1   **GLUTARALDEHYDE IN BIO-CATALYSTS DESIGN:**

2   **A useful crosslinker and a versatile tool in enzyme immobilization**

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Abstract

27

28   Glutaraldehyde is one of most widely used reagents in the design of biocatalysts. It is a

29   powerful crosslinker, able to react with itself, with the advantages that this may bring forth. In

30   this review, we intend to give a general vision of its potential and the precautions that must be

31   taken when using this effective reagent. First, the chemistry of the glutaraldehyde/amino

32   reaction will be commented. This reaction is still not fully clarified, but it seems to be based on

33   the formation of 6-membered heterocycles formed by 5 C and one O. Then, we will discuss the

34   production of intra and inter-molecular enzyme crosslinkings (increasing enzyme rigidity or

35   preventing subunit dissociation in multimeric enzymes). Special emphasis will be placed on the

36   preparation of cross-linked enzyme aggregates (CLEAs), mainly in enzymes that have low

37   density of surface reactive groups and, therefore, may be problematic to obtain a final solid

38   support. Next, we will comment on the uses of glutaraldehyde in enzymes previously

39   immobilized on supports. First, the treatment of enzymes immobilized on supports that cannot

40   react with glutaraldehyde (only inter and intramolecular cross-linkings will be possible) to

41   prevent enzyme leakage and obtain some enzyme stabilization via cross-linking. Second, the

42   cross-linking of enzymes adsorbed on aminated supports, where together with other reactions

43   enzyme/support crosslinking is also possible; the enzyme is incorporated to the support. Finally,

44   we will present the use of aminated supports preactivated with glutaraldehyde. Optimal

45   glutaraldehyde modifications will be discussed in each specific case (one or two glutaraldehyde

46   molecules for amino group in the support and/or the protein). Using preactivated supports, the

47   heterofunctional nature of the supports will be highlighted, with the drawbacks and advantages

48   that the heterofunctionality may have. Particular attention will be paid to the control of the first

49   event that causes the immobilization depending on the experimental conditions to alter the

50   enzyme orientation regarding the support surface. Thus, glutaraldehyde, an apparently old

51   fashioned reactive, remains as the most widely used and with broadest application possibilities

52   among the compounds used for the design of biocatalyst.

53   **Key words:** enzyme immobilization, enzyme stabilization, inter and intramolecular

54   crosslinking, CLEAs, heterofunctional supports, glutaraldehyde.

55

1. Introduction

Enzymes are biocatalysts which catalyze most metabolic reactions in living beings. *In vivo,* they are highly specific (modifying just one substrate among a collection of similar ones), chemo/enantio/regio-selective (yielding just one substrate among several possible) and very active under very mild environmental conditions (atmospheric pressure, room temperature, aqueous medium). Thus, enzymes have been considered the ideal catalyst from an environmental point of view, in reactions involving complex or labile compounds.[1-5]

However, enzymes perform their function inside living beings, under complex regulations and stress. This causes enzymes to become inhibited by many compounds. Thus, their *in vivo* stability can hardly be measured in days, and their exceptional properties may not be found when they are utilized for modifying other substrates (different to the physiological ones) or even performing other reactions (e.g., using hydrolases as transferases in kinetically controlled synthesis). Moreover, the soluble nature of the enzymes avoids their extended use (and subsequent re-use) in industry. These properties are far from the requirements of an industrial catalyst.[6]

The solution to these limitations is the main subject of enzyme technology. The researcher may use a handful of different tools, such as microbiology,[2] genetic approaches,[7-10] immobilization,[11-17] chemical modification,[18,19] and medium and reactor engineering to shortcut these enzyme limitations. Many of these tools may be used in an integrated way.[20-23]

Physicochemical tools, such as chemical modification or enzyme immobilization, have special interest in this context. Due to the requirements of producing a heterogeneous catalyst, enzyme immobilization becomes a necessity from this perspective.[14] This strategy has been investigated by many authors as a way to improve enzyme properties. It has been shown that enzyme stability may be significantly improved if an intense multipoint covalent attachment (MCA) between an enzyme molecule and a rigid support, via a short spacer arm, is achieved.[17]

81 This phenomenon causes all groups involved in the immobilization to keep their relative

82 positions when the enzyme is submitted to any conformational change. The area of the enzyme

83 involved in the immobilization may be also critical to maximize the stabilizing effect of the

84 MCA.[22] Immobilization may also tune some other enzyme properties, such as activity,

85 resistance to inhibition, selectivity or specificity.[17] Chemical modification of enzymes may be

86 used to further improve enzyme stability (e.g., via chemical crosslinking),[24] and may be also

87 used as another tool to alter enzyme catalytic features.[18] Moreover, as it has been revised,

88 chemical modification or immobilization of enzymes may be designed to simplify or improve

89 one another.[21]

90 Glutaraldehyde is the reagent that the present review is devoted to. It has been used in

91 many instances as protein cross-linker, as an activator of supports, and as crosslinker of enzymes

92 and supports.[25,26] In this review, we intend to give a wide vision of the prospects of this very

93 interesting and versatile molecule in the design of biocatalysts.

94

95 **2. Chemistry of glutaraldehyde**

96 Glutaraldehyde is a bi-functional reagent with the capacity to polymerize.[27,28]

97 Glutaraldehyde may react with different enzyme moieties, mainly involving primary amino

98 groups of proteins, although it may eventually react with other groups (thiols, phenols, and

99 imidazoles).[27-30] However, the exact structure of the main structures related to protein

100 crosslinking or enzyme immobilization is still not fully clarified. Figure 1 shows some of these

101 proposed structures. It is clear that the structure of the glutaraldehyde relevant for the

102 modification of enzymes and supports is not a linear one, but some kind of fairly stable cycles

103 (activated support may be washed with an excess of distilled water without losing

104 glutaraldehyde moieties).

105

106

107   The reaction mechanism of glutaraldehyde with proteins implies that it is not limited to just one

108   mechanism. This is because the main reactive species of glutaraldehyde are found in equilibrium

109   between their monomeric and polymeric conformations.[27,28,31,32] Moreover, every structure can

110   react in a different way with the protein. For instance, under both acidic and neutral conditions,

111   aldehyde groups from glutaraldehyde can react with proteins by formation of Schiff bases. In

112   this case, a nucleophilic attack takes place by the ε-amino group from lysine to glutaraldehyde

113   (See Figure 2a). However, Schiff bases are unstable at acidic conditions and are broken up,

114   regenerating both the aldehyde and the amine groups. For this reason, several procedures have

115   recommended reduction by $NaBH_4$ or $NaBCNH_3$ as a final step in order to stabilize the Schiff

116   base into a secondary amine. Nevertheless, some studies suggest that protein preparations

117   treated with a reducing agents does not cause an striking increase in the enzyme stability.[33]

118   Additionally, lysine residues of protein treated with glutaraldehyde without further chemical

119   reduction are not regenerated by incubation in HCl 6M at 110 ºC by 24 h.[34] Consequently, it is

120   possible that a mechanism of formation of Schiff bases between proteins and glutaraldehyde

121   could not be carried out. In this sense, an additional reduction step would not be necessary in

122   order to stabilize the reaction product.[27,28,35] Therefore, the mechanism of glutaraldehyde at

123   neutral and acidic conditions would be mediated by hemiacetal cyclic conformations from both

124   the monomer and polymer of glutaraldehyde. This cyclic hemiacetal of glutaraldehyde reacts by

125   nucleophilic substitution of the amino groups from lysines with the hydroxy group of

126   glutaraldehyde according to the Figure 2.

127   On the other hand, under basic conditions it has been proposed that glutaraldehyde quickly

128   suffers intramolecular aldolic condensations, producing a polymeric form of an a α,β-

129   unsaturated aldehyde, which may react with amino groups from proteins through two

130   mechanisms: Firstly by formation of Schiff bases between internal aldehyde groups from the

131   polymeric form of glutaraldehyde and primary amino groups from the protein (Figure 3,

132   reaction 1). In this case, the obtained product could be stabilized by a resonance effect of

133 conjugated double C-C bonds.[32] The second mechanism involves a Michael addition to the

134 double C-C linkage.[27] However, this reaction results in a less stable product due to loss of

135 resonance effect of the conjugated double C-C bonds, which would be labile under acidic

136 conditions (Figure 3,).

137 Prof. Monsan,[36,37] has shown that it is relatively simple to only have one or two

138 molecules of glutaraldehyde per amino group in a support. This result, coupled to some more

139 recent ones that confirm this, suggested a different reactivity of the amino/glutaraldehyde

140 moiety with free glutaraldehyde compared to the capacity of free glutaraldehyde molecule to

141 polymerize, as under the described conditions to get this amino/glutaraldehyde/glutaraldehyde

142 moiety there is not a massive precipitation of glutaraldehyde polymers.[38] Amino/glutaraldehyde

143 reacted with free glutaraldehyde much more rapidly that free glutaraldehyde with free

144 glutaraldehyde molecules. It also showed that the two glutaraldehyde molecules/amino moiety

145 groups in the support possessed the structure that exhibited the highest reactivity versus amino

146 groups in a protein. If just one glutaraldehyde molecule was attached to the amino group, this

147 moiety exhibited a low reactivity versus amino groups. However, this group, together with the

148 already commented reactivity versus free glutaraldehyde molecules, exhibited a vey high

149 reactivity versus other similar amino/glutaraldehyde moieties, being the activation of a protein

150 the preferred way to get crosslinkings as we will discuss later. This different reactivity of the

151 amino/glutaraldehyde moiety compared to the glutaraldehyde or the

152 amino/glutaraldehyde/glutaraldehyde moiety should be found in the presence of an amino group

153 in the heterocycle of the glutaraldehyde ring.[39,40] Amino/glutaraldehyde/glutaraldehyde has a

154 similar reactivity when compared to free glutaraldehyde, as the second glutaraldehyde group

155 will be very similar to free glutaraldehyde. If the conditions were forced (e.g., increasing the pH

156 value or the glutaraldehyde concentration) to get a higher degree of polymerization on the amino

157 group, the free glutaraldehyde may also react; giving large glutaraldehyde aggregates that may

158 precipitate and the suspension will turn white.

159

3. Glutaraldehyde as protein crosslinker

161   The first use of glutaraldehyde was to preserve and fix tissues[41,42] in some instances

162 combined with formaldehyde.[25] This is achieved through the formation of intermolecular

163 crosslinking. Nowadays, glutaraldehyde remains as one of the most potent crosslinker reagents,

164 even with clinical applications.[43-45] In part, these very good crosslinker features are a

165 consequence of the capacity of glutaraldehyde to react with itself or with protein groups already

166 modified with a glutaraldehyde molecule.[28]

167   The protein crosslinkings may be among groups placed on different protein molecules

168 (intermolecular crosslinking) or between groups placed in the same molecule (intramolecular

169 crosslinkings).[24,46] Both kinds of crosslinkings have interest in specific cases.

170

3.1- Glutaraldehyde as intermolecular crosslinker of proteins

172   As previously commented, this was the first use of glutaraldehyde, using its crosslinker

173 potential to fix tissues.[41] However, in this review we will focus on the use of glutaraldehyde in

174 the design of biocatalysts.

175   The addition of glutaraldehyde to a protein solution may produce the chemical

176 aggregation of the enzyme, causing protein molecules to react among themselves, and can

177 directly yield a "solid biocatalyst" (Figure 4). Although this immobilization strategy has not

178 been widely used, it is possible to find diverse examples in literature. For example, insoluble

179 trypsin was prepared by the use of glutaraldehyde to produce intermolecular crosslinks and the

180 insoluble trypsin thus prepared exhibited enzymatic activity toward casein.[47] Later,

181 glutaraldehyde was reacted in aqueous solutions with papain to form a water-insoluble product

182 with enzymatic activity after activation by reducing agents. A rapid reaction of glutaraldehyde

183 with the essential sulfhydryl of papain was not involved in the reaction since after activation the

184 insoluble enzyme retained esterolytic and proteolytic activity.[48]

185   In another example, a purified preparation of extracellular alkaline proteinase of

186   *Trichoderma koningii* was insolubilized by intermolecular crosslinking with glutaraldehyde.[49]

187   The optimum operational temperature of the insolubilized enzyme increased by 20 ºC. The

188   immobilized enzyme was also relatively more stable and activity was less depended on the

189   presence of ions or detergents than the soluble enzyme. More surprisingly, an enhanced affinity

190   to casein, hemoglobin and bovine serum albumin was found, although with a lower V max

191   values.

192   Intermolecular cross-linking of the protease stem bromelain with 0.25 and

193   1.25% glutaraldehyde results in the formation of a large molecular mass, multimeric and

194   soluble aggregate having comparable activity to the unmodified bromelain. Both 0.25 and

195   1.25% glutaraldehyde cross-linked bromelain preparations were more stable against urea,

196   guanidine hydrochloride and temperature-induced inactivation, and exhibited slightly better

197   storage stability compared to the unmodified protease. Such a high molecular weight, soluble,

198   active and stable preparation may be useful in industry, i.e. in the textile industry for improving

199   the properties of a fabric without loss of fabric strength and shape.[50]

200   On the other hand, glutaraldehyde is the intermolecular crosslinker of some of the

201   recent carrier-free immobilization protocols, such as crosslinked enzyme crystals (CLECs)[51,52]

202   (Figure 5) or aggregates (CLEAs) (Figure 6).[53-56] While using a conventional homo-bi-

203   functional reagent the ideal strategy seems to modify around 50% of the reactive groups of the

204   protein to maximize the possibility of crosslinking, the chemistry of glutaraldehyde causes this

205   option not to be the optimal one, as amino/glutaraldehyde moieties reacts better with other

206   amino/glutaraldehyde groups than one amino group.[33] Moreover,

207   amino/glutaraldehyde/glutaraldehyde did not react easily with other amino/ (glutaraldehyde)$_2$

208   groups, making it inconvenient to excessively modify the proteins. Thus, to reach protein

209   crosslinking using glutaraldehyde, it seems adequate to use moderate concentrations of

210   glutaraldehyde, high enough to ensure the activation of most amino groups with one molecule of

glutaraldehyde, but not too high so as not to reach activations with two molecules of glutaraldehyde per amino group (e.g., 0.1-1% (v/v) glutaraldehyde at pH 7 for 1 hour).[36] Milder activation of the amino groups of the enzyme may produce a milder crosslinking. However, in some instances it may be necessary to use suboptimal crosslinking conditions if the protein is especially sensible to glutaraldehyde modifications. In certain cases, as if the enzyme has a Lys group in its catalytic site (e.g., some aldolases), glutaraldehyde must be discarded and other crosslinking reagent should be used, such as aldehyde dextran.[57]

To achieve a proper crosslinking in this kind of immobilization strategies may be a problem, mainly if the protein has not many Lys on its surface (Figure 7). This may make the formation of large aggregates difficult and enable the release of enzyme molecules (individual molecules or small aggregates) from the solid. In some cases, it is even not possible to get a solid in aqueous medium. Using CLEAs some solutions have been offered to overcome this situation. Taking advantage of the fact that the enzyme did not need to be pure to prepare CLEAs, the co-precipitation of the target enzyme with inert proteins having a high density of superficial Lys groups (e,g, bovine serum albumin)[58-63] or with polymers having many amino groups (e.g., polyethylenimine)[64-67] has been proposed (Figure 8). Both strategies permit the formation of physically stable CLEAs, but they also produce a decrease in the volumetric loading of the target enzyme, as part of the volume will be occupied by the inert protein or the polymer. As an alternative, a strategy based on the use of chemically aminated enzymes with their protein surface enriched on reactive groups has been proposed (Figure 9).[68] This way, it is not necessary to mix the target protein with any other molecule or polymer and the volumetric loading of the CLEA is similar to that of proteins that may be directly used to prepare CLEAs.

3.2. Glutaraldehyde as intramolecular crosslinker of proteins

The introduction of intramolecular crosslinkings in an enzyme structure is one of the most widely used techniques to increase enzyme stability, using genetic or chemical

237   routes.[24,69,70] If the crosslinking agent is short enough, the relative mobility of one group relative

238   to the other crosslinked group should be minimized.[71] Furthermore, if the researcher is able to

239   introduce several crosslinkings on the enzyme surface, the final result should be a global

240   rigidification of the overall structure of the enzyme (Figure 10).

241   However, to introduce several intramolecular crosslinkings in a protein molecule and to

242   get a significant stabilization effect is not a simple task, as many problems may rise. We will

243   detail some of the most relevant ones.

244   - *Distance between reactive groups in the protein.* The crosslinking formation requires the

245   existence of at least two reactive moieties in the protein surface at the adequate distance.[72,73] If

246   there are no groups in the range of distances covered by the crosslinker, crosslinking will not be

247   possible. This may be solved if the researcher somehow increases the number of reactive groups

248   on the protein surface, e.g. via amination (Figure 11). Site-directed mutagenesis, changing an

249   aminoacid of the protein by Lys next to other Lys or the terminal amino group, may permit to

250   increase the prospects of crosslinking just in a desired place (e.g., an unstable area of the

251   protein). However, although the site directed introduction of Cys residues has been used to

252   establish disulfide bonds in a protein,[74-76] these reviewers have been unable to find the use of

253   introduce Lys to improve the chemical crosslinking with glutaraldehyde, only to improve the

254   multipoint covalent attachment via immobilization.[77,78] Another possibility is the chemical

255   amination of the enzyme with ethylenediamine via activation of the carboxylic groups of the

256   protein with carbodiimide.[79,80] This strategy implies the general modification of the enzyme

257   surface, but the number of reactive groups may be increased by a factor between 2 and 5,

258   augmenting the possibilities that several groups in the protein surface may be at an adequate

259   crosslinking distance. This strategy has been utilized to introduce intramolecular crosslinking in

260   immobilized penicillin G acylase, with stabilization factor of 30-50, depending on the

261   crosslinking conditions and inactivation cause.[33]

262   - *Competition between one-point chemical modification and crosslinking*. The first one-point

263   chemical modification involves a soluble reactive that must modify a group placed in the surface

264   of a protein. In principle, this one-point modification may be very rapid. However, the

265   crosslinking reaction involves the correct alignment of two groups located on a moderately rigid

266   surface, such as that of an enzyme. Using other homo-bifunctional crosslinkers, this competition

267   may produce serious hindrances to the promotion of an intense crosslinking in the enzyme

268   surface, using glutaraldehyde, as stated above, there is not a real competition between one-point

269   modification and crosslinking, as the best solution is an activation of all amines in the protein

270   with just one glutaraldehyde group. However, there is no guarantee that the modified amino

271   groups participate in crosslinking. In the best scenario, the final effect of the glutaraldehyde

272   treatment will be a mixture of crosslinking and chemical modification. The first one should

273   produce a rigidification of the enzyme surface; the second may have unpredictable effects on the

274   enzyme features. Some times these effects may be very negative, even overcoming the positive

275   effects of the crosslinking bridges. Some times, it may be very positive, increasing enzyme

276   stability even without the formation of any crosslinking.[81,82] This effect of the one-point

277   chemical modification needs to be considered when explaining the effects of the glutaraldehyde

278   treatment on the stability of a particular enzyme.

279   - *Competition between inter and intra molecular crosslinking*. Another possibility is the

280   competition between intra and intermolecular crosslinking. In fact, using free enzyme, to just

281   have the desired intramolecular crosslinking may be nearly impossible, as inter molecular

282   crosslinking is a faster reaction. Although this may also produce enzyme stabilization (see

283   above), it may complicate the understanding of the results, and also make reproducibility

284   complex, because the intermolecular crosslinking will depend on the protein concentration, kind

285   and percentage of contaminant proteins, etc. The use of simple analytical techniques applied to

286   glutaraldehyde treated samples after reduction with borohydride, such as SDS-PAGE, may

287   permit to visualize the presence and extension of the intermolecular crosslinking.

288   Even when using immobilized enzymes, to fully prevent enzyme crosslinking may be

289   hard. Using porous supports, if the immobilization rate is much higher than the diffusion rate,

290   the enzymes may be packed so near each other that the enzyme molecules may be crosslinked

291   with each other even using short reagents like glutaraldehyde (Figure 12). If the immobilization

292   rate is slow enough, the distance between enzyme molecules may be enough to almost fully

293   prevent enzyme intermolecular crosslinking.[83] Recently, by changing the immobilization

294   conditions, a lipase has been immobilized on octyl agarose and treated with glutaraldehyde.[84]

295   Under one immobilization condition, immobilization was quite slow (in presence of ethanol)

296   and intermolecular crosslinking was almost negligible and it was possible to analyze the effect

297   of intramolecular modifications. Under another condition, immobilization rate was vey high and

298   the enzyme molecules were so near that both modifications could be achieved. The comparison

299   between both derivatives (one having only intramolecular modifications, the other having both

300   intramolecular and intermolecular modifications) permitted to determine the positive effects of

301   the intermolecular crosslinking in the enzyme stability.

302

303   **3.3. Crosslinking of multimeric enzymes.**

304   Many enzymes are formed by several subunits, which are in association/dissociation

305   equilibrium, usually being the associated form the most active and stable.[85-87] In other cases,

306   some cascade reactions may require the joint action of several different enzymes that require

307   their being associated.[88-90] If the researcher is able to crosslink these structures, the system will

308   work in a more adequate fashion. However, the difficulties commented for the intramolecular

309   crosslinking remain valid here, even with one additional key point: the reactive groups at the

310   crosslinking distance need to be placed in different enzyme subunits (Figure 13).[23] This is why

311   crosslinking of these structures may be achieved in a better way using polymers, as they should

312   not confer an intense structural rigidity, but may be useful to keep together the different enzyme

313   subunits or maintain the multi enzymatic complex assembled (e.g., dextran aldehyde,[91]

314 polyehtylenimine[92,93]). Nevertheless, glutaraldehyde has been used in certain cases to stabilize,

315 even though only in a small percentage of the total enzyme structures, the subunit-subunit

316 interactions. For example, the crosslinking of the hetero-oligomeric glucose dehydrogenase from

317 a moderate thermophilic bacterium, SM4, using glutaraldehyde as crosslinking reagent has been

318 reported.[94] The treatment permitted glucose dehydrogenase to gain high thermal stability

319 without loss of its catalytic activity and thus increase the activity of the enzyme at high

320 temperature. The authors concluded that by chemical cross-linking of the subunits, the

321 dissociation of alpha and beta subunits was prevented. Consequently, its quaternary structure

322 was stabilized, and thus the thermal stability of the glucose dehydrogenase was enhanced. Also,

323 D-Lactate dehydrogenase from *Limulus polyphemus* is a homodimer which is composed of

324 identical subunits that has been crosslinked with glutaraldehyde to show a relation of

325 reactivation with reassociation of the dimer.[95]

326   In another paper, primase/helicase produced by bacteriophage T7 that can form both

327 hexamers and heptamers. These oligomers were stabilized via cross-linking with glutaraldehyde

328 and purified.[96] The authors detected how the percentage of each oligomeric form could be

329 altered by the presence of either dTTP or β,γ-methylene. Heptamers are unable to efficiently

330 bind either single-stranded DNA or double-stranded DNA, thus the authors postulated that a

331 switch between heptamer to hexamer may provide a ring-opening mechanism for the single-

332 stranded DNA binding pathway.

333   NTPDase1 and NTPDase2 enzymes from rats were expressed in *Xenopus laevis*

334 oocytes and their quarternary structure was analyzed.[97] The treatment with glutaraldehyde

335 permitted to detect that native NTPD-ase1 and NTPD-ase2 occur in oligomeric form. Dynamic

336 alterations in oligomeric state may induce changes in substrate preference and thus influence the

337 pattern of *in situ* extracellular nucleotide degradation.

338   In some instances, glutaraldehyde may have an indirect use to stabilize a multimeric

339 enzyme, by crosslinking a polymer to the enzyme surface avoiding the polymer release. The

340 coating of the surface of multimeric enzymes with a poly-ionic polymer that may

341 simultaneously interact with several enzyme subunits, preventing enzyme dissociation has

342 proved to prevent enzyme subunit dissociation if the polymer can become adsorbed on the

343 enzyme (Figure 14).[93] This strategy has been recently utilized to stabilize the enzyme glutamate

344 dehydrogenase from *Thermus thermophilus* and formate dehydrogenase from *Pseudomonas* sp.*,*

345 using polyethyleneimine as the "crosslinking" polymer. Both enzymes were inactivated by

346 dissociation at acidic pH value, and the coating of their surface with polyethyleneimine

347 prevented this (Figure 14). However, the reversible nature of the polymer adsorption permitted

348 the polymer to become desorbed under certain conditions (e.g., high ionic strength, drastic pH

349 value), and the protective effect of the polymer coating was lost. This problem was solved by a

350 further treatment with glutaraldehyde, crosslinking the enzyme and the polymer: the new

351 composite was fully stable under enzyme dissociation conditions. This composite could be used

352 in soluble form, or ionically exchanged on a cation exchanger.[93] When a similar strategy was

353 applied to the hexameric glutamate dehydrogenase from *E. coli*, the polymer protection was

354 successful, but the enzyme was rapidly inactivated by incubation with glutaraldehyde.[92] Thus,

355 this strategy not only requires that the polyethyleneimine may stabilize the multimeric structure

356 of the enzyme without inactivating it, but also requires that the enzyme was resistant to the

357 treatment with glutaraldehyde.

358

359 **4. Intermolecular crosslinking of immobilized proteins via reversible immobilization using**

360 **supports unreactive towards glutaraldehyde**

361   Enzyme immobilization by physical adsorption (using ion exchange, immobilized

362 chelates or hydrophobic adsorption) is the simplest protocol to immobilize enzymes, does not

363 require enzyme nor support treatment, immobilization is rapid, and the support does not require

364 special storage conditions.[14] However, this enzyme immobilization strategy has two

365 disadvantages. The first one, the enzyme may be released from the support under certain

366 experimental conditions.[98,99] Using ion exchangers, this may occur when changing the pH value

367 or increasing the ionic strength. Using hydrophobic adsorption, if the reaction requires the

368 presence of co-solvents or detergents the enzyme may be released to the medium. The second

369 problem is that physical adsorption may have no positive effects on enzyme stability, even as the

370 support retains its capacity to interact with the enzyme, the immobilization may produce a

371 certain destabilization of the enzyme.[14]

372   Thus, to prevent enzyme desorption and improve enzyme stability, it is relatively

373 common to find reports where the researcher, after enzyme reversible immobilization, treats the

374 immobilized enzyme with glutaraldehyde. In most cases, a certain enzyme stabilization is

375 observed and enzyme desorption is at least partially avoided under conditions where previously

376 all the immobilized enzyme became desorbed (Figure 12). Furthermore, this occurs using

377 supports with which the glutaraldehyde can not react, thus the enzyme cannot become

378 immobilized on the support. This is the case of supports having aliphatic acyl moieties or

379 quaternary amine groups as active groups on the support; glutaraldehyde hardly can react with

380 these groups in the support.

381   To explain these results, we should go back to point 3.2 of this review. Immobilization

382 via physical adsorption is usually much faster than enzyme diffusion inside the support pores

383 and in most cases the treatments of the biocatalyst involving glutaraldehyde described in the

384 literature are performed using fully protein loaded biocatalysts. Under these conditions, the

385 enzymes are packed together, so close to each other that the distance is in the range of

386 glutaraldehyde crosslinking. Furthermore, if two reactive groups are conveniently confronted,

387 intermolecular crosslinking will occur (Figure12).[84] Moreover, intramolecular modifications

388 (one point or crosslinkings) will also take place, reinforcing in some cases the stabilization

389 effects. Regarding enzyme release, it now becomes necessary to simultaneously release all

390 adsorbed enzymes, otherwise the aggregate will remain adsorbed. This makes that the "strength"

391 of the adsorption increases exponentially with the size of the aggregate, and under conditions

392   where individual molecules were fully desorbed, now the enzyme remains fully adsorbed on the

393   support.[84]

394   As in certain cases the reactivity of the support with the glutaraldehyde cannot be fully

395   discarded due to its unknown nature, the lack of glutaraldehyde reaction with the support may be

396   verified via different strategies. For example, it is possible to check if we can "preactivate" the

397   support, to achieve a covalent attachment between the enzyme molecules and the support after

398   washing the support with an excess of water to eliminate all free glutaraldehyde molecules and

399   later offering the enzyme. The use of Schiff reactive may also help to verify if some reactive

400   glutaraldehyde remain bound to the support after the washings. Second, slowing down the

401   immobilization rate of the enzyme to increase the distance between enzyme molecules, and

402   checking if the glutaraldehyde treatment still avoids desorption of a significant percentage of the

403   enzyme molecules.

404   One drawback of this strategy is that desorption of very large chemical aggregates may

405   be fully impossible even under the most drastic conditions, transforming a reversible

406   immobilization method in an irreversible one. The researcher should carefully evaluate the

407   convenience of this treatment, considering the stability requirements of the enzyme and the real

408   risks of enzyme desorption during the use of the biocatalyst versus the possibility of having to

409   discard the support after enzyme immobilization.[14]

410   This strategy has been used in several examples to prepare crosslinked enzyme

411   aggregates in an immobilized form, that some authors call CLEAs (see above), but which really

412   are an altogether different thing. Hierarchically ordered mesocellular mesoporous carbon was

413   used as a host for enzyme immobilization.[100] To improve the retention of enzymes, the adsorbed

414   enzymes were cross-linked using glutaraldehyde. The resulting preparation showed a significant

415   stabilization with high enzyme loadings. For example, 0.5 g chymotrypsin could be loaded in 1

416   g of silica with no activity decrease observed with rigorous shaking over one month. In contrast,

417   adsorbed chymotrypsin without any crosslinking treatment resulted in a lower loading, which

418 further decreased due to continuous leaching of adsorbed chymotrypsin under shaking. The

419 activity of crosslinked enzyme aggregates was 10 times higher than that of the adsorbed

420 chymotrypsin..

421   In two papers, both α-chymotrypsin and a lipase were immobilized in SBA-15

422 mesoporous silica by crosslinking adsorbed enzymes to give the so-called one-dimensional

423 crosslinked enzyme aggregates.[101,102] This simple approach resulted in one-dimensional

424 crosslinked enzyme aggregates in the linear pore channels of SBA-15, which was very effective

425 in preventing the enzyme leaching and consequently improving the enzyme stability.

426   In another research effort, meso-structured onion-like silica was produced with a 200-

427 300 nm sized primary meso-structured onion building unit, with each onion unit having highly

428 curved mesopores of 10 nm diameter in a multishell structure.[103] Nanoscale enzyme reactors in

429 these supports were prepared via a two-step process of enzyme adsorption and subsequent

430 enzyme cross-linking with glutaraldehyde, which effectively prevents the leaching of cross-

431 linked enzyme aggregates from highly curved mesopores. This improved the enzyme stability as

432 well as the enzyme loading.

433   In another example, β-glucosidase was immobilized onto mesocellular silica foams

434 and later crosslinked with glutaraldehyde.[104] This resulted in the formation of crosslinked

435 enzyme aggregates of nanometer scale. The final catalyst was more stable and presented a lower

436 Km than that of its free counterpart.

437   Other authors really produced CLEAs inside the pores of supports, taking advantage of

438 the increase in size of the enzyme when an aggregate was formed. However, they are neither

439 real CLEAs, as the enzyme is not previously precipitated (Figure 15). This is the case of the

440 examples described by Prof Hartman´s group. Using chloroperoxidase, they showed that the

441 formation of cross-linked chloroperoxidase aggregates in the pores of mesocellular foam

442 materials results in active biocatalysts that are more resistant to leaching than the conventional

443 catalyst prepared by physisorption of chloroperoxidase. Small-angle neutron scattering

444   experiments clearly confirm that the chloroperoxidase -CLEAs are located in the pores of the

445   mesocellular foams.[105] Later, they extended the studies to glucose oxidase. The formation of

446   cross-linked enzyme aggregates of glucose oxidase in the pores of mesocellular foams was

447   obtained. The enzymes can enter the ultra-large cavities connected through the smaller windows,

448   where their agglomeration and cross-linking with glutaraldehyde will take place. After cross-

449   linking, the diameter of the aggregates is larger than the diameter of the pore entrance and, thus,

450   the enzymes are trapped in the pores of the support.[106] Finally, glutaraldehyde cross-linked

451   enzyme aggregates of chloroperoxidase and glucose oxidase were grown in large-pore

452   mesocellular foams, improving operational stability in the oxidation of indol.[107]

453

454   **5. Crosslinking of supports bearing primary amino groups and ionically exchanged**

455   **proteins**

456   In this new example of use of glutaraldehyde to immobilize enzymes in preexisting

457   supports, as in the case explained above, the treatment with glutaraldehyde is performed after

458   the enzyme is adsorbed in the support, in this case via ion exchange. However, this is a fully

459   different case from the one described above. Now, the researcher knows that the support has

460   primary amino groups, and that, therefore, it is likely to modify the support with glutaraldehyde,

461   and finally obtain enzyme-support covalent bonds.[108] Thus, in this case, the enzyme may

462   experience three different kinds of modifications caused by glutaraldehyde (Figure 16):

463   1- Intramolecular modifications (one point modifications or crosslinking, see above).

464   2- Interprotein crosslinking. If the ion exchange immobilization has been rapid enough,

465   the enzymes will be very near each other and it is likely to have enzyme-enzyme crosslinking

466   (see above).

467   3- Support-enzyme reaction. The support and the enzyme molecules may react with each

468   other. In fact, if the treatment with glutaraldehyde is performed in a way that enables the

469   modification of each reactive group in the support and the enzyme with one glutaraldehyde

470   molecule, we will have the situation where the highest prospects of enzyme support reaction

471   may occur. And this reaction will take place in a relative wide range of pH value, as the reaction

472   will occur between amino/glutaraldehyde moieties; although considering the stability of the

473   groups and its reactivity, some reports suggest that pH values around 8.5 may be the most

474   adequate.[33] That way, the support will behave as a large multi-crosslinking reagent, fixing the

475   positions of all the protein groups involved in the reaction with the support, whose relative

476   position must remain unaltered under any distorting condition, promoting an increase of the

477   overall enzyme structure, which becomes more intense when a more intense multipoint covalent

478   attachment is achieved.[17,109-111]

479   Thus, this strategy has some positive points, as the good reactivity of the amino

480   glutaraldehyde moieties with similar groups in a relatively wide range of pHs, that include

481   neutral pH, the fact that the glutaraldehyde treatment is performed on a previously immobilized

482   enzyme, and the possibilities of having some positive inter or intra-modifications. This last point

483   is also the drawback of the strategy, as the enzyme should be fully modified, not only in the

484   groups involved in the immobilization, and in some cases these modifications may be negative

485   for enzyme stability or activity. However, in many instances when there is a comparison

486   between immobilization on preactivated supports (see below) or treatment with glutaraldehyde

487   after enzyme in exchange, stabilization improves using this strategy.[108,112,113]

488   In other many cases, a comparison was not performed but results using the treatment

489   of previously adsorbed enzymes were very positive regarding stabilization and prevention of

490   enzyme leakage.[114-119]

491   The treatment of lipases adsorbed on anion exchanger supports has been in some cases

492   used to modulate enzyme properties. Thus, the open form of some lipases was fixed by

493   glutaraldehyde treatment of adsorbed lipases in the presence of detergents.[120] In other cases, the

494   random chemical modification was enabled to modulate the lipase selectivity or specificity.[121]

495   This strategy may be only used when the support is able to immobilize the protein via

496   ionic exchange. That means that the support should present a high enough number of ionic

497   groups to permit the adsorption of the enzyme on the support. Moreover, the enzyme should

498   have the capacity to become adsorbed to anion exchangers. Considering that this is a multipoint

499   process (several ion bridges need to be produced to fix the enzyme to the support),[122-124] the

500   strategy presents the problem that the support should never be physically inert. Furthermore, the

501   inertness of the support surface may be in many instances a desired feature of the support, to

502   prevent uncontrolled support-enzyme interactions that may affect enzyme stability (sometimes

503   in a positive sense, but in another may have a negative impact in the enzyme stability).[14,125]

504   Another negative point to be considered is the possibility of reactivating the enzyme after

505   inactivation, the folding-unfolding strategy may not work if the unfolded enzyme may interact

506   with the support.[14]

507   Moreover, the strategy does not offer a large versatility. It may be possible that the

508   immobilization via ionic exchange under different conditions of pH and ionic strength may

509   involve different areas, but that is the only way of altering the enzyme orientation regarding the

510   support.[126-128] The immobilization of an enzyme via different areas may be interesting to reach

511   optimum stabilization (involving the most labile area of the protein in the immobilization)[22,129-132]

512   or to tune the enzyme catalytic properties (selectivity, specificity, activity).[17]

513

514   **6. Activation of supports with glutaraldehyde**

515   In this last case, the support is pre-activated with glutaraldehyde. Following the results

516   from Monsan, the optimal activation regarding the chemical reactivity of the support should be

517   two molecules of glutaraldehyde per amino group in the support (obtained, e.g., by incubating

518   the support in 1 M glutaraldehyde at pH 7 by 12-16 h).[37] It is very likely the most widespread

519   form of using glutaraldehyde to immobilize enzymes (see for example[133-141]) and one of the first

520   papers concerning the use of glutaraldehyde to prepare immobilize enzymes may be found in .[142]

521   Compared to the strategy described above, this method has some advantages and

522   drawbacks. As a drawback, we can consider the higher difficulty on having an intense

523   multipoint covalent attachment, as now the reaction is between

524   amino/glutaraldehyde/glutaraldehyde moieties and the ε amino groups of the Lys residues (and

525   the terminal amino group). The Lys groups have a pK of 10.7, and the stability of the

526   glutaraldehyde groups in the support is not good at alkaline pH value while at neutral pH value

527   the reactivity of the amino groups is relatively low. At neutral pH value, the most reactive amino

528   group is the terminal one, with a pK value between 7 and 8, being much more reactive that the

529   addition of the reactivities of all Lys external groups.[143] However, it should be considered that

530   after the first immobilization, if some nucleophiles of the protein are in the area exposed to the

531   support, the high apparent concentration of the different groups may permit the establishment of

532   some new covalent enzyme-support bonds.[144] Moreover, the preactivated support may not be

533   stored for a long time under wet conditions due to the relatively low stability of the groups.

534   The first advantage compared to the case described in Section 5, is that now only the

535   groups involved in the covalent immobilization are modified by glutaraldehyde. Moreover, the

536   support may immobilize enzymes even if they are very poorly activated, because the enzyme is

537   covalently fixed to the support just with one point as the glutaraldehyde-protein bonds are

538   stable. Thus, using very lowly activated amino supports (e.g., 1 reactive group under each

539   projected area of the protein) immobilization using glutaraldehyde activated supports will be

540   directly performed by a covalent reaction by the most reactive amino group on the enzyme (very

541   likely the amino group of the enzyme if immobilization is performed at neutral pH value).[143]

542   However, immobilization will be very slow due to the low activation of the support, and will

543   offer no chance of reaching an intense multipoint covalent attachment.[14]

544   On the other hand, using highly activated supports; the features of the spacer arm

545   (amino/glutaraldehyde/glutaraldehyde) convert the supports in a heterofunctional support. The

546   term "heterofunctional support" may be applied to supports that present several functionalities

547   on its surface that are able to interact with different groups of an enzyme, and these interactions

548   may be different under different conditions (Figure 17).[145,146]

549   Immobilization of enzymes on a heterofunctional support may be more versatile. If the

550   researcher is able to benefit from this fact it may be an advantage, but if this fact is not

551   considered, the understanding of the results may be really complex. The use of heterofunctional

552   supports to immobilize proteins has been recently discussed in the literature.[147] In this review we

553   will focus on glutaraldehyde activated supports.

554   The multifunctionality of the supports having a high surface density of amino groups and

555   activated with glutaraldehyde is a direct consequence of the way they are prepared. Their

556   preparation requires the modification of supports bearing primary amino groups with two

557   glutaraldehyde molecules, if the optimal protocol is followed.[28,36,37] The final result is a support

558   having spacer arms bearing one or two amino groups (cationic groups that may function as

559   anion exchangers), a fairly hydrophobic moiety formed by the glutaraldehyde dimer and the

560   covalent reactive group.

561   In this way, a support highly activated with glutaraldehyde may give three different kinds

562   of interactions with a protein: hydrophobic, anionic exchange and covalent (Figure 18).[84]

563   Biomacromolecules are only immobilized on supports via ionic exchange or hydrophobic

564   interactions when several enzyme-support interactions may be established, being the one point

565   interaction insufficient to immobilize a protein.[128] Thus, these interactions will only have a real

566   impact on enzyme immobilization when using supports bearing several amino-(glutaraldehyde)$_n$

567   moieties under each enzyme molecule (Figure 18). [148-153] Thus, using highly activated

568   glutaraldehyde supports, the three immobilization causes may be able to immobilize a protein

569   molecule. If the researcher is aware of this fact, it is possible to permit that one or the other

570   immobilization cause may be the dominant one, by playing with the experimental conditions.[38]

571   This permits to immobilize enzymes following different orientations regarding the support

572   surface. If there is a very small amount of groups on the support, (e.g. just one spacer arm per

573   projected area of the enzyme), this multi-interaction will no longer be possible or will be so

574   slow, that the only relevant cause of immobilization will be the one point covalent

575   immobilization, as explained above.[143,148-153]

576   This means that even if all enzyme molecules are immobilized (incorporated to the

577   support) in a very rapid fashion using a highly activated glutaraldehyde support, there is no

578   guarantee that covalent immobilization of the enzyme on the support has taken place. In fact, the

579   immobilization may be very strong, and the enzyme may remain immobilized under conditions

580   where the enzyme may be fully released from the mother amino support, and still the enzyme

581   molecules may be just adsorbed, as now the adsorption may be mixed, ionic/hydrophobic.[84] To

582   ensure that covalent immobilization has taken place, the enzyme should remain immobilized

583   under conditions where both ionic and hydrophobic interactions may be broken (e.g., using

584   cationic detergents, using guanidine, increasing the ionic strength in the presence of non-ionic

585   detergents).[84]

586   Using highly activated supports, it has been shown that in most cases an ionic exchange

587   with the amino groups in the support is the first step in the immobilization of most

588   enzymes.[22,38,84,154-156]

589   One effect of this first ionic adsorption is that, even though glutaraldehyde is able to

590   immobilize enzymes via just one attachment due to the stability of the bond formed, the

591   activation degree of the support has an exponential effect on the immobilization reaction rate of

592   proteins, not the expected linear effect using reactive groups in the support able to immobilize

593   the proteins via just one point.[143] This is because the researcher is measuring the rate of ionic

594   exchange of the enzyme on the support, which requires the establishment of several enzyme-

595   support interactions, and thus it is exponentially dependent on the surface density of amino

596   groups on the support although the covalent reaction should be of order 1.[38,148-153] This

597   immobilization mechanism is much faster that the direct covalent attachment via

598   glutaraldehyde-enzyme covalent reaction, being this way the first cause of immobilization for

599   most enzyme molecules.

600   This multifunctionality of the glutaraldehyde supports is an advantage in certain cases.

601   The rapid ionic exchange of the enzyme on the support keeps the enzyme from being in soluble

602   form before being covalently immobilized (thus avoiding some inactivation causes, such as

603   interaction with interfaces or autolysis).[16,17] If enzyme immobilization via ion exchange has a

604   positive effect on enzyme stability, enzyme inactivation by distortion will also be slowed down.

605   However, the main advantage of the multifunctionality of glutaraldehyde is that we can

606   alter the enzyme orientation on the support by changing the immobilization conditions, favoring

607   one mechanism or another as the first immobilization cause (Figure 18).

608   If the researcher wishes to have a first hydrophobic adsorption, this can be achieved

609   using a high enough ionic strength. The glutaraldehyde dimer is not very hydrophobic, but may

610   be hydrophobic enough if the surface density of the groups in the support is high enough. Using

611   very high ionic strength, the ionic exchange will be rolled out, and the areas of the protein with

612   high concentration of external hydrophobic groups may be involved in the first enzyme

613   adsorption and delimit the area where the reactive groups of the enzyme which will react with

614   the support should be located. After enzyme hydrophobic adsorption, the reactive groups of the

615   enzyme near the support surface may produce some covalent reactions. However, this will be

616   produced *after* enzyme immobilization, and there is no guarantee that the enzyme will finally

617   have any covalent attachment with the support (e.g., if there are no reactive groups on the

618   enzyme surface in that area).

619   The second possibility is to permit ionic exchange of the enzyme prior to covalent

620   immobilization. Using most enzymes, the use of low ionic strength is enough to reach this

621   situation (an ionic strength that permits ionic exchange of the proteins on the non-activated

622   glutaraldehyde amino support).[38,84] In this case, the enzyme will be first immobilized on the

623   support by ionic exchange and this area will be the one where nucleophiles capable of reacting

624 with the glutaraldehyde moieties should be located. Ionic exchange may also involve different

625 enzyme regions depending on the experimental conditions and activation degree of the support.

626 Ionic exchange at different pH values may in certain enzymes change the area where the highest

627 concentration of available anionic charged groups may be found. Furthermore, the ionic strength

628 may determine the area involved because the higher the ionic strength, the more restrictive the

629 immobilization becomes (requiring more enzyme support-interactions).[126,127]

630 Finally, it is possible to immobilize the enzyme via a direct first covalent attachment,

631 involving the most reactive exposed group of the enzyme (usually the terminal amino group).

632 Using most enzymes, the moderate ionic strength used to prevent ionic exchange (100-250 mM

633 of NaCl) is not enough to force the hydrophobic adsorption of the protein on the support (that is

634 not very hydrophobic) and a direct covalent immobilization may be the first cause for the

635 enzyme immobilization.[38,84]

636 Lipases are a special case of enzymes that permit a new alternative to the

637 immobilization on glutaraldehyde activated supports. This has special interest as they are

638 perhaps the most used enzymes in industry and academic studies due to their broad range of

639 substrates and reactions, accompanied of a high enantio- and regio- selectivity or specificity,

640 together to good stability in different reaction medium and wide availability.[157-159]

641 Lipases are complex enzymes that usually have two conformations, a closed one where

642 the active center is secluded from the medium by a polypeptide chain (lid or flap), and an open

643 form, where the lid is displaced and the active center of the lipase is exposed to the medium

644 (Figure 19).[160] This open form presents a very large hydrophobic area exposed to the medium,

645 formed by the hydrophobic residues around the active center of the lipase and the hydrophobic

646 groups in the internal face of the lid.[161] The exposition to a hydrophilic medium (e.g., an

647 aqueous buffer) of this large hydrophobic area is unfavorable, thus the enzyme in aqueous

648 homogeneous media will be mainly in the closed form. However, this open form is readily

649   adsorbed on the hydrophobic surface of the oil drops even at very low ionic strength (interfacial

650   activation) (Figure 19)..[162,163]

651   It has been shown that lipases may become adsorbed via a similar mechanism on any

652   other hydrophobic surface (hydrophobic supports, hydrophobic proteins, other "open" lipases).

653   [164-166] Thus, the use of hydrophobic supports to immobilize lipases at low ionic strength has been

654   suggested as a simple way to obtain the open and stabilized form of the immobilized lipases.[167]

655   Although this interfacial activation is the specific feature of lipases, the lid may be quite

656   different from one lipase to another, e.g., lipase B from *Candida antarctica* has a very small lid

657   that can not fully seclude the active center from the medium,[168] while the lipase from *Bacillus*

658   *thermocatenulatus* has a double lid and a very complex movement governing its activation.[169]

659   This immobilization is based on the fact that the hydrophobic support mimics the natural

660   substrate of lipases. And highly activated glutaraldehyde activated supports offers a fairly

661   hydrophobic surface to this interfacial activation of lipases.

662   In fact, it has been shown that using lipases, it is at least possible to immobilize the

663   enzyme via 4 different mechanisms on highly activated glutaraldehyde supports.[84]

664   Thus, the control of the immobilization on these supports using lipases is more

665   complex, but the support offers a new immobilization orientation.

666   Due to the tendency of the open form of the lipases to become adsorbed versus

667   hydrophobic interfaces,[164,170,171] if the immobilization is just performed at low ionic strength, the

668   enzyme will be immobilized by both immobilization mechanisms: interfacial activation and

669   ionic exchange (Figure 20).[84] Depending on the enzyme, the support and the immobilization

670   conditions, one or the other immobilization cause may be predominant, but most likely the other

671   cause will always be present in a major or minor percentage. This mixture of lipase orientations

672   is not the ideal situation if the researcher wishes to tune lipase properties via immobilization and

673   may also make difficult to understand the results. In fact, this is the usual situation that we may

674   find in the literature.

675   This situation may be avoided by using non-ionic detergents, which prevent the

676   interfacial activation of the lipase versus a hydrophobic support (Figure 21). [164] Performing the

677   immobilization in the presence of Triton X-100 at low ionic strength, lipases are mainly

678   immobilized on the glutaraldehyde supports via a first ionic exchange.[84] Using moderate ionic

679   strength, the enzyme immobilization will mainly proceed via interfacial activation (Figure 22).

680   The use of very high ionic strength will cause the lipase to adopt mainly the closed

681   form, permitting as a first immobilization step the conventional hydrophobic adsorption via

682   hydrophobic groups located on the lipase surface (Figure 23). It has been reported that

683   interfacial activation of lipases on hydrophobic supports is slowed at high ionic strength.[164] That

684   way, using an ionic strength just high enough able to prevent the ionic exchange of the lipase,

685   lipases will become immobilized on the support first via a rapid interfacial activation on the

686   support, which is much faster than the direct covalent attachment.[84]

687   The only way to ensure that the first step in the immobilization of a lipase in these

688   supports is a covalent attachment is to simultaneously prevent ionic exchange and interfacial

689   activation. This may be achieved using simultaneously moderately high ionic strength and

690   detergents, or ionic detergents (Figure 24).[84]

691   Whatever the enzyme used, if we have a situation where the first phenomenon is

692   covalent immobilization, the surface density of groups in the support will have a first order

693   effect on the rate of enzyme immobilization.[143] If this is not the case (mainly at the highest

694   support activation degree), another cause may be the responsible for the first enzyme

695   immobilization.

696   Thus, it is possible to immobilize enzymes on glutaraldehyde supports via different first

697   events. This may lead to different orientations of the enzyme on the support, offering different

698   stabilities.[38] In the case of lipases, they also exhibited different catalytic behavior (e.g.,

699   specificity was altered).[84] This way, it is possible to have, using the same immobilization

700   support, enzymes immobilized by different areas, with different numbers of enzyme molecule-

701 support covalent bonds and different enzyme-support unspecific interactions.[147] We should bear

702 in mind that, due to the proximity between the groups of the support and of the enzyme,

703 interactions between immobilized enzyme and support will be produced over time even though

704 they may not be enough to be the only cause for immobilization.[144]

705   In other, more difficult to classify cases, a lipase was immobilized on electrospun and

706 ethanol-dispersed polystyrene-poly(styrene-co-maleic anhydride) nanofibers in the form of

707 enzyme precipitate coatings.[172] Lipase precipitate coatings were prepared in a three-step process,

708 consisting of lipase covalent attachment, lipase precipitation, and crosslinking of precipitated

709 lipases onto the covalently attached lipases via glutaraldehyde treatment.

710   A similar approach was used to immobilize β-glucosidase. The enzyme was

711 immobilized on polymer nanofibers. Then, additional enzyme molecules were crosslinked onto

712 the covalently attached enzyme molecules via glutaraldehyde treatment.[173]

713

714 **Conclusions**

715   Glutaraldehyde, an apparently old fashioned reagent use for a long time in design of

716 biocatalyst, remains as one of the most interesting tools in enzyme crosslinking and

717 immobilization (Figure 25). However, to achieve optimal results, it is necessary to understand

718 the different reactivities of the amino/glutaraldehyde and amino/glutaraldehyde/glutaraldehyde,

719 together with the fact that, in immobilization of enzymes on glutaraldehyde pre-activated

720 supports, it is a heterofunctional support that permits altering the enzyme orientation on the

721 support surface. Thus, glutaraldehyde remains one of the most potent and versatile tools in

722 Enzyme technology, and the new knowledge on its reactivity and possibilities open even new

723 opportunities for the future.

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FIGURE LEGENDS

FIGURE 1. Possible structures of glutaraldehyde in aqueous solution.

FIGURE 2. Reactions of glutaraldehyde with proteins under acidic or neutral conditions.

FIGURE 3. Schiff base (1) and Michael-type (2) reactions of glutaraldehyde with proteins under basic conditions

FIGURE 4. Immobilization of enzyme via copolymerization with glutaraldehyde.

FIGURE 5. Preparation of Crosslinked Enzyme Crystals (CLECs).

FIGURE 6. Preparation of Crosslinked Enzyme Aggretates (CLEAs).

FIGURE 7. Problems in the crosslinking step during CLEAs preparation using enzymes poor in Lys residues.

FIGURE 8. Preparation of combi-CLEAs using proteins having many Lys residues to facilitate the crosslinking step.

FIGURE 9. Amination of enzymes that are poor in Lys residues to facilitate the crosslinking step in CLEA preparation.

FIGURE 10. Effect of intramolecular crosslinking on the stability of enzymes.

FIGURE 11. Amination of enzyme surfaces to facilitate the promotion of an intense intramolecular crosslinking.

FIGURE 12. Effect of the immobilization rate on the possibilities of intermolecular crosslinking between immobilized enzyme molecules.

FIGURE 13. Chemical crosslinking of subunits in multimeric enzymes.

FIGURE 14. Stabilization of polyethyleneimine/enzyme composites via glutaraldehyde crosslinking to prevent subunit dissociation.

FIGURE 15. Production of enzyme chemical aggregates to prevent enzyme leakage on mildy adsorbed enzymes.

FIGURE 16. Crosslinking of enzymes and aminated supports after enzyme ionic adsorption.

Figure 1

1059

1060

1061

1062

(2a)

(2b)

(2c)

Figure 2

1063

1064

Figure 3

GLUTARALDEHYDE

FREE ENZYME

**COPOLYMER
GLUTARALDEHYDE-ENZYME**

Figure 4

1067

1068

FREE PURE ENZYME

CRYSTALLIZATION

Enzyme crystal

glutaraldehdye

CLEC

Figure 5

1069

1070

FREE ENZYME

precipitant
PEG
Salts, solvents

PROTEIN
AGGREGATE

glutaraldehyde

CLEA

Figure 6

FREE ENZYME

PROTEIN AGGREGATE

**precipitant
PEG
Sallts, solvents**

glutaraldehyde

SOLUBLE MODIIFIED ENZYME

CLEA??

RESUSPENSION

Figure 7

FREE ENZYME

**PROTEIN
AGGREGATE**

precipitant
**PEG
Salts, solvents**

+

glutaraldehyde

COMBI-CLEA

RESUSPENSION

Figure 8

1075

1076

FREE ENZYME

Amination

precipitant
PEG
Sallts, solvents
+

FREE
AMINATED
PROTEIN

AMINATED
PROTEIN
AGGREGATE

CLEAs

glutaraldehyde

Figure 9

ENZYME INACTIVATION

NATIVE ENZYME　　　**DISTORTED ENZYME**

INACTIVE ENZYME

REVERSIBLE CONFORMATIONAL CHANGES

Enzyme crosslinking

CONFORMATIONAL CHANGES ARE RESTRICTED

Figure 10

CROSSLINKER

**INTRAMOLECULAR
CROSSLINKING
IS NOT POSSIBLE**

AMINATION

**AN INTENSE
INTRAMOLECULAR
CROSSLINKING
IS NOW POSSIBLE**

Figure 11

Figure 12

1081

1082

Rapidly immobilized Enzyme

Glutaraldehyde modification

Intermolecular crosslinking plus intramolecular chemical modifications

IMPROVED ACTIVITY
IMPROVED STABILITY

Slowly immobilized Enzyme

Glutaraldehyde modification

Intramolecular chemical modifications

1083

1084

SMALL
BIFUNCTIONAL
REAGENT

**No intersubunit
crosslinking**

LARGE
MULTIFUNCTIONAL
REAGENT

**Intersubunit
crosslinking**

Figure 13

1085

1086

PEI

COO⁻
COO⁻
COO⁻
COO⁻
COO⁻
COO⁻
COO⁻

NH₂
NH₂
NH₂
NH₂
NH₂

Stabilized Quaternary Structure

NH₂
NH₂
NH₂
NH₂
NH₂
NH₂

Glutaraldehyde

Dissociation and inactivation

Salts, drastic pH value

NH₂
NH₂
NH₂
NH₂
NH₂
NH₂

Figure 14

52

1088

Risk of enzyme leakage

Glutaraldehyde

No risk of enzyme leakage

Large covalent aggregates STABILIZATION??

Figure 15

1089

1090

Ionically exchanged enzyme
in an aminated support

Glutaraldehyde

One point modification

Intramolecular crosslinking

Intermolecular
crosslinking

Support-enzyme
crosslinking

Figure 16

54
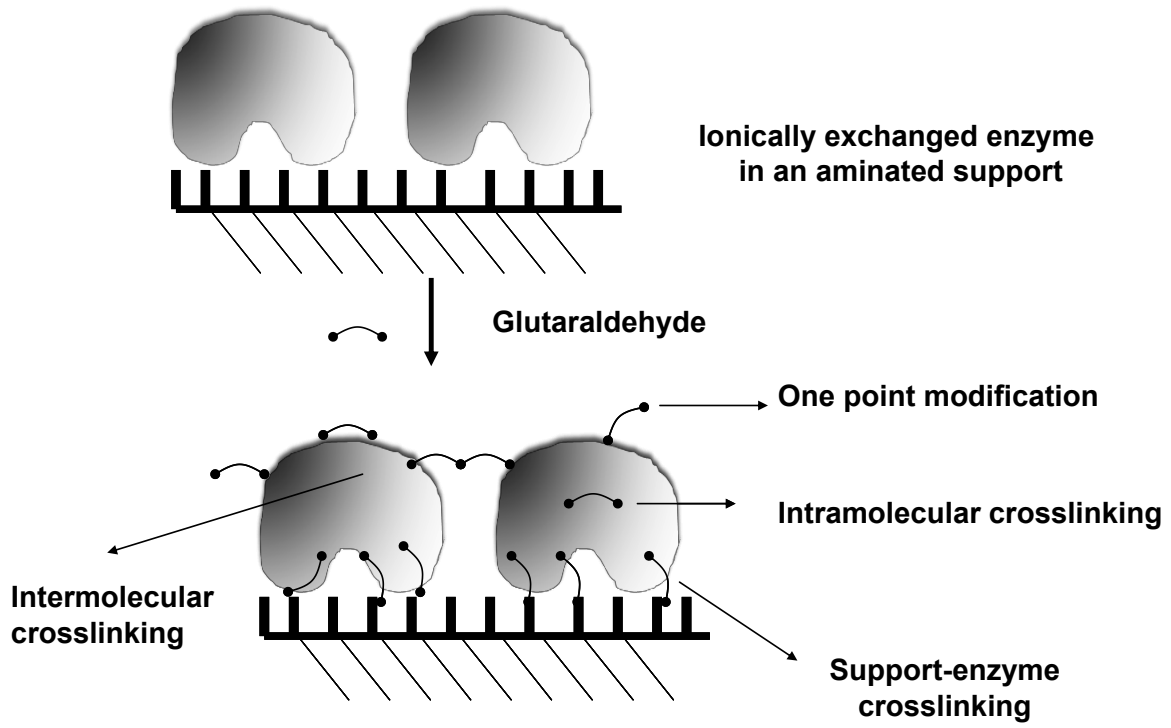
1091

1092

MULTIFUNCTIONAL SUPPORTS
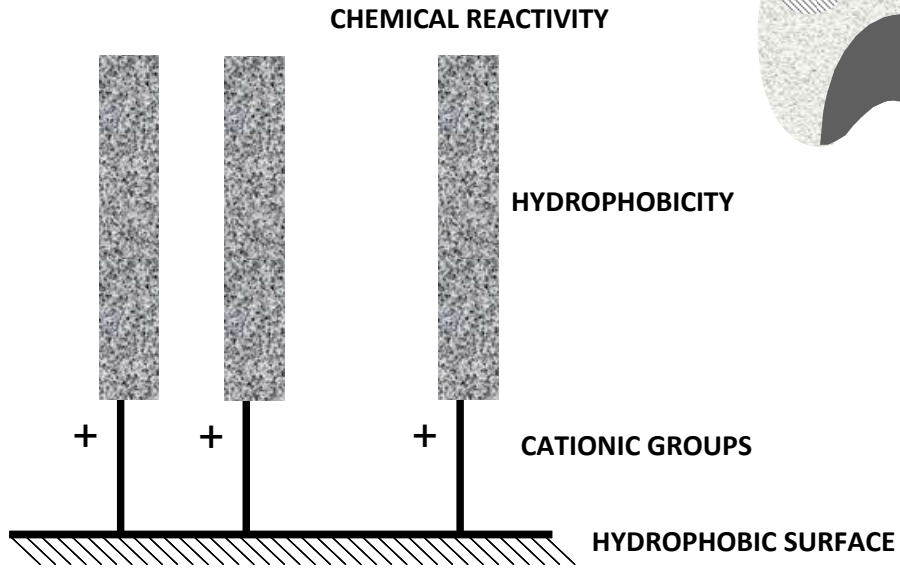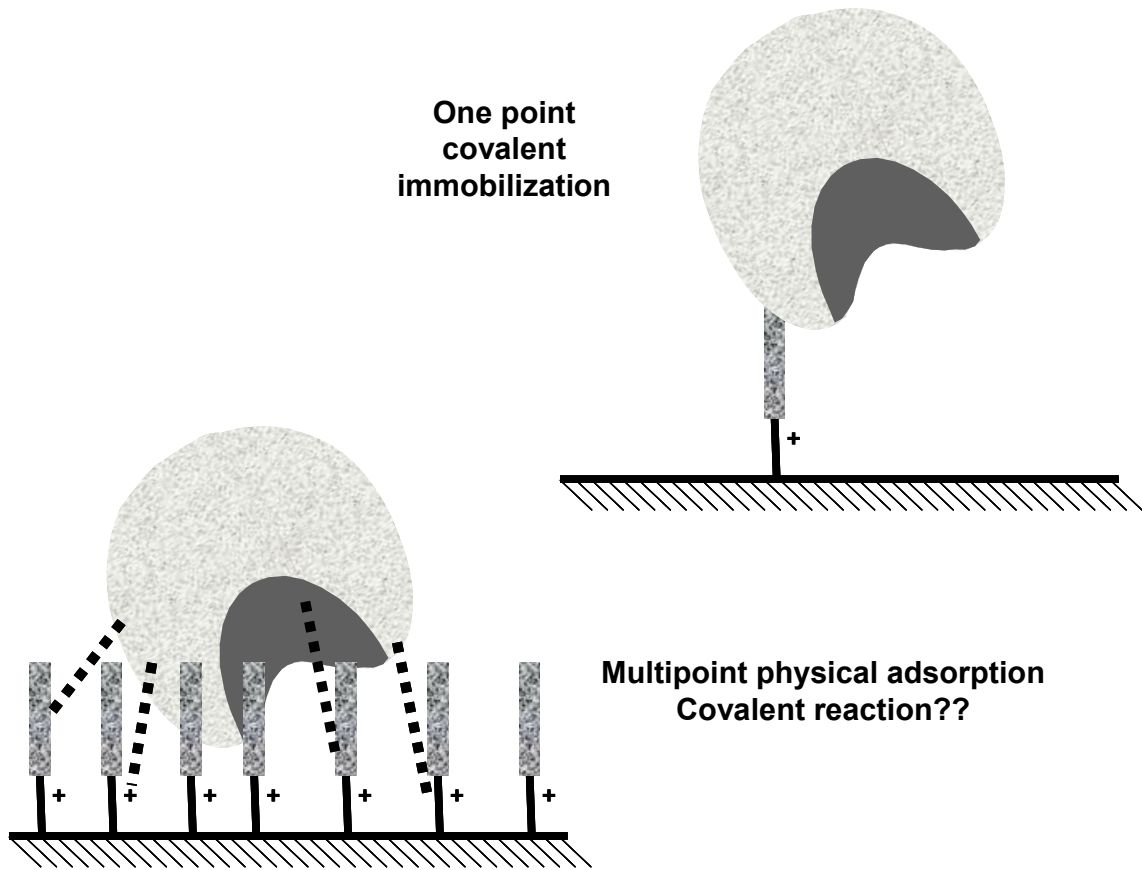
CHEMICAL REACTIVITY

HYDROPHOBICITY

+   +   +   CATIONIC GROUPS

HYDROPHOBIC SURFACE

Figure 17

1093

1094

**One point
covalent
immobilization**

**Multipoint physical adsorption
Covalent reaction??**

Figure 18

THE CASE OF LIPASES

Interfacial adsorption on hydrophobic supports

Large hydrophobic pocket

Figure 19

LOW IONIC STRENGTH

Possible covalent attachments

LIPASE ADSORPTION VIA INTERFACIAL ACTIVATION
IONIC EXCHANGE
NO INSPECIFC HYDROPHOBIC ADSORPTION
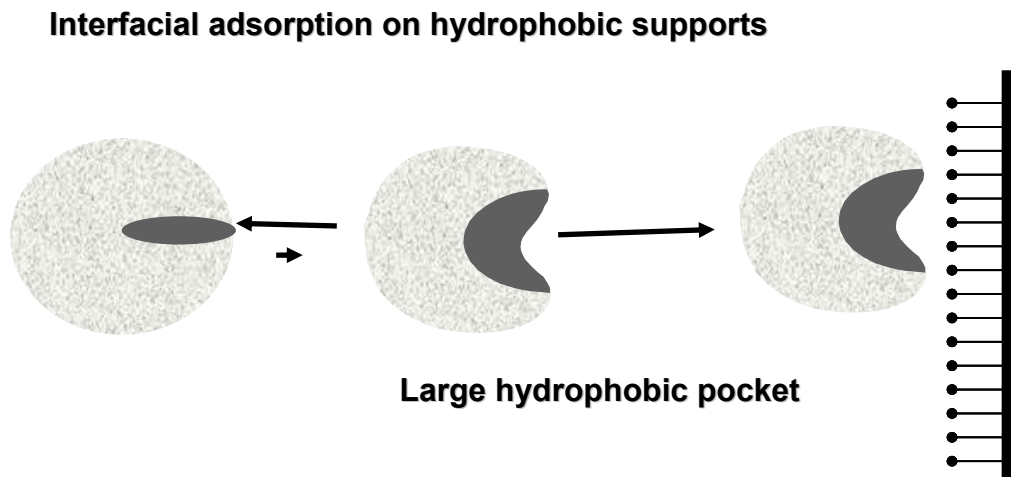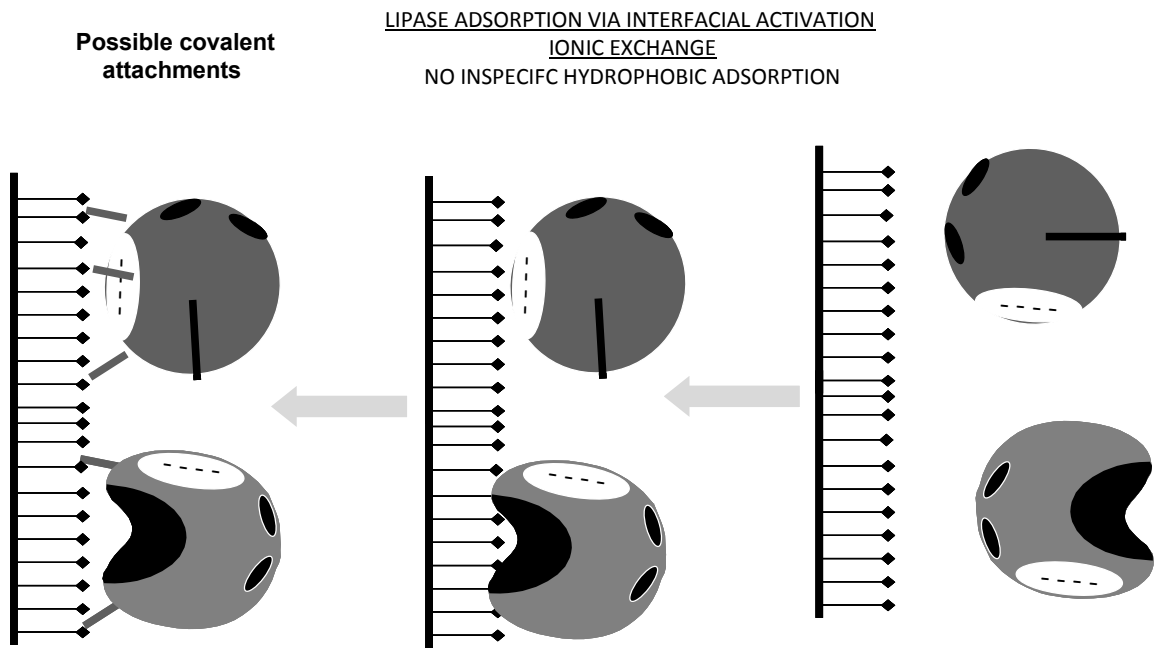
Figure 20

**Low ionic strength+
DETERGENT**

**1. Ionic
exchange**

IONIC EXCHANGE
NO INSPECIFC HYDROPHOBIC ADSORPTION
NO ADSORPTION VIA INTERFACIAL ACTIVATION
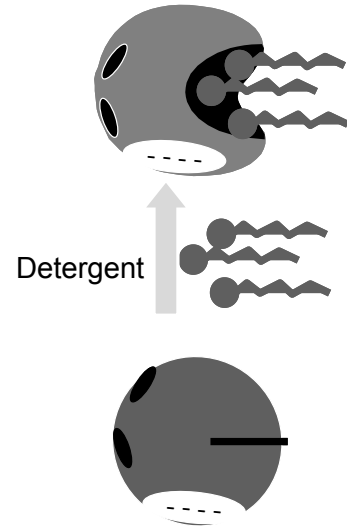
Detergent
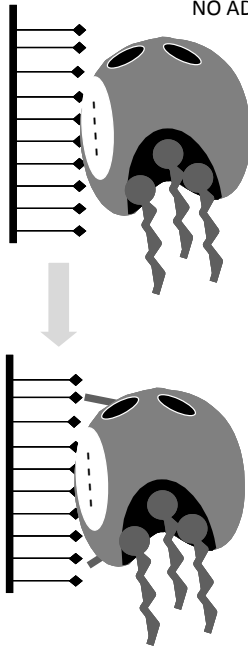
**2. Possible
covalent
attachments**

Figure 21

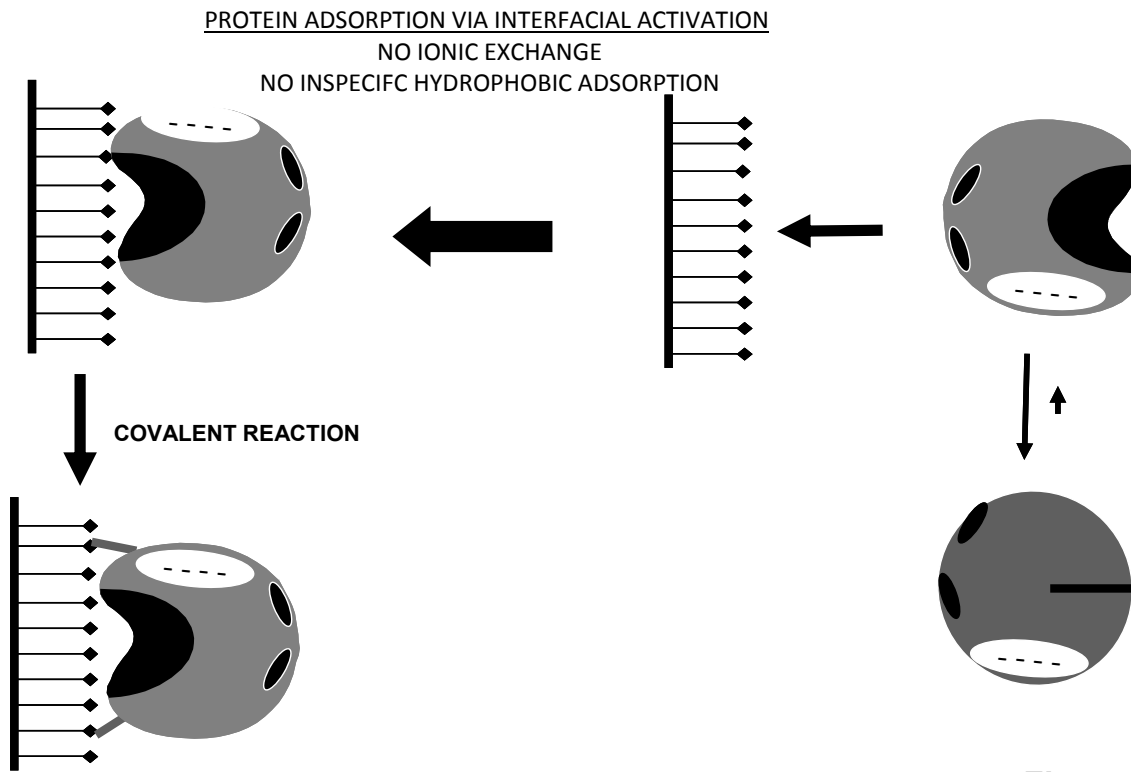**IONIC STRENGTH HIGH ENOUGH
TO PREVENT IONIC EXCHANGE**

PROTEIN ADSORPTION VIA INTERFACIAL ACTIVATION
NO IONIC EXCHANGE
NO INSPECIFC HYDROPHOBIC ADSORPTION

COVALENT REACTION

Figure 22

Very high ionic strength

1. Hydrophobic adsorption

LIPASE
CLOSED FORM

HYDROPHOBIC ADSORPTION
NO IONIC EXCHANGE
NO ADSORPTION VIA INTERFACIAL ACTIVATION

2. Possible covalent attachments

Figure 23