GLUTARALDEHYDE IN BIO-CATALYSTS DESIGN:

A useful crosslinker and a versatile tool in enzyme immobilization

Oveimar Barbosa[a], Claudia Ortiz[b], Ángel Berenguer-Murcia[c], Rodrigo Torres[a], Rafael C. Rodrigues*[,d] Roberto Fernandez-Lafuente[e,]*.

[a] Escuela de Química, Grupo de investigación en Bioquímica y Microbiología (GIBIM), Edificio Camilo Torres 210, Universidad Industrial de Santander, Bucaramanga, Colombia.

[b] Escuela de Bacteriología y Laboratorio Clínico, Universidad Industrial de Santander, Bucaramanga, Colombia.

[c] Instituto Universitario de Materiales, Departamento de Química Inorgánica, Universidad de Alicante, Campus de San Vicente del Raspeig, Ap. 99 - 03080 Alicante, Spain.

[d] Biocatalysis and Enzyme Technology Lab, Institute of Food Science and Technology, Federal University of Rio Grande do Sul, Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil.

[e] Departamento de Biocatalisis. ICP-CSIC. Campus UAM-CSIC. Cantoblanco. 28049 Madrid. Spain.

* Co-corresponding authors.

Dr Rafael Costa Rodrigues

Biocatalysis and Enzyme Technology Lab, ICTA-UFRGS

Av. Bento Gonçalves, 9500, P.O. Box 15090, ZC 91501-970, Porto Alegre, RS, Brazil.

e-mail: rafaelcrodrigues@ufrgs.br

Prof. Dr Roberto Fernández-Lafuente

Departamento de Biocatálisis. Instituto de Catálisis-CSIC.

C/ Marie Curie 2. Campus UAM-CSIC. Cantoblanco.

28049 Madrid (Spain).

e-mail: rfl@icp.csic.es

Abstract

Glutaraldehyde is one of most widely used reagents in the design of biocatalysts. It is a powerful crosslinker, able to react with itself, with the advantages that this may bring forth. In this review, we intend to give a general vision of its potential and the precautions that must be taken when using this effective reagent. First, the chemistry of the glutaraldehyde/amino reaction will be commented. This reaction is still not fully clarified, but it seems to be based on the formation of 6-membered heterocycles formed by 5 C and one O. Then, we will discuss the production of intra and inter-molecular enzyme crosslinkings (increasing enzyme rigidity or preventing subunit dissociation in multimeric enzymes). Special emphasis will be placed on the preparation of cross-linked enzyme aggregates (CLEAs), mainly in enzymes that have low density of surface reactive groups and, therefore, may be problematic to obtain a final solid support. Next, we will comment on the uses of glutaraldehyde in enzymes previously immobilized on supports. First, the treatment of enzymes immobilized on supports that cannot react with glutaraldehyde (only inter and intramolecular cross-linkings will be possible) to prevent enzyme leakage and obtain some enzyme stabilization via cross-linking. Second, the cross-linking of enzymes adsorbed on aminated supports, where together with other reactions enzyme/support crosslinking is also possible; the enzyme is incorporated to the support. Finally, we will present the use of aminated supports preactivated with glutaraldehyde. Optimal glutaraldehyde modifications will be discussed in each specific case (one or two glutaraldehyde molecules for amino group in the support and/or the protein). Using preactivated supports, the heterofunctional nature of the supports will be highlighted, with the drawbacks and advantages that the heterofunctionality may have. Particular attention will be paid to the control of the first event that causes the immobilization depending on the experimental conditions to alter the enzyme orientation regarding the support surface. Thus, glutaraldehyde, an apparently old fashioned reactive, remains as the most widely used and with broadest application possibilities among the compounds used for the design of biocatalyst.

Key words: enzyme immobilization, enzyme stabilization, inter and intramolecular crosslinking, CLEAs, heterofunctional supports, glutaraldehyde.

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]

This phenomenon causes all groups involved in the immobilization to keep their relative positions when the enzyme is submitted to any conformational change. The area of the enzyme involved in the immobilization may be also critical to maximize the stabilizing effect of the MCA.[22] Immobilization may also tune some other enzyme properties, such as activity, resistance to inhibition, selectivity or specificity.[17] Chemical modification of enzymes may be used to further improve enzyme stability (e.g., via chemical crosslinking),[24] and may be also used as another tool to alter enzyme catalytic features.[18] Moreover, as it has been revised, chemical modification or immobilization of enzymes may be designed to simplify or improve one another.[21]

Glutaraldehyde is the reagent that the present review is devoted to. It has been used in many instances as protein cross-linker, as an activator of supports, and as crosslinker of enzymes and supports.[25,26] In this review, we intend to give a wide vision of the prospects of this very interesting and versatile molecule in the design of biocatalysts.

2. Chemistry of glutaraldehyde

Glutaraldehyde is a bi-functional reagent with the capacity to polymerize.[27,28] Glutaraldehyde may react with different enzyme moieties, mainly involving primary amino groups of proteins, although it may eventually react with other groups (thiols, phenols, and imidazoles).[27-30] However, the exact structure of the main structures related to protein crosslinking or enzyme immobilization is still not fully clarified. Figure 1 shows some of these proposed structures. It is clear that the structure of the glutaraldehyde relevant for the modification of enzymes and supports is not a linear one, but some kind of fairly stable cycles (activated support may be washed with an excess of distilled water without losing glutaraldehyde moieties).

The reaction mechanism of glutaraldehyde with proteins implies that it is not limited to just one mechanism. This is because the main reactive species of glutaraldehyde are found in equilibrium between their monomeric and polymeric conformations.[27,28,31,32] Moreover, every structure can react in a different way with the protein. For instance, under both acidic and neutral conditions, aldehyde groups from glutaraldehyde can react with proteins by formation of Schiff bases. In this case, a nucleophilic attack takes place by the ε-amino group from lysine to glutaraldehyde (See Figure 2a). However, Schiff bases are unstable at acidic conditions and are broken up, regenerating both the aldehyde and the amine groups. For this reason, several procedures have recommended reduction by $NaBH_4$ or $NaBCNH_3$ as a final step in order to stabilize the Schiff base into a secondary amine. Nevertheless, some studies suggest that protein preparations treated with a reducing agents does not cause an striking increase in the enzyme stability.[33] Additionally, lysine residues of protein treated with glutaraldehyde without further chemical reduction are not regenerated by incubation in HCl 6M at 110 ºC by 24 h.[34] Consequently, it is possible that a mechanism of formation of Schiff bases between proteins and glutaraldehyde could not be carried out. In this sense, an additional reduction step would not be necessary in order to stabilize the reaction product.[27,28,35] Therefore, the mechanism of glutaraldehyde at neutral and acidic conditions would be mediated by hemiacetal cyclic conformations from both the monomer and polymer of glutaraldehyde. This cyclic hemiacetal of glutaraldehyde reacts by nucleophilic substitution of the amino groups from lysines with the hydroxy group of glutaraldehyde according to the Figure 2.

On the other hand, under basic conditions it has been proposed that glutaraldehyde quickly suffers intramolecular aldolic condensations, producing a polymeric form of an a α,β-unsaturated aldehyde, which may react with amino groups from proteins through two mechanisms: Firstly by formation of Schiff bases between internal aldehyde groups from the polymeric form of glutaraldehyde and primary amino groups from the protein (Figure 3, 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.

3. Glutaraldehyde as protein crosslinker

The first use of glutaraldehyde was to preserve and fix tissues[41,42] in some instances combined with formaldehyde.[25] This is achieved through the formation of intermolecular crosslinking. Nowadays, glutaraldehyde remains as one of the most potent crosslinker reagents, even with clinical applications.[43-45] In part, these very good crosslinker features are a consequence of the capacity of glutaraldehyde to react with itself or with protein groups already modified with a glutaraldehyde molecule.[28]

The protein crosslinkings may be among groups placed on different protein molecules (intermolecular crosslinking) or between groups placed in the same molecule (intramolecular crosslinkings).[24,46] Both kinds of crosslinkings have interest in specific cases.

3.1- Glutaraldehyde as intermolecular crosslinker of proteins

As previously commented, this was the first use of glutaraldehyde, using its crosslinker potential to fix tissues.[41] However, in this review we will focus on the use of glutaraldehyde in the design of biocatalysts.

The addition of glutaraldehyde to a protein solution may produce the chemical aggregation of the enzyme, causing protein molecules to react among themselves, and can directly yield a "solid biocatalyst" (Figure 4). Although this immobilization strategy has not been widely used, it is possible to find diverse examples in literature. For example, insoluble trypsin was prepared by the use of glutaraldehyde to produce intermolecular crosslinks and the insoluble trypsin thus prepared exhibited enzymatic activity toward casein.[47] Later, glutaraldehyde was reacted in aqueous solutions with papain to form a water-insoluble product with enzymatic activity after activation by reducing agents. A rapid reaction of glutaraldehyde with the essential sulfhydryl of papain was not involved in the reaction since after activation the insoluble enzyme retained esterolytic and proteolytic activity.[48]

In another example, a purified preparation of extracellular alkaline proteinase of *Trichoderma koningii* was insolubilized by intermolecular crosslinking with glutaraldehyde.[49] The optimum operational temperature of the insolubilized enzyme increased by 20 ºC. The immobilized enzyme was also relatively more stable and activity was less depended on the presence of ions or detergents than the soluble enzyme. More surprisingly, an enhanced affinity to casein, hemoglobin and bovine serum albumin was found, although with a lower V max values.

Intermolecular cross-linking of the protease stem bromelain with 0.25 and 1.25% glutaraldehyde results in the formation of a large molecular mass, multimeric and soluble aggregate having comparable activity to the unmodified bromelain. Both 0.25 and 1.25% glutaraldehyde cross-linked bromelain preparations were more stable against urea, guanidine hydrochloride and temperature-induced inactivation, and exhibited slightly better storage stability compared to the unmodified protease. Such a high molecular weight, soluble, active and stable preparation may be useful in industry, i.e. in the textile industry for improving the properties of a fabric without loss of fabric strength and shape.[50]

On the other hand, glutaraldehyde is the intermolecular crosslinker of some of the recent carrier-free immobilization protocols, such as crosslinked enzyme crystals (CLECs)[51,52] (Figure 5) or aggregates (CLEAs) (Figure 6).[53-56] While using a conventional homo-bi-functional reagent the ideal strategy seems to modify around 50% of the reactive groups of the protein to maximize the possibility of crosslinking, the chemistry of glutaraldehyde causes this option not to be the optimal one, as amino/glutaraldehyde moieties reacts better with other amino/glutaraldehyde groups than one amino group.[33] Moreover, amino/glutaraldehyde/glutaraldehyde did not react easily with other amino/ $(glutaraldehyde)_2$ groups, making it inconvenient to excessively modify the proteins. Thus, to reach protein crosslinking using glutaraldehyde, it seems adequate to use moderate concentrations of 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]

- *Competition between one-point chemical modification and crosslinking.* The first one-point chemical modification involves a soluble reactive that must modify a group placed in the surface of a protein. In principle, this one-point modification may be very rapid. However, the crosslinking reaction involves the correct alignment of two groups located on a moderately rigid surface, such as that of an enzyme. Using other homo-bifunctional crosslinkers, this competition may produce serious hindrances to the promotion of an intense crosslinking in the enzyme surface, using glutaraldehyde, as stated above, there is not a real competition between one-point modification and crosslinking, as the best solution is an activation of all amines in the protein with just one glutaraldehyde group. However, there is no guarantee that the modified amino groups participate in crosslinking. In the best scenario, the final effect of the glutaraldehyde treatment will be a mixture of crosslinking and chemical modification. The first one should produce a rigidification of the enzyme surface; the second may have unpredictable effects on the enzyme features. Some times these effects may be very negative, even overcoming the positive effects of the crosslinking bridges. Some times, it may be very positive, increasing enzyme stability even without the formation of any crosslinking.[81,82] This effect of the one-point chemical modification needs to be considered when explaining the effects of the glutaraldehyde treatment on the stability of a particular enzyme.

- *Competition between inter and intra molecular crosslinking.* Another possibility is the competition between intra and intermolecular crosslinking. In fact, using free enzyme, to just have the desired intramolecular crosslinking may be nearly impossible, as inter molecular crosslinking is a faster reaction. Although this may also produce enzyme stabilization (see above), it may complicate the understanding of the results, and also make reproducibility complex, because the intermolecular crosslinking will depend on the protein concentration, kind and percentage of contaminant proteins, etc. The use of simple analytical techniques applied to glutaraldehyde treated samples after reduction with borohydride, such as SDS-PAGE, may permit to visualize the presence and extension of the intermolecular crosslinking.

Even when using immobilized enzymes, to fully prevent enzyme crosslinking may be hard. Using porous supports, if the immobilization rate is much higher than the diffusion rate, the enzymes may be packed so near each other that the enzyme molecules may be crosslinked with each other even using short reagents like glutaraldehyde (Figure 12). If the immobilization rate is slow enough, the distance between enzyme molecules may be enough to almost fully prevent enzyme intermolecular crosslinking.[83] Recently, by changing the immobilization conditions, a lipase has been immobilized on octyl agarose and treated with glutaraldehyde.[84] Under one immobilization condition, immobilization was quite slow (in presence of ethanol) and intermolecular crosslinking was almost negligible and it was possible to analyze the effect of intramolecular modifications. Under another condition, immobilization rate was vey high and the enzyme molecules were so near that both modifications could be achieved. The comparison between both derivatives (one having only intramolecular modifications, the other having both intramolecular and intermolecular modifications) permitted to determine the positive effects of the intermolecular crosslinking in the enzyme stability.

3.3. Crosslinking of multimeric enzymes.

Many enzymes are formed by several subunits, which are in association/dissociation equilibrium, usually being the associated form the most active and stable.[85-87] In other cases, some cascade reactions may require the joint action of several different enzymes that require their being associated.[88-90] If the researcher is able to crosslink these structures, the system will work in a more adequate fashion. However, the difficulties commented for the intramolecular crosslinking remain valid here, even with one additional key point: the reactive groups at the crosslinking distance need to be placed in different enzyme subunits (Figure 13).[23] This is why crosslinking of these structures may be achieved in a better way using polymers, as they should not confer an intense structural rigidity, but may be useful to keep together the different enzyme subunits or maintain the multi enzymatic complex assembled (e.g., dextran aldehyde,[91]

polyehtylenimine[92,93]). Nevertheless, glutaraldehyde has been used in certain cases to stabilize, even though only in a small percentage of the total enzyme structures, the subunit-subunit interactions. For example, the crosslinking of the hetero-oligomeric glucose dehydrogenase from a moderate thermophilic bacterium, SM4, using glutaraldehyde as crosslinking reagent has been reported.[94] The treatment permitted glucose dehydrogenase to gain high thermal stability without loss of its catalytic activity and thus increase the activity of the enzyme at high temperature. The authors concluded that by chemical cross-linking of the subunits, the dissociation of alpha and beta subunits was prevented. Consequently, its quaternary structure was stabilized, and thus the thermal stability of the glucose dehydrogenase was enhanced. Also, D-Lactate dehydrogenase from *Limulus polyphemus* is a homodimer which is composed of identical subunits that has been crosslinked with glutaraldehyde to show a relation of reactivation with reassociation of the dimer.[95]

In another paper, primase/helicase produced by bacteriophage T7 that can form both hexamers and heptamers. These oligomers were stabilized via cross-linking with glutaraldehyde and purified.[96] The authors detected how the percentage of each oligomeric form could be altered by the presence of either dTTP or β,γ-methylene. Heptamers are unable to efficiently bind either single-stranded DNA or double-stranded DNA, thus the authors postulated that a switch between heptamer to hexamer may provide a ring-opening mechanism for the single-stranded DNA binding pathway.

NTPDase1 and NTPDase2 enzymes from rats were expressed in *Xenopus laevis* oocytes and their quarternary structure was analyzed.[97] The treatment with glutaraldehyde permitted to detect that native NTPD-ase1 and NTPD-ase2 occur in oligomeric form. Dynamic alterations in oligomeric state may induce changes in substrate preference and thus influence the pattern of *in situ* extracellular nucleotide degradation.

In some instances, glutaraldehyde may have an indirect use to stabilize a multimeric enzyme, by crosslinking a polymer to the enzyme surface avoiding the polymer release. The

coating of the surface of multimeric enzymes with a poly-ionic polymer that may simultaneously interact with several enzyme subunits, preventing enzyme dissociation has proved to prevent enzyme subunit dissociation if the polymer can become adsorbed on the enzyme (Figure 14).[93] This strategy has been recently utilized to stabilize the enzyme glutamate dehydrogenase from *Thermus thermophilus* and formate dehydrogenase from *Pseudomonas* sp*.,* using polyethyleneimine as the "crosslinking" polymer. Both enzymes were inactivated by dissociation at acidic pH value, and the coating of their surface with polyethyleneimine prevented this (Figure 14). However, the reversible nature of the polymer adsorption permitted the polymer to become desorbed under certain conditions (e.g., high ionic strength, drastic pH value), and the protective effect of the polymer coating was lost. This problem was solved by a further treatment with glutaraldehyde, crosslinking the enzyme and the polymer: the new composite was fully stable under enzyme dissociation conditions. This composite could be used in soluble form, or ionically exchanged on a cation exchanger.[93] When a similar strategy was applied to the hexameric glutamate dehydrogenase from *E. coli*, the polymer protection was successful, but the enzyme was rapidly inactivated by incubation with glutaraldehyde.[92] Thus, this strategy not only requires that the polyethyleneimine may stabilize the multimeric structure of the enzyme without inactivating it, but also requires that the enzyme was resistant to the treatment with glutaraldehyde.

4. Intermolecular crosslinking of immobilized proteins via reversible immobilization using supports unreactive towards glutaraldehyde

Enzyme immobilization by physical adsorption (using ion exchange, immobilized chelates or hydrophobic adsorption) is the simplest protocol to immobilize enzymes, does not require enzyme nor support treatment, immobilization is rapid, and the support does not require special storage conditions.[14] However, this enzyme immobilization strategy has two 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

where individual molecules were fully desorbed, now the enzyme remains fully adsorbed on the support.[84]

As in certain cases the reactivity of the support with the glutaraldehyde cannot be fully discarded due to its unknown nature, the lack of glutaraldehyde reaction with the support may be verified via different strategies. For example, it is possible to check if we can "preactivate" the support, to achieve a covalent attachment between the enzyme molecules and the support after washing the support with an excess of water to eliminate all free glutaraldehyde molecules and later offering the enzyme. The use of Schiff reactive may also help to verify if some reactive glutaraldehyde remain bound to the support after the washings. Second, slowing down the immobilization rate of the enzyme to increase the distance between enzyme molecules, and checking if the glutaraldehyde treatment still avoids desorption of a significant percentage of the enzyme molecules.

One drawback of this strategy is that desorption of very large chemical aggregates may be fully impossible even under the most drastic conditions, transforming a reversible immobilization method in an irreversible one. The researcher should carefully evaluate the convenience of this treatment, considering the stability requirements of the enzyme and the real risks of enzyme desorption during the use of the biocatalyst versus the possibility of having to discard the support after enzyme immobilization.[14]

This strategy has been used in several examples to prepare crosslinked enzyme aggregates in an immobilized form, that some authors call CLEAs (see above), but which really are an altogether different thing. Hierarchically ordered mesocellular mesoporous carbon was used as a host for enzyme immobilization.[100] To improve the retention of enzymes, the adsorbed enzymes were cross-linked using glutaraldehyde. The resulting preparation showed a significant stabilization with high enzyme loadings. For example, 0.5 g chymotrypsin could be loaded in 1 g of silica with no activity decrease observed with rigorous shaking over one month. In contrast, 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

molecule, we will have the situation where the highest prospects of enzyme support reaction may occur. And this reaction will take place in a relative wide range of pH value, as the reaction will occur between amino/glutaraldehyde moieties; although considering the stability of the groups and its reactivity, some reports suggest that pH values around 8.5 may be the most adequate.[33] That way, the support will behave as a large multi-crosslinking reagent, fixing the positions of all the protein groups involved in the reaction with the support, whose relative position must remain unaltered under any distorting condition, promoting an increase of the overall enzyme structure, which becomes more intense when a more intense multipoint covalent attachment is achieved.[17,109-111]

Thus, this strategy has some positive points, as the good reactivity of the amino glutaraldehyde moieties with similar groups in a relatively wide range of pHs, that include neutral pH, the fact that the glutaraldehyde treatment is performed on a previously immobilized enzyme, and the possibilities of having some positive inter or intra-modifications. This last point is also the drawback of the strategy, as the enzyme should be fully modified, not only in the groups involved in the immobilization, and in some cases these modifications may be negative for enzyme stability or activity. However, in many instances when there is a comparison between immobilization on preactivated supports (see below) or treatment with glutaraldehyde after enzyme in exchange, stabilization improves using this strategy.[108,112,113]

In other many cases, a comparison was not performed but results using the treatment of previously adsorbed enzymes were very positive regarding stabilization and prevention of enzyme leakage.[114-119]

The treatment of lipases adsorbed on anion exchanger supports has been in some cases used to modulate enzyme properties. Thus, the open form of some lipases was fixed by glutaraldehyde treatment of adsorbed lipases in the presence of detergents.[120] In other cases, the random chemical modification was enabled to modulate the lipase selectivity or specificity.[121]

This strategy may be only used when the support is able to immobilize the protein via ionic exchange. That means that the support should present a high enough number of ionic groups to permit the adsorption of the enzyme on the support. Moreover, the enzyme should have the capacity to become adsorbed to anion exchangers. Considering that this is a multipoint process (several ion bridges need to be produced to fix the enzyme to the support),[122-124] the strategy presents the problem that the support should never be physically inert. Furthermore, the inertness of the support surface may be in many instances a desired feature of the support, to prevent uncontrolled support-enzyme interactions that may affect enzyme stability (sometimes in a positive sense, but in another may have a negative impact in the enzyme stability).[14,125] Another negative point to be considered is the possibility of reactivating the enzyme after inactivation, the folding-unfolding strategy may not work if the unfolded enzyme may interact with the support.[14]

Moreover, the strategy does not offer a large versatility. It may be possible that the immobilization via ionic exchange under different conditions of pH and ionic strength may involve different areas, but that is the only way of altering the enzyme orientation regarding the support.[126-128] The immobilization of an enzyme via different areas may be interesting to reach optimum stabilization (involving the most labile area of the protein in the immobilization)[22,129-132] or to tune the enzyme catalytic properties (selectivity, specificity, activity).[17]

6. Activation of supports with glutaraldehyde

In this last case, the support is pre-activated with glutaraldehyde. Following the results from Monsan, the optimal activation regarding the chemical reactivity of the support should be two molecules of glutaraldehyde per amino group in the support (obtained, e.g., by incubating the support in 1 M glutaraldehyde at pH 7 by 12-16 h).[37] It is very likely the most widespread form of using glutaraldehyde to immobilize enzymes (see for example[133-141]) and one of the first papers concerning the use of glutaraldehyde to prepare immobilize enzymes may be found in .[142]

Compared to the strategy described above, this method has some advantages and drawbacks. As a drawback, we can consider the higher difficulty on having an intense multipoint covalent attachment, as now the reaction is between amino/glutaraldehyde/glutaraldehyde moieties and the ε amino groups of the Lys residues (and the terminal amino group). The Lys groups have a pK of 10.7, and the stability of the glutaraldehyde groups in the support is not good at alkaline pH value while at neutral pH value the reactivity of the amino groups is relatively low. At neutral pH value, the most reactive amino group is the terminal one, with a pK value between 7 and 8, being much more reactive that the addition of the reactivities of all Lys external groups.[143] However, it should be considered that after the first immobilization, if some nucleophiles of the protein are in the area exposed to the support, the high apparent concentration of the different groups may permit the establishment of some new covalent enzyme-support bonds.[144] Moreover, the preactivated support may not be stored for a long time under wet conditions due to the relatively low stability of the groups.

The first advantage compared to the case described in Section 5, is that now only the groups involved in the covalent immobilization are modified by glutaraldehyde. Moreover, the support may immobilize enzymes even if they are very poorly activated, because the enzyme is covalently fixed to the support just with one point as the glutaraldehyde-protein bonds are stable. Thus, using very lowly activated amino supports (e.g., 1 reactive group under each projected area of the protein) immobilization using glutaraldehyde activated supports will be directly performed by a covalent reaction by the most reactive amino group on the enzyme (very likely the amino group of the enzyme if immobilization is performed at neutral pH value).[143] However, immobilization will be very slow due to the low activation of the support, and will offer no chance of reaching an intense multipoint covalent attachment.[14]

On the other hand, using highly activated supports; the features of the spacer arm (amino/glutaraldehyde/glutaraldehyde) convert the supports in a heterofunctional support. The 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

projected area of the enzyme), this multi-interaction will no longer be possible or will be so slow, that the only relevant cause of immobilization will be the one point covalent immobilization, as explained above.[143,148-153]

This means that even if all enzyme molecules are immobilized (incorporated to the support) in a very rapid fashion using a highly activated glutaraldehyde support, there is no guarantee that covalent immobilization of the enzyme on the support has taken place. In fact, the immobilization may be very strong, and the enzyme may remain immobilized under conditions where the enzyme may be fully released from the mother amino support, and still the enzyme molecules may be just adsorbed, as now the adsorption may be mixed, ionic/hydrophobic.[84] To ensure that covalent immobilization has taken place, the enzyme should remain immobilized under conditions where both ionic and hydrophobic interactions may be broken (e.g., using cationic detergents, using guanidine, increasing the ionic strength in the presence of non-ionic detergents).[84]

Using highly activated supports, it has been shown that in most cases an ionic exchange with the amino groups in the support is the first step in the immobilization of most enzymes.[22,38,84,154-156]

One effect of this first ionic adsorption is that, even though glutaraldehyde is able to immobilize enzymes via just one attachment due to the stability of the bond formed, the activation degree of the support has an exponential effect on the immobilization reaction rate of proteins, not the expected linear effect using reactive groups in the support able to immobilize the proteins via just one point.[143] This is because the researcher is measuring the rate of ionic exchange of the enzyme on the support, which requires the establishment of several enzyme-support interactions, and thus it is exponentially dependent on the surface density of amino groups on the support although the covalent reaction should be of order 1.[38,148-153] This 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-

support covalent bonds and different enzyme-support unspecific interactions.[147] We should bear in mind that, due to the proximity between the groups of the support and of the enzyme, interactions between immobilized enzyme and support will be produced over time even though they may not be enough to be the only cause for immobilization.[144]

In other, more difficult to classify cases, a lipase was immobilized on electrospun and ethanol-dispersed polystyrene-poly(styrene-co-maleic anhydride) nanofibers in the form of enzyme precipitate coatings.[172] Lipase precipitate coatings were prepared in a three-step process, consisting of lipase covalent attachment, lipase precipitation, and crosslinking of precipitated lipases onto the covalently attached lipases via glutaraldehyde treatment.

A similar approach was used to immobilize β-glucosidase. The enzyme was immobilized on polymer nanofibers. Then, additional enzyme molecules were crosslinked onto the covalently attached enzyme molecules via glutaraldehyde treatment.[173]

Conclusions

Glutaraldehyde, an apparently old fashioned reagent use for a long time in design of biocatalyst, remains as one of the most interesting tools in enzyme crosslinking and immobilization (Figure 25). However, to achieve optimal results, it is necessary to understand the different reactivities of the amino/glutaraldehyde and amino/glutaraldehyde/glutaraldehyde, together with the fact that, in immobilization of enzymes on glutaraldehyde pre-activated supports, it is a heterofunctional support that permits altering the enzyme orientation on the support surface. Thus, glutaraldehyde remains one of the most potent and versatile tools in Enzyme technology, and the new knowledge on its reactivity and possibilities open even new opportunities for the future.

ACKNOWLEDGMENTS

726 This work has been supported by grant CTQ2009-07568 from Spanish Ministerio de Ciencia e
727 Innovacion, Grant No.1102-489-25428 from COLCIENCIAS and Universidad Industrial de
728 Santander (VIE-UIS Research Program) and CNPq and FAPERGS (Brazil). Á. Berenguer-
729 Murcia thanks the Spanish Ministerio de Ciencia e Innovacion for a Ramon y Cajal fellowship
730 (RyC-2009-03813). The authors would like to thank Mr Ramiro Martinez (Novozymes, Spain
731 S.A) for his kind support to our research.
732

References

1. A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts and B. Witholt, *Nature*, 2001, **409**, 258-268.
2. S. G. Burton, D. A. Cowan and J. M. Woodley, *Nat. Biotechnol.*, 2002, **20**, 37-45.
3. J. M. Woodley, *Trends Biotechnol.*, 2008, **26**, 321-327.
4. A. Liljeblad and L. T. Kanerva, *Tetrahedron*, 2006, **62**, 5831-5854.
5. S. Panke, M. Held and M. Wubbolts, *Curr. Opin. Biotechnol.*, 2004, **15**, 272-279.
6. H. E. Schoemaker, D. Mink and M. G. WubboLts, *Science*, 2003, **299**, 1694-1697.
7. F. H. Arnold, *Nature*, 2001, **409**, 253-257.
8. F. H. Arnold, *Chem. Eng. Sci.*, 1996, **51**, 5091-5102.
9. J. M. Woodley, *Curr. Opin. Chem. Biol.*, 2013, **17**, 310-316.
10. A. R. Fersht and L. Serrano, *Curr. Opin. Struct. Biol.*, 1993, **3**, 75-83.
11. R. C. Rodrigues, C. Ortiz, A. Berenguer-Murcia, R. Torres and R. Fernandez-Lafuente, *Chem. Soc. Rev.*, 2013, **45**, 6290-6307.
12. M. L. Verma, C. J. Barrow and M. Puri, *Appl. Microbiol. Biotechnol.*, 2013, **97**, 23-39.
13. E. T. Hwang and M. B. Gu, *Eng. Life Sci.*, 2013, **13**, 49-61.
14. C. Garcia-Galan, A. Berenguer-Murcia, R. Fernandez-Lafuente and R. C. Rodrigues, *Adv. Synth. Catal.*, 2011, **353**, 2885-2904.
15. D. Brady and J. Jordaan, *Biotechnol. Lett.*, 2009, **31**, 1639-1650.
16. P. V. Iyer and L. Ananthanarayan, *Process Biochem.*, 2008, **43**, 1019-1032.
17. C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2007, **40**, 1451-1463.
18. A. Díaz-Rodríguez and B. G. Davis, *Curr. Opin. Chem. Biol.*, 2011, **15**, 211-219.
19. J. M. Chalker, G. J. L. Bernardes, Y. A. Lin and B. G. Davis, *Chem.-Asian J.*, 2009, **4**, 630-640.
20. D. A. Cowan and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2011, **49**, 326-346.
21. R. C. Rodrigues, Á. Berenguer-Murcia and R. Fernandez-Lafuente, *Adv. Synth. Catal.*, 2011, **353**, 2216-2238.
22. K. Hernandez and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2011, **48**, 107-122.
23. R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2009, **45**, 405-418.
24. S. S. Wong and L. J. C. Wong, *Enzyme Microb. Technol.*, 1992, **14**, 866-874.
25. E. A. Woodroof, *J. Bioeng.*, 1978, **2**, 1-9.
26. A. D. Russell, *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America*, 1994, **15**, 724-733.
27. I. Migneault, C. Dartiguenave, M. J. Bertrand and K. C. Waldron, *Biotechniques*, 2004, **37**, 790-802.
28. D. R. Walt and V. I. Agayn, *TrAC, Trends Anal. Chem.*, 1994, **13**, 425-430.
29. A. F. S. A. Habeeb and R. Hiramoto, *Arch. Biochem. Biophys.*, 1968, **126**, 16-26.
30. Y. Wine, N. Cohen-Hadar, A. Freeman and F. Frolow, *Biotechnol. Bioeng.*, 2007, **98**, 711-718.
31. C. Aso and Y. Aito, *Die Makromolekulare Chemie*, 1962, **58**, 195-203.
32. F. M. Richards and J. R. Knowles, *J. Mol. Biol.*, 1968, **37**, 231-233.
33. R. Fernandez-Lafuente, C. M. Rosell, V. Rodriguez and J. M. Guisan, *Enzyme Microb. Technol.*, 1995, **17**, 517-523.
34. A. H. Korn, S. H. Feairheller and E. M. Filachoine, *J. Mol. Biol.*, 1972, **65**, 525-529.
35. T. J. Johnson, *Eur. J. Cell Biol.*, 1978, **45**, 160-169.
36. P. Monsan, G. Puzo and H. Mazarguil, *Biochimie*, 1975, **57**, 1281-1292.
37. P. Monsan, *J. Mol. Catal.*, 1978, **3**, 371-384.

38. L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, C. Mateo, R. Fernández-Lafuente and J. M. Guisán, *Enzyme Microb. Technol.*, 2006, **39**, 877-882.
39. K. Ziegler, I. Schits and H. Zahn, in *Protein Crosslinking: Biochemical and Biological Aspects*, ed. M. Friedman, Plenum, New York, 1977, vol. 86a, pp. 345-354.
40. T. Imoto and H. Yamada, in *Protein Function: A Practical Approach*, ed. T. E. Creighton, IRL Press, Oxford, UK, 1989, pp. 247–278.
41. D. T. Cheung, N. Perelman, E. C. Ko and M. E. Nimni, *Connect. Tissue Res.*, 1985, **13**, 109-115.
42. E. A. Talman and D. R. Boughner, *Ann. Thorac. Surg.*, 1995, **60**, S369-S373.
43. S. Mathapati, D. K. Bishi, S. Guhathakurta, K. M. Cherian, J. R. Venugopal, S. Ramakrishna and R. S. Verma, *Mater. Sci. Eng., C*, 2013, **33**, 1561-1572.
44. Y. Liu, L. Ma and C. Gao, *Mater. Sci. Eng., C*, 2012, **32**, 2361-2366.
45. P. Sinha, D. Zurakowski, T. K. Susheel Kumar, D. He, C. Rossi and R. A. Jonas, *J. Thorac. Cardiovasc. Surg.*, 2012, **143**, 224-227.
46. C. P. Govardhan, *Curr. Opin. Biotechnol.*, 1999, **10**, 331-335.
47. A. F. S. A. Habeeb, *Arch. Biochem. Biophys.*, 1967, **119**, 264-268.
48. E. F. Jansen and A. C. Olson, *Arch. Biochem. Biophys.*, 1969, **129**, 221-227.
49. H. K. Manonmani and R. Joseph, *Process Biochem.*, 1993, **28**, 325-329.
50. T. Anwar, B. Ahmad and H. Younus, *Biocatal. Biotransform.*, 2007, **25**, 453-458.
51. F. A. Quiocho and F. M. Richards, *Proc. Natl. Acad. Sci. U. S. A.*, 1964, **52**, 833-839.
52. N. L. St. Clair and M. A. Navia, *J. Am. Chem. Soc.*, 1992, **114**, 7314-7316.
53. L. Cao, F. Van Rantwijk and R. A. Sheldon, *Org. Lett.*, 2000, **2**, 1361-1364.
54. R. A. Sheldon, *Adv. Synth. Catal.*, 2007, **349**, 1289-1307.
55. R. A. Sheldon, R. Schoevaart and L. M. Van Langen, *Biocatal. Biotransform.*, 2005, **23**, 141-147.
56. R. A. Sheldon, *Appl. Microbiol. Biotechnol.*, 2011, **92**, 467-477.
57. C. Mateo, J. M. Palomo, L. M. Van Langen, F. Van Rantwijk and R. A. Sheldon, *Biotechnol. Bioeng.*, 2004, **86**, 273-276.
58. S. Shah, A. Sharma and M. N. Gupta, *Anal. Biochem.*, 2006, **351**, 207-213.
59. I. Matijošyte, I. W. C. E. Arends, S. de Vries and R. A. Sheldon, *J. Mol. Catal. B: Enzym.*, 2010, **62**, 142-148.
60. F. Šulek, D. P. Fernández, Z. Knez, M. Habulin and R. A. Sheldon, *Process Biochem.*, 2011, **46**, 765-769.
61. T. Dong, L. Zhao, Y. Huang and X. Tan, *Bioresour. Technol.*, 2010, **101**, 6569-6571.
62. J. J. Karimpil, J. S. Melo and S. F. D'Souza, *J. Mol. Catal. B: Enzym.*, 2011, **71**, 113-118.
63. J. Cruz, O. Barbosa, R. C. Rodrigues, R. Fernandez-Lafuente, R. Torres and C. Ortiz, *J. Mol. Catal. B: Enzym.*, 2012, **80**, 7-14.
64. J. Pan, X. D. Kong, C. X. Li, Q. Ye, J. H. Xu and T. Imanaka, *J. Mol. Catal. B: Enzym.*, 2011, **68**, 256-261.
65. L. Wilson, G. Fernández-Lorente, R. Fernández-Lafuente, A. Illanes, J. M. Guisán and J. M. Palomo, *Enzyme Microb. Technol.*, 2006, **39**, 750-755.
66. F. López-Gallego, L. Betancor, A. Hidalgo, N. Alonso, R. Fernández-Lafuente and J. M. Guisán, *Biomacromolecules*, 2005, **6**, 1839-1842.
67. J. Yan, X. Gui, G. Wang and Y. Yan, *Appl. Biochem. Biotechnol.*, 2012, **166**, 925-932.
68. M. Galvis, O. Barbosa, M. Ruiz, J. Cruz, C. Ortiz, R. Torres and R. Fernandez-Lafuente, *Process Biochem.*, 2012, **47**, 2373-2378.
69. V. P. Torchilin and K. Martinek, *Enzyme Microb. Technol.*, 1979, **1**, 74-82.
70. A. E. Habibi, K. Khajeh, H. Naderi-Manesh, B. Ranjbar and M. Nemat-Gorgani, *J. Biotechnol.*, 2006, **123**, 434-442.

71. K. Martinek and V. P. Torchilin, in *Methods Enzymol.*, 1988, vol. 137, pp. 615-626.
72. V. P. Torchilin, A. V. Maksimenko, V. N. Smirnov, I. V. Berezin, A. M. Klibanov and K. Martinek, *Biochim. Biophys. Acta*, 1978, **522**, 277-283.
73. V. P. Torchilin, A. V. Maksimenko, V. N. Smirnov, I. V. Berezin and K. Martinek, *Biochim. Biophys. Acta, Enzymol.*, 1979, **568**, 1-10.
74. N. M. Young, C. R. MacKenzie, S. A. Narang, R. P. Oomen and J. E. Baenziger, *FEBS Lett.*, 1995, **377**, 135-139.
75. U. Arnold and M. Sch√∂pfel, *FEBS J.*, 2012, **279**, 2508-2519.
76. V. G. H. Eijsink, A. Bjork, S. Gaseidnes, R. Sirevag, B. Synstad, B. V. D. Burg and G. Vriend, *J. Biotechnol.*, 2004, **113**, 105-120.
77. V. Grazu, F. López-Gallego and J. M. Guisán, *Process Biochem.*, 2012, **47**, 2538-2541.
78. O. Abian, V. Grazú, J. Hermoso, R. González, J. L. García, R. Fernández-Lafuente and J. M. Guisán, *Appl. Environ. Microbiol.*, 2004, **70**, 1249-1251.
79. K. L. Carraway and D. E. Koshland Jr, *Methods Enzymol.*, 1972, **25**, 616-623.
80. K. L. Carraway, P. Spoerl and D. E. Koshland Jr, *J. Mol. Biol.*, 1969, **42**, 133-137.
81. V. P. Torchilin, A. V. Maksimenko, V. N. Smirnov, I. V. Berezin, A. M. Klibanov and K. Martinek, *Biochim. Biophys. Acta, Enzymol.*, 1979, **567**, 1-11.
82. R. Tyagi and M. N. Gupta, *Biochemistry (Mosc.)*, 1998, **63**, 334-344.
83. J. M. Bolivar, A. Hidalgo, L. Sánchez-Ruiloba, J. Berenguer, J. M. Guisán and F. López-Gallego, *J. Biotechnol.*, 2011, **155**, 412-420.
84. O. Barbosa, R. Torres, C. Ortiz and R. Fernandez-Lafuente, *Process Biochem.*, 2012, **47**, 1220-1227.
85. O. M. Poltorak, E. S. Chukhray and I. Y. Torshin, *Biochemistry (Mosc.)*, 1998, **63**, 303-311.
86. R. W. Lencki, J. Arul and R. J. Neufeld, *Biotechnol. Bioeng.*, 1992, **40**, 1427-1434.
87. O. M. Poltorak, E. S. Chukhrai and I. Y. Torshin, *Russ. J. Phys. Chem. A*, 2000, **74**, S400-S410.
88. A. J. D. Silva, D. P. Gómez-Mendoza, M. Junqueira, G. B. Domont, E. Ximenes Ferreira Filho, M. V. de Sousa and C. A. O. Ricart, *Proteomics*, 2012, **12**, 2729-2738.
89. T. Ohtsuki, Suyanto, S. Yazaki, S. Ui and A. Mimura, *Lett. Appl. Microbiol.*, 2005, **40**, 111-116.
90. H. Schagger, W. A. Cramer and G. Von Jagow, *Anal. Biochem.*, 1994, **217**, 220-230.
91. M. Fuentes, R. L. Segura, O. Abian, L. Betancor, A. Hidalgo, C. Mateo, R. Fernandez-Lafuente and J. M. Guisan, *Proteomics*, 2004, **4**, 2602-2607.
92. C. Garcia-Galan, O. Barbosa and R. Fernandez-Lafuente, *Enzyme Microb. Technol.*, 2013, **52**, 211-217.
93. J. M. Bolivar, J. Rocha-Martin, C. Mateo, F. Cava, J. Berenguer, R. Fernandez-Lafuente and J. M. Guisan, *Biomacromolecules*, 2009, **10**, 742-747.
94. T. Yamazaki, W. Tsugawa and K. Sode, *Biotechnol. Lett.*, 1999, **21**, 199-202.
95. M. Gerl, R. Rudolph and R. Jaenicke, *Biol. Chem. Hoppe. Seyler*, 1985, **366**, 447-454.
96. D. J. Crampton, M. Ohi, U. Qimron, T. Walz and C. C. Richardson, *J. Mol. Biol.*, 2006, **360**, 667-677.
97. B. U. Failer, A. Aschrafi, G. Schmalzing and H. Zimmermann, *Eur. J. Biochem.*, 2003, **270**, 1802-1809.
98. R. A. Sheldon and S. Van Pelt, *Chem. Soc. Rev.*, 2013, **42**, 6223-6235.
99. P. Jochems, Y. Satyawali, L. Diels and W. Dejonghe, *Green Chem.*, 2011, **13**, 1609-1623.
100. J. Lee, J. Kim, H. Jia, M. I. Kim, J. H. Kwak, S. Jin, A. Dohnalkova, H. C. Park, H. N. Chang, P. Wang, J. W. Grate and T. Hyeon, *Small*, 2005, **1**, 744-753.
101. M. I. Kim, J. Kim, J. Lee, S. Shin, H. B. Na, T. Hyeon, H. G. Park and H. N. Chang, *Microporous Mesoporous Mater.*, 2008, **111**, 18-23.

102. I. K. Moon, J. Kim, J. Lee, H. Jia, B. N. Hyon, K. Y. Jong, H. K. Ja, A. Dohnalkova, J. W. Grate, P. Wang, T. Hyeon, G. P. Hyun and N. C. Ho, *Biotechnol. Bioeng.*, 2007, **96**, 210-218.
103. S. H. Jun, J. Lee, B. C. Kim, J. E. Lee, J. Joo, H. Park, J. H. Lee, S. M. Lee, D. Lee, S. Kim, Y. M. Koo, C. H. Shin, S. W. Kim, T. Hyeon and J. Kim, *Chem. Mater.*, 2012, **24**, 924-929.
104. R. Reshmi and S. Sugunan, *J. Mol. Catal. B: Enzym.*, 2013, **85-86**, 111-118.
105. D. Jung, M. Paradiso, D. Wallacher, A. Brandt and M. Hartmann, *ChemSusChem*, 2009, **2**, 161-164.
106. D. Jung, M. Paradiso and M. Hartmann, *J. Mater. Sci.*, 2009, **44**, 6747-6753.
107. D. Jung and M. Hartmann, *Catal. Today*, 2010, **157**, 378-383.
108. F. López-Gallego, L. Betancor, C. Mateo, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, J. M. Guisán and R. Fernández-Lafuente, *J. Biotechnol.*, 2005, **119**, 70-75.
109. A. S. Bommarius and M. F. Paye, *Chem. Soc. Rev.*, 2013, **42**, 6534-6565.
110. U. Hanefeld, L. Cao and E. Magner, *Chem. Soc. Rev.*, 2013, **42**, 6211-6212.
111. S. Cantone, V. Ferrario, L. Corici, C. Ebert, D. Fattor, P. Spizzo and L. Gardossi, *Chem. Soc. Rev.*, 2013, **42**, 6262-6276.
112. L. Betancor, F. López-Gallego, A. Hidalgo, N. Alonso-Morales, G. Dellamora-Ortiz, J. M. Guisán and R. Fernández-Lafuente, *J. Biotechnol.*, 2006, **121**, 284-289.
113. M. Filho, B. C. Pessela, C. Mateo, A. V. Carrascosa, R. Fernandez-Lafuente and J. M. Guisán, *Enzyme Microb. Technol.*, 2008, **42**, 265-271.
114. D. Radva, J. Spanyol and J. Kosáry, *Food Technol. Biotechnol.*, 2011, **49**, 257-262.
115. C. Z. Guidini, J. Fischer, L. N. S. Santana, V. L. Cardoso and E. J. Ribeiro, *Biochem. Eng. J.*, 2010, **52**, 137-143.
116. C. Pan, B. Hu, W. Li, Y. Sun, H. Ye and X. Zeng, *J. Mol. Catal. B: Enzym.*, 2009, **61**, 208-215.
117. C. Sisak, Z. Csanádi, E. Rónay and B. Szajáni, *Enzyme Microb. Technol.*, 2006, **39**, 1002-1007.
118. A. Kumar and S. S. Kanwar, *Enzyme Research*, 2011, **2011**, 718949.
119. A. Kumar and S. S. Kanwar, *Bioresour. Technol.*, 2011, **102**, 2162-2167.
120. G. Fernández-Lorente, J. M. Palomo, C. Mateo, R. Munilla, C. Ortiz, Z. Cabrera, J. M. Guisán and R. Fernández-Lafuente, *Biomacromolecules*, 2006, **7**, 2610-2615.
121. J. M. Palomo, R. L. Segura, G. Fernandez-Lorente, R. Fernandez-Lafuente and J. M. Guisán, *Enzyme Microb. Technol.*, 2007, **40**, 704-707.
122. M. Filho, B. C. Pessela, C. Mateo, A. V. Carrascosa, R. Fernandez-Lafuente and J. M. Guisán, *Process Biochem.*, 2008, **43**, 1142-1146.
123. M. Fuentes, P. Batalla, V. Grazu, B. C. C. Pessela, C. Mateo, T. Montes, J. A. Hermoso, J. M. Guisan and R. Fernandez-Lafuente, *Biomacromolecules*, 2007, **8**, 703-707.
124. B. C. C. Pessela, R. Munilla, L. Betancor, M. Fuentes, A. V. Carrascosa, A. Vian, R. Fernandez-Lafuente and J. M. Guisán, *J. Chromatogr. A*, 2004, **1034**, 155-159.
125. C. Mateo, O. Abian, G. Fernández-Lorente, J. Pedroche, R. Fernández-Lafuente, J. M. Guisan, A. Tam and M. Daminati, *Biotechnol. Prog.*, 2002, **18**, 629-634.
126. B. C. C. Pessela, M. Fuentes, C. Mateo, R. Munilla, A. V. Carrascosa, R. Fernandez-Lafuente and J. M. Guisan, *Enzyme Microb. Technol.*, 2006, **39**, 909-915.
127. B. C. C. Pessela, L. Betancor, F. Lopez-Gallego, R. Torres, G. M. Dellamora-Ortiz, N. Alonso-Morales, M. Fuentes, R. Fernández-Lafuente, J. M. Guisán and C. Mateo, *Enzyme Microb. Technol.*, 2005, **37**, 295-299.
128. M. Fuentes, C. Mateo, B. C. C. Pessela, P. Batalla, R. Fernandez-Lafuente and J. M. Guisán, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2007, **849**, 243-250.
129. A. Schellenberger and R. Ulbrich, *Biomed. Biochim. Acta*, 1989, **48**, 63-67.

130. J. Mansfeld, G. Vriendl, O. R. Veltman, B. Van Den Burg, G. Venema, V. G. H. Eijsink and R. Utbrich-Hofmann, *FASEB J.*, 1997, **11**.
131. R. Ulbrich-Hofmann, U. Arnold and J. Mansfeld, *J. Mol. Catal. B: Enzym.*, 1999, **7**, 125-131.
132. J. Mansfeld, G. Vriend, B. Van Den Burg, V. G. H. Eijsink and R. Ulbrich-Hofmann, *Biochemistry*, 1999, **38**, 8240-8245.
133. S. Pahujani, S. S. Kanwar, G. Chauhan and R. Gupta, *Bioresour. Technol.*, 2008, **99**, 2566-2570.
134. A. Pal and F. Khanum, *Process Biochem.*, 2011, **46**, 1315-1322.
135. S. Nagar, A. Mittal, D. Kumar, L. Kumar and V. K. Gupta, *Process Biochem.*, 2012, **47**, 1402-1410.
136. T. N. Nwagu, B. N. Okolo and H. Aoyagi, *J. Microbiol. Biotechnol.*, 2012, **22**, 628-636.
137. G. Yang, J. Wu, G. Xu and L. Yang, *Colloids and Surfaces B: Biointerfaces*, 2010, **78**, 351-356.
138. M. Yiitolu and Z. Temo√ßin, *J. Mol. Catal. B: Enzym.*, 2010, **66**, 130-135.
139. M. P. Klein, L. P. Fallavena, J. D. N. Schöfer, M. A. Z. Ayub, R. C. Rodrigues, J. L. Ninow and P. F. Hertz, *Carbohydr. Polym.*, 2013, **95**, 465-470.
140. M. P. Klein, M. R. Nunes, R. C. Rodrigues, E. V. Benvenutti, T. M. H. Costa, P. F. Hertz and J. L. Ninow, *Biomacromolecules*, 2012, **13**, 2456-2464.
141. S. G. Valerio, J. S. Alves, M. P. Klein, R. C. Rodrigues and P. F. Hertz, *Carbohydr. Polym.*, 2013, **92**, 462-468.
142. W. L. Stanley, G. G. Watters, S. H. Kelly, B. G. Chan, J. A. Garibaldi and J. E. Schade, *Biotechnol. Bioeng.*, 1976, **18**, 439-443.
143. C. Mateo, O. Abian, M. Bernedo, E. Cuenca, M. Fuentes, G. Fernandez-Lorente, J. M. Palomo, V. Grazu, B. C. C. Pessela, C. Giacomini, G. Irazoqui, A. Villarino, K. Ovsejevi, F. Batista-Viera, R. Fernandez-Lafuente and J. M. Guisán, *Enzyme Microb. Technol.*, 2005, **37**, 456-462.
144. J. M. Bolivar, C. Mateo, C. Godoy, B. C. C. Pessela, D. S. Rodrigues, R. L. C. Giordano, R. Fernandez-Lafuente and J. M. Guisan, *Process Biochem.*, 2009, **44**, 757-763.
145. C. Mateo, G. Fernández-Lorente, O. Abian, R. Fernández-Lafuente and J. M. Guisán, *Biomacromolecules*, 2000, **1**, 739-745.
146. C. Mateo, V. Grazu, J. M. Palomo, F. Lopez-Gallego, R. Fernandez-Lafuente and J. M. Guisan, *Nat. Protoc.*, 2007, **2**, 1022-1033.
147. O. Barbosa, R. Torres, C. Ortiz, Á. Berenguer-Murcia, R. C. Rodrigues and R. Fernandez-Lafuente, *Biomacromolecules*, 2013, **in press**, DOI: 10.1021/bm400762h.
148. A. Kumar, I. Y. Galaev and B. Mattiasson, *J. Chromatogr. B Biomed. Sci. Appl.*, 2000, **741**, 103-113.
149. B. C. C. Pessela, R. Torres, M. Fuentes, C. Mateo, R. Munilla, A. Vian, A. V. Carrascosa, J. L. Garcia, J. M. Guisán and R. Fernandez-Lafuente, *J. Chromatogr. A*, 2004, **1055**, 93-98.
150. F. H. Arnold, *Biotechnology. (N. Y.)*, 1991, **9**, 151-156.
151. E. Hochuli, H. Döbeli and A. Schacher, *J. Chromatogr. A*, 1987, **411**, 177-184.
152. V. Gaberc-Porekar and V. Menart, *J. Biochem. Biophys. Methods*, 2001, **49**, 335-360.
153. M. Fuentes, C. Mateo, R. Fernández-Lafuente and J. M. Guisán, *Biomacromolecules*, 2006, **7**, 540-544.
154. W. S. Adriano, E. H. C. Filho, J. A. Silva, R. L. C. Giordano and L. R. B. Gonçalves, *Brazilian Journal of Chemical Engineering*, 2005, **22**, 529-538.
155. W. S. Adriano, E. H. C. Filho, J. A. Silva and L. R. B. Gonçalves, *Biotechnol. Appl. Biochem.*, 2005, **41**, 201-207.
156. W. S. Adriano, D. B. Mendonça, D. S. Rodrigues, E. J. Mammarella and R. L. C. Giordano, *Biomacromolecules*, 2008, **9**, 2170-2179.

157. K. E. Jaeger and T. Eggert, *Curr. Opin. Biotechnol.*, 2002, **13**, 390-397.
158. K. E. Jaeger and M. T. Reetz, *Trends Biotechnol.*, 1998, **16**, 396-403.
159. R. D. Schmid and R. Verger, *Angew. Chem., Int. Ed.*, 1998, **37**, 1609-1633.
160. R. Verger, *Trends Biotechnol.*, 1997, **15**, 32-38.
161. Z. S. Derewenda, U. Derewenda and G. G. Dodson, *J. Mol. Biol.*, 1992, **227**, 818-839.
162. N. Miled, F. Beisson, J. De Caro, A. De Caro, V. Arondel and R. Verger, *J. Mol. Catal. B: Enzym.*, 2001, **11**, 165-171.
163. P. Reis, K. Holmberg, H. Watzke, M. E. Leser and R. Miller, *Adv. Colloid Interface Sci.*, 2009, **147-148**, 237-250.
164. R. Fernandez-Lafuente, P. Armisén, P. Sabuquillo, G. Fernández-Lorente and J. M. Guisán, *Chem. Phys. Lipids*, 1998, **93**, 185-197.
165. J. M. Palomo, C. Ortiz, M. Fuentes, G. Fernandez-Lorente, J. M. Guisan and R. Fernandez-Lafuente, *J. Chromatogr. A*, 2004, **1038**, 267-273.
166. J. M. Palomo, M. M. Peñas, G. Fernández-Lorente, C. Mateo, A. G. Pisabarro, R. Fernández-Lafuente, L. Ramírez and J. M. Guisán, *Biomacromolecules*, 2003, **4**, 204-210.
167. J. M. Palomo, G. Muoz, G. Fernández-Lorente, C. Mateo, R. Fernández-Lafuente and J. M. Guisán, *J. Mol. Catal. B: Enzym.*, 2002, **19-20**, 279-286.
168. J. Uppenberg, S. Patkar, T. Bergfors and T. A. Jones, *J. Mol. Biol.*, 1994, **235**, 790-792.
169. C. Carrasco-López, C. Godoy, B. de las Rivas, G. Fernández-Lorente, J. M. Palomo, J. M. Guisán, R. Fernández-Lafuente, M. Martínez-Ripoll and J. A. Hermoso, *J. Biol. Chem.*, 2009, **284**, 4365-4372.
170. J. M. Palomo, M. Fuentes, G. Fernández-Lorente, C. Mateo, J. M. Guisan and R. Fernández-Lafuente, *Biomacromolecules*, 2003, **4**, 1-6.
171. G. Fernández-Lorente, J. M. Palomo, M. Fuentes, C. Mateo, J. M. Guisán and R. Fernández-Lafuente, *Biotechnol. Bioeng.*, 2003, **82**, 232-237.
172. H. J. An, H. J. Lee, S. H. Jun, S. Y. Hwang, B. C. Kim, K. Kim, K. M. Lee, M. K. Oh and J. Kim, *Bioprocess Biosyst. Eng.*, 2011, **34**, 841-847.
173. S. M. Lee, L. H. Jin, J. H. Kim, S. O. Han, H. B. Na, T. Hyeon, Y. M. Koo, J. Kim and J. H. Lee, *Bioprocess Biosyst. Eng.*, 2010, **33**, 141-147.

1017 **FIGURE LEGENDS**

1018

1019 **FIGURE 1.** Possible structures of glutaraldehyde in aqueous solution.

1020 **FIGURE 2.** Reactions of glutaraldehyde with proteins under acidic or neutral conditions.

1021 **FIGURE 3.** Schiff base (1) and Michael-type (2) reactions of glutaraldehyde with proteins
1022 under basic conditions

1023 **FIGURE 4.** Immobilization of enzyme via copolymerization with glutaraldehyde.

1024 **FIGURE 5.** Preparation of Crosslinked Enzyme Crystals (CLECs).

1025 **FIGURE 6.** Preparation of Crosslinked Enzyme Aggretates (CLEAs).

1026 **FIGURE 7**. Problems in the crosslinking step during CLEAs preparation using enzymes poor
1027 in Lys residues.

1028 **FIGURE 8**. Preparation of combi-CLEAs using proteins having many Lys residues to
1029 facilitate the crosslinking step.

1030 **FIGURE 9**. Amination of enzymes that are poor in Lys residues to facilitate the crosslinking
1031 step in CLEA preparation.

1032 **FIGURE 10**. Effect of intramolecular crosslinking on the stability of enzymes.

1033 **FIGURE 11**. Amination of enzyme surfaces to facilitate the promotion of an intense
1034 intramolecular crosslinking.

1035 **FIGURE 12**. Effect of the immobilization rate on the possibilities of intermolecular
1036 crosslinking between immobilized enzyme molecules.

1037 **FIGURE 13**. Chemical crosslinking of subunits in multimeric enzymes.

1038 **FIGURE 14**. Stabilization of polyethyleneimine/enzyme composites via glutaraldehyde
1039 crosslinking to prevent subunit dissociation.

1040 **FIGURE 15**. Production of enzyme chemical aggregates to prevent enzyme leakage on mildy
1041 adsorbed enzymes.

1042 **FIGURE 16**. Crosslinking of enzymes and aminated supports after enzyme ionic adsorption.

FIGURE 17. Multifunctional supports.

FIGURE 18. Effect of the superficial density of glutaraldehyde groups on the possibilities of physical adsorption of the enzyme on the support surface.

FIGURE 19. Interfacial activation of lipases.

FIGURE 20. Immobilization of lipases at low ionic strength on glutaraldehyde activated supports.

FIGURE 21. Immobilization of lipases at low ionic strength in the presence of a detergent on glutaraldehyde activated supports.

FIGURE 22. Immobilization of lipases at high enough ionic strength to prevent ionic exchange on glutaraldehyde activated supports.

FIGURE 23. Immobilization of lipases at very high ionic strength (more than 1 M) on glutaraldehyde activated supports.

FIGURE 24. Immobilization of lipases at moderate ionic strength and in the presence of detergents on glutaraldehyde activated supports.

FIGURE 25. Glutaraldehyde, a versatile reagent in enzyme biocatalyst design.

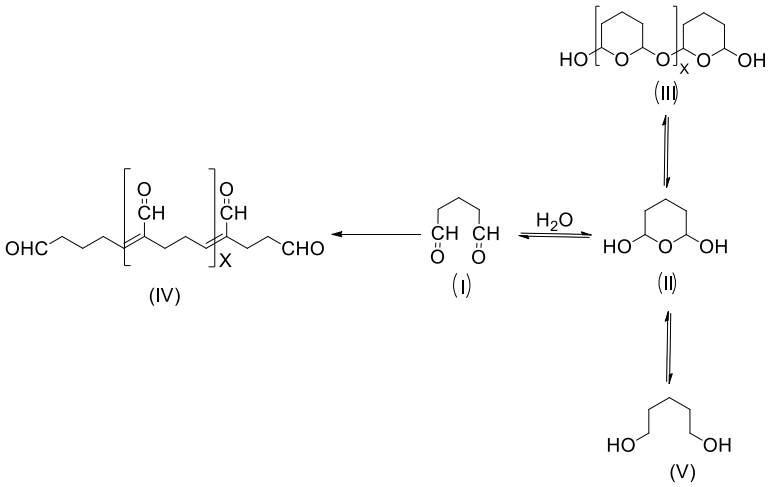


Figure 1

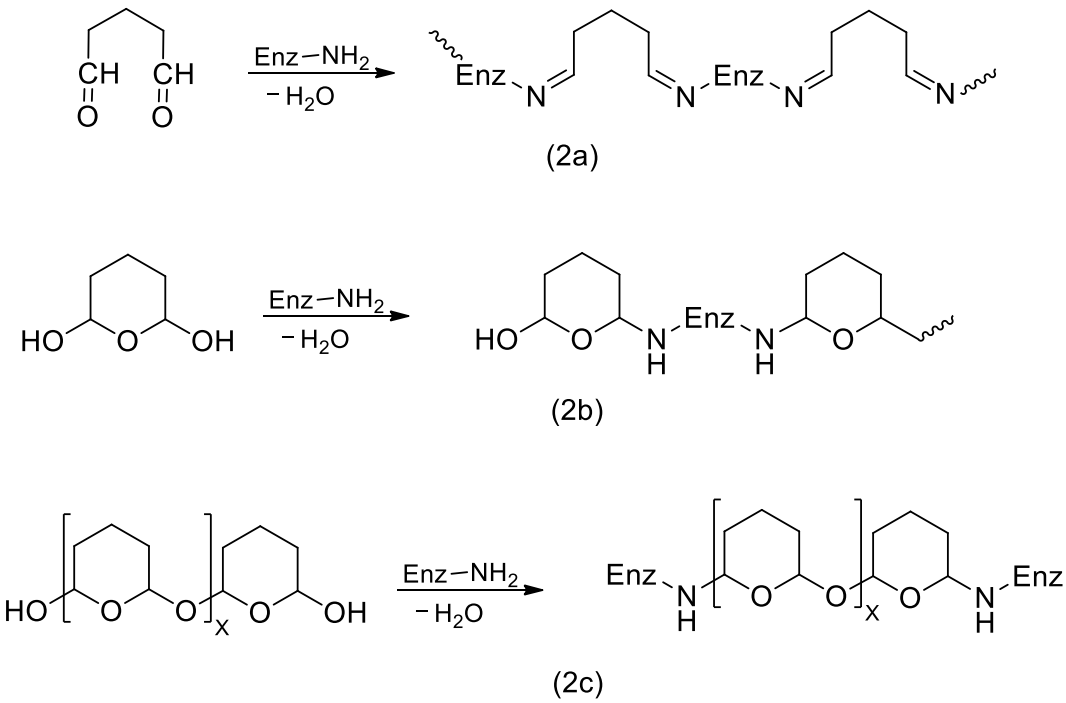

Enz–NH2
– H2O
Enz
N
Enz
N
N
(2a)
HO
O
OH
Enz–NH2
– H2O
HO
O
N
H
Enz
N
H
O
(2b)
HO
O
O
X
O
OH
Enz–NH2
– H2O
Enz
N
H
O
O
X
O
N
H
Enz
(2c)

Figure 2

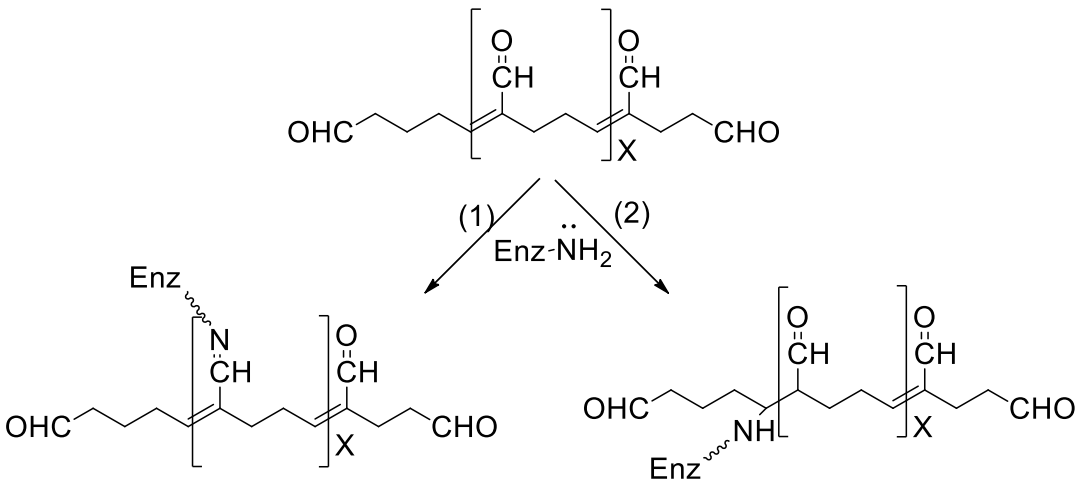

O
CH
O
CH
OHC
CHO
X
(1)
(2)
Enz-NH2
Enz
N
CH
O
CH
OHC
CHO
X
O
CH
O
CH
OHC
CHO
NH
X
Enz

Figure 3

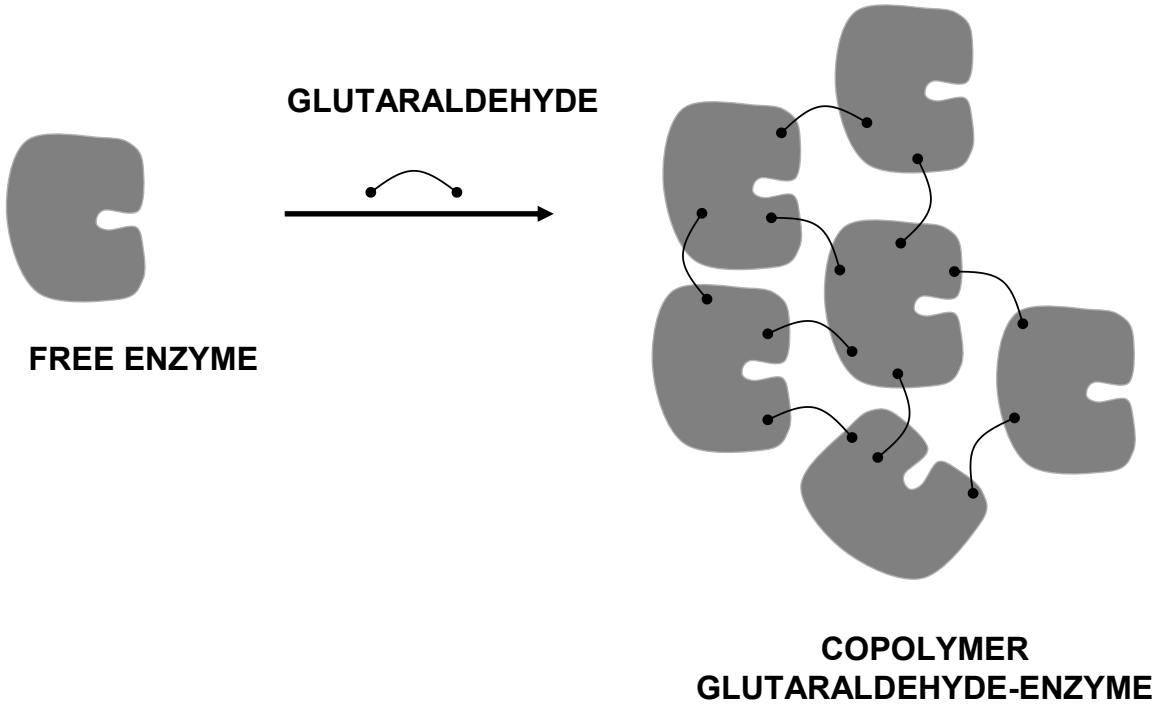

GLUTARALDEHYDE
FREE ENZYME
COPOLYMER
GLUTARALDEHYDE-ENZYME

Figure 4

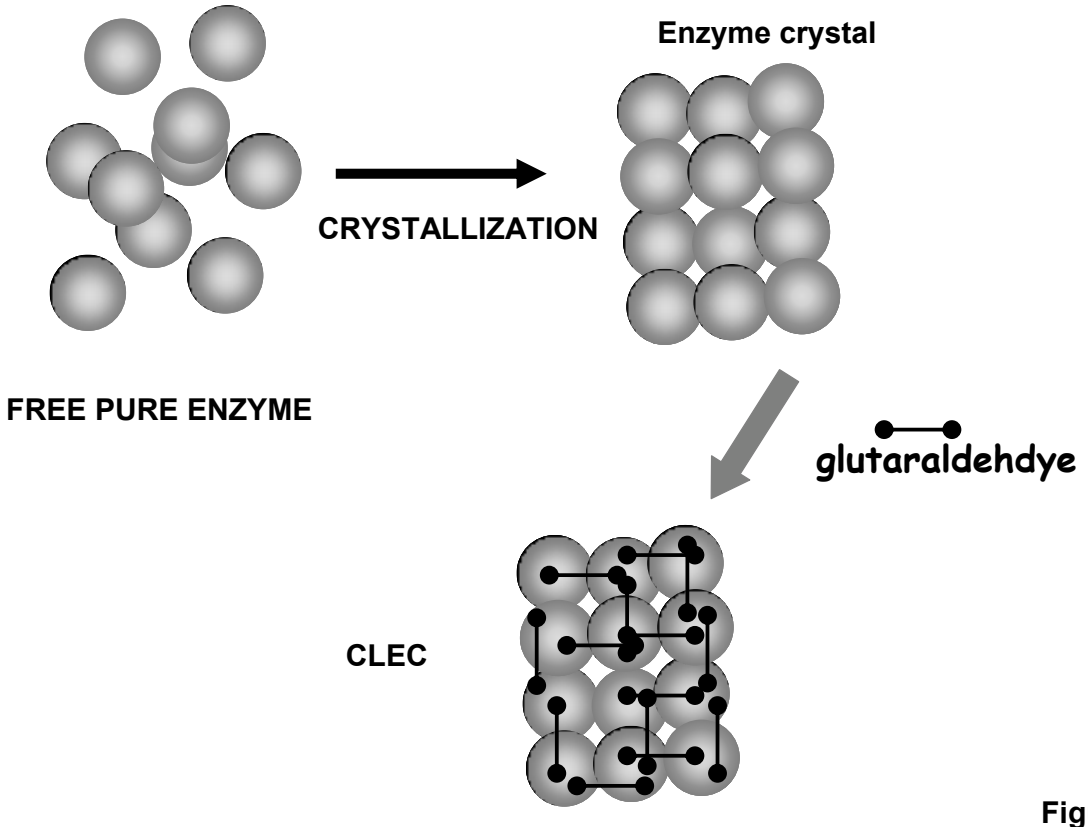

Enzyme crystal
CRYSTALLIZATION
FREE PURE ENZYME
glutaraldehdye
CLEC

Figure 5

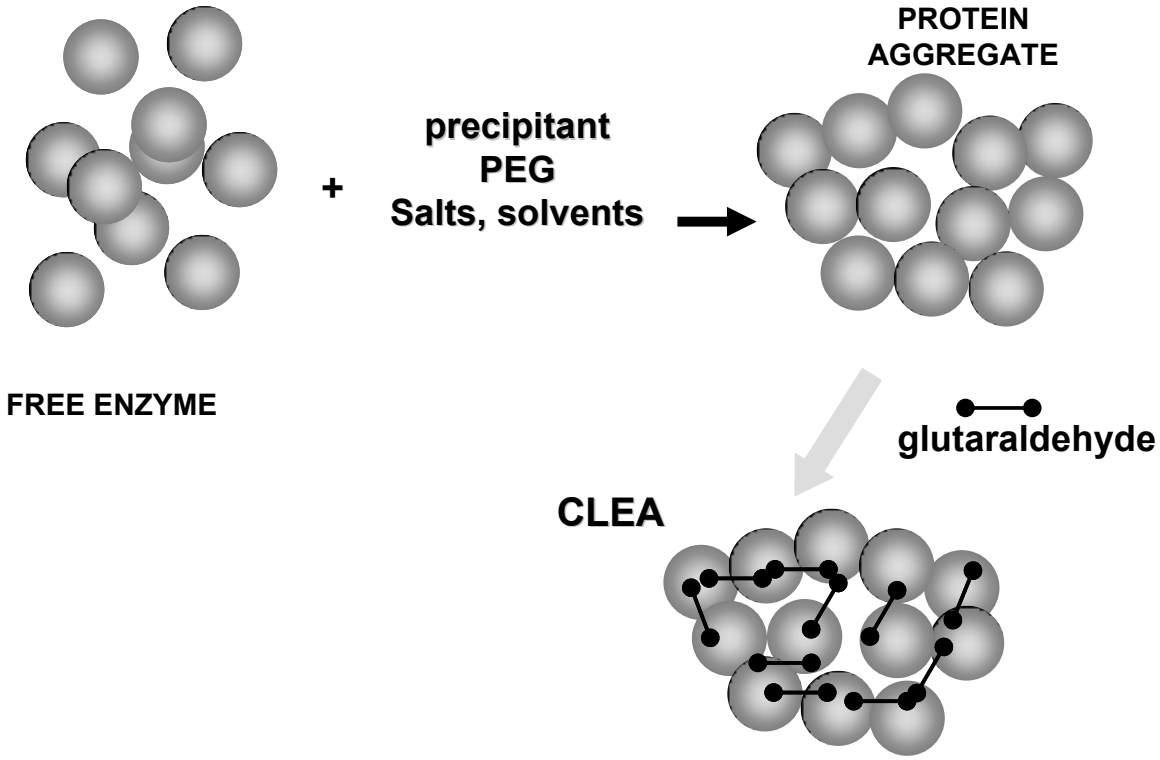

PROTEIN
AGGREGATE
precipitant
PEG
Salts, solvents
+
FREE ENZYME
glutaraldehyde
CLEA

Figure 6

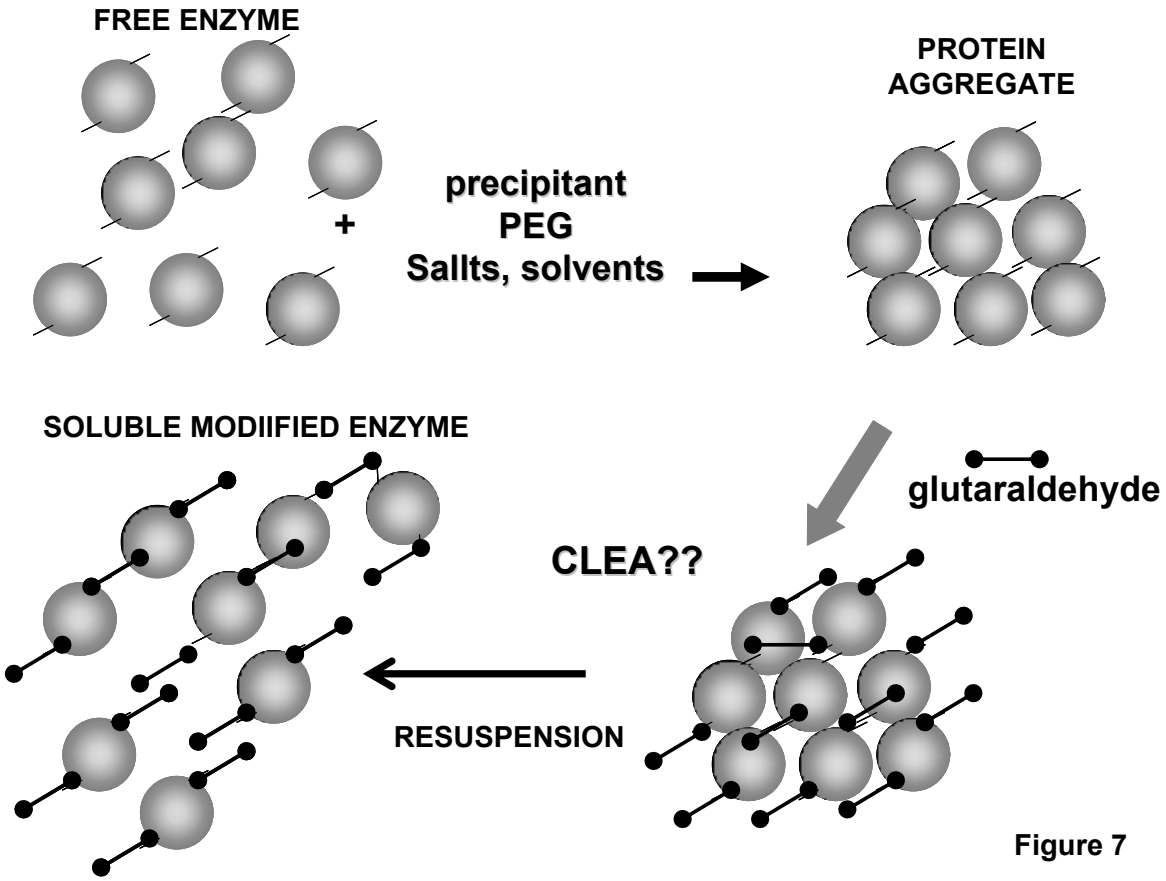

FREE ENZYME
PROTEIN AGGREGATE
precipitant
PEG
Sallts, solvents
+
SOLUBLE MODIIFIED ENZYME
glutaraldehyde
CLEA??
RESUSPENSION

Figure 7

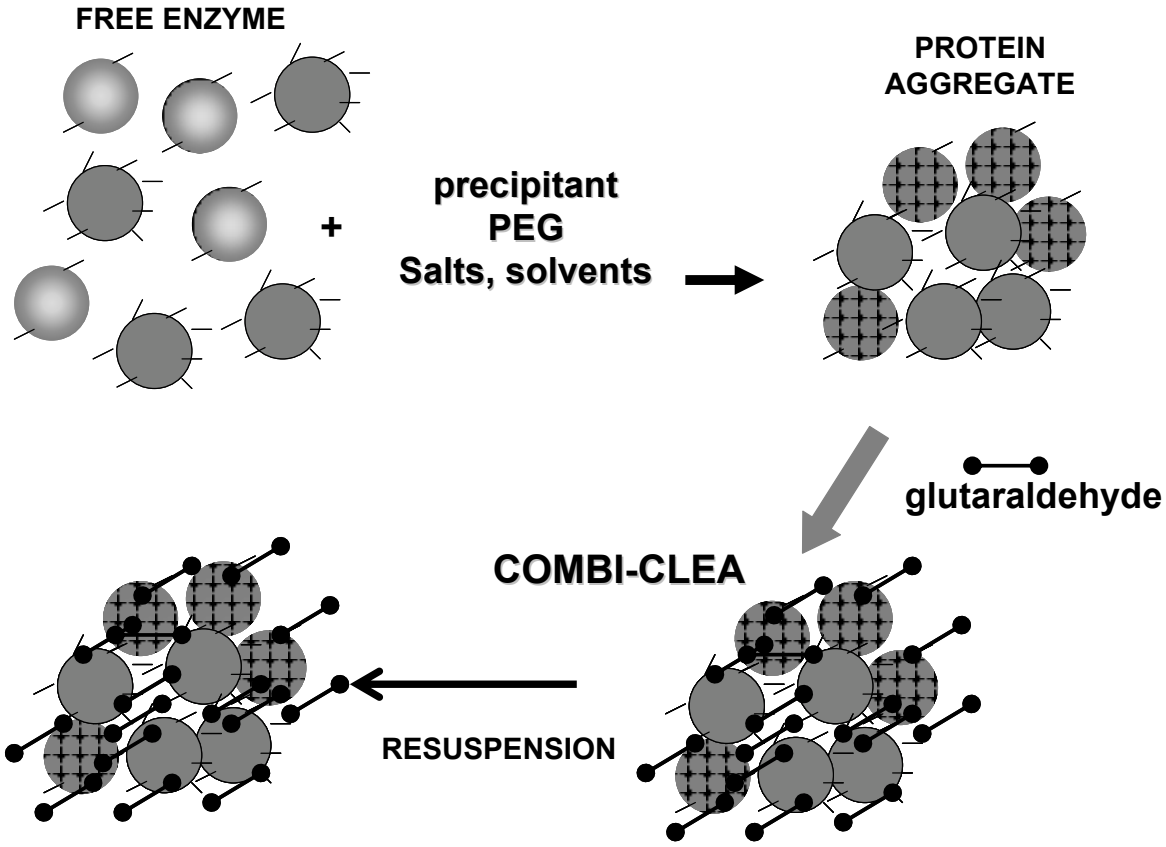

FREE ENZYME
PROTEIN AGGREGATE
+
precipitant
PEG
Salts, solvents
glutaraldehyde
COMBI-CLEA
RESUSPENSION

Figure 8

1075

1076

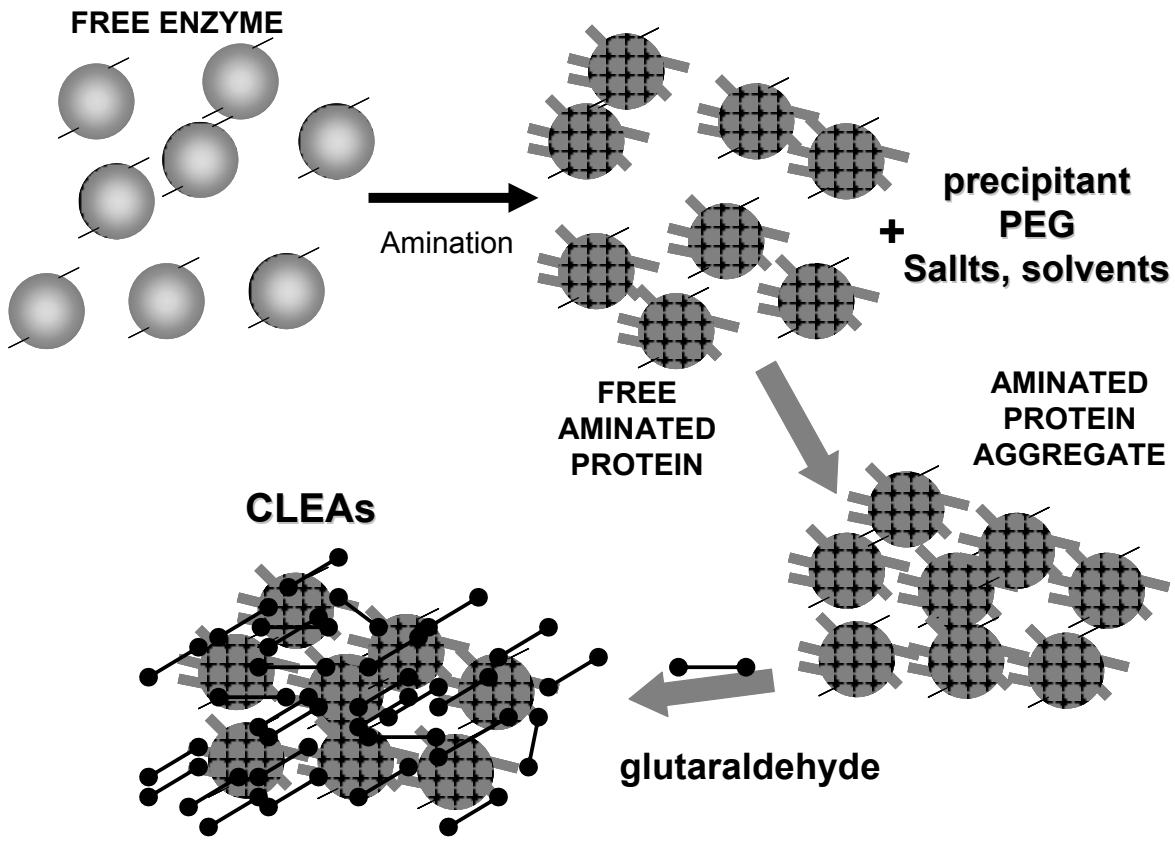


Figure 9

1077

ENZYME INACTIVATION

1078

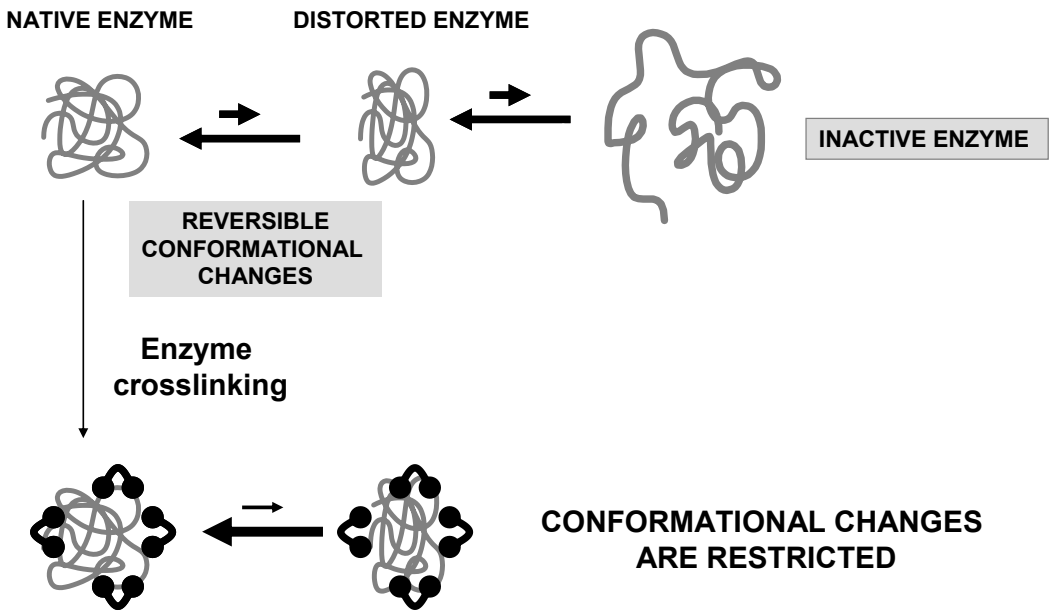


Figure 10

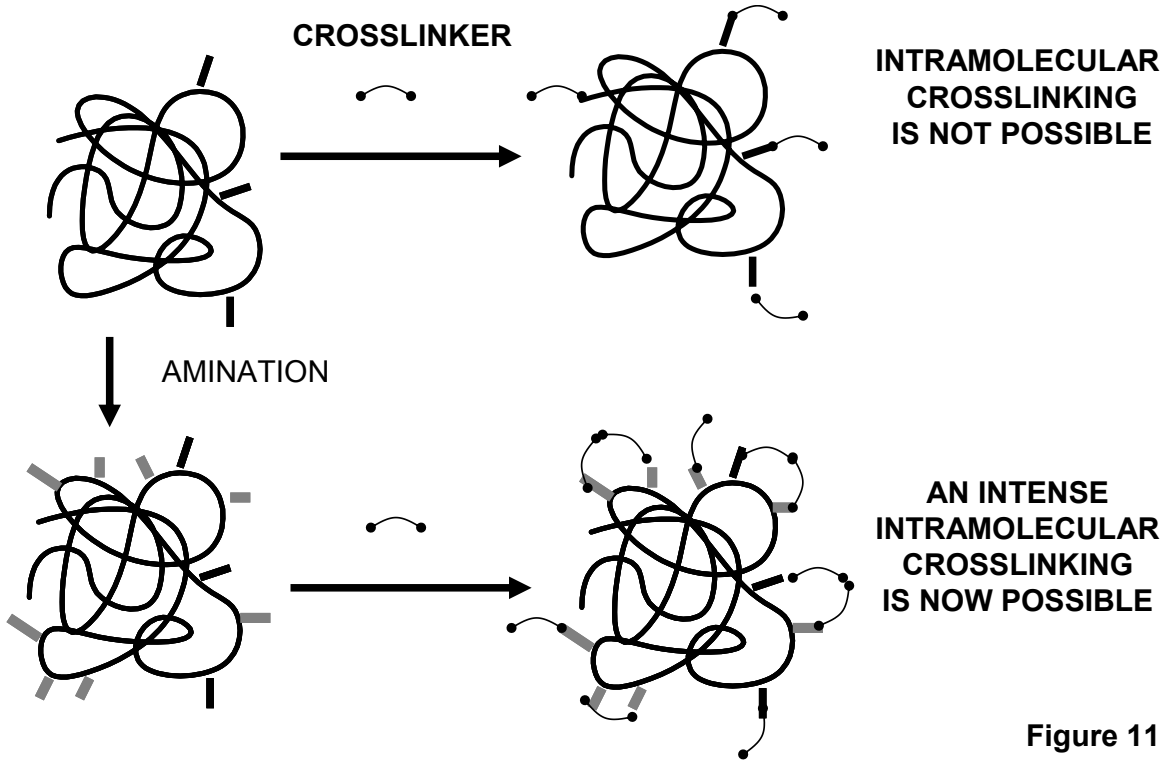
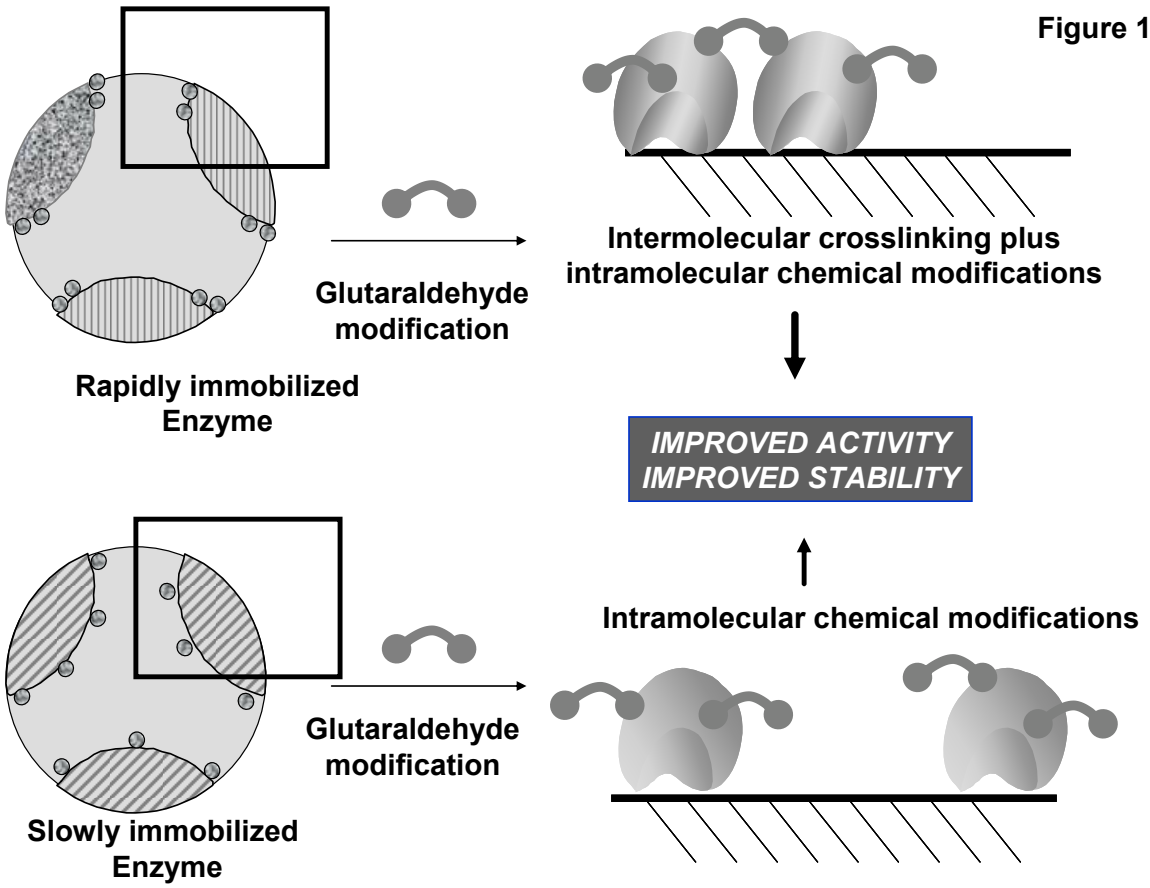

CROSSLINKER
INTRAMOLECULAR
CROSSLINKING
IS NOT POSSIBLE
AMINATION
AN INTENSE
INTRAMOLECULAR
CROSSLINKING
IS NOW POSSIBLE

Figure 11

1081

1082

Figure 12



1083

1084

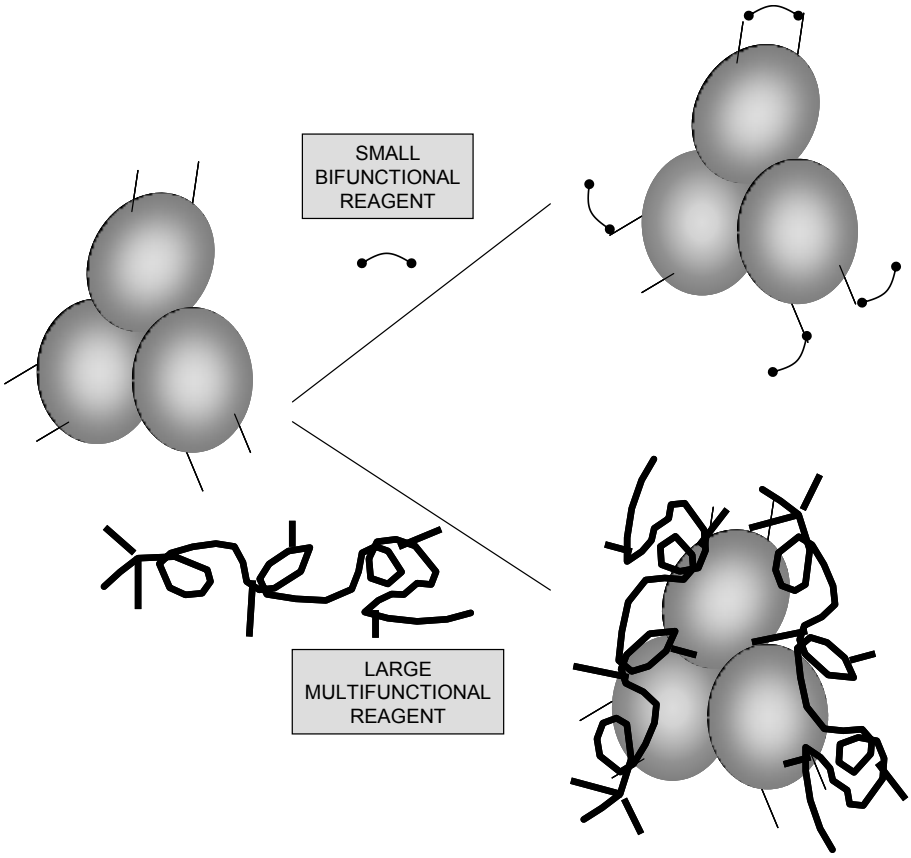


Figure 13

1085

1086

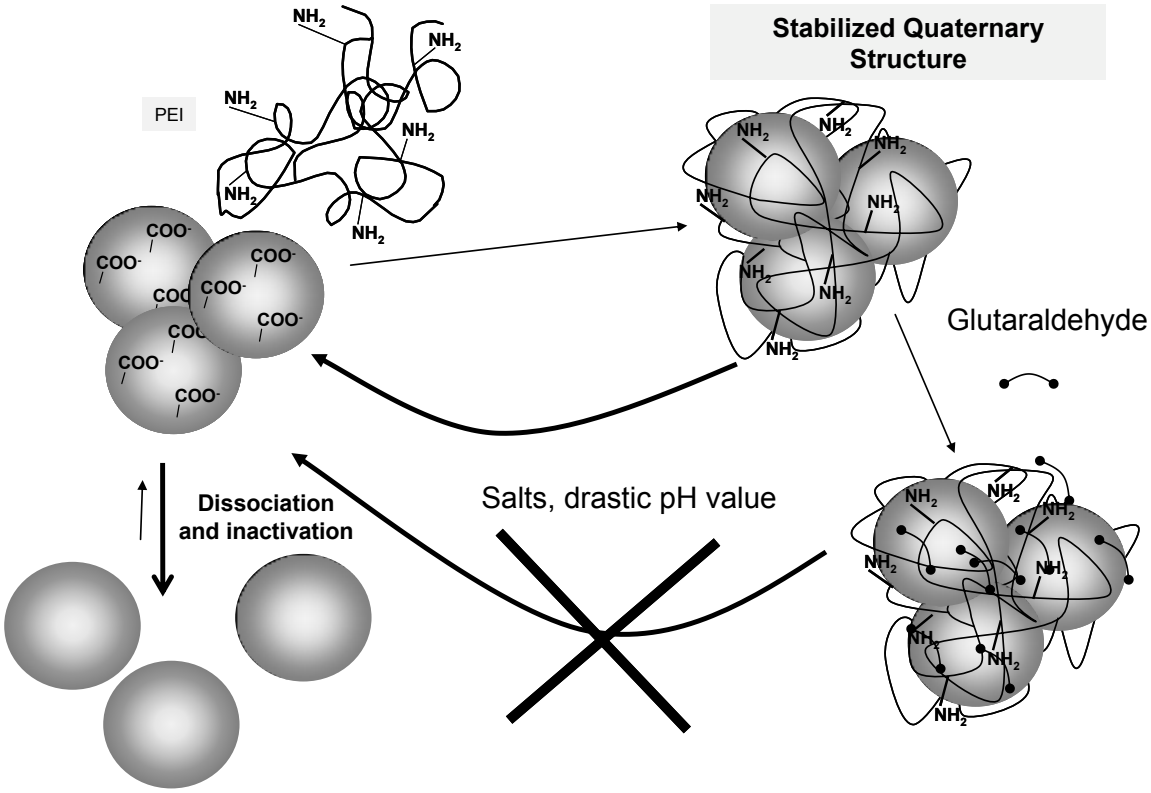


Figure 14

1087

1088

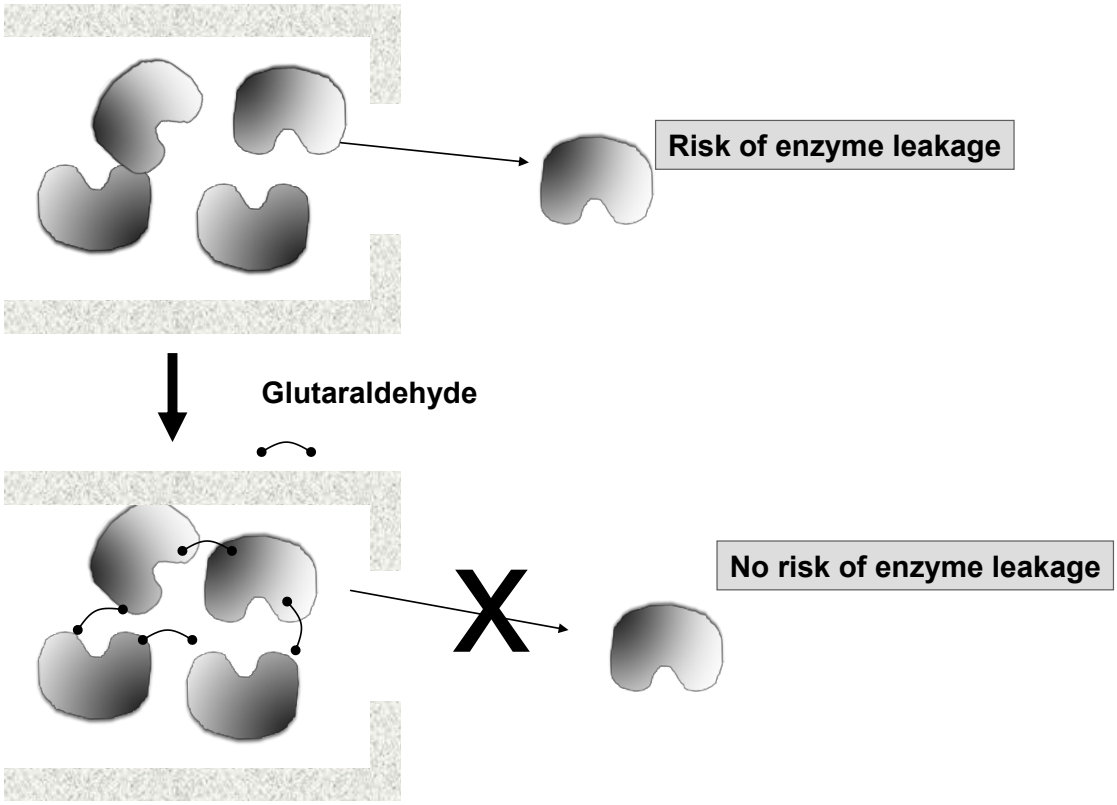


Figure 15

1089

1090

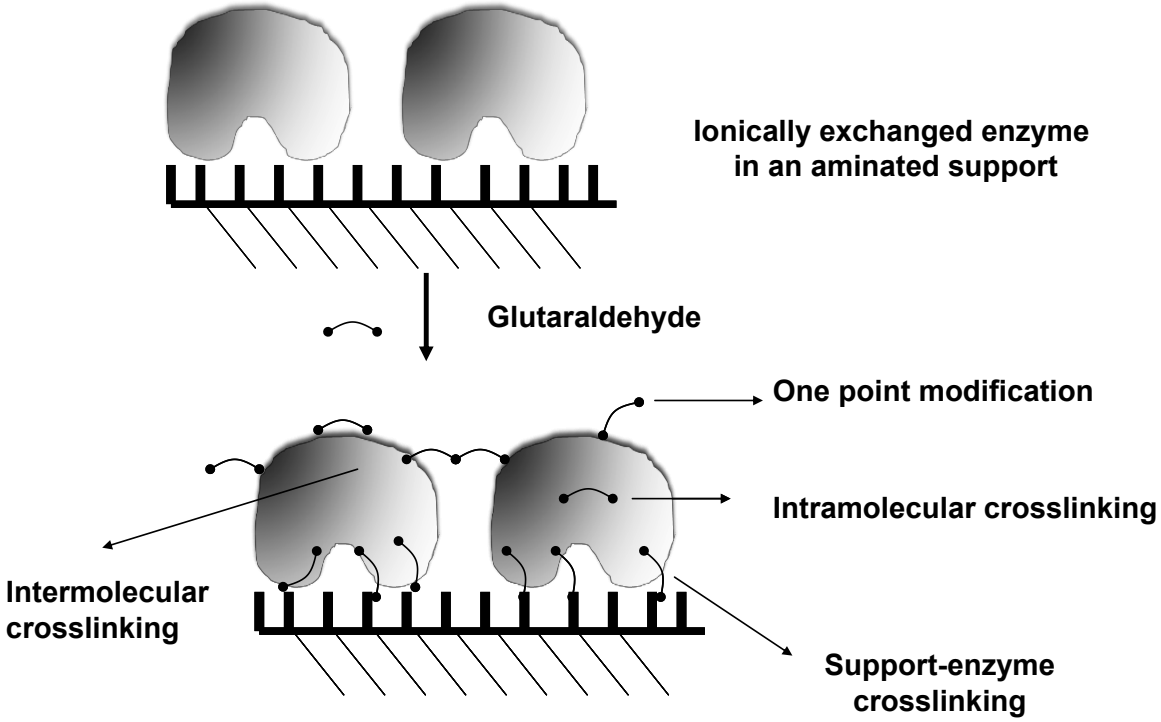


Figure 16

1091 **A PROTEIN IS A MULTIFUNCTIONAL STRUCTURE**

1092

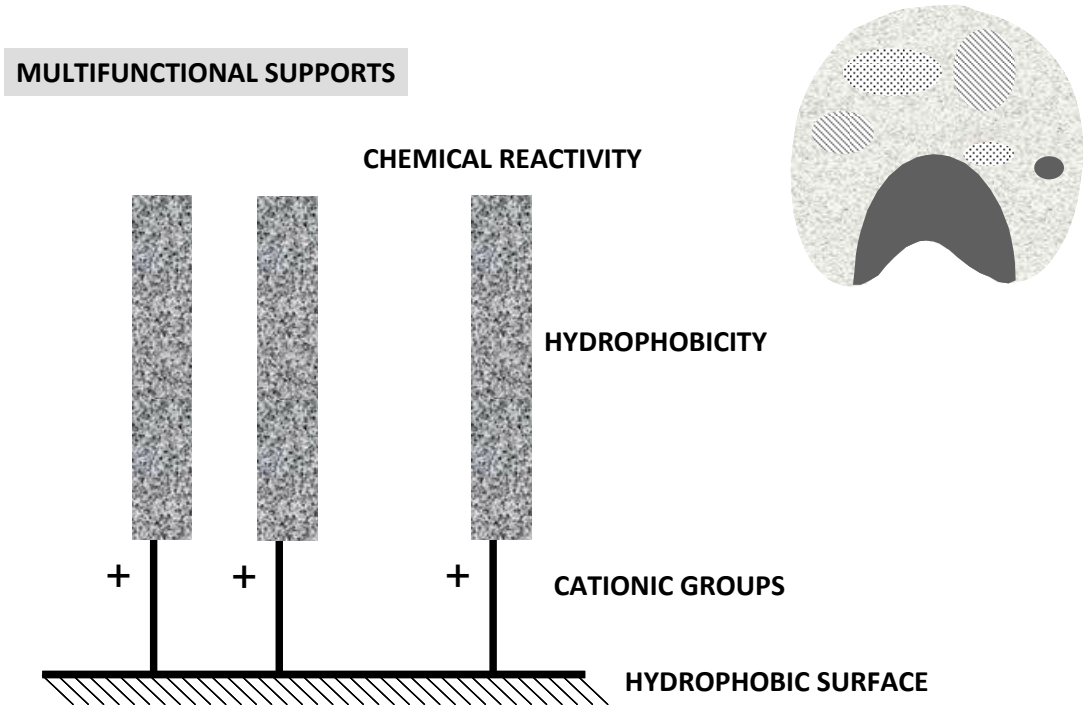


Figure 17

1093

1094

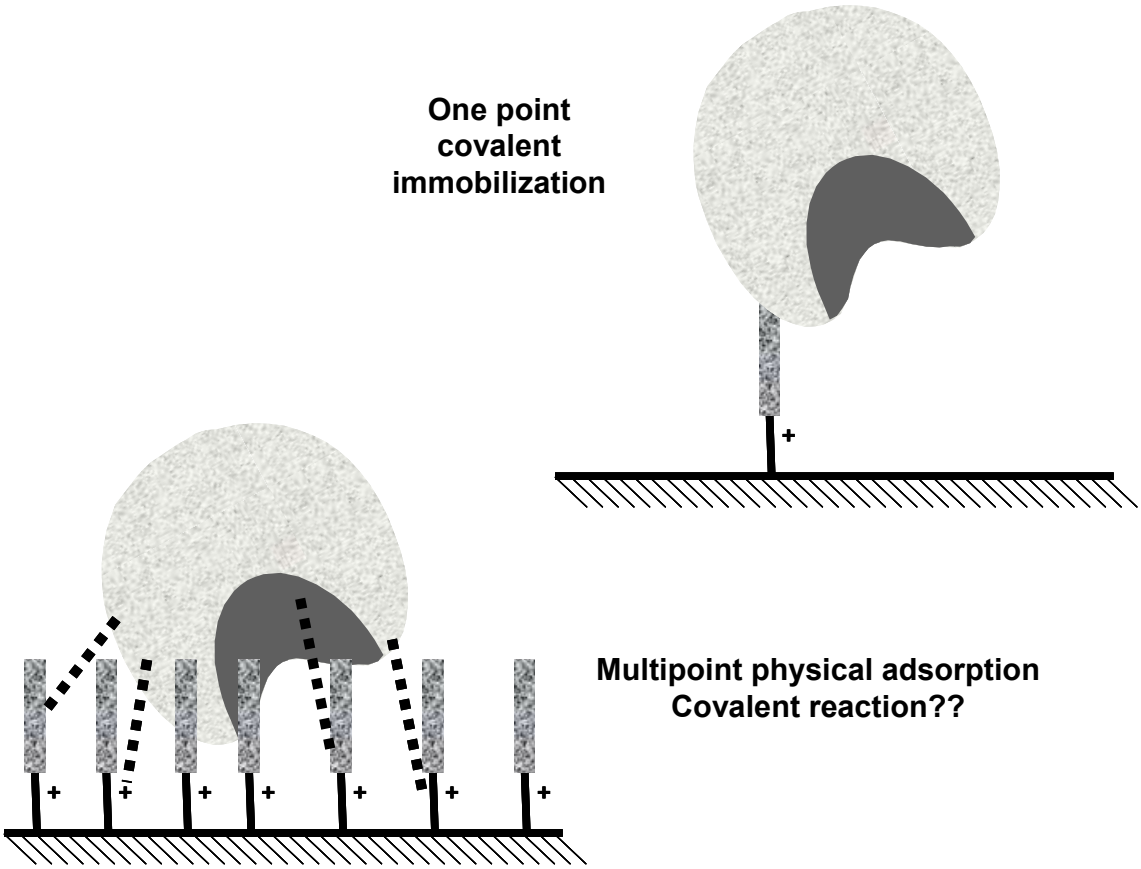


Figure 18

1095 **THE CASE OF LIPASES**

1096

Interfacial adsorption on hydrophobic supports

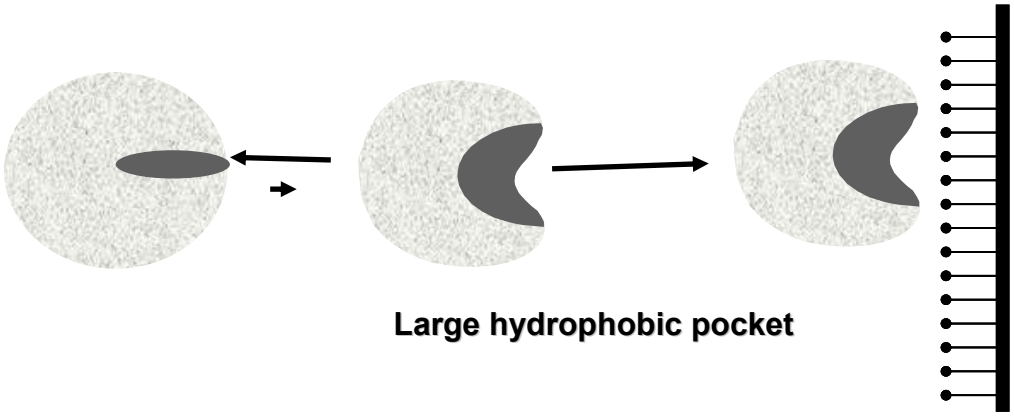


Figure 19

1097

LOW IONIC STRENGTH

1098

Possible covalent attachments

LIPASE ADSORPTION VIA INTERFACIAL ACTIVATION
IONIC EXCHANGE
NO INSPECIFC HYDROPHOBIC ADSORPTION

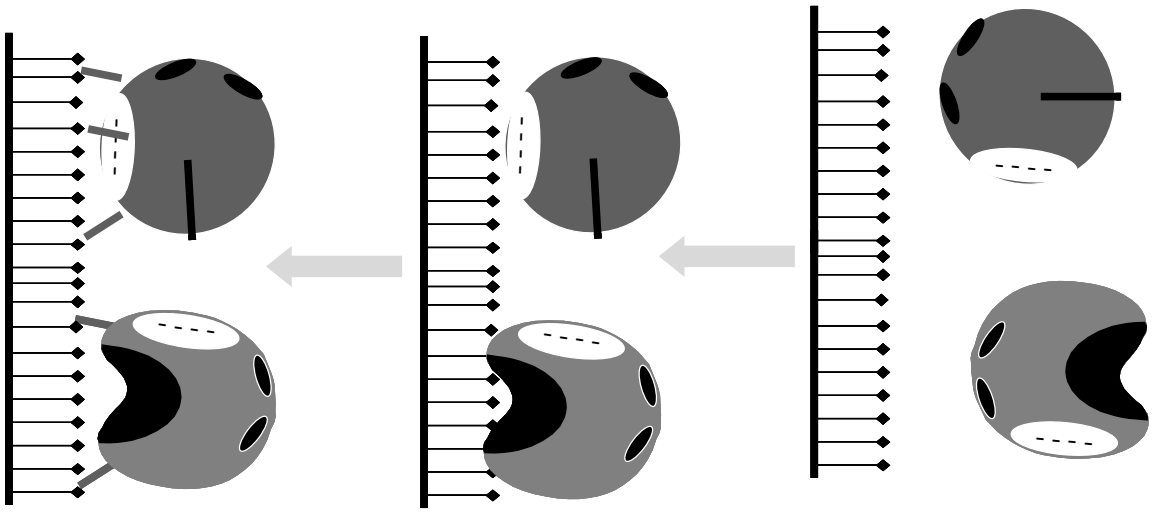


Figure 20

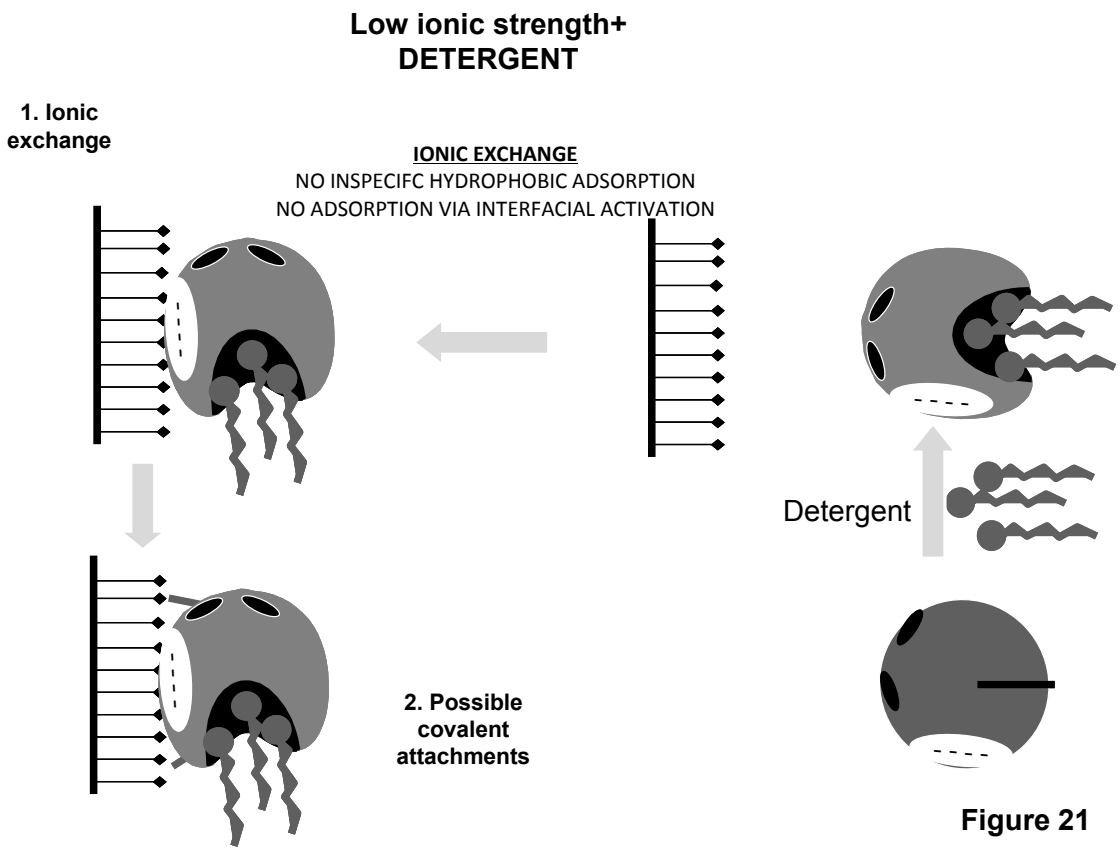

Low ionic strength+
DETERGENT
1. Ionic exchange
IONIC EXCHANGE
NO INSPECIFC HYDROPHOBIC ADSORPTION
NO ADSORPTION VIA INTERFACIAL ACTIVATION
Detergent
2. Possible covalent attachments

Figure 21

1101

IONIC STRENGTH HIGH ENOUGH TO PREVENT IONIC EXCHANGE

1102

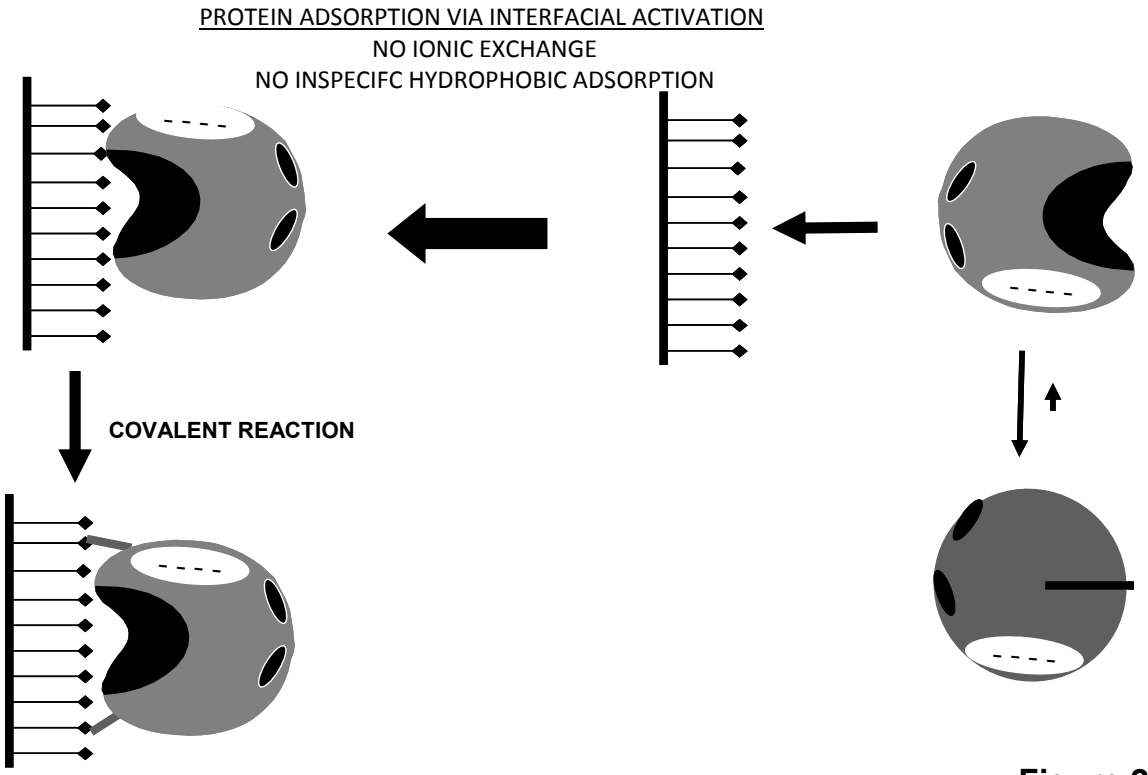


Figure 22

1103

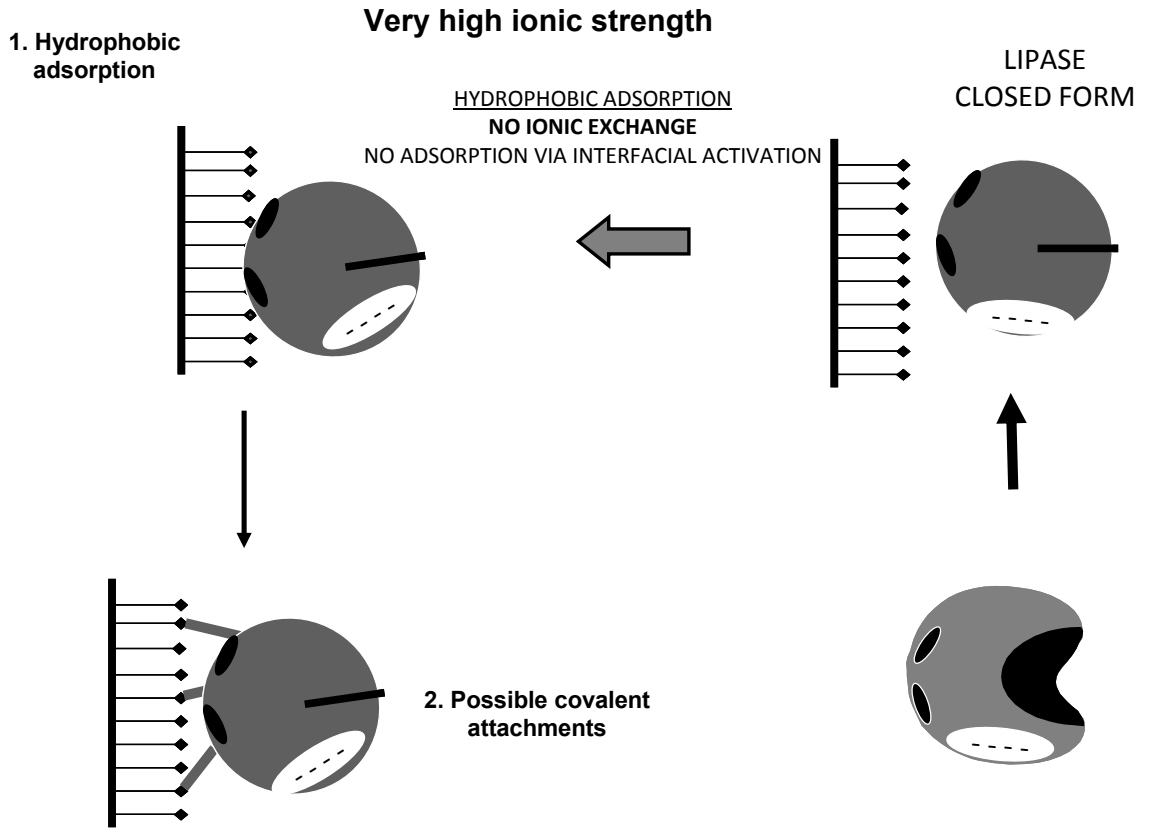


Figure 23