





Review

Enzyme-Coated Micro-Crystals: An Almost Forgotten but Very Simple and Elegant Immobilization Strategy

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Abstract: The immobilization of enzymes using protein coated micro-crystals (PCMCs) was reported for the first time in 2001 by Kreiner and coworkers. The strategy is very simple. First, an enzyme solution must be prepared in a concentrated solution of one compound (salt, sugar, amino acid) very soluble in water and poorly soluble in a water-soluble solvent. Then, the enzyme solution is added dropwise to the water soluble solvent under rapid stirring. The components accompanying the enzyme are called the crystal growing agents, the solvent being the dehydrating agent. This strategy permits the rapid dehydration of the enzyme solution drops, resulting in a crystallization of the crystal formation agent, and the enzyme is deposited on this crystal surface. The reaction medium where these biocatalysts can be used is marked by the solubility of the PCMC components, and usually these biocatalysts may be employed in water soluble organic solvents with a maximum of 20% water. The evolution of these PCMC was to chemically crosslink them and further improve their stabilities. Moreover, the PCMC strategy has been used to coimmobilize enzymes or enzymes and cofactors. The immobilization may permit the use of buffers as crystal growth agents, enabling control of the reaction pH in the enzyme environments. Usually, the PCMC biocatalysts are very stable and more active than other biocatalysts of the same enzyme. However, this simple (at least at laboratory scale) immobilization strategy is underutilized even when the publications using it systematically presented a better performance of them in organic solvents than that of many other immobilized biocatalysts. In fact, many possibilities and studies using this technique are lacking. This review tried to outline the possibilities of this useful immobilization strategy.

Keywords: enzyme immobilization; enzymes in organic media; crosslinking of immobilized enzymes; enzyme stabilization; enzyme hyperactivation; solid buffers

1. Introduction: Enzyme Biocatalysis

Increasing growth of the human population makes the urgent development of greener chemical procedures necessary in order to fulfill the overall demand of products with an increasing complexity produced under environmentally friendly conditions [1–3]. In this context, biocatalysis is gaining increased interest because of its high activity under very mild environmental conditions coupled to high substrate specificity or product selectivity. These qualities reduce the generation of side-products and simplify the downstream. Unfortunately, enzymes have been designed by nature to fulfill some

requirements necessary for life but not for industrial catalysts. In this way, enzymes can be unstable and suffer inhibition by different compounds; additionally, their excellent catalytic properties are generally limited by their physiological products, and, finally, enzymes are hard to separate from the reaction media for developing the workup [4]. In industry, ideal requirements would demand an enzymatic stability of several months; moreover, biocatalyst inhibition will always be a problem, and, in many instances, we intend to use enzymes to convert non-physiological substrates and under non-physiological conditions. Fortunately, there are a growing number of tools to solve these enzyme limitations; thus, metagenomics enables us to find the best available enzyme for the target process, even if produced by a non-cultivable or a no longer existing microorganism [5–7]; directed evolution permits us to enhance the selected enzyme features mimicking natural evolution, but in a much faster way [8,9]; protein chemical modification permits a growing control of the modification [10–14] and immobilization may solve the problem of enzyme recovering [15,16]. Furthermore, some strategies may be used in a coupled way to get an optimal biocatalyst, in some cases with a functionality very far from the natural one. For example, recently an enzyme obtained using metagenomics techniques was submitted to site directed mutagenesis to create an enzyme bearing two active so-called plurizymes [17]. Using modelling and dynamic simulation, this active center was improved and an irreversible inhibitor bearing an organometallic catalyst was designed for one of the active centers. This strategy permitted us to generate an enzyme with two different catalytic activities, which was subsequently used in a cascade reaction utilizing both, one of the plurizyme catalytic active centers and the newly inserted metal catalyst [18]. Moreover, the demand for cleaner processes has extended to the preparations of immobilized enzymes, that now is required to use in non-contaminating reagents. This way, some contaminant compounds used in the supports activations are being changed by greener ones (e.g., using genipin as crosslinking agent instead of the more toxic glutaraldehyde) [19].

Focusing on immobilization, it may also be coupled to any other strategy for better biocatalyst design. For instance, the chemical modification of immobilized enzymes will benefit from the advantages of the solid phase chemical modification, while enrichment of the target groups of the enzyme may permit a more intense enzyme-support interaction [20–22]. Similarly, site-directed mutagenesis may be addressed to improve enzyme immobilization and not to improve the enzyme features in a straightforward manner [23,24].

Enzymes may be utilized in a wide diversity of reaction medium. From the initial use in aqueous media where the enzymes have a moderate stability, the enzymes have been later utilized in fully anhydrous media formed by organic solvents to shift thermodynamic equilibrium, improve substrate solubility, etc. [25]. Novel media has been designed to solve some limitations of enzymes on this media, such as ionic liquids [26–28], supercritical fluids [29,30], or deep-eutectic-solvents [31–33].

2. Enzyme Immobilization

Immobilization was initially designed with the aim of solving problems associated with the reuse of the enzymes, but nowadays it has become a very powerful tool to solve many other enzyme limitations [34–41]. After enzyme immobilization inside a porous support, enzyme inactivation by enzyme aggregation, interaction with external hydrophobic interfaces, or autolysis no longer become possible [35]. If the enzyme structure is rigidified via multipoint covalent attachment, it will become more stable, and the range of conditions where it may be used will be enlarged [42–44]. Similarly, multisubunit immobilization of multimeric enzyme makes subunit dissociation impossible [45], increasing the enzyme stability and the range of conditions where the enzyme may be used [46]. The support surface properties may also tune the enzyme environment, producing some positive effects on the enzyme stability in the presence of deleterious compounds via a partition effect [47].

Moreover, a proper immobilization protocol may improve enzyme activity, selectivity, or specificity, reduce inhibition, and even purify the target enzyme [34]. That way, enzyme immobilization has become a critical step in the development of immobilized enzyme biocatalysts. There are many commercially available immobilized enzymes, but they usually use already quite stable enzymes,

and they may be easily improved using advanced immobilization protocols. To date, there are no “universally suitable” immobilization protocols, as each enzyme has their own peculiarities. In that way, this subject deserves continuous research to take full advantage of the immobilization process for any enzyme. Recently, Prof. Woodley suggested that enzyme stability should be measured not in terms of conventional half-life time, but considering the mass of product produced per mass of the biocatalyst before the catalysts must be discarded [48]. That idea states that in the enzyme “stabilization” achieved after immobilization, the researcher should consider not only the increase in conventional half-lives, but also any increase in enzyme activity during its utilization under the process conditions. This is relevant, for example, in the case of lipases immobilized on hydrophobic supports where a significant hyperactivation is observed in many instances [48].

Enzyme immobilization advantages must compensate the immobilization expenses. This will be mainly the support costs (including the support price, but also the support transporting, storage, and support discarding if it needs some treatment to be discarded) and the immobilization process costs (personal, reactor, etc.). That way, from an industrial point of view, an immobilization process should be as simple as possible. The support costs may be relevant, but they should be just one parameter to be considered among many other ones. In fact, support enzyme loading, possibility of improving the enzyme properties during immobilization, mechanical resistance of the biocatalysts particles, etc. are parameters more relevant than only the support price [35,47].

Many different immobilization strategies are available. Immobilization on preexisting supports remains as one of the most used immobilization strategies, via physical adsorption or covalent bonds, using porous supports or nonporous nano-supports [35]. These kinds of supports are very versatile and become optimal for some applications as multipoint covalent attachment [35]. The price of the support may be decreased if reversible immobilization techniques are used. These techniques allow for the re-use of the support after releasing the inactivated enzyme, and that way the global incidence of the support price may be decreased. However, this reversible immobilization strategy usually gives worse results in terms of enzyme stabilization than covalent immobilization [49]. A remarkable exception to this low stabilization effect of enzyme immobilization by physical methods is the lipase immobilization via interfacial activation on hydrophobic supports [50]. In an effort to save on support costs, there are many examples of enzyme immobilization without supports. Enzyme copolymerization is one of these techniques, a quite old strategy of enzyme immobilization [51–53]. In this strategy, the enzyme is mixed with a monomer, and, after activation, the polymer starts to polymerize and the enzyme becomes a part of the structure (Figure 1).

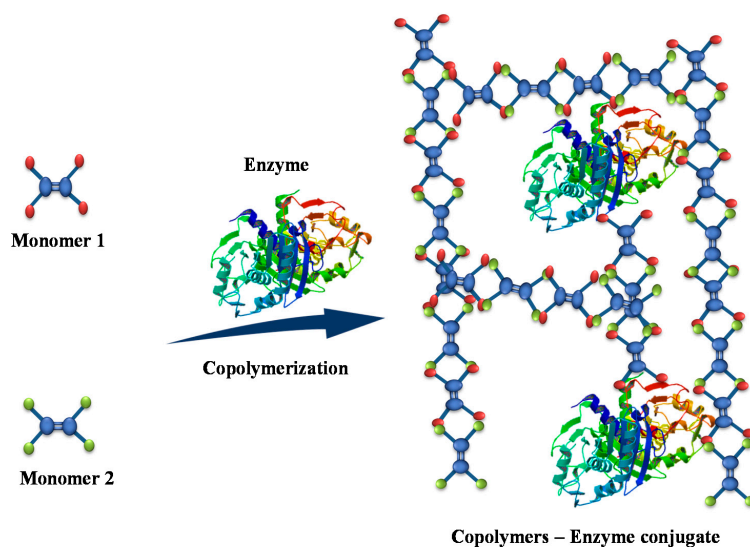


Figure 1. Schematic representation of enzyme immobilization via copolymerization.

The use of chemically crosslinked enzyme crystals is another strategy to save the support, but it has higher costs, enzyme purity must be very high, and the enzyme itself needs to be crystallized [54,55], Figure 2.

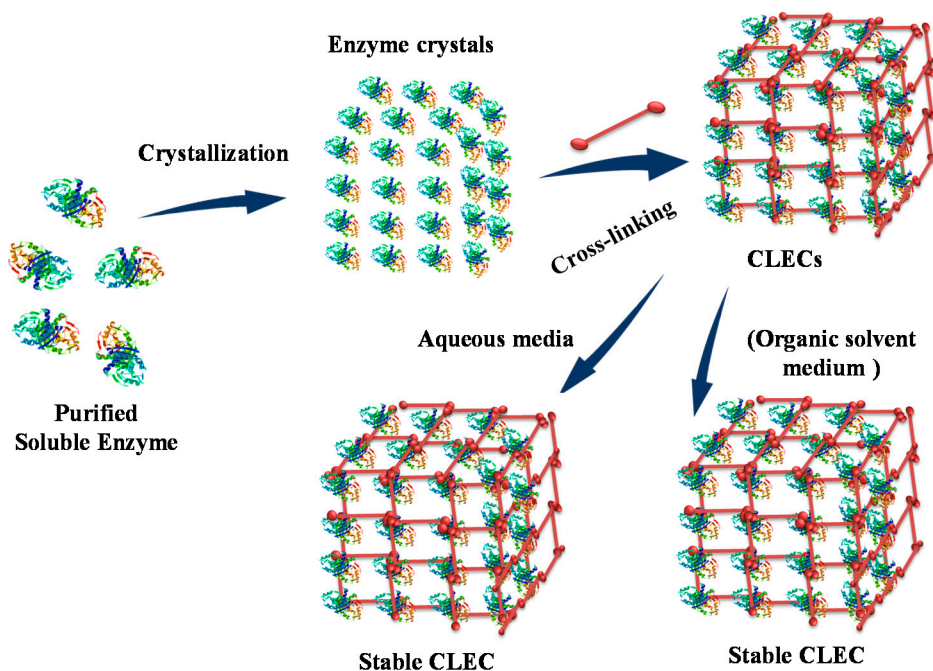


Figure 2. Schematic representation of the enzyme immobilization via cross-linked enzyme crystals (CLECs).

The production of nano-flowers is a more recent strategy that has rapidly expanded and been readily applied in many cases, as it may be observed in the many recent reviews on this matter [56,57], shown in Figure 3. In this strategy, the enzyme is mixed with a metal salt that forms a structure involving the enzyme and in many instances give as result a particle with a flower shape.

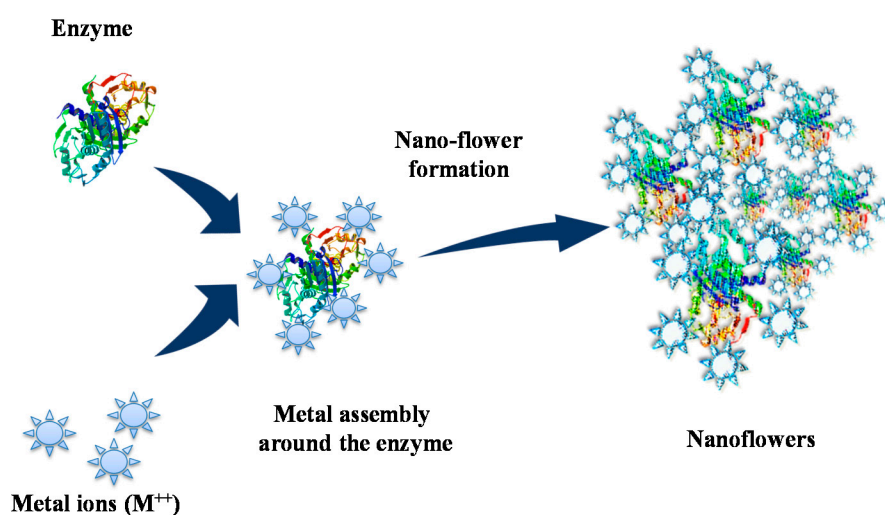


Figure 3. Schematic enzyme representation of enzyme immobilization using nanoflowers formation strategy.

However, the oldest strategy of enzymes immobilized without support is enzyme aggregation, and it was utilized to prepare immobilized enzyme biocatalysts to be used in organic media [56,57] (Figure 4). As the enzyme was not soluble in this media, the aggregates particles remained stable for a long time. However, they cannot be used in aqueous medium.

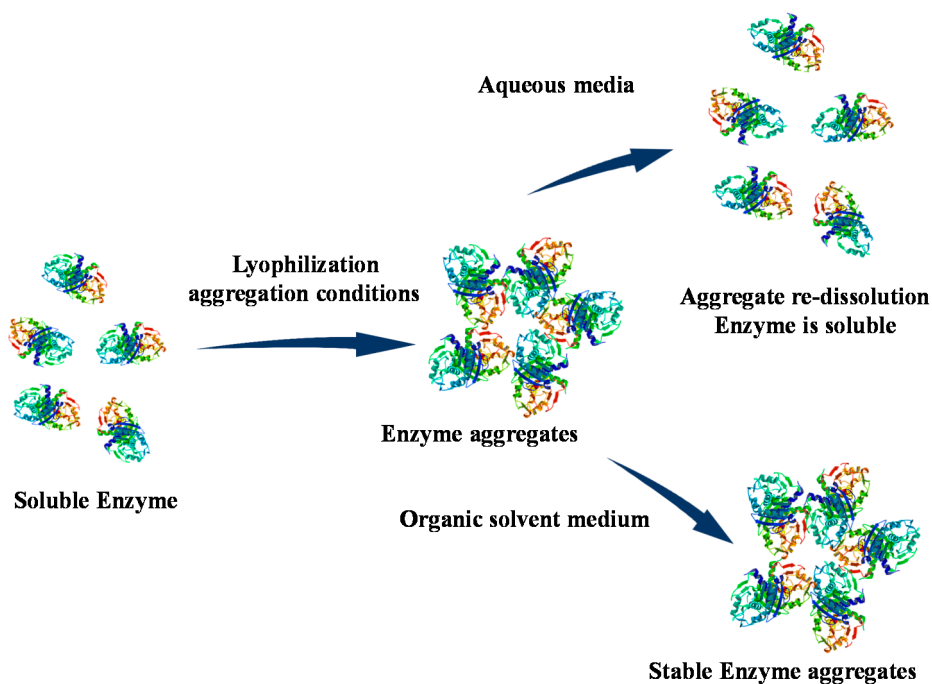


Figure 4. Schematic representation of the immobilization of enzymes using aggregates.

Prof. Sheldon solved this problem of the limitations of the use of aggregates to organic medium by the immobilization strategy called crosslinked enzyme aggregates, where the enzyme in aggregated form was chemically crosslinked [58,59] (Figure 5). This way, the enzyme remained immobilized under any reaction media.

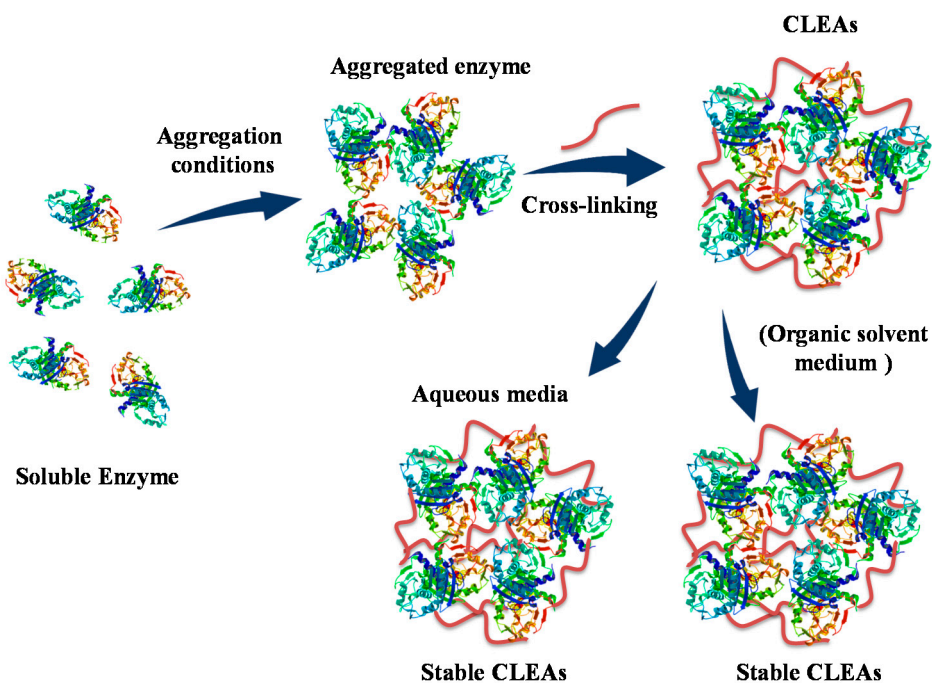


Figure 5. Schematic representation of enzyme immobilization via cross-linked enzyme aggregates technique (CLEAs).

In this context, in 2001, a novel immobilization strategy was developed by Kreiner and coworkers, mainly addressed towards the use of enzymes in organic solvent medium with low water activity: the protein-coated microcrystals (PCMCs) [60]. This technique presents some interesting prospects,

but its implementation is not as high as it may be expected from its great possibilities, as will be presented in this review. This will be the objective of the current review.

3. Use of Enzyme in Organic Solvents with Low Water Activity

PMCMs are only valid to be used in systems with low water content. However, this kind of reaction media is highly utilized in biocatalysis. The use of biocatalysts in nearly anhydrous media was thought to be impossible until the end of last century; more concretely, starting from 1985 through the articles published from Klivanov [61–73] and other researchers [74–89], which showed how some enzymes (hydrolases, mainly lipases) were perfectly capable of working in water-insoluble organic solvents such as toluene or cyclohexane. Until that moment, the generalized dogma that enzymology should be limited to aqueous solutions hampered the development of this research area, firmly recognized nowadays [90–92]; in fact, this dogma had stated that proteins would become denatured, losing their native structure and their catalytic activity in organic solvents, and even today this concept can be found in many textbooks [93]. However, this belief came from examining proteins in aqueous-organic mixtures and the concomitant direct extrapolation of those observations to the behavior in neat organic solvents [34]. In fact, as water acts as a molecular lubricant [94–96], enzyme structure is very rigid in those media with an almost null water activity (this thermodynamic parameter is better than water amount to define the water-dependence of enzyme behavior [97,98]), so that in pure organic solvents crystalline enzymes do retain their native structures [99]. Another fascinating peculiarity associated with the use of enzymes in organic solvent is the concept of “pH memory” [100–105]; that is, the enzymatic conformation in an aqueous solution at a certain pH value can be “solidified” by lyophilisation, as protein ionogenic residues would retain the previous ionization state, which would be retained in the organic solvent. This fact, initially observed with water, promoted the development of ligand bio-imprinting technology, initially described by Mosbach et al. [106,107], through the lyophilization of the enzyme and a substrate analogue (forming a complex similar to the enzyme-substrate complex) in the aqueous solution and a further removing of the substrate analogue. Thus, the enzyme will retain the structure of the substrate analogues in the organic medium. Subsequently, some new bio-imprinting technologies such as interface activation [108–110] were developed, and bio-imprinted enzymes have been extensively applied in organic media [111–115].

In any case, enzymes are not soluble in almost any organic solvents. That means that if the researcher intends to use them as free enzymes, it becomes mandatory to modify their surface to improve their solvent solubility, thus dispersing the enzyme in the reaction media and increasing the activity by reducing the diffusional problems of the enzyme aggregates [116,117]. Otherwise, the enzyme will aggregate. That is, comparison in organic medium between immobilized and “free” enzyme is really a comparison between immobilized and dispersed enzyme and immobilized “aggregated enzymes particles”, and this aggregates lead to strong mass-transfer limitations, not always overcome by a simple stirring increase [116,117].

4. Immobilization of Enzyme by Protein-Coated Microcrystals (PCMCs)

As explained above, the PCMC technique was developed by Kreiner and coworkers [60]. It was based on a previous patent from the group to rapidly dehydrate proteins without seriously affecting the enzyme conformation [118]. Here, they observed that by releasing drops of an enzyme solution in an organic solvent, the enzyme dried immediately and gave a precipitate. However, the proposed immobilization technique goes further. The authors proposed to prepare an enzyme solution containing an additive at a very high concentration, which they called the “crystal forming component” [60] (Figure 6).

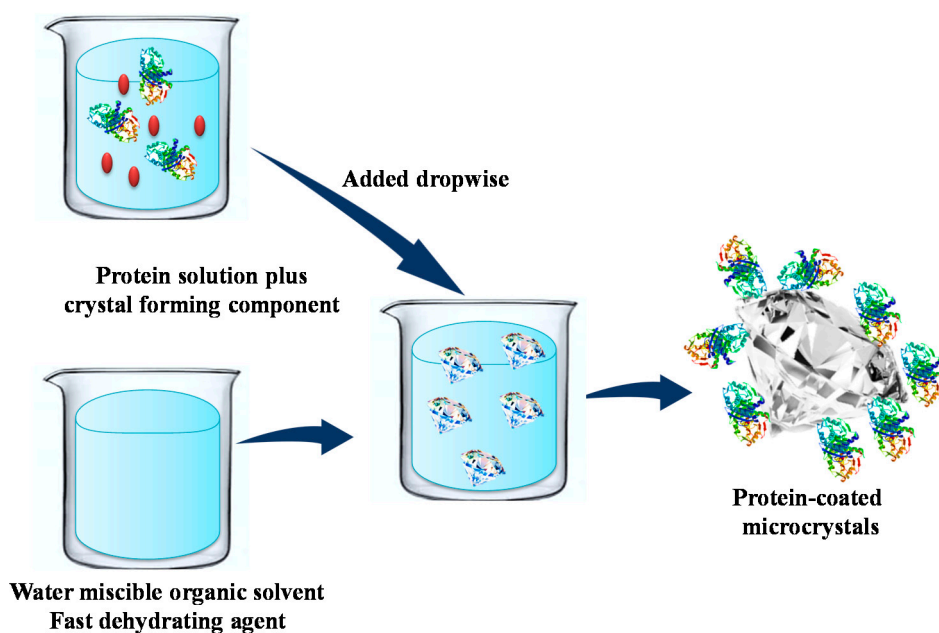


Figure 6. Schematic representation of enzyme immobilization via protein coated microcrystals strategy (PCMC).

This crystal forming component could be a salt, a sugar, or an amino acid; the requirements are that they should be very soluble in water and very poorly (better not at all) soluble in the solvent media. Then, they added under a rapid mixture this enzyme solution dropwise into a water solvent miscible solvent (e.g., propanol, ethanol, acetonitrile, acetone, tetrahydrofuran) to get a rapid enzyme precipitation (Figure 6). Initially, they used as crystal forming component K_2SO_4 and subtilisin Carlsberg as model enzyme. Using these components, the precipitated particle size ranged from 0.1 to 5 μm . When they analyzed the precipitate by transmission electron microscopy, they found that they did not have a random precipitate, but a crystalline structure formed by the salt. Applying tapping-mode atomic force microscopy to the crystals, they found that the protein molecules were located on the surface of the crystals forming a uniform layer. If only the salt (without enzyme) was added to the dehydrating solvent solution, the crystals were bigger, suggesting that the enzyme layer over the salt crystals avoids the salt crystal growth. The enzyme loading was 8% of protein [60]. They suggested that the rapid sample dehydration produced by the solvent occurred via a very rapid dehydration mechanism that permits keeping the active enzyme conformation [60].

The solvent used to dehydrate the enzyme solution in the crystal forming agent solution could be the final reaction medium in which the enzyme is intended to be used, or the PCMCs could rather be recovered and re-suspended in another solvent (now not necessarily water-soluble). Eventually, it could also be one of the substrates of the reaction.

The authors used the different subtilisin PCMCs produced in this first approach in the transesterification of *N*-acetyl-*L* tyrosine ethyl ester and propanol in acetonitrile, comparing the performance with that of the enzyme only submitted to freeze-drying: the new biocatalysts were 3 orders of magnitude more active than the just lyophilized enzyme [60]. Results were better using propanol or ethanol; and, with up to 20% water in the system, the presence of water had no significant effects on enzyme activity. However, if the water content exceeded 20%, the PCMC activity was reduced. They extended the study to the lipase A from *Candida antarctica*, the lipases from *Pseudomonas* sp., *Mucor miehei* or *Alcaligenes* sp. using the biocatalysts in the kinetic resolution of (*R,S*) phenylethanol via transesterification with vinyl acetate as activated acyl donor and *tert*-butyl methyl ether as reaction medium. Once again, significant improvements in enzyme activity were observed for the PCMC biocatalysts (from 5 to 200-folds), except for the PCMCs of the lipase from *Alcaligenes* sp. [60]. Furthermore, they showed that the PCMCs could be stored as a suspension in the solvent or as a dried

powder. That is, the first presentation of the results suggested a very promising strategy to enzyme immobilization to be used in organic medium [60].

This has some additional advantages. They had shown how the use of solid buffers could be a very appropriate option to tuning the enzyme selectivity and specificity of lipases in organic media, and the importance of these buffers increased if the reaction released or consumed some ionizable compound [119]. They showed this idea using the lipase B from *Candida antarctica* and the lipase from *Mucor miehei* in the enantioselective production of (*S*)-*N*-benzoylphenylalanine butyl ester by the enantioselective butanolysis of (\pm)-2-phenyl-4-benzyloxazol-5(4H)-one. This way, the new strategy, if using as crystal forming component a compound that can act as a buffer, could couple the enzyme immobilization to the generation of a solid buffer that can control the pH value during the reaction. This was the goal of a second paper on the use of PCMCs by the Kreiner group [120]. They extended the possibilities of compounds to be used as crystal forming components Na-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid, Na₂CO₃ or NaHCO₃. In this study, they used subtilisin Carlsberg and α -chymotrypsin as model enzymes. After preparing the PCMCs biocatalysts, they were employed in the transesterification of *N*-acetyl-*L*-tyrosine ethyl ester with 1-propanol in a medium composed of acetonitrile/1 wt.% H₂O. Even 3-fold more activity was found compared to an unbuffered PCMC (that was prepared using subtilisin Carlsberg as enzyme and K₂SO₄ as crystal forming component) [120]. Compared to freeze-dried enzyme preparations, ~3000-fold increase in catalytic activity was detected. When lipases from different sources (lipases A and B from *Candida antarctica*, lipase from *Mucor miehei*, *Thermomyces lanuginosa*, *Pseudomonas cepacia* and *Pseudomonas sp*) were immobilized on these PCMCs, its activity was much less improved when compared to not buffering PCMCs (ranging between 0.6 and 1.1) or just lyophilized powders (ranging from 5 to more than 125 times). Later, these researchers investigated the stabilities of the PCMCs biocatalysts [121]. In this context, PCMCs prepared using lipase B from *Candida antarctica* and subtilisin Carlsberg showed very good long-term stability when maintained as suspensions in 1-propanol/1 wt.% H₂O at room temperature (remarkably, in the case of subtilisin Carlsberg around 90% of their initial activity was maintained after 1 year at room temperature). As expected, the effect of the temperature on the subtilisin Carlsberg PCMCs activity depended on the solvent used, using propanol/1 wt.% H₂O, the enzyme activity increased until 60 °C, whereas in acetonitrile/1 wt.% H₂O, the activity decreased when the temperature increased from the lower temperatures assayed. In addition, the operational stabilities of the PCMCs depended on the solvent utilized for storage or usage. Thus, in propanol/1 wt.% H₂O, subtilisin Carlsberg PCMC could be reused for 5 cycles maintaining almost 85% of the initial activity while using acetonitrile/1% (*v/v*) H₂O or tetrahydrofuran/1% (*v/v*) H₂O, a fast loss of activity could be observed within 4 h in a continuous flow reactor. In this medium, the inactivation rate slowed down after these first 4 h and after 96 h of continuous use only a small additional deactivation of the PCMCs was observed. That is, PCMCs stability were very adequate under certain conditions [121]. In a further research effort [122], the same group prepared PCMCs of horse liver alcohol dehydrogenase, catalase, soybean peroxidase, and horseradish peroxidase, using as salt K₂SO₄ and used to catalyzed reactions in organic medium. PCMCs of most of the studied enzymes increased their activity in most of the reactions when compared to the enzyme powder (e.g., 50-fold using horse liver alcohol dehydrogenase, 25 fold using catalase). Horseradish peroxidase was an exception, maintaining the activity of the powder in thioanisole oxidation, while increasing the activity 18-fold in the case of guaiacol oxidation in propanol. Moreover, the observed enzyme hyperactivation depended on the solvent used in the reaction. The stability of the different PCMCs was also studied. Catalase PCMC in a propanol suspension containing 1% (*w/v*) H₂O for 4 months at room temperature maintained only 15% of the initial activity. However, this activity was higher than that of fresh catalase powder [122]. One further advantage of this immobilization system is that it is possible to add NAD⁺ to the enzyme initial solution because it co-precipitated with the enzyme and the salt, and then remains in the enzyme nano-environment. This co-immobilization of enzymes and cofactors in organic medium had been previously reported [123], and although nowadays there are systems to co-immobilize

them even to be used in aqueous media [124], this possibility retains interest [122]. This research group also developed a technique to determine the number of intact active sites in proteins after immobilization using PCMC strategy [125]. As model enzyme, they employed subtilisin Carlsberg that was covalently inhibited employing phenylmethylsulfonyl fluoride using lyophilized enzymes and PCMCs, using the electrospray ionization mass spectrometry in order to calculate the percentage of modified enzyme molecules.

Professor Gupta's group took over the research using this immobilization strategy. In an initial publication [126] lipase from *Pseudomonas cepacia* was immobilized using different strategies, such as immobilization on Accurel via interfacial activation [50], cross-linked enzyme aggregates [58,127], lyophilization and PCMCs [126]. Then, they used their home-made biocatalyst and the commercial preparation of the enzyme to perform the synthesis of biodiesel using the oil from *Madhuca indica*, which contains a high free fatty acid content in a solvent-free reaction system. The enzyme immobilized in Accurel gave 96% conversion in 6 h, the cross-linked enzyme aggregates led to 92% conversion in 2.5 h and the PCMCs provided 99% conversion in 2.5. That way, PCMC biocatalysts permitted higher yields and more rapid reaction courses than any of the other biocatalysts, using equivalent amounts of immobilized enzymes [126]. After this success, the research group prepared enzyme precipitated, PCMCs and crosslinked enzyme aggregates of lipases from *Candida rugosa* and *Burkholderia cepacia* and tested them in the kinetic resolution of (\pm)-1-phenylethanol using ionic liquids as reaction media ([Bmim][PF₆]) [128]. They studied the transesterification using vinyl acetate as activated acyl donor. Lipase from *Candida rugosa* was the one that exhibited the best performance and the best results were obtained using its PCMCs, with a conversion yield of 53% and an enantiospecificity value of 79 [128]. Later on, they immobilized the lipase from *Pseudomonas cepacia* using PCMCs and ammonium sulfate as crystal forming component [129]. The structures of the particles were analyzed by atomic force microscopy, confirming the initial results on this technique: there were crystals of potassium sulfate coated by the lipase. The dimensions of the particles ranged between 500 and 1000 nm. Then, the PCMCs biocatalysts were used in biodiesel production. The enzyme activities were significantly improved after immobilization (96% conversion versus 8% using the aggregated enzyme at the same reaction time). The free enzyme was deactivated at 60 °C, whereas the PCMC preparation retained almost all enzyme activity after 10 h at this temperature [129]. In a further research effort, they compared enzyme precipitated and rinsed with *n*-propanol and PCMC with lyophilized chymotrypsin, finding much higher activities for the first immobilized preparation forms [130]. They also described that trehalose co-precipitated with the enzyme, even though this sugar did not precipitate alone in propanol. This trehalose co-precipitation improved by more than 2.5-fold the transesterification activity of the enzyme PCMC in octane, but not in acetonitrile. Using higher concentrations of enzyme precipitated and rinsed with *n*-propanol, it was possible to produce PCMCs with the sugar, leading to higher transesterification rates of the biocatalyst in both acetonitrile and octane media. The authors found that the trehalose co-precipitation produced smaller PCMC particles sizes. In the case of PCMC, the increase in enzyme activity was attributed to an increase in the surface area of the biocatalyst [130].

Some other research groups have also used this immobilization strategy. For example, PCMCs of the lipase from *Humicola* sp were used in the preparation of chiral bicyclo [2.2.2]octane-2,5-dione via Diels-Alder reaction and lipase resolution of an enol acetate derivative [131]. Lyophilized lipase led to low specificity and activity, while PCMCs improved the results, but still the enzyme specificity was not good enough [131]. In another paper, lipase from *Pseudomonas aeruginosa* was immobilized following the PCMC strategy, using *n*-propanol as solvent and K₂SO₄ as crystal forming agent [132]. The formation of real PCMCs was confirmed by transmission electron micrographs and it was observed that the immobilized enzyme was more thermostable than the free enzyme. Using the hydrolysis of *p*-nitrophenyl palmitate in *n*-heptane as model reaction, the optimal temperature increased from 40 °C to 45 °C after immobilization and the catalytic efficiency was enhanced tenfold [132]. In another research effort, lipase from *Mucor javanicus* was immobilized via PCMC strategy using different

salts as crystal growing agents [133]. These PCMCs were not active in the esterification reaction between propanol and lauric acid in *isooctane* when Na_2SO_4 or NaCl were used as the crystal forming agents. However, they were more active than the enzyme powder when K_2SO_4 (5.8 times) or KCl (10.0 times) were used as the crystal forming agents and propanol was used as dehydration agent. Maximal activities were found using acetonitrile as precipitating and dehydrating solvent (22.4 fold using K_2SO_4 and 26.2 fold employing KCl). These biocatalysts maintained the activity shown at 40 °C when using 80 °C, while free enzyme was almost fully inactive under these drastic conditions. The strategy was also applied to the lipase from *Candida rugosa*, where changes in the enantiospecificity in the transesterification of 1-phenylethanol with vinyl acetate in hexane were not detected [133].

Later, lipase from *Thermomyces lanuginosus* was immobilized following this strategy and utilized in biodiesel production [134]. PCMCs were prepared using acetone as solvent and K_2SO_4 as crystal forming agent. Optimal PCMCs were obtained using a solution at 60% of the saturation concentrations of K_2SO_4 . The authors optimized the biodiesel production, using refined edible grade palm oil, crude palm oil, or palm fatty acid distillate as raw materials. Thus, they reported that the use of *tert*butanol increased the biocatalysts activity and stability, enabling a higher production of esters (89.9% yield using the most refined oil and equimolecular amounts of methanol and fatty acid esters). Using crude palm oil, the yields decreased to 82.1%, while using palm fatty acid distillate (the one contained the highest amount of free fatty acids), the yields decreased to 75.5% yield. Rinsing the biocatalysts with *tert*butanol after each reaction cycle allowed to increase the operational stability of the biocatalysts (after 8 reaction cycles, only a slight decrease in enzyme activity was observed) [134]. In another paper, lipase from *Pseudomonas cepacia* was immobilized following the PCMC strategy, using K_2SO_4 as crystal forming agent [135]. The biocatalyst preparation was optimized via single factorial experiments and response surface methodology. The optimized PCMC showed high stability at high temperatures and in the presence of organic solvents, and it also presented a high transesterification activity. The actual production of PCMC was confirmed via scanning electron microscopy (SEM). This PCMC was used in the biodiesel production, giving more than 83% yield for most of the seven oils tested, and in some instances values over 99% were achieved. The use of the biocatalysts for eight reaction cycles produced a decrease in the conversion of only 15% [135]. Again, very good results could be found using this immobilization technique in biodiesel production.

In another research effort, PCMCs of the lipase from *Rhizomucor miehei* were prepared and characterized [136]. The crystal forming agent employed was K_2SO_4 , and the dehydrating solvent was acetone. The production of lipase-PCMCs was verified by scanning electron microscopy. After optimization response surface methodology, the immobilized biocatalyst was more active and stable during *p*-nitrophenyl palmitate hydrolysis in *n*-hexane than the free enzyme, increasing the optimal temperature from 30 to 37 °C (this means an increase in maximal activity of almost 800-fold). The biocatalyst was used to produce 2-phenethyl octanoate. After optimization, a yield of 80% in 1 h was obtained, clearly surpassing the results obtained with the commercial Lipozyme RM-IL [136]. In another contribution, lipase from *Candida rugosa* was used to produce PCMCs [137]. The preparation of the biocatalysts was optimized via single-factorial experiments (the studied variables were precipitating solvents, pH, saturated K_2SO_4 solution, and water content). The optimized biocatalyst was used to catalyze the esterification of *isooctanol* and (*R*, *S*)-ibuprofen, giving a yield of 49.83% (50% should be the maximum conversion for a perfect kinetic resolution) and an enantiomeric excess of 97.34%. The activity was almost 5.8-fold higher than using lipase powder. The operational, thermal, and cosolvent stabilities were also highly increased. Fourier transform infrared spectroscopy revealed the correlation between enzyme conformation and enzyme activity enhancement. This PCMC biocatalyst could be reused for 15 reaction batches without suffering any decrease in activity [137].

Yildirim and co-workers reported some examples of the utilization of this immobilization strategy. In the first one, hydroxynitrile lyase from *Prunus armeniaca* was used to produce PCMCs using K_2SO_4 as crystal growing agent and acetone as dehydrating solvent [138]. The biocatalyst was used in the synthesis of (*R*)-mandelonitrile (yield 100%, enantiopurity 99.9%) in buffer-saturated

(at pH 4.0) methyl *tert*butyl ether. These results improved those obtained using the precipitated enzyme. The thermal and storage stabilities of the enzyme were also improved after immobilization. The crystals formation was confirmed and they presented a rectangular shape. The biocatalyst was reused five times, maintaining 75% of the initial activity, without significant changes in enantiomeric excess. Later, this research group immobilized the lipase from *Rhizomucor miehei* by two different techniques: PCMC production and adsorption on hexagonally-ordered nanoporous aluminium oxide membranes [139]. For PCMC, K_2SO_4 was used as crystal forming agent and pre-chilled acetone as dehydrating solvent. In hydrolytic reactions, PCMC was not only more active than both free enzyme and the enzyme immobilized in the membrane, but also more stable than the other preparations at 50 °C. The biocatalysts were used in the synthesis of geranyl acetate, heptyl acetate, hexyl acetate, *iso*amyl acetate, and butyl acetate, using vinyl acetate as activated acyl donor, to produce esters that can be used as aroma.

The interest of this immobilization protocol caused a research group to report the development of a pilot plant-scale for the industrial production of PCMCs of therapeutic proteins [140]. The authors describe a continuous manufacturing process able to produce grams to kilograms of PCMCs. The procedure chain encompasses three steps: mixing/precipitation, solvent reduction, and final drying. The overall process was intended to be published in two parts. Hence, in a first report, they described the mixing and precipitation steps employing continuous impingement jet mixers. The division of the anti-solvent flow into two or four jets improved the mixing efficiency, which were combined again inside the mixer to obtain a “sandwich effect” facilitating the embracing of the aqueous solution. The anti-solvent jets competently mixed the protein-carrier containing 5% water. The enhanced mixing performance of the double or quadruple jet impingement mixers exhibited positive effects on easily crystallizing crystal forming agent (e.g., *DL*-valine) at laminar flow rates. If using low flow rates, mixing could be improved by ultrasound [140]. Unfortunately, we have been unable to find the second part of this engineering paper.

In one interesting paper, the authors developed a system to measure immobilization of the enzyme in a microtiter plate to utilize high-throughput screening using heterogeneous biocatalysts [141]. This has special interest because immobilization may tune the enzyme properties in unpredictable ways [44], and the best immobilized enzyme for one application may not be the most adequate for another application since immobilization may greatly alter enzyme specificity and selectivity [142–144]. The concept was developed using esterases from *Pseudomonas fluorescens*, *Streptomyces diastatochromogenes* and *Bacillus subtilis*, produced in *E. coli*. To check the “universality” of the technique, the enzymes were immobilized on a battery of different supports including supports where covalent attachment occurs and other supports where just physical adsorption caused the enzyme immobilization, (Eupergit C, an epoxy activated matrix, bentonites, Celite and Hyflo Super Cel) and also some carrier-free techniques, such as crosslinking enzyme aggregates and PCMCs methodologies [141]. The synthetic activities of the immobilized esterase preparations were analyzing the alcoholysis reaction of *p*-nitrophenyl acetate in propanol, the hydrolytic activity was determined in the hydrolysis of the same substrate in aqueous buffer. The main problem in this strategy was to get a uniform distribution of the solid biocatalysts in each well of a microtiter plate. [141]. This was solved using a device prepared from Plexiglass[®], which contained 3 × 8 holes arranged in a similar fashion to the wells of a 96-well microtiter plate. For application of the samples, this device was located on top of the microtiter plates separated by a paper sheet and the holes were filled with the carrier material. After elimination of the excess of support, the sheet of paper was detached to transfer the carrier material to the microplate [141].

Focusing on PCMCs optimization, they were prepared by mixing the enzyme and the saturated salts and later adding this solution to the dehydrating solvent previously located in the plate, under gentle stirring [141]. As salts or crystal forming agent, they used potassium oxalate, Na_2EDTA , $NaCl$ K_2SO_4 and KNO_3 , while as solvents they employed propanol or ethanol. The specific activity of the PCMCs decreases when the enzyme loading increases. The highest activities were obtained using Na_2EDTA as

crystal growing agent and ethanol as dehydrating solvent. That way, this technique could be applied to optimize this immobilization protocol, analyzing all the variables that can affect the final results. In fact, the PCMCs were the most active catalysts among the analyzed ones [141]. The authors declared that they could not use this strategy using some enzyme immobilization strategies, such as the sol-gel technique. Moreover, the adsorption of the enzymes onto EP100 failed because the centrifugations did not work to recover the immobilized biocatalysts. The authors even showed that it was possible to alter the immobilization conditions using Eupergit C as a model support, where the immobilization and the incubation conditions to get an intense multipoint covalent attachment differs [145,146].

That way, this technology may be applied to most immobilization strategies, even if they required some multi-step protocols, such as multipoint covalent attachment on glyoxyl-supports where a final reduction step is required [147]. This technique may also be applied to immobilization protocols where immobilization conditions and incubation conditions may not be identical, such as immobilization using glutaraldehyde [42,148–152], vinyl sulfone [153,154] or the already mentioned epoxide supports. If the immobilization protocol requires a blocking step, this may expand the range of likely immobilization protocols applied to the enzyme, and that way take profit of this high-throughput screening strategy. The enzyme tuning through blocking of epoxy [155] and vinyl sulfone supports with different compounds having very different physical properties has been recently reported [156–159]. In any case, the system worked in the PCMCs immobilization in a very efficient fashion, facilitating its optimization [141].

PCMCs have been used for other applications different from the preparation of catalysts. For example, the Parker group utilized this strategy for a fully different goal. Adenylate cyclase toxin, a virulence factor of *Bordetella pertussis* was formulated as a PCMC as cellular pertussis vaccines [160]. The PCMCs were prepared using D/L-valine as crystal forming component. They maintained adenylate cyclase and cell invasive activities after its solubilization in urea buffer, and could be stored at 37 °C for 2 weeks. However, to get the adenylate cyclase activity, it was required to use buffers with urea in the reaction. This could be solved if bovine serum albumin and/or calmodulin were added during the preparation of PCMC. However, this gave a low cytotoxicity of the re-dissolved toxin. In any case, after reconstitution in an aqueous buffer, the PCMCs induced a strong serum IgG response to adenylate cyclase toxin when injected subcutaneously into mice [160]. In another paper, several model antigens were analyzed as potential vaccines when used as PCMC [161]. Results suggested that the use of antigens-PCMCs increased the antigen-specific IgG responses compared to the use of soluble antigens. PCMCs were modified with calcium phosphate to compare this formulation to a conventional aluminium-adsorbed one. They showed a decreased antigen-specific IgG1:IgG2a ratio and enhanced antigen-specific IgG responses, indicating a more balanced Th1/Th2 response. PCMCs modified with CaP increased the phagocytosis by monocyte/macrophage cells compared to soluble antigen or standard PCMCs.

In another paper, bacteriophages were processed by preparing PCMC [162]. To reach this goal, *Siphoviridae* was mixed with glycine or glutamine as crystal forming agent, and added to *isobutanol* or *isopropanol*. The phage-PCMC was collected by filtration and the solvent was eliminated by just air-drying. The feeding of the precipitation mixture with albumin or trehalose permitted a higher phage stability during co-precipitation. The PCMC phage composite was stable at room temperature for one month, leading to better results than the ones obtained using the lyophilization or the immobilization into polyester microcarriers [162].

That way, PCMC preparation seems to be an interesting way to immobilize the enzyme to be used in organic media with a low to moderate content in water (even 20%). Moreover, the performance of these PCMCs could be enlarged by coupling the immobilization to the chemical modification, as will be discussed in the next section.

4.1. Chemical Modification of PCMCs

As pointed out in our introduction, in some instances chemical modification and enzyme immobilization may be integrated to get an optimized enzyme biocatalyst [20–22], e.g., to get some intramolecular crosslinking that can increase the enzyme stability [163,164] or some intermolecular crosslinking that prevents the enzyme release from the support [165,166]. In other cases, just one point modification of some key groups is intended [10–12].

This has also been the case in using PCMCs; chemical modification has been used for different objectives, even if the biocatalysts should always be managed in organic media.

4.2. Chemically Crosslinked PCMCs

As an evolution of the PCMCs, Professor Gupta's group proposed a new strategy consisting of the treatment of the PCMCs with glutaraldehyde to get the production of crosslinked PCMCs (CLPCMCs) (Figure 7). This protocol was first the subject to a patent [167] and lately shown in a research paper [168]. In this publication, the researchers prepared PCMCs of subtilisin Carlsberg and lipases from *Burkholderia cepacia* and *Candida rugosa*, using as crystal forming component K_2SO_4 and propanol (for subtilisin) or acetone (for lipases) as dehydrating solvent. Then, glutaraldehyde was added to get the crosslinking. Subtilisin was assayed in the transesterification of *N*-acetyl-*L*-phenylalanine ethyl ester with propanol, obtaining an increase in the activity by 1.23 or 2.33 factor (using *tert*amyl alcohol) after PCMC preparation. Lipases were assayed in the transesterification of 1-hexanol and tributyrin. Using the lipase from *Candida rugosa*, the activity increased almost 4.5-fold using octane as reaction medium, while for other solvents the activity improvements were marginal. When analyzing the lipase from *Burkholderia cepacia*, the improvement in enzyme activity was clear in all solvents by a factor of around 2. The authors proved that this increase in activity was related to the higher stability of CLPCMCs, as differences in initial rates increased when the reaction temperature increased [168]. This research group immobilized lipase B from *Candida antarctica* as cross-linked enzyme aggregates, PCMCs (using potassium sulfate as crystal forming agent) or CLPCMCs [169]. The biocatalysts were employed as catalysts in the esterification of glycerol and palmitic acid in acetone at low water activity. The highest monoglycerides yields were obtained CLPCMC (87% versus 81–82%), with lower production of diglyceride (3.3% versus 4.5–4%) [169]. These authors investigated the structures of chymotrypsin and subtilisin immobilized as enzymes precipitated and rinsed with propanol, crosslinked enzyme aggregates, PCMCs or CLPCMCs [170]. The catalytic activity of all these preparations was analyzed in the transesterification of *N*-acetyl-*L*-phenylalanine ethyl ester and *n*-propanol in a solvent free system or using 1 M propanol in octane. Then, the structure of the different immobilized preparations was determined by infra-red spectroscopy or circular dichroism. They found that adding the aqueous enzyme solution to the propanol was better than those obtained when inverting the addition order in the PCMC preparation [170]. They also found that the glutaraldehyde addition to the aggregated enzymes produced a decrease in activity, while the production of CLPCMC permitted to get an increased activity. This was attributed to the fact that, by crosslinking the aggregates, the structure of the particles became denser and the diffusional problems increased. The crosslinking the PCMCs produced greater conformational changes, but their activities were preserved, perhaps because of the higher enzyme stability. PCMC presented better maintenance of the enzyme conformation than the precipitated enzyme, explaining the high activity of these enzyme formulations [170]. In another paper, this research group compared different formulations of the lipase B from *Candida antarctica* (the commercial immobilized preparation Novozyme-435, lipase precipitated and rinsed with acetone, PCMC and CLPCMC) in a promiscuous reaction, a decarboxylative aldol reaction for the synthesis of 4-hydroxy-4-(4-nitro-phenyl)-butan-2-one [171]. The substrates were 4-nitrobenzaldehyde and ethyl acetoacetate. After showing that the reaction proceeds using the active enzyme but almost did not proceed using denatured enzyme, they compared the different enzyme immobilized biocatalysts. The use of CLPCMC or PCMC gave the highest reaction rates, 90% conversion was obtained in

acetonitrile after 24 h in the presence of imidazole. However, the enantioselectivity of the reaction was poor [171].

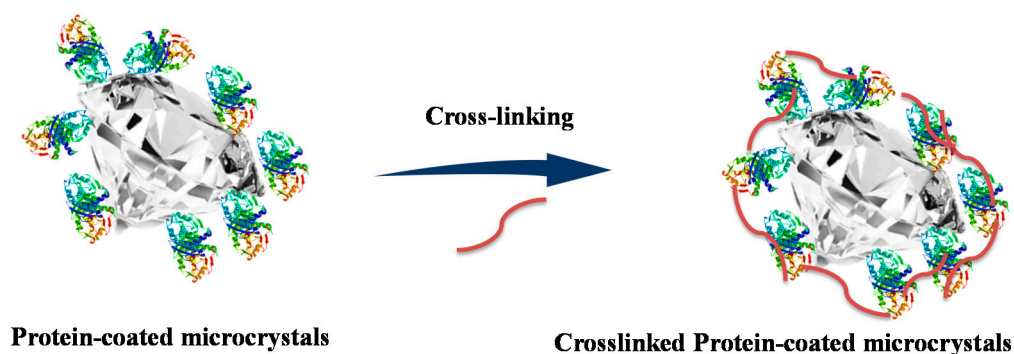


Figure 7. Schematic representation of the production of CLPCMC.

Later, the lipase from *Thermomyces lanuginosus* was used to produce CL-PCMC and utilized in the esterification of palm fatty acids and ethanol [172]. They optimized the preparation of the biocatalysts, finding that the best activity was obtained using glycine as crystal forming agent and acetone as dehydrating solvent. This optimized PCMC was later crosslinked with glutaraldehyde. This optimal biocatalyst permitted us to reach 87.2% ester yield using palmitic acid and 81.4% using industrial palm fatty acid distillate. These results surpassed those obtained using Novozyme[®]435 (one usual golden biocatalysts [173]) or Lipolase 100T (immobilized commercial form of this lipase). The CLPCMC-lipase could be employed for 8 consecutive reaction cycles without decreasing its activity. The glycine-based microcrystalline lipase is thus a promising alternative for this reaction. The research was continued, analyzing the importance of the pK of the buffering element used as crystal forming agents [174]. The biocatalysts were used in the esterification of palmitic acid and transesterification of refined palm oil, with the final objective of using the biocatalysts in the production of biodiesel using crude vegetable oil with high free fatty acid content, where both esterification and transesterifications reactions play a relevant role. This way, the lipase was immobilized following the CLPCMC strategy using different forming crystal forming agents, reporting the best results using glycine, 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid or [(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)amino]-1-propanesulfonic acid. The use of *tert*butanol as a cosolvent prevented the adsorption of glycerin on the biocatalysts surface produces its inactivation. Using an excess of 4 mols of methanol per mol of fatty acid, maximal ester yields of around 95% were obtained for the 3 substrates in only 6 h. The biocatalysts could be reused for five cycles retaining around 80% of the initial activity [174].

This technology was assayed to immobilize a bioimprinted lipase from *Burkholderia cepacia* [175]. The enzyme bioimprinting was performed by adding free fatty acids before the precipitation of the enzyme. The fatty acids could act as detergent-like compounds, facilitating the stabilization of the open form of the lipase, as described in many papers [176–180]. *p*-Benzoquinone was used as the cross-linker for preparing the bioimprinted CLPCMC. This biocatalyst maintained 84% of initial activity after 4 h of being incubated at 80 °C in *n*-hexane/ethanol, while the non-imprinted biocatalyst only retained 63% of its initial activity. Scanning electron microscopy confirmed the production of crystals, with a smaller size than that of the non-imprinted biocatalysts. Imprinted and non-imprinted biocatalysts were used in the production of biodiesel from ethanol and *Jatropha curcas* L. oil, including in the study Novozym 435 (as “golden” biocatalysts). Furthermore, imprinted-CLPCMC was used as biocatalysts for producing biodiesel from *Jatropha curcas* L. oil. The imprinted biocatalyst gave similar yields to Novozym 435 (around 95%), while the non-imprinted biocatalyst gave only 85%. However, both home-made biocatalysts showed a poor operational stability. The authors observed that just the stirring of the enzymes in the reaction medium promoted an inactivation, which was

attributed to the action of shearing force on the enzyme molecules because the enzyme is located on the support surface [175]. This problem is general for any nanomaterial where the enzyme is located on the surface [181] and could be at least partially solved if coating the enzyme molecules with some polymers [181–185].

However, the researchers did not analyze some possibilities of these CLPCMCs biocatalysts. For instance, they did not analyze if the glutaraldehyde treatment produced just crosslinking between immobilized enzyme molecules in the same particle or if it produced crosslinking between enzymes located in different crystal particles, producing a superstructure of PCMCs. Neither has it been checked if these CLPCMCs could be used in aqueous media in case the crosslinking were efficient perhaps a monolayer of crosslinked enzyme molecules could be retained even in aqueous media.

4.3. Modification of the Enzyme before PCMCs Production

In just one example, the enzyme molecules were chemically modified before being immobilized using the PCMC strategy, and also to alter the CLPCM production strategy. In this example, the lipase from *Pseudomonas cepacia* was acylated with pyromellitic dianhydride, modifying 72% of primary amino groups in the enzyme [186]. The use of 1,2-Dimethoxyethane as dehydrating solvent permitted to precipitate 97% of the protein and the precipitate retained the full enzyme activity. Then, the researcher prepared PCMC, CLPCMC, and enzyme precipitated and rinsed with organic solvents. The different biocatalysts of modified and unmodified lipase were employed as catalysts of transesterification reactions, using dimethyl formamide or octane as reaction medium. In *n*-octane, the most active biocatalyst was the one prepared using CLPCMC of modified lipase (more than 10 folds more active than the least active one). In dimethyl formamide, CLPCMC of modified enzyme was the most active, while the unmodified lipase was fully inactive [186].

4.4. Enzyme Co-immobilization

Enzyme co-immobilization is becoming increasingly important in enzyme technology, growing its interest when the number of cascade reactions growth: in these reactions, the substrate of the second enzyme is the product of the first one, and that way a reaction chain may be armed, simulating the metabolic chains [187–189]. The interest of enzyme coimmobilization lays in the fact that both enzymes are working in a confined space, therefore the second enzyme can be under substrate saturation conditions almost from the first reaction moments, avoiding the lag time that occurred using individually immobilized enzymes or even free enzymes. This lag time is the time where the second enzyme is working at concentrations lower than the saturation ones (that even perhaps cannot be reached in the whole process in certain cases). This fact greatly alters the reaction initial rates, but the incidence in the whole reaction course may be reduced [35,44]. However, in some instances, this is a requirement not related to the shortening of the reaction course, but with the final yields, e.g., when some of the intermediate products are not stable and can lead to another compound different from the target, producing a decrease in the reaction yield and a complication on the final purification of the target compound, as contaminant compounds will appear in the final reaction medium [35,44]. However, as recently reviewed, co-immobilization presents many problems (e.g., necessity of using the same support surface, different stability of the coimmobilized enzymes) [190,191], and the researcher must analyzed if the co-immobilization problems compensate the advantages (for a revision of this topic, consult [192]).

Professor Gupta's group used PCMC immobilization protocol to coimmobilize several enzymes [193]. They used this protocol to produce PCMC and CLPCMC of subtilisin Carlsberg and lipase B from *Candida antarctica* and lipase from *Rhizomucor miehei* or the coimmobilization of some of these enzymes. In the case of full modification of multifunctional substrates, such as oils, enzyme specificity can become a problem, and the use of several enzymes with different specificities may be an optimal solution, as show in the development of the concept of the so-called combilipases [194]. The combilipase obtained using by co-immobilizing both lipases was utilized to produce biodiesel from

oil of spent coffee grounds, with better results than those obtained using the individual biocatalysts. However, the authors did not show the advantage of using the co-immobilized combilipases and not just the mixture of both individually immobilized PCMC enzymes. Moreover, also some coimmobilized biocatalysts of protease and lipases were prepared, for non-cascade reactions [193]. These biocatalysts, called multipurpose biocatalyst [195–197], have unclear advantages and many disadvantages [192].

5. Conclusions

The immobilization of enzymes via PCMC is a very simple and apparently useful strategy to immobilize enzymes mainly to be applied in organic medium. This strategy has some versatility, as the final properties of the biocatalysts will be defined by the growing crystal agent and the solvent used as dehydrating agent, together with other factors such as enzyme concentration. The salt used in the immobilization may act as water reservoir and solid pH buffer, both very important features in enzymes used in organic media. Very interestingly, the medium where the enzyme is precipitated may also be tuned, adding some additives to preserve better the enzyme activity during the preparation of the PCMC. The final results are crystals where the enzyme is deposited on their surface. However, there are no proper studies to understand the nature of the forces that immobilize the enzyme on the surface of the enzyme, or the reason that makes the enzyme only able to be located on the surface of the crystal and not in the core. This enzyme location has some interest, as it reduces the likely diffusional limitations. The PCMC strategy could be applied to any enzyme that can remain active and stable in the presence of high salt concentrations and dehydrating agents, and may be later used in any organic medium with a water content where neither the crystal growing agent nor the enzyme are soluble. This immobilization strategy has been mainly utilized for enzymes usually employed in organic media, such as proteases or lipases, but it has been extended to other enzymes such as redox enzymes. The enzymes immobilized using this strategy used to have higher activity and stability than other enzyme preparations in organic media; a proper study on the reasons of this is also lacking. For example, perhaps the enzymes are in the surface of the crystal but partially surrounded by the salt crystal. This could decrease enzyme mobility, and in that way increase enzyme stability. The technique has also been shown to permit the coimmobilization of enzymes and cofactors, with the positive impact that this can present in the implementation of many reactions requiring these complex and expensive molecules. Furthermore, this technique may be used to coimmobilize enzymes, as shown in the preparation of combilipases utilized in the production of biodiesel, or the multifunctional lipase/protease biocatalyst. Additionally, PCMC technique is compatible with the chemical modification of the immobilized enzyme, which has been used to produce CLPCMC, a way to further increase the enzyme stability via inter and intramolecular crosslinking. Moreover, the only residue generated after enzyme inactivation and biocatalysts disposal will be inactivated protein, and the growing crystal agent, although very likely this can be recovered if adequate strategies are developed. The solvent used as dehydrating agent may be also recovered and reused, although there are no studies in this regard.

That way, PCMC appears as an immobilization technique that should have a great impact in enzyme immobilization and biocatalysis; it is versatile and cheap, it does not produce residues and it may be used with many enzymes. However, the strategy is under-utilized, in fact many investigations on the potential of this technique are lacking, and even the understanding on this immobilization strategy is not fully understood.

Among unexplored possible applications, we can remark, for example, that the possibility of preparing CLPCMC by crosslinking of PCMC using different crosslinkers has been described, and this strategy has proved to increase the enzyme stability. However, there are no investigations on the possibility of using these crosslinked immobilized biocatalysts in aqueous media, some kind of “crosslinked planar enzyme” structure could be obtained after dissolution of the crystal growing factor. If the intermolecular crosslinking is properly developed to include most of the enzymes in the PCMC, this could give a kind of “empty globe” formed by crosslinked enzyme molecules, which could

find many different applications. This massive inter-protein crosslinked may be achieved using dextran aldehyde instead of glutaraldehyde [182], and fully protein loaded PCMC to make easier this crosslinking.

Moreover, we have been unable to find any paper where the PCMC has been prepared by mixing and co-precipitating the enzyme and some ionic polymers, (such as polyethylenimine [183]) when the polymers have been shown as a very good strategy to have an intense crosslinking in the CLEAs production [198–200], and in many immobilized enzymes, the coating with ionic polymers has proved to be an efficient way to avoid the enzyme exposition to external interfaces [201,202], an strategy to produce partition of deleterious compounds, or as a strategy to prevent enzyme release from the particle via intermolecular enzyme crosslinking [165,203].

These biocatalysts have been assayed only in organic solvents and not in other non-aqueous media systems, such as supercritical fluids or deep eutectic solvents. Neither the possibilities of trapping these crystals in larger particles of a material easier to manipulate have been assayed, as it has been performed using crosslinking enzyme aggregates [204–206]. Neither magnetic PCMC has been reported. This way, a large way remains ahead to take full advantage of this immobilization technique.

The reasons for this underutilization of PCMC strategy may be due to the fact that they were launched almost simultaneously to the very successful crosslinking enzymes aggregates (Figure 5), and that could have eclipsed them [206]. Another possibility is that the word “crystals” in the name causes many researchers to think that it is another version of the expensive and complex chemically crosslinked enzyme crystals (Figure 2). However, they have such high potential that we are sure that they should become one of the most used strategies to prepare immobilized enzymatic biocatalysts to be used in organic medium in the medium term. The full extent of the possibilities of this strategy, almost 20 years after its initial launching, are difficult to foresee.

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