

1           **HETEROFUNCTIONAL SUPPORTS IN ENZYME IMMOBILIZATION:**  
2           **FROM TRADITIONAL IMMOBILIZATION PROTOCOLS TO OPPORTUNITIES IN**  
3           **TUNING ENZYME PROPERTIES**

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27 **Abstract**

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

50

51 **Keywords:** heterofunctional supports; enzyme immobilization; enzyme stabilization; multipoint  
52 covalent attachment;

## 53 1. INTRODUCTION

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

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

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

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

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

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

94           In the case of multimeric enzymes, in many instances, enzyme inactivation starts via  
95 subunit dissociation.<sup>24</sup> If this is the case, prevention of this dissociation may be achieved also by  
96 multi-subunit immobilization among other strategies.<sup>24</sup>

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

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

104 produces a global rigidification of the enzyme structure, and the effects are more relevant when  
105 they directly involve the area of the enzyme structure where the inactivation starts.<sup>18, 27, 28</sup>

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

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

129

## 130 2. STANDARD MULTIFUNCTIONAL SUPPORTS

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

134

### 135 2.1. Glutaraldehyde activated supports: an old heterofunctional support

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

145 <sup>49</sup>

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

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

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

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

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

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

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



207 thus it is exponentially dependent on the surface density of amino groups on the support  
208 although the covalent reaction should be of order 1.<sup>34, 36, 38-41, 49</sup>

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

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

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

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

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

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

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

253 Finally, it is possible to immobilize the enzyme via a direct first covalent attachment,  
254 involving the most reactive exposed group of the enzyme (usually the terminal amino group).<sup>7</sup>  
255 Using most water soluble enzymes, the moderate ionic strength used to prevent ionic exchange  
256 (100-250 mM of NaCl) is not enough to force the hydrophobic adsorption of the protein on the  
257 support and a direct covalent immobilization may be the first cause for the enzyme  
258 immobilization.<sup>53</sup> In most enzymes, the use of an ionic strength which is sufficient to prevent

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

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

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

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

282

## 283 **2.2 Standard epoxy supports**

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

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

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

308 Nevertheless, the fact is that epoxy supports have been available for immobilizing  
309 industrial enzymes for a long time, and they have proven their efficiency in certain cases.<sup>58, 61-64</sup>

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

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

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

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

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

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

357

### 358 **2.3. Other multifunctional supports**

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

362 hydrophobic.<sup>58-60</sup> In some specific cases, the spacer arm that we introduce having the covalent  
363 functionality may be enough to provide this multifunctionality, since many of these groups are  
364 not physically inert.<sup>49, 53</sup> For example, long spacer arms composed of just CH<sub>2</sub> chains will have  
365 a hydrophobic character. Similarly tosyl chloride may be considered hydrophobic.<sup>71, 72</sup>

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

372

### 373 **3. New tailor-made heterofunctional supports**

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

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

387 groups that result in adsorption will be revised. Finally, some specific uses of these new tailor-  
388 made requirements will be presented.

389

### 390 **3.1. Designing an ideal heterofunctional support to obtain an intense multipoint covalent** 391 **attachment**

392 In order to have an intense multipoint covalent attachment, whatever the support  
393 groups used, it is necessary to use supports offering large internal surfaces (Figure 10) .<sup>6</sup> Only if  
394 these supports offer a high enough geometrical congruence with the enzyme, the enzyme–  
395 support interaction may involve many groups of the support and the enzyme, and thus produce  
396 an intense multipoint covalent attachment.<sup>7</sup> Thus, supports formed by thin chains, of a diameter  
397 similar or smaller to that of the protein, can hardly yield many enzyme-support bonds, while  
398 supports having large internal surfaces, like very thick cylindrical chains (agarose),<sup>30</sup> or pores in  
399 solid materials (porous glass, Sepabeads) may permit intense enzyme support interactions.<sup>14, 62</sup>

400 Another requisite is that many reactive groups of the support should be under the surface  
401 of each protein molecule.<sup>7</sup> Only if there are many reactive groups of the support under the  
402 enzyme surface, the involvement of most of the available enzyme groups on the enzyme-support  
403 multipoint covalent attachment can be expected (Figure 10). Thus, only supports having very  
404 high surface densities of reactive groups will be useful to produce an intense multipoint covalent  
405 attachment.<sup>6, 7</sup>

406 However, even using an adequate support, the heterofunctional support may only give an  
407 intense multipoint covalent attachment if the reactive groups fulfill some features, as discussed  
408 below.

409

### 410 **3.1. The main chemical group**

#### 411 **3.1.1. The ideal group**



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

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

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

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

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

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

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

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

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

452

### 453 **3.1.2. Epoxy supports**

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

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

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

480

### 481 **3.1.3. Glyoxyl supports**

482            Glyoxyl supports have been described as very suitable supports to give an intense  
483 enzyme-support multipoint covalent attachment.<sup>7, 29, 30, 80</sup>

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

490 secondary bonds.<sup>30</sup> For some companies, this reduction step using sodium borohydride may  
491 become a problem.<sup>30</sup>

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

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

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

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

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

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

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

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

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

540

### 541 **3.2. The secondary group**

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

548

### 549 **3.2.1. The ideal secondary groups**

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

556 <sup>18</sup>

557

### 558 **3.2.2. Groups able to immobilize proteins via general interactions between enzyme and** 559 **support**

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

564 Physical adsorption of proteins is a quite rapid phenomenon. As stated above, the  
565 original epoxy supports already use the concept of heterofunctional supports by using  
566 hydrophobic adsorption of the protein on their hydrophobic matrix.<sup>62, 63, 67, 73-75</sup>

567 Cationic or anionic groups may produce enzyme adsorption via ionic exchange.<sup>32, 58, 92-</sup>  
568 <sup>95</sup> As explained for the glutaraldehyde supports, ionic exchange requires the involvement of  
569 several groups of the enzyme and the support to fix the enzyme to the support.<sup>34, 49</sup>

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

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

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

590 Initially, the groups were introduced by modification of the support main group (e.g.  
591 epoxy groups).<sup>58</sup> A preliminary optimization of the support modification degree was necessary:  
592 the higher the modification, the faster the protein adsorption.<sup>58, 69</sup> However, the covalent

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

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

607

### 608 **3.2.2.1. Second generation of supports**

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

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



619 competition between secondary and primary groups in the support, the adsorption rate and  
620 covalent reaction may be maximized. Second, the support-enzyme reaction does not have any  
621 steric hindrances generated by the adsorbing groups.<sup>67</sup>

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

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

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

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

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

652 Thus, even though the results are promising, more efforts are necessary to get  
653 heterofunctional supports that are nearer to fulfilling the whole set of requirements.<sup>18</sup>

654

### 655 **3.2.3. Site-directed immobilization/rigidification of enzymes**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

755

#### 756 **4.- Uses of heterofunctional supports**

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

766

#### 767 **4.1. Immobilization/purification of enzymes by using tailor-made heterofunctional** 768 **supports.**

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

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

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

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

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

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

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

802

#### 803 **4.1.1 One step purification-immobilization-stabilization of multimeric enzymes**

804 As it has been discussed in this review, ionic exchange of proteins on anionic exchangers  
805 or adsorption of proteins on IMAC supports is generally performed via multipoint adsorption.<sup>18,</sup>  
806 <sup>34, 36</sup>

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

820 Once this multipoint nature of ionic exchange is established, it seems obvious that a  
821 large protein may establish interactions at a longer distance than small proteins.<sup>94</sup> It was shown  
822 that using supports having a very low density of cationic groups on the support surface (around  
823 2 residues / 1000Å<sup>2</sup>), only large multimeric proteins could become adsorbed on the support,  
824 even though they can become desorbed at very low ionic exchange levels.<sup>94</sup> The next step was  
825 the development of heterofunctional amino and epoxy supports first and amino and glyoxyl



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

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

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

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

858

#### 859 **4.1.2. One step purification-immobilization-stabilization of poly-His tagged proteins.**

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

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

878 stabilize enzymes of glyoxyl groups,<sup>91</sup> results may be expected to be even better than the  
879 reported using epoxy supports.

880

## 881 **4.2. Rigidification of different areas of the enzyme**

882 The use of heterofunctional supports to immobilize enzymes may permit to alter enzyme  
883 orientation on the support surface, involving different regions of the enzyme on the  
884 immobilization process (Figures 9 and 21).<sup>18, 58, 67</sup> This means that different areas of the enzyme  
885 may be protected or blocked by the support surface while other areas of the protein will be  
886 oriented towards the reaction medium.<sup>18</sup> The protein area in contact with the support is the one  
887 that may increase the rigidity via multipoint covalent attachment (rigidification that will be  
888 transmitted to the whole protein structure), and also the most affected by the reaction with the  
889 support groups. Orientation of the enzyme on the support may produce changes in enzyme  
890 activity, stability, but also on the selectivity or specificity, as different regions of the enzyme  
891 will suffer different distortions.<sup>18</sup>

892

### 893 **4.2.1. Effect on enzyme activity**

894 The orientation of the enzyme is a key point when Redox enzymes are involved and  
895 the current of electrons must go via the support. This may work only if the active center is  
896 properly oriented. The review from Hernandez and Fernandez-Lafuente<sup>18</sup> shows many examples  
897 of this effect. However, they are mainly related to the use monofunctional supports to modify  
898 enzymes, not to the use of heterofunctional supports. Nevertheless, enzyme orientation may  
899 affect enzyme activity in many other cases.<sup>108, 160</sup>

900 The effect of orientation on enzyme activity is quite evident if the substrate is very large:  
901 if the active center is not oriented towards the reaction medium, and depending on the protein  
902 loading of the support, the expressed activity may be quite different (Figure 27).<sup>18</sup> If the  
903 substrate is small, it is very likely that even if the active center is facing the support surface, the

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

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

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

920

#### 921 **4.2.2 Effect on enzyme stability**

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

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

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

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

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

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

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

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

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

#### 975 **4.2.3. Effect on enzyme specificity and/or selectivity**

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

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

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

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

996 In a first example, the enzyme penicillin G acylase from *E. coli* was submitted to site-  
997 directed mutagenesis and each mutant immobilized-stabilized via site-directed  
998 immobilization.<sup>137</sup> The enzyme was used in a kinetic resolution of chiral esters by hydrolysis.  
999 Using monofunctional thiol reactive supports, where no rigidification was observed, all the  
1000 immobilized mutant enzymes exhibited the same specificity. Using thiol-epoxy or thiol-glyoxyl  
1001 supports, most enzymes remained unaltered in its enantiospecificity, but one mutant doubled the  
1002 value.<sup>137</sup> This result pointed out two important facts:

1003 First, only enzyme immobilization via one point (the thiol exchange) has no effect on  
1004 enzyme mobility or conformation and, therefore, maintains the enzyme features, even when  
1005 altering the position of the enzyme regarding the support surface, in the case where the support  
1006 did not promote any uncontrolled interaction with the enzyme.

1007 Second, site-directed rigidification of an enzyme may permit to modulate the enzyme  
1008 properties and identify the most relevant areas for the process.<sup>137</sup>

1009 The same battery of immobilized Cys mutant enzymes was used in a more  
1010 sophisticated reaction, a kinetically controlled synthesis.<sup>137</sup> This process involves the use of an  
1011 activated acyl donor (in this case, as an ester), and the yields came from the balance between  
1012 three reactions: the synthesis of the target product, the hydrolysis of the ester substrate and the  
1013 hydrolysis of the product. The yields reach a maximum and then, they decrease as the medium  
1014 may be even fully aqueous and the thermodynamic constant of the process may offer very low  
1015 yield at equilibrium.<sup>16, 173</sup> Therefore, the yields are strictly determined by the kinetic  
1016 properties of the enzyme (affinity by the nucleophile, ester and product, activity in the 3 likely  
1017 substrates). Again, while all the one-point attached Cys mutant enzymes remained with almost  
1018 identical behavior, one of the site-directed immobilized-rigidified enzymes preparations  
1019 permitted a significant increase in the yields.<sup>137</sup> This mutant is the same that permits to increase  
1020 the enantioselectivity and it holds the same position that produces a higher stabilization; the new  
1021 Cys was introduced in the position 380 of the B chain of penicillin G acylase.<sup>137</sup>

1022 Similar studies were performed using the lipase from *Bacillus thermocatenolatus*  
1023 (BTL2).<sup>138, 174</sup> In this case, the immobilization via one-point permitted to improve enzyme  
1024 features in some instances.<sup>134</sup> This may be based on the drastic conformational changes of this  
1025 lipase during catalysis, the enzyme has a double lid and any hindrance to the movement of this  
1026 complex structure may alter the enzyme properties.<sup>175</sup>

1027 However, if thiol-glyoxyl supports were used, permitting a certain rigidification of the  
1028 areas involved in the immobilization, the changes were more significant.<sup>138</sup> For example, the  
1029 simple orientated immobilization by the BTL2-S334C on monofunctional disulfide supports  
1030 gave ee > 99% in the asymmetric hydrolysis of phenylglutaric acid dimethyl diester but not in  
1031 the kinetic resolution of *rac*-2-*O*-butyryl-2-phenylacetic acid (ee = 27%). On the contrary, the



1032 site-directed rigidification of the BTL2-S334C variant on disulfide-aldehyde supports generated  
1033 a fully enantioselective biocatalyst in both processes (ee > 99%).<sup>138</sup>

1034

#### 1035 **4.2.4. Co-immobilization of enzymes**

1036 This is the last example of the advantages of heterofunctionality of supports that we  
1037 will include in this review. Co-immobilization of enzymes, working in cascade reactions, has  
1038 advantages and drawbacks.<sup>6, 176, 177</sup> From a kinetic point of view, the second enzyme will be  
1039 working using higher concentrations of product from the first enzyme, increasing the global  
1040 reaction course (Figure 29).<sup>178-185</sup> However, co-immobilization of two enzymes causes the life of  
1041 the biocatalyst to be determined by the stability of the weaker component.<sup>6</sup> Moreover, co-  
1042 immobilization results in both enzymes needing to be immobilized on the same support, and in  
1043 some cases optimal immobilization conditions for an enzyme may be quite far from the optimal  
1044 immobilization conditions and support for the other enzyme.<sup>6</sup>

1045 The use of a bifunctional or even a multifunctional support may be a very suitable  
1046 alternative to immobilize two enzymes whose immobilization on the same monofunctional  
1047 support may be complex. In this case, we do not intend that one of the groups on the support  
1048 makes a first immobilization and then the other produces a covalent reaction. In this case, we  
1049 intend that the support may be able to immobilize one enzyme using one kind of groups and the  
1050 other enzyme using another kind of groups (Figure 30). The idea may involve two different  
1051 reversible immobilization protocols (IMAC and ionic exchange, for example), or a combination  
1052 of the groups from that support with groups able to covalently immobilize the enzyme. The  
1053 advantages may be many. First, it is possible to immobilize the enzyme that requires the most  
1054 drastic immobilization conditions, and in a following step, the second enzyme may be  
1055 immobilized under milder conditions. This may not be an ideal strategy if both enzymes require  
1056 to be very stabilized by immobilization to be usable, but it may be a good alternative when one  
1057 of the enzymes is much more stable than the second under operation conditions, and this may be

1058 immobilized on a support that may permit a high stabilization via multipoint or multisubunit  
1059 immobilization.

1060           We have been able to find just one example of this very nice strategy. In that paper, the  
1061 researchers intend to co-immobilize different Redox enzymes, one to produce the target product  
1062 and other to regenerate the consumed cofactor.<sup>186</sup> One of the enzymes was a poly-His tagged  
1063 enzyme that becomes deactivated when immobilized on glyoxyl supports, while the other Redox  
1064 enzyme was immobilized-stabilized via immobilization on this support.<sup>186</sup> The poly-His tagged  
1065 enzyme could be readily immobilized on IMAC supports, preserving high activity.<sup>186</sup> Thus, both  
1066 enzymes could be immobilized on the same particle using an IMAC-glyoxyl support, with good  
1067 activity recovery. The authors went further. They used very low enzyme loadings compared to  
1068 the capacity of the support. Using confocal measurements,<sup>187-191</sup> they showed that while the  
1069 enzyme immobilized on glyoxyl supports was slowly attached and gave a homogenous  
1070 distribution along the pores of the support particles, the poly-His tagged protein became  
1071 immobilized very quickly and was placed on the outer part of the particle pores (Figure 31).<sup>186</sup>  
1072 The immobilization rate of this enzyme could be controlled adding imidazol, a competitor of the  
1073 adsorption of proteins to IMAC supports.<sup>35</sup>

1074           This permitted to prepare co-immobilized biocatalysts of both proteins where enzyme  
1075 distribution varied. It was shown that when both enzymes were slowly and, therefore,  
1076 homogeneously immobilized along the pores of the support, the global activity of the reaction  
1077 was higher than immobilizing one in a homogenous way and the other forming a crown. In fact,  
1078 the homogeneously distributed co-immobilized preparations gave more activity even than the free  
1079 enzymes, thanks to the high cofactor concentration, although the individual determination of the  
1080 activity of both enzymes showed a decrease on enzyme activity.<sup>186</sup>

1081

## 1082 **5. Future Prospects**

1083 Heterofunctional supports constitute a potent tool to improve enzyme performance.  
1084 However, researchers should consider that many of the oldest immobilization techniques are  
1085 really based on heterofunctional supports, as we have discussed in section 2 of this review. This  
1086 may complicate the understanding of the experiments and may require the use of adequate  
1087 reference supports and immobilization conditions to really seclude and identify the different  
1088 effects and causes of the immobilization on the different groups of the support. But if properly  
1089 controlled, heterofunctionality will increase the versatility of any immobilization protocols, as  
1090 we can alter the first cause of immobilization and that way the final performance of the final  
1091 biocatalyst.<sup>18</sup>

1092 However, the most important expectations lay on the side of the tailor-made  
1093 heterofunctional supports, where we can fulfill the enzyme technologist dream of a full control  
1094 over enzyme immobilization, orientation of the enzyme on the support surface and intensity of  
1095 the enzyme-support interactions. All these may be controlled using tailor made-heterofunctional  
1096 supports and site directed mutagenesis. There are only a handful of examples on the use of these  
1097 techniques, but they have shown the potential for both, preparation of industrial biocatalyst, and  
1098 some academic studies, as the detection of the most relevant areas for enzyme stability under  
1099 different conditions.<sup>137</sup> Coupling tailor-made heterofunctional supports to site-directed  
1100 mutagenesis we can go from one step immobilization-stabilization-purification processes (e.g.,  
1101 using poly-His tagged enzymes) to site-directed rigidification of the enzyme.<sup>18</sup> However, it is  
1102 still necessary to further improve the features of the supports to take full advantages of the  
1103 possibilities of the heterofunctionality. In general rigidification of the enzyme structures to its  
1104 fullest extent will be positive to improve their stability and also to improve the effects of the  
1105 immobilization on other enzyme features.<sup>7</sup>

1106 The design of new concepts involving tailor-made heterofunctionality of the supports  
1107 very likely will go further in the near future. The co-immobilization of two enzymes on a  
1108 heterofunctional support using different groups for each enzyme is one of these new

1109 developments. However, this idea may be exploited further if combined with nanotechnology. In  
1110 this case heterofunctionality may come from the integration of different nanostructures bearing  
1111 each of them different functional groups.

1112 Thus, it may be expected that the use of new ideas based on tailor-made heterofunctional  
1113 supports may be a key to fulfill the requirements of an enzyme as an industrial catalyst,  
1114 permitting good activity recovery, good stability, and even improved selectivity or specificity.

1115

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1123

- 1126 (1) Hartmeier, W. *Trends Biotechnol.* **1985**, *3*, 149-153.  
1127 (2) Katchalski-Katzir, E. *Trends Biotechnol.* **1993**, *11*, 471-478.  
1128 (3) Sheldon, R. A. *Adv. Synth. Catal.* **2007**, *349*, 1289-1307.  
1129 (4) Schmid, A.; Dordick, J. S.; Hauer, B.; Kiener, A.; Wubbolts, M.; Witholt, B. *Nature* **2001**,  
1130 *409*, 258-268.  
1131 (5) Schoemaker, H. E.; Mink, D.; WubboLts, M. G. *Science* **2003**, *299*, 1694-1697.  
1132 (6) Garcia-Galan, C.; Berenguer-Murcia, A.; Fernandez-Lafuente, R.; Rodrigues, R. C. *Adv.*  
1133 *Synth. Catal.* **2011**, *353*, 2885-2904.  
1134 (7) Mateo, C.; Palomo, J. M.; Fernandez-Lorente, G.; Guisan, J. M.; Fernandez-Lafuente, R.  
1135 *Enzyme Microb. Technol.* **2007**, *40*, 1451-1463.  
1136 (8) Iqbal, J.; Iqbal, S.; Müller, C. E. *Analyst* **2013**, *138*, 3104-3116.  
1137 (9) Verma, M. L.; Barrow, C. J.; Puri, M. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 23-39.  
1138 (10) Singh, R. K.; Tiwari, M. K.; Singh, R.; Lee, J. K. *Int. J. Mol. Sci.* **2013**, *14*, 1232-1277.  
1139 (11) Ansari, S. A.; Husain, Q. *Biotechnol. Adv.* **2012**, *30*, 512-523.  
1140 (12) Tran, D. N.; Balkus, K. J. *ACS Catalysis* **2011**, *1*, 956-968.  
1141 (13) Brady, D.; Jordaan, J. *Biotechnol. Lett.* **2009**, *31*, 1639-1650.  
1142 (14) Iyer, P. V.; Ananthanarayan, L. *Process Biochem.* **2008**, *43*, 1019-1032.  
1143 (15) Polizzi, K. M.; Bommarius, A. S.; Broering, J. M.; Chaparro-Riggers, J. F. *Curr. Opin.*  
1144 *Chem. Biol.* **2007**, *11*, 220-225.  
1145 (16) Rodrigues, R. C.; Ortiz, C.; Berenguer-Murcia, A.; Torres, R.; Fernandez-Lafuente, R.  
1146 *Chem. Soc. Rev.* **2013**, *in press*, doi: 10.1039/C2CS35231A.  
1147 (17) Cowan, D. A.; Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2011**, *49*, 326-346.  
1148 (18) Hernandez, K.; Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2011**, *48*, 107-122.  
1149 (19) Rodrigues, R. C.; Berenguer-Murcia, Á.; Fernandez-Lafuente, R. *Adv. Synth. Catal.* **2011**,  
1150 *353*, 2216-2238.  
1151 (20) Hernandez, K.; Berenguer-Murcia, A.; Rodrigues, R. C.; Fernandez-Lafuente, R. *Curr.*  
1152 *Org. Chem.* **2012**, *16*, 2652-2672.  
1153 (21) Abian, O.; Wilson, L.; Mateo, C.; Fernández-Lorente, G.; Palomo, J. M.; Fernández-  
1154 Lafuente, R.; Guisán, J. M.; Re, D.; Tam, A.; Daminatti, M. *J. Mol. Catal. B: Enzym.* **2002**, *19*-  
1155 *20*, 295-303.  
1156 (22) Mateo, C.; Fernandes, B.; Van Rantwijk, F.; Stolz, A.; Sheldon, R. A. *J. Mol. Catal. B:*  
1157 *Enzym.* **2006**, *38*, 154-157.  
1158 (23) Wilson, L.; Illanes, A.; Abián, O.; Pessela, B. C. C.; Fernández-Lafuente, R.; Guisán, J. M.  
1159 *Biomacromolecules* **2004**, *5*, 852-857.  
1160 (24) Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2009**, *45*, 405-418.  
1161 (25) Kaddour, S.; López-Gallego, F.; Sadoun, T.; Fernandez-Lafuente, R.; Guisan, J. M. *J. Mol.*  
1162 *Catal. B: Enzym.* **2008**, *55*, 142-145.  
1163 (26) Palomo, J. M. *Curr. Org. Synth.* **2009**, *6*, 1-14.  
1164 (27) Mansfeld, J.; Vriend, G.; Van Den Burg, B.; Eijsink, V. G. H.; Ulbrich-Hofmann, R.  
1165 *Biochemistry* **1999**, *38*, 8240-8245.  
1166 (28) Mansfeld, J.; Vriendl, G.; Veltman, O. R.; Van Den Burg, B.; Venema, G.; Eijsink, V. G.  
1167 H.; Utbrich-Hofmann, R. *FASEB J.* **1997**, *11*.  
1168 (29) Mateo, C.; Abian, O.; Bernedo, M.; Cuenca, E.; Fuentes, M.; Fernandez-Lorente, G.;  
1169 Palomo, J. M.; Grazu, V.; Pessela, B. C. C.; Giacomini, C.; Irazoqui, G.; Villarino, A.; Ovsejevi,  
1170 K.; Batista-Viera, F.; Fernandez-Lafuente, R.; Guisán, J. M. *Enzyme Microb. Technol.* **2005**, *37*,  
1171 456-462.

- 1172 (30) Mateo, C.; Palomo, J. M.; Fuentes, M.; Betancor, L.; Grazu, V.; López-Gallego, F.;  
1173 Pessela, B. C. C.; Hidalgo, A.; Fernández-Lorente, G.; Fernández-Lafuente, R.; Guisán, J. M.  
1174 *Enzyme Microb. Technol.* **2006**, *39*, 274-280.
- 1175 (31) Pessela, B. C. C.; Betancor, L.; Lopez-Gallego, F.; Torres, R.; Dellamora-Ortiz, G. M.;  
1176 Alonso-Morales, N.; Fuentes, M.; Fernández-Lafuente, R.; Guisán, J. M.; Mateo, C. *Enzyme*  
1177 *Microb. Technol.* **2005**, *37*, 295-299.
- 1178 (32) Pessela, B. C. C.; Fuentes, M.; Mateo, C.; Munilla, R.; Carrascosa, A. V.; Fernandez-  
1179 Lafuente, R.; Guisan, J. M. *Enzyme Microb. Technol.* **2006**, *39*, 909-915.
- 1180 (33) Pessela, B. C. C.; Mateo, C.; Carrascosa, A. V.; Vian, A.; García, J. L.; Rivas, G.; Alfonso,  
1181 C.; Guisan, J. M.; Fernández-Lafuente, R. *Biomacromolecules* **2003**, *4*, 107-113.
- 1182 (34) Kumar, A.; Galaev, I. Y.; Mattiasson, B. *J. Chromatogr. B Biomed. Sci. Appl.* **2000**, *741*,  
1183 103-113.
- 1184 (35) Pessela, B. C. C.; Mateo, C.; Filho, M.; Carrascosa, A.; Fernández-Lafuente, R.; Guisan, J.  
1185 M. *Enzyme Microb. Technol.* **2007**, *40*, 242-248.
- 1186 (36) Pessela, B. C. C.; Torres, R.; Fuentes, M.; Mateo, C.; Munilla, R.; Vian, A.; Carrascosa, A.  
1187 V.; Garcia, J. L.; Guisán, J. M.; Fernandez-Lafuente, R. *J. Chromatogr. A* **2004**, *1055*, 93-98.
- 1188 (37) Vançan, S.; Miranda, E. A.; Bueno, S. M. A. *Process Biochem.* **2002**, *37*, 573-579.
- 1189 (38) Arnold, F. H. *Biotechnology. (N. Y.)* **1991**, *9*, 151-156.
- 1190 (39) Hochuli, E.; Döbeli, H.; Schacher, A. *J. Chromatogr. A* **1987**, *411*, 177-184.
- 1191 (40) Gaberc-Porekar, V.; Menart, V. *J. Biochem. Biophys. Methods* **2001**, *49*, 335-360.
- 1192 (41) Fuentes, M.; Mateo, C.; Fernández-Lafuente, R.; Guisán, J. M. *Biomacromolecules* **2006**,  
1193 *7*, 540-544.
- 1194 (42) Monsan, P. *J. Mol. Catal.* **1978**, *3*, 371-384.
- 1195 (43) Monsan, P.; Puzo, G.; Mazarguil, H. *Biochimie* **1975**, *57*, 1281-1292.
- 1196 (44) Walt, D. R.; Agayn, V. I. *TrAC, Trends Anal. Chem.* **1994**, *13*, 425-430.
- 1197 (45) Bolivar, J. M.; Mateo, C.; Godoy, C.; Pessela, B. C. C.; Rodrigues, D. S.; Giordano, R. L.  
1198 C.; Fernandez-Lafuente, R.; Guisan, J. M. *Process Biochem.* **2009**, *44*, 757-763.
- 1199 (46) Adriano, W. S.; Filho, E. H. C.; Silva, J. A.; Giordano, R. L. C.; Gonçalves, L. R. B.  
1200 *Brazilian Journal of Chemical Engineering* **2005**, *22*, 529-538.
- 1201 (47) Adriano, W. S.; Filho, E. H. C.; Silva, J. A.; Gonçalves, L. R. B. *Biotechnol. Appl.*  
1202 *Biochem.* **2005**, *41*, 201-207.
- 1203 (48) Adriano, W. S.; Mendonça, D. B.; Rodrigues, D. S.; Mammarella, E. J.; Giordano, R. L. C.  
1204 *Biomacromolecules* **2008**, *9*, 2170-2179.
- 1205 (49) Betancor, L.; López-Gallego, F.; Hidalgo, A.; Alonso-Morales, N.; Dellamora-Ortiz, G.;  
1206 Mateo, C.; Fernández-Lafuente, R.; Guisán, J. M. *Enzyme Microb. Technol.* **2006**, *39*, 877-882.
- 1207 (50) Migneault, I.; Dartiguenave, C.; Bertrand, M. J.; Waldron, K. C. *Biotechniques* **2004**, *37*,  
1208 790-802.
- 1209 (51) Wine, Y.; Cohen-Hadar, N.; Freeman, A.; Frolow, F. *Biotechnol. Bioeng.* **2007**, *98*, 711-  
1210 718.
- 1211 (52) Fernandez-Lafuente, R.; Resell, C. M.; Rodriguez, V.; Guisan, J. M. *Enzyme Microb.*  
1212 *Technol.* **1995**, *17*, 517-523.
- 1213 (53) Barbosa, O.; Torres, R.; Ortiz, C.; Fernandez-Lafuente, R. *Process Biochem.* **2012**, *47*,  
1214 1220-1227.
- 1215 (54) Brzozowski, A. M.; Derewenda, U.; Derewenda, Z. S.; Dodson, G. G.; Lawson, D. M.;  
1216 Turkenburg, J. P.; Bjorkling, F.; Hüge-Jensen, B.; Patkar, S. A.; Thim, L. *Nature* **1991**, *351*,  
1217 491-494.
- 1218 (55) Fernandez-Lafuente, R.; Armisen, P.; Sabuquillo, P.; Fernández-Lorente, G.; Guisán, J. M.  
1219 *Chem. Phys. Lipids* **1998**, *93*, 185-197.
- 1220 (56) Miled, N.; Beisson, F.; De Caro, J.; De Caro, A.; Arondel, V.; Verger, R. *J. Mol. Catal. B:*  
1221 *Enzym.* **2001**, *11*, 165-171.
- 1222 (57) Verger, R. *Trends Biotechnol.* **1997**, *15*, 32-38.

- 1223 (58) Mateo, C.; Fernández-Lorente, G.; Abian, O.; Fernández-Lafuente, R.; Guisán, J. M.  
1224 *Biomacromolecules* **2000**, *1*, 739-745.
- 1225 (59) Turková, J.; Bláha, K.; Malaníková, M.; Vančurová, D.; Švec, F.; Kálal, J. *Biochim.*  
1226 *Biophys. Acta, Enzymol.* **1978**, *524*, 162-169.
- 1227 (60) Hilterhaus, L.; Minow, B.; Müller, J.; Berheide, M.; Quitmann, H.; Katzer, M.; Thum, O.;  
1228 Antranikian, G.; Zeng, A. P.; Liese, A. *Bioprocess Biosyst. Eng.* **2008**, *31*, 163-171.
- 1229 (61) Mateo, C.; Abian, O.; Fernández-Lafuente, R.; Guisan, J. M. *Enzyme Microb. Technol.*  
1230 **2000**, *26*, 509-515.
- 1231 (62) Mateo, C.; Abian, O.; Fernández-Lorente, G.; Pedroche, J.; Fernández-Lafuente, R.;  
1232 Guisan, J. M.; Tam, A.; Daminati, M. *Biotechnol. Prog.* **2002**, *18*, 629-634.
- 1233 (63) Katchalski-Katzir, E.; Kraemer, D. M. *J. Mol. Catal. B: Enzym.* **2000**, *10*, 157-176.
- 1234 (64) Grazu, V.; López-Gallego, F.; Guisán, J. M. *Process Biochem.* **2012**, *47*, 2538-2541.
- 1235 (65) Ovsejevi, K.; Grazú, V.; Cuadra, K.; Batista-Viera, F. *Enzyme Microb. Technol.* **2004**, *35*,  
1236 203-209.
- 1237 (66) Grazú, V.; Ovsejevi, K.; Cuadra, K.; Betancor, L.; Manta, C.; Batista-Viera, F. *Appl.*  
1238 *Biochem. Biotechnol.* **2003**, *110*, 23-32.
- 1239 (67) Mateo, C.; Grazú, V.; Pessela, B. C. C.; Montes, T.; Palomo, J. M.; Torres, R.; López-  
1240 Gallego, F.; Fernández-Lafuente, R.; Guisán, J. M. *Biochem. Soc. Trans.* **2007**, *35*, 1593-1601.
- 1241 (68) Bastida, A.; Sabuquillo, P.; Armisen, P.; Fernández-Lafuente, R.; Huguet, J.; Guisán, J. M.  
1242 *Biotechnol. Bioeng.* **1998**, *58*, 486-493.
- 1243 (69) Barbosa, O.; Ortiz, C.; Torres, R.; Fernandez-Lafuente, R. *J. Mol. Catal. B: Enzym.* **2011**,  
1244 *71*, 124-132.
- 1245 (70) Krajewska, B. *Enzyme Microb. Technol.* **2004**, *35*, 126-139.
- 1246 (71) Albayrak, N.; Yang, S. T. *Enzyme Microb. Technol.* **2002**, *31*, 371-383.
- 1247 (72) Nilsson, K.; Mosbach, K. *Eur. J. Biochem.* **1980**, *112*, 397-402.
- 1248 (73) Melander, W. R.; Corradini, D.; Horvath, C. *J. Chromatogr.* **1984**, *317*, 67-85.
- 1249 (74) Smalla, K.; Turkova, J.; Coupek, J.; Hermann, P. *Biotechnol. Appl. Biochem.* **1988**, *10*, 21-  
1250 31.
- 1251 (75) Wheatley, J. B.; Schmidt Jr, D. E. *J. Chromatogr. A* **1999**, *849*, 1-12.
- 1252 (76) Fernandez-Lorente, G.; Godoy, C. A.; Mendes, A. A.; Lopez-Gallego, F.; Grazu, V.; de las  
1253 Rivas, B.; Palomo, J. M.; Hermoso, J.; Fernandez-Lafuente, R.; Guisan, J. M.  
1254 *Biomacromolecules* **2008**, *9*, 2553-2561.
- 1255 (77) López-Gallego, F.; Montes, T.; Fuentes, M.; Alonso, N.; Grazu, V.; Betancor, L.; Guisán, J.  
1256 M.; Fernández-Lafuente, R. *J. Biotechnol.* **2005**, *116*, 1-10.
- 1257 (78) Rodrigues, R. C.; Godoy, C. A.; Volpato, G.; Ayub, M. A. Z.; Fernandez-Lafuente, R.;  
1258 Guisan, J. M. *Process Biochem.* **2009**, *44*, 963-968.
- 1259 (79) Grazu, V.; Betancor, L.; Montes, T.; Lopez-Gallego, F.; Guisan, J. M.; Fernandez-  
1260 Lafuente, R. *Enzyme Microb. Technol.* **2006**, *38*, 960-966.
- 1261 (80) Pedroche, J.; del Mar Yust, M.; Mateo, C.; Fernández-Lafuente, R.; Girón-Calle, J.; Alaiz,  
1262 M.; Vioque, J.; Guisán, J. M.; Millán, F. *Enzyme Microb. Technol.* **2007**, *40*, 1160-1166.
- 1263 (81) Mateo, C.; Fernández-Lorente, G.; Cortés, E.; Garcia, J. L.; Fernández-Lafuente, R.;  
1264 Guisan, J. M. *Biotechnol. Bioeng.* **2001**, *76*, 269-276.
- 1265 (82) Bolivar, J. M.; Rocha-Martin, J.; Mateo, C.; Cava, F.; Berenguer, J.; Vega, D.; Fernandez-  
1266 Lafuente, R.; Guisan, J. M. *J. Mol. Catal. B: Enzym.* **2009**, *58*, 158-163.
- 1267 (83) Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **1995**, *17*, 366-372.
- 1268 (84) Bolivar, J. M.; López-Gallego, F.; Godoy, C.; Rodrigues, D. S.; Rodrigues, R. C.; Batalla,  
1269 P.; Rocha-Martín, J.; Mateo, C.; Giordano, R. L. C.; Guisán, J. M. *Enzyme Microb. Technol.*  
1270 **2009**, *45*, 477-483.
- 1271 (85) Borch, R. F.; Bernstein, M. D.; Durst, H. D. *J. Am. Chem. Soc.* **1971**, *93*, 2897-2904.
- 1272 (86) Abian, O.; Grazú, V.; Hermoso, J.; González, R.; García, J. L.; Fernández-Lafuente, R.;  
1273 Guisán, J. M. *Appl. Environ. Microbiol.* **2004**, *70*, 1249-1251.

- 1274 (87) Serra, I.; Ceechini, D. A.; Ubiali, D.; Manazza, E. M.; Albertini, A. M.; Terreni, M. *Eur. J.*  
1275 *Org. Chem.* **2009**, 1384-1389.
- 1276 (88) Scaramozzino, F.; Estruch, I.; Rossolillo, P.; Terreni, M.; Albertini, A. M. *Appl. Environ.*  
1277 *Microbiol.* **2005**, *71*, 8937-8940.
- 1278 (89) Cecchini, D. A.; Serra, I.; Ubiali, D.; Terreni, M.; Albertini, A. M. *BMC Biotechnol.* **2007**,  
1279 *7*.
- 1280 (90) Ryan, B. J.; Ó'Fágáin, C. *BMC Biotechnol.* **2007**, *7*.
- 1281 (91) Bolivar, J. M.; Mateo, C.; Grazu, V.; Carrascosa, A. V.; Pessela, B. C.; Guisan, J. M.  
1282 *Process Biochem.* **2010**, *45*, 1692-1698.
- 1283 (92) Fuentes, M.; Pessela, B. C. C.; Maquiese, J. V.; Ortiz, C.; Segura, R. L.; Palomo, J. M.;  
1284 Abian, O.; Torres, R.; Mateo, C.; Fernández-Lafuente, R.; Guisán, J. M. *Biotechnol. Prog.* **2004**,  
1285 *20*, 1134-1139.
- 1286 (93) Mateo, C.; Abian, O.; Fernandez-Lafuente, R.; Guisan, J. M. *Biotechnol. Bioeng.* **2000**, *68*,  
1287 98-105.
- 1288 (94) Pessela, B. C. C.; Munilla, R.; Betancor, L.; Fuentes, M.; Carrascosa, A. V.; Vian, A.;  
1289 Fernandez-Lafuente, R.; Guisán, J. M. *J. Chromatogr. A* **2004**, *1034*, 155-159.
- 1290 (95) Fuentes, M.; Maquiese, J. V.; Pessela, B. C. C.; Abian, O.; Fernández-Lafuente, R.; Mateo,  
1291 C.; Guisán, J. M. *Biotechnol. Prog.* **2004**, *20*, 284-288.
- 1292 (96) Carlsson, J.; Mosbach, K.; Bülow, L. *Biotechnol. Bioeng.* **1996**, *51*, 221-228.
- 1293 (97) Chaga, G. S. *J. Biochem. Biophys. Methods* **2001**, *49*, 313-334.
- 1294 (98) Ho, L. F.; Li, S. Y.; Lin, S. C.; Hsu, W. H. *Process Biochem.* **2004**, *39*, 1573-1581.
- 1295 (99) Porath, J.; Carlsson, J.; Olsson, I.; Belfrage, G. *Nature* **1975**, *258*, 598-599.
- 1296 (100) Armisen, P.; Mateo, C.; Cortés, E.; Barredo, J. L.; Salto, F.; Diez, B.; Rodés, L.; García, J.  
1297 L.; Fernández-Lafuente, R.; Guisán, J. M. *J. Chromatogr. A* **1999**, *848*, 61-70.
- 1298 (101) Mateo, C.; Fernandez-Lorente, G.; Pessela, B. C. C.; Vian, A.; Carrascosa, A. V.; Garcia,  
1299 J. L.; Fernandez-Lafuente, R.; Guisan, J. M. *J. Chromatogr. A* **2001**, *915*, 97-106.
- 1300 (102) Abad, J. M.; Vélez, M.; Santamaría, C.; Guisán, J. M.; Matheus, P. R.; Vázquez, L.;  
1301 Gazaryan, I.; Gorton, L.; Gibson, T.; Fernández, V. M. *J. Am. Chem. Soc.* **2002**, *124*, 12845-  
1302 12853.
- 1303 (103) Basso, A.; Braiuca, P.; Cantone, S.; Ebert, C.; Linda, P.; Spizzo, P.; Caimi, P.; Hanefeld,  
1304 U.; Degrassi, G.; Gardossi, L. *Adv. Synth. Catal.* **2007**, *349*, 877-886.
- 1305 (104) Bouriotis, V.; Galpin, I. J.; Dean, P. D. G. *J. Chromatogr. A* **1981**, *210*, 267-278.
- 1306 (105) Gutarra, M. L. E.; Mateo, C.; Freire, D. M. G.; Torres, F. A. G.; Castro, A. M.; Guisan, J.  
1307 M.; Palomo, J. M. *Catalysis Science and Technology* **2011**, *1*, 260-266.
- 1308 (106) Singhal, R. P.; DeSilva, S. S. *Adv. Chromatogr.* **1992**, *31*, 293-335.
- 1309 (107) Li, Y.; Larsson, E. L.; Jungvid, H.; Galaev, I. Y.; Mattiasson, B. *J. Chromatogr. A* **2001**,  
1310 *909*, 137-145.
- 1311 (108) Rusmini, F.; Zhong, Z.; Feijen, J. *Biomacromolecules* **2007**, *8*, 1775-1789.
- 1312 (109) Jelinek, R.; Kolusheva, S. *Chem. Rev.* **2004**, *104*, 5987-6015.
- 1313 (110) Jin, S.; Cheng, Y.; Reid, S.; Li, M.; Wang, B. *Med. Res. Rev.* **2010**, *30*, 171-257.
- 1314 (111) Lim, R. K. V.; Lin, Q. *Chem. Commun.* **2010**, *46*, 1589-1600.
- 1315 (112) Stolowitz, M. L.; Ahlem, C.; Hughes, K. A.; Kaiser, R. J.; Kesicki, E. A.; Li, G.; Lund, K.  
1316 P.; Torkelson, S. M.; Wiley, J. P. *Bioconjug. Chem.* **2001**, *12*, 229-239.
- 1317 (113) Torres, R.; Pessela, B.; Fuentes, M.; Munilla, R.; Mateo, C.; Fernández-Lafuente, R.;  
1318 Guisán, J. M. *J. Biotechnol.* **2005**, *120*, 396-401.
- 1319 (114) Mohler, L. K.; Czarnik, A. W. *J. Am. Chem. Soc.* **1993**, *115*, 7043-7044.
- 1320 (115) Denizli, A.; Pişkin, E. *J. Biochem. Biophys. Methods* **2001**, *49*, 391-416.
- 1321 (116) Clonis, Y. D.; Labrou, N. E.; Kotsira, V. P.; Mazitsos, C.; Melissis, S.; Gogolas, G. *J.*  
1322 *Chromatogr. A* **2000**, *891*, 33-44.
- 1323 (117) Lowe, C. R.; Burton, S. J.; Burton, N. P.; Alderton, W. K.; Pitts, J. M.; Thomas, J. A.  
1324 *Trends Biotechnol.* **1992**, *10*, 442-448.



- 1325 (118) Mateo, C.; Torres, R.; Fernández-Lorente, G.; Ortiz, C.; Fuentes, M.; Hidalgo, A.; López-  
1326 Gallego, F.; Abian, O.; Palomo, J. M.; Betancor, L.; Pessela, B. C. C.; Guisan, J. M.; Fernández-  
1327 Lafuente, R. *Biomacromolecules* **2003**, *4*, 772-777.
- 1328 (119) Fuentes, M.; Batalla, P.; Grazu, V.; Pessela, B. C. C.; Mateo, C.; Montes, T.; Hermoso, J.  
1329 A.; Guisan, J. M.; Fernandez-Lafuente, R. *Biomacromolecules* **2007**, *8*, 703-707.
- 1330 (120) Torres, R.; Mateo, C.; Fernández-Lorente, G.; Ortiz, C.; Fuentes, M.; Palomo, J. M.;  
1331 Guisan, J. M.; Fernández-Lafuente, R. *Biotechnol. Prog.* **2003**, *19*, 1056-1060.
- 1332 (121) Hefti, M. H.; Van Vugt-Van Der Toorn, C. J. G.; Dixon, R.; Vervoort, J. *Anal. Biochem.*  
1333 **2001**, *295*, 180-185.
- 1334 (122) Madoz-Gúrpide, J.; Abad, J. M.; Fernández-Recio, J.; Vélez, M.; Vázquez, L.; Gómez-  
1335 Moreno, C.; Fernández, V. M. *J. Am. Chem. Soc.* **2000**, *122*, 9808-9817.
- 1336 (123) Todd, R. J.; Van Dam, M. E.; Casimiro, D.; Haymore, B. L.; Arnold, F. H. *Proteins:*  
1337 *Struct., Funct., Genet.* **1991**, *10*, 156-161.
- 1338 (124) Andreescu, S.; Magearu, V.; Lougarre, A.; Fournier, D.; Marty, J. L. *Anal. Lett.* **2001**, *34*,  
1339 529-540.
- 1340 (125) Csoregi, E.; Jonsson-Pettersson, G.; Gorton, L. *J. Biotechnol.* **1993**, *30*, 315-337.
- 1341 (126) Batista-Viera, F.; Barbieri, M.; Ovsejevi, K.; Manta, C.; Carlsson, J. *Appl. Biochem.*  
1342 *Biotechnol.* **1991**, *31*, 175-195.
- 1343 (127) Ovsejevi, K.; Cuadra, K.; Batista-Viera, F. *J. Mol. Catal. B: Enzym.* **2009**, *57*, 188-193.
- 1344 (128) Ferraz, N.; Leverrier, J.; Batista-Viera, F.; Manta, C. *Biotechnol. Prog.* **2008**, *24*, 1154-  
1345 1159.
- 1346 (129) Giacomini, C.; Irazoqui, G.; Batista-Viera, F.; Brena, B. M. *Biocatal. Biotransform.* **2007**,  
1347 *25*, 373-381.
- 1348 (130) Ovsejevi, K.; Grazú, V.; Batista-Viera, F. *Biotechnol. Tech.* **1998**, *12*, 143-148.
- 1349 (131) Batista-Viera, F.; Manta, C.; Carlsson, J. *Biotechnol. Appl. Biochem.* **1996**, *24*, 231-239.
- 1350 (132) Ovsejevi, K.; Brena, B.; Batista-Viera, F.; Carlsson, J. *Enzyme Microb. Technol.* **1995**, *17*,  
1351 151-156.
- 1352 (133) Batista-Viera, F.; Manta, C.; Carlsson, J. *Appl. Biochem. Biotechnol.* **1994**, *44*, 1-14.
- 1353 (134) Godoy, C. A.; de las Rivas, B.; Filice, M.; Fernández-Lorente, G.; Guisan, J. M.; Palomo,  
1354 J. M. *Process Biochem.* **2010**, *45*, 534-541.
- 1355 (135) Grazú, V.; Abian, O.; Mateo, C.; Batista-Viera, F.; Fernández-Lafuente, R.; Guisán, J. M.  
1356 *Biomacromolecules* **2003**, *4*, 1495-1501.
- 1357 (136) Grazú, V.; Abian, O.; Mateo, C.; Batista-Viera, F.; Fernández-Lafuente, R.; Guisán, J. M.  
1358 *Biotechnol. Bioeng.* **2005**, *90*, 597-605.
- 1359 (137) Grazú, V.; López-Gallego, F.; Montes, T.; Abian, O.; González, R.; Hermoso, J. A.;  
1360 García, J. L.; Mateo, C.; Guisán, J. M. *Process Biochem.* **2010**, *45*, 390-398.
- 1361 (138) Godoy, C. A.; Rivas, B. D. L.; Grazú, V.; Montes, T.; Guisán, J. M.; López-Gallego, F.  
1362 *Biomacromolecules* **2011**, *12*, 1800-1809.
- 1363 (139) Segura, R. L.; Palomo, J. M.; Mateo, C.; Cortes, A.; Terreni, M.; Fernández-Lafuente, R.;  
1364 Guisan, J. M. *Biotechnol. Prog.* **2004**, *20*, 825-829.
- 1365 (140) Melton, R. G. *Adv. Drug Delivery Rev.* **1996**, *22*, 289-301.
- 1366 (141) Toribio, F.; Martínez-Lara, E.; Pascual, P.; López-Barea, J. *J. Chromatogr. B Biomed.*  
1367 *Appl.* **1996**, *684*, 77-97.
- 1368 (142) Fuentes, M.; Mateo, C.; Pessela, B. C. C.; Guisán, J. M.; Fernandez-Lafuente, R.  
1369 *Proteomics* **2005**, *5*, 4062-4069.
- 1370 (143) Palomo, J. M.; Segura, R. L.; Fernández-Lorente, G.; Pernas, M.; Rua, M. L.; Guisán, J.  
1371 M.; Fernández-Lafuente, R. *Biotechnol. Prog.* **2004**, *20*, 630-635.
- 1372 (144) Fuentes, M.; Pessela, B. C. C.; Mateo, C.; Palomo, J. M.; Batalla, P.; Fernández-Lafuente,  
1373 R.; Guisán, J. M. *Biomacromolecules* **2006**, *7*, 1357-1361.
- 1374 (145) Palomo, J. M.; Muoz, G.; Fernández-Lorente, G.; Mateo, C.; Fernández-Lafuente, R.;  
1375 Guisán, J. M. *J. Mol. Catal. B: Enzym.* **2002**, *19-20*, 279-286.

1376 (146) Fernandez-Lorente, G.; Filice, M.; Lopez-Vela, D.; Pizarro, C.; Wilson, L.; Betancor, L.;  
1377 Avila, Y.; Guisan, J. M. *J. Am. Oil Chem. Soc.* **2010**, 1-7.

1378 (147) Barbosa, O.; Torres, R.; Ortiz, C.; Fernandez-Lafuente, R. *Process Biochem.* **2012**, *47*,  
1379 766-774.

1380 (148) Akgöl, S.; Denizli, A. *J. Mol. Catal. B: Enzym.* **2004**, *28*, 7-14.

1381 (149) Tüzmen, N.; Kalburcu, T.; Denizli, A. *Process Biochem.* **2012**, *47*, 26-33.

1382 (150) Baydemir, G.; Derazshamshir, A.; Andaç, M.; Andaç, C.; Denizli, A. *J. Appl. Polym. Sci.*  
1383 **2012**, *126*, 575-586.

1384 (151) Labrou, N. E. *Journal of Chromatography B: Analytical Technologies in the Biomedical*  
1385 *and Life Sciences* **2003**, *790*, 67-78.

1386 (152) Fitzgerald, J.; Leonard, P.; Darcy, E.; O'Kennedy, R. *Methods in molecular biology*  
1387 *(Clifton, N.J.)* **2011**, *681*, 35-59.

1388 (153) Vishwanath, S. K.; Watson, C. R.; Huang, W.; Bachas, L. G.; Bhattacharyya, D. *J. Chem.*  
1389 *Technol. Biotechnol.* **1997**, *68*, 294-302.

1390 (154) Jan, U.; Husain, Q.; Saleemuddin, M. *Biotechnol. Appl. Biochem.* **2001**, *34*, 13-17.

1391 (155) Solomon, B.; Koppel, R.; Pines, G.; Katchalski-Katzir, E. *Biotechnol. Bioeng.* **1986**, *28*,  
1392 1213-1221.

1393 (156) Finette, G. M. S.; Mao, Q. M.; Hearn, M. T. W. *J. Chromatogr. A* **1997**, *763*, 71-90.

1394 (157) Dimer, F.; Petzold, M.; Hubbuch, J. *J. Chromatogr. A* **2008**, *1194*, 11-21.

1395 (158) Bolivar, J. M.; Mateo, C.; Rocha-Martin, J.; Cava, F.; Berenguer, J.; Fernandez-Lafuente,  
1396 R.; Guisan, J. M. *Enzyme Microb. Technol.* **2009**, *44*, 139-144.

1397 (159) Bolivar, J. M.; Rocha-Martin, J.; Godoy, C.; Rodrigues, R. C.; Guisan, J. M. *Process*  
1398 *Biochem.* **2010**, *45*, 107-113.

1399 (160) Turková, J. *J. Chromatogr. B Biomed. Sci. Appl.* **1999**, *722*, 11-31.

1400 (161) Mateo, C.; Archelas, A.; Fernandez-Lafuente, R.; Guisan, J. M.; Furstoss, R. *Org. Biomol.*  
1401 *Chem.* **2003**, *1*, 2739-2743.

1402 (162) Schellenberger, A.; Ulbrich, R. *Biomed. Biochim. Acta* **1989**, *48*, 63-67.

1403 (163) Ulbrich-Hofmann, R.; Arnold, U.; Mansfeld, J. *J. Mol. Catal. B: Enzym.* **1999**, *7*, 125-  
1404 131.

1405 (164) Pessela, B. C. C.; Mateo, C.; Fuentes, M.; Vian, A.; García, J. L.; Carrascosa, A. V.;  
1406 Guisán, J. M.; Fernández-Lafuente, R. *Biotechnol. Prog.* **2004**, *20*, 388-392.

1407 (165) Terreni, M.; Pagani, G.; Ubiali, D.; Fernández-Lafuente, R.; Mateo, C.; Guisán, J. M.  
1408 *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2429-2432.

1409 (166) Pessela, B. C. C.; Dellamora-Ortiz, G.; Betancor, L.; Fuentes, M.; Guisán, J. M.;  
1410 Fernandez-Lafuente, R. *Enzyme Microb. Technol.* **2007**, *40*, 310-315.

1411 (167) Palomo, J. M.; Fernandez-Lorente, G.; Mateo, C.; Ortiz, C.; Fernandez-Lafuente, R.;  
1412 Guisan, J. M. *Enzyme Microb. Technol.* **2002**, *31*, 775-783.

1413 (168) Palomo, J. M.; Muñoz, G.; Fernández-Lorente, G.; Mateo, C.; Fuentes, M.; Guisan, J. M.;  
1414 Fernández-Lafuente, R. *J. Mol. Catal. B: Enzym.* **2003**, *21*, 201-210.

1415 (169) Volpato, G.; Filice, M.; Rodrigues, R. C.; Heck, J. X.; Guisan, J. M.; Mateo, C.; Ayub, M.  
1416 A. Z. *J. Mol. Catal. B: Enzym.* **2009**, *60*, 125-132.

1417 (170) Palomo, J. M.; Segura, R. L.; Mateo, C.; Terreni, M.; Guisan, J. M.; Fernández-Lafuente,  
1418 R. *Tetrahedron: Asymmetry* **2005**, *16*, 869-874.

1419 (171) Chaubey, A.; Parshad, R.; Koul, S.; Taneja, S. C.; Qazi, G. N. *J. Mol. Catal. B: Enzym.*  
1420 **2006**, *42*, 39-44.

1421 (172) Yu, H.; Wu, J.; Chi, B. C. *Biotechnol. Lett.* **2004**, *26*, 629-633.

1422 (173) Kasche, V. *Enzyme Microb. Technol.* **1986**, *8*, 4-16.

1423 (174) Godoy, C. A.; Romero, O.; De Las Rivas, B.; Mateo, C.; Fernandez-Lorente, G.; Guisan,  
1424 J. M.; Palomo, J. M. *J. Mol. Catal. B: Enzym.* **2013**, *87*, 121-127.

- 1425 (175) Carrasco-López, C.; Godoy, C.; de las Rivas, B.; Fernández-Lorente, G.; Palomo, J. M.;  
1426 Guisán, J. M.; Fernández-Lafuente, R.; Martínez-Ripoll, M.; Hermoso, J. A. *J. Biol. Chem.*  
1427 **2009**, *284*, 4365-4372.
- 1428 (176) Schoffelen, S.; Van Hest, J. C. M. *Soft Matter* **2012**, *8*, 1736-1746.
- 1429 (177) Lopez-Gallego, F.; Schmidt-Dannert, C. *Curr. Opin. Chem. Biol.* **2010**, *14*, 174-183.
- 1430 (178) Ogawa, K.; Nakajima-Kambe, T.; Nakahara, T.; Kokufuta, E. *Biomacromolecules* **2002**,  
1431 *3*, 625-631.
- 1432 (179) Vejvoda, V.; Martínková, L.; Veselá, A. B.; Kaplan, O.; Lutz-Wahl, S.; Fischer, L.;  
1433 Uhnáková, B. *J. Mol. Catal. B: Enzym.* **2011**, *71*, 51-55.
- 1434 (180) Wang, L.; Li, C.; Wang, N.; Li, K.; Chen, X.; Yu, X. Q. *J. Mol. Catal. B: Enzym.* **2010**,  
1435 *67*, 16-20.
- 1436 (181) Wu, Q.; Liu, B. K.; Lin, X. F. *Curr. Org. Chem.* **2010**, *14*, 1966-1988.
- 1437 (182) Jia, F.; Narasimhan, B.; Mallapragada, S. K. *AIChE J.* **2013**, *59*, 355-360.
- 1438 (183) Fornera, S.; Kuhn, P.; Lombardi, D.; Schlüter, A. D.; Dittrich, P. S.; Walde, P.  
1439 *ChemPlusChem* **2012**, *77*, 98-101.
- 1440 (184) Ricca, E.; Brucher, B.; Schrittwieser, J. H. *Adv. Synth. Catal.* **2011**, *353*, 2239-2262.
- 1441 (185) Bolivar, J. M.; Wiesbauer, J.; Nidetzky, B. *Trends Biotechnol.* **2011**, *29*, 333-342.
- 1442 (186) Rocha-Martín, J.; Rivas, B. d. I.; Muñoz, R.; Guisán, J. M.; López-Gallego, F.  
1443 *ChemCatChem* **2012**, *4*, 1279-1288.
- 1444 (187) Bolivar, J. M.; Hidalgo, A.; Sánchez-Ruiloba, L.; Berenguer, J.; Guisán, J. M.; López-  
1445 Gallego, F. *J. Biotechnol.* **2011**, *155*, 412-420.
- 1446 (188) Hubbuch, J.; Kula, M. R. *Bioprocess Biosyst. Eng.* **2008**, *31*, 241-259.
- 1447 (189) Jokerst, J. V.; Chou, J.; Camp, J. P.; Wong, J.; Lennart, A.; Pollard, A. A.; Floriano, P. N.;  
1448 Christodoulides, N.; Simmons, G. W.; Zhou, Y.; Ali, M. F.; McDevitt, J. T. *Small* **2011**, *7*, 613-  
1449 624.
- 1450 (190) Ljunglöf, A.; Thömmes, J. *J. Chromatogr. A* **1998**, *813*, 387-395.
- 1451 (191) Ma, Y.; Rajendran, P.; Blum, C.; Cesa, Y.; Gartmann, N.; Brühwiler, D.; Subramaniam,  
1452 V. *J. Colloid Interface Sci.* **2011**, *356*, 123-130.

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1457 **Figure 1. Multifunctionality of glutaraldehyde activated supports.**

1458

1459 **Figure 2. Enzyme immobilization on lowly activated glutaraldehyde supports.**

1460

1461 **Figure 3. Theoretical effect of the activation degree on immobilization rates of proteins on**  
1462 **glutaraldehyde activated supports under conditions where the first event is the ionic**  
1463 **adsorption (the first immobilization is a multipoint process) or the covalent attachment**  
1464 **(the first immobilization is a one-point process).**

1465

1466 **Figure 4. Different mechanisms of immobilization on glutaraldehyde supports of standard**  
1467 **proteins**

1468

1469 **Figure 5. Different mechanisms of immobilization on glutaraldehyde supports of lipases.**

1470

1471 **Figure 6. Heterofunctionality of standard epoxy supports.**

1472

1473 **Figure 7. Steps in protein immobilization and stabilization via multipoint covalent**  
1474 **attachment on standard epoxy-activated supports: protein adsorption, first covalent bond,**  
1475 **multipoint covalent attachment and blocking of the remaining epoxy groups with**  
1476 **hydrophilic molecules.**

1477

1478 **Figure 8. Different possibilities of immobilizing lipases on hydrophobic epoxy-supports.**

1479

1480 **Figure 9. Tailor made heterofunctional supports using secondary groups able to produce a**  
1481 **first enzyme immobilization. One enzyme, one immobilization chemistry but different**  
1482 **orientations of the enzyme on the support.**

1483

1484 **Figure 10. Effect of the internal geometry of the support microspheres and activation**  
1485 **degree on the possibilities of getting an intense multipoint covalent attachment (MCA).**

1486

1487 **Figure 11. Effect of the steric hindrances of the reactive group on the support on the**  
1488 **immobilization rate and on the prospects of getting an intense multipoint covalent**  
1489 **attachment.**

1490

1491 **Figure 12. Necessity of the correct alignment of the reactive groups in the enzyme and the**  
1492 **support to get an intense multipoint covalent attachment: need of long term incubations**  
1493 **even if immobilization is very rapid.**

1494

1495 **Figure 13. Effect of the spacer arm in the support on the possibilities of achieving an**  
1496 **intense multipoint covalent attachment and the rigidification effect.**

1497

1498 **Figure 14. Multipoint immobilization of proteins on glyoxyl-agarose supports.**

1499

1500 **Figure 15. Possibilities to increase protein reactivity versus glyoxyl supports:**

1501 **1.- Chemical amination that produces a global modification of the protein and uses the**  
1502 **carboxylic groups of the protein.**

1503 **2.- Genetic amination: site-directed modification of the enzyme only on the desired area**  
1504 **and without strict limitations on amount of amino groups introduced.**

1505

1506 **Figure 16. Effect of the modification of the epoxy groups (during the preparation of**  
1507 **heterofunctional epoxy supports) on the immobilization rate and covalent immobilization**  
1508 **rate.**  
1509

1510 **Figure 17. Steric hindrances for the enzyme/support chemical reaction generated by the**  
1511 **secondary groups introduced on the heterofunctional supports.**  
1512

1513 **Figure 18. Building the second generation of heterofunctional supports: the primary group**  
1514 **in the same arm as the secondary group, and the secondary group under the primary one.**  
1515

1516 **Figure 19. Immobilization/stabilization of proteins by immobilization on second generation**  
1517 **of heterofunctional supports.**  
1518

1519 **Figure 20. An optimal heterofunctional support: the matrix is able to adsorb proteins, and**  
1520 **a layer of protein reactive groups is placed over this matrix.**  
1521

1522 **Figure 21. Heterofunctional supports and site-directed mutagenesis: one support and a**  
1523 **collection of mutated enzymes produce different orientations on the immobilization.**  
1524

1525 **Figure 22. IMAC-epoxy or glyoxyl supports for the directed immobilization of proteins.**  
1526 **Use of poly-His tags or introduction of a couple of His on different areas of the protein**  
1527 **surface.**  
1528

1529 **Figure 23. Site directed rigidification of Cys- mutant enzymes on thiol heterofunctional**  
1530 **supports.**  
1531

1532 **Figure 24. Site directed rigidification of Cys- mutant enzymes on thiol heterofunctional**  
1533 **supports: use of disulfide enzymes or disulfide supports.**  
1534

1535 **Figure 25. Heterofunctional amino supports: the control of the amination permits the**  
1536 **selective adsorption of large proteins.**  
1537

1538 **Figure 26. Heterofunctional IMAC supports and poly His-tagged proteins: the control of**  
1539 **the IMAC density on the support permits the selective adsorption of poly His tagged**  
1540 **proteins.**  
1541

1542 **Figure 27. Effect of enzyme orientation and loading degree on the activity of the enzyme**  
1543 **molecules as a function of the substrate size.**  
1544

1545 **Figure 28. Ideal support for the site directed rigidification of proteins integrating tailor**  
1546 **made-heterofunctional supports and site directed mutagenesis.**  
1547

1548 **Figure 29. Kinetic advantages of enzyme co-immobilization on cascade reactions.**  
1549

1550 **Figure 30. Use of heterofunctional supports to co-immobilize two proteins with very**  
1551 **different requirements.**  
1552

1553 **Figure 31. Controlling the enzyme distribution in the support particle pores by controlling**  
1554 **the immobilization rate.**