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Strategies in Making Cross-Linked Enzyme Crystals

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1. Introduction

Enzymes are recognized as useful tools for accomplishing industrially important chemical reactions in stereo-, regio-, and chemoselective ways. Biocatalytic routes that have emerged over the past few decades offer the promise of radically altering chemical manufacturing processes. Industry needs enzymes for extended periods of use in organic solvent environments for the production of fine chemicals such as pharmaceuticals, agrochemicals, fragrances and flavors, food additives, and consumer care products. One of the major challenges in biocatalytic processes is to improve the activity and stability of the enzymes. Biomolecular engineering[1] techniques are nowadays employed to enhance enzymes' useful properties such as pH stability, thermal stability, increased activity, and so forth. The direct evolution[2] approach is favored for many industrial enzymes owing to the difficulties of relating the desired applications to required properties. Direct evolution, also called molecular evolution through DNA shuffling, involves preparation of protein variants by recombining gene fragments using *in vitro* methods. Other techniques include changing a single or a few amino acid residues,[3] either by exchanging functional domain or by introducing a small protein

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fragment scaffold enzyme. Even though the properties of the enzymes can be improved by any of these methods, enzyme recycling is important in continuous processes for high productivity.

Immobilization circumvents this problem and improves the economy of the process by the reuse of the biocatalyst. However, immobilization is costly and requires an inert matrix on which the enzyme can be immobilized. Moreover, that matrix must often be chemically modified to couple the enzyme. The interaction between the enzyme and the matrix dilutes the effective concentration of the enzymes. The activity loading[4] of the enzyme on the support is usually 0.1−10% w/w. Hence, immobilization has only partially solved the problem of low enzyme activity for biotransformations in nonaqueous media. Cross-linked enzyme crystal (CLEC) technology provides a unique approach to ameliorating the above disadvantages of immobilization. Table 1 provides a comparison of the properties of soluble enzyme, immobilized enzyme, and CLEC. Cross-linking of enzymes results in both stabilization and immobilization of the enzyme without dilution of activity.[5] This review provides a comprehensive account of the different strategies available for the fabrication and characterization of cross-linked enzyme crystals. A detailed description of the techniques employed for activity enhancement of CLECs in nonaqueous media is also included.

2. Features of Cross-Linked Enzyme Crystals for Bioprocessing

CLECs are chemically cross-linked enzyme crystals having special advantages over soluble or conventionally immobilized enzymes for bioprocessing.

Compared to immobilized enzymes or soluble enzymes, CLECs have a higher activity per unit volume. The enzyme concentrations within a CLEC are close to theoretical limits. CLEC particles are uniform and microporous,[6] in contrast to soluble enzymes, and remain monodisperse on reconstitution, even in organic solvents. Owing to their insoluble nature in both organic and aqueous media, separation from product is easy by settling or filtration, thereby eliminating a chance for contamination.

CLECs can withstand the shear forces associated with processing equipment such as stirred tanks, cross-flow microfilters, and pumps, all of which cause particle attrition and fragmentation. CLECs are robust biocatalysts, and extended agitation in suspension at high mixing speeds using either a turbine (high shear) or a propeller blade (moderate shear) does not lead to particle breakage.[7]

CLECs are heterogeneous catalysts and can readily be isolated, recycled, and reused many times. The high operational stability allows reactions at higher temperatures and in aqueous organic solvent mixtures or neat organics, thus increasing the substrate solubility. The ability to withstand proteolysis and autolysis makes possible the use of high concentrations of this modified enzyme in hydrolytic reactions. The intermolecular contacts and cross-links between enzymes in the crystal lattice of a CLEC stabilize the enzyme and prevent denaturation. CLECs can easily be freeze-dried or air-dried and in that form can be stored indefinitely at room temperature.[9] Long shelf life[8] solves storage problems and eases handling of enzymes as ordinary chemicals. CLECs give improved yield under harsh conditions or in situations requiring high throughput and enable process chemists to concentrate on maximizing yields.

CLEC catalysts are basically purified enzymes. Pure enzymes have maximal selectivity but are costly and have low stability, so crude enzyme preparations are used commercially even though they normally contain cell debris, nucleic acids, inactive proteins, and pigments. Such catalyst cocktails may sometimes catalyze competing reactions, interfere with purifica-

Table 1. Comparison of the Characteristic Features of Soluble and Immobilized Enzymes and CLEC

character	soluble enzyme	immobilized enzyme	cross-linked enzyme crystal
enzyme purity	enzyme of any purity	even crude enzyme can be immobilized	only pure enzymes can be used
stability	can be stored at concentrated form at refrigerated temperature	store at refrigerated temperature	higher stability due to cross-linking; can be stored at room temperature
specific activity	high specific activity	dilutes the activity due to the interaction with the matrix	high specific activity due to high volumetric activity
reaction in aq/org media	only in aqueous media	react in both aqueous and less in organic media	react in both aqueous and organic media
separation from the reaction mixture	difficult to separate from reaction mixture	can be separated by filtration or centrifugation	easily separated by filtration or centrifugation
pH and thermal stability	not stable over a range of pH and temperature	not stable over a range of pH and temperature	stable over a range of pH and temperature
productivity	low productivity	high productivity	very high productivity

tion of the final product, and thus make the process difficult and expensive. Contamination of the reaction mixtures by proteins and the products of protein self-digestion is a serious problem in the synthesis of peptides or other pharmaceuticals since such contaminants can cause anaphylactic shock. Thus, in these applications, a thorough purification of the product is required. Cross-linked enzyme crystals eliminate these problems since they are pure, heterogeneous, and free of unwanted protein contaminants.

CLECs have high specific activity. The entire volume of a CLEC consists of an active catalyst with no inert carrier as opposed to the situation for immobilized enzymes. Enzyme concentrations within the crystal approach the theoretical packing limit[10] for molecules of a given size. Thus, the volumetric activity of CLECs is 2−4 orders of magnitude higher than that of both conventional and immobilized enzymes, which reduces both the reaction time and the volume of enzyme required, thereby maximizing the volumetric productivity.

CLECs are environmentally benign and easy to dispose of compared to traditional chemical catalysts (precious metals), resolving agents, or coupling agents. The cost savings from simplified product workup and catalyst disposal procedures alone can be very significant. In a batch configuration, CLECs can be recycled and used many times, and in column configuration they can be used for a long time. Thus, this technology is cost-effective. The increased selectivity of CLECs and their high stability at elevated temperature and in the presence of organic solvents significantly broaden the synthetic potential of enzyme catalysts. Many biotransformation processes that chemists could not even contemplate before are now possible with CLECs.

CLECs do not require expensive supports or complex immobilization procedures, and they exhibit the highest possible protein density. The high specific activity means a CLEC will generate the largest possible signal[11] from even the smallest substrate (analyte) concentration. Proteins in crystal form are uniformly arranged, which produces a linear and predictable signal.

Soluble enzymes are vulnerable to proteolysis as well as contamination in many biosensor environments. CLECs are very stable toward degradation by protease since both the protein−protein interactions necessary for this process are hindered, and also access of the protease to the crystal lattice is limited.

3. Preparation of Cross-Linked Enzyme Crystals

CLECs are prepared stepwise, with the first step being controlled precipitation of enzymes into microcrystals. The second step is cross-linking with bifunctional agents to form strong covalent intra- and intermolecular bonds between the ϵ-amino group of lysine residues[12] or by cross-linking between carbohydrate moieties[13] of the enzymes. Inexpensive chemical cross-linking locks the enzyme in the crystalline form, thus enhancing both thermal and proteolytic stability as well as insolubility in both aqueous and organic solvents.

$$\text{pure enzyme} \xrightarrow{\text{crystallization}} \text{enzyme crystals} \xrightarrow{\text{chemical cross-linking}} \text{CLEC}$$

3.1. Enzyme Crystallization

Crystallization is a powerful tool for the purification,[14] isolation, and long-term storage of enzymes. The effectiveness of crystallization for purification is usually greater than that of any other method. In general, enzymes are much more stable in crystalline form than in soluble or amorphous form. Also, crystallized enzymes are more concentrated and are purer. Crystallization of macromolecules requires the creation of a supersaturated state. This is a nonequilibrium condition in which some quantity above the solubility limit of the macromolecules is present in solution. Crystallization strategies for enzymes have been extensively reviewed in research papers[15−20] and books.[21,22] Crystallization is an important step in CLEC preparation because the quality of the crystal determines the stability of the cross-linked enzyme since the enzyme crystal itself acts as its own support.[8] Crystallization of enzymes is achieved by adjusting the rate of solvent evaporation, pH, and temperature and by manipulation of protein and precipitant concentration. During the preparation of a CLEC, the crystallization conditions should be optimized to get CLECs of size appropriate to the intended applications. The size of the crystals[23,24] can be changed by controlling the kinetics of crystalliza-

tion. Crystals of 50−150 μm size are preferable for biocatalysis applications since they offer a combination of good filtration properties and activity. A particle size of around 10 μm is preferred for biosensor and cosmetic applications.[25] In these cases, it is crucial to define a set of crystallization conditions that are amenable to scale-up, can tolerate some variation of input, provide a high yield of crystals with uniform size and shape, and have minimum loss of functional activity.[26]

3.1.1. Standardization and Screening of the Crystallization Process

When attempting the crystallization of macromolecules, first find some set of initial conditions that yield crystals, even if they are too small or misshapen. Then, using those conditions as a starting point, optimize crystal growth by varying all the parameters, such as precipitant type (inorganic salts,[21,22,27,28] organic solvents,[22,29,30] or polymers such as poly(ethylene glycol) (PEG)[15,16,31,32] of different molecular weight), pH, temperatures, and buffer system. It is also important to determine the solubility properties and to screen additives and other variables[17,18] which affect the crystallization process such as temperature,[33,34] pH,[35] and enzyme concentration.[36,37] Mutant proteins may crystallize under very similar conditions with only minor changes in precipitant concentrations and pH, which are needed to compensate for changes in solubility and charge. There are several techniques for the screening process of crystallization experiments such as vapor diffusion,[38] batch crystallization,[39] microdialysis,[40] and microbatch under oil.[41] Once screening is completed, further optimization is required, which is amenable to the tried and true statistical method.[42]

3.1.2. Sparse Matrix Crystallization

In the crystallization of enzymes, it is very difficult to do a complete screening of crystallization parameters by fixing the precipitant, pH, temperature, and so forth. The best method to arrive at crystallization conditions is sparse matrix[43] screens, in which samples selected randomly from a complete combinatorial matrix are used to narrow down the parameters for subsequent optimization experiments. In the process of optimization, the variables such as precipitant concentration, pH, temperature, and so forth are expanded into a finely sampled multidimensional matrix with conditions centered on the initial condition that produced crystals.

3.1.3. Purity of the Enzyme for Crystallization

The most commonly used commercial enzymes have less than 25% protein, which in turn may contain only a fraction of the enzyme of interest, probably contaminated with other impurities. How pure should an enzyme be for crystallization? The answer is that it should be as pure as possible, but in any case at least 90−95% pure. Purification of an enzyme also improves its enantioselectivity. Most of the commercially available enzymes are not very pure and may contain additives, salts, sugars, and so forth. Many of the problems related to purity and purification can be minimized when working with overexpressed recombinant proteins.[44] Recombinant DNA techniques[45] can modify the solubility characteristics or some other crucial physicochemical property of the macromolecule that enhances its probability of crystallizing.

3.1.4. Bulk Crystallization of Enzymes

Bulk crystallization of enzymes from crude protein mixtures is a highly efficient scalable process and reduces purification costs. The goal is to promote the crystallization of the enzyme in large volumes, commonly reaching several thousands of liters, of rather impure solutions from culture broth.[46] In most industrial processes, the aim of crystallization is to generate a concentrated,[47] highly purified stable form of the enzyme in a single economical step within a short time, at a lower production cost. Most of the laboratory purification methods such as HPLC, gel permeation, affinity chromatography, and so forth are not suitable for the large-scale purification of the enzymes, and the reagents used for these procedures are too expensive. In a well-developed process, the protein yield can be very high and also give the best purification (>99%) in a single step. Control of crystal size[47] is important during the process, since crystalline products obtained from bulk protein crystallization are often of small average size with a wide crystal size distribution. Optimization of crystallization with respect to the target crystal size is based on a fundamental understanding of the process, and in particular, on the knowledge of phase equilibrium, and control of crystallization kinetics[24] and supersaturation.[48] The number, size, and properties of the enzyme crystals formed depend on the location of the initial conditions on the solubility phase diagram.[15,46] The solution should be at a level of sufficient supersaturation at which nucleation occurs spontaneously and the crystals formed grow significantly larger. At lower supersaturation, crystals will not be formed at all. At higher supersaturations, the nucleation will be so prolific that the protein available for crystallization will be spread over the large number of crystals formed thus leading only to microcrystals. At very high supersaturations, protein will just precipitate as an amorphous powder. Crystallization of the enzymes is usually done using clarified fermentation broth that has been concentrated, normally by ultrafiltration. Before concentration, the solution should be filtered or centrifuged to perfect clarity to eliminate any amorphous material or debris, including materials such as glycogen, starch, and other macromolecular contamination. The concentrated enzyme should have a high amount of protein (>50 mg/mL), and supersaturation is attained on addition of precipitant. The pH for crystallization is generally adjusted to values at or near the isoelectric point[46] of the protein to take advantage of the lower solubility at these pH values.

Batch crystallization (in a batch stirred reactor) can be used for large-scale production of protein crystals because of its inherent simplicity and reproducibility. During isothermal batch crystallization, the supersaturation falls rapidly from an initial high value to

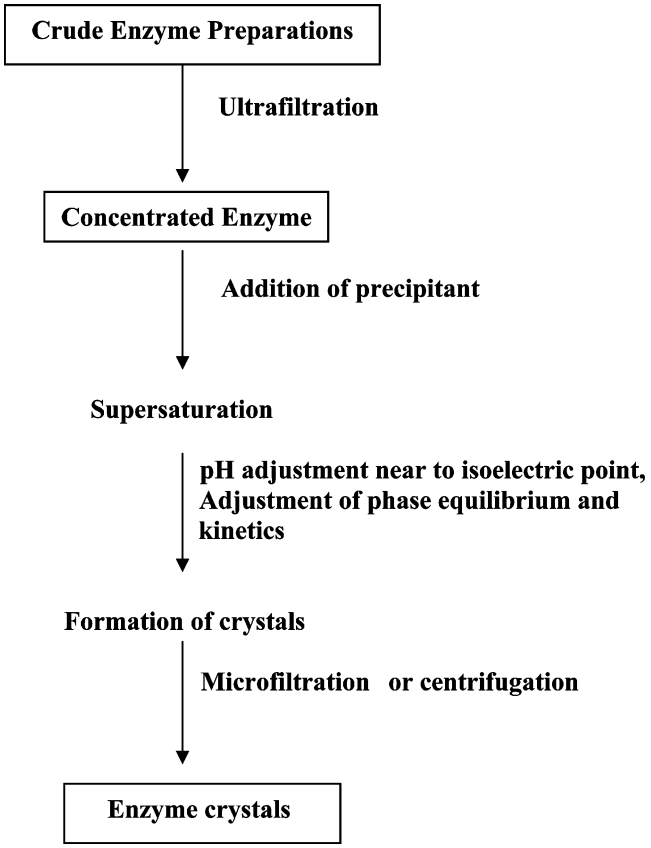


Figure 1. Schematic representation of bulk crystallization of enzymes.

much lower values, especially when supersaturation is achieved by the addition of a precipitant. In most large-scale crystallizations, halide salts[24] or PEG is preferred for producing supersaturation. Bulk crystallization of some of the commercial enzymes,[46] such as insulin, glucose isomerase, asparginase, subtilisin, lipases, thermolysin, and penicillin acylase, has been reported in patents. A schematic representation of the bulk crystallization process is given in Figure 1.

3.2. Limitations of Crystallization

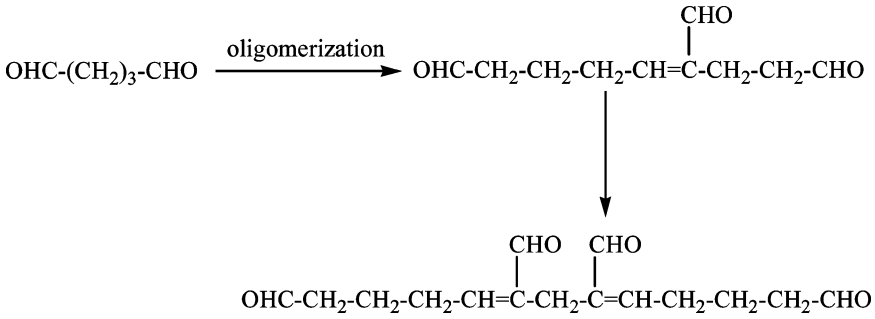
Some enzymes are difficult to purify and crystallize because their molecules can exist in several conformations,[34] which prevents the formation of a highly ordered crystal. Enzymes have a large number of sites capable of intermolecular interactions, but only relatively few sites will produce the precise alignment of molecules necessary for crystal formation. The binding energies of protein−protein contacts are comparable to those between small molecules, but there are fewer contacts for protein crystals in proportion to their molecular weight, so this energy is apparently weak. Hence, the supersaturation stage is also far higher for proteins when compared with small molecules. By varying the crystallization conditions and using different precipitants, one can produce crystals with a most favorable conformation. Some crystallization conditions can have specific detrimental effects, such as the loss of cofactor. In the crystallization, some crystal forms are functionally inactive or less active than other crystal forms of the same enzyme. This happens when the active centers are not exposed to the solution volume available for substrates or the channels in a given crystal form are too narrow for the substrate to enter.

3.3. Cross-Linking of Enzyme Crystals

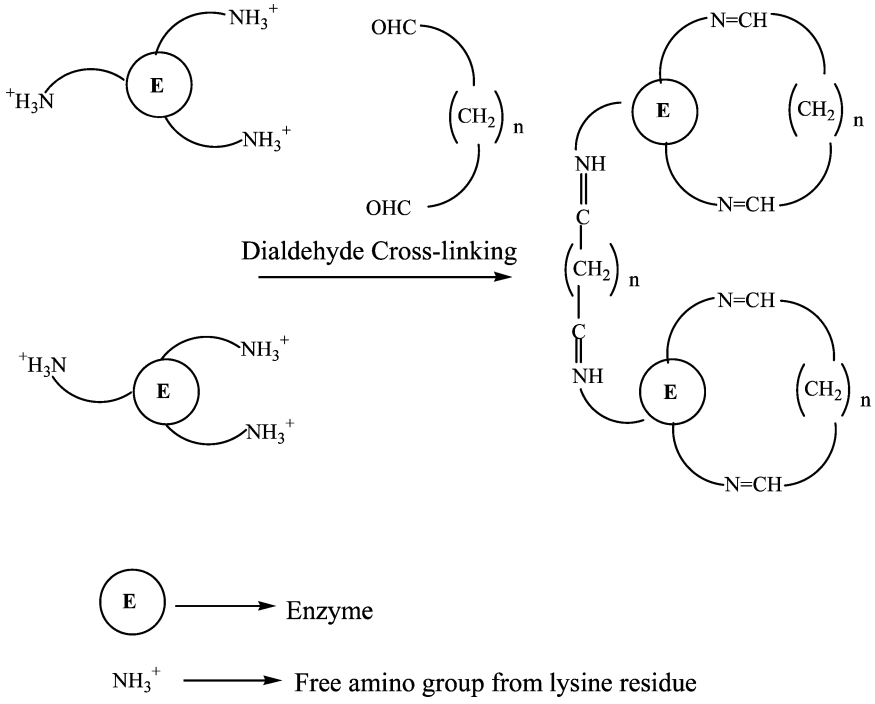
A chemical cross-link, which does not disturb the crystal lattice structure, provides additional stabilization to an enzyme. In an immobilized enzyme, the enzyme is linked by a point attachment to a two-dimensional solid surface, but a protein in a cross-linked crystal is stabilized by links throughout its three-dimensional structure.[49] In cross-linked enzyme crystals, the lattice interactions, when fixed by chemical cross-links, are particularly important in preventing denaturation, especially in organic solvents. The combination of the crystalline lattice contacts and the covalent cross-linking results in a 100−1000-fold increase in protein stability toward thermal deactivation and organic solvent denaturation.[49] The inter- and intramolecular covalent cross-links provide an additional barrier to catalyst deactivation. The cross-linking interactions prevent the constituent enzyme molecules in the crystal from redissolving, thus effectively immobilizing the enzyme molecules into microcrystalline particles. Uniformity[50] is also maintained by the intermolecular contacts and the chemical cross-links between the protein molecules constituting the crystal lattice. Intermolecular[51] cross-linking is necessary to maintain crystal structures in environments different from the crystallization liquor and also increases stability to storage, even at elevated temperatures. The crystals are highly active, easy to handle, recyclable, capable of being incorporated in fabrics, and indifferent to shear and foaming.[25]

Chemical cross-linking of an enzyme crystal results in stabilization of the crystal lattice by introducing covalent links between the constituent enzyme molecules of the crystal. This makes it possible to transfer the enzyme into an alternative reaction environment that might otherwise be incompatible with the intact soluble protein. While crystallization of a protein creates a precise spatial arrangement of the molecules, subsequent cross-linking inside the crystal locks the proteins in place. Many reagents and newer methods are now available for chemical cross-linking. Cross-linkers have been used to brace a protein and to connect it intermolecularly with another protein molecule. Two types of chemical cross-linkers are used for the cross-linking[52] of biomacromolecules: homobifunctional and heterobifunctional cross-linkers. Homobifunctional reagents are commonly used for cross-linking of enzyme crystals having similar reactive groups. These reagents couple functional groups such as two aldehydes, two amines, or two thiols. The commonly used homobifunctional cross-linking agents include dialdehyde cross-linkers (glyoxal, glutaraldehyde, succinaldehyde), diamine cross-linkers (ethylenediamine, hexamethylenediamine, octanediamine, etc.), bis(imido esters), and bis(succinimidyl esters). In heterobifunctional reagents, the reactive groups are different, allowing the formation of cross-links between unlike functional groups. Heterobifunctional reagents are used to

Scheme 1. Oligomerization of Glutaraldehyde



Scheme 2. Schematic Representation of Inter- and Intramolecular Imine Cross-Linking of Enzyme Crystals through Dialdehyde Coupling

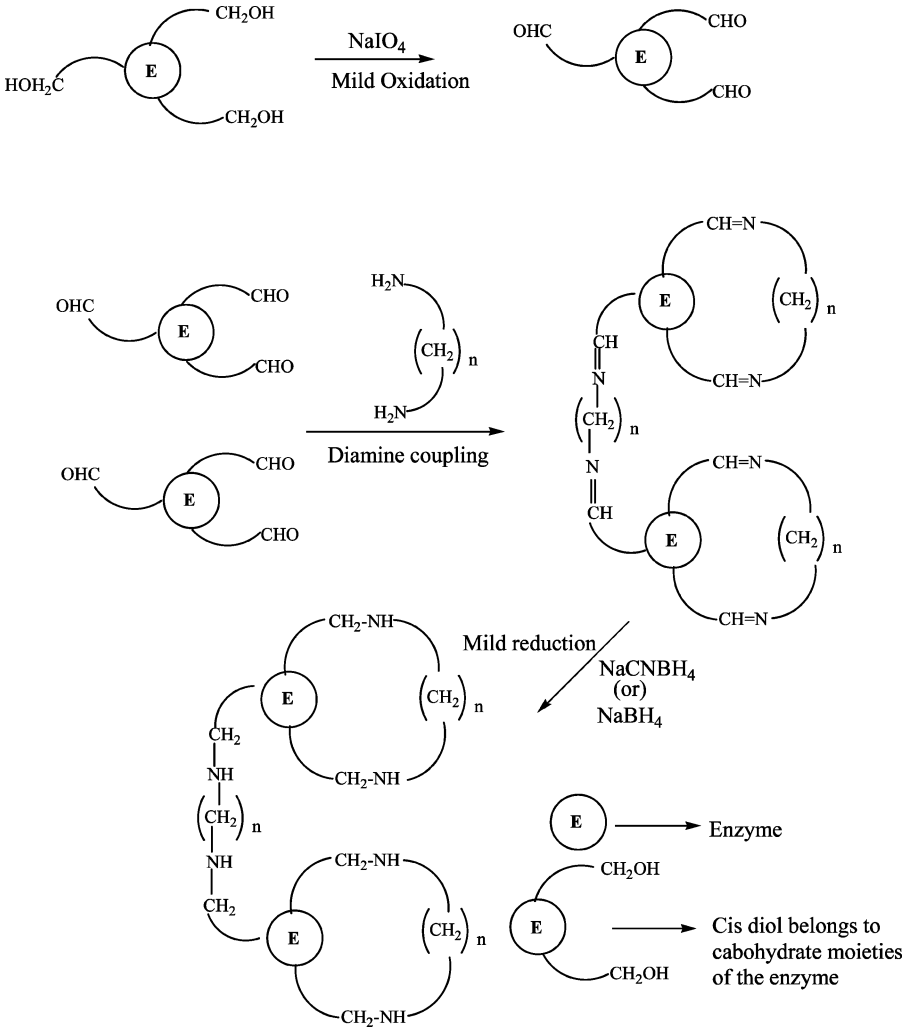


cross-link two different molecules such as enzyme with antibody, nucleic acid with drug or peptide, and so forth. Increasing the chain length of the cross-linking agent increases the flexibility[53] of the cross-linked enzyme crystal. The cross-linking conditions such as pH, temperature, protein concentration, reagent concentration, cross-linking time, and reagent addition rate must be carefully optimized. The cross-linking can be done in an organic solvent such as acetone[107] or dimethyl sulfoxide (DMSO)[108] to reduce reaction time, since only a few minutes is needed for cross-linking in these solvents. Excessive cross-linking may lead to aggregation, protein precipitation, loss of activity, and distortion of the crystal lattice.[8]

3.3.1. Glutaraldehyde Cross-Linking

Glutaraldehyde, a dialdehyde reagent, is the most popular cross-linking agent for enzyme crystals. Glutaraldehyde is safe, inexpensive, and easily handled. The ability of glutaraldehyde to form a mixture of oligomers of different lengths and structures in aqueous solutions makes the use of this cross-linker somewhat unpredictable.[54] The long chain of oligomers of glutaradehyde formed in aqueous solutions is given in Scheme 1. Cross-linking with glutaraldehyde forms strong covalent bonds between the ϵ-amino groups of lysine residues within and between the enzyme molecules (i.e., intra- and intermolecular imine cross-linking) in a crystal lattice.[12] Because many of the linkages are between adjacent macromolecules in the lattice, the crystals become cross-linked throughout. The cross-linking is also irreversible and therefore cannot be explained by simple imine or Schiff base formation (Scheme 2), and the mechanism is not fully understood. Cross-linking with glutaraldehyde confers several advantages. The enzyme crystals, which are normally very fragile, become more sturdy and robust after cross-linking so that there is much less chance of damage in handling. They become completely insoluble under a variety of conditions but remain permeable to solute and are insensitive to pH and temperature, which can be varied over a wide range without dissolution or deterioration of the cross-linked crystals.[55] The advantage of glutaraldehyde cross-linking is that it gives rise to a stable, three-dimensional crystal net that is resistant to denaturation by urea. The cross-linking conditions should be carefully optimized with different glutaraldehyde concentrations at various

Scheme 3. Schematic Representation of Carbohydrate Cross-Linking of Enzyme Crystals



cross-linking times. A concentration of 0.1−5% of glutaradehyde is reported to give better cross-linking. Crystals cross-linked with excessive glutaraldehyde are yellow-brown in color and mechanically hard. Analysis of the amino acid sequence after cross-linking gives the number of lysine residues that have been cross-linked. Rose et al. have reported that in the case of thermolysin[51] CLEC, approximately 8 of the 11 lysine residues are found to be modified. The other 3 lysine[22] residues that are involved in the intra- and intermolecular interactions are not accessible to glutaraldehyde.

3.3.2. Carbohydrate Cross-Linking

Another method for stabilization of the crystal lattice is exclusive cross-linking of the carbohydrate moieties of glycoproteins[13] or a combination of carbohydrate and amino acid side chains. In such carbohydrate cross-linked glycoprotein crystals, the lattice interactions are fixed by chemical cross-links, which are particularly important in providing stability to storage under harsh environments and in preventing denaturation. In the case of enzymes having a lysine residue in their active sites, carbohydrate cross-linking is preferred, since glutaraldehyde has a great affinity for lysine, which may lead to enzyme inactivation by active site fixation. This method advantageously accomplishes the crystallization of glycoproteins on a large scale, without the need for the cumbersome and potentially denaturing effects of chemical deglycosilation.

Carbohydrate cross-linked crystals are produced by the initial oxidation of the carbohydrate moieties, which cleaves *cis*-diol groups to produce a dialdehyde. This is followed by cross-linking with a bifunctional reagent such as a diamine thereby forming a Schiff base which can be reduced under mild conditions using $NaCNBH_4$ or $NaBH_4$ (Scheme 3). The diamine cross-linking agents can be ethylenediamine, hexamethylene- diamine, diaminooctane, adipic acid dihydrazide,[56] and so forth. CLEC glucose oxidase and lipase have been prepared by this carbohydrate cross-linking[13] method (Table 2).

3.3.3. Thiol Cross-Linking

Another possible cross-linking method is through the thiol groups in the enzyme crystals. Here a heteroconjugate is formed, which involves indirect coupling of an amine group in one protein molecule to a thiol group of a second biomolecule, usually by two or three reaction steps. The first step is the introduction of thiol groups in the enzyme crystals. If the enzyme does not have enough free thiol groups, they can be introduced by selectively reducing cystine

Table 2. Conditions for the Preparation of Cross-Linked Enzyme Crystals

enzyme name	crystallization conditions	cross-linking conditions	reference
fructose diphosphate aldolase	45% NH_4SO_4 solution at 22 °C for 1−2 weeks	1−16 mM concn glutaraldehyde in 0.5 mM triethanolamine buffer at 0 °C for 1 h	104
glucose isomerase	NH_4SO_4 as precipitant at pH 7.0 for 20 h at 16 °C	12.5% glutaraldehyde (v/v) with 20% lysine solution for 3.5 h at 100 rpm	105, 106
hydroxynitrile lyase	46 mg/mL of protein, 5% PEG 8000, and 25% MPD in citrate buffer at pH 5.4 for 5 days at 23 °C	0.1−1% glutaraldehyde in acetone at 300 rpm for 5 min	107
glucose oxidase	18% PEG 6000 and 32% 2-propanol in 0.2 M acetate buffer pH 5.0 at 6 °C at 100 rpm for 24 h	$NaIO_4$ oxidation in the dark; cross-linked using 0.25 M 1,8-diamino- octane with 9% PEG 6000 and 16% 2-propanol in 0.1 M acetate buffer pH 5.0	13
lipase	1.93−4.63 mg/mL protein, 47.5% v/v MPD, and 0.4 mM $CaCl_2$ in MES buffer at pH 5.9 at 200 rpm	5% glutaraldehyde v/v in MES buffer pH 5.9 for 3 h	66, 13
α-chymotripsin	2.4 M NH_4SO_4 and 5.7 mg/mL protein concn in 0.1 M citrate buffer at pH 4	5% glutaraldehyde v/v in 0.1 M citrate buffer at pH 4	66
yeast alcohol dehydrogenase	14 mg/mL protein concn and PEG 4000 16% v/v in 50 mM Tris buffer at pH 8 with 2 mM β-nicotinamide adenine dinucleotide (β-NAD) at 100 rpm at room temperature for 3 days	5% glutaraldehyde v/v in 50 mM Tris buffer at pH 8 for 2 h	66, 108, 109
lactate dehydrogenase	5 mg/mL protein concn and 35% NH_4SO_4 in 0.1 M phosphate buffer at pH 7.5 at 25 °C	15 mM glutaraldehyde in 0.5 M triethanolamine buffer pH 7.5 at 4 °C overnight	108
thermolysin	100 mg/mL protein concn in 1 M calcium acetate solution, 30% DMSO in 50 mM Tris buffer pH 7.0	12.5% glutaraldehyde in 50 mM Tris buffer pH 6.5 for 1 h	110
chloroperoxidase	7 mg/mL of protein and 14% PEG 8000 in 10 mM phosphate buffer at pH 5.0 at 18 °C	1000−6000 molar excess of glutaraldehyde at pH 6.5 in 0.1 M sodium cacodylate buffer for 1 h at room temperature	111
subtilisin	Na_2SO_4 as precipitant, enzyme in 0.33 M cacodylate buffer at pH 5.6 at 30 °C	1.5% glutaraldehyde solution in 30 mM cacodylate buffer at pH 7.5, with 13% Na_2SO_4	98
horseradish peroxidase	30 mg/mL protein in 10 mM phosphate buffer at pH 7.2 with 1,5-pentanediol (10%) NH_4SO_4	1% glutaraldehyde in acetone (v/v) for 5 min at 200 rpm at 4 °C	113
glucoamylase	65% NH_4SO_4 with 20% 2-propanol in 0.5 M acetate buffer at pH 4.5 for 16 h at 4 °C	2% glutaraldehyde (v/v) in 0.2 M phosphate buffer at pH 7.0 for 1 h	118

disulfides with reagents such as dithiothreitol (DTT),[57] β-mercaptoethanol, tris-(2-carboxyethyl) phosphine (TCEP), or tris-(2-cyanoethyl) phospine. Amines can be indirectly thiolated by reaction with succinimidyl 3-(2-pyridyldithio) propionate (SPDP)[58] (Scheme 4). Excess of these reagents must be removed by dialysis or gel filtration.

The second step involves the coupling of the thiol-containing enzyme molecule with another enzyme molecule containing amine groups using heterobifunctional reagents such as maleimides or iodoacetamides. Succinimidyl *trans*-4-(maleimidylmethyl) cyclohexane-1-carboxylate (SMCC)[59] is one of the maleimide heterobifunctional reagents used to introduce a thio-reactive group at an amine site of an enzyme followed by cross-linking with a thiol-containing enzyme molecule (Scheme 4).

3.3.4. Cross-Linking with Dithiobiimidates

The reaction of the imidate[60] functional group with a lysine residue of the protein leads to the retention of positive charge, and the cross-links can be cleaved very easily and quantitatively by mild reduction. The reaction of imidates with proteins is carried out at nearly physiological pH, but it is not preferred for cross-linking of enzyme crystals since the imidoesters thus formed are unstable and slowly hydrolyze in aqueous solution to the corresponding amine and alcohol.

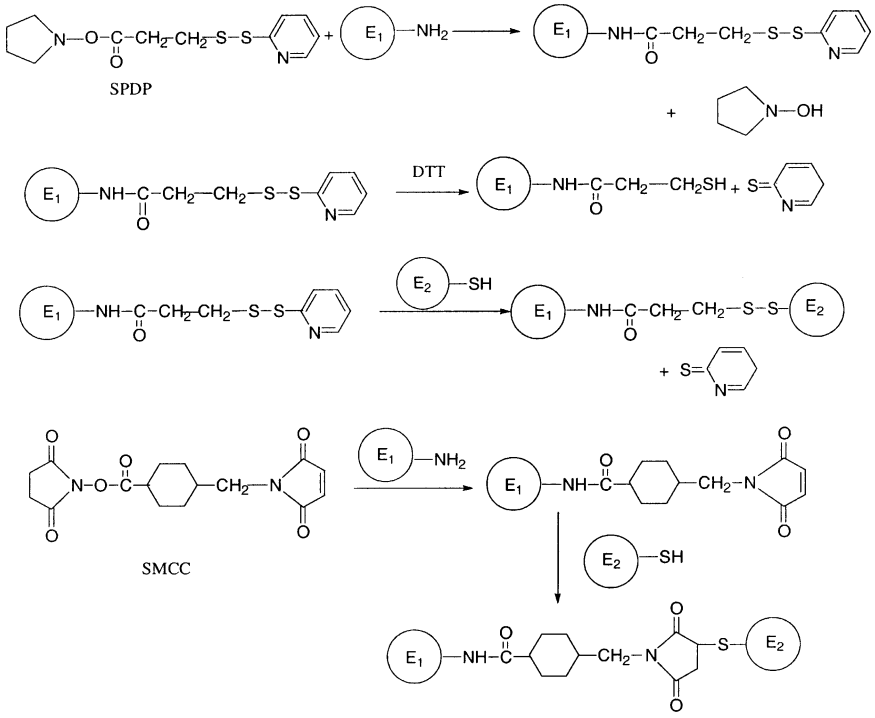
3.3.5. Other Cross-Linking

Other cross-linking methods, such as metal chelation, carbodiimide coupling, and diazo coupling which are used for the modification of immobilization matrixes, cannot be used for the cross-linking of protein crystals because the reagents may inhibit or inactivate the enzymes when added directly to the crystals.

3.4. Limitations of Cross-Linking of Enzyme Crystals

When enzyme crystals are cross-linked in a three-dimensional lattice by a cross-linking agent, microscopic channels of about 20−55 Å are formed between the two crystals. The diffusion limitations[8] of CLECs

Scheme 4. Schematic Representation of Amine−Thiol Cross-Linking of Enzyme Crystals



are defined by size, length, and microenvironment (surface charge, etc.) of these channels. Cross-linkers of sufficient length could block the channels wall-to-wall resulting in particles with peculiar properties. In some cases, crystals remain under-cross-linked, which results in having the outer layers fixed, but without much cross-linking in the interior. This type of CLEC will look normal in the mother liquor but expand in low ionic strength buffers and shrink in concentrated ones, thereby losing storage and mechanical stability. The diffusion inside the expanded CLEC is less hindered, although it may be that the cross-linked "rind" on the surface determines the actual rate of entrance and departure of the substrate. The size of the substrate[52] is also important, because substrates with high molecular weight may not be able to diffuse through the channels and hence have reduced reactivity.

Different lattice packing in enzyme crystals can dramatically change the cross-linking pattern of CLECs, presenting different numbers of reactive residues to other cross-linker molecules with different distances between them. Thus, the crystal structure, the crystal packing information, and the nature and length of cross-linking reagents are important factors in rationally engineering CLECs. A CLEC with good activity can be obtained only by proper standardization of crystallization and cross-linking conditions.

4. Cross-Linked Enzyme Aggregates (CLEAs)

The physical aggregation[61] of enzyme molecules into supermolecular structure can be induced, without perturbation of the original three-dimensional structure of the protein by the addition of salts, organic solvents, or nonionic polymers to protein solution. These solid aggregates are held together by noncovalent bonding and readily collapse and redissolve when dispersed in an aqueous medium. The chemical cross-linking of these physical aggregates would produce cross-linked enzyme aggregates in which the reorganized superstructure of the aggregates and their activity would be maintained. Cross-linked enzyme aggregates can also be prepared by a simple method using cross-linking agents. Cross-linked enzyme aggregates of penicillin acylase[62] (E.C.3.5.1.11) and lipase[117] have been prepared by Linqiu Cao et al. by glutaraldehyde cross-linking.

5. Stability of CLECs

5.1. Chemical Stability

Biocatalytic stability under chemical process conditions is critical for the large-scale commercial applications of CLECs. Stability toward organic substrates, solvents, heat, mechanical shear, and pressure is a requirement for long catalyst lifetime.[49] The increased stability of a protein molecule in a crystal is due to additional ionic and hydrophobic contacts between the protein molecules. When a protein is transferred from a solution to crystalline form, an increase in the number of both polar (electrostatic) and hydrophobic interactions[63] among the protein molecules may significantly enhance protein stability against heat and other denaturants by preventing unfolding, aggregation, or dissociation. The crystalline enzyme maintains its native conformation at elevated temperature and has a lower tendency to aggregate. The chemical cross-linking of these protein crystals provides additional stabilization. Figure 2 is a schematic representation of the relative stability of a CLEC, a soluble enzyme, and an immobilized enzyme.

Cross-linked soluble enzymes do not show increased stability. Tolerance of temperature, pH, and

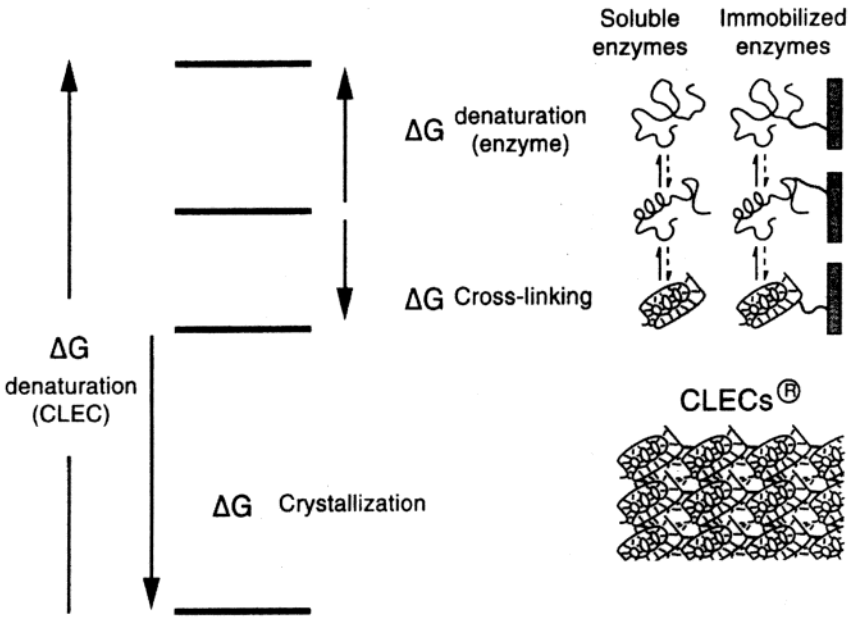


Figure 2. Schematic representation of comparison of enzyme stability. Reprinted from ref 95 with permission from Pharma Press Ltd. and Jim J. Lalonde (Altus Biologics, Inc.). Copyright 1998 Pharma Press Ltd.

organic solvents probably arises from protein−protein interactions and the contacts that occur in the crystal lattice which are also maintained in CLECs. In a CLEC, the enzyme molecules are linked together in a three-dimensional lattice with ordered microscopic channels between them. This lattice provides structural strength and durability, since the total energy of crystallization as well as the energy needed to denature the cross-linked protein will be high. The microscopic channels allow the substrate to pass readily through the crystal lattice and access the active sites of the enzyme. The degree of stabilization[64] depends on the number of contacts, both intermolecular and intramolecular, among the enzyme molecules. Cross-linking of protein crystals also provides additional stabilization against unfolding and thus leads to both chemical and mechanical stability.

5.2. Mechanical Stability

For the industrial application of CLECs as biocatalysts, it is important to understand their mechanical properties in relation to process design and operation. A CLEC must withstand the shear forces associated with processing equipment such as stirring tanks and cross-flow microfilters, which may cause particle attrition and fragmentation. Stability of the cross-linked enzyme crystals has been reviewed by Margolin et al.[52] The enhanced stability of protein crystals by chemical cross-linking also extends to mechanical stability. Mechanical stability of a CLEC also depends on the shape of the crystals. Lee et al.[7] have studied the mechanical stability of both hexagonal and rod-shaped crystals of alcohol dehydrogenase (YADH). Hexagonal crystals exhibited some breakage during mechanical shearing at a disk rotational speed of 27 000 rpm, which may be due to the shear-induced attrition, but no breakage was found in rod-shaped crystals. Both crystal forms showed no significant change in catalytic activity induced by shearing. The controlled exposure of protein crystals to lower concentrations of cross-linkers, followed by treatments with butylamine that remove excess reagent, has produced more mechanically stable crystals.[22] The formation of heavy atom derivatives without crystal disruption showed a notable reduction in radiation damage. CLEAs are too soft and hence may exhibit poor stability when used in stirred tanks or in packed bed reactors. For the production of a sturdy process biocatalyst, this can be overcome by encapsulation of CLEAs into a very rigid poly(vinyl alcohol) network or a sol gel matrix by a suitable immobilization technique.

6. Characterization of a New CLEC

6.1. Catalytic Properties

A new CLEC can be characterized as similar to an immobilized enzyme as proposed by Van Ginkel et al.[65] The catalytic activity and stability at different temperatures and pHs, in various organic solvents, and in aqueous−organic mixtures is measured by assaying the activity of the CLEC after a long-term exposure to the above conditions. Stability studies of the CLEC enable us to determine the robustness of the catalyst under different operational conditions. A study of the stability of the CLEC in the presence of proteases should be examined in order to apply to a crude preparation that may include even traces of microbial proteases. Kinetic properties of the CLEC such as catalytic efficiency, K_{cat}, K_m, and substrate specificity have to be determined for comparison to both the soluble and the immobilized form.

6.2. Physical and Mechanical Properties

The crystal size, shape, and density give the basic data related to the mechanical properties of the crystal. This may also indicate the existence of any diffusion limitation. The mechanical stability of the

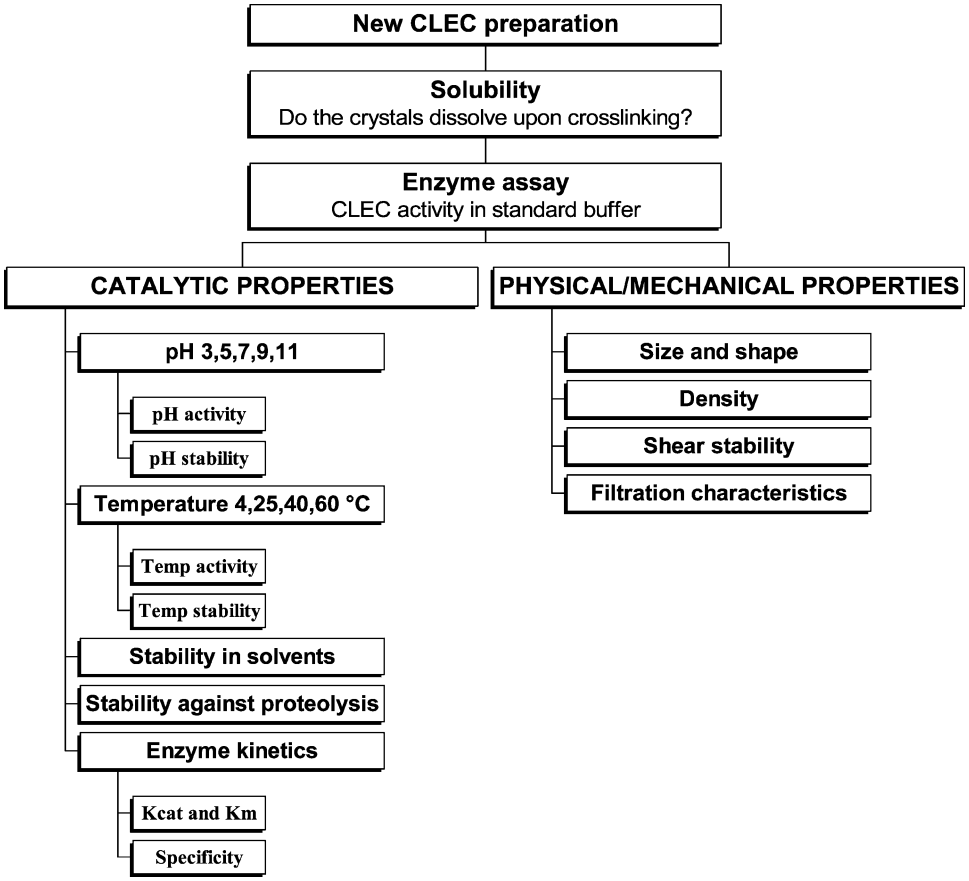


Figure 3. Protocol for the characterization of a new CLEC. Reprinted from ref 66 with permission from Taylor and Francis Ltd. (http://www.tandf.co.uk/journals). (Courtesy: Gary J. Lye). Copyright 2000 Taylor and Francis Ltd.

CLEC is also important under physical conditions that are found in the most frequently used reactor configuration, that is, a mechanically stirred vessel. The porosity of the CLEC should also be determined. A CLEC has well-defined pores of limited size (15−100 Å) leading to the enzyme molecules within its body. As a result, a substrate larger than the available pore size will not be able to penetrate the body of the CLEC particle. Hence, a CLEC with limited pore size would be unsuitable for many enzymatic reactions of commercial interest involving substrates larger than the pore size of the CLEC. Determination of the number of filtration cycles needed for product recovery allows prediction of the processing time. Figure 3 represents the protocol for the characterization of a CLEC, as proposed by Jeetendra[66] et al.

7. CLEC in Organic Solvents

The enzyme activity in organic solvents is intimately related to the water content, size, and morphology of the catalyst particle and to the enzyme microenvironment.[67] Generally, the catalytic activity of enzymes in neat organic solvents[68] is far lower than that in water. The organic solvents lack the ability to engage in multiple hydrogen bonds with water molecules and also have lower dielectric constants, leading to stronger intraprotein electrostatic interactions which lead to the loss of activity. The effect of different organic solvents on the catalyst is not always uniform, and properties such as solvent hydrophobicity, hydrogen bonding capacity, and water miscibility have a profound influence on the structural integrity and catalytic activity of enzymes. Proteins in hydrophobic solvents[69] are thought to retain their native structure as a result of kinetic trapping, resulting from stronger hydrogen bonding between the protein atoms and a more rigid structure in the absence of water. Polar solvents can easily strip water from the surface of the protein by competing strongly for hydrogen bonds between protein atoms. Solvents such as DMSO and dimethylformamide (DMF) usually denature the structure to a largely unfolded state.[70] However, this denaturation will not happen to either crystalline or lyophilized enzymes.[71]

Protein crystal structure in organic solvents suggested the idea that organic solvents cannot be used to map binding surfaces of proteins, since organic solvents bind only at few positions, primarily at the active site and in crystal contacts. In both cases, the anhydrous environment presumably locks the enzyme molecule kinetically in its prior conformation. The insolubility of CLEC catalysts in water and organic solvents allows recovery by simple filtration or use in a column reactor. CLECs need to be formulated as a free flowing dry powder with low moisture content (1−10%) to function in near-anhydrous organic solvents. This has been done by adding small amounts of surfactants[17] during the drying process. Surfactants often possess strong denaturing potential, which when used in small concentrations nevertheless can generate enzyme preparations with

extraordinary properties. Surfactants are usually mixed with an aqueous solution of an enzyme, and the mixture is dewatered[72,73] by lyophilization, vacuum-drying, and so forth. The resulting enzyme preparation is used as a catalyst having enhanced activity in organic solvents. Surfactants may help to maintain a better water balance and the native conformation of enzymes. Surfactants may also facilitate the transfer of hydrophobic substrate molecules through the layer of tightly bound water to the binding site of the enzyme. Enzymes can be extracted into hydrophobic organic phases in the presence of surfactants such as Aerosol OT (AOT)[74] or others[75] in small concentration (1 mM) to give highly active preparations more stable in organic solvents than the soluble enzyme preparations. Like surfactants, some additives may also improve the activity of the enzymes in organic solvents. Additives such as methyl β-cyclodextrin[76] (MβCD) dramatically activate the serine protease subtilisin Carlsberg in THF and in 1,4-dioxane. The transesterification rate of *sec*-phenethyl alcohol with vinyl butyrate is reported to be increased 164-fold in THF by subtilisin Carlsberg[77] lyophilized with MβCD.

7.1. Crown Ether Activation of CLECs in Organic Solvents

Crown ethers are macrocyclic organic molecules that have been shown to activate enzymes following their colyophilization from an aqueous solution. The effects of crown ether[78] treatment under various conditions give support to the hypothesis that removal of bound water molecules from the active enzyme during the drying process is the origin of the observed enzyme activation. Crown ethers might prevent the formation of deactivating inter- and intramolecular salt bridges in organic solvents by complexation of the ammonium functions of lysine residues or may contribute to an enhanced substrate binding and consequently to a higher enzymatic activity by facilitating the transport of water molecules from the active site into the bulk organic solvent.[79] The capability of crown ethers to form complexes with ammonium groups[80] (lysine residues) and water molecules by hydrogen bonding is well-defined.

In aqueous solutions, the transfer of water molecules from an active site to the bulk solvent is entropically favorable due to the increase in transitional and rotational freedom. Increased K_m[81] values and thus lowered enzymatic activity are found after the transfer of enzymes from an aqueous solution to organic solvents. Crown ethers, which are able to complex with water molecules in organic solvents, will facilitate the transport of water from the active site to the organic solvent during the process of drying the enzyme crystal. Lyophilization of subtilisin Carlsberg crystals in the presence of 18-crown-6[78] (50 μmol) increased its activity by 8.5 times, but lyophilization in the absence of crown ether resulted in a decrease of about 10% of the original crystal activity. This reduction of activity in the absence of crown ether during lyophilization of enzyme crystals might originate from a distortion of the enzyme conformation by crystallization of the water in the solvent, which fills the channels of the crystals. The presence of 18-crown-6 may act as a lyophilization-protecting agent.

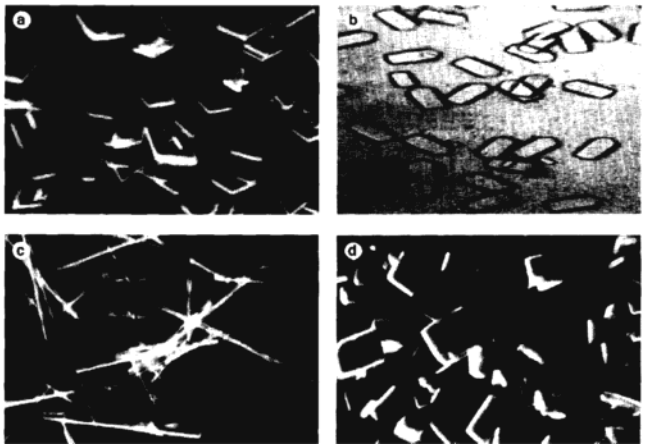


Figure 4. Cross-linked enzyme crystals (CLEC) of (a) *C. rugosa* lipase, (b) *Pseudomonas cepacia* lipase, (c) subtilisin, and (d) penicillin acylase (magnification, ×400). Reprinted from ref 8 with permission from Elsevier Science (Courtesy: A. L. Margolin (Altus Biologics, Inc.)). Copyright 1996 Elsevier Science.

In cross-linked enzyme crystals, most of the lysine residues react with the cross-linking agent and consequently get converted into imine functions. Margolin[51] et al. have observed that in the case of thermolysin CLEC, amino acid analysis reveals that 8 out of 11 lysine residues are modified. Moreover, unreacted free lysine residues in the CLEC may seem to be of minor importance for the activation effect of crown ether in organic solvent. But in the case of enzyme crystals, crown ether activation is very important to prevent the loss of activity during lyophilization, since the properties of cross-linked enzyme crystals depend only on active enzyme crystals.

7.2. Lyophilized CLEC

Lyophilizing enzymes in the presence of structure-preserving lyoprotectants[82] such as sugars and poly(ethylene glycol)s and some inorganic salts and crown ethers minimize the denaturing effects of organic solvents. These lyoprotectants prevent protein aggregation in organic solvents. Similarly, CLECs when subjected to lyophilization with various surfactants or additives and inorganic salts or crown ethers produced lyophilized CLEC with minimum denaturation. Most of the CLECs (subtilisin, thermolysin, protease, lipase) (Figure 4) retain their activity in organic solvents.[83−86] These cross-linked enzyme crystals in organic solvents may be used in a number of chemical processes such as organic synthesis of specialty chemicals and pharmaceuticals, synthesis of intermediates, and also the chiral synthesis and resolution of optically pure pharmaceuticals. Enzymatic conversion processes such as oxidation, reduction, esterification, coupling reactions, and asymmetric conversion including stereoselective, stereospecific, and regioselective reactions can be done with CLECs for the production[51,87−90] of organic molecules, peptides, carbohydrates, and so forth.

8. Productivity of CLEC

The productivity of a catalyst is the quantity of product per kilo of the catalyst. For a biocatalytic process, the catalyst cost should be less than 5−10% of the product value. The process development and scale-up are accelerated by recommendations on catalyst loading and depend on catalyst recovery and reusability. A biocatalyst which performs in organic solvents, in aqueous−organic mixtures, at elevated temperatures, and under high shear conditions results in increased catalyst productivity.[49] The final productivity of a CLEC catalyst is further increased by the number of times the catalyst can be recycled. The increased volumetric activity is essential for commercial scale-up since it extends to an increased reactor productivity.[10] The entire volume of the CLEC catalyst consists of active material, unlike an immobilized enzyme on an inert carrier. The CLEC catalysts are highly stable, active, and recyclable due to easy filtration or centrifugation properties, but routine immobilization methods do not guarantee enzyme stability and have low specific activity. The enzyme loading[4] on an inert matrix will be only 0.1−10% w/w of the total, and hence a large amount of immobilized enzyme is needed for high reaction rates. However, in CLEC catalysts, the enzyme is packed as crystals and hence the volumetric activity is orders of magnitude higher than that of conventional soluble or immobilized enzymes. For biotransformation reactions, small amounts of CLEC are required to produce large amounts of products, and the reaction will be completed within a shorter time.

The catalyst-to-product ratio[49] for CLEC reactions normally ranges from 1:100 to 1:5000 for a single reaction cycle. If the enzyme crystals can be reused 10−20 times, the final productivity will be in the 1:1000 to 1:100 000 range. The cost of a commercially[49] available CLEC catalyst ranges from $12 000 to $350 000/kg (according to the enzyme used). Thus, for example, if the productivity is about 1:10 000 then 1 kg of product would be formed by using 100 mg of CLEC costing $5. The resolution of 1-phenylethanol with vinyl acetate in toluene catalyzed by CLEC lipase (PS) is the best example for CLEC productivity. In this reaction, the substrate-to-catalyst ratio is 4600. Thus, we see that CLEC catalysts have high productivity for biotransformation reactions when compared to soluble or immobilized enzymes.

9. Application of CLEC Enzymes

The major applications of CLEC catalysts involve high concentrations of organic solvents and substrates for which the soluble enzyme cannot be used. These occur mainly in the areas of synthetic organic chemistry, biomedical applications, and environmental catalysis.

9.1. Synthetic Chemistry

Applications of CLECs in synthetic chemistry[116] arise from their enhanced stability without loss of specific activity in both aqueous and organic media. CLEC catalysts are more enantioselective than a crude enzyme mixture. CLECs remain insoluble throughout the process and can be recycled many times, which increases their productivity.

9.1.1. Synthesis of Peptides and Peptidomimetics

Peptides and peptidomimetics are an important group of modern drugs. Peptides are typically prepared either by the stepwise coupling of the individual amino acids or by the condensation of amino acids and/or peptide fragments in solutions. The proteases catalyzes the peptide bond hydrolysis, but the reaction equilibrium can be reversed to peptide bond formation in the presence of organic solvents or byproduct precipitation. However, proteases are unstable in the presence of organic solvents, and the reaction mixture contains peptide and protein fragments, which are difficult to separate from the product. CLEC proteases circumvent these problems in peptide synthesis[91] and hence are better suited for large-scale synthesis of peptides and peptidomimetics (Scheme 5). Subtilisin-CLEC[89,92] effectively catalyzes the synthesis of peptides giving >90% yield by accepting both L and D amino acid amides as nucleophiles. Thermolysin-CLECs[51] were efficient in the synthesis of several peptides in 90% EtOH and were used in a multicycle preparation of aspartame in ethyl acetate for 18 cycles, but the soluble enzyme can hardly be used for 2 cycles. The above two CLEC proteases are commercially available as pepti CLEC-BL (subtilisin from *Bacillus licheniformis*) and pepti CLEC-TR (thermolysin from *Bacillus thermoproteolyticus*), respectively, from Altus Biologics, Cambridge, MA.

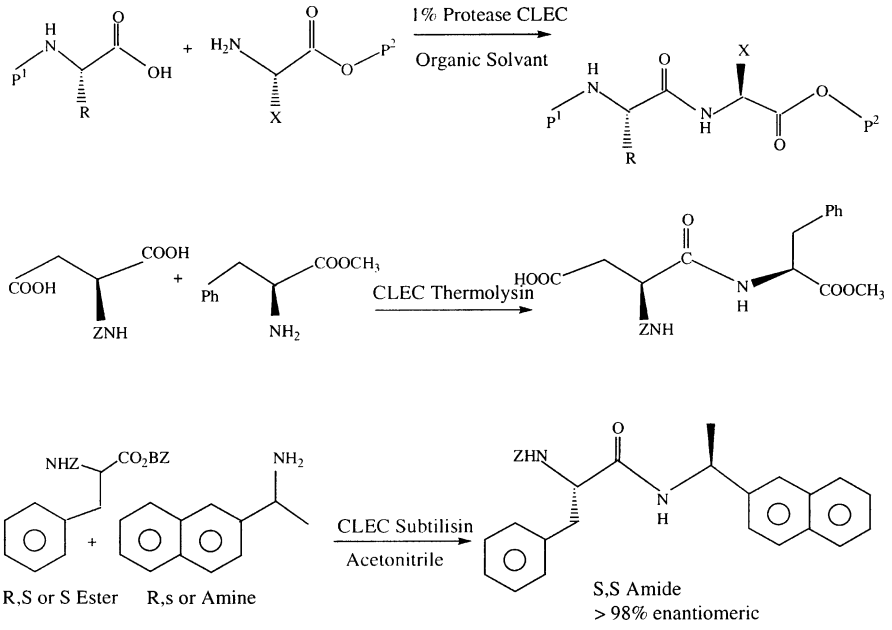
9.1.2. Chiral Resolution Applications

The major use of enzymes in biocatalysis is for the resolution of racemates to obtain enantiomerically pure compounds. The commercial CLEC forms of lipases and esterases are important in kinetic resolution in organic synthesis[93,94] (Scheme 6).These enzymes are used to catalyze the enantioselective hydrolysis or synthesis of esters. The chief advantage of the CLEC form is in the resolution of alcohols via the stereospecific transesterification in organic media or the hydrolysis of racemic esters, where a high concentration of organic solvent or substrate is required. The rate of organic phase transesterification can be 2 orders of magnitude greater than that of the crude enzyme. CLEC lipase[88,114,115] (*Candida rugosa*) catalyzes the acylation of menthol and hydrolyzes the arylpropionate esters, with a 4−50-fold increase in enantioselectivity over the crude lipase[95] preparation. CLEC-subtilisin catalyzed resolution of *sec*-phenethyl alcohol and *trans*-sobrerol was studied by Colombo[96] et al. in organic solvents. Large-scale resolution[97] of the racemic mixture (*R*)(*S*)-*sec*-phenethyl acetate to *R*-*sec*-phenethyl alcohol was studied by Collin et al. using Chiro CLEC-lipase (PS) (Altus).

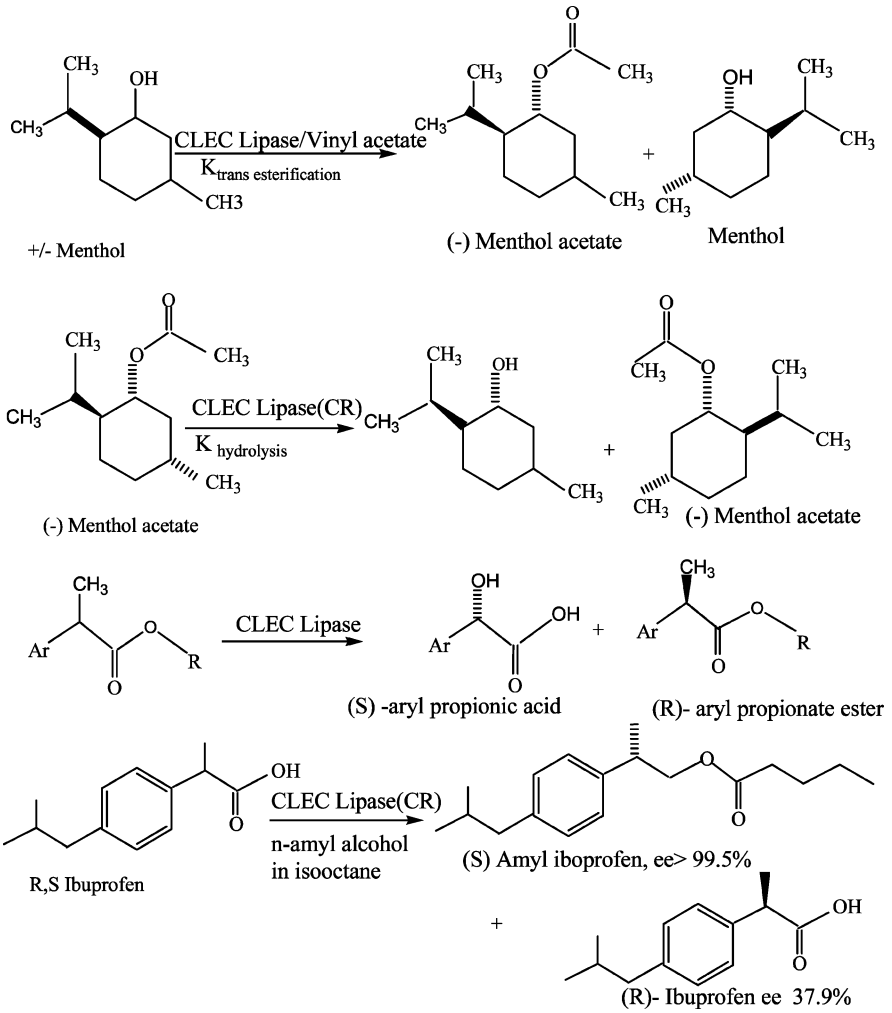
9.1.3. Chemoselective and Regioselective Transformations

The chemical process for semisynthetic antibiotic manufacture requires extremely low temperatures

Scheme 5. Synthesis of Peptides and Peptidomimetics

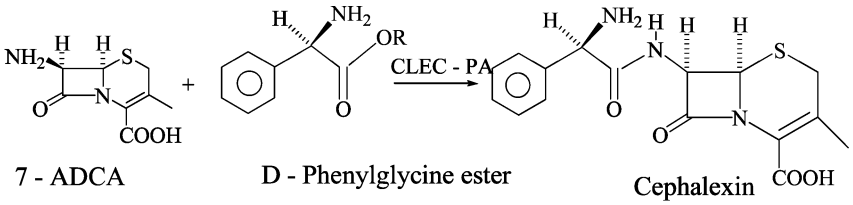


Scheme 6. Chiral Resolution Applications

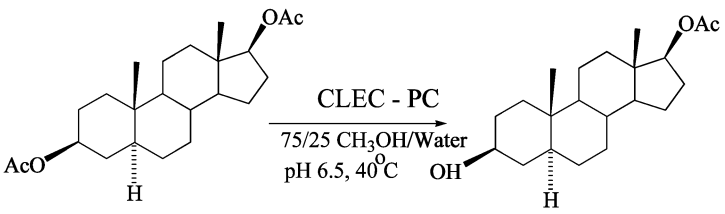


and includes several steps for protection, activation, and deprotection that generate many times more waste than the product. The catalytic coupling of D-amino acids with 6-aminopenicillanic acid or 7-aminodesacetoxycephalosporanic acid (7-ADCA) derivatives exploits the chemoselectivity of the enzyme penicillin acylase[98] (PA). CLEC-PA catalyzes the direct coupling[49] of D-phenylglycine ester or amine with 7-ADCA at room temperature in water at near-neutral pH (Scheme 7).The CLEC form of PA is used

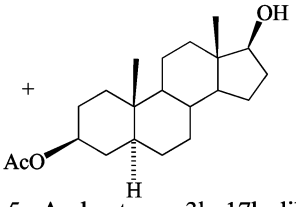
Scheme 7. Chemoselective and Regioselective Transformations



7 - ADCA D - Phenylglycine ester Cephalexin



-Androstane-3b, 17b-dihydroxy diacetate 5a-Androstane-3b, 17b-dihydroxy (17 - O) monoacetate
> 98%



5a-Androstane - 3b, 17b-dihydroxy (3-O) monoacetate
= 0%

at 1−2 wt % of enzyme, and even after 1000 batch reactions, the catalyst retained 70% of its original activity.[95]

9.2. Biosensor Applications of CLEC

A biosensor is a device that detects, transmits, and records information regarding a biological change. Biosensors integrate a biological component with an electronic transducer, thereby converting a biochemical signal into an electronic response. Generally, biosensors are immobilized molecules connected to some type of optical, electrical, electromagnetic, or chemical signal transducer that produces a signal in the presence of an analyte biomolecule. The function of a biosensor depends on the biochemical specificity of the bioactive material. CLECs exhibit the highest protein density and specific activity and will produce a clear signal even in the presence of small amounts of substrate. In the analysis of samples with a biosensor, it is particularly desirable to produce the largest possible detectable signal from the smallest possible quantity of substrate and catalyst. CLEC formulations[11] may be used as the biosensor component, which detects an analyte of interest in a sample. A CLEC allows improved contact between itself and an analyte of interest in both aqueous and organic media. A CLEC-based glucose oxidase[99] biosensor can be used to measure the glucose level in blood. CLEC urease[11] from Jack-bean can be used in clinical biosensor applications to measure urea levels in the circulatory fluid as an early indication of renal disease. In our laboratory, we have developed cross-linked enzyme crystals of horseradish peroxidase (Figure 5) which can be used in a biosensor to monitor organic pollutants.

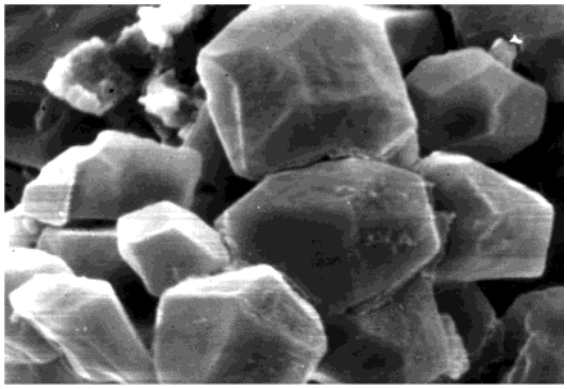


Figure 5. Cross-linked enzyme crystals of horseradish peroxidase produced in our laboratory (Courtesy: L. V. Bindhu).

9.3. Biomedical Applications

Most of the drugs in research and development are peptides or peptide-like synthetic organic compounds. These peptide drugs are quickly broken down into smaller units in the gut, mostly by proteolytic enzymes in the stomach. The improved stability of CLECs toward proteolysis may make these compounds attractive alternatives to intravenously administered peptide and peptide-like drugs.

Protein/peptide crystalline drugs are carrier-free, pure, stable, and storable at room temperature. Crystals are the most concentrated form of proteins and are useful as certain drugs, such as antibodies,[100] which require high concentrations at the delivery stage. CLECs are ideally suited for diagnostic application because of their greatly enhanced stability and excellent bioactivity. The CLEC system functions as a self-delivery vehicle for drugs incorporating a high concentration of therapeutic agents into a nondissolvable or biodegradable crystal matrix.

Enzyme therapy such as lipase therapy[101] can be performed by administering cross-linked lipase crystals orally. The digestive disorders linked with cystic fibrosis or pancreatitis can be traced to improper levels of lipase enzymes in the duodenum. But CLEC formulations of such therapeutically beneficial enzymes and other proteins can be used in the gut lumen without being degraded by extremes of pH or endogenous protease action. CLEC glucose oxidase/peroxidase test strips can be used as a diagnostic reagent to detect the level of glucose in blood.

10. Conclusions

Chemically cross-linked enzyme crystals are significantly very stable against denaturation by heat and proteolysis and have good mechanical and storage stability. The cross-linked enzyme crystals can be used in organic solvents even with concentrated organic substrates for biotransformation reactions. CLECs are organic zeolites, the size and shape of which can be controlled through the crystallization process. Bulk crystallization processes are used to produce pure crystals of enzymes from crude mixtures in a single step. CLEC could also be tailored to exhibit much higher activity toward macromolecular substrates. Though the stabilized enzyme crystals are macroporous, they can be used as novel chromatographic separation materials and can easily be packed into a column. Chemical modification[102] of the enzymes is carried out to stabilize and modify their catalytic activity. By cross-linking the chemically modified enzyme, a new type of chemically engineered[103] enzyme is produced. Cross-linked enzyme technology can be extended very well to genetically engineered enzymes. Lowering the cost of CLECs by future developments in this area and tailoring the existing CLECs for specific applications will make this technology highly attractive. Thus the improved performance of CLECs may expand the commercial manufacturing potential of peptides, peptidomimetics, enzymes, proteins, and antibodies severalfold in the coming years.

11. Acknowledgments

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