



Research review paper

Stabilization of enzymes via immobilization: Multipoint covalent attachment and other stabilization strategies

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ABSTRACT

The use of enzymes in industrial processes requires the improvement of their features in many instances. Enzyme immobilization, a requirement to facilitate the recovery and reuse of these water-soluble catalysts, is one of the tools that researchers may utilize to improve many of their properties. This review is focused on how enzyme immobilization may improve enzyme stability. Starting from the stabilization effects that an enzyme may experience by the mere fact of being inside a solid particle, we detail other possibilities to stabilize enzymes: generation of favorable enzyme environments, prevention of enzyme subunit dissociation in multimeric enzymes, generation of more stable enzyme conformations, or enzyme rigidification via multipoint covalent attachment. In this last point, we will discuss the features of an "ideal" immobilization protocol to maximize the intensity of the enzyme-support interactions. The most interesting active groups in the support (glutaraldehyde, epoxide, glyoxyl and vinyl sulfone) will be also presented, discussing their main properties and uses. Some instances in which the number of enzyme-support bonds is not directly related to a higher stabilization will be also presented. Finally, the possibility of coupling site-directed mutagenesis or chemical modification to get a more intense multipoint covalent immobilization will be discussed.

1. Enzyme biocatalysis

Enzymes seem to be almost ideal biocatalysts for their application in different industrial areas. After all, they are the most efficient catalysts in Nature. They exhibit a high activity under mild conditions (aqueous media, room temperature and atmospheric pressure), and are very selective (which reduces the production of side-products) and specific (which avoids the modification of molecules similar to the substrate, saving purification steps) catalysts (Choi et al., 2015; Pollard and Woodley, 2007; Reetz, 2013; Schmid et al., 2001; Sheldon and Woodley, 2018). However, enzymes have been designed by natural evolution to fulfill their physiological function. That means that, in a living organism, enzymes must be able to adapt to changes in the medium and provide a rapid answer under stress situations.

In this context, many enzyme features, necessary to be adequate in

vivo, are far from industrial requirements (Schoemaker, 2003). That way, enzymes are relatively unstable, prone to inhibition by different compounds and the good features are manifested versus the natural substrates, when industry wants to use enzymes with substrates that are very far from the natural substrate in most instances. For example, enzyme specificity is a key feature when the enzymes are used in the resolution of racemic mixtures (de Miranda et al., 2015; Hoyos et al., 2012; Kamal et al., 2008; Martín-Matute and Bäckvall, 2007) or to save some purification steps (Fernández-Lafuente et al., 2001; Terreni et al., 2001), but it may reduce the use of one specific enzyme in a unique process, making it necessary to search for another enzyme if a slightly different substrate is employed (Fernández-Lafuente et al., 2001). Thus, it is becoming popular to direct efforts to find and design enzymes with a wide specificity. This is contrary to the first conception in which restricted enzyme specificity was considered a positive enzyme feature

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(Copley, 2015; Freund et al., 2017; Hult and Berglund, 2007; Nobeli and Favia, 2009; Roda et al., 2021). In fact, lipases, which are enzymes with very wide enzyme specificity, are the most widely used enzymes (Borrelli and Trono, 2015; Carvalho et al., 2015; de Miranda et al., 2015; Dhake et al., 2013; Ghanem, 2007; Gotor-Fernández et al., 2006; Jaeger and Eggert, 2002; Stergiou et al., 2013).

Nowadays, the implementation of enzymes is being pushed forward by the quick and profound development of very different scientific areas that can be used to improve their features. Some decades ago researchers could only use enzymes from cultivable microorganisms. Nowadays metagenomics has opened up the door to the use of all current enzyme biodiversity and even enzymes of no longer existing organisms (Ferrer et al., 2008; Schloss and Handelsman, 2003; Vieites et al., 2009). Advances in site-directed mutagenesis and enzyme modelling permit the creation of enzymes with improved properties (Andorfer and Lewis, 2018; Drufva et al., 2020; Ravikumar et al., 2015). Furthermore, directed evolution allows the rapid improvement of the selected enzyme features in the industrially required direction. This is done by mimicking an accelerated natural evolution-like process (Bornscheuer and Pohl, 2001; Eijnsink et al., 2005; Renata et al., 2015; Romero and Arnold, 2009). Protein chemical modification has evolved, aiming to a more controlled and directed enzyme modification to achieve further enzyme improvements (Boutureira and Bernardes, 2015; Chalker et al., 2009; Spicer and Davis, 2014). In fact, the combined use of these different strategies allows the researcher to go beyond the highest expectations that may be dreamt of some decades ago. For example, nowadays it is possible to create an enzyme bearing two active centers (the so-called plurizymes) combining site-directed mutagenesis and enzyme modelling (Santiago et al., 2018). Later on, it is possible to modify one of them with a specific irreversible inhibitor bearing an organometallic catalyst specially designed for one of the active centers (Alonso et al., 2020). Thus, an enzyme bearing two very different active centers may be created and used as catalyst in a cascade reaction (Alonso et al., 2020).

One further opportunity to improve enzymes is enzyme immobilization. Initially, this was designed to solve the problem of enzyme recovery and reuse (Dicosimo et al., 2013; Liese and Hilterhaus, 2013; Monteiro et al., 2021), as enzymes were very expensive catalysts (this is no longer an universal reality, as the price of some enzymes has kept going down in the last years) (Monteiro et al., 2021). The use of heterogeneous catalysts enables the continuous operation of the enzyme and downstream processing (Sheldon and van Pelt, 2013). From this initial necessity, many researchers tried to couple immobilization to the solution of other enzyme limitations, such as enzyme stability, activity, selectivity or specificity, purity, inhibitions, etc. (Barbosa et al., 2015; Bilal et al., 2019; Chapman et al., 2018; Garcia-Galan et al., 2011; Iyer and Ananthanarayan, 2008; Mateo et al., 2007c; Rodrigues et al., 2013; Silva et al., 2018; Valikhani et al., 2021; Wahab et al., 2020). One point to be considered is that the use of immobilization does not rule out the use of all the other stabilization techniques described above. In fact, the use of some of them to improve the results of enzyme immobilization has proved to produce significantly improved industrial biocatalysts. This will be discussed later in this review (Bernal et al., 2018b; Fernandez-Lafuente, 2009; Hernandez and Fernandez-Lafuente, 2011a; Rodrigues et al., 2014; Rodrigues et al., 2011; Rueda et al., 2016b; Singh et al., 2013).

In this review, we will focus on how immobilization may improve enzyme stability, and the main parameters that should be considered at the different levels to take the maximum profit of immobilization in this specific point. In this review, stabilization refers to the maintenance of the enzyme activity, usually when submitted to stress conditions that cause inactivation, as this is parameter studied in most papers. In most cases, this could be closely related to the stability of the structure of the polymer, but it should be considered that in most cases, the enzyme activity is lost long before the enzyme has been fully unfolded. Some enzymes can retain a large percentage of activity even when their structure has been severely affected, while others can lose all activity

after some minor change in the tridimensional structure. That way, this will be more related to the operational stability than to the thermodynamic stability. Although this is not used in this discussion, if measuring enzyme stability as produced product per biocatalysts unit, the enzyme activity and operational stability may be closely related concepts. Under this concept a more active enzyme (e.g., an hyperactivated one) may produce more target product than an enzyme with a loss of activity after immobilization (Gomes and Woodley, 2019). That way, residual activity and stability may be considered related concepts. It should be considered that stability is an “absolute” property of an enzymatic biocatalyst (measured for example as inactivation constant or half-life under specific conditions), while stabilization refers to the increase of the stability of an enzyme after some treatment. It is therefore a relative term. It is possible that a high stabilization of an initially very unstable enzyme may produce a less stable final biocatalyst than that prepared using another initially much more stable enzyme. This may happen although the stabilization strategy via immobilization with this enzyme may not work so well.

Immobilization may improve the stability in any reaction media. In aqueous media, the main focus will be on reducing the enzyme structural mobility without affecting the enzyme activity (Mateo et al., 2007c; Garcia-Galan et al., 2011; Sheldon and van Pelt, 2013). In organic anhydrous media, enzyme mobility is reduced due to the lack of water. This can improve enzyme stability in some cases (if the solvent did not take the essential water) (Bell et al., 1995; Bell et al., 1997; Halling, 1989; Halling, 1994, 2000; Toba et al., 1996; Zaks and Klivanov, 1988). The enzymes may be inactivated by hydrophobic interaction with the organic phase, or if the organic solvent is able to take the water molecules that are in the essential water layer (the water molecules that permit the enzyme mobility) (Halling, 1994, 2000; Toba et al., 1996). Some molecules of solvent can also be dissolved in the water and interact with the enzyme. Moreover, enzymes are not soluble in organic solvents, and can aggregate, and even if the enzyme remains active, the diffusional limitations generated will produce a decrease in enzyme activity. In this case, the fact of using immobilized enzymes dispersed on the support surface has a positive effect, as it avoids enzyme precipitations. Still, this approach has a problem, all the enzyme molecules are fully exposed to the anhydrous solvent. The support can act as storage of water to maintain the essential layer of water in the enzyme, and some of the below strategies has been specifically designed to prevent the interaction of the individual enzyme molecules with the solvent interface (Guisan et al., 2001). Some nonconventional media are supposed to stabilize enzyme such as some ionic liquids (while others can inactivate the enzymes) (De Diego et al., 2005; Domínguez de Marfa and Maugeri, 2011; Huang et al., 2014; Kaar, 2017; Lozano et al., 2001 and Lozano et al., 2002; Sheldon, 2005; Stepankova et al., 2013; van Rantwijk and Sheldon, 2007; Wagle et al., 2014), while other such as supercritical media have shown strong enzyme inactivating capacity (Habulin and Knez, 2001; Lozano et al., 2007; Lozano et al., 2011; Lozano et al., 2001; Striolo et al., 2003). Using water-soluble organic solvents, the direct interaction of the enzyme molecules with the enzyme may lead to enzyme inactivation, and may easily alter the essential water layer of the enzyme, in general a decrease in enzyme stability is expected (Bonneau et al., 1993; Khmelnsky et al., 1991; Kuper et al., 2007; Prakash and Upadhyay, 2006). However, some solvents may even have positive effects for enzyme stability in certain cases (e.g., glycerin, sorbitol) (Braham et al., 2021a, 2021b; Ghosh and Kishore, 2018; Nazari-Robati et al., 2016; Xie and Timasheff, 1997) Many of the points below means that the enzyme structure is more rigid, and that means that the stabilization is expected versus any agent able to produce enzyme distortion.

2. Enzyme stabilization by just preventing enzyme exposition to some inactivation causes

Enzyme stabilization after immobilization may be derived from different reasons. It is not always related to a real improvement of the

enzyme molecular features (Garcia-Galan et al., 2011). Immobilization may prevent an immobilized enzyme from becoming exposed to some inactivation causes, causing its operational stabilization without really improving the enzyme features (Garcia-Galan et al., 2011). This does not mean that this stabilization is not “real”. It is just inherent to the fact of having an immobilized enzyme molecule.

Using porous supports of a pore diameter 10-40 fold larger than the proteins, most of the enzyme molecules will be inside the particle, which is isolated from the medium (Garcia-Galan et al., 2011) (Fig. 1). Similarly, using strategies such as crosslinked enzyme aggregates (Cao et al., 2000; Sheldon, 2011) or crystals (Khalaf et al., 1996; Margolin, 1996; Margolin and Navia, 2001) most of the enzyme molecules will be isolated from the medium. That means that the enzyme cannot interact with external interfaces (e.g., gas bubbles, drops of immiscible organic solvents or substrates) (Fig. 1). Proteins are distorted when exposed to hydrophobic interfaces, such as gas bubbles or drops of organic solvents (forming foams), and this interaction is no longer possible if the enzyme is on the inner surface of a porous solid (Garcia-Galan et al., 2011).

Enzymes fully immobilized on the external surface of a support, for example if they are immobilized on nonporous nanoparticles (Cipolatti et al., 2016) or crystals coated of proteins (Kreiner et al., 2001; Kreiner and Parker, 2004; Monteiro et al., 2020; Shah et al., 2008) are not protected from this negative effect derived from the interaction with hydrophobic surfaces (Fig. 2). To get this protection, it has been shown that the coating of the immobilized enzyme molecules with hydrophilic polymers may be an alternative (Betancor et al., 2005) (Fig. 2).

The stabilization value versus this inactivation cause depends on the concentration of free enzyme and of the stirring or gas bubbling rate: a high enzyme concentration can increase the stability of the free enzyme, reducing the stabilization effect of enzyme immobilization.

Immobilization may also protect the enzyme from irreversible inactivation caused by aggregation of partially unfolded protein molecules (Garcia-Galan et al., 2011). When immobilized on a porous support, protein-protein interactions are only possible when the support is loaded with the maximum amount of protein possible (i.e. that allowed by the support). These enzyme molecules interactions may also occur if the enzyme molecules are immobilized so rapidly that the enzyme cannot diffuse in the pores of the support and they are packed together forming a crown in the pore mouth (Fig. 3). The effect of these protein-protein interactions in immobilized enzymes may be positive or negative for enzyme stability (Arana-Peña et al., 2020b; Fernandez-Lopez et al., 2017a; Zaak et al., 2017d). Again, the stabilization value depends on the inactivation conditions for the free enzyme, in this case, enzyme aggregations will be favored by using higher enzyme concentrations, and a higher “stabilization” may be found using high concentrations of protein, or even depend on the purity of the enzyme sample.

Compared to the advantages described using porous supports, using

non-porous biocatalysts the interaction of the enzyme molecules with enzyme molecules immobilized on another particle is still possible (Garcia-Galan et al., 2011) (Fig. 4). Again, this may be solved by coating the immobilized enzymes with some polymers (Fig. 4).

If the enzyme is a protease or the researcher is using an impure sample, containing some proteases, immobilization may prevent autolysis or proteolysis (Morellon-Sterling et al., 2020; Tacias-Pascacio et al., 2020; Tavano et al., 2018). In the case of the use of porous supports, this protection will be effective in most cases (Fig. 1). The risks involve the possibility of enzyme release from the support, as in that case the protease will regain the capacity to attack other free proteases or even immobilized molecules. The use of very highly loaded biocatalysts using short spacer arms, may fully avoid proteolysis if the active center is oriented towards the media. If the active center is looking towards one of the other immobilized enzyme molecules, it may be possible that under certain conditions a point of breakage is produced, but if the enzyme has no mobility on the support surface, this may have a limited effect on enzyme stability. If the immobilization is not via irreversible bonds and the enzyme is not released, but can move on the support surface, autolysis may remain a problem even after enzyme immobilization. Highly loaded biocatalysts having long spacer arms (e.g., an extreme case will be the use of long polymers) will also permit autolysis. Using non-porous biocatalysts, proteolysis will be avoided regarding other protease molecules immobilized on the same particle (Cipolatti et al., 2016) (Fig. 5). However, proteolysis of the enzyme molecules by other protease molecules immobilized on another particle will only be reduced if the enzymes are not properly oriented (e.g., with the active center not fully exposed to the medium) (Fig. 5). If the protease active center is fully exposed to the medium, the only way to ensure the protection of the biocatalysts versus autolysis will be the coating of the enzymes with some polymers (Fig. 6), but this can also reduce or eliminate its proteolytic activity versus protein substrate (Morellon-Sterling et al., 2020; Tacias-Pascacio et al., 2020; Tavano et al., 2018) (Fig. 6). Autolysis or proteolysis will increase when the concentration of enzyme increases. That way the stabilization value may be increased or decreased by altering the protein concentration.

Immobilization may also reduce some inactivation causes by partition of some deleterious compounds away from the enzyme environment (Virgen-Ortiz et al., 2017a). Most of the cases of enzyme stabilization by this cause involve the generation of a hydrophilic environment around the enzyme after immobilization. This is the case of the immobilization of enzymes on supports coated with hydrophilic polymers (Fig. 7). This can prevent or reduce enzyme inactivation by hydrophobic distorting substrates when used at high concentrations and can inactivate the enzyme by interacting with different areas of the enzyme surface (e.g., catechol or phenol), enzyme inactivation by hydrophobic distorting medium components (e.g., hydrophobic solvents)

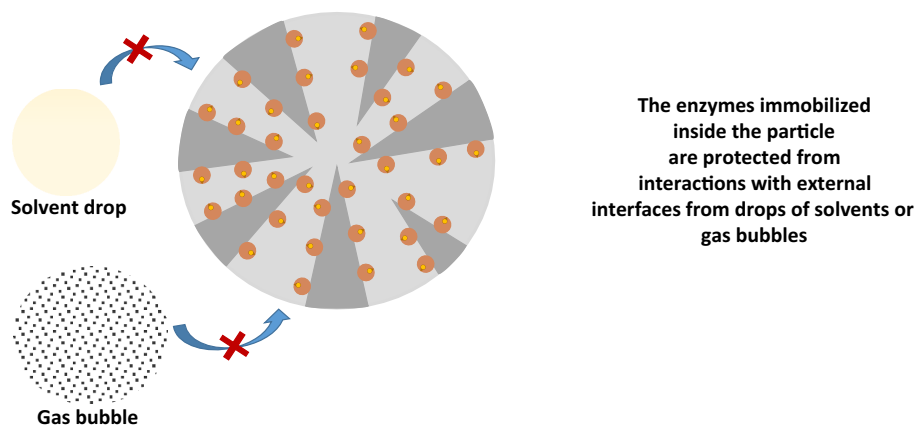


Fig. 1. Protection of enzyme molecules immobilized inside a porous particle from interactions with external interfaces.

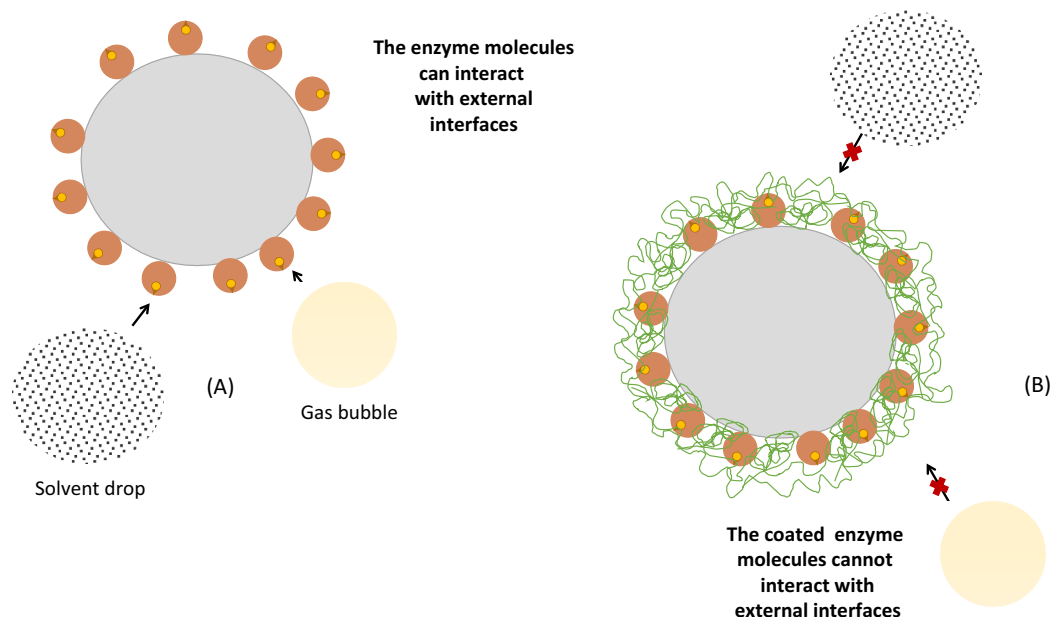


Fig. 2. The enzymes immobilized on non-porous supports can be inactivated by interaction with external interfaces (A), but the coating with hydrophilic polymers may prevent this interaction (B).

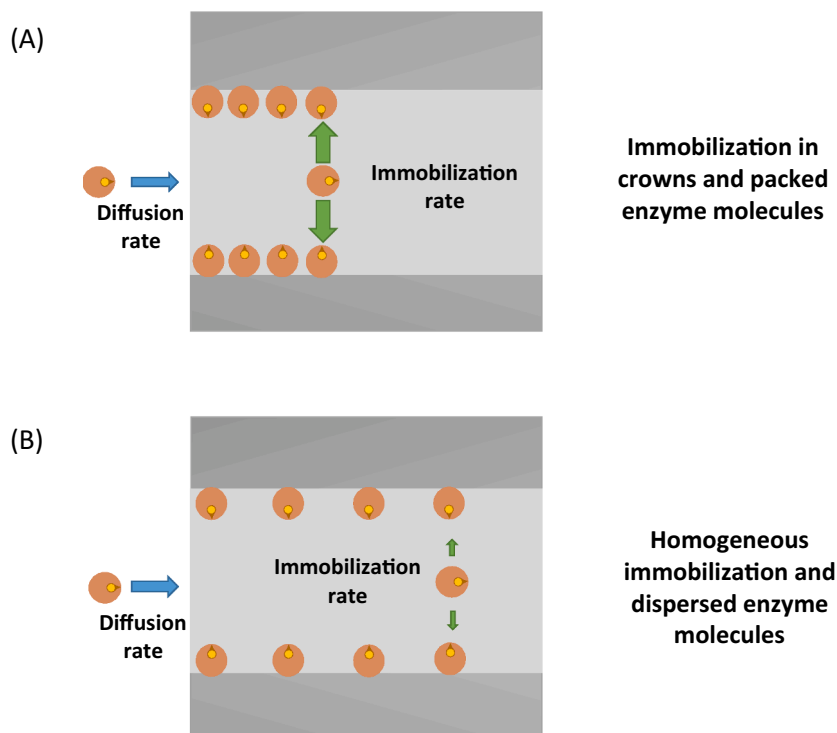


Fig. 3. Immobilized protein-protein interactions depend on the enzyme immobilization/diffusion rates. If the immobilization rate exceeds enzyme diffusion rate, and a high loading is used, enzyme molecules are packed together and protein-protein interactions may exist (A). If the situation is the reverse, enzymes are more homogeneously immobilized and there is some free space between immobilized enzyme molecules (B)

(Dasgupta et al., 2007; Huang et al., 2000; Vaillancourt et al., 2006; Ximenes et al., 2011; Fernandez-Lafuente et al., 1999; Irazoqui et al., 2007; Irazoqui et al., 2002; Wilson et al., 2004b) or oxygen (Mateo et al., 2006; Klibanov, 1979). For example, immobilized 1,2 catechol dioxygenase presented maximum activity in 1 mM of catechol, while the free enzyme presented this maximum in 0.1 mM. Penicillin G acylase multipoint covalently immobilized plus coated with polyethylenimine and dextran sulfate was much more stable in 90% dioxane than the one-point

immobilized enzyme in 50% of the cosolvent. This means that a proper immobilization can not only improve the enzyme stability in a quantitative way, but it may permit to give a qualitative leap and enable to perform reactions under conditions where the non-stabilized biocatalysts was not active/stable, allowing to make wider academic studies or even have a biocatalyst that could be suitable for industrial implementation when the non-stabilized enzyme was unsuitable even to be used in academic studies. (Abian et al., 2001, 2002, 2003, 2004a; b).

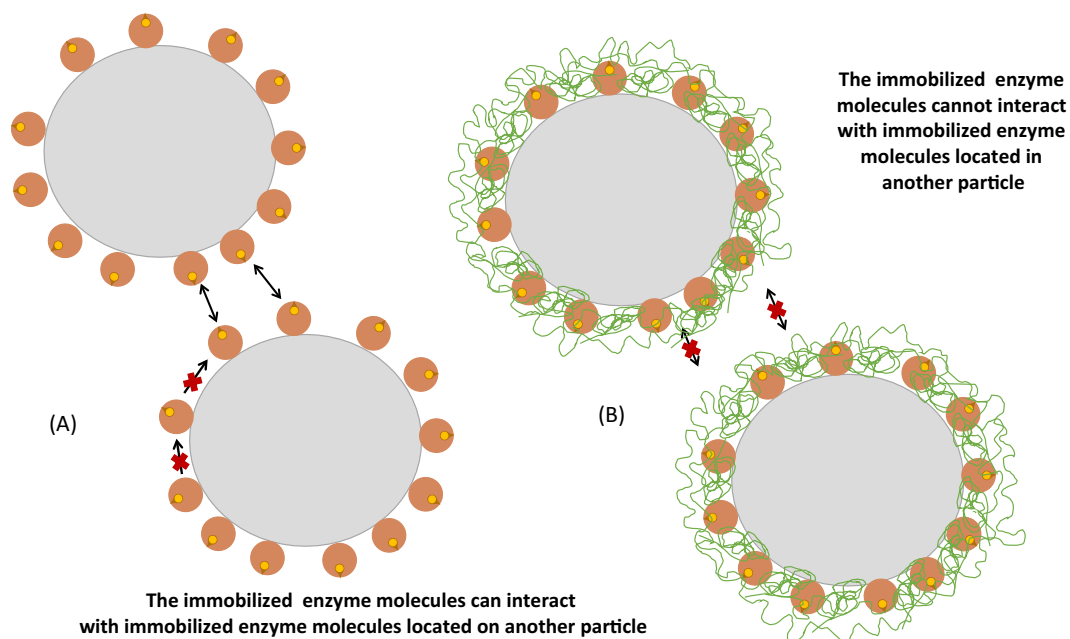


Fig. 4. Enzyme molecules immobilized on non-porous supports can interact with enzyme molecules immobilized on another support particle (A). This may be solved by coating the enzymes with a polymer (B).

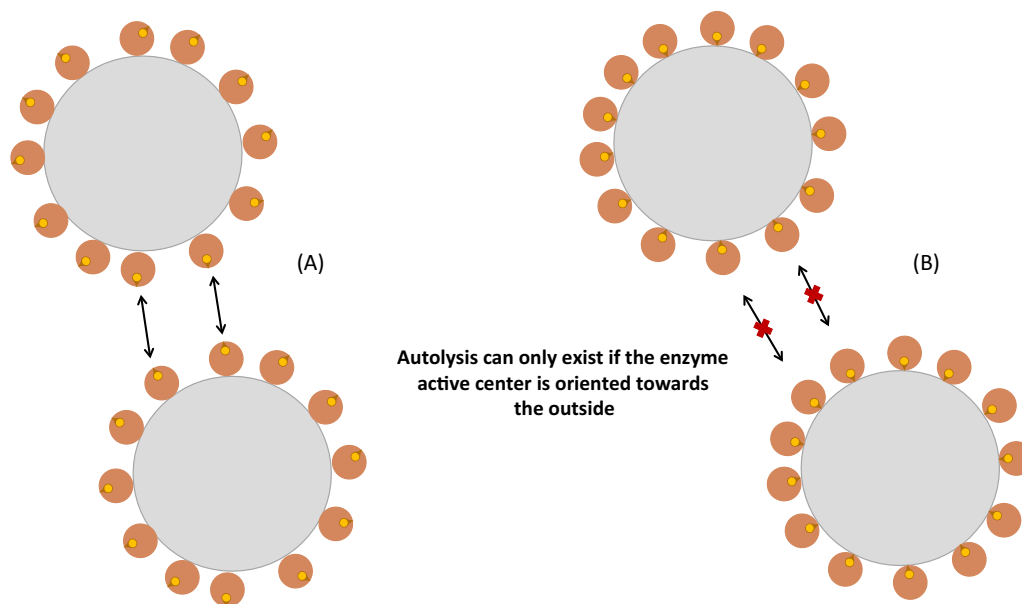


Fig. 5. When proteases are immobilized in non-porous supports, enzyme autolysis is only possible if the active center is oriented towards the medium (A), while this is not possible if the enzyme active center is oriented towards the support surface (B).

Exposure of the enzyme to a hydrophobic surface is generally negative for the thermal stability of an enzyme. However in the presence of some hydrophilic deleterious compounds, such as hydrogen peroxide, the final balance on enzyme stability may be positive. This occurs even if the stability of the immobilized enzyme in absence of the inactivating reagent is lower than that of the free enzyme (Hernandez et al., 2012; Hernandez and Fernandez-Lafuente, 2011b). The combination of immobilization and further coating of the immobilized enzyme molecules with polymers of the desired physical features may reinforce this effect (Fernández-Lafuente et al., 1999). For example, the lipase B from *Candida antarctica* immobilized on a hydrophobic support presented a similar stability in 10 M H₂O₂ to the stability of Novozym, 435 in 1 M of this reagent (Hernandez and Fernandez-Lafuente, 2011b), where the

enzyme was immobilized in a relatively hydrophilic support (Ortiz et al., 2019).

In all these cases, the rigidity of the enzyme structure after immobilization may be identical to that of the free enzyme. The benefits of its immobilization will only be found under specific conditions, where these other inactivation causes become relevant. In any case, the magnitude of enzyme stabilization may be huge under those conditions, e.g., if compared to diluted free enzyme. For this reason, discriminating between the effects of the “mere” immobilization of the enzyme inside a solid from a real alteration of the enzyme features is not straightforward. It is recommended to compare the enzyme stability of the target biocatalyst with an enzyme that has been immobilized via just one point to the support. After, it must be verified that the properties of this reference

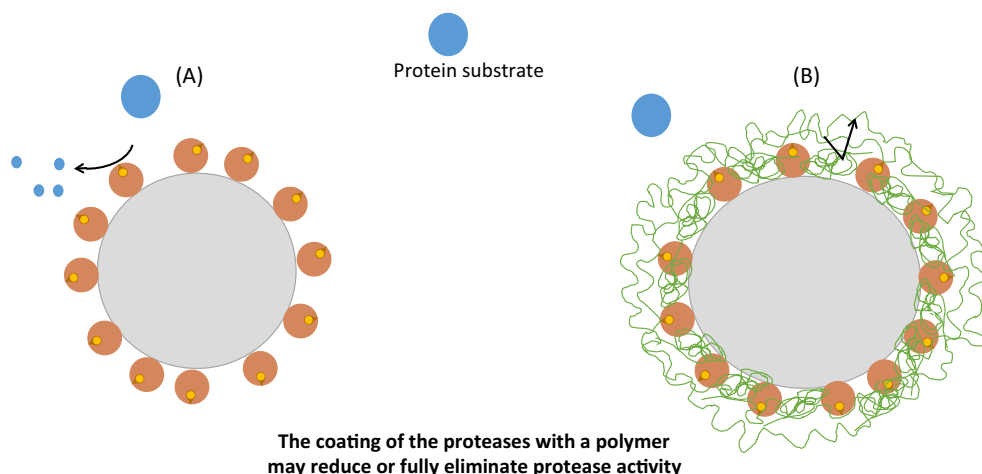


Fig. 6. Properly oriented proteases in non-porous supports may hydrolyze large protein substrates (A), but the coating of the enzymes with a polymer may avoid this by steric hindrances (B).

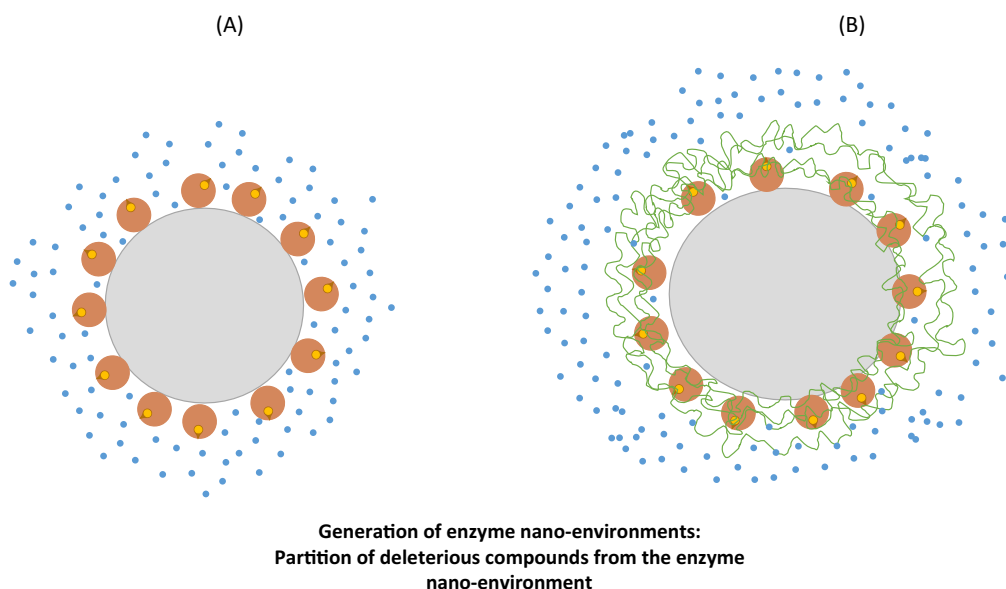


Fig. 7. Deleterious compounds may inactivate enzymes by direct interaction with the immobilized enzyme molecules (A), but the promotion of artificial nano-environments may produce a partition effect, reducing the concentration of the immobilized enzyme to these deleterious compounds.

immobilized biocatalyst are identical to those of the free enzyme in absence of some inactivating reagent, aggregation, proteolysis or exposition to external interfaces. This reference immobilized enzyme preparation will later indicate the effects of the immobilization on enzyme stability under the diverse inactivation conditions where the enzyme stability may be studied. This will permit to discriminate between stabilization by effect on enzyme molecular features and those caused by immobilization inside a porous solid (Alvaro et al., 1990; Bes et al., 1995; Guisán et al., 1993).

3. Multimeric enzyme stabilization by involving the immobilization of all enzyme subunits

The first step in the inactivation of some multimeric enzymes (but not all) is the enzyme subunit dissociation (Lencki et al., 1992; Poltorak et al., 2000; Poltorak et al., 1998), induced by heat or some organic solvents. When this occurs, enzyme stability increases with enzyme concentration. However, even if this correlation is found, explanations may be based on some other dissociation phenomena, e.g., the

dissociation from the enzyme of a cofactor or a cation that may be essential for the catalytic activity or for the preservation of the enzyme structure (Fig. 8). That way, profound studies are required before concluding that the first cause for enzyme inactivation is the dissociation of the enzyme subunits. If this is the case, the immobilization of the enzyme via all enzyme subunits will prevent the possibility of enzyme dissociation (Fig. 9). As a result, this will have a direct impact on enzyme stabilization, that will be larger when more diluted free enzyme solutions are used as reference (Bolivar et al., 2008; Bolivar et al., 2006a; 2009; Fernandez-Lafuente, 2009; Kaddour et al., 2008; Lopez-Gallego et al., 2007) (Figs. 8 and 9).

Using pre-existing supports and dimeric enzymes, the immobilization of the enzyme via both enzyme subunits is relatively simple. This is because a flat surface may involve both enzyme subunits, and usually the largest surface of the enzyme will be that involving both of them. That will in turn facilitate the multi-subunit immobilization (Fernandez-Lafuente, 2009) (Fig. 9). Stabilization of multimeric enzymes with larger oligomerization stages upon immobilization requires that all enzyme subunits may interact with a flat surface (Betancor et al., 2003; Juan M

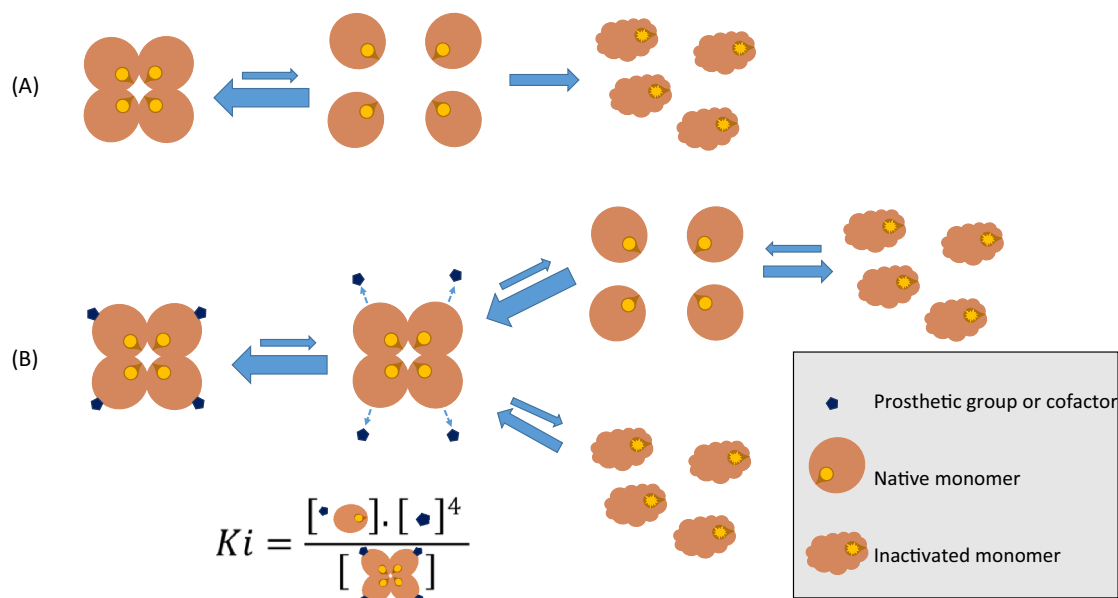


Fig. 8. Schematic representation of the inactivation of multimeric enzymes, when subunit dissociation is the first step of the inactivation (A) or when the first step the dissociation of some prosthetic groups, cofactor or ion (B). The fig. shows the different association/dissociating equilibria on multimeric enzymes and multimeric enzymes containing some no protein group that can be dissociate from the enzyme,

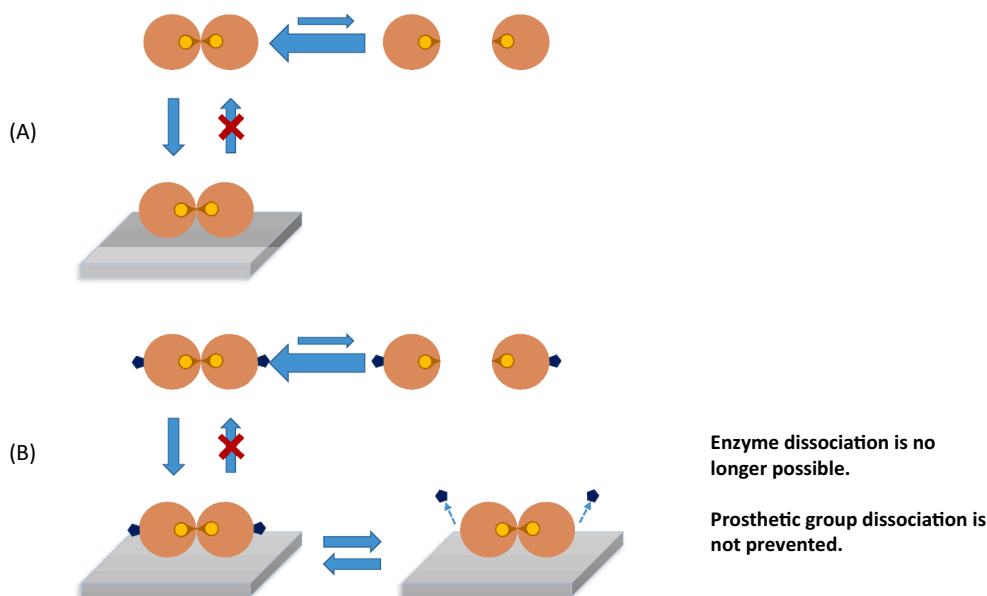


Fig. 9. Multi-subunit immobilization prevents enzyme subunit dissociation, but it cannot prevent the dissociation of some prosthetic groups, cofactor or ion.

Bolivar et al., 2009; Fernandez-Lafuente, 2009; Fernández-Lafuente et al., 2001; Hidalgo et al., 2003). That is not possible in the case of tetrahedral enzymes, (Fig. 10) although a flat surface may involve all enzyme subunits of other tetrameric enzymes in the immobilization, or even of larger oligomerization states. If the support is activated with a polymeric bed and the enzyme may be accommodated on the bed to involve all enzyme subunits in the immobilization (e.g., immobilizing the enzyme at high ionic strength on an ionic polymer to increase the number of enzyme-support interactions required to immobilize the enzyme) (Fernandez-Lafuente, 2009) (Fig. 11).

When all the enzyme subunits are in the same plane, there are some strategies that ensure the immobilization of the enzyme involving the surface where all enzyme subunits are involved (that will be the largest one) (Bolivar et al., 2009). These strategies involve the use of hetero-functional supports (discussed later) (Barbosa et al., 2013). These are

based on the fact that most physical adsorptions require a multipoint support-enzyme interaction. Thus, by combining a support bearing the minimal superficial density of groups able to adsorb the multimeric enzyme with groups able to give a multipoint covalent immobilization, the enzymes may be immobilized by the area involving all enzyme subunits (Bolivar et al., 2009) (Fig. 12).

If the enzyme oligomerization stage is very large or some subunits are not on the same plane (tetrahedral enzymes), there is one possibility of preventing the enzyme subunit dissociation in the biocatalyst, yet. This is done by further coating the immobilized enzyme with a multifunctional polymer to crosslink all enzymes subunits with, at least, one enzyme subunit attached to the support (Fernandez-Lafuente, 2009) (Fig. 13). One alternative strategy for these very complex enzymes is to coat the free multimeric enzyme with an ionic polymer. This usually permits the crosslinking of the enzyme subunits (Garcia-Galan et al.,

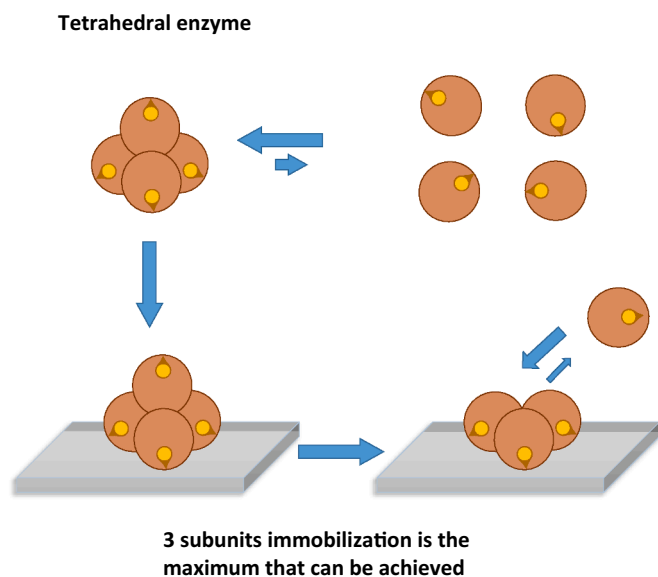


Fig. 10. The geometrical distribution of the enzyme subunits conditions the stabilization of the enzymes in preexisting supports; if all enzyme subunits are not in the same plane, the immobilization of all enzyme subunits will be not possible.

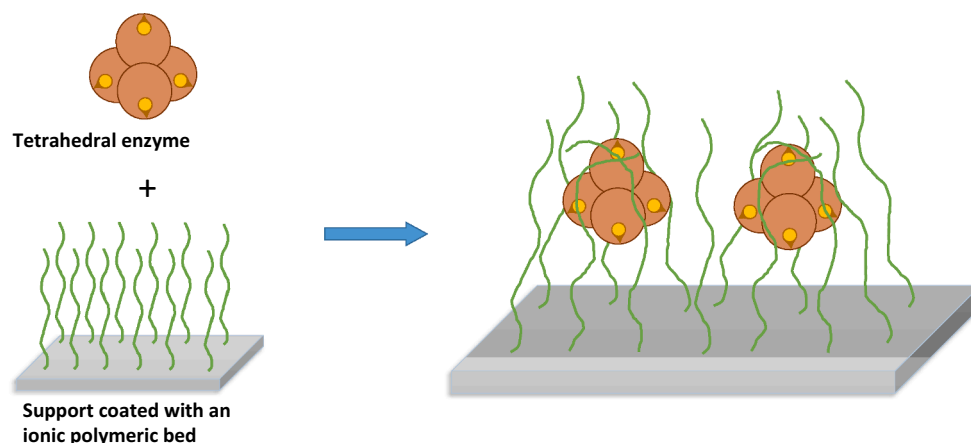


Fig. 11. Immobilization of a tetrahedral enzyme on a support coated with a polymer forming a volumetric bed can involve all enzyme subunits in the immobilization.

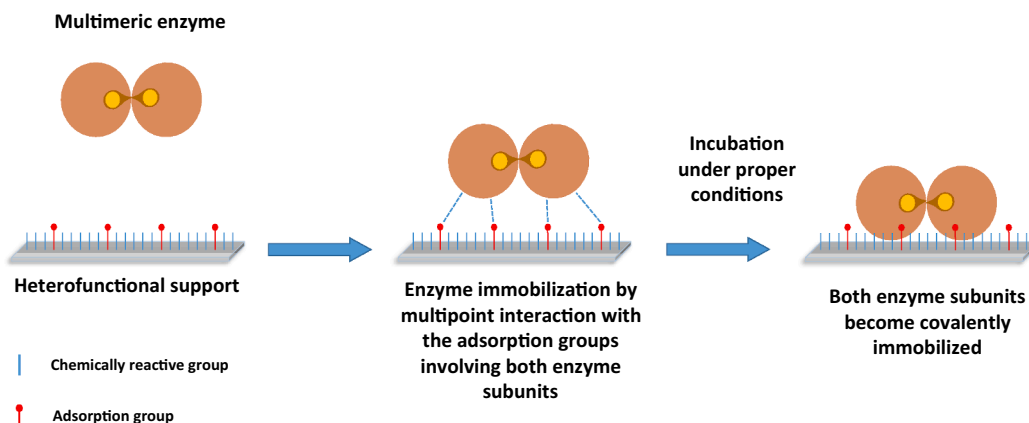


Fig. 12. Use of tailor-made heterofunctional supports to stabilize multimeric enzymes.

2013), and later to immobilize this composite on a support (Fig. 14).

Other immobilization strategies, not using preexisting supports, ensures the prevention of enzyme dissociation by the way they are prepared, like the crosslinked enzyme aggregates (Schoevaart et al., 2004; Sheldon et al., 2005), the crosslinked enzyme crystals (Abraham et al., 2004; Navia et al., 1993) or the copolymers (Johansson and Mosbach, 1974; Müller et al., 2018; Pollak et al., 1980). If they are physically stable solids, they should ensure the immobilization via all enzyme subunits (Wilson et al., 2004a) (Fig. 15).

The production of enzyme-metal nanoflowers (Altinkaynak et al., 2016; Han et al., 2020; Wu et al., 2015; Zhang et al., 2021) may, in some instances, also ensure that the enzyme multimeric structure is coated by the metal salt and dissociation may be no longer possible (Fig. 16).

To ensure that the immobilization achieved the stabilization of the multimeric enzyme structure, a first clue is to check on the dependence of enzyme stability with enzyme concentration has disappeared (Fernandez-Lafuente, 2009) (Fig. 9). A more elegant way to prove this (only valid if the immobilization is performed by stable bonds) is to boil the immobilized biocatalyst in breaking buffer and submit the supernatant to a study by SDS-poly-acryl amide electrophoresis analysis, to check if all enzyme subunits remain immobilized (Fernandez-Lafuente, 2009). If this experiment confirms that all enzyme subunits are attached to the support, but the enzyme stability is still enzyme concentration dependent, it becomes a proof of some other dissociation phenomena affecting enzyme stability (e.g., some unknown ion) (Kaddour et al., 2008) (Fig. 9).

Immobilization of all enzyme subunits will be positive even for

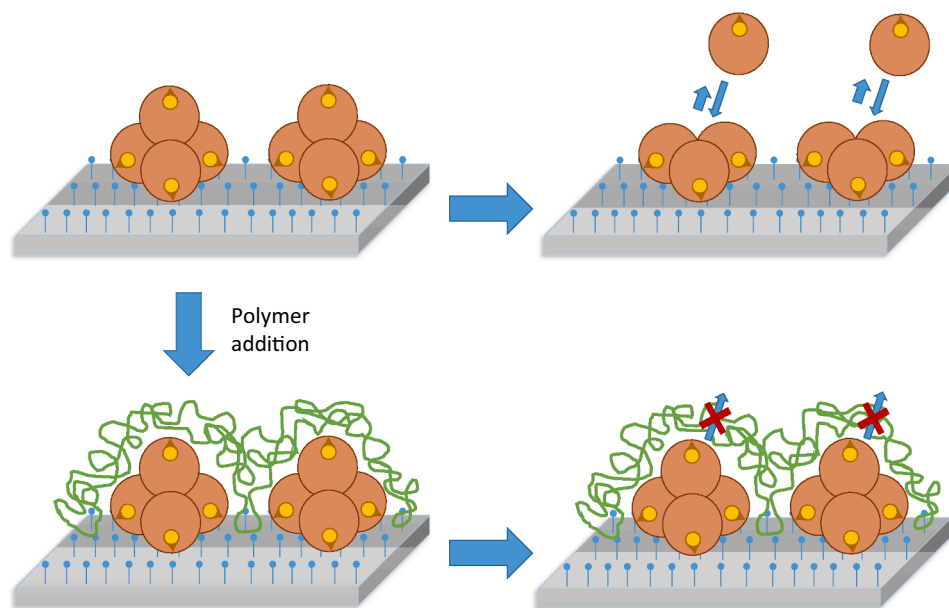


Fig. 13. Stabilization of multimeric enzymes by multi-subunit immobilization plus crosslinking with polymers.

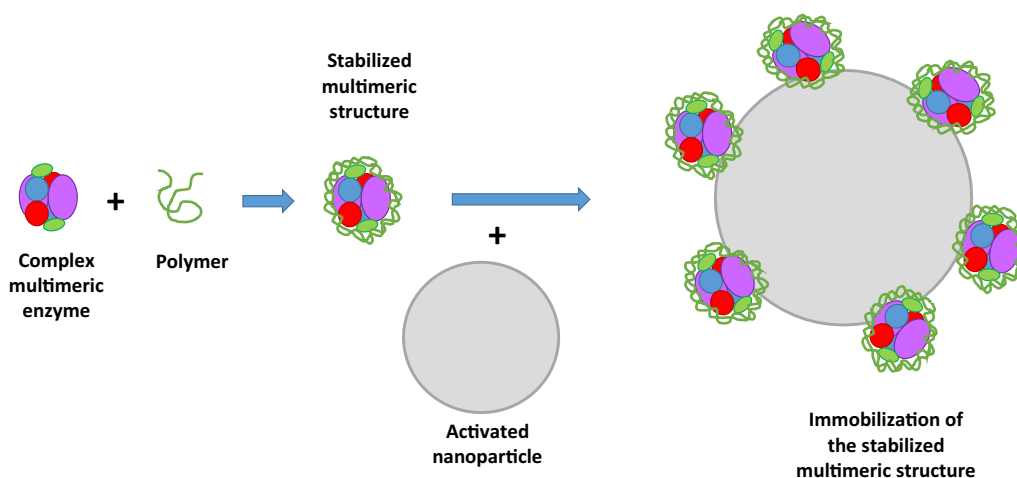


Fig. 14. Stabilization of complex multimeric enzymes by coating with ionic polymers and further immobilization of the coated-stabilized composite on a support.

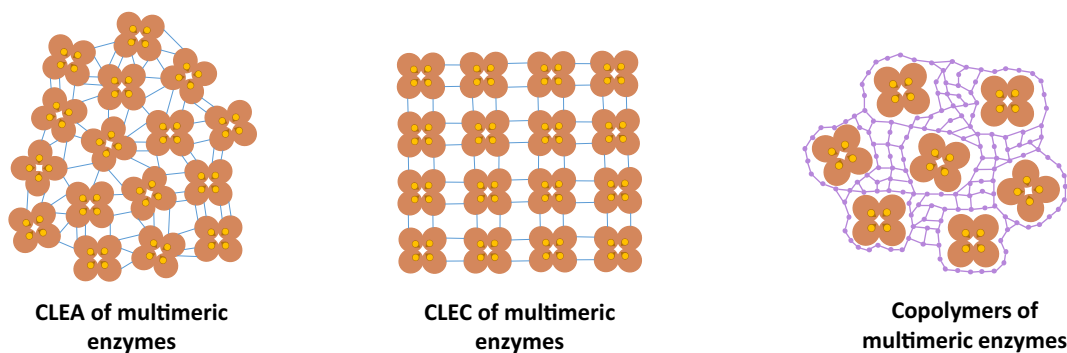


Fig. 15. Enzyme subunit dissociation may be prevented by the use of crosslinked enzyme crystals (CLECs) or aggregates (CLEAs) or the preparation of copolymers.

multimeric enzymes where the monomers are attached to each other by disulfide bonds. That way even if these bonds are broken, there are no risks of contamination of the medium by the enzyme monomers. However, enzyme stabilization will be derived more of the each monomer

rigidification than on the prevention of enzyme dissociation. In this instance, it is very likely that the dissociated enzymes may be already inactivated.

The value of stabilization after multi-subunit immobilization

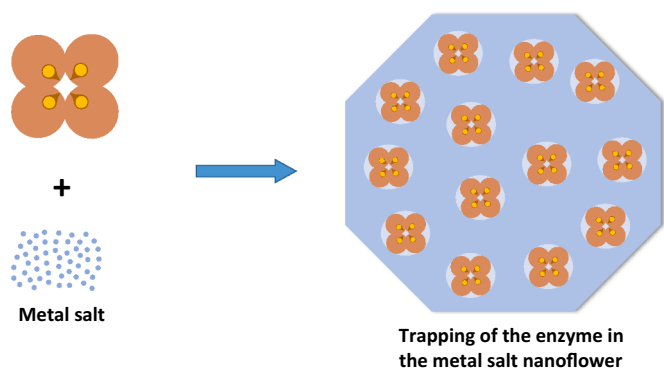


Fig. 16. Prevention of enzyme dissociation by the production of enzyme nanoflowers.

depends on the concentration of the soluble enzyme used in the studies, as this enzyme form will be more stable when the enzyme concentration increases. However, independently on this quantitative value, the effects on operational stability will be remarkable, and in some instances, may be even the possibility or impossibility of using some dissociation conditions that can favor enzyme performance (Fernández-Lafuente et al., 2001).

4. Enzyme stabilization by generating a more stable enzyme structure

Some enzymes undergo drastic conformational changes during its catalytic mechanisms or in response to changes in the experimental conditions, and some of these different conformations may be more stable than others. If we can select the most stable conformations during immobilization, this can have a positive impact on the final immobilized enzyme stability in thermal or organic solvents induced inactivations.

One of the first examples reported in the literature in this regard was using enzymes having different oligomerization stages under different conditions, with different stabilities. This is the case of the multimeric invertase (Esmont et al., 1987; Kern et al., 1992). The oligomerization of the enzyme depends on the pH (Chu et al., 1983). At alkaline pH it tends to be a tetramer, while at acidic pH values, it is a dimer. When the enzyme is immobilized at pH 8.5 on PEI-coated supports, the enzyme is immobilized as a tetramer, while when immobilized at pH 5 it is a dimer (Torres et al., 2002) (Fig. 17). The enzyme immobilized as a tetramer at pH 8.5 was more stable than the enzyme immobilized as a dimer at pH 5 in all the range of inactivation pH values studied.

However, perhaps the most remarkable example of this immobilization of different enzyme conformations is the case of lipases. Lipases

exist in equilibrium between two different conformations, depending on the experimental conditions (Brzozowski et al., 1991; Grochulski et al., 1993; Martinelle et al., 1995; Van Tilbeurgh et al., 1993; Wang et al., 2021). Lipases may present their very hydrophobic active center blocked by a polypeptide chain called lid or tap (closed form), or the lid may move exposing a huge hydrophobic pocket (formed by the hydrophobic surroundings of the active center and the inner face of the lid) (open form) (Brzozowski et al., 1991; Grochulski et al., 1993; Martinelle et al., 1995; Van Tilbeurgh et al., 1993; Wang et al., 2021) (Fig. 18).

Obviously, this capacity of movement confers a great plasticity to the active center of lipases and their catalytic properties may be easily tuned via immobilization (Cabrera et al., 2009; Chaubey et al., 2006; Jose C. S. dos Santos et al., 2015e; Fernandez-Lorente et al., 2008a; Manoel et al., 2015; Palomo et al., 2002b, Palomo et al., 2002a; Rodrigues et al., 2019, Rodrigues et al., 2013; Takaç and Bakkal, 2007). In the presence of a hydrophobic surface (e.g., a drop of oil), a lipase becomes adsorbed on it, involving its open form, that becomes stabilized (Verger, 1997). This mechanism is called interfacial activation, as the conformational equilibrium shifted towards the lipase adsorbed in its open form, usually promoting an increase in enzyme activity. However, in the context of this review, the most relevant point is that this adsorbed open lipase form is more stable than the enzyme in the conformational equilibrium (Cygler and Schrag, 1999; Jaeger et al., 1993; Kim et al., 1997).

Very likely for this reason, the most used lipase immobilization method is the immobilization of lipases on hydrophobic supports (Rodrigues et al., 2019) (Fig. 18). This mimics the interfacial activation mechanism of these enzymes (Manoel et al., 2015). These immobilized enzymes are more stable than the multipoint covalently immobilized lipases maintaining the conformational equilibrium, at least in thermal inactivation by a factor 5-100, (dos Santos et al., 2015e; dos Santos et al., 2015d, 2015c) being 10-1000 folds more stable than the free lipases internal inactivations, depending on the enzyme and the support (Rodrigues et al., 2019)

One weak point of this immobilization strategy is the possibility of enzyme desorption under certain conditions, like presence of hydrophobic co-solvents (not in anhydrous media, where the enzyme is not soluble) or detergents (or detergent-like) substances (Rueda et al., 2015; Virgen-Ortiz et al., 2017c). This causes that while the lipases immobilized via interfacial activation on hydrophobic supports are very stable in thermal inactivations, they may be poorly stable in these unfavorable media. This has been solved by using strategies that prevent enzyme desorption, like intermolecular protein crosslinking (Fig. 19) (Fernandez-Lopez et al., 2018; Fernandez-Lopez et al., 2017b; Fernandez-Lorente et al., 2011; Zaak et al., 2017a) or the use of heterofunctional acyl-chemical reactive agents (to give at least one covalent bond) (Fig. 20) (de Albuquerque et al., 2016; Bernal et al., 2015; Bernal et al., 2014; Guajardo et al., 2015a, 2015b; Hirata et al., 2016a, 2016b; Rios et al.,

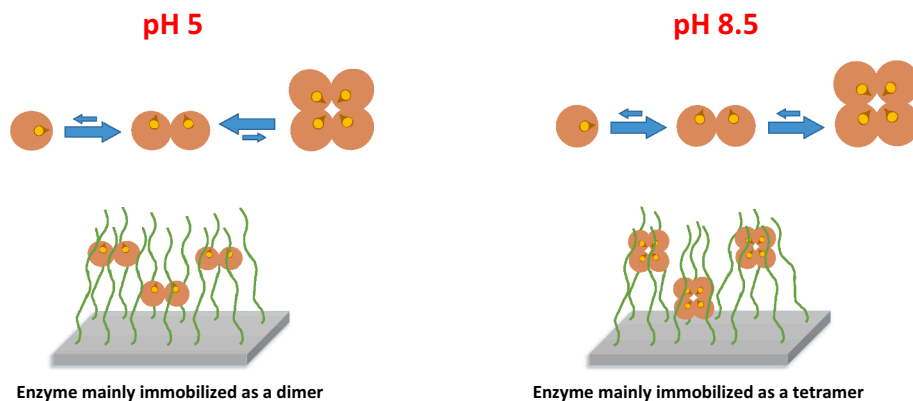


Fig. 17. The oligomeric structure of amylase depends on the pH, that way, depending on the immobilization pH, the main immobilized form will be a dimer or a tetramer.

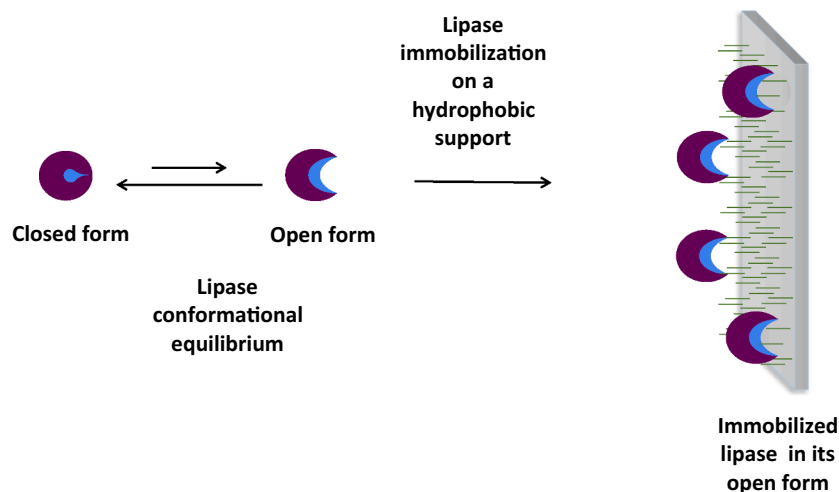


Fig. 18. The conformational equilibrium between open and closed form is shifted towards the open form in the presence of a hydrophobic support, yielding the lipase immobilized in its open form.

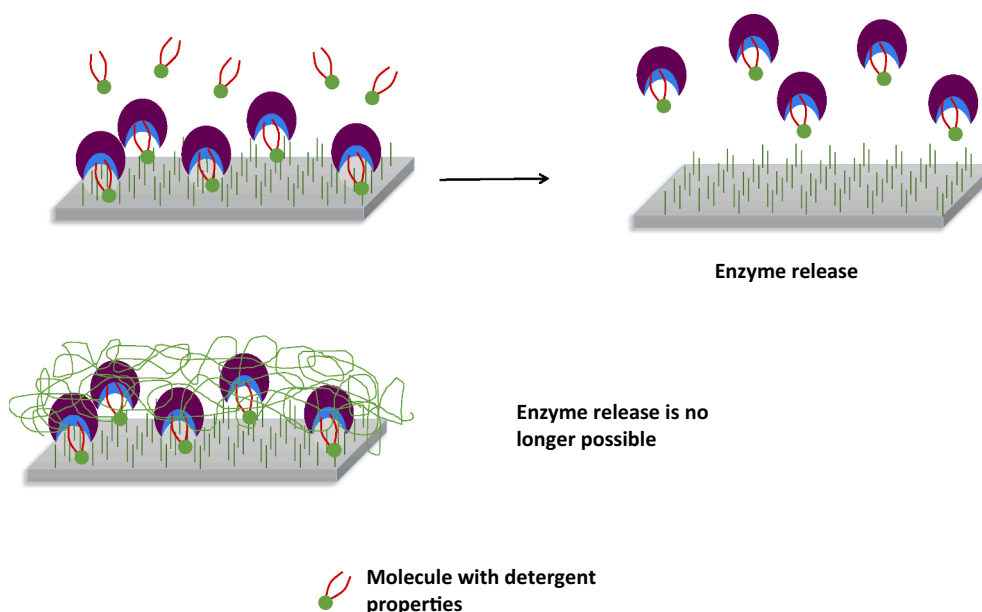


Fig. 19. Prevention of lipase release from hydrophobic supports via physical or chemical crosslinking with polymers.

2019; Rueda et al., 2015).

Also, the combination of interfacial activation with another physical immobilization event (e.g., an ionic group) may prevent (or at least make more difficult) enzyme release from the support, in this case maintaining the reversibility of the immobilization protocol and enabling the reuse of the support after enzyme inactivation (Rueda et al., 2016c; Rueda et al., 2016a).

Recently, it has been shown that the immobilization of lipases via interfacial activation in different media may also greatly alter the final stability of the biocatalysts, even being a reversible immobilization method. In some instances, after washing and inactivating under identical conditions, one immobilized lipase maintained the initial activity almost intact while another lipase immobilized under different conditions was fully inactivated. This suggests that the medium is able to induce large conformational changes in the lipase structure that are maintained after immobilization even in absence of these distorting conditions at high temperatures (Arana-Peña et al., 2021; Arana-Peña et al., 2020b; Lokha et al., 2020; Silveira et al., 2019; Silveira et al., 2017). In fact, the immobilized enzymes maintained different enzyme

conformations after several days incubated under identical conditions.

Moreover, it should be pointed out that this immobilization protocol produces immobilized lipases with an especially intense response to changes in the medium, e.g., they are stabilized by certain cations (Fernandez-Lopez et al., 2016; Fernandez-Lopez et al., 2015) or they are destabilized by phosphate ions, while other immobilized lipases forms are not so sensitive (Kornecki et al., 2020; Zaak et al., 2017b). They are also significantly stabilized in glycerin (Braham et al., 2021b) or high concentrations of ammonium sulfate (Braham et al., 2021a). That way, the differences in stability comparing this and other protocols may depend on the exact inactivation conditions.

Thus, the immobilization of the open form of lipases via interfacial activation on hydrophobic supports produces very stable biocatalysts, although the presence of a near hydrophobic surface and the weak nature of each individual enzyme-support linkage should be even negative for enzyme stability (as it will be discussed later) (dos Santos et al., 2015a, 2015b, 2015c, 2015d, 2015e, 2015f).

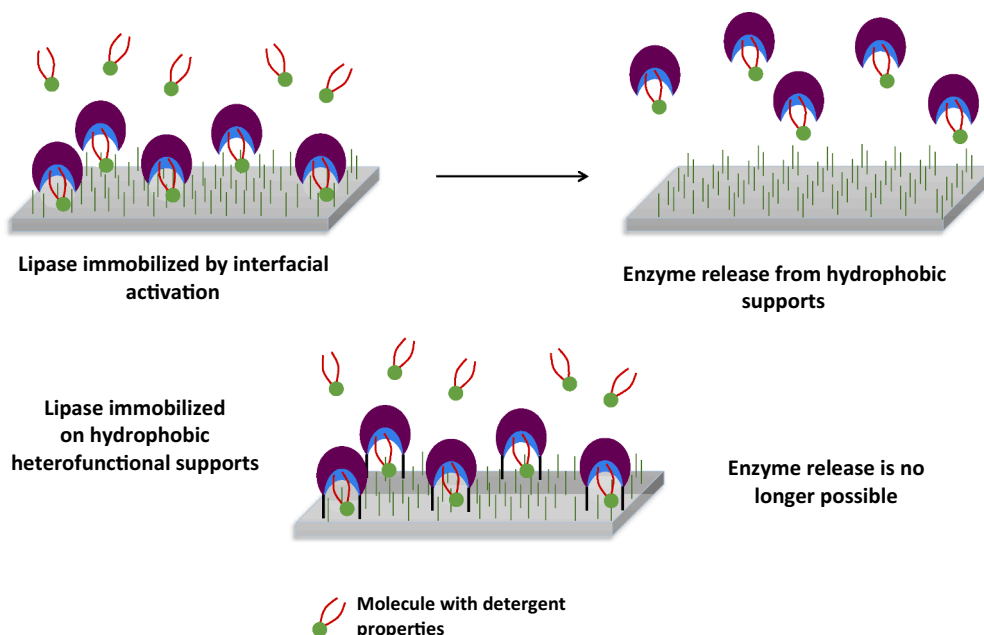


Fig. 20. Prevention of lipase release from hydrophobic supports via immobilization on heterofunctional hydrophobic supports.

5. Enzyme stabilization by multipoint covalent attachment

Enzyme immobilization via multipoint covalent attachment on pre-existing supports is one of the most effective ways to produce a more rigid enzyme, and at first glance, to produce a more stable enzyme, at least a more rigid enzyme polymer. This idea was launched by the Russian groups, although they stressed the poor geometrical congruence between pre-existing solids and enzyme as a negative aspect that will reduce the impact of multipoint covalent immobilization on enzyme stability (Klibanov, 1983; Klibanov, 1979; Martinek et al., 1977; Mozhaev, 1993; Mozhaev et al., 1983; Mozhaev et al., 1990). Currently, the potential of multipoint covalent immobilization to stabilize enzymes is accepted by the majority of the scientific community (Mateo et al., 2007c; Weltz et al., 2020) (Fig. 21). This enzyme stabilization strategy is based on the fact that the relative positions of all groups involved in the immobilization must remain fixed under any experimental condition, only permitting the movement determined by the length of the spacer arm. In a simplified view, it is to achieve a multiple intramolecular crosslinking, where the crosslinking agent is a flat and multifunctional structure constituted by the activated support surface (Fig. 21). To get this effect, the support must be significantly more rigid than the enzyme

otherwise the enzyme will rigidify the support. This should improve enzyme stability versus any distorting reagent, including heat, solvents, chaotropic reagents, etc. Cold denaturation of both monomeric and multimeric enzymes, albeit a fully different phenomenon from thermoinactivation, also involves the existence of some conformational changes that give inactive forms of the enzymes as a result (Agashe and Udgaonkar, 1995; Inoue et al., 2019; Menon and Sengupta, 2019; Nishii et al., 1994; Parui and Jana, 2021; Privalov, 1990; Yang et al., 2021). Although studies on the effect of multipoint covalent immobilization on this inactivation mechanism have not been found, we can guess that it could also prevent enzyme inactivation due to this cause.

The use of other immobilization strategies, such as CLECs, CLEAs, copolymers, etc. may involve the entire enzyme surface in the immobilization, but the rigidity of the “support” (other enzyme molecule) may not be large enough to give a very high enzyme rigidification. This enzyme stabilization will be extended to any inactivation cause: high temperatures, presence of organic solvents and other inactivating molecules, drastic pHs values, presence of inactivating compounds, etc. (Mateo et al., 2007d).

However, only a proper immobilization system will permit an intense multipoint covalent attachment (Barbosa et al., 2013). The

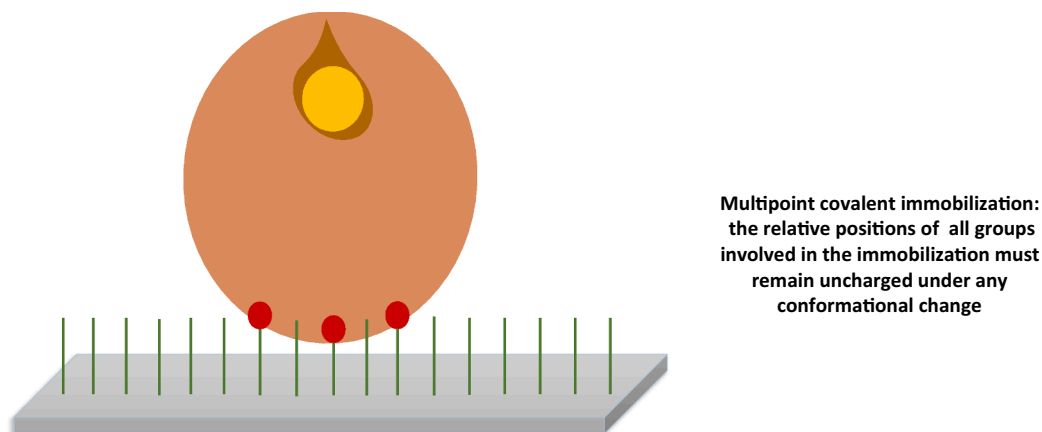


Fig. 21. Stabilization of enzymes by multipoint-covalent attachment.

immobilization system should consider several points: the final physical nature of the support surface, the support surface geometric constitution, the support activation possibilities, the reactive group features, the spacer arms of these groups, and the immobilization protocol (dos Santos et al., 2015f). Next we will discuss each of these parameters.

5.1. Final support surface physical and chemical features

At first glance, the ideal immobilization system, if it is to be universal, should permit to have at the end of the immobilization protocol a support surface as inert as possible, without the capacity to form any additional physical or covalent enzyme-support interaction (dos Santos et al., 2015f) (Fig. 22). This is because these uncontrolled interactions can stabilize incorrect enzyme structures. At the end the enzyme may be fully unfolded to maximize the enzyme-support interactions (Virgen-Ortíz et al., 2017b; Virgen-Ortíz et al., 2016) (Fig. 22).

Although the advantages of a support final inertness may be considered a general rule, in some instances, some enzymes, may become stabilized by the generation of an enzyme environment positive for its stability. This is mainly so if this environment can decrease the concentration of some deleterious compounds, as explained in section 2. Lipase immobilization in hydrophobic supports is another exception, but this is because the enzyme is immobilized via its open form, that when stabilized by interaction with a hydrophobic surface become more stable than the enzyme in conformational equilibrium (see point 4) (Rodrigues et al., 2019). When the immobilization is covalent and the support has a hydrophobic nature, the expected final result is a lower enzyme stability because the proximity of this hydrophobic surface may stabilize incorrect conformations of the enzymes, when hydrophobic internal groups become exposed to the medium by the enzyme structural movements caused by heat, drastic pH, solvents, etc. (dos Santos et al., 2015c; Mateo et al., 2000a, 2000b).

This final inertness involves both the matrix and the active groups. As a general rule, the ideal support should be as similar to water as possible. Agarose beads are one outstanding support in this regard (Zucca et al., 2016), also cellulose beads (Lam et al., 2012; Shah et al., 2013) may be adequate for this purpose, as well as any other support formed by hydrophilic and physically inert polysaccharides (Bezerra et al., 2015a, 2015b).

However, many supports do not have the possibility of becoming fully inert. Many of them are intrinsically hydrophobic in their matrix, like polystyrene (Bahar and Çelebi, 1998; Chen et al., 2007; Ho et al., 1998), poly-styrene-divinylbenzene (Rodrigues et al., 2015) or

polyurethane (LeJeune and Russell, 1996; Phadtare et al., 2003; Pires-Cabral et al., 2010) beads. Epoxyacrylic resins have this problem (Boller et al., 2002; Hilterhaus et al., 2008; Katchalski-Katzir and Kraemer, 2000), although they are commercialized stating that they are hydrophilic. Although they are hydrophilic when compared to polyurethane, they are hydrophobic if compared to agarose beads, and may have negative effects on enzyme stability (we will discuss in a deeper way this later in this review). The modification of the support after enzyme immobilization, that is a requirement in certain immobilization protocols as explained later, may be an opportunity to tailor the final properties of the support surface. Furthermore, this may enable reducing undesired enzyme-support interactions. Other supports, such as chitosan, have an inherent cationic nature (Muzzarelli, 1980; Verma et al., 2020), which may also produce positive or negative effects on the final enzyme stability. The problem may become very complex when using a commercial matrix, as the exact composition of their surface may be not detailed and some unexpected negative results may be found on enzyme stability (and activity) after enzyme immobilization. In some instances, these negative results may be due to the interaction with the matrix or with this unknown and minority groups, not with the reported active groups on the support. For this reason, it is recommended to assay the immobilization of the enzymes in some support where the only existing physical or chemical groups are those introduced by the researcher (e.g., agarose beads) (Zucca et al., 2016). After studying the immobilization effects on this support using an activation protocol, if agarose is not adequate for our final purposes, the protocol may be extrapolated to other supports. If results are poorer than using agarose, the failure is on the support, not on the activation method.

Even using an adequate support, the active group is also very important (Barbosa et al., 2013). If the immobilization is based in some physical adsorption (Jesionowski et al., 2014), the support may be never inert, as this will release the enzyme from the support, and always some additional enzyme-support interactions may be established during enzyme inactivation (Garcia-Galan et al., 2011) (Fig. 22).

If the enzyme immobilization is covalent, it is convenient to establish some strategy that can permit to eliminate the chemical reactivity of the support to prevent the formation of new enzyme-support covalent bonds during enzyme inactivation or operation. Moreover, the reaction of the support surface (is still present some chemical reactivity) with some components of the reaction medium may alter the physical properties of the support surface in an undesired way (dos Santos et al., 2015f). Aldehyde groups (e.g., glyoxyl) can be reduced using borohydride to produce inert hydroxyl groups (Blanco and Guisán, 1989), other active

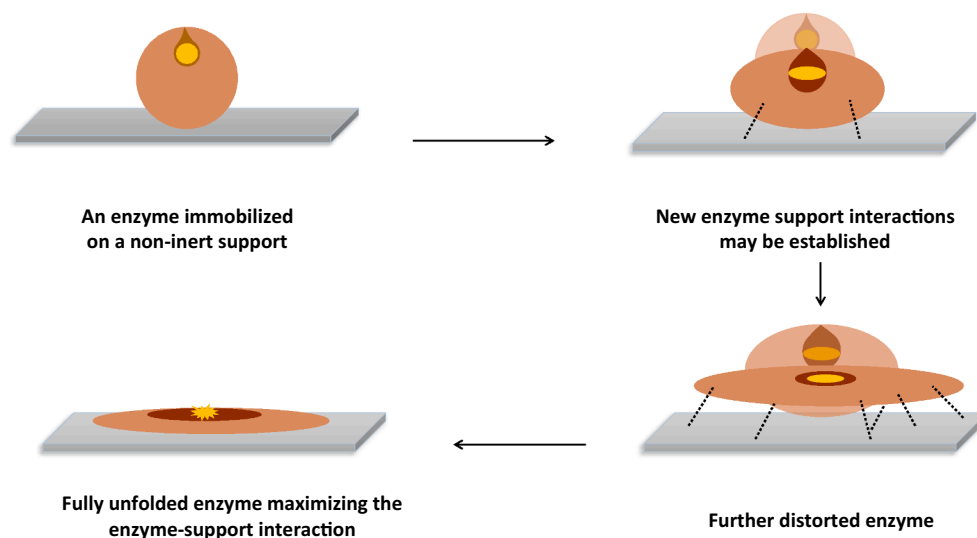


Fig. 22. The possibility of uncontrolled enzyme-support interactions during operation may favor the stabilization of incorrect enzyme-support structures and the final maximization of the enzyme unfolded polymer-enzyme interactions.

groups (such as bromocyanogen, epoxide, vinyl sulfone) must be blocked with some nucleophiles to eliminate the chemical reactivity (Barbosa et al., 2013). This can hardly produce a fully physically inert surface. Even using glycine, it should be considered that a mixed cation/anion exchanger is produced, and it can still interact and immobilize proteins, in some instances even better than monofunctional ionic supports (Fuentes et al., 2007). However, this blocking step opens new possibilities to tailoring the enzyme features (activity, stability, but also specificity and selectivity) (Albuquerque et al., 2016; Bonomi et al., 2013; Jose C. S. dos Santos et al., 2015c, 2015d, 2015e; Souza et al., submitted; Zaak et al., 2018), and has been recently utilized to permit the coimmobilization of enzymes with dissimilar stabilities reusing the most stable ones (Morellon-Sterling et al., 2021a) (Fig. 23). The existence of physical enzyme-support interactions may be negative in some conditions and positive in some others, but general rules have not been established to date.

Aminated supports activated with glutaraldehyde are an example of supports bearing many different features (Barbosa et al., 2014): the amine group gives anion exchanger capacity to the support, the glutaraldehyde rings give some hydrophobicity character and chemical reactivity. However, the chemical reactivity of glutaraldehyde disappeared after some time by diverse and still unknown mechanisms. Thus, during the biocatalyst uses, the only problem is the fact that some hydrophobic and ionic interactions between the immobilized enzyme and the support may exist. This multifunctionality of the active support makes amino-glutaraldehyde supports a very versatile immobilization strategy as it will be discussed later (Barbosa et al., 2014).

Thus, the understanding of the effect of the immobilization of an enzyme using a defined protocol should consider whether the final support surface is really inert or not (dos Santos et al., 2015f).

5.2. Support internal geometry

The enzyme can only react with the support by the support-enzyme contact area. That way, the internal morphology of the support surface

determines the possibilities of support-enzyme interaction, and this determines the possibility of getting an intense enzyme-support multi-point attachment (dos Santos et al., 2015f; Garcia-Galan et al., 2011; Mateo et al., 2007c; Rodrigues et al., 2013) (Fig. 24). Using supports formed by fibers of a diameter similar or thinner than the enzyme diameter, the possibilities of an intense multipoint covalent attachment are low, that way the increase in the enzyme rigidity should be low. Although most supports are convex (e.g., agarose) (Zucca et al., 2016) or concave (e.g., silicates) from a macroscopic point of view, considering the size of the enzyme many of them may be considered a surface similar to a flat surface when reacting with an enzyme (by comparing the size of the support and that of the enzyme., the possibilities of intense enzyme support interactions may become maximized (Fig. 24). This higher geometrical congruence is even clearer if the support pores surfaces are concave. Pores of a similar size to that of the enzyme should not permit enzyme immobilization inside the pore. Thus, to increase the interaction by using a support bearing pores with a diameter similar to the enzyme is not feasible if a high support loading is desired. It should be considered that the textural properties of the support are determined under dry conditions. This may be fully different to those wet conditions, that are the conditions where the enzyme is immobilized, this can explain why in some papers, pores with dimeters similar to the enzyme size are utilized with good results in terms of loading and stabilization (Coscolín et al., 2018; Ferdousi et al., 2016; Jia et al., 2015; Weber et al., 2010).

For example, agarose beads are formed by trunks of packed polymer, and these trunks are thicker when the percentage of agarose increases (Zucca et al., 2016). This permits to get a higher geometric enzyme-support congruence when using agarose beads produced with higher agarose percentage, and permits to get a higher number of enzyme-support bonds and a higher enzyme stabilization (Pedroche et al., 2007).

However, the negative effects of a non-inert surface are also lower when the enzyme-support geometric congruence is poor. That way, if the enzyme is immobilized in a support where the possibilities of an intense multipoint covalent attachment are low, but the negative

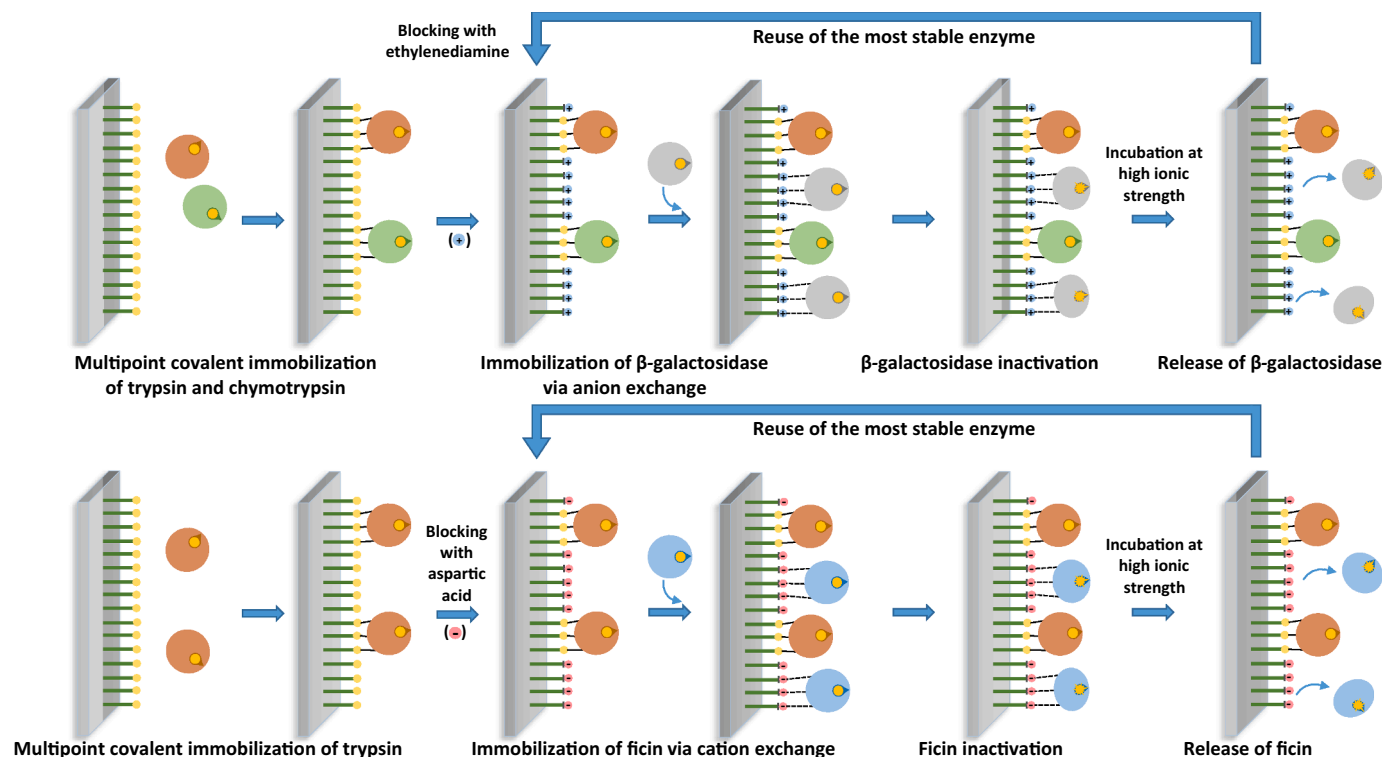


Fig. 23. Use of vinyl sulfone activated supports to coimmobilize enzymes with diverse stability, enabling the reuse of the most stable enzymes. The most stable enzymes are immobilized via multipoint covalent immobilization while the least stable enzymes are immobilized via ion exchange.



Fig. 24. Importance of the enzyme-support geometric congruence in the multipoint covalent immobilization.

physical enzyme-support interactions are also poor, its stability may be higher than using a support where the possibilities of an intense multipoint covalent attachment (but also the negative effects of the enzyme-support interactions) are high. This is the case of the immobilization of penicillin G acylase on different epoxy acrylic matrices. The enzyme was immobilized on Eupergit C (a support formed by relatively thin fibers) and Sepabeads supports (a porous support where the pores are tunnels formed by porogenic agents) (Mateo et al., 2000a, 2002). Both supports are epoxy-acrylic supports, which covalently immobilize the enzymes via epoxides. While Eupergit C permits a weak enzyme-support interaction, Sepabeads permits an intense one. The result is that the stability of the enzyme immobilized on Eupergit is similar independently of the blocking agent employed (Mateo et al., 2000a). However, the enzyme immobilized on Sepabeads was much less stable than that immobilized in Eupergit when it was blocked using mercaptoethanol, the blocking agents recommended by the manufacturers of Eupergit C. Nevertheless, using blocking agents able to reduce the hydrophobic interactions of the enzyme with the support surface, the stability of the enzyme immobilized on Sepabeads greatly increased, surpassing the stability achieved when the enzyme was immobilized on Eupergit C (Mateo et al., 2002).

5.3. Surface density of the reactive groups in the support

One critical point to get an intense enzyme-support multipoint covalent attachment is that the enzyme needs to interact with many groups in the support. This is determined by the surface density of the reactive groups in the support, that is, the amount of groups per surface unit (Fig. 25). To compare supports with different specific surface bearing similar amount of groups can drive to incorrect conclusions. The surface density is the correct parameter to be considered. An intense multipoint covalent attachment may be expected only if under the projected enzyme surface there are many active groups in the support. Using agarose, it has been calculated that 17-20 reactive groups/1000 Å² may be introduced (modifying only the anomeric group of glucose in the

agarose), and this is around the projected area of a small protein like trypsin or chymotrypsin (Pedroche et al., 2007; Zucca et al., 2016).

The maximum support activation will offer the maximum possibilities of enzyme-support reaction, however if this intense enzyme multipoint covalent immobilization may drive to an excessive loss of enzyme activity, a simple way to control the intensity of the enzyme-support interaction is to lower the surface density of active groups in the support. That is, it is important to have the opportunity of achieving high support activation, but it is not compulsory to use this maximum activation. However, we can also imagine that a support with an extremely high activation may permit an intense multipoint attachment without making a great distortion of the enzyme necessary, while if the distance among groups in the support is higher, it is more likely that the enzyme will suffer some distortion to establish several enzyme-support linkages. Unfortunately, we have not found studies on this matter in the literature.

The maximal surface density of reactive groups will also determine the level of support surface modification if the reaction end point is based in a blocking step using some nucleophile, the higher the superficial density of the reactive groups, the higher the change of the support properties after the blocking step. And this is important if the support surface has some hydrophobicity, as discussed above (Mateo et al., 2002).

It must be considered that it is most unlikely that all reactive groups in the support are involved in enzyme immobilization. First, even using a support fully loaded with an enzyme that has been so quickly immobilized that the enzymes are packed together (Fig. 3), a significant percentage of the support surface will not be under the enzyme molecules. Moreover, a significant percentage of groups under the projected area of the enzyme will be too far from the enzyme surface to can react with them. That way, if all reactive groups in the support disappear after enzyme immobilization but before the blocking step, it must be considered that the reactive groups have been inactivated by the immobilization conditions, and therefore, many of them are not

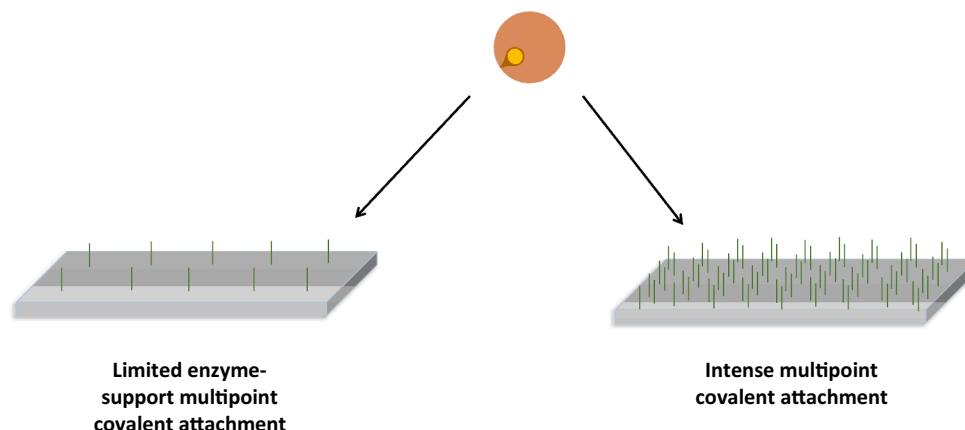


Fig. 25. Importance of the superficial density of reactive groups in the support in the multipoint covalent immobilization.

involved in enzyme immobilization. A reference of the support submitted to exactly the same protocol, but in absence of enzyme, should be used as a reference to check this possibility.

5.4. The spacer arm

The spacer arm is another critical parameter that must be considered in the “ideal” immobilization protocol (Barbosa et al., 2013; Garcia-Galan et al., 2011; Mateo et al., 2007c). Enzyme rigidification is obtained because the mobility of the involved groups is decreased to the length of the spacer arm (Klibanov, 1983; Klibanov, 1979; Martinek et al., 1977). This means that the shorter the spacer arm is, the higher the obtained reduction of the mobility of the enzyme groups involved in the immobilization by each additional enzyme-support bond (Fig. 26). However, the length of the spacer arm permits some mobility of the reactive group of the support, and that means that the longer the spacer arm is, the higher the possibility of achieving more enzyme-support bonds, and the higher the intensity of the enzyme-support reaction. Moreover, longer spacer arms may involve in the immobilization a larger percentage of the enzyme surface, as it can access protein groups farther from the support surface (Fig. 26). That is, longer spacer arms permit a more intense multipoint covalent attachment because they have more mobility and can access a large percentage of the enzyme molecule. However, the final enzyme stabilization achieved by each additional attachment may be lower than using a shorter spacer arm because the reduction of the enzyme mobility will be smaller (Barbosa et al., 2013; Garcia-Galan et al., 2011; Mateo et al., 2007c) (Fig. 26).

Moreover, it must be considered that a long spacer arm may also produce some physical enzyme-support interactions, as it will hardly be fully physically inert (e.g., aldehyde dextran may be used a long, flexible and inert spacer arm). Thus, a reactive group directly located on the support surface will greatly reduce the mobility of the attached enzyme moiety, but may not be easy to have a very intense multipoint covalent attachment (Fig. 26).

The optimal spacer arm should be studied for each enzyme. In some cases the increase in the number of enzyme-support bonds may compensate the lower induced rigidity per additional enzyme-support bond, while in other cases it may not.

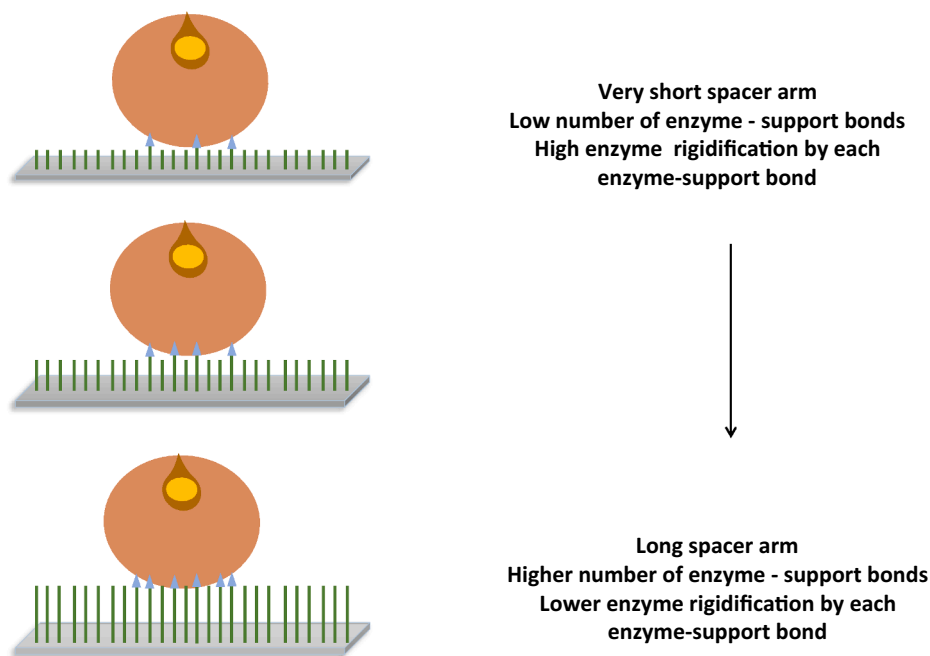


Fig. 26. Effect of the spacer arm length in the stabilization of enzymes via multipoint covalent attachment. The longer the spacer arm, the higher the number of enzyme-support bonds but the lower the impact in the polymer rigidity.

Another important topic is when the layer of reactive groups in the support is under a layer of other groups existing in the support (Fig. 27). These are the reactive groups which are not like those at the level of the support surfaces, but in holes under the other group layer. These groups make the enzyme-support multipoint reaction very difficult (Fig. 27). This is the case of the first generation of epoxy and glyoxyl heterofunctional supports, where some of the reactive groups in the support are modified to permit the enzyme first immobilization and that way control the enzyme orientation. These groups are above the layer of reactive groups (Barbosa et al., 2013). Using these supports, the final enzyme stability is greatly reduced (Bolivar et al., 2010; Grazú et al., 2010; Grazú et al., 2005; Mateo et al., 2007a, 2007b, 2007d). This problem was greatly overcome when the adsorbing groups were located on the support surface, and then modified to introduce the epoxide groups (Mateo et al., 2003). Although this enlarged the final spacer arm (reducing the stabilizing effect of each additional enzyme-support bond), a much higher stabilization was obtained using this second generation of heterofunctional supports. That way, the existence of steric hindrances for the enzyme-support reaction was found to be a critical factor when the enzyme-support interaction intended to be maximized (Fig. 27).

In this regard, some reports show how the use of dendrimers as spacer arms, offering a very high geometrical congruence with the enzyme that can be partially embedded in this polymer (and that way increasing the percentage of the protein that can participate in the immobilization), many reactive groups and some rigidity (Wang et al., 2020; Weltz et al., 2019), not like flexible polymers such as polyethylenimine (Virgen-Ortiz et al., 2017a), have given impressive stabilization factors. However, the complexity of the preparation of these spacer arms has maintained the number of examples of the use of these spacer arms in literature relatively low.

5.5. The immobilization protocol

Even using the best support and reactive group, only the use of a proper immobilization protocol will allow fully utilizing the possibilities of the multipoint covalent immobilization to stabilize an enzyme (Pedroche et al., 2007).

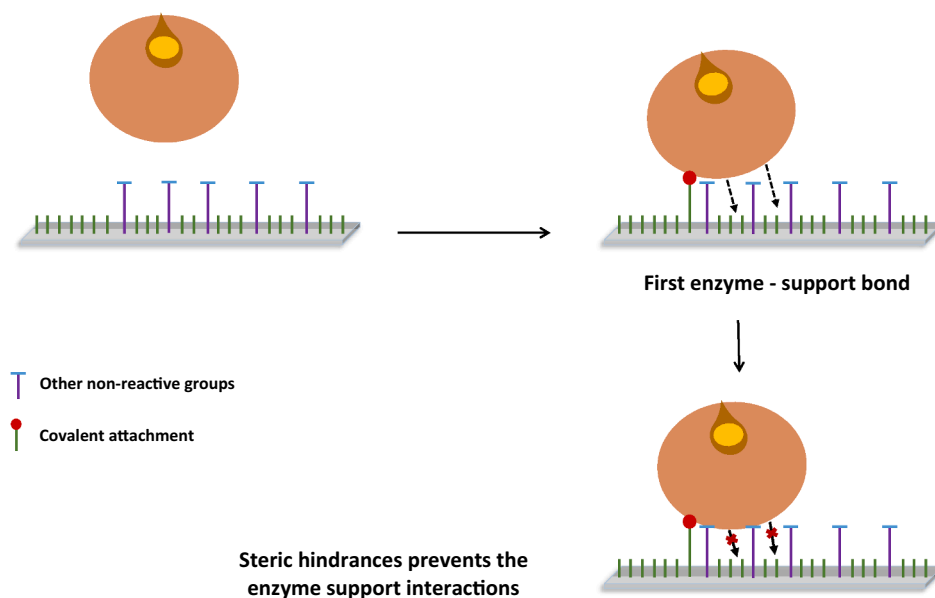


Fig. 27. Negative effects of the steric hindrances in the support-enzyme reaction to maximize the intensity of the multipoint covalent immobilization.

First, we must seclude in our mind enzyme immobilization (the enzyme has been incorporated to the support) with the process of multipoint covalent attachment, a much slower process. It may be even separated in two different steps run under different conditions (Mateo et al., 2000a, 2000b, 2003, 2002, 2007b;). The enzyme incorporation to the support may mean a single covalent bond or even may be via some physical event (e.g., this is very usual using glutaraldehyde activated supports and other heterofunctional supports). After the first enzyme immobilization, a protocol to favor the enzyme multipoint covalent attachment needs to be developed (Pedroche et al., 2007), and this requires to use longer times that just needs for immobilization (Fig. 28).

The immobilization protocol should consider that we must maximize the reactivity of the enzyme with the support. They are not complementary and rigid structures, and the rigidity of the enzyme polymer will be increasing when more and more enzyme-support bonds are formed. That way to get the maximum multipoint reaction the reaction

conditions need to be established to favor this reaction. A high temperature will increase the mobility of both, enzyme and support groups, but may have a negative effect on the enzyme stability and also in the support reactive group stability. The pH must be also selected in a way that maximizes the enzyme-support reactivity. For example, if we intend to use the ϵ -amine of Lys, we need to consider that only the non-protonated groups will be reactive, that means that an alkaline pH may be convenient (Mateo et al., 2005, 2006). Again, this may be detrimental for the enzyme or support reactive group stability that must be deeply studied before immobilization. In some cases, the use of inadequate pH values can not only reduce the enzyme stabilization by multipoint covalent immobilization, but also may even entirely prevent enzyme immobilization.

Reaction time is another critical parameter. After enzyme immobilization, the formation of new enzyme support bonds may be comparatively a much slower process, as it needs the alignment of two groups

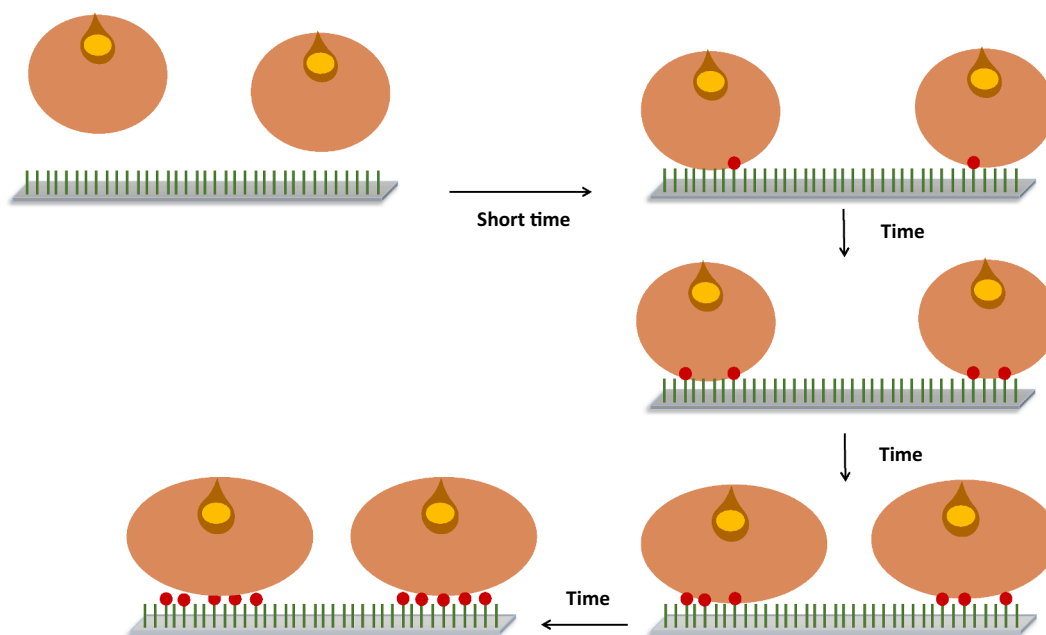


Fig. 28. Effect of enzyme-support reaction time in the multipoint covalent immobilization.

located in two non-complementary and rigid structures (Fig. 28). This process can take hours or even some days to reach the maximum level of enzyme-support reaction (Pedroche et al., 2007).

The medium composition is also a critical point. The use of some buffers may produce some interferences in the enzyme-support reaction. For example, it has been shown that the use of borate can block aldehyde groups, reducing the intensity of the multipoint covalent attachment (Alvaro et al., 1990). If the support is able to react with primary amino groups, the use of Tris buffer (Braham et al., 2021a, 2021b, 2021c) or the use of an impure protein extract containing compounds bearing primary amino groups (Morellon-Sterling et al., 2021b), can reduce the intensity of the multipoint covalent immobilization by two ways: first, by reducing the amount of available support reactive groups; second, by producing steric hindrances to the enzyme support reaction (see point 5.4) (Fig. 29).

The use of some additives with stabilizing effects (e.g., competitive inhibitors (Alvaro et al., 1991; Blanco and Guisán, 1988), glycerin or dextran (Braham et al., 2021b; de Cordt et al., 1994; Haque et al., 2005; Khoshnevisan et al., 2018; Kumar et al., 2011; Liu et al., 2010; Lozano et al., 1994; Pazhang et al., 2016; Romero and Albis, 2010; Tiwari and Bhat, 2006)) may be important to be able to enlarge the immobilization conditions window, decreasing the impact of the immobilization conditions and multipoint enzyme support reaction on the decrease of enzyme activity. However, it may produce a lower mobility of the enzyme surface, and that way, a lower final number of enzyme support bonds, and a final lower enzyme stability (Alvaro et al., 1991; Blanco and Guisán, 1988). That way, the researcher should select the level of enzyme activity/stability required for the process.

Finally, the design of an appropriate support-enzyme reaction end point process is important, as remarked in point 5.1 (dos Santos et al., 2015f; Garcia-Galan et al., 2011; Mateo et al., 2007c). When the active groups in the support are very unstable, it may be likely that the end point process is not necessary. When it is required, the nature and concentration of the blocking or reducing agent must be optimized to have the lower impact in the enzyme activity. It should be considered that the critical support groups to be modified are those under the immobilized enzyme molecules, where the access may be more complicated for steric reasons. That way, it may be expected that the modification of these groups may be harder than the modification of the naked surface support, requiring longer reaction times.

5.6. The reactive groups

The properties of the reactive groups in the support are one of the most important points to get an optimal enzyme-support multipoint covalent immobilization (Barbosa et al., 2013). These groups must be stable enough under the immobilization conditions, as multipoint covalent attachment requires some time, as previously discussed (Pedroche et al., 2007). Moreover, they should not present steric hindrances to the enzyme-support reaction, as the reaction between two rigid structures as a support surface and an enzyme surface is difficult enough. Finally, they should have the capability to react with the maximum amount of groups that are present in the enzyme surface.

With these requirements, immobilization of enzymes via their carboxylic groups seems an ideal solution, as these groups are the most abundant on the enzyme surface (those from Asp, Glu and carboxy terminal moiety) (Gao and Kyratzis, 2008; Gombin et al., 1999; Kumar et al., 2014; Milani et al., 2017). However, these groups need to be activated to be reactive, and the produced reactive groups are not very stable, making it very difficult to get an intense multipoint covalent immobilization (Carraway and Koshland Jr, 1972; Rodrigues et al., 2014). This means that the different strategies of immobilization via their carboxylic groups, usually utilizing carbodiimide routes, cannot be considered a recommendable method to get an intense multipoint covalent immobilization, although it may be a valid method for enzyme immobilization.

The most intense multipoint covalent immobilization tends to be achieved using supports able to react with primary amino groups (those from the ϵ amino groups of the Lys and the terminal amino group). However, it must be considered that the pK of the ϵ -amine of Lys is over 10.5, and the pK of the terminal amino groups may be between 7 and 8, depending on the environment and vicinal groups. This way, at neutral pH, only the terminal amino groups will be highly reactive with the support, permitting the enzyme immobilization but not an intense enzyme-support multipoint reaction.

From the literature, we can remark four different active groups that are very suitable to get an intense multipoint covalent attachment: glutaraldehyde, glyoxyl, epoxide and vinyl sulfone. Table 1 summarizes the main differences among these active groups. Next, we will discuss the *pros* and *cons* of each of these groups.

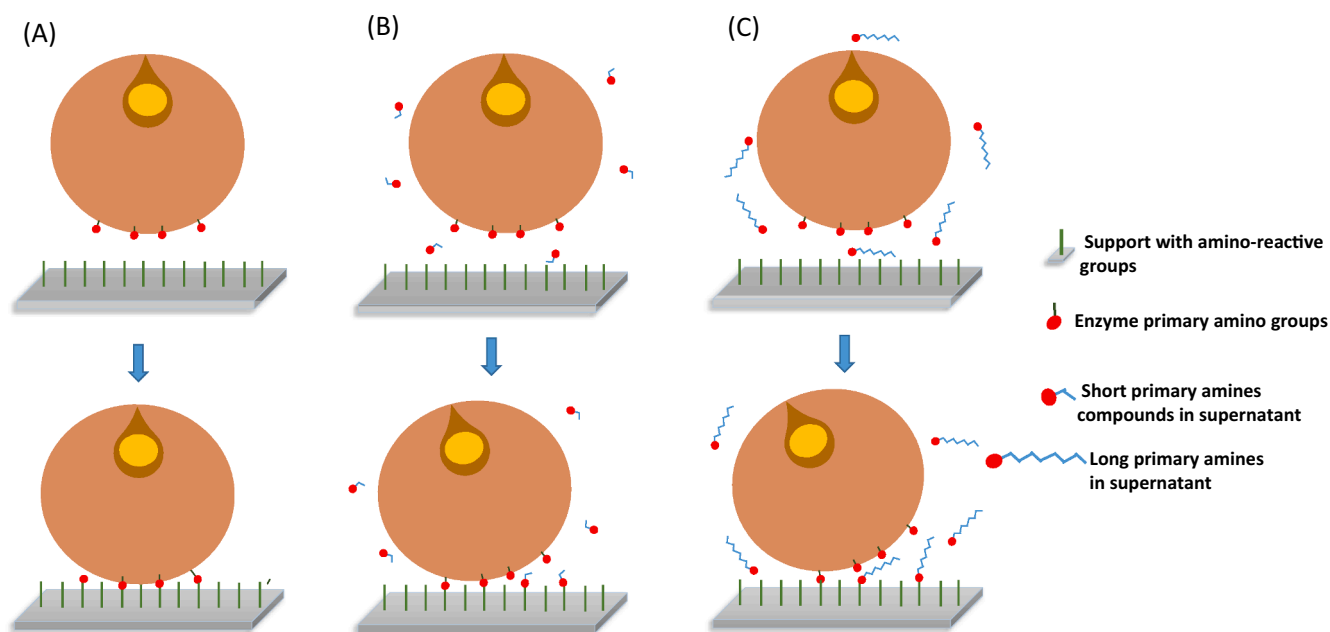


Fig. 29. Effect of short (B) or long aminated (C) compounds in the enzyme solution on the multipoint covalent enzyme immobilization.

Table 1
Main differences among the most used active groups of the supports

	Protein groups that can react with them	Reactivity	pH range to give covalent immobilization	Size of the spacer arm	Innerness of the final support surface	Reaction end point
Glutaraldehyde Epoxide	Primary amino groups	Low	pH 7-8	Long	Very poor	Not necessary
	Primary amino groups	Very low	pH 7-10.5			
	Carboxylic groups					
	Phenol	Extremely low	pH under 5			
	Imidazole	Low	pH 5-10			
Glyoxyl	Thiol	Low	pH 5-10	Depends on the activation protocol	Moderately poor	Blocking
		Moderately low	pH 5-10			
		High	pH 10.05			
Vinylsulfone	Primary amino group		pH7 (in case there is several terminal amino groups)	Moderately long	Moderately poor	Blocking
	Phenol	Moderate to high	pH 5-10			
	Imidazole	High	pH 5-10			
	Thiol	Very high	pH 5-10			
		Very high				

5.6.1. Enzyme stabilization by its immobilization using the glutaraldehyde chemistry

The use of glutaraldehyde to immobilize enzymes is well established (Stanley et al., 1976). However, this reagent is very complex and versatile, and its possibilities have not been properly evaluated until very recently. In fact, still some questions remain unsolved (Barbosa et al., 2014). Usually, supports bearing primary amino groups are activated with this reagent. Depending on the activation conditions (mainly concentration of glutaraldehyde, activation pH, and time), Monsan showed some time ago that it was possible to get a modification of the primary amino groups in the support by just one glutaraldehyde molecule, by two glutaraldehyde molecules, or permit the uncontrolled polymerization of the glutaraldehyde (Monsan, 1978). He showed that the best reactivity of the glutaraldehyde pre-activated support versus primary amino groups was obtained using conditions where 2 molecules of glutaraldehyde were introduced per amino group in the support. This reactive group is not very stable and mainly reacts with primary amino groups of the proteins, very likely forming stable cycles that are still under discussion (Barbosa et al., 2014). That way, the amino groups in the enzyme still remain with ionization capacity. The cycles produce reactive groups that can generate some steric hindrances to the enzyme-support multipoint attachment, as previously discussed (Barbosa et al., 2014). The low stability of the glutaraldehyde groups at alkaline pH values causes that usually the enzymes are immobilized at pH 7-8, and at these pH values, the main enzyme group that participates in the covalent immobilization is the terminal amino group. However, the situation is far more complex, as these supports are really heterofunctional ones, as they bear amino groups (with ion exchange capacity), hydrophobic moieties (the glutaraldehyde cycles), and chemical reactivity (Barbosa et al., 2014). That way, they can immobilize most enzymes under a wide range of conditions, but enzyme immobilization does not mean the enzyme has been attached to the support even by a single covalent bond. It may be immobilized via anionic exchange, hydrophobic interactions, or both of them (Barbosa et al., 2014). In fact, the direct covalent immobilization of enzymes on glutaraldehyde pre-activated supports is much slower than the immobilization via the other mechanisms. Playing with the immobilization conditions (e.g., ion strength), the importance of the events that produce the first enzyme immobilization may be altered, altering the orientation of the enzyme regarding the support. This has been described in different papers and used to improve the biocatalyst performance (Barbosa et al., 2012; Betancor et al., 2006b; Dal Magro et al., 2020; Dal Magro et al., 2019; Siar et al., 2018a; Vazquez-Ortega et al., 2018; Zaak et al., 2017c).

Moreover, it has been recently shown that ion exchange may produce different biocatalyst properties depending on the immobilization conditions (ion strength, pH), increasing the versatility of this

immobilization method (de Albuquerque et al., 2016; Pessela et al., 2006; Pessela et al., 2005).

Nevertheless, although some level of multipoint covalent immobilization may be obtained, the expected intensity of the multipoint covalent immobilization may not be very high using aminated supports pre-activated with glutaraldehyde: the relatively low stability of the glutaraldehyde groups, the low reactivity of amine from Lys at pH 7-8 and the steric hindrances makes to get the maximum multipoint covalent immobilization very difficult (Barbosa et al., 2014).

There is an alternative to the use of the aminated glutaraldehyde pre-activated supports: the treatment with glutaraldehyde of the enzymes previously immobilized on the aminated supports just via ion exchange (López-Gallego et al., 2005a). In this case, the objective is to modify the amino groups in the support and in the enzyme with just one glutaraldehyde molecule, as the reactivity of this amino-glutaraldehyde with other similar group is relatively high even at pH 7-8 (Fernandez-Lafuente et al., 1995b). The groups modified with two glutaraldehyde molecules are poorly reactive with similar groups. This strategy has a significant problem: the whole enzyme surface needs to be modified with glutaraldehyde, and in some instances this can have inactivating or destabilizing effects (Carballares et al., 2021.; Siar et al., 2018a). However, it has some interesting advantages. The multipoint covalent attachment is now more likely, as long as the area of the enzyme in contact with the support has several Lys groups. This is likely to occur in some instance, as by altering the ionic exchange conditions, it is possible to alter the area of the enzyme interacting with the support. Moreover, it is possible to further increase the enzyme stability by achieving some protein inter or intra molecular crosslinking. Thus, generally a higher level of stabilization is achieved using this strategy than using glutaraldehyde pre-activated supports (López-Gallego et al., 2005a), although in many instances it is not possible to use it due to the effects of the glutaraldehyde modification on the enzyme activity.

The reduction of biocatalysts prepared using glutaraldehyde is used in some papers, but it does not appear fully necessary, as the glutaraldehyde cycles suffer some reactions that give stable enzyme-glutaraldehyde-support bonds and a very low chemical reactivity. Moreover, the reduction of the biocatalyst with 1 mg/ml sodium borohydride did not fully eliminate the support reactivity (unpublished results).

In any case, many enzymes have been greatly stabilized by using the glutaraldehyde immobilization (Barbosa et al., 2014). Although this is the optimal protocol in certain cases (e.g., when the enzyme cannot be immobilized on glyoxyl supports due to their instability under alkaline conditions, or when the enzyme has multimeric nature or require some cofactor), stabilization factors are scarcely over 200-300 (Alonso et al., 2005; Betancor et al., 2006a; b; Lopez-Gallego et al., 2005a and b, c).

5.6.2. Enzyme stabilization by immobilization on epoxide-supports

Epoxide is another reactive group used to stabilize enzymes via multipoint covalent attachment for a long time (Mateo et al., 2007d; Tadzhev et al., 1978; Turková et al., 1978; Ulu et al., 2018). They can react with primary amino groups, but also with thiol (from Cys), imidazole (from His), phenol (from Tyr) and even with carboxylic acids (from Asp or Glu) (Turková et al., 1978).

They are directly produced as acrylic epoxy resins, and they are stable even at alkaline pH value, have low steric hindrance for the enzyme-support reaction, and the enzyme-support bonds are stable (secondary amino, thio-ether, ether, ester). As a reaction end point, the blocking of the supports is a good option (Boller et al., 2002; Hilterhaus et al., 2008; Katchalski-Katzir and Kraemer, 2000).

That way, apparently they are “ideal” supports to get an intense multipoint covalent immobilization. However, the reactivity of epoxy supports with enzymes is very poor, only immobilization via Cys has a rate relevant enough to directly covalently immobilize a significant percentage of enzyme (Grazu et al., 2012; Grazú et al., 2010).

Generally, protein immobilization on these supports follows a two-step mechanism: first the enzyme is incorporated to the support through some other mean, then, the enzyme reactive groups can react with the very near epoxy groups (Mateo et al., 2000b) (Fig. 30). The initial commercial epoxy-activated resins had a hydrophobic nature, and the protocol for immobilization suggested the use of high ionic strength (to force the immobilization of the enzyme by hydrophobic interactions) (Melander et al., 1984; Smalla et al., 1988; Wheatley and Schmidt Jr, 1999; Wheatley and Schmidt Jr, 1993). In the 2000s, Prof. Guisán's group suggested modifying some epoxy groups on the support to introduce moieties able to favor enzyme adsorption or immobilization (amino, carboxy, boronate, immobilized metal chelate, thiol) (Fig. 31) (Mateo et al., 2000b). Later, they observed some covalent immobilization via the remaining epoxy moieties (Mateo et al., 2000b). Afterwards, the company Resindion launched an amino-epoxy support that permitted to have maximal adsorption capacity of the support and

maximum capacity to establish covalent immobilization (the epoxides were introduced modifying amino supports) (Mateo et al., 2000b) (Fig. 31). To get a multipoint covalent attachment, a third step was necessary, to incubate the already immobilized enzyme at alkaline pH value, in many instances for days (Mateo et al., 2002; Mateo et al., 2000a). And finally, the blocking step permitted to tune the enzyme features and eliminate the chemical reactivity of the support (Bonomi et al., 2013; Mateo et al., 2000a, 2002).

The use of tailor-made heterofunctional epoxy supports found some applications: purification/immobilization/stabilization of multimeric large proteins (Barbosa et al., 2015; Bolivar et al., 2009a, 2010) and site-directed rigidification (Grazú et al., 2010). This has been already reviewed (Barbosa et al., 2013) and we will not further discuss these possibilities. As previously indicated in this review, the steric hindrances generated by the additional groups reduce the intensity of the multipoint covalent immobilization (Fig. 27).

Moreover, the low reactivity of the epoxy groups makes the process a very long one (Bolivar et al., 2010), even using mono-functional epoxy supports. That way, these supports are not so “ideal” to get an intense multipoint attachment as previously expected. However, there are some examples where the enzyme stabilization using this kind of supports is quite significant (Barbosa et al., 2013). That is the case of the immobilization of the enzyme penicillin G acylase on Eupergit or Sepabeads, (with stabilization values ranging 10,000 for the latter) (Bonomi et al., 2013; Mateo et al., 2000a, 2002) giving stabilization values similar to those achieved using glyoxyl-supports (see below), however these stabilization values are more the exception than the rule, glyoxyl supports (see below) usually largely increasing the obtained results using epoxy activated supports. However, there are exceptions. The β -galactosidase from *Aspergillus niger* immobilized on amino-epoxy supports gave the highest stabilization values achieved by the immobilization of this enzyme in that moment (a hundred-folds more stable than the free enzyme under certain conditions) (Torres et al., 2002).

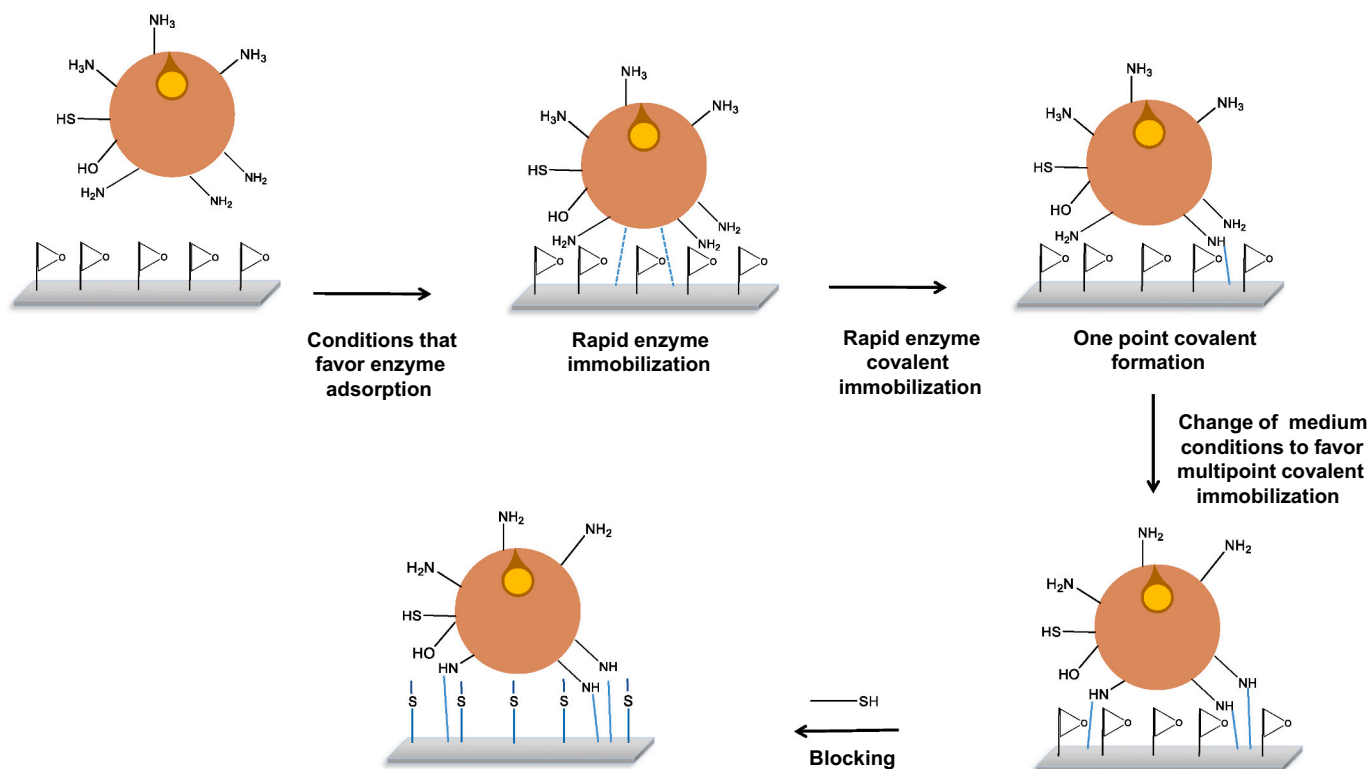


Fig. 30. Stabilization of enzyme by multipoint covalent immobilization on epoxy supports: first enzyme immobilization, first epoxy covalent immobilization, multipoint covalent immobilization and blocking steps.

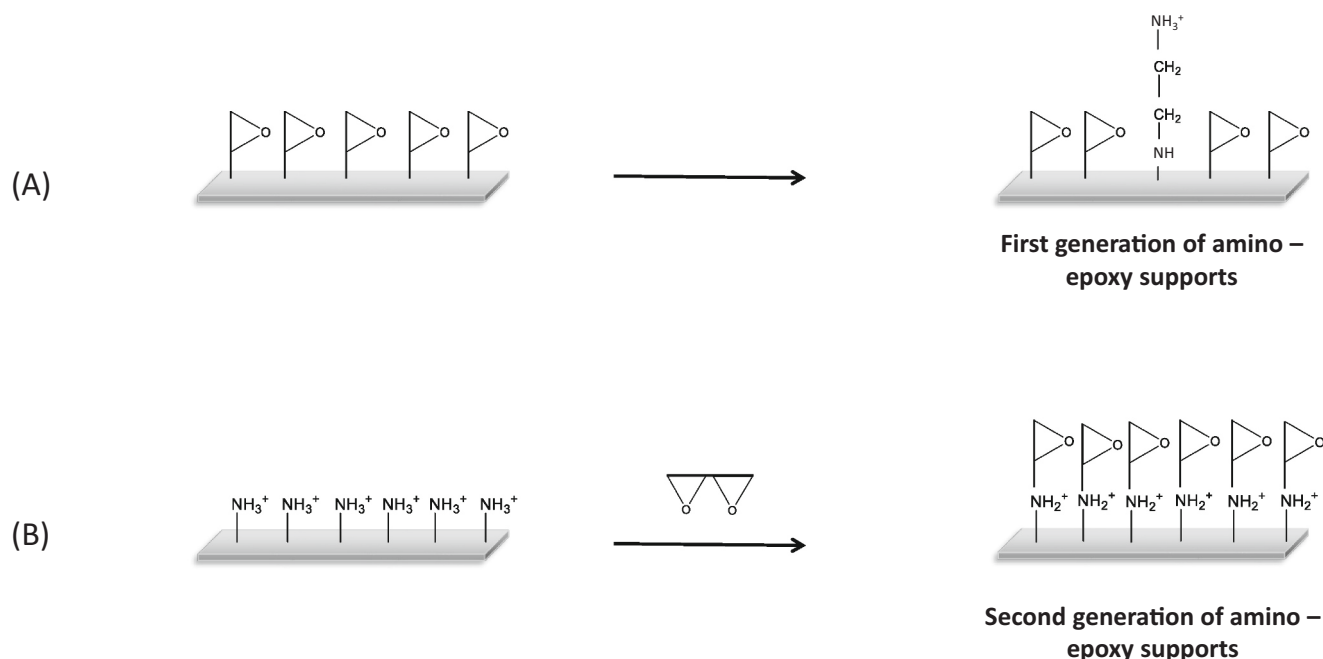


Fig. 31. Differences in the two generations of amino epoxy supports. In the first one, some epoxy groups are modified to introduce amino groups, and this produces some steric hindrances to the enzyme-epoxy reaction (A). In the second generation, the epoxy groups are introduced by modification of an aminated support (B). Steric hindrances are not produced, but the spacer arm is longer.

5.6.3. Stabilization of enzyme by its immobilization on glyoxyl supports

Glyoxyl activated supports have been proposed some time ago as very adequate ones to give a very intense multipoint covalent enzyme immobilization (Guisán, 1988). These groups are able to react only with non-protonated primary amino groups, giving as final result secondary amino bonds, also with ionizable properties (Mateo et al., 2005, 2006) (Fig. 32).

They have some very interesting features: high stability at pH 10 and room temperature (half-life is over one week), there are no steric hindrances to the enzyme-support reaction, the spacer arm is very short, it may be easily generated in several supports, and the reduction with borohydride is a suitable reaction end point (Mateo et al., 2005, 2006). This in turn converts the imine bonds in irreversible secondary amino bonds and the remaining and unreacted aldehyde in the support in inert hydroxyl groups. They have permitted between 50 and 2,000,000 increases of the half-life of a wide collection of enzymes, maintaining high levels of activity (Abian et al., 2004; Becaro et al., 2020; Bernal et al., 2018a, Bernal et al., 2013; Betancor et al., 2006a, Betancor et al., 2003; Bezerra et al., 2015a, 2015b; Bolivar et al., 2008, Bolivar et al., 2007, Bolivar et al., 2006b, 2006a, Bolivar et al., 2009b, 2009c, Bolivar et al., 2010, 2012, Bruni et al., 2020; Fernandez-Lafuente et al., 1995a;

Fernandez-Lafuente et al., 1995b; Fernandez-Lorente et al., 2008b; Fernández-Lorente et al., 2015; García-García et al., 2020; Guerrero et al., 2019; Guisán, 1988; Huerta et al., 2011; Kuroiwa et al., 2005; Lopes et al., 2020; Lopez-Gallego et al., 2007; López-Gallego et al., 2005b; Manrich et al., 2010; Martins de Oliveira et al., 2021; Mateo et al., 2005, 2006; Megías et al., 2006; Mendes et al., 2011a,; Mendes et al., 2011b; Morais Junior et al., 2021; Orrego et al., 2020, Orrego et al., 2018; Pedroche et al., 2007; Rios et al., 2016; Rocchietti et al., 2004; Rodrigues et al., 2008, 2009; Romero-Fernández et al., 2018a, 2018b; Siar et al., 2020; Suárez et al., 2018; Tardioli et al., 2003a and b; Tardioli et al., 2005, Tardioli et al., 2006, Tardioli et al., 2011; Toogood et al., 2002; Ubilla et al., 2020; Urrutia et al., 2013; Yust et al., 2010). This places it as the most potent method to stabilize enzymes at least until vinylsulfone supports were proposed as an alternative.

Later, a critical feature of this support was found, which is apparently an inconvenient: the individual imine bond is extremely unstable, making the enzyme only fixed on the support via several enzyme-support bonds (Mateo et al., 2005). Thus, the enzyme is directly immobilized on the support by the region that is the richest one in enzyme reactive groups, that is, where the enzyme-support multipoint attachment is more likely. The low energy of the imine bonds also

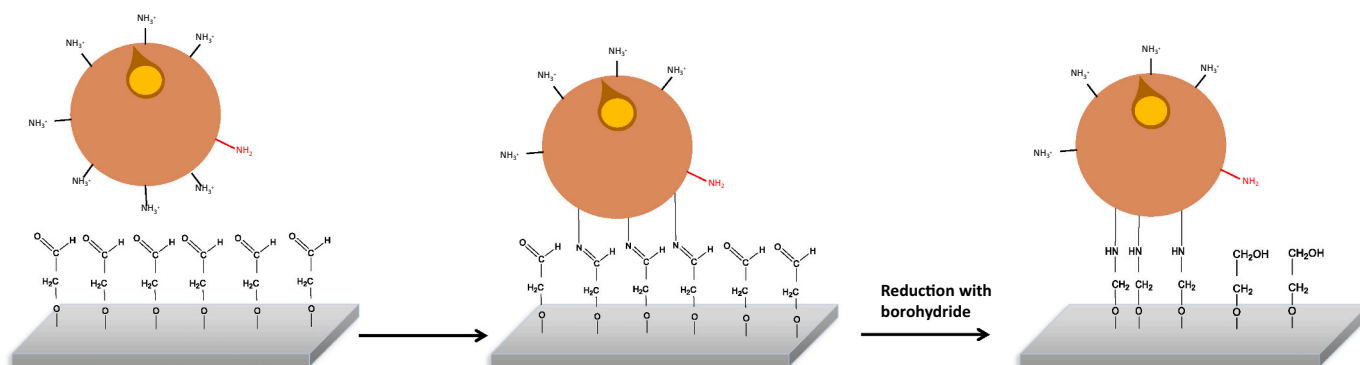


Fig. 32. Enzyme multipoint covalent immobilization on glyoxyl supports.

prevents very strong distortions of the enzyme structure caused by the formation of a single individual new bond. That way, the apparent problem created by the low energy of the imine bond becomes one of the reasons of the very good results using glyoxyl supports in enzyme immobilization (Mateo et al., 2005).

This immobilization mechanism has been also utilized to couple enzyme immobilization with enzyme purification (Bolivar et al., 2009b). If the immobilization is performed at neutral pH value, only enzymes bearing several primary amino groups in the same plane can become immobilized (Grazu et al., 2006; Pessela et al., 2007) (Fig. 33). This involves multimeric enzymes, or enzymes that have suffered some proteolysis (Grazu et al., 2006) (Fig. 14). Later, after eliminating the non-immobilized enzymes, the pH may be increased to 10.05 to favor the multipoint covalent attachment (with the advantage that the already immobilized enzyme cannot dissociate). That way, the immobilization at neutral pH values using these supports enables the one step partial purification, rigidification and stabilization of the multimeric structure of oligomeric enzymes that have several terminal amino groups available to be immobilized (Fig. 33).

However, this low energy of the imino bond raises some problems. First, it is necessary to use alkaline pH values even to just immobilize the enzymes (Mateo et al., 2005). This may be solved using some imino-stabilizer agents, but this way, the system loses the advantage of the immobilization via the richest area in Lys (Bolivar et al., 2009c). Second, enzymes that are poor in Lys groups or supports that cannot be highly activated may fail in even giving an immobilized biocatalyst.

The use of sodium borohydride may be also a problem (Blanco and Guisán, 1989). Although at laboratory level its handling is not a great problem, the storage and management of this explosive compound may not be simple at industrial level, if the factory does not have the proper facilities. Moreover, some enzymes may be sensible against this agent. At high concentrations (higher than recommended to reduce the glyoxyl-enzyme biocatalysts, 1 mg/ml), it can break peptide bonds and disulfide bridges, but even at low concentrations it may reduce some metals, which may be a key for the enzyme activity/stability (Blanco and Guisán, 1989). Some alternative reducing agents have been proposed to avoid this problem such as cyano-borohydride (Gemeiner and Breier, 1982; Hansen and Mikkelsen, 1991; Kim and Wainer, 2005) or 2-picoline borane (Orrego et al., 2018).

That way, this support has remained for 40 years as a very

recommendable strategy for enzyme stabilization via multipoint covalent attachment, and usually (although not always) offers higher stabilization factors than the previously described methods (Fernández-Lorente et al., 2015).

5.6.4. Enzyme stabilization by immobilization on vinyl sulfone supports

Supports activated with divinyl sulfone have been used for a relatively long time for immobilization of different compounds and biomacromolecules (Arana-Peña et al., 2020a; Begara-Morales et al., 2013; Bryjak et al., 2008; Li et al., 2019; Medina-Castillo et al., 2012; Ortega-Muñoz et al., 2010; Santos-Moriano et al., 2016) (Fig. 34). However, only very recently they have been proposed as a suitable protocol for enzyme stabilization via multipoint covalent attachment (dos Santos et al., 2015a, 2015b, 2015c, 2015d, 2015e), with stabilization values sometime surpassing those obtained using glyoxyl supports (e.g., using chymotrypsin, 4-5 fold more stable than glyoxyl preparations (dos Santos et al., 2015a), that was 60,000 folds more stable than the free enzyme (Guisán et al., 1991)). However, using trypsin, even though a more intense enzyme-support multipoint covalent attachment was achieved, the stabilities of the new biocatalysts remained under those achieved using glyoxyl supports (dos Santos et al., 2015b).

These groups can react with primary and secondary amines, thiol and hydroxyl groups, etc. (dos Santos et al., 2015a). They are quite stable, enabling long enzyme-support reaction periods, the reaction has low steric hindrances, the bonds are stable and as a reaction end point, it is possible to block the support with different nucleophiles. The spacer arm is not too long, but it is longer than that of the glyoxyl support (Fig. 35). This length produces some hydrophobicity on the support surface, enough to permit the interfacial activation of some lipases on the activated support (dos Santos et al., 2015d). The reactivity is much higher than that of the epoxide groups, enabling to immobilize enzymes at pH from 5 to 10 (Fig. 35). Nevertheless moderately alkaline pH values are necessary to have an intense multipoint covalent attachment (dos Santos et al., 2015d) (Fig. 35). The immobilization pH alters the main enzyme groups involved in its immobilization, enabling to immobilize the enzymes by different areas (dos Santos et al., 2015e; dos Santos et al., 2015b; dos Santos et al., 2015d, 2015a) (Fig. 34). Again, it is possible to use different conditions in the immobilization step and in the multipoint covalent attachment step (dos Santos et al., 2015d) (Fig. 35). The blocking step is a very important one, as it may be used to tune the

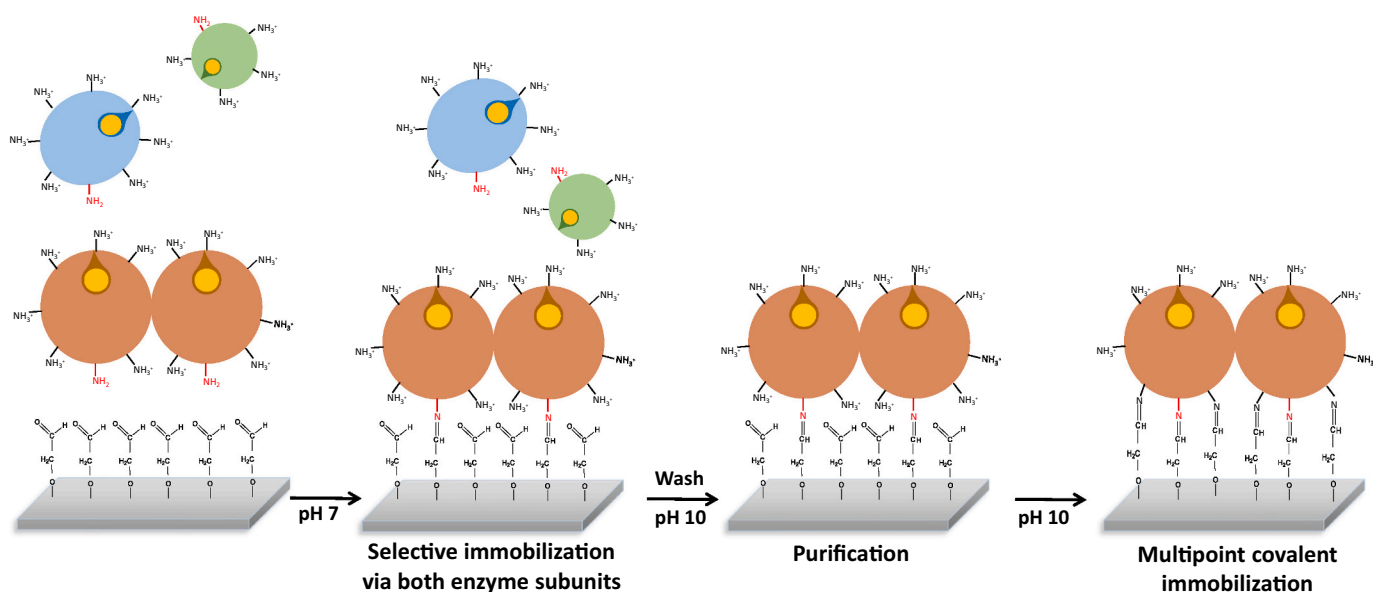


Fig. 33. Purification of multimeric enzymes by immobilization on glyoxyl supports at neutral pH. At this pH, all monomeric enzymes can only react via one point with the support, and the enzymes are not immobilized. However, multimeric enzymes having several terminal amino groups in the same plane can multipoint react with the support and become immobilized.

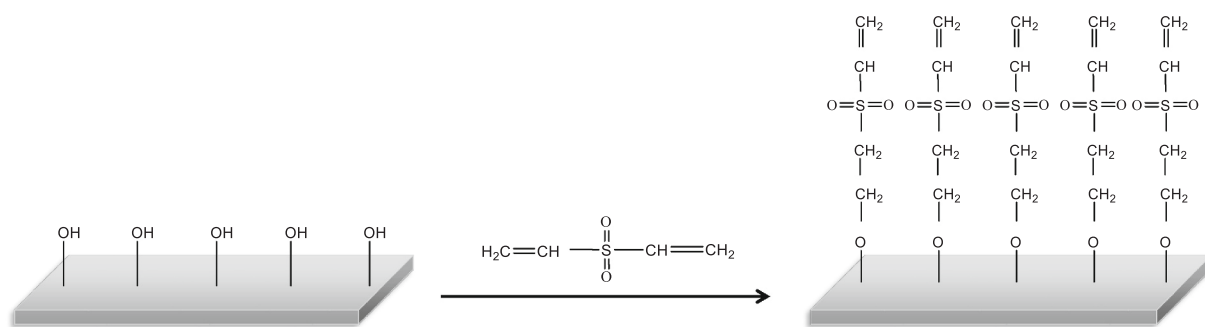


Fig. 34. Activation of hydroxyl-supports with divinyl sulfone.

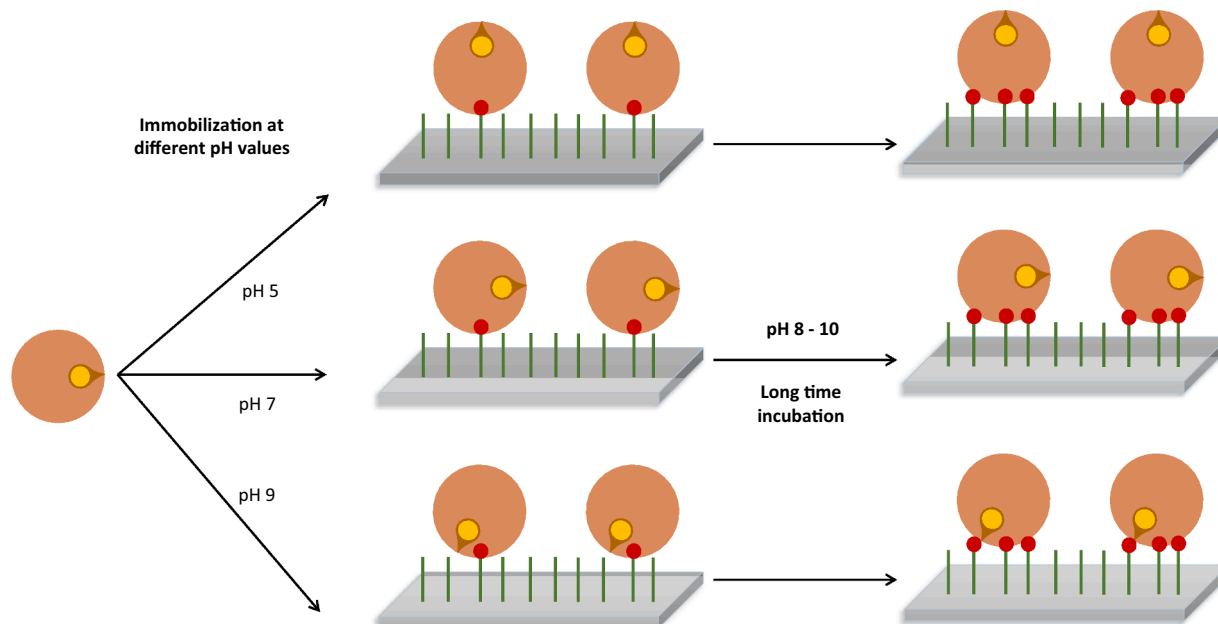


Fig. 35. Versatility of vinyl sulfone activated supports to immobile enzymes. The possibility of immobilizing the enzymes at different pH values may permit immobilizing the enzymes following different enzyme orientations.

final enzyme properties (dos Santos et al., 2015d) (Fig. 36), or even open new possibilities for enzyme coimmobilization (Morellon-Sterling et al., 2021a.) (Fig. 23). This support permits a much more intense multipoint covalent immobilization than the glyoxyl supports (involving more Lys groups and other enzyme groups like His, Cys or Tyr), however the effects on enzyme stability are not always higher than the immobilization on glyoxyl supports (dos Santos et al., 2015a, 2015b). This may be caused because of the longer spacer arm (lower reduction of the enzyme groups mobility) or the incomplete inertness of the final support surface.

Nevertheless, the features of this support have not been studied as in depth as the previously presented supports. For example, some enzymes cannot be immobilized on it while they are immobilized on glyoxyl supports, where it requires simultaneous immobilization by several non-protonated primary amino groups. The use of heterofunctional supports (amino-vinyl sulfone, octyl-vinyl sulfone) solves this problem. Enzymes that are not immobilized on monofunctional vinyl sulfone supports are rapidly and covalently immobilized on these heterofunctional supports and can give excellent stabilizations (Albuquerque et al., 2016; Pinheiro et al., 2019; Zaak et al., 2018).

Thus, this kind of activated supports are very promising ones to stabilize enzymes via multipoint covalent attachment, but this support still requires some intense research to fully understand its enzyme immobilization mechanism, and there are few examples where the multipoint covalent attachment of the enzymes have been pursued and

analyzed.

5.7. Determination of the number of enzyme-support bonds

After enzyme immobilization, it is interesting to determine the number of enzyme-support bonds, to check if really a correlation between the effects on enzyme activity/stability and number of enzyme linkages exists (Blanco et al., 1989; dos Santos et al., 2015b; dos Santos et al., 2015a; Morellon-Sterling et al., 2021b; Orrego et al., 2020; Pedroche et al., 2007). This can be only performed using pure enzymes, using impure protein extracts; this can only give some indication on the increase of the average number of proteins-support linkages, but not of our specific enzyme.

The colorimetric titration of the free primary amino groups (e.g., using picrylsulfonic acid) (Xue et al., 2009; Yewle et al., 2012; Snyder and Sobocinski, 1975) may be useful, but only when primary amino groups of the enzyme are the only groups involved in the immobilization, and must consider that the extinction concentration of the chromospheres is identical independently of the environment (and this may be not real). This is not valid to indicate the participation of other enzyme moieties in the immobilization. Moreover, this is only applicable if the support is transparent (e.g., agarose beads).

If the enzyme-support bonds are very stable, more stable than the peptide bonds (secondary amino bonds, ether, thioether, but not amide

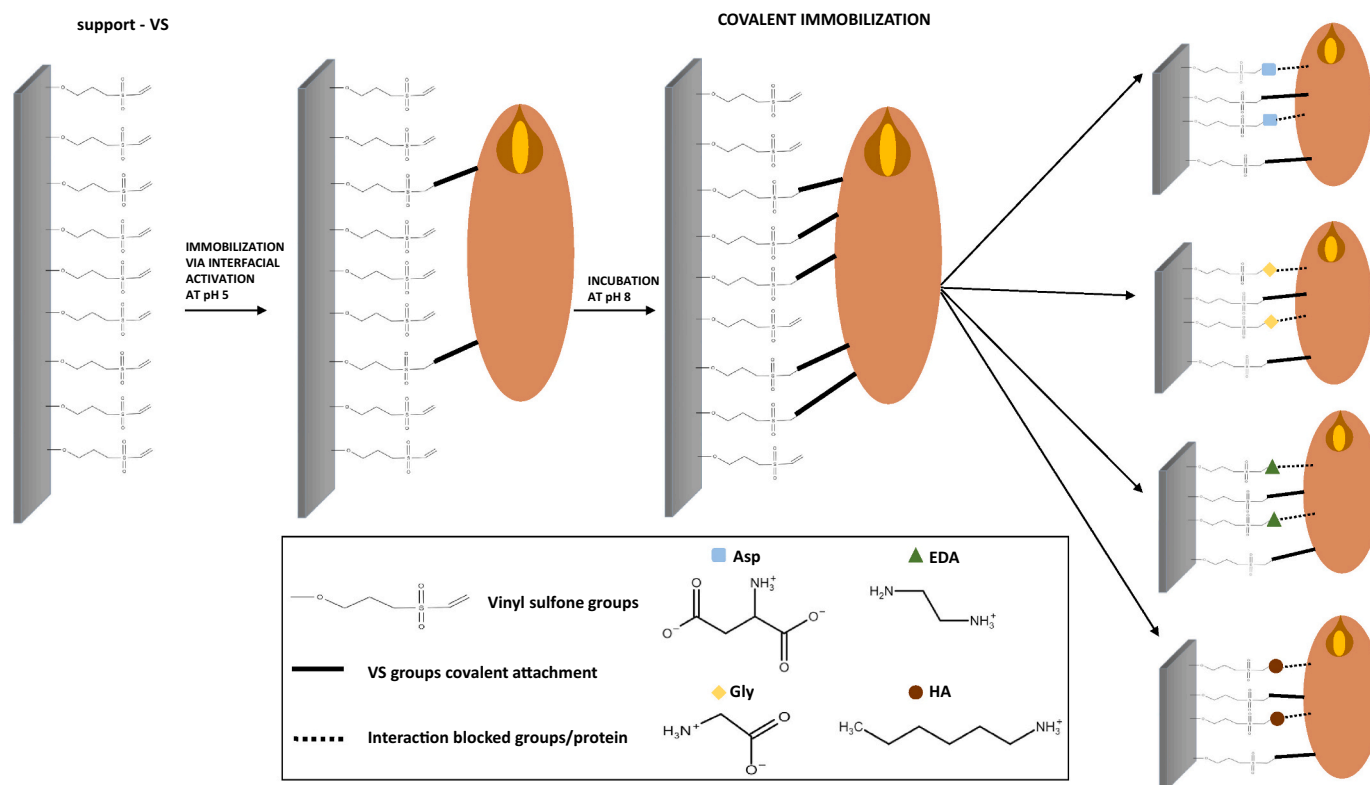


Fig. 36. The blocking step of the enzyme immobilization in supports activated with vinyl sulfone enables the tuning of the support surface properties.

or ester bonds), a strategy that is of more general utility to determine the groups involved in the immobilization is the determination of the amino acid composition of the enzyme and that of the immobilized enzyme, after acid hydrolysis. Some amino acids are lost by this treatment (e.g., Trp), but in general this may provide useful information on the percentage of different groups involved in protein immobilization. Some reference amino acids should be selected, they must not be in the amino nor carboxylic terminal positions and should lack of reactive moieties to ensure that they are not involved in the immobilization (dos Santos et al., 2015b, dos Santos et al., 2015a; Morellon-Sterling et al., 2021b; Orrego et al., 2020; Pedroche et al., 2007). This way, the intensity of the multipoint covalent immobilization may be determined. However, nowadays this is based on the decrease of the percentage of the unmodified involved amino acids, and this means that small differences in the intensity of the covalent immobilization may not be easily detected. It should be very interesting to establish strategies where the modified amino acid is directly determined, this can increase the precision of the protocol.

One problem of this technique is if the support, under acid hydrolysis conditions (high pressure and temperature, very acid pH value) can generate some groups able to react with the amino acids. To check this, it is convenient to use a blocked or reduced support added to free enzyme in the acid hydrolysis, and check if the presence of the support decreases or not the amount of detected amino acids. For example, using some epoxy acrylic resins, this technique is not possible because free amino acids can react with the support under acid hydrolysis conditions (Mateo et al., 2002).

6. Multipoint covalent attachment intensity versus stabilization of the enzyme structure

Multipoint covalent immobilization should increase enzyme stability under any inactivating condition that involves an enzyme distortion, as we can assume that the polymer should be more rigid. It can be assumed

that the enzyme molecules rigidity achieved via immobilization will be maximized near the support surface and will be lower in the protein structure that is farther from the support surface. Moreover, it has been shown that the inactivation conditions determine the pathway of the inactivation, promoting different changes on the enzyme structure (Sanchez et al., 2016; Souza et al., submitted.). Thus, the enzyme stabilization promoted by its rigidification due to the multipoint covalent attachment using different immobilization protocols may be qualitatively different depending of the inactivation conditions.

However, there are some reports where the number of the multipoint covalent bonds is not directly related to a higher stabilization of the enzyme. Next, we will discuss some examples of this situation.

6.1. Cases where the enzyme distortion promoted by the multipoint covalent attachment may produce a negative effect on enzyme stability

It can be assumed that the formation of an additional enzyme-support bond may produce a more rigid polymer, but it also may produce some enzyme distortion. Using monomeric enzymes that do not depend on cofactors or ions and where some key group oxidation is not the first step of the enzyme inactivation, the enzyme stability should increase when the incubation time does, reaching a maximum of stability that should be maintained if the incubation time is prolonged (Pedroche et al., 2007) (from biocatalysts with just enzyme-support bond and stability similar to the free enzyme to immobilized enzyme thousands fold more stable than this biocatalyst). However, in enzymes where these alternative inactivation causes can occur, it is possible that an optimal value of the incubation time (that is related to an optimal number of enzyme-support bonds) exists, and after that moment, the enzyme stability starts to decrease (García-García et al., 2020; Morellon-Sterling et al., 2020; Siar et al., 2019; Siar et al., 2018b; Siar et al., 2017; Zaak et al., 2018). This phenomenon has not been studied in depth. This may not be because the protein polymer becomes less rigid, but because the enzyme distortion produces a larger exposure of their sensitive

groups to the medium (making oxidation simpler), or the adsorption of a key cofactor or metal ion to the enzyme may be weakened, or the multimeric enzyme assembly may be under stress. That means that the optimization must be carefully performed, and it should not be taken for granted that always a more intense multipoint covalent attachment will produce higher enzyme stabilization. In these cases, the stabilization achieved by rigidification via multipoint covalent hardly exceed 100 folds.

6.2. Importance of the area of the enzyme involved in the immobilization

In the last decade of the previous century, Mansfeld (Mansfeld et al., 1999; Mansfeld and Ulbrich-Hofmann, 2000; Ulbrich-Hofmann et al., 1999) developed the concept of protein “unfolding region”, showing that not all the enzyme zones have the same relevance for enzyme stabilization after their multipoint covalent immobilization (in these initial publications stabilization were in the range of tenths). Later on, Guisán’s research group, using thiol-epoxy or glyoxyl heterofunctional supports, showed that the rigidification of an enzyme via different areas may produce very different effects on the enzyme stability (Godoy et al., 2011; Grazu et al., 2012; Grazú et al., 2010). The use of heterofunctional epoxy supports maintained the stabilization factors under 100, but using glyoxyl agarose and an enzyme enriched in Lys groups the area, stabilization factors of two-million could be achieved (Abian et al., 2004).

That way, to prepare an optimally stabilized enzyme, not only is the number of enzyme-support bonds relevant, but also the area of the protein involved in the rigidification. A lower number of enzyme-support bonds in a more adequate area of the protein may produce higher final enzyme stabilization than more enzyme-support bonds in a non-relevant area. This way the possibility of immobilizing an enzyme by different areas becomes a very important point in the design of immobilized enzyme biocatalysts, also from a stability point of view.

7. Possibilities of increasing the intensity of multipoint covalent attachment

If the stability of the immobilized enzyme did not reach the desired value after multipoint covalent attachment immobilization, there are still possibilities to further improve the enzyme properties. One possibility is to use site directed mutagenesis or directed evolution to have an initially more stable enzyme (Kurahashi et al., 2020; Liu et al., 2019; Miller, 2017; Moore et al., 2017; Rojkova et al., 1999; Sung and Kang, 1998; Wijma et al., 2014), that very likely after optimal immobilization may maintain this improved stabilization compared to the original immobilized biocatalyst (if the changes in the enzyme structure did not affect the groups relevant for enzyme multipoint covalent immobilization). Another alternative is to modify the enzyme with the objective of producing not a more stable enzyme, but to permit a more intense multipoint covalent immobilization (Abdul Wahab et al., 2019; Abian et al., 2004; Ashkan et al., 2021; Bernal et al., 2018b; Bilal et al., 2018; Cowan and Fernandez-Lafuente, 2011; Fernandez-Lafuente et al., 1992; Fernandez-Lorente et al., 2008b; Hernandez and Fernandez-Lafuente, 2011a; Kamal et al., 2021; Rodrigues et al., 2014; Rodrigues et al., 2011; Rodrigues et al., 2009; Rueda et al., 2016b). This may be achieved via chemical or genetic modification of the enzyme, mainly by enriching the enzyme surface in primary amino groups. This way, the number of enzyme-support bonds may be increased, and likely this may allow increasing enzyme stability, at least rigidity. This enzyme surface amination may have effects on the free enzyme activity/stability, in some instance negative, but in some other cases they have even positive (Abdul Wahab et al., 2019; Abian et al., 2004; Ashkan et al., 2021; Bernal et al., 2018b; Bilal et al., 2018; Cowan and Fernandez-Lafuente, 2011; Fernandez-Lafuente et al., 1992; Fernandez-Lorente et al., 2008b; Hernandez and Fernandez-Lafuente, 2011a; Kamal et al., 2021; Rodrigues et al., 2014; Rodrigues et al., 2011; Rodrigues et al., 2009; Rueda et al., 2016b).

Protein chemical modification is rapid. The modification degree may be easily controlled and it allows using unnatural groups with more favorable properties than natural amino acids (e.g., amino groups with a lower pK than that of the ϵ -amino group of the Lys groups) (Rodrigues et al., 2011). However, chemical modification involves all enzyme surface, not only on the area involved in the immobilization. The modification may have positive or negative effects on enzyme stability/activity. Moreover, the modification must be performed each time that a new batch of biocatalysts is prepared.

On the contrary, genetic modification requires longer studies and the initial preparation may take longer times. However, after preparing the modified enzyme, the enzyme will be always produced with this modification. This modification will be limited to the enzyme area involved in the immobilization (Hernandez and Fernandez-Lafuente, 2011a). Although the variety of groups to be included will be more limited than the chemical modification, in the context of increasing the amount of enzyme reactive groups, the alternatives are enough.

Both strategies have shown their feasibility, as they have recently revised, we do not extent longer in this point (Hernandez and Fernandez-Lafuente, 2011a; Rueda et al., 2016b; Bernal et al., 2018b; Bilal et al., 2018).

8. Operational stabilization of by having the enzyme inside a macrostructure

Using porous supports, diffusional limitations are traditionally considered just a problem that reduces the enzyme activity. However, although this has not been properly studied in the literature, we can imagine some situations where the diffusional limitations may have a positive effect on enzyme stability.

The first example may be just the substrate diffusional limitations. If the substrate has some enzyme inactivating effects (e.g., hydrogen peroxide) and the reaction product has not this inactivating effects (Rodrigues et al., 2013). In this case, if the enzyme volumetric activity is high enough to reduce the concentration of the substrate inside the biocatalyst particle, an enzyme immobilized in the core of the support particle will be exposed to lower concentrations of the substrate. Internal enzyme molecules will be exposed to a lower concentration of the inactivating substrate (Fig. 37). This can increase the operational stability of the biocatalysts because these enzyme molecules will be not inactivated by the substrate. However, this will not be caused by any real change in the enzyme molecule properties, it will be just related to the fact that the enzyme is inside a porous structure where substrate diffusion limitations have been generated (Bolívar et al., 2013; Boniello et al., 2010; Pronk et al., 2014; Regan et al., 1974; Shen and Chen, 2007).

Another instance where the enzyme stability may increase due to its immobilization inside a porous structure is the existence of pH gradients (Boniello et al., 2010; Bourdillon et al., 1999; Luo, 2018; Luo et al., 2017; Neira and Herr, 2017; Schroën et al., 2002; Valencia et al., 2011; Valencia et al., 2010; Zavrel et al., 2010). If the reaction produces or consumes H⁺, it is possible that the medium external pH value may be not like the pH inside the particle. Inside the particle, a pH gradient will exist, more similar to that of the medium near the surface of the particle, farther from this one the enzyme molecules are located more inside the particle (Fig. 38). If the reaction pH is selected by the enzyme activity, it is possible that this will not fit the optimal pH for the enzyme stability. This is the case of the penicillin G acylase in the hydrolysis of penicillin G, the external pH is around 8 by thermodynamic, substrate/product stability and enzyme activity (Guisan et al., 1994). However, the stability of the enzyme is optimal at pH 5. During the reaction, the pH decrease by the release of phenyl acetic acid, and it has been suggested that this may be used to enlarge the operational stability of the immobilized enzyme. These stabilizing effects will be higher when the particle size and the enzyme volumetric activity increase.

Another situation where the enzyme stability may increase by immobilization is considering the pH adjustment during a reaction that

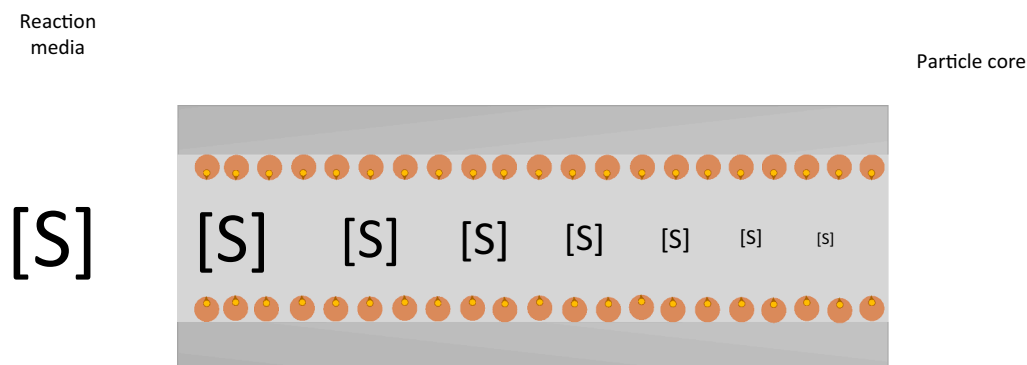


Fig. 37. Substrate diffusional limitations may reduce the exposition of enzyme molecules to high concentrations of deleterious substrate.

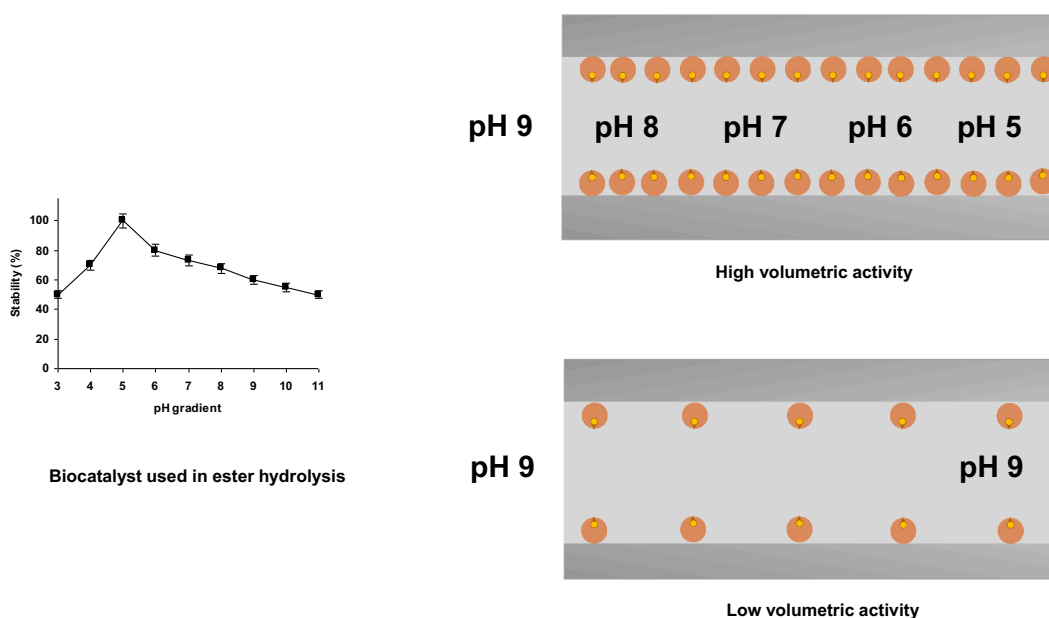


Fig. 38. pH gradients may enhance the operational stability of an immobilized enzyme.

produces the pH change, for example an ester hydrolysis. Even using the best dispersion system, some micro drops of titrating agent will exit, and this may produce a drop in the pH of the enzyme molecules that are in this drop (Fig. 39). If the enzyme is immobilized in a support with groups bearing buffering properties, it may be expected that a stabilizing affect may be found (Virgen-Ortiz et al., 2017a) (Fig. 40). We have not been able to find any reference on this regard in literature.

9. Conclusion and future trends

The stabilization of enzymes via multipoint covalent immobilization is still an “in development” scientific area. Although there are many immobilization protocols, few of them have proved to be really effective in the enzyme stabilization via multipoint covalent attachment. Even among the few that have proved their suitability, some have not been fully characterized in their action mechanism. That way, the design of new active groups useful for this target is expected in the future. Among this new active groups, genipin activated supports are very promising, genipin is as a non-toxic and Generally Recognized as Safe from the Federal Food, Drug, and Cosmetic Act (USA) crosslinking reagent that could become a good alternative to glutaraldehyde, but the exact mechanism of action of this reactive is still unknown, and its price is excessive for activation of enzyme immobilization supports in industrial biocatalysis (but no in biomedicine or biosensors) (Flores et al., 2019;

Hong et al., 2021; Klein et al., 2016; Lima et al., 2021; Ma et al., 2018; Tacias-Pascacio et al., 2019). Alternative groups to glyoxyl, epoxide or vinyl sulfone activated supports should also appear to widen the range of opportunities to find suitable immobilization protocols. Moreover, the development new supports matrices, very hydrophilic and with very high activation possibilities may open new opportunities.

The possibility to get site-directed immobilization (using Cys variants of the enzymes and immobilization via disulfide bridges) together with the enrichment of the protein surface on groups that are reactive with the support, and the use of heterofunctional supports, have permitted to greatly increase the complementarity of the enzyme and support surface, increasing the opportunities to get an intense multipoint covalent attachment (Grazu et al., 2012; Grazú et al., 2010). This strategy has not been studied nor used in depth in literature, but it is expected that in the future more enzymes may become stabilized using similar strategies.

The development of new techniques to determine the effect of immobilization on the enzyme features and structure (e.g., single particle or molecule analysis) and the importance of the enzyme environment, including the development of specific tools to perform these studies (Benítez-Mateos et al., 2018; Bolivar et al., 2016; Bolivar and López-Gallego, 2020; Bolivar and Nidetzky, 2020; Bolivar and Nidetzky, 2019; Bolivar and Nidetzky, 2020; Chaparro Sosa et al., 2020; Chaparro Sosa et al., 2021; Coglitore et al., 2019; Dalkas and Euston, 2019; Faulón

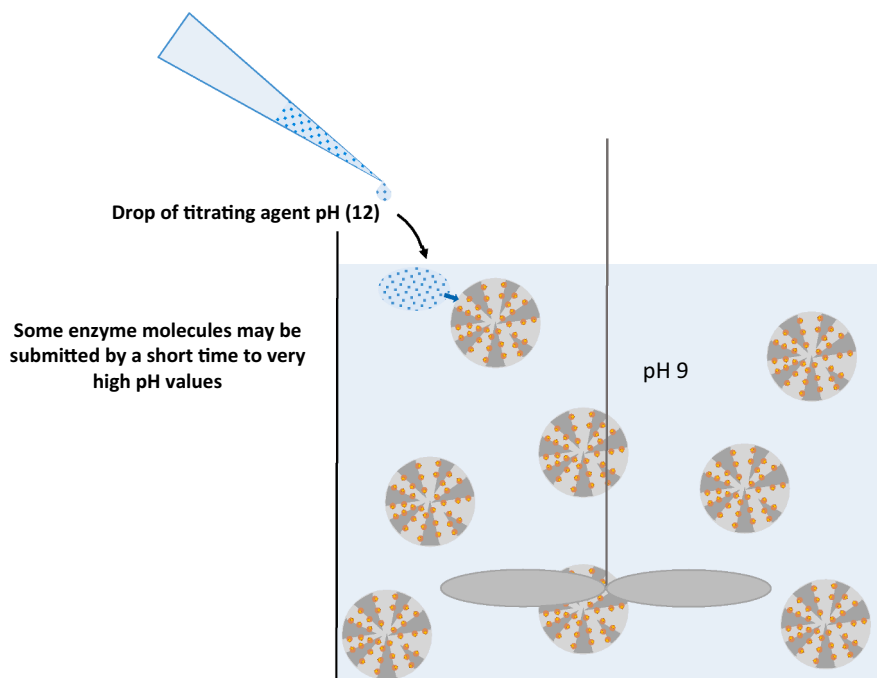


Fig. 39. Operational enzyme inactivation due to the drops of titrating agent in reactions where the pH is altered.

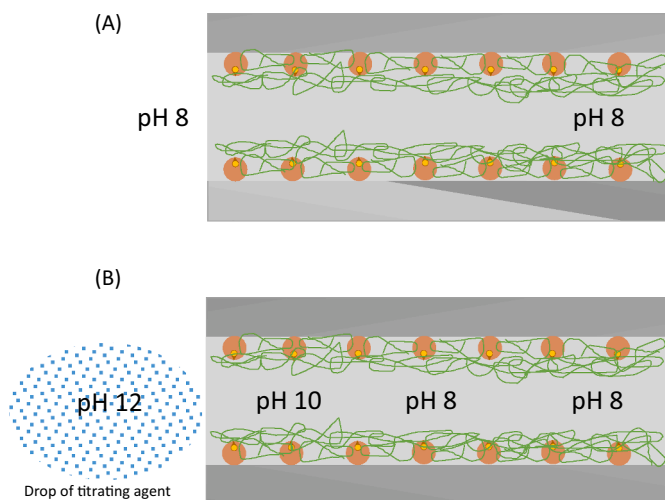


Fig. 40. If the support is coated with a molecule with buffering capacities (e.g., a polymer like PEI), the internal and external pH values may be very similar (A) and if the particle of biocatalysts is in the drop of titrating agent, only some enzyme molecules will suffer exposure to these drastic conditions.

Marruecos et al., 2018; Ogorzalek et al., 2015; Sánchez-Morán et al., 2021; Siefker et al., 2018; Zou et al., 2018). This could be the subject of a full review, albeit different from the scope of the current one.

Thus, far from being a closed topic, the design of protocols to get enzymes stabilized via multipoint covalent attachment will remain an interesting topic for a long time, involving efforts from materials science, organic chemistry, chemistry of proteins, enzyme genetic modification, etc. to take the maximum profit to the enzyme immobilization.

Author credit statement

Rafael C. Rodrigues, Diego Carballeares and Roberto Morellon- Sterling performed the initial literatures search and edited the final version of the paper and prepared the figures.

Angel Berenguer-Murcia edited the final version of the paper and figures.

Roberto Fernandez-Lafuente designed and wrote the initial draft of the paper, edited the figures and the final version of the manuscript.

Declaration of Competing Interest

None.

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