be able to immobilize one enzyme using one kind of groups and the other enzyme using another kind of groups (Figure 30). The idea may involve two different reversible immobilization protocols (IMAC and ionic exchange, for example), or a combination of the groups from that support with groups able to covalently immobilize the enzyme. The advantages may be many. First, it is possible to immobilize the enzyme that requires the most drastic immobilization conditions, and in a following step, the second enzyme may be immobilized under milder conditions. This may not be an ideal strategy if both enzymes require to be very stabilized by immobilization to be usable, but it may be a good alternative when one of the enzymes is much more stable than the second under operation conditions, and this may be

immobilized on a support that may permit a high stabilization via multipoint or multisubunit immobilization.

We have been able to find just one example of this very nice strategy. In that paper, the researchers intend to co-immobilize different Redox enzymes, one to produce the target product and other to regenerate the consumed cofactor.[186] One of the enzymes was a poly-His tagged enzyme that becomes deactivated when immobilized on glyoxyl supports, while the other Redox enzyme was immobilized-stabilized via immobilization on this support.[186] The poly-His tagged enzyme could be readily immobilized on IMAC supports, preserving high activity.[186] Thus, both enzymes could be immobilized on the same particle using an IMAC-glyoxyl support, with good activity recovery. The authors went further. They used very low enzyme loadings compared to the capacity of the support. Using confocal measurements,[187-191] they showed that while the enzyme immobilized on glyoxyl supports was slowly attached and gave a homogenous distribution along the pores of the support particles, the poly-His tagged protein became immobilized very quickly and was placed on the outer part of the particle pores (Figure 31).[186] The immobilization rate of this enzyme could be controlled adding imidazol, a competitor of the adsorption of proteins to IMAC supports.[35]

This permitted to prepare co-immobilized biocatalysts of both proteins where enzyme distribution varied. It was shown that when both enzymes were slowly and, therefore, homogenously immobilized along the pores of the support, the global activity of the reaction was higher than immobilizing one in a homogenous way and the other forming a crown. In fact, the homogenously distributed co-immobilized preparations gave more activity even than the free enzymes, thanks to the high cofactor concentration, although the individual determination of the activity of both enzymes showed a decrease on enzyme activity.[186]

5. Future Prospects

Heterofunctional supports constitute a potent tool to improve enzyme performance. However, researchers should consider that many of the oldest immobilization techniques are really based on heterofunctional supports, as we have discussed in section 2 of this review. This may complicate the understanding of the experiments and may require the use of adequate reference supports and immobilization conditions to really seclude and identify the different effects and causes of the immobilization on the different groups of the support. But if properly controlled, heterofunctionality will increase the versatility of any immobilization protocols, as we can alter the first cause of immobilization and that way the final performance of the final biocatalyst.[18]

However, the most important expectations lay on the side of the tailor-made heterofunctional supports, where we can fulfill the enzyme technologist dream of a full control over enzyme immobilization, orientation of the enzyme on the support surface and intensity of the enzyme-support interactions. All these may be controlled using tailor made-heterofunctional supports and site directed mutagenesis. There are only a handful of examples on the use of these techniques, but they have shown the potential for both, preparation of industrial biocatalyst, and some academic studies, as the detection of the most relevant areas for enzyme stability under different conditions.[137] Coupling tailor-made heterofunctional supports to site-directed mutagenesis we can go from one step immobilization-stabilization-purification processes (e.g., using poly-His tagged enzymes) to site-directed rigidification of the enzyme.[18] However, it is still necessary to further improve the features of the supports to take full advantages of the possibilities of the heterofunctionality. In general rigidification of the enzyme structures to its fullest extent will be positive to improve their stability and also to improve the effects of the immobilization on other enzyme features.[7]

The design of new concepts involving tailor-made heterofunctionality of the supports very likely will go further in the near future. The co-immobilization of two enzymes on a heterofunctional support using different groups for each enzyme is one of these new

developments. However, this idea may be exploited further if combined with nanotechnology. In this case heterofunctionality may come from the integration of different nanostructures bearing each of them different functional groups.

Thus, it may be expected that the use of new ideas based on tailor-made heterofunctional supports may be a key to fulfill the requirements of an enzyme as an industrial catalyst, permitting good activity recovery, good stability, and even improved selectivity or specificity.

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Figure legends

Figure 1. Multifunctionality of glutaraldehyde activated supports.

Figure 2. Enzyme immobilization on lowly activated glutaraldehyde supports.

Figure 3. Theoretical effect of the activation degree on immobilization rates of proteins on glutaraldehyde activated supports under conditions where the first event is the ionic adsorption (the first immobilization is a multipoint process) or the covalent attachment (the first immobilization is a one-point process).

Figure 4. Different mechanisms of immobilization on glutaraldehyde supports of standard proteins

Figure 5. Different mechanisms of immobilization on glutaraldehyde supports of lipases.

Figure 6. Heterofunctionality of standard epoxy supports.

Figure 7. Steps in protein immobilization and stabilization via multipoint covalent attachment on standard epoxy-activated supports: protein adsorption, first covalent bond, multipoint covalent attachment and blocking of the remaining epoxy groups with hydrophilic molecules.

Figure 8. Different possibilities of immobilizing lipases on hydrophobic epoxy-supports.

Figure 9. Tailor made heterofunctional supports using secondary groups able to produce a first enzyme immobilization. One enzyme, one immobilization chemistry but different orientations of the enzyme on the support.

Figure 10. Effect of the internal geometry of the support microsurfaces and activation degree on the possibilities of getting an intense multipoint covalent attachment (MCA).

Figure 11. Effect of the steric hindrances of the reactive group on the support on the immobilization rate and on the prospects of getting an intense multipoint covalent attachment.

Figure 12. Necessity of the correct alignment of the reactive groups in the enzyme and the support to get an intense multipoint covalent attachment: need of long term incubations even if immobilization is very rapid.

Figure 13. Effect of the spacer arm in the support on the possibilities of achieving an intense multipoint covalent attachment and the rigidification effect.

Figure 14. Multipoint immobilization of proteins on glyoxyl-agarose supports.

Figure 15. Possibilities to increase protein reactivity versus glyoxyl supports:
1.- Chemical amination that produces a global modification of the protein and uses the carboxylic groups of the protein.
2.- Genetic amination: site-directed modification of the enzyme only on the desired area and without strict limitations on amount of amino groups introduced.

Figure 16. Effect of the modification of the epoxy groups (during the preparation of heterofunctional epoxy supports) on the immobilization rate and covalent immobilization rate.

Figure 17. Steric hindrances for the enzyme/support chemical reaction generated by the secondary groups introduced on the heterofunctional supports.

Figure 18. Building the second generation of heterofunctional supports: the primary group in the same arm as the secondary group, and the secondary group under the primary one.

Figure 19. Immobilization/stabilization of proteins by immobilization on second generation of heterofunctional supports.

Figure 20. An optimal heterofunctional support: the matrix is able to adsorb proteins, and a layer of protein reactive groups is placed over this matrix.

Figure 21. Heterofunctional supports and site-directed mutagenesis: one support and a collection of mutated enzymes produce different orientations on the immobilization.

Figure 22. IMAC-epoxy or glyoxyl supports for the directed immobilization of proteins. Use of poly-His tags or introduction of a couple of His on different areas of the protein surface.

Figure 23. Site directed rigidification of Cys- mutant enzymes on thiol heterofunctional supports.

Figure 24. Site directed rigidification of Cys- mutant enzymes on thiol heterofunctional supports: use of disulfide enzymes or disulfide supports.

Figure 25. Heterofunctional amino supports: the control of the amination permits the selective adsorption of large proteins.

Figure 26. Heterofunctional IMAC supports and poly His-tagged proteins: the control of the IMAC density on the support permits the selective adsorption of poly His tagged proteins.

Figure 27. Effect of enzyme orientation and loading degree on the activity of the enzyme molecules as a function of the substrate size.

Figure 28. Ideal support for the site directed rigidification of proteins integrating tailor made-heterofunctional supports and site directed mutagenesis.

Figure 29. Kinetic advantages of enzyme co-immobilization on cascade reactions.

Figure 30. Use of heterofunctional supports to co-immobilize two proteins with very different requirements.

Figure 31. Controlling the enzyme distribution in the support particle pores by controlling the immobilization rate.