# SECTION I: INTRODUCTION

# 1. INTRODUCTION

***Enzymes*** can be defined as the reaction-specific protein catalysts for chemical reactions in biological systems. *Coenzymes* are specific heat-stable, low-molecular-weight organic molecules (non-protein). The functional enzymes (holoenzyme) is formed by a proper combination of the apoenzyme (non-functional enzyme) and a coenzyme can be represented as:

| | | | | |
|---|---|---|---|---|
| Apoenzyme | + | Coenzyme | → | Holoenzyme (Enzyme) |
| (Protein) | | (Non-protein) | | |

The enzymes are used for brewing, food production, textiles, tanning, medicines, fermentation process, etc. Enzymes display great specificity and they are not changed during the reactions, so it is cost-effective to use them more than once. Most enzymes are labile under normal operating conditions and therefore have only a very limited shelf-life. This is uneconomical, as active enzyme is lost after a short time span. Therefore, to eliminate above disadvantages inherent in enzymes, they alternatively can be physically confined or localized (i.e. immobilized) with retention of their catalytic activities.

***Immobilized enzymes*** are the enzymes which are physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously (Zaborsky, 1973; Chibata, 1978).

Immobilized enzymes can have the following advantages: (a) better and efficient utilization of enzymes, (b) improved stability, (c) possible room temperature storage, (d) improved shelf-life, (e) avoided incompatibilities with excipients, vitamins and carminatives, (f) development of better economical formulations, etc.

# 1.1. Enzyme Immobilization

Method for enzyme immobilization can be broadly classified into: (i) ***Carrier-binding***, where in the enzymes are bound to water-insoluble carriers either by physical adsorption, ionic binding, or covalent binding. (ii) ***Cross-linking***, where in the enzymes are cross-linked to the matrix (polymers) using bi-functional or multi-functional reagents. (iii) ***Entrapment***, where in the enzymes are incorporated into the lattices of a semi-permeable gel or enclosed in a semi-permeable polymer membrane. The economic factors that have to be taken into account for immobilization are: (i) cost of enzymes, (ii) extent of enzyme purification required, (iii) cost of immobilization chemicals and immobilization process, (iv) enzyme stability, and (v) inhibition and poisoning effects.

### 1.1.1. CARRIER-BINDING METHOD

The carrier-binding method is the oldest immobilization technique for enzymes. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier. The selection of the carrier depends on the nature of the enzyme itself, as well as the: (a) particle size, (b) surface area, (c) molar ratio of hydrophilic to hydrophobic groups and (d) chemical composition.

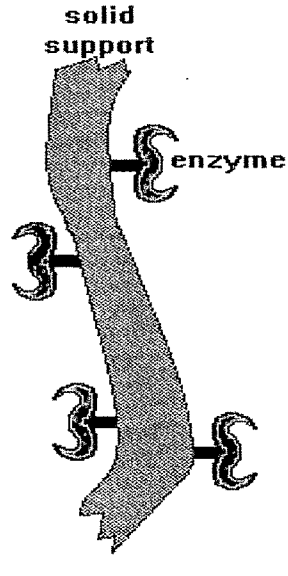


**Figure 1.1:** Schematic representation of carrier-binding method.

Some of the most commonly used carriers for enzyme immobilization are polysaccharide derivatives such as cellulose, dextran, agarose, and polyacrylamide gel. According to the binding mode of the enzyme, the carrier-binding method can be further sub-classified into: (i) physical adsorption, (ii) ionic binding, and (iii) covalent binding.

#### 1.1.1.1. Physical Adsorption Method

This method for the immobilization of an enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. Hence, the method causes little or no conformational change of the enzyme or destruction of its active center. A major advantage of adsorption as a general method of immobilizing enzymes is that usually no reagents and only a minimum of activation steps are required. Adsorption tends to be less disruptive to the enzymatic protein than chemical means of attachment because the binding is mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. Because of the weak bonds involved, desorption of the protein resulting from changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed. Another disadvantage is non-specific, adsorption of other proteins or other substances. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate.

#### 1.1.1.2. Ionic Binding Method

The ionic binding method relies on the ionic binding of the enzyme protein to water-insoluble carriers containing ion-exchange residues. Polysaccharides and synthetic

polymers having ion-exchange centers are usually used as carriers. The binding of an enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the conformation and the active site of the enzyme. Therefore, this method yields immobilized enzymes with high activity in most cases. Leakage of enzymes from the carrier may occur in substrate solutions of high ionic strength or upon variation of pH. This is because the binding forces between enzyme proteins and carriers are weaker than those in covalent binding. The main difference between ionic binding and physical adsorption is that the enzymes to carrier linkages are much stronger for ionic binding although weaker than in covalent binding.

#### 1.1.1.3. Covalent Binding Method

When trying to select the type of reaction by which a given protein should be immobilized, the choice is limited by two characteristics: (1) the binding reaction must be performed under conditions that do not cause loss of enzymatic activity, and (2) the active site of the enzyme must be unaffected by the reagents used. The functional groups that may take part in this binding are listed below: amino group, carboxyl group, sulfhydryl group, hydroxyl group, imidazole group, phenolic group, thiol group, threonine group, indole group, etc. This method can be further classified into diazo, peptide and alkylation methods according to the mode of linkage. Covalent binding may alter the conformational structure and active center of the enzyme, resulting in major loss of activity and/or changes of the substrate. However, the binding force between enzyme and carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength. Higher activities result from prevention of inactivation reactions

with amino acid residues of the active sites. Covalent binding can be brought about by the following:

| | |
|---|---|
| Diazotization | Support--N=N--Enzyme |
| Amide bond formation | Support--CO-NH--Enzyme |
| Alkylation and Arylation | Support--$CH_2$-NH-Enzyme |
| | Support--$CH_2$-S--Enzyme |
| Schiff's base formation | Support--CH=N--Enzyme |
| Amidation reaction | Support--CNH-NH--Enzyme |
| Thiol-Disulfide interchange | Support--S-S--Enzyme |

UGI reaction, Mercury-Enzyme interchange, Gamma-Irradiation induced coupling, Carrier binding with bifunctional reagents, etc.

### 1.1.2. CROSS-LINKING

Immobilization of enzymes has been achieved by intermolecular cross-linking of the protein, either to other protein molecules or to functional groups on an insoluble support matrix.

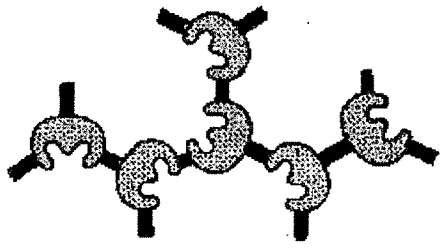

**Figure 1.2:** Schematic diagram of cross-linking method.

Cross-linking an enzyme to itself is both expensive and insufficient, as some of the protein material will inevitably be acting mainly as a support. This will result in relatively low enzymatic activity. Generally, cross-linking is best used in conjunction with one of the other methods. It is used mostly as a means of stabilizing adsorbed enzymes. Since the enzyme is covalently linked to the support matrix, very little desorption is likely using this method. The most common reagent used for cross-linking is glutaraldehyde (other

reagents used are: bisdiazobenzidine, *N,N'*-ethylene bismaleimide, hexamethylene diisocyanate, *N,N'*-polymethylene bisiodoacetamide). Cross-linking reactions are carried out under relatively severe conditions. These harsh conditions can change the conformation of active center of the enzyme; and so may lead to significant loss of activity.

### 1.1.3. ENTRAPMENT OF ENZYMES

The entrapment method of immobilization is based on the localization of an enzyme within the lattice of a polymer matrix or membrane. It is done in such a way as to retain protein while allowing penetration of substrate. It can be classified into lattice and micro capsule types.

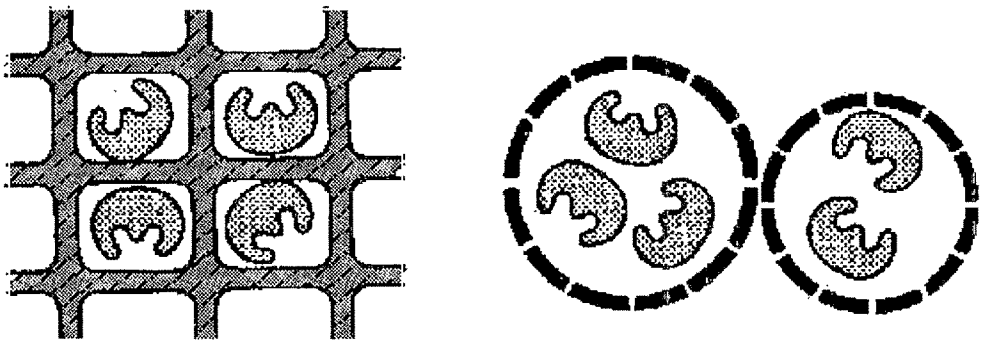


**Figure 1.3:** Lattice type and microcapsule type entrapment.

This method differs from the covalent binding and cross linking in that the enzyme itself does not bind to the gel matrix or membrane. This results in a wide applicability. The conditions used in the chemical polymerization reaction are relatively severe and result in the loss of enzyme activity. Therefore, careful selection of the most suitable conditions for the immobilization of various enzymes is required.

**Lattice-Type** entrapment involves entrapping enzymes within the interstitial spaces of a cross-linked water-insoluble polymer. Some synthetic polymers such as polyarylamide, polyvinylalcohol, and natural polymer (starch), etc have been used to immobilize enzymes using this technique.

**Microcapsule-Type** entrapping involves enclosing the enzymes within semi permeable polymer membranes. The preparation of enzyme micro capsules requires extremely well-controlled conditions and the procedures for micro capsulation of enzymes can be classified as: (i) *Interfacial Polymerization Method.* An aqueous mixture of the enzyme and hydrophilic monomer are emulsified in a water-immiscible organic solvent. Then the same hydrophilic monomer is added to the organic solvent by stirring. Polymerization of the monomers then occurs at the interface between the aqueous and organic solvent phases in the emulsion. The result is that the enzyme in the aqueous phase is enclosed in a membrane of polymer. (ii) *Liquid Drying.* In this process, a polymer is dissolved in a water-immiscible organic solvent which has a boiling point lower than that of water. An aqueous solution of enzyme is dispersed in the organic phase to form a first emulsion of water-in-oil type. The first emulsion containing aqueous micro droplets is then dispersed in an aqueous phase containing protective colloidal substances such as gelatin, and surfactants, and a secondary emulsion is prepared. The organic solvent in then removed by warming in vacuum. A polymer membrane is thus produced to give enzyme micro capsules. (iii) *Phase Separation.* This method involves purification of polymer by dissolving in an organic solvent and re-precipitating it.

The solid supports used for enzyme immobilization can be inorganic or organic . Some organic supports include: polysaccharides, proteins, carbon, polystyrenes, polyacrylates, maleic anhydride based copolymers, polypeptides, vinyl and allyl polymers, and polyamides.

# 1.2. Neural Network

NN has been successfully applied to many pharmaceutical areas in recent years (Takayama et al., 2003; Yamamura, 2003) e.g.: QSAR analysis and drug modeling (Winkler and Burden, 2004), pharmacokinetic (Chen et al., 1999; Yamamura, 2003)– pharmacodynamic studies (Veng-Pedersen and Modi, 1993; Gobburu and Shelver, 1995; Gobburu and Chen, 1996), optimization and pharmaceutical formulation development (Takayama et al., 2003), powder flow (Kachrimanis et al., 2003), compound determination using HPLC (Agatonovic-Kustrin et al., 1998), analysis of NMR spectra (Dow et al., 2004), prediction of drug release profile (Lim et al., 2003), prediction of physicochemical properties (Taskinen and Yliruusi, 2003), prediction of octanol-water partition coefficient (Molnar et al., 2004), prediction of solubility (Jouyban et al., 2004), etc. Hence was used in the present study to predict the response surface.

Neural network (NN) models might generalize better than regression models since regression analyses are dependent on predetermined statistical significance levels (i.e. less significant terms are not included in the model). With the NN method all data are used potentially making the models more accurate. Hence NN was selected for modeling and evaluating tool in this paper.

NN operation is based upon the simulation of biological neural process abilities in the human brain. NN are very useful in modeling of systems where independent and dependent variable relationships are not well known. They are characterized by architecture, transfer function and learning paradigm.(Hussain et al., 1991; Erb, 1993) NN consists of a number of processing elements (neurons) which are interconnected forming input and output layers and one or more hidden layers (Figure 1). The use of at least one

hidden layer enables the NNs to describe nonlinear systems. One layer is usually sufficient to provide adequate prediction even if continuous variables are adopted as the units in the output layer and also there is a little evidence to suggest that a larger number of hidden layers improves performance. Processing elements on the input layer receive input signals, process them and send them to the output layer through hidden layers by the network connections (synapses). Each connection is characterized by a synaptic strength (weight). Learning of NN with known independent and dependent variables based on an experimental design results in a condition for using that network for modeling. It begins with a random set of synaptic weights and proceeds in epoch (iterations). During each epoch connection weights are adapted via back propagation to minimize so-called 'error $e_i(n)$' which is the difference between the momentary network signal at neuron $i$ at iteration $n$, $y_i(n)$, and the aimed signal based on experimental results $d_i(n)$.

**Eq. 1.1** $$e_i(n) = d_i(n) - y_i(n)$$

In each hidden layer and output layer the processing unit sums its input from the previous layer and then applies the non-linear sigmoidal function to compute its output to the next layer according to equations:

**Eq. 1.2** $$y_j = \sum w_{ij} x_i$$

**Eq. 1.3** $$f(y_j) = \frac{1}{1 + e^{(-\sigma y_j)}}$$

where $w_{ij}$ is the weight of the connection between neuron $j$ in the current layer to neuron $i$ in the previous layer, $x_i$ is the output value from the previous layer, $f(y_j)$ is conducted to the next layer as an output value, and $\sigma$ is a parameter relating to the shape of the sigmoidal function. Nonlinearity of the sigmoidal function is strengthened with an increase in $\sigma$.

The three most common criterions to stop training are: to cap the number of iterations, to threshold the output mean square error, or to use cross validation. If a network is left to train for too long, it will overtrain and will lose the ability to generalize. Cross validation is more powerful of the three since it stops the training at the point of best generalization. When the performance starts to degrade in the validation set, training is stopped and connection weights become the memory units. Then trained NN can be used for output prediction on the basis of new input values.

The multilayer perceptron (MLP) is one of the most widely implemented neural network topologies and is important in the study of nonlinear dynamics. MLPs are normally trained with the backpropagation algorithm. Two important characteristics of the multilayer perceptron are: its nonlinear neurons which have a nonlinearity that must be smooth (the logistic (sigmoidal) function is the most widely used); and their massive interconnectivity (i.e. any element of a given layer feeds all the elements of the next layer).

In present study, MLP with a training rule of momentum learning was applied which uses a memory term (the past increment to the weight) to speed up and stabilize convergence. The equation to update the weights ($w_{ij}$) can be represented as:

**Eq. 1.4** $$w_{ij}(n+1) = w_{ij}(n) + \eta\delta_i(n)x_j(n) + \alpha(w_{ij}(n) - w_{ij}(n-1))$$

The local error $\delta_i(n)$ can be directly computed from $e_i(n)$ at the output neuron or can be computed as a weighted sum of errors at the internal neurons. The constant $\eta$ is called the step size and $\alpha$ is the momentum. Normally $\alpha$ should be set between 0.1 and 0.9.

NN has been successfully applied to many pharmaceutical areas in recent years e.g.: quantitative structure activity relationship analysis, pharmacokinetic–pharmacodynamic studies, pharmaceutical formulation development (Hussain et al., 1991), optimization of manufacturing processes, in vitro–in vivo correlations, etc.

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