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Cross-linked enzyme aggregates (CLEA®s): stable and recyclable biocatalysts

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Abstract

The key to obtaining an optimum performance of an enzyme is often a question of devising an effective method for its immobilization. In the present review, we describe a novel, versatile and effective methodology for enzyme immobilization as CLEAs (cross-linked enzyme aggregates). The method is exquisitely simple (involving precipitation of the enzyme from aqueous buffer followed by cross-linking of the resulting physical aggregates of enzyme molecules) and amenable to rapid optimization. We have shown it to be applicable to a wide variety of enzymes, including, in addition to a wide variety of hydrolases, lyases, e.g. nitrile hydratases and oxynitrilases, and oxidoreductases such as laccase and galactose oxidase. CLEAs are stable, recyclable catalysts exhibiting high catalyst productivities. Because the methodology is essentially a combination of purification and immobilization into one step, the enzyme does not need to be of high purity. The technique is also applicable to the preparation of combi-CLEAs, containing two or more enzymes, for use in one-pot, multistep syntheses, e.g. an oxynitrilase/nitrilase combi-CLEA for the one-pot conversion of benzaldehyde into (*S*)-mandelic acid, in high yield and enantiomeric purity.

Introduction

Biocatalysis has much to offer in the context of green, sustainable chemistry [1]: mild reaction conditions (ambient temperature and pressure at physiological pH), high activities and chemo-, regio- and stereo-selectivities. Furthermore, biocatalytic syntheses are generally shorter, less energy-intensive and generate less waste than conventional organic syntheses. However, commercialization is often hampered by the lack of operational stability of enzymes, coupled with their relatively high price. One way to overcome this obstacle is by immobilization of the enzyme ([2] and references cited therein), affording improved operational stability and providing for its facile separation and reuse.

Immobilization methods

Immobilization methods can be conveniently divided into three types: binding to a carrier, encapsulation in an inorganic or organic polymeric gel, or by cross-linking of the protein molecules [2]. Binding to a carrier inevitably leads to dilution of catalytic activity resulting from the introduction of a large proportion (90–99% of the total) of non-catalytic mass. This translates to lower volumetric and space-time yields and lower catalyst productivities. In contrast, immobilization via cross-linking of enzyme molecules with a bifunctional cross-linking agent is a carrier-free method and the resulting biocatalyst essentially comprises 100% active enzyme.

Key words: *Candida antarctica* lipase B (CaLB), cross-linked enzyme aggregate (CLEA®), enzyme immobilization, hydrolase, oxidoreductase, recyclable biocatalyst.

Abbreviations used: CaLB, *Candida antarctica* lipase B; CLEA, cross-linked enzyme aggregate; CLEC, cross-linked enzyme crystal; NHase, nitrile hydratase; s.c.CO_2, supercritical CO_2.

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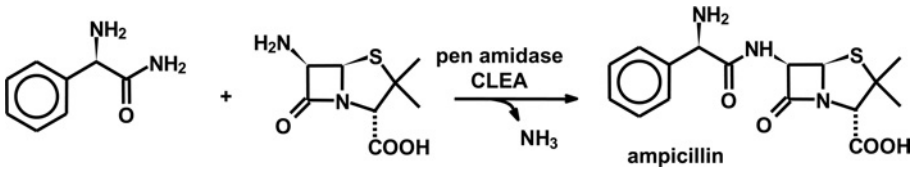
The technique of protein cross-linking, via reaction of e.g. glutaraldehyde with reactive NH_2 groups on the protein surface, was originally developed more than 40 years ago [2]. However, the cross-linked enzymes exhibited low activity retention, poor reproducibility and low mechanical stability and, owing to their gelatinous nature, were difficult to handle. Consequently, binding to a carrier became the most widely used methodology for enzyme immobilization. The use of CLECs (cross-linked enzyme crystals) as industrial biocatalysts was introduced in the early 1990s and subsequently commercialized by Altus Biologics [3–6]. 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 biotransformations.

CLEAs (cross-linked enzyme aggregates)

An inherent disadvantage of CLECs is the need to crystallize the enzyme, a laborious procedure requiring an enzyme of high purity. On the other hand, it is well known [7] that the addition of salts, or water-miscible organic solvents or non-ionic polymers, to aqueous solutions of proteins leads to their precipitation as physical aggregates, held together by non-covalent bonding without perturbation of their tertiary structure. We reasoned that cross-linking of these physical aggregates would render them permanently insoluble while maintaining the preorganized superstructure of the aggregates and, hence, their catalytic activity. This indeed proved

Figure 1 | **Ampicillin synthesis**
pen amidase, penicillin G amidase.



to be the case and led to the development of a new family of immobilized enzymes: CLEAs. Since precipitation is often used to purify enzymes, the CLEA methodology essentially combines purification and immobilization into a single unit operation. CLEAs are commercially available from CLEA Technologies (http://www.cleatechnologies.com).

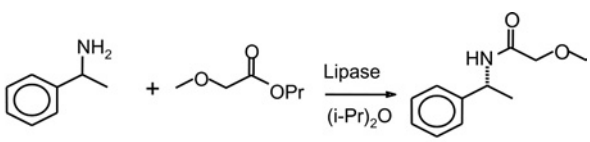
Initially, we studied [8,9] the synthesis of CLEAs from penicillin G amidase (EC 3.5.1.11), an industrially important enzyme used in the synthesis of semi-synthetic penicillin and cephalosporin antibiotics [10]. The free enzyme has limited thermal stability and a low tolerance to organic solvents, which makes it an ideal candidate for stabilization as a CLEA. Indeed, penicillin G amidase CLEAs, prepared by precipitation with, for example, ammonium sulfate or t-butanol, proved to be effective catalysts for the synthesis of ampicillin (Figure 1) [8,9]. Remarkably, the productivity of the CLEA was even higher than that of the free enzyme from which it was made and substantially higher than that of the CLEC. Not surprisingly, the productivity of the commercial catalyst was much lower, a reflection of the fact that it mainly consists of non-catalytic 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 [11].

Lipase CLEAs and hyperactivation

Since lipases (EC 3.1.1.3) are widely used in industrial biotransformations, we selected seven commercially available lipases for an investigation of the effect of various parameters, such as the precipitant and the effect of additives such as surfactants and crown ethers, on the activities of the resulting CLEAs [12]. The activation of lipases by additives, such as surfactants, crown ethers and amines, is well documented and is generally attributed to the lipase being induced to adopt a more active conformation [13]. We reasoned that cross-linking of enzyme aggregates, resulting from precipitation in the presence of such an additive, would 'lock' the enzyme in this more favourable conformation. Since the additive is not covalently bonded to the enzyme, it can subsequently be washed from the CLEA. In this way, several hyperactive lipase CLEAs were prepared, exhibiting activities even higher than the corresponding free enzyme [12]. The experimental procedure was further simplified by combining precipitation, in the presence or absence of additives, with cross-linking into a single operation [12].

Optimization of the protocols for CLEA preparation, with regard to parameters such as temperature, pH, concentration,

Figure 2 | **Relative activities of lipase CLEAs in aqueous and organic media**
[a]AM, aqueous medium; $(i\text{-Pr})_2O$, di-isopropyl ether; OM, organic medium.



	"Aqueous" activity (Ug -1)	"Organic" activity (Ug -1)	Ratio
CALB (lyophilized)	22000	-	
Novozym 435	7300	250	2
CLEA-*AM* [a]	38000	50	760
CLEA-*OM* [a]	31000	1500	21

stirring rate, precipitant, additives and cross-linking agent, is a relatively simple operation that lends itself to automation, e.g. using 96-well plates [14]. The enzyme and glutaraldehyde concentrations were important factors in determining the particle size of *Candida rugosa* lipase CLEAs [15]. Particle size is an important property from the point of view of large-scale applications, since it directly affects mass transfer and filterability under operational conditions.

Interestingly, CLEA preparations of the popular CaLB (*Candida antarctica* lipase B) performed better than Novozym® 435 (CaLB immobilized on a macroporous acrylic resin) in water but this superior activity could not be directly translated to organic media. Consequently, we modified the preparation to produce a more lipophilic CLEA [16]. This afforded a dramatic improvement in the activity of CaLB CLEA in the enantioselective acylation of 1-phenethylamine in di-isopropyl ether as a solvent (Figure 2). The optimized CaLB CLEAs displayed activities surpassing those of Novozym® 435 in both aqueous and organic media. We also note that enzyme can be leached from the surface of Novozym® 435 in water, whereas the CaLB CLEA, being covalently bound, is completely stable.

It was subsequently shown that CaLB CLEAs exhibit excellent activities in s.c.CO_2 (supercritical CO_2) and ionic liquids. Thus CaLB CLEA displayed superior activity to that of Novozym® 435 in the kinetic resolution of 1-phenylethanol by acylation with vinyl acetate in s.c.CO_2 [17,18]. Similarly, CaLB CLEA preparations were

effective in the kinetic resolution of 1-phenylethanol and 1-phenylethylamine in the ionic liquids, [bmim][NO_3] (1-butyl-3-methylimidazolium nitrate) and [bmim][$N(CN)_2$] (1-butyl-3-methylimidazolium dicyanamide), whereas Novozym® 435 was completely inactive under these conditions [19].

Cross-linking agents

Glutaraldehyde is generally the cross-linking agent of choice as it is inexpensive and readily available in commercial quantities. However, with some enzymes, e.g. nitrilases, we sometimes observed low or no retention of activity when glutaraldehyde was used as the cross-linker. We reasoned that this could be due to reaction of the cross-linker with amino acid residues that are crucial for the activity of the enzyme. This would be particularly severe with glutaraldehyde owing to its high reactivity and small size, which allows it to penetrate the interior of the protein. In order to circumvent this, we used bulky polyaldehydes, obtained by periodate oxidation of dextrans, as the cross-linkers [20], followed by reduction of the Schiff base moieties with sodium borohydride to form irreversible amine linkages. The activity retention of these CLEAs was generally much higher than that observed with CLEAs prepared by using glutaraldehyde. Dramatic results were obtained, for example, with two different nitrilases (EC 3.5.5.13). Cross-linking with glutaraldehyde produced a completely inactive CLEA, while with dextran polyaldehyde 50–60% activity retention (not optimized) was observed [20].

Scope of the CLEA methodology (for recent reviews, see [21,22])

Hydrolases

Examples of hydrolases include, in addition to the above-mentioned penicillin amidase and several lipases, pig liver esterase, aminoacylase, proteases, nitrilases (see above) and glycosidases. We have prepared CLEAs from (chymo) trypsin (EC 3.4.21.4) [14] and from the alkaline protease from *Bacillus licheniformis* (alcalase, EC 3.4.21.62, also known as subtilisin Carlsberg), an inexpensive enzyme used in laundry detergents, has been widely used in organic synthesis, e.g. in the resolution of (amino acid) esters [23], and amines [24] and peptide synthesis [25]. The alcalase CLEA showed excellent activities and enantioselectivities in amino acid ester hydrolyses (M.H.A. Janssen and R.A. Sheldon, unpublished work).

A CLEA from aminoacylase (EC 3.5.1.14) derived from *Aspergillus* sp. was unable to catalyse the hydrolysis of esters observed with the crude enzyme [26]. A plausible explanation is that the esterolytic activity is derived from a protein impurity. It demonstrates the power of the CLEA methodology for performing purification and immobilization in a single operation.

A CLEA was also prepared from the glycosidase, β-galactosidase (lactase; EC 3.2.1.23) from *Aspergillus oryzae*, which catalyses the hydrolysis of lactose in dairy products and is administered as a digestive supplement to alleviate the symptoms of lactose intolerance [27]. The galactosidase CLEA was recycled with no loss of activity [14]. Similarly, a CLEA was successfully prepared from phytase (EC 3.1.3.26) from *Aspergillus niger*, an acid phosphatase, a nutritional supplement that is added to animal feed [14].

Oxidoreductases

Recyclable CLEAs were prepared from a variety of oxidases: glucose oxidase (EC1.1.3.4), galactose oxidase (EC 1.1.3.9) and laccase (EC 1.10.3.2) [14,16]. Laccase, in particular, has many potential applications, e.g. in combination with the stable radical TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) for the catalytic aerobic oxidation of starch to carboxy starch [16]. 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 [16].

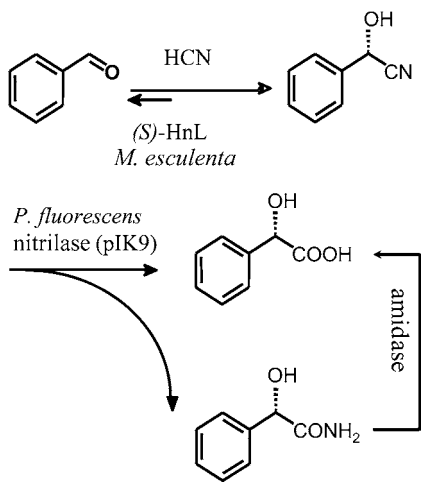
An additional benefit of the CLEA technology is that it can stabilize the quaternary structures of multimeric enzymes, a structural feature often encountered with redox metalloenzymes. For example, the stability of CLEAs from two tetrameric catalases (EC 1.11.1.6) exhibited improved stability compared with the free enzyme [28].

Lyases

The CLEA methodology has also been successfully applied to several C–C-bond-forming lyases, notably the *R*- and *S*-specific oxynitrilases (hydroxynitrile lyases, EC 4.1.2.10) which catalyse the hydrocyanation of a wide range of aldehydes. For example, a CLEA prepared from the (*R*)-specific oxynitrilase from *Prunus amygdalus* (almond) (PaHnL, EC 4.1.2.10) was a highly effective catalyst for the hydrocyanation of aldehydes under microaqueous conditions and was recycled ten times without loss of activity [29]. CLEAs were similarly prepared from the (*S*)-specific oxynitrilases from *Manihot esculenta* and *Hevea brasiliensis* [30,31]. Because these oxynitrilase 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 non-enzymatic hydrocyanation under these conditions [32]. Other C–C-bond-forming lyases that have been successfully CLEAted include pyruvate decarboxylase (EC 4.1.1.1) and DERA (deoxy-D-ribose phosphate aldolase; EC 4.1.2.4) [27].

Another class of lyases that has considerable industrial relevance comprises the NHases (nitrile hydratases) [33]. NHases are metalloenzymes (Fe- or Co-dependent) that usually consist of multimeric structures. They are generally used as whole-cell biocatalysts as the free enzymes have

Figure 3 | Cascade catalysis with a combi-CLEA
(*S*)-HnL, *S*-specific oxynitrilase.



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 into acrylamide and was active with a variety of aliphatic nitriles (S. van Pelt, S. Quignard, D. Kubac, D.Y. Sorokin, F. van Rantwijk and R.A. Sheldon, unpublished work). Moreover, the NHase CLEA was recycled 36 times with little loss of activity.

Combi-CLEAs and cascade processes

Catalytic cascade processes [34] have numerous potential benefits: fewer unit operations, less reactor volume, and higher volumetric and space-time yields, shorter cycle times and less waste generation. Furthermore, by coupling steps together, unfavourable equilibria can be driven towards product. We have achieved this by immobilizing two or more enzymes in 'combi-CLEAs', e.g., containing catalase in combination with glucose oxidase or galactose oxidase respectively.

We recently used a combi-CLEA containing an *S*-selective oxynitrilase (from *M. esculenta*) and an aselective nitrilase from *Pseudomonas fluorescens* for the one-pot conversion of benzaldehyde into (*S*)-mandelic acid (Figure 3) [35]. The enantioselectivity is provided by the oxynitrilase, 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 non-enzymatic hydrocyanation (as we have previously shown with an *R*-nitrilase) 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 catalyse the hydrolysis of the amide. In this way, using an oxynitrilase/nitrilase combi-CLEA, in combination with an immobilized penicillin G amidase, (*S*)-mandelic acid was obtained as the sole product in high yield and enantiopurity (A. Chmura, F. van Rantwijk and R.A. Sheldon, unpublished work). The next step is to show that a combi-CLEA containing all three enzymes can be used.

Conclusions and prospects

The CLEA technology has many advantages in the context of industrial applications. The method is exquisitely simple and amenable to rapid optimization, which translates to low costs and short time-to-market. It is applicable to a wide variety of enzymes, including crude preparations, affording stable, recyclable catalysts with high retention of activity and tolerance to organic solvents. The technique is applicable to the preparation of combi-CLEAs containing two or more enzymes, which can be advantageously used in catalytic cascade processes. CLEAs have also been used in microchannel reactors (see, for example, [36]). In short, we believe that CLEAs will be widely applied in industrial biotransformations and other areas requiring immobilized enzymes.

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