# Protein Containing Porous Polymers For Biocatalytic Applications

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**This research programme was carried out in collaboration with the University of Liverpool, Department of Chemistry**

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# Contents

**Chapter 2:**

# Declaration

I declare that, except where otherwise stated, the work conducted within this thesis is my own research, carried out from September 2001 until December 2004, at the School of Pharmacy and Chemistry, Liverpool John Moores University and at the Department of Chemistry, University of Liverpool.

James Long

May 2005

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# Abbreviations

| | |
|---|---|
| AIBN | 2,2'-azo-bis-isobutyronitrile |
| AM | acrylamide |
| AOT | Aerosol-OT |
| APS | ammonium persulfate |
| ATEE | N-acetyl tyrosine ethyl ester |
| atm | atmospheres |
| ATPE | N-acetyl tyrosine propyl ester |
| $a_w$ | water activity |
| BET | Brunauer Emmett Teller method |
| BSA | bovine serum albimum |
| CALB | Candida Antarctica lipase B |
| CLEC | cross-linked enzyme crystals |
| CT | chymotrypsin |
| C/W | carbon dioxide-in-water emulsion |
| Da | Daltons |
| Dex | dextran |
| DMSO | dimethyl sufoxide |
| EPSRC | Engineering and Physical Sciences Research Council |
| ESR | electron spin resonance |
| EVE | ethyl vinyl ether |
| GPC | gel permeation chromatography |
| HEMA | hydroxyethyl methacrylate |
| Hex | hexane |
| HIPE | high internal phase emulsion |

| | |
|---|---|
| HPLC | high performance liquid chromatography |
| HS30 | Ludox® silica nanoparticles |
| i.d. | internal diameter |
| IR | infra red |
| LC-MS | liquid chromatography mass spectrometry |
| LD-MS | laser desorbtion mass spectrometry |
| MALDI | matrix assisted laser desorbtion ionisation |
| MBAM | methylene bis-acrylamide |
| MMA | methyl methacrylate |
| MUTMAC | 4-Methylumbelliferyl p-trimethylammoniocinnamate chloride |
| $M_w$ | molecular weight |
| O/W | oil-in-water emulsion |
| PAA | poly(acrylic acid) |
| PAM | poly(acrylamide) |
| $P_c$ | critical pressure |
| PDDA | poly(diallydimethyl ammonium chloride) |
| PEG | poly(ethylene glycol) |
| PFOA | poly(1,1-dihydroperfluorooctyl acrylate) |
| PIT | phase inversion temperature |
| PLS | partial least squares |
| PNaA | poly(sodium acrylate) |
| psi | pounds per square inch |
| PSSS | poly(styrene sodium sulfonate) |
| PVA | poly(vinyl alcohol) |
| PVP | poly(vinyl pyrrolidone) |

| | |
|---|---|
| R134a | 1,1,1,2-tetrafluoroethane |
| RALS | right angle light scattering |
| RDH | rhomboidal dodecahedrons |
| Rhw | hydrodynamic radius |
| SCF | supercritical fluid |
| SDBS | dodecyl sulfonic acid sodium salt |
| SDS-PAGE | sodium dodecyl sulfate-poly acrylamide gel electrophoresis |
| SEM | scanning electron microscope |
| St | styrene |
| $T_c$ | critical temperature |
| THF | tetrahydrofuran |
| TKDH | tetrakaidecahedrons |
| TM50 | Ludox® silica nanoparticles |
| TMEDA | tetramethyl ethyldiamine |
| TNBS | 2,4,6-trinitro benzene sulfonate |
| TOF | time of flight |
| Tol | toluene |
| TRIM | trimethylolpropane trimethacrylate |
| US-FDA | United States Food and Drug Administration |
| UV | ultra violet |
| V-65 | 2,4-dimethyl valeronitrile |
| W/C | water-in-carbon dioxide emulsion |
| W/O | water-in-oil emulsion |

# Abstract

Over the last twenty years there has been a great deal of research conducted into the use of enzymes as catalysts for reactions conducted in non-aqueous media. Not only has it been shown that enzymes can function effectively in organic media, but also that the range of reactions that they can catalyse has been vastly increased.

The major disadvantages associated with non-aqueous enzymatic catalysis are relatively slow reaction rates compared to traditional aqueous catalysis, poor enzyme stability towards polar organic solvents and protein agglomeration, which leads to reduced efficiency, making recovery and reuse of the enzyme very difficult.

Immobilisation of the enzyme on a suitable support material has been shown to be an effective method in overcoming these problems. This study examined several different methods for immobilising α-chymotrypsin on novel support materials. The catalytic activities of the preparations were assayed by following the transesterification reaction between N-acetyl-L-tyrosine ethyl ester (ATEE) and propan-1-ol by high performance liquid chromatography (HPLC). Immobilised enzyme activities were compared to those obtained for simple unsupported lyophilised preparations of α-chymotrypsin.

Uniform porous poly(acrylamide) beads were loaded with various quantities of enzyme via an adsorption procedure. Catalytic activity was measured over a wide range of thermodynamic water activities and was found to be similar to the lyophilised preparations. However, the immobilised enzyme was shown to be more resistant to changes in pH and temperature, and could easily be recovered from the reaction mixture and reused.

Design of experiment methodology was employed to optimise support matrices constructed from six component materials. The enzyme was immobilised via a non-covalent entrapment method. The best composites prepared displayed a fifty-fold increase in catalytic activity and a three-fold increase in mechanical strength relative to the equivalent α-chymotrypsin controls. These materials could be reused more than ten times whilst still retaining useful catalytic activity.

Porous poly(acrylamide) monoliths containing entrapped α-chymotrypsin were synthesised using a novel carbon dioxide high internal phase emulsion templating technique. The effects of enzyme loading, carbon dioxide pressure and monomer to crosslinker ratio were examined. The corresponding enzyme activity of all the emulsion templated materials was shown to be higher than for the unsupported lyophilised preparations, with the best materials exhibiting a ten-fold increase in activity.

Multipoint covalent enzyme immobilisation was also studied. The structure of the enzyme was first modified so as to include a polymerisable functionality. This modified enzyme was then dissolved in organic solvents via the formation of ion-pairs with various anionic surfactants. It was shown that the enzyme remained in solution when transferred from organic solvents to a mixture of monomers. The dense gas porogen R134a was then added to the solution of enzyme in monomer, prior to the initiation of a polymerisation reaction. The resulting crosslinked enzyme-containing polymers were shown to possess useful catalytic activity.

# Chapter 1

# Introduction

## 1.1 Enzymatic catalysis in aqueous media

Enzymes are large polypeptides, constructed from a linear sequence of amino acids, linked together via amide bonds. The molecular weight of these macromolecules can vary between 5 and 5000 KDa.[1] All enzymes are proteins, but not all proteins are enzymes, the difference being that enzymes possess catalytic activity and other proteins do not. This catalytic activity arises from the spontaneous folding of the polypeptide chain to form a very specific three dimensional, tertiary structure.[1] This complex three-dimensional shape contains a cavity (termed the active site) formed from the very precise stereochemical arrangement of amino acids. The active site is where the chemistry takes place. Substrate molecules of a certain shape fit into this pocket and undergo catalysis. This "lock and key" relationship between the enzyme and substrate allows the enzyme to catalyse these reactions with great specificity and efficiency, under very mild conditions, resulting in the formation of stereospecific products.[2] It is for these reasons that enzymes are advantageous as catalysts for both biochemical reactions *in vivo* and also in organic synthesis.

The exact three-dimensional conformation of the enzyme is maintained via non-covalent interactions between the amino acid residues that make up the proteins backbone. These non-covalent interactions include hydrogen bonding, electrostatic and Van der Waals

forces as well hydrophobic and hydrophilic interactions with the solvent (water).[3-5] As enzymes are naturally occurring biomolecules, they are typically employed under biological conditions, i.e. in aqueous media, at comparatively neutral pH and at temperatures below 40 °C. Under these conditions enzymes can achieve extremely rapid catalysis, typically $10^4$ to $10^6$ times faster than for the equivalent uncatalysed reaction rates.[1] However, when enzymatic catalysis is conducted in aqueous media, the enzyme can become extremely vulnerable to deactivation by changes in the reaction conditions. Altering the pH of the reaction media will affect how the charged amino acid residues contained within the enzyme's primary structure interact with one another. As the amino acid interactions are responsible for maintaining the enzyme's conformation, which is in turn critical to the enzyme activity, changes to these interactions can result in deactivation of the enzyme.[6] Similarly, exposure of the enzyme to high temperatures can also result in deactivation. Once the attractive forces between the amino acid residues have been overcome by the increased thermal energy, the protein structure can unfold, disturbing the active site and preventing catalysis.[6]

Each different type of enzyme is a catalyst for a particular type of reaction.[7] Examples of these include hydrolysis, esterification, carbon-carbon bond formation, oxidation, reduction, addition, elimination and isomerisation reactions, as well as many others.

### 1.2 Enzymatic catalysis in non-aqueous media

Alexander Kilbanov's work conducted in the early 1980's concerning heterogeneous suspensions of enzymes in organic solvents, demonstrated that enzymes do retain a proportion of their catalytic activity in non-aqueous environments.[8] Over the subsequent

twenty years a great deal of research has been conducted into the use of enzymes in non-aqueous media.[8-13] The main driving forces behind this research have been to increase the range of reactions that can be catalysed by enzymes, to utilise the altered specificities of enzyme in organic media, as well as to increase the stability of the catalysts.

Initially organic solvents were required in order to solvate hydrophobic substrates that could not be dissolved in water. This could be achieved by adding small quantities of a water miscible organic solvent to an aqueous solution of enzyme. In theory this should facilitate the homogeneous catalysis of non water-soluble substrates, but in practice highly polar water miscible solvents such as short chain alcohols and acetone are often deleterious towards enzyme activity.[14, 15] It is postulated that in a homogeneous two solvent reaction medium, the polar organic molecules may interact with charged enzyme active sites. An alternative strategy is to dissolve the sparingly water soluble substrate in a water immiscible hydrophobic solvent. The addition of this to an aqueous solution of enzyme creates a biphasic system (figure 1.1).[16, 17]

Some control over the partitioning of substrates and products between the two phases can be achieved by careful manipulation of the aqueous layer (e.g. by changing the pH and ionic strength of the solution). This can be advantageous for the recovery and separation of the reaction products.[6] However, as the bi-phasic system is intrinsically heterogeneous the mass transfer of substrate materials from the organic phase to the enzyme containing aqueous phase can become the rate-limiting step. This can be overcome by increasing the contact surface area between the two phases, but it is known that enzymes can be deactivated by contact with a heterogeneous hydrophobic medium. This is known as "phase toxicity" and most likely occurs when one part of an enzyme's structure is

exposed to a hydrophobic medium whilst the rest of the structure is still surrounded by water molecules. The differing solvent interactions with the amino acid residues of the enzyme's structure occurring simultaneously on the same molecule may cause an unfavourable change in its conformation.[18, 19] The increase in mass transfer that can be achieved by increasing the contact area between the two phases must be balanced against the possible decrease in enzyme activity caused by the increase in phase toxicity.

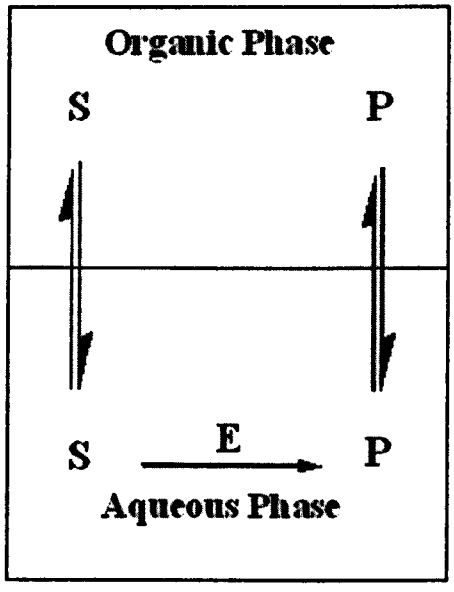


*Figure 1.1 Schematic representation of an enzymatic reaction conducted in a biphasic system.*

*S = substrate, P = product and E = enzyme.*

Although both these methodologies do represent viable pathways for the use of enzymes with organic solvents, both still require the enzyme to be dissolved in a significant quantity of water. As such the enzyme is still vulnerable to deactivation by exposure to extremes of pH and temperature.[6] Enzyme catalysis will also be affected by the large

quantities of water present, with reactions that involve water as a substrate dominating over alternative reaction pathways.

The effects of changing pH are easily overcome by employing the enzyme in a totally organic environment. Klibanov *et al* demonstrated that the ionisation state of the enzyme does not change when it is transferred from aqueous to organic environments.[12] This has been called "pH memory" and means that the pH of the enzyme can be set by lyophilising it from a buffered aqueous solution prior to use in the organic phase. The ionisation state of the enzyme can be controlled to some extent whilst in non-aqueous media, via the addition of organic phase buffers such as trisoctylamine and triphenyl acetic acid.[20]

The thermal stability of enzymes employed in organic solvents has also been examined in numerous studies.[21-24] Generally enzymes are resistant to far higher temperatures when suspended in organic solvents compared to aqueous solutions. The half-life of α-chymotrypsin dissolved in aqueous buffer at 55 °C is less than 15 minutes.[22] However, the same enzyme suspended in octane at 100 °C displayed a half-life of 80 minutes.[21] Ahern *et al* examined the stability of lysozyme and found that its half-life in boiling aqueous solutions was less than 10 minutes, whereas in cyclohexane (110 °C) the half-life was increased to 140 hours.[25]

When the flexibility of enzymes was studied in organic media, they were found to adopt a far more rigid conformation than when in aqueous solution.[19, 26, 27] This reduction in flexibility was caused by increases in the relative strength of the non-covalent intramolecular interactions between the amino acid residues of the proteins primary structure. In aqueous solutions the hydrogen bonding interactions between the enzyme's

functional groups and the surrounding solvent are dominant. However, removal of the majority of the water that surrounds the enzyme's structure allows for stronger intramolecular interactions (in relative terms). It is generally accepted that these stronger interactions hold the enzyme in a more rigid conformation and help to prevent the unravelling of its tertiary structure under increased thermal conditions.[6]

The increased rigidity of enzymes in organic solvents has also been shown to affect the stereospecificity of the catalysed reactions products.[6, 28] The reduced flexibility of the enzyme's tertiary structure, and hence its active site, can mean that only one stereoisomer of a particular substrate is able to undergo catalysis. This results in the production of only product isomer.

Many studies have been conducted to investigate the effects on catalysis when enzymes are employed in low-water organic environments. However, these studies generally use one of two methodologies. Firstly, the enzyme can be suspended in the organic solvent to form a heterogeneous system. In this methodology the enzyme can be used in its native form as a lyophilised powder, or after being immobilised on a support material. Alternatively, the enzyme can be dissolved in the organic solvent to form a totally homogeneous catalysis system. This is generally achieved via either the covalent modification of the enzyme with a suitably hydrophobic moiety, or by the non-covalent association of the enzyme with a surfactant. Both of these general strategies will be discussed in more detail.

### 1.2.1 Effects of protein hydration

The catalytic activities of enzymes suspended in anhydrous organic solvents are generally reduced by several orders of magnitude compared to the equivalent enzymatic catalysis conducted in traditional aqueous solutions.[6] It has been shown that the flexibility of the enzyme is greatly reduced when it is exposed to environments with very low water contents.[6, 26, 28] This rigid conformation makes it harder for the enzyme to accommodate the incoming substrate molecules and so reduces the catalytic reaction rate. Numerous studies have now been conducted investigating the effects of protein hydration on the corresponding catalytic rate.[29-33] In general, the addition of small quantities of water results in large increases in the observed enzyme activity. For example Koshinen *et al* have reported that the α-chymotrypsin catalysed esterification of N-acetyl-L-phenylalanine with ethanol, conducted in anhydrous ethyl acetate, is strongly affected by the quantity of water added.[6] When no water was added to the anhydrous system, no catalytic activity was observed. When 2 $\%^{v}/_{v}$ water was present the enzyme's catalytic activity rose to approximately 100 nmol/mg CT.min. Doubling the concentration of water in the system equated to an approximate doubling of the observed catalytic activity. It can be reasoned that these increases in catalytic activity that correspond to greater levels of hydration, are a result of the enzyme's increased flexibility.[19] However, the study also demonstrated that increasing the water content of the system beyond 5 $\%^{v}/_{v}$ caused a rapid decline in catalytic activity. This reduction in catalytic activity may be due to a fundamental change in the nature of the system. Initially the reaction medium is completely organic and the addition of small quantities of water influences the enzyme's flexibility and activity. However, with the addition of large quantities of water (> 5 $\%^{v}/_{v}$)

the nature of the system changes from completely organic to aqueous with a miscible polar organic co-solvent, which has been postulated to be deleterious towards enzyme activity.[6]

A similar effect has been observed with enzymatic catalysis conducted in hydrophobic solvents.[32, 34] The addition of very small quantities of water is generally beneficial to enzyme activity, but larger volumes of water cause a phase separation between the aqueous and the organic media. Enzymes present at the interface of these two layers are then affected by phase toxicity, which deactivates the enzyme and reduces the observed catalytic activity.[6, 16]

It is clear that the concentration of water present in an organic system has a significant influence over enzymatic catalysis. As such it is essential that the hydration level of any system is known and can be controlled in order to accurately assess the function of enzymes in the non-aqueous media. The exact amount of water present in a particular organic system (below its saturation point) can be defined as its thermodynamic water activity ($a_w$).[32, 35] The thermodynamic water activity of two different systems containing exactly the same amount of water may not necessarily be the same. Very hydrophobic organic solvents will absorb less water at a given thermodynamic water activity than other more hydrophilic solvents. Control over a system's thermodynamic water activity can be achieved via equilibration over saturated salt solutions.[36] Different saturated salt solutions give rise to different water vapour pressures. By equilibrating the reaction components in a sealed atmosphere the water content of a system can be set at various controlled levels. It is also important to maintain a constant ambient temperature, as changes in temperature will greatly affect the vapour pressure of each system.

Thermodynamic water activity is measured over a scale of zero to one. Zero is defined as a completely anhydrous system and is achieved by equilibrating the reaction components in a sealed environment with phosphorus pentoxide. Equilibration over distilled water containing no dissolved compounds results in a thermodynamic water activity of one.[36] An alternative method for the control of a system's thermodynamic water activity is the direct addition of heterogeneous salt hydrates.[37] By selecting pairs of salt hydrates with different degrees of hydration, the amount of water contained in an organic reaction system can effectively be buffered.

### 1.2.2 Dissolving enzymes in organic media

Several studies have been conducted in order to examine the use of enzymes for homogeneous catalysis in organic media.[38-44] Enzymes are large hydrophilic molecules that are not naturally soluble in organic solvents. However through covalent modification of the enzyme with a suitably hydrophobic moiety, or via non-covalent complexation with surfactants, enzymes can be dissolved in non-aqueous media. The advantage of this approach to enzymatic catalysis is that the reaction medium is homogeneous and so the mass transfer limitations of substrates and products to and from the enzyme's active sites are greatly reduced.[6, 42, 43] This can lead to catalytic rates that approach those of traditional aqueous enzymatic catalysis.[42, 43] The major disadvantage of dissolving enzymes in organic solvents is the increased difficultly in the recovery of the enzyme from the reaction medium after completion of the reaction.

Enzymes have frequently been "dissolved" in organic solvents via the formation of reverse micelles.[39] These micelles are usually formed by encapsulating very small

droplets of water in surfactant shells, which are then dispersed in the organic phase. As such the enzyme is really dissolved in water, and the reaction medium is biphasic in nature. Although this is a relatively simple method of achieving a "homogeneous" dispersion of enzyme in largely organic media, the enzyme is still dissolved in the aqueous phase and is vulnerable to the same changes in pH and temperature that can easily deactivate traditional aqueous solutions of enzymes.

Takahashi *et al* reported the first truly organic solutions of enzyme in the early 1980's.[45] This technique involved the formation of a covalent linkage between the primary amine residues present on the surface of the enzyme and cyanuric chloride activated poly(ethylene glycol) (PEG) (figure 1.2). Following this modification procedure the enzyme could be dissolved in organic media and assayed for catalytic activity. The results indicated that the enzyme did still retain some catalytic activity, however only low concentrations of the enzyme in solution could be achieved (approximately 0.15 to 0.5 mg/ml in toluene and DMSO). Numerous other methodologies for dissolving enzymes in organic solvents have since been reported, although many of these techniques vary only in the coupling chemistry between the PEG groups and the enzyme's primary amine residues.[45-47] These "PEGylation" techniques are now so well established that several different enzymes are now commercially available in a PEGylated form, suitable for direct use in organic solvents.

Enzymes have also been coupled to polymers other than PEG for the purposes of dissolving the enzyme in organic solvents. Polyacrylates, polystyrenes, and poly(n-vinylpyrolidone) have all been employed to this end.[48, 49] However all these methods generally produce enzyme preparations of relatively low solubility and catalytic activity.

It is reasoned that the low catalytic activity is due to the some distortion of the shape of the enzyme's active site as well as a possible blockage of the active sites by the large polymeric moieties. Other covalent modification procedures involving hydrophobic long-chain aliphatic compounds have also been reported.[44, 50] However, these preparations are also sparingly soluble in organic media.

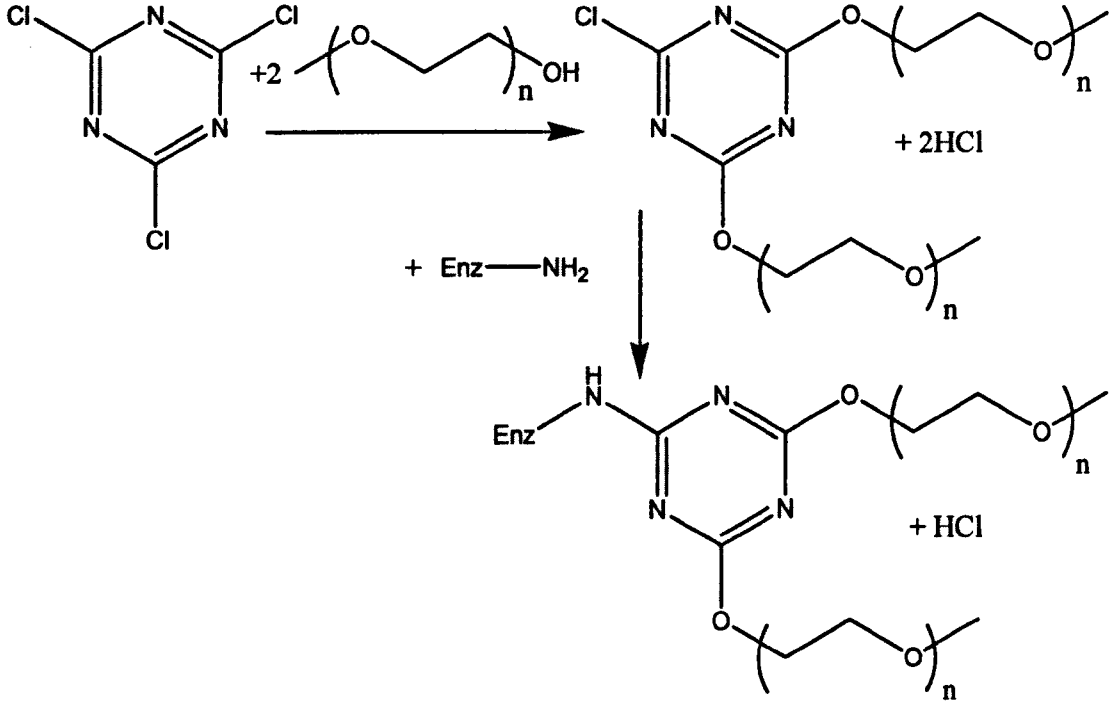


*Figure 1.2 A potential reaction scheme for the activation of monoethoxy poly (ethylene glycol) with cyanuric chloride and the subsequent coupling of the activated polymer to the primary amine groups present on the surface of the enzyme. Enz = Enzyme*

A common alternative strategy to covalent modification, is to dissolve the enzyme via non-covalent complexation with surfactant molecules.[38, 40, 41, 51, 52] The most successful of these techniques was first reported by Dordick *et al* in the mid 1990's.[42, 43] This involved

the formation of enzyme-surfactant complexes stabilised by the electrostatic interaction between the anionic surfactant sodium bis (2-ethylhexyl) sulfosuccinate (AOT) and the charged amine residues present on the surface of the enzyme. A buffered aqueous solution of the enzyme is contacted with an organic solution of AOT. After vigorous stirring an emulsion is formed between the two phases. During this stage enzyme molecules present at the interface between the aqueous and organic layers form ion pairs with surfactant molecules. As more ion pairs are formed the enzyme becomes less hydrophilic, until it becomes sufficiently hydrophobic to cross the boundary between the two phases. Separation of the emulsion under centrifuge allows the enzyme containing organic phase to be isolated. The organic solvent can then be removed to yield a solid enzyme/surfactant preparation that can be re-dissolved in a variety of organic solvents. These preparations have been shown to possess far higher catalytic activities than for the enzymes dissolved via a covalent modification method. Activities approaching those of enzymes dissolved in traditional aqueous media have been reported.[42, 43] The solubility of these preparations is also much improved when compared to the covalently modified enzymes. Concentrations as high as 500 mg/ml have been reported.[51] The general disadvantage of these non-covalent surfactant complexation methodologies is that the separation of the enzyme from the reaction medium becomes even more difficult as there are now numerous surfactant molecules present in addition to the enzyme.

### 1.3 Immobilised enzyme preparations

The most commonly employed technique for using enzymes in both non-aqueous and aqueous enzymatic catalysis is to immobilise the enzyme on suitable support materials,

prior to use in a heterogeneous suspension.[6] Homogeneous solutions of enzyme in organic solvents are not frequently used in industrial synthetic reactions owing to the difficulties of product and catalyst recovery.[6] Heterogeneous suspensions of native and lyophilised enzymes are also often impractical due to the problems associated with protein agglomeration.[6, 9, 51, 53] When unsupported enzymes are suspended in organic solvents the relative strengths of the non-covalent intramolecular and intermolecular interactions, such as hydrogen bonding, electrostatic and Van der Waal's forces, become greater.[6, 19] This is because the overwhelming dominance of the hydrogen bonding aqueous medium has been removed. This can be beneficial to enzyme stability as the molecule's flexibility is reduced and as such it is less likely to flex out of its catalytically active conformation.[19, 26, 27] However, the relative increase in the attractive forces between enzyme molecules often results in their agglomeration, forming sticky films that adhere to the walls of reaction vessels, making the enzyme very difficult to recover and reuse.[6, 54] As the enzyme molecules agglomerate, catalytic activity is often reduced due to the blockage of active sites through protein-protein contacts.[6] A solution to these problems is to immobilise the enzyme on a support material. This helps prevent the enzyme molecules from sticking together and can make their recovery from the reaction vessel much easier. In addition to these benefits, immobilising the enzymes on a support material can increase their stability towards deactivation by changing pH, elevated temperatures and exposure to polar solvents.[6, 51, 54] Contacting the enzyme with a rigid, inflexible material can reduce the degree of conformational change that the enzyme is able to undergo, helping to maintain a catalytically active shape.[6]

The nature of the support material is extremely important to the resulting catalytic activity. Ideally the support should have a large surface area, spreading out the enzyme molecules and reducing the number of protein-protein contacts.[6, 13, 55] A high degree of porosity is also beneficial, as this reduces the mass transfer limitations of substrates and products to the active sites of enzymes immobilised within the interior of a support material, increasing the observed catalytic rate.[56, 57] Finally the chemical nature of the support material is also important as its ability to adsorb water will strongly affect the catalytic activity of the supported enzyme.[58]

### 1.3.1 Methods of enzyme immobilisation

There are several methods available for the immobilisation of enzymes. These methods can broadly be separated into two categories covalent and non-covalent. In general, covalent methods of immobilisation are harder to achieve, as they require some sort of chemical reaction to be performed on the enzyme. Non-covalent methods are usually easier to accomplish, but these preparations often suffer from enzyme leaching, as there is no chemical bond preventing the enzyme from being washed from the support material. Figure 1.3 outlines the most common methods employed for enzyme immobilisation as well as a brief description of the major advantages and disadvantages of the covalent and non-covalent techniques.

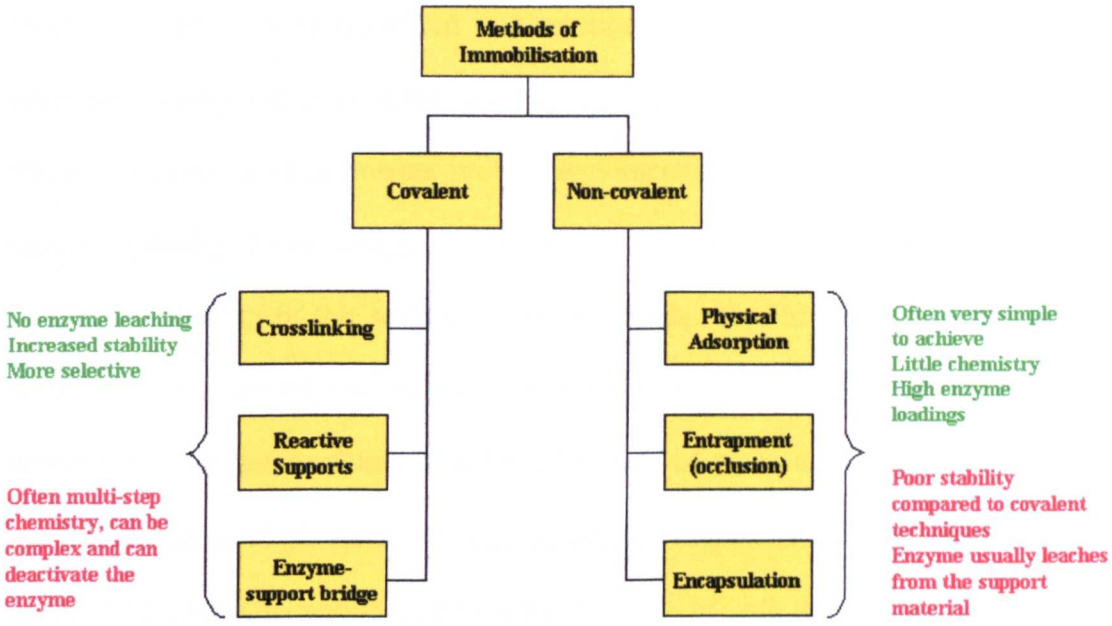


*Figure 1.3 Schematic illustrating the general strategies for the immobilisation of enzymes on support material. The main advantages (green) and disadvantages (red) of both covalent and non-covalent immobilisation techniques are also listed.*

### 1.3.1.1 Physical adsorption

Immobilisation of an enzyme via its physical adsorption to a support material is the simplest method and is well reported in the literature.[29, 59-62] The enzyme is only associated with the support material through non-covalent interactions such as hydrogen bonding and Van der Waal's forces. This means that the immobilised preparation is unsuitable for use in traditional aqueous catalysis, as the enzyme would simply dissolve. However, when used in organic media the non-covalent hydrophobic repulsive forces that exist between the enzyme and the solvent can help to "pin" the enzyme to the support material.[19] This helps reduce the amount of enzyme that leaches from the support, however the immobilised catalyst is still easily dislodged from the support by the

mechanical action of being stirred in a reaction vessel, or by a strong flow of solvent when being employed in a continuous-flow catalytic reaction.[6]

Physical adsorption does prevent protein agglomeration and can offer some increase in enzyme stability. Even though the enzyme is only adsorbed to a single surface, the mechanical stability of this surface can result in a decrease in the immobilised enzyme's flexibility. This can help to maintain the correct active site conformation, even during elevated reaction temperatures. Another advantage of physical adsorption as an enzyme immobilisation method is that the enzyme can be completely removed from the support material without deactivation, allowing for the reuse of both the enzyme and the support material in another application.

There are two popular methods for the immobilisation of proteins via physical adsorption. The support material is suspended in an aqueous buffered enzyme solution and enzyme is then either precipitated from solution onto the support by addition of a water miscible organic solvent such as acetone,[63] or the saturated support material is freeze dried to remove the water and deposit the enzyme onto the support material.[29]

The most commonly reported materials for enzyme adsorption are porous, water absorbent polymers such as poly(acrylamides),[64, 65] glass beads[56] and naturally occurring clays such as celite.[29, 61]

### 1.3.1.2 Enzyme entrapment

A more sophisticated method for the non-covalent immobilisation of enzymes is mechanical entrapment (also known as occlusion). This technique often involves the formation of the support material in the presence of an enzyme, and relies on the physical

constraint of the enzyme provided by the support material in order to limit leaching. Chemical reactions in homogeneous aqueous solution that yield solid, insoluble products, such as polymers, are often used for the entrapment of enzymes.[66-71] Freeze dried aqueous solutions of polymers and enzyme also yield immobilised preparations suitable for use in non-aqueous catalysis reactions.[72, 73] This method yields materials in which the enzyme is usually dispersed evenly throughout the support matrix. Enzyme molecules present on or very near the surface of the immobilisation material are only physically adsorbed to the support, since no covalent bond has been formed between them. As such, these enzyme molecules are able to leach from the support. However, much of the enzyme immobilised via this method is entrapped within the interior of the support material and is unable to leach away during use. This is an advantage because it enables entrapped enzyme preparations, formed from insoluble materials such as crosslinked polymers, to be used in aqueous catalysis reactions as well as for non-aqueous catalysis.[68] Alternative entrapment strategies have also been developed that rely on the use of swellable materials such as natural rubbers and poly(acrylamides).[66] Generally, the support materials are swollen in an aqueous buffered solution of enzyme. This allows the enzyme to diffuse into the interior of the support material. Subsequent removal of the water under vacuum causes the enzyme to precipitate and the support material to shrink, resulting in the entrapment of much of the enzyme. These preparations are then suitable for catalysis in solvents that do not swell the support material.

An advantage of enzyme immobilisation via an entrapment method is that the stability of the preparations is often significantly increased.[68, 70] Better resistance to changes in temperature, pH and reaction solvent have been observed, compared to enzymes

immobilised via a physical adsorption methodology.[67, 70] This is due to the increased rigidity of the enzymes conformation provided by the entrapment process. The enzyme is often constrained on several sides when entrapped, instead of just one side when physically adsorbed.

A disadvantage of entrapment mechanisms is that a large proportion of the immobilised enzyme is often rendered inaccessible to the incoming substrate molecules, as they are situated within the interior of the support matrix. This problem is encountered with entrapment materials that possess low porosity.

### 1.3.1.3 Enzyme encapsulation

Enzyme adsorption and entrapment can be viewed as single, discrete enzyme molecules immobilised on a surface or within a matrix. However, enzymes can be immobilised by isolating them by means of a physical barrier. This is termed enzyme encapsulation and large numbers of enzyme molecules are often contained together within a matrix in this way. Several methods are available for the encapsulation of enzymes.[74-76] Membranes and hollow fibres (figure 1.4) that possess pore sizes small enough to retain the enzyme and yet large enough to allow the diffusion of substrates and products are suitable for enzyme encapsulation. Enzyme encapsulation has also been achieved via the electrostatic coating of enzyme crystals with multiple layers of polymer, which provide the porous barrier necessary to retain the enzyme.[74]

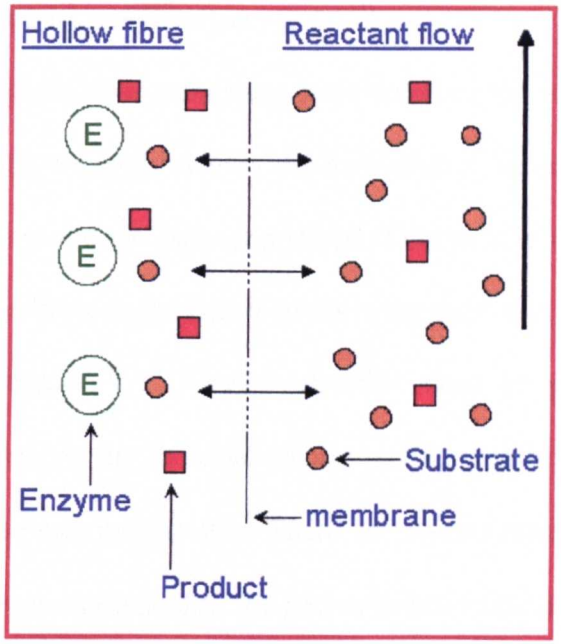


*Figure 1.4 A schematic representation of catalytic reaction conducted using an encapsulated enzyme. A barrier is used to retain the enzyme molecules. Pores in the barrier are large enough to allow the diffusion of substrates and products, but small enough to prevent the escape of the enzyme.*

The main advantage of enzyme encapsulation is that it can be relatively easy to achieve and can often facilitate the simple separation of the enzyme from the reaction products. However, the encapsulation process can mean that the enzyme molecules are retained by the porous barrier, whilst suspended or dissolved in a liquid medium (aqueous or organic). As such, the enzyme's flexibility is not reduced by the additional physical constraint observed with adsorption and entrapment immobilisation techniques. Therefore the enzyme's stability towards changing reaction temperature and pH can be lower than for the alternative methodologies.

### 1.3.1.4 Covalent enzyme immobilisation

Covalent enzyme immobilisation techniques have received the most attention in recent years.[53, 77-81] Broadly, this method involves the formation of an actual covalent chemical bond between the enzyme and the support material. This form of enzyme immobilisation is the most difficult to achieve as it often requires many steps and requires the enzyme to undergo a chemical reaction.[51, 52] Careful attention must be paid to any chemistry performed on the enzyme, as its catalytically active conformation can easily be denatured, rendering the enzyme inactive. There are several advantages to the covalent immobilisation of enzymes compared to the non-covalent methods described previously. Enzyme leaching is eliminated, as the presence of a chemical linkage between the enzyme and the support prevents the separation of the two groups. This allows for the use of covalently immobilised enzymes in traditional aqueous reaction media.[79] Covalently immobilised preparations also frequently exhibit much higher stabilities and catalytic activities when compared to other immobilised preparations.[82, 83]

Enhanced stability towards changes in reaction temperature, pH and solvent is provided by the increased conformational rigidity that is associated with covalent attachment to supports. It has been shown that as the number of attachment points between the enzyme and the support are increased, the corresponding stability of the enzyme is also increased.[77, 78, 82, 83, 84] This is because multipoint attachment of an enzyme to a support significantly reduces its conformational flexibility, preventing the active site from being twisted out of shape. Catalytic activity has also been shown to increase with an increase in the number of covalent attachment points, provided the chemistry required to form the linkages is tightly controlled so as not to deactivate the enzyme prior to immobilisation.

Again, this is thought to be due to the decreased conformational flexibility of the immobilised enzyme. Once an enzyme has been pinned in position by covalent immobilisation, its active site conformation and orientation are also fixed. Provided this site is accessible to the incoming substrate and has not been twisted out of a catalytically active conformation, the rate of catalysis for that specific active site can increase. This is because the number of correctly orientated collisions that occur between the catalyst and the substrates will greatly increase when the enzyme is held in an active and fixed position. Overall, covalent immobilisation may reduce the total number of enzyme active sites available to substrates, but can also increase the turnover number for those active sites that remain accessible and in the correct conformation. Studies have recently been reported that examine methods for the "site-specific" orientation of immobilised enzymes, maximising the number of accessible and catalytically active sites available to incoming substrates.[79]

One strategy employed for the covalent immobilisation on enzymes, is to first modify its structure so as to provide chemically reactive moieties that can then undergo covalent bond formation with a suitable support material.[51, 52] For example this can be achieved via the reaction of the primary amine functionalities present on the enzyme's surface with a chemical reagent such as acryloyl chloride. After suitable purification, an enzyme preparation is obtained where the surface of each molecule is covered with polymerisable vinyl groups. The reaction of this modified enzyme with suitable monomers and crosslinkers can produce a rigid, porous support structure with the enzyme covalently attached to the polymer.[51, 52] It has been shown that the enzyme can successfully be modified in this way, without significant deactivation. However very strict control must

be maintained over all the experimental steps in which the enzyme is involved, in order to minimise any enzyme deactivation.

A more popular alternative strategy for the covalent immobilisation of enzymes is to perform as many steps as possible, prior to the addition of the enzyme to the reaction mixture.[78, 83] This involves the functionalisation of the support material to produce reactive moieties such as epoxides. These are capable of reacting with groups already present on the enzyme, such as primary amines.[78, 83] This strategy minimises the enzyme's exposure to chemical reactions, and so reduces the likelihood of deactivation.

An alternative to the functionalisation of either the enzyme or the support material, is to include a difunctional crosslinking reagent such as gluteraldehyde.[85] These crosslinkers contain reactive groups capable of covalent bond formation with both the support material and the naturally occurring functionalities of the enzyme. This can afford a relatively simple one-step multipoint immobilisation procedure.

Recently there has been much research conducted into the covalent immobilisation of enzymes without the use of a support material. The gradual addition of aqueous enzyme solutions to miscible organic solvents such as acetone causes the precipitation of the enzyme. With careful control over both the precipitation procedure and the enzyme purity, protein crystals can be produced. Subsequent covalent crosslinking of these protein crystals results in a preparation of enzymes immobilised on other enzyme molecules via several covalent chemical bonds. These materials are known as cross-linked enzyme crystals (CLECs).[86-90] CLECs have been shown to be highly catalytically active, with reaction rates in excess of 3000 times those observed with preparations of enzyme lyophilised from aqueous buffers.[86] The stability of cross-linked enzyme crystals

towards increased temperatures has also shown to be improved when compared to conventional lyophilised preparations.[88]

### 1.4 Supercritical fluids

A substance can be defined as a supercritical fluid when both its temperature and pressure surpass the critical values.[91] These critical values are unique to each compound and can be derived from the critical point. The critical point represents the highest temperature and pressure at which the substance can exist in equilibrium as a vapour and a liquid (figure 1.5).

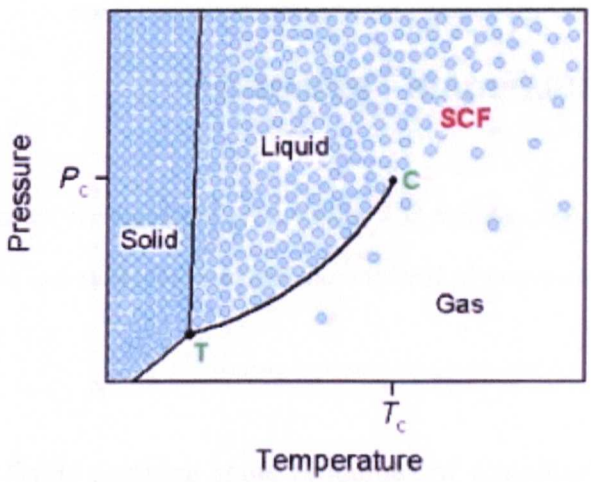


*Figure 1.5 Schematic pressure-temperature phase diagram for a pure component. The circles represent the variation in density of the substance in the different regions of the phase diagram. The triple point (T) and the critical point (C) are marked. Note that the density varies continuously between the liquid state and the gaseous state, providing the boiling curve (T-C) is not crossed. $T_c$ = critical temperature, $P_c$ = critical pressure.*

The boundary between the gaseous and liquid phases is known as the boiling curve. As the temperature of the system is increased, the liquid density decreases due to thermal

expansion and the density of the gaseous phase is increased, as more and more liquid enters the vapour state.[92] The densities of the two phases continue to converge until parity is reached. At this point, the meniscus between the two phases disappears and it is no longer possible to distinguish between them (figure 1.6).

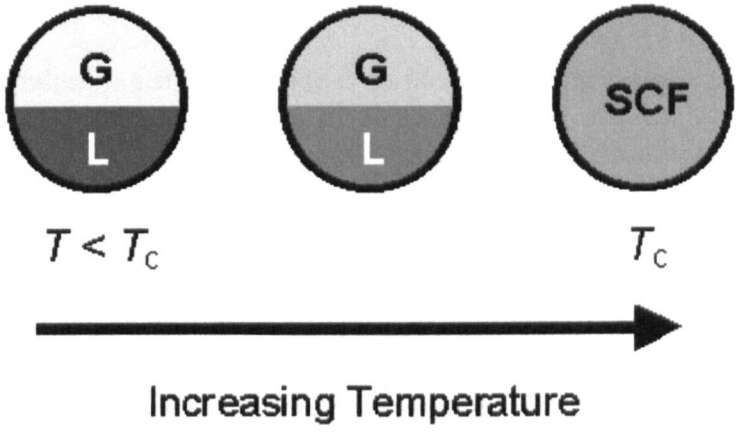


*Figure 1.6 Schematic representation of the change from liquid-gas equilibrium ($T<T_c$) to supercritical fluid ($T \geq T_c$) conditions as a substance is heated above its critical temperature at a pressure in excess of $p_c$. G = Gas, L = Liquid.*

Supercritical fluids combine some properties of traditional organic solvents with those properties usually associated with gases. The liquid-like densities mean that supercritical fluids can be used to dissolve a wide range of compounds, whilst retaining gas-like diffusivities, due to their very low viscosities.[92, 93] An interesting property of many supercritical fluids is that their density can be "fine-tuned" by varying the reactor vessel's pressure. Carbon dioxide is by far the most commonly employed supercritical fluid,[91-93] however there are numerous alternative compounds the have successfully been employed for supercritical applications (table 1.1).

The most obvious advantage in using supercritical carbon dioxide as an alternative reaction medium is its environmental benefits. The large scale use of traditional volatile organic solvents by the chemical industry (approximately 15 million tonnes per year) has caused serious concern over their detrimental effect on the environment.[94] If suitable supercritical solvents can be found to replace volatile organics for some of these chemical processes, it could result in a significant reduction in the environmental damage caused by the chemical industry. Carbon dioxide is an ideal choice as a supercritical replacement for traditional solvents, as it is non-toxic, non-flammable and greenhouse neutral.

| Solvent | Critical Temperature $T_c$ (°C) | Critical Pressure $P_c$ (bar) |
|---|---|---|
| Carbon Dioxide | 31.1 | 73.8 |
| Ethane | 32.2 | 48.8 |
| Propane | 96.7 | 42.5 |
| Cyclohexane | 280.3 | 40.7 |
| Isopropanol | 235.2 | 47.6 |
| Benzene | 289.0 | 48.9 |
| Chlorotrifluoromethane | 28.9 | 39.2 |
| 1,1,1,2-Tetrafluoroethane | 101.0 | 40.6 |
| Ammonia | 132.5 | 112.8 |
| Water | 374.2 | 220.5 |

*Table1.1 Critical parameters for selected substances. $T_c$ = critical temperature, $P_c$ = critical pressure.*

Supercritical fluids also offer other advantages such as pressure dependent density and in some cases, pressure dependent dielectric constant.[95] This means that the physical parameters of the solvent can be adjusted without the need to change the intrinsic chemical nature of the solvent.

Numerous applications have now been reported for the use of supercritical fluids as alternative reaction solvents. These include, organic synthesis, supercritical extractions, fractionations, separations, chromatography, plasticisation of polymers, doping and impregnation procedures, enzymatic reactions and as a polymerisation medium.

By far the largest industrial application of supercritical fluid technology is in the decaffeination of coffee beans.[93] Due to the low solubility of caffeine in supercritical carbon dioxide (approximately 0.2 $\%^{w}/_{w}$ at 60 °C and 300 bar) large excesses of the solvent are required. Nevertheless, in excess of half a million tonnes of coffee are decaffeinated via supercritical carbon dioxide extraction each year.[93] Following the United States Food and Drug Administration's (US-FDA) decision not re-evaluate the use of carbon dioxide in the processing and synthesis of materials for human consumption; much research has been conducted in order to examine the potential of carbon dioxide as a replacement for traditional organic solvents.[95] This decision reflects the non-toxic nature of carbon dioxide as well as the extremely low residual quantities that remain after depressurisation of the reaction vessel. Water is the only other medium that is deemed to be suitable for use without evaluation by the FDA.[95]

For the enzyme immobilisation procedures conducted in this study, the most relevant applications of supercritical fluid technology are for the production of porous polymer supports and as replacement reaction media for enzymatic catalysis.

### 1.4.1 Supercritical fluids as polymerisation media

The majority of polymerisation reactions carried out in supercritical media have been conducted in carbon dioxide.[91] This is due to carbon dioxide being inexpensive, non-

toxic, non-flammable, readily available in high purity and relatively chemically inert.[91, 96, 97] Relatively large volumes of carbon dioxide can be employed in synthesis reactions without the environmental problems or energy intensive drying steps associated with conventional organic solvents.[91, 96, 97] In addition to this, carbon dioxide does not support chain transfer to solvent during free-radical polymerisation reactions.[91, 95] This is advantageous, as chain transfer can result in lower reaction rates and can reduce the molecular weight of the polymers produced.

However, there are disadvantages associated with the use of supercritical carbon dioxide. As with most supercritical processes, expensive and specialised high-pressure equipment is required to ensure their safe use.[91, 95, 96] In addition to this, supercritical carbon dioxide is a relatively poor solvent when used at moderate and economic temperatures and pressures (<100 °C and <1000 bar).[91] High molecular weight polymers are generally insoluble in carbon dioxide under these conditions, with the exception of some fluorinated polymers such as poly(1H, 1H-perfluorooctyl acrylate, PFOA).[91, 94]

Numerous polymerisation methodologies have been conducted employing supercritical carbon dioxide as the reaction medium, or as a component of the reaction medium.[91, 95] These methods include, homogeneous polymerisations, precipitation polymerisations, dispersion polymerisations and emulsion polymerisations. Carbon dioxide had also been shown to plasticise certain polymers formed from bulk polymerisations and can be used in the formation of highly porous crosslinked polymeric materials.[91, 97-100]

Carbon dioxide has been used as the reaction medium for many homogeneous polymerisation reactions. However, due to the relatively low solvating power of supercritical carbon dioxide when employed under readily achievable conditions (i.e. at

pressures under 1000 bar), very few non-fluorinated polymerisations have been carried out. The solvent power of carbon dioxide when used under these "achievable" conditions has been likened to that of n-alkanes.[101] Beuermann *et al* have reported the free-radical polymerisation of styrene in supercritical carbon dioxide under homogeneous conditions.[102] However, the reaction conditions employed involved the use of high temperatures and pressures (80 °C and 200-1500 bar), as well as the presence of a chain transfer agent (carbon tetrabromide). It is likely that these reactions remained homogeneous due to the high temperatures and pressures employed as well as the inclusion of a chain transfer agent in the reaction mixture. This would reduce the average molecular weight of the poly(styrene) produced, making it soluble in the supercritical carbon dioxide.[91] Although most polymers are insoluble in carbon dioxide, its ability to solvate high molecular weight fluorinated polymers is still advantageous, as these polymers would otherwise be synthesised in chlorofluorocarbons that are known to be very damaging to the environment.[103, 104]

The insolubility of most non-fluorinated high molecular weight polymers might be thought of as a disadvantage, however the insolubility of the polymer product is often a necessity for other heterogeneous polymerisation techniques such as suspension, emulsion and dispersion polymerisation.[91]

Numerous examples of precipitation polymerisation reactions conducted in supercritical fluids have been reported in the literature.[105] The most commonly cited advantage for using supercritical carbon dioxide as an alternative reaction medium to traditional organic solvents is the ease of product recovery. The precipitated polymers are simply removed from the reaction vessel as dry, free-flowing powders, upon depressurisation.[91] Romack

*et al* synthesised poly(acrylic acid) via a precipitation polymerisation methodology, conducted in supercritical carbon dioxide.[106] The polymer formed was readily isolated as a dry powder and was shown to have a comparable molecular weight, polydispersity and particle size as was produced by polymerisations conducted in conventional organic solvents. Molecular weights as high as 150 KDa could be achieved, and the molecular weight could be manipulated via the addition of various quantities of a suitable chain transfer agent, such as ethyl mercaptan. DeSimone *et al* investigated the cationic polymerisation of vinyl ethers in liquid and supercritical carbon dioxide.[111] A major advantage of cationic polymerisations is that the high reactivity of carbocations results in fast kinetics.[107] However this reactivity can lead to side reactions, chain transfer and termination.[91] Carbon dioxide has been shown to be advantageous compared to traditional organic solvents. In addition to the environmental benefits, carbon dioxide is relatively chemically inert towards cationic species. Polymerisations have been conducted that produce materials that show no evidence for the incorporation of $CO_2$ into the growing polymer backbone.[108, 109]

In dispersion polymerisation the monomers are soluble in the reaction medium, whilst the polymers formed are not.[91] Supercritical carbon dioxide is a useful medium for this polymerisation methodology as numerous monomers can be dissolved, but very few polymers are soluble (particularly at high molecular weights). The addition of suitable surfactants to the polymerisation reaction can help to stabilise the polymers formed, preventing them from forming an agglomerated mass.[91] Careful selection of surfactant as well as control over its quantity and the reaction conditions, can lead to the formation of uniform polymer microspheres. A significant advantage of dispersion polymerisations

conducted in supercritical carbon dioxide compared to conventional organic solvents, is that depressurisation of the reaction vessel yields the dry polymer products. This eliminates the need for a drying step that can often cause the polymer microspheres to agglomerate. A major disadvantage of carbon dioxide is that there are only a limited number of surfactants that are soluble.[110] To this end specialised fluorinated $CO_2$ soluble surfactants were synthesised.[94, 111] DeSimone *et al* demonstrated that these surfactants could be used to stabilise polymers formed via dispersion polymerisation reaction conducted in carbon dioxide, producing spherical particles of between 0.9 and 2.7 μm in diameter. Both the total conversion of monomers and the molecular weight of the products were shown to be greater than for the same polymerisation reaction conducted in the absence of a surfactant.

Carbon dioxide is not the only supercritical medium employed for dispersion polymerisations. 1,1,1,2-tetrafluoroethane (R134a) has also been successfully used in the production of relatively uniform spherical polystyrene microspheres.[110] Wood *et al* demonstrated that inexpensive poly(vinyl acetate) macromonomer stabilisers were soluble in R134a and could be employed in dispersion polymerisations conducted at relatively low temperatures and pressures (60 °C and <20 bar). At these temperatures R134a exists as a liquefied gas, as its critical temperature is 101.1 °C.[91] R134a does have some disadvantages when compared to carbon dioxide. It is more expensive and is a potent greenhouse gas (1300 times the effect of carbon dioxide). However, it is also non-toxic, non-flammable and is not ozone depleting.

Both emulsion and suspension polymerisation reactions require monomers that have a very low solubility in the continuous phase. Carbon dioxide is a relatively good solvent

for most common monomers, even when employed under moderate temperatures and pressures.[91] As such, few examples exist of carbon dioxide based emulsion or suspension polymerisations. Cooper *et al* have formed oil-in-water emulsions with a supercritical carbon dioxide internal phase.[97-99] Emulsions were readily formed in the presence of Tween-40 and PVA co-surfactants. Acrylamide and bis-acrylamide monomers dissolved in the continuous aqueous phase were then polymerised at low temperature in the presence of a redox co-initiator, "locking-in" the structure of the emulsion. Depressurisation of the reaction vessel yielded highly porous emulsion templated polymer monoliths. The most significant advantage of using a supercritical carbon dioxide internal phase over a conventional organic solvent was the facile removal of the dispersed phase. The removal of large quantities of organic solvents trapped within the internal pore structure of a polymer monolith is a very energy intensive and time-consuming process. The materials must either by dried under vacuum for long periods, or the organic solvent displaced by progressive washings with a more volatile miscible solvent.[99]

In general emulsions formed with larger volumes of internal phase will produce templated polymers with a greater degree of porosity. When the concentration of the internal phase exceeds 74.05 $\%^{v}/_{v}$ (the close packed limit for non-deformable uniform spheres) the emulsion is termed a High Internal Phase Emulsion (HIPE). Polymers formed from HIPEs are called polyHIPEs.[112-114] Butler *et al* have synthesised polymers from high internal phase emulsions formed with a carbon dioxide internal phase.[98, 99] The materials produced are highly porous and display a high degree of interconnectivity. As such these materials are suitable for use as enzyme supports. The high porosity and

interconnectivity allows substrates and products to diffuse throughout the entire polymer structure, accessing the enzymes active sites, even when the enzyme is immobilised in the interior of the polymers structure. Some preliminary investigations regarding the immobilisation of enzymes in porous polyHIPE materials synthesised from traditional organic solvents have been conducted.[63, 115] However, no work has yet been reported concerning the immobilisation of enzymes of polymer supports synthesised from HIPEs formed with supercritical internal phases.

### 1.4.2 Supercritical biocatalysis

Supercritical fluids can be used as the reaction medium for enzyme catalysed reactions, this is known as supercritical biocatalysis. Supercritical fluids offer several unique advantages over traditional solvents when employed for enzymatic catalysis. In their native state enzymes are insoluble in supercritical fluids, resulting in a heterogeneous reaction system.[116, 117] This allows for the relatively simple separation of the reaction products by filtration of the reaction medium (whilst still under pressure) to remove the insoluble enzyme particles. Depressurisation of the reactor causes the precipitation of the products from the supercritical solution, facilitating their recovery. Unlike traditional organic solvent methodologies, the products yielded are free from organic solvent residues, which is particularly important for compounds synthesised for biomedical applications.[80]

The nature of the solvent used as the biocatalysis medium is critical to the catalytic activity of the suspended enzyme. Solvents can affect the enzyme directly by chemically inhibiting the function of the enzyme's active site or by disturbing the overall

conformation, thereby influencing catalysis. The solvent can also influence the enzyme's activity indirectly, through solvent parameters such as, dielectric constant,[118] Hildebrand solubility parameters[119] and partition coefficient.[120] Using traditional organic media, it is virtually impossible to study each of these effects individually, as solvents with differing chemical structures will also have different direct and indirect effects on the enzyme. The prediction of an enzyme's properties for reactions conducted in non-aqueous media is very difficult due to the multiple effects encountered when changing the reaction medium.

However the use of supercritical fluids offers a unique methodology for the study of various solvent parameters and their effect on enzyme activity, without the need to change the intrinsic chemical nature of the reaction medium. Subtle changes in the solvent's properties can be achieved through variation of the reactor's pressure and temperature.

The very low gas-like viscosities of supercritical fluids are generally at least an order of magnitude lower than traditional organic solvents, making them advantageous for use as the reaction media in biocatalytic reactions.[117, 121] Substrates dissolved in liquefied gases are able to quickly diffuse throughout nanoporous materials, reducing the mass transfer limitation to the active sites of enzymes immobilised on such materials.[116]

The diffusional limitation of substrates and products to and from the enzyme's active sites can broadly be divided into two steps. The external mass transfer limit governs the rate at which substrates and products diffuse to and from the bulk solvent and the surface of any heterogeneous catalytic particles. The internal mass transfer limit describes the rate at which substrates and products diffuse to and from the exterior surface of a heterogeneous

particle and the particle's interior surfaces. Kamat *et al*[122] calculated that the intrinsically high diffusivities of supercritical fluids enabled approximately two orders of magnitude less agitation, to achieve the same diffusional rates as found in conventional organic solvents. Numerous practical studies involving enzymatic catalysis in supercritical fluids have shown that there is no detectable external mass transfer limit.[123-125] Internal mass transfer limits for supercritical carbon dioxide have also be calculated to be higher than for hexane.[124] Nakaya *et al* also concluded that for the transesterification reaction of laurin with palmitic acid in supercritical carbon dioxide, the reaction rate was not diffusionally limited.[130] Iborra *et al* have reported the use of a biphasic reaction medium consisting of supercritical carbon dioxide and an ionic liquid.[80] *Candida antarctica* lipase B was employed for the catalysis and was shown to be stable under extreme conditions of temperature and pressure. It is argued that under these conditions the ionic liquid can be regarded as an immobilisation matrix due to the multipoint stabilising interactions of the fluid with the enzyme's polar and hydrophobic amino acid residues. This study demonstrates that the advantageous attributes of supercritical fluids, such as high diffusivities can be combined with the stabilising benefits of an ionic liquid.

The use of supercritical biocatalysis also offers a new route to the enzymatic reaction of gaseous substrates, such as hydrogen and oxygen. Under traditional reaction conditions these gases can be used as substrate materials, but comparatively low reaction rates are observed. However, supercritical methodology should allow for increased reaction rates due to the solubility of these substrates in other supercritical media.[117, 121] Hammond *et al* studied the enzymatic oxidation of p-cresol and p-chlorophenol using oxygen in supercritical fluoroform.[126] Conversions of 70-80 % were typically obtained.

The major disadvantage of using supercritical fluids as reaction media for biocatalytic reactions is the expense of the equipment necessary for conducting the reactions. High-pressure equipment is always associated with high costs, however this must be balanced against the value of the experiments conducted or products synthesised.[116]

Another disadvantage of supercritical fluids is their relatively low solubility of conventional substrates. This can make comparing pure supercritical systems with traditional organic solvent systems difficult, as the assays employed for this purpose are no longer comparable.[116, 117, 121] Commonly used substrates such as p-nitrophenyl phosphate have been shown to be insoluble in supercritical fluids such as carbon dioxide.[127] This problem can usually be overcome by the addition of small quantities of a suitable cosolvent. Primary alcohol cosolvents have been shown to enhance the observed reaction rate by as much as 200 fold, when compared to a pure carbon dioxide system.[128]

Most biocatalytic studies conducted in supercritical media have involved following the esterification and transesterification reactions of various lipases.[116] This is because the relative solubility of typical lipase substrates such as fats and oils, are higher in common supercritical solvents such as carbon dioxide, when compared to the polar aromatic substrates frequently employed for the activity assay of other enzymes.

Carbon dioxide is the most commonly used medium for supercritical biocatalytic experimentation.[80, 117, 121, 129-133] The material itself is readily available, cheap, non-toxic and can easily be produced in very high purity. Carbon dioxide is a logical choice for supercritical biocatalytic reactions because its critical pressure is relatively low (72.8 atm) and its critical temperature is within the best working range of most enzymes (31.1 °C). Several enzymatic reactions have been studied in supercritical carbon dioxide,

employing a variety of enzymes and substrates. It has been noted that many enzymes such as *rhizopus delemar* lipase[134] and *cholesterol oxidase*[135] display significantly increased reaction rates for catalysis carried out in supercritical carbon dioxide (as compared to traditional organic solvents). It is likely in these systems that either the concentration of water in the enzyme's microenvironment has been increased, or that the diffusivity of substrates to the enzyme's active sites has been enhanced.

However, several studies have shown that enzymes can be deactivated by exposure to supercritical carbon dioxide.[121, 128, 131, 132] The enzyme from *streptomyces sp.* was demonstrated to possess a half-life of only one hour when exposed to supercritical carbon dioxide. In these cases enzyme activity was usually regained after separation of the catalyst from the reaction media followed by rehydration. It had been postulated that this deactivation might have been caused by the very rapid dehydration of the enzyme's microenvironment that can occur during the depressurisation step necessary to vent the carbon dioxide.[131, 132, 136] However, the general consensus is that the enzyme undergoes chemical modification on exposure to supercritical carbon dioxide. The lysine residues present in the protein's amino acid chain contain primary amine groups. These amine groups could react with the carbon dioxide to form carbamates.[129] If the particular enzyme employed for the catalysis required the primary amine groups in order for the active site's catalytic mechanism to function, then the enzymes with these carbamates modifications would not be observed to function as well as those without. Depressurisation of the reactor results in the disintegration of these unstable carbamates, allowing the enzyme to regain its activity. This instability of the carbamate groups formed makes validation of their presence difficult to ascertain. However one qualitative

study has found evidence for the formation of carbamates. Kamat *et al* employed Laser Desorption Mass Spectrometry (LD-MS) to probe possible reaction products formed when Subtilisin Carlsberg was suspended in supercritical carbon dioxide.[137] The observed mass of the protein increased by 176 Da, equivalent to four carbon dioxide units. However this does not definitively prove that enzyme activity is directly affected by carbamate formation on contact with supercritical carbon dioxide.

Lozano *et al* studied the effect of carbon dioxide density on the stability of a suspension of enzyme.[131] α-Chymotrypsin was shown to be deactivated by prolonged exposure to carbon dioxide. Strangely, the rate of deactivation was found to be inversely proportional to the density of the incubating medium with the best stability obtained on suspension of the enzyme in supercritical carbon dioxide with a density of 0.8 g/cm$^3$. A separate study also found that the deactivation of enzymes suspended in carbon dioxide was alleviated by increases in the reactor pressure, and so solvent density.[132]

Another alternative explanation for the apparent reversible deactivation of some enzymes on exposure to supercritical carbon dioxide may be due to changes in the pH of the enzyme's microenvironment. Enzymes are well known to be sensitive to changes in pH, with deactivation commonplace at extremes of acidity or alkalinity. Although the enzyme is suspended in a non-aqueous medium, a monolayer or more of water in still likely to exist around its structure. Contact between this water layer and the supercritical carbon dioxide may result in a localised reduction in pH lowering the corresponding enzyme activity.[123, 138] However, it was determined that the simple lyophilisation of enzymes from buffered solution could provide a sufficient localised concentration of buffer salts to overcome deactivation by changes in localised pH.[123]

For these reasons several other gases have been investigated for their effects on enzyme activity when used for supercritical biocatalysis. These include ethylene, propane, fluoroform, sulfur hexafluoride and ethane.[129, 132, 138-140] Russell *et al* conducted biocatalytic reactions in these supercritical media.[121] *Candida cylindracea* was employed for the catalysis of the transesterification reaction between methyl methacrylate and 2-ethyl hexanol. The nature of the supercritical fluid was shown to have a profound effect on the catalysed reaction rate. The order of activity for the various media was found to be sulfur hexafluoride > propane > ethane > ethylene > fluoroform > carbon dioxide. It was postulated that this trend in activity may correspond to the supercritical medium's ability to maintain a monolayer of water covering the enzyme.[116] It is well known that the amount of water contained within the enzyme's microenvironment is critical to its catalytic activity.

Supercritical ethane has been used as the reaction medium for enzymatic catalysis using Cross-Linked Enzyme Crystals (CLEC).[141] It was shown that these enzyme preparations were vulnerable to deactivation by acidic reaction products. Halling *et al* have demonstrated that the ionisation state of the enzyme and hence its activity, can be controlled by the addition of solid-state buffers to the reaction medium.[20]

Kamet *et al* have conducted numerous experiments exploiting the pressure dependent properties of fluoroform when used as a supercritical medium for biocatalysis.[117] As the pressure of supercritical fluoroform is raised from 500 to 5000 psi, the corresponding dielectric constant of the solvent changes from 1 to 8. It is known that the conformation and flexibility of proteins is affected by suspension in organic solvents of various dielectric constants, ranging between 1 and 10.[118] This change in dielectric constant was

shown to have a significant effect of the catalytic activity of a lipase (*Candida cylindracea*) suspended in the medium. For the transesterification reaction between 2-ethyl hexanol and methyl methacrylate, it was shown that the reaction rate was significantly reduced as the pressure (and so dielectric constant) of the system was increased. The corresponding reaction conducted in propane showed no variation in overall catalysis with changing reactor pressure (propane's dielectric constant does not change with varying reaction pressure). This illustrates the enzyme's dependence on the solvent properties of the supercritical medium.

The changing dielectric constant of the reaction medium is also shown to influence the enantioselectivity of the enzymes suspended in it. *Subtilisin Carlsberg* and *Aspergillus* protease were both studied for the transesterification reaction between N-acetyl-(L or D)-phenylalanine ethyl ester and methanol.[117] It was found that increasing the reactor pressure from 500 to 5000 psi resulted in a significant increase in the enantioselectivity of both enzymes.

Okahata *et al* also used fluoroform as a reaction medium for enzyme catalysed transglycosylations.[142] Manipulation of the reactor pressure, and hence the dielectric constant of the reaction medium, was shown to have a profound effect on the initial reaction rate. The best conversion rates were obtained at intermediate pressures corresponding to a dielectric constant of between four and five.

These experiments demonstrate that the pressure of the reactor (and so solvent properties) can be manipulated to influence the enzyme directly, i.e. enzyme activity and selectivity. However, it was also shown that the variable solvent properties of supercritical fluids could also be used to affect enzyme-catalysed reactions via solvent interactions with the

products formed in the reaction. Fluoroform can be used as the reaction medium for the lipase catalysed polymerisation of 1,4–butanediol and bis (2,2,2–trichloroethyl) adipate. As the polymer chain becomes longer, the product of the reaction precipitates from solution. As both the enzyme and the precipitated polymer are no longer in solution with the monomer feed stocks, the reaction stops. Increasing the reactor pressure results in an increase in the solvating power of the supercritical fluid. Therefore, the reaction produces polymers of a discrete molecular weight range that can be tuned by changing the reactor pressure. At pressures of around 900 psi, polymers were produced with an average molecular weight of 1020 Da and a poly dispersity of only 1.02. Increasing the reactor pressure to 3000 psi resulted in the precipitation of polymers with an average molecular weight of 3357 Da and a poly dispersity of 1.05.

One of the major problems associated with enzymatic catalysis in non-aqueous media is protein agglomeration. This is due to the relative increase in non-covalent intermolecular attractive forces that occurs when water is removed from the reaction system. This problem is also encountered when enzymatic catalysis is conducted in supercritical media. The use of reactors fitted with high-pressure view cells enables the visualisation of protein particle's agglomeration.[121] This problem can be overcome using the same enzyme immobilisation techniques employed for reactions conducted in conventional organic solvents. Several groups have now studied the effects on catalytic activity of enzyme immobilisation.[143, 144] The immobilisation of the enzyme also enables them to be employed in continuous flow applications. The very low viscosities of supercritical fluids means that support materials with nanoporous morphologies can be used as enzyme immobilisation matrices. Substrates and products can then be transported to and from the

enzyme's active sites, positioned on small structural features that would be inaccessible to conventional organic solvents.

## 1.5 Aims

The aim of this study is to investigate the various methods available for the immobilisation of enzymes on porous polymer supports. The effects of these immobilisation methods on enzymatic catalysis in organic media will be assessed. The end goal of the project is to synthesise highly active, re-usable, porous polymer supports containing covalently immobilised enzymes. Supercritical fluids will be employed as environmentally friendly alternatives to traditional organic solvents, for the polymer synthesis. In addition to this, it is hoped that the use of a supercritical reaction media will enable the porosity of the polymer supports to be "fine tuned" for optimum enzyme activity.

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# Chapter 2

## Novel Polyacrylamide Beads For Use As Reusable Enzyme Supports

### 2.1 Introduction

Over the last twenty years much research has been conducted to examine the properties of enzymes suspended in organic media.[1-5] The use of organic solvent's as reaction media offers several advantages over the enzymes traditional aqueous environment. The number of substrates that can be employed for enzymatic catalysis is increased, due to the improved solubility of these substrates in organic solvents, compared to water.[6] This increase in the variety of soluble substrates results in a greater number of reactions that are suitable for enzymatic catalysis. The nature of the reaction catalysed by the enzyme can also be altered when conducted in organic media. For example, esterification and transesterification reactions can be catalysed instead of the hydrolysis reaction that is usually observed when proteolytic enzymes are employed in aqueous solutions.[7-10]

In addition to the increased number of substrates available to the enzyme, the specificity of the catalyst has also been shown to change when employed in organic media.[11-14] This altered specificity can be used for the synthesis of chiral products.[15] Furthermore, the stability of enzymes suspended in organic media has been shown to be enhanced when compared to traditional aqueous environments.[16, 17] Several groups have reported enzymatic catalysis at elevated temperatures of 100 °C and even 150 °C,[4, 18] whereas enzymatic catalysis conducted at 100 °C in an aqueous environment results in the rapid deactivation of the enzyme.[19]

However, there are also numerous disadvantages associated with the use of enzymes in organic media. Enzymes can be deactivated on exposure to organic solvents.[20-23] The removal of water from the enzyme's microenvironment promotes the relative strength of the hydrogen bonding interactions between the enzyme molecules, causing them to aggregate.[24] This aggregation can be exacerbated by the repulsive interactions between amino acid residues present in the enzymes structure and certain organic solvents. As the protein agglomerates, the active sites that perform the desired chemistry can become blocked to the substrate molecules, reducing the enzyme catalytic efficiency. These repulsive and attractive forces can also cause a change in the enzyme conformation, which can result in deactivation.

Protein agglomeration is a major industrial problem; often making large-scale biosynthesis in non-aqueous media impractical. Reactors and equipment can become covered in a sticky film of inactive enzyme, extraction of the reaction products can be difficult and reuse of the enzyme is also limited. Overall, large-scale, enzyme catalysis is often rendered uneconomic by these practical problems.[19, 25]

A solution to this problem is to immobilise the enzyme on a support, preventing the particles from sticking together thus making the preparation easier to recover and separate from the reactor and the reaction products. Spreading the enzyme out over a large area also reduces the inherent protein-protein contacts that are experienced with the use of enzymes in heterogeneous organic suspensions. Increases in stability and reusability can also be achieved via enzyme immobilisation.

Of all the various methods of protein immobilisation, the simplest is adsorption. In this case no covalent chemical bond is present linking the enzyme to the support. Only non-

covalent, attractive forces between the support and the enzyme, and repulsive forces between the enzyme and the organic reaction medium prevent the enzyme from leaching away from the support matrix. For large-scale industrial synthesis, a simple, inexpensive and straightforward enzyme immobilisation procedure is highly desirable.

The enzyme adsorption procedure employed for this study was achieved via the facile lyophilisation of the swollen support material, which had previously been immersed in a buffered enzyme solution. Poly(acrylamides) have been used extensively to immobilise enzymes as the monomers are readily polymerised and the products are stable and non-toxic.[26-28] In this study novel porous poly(acrylamide) beads were employed as enzyme supports. These materials are very easy to handle due to their comparatively large size and good mechanical strength. They are easily separated from the reaction mixture by simple decanting, can be washed to remove substrate and product residues and are then suitable for reuse. The interconnectivity of these porous materials allows for the diffusion of substrates and products to and from the active sites of the enzymes which are immobilised within the internal structure of the poly(acrylamide) beads.[29]

In this study α-chymotrypsin was chosen as a model enzyme as its properties in organic media have been well studied.[26-28, 30-32]The full crystal structure of the α-chymotrypsin is available enabling a better understanding of how the substrates interact with the enzymes active site. α-Chymotrypsin is also a relatively inexpensive and industrially useful enzyme that is widely employed for peptide synthesis.[30-32]

## 2.2 Materials

For the production of porous polymer beads, the following chemicals were used: acrylamide (AM) (99+ %, Aldrich), *N,N'*-methylene bisacrylamide (MBAM) (99 %, Aldrich), Triton X-405 (70 % solution in water, Aldrich), poly (vinyl alcohol) (PVA) ($M_w$ 9-10 kDa, 80 % hydrolysed, Aldrich), ammonium persulfate (APS) (98 +%, Aldrich), *N,N,N,N*-tetramethylethylenediamine (TMEDA) (99.5 +% redistilled, Aldrich) and mineral oils (heavy and light grades, Aldrich). For the enzyme loading and activity assays the following chemicals were used: α-chymotrypsin (type II from bovine pancreas, Sigma), subtilisin Carlsberg (type VIII from *bacillus licheniformis*, Sigma), bromelain (from pineapple stem, Fluka) and N-acetyl-L-tyrosine ethyl ester (ATEE) (Sigma grade, Sigma). Solvents were obtained from the VWR Company and were of HPLC grade. All other chemicals were obtained from Aldrich and were of general-purpose grade.

## 2.3 Experimental

### 2.3.1 Preparation of porous poly(acrylamide) beads via sedimentation polymerisation

The preparation of porous poly(acrylamide) (PAM) beads was achieved by following a synthetic procedure first reported by Zhang *et al.*[29]

Three sets of PAM beads were synthesised with differing ratios of monomer to crosslinker (acrylamide (AM) monomer and *N,N'*-methylene bisacrylamide (MBAM)

crosslinker). These ratios were fixed at 70:30, 80:20 and 95:5 $^{w}/_{w}$ monomer to crosslinker. All subsequent enzyme related experiments were conducted using a single batch of each of the various PAM beads, to eliminate the effect of any batch-to-batch variations.

The monomers were dissolved in the aqueous phase by stirring at room temperature (40 $\%^{w}/_{w}$ total monomer content in aqueous solution containing 2 $\%^{w}/_{w}$ poly(vinyl alcohol) co-surfactant, 6.0 ml total volume). The surfactant Triton X-405 (1.3 ml of a 70 % solution in water) was added to the mixed monomer solution, along with an aqueous solution of ammonium persulfate (APS) initiator (60 mg of APS in 0.6 ml water). The resulting solution was stirred (approximately 600 rpm) with an overhead paddle stirrer (RW11 Basic IKA paddle stirrer) during the gradual addition of the internal oil phase of the emulsion, containing a redox initiator (30.0 ml of light mineral oil containing 16.4 mg of *N,N,N,N*-tetramethylethylenediamine (TMEDA)). Stirring was continued until a stable high internal phase emulsion was formed.

The stirred high internal phase emulsion was then injected into the sedimentation column at a flow rate of 0.5 ml/min using a A-99 FZ Razel syringe pump, fitted with a 0.6 mm x 25 mm needle.

The glass sedimentation column had dimensions of 53 cm in length x 5.6 cm outside diameter x 4.6 cm internal diameter. Heavy mineral oil was used as the sedimentation medium. The level of the oil was approximately 5 cm from the top of the glass column. The sedimentation medium was heated to 60 °C with an electrical heating tape. The temperature at the base of the column was maintained by means of an oil bath.

The heavy mineral oil sedimentation medium was chosen to provide a density such that uniform spherical droplets were formed during sedimentation. Sedimentation was typically completed within 10-20 seconds. Complete polymerisation was achieved by heating the column for a further 3 hours. The internal oil phase was removed from the beads by immersing them in *n*-hexane for at least 24 hours at room temperature. The beads were then washed 10 times with alternating portions of acetone and *n*-hexane, before being allowed to air dry at room temperature. Finally, the beads were dried overnight under vacuum at 50 °C.

### 2.3.2 Enzyme immobilisation on porous PAM beads

α-Chymotrypsin was dissolved in 0.2 M phosphate buffer adjusted to pH 7.8 with sodium hydroxide. Solutions with concentrations between 10 mg/ml and 400 mg/ml were prepared. A known mass of dry polymer beads was then soaked in the various solutions. The swollen beads were freeze dried (>48 hours, Lyolab 3000, Heto) to remove the aqueous phase, leaving the enzyme adsorbed to the support. Equivalent lyophilised preparations of unsupported enzyme were prepared by freeze-drying the buffer solutions used to load the polymer beads with enzyme. All enzyme preparations were stored under vacuum, over #4 molecular sieves at room temperature when not in use.

The effect of pH of the catalytic activity of the immobilised enzyme was also studied. PAM beads were loaded with enzyme solutions of 10 mg/ml in 20 mM $KH_2PO_4$ buffer solutions. The pH of these buffered solutions ranged from pH 4 to pH 11. Equivalent lyophilised preparations of unsupported enzyme were also prepared from buffered enzyme solutions of various pH, by freeze-drying the solution for >48 hours.

Two alternative protease enzymes were also selected for comparison with α-chymotrypsin; Subtilisin Carlsberg and Bromelain. Loading of the PAM beads with each of these two enzymes was achieved via a similar method to that described above. Enzyme concentrations were fixed at 10 mg/ml in 20 mM $KH_2PO_4$ buffer and the pH set to the optimum reported level for each of the enzymes; α-chymotrypsin pH 7.8[33], subtilisin Carlsberg pH 7.5[34] and bromelain pH 4.6[35, 36]. Equivalent lyophilised preparations of these enzymes were also produced via the freeze-drying of the buffered solutions for >48 hours.

### 2.3.3 Physical characterisation

Pore size distributions were recorded by mercury intrusion porosimetry using a Micrometrics AutoPore IV 9500 porosimeter. Samples were subjected to a pressure cycle starting at approximately 0.5 psi, increasing to 60,000 psi in predefined steps to give pore size / pore volume information. Intrusion volumes were calculated for the samples by subtracting the intrusion volume arising from the mercury interpenetration between beads (pores >200 μm) from the total intrusion volume. Polymer surface areas were measured using the BET method with a ASAP 2010 nitrogen adsorption analyser. Samples were degassed for 2 hours at 110 °C under vacuum before analysis. Absolute densities were measured via helium pycnometry with a Micrometrics AccuPyc 1330 instrument. Scanning Electron Microscope (SEM) images were captured using a Jeol 840 instrument. Samples were sputter-coated with approximately 2 nm of gold (Polaron E5000 coating unit) prior to analysis and mounted on 12 mm carbon coated aluminium stubs (Agar

Scientific). SEM images were captured for the exterior surfaces of the beads as well as the interior of individual beads previously sectioned with a razor blade.

### 2.3.4 Measurement of water adsorption

A known mass of enzyme-loaded beads was hydrated to various thermodynamic water activities through equilibration in closed vessels containing saturated salt solutions.[37] The samples were allowed to equilibrate until a constant mass was observed. Typically this was achieved within 24 hours, however preparations being hydrated to very low water activities did require longer to reach equilibrium. The samples were transferred to dry vials before being re-weighed. The mass increase observed was attributed to the water content of the beads.

### 2.3.5 Enzyme activity assay

The catalytic activity of the immobilised enzyme-loaded beads was assayed by monitoring the transesterification reaction between ATEE and propan-1-ol using HPLC. The reactions were carried out in 3 ml of hexane (hydrated to the same water activity as the immobilised enzyme support) containing a known mass of enzyme preparation. The reaction was initiated with the addition of ATEE (0.028 mmol) dissolved in dry propan-1-ol (1.5 mmol) and was accompanied by orbital shaking at 100 rpm using an IKA-vibrax-VXR orbital shaker. All enzyme reactions were carried out at a constant temperature of 22 °C. 50 μl aliquots were removed from the reaction mixture at regular time intervals and washed through a 0.22 μm syringe filter with 0.5 ml of acetonitrile to remove any enzyme particles, stopping the catalytic reaction. The determination of the

relative proportions of starting material (ATEE) and N-acetyl-L-tyrosine propyl ester (ATPE) was carried out by reverse phase HPLC. The instrument employed for this analysis consisted of a Spectra-Physics SP8800 pump, Kontron 465 autosampler and Applied Biosystems 757 UV absorbance detector (245 nm) processed with Datalys Azur v3.0 software. Eluent consisted of 70:30 water : acetonitrile mixture adjusted to pH 2 with orthophosphoric acid. Flow rate was set to 1 ml/min. An injection volume of 10 µl was passed through a 250 x 4.6mm i.d. Hichrom column packed with Waters Spherisorb ODS2. Typically all reaction components were separated and detected within 10 minutes. Determination of which compound (ethyl ester, propyl ester or carboxylic acid) corresponded to each individual peak was achieved with the use of Liquid Chromatography-Mass Spectrometry (LC-MS). The instrument employed was a Micromass LCT electrospray hexapole.

### 2.3.6 Thermal stability of enzymes immobilised on PAM beads

PAM beads loaded with a 10 mg/ml α-chymotrypsin solution in 20 mM $KH_2PO_4$ pH 7.8 buffer were immersed in cyclohexane and heated to 80 °C. 10 beads were removed at 24 hour intervals, frozen in liquid nitrogen and freeze dried for >48 hours to remove the organic solvent. The beads were then assayed for catalytic activity at a thermodynamic water activity of 0.43 using the transesterification assay described above (section 2.2.5). The equivalent unsupported lyophilised enzyme preparation was also heated to 80 °C in cyclohexane before being sampled, freeze-dried and assayed in the same way as for the immobilised preparations.

### 2.3.7 Re-utilisation of the supported enzyme preparations

Two re-utilization studies were conducted. For the first study PAM beads were loaded with enzyme using a 10 mg/ml α-chymotrypsin in 20 mM $KH_2PO_4$ pH 7.8 buffer solution. 10 beads were accurately weighed and hydrated to a constant water activity of 0.43. Initial catalytic activity was determined using the transesterification assay (section 2.2.5). After their initial use the beads were washed with 3 x 3 ml portions of dry hexane (stored over # 4 molecular sieves). The transesterification assay was then repeated, using hexane that had previously been hydrated to a constant water activity of 0.43. This process of washing and rehydration was repeated for each subsequent use of the beads. All reactions were carried out at 22 °C and in triplicate, in order to obtain concordant results. For the second re-utilization study, beads of each different enzyme loading (section 2.2.2) were hydrated to a constant water activity of 0.43 before being assayed using the transesterification assay (section 2.2.5). After their initial use the beads were washed extensively with 3 x 50 ml volumes of acetone and dried under vacuum at 40 °C for at least 12 hours. The beads were then rehydrated over saturated salt solutions (>24 hours) to $a_w$ 0.43 prior to the next assay. This process was repeated for each use of the beads. At each stage one bead was removed from each set of different enzyme loadings and imaged with SEM. The procedure of washing and vacuum drying was repeated for the equivalent unsupported enzyme, prepared from lyophilised 0.2 M $KH_2PO_4$ pH 7.8 buffer solutions of various enzyme concentrations.

## 2.4 Results and discussion

### 2.4.1 Enzyme loading of porous beads

Highly porous PAM beads were produced via a novel emulsion templating sedimentation polymerisation procedure[29] described previously (section 2.2.1). The beads produced were uniform white spheres (figure 2.1), with a typical diameter of 2.16 mm and a standard deviation of 2.6 % (56 μm). Figure 2.1 also illustrates the highly porous nature of the internal structure, as well as the porous exterior surface of the unloaded beads.

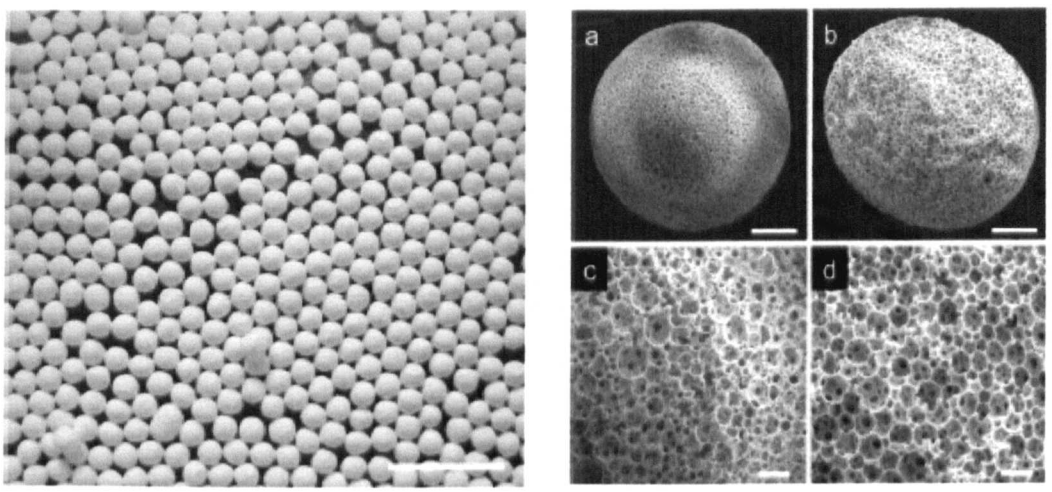


*Figure 2.1. Optical image (left) of porous PAM beads. Scale bar = 10 mm. Electron microscope images (right) of porous poly acrylamide beads. (a) whole bead (scale bar = 500 μm). (b) Sectioned "half bead" showing internal pore structure (scale bar = 500 μm). (c) Magnified image of bead surface showing pore structure (scale bar = 100 μm). (d) Magnified image of a fracture surface in a sectioned bead showing internal pore structure (scale bar = 100 μm).*

The dry beads had an average mass of 0.65 mg before the enzyme was loaded. After the enzyme was adsorbed the residual mass of the beads varied according to the concentration of the loading solution (figure 2.2).

Figure 2.2 illustrates that the polymer beads are capable of adsorbing very large quantities of catalyst. Loadings in excess of three times the mass of the support material were easily achieved. With very highly water-soluble catalysts it may be possible to load even greater masses on each bead. This may be of considerable advantage in applications where the catalyst is poisoned, deactivated or lost from the support matrix during a reaction procedure.

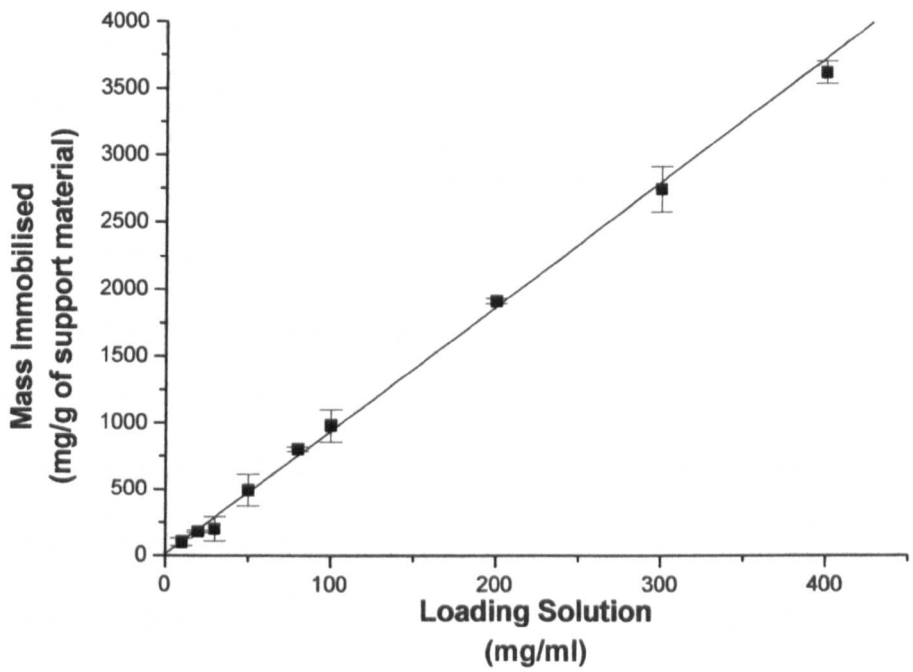


*Figure 2.2 Relationship between the concentration of the buffered enzyme solution and the resulting mass of enzyme and buffer salts immobilised on the porous PAM beads.*

### 2.4.2 Physical characterisation of enzyme loaded PAM beads

In order to investigate the influence of bead morphology on the corresponding enzyme activity, three sets of beads were synthesised, each with different monomer to crosslinker

ratios. As the monomer to crosslinker ratio was increased from 5 % crosslinker to 30% crosslinker, the corresponding surface area of the unloaded beads increased (5 % crosslinker = 1.336 $m^2/g$, 20 % crosslinker = 9.590 $m^2/g$ and 30 % crosslinker = 15.981 $m^2/g$). Increased surface area of enzyme supports is known to result in higher catalytic activities,[38] as the enzyme is spread out over a larger area, increasing the accessibility of its active sites to the incoming substrate. All three variations of bead were shown to adsorb very similar quantities of enzyme, when immersed in the buffered enzyme solutions and little or no difference between the catalytic activities of α-chymotrypsin immobilised on these different supports was observed. This is most likely because all three of the support materials exhibit relatively low surface areas (<20 $m^2/g$). Hence, it was assumed that the change in bead porosity associated with the differing crosslinker levels did not significantly influence the diffusion of substrates and products to and from the enzyme's active sites.

It was observed that the beads formed with 5 % crosslinker exhibited little mechanical stability, tending to disintegrate during the enzyme activity assay. Surprisingly, the same disintegration was observed with the beads formed from the highest crosslinking ratio (30 % crosslinker). This may be due to poor solubility of MBAM in water at these concentrations. Precipitation of the crosslinker prior to and during the polymerisation reaction could result in a mechanically unstable product. However, the 20 % crosslinker variety of bead appeared sufficiently mechanically robust to be easily handled and showed no signs of wear during the initial enzyme activity assays. As such, these beads were employed for all subsequent experiments performed in this study.

| Enzyme Loading mg/g of polymer | Surface Area $m^2g^{-1}$ | Average Density g/ml | Total intrusion vol. ml/g |
|---|---|---|---|
| 0 | 10.4 | 5.0 | 3.1 |
| 79 | 8.7 | 3.9 | 3.1 |
| 132 | 8.3 | 2.1 | 2.3 |
| 171 | 7.5 | 4.8 | 2.5 |
| 408 | 6.6 | 3.7 | 2.3 |
| 790 | 3.4 | 3.0 | 2.9 |
| 1066 | 2.9 | 2.7 | 2.8 |
| 1895 | 4.0 | 1.8 | 2.1 |
| 2619 | 3.0 | 1.8 | 1.7 |
| 3553 | 1.2 | 1.9 | 1.3 |

*Table 2.1 Physical characterisation data for enzyme loaded PAM beads formed from a 20:80 crosslinker : monomer ratio. Surface areas calculated using nitrogen desorption (BET method). Absolute densities determined with helium pycnometry. Intrusion volumes measured via mercury intrusion porosimetry.*

As the mass of catalyst loaded on each bead is increased, the physical morphology of the beads changes significantly (table 2.1 and figure 2.3). The bead's surface area is considerably reduced as the enzyme loading is augmented. This is particularly noticeable when loading is increased to very high levels (figure 2.3).

SEM images show that as the enzyme loading is increased the internal structure of the support changes from a highly porous state to being densely packed and highly crystalline. Total intrusion volume as measured by mercury porosimetry also exhibits an overall trend of decreasing pore volume with increased enzyme loading. As the internal pore structure of the support material is packed with more and more catalyst the porous structure becomes blocked, reducing pore volumes (figure 2.4). A trend toward reduction of absolute density is also observed with increasing enzyme loading. This is because of the differences in absolute densities between the enzyme and the support material. Due to the very high loadings achieved, a change in absolute density can also be detected.

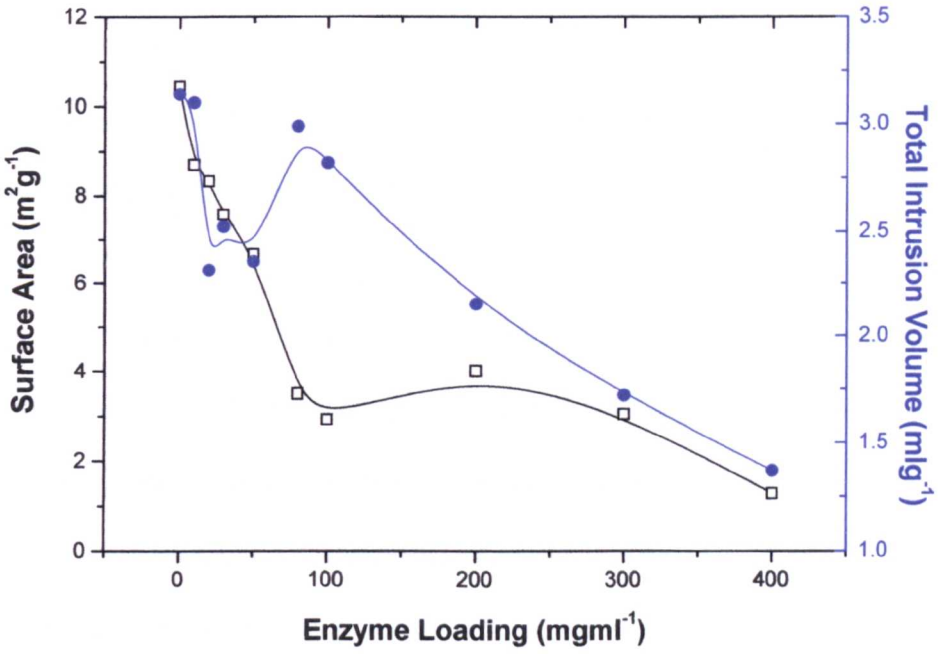


*Figure 2.3 Relationship between the surface areas and intrusion volumes of the porous PAM beads, and enzyme loading, at various levels of enzyme loading. Black squares = surface area. Blue circles = total intrusion volume.*

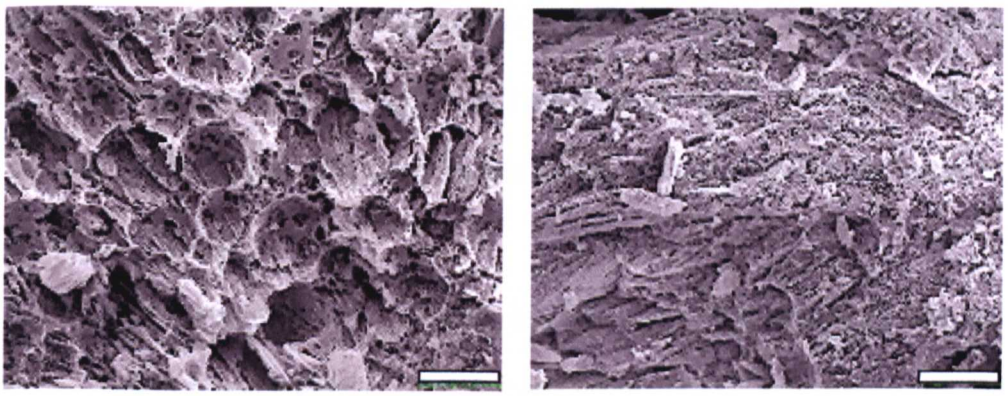

*Figure 2.4 SEM images of sectioned porous PAM beads demonstrating the changes in the beads internal structure that correspond to differing enzyme loading levels. Low enzyme loading, 79.9 mg/g (left) and high enzyme loading, 3553 mg/g (right). Scale bars = 100 μm.*

### 2.4.3 Effect of pH on immobilised enzyme catalytic activity.

The ionisation state at which the enzyme operates is critical to its catalytic activity. In traditional aqueous media, enzymes classically exhibit a bell shaped curve describing how catalytic activity varies with pH. However, since the protonation and deprotonation of the enzyme is very rarely observed when catalysis is conducted in organic media, the pH of the enzyme is usually preset whilst still in aqueous solution before the immobilisation/lyophilization process. Generally the enzyme will retain its pH from the aqueous environment when transferred for use in organic media. This retention of pH is known as "pH memory".[39] However, it should be noted that some "organic phase" buffers such as trisocylamine and triphenyl acetic acid do exist, which enable the fine-tuning of the enzymes ionisation state whilst in the organic reaction media.[40]

Enzyme-containing buffered solutions were prepared with the pH set between 4 and 11. PAM beads were loaded with these solutions prior to freeze-drying. The equivalent unsupported lyophilised preparations were also synthesised from the same solutions and used for comparison. The effect of these pH changes on enzyme catalytic activity in supported and unsupported reaction systems was measured. The results (figure 2.5) show that the lyophilised, unsupported enzyme activity is significantly affected by the pH of the buffer solution.

Generally, the catalytic activity of the lyophilised enzyme increased with increasing pH to an observed maximum at pH 7.8 that corresponds to the optimum reported for α-chymotrypsin[33] before gradually decreasing with further increases in the pH of the loading solution. However, the equivalent immobilised preparations not only offer considerably greater initial catalytic rates, but also appear less affected by the pH of the

enzyme loading solution. Catalytic rates remained comparatively high over the entire pH range studied. It may be that the basic amide surface of the polymer beads provides a buffering effect, counteracting the more acidic enzyme loading solutions. It is apparent from figure 2.5 that the deactivation effect observed for the lyophilised preparations is less severe at higher pH environments (7-11) than for the lower range (4-7). As such, the basic surface of the PAM beads may not have a very significant effect on the enzyme activity.

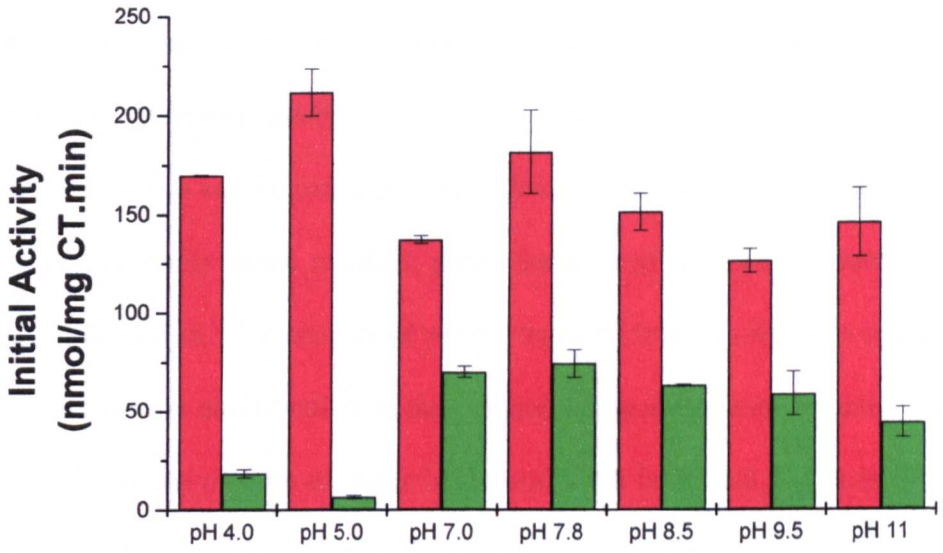


*Figure 2.5. Relationship between the immobilisation pH of the enzyme and catalytic activity in organic media. The initial rate of the reaction catalysed by the enzyme containing preparations was determined for the transesterification of ATEE with propan-1-ol in hexane. Red bars = α-chymotrypsin immobilised on PAM beads. Green bars = equivalent lyophilised unsupported α-chymotrypsin preparations.*

The enzyme supporting beads were designed for use in organic media, where the initial pH of the enzyme loading solution can be controlled and where the ionisation state of the

enzyme is unlikely to be affected. However, if the enzyme were to be crosslinked in a subsequent synthetic step, rendering the enzyme insoluble, the catalytically active beads could then be used in traditional aqueous media. Enhanced pH stability would be of considerable advantage, widening the range of reaction conditions in which the immobilised enzyme could be employed.

### 2.4.4 Water adsorption of porous polymer support

The quantity of water contained within the microenvironment of the enzyme is critical to its resultant catalytic activity.[17, 41-43] It has been shown that the flexibility of the enzyme's structure increases as the level of hydration rises.[6] It is this increased flexibility that gives rise to the enhanced catalytic rates. When used in traditional aqueous environments, enzyme catalysis can be extremely fast. However, when used in organic media, catalytic rates are generally much reduced, partly because of the lack of water in the enzyme's microenvironment. The amount of water contained in a non-aqueous reaction system can be controlled via equilibration of the reaction components over saturated salt solutions.[37] Generally, as the amount of water in the system is increased, the corresponding enzyme activity is also increased. However, the presence of large quantities of water can also lead to undesirable side reactions. For enzyme-catalysed condensation and transesterification reactions, large quantities of water can shift the reaction equilibrium towards production of an unwanted hydrolysis product, (carboxylic acid) instead of the desired ester.

The porous nature of these PAM supports means that very large quantities of water can be adsorbed by the material, when equilibrated over the appropriate saturated salt solution (figure 2.6).

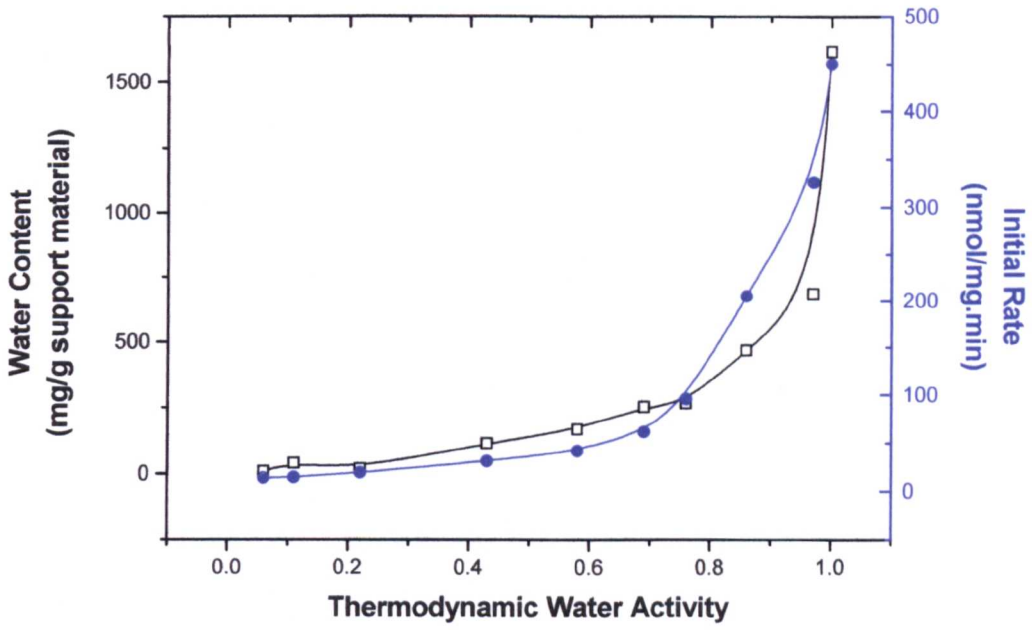


*Figure 2.6 Relationship between water activity and quantity of water adsorbed per gram of the enzyme loaded polymer support (open black squares). Also shown is a typical plot of initial enzyme activity for beads of a fixed enzyme loading (10 mg/ml loading solution) assayed at the corresponding water activities (blue circles).*

It was found that the reaction rate did indeed increase dramatically with increasing thermodynamic water activity. However HPLC analysis of the reaction products over time indicated that even at high water activities, the reaction product was predominantly the desired ester (figure 2.7). The quantity of the hydrolysis product produced appeared relatively unaffected by the amount of water contained within the enzyme's microenvironment.

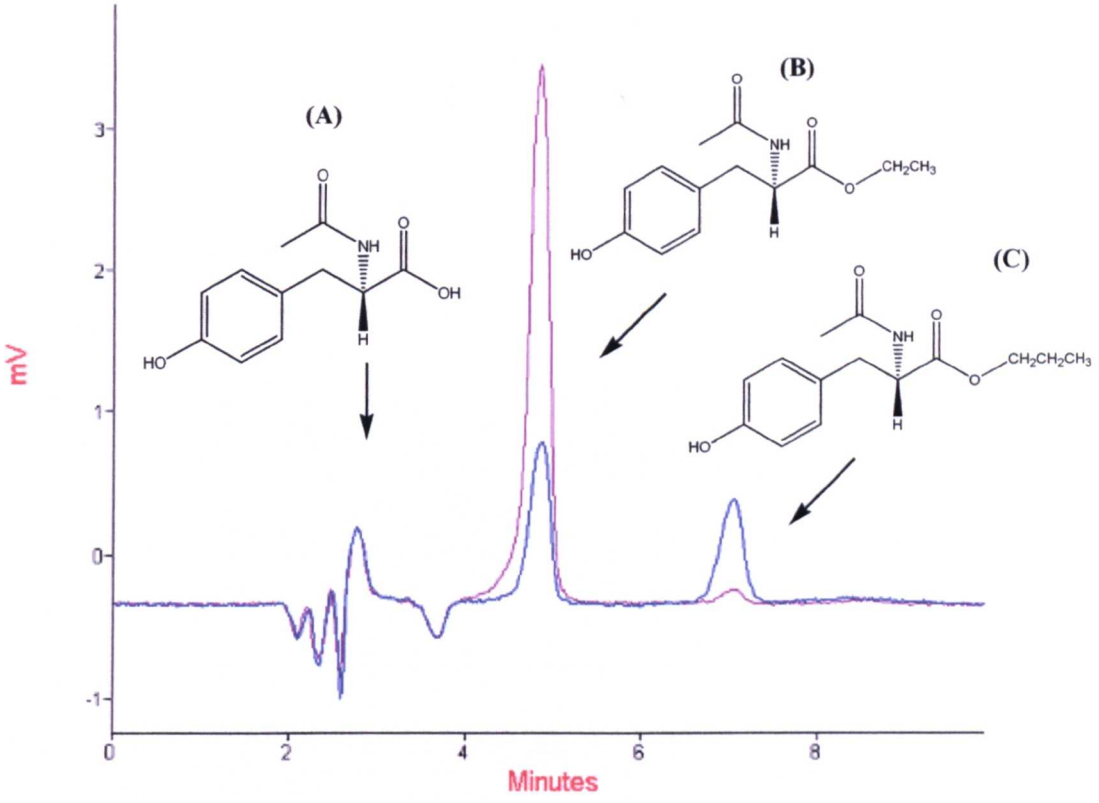


*Figure 2.7. HPLC chromatograms illustrating the typical effects of reaction hydration on the production of hydrolysis and esterification products. Red trace = water activity 0.43, Blue trace = water activity 1.0. Hydrolysis product, 2-acetylamino-3-(4-hydroxy-phenyl)-propionic acid (A) = peak at 2.75 minutes, Starting material, ATEE (B) = peak at 4.83 minutes and ester product, ATPE (C) = peak at 7.03 minutes.*

It has been postulated that supports that adsorb large amounts of water can actually be beneficial to the specificity of the immobilised enzyme catalysed reaction.[30] Although the enzyme and support material are in intimate contact with each other, the hydroscopic support material acts like a sponge, draining water away from the enzyme molecules. It could therefore be reasoned that although large quantities of water are contained within the reaction system at high thermodynamic water activities, only a relatively small amount of water is present in the enzyme's microenvironment, i.e. within the internal structure, or in monolayer coverage on the exterior of the enzyme molecules. This small

quantity of water acts as a sort of "molecular lubricant" facilitating the necessary structural flexibility required by the enzyme to catalyse the transesterification reaction.[2, 17]

This minimal quantity of water is then not acting as a substrate feedstock, but being consumed in the hydrolysis reaction, and so forming the carboxylic acid product. The production of the ester would be much greater because of the increased quantity of propanol, relative to water, in the microenvironment of the enzyme. Inevitably some water must be consumed in the reaction in order to generate the carboxylic acid peak detected in the HPLC separations. It can be seen (figure 2.7) that the quantity of the carboxylic acid produced does not change significantly from when the reaction is carried out at a water activity of 0.43 to when the same reaction is conducted at a water activity of 1.0, even though the total amount of water adsorbed by the support does increase dramatically from 114 mg/g at $a_w$ 0.43 to 1616 mg/g at $a_w$ 1.0.

It may be that as the water contained in the enzyme's microenvironment is consumed in the reaction, the water contained in its macroenvironment, i.e. water adsorbed to the support material, gradually replenishes it. Therefore at low water activities where very little water is present, it could be significantly slower to equilibrate water from the macroenvironment to the microenvironment, than for the same reaction system equilibrated to higher water activities, where the amount of water in the enzyme's macroenvironment is much more abundant. Overall this could result in the comparatively fast reaction rates observed at high water activities compared to low water activities, without any great increase in the catalysis of the undesired hydrolysis reaction.

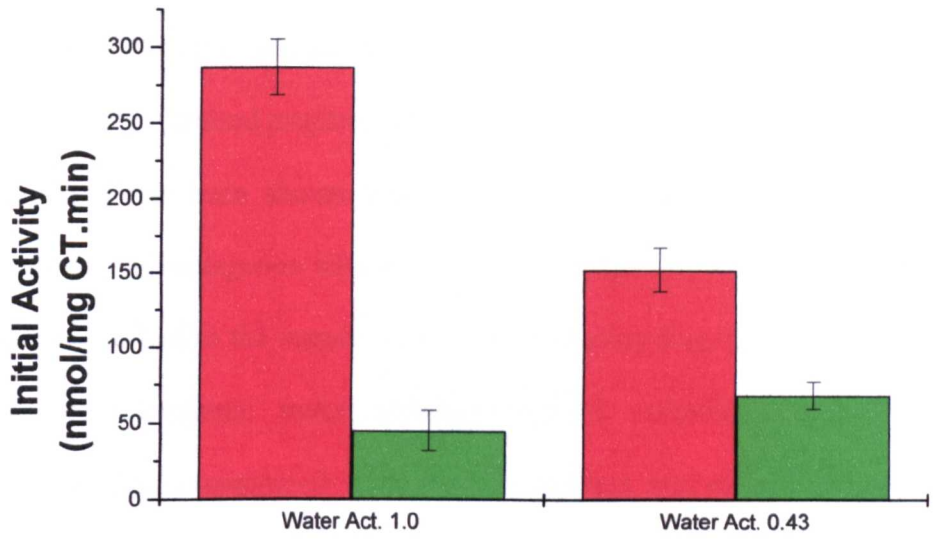


*Figure 2.8 Illustrating the effects of water activity on the initial catalytic activity of an immobilised enzyme preparation (red bars) vs. the equivalent unsupported lyophilised enzyme preparation (green bars). Initial activities determined by following the transesterification reaction between ATEE and propan-1-ol in hexane.*

Another problem frequently encountered when using enzymes in non-aqueous media at high water activities is that of agglomeration.[6] Protein particles often coalesce forming sticky films that cling to the reactor walls. This agglomeration can reduce the number of active enzyme sites available to the incoming substrates, reducing the observed catalytic rate. Agglomeration can also make the enzyme difficult to recover, limiting the reusability of the preparation. Figure 2.8 illustrates the reduction in catalytic rate that can be observed at high water activities.

The unsupported lyophilised enzyme preparation actually shows a reduction in catalysis at a water activity of 1.0 when compared to the same reaction carried out at a water

activity of 0.43. However, the enzyme immobilised on the porous PAM beads displays an increase in catalytic rate, as the supported enzyme is unable to agglomerate in the same way as the lyophilised preparations.

Overall, it has been shown that the very hydroscopic nature of these porous PAM supports is advantageous for several reasons; large quantities of water-soluble catalysts can be adsorbed to the support in the initial loading step, the preparation can be used at high thermodynamic water activities without suffering the problems of protein agglomeration encountered with the unsupported equivalents and that the undesired hydrolysis side reaction is limited, even in reaction systems with high water contents.

### 2.4.5 Influence of hydration level and enzyme loading on catalytic activity.

The influence of two important variables affecting enzyme activity was studied. Nine different concentrations of buffered enzyme solution (10 mg/ml to 400 mg/ml in 0.2 M $KH_2PO_4$ pH 7.8 buffer) were used to load PAM beads with α-chymotrypsin. Each of these nine different enzyme loadings were then hydrated to ten different thermodynamic water activities via equilibration over saturated salt solutions. Each of the preparations was then assayed for catalytic activity by following the initial reaction rate of the transesterification reaction (section 2.2.5).

The results clearly show (figure 2.9) the dramatic increase in enzyme activity associated with the increase in hydration level. This relationship is well documented in the literature [17,41-43] for a variety of different enzyme containing preparations.

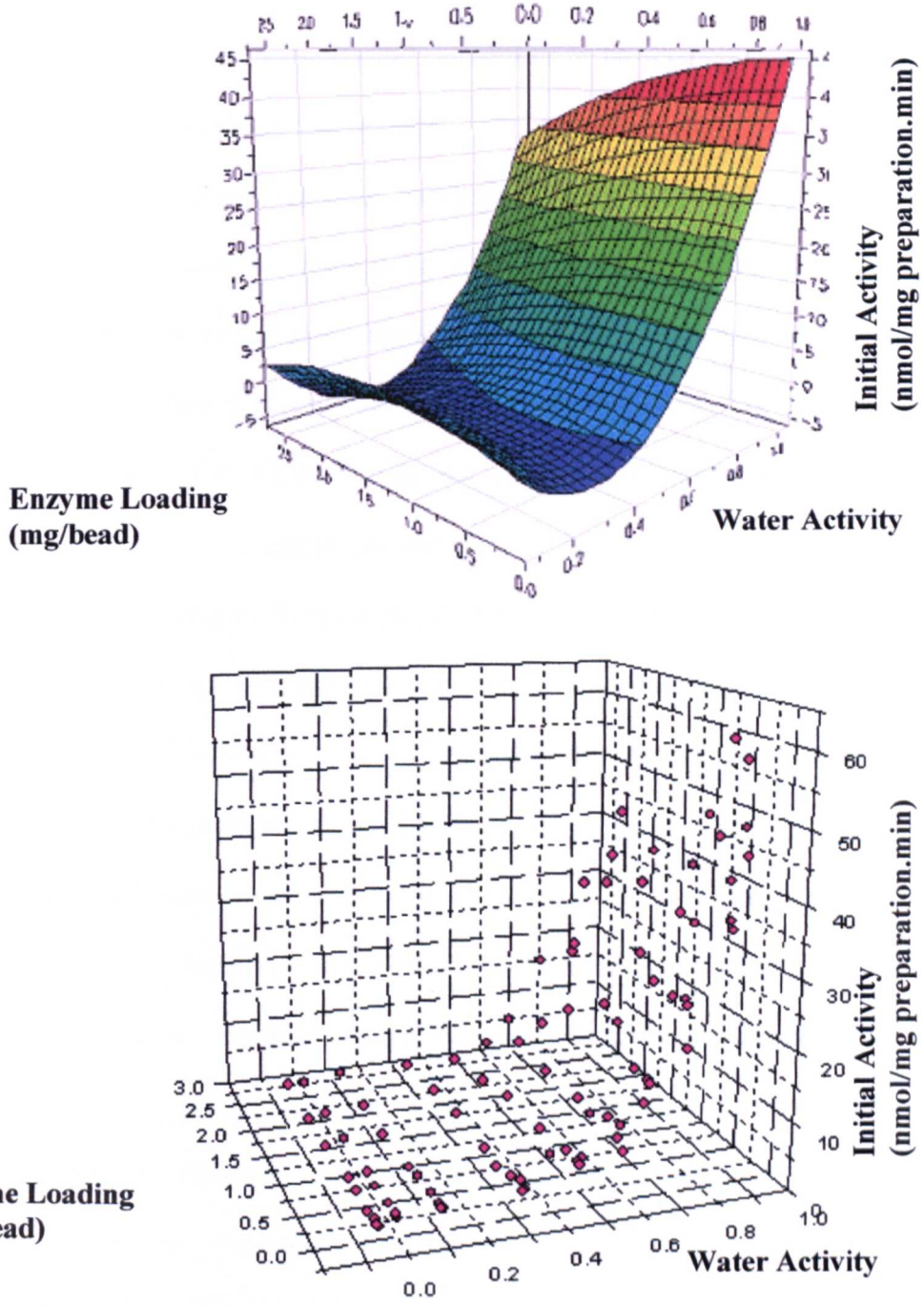


*Figure 2.9 Relationship between the enzyme loading level of the porous PAM beads, the thermodynamic water activity at which the reaction was carried out and the corresponding initial catalytic activity. Actual experimental data are displayed (bottom) alongside a smooth surface representation (top) calculated using MODDE Design of Experiment software.*

However, as discussed above (section 2.3.4) the increased catalytic rate observed at high thermodynamic water activities did not appear to be accompanied by the characteristic increase in the hydrolysis side reaction.

It is clear from these results that, although we can load a large amount of enzyme onto these materials, large increases in the quantity of enzyme immobilised have a negative effect on the observed catalytic activity (per unit mass of enzyme loaded on the support). This negative trend is exaggerated at higher water activities. The reasons for this decline in activity are most likely due to the effects of increased enzyme loading on the morphology of the catalytic preparation. As the quantity of immobilised catalyst is increased, it is apparent from the physical characterisation data (table 2.1) that the overall surface area of the preparation decreases from approximately 10 $m^2/g$ to 1 $m^2/g$ of support material. Although these surface areas are very low, any reduction in the surface area of enzyme available to the incoming substrates is likely to affect the observed reaction rate. In addition to this, the total intrusion volume of the preparation is shown to reduce with increased enzyme loading. The total intrusion volume is a measure of the material's porosity. As this reduces, the average pore size connecting the various cells contained within the material also reduces. At some point this pore size will decrease to a point where the diffusion of substrate from one cell to another is hindered. Bosley et al have reported that the catalytic activity of immobilised lipase is affected by substrate diffusion limitations at support pore sizes of less than 100 nm.[44]

When larger quantities of enzyme are immobilised on a support, the protein molecules will quickly reach a point where they exist as a closely packed monolayer covering the material's surface. Further increases in enzyme loading will result in additional layers

being deposited over the first, preventing the diffusion of substrates to the active sites of the enzyme molecules immobilised closest to the supports surface. This blocking of catalytically active sites is termed "protein-protein" contacts[19] and results in reduced catalytic efficiency.

It is clear from this study that the optimum conditions for enzyme catalysis are found at the highest thermodynamic water activities and the lowest enzyme loadings. Hydration of the catalytic preparation via the equilibration over saturated salt solutions method[37] can only result in a maximum hydration level of 1.0 (i.e. equilibration over distilled water containing no salts). Therefore without altering the methodology, further increases in hydration cannot be measured for this system. However, the quantity of enzyme immobilised can be reduced below the minimum level studied in figure 2.8. A further set of enzyme containing buffered solutions (0 mg/ml to 8 mg/ml α-chymotrypsin in 0.2 M $KH_2PO_4$ pH 7.8) were prepared and used to immobilise low levels of enzyme on the porous PAM beads. These beads were assayed for catalytic activity along with the equivalent unsupported lyophilised enzymes prepared from the same solutions as were used to immobilise the enzyme (figure 2.10).

There was little difference observed in catalytic activity between the supported and lyophilised preparations at a thermodynamic water activity of 0.43. However as demonstrated previously (figure 2.8), at higher water activities the supported α-chymotrypsin preparations do not suffer from the problems of protein agglomeration which deactivates the unsupported lyophilised preparations. At high enzyme concentrations both the supported and "unsupported" systems display very similar catalytic activities. This is because the quantity of catalyst in the system is so large that

the enzyme is effectively being immobilised on other layers of enzyme, and not the support material.[44] This reduces the influence of the chemical nature of the immobilisation matrix.

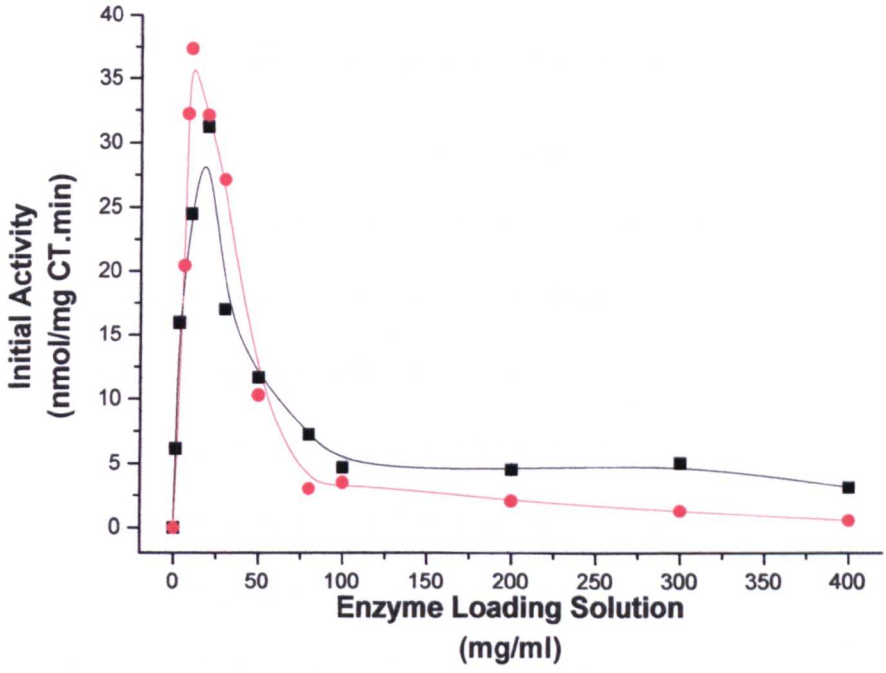


*Figure 2.10 Comparison of supported (red circles) and lyophilised (black squares) enzyme preparations from buffered solutions of different enzyme concentrations. Initial catalytic rate was determined by following the transesterification reaction between ATEE and propan-1-ol in hexane.*

The enzyme activity in both systems was observed to decrease rapidly below an optimum immobilisation concentration of around 10 mg/ml. This effect has been documented previously for different immobilisation systems.[41] At loading levels that equate to less than monolayer coverage of protein, enzymes have been shown to be extremely vulnerable to the effects of small impurities and imperfections on the support surface as

well as to the chemical nature of the support. It is possible that at these very low enzyme-loading levels (< 8 mg/ml α-chymotrypsin loading solutions) the support material is toxic to the catalyst or influences the substrate. The equivalent lyophilised system displays the same trend in activity. It is likely that a similar process is responsible for this trend. As the concentration of protein in buffer is reduced to very low levels, the quantity of enzyme is far outweighed by the amount of buffer salts in the system.

It has been shown that the strength of the buffer solution has a significant effect on the corresponding activity of the enzyme.[45] Immobilisation of the enzyme on essentially neat buffer salts is probably the cause of the observed reduction in catalytic rate at low enzyme levels. From this additional information it can be deduced that the optimum conditions for transesterification catalysis in this system are indeed found with an immobilisation concentration of 10 mg/ml and at a thermodynamic water activity of 1.0. However it is also clear that the concentration of the buffer solution is likely to significantly affect the resulting supported enzyme activity. To examine this, two sets of enzyme supporting PAM beads (and their equivalent lyophilised preparations) were prepared from different strength buffers of the same type and pH. These preparations were then assayed for their catalytic activity (figure 2.11). The results clearly show that the preparations derived from the stronger buffer solution (0.2 M $KH_2PO_4$ pH 7.8) are considerably less active than those prepared from a buffer ten times weaker (0.02 M $KH_2PO_4$ pH 7.8). This indicates that the enzyme is strongly influenced by the concentration of the buffer in which it is dissolved. These results support the idea that the lyophilised enzyme preparations synthesised from very weak solutions of α-

chymotrypsin in buffer are likely to suffer deactivation through contact with the ionic buffer salts.

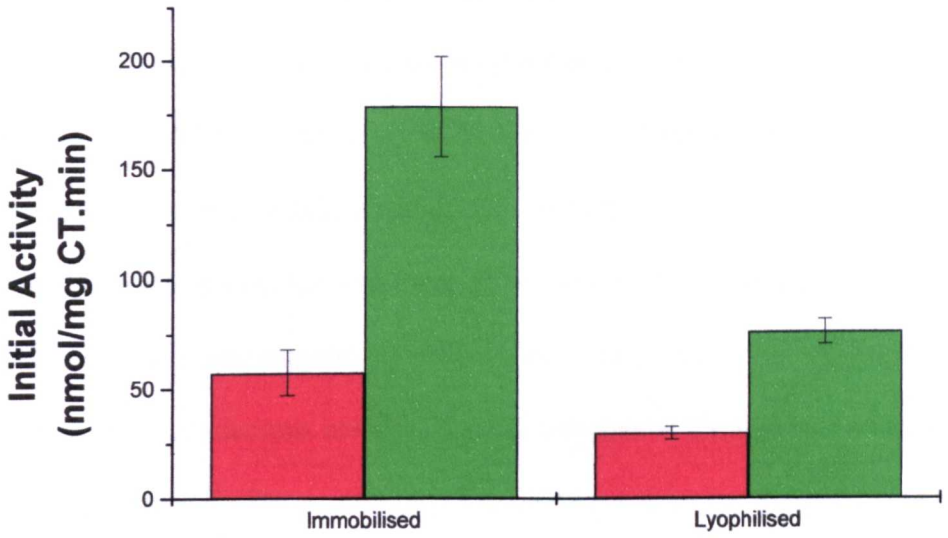


*Figure 2.11. Illustrating the effect of buffer concentration on the resulting activity of the corresponding preparation. Catalytic activity was measured by following the transesterification reaction between ATEE and propan-1-ol in hexane. Red bars = 0.2 M buffer concentration. Green Bars = 0.02 M buffer concentration. Both sets of transesterification assays conducted at a water activity of 0.43.*

### 2.4.6 Thermal stability of supported vs. unsupported α-chymotrypsin

In traditional aqueous media, enzymes are generally very thermally labile,[46] small increases in temperature above 37 °C can often result in rapid deactivation. However, it has long been known that enzymes are generally much more thermally stable when used in organic media.[4] It has been shown that lysozyme loses half its activity when heated to 100 °C in aqueous solution in less than 10 minutes.[47] The same enzyme has an activity half-life of 140 hours when immersed in cyclohexane at 110 °C. It is thought that the

enzymes stability is related to its conformational flexibility. The more flexible an enzyme's structure, the more likely it is to unfold and become totally catalytically inactive. Most enzymes are naturally more stable in organic media due to the reduced water content present within their microenvironment. When in the presence of large quantities of water (i.e. in aqueous solution or in a bi-phasic system) the electrostatic interactions (hydrogen bonding and Van der Waal's forces) that exist between functionalities in the protein backbone are significantly weakened by the interaction of clusters of water molecules with these polar groups. However if the majority of the water is removed and the enzyme is utilised in a non-polar, low dielectric medium, the intramolecular interactions are significantly stronger as there is less interference between attracting groups.[24] These stronger interactions make for a more rigid, less flexible structure that can better withstand an increase in temperature. Zaks and Klibanov demonstrated the dramatic effect that relatively small quantities of water can have on an enzyme's stability at high temperature.[4] In a dry organic medium at 100 °C porcine pancreatic lipase possessed a half-life of 12 hours. Addition of up to 0.4 $\%^{v}/_{v}$ water to the system was shown to have no effect on enzyme stability. However as the water content was increased to 0.8 $\%^{v}/_{v}$, the half-life of the enzyme was reduced to just a few minutes.

In this study α-chymotrypsin immobilised on porous PAM beads was compared to the equivalent lyophilised enzyme prepared from the same concentration of buffered enzyme solution (10 mg/ml α-chymotrypsin in 20 mM $KH_2PO_4$ pH 7.8). The preparations were heated to 80 °C in dry cyclohexane. The results (figure 2.12) show that the half-life of the lyophilised enzyme was approximately 140 hours, whilst the equivalent immobilised preparation retained more than 90 % of its activity after the same period.

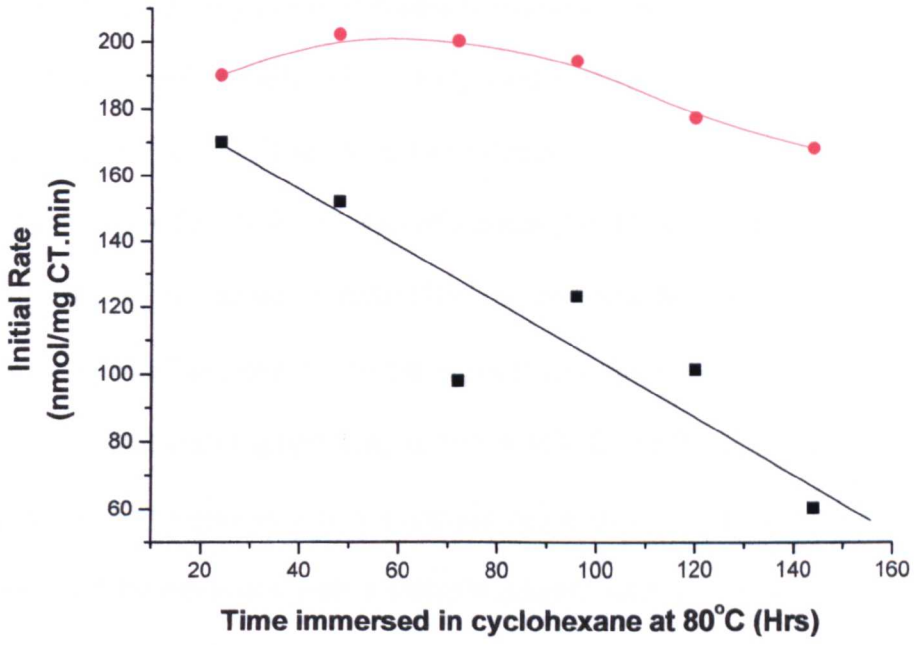


*Figure 2.12. Comparison of supported (red circles) and equivalent unsupported (black squares) enzyme preparations obtained from 10 mg/ml α-chymotrypsin in 0.2 M $KH_2PO_4$ pH 7.8 buffer. Samples heated to 80 °C in dry cyclohexane prior to freeze drying and catalytic activity measured by following the transesterification reaction between ATEE and propan-1-ol in hexane.*

Both these results compare well with previous studies. When heated in octane to 100 °C, Zaks *et al* reported that unsupported lyophilised α-chymotrypsin exhibited a half-life of 80 minutes.[19]

As the flexibility of the enzyme's structure is directly related to its thermal stability, it is unsurprising that the results of this study show the immobilised enzyme to be more stable than the lyophilised equivalent. Even though the supported enzyme is not covalently

bound to the PAM matrix the adsorption of the enzyme to the support surface is likely to constrain the flexibility of the molecule to some degree. This relatively small reduction in the conformational mobility of the supported enzymes may account for their enhanced stability relative to their lyophilised equivalents.

A better strategy for the production of thermally stable enzyme preparations would be to further reduce the protein's flexibility via covalent attachment to the support. This effectively "pins" the enzyme to the support in a more rigid conformation. It has been shown that multipoint attachment is better still for enzyme stability than single point attachment.[48] The porous poly acrylamide beads used in this study could be covalently immobilised by treatment with a suitable reagent such as glutaraldehyde, in a separate crosslinking step.[49, 50]

Interestingly, both the supported and unsupported preparations studied show slightly better catalytic activity after immersion in cyclohexane at 80 °C for one day, compared to the "fresh" preparations that had not been through the same process. It is possible that the cyclohexane is washing some impurity away form the enzymes microenvironment, which would otherwise reduce the observed activity.

### 2.4.7 Re-utilisation of enzyme loaded PAM beads

Unsupported enzymes are rarely used in industrial applications as they are easily deactivated by polar organic solvents and can be extremely difficult to recover from the reactor.[51] In applications where enzymes are required for catalysis, they are almost invariably immobilised on a support.[52] This facilitates recovery and allows for the reuse of the enzyme.

Two studies were conducted to evaluate the reusability of the PAM supports. A batch of the enzyme supporting beads were prepared from a 10 mg/ml α-chymotrypsin 0.02 M $KH_2PO_4$ pH 7.8 buffer solution. These beads were hydrated to a constant water activity of 0.43 before being assayed for catalytic activity. After the first use, the beads were simply decanted from the reaction medium and washed with a small quantity of dry hexane. The beads were then assayed again, using pre-hydrated hexane as the reaction solvent. The results (figure 2.13) show that the α-chymotrypsin immobilised on the porous PAM beads does lose a significant amount of its catalytic activity over the first three uses, but then appears to stabilise, still retaining useful activity after the sixth consecutive use.

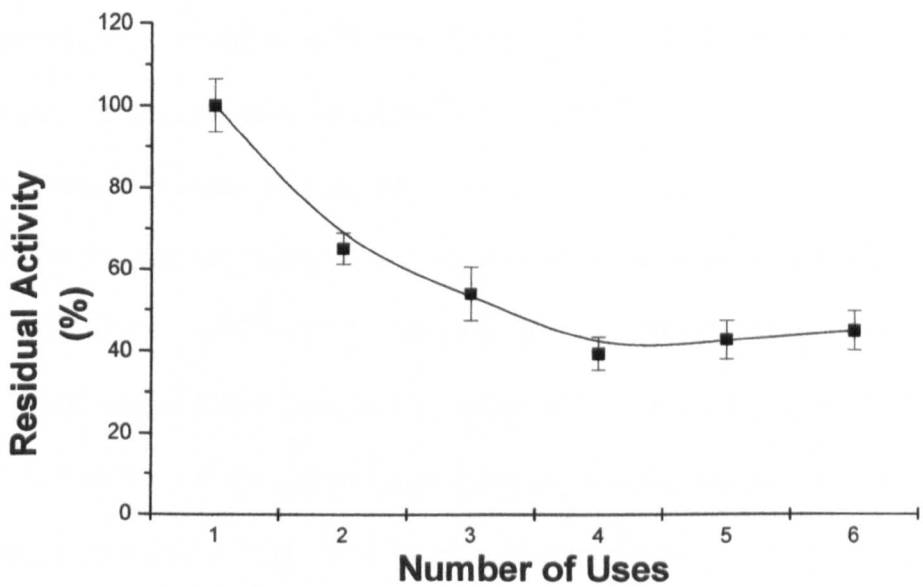


*Figure 2.13. Residual activity of α-chymotrypsin immobilised of PAM beads, over six consecutive uses of the activity assay. Catalytic activity was measured by following the transesterification reaction between ATEE and propan-1-ol in hexane. All enzyme reactions carried out at a fixed water activity of 0.43.*

In contrast the lyophilised enzyme, prepared from the same buffered solutions as was used to immobilise the catalyst on the PAM beads, was very difficult to recover from the reactor, and displayed no measurable catalytic activity on its second use. Clearly the immobilised preparation offers a much more practical method for using enzymes in non-aqueous media.

A second re-utilisation study was carried out using the nine different enzyme-loading levels (section 2.2.2). The beads were hydrated to a water activity of 0.43 and assayed for activity. After their first use the beads were decanted from the reaction medium and rigorously washed with acetone. Acetone is known to be deleterious to enzyme activity, as are most low molecular weight polar solvents.[53-55] However this rigorous washing does remove all traces of the substrates and products from the previous reaction, enabling the immobilised preparation to be reused in an entirely different reaction if so desired. The results (figure 2.14) show that after five consecutive uses of the supported enzyme, all the preparations retained some activity.

The beads with very high enzyme loadings were only slightly deactivated after exposure to these harsh conditions. In contrast, none of the equivalent lyophilised enzymes prepared from buffered enzyme solutions of less than 100 mg/ml (α-chymotrypsin in 0.2 M $KH_2PO_4$ pH 7.8 buffer) were completely deactivated after only one cycle of use.

After each cycle of use, an enzyme-supporting bead was removed from the reaction, washed in acetone, dried under vacuum and imaged with SEM. These SEM images (figure 2.15) offer a possible explanation as to the supported enzyme's superior reusability compared to the unsupported equivalents.

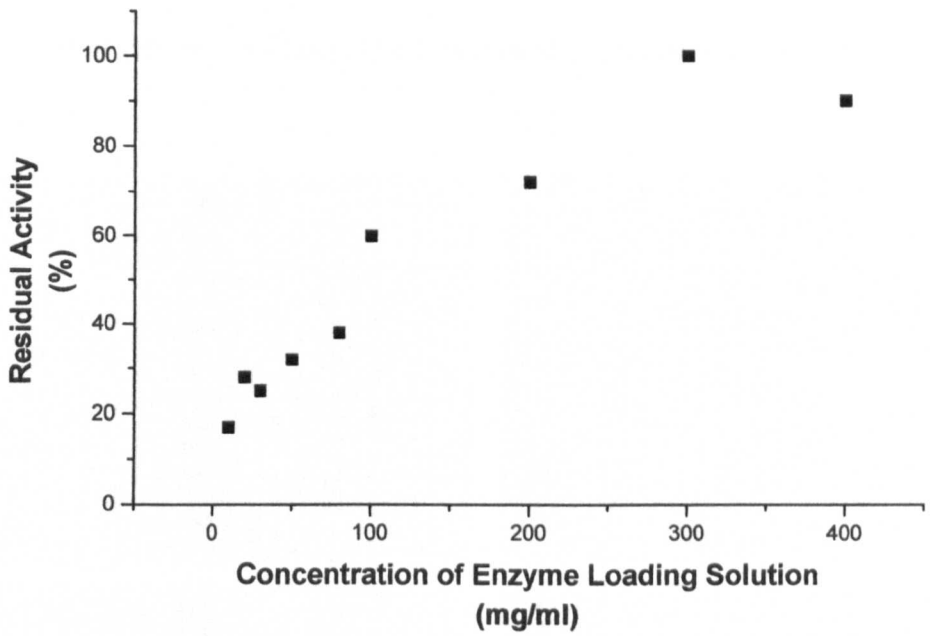


*Figure 2.14. Residual activity of α-chymotrypsin supporting beads of various enzyme loadings, after five consecutive cycles of use. Each cycle of use involved the rigorous washing of the porous PAM beads with acetone, followed by vacuum drying.*

At low enzyme loadings the surface of the beads possess an intricate fine pore structure. However after five cycles of use this fine pore structure has been completely stripped away. The corresponding activity of the preparation has fallen to around 20 % of its original value. Medium enzyme loadings produce a relatively compacted surface to the bead, prior to the first use. After five cycles this compacted surface has been completely lost, revealing a very similar substructure to that observed with lower enzyme loadings. The activity of these beads had fallen to around 30 % of the original value after five uses. SEM images of the most heavily loaded beads reveal an extremely dense and compacted surface of enzyme prior to any activity assay. After five uses this surface has become

highly fractured, but large sections of the original material appear intact. The corresponding activity of the preparation has only dropped to around 90 % of its original value.

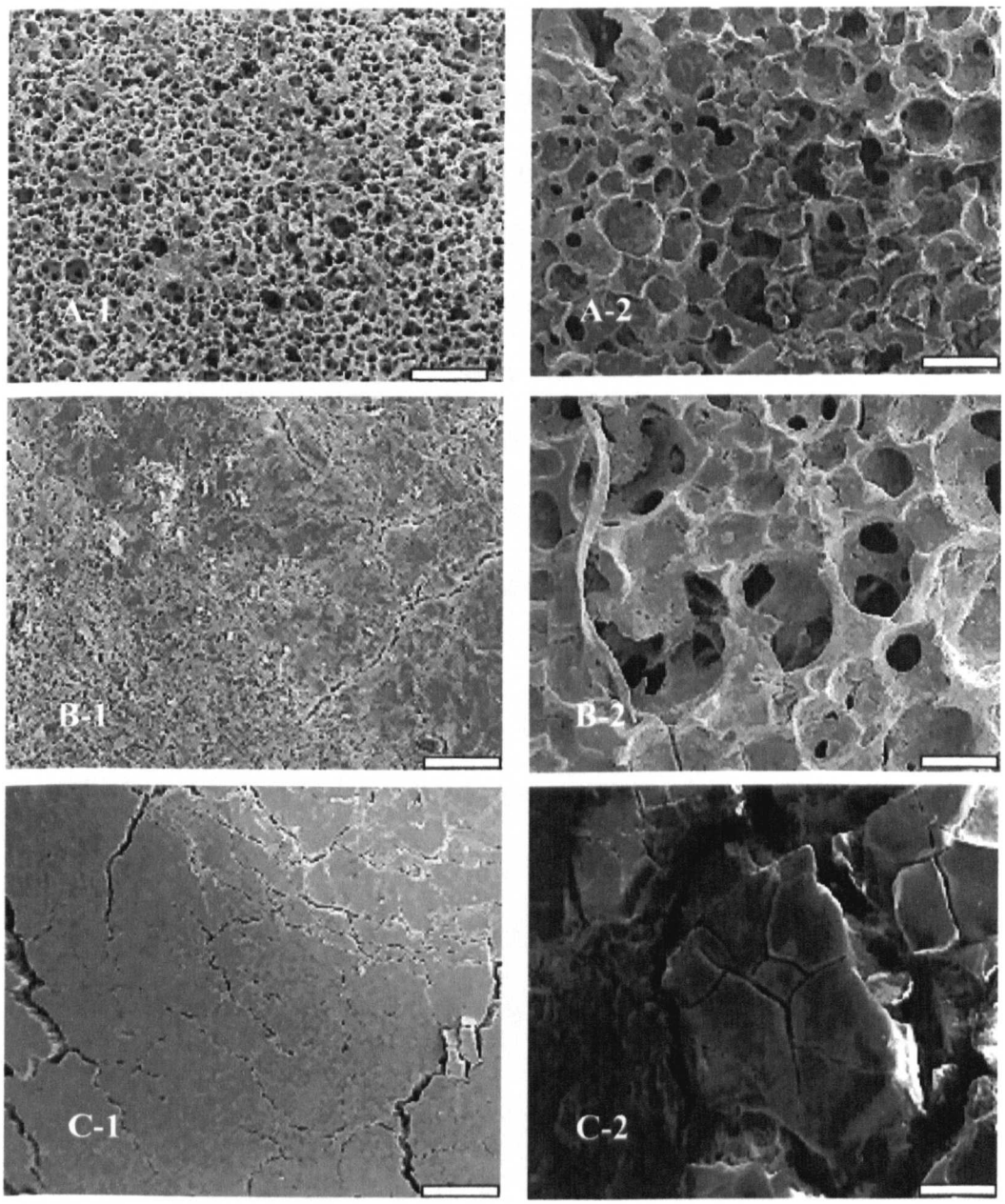


*Figure 2.15 SEM images before (1) and after (2) five consecutive cycles of use (A = 10mg/ml, B = 50mg/ml, C = 400mg/ml enzyme loading solutions). All scale bars = 100 μm.*

An explanation for this observed deactivation is that most of the enzyme catalysis is taking place at the surface of the bead. At low enzyme loadings, where the surface of the bead is very friable and completely removed after several uses, the enzyme deactivation is most severe. At the highest enzyme loadings, layer upon layer of catalyst cover the surface of the bead. As the preparation is reused the uppermost layers of enzyme are either deactivated by contact with the polar solvent, or completely removed by the mechanical effects of the activity assay. However, this exposes new layers of active enzyme and so the observed catalysis rate is not significantly affected. Should these beads be used continuously, it is likely that they would eventually suffer the same deactivation as the preparations formed from lower enzyme loadings. This highlights the advantage of being able to immobilise very large quantities of catalyst on the porous PAM beads, making them useful in applications where the catalyst is easily deactivated.

### 2.4.8 Immobilisation of alternative enzymes.

Two alternative protease enzymes were also supported on the PAM beads for comparison with α-chymotrypsin. Bromelain and Subtilisin Carlsberg were immobilised using a 10 mg/ml solution of enzyme in 20 mM $KH_2PO_4$ buffer set to the optimum reported pH for each protein. Lyophilised equivalents were also prepared from the same buffered solutions.

Although the same transesterification reaction assay was employed for all the preparations synthesised, it should be noted that due to the enzyme's differing specificity, the results obtained are not necessarily comparable. However, the immobilised and lyophilised preparations of the same enzyme can be equated to one another. The results

(figure 2.16) show that in each case the immobilised enzyme exhibits better activity than for the equivalent lyophilised preparation. Bromelain was only active when immobilised, even when the lyophilised preparation was assayed over several days.

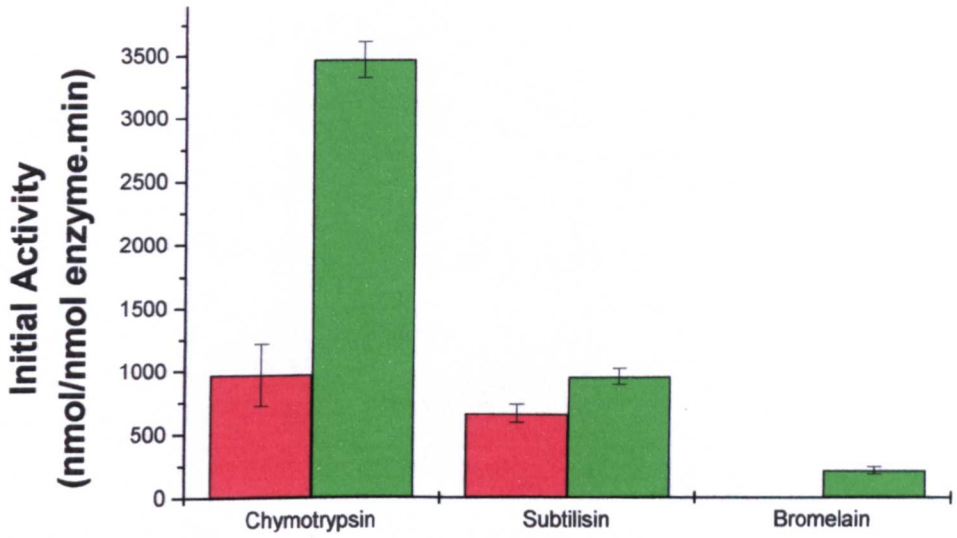


*Figure 2.16. Comparison between the activities of various lyophilised and immobilised enzymes. Green bars = Immobilised. Red bars = Lyophilised. Catalytic activity was measured by following the transesterification reaction between ATEE and propan-1-ol in hexane. All transesterification reactions carried out at a fixed water activity of 0.43.*

This study demonstrates that these porous PAM beads are applicable to the immobilisation of various enzymes, not just α-chymotrypsin.

## 2.5 Conclusions

This study has shown that novel, porous PAM beads can be synthesised and loaded with enzyme relatively easily. The beads can adsorb very large quantities of catalyst and because of their large uniform size, they are very simple to handle and use for enzyme-

catalysed reactions. The material has also been shown to adsorb large quantities of water when equilibrated over the appropriate saturated salt solutions. This enhances the reaction rate for the desired esterification product without significantly increasing the rate of the unwanted hydrolysis side reaction, or suffering the problems of agglomeration experienced with unsupported enzymes.

The immobilised preparations display excellent pH resistance when compared to their lyophilised counterparts, as well as enhanced thermal stability. Alternative enzymes have also been studied, demonstrating that this immobilisation technique is not just applicable to α-chymotrypsin, but potentially to all enzymes.

The most desirable attributes of these supports is their reusability. Without immobilisation the reuse of enzymes is extremely difficult and inefficient. However these preparations can be used more than six times whilst still retaining useful catalytic activity.

## 2.6 Future work

Although the immobilisation of enzymes onto the porous PAM supports is achieved through a relatively simple procedure, it is important to remember that the enzyme is only physically adsorbed to the support. No covalent bond exists to prevent the enzyme from leaching out of the immobilisation matrix. As such, these PAM beads can only be used for enzymatic catalysis in organic solvents (preferably hydrophobic organic solvents, where there is a repulsive interaction between the enzyme and the support). By crosslinking the enzyme with a suitable di-functional agent, such as glutaraldehyde, it

may be possible to use these preparations in aqueous media.[49] As the polymer support is already crosslinked and so insoluble in water, very light crosslinking could be used to covalently bind the enzyme molecules, enabling the best possible catalytic activity to be retained. When employed in aqueous media the enzyme would be able to catalyse hydrolysis reactions and may benefit from the enhanced pH and thermal stability provided by the support. Initial experiments suggest that these enzyme loaded PAM beads can be covalently crosslinked by exposure to glutaraldehyde vapour without the total loss of enzyme activity. However, more work needs to be conducted in order to determine the effects of the crosslinking methodology on the residual enzyme activity.

The versatility of the support has already been demonstrated in that different enzymes have been effectively adsorbed. Future work could include the immobilisation of alternate classes of enzymes such as lipases. This may enable the catalysis of more industrially useful reactions such as the acylation of oils and fats.[53, 55]

The porous PAM beads are not only limited to the immobilisation of enzymes. It has been shown (section 2.3.4) that the beads are capable of adsorbing very large quantities of water. Therefore it should be possible to immobilise any water-soluble catalyst, such as inorganic salts and transition metal complexes.

In this study enzymes were lyophilised directly from buffered solutions onto the support's surface. It has been shown that the addition of a suitable lyoprotectant such as sorbitol to the buffered solution prior to freeze drying, can enhance the catalytic activity of the resulting preparations.[56] As such the effects of various additives to the immobilised enzyme could be investigated for their effects on catalytic activity and stability.

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# Chapter 3

## Optimisation of Enzyme Entrapment In Reusable Supports: A Multivariate Approach

### 3.1 Introduction

Experimental design is a well-established and proven method of improving and optimising various synthetic methods and processes.[1-4] It offers an efficient way of identifying critical parameters that have the most significant influence over the experimental result. The first step in the design process is to define which responses are important (e.g. yield, cost, time etc.) and which factors have a significant influence over these responses (e.g. temperature, solvent, stir speed, molar ratio of starting materials etc.). A set of experiments can then be produced that allows the effects of all the factors on the responses to be examined simultaneously. The design of experiment methodology relies on advanced statistical analysis to separate out and quantify the effects of each factor singularly. A model can be constructed from this information to represent how the experimental parameters combine to influence the results obtained. This model can then be used to optimise the experimental conditions to maximise results. This offers a significant advantage over the traditional "step-by-step" methods for process optimisation, which can be expensive, wasteful and time consuming.[5] The design of experiment software (MODDE) employed for this study was simple to use, yet highly effective, and required no specialist statistical knowledge.

In chapter two it was demonstrated that α-chymotrypsin could be successfully adsorbed to porous PAM beads. These immobilised enzyme preparations were shown to be catalytically active, stable and reusable. However, as the enzyme is only absorbed to the support material, the mechanical forces involved in using the enzyme can displace it relatively easily. Also, it is known that the stability of immobilised enzyme preparations is related to the flexibility of the enzyme's conformation.[6, 7] Generally, the more rigidly constrained an enzyme's structure, the higher its corresponding stability will be. Physically adsorbed enzymes are only constrained in one dimension, i.e. by contact with the support's surface. A better strategy for enzyme immobilisation is mechanical entrapment. By forming a support matrix around an enzyme, its flexibility can be limited by contact with the support material on all sides. This additional constraint should improve the enzyme's stability and help prevent the catalyst from being dislodged from the support when employed in enzymatic catalysis applications. Although mechanical entrapment should increase the enzyme's stability, the process can also result in the total deactivation of some enzyme molecules, as they can become entrapped in the interior of the support matrix, preventing the access of small substrates to the enzyme's active site.
It is known that the chemical nature of the support material influences the properties of immobilised enzymes.[7-10] Different support materials will have differing affinities for water adsorption, and it is well known that the quantity of water contained in the enzyme's microenvironment strongly influences its catalytic activity.[11-15]

The aim of this study was to produce uniform enzyme-containing beads suitable for repeated use in α-chymotrypsin catalysed reactions. The enzyme is to be immobilised via

an entrapment mechanism during the preparation of the catalytic beads. Design of experiment methodology is to be employed in order to optimise the support materials chemical composition. The goal is to produce highly catalytically active enzyme-containing beads that are mechanically robust enough to be reutilised.

### 3.2 Materials

Poly(styrene sodium sulphonate) (100 kDa, Arcos Chemicals, PSSS-s), poly(vinyl alcohol) (9-10 kDa, Aldrich, PVA), dodecyl sulfonic acid sodium salt (Aldrich, SDBS), poly(vinyl pyrrolidone) (8 kDa, Aldrich, PVP), dextran (100 kDa, Fluka, Dex), poly(sodium acrylate) (2100 Da, Aldrich, PNaA), poly(styrene sodium sulfonate) (30 $\%^{w}/_{w}$ aqueous commercial solution, Aldrich, PSSS-l), cerium oxide nanoparticles (20 $\%^{w}/_{w}$ aqueous colloidal suspension, Aldrich, $CeO_2$), Ludox HS30 silica nanoparticles (30 $\%^{w}/_{w}$ aqueous colloidal suspension, Aldrich, HS30), Ludox TM50 silica nanoparticles (50 $\%^{w}/_{w}$ aqueous colloidal suspension, Aldrich, TM50), zinc oxide nanoparticles (50 $\%^{w}/_{w}$ aqueous colloidal suspension, Nanophase Technologies Corporation, ZnO), poly(acrylic acid) (240 kDa, Aldrich, PAA), poly(acrylamide) (10 kDa, Aldrich, PAM), poly(diallydimethyl ammonium chloride) (200-350 kDa, Aldrich, PDDA), poly(ethylene glycol) (1.5, 2, 3.4, 6 and 8 kDa, Aldrich, PEG), sodium dodecyl sulfate (P & R Laboratory Supplies, SDS), N-acetyl-L-tyrosine ethyl ester (Sigma grade, Sigma, ATEE), propan-1-ol (99+ %, Aldrich) and α-chymotrypsin (type II from bovine pancreas, Sigma, α-CT). All other chemicals and reagents were obtained from the Aldrich chemical company and were of general purpose grade. All solvents were obtained from VWR and were of HPLC grade.

## 3.3 Experimental

### 3.3.1 Bead preparation

Solutions of the various chemical components of the support materials SDS, Dex, PSSS, PEG, HS30 and PVA (570 mg of each, in 2.7 ml HPLC water) were prepared prior to the addition of 300 µl of α-chymotrypsin (α-CT) in 20 mM phosphate buffer, pH 7.8 at a concentration of 100 mg/ml. In order to obtain consistent and comparable results, the total mass of solid dissolved (enzyme and support material) in the solution was kept constant at 20 $\%^{w}/_{w}$ and the total proportion of α-CT to support material was fixed at 5 $\%^{w}/_{w}$, for the α-CT control experiment, α-CT was used at a 20 $\%^{w}/_{w}$ concentration in 20 mM $KH_2PO_4$ pH 7.8 buffer solution. It should be noted that in some cases precipitation was observed upon addition of the buffered α-CT solution to the solution of the support materials. This precipitate normally re-dissolved after a few minutes agitation, but in the few cases where it did not, the remaining solid particles were fine enough not to cause any practical problems in the bead formation step. To form emulsion templated preparations, aliquots of cyclohexane were added to form stable emulsions. Otherwise the solutions were used directly in the next step of the procedure. A peristaltic pump (205u Watson Marlow) was used to drip the solution or emulsion into liquid nitrogen at such a rate as to prevent the agglomeration of the freezing particles. Once frozen, the materials were transferred to a freeze drier (Lyolab 3000, Heto) where they were dried under vacuum for at least two days.

### 3.3.1.1 Initial screening experiments

Beads composed of various molecular weights (8 k, 6 k, 3.4 k, 2 k and 1.5 k) poly(ethylene glycol) (PEG) were prepared individually and in combination with sodium dodecyl sulphate (SDS, present in 20 %, 40 % 60 %, 80 % and 100 % proportions with respect to the amount of 8 k PEG). A series of emulsions were also formed between 8 k PEG (68 $\%^{w}/_{w}$) and SDS (32 $\%^{w}/_{w}$), varying the amount of the cyclohexane internal phase (100:0 Emulsion (E) no internal phase, 50:50 E 50 % internal phase, 33:67 E 67 % internal phase and 25:75 E 75 % internal phase). These emulsions were then used to form the corresponding enzyme containing beads. All beads were assayed for catalytic activity by following the transesterification reaction between ATEE and propan-1-ol, described previously (chapter two, section 2.2.5). The mechanical strength of these beads was also assayed using the procedure described below (section 3.2.2).

### 3.3.1.2 Further initial screening experiments

A second screening experiment was conducted to expand the range of materials studied. Numerous materials were examined, however only those found to be soluble enough to meet the 20 $\%^{w}/_{w}$ requirement necessary for parity with the other composites previously studied, were prepared as beads. Some of the compounds did not yield uniform beads, instead producing agglomerated and amorphous materials (probably due to the depression of the frozen solution's melting point, causing the preparations to thaw too quickly during the freeze drying process). These preparations were assayed for enzyme activity, but not for mechanical strength, as they were unsuitable for the deformation assay. Only

compositions that yielded uniform spherical beads were assayed for both catalytic activity and mechanical strength.

### 3.3.2 Physical characterisation

The mechanical strength of the beads was determined using a Lloyd instruments LR30K series tensile testing machine. The instrument was used in compression mode with a 100 Newton probe. The energy required to deform each bead by 1 mm was calculated (units of N-mm) by measuring the area under a force/distance plot. The "strength" of each set of beads was averaged from three concordant results.

The surface area of the various composite beads was characterised with a Micrometrics ASAP 2010 using the BET method. Pore volumes were measured by the use of mercury porosimetry with a Micrometrics AutoPore IV. Absolute density was obtained through Helium pycnometry, with a Micrometrics AccuPyc 1330 instrument. Scanning Electron Microscope (SEM) images were captured using a Jeol 840 instrument. Samples were sputter-coated with approximately 2 nm of gold (Polaron E5000 coating unit) prior to analysis and mounted on 12 mm carbon coated aluminium stubs (Agar Scientific).

Energy dispersive analysis by X-ray was carried out for selected sets of composite beads using a JEOL 840 SEM instrument equipped with a Rontec EDAX system. Two types of detector were employed; a SiLi drifted crystalline detector (cooled with liquid nitrogen) and a "dry" Multimax detector to produce quick X-ray dot maps.

### 3.3.3 Enzyme activity assay

Enzyme catalytic activity was assayed by following the transesterification reaction between ATEE and propan-1-ol in hexane (chapter two, section 2.2.5). The beads were assayed for catalytic activity as whole particles and after being ground to a fine powder.

### 3.3.4 Enzyme re-utilisation study

Selected composites were assayed for their reusability via the enzyme activity assay. Samples of five whole beads and the reaction media were hydrated to a constant water activity of 0.43. After determination of the initial catalytic activity, the reaction media was decanted from the reaction vessel and the beads washed with 3 x 3 ml portions of dry hexane (stored over #4 molecular sieves). The transesterification assay was then repeated using fresh hexane with a water activity of 0.43. This process of washing and rehydration was repeated for each subsequent use of the preparations. All reactions were carried out at 22 °C and in duplicate.

## 3.4 Results and discussion

Two initial screening experiments were conducted prior to a large-scale examination of composite supports, using design of experiment methodology. The aim of these screening experiments was to gain more information about the effects of support characteristics (differing chemical composition) on enzyme activity and on the beads mechanical strength. The effects of bead morphology on these two responses were also examined. The results of these initial screening experiments were used to determine which of the

materials and methods examined would be included in the main design of experiment study.

### 3.4.1 Initial screening of materials

The first study was designed to investigate how the emulsion templating of the enzyme support material might affect the corresponding enzyme activity. It was postulated that increased surface area and porosity of the material might have a beneficial effect on the enzymatic catalysis, because increased surface area and porosity have previously been shown to facilitate the mass transfer of substrates to the enzyme's active sites.[16]

In order to form a stable emulsion, it was necessary to add a suitable surfactant to the support matrix. Sodium dodecyl sulphate (SDS) was chosen for this role as it is cheap, widely available and has been previously used to stabilise emulsions.[17] SDS is also a material frequently used in conjunction with enzymes.[18] Poly(ethylene glycol) (PEG 8 kDa) was chosen to make up the bulk of the support matrix, as it has also been widely used to both modify and immobilise enzymes.[19-21] Six sets of composite beads were synthesised with different ratios of PEG to SDS (ranging from 100 % PEG to 100 % SDS). It was thought that the polymer's molecular weight might be directly related to the strength of the bead formed so the molecular weight of the PEG used was also varied (ranging from the lowest molecular weight that would produce a solid bead, 1.5 kDa, to the highest molecular weight that would be soluble to 20 $\%^{w}/_{w}$, 8 kDa). Finally, emulsions were formed with various ratios of internal (aqueous) phase to external (organic) phase, in order to influence the porosity of the resulting beads.

The strength of all the composite beads formed was assayed using a Lloyd's tensile testing instrument. Results show (figure 3.1) that there appears to be little correlation between the beads strength and the PEG molecular weight. As the molecular weight is lowered, the materials strength is not significantly affected.

However, varying the ratio of the two components did affect the resulting beads mechanical strength. Addition of 20 % SDS increases mechanical strength c.f. 100 % PEG. Further increasing the proportion of SDS results in a small decrease in strength, with 100 % SDS being slightly weaker than 100 % PEG. This may be because the addition of a small quantity of the ionic surfactant produces a positive non-covalent interaction with the polymer chain resulting in a stronger material. However further increasing the proportion of SDS decreases the matrix strength.

It is clear that the beads formed from freeze dried emulsions become weaker as the ratio of internal to external phase is increased. This is not surprising, as the beads synthesised from low external phase emulsions would be expected to be less dense and more porous, hence easier to crush. The mechanical strength of the emulsion templated beads formed with no internal phase was lower than expected, as the composition of these beads is very similar to those formed with differing SDS : PEG ratios. This may be due to the slightly different methodology employed for the preparation of these beads, as they were produced on a much smaller scale than were the other compositions.

The catalytic activity of all these beads was also determined (figure 3.2) using the transesterification assay.

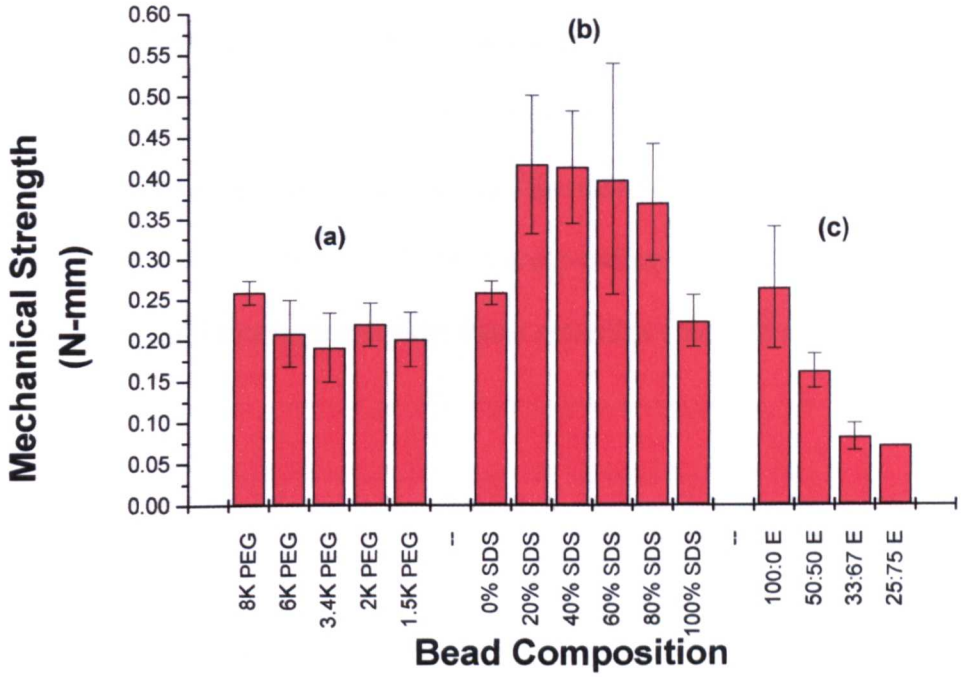



*Figure 3.1 Mechanical strength of beads formed from various combinations of PEG and SDS. (a) = Effect of PEG molecular weight (8 k to 1.5 k). (b) = Effect of SDS : PEG (8 k) ratio (0 % to 100 %). (c) = Emulsion templated beads, affect of varying internal : external phase ratio of the emulsion for beads formed with a constant SDS : PEG composition of 32 % SDS and 68 % PEG. All mechanical strength measurements were conducted with a Lloyd instruments LR30K tensile testing machine in triplicate.*

The enzyme activity was much greater with the lower molecular weight PEG beads than with the higher molecular weights. The reasons for this are not obvious because there is very little chemical difference between the various materials. Two possible explanations were proposed for this trend in catalytic activity. It was noticed that the slightly weaker, low molecular weight PEG beads partially disintegrated in the reaction vessel. The resulting increase in surface area from this may have exposed a greater number of the enzyme's active sites to the substrate molecules, resulting in a higher observed catalytic rate. To investigate this possibility the enzyme assay was repeated using beads that had

been ground to a fine powder. It was assumed that there would now be little change in the number of accessible active sites between the different materials. However the data obtained (figure 3.3) do not appear to support this theory, although using powdered materials for the assay did result in a small increase in enzyme activity compared to using whole beads. The catalytic activity corresponding to the lowest molecular weight polymers was still much greater than those activities observed with the higher molecular weight supports.

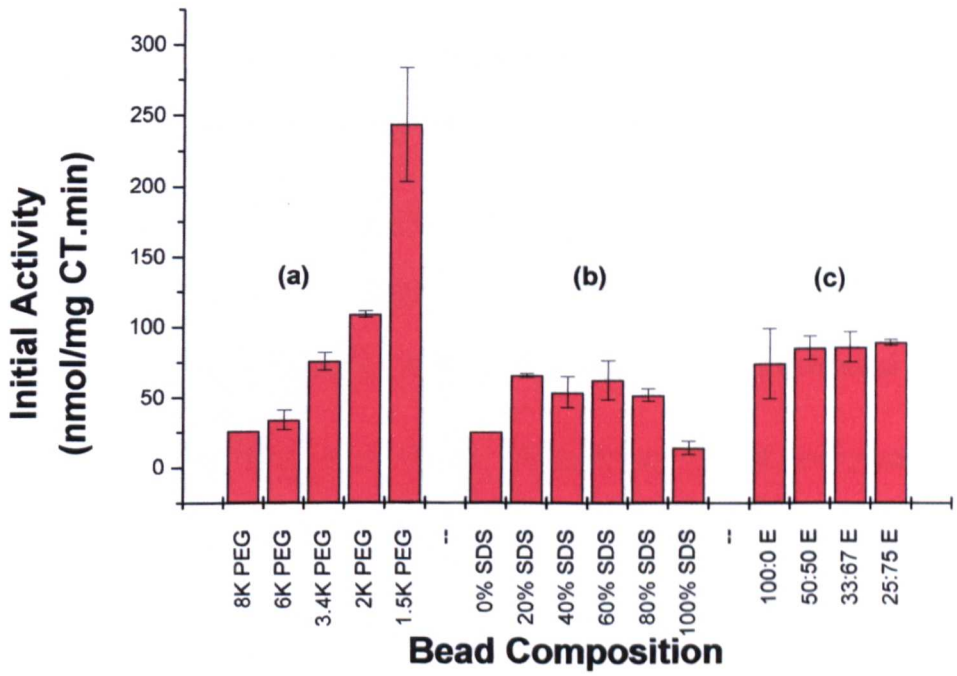



*Figure 3.2 Catalytic activity of beads formed from various combinations of PEG and SDS assayed by following the transesterification reaction between ATEE and propan-1ol in hexane. (a) = Effect of PEG molecular weight (8 k to 1.5 k). (b) = Effect of SDS : PEG (8 k) ratio (0 % to 100 %). (c) = Emulsion templated beads, affect of varying internal : external phase ratio of the emulsion for beads formed with a constant SDS : PEG composition of 32 % SDS and 68 % PEG.*

An alternative explanation for the increase in enzyme activity with decreasing PEG molecular weight may be found by examining the structure of the dissolved PEG used to prepare the beads. It has been shown that in solution, higher molecular weight PEGs exhibit comparatively closed and compact structures.[22] The polymer folds in on itself resulting in a dense and squashed arrangement. As the molecular weight of the polymer is lowered the chain length is reduced, making it harder for the chain to fold up. Removal of the solvent results in a more open-structured polymer matrix, which may allow for better diffusion of substrates and products to and from the enzymes active sites, hence resulting in increased catalytic activities. This change in structure occurs at a threshold molecular weight of around 4 kDa, which corresponds to the sharp increase in enzyme activity that begins at the 3.4 kDa molecular weight level (figures 3.2 and 3.3).

For the purposes of this study, we were satisfied that the low molecular weight PEG produces beads with much higher activity than those formed with higher molecular weight PEGs, without causing a large reduction in the beads mechanical strength.

The effect of SDS/PEG ratio on the catalytic activity of α-chymotrypsin was also investigated for whole and powdered beads. Figures 3.2 and 3.3 show that as for mechanical strength, the addition of some SDS to the polymer is beneficial to α-chymotrypsin activity, but that using 100 % SDS reduces the activity. Overall it appears that there is a positive synergy between the polymer and the surfactant, in that a 20 % addition of SDS to 8 KDa PEG produces beads that exhibit significantly higher strength and catalytic activity than for either 100 % PEG or 100 % SDS preparations.

Emulsion templating is a technique that allows for the formation of rigid porous structures based on the morphology of the original emulsion.[17] Therefore, it was thought

that emulsion templating might produce beads with increased catalytic activity, due to an increase in the accessibility of the substrate to the enzyme active sites. However it was observed (figure 3.3) that there is very little difference between beads formed with a high internal phase ratio and those formed without any emulsion templating, in terms of either enzyme activity or mechanical strength.

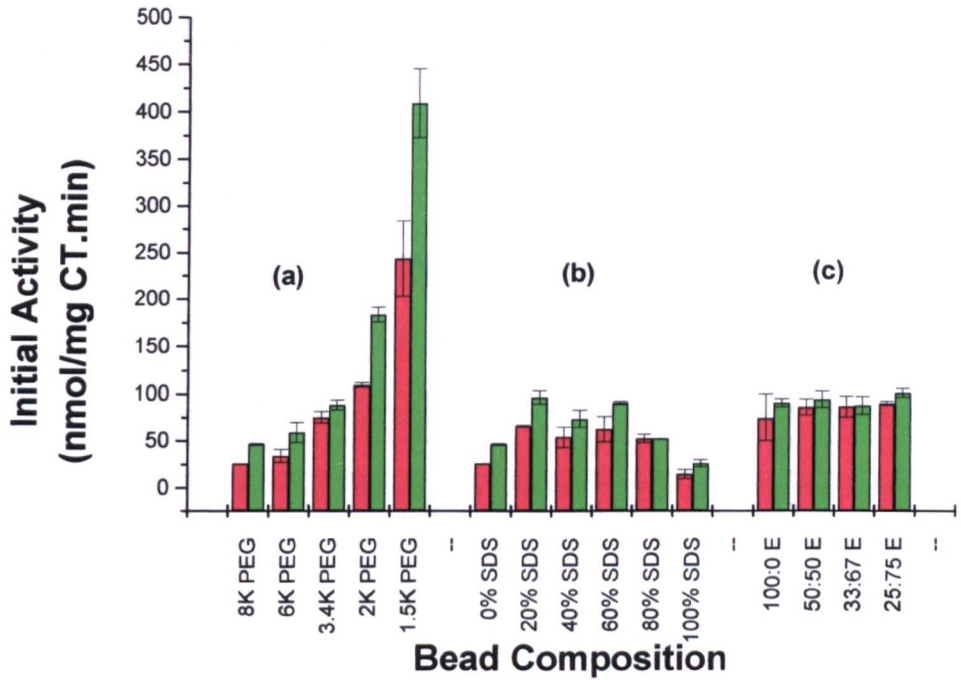



*Figure 3.3 Catalytic activities of beads formed from various combinations of PEG and SDS assayed by following the transesterification assay between ATEE and propan-1-ol in hexane. Red bars = whole beads, Green bars = powdered beads. (a) = Effect of PEG molecular weight (8 K to 1.5 K). (b) = Effect of SDS : PEG (8 k) ratio (0 % to 100 %). (c) = Emulsion templated beads, affect of varying internal : external phase ratio of the emulsion for beads formed with a constant SDS : PEG ratio of 32 : 68 $^{w}/_{w}$.*

Overall, this initial study demonstrated that the highest immobilised enzyme activities were found when α-chymotrypsin is immobilised in the presence of low molecular

weight PEG. Changing the PEG molecular weight did not significantly affect the corresponding mechanical strength of the beads. Emulsion templating did not significantly affect either the enzyme activity or the bead's mechanical strength. The addition of small quantities of SDS to PEG resulted in a synergistic increase in both the enzyme's catalytic activity and the bead's mechanical strength.

### 3.4.2 Second screen of materials for use as enzyme supports

To better direct the subsequent design experiment, a further screening study was carried out in order to expand the range of materials tested. A diverse array of compounds was selected including various polymers, polysaccharides, polyelectrolytes and inorganic nano-particles. All the materials were used to form beads containing entrapped α-chymotrypsin, under the same conditions as for those prepared in the initial study (section 3.3.1). The results (figure 3.4) indicated that several of the materials tested in the second screen were considerably stronger than any of the beads examined in the first assay, notably Dex, PAA, PVA and $CeO_2$.

Dextran, PAA, PVA and PAM were all amongst the strongest beads tested. This is probably due to the added stability provided by intermolecular hydrogen bonding. Cerium oxide nanoparticles ($CeO_2$) also exhibited a relatively high degree of strength, which is slightly unusual as the other inorganic metal oxide nanoparticles included in the study (ZnO) displayed a relatively low level of mechanical stability. Beads formed from PVA appeared to demonstrate elastic properties. Not only were the beads comparatively strong, but they also regained some of their shape after deformation.

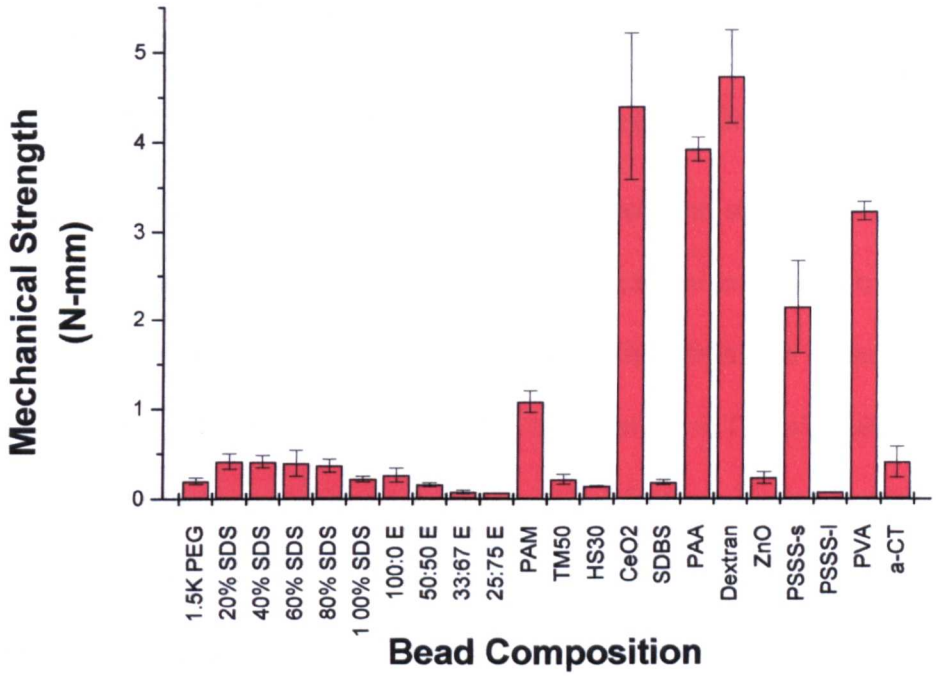



*Figure 3.4 Mechanical strength of beads formed from the support materials used in the first and second screening experiments. All mechanical strength measurements were conducted in triplicate with a Lloyd instruments LR30K tensile testing machine.*

There was also a noticeable difference between the two variants of silica bead studied (Ludox® HS30 and Ludox® TM50). Given that both these materials are intrinsically the same, it would have been expected that they would both exhibit similar mechanical properties. It should also be noted that the beads formed from a freeze-dried 20 $\%^{w}/_{w}$ solution of α-chymotrypsin possessed better mechanical strength than many of the other materials tested in the second screen, and all of the materials tested in the first screening. Strength tests were only conducted on materials that formed discrete, uniform beads. Some support materials, such as SDBS, resulted in poorly shaped or agglomerated particles, probably caused by premature thawing in the freeze-drying process. This may

be due to the depressed melting point of the solutions caused by their high salt contents. However all the materials were assayed for catalytic activity using the transesterification assay (chapter two, section 2.2.5) in order to assess their effects on enzyme activity

The activity assay for the various materials also produced some surprising results (figure 3.5). The catalytic activity values in figure 3.5 can be compared to an activity value recorded for unsupported α-chymotrypsin lyophilised from the same phosphate buffer, and hydrated to the same water activity, of 33 nmol/mg CT.min (chapter two, section 2.3.5). This value was obtained for a freeze-dried 10 mg/ml solution of the enzyme, and is lower than most of the other composites assayed. However the "control" beads formed with α-chymotrypsin only (i.e. a freeze dried 200 mg/ml solution of enzyme in 20 mM $KH_2PO_4$ pH 7.8 buffer) exhibited very low activity.

The highest enzyme activities were observed with the 1.5 kDa PEG and Silica HS30 beads. These activities are approximately 50 times greater than was observed for the 100 % α-chymotrypsin equivalent beads.

The difference between the two silica composites (HS30 and TM50) was very noticeable, even though there is little chemical difference between the commercial solutions, especially after dilution to the same $^{w}/_{w}\%$. Although the surface area of the HS30 is higher ($\approx 220\ m^2g^{-1}$) than for the TM50 ($\approx 140\ m^2g^{-1}$), the pH of the TM50 solution (pH 9.0) is slightly closer to the enzyme's optimum pH of 7.8[23] than the HS30 solution (pH 9.8). The greater surface area of HS30 may more than counter its slightly unfavourable pH, when compared to TM50, but it would not be expected to account for the 10-fold increase in enzyme activity observed.

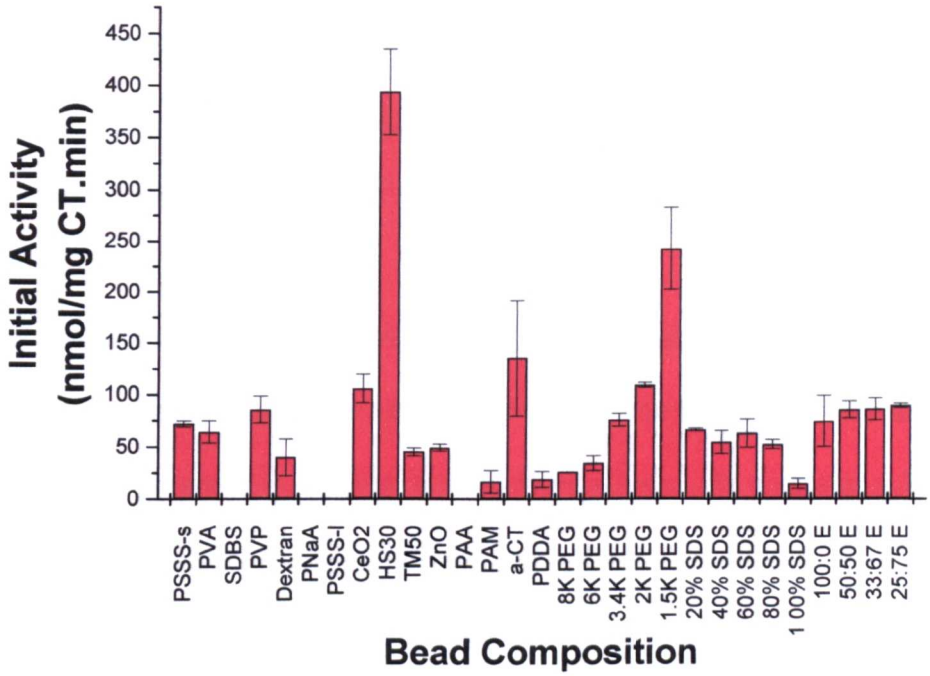



*Figure 3.5 Catalytic activities of beads formed from the various support materials used in the first and second screening experiments. Catalytic activity assayed by following the transesterification reaction between ATEE and propan-1-ol conducted in hexane.*

Cerium Oxide nanoparticles are a novel material for use in enzyme immobilisations, yet it achieves the fourth highest observed activity. This is despite the $CeO_2$ suspension being stabilised with 2.5 % acetic acid, which would be thought to have a very unfavourable effect on the enzymes activity.

Both metal nanoparticulate suspensions examined in this study may also contain solvated metal ions, which might be responsible for the considerable difference in enzyme activity observed between the zinc and cerium containing beads. The effect of metal ions on enzyme activity has been investigated previously.[24] Transition metals such as titanium have been shown to be potent inhibitors of serine protease, via irreversible coordination

to the enzymes active site. It has also been postulated that other metal ions could also inhibit various serine protease. However Duffy *et al* did not examine the effects of cerium ions on enzyme activity and concluded that zinc ions had no effect on the catalytic rate, indicating that this hypothesis is unlikely to be the cause of the results observed in this work.[24]

Beads formed from solid 100 kDa PSSS possessed significant activity, yet the commercially available solution produced beads with no enzyme activity at all. This may be due to the presence of some stabiliser in the commercially available solution that is a particularly potent inhibitor of α-chymotrypsin.

PDDA was one of the more unusual materials studied, but exhibited very low catalytic activity. This might be due the highly charged nature of the polyelectrolyte material. The same explanation may also account for the lack of activity observed for the SDBS and PNaA composites. The intrinsic acidic nature of poly(acrylic acid) was most likely the reason for the inactivity of the enzyme immobilised in this material.

### 3.4.3 Modelling and design of experiments

The overall aim of this study was to find composites of support materials suitable for enzyme immobilisation. The goal is to find materials that result in strong, reusable beads that also display the best possible enzyme activity. Multivariate design of experiment methodology allows us to design a series of experiments that enables the maximum amount of information to be extracted from the data produced using the fewest number of experimental runs. Ideally our study would examine how all the materials considered in the first two screens interacted with each other. In practice this would mean forming

composite beads containing all possible combinations of more than a dozen materials. This could result in a prohibitively large number of experiments to be included in the design, making the study impractical.

### 3.4.3.1 Selection of materials for further examination

In order to lessen the total number of experiments to a more realistic level, the number of component materials was reduced to six by analysis of the results obtained from the first two screening studies. In general, our previous results demonstrated that the materials with the best catalytic properties were mechanically very weak, while the strongest materials exhibited poor catalytic rates. These trends made this system ideal for a design experiment to produce strong composites with good activities.

Materials that displayed both poor mechanical strength and low catalytic activity were excluded, as these seemed unlikely to contribute to the desired objectives.

Polyethylene glycol (PEG, 1.5 kDa) and silicon oxide nano-particles (HS30) were both selected as they displayed by far the greatest enzyme activity, although they both exhibited poor mechanical strength. Dextran (Dex) was chosen because it produced the strongest beads. Sodium dodecyl sulfate (SDS) was not shown to be particularly strong or active, but did demonstrate a positive synergy with PEG for both these responses. The inclusion of this surfactant would also allow for the subsequent formation of emulsion templated materials. Although the PEG/SDS emulsion templates were not found to be superior to the non-emulsion equivalents, it may be that other composites would be enhanced by the increase porosity offered by the emulsion templating process. Poly(vinyl alcohol) (PVA, 9-10 kDa, 80 % hydrolysed) demonstrated good mechanical strength and

reasonable enzyme activity. Furthermore, the elastic nature of the PVA beads was an interesting property lacking in all the other materials examined, which may have a positive effect on the re-usability of the composites containing it. Poly(styrene sodium sulphonate) (PSSS 100 kDa) was selected because of its better than average strength and activity, but also because the metal counter ions may demonstrate similar positive synergies to those observed with SDS.

The most obvious omission from the list of materials for further study was the cerium oxide nanoparticles ($CeO_2$). These displayed the fourth highest enzyme activity and the second highest mechanical strength. However, the strongly acidic nature of the stabilising solution caused severe precipitation of the enzyme. Given the unknown effects of this precipitation on the properties of the beads formed and the practical difficulties of dealing with these thick suspensions, $CeO_2$ was excluded from further study.

### 3.3.3.2 Preparation of beads from selected materials

As a control all six of the materials selected for further study were prepared as beads from a 20 $\%^{w}/_{w}$ aqueous solution, both in the presence and absence of α-chymotrypsin. The catalytic activity of the beads that did not contain any α-chymotrypsin was measured using the transesterification assay (section 3.2.3). No catalysis was observed in the absence of enzyme.

Design of experiment software was employed to generate an 80-experiment screen evaluating the various combinations of the six materials selected. All combinations were synthesised according to the design and both the mechanical strength and enzyme activity measured.

The responses from these 80 experiments (appendix 1) were then used to construct a model representing the 6-factor system using the method of partial least squares (PLS). PLS is a multivariate method of estimating the models for several responses simultaneously. An overall "summary of fit" plot was produced (figure 3.6) illustrating how well the model produced interprets the experimental results obtained. Briefly, the four components of the model fit plot are: $R^2$ is a representation of how well the model interprets the actual experimental data. $Q^2$, an indication of the predictive ability of the model. A summary of model validity is also produced along with its reproducibility. The $R^2$ and $Q^2$ values are by far the most important indicators of model fit. $R^2$ is an overestimation and $Q^2$ is an underestimation of fit. The true value lies somewhere in between these components.

The fit of the model can be improved by adding more PLS components. When a PLS component is added, a new variable is created as a weighted combination of the original factor variables. These new components can be used to improve the models ability to interpret the experimental results ($R^2$) however, as no "new" data is being added to the model, its predictive ability ($Q^2$) remains unchanged. As can be seen from figure 3.7, a diminishing return in the beneficial effects of adding new PLS components was observed. For the model created in this study a total of ten PLS components were added, improving the $R^2$ fit considerably.

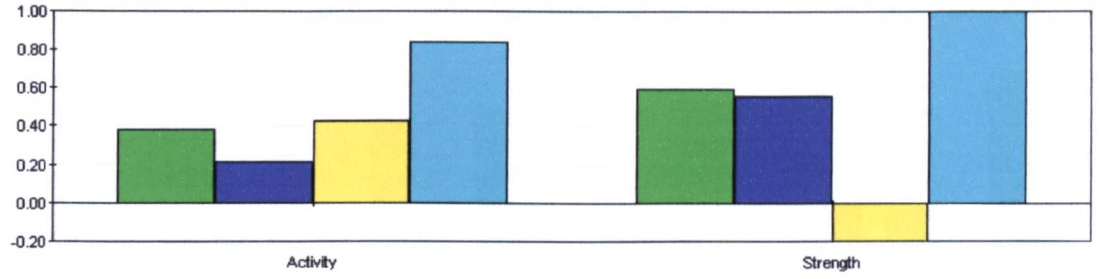



*Figure 3.6a*

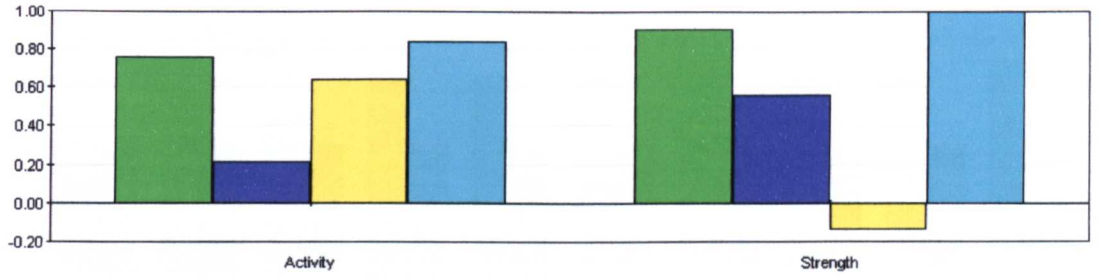



*Figure 3.6b*

*Figure 3.6 Overall summary of fit for the Design of Experiment model. Fit shown for both enzyme activity and mechanical strength responses. Figure 3.6a represents the original model without additional PLS components. Figure 3.6b represents the modified model, containing a total of ten additional PLS components. Green Bars = $R^2$ interpretive value. Dark Blue Bars = $Q^2$ predictive value. Yellow Bars = Indication of model fit. Pale Blue Bars = Reproducibility of the model.*

The model can be used to produce standardised residual plots for both the enzyme activity and mechanical strength responses (figures 3.8). These plots allow for the facile detection of outliers in the results. Outliers are judged to lie outside the range of ±4 standard deviations from the median. Normally distributed results will lie on a straight line within this standard deviation range.

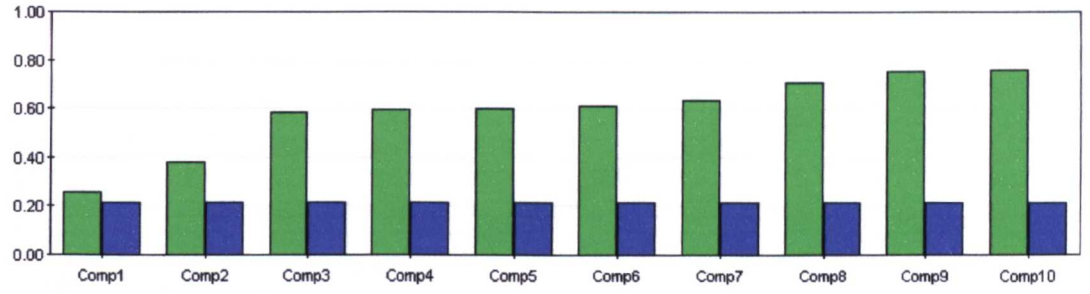



*Figure 3.7a*

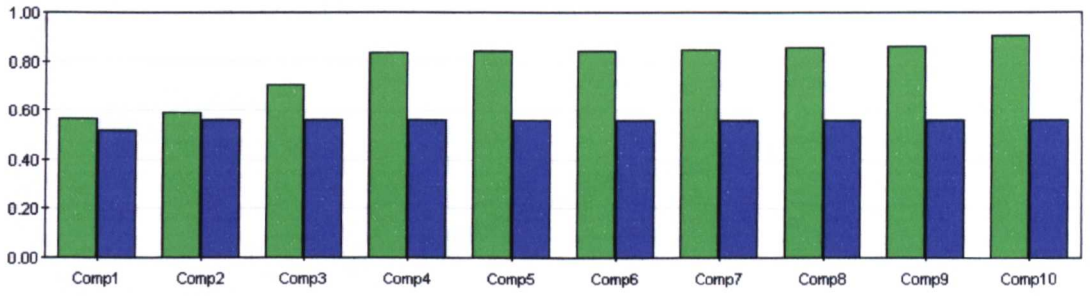



*Figure 3.7b*

*Figure 3.7 Summary of effects of additional PLS components of the $R^2$ and $Q^2$ values generated by the model. Figure 3.7a shows effects on enzyme activity component. Figure 3.7b shows effects on mechanical strength component. Green Bars = $R^2$ interpretive values. Blue Bars = $Q^2$ predictive values.*

As can be seen (figure 3.8) the results for both activity and strength responses appear normally distributed. Although there are no technical outliers (i.e. beyond the ±4 standard deviations range) the residuals plot does indicate that some of the results obtained do deviate considerably from the normal distribution. For example, the enzyme activity results for experiments 52, 56 and 67 do deviate from the bulk of the results. Excluding these data points from the model would improve the overall fit significantly. However as with most experiments some scatter in the results is common, and in order to keep the model as valid and realistic as possible, all the data obtained from the 80 screening experiments was used in the construction of the model.

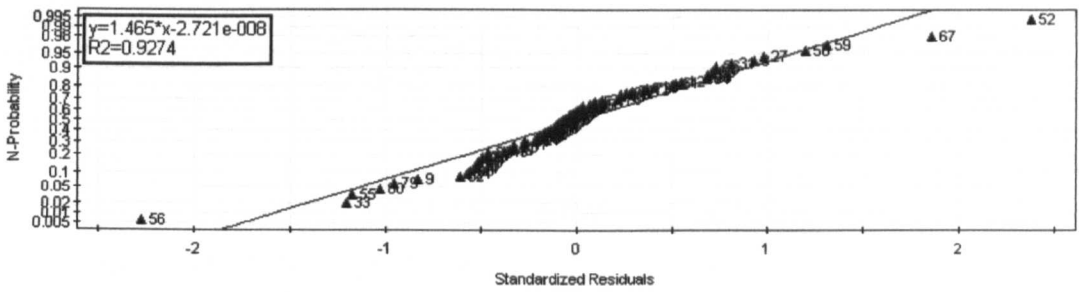



*Figure 3.8a*

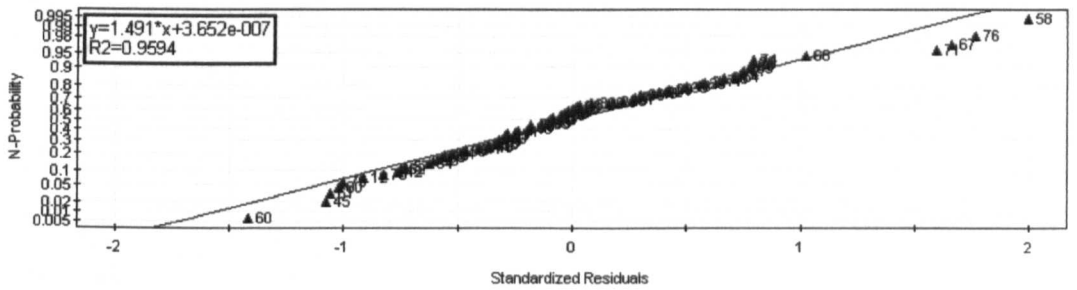



*Figure 3.8b*

*Figure 3.8 Double log plots showing experimental number vs. standardised residuals. Figure 3.8a is a residuals plot for the enzyme activity response. Figure 3.8b is a residuals plot for the mechanical strength plot.*

A plot of residuals against run order can be used to identify systematic errors present in the experimental method. Figure 3.9 indicates that results for both responses are evenly scattered indicating that there are no obvious errors in the experimental data. If for example, the scatter in these plots had been skewed, producing an observable trend, this may indicate some sort of consistent error, such as changing laboratory temperature etc.

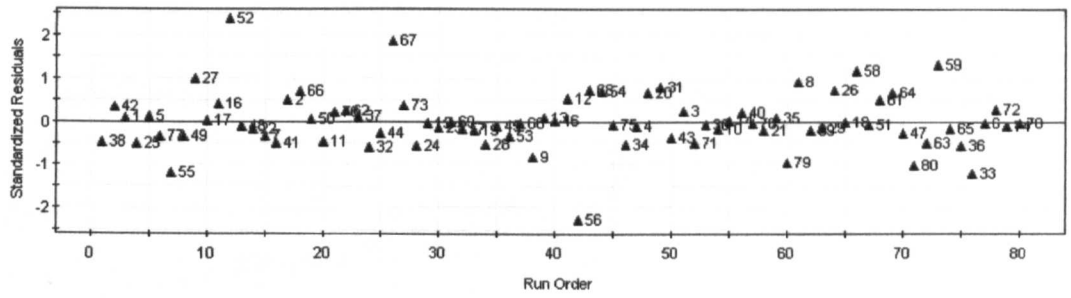



*Figure 3.9a*

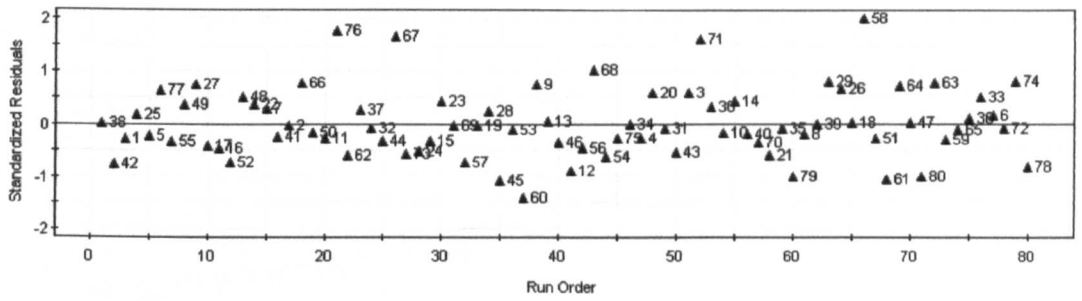



*Figure 3.9b*

*Figure 3.9 Standardised residuals vs. experimental run order. Figure 3.9a shows residuals from enzyme activity response. Figure 3.9b shows residuals from mechanical strength response.*

The model can be used to assay the responses that were used to construct it, by plotting a graph of the experimentally obtained data against the values predicted by the model for the same experiments (figure 3.10).

Ideally the result should lie on the 45 degree line drawn between the observed and predicted axis. For the enzyme activity response, the observed vs. predicted graph (figure 3.10a) shows a considerable degree of scatter around the regression line, providing another indication that the fit of the model for these results is inferior to the fit found with the mechanical strength response (figure 3.10b). Although a certain amount of scatter is found with the experimental results, both responses seem well fitted to the predictive model.

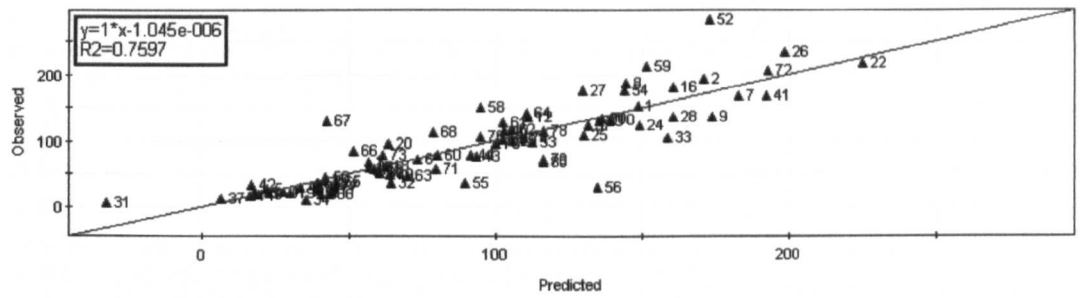



*Figure 3.10a*

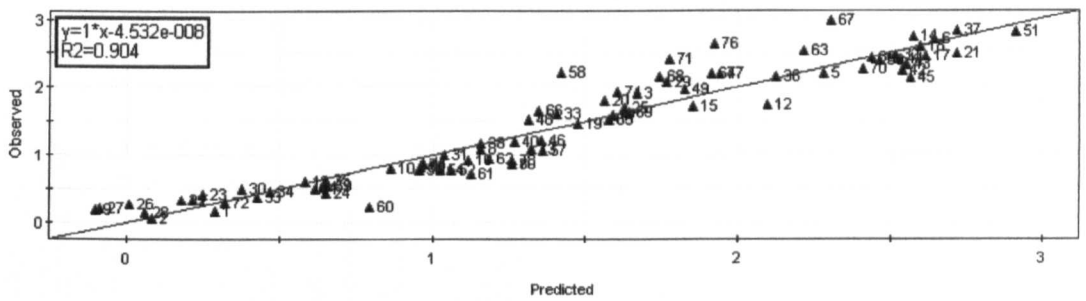



*Figure 3.10b*

*Figure 3.10 Observed experimental results vs. predicted results. Figure 3.10a shows observed vs. predicted results for the enzyme activity response measured in units of nmol/mg CT.min. Figure 3.10b show the observed vs. predicted results for the mechanical strength response measured in units of N-mm.*

Once the experimental data has been satisfactorily fitted to a model, it can be used to analyse which factors or combinations of factors have the greatest influence over the desired responses. Figure 3.11 illustrates these effects, clearly indicating that some factors are much more influential, over the strength and activity of the products than others.

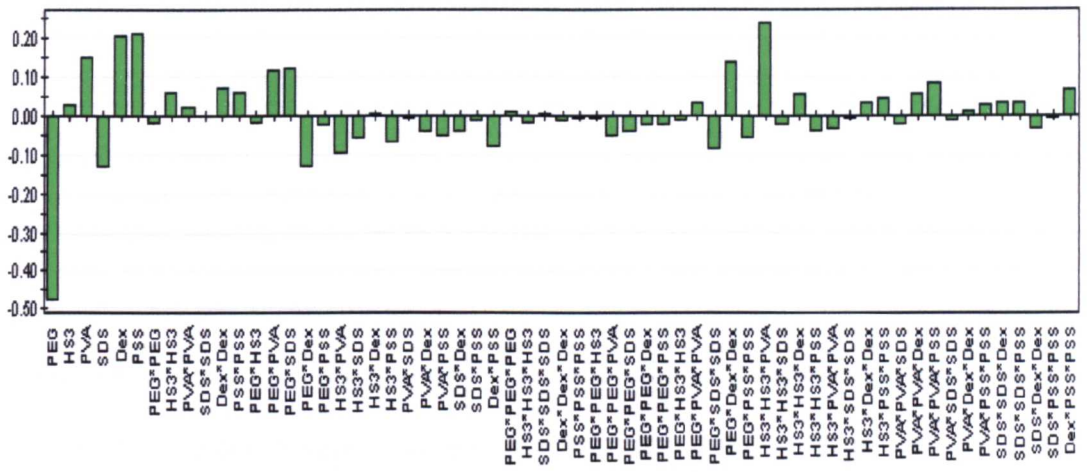



*Figure 3.11a*

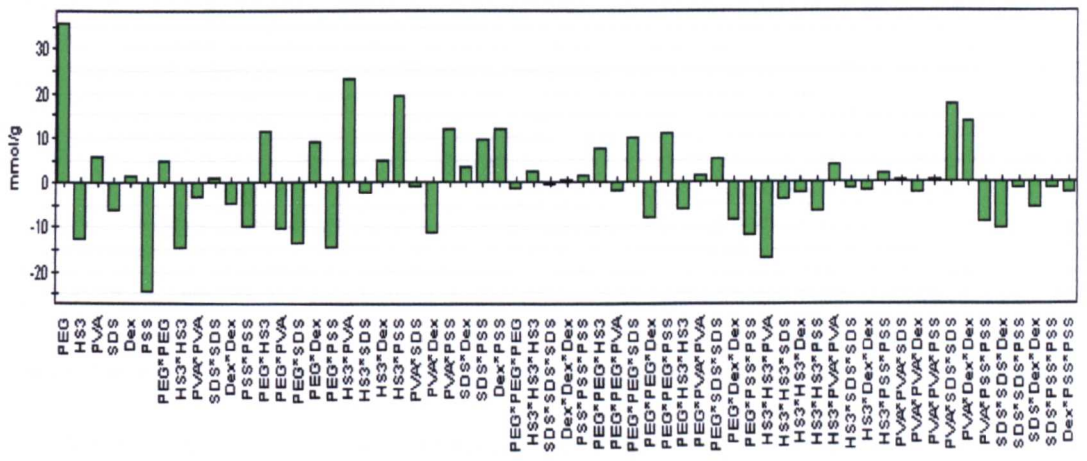



*Figure 3.11b*

*Figure 3.11 Demonstrating the effects of single and combined factors on response. Figure 3.11a shows effects on mechanical strength. Figure 3.11b shows effects on enzyme activity. Plots show the effects of single materials, combinations of two materials and combinations of three materials. Materials or combinations of materials with an overall positive affect on either of the two responses correspond to a positive response on the graph, whereas those with an overall negative affect correspond to a negative value on the graph. The size of the response is proportional to the magnitude of the affect.*

In general, it can be seen from these plots that the factors that are most beneficial to one response have the most deleterious effect on the other response. For example PEG is

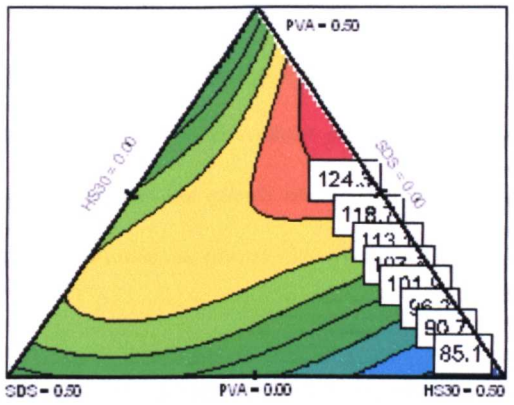
shown to be by far the worst material for bead strength (figure 3.11a) but is the best material for increasing the enzyme activity (figure 3.11b). Conversely PSS is a good material for enzyme activity but poor for mechanical strength. PVA displays positive contributions to both strength and activity, while SDS clearly has a negative effect on both the desired responses.

These effects plots can also be used to identify synergistic and antagonistic interactions. Examination of the strength response (figure 3.11a) shows that the interaction between silica and PVA (in a two thirds silica to one third PVA ratio) produces a strong positive effect. The value of the response is considerably greater than for either the single factor silica or PVA effects. The interaction of PVA with SDS (1:2 PVA to SDS ratio) also demonstrates a clear synergy for enzyme activity (figure 3.11b) producing a response far higher than for either PVA or SDS measured on their own.

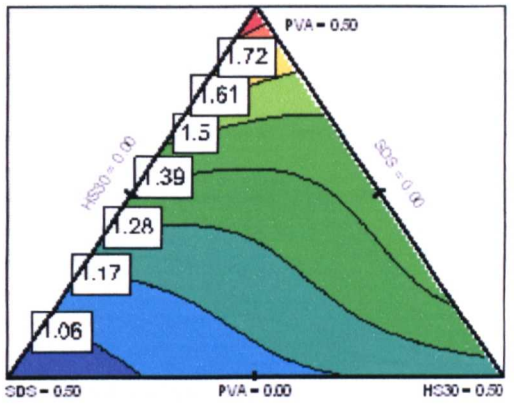
Not all the interactions result in a positive synergy. It can be seen (figure 3.11b) that the combination of PVA and DEX (in a 50 : 50 ratio) results in a negative response for enzyme activity whilst both compounds produce positive responses when measured singularly.

The purpose of constructing a model to represent this six-factor system is to provide a continuous representation of all possible combinations of the factors, not just the discrete integers covered by the actual experimentation. Ideally a well-fitted model can be used to accurately predict composites that produce the best responses.

Activity

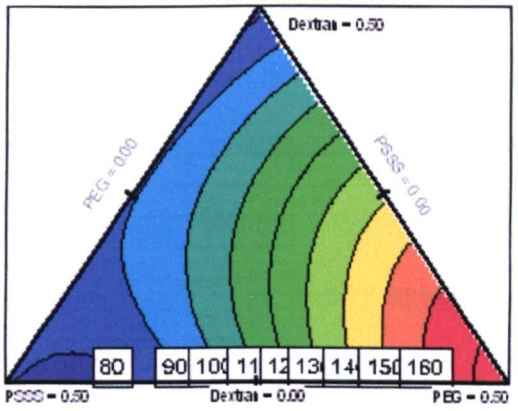


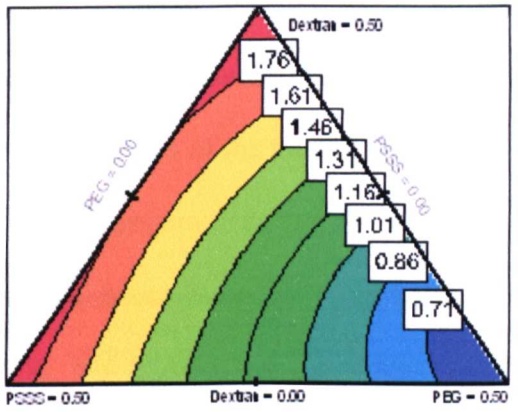

Strength




PEG = 0.166667 Dextran = 0.167 PSSS = 0.167

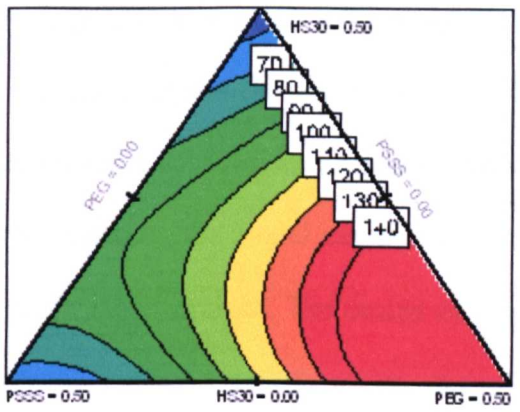
*Figure 3.12a*

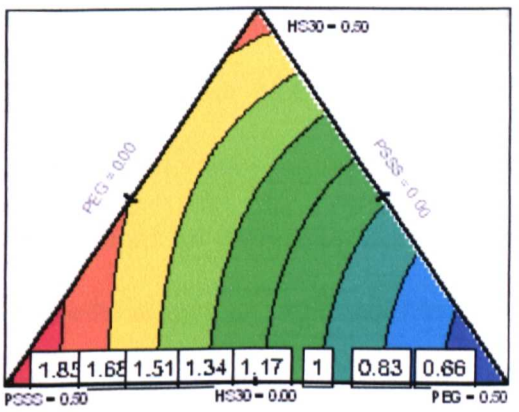



HS30 = 0.166667 PVA = 0.166667 SDS = 0.167

*Figure 3.12b*




PVA = 0.166667 SDS = 0.167 Dextran = 0.167

*Figure 3.12c*

*Figure 3.12 Contour plots illustrating the effects of varying three factors whilst keeping the remaining three factors constant at a fixed ratio of one sixth of the total mass of the material. The constant factors are defined beneath each contour plot. Left hand plots represent the effects on enzyme activity. Right hand plots represent the effects on mechanical strength. Red areas indicate the highest response, whilst blue areas represent the lowest response.*

The model generated in this study can be used to make predictions with either no restrictions on any of the six variables, or with certain factors set to a constant value. Figure 3.12 displays typical response contour plots obtained when three of the six factors are set to a constant reference level (one sixth of the total content of the mixture) and the remaining three variables are allowed to range from 0-50 % of the total content.

These contour plots allow the optimum areas for each system to be quickly identified. For example, figure 3.12b clearly shows that the optimum enzyme activity (with silica, PVA and SDS factors kept constant) is found at the highest concentrations of PEG. Conversely, the same high concentrations of PEG result in beads with the lowest mechanical strength. It can also been seen that the strongest beads predicted for this system can be formed with either high levels of DEX or PSSS. The same negative correlation of effects can be seen in the figure 3.12c contour plots where the highest numerical values for one response correspond to the lowest values for the other response.

Alternatively, the model can be used to make predictions for both responses with all six factors unconstrained. The design of experiment model allows for predictions to be made for the optimisation of one or other responses, both responses simultaneously or for targeted values of either response. The only sure way of testing any model's predictive ability is to synthesise and test some of the predicted formulations. To this end the model

was used to predict nine composites (table 3.1) designed to yield beads with various enzyme activities and mechanical strengths.

| Exp. No. | PEG | HS30 | PVA | SDS | Dextran | PSSS | Activity | Strength |
|---|---|---|---|---|---|---|---|---|
| 1 | 0.1991 | 0.1406 | 0.1862 | 0 | 0.4108 | 0.0633 | 153.1 | 1.5332 |
| 2 | 0.5879 | 0.2324 | 0.1797 | 0 | 0 | 0 | 238.6 | 0.3749 |
| 3 | 0.6667 | 0 | 0 | 0.3333 | 0 | 0 | 210.6 | 0.3183 |
| 4 | 0 | 0.1477 | 0.1 | 0 | 0.3313 | 0.421 | 70.9 | 2.7822 |
| 5 | 0 | 0.15 | 0 | 0 | 0.3333 | 0.5167 | 55.5 | 2.9535 |
| 6 | 0.14 | 0.2 | 0.4 | 0 | 0.2 | 0.06 | 155.0 | 1.5185 |
| 7 | 0.0015 | 0.3076 | 0.6252 | 0.0124 | 0.0129 | 0.0403 | 140.6 | 1.6076 |
| 8 | 0.1847 | 0.2038 | 0.5 | 0 | 0 | 0.1115 | 203.1 | 1.604 |
| 9 | 0.1207 | 0.1442 | 0.7 | 0 | 0 | 0.0349 | 172.3 | 1.5821 |

*Table 3.1 Ratios of the six components used to form the nine predicted composites synthesised to validate the design of experiment model. The predicted enzyme activity values (units of nmol/mg CT.min) and the predicted mechanical strength values (units of N-mm) are also shown.*

Experiment 1 was designed for maximum activity with a target strength value of 1.5 N-mm to produce beads with good catalytic rates but with a level of strength that was judged to be practical for re-use. Experiments 2&3 were designed for maximum activity with no regard to the beads strength. Experiments 4&5 were designed for maximum strength with no regard to the beads activity. Experiments 6&7 were designed to achieve specific strength and activity targets so as to demonstrate the accuracy and versatility of the model. Experiments 8&9 were designed to produce beads with the maximum combination of both strength and activity, whilst having one parameter constrained. The PVA component was fixed at ratios of 0.5 and 0.7 respectively, to examine the effects of this elastic material on the beads reusability.

All nine predicted composites were synthesised and assayed for mechanical strength and enzyme activity.

### 3.4.4 Analysis of predicted composites

As can be seen from the optical images (figure 3.13), all nine of the predicted formulations yielded beads of similar appearance, regardless of their composition.

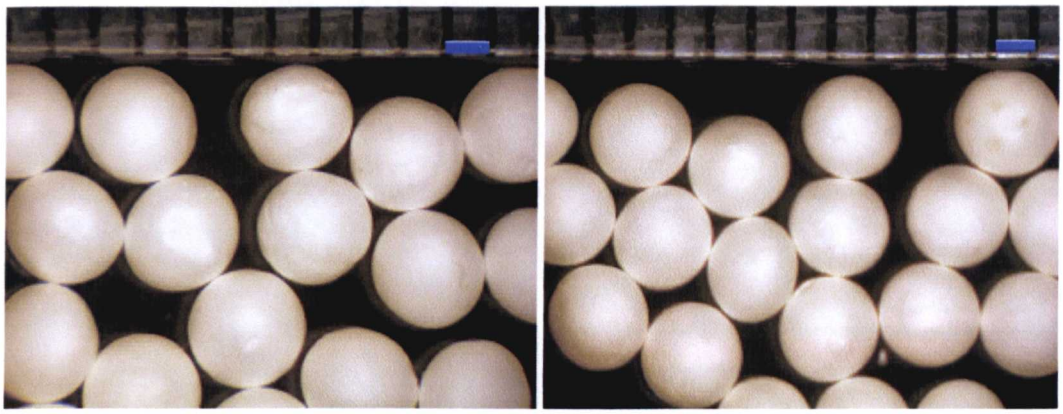


*Figure 3.13 Optical images of two sets of beads with differing compositions. Scale bars = 1 mm. The images are typical of the beads produced. Specifically, the right hand image refers to the beads formed in experiment 2 while the left hand image refers to the beads formed in experiment 9.*

High magnification SEM imagery of the beads surfaces and internal structure also illustrates their similar appearance (figure 3.14).

However, some of the SEM images indicated the presence of small particles embedded within the beads substructure (figure 3.15).

During the experimental procedure it was noted that some of the solutions made up prior to the bead synthesis turned opaque upon standing for a few minutes, after addition of the colloidal silica nanoparticle suspension (HS30). This opaque appearance to the liquid is presumably due to the agglomeration of some of the solution components.

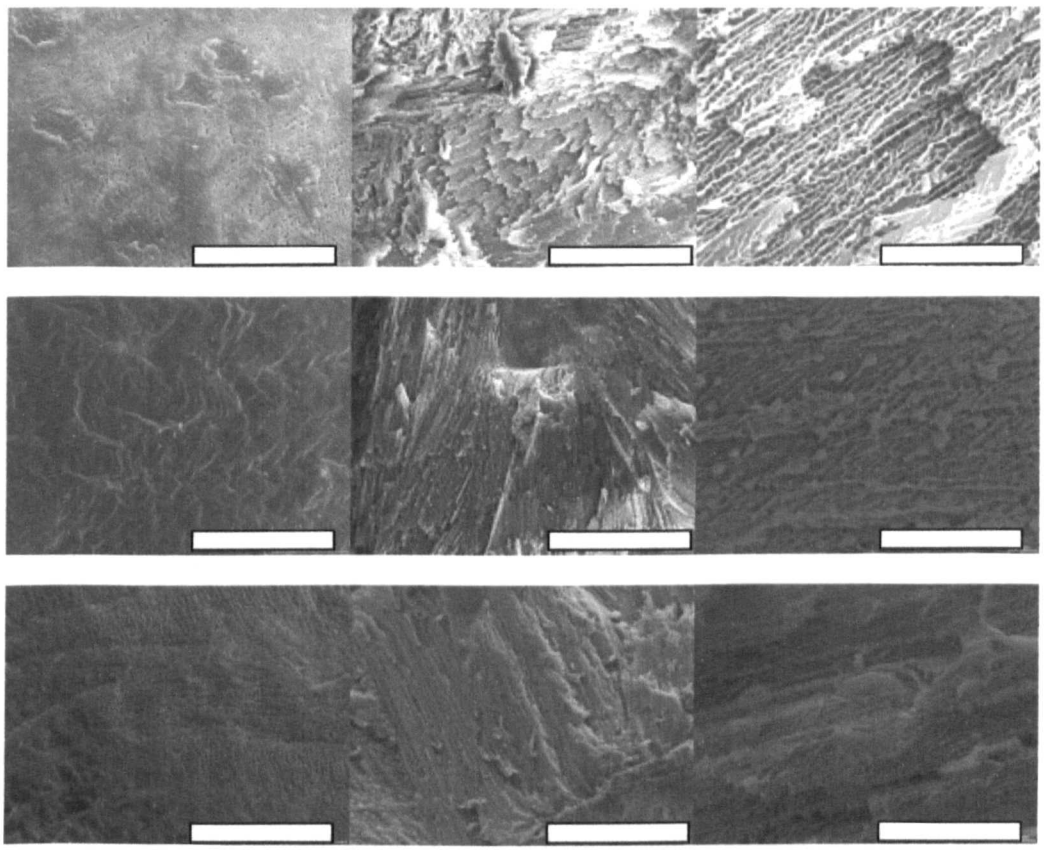


*Figure 3.14 SEM images of three sets of beads with differing compositions. Left = surface image. Scale bar = 100 μm. Centre = interior structure of the bead. Scale bar = 500 μm. Right = interior structure of the bead. Scale bar = 50 μm. Top = experiment 1, middle = experiment 2 and bottom = experiment 3.*

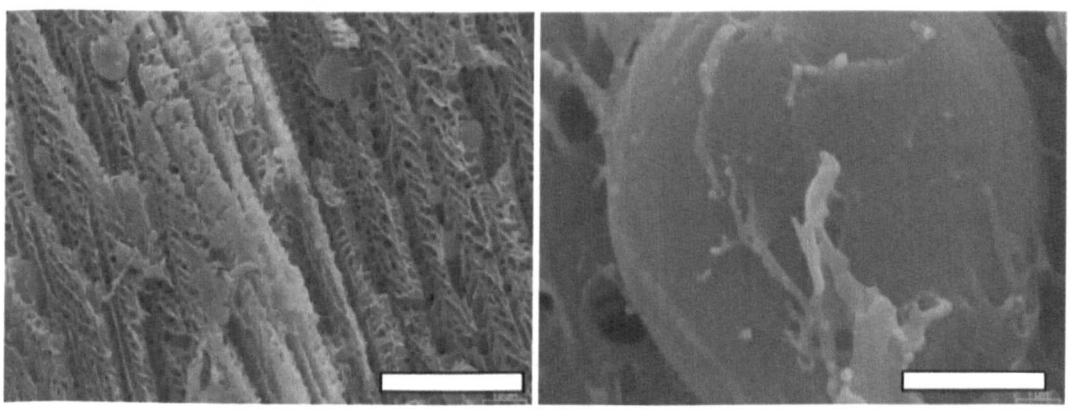


*Figure 3.15 SEM images of the beads formed in experiment 7. Left = SEM image showing interior fine pore structure of a bead, scale bar = 30 μm. Right = Close up SEM image of agglomerated particle within the internal bead structure, scale bar = 3 μm.*

The effects of protein adsorption to silica nanoparticles have been studied previously.[25-27] Lysozyme has been shown to change its structural conformation when adsorbed to colloidal silica.[27] The change in conformation increases as the size of the silica nanoparticle support increases. The change in the number of protein α-helices was measured spectroscopicaly and was found to be directly linked to the enzymes catalytic activity. As the number of α-helices is reduced, the corresponding enzyme activity is also lowered. It was postulated that the shallower curvature of the larger nanoparticles resulted in an increase in the deformation of the proteins secondary structure. Lysozyme has a molecular mass of around 14.3 kDa[28] and molecular dimensions of approximately 4.5 x 3.5 x 3.5 nm.[29] This corresponds to the same approximate size as the smallest 4 nm nanoparticles studied. As the enzyme and the support are approximately the same size, very few of the enzyme molecules are able to pack around the nanoparticle. In this system the enzyme could be thought of as a hard sphere, unable to deform around the tight radius of the nanoparticle. However, as the diameter of the nanoparticle is increased to 100 nm the surface area increases and the surface curvature relative to the enzyme is reduced. This means that there is less restriction in the packing arrangement of the enzyme molecules on the support surface. As a result more of the charged groups on the enzyme's outer surface can overcome the reduced physical packing restraints and stick to the oppositely charged surface of the support. The more charged groups of the enzyme that are attracted to the nanoparticle, the more the enzyme twists out of shape. The more the enzyme is deformed from its native conformation, the greater the loss of catalytic activity. Figure 3.16 illustrates this deformation process.

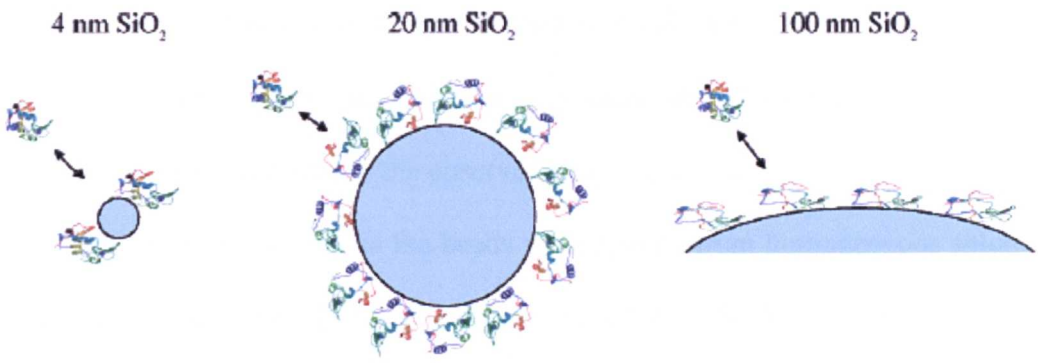



*Figure 3.16 Schematic representing the increased deformation of the enzymes conformation, on adsorption to larger diameter silica nanoparticle surfaces.*[27]

Although lysozyme is a smaller enzyme than the α-chymotrypsin studied in this work, it is possible that a similar adsorption process is occurring in this system. However, it is reported in the literature that the adsorption of the enzyme to the silica support results in an overall deactivation, whereas in this study the catalytic activity of α-chymotrypsin is shown to increase dramatically.[27] It may be that the deformation of the α-chymotrypsin occurring during the adsorption process does not effect the area of the enzyme containing the active site, and therefore does not deactivate the enzyme.

The effect of the curvature of silica surfaces on the activity of enzymes supported on them, may offer an explanation as to why the two types of silica nanoparticle examined in the second screening of materials experiment (Ludox® HS30 and TM50, section 3.3.2) displayed such differences in enzyme activity. α-Chymotrypsin immobilised on TM50 possessed very little activity whilst the equivalent HS30 system was one of the most catalytically active preparations examined in the whole study. Although the pH and surface area of the two materials are slightly different, the biggest factor affecting the immobilised enzyme activity may be the size of the silica nanoparticles. TM50 particles are significantly larger (35 nm) than the HS30 particles (13 nm). HS30 nanoparticles may

be so small that the adsorbed α-chymotrypsin is not deformed at all, whereas the "softer" radius of the larger TM50 nanoparticles may cause an unfavourable deformation in the enzymes structure, resulting in the observed lack of catalytic activity.

It was initially assumed that as the beads were formed from homogeneous solutions that the composition of the resulting supports would also be homogeneous and uniform. However, it appears that the enzyme has differing affinities for complexation with the various materials that make up the composite. Adsorption of the enzyme to the surface of the silica nanoparticles is preferential to any of the other materials, causing the nanoparticles to agglomerate. Evidence for this can be seen by imaging the secondary electrons refracted from the composites surface (figure 3.18). When bombarded with electrons from a scanning electron microscope, different elements emit electrons with differing energies (figure 3.17).

The electron microscope can then be used to produce an image from this pattern of secondary electrons (figure 3.18).

Not all elements produce a strong enough secondary electron signal to be imaged accurately (figure 3.17). Ideally, it would be possible to image the position of the entrapped α-chymotrypsin directly. This could be achieved by examining the response due to the element sulfur. α-Chymotrypsin is the only component of the composite beads that contains this element, and so it may be possible to determine whether the enzyme is aggregating in solution prior to the formation of the bead, or whether it is evenly dispersed throughout the support material. However, the low response of sulfur to this technique (figure 3.17) coupled with the very low abundance of this element in the composite, meant that the position of the α-chymotrypsin could not be determined via

this method. It may be possible to "fix" the enzyme in the support via a subsequent covalent crosslinking step, such as treatment with glutaraldehyde solution. The enzyme could then be stained with a material such as silver, which is far more sensitive to the x-ray technique (figure 3.17). However, as all the components of the support are highly water soluble, the effect of an aqueous glutaraldehyde solution on the morphology of the bead would most likely disrupt its fine pore structure.

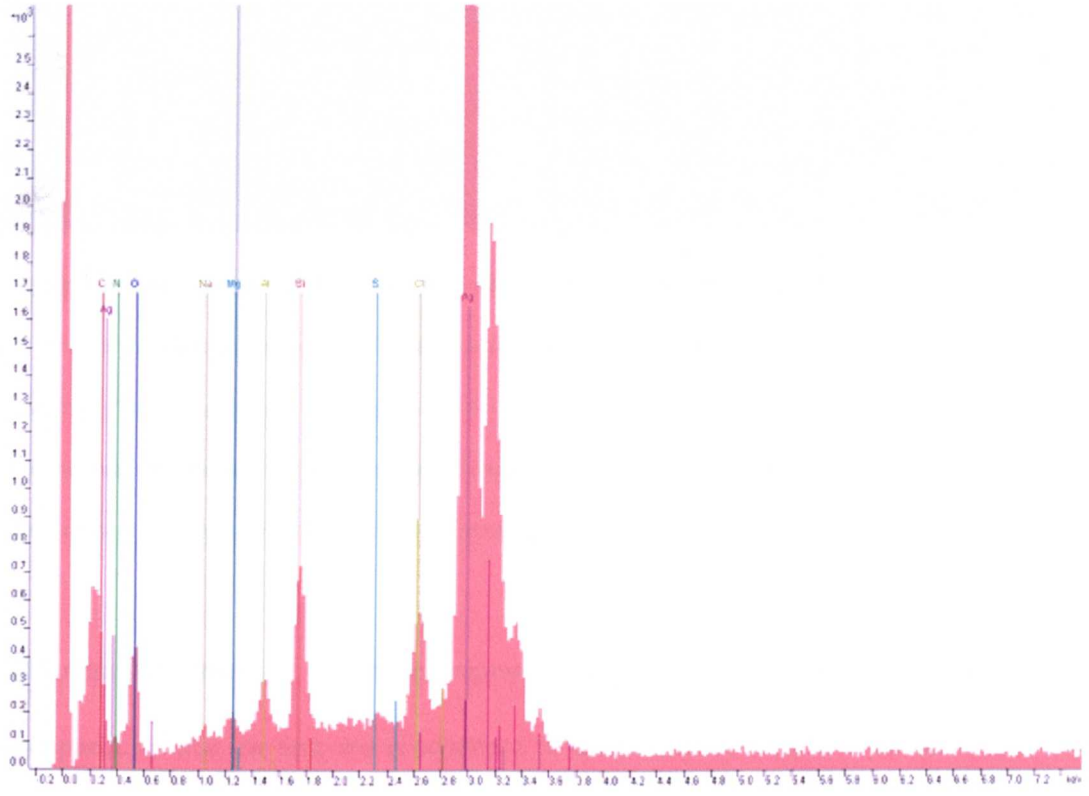



*Figure 3.17 Typical intensity trace for the secondary electrons refracted from the surface of the composite beads. Note the intensities of the sulfur, silver and silicon peaks. X-axis = electron energy (keV). Y-axis = electron intensity (counts/min).*

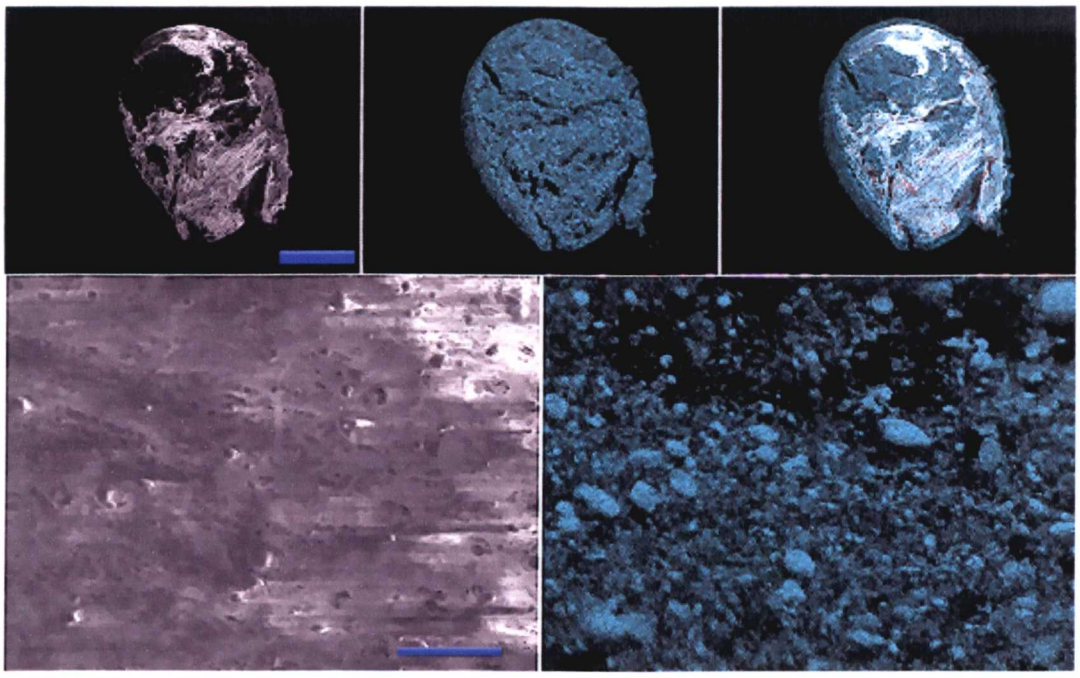


*Figure 3.18 Energy dispersive analysis by x-ray of a bead formed in experiment 7. Top left = SEM image of the interior surface of an uncoated bead, scale bar = 1 mm. Top middle = x-ray dot map illustrating the intensity of silicon deposits. Top right = x-ray dot map overlaid on electron microscope image. Bottom left = SEM image of the surface of an uncoated bead. Scale bar = 100 μm. Bottom right = x-ray dot map of the same section of bead surface, showing the intensity of silicon.*

Although the imaging of the entrapped α-chymotrypsin is very difficult, the silica nanoparticles that the enzyme is adsorbed to are relatively simple to image. It can be seen (figure 3.18) that the silica nanoparticles are not dispersed uniformly throughout the bead, but have in fact agglomerated to form much larger irregular particles of around 50 μm in size. It is probable that the majority of the entrapped α-chymotrypsin is also contained in the vicinity of these silicon deposits.

The mechanical strength and enzyme activity of all nine predicted composite beads was also assayed. The results (figure 3.19) show that although there is some experimental scatter, the model does predict the actual strength and activity values very well.

An $R^2$ value of 0.906 was obtained for the enzyme activity results and $R^2 = 0.930$ for the mechanical strength assay. It can be seen (figure 3.19a) that the observed enzyme activity results are generally slightly higher than those predicted by the model. The three targeted responses are clearly identifiable in the mechanical strength plot; maximum strength, maximum activity (no regard to strength) and targeted strength values of 1.5 N-mm (figure 3.19b).

Although the design of experiment methodology has not produced any composite beads with significantly greater enzyme activity or mechanical strength than were produced in the initial screening experiments, it has resulted in materials with an excellent compromise between the two responses.

A plot of observed enzyme activity against observed mechanical strength for all the composites synthesised, allows the "best" materials to be visualised figure 3.20).

In practical terms, a bead with a mechanical strength value of around 0.5 N-mm is difficult to handle and can easily be crushed between the fingertips. However, a bead with a mechanical strength of around 3 N-mm is far more robust. They are easily handled and extremely difficult to deform by hand. It was for this reason that the "targeted" materials were designed to have a mechanical strength of 1.5 N-mm. This represents a material that is still easily handled and strong enough to reuse. The design of experiment methodology allowed for the synthesis of these strong and robust materials, whilst retaining some of the highest enzyme activity levels recorded for any of the composites examined.

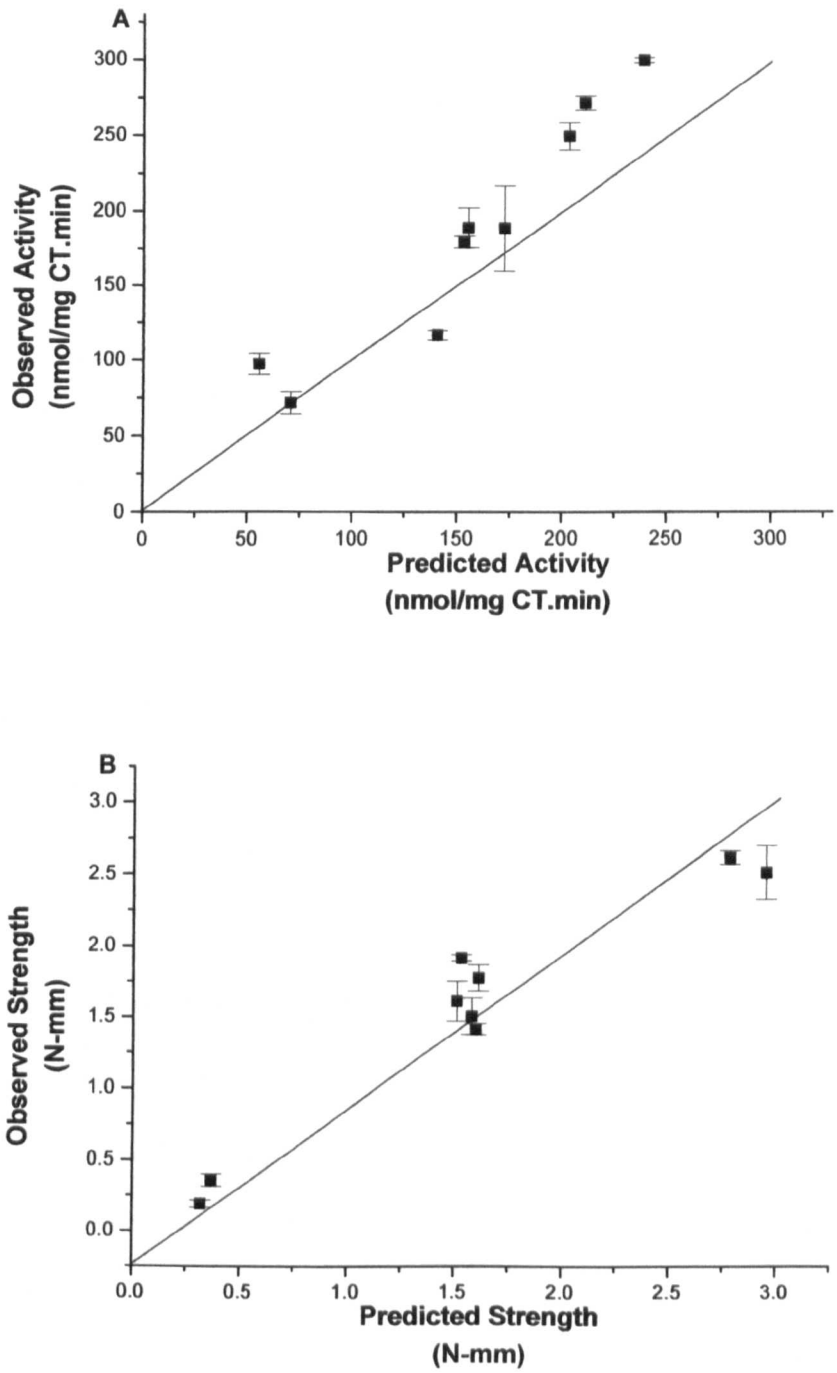



*Figure 3.19 Showing the correlation between the predicted and observed responses for enzyme activity (figure 3.19a) and mechanical strength (figure 3.19b) for all nine optimised formulations. A linear regression line is also displayed in both cases.*

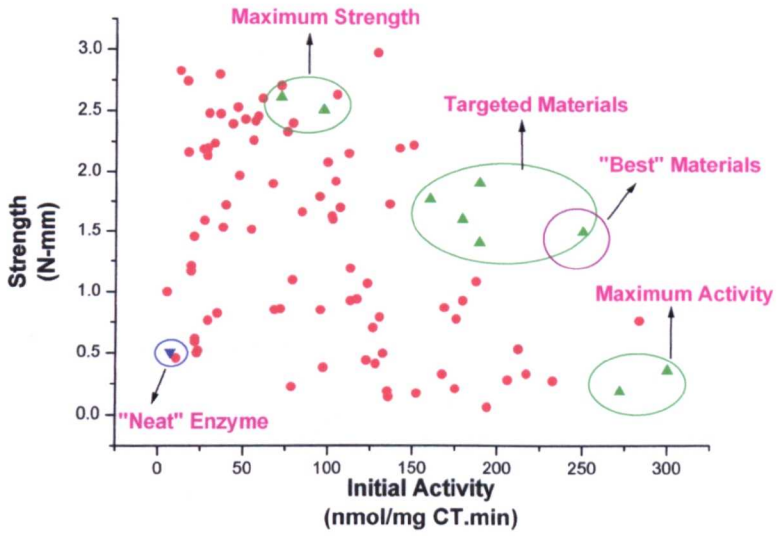



*Figure 3.20 Summary of all results obtained from the design of experiment study. Red circles = Data used to construct the model of the six component system. Green triangles = Data obtained from the "predicted" compositions. Blue triangles = Beads formed from 100 % α-chymotrypsin (for comparison).*

For comparison, the strength and activity responses for beads made from 100% α-chymotrypsin are included. It can be seen that nearly all the composites are both stronger and more active than this "neat" enzyme bead (figure 3.20). It is also clear that there are no compositions with strength and activity values that lie in the upper right quadrant of the graph (i.e. high strength and high activity). This is because the two responses are negatively correlated. Although the design of experiment method can exploit synergies found between the various materials, it cannot find mixtures that produce unrealistically high responses. Given that most of the recorded responses lie on the lower side of a regression line between maximum activity and maximum strength, any model that

predicted formulations with responses so far superior than anything previously observed, would be of doubtful validity. The only way to prepare composites with these very high response values would be to include new materials in the original set of six factors.

The best materials synthesised were observed to be three times stronger and up to 50 times more active than for the "neat" enzyme.

### 3.4.5 Why are enzymes immobilised on various composites more active than equivalent lyophilised preparations?

The use of design of experiment methodology has been shown to be very useful in the optimisation of the six-component system studied here. Composite beads have been synthesised possessing enhanced mechanical strength and enzyme activity, compared to both lyophilised α-chymotrypsin preparations and "composite" beads formed from 100 % enzyme. However, it is important to realise that the model is constructed from empirical observations only. The original work sheet of screening experiments used to build the model was designed so that trends in the response data (appendix 1) could be readily identified. The model produced does not provide any explanation as to why the changes in composition are effecting the mechanical strength and enzyme responses.

The most probable reasons for the enhancement of α-chymotrypsin's activity can broadly be separated into two categories; physical effects of the supports morphology and chemical effects on the enzymes conformation. The two most important parameters effecting enzyme activity, which make up the supports morphology are porosity and surface area. It can be seen from the SEM images (figures 3.14 and 3.15) that the average pore sizes of the beads synthesised in this study are far larger than the typical threshold

limit for the diffusion of small substrate molecules.[16] If the mass transfer rate of substrates throughout the interior of the bead is not a rate-limiting step, then the number of enzyme molecules accessible to the substrate will be more important. The amount of accessible enzyme will be related to the surface area of the support material. In order to study this effect, the surface area of all nine predicted composites was determined via the BET method (figure 3.21).

All the composite beads displayed relatively low surface areas and the results do not show any correlation between the support's surface area and the corresponding enzyme activity.

As the change in physical morphology of the composite support materials studied does not appear to affect the catalytic activity of the enzymes entrapped within them, the explanation for the observed results is most probably to do with the chemical effects of the supports on the enzyme.

There are two main chemical effects occurring within the enzyme's microenvironment that influence its catalytic activity; water content and proximity to different chemical functionalities. It has been well reported that the quantity of water contained within the enzymes microenvironment is critical to its catalytic activity.[11-14, 30] Although all of the composite beads were hydrated over saturated salt solutions to the same constant water activity,[31] the actual quantity of water in the system will depend on the composition of the bead. This is because each of the six component materials will have a different affinity for water and so will adsorb different quantities of water (weight for weight) at the same water activity. These differing affinities toward water are defined as the

materials aquaphilicity.[13] In general compounds containing more hydroxyl functionalities will be more hydrophilic and so will have a greater aquaphilicity.[9]

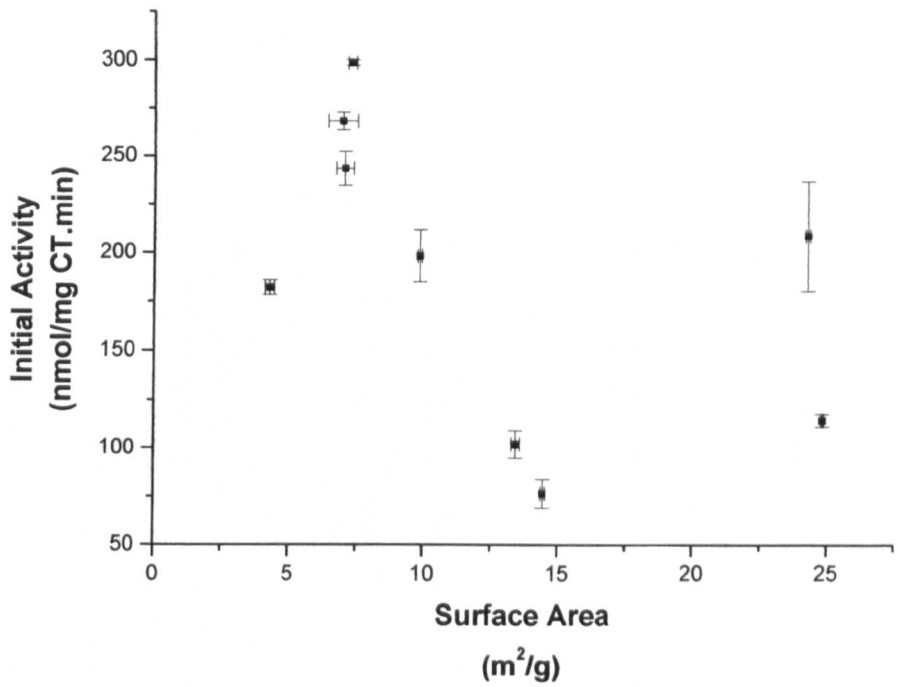



*Figure 3.21 Relationship between the observed catalytic activity of the immobilised enzyme and the surface area of the support material. Catalytic activity was measured by following the transesterification reaction between ATEE and propan-1-ol in hexane.*

However this will also depend on other reaction parameters such as solvent polarity. As a result of this phenomenon, it is possible that some of the high enzyme activities observed were due to the different amounts of water in the reaction system at the time of the assay. To examine this postulation, beads were synthesised from each material singularly (with the addition of the standard 5 $\%^{w}/_{w}$ of α-chymotrypsin), hydrated over a saturated solution of potassium chloride ($a_w$ 0.86) or dried rigorously over phosphorus pentoxide ($a_w$ 0.0) before being assayed for catalytic activity. The results show that the six

components that make up the supports exhibit very different effects on the catalytic activity of the enzyme entrapped within them, when hydrated to differing water activities (figure 3.22).

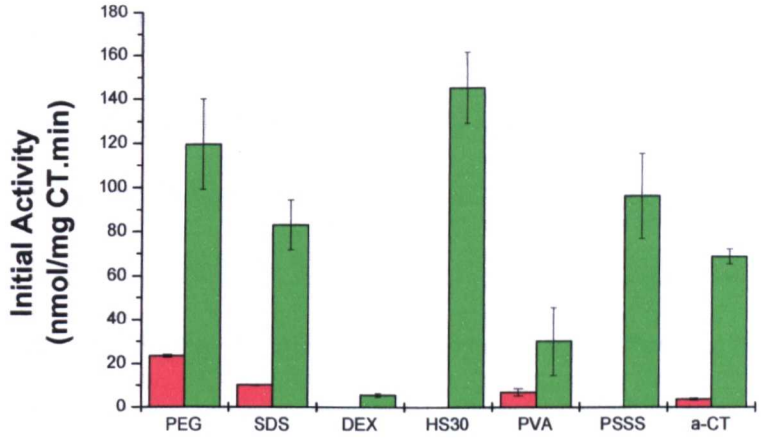



*Figure 3.22 Effect on enzyme activity of various support materials hydrated to different water activities. Catalytic activity was measured by following the transesterification reaction between ATEE and propan-1-ol in hexane. Green bars = water activity of 0.86. Red bars = water activity of 0.0. The most catalytically active lyophilised enzyme preparation is also displayed for comparison (a-CT).*

It can be seen that the α-chymotrypsin immobilised in SDS and PEG supports exhibit similar properties to the most catalytically active lyophilised α-chymotrypsin preparations, i.e. low catalytic activity at low hydration levels and much higher activities at the increased water activities. However, the activity of α-chymotrypsin immobilised on the silica nanoparticle support material appears even more water dependent. At low water levels no enzyme activity can be detected, whereas at $a_w$ 0.86 the material exhibits the highest catalytic rate of all six individual materials.

The other major chemical effect of the support material on the immobilised enzyme is the proximity of the protein to the supports functional groups. These functional groups interact with the enzyme, primarily through hydrogen bonding and hydrophilic/hydrophobic attractions and repulsions. Any of these forces that exert a distorting effect in the enzyme are likely to change its conformation and so reduce its catalytic activity.[6]

It has been shown that certain enzymes co-lyophilised with ethylene glycol and poly(ethylene glycol) exhibit enhanced catalytic activity when assayed in organic media.[8, 9] It is reasoned that when the levels of water contained within the reaction system are reduced to very low levels, the effects of other hydrogen bonding moieties become more dominant. The relative effect of this hydrogen bonding is greatly increased when the very strongly hydrogen bonded water molecules are removed from the enzyme's microenvironment. In this case PEG is thought to act as a "water mimic". It was found in the initial screening study (figure 3.2) that the enzyme activity greatly increased when the molecular weight of the PEG support material was reduced from 8 kDa to 1.5 kDa. If indeed the PEG is acting as a water mimic then the observed trend is the expected one. As the molecular weight of the PEG is reduced the ratio of hydroxyl groups present will increase. The additional hydrogen bonding resulting from these hydroxyl groups should act in a similar way to the progressive addition of water to the reaction system. This is illustrated by the comparatively high catalytic activities observed with α-chymotrypsin immobilised on pure PEG supports at very low water activity (figure 3.22). The initial rate observed is considerably higher than for any other enzyme containing system at this water activity. It has been found that when PEG (2 kDa) was co-lyophilised from solution

with *Canada antarctica lipase-B* (CALB) in equimolar equivalents, an increase in enzyme activity was observed.[9] A linear correlation between enzyme activity and quantity of PEG incorporated was found, to a maximum of six molar equivalents, which corresponded to a six-fold increase in catalysis.

Conflicting literature reports imply that the hydroxyl groups of polyols do not form favourable hydrogen bonds with enzyme molecules.[7] Various cyclodextrins containing hydroxypropyl, di-methyl and tri-methyl moieties, were co-lyophilised with α-chymotrypsin. It was found that the more hydrophobic cyclodextrins actually produced enzyme preparations with greater activity than the hydrophilic hydroxypropyl-β-cyclodextrin. It may be that the presence of more hydrophobic groups in close proximity to the enzyme actually repel water, increasing the water concentration in the localised microenvironment of the enzyme.

However it is still possible that the theory of changing PEG crystallinity with molecular weight (section 3.3.1) is still valid. As the molecular weight of PEG is reduced from 8 kDa to 1.5 kDa its structure does change. At higher molecular weights the polymer is able to fold up into a small and compact arrangement. At lower molecular weights (<4 kDa) the polymer chain length becomes too short for this process to be favourable. Overall this leads to a different packing arrangement around the protein, with the higher molecular weights able to pack more densely, hindering the mass-transfer of substrates. A similar effect has been reported in the literature,[7, 10] where the compact structure (figure 3.23) of cyclodextrin was shown to impart far greater catalytic activity to materials when co-lyophilised with α-chymotrypsin than the equivalent preparation synthesised with long linear chains of dextrin.

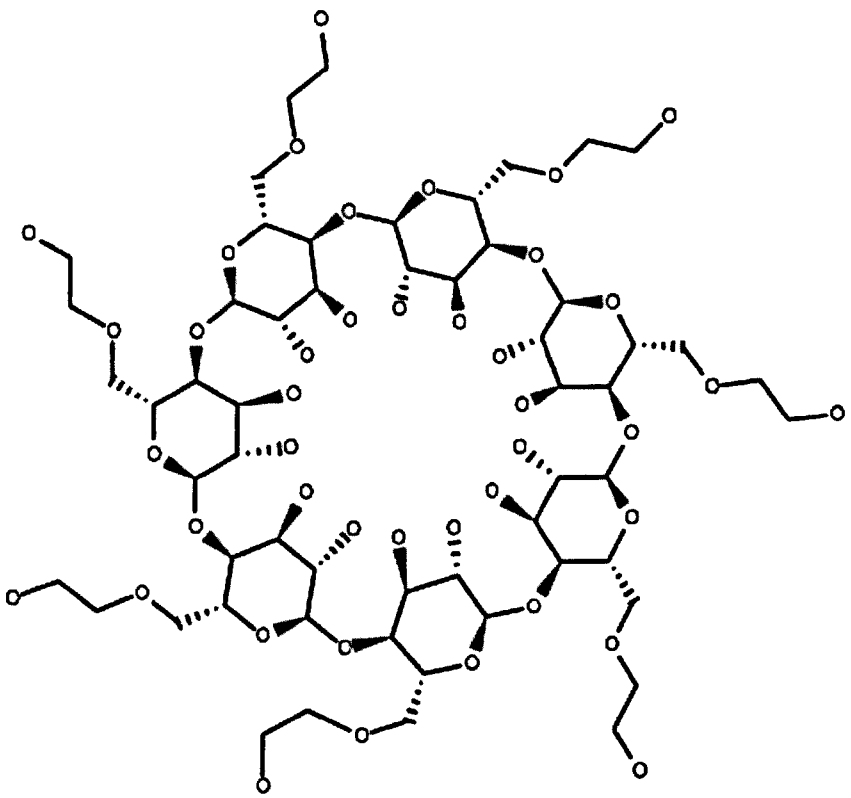


*Figure 3.23 Structure of Hydroxypropyl-β-cyclodextrin. Molecular diameter of cylinder as shown as approximately 0.7 nm.[40] The molecular diameter of α-chymotrypsin (assumed to be a uniform sphere) is approximately 4 nm.*

Again it is reasoned that the comparatively small cyclic dextrin structures allow for better diffusion of substrate molecules to the enzymes active sites, through the "hole" intrinsically present in the cyclic structure. These literature reports may explain why the co-lyophilization of α-chymotrypsin with dextran produced materials with low catalytic activity. The comparatively long and branched dextran chains may be hindering the mass transfer of substrates to the enzyme's active sites, reducing catalytic activity.

In addition to the enhanced enzyme activity due to the increased water content of aquaphilic systems and the action of "water mimicking" materials, other chemical effects of support materials co-lyophilised with enzymes have also been postulated. When enzymes are employed in non-aqueous media they tend to aggregate due to repulsive hydrophilic interactions with the organic solvent. By co-lyophilising any support material with the enzyme, some separation of the enzyme molecules results. This reduces the blockage of enzyme active sites through protein-protein contacts.[32] Organic solvents also distort the enzyme native conformation through hydrophilic/hydrophobic interactions, causing a reduction in enzyme activity. Spectroscopic studies have shown that cyclodextrins co-lyophilised with α-chymotrypsin reduce these changes in the enzymes secondary and tertiary structure, increasing its stability in these polar solvents.[7]

The freeze-drying process is also known to reduce enzyme activity.[33] Additives co-lyophilised with the enzyme may act as cryoprotectants, reducing the enzymes relative deactivation, rather than enhancing its activity.

It has been shown that almost all the enzyme containing composite beads display better activity than the beads formed from 100 % "neat" α-chymotrypsin (figure 3.20). These beads can be thought of as "enzyme immobilised on enzyme". It is possible that α-chymotrypsin is simply a poor support material. Its complex three-dimensional structure, containing many hydrophobic and hydrophilic amino acid residues may distort the conformation of other enzymes that come into close proximity, resulting in the deactivation of both molecules. Therefore the addition of the polymer support materials may not significantly enhance the enzyme's activity, just alleviate the deactivation caused by the enzyme itself.

The mechanical strength of the beads has also been shown to vary considerably with their differing compositions. It was noted during the enzyme activity assays of the nine predicted formulations that although the sizes of the various sets of beads were very similar, the average mass of each set did vary. However, no correlation between the average bead mass and its corresponding mechanical strength was found (figure 3.24).

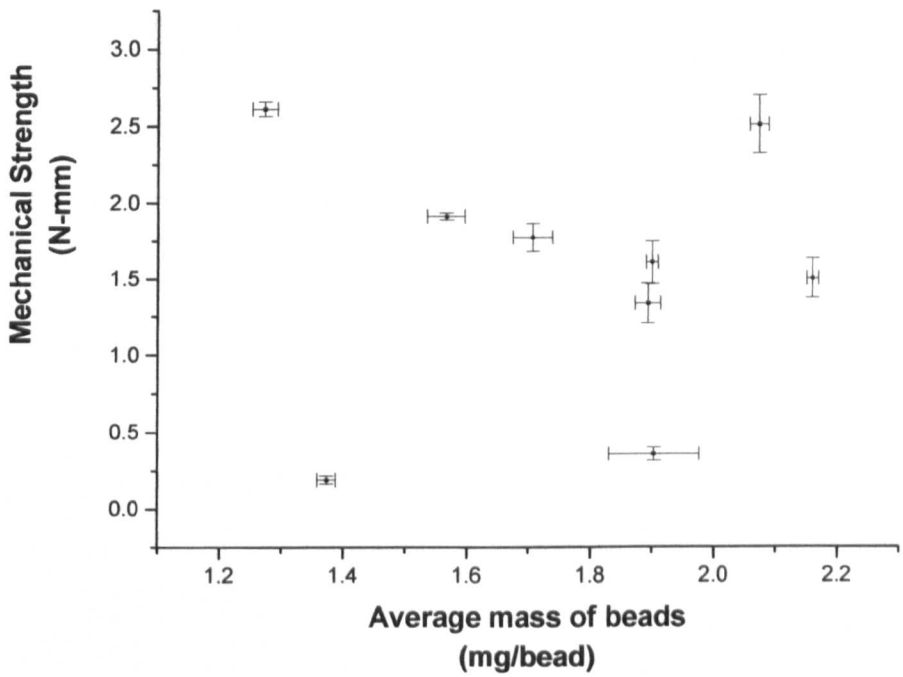


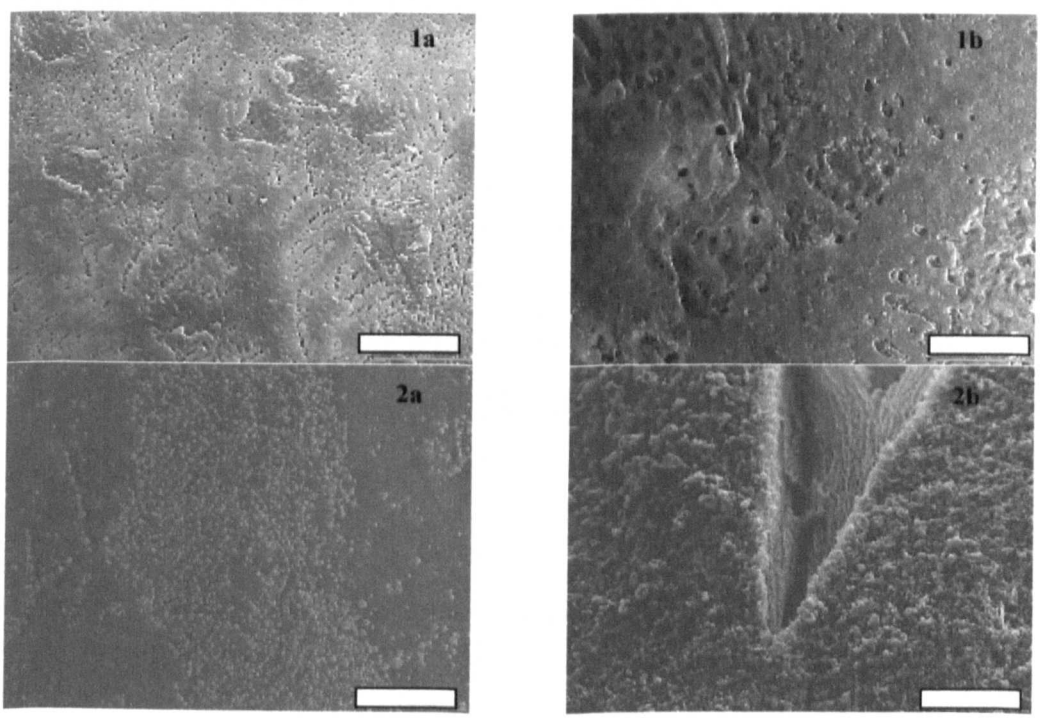

*Figure 3.24 Demonstrating the lack of correlation between the average bead mass and its corresponding mechanical strength. Data shown for the nine predicted compositions.*

Similarly no correlation was found between the bead's catalytic activity and the corresponding surface areas (not shown). As the measured physical properties of beads did not appear to be related to their mechanical strengths, it is assumed that non-covalent chemical interactions between the support materials are responsible for the variations in mechanical strength. Materials that are able to form multiple hydrogen bonds with both

the enzyme and the other support materials, such as dextran, were found to produce the strongest composites.

### 3.4.6 Re-utilisation study of predicted composites

One of the main aims of the optimisation study was to produce beads that were not only catalytically active but also mechanically strong enough to be re-used. Although the strength of each bead type can be assayed, the only definitive method of assessing their reusability is experimentally. Five of the nine sets of optimised beads were selected for this study (experiments 1,2,4,6 and 8) representing each of the different features (e.g. maximum strength, maximum enzyme activity etc, section 3.3.3) that the beads were designed for (figure 3.25).



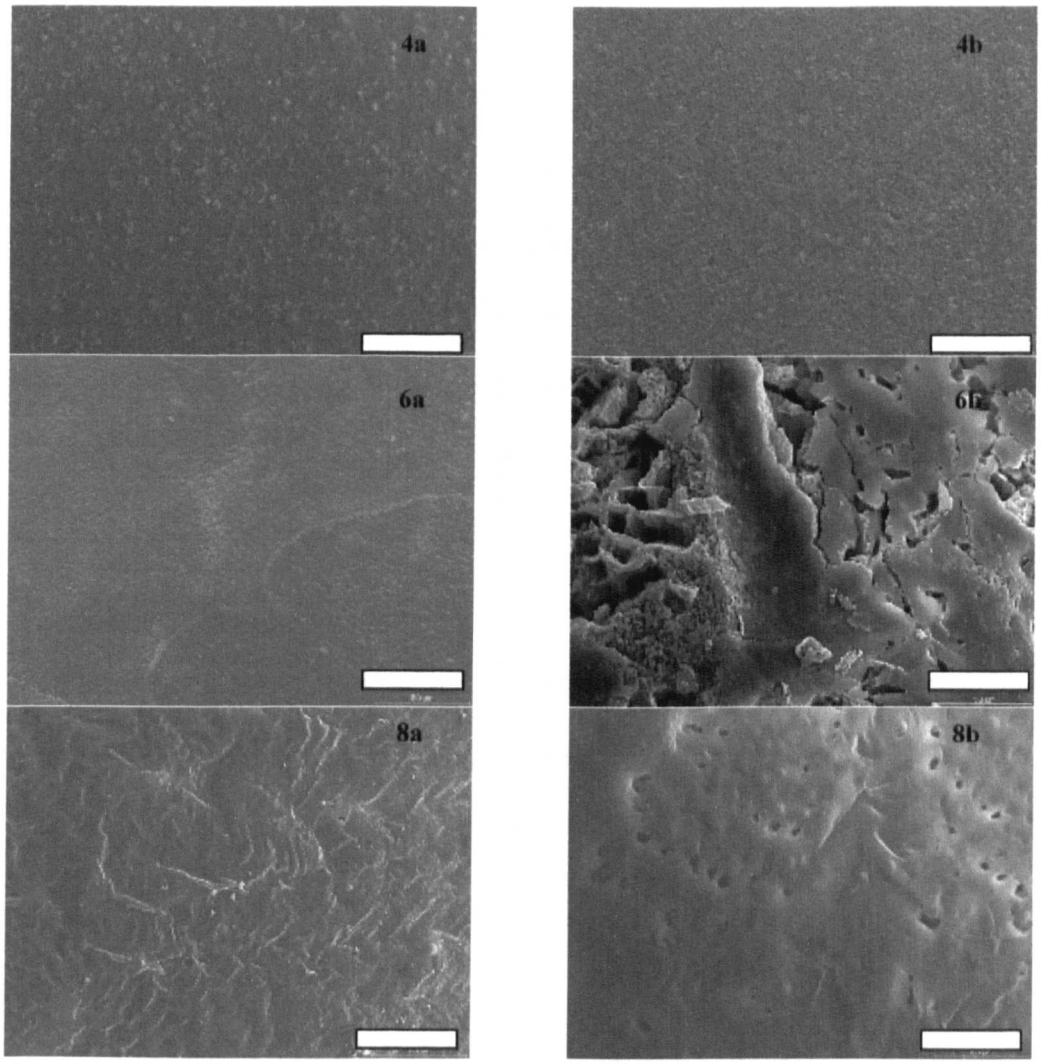



*Figure 3.25 SEM images of the surfaces of composite beads before (a) and after (b) the equivalent of 100 consecutive uses. Scale bar = 50μm. Images of beads formed in experiments 1,2,4,6 and 8 are displayed. The exact composition of these beads can be found in table 3.1.*

The beads were subjected to simulated reaction conditions (i.e. vigorous shaking in hexane) equivalent to more than 100 consecutive uses. Electron microscopy was employed to capture images of the beads surface before and after use (figure 3.25).

Set 1 were designed for maximum enzyme activity with a targeted mechanical strength value of 1.5 N-mm. SEM imaging shows that there is very little damage to the surface of

the bead, which still appears to have the characteristically smooth "skin" common to most of these composite preparations. The major component of this material was dextran (41 $\%^{w}/_{w}$), which has been shown to be the strongest of the materials.

Set 2 were optimised for maximum activity with no regard for the beads mechanical strength. As can be seen from the SEM images, the smooth skin of the unused beads has been completely stripped away during the reactions exposing the subsurface. Large cracks have also appeared, through which the "herring-bone" interior structure is visible. The major component of these beads was PEG (58 $\%^{w}/_{w}$), which has been shown to be the weakest of the materials studied.

Set 4 were optimised for maximum mechanical strength with no regard for catalytic activity. The results show that the apparently densely compacted bead surface was still very much intact after 100 uses. In this case PSSS (42 $\%^{w}/_{w}$) and Dextran (33 $\%^{w}/_{w}$) represented the majority of the composite. Both these materials were shown to be amongst the strongest tested.

Set 6 were targeted for a specific enzyme activity and a specific mechanical strength. Close inspection of the bead surface after use showed that although sections of the original skin were still present, large areas had been lost, uncovering the more porous interior structure of the bead. PVA was the largest single component present in this composite (40 $\%^{w}/_{w}$). Although this material was demonstrated to be strong, it also appeared to have elastic properties that may have helped to hold some of the surface together.

Set 8 was designed for maximum enzyme activity and maximum mechanical strength whilst keeping the level of the PVA component fixed (50 $\%^{w}/_{w}$). SEM imaging shows the

smooth skin of the bead to be relatively unaffected after 100 consecutive uses. Again, this may be due the presence of the "rubbery" PVA material.

The catalytic activity of the enzyme-loaded beads was also studied over several uses. The results (figure 3.26) show that in general, enzyme activity decreases over a number of cycles.

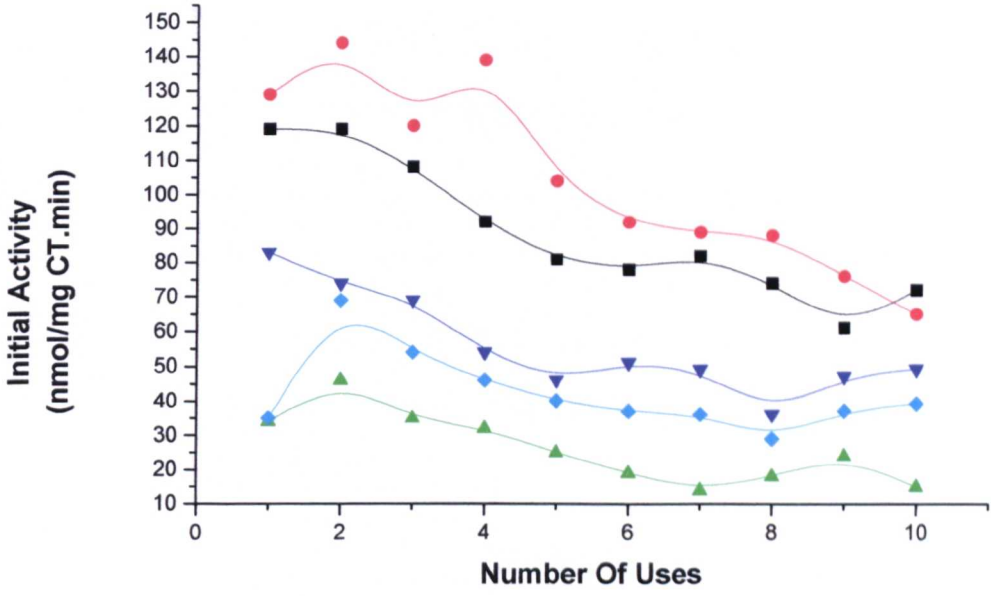



*Figure 3.26 Catalytic activity of composite beads over ten consecutive cycles of use. Set 1 = Black Squares, Set 2 = Red Circles, Set 4 = Green Triangles, Set 6 = Blue Triangles, Set 8 = Blue Diamonds. Catalytic activity was measured by following the transesterification reaction between ATEE and propan-1-ol in hexane.*

Overall, the highest enzyme activities were observed for those beads designed with maximum activity as one of the objectives (sets 1 and 2) whereas the lowest enzyme activities were found with the beads optimised for mechanical strength (set 4). It can be seen that some of the beads displayed an increase in activity on their second use (sets 2, 4

and 8). This delay in reaching maximum catalytic activity could either be attributed to the time taken for the substrate to penetrate into the interior of the bead, to the gradual removal of impurities that may have otherwise inhibited enzyme catalysis. The diffusion of substrates to the interior of the beads may not be completed during the first use of the beads if only the enzyme active sites positioned near the surface are accessible to the substrates. Once more of the active sites become available, the observed reaction rate increases. The only clear outlier observed in this study occurred at use 4 of set 2. As can be seen from the above SEM images (figure 3.25) this set of beads displayed the highest level of physical degradation. It is possible that as the surface of the bead disintegrates a temporary increase in surface area is produced. This may expose catalytic sites that are inaccessible when the bead is intact, increasing the reaction rate. However, these additional active sites would be lost when the reaction solvent is decanted and the beads washed prior to their subsequent reuse.

A plot of residual activity (figure 3.27) demonstrates that most of the beads examined lose their catalytic activity at approximately the same rate, with activity half-lives projected to be approximately twelve uses.

However it can be seen that set 8 still possesses approximately 100 % of its original activity even after 10 consecutive uses. This is due in part to the very large increase (~100 %) in activity upon its second use, after which the activity appears to fall off at a similar rate to the other sets of beads.

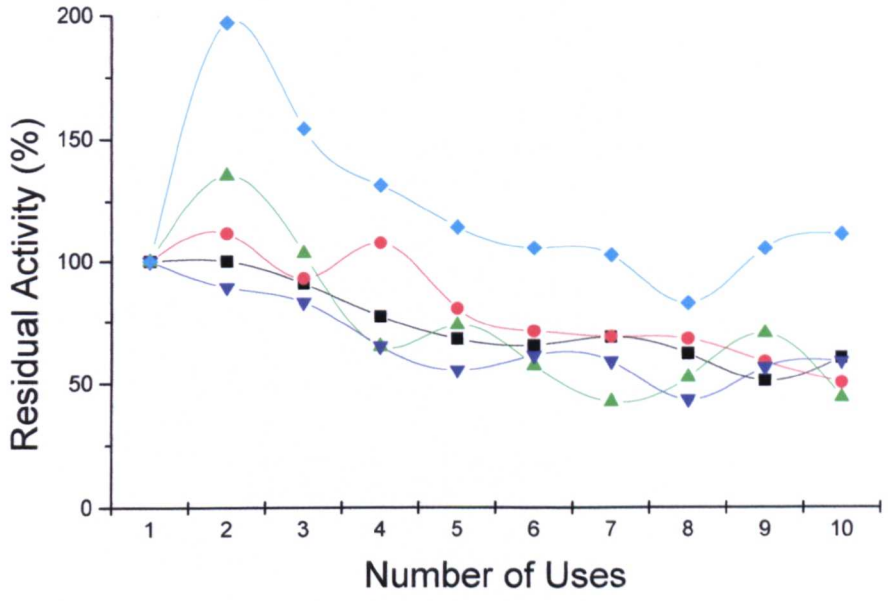



*Figure 3.27 Residual activity of composite beads over 100 consecutive uses. Set 1 = Black Squares, Set 2 = Red Circles, Set 4 = Green Triangles, Set 6 = Blue Triangles, Set 8 = Blue Diamonds. Catalytic activity was measured by following the transesterification reaction between ATEE and propan-1-ol in hexane.*

## 3.5 Conclusions

The use of design of experiment methodology has allowed for the efficient screening of enzyme containing composites of six different materials with the minimum amount of experimentation. The large quantity of information gained has been used to construct a model, representing this six-component system. The model has been shown to be reasonably accurate in predicting the experimental responses for any combination of the six materials. Materials have been synthesised that possess three times the mechanical strength and up to fifty times the enzyme activity of the original 100 % enzyme control. In addition to this, the best materials synthesised were shown to be reusable, retaining useful activity after more than 10 consecutive uses. The beads were also shown to be

physically strong enough to be used in more than one hundred cycles of the enzyme reaction.

In general it was found that the materials producing the strongest beads were also associated with the lowest catalytic activity. It is this negative correlation between responses that makes this system so suitable for examination with design of experiment methodology. Using traditional methods to evaluate the various combinations of these six components would have been laborious, time consuming and wasteful of materials. The "best" material produced (table 3.2) demonstrates the complexity of the composition, yet was attained relatively quickly once an accurate model had been constructed.

| Component | Wt. % |
|---|---|
| PEG | 19.9 |
| HS30 | 14.1 |
| PVA | 18.6 |
| SDS | 0 |
| DEX | 41.1 |
| PSS | 6.3 |

*Table 3.2 Composition of the "best" material synthesised during the study. This formulation produced a mechanically strong composite (strength value = 1.5 N-mm) with excellent enzyme activity (initial rate = 250 nmol/mg CT.min).*

In summary, design of experiment methodology (in conjunction with high through-put parallel synthesis techniques) represents a powerful tool in the analysis of multivariate systems.

### 3.6 Future work

Although the design of experiment methodology has successfully been shown to optimise the six-component system studied in this work, the best responses will always be limited by the components themselves. The study could be expanded to include other materials, which may enable higher responses to be achieved. SDS could be eliminated as a component, as it adds little in terms of mechanical strength or enzyme activity to the optimised beads. The inclusion of cerium oxide nanoparticles could prove interesting, as this material possessed some of the best properties when examined in the initial screens. Alternatively new components such as cyclodextrin could be included in the screening experiments, as they have been reported to impart increased activity to preparations, when co-lyophilised with various enzymes.

In addition to this expansion of materials, it could be advantageous to produce covalently crosslinked composites. Currently, the beads produced in this study are unsuitable for enzymatic catalysis in aqueous media, as they are soluble. Covalent crosslinking could produce insoluble beads as well as enhancing the stability of the immobilised enzyme.[34-37] There are two different approaches to achieving this covalent immobilisation; the beads can be exposed to a crosslinking reagent after their initial synthesis, or the crosslinking reaction can be performed during the bead synthesis in a one step reaction.

The use of glutaraldehyde vapour is a traditional method of crosslinking enzyme preparations.[38] However even rapid exposure of the beads to high water content environments may result in some loss of the support's porous morphology. Alternatively, the inclusion of a reactive, crosslinking compound in the initial list of support materials may allow from the covalent immobilization of the enzyme during the bead synthesis.

Materials similar to those already studied, such as dextran polyaldehyde may be the most suitable for addition to the original design of experiment screen.[39]

## 3.7 References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## 3.8 Appendix 1

| Exp No | Exp Name | Run Order | Incl/Excl | PEG | HS30 | PVA | SDS | Dextran | PSSS | Activity | Strength |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | N1 | 3 | Incl | 1 | 0 | 0 | 0 | 0 | 0 | 152.4 | 0.174 |
| 2 | N2 | 17 | Incl | 0 | 1 | 0 | 0 | 0 | 0 | 193.8 | 0.062 |
| 3 | N3 | 51 | Incl | 0 | 0 | 1 | 0 | 0 | 0 | 67.3 | 1.899 |
| 4 | N4 | 47 | Incl | 0 | 0 | 0 | 1 | 0 | 0 | 23.2 | 0.521 |
| 5 | N5 | 5 | Incl | 0 | 0 | 0 | 0 | 1 | 0 | 26.5 | 2.187 |
| 6 | N6 | 77 | Incl | 0 | 0 | 0 | 0 | 0 | 1 | 72 | 2.704 |
| 7 | N7 | 15 | Incl | 0.5 | 0.5 | 0 | 0 | 0 | 0 | 167.3 | 0.328 |
| 8 | N8 | 61 | Incl | 0.5 | 0 | 0.5 | 0 | 0 | 0 | 187.1 | 1.084 |
| 9 | N9 | 38 | Incl | 0.5 | 0 | 0 | 0.5 | 0 | 0 | 135.2 | 0.192 |
| 10 | N10 | 54 | Incl | 0.5 | 0 | 0 | 0 | 0.5 | 0 | 130.3 | 0.79 |
| 11 | N11 | 20 | Incl | 0.5 | 0 | 0 | 0 | 0 | 0.5 | 22.6 | 0.5 |
| 12 | N12 | 41 | Incl | 0 | 0.5 | 0.5 | 0 | 0 | 0 | 136.3 | 1.73 |
| 13 | N13 | 39 | Incl | 0 | 0.5 | 0 | 0.5 | 0 | 0 | 21.5 | 0.596 |
| 14 | N14 | 55 | Incl | 0 | 0.5 | 0 | 0 | 0.5 | 0 | 17.1 | 2.742 |
| 15 | N15 | 29 | Incl | 0 | 0.5 | 0 | 0 | 0 | 0.5 | 39.7 | 1.718 |
| 16 | N16 | 11 | Incl | 0 | 0 | 0.5 | 0.5 | 0 | 0 | 179.4 | 0.929 |
| 17 | N17 | 10 | Incl | 0 | 0 | 0.5 | 0 | 0.5 | 0 | 58.5 | 2.454 |
| 18 | N18 | 65 | Incl | 0 | 0 | 0.5 | 0 | 0 | 0.5 | 61.3 | 2.601 |
| 19 | N19 | 33 | Incl | 0 | 0 | 0 | 0.5 | 0.5 | 0 | 21 | 1.456 |
| 20 | N20 | 48 | Incl | 0 | 0 | 0 | 0.5 | 0 | 0.5 | 94.9 | 1.789 |
| 21 | N21 | 58 | Incl | 0 | 0 | 0 | 0 | 0.5 | 0.5 | 29.8 | 2.479 |
| 22 | N22 | 14 | Incl | 0.666667 | 0.333333 | 0 | 0 | 0 | 0 | 216.9 | 0.332 |
| 23 | N23 | 30 | Incl | 0.333333 | 0.666667 | 0 | 0 | 0 | 0 | 128.1 | 0.414 |
| 24 | N24 | 28 | Incl | 0.666667 | 0 | 0.333333 | 0 | 0 | 0 | 122.5 | 0.441 |
| 25 | N25 | 4 | Incl | 0.333333 | 0 | 0.666667 | 0 | 0 | 0 | 107.1 | 1.698 |
| 26 | N26 | 64 | Incl | 0.666667 | 0 | 0 | 0.333333 | 0 | 0 | 232.4 | 0.276 |
| 27 | N27 | 9 | Incl | 0.333333 | 0 | 0 | 0.666667 | 0 | 0 | 175 | 0.211 |
| 28 | N28 | 34 | Incl | 0.666667 | 0 | 0 | 0 | 0.333333 | 0 | 135.8 | 0.146 |
| 29 | N29 | 63 | Incl | 0.333333 | 0 | 0 | 0 | 0.666667 | 0 | 99.4 | 2.078 |
| 30 | N30 | 53 | Incl | 0.666667 | 0 | 0 | 0 | 0 | 0.333333 | 132.5 | 0.498 |
| 31 | N31 | 49 | Incl | 0.333333 | 0 | 0 | 0 | 0 | 0.666667 | 5 | 1 |
| 32 | N32 | 24 | Incl | 0 | 0.666667 | 0.333333 | 0 | 0 | 0 | 36.4 | 2.475 |
| 33 | N33 | 76 | Incl | 0 | 0.333333 | 0.666667 | 0 | 0 | 0 | 102.7 | 1.603 |
| 34 | N34 | 46 | Incl | 0 | 0.666667 | 0 | 0.333333 | 0 | 0 | 10.5 | 0.458 |
| 35 | N35 | 59 | Incl | 0 | 0.333333 | 0 | 0.666667 | 0 | 0 | 21.5 | 0.616 |
| 36 | N36 | 75 | Incl | 0 | 0.666667 | 0 | 0 | 0.333333 | 0 | 17.7 | 2.159 |
| 37 | N37 | 23 | Incl | 0 | 0.333333 | 0 | 0 | 0.666667 | 0 | 12.7 | 2.825 |
| 38 | N38 | 1 | Incl | 0 | 0.666667 | 0 | 0 | 0 | 0.333333 | 19.3 | 1.168 |
| 39 | N39 | 62 | Incl | 0 | 0.333333 | 0 | 0 | 0 | 0.666667 | 50.8 | 2.431 |
| 40 | N40 | 56 | Incl | 0 | 0 | 0.666667 | 0.333333 | 0 | 0 | 113.2 | 1.191 |
| 41 | N41 | 16 | Incl | 0 | 0 | 0.333333 | 0.666667 | 0 | 0 | 168.4 | 0.871 |
| 42 | N42 | 2 | Incl | 0 | 0 | 0.666667 | 0 | 0.333333 | 0 | 33.1 | 2.234 |
| 43 | N43 | 50 | Incl | 0 | 0 | 0.333333 | 0 | 0.666667 | 0 | 75.6 | 2.33 |
| 44 | N44 | 25 | Incl | 0 | 0 | 0.666667 | 0 | 0 | 0.333333 | 78.9 | 2.4 |
| 45 | N45 | 35 | Incl | 0 | 0 | 0.333333 | 0 | 0 | 0.666667 | 28.7 | 2.132 |
| 46 | N46 | 40 | Incl | 0 | 0 | 0 | 0.666667 | 0.333333 | 0 | 19.3 | 1.21 |
| 47 | N47 | 70 | Incl | 0 | 0 | 0 | 0.333333 | 0.666667 | 0 | 27 | 1.592 |
| 48 | N48 | 13 | Incl | 0 | 0 | 0 | 0.666667 | 0 | 0.333333 | 54.6 | 1.516 |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 49 | N49 | 8 | Incl | 0 | 0 | 0 | 0.333333 | 0 | 0.666667 | 47.5 | 1.967 |
| 50 | N50 | 19 | Incl | 0 | 0 | 0 | 0 | 0.666667 | 0.333333 | 43.6 | 2.393 |
| 51 | N51 | 67 | Incl | 0 | 0 | 0 | 0 | 0.333333 | 0.666667 | 35.9 | 2.796 |
| 52 | N52 | 12 | Incl | 0.333333 | 0.333333 | 0.333333 | 0 | 0 | 0 | 283.2 | 0.763 |
| 53 | N53 | 36 | Incl | 0.333333 | 0.333333 | 0 | 0.333333 | 0 | 0 | 97.2 | 0.383 |
| 54 | N54 | 44 | Incl | 0.333333 | 0.333333 | 0 | 0 | 0.333333 | 0 | 175.5 | 0.775 |
| 55 | N55 | 7 | Incl | 0.333333 | 0.333333 | 0 | 0 | 0 | 0.333333 | 34.8 | 0.821 |
| 56 | N56 | 42 | Incl | 0.333333 | 0 | 0.333333 | 0.333333 | 0 | 0 | 29.1 | 0.763 |
| 57 | N57 | 32 | Incl | 0.333333 | 0 | 0.333333 | 0 | 0.333333 | 0 | 123.1 | 1.068 |
| 58 | N58 | 66 | Incl | 0.333333 | 0 | 0.333333 | 0 | 0 | 0.333333 | 150.1 | 2.223 |
| 59 | N59 | 73 | Incl | 0.333333 | 0 | 0 | 0.333333 | 0.333333 | 0 | 212 | 0.535 |
| 60 | N60 | 37 | Incl | 0.333333 | 0 | 0 | 0.333333 | 0 | 0.333333 | 78.4 | 0.226 |
| 61 | N61 | 68 | Incl | 0.333333 | 0 | 0 | 0 | 0.333333 | 0.333333 | 126.4 | 0.704 |
| 62 | N62 | 22 | Incl | 0 | 0.333333 | 0.333333 | 0.333333 | 0 | 0 | 117 | 0.942 |
| 63 | N63 | 72 | Incl | 0 | 0.333333 | 0.333333 | 0 | 0.333333 | 0 | 46.4 | 2.527 |
| 64 | N64 | 69 | Incl | 0 | 0.333333 | 0.333333 | 0 | 0 | 0.333333 | 141.9 | 2.197 |
| 65 | N65 | 74 | Incl | 0 | 0.333333 | 0 | 0.333333 | 0.333333 | 0 | 38.1 | 1.533 |
| 66 | N66 | 18 | Incl | 0 | 0.333333 | 0 | 0.333333 | 0 | 0.333333 | 84.5 | 1.662 |
| 67 | N67 | 26 | Incl | 0 | 0.333333 | 0 | 0 | 0.333333 | 0.333333 | 128.6 | 2.972 |
| 68 | N68 | 43 | Incl | 0 | 0 | 0.333333 | 0.333333 | 0.333333 | 0 | 112.1 | 2.151 |
| 69 | N69 | 31 | Incl | 0 | 0 | 0.333333 | 0.333333 | 0 | 0.333333 | 102.1 | 1.629 |
| 70 | N70 | 57 | Incl | 0 | 0 | 0.333333 | 0 | 0.333333 | 0.333333 | 55.8 | 2.26 |
| 71 | N71 | 52 | Incl | 0 | 0 | 0 | 0.333333 | 0.333333 | 0.333333 | 56.9 | 2.416 |
| 72 | N72 | 78 | Incl | 0.583333 | 0.0833333 | 0.0833333 | 0.0833333 | 0.0833333 | 0.0833333 | 205.9 | 0.283 |
| 73 | N73 | 27 | Incl | 0.0833333 | 0.583333 | 0.0833333 | 0.0833333 | 0.0833333 | 0.0833333 | 78.8 | 1.096 |
| 74 | N74 | 79 | Incl | 0.0833333 | 0.0833333 | 0.583333 | 0.0833333 | 0.0833333 | 0.0833333 | 104.3 | 1.919 |
| 75 | N75 | 45 | Incl | 0.0833333 | 0.0833333 | 0.0833333 | 0.583333 | 0.0833333 | 0.0833333 | 95.5 | 0.85 |
| 76 | N76 | 21 | Incl | 0.0833333 | 0.0833333 | 0.0833333 | 0.0833333 | 0.583333 | 0.0833333 | 104.9 | 2.631 |
| 77 | N77 | 6 | Incl | 0.0833333 | 0.0833333 | 0.0833333 | 0.0833333 | 0.0833333 | 0.583333 | 29 | 2.196 |
| 78 | N78 | 80 | Incl | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 113.2 | 0.928 |
| 79 | N79 | 60 | Incl | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 71.8 | 0.858 |
| 80 | N80 | 71 | Incl | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 0.166667 | 68.4 | 0.85 |

*Appendix 1. Worksheet used to perform screening experiment. Data used to construct the model is also included. Strength is measured in units of N-mm. Catalytic activity is measured in units of nmol/mg CT.min.*

# Chapter 4

## Protein-Containing Porous Polymers from High Internal Phase $CO_2$-in-Water (C/W) Emulsion Templates

### 4.1 Introduction

The use of high internal phase emulsions for the preparation of rigid polymer supports offers a novel method for introducing a very high degree of porosity and interconnectivity to the products. This porosity and interconnectivity allows for excellent mass transfer of small molecules throughout the entire internal structure of the polymer. It is this property that makes the use of polymers formed from high internal phase emulsions (polyHIPEs) ideal for the immobilisation of biomolecules.

Several studies have shown that the morphology of the support material strongly influences the catalytic activity of the immobilised enzyme.[1, 2] Bosley *et al* have shown that a support matrix with an average pore size of less than 100 nm can reduce the catalytic activity of an immobilised lipase through the mass transfer limitation of small substrate molecules to the enzymes active sites.[1]

High internal phase emulsions (HIPEs) were originally defined as emulsions containing an internal phase in excess of 70 % by volume.[3] However it would be more accurate to define them as having an internal phase volume of greater than 74.05 %. This represents the maximum volume occupied by close packed uniform spheres.[4] It was thought that

exceeding this close packed arrangement would be impossible,[5] however numerous examples of polyHIPEs have now been reported in the literature.[6-12]

The first step in this type of polymerisation reaction is the formation of a stable high internal phase emulsion. This requires the presence of two immiscible liquids and at least one surfactant. One of the immiscible liquids is usually water and the other is usually a hydrophobic organic solvent.[5] Although it is possible for both phases of the emulsion to be non-aqueous, it is very rare. Some work has been conducted in order to find a method for preparing highly concentrated emulsions of jet fuel in formamide, for use as safety fuels.[13, 14] Generally the emulsion will be formed with the water acting as the internal phase, i.e. a water-in-oil (W/O) emulsion.[11, 15] It is also possible to form an emulsion with an organic internal phase, i.e. an oil-in-water (O/W) emulsion with careful selection of surfactants.[6-10] The choice of surfactant is crucial to the structure and stability of the emulsion formed. Without the correct surfactant, emulsions can spontaneously invert from water-in-oil to oil-in-water, when the ratio of internal phase to external phase reaches a critical point.[5]

Most methods of forming high internal phase emulsions involve the slow and gradual addition of the internal phase to a stirred solution of surfactant in the external phase. However, it has also been shown[16] that emulsions with very high internal phase volumes can be created by centrifuging an emulsion formed from a lower volume of the internal phase component. The force acting on the emulsion during centrifugation drives the internal phase droplets together, compressing them into a more compact arrangement that exceeds that of close packed uniform spheres. The excess external phase present on top of the high internal phase emulsion is removed. This process is known as "creaming".

There are other methods for "creaming" emulsions to form HIPEs. The forces exerted on the internal phase droplets during centrifugation can be replaced by simple phase separation under gravity.[17] This technique is simple but more time consuming than the centrifuge method. An enrichment in the internal phase of an emulsion can also be achieved via osmotic pressure.[18] An oil-in-water emulsion confined in a dialysis membrane and immersed in a large mass of water containing a dissolved polymer, will gradually become more concentrated as the water phase of the emulsion diffuses through the membrane to dilute the polymeric solution.

The close packing limit for uniform spheres is 74.05 %.[4] To form emulsions with internal phase volumes greater than this limit, the dispersed droplets cannot be uniform spheres. To reach very high internal phase ratios the dispersed phase must either consist of non-uniform droplets or polyhedral mono-disperse droplets.

It has been calculated that the formation of mono-disperse, polyhedral internal phase droplets should be more favourable than an equivalent polydisperse arrangement.[19] As such the geometric shape of the internal phase droplets can be derived.[20] At internal phase ratios less than 74.05 % uniform spheres will be formed. As the internal phase volume is increased, these spheres will gradually deform into polyhedrons. At internal phase volumes of between 74 % and 94 % of the total emulsion volume, rhomboidal dodecahedrons (RDH) droplet shapes are favoured. At internal phase concentrations greater than 94 % the droplet deform again, into truncated octahedrons, or tetrakaidecahedrons (TKDH).

These theoretical calculations were later confirmed experimentally when cured high internal phase emulsions formed from water in a styrene based resin were imaged with

SEM.[21] The images showed that all the emulsions internal phase droplets were indeed relatively uniform in size. Evidence for the droplets deformation into polyhedra can also be seen in the shape of the polymerised cells. The deformation of internal phase droplets into polyhedra is very similar to the process observed during the formation of foams. In these systems where the internal phase is a gas, droplets readily deform into large, visible polyhedra, enabling very high internal phase ratios (<99 $\%^{v}/_{v}$) to be formed.

One physical property of HIPEs that arises from their unusual structure, is their viscosity. The viscosity of HIPEs are usually very high compared to lower internal phase emulsions. This is due to their uniform polyhedral structure. In order for the emulsion to "flow" the internal phase droplets must be able to pass over one another easily. For a low internal phase emulsion (<74 % internal volume) where the mono-disperse droplets are spheres, this motion is relatively easy to achieve. However, the polyhedral droplets that make up high internal phase emulsions (>74 $\%^{v}/_{v}$ internal phase) occupy a much more rigid, interlocking pattern that does not allow for the necessary deformation of the droplets required for them to flow past each other. This results in their high observed viscosities.[22] It has also been shown that the size of these polyhedral droplets is also important for the emulsions resulting viscosity.[23] Emulsions formed with smaller droplets become increasingly viscous. This is because the smaller droplets must be deformed by a proportionally greater amount in order for them to flow past one another, when compared to HIPEs formed with larger droplets. This can be studied via the formation of HIPEs with differing amounts of agitation. The more stirring that accompanies the formation of an emulsion, the smaller the resulting internal phase droplet size. This effect can be

examined with freeze-fracture SEM imaging of the emulsion, which allows the droplet size to be quantified.

Another important requirement necessary for a HIPE to be used as a polymerisation medium is stability. The structure of the emulsion must remain stable long enough for the polymerisation reaction to be conducted. There are a number of factors that influence the stability of high internal phase emulsions. These include the type of surfactant, the surfactant concentration, the type of continuous phase, the emulsion temperature and the presence of salts in the aqueous phase.

It has been postulated that for a surfactant to be a good emulsifier it must possess three properties.[24] Firstly, it needs to be able to lower the interfacial tension between the water and oil phases. Secondly, it must form a rigid interfacial film between the two phases and thirdly, must show rapid adsorption at the interface.[5] Many studies have been conducted using non-ionic surfactants to stabilise oil-in-water emulsions. These surfactants have been shown to stabilise increasingly oil rich O/W emulsions, as their concentration is increased. This effect is most likely due to the decreasing interfacial tension that accompanies the increased surfactant concentration.[25] Emulsions stabilised by non-ionic surfactants have also been shown to be temperature sensitive.[26] Some of these systems exhibit a phase inversion temperature (PIT). At temperatures below this point the non-ionic surfactants are water-soluble and an oil-in-water emulsion is formed. However, at temperatures above the PIT the surfactant becomes oil soluble and the system spontaneously inverts to form a water-in-oil emulsion. Generally high internal phase emulsions are destabilised by increases in the systems temperature.[27, 28] It is thought that this is due to an increase in the coalescence of the dispersed phase droplets with

increasing thermal energy. Increasing internal phase droplet size facilitates the flow of one droplet past another, reducing the emulsions viscosity and decreasing its stability.[29]

The stability of the high internal phase emulsion is also affected by the nature of the two immiscible phases. It was shown that the more stable emulsions are formed when the difference in hydrophobicity between the two solvents is greatest, i.e. one phase should be very hydrophobic and the other should be very hydrophilic.[30] This leads to the greatest interfacial tension between the two phases in the absence of surfactant, and so results in the most stable emulsions.

It has also been demonstrated that the addition of salts to the aqueous phase of a water-in-oil emulsion can enhance its stability.[31, 32] It is thought that the addition of salts decreases the cloud point of the non-ionic surfactant in the aqueous phase. Salts that produce a greater "salting-out" effect were shown to offer greater emulsion stability.[33] Electron spin resonance experiments revealed that the interactions between surfactant molecules present at the interface between the two fluids were greatly increased, due to their dehydration by the addition of salts. The interfacial surfactant molecules became more ordered as the amount of salt added was increased.

If a HIPE is prepared containing one or more monomers dissolved in, or acting as, the internal phase, then the structure of the emulsion can be "locked-in" by polymerising the system. This method is known as templating, and polymers formed using this process are termed "polyHIPEs". The first synthesis of a polyHIPE material was carried out in 1982.[34] This involved the water-in-oil polymerisation of an emulsion formed between water, non-ionic surfactant and a mixture of styrene and divinyl benzene. Two initiators were employed, potassium persulfate dissolved in the aqueous phase and 2,2'-azo-bis-

isobutyronitrile (AIBN) dissolved in the oil phase. Polymerisation was then initiated thermal by heating the emulsion to 50°C for 24 hours. This reaction produced a highly porous monolithic polymer, with the aqueous dispersed phase remaining trapped within the rigid, microporous structure. The water was then displaced through exhaustive soxhlet extraction with a lower alcohol, which in turn was removed under vacuum.

Further research has indicated that the concentration of surfactant used to form the emulsions of water and monomer is critical to the polymer structure produced.[35, 36] A minimum of 4 % surfactant (relative to the total volume of the oil phase) was necessary for the formation of a stable polymer. However, increasing the surfactant concentration to levels greater than 80 % produced "closed" polymers with little or no interconnectivity between cells. The best surfactant concentrations for this polymerisation system were found to be between 20 % and 50 %. Another study also found that the cell structure of the polymer produced was heavily influenced by the concentration of the surfactant.[36] Closed cell structures were produced at concentrations of <7 %. These closed cell structures possessed a high density, due to the internal aqueous phase being trapped within the polymer structure. However, at higher surfactant concentrations (>7 %) the cell structure of the polymer produced was completely interconnected. This allowed for the comparatively simple removal of the aqueous phase. The results of the same study also indicated that the concentration of the surfactant was even more important in determining the resulting polymer structure than was the ratio of internal to external phases used to form the emulsion.

It is reasoned that the changes in surfactant concentration cause a thinning of the cell walls of the continuous phase that separates the internal phase droplets. At some point the

concentration of the surfactant may drop to a level where the cell walls become so thin that holes begin to appear connecting adjacent cells. It is these holes that provide the materials interconnectivity. However, the cell walls must remain intact for at least part of the polymerisation reaction, otherwise the emulsion will destabilise. It seems likely that the slight shrinkage during polymerisation observed with many vinyl monomers may cause holes to appear in the thinnest sections of cell walls.[5] Other reaction parameters such as the nature of the initiator used also have an effect of the resulting polymers formed. It was found that non-electrolyte initiators such as AIBN generally produced polymers with larger cell sizes than potassium persulfate initiated polymers.[35]

Although polymerisation of high internal phase emulsions does produce porous polymers with a very high degree of interconnectivity, removal of the internal phase can still be difficult. The porogen can be removed by either extensive vacuum drying or successive displacement with progressively more volatile solvents, which are then easier to remove under vacuum. However these processes are time consuming, energy intensive and involve the use of large quantities of organic solvent. A recent study has shown that these problems can be overcome by the use of supercritical fluids as the internal phase, during the formation of the HIPE.[37] Carbon dioxide-in-water emulsions can be stabilised by inexpensive hydrocarbon surfactants, resulting in a polymerisation medium that contains no organic solvents at all. Depressurisation of the reaction vessel causes the liquid phase $CO_2$ to revert to the gaseous phase, enabling the facile remove of the porogen from the porous polymer structure. The small quantity of continuous aqueous phase remaining in the open polymer structure is then easily removed under vacuum.

The aim of this study was to employ concentrated carbon dioxide-in-water emulsions for the environmentally sound synthesis of highly porous enzyme containing polymers. Ideally these polymers should possess a high level of interconnectivity facilitating the mass transfer of substrates and products to and from the enzymes active sites, as well as allowing for the used of these enzyme-containing materials in continuous flow applications. The effects of various synthetic parameters on polymer morphology will be examined in order to optimise the corresponding enzyme activity of the materials.

## 4.2 Materials

Acrylamide (99 +%, Aldrich), *N,N'*-methylene-bis-acrylamide (99 %, Aldrich), Tween-40 (Aldrich), *N,N,N,N*-tetramethylethylenediamine (TMEDA, 99.5 +%, Aldrich), potassium persulfate (99 +%, Aldrich), poly(vinyl alcohol) ($M_w$ = 9,000-10,000 $gmol^{-1}$, 80 % hydrolyzed, Aldrich), α-chymotrypsin (Type II, Sigma), *N*-acetyl-L-tyrosine ethyl ester (ATEE, Sigma grade, Sigma), *n*-propanol (99.7 %, Aldrich), carbon dioxide (99.9995 %, Messer), water (HPLC, BDH), acetonitrile (HPLC, BDH). All other solvents and chemicals were of reagent grade and were used as supplied, without further purification.

## 4.3 Experimental

### 4.3.1 $CO_2$-in-water (C/W) emulsion templating by free-radical polymerisation

In a typical polymerisation, a 10 ml view cell reactor (Baskerville, UK)[1,2] was charged with an aqueous solution of monomers (2.0 ml 40 $\%^{w}/_{v}$, acrylamide and MBAM crosslinker), initiator ($K_2S_2O_8$, 2 $\%^{w}/_{v}$ based on monomer), surfactant (Tween-40, 10 $\%^{v}/_{v}$ based on aqueous phase), cosurfactant (PVA, 2 $\%^{w}/_{w}$ based on monomer content) and enzyme (100 µl of a 20 mM $KH_2PO_4$ pH 7.8 buffer solution containing α-chymotrypsin, 100 mg/ml), before purging with a slow flow of $CO_2$ for 5 minutes in order to expel oxygen from the vessel. For the thermally initiated polymerisations, the reactor was pressurised with liquid $CO_2$ (22 °C, 100 ±5 bar, unless otherwise stated) and stirred using a magnetic stir bar for 30 minutes to form a milky-white C/W emulsion. The temperature was then raised to 60 °C in 10 °C steps, thus allowing careful monitoring of the pressure increase and preventing autoacceleration of the reaction and resultant over-pressurisation. The reaction was left overnight at 60 °C, cooled to room temperature, and then the $CO_2$ vented. The reactor was dismantled and the product removed as a solid continuous monolith. All thermally initiated samples were dried for at least 24 hours in air and then under vacuum at 60 °C overnight. For the samples that were prepared by redox initiation, a similar procedure was followed but a small headspace was left in the reaction vessel when the reactor was first charged with liquid $CO_2$. The reaction mixture was stirred until a stable milky white emulsion was observed (usually less than 60 seconds), whereupon TMEDA (50 µl) was injected into the reactor by flushing $CO_2$ through a stainless steel

addition tube containing the redox coinitiator (TMEDA is freely soluble in $CO_2$). The reactor was then filled with $CO_2$ to the desired pressure (usually 100 ±5 bar) and stirred continuously for 60 minutes. Completion of the polymerisation reaction was accompanied by a characteristic jump in both temperature and pressure (from 22 °C, 100 ±5 bar to 45 °C, 250 ±10 bar) as a result of the exothermic nature of the polymerisation. The $CO_2$ was vented and the product removed from the reactor, as before. The resulting monolith was frozen in liquid nitrogen and freeze dried for >48 hours to remove the residual aqueous phase.

### 4.3.2 Oil-in-water (O/W) emulsion templating by free radical polymerisation

Typically, a boiling tube was charged with an aqueous solution of monomers (40 $\%^{w}/_{v}$, acrylamide and MBAM crosslinker), initiator ($K_2S_2O_8$, 2 $\%^{w}/_{v}$ based on monomer), surfactant (Tween-40, 10 $\%^{v}/_{v}$ based on aqueous phase), cosurfactant (PVA, 2 $\%^{w}/_{w}$ based on monomer) and enzyme (100 µl of a 20 mM $KH_2PO_4$ pH 7.8 buffer solution containing α-chymotrypsin, 100 mg/ml). The reaction mixture was stirred at 100 rpm with an overhead paddle stirrer. Cyclohexane (8 ml) was added slowly (approx. 1 ml/min) at 22 °C and the formation of a milky-white oil-in-water (O/W) emulsion was observed. Stirring was continued for 30 minutes prior to the addition of neat TMEDA (50 µl). Polymerisation was completed within 60 minutes, at which time the polymer product was removed from the reaction vessel as a solid monolith. The polymer was frozen in liquid nitrogen and freeze dried for >72 hours to remove both the aqueous and the cyclohexane phase.

### 4.3.3 Non-emulsion templated polymers

In a typical polymerisation, a boiling tube was charged with an aqueous solution of monomers (40 $\%^{w}/_{w}$, acrylamide and MBAM crosslinker), initiator ($K_2S_2O_8$, 2 $\%^{w}/_{v}$ based on monomer), surfactant (Tween-40, 10 $\%^{v}/_{v}$ based on aqueous phase), cosurfactant (PVA, 2 $\%^{w}/_{w}$ based on monomer) and enzyme (100 μl of a 20 mM $KH_2PO_4$ pH 7.8 buffer solution containing α-chymotrypsin, 100 mg/ml). The resulting homogeneous solution was magnetically stirred during the addition of neat TMEDA (50 μl). Stirring was continued until completion of the polymerisation, usually 30 minutes. The product was removed from the reactor as a solid monolith which was frozen in liquid nitrogen and freeze dried for >48 hours to remove the aqueous phase.

### 4.3.4 Polymer characterisation

The surface area of the α-chymotrypsin-loaded polymers was characterised using a Micrometrics ASAP 2010 $N_2$-sorption analyser (BET method). Pore volumes were measured by mercury intrusion porosimetry using a Micrometrics AutoPore IV. Absolute densities were obtained using a Micrometrics AccuPyc 1330 instrument. Scanning Electron Microscope (SEM) images were captured using a Jeol 840 instrument. The samples were sputter-coated with approximately 2 nm of gold (Polaron E5000 coating unit) prior to analysis and mounted on 12 mm carbon coated aluminium stubs (Agar Scientific).

### 4.3.5 Enzyme leaching

A suspension of 200 mg of each enzyme-containing polymer (ground to a particle size of 90-100 μm) in 10 ml water was shaken for 60 minutes. Aliquots (0.5 ml) of these suspensions were passed through a 0.22 μm filter and treated with Bradfords reagent.[25,26] Bradfords stock reagent was made by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95 % ethanol. To this 100 ml of 85 %$^{w}/_{v}$ orthophosphoric acid was added. The mixture was then diluted to 1000 ml with distilled water. UV absorbance analysis at 595 nm was used to calculate the protein content by comparison with a calibration curve constructed from solutions of known α-chymotrypsin concentration.

### 4.3.6 Enzyme activity assay

The catalytic activity of the immobilised α-chymotrypsin was determined by following the transesterification assay described in section 2.2.2. Prior to the assay all polymers were ground to fine particles and sieved. Only particle sizes between 90 and 100 μm were assayed for catalytic activity.

### 4.3.7 Enzyme active site determination

Quantitative determination of the number of active sites that remain available after immobilisation in the polymer matrix was achieved by titration with a fluorogenic reagent.[38] Samples of native α-chymotrypsin were dissolved in 2.85 ml of buffer

(100 mM Sodium tetraborate decahydrate (Borax) adjusted to pH 7.5) and allowed to stand for five minutes prior to the addition of 150 μl of a 0.2 mM aqueous solution of the fluorogenic reagent MUTMAC. These solutions were transferred to polystyrene fluorescence cuvettes and illuminated at a wavelength of 360 nm. The maximum fluorescence intensity over the 430 to 480 nm range was recorded. The quantity of α-chymotrypsin active sites was calculated by comparison to a calibration curve constructed from the fluorescence intensities of aqueous 4-methylumbelliferone solutions with concentrations between 0.2 mM and 0.002 mM. The instrument employed for these measurements was a Varian Cary-eclipse fluorescence spectrophotometer. Samples of immobilised α-chymotrypsin were ground to a fine powder and sieved to separate the 90 to 100 μm particle size range. These particles were suspended in 2.85 ml of Borax buffer for ten minutes prior to the addition of 150 μl of 0.2 mM MUTMAC solution. The suspensions were rigorously shaken and then allowed to stand for five minutes. The fluorescence intensity of each sample was measured after the heterogeneous polymer suspension had settled to the bottom of the cuvettes.

### 4.3.8 Synthesis of polymer-enzyme grafts

Two sets of experiments were conducted in order to investigate the interaction of α-chymotrypsin with various growing polymers under free radical conditions. Firstly a set of five non-crosslinked enzyme-contained polymers was synthesised. Typically, a solution of 0.5 g sodium acrylate in 5.0 ml water was prepared. Various quantities of α-chymotrypsin were then added to these solutions (table 4.1) in order to alter the molar

ratio of enzyme to polymer. The actual mass of α-chymotrypsin added to the reaction is displayed along with the mass calculated to achieve the desired molar ratio. Potassium persulfate (5 mg) was added to each reaction solution and polymerisation was initiated thermally by heating the reaction mixtures to 60 °C for 18 hours. All five samples were then freeze dried (Lyolab 3000, Heto) >72 hours to remove the reaction solvent. Each polymer was then analysed by GPC, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electrospray mass spectrometry (sections 4.2.9, 4.2.10 and 4.2.11).

| Polymer | Na Acrylate | Molar ratio | CT calc | CT actual |
|---|---|---|---|---|
| 1 | 0.5g | 10000:1 | 0.0130g | 0.0141g |
| 2 | 0.5g | 1000:1 | 0.1295g | 0.1160g |
| 3 | 0.5g | 500:1 | 0.2585g | 0.2529g |
| 4 | 0.5g | 200:1 | 0.6463g | 0.5783g |
| 5 | 0.5g | control | 0.0000g | 0.0000g |

*Table 4.1 Calculated and actual quantities of α-chymotrypsin used in the synthesis of various enzyme-containing sodium acrylate polymerisations.*

A second set of three non-crosslinked enzyme-containing polymers was also synthesised. Solutions of acrylamide (0.5 g) in 10 ml water were prepared. Various quantities of α-chymotrypsin (0.0 g, 0.1 g and 0.5 g) were added to these solutions along with 50 mg of potassium persulfate. Polymerisation was initiated by the addition of 100 µl of TMEDA to each reaction vessel. The solutions were stirred at room temperature (22 °C) for 18 hours. At this point the reaction solutions were dialysed for two hours against three successive volumes (500 ml) of water. All three samples were then freeze-dried (Lyolab 3000, Heto) for >72 hours to remove the reaction solvent. These polymers were then

analysed with SDS-PAGE (section 4.2.9) and electrospray mass spectrometry (see section 4.2.10).

### 4.3.9 SDS-PAGE protein separation and analysis procedures

Separation of the various components of the commercially supplied α-chymotrypsin was achieved via sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE). Two separate types of gel with differing separation capabilities were prepared according to the composition described in table 4.2, using Biorad kits.

| | 15% SDS-Polyacrylamide Gel (ml) | 20% SDS-Polyacrylamide Gel (ml) | 5% SDS-Polyacrylamide Stacking Gel (ml) |
|---|---|---|---|
| 30 wt.% Acrylamide soln. | 7.5 | 10 | 2.5 |
| 1 wt.% Bis-Acrylamide soln. | 1.3 | 0.975 | 3.9 |
| 1.5 M Tris-HCl, pH8.8 | 3.75 | 3.75 | - |
| 20 wt.% Sodium dodecylsulphate | 0.15 | 0.15 | 0.15 |
| Distilled Water | 2.2 | 0.025 | 4.0 |
| 10 wt.% Ammonium persulphate soln. | 0.1 | 0.1 | 0.1 |
| TMEDA | 0.01 | 0.01 | 0.01 |
| 1.0 M Tris-HCl, pH6.8 | - | - | 3.75 |
| 0.1 wt.% Bromophenol Blue | - | - | 0.6 |

*Table 4.2 Composition of the two different types of poly(acrylamide) gels used for the separation of α-chymotrypsin's various components. Composition of the stacking gel used to load the protein containing solutions onto the separating gel is also shown.*

Typically, 30 μg of α-chymotrypsin (or preparation with equivalent protein content) was dissolved in reducing buffer (table 4.3) prior to being loaded onto the separation gel. The

running buffer employed for the protein separations consisted of glycine (28.8 g/L), tris (6.06 g/L) and sodium dodecyl sulphate (1.0 g/L).

| | Reducing Sample Buffer | Non-reducing Sample Buffer |
|---|---|---|
| 2-Mercaptoethanol | 0.5 ml | - |
| Glycerol | 10 ml | 10 ml |
| Sodium dodecylsulphate | 1.0 g | 1.0 g |
| 1.0M Tris-HCl, pH 6.8 | 4.0 ml | 4.0 ml |
| Bromophenol Blue | 0.10 % | 0.10 % |
| Distilled Water | 50 ml | 50 ml |
| | Boil with samples (10 min) | Incubate with samples (20 min) |

*Table 4.3 Composition of the two different buffers employed the sample preparation prior to protein separation (reducing buffer) or zymography (non-reducing buffer).*

After loading with protein containing preparations, the gels were electrophorised at a constant current of 26 mA per gel for approximately 90 minutes or until the bromophenol blue tracking dye reached to within 1 cm of the bottom of the gel. After the completion of the electrophoresis step, the gels were stained with Coomassie Blue. The Coomassie Blue staining solution was composed from 2.0 g of Coomassie Brilliant Blue G-250 dissolved in ethanol (500 ml). This solution was then added to 70 ml of glacial acetic acid and 430 ml of distilled water. The gels were then de-stained in a solution of ethanol (450 ml), glacial acetic acid (100 ml) and distilled water (450 ml).

Some gels were stained with a more sensitive silver staining method, capable of detecting very weak protein bands. The silver staining reagents and methodology are described in table 4.4.

| Procedure | Reagents | Time (minutes) |
|---|---|---|
| Fixing | 40 % Ethanol<br>10 % Glacial acetic acid<br>50 % Distilled water | >60 |
| Wash | Distilled water | 5 |
| Sensitising | 17 g Sodium acetate (anhydrous)<br>0.75 g Sodium thiosulfate pentahydrate<br>5 ml 25 %$^{w}/_{w}$ Gluteraldehyde<br>700 ml Distilled water<br>300 ml Ethanol | 60 |
| Wash | Distilled water | 5 |
| Silver Stain | 0.125 g Silver nitrate<br>62.5 µl 37 %$^{w}/_{w}$ Formaldehyde<br>125 ml Distilled water | 30 |
| Wash | Distilled water | 1 |
| Development | 7.5 g Sodium carbonate<br>62.5 µl 37 %$^{w}/_{w}$ Formaldehyde<br>250 ml Distilled water | 3-5 |
| Wash | Distilled water | 0.5 |
| Stop | 2.5 g Glycine<br>250 ml Distilled water | 5 |
| Wash | Distilled water | >10 |

*Table 4.4 Reagents and methodology employed in the silver staining procedure required for the imaging of protein bands of very low abundance.*

### 4.3.10 Electrospray mass spectrometry determination

Accurate mass infusion mass spectrometry experiments were carried out on a Micromass LCT hybrid hexapole orthogonal acceleration time-of-flight mass spectrometer that was equipped with a LockSpray dual electrospray ion source: the LockSpray allows exact mass measurement using an external reference material. The instrument was operated in positive ion electrospray mode (ES+) with both reference and analyte probes at a capillary potential of 2800 V. The source and desolvation temperatures were set to 100 °C and 150 °C respectively. Nitrogen was used as a desolvation and nebulisation gas with the desolvation flow rate set at 650 L/hour. The sample and extraction cone voltages were set to 30 V and 3 V respectively. The hexapole RF lens potential was set at 400 V (peak-to-peak). The instrument was calibrated using a mixture of PEG 200, 400 and 600 (Sigma) over the mass range 260 to 1000 Da. Leucine enkephalin (Sigma) was used as the external LockSpray reference compound with $[M+H]^+$ set at 556.2771 Da. MassLynx v4.0 SP2 was used for both control of instrumental parameters and data acquisition. 1 % formic acid solutions were added to various samples to assist in the ionisation of the material.

### 4.3.11 Gel permeation chromatography procedures

Two sets of gel permeation chromatography (GPC) analyses were conducted on the α-chymotrypsin-sodium acrylate composites synthesised (see section 4.2.8). Independent analytical companies performed both sets of experiments. Rapra Technology Ltd conducted the first aqueous GPCs. Typically, an aqueous solution of each reaction

product was prepared (20 mg of solid in 10 ml of water) and then filtered through a 0.45 μm filter. The instrument employed for the analysis was a Viscotek "Evolution" with associated pump and autosampler, fitted with a Plaquagel guard column and two Plaquagel mixed-OH separation columns (30 cm x 8 μm). The eluent was 0.3 M $NaNO_3$/0.01 M $NaH_2PO_4$ buffer adjusted to pH 7.0. Flow rate was set at 1.0 ml/min at a constant temperature of 30 °C. Separated compounds were detected via refractive index. Data capture and subsequent data handling was carried out using Viscotek "Trisec 2000" and "Trisec" 3.0 software. Calibration of the GPC system was achieved with a series of narrow molecular weight poly(acrylic acid) calibrants obtained from Polymer Laboratories Limited. All analyses were conducted in duplicate.

Viscotek Europe Ltd conducted the second set of aqueous GPC experiments. Typically, samples were dissolved in phosphate buffer and allowed to equilibrate for one hour prior to filtration with a cellulose filter and injection into the GPC system. The injection system consisted of a VE2001 GPCmax GPC solvent/sample module. Injection volume was set at 200 μl. Flow rate was constant at 0.7 ml/min with a fixed temperature of 25 °C. The column employed for the separation was a Superdex 200. A TDA302 triple detector array was employed for the detection of the eluted compounds. The detection system measured UV absorbance, solution viscosity, refractive index and Right Angle Light Scattering (RALS). The GPC system was previously calibrated with a Bovine Serum Albumin UV standard.

### 4.3.12 IR and UV spectroscopic analysis

Infra red spectroscopy was performed on a Perkin Elmer FT-IR spectrum BX instrument. Samples were prepared via the potassium bromide disc method.

Ultra violet spectroscopy was performed on a Perkin Elmer Lambda 25 UV/Vis spectrometer.

### 4.3.13 Aqueous protein HPLC

Separation of proteins from the non-crosslinked polymerisation reactions (section 4.2.8) was achieved with the use of a gradient HPLC system. An HP1050 quaternary pump was employed to deliver the mobile phase. An initial mobile phase consisting of 90 % 10 mM phosphate buffer (pH 7.5) with 10 % acetonitrile, was altered over a period of 20 minutes to 30 % buffer, 70 % acetonitrile. Separation occurred on a standard ODS-2 column. Detection was achieved by following UV adsorption at 280 nm.

## 4.4 Results and discussion

### 4.4.1 Non-emulsion-templated polymers

Aqueous solutions of acrylamide (AM) monomer, methylene bisacrylamide (MBAM) crosslinker and α-chymotrypsin (dissolved in 0.02M $KH_2PO_4$ pH 7.8 buffer at a concentration of 100 mg/ml) were polymerised via free radical initiation in the presence

of a redox catalyst (TMEDA). These polymers contained the same amounts of the co-surfactants used in all the other polymerisations, however no porogen was present. These polymers were used as controls for the subsequent emulsion templating experiments. These "solution-templated" or "non-emulsion templated" polymers displayed no detectable catalytic activity when assayed with the same transesterification assay used previously (section 2.2.5). There are two likely explanations for this apparent deactivation of the enzyme. Firstly it was noted during the experiment that the reaction vessels heated up during the exothermic polymerisation reactions. It is well known that enzymes are extremely thermally labile in aqueous solution with most enzymes losing all their catalytic activity almost instantly on exposure to boiling water.[39, 40] The heat build up in the solution polymerisations that contained no porogen was more severe than for the polymerisations conducted in the presence of a dispersed phase (hexane or $CO_2$). A rise in temperature from 22 °C (room temperature) to approximately 60 °C was observed for the solution polymerisation controls, whereas the emulsion templating polymerisations were only observed to rise in temperature by around 15 °C (i.e. room temperature rising to 35-40 °C). In all cases the temperature jump corresponded to the gel phase of the polymerisation. It may be that the porogen acts as a heat sink, removing thermal energy from the aqueous phase containing the α-chymotrypsin. Although the exposure of the enzymes to temperatures of around 60 °C while in solution will cause some deactivation, it is unlikely that the enzyme would be totally deactivated in the few minutes taken to complete the polymerisation reaction.

A second possible explanation for the total deactivation of the α-chymotrypsin containing polymers formed from solution may be found by examining their morphology (figure 4.1).

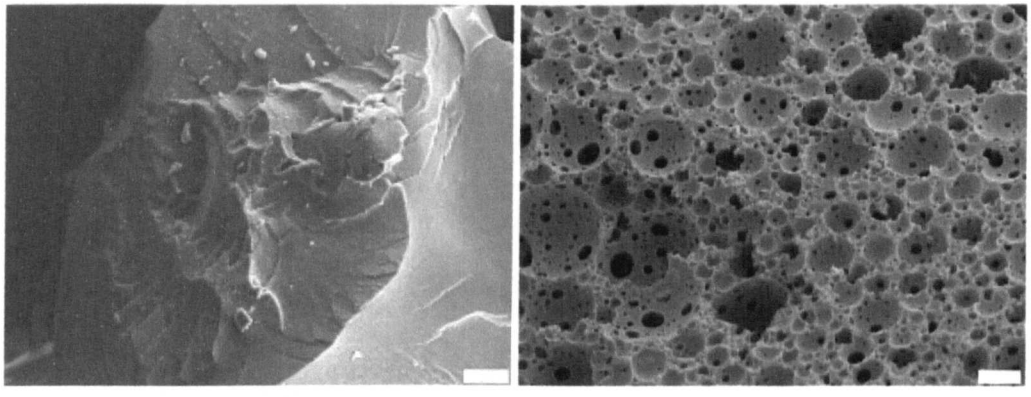

*Figure 4.1 SEM images of α-chymotrypsin containing poly(acrylamides). Both polymers are formed from the same stock solution of mixed acrylamides and surfactants, both polymers contain 20 %$^{w}/_{w}$ crosslinker (see section 4.2.3). Left = A typical image of the dense and compact structure of polymers formed from solution, with no porogen added. Right = A typical image illustrating the highly porous structure of polymers formed in the presence of a porogen (hexane). Small pores interconnecting the large cell are visible. All scale bars = 100μm.*

The polymers prepared from solution appear very dense and compacted, when compared to the highly porous structure resulting from the addition of a porogen. It is possible that the non-porous morphology of these supports prevents the diffusion of substrates and product to and from the immobilised enzyme's active sites. However, even though enzymes entrapped within the interior of the polymer matrix may not be accessed by the substrate molecules, the enzymes present on the surface of the polymer should still be available to the substrates, resulting in some degree of observable activity. Figure 4.2 shows that at high magnification both the polymers prepared from solution and the

emulsion templated polymers do appear to exhibit some porosity. Very small holes (<100 nm) are visible amongst the flocculated polymer surfaces.

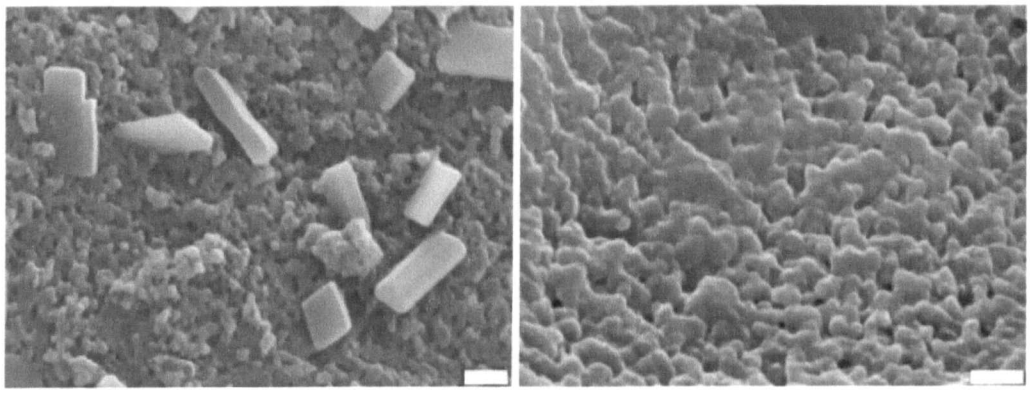

*Figure 4.2 High magnification SEM images of α-chymotrypsin containing poly(acrylamides). Both polymers are formed from the same stock solution of mixed acrylamides and surfactants, both polymers contain 20 $\%^{w}/_{w}$ crosslinker (see section 4.2.3). Left = A typical image of the flocculated surface of polymers formed from solution, with no internal phase added. Some crystallisation of one of the reaction components is visible on the surface of the material. Right = A typical image of the surface of a polymer produced from a HIPE, illustrating similar flocculation to that observed with the solution polymerisation products. All scale bars = 1 μm.*

Although the polymers prepared from solution do not exhibit the obvious large pores caused by the dispersed phase droplets, as in the emulsion templated materials (figure 4.1), both types of polymer should display some sort of porosity as a result of the aqueous phase. When the polymer precipitates out of solution during its formation, the pockets of water that are present within the matrix will create some morphology. Removal of this aqueous phase during freeze-drying would be expected to reveal some degree of fine structure.

However, no catalytic activity was observed with any of the support materials prepared from solution. Samples of equivalent polymers formed both from solution and in the presence of a porogen (cyclohexane), were ground to a fine powder and sieved to remove the 90 to 100 μm particle range. These particles were then subjected to an active site determination procedure using the fluorogenic reagent MUTMAC, which is specific to the active site of α-chymotrypsin.[38] Preliminary results suggest that the polymers formed in the presence of a porogen possess more than twice as many enzyme active sites that are accessible to small incoming substrates, than for the polymers formed from solution. Comparisons with aqueous solutions of α-chymotrypsin indicate that, for the quantity of enzyme immobilised, only around 5 % of the total number of active sites are available to substrates for the emulsion templated polymers.

As it has been determined that some enzyme active sites (approximately 2 %) are accessible for the polymers formed from solution, it seems likely that a combination of the poor diffusivity of substrate materials through the polymer matrix and the thermal deactivation of enzyme positioned on or near the surface, results in an overall reduction in enzyme activity to a point where none is detected.

### 4.4.2 Emulsion-templated polymers

The aqueous solutions of mixed acrylamide monomers (acrylamide and methylene bisacrylamide) and cosurfactants (PVA and Tween-40) were employed as the continuous phases in the formation of stable high internal phase emulsions. Two types of dispersed phase were examined for their effects on the catalytic activity of enzyme containing polymers formed from these emulsions. A traditional organic solvent (cyclohexane) was

compared to a liquefied gas medium (liquid $CO_2$, however during the exothermic polymerisation reaction, the critical point of 31.1 °C and 73.8 bar was usually passed, meaning that at the gel point of the polymerisation the reaction medium was in the supercritical phase). Polymerisation was initiated via the addition of a redox catalyst (TMEDA) to the monomer solution containing potassium persulfate free radical initiator. The effects of enzyme loading and variation in monomer to crosslinker ratio were examined for both the cyclohexane and the carbon dioxide emulsion templated polymers. The effect of reactor pressure was also examined for the $CO_2$ templated materials. Varying the reactor pressure prior to the initiation of the polymerisation reaction allows the effects of changing $CO_2$ density to be examined.

#### 4.4.2.1 Effect of enzyme loading on emulsion templated polymers

Polymers were prepared with various enzyme loadings. The α-chymotrypsin content of the polymers was between 0.5 $\%^{w}/_{w}$ and 4.0 $\%^{w}/_{w}$ relative to the total monomer content of the system. Figure 4.3 illustrates the effects of the enzyme loading on the catalytic activity of the polymer, i.e. the results are displayed in terms of catalytic activity per unit mass of polymer.

The catalytic activity of both sets of emulsion templated polymers appeared relatively unaffected by the changing enzyme loading. It could be reasoned that the polymers formed with the liquefied gas do show a slight increase in catalytic activity with increased enzyme loading (figure 4.3a). However, this trend may not be significant given the considerable errors encountered during the enzymatic transesterification assay. These activity assay errors are more significant in this study (compared to the previous work

carried out with α-chymotrypsin adsorbed to porous poly(acrylamide) beads, or entrapped within various composite matrices) because of the polymer particle size examined.

The monolithic nature of these materials means that the polymers are fragmented during their recovery from the reactor. The randomness of these particle sizes meant that the enzyme activity assays would not be comparable, due to the differing times required for the substrate molecules to diffuse throughout the internal structure of the polymer particles. Therefore the polymers were ground down to a uniform size of between 90 and 100 μm prior to the enzyme activity assay. This meant that the larger macroscopic morphology of the polymer caused by the internal phase droplets was lost. However the much smaller pores that interconnect the adjacent cells as well as any fine structure resulting from the removal of the aqueous phase remained. It has been shown[1,2] that the mass transfer of substrates to enzymes active sites, when immobilised on porous materials is important to the resulting activity. However this mass transfer process was only found to be seriously rate limiting in the case of porous materials with a mean pore diameter of less than 100 nm. As the pore size was reduced, the catalytic activity was also reduced until a complete lack of activity was observed with pore sizes less than 35 nm. From this it is reasonable to assume that the very large cells present in the emulsion templated structures are not very relevant to enzyme activity, but that the smaller structural features are. Grinding the polymer down to smaller particle sizes should remove these relatively minor "macro" structural effects whilst still allowing any significant response due to the small features to be detected.

Although the polymers have been ground down, the particle size is still much larger than was assayed in the previous emulsion/polymer composite work (chapter three). As such, the scope for error in the transesterification assay will be larger as can be seen from the results (figure 4.3).

The enzyme activity observed with the polymers formed from high internal phase emulsions using cyclohexane as the internal phase also show little difference in enzyme activity (figure 4.3b). With the possible exception of the first data point, it is reasonable to assume that no increase in polymer activity is observed with increasing enzyme activity.

When the data is re-plotted in terms of transesterification activity per unit mass of enzyme, not preparation, the effects of enzyme loading become clearer (figure 4.4).

This plot gives an indication of the preparation's efficiency. As the enzyme loading is increased the corresponding activity declines. This indicates that although the amount of enzyme immobilised is increasing, there is actually very little difference in the number of active sites available to the incoming substrate. From this it is reasonable to assume that a maximum enzyme efficiency (i.e. maximum activity per unit mass of enzyme immobilised) is attained with an enzyme loading of less than 0.5 $\%^{w}/_{w}$. The data also indicates that to within experimental error, there is no difference in catalytic activity between the polymers formed with cyclohexane or carbon dioxide dispersed phase HIPEs.

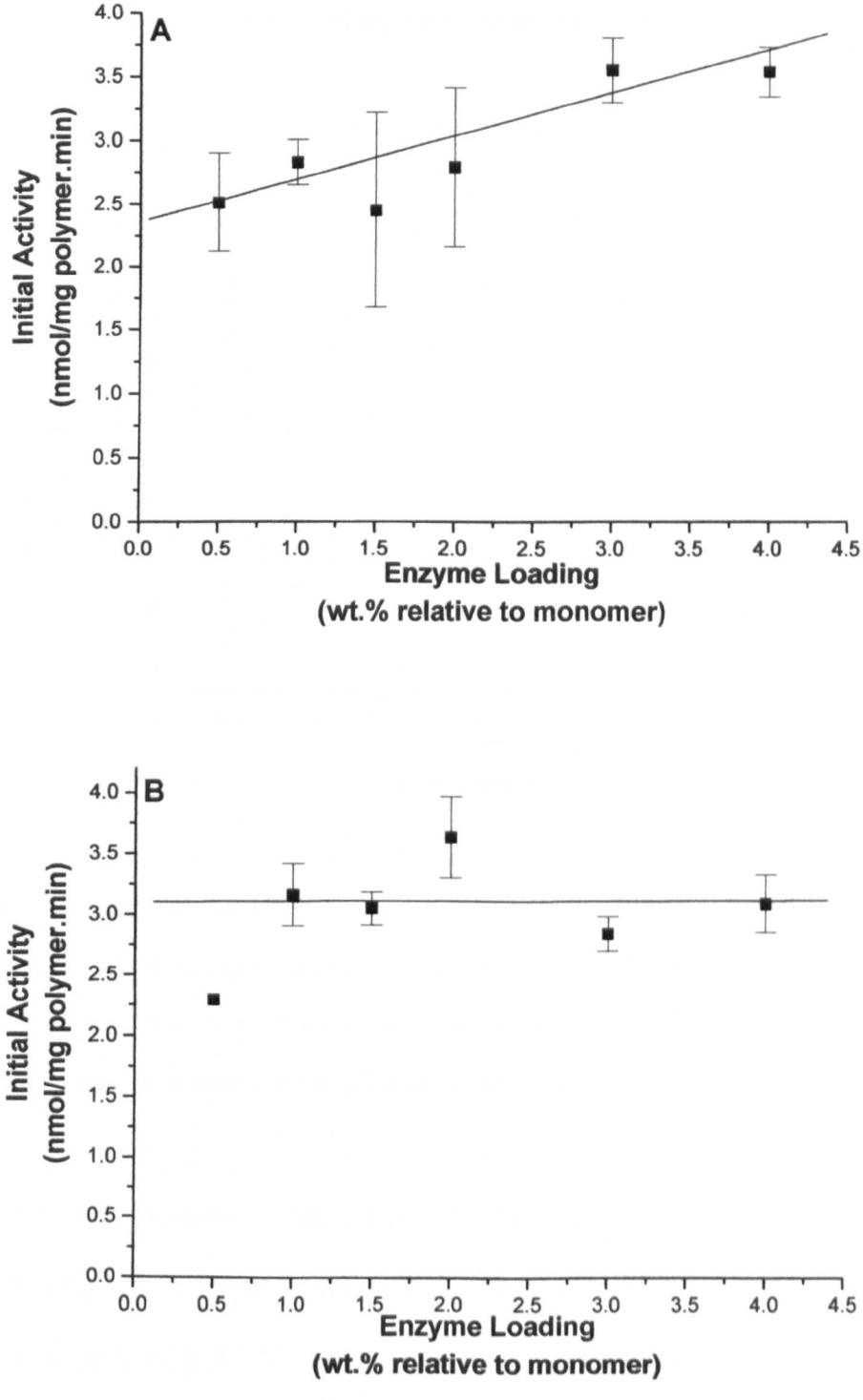


*Figure 4.3 Illustrating the effect of enzyme loading of the catalytic activity of the enzyme-containing polymer. Catalytic activity measured via the transesterification of ATEE and propan-1-ol in hexane. Figure*

*4.3a = Emulsion templated polymers formed with a carbon dioxide internal phase. Figure 4.3b = Emulsion templated polymers formed with a cyclohexane internal phase.*

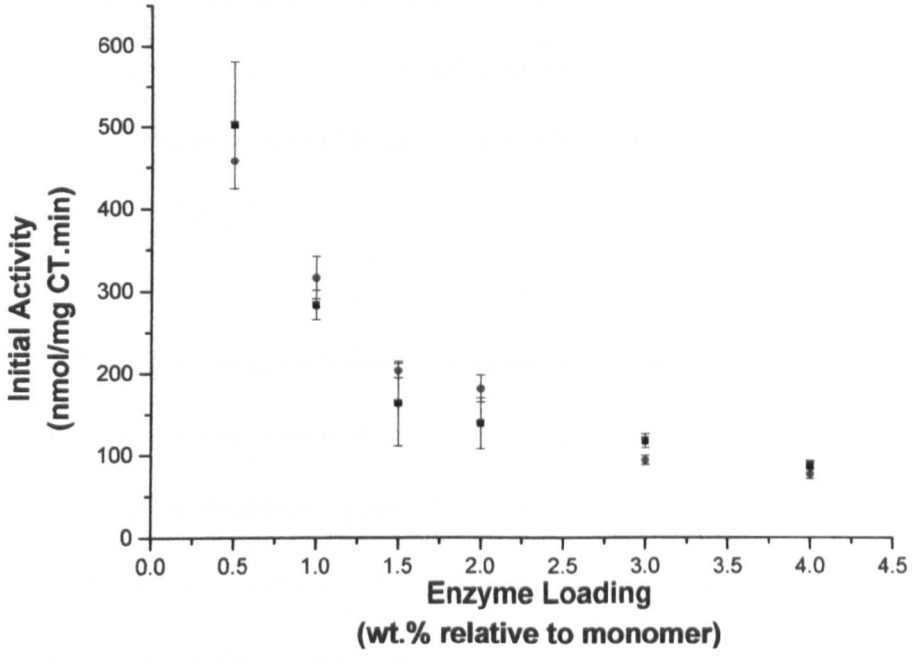


*Figure 4.4 Illustrating the effects of enzyme loading on the catalytic activity of the immobilised α-chymotrypsin. Catalytic activity measured via the transesterification of ATEE and propan-1-ol in hexane. Black squares = Emulsion templated polymers formed with a carbon dioxide internal phase. Red circles = Emulsion templated polymers formed with a cyclohexane internal phase.*

These α-chymotrypsin containing polymers exhibit very good catalytic activity when compared to the materials synthesised to chapters two and three. Emulsion templated polymers containing 0.5 $\%^{w}/_{w}$ α-chymotrypsin possessed a transesterification activity of approximately 500 nmol/mg CT.min, which compares favourably with α-chymotrypsin immobilised of porous poly(acrylamide) beads (approx. 50 nmol/mg CT.min when

assayed at a water activity of 0.43). Although it should be noted that the porous beads (chapter two) were assayed as whole particles, and were not ground down to the same particle size range as the emulsion templated polymers. Given that the two sets of materials are basically the same (porous poly(acrylamide) formed from high internal phase emulsions) it may be that this indicates some mass transfer limitation for substrates diffusing through these much larger particles (100 μm compared to 2 mm for the porous poly(acrylamide) beads).

#### 4.4.2.2 Effect of varying crosslinker to monomer ratio

The effect of polymer morphology on enzyme activity was examined by preparing a series of emulsion templated materials with differing levels of crosslinking, but with a fixed α-chymotrypsin content of 4 $\%^{w}/_{w}$. It was assumed that changing the ratio of monomer to crosslinker in the initial aqueous solution of mixed acrylamides would result in a change in the morphology of the resulting polymers. This changing morphology may then influence the catalytic activity of the immobilised enzyme. Figure 4.5 illustrates the differences in polymer morphology observed with changing crosslinker concentration.

It can be seen that as the ratio of crosslinker to monomer in the initial monomer mix is increased from 5 $\%^{w}/_{w}$ to 25 $\%^{w}/_{w}$ (around the maximum solubility of MBAM in a 40 $\%^{w}/_{w}$ total monomer content aqueous solution, at room temperature), the cell size of the porous polymer decreases.

Polymers with various levels of crosslinking were formed, employing both cyclohexane and carbon dioxide as the dispersed phase during the formation of the stable high internal

phase emulsion. The resulting polymers were assayed for catalytic activity by following the transesterification assay (figure 4.6).

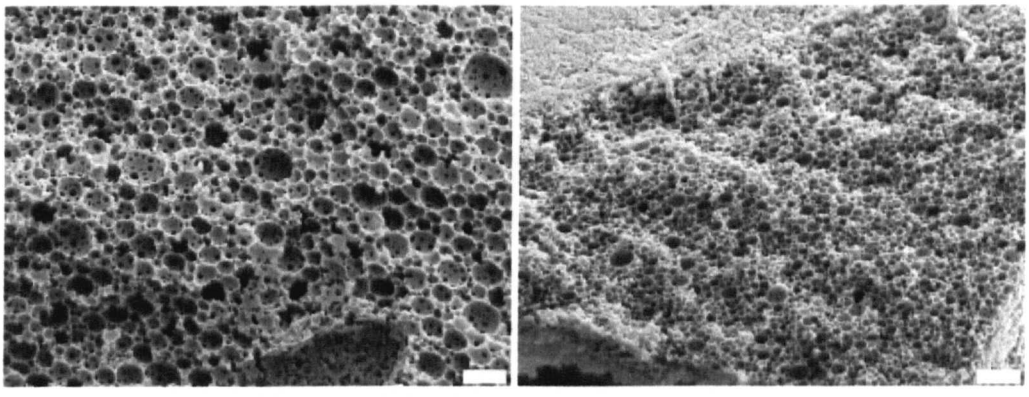

*Figure 4.5 Typical SEM images of emulsion templated poly(acrylamides) illustrating the differences in polymer morphology encountered with changing monomer to crosslinker ratio. Left = Emulsion templated polymer formed from a 5 %$^{w}/_{w}$ crosslinker content, based on the total monomer content of the aqueous phase. Right = Emulsion templated polymer formed from a 25 %$^{w}/_{w}$ crosslinker content, based on the total monomer content of the aqueous phase. All scale bars = 100 μm.*

It can be seen from the results (figures 4.6a and 4.6b) that neither the polymers formed from the cyclohexane nor carbon dioxide dispersed phase emulsions show any significant difference in catalytic activity. Although it could be argued that with the exception of the first data point a very slight trend toward increasing catalytic activity with increasing crosslinking of the support can be detected. However, given the experimental errors involved, it is assumed that there is no significant difference between the polymers in terms of their influence on enzyme activity.

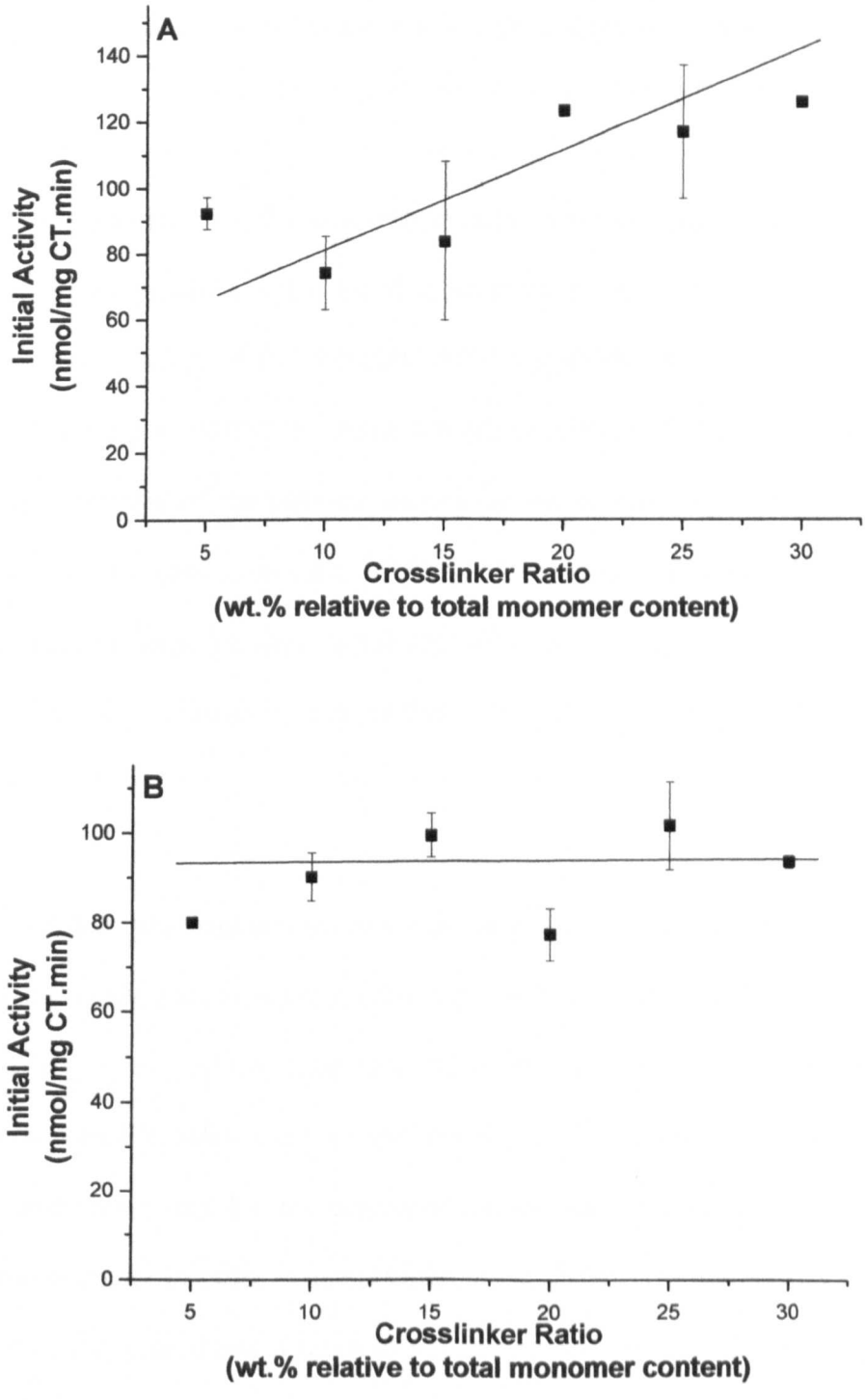


Figure 4.6 *Illustrating the effects of changing crosslinker concentration in the initial monomer mix on the catalytic activity of the α-chymotrypsin immobilised in the resulting polymer. Catalytic activity measured*

*via the transesterification of ATEE and propan-1-ol. Figure 4.6a = Emulsion templated polymers formed with a carbon dioxide internal phase. Figure 4.6b = Emulsion templated polymers formed with a cyclohexane internal phase. Both sets of polymers were prepared with a fixed enzyme loading of 4 $\%^{w}/_{w}$.*

A likely explanation for this lack of correlation between enzyme activity and crosslinking of the support material is that the changes in the polymer morphologies are not relevant to the mass transfer of the substrates to and from the enzymes active sites. The SEM images of the polymers with various crosslinking levels (figure 4.5) clearly show that the average cell size of the polymer caused by the droplet size of the emulsions internal phase, does decrease as the ratio of MBAM is increased. However these cell sizes are still in the micron range. To have significant effects on the immobilised enzyme's activity (for the 90 to 100 μm particle sizes assayed) the cell size would need to be reduced still further.

#### 4.4.2.3 Effect of initial reactor pressure on polymer morphology

Carbon dioxide does have some advantages over traditional organic solvents, when used as a porogen for emulsion templating polymerisations. Not only does it offer significant environmental benefits such as low toxicity and removal of the need for an energy intensive drying step, but the density of the solvent can also be "tuned" by altering the reactor pressure. In order to examine the effects of solvent density (i.e. reactor pressure) on the resulting products, a series of polymers were synthesised from high internal phase $CO_2$-in-water emulsions. Initial reactor pressure was varied from 60 bar (lower limit of liquid carbon dioxide) up to 300 bar (maximum safe pressure). All other reaction parameters were kept constant (4 $\%^{w}/_{w}$ α-chymotrypsin loading and 20 $\%^{w}/_{w}$ crosslinker,

relative to the total monomer content of the aqueous phase). The resulting polymers were imaged with SEM and assayed for their catalytic activity with the transesterification assay. Figure 4.7 illustrates the effect of changing internal phase density on the morphology of the polymers formed.

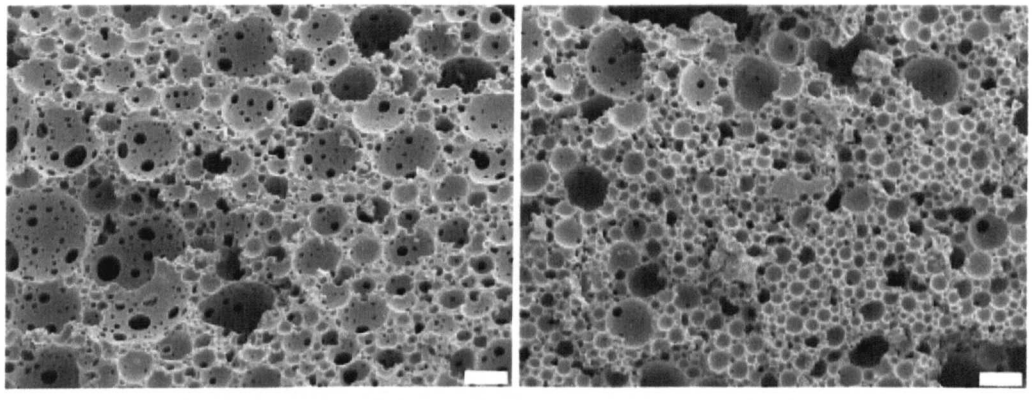

*Figure 4.7 Typical SEM images of emulsion templated poly(acrylamides) illustrating the differences in polymer morphology encountered with changing initial reactor pressure. All polymers formed with a fixed 4 $\%^{w}/_{w}$ enzyme loading and a 20 $\%^{w}/_{w}$ crosslinker ratio, relative to the total monomer content of the aqueous phase. Left = Emulsion templated polymer formed with an initial reactor pressure of 60 Bar. Right = Emulsion templated polymer formed with an initial reactor pressure of 150 Bar. All scale bars 100 μm.*

The images show that as the reactor pressure/solvent density is increased, the average cell size of the polymer formed is reduced from approximately 100 μm to around 50 μm. The effect of pressure over this range does not appear to be large. Greater changes in polymer morphology were achieved by varying the monomer to crosslinker ratio.

The catalytic activity of the immobilised enzyme also demonstrated that the effects of reactor pressure during the polymerisation are not dramatic (figure 4.8).

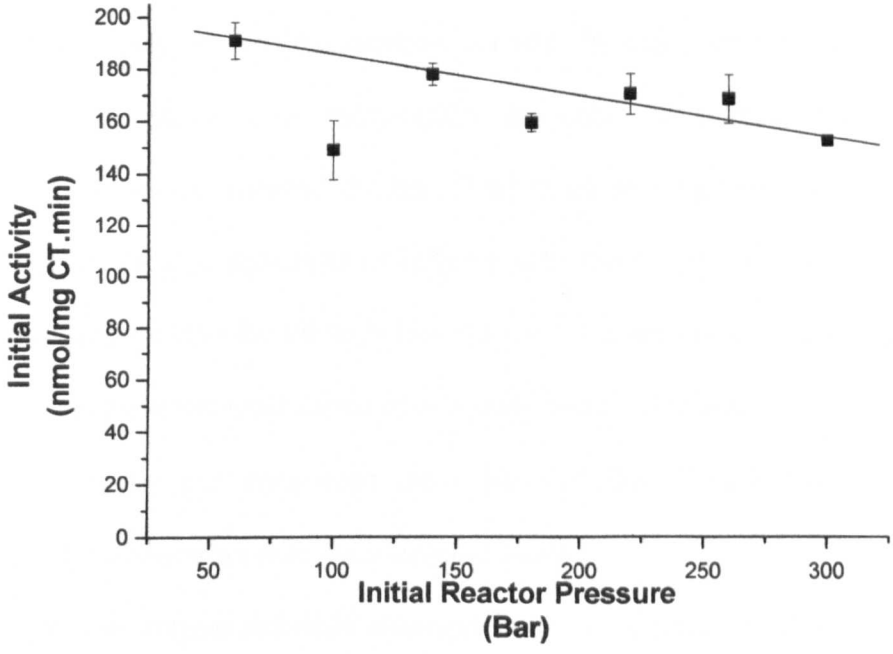


*Figure 4.8 Illustrating the effects of changing initial reactor pressure on the catalytic activity of the α-chymotrypsin immobilised in the crosslinked polymer formed. All polymers formed with a fixed 4 $\%^{w}/_{w}$ α-chymotrypsin content and a 20 $\%^{w}/_{w}$ crosslinker ratio, based on the total monomer content of the aqueous phase. Catalytic activity measured via the transesterification of ATEE and propan-1-ol in hexane.*

The results have been shown with a slight negative correlation between enzyme activity and initial reactor pressure. However, given the experimental errors involved, it would also be reasonable to assume that the changing reactor pressure has no significant effect on enzyme activity.

It is likely that the explanation for this is similar to that found with the changing crosslinker ratio experiment (section 4.3.2.2). The changing reactor pressure does affect the morphology of the polymer, as can be seen from the SEM images (figure 4.7). However the changes are occurring in the macro structure of the material, which makes

very little difference to the diffusion of substrates to and from the enzyme, for the particle size range being assayed for catalytic activity. In order for the enzyme activity to be influenced by the polymer morphology, the pore size interconnecting adjacent cells would have to be reduced further. The literature suggests that in order for the immobilised enzyme activity to be influenced by the morphology of the support material, the average pore size should be below 100 nm.[1,2] It has been demonstrated that the pore sizes of the materials synthesised in this study were influenced by various factors such as reactor pressure and crosslinker ratio, but that the changes were occurring on the micrometer scale rather than the nanometer scale.

However, the enzyme activities observed for these immobilised enzyme preparations do compare favourably with the alternative immobilisation methods examined in chapters two and three. The highest enzyme activities recorded with the emulsion templated-preparations (at a fixed water activity of 0.43) were approximately 500 nmol/mg CT.min which is significantly higher than for the preparations; lyophilised from buffer (33 nmol/mg CT.min), adsorbed to poly(acrylamide) beads (50 nmol/mg CT.min) and entrapped in the composite beads (300 nmol/mg CT.min).

### 4.4.3 Does α-chymotrypsin bind to the growing polymer?

In order to examine how effectively the α-chymotrypsin was incorporated into the growing polymer enzyme, leaching experiments were performed on various enzyme containing emulsion-templated materials. Polymers formed with different crosslinking ratios and enzyme-loading levels were ground to a fine powder and shaken in water for several hours at room temperature. The suspensions of polymer in water were filtered

through a 0.22 μm syringe filter to remove the polymer particles and the washings collected for protein content analysis. Bradford's assay was used to determine the protein content of the washing solutions. It was calculated that the maximum amount of enzyme that leached out of any of the polymers examined was less than 5 % of the material's total enzyme content, and in many cases, no protein was detected leaching from the supports. Bradfords assay is a sensitive technique capable of detecting nano-mole quantities of enzyme. It works by spectroscopicaly measuring the change in UV absorbance that occurs when a dye (Coomassie Brilliant Blue) chelates to the lysine amino acid residues (primary amines) present on the surface of most proteins. The sensitivity of the Bradford's assay coupled with the low quantities of α-chymotrypsin detected in the washing of the polymers, indicated that the vast majority of the enzyme present at the start of the polymerisation reaction was successfully entrapped within the polymer structure. If the enzyme were just adsorbed to the surface of the polymers, it would be quickly dissolved during the washing procedure, and so detected with the Bradford's assay.

There are two probable explanations as to why the enzyme was so efficiently immobilised within the support material. Either the enzyme has been physically entrapped within the growing polymer matrix, mechanically preventing the enzyme from leaching from the support, or some covalent linkage has been formed between the α-chymotrypsin and the polymer, preventing the detachment of the enzyme from the support.

Mechanical entrapment seems the less likely of the two postulated immobilisation mechanisms. It is reasonable to assume that some of the protein's structure becomes

enmeshed within the growing polymer. This entanglement could prevent the α-chymotrypsin from leaching out of the support matrix, without deactivating the enzyme (it has been shown that the porous poly acrylamide supports still retain excellent activity). However, after being ground to a fine powder, it would be expected that a significant amount of the enzyme would be able to leach from the support. The Bradford's assay indicates that this is not the case.

The alternative immobilisation mechanism of covalent bond formation between the α-chymotrypsin and the support would prevent the leaching of the enzyme from the polymer. However, this would require the presence of a functional moiety within the enzyme's structure capable of participating in the free radical reaction.

It is well known that thiol groups are capable of acting as potent chain transfer agents during free radical polymerisations.[41, 42] Cysteine is the only amino acid that contains a free thiol group. α-Chymotrypsin does contain ten cysteine residues, however the thiol groups contained in the amino acid residues form structural disulfide bonds that influence the conformation of the enzyme. The crystal structure of α-chymotrypsin has been resolved, revealing the position of these disulfide bonds (figure 4.9).[43]

It is known that disulfide containing compounds can act as chain transfer agents, although they are not as potent as thiol containing compounds.[44, 45] It was reasoned that the disulfide bonds contained within α-chymotrypsin's structure may undergo covalent incorporation into the growing polymer, in the presence of free radicals.

For the α-chymotrypsin containing polymers synthesised in this study, there is no direct method for determining whether the enzyme has actually undergone a covalent

interaction with the growing polymer, as the levels of crosslinking employed render the preparation insoluble in all solvents.

*Figure 4.9 X-ray crystal structure of α-chymotrypsin.*[43] *Ten cysteine residues are present within the enzymes structure (highlighted in red). It can be seen that these residues are arranged in pairs, forming disulfide linkages. None of these disulfide bonds directly participate in the chemistry of α-chymotrypsin's active site.*

In order to examine the postulated interaction between α-chymotrypsin and the free radically initiated polymerisation, two series of experiments were conducted. These involved the free radical polymerisation of water-soluble monomers in the presence of α-chymotrypsin. No crosslinking agent was added to any of these reactions, resulting in preparations that were soluble in water, and so suitable for analysis with a variety of techniques, including gel permeation chromatography, sodium dodecyl sulphate

polyacrylamide gel electrophoresis, electrospray mass spectrometry and HPLC protein separation.

#### 4.4.3.1 Gel Permeation Chromatography analysis

The thermally initiated α-chymotrypsin/sodium acrylate grafts (synthesised in section 4.2.8) were submitted for gel permeation chromatography analysis by an independent analytical company (Rapra Technology Ltd, section 4.2.11). The purpose of this analysis was to determine whether the molecular weight of the enzyme had changed relative to a sample of native α-chymotrypsin, during the polymerisation reaction. Analysis was conducted using refractive index detection only. The results (figure 4.10 and table 4.2) did indicate that the molecular weight of the preparations did change as the ratio of enzyme to monomer present in the reaction mixture was altered. However, the molecular weights calculated were far lower than expected. No samples were calculated to have a molecular weight greater than 1240 Da. The molecular weight of α-chymotrypsin is approximately 23,000 Da. The analysis of proteins with GPC is often difficult due to their compact conformation. Techniques that rely on the molecular radius of the compounds being analysed can be easily confused by these relatively small compact structures.

Although the results obtained (table 4.2) did not appear representative of the actual molecular masses of the preparations, a trend in molecular weight is observed. As the quantity of α-chymotrypsin present in the polymerisation mixture was increased, the corresponding molecular weights of the composites formed are reduced. This is the trend that would be expected if increasing levels of a chain transfer agent were added to a free radical polymerisation.

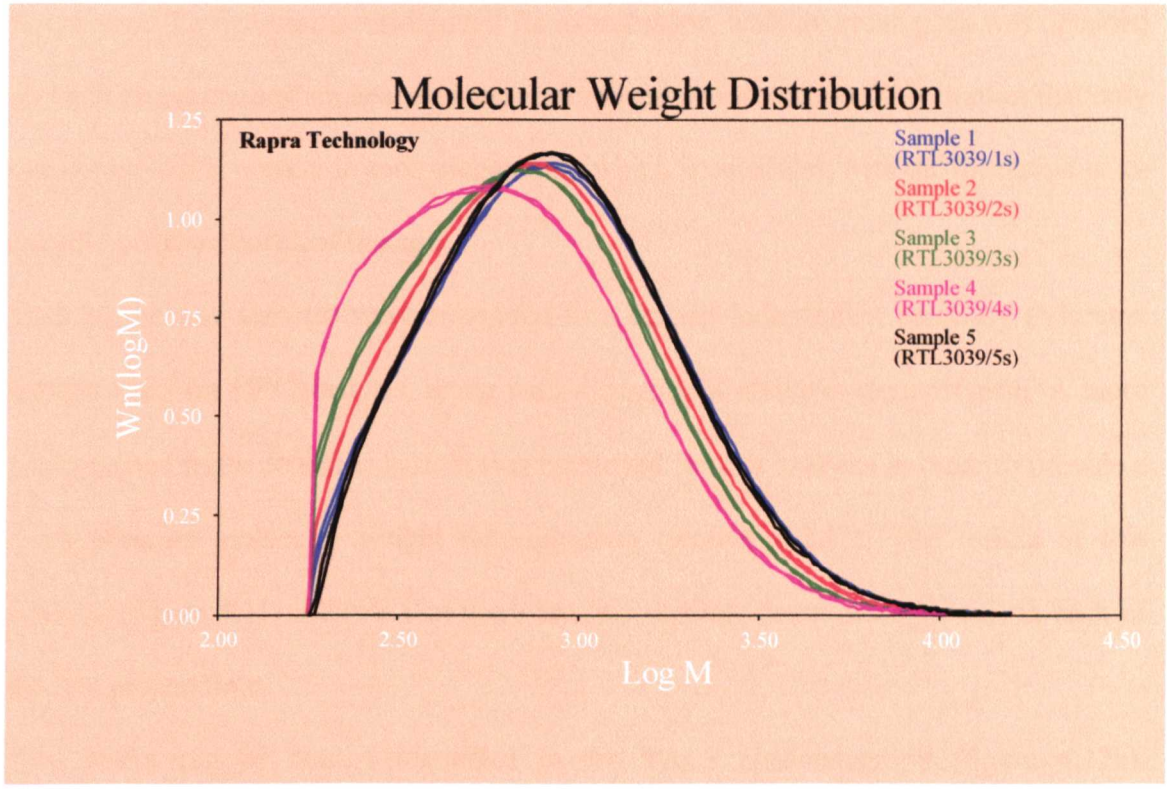


*Figure 4.10 Molecular weight distribution of various enzyme-polymer grafts, as determined by aqueous gel permeation. Samples analysed with refractive index detection only (see section 4.2.11). Data supplied by Rapra Technology Ltd.*

| Molar ratio | Run | Mw | Mn | Polydispersity |
|---|---|---|---|---|
| **10000: 1** | 1 | 1160 | 680 | 1.7 |
| **10000: 1** | 2 | 1160 | 670 | 1.8 |
| **1000: 1** | 1 | 1050 | 610 | 1.7 |
| **1000: 1** | 2 | 1020 | 610 | 1.7 |
| **500: 1** | 1 | 950 | 570 | 1.7 |
| **500: 1** | 2 | 930 | 560 | 1.7 |
| **200: 1** | 1 | 860 | 500 | 1.7 |
| **200: 1** | 2 | 830 | 500 | 1.7 |
| **No enzyme** | 1 | 1170 | 690 | 1.7 |
| **No enzyme** | 2 | 1240 | 700 | 1.8 |

*Table 4.2 Molecular weight, molecular number and poly dispersity of α-chymotrypsin/sodium acrylate grafts. All samples run in duplicate. Molar ratio indicates the ratio of monomer to enzyme present in the reaction mixture prior to polymerisation. Data supplied by Rapra Technology Limited.*

For each of the five samples submitted for examination, a single broad peak was obtained for each preparation when analysed with refractive index detection. This implies that only one compound is present in each preparation, which is consistent with the formation of an enzyme-polymer covalent linkage.

Each of the five samples were submitted to a second independent company (Viscotek Europe Ltd.) for GPC analysis, along with a sample of native α-chymotrypsin. A more sophisticated triple detection system was employed for this analysis in order to provide a more accurate molecular weight determination (section 4.2.11). The results of this analysis (figure 4.11) indicate that there are two separate compounds present in each of the five preparations.

Two peaks can be readily identified in the RALS chromatograph (figure 4.12b). However, it is unclear as the whether these peaks are due to enzyme and polymer, or to enzyme-polymer composites and "unreacted" polymer. Although these GPC results do imply the presence of two separate compounds, the molecular weights determined for the preparations are still very low (table 4.3).

The maximum recorded weight for the enzyme-polymer grafts was 2384 Da, far lower than the literature mass of α-chymotrypsin (approximately 23,000 Da). The corresponding observed hydrodynamic molecular radius for the various preparations was also lower than was found for the native enzyme (2.42 nm). It is possible that the exposure of the enzyme to prolonged heating whilst in aqueous solution during the polymerisation reaction, may have caused the breakdown of its structure through hydrolysis. However, it seems unlikely that all the enzyme present would have hydrolysed and that none of the original higher molecular weight protein would remain. It

is much more likely that aqueous GPC is just a poor method for the analysis of enzymes. As such, further analysis of the reaction products was conducted with various alternative techniques.

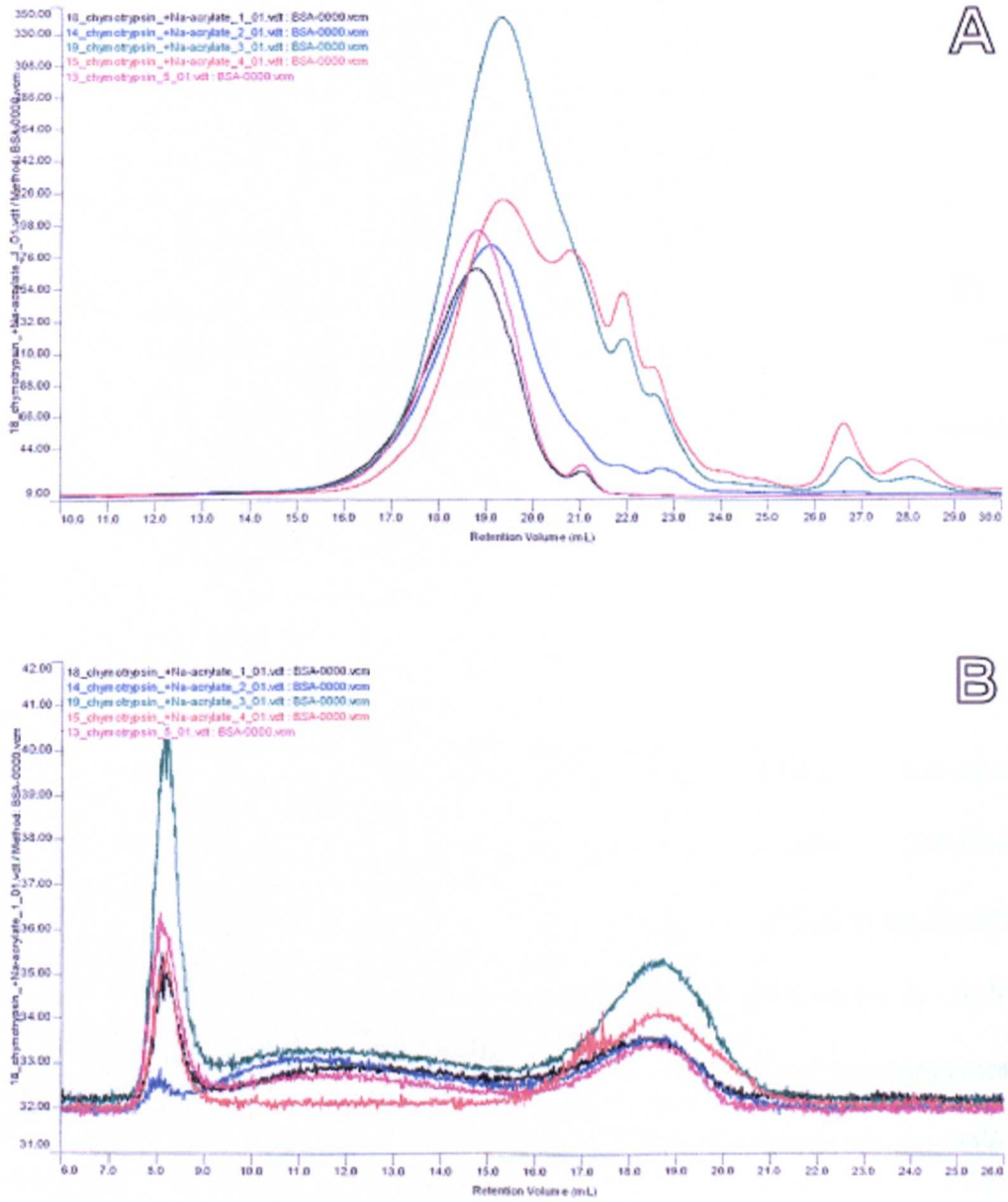


*Figure 4.11 GPC analysis of α-chymotrypsin-polymer composites. Figure 4.12a overlay of all five chromatographs recorded with UV detection. Figure 4.12b overlay of all five chromatographs recorded*

*with right angle light scattering (RALS) detection. RALS plot clearly indicates the presence to two compounds with different molecular weights. Data supplied by Viscotek Europe Limited.*

| Molar ratio | Mw (Da) | Rhw (nm) | Poly Dispersity |
|---|---|---|---|
| **10000: 1** | 1561 | 0.898 | - |
| **1000: 1** | 2384 | 1.05 | - |
| **500: 1** | 2117 | 1.01 | - |
| **200: 1** | 1316 | 0.796 | - |
| **Polymer** | 1375 | 0.834 | - |
| **Enzyme** | 29983 | 2.42 | 1.02 |

*Table 4.3 Molecular weight (Mw) and hydrodynamic radius (Rhw) of the five α-chymotrypsin-polymer composites, as determined by triple detection GPC. Also shown is data obtained for native α-chymotrypsin. Data supplied by Viscotek Europe Limited.*

### 4.4.3.2 SDS-PAGE analysis

The set of five α-chymotrypsin-sodium acrylate polymerisation products were analysed with sodium dodecyl sulphate poly(acrylamide) gel electrophoresis (SDS-PAGE section 4.2.9). If the growing polymer was successfully covalently linked to the α-chymotrypsin, then a shift in position of one of the protein bands should be visible in the SDS-PAGE separation, relative to the native α-chymotrypsin. However, as the concentration of α-chymotrypsin was very low in some of the preparations (particularly the 10000: 1 molar ratio of monomer to enzyme experiment) the amount of sample initially loaded onto the gel had to be relatively large. The presence of large quantities of poly (sodium acrylate) caused a lifting in the baseline positions of these low enzyme concentration samples. This

made the subsequent comparison of the separated protein bands impossible. Various poly(acrylamide) gel concentrations were employed along with the variation of other experimental parameters, in order to generate a flat baseline. However, at the minimum protein concentrations required for detection during the gel development step, a stable baseline could not be achieved.

In order to overcome this problem, a second set of α-chymotrypsin-polymer composites was synthesised (section 4.2.8) employing acrylamide monomer, to produce the polymer for covalent linkage to the enzyme. The concentration of α-chymotrypsin was also altered. Reactions were conducted in the absence of enzyme, as well as at intermediate and high enzyme concentrations (20 $\%^{w}/_{w}$ and 50 $\%^{w}/_{w}$ relative to the total monomer content). These preparations did not contain any of the sodium salts present in the previous set of monomer-enzyme polymerisations. Milder reaction conditions were also achieved by conducting the polymerisation in the presence of a redox co-initiator (TMEDA). This enabled the reaction to be carried out at room temperature (23 °C) reducing the possible hydrolysis problem discussed previously (section 4.3.3.1). These preparations were then analysed using the same SDS-PAGE procedure. The gels produced (figure 4.12) did not typically display any measurable difference between the separated bands of α-chymotrypsin present in any of the reactions.

A second SDS-PAGE method was also employed for the separation of the various components of the enzyme containing preparation. A non-reducing gel was used to separate the protein bands without unravelling the enzyme's conformation. This allowed for the subsequent analysis of the various protein bands in order to determine which was responsible for the enzymatic catalysis. It can be seen (figure 4.13) that the relative

position of the active protein band has shifted for the α-chymotrypsin exposed to the free radical polymerisation. However, this small shift in position is not conclusive evidence for a change in the molecular weight of the enzyme, as non-reducing SDS-PAGE separation is not as accurate as for the standard SDS-PAGE technique.

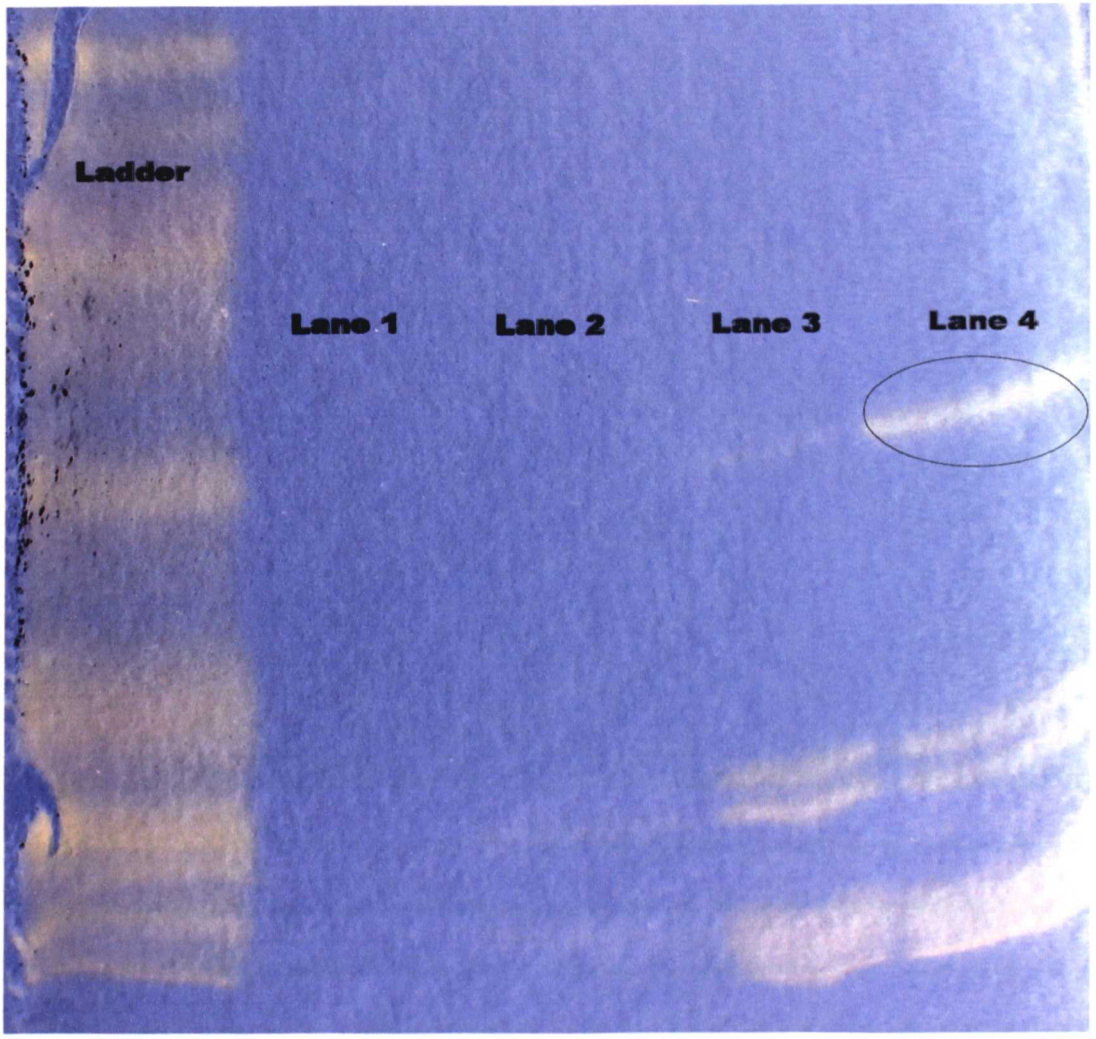


*Figure 4.12 A typical image of a dried SDS-PAGE gel, stained with Coomassie Blue (negative image shown). Ladder = Dalton mark VI. Lane 1 = 100 % poly(acrylamide). Lane 2 = 5: 1 poly(acrylamide): α-chymotrypsin. Lane 3 = 50: 50 poly(acrylamide): α-chymotrypsin. Lane 4 = native α-chymotrypsin. The α-chymotrypsin protein band corresponding to a molecular weight of approximately 23 kDa in highlighted in lane 4. Some edge lifting is observed in lanes 3 and 4.*

Although no difference was measurable in the mass of native α-chymotrypsin and α-chymotrypsin that had been exposed to the free radical polymerisation reaction, it may be that SDS-PAGE is simply not a sensitive enough technique for the detection of the relatively small mass changes associated with the linkage of polymer to an enzyme.

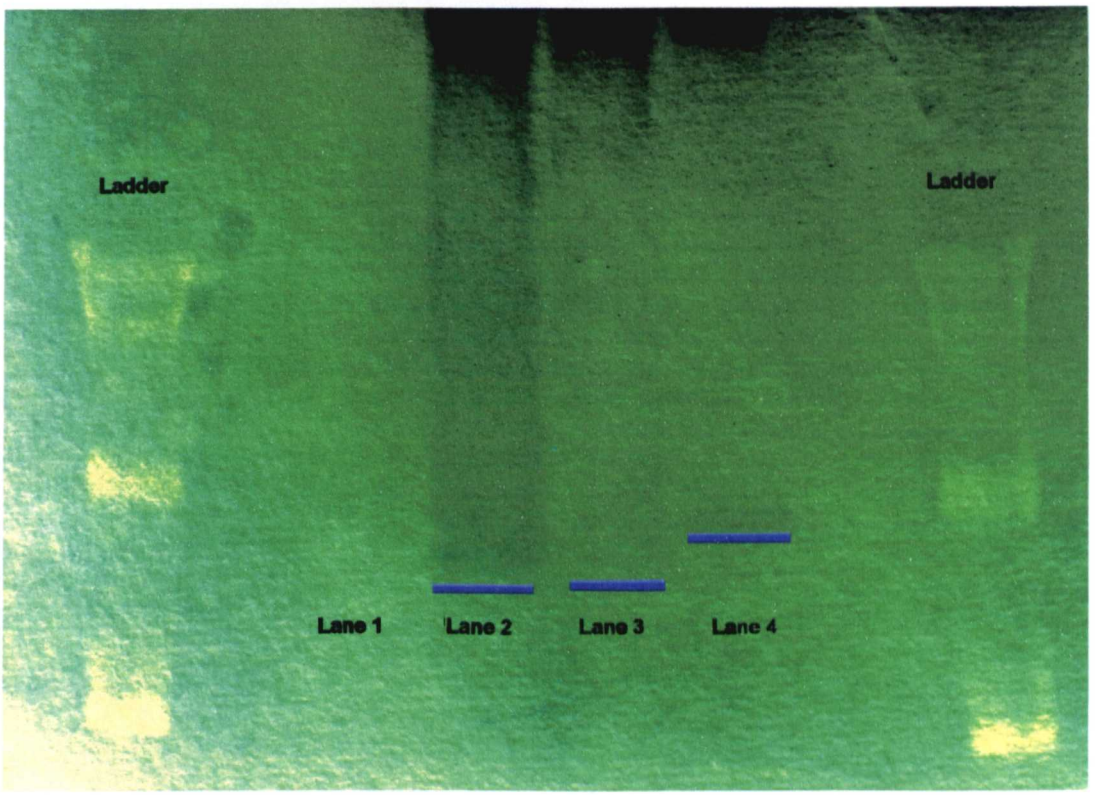


*Figure 4.13 SDS-PAGE activity gel results. α-chymotrypsin was separated using a non-reducing SDS-PAGE technique. i.e. the proteins conformation is not destroyed. This enable the protein band responsible for enzymatic catalysis to be identified by the use of a subsequent aqueous activity assay. Lane 1 = 100 % poly(acrylamide). Lane 2 = 5: 1 poly(acrylamide): α-chymotrypsin. Lane 3 = 50: 50 poly(acrylamide): α-chymotrypsin. Lane 4 = native α-chymotrypsin.*

#### 4.4.3.3 Spectroscopic and HPLC analysis of the enzyme-containing polymers

Both IR and UV techniques were found to be unsuitable for the analysis of α-chymotrypsin. The complex structure of the enzyme prevented any determination as to whether the protein had undergone a covalent reaction with the growing polymer.

Separation of the enzyme from the enzyme-containing polymer preparation by gradient HPLC was also inconclusive. Although the protein bands could be separated via this technique, the reproducibility of the experiment was limited and so the difference in the retention times associated with different molecular weights did not show any definitive evidence for protein modification.

#### 4.4.3.4 Electrospray mass spectrometry analysis

Both sets of α-chymotrypsin-acrylamide composites that were synthesised in order to determine whether a covalent linkage was created between the two moieties, were analysed with electrospray mass spectrometry. Mass spectrometry should have been able to provide a definitive answer to this question. However, no result was obtainable for either set of preparations, due to the very poor ionisation of the enzyme. The ionisation of the enzyme could not be improved, even with the addition of small quantities of formic acid.

The result of the numerous experiments conducted in order to determine whether α-chymotrypsin is covalently incorporated into the growing polymer under free radical conditions, are inconclusive. No definitive evidence can be found for a covalent linkage between the enzyme and the polymer. As such, it is reasoned that the absence of any

observed enzyme leaching from the crosslinked support materials, even during rigorous washing cycles, is due to very high mechanical entrapment efficiency. It is possible that the relatively small monomer molecules are able to diffuse into the three dimensional conformation of α-chymotrypsin. After polymerisation these monomers form a rigid, crosslinked structure that is enmeshed within the structure of the enzyme, preventing it from detaching from the support material during the washing cycles.

### 4.5 Emulsion templated polymers for continuous flow reactions

It has been shown that high internal phase emulsions containing suitable monomers and crosslinkers dissolved in the continuous aqueous phase can be used to produce polymers with highly porous morphologies. The interconnectivity of the structures allows for the diffusion of substrate molecules throughout the entire matrix. The use of these materials for the immobilisation of enzymes allows for their potential use in continuous flow reactions.

The emulsions formed with organic (or supercritical) porogens prior to initiation of the polymerisation reaction, can be transferred to any reaction vessel. The resulting crosslinked polymer should then occupy the entire volume of the reaction vessel. In order to produce continuous flow reactors, these emulsions were cast in empty HPLC columns. After completion of the polymerisation reaction, the HPLC columns were freeze dried to remove the remaining aqueous phase. However, even very slow freeze-drying resulted in some shrinkage of the polymer, causing gaps to appear between the polymer monolith and the column walls. Displacement of the aqueous phase with miscible organic solvents

such as acetonitrile and propan-1-ol also results in some shrinkage of the polymer (figure 4.14).

The shrinkage observed upon removal of the aqueous phase renders the column unusable for catalysis reactions, as the eluent can simply flow through the gap between the polymer and the column wall rather than through the porous polymers structure. However, as it has been demonstrated that the enzyme does not leach from the support (section 4.3.3) it may be possible avoid displacing the aqueous phase and simply employ the column for traditional aqueous catalysis reactions. (N.B. recent unpublished results obtained by our research group, indicate that it may be possible to use alternative freeze-drying techniques to produce monolithic materials that entirely fill HPLC columns).

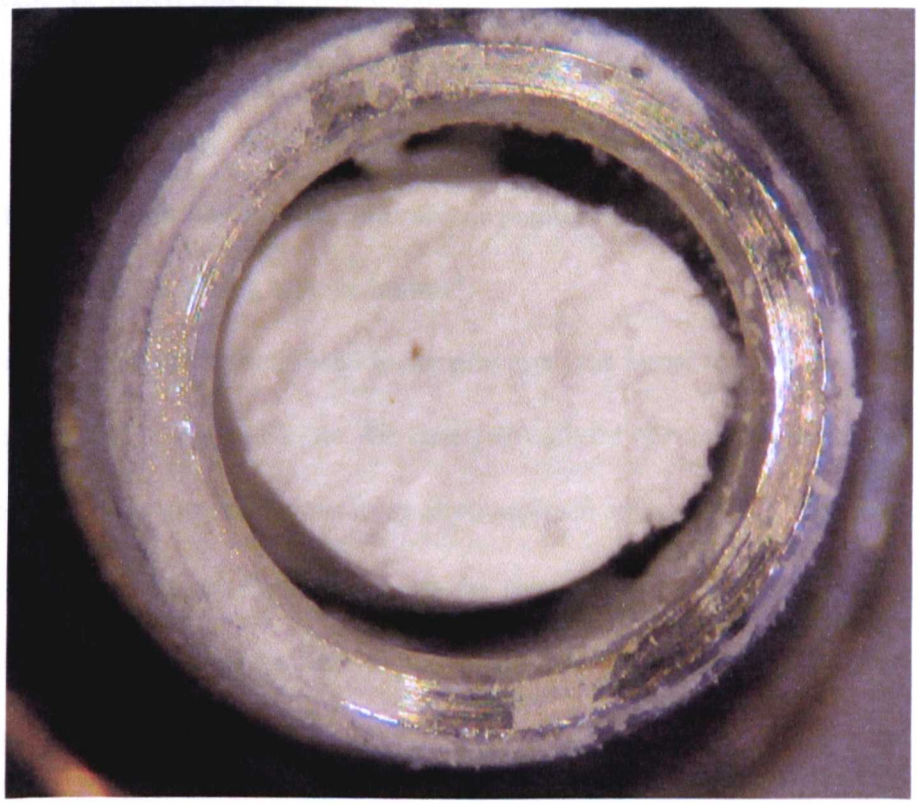

*Figure 4.14 A typical image of a "cast" polymer. An emulsion of cyclohexane and water, containing AM and MBAM monomers and crosslinkers was used to completely fill an empty HPLC column. After polymerisation the polymer appeared to fill the entire internal volume of the column. However, removal of*

*the remaining aqueous phase of the original emulsion caused some shrinkage of the polymer. This resulted in gaps between the polymer monolith and the column walls, which can be clearly seen in the image above. Internal diameter of the column = 4.6 mm.*

## 4.6 Conclusions

A novel high internal phase emulsion templating technique was employed for the synthesis of a variety of α-chymotrypsin containing crosslinked polymers. Emulsions were formed from an aqueous external phase, containing the various monomers and the enzyme, and an organic internal phase. This organic phase consisted of either cyclohexane, or supercritical carbon dioxide. The equivalent polymers were also formed from solution, i.e. in the absence of any internal phase. The effects on immobilised enzyme activity arising from different monomer/crosslinker compositions, various enzyme-loading levels and of differing reactor pressures, in the case of polymers formed from supercritical emulsions, were examined.

The quantity of enzyme immobilised in the polymer was found to have the greatest effect on the specific enzyme activity (i.e. the catalytic activity normalised to the initial rate of substrate conversion per milligram of α-chymotrypsin immobilised). Generally enzyme activities were found to be high, relative to the lyophilised α-chymotrypsin preparations. The optimum enzyme loading was shown to be less than 0.5 $\%^{w}/_{w}$ based on the total monomer content.

The initial reactor pressure and the monomer to crosslinker ratio were shown to influence the morphology of the polymer formed. However, these changes in polymer morphology were not found to influence the catalytic activity of the immobilised enzyme. It is

postulated that the variations in polymer porosity were occurring on too large a scale to have any significant effect on the diffusion of substrates and products to and from the enzyme's active sites. As such, under the conditions employed for the enzyme activity assay, all the emulsion templated enzyme-containing polymer preparations displayed similar activity. No significant difference was observed in the catalytic activities of enzymes immobilised in emulsion templated polymers formed with either supercritical carbon dioxide, or cyclohexane internal phases.

The equivalent enzyme-containing polymers formed in the absence of any internal phase, were shown to possess no detectable catalytic activity. It was postulated that these enzyme-containing preparations were deactivated by either the polymerisation conditions or by the resulting polymer's morphology. A greater temperature rise was observed during the final stages of the solution polymerisations, when compared to the equivalent emulsion templating reactions. The exposure of the enzyme to elevated temperatures whilst still in aqueous solution may have resulted in some deactivation. Alternatively, it was shown that the solution templated polymers possessed a far more densely compacted morphology than the polymers formed from high internal phase emulsions. It was postulated that this lack of porosity may have hindered the mass transfer of substrates to the enzyme's active sites, resulting a reduction in the observed catalytic rate. Active site titrations with the fluorescent, α-chymotrypsin specific reagent, MUTMAC, demonstrated that the number of active sites accessible to small substrate molecules in the solution templated polymers was approximately half the number available in the emulsion templated polymers.

It appears that the total observed deactivation of the enzymes contained in the polymers formed in the absence of any internal phase, is due to a combination of the higher polymerisation temperatures and the poor substrate diffusion, relative to the emulsion templated polymers.

Extensive washing of the enzyme containing emulsion templated polymers indicated that no enzyme was detected leaching from the support material. Hence, it was postulated that α-chymotrypsin might undergo covalent attachment to the growing polymer, possibly through a chain transfer mechanism involving the enzyme's disulfide bonds. The presence of a covalent bond between the enzyme and the crosslinked polymer would explain why no enzyme leaching could be detected. Subsequent investigation failed to provide any conclusive evidence for this theory. As such it is proposed that the enzyme is immobilised via an entrapment mechanism, becoming enmeshed within the growing polymer with near total efficiency.

## 4.7 Future work

It has been shown that the morphology of the crosslinked polymer produced from a high internal phase emulsion can be influenced by factors such as the monomer to crosslinker ratio and the initial pressure of the reaction vessel (in the case of emulsions formed with a supercritical internal phase). However, the changes are not occurring on a small enough scale to affect the immobilised enzymes activity. Instead of being used as enzyme supports, these "tuneable" materials may have applications for use as support materials

for the immobilisation of cells. These larger moieties should be of a more suitable size to be influenced by the changes in porosity that can be achieved with these materials.

Attempts have been made to use these emulsion templated monolithic polymers for continuous flow applications. It was found that a small amount of shrinkage occurred on displacement of the water remaining in the crosslinked polymer. This shrinkage caused gaps between the edge of the polymer monolith and the walls of the column to appear. These gaps prevented the packed columns use in continuous flow enzyme catalysis reactions. The shrinkage that occurs is due to the swelling action of the water on the poly(acrylamide) support. If these materials can be synthesised in the absence of an aqueous phase, the resulting shrinkage may be alleviated. High internal phase emulsions formed between two organic phases have been reported and it might be possible to adapt these methodologies to allow for the use of a supercritical internal phase, in the production of porous polymers.[13, 14] However, this approach would necessitate the solubilisation of an enzyme in non-aqueous media.

## 4.8 References


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# Chapter 5

## Multipoint Covalent Enzyme Immobilisation Using Supercritical and Dense Gas Solvents

### 5.1 Introduction

The physical adsorption of α-chymotrypsin to a support has been shown to be a simple and effective immobilisation method (chapter two). Enzyme activity and stability is better than for the equivalent unsupported, lyophilised preparations. However, the physical entrapment of α-chymotrypsin has been shown to offer even greater stability, reusability and catalytic activity (chapter three). This is due in part to reduction in the flexibility of the protein.[1-5] Physically adsorbed proteins are only constrained in one dimension, i.e. the surface they are adsorbed to. The entrapped proteins can be mechanically constrained on all sides. This prevents large changes in the protein's conformation, helping it to remain in a catalytically active shape. A disadvantage of both these immobilisation strategies is that the enzyme is often quickly washed from the support on exposure to water. As such, this immobilisation method is not usually applicable to catalysis in traditional aqueous media. It has been shown that this problem can be overcome by the entrapment of a protein during a free radical polymerisation reaction (chapter four). Small monomers can penetrate the enzyme's structure prior to the polymerisation, which then locks them in a rigid conformation. The resulting rigid polymer is enmeshed within the enzyme's structure, preventing any leaching from the support material.[5]

An alternative strategy for effective enzyme immobilisation is the formation of a covalent chemical bond, linking the enzyme to the support matrix.[6-8] This not only prevents

enzyme leaching, but also provides a fixed, rigid point of attachment. This reduces the enzyme's flexibility by effectively "pinning" it to the support. Increasing the number of attachment points between the enzyme and the support reduces the enzyme's flexibility further and can result in a more stable preparation.[6, 9] Multiple attachment points can prevent the protein from unfolding in extreme environments of temperature, pH and solvent polarity.[6, 9-15] Studies have shown that multipoint covalent immobilisation of enzymes can increase their stability by up to 600 fold, relative to unsupported equivalents.[6] The catalytic activity of the supported protein can also be enhanced, if the conformation of the protein prior to covalent immobilisation is such that the active site is accessible and in the correct orientation to interact with incoming substrate molecules.[16, 17]

A disadvantage of this process is that several chemical modification steps need to be performed on the enzyme in order to achieve multipoint attachment. This exposure to chemical reagents can cause deactivation of the enzyme.[6, 10, 15, 18] In addition to this, the enzyme may be immobilised in an unfavourable conformation or orientation for catalysis. Once immobilised, the enzyme is then fixed in an inactive state and cannot be regenerated.

In addition to the immobilisation procedure, enzyme activity is also affected by the nature of the support material. It is desirable for the matrix to have a large surface area and high degree of porosity.[19] This enables the maximum number of enzyme active sites to be accessed by the incoming substrates. Supports need to be mechanically strong enough to be used in a stirred reactor, recovered and washed, and then reused. Porous, crosslinked polymers are ideal as support materials, as they are readily synthesised and the resulting

morphology can be controlled. The addition of various quantities of solvent to the polymerisation reaction can influence the porosity of the polymers formed.[7] However, this process is environmentally unfriendly, as it requires large quantities of organic solvent, the removal of which is an energy intensive process. It has been demonstrated that supercritical fluids can be used to replace traditional organic porogens.[20-23] The most commonly used supercritical fluid is carbon dioxide.[20] It is cheap, non-toxic, readily available and its density is pressure dependent. The solvent strength of carbon dioxide is proportional to its density; as such changes in the pressure of the reactor alter the interaction between the solvent and the reagents. It is possible to "fine tune" the porosity of polymers formed in a supercritical medium by controlling the reactor pressure. In addition to this, removal of the reaction solvent is achieved by simply venting the reactor, eliminating the need for extensive drying of the polymer products.

The general strategy employed for this study was adapted from work originally conducted by Wang *et al.*[8] Firstly, the enzyme has to be chemically modified so as to introduce polymerisable moieties into its structure. This is achieved by the reaction of the enzyme's primary amine groups (present in the lysine amino acid residues contained within the enzyme's backbone) with acryloyl chloride to produce a vinyl amide that can react with the growing polymer. The greater the degree of vinyl modification, the more covalent attachment points become available for the immobilisation of the enzyme.

The next step in the immobilisation strategy involves the transfer of the enzyme from aqueous buffer to an organic solvent. It is well known that enzymes are more stable in organic media than in traditional aqueous environments.[24, 25] As such the enzyme should be better able to withstand exposure to the subsequent polymerisation reaction. In

addition to this, the number of monomers and crosslinkers soluble in organic solvents is far greater than for aqueous media. This increases the number of different enzyme containing polymers that can be formed from a homogeneous reaction mixture. The extraction of enzymes into organic solvents can be achieved via the formation of ion-pairs between the enzyme's lysine residues and a suitable anionic surfactant such as AOT.[8, 24, 26, 27]

In Wang's original study catalytically active immobilised enzyme preparations were synthesised (initial activities of up to 10 nmol/mg CT.min under anhydrous conditions).[8] The effects of monomer type, crosslinker concentration, crosslinker length and solvent to monomer ratio were examined for their effects on enzyme activity. It was found that the most catalytically active preparations were formed with hydroxyethyl methacrylate (HEMA), high solvent to monomer ratios and low levels of crosslinking. No clear trends were observed with changing crosslinker lengths. All changes in enzyme activity were attributed to changes in the morphology of the polymer supports. However, no physical characterisation of the support materials was conducted, and it is much more likely that the observed changes in enzyme activity were due to the chemical nature of the differing support materials.

The aim of this study was to prepare enzyme-containing polymers via a similar method to that of Wang *et al*,[8] i.e. chemical modification of the enzyme, transfer of this modified enzyme to the organic phase by ion-pairing with an anionic surfactant, followed by the enzyme's covalent incorporation to a crosslinked polymer, during a free radically initiated polymerisation reaction. However, the organic phase employed as a porogen (hexane) by Wang *et al* will be replaced with supercritical carbon dioxide. Not only will this eliminate

the need for large quantities of organic solvent and simplify the drying of the polymer products, but should also provide some control over the pore structure of the crosslinked polymers. It has been demonstrated that changes in the reactor pressure and hence solvent density of supercritical carbon dioxide reactions can produce changes in the morphology of the polymer product.[21,22] The effects of these changes to the polymer fine structure on immobilised enzyme catalysis will be examined.

## 5.2 Materials

2,2'-azobis (2,4-dimethyl valeronitrile) (Wako pure chemicals, (V-65)), 2,4,6-trinitrobenzene sulfonic acid (BDH chemicals, (TNBS)), α-naphthol (AnalaR, 99.5 %), trimethylolpropane trimethylacrylate (Avocado chemicals, 96 % (TRIM)), methyl methacrylate (Avocado chemicals, 99 % (MMA)), potassium dihydrogen orthosulfate (Fisons, 99.5 %), sodium metabisulfite (Avocado chemicals, 97 %), thionyl chloride (Riedel-de Haen, 98 %), fumaric acid (Hopkins & Williams, 98 %), fumaryl chloride (Fluka, 99 %), β-alanine (BDH chemicals, 99 %), 2,5,5-trimethyl-hexan-1-ol (Aldrich, 85 %), 2-propyl-hexan-1-ol (Aldrich, 96 %), 5,5-dimethyl-hexan-1-ol (Aldrich, 98 %), 4-methyl-heptan-3-ol (Aldrich, 96 %), 6-methyl-heptan-2-ol (Aldrich, 96 %), hexan-1-ol (Aldrich, 98 %), nonan-1-ol (Aldrich, 98 %), benzyl alcohol (Aldrich, 99 %), α-chymotrypsin (Sigma, type II from bovine pancreas), N-acetyl-L-tyrosine ethyl ester (Sigma, Sigma grade (ATEE)), acryloyl chloride (Aldrich, 96 %), sodium bis(2-ethylhexyl) sulfosuccinate (Alidrich, 99 % (AOT)), carbon dioxide (BOC supercritical grade, 99.9995%), 1,1,1,2-tetrafluoroethane (Harp International (R134a)). All solvents

were obtained from BDH chemicals and were of General Purpose grade unless specified as HPLC or anhydrous. All other reagents were obtained from the Aldrich chemical company, unless otherwise stated.

## 5.3 Experimental

### 5.3.1 Enzyme modification

α-Chymotrypsin (600 mg) was dissolved in 80 ml of phosphate buffer (0.2 M pH 7.8). The solution was cooled in an ice bath and acryloyl chloride (326 μl) added in five equal portions over ten minutes, accompanied by stirring. The modified enzyme was purified and buffer exchanged (with a running buffer of 10 mM tris (hydroxymethyl) methylamine pH 7.8 containing 2 mM calcium chloride and 1 $\%^{v}/_{v}$ isopropanol) via gel permeation column chromatography (sephadex G-25 gel, 100-300μm). The resulting solution was adjusted to pH 7.8, and then lyophilised using an Edwards super modulyo freeze drier. A control was produced, following the same procedure as described above, but without the addition of acryloyl chloride.

One batch of unmodified α-chymotrypsin was lyophilised from 10 mM phosphate buffer (pH 7.8) at a concentration of 5 mg/ml, and a second lyophilised from 10 mM tris buffer (pH 7.8), also at 5 mg/ml.

To produce preparations of α-chymotrypsin with various degrees of modification the general procedure described above was followed, but the quantity of acryloyl chloride was altered. Thus 27 μl, 54 μl, and 81 μl respectively were added to the enzyme solution in one single portion (not in five separate portions as in the previous method). This was

allowed to react for 10 minutes before being passed though a sephadex G-25 column (stopping the reaction by separating the acryloyl chloride from the α-chymotrypsin). The pH of the enzyme solution was readjusted to 7.8 with sodium hydroxide solution, prior to freeze-drying.

### 5.3.2 Enzyme activity assay

The catalytic activity of the α-chymotrypsin containing preparations was quantified by following the transesterification assay between ATEE and propan-1-ol in hexane. Aliquots of the reaction mixture were removed at regular time intervals and analysed with HPLC to determine the relative quantities of products and starting materials (chapter two, section 2.2.5).

### 5.3.3 Enzyme extraction into organic solvents

The general method employed for measuring the effects of variables on the efficiency of extracting enzymes into organic media was as follows; 4 ml of buffer (10 mM Tris, 2 mM $CaCl_2$, 1 $\%^{v}/_{v}$ isopropanol pH 8.0) containing 1 mg/ml enzyme (determined by UV absorbance) was contacted with 4 ml of a 2 mM solution of AOT in hexane. This mixture was then stirred magnetically at 600 rpm with a 20 mm cross stirrer bar for 20 minutes to form an emulsion. A Radleys 12-well reaction carousel was used to provide control and reproducibility over the temperature and stirring speed of the reactions. The emulsions were then centrifuged at 2000 g for 10 minutes to separate the two phases, and the absorbance of the organic layer measured at 280 nm using a Perkin-Elmer Lamda-25 UV/VIS instrument. All reactions carried out with at least 6 duplicates. Solutions of

various AOT concentrations were prepared via dilution of a 20 mM stock solution with hexane.

### 5.3.4 Protein concentration determination

Two general methods to determine protein concentration were employed. When a significant quantity of protein dissolved in aqueous solution was available for analysis (several milliliters), the UV absorbance at 280 nm due to the protein present could be measured directly. This absorbance was then compared to a calibration curve constructed from a series of twenty known concentrations of enzyme prepared from commercially available α-chymotrypsin. All absorbance measurements were taken using a Perkin-Elmer Lambda 25 UV/Vis instrument. The Bradford's assay was employed when the concentration of protein was too low for direct detection with UV/Vis.[28] Bradford's assay is known to be able to detect nano-mole quantities of protein and works by monitoring the change in absorbance that occurs when a UV active dye chelates to the primary amine groups present on the surface of proteins. 100 µl aliquots of aqueous protein solution were added to 5 ml of Bradford's stock reagent. Bradford's stock reagent was made by dissolving 100 mg of Coomassie Brilliant Blue G-250 in 50 ml of 95 % ethanol. To this solution 100 ml of 85 % ($^{w}/_{v}$) orthophosphoric acid was added. The mixture was then diluted to 1000 ml with distilled water. Solutions were allowed to stand for five minutes. The change in UV absorbance at 595 nm that occurs on the addition of protein solutions to the stock reagent was used to calculate the protein content by comparison with a calibration curve, constructed from solutions of known α-chymotrypsin concentration. All UV measurements were completed within one hour of the addition of the protein

solution to the Bradford's stock reagent, as it is known that the complexes formed degrade over time. N.B. Bradford's technique was not employed for protein concentrations greater than 0.5 mg/ml as the relationship between absorbance and concentration is no longer linear at these higher concentrations. Solutions with protein contents greater than 0.5 mg/ml were diluted prior to the application of the Bradford's assay, or were measured directly with UV/Vis spectrometry at 280 nm.

### 5.3.5 Primary amine group determination

The extent to which the primary amine groups of the enzyme had been modified was examined using one of two methods. Samples of the enzyme (buffer salts removed using a sephadex-G25 column, followed by freeze drying) were submitted to the EPSRC National Mass Spectrometry Service Centres at Swansea and Warwick. The instrument used to analyse these samples was an Applied Biosystems Mariner 5271 electro-spray mass spectrometer. Samples of buffer free, freeze-dried enzyme were also submitted to Liverpool University Mass Spectrometry Service. These samples were analysed with a Micromass LCT mass spectrometer and a Micromass TOF-SPEC/DEC VAX instrument. Matrix Assisted Laser Desorption Ionisation (MALDI) mass spectrometry was also employed for the analysis of the protein samples.

Chemical analysis of the remaining primary amine groups present on the enzyme after modification was also conducted. In order to accurately determine the number of primary amine groups that remain on the enzyme, a method of standard addition was employed. A series of alanine standards were prepared as follows; a stock solution of 0.2 M alanine in water was prepared, and then diluted to produce concentrations of 0.2 mM, 0.16 mM,

0.12 mM, 0.08 mM and 0.04 mM. 50 µl of water was then added to 1.5 ml of each solution. These solutions were transferred to UV cuvettes and 50 µl of 0.03 M 2,4,6-trinitrobenzene sulfonic acid (TNBS) added to each.[29-31] The UV absorbance at 420 nm was measured after 150 minutes.

This procedure was repeated, but with the addition of 50 µl of 1 mg/ml (accurate concentration determined via UV absorbance at 280 nm) in place of the 50 µl aliquot of water. The UV absorbance for each reaction was determined in the same way.

### 5.3.6 Surfactant synthesis

Several novel surfactants were synthesised based on the di-alkyl, anionic morphology of AOT, according to the methodology reported by Eastoe *et al.*[32] The general synthesis and work-up procedure is included below. The differences in branching were achieved by using the corresponding branched alcohol.

Typically, an alcohol (10ml, excess) was refluxed with 7.0 g of pyridine in dry THF. To this solution, fumaryl chloride (5.0 g) was added dropwise. The resulting purple/black solution was then refluxed overnight. The reaction mixture was filtered to remove the solid pyridinium chloride. The THF was removed under vacuum and the resulting black oil re-dissolved in diethyl ether. This solution was washed several times with alternating sodium carbonate and hydrochloric acid solutions. The ethereal layer was separated and dried over magnesium sulphate. The ether was removed under vacuum and the resulting oil distilled under high vacuum. The di-ester formed was usually obtained as a pale yellow/green oil at between 160-240 °C at $>10^{-4}$mmHg depending on which ester was being separated. The distilled di-ester was then dissolved in a 1:1 mixture of

water/absolute ethanol and refluxed with an excess of sodium metabisulfite for 24 hours. The resulting solution was filtered and the solvents removed under vacuum. Typically a white solid was formed which was then dissolved in dry methanol. Unreacted sodium metabisulfite does not dissolve in dry methanol, and so formed a suspension. This suspension was separated with several centrifuge cycles. Removal of the methanol under vacuum yielded the anionic surfactant.

### 5.3.7 Polymerisations conducted in conventional organic solvents

The acryloylated ion-paired α-chymotrypsin, monomer, crosslinker and initiator were dissolved in hexane. The concentration of initiator (V-65) was fixed at 9.0 mg/ml, whereas the concentration of the other species was varied according to the conditions employed. Polymerisation was initiated by irradiation with a filtered mercury arc lamp (the 365 nm wavelength decomposes V-65) for 18 hrs at room temperature. Subsequently, it was found that thermal initiation at 60 °C for 18 hrs represented a much improved initiation method. A twelve well Radley's reaction carousel proved to be an ideal reaction vessel for these polymerisations, providing excellent temperature control as well in addition to good reproducibility and uniform conditions for all twelve wells. The resulting polymers were ground into a fine powder and washed extensively with hexane, then vacuum dried overnight at 35 °C.

### 5.3.8 Polymerisations conducted in supercritical carbon dioxide

All polymerisations and solubility tests were carried out in a 10 ml sealed steel vessel, fitted with 20 mm solid sapphire window that enables the status of the reaction mixture to

be visualised. Heating was achieved by means of an external heating coil and fuzzy logic temperature controller. Pressure was monitored with a pressure transducer fitted to the rear of the reactor.

The polymerisations carried out typically involved the addition of 0.66 g of TRIM, 0.062 g of recrystalised AIBN and 5.00 g of MMA (containing 1.00 mg/ml α-chymotrypsin ion-paired to AOT). The reaction vessel was then filled with liquid $CO_2$ at an initial pressure of approximately 1750 psi. The vessel was sealed and heated in 5 °C stages over one hour to a constant temperature of 60 °C with a corresponding pressure of approximately 5500 psi. A monolith was recovered on venting the reactor after a total reaction time of four hours.

Solubility tests were conducted for a solution of chymotrypsin in "wet" hexane, as well as for α-chymotrypsin in rigorously dried hexane (#4 molecular sieves). The solubility of AOT in dry hexane was also tested in supercritical carbon dioxide.

### 5.3.9 Polymerisations conducted in 1,1,1,2-tetrafluoroethane (R134a)

The same reaction vessel was used as for the polymerisations conducted in supercritical carbon dioxide. Typically, 2.85 g of MMA (containing 1.50 mg/ml of modified chymotrypsin ion paired to AOT), 3.20 g of TRIM and 0.10 g of recrystalised AIBN were added to the reactor. Approximately 5.30 ml of liquid tetrafluoroethane (R134a) was added to the reactor at an initial pressure of approximately 140 psi. The reactor was heated to 60 °C over one hour, with a final pressure of approximately 2100 psi. The reactor was vented after four hours, yielding a solid white monolith.

A similar procedure was followed using a solution of unmodified enzyme in monomer. The reaction mixture consisted of 3.00 g of TRIM, 2.50 g of MMA (containing 1.00 mg/ml of solubilised ion-paired α-chymotrypsin) and 0.10 g of recrystalised AIBN.

## 5.4 Results and discussion

### 5.4.1 Enzyme modification and activity analysis

The first stage in the proposed mechanism for the formation of multipoint covalently immobilised enzymes in porous polymer matrices is the modification of the enzyme. Polymerisable moieties need to be introduced to the surface of the enzyme in order for them to undergo covalent reaction during the subsequent free radical polymerisation. The method chosen for this modification step was first reported in the literature by Wang *et al.*[7, 8] Acryloyl chloride is a vinyl containing compound that readily undergoes reaction with nucleophiles, such as the primary amine groups present on the surface of the enzyme. Primary amine groups are only contained in the side chain of lysine, which is one of the amino acids that make up the structure of α-chymotrypsin. As the crystal structure of α-chymotrypsin has already been reported, it is possible to determine the exact number of lysine residues present in the enzyme, as well as their relative position in the enzyme's three-dimensional structure (figure 5.1). By reorienting a three-dimensional computer generated model of α-chymotrypsin it is also possible to make a judgement as to how many of these lysine residues are present on the exterior surface of the enzyme, and so are accessible to the incoming reagents. All sixteen lysine residues present in α-

chymotrypsin's three-dimensional structure appear to be on or near the surface of the structure, making them accessible for vinyl modification by acryloyl chloride.

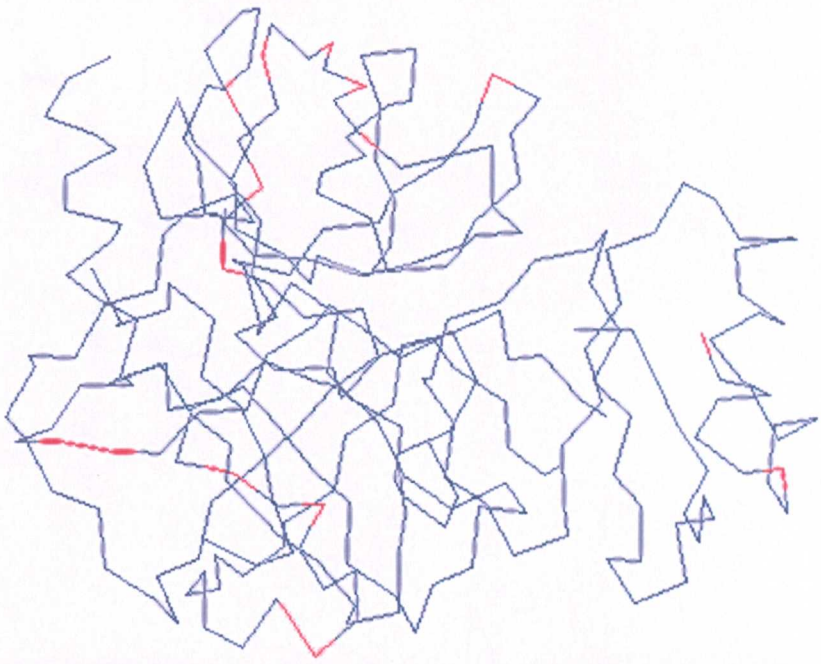


*Figure 5.1 Crystal structure of α-chymotrypsin represented by a linear amino acid sequence.*[42] *Primary amine containing lysine residues are highlighted in red. There are sixteen lysine residues present in the enzymes structure. Manipulation of the three dimensional structure allows the relative position of these lysine residues to be determined.*

It is also important that the modification chemistry does not interfere with the chemistry that occurs at the enzyme's active site. This would most likely result in inhibition of the active site and deactivation of the enzyme. Fortunately α-chymotrypsin is a serine-based protease, with an active site consisting of aspartate, histidine, serine and glycine amino acids.[33] Lysine residues do not directly participate in the active site's chemistry (figure 5.2).

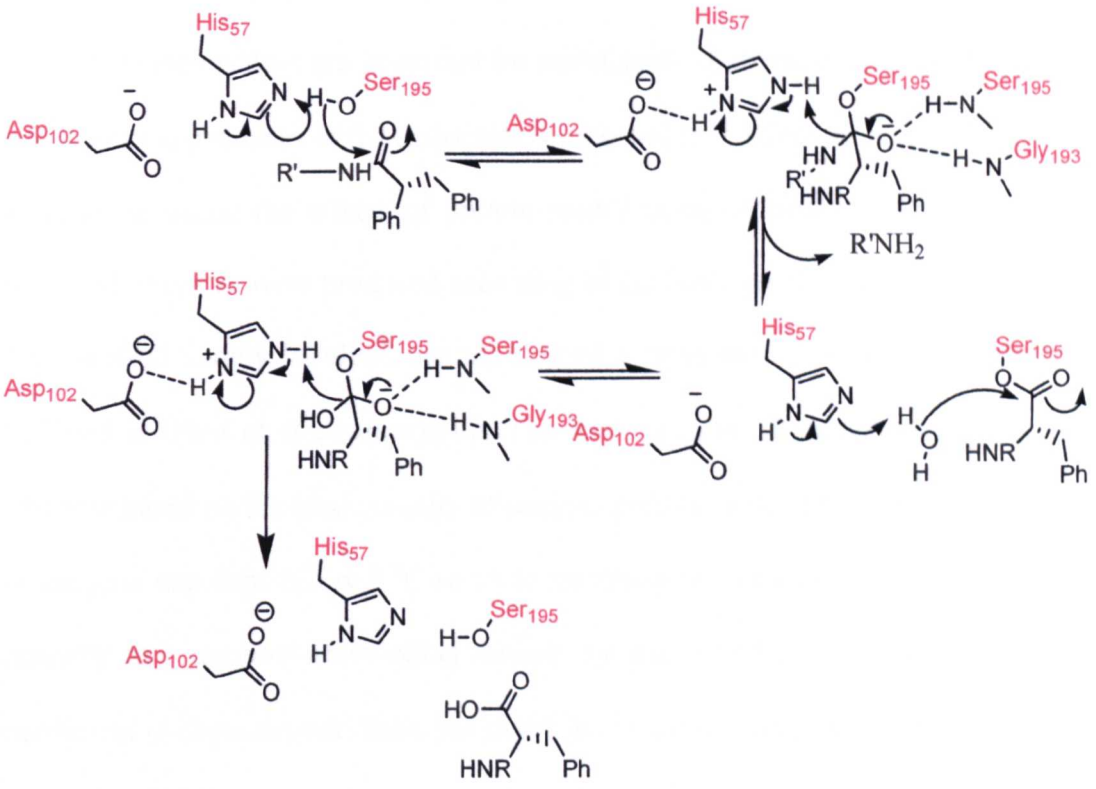



*Figure 5.2 A typical mechanism for the hydrolysis of an amino acid that occurs at the active site of α-chymotrypsin. This illustrates the absence of any lysine residues participating in the catalysis.*[33]

Although it has been shown that lysine residues do not directly participate in α-chymotrypsin's catalytic cycle, it does not necessarily mean that lysine residues are not important for the enzyme's activity. The catalytic activity of enzymes is largely based on their complex three-dimensional structure. This conformation is maintained through non-covalent attractive and repulsive interactions that occur between functional group present in the enzyme's amino acid chain. It is known that at pH 7.0 negatively charged carboxylate anions resulting from carboxylic acid residues, often bind to the protonated amine residues of lysine groups. In aqueous solution, these electrostatic interactions are comparatively strong (25-50 $kJmol^{-1}$) and become even stronger (in relative terms) when

the enzyme is employed in a non-aqueous environment.[33, 34] It may be that lysine's primary amine residues are important for maintaining α-chymotrypsin's tertiary structure and hence the geometry of the active site, which will then effect the enzyme's catalysis.

In order to assess the effects of protein modification on catalytic activity, a range of modified enzymes were produced according to the basic methodology of Dordick *et al.*[7] This method simply involved the addition of a large excess of acryloyl chloride to a buffered solution of α-chymotrypsin. The molar excess of reagent was approximately 150-fold based on the total quantity of enzyme present in solution. The buffered solution of enzyme was kept below 5 °C so as to minimise any deactivation of the enzyme. To quantify any enzyme deactivation caused by the modification procedure, additional batches of α-chymotrypsin were subjected to the same modification procedure but with differing amounts of acryloyl chloride. Batches were synthesised with 0, 15, 30 and 45 fold molar excesses of reagent. It was assumed that these reduced levels of reagent would produce batches of enzyme with differing levels of modification.

The transesterification assay described previously (chapter two section 2.2.5) was employed to determine the catalytic activity of the modified enzyme batches. It was found that all the batches of enzyme possessed approximately twice the catalytic activity of the unmodified equivalent lyophilised from a phosphate buffer. This is most likely due to the buffer exchange step of the modification procedure. The initial phosphate buffer is replaced with a more favourable tris buffer, resulting in increased enzyme catalysis.

However, the transesterification assay demonstrated that all the different batches of modified enzyme (including the unmodified control lyophilised from the tris buffer)

possessed approximately the same catalytic activity. This implies that the modification procedure did not induce any significant enzyme deactivation.

### 5.4.2 Enzyme solubilisation

The next stage of the proposed covalent immobilisation mechanism involved dissolving the modified enzyme in non-aqueous media. As enzymes will not dissolve in organic solvents whilst in their native state, it is necessary to first modify the enzyme in some way, or find a suitable surfactant system in order to make them more soluble. The method chosen for the solvation of α-chymotrypsin in non-aqueous media was first reported by Paradkar *et al.*[26, 27] Anionic surfactants were found to associate with the primary amines of the lysine amino acid groups of the enzyme, via a non-covalent electrostatic interaction. There are several advantages to this method of dissolving enzymes, when compared to common alternative strategies such as poly(ethylene-glycol) modification.[35] The enzyme is not subjected to any covalent bond forming chemistry, which can lead to deactivation. The extraction efficiency for unmodified enzyme is reportedly very high (>90 %).[27] High catalytic activities are retained upon resolvation in aqueous media.[26] It has also been demonstrated that very high concentrations of enzyme in non-aqueous media can be achieved through this method, compared to most other methodologies (~500 mg/ml) which allows for the subsequent formation of enzyme containing polymers with very high levels of protein loading (~50 $\%^{w}/_{w}$).[7, 8]

Although extraction efficiencies as high as 90 % have been reported in the literature, the extraction efficiency obtained in this study was typically around 50 % for unmodified α-chymotrypsin, when the same procedures were employed. An extraction efficiency of

only 50 % would still be sufficient for the enzyme immobilisation procedure, as the enzyme can be concentrated by evaporation of most of the non-aqueous media under vacuum. However, when the same extraction methodology was applied to the modified enzyme (using the 150-fold molar excess of acryloyl chloride reported in the literature[7, 8]), very little protein was detected in the organic phase (<3 % extraction efficiency). This inconsistency with the published results has also been experienced by other research groups (unpublished results).

A drop in extraction efficiency would be expected, as both the modification procedure and the surfactant ion-pairing mechanism compete for the same primary amine groups present on the surface of the enzyme. Once an amine group has been converted to an amide during the modification reaction, it is no longer able to form ion-pairs with the surfactant. However, it is reported in the literature that this modification procedure results in approximately half the enzyme's lysine groups being converted to amides.[7] This "half-modified" enzyme is then extracted into the organic phase with an efficiency of around 55 %. Hence given our previous results, we would expect an extraction efficiency of at least 25 %.

In order to determine whether α-chymotrypsin with lower levels of modification could be extracted into the organic phase, the alternative batches of enzyme that were reacted with less acryloyl chloride were also examined for their solubility (table 5.1).

It can be seen that the extraction efficiency of the modified enzyme is related to the quantity of reagent employed during the reaction procedure, and hence the degree of modification. Better enzyme extraction is observed with lower levels of modification. The number of primary amine groups present after modification was determined by

following the change in UV absorbance that accompanies the reaction of these amine groups with trinitrobenzene sulfonic acid.[29-31] It was found that the reaction of α-chymotrypsin with a 45-fold molar excess of acryloyl chloride resulted in the detection of approximately 17 % fewer primary amine groups. This corresponds to the modification of only three of the enzyme's lysine residues. Therefore, the extraction efficiency of the ion-pairing process decreases from around 60 % to only 30 % when the number of lysine groups is reduce from 16 to 13.

| Degree of Modification | % Extraction |
|---|---|
| Unmodified | 60.4 |
| 150 fold molar excess | 2.7 |
| 45 fold molar excess | 28.8 |
| 30 fold molar excess | 29.9 |
| 15 fold molar excess | 35.5 |

*Table 5.1 Extraction efficiency of α-chymotrypsin subjected to various degrees of modification by treatment with different molar excesses of acryloyl chloride. 4 ml of buffer (10 mM tris, 2 mM $CaCl_2$, 1 %v/v isopropanol pH 8.0) containing 1 mg/ml α-chymotrypsin was stirred with 4 ml of a 2 mM AOT solution in hexane. Emulsions were separated by centrifuge and the protein content of the organic phase determined by UV absorbance at 280 nm. Six replicates of each extraction were carried out at room temperature (22 °C).*

Although the concentration of the modified enzyme could be increased via the evaporation of the organic solvent, the dissolved enzyme would only have three modified

lysine groups. Subsequent immobilisation through exposure to a free radical polymerisation reaction could only result in an enzyme covalently attached to the polymer via three anchor points. This would be the greatest number of immobilisation points achievable, assuming all the enzyme's polymerisable functionalities undergo reaction. In order to increase the number of immobilisation points it would be necessary to dissolve α-chymotrypsin with a greater degree of modification, in organic media. Therefore the extraction methodology was examined in order to optimise the procedure and hence be able to extract more heavily modified enzyme.

Three variables of the extraction methodology were examined for their effects on the efficiency of the extraction process. The pH of the aqueous buffer phase (figure 5.3), the temperature at which the extraction was carried out (figure 5.4) and the concentration of surfactant in the aqueous phase (figure 5.5) were all shown to significantly influence the efficiency of the extraction process.

The results of these experiments demonstrate that each of the three variables examined has a clear optimal range for enzyme extraction. The optimal pH was found to be around 7.5, which is almost ideal for the catalytic activity of α-chymotrypsin. Changing the pH of the buffer solution will influence the ionisation state of the enzyme and hence the electrostatic ion-pairing interaction of the anionic surfactant. It was expected that the association between the enzyme and the surfactant would be greatest at lower pH levels, when the enzyme's lysine groups are fully protonated. However this experiment clearly shows that neutral pH levels are best for the ion-pairing interaction.

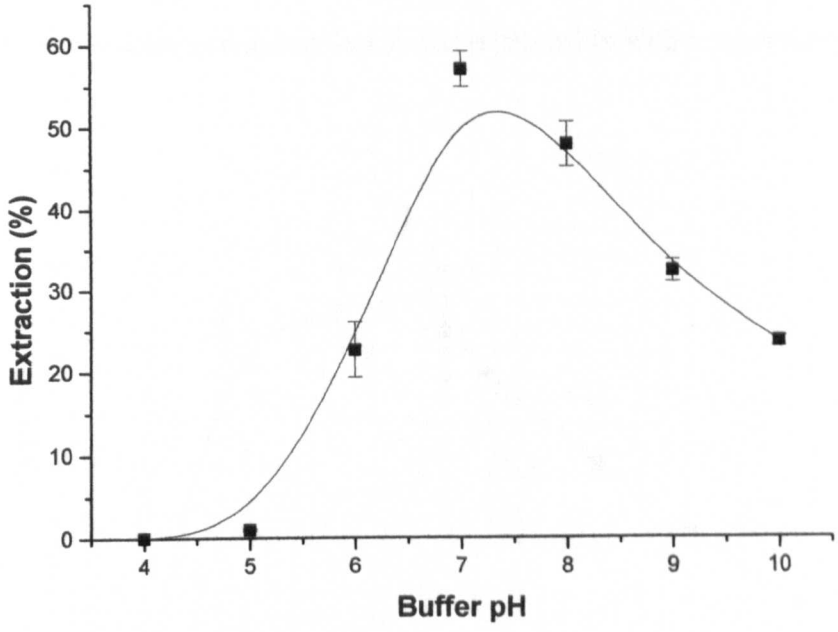



*Figure 5.3 Influence of aqueous buffer pH on the extraction efficiency of unmodified α-chymotrypsin. 4 ml of buffer (10 mM tris, 2 mM $CaCl_2$, 1 %$^v/_v$ isopropanol pH was varied between 4.0 and 10.0) containing 1 mg/ml α-chymotrypsin was stirred with 4 ml of a 2 mM AOT solution in hexane. Emulsions were separated by centrifuge and the protein content of the organic phase determined by UV absorbance at 280 nm. Six replicates of each extraction were carried out at room temperature (22 °C).*

The temperature at which the extraction procedure was conducted was also shown to be important for the extraction efficiency. The optimum temperature was found to be around 20 °C, which was marginally below the laboratory temperature (22 °C). It was expected that lowering the temperature of the extraction system would increase the stability of the non-covalent electrostatic interactions between the enzyme and the surfactant, enhancing the overall extraction efficiency. However the results show that the extraction efficiency

declines rapidly at temperatures below 20 °C. At higher temperatures, the enzyme extraction efficiency declines in a linear relationship with temperature.

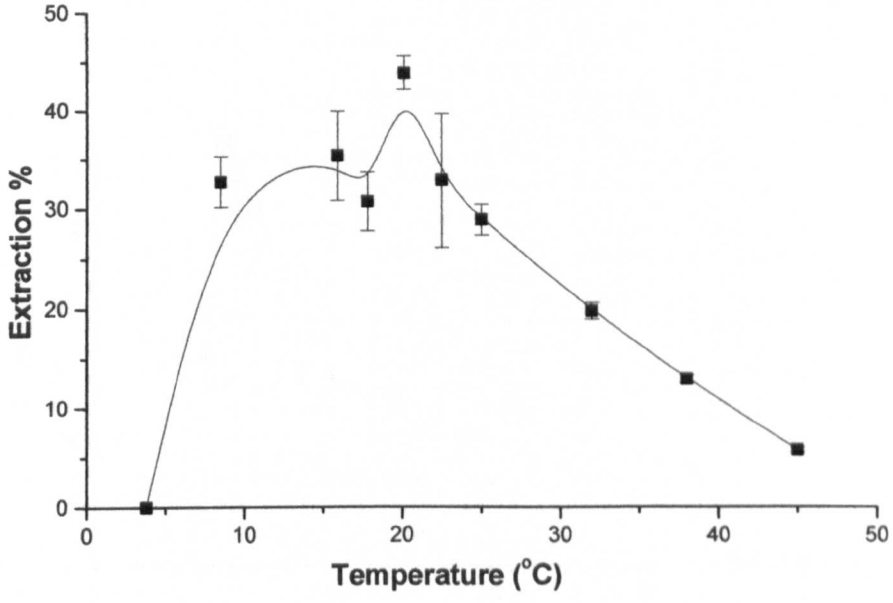



*Figure 5.4 Influence of reaction temperature on the extraction efficiency of unmodified α-chymotrypsin. 4 ml of buffer (10 mM tris, 2 mM $CaCl_2$, 1 %$^v/_v$ isopropanol pH 8.0) containing 1 mg/ml α-chymotrypsin was stirred with 4 ml of a 2 mM AOT solution in hexane. Emulsions were separated by centrifuge and the protein content of the organic phase determined by UV absorbance at 280 nm. Six replicates of each extraction were carried out at temperatures of between 4 °C and 45 °C.*

This is most likely due to the thermal destabilisation of the surfactant-enzyme interaction, preventing the transfer of the enzyme to the organic phase. The results show an apparent spike in extraction efficiency between the temperatures of 17 and 23 °C. These extractions were repeated six times in order to gain accurate information as to the extent of experimental errors. The results show that even at the maximum limit of these errors, the "20 °C" extraction is still significantly better than any others. It is likely that the

extraction efficiency is being affected by subtle changes in pH as well as the changes in temperature, as it is known that the pH of the Tris buffer used in the extraction procedure is temperature sensitive.

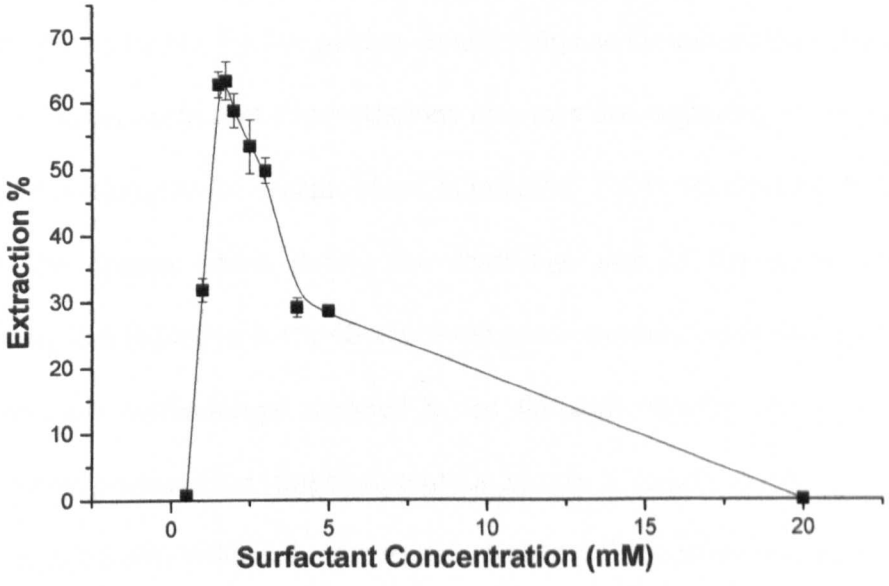


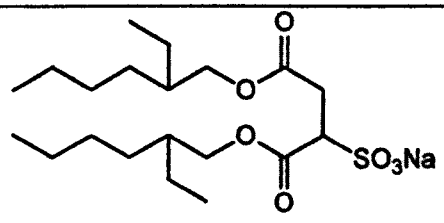
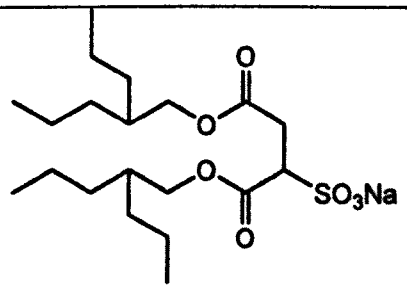
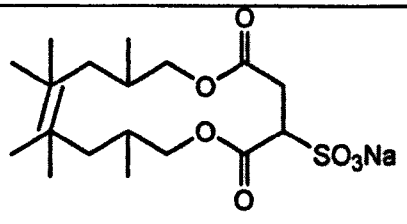
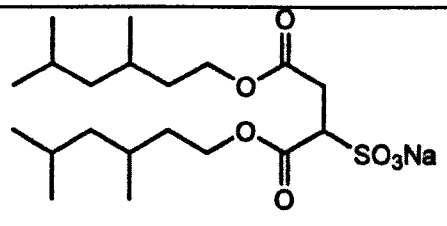
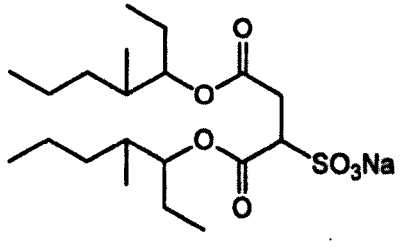
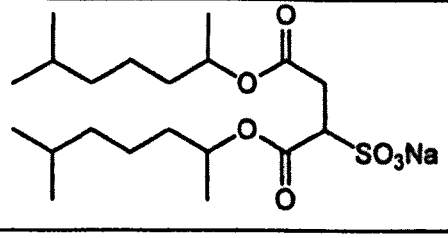

*Figure 5.5 Influence of surfactant concentration on the extraction efficiency of unmodified α-chymotrypsin 4 ml of buffer (10 mM tris, 2 mM $CaCl_2$, 1 %$^v/_v$ isopropanol pH 8.0) containing 1 mg/ml α-chymotrypsin was stirred with 4 ml of an AOT solution (varying in concentration between 0.5 mM and 20 mM) in hexane. Emulsions were separated by centrifuge and the protein content of the organic phase determined by UV absorbance at 280 nm. Six replicates of each extraction were carried out at room temperature (22 °C).*

The effect of surfactant concentration in the organic phase on enzyme extraction efficiency was also examined. The results show that below AOT concentrations of 0.5 mM, no enzyme extraction is observed. This is probably because there are simply too few AOT molecules available for ion pairing, preventing any of the enzyme molecules from associating with sufficient numbers of surfactants to enable their transfer to the

organic phase. Extraction efficiency increases to a maximum of 63 % (when conducted at 30 °C) with a surfactant concentration of 1.75 mM. Above this level, the extraction efficiency was observed to decline, with no enzyme transferred to the organic phase at surfactant concentrations of 20 mM. This is peculiar, as a greater number of surfactant molecules available for ion pairing should enhance the extraction efficiency. It may be that at higher surfactant concentrations enzymes are beginning to agglomerate and are being transferred to the organic phase in micelles. These micelles would then be removed from the organic phase during the centrifuge step of the extraction methodology, resulting in a reduction in the observed enzyme concentration in the organic phase.

Overall, the methodology reported in the literature already offers the best extraction efficiency, provided the temperature of the system is closely monitored. In order to attain greater extraction efficiencies the nature of the AOT surfactant was examined. A series of dialkyl anionic surfactants based on AOT were synthesised according to the general method described by Eastoe *et al.*[32]

After synthesis and purification, these surfactants were dissolved in hexane at a concentration of 2 mM and employed in the same extraction methodology as was used for the original AOT surfactant examined previously (table 5.2).

| Compound | Structure | Relative Efficiency |
|---|---|---|
| Sodium bis (2-ethyl hexyl) sulfosuccinate **AOT** |  | 100% |
| Sodium bis (2-propyl pentyl) sulfosuccinate **"A"** |  | 99.1% |
| Sodium bis (2,4,4-trimethyl pentyl) sulfosuccinate **"B"** |  | 2.6% |
| Sodium bis (3,5-dimethyl hexyl) sulfosuccinate **"C"** |  | 37.5% |
| Sodium bis (1-ethyl 2-methyl pentyl) sulfosuccinate **"D"** |  | 45.2% |
| Sodium bis (1,5-dimethyl hexyl) sulfosuccinate |  | 114% |

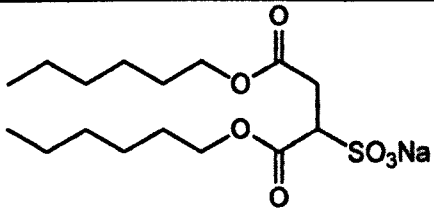
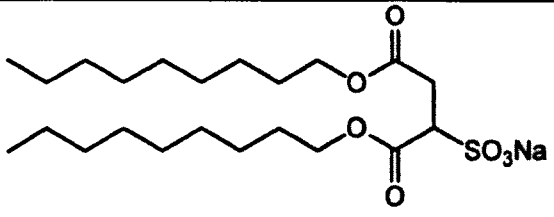
| | | |
|---|---|---|
| "E" | | |
| Sodium bis hexyl sulfosuccinate "F" |  | 0.0% |
| Sodium bis nonyl sulfosuccinate "G" |  | 0.0% |

*Table 5.2 α-chymotrypsin extraction efficiencies of a series of anionic dialkyl surfactants. All extraction efficiencies are measured relative to AOT studied under the same conditions. 4 ml of buffer (10 mM tris, 2 mM $CaCl_2$, 1 %v/v isopropanol pH 8.0) containing 1 mg/ml α-chymotrypsin was stirred with 4 ml of a 2 mM surfactant solution in hexane. Emulsions were separated by centrifuge and the protein content of the organic phase determined by UV absorbance at 280 nm. Six replicates of each extraction were carried out at a temperature of 30 °C.*

It is clear from the results obtained that subtle changes in the alkyl chain of the surfactants structure have profound effects of the corresponding enzyme extraction efficiency. Only the surfactants with branched alkyl chains display any ability to extract α-chymotrypsin into organic media. It is possible that if the two surfactants synthesised with linear alkyl chains do ion-pair to the enzyme, their linear chains wrap around the protein reducing the overall complex's solubility in organic media. The chemical structures of AOT and surfactant "A" are similar, as are their corresponding extraction efficiencies. However surfactant "D" also has a similar structure, with the exception of

the position of one methyl group, yet the extraction efficiency was halved. Only one of the novel surfactants synthesised was shown to be better than the commercially available AOT for the extraction of α-chymotrypsin into organic media (surfactant "E"). However, when the methyl group present at the #1 position in the alkyl chains of surfactant "E" were moved to the #3 positions to form surfactant "C", the enzyme extraction efficiency was significantly reduced. These comparatively small changes to the surfactants structure appear to have a large effect on the solubility of the enzyme-surfactant complexes that were formed by the ion-pairing interaction.

The two most promising surfactants ("A" and "E") were examined for their ability to dissolve the various partially modified α-chymotrypsin preparations in organic media (table 5.3).

| **Degree of modification** | **% Extraction AOT** | **% Extraction AOT A** | **% Extraction AOT E** |
|---|---|---|---|
| Unmodified | 60.4 | 57.2 | 68.8 |
| 150 fold molar XS | 2.7 | 1.0 | 2.8 |
| 45 fold molar XS | 28.8 | 20.4 | 18.8 |
| 30 fold molar XS | 29.9 | 23.4 | 19.9 |
| 15 fold molar XS | 35.1 | 29.9 | 22.5 |

*Table 5.3 Extraction efficiency of modified α-chymotrypsin when ion-paired with various anionic surfactants. 4 ml of buffer (10 mM tris, 2 mM $CaCl_2$, 1 %$^v/_v$ isopropanol pH 8.0) containing 1 mg/ml α-chymotrypsin was stirred with 4 ml of a 2 mM surfactant solution in hexane. Emulsions were separated by centrifuge and the protein content of the organic phase determined by UV absorbance at 280 nm. Six replicates of each extraction were carried out at room temperature (22 °C).*

The results indicated that none of the synthesised surfactants were significantly better at extracting the enzyme into the organic phase than the commercially available AOT. Therefore all subsequent experiments were conducted using AOT and using experimental conditions similar to those found in the literature.[7, 8, 26, 27]

### 5.4.3 Polymerisations conducted in non-supercritical media

The next stage in the enzyme immobilisation strategy involved the incorporation of the α-chymotrypsin dissolved in organic media, during a free radically initiated polymerisation. Unmodified, native α-chymotrypsin was dissolved in hexane via the ion pairing mechanism described previously.[7, 8, 26, 27] The enzyme containing solvent was then concentrated via evaporation under vacuum. This concentrated solution was then used as a stock solution for the addition of measured quantities of enzyme to polymerisation reactions involving two different monomers. Various enzyme-loading levels between 0 and 3.17 $\%^{w}/_{w}$ were studied for crosslinked methyl methacrylate (MMA) and ethyl vinyl ether (EVE) monomers. Crosslinking was achieved by the inclusion of a fixed quantity of trimethylolpropane trimethacrylate (TRIM). A constant volume of organic solvent (hexane) was added to each polymerisation reaction, consisting of a known amount of dissolved α-chymotrypsin stock solution diluted with fresh hexane. Free radical polymerisation was initiated thermally. The EVE and MMA polymers were formed by heating the reaction mixture to 60 °C overnight. In each case, the resulting polymers were removed from the reaction vessel and dried under vacuum at 45 °C overnight to remove the reaction solvent. All polymers were ground to a fine powder before being sieved to remove the 90 to 100 μm particle size range. This particle size range was then assayed for

the immobilised enzymes catalytic activity, by following the transesterification assay described previously (chapter two, section 2.2.5). It was necessary to assay only discrete particle sizes so as to eliminate the diffusional effects of the substrates and products to and from the enzymes active sites. The results clearly show that the catalytic activity of the polymers is directly related to the quantity of enzyme immobilised during the polymerisation reaction (figures 5.6 and 5.7).

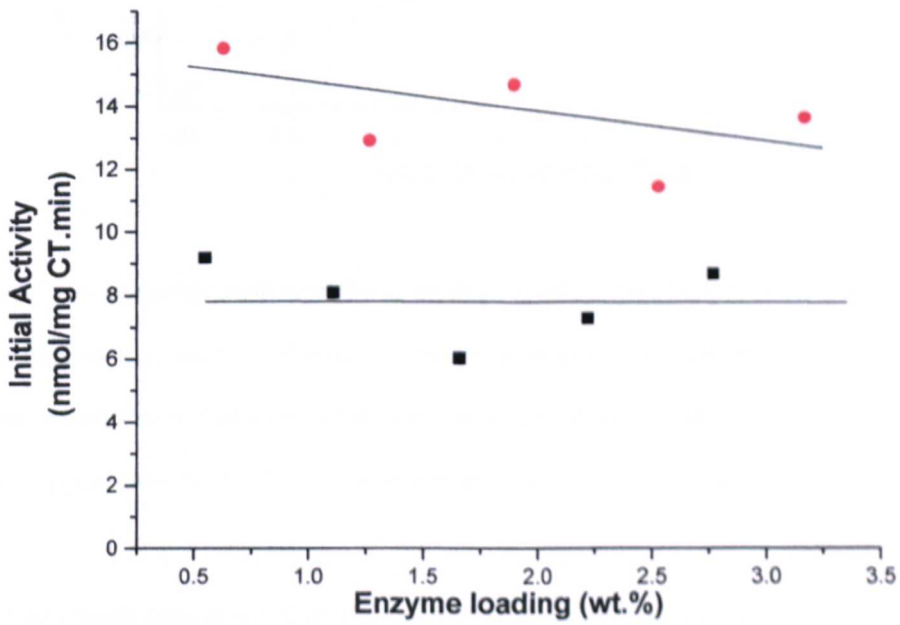



*Figure 5.6 Transesterification activities of α-chymotrypsin containing polymers. Red circles = poly(EVE). Black squares = poly(MMA). Catalytic activities of preparations containing between 0.5 and 3.17 $\%^{w}/_{w}$ enzyme are displayed. No catalytic activity was observed for any polymer preparations with no enzyme loading (data not shown). All catalytic activities are displayed in units of nmol/mg CT.min, i.e. normalised to the quantity of α-chymotrypsin immobilised.*

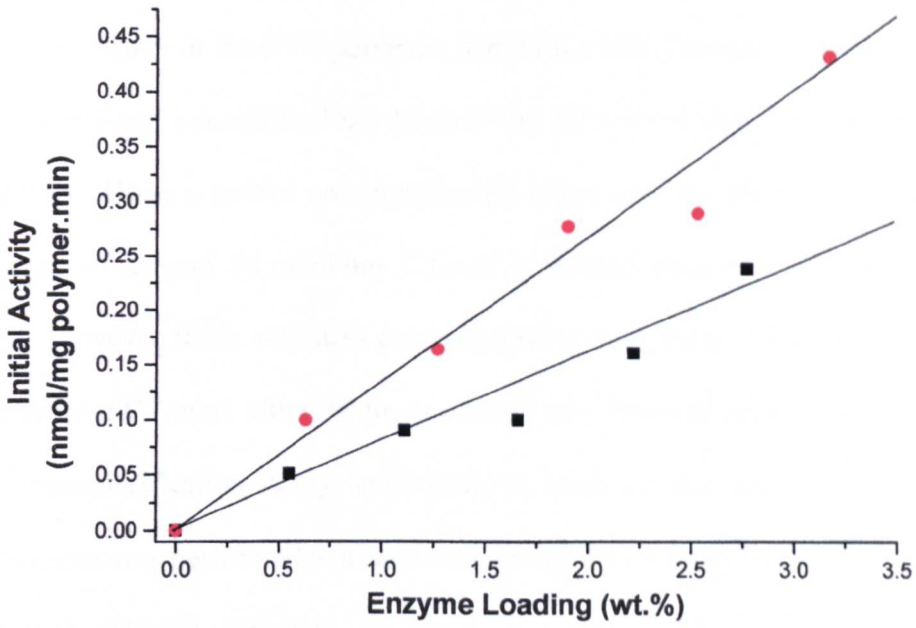



*Figure 5.7 Transesterification activities of α-chymotrypsin containing polymers. Red circles = poly(EVE). Black squares = poly(MMA). Catalytic activities of preparations containing between 0.5 and 3.17 $\%^{w}/_{w}$ enzyme are displayed. Catalytic activities are displayed in units of nmol/mg polymer.min, i.e. activity per mass of preparation, not normalised to the quantity of enzyme immobilised.*

The enzyme-containing polymers formed from MMA and EVE monomers both displayed catalytic activity directly proportional to the amount of enzyme incorporated (figure 5.7). This indicates the polymers are not overloaded with catalyst at these levels and that greater catalytic rates could be achieved by the addition of more α-chymotrypsin to the polymerisation mixture. This is confirmed by the re-plotted data (figure 5.6) displaying catalytic activity as a function of enzyme content, instead of mass of polymer. These results indicate that as the quantity of immobilised enzyme is increased, the enzyme efficiency does not decrease significantly.

Overall, it can be seen that under the polymerisation conditions employed, enzyme activity is greater in the EVE polymers than in the MMA polymers. The catalytic rate per mass of enzyme immobilised is ~14 nmol/mg CT.min of EVE and ~8 nmol/mg CT.min for MMA. These activities are significantly lower than for the best lyophilised enzyme activities of around 30 nmol/mg CT.min measured previously (chapter two, section 2.3.5). However these activities are comparable with those obtained by Dordick *et al* (10 nmol/mg CT.min), although those results were obtained under anhydrous conditions.[7] The transesterification assay employed to examine the catalytic activity of these preparations was performed at a water activity of 0.43, and so would be expected to result in higher catalytic activities. In order to attribute this deactivation to either the immobilisation procedure, or to the dissolved enzyme's contact with the various monomers, the catalytic activity of a lyophilised α-chymotrypsin preparation suspended in a variety of different media was determined. All reactions were carried out at the same fixed water activity and temperature as was used to assay the immobilised enzyme preparations (figure 5.8).

The results of this experiment show that the lyophilised enzyme is catalytically active when suspended in a variety of organic media. It is also clear that under these conditions EVE is as good a medium as hexane for enzyme catalysis. This is surprising, as it is well known that enzymes are often deactivated when exposed to polar organic solvents.[36] The low viscosity of ethers and hence better diffusivity of substrates to and from the enzyme's active sites, may compensate for any deactivation caused by the solvent's polarity.

Hydroxyethyl methacrylate was also examined as a potential medium for enzymatic catalysis. No activity was observed, although this is most likely due to the solvent

participating in an enzyme-catalysed esterification. Due to the very high molar excess of the solvent, compared to the other substrates present in the reaction mixture, it may be that the intended transesterification products are not formed, and hence not observed in the HPLC assay.

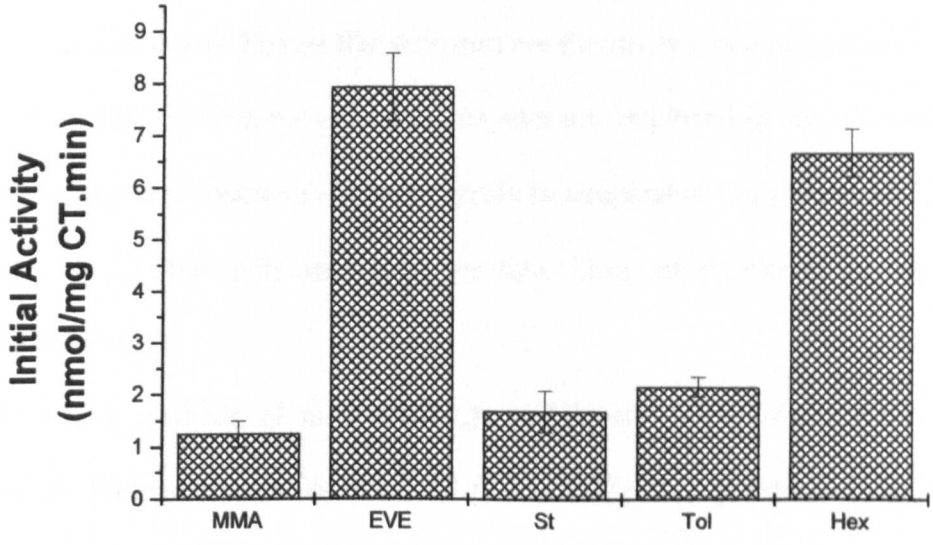



*Figure 5.8 Catalytic activities for a single batch of α-chymotrypsin lyophilised from a phosphate buffer and suspended in various organic media. MMA = methyl methacrylate. EVE = ethyl vinyl ether. St = styrene. Tol = toluene. Hex = hexane. All reactions carried out at a fixed water activity of 0.43 and at a constant temperature of 22 °C.*

Overall it is clear that α-chymotrypsin is not deactivated on exposure to the various monomers examined. Therefore, it is assumed that the lower enzyme activities observed with the enzyme immobilised in different polymers are a result of the immobilisation methodology. It has already been established that exposure to the enzyme modification procedure does not deactivate the enzyme. It is possible that the solubilisation via ion-pair formation with anionic surfactants causes some change in the enzyme conformation,

and so affects the corresponding activity. However, the catalytic activity of enzyme dissolved in organic media via ion pairing has already been reported in the literature.[7, 8, 26, 27] Results show that very high catalytic activities can be achieved via this method, approaching the rates observed for traditional aqueous catalysis.[26] As such, the most likely cause of the enzyme deactivation observed in this study is the polymerisation procedure. It is well known that enzymes are thermally labile in aqueous environments.[37] Thermal stability is improved when enzymes are employed in organic media,[38] however, the prolonged exposure of α-chymotrypsin to temperatures of around 60 °C necessary for initiation of the polymerisation reaction have most likely contributed to their deactivation.

Alternative methods of initiation such as UV and microwave techniques have been studied. Preliminary results indicated that both these polymerisation methodologies are more favourable to the immobilised enzyme activity, when compared to traditional thermal initiation. Microwave initiation appears particularly promising, as not only is the reaction temperature minimised, but also the duration of the polymerisation is reduced from several hours to only a few minutes. This should allow for the minimum enzyme deactivation.

It was also noted that when the finely ground enzyme containing polymers were washed extensively with aqueous buffer; no enzyme leaching was detected in these washings upon analysis with Bradford's reagent. This offers further conformation that even unmodified native α-chymotrypsin is effectively entrapped with near total efficiency when immobilised from homogeneous solution via a polymerisation reaction (chapter

four). As yet no studies have been conducted examining the multipoint covalent incorporation of the modified enzyme into a growing polymer.

### 5.4.4 Polymerisations conducted in supercritical media

It has been demonstrated that α-chymotrypsin can be modified with polymerisable moieties, and can also be dissolved in organic solution via an ion pairing mechanism with a suitable anionic surfactant. The next step in the proposed immobilisation strategy is to conduct the polymerisation reaction in a supercritical fluid. This involves the transfer of the ion-paired α-chymotrypsin from its initial extraction solvent (hexane) to a suitable monomer. Initial experiments demonstrated that if the ion-paired enzyme were separated from hexane via evaporation of the organic solvent under vacuum, the resulting solid preparation could not then be re-dissolved in another solvent. Therefore, in order to keep the ion-paired enzyme in solution, monomer (MMA) was slowly added to a solution of the enzyme in hexane. The hexane was then removed by evaporation under vacuum. Continued addition of small quantities of MMA followed by rotary evaporation allowed for the complete removal of hexane. The solution of ion-paired enzyme in MMA could then be concentrated by further rotary evaporation. The concentration of enzyme in MMA was calculated from the initial concentration of α-chymotrypsin in hexane that was determined spectroscopically. As no precipitation was observed, it was assumed that the transfer of enzyme from hexane to MMA was 100 % efficient.

A better method for the solvation of α-chymotrypsin in organic media may be to extract the enzyme directly into a monomer phase, via the formation of surfactant ion-pairs. This would not only simplify the process, but would also remove the need for the use of large

quantities of hexane. However monomers suitable for this task must be immiscible with the water in order to extract the enzyme, yet miscible with the supercritical fluid, so as to produce a homogeneous reaction medium.

A suitable concentration of initiator (V-65) and crosslinker (TRIM) was then added to the ion-paired solution of α-chymotrypsin in MMA. Again, no precipitation of the enzyme was observed. This solution was then transferred to a stainless steel high-pressure reaction vessel, fitted with a sapphire view cell. The remaining volume of the reactor was filled with liquid carbon dioxide. A thick white precipitate was observed on contact of the liquid carbon dioxide with the mixed monomers. This precipitate persisted as the reactor was heated to 60 °C to initiate polymerisation. The resulting polymer monolith extracted from the reactor was observed to contain heterogeneous yellow particles. Repeat experiments conducted with various concentrations of dissolved enzyme also yielded similar results. No catalytic activity was recorded for these polymers.

It has been previously reported that enzymes are reversibly deactivated on contact with supercritical carbon dioxide.[39, 40] There are three main rationales for this process. The enzyme may be deactivated by a change in the pH of its microenvironment, caused by the partial solvation of carbon dioxide in the aqueous monolayer surrounding the enzyme. Rapid depressurisation of the reaction vessel has been reasoned to cause enzyme deactivation by the sudden removal of water from its microenvironment. Chemical reaction of the enzyme's lysine amino acid residues with the carbon dioxide medium has also been shown to deactivate enzymes.

The precipitation of the enzyme observed on contact with carbon dioxide could be due to either a change in pH of its microenvironment, or to reaction of the enzyme's lysine

groups. It has been shown that the extraction efficiency of the ion-pairing process employed to dissolve α-chymotrypsin in organic media is greatly affected by the changing pH of the enzymes environment. Changing the enzyme's pH will affect the ionisation state of the primary amine groups present in the lysine amino acid residues. These primary amines are responsible for maintaining the electrostatic interaction with the anionic surfactant. If the addition of carbon dioxide causes the enzyme's microenvironment to become more acidic, the interaction between the enzyme and the surfactant will reduce, causing the enzyme to precipitate from solution.

Alternatively, direct chemical reaction of the enzyme's primary amines to form carbamates would also result in the precipitation of the enzyme from solution (figure 5.9), as electrostatic interaction between the enzyme and the surfactant are no longer possible.

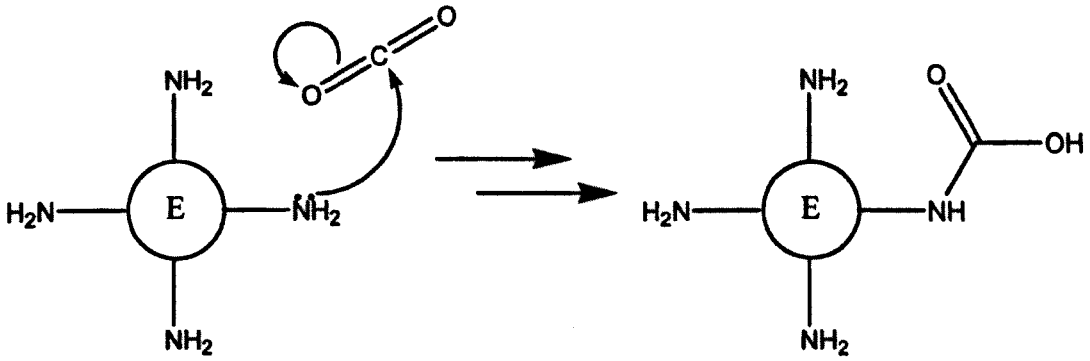



*Figure 5.9 A proposed mechanism for the reaction between the primary amine groups present on the surface of α-chymotrypsin and supercritical carbon dioxide. The formation of unstable carbamates may disrupt the electrostatic interaction between AOT and the primary amine groups, resulting in the precipitation of the enzyme from solution, on contact with the supercritical carbon dioxide.*

Whichever mechanism is responsible for the precipitation of α-chymotrypsin, it is clear that carbon dioxide is an unsuitable supercritical medium for the formation of enzyme containing polymers, from homogeneous solution.

Similar experiments were conducted, employing 1,1,1,2-tetrafluoroethane (R134a) as an alternative dense gas medium. R134a benefits from being non-toxic, FDA approved, non-flammable, non-ozone depleting and has a neutral pH in aqueous media. It is commonly used as the propellant in asthma inhalers and as a refrigerant in air-conditioning systems. When employed in these enzyme immobilisation experiments, R134a was present as a dense gas due to its high critical temperature of 104 °C. A dilute solution of α-chymotrypsin in MMA was prepared as before and added to a high-pressure reactor fitted with a sapphire view cell. No precipitation was observed on contact of the dense gas with the monomer solution. Polymerisation was initiated thermally by heating the reactor to 60 °C. When the reaction was completed, the enzyme-containing polymer could be removed from the reactor as a single homogeneous white monolith. This monolith was ground to a fine powder prior to being assayed for enzyme activity using the transesterification assay described previously (chapter two, section 2.2.5). Very low levels of enzyme activity were observed.

In order to examine why the immobilised enzyme activity was so poor, a lyophilised preparation of α-chymotrypsin was suspended in R134a and used to catalyse the transesterification reaction between ATEE and *n*-propanol. After a period of several hours, the reactor was vented and the precipitated reactants and products redissolved in dry ethanol. A syringe filter was used to remove the enzyme from this mixture. HPLC analysis indicated that some catalysis had taken place, with the rate estimated to be

around one fifth of that observed in hexane. This indicates that the enzyme is not deactivated on contact with R134a. Therefore the most likely cause of the low catalytic activity of the immobilised enzyme containing preparations formed in R134a is a combination of the low enzyme loading levels examined, coupled with the thermal deactivation incurred during the polymerisation reaction. UV and microwave initiation of polymerisations conducted under high-pressure conditions is impractical. However through the use of low temperature free radical initiators, it may be possible to alleviate some of the thermal deactivation experienced with this methodology.

## 5.5 Conclusions

A new methodology has been developed that allows for the limited modification of α-chymotrypsin's primary amine functionalities with a polymerisable vinyl based moiety. This modified enzyme has been successfully extracted from aqueous buffer, into hexane. From this organic solution, it was demonstrated that α-chymotrypsin could be transferred to other organic solvents, whilst retaining catalytic activity. The factors affecting this extraction process were examined and optimised. These factors included the pH of the aqueous buffer, the temperature at which the extraction was carried out and the concentration of the surfactant solution. In order to improve the efficiency of the extraction process a series of branched dialkyl surfactants were synthesised based on the commercially available AOT surfactant. These surfactants were assessed for their ability to extract α-chymotrypsin into organic media. Small variations in the structure of the

surfactant's dialkyl chains were shown to have a significant influence over the corresponding enzyme extraction efficiency.

Polymerisations were conducted in totally organic systems involving both EVE and MMA monomers. Enzymes solvated in hexane were shown to be effectively immobilised in the polymers formed, with no leaching observed after extensive washing with aqueous buffer. The catalytic activity of both sets of enzyme containing polymers was examined using the transesterification assay described previously. The immobilised enzymes were shown to be less active than the equivalent lyophilised preparations. This deactivation was attributed to the prolonged heating required to initiate the polymerisation reaction.

Experiments were conducted using supercritical carbon dioxide as a replacement porogen for hexane. The enzyme was observed to precipitate from solution on contact with carbon dioxide. It was postulated that the precipitation might have occurred as a result of the chemical modification to the enzyme's lysine groups by the solvent. Alternatively changes in the pH of the enzyme's microenvironment may have inhibited the interaction between the anionic surfactant and the enzyme, causing it to precipitate from solution.

1,1,1,2-Tetrafluoroethane (R134a) was investigated as an alternative dense gas solvent. It was demonstrated that homogeneous enzyme-containing solutions of R134a, MMA and TRIM could be polymerised to produce homogeneous polymer monoliths that retained some enzyme activity.

It was also demonstrated that α-chymotrypsin was not deactivated by exposure to the various non-aqueous media employed in the polymerisation reactions. These media included monomers such as MMA and EVE as well as the dense gas R134a.

## 5.6 Future work

The most complex step in this strategy for the immobilisation of enzymes in free radical initiated polymers, is the solubilisation of the enzyme in organic media after modification to its exterior lysine residues. The primary amine groups of lysine residues are required for both the modification and ion-pairing steps. This means that the modification step must be controlled in order to prevent total modification of all of the amine groups. A balance must be achieved in order to produce an enzyme that can be covalently incorporated into the polymer, but can still form enough ion-pairs to make it soluble in organic media. This study has shown that the modification of only three of the enzyme's lysine residues seriously affects the extraction efficiency of the ion-pairing process. A better strategy would be to find an alternative methodology that does not rely on the presence of primary amines, for solvating the enzyme in organic media. Wong *et al* have successfully modified ferritins via a carbodiimide activated coupling between the enzyme's carboxylic acid residues and long-chain primary amines, rendering the protein soluble in organic media.[41] This modified enzyme can be removed from solution by complete evaporation of the reaction solvent, and then re-dissolved in most organic solvents. This method would then leave the enzyme's lysine residues free for modification with polymerisable moieties.

Preliminary experiments involving the carbodiimide activated coupling of α-chymotrypsin to long-chain primary amines have been conducted. The results indicate that some α-chymotrypsin is transferred to the organic phase. However, no in depth study has yet been carried out to assess the enzyme's residual catalytic activity.

If this method for enzyme solvation and modification can be developed without seriously deactivating the enzyme, the subsequent immobilisation during polymerisation reactions ought to be straightforward. It should also allow for the use of supercritical carbon dioxide as a replacement porogen, without the associated problems of carbamate formation. This might lead to the formation of active, stable, multipoint covalently immobilised enzymes, in porous polymer supports. The thermal deactivation of the dissolved enzyme experienced during the initiation of the polymerisation reaction could be alleviated by the used of an alternative initiation mechanism. Both UV and microwave initiated polymerisations have been successfully conducted, producing catalytically active preparations. However, more work needs to be carried out in order to optimise these techniques.

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# Chapter 6

## Summary

The work conducted has explored three of the main immobilisation techniques that are commonly employed to render enzymes suitable for use in non-aqueous reaction media. Chapter two investigated a simple adsorption procedure that allowed for the immobilisation of three different proteolytic enzymes: α-chymotrypsin, bromelain and subtilisin Carlsberg. Although the methodology of adsorbing the enzymes to a novel porous polymeric support was relatively simple, the resulting preparations were shown to be catalytically active, stable at elevated temperatures, resistant to changes in pH and could be used for several cycles of non-aqueous catalysis whilst still retaining useful activity. A thorough examination of the affects of hydration and enzyme loading were conducted. This allowed for a good understanding of the affects of these parameters on the adsorbed system, and provided a better understanding of the general principles of enzyme hydration and substrate diffusion.

The work conducted in chapter three concerned a slightly more complex immobilisation procedure. α-Chymotrypsin was mechanically entrapped in a rigid composite support material via the colyophilisation of the enzyme and the immobilisation matrix, from solution. In this study, a multivariate design of experiment methodology was employed in order to formulate support composites that provided both optimum enzyme activity and optimum mechanical strength. This technique produced a comprehensive model, capable

of predicting the effects on both enzyme activity and the supports mechanical strength, caused by the interactions between up to six component materials, present in various ratios. This model was tested by actually preparing nine of the best predicted formulations, and then experimentally determining their relative strengths and activities. The observed values correlated very well with those predicted by the model. The most active of these preparations displayed more than a fifty fold increase over the values obtained for the control, whilst being at least three times stronger. Reusability studies of the best composites demonstrated that they could be used in more than ten cycles of catalysis without any significant mechanical damage, and still retained useful catalytic activity.

Chapter four examined the effect of changing porosity on immobilised enzyme activity. Immobilisation was achieved by entrapping the enzyme within a growing polymerisation reaction. The polymerisation was conducted in a HIPE, which allowed the templated structure of the emulsion to be transferred to the monolithic polymer product. In order to reduce the environmental impact of the large volumes of organic solvent required for traditional emulsion templating procedures, supercritical carbon dioxide was employed as the emulsions internal phase. It was thought that the use of supercritical carbon dioxide at various densities would also provide some control over the fine structure of the enzyme support matrix. These changes in porosity should then influence the diffusion of substrates to and from the immobilised enzyme. However, after synthesising and examining numerous enzyme containing polymers, it was found that although the porosity of the crosslinked polymers could be altered significantly, the changes were not taking place on a sufficiently small scale to influence the enzymes activity, over the

specific particle size range examined. Although the enzyme activity was not influenced by the porosity of its environment, the catalytic rates observed for these materials were still more than ten fold higher than for the equivalent unsupported control preparations. It was also noted that after several cycles of aqueous washing, little or no enzyme was found to leach from the support. Initially it was postulated than the enzyme may be covalently incorporated into the growing polymer in the presence of free radicals. However, after conducting several sets of GPC and SDS-PAGE experiments, it was concluded that the enzyme was simply very efficiently entrapped within the crosslinked polymer network.

Finally, the most complex enzyme immobilisation method was studied in chapter five. It is well known that multipoint covalent immobilisation affords the most stable, and sometimes the most catalytically active preparations. The method chosen involved first covalently modifying the enzyme via chemical reaction with acryloyl chloride, in order to introduce some polymerisable functionalities. The modified enzyme was then dissolved in hexane via an ion-pairing interaction with the anionic surfactant, AOT. This solubilised, modified enzyme was then added to one of several liquid monomers, such as MMA and EVE. The addition of a cross linking agent prior to polymerisation in the presence of a free radical initiator produced a monolithic, enzyme containing polymer. These polymers were shown to possess similar catalytic activity to equivalent preparations recorded in the literature.

The next step in the proposed method, involved the elimination of organic solvents during the polymerisation stage. This was achieved by transferring the dissolved enzyme from hexane to MMA. The enzyme containing monomer was then polymerised in the

presence of a miscible supercritical fluid. Initially supercritical carbon dioxide was chosen for this role. However it was found that the enzyme precipitated from solution on addition of the carbon dioxide. The most likely explanation of this is that the lysine groups present on the exterior surface of the enzyme, forming the ion-pair with the anionic surfactant, undergo a covalent reaction with the carbon dioxide to form an unstable carbamate. This causes the ion-pair to break, precipating the enzyme from solution. Fortunately alternative dense gas solvents exist that are also miscible with the monomers and can be used in place of carbon dioxide. Initial experiments have demonstrated that R134a can be used to produce uniform, catalytically active enzyme containing preparations.

It is hoped that this work can be continued in order to produce highly active and stable supported enzyme preparations that can be used for catalysis in both aqueous and non-aqueous media.