REACTIVE HYDROGELS EXHIBITING CATALYTIC ACTIVITY

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by

ROHINI P. PATWARDHAN
M.Sc

POLYMER SCIENCE AND ENGINEERING GROUP
CHEMICAL ENGINEERING DIVISION
NATIONAL CHEMICAL LABORATORY
PUNE 411 008 (INDIA)

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Dedicated to my father

CERTIFICATE

Certified that the work incorporated in the thesis entitled "**Reactive Hydrogels Exhibiting Catalytic Activity**" submitted by Ms. R.P. Patwardhan, was carried out under my supervision. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

National Chemical Laboratory
Pune 411 008

Dr. R.A. Mashelkar
(Research Guide)

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R.P. Patwardhan

(R.P. Patwardhan)

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ABSTRACT

A polymer gel is a crosslinked polymer network swollen in a liquid medium. Gels are found everywhere in nature. In human bodies the cornea, vitreous and connective tissues are gels. They have a wide range of applications.

Hydrogels are a type of gel in which the polymer network is hydrophilic and the liquid component is aqueous. An important class of hydrogels are those known as stimuli-responsive gels. These have potential as sensors, switches, membranes for separations, and are widely employed in the biomedical field. An increasing interest has evoked in developing gels as active materials. By introducing functional groups and by choosing an appropriate polymerization technique, reactive hydrogels can be synthesized.

The thesis describes the studies undertaken in designing catalytic hydrogels for the hydrolysis of esters and amides. These could find applications in controlled release drug delivery systems. Catalytic hydrogels that mimic the catalytic activity of enzymes were investigated. These mimics could replace enzymes in industrial applications.

The work is presented in seven chapters and a brief outline of the studies carried out is as follows :

CHAPTER I : Literature survey

This chapter describes the synthesis, properties and applications of hydrogels in different areas. The literature on the efforts to synthesize polymeric catalysts using different techniques has been reviewed.

CHAPTER II : Objectives and scope of work

The objectives in undertaking the investigation have been summarized.

CHAPTER III : Catalytic Hydrogels as enzyme mimics : Anchimeric effects

This chapter describes the synthesis of catalytic hydrogels by the polymerization of a charge transfer complex between the substrate and the catalyst. The substrate and the catalyst were present on the same chain next to each other. This led to enhanced rate of hydrolysis of the ester by the anchimeric effect of the catalyst in the catalytic hydrogels , compared to the hydrolysis of the ester from the conventional hydrogel.

Chapter IV : Catalytic Hydrogels as enzyme mimics : Molecular imprinting effects

This chapter deals with the synthesis of catalytic hydrogels based on template polymerization and molecular imprinting. The ester and amide substrates were brought in vicinity either by coordination with a metal ion or the substrate was polymerized in preformed cavities prepared by molecular imprinting. These catalytic hydrogels also exhibited enhanced rate of hydrolysis of the ester and amide substrates, compared to the rate in the conventional polymers. The catalytic activity was found to be pH sensitive. These hydrogels could therefore be used for oral controlled drug delivery systems.

CHAPTER V : Catalytic hydrogels exhibiting α-chymotrypsin like activity

This chapter deals with the synthesis of hydrogels that mimic the catalytic activity of α-chymotrypsin. A model mimicing the active site of α-chymotrypsin was prepared using monomers bearing the functional groups present in the active site of the enzyme. The polymer mimic exhibited hydrolytic activity similar to that of the enzyme for phenylalanine ester and amide substrates. Characteristic features of enzyme like activity could also be demonstrated. The approach employed can be used to synthesize 'tailor-made' catalysts for industrial applications.

CHAPTER VI : Summary & conclusions

This chapter summarizes the important findings of the investigations and the conclusions derived.

CHAPTER VII : Suggestions for future work

This chapter describes the possibilities for extending the work further. They include designing pH sensitive, chemically linked controlled release drug delivery systems and synthesizing polymer mimics of other hydrolytic enzymes.

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CHAPTER I

Literature survey

1.0.0 Introduction

A polymer gel is a crosslinked polymer network swollen in a liquid medium. The polymer network of a gel can be formed in two ways 1) by polymerizing bifunctional units with polyfunctional units 2) by crosslinking polymers formed from bifunctional monomers. Gels are characterized by their equilibrium, dynamic and kinetic properties. These properties depend on the gel state represented by osmotic pressure, temperature, solvent composition and degree of swelling.

Gels are found in human bodies. The cornea, vitreous and connective tissues are gels. The surface of the internal tracts such as the stomach and lung are covered with gels. Naturally occurring polymers such as gelatin, sepharose, carragenan, cellulose and albumin are also gels. Since the mechanical strength of polymer gels can match that of living muscle, they are good candidates for biomimetic materials. They are widely employed in the biomedical fields for drug delivery, as water absorbents in disposable diapers, for agricultural purposes as retainers of water and solutes. They are important intermediates in the manufacture of polymers such as rubber, plastics, glues, films and membrane.

The gels are no more restricted to a passive role in these applications. An increasing interest has evoked in developing gels as active elements. Stimuli-responsive motion of polymer gels is attracting an intensive attention in wide areas. Stimuli responsive gels find applications in various fields like sensors, switches, membranes for separations, adsorbents, transducers, dehydrants and matrices for drug delivery systems, etc.

1.1.0 Hydrogels

Hydrogels are coherent systems rich in water. Essentially hydrogels are a type of gel in which the polymer network is hydrophilic and the liquid component is aqueous. They are defined as infinite three-dimensional polymeric networks containing a considerable amount of water. Hydrogels swell to equilibrium in water but preserve their shape.

Their general properties include :- i) softness ii) permeability to metabolites iii) resemblance to human tissues iv) hydrophilicity v) biocompatibility.

There are two kinds of hydrogels a) naturally occurring and b) synthetic. Naturally occurring hydrogels are polysaccharides, carragenan, gum arabic, agarose etc. They are mainly used in pulp and paper production, artificial silk, cellulosic membranes. The limitations of naturally occurring hydrogels are their poor mechanical properties, low temperature tolerance and poor resistance to microbial attack.

Synthetic hydrogels on the other hand have better mechanical properties, good temperature tolerance and resistance to microbial attack. They are therefore used as prosthetic materials, soft lenses and as matrices for controlled drug release (Andrade 1976).

1.2.0 Classification of hydrogels

Synthetic hydrogels can be classified on the basis of their monomer composition and structure.

1. Hydrogels based on methacrylic and acrylic esters
2. Hydrogels based on hydroxyalkyl methacrylates or acrylates
3. Hydrogels based on higher hydroxyalkyl esters of methacrylic acid.
4. Hydrogels based on acrylamide.
5. Hydrogels based on substituted and unsubstituted amides.
6. Hydrogels based on polyelectrolytes
7. Hydrogels based on poly vinyl alcohol.

Monomers used in hydrogels are listed in Table 1.1.

Table 1.1

Monomers used in the synthesis of hydrogels

Neutral	**Acidic or anionic**
Hydroxyalkyl methacrylates	Acrylic acid derivatives
2,4 pentadiene-1-ol	Crotonic acid
Acrylamide derivatives	Sodium styrene sulfonate
N-vinyl pyrrolidinone	
Hydrophobic acrylics	
	Basic or cationic
	Aminoethyl methacrylate
	Vinyl pyridine
	Crosslinkers
	Ethylene glycol dimethacrylate
	Methylene bisacrylamide

1.3.0 Structure and physical properties of hydrogels

The fundamental building block of a hydrogel is the water-soluble monomer. A wide range of monomers can be used, ranging from hydrophilic vinyl monomers to sugars and amino acids. The latter are obtained from natural resources.

1.3.1 A) Thermodynamics and swelling behaviour

In a dry state hydrogels (xerogels) are hard with properties ranging from leathery to glassy. In aqueous medium the polymer network imbibes water and the gel swells to equilibrium which is defined by the point at which the swelling pressure is balanced by the retractive force of the network. The swelling pressure is the result of the difference in water activity inside and outside the polymer and is analogous to osmotic pressure. The equilibrium water content depends on the hydrophilicity of the polymer and the degree of crosslinking and can range up to well over 90%.

1.3.2 B) Surface properties and biocompatibility

Biocompatibility implies the ability of the body to tolerate the material without unacceptable histological consequences for an extended period of time.

A hydrogel presents a diffuse surface with a more or less gradual transition between the bulk gel and the surrounding bulk water. An important feature of the hydrogel surface is the extremely high mobility of the polymer chain. This chain mobility allows the surface to adapt to its environment. Contact angle measurements showed that the polymer surface appears hydrophobic when measured in air but hydrophilic when measured in water (Holly et al 1975).

Protein adsorption measurements showed that hydrogels tend to adsorb less protein than the mostly hydrophobic materials. In absence of any strong binding sites the high chain mobility may actually reduce the chance of forming a multipoint attachment.

1.3.3 C) Water in hydrogels

The state of water in hydrogels is a subject of some controversy. DSC, NMR and permeability measurements showed only two kinds of water in DSC experiments and an existence of an intermediate state of water in the gel in their permeability measurements. Haldankar et al (1989) and Ohno et al (1983) studied poly (acrylic acid) by DSC. They explained their results in terms of a three-state model "nonfreezing", freezing with "constant melt temperature" and freezing with a "melt temperature dependent on water content".

In contrast, Roorda et al (1988) studied PHEMA gels by DTA and adiabatic calorimetry and concluded that their results were not consistent with discrete thermodynamic classes of water but the water molecules were continuously distributed over all possible orientation and interacted with the polymer.

1.4.0 Some important hydrogels

A) Poly (2-hydroxyethyl methacrylate)

B) Poly (N-vinyl pyrrolidinone)

C) Poly (vinyl alcohol)

D) Poly (ethylene oxide)

E) Cellulose

F) Agarose and carragenan

G) Polysaccharides

1.5.0 Stimuli-responsive hydrogels

Another important class of hydrogels are those known as the stimuli-responsive gels.

There are many possible environmental changes which can stimulate interesting responses in water soluble polymers and their crosslinked hydrogels. The motion of the polymer networks and the diffusion of ions takes place easily by an external medium (stimulus). These can be brought about by introducing certain functional groups in the polymer network.

By supplying thermal, chemical or electrical energy, large volume or shape changes may be induced in the swollen gels.

The various environmental stimuli and the different responses of the polymers are listed in Table 1.2.

1.5.1 Thermosensitive hydrogels

Some water-soluble polymers or copolymers exhibit a phenomenon known as lower critical solution temperature (LCST). When dissolved in aqueous solution such polymers will precipitate as the temperature is raised to and above the LCST. Hydrogels composed of an LCST polymer will shrink significantly over a relatively narrow temperature range as the temperature is raised to the LCST and above. These phenomena reverse when the gel is cooled below the LCST where the gel returns to its swollen, soft and transparent state. This behaviour is attributed to the attractive and repulsive forces acting on the polymer network and the solvent polymer and polymer-polymer interactions.

Thermally reversible hydrogels find diverse applications. They have been used in the selective removal and delivery of species in aqueous solution. Specific binding pair ligands, such as antibodies, antigens and haptens may be immobilized on the polymer backbone used to prepare the gels. Substances which specifically bind to these ligands can be successfully removed from the surrounding medium (Hoffman et al 1986, Monji et al 1987).

Table 1.2

Stimuli responsive hydrogels

Stimulus	Response
pH	Chemical / Biochemical
Temperature	Phase separation
Chemical or biochemical agents, solvents, salts	Shape (shrinks or swells)
Electric field	Surface
Electro-magnetic radiation	Permeability
Mechanical stress	Hardening or softening

A thermosensitive hydrogel based on N-isopropyl acrylamide (NIPAAm), butyl methacrylate (BMA) and ethylene dimethacrylate (EDMA) was studied for a thermal on-off switch for a pulsatile drug release system. The pulsatile release of the drug between 20°C and 30°C correlated with the different swelling rates observed for the two temperatures (Bae et al 1987). Poly(vinyl methyl ether) gels were investigated for dewatering biological sludge (Huang et al 1989).

When an enzyme is immobilized within a gel which exhibits reversible shrinking and swelling as the temperature is raised and lowered through the LCST of the gel, the enzyme activity may be switched off and on as the substrate diffusion rate is regulated by the gel pore size. In addition to enzymes, a variety of catalysts, cocatalysts or reactants may be immobilized within the LCST hydrogels. Dong et al (1987) immobilized the enzyme asparaginase within crosslinked copolymers of N-isopropyl acrylamide and acrylamide. When the hydrogels were warmed above their LCST's the enzyme activity was significantly reduced as the substrate diffusion was hindered. This phenomena was found to be reversible.

1.5.2 pH-sensitive hydrogels

pH-sensitive hydrogels usually contain pendent acidic or basic groups such as carboxylic acids and primary amines or strong acid and bases such as sulfonic acid and quaternary ammonium salts, which change ionization in response to changes in pH, thus changing the properties of the gel. Table 1.3 lists some of the monomers used in the preparation of pH-sensitive hydrogels. Among the first ionic hydrogels investigated were gels based on acrylic acid and methacrylic acid (Katchalsky et al 1955, Michaeli et al 1957). It was observed that the equilibrium degree of swelling of these gels responded to changes in pH.

When the pH of the solution was increased the gels showed an increase in swelling. The ionized gels often exhibit very high degrees of swelling. A high concentration of ions exists inside the gel due to the dissociation of acidic or basic groups and the diffusion of counterions into the

Table 1.3

pH-sensitive hydrogels

Type	Monomer	pH-sensitive group
Acidic	Acrylic acid Methacrylic acid	- COOH
	Sodium styrene sulfonate	- SO^-Na^+
	Sulfoxy ethyl methacrylate	- SO_3H
Basic	Aminoethyl methacrylate	- NH_2
	N,N' dimethyl aminoethyl methacrylate	- $N(CH_3)_2$
	Vinyl pyridine	- Pyridine ring
	Vinyl benzyl trimethyl ammonium chloride	- $N(CH_3)_3^+Cl^-$

gel from the surrounding medium. The high concentration of ions increases the water flow into the gel due to osmosis. Another factor contributing to increase in swelling is the interaction and repulsion of charges along the polymer chain.

The equilibrium degree of swelling of pH-sensitive hydrogels is mainly influenced by the charge of the ionic monomer, pk_a of the ionizable group, degree of ionization, concentration of the ionizable monomer in the network, pH, ionic strength and composition of the swelling solution. Also, factors such as crosslinking density and hydrophilicity / hydrophobicity of the polymer influences the degree of swelling and the pH-sensitivity.

The charge of the ionic monomer affects the pH-sensitivity of the gel. An acidic hydrogel will be ionized at high pH but unionized at low pH, thus, the equilibrium degree of swelling will increase at high pH (Kou et al 1988). A cationic / basic hydrogel has the opposite pH-dependence. Siegel et al (1988) developed a drug delivery system for oral delivery using basic poly (methyl methacrylate-co- N,N diethyl amino ethyl methacrylate). An acidic gel developed by Kou et al (1988) has the potential for drug delivery of acidic drugs to the small intestine. Presently Dong et al (1991) have developed an enteric delivery system based on pH and thermosensitive polymers. In vitro release studies were performed using a model drug, indomethacin which causes severe gastric irritation. No drug was released at pH 1.4 but more than 90% was released at pH 7.4 during 5 hours. Devices which release insulin in response to glucose levels were developed by several research groups (Ishihara et al 1984[a]). These systems were composed of a basic polyamine hydrogel containing glucose-oxidase. As the glucose concentration increased, glucose diffused into the membrane. There it was converted to gluconic acid by the action of glucose-oxidase. This resulted in lowering the pH and ionization of the basic groups increased the degree of swelling. Insulin loaded in the gel was then released. The swelling of these membranes was reversible and very sensitive to changes in pH. The release of insulin could be controlled in response to changes in glucose concentrations.

Hydrogels containing acidic comonomers and enzymatically degradable azoaromatic crosslinks were developed for the delivery of drugs to the colon (Saffran et al 1986). In the low pH-range the gels have a low degree of swelling and the drug loaded is protected against digestion by enzymes. As the pH increases down the GI-tract the degree of swelling increases due to increased degree of ionization. In the colon the gels are sufficiently swollen which makes the crosslinks accessible to enzymes. The gels degrade and the drug is released. Hydrogels based on poly (acrylic acid- co -N,N- dimethylacrylamide- co -N- tert-butyl acrylamides were developed. The response of these hydrogels to pH change was very fast and reversible. These could be used for on-off type delivery systems. pH-sensitive hydrogels have potential for use in delivery, where a target site may have a different pH than its surroundings. For oral delivery, pH sensitive hydrogels are useful because of the pH variation throughout the G-I tract. Also by combining the pH sensitive properties with enzyme degradable crosslinks it is possible to formulate hydrogels for drug delivery to the colon.

1.5.3 Electric field sensitive hydrogels

Hydrogels that respond to electric field were reported (Osada 1992). These were based on weakly crosslinked poly (2-acrylamido-2-methyl propane sulphonic acid). The anionic gel was dipped in water containing positively charged surfactant molecules. The positively charged surfactant molecules were bound to the gel surface and induced a local shrinkage. When an electric field was applied, the surfactant binding took place selectively to one side of the gel. When the field was reversed, the contraction occurred on the opposite side. By doing this repeateadly, the polymer gel could be made to bend and stretch repeatedly. This caused the gel to move in a worm-like motion. Kwon et al (1991) reported the synthesis of electrically erodible polymer hydrogel. The hydrogels were based on poly (ethyloxazoline) and poly (methacrylic acid) or poly (acrylic acid). These are known to form complexes by means of intermolecular hydrogen bonding between carboxyl and oxazoline groups. When the solid polymer complex was exposed to small electrical currents, it disintegrated into two water soluble polymers. These hydrogels can be

exploited for the pulsed release of drugs, especially polypeptide molecules. Tanaka et al (1982) investigated the phase transitions of a hydrolyzed poly acrylamide gel in 50% acetone-water mixtures.

1.5.4 Light-sensitive gels

By incorporating photo-sensitive molecules in the hydrogel network, they can be made photo-responsive. Photo sensitive molecule like bis (4-(dimethylamino) phenyl (4-vinyl phenyl) methyl leucocyanide was incorporated in N-isopropyl acrylamide gels. When the polymer gels were exposed to U.V. light, the photosensitive molecules were ionized creating an internal osmotic pressure (Mamada et al 1990). A visible light sensitive gel based on N-isopropyl acrylamide and the light sensitive chromophore, trisodium salt of copper chlorophyllin was reported by Suzaki et al (1990).

Similarly hydrogels containing azo linkages in the side chains exhibited a photo sensitive behaviour (Ishihara et al 1982, 1984[b]). The azobenzene moiety exhibited reversible isomerization from the trans to cis form by U.V. irradiation and from the cis to the trans form by thermal or visible light irradiation. This induced a change in the equilibrium swelling of the hydrogel which was reversible. The hydrogel shrinks when exposed to U.V. light and attains the original swelling in darkness.

In addition to these, gels sensitive to solvents, stress and specific molecules have been reported (Tanaka et al 1982, Kokufuta et al 1991).

1.6.0 Phase transition in hydrogels

Polymer gels are known to exist in two distinct phases, swollen and collapsed. Volume transition occurs between the phases, either continuously or discontinuously in response to chemical and physical stimuli. Naturally occurring polymers like gelatin, agarose and DNA also exhibit phase transition (Amiya et al 1987). Verdugo (1986) found a fascinating example of the gel phase

transition in biological system. Slug muccin is stored in the body in an extremely compact form. When secreted out of the body, it absorbs water and swells more than 1000 times. The slugs are thus able to retain water and maintain the moist environment necessary for survival. When the calcium concentration around the muccins increases, they deswell. Gel phase transition is a result of a competitive balance between a repulsive force that acts to expand the polymer network and an attractive force that acts to shrink the network. The most effective repulsive force is the electrostatic interaction between the polymer charges of the same kind and the osmotic pressure by counter ions. The attractive interaction can be Van der Waals, hydrophobic interaction, ionic and hydrogen bonding.

Each one of the interactions induces a transition between the different phases in water. Nature uses these interaction to create extremely effective and specific molecular recognition mechanisms.

1.7.0 Hydrogels as biomaterials

Hydrogels exhibit superior compatibility with blood *in vitro* and *ex vivo*. They are non-thrombogenic and used for vascular implant interfaces. Hydrogels have been extensively used in ophthalmology mainly for the manufacture of soft corneal contact lenses due to their transparency (Refojo et al 1980). Wichterle et al (1960) proposed 2-hydroxyethyl methacrylate polymers for use as surgical implants.

Hydrogels composed of poly (HEMA) doped with small amounts of methacrylic acid and a crosslinker, demonstrate large volume changes between pH 6 and pH 7. These polymers have demonstrated resistance to calcification in the urinary tract (Eckstein et al 1984).

In addition, poly (HEMA) hydrogels have been used in prostheses, occular surgery, suture coatings and for the manufacture of artificial internal organs. Swollen crosslinked poly vinyl alcohol networks were prepared by electron beam irradiation of aqueous poly vinyl alcohol solutions

at various temperatures and doses of irradiation. The membranes were used for selective transport of macromolecules and as biomaterials for synthetic articular cartilage application (Peppas et al 1977).

1.7.1 Hydrogels for controlled drug delivery applications

Hydrogels being biocompatible can be used to design controlled release drug delivery systems. The drug can be released by erosion of the hydrogel backbone, hydrolysis of the drug from the polymer backbone and by diffusion.

In swelling controlled delivery system, the active ingredient is uniformly dispersed within the polymer matrix. As the penetrant enters the matrix, it swells the polymer and allows the active ingredient to diffuse out. Davidson and Peppas (1986) have studied the release of theophylline from P(HEMA-MMA) hydrogels at a constant rate. In the ongoing research in our group, different kinds of drug delivery systems based on hydrogels have been developed (Vyavahare et al 1990, Shah et al 1991, Vadalkar et al 1993)

In drug delivery systems where the active ingredient is chemically linked to the polymer, two main approaches have been employed : 1) The active ingredient is chemically linked to the polymer via a functional group, 2) The active ingredient is chemically linked to the monomer which is then polymerized. The active ingredient could be released either by the degradation of the polymer backbone or as a result of the cleavage of the bond between the polymer and the active ingredient. Scholsky and Fitch (1986) studied the release of salicylic acid/chloramphenicol from pendent linked acrylate based polymers. Akashi et al (1986) synthesized water soluble and water dispersible hydrogel copolymers containing chemically linked 5-fluorouracil, thymine or adenine. Shah et al (1990) investigated the release kinetics of covalently linked, substituted benzoic acids from swellable hydrogels.

1.8.0 Catalytic hydrogels

Certain hydrophilic polymers, such as poly-N-alkylacrylamides show unique thermal reversibility as described earlier. When crosslinked, such polymers form hydrogels that collapse and deswell above LCST and reswell below LCST. Dong et al (1986, 1987) immobilized asparaginase, an enzyme used in leukaemia treatment, in poly (NIPAAm-Am) copolymer LCST hydrogels. The enzyme activity was shut off above the LCST, but was regained when the temperature was lowered below the LCST. The effect was found to be reversible. Similarly β-galactosidase was immobilized within thermally reversible hydrogel beads that exhibited LCST behaviour. It was observed that the average conversion, the activity, and the productivity increased by thermally cycling the enzyme within the hydrogels (Park et al 1988). These were termed as ' catalytic hydrogels'. The catalytic activity in these cases is due to the enzyme present and not due to the hydrogel backbone. Kokufuta et al (1992) reported the synthesis of a hydrogel based on poly (vinyl alcohol). The hydrogel contained entrapped concanavalin A (Con A) and the enzyme glucoamylase. The substrate for the enzyme was starch. Starch possesses a binding affinity towards Con A. It was observed that this binding affinity caused an increase in the diffusion of starch in the gel matrix which increased the activity of glucoamylase.

Belokon et al (1980, 1982) employed an entirely different approach to design hydrogels which exhibit catalytic activity. A polymeric gel in which the salicylaldehyde and lysine moieties are capable of forming an 'internal aldimine' was prepared by copolymerizing N^{α} -5- methacryloyl - aminosalicylidene -N^{ϵ}-methacryloyl -(S)- lysinato copper (II) with acrylamide and N, N' methylenebisacrylamide as a pyridoxal enzyme model. The copper ions were extracted in 0.1 M HCl. The equilibrium constants of the internal aldimine were 100 times higher than the equilibrium constants of the model reaction of Schiff base formation between 5-isobutyryl aminosalicylidene and N-isobutyryl-(S)-lysine. This effect was also observed in the non-crosslinked polymers. It was thus concluded that the rate of cyclocopolymerization of N^{α} -5- methacryloyl aminosalicylidene-N^{ϵ}-methacryloyl -(S)- lysinato) (pyridine) copper(II) was much higher than the

rate of crosslinked polymer formation. After removal of copper ions from the polymer, the lysine and salicylaldehyde moieties remained attached to the same polymer chain in the immediate vicinity of each other, thus favouring the formation of internal aldimine.

These examples indicate the possibility of designing catalytic hydrogels where the catalytic activity is due to the functional groups present in the hydrogel. In order to design such hydrogels the polymer structure has to be appropriately tailored to obtain a predefined arrangement of functional groups. Recently different techniques have been developed which enables the localization of the functional groups. The next section describes the different approaches used for the synthesis of both low molecular weight and macromolecular catalysts.

1.9.0. Molecular recognition

Molecular recognition governs the basis for most biological processes and is therefore of universal interest. In biological systems molecular recognition is achieved by collective weak intermolecular forces that act over complementary surfaces of the host and guest. The design of binding sites that mimic the specificity of biological macromolecules remains an important challenge to chemists. The interest in this area stems from two sources; (i) in developing deeper insights into the different parameters like cooperativity of functional groups, anchimeric effects and their application as specific catalysts behaving similar to natural enzymes, (ii) for isolation and purification of stereoisomers. A synthetic binding site should possess a three dimensional scaffolding that creates a binding site and provides a framework for positioning functional groups for tuning specificity or catalytic activity. Considerable efforts are being made to develop synthetic receptors for specific molecules.

These can be grouped arbitarily into two categories (a) those based on small molecule and (b) those based on macromolecules.

1.9.1. Small molecule receptors and catalysts

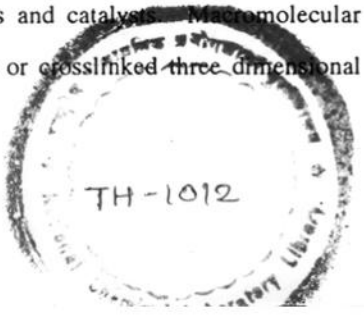
The design of small molecular receptors has been a successful area of research. These involve three dimensional 'host' structures such as cyclodextrins, crown ethers and cryptands and other supramolecular assemblies, which selectively bind the guest molecule.

These macropolycyclic structures meet the requirements for designing artificial receptors. Since they are large in size they may contain cavities and clefts of appropriate size and shape. They allow the arrangement of functional groups and binding sites. These structures are able to complex a substrate, react with it and release the products, thus regenerating the reagent for a new cycle.

Cyclodextrin based molecular receptors have been discussed in details by Bender et al (1978). Paracyclophanes having catalytic groups are inclusion catalysts. Murakami et al (1977) have demonstrated that 10-amino-[20] paracyclophane binds p-nitrophenyl carboxylates by a hydrophobic interaction and deacylates them efficiently. The unique property of the macrocyclic polyethers as complexing agents is their preference for alkali metal ions. The solubilizing power of the saturated macrocyclic polyethers permits ionic reactions to occur in aprotic media. This property has applications in catalysis, separation, recovery of salts and in analytical chemistry (Pederson 1988).

1.9.2. Macromolecular receptors and catalysts

The functional properties of biological macromolecules arise from their polymeric nature. The structures are derived from particular sequences of amino acids in polypeptide chains. The primary amino acid sequence of proteins are not always well established. Therefore attempts were made to synthesize synthetic macromolecular receptors and catalysts. Macromolecular receptors and catalysts are based either on soluble polymers or crosslinked three dimensional structures.



1.9.3. Soluble polymers as catalysts

Different types of synthetic polymer catalysts carrying the active groups and the binding groups were synthesized by random copolymerization or grafting onto a polymer backbone. The different amino acids residues which participate in the catalytic reactions of enzymes are given in figure 1.1. A number of enzyme models have been prepared by using functional groups singly or in combination (Kunitake 1976*). Substrate binding in these polymers is achieved through the secondary valence forces like hydrophobic interactions, hydrogen bonding, charge transfer interactions.

Polymers comprising imidazolyl groups and long chain alkyl or acyl chains exhibited substrate binding by hydrophobic interactions which led to catalysis (Kunitake et al 1972).

A polymer catalyst based on branched polyimino ethylene (M.W = 600) in which 10% of the primary amino groups were laurylated and approximately 15% imidazolyl methylated was reported. This polymer was found to be 75 times as effective as a specific enzyme type II sulfatase in the hydrolysis of 2-hydroxy-5-nitrophenyl sulfate at 20°C and pH 9.2 (Kiefer et al 1972).

Copolymers of dodecyl methacrylate and N-methacryloyl L-histidine or N-methacryloyl L-histidine methyl ester exhibited enantiomer selective catalysis in the solvolysis of N-protected phenyalanine p-nitrophenyl ester. (Cho et al 1982). In enzymes like acetylcholine esterase the electrostatic interaction has a definite importance. It possesses an anionic site and attracts cationically charged species.

Morawetz et al (1968) synthesized cationically charged, nucleophilic catalyst by quaternizing the copolymer of N-vinyl imidazole and 4-vinyl phenol. This catalyst selectively catalyzed the hydrolysis of the anionic ester sodium-3-nitro 4-acetoxy benzene sulfonate (NABS) and not the neutral or cationic esters p-nitrophenyl acetate and 3-acetoxy-N-trimethyl 1-anilium iodide respectively.

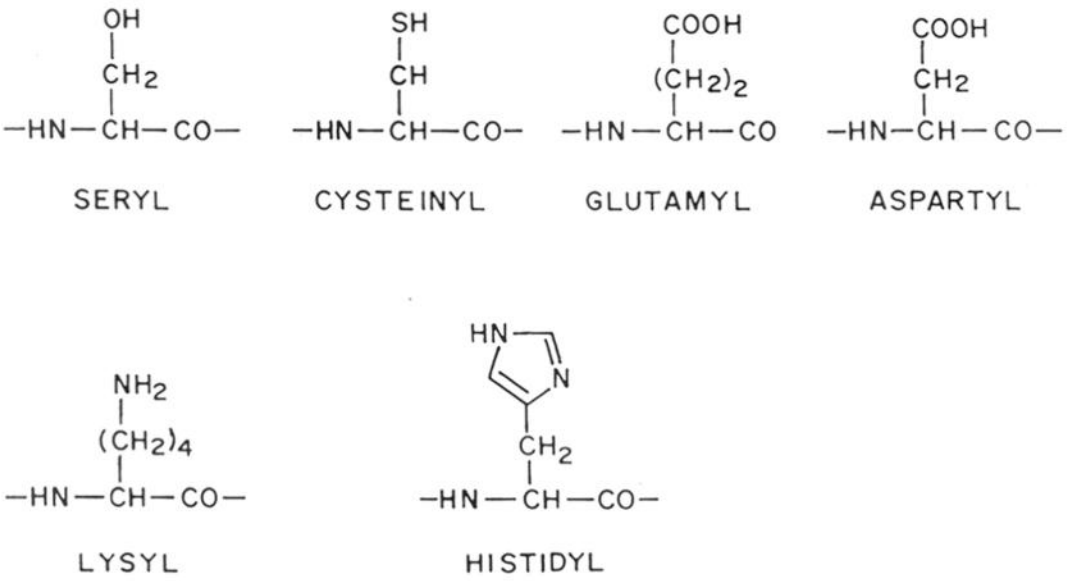


Figure 1.1 : Amino acid residues commonly involved in enzyme catalysis.

Lysozyme catalyzes the cleavage of the β-1,4 glycoside bond of mucopolysaccharide by hydrogen bonding between the substrate and the enzyme. Arai et al (1979) investigated the hydrolysis of polysaccharides by copolymers having sulfonic acid groups and alcoholic groups.

1.9.4. Charge transfer interactions

Imidazole group of the histidyl residue is responsible for the catalytic activity of many hydrolytic enzymes. A number of investigations based on the catalytic esterolysis by soluble imidazole containing vinyl polymers has been reported (Kunitake 1980). The structures of some of these polymers are shown in figure 1.2.

These polymers selectively catalyzed the hydrolysis of cationic or anionic substrates depending on the charge residing on the polymer. (Letsinger et al 1962, Overberger et al 1970). They also exhibited enzyme like or Michaelis-Menten type kinetics. The hydrophobic interactions were considered to be responsible for substrate binding (Kunitake et al 1969).

1.9.5. Multifunctional catalysis by soluble polymers

Water soluble bifunctional copolymers of N-methyl hydroxamate (MHA) and 4(5) vinyl imidazole were synthesized and the catalytic activity of these polymers on the hydrolysis of p-nitrophenyl acetate was investigated. The imidazole group was found to be less efficient than the hydroxyl group of hydroxamate. The catalytic activity was due to hydroxyl group which was more nucleophilic (Kunitake et al 1976[b]).

Soluble polymers described above suffer from certain disadvantages like :- (a) the reaction products cannot be easily separated from the reaction mixture (b) recovery and regeneration of the catalyst is difficult (c) these catalysts are not stereoselective. On the other hand heterogeneous or insoluble catalysts possess certain advantages like (a) ease of separation of the supported species

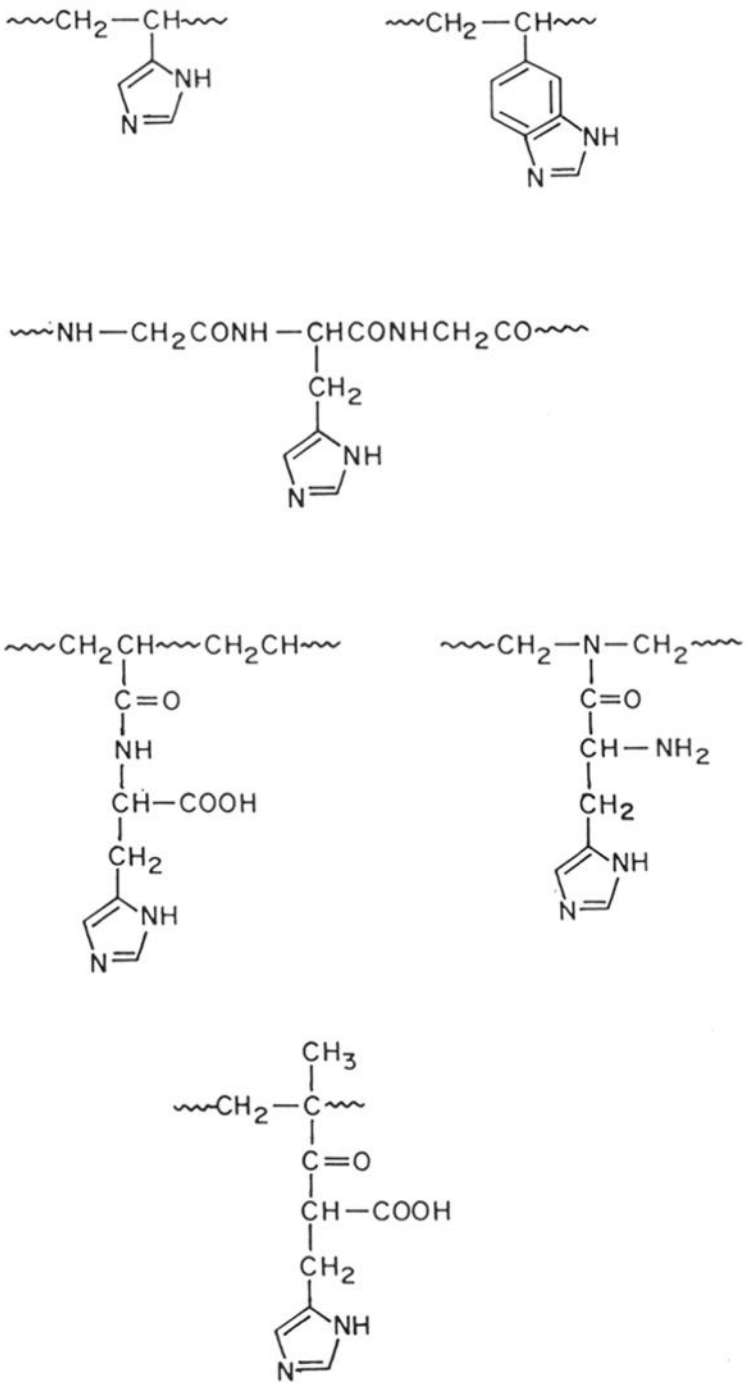


Figure 1.2 : Imidazole group containing soluble polymers.

from the reaction mixture (b) reuse of the catalyst after regeneration (c) adaptability to continuous flow processes (d) prolonged activity and selectivity of the catalyst (e) transfer of 3-dimensional information.

Various groups all over the world have been working on the development of polymeric reagents and catalysts. These have been described in details in the literature (Ford 1986, Sherrington 1988). These polymer based reagents and catalysts have been used in organic syntheses, ion-exchange resins, phase transfer catalysis and selective separation of organic and inorganic compounds. Applications involving both aqueous and organic media have been described.

1.10.0. Molecularly imprinted catalysts

Amongst the various techniques employed in the synthesis of network polymers, molecular imprinting provides a novel approach for the construction of macromolecular binding and catalytic sites. This concept was explored experimentally by Dickey (1949) and Curti (1952) who attempted to create shape selective sites in silica gel by the precipitation of sodium silicate in the presence of organic dyes. The resulting silicates exhibited a slight affinity for the dye.

The polymerization process involves a template and associated functional monomers together with a large excess of crosslinking monomer, an equal volume of inert solvent and free-radical initiator. The template was used to organize polymerizable monomers around it prior to their incorporation into the network polymer. This is described schematically in figure 1.3.

Wulff et al (1972) first demonstrated that a highly crosslinked organic polymer serves as the scaffolding for the imprinting technique. They polymerized 2,3-O-p-vinyl phenyl boronic ester of D-glyceric acid p-vinyl anilide (I) with divinyl benzene in acetonitrile. 50% D-glyceric acid was split off. It was also observed that the binding of D-form of glyceric acid was significantly better.

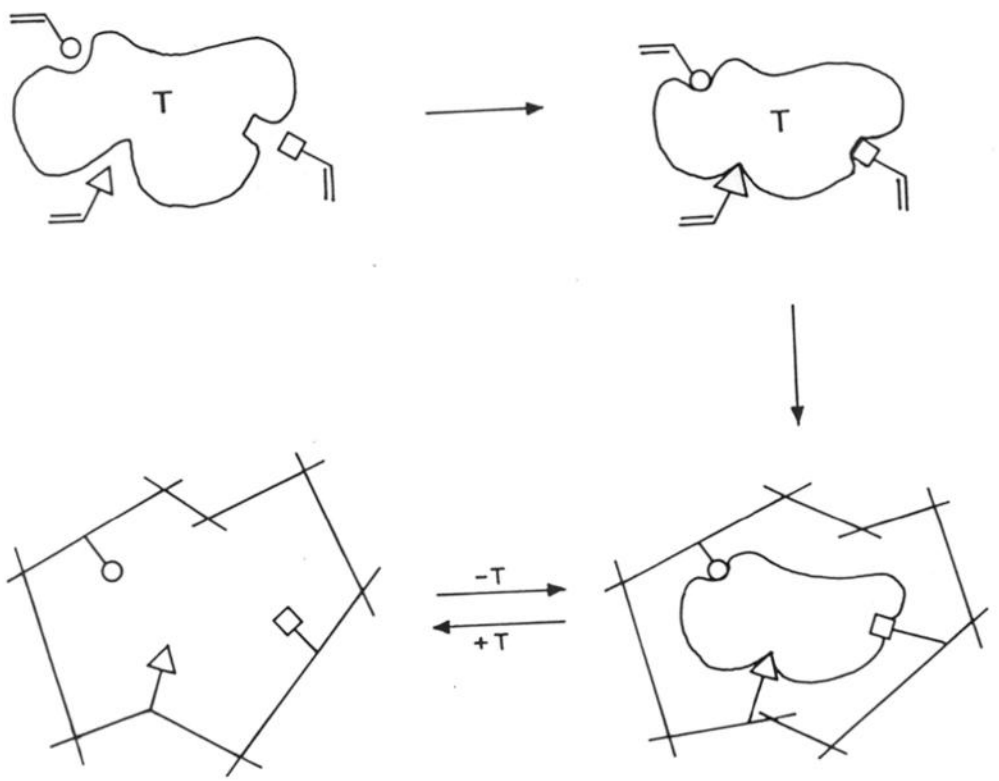


Figure 1.3 : A schematic diagram for the preparation of defined functional cavities by an imprinting approach.

The organization of polymerizable monomers by the template is achieved either by covalent bonds and/or weak intermolecular forces, including electrostatic, hydrogen bonding and hydrophobic interactions. The resulting polymers are crosslinked insoluble networks with high internal surface area (200-500 m^2/g) and broad distribution of pores, which ensure exposure to solvent and reagents.

An example illustrating this is shown in figure 1.4. The template phenyl α-D-mannopyranoside was linked with two molecules of 4-vinyl phenyl boronic acid by ester linkages to the hydroxyl groups to give (a). Boronic acid was chosen because it undergoes reversible interactions with diol groups easily. The monomer (a) was polymerized as described above with large amounts of bifunctional crosslinking agents in an inert solvent. 90% of template was split off by treatment with water or alcohol. When the polymer was treated with the racemate of the template, the D-enantiomer was preferably incorporated in the polymers. The cavities retained their shape after splitting off the template.

The performance of these imprinted polymers as the chromatographic supports in HPLC was optimized.

1.10.1 Origin of the memory effect

The selectivity of the imprinted polymers for the preferred adsorption of their templates can be explained by crosslinking and stabilizing a favourable conformation of the polymer chain around the template. Chiral templates give rise to asymmetric cavities where the resolution takes place. The shape of the cavity and arrangement of the binding groups give rise to specificity (Sarhan et al 1982[a], 1982[b], Wulff et al 1984). In order to determine the specificity of the polymer to resolve racemates, Wulff et al (1979) synthesized polymers with different ester of glyceric acid phenyl boronate. The polymers were most specific for resolution of the racemate of their own templates. Small differences in structure led to a decrease in specificity and higher differences resulted in complete disappearance of specificity (Sarhan et al 1982[a]).

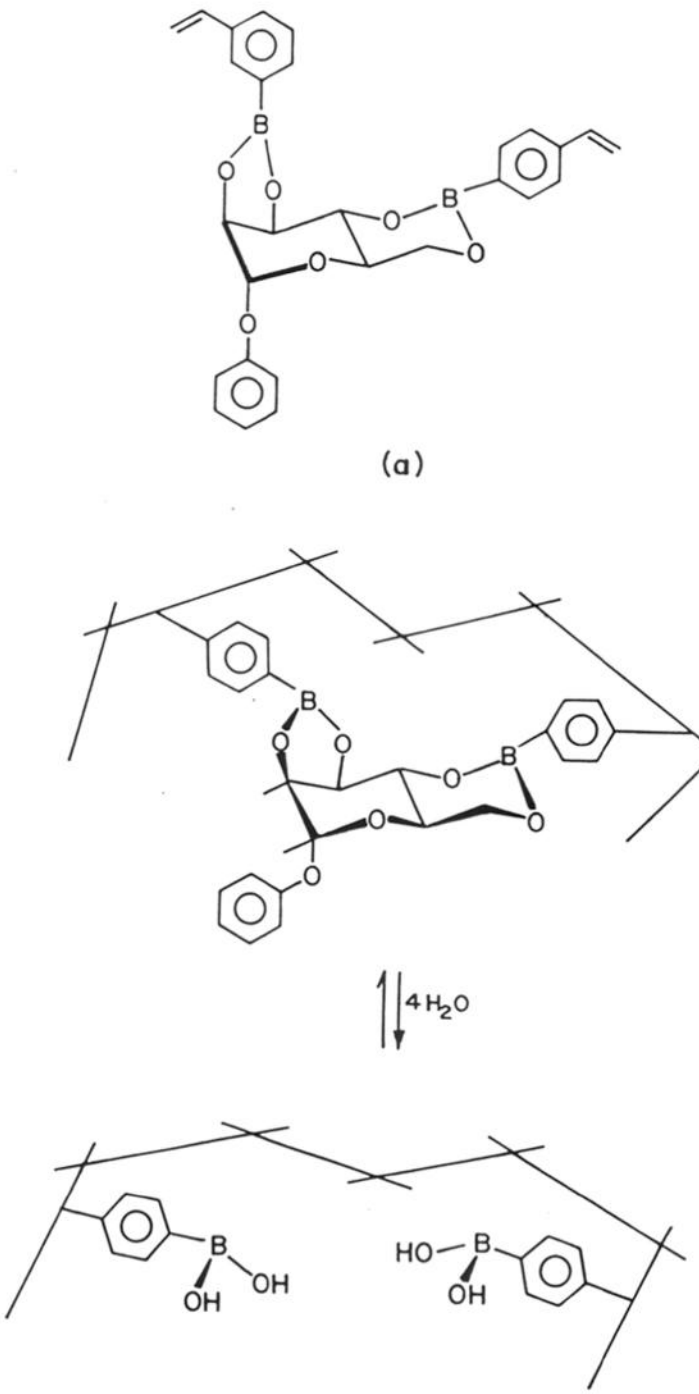


Figure 1.4 : Organization of polymerizable monomers by the template.

1.10.2 Different kinds of binding interactions

Poly (vinyl boronic acids) are commercially available and they have been used for the purification and chromatographic separation of diols (Ferrier 1978). Several template monomers have been polymerized by using just one boronic acid. They are glyceric esters, propane diol and mandelic acid (Sarhan et al 1982[a], 1982[c], Wulff et al 1980, 1982). Generally with one binding interaction, the selectivity of the polymer was moderate while two interactions gave much higher selectivity (Wulff et al 1982). But three binding interaction gave lower specificity. A polymer from D-mannitol-tris-0-1, 2, 3, 4, 5, 6 -(4-vinyl phenyl boronate) showed a selectivity of $\alpha = 1.09$ for the optical resolutions of D, L-mannitol (Wulff et al 1980).

In another case, 4-vinyl phenyl boronic acid was bound to two hydroxyl groups of L-DOPA methyl ester. In addition, 5-vinyl salicylaldehyde was linked to the amino group through an azomethine bond. On polymerization and splitting off of the template L-DOPA methyl ester, yielded a polymer that showed a separation factor $\alpha = 1.98$ for the solution of the racemate of the template. The polymer did not resolve the racemate of tyrosine methyl ester or phenylalanine methyl ester (Wulff et al 1978).

1.10.3 Influence of the structure of the binding site

The template molecule phenyl α-D-mannopyranoside was used as the template to which two molecules of boronic acid were bound. The structure of the boronic acid was varied and the influence of the flexibility and/or fixed orientation or selectivity was investigated. It was found that increased structural flexibility of the binding groups reduced the selectivity (Wulff et al 1987[a]).

1.10.4 Selectivity and stability of the imprinted polymers

When polymers prepared using a chiral template were reacted with the racemate of the template in a batch procedure under equilibrium conditions, the enantiomer which was used as the template was taken up preferentially. Distribution coefficients α were in the range 1.2 to 4.76 (Wulff et al 1987[a], 1987[b], 1987[c]).

The enantiomer selectivity was also influenced by the type and amount of crosslinking agent used during polymerization. Polymers with more than 50% crosslinking exhibited a higher selectivity. It was also observed that polymers prepared with ethylene glycol dimethacrylate retained their specificity for a longer period under high pressure and temperature conditions. In addition to the requirement of rigidity for the stability of the cavity, the polymers should at the same time possess some degree of flexibility to enable fast reversible binding of the substrates within the cavities.

Ethylene dimethacrylate crosslinked polymers showed less non-specific hydrophobic interactions with the racemate. Due to the higher flexibility of the polymer chain, fast and efficient splitting of the template was observed. Other crosslinkers like butanediol dimethacrylate and p-divinyl benzene showed a much lower specificity and stability (Wulff et al 1987[d]).

1.10.5. Applications

Polymers comprising chiral cavities obtained by polymerization in the presence of suitable templates can be used for the chromatographic separation of the racemates of the template molecules, as catalysts for reactive separations, for synthesis, metal ion sorption and affinity chromatography.

New types of macroporous polymers containing chiral cavities were prepared for the racemic resolution of free sugars using template monomers 6-O-methyl and 6-O benzyl

α-O-galactopyranose 1, 2, 3, 4 tetra-O-bis (4-vinyl phenyl boronate). After splitting of the templates these polymers were used for the racemic resolution of D, L galactose and D, L fructose (Wulff et al 1991[a]).

The release and uptake of the template α-phenyl mannoside in microcavities prepared by imprinting was studied in liquid chromatography. Baseline separations of α-phenyl mannoside was observed. The resolution was completed in 25 minutes (Wulff et al 1990). A number of chiral, polymerizable Schiff base templates were prepared to construct polymers for the asymmetric synthesis of α-amino acids. A scheme showing the various steps involved in the process can be seen in figure 1.5. Diastereomeric aldol adducts (Threo : allo, x:y = 3:1) were formed. The threo diastereomer was 35 % enantiomerically enriched. The origin of the chiral excess was in the chiral template used during the polymerization reaction (Wulff et al 1989). The synthesis of some rare sugars was accomplished using polymer bound 1,3,2-dioxaboroles (Wulff et al 1987[a]). L-ribose which is very difficult to obtain was synthesized by the reaction of 2, 3-O-cyclohexylidene -L-glyceraldehyde to give 60% L-ribose along with other sugars (See figure 1.6).

1.10.6. Exact placement of functional groups on the surfaces of rigid matrices

The selectivity observed in the type of racemic resolution discussed above is an outcome of the combination of an accurate cavity shape fitting and the precision of the arrangement of the functional groups. In order to establish whether the arrangement of the functional groups alone can give rise to selectivity, attempts were made to introduce amino groups arranged at a specific distance in crosslinked polymers with the aid of a template.

A similar procedure was adopted to locate two functional groups on the plane surface of silica (See figure 1.7). The studies suggested that by the distance selectivity alone, differences of only 0.33 nm between the two templates could be resolved (Wulff et al 1986[b], 1987).

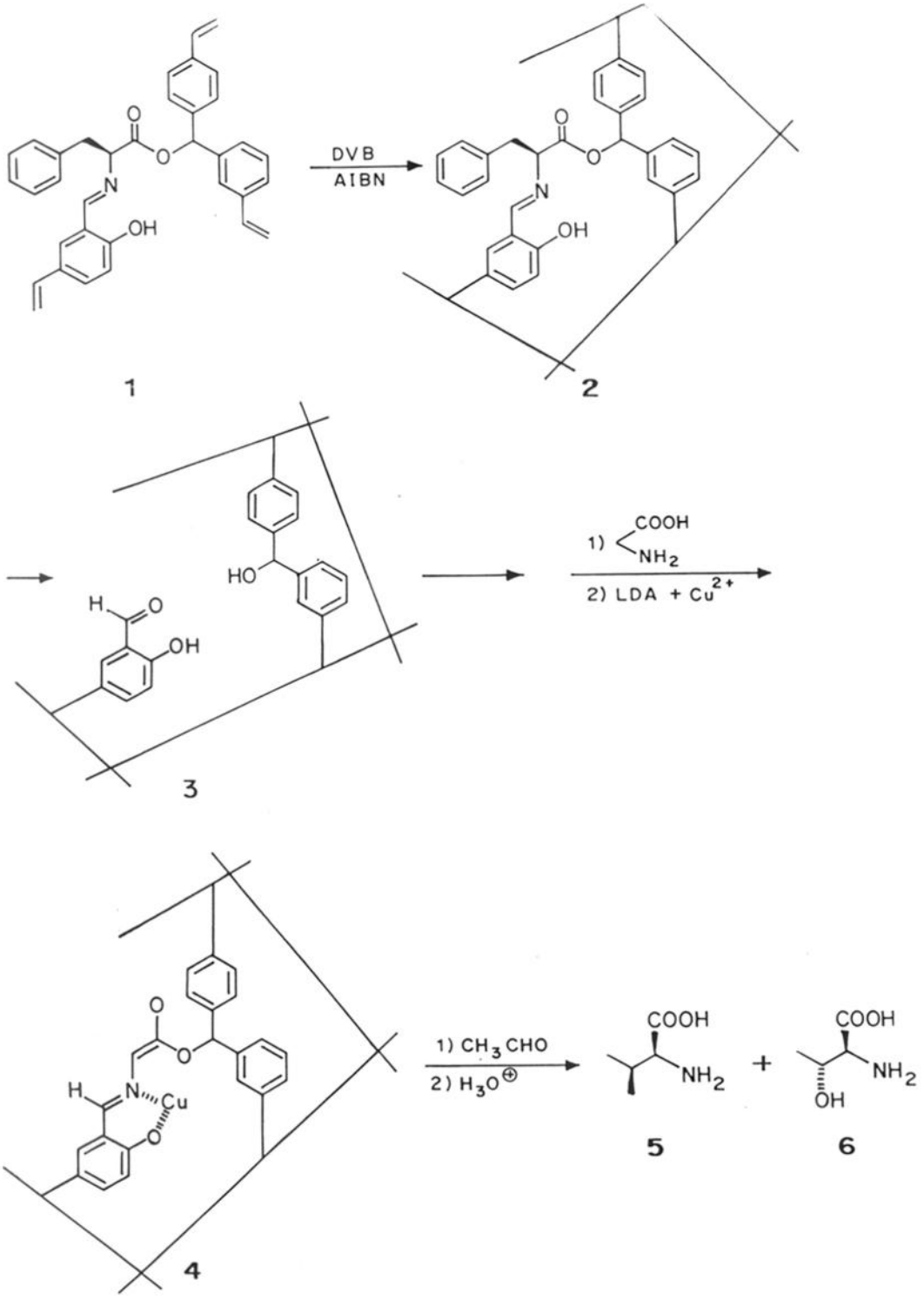


Figure 1.5 : Chiral polymers prepared by molecular imprinting for the synthesis of α-amino acids.

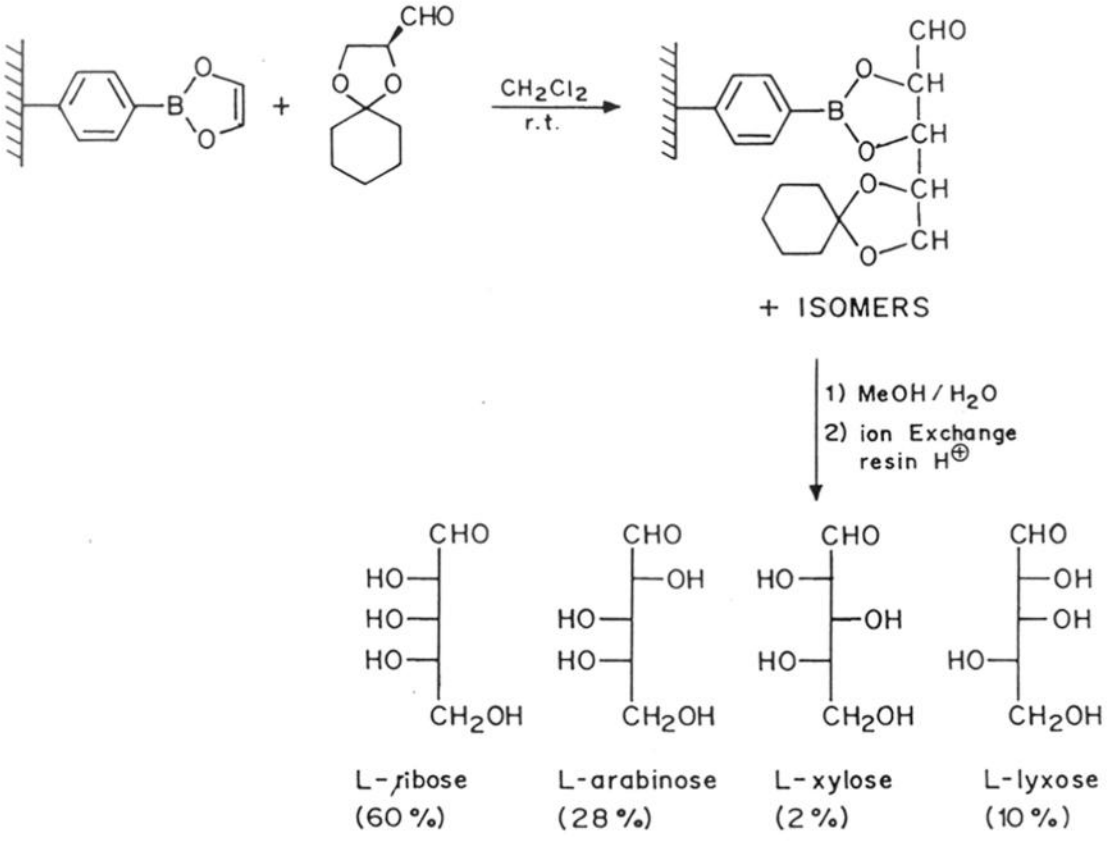
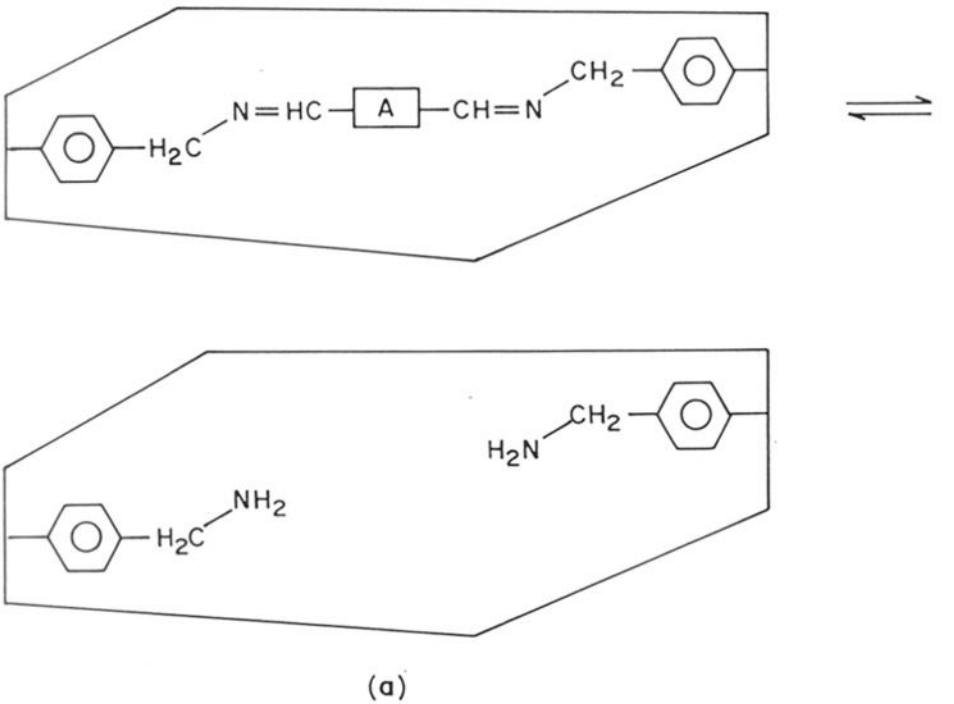


Figure 1.6 : Preparation of L-ribose via polymer bound 1,3,2-dioxaboroles.



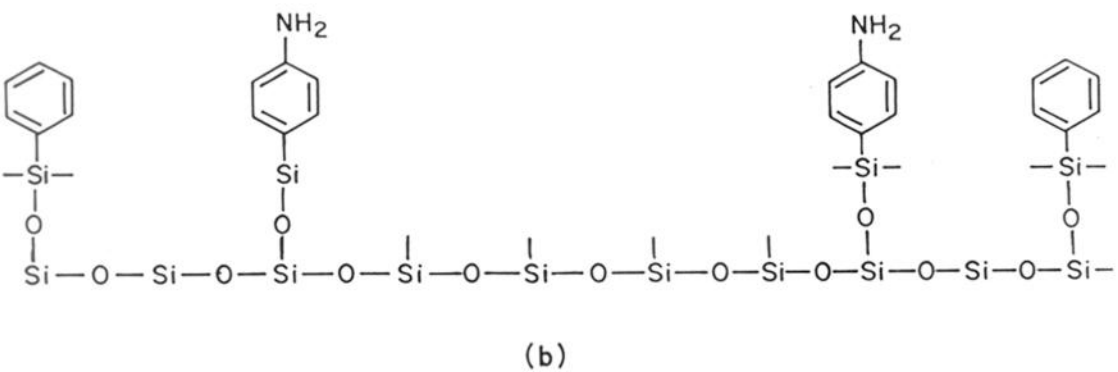


Figure 1.7 : Introduction of two amino groups at a distinct distance apart in ; (a) crosslinked polymer and (b) on a plane surface of silica.

1.10.7. Metal-ion separations

Nishide et al (1976) reported the crosslinking of a metal complex between a poly (4-vinyl pyridine) ligand (partially quaternized) and a metal ion, by adding 1,4-dibromobutane to the solution. The resins showed a preference for adsorbing the metal ions used as a template. Similarly, Gupta et al (1982) used complexing monomer 4-methyl-4-vinyl-2,2'-bipyridine with divinyl benzene in the presence of metal ions Ni^{2+}, Co^{2+} and Cu^{2+}. After removing the metal, the polymers retained some memory for the template metal. The selectivity for metal ions was not great enough for practical applications.

1.11.0. Limitations of the covalently imprinted polymers

(a) In chromatographic separations the mass transfer is slow and reactions inside the cavity show only moderate yields.

(b) Only a few suitable binding reactions are known.

(c) Due to the high selectivity of these polymers broader applicability is lost. For templates of different structures different polymers have to be synthesized.

(d) The formation and hydrolysis of the covalent bonds during chromatography is slow.

1.12.0. Non-covalent interactions

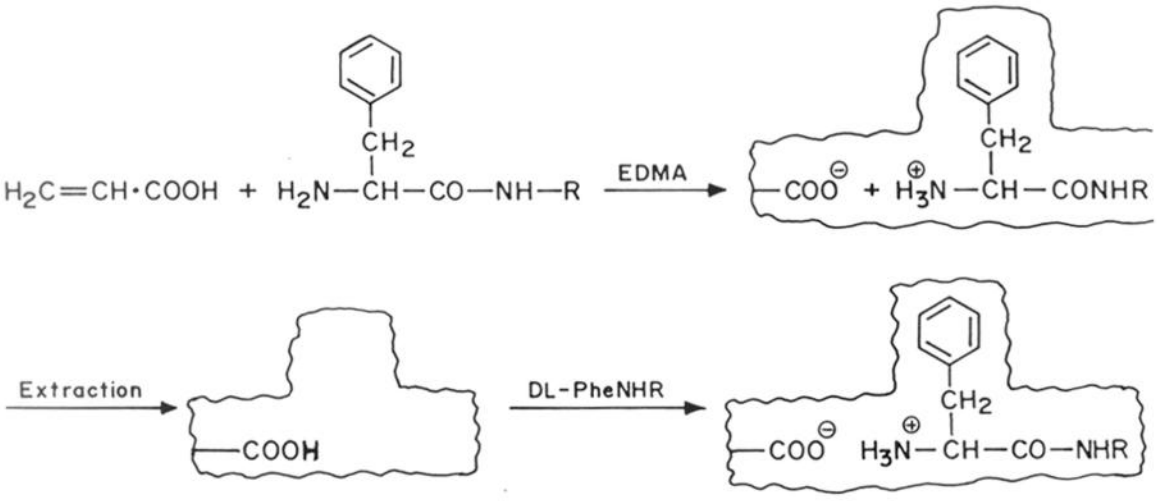
Molecularly imprinted polymers prepared by covalent interactions have certain drawbacks. This technique was extended further by using non-covalent interactions for the organization of functional monomers by the imprinting method. This simplified the preparation of many imprinted polymers. These system are more versatile because many more monomer-print molecule interactions can be exploited compared to the reversible covalent binding approach. The interactions may be ionic, hydrogen bonding, hydrophobic, change transfer etc. The print molecule may be removed by simple extraction.

Arshady et al (1981) prepared copolymers of methyl methacrylate, N,N' methylenediacrylamide in presence of dyes like Rhodanile Blue and Safframine O. The dye was then extracted from the polymer. These polymers were found to be selective for the dye present during polymerization. This technique was later used in chromatography by imprinting a thin shell of acrylic polymers on porous silica (Norrlow et al 1984).

In the initial stages, amino acid derivatized on the carboxyl group (esters of anilides) were used as the print molecules to separate amino acid derivatives on basis of both substrate and enantio selectivity.

Sellergren et al (1988) prepared polymers using methacrylic acid, ethylene glycol dimethacrylate and print molecules like D and L-phenyl alanine anilide, p-amino phenyl alanine ethyl ester, phenyl alanine ethyl amide and phenyl alanine ethyl ester. The print molecules were extracted in acetonitrile. Chromatographic investigation were performed using the DL-racemates. In the case of L-phenyl alanine anilide almost base line separation of the enantiomers on a L-phenyl alanine anilide selective polymer was obtained with $\alpha = 3.5$. This polymer also exhibited high capacity for resolution. The selectivities could be explained on the basis of hydrogen bonding between carboxylic acid and the amide functional groups. (See figure 1.8). Substrates other than the respective print molecule were poorly resolved. In order to investigate these interactions, a chromatographic ^1H NMR titration of the print molecule phenyl alanine anilide with carboxylic acid was carried out. It was demonstrated that complexes between L-phenyl alanine anilide and a maximum of three methacrylic monomers existed in solution prior to polymerization.

Three main areas of application of non-covalently imprinted polymers are :- (1) As tailor-made separation materials (2) Use in organic synthesis as catalytically active polymers or enzyme mimics (3) As sensors.



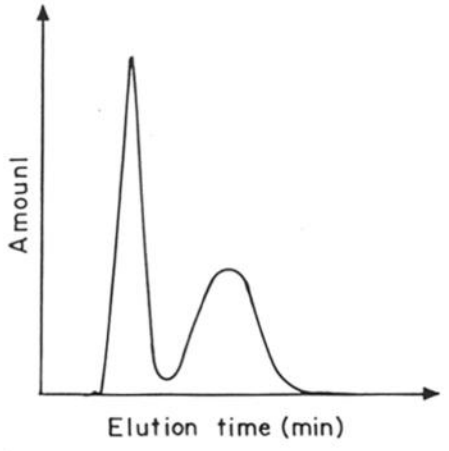


Figure 1.8 : (a) Molecular imprinting using non-covalent interactions between L-phenylalanine anilide and acrylic acid.
(b) The racemic resolution of DL-phenylalanine anilide.

1.12.1. Chiral separations

The resolution of synthetic pharmaceuticals is a vexing problem for the drug industry. The enantiomer dependent differences in biological activities are well documented through the horrific consequences of phthallidomide and penicillamine. Though there is a dramatic improvement made in asymmetric synthetic methodologies, the production of chiral compounds in research and industry still relies heavily on chromatographic methods for analysis and preparative separations. Traditional commercial chiral stationary phases (CSP's) utilize immobilized chiral compounds, which engage in diastereomeric complex formation with the analyte to obtain separation. An alternative approach to CSP's is the use of molecular imprinted polymers that are selective for a predetermined ligand.

The enantiomeric resolution of a range of β-adrenergic blocking agents, timolol, propanolol and atenolol was reported (Fischer et al 1991). When (-)-S-timolol was used as the imprinting molecule the imprinted polymer gave baseline separation of racemic timolol (R_s = 2.0). The structurally related derivatives atenolol and propanolol were not separated into their enantiomers nor were they retained on the column. Similarly the resolution of nonsteroidal anti-inflammatory agent naproxen was reported. The separation of naproxen from the structurally related ibuprofen and ketoprofen was also studied (Kempe et al 1994).

In addition to the drugs, other biologically important molecules have been resolved using molecularly imprinted polymers. They include nucleotide bases (Shea et al 1993), sugars (Mayes et al 1994), steroids (Rystrom et al 1993), peptides (Ramstrom et al 1994). Not only do these molecular imprinted polymers show regio and stereoselectivities but they have been tested for their suitability as antibody mimics. Anti theophylline MIP's were used for the determination of theophylline concentrations in patient serum samples. The cross reactivity profiles of these MIP's was practically identical to those reported for monoclonal antibodies against theophylline (Vlatakis et al 1993).

Though there are large number of examples in resolution of different classes of molecules, application to the field of protein separation still remains a challenge.

There are a few reports on protein imprints by surface imprinting on silica (Glad et al 1985). After allowing the protein to interact with the functional monomers, the protein is 'surface imprinted' to a carrier. Subsequent protein recognition is specific for the print protein owing to the complementary binding sites (See figure 1.9).

1.12.2. Protein separation by metal-ion coordination

In an entirely different approach to producing protein selective recognition systems, use of small molecules to control the geometry of protein MIP recognition sites, such as bisimidazoles has been reported. It is known that interactions between biological molecules and metal ions can be highly specific and are formed and broken under mild conditions. Immobilized metal-affinity chromatography is a known technique used extensively for protein purification. Protein discrimination is achieved based on the nature and multiplicity of the surface exposed ligands, usually the imidazole moiety of histidine. In a similar approach the synthesis of rigid macroporous polymers containing strategically distributed Cu (II)-iminodiacetate (Cu II IDA) complexes was reported. These polymers exhibited selectivity for bisimidazole "protein analogues" that were not distinguishable by reverse phase HPLC (Dhal et al 1991, 1992).

1.12.3. Advantages of the MIP's over chiral stationary phases

a) The order of enantiomer elution can be predicted a priori.

b) They can be successfully used as substitutes for biological recognition in immuno assay protocols.

c) These materials possess remarkable chemical, mechanical and thermal stabilities that are not observed in biomolecules.

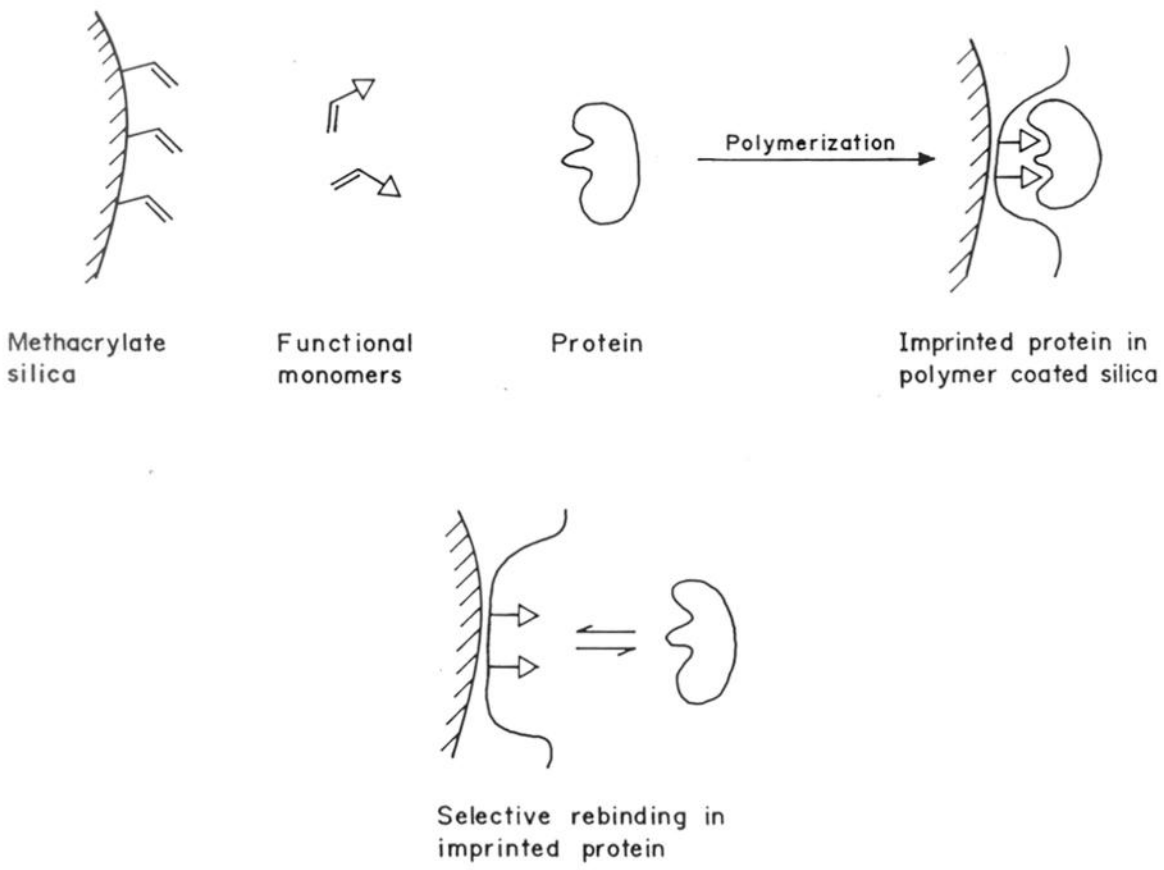


Figure 1.9 : Schematic presentation of the concept utilized in a protein imprinting protocol.

d) They are well suited to long term use and applications that require extreme conditions such as very high or very low temperatures and non-aqueous media.

e) The materials used for the synthesis of MIP's are extremely cheap, relative to the cost of most protein and synthetic chiral CSPs. Cost of the template may vary substantially, although this material can often be recovered in excellent yield.

1.12.4. Substrate selective sensors

Molecular imprinted polymers can be used as sensor components. On substituting the biopart in the enzyme or antibody based sensor with catalytically active or ligand specific polymers prepared from specific molecular imprints, a more robust sensing element system can be obtained. Further in cases where no suitable biomolecule is available, tailor-made binding site for a given molecule can be created in these polymers. Andersson et al (1990[a]) prepared a flow through column electrode, based on polymers imprinted against L-phenyl alanine anilide. The flow through stream potential across the column was continuously recorded as the solvent was pumped through the system. The column resolved the enantiomers of phenylalanine anilide as detected by both U.V. absorption and potentiometric measurements and the recorded signals could be correlated with the concentration of the phenylalanine anilide.

1.12.5. MIP's for studying mechanisms underlying molecular recognition

Molecular imprints were prepared utilizing only weak bonds between the print molecule and functional monomers. The interactions, during both polymerization and the subsequent recognition event, were based solely on hydrogen bonds and other weak forces, such as hydrophobic interactions and dipole-dipole interactions. These polymers had the abi ity to bind selectively the print molecule from a mixture of both racemates (Andersson et al 1990[b]). Wulff et al (1991[b]) synthesized molecular imprinted polymers for racemic resolution of free sugars. Polymers prepared from a D-fructose template preferably absorbed D-fructose from D,L-fructose but absorbed

L-galactose from D,L-galactose. Similarly polymers prepared with D-galactose template absorbed D-galactose and L-fructose from the racemate. These studies indicated that the orientation of the functional groups inside the cavity was the dominating factor while shape selectivity was only of secondary importance. Studies carried out by Shea et al. (1989) indicated that the template shapes the microenvironment during polymerization and shape selectivity may be the most important recognition factor.

1.12.6. MIP's as enzyme mimics and designer catalysts

Scientists have long attempted to create synthetic polymers with enzyme like properties, though the progress till date has been modest. In some of the earlier studies, Robinson et al (1989) prepared polymer imprints against transition state analogues. A polymer was prepared with sites for p-nitrophenyl methyl-phosphonate, a transition state analogue for the hydrolysis of p-nitro phenyl acetate (See figure 1.10). The polymers demonstrated preferential binding and induced a small increase in the rate of hydrolysis of p-nitrophenyl acetate. The rate enhancement was inhibited by the transition state analogue indicating that the catalysis achieved was due to specific binding sites provided by molecular imprinting. Polymers using 4(5) vinyl imidazole as the functional monomer were prepared using amino acid derivatives as templates. The monomer and the template were chelated with a metal ion cobalt. These polymers were capable of selectively hydrolyzing amino acid ester substrates related to the print species with a modest turnover (Leonhardt et al 1987).

Pyridoxal-5'-phosphate is a coenzyme involved in many of the enzymatic transformations of amino acids occurring in nature such as α-decarboxylation, α,β-elimination reactions, racemization and transamination. Imprinting was carried out with co-enzyme substrate analogue, N-pyridoxyl-L-phenyl alanine anilide in methacrylic acid and ethyleneglycol dimethacrylate polymers. Studies were performed to determine if the presence of the polymer in

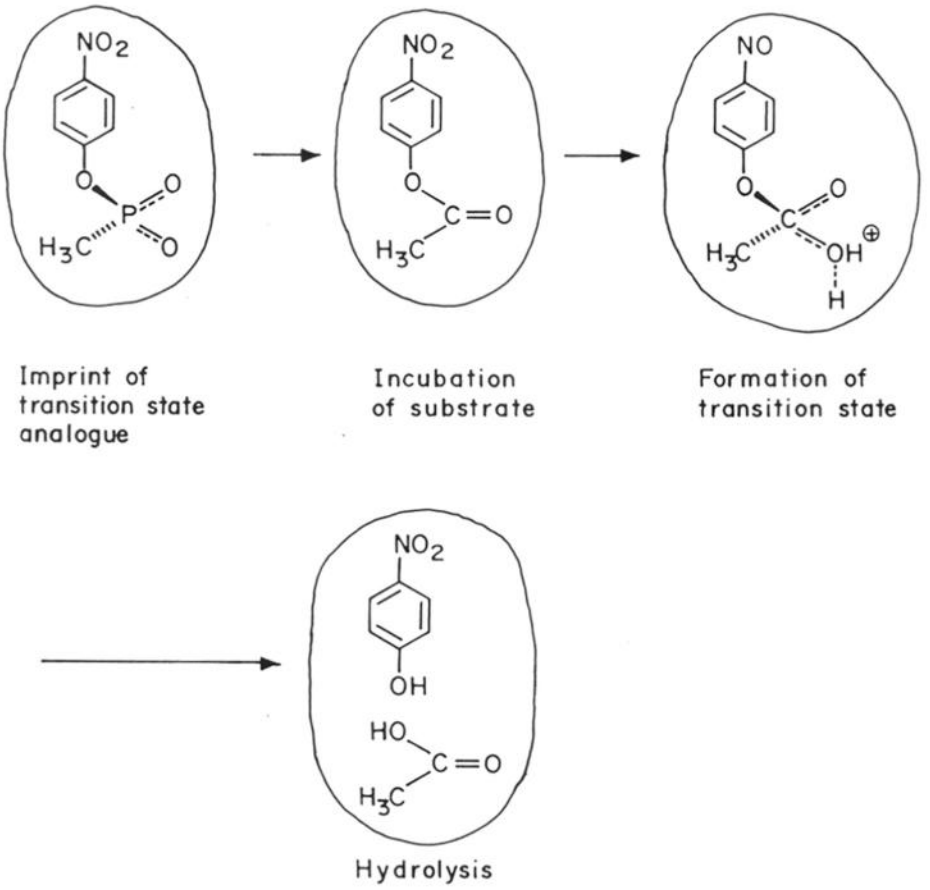


Figure 1.10 : Molecularly imprinted polymers using transition state analogues for hydrolysis of esters.

the reaction mixture would enhance the condensation reaction. It was observed that the imprinted polymer modestly enhanced the rate of pyridoxal catalyzed α-proton exchange on ^{3}H labelled phenylalanine anilide (Andersson et al 1989).

Muller et al (1993) prepared molecular imprints for β-elimination of HF from 4-fluoro-4 (p-nitrophenyl) 2-butanone. A similar catalyst for β-elimination was reported by Shea et al (1993). A 1,3 dicarboxylic acid template was used to organize two polymerizable amines prior to their copolymerization. The amine-functionalized network polymer was found to catalyze the dehydrofluorination of the fluoroketone. Control polymer, containing an equal number of amine functional groups but randomly dispersed in the polymer were found to be only slightly active. In presence of excess substrates, the polymer catalyst exhibited Michaelis-Menten kinetics (K_m = 27 mm, K_{cat} = 1.1. x 10^{-2} min^{-1}). Sellergren et al (1994) have reported the synthesis of a molecularly imprinted polymer containing phenol imidazole and carboxyl group in the cavities. The phenol imidazole group was found to be responsible for the hydrolysis of amino acid esters and the catalysts also exhibited enantioselectivity The catalytic activity was lost above pH 7.0. At higher temperature the enantioselectivity was lost but the rate enhancement relative to the control was unaffected.

1.13.0 Concluding remarks

This chapter summarizes the synthesis and applications of hydrogels in different areas. The biomimetic approach used to design catalytic hydrogels has been described. In particular the literature on the past efforts to synthesize polymers, using the molecular imprinting technique for a wide range of applications has been reviewed. Soluble polymeric catalysts reported earlier exhibited cooperative interactions among the functional groups. Our work aims at combining the effects of cooperative interactions with the imprinting techniques to synthesize new reactive hydrogels.

CHAPTER II

Objectives & scope of work

2.0.0 Introduction

Hydrogels represent an emerging class of smart materials. They can be made to respond to diverse stimuli like pH, temperature, voltage, light, etc. as described in the earlier chapter. Such systems can find wide applications as control processes in medicine, industry and pharmacy. Over the years many efforts have been directed towards the development of such hydrogels.

Park et al (1988) demonstrated that the catalytic activity of an enzyme, immobilized within a thermally reversible hydrogel, could be reversibly activated and deactivated by cycling above and below the lower critical solution temperature (LCST) of the hydrogel. Below the LCST the hydrogel remained in a swollen state leading to an increase in the mass transfer rates of the solutes giving rise to the enzyme activity. When the temperature was increased above the LCST, the hydrogel was deswollen which inhibited the mass transfer leading to loss of the catalytic activity.

Kokufuta et al (1992) reported the synthesis of a hydrogel based on poly(vinyl alcohol) containing concanavalin A (Con A) and glucoamylase. The substrate for glucoamylase i.e. starch had a binding affinity towards starch to be diffused through the gel pores. The activity of the enzyme entrapped in the gel matrix increased due to the facilitated diffusion of the substrate (starch) which was induced by con A.

The hydrogels described above are called 'catalytic hydrogels' but the catalytic activity does not arise due to the hydrogel itself but arises from the enzymes entrapped in the matrix. The hydrogel serves only as a support.

In a different approach, Belokon et al (1980) prepared a pyridoxal enzyme model by cyclocopolymerization of N^{α}-5-methacryloyl amino salicylidene N^{ϵ}-methacryloyl-(S)-lysinato copper (II), with acrylamide and N, N' methylene bisacrylamide. After the removal of the copper ions, the internal aldimine formation between the salicylaldehyde and the lysine moieties bound to

the polymer backbone at pH 9.2 was 100 times higher than that for the monomer analogues. This example illustrated that by introducing appropriate functional groups in the hydrogel and by choosing an appropriate polymerization technique, reactive hydrogels can be synthesized.

In this work two types of systems have been investigated :- (1) catalytic hydrogels for the facile hydrolysis of esters and amides, (2) hydrogels exhibiting enzyme like activity.

2.1.0. Catalytic hydrogels for facile hydrolysis

2.1.1 Hydrogels comprising charge transfer complexes

As described by Belokon et al (1980) the key to achieve catalytic activity lies in bringing the reacting groups in proximity prior to polymerization. The conformation can then be frozen by polymerization.

In order to bring the reactive groups in proximity, they must possess some binding interaction or they must be forced into proximity. Binding interactions can be of different kinds viz. electrostatic, hydrophobic, charge transfer etc.

It was therefore proposed to study the catalytic activity of a donor-acceptor complex of a monomeric ester p-nitro phenyl p-vinyl benzoate and a monomeric catalyst viz. N-vinyl imidazole incorporated in HEMA.

Due to the binding interactions, the groups are expected to remain in proximity after polymerization (Jones et al 1986). The imidazole catalyzed hydrolysis of the ester was investigated.

The hydrolysis of such pendent chain linked esters or amides has been studied by various researchers for controlled release applications (McCormick et al 1980, 1988, Shah et al 1990[b]). It was demonstrated that the release kinetics was controlled by either the rate of hydrolysis of the labile bond or by the diffusivity of the product through the matrix. But the hydrolysis took place under alkaline conditions (pH = 11) which did not have in physiological relevance.

In order to bring about the hydrolysis in the intestinal pH range (6.8 - 7.5) Vadalkar et al (1995) investigated the imidazole catalyzed heterogeneous hydrolysis of the polymer bound p-nitrophenyl esters. Though the hydrolysis took place at pH = 8.0 the rate of hydrolysis was dependent on the rate of diffusion of the catalyst in the polymer matrix.

It was therefore proposed to investigate the various aspects of intramolecular catalysis in the catalytic hydrogels. The effect of pH on the catalytic activity was studied and the implications of using these hydrogels as controlled drug delivery systems and as alternative to enteric coatings has been demonstrated.

2.1.2 Hydrogels by molecular imprinting

In the hydrogels described above, the reactive groups are brought in proximity by non-covalent interactions. Such interactions may not be possible for all the catalyst substrate pairs. The techniques of molecular imprinting and template polymerization by metal coordination described in the earlier chapter can be applied to synthesize novel catalytic hydrogels. This investigation was undertaken with a view to explore the possibility of applying the techniques of molecular imprinting and template polymerization for the hydrolysis of polymer bound esters and amides.

As a first step in efforts to design such systems, investigation of imidazole catalyzed hydrolysis of polymeric 2-methacryloyl ethyl p-amino benzoate ester was undertaken. A method has been developed to provide guidelines for tailoring hydrogels to achieve catalytic hydrolysis of the ester. The effect of pH, and structure of the polymer on the catalytic activity and the mechanism of hydrolysis is explained.

Further the imidazole catalyzed hydrolysis of polymeric 2-methacryloyl ethyl p-nitro benzoate has been studied to explore the possibility of tailoring the hydrogel structure using

imprinting techniques. The role of the print molecule, enhanced catalytic rate due to N-methacryloyl histidine and the pH dependent activity which led to on/off release of p-nitro benzoic acid from P(HEMA-PNP-MA-His-MAA) were investigated and the results explained.

2.2.0 Hydrogels exhibiting enzyme like activity

In the past there have been many attempts to mimic enzymes especially chymotrypsin by the functional modifications of cyclodextrins, crown ethers, monoclonal antibodies and cryptands (D'Souza et al 1985, Lehn 1988, Pollock et al 1986).

Polymers mimicking substrate binding and esterolytic activity of hydrolytic enzymes were synthesized using molecular imprinting techniques (Leonhardt et al 1987, Robinson et al 1989, Sellergren et al 1994). But these polymers were not exact replicas of the active site nor was their activity comparable to the enzymes.

This investigation was undertaken with a view to synthesize a polymer model that would mimic the active site of α-chymotrypsin and exhibit catalytic activity. Systematic efforts have been made to study the factors affecting the catalytic activity of these polymers. Characteristic features of enzyme catalysis viz. active site inhibition, substrate binding, enhanced catalytic activity were demonstrated for the polymer mimic. In addition the enhanced stability of these polymeric catalysts towards temperature, pH and repeated use, as compared to chymotrypsin is demonstrated.

Further efforts were made to improve the catalytic activity of the polymers by using the surface imprinting techniques. The hydrolysis of ester and amide substrates was investigated and compared to that exhibited by native α-chymotrypsin. U. V. and pH sensitive catalytic activity of the mimic was demonstrated. The kinetic constants in the Michaelis-Menten equation for ester and amide substrates were computed over a wide range of substrate concentrations for the polymer mimic and the enzyme.

2.3.0 Summary

The objectives of this work are summarized below.

- To develop catalytic hydrogels as enzyme mimics for the facile hydrolysis of esters and amides.
- To study the anchimeric effects in the hydrolysis of reactive esters from catalytic hydrogels.
- To investigate imidazole and NVIm catalyzed hydrolysis of HEMA esters from these hydrogel matrices.
- To explore the possibility of achieving a pH sensitive release of the product for controlled drug delivery applications.
- To develop a model for the active site of α-chymotrypsin using the techniques of molecular imprinting.
- To study the kinetics of hydrolysis for model ester and amide substrates using the polymer mimic and compare its performance with that of the native enzyme.
- To investigate the pH and U.V responsive catalytic activity of the polymer mimic.

CHAPTER III

Catalytic hydrogels as enzyme mimics : Anchimeric effect

3.0.0 Introduction

Enzyme catalysis is explained on the basis of chemical catalysis with an ability of enzymes to reroute intermolecular processes through intramolecular pathways by binding substrates to preorganized active sites. It is very well established that intramolecular reactions are usually faster than intermolecular reactions. In intramolecular reactions the reactants are always in proximity and hence they have already lost the required translational and rotational degrees of freedom prior to the rate determining step. Intramolecular catalysis can then serve as a simple model of the intracomplex catalysis exhibited by enzymes. Various types of intramolecular reactions have been made investigated in the past (Bruice et al 1966).

In unimolecular intramolecular reactions, the reactive groups are present on the same molecule. The most simple example is that of the hydrolysis of aspirin (ortho acetyl salicylic acid). The hydrolysis of aspirin is fifty times faster than that of its para-isomer (Schmir et al 1958).

Intramolecularly catalyzed reactions in polymeric systems have also been investigated (Zimmering et al 1957, Gaetjens 1961). Copolymers of acrylic acid with less than 10 % of p-nitrophenyl methacrylate were chosen. The rate of ester hydrolysis was accelerated by many orders of magnitude when the ester was attached to the polymeric acid. The rapid hydrolysis of the copolymer was due to the formation of a six-membered cyclic intermediate through attack by a neighbouring carboxylate ion on the ester group.

Fitch et al (1986) reported the preparation of polyacrylate ester latex particles possessing pendent drug moieties attached to the polymer by ester linkages. These particles self-catalyzed the hydrolysis of the pendent ester linkages by ion-exchange. Akashi et al (1986) reported the synthesis of water soluble or dispersible drugs containing pendent theophylline or 5-fluorouracil moieties which are hydrolyzed by imidazole groups in the polymer.

In an effort to design a polymeric mimic of pyridoxal enzyme Belokon et al (1982) copolymerized N^{α}-5-methacryloyl amino salicylidene - N^{ϵ} - methacryloyl - (S) - lysinato) (pyridine) copper (II) with acrylamide. Extraction of Cu (II) with EDTA, led to a polymer in which the lysine and salicylaldehyde moieties were in juxtaposition forming an "internal aldimine".

This work describes the polymerization of a charge transfer complex between N-vinyl imidazole and p-nitrophenyl p-vinyl benzoate with 2-hydroxy ethyl methacrylate. This leads to catalytic hydrogels in which the catalytic and substrate sites are on the same chain next to each other. Enhanced hydrolysis of the ester due to intramolecular catalysis is observed.

3.1.0 Experimental work

2-hydroxyethyl methacrylate (HEMA), N-vinyl imidazole (NVIm) and 6-amino caproic acid were obtained from Aldrich Chemical Co., USA. p-nitrophenol, 2,4 dinitrophenol, L-histidine, thionyl chloride, p-toluic acid, N-bromosuccinimide, benzoyl peroxide, formaldehyde (37% aq. solution) tert-butyl hydroperoxide were obtained from local suppliers. All the above compounds were purified by standard methods (Perrin et al 1981).

3.2.0 Monomer synthesis

3.2.1 Synthesis of p-vinyl benzoic acid

P-vinyl benzoic acid was prepared from p-carboxyl benzyl bromide as reported by Broos et al (1978).

Molecular formula : $C_9H_8O_2$ (mol. wt 148)

M.P. : 140°C

IR (Nujol) 1610 and 920 cm^{-1} (vinyl $C=CH_2$), 1670 cm^{-1} (acid-carboxyl), 1500-1600 cm^{-1} (aromatic ring)

^{1}H NMR ($CDCl_3$) : 5.5 δ (d, 1H, C=CH), 5.9 δ (d, 1H, C=CH) 6.8 δ (dd, 1H, HC=C), 7.6-8.2 δ (4H, aromatic protons).

3.2.2 Synthesis of p-nitrophenyl p-vinyl benzoate

P-vinyl benzoic acid (0.1 mol) was converted into its acid chloride using thionyl chloride (0.15 mol) at 0-5°C. Excess thionyl chloride was removed under vacuum.

To a solution of p-nitrophenol (0.12 mol) and triethylamine (0.15 mol) in 100 ml dry benzene, p-vinyl benzoyl chloride (0.1 mole) was added slowly at 0-5°C. After the addition was complete, the solution was stirred for 2-3 hours at room temperature. The solution was washed repeatedly with ice cold water and then with dilute Na_2CO_3 twice and once again with cold water. The benzene layer was dried for 24 hours over anhydrous sodium sulfate. Benzene was removed under vacuum at 4°C. The crude monomer was purified by recrystallization in benzene/petroleum ether. The yield obtained was 85%.

Molecular formula : $C_{15}H_{11}O_4N$ (mol. wt. 269).

IR (nujol) : 1609 and 937 cm^{-1} (vinyl C=CH_2), 1736 cm^{-1} (ester, carbonyl), 1463 cm^{-1} (-NO_2, stretching).

^{1}H NMR ($CDCl_3$) : 5.5 δ (d, 1H, C=CH), 5.9 δ (d,1H, C=CH), 6.8 δ (dd, 1H, HC=C), 7.5-8.3 δ (4H, aromatic vinyl benzoate protons), 8.5-8.9 δ (4H, aromatic nitrophenyl protons).

3.2.3 Synthesis of 2,4-dinitrophenyl p-vinyl benzoate

This compound was synthesized as described previously, using 2,4- dinitrophenol (0.12 mol), triethyl amine (0.15 mol) and p-vinyl benzoyl chloride (0.1 mol) in 100 ml dry benzene. The yield obtained was 82%.

Molecular formula : $C_{15}H_{10}O_6N_2$ (mol. wt. 314)

IR (Nujol) : 1609 and 926 cm^{-1} (vinyl $C=CH_2$), 1738 cm^{-1} ester, carbonyl), 1456 cm^{-1} ($-NO_2$, stretching).

^1H NMR ($CDCl_3$) : 5.5 δ (d H, C=CH), 5.9 δ (d, 1H, C=CH), 6.8 δ (dd, 1H, HC=C), 7.5-8.3 δ (4H, aromatic vinyl benzoate protons), 8.7-8.9 δ (3H, aromatic dinitrophenyl protons).

3.3.0 Synthesis of 4-nitrophenyl 6-(-N-vinyl benzoyl amino) caproate

P-vinyl benzoyl chloride was reacted with 6-amino caproic acid under Schotten-Baumann conditions to yield 6-p-vinyl benzoyl amino caproic acid. The yield obtained was 60%.

Molecular formula : $C_{15}H_{19}O_3N$ (mol. wt. 261)

IR (Nujol) : 1599 cm^{-1} (amide C=O), 1710 cm^{-1} (acid C=O), 2926 cm^{-1} (CH_2, stretching), 3400 cm^{-1} (-OH of acid).

^1H NMR ($DMSOd_6$) : 5.4 δ (d, 1H, C=CH), 5.86 δ (d, 1H, C=CH), 7.45-8.2 δ (4H, aromatic protons), 1.25 δ (m, 6H, methylene), 1.70 δ (m, 4H, methylene).

6-p-vinyl benzoyl amino caproic acid (0.1 mol), p-nitro phenol (0.15 mol), dicyclohexyl carbodiimide (0.12 mol) in 100 ml dry ethyl acetate, was stirred at 0-5°C for 5 hours and then at room temperature for 12 hours. The urea salt was filtered. Ethyl acetate solution was washed with cold dilute Na_2CO_3 solution twice and twice with cold distilled water. The ethyl acetate layer was dried for 24 hours over anhydrous sodium sulfate. Ethyl acetate was removed under vacuum at 4°C. The crude monomer obtained was recrystallized from ethyl acetate/petroleum ether.

Molecular formula : $C_{21}H_{22}O_5N_2$ (mol. wt. 382)

IR (Nujol) : 1745 cm^{-1} (ester, C=O), 1610 cm^{-1} (amide, C=O), 2926 cm^{-1} (CH_2, stretching)

^1H NMR ($CDCl_3$) : 5.5 δ (1H, C=CH), 5.9 δ (1H, C=CH), 7.4-8.2 δ (4H, aromatic protons), 1.25 δ (m, 6H, methylene), 1.70 δ (m, 4H, methylene), 7.9-8.4 δ (4H, aromatic nitrophenyl protons).

3.4.0 Synthesis of N-methacryloyl histidine

N-methacryloyl histidine was synthesized from methacryloyl chloride (0.1 mol) and L-histidine (0.15 mol) under the Schotten-Baumann conditions as reported (Okuda et al 1987).

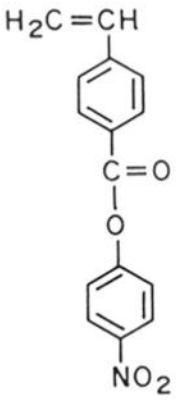
Molecular formula : $C_{10}H_{13}O_3N_3$ (mol wt 223)

^1H NMR ($DMSOd_6$) : 5.3 δ (d, 1H, C=CH), 5.7 δ(d, 1H, C=CH), 2.1 δ (s, 3H, CH_3), 2.5 δ (m, 3H, CH-CH_2), 6.8 δ, 7.9 δ, 8.2 δ (3H, aromatic imidazole protons).

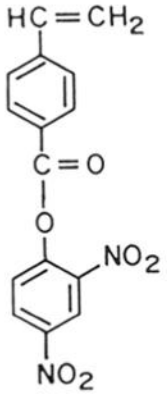
The structures of the monomeric substrates and the catalysts are shown in figure 3.1.

3.5.0 Spectral analysis of the charge-transfer complex

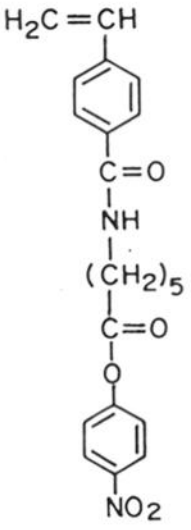
The formation of the complex between p-nitrophenyl p-vinyl benzoate and N-vinyl imidazole was detected in the U.V region at λ_{max} = 280 nm. The change in the absorbance of the complex resulting from the variation in the compositions of the N-vinyl imidazole is shown in figure 3.2. It was observed that the complex had the highest absorbance when the ratio of PNPVB to NVIm was 1:1. Similar complexes were also observed with N-methacryloyl histidine and 2,4-dinitrophenyl p-vinyl benzoate and 4-nitrophenyl 6-(p-vinyl benzoyl amino) caproate.



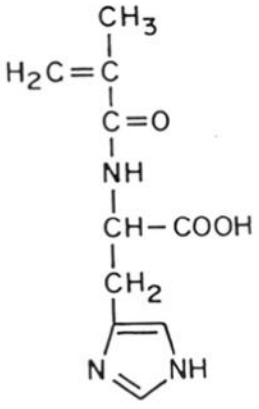
P-nitrophenyl p-vinyl benzoate



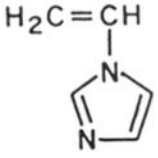
2,4 dinitrophenyl p-vinyl benzoate



4-nitrophenyl 6-(-N-vinyl benzoyl amino) Caproate



N-methacryloyl histidine



N-vinyl imidazole

Figure 3.1 : Structures of the various substrates and catalysts used.

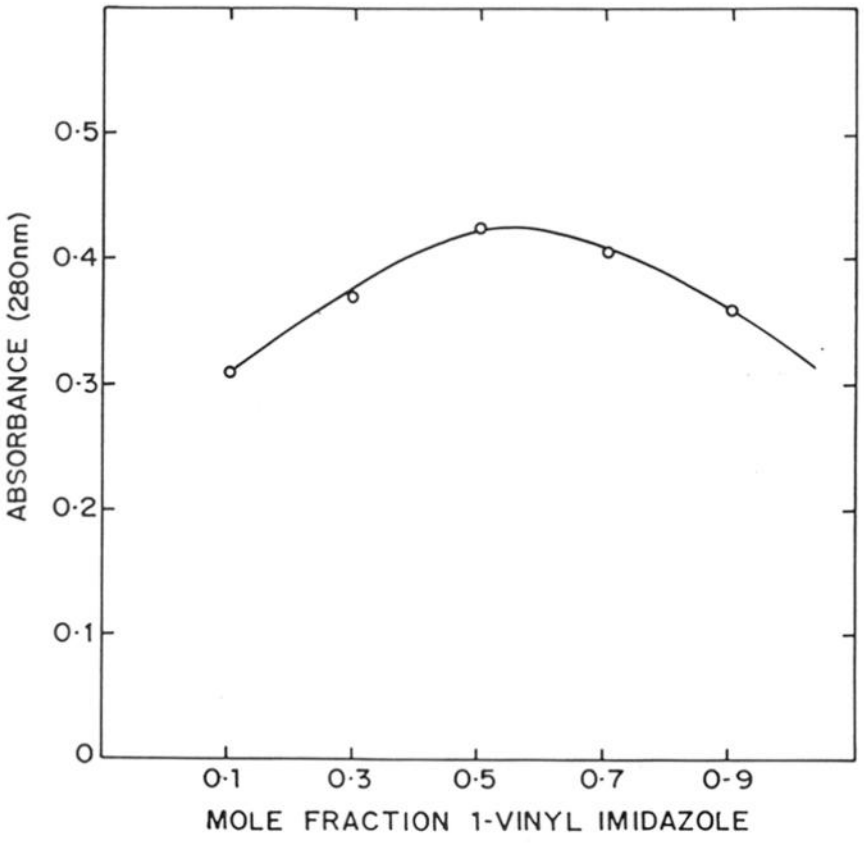


Figure 3.2 : Evidence for the complex formation between PNPVB and NVIm at λmax = 280 nm.

3.6.0 Polymer synthesis

To the charge-transfer complex of N-vinyl imidazole or N-methacryloyl histidine with the substrates PNPVB, 2,4 DNPVB or 4CS-6ACA-PNP, appropriate quantities of HEMA were added. Bulk polymerization of all the monomer mixtures was carried out using 0.8% t-butyl hydroperoxide as the initiator at 60°C for 16 hours. Since HEMA used contained a small amount of EGDMA, a crosslinked glassy polymer in the form of a rod was obtained which was recovered by breaking the test tube. Discs of thickness 0.090 - 0.11 cm were obtained by cutting the cylinder on a lathe. The discs were stored in a desiccator. The detailed description of different polymers synthesized is given in Tables 3.1 and 3.2. Soluble polymers of identical composition were also synthesized. HEMA was purified as reported to get rid of EGDMA for the synthesis of soluble polymers (Pinchuk et al 1984).

3.7.0 In vitro hydrolysis studies

Hydrolysis experiments were carried out in a jacketed vessel maintained at 37°C. Hydrogel discs were immersed in appropriate buffers, which were constantly stirred. The amount of p-nitrophenol released in the solution was followed by monitoring the absorbance of the medium at λ_{max} = 400 nm and 2,4-dinitrophenol at λ_{max} = 320 nm on a U.V. spectrophotometer. The extent of hydrolysis at time t viz. (Mt) determined from the appropriate calibration curve. The total amount of p-nitrophenyl ester incorporated in the disc was taken as M_{oo}.

3.8.0 Results and discussion

3.8.1 Diffusion and reaction effects in hydrogels

In the ongoing efforts in our group to design hydrogel based drug delivery system Shah et al (1990[b]) reported the hydrolysis of a series of substituted benzoate esters in alkaline media from poly (2-hydroxyethyl methacrylate) hydrogels. It was observed that the rates of hydrolysis of the benzoate esters vary substantially depending on the nature of the substituent but their diffusion

Table 3.1

Feed compositions of monomers for hydrogel syntheses[a)]

Polymer composition	HEMA	PNPVB	NVIm
HEMA-PNPVB	4.50	0.5	--
HEMA-PNPVB-NVIm	4.32	0.5	0.174
	4.15	0.5	0.348
	3.98	0.5	0.522
	3.82	1.0	0.174
	3.33	1.5	0.174

a) All the quantities are in gm. wt.

Table 3.2

Feed compositions of monomers for the hydrogel syntheses[a)]

Polymer composition	HEMA	PNPVB	MA-His	NVIm	4CS-6ACA-PNP	2,4 DNPVB
HEMA-PNPVB-MA-His	4.08	0.5	0.414	--	--	--
HEMA-2,4 DNPVB-NVIm	4.35	--	--	0.149	--	0.5
HEMA-4CS-6ACA-PNP-NVIm	4.37	--	--	0.122	0.5	--
HEMA-4CS-6ACA-PNP	4.50	--	--	--	0.5	--
HEMA-2,4 DNPVB	4.50	--	--	--	--	0.5

a) All the quantities are in gm .wt

coefficients varied only within a narrow range. While observed release of p-methoxy or p-amino benzoate esters was controlled by the kinetics of the hydrolysis step, the release of p-nitro benzoic acid was controlled by diffusion. Yean et al (1990) observed that the hydrolysis of chloramphenicol ester of HEMA governed its release from P(HEMA) matrices. Therefore it is essential to identify the rate controlling parameter. If the kinetics of hydrolysis is the governing step, the enhanced rate of hydrolysis should lead to faster release rates. Efforts to enhance the rates of hydrolysis of such conjugates were made by increasing the degree of hydration (Pitt et al 1995) or by using enzymes and enzyme mimicking polymers (Vadalkar et al 1995). The use of enzymes or enzyme mimicking polymers had a limited success because of the diffusional limitations of these polymeric catalysts.

In order to overcome some of these problems we considered it appropriate to incorporate functional groups responsible for the catalytic hydrolysis in the hydrogel itself. It is crucial that the catalytic and the substrate groups be in the vicinity of each other so that a high catalytic activity is achieved.

Systems of this kind have not been extensively studied. Belokon et al 1982 copolymerized N^{α}-5-methacryloyl amino salicylidene - N^{ε}-methacryloyl-(S)-lysinato) (pyridine) copper (II) with acrylamide. Extraction of Cu (II) with EDTA, led to a polymer in which the lysine and salicylaldehyde moieties were in juxtaposition due to high rates of intramolecular copolymerization. The rate of formation of the Schiff base or an "internal aldimine" was found to be 100 times that for the free amino acid and salicylaldehyde. This high rate of copolymerization within the complex could be exploited to synthesize catalytic hydrogels.

3.8.2 Catalytic hydrogels based on charge transfer complexes

We chose p-nitrophenyl esters as substrates and N-vinyl imidazole and N-methacryloyl histidine as the catalysts. Letsinger and Klaus (1965) investigated the imidazole and poly (N-vinyl imidazole) catalyzed hydrolysis of polymeric p-nitrophenyl esters. The rate constant for the hydrolysis was 2×10^{-6} sec^{-1}. The diffusion coefficient of p-nitrophenol from hydrogel selected in

this work is $\simeq 2.7 \times 10^{-7}$ cm^2/sec. The diffusion time of p-nitrophenol from a 1 mm thick disc is forty times smaller than the reaction time. Thus enhancing the rate of hydrolysis step would lead to enhancement in the observed release kinetics of p-nitrophenol.

It was observed that the esters and N-vinyl imidazole and N-methacryloyl histidine formed donor-acceptor complexes. These could be detected in U.V. region. It was also observed that the absorbance of the complex resulting from variation in the composition was maximum when the ratio of the ester to the catalyst was 1:1 (See figure 3.2). This indicates a 1:1 complex formation between the catalyst and the substrate.

Bender et al (1971) have reported such donor-acceptor complexes between nucleophiles comprising nitrogen, oxygen with compounds containing carbonyl groups. It has also been established that if these complex forming pairs are polymerized the polymer formed has an alternating nature (Jones et al 1986). When polymerized in the presence of HEMA, the resulting hydrogel would contain the donor acceptor complex distributed randomly along the length of the polymer chain, the substrate and the catalyst group always next to each other. The hydrolysis of the ester linkage and the release of p-nitrophenol from these hydrogels is expected to be much faster vis-a-vis hydrogels of identical composition, when the catalyst is absent.

3.8.3 Enhanced reactivity of catalytic hydrogels

The results of hydrolysis of PNPVB from the hydrogel based on P(HEMA-PNPVB-NVIm) and P(HEMA-PNPVB) are shown in figure 3.3. The mole ratio of PNPVB to NVIm is 1:1 mole/mole (See Table 3.1). The observed rate of release of p-nitrophenol in this case is governed by the hydrolysis step. It can be clearly seen that in P(HEMA-PNVB-NVIm) the release rate of p-nitrophenol is higher than that in the case of P(HEMA-PNPVB). This is because in the terpolymer, the neighbouring imidazole group catalyzes the hydrolysis of the ester.

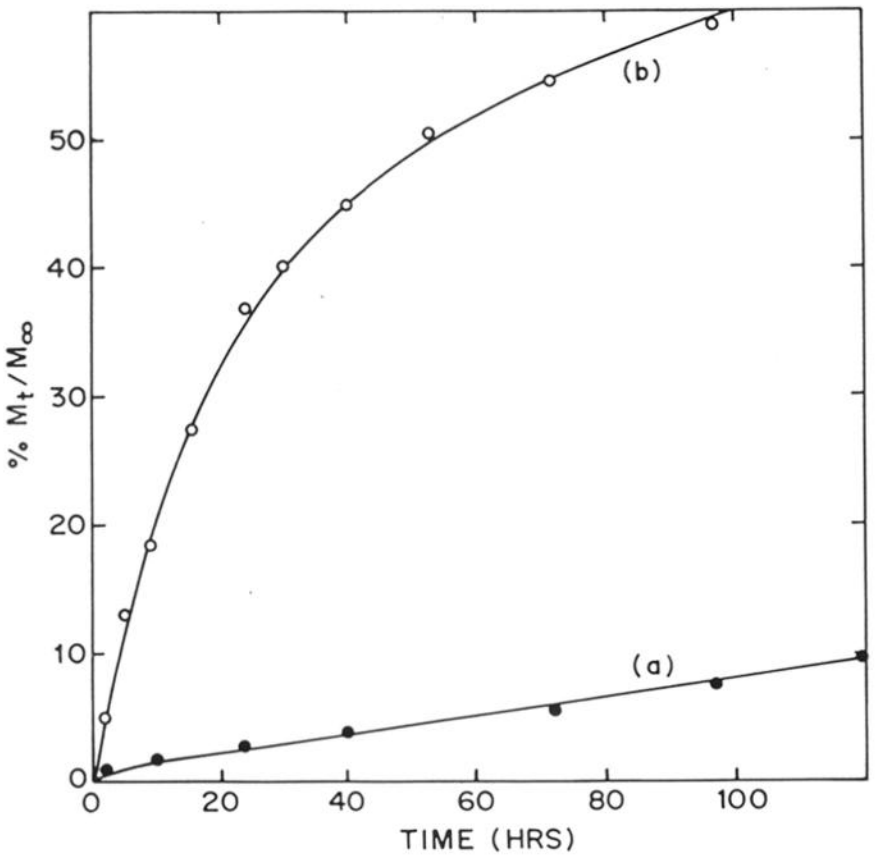


Figure 3.3 : The hydrolysis of PNPVB and the observed release of p-nitrophenol from (a) P(HEMA-PNPVB) uncatalyzed hydrogel compared to (b) P(HEMA-PNPVB-NVIm) catalytic hydrogel in phosphate buffer (0.01M, pH = 8).

3.8.4 Evidence for intramolecular catalysis and the structure of the complex in the hydrogel

In order to demonstrate the anchimeric effect of the imidazole group, it would be desirable to compare the observed rate of release of p-nitrophenol from P(HEMA-PNPVB-NVIm) with that of the hydrogel where PNPVB and NVIm are randomly distributed along the polymer chain. However, such a hydrogel cannot be synthesized since N-vinyl imidazole and p-nitrophenyl p-vinyl benzoate form a 1:1 complex. Soluble polymers P(HEMA-PNPVB-NVIm) and P(HEMA-PNPVB) were synthesized. The resulting polymers were soluble in a mixture of buffer and ethanol (60:40 vol/vol). The higher rate of hydrolysis of PNPVB from the terpolymer vis-a-vis the copolymer is seen in figure 3.4. It is therefore clear that crosslinking is not essential for stabilizing the conformation of the substrate and the catalytic groups. A similar observation was made by Belokon et al (1980). The catalyst and the substrate groups remain in juxtaposition as result of charge transfer complex formation. High rates of intramolecular copolymerization result in the formation of the terpolymer in which the two monomers are always situated next to each other.

In order to compare the intramolecular catalysis in the hydrogel with intermolecular reactions, the hydrolysis of PNPVB from P(HEMA-PNPVB) was carried out in buffers containing increasing N-methyl imidazole concentration (See figure 3.5). It was observed that rate of hydrolysis and therefore the rate of release of p-nitrophenol increased with increasing concentration of N-methyl imidazole. In contrast, when the ratio of PNPVB : NVIm was increased in P(HEMA-PNPVB-NVIm), there was no further enhancement in the hydrolysis. This is because the excess NVIm incorporated in the hydrogel which is not in the vicinity of the substrate cannot offer any anchimeric assistance (figure 3.6).

When the catalytic hydrogel contains excess substrate, the observed release of p-nitrophenol showed a biphasic profile (figure 3.7). The hydrolysis of PNPVB which forms a 1:1

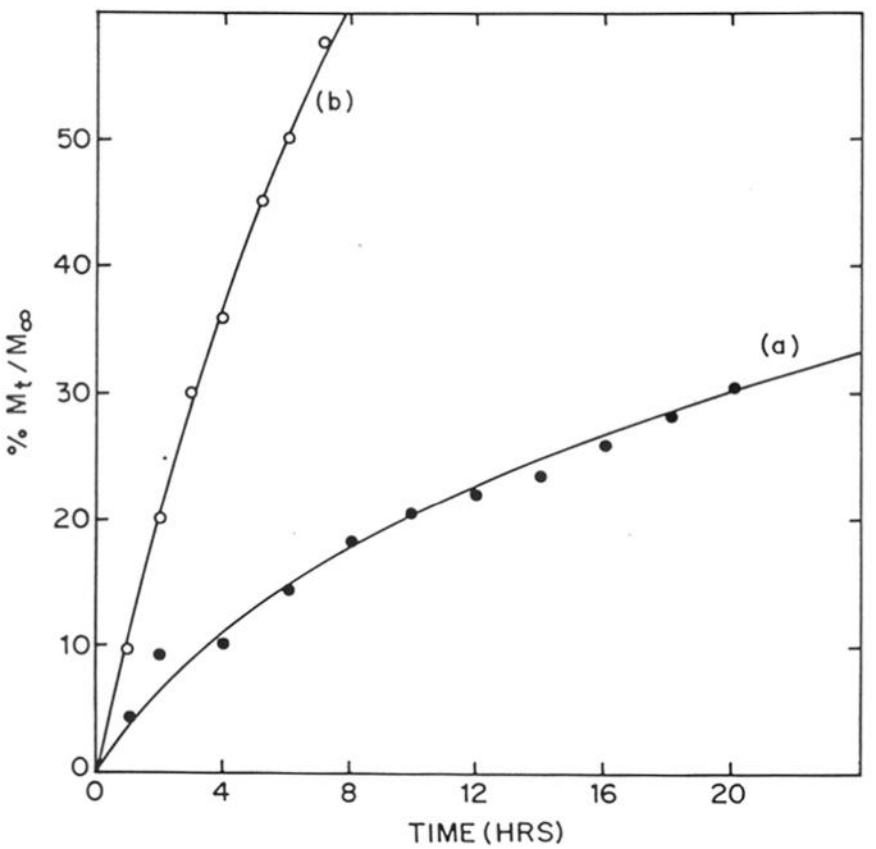


Figure 3.4 : Hydrolysis of PNPVB from the soluble polymers (a) P(HEMA-PNPVB) and (b) P(HEMA-PNPVB-NVIm), in 40:60 vol / vol ethanol / phosphate buffer (0.01 M, pH = 8).

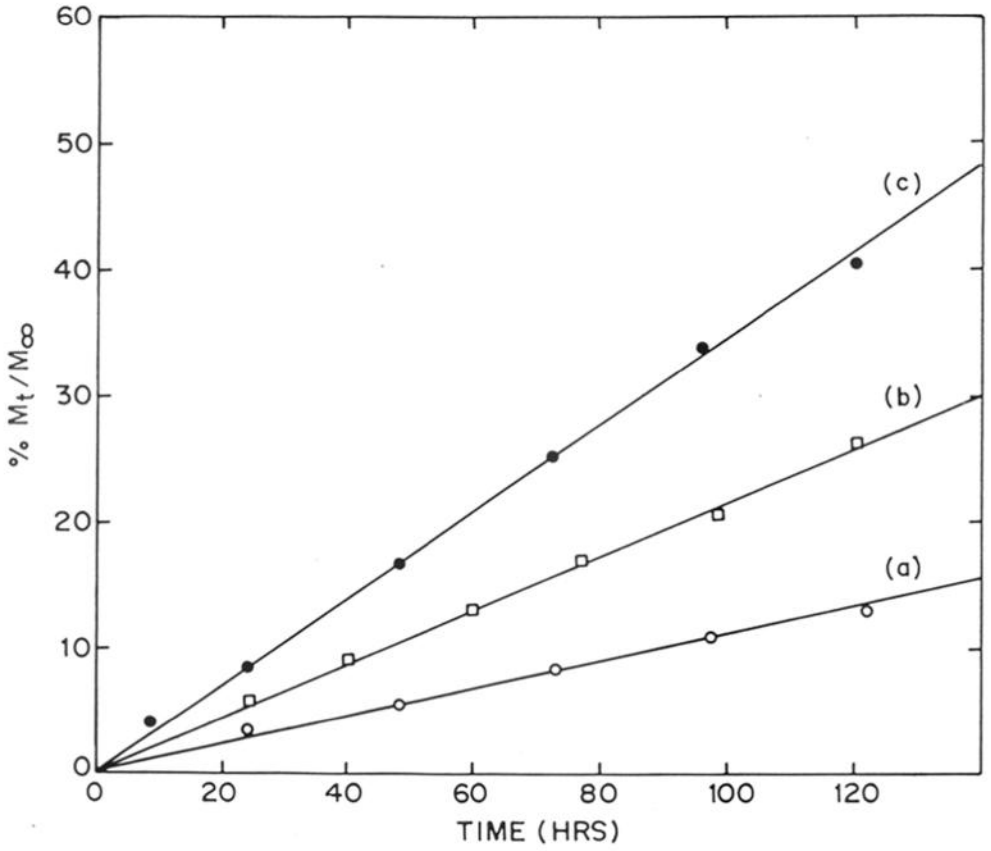


Figure 3.5 : The effect of changing the concentration of the catalyst in the reaction medium on the hydrolysis of PNPVB from P(HEMA-PNPVB) in phosphate buffer containing : (a) 0.68%, (b) 0.1% and (c) 0.136% N-methyl imidazole respectively.

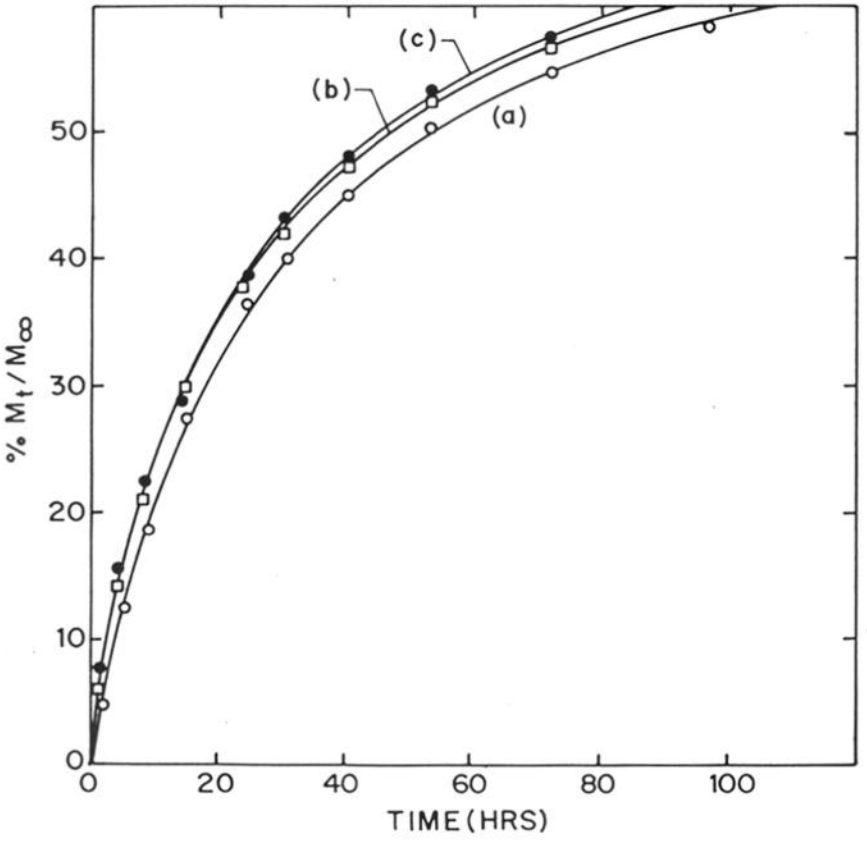


Figure 3.6 : The effect of changing the concentration of the catalyst in the catalytic hydrogels on the hydrolysis of PNPVB from P(HEMA-PNPVB-NVIm). The mole ratio of the substrate to catalyst is : (a) 1:1, (b) 1:2 and (c) 1:3 respectively.

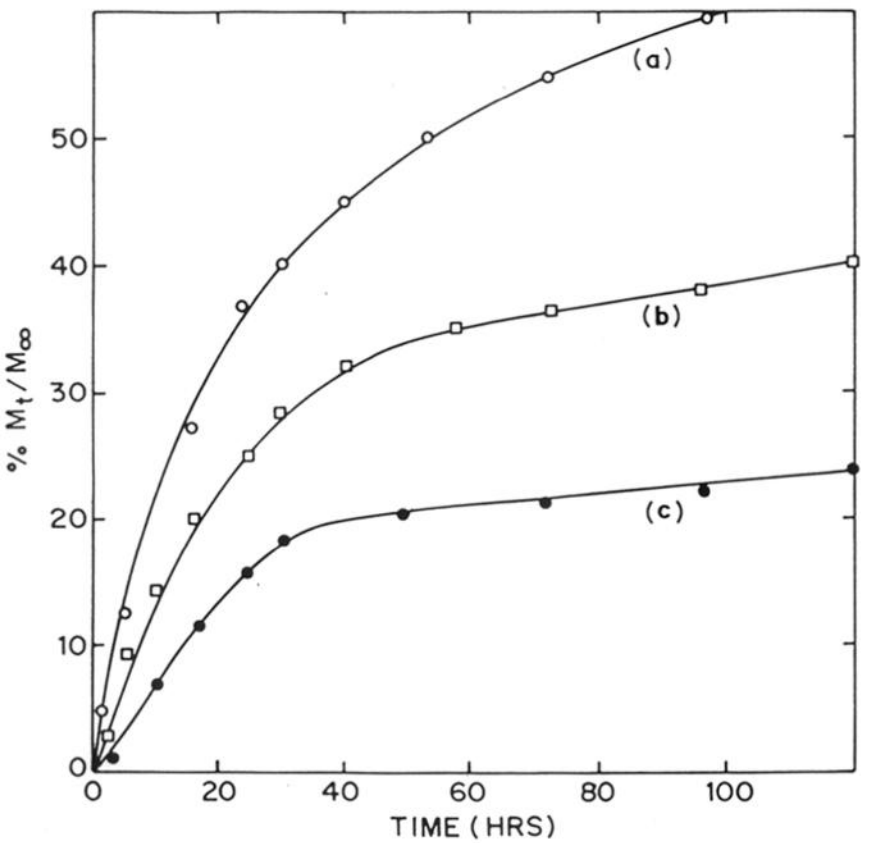


Figure 3.7 : The effect of changing the concentration of the substrate PNPVB on the release of p-nitro phenol from P(HEMA-PNPVB-NVIm). The mole ratio of the substrate to the catalyst in the polymers is : (a) 1:1, (b) 2:1 and (c) 5:1 respectively.

complex with NVIm was enhanced while the PNPVB that did not form a part of the complex was catalyzed only by the buffer. Since the rate of base catalyzed hydrolysis is much slower, a decline in the rate was observed.

3.9.0 Manipulating the catalytic activity : structural effects

It is obvious that the structure/composition variation in the hydrogel which would enhance the rate of hydrolysis further, would also lead to enhanced release of p-nitrophenol.

It is known that imidazole is 25% a better catalyst than N-methyl imidazole (Bender et al 1957). Since N-vinyl imidazole is analogous to N-methyl imidazole we chose N-methacryloyl histidine (MA-His) containing a free imidazole group as the catalyst to represent imidazole. A 1:1 complex of MA-His with PNPVB was copolymerized with HEMA (See Table 3.2). The catalytic activity of this hydrogel was compared to that of P(HEMA-NVIm-PNPVB). The enhanced catalytic effect of MA-His is reflected in the enhanced rate of release of p-nitrophenol (See figures 3.8 b and 3.3 b).

The rate of hydrolysis can also be enhanced by increasing the reactivity of the ester group undergoing hydrolysis. We chose a more reactive ester namely 2,4-dinitrophenyl p-vinyl benzoate and compared the catalytic effect of NVIm on the release of 2,4-dinitrophenol vis-a-vis p-nitrophenol. The enhanced release of 2,4 dinitrophenol is clearly seen in figure 3.9.

When 6-amino caproic acid was introduced as a spacer between p-vinyl benzoic acid and p-nitrophenol, rate of release of p-nitrophenol from P(HEMA-4CS-6ACA PNP-NVIm) was further enhanced. This could be attributed to the flexibility of the spacer group which facilitates the accessibility of the reaction site and thereby enhances the reaction rate (figure 3.10).

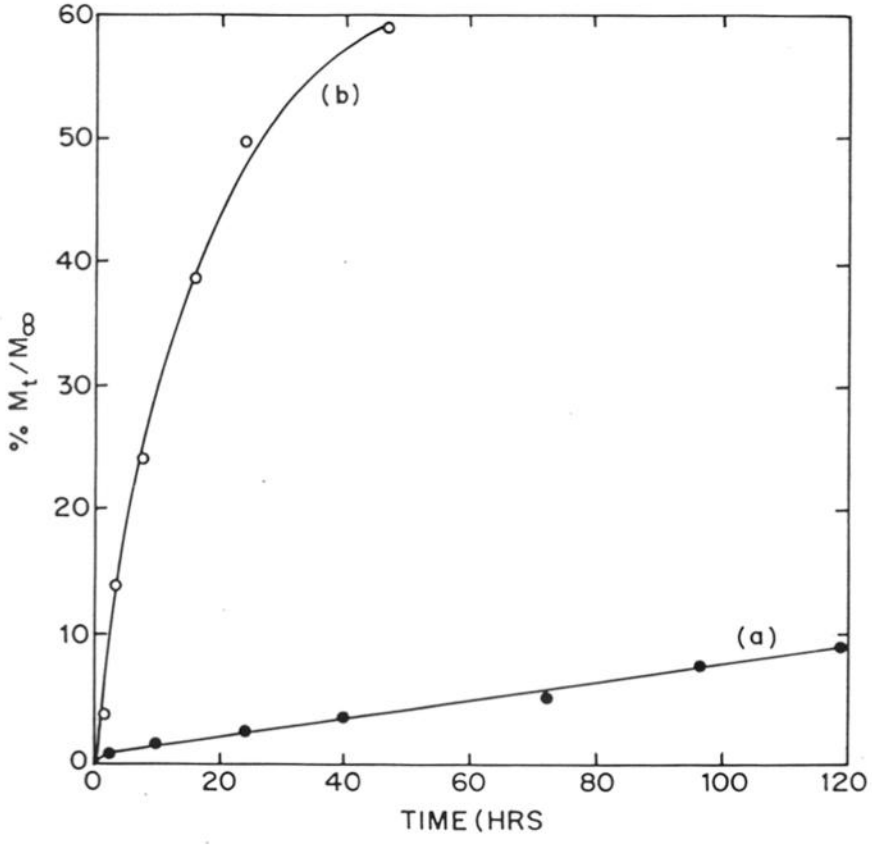


Figure 3.8 : The enhanced rate of hydrolysis of PNPVB from (a) P(HEMA-PNPVB-MA-His) compared to (b) P(HEMA-PNVB) in phosphate buffer.

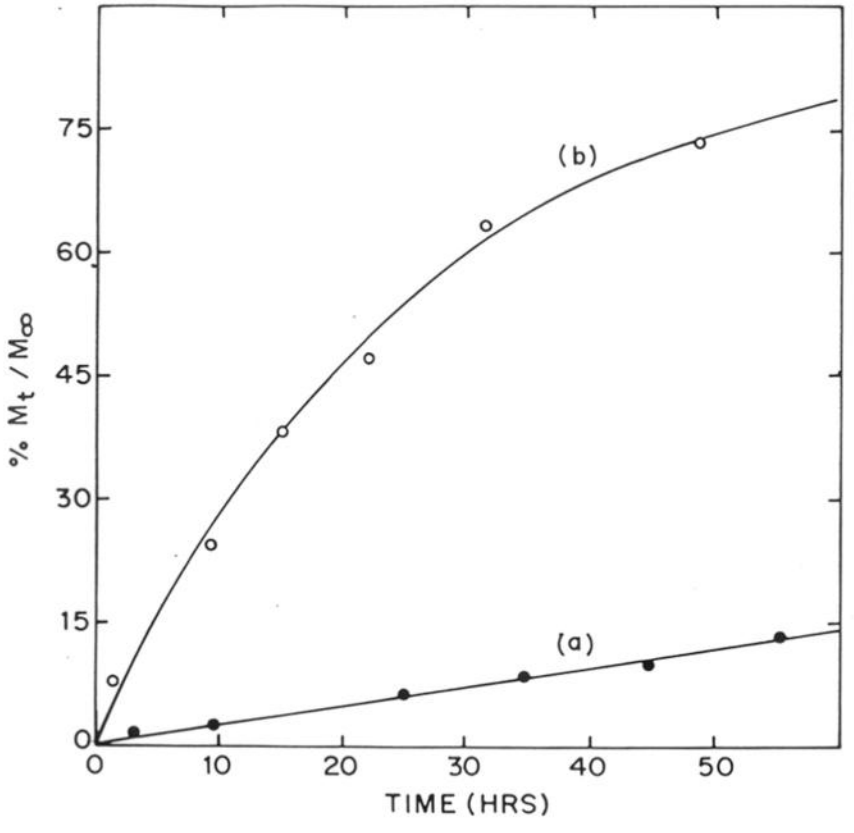


Figure 3.9 : The enhanced rate of hydrolysis of the ester by increasing the reactivity of the ester. The release of 2,4- dinitrophenol from (a) P(HEMA-2,4 DNPVB) and P(HEMA-2,4 DNPVB-NVIm).

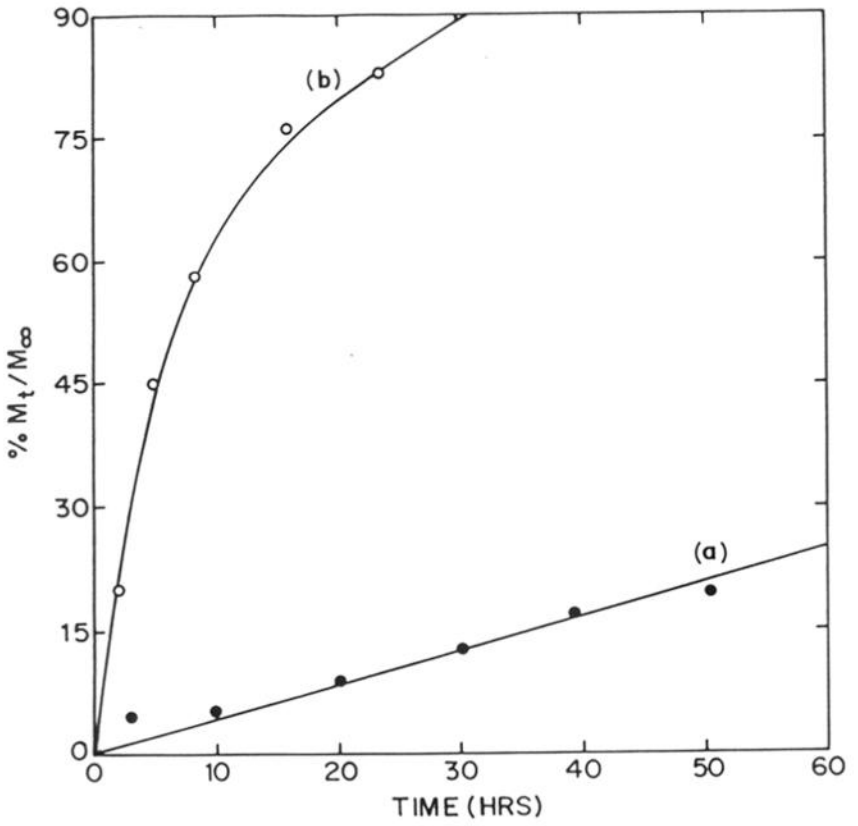


Figure 3.10 : The enhanced rate of hydrolysis of the ester by introducing a spacer. The release of p-nitrophenol from : (a) P(HEMA-4CS-6ACA-PNP) and (b) P(HEMA-4CS-6ACA-PNP-NVIm).

3.10.0 pH responsive catalytic hydrogels

Extensive efforts have been directed in the past to devise hydrogels which respond to diverse stimuli such as pH, temperature, light etc. (Osada, 1993). pH and temperature responsive release of active ingredients from hydrogels has been demonstrated. pH sensitive hydrogels are based on polyelectrolytes which swell or shrink in response to pH. The active ingredient to be released is uniformly dispersed in the polymer matrix. At an appropriate pH, the polymer swells and releases the active ingredient. In the present case the molecule that is released is chemically linked and its release is preceded by the hydrolysis. The catalytic activity of imidazole depends on the number of neutral imidazole atoms which in turn depends on the pH. Thus the release of p-nitrophenol can be kinetically controlled by varying the pH. The release profiles of p-nitrophenol from P(HEMA-PNPVB-NVIm) in media of decreasing pH are shown in figure 3.11. It is evident that in the acidic range the imidazole molecules are protonated and lose their catalytic activity resulting in negligible hydrolysis of the ester.

3.11.0 Concluding remarks

Understanding the catalytic activity of enzymes such as chymotrypsin and lysozyme has guided the efforts to design hydrolytic polymer catalysts which would eventually replicate enzyme activity.

In this chapter, we have directed our efforts to design catalytic hydrogels. These have been based on organizing the polymer structure as to bring the substrate and the catalytic group in proximity by polymerization of the charge transfer complex between N-vinyl imidazole and different active p-nitrophenyl esters with 2-hydroxyethyl methacrylate. These groups are located on the same chain next to each other. It has been shown that these hydrogels exhibit enhanced hydrolysis rates as compared to the hydrogels without a catalytic group. Moreover, it has also been

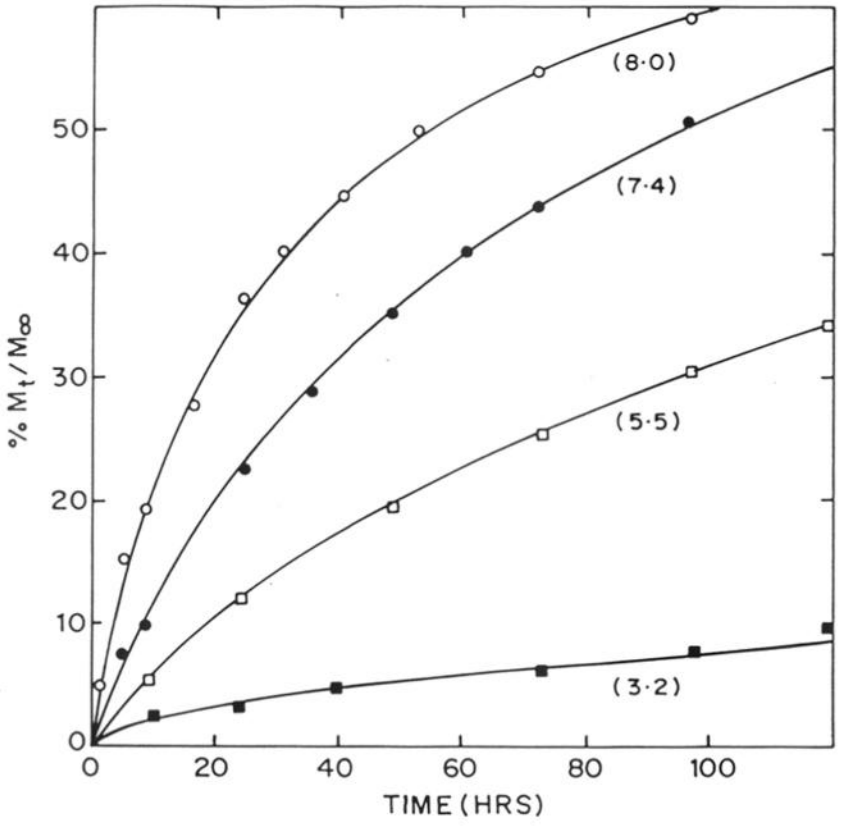


Figure 3.11 : The pH dependent release of p-nitrophenol from the catalytic hydrogels P(HEMA-PNPVB-NVIm).

demonstrated that the hydrolysis takes place under mild alkaline conditions and the catalytic activity of imidazole is a function of pH. The framework developed in this work would be useful for the design of pH responsive drug delivery systems.

CHAPTER IV

Catalytic hydrogels as enzyme mimics : Molecular imprinting effects

4.0.0 Introduction

Enzyme catalyzed reactions are characterized by high substrate specificity and rates, resulting from the chain conformation and cooperative effects that enhance the activity of the functional groups present at the active site (Czarnik, 1988).

In the earlier chapter we have discussed the synthesis and catalytic activity of reactive hydrogels prepared by polymerizing a charge transfer complex between the substrate and the catalyst with HEMA. These catalytic hydrogels exhibited enhanced activity but did not have any substrate specificity, nor were cooperative effects among the catalytic groups present. Therefore they cannot be looked upon as enzyme mimics in the true sense.

The creation of enzyme models with the aid of synthetic macromolecules involves the synthesis of polymers with a defined arrangement of functional groups. A synthetic binding site should possess a three-dimensional scaffolding that creates a binding pocket and provides a framework for positioning functional groups. To create such binding sites in polymers, the methods of template polymerization and molecular imprinting were developed by various researchers (Wulff 1986[a], Mosbach 1987, Belokon 1982). These have been described in details in the chapter I.

We have modified these techniques to design catalytic hydrogels for the hydrolysis of ester and amides. The substrates chosen were substituted benzoate esters of 2-hydroxyethyl methacrylate. They were copolymerized with the catalytic groups containing monomer and excess HEMA by manipulating the synthetic procedures, to design catalytic hydrogels.

4.1.0 Experimental work

4.1.1 Materials

P-amino benzoic acid, p-nitro benzoic acid, p-nitroaniline, p-nitrophenol, β-alanine, nicotinic acid, L-Histidine, ethylene glycol, diethylene glycol, isobutyric acid, $CoCl_2.6H_2O$ were obtained from local suppliers. 2-hydroxyethyl methacrylate, N-vinyl imidazole, methacrylic acid, Azobisisobutyronitrile were obtained from Aldrich Chemical Co., USA

4.1.2 Monomer synthesis

4.1.3 Synthesis of 2-methacryloyl ethyl p-nitrobenzoate (PNP) and 2-methacryloyl ethyl p-aminobenzoate (PAP)

2-methacryloyl ethyl p-nitro benzoate and 2-methacryloyl ethyl p-amino benzoate were synthesized as reported (Shah et al 1990[b]).

4.1.4 Synthesis of N-methacryloyl β-alanyl p-nitroanilide (PNA)

β-alanine was reacted with methacryloyl chloride under the Schotten-Baumann conditions to yield N-methacryloyl β-alanine. This was then condensed with p-nitroaniline using dicyclohexyl carbodiimide.

Molecular formula : $C_{13}H_{15}N_3O_4$ (mol. wt. 277)

IR (nujol) : 1650 cm^{-1} (amide carbonyl), 1760 cm^{-1} (acid carbonyl), 3550 cm^{-1} (acid hydroxyl, stretching), 1170 cm^{-1} (CH_3 rocking).

^1H NMR ($CDCl_3$): 5.3 δ (dd, C=CH), 5.45 δ (dd, C=CH), 2.1 δ (3H, CH_3), 1.7 δ (m, 4H, CH_2 groups), 7.6 δ (s, 1H, NH proton)

4.1.5 Synthesis of print molecules

4.1.6 Synthesis of 2-isobutyryl ethyl nicotinate (print molecule for PNP)

Monoesters of ethylene glycol and diethylene glycol with isobutyric acid were synthesized by the following procedure. In a 500 ml round bottom flask, 200 ml chloroform, 16.6 gm ethylene glycol, 37.8 gm Na_2SO_4, 30.8 gm Na_2SO_3 were mixed together and stirred. To this, 20 gm isobutyryl chloride in the form of 50% chloroform solution was added dropwise over a period of 30 minutes. The temperature of the reaction mixture was maintained between 35-40°C. The salts were filtered and the chloroform solution was concentrated under reduced pressure to yield an oily liquid.

Molecular formula : $C_6H_{12}O_3$ (mol. wt. 132)

IR (neat) : 1725 cm^{-1} (ester carbonyl), 3400 cm^{-1} (hydroxyl group).

6.6 gm 2-hydroxy ethyl isobutyrate was taken in 75 ml dry ethyl acetate. To it 9.225 gm nicotinic acid and 10.5 ml triethyl amine were added and the mixture was cooled to 0°C. 14.28 gm dicyclohexyl carbodiimide was added in portion and the temperature was maintained between 0-5°C for 5 hrs. and then at room temperature for 24 hrs. The urea salt was filtered. The ethyl acetate solution was washed with cold dilute Na_2CO_3 twice, then with cold water and dried over anhydrous sodium sulfate for 24 hrs. Ethyl acetate was concentrated under reduced pressure to yield an oily liquid. Yield obtained was 70%.

Molecular formula : $C_{12}H_{15}O_4N$ (mol. wt. 237)

IR (Neat) : 1735 cm^{-1} and 1740 cm^{-1} (ester carbonyls)

^1H NMR ($CDCl_3$) : 2.8 δ (m, CH_3), 4.3 δ (m. CH_3-CH), 5.5 δ and 6.1 δ (dd, methylene protons), 7.2-8.7δ (3H, aromatic, pyridine protons).

4.1.7 Synthesis of N-isobutyryl β-alanyl 2-aminopyridine (print molecule for PNA)

β-alanine was condensed with isobutyryl chloride under the Schotten-Baumann conditions and the resulting amide was then condensed with 2-aminopyridine using dicyclohexyl carbodiimide

Molecular formula	:	$C_{12}H_{17}N_3O_2$ (mol. wt. 235)
IR (Nujol)		1650 cm^{-1} (amide carbonyl), 1645 cm^{-1} (amide carbonyl), 1170 cm^{-1} (m, CH_3 rocking)
^1H NMR ($CDCl_3$)		2.8 δ (m, CH_3 protons), 4.3 δ (m, CH_3-CH), 5.5 δ and 5.8 δ (dd, methylene), 7.5-8.0 δ (3H, aromatic, pyridine protons), 7.6 δ (s, 1H, NH proton).

4.1.8 Polymer synthesis

4.1.9 Soluble polymers

P(HEMA-PAP-VIm), P(HEMA-PAP) with and without $CoCl_2.6H_2O$ were synthesized as follows:

HEMA was purified using standard procedures for removing trace amount of ethylene glycol dimethacrylate (Pinchuk et al 1984). Solution polymerization in methanol with 20% monomer concentration and 0.1 % AIBN as the initiator was carried out. The polymers were obtained by precipitation in petroleum ether. The initial monomer composition taken for polymerization of the individual polymers was the same as described for the gels in Table 4.1. Co^{++} was extracted with cold aqueous dilute HCl.

Table 4.1

Feed compositions of monomers for hydrogel syntheses[a)]

Polymer	HEMA	PAP	NVIm	$CoCl_2.6H_2O$
PAP_1	4.22	0.5	0.170	0.106
PAP_2	4.30	0.5	0.170	--
PAP_3	4.50	0.5	--	--

a) All the quantities are in gm. wt

Table 4.2

Feed compositions of monomers for hydrogel syntheses[a)]

Polymer	HEMA	NVIm	$CoCl_2.6H_2O$	Print molecule I	Print molecule II	MA-His	MAA
PNP_1	2.88	0.30	--	0.750	--	--	--
PNP_2	3.34	0.30	--	0.750	--	--	--
PNP_3	2.74	0.30	--	--	0.889	--	--
PNP_4	1.31	0.60	0.376	1.50	--	--	--
PNP_5	2.17	--	0.249	0.750	--	--	--
PNP_6	2.43	--	--	0.750	--	0.705	0.2

a) All the quantities are in gm. wt

4.1.10 Synthesis of catalytic hydrogels by template polymerization

PAP, NVIm and $CoCl_2$ were mixed together in 10 ml methanol and stirred for 1 hour. The mixture was added to a test tube and methanol was evaporated under reduced pressure. A blue coloured complex was isolated. To this complex, appropriate quantities of HEMA and AIBN were added. The test tube was purged with nitrogen gas for 10 minutes and polymerized at 65°C for 16 hours. The polymers were isolated by breaking the test tube. They were cut into discs of 1 mm nominal thickness.

Co^{++} was extracted from the discs in 1% bipyridyl solution in methanol. The discs were then dried at 60°C for 24 hours.

Hydrogels synthesized similarly excluding $CoCl_2.6H_2O$ and NVIm were used as controls. The individual monomer feed composition for different polymers is described in Table 4.1.

4.1.11 Synthesis of catalytic hydrogels by molecular imprinting

A complex comprising print molecules, the catalyst (NVIm or MA-His) with Co was prepared in methanol as described earlier. The deep blue complex was isolated and copolymerized with HEMA at 65°C, using 0.1 % AIBN (based on the total wt of the monomers) as the initiator. The polymer discs were subjected to repeated extraction in methanol and 2,2' bipyridyl solution. These discs were soaked in a 1% acetone solution of the substrates PNP or PNA for 12 hours. The PNP and PNA that was absorbed in the discs was polymerized by exposing the discs to γ irradiation from a Co^{60} source (0.25 Mrad/hr) for 6 hours. The monomer polymerized with the polymer through the unreacted vinyl groups present in the backbone (Dhal et al 1995). The unreacted monomers were extracted in acetone. The initial monomer and the print molecule compositions taken for preparation of the catalytic hydrogels are summarized in Table 4.2.

4.1.12 Hydrolysis studies

The hydrolysis of p-amino benzoate ester from soluble polymers was studied in a mixed solvent system composed of ethanol and phosphate buffer (0.01 M) in the ratio 40:60 vol/vol, at 37°C. The p-aminobenzoic acid liberated was detected at λ_{max} = 260 nm on a Shimadzu 240 UV spectrophotometer.

Hydrolysis of the substrates from the hydrogel discs was carried out in a jacketed vessel maintained at 37°C. The hydrolyzed products p-nitrobenzoic acid, p-amino benzoic acid and p-nitroaniline were monitored at λ_{max} = 270 nm, 265 nm and 380 nm respectively on a Shimadzu 240 UV spectrophotometer. The total amount of the product that was released in the solution at time t, viz (M_t) was determined from the appropriate calibration curve. The amount of substrate incorporated in the disc was taken as M_∞. The percentage hydrolysis % M_t / M_∞ was plotted against time.

4.2.0 Results and discussion

4.2.1 Catalytic hydrogels through template polymerization

2-methacryloyl ethyl p-amino benzoate (PAP) and N-vinyl imidazole and $CoCl_2.6H_2O$ in the mole ratio 2:2:1 were added in methanol. PAP and NVIm possess a donor nitrogen group which can coordinate with cobalt giving rise to a blue coloured complex. Cobalt can bind to two PAP and two NVIm molecules. This complex was isolated by removing methanol under reduced pressure. The complex was then polymerized with excess HEMA containing a small amount of crosslinker EGDMA (1 %) to give an insoluble polymer. Cobalt was eluted from the polymer discs using methanolic solution of 2, 2' bipyridyl. Total removal of cobalt was confirmed by spectroscopic measurements. The discs were then suspended in phosphate buffer of pH = 8 and the hydrolysis of poly (PAP) was followed by monitoring the p-amino benzoic acid that was released in the solution. This was compared with the release from P(HEMA-PAP) and P(HEMA-NVIm-PAP) synthesized

without using cobalt. As is very clear from figure 4.1 (a,b) there was only a marginal enhancement in the release of p-amino benzoic acid from P(HEMA-PAP-NVIm) prepared without using cobalt as compared to P(HEMA-NVIm) indicating that no interaction between the substrate and the catalyst occur in the polymer. The imidazole groups and the ester groups are randomly distributed in the polymer and no catalytic activity is observed

On the other hand when the hydrolysis of poly (PAP) from the polymer P(HEMA-PAP-NVIm) prepared in presence of cobalt was carried out, it was observed that there was an enhancement in the rate of release of p-amino benzoic acid (figure 4.1 c). This indicated that in presence of cobalt, the substrate and the catalytic groups are brought in vicinity and this conformation is retained during polymerization.

To elucidate the structure of the catalytic hydrogel, we prepared three soluble polymers of identical compositions as that of the hydrogels P(HEMA-PAP), P(HEMA-PAP-NVIm) without $CoCl_2$ and P(HEMA-PAP-NVIm) with $CoCl_2.6H_2O$ respectively. These polymers were soluble in a mixed solvent system comprising ethanol and phosphate buffer (0.01 M). The hydrolysis of the ester was followed in ethanol/buffer, 40:60 vol/vol at 37°C. It was observed that there was no significant catalytic activity in the templated polymer as compared to the conventional polymers (figure 4.2). This indicated that in the hydrogels prepared using cobalt, a small amount of crosslinker was essential to stabilize the conformation.

To substantiate this further, we reconstituted the hydrogel by complexing the soluble polymers in presence of cobalt and 1 % EGDMA. The catalytic activity of the regenerated hydrogel is evident (figure 4.3 a,b). Soluble polymers P(HEMA-PAP) and P(HEMA-NVIm) when mixed together, complexed with Co^{++} and crosslinked, did not yield similar catalytic activity. Soluble polymer P(HEMA-PAP-NVIm) prepared in the absence of cobalt was also constituted by adding the same amount of cobalt and EGDMA. This polymer did not show any significant catalytic activity (figure 4.3 c).

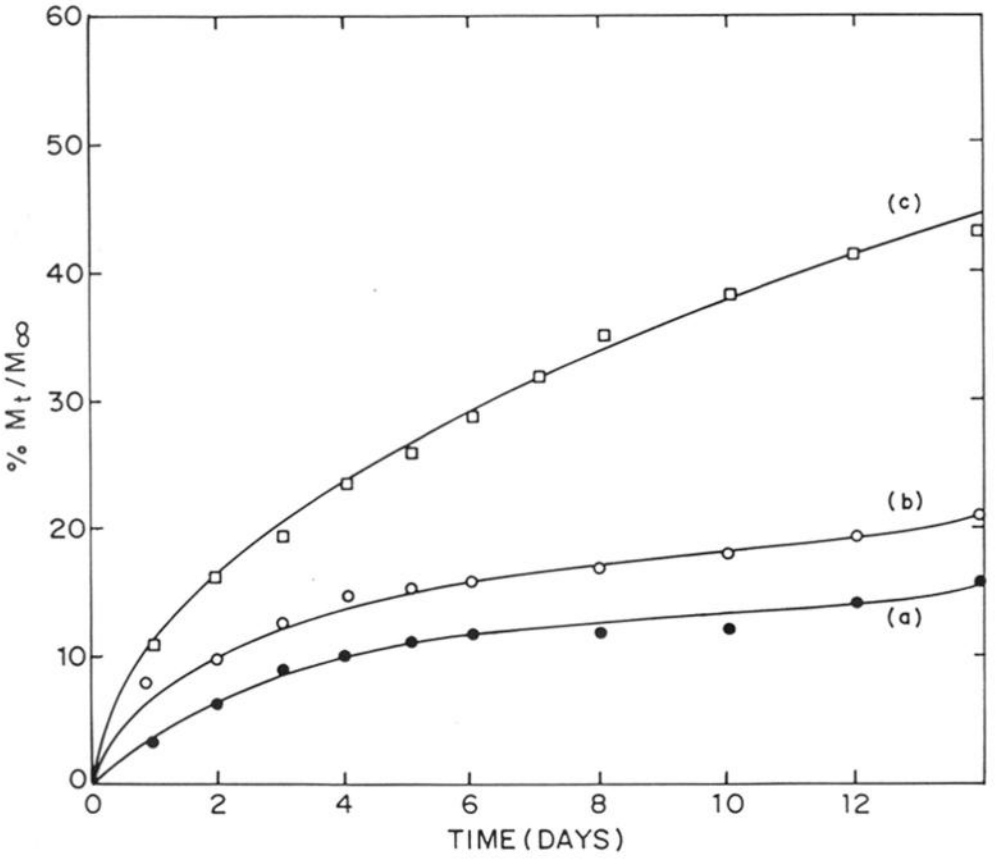


Figure 4.1 : The hydrolysis of PAP from the hydrogels (a) P(HEMA-PAP) conventional polymer, (b) P(HEMA-PAP-NVIm) prepared in the absence of cobalt and (c) P(HEMA-PAP-NVIm) prepared by template polymerization.

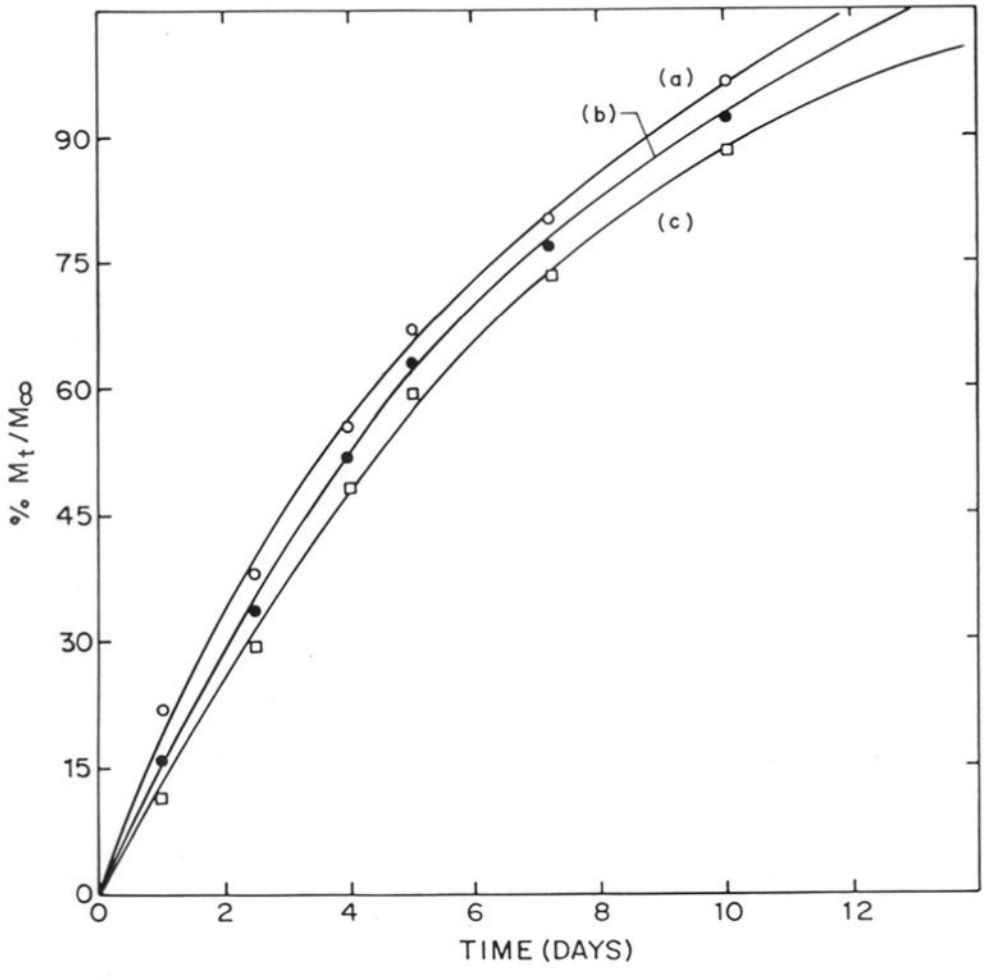


Figure 4.2 : The hydrolysis of PAP from the soluble polymers in 40 : 60 vol / vol, ethanol / phosphate buffer.

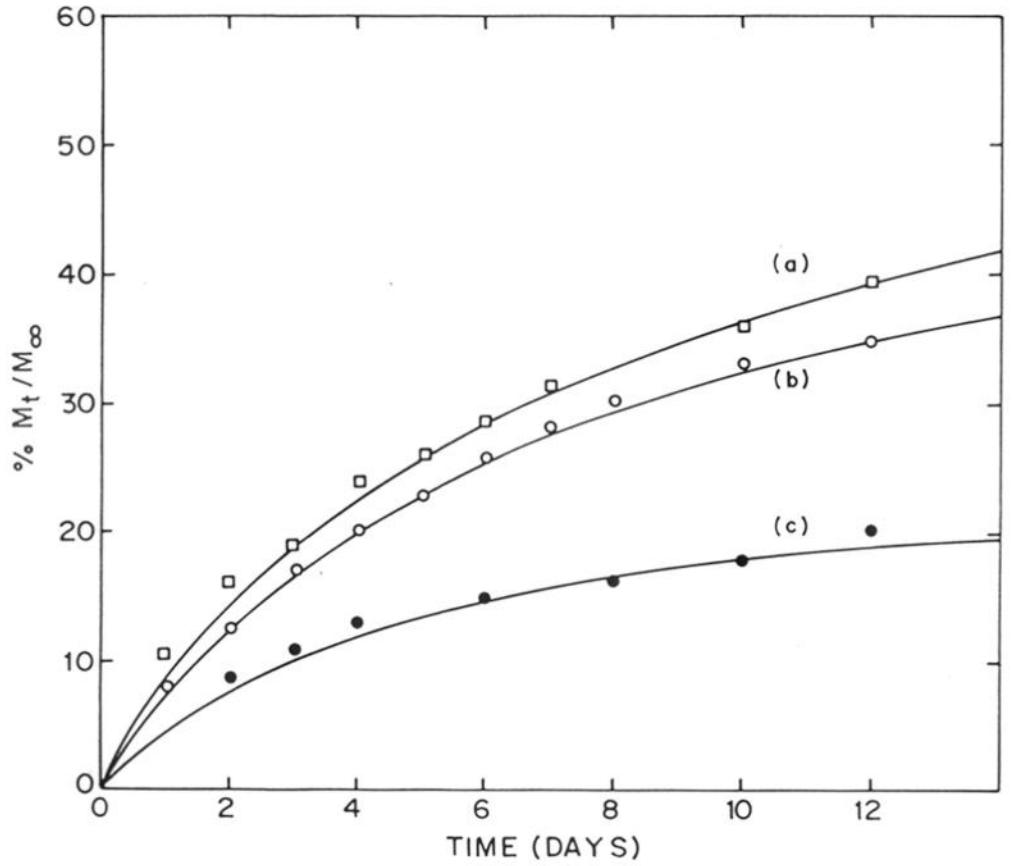


Figure 4.3 : The hydrolysis of PAP from the hydrogels reconstituted from the solutions by crosslinking in the presence of cobalt :- (a) P(HEMA-PAP-NVIm) original catalytic hydrogel, (b) P(HEMA-PAP-NVIm) reconstituted from the soluble templated polymer and (c) reconstituted hydrogel from soluble P(HEMA-PAP) and P(HEMA-NVIm).

These results indicate that crosslinking is essential for the stability of the conformation. NVIm and PAP have to be present on the same chain.

4.2.2 pH sensitive catalytic activity

As described in the earlier chapter, the catalytic activity of imidazole depends on the number of neutral imidazole groups present. The effect of pH on the rate of hydrolysis of the p-amino benzoate ester was investigated. As is clearly seen in figure 4.4, the rate of release of p-amino benzoic acid decreased as the pH decreased. In the acidic pH range there was practically no release. This indicated that the rate of hydrolysis in the acidic pH range was negligible and the imidazole groups were protonated.

4.2.3 Synthesis of catalytic hydrogels by molecular imprinting

In the above example, the p-amino benzoate ester and the catalyst were brought in proximity by complex formation with cobalt. This was because the amino group of the ester and the imidazole nitrogen of the catalyst could bind simultaneously to cobalt. If a substrate does not possess any binding groups, the above technique is not applicable. For example, p-nitro benzoate ester of HEMA, PNP, cannot form a complex with any metal ion. To bring such substrates and catalysts in proximity we modified the technique of molecular imprinting reported by Leonhardt et al (1987).

We synthesized a print molecule namely N-isobutyryl ethyl nicotinate for the substrate 2-methacryloyl ethyl p-nitro benzoate. The structural similarity between the two is evident (figure 4.5).

A cobalt complex comprising N-vinyl imidazole, the print molecule and $CoCl_2.6H_2O$ in the mole ratio 2:2:1 was formed in methanol. This was then copolymerized with HEMA. Cobalt and the print molecule were extracted from the polymer discs in methanol and 2,2' bipyridyl. The dry hydrogel discs were soaked in an acetone solution of PNP for 12 hours. The discs were removed

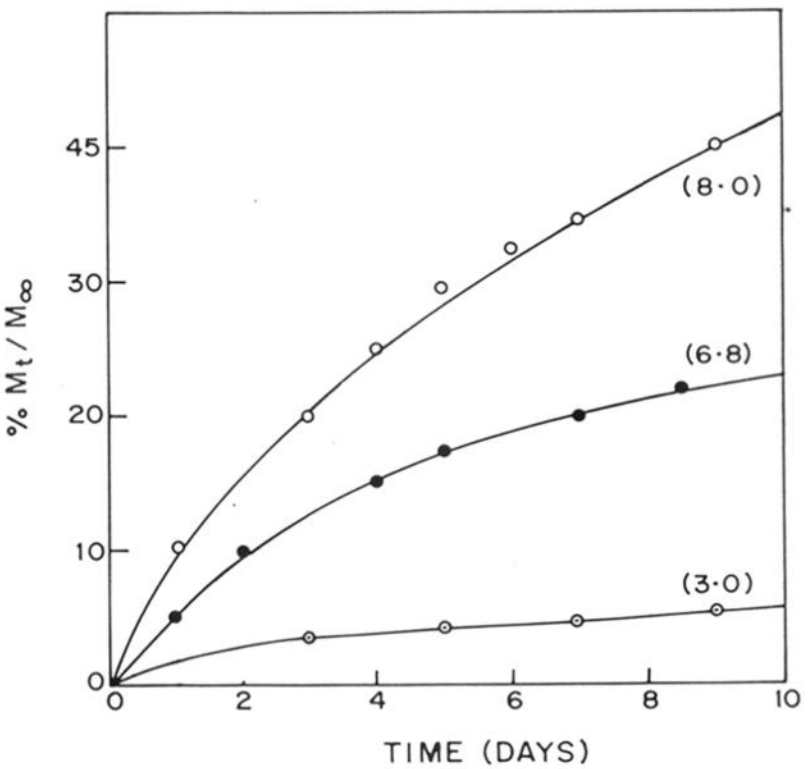
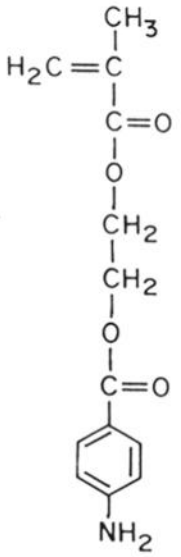
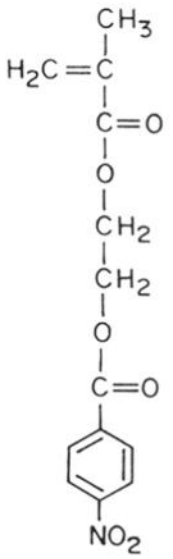


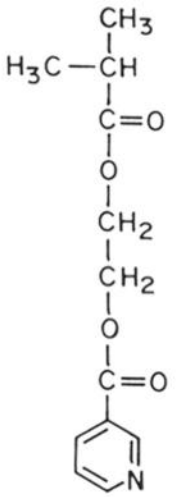
Figure 4.4 : The pH dependent hydrolysis of PAP from P(HEMA-PAP-NVIm).



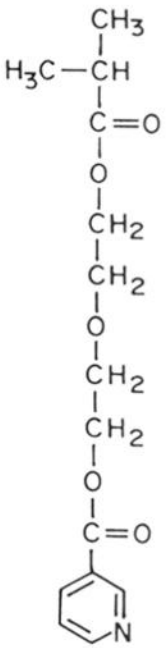
2-methacryloyl ethyl p-amino benzoate
(PAP)



2-methacryloyl ethyl p-nitrobenzoate
(PNP)



N-isobutyryl ethyl nicotinate
(Print I)



N-isobutyryl diethyl nicotinate
(Print II)

Figure 4.5 : Structures of different substrates and the print molecules.

and exposed to a Co^{60} source of 0.25 Mrad/hr for 6 hrs. The unreacted monomer, if any, was extracted in acetone. To emphasize the role of complexation, hydrogels excluding $CoCl_2$ from the reaction mixture were synthesized and used as controls. A comparison of the rates of hydrolysis of PNP from the two hydrogels indicated difference in the catalytic effect of imidazole in the two polymers. Hydrogels prepared using $CoCl_2$ exhibited a marked enhancement in the rate of hydrolysis as indicated from the appearance of p-nitro benzoic acid in solution, compared to the control prepared in the absence of cobalt (figure 4.6 a,b). The imprint molecule and NVIm bind to cobalt to give a blue coloured complex which on copolymerization with HEMA gave crosslinked hydrogels. Extraction of the print molecule creates cavities that would correspond to the size of the print and hence that of PNP. PNP was absorbed within the hydrogel from an acetone solution. It occupies the cavities formed by the elution of the print molecule. These cavities already contained the NVIm group which resulted in the enhanced rates of hydrolysis. The evidence for PNP occupying the cavities was obtained indirectly by comparing the amount of print molecule that was used during the complex formation and the amount of PNP that was loaded in the hydrogel. It was observed that the amount of PNP in the catalytic hydrogel coincided with the amount of print molecule used during polymerization.

To illustrate the generality of the concept, hydrogels containing a pendent amide linkage were synthesized using N-isobutyryl β-alanyl 2-amino pyridine as the print molecule and 2-methacryloyl β-alanyl p-nitroanilide as the substrate. The enhanced rate of hydrolysis of the amide (figure 4.7 a,b) illustrates the activity of the catalytic hydrogel for the amide hydrolysis as well.

To investigate the selectivity of these hydrogels, two catalytic hydrogels were synthesized using an identical procedure. In one case N-isobutyryl ethyl nicotinate was used as the imprint and in the other N-isobutyryl diethyl nicotinate was used as the imprint (See figure 4.5).

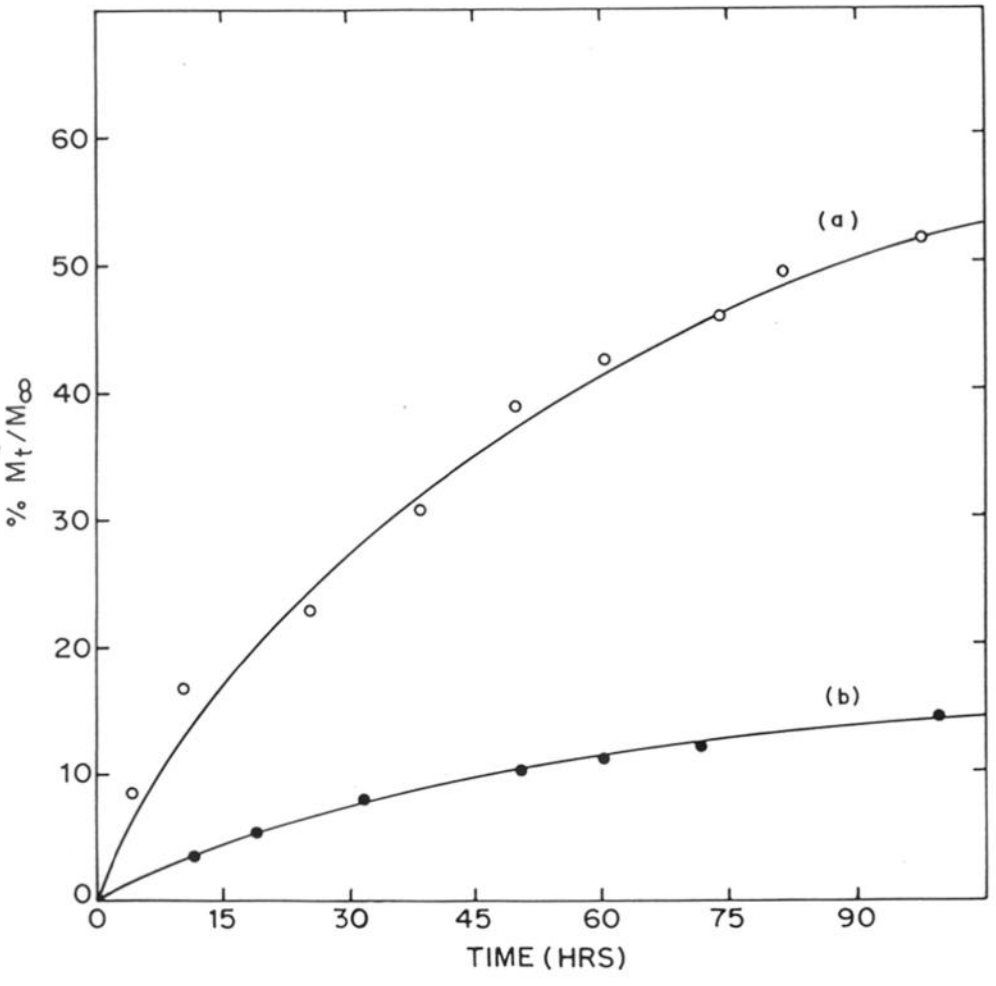


Figure 4.6 : Hydrolysis of PNP from hydrogels in phosphate buffer : (a) P(HEMA-PNP-NVIm) prepared by imprinting and (b) P(HEMA-PNP-NVIm) prepared by conventional polymerization.

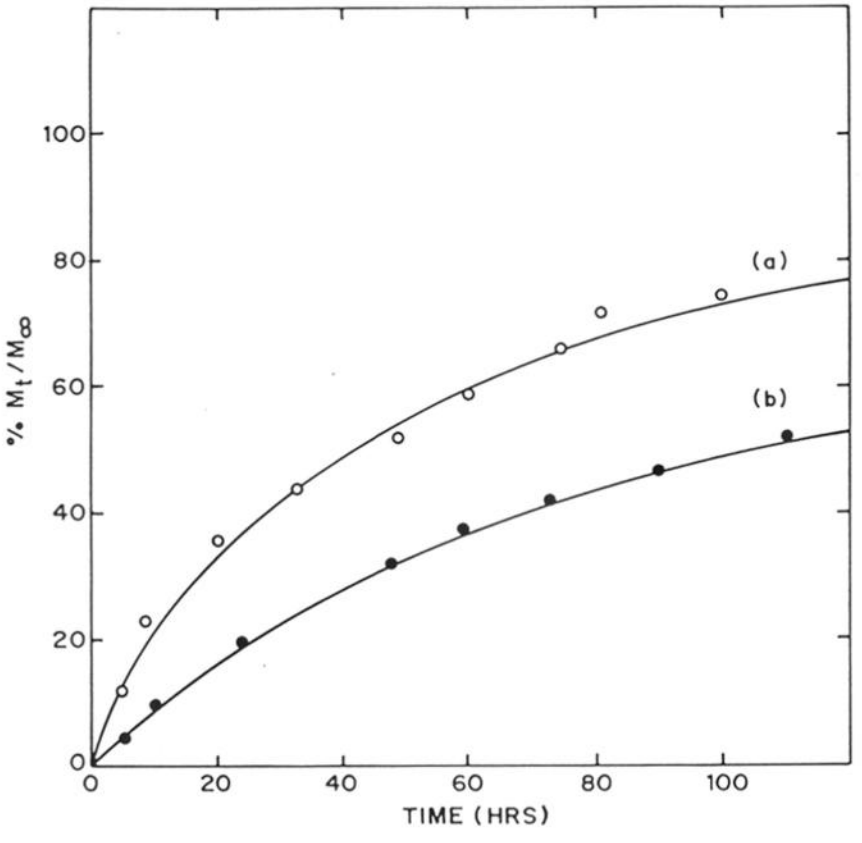


Figure 4.7 : Hydrolysis of PNA from hydrogels in phosphate buffer (0.01M, pH = 8). (a) P(HEMA-MA-His-PNA) prepared by imprinting and (b) P(HEMA-MAA-MA-His-PNA) prepared by conventional polymerization.

The substrate was PNP for both the hydrogels. The observed rate of hydrolysis was higher when N-isobutyryl ethyl nicotinate was used as the print molecule (figure 4.8 a,b), compared to when N-isobutyryl diethyl nicotinate was the imprint.

The selectivity could arise from the shape of the cavity and binding site, or from the spatial arrangement of functional groups in the binding site (Wulff et al 1991, Shea et al 1989). In our case the interactions between the metal ion and the print molecule is non-covalent, the selectivity is expected to result from the size and shape of the cavity. Since N-isobutyryl ethyl nicotinate and PNP have identical size and shape higher rates of hydrolysis was observed.

4.2.4 Enhanced catalytic activity due to cooperative effects

While the catalytic hydrogels reported herein exhibit hydrolytic activity, the rates are too slow in comparison to the rates of enzyme catalyzed reactions. In enzyme, a cooperative effect among various functional groups leads to enhanced reaction rates. It has been demonstrated that by introducing hydroxyl and carboxyl functional groups in soluble imidazole polymers the esterolytic activity could be enhanced (Overberger et al 1965, 1967). By incorporating similar functional groups during the complex formation with cobalt, it should be possible to enhance the reaction rate. A hydrogel comprising N-methacryloyl histidine (MA-His), methacrylic acid (MAA), $CoCl_2.6H_2O$ and the print molecule in the mole ratio 1:1:2:1 was synthesized. Methacrylic acid can also form a complex with cobalt. After incorporating PNP in the hydrogel hydrolysis was carried out. Clearly the rate of hydrolysis is enhanced by the incorporation of MA-His and MAA (figure 4.9 a,b). Thus as seen in enzymatic reactions it is possible to enhance the catalytic activity by cooperative effects.

4.2.5 Modulating catalytic activity of the hydrogels

In many of the industrial applications of enzymes it is essential to switch on and off the activity of the enzyme at will. This can be achieved by changing the pH of the reaction but there is a danger of enzyme denaturation. We have clearly demonstrated that the activity of the hydrogel

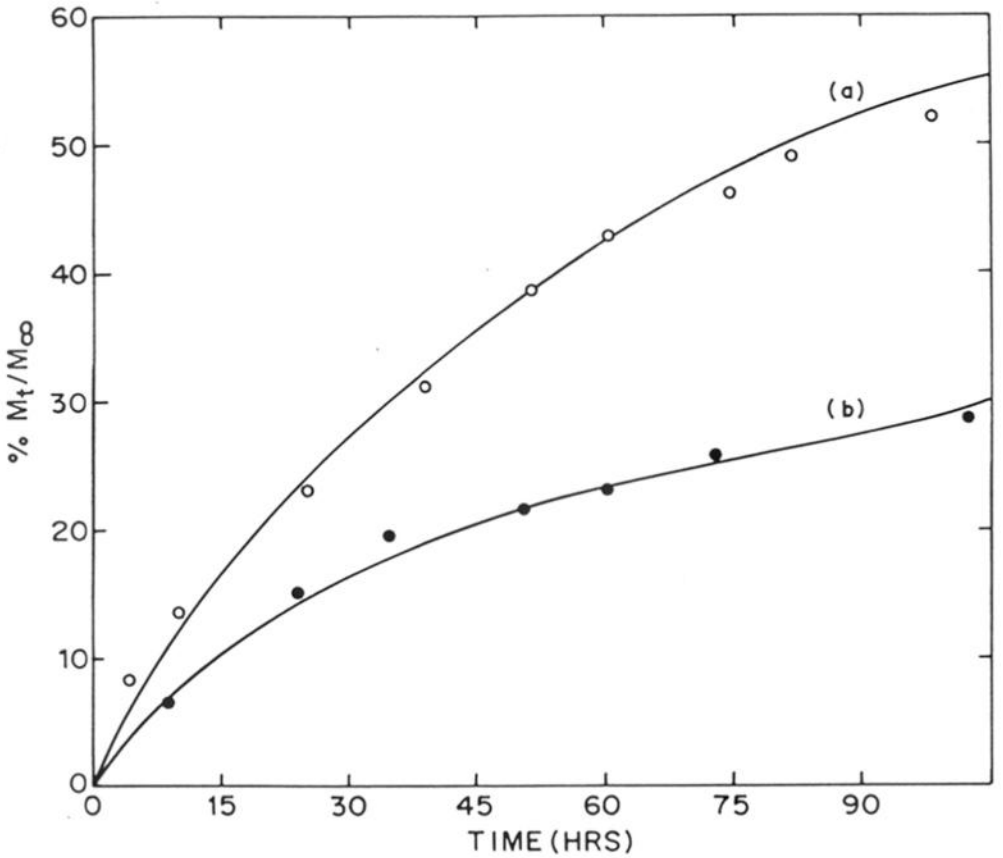


Figure 4.8 : The effect of shape of the print molecule used on the hydrolysis of PNP from hydrogels (a) P(HEMA-PNP-NVIm) prepared using N-isobutyryl ethyl nicotinate as the print molecule and (b) P(HEMA-NVIm-PNP) prepared using N-isobutyryl diethyl nicotinate as the print molecule.

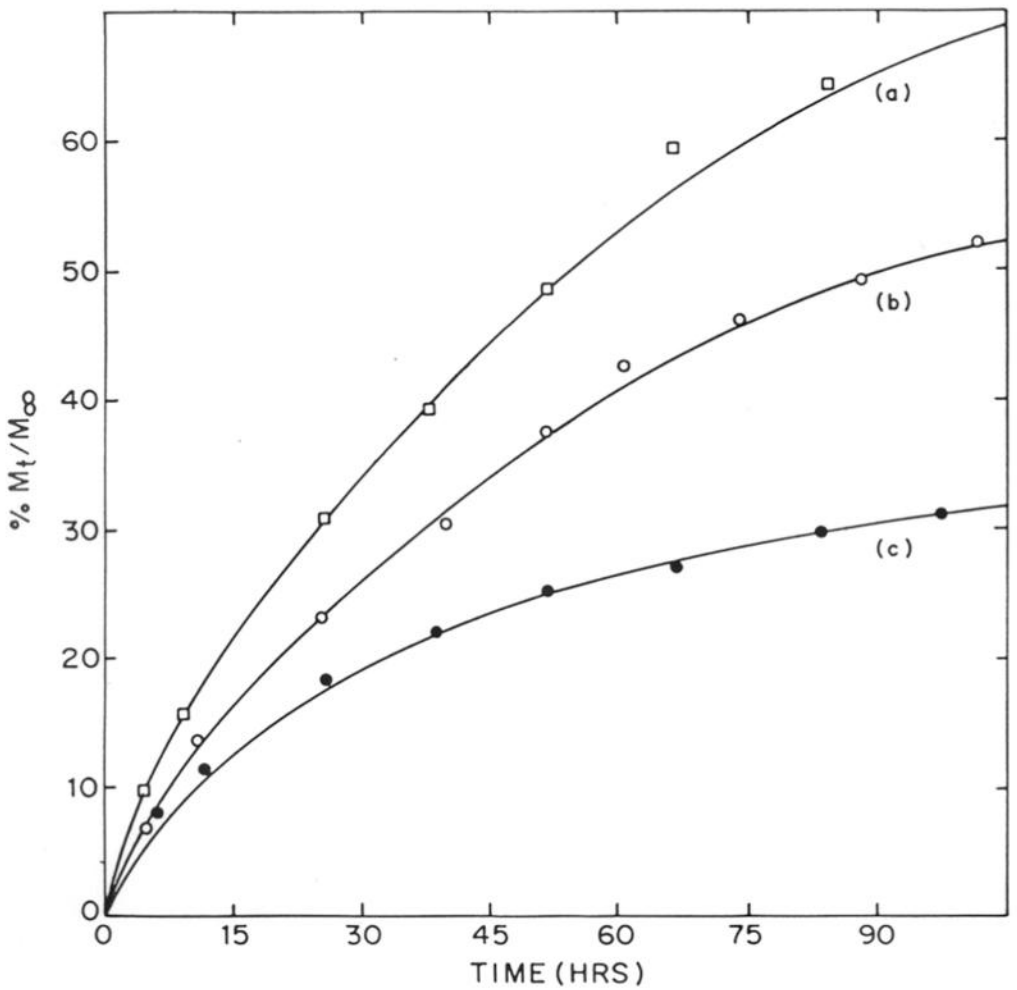


Figure 4.9 : The enhanced rate of hydrolysis of PNP by incorpora ing methacrylic acid and methacryloyl-histidine during the complex formation (a) P(HEMA-PNP-MA-His-MAA) imprinted polymer, (b) P(HEMA-PNP-NVIm) imprinted polymer and (c) P(HEMA-PNP-MA-His-MAA) conventional polymer.

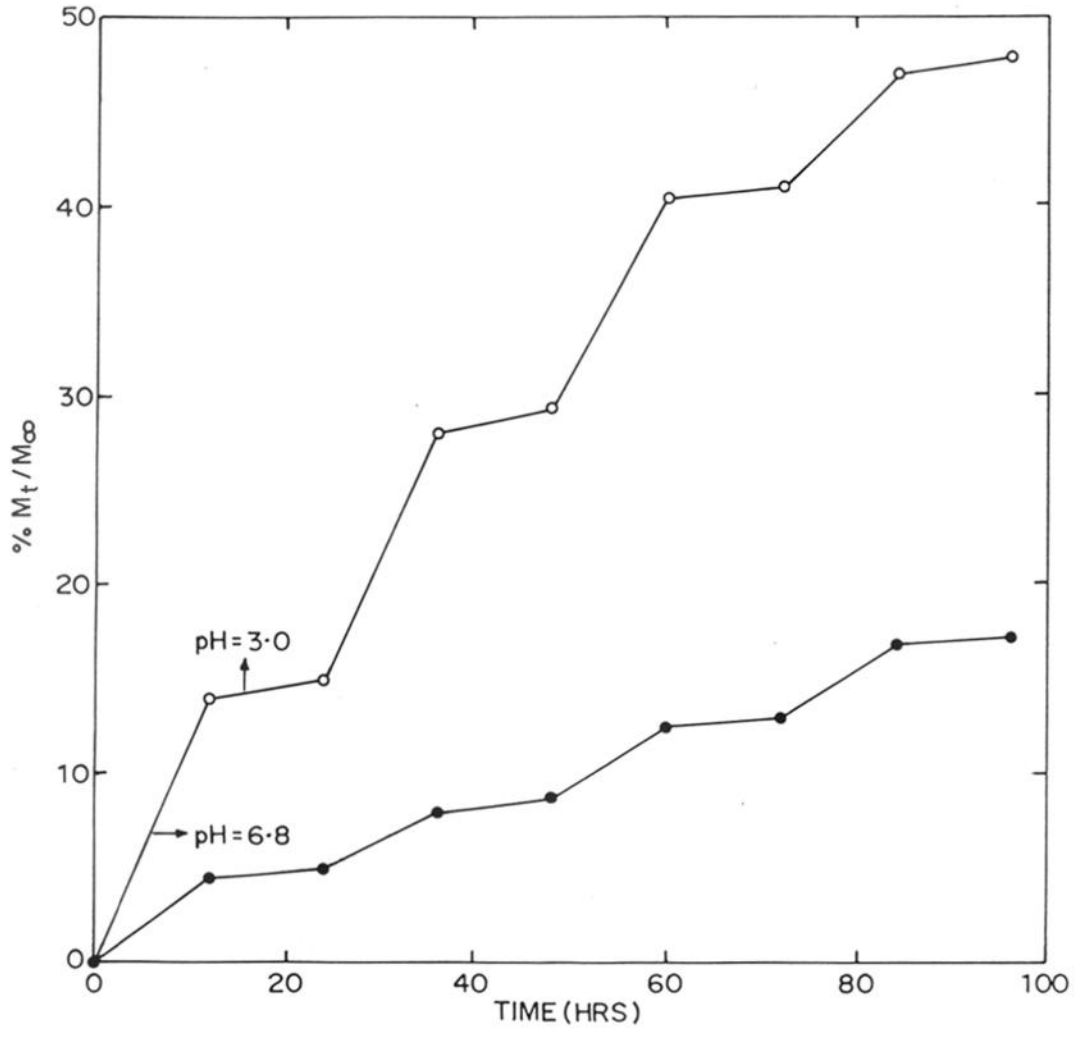


Figure 4.10 : The pH responsive catalytic activity of the hydrogels. (a) P(HEMA-PNP-MAA-MA-His) imprinted polymer and (b) P(HEMA-PNP-MAA-MA-His) conventional polymer.

arises from the neutral imidazole groups. Since the substrate molecule are bound to the polymer it would be easier to change the pH of the reaction. We carried out the hydrolysis reaction of the catalytic hydrogel and the control in pH = 6.8 and pH = 3.0 alternately. In case of the catalytic hydrogel, the activity could be switched on-off reversibly by switching the pH (figure 4.10 a,b). Moreover, the response of the catalytic hydrogel was sharper than that for the control.

4.3.0 Concluding remarks

In this chapter, the synthesis of catalytic hydrogels based on template polymerization and molecular imprinting was undertaken. The imidazole catalyzed hydrolysis of polymeric ester and amide substrates from the catalytic hydrogels and the control polymers was investigated. The catalytic hydrogels exhibit features similar to enzymes like enhanced reaction rates, substrate specificity, cooperative effects and pH dependent activity. The studies could be extended to design catalytic hydrogels for the hydrolysis of pendent chain drug delivery systems.

CHAPTER V

Catalytic hydrogels exhibiting α-chymotrypsin like activity

5.0.0 Introduction

In the earlier chapters we have reported the synthesis and the hydrolytic activity of hydrogels containing imidazole groups. The substrate and the catalytic groups were brought in proximity by the formation of a charge transfer complex, by template polymerization with a metal ion or by molecular imprinting and their conformation was frozen by polymerization. The activity of imidazole containing hydrogel was further enhanced by involving carboxyl groups during the complex formation step. These hydrogels exhibited features of enzyme like catalytic activity but they cannot be called as true enzyme mimics, since they do not exhibit mechanistic analogy nor match the catalytic activity of any enzyme.

Enzymes are considered to be nature's most efficient catalysts because of their specificity and very high reaction rates under mild conditions. The enzyme creates a binding pocket for the substrate by chain folding and the cooperative effects among the functional groups at the active site leads to very high reaction rates. Any enzyme mimic should therefore possess a substrate binding site and the functional groups responsible for the catalytic activity in a defined position.

In the past there have been efforts to mimic enzymes using cyclodextrins, crown ethers, cryptands and monoclonal antibodies (D'Souza et al 1985, Lehn, 1988, Pollock et al 1986).

Enzyme activity in soluble polymers or polymer micelles has also been demonstrated (Kunitake et al 1976*, Van den Berg 1988). Molecularly imprinted polymers have emerged as potential enzyme mimics (Wulff 1986, Mosbach 1994, Shea 1994, Robinson et al 1989). These have been described in details in the chapter I. But in all these the enzyme activity is not always replicated nor the mechanistic analogy to it demonstrated. In a recent report Parton et al (1994) designed polymer encapsulated zeolite mimic of cytochrome C which replicated the enzyme activity and exhibited mechanistic analogy. A hydrogel based mimic of pyridoxal enzyme was reported earlier (Belokon et al 1980, 1982).

In this chapter we show how catalytic hydrogels can be further modified to mimic α-chymotrypsin, both in mechanistic terms and catalytic activity. α-chymotrypsin was chosen because the active site is well characterized and the mechanism of the catalytic activity is well defined. α-chymotrypsin cleaves the peptide bond neighbouring the aromatic amino acids like L-phenyl alanine, L-tyrosine and L-tryptophan. It also has a specificity towards the L isomers. In the active site of chymotrypsin, a decisive role is played by three amino acids. They are Serine-195, Histidine-57 and Aspartic acid-102. This triad represents the charge relay system. The functional groups present in the hydrogels studied so far are the hydroxyl group in HEMA, imidazole in MA-His and the carboxylic group in MAA. They can constitute the active site of chymotrypsin, viz. Serine-195, histidine-57 and aspartic acid-102 respectively. Thus the catalytic hydrogels have the potential to mimic α-chymotrypsin.

5.1.0 Experimental work

5.1.1 Monomer synthesis

5.1.2 Synthesis of N-methacryloyl 6-amino caproyl L-phenyl alanine p-nitro phenol (MA-6ACA-L-PheAl-PNP)

a) Synthesis of 6-methacryloyl amino caproic acid.

13.8 gm 6-aminocaproic acid was dissolved in 150 ml water. A pH meter electrode was dipped into this solution. In another flask 17.6 gm sodium hydroxide was dissolved in 75 ml water. 9.8 ml of methacryloyl chloride was added dropwise from a dropping funnel to the amino acid solution. The pH of the reaction medium was maintained by adding the concentrated sodium hydroxide solution by a micropipette. After completing the addition the reaction mixture was stirred at room temperature for 2 hours. The aqueous solution was washed with diethyl ether twice to remove methacrylic acid. 250 ml ethylacetate was used to extract the product from the aqueous layer and dried over anhydrous sodium sulphate for 24 hours. Ethylacetate was concentrated under

reduced pressure at 4°C to half the volume and then added to excess petroleum ether. Oily layer was separated which was treated with excess petroleum ether and kept at -20°C. White solid separated out which was recrystallized from ethyl acetate/petroleum ether.

Molecular formula	:	$C_{10}H_{17}O_3N$ (mol. wt. 199)
M.P.	:	52°C
^1H NMR ($CDCl_3$)	:	5.45 δ and 5.5 δ (dd, 2H, CH_2=CH, 2.00 δ (s, 3H,CH_3), 1.70 δ (m, 4H, methylene), 1.25 δ (m, 6H, methylene), 6.4 δ (1H, NH proton).

b) **Synthesis of 4-nitrophenyl 6-methacryloyl amino caproate MA-6ACA-PNP**

9.95 gm N-methacryloyl 6-amino caproic acid, 10.4 gm p-nitrophenol was added to 100 ml dry ethyl acetate. The temperature was maintained between 0-5°C. 12.5 gm dicyclohexyl carbodiimide was added in portions and the mixture was stirred below 5°C for 3 hours. The urea salt that precipitated was filtered. The ethylacetate layer was washed with ice cold water and cold dilute HCl twice and dried over anhydrous sodium sulphate for 24 hours. The ethylacetate layer was concentrated under reduced pressure at 4°C to 20 ml and then poured in excess petroleum ether. Pale yellow amorphous solid separated out which was recrystallized from ethanol/hexane.

Molecular formula	:	$C_{16}H_{20}O_5N_2$ (mol. wt. 320)
M.P.	:	77°C
^1H NMR ($CDCl_3$)	:	5.5 δ and 5.6 δ (dd, 2H, CH_2=CH), 2.00 δ (3H, CH_3), 1.70 δ (m, 4H, methylene), 1.3 δ (m, 6H, methylene), 7.9-8.2 δ (4H, aromatic protons).

c) **Sodium salt of L-phenylalanine**

16.5 gm L-phenylalanine and 8.4 gm $NaHCO_3$ was dissolved in distilled water and stirred for 2 hours. The solution was concentrated under reduced pressure to give sodium salt of L-phenylalanine.

d) **Synthesis of 6-methacryloyl amino caproyl phenyl alanyl 4-nitrophenol (MA-6ACA-L-PheAl-PNP)**

8 gm 4-nitrophenyl 6-methacryloyl amino caproate was dissolved in 80 ml DMSO/dioxane/water (1.5:1:3). 5 gm of the sodium salt of L-phenylalanine was added and stirred at room temperature for 48 hours. The mixture was acidified with concentrated citric acid solution to pH 3.0. N-methacryloyl 6-aminocaproyl L-phenyl alanine (MA-6ACA-L-PheAl) that precipitated out was filtered and washed with diethyl ether. 8.65 gm MA-6ACA-L-PheAl was dissolved in 75 ml dry ethyl acetate. 5.2 gm p-nitrophenol and 6.18 gm, dicyclohexyl carbodiimide was added and stirred at 0-5°C for 3 hours. The ethyl acetate layer was washed with cold water and cold dilute HCl and dried over anhydrous sodium sulphate for 24 hours. Ethyl acetate was evaporated under reduced pressure. Crude MA-6ACA-L-PheAl-PNP was recrystallized from ethyl acetate/petroleum ether.

Molecular formula	:	$C_{25}H_{29}O_6N_3$ (mol. wt. 467)
M.P.	:	115°C

^{1}H NMR ($CDCl_3$) : 5.4 δ and 5.6 δ (dd, 2H, CH_2=CH), 2.00 δ (3H, CH_3), 1.7 δ (m, 4H, methylene), 1.3 δ (m, 6H, methylene), 3.55 δ (-CH_2 group of phenyl alanine), 2.4 δ (m, CH-CH_2), 6.9-7.4 δ (5H, aromatic phenyl alanine protons), 7.3-8.3 δ (4H, aromatic nitrophenyl protons).

5.1.3 Synthesis of the print molecule N-isobutyryl 6-amino caproyl L-phenylalanyl 2-amino pyridine (IBA-6ACA-L-PheAl-2AP)

a) Synthesis of 6-isobutyryl amino caproic acid (IBA-6ACA)

13.8 gm 6-amino caproic acid was reacted with 10.5 ml isobutyryl chloride as described previously in 5.1.2 a). The crude product was recrystallized from ethanol/water.

Molecular formula : $C_{10}H_{19}O_3N$ (mol. wt. 201)

M.P. : 53°C

b) Synthesis of 6-isobutyryl amino caproyl p-nitrophenol

10.4 gm IBA-6ACA, 10.4 gm p-nitrophenol was added to 80 ml dry ethylacetate, 12.3 gm dicyclohexyl carbodiimide was added in portion and the mixture was stirred for 3 hours at 0-5°C. The precipitated urea salt was filtered. Ethyl acetate was washed with ice cold water and cold dilute HCl to remove excess p-nitrophenol, and dried over anhydrous sodium sulphate for 24 hours. Ethyl acetate was evaporated under reduced pressure to yield crude IBA-6ACA-PNP which was recrystallized from ethyl acetate/petroleum ether.

Molecular formula : $C_{16}H_{22}O_5N_2$ (mol. wt. 322)

M.P. : 81°C

c) **Synthesis of Isobutyryl 6-amino caproyl L-phenyl alanyl 2-amino pyridine**

7.85 gm IBA-6ACA-PNP was dissolved in a mixture of DMSO/dioxane/water (1.5:1:3) and 5 gm of sodium salt of L-phenyl alanine was added and the mixture was stirred at room temperature for 48 hours. The mixture was acidified with concentrated citric acid solution to pH 3.0. The precipitate of IBA-6ACA-L-PheAl was washed with diethyl ether and recrystallized from THF/ether.

8.7 gm IBA-6ACA-LPheAl was dissolved in 75 ml dry ethyl acetate. To it 3 gm of 2-amino pyridine and 6.18 gm dicyclohexyl carbodiimide was added. This was stirred at 0-5°C for 3 hours. Ethyl acetate was washed with cold dilute HCl and then twice with cold water and dried over anhydrous sodium sulphate and concentrated under reduced pressure.

Molecular formula	:	$C_{24}H_{32}O_3N_4$ (mol wt. 424)
^{1}H NMR ($CDCl_3$)	:	2.8 δ (m, 3H, methyl group), 4.3 δ (m, 4H, CH_3-CH), 1.7 δ (m, 4H, -CH_2 groups), 1.3 δ (m, 6H, -CH_2 group), 2.3 δ and 3.2 δ (4H, phenyl alanine group), 7.2-8.7 δ (4H, aromatic pyridine protons).

5.1.4 Synthesis of 6-methacryloyl amino caproyl L-phenyl alanyl 4-nitro anilide (MA-6ACA-L-PheAl-PNA)

The procedure for the synthesis of the amide was identical to that of the ester MA-6ACA-L-PheAl-PNP.

5.1.5 Polymer synthesis

5.1.6 Synthesis of catalytic hydrogel

0.189 (0.0022 M) gm methacrylic acid (MAA), 0.490 gm (0.0022 M) N-methacryloyl histidine (MA-His) and 0.864 (0.0044 M) N-isobutyryl 6-amino caproyl L-phenylalanine 2-amino pyridine (IBA-6ACA-L-PheAl-2AP) were taken in a 25 ml beaker. 5 ml methanol and 0.523 gm $CoCl_2.6H_2O$ (0.0022 M) were added and the mixture was stirred at room temperature for 1 hour. Methanol was evaporated under reduced pressure to give a deep blue coloured complex. 2.5 gm complex was taken in a test tube. To it 2 gm HEMA, 0.5 ml ethylene glycol dimethacrylate (EGDMA) and 0.8 ml t-butyl hydroperoxide was added. The test tube was purified with nitrogen for 10 minutes and then immersed in a water bath maintained at 65°C. Polymerization was carried out for 16 hours. The polymer was isolated in the form of a cylindrical rod by breaking the test tube and cut into discs of 0.09 - 0.11 cm thickness on a lathe. Co^{++} and the print molecule was extracted in methanol containing 1% 2,2' bipyridyl. The complete removal of cobalt and the print molecule was confirmed by spectroscopic measurements. The discs were dried in vacuum oven at room temperature for 48 hours. Discs were then soaked in an acetone solution of MA-6ACA-L-PheAl-PNP. The monomer that was sorbed was polymerized by γ irradiation from a Co^{60} source of 0.25 Mrad/hr for 6 hours. The unreacted monomer was extracted in acetone. A hydrogel of a similar composition but without using $CoCl_2$ was synthesized and used as the control.

5.1.7 Synthesis of the polymer mimic microspheres

A complex of HEMA (0.0022 M), MAA (0.0022 M), MA-His (0.0022 M) and IBA-6ACA-L-PheAl-2AP (0.0022 M) with $CoCl_2.6H_2O$ (0.0022 M) was synthesized as described earlier. 2.5 gm complex, 2 gm HEMA, 0.5 ml EGDMA and 0.125 gm AIBN were mixed together in a 25 ml beaker. Air was expelled from the mixture using nitrogen.

In a three neck round bottom flask equipped with a stirrer, 47 ml, 35% NaCl solution was added. To it 3 ml 1 N NaOH and 2 gm $MgCl_2.6H_2O$ was added to give a uniform suspension of $Mg(OH)_2$. The monomer mixture was added dropwise to the suspension and stirred at 1000 rpm at 75°C for 3 hours to yield microspheres. Yield obtained was 3.8 gm. Cobalt and the print molecule were extracted from the microspheres as described earlier. The microspheres were dried in a vacuum oven at room temperature. Microspheres having an identical composition of HEMA, MA-His, MAA and the print molecule, without $CoCl_2.6H_2O$ were synthesized as the controls.

5.1.8 Synthesis of a U.V. sensitive polymer mimic

2.5 gm of the complex prepared as described earlier, 0.8 gm 2-methacryloyl ethyl p-phenyl azobenzoate (HEAZ), 1.2 gm HEMA, 0.5 ml EGDMA and 0.125 gm AIBN were mixed together and purged with nitrogen for 10 minutes. The monomer mixture was then added to the $Mg(OH)_2$ suspension as described earlier. Microspheres were isolated by filtration. Cobalt and the print molecule were extracted as described earlier.

5.1.9 Synthesis of surface imprinted mimic

The macroporous support of P(GMA-EGDMA) for the mimic was synthesized as reported by Svec et al (1975). 3.6 gm glycidyl methacrylate (GMA), 8.4 gm EGDMA and 0.120 gm AIBN was mixed with 16 gm cyclohexanol. In a three neck round bottom flask 88 ml 1% polyvinyl pyrrolidone (M.W. 3.6×10^5) was stirred at a speed of 1000 rpm at 70°C. The monomer mixture was added dropwise and the polymerization was performed at 70°C for 2 hours and at 80°C for 6 hours. The mixture was allowed to cool for 2 hours. The spheres were isolated by filtration and washed with water and alcohol repeatedly and dried in a vacuum oven at 40°C for 48 hours. Microspheres in the range 37-45 μ were chosen for all the studies.

HEMA (0.0022 M), MA-His (0.0022 M), MAA (0.0022 M), aIBA-6ACA-L-PheAl-2AP (0.0022 M) were added in 5 ml methanol. To it 0.0022 M $CoCl_2.6H_2O$ and 0.1 % AIBN was added

and the mixture was stirred at room temperature for 1 hour. 1 gm of the P(GMA-EGDMA) microspheres were added to this and gently stirred for 24 hours. The monomer mixture that was adsorbed in the internal surfaces of the microsphere was polymerized either thermally at 75°C for 48 hours or by U.V. light (λ= 280-350 nm), at 4°C for 6 hours. Control polymers without using $CoCl_2.6H_2O$ were also synthesized.

5.1.10 Synthesis of soluble polymers

HEMA was purified to remove EGDMA by the standard procedure (Pinchuk et al 1984). Polymers using the same composition of HEMA, MAA, MA-His, IBA-6ACA-L-PheAl-2AP and $CoCl_2$-$6H_2O$ as described in 5.1.6 were synthesized. The polymerization was carried out in methanol containing 20 % monomer concentration and AIBN as the initiator at 65°C. The polymers were precipitated in petroleum ether and dried in a vacuum dessicator. Cobalt was extracted using cold aqueous dilute HCl.

5.1.11 Intrinsic viscosity measurements

The intrinsic viscosity of the soluble polymers prepared with and without $CoCl_2$ was measured in methanol at 25°C. Viscosity was also measured after adding cobalt chloride externally.

5.1.12 Monomer reactivity ratio measurements

The monomer reactivity ratios for the binary systems HEMA, MAA, HEMA-MA-His and MA-His-MAA were determined using the technique described by Collins et al (1973).

5.1.13 Estimation of the catalytic group concentration in the polymers

The catalytic group concentration in the polymers was based on moles of imidazole present. This was estimated by hydrolyzing a known quantity of the polymer using hydrochloric acid to liberate free histidine which was then estimated by the ninhydrin test for amino acids.

5.1.14 Surface area measurements

The surface area of the macroporous microspheres before and after polymerization of the complex were measured using a mercury porosimeter.

5.1.15 Spectroscopic analysis of the cobalt complex

The cobalt complex of HEMA, MAA, MA-His and IBA-6ACA-L-PheAl-2AP was characterized by the shift in the electronic absorption spectra using a UV-VIS spectrophotometer. The shift in the λ_{max} values on sequential addition of the ligands are summarized in Table 5.1.

5.1.16 ESR spectra of the complex

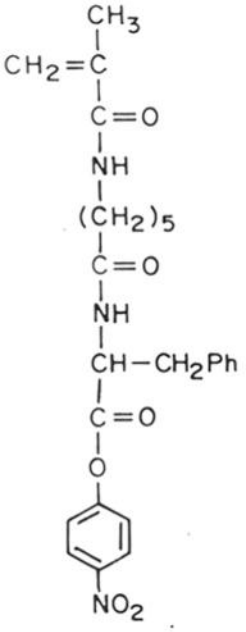
The ESR spectra of the polymerized complex was measured after addition of individual ligands to cobalt at 298°K. The paramagnetic g values of the spin coupling in cobalt are summarized in Table 5.1.

5.1.17 Hydrolysis studies of MA-6ACA-L-PheAl-PNP from the hydrogel

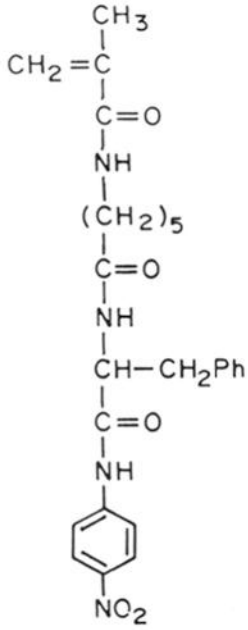
The hydrolysis of the ester from the hydrogel was carried out in a jacketed vessel at 37°C in phosphate buffer of pH 8.0. The extent of hydrolysis was monitored by the release of p-nitrophenol at 400 nm.

5.1.18 Hydrolysis of the ester and amide substrates using the polymer mimic

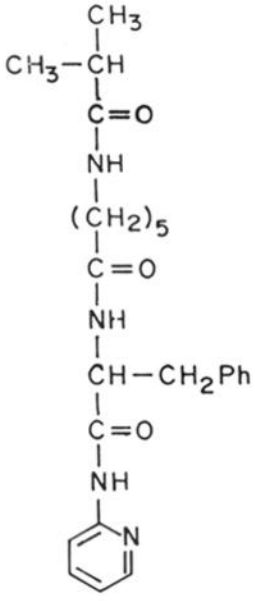
Hydrolysis of the substrates was carried out in ethanol/buffer (40:60 vol/vol) at 37°C. The substrate stock solutions were prepared in ethanol. The hydrolyzed products namely, p-nitrophenol and p-nitroaniline were detected in solution by spectroscopic measurements at λ_{max} 400 nm and 380 nm respectively. αchymotrypsin obtained from Sigma Chemical Co. was used. Hydrolysis of the substrates using U.V. sensitive polymer mimic was carried out in the presence of U.V. light and in the absence of light.



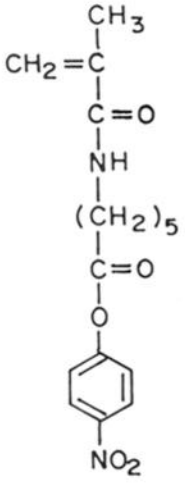
Methacryloyl 6-amino caproyl
L-phenylalanyl p-nitrophenol
(MA-6ACA-L-PheAl-PNP)



Methacryloyl 6-amino caproyl
L-phenylalanyl p-nitroanilide
(MA-6ACA-L-PheAl-PNA)

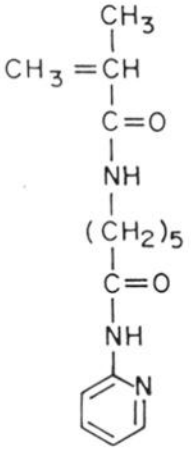


Isobutyryl 6-aminocaproyl
L-phenylalanyl 2-aminopyridne
(IBA-6ACA-L-PheAl-2AP)
Print molecule

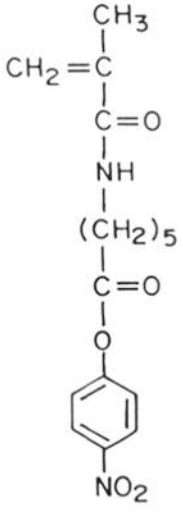


Methacryloyl 6-aminocaproyl
p-nitrophenol
(MA-6ACA-PNP)

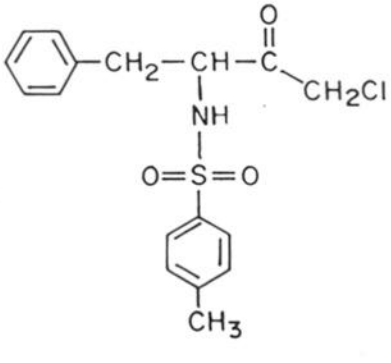
Figure 5.1 : Structures of the substrates and print molecules.



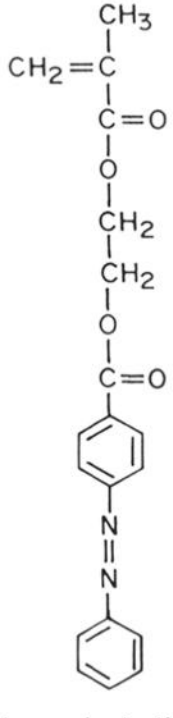
Isobutyryl 6-amino caproyl
2-amino pyridine (IBA-6ACA-2AP)



Methacryloyl 6-amino caproyl
p-nitrophenol (MA-6ACA-PNP)



Tosyl-phenylalanine chloromethyl
Ketone (TPCK)



2-methacryloyl ethyl p-phenyl
azobenzoate (HEAZ)

Figure 5.2 : Structures of the inhibitor, the U.V. sensitive monomer and other substrates and print molecules.

Table 5.1

The shift in the λ_{max} values of cobalt on addition of different ligands and the g values obtained from the ESR spectra*

	Ligands	λ_{max}	g average
1.	$CoCl_2.6H_2O$ (0.001 M) + Methanol (10 ml)	670 nm	2.090
2.	$CoCl_2.6H_2O$ (0.001 M) + Methacrylic acid (0.001 M) + Methanol (10 ml)	665 nm	2.199
3.	$CoCl_2.6H_2O$ (0.001 M) + Methacrylic acid (0.001 M) + 2-Hydroxyethyl methacrylate (0.001 M) + 10 ml methanol	660 nm	2.197
4.	$CoCl_2.6H_2O$ (0.001 M) + Methacrylic acid (0.001 M) + 2-Hydroxy ethyl methacrylate (0.001 M) + N-Methacryloyl histidine (0.001 M) + 10 ml methanol	632 nm	2.180
5.	$CoCl_2.6H_2O$ (0.001 M) + Methacrylic acid (0.001 M) + 2-Hydroxyethyl methacrylate (0.001 M) + N-Methacryloyl histidine (0.001 M) + Isobutyryl 6-amino caproyl L-phenyl alanyl 2-amino pyridine (0.001 M)	628 nm	2.140

* The ESR spectra were measured at 298°K operating at 9.72 GHz.

5.2.0 Results and discussion

5.2.1 Catalytic hydrogel

In the earlier chapter we have seen how the technique of molecular imprinting can be used to design catalytic hydrogels. The substituted benzoate esters of HEMA are not very reactive. In order to investigate the hydrolysis of an active ester we chose N-methacryloyl 6-amino caproyl L-phenylalanyl p-nitro phenol (MA-6ACA-LPheAl-PNP) as the substrate and N-isobutyryl 6-amino caproyl L-phenyl alanine 2-aminopyridine (IBA-6ACA-L-PheAl-2AP) as the print molecule. The structures of the different substrates and the print molecules used are given in figure 5.1 and 5.2. The hydrolysis of the ester was studied in phosphate buffer of pH 8.0, at 37°C. A complex comprising MAA, MA-His, IBA-6ACA-L-PheAl-2AP and $CoCl_2$ in the mole ratio 1:1:2:1 respectively, was prepared. This was further diluted with HEMA and EGDMA and polymerized at 65°C using azobisisobutyronitrile. The print molecule and cobalt were extracted and the substrate MA-6ACA-L-PheAl-PNP was sorbed into the polymer discs and polymerized by γ irradiation.

Figure 5.3 shows the release profile of p-nitrophenol from these catalytic hydrogels as compared to those prepared by conventional polymerization without using cobalt chloride. The rate of hydrolysis of the ester is enhanced in the catalytic hydrogel. In this case MAA, MA-His IBA-6ACA-L-PheAl-2AP were complexed with $CoCl_2$ and polymerized with excess HEMA. After extraction of the print molecule and cobalt, the substrate was polymerized in the cavities created by the print molecule. The enhanced hydrolysis results from the cooperative effects of imidazole and carboxyl groups as described by Overberger et al (1965, 1967). In the hydrogels prepared without using $CoCl_2$ the functional groups are randomly distributed. The cooperative effects cannot be seen and therefore the rates of hydrolysis are lower. The diffusivity of p-nitrophenol from similar HEMA based hydrogels is $\simeq 2.7 \times 10^{-7} cm^2/sec$. The time for diffusion of PNP estimated

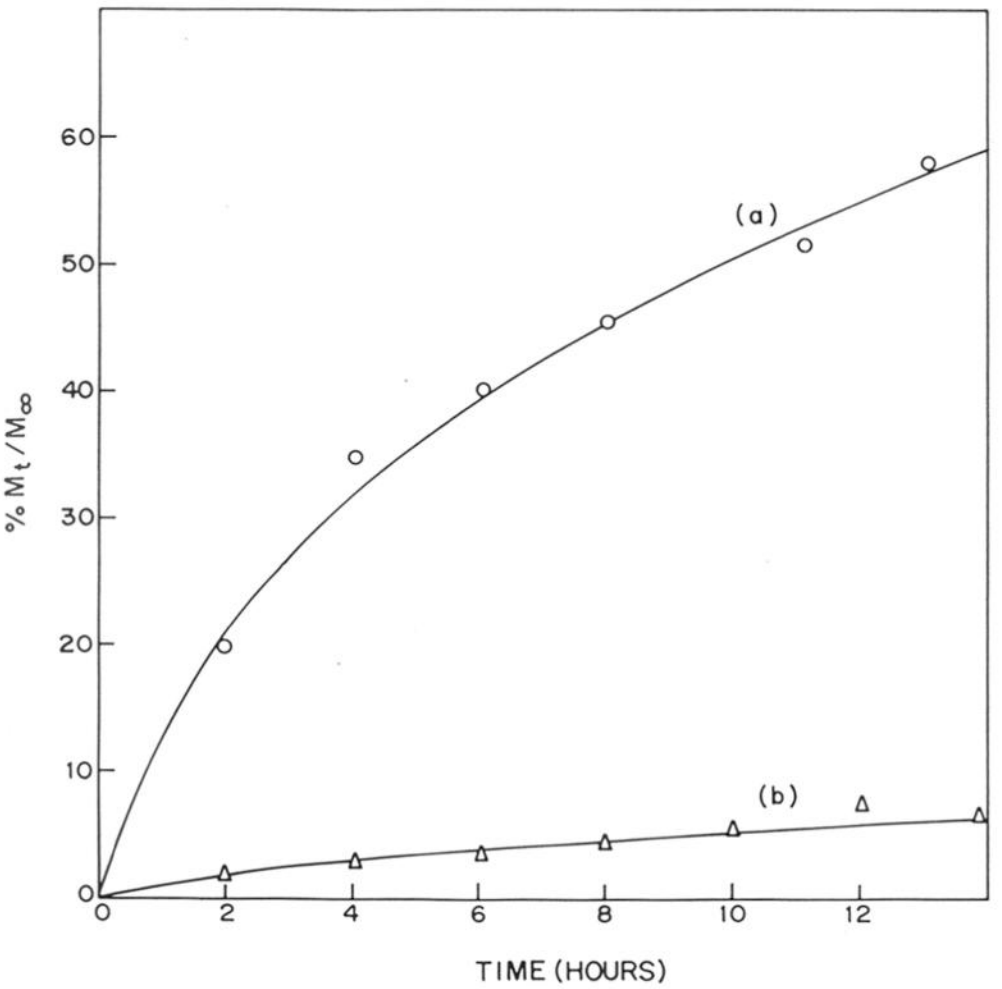


Figure 5.3 : Ester hydrolysis by the catalytic hydrogel synthesized by (a) template polymerization and (b) conventional polymerization.

for a hydrogel disc of thickness 1 mm in 3.5 hrs. 60 % of PNP is released in 14 hours indicating that the rate of hydrolysis is slow. The catalytic activity of imidazole is enhanced by the presence of a carboxyl group.

5.2.2 Catalytic hydrogels exhibiting α-chymotrypsin like catalytic activity

In the catalytic hydrogels designed so far the imidazole group is the catalytically active group whose activity is enhanced by the carboxyl and hydroxyl groups. But in the case of α-chymotrypsin and other serine proteases it in the serine hydroxyl group which is responsible for the activity. In α-chymotrypsin the nucleophilicity of the serine hydroxyl is enhanced by the imidazole group of histidine and the carboxyl group of aspartic acid. These three together constitute the change relay system (Fersht 1985).

Secondly, the catalytic hydrogels used were in the form of polymer discs where the appearance of the reaction product in solution is limited by diffusion. To overcome these limitations, we modified the procedure used for the synthesis of the hydrogel and used these in the form of microspheres.

We prepared a complex of HEMA, MAA, MA-His and IBA-6ACA-L-PheAl-2AP and $CoCl_2$-$6H_2O$ in the mole ratio 1:1:1:1:1. This was diluted with HEMA containing a known amount of EGDMA and AIBN. Dense microspheres (150-210 μm) were prepared by suspension polymerization. Cobalt and the print molecule were extracted from the microspheres. Here HEMA is part of the complex. An analogy between the active site of α-chymotrypsin and the mimic is shown in figure 5.4. The hydroxyl group of serine forms a hydrogen bond with the nitrogen atom of the imidazole nucleus of histidine. Another nitrogen atom of the nucleus is connected with the carboxylic group of aspartic acid through a hydrogen bond. The charge may be displaced along these bonds.

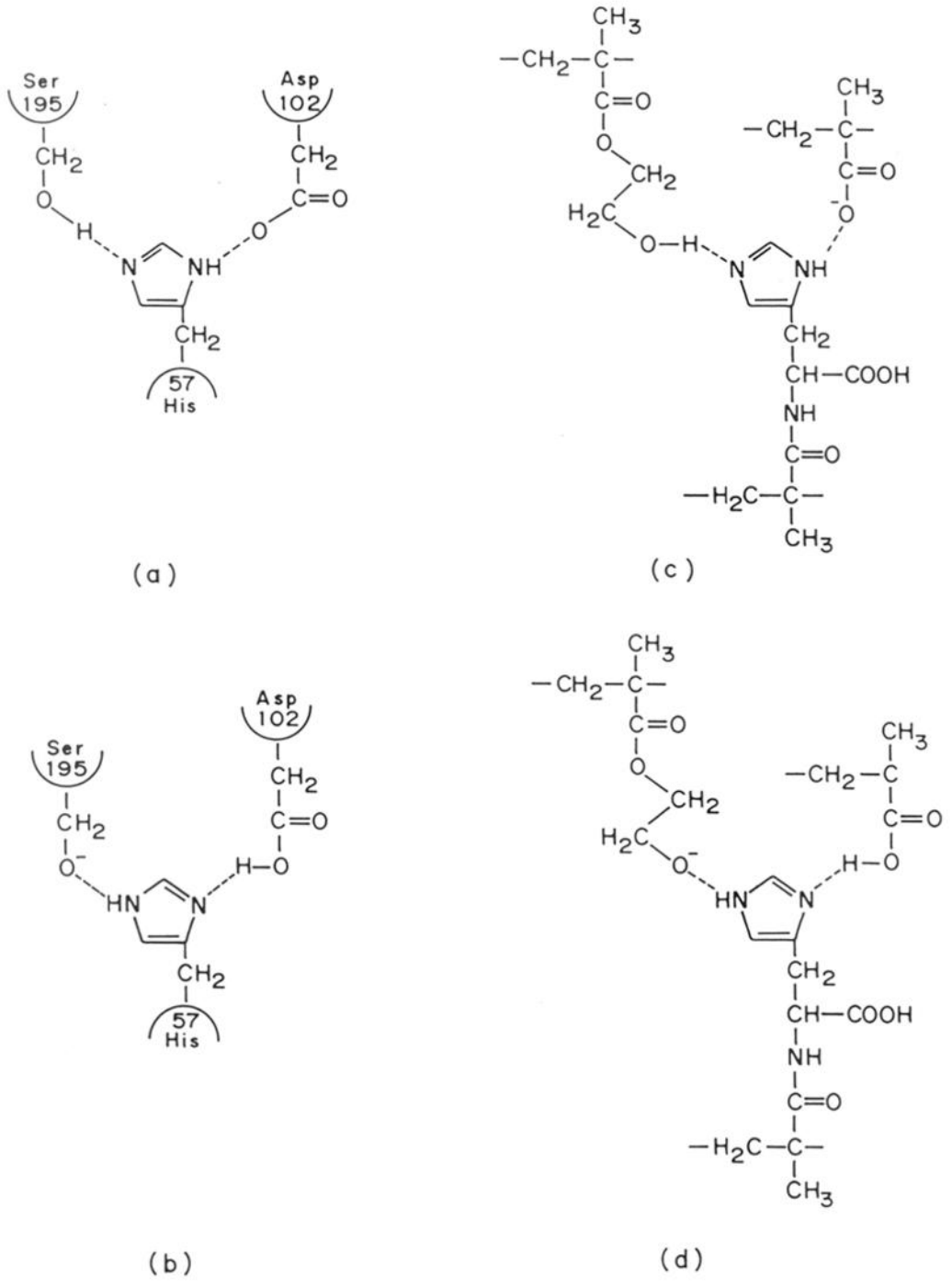


Figure 5.4 : (a,b) The charge relay system in chymotrypsin and (c,d) the catalytic hydrogel.

In the catalytic hydrogels described so far we have polymerized the substrate in the cavities created by the print molecule. In order to compare the catalytic activity with the native enzyme, the hydrolysis of the substrate MA-6ACA-L-PheAl-PNP was carried out in its monomeric form. The microspheres (150-210μm) were added to ethanol / buffer mixture. A known amount of the substrate was added and the hydrolysis was followed till no further p-nitrophenol could be detected. For a comparison the hydrolysis of the ester using control polymer was investigated (figure. 5.5 c). The hydrolysis of same substrate using the native chymotrypsin at identical active site concentration was also studied (figure. 5.5 e). Though the modified template polymerization technique significantly enhanced the observed rate of hydrolysis of the ester, the rates were lower as compared to the chymotrypsin (figure 5.5 d,e). We repeated the experiment using smaller microspheres of particle size (37-45 μm) diameter. The rate of hydrolysis of the substrate was further enhanced (figure. 5.5 b) and it approached nearer to that of α-chymotrypsin.

Since we observed a rate enhancement on decreasing the microsphere size we concluded that the rate of hydrolysis of the ester is limited by the diffusion of the substrate through the microsphere.

5.2.3 The structure of the active site and distribution of the functional groups

To study the complex of cobalt with the different ligand monomers the shift in the electronic absorption spectra of the complex on addition of stoichiometric quantities of the ligands was determined by a UV-VIS spectrophotometer. The structure of the cobalt complex is shown schematically in figure 5.6. In order to study the distribution of the functional groups in the polymer mimic and those in the conventional polymers we synthesized soluble polymers of identical compositions. The intrinsic viscosities of the polymers synthesized in the presence and in the absence of cobalt chloride after removal of cobalt was measured in methanol at 25°C. The viscosity for the polymer prepared in the presence of cobalt was found to be 0.15 dl/g and that of the polymer prepared in the absence of cobalt chloride was 0.13 dl/g. We then added cobalt chloride externally

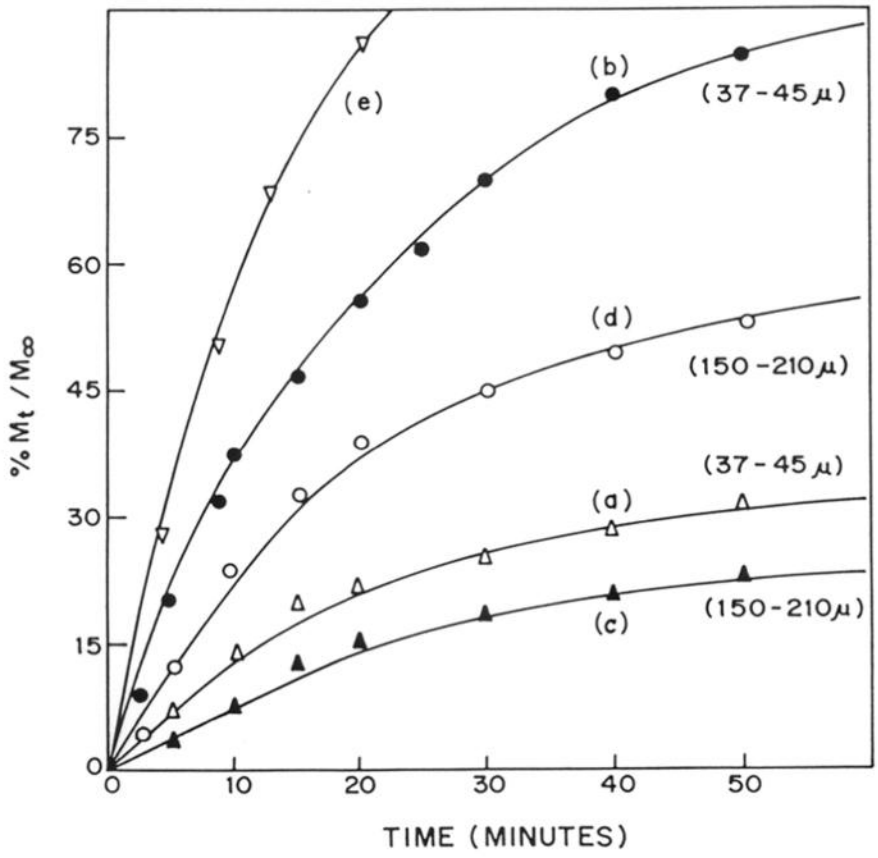


Figure 5.5 : Ester hydrolysis using the catalytic hydrogel microspheres : (a) 37-45 μm, prepared by conventional polymerization, (b) 37-45 μm, prepared by imprinting, (c) 150-210 μm, prepared by conventional polymerization, (d) 150-210 μm prepared by imprinting and (e) using native α-chymotrypsin.

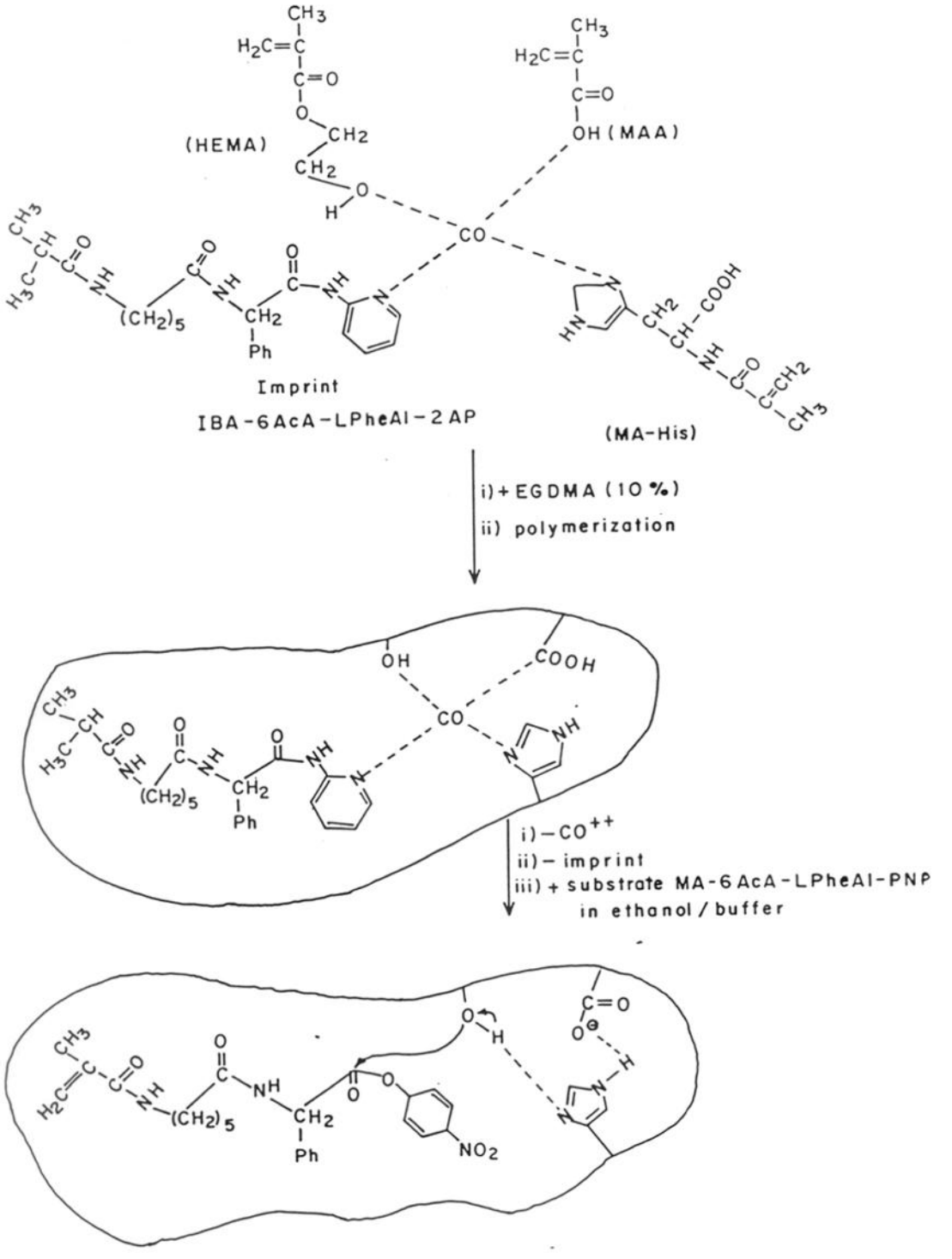


Figure 5.6 : Schematic figure showing the method of polymerization and the hydrolysis of the ester.

to the polymer solution and again measured the viscosities. It was observed that for the polymer prepared in presence of cobalt the viscosity dropped to 0.04 dl/g. Correspondingly the intrinsic viscosity of the polymer prepared in the absence of cobalt chloride decreased only marginally to 0.11 dl/g. This is because during template polymerization the hydroxyl, carboxyl and the imidazole groups form a complex with cobalt. On polymerization these groups are so distributed along the polymer chain that they are brought in close proximity again on adding cobalt. This leads to a collapse of the polymer chain and a decrease in the chain dimension indicated by the drop in viscosity. When the polymerization is carried out in the absence of cobalt, the functional groups are so distributed that they cannot be brought together by adding cobalt externally. Therefore the viscosity of the polymer did not decrease significantly.

To justify this further, we measured the monomer reactivity ratios ($r_1 : r_2$) for the binary systems HEMA : MAA, HEMA : MA-His and MA-His : MAA. They are found to be 1.5 : 0.6, 0.95 : 0.68 and 0.75 : 0.51 respectively. These values indicate a preference for homopolymerization of HEMA but a random copolymer for MAA and MA-His. This clearly indicates that in the conventional polymer the functional groups are randomly distributed and it is only during template polymerization with cobalt, that they are brought in proximity.

Thus in these catalytic hydrogels complexation brings the functional groups in proximity and the conformation is frozen by crosslinking and polymerization, while the print molecule creates the cavity which confers substrate specificity. Therefore these hydrogels mimic enzymes more closely than those described in the earlier chapters.

5.2.4 Catalytic activity of soluble polymers

The hydrolysis of MA-6ACA-L-PheAl-PNP by the soluble polymers was investigated. It was observed that these polymers possess very low activity compared to the hydrogel (figure.

5.7a,b). This is because although the necessary functional groups are present in the soluble polymers, they cannot assume the conformation needed to bring them in vicinity in solution. Moreover, there is no binding site for the substrate in these polymers.

5.3.0 U.V. sensitive catalytic activity of the mimic

It has been well established that polymers containing azo linkages shrink when exposed to U.V. light. Such polymers have been used for enzyme immobilization or encapsulation in order to get a photoswitchable enzyme activity (Hoshaka et al 1994, Westmark et al, 1993, Willner et al 1993). We polymerized the complex with 2-methacryloyl ethyl p-phenyl azobenzoate a photosensitive monomer, HEMA and EGDMA. When this polymer was exposed to U.V. light the azobenzene ring which exists in the stable trans form isomerizes to the cis form which leads to loss of the bound water and hence collapse of the matrix (equilibrium swelling 13%) (Ishihara et al 1984[b]). In darkness the cis form again returns to the trans form, leading to enhanced swelling of the polymer (equilibrium swelling 32%).

The hydrolysis of MA-6ACA-L-PheAl-PNP was carried out at 30°C darkness for the first 20 minutes and then the microspheres were exposed to U.V. light (λ = 280 - 350 nm) for next 20 minutes. It was observed that the hydrolysis of the substrate was suppressed considerable in the presence of U.V. light (figure 5.8). This is due to the hindered diffusion of the substrate into the collapsed matrix under U.V. light. However, after removing the U.V. light, the catalytic activity is regained. This provides a simpler alternative to switch enzyme activity.

5.4.0 Stability of the catalytic hydrogels

In industrial application of enzymes they are more often exposed to drastic pH and temperature conditions. This may lead to denaturation of the enzyme and irreversible loss. Enzyme mimics are synthetic crosslinked molecules and therefore are more stable. The catalytic hydrogels prepared were exposed to 75°C for 2 hours and after cooling the hydrolysis of the ester was carried

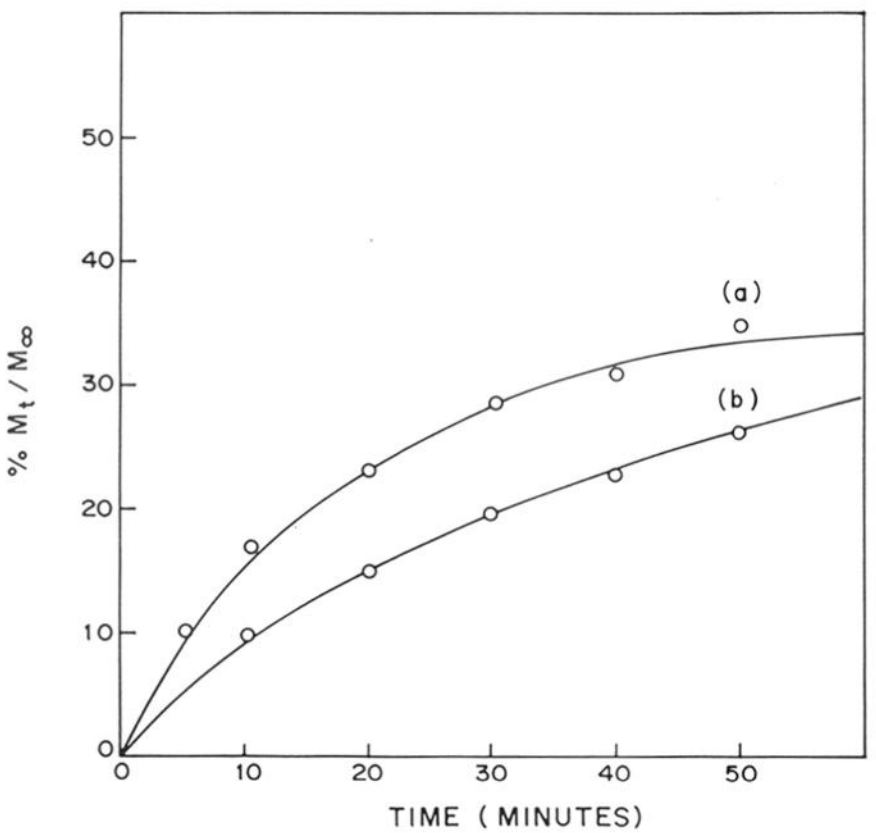


Figure 5.7 : Ester hydrolysis by soluble polymers synthesized by (a) template polymerization and (b) conventional polymerization

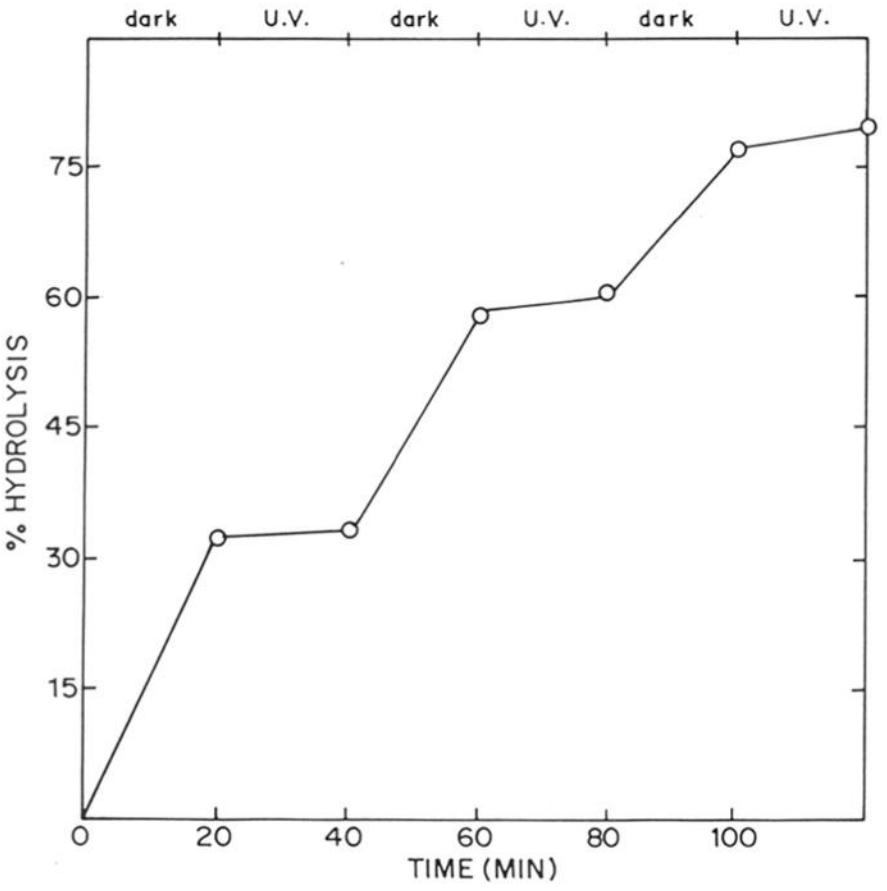


Figure 5.8 : The U.V sensitive hydrolysis of the ester.

was found that catalytic activity of the mimic was retained. Similarly the microspheres were exposed to a concentrated alkali solution, at room temperature for 2 hours. After washing all the excess alkali the hydrolysis of the ester was carried out. It was observed that the catalytic activity of the microspheres was retained.

The same polymer mimic was used repeatedly for the hydrolysis of the substrate for 45 cycles. The activity of the hydrogel was retained within 98% of the original after 20 recycles and within 95% after 45 recycles (See table 5.2).

5.5.0 pH sensitive catalytic activity

Normally α-chymotrypsin shows a bell-shaped pH profile for hydrolysis with the pH optima at 7.9. On either side of the curve there is a loss of catalytic activity. In order to study the pH dependent activity of the mimic we studied the hydrolysis of the ester MA-6ACA-L-PheAl-PNP at pH = 6.8 and pH = 3.0. At pH = 3.0 there was negligible hydrolysis of the ester indicating loss of activity of the polymer. This is due to the protonation of the imidazole nucleus of histidine at pH = 3.0 which disrupts the charge relay system. But the catalytic activity can regained by changing the pH of the reaction medium. Thus the catalytic activity can be reversibly switched on or off (figure 5.9). Therefore the mimic exhibited a pH responsive catalytic activity. There was no loss of catalytic activity in the recycling process indicating the stability of the hydrogels.

5.6.0 Enhanced catalytic activity of the hydrogels due to surface imprinting

The microspheres synthesized so far exhibited enzyme like esterolytic activity. The rates of hydrolysis were much lower as compared to those of the native enzyme. The microspheres were dense, therefore most of the catalytic sites would be buried in the bulk of the polymer and not accessible to the substrate. In order to improve the efficiency of the polymer catalyst the accessibility of the catalytic sites should be enhanced (Guyot et al 1992). We therefore synthesized macroporous poly (GMA-EGDMA) microspheres as supports. The complex comprising the monomers and the

Table 5.2

The repeated use of the surface imprinted polymer for the hydrosis of an ester

No. of cycles	Catalytic Activity % of the virigin catalyst
5	99.98
10	99.54
15	99.09
20	98.87
25	97.84
30	97.42
35	97.06
40	96.86
45	95.80

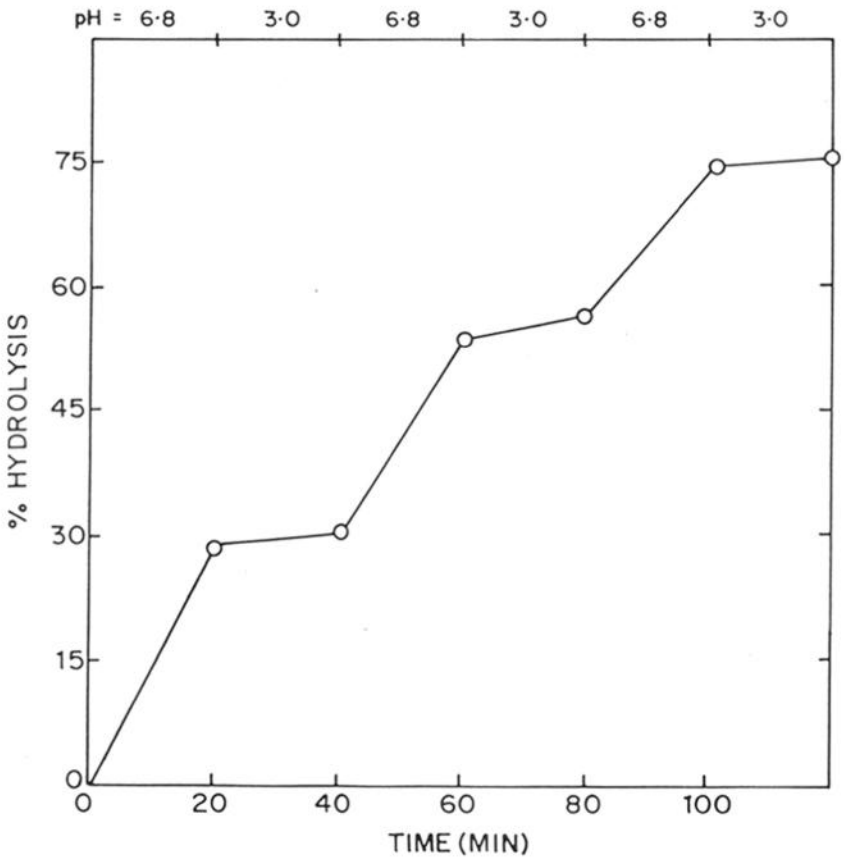


Figure 5.9 : The pH sensitive hydrolysis of the ester.

print molecule was adsorbed on the internal surface of the pores and then polymerized. Microspheres of surface area 282 m^2/g and 350 m^2/g were synthesized. After polymerization of the complex the surface areas decreased to 265 m^2/g and 320 m^2/g. The hydrolysis of MA-6ACA-L-PheAl-PNP was studied using the surface imprinted polymers. The hydrolysis rate profiles are seen in figure 5.1C. It is evident that the microspheres of surface area 265 m^2 / g have nearly the same activity as that of chymotrypsin. Surprisingly the catalytic activity of the mimic with surface area 320 m^2/g surpassed the activity of the enzyme. This was a clear indication that by improving the accessibility of the catalytic sites very high reaction rates can be obtained.

We also investigated the hydrolysis of an amide substrate MA-6ACA-L-PheAl-PNA using the same catalyst. Here the catalytic activity of chymotrypsin was higher than that of the mimic. This indicated that the nucleophilicity of the hydroxyl group of HEMA was lower than that of serine hydroxyl group (figure. 5.11).

5.7.0 Chymotrypsin like features of the polymer mimic.

5.7.1 Substrate specificity

α-chymotrypsin is selective for hydrolysis of the peptide bonds on the carboxyl side of the aromatic side chains tyrosine, tryptophan and phenylalanine and hydrophobic residues like methionine. We carried out the hydrolysis of two substrates MA-6ACA-L-PheAl-PNP and MA-6ACA-PNP using the polymer catalyst prepared by imprinting with IBA-6ACA-L-PheAl-2AP and IBA-6ACA-2AP respectively. MA-6ACA-L-PheAl-PNP was cleaved at a faster rate than MA-6ACA-PNP (figure. 5.12). This clearly demonstrated the specificity of the polymer for phenyl alanine containing side chains similar to that of chymotrypsin.

Further, to investigate the enantioselectivity of the mimic we studied the hydrolysis of MA-6ACA-D-PheAl-PNP using the surface imprinted mimic imprinted with the L-isomer. Initial studies showed that the mimic did not exhibit selectivity. We modified the polymerization procedure

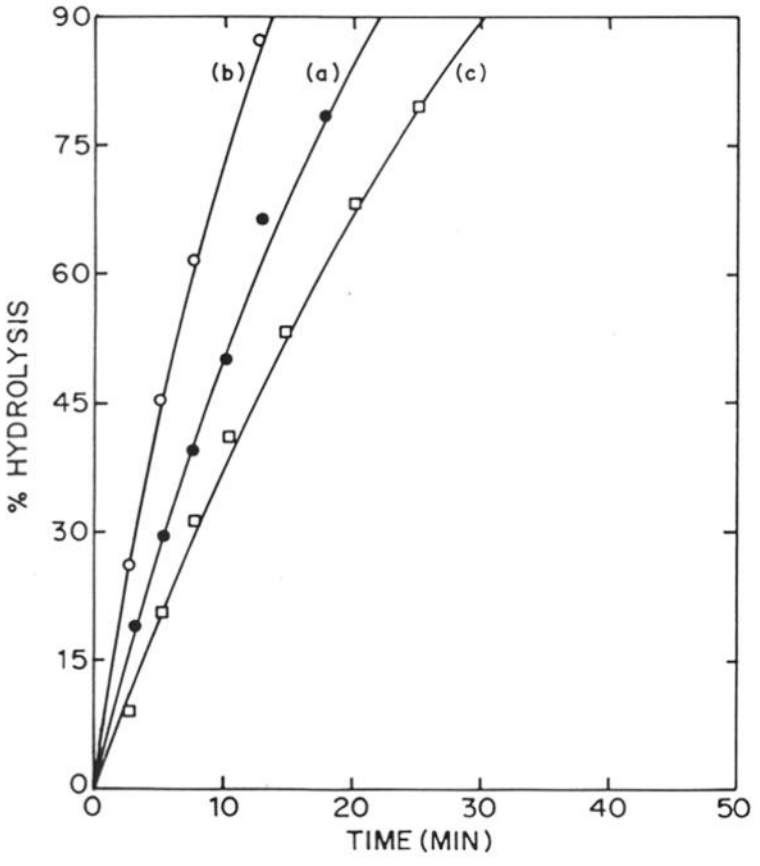


Figure 5.10 : The enhanced catalytic activity for hydrolysis of ester due to surface imprinting using (a) α-chymotrypsin (b) microspheres of surface area 320 m^2/g and (c) of surface area 265 m^2/g.

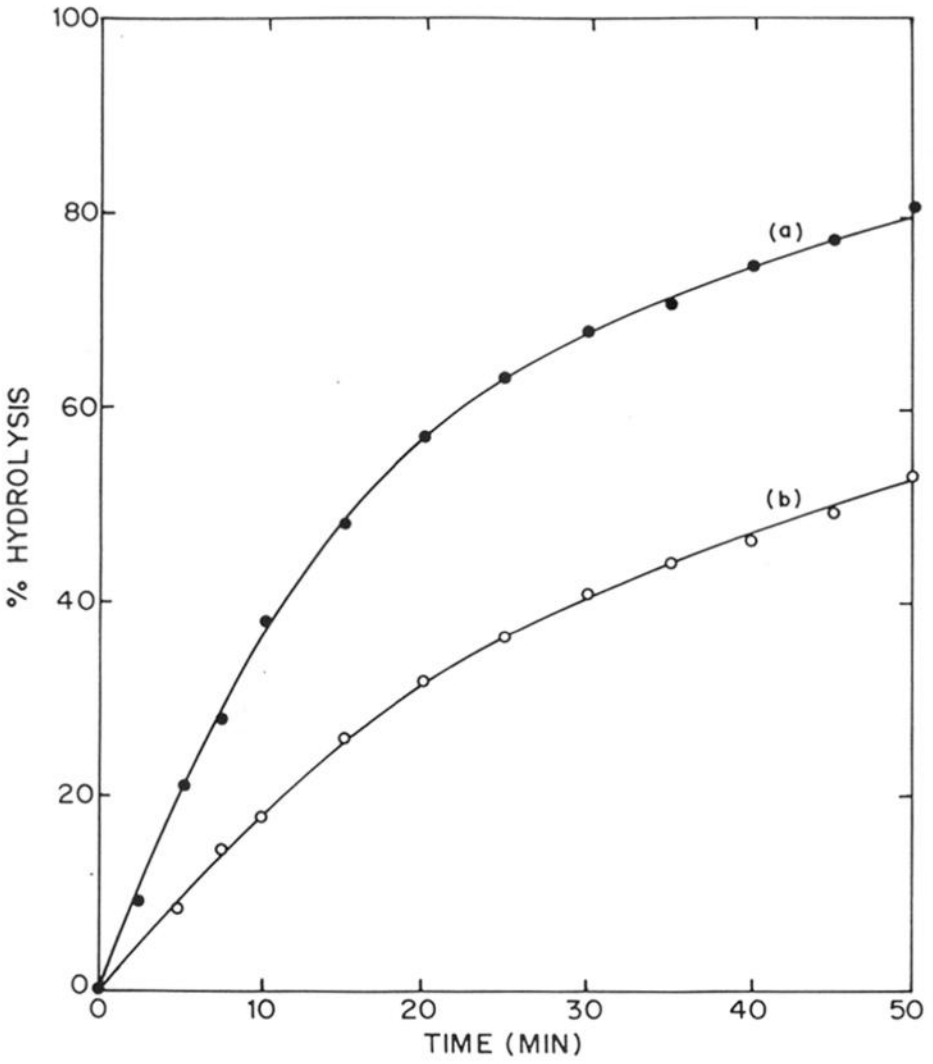


Figure 5.11 : Hydrolysis of the amide MA-6ACA-L-PheAl-PNA using (a) α-chymotrypsin and (b) surface imprinted catalyst (320 m^2/g).

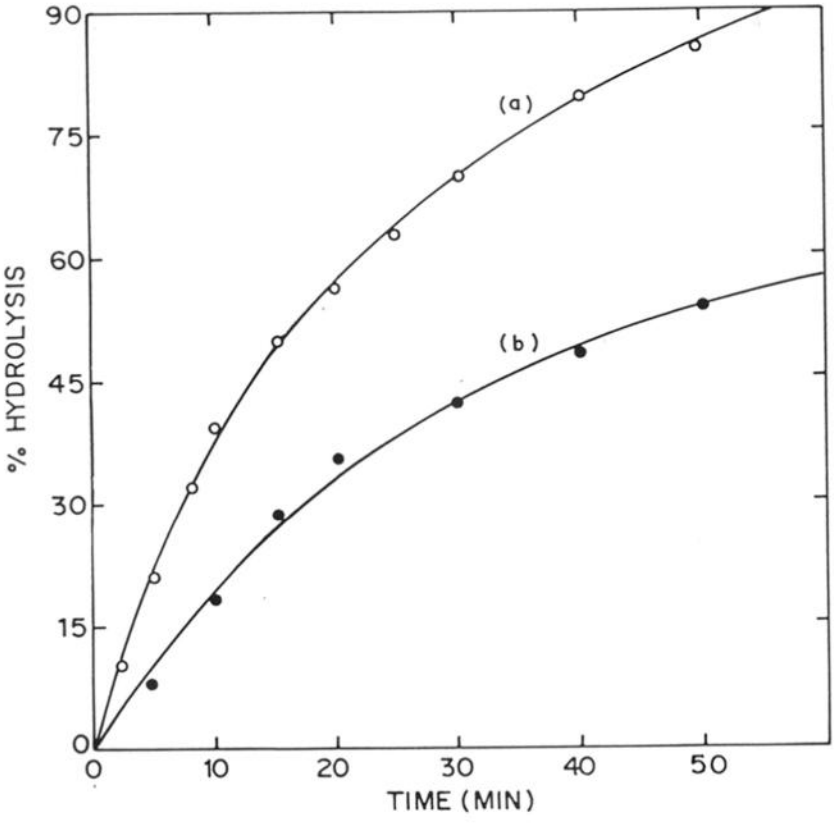


Figure 5.12 : The hydrolysis of the esters : (a) MA-6ACA-L-PheAl-PNP and (b) MA-6ACA-PNP using catalytic hydrogel microspheres.

and carried out the polymerization of the cobalt complex at a lower temperature (4°C) using U.V. light and azobisisobutyronitrile as the photoinitiator. The hydrolysis of the D and L isomers was repeated. The rate of hydrolysis of the L-isomer was considerably higher than the D-isomer (figure 5.13). Polymerization at a lower temperature enhanced the enantioselectivity of the polymer mimic. This observation was similar to that made by O' Shannessy et al (1989).

5.7.2 Active site inhibition and regeneration of the activity

Tosyl L-phenyl alanine chloro methyl ketone (TPCK) is a known inhibitor for chymotrypsin. It binds irreversibly to the imidazole group of histidine in the mole ratio 1 : 1 which leads to irreversible inhibition of the enzyme (Schoellmann et al 1963). The polymer mimic was treated with excess TPCK and the hydrolysis of the ester was followed. Nearly complete loss of activity was observed (figure 5.14 a,b). This indicated that the mimic exhibited active site inhibition similar to the enzyme.

Regeneration of the enzyme activity is not possible after irreversible inhibition. Treatment of the mimic with dilute alkali (0.01 N NaOH) extracted all the TPCK. The polymer was then washed thoroughly with distilled water to remove all traces of alkali. The activity of the mimic was partially restored (figure 5.14 c). The mimic was more sturdy compared to the enzyme and is not easily affected by drastic conditions.

5.7.3 Michaelis-Menten kinetics

The catalytic activity of enzymes arises from their ability to bring the substrate in a favourable orientation in the enzyme-substrate complex (ES). The substrate is bound to a specific region of the enzyme called the active site. The rate of catalysis V, varies with the substrate concentration [S]. V is defined as number of moles of product formed per second at a fixed

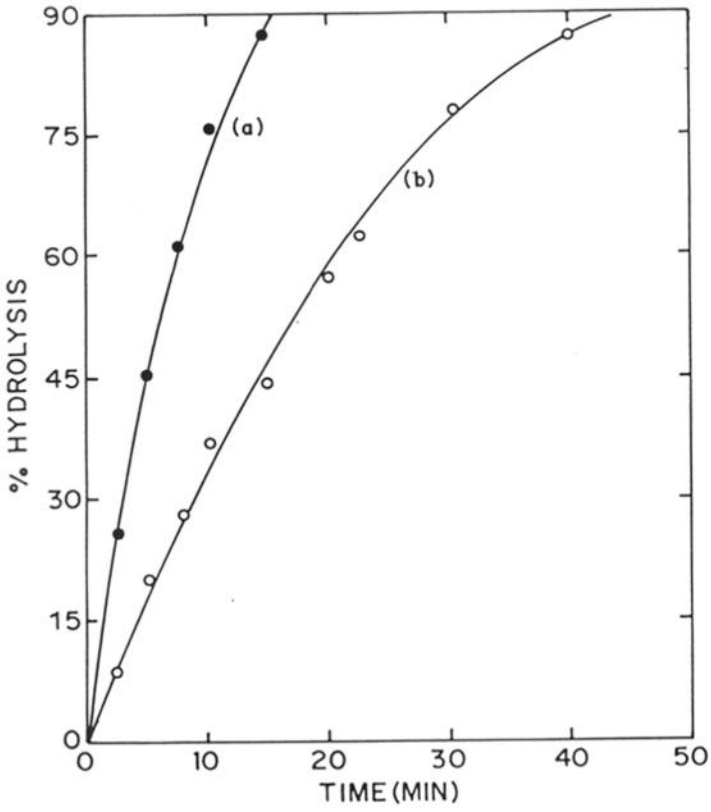


Figure 5.13 : The stereoselective hydrolysis of the esters by the surface imprinted polymer : (a) MA-6ACA-L-PheAl-PNP, the L-isomer and (b) MA-6ACA-D-PheAl-PNP, the D-isomer.

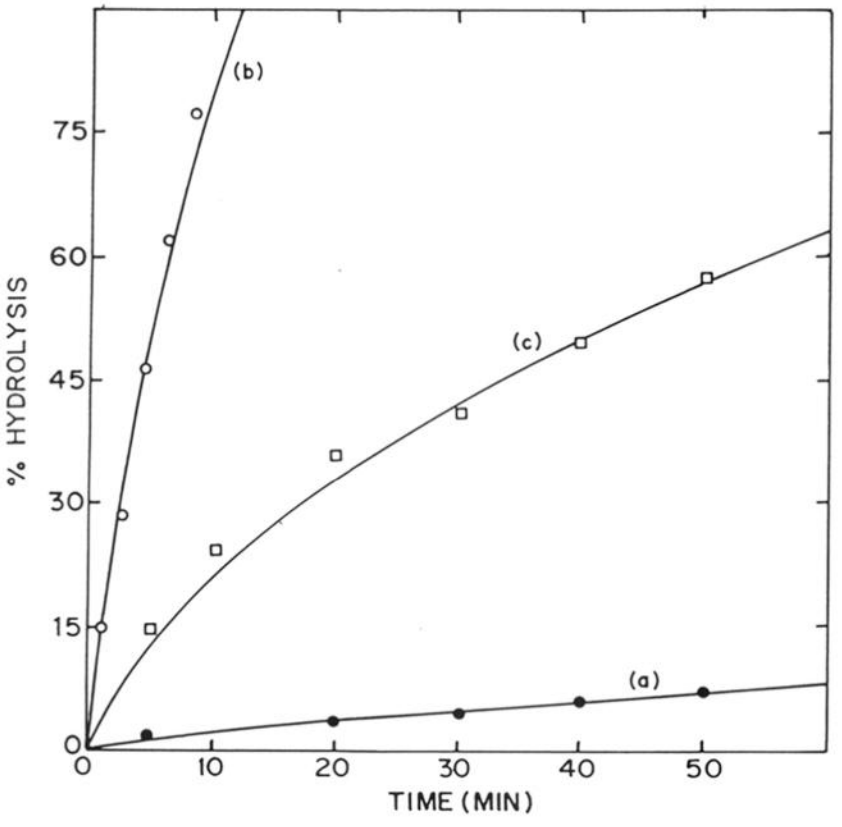


Figure 5.14 : Inhibition of the activity of the mimic by TPCK. (a) Hydrolysis of the ester after inhibition, (b) original activity before inhibition and (c) revival of the activity on dilute alkali treatment.

concentration of the enzyme. V is linearly proportional to [S] when [S] is small. At high [S], V is independent of [S]. Michaelis and Menten (1913) have proposed a simple equation describing the kinetics of enzyme catalyzed reactions.

$$V = \frac{[E]_o[S]\ K_{cat}}{K_m + [S]}$$

where $K_{cat}\,[E]_o = V_{max}$

V = velocity M/lit/sec

[S] = substrate concentration M/lit

$[E]_o$ = enzyme concentration

K_{cat} = catalytic rate constant

K_m = constant which determines the binding of the substrate

K_{cat} / K_m = specificity constant

Simplifying the equation and replacing,

$$K_{cat} = \frac{V_{max}}{[E]_o}$$

we get

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}}[S]_o$$

Plotting 1/V against 1/[S] gives an intercept of $1/V_{max}$ on the Y-axis as 1/[S] tends towards zero and 1/[S] intercept on the X-axis. $1/[S] = -1/K_m$

The slope of the line is K_m / V_{max}

We computed the Michaelis-Menten kinetics for the hydrolysis of the ester and amide substrates in a wide concentration range. The values are listed in table 5.3. The polymer mimic was a better catalyst for the hydrolysis of the ester but the rate of amide hydrolysis was lower than that of the enzyme. The value of K_m which indicated the binding constants for the substrates were comparable to the enzyme under identical conditions.

5.8.0 Concluding remarks

The hydrogels synthesized by molecular imprinting provide a methodology to synthesize enzyme mimics. Further by surface imprinting catalytic activity comparable to that of the enzyme can be achieved. The polymer mimic also exhibited characteristic features of enzymes like stereoselectivity, active site inhibition, Michaelis-Menten type kinetics In addition to these the activity can be externally controlled by incorporating stimuli responsive features in the polymer. These catalytic hydrogels are more robust in that their activity is retained after temperature and pH shocks.

This approach will lead to designer "GELZYMES" having tailored and externally controllable activity and substrated specificty.

Table 5.3

The Michaelis-Menten constants for the hydrolysis of substrates using surface imprinted polymers

Mimic	Enzyme
Ester	
$K_m = 1.021 \times 10^{-4}$ M/lit	$K_m = 1.095 \times 10^{-4}$ M/lit
$K_{cat} = 0.272$ sec^{-1}	$K_{cat} = 0.243$ sec^{-1}
$K_{cat}/K_m = 2664$ M^{-1} lit sec^{-1}	$K_{cat}/K_m = 2219$ M^{-1} lit sec^{-1}
Amide	
$K_m = 2.12 \times 10^{-4}$ M/lit	$K_m = 1.933 \times 10^{-4}$ M/lit
$K_{cat} = 0.178$ sec^{-1}	$K_{cat} = 0.184$ sec^{-1}
$K_{cat}/K_m = 839$ M^{-1} lit sec^{-1}	$K_{cat}/K_m = 951$ M^{-1} lit sec^{-1}

CHAPTER VI

Summary & conclusions

Rationale

This work was undertaken to explore the possibility of designing catalytic hydrogels in general and especially those which would mimic the catalytic activity of enzymes. A model of the active site of α-chymotrypsin by choosing monomers bearing functional groups participating in the catalytic activity of the native enzyme was proposed. The activity of these catalysts for the hydrolysis of esters and amides was investigated and compared with the native enzyme.

Catalytic hydrogels : intramolecular catalysis

Intramolecular cooperative catalysis is the basis of enzyme catalyzed reactions. Catalysis by soluble polymeric systems has been investigated in the past (Zimmering et al 1957, Gaetjens 1961, Akashi et al 1986).

In this work imidazole catalyzed intramolecular hydrolysis of activated esters from catalytic hydrogels has been demonstrated. The substrate and the catalytic groups were brought in proximity by polymerization of the charge transfer complex formed between the substrate and the catalytic group. The higher rates of hydrolysis have been explained on the basis of imidazole catalyzed intramolecular catalysis of the ester. The effect of the polymer structure, the nature of the catalyst, the reactivity of the substrate and pH on the catalytic activity has been explained.

Catalytic hydrogels : molecular imprinting effects

The techniques of template polymerization and molecular imprinting have been employed to create binding sites in the polymer network and for positioning the functional groups in the binding site (Wulff 1986*, Mosbach 1994).

These techniques were used to synthesize new catalytic hydrogels. The imidazole catalyzed hydrolysis of polymeric ester and amide substrates from these hydrogels was investigated. The hydrogels exhibited enhanced catalytic activity due to the presence of the functional groups. Catalytic activity was substrate specific and pH dependent. The studies could be used in designing hydrogel based pendent chain drug delivery systems for oral drug delivery.

Enzyme-mimicing hydrogels

Enzymes are nature's most efficient catalysts because of their specificity and very high reaction rates (Fersht 1985). Enzymes achieve these rates by creating a binding site for the substrate resulting from chain folding and cooperative effects among the functional groups. There have been many attempts in the past to mimic enzymes using supramolecular assemblies, soluble polymers as well as highly crosslinked polymers (D'Souza et al 1985, Kunitake 1980, Wulff 1986[a], and Mosbach 1994). However very few enzyme mimics were able to replicate the enzyme activity and exhibit mechanistic analogy.

The functional groups present in the hydrogels studied by us are, the hydroxyl group in 2-hydroxyethyl methacrylate, the imidazole group in N-methacryloyl histidine and the carboxylic group in methacrylic acid. These groups also constitute the active site of α-chymotrypsin viz. serine-195, histidine-57 and aspartic acid-102. Therefore the objective of our work was to modify the catalytic hydrogels further so as to mimic the active site of α-chymotrypsin.

The functional groups and a print molecule were complexed with cobalt and polymerized with 2-hydroxyethyl methacrylate or polymerized on the surface of macroporous poly (GMA-EGDMA) microspheres. The hydrolysis of L-phenyl alanine ester and amide substrate was investigated. The rate of hydrolysis of the substrate by the imprinted polymer catalyst was found

to be considerably higher as compared to the polymers prepared without imprinting. In the dense micropsheres, the rate of hydrolysis of the substrate was controlled by its diffusion in the bulk of the polymer. The complex was adsorbed on the internal surfaces of poly (GMA-EGDMA) microspheres. The catalytic activity of the microspheres was comparable to the enzyme under identical conditions. The polymer catalyst also exhibited stereoselectivity, active site inhibition and obeyed the Michaelis-Menten kinetics for hydrolysis. By incorporating a U.V. sensitive monomer in the polymer the catalytic activity could be externally controlled by U.V. light. The catalytic activity was also found to be pH dependent. The hydrogel based catalysts are more rugged and can withstand pH and temperature variations without any loss of catalytic activity. These catalysts can also be used repeatedly for more than 50 times and can easily be recovered from the reaction mixture.

The conclusions of this work can be summarized as follows :

* Catalytic hydrogels where the substrate and the catalytic groups are brought in proximity have been shown to exhibit enhanced catalytic activity.

* A catalytic hydrogel based on a charge transfer complex of the substrate and the catalyst has been demonstrated. The enhanced rates of hydrolysis in these hydrogels were explained on the basis of intramolecular catalysis. A comparison of the intramolecular and intermolecular rates of reaction was made and it was shown that the rate of the intramolecular reaction was far greater than the intermolecular reaction in the catalytic hydrogels.

* It was shown that the enhanced hydrolysis of pendent chain linked esters and amides could be brought about under physiological conditions. The catalytic hydrogels were designed

based on metal coordination and molecular imprinting techniques. These hydrogels exhibited features of enzyme activity viz. substrate specificity, cooperative effects among functional groups and pH dependent activity.

* A polymer model of the active site of α-chymotrypsin was developed. The catalytic hydrolysis of L-phenylalanine esters was investigated. In order to improve the accessibility of the active site, a surface imprinted polymerization method was used.

* The polymer catalysts surpassed enzyme activity under identical conditions in some cases. They also exhibited stereoselectivity, active site inhibition and obeyed Michaelis-Menten kinetics. The catalytic hydrogels are more robust and the activity is externally controllable. This approach can be applied to designer " **GELZYMES** " for industrial applications.

CHAPTER VII

Suggestions for future work

This work describes the synthesis of catalytic hydrogels for controlled drug delivery applications and as enzyme mimicing polymers. The work demonstrates the concept of reactive hydrogels and is only a beginning. This work could be further extended to the following systems:

1) The frame work developed in this work based on the catalytic hydrogels can be useful for the design of pH sensitive drug delivery systems for enteric coating applications using drug molecules.

2) The hydrogels in this were based on the matrix system. This could be extended to the synthesis of microspheres which could minimize the diffusional limitations.

3) The hydrolysis of reactive esters and amides was investigated. This is not always likely to be the case; especially for industrial reactions. It is therefore important to evaluate the efficacy of these mimics in the hydrolysis of less active esters and especially industrially relevant substrates.

4) These enzyme mimics have an advantage over the enzymes in that the activity is retained in organic solvents. In order to develop better enzyme mimics it is important to understand the origin of the stability in organic media. It will be therefore desirable to investigate the Michaelis-Menten constants for the mimic for specific reactions in media of carying solvent compositions.

5) The photosensitive activity of the enzyme mimic can be utilized in development of new photographic material based on photoactivation of enzymes for detection of light.

6) The criteria developed for the synthesis of enzyme mimicing polymers can be applied to the synthesis of enzyme mimics of other hydrolytic enzymes like papain, penicillin acylase, β-galactosidase, amylase etc.

7) The technique of surface imprinting of macroporous supports can be useful to design new polymeric catalysts.

8) The catalysts can be useful in chromatographic separations, reactive separations in organic synthesis and can replace enzymes in industrial reactions.

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LIST OF PUBLICATIONS / PATENTS

PUBLICATIONS

1. Catalytic hydrogels as enzyme mimics I : Facile hydrolysis through anchimeric effect. R.N. Karmalkar, M.G. Kulkarni & R.A. Mashelkar, Makromol Chem (communicated).

2. Catalytic hydrogels as enzyme mimics II : Molecular imprinting effects. R.N. Karmalkar, M.G. Kulkarni & R.A. Mashelkar, Makromol Chem (communicated).

3. Surface imprinted polymer mimic surpasses chymotrypsin activity. M.G. Kulkarni, R.N. Karmalkar & R.A. Mashelkar, Nature (communicated).

PATENTS FILED

1. A process for the preparation of polymeric composition useful for the conversion of esters and amides to corresponding alcohols and amines., R.A. Mashelkar, M.G. Kulkarni & R.N. Karmalkar. Indian Patent file no. NF-115/95.

2. An improved process for the conversion of esters and amides to corresponding alcohols and amines., R.A. Mashelkar, M.G. Kulkarni & R.N. Karmalkar. Indian Patent file no. NF-116/95.

3. A process for the preparation of new polymeric composition for the controlled release of an active ingredient in response to pH., R.A. Mashelkar, M.G. Kulkarni & R.N. Karmalkar. Indian Patent file no. NF-117/95.

SYNOPSIS

1.0 Introduction

Hydrogels represent an emerging class of smart materials. Researchers have endowed them with a kind of intelligence, the ability to respond to stimuli, by incorporating stimuli reponsive moieties in the polymer network. Gels that swell or shrink, absorbing or expelling water, in response to changes in light, pH, temperature, specific molecules and electric field have been reported (Tanaka 1992). They have potential as actuators or artificial muscles for robots or prostheses (Kajiwara & Ross-Murphy, 1992). These systems have potential in control processes in medicine, industry and pharmacy.

Present investigation was undertaken to elucidate the possibility of designing catalytically active hydrogels for applications in controlled release drug delivery and in enzyme mimicking.

2.0 Reactive hydrogels drug delivery systems

2.1 Intramolecular catalysis by catalyst-substrate complex formation

Neighbouring groups catalyze the hydrolysis of pendent esters or amides (McCormick and Kim, 1988, Akashi et.al, 1986 & Chaves et.al 1989). Due to the presence of carboxyl or amide groups the hydrolysis of the neighbouring ester or amide can be accelerated and induced under mild conditions. This is mainly attributed to the increase in hydrophilicity of the polymer and to some extent to the anchimeric assistance.

In this work we have modified the above mentioned approach. A charge transfer complex of the activated ester substrate p-nitrophenyl p-vinyl benzoate (PNPVB) and the catalyst 1-vinyl imidazole (VIm) was copolymerized with 2-hydroxyethyl methacrylate (HEMA). The 1-vinyl imidazole catalyzed hydrolysis of the ester from the hydrogel matrices was investigated. It was shown that in case of P(HEMA-PNPVB-VIm) the release of p-nitrophenol was enhanced at pH 8

due to the higher hydrolysis rates as compared to that in P(HEMA-PNPVB). The catalytic activity of 1-vinyl imidazole was dependent on the pH of the release medium. Factors affecting the release of p-nitrophenol like pH, the length of the pendent chain, or the catalyst to substrate ratio in the polymer were investigated. The release of the active ingredient takes place in the pH range prevalent in the intestinal region. Since the catalyst is present in the polymer matrix the problem of diffusivity of the catalysts like enzymes or enzyme mimicking polymers into the polymer is eliminated (Vadalkar et. al). This approach should be useful in designing of improved enteric coatings.

2.2 Molecular imprinting in hydrogels : Catalytic hydrolysis of substrates

Facile hydrolysis of the kind described above can be exploited only for systems which form a donor-acceptor complex. It is necessary to design a system which will have a wider application.

Molecular imprinting involves the use of specific template molecules to co-ordinate the assembly of synthetic functional monomers around it. Two different approaches have been developed namely, i) non-covalent interactions between the functional groups and the template molecule (Mosbach et.al 1994). ii) covalent bond formation between the functional groups and the template molecule (Wulff et.al 1986). The functional monomers are polymerized in presence of the template and a crosslinker. After the template molecule is leached out, cavities are formed in the polymer network which contain the functional groups.

This investigation was undertaken with a view to investigate the catalytic hydrolysis of pendent chain ester/amide which were polymerized within cavities created by molecular imprinting.

In the first case the p-amino benzoate ester of HEMA (PAP) and 1-vinyl imidazole were complexed with Co^{++} in methanol and then copolymerized with HEMA. After removing cobalt ions from the polymer discs the hydrolysis of poly (2-methacryloyl ethyl p-aminobenzoate, PAP) was investigated. A random copolymer P(HEMA-VIm-PAP) was also synthesized. It was observed that the release of p-amino benzoic acid from the tailored polymer was enhanced considerably as compared to that in the random polymer. This was attributed to the effect of the neighbouring imidazole group in the tailored polymer. Soluble polymers of the same compositions did not exhibit

differences in rates of hydrolysis indicating the importance of freezing the conformation of the ester and the catalyst by crosslinking.

In the second case, the p-nitro benzoate ester of HEMA (PNP) was used as the substrate. Since the nitro group cannot form a complex with Co^{++}, a template molecule N-isobutyryl ethyl nicotinate was synthesized. A complex of this template and 1-vinyl imidazole with Co^{++} was prepared and polymerized with HEMA. After removing cobalt and the template, the PNP ester was absorbed in the polymer matrix and polymerized by γ irradiation. These polymers exhibited higher catalytic hydrolysis of poly(PNP) when compared with the random polymer P(HEMA-VIm-PNP). On introducing N-methacryloyl histidine and methacrylic acid in the complex the activity could be further enhanced. The catalytic activity of the tailored polymers was pH dependent.

The framework developed in this work would be useful for the design of reactive hyrogels for the release of an active ingredient linked to a polymer backbone in response to pH under physiological conditions. These systems too provide a superior alternative to enteric-coatings.

3.0 Enzyme-mimicking hydrogels

Synthesis of enzyme mimicks by the functional modification of cyclodextrin, crown ethers, monoclonal antibodies and cryptands is well known (D'Souza et. al, 1985, Leh
n, 1988, Pollock et. al, 1986). Enzyme mimicking polymers have been synthesized by different researchers (Kunitake et. al, 1976, Wulff et. al, 1986 and Mosbach, 1989). But these efforts were not direacted to mimick the active site of specific enzyme nor the mimick activity was comparable to the enzyme.

This investigation was undertaken with a view to synthesize a polymer model which would mimick the active site of α-chymotrypsin and have a comparable catalytic activity. Functional monomers bearing the groups responsible for the activity of α-chymotrypsin were chosen. Suitable template molecules were synthesized for the given substrates. A complex of the monomers and the template molecule with Co^{++} was prepared and this was copolymerized with HEMA by suspension polymerization to yield microspheres. Cobalt and the template molecule were extracted from these

microspheres and the hydrolysis of an ester solubilized in a ethanol/buffer mixture was studied. At equimolar concentration the polymer mimick exhibited lower catalytic activity than that of the native enzyme. When a U.V. sensitive azobenzene monomer was copolymerized along with the other functional monomers the catalytic activity of the mimick was found to be U.V. responsive.

The lower catalytic activity of the polymer mimick was attributed to the inaccessibility of the active sites which are buried in the polymer bulk. To enhance accessibility of the active sites, the complex was adsorbed on a preformed macroporous high surface area poly(GMA-EGDMA) microparticle support and polymerized. The hydrolysis of ester and amide substrates was investigated. The polymer mimick had higher catalytic activity as compared to the native enzyme for the ester but the activity was found to be lower for the amide. The hydrolysis of the ester and amide substrates by the mimick followed the Michaelis-Menten kinetics with parameters K_{cat} and K_m comparable to those for the native enzyme under identical conditions.

The polymer mimick demonstrated many of the characteristic features of enzymes. This strategy could be useful in the synthesis of polymer models for enzymes that do not have a cofactor, for industrial applications.

4.0 Conclusions

In summary, this work highlights the synthesis and applications of different hydrogels in the area of controlled drug delivery and enzyme mimicking.

In has been demonstrated that the catalytic hydrolysis of pendent chain esters can be achieved by the intramolecular pathway or by using tailored hydrogels.

A polymer mimick of α-chymotrypsin has been synthesized and the catalytic activity that is comparable to the native enzyme is demonstrated.

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(Signature of the Guide) (Signature of the candidate)

CURRICULAM VITAE

Name	R.P. Patwardhan (Mrs.R.N. Karmalkar)
Date of birth	13th February 1966
Address	Flat No. 18 Nishigandh Park Ideal Colony, Paud Rd Kothrud 411 029.

Qualifications :

Education :

Degree and Year	University	Class obtained	Subjects and Scientific field
Ph.D*	Pune	Thesis*	Catalytic hydrogels as enzyme mimicks, and hydrogels for controlled drug delivery applications
M.Sc (1988)	Pune	First Class	Organic Chemistry Physical Chemistry Inorganic Chemistry
B.Sc (1986)	Pune	First Class	Chemistry Physics Botany

* Thesis entitled " Reactive Hydrogels Exhibiting Catalytic Activity " to be submitted to the University of Pune in July 1995.

Scholarships / Fellowships Awarded :

6.11.91 to 6.11.94	Senior Reserch Fellowship by University Grants Comission, New Delhi, INDIA.
6.11.89 to 6.11.91	Junior Research Fellowship by University Grants Comission, New Delhi, INDIA.
	National Merit Loan Scholarship by Government of Maharashtra, INDIA

Experience and Research Activities

Nov 1989 till date

Working as a Research Fellow for Ph.D degree under able guidance of Dr. R.A. Mashelkar, Director, National Chemical Laboratory at N.C.L (Pune). The work involved development of novel catalytic hydrogels for hydrolysis of esters and amides. These hydrogels have applications in the controlled release delivery systems, especially for pH sensitive release of drugs. The hydrogels also exhibited enzyme like catalytic activity and therefore can be used for industrial applications. Synthesis of biodegradable polymers based on poly (lactic acid-co-glycolic acid) and other copolymers for controlled release of pharmaceuticals, especially macromolecular solutes was investigated.

May 1989 to October 1989 :

Worked as a Trainee Chemist in Indian Organic Chemicals Ltd. Khopoli. The work involved the isolation of anthracene and phenanthrene from waste mud obtained from refineries.