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Cross-Linked Enzyme Aggregates as Industrial Biocatalysts

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Abstract:

The immobilization of enzymes as cross-linked enzyme aggregates (CLEAs) and their applications in industrially relevant biotransformations are reviewed. The preparation of CLEAs involves precipitation from aqueous buffer followed by cross-linking with a bifunctional reagent, usually a dialdehyde such as glutaraldehyde or dextran polyaldehyde. The technique is exquisitely simple and broadly applicable. CLEAs have several benefits in the context of industrial biocatalysis. They have a significantly enhanced shelf life and operational stability, are easy to recover and reuse, and are completely stable towards leaching in aqueous media. They circumvent the use of, often expensive, carriers and have high volumetric and catalyst productivities as a result of the absence of noncatalytic ballast. The applications of CLEAs for a wide variety of enzymes, including various types of hydrolases, oxidoreductases, and lyases in industrially relevant biotransformations, are discussed. Co-aggregation and cross-linking of two or more enzymes affords combi-CLEAs that are ideally suited for catalyzing one-pot, enzymatic cascade processes, for example, the successful application of a triple-decker combi-CLEA comprising a hydroxynitrile lyase, a nitrilase, and an amidase in the one-pot conversion of benzaldehyde to *S*-mandelic acid in >99% ee at 96% conversion. Finally, reactor concepts are discussed in the context of industrial applications.

1. Introduction

Biocatalysis has many benefits from the viewpoint of developing green and sustainable processes for the chemical industry.[1,2] Enzymes are derived from renewable resources and are 'natural', biocompatible, and biodegradable, thus avoiding the costs associated with removing residues of metal catalysts from products. Reactions are performed under mild conditions (ambient temperature and pressure at physiological pH) in water as solvent, in high chemo-, regio-, and enantioselectivities. Biocatalytic syntheses are generally more atom and step economical as well as less energy intensive and generate less waste than conventional organic syntheses. Notwithstanding these advantages, commercialization is often hampered by the lack of operational and storage stability of enzymes coupled with their cumbersome recovery and recycling and product contamination. These obstacles can generally be overcome by immobilization of the enzyme,[3,4] affording improved storage and operational stability and providing for its facile separation and reuse. Moreover, immobilized enzymes, in contrast to free enzymes which can penetrate the skin, are hypoallergenic.

Methods for enzyme immobilization can be conveniently divided into three types:[4] binding to a carrier, encapsulation in an inorganic or organic polymeric matrix, or by cross-linking of the protein molecules. Binding to or encapsulation in a carrier inevitably leads to dilution of catalytic activity and, hence, lower productivities (kg of product per kg of enzyme) owing to the introduction of a large proportion (90→99% of the total) of a noncatalytic mass. In contrast, cross-linking of enzyme molecules is a carrier-free method, and the resulting immobilized biocatalyst contains a high proportion of active enzyme.

Cross-linking of proteins via reaction of glutaraldehyde with reactive NH_2 groups on the protein surface was originally developed more than 40 years ago.[5] Such cross-linked enzymes (CLEs) are produced by mixing an aqueous solution of the enzyme with an aqueous solution of glutaraldehyde.[6] However, the CLEs exhibited low activity retention, poor reproducibility, and low mechanical stability and, owing to their gelatinous nature, were difficult to handle. Consequently, binding to a carrier became the most widely used methodology for enzyme immobilization. In the early 1990s Altus Biologics introduced the use of cross-linked enzyme crystals (CLECs) as industrial biocatalysts.[7,8] The method was applicable to a broad range of enzymes, and CLECs proved significantly more stable to denaturation by heat, organic solvents, and proteolysis than the corresponding soluble enzyme or lyophilized (freeze-dried) powder. Their operational stability, controllable particle size, and ease of recycling, coupled with their high catalyst and volumetric productivities, made them ideally suited for industrial biocatalysis. However, an inherent limitation of CLECs is the need to crystallize the enzyme, a laborious and costly procedure requiring an enzyme of high purity.

Several years ago we reasoned that crystallization could perhaps be replaced by precipitation of the enzyme from

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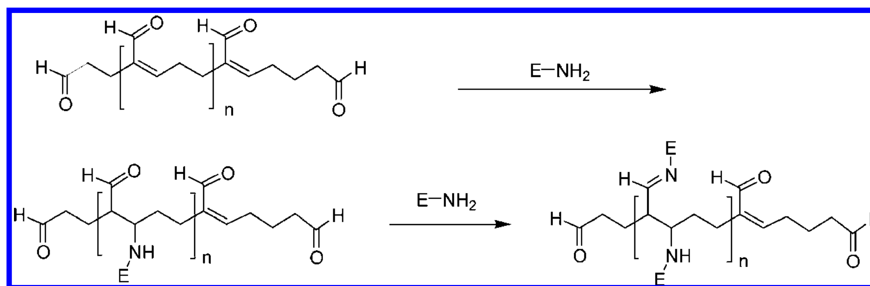

Figure 1

aqueous buffer, a simpler and less expensive method not requiring highly pure enzymes. This led us to develop a new class of immobilized enzymes which we called cross-linked enzyme aggregates (CLEAs).[9,10]

2. Cross-Linked Enzyme Aggregates: The Method

Addition of salts, or water miscible organic solvents or nonionic polymers, to aqueous solutions of proteins leads to their precipitation as physical aggregates that are held together by noncovalent bonding without perturbation of their tertiary structure. Addition of water to this precipitate results in dissolution of the enzyme. In contrast, cross-linking of these physical aggregates by reaction of reactive groups on the enzyme surface (e.g., free amino groups of lysine residues) with a bifunctional reagent such as glutaraldehyde renders the aggregates permanently insoluble while maintaining their pre-organized superstructure and, hence, their catalytic activity. Since precipitation is a commonly used method for enzyme purification, CLEA formation essentially combines purification and immobilization into a single unit operation.

Glutaraldehyde is generally the cross-linking agent of choice since it is inexpensive and readily available in commercial quantities. It has been used for decades for cross-linking proteins.[11,12] However, the chemistry is complex and not fully understood.[11] Cross-linking occurs via reaction of the free amino groups of lysine residues, on the surface of neighbouring enzyme molecules, with oligomers or polymers resulting from aldol condensations of glutaraldehyde. This can involve both Schiff's base formation and a Michael-type 1,4 addition to α,β-unsaturated aldehyde moieties[12] (Figure 1). The exact mode of cross-linking is also dependent on the pH of the mixture.[12]

Other dialdehydes, involving less complicated chemistry, can be used as cross-linkers. With some enzymes, e.g. with nitrilases, we sometimes observed low or no retention of activity when glutaraldehyde was used as the cross-linker but good activity retention was obtained using dextran polyaldehyde as a cross-linker,[13] followed by reduction of the Schiff's base moieties with sodium borohydride to form irreversible amine linkages. Galactose dialdehyde, formed by galactose oxidase catalyzed aerobic oxidation of galactose, has also been proposed as a potential protein cross-linker.[14]

Since every enzyme is a different molecule with a different number of accessible lysine residues, one would expect every enzyme to behave differently under cross-linking conditions. Indeed, cross-linking may be ineffective with enzymes containing few or no accessible lysine residues and lead to CLEAs that are unstable towards leaching in aqueous media. One approach that has been successfully used to overcome this problem is to cross-link coaggregates of the enzyme with a polyamine, such as polyethyleneimine.[15]

2.1. Optimization Protocols. Optimization of CLEA preparations involves examination of the effects of varying parameters such as temperature, pH, concentration, stirring rate, precipitant, additives. and cross-linking agent. This lends itself to automation, e.g. using 96-well plates.[16] The nature of the precipitant predictably has an important effect on the activity recovery, and hence, it is necessary to screen a number of water miscible salts, organic solvents, and polymers such as polyethylene glycols (PEGs). In the initial screening of precipitants, the amount of aggregates formed is determined and then they are redissolved in aqueous buffer and their activities are measured. However, we note that a high activity retention on redissolution in buffer does not necessarily mean that they will retain this high activity after cross-linking. The aggregates could contain the enzyme in an unfavorable conformation which is not retained when they are redissolved in buffer but will be preserved when they are cross-linked. Hence, it is advisible to choose a few precipitants which give good yields of aggregates for further screening in cross-linking. The optimum precipitant may not be the one that ultimately gives the optimum CLEA.

The ratio of cross-linker to enzyme is obviously an important factor. If the ratio is too low, sufficient cross-linking does not occur and insoluble CLEAs are not formed, and if it is too high, too much cross-linking occurs resulting in a complete loss of the enzyme's flexibility which is necessary for its activity. Since

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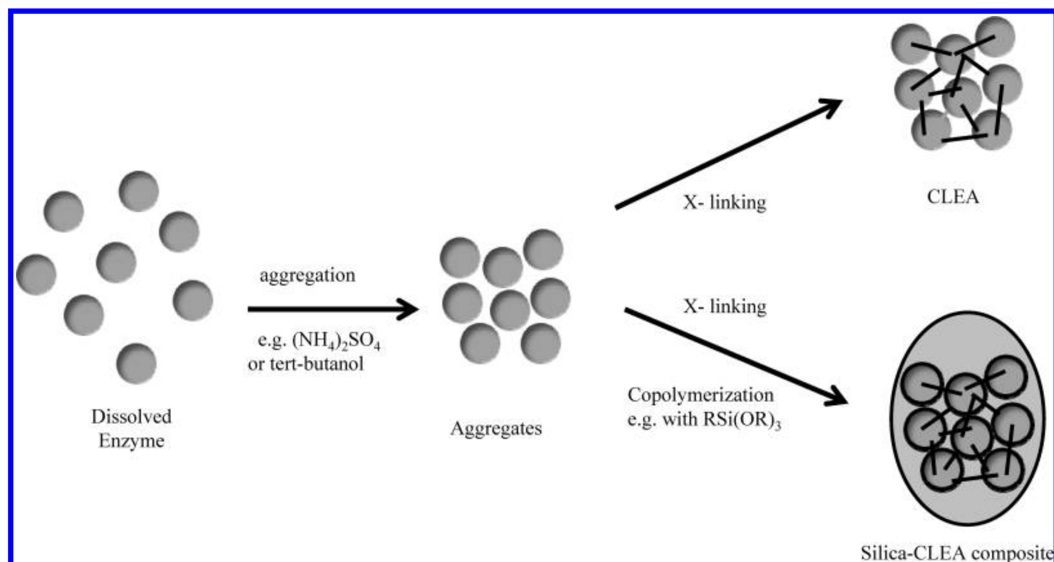

Figure 2

every enzyme has a unique surface structure, containing varying numbers of lysine residues, for example, the optimum ratio has to be determined for each enzyme.

The ratio of cross-linker to enzyme is also important in determining the particle size of CLEAs.[17] Particle size is an important property from the point of view of large scale applications since it directly effects mass transfer and filterability under operational conditions. A typical particle size of CLEAs is 5−50 μm, and their filterability is generally sufficient for batch operation. However, for certain large-scale applications it may be necessary to perform the reaction in a continuous operation mode over a packed bed of biocatalyst. This will require relatively large particles in order to avoid a large pressure drop over the column. One approach to preparing CLEAs with increased particle size and mechanical stability is to encapsulate them in a polyvinyl alcohol matrix (so-called Lentikats).[18] The resulting dilution of activity observed with a penicillin amidase CLEA was an acceptable 40%. In another variation on the standard CLEA protocol, hybrid silica−CLEA polymer composites were prepared by performing the cross-linking of the enzyme aggregates in the presence of alkoxysilanes as silica precursors (see Figure 2)[19] The hydrophobicity of the resulting composites can be tuned by an appropriate choice of alkoxysilane. This methodology can also be applied to the synthesis of CLEA−polymer composites from other inorganic or organic polymer precursors. In addition to providing the possibility to prepare CLEAs with the desired hydrophobic or hydrophilic microenvironments, the methodology also allows for tuning of their particle size.

An important advantage of CLEAs is that they can be prepared from very crude enzyme abstracts, probably even extracts obtained directly from fermentation broth. However, sometimes it is difficult to achieve CLEA formation from enzyme preparations containing low protein content. In such cases CLEA formation can be promoted by the addition of a second protein, such as bovine serum albumin, as a so-called proteic feeder.[20]

2.2. The Metrics of Enzyme Immobilization. Two parameters are of importance when comparing different immobilized enzyme preparations to the corresponding free enzyme: *activity recovery* and *enzyme loading*. Activity recovery is quite simply activity recovered divided by the activity charged expressed as a percentage and could involve an increase or decrease in weight of the CLEA compared to that of the free enzyme sample charged. Specific activity, on the other hand, is defined as an activity per unit weight of protein. Some authors divide the specific activity of the immobilizate by that of the free enzyme and report this percentage as activity recovery. We emphasize that this is not the true activity recovery. For example, in this way one could obtain an apparent high "activity recovery" owing to a substantial decrease in weight of the sample as a result of enzyme purification. We also note that a meaningful comparison of activities of free and immobilized enzymes is possible only when it involves *the same reaction under the same conditions*. This is relatively straightforward for a hydrolytic process in which the free enzyme is dissolved in water, e.g. tributyrin hydrolysis for a lipase. However, comparison becomes more problematical for a synthesis reaction, such as a lipase catalyzed (trans)esterification, in nonaqueous media where the free enzyme is not soluble. In this case many authors compare the specific activity of the immobilized lipase with that of the so-called acetone powder obtained by precipitation of the enzyme from aqueous solution with acetone. Another possibility is to compare it with the freeze-dried enzyme.

The variable production costs of a CLEA production are largely determined by the cost of the free enzyme, the labour costs, and the activity recovery. Obviously the activity recovery should preferably be close to (or more than) 100%. We hasten to add however that the enzyme costs per kg of product are

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also determined by how many times the CLEA can be recycled. The enzyme loading (weight percentage of enzyme in the sample) is important from the viewpoint of productivity (see below), and CLEAs have the advantage of high productivities compared to classical carrier-bound enzymes because of the much higher enzyme loading.

2.3. Advantages of CLEAs. CLEAs have many economic and environmental benefits in the context of industrial biocatalysis. They are easily prepared from crude enzyme extracts, and the costs of (often expensive) carriers are circumvented. They generally exhibit improved storage and operational stability towards denaturation by heat, organic solvents, and autoproteolysis and are stable towards leaching in aqueous media. Furthermore, they have high catalyst productivities (kg of product per kg of biocatalyst) and are easy to recover and recycle. Yet another advantage derives from the possibility to coimmobilize two or more enzymes to provide CLEAs that are capable of catalyzing multiple biotransformations, independently or in sequence as catalytic cascade processes.

As with all immobilized enzymes one might expect loss of activity owing to diffusional limitations in a solid catalyst. However, CLEAs are highly porous materials and we have generally not observed any diffusional limitations when using them in the biocatalytic transformations used in organic synthesis. In contrast, we have observed diffusional limitations in colorimetric assays which are usually very fast reactions. Consequently, activities based on such assays may lead one to the wrong conclusion that the CLEA has a low activity in the envisaged biotransformation. Obviously, the rate of diffusion is also influenced by the particle size, and as we noted above, this is in turn influenced by e.g. the cross-linker/enzyme ratio. Optimum rates are observed with smaller particles, but practical considerations, e.g. ease of filtration, dictate the use of larger particles. Hence, one is always looking for a compromise of good activity coupled with good filterability. Similarly, other physical attributes that effect recovery, handling, and recycability, such as hardness, density, porosity, and robustness of the CLEA particles, can be modified by preparing the silica−CLEA composites (see earlier) with varying hydrophobicity and particle size. This constitutes a trade off between enzyme loading and the desired physical properties. However, we note that the dilution of activity in a silica−CLEA composite is at least an order of magnitiude less that that observed in a typical carrier-immobilized enzyme.

2.4. Multi-CLEAs and Combi-CLEAs. Additional proteins can be incorporated into CLEAs by coprecipitation and cross-linking. This notion leads to formation of multipurpose CLEAs from crude enzyme extracts comprising multiple enzymes. For example, Gupta and co-workers[21] prepared a CLEA from a porcine pancreatic acetone powder extract containing lipase, phospholipase A_2, and α-amylase activity. All three enzyme activities were completely retained in the CLEA, and the latter could be recycled 3 times without appreciable loss of activity. Similarly a multipurpose CLEA exhibiting pectinase, xylanase, and cellulose activities was prepared[22] from the commercial preparation, Pectinex Ultra SP-L. The latter is used in the food processing industry for hydrolyzing pectin. This multipurpose CLEA could be used for carrying out three independent reactions: the hydrolysis of polygalacturonic acid (pectinase activity), xylan (xylanase activity), or carboxymethyl cellulose (cellulose activity). With all three enzymes the CLEA exhibited increased thermal stability compared to the free enzyme and it could be used three times without activity loss. We propose the name Multi-CLEA for such CLEAs that are made from heterogeneous populations of enzymes and can be used for performing different biotransformations independently.

On the other hand, we envisaged the deliberate coimmobilization of two or more enzymes in a single CLEA for the sole purpose of performing two or more biotransformations in sequence, i.e. as multienzyme cascade processes. We gave the name combi-CLEAs to such catalysts, and examples of cascade processes performed with such combi-CLEAs will be discussed later.

2.5. CLEAs from Hydrolases. The majority of the CLEAs that have been reported to date involve hydrolases, mainly because they are the enzymes that have the most industrial applications and are, hence, readily available in commercial quantities but also because they are probably the simplest enzymes to work with.

2.6. Protease CLEAs. We have prepared CLEAs from (chymo) trypsin (E.C. 3.4.21.4),[16] papain (E.C. 3.4.22.2), and the alkaline protease from *Bacillus licheniformis* (alcalase, E.C. 3.4.21.62, also known as subtilisin Carlsberg). The latter is an inexpensive enzyme used in laundry detergents. It has also been widely used in organic synthesis, e.g. in the resolution of (amino acid) esters,[23] and amines[24] and peptide synthesis.[25] It is perhaps not surprising, therefore, that alcalase CLEA has been used in amino acid and peptide biotransformations.[26−29] For example, Quaedflieg et al.[26] reported a versatile and highly selective synthesis of α-carboxylic esters of N-protected amino acids and peptides (Figure 3) using alcalase CLEA.

Alcalase CLEA was similarly used[27] in the synthesis of α-protected aspartic acid β-esters and glutamic acid γ-esters via selective α-hydrolysis of symmetrical aspartyl or glutamyl diesters or in a three-step protocol involving enzymatic formation of the α-methyl ester followed by chemical β-esterification and selective α-hydrolysis (Figure 4).

The same group also described[28] the use of alcalase CLEA, under near anhydrous condtions, to catalyze the mild and cost-efficient synthesis of C-terminal arylamides of amino acids and peptides by reaction of the corresponding free carboxylic acid, or the methyl or benzyl ester, with an aromatic amine (Figure

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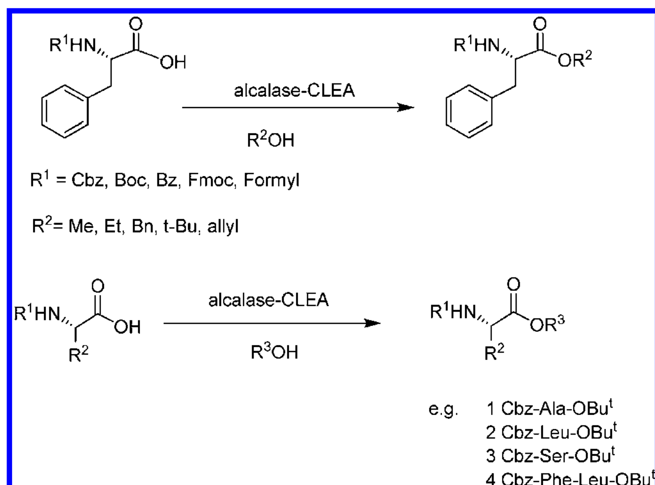

Figure 3

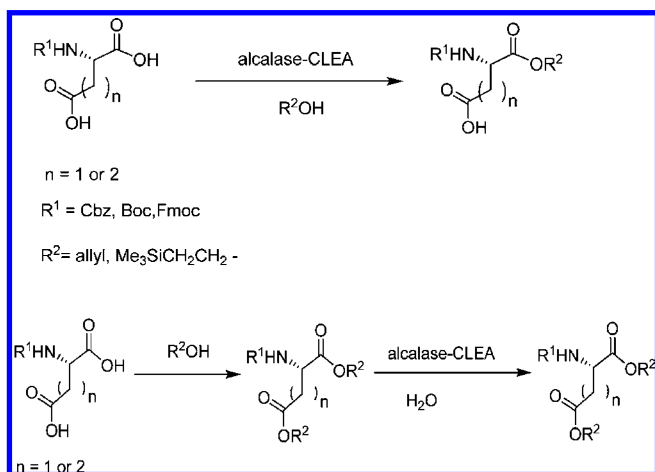

Figure 4

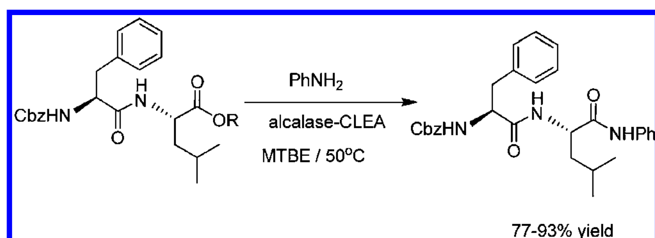

Figure 5

5). The products were obtained in high chemical and enantio- and diastereomeric purities without any racemization being observed.

Eggen and Boeriu[29] similarly reported the use of Alcalase CLEA for the selective enzymatic hydrolysis and amidation of C-terminal esters or acids of peptides.

2.7. Amidase CLEAs. Our initial studies of CLEAs[9,30] addressed the preparation of CLEAs from penicillin G amidase (E.C. 3.5.1.11), an industrially important enzyme used in the synthesis of semisynthetic penicillin and cephalosporin antibiotics.[31] The free enzyme has limited thermal stability and low tolerance to organic solvents, making it an ideal candidate for stabilization as a CLEA. Indeed, a penicillin G amidase CLEA, prepared by precipitation with *tert*-butanol, and cross-linking with glutaraldehyde, proved to be an effective catalyst for the synthesis of ampicillin (Figure 6).[9,30] Remarkably, the productivity of the CLEA was higher even than that of the free enzyme that it was made from and substantially higher than that of the CLEC. Not surprisingly, the productivity of the commercial catalyst was much lower, reflecting the fact that it mainly consists of noncatalytic ballast in the form of the polyacrylamide carrier. Analogous to the corresponding CLECs, the penicillin G amidase CLEAs also maintained their high activity in organic solvents.[32]

Similarly, a CLEA produced from a partially purified penicillin G amidase from a recombinant *Escherichia coli* strain was used by Illanes et al.[33] in the kinetically controlled synthesis of ampicillin in ethylene glycol−water (60:40 v/v). The same group[34] was used a penicillin amidase CLEA for the synthesis of the semisynthetic cephalosporin cephalexin in an aqueous medium (Figure 7). They also recently reported a study of the influence of the degree of cross-linking (0.15 or 0.25) on the performance of penicillin amidase CLEAs in cephalexin synthesis in aqueous ethylene glycol.[35] The cross-linker to enzyme ratio had only a minor effect on the specific activity of the CLEA but significantly influenced the activity recovery, the thermal stability, and the productivity in cephalexin synthesis, all being higher at the lower glutaraldehyde to enzyme ratio.

Svedas and co-workers[36] recently reported a study of the effect of "ageing" on the catalytic properties of penicillin amidase CLEAs in the hydrolysis of (*R*)-phenylglycine amide and the synthesis of ampicillin. The period of time between enzyme precipitation and cross-linking was found to influence the structural organization of the resulting CLEA, the "mature" CLEAs consisting of larger particles that were more effective in both the hydrolytic and synthetic process. They suggested that the aggregate size might regulate the extent of covalent modification and thereby the catalytic activity of the CLEAs.

We prepared a CLEA[37] from aminoacylase (EC 3.5.1.14) derived from an *Aspergillus sp.* which is the catalyst in the Evonik process for the manufacture of (*S*)-amino acids by highly enantioselective hydrolysis of *N*-acetyl amino acids (Figure 8). The aminoacylase CLEA was an active and recyclable catalyst for this reaction. Interestingly, it was unable to catalyze the hydrolysis of simple esters which is known to occur with the free enzyme preparation from which it was derived. A plausible explanation for this observation is that the esterolytic activity is derived from a protein impurity in the free enzyme preparation. We conclude that it demonstrates the power of the CLEA methodology for combining enzyme purification and immobilization into a single operation.

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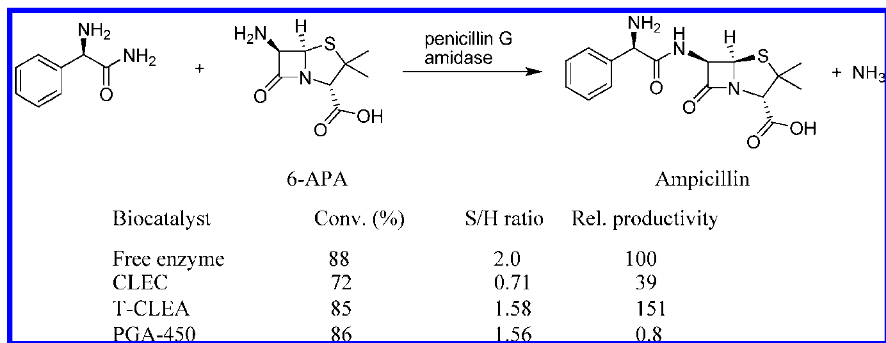

Biocatalyst	Conv. (%)	S/H ratio	Rel. productivity
Free enzyme	88	2.0	100
CLEC	72	0.71	39
T-CLEA	85	1.58	151
PGA-450	86	1.56	0.8

Figure 6

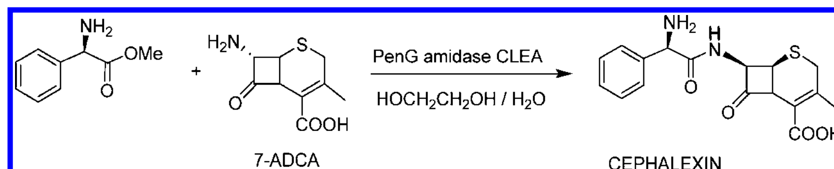

Figure 7

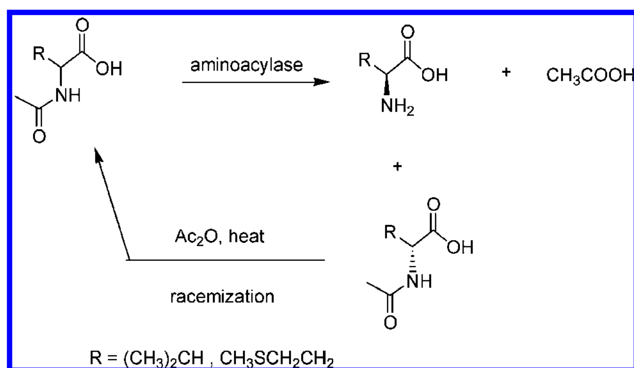

Figure 8

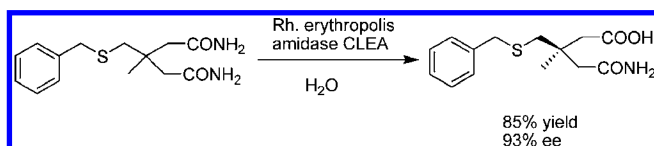

Figure 9

A *Rhodococcus erythropolis* amidase-CLEA was successfully applied to the synthesis of a pharmaceutical intermediate by enantioselective hydrolysis of the prochiral diamide shown in Figure 9.[38] The product was obtained in 85% yield and 93% ee.

2.8. Lipase CLEAs. Lipases (E.C. 3.1.1.3) are widely used in industrial biotransformations, and it is not surprising, therefore, that the preparation and application of lipase CLEAs have attracted much attention[39−51] We selected seven commercially available lipases for an investigation of the effect of various parameters, such as the precipitant and the addition of additives such as surfactants and crown ethers, on the activities of the resulting CLEAs.[39] Activation of lipases by additives, such as surfactants and crown ethers, is generally attributed to the lipase being induced to adopt a more active conformation. We reasoned that cross-linking of enzyme aggregates, in the presence of such an additive, would 'lock' the enzyme in this more favorable conformation. Since the additive is not covalently bonded to the enzyme, it can subsequently be washed from the CLEA. In this way lipase CLEAs were prepared that, in some cases, exhibited activities higher than that of the corresponding free enzyme. The experimental procedure was further simplified by combining precipitation, in the presence or absence of additives, with cross-linking into a single operation.[39]

Xu and co-workers[40] prepared CLEAs from a range of commercially available lipases and compared their activities with those of the corresponding acetone powders in the esterification of lauric acid with *n*-propanol in a solvent-free system. The nature of the precipitant, as would be expected, had a profound effect on the specific activities of the CLEAs. For example, *Candida antarctica* B lipase (CaLB) CLEAs were obtained using ammonium sulfate, acetone, and PEG 600 or PEG200. The PEG200 precipitated CLEA showed the highest specific activity: 139% compared to acetone powder. Similarly, a *Candida rugosa* lipase CLEA, prepared by precipitation with

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PEG200, exhibited 270% esterification activity relative to the corresponding acetone powder.

Different CLEA preparations of the popular *Candida antarctica* lipase B (CaLB) have been optimized for reactions in water or organic media by fine-tuning the hydrophobicity as mentioned earlier).[19] They were completely stable towards leaching in aqueous media. CaLB CLEAs also exhibit excellent activities in supercritical carbon dioxide[41−43] and ionic liquids[44]

As noted above, particle size is an important property from the point of view of large scale applications since it directly effects mass transfer and filterability under operational conditions. Yu and co-workers showed[45] that the nature and concentration of the precipitant, enzyme, and glutaraldehyde and pH all play a role in determining the particle size of *Candida rugosa* lipase CLEAs. The most important of these parameters was the enzyme to glutaraldehyde ratio. Interestingly, the *C. rugosa* CLEAs, prepared using ammonium sulfate as precipitant, also showed a ca. 2-fold increase in enantioselectivity in the kinetic resolution of racemic ibuprofen by esterification with 1-propanol.[45]

Saxena and co-workers[46] prepared CLEAs from the alkaline and thermostable *Thermomyces lanuginosa* lipase. Efficient CLEA formation was observed using ammonium sulfate as precipitant along with a 2-fold increase in activity in the presence of the anionic surfactant, sodium dodecyl sulfate (SDS). The CLEA preparation was highly stable and could be used 10 times without appreciable loss of activity in the hydrolysis of olive oil in isopropyl alcohol.

Gupta and co-workers[47] prepared CLEAs from *Burkholderia cepacia* lipase (formerly known as *Pseudomonas cepacia* lipase) by precipitation with acetone, in the presence of bovine serum albumin as the proteic feeder, and cross-linking with glutaraldehyde. They subsequently used the CLEAs as the catalyst in the transesterification of triglycerides from *Madhuca indica* oil, containing a high free fatty acid content, with ethanol to afford biodiesel in the form of fatty acid ethyl esters. Triglycerides containing high amounts of free fatty acids are difficult to convert into biodiesel using the classical (alkali) chemical catalyst. The *B. cepacia* CLEAs afforded a 92% conversion in 2.5 h at 40 °C. The same group also reported[48] the successful use of *B. cepacia* lipase CLEAs in the resolution of racemic citronellol by enzymatic transesterification with vinyl acetate. They further showed, with the aid of scanning electron microscopy (SEM), that the morphology of the CLEA particles is dependent on the extent of cross-linking. Similarly, *B. cepacia* lipase CLEA was used by Kanerva et al.[49] for the highly enantioselective resolution of 1-phenyl ethanol (Figure 9).

Cao and co-workers[50] employed a *Pseudomonas sp*. Lipase (PSL) CLEA for the resolution of *N*-(2-ethyl-6-methylphenyl)alanine (Figure 10). The PSL-CLEA exhibited a higher activity and thermal stability than the free enzyme while maintaining an excellent enantioselectivity ($E > 100$). Furthermore, it was recycled 10 times with only 19% loss of activity.

Özdemirhan and co-workers reported[51] that CaLA CLEA was an effective catalyst for the resolution of aromatic ring fused cyclic tertiary alcohols by transesterification with vinyl acetate (Figure 11). *Candida antarctica* lipase A (CaLA) is a rather

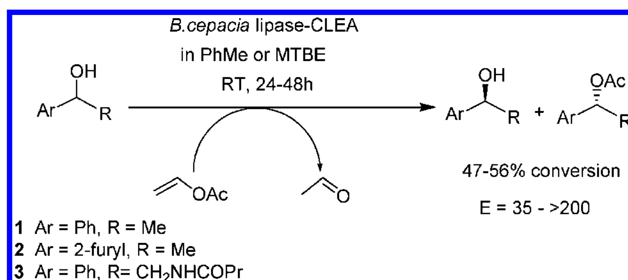

Figure 10

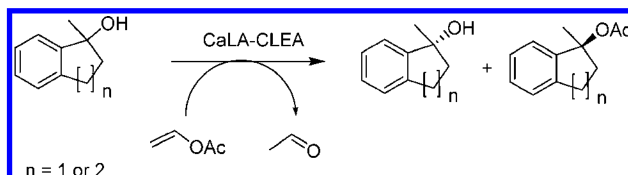

Figure 11

exceptional lipase that is known to accept bulky tertiary alcohols as substrates and, therefore, has considerable potential in organic synthesis.[52]

2.9. Esterases. In contrast to the extensive studies devoted to lipase CLEAs, there have been only sporadic reports of CLEAs from other esterases. Feruloyl esterases (E.C. 3.1.1.73) play a key role in the degradation of plant cell walls by catalyzing the hydrolysis of ferulate (4-hydroxy-3-methoxycinnamate) ester groups involved in the cross-linking of hemicellulose and lignin. Consequently, they are of commercial importance in the context of biomass conversion processes. Christakopoulos et al. described[53] the synthesis of a recombinant *Aspergillus niger* feruloyl esterase CLEA and its use in the enzymatic synthesis of various hydroxycinnamate esters[54] including the synthesis of esters of glycerol in ionic-liquid water mixtures.[55] Ju and co-workers similarly reported[56] the preparation of a CLEA from an *Aspergillus awamori* feruloyl eseterase and studied its properties using differential scanning calorimetry and scanning electron microscopy.

Similarly, Sanchez-Ferreira and co-workers[57] prepared a CLEA from a recombinant acetyl xylan esterase from *Bacillus pumilus*, another enzyme which *in vivo* is involved in the degradation of lignocellulose. *In vitro* this enzyme catalyzes the hydrolysis of the acetate moiety in cephalosporin C and 7-aminocephalosporanic acid (7-ACA) to form advanced intermediates for the production of semisynthetic cephalosporins (Figure 12). A novel dispersing technology was used to obtain CLEAs with high activity and operational stability.

2.10. Nitrilases. Nitrilases (EC 3.5.5.1) catalyze the hydrolysis of nitriles to the corresponding carboxylic acids and

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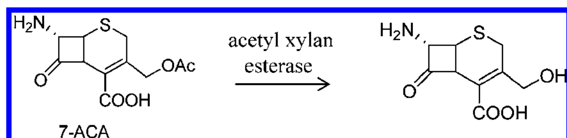

Figure 12

are, hence, potentially interesting catalysts for the enantioselective synthesis of carboxylic acids from readily available nirile precursors. It is well-known that nitrilases are rather sensitive enzymes owing to the presence of an oxygen-sensitive cysteine residue in the active site. As mentioned earlier, we experienced difficulties in obtaining CLEAs from nitrilases using the standard protocol of cross-lnking with glutaraldehyde.[13] However, we were able to successfully prepare a nitrilase CLEA by using dextran polyaldehyde as the cross-linker.[13] On the other hand, Banerjee et al.[58] reported the preparation of a CLEA from a recombinant nitrilase, overexpressed in *E. coli*, using the standard cross-linking with glutaraldehyde. We reasoned that the oxygen sensitivity of nitrilases could be suppressed by surrounding the enzyme molecules with a hydrophilic shell that would reduce the solubility of oxygen in the enzyme environment (N.B. this could be the explanation for the positive effect of dextran polyaldehyde as a cross-linker). Indeed, we found that coaggregates of nitrilases with polyethyleneimine (PEI) are much more oxygen tolerant than the dissolved enzyme.[59]

2.11. Glycosidases. Khare and co-workers[60] reported the preparation of a CLEA of the β-galactosidase from *Aspergillus oryzae* and applied it successfully in the synthesis of galacto-oligosaccharides. The latter are of interest as prebiotic food ingredients. *In vivo* this enzyme catalyzes the hydrolysis of lactose and is used to alleviate the symptoms of lactose intolerance, but it can also be used to synthesize oligosaccharides by transgalactosylation. Similarly, Lopez-Munguia and co-workers[61] prepared a CLEA from *Bacillus subtilis* levansucrase and showed that it was an effective and robust catalyst for the synthesis of oligofructosides by transfructosylation. The latter are also of interest because of their nutraceutical properties.

2.12. Oxidoreductases. *2.12.1. Oxidases.* Recyclable CLEAs were prepared from a variety of oxidases: glucose oxidase (E.C.1.1.3.4),[16] galactose oxidase (E.C. 1.1.3.9),[16] and laccase (E.C. 1.10.3.2).[19] Laccase has many potential applications, e.g. in combination with the stable radical TEMPO for the catalytic aerobic oxidation of starch to carboxy starch.[19] The latter is of interest as a biodegradable substitute for polyacrylates as a super water absorbent. However, the enzyme costs are too high, owing to the instability of the laccase under the reaction conditions, which is assumed to be a direct result of the oxidation of the surface of this heavily glycosylated enzyme. Cross-linking would be expected to increase the stability of the laccase by protecting reactive groups on the surface, and a CLEA prepared from the laccase from *Coriolus versicolor* indeed exhibited improved stability and a better performance in starch oxidation.[19] Similarly, CLEAs derived from laccases from three different sources—*Trametes versicolor*, *Trametes villosa*, and *Agaricus bisporus*—were tested in the oxidation of linear primary alcohols.[62] Rates were an order of magnitude higher than those observed with the corresponding free enzyme, and the CLEAs could be recycled several times without appreciable loss of activity. Another potential application of laccases is in bioremediation of wastewater, but in order to be commercially viable the enzyme costs must be very low and, hence, it is important to immobilize the laccase to improve stability and facilitate recycling. Interestingly, Agathos and co-workers[63] reported the use of laccase CLEAs in a perfusion basket reactor for the continuous removal of endocrine-disrupting chemicals such as bisphenol A, 4-nonyl phenol, and triclosan from urban wastewater. Tyrosinase (E.C. 1.14.18.1) is a copper-dependent oxidase with properties very similar to laccase. It catalyzes the ortho hydroxylation of phenols and has potential applications in the same areas as those for laccase. Aytar and Bakir[64] prepared a CLEA from mushroom tyrosinase with 100% activity recovery, via precipitation with ammonium sulfate and cross-linking with glutaraldehyde, and showed that it had enhanced thermal stability, both on storage and under operational conditions, compared to the free enzyme.

2.12.2. Peroxidases. An additional benefit of the CLEA technology is that it can stabilize the quaternary structures of multimeric enzymes, a structural feature encountered with many redox metalloenzymes. For example, CLEAs from two tetrameric catalases (E.C. 1.11.1.6) exhibited improved stability compared to the free enzyme.[65] Similarly, Torres and co-workers[66] reported that the stability of the CLEA of royal palm (*Roystonia regia*) peroxidase exhibited a 5000-fold increase in thermal stability compared to the free enzyme. It was used in the decolorisation of wastewater containing azo dyes where it could be recycled several times times without appreciable loss of activity.

We recently reported[67,68] the successful preparation of CLEAs from the heme-dependent chloroperoxidase (CPO; E.C.1.11.1.10) from *Caldariomyces fumago. In vivo* CPO catalyzes the oxidation of chloride ion, by hydrogen peroxide, to hypochlorite, but in the absence of chloride ion it can catalyze various regio- and enantioselective oxygen transfer processes which endow it with enormous synthetic potential.[69] However, a major obstacle to commericial applications of CPO is its low stability owing to its facile oxidative degradation by hydrogen peroxide,[70] even at relatively low peroxide concentrations.

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Coprecipitation with bovine serum albumin or pentaethylene hexamine was needed for optimum results, presumably because of the paucity of surface lysine residues available for cross-linking. Under optimized conditions an activity recovery of 68% was obtained and the CLEA exhibited enhanced thermal stability and tolerance towards hydrogen peroxide during sulfoxidation of anisole.

We previously reported[71] the design of a semisynthetic peroxidase by addition of vanadate to the acid phosphatase, phytase. Recently, we described[72] the preparation, characterization, and performance of a CLEA of this semisynthetic peroxidase in sulfoxidations.

2.13. Lyases. *2.13.1. Nitrile Hydratases.* A class of lyases that has considerable industrial relevance comprises the nitrile hydratases (NHases; E.C. 4.2.1.84)[73,74] that catalyze the addition of water to nitrile moieties. NHases are Fe- or Co-dependent metalloenzymes that usually consist of multimeric structures. They are generally used as whole-cell biocatalysts because the free enzymes have limited operational stability outside the cell, possibly owing to dissociation of tetramers resulting in deactivation. Hence, we reasoned that CLEA formation could have a beneficial effect by holding the catalytically active tetramer together, analogous to that observed with catalase (see above). This indeed proved to be the case; a CLEA prepared from a cell-free extract of an NHase isolated from an alkaliphilic bacterium showed excellent activity in the conversion of acrylonitrile to acrylamide and was active with a variety of aliphatic nitriles.[75,76] Moreover, the NHase-CLEA could be recycled 36 times with little loss of activity.

2.13.2. Hydroxynitrile Lyases. Hydroxynitrile lyases (E.C. 4.1.2.10) catalyze the enantioselective hydrocyanation of a wide range of aldehydes. For example, CLEAs prepared from the (*R*)-specific oxynitrilase from almonds, *Prunus amygdalis* (PaHnL), by cross-linking with glutaraldehyde[77] or dextran polyaldehyde[13] were highly effective catalysts for the hydrocyanation of aldehydes under microaqueous conditions and could be recycled several times without loss of activity. CLEAs were similarly prepared from the (*S*)-specific oxynitrilases from *Manihot esculenta* and *Hevea brasiliensis*.[78,79] Because these CLEAs perform exceptionally well in organic solvents, they can afford higher enantioselectivities than observed with the free enzymes owing to the essentially complete suppression of competing nonenzymatic hydrocyanation under these conditions.[80] For example, Roberge and co-workers[80] obtained high enantioslectivities in the hydrocyanation of pyridine-3-aldehyde (Figure 13). The latter is a difficult substrate for enantioselective hydrocyanation owing to the relatively facile nonenzymatic background reaction as a result of the electron-attracting properties of the pyridine ring. The high enantioselectivities observed with the HnL-CLEAs could not be obtained with the free enzyme or other immobilized forms.

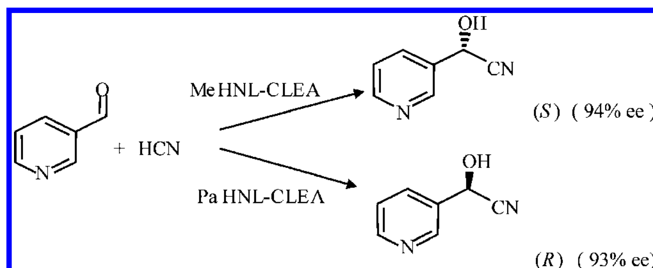

Figure 13

We recently reported[81] the preparation of a CLEA from the relatively unknown *R*-selective hydroxynitrile lyase from *Linus usitatissimum* (*Lu*HNL) and used it in the conversion of butanone to the *R*-cyanohydrin with 87% ee. Interestingly, addition of the 2-butanone substrate prior to formation of the CLEA enhanced its synthetic activity.

2.14. Combi-CLEAs and Cascade Processes. Catalytic cascade processes[82] have numerous potential benefits from both an economic and an environmental viewpoint. They involve fewer unit operations, less reactor volume, higher volumetric and space-time yields, shorter cycle times, and less waste generation compared to more conventional multistep syntheses. Furthermore, by coupling steps together unfavorable equilibria can be driven towards product. Biocatalytic processes lend themselves for combination in cascade processes because they are performed under roughly the same conditions of temperature and pressure. This can be achieved by immobilizing two or more enzymes in 'combi-CLEAs', e.g., catalase in combination with glucose oxidase or galactose oxidase, respectively.[16]

We recently used a combi-CLEA containing the *S*-selective hydroxynitrile lyase from *Manihot esculenta* and an aselective nitrilase from *Pseudomonas fluorescens* for the one-pot conversion of benzaldehyde to *S*-mandelic acid (Figure 14).[83] The enantioselectivity is provided by the HnL, and *in situ* conversion by the nitrilase serves to drive the equilibrium of the first step towards product. This could, in principle, also be achieved by using an *S*-selective nitrilase in combination with nonenzymatic, but unfortunately, there are no nitrilases that exhibit *S*-selectivity with mandelonitriles.

Interestingly, substantial amounts of the corresponding *S*-amide were also formed which led to the idea of using a third enzyme, penicillin G amidase, to catalyze the hydrolysis of the amide. Consequently, we prepared a combi-CLEA containing the *Manihot esculenta* hydroxynitrile lyase, in combination with

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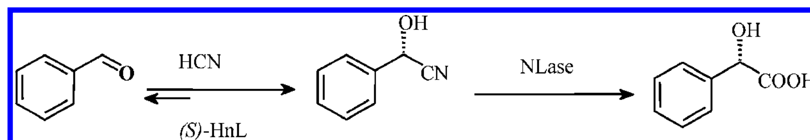

Figure 14

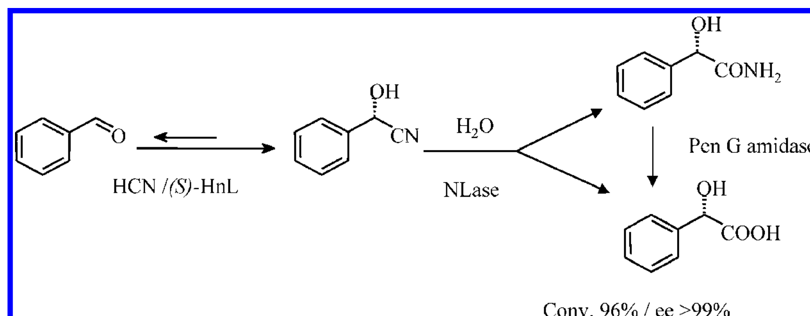

Figure 15

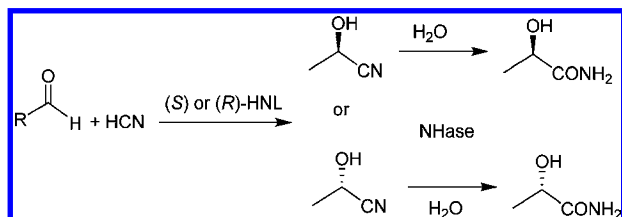

Figure 16

the nitrilase and penicillin G amidase. This triple decker CLEA afforded *S*-mandelic acid in 99% ee at 96% benzaldehyde conversion (Figure 15).[84]

We also prepared a combi-CLEA from *Manihot esculenta* hydroxynitrile lyase and the alkaliphilic nitrile hydratase from *Nitriliruptor akaliphilus* (EC 4.2.1.84) and used it to catalyze the bienzymatic cascade process for the conversion of aldehydes to *S*-α-hydroxycarboxylic acid amides (Figure 16).[85]

2.15. Reactor Design. The application of immobilised enzymes in pharmaceutical and fine chemical industries usually involves batch processes with recovery by filtration or centrifugation. In other industries, e.g the processing of fats and oils, continuous processes are more the norm and the immobilised enzyme is used in a packed bed reactor. This generally requires fairly large particles in order to avoid a large pressure drop over the column. However, the use of large particles can lead to diffusion limitations, resulting in lower rates of reaction, and hence, a compromise has to be found.

One alternative is to use a fluidized bed which can contain very small particles which must, however, be relatively dense; otherwise they will be blown out of the column. More recently, we have developed magnetic CLEAs by performing the cross-linking in the presence of functionalized magnetic nanoparticles.[86] The resulting magnetic CLEAs can be separated by magnetic decantation or can be used in a magnetically stabilized fluidized bed.

2.16. Membrane Slurry Reactor. Alternatively, a Membrane Slurry Reactor (MSR)[87] can be used, whereby the CLEA is retained in the reactor because it is too large to pass through the pores of the membrane. In contrast, the substrate and product can be pumped in and out of the reactor. CLEAs of small and/or broad particle size can be used, thus enabling a combination of high rates of reaction with ease of separation. This also enables better control of process conditions, eliminates down stream processing steps, and ensures a highly efficient use of the biocatalyst. High catalyst loadings are possible, resulting in high space-time yields. In principle, any standard stirred tank reactor, continuous or batch, can be converted into a membrane slurry reactor by applying only minor changes, and many inexpensive, size-selective membranes are commercially available and can be used in the MSR.

Combination of the high volumetric and catalyst productivities and ease of preparation of CLEAs with the high catalyst loading capacity of an MSR and the retention of the catalyst within the reactor offers a very cost-effective system for performing continuous biotransformations on an industrial scale.

The practical utility of the MSR was demonstrated by performing the industrially important hydrolysis of penicillin G to 6-amino penicillanic acid (6-APA), the key intermediate in the synthesis of semisynthetic penicillins and cephalosporins, catalyzed by a penicillin amidase CLEA. The phenylacetic acid coproduct is an inhibitor and needs to be neutralised with a base. The conversion in time was monitored by measuring the amount of base consumed. The 6-APA product was isolated from the reactor effluent by crystallization at the isoelectric point (pH 4.3). Space-time yields up to 30.5 g/L/h were achieved using a 10% catalyst loading at 20 °C compared to 18 g/L/h at 35 °C in current industrial processes based on batch operation. The MSR/CLEA system was allowed to operate over a period of two weeks without any loss in catalyst efficiency or membrane fouling.

2.17. CLEAs in Microchannel Reactors. Microreactor technologies are rapidly gaining in popularity for the production

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of fine chemicals.[88] Key benefits are the extremely efficient heat exchange and mass transfer as a consequence of the much larger surface area to volume ratios compared to conventional reactors. Microchannel enzyme reactors can also enable the rapid screening and optimization of biotransformations. Consequently, there is a rapidly developing interest in the application of microchannel enzyme reactors which generally require immobilization of the enzyme, either separately or attached to the microchannel surface.[89,90] For example, Miyazaki and Maeda et al.[91] immobilized α-chymotrypsin CLEAs as an enzyme-polymer membrane on the inner surface of polytetrafluoroethylene (PTFE) tubing of 500 μm diameter and 6 cm length by cross-linking with a mixture of formaldehyde and glutaraldehyde. The enzyme-immobilized microchannel reactor prepared in this way was tested in the hydrolysis of *N*-glutaryl-L-phenyl alanine and shown to be stable for 40 days of operation. Similarly, the same type of enzyme-immobilized microchannel reactor containing aminoacylase in an integrated microfluidic system was used for the continuous resolution of racemic *N*-acyl amino acids.[92]

Littlechild and co-workers[93] followed a different strategy. They prepared CLEAs from a thermophilic L-aminoacylase from *Thermococcus litorali*, which had been overexpressed in *E. coli*. The CLEAs were subsequently mixed with controlled pore glass and packed in capillary reactors fitted with a silica frit to contain them in the reactor. The CLEA microchannel reactor retained activity for at least two months during storage at 4 °C.

3. Conclusions and Future Prospects

The CLEA technology has many advantages in the context of industrial biotransformations. It is exquisitely simple and amenable to rapid optimization, which translates to low costs and short time-to-market. It has a broad scope, and crude enzyme extracts can be used as the feedstock to afford robust, recyclable catalysts that exhibit high activity retention, low or no allergenicity, enhanced thermal stability, better tolerance to organic solvents, and enhanced resistance to proteolysis. The technique is applicable to the preparation of combi-CLEAs containing two or more enzymes, which can be advantageously used in catalytic cascade processes. The use of CLEAs immobilized in microchannel reactors has obvious potential in the rapid screening and optimization of biotransformations and, ultimately, in continuous production processes. In short, we believe that CLEAs will be widely applied in in the future in industrial biotransformations and other areas requiring immobilized enzymes.

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