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Parameters in preparation and characterization of cross linked enzyme aggregates (CLEAs)

Sachin Talekar,* Asavari Joshi, Gandhali Joshi, Priyanka Kamat, Rutumbara Haripurkar and Shashikant Kambale

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In the past couple of decades, cross linked enzyme aggregates (CLEAs) have emerged as a novel and versatile carrier free immobilization technique. The immobilization of enzymes as cross linked enzyme aggregates (CLEAs) involves precipitation of an enzyme from aqueous solution followed by cross linking with a bi-functional reagent. It is worth noting that many parameters alter the enzyme precipitation and the aggregate cross linking and hence affect the activity and stability of CLEAs. Therefore to endorse CLEAs for industrial application, each newly synthesised CLEA is characterized. This review intends to investigate the effects of various parameters, such as the nature and purity of the enzyme, the nature and amount of precipitant, the nature and amount of cross linker, the cross linking time, the pH and temperature during CLEA preparation and washing and separation techniques on the activity and stability of CLEAs. The major parameters such as catalytic properties, particle size and morphology, stability and reusability required for approval of industrial applicability of newly synthesized CLEAs are critically reviewed. Furthermore the scope of CLEAs in non-aqueous solvent, the development of one pot cascade processes and the design of different types of enzyme reactors is also discussed.

1 Introduction

The need for the development of clean, reliable, biocompatible and benign processes for the industrial manufacture of chemicals has led to more and more researchers exploiting biocatalysts as possible eco-friendly catalysts. Therefore biocatalysts have become important tools for industrial chemical synthesis and reached a particularly exciting time. Biocatalysts are mainly fall into two types: whole cell and purified enzymes. Compared to whole cells, purified enzymes offer several benefits, including simpler reaction apparatus, higher productivity due to higher catalyst concentration and simpler product purification owing to the elimination of undesirable side reactions during growth.[1] Enzymes are recognized as useful tools for catalysing industrial chemical synthesis reactions in stereo, regio, and chemo selective ways. As such, they are being used extensively in the industrial production of bulk chemicals, pharmaceutical and agrochemical intermediates and active pharmaceuticals where the need for optically pure molecules is critical.[2,3] Although due to recombinant DNA technology[4] it has become possible to produce large amounts of enzymes for industrial applications, one of the major challenges in enzymatic processes is to improve the activity and stability of the enzymes. Biomolecular engineering techniques such as *in vitro* direct evolution *via* gene shuffling[5,6] and site directed mutagenesis[7,8] have made it possible to enhance the useful properties of enzymes such as pH stability, thermal stability, increased activity, and so forth. Even though the properties of the enzymes can be improved by any of these techniques, commercialization of enzymes is often hampered by cumbersome recovery and recycling and product contamination.[9]

These obstacles can be circumvented by the immobilization of enzymes.[10–17] In addition, immobilization also improves enzyme activity and stability under optimal process conditions, a requirement that has often retarded enzyme applications in industrial chemical synthesis.[18] However, enzyme immobilization on a solid carrier results in the non-catalytic carrier occupying a significant amount of the space (about 90–99% of total mass) which dilutes the volumetric activities, decreases space–time yields and lowers the catalyst productivity.[19] In addition, immobilization with a solid carrier is often expensive and generally requires chemical modification of an inert matrix to enable covalent coupling of the enzyme.[20] Therefore new approaches for immobilizing enzymes without solid carriers are gaining importance. Methods for enzyme immobilization without solid carriers can be conveniently divided into four types:[19] (i) cross linking of dissolved enzymes which yields cross linked enzymes (CLEs); (ii) cross linking of spray dried enzymes which yields cross linked spray dried enzymes (CLSDs); (iii) cross linking of crystalline enzymes which yields cross linked enzyme crystals (CLECs) and (iv)

Department of Biotechnology Engineering, Kolhapur Institute of Technology's College of Engineering, Kolhapur 416 234, India. E-mail: sachintalekar7@gmail.com

cross linking of physically aggregated enzymes which yields cross linked enzyme aggregates (CLEAs).

Cross linked enzymes (CLEs) were obtained by direct cross linking of a dissolved enzyme.[21,22] However, the resulting enzyme preparation exhibited low activity retention, poor reproducibility and mechanical stability, irregular geometry and, owing to its gelatinous nature, was difficult to handle.[9] Reasonable activity was retained when the spray dried enzyme was cross linked, yielding cross linked spray dried enzymes (CLDs).[23] Due to the fact that the spray drying process reversibly deactivates the enzymes, CLDs have not been exploited until now. The approach of cross linking crystalline enzymes yielded cross linked enzyme crystals (CLECs)[24] and were found to give better results in terms of activity retention, stability to denaturation by heat, organic solvents, and proteolysis, mechanical stability, particle geometry and therefore have been successfully commercialized.[25–30] However, due to the requirement of laborious, expensive and time consuming crystallization protocols[31] which require highly pure enzyme, further growth of the CLEC methodology has been discouraged. In recognition of the limitations of CLEC, it was reasoned that crystallization could be replaced by simpler and less expensive precipitation protocols to form physical aggregates of enzyme molecules without denaturation. Subsequent cross linking of these aggregates resulted in the development of a new enzyme immobilization strategy called cross linked enzyme aggregates (CLEAs).[32] Later, this strategy was commercialised by CLEA Technologies (Netherlands). In this review we intend to provide a comprehensive account of the different parameters in the preparation and characterization of cross linked enzyme aggregates. A detailed description of the application of CLEAs for catalysis in organic solvent, immobilization of multiple enzymes for bio-catalytic cascades and design of enzyme reactors is also included. If we succeed in our goal, after reading this review the researcher will have a complete picture of the parameters which should be considered while preparing and characterizing CLEAs.

2 Preparation method for cross linked enzyme aggregates

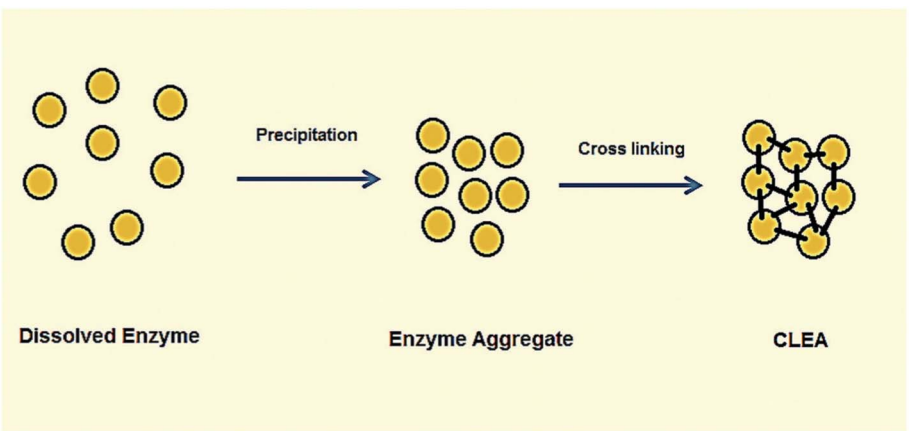
The principal method of enzyme immobilization using the CLEA technique involves two steps: (i) Precipitation of soluble enzyme; (ii) Cross linking of the formed aggregates (Scheme 1). In the first step the soluble enzyme is aggregated by the addition of precipitating agents such as salts, water-miscible organic solvents and non-ionic polymers to an aqueous solution of enzyme. In the aggregate, the enzyme molecules are held together by non-covalent bonding without perturbation of their tertiary structure, *i.e.* without denaturation.[33] In the second step, the formed aggregates are chemically cross linked to each other by a bifunctional reagent *via* the reaction of amino groups of Lys residues on the external surface of the enzyme.[32,34,35] After chemical cross linking, the aggregates become permanently insoluble while maintaining their pre-organized superstructure, and, hence their catalytic activity. Since precipitation is often used to purify enzymes, enzyme immobilization using the CLEA technique combines purification and immobilization into single unit operation. Therefore it is possible to isolate an enzyme in its immobilized form directly from a fermentation broth.

3 Preparation parameters for cross linked enzyme aggregates

While CLEAs do eliminate the need to crystallize the enzyme of interest, a substantial effort must be devoted to the consideration of following important parameters involved in the preparation of CLEAs to obtain CLEAs with high activity retention and operational stability. This will pave the way for changing the CLEA from an interesting phenomenon into a mature, well-defined catalytic particle.

3.1 Nature and purity of the enzymes

Since cross linking of aggregated enzyme molecules largely involves the reaction of a bi-functional agent such as



Scheme 1 Enzyme immobilization using CLEAs technique.

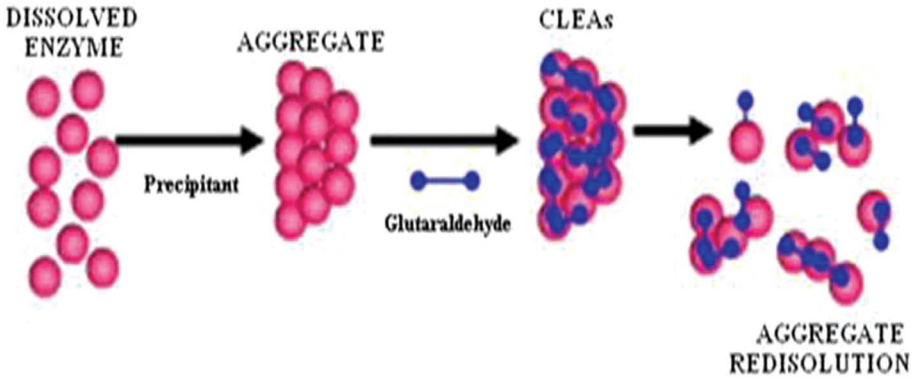
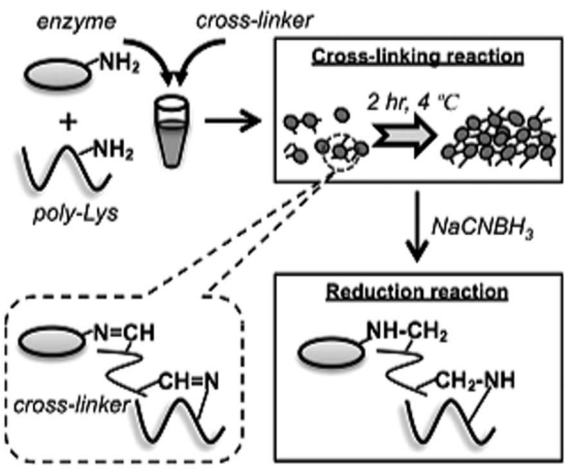


Fig. 1 Mechanically fragile CLEAs releasing enzyme in reaction medium. Reprinted from ref. 48 with permission from Elsevier. Copyright 2012 Elsevier B.V.

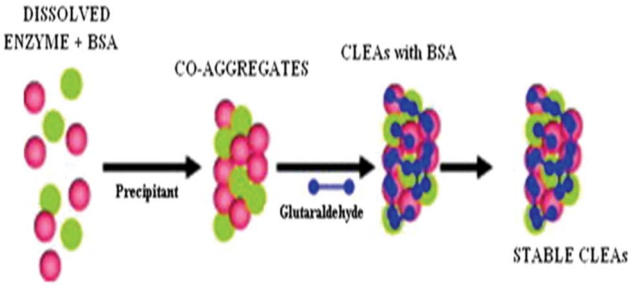
glutaraldehyde with reactive amino groups (mainly Lys) on the enzyme surface, the nature of enzyme in terms of Lys content certainly affects immobilization by the CLEA technique. Every enzyme would be expected to contain differing amounts of accessible Lys residues.[9] Thus, immobilization using the CLEA technique might not be as efficient as expected, typically for electronegative enzymes that contain a paucity of surface reactive amino groups.[16] CLEAs of such enzymes do not recover all of the free enzyme activity and are mechanically fragile, releasing enzymes into the reaction medium during a reaction[36] (Fig. 1). On the other hand, if the amino groups involved in cross linking are crucial for the activity of an enzyme, then even for enzymes with adequate reactive amino groups it is not always possible to prepare active CLEAs, as observed in case of nitrilases.[37,38]

Several interesting approaches for the preparation of CLEAs have been successfully used to overcome the problem of enzymes with low surface reactive amino groups. In 2005, Lopez-Gallego *et al.*[36] and Wilson *et al.*[39] proposed that co-aggregation of enzymes and polyethyleneimine, a polyionic polymer containing numerous free amino groups could be used for the preparation of highly stable CLEAs of glutaryl acylase and penicillin G acylase, respectively. Although these enzymes have a low number of surface reactive amino groups, the primary amino groups on the polymer led to adequate intermolecular cross linking between polymers and enzyme molecules resulting in sufficient activity recovery in CLEAs. Moreover, polyethyleneimine induced CLEAs of penicillin G acylase showed 25 fold higher stability in organic solvent than the CLEAs prepared by the conventional method. Afterwards, Pan *et al.*[40] and Vaidya *et al.*[41] further examined this approach for the preparation of CLEAs of *Serratia marcescens* lipase and L-aminoacylase, respectively and found that co-aggregation with polyethyleneimine resulted in CLEAs of lipase and L-aminoacylase with high activity recovery and excellent operational stability without enzyme leakage during reactions. *Geotrichum* sp. Lipase when immobilized as CLEAs based on polyetheleneimine showed improved stability and activity in the hydrolysis of fish oil for the enrichment of polyunsaturated fatty acid.[42] In another approach, poly-lysine, a polymer containing a large number of primary amino groups was used as an amino group donor to prepare CLEAs of acylase[43] and proteases[44] with improved cross linking yield using a lower concentration of cross linker (Scheme 2). These poly-lysine supported CLEAs had efficient enzyme activity and high operational stability. Based on a similar idea, pentaethylene-hexamine was also used to enhance the cross linking yield of the chloroperoxidase enzyme.[45]

In a variation on this theme, Shah *et al.*[46] proposed the approach of improving the cross linking by co-aggregation of a low Lys containing enzyme and certain feeder proteins rich in Lys residue such as bovine serum albumin (BSA) (Scheme 3). In the presence of BSA, they obtained 100% and 86% retention of activity in the case of CLEAs of lipase and penicillin acylase, respectively while in the absence of BSA, only 0.4% and 50% activity retention was obtained in the case of lipase and penicillin acylase, respectively. While studying the effect of BSA addition on the cross linked enzyme aggregates (CLEAs) of aminoacylase from *Aspergillus melleus* (EC 3.5.1.14), Dong *et al.*[47] observed CLEA of aminoacylase prepared with 10 mg BSA per 100 mg enzyme retained 82% activity recovery (named CLEA-E-BSA) whereas CLEA prepared without BSA retained only 24% activity recovery (named CLEA-E) due to the low content of amine residues of aminoacylase. CLEA-E-BSA also



Scheme 2 Preparation of the poly-Lys supported CLEA. Reproduced from ref. 44.



Scheme 3 Preparation of stable CLEAs using BSA as feeder protein. Reprinted from ref. 48 with permission from Elsevier. Copyright 2012 Elsevier B.V.

showed excellent operational stability, retaining 82.4% residual activity even after 10 cycles of repeated use. After optimization of the preparation of CLEAs of lipase in the presence of BSA, Cruz *et al.*[48] achieved effective cross linking of lipase with a very high stability. In one more report, hen egg white has been used as the feeder protein to prepare lipase CLEAs and was found to stabilize enzymes to changes in the system parameters such as pH, temperature and denaturants such as urea and GndHCl.[49] However, recently CLEAs of *Thermomyces lanuginose* lipase (TLL) formed by the addition of BSA as the feeder protein were found with occluding lipase resulting in less activity due to mass transfer limitations. To avoid this, a modification of the traditional CLEAs synthesis protocol with feeder protein was designed to develop ''layered CLEAs''. The approach implied the addition of lipase only after the feeder protein aggregates were formed and cross linked. Lipase was expected to form a slightly cross linked layer over BSA cores resembling an onion-like structure, which might lead to enhanced lipase substrate contact (Fig. 2).[50]

Recently, in a more exciting approach of magnetic CLEAs preparation, our group has shown that the addition of amino coated magnetite nanoparticles into the enzyme solution with low Lys residue content can lead to mechanically stable and non-leachable CLEAs due to sufficient cross linking of enzyme aggregates[51] (Scheme 4). Galvis *et al.*[52] proposed a novel way of enriching lipase B content in amino groups by chemical amination of enzymes using ethylenediamine and carbodiimide. After amination, lipase B was intensively cross linked and the CLEA did not release enzyme molecules even if boiled in SDS.

One other consideration is the multi-subunit nature of enzymes which is often a challenge for most immobilization techniques. If some subunits are not immobilized, they get dissociated from the immobilized biocatalyst, resulting in inactivation of the biocatalyst and contamination of the product, which is not a desired situation. However, for the CLEA technique, the chemical cross linking increases the likelyhood that any multi-subunit enzyme was cross linked to another component of the biocatalyst, preventing enzyme dissociation and also stabilizing the quaternary structure of enzyme.[53] There are reports of successful immobilization and

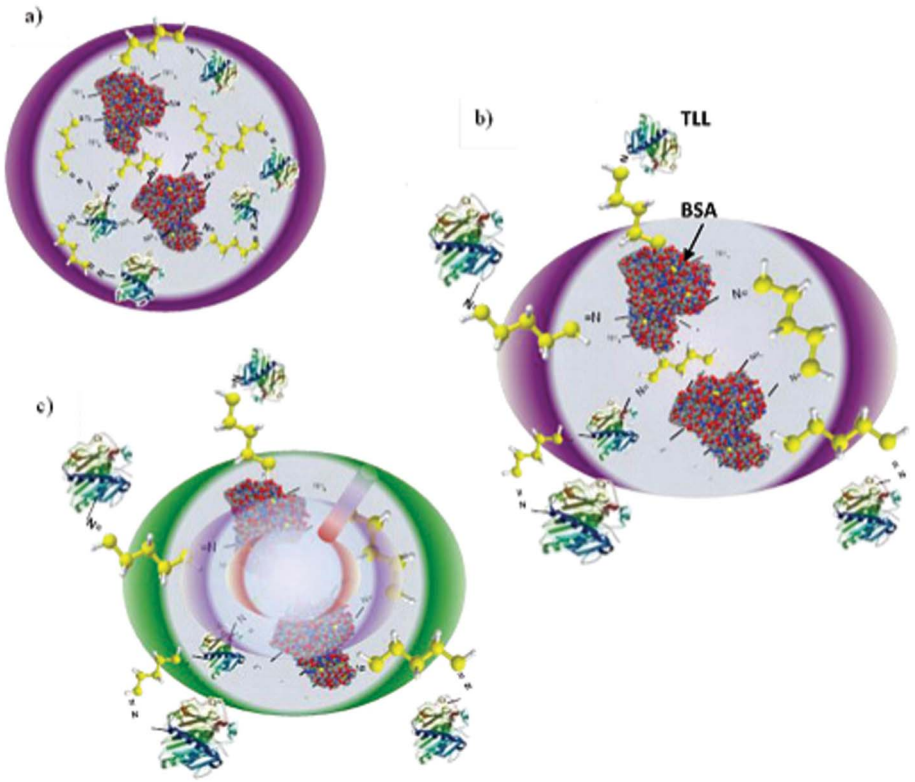
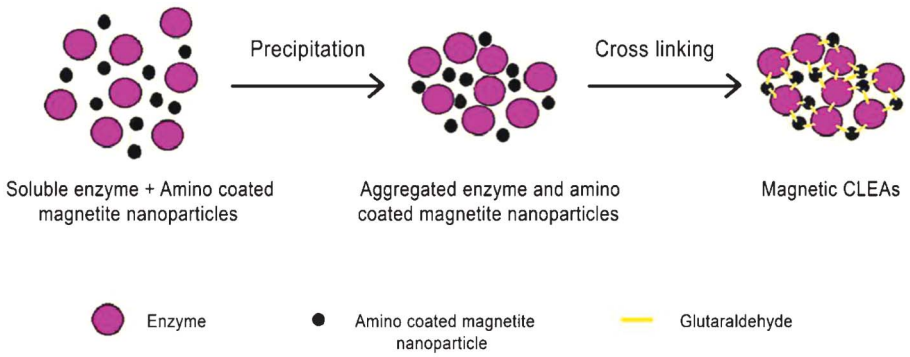


Fig. 2 Scheme of CLEAs (a) Traditional CLEAs with BSA used as protein co-feeder; (b) one lipase layer, (c) three lipase layers. Reprinted from ref. 50 with permission from Elsevier. Copyright 2013 Elsevier B.V.



Scheme 4 Preparation of magnetic CLEAs.

stabilization of multi-subunit enzymes like catalases,[54] nitrile hydratases,[55,56] alcohol dehydrogenase,[57] formate dehydrogenase[57] using CLEA technique.

The enzyme purity requirement is the most vital parameter in any immobilization technique. All solid carrier enzyme immobilization techniques have the inherent drawback of lower enzyme loading and hence less specific/volumetric activity. One way to overcome this drawback is to use purified enzymes to avoid loading of contaminant proteins/enzymes. But the price of enzyme purification is high, which further increases the cost of the final immobilized biocatalyst. As previously mentioned, although CLEC gives high enzyme loading and high specific/volumetric activity, it also requires purified enzymes for the production of crystalline enzymes. Since CLEAs replace crystallization by the less expensive precipitation, preparation of CLEAs does not require purified enzyme and is incompatible with most contaminant proteins, because the target enzyme may precipitate under appropriate conditions, specific for each enzyme.[53] In fact as mentioned previously, CLEA methodology has the potential to purify and immobilize in a single operation. Indeed, several enzymes such as aminoacylase,[58] penicillin G amidase,[59] invertase,[60,61] alpha amylase,[62] lipase,[40,63,64] phenylalanine ammonia lyase[65] have been successfully immobilized as CLEAs with high activity recovery and specific activity directly from crude fermentation broth. Hence, one can envisage the direct immobilization of an enzyme at the production site without any need for intermediate work-up or purification.

3.2 Nature and amount of precipitant

Recovery of the enzyme activity is a key cost determining parameter of CLEAs. It should be preferably close to or more than 100%. The precipitation step predictably has an important effect on the activity recovery in CLEAs as it causes physical aggregation of enzyme molecules into supramolecular structures, which are subsequently cross linked to lock enzyme in CLEAs. Generally, precipitation is done by the addition of salts, organic solvents or non-ionic/ionic polymers to aqueous solutions of proteins. Hence, it is necessary to screen a number of precipitants to recover the maximum enzyme activity in CLEAs. The initial screening of a precipitant is done by determining the enzyme activity of the redissolved precipitates obtained by a particular precipitant. But, observation of a high activity on redissolution of the precipitate in buffer does not automatically mean that the aggregates will retain this high activity after cross linking. This is because an enzyme may precipitate in an inactive conformation but when redissolved it will exhibit its normal activity. On the other hand, when it is cross linked it will remain in the inactive conformation and display a lower activity. Therefore, it is advisable to select a few precipitants at the start which give good yields of aggregates but final selection of precipitant should be based on the activity recovery in CLEAs and not on the redissolved precipitate. Ammonium sulphate has been most successfully applied for precipitation during CLEA preparation. Several reports on CLEAs such as CLEAs of *Candida rugosa* lipase,[66] β-galactosidase,[67] penicillin G acylase,[68] tyrosinase,[69–71] subtilisin,[72] phytase,[73] feruloyl esterase,[74] acetyl xylan esterase,[75] *Rhodococcus erythropolis* amidase,[76] *Pseudomonas putida* nitrilase,[77] invertase,[60,61] alpha amylase,[51,62] *N*-acetyl-D-neuraminic acid aldolase,[78] SGNH hydrolase,[79] laccase,[80] hydrolase (BL28),[81] *etc.* showed ammonium sulphate acting as the best precipitating agent. Despite the fact that denaturation of enzymes occurs upon precipitation with organic solvent due to hydrophobic interactions between the solvent and the nonpolar groups of the enzyme,[82,83] numerous reports underscore the preparation of active CLEAs using water miscible organic solvents like acetone, ethanol, dimethoxyethane, *tert*-butyl alcohol, isopropyl alcohol, acetonitrile, *etc.* CLEAs of lipase,[46,84–86] horseradish peroxidase[87] and poly-3-hydroxybutyrate depolymerase[88] using acetone, papain[89] and lipase [90] using ethanol, (*R*)-oxynitrilase,[91] penicillin acylase,[46] hydroxynitrile lyase[92] and lipase-α-amylase-phospholipase A_2[93] using dimethoxyethane, penicillin G acylase,[34] penicillin acylase[94] and aminoacylase[47] using *tert*-butyl alcohol, nitrilase[95] using isopropyl alcohol, chloroperoxidase[96] using acetonitrile as precipitant have been prepared. Furthermore, for the enzymes which are denatured when precipitated with water miscible organic solvents, Wang *et al.*[97] developed a sugar-assisted precipitation strategy to avoid this denaturation by adding sugar as the stabilizer during precipitation. Similar to ammonium sulphate and water miscible organic solvents, a non-ionic polymer polyethelene glycol aggregated many enzymes such as penicillin acylase,[34] subtilisin,[72] lipase,[98] laccase[83] and lipozyme TL 100 L[99] during the preparation of CLEAs to retain maximum enzyme activity.

Table 1 Activity of resolved aggregates using 90% (v/v) precipitant and activity after cross linking. Reprinted from ref. 57 with permission from John Wiley & Sons, Inc. Copyright 2004 Wiley Periodicals, Inc

Precipitant (90%)		CaLA	CaLB	TIL	RmL	GlcOx	Laccase	Gal-ase	Trypsin	ADH	FDH	Phytase	Average[a]
1	Buffer	100	100	100	100	100	100	100	100	100	100	100	100
2	Methanol	3	64	43	21	0	0	0	89	0	2	19	18
3	Ethanol	45	66	258	187	15	47	0	135	1	23	97	51
4	1-Propanol	48	30	1511	223	85	85	80	129	1	13	66	66
5	2-Propanol	43	77	169	95	104	99	82	144	6	55	93	79
6	*tert*-Butyl alcohol	142	100	1779	934	116	139	99	148	13	90	88	91
7	Acetone	107	52	178	706	94	58	65	185	24	95	77	79
8	Acetonitrile	100	75	561	428	116	27	88	151	21	84	79	81
9	DME	231	100	1013	561	113	78	79	142	7	50	95	83
10	Ethyl lactate	86	39	108	14	127	108	82	142	4	32	123	71
11	Sat.$(NH_4)_2SO_4$	101	131	113	133	101	139	83	186	100	88	52	91
12	DMF	6	72	62	58	1	0	0	85	0	0	19	25
13	DMSO	5	107	95	43	2	0	0	131	0	0	5	29
14	PEG	115	138	141	102	114	186	100	153	44	81	80	88
	After cross linking	263	177	327	934	100	50	100	51	20	7	100	77

[a] Maximum contribution per enzyme = 100%.

Polyetheleneimine, an ionic polymer, has also been used as the sole precipitating agent for the CLEAs of glutaryl acylase,[36] *Serratia marcescens* lipase,[40] L-aminoacylase.[41]

From these reports and as shown in Table 1, it is seen that wide variety of precipitants has been applied successfully to prepare CLEAs of a wide variety of enzymes and in some cases, more than one precipitant to a single enzyme. This could be because of different biochemical and structural properties of proteins due to which the best precipitant can vary from one enzyme to another and even for the same enzyme from different sources. Hence, it is difficult to make some generalizations about the nature of precipitant for the preparation of active CLEAs. But it is worth mentioning that it was found that the catalytic behaviour of CLEAs differs depending on the properties of the precipitants: CLEAs of penicillin G acylase precipitated by ammonium sulphate performed in a similar manner to the native enzyme in the synthesis of ampicillin, whereas CLEAs prepared using *tert*-butanol as precipitant displayed a relatively constant selectivity (S/H ratio) during the reaction course.[32] The CLEAs of acetyl xylan esterase obtained with ammonium sulfate were found to be of uniform porosity and hence highly active, whereas with acetonitrile or *tert*-butanol, the CLEAs of acetyl xylan esterase were less porous and hence less active.[75] Among the aliphatic alcohols tested, methanol caused a near total loss of activity whereas *tert*-butanol worked as the best precipitant during the preparation of multipurpose CLEAs with feruloyl esterase activity which indicates that increase of alcohol's aliphatic chain resulted in higher retention of enzyme activity.[100] Moreover, the upward tendency of precipitated activity from methanol (14.05%) to isopropanol (30.40%) was recently observed during the preparation of β-mannanase CLEAs.[101] With the increase in hydrophobicity from methanol to isopropanol (from −0.66 to 0.14) the capability of capturing necessary water from enzyme molecules decreases. Since the necessary water is very important for the activity of enzyme, the conformation of the enzyme would be changed and the activity of enzyme would be decreased when the necessary water is lost. In line with these reports, CLEAs of seven lipases (*e.g. C. antarctica* lipase A [CAL-A], *C. antarctica* lipase B [CAL-B], *C. rugosa* lipase [CRL] *etc.*) were prepared with high activity retention by changing the type of precipitant.[35] In addition, precipitation using an ionic polymer, polyetheleneimine, has been proposed to generate a hydrophilic microenvironment surrounding the enzymes, thus reducing the concentration of organic solvent molecules in the enzyme environment and improving the stability of the enzyme in the presence of organic solvents.[39–41,54,98] These changes in catalytic behaviour of CLEAs based on the properties of the precipitants could be due to induction of different enzyme conformations by different precipitants, that are subsequently locked by the cross linking. Therefore the choice of the best applicable precipitant is usually empirical.

Furthermore, the dosage of the precipitation agent also affects the recovered activity of CLEAs. Activity recovery in CLEAs of *Candida rugosa* lipase,[66] tyrosinase,[69] invertase[60] and acetyl xylan esterase[75] was increased with increasing ammonium sulphate saturation of the enzyme solution during precipitation. Moreover, greater than 100% activity recovery, called ''hyper activation'', has been obtained at high ammonium sulfate concentration due to the formation of fine grained and more structured CLEAs[60,69] (Fig. 3 and Fig. 4). Similarly, lipases[84] and lipozyme TL 100 L[99] were gradually precipitated by increasing the amount of acetone and PEG600, respectively and at high concentration of precipitants, CLEAs retained almost all of the free enzyme's activity. When ammonium sulfate and *tert* butanol were used in amounts greater than twice (v/v) the volume of commercial *Linum usitatissimum* hydroxynitrile lyase solution, a fair amount of precipitate with little deactivation and soluble enzyme left was obtained during CLEAs preparation.[102] As seen from these reports, precipitating the enzyme with high precipitant concentrations always gave a completely active aggregate in cases of poor activity recovery. The reason for this high retention of activity can be found in a different outcome of the competition between aggregation and denaturation due to severe forces exerted on the enzyme tertiary structure during precipitation.[57] When the process of precipitation is slow, the enzyme denatures. If it is fast then the enzyme molecule is

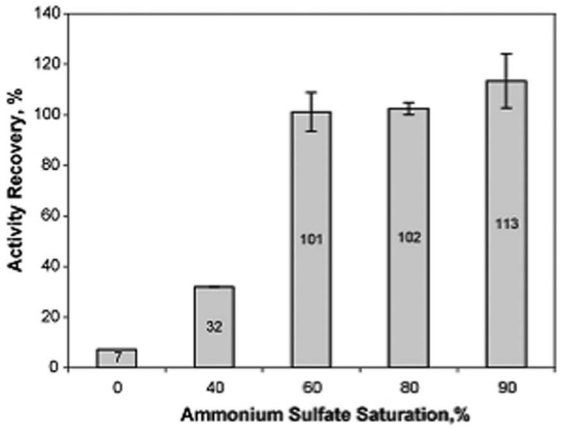


Fig. 3 Effect of ammonium sulfate concentration on CLEAs' activity recovery. Reprinted from ref. 69 with permission from Elsevier. Copyright 2007 Elsevier Ltd.

able to find neighbouring molecules in time to surround it and chances are fairly good that it will retain its tertiary structure. Therefore, it is worth noting that a high concentration of precipitant results in a shock wise aggregation in which time there is little chance for the enzyme to denature (Fig. 5).

3.3 pH and temperature

Principally, cross linking of protein molecules using glutaraldehyde largely involves the reaction between highly reactive surface unprotonated amino groups of lysyl residues and aldehyde groups of glutaraldehyde.[103–106] Therefore the charge of the surface lysyl amino groups regulates intermolecular cross linking. It should be noted that lysyl amino groups have an acid dissociation constant (pK_a) > 9.5 and hence there would be an optimum pH or pH range at which maximum cross linking occurs between protein molecules. In most of the reports, the preparation of CLEAs has been carried out at around neutral pH or slightly alkaline pH range. This is because of the high reactivity of glutaraldehyde toward proteins at around neutral pH due to the presence of several reactive residues in proteins and molecular forms of glutaraldehyde in aqueous solution, leading to many different possible reaction mechanisms.[107] Therefore, the choice of pH should also be taken into account regarding the reactivity of glutaraldehyde. Glutaraldehyde reacts reversibly with amino groups over a wide pH range above pH 3.0, except between pH 7.0 to 9.0 where only a little reversibility is observed.[108] Consequently, cross linking at pH 7.0 to 9.0 leads to complete cross linking of all enzyme molecules, hence the high activity recovery in CLEAs. Moreover, glutaraldehyde in an aqueous solution at acidic pH is present predominantly in a monomeric form whereas alkaline pH favours the existence of a polymeric form.[109–111] Due to this, lipase CLEAs prepared with glutaraldehyde at pH 4.5 were found to be compact CLEAs with less space between enzyme aggregates because the cross linking reagent is a monomer. On the other hand, in CLEAs produced with glutaraldehyde at pH 9.5, the equilibrium shifted towards the formation of polymers of glutaraldehyde, which gave greater CLEAs with more space between the enzyme aggregates, thereby preventing the occurrence of diffusional restrictions.[87] In addition, adequate cross linking occurred at pH 9.5, resulting in high activity recovery and stability of lipase in CLEAs. Similar to cross linking, the precipitation step is also affected by the pH. When the pH of the enzyme solution is equal to pI of the enzyme, the electrostatic forces are reduced and dispersive forces dominate, causing the enzyme molecules to aggregate and precipitate[112] which after subsequent cross linking can result in highly active CLEAs. For instance, the *A. bisporus* laccase gave high activity recoveries at higher pH (pH 8), owing to the pI in the alkaline range due to more strongly basic Arg and Lys residues compared with the other two laccases from *T. versicolor* and *T. villosa*.[113]

Although Ottesen *et al.*[114] and Bullock[115] suggested that the cross linking reaction of glutaraldehyde with lysine residues progresses with time depending on the accessibility of the lysyl amino groups, its rate will depend on the temperature for an enzyme with a typical number of accessible lysyl amino

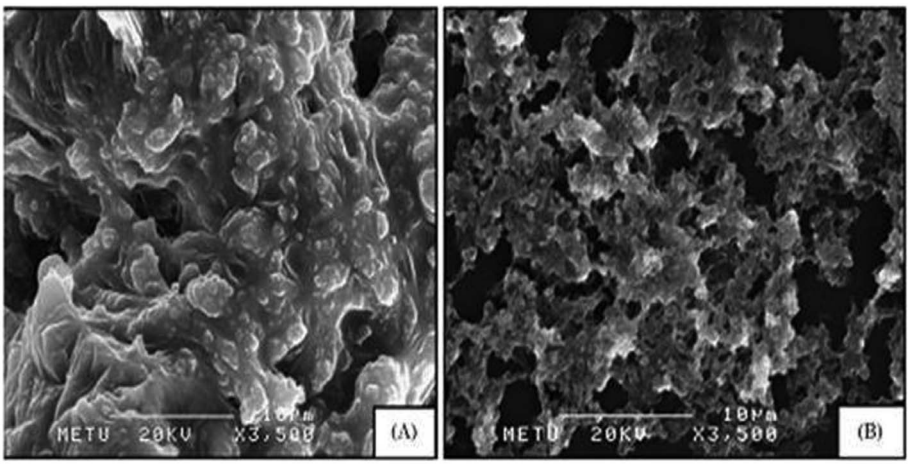


Fig. 4 Scanning electron microscope images of CLEAs prepared with 40% (A) and 80% (B) ammonium sulfate saturations (magnification 3500 ×). Reprinted from ref. 69 with permission from Elsevier. Copyright 2007 Elsevier Ltd.

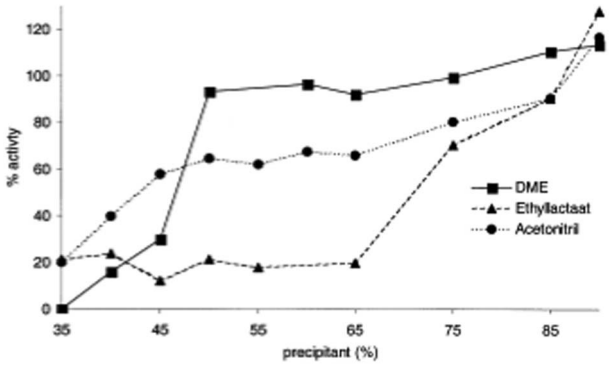


Fig. 5 Precipitation of glucose oxidase. The activity shown is measured after resolvation of the aggregate in buffer. Reprinted from ref. 57 with permission from John Wiley & Sons, Inc. Copyright 2004 Wiley Periodicals, Inc.

groups. At lower temperatures, cross linking requires long reaction times whereas at higher temperatures it occurs within short times. In the majority of reports, the formation of CLEAs has been carried out at low temperature (4 °C) due to the heat labile nature of the enzymes. Formation of CLEAs at high temperature resulted in less active CLEAs. For instance, the activity of Lipozyme TL 100 L CLEAs decreased with increasing glutaraldehyde cross linking temperature and the colour of the aggregated enzyme turned into taupe at temperatures higher than 20 °C (Fig. 6).[99] Similarly, laccase CLEAs prepared at 4 °C with chitosan as cross linker showed highest activity to those prepared at 30 °C.[116] It is probably because of the thermal degradation of the enzymes at high temperature, therefore yielding less active CLEAs. However, cross linking of tyrosinase aggregates at room temperature recovered 100% activity of the free enzyme in CLEAs, perhaps due to the higher thermal stability of tyrosinase.[68] Therefore it is advisable to select the temperature of formation of CLEAs based on thermal stability of enzyme.

3.4 Nature of cross linker

Traditionally, among the many available protein cross linking agents, glutaraldehyde has been undoubtedly used as the cross

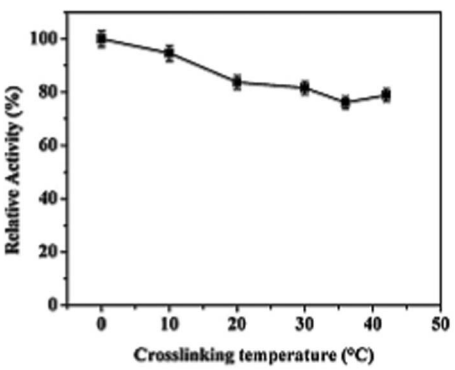
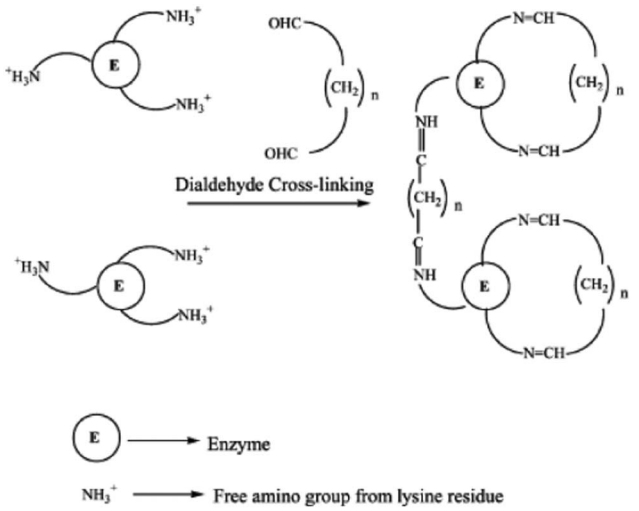


Fig. 6 Effect of cross linking temperature on the relative activity of CLEAs. Reprinted from ref. 99 with permission from Elsevier. Copyright 2012 Elsevier B.V.



Scheme 5 Schematic representation of inter and intramolecular imine cross linking through dialdehyde (like glutaraldehyde) coupling. Reprinted from ref. 117 with permission from American Chemical Society. Copyright 2004 American Chemical Society.

linking agent to prepare CLEAs (Scheme 5). However, with some enzymes, *e.g.* nitrilases, low or no retention of activity has been observed when glutaraldehyde was used as the cross linker. It may be due to the small size of the glutaraldehyde which allows it to penetrate the interior of the protein, causing the reaction of the cross linker with amino groups that are crucial for the catalytic activity of the enzyme. In order to circumvent this, dextran polyaldehyde, a bulky polyaldehyde, was used as the cross linker. Due to its large size, it could not penetrate the interior of the protein which precludes its possible reaction with catalytically relevant amino groups in the active cleft. Indeed, 50–60% of nitrilase activity was recovered in CLEAs prepared using dextran polyaldehyde as cross linker whereas the use of glutaraldehyde caused complete inactivation of these enzymes. Similarly, a significant percentage of active sites of penicillin G acylase CLEAs were irreversibly lost when glutaraldehyde was used, whereas >90% of the active sites were preserved when using dextran polyaldehyde (Table 2).[38] In addition, the enzyme aggregates cross linked by micro-molecular cross linker like glutaraldehyde form a compact super-molecular structure and hinders the mass transfer of substrates within the enzyme molecules, which would lead to the low activity of CLEAs especially when the substrates were macromolecules (Fig. 7 and Table 3).[101] Therefore, for the preparation of CLEAs of enzymes in which lysyl amino groups are essential for their activity and when the

Table 2 Active site titration of penicillin G acylase CLEAs. Reprinted from ref. 38 with permission from John Wiley & Sons, Inc. Copyright 2004 Wiley Periodicals, Inc

Cross linker	Activity recovery (%)	Active site recovery (%)
Glutaraldehyde	48	49
Dextran polyaldehyde	85–90	92

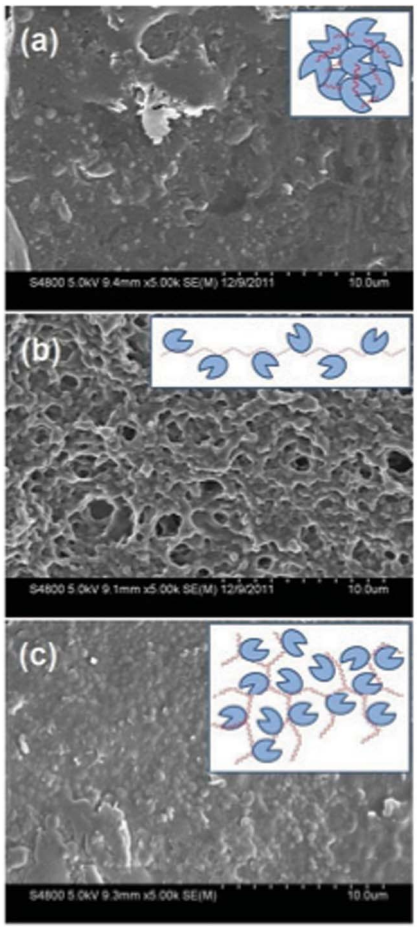


Fig. 7 The morphology of β-mannanase CLEAs cross linked by (a) glutaraldehyde, (b) dextran (2000 kDa) and (c) dialdehyde starch (magnified 5 k times). Reproduced from ref. 101.

substrates are macromolecules, macromolecular cross linkers are generally superior to small dialdehydes such as glutaraldehyde.

Recently some researchers embarked on attempts to replace glutaraldehyde as a cross linking agent to construct a flexible technology platform for designing robust CLEAs for broad applications. Wang *et al.*[118] have proposed a facile technique to prepare cross linked enzyme aggregates of lipase using *p*-benzoquinone as the cross linking agent (Scheme 6). In this technique they have prepared more stable CLEAs of lipase than when using glutaraldehyde as the cross linking agent because of the formation of C–O and C–N bonds tolerant to pH variation using *p*-benzoquinone as cross linking agent. To avoid the adverse effects on aquatic ecosystems and human health caused by leaching of glutaraldehyde to the environment, Arsenault *et al.*[116] used chitosan as a novel cross linking agent for the preparation of laccase CLEAs destined for environmental processes. The amino groups present on chitosan reacted with activated carboxylic group present in the nonessential amino acids of the enzyme molecules and formed amide bond cross links. In similar lines, to overcome the potential issues of toxicity and biocompatibility of glutaraldehyde, Ayhan *et al.*[119] demonstrated a simple and successful routine to obtain robust and biocompatible glucose oxidase, peroxidase and urease CLEAs using a biocompatible and non-toxic biomolecule L-Lysine as the cross linker and bovine serum albumin (BSA) as a proteic feeder. Hydroxyl groups of serine and threonine residues in proteins were oxidized with periodate to yield aldehydes and then cross linking was carried out by adding L-Lysine to recover around 60% of the free enzyme activity (Scheme 7).

Although some researchers recently succeeded in replacing glutaraldehyde as cross linking agent due to its toxic effects, many researchers have continued to use glutaraldehyde to develop CLEAs for the catalysis of several reactions. It may be because of its low toxicity in the relevant reactions, commercial availability, low cost and ease of manipulation in addition to its high reactivity compared with other reported cross linkers. It also reacts rapidly with amine groups at around neutral pH and is more efficient than other aldehydes in generating highly stable intra and intermolecular cross links.[120] It could be advisable, therefore, to select the cross linker based on the final application of CLEAs and severity of glutaraldehyde toxicity. In our opinion, further research should be continued to find alternative safe cross linkers, giving priority to high activity recovery and operational stability of enzymes in CLEAs.

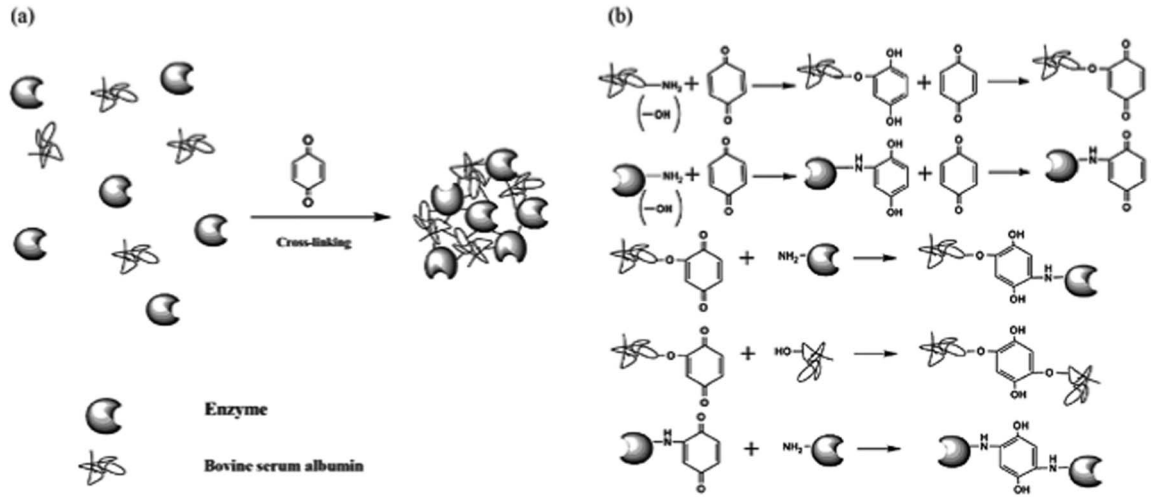
Table 3 The activity of β-mannanase CLEAs cross linked by glutaraldehyde, dextran polyaldehyde and dialdehyde starch[a]. Reprinted from ref. 101. Copyright 2013 The Royal Society of Chemistry

Cross linker	Molecular weight (kD)	Activity (%)[b]
glutaraldehyde	0.1	4.68
dextran polyaldehyde	42	11.12
	466	25.94
	2000	32.01
dialdehyde starch	350–750	20.98

[a] The CLEAs were prepared by precipitating β-mannanase with isopropanol/enzyme solution of 5(v/v) for 30 min and cross linking at 4 °C for 16 h with 0.01 mL of glutaraldehyde (50%, *w*/*v*), 0.1 mL of dextran polyaldehyde (3%, *w*/*v*) and 0.1 mL of dialdehyde starch (3%, *w*/*v*) respectively. [b] Assuming the relatively catalytic efficiency of free β-mannanase on carob bean gum was 100%.

3.5 Amount of cross linker

One key parameter in the production of CLEAs is the concentration of cross linker as it influences the activity, operational stability, and particle size (morphology) of the resulting CLEAs. As is evident from previous literature, the concentration of cross linker has an optimum point. It is observed that the activity recovery of enzymes increases with an increase in cross linker concentration to a maximum value and then reduces with further increase in cross linker concentration. At lower cross linker concentration insufficient cross linking occurs, affording very little insoluble aggregates that would result in operationally unstable CLEAs releasing free enzyme into the reaction medium. On the other hand when the cross linker concentration is high, rigidification of the enzyme molecule occurs due to excessive cross linking, resulting in a loss of the enzyme's flexibility which is essential



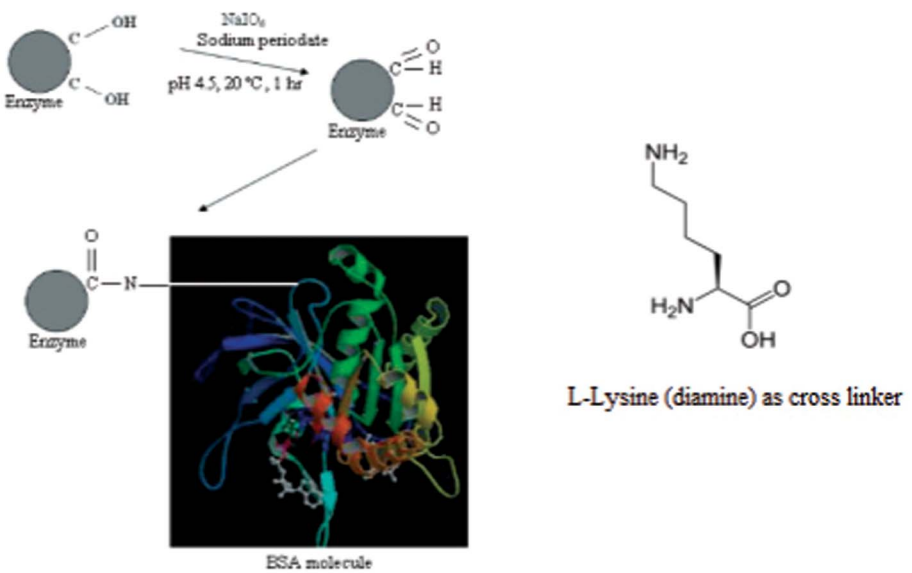
Scheme 6 Facile route to prepare CLEAs and possible mechanism for the cross linking reaction using *p*-benzoquinone as cross linker. (a) Route to prepare CLEAs (b) Possible mechanism for the cross linking reaction using *p*-benzoquinone as cross linker. Reprinted from ref. 118 with permission from Springer. Copyright 2011 Springer.

for its activity. Steric hindrance is also created due to rigidification which prevents the substrate from reaching the active site.[121] Therefore there will be an optimum cross linker concentration at which sufficient cross linking occurs while retaining an enzyme's flexibility with high activity recovery and operational stability (Fig. 8). As the number of free amino groups on the surface varies from enzyme to enzyme, one would expect there to be a unique optimum cross linker concentration for an enzyme with a given number of free amino groups. Thus the optimum concentration has to be determined for each enzyme.

The concentration of cross linker is also important in determining the morphology and hence catalytic properties of CLEAs. The amount of cavities was increased by increasing the cross linker concentration and fine grained, more structured CLEAs were obtained at around optimum cross linker concentration[66,69,87] whereas when the cross linker concentration was increased, course grained, larger CLEAs with less cavities were obtained.[66] As a result, internal mass transfer limitations and hence decreased catalytic activity are unavoidable at higher cross linker concentrations (Fig. 9).

3.6 Cross linking time (aging effect)

Since cross linking is a reaction, the time required to recover maximum activity of enzymes in CLEAs during cross linking is important.[122] As for the cross linker to enzyme ratio, the cross linking time also passes through an optimum (Fig. 10). A very short cross linking time results in inadequate cross linking,



Scheme 7 Schematic representation of $NaIO_4$ oxidation and L-Lys cross linking. Reprinted from ref. 119.

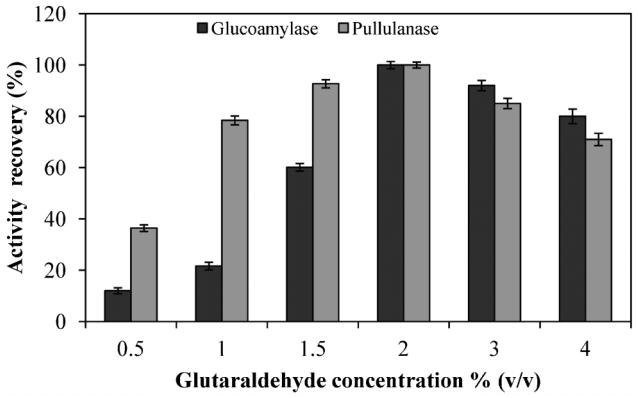


Fig. 8 Effect of glutaraldehyde concentration on the activity recovery of glucoamylase and pullulanase in combi-CLEAs. Reproduced from ref. 173.

leading to poor activity recovery and operational stability of CLEAs. As higher cross linker to enzyme ratio favours faster cross linking, one might expect efficient cross linking with a shorter cross linking time with a higher cross linker to enzyme ratio. However, a high cross linker to enzyme ratio is detrimental to enzyme activity. Prolonged cross linking time restricts the enzyme flexibility, abolishing enzyme activity due to more intensive cross linking. However, the optimum cross linking time can involve a compromise between efficient cross linking and enzyme stability. For instance, the lipase CLEAs showed a decrease in activity from 100% to 85% to 50% upon increasing cross linking times of 4, 6 and 8 h, respectively whereas thermal stability (Fig. 11) and reusability (Fig. 12) of lipase CLEAs were substantially increased upon increasing cross linking time.[93] Similar observations of cross linked enzymes were obtained earlier.[123,124]

3.7 Washing and separation of CLEAs

After cross linking, in order remove unbound proteins and glutaraldehyde, CLEAs are suspended in buffer and washed by centrifugation. Furthermore, for the separation of CLEAs from the reaction mixture, centrifugation or filtration is used. Since due to a cross linking reaction, the enzyme molecules are packed together in a small volume which results in a small pore size in the CLEAs. As a result, one might expect internal mass-transport limitations.[57] Also due to less compression resistance of CLEAs, washing and separation by centrifugation or filtration leads to further squeezing of the CLEA particles, elevating mass-transport limitations to a noticeable level which is serious, particularly in the case of enzymes acting on macromolecular substrates.[75] This reduces the global activity and broad applicability of CLEAs. Therefore, controlling the mass transport limitations created due to washing and separation by centrifugation or filtration treatments should be the focus of further research in designing CLEAs with high catalytic efficiency.

Recently, to overcome the problem of small pore size in CLEAs, a novel design called porous-CLEAs (*p*-CLEAs) has been proposed to increase the porosity of CLEAs.[61,125,126] It involves the addition of starch as a suitable pore making agent due to its large molecular size into an enzyme solution to prepare a co-dissolved homogeneous mixture followed by co-precipitation of the enzyme and starch. The starch is then removed after glutaraldehyde cross linking by alpha amylase, as alpha amylase can hydrolyse starch into maltose and glucose which can be washed away easily (Scheme 8). After characterization of *p*-CLEAs in terms of surface morphology and effectiveness factor, *p*-CLEAs showed improved mass transport of substrate even at low substrate concentration (Table 4) due to greater porosity compared to conventional CLEAs (Fig. 13).[61] In line with this idea of increasing the porosity of CLEAs, porous β-mannanase CLEAs were prepared using linear dextran polyaldehyde (MW 2000 kDa) as a macromolecular cross

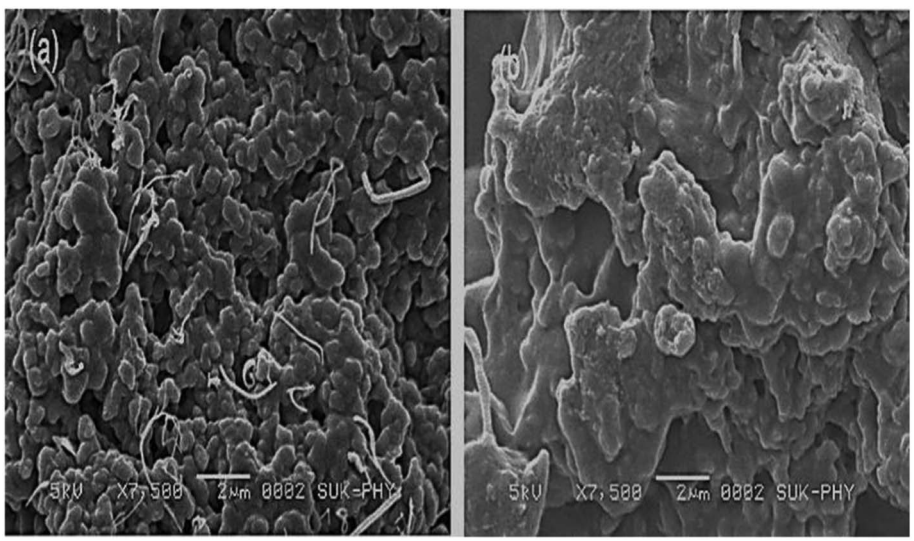


Fig. 9 Scanning electron microscope images of combi-CLEAs of alpha amylase, glucoamylase and pullulanase prepared with (a) 0.2% and (b) 2% glutaraldehyde. Reprinted from unpublished data (Courtesy: S. Talekar).

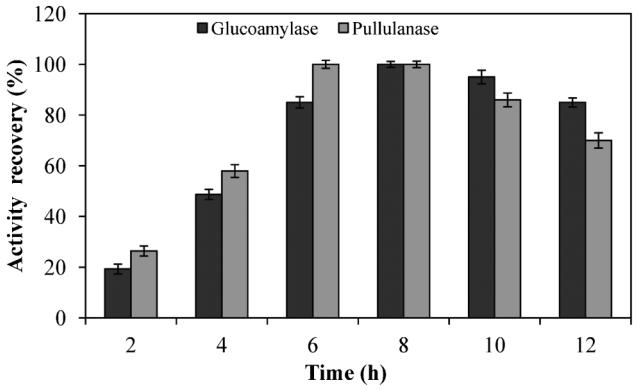


Fig. 10 Effect of cross linking time on the activity recovery of glucoamylase and pullulanase in combi-CLEAs. Reproduced from ref. 173.

linker instead of traditional glutaraldehyde.[101] Scanning electron microscope revealed that CLEAs prepared by linear dextran polyaldehyde (MW 2000 kDa) presented a porous structure (Fig. 7) with low steric hindrance, and thus exhibits excellent activity on macromolecular substrates, 16 times higher than when prepared by glutaraldehyde. Due to this extra porosity created by this method, one can expect to minimize the mass transport limitations generated by squeezing of the CLEAs by washing and separation treatments. Developing an alternative method for centrifugation or filtration for the separation and washing of CLEAs could be an ultimate solution to avoid squeezing of the CLEAs. Recently we have proposed magnetic decantation as an alternative to centrifugation or filtration by developing smart CLEAs called ''magnetic CLEAs'' (Scheme 4). More recently, CLEAs with similar magnetic properties called ''cross linked enzyme aggregate onto magnetic particles (CLEMPA)'' have been prepared by conducting the cross linking of enzyme aggregates

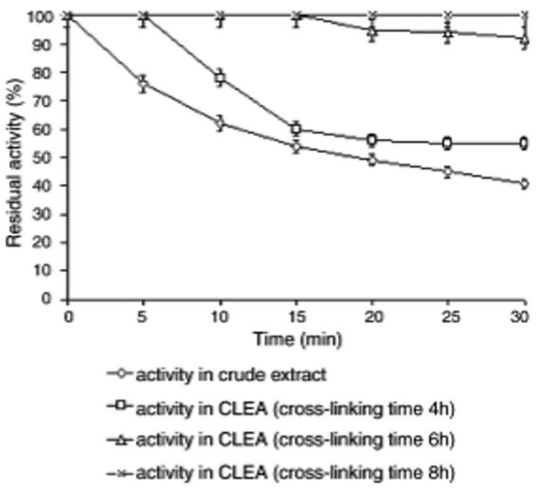
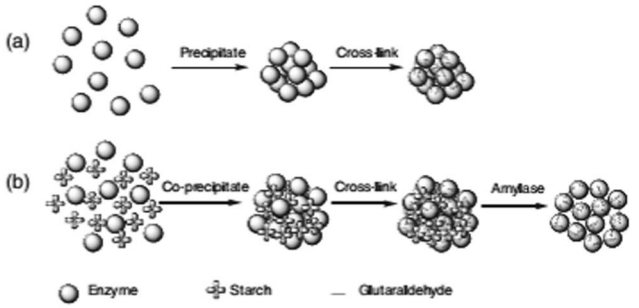


Fig. 11 Thermal stability of lipase in combi-CLEA. Thermal stability of lipase in free enzyme and CLEA was checked at 50 °C, using *p*-NPP as a substrate. Reprinted from ref. 93 with permission from Elsevier. Copyright 2006 Elsevier B.V.



Scheme 8 Preparation of (a) CLEAs and (b) *p*-CLEAs. Reprinted from ref. 125 with permission from Elsevier. Copyright 2010 Elsevier Ltd.

onto magnetic particles with $-NH_2$ terminal groups (Scheme 9).[128] Due to their magnetic nature, magnetic CLEAs and CLEMPA could be washed and separated by magnetic decantation, eliminating the need for centrifugation and filtration and thus circumventing the problem of squeezing of the CLEAs (Fig. 14 and Scheme 9).[51,128]

4 Characterization of new cross linked enzyme aggregates

To construct a flexible technology platform for screening and designing robust CLEAs for large scale applications, newly synthesized CLEAs can be characterized with respect to the following parameters. The protocol for the characterization of new CLEAs is given in Scheme 10.

4.1 Catalytic properties

The study of enzyme kinetics is important for the formulation of kinetic models for the design and evaluation of enzyme reactor performance. Immobilization of enzymes as CLEAs may produce both conformational and micro-environmental effects that will affect the kinetics of the enzyme catalysed reaction. Conformational effects refer to the alteration of the native three dimensional structure of the enzyme protein and the generation of steric effects due to the CLEA procedure which may produce differences in kinetic behaviour with respect to the free enzyme (Table 5). Micro-environmental effects refer to the partition of substrates (and products) to the enzyme phase and mass transport of substrates (and products). Therefore, kinetic properties of the CLEAs such as V_{max}, K_m and catalytic efficiency have to be determined for comparison with the free enzyme.

The catalytic potential of enzymes is affected by several environmental factors among which pH and temperature are the most significant. The change in pH changes the distribution of charges in the active site and in the whole surface of the enzyme molecule whereas a change in temperature causes an alteration of the native structure of enzymes. It is a well-known fact that the rates of enzyme catalysed reactions tend to decrease at extremes of pH and temperature, usually exhibiting maxima at intermediate values. Immobilization usually results in a shift of the optimum pH and temperature

Table 4 Effectiveness factor (η) for free, CLEA and *p*-CLEA forms of invertase. Reprinted from ref. 61. Copyright 2012 The Royal Society of Chemistry

Form of invertase	Concentration of sucrose											
	2 mM		5 mM		25 mM		300 mM		600 mM		800 mM	
	Velocity (mM min^{-1})	η	Velocity (mM min^{-1})	η	Velocity (mM min^{-1})	η	Velocity (mM min^{-1})	η	Velocity (mM min^{-1})	η	Velocity (mM min^{-1})	η
Free	0.72 ± 0.03	1 ± 0.04	2.8 ± 0.2	1 ± 0.07	18 ± 1.2	1 ± 0.06	227 ± 2.6	1 ± 0.01	526 ± 2.9	1 ± 0.005	526 ± 3.1	1 ± 0.005
CLEA	0.11 ± 0.05	0.15 ± 0.06	0.90 ± 0.1	0.32 ± 0.02	10 ± 0.8	0.55 ± 0.01	178 ± 1.0	0.78 ± 0.01	439 ± 1.4	0.83 ± 0.002	440 ± 2.7	0.83 ± 0.006
p-CLEA	0.30 ± 0.04	0.41 ± 0.04	2.0 ± 0.2	0.71 ± 0.03	16 ± 0.6	0.88 ± 0.02	222 ± 3.0	0.97 ± 0.01	523 ± 2.8	0.99 ± 0.004	523 ± 2.2	0.99 ± 0.002

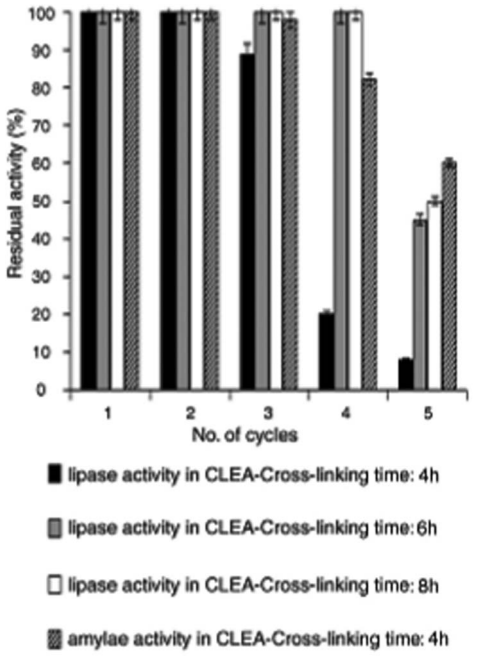


Fig. 12 Reusability of lipase and α-amylase in combi-CLEA. Reprinted from ref. 93 with permission from Elsevier. Copyright 2006 Elsevier B.V.

compared to the free enzyme. Formation of a covalent bond between basic residues of the enzyme and glutaraldehyde during cross linking results in changes in the microenvironment around the active site and a decrease in the conformational flexibility of enzyme.[69,72] Due to this, the optimal pH and temperature of enzyme could be shifted when it is immobilized as CLEAs. Moreover, interestingly it was found that the enantioselectivity (E-values) of soluble nitrilase was increased at higher temperature when it was immobilized as CLEAs.[95] It was due to the same decrease in conformational flexibility of enzymes in CLEAs which results in a reduced conformational perturbation at high temperature compared to soluble enzymes imparting higher enantioselectivity than soluble enzyme. Therefore, each newly synthesized CLEAs must be characterized in terms of optimal pH and temperature required for enzyme activity.

4.2 Particle size and morphology

Particle size and morphology are important properties of CLEA in the context of industrial applications since they directly influence mass transfer and filterability under operational conditions. Mass transfer limitations severely restrict the catalytic potential of CLEAs and is particularly serious in cases of enzymes acting on macromolecular substrates. If the particle size of the CLEA is big, the inner enzymes will lose the opportunity to form complexes with substrates and these enzymes waste their activity. If the particle size of the CLEA is small, all enzyme molecules will have a direct contact with substrate. Based on morphology, CLEAs are classified as type 1 and type 2 aggregates. In case of type 1 aggregates, CLEAs appear fine grained structures with many cavities whereas in case of type 2 aggregates, they are course grained structures

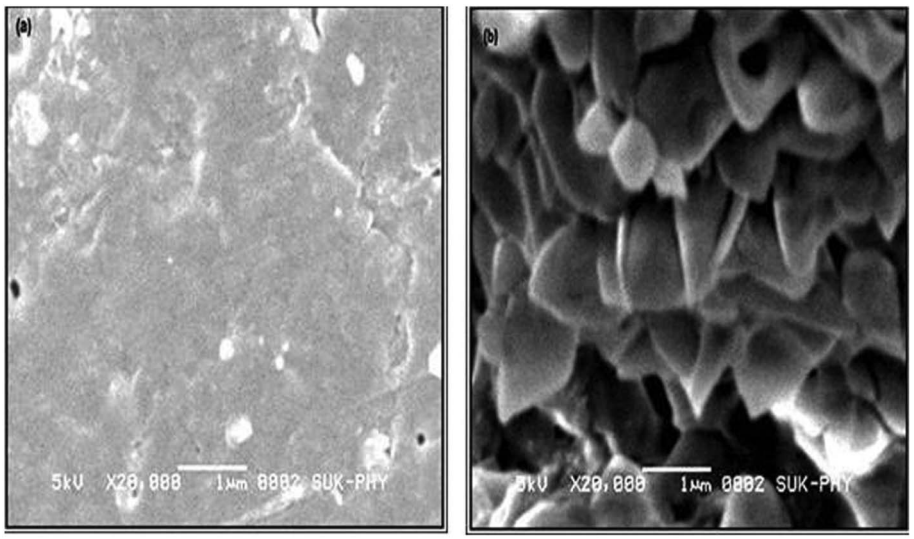
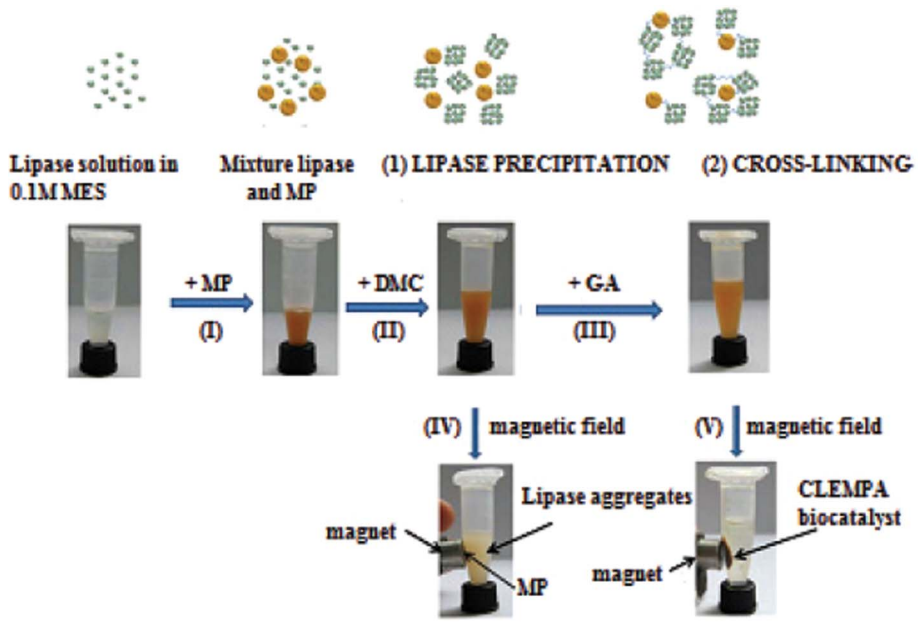


Fig. 13 The SEM images of (a) CLEAs and (b) *p*-CLEAs. Reproduced from ref. 61.

with fewer cavities (Fig. 15). Consequently, CLEAs with type 1 morphology aggregates permit better mass transfer.[57,69] Therefore the particle size and morphology of CLEA must be controlled. This could be done by controlling certain parameters in the synthesis, such as the cross linker to enzyme ratio,[66] aging of CLEAs,[127] the stirring rate, precipitant addition rate, protein concentration,[53] *etc.* Although optimum mass transfer rates are observed with smaller CLEA particles, practical considerations such as the ease of filtration for recycling in batch operations and the large pressure drop over the column in continuous operation observed with small particles dictate the use of larger CLEA particles. Xu and Yang[130] recently encapsulated tyrosinase CLEAs into calcium alginate gels to increase their size, which avoids clumping of fine CLEAs particles and used them for continuous dephenolization processes in a continuously stirred tank reactor. Therefore, this necessitates a compromise between some practical considerations and good activity for large scale application of CLEAs. Due to this fact each newly synthesized CLEA must be characterized in terms of its particle size before large scale use.

4.3 Stability

Despite their many potential advantages, the marginal stability of enzymes is the main problem standing in the way of



Scheme 9 CLEMPA approach for biocatalyst preparation. (MP - magnetic particles; DMC - dimethyl carbonate and GA - glutaraldehyde). Reproduced from ref. 128.

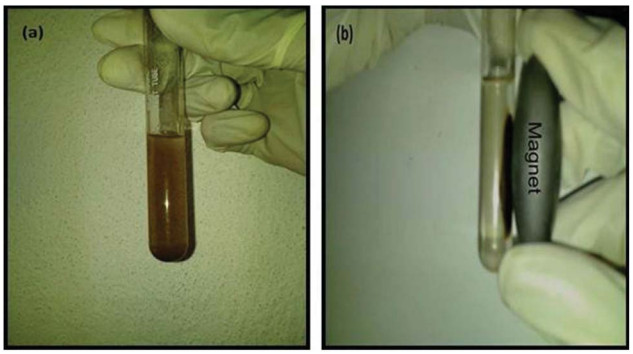


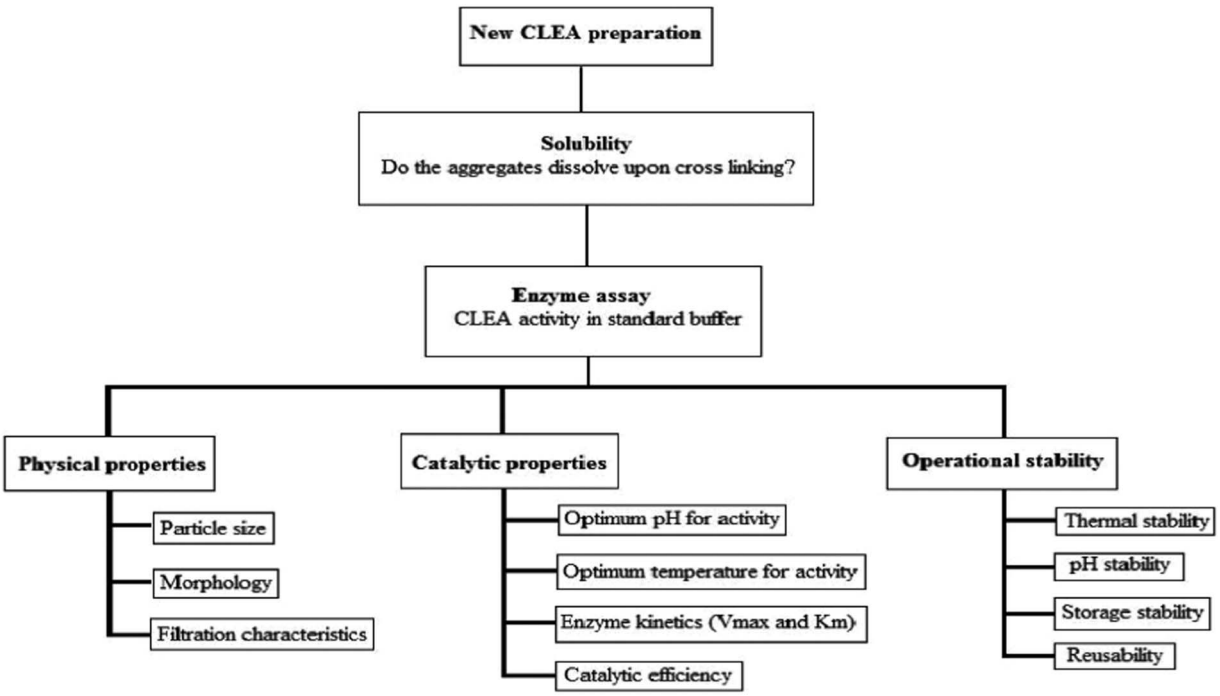
Fig. 14 (a) Magnetic CLEAs dispersed in reaction medium (b) Magnetic CLEAs attracted by magnet. Reprinted from ref. 51 with permission from Elsevier. Copyright 2012 Elsevier Ltd.

expanding the industrial enzyme arsenal. Consequently, there is great interest in developing strategies and procedures for stabilizing enzymes. Immobilization of enzymes is perhaps the most preferred strategy to improve stability of enzymes.[129] This is a direct result of the decrease in flexibility upon immobilization which suppresses the susceptibility of enzymes towards denaturation and accompanying loss of tertiary structure necessary for activity. However, in some cases the enzyme stability has been decreased after immobilization.[131–139] Therefore, as like other immobilized enzymes, the stability of enzymes immobilized as CLEAs should be measured. The stability of enzymes is assessed based on their stability to operational conditions such as temperature, pH, solvents, impurities, other factors that contribute to protein denaturation, and storage conditions.

In the majority of published work on CLEAs, enzyme stability has been determined by assaying the activity decay over time under above mentioned operational and storage conditions. When the activity decay proceeds according to simple kinetic rules, as in thermal inactivation, which obeys first-order kinetics, the half-life values can be calculated by plotting ln(residual activity of enzyme) *vs.* time[140] (Fig. 16 and Table 6). The half-life of an enzyme is a very important parameter that governs the economic feasibility of the concerned bioprocess. However, complications occur when mass transfer limitations in CLEAs lead to low activity. In this case, only a small fraction of the total enzyme in CLEA is working. If in the course of operation the actively working enzyme fraction is inactivated, some other previously ''resting'' fraction may substitute to some extent. In other words, due to mass transfer limitation,[10] CLEAs appear to be much less sensitive to the decay of enzyme activity, thus falsely creating an impression of stabilization. To avoid such misinterpretation, it is advisable not only to determine the stability of CLEA by tracing the time course of activity (half-life) but also to follow its productivity (units/kg of the product) or, alternatively, its consumption in relation to the product formed (kg of the product/unit).

4.4 Reusability

Reusability is the number of times the enzyme can be recycled. Reusability is another important reason to immobilize costly enzymes which facilitates their recovery from the reaction medium, resulting in the simplification of downstream



Scheme 10 Protocol for the characterization of new CLEAs.

Table 5 Kinetic parameters of native and CLEA subtilisin. Reprinted from ref. 72 with permission from Elsevier. Copyright 2008 Elsevier B.V

Enzyme	K_m (moles/ml)	V_{max} (moles/g g^{-1})	K_{cat} (moles/s/mole of enzyme)	K_{cat}/K_m
Native	1.07×10^{-5}	4.21×10^{-6}	0.147	1.4×10^{-4}
CLEA	5.03×10^{-6}	3.22×10^{-4}	8.71	1.73×10^{5}

processing. It also reduces enzyme cost contribution to the product. As a rule of thumb for economic viability of the process, the enzyme costs per kg of the product should not amount to more than a few percent of the total production costs. Indeed, the development of an effective method for enzyme immobilization is crucial for its repetitive usage in large scale industrial application. Therefore, after immobilization, the enzyme should be characterized in terms of how many times it can be recycled.

Reusability of CLEA has been studied in sequential batch operations at optimum reaction conditions for a fixed period of time. After every batch reaction, the enzyme activity in CLEA has been measured and expressed as the residual activity by taking the activity of first batch as 100%. Sometimes conversion achieved after each batch has been measured. However, sequential batch operation for a fixed period of time makes very little sense when enzyme inactivation is significant. Because, in all batches; maximum conversion can be attained by increasing the reaction time.[141] The inactivation due to reuse is likely to be due to long-term mechanical stirring under continuous reactions causing conformational changes which distort the active site. This type of inactivation of CLEAs may be sometimes accompanied by a substantial change in morphology (Fig. 17).[93] Therefore in such cases, the morphology of CLEAs before and after reuse can be compared by taking scanning electron microscopic (SEM) pictures. The activity loss in the recycle experiments has also been resulted from leaching of the enzyme into the reaction medium (Fig. 18).[20] For this reason, leaching of enzyme into a reaction medium should be studied during repetitive use to confirm the reason for the loss of CLEAs activity. It can be done by directly assaying the samples of reaction mixtures for enzyme activity.

Characterization of newly synthesized CLEAs especially in terms of stability and reusability is extremely valuable for its featured industrial applications. Poor stability and reusability have driven researchers to combine CLEA technology with other traditional immobilization methods in order to enhance the robustness and operability of CLEAs. For instance, Hilal *et al.*[142] have immobilized lipase CLEAs within microporous polymeric membranes which can be applied to a cross-flow membrane reactor with greater stability; Wilson *et al.*[143] encapsulated penicillin G acylase CLEAs into very rigid lens-shaped polyvinyl alcohol hydrogel particles (LentiKats), which have successfully improved the mechanical properties of CLEAs; CLEAs of α-chymotrypsin[144,145] and lipase[145] were entrapped in hierarchically ordered mesocellular mesoporous silica, which has proven to be a simple and effective method for enzyme stabilization; Jung *et al.*[146] immobilized chloroperoxidase in the cages of mesocellular foams resulting in leaching resistant, highly stable biocatalyst in a flow-type fixed-bed reactor; and Wang *et al.*[89] have developed a simple strategy for preparing a new kind of CLEA by cross linking papain into the pores of macro-porous silica gel, thus significantly improving the operational and mechanical stability of CLEAs.

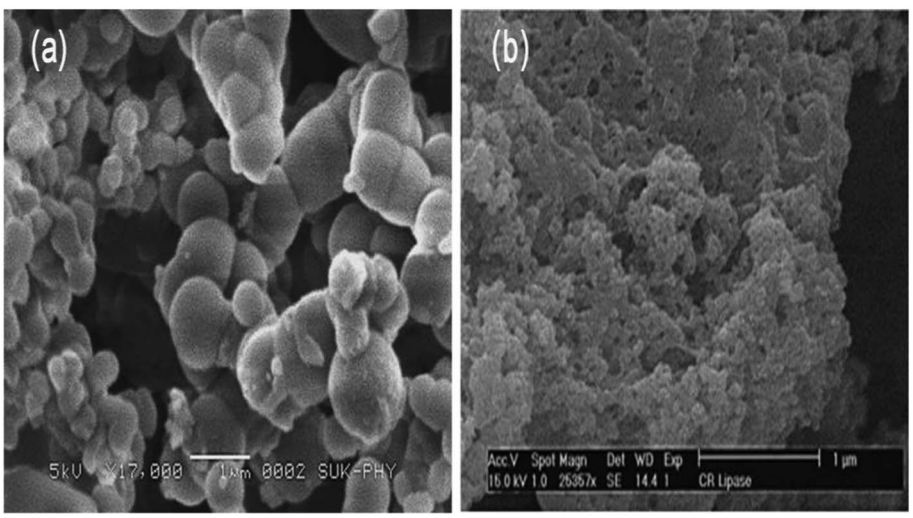


Fig. 15 (a) Type 1 aggregates of alpha amylase CLEAs (Magnification 17 000×). Reprinted from unpublished data (Courtesy: S. Talekar). (b) Type 2 aggregates of *Candida rugosa* lipase (Magnification 25 000×). Reprinted from ref. 57 with permission from John Wiley & Sons, Inc. Copyright 2004 Wiley Periodicals, Inc.

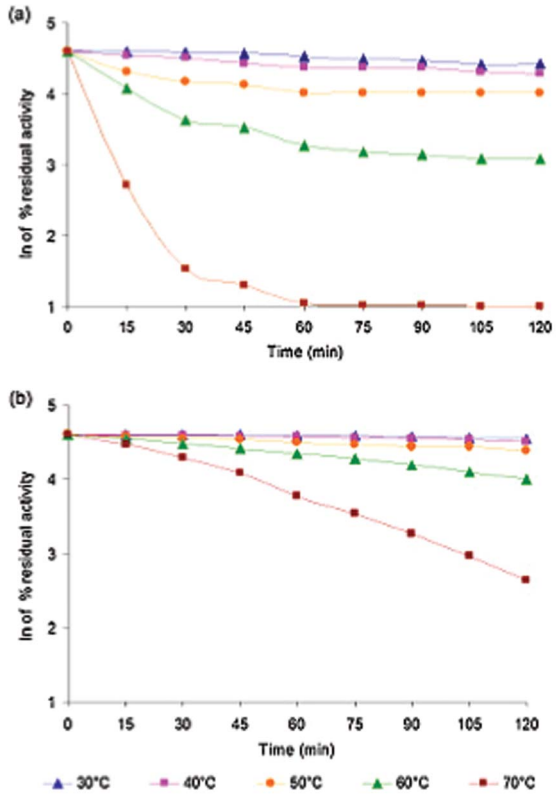


Fig. 16 Thermal deactivation of (a) free aminoacylase and (b) aminoacylase-polyethyleneimine CLEA in temperature range of 30–70 °C. Reprinted from ref. 41 with permission from Elsevier. Copyright 2011 Elsevier B.V.

5 CLEAs in non-aqueous media

Aqueous medium is undeniably the natural milieu for enzyme action. However, it is often advantageous to shift to a non-aqueous milieu when employing enzymes as practical biocatalysts in synthetic chemistry or biotechnology. There are numerous reasons that underlie such a shift, including the insolubility of many commercially relevant substrates in water, various side reactions promoted by water, the unfavourable thermodynamic equilibrium of numerous reactions in water, the difficulties of product recovery from aqueous solutions and microbial contamination often occurring in aqueous medium.[147,148] Exploiting the advantages of non-aqueous catalysis is often limited by the low stability and/or activity of enzymes in non-aqueous system. There are many enzymes which are required to function in non-aqueous media.[149] Therefore, much of the current focus in enzyme technology involves enhancement of enzyme activity and stability in non-aqueous systems. CLEAs have become widely known in the field of biocatalysis and have proven their usefulness for preparative organic synthesis on small scale of operation as discussed below.

5.1 Protease CLEAs

The inexpensive alkaline protease from *Bacillus licheniformis* (alcalase, EC 3.4.21.62, also known as subtilisin Carlsberg) has been widely used in organic synthesis *e.g.*, in the resolution of (amino acid) esters, and amines and peptide synthesis. When it was immobilized as a CLEA, it showed high efficiency in the synthesis of amino acid derivatives and peptides in anhydrous organic solvents, notably methyl *tert*-butyl ether by coupling of N-terminally protected amino acid C-terminal methyl esters with C-terminally protected amino acid nucleophile.[150] Encouraged from these results, the same group[151] explored the scope of coupling reactions in which the poor nucleophile proline was used. Coupling of Cbz-L-Phe-OMe with H-L-*Pro*-O^{t}Bu in the presence of alcalase CLEA in anhydrous tetrahydrofuran gave an almost 98% conversion to the dipeptide after 24 h. Similarly, Vossenberg *et al.*[152] synthesized dipeptides by coupling of the carbamoyl-methyl ester of phenylalanine (of which the amino group was benzyloxycarbonyl-protected, Z-Phe-OCam), and phenylalanine amide (Phe-NH_2) using alcalase CLEA-OM as catalyst in tetrahydrofuran.

5.2 Amidase CLEAs

Penicillin G amidase (E.C. 3.5.1.11) is an industrially important enzyme used in the synthesis of semisynthetic penicillin and cephalosporin antibiotics.[153] This reaction involves enzyme catalysed condensation of the β-lactam nucleus to an acyl donor in aqueous medium. A major problem of such schemes is the competing irreversible hydrolysis of the acyl donor as well as the antibiotic product. One option to reducing the competing hydrolysis is to replace the water by an organic solvent. However, such reaction conditions are not suitable for penicillin G amidase due to its low tolerance to organic solvents, requiring more robust form of enzyme. Cross linked enzyme aggregates (CLEAs), are particularly promising robust biocatalysts for organic synthesis. Indeed, a penicillin G amidase CLEA proved to be an effective catalyst for the synthesis of semi-synthetic penicillins[59,154] and cephalos-

Table 6 Thermal deactivation coefficient (k_d) and half-life ($t_{1/2}$) of free enzyme and AP-CLEA. Reprinted from ref. 41 with permission from Elsevier. Copyright 2011 Elsevier B.V

T/°C	K_d (min^{-1})		$t_{1/2}$(min)		Fold increase in $t_{1/2}$
	Free enzyme	AP-CLEA	Free enzyme	AP-CLEA	
30	−0.00141	−0.00038	496.45	1842.11	3.7
40	−0.00319	−0.00081	219.43	864.19	3.9
50	−0.00840	−0.00189	8333	370.37	4.4
60	−0.01979	−0.00404	35.37	173.26	4.9
70	−0.05262	−0.01015	13.30	68.96	5.1
Average of fold increase in the half-life over the range of 30–70 °C					4.4

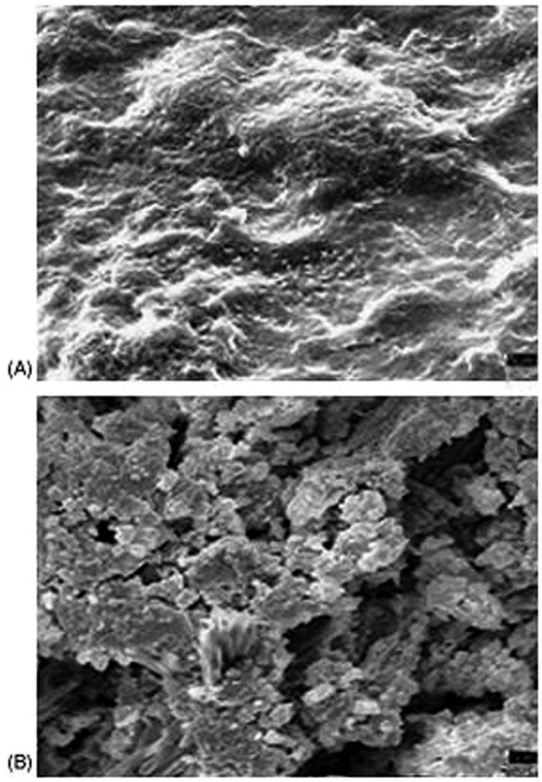


Fig. 17 (a) SEM image of combi-CLEA and (b) SEM image of combi-CLEA after five cycles of use (inactivated CLEA) (Magnification 1500×). Reprinted from ref. 93 with permission from Elsevier. Copyright 2006 Elsevier B.V.

porin.[155] Remarkably, the productivity of the CLEA was higher even than that of the free enzyme from which it was derived and substantially higher than that of the CLEC also.

5.3 Lipase and esterase CLEAs

By far the most popular type of enzymes for industrial applications are lipases (E.C. 3.1.1.3). Therefore, it is not surprising that a variety of lipase CLEAs have been prepared and used as robust catalysts in non-aqueous synthesis. For example, *Candida antarctica* lipase B (CaLB) CLEAs displayed excellent activity in the kinetic resolution of 1-phenyl-ethanol and 1-tetralol, by acylation with vinyl acetate in supercritical carbon dioxide.[156–158] Furthermore, *Candida antarctica* lipase B (CaLB) CLEAs also exhibit excellent activities in ionic liquids.[159] Interestingly, the *C. rugosa* lipase CLEAs showed a 2-fold increase in enantioselectivity in the kinetic resolution of racemic ibuprofen by esterification with1-propanol in iso-octane.[66] Gupta *et al.*[160] prepared highly stable CLEAs from the alkaline and thermostable *Thermomyces lanuginose* lipase which could be used 10 times without appreciable loss of activity in the hydrolysis of olive oil in isopropyl alcohol.

In contrast to the extensive studies devoted to lipase CLEAs, little attention has been paid to other esterases CLEAs in non-aqueous catalysis. Vafiadi *et al.*[100] described the transesterification of methyl ferulate to 1-butyl ferulate using feruloyl esterase CLEAs in ternary water-organic mixture containing more amount of organic solvent. The same group also reported enzymatic synthesis of hydroxycinnamate esters including the synthesis of esters of glycerol in ionic-liquid water mixtures.[161]

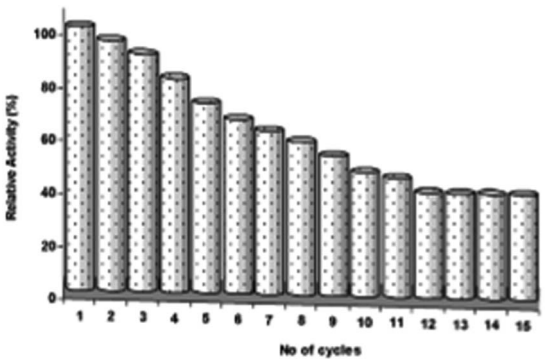


Fig. 18 Reusability of CLEAs. The activity loss in the recycle experiment results from leaching of enzyme into the reaction medium. Reprinted from ref. 20 with permission from Elsevier. Copyright 2011 Elsevier B.V.

5.4 Lyase CLEA

The CLEA methodology has also been successfully applied to various C–C bond forming lyases, notably the *R*- and *S*-specific oxynitrilases (hydroxynitrile lyases, EC 4.1.2.10) for catalysing the hydrocyanation of a wide range of aldehydes in organic solvent. For example, CLEAs prepared from the (*S*)-specific oxynitrilases from *Manihot esculenta* and *Hevea brasiliensis* performed exceptionally well in organic solvents, affording higher enantioselectivities than observed with the free enzymes.[92,162] Similarly CLEAs prepared from the (*R*)-specific oxynitrilase from almonds, *Prunus amygdalis* (PaHnL) were highly effective in the hydrocyanation of aldehydes under micro-aqueous conditions and could be recycled ten times without loss of activity.[91]

6 Combi-CLEAs: Opportunity in catalytic cascade processes

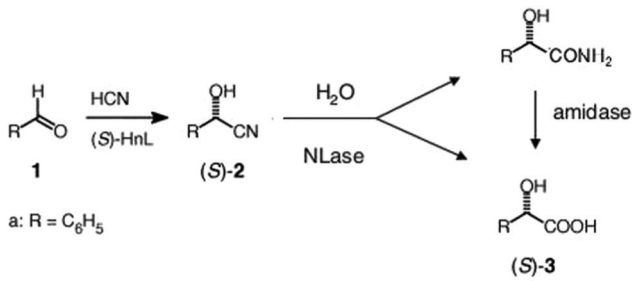
The progress of bio-catalytic methodologies in the synthesis of fine chemicals is rapid. New compounds are being synthesized every day, and it is no longer a question of what we can synthesize, but how we can synthesize it. The traditional approach of synthesizing fine chemicals usually involves multi-step reactions that are carried out in separate stages. Each intermediate product is isolated and purified from the reaction mixture and serves as a substrate for the next step.[163] Such a synthetic approach results in low yield and expensive products as it lasts a long time, needs high operating costs and uses too many chemicals for downstream processing. So there is a need for new kinds of processes which will resolve these issues. Combining catalytic steps in multistep synthetic processes into a one-pot, catalytic cascade process without the need for separation of intermediates could be an ultimate solution.[164] This interesting concept of one-pot, catalytic cascade process truly emulates metabolic pathways in living cells, which involve an elegant orchestration of a series of multi-enzymatic steps into an exquisite multi-catalyst cascade,

without the need for separation of intermediates. Such 'telescoping' of multi-step synthesis process into a one-pot catalytic cascade process has several benefits such as fewer unit operations, less solvent, small reactor volume, shorter cycle times, higher volumetric and space time yields and less waste generation—which translates to substantial economic and environmental efficiency. Furthermore, in a one-pot reaction, as the intermediates can participate in other reactions catalysed by other enzymes simultaneously, their concentrations are usually maintained at a low level and in this way the possibility of product inhibition of one enzyme or substrate inhibition of another enzyme can be reduced, which can drive the equilibrium towards the product.[165–167] However, there are several problems associated with the construction of enzymatic cascades: conditions (such as media, temperature, pH, and enzyme stability) required for each enzyme are different, the rates of each enzymatic reaction are very different, enzyme recovery and recycle is complicated and downstream processing is difficult. Nature has solved this problem by compartmentalisation of the various enzymes inside the cell due to which reactions proceed roughly under the same conditions in water. Hence, compartmentalisation *via* immobilization can be a conceivable way of solving these problems in cascade processes.

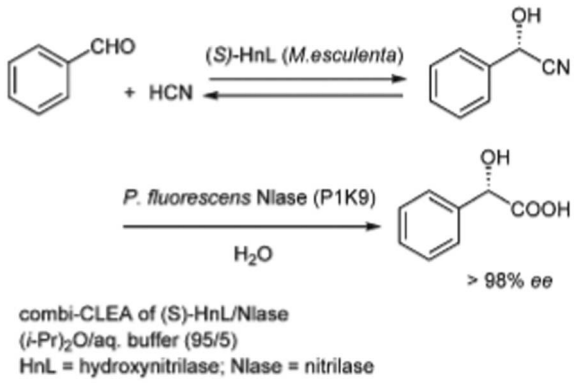
As additional proteins can be incorporated into CLEAs by co-precipitation and cross linking, CLEAs are of interest in the context of immobilisation of multiple enzymes for bio-catalytic cascades. In principle, this can be achieved by co-precipitation and cross linking of two or more enzymes to give combi-CLEAs.[165] We have discussed below examples of cascade processes performed with such combi-CLEAs.

6.1 Combi-CLEAs of hydroxynitrile lyase, nitrilase and penicillin G amidase

A combi-CLEA containing *S*-selective hydroxynitrile lyase(*S*-HnL) in combination with aselective nitrilase (NLase) and penicillin G amidase was used to catalyse one pot conversion of benzaldehyde to *S*-mandelic acid (Scheme 11).[168] The role of hydroxynitrile lyase was to provide enantioselectivity and nitrilase served to drive the equilibrium of the first step towards product by hydrolysing the intermediate nitrile.[169] The penicillin G amidase catalysed the hydrolysis of the *S*-amide by product formed in the nitrilase step. By this process 96% conversion of benzaldehyde to *S*-mandelic acid in 99% ee was obtained. Interestingly, the combi CLEA was more effective than a mixture of the two separate CLEAs due to more close proximity of the two enzymes inside the combi CLEA, compared to the case with two separate CLEAs, which is favourable for transfer of the product of the first step to the active site of the enzyme for the second step.



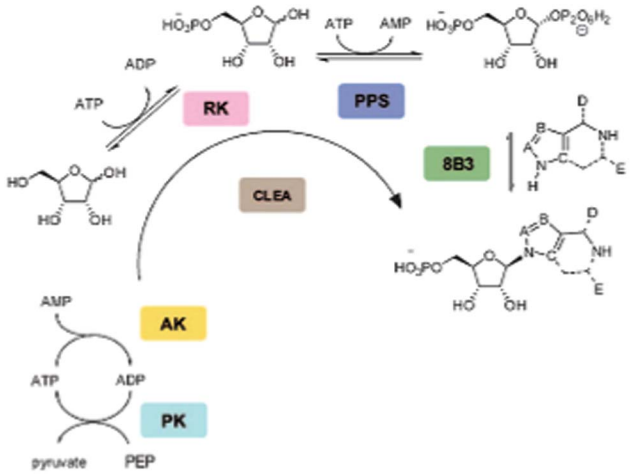
Scheme 11 One pot conversion of benzaldehyde to (*S*)-mandelic acid with combi-CLEAs of hydroxynitrile lyase, nitrilase and penicillin G amidase.



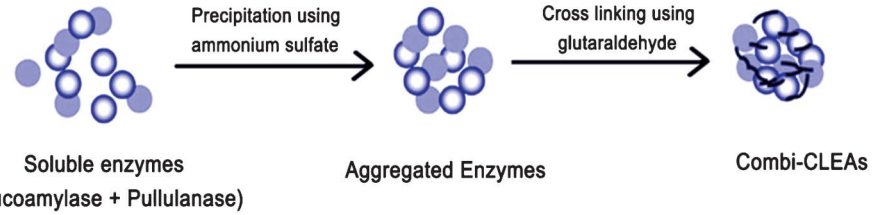
Scheme 12 One pot conversion of benzaldehyde to (*S*)-mandelic acid. Reprinted from ref. 16 with permission from John Wiley & Sons, Inc. Copyright 2007 Wiley-VCH Verlag GmbH& Co.

6.2 Combi-CLEAs of hydroxynitrile lyase and nitrile hydratase

Similarly, van Pelt *et al.*[170] used a combi-CLEA of *M. esculenta* hydroxynitrile lyase and the alkaliphilic nitrile hydratase from *N. akaliphilus* to catalyse the one pot bi-enzymatic cascade conversion of aldehydes to (*S*)-α-hydroxycarboxylic acid amides (Scheme 12).



Scheme 13 Ribose to nucleotide analogues pathway using combi-CLEAs of RK, PPS, 8B3, AK and PK. Reprinted from ref. 171 with permission from John Wiley & Sons, Inc. Copyright 2010 Wiley-VCH Verlag GmbH& Co.



Scheme 14 Preparation of combi-CLEAs of glucoamylase and pullulanase. Reproduced from ref. 173.

6.3 Combi-CLEAs of ribokinase (RK), phosphoribosyl pyrophosphate synthetase (PPS), engineered hypoxanthine phosphoribosyl transferase 8B3PRT (8B3), adenylate kinase (AK) and pyruvate kinase (PK)

A self-immobilized, five enzyme system was developed using combi-CLEAs of RK, PPS, 8B3, AK and PK.[171] Combi-CLEAs were formed using crude cell lysates of recombinant *Escherichia coli* K12 and *Bacillus cereus* and used to develop a five step nucleotide analogue pathway involving a three-step cascade reaction and a two-step ATP regeneration system (Scheme 13). In this pathway, ribokinase phosphorylates the 5′ hydroxyl of D-ribose to form D-ribose-5-phosphate, which is subsequently anomerically pyro-phosphorylated by phosphoribosyl pyrophosphate synthetase, generating PRPP and consuming two equivalents of ATP. The transferase 8B3PRT then catalyzes the addition of purine nucleobases to the activated sugar, and this potentially provides a variety of nucleotide analogues. ATP hydrolysis products ADP and AMP are recycled to ATP by including adenylate kinase and pyruvate kinase, which uses phosphoenol pyruvate (PEP) as a source of activated phosphate. Without extensive optimization, the combi-CLEA was found to be robust, reusable, and demonstrated greatly improved stability in comparison to the soluble enzyme pathway.

6.5 Combi-CLEAs of versatile peroxidase and glucose oxidase

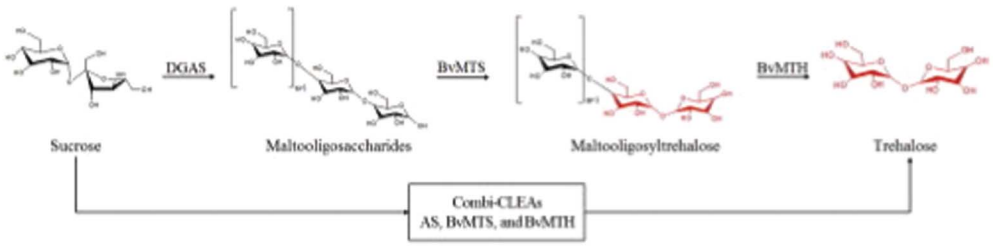
Combi-CLEAs of *Bjerkandera adusta* versatile peroxidase (VP) and *Aspergillus niger* glucose oxidase (GOD) were applied for the elimination of endocrine disruptors.[172] The concentration of hydrogen peroxide has the most crucial effect on VP activity. High concentration of hydrogen peroxide causes the inactivation of the enzyme, whereas too low concentration limits the reaction rate. Hence there is need to control the concentration of hydrogen peroxide. Therefore, a functional catalytic cascade of VP and GOD was developed as combi-CLEAs in which hydrogen peroxide produced *in situ* by GOD upon glucose addition serves as a substrate for VP.

6.6 Combi-CLEAs of glucoamylase and pullulanase

Glucoamylase and pullulanase are used as a blend for the saccharification of starch. Glucoamylase hydrolyses the α (1–4) and α (1–6) glycosidic bonds of starch from the non-reducing ends successively to produce glucose. However glucoamylase is slower in hydrolyzing α-1, 6 branch links. Therefore, it is supplemented with a debranching enzyme, pullulanase, which has the capability to hydrolyse α-1, 6 glycosidic bonds. Recently, we have co-immobilized glcoamylase and pullulanase as combi-CLEAs using commercial multi-enzyme preparation OPTIMAX® 7525 HP and applied for saccharification of starch (Scheme 14).[173] The glucoamylase products containing α-1, 6 branch links are further hydrolysed by pullulanase to release glucose. Interestingly after starch hydrolysis reaction in batch mode, 100, 80 and 30% conversions were obtained with combi-CLEAs, mixture of separate CLEAs and free enzymes, respectively. This could be probably due to close proximity effect as discussed previously. Furthermore, the stability of both enzymes was increased upon combi-CLEA preparation.

6.7 Combi-CLEAs of Amylosucrase (AS), maltooligosyltrehalose synthase (MTS) and maltooligosyltrehalose trehalohydrolase (MTH)

The multi-step bioconversion of sucrose to trehalose was successfully performed in one pot with combi-CLEAs made with AS, MTS and MTH[174] (Scheme 15). Amylosucrase is responsible for the generation of malto-oligosaccharides that are used as substrates for MTS and MTH to form trehalose. The first enzyme, MTS, reacts with malto-oligosaccharides to



Scheme 15 One pot multi-step bioconversion of sucrose to trehalose with combi-CLEAs of AS, MTS, and MTH. Reprinted from ref. 174 with permission from Elsevier. Copyright 2013 Elsevier Ltd.

form malto-oligosyl trehalose by intramolecular transglucosylation of the terminal glucose. The trehalose part of the intermediate is then cleaved by a second enzyme, MTH, to give trehalose and a shorter malto-oligosaccharide. The catalytic activity of enzymes in one pot combi-CLEAs was well-maintained up to five cycles without loss of activity.

Although combi-CLEAs have been mostly described for designing cascade process for one pot bioconversion/biotransformation, some approaches of combi-CLEA were aimed at designing a multipurpose one biocatalyst preparation capable of catalysing pre-determined, unrelated, non-cascade biotransformations/bioconversions. For example, Gupta *et al.*[93] prepared a combi-CLEA from a porcine pancreatic acetone powder extract containing lipase, phospholipase A2, and α-amylase activity. All three enzyme activities were completely retained in the combi-CLEA, and the latter could be recycled 3 times without appreciable loss of activity. The same group[175] also prepared a multipurpose combi-CLEA exhibiting pectinase, xylanase, and cellulase activities from the commercial preparation, Pectinex™ Ultra SP-L. This multipurpose combi-CLEA could be used for carrying out three independent reactions: the hydrolysis of polygalacturonic acid (pectinase activity), xylan (xylanase activity), or carboxymethyl cellulose (cellulase activity). All three enzymes in combi-CLEA exhibited increased thermal stability compared to the free enzyme and could be used three times without activity loss. Similarly multipurpose combi-CLEAs with feruloyl esterase activity were prepared from commercial multicomponent enzyme preparations and used for transesterification of methyl ferulate to 1-butyl ferulate.[100]

7 Scope in the design of enzyme reactors

More than 80% of the commercial value of enzymes is linked to their applications as process catalysts in the fields of food industries, fine chemicals synthesis (particularly in the pharmaceutical area) and even for environmental purposes. In any enzymatic process, poor stability of enzymes is usually the limiting factor during reactor operation. Among the many strategies applied for enzyme stabilization, enzyme immobilization is the most relevant. Most of the time the enzymatic reactions are carried out in a classical batch reactor, of course the application of immobilised enzymes in pharmaceutical and fine chemical industries requires batch processes with recovery by filtration or centrifugation. Despite the advantages of new reactor designs with immobilized enzymes, other industries have been reluctant to implement it and modify existing conventional batch processes. This situation is changing as new strategies of enzyme immobilization are developed. Cross linked enzyme aggregates (CLEAs) technology has emerged as a novel, versatile and highly advantageous immobilization technique since the early 2000. Below we have discussed the scope of CLEA technology in design of enzyme reactors.

7.1 Micro-reactor for process intensification

The tendency towards higher added-value chemicals, with increased product purity at reducing capital and energy costs along with less environmental impact presents one of the major challenges of a process engineer. Which is why process intensification based on micro-reactors, a new concept in chemical engineering which aims at the above challenges has been attracting increasing attention of academia and industrial R&D departments.[176] The excellent performance of micro-reactor systems is achieved by rapid heat and mass transfers as a consequence of the much larger surface area to volume ratios compared to conventional reactors and moreover, the streams in microfluidics mainly form a laminar flow which allows strict control of the reaction conditions.[177] These attractive features are favourable to enzymatic reactions which generally require immobilization of the enzyme, either separately or attached to the microchannel surface in the application of microchannel enzyme reactors.[178,179] Honda *et al.*[180] immobilized α-chymotrypsin as CLEAs on the inner surface of polytetrafluoroethylene (PTFE) tubing of 500 μm diameter and 6 cm length by mixing the solution of α-chymotrypsin with glutaraldehyde and formaldehyde as cross linkers (Fig. 19). This α-chymotrypsin CLEAs microchannel reactor was tested for proteolytic activity and shown to be stable for 40 days of operation while the proteolytic activity of the free α-chymotrypsin solution became progressively lower. Also, the microreactor showed resistance to a chemical denaturant like urea and an organic solvent like dimethyl sulfoxide (DMSO) as compared to free chymotrypsin. In addition, this microreactor could maintain the hydrolysis yield at 90% and above in a continuous flow (4 μl min^{-1}) of substrate solution for a few days. More recently, a similar procedure has been adapted for the immobilization of laccase on the inner wall of poly(tetrafluoroethylene) (PTFE) microtubes to develop laccase-immobilized microreactors which were applied for the biotransformation of model compounds

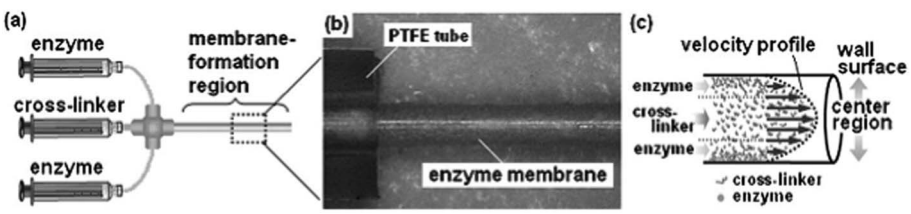


Fig. 19 Preparation of enzyme-membrane on the inner wall of a PTFE tube. a) Enzyme and aldehyde solutions were each charged into a 1 ml syringe, the solutions were supplied to a PTFE tube using a syringe pump. b) Cylindrical enzyme-membrane (dry state) exposed from PTFE tube, which forms on the inner wall of the tube. c) Possible mechanism of polymerization process of enzyme and cross linker reagent in a microchannel. Reproduced from ref. 180.

to demonstrate their efficiency and performance.[181] These laccase-immobilized microreactors exhibited a broader range of optimum pH and temperature and excellent stability under different conditions of pH, temperature, chemical inactivating agent, storage and long-term operation. Important reaction yields were obtained, even at lower residence times compared with conventional bioreactors. Similarly, CLEA based microreactor containing aminoacylase was prepared[182] and used for the continuous resolution of racemic N-acyl amino acids.[183]

In a different strategy, Hickey *et al.*[184] prepared CLEAs of thermophilic L-aminoacylase subsequently mixed with controlled pore glass and packed in capillary reactors fitted with a silica frit to retain them in the reactor. The CLEA microchannel reactor prepared in this way retained enzyme activity for at least two months during storage at 4 °C. Latter, the same group developed a highly miniaturized reaction system based on a γ-lactamase CLEA flow microreactor built from a capillary column for substrate screening and enzyme kinetic characterization, and this CLEA microreactor showed useful stability under conditions of continuous biotransformation.[185] These reports show that the use of such CLEA based microchannel reactors clearly has considerable potential for the design of green and sustainable biotransformations.

7.2 Enzyme membrane rector

The enzyme membrane reactor (EMR) is a specific mode for running continuous processes in which the enzymatic reaction and the separation step take place separately with the help of a selective membrane. An integration of membranes into enzyme membrane reactors is multipurpose, providing enzyme support, control of reactant/product transport and feeding, phase separation for biphasic reactions, reactant/product separation, *etc.* As a result, membrane reactors show various advantages such as easy control and straightforward scaling-up, high enzyme loads, prolonged enzyme activity, high flow rates, and reductions in costs, energy and waste products.[186] The membrane selectivity has been often associated with the relative size of the molecules and membrane pores. As CLEAs are too large to pass through the pores of the membrane, they can be retained in the reactor whereby the substrate and product can be pumped in and out of the reactor. Furthermore, due to large size of CLEAs, membranes with large pore size can be used which avoids fouling and clogging of membranes during continuous reaction. This enables better control of process conditions, eliminates downstream processing steps, and ensures a highly efficient use of the CLEAs. In addition, due to high volumetric activity of CLEAs and high catalyst loading capacity of membranes, membrane reactors with CLEAs can offer a very cost effective system for performing continuous biotransformations with increased space time yields on an industrial scale.

Hilal *et al.*[142] first proposed to immobilize lipase into a microporous polymeric membrane *via* embedding cross linked enzyme aggregates within hydrophilic cellulose and hydrophobic PTFE membranes pores. They formed cross linked lipase aggregates inside the pores of microfiltration membranes by precipitation using organic solvents with simultaneous cross linking by glutaraldehyde. The membrane samples were saturated by lipase solution and then transferred

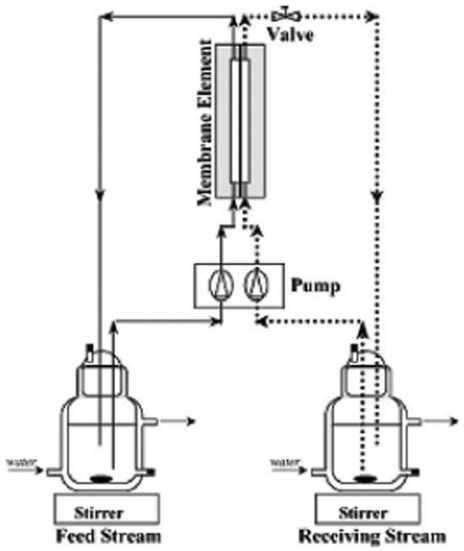


Fig. 20 Schematic diagram of membrane reactor system with cross flow membrane element. Reprinted from ref. 142 with permission from Elsevier. Copyright 2004 Elsevier B.V.

to the solution of glutaraldehyde in acetone. Prepared biocatalytic membranes were studied in a cross flow membrane bioreactor to synthesize butyloleate through esterification of oleic acid with *n*-butanol (Fig. 20).Latter, the industrial utility of the enzyme membrane reactors based on CLEAs 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 in continuous mode.[187] 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^{-1} h^{-1} were achieved using 10% catalyst loading, 70 min. residence time and 20 °C compared to current industrial processes based on repetitive batch operation, followed by extraction of phenyl acetic acid and crystallisation of 6-APA with typical space time yields in the order of 18 g L^{-1} h^{-1} at 35 °C. Furthermore, this CLEA based enzyme membrane reactor system when operated over a period of two weeks, showed no loss in catalyst efficiency or membrane fouling. Recently, combi-CLEAs of versatile peroxidase and glucose oxidase have been applied for continuous removal of endocrine disrupting chemicals using membrane bioreactor.[172] In membrane bioreactor set up combi-CLEAs were retained in cellulose acetate membrane. More than 90% degradation of bisphenol A from a 10 mg L^{-1} solution was obtained at a hydraulic retention time of 90 min constantly for 43 h.

7.3 Enzyme nanoreactors

The individual particle size of mesoporous material is a few hundred nano-meters, which is favourable for the development of nanometer scale reactor for biomolecules.[188] Lee *et al.*[144] have developed a ''ship-in-a-bottle'' approach to obtain active and stable CLEA based enzyme reactors in the pores of a uniquely designed mesocellular mesoporous silica materials

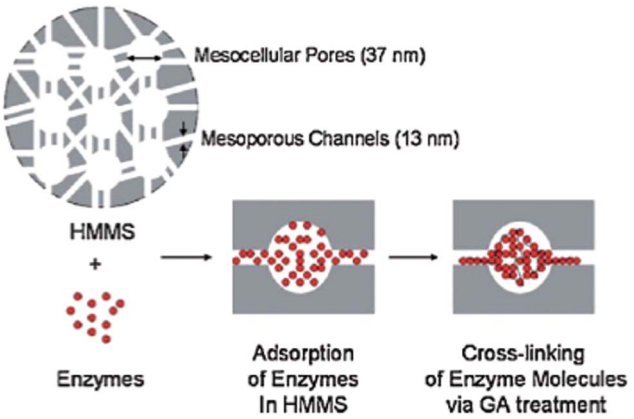


Fig. 21 Schematic representation for CLEAs in HMMS (cross linked enzyme aggregates in hierarchically ordered mesocellular mesoporous silica). Reprinted from ref. 145 with permission from John Wiley & Sons, Inc. Copyright 2006 Wiley Periodicals, Inc.

(HMMS) (Fig. 21). They prepared α-chymotrypsin CLEAs in HMMS by a two-step process. In the first step the enzyme was adsorbed into HMMS, which proceeded with a high degree of enzyme loading within a short time. In the second step, cross linking of enzyme molecules to create aggregates within the pores of HMMS was carried out with glutaraldehyde treatment. Glutaraldehyde cross linking was performed promptly after the enzyme adsorption in order to enhance the high enzyme loading. This approach yielded stable enzyme activity by preventing enzyme leaching, since the enzyme aggregates created in the larger mesocellular pores (37 nm) are not expected to leach out through the smaller mesoporous channels (13 nm). To intensively verify the applicability of HMMS in enzyme immobilization in nanometer scale pores, the same group further described a simple and effective strategy for enzyme stabilization using CLEAs of α-chymotrypsin and lipase.[145] CLEAs of α-chymotrypsin in HMMS showed a high enzyme loading capacity and significantly increased enzyme stability. No activity decrease of α-chymotrypsin CLEAs and autolysis was observed for 2 weeks under even rigorously shaken conditions, while only adsorbed α-chymotrypsin in HMMS and free α-chymotrypsin showed a rapid inactivation due to the enzyme leaching and presumably autolysis, respectively. Moreover, CLEAs of lipase in HMMS retained 30% specific activity of free lipase with greatly enhanced stability.

7.4 Fixed bed and fluidised bed reactor

In industries like the processing of fats and oils, continuous processes with fixed bed of immobilized enzymes are used. This generally requires fairly large particle size of immobilized enzymes in order to avoid a large pressure drop over the column. Therefore, the size of CLEAs must be large enough for the design of a fixed bed of CLEAs in continuous operation mode. In one approach, chloroperoxidase CLEAs and glucose oxidase CLEAs were formed in the cages of mesocellular siliceous foams and the oxidation of indole to 2-oxindole was performed under continuous flow in a fixed bed reactor

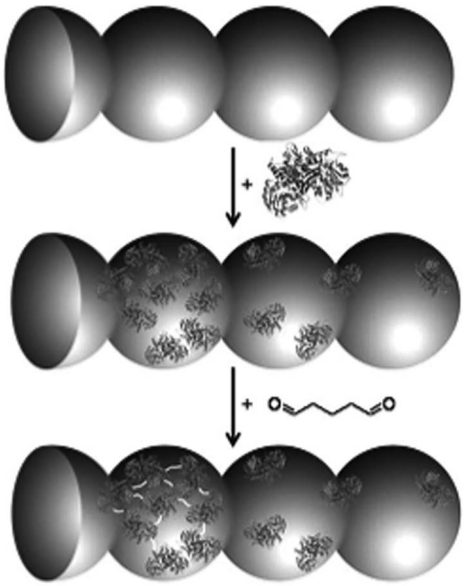


Fig. 22 Preparation of the CLEA in mesocellular foams (MCFs). Reprinted from ref. 146 with permission from John Wiley & Sons, Inc. Copyright 2009 Wiley-VCH Verlag GmbH& Co.

(Fig. 22).[146] The initial activity of the CLEA catalyst was about 40% and dropped to around 14% within 24 h. Thereafter, the yield of 2-oxindole remained constant for another 57 h. For the catalyst prepared by physical sorption, the initial activity was only 17% and dropped to almost 0% after 12 h. These results clearly demonstrated that the use of CLEAs in the pores of mesocellular siliceous foams resulted in highly active and stable biocatalysts for the use in flow type fixed bed reactor. Similarly, glucose oxidase CLEAs were formed in mesocellular foams to enable their use in continuous-flow fixed bed reactors.[189] However, formation of CLEAs in MCF pores results in large particle size which leads to the increased mass transfer limitation for the substrate in MCF containing CLEA,[121] 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, however it requires particles to be of sufficient density; otherwise they will be blown out of the reactor. More recently, we have developed ''smart magnetic CLEAs'' by performing the cross linking in the presence of amino functionalized magnetic nanoparticles.[51] The resulting magnetic CLEAs can be separated by magnetic decantation or can be held in place in a magnetically stabilized fluidized bed.

8 Summary

The addition of a carrier free enzyme immobilization methodology ''CLEAs'' to the biocatalysis tool-box offers several advantages in the context of industrial applications. Enzyme immobilization by CLEA methodology is exquisitely simple. As it does not require a highly pure enzyme, immobilization can be directly started with a crude fermenta-

tion broth, combining purification and immobilization into a single step which translates to low costs and a short time to market. In addition to the benefits obtained by carrier free immobilization, the CLEA methodology offers a low cost, robust and reusable biocatalyst that exhibits high activity retention, enhanced operational stability and better tolerance to non-aqueous solvents. The technique is applicable for the co-immobilization of two or more enzymes as combi-CLEAs which can be used for the development of single pot catalytic cascade processes 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 and nanoreactors has obvious prospective in the rapid screening and optimization of biotransformations. Use of CLEAs in enzyme membrane reactors, packed bed and fluidised bed reactors ultimately proves their potential in development of continuous production processes. Therefore CLEAs will be widely applied in the future development of sustainable products and processes for the chemical and allied industries.

Hopefully, it is clear from this review that these advantages of CLEA methodology could be harnessed by focusing on various parameters involved in CLEA preparation and its characterization. Every enzyme is different and, consequently, there is no all-encompassing, ''one size fits all'' protocol for the development of highly active and stable CLEAs. In this regard, from the past few years, the research on CLEAs has been focused on obtaining insights into the effect of different parameters like the nature and purity of enzyme, the nature and amount of precipitant, the nature and amount of cross linker, the cross linking time, pH and temperature during CLEA preparation and the washing and separation techniques on activity recovery and stability of CLEAs. The newly synthesized CLEAs have been characterized in terms of catalytic properties, particle size and morphology, stability and reusability to confirm their usefulness in industrial application. This provides the rational basis for the synthesis of CLEAs of wide variety of enzymes as a well-defined catalytic particle applicable on large scale industrial processes.

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