Chapter 2
Basics of Enzyme Immobilization

2.1 Introduction

Immobilization of enzymes is not a new concept but one that has been around for over 100 years. However, wide applications for immobilized enzymes came only in the past four decades in the form of synthesis of various complex drug intermediates; chemical synthesis under mild conditions without production of toxic by-products; remediation of polluted water, air and soil by removal of recalcitrant pollutant in an effective way; disease diagnosis; and correction of various genetic diseases due to the absence of metabolic enzymes, etc. In its immobilized state, an enzyme has convenient handling, provides easier product separation by eliminating protein contaminants, has reusability (useful particularly for costly enzymes), higher stability under extreme physical and chemical conditions, easier shipment of enzyme from one place to another, makes the complete process more viable economically, is applicable for all types of reactors (e.g., continuous, fixed-bed) with varied interior design, and provides easier process control [90, 91]. Thus, immobilized enzymes are adaptable for all type of industrial processes. Furthermore, enzyme immobilization is useful for multienzyme and chemoenzymatic cascade processes [92]. In its lyophilized state (i.e. freeze-dried powders), enzymes have various properties that are similar to immobilized enzymes such as stability during storage, easier shipment, and not being affected by the presence of an extreme physicochemical environment. However, once solubilized in appropriate media it resembles completely to a soluble enzyme, which is associated with the problem of product contamination with the enzyme molecules and not allowing enzyme reusability. Furthermore, lyophilization is not applicable for all types of enzymes [93]. In addition to enzyme immobilization, there are various other techniques available that also permit improvement in the enzyme features, including recombinant DNA technology, protein engineering, high throughput technology, genomics and proteomics. The combination of recombinant DNA and protein engineering have

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been significantly helpful for large-scale enzyme production with desirable properties. Protein engineering using techniques like site-directed mutagenesis and in vitro evolution *via* gene shuffling has helped in enzyme manipulation to exhibit desired properties (e.g., chemoselectivity, regioselectivity, stereoselectivity long-term stability, activity in the presence of high substrate concentrations, and tolerance towards organic solvents). However, these techniques are laborious, costly, lack long-term operational stability, and are difficult for enzyme recovery and reusability. These drawbacks can be overcome by enzyme promiscuity as a result of immobilization. Debate on enzyme immobilization has also raised various issues such as lowering enzyme activity due to conformational change, the possibility of enzyme denaturation and changes in kinetic properties in some cases, mass transfer limitations, and lower efficacy in the presence of insoluble substrates. The issues mentioned are not applicable to all types of immobilization systems. It has been recommended that protein engineering methodologies can be used, followed by immobilization for establishing robust processes in various immobilization systems [94, 95]. Therefore, the choice of method is still a case-by-case decision, depending on existing structural and mechanistic knowledge, interest as well as practical considerations.

There are numerous methods for enzyme immobilization onto a variety of different materials that have been developed over the last 100 years. Enzyme immobilization involves inclusion of enzymes into various matrices or binding onto their surface. Several modifications (e.g., pre-fabrication of matrices, enzyme cross-linking without addition of matrix) have been made and are still continuously evolving to update the protocols of immobilization so that immobilized enzymes become compatible for various emerging applications [96]. Presently, immobilized enzymes are of considerable interest due to their expected benefits over soluble enzymes or alternative technologies and their increasing applications. Enzyme immobilization is the simplest technique and allows convenient handling of enzyme preparations. It has two main benefits that are not offered by any other technique known to date: easier enzyme separation from the product, making it compatible for a wide range of applications; and it minimizes downstream product processing, making the process cost effective, reliable and efficient. Furthermore, enzyme reusability is the major reason for dramatic cost reduction of any process employing immobilized enzyme. The difference in cost from process to process based on immobilized enzymes is estimated by the source of the enzyme (plant, animal or microbes), its level of expression in the source, isolation and purification of enzyme, chosen matrix for immobilization and immobilization procedure involving various chemicals. However, limitations by mass transfer effects are the major concern while using immobilized enzyme. This can be obviated to a large extent by intensive optimization of the immobilization process and continuous stirring of the solution containing immobilized enzyme, substrate and released product at optimum velocity [97].

The properties of immobilized enzymes are determined by the type of enzyme and matrix used for the immobilization. Evolution of any new immobilization protocols focus primarily on: percentage enzyme recovery, operational stability, selectivity, and reduction in inhibition by products or any other component present in the media. It involves intensive optimization of immobilization solutions to

control interaction between the matrix and the enzyme and proper orientation of the enzyme to maintain its catalytic properties. Most importantly, use of toxic and highly unstable reagents during the immobilization processes should be kept to a minimum, making it eco-friendly with respect to other alternative technologies. Furthermore, reactors based on immobilized enzymes have the simplest design and the reaction is easier to control. Sometimes, during random immobilization, unexpected results arise such as a significant reduction or an increase in activity by several folds, or a decrease in enzyme stability with respect to soluble enzyme etc. For example, cross-linked crystals of subtilisin have activity 27 times less than that of the soluble enzyme; immobilized lipoprotein lipase using solvent-mediated synthesis of esters has a higher activity by 40-fold as compared with enzyme powder [98, 99]. The present chapter focuses on recent developments in immobilization techniques with respect to the type of matrices used (introduction of novel support like smart polymers), immobilization methods, structural changes during enzyme immobilization and the recent development of cross-linked enzyme aggregates (CLEAs) and cross-linked enzyme crystals (CLECs).

2.2 Methods of Enzyme Immobilization

2.2.1 Physical Method

Enzyme attachment onto different matrices *via* physical forces involving van der Waals forces, hydrophobic interactions and hydrogen bonding. The process is reversible in nature by controlling physicochemical parameters [100] (Fig. 2.1).

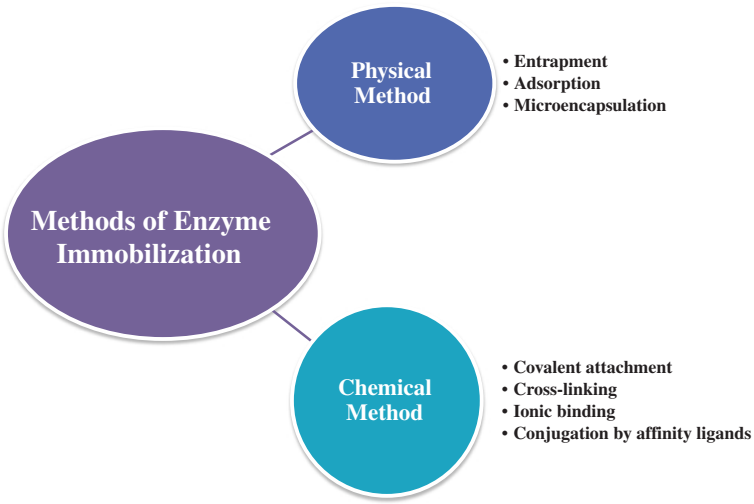

Fig. 2.1 Overview on the techniques being used for enzyme immobilization

(i) *Entrapment*: This involves cross-linking of the enzyme to a polymer (polyacrylamide, alginate etc.) in every direction, covering almost every side chain present on the surface of the enzyme by physical entrapment within the polymer lattice. It allows permeation of appropriately sized substrate and release of product molecules, which ensures continuous transformation. However, this method can only be used in a limited number of enzymes. This method has several advantages: simplicity, no change in intrinsic enzyme properties, involves no chemical modification, minimal enzyme requirement and matrices are available in various shapes. The various disadvantages of this method are; enzyme leakage, only small sized substrate/products can be used, requires delicate balance between mechanical properties of the matrix and its effect on enzyme activity and presence of diffusional constraints [101, 102].

(ii) *Adsorption*: Enzyme is attached to the support material by non-covalent linkages including ionic or hydrophobic interactions, hydrogen bonding, and van der Waals forces without any pre-activation of support. The matrices used are either organic or inorganic in nature, *viz*. ceramic, alumina, activated carbon, kaolinite, bentonite, porous glass, chitosan, dextran, gelatin, cellulose, starch. The immobilization method involves optimization of variables including pH, temperature, nature of the solvent, ionic strength, concentration of enzyme and adsorbent. Here, the enzyme is directly added to the surface (active adsorbent) without the removal of any non-adsorbed enzyme during washing. The method is simple and mild with a vast variety of carriers helpful for simultaneous purification as well as enzyme immobilization (e.g. Asparginase on CM-cellulose) without any conformational change. However, it involves intensive optimization due to the involvement of a number of factors that play a role in enzyme desorption following slight changes in its micro-environment (e.g., pH, temperature, solvent, ionic strength and high substrate concentrations) [103–105].

(iii) *Microencapsulation*: Enzymes are immobilized by enclosing them within spherical semi-permeable polymer membranes with controlled porosity (1–100 μm) [106]. Semi-permeable membranes can either be permanent or non-permanent membranes based on the constituents. Permanent membranes are made of cellulose nitrate and polystyrene while non-permanent membranes are made of liquid surfactant. These membranes are also used in the encapsulation of dyes, drugs, and other chemicals. Enzymes immobilized by encapsulation have extremely large surface areas due to which they have higher catalytic efficiency. However, there are several reports on occasional inactivation of the enzyme; despite presence of high enzyme concentration [107–109].

2.2.2 Chemical Method

This involves attachment of enzymes onto different matrices using covalent or ionic bonds and the process is irreversible [100] (Fig. 2.1).

(a) *Covalent attachment*: The enzyme is attached to the matrix by means of covalent bonds (diazotation, amino bond, Schiff's base formation, amidation reactions, thiol-disulfide, peptide bond and alkylation reactions). Enzyme molecules are attached either directly to the reactive groups (e.g., hydroxyl, amide, amino, carboxyl groups) present on the matrix or by a spacer arm, which is artificially attached to the matrix through various chemical reactions (e.g., diazotization, schiff base, imine bond formation). Matrices commonly used are either natural (e.g., glass, Sephadex, Agarose, Sepharose) or synthetic (e.g., acrylamide, methacrylic acid, styrene). The selection of a particular matrix depends on its cost, availability, binding capacity, hydrophilicity, structural rigidity and durability during various applications. This method of immobilization involves non-essential amino acids (other than active site groups) leading to minimal conformational changes. It helps to promote the higher resistance of immobilized enzymes towards extreme physical and chemical conditions (e.g., temperature, denaturants, organic solvents). However, this method of immobilization leads to greater strain on the enzyme and sometimes leads to drastic changes in conformational and catalytic properties of the enzyme, due to harsh immobilization conditions and concurrence of similar amino-groups at the active site being involved during interaction of enzyme with the matrix [110–114].

(b) *Cross-Linking*: This involves formation of a number of covalent bonds between enzyme and the matrix using bi- or multi-functional reagents (e.g., glutardialdehyde, glutaraldehyde, glyoxal, diisocyanates, hexamethylene diisocyanate, toluene diisocyanate). Generally, amino groups of lysine, sulfhydryl groups of cysteine, phenolic OH groups of tyrosine, or imidazol group of histidine are used for enzyme binding under mild conditions. The main advantage of this method is its simplicity. However, it leads to the loss of a large amount of enzyme due to the non-regulation of the reaction. Further, this method of enzyme immobilization suffers limitations caused by diffusion [115–119].

(c) *Ionic binding*: This is based on ionic interactions between enzyme molecules with a charged matrix. Here, higher the surface charge density on the matrix, the greater would be the amount of enzyme being bound to the matrix. Sometimes, in addition to ionic interactions, enzyme molecules are also physically adsorbed to the matrix. The method of enzyme immobilization is similar to that discussed in the section on physical adsorption. Enzyme binding *via* ionic interactions during immobilization depends on the pH of the solution, the concentration of the enzyme and temperature. Commonly used matrices are: polysaccharide derivatives (e.g., diethylaminoethylcellulose, dextran, carboxymethylcellulose, chitosan), synthetic polymers (e.g., polystyrene derivatives, polyethylene vinylalcohol) and inorganic materials (e.g., Amberlite, alumina, silicates, bentonite, sepiolite, silica gel). This method of immobilization leads to minimal changes in enzyme conformation. However, special attention is given to the maintenance of accurate ionic strength and the pH of the solution in which the immobilized enzyme undergoes catalysis since there is increased chance of enzyme detachment from the matrix under suboptimal conditions [120–123].

(d) *Conjugation by affinity ligands*: Attachment of the enzyme to the matrix using specific ligands; *viz*, his-tag on enzyme to a metal-containing matrix, lectin-containing domain to carbohydrate moieties present on the matrix or sometimes substrate-mimicking chemical compounds are also used as ligands. In some cases, ligands are naturally present on the enzyme, while in other cases they are attached artificially by fusing a nucleotide sequence corresponding to the tag with the DNA encoding a polypeptide of the given enzyme. This method of immobilization leads to minimal changes in the conformation of enzyme, with high stability and catalytic efficiency of the immobilized enzyme due to non-involvement of active site residues and higher immobilization efficiency due to the presence of high densities of ligands on the matrix. This method is not only useful for enzyme immobilization but also for several proteins including antibodies, cytokines, streptavidin etc. Enzymes immobilized by this method have found various applications in biotechnology, diagnostics and medicine. The basis of this method has also been used in animal cell culture by attachment of various types of mammalian cells to different matrices containing a variety of peptides, growth factors and cytokines to a specific binding domain present on the cell as well as in their activation [124–128].

2.3 Properties of Immobilized Enzymes

During enzyme immobilization, there is a change in its microenvironment due to the varied interactions of the matrices with the substrates/products based on their physical and chemical properties. The following are the important parameters that are affected during enzyme immobilization (Fig. 2.2):

(a) *Optimum pH*: This is based on the pK_a of amino-acids in the vicinity of enzyme's active site. The change in optimum pH has been observed in the case of immobilized enzymes (it is either broadened or shifts to acidic or basic side) with respect to soluble enzyme. The extent of change depends on the type of immobilization method used as well as the physical and chemical properties of the matrix (*viz*. charge and surface area of the matrix) [100].

The change in optimum pH profile is defined by the equation: $\Delta pH = pH_i - pH_o = 0.43\frac{\varepsilon\varphi}{kT}$ when matrices are charged (either negatively or positively charged).

pH_i pH in vicinity of the immobilized enzyme
pH_o pH of the bulk solution
ε positive charge on the proton
φ average electrostatic potential
k Boltzmann's constant
T temperature

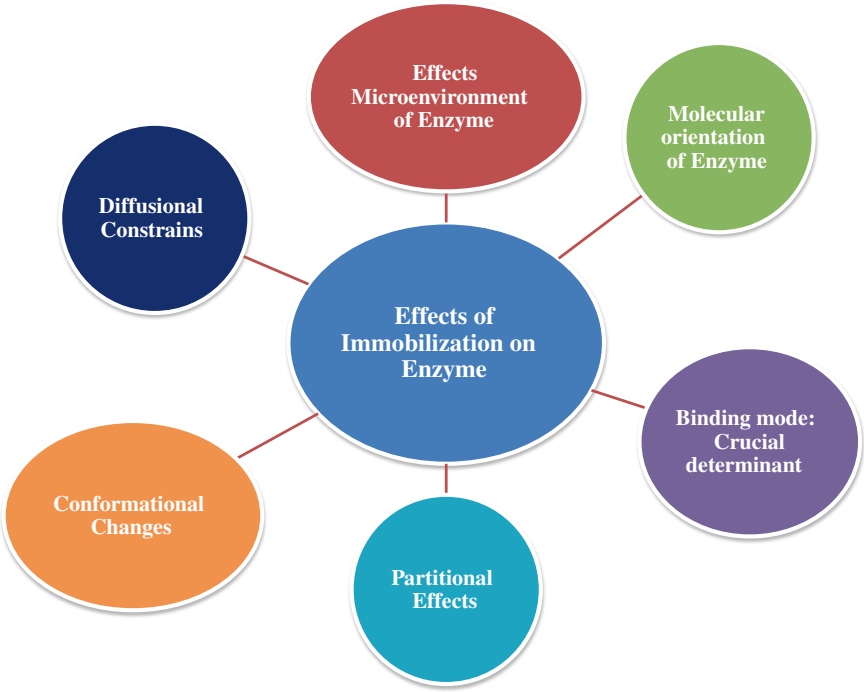

Fig. 2.2 Effects on enzymes being immobilized on various matrices by either method of immobilization

When the matrix is positively charged, it leads to the repulsion of protons while attraction of electrons shifts the optimum pH towards the basic side. Similarly, optimum pH is shifted to the acidic side, when the matrix is negatively charged. The extent of shifting depends on the surface charge density of the matrix. Broadening of the optimum pH is observed when there is very high loading of enzyme, i.e. a large quantity of enzyme activity per unit of matrix. It leads to high intrinsic specific activity (Zulu effect), which increases the concentration of released product molecules near to the immobilized enzyme. Movement of substrate molecules towards the active site of the immobilized enzyme is obstructed due to the high concentration of enzyme (present on the surface or inside the matrix) and product molecules. Therefore, the rate of the reaction is observed to be constant for a longer duration in spite of the change in the pH of the solution [115–119, 129].

(b) *Optimum Temperature*: There is shift in the optimum temperature of immobilized enzymes towards higher temperatures. During enzyme immobilization, free movement of enzyme molecules is obstructed, even at higher temperatures. Thus, enzyme denaturation is not observed due to protection of amino acids at the active site as well as on the surface, while substrate conversion

continues at higher temperatures. As the temperature increases, substrate molecules gain kinetic energy and reach the active site of the immobilized enzyme rapidly, which shifts the optimum temperature towards higher temperatures. The extent of optimum temperature displacement for immobilized enzymes depends on the type of matrix as well as on interactions between the enzyme and the matrix. Broadening of the optimum temperature range has been also observed in various cases due to the high intrinsic specific activity (Zulu effect) with discussion similar to optimum pH [115–119].

(c) Kinetic Parameters: Changes in kinetic parameters (K_m, V_{max}, k_{cat}) are observed during enzyme immobilization. Enzyme immobilization does not ensure that enzyme molecules are attached in their correct conformation (i.e. involving residues away from active site), which strongly affects the V_{max} of the enzyme. Furthermore, diffusion barriers (*internal* and *external*) are other important reasons for changes in kinetic parameters [115–123]. In case of an internal diffusion barrier, the enzyme is present inside the matrix, which limits diffusion of substrate molecules into the matrix, which affects K_m followed by V_{max}. In the case of external diffusion where the enzyme is present on the surface of the matrix and has a thin unstirred layer of solvent called 'Nernst layer' around the immobilized enzyme which obstructs diffusion of substrate molecules. It has been found that the higher the concentration of substrate, greater would be the barrier caused by external diffusion. The Nernst layer is thickened with time, which subsequently decreases the V_{max} of the immobilized enzyme [130]. However, increase in stirring would help in reducing the effects caused by external diffusion due to the following relation:

$$\alpha = \frac{V_{max}}{K_m}$$

where, α is the diffusion constant.

Furthermore, polyionic matrices also affect kinetic parameters of immobilized enzymes due to partitioning effects, which involves interaction between the matrix and the ionic substrate as well as product molecules [130]. In presence of partitioning effects, Michaelis-Menten equation becomes:

$$v = \frac{V_{max} \times S_o \times p}{K_m + S_o \times p} \text{ and } K_{m(app.)} = \frac{K_m}{p}$$

where, p is partition coefficient ($p = S_i/S_o$, where S_i is the concentration of ions around enzyme and S_o is the concentration of ions in bulk phase). Therefore, increase in partitioning leads to decrease in K_m and an increase in V_{max}.

(d) Stability: The stability of the immobilized enzymes can increase or decrease depending on the type of matrix as well as on the interaction between the enzyme and the matrix. The following are three important parameters that are the determinants of enzyme stability during immobilization.

- Immobilizing matrix: This should have a large surface area to have good geometrical congruence with the enzyme surface and high superficial density of reactive groups. The groups present on the matrix being used for enzyme immobilization should be highly reactive, stable and present minimal steric hindrances during the reaction. Once the enzyme is properly immobilized, the matrix should be made inert in order to avoid any interference during enzymatic catalysis by blocking the remaining reactive groups present on the matrix using suitable chemical reagents [115–123].
- Immobilization conditions: This includes: reaction time, pH value, temperature and buffers. They should be properly optimized for the best immobilizing efficiency and stability of the immobilized enzyme.

  1. *Reaction time*: This should be optimum, allowing accurate alignment of groups present on the surface of the matrix, partially rigidified enzyme and already immobilized enzyme.
  2. *pH*: This should be optimized with each immobilization system as it varies from enzyme to enzyme, carrier and type of immobilization.
  3. *Temperature*: Similar to pH, this should be optimized for different immobilization systems. Furthermore, it has been found that reasonably high temperatures have a higher possibility of getting more enzyme-support linkages due to higher rate of vibration in enzyme molecules as well as the matrix.
  4. *Buffers*: It has been found that sometimes it is not the pH that affects stability and efficiency of immobilized enzyme but the type of buffer being used for the reaction. For example, borate interferes in the reaction between –CHO and $–NH_2$ but tris ethanolamine helps in it; however, this is not applicable when Lys on the enzyme molecules is chosen for attachment [123].

- Rigidification by multipoint attachment: Enzymes that are attached by various covalent bonds either directly or by activated matrix (by attaching short spacer arms) are found to be very stable with higher reusability. The number of reactive groups present on the surface of the matrix and immobilization conditions should be properly characterized in order to achieve effective multipoint attachment [115–117]. However, it is problematic for multimeric enzymes due to dissociation of subunits caused by their inactivation. Furthermore, release of enzyme subunits contaminate the final product (which is most important when immobilized enzymes are used in food industry) [131]. Therefore, the immobilizing strategy should be such that it involves not only multipoint attachment of the enzyme onto highly activated supports in addition to in between attachment of enzyme subunits. It can be done by chemical cross-linking involving surface groups away from the active site, which protect release of enzyme subunits and provides complete enzyme attachment onto the matrix [132].

2.4 Matrices for Enzyme Immobilization

During enzyme immobilization, enzymes are either attached to the surface of matrices or entrapped inside them by physical or chemical methods. The following are details of the matrices being used for enzyme immobilization:

(a) *Surface-Bound Enzymes*: The physical and chemical properties of the matrices used for enzyme immobilization are very important as they are major governing factors of chemical, biochemical, mechanical and kinetic properties of immobilized enzymes. The matrix can be biopolymer, synthetic organic polymer, hydrogels, smart polymer or inorganic solid.

- Biopolymers: They are water-insoluble polysaccharides (e.g., cellulose, starch, agarose, chitosan, and proteins such as gelatin and albumin). The first industrial application of a biopolymer came in 1960, using immobilized aminoacylase from *Aspergillus oryzae* onto DEAE-Sepahdex (modified cellulose with diethylaminoethyl) by ionic adsorption for production of amino acids by resolution of racemic acylamino acids (Tanabe process) [133]. The process was performed in a fixed-bed reactor under continuous operation. Since then, several other commercial enzymes were immobilized onto DEAE-Sephadex [134]. In case of immobilized recombinant epoxide hydrolase (from *Aspergillus niger*) onto DEAE-Sephadex, there was retention of 70 % enzyme activity with respect to the soluble enzyme. Immobilized enzyme has activity even at very high substrate concentrations (306 g/L) with a reusability of 7 washes [135].
- Synthetic organic polymers: The various synthetic organic polymers are being used for enzyme immobilization are; Eupergit-C (acrylic resins), Sepa beads FP-EP, Amberlite XAD-7 (porous acrylic resins) etc. Eupergit-C has surface diameter of 170 mm and pore diameter of 25 nm. It is prepared by using compounds: *N*,*N*′-methylene-bi-(methacrylamide), methacrylamide, allyl glycidyl ether and glycidyl methacrylate. It is hydrophilic in nature with chemical and mechanical stability over a pH range of 0–14. It has a high density of oxirane moieties on its surface, which is responsible for multi-point enzyme binding *via* covalent bonds at either neutral or alkaline pH. Thus, it gives long term operational stability in wide pH range (1–12). After enzyme attachment, the remaining epoxy groups of oxirane are blocked by mercaptoethanol, ethanolamine or glycine to prevent non-specific interactions [136]. Eupergit-C has been used for several industrial enzymes. Major success has been achieved in case of Penicillin amidase with a reusability of more than 800 washes [137]. However, due to diffusional limitations by Eupergit-C, kinetic properties of immobilized enzymes have been greatly interpolated. Sepa beads FP-EP have been commercialized by Resindion (Milan, Italy) [138]. It consists of functionalized polymethacrylate-based resin with oxirane groups, similar to Eupergit C. Amberlite XAD-7 is another synthetic matrix used to

immobilize enzymes by simple adsorption, for example lipases from *Humicola lanuginosa*, *Candida antarctica* and *Rhizomucor miehei* [139, 140]. Sepa-beads are best immobilizing matrix with respect to Eupergit-C and Amberlite XAD-7 due to excellent operational stability, reusability and kinetic properties of immobilized enzymes.

- Hydrogels: They are of two types: natural and synthetic hydrogels. Enzymes are not only attached to their surface but can also be entrapped by a slight change in immobilization method. PVA (polyvinyl alcohol) hydrogel is the most commonly used matrix for enzyme immobilization. It has very good mechanical stability, with a diameter of 3–5 mm and thickness of 200–400 mm. Enzymes immobilized onto PVA hydrogel have ameliorated thermal stability, excellent operational stability and undergo catalysis in organic solvent. Alcohol dehydrogenase from *Lactobacillus kefir* has been immobilized onto beads of PVA hydrogels along with its co-factor, NADP. Immobilized enzyme have been used to reduce hydrophobic prochiral ketones to secondary alcohols when *n*-hexane is a solvent. However, in the presence of water as a solvent, enzyme leaching has been observed [141]. It can be rectified by cross-linking enzyme to chitosn *via* glutaraldehyde followed by entrapment inside PVA hydrogel. This has resulted in an immobilized enzyme with well-defined particle size (3–5 mm) and a reusability of 20 washes [142]. Enzymes such as horseradish peroxidase and chloroperoxidase are immobilized by novel method onto PHEA (poly 2-hydroxyethyl acrylate) hydrogel [143]. Immobilized enzyme has amphiphilic network i.e. hydrophobic and hydrophilic phase allowing it to work in both aqueous and organic solvent. This amphiphilic network has been synthesized by copolymerization of silylated 2-hydroxyethyl acrylate and methacroyloxypropyl-poly(dimethylsiloxane) in the presence of UV followed by enzyme immobilization. Immobilized enzyme has considerably higher catalytic activities and operational stabilities as compared to soluble enzyme [144].
- Inorganic solids: They include alumina, silica, zeolites and mesoporous silicas (MCM-41, and SBA-15). They are known to be the cheapest matrix being used for the immobilization of several industrial or non-industrial enzymes. CaLB (lipase from *Candida antartica*) (detergent industry) has been immobilized onto silica granules prepared by adsorbing lipase on silica powder followed by its aggregation into small granules called granulates [145]. Immobilized enzymes which are specifically used in organic solvents have been very helpful for various industrial processes, most importantly in packed bed reactors based on packed granules. Epoxide hydrolase from *Aspergillus niger* has been immobilized onto silica by covalent linkage leading to an immobilized enzyme with retention of 90 % of enzyme activity with respect to soluble enzyme. The immobilized enzyme is stable for several months under dried conditions and stability can further be enhanced by keeping it in 20 % DMSO (Dimethyl sulfoxide) [146]. Silica has been found to be an excellent matrix

for enzyme immobilization due to their small size (dia. 2–40 nm) and high surface area ranging from 300–1500 m^2 g^{-1} with very high stability at elevated temperatures. Its surface can be easily functionalized with any moiety, due to which it can attach any enzyme. Immobilized enzymes are either present on the surface or inside silica based on immobilization protocols. α-Chymotrypsin has been immobilized by covalent linkage onto mesoporous sol-gel glass modified with 3,3,3-trimethoxypropanal. Immobilized enzymes have a shelf life of thousand times greater than that of soluble enzymes [147]. Inorganic solids have been used in the generation of protein-coated microcrystals (PCMCs). They are prepared by mixing enzyme solutions with a concentrated salt solution (potassium sulfate) followed by its drop-wise addition to water-miscible solvent (isopropyl alcohol) generating micron-sized crystals containing enzyme on their surface. PCMCs have very high stability in the absence of any solvent for a long period with applicability for wide range of solvents. This method of immobilization causes the least damage to the three-dimensional structure of the enzyme. The technique has been successfully used for various enzymes such as lipase, subtilisin Carlsberg, alcohol dehydrogenase, catalase, soybean peroxidase and horseradish peroxidase [148–150].

- Smart Polymer: The most studied example of smart polymer is thermostable biocompatible polymer [poly-*N*-isopropylacrylamide (polyNIPAM)]. PolyNIPAM exhibits the unique property of existing in two states; the solution state when the temperature is lower than 32 °C, and the polymer state when the temperature is 32 °C. Enzyme immobilization is done by mixing the enzyme solution with solution state of polyNIPAM, i.e. <32 °C followed by an increase in temperature, which precipitates immobilized enzyme and subsequently filtration to get immobilized enzyme. This method of immobilization causes the least damage to enzyme conformation. The enzyme is attached by covalent linkage with polyNIPAM involving its vinyl groups with NH_2 groups present on enzyme surface. Penicillin G amidase has been successfully immobilized onto polyNIPAM leading to an immobilized enzyme with a higher stability and activity almost similar to soluble enzyme [151]. Recently, a thermostable polymer has been made using 2-(2-methoxyethoxy) ethyl methacrylate and oligo(ethylene glycol) methacrylate (OEGMA) [152]. The formed polymer resembles poly(ethylene glycol) in terms of low toxicity and it is anti-immunogenic. It is similar to polyNIPAM in terms of existing in solution and polymer states as a function of temperature. Solution state temperature can vary from 26–90 °C depending on the amount of OEGMA used [153]. It is used for the immobilization of a wide range of enzymes for various processes [154–156].

(b) *Entrapment*: Enzymes can be immobilized by enclosing them inside the matrices. Sol-gel is a metal alkoxides that has been used for the entrapment of several enzymes. Enzyme immobilization into silica sol-gel is prepared by hydrolytic polymerization of tetraethoxysilane followed by drying. The immobilization method involves drying, which is the determining

factor in the morphology of sol-gel. For example, drying by evaporation produces xerogels with nano-cages and pores while drying in the presence of CO_2 produces aerogel with a delicate structure with phenomenal porosity [157, 158]. Silica aerogel is known to be the lightest solid on earth with a density of 0.001. It has been found that when lipases were entrapped in sol-gels produced from SiACHTUNGTRENUNG$(OEt)_4$, immobilized enzymes has significantly less activity than soluble enzymes. However, when sol-gel is prepared by mixing Si-ACHTUNGTRENUNG$(OMe)_4$ and RSiACHTUNGTRENUNG$(OMe)_3$ and used for lipase immobilization, increase in activity of 8-fold have been observed due to the increase in matrix hydrophobicity. Furthermore, the addition of Celite to sol-gel has led to increased thermal stability of immobilized lipase [159]. When higher alkyl groups are used for sol-gel preparation in the presence of additives (e.g., isopropyl alcohol, crown ethers, surfactants and KCl), an increase in V_{max} by a factor of 10 has been observed [160]. In case of lipases from *Burkholderia cepacia* and *Candida Antarctica*, it has been found that the addition of silica quartz fiber has improved the mechanical properties of immobilized enzyme [161, 162]. Sol-gel immobilized lipases have been used for the synthesis of biodiesel by esterifying oil from sunflower seed. However, they cannot be used at high substrate concentrations due to diffusion constraints. Experiments are still ongoing that aim to improve the activity of immobilized enzymes *via* entrapment by various additives [162, 163]. Enzymes like catalase and horseradish peroxidase (HRP) have been entrapped using biosilification process. This involves polymerization of silica nanoparticles produced by diatoms to silicic acid in the presence of the enzymes to be immobilized. The process of polymerization requires a catalyst called silicateins produced by *Cylindrotheca fusiformis* [164].

2.5 Structure Based Immobilization

Studies on structural changes brought about during enzyme immobilization are scarce. Correct orientation of enzymes during immobilization is the key factor in efficient catalysis by immobilized enzymes. It has been found that enzymes immobilized at the surface of the matrix are more prone to conformational changes by immobilization than when present inside the matrix. There are no effective tools available to date that can accurately characterize surface-bound enzymes. There are over 70,000 solved protein structures with or without bound substrates that are available in protein database bank and are still growing continuously. However, there are none of the reported structures of bound proteins/enzymes onto the matrix. This is due to the fact that these reported data are based on X-ray diffraction and nuclear magnetic resonance (NMR) based on atomic positions of molecules present in protein/enzyme in their crystal or solution states. Due to the limitations of these techniques with respect to sample preparations, structural

details of immobilized cannot be deciphered. There is no single technique that can provide molecular high-resolution structural details of proteins/enzymes as well as their conformational details while present on the surface of various matrices. Recently, few techniques have claimed to give various structural and conformational details of immobilized enzyme/proteins present on the matrix surface. This would help not only in understanding the phenomenon but in screening for the best matrix for perfect immobilization of enzymes/proteins with the highest operational efficiencies.

- *Thermal gravimetric analysis (TGA)*: This involves thermal analysis of the given substance and its reaction products, which are measured as the function of temperature under controlled conditions. The sample size can vary from a few mg to 10 g as experimental requisite. The measurements are done based on changes in the weight of the sample subjected to varied temperature using a furnace. Analysis of thermograms weight *versus* temperature in a given time frame helps in generating information related to sample thermal stability, rate of reaction, oxidation and kinetics of decomposition [165]. In case of enzyme immobilization studies, it is used to assess thermal stability of the immobilized enzyme, screening of immobilizing matrices, structural details of immobilized enzyme and determining the number of functional groups (degree of functionalization) present on the given matrix [166]. Thus, it helps in reducing the number of trials used for the assessment of efficacy of immobilization method. TGA has been used in various industries as well as other sectors including environmental, pharmaceutical, food science, biotechnology and petrochemical applications.
- *Electron microscopy [field emission scanning electron microscopy (FESEM), scanning electron microscopy (SEM), transmission electron microscopy (TEM)]*: They are used to obtain structural details of the given sample at a resolution of 10^{-9} m. SEM and FESEM are used for collecting structural details at the surface with little information on internal details. In the case of enzyme immobilization, SEM is used to observe morphology and distribution of enzyme immobilized onto the matrix while FESEM is used in visualizing morphological details at a particular portion of the matrix [167–169]. Thus, this helps in giving confirmation of enzyme presence on the given matrix and efficacy of the given immobilization method based on the density of enzyme molecules on the matrix surface. SEM is an analytical technique with spectacular improvisation over optical microscope due to its resolution ($3 \times 10^4 - 6 \times 10^4$ X) and field depth (factor of 300). SEM is based on collecting a formed image on cathode ray tube by the electron beam reflected from the surface of the given sample [170]. FESEM is an advanced version of SEM technology due to improvisation in secondary electron detector technology, producing sample image at ultrahigh resolution [171].

  TEM provides structural information of the given sample in two-dimensional projections. It works by transmitting a beam of electrons produced by tungsten filament in a vacuum chamber onto the sample and collecting the emitted electrons into a narrow beam using electromagnetic field. The emitted beam from

the sample is allowed to hit phosphor screen/film/charge coupled device (CCD) and produces corresponding image of the sample. The brightness of the image depends on the density of the sample, i.e. higher the density, greater would be the darkness. In case of biological samples, positive staining is done at resolution <0.2 nm (in range of atomic size) to produced resolved sample image giving in depth analysis on atomic structural arrangement in the given sample [172]. TEM has been widely used in material science, particularly in the characterization of materials with nanometer scale length. In case of enzyme immobilization, TEM is used to study enzyme distribution onto the matrix, and provide information on particle size and morphology of immobilized sample. It is especially useful for enzyme immobilization onto nano- or micro-scale matrices (e.g.,carbon nanotubes, micron-size magnetic beads, nanoparticles) (Fig. 2.3) [173–175].

- *X-ray photoelectron spectroscopy (XPS)*: This is also called electron spectroscopy and is used to provide total elemental analysis (excluding hydrogen and helium) of surface layers or thin film structures at 10–200 Å. It is used in a wide range of industries including catalysis, corrosion, adhesion, polymer surface modification, magnetic media, electronics, dielectric materials, semiconductors and thin film coatings. [176]. XPS gives the most quantitative, informative and readily interpretable information with respect to the various elements present in the given sample. Here, a sample surface is irradiated with low-energy X-ray leading to electron excitation from the sample atoms. It is based on the photoelectric effect where atoms present on the surface eject electrons upon being irradiated with X-rays. The ejected electrons present on the sample surface with their binding energy lower than the energy of X-ray energy contains information regarding the chemical oxidation state and electronic structure. Angle resolved XPS has been used for analysis of immobilized enzyme present at the surface of functionalized polyaniline films. In case of enzyme immobilization, sample is irradiated with X-rays followed by ejection of electrons. The peak corresponding to the photoelectron is used in elemental identification of the sample surface. Obtained high-resolution spectra helps in establishing the presence or absence of specific functional groups as well as their quantification. Generally, chemical compositions of nitrogen, oxygen and carbon present on the surface of the immobilized enzyme are estimated. Furthermore, thickness of the enzyme layer present at the matrix surface can also be found by assessment of elemental depth distribution and thickness of ring containing amino acids such as histidine, tryptophan, tyrosine etc. [177, 178].
- *Surface plasmon resonance (SPR)*: This is used in studying various biological processes including conformational and interaction studies. It can be used for small biological molecules with diameter $\geq$20 nm. It is based on collecting information from oscillating resonant electrons at the interface between negative and positive permittivity material stimulated by incident light. Only electrons present at the surface are made resonant by incident light. SPR has been used for various studies based on adsorption of material onto planar metal surface, *viz.* silver and gold. Silver has larger deviation with respect to gold, therefore the later is being used for various interaction studies including

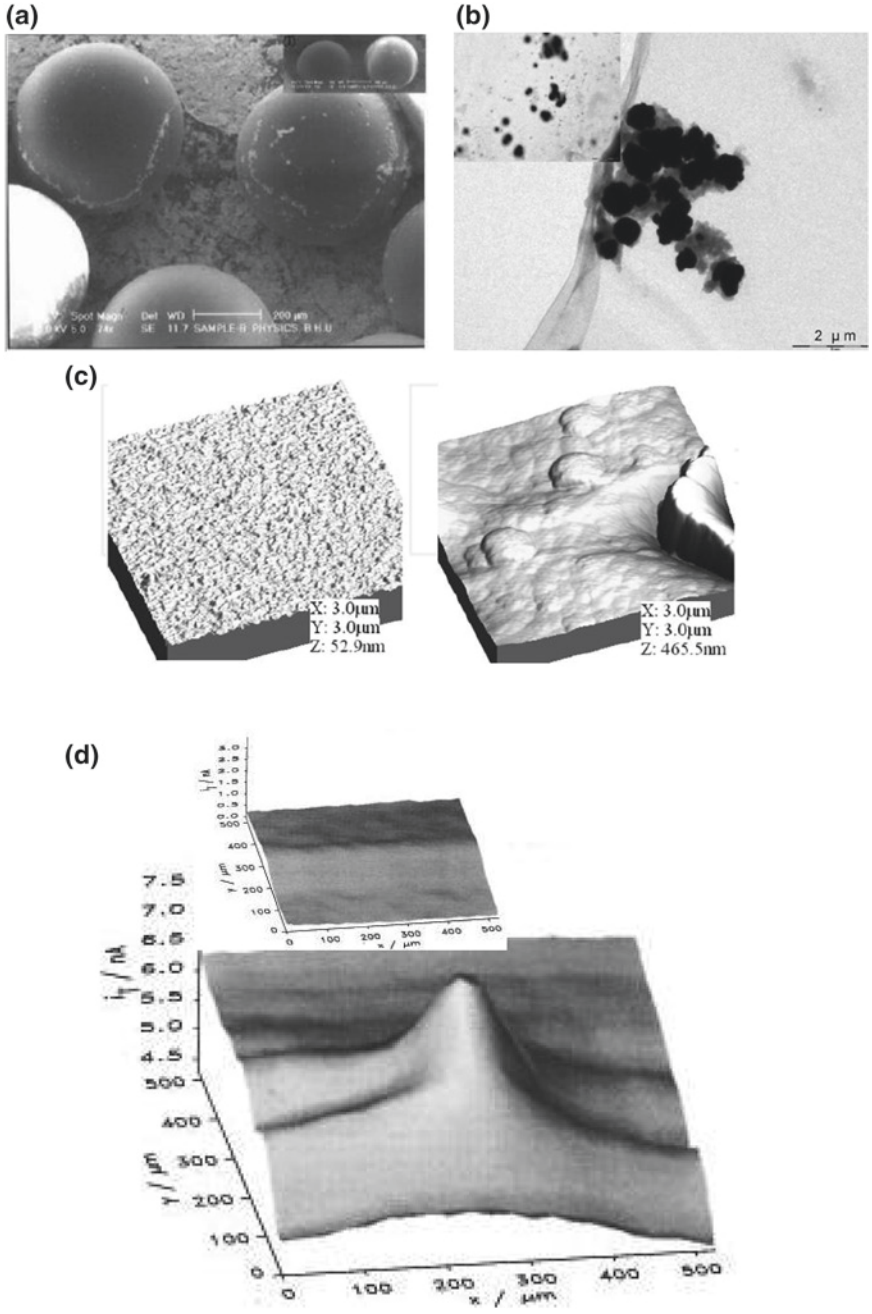

Fig. 2.3 Microscopic images of immobilized enzymes; **a** SEM of immobilized β-galactosidase onto Amberlite beads. **b** TEM of immobilized β-galactosidase onto gold nanoparticles. **c** AFM of immobilized invertase into lipid film. **d** SECM glucose oxidase immobilized onto self assembled monolayers (Adapted from refs. [167, 173, 183, 187])

protein-drug, antigen-antibody, protein-protein, protein-DNA etc. It can also be used in immobilization studies based on attachment on enzyme onto functionalized gold surface. It includes enzyme orientation, their altered catalytic properties, identification of active surface groups used for enzyme immobilization, interaction of enzyme with various matrices (*viz*. membranes, vesicles) etc. The surface coverage of biological molecules can be found by plotting concentration *versus* adsorption isotherm, which gives equilibrium dissociation constant (K_d). SPR has also been used in the identification of various enzyme inhibitors and substrates, which helps in creating immobilization protocols with minimum enzyme wastage [179, 180].

- *Circular dichroism (CD) spectroscopy*: This is one of the oldest spectroscopic techniques being used by structural biologists in examining 2° and 3° structure of peptides, polypeptides and proteins. Furthermore, it helps in the assessment of proteins conformation and stability in various environmental conditions (temperature, ionic strength/charge, presence of small molecules). CD spectroscopy uses circularly polarized light as the source that oscillates rotationally right or left around the axis of propagation depending on the type of sample. The extent of the deviation and direction of oscillation helps in extracting information on structural properties of the given protein samples. Secondary structural details of the given protein sample is collected in the far UV regions (180–250 nm) corresponding to n–p and p–p transitions of amide, which gives geometrical details of polypeptide backbones. The tertiary structure of protein is characterized by near UV regions (250–320 nm) corresponding to p–p transition of polypeptides. Furthermore, near UV spectra is also contributed by aromatic amino acids residues with absorption >250 nm. CD is also used to study cystine linkages that present inter- or intraspecifically between polypeptides using n–s transitions corresponding to 260 nm. In case of enzyme immobilization, CD spectroscopy provides structural information with respect to enzyme-matrix interactions, structural composition of immobilized enzyme, conformational changes, kinetic and thermodynamic information etc. [181, 182].
- *Atomic force microscopy (AFM)*: This is a microscopic technique that allows visualization of the surface topography of biomolecules on the surface of carrier, adhesion, elasticity, association processes and dynamics at sub-nanometre scale. It is being widely used in surface-based interactions studies, protein-protein interactions, biosensors, single-molecule analysis, bioelectronics or drug screening. AFM has a probe consisting of microfabricated cantilever that tapers into a sharp nanotip. A probe can be moved in 3D with accuracy at sub-nm scale using several piezoelectric scanners. The quantification of the interaction between given molecules is done by placing one at the sample surface and other at the tip of the AFM probe, which leads to the generation of a laser beam from the top of the cantilever, reflected onto photodiode. AFM operates in two modes including single-molecule force spectroscopy (SMFS) and jumping mode (JM). In case of SMFS, cantilever is deflected vertically using piezo scanner. Extent of deflection quantifies level on interactions between two given molecules. However, in the case of JM-based scanning, it involves both topographical

assessment as well as quantification of binding between given molecules based on sample tip adhesion maps. AFM has been used in the assessment of enzymatic activity of various immobilized systems, such as in immobilized glutamate dehydrogenase onto the surface of the biosensor by evaluating the correct orientation of ordered enzymatic monolayers (Fig. 2.3) [183, 184].

- *Microcalorimetry*: This is a versatile technique based on calorimetric measurements (heat flow, heat capacity, molar and specific enthalpy) used for interaction studies. It works on the basis that all processes are brought about by heat exchange with respect to surroundings. There are various calorimetric techniques being used to investigate the various protein aspects. In case of differential scanning calorimetry, thermodynamic parameters (mid vales of temperatures maxima, enthalpy change) are used to study protein unfolding, protein stability in its soluble and immobilized states as well as matrix efficiency (*viz.* lipid, polynucleotide). In case of enzyme immobilization, it is helpful in the assessment of protein (enzyme) stability after immobilization, effect of microenvironment on immobilized enzyme (using isothermal batch and flow calorimetry) and catalytic efficiency of immobilized enzyme. Furthermore, calorimetry is non-destructive to the sample and does not require any prior sample treatment. It can be used for any sample states (solids, liquids and gases) [185].
- *Forster resonance energy transfer (FRET)*: This is based on fluorescent molecules transferring their energy from one molecule to another through intermolecular long-range dipole-dipole coupling. The donor and acceptor fluorophores should be adequately aligned by covalent attachment at the distance from 10–100 Å for proper induction. Generally, the emission spectrum of donor fluorophore coincides with absorption spectrum of acceptor fluorophore. There are a number of fluorophores that have been made with a small molecular mass, that are organic in nature and photostable. Even activated nanoparticles are also being used as fluorophores. During sample measurements by FRET, it is irradiated at absorption wavelength of the donor, which is being temporarily excited to higher energetic electronic state followed by transfer of energy to acceptor fluorophore by dipole-dipole interaction. If the sample is not fluorescent in nature then it is being tagged with suitable fluorophore. FRET is helpful in monitoring conformational changes of molecules in solution with respect to structural and dynamic properties at atomic scale. It is being used in the analysis of molecular interactions at the cellular level, and in understanding the trajectory followed by proteins during folding. In the case of enzyme immobilization, it is used in comparative analysis of soluble and immobilized enzymes with respect to their orientation and changes in chemical environment. The most important point to be taken care during studies by FRET is protection by bleeding during excitation by donor fluorophore and controlling the background to avoid zero signals during emission by acceptor fluorophores [186].
- *Scanning electrochemical microscopy (SECM)*: This consists of electrochemical components, actuator and computer control that allows directional flowing of current through a microelectrode immersed in an electrolytic solution closed to substrate (conductive or semi-conductive, sometimes insulating). SECM is

used for surface analysis of a given sample by scanning sample surface using ultramicroelectrode (at the tip of microelectrode) followed by generation of an electrochemical response, thus providing quantitative information about interfacial region. Thus, this is helpful for probing a variety of electrochemical processes, energy storage, materials science, corrosion science, biosensors research and biophysics. In the case of enzyme immobilization, it is used to characterize modified matrix surface with enzyme; transport of molecules between enzymatic layers present on the surface of matrix, which is helpful in the assessment of microenvironment around the immobilized enzyme; and apparent kinetics of the immobilized enzyme. In particular, it is used in the assessment of biosensors' efficacy, prior to their use in various applications (Fig. 2.3) [187].

- *Fourier transform infra-red spectroscopy (FTIR)*: This is based on collecting information based on its infra-red absorption (light with longer wavelength and lower frequency than visible light) information by the sample using fourier transform infrared spectrometer. The sample can be used in any physical state (solid, liquid or gas). Infrared light is directed by interferometer through the sample using a properly oriented moving mirror inside the apparatus and this is then processed to achieve the desired result. Data are always represented as infra-red absorbance *versus* wave-number (frequency reciprocal of wavelength in centimeter). Sample analysis is always carried out using a standard reference. The infrared portion of electromagnetic spectrum that is used for sample analysis: near IR (14000–4000 cm^{-1}), mid IR (4000–400 cm^{-1}) and far IR (400–10 cm^{-1}) corresponds to harmonic, fundamental and rotational vibrations, respectively. FTIR is similar to UV-spectroscopy in terms of the absorption parameter. However, it is more informative, with a very high signal-to-noise ratio. FTIR is being used for studying conformational changes during protein folding, distinction between wild and mutant proteins etc. In case of enzyme immobilization, FTIR can confirm the presence of enzyme attachment on the surface of matrix, and allows assessment of the nature of the bonds used for enzyme attachment and conformational changes brought about during enzyme immobilization [188–190].
- *Sum Frequency Generation spectroscopy (SFG)*: This is similar to FTIR in terms of being a vibrational spectroscopic technique. It assesses N–C=O, N–H, C–H, and O–H vibrational modes, which helps in the identification of secondary structures such as α-helices, β-sheets and β-turns as well as amino acid side chains present in proteins. Here, an incident photon source at a fixed frequency (within visible range) and tunable broadband IR are used to analyze sample conformation based on reflected summed beam coming from the sample. In case of immobilized enzymes, SFG is used to assess the presence of ordered secondary structures corresponding to amide I stretching region, which confers correct orientation of the enzyme during immobilization [191].
- *Time of flight secondary ion mass spectrometry (TOF-SIMS)*: This is an analytical technique that can be used for mapping sample surface with respect to its composition, structure, orientation, spatial distribution of molecules and chemical structures. Therefore, it can be very useful in studying protein-adsorbed materials and imaging of the distribution pattern of a particular protein

at sub-micron scale on the matrix surface. However, TOF-SIMS has not being used to date for enzyme immobilization due to complicated spectrum interpretation. Details of TOF-SIMS have been briefly described here. It consists of ultrahigh vacuum system, particle gun, circular designed flight path and mass spectrometer, equipped with electrostatic analysers. A pulsed beam of primary ions is used to bombard sample surface followed by emission of secondary ion (both positive and negative) particles including photons, neutrons, and secondary electrons. These secondary ion particles are accelerated to TOF mass analyzer at potential, 222 keV and subsequently reach detector in given time span. Generally, ions having lower mass to charge ratio reach faster than those ions with higher mass to charge ratio. It operates in three modes: surface imaging, surface spectroscopy and depth profiling. Thus, it is useful for visualization of distribution of individual species on a surface, analysis of elemental and molecular species on a surface as well as depth analysis of different chemical species. Studies related to sample surface requires a depth of <2 nm, usually helpful for large proteins. During enzyme immobilization, this helps in analyzing the orientation of the enzyme onto matrix surface and analyses of the various monolayers of the enzyme and the type of linkages involved for their attachment onto the matrix surface [192, 193].

- *Near Edge X-ray Adsorption Fine Structure (NEXAFS) spectroscopy*: This is helpful for analyzing the immobilized enzyme by giving detailed information about enzyme orientation, bond order and microenvironment of immobilized enzyme. It consists of polarized synchrotron X-rays directed at a specific angle using an electric field, which are being absorbed by the surface molecules of the given sample, generating photoelectrons. It leads to the formation of holes at core levels, which are filled by an electron at a higher energy level, and leads to emission of an Auger electron or photon. Further, emitted electrons encountering inelastic scattering processes are collected at the detector and analyzed. Generally, a sample with a width of ~10 nm is used for analysis by NEXAFS. It can also be used for specific bonds studies of DNA oligomers and model peptides [194].

2.6 Cross-Linked Enzyme Aggregates and Crystals

Immobilization of enzyme onto solid matrices has been carried out since 1916. The basic idea behind this is to make enzymes stable in the presence of a broad range of physical and chemical factors to make it widely applicable. However, in early days of 1960, enzyme immobilization without solid support was highlighted, which involved enzyme cross-linking using glutaraldehyde (bifunctional chemical cross-linker) by forming amide linkage between surface NH_2 groups of the enzyme and the cross-linker. Enzymes so prepared were called cross-linked enzymes (CLEs) with almost complete retention of catalytic activity. Nevertheless, CLEs had lower mechanical stability, poor reproducibility and were difficult to handle [195].

In 1964, Quiocho and Richards cross-linked enzyme crystals (CLECs) using glutaraldehyde to stabilize them for X-ray diffraction studies. They found that cross-linking not only helped in stabilization but also retained catalytic activity [196]. Thus, it was helpful for studying enzyme catalysis of enzyme crystals and elucidation of functional enzyme structure. CLECs had been adopted by various industries like Vertex Pharmaceuticals, Altus Biologics etc. for various applications. The first commercial application of CLECs was in 1990 using thermolysin for manufacturing aspartame (artificial sweetener). Subsequently, several CLECs of various enzymes were prepared and used for a number of applications. CLECs were stable to denaturation by extreme physicochemical conditions such as heat, organic solvents and proteolysis. Furthermore, their size can be regulated within range from 1–100 mm and they can be recycled, similar to immobilized enzymes. For example, CLEC of chloroperoxidase (CPO) from *Caldariomyces fumago* has exceptionally very high thermal stability with tolerance to organic solvents and reusability of 4 washes [197]. The major drawback of CLECs is the requirement for an enzyme in its crystalline state, which is laborious, time consuming, and requisite of an enzyme with very high purity. Furthermore, it is not possible to crystallize all types of enzymes (Fig. 2.4).

Subsequently, a new class of immobilized enzymes was developed known as cross-linked enzyme aggregates (CLEA). This was prepared by precipitating enzyme as physical aggregates from its solution by the addition of salts (ammonium

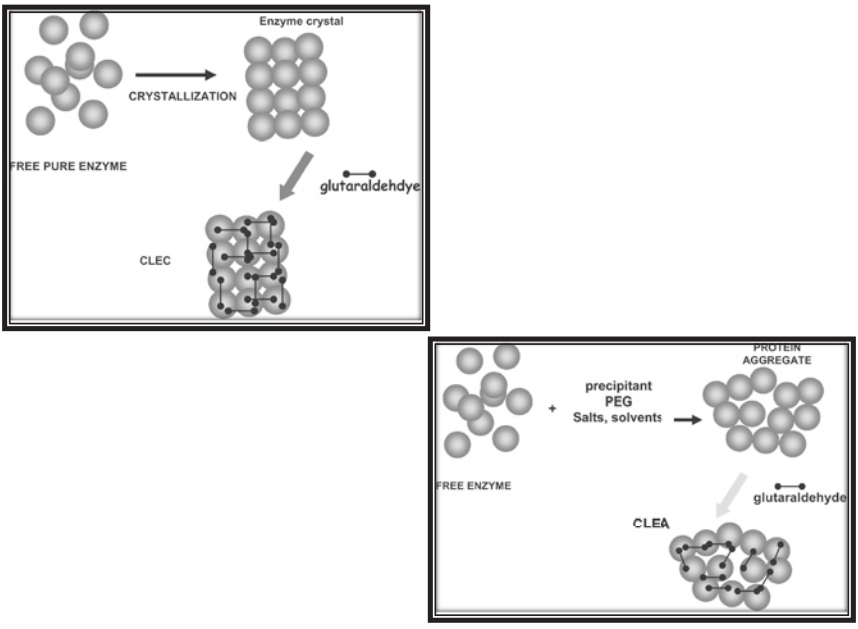

Fig. 2.4 Mechanism of enzyme immobilization without matrices, CLEC (cross-linked enzyme crystals) and CLEA (cross-linked enzyme aggregates)

sulfate), or water-miscible organic solvents or non-ionic polymers (polyethyleneglycol) followed by their cross-linking (glutaraldehyde). This methodology causes the minimum damage to the three-dimensional tertiary structure of the enzyme without any requisite of purified enzyme as the process allows purification during enzyme immobilization. The size of CLEA generally ranges from 0.1–200 μm, which is controlled by optimization of enzyme concentrations and amount of glutaraldehyde. In case of some enzymes such as nitrilases from *P. fluorescens*, there is complete loss of enzyme activity after synthesizing its CLEA when glutaraldehyde has been used as a cross-linker due to the involvement of residues present at the active site of the enzyme [198, 199]. Therefore, glutaraldehyde is not applicable for cross-linking all types of enzymes. In these cases, dextran polyaldehyde (formed by periodate oxidation of dextrans followed by Schiff's base reduction using sodium borohydride) can be used, which is less bulky than glutaraldehyde [200]. It has been found that enzymes with a smaller number of lysines are more prone for inactivation by glutaraldehyde. Another method of synthesizing CLEA without glutaraldehyde is by co-precipitating an enzyme with a polymer with numerous free amino groups (poly-L-lysine, polyethylene imine) [201]. In the case of dilute enzyme preparations, BSA (bovine serum albumin) is added, which facilitates CLEA formation [202]. CLEA technology is helpful in stabilizing quaternary structures of multimeric enzymes, an important feature that is a major limitation of number of immobilization methods known till date. Penicillin G amidase used in the synthesis of antibiotics (penicillin and cephalosporin) was the first known industrial enzyme used for CLEA formation. Amidase CLEA was found to have higher thermal stability and lower tolerance to organic solvents than the soluble enzyme. It was prepared by precipitation using ammonium sulfate or tertbutyl alcohol followed by cross-linking by glutaraldehyde [203]. Thermal stability and reusability was further enhanced by immobilizing amidase CLEA onto polyacrylamide (PGA-450), which had lowered the cost of antibiotic synthesis by several hundred fold [204]. Subsequently, CLEA of seven commercially available lipases were prepared by optimizing the type of precipitant and additives as well their concentrations. The most important factor taken into consideration during preparation of enzyme CLEAs is cross-linking of the enzyme aggregates in their most favorable conformation. Out of seven lipases, three were hyperactive with activities up to twelve times than that of soluble enzyme [205]. The process of CLEA synthesis has not been fully automated using 96-well plates. It was found that lipase B from *Candida antarctica* (CaLB) has varied commercial applications [206]. Novozym 435 is a commercially used immobilized CaLB (onto macroporous acrylic resin) that has been found to have less operational stability in organic solvents than CaLB CLEAs. Furthermore, CaLB CLEA is enantioselective causing specific acylation of 1-phenethylamine in diisopropyl ether as a solvent [207]. CLEAs of several hydrolases (pig liver esterase, aminoacylase, proteases, and glycosidases) have been successfully prepared for various industrial applications. Alkaline protease from *Bacillus licheniformis* (alcalase or subtilisin Carlsberg) is used in laundry detergents, resolution of amino acid esters as well as synthesis of amines and peptides. An alcalase CLEA has excellent activities; especially in amino acid ester hydrolyses. The synthesis of CLEA is helpful for

both enzyme purification and immobilization in a single operation [208]. The best known example is aminoacylase (*Aspergillus niger*), which has negligible esterase activity in its crude state while very high esterolytic activity by its CLEA [209]. CLEAs of various commercially used enzymes have been done: β-galactosidase from *Aspergillus oryzae* in lactose hydrolysis helpful for lactose intolerants; phytase from *Aspergillus niger* in hydrolyzing phytate (inositol hexaphosphate) is useful for animal feed; laccase in effluent treatment is useful for oxidizing starch to carboxystarch and for bleaching pulp and paper; alcohol dehydrogenase from *Rhodococcus erythropolis* and formate dehydrogenase from *Candida boidini* is useful in the hydrolysis of the C-C bond; and oxynitrilase from *Prunus amygdalis* is helpful in the hydrocyanation of aldehydes etc. In all cases, enzymes CLEAs are recyclable for more than ten times without loss of activity (Fig. 2.4) [210–212].

Various innovative modifications have been done in the CLEA technology for versatile applications. Lipase CLEAs are enclosed in hydrophobic polytetrafluoroethylene membranes to be used in membrane bioreactors [213]. Similarly, glucose oxidase CLEAs is enclosed in magnetic mesocellular carbon foam useful in bioelectrocatalytic system [214]. Co-precipitation and cross-linking of two or more enzymes is used to prepare combi CLEAs helpful for catalytic cascade processes (without the need for separation of intermediates). It has numerous benefits such as fewer unit operations, lower reactor volume, higher volumetric and space-time yields, shorter cycle times and less waste generation. Combi CLEA of catalase, glucose oxidase and galactose oxidase is used for the rapid removal of H_2O_2 formed during aerobic oxidation of glucose and galactose rapidly. Thus, it finds tremendous applications in disease diagnostics [215, 216]. Combi CLEA of S-selective oxynitrilase (*Manihot esculenta*) and an aselective nitrilase (*Pseudomonas fluorescens*) is used to convert benzaldehyde into *S*-mandelic acid in the solution at pH 5.5 containing di-isopropyl ether/water (9:1). Furthermore, combi CLEA was more effective than a mixture of the two separate CLEAs [217]. Microreactor technology involves usage of microchannel reactors (microfluidic devices) containing immobilized enzymes onto polymer membrane present inside walls of the microchannels. It was found that using enzyme CLEA prepared by mixing enzyme solution with glutaraldehyde and formaldehyde as cross-linkers followed by attachment onto polytetrafluoroethylene (PTFE) tubing with an inner diameter of 500 mm helps in the development of stirrer reactor with specialized enzyme configuration, including honeycomb, ceramic monolith, etc., as per requirement. Furthermore, it enhances operational stability of the enzyme for several weeks without a significant loss of activity [218].

2.7 Summary

Enzyme immobilization has been appealing to researchers in every branch of science, as well as industrialists. Several methods are known to date for effective enzyme immobilization onto various matrices; however, it is still known to be

method of trial and error as there is no universal protocol applicable to all types of enzymes. It is not known which type of matrix is best suited for the given enzyme and its performance for a given application. Intensive research on immobilized enzymes has concluded that the immobilized state is the natural state of enzymes, i.e. inside the cell. The attachment of enzymes to the appropriate surface constricts its activity to a particular site and enhances its concentration at that location by several thousand fold. Thus, it is the major reason for its stability in the presence of extreme physical and chemical conditions. It has been found that the smaller the size of the matrix greater would be the stability and catalytic efficiency of immobilized enzyme. Nanoparticles (Au, Ag, Zn, Fe etc.) have been found to be useful immobilizing matrices and found wide range of applications, most importantly in drug delivery, tumor location analysis, biosensors and bioreactors [219, 220]. Implementation of immobilized enzymes for therapeutic applications has now been accepted after long debate and experimentations. Immobilized enzymes are also extensively used in the synthesis of biosensors due to accurate enzyme sensitivity towards various molecules. Further, in development of microprocessing and microelectronic devices being used in miniaturized biosensors having several thousand fold higher activity due to presence of very large surface area. Biosensor has also helped in the analysis of even most complicated substrates. The increasing applications of immobilized enzymes in almost every field has created huge interest for its further improvement, particularly their operational performance. It will not only increase its pertinence in various known fields but also give a platform in various unexplored new fields.

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