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ENZYME IMMOBILIZATION ON WOOLEN CLOTH

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A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Engineering

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Abstract

The objective of this research was to determine the feasibility and the limitation of using woollen cloth as a support matrix for enzyme immobilization. Lipase from pseudomonas fluorescens was selected as the model enzyme. Covalent binding protocol (CVB) with a polyethyleneimine (PEI) spacer and glutaraldehyde (GA) cross-linker was used as benchmark protocol.

It was found that lipases could be effectively immobilized onto woollen cloth by the benchmark CVB protocol. The immobilized activity was independent of the GA concentration and soaking time in the GA solution, but dependent on the GA solution pH (optimal at pH 9). The treatment time in lipase solution was found to be the rate-determining step in the immobilization: the immobilized activity increased steadily within 5 to 10 hours of soaking in lipase solution, after that, the increase plateaued to a maximum of 4 µmol pNPP.min⁻¹.(g cloth)⁻¹.

The immobilized lipase was reasonably stable: when the immobilized cloth was stored in 0.05 M Tris buffer (pH 8.5) for more than 80 days in a refrigerator, more than 80% of the lipase activity remained. The optimum pH for both free and immobilized lipase is approximately the same, which may indicate that microenvironment for the free and immobilised lipase was not considerably different. The Michaelis Menten parameters for the free and immobilized lipase were determined: $K_M$ and $v_{max}$ for the free and immobilized lipase were 2.40, 3.16 mM and 153.76, 5.16 µmol pNPP.min⁻¹.(mg lipase)⁻¹ respectively. The lipase-immobilized cloth exhibited excellent oily stain removal ability: after being stained with olive oil and stored for one day in air at room temperature, the oily stain could be easily removed by 0.05 M pH 8.5 Tris buffer without any detergent addition. This enhanced cleaning was stable also, after cloth was stored in air for almost one month, similar cleaning performance was still observed.

Despite the successfulness in the benchmark CVB method, it was found to be a challenge to further improve the immobilised activity by variation of the CVB protocol and using different cross-linkers, although the ESEM images and measurement of protein load showed there was potential to do so. This was probably due to the fact that there are a number of reactive groups on wool surface which are not very reactive with the cross-linker used, thereby reducing the extent of surface activation, resulting in limited immobilized activity.

To further reduce the lipase deactivation during immobilization, immobilization of the lipase via assistance of another enzyme was tried in order to eliminate the use of GA. Thus, lipase was
entrapped inside a casein gel formed on woollen cloth in the presence of Transglutaminase (mTGase). Lipases were successfully immobilized by this protocol. Unavoidable lipase leakage and high mass transfer resistance from the casein gel however made this protocol not very promising compared to the CVB benchmark protocol.

The benchmark CVB immobilization protocol was also applied to galactosidase. The overall performance of the immobilized galactosidase was poor compared to the lipase however. This may indicate that the CVB immobilization method used is not immediately transferable to other enzymes and that modifications are required to do so. Further investigation is therefore needed.

Overall, the results from this project have shown that it is feasible to immobilize enzymes onto woollen cloth, although surface treatment of the wool to produce more binding groups would improve the surface coverage.
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List of Abbreviations

AU    Absorbance unit
BCA kit  Bicinchoninic acid protein assay kit
CVB   immobilization via covalent binding method
CRL   immobilization via cross-linking method
DCCA   Dichlorocyanuric acid
DI water  Deioned water
GA   Glutaraldehyde
K_M   Parameter of Michaelis-Menten kinetic model
M-M kinetics  Michaelis-Menten kinetics
mTGase   Microbial Transglutaminase
PEI   polyethyleneimine
PFL   Lipase from *pseudomonas fluorescens*
pNPP   p-nitrophenol palmitate
pNP   p-nitrophenol
pNPA   p-nitrophenyl acetate
[S]   The concentration of the substrate
Tris buffer  Trizma buffer
UV spectra  ultraviolet and visible adsorption spectra
v   Reaction rate
v_{max}   Maximum reaction velocity in Michaelis-Menten kinetic model
1. Introduction

1.1 Why use Enzymes? Advantages and Disadvantages

Enzymes are used for chemical reactions instead of chemical catalysts because of several advantages that are provided [1]:

- Reactions occur at a low temperature and atmospheric pressure.
- The possibility of using water as the reaction solvent instead of toxic organic chemicals.
- High efficiency, which enzymes can catalyse up to several million reactions per second.
- Stereospecificity, i.e. enzyme can only catalyse those substrates that fit in its biological structure.

The current high cost of enzymes prevents a broad application in industries. Consequently, there are many techniques available to improve enzyme applicability and reduce their cost. Enzyme immobilization is one of the most powerful tools to solve this problem. The principle of enzyme immobilization is to keep the enzyme at a fixed point within a bioreactor, thereby enabling the reuse of the relatively expensive biocatalyst in order to make the process more economically feasible. This is done by attaching the enzyme onto a solid support matrix, which then can be engineered into the structure of the reactor. Moreover, the utilization of immobilized enzymes can simplify the reactor design and process control: there is a strong possibility that the reaction will be stopped when the immobilized enzyme is filtered out. The two key factors, which are used to determine whether an enzyme immobilization is successful or not are: the stability of immobilized enzyme and the cost.

The stability here is both in terms of residual activity during storage (storage stability) as well as the amount of enzyme that remains immobilized during chemical reaction (operational stability). The immobilized enzyme must be robust enough to withstand multiple recoveries so that it can be reused many times, making the immobilization cost effective. Many immobilized enzymes are not stable for long-term operation [2], so cannot be commercialised.

The other important factor for a successful immobilization is the cost. Many of the immobilization techniques that are discussed in academic literature can only remain within laboratories and the research domain, since they have been too expensive. Besides, there are several other factors that
also must be met when developing an enzyme immobilization procedure—i.e. activity, selectivity and reduction of inhibition. It is not an easy job to balance all these features. Consequently, a major challenge currently still exists to develop a cheaper and stable immobilization approach, whilst balancing all these factors mentioned above.

A number of different methods have therefore been developed for enzyme immobilization, of which the major ones are: physical adsorption, encapsulation, cross-linking and covalent binding [3-5]. Among all the factors affecting immobilization, the selection of support matrix is very important for successful enzyme fixation. A wide range of materials have been studied as enzyme supports, including: chitosan [6], polymer particles [7], membranes [8], animal bone powders [9] and cellulous fabrics [10]. Recently, fabrics as support materials have drawn much research attention, due to their advantages, which include a cheap price, high porosity, large specific surface area and excellent mechanical stability [10]. The most popular fabrics used in enzyme immobilization are cotton cloth [11, 12] and silk [13]. Wool, despite its wide application in apparel and carpets, has not been as widely studied in enzyme immobilization as cotton. To fill the research gap, this thesis therefore detailed the study on enzyme immobilization on woollen cloth [14].

As one of the major sheep raising countries in the world, the wool industry is very important to the New Zealand economy. Therefore, the development of value-added woollen products to expand the application of wool is both desirable and relevant for New Zealand based research. The wide availability and rigid structure makes wool a potential candidate as an immobilization matrix. However, as a charged keratin type protein, wool possesses a number of reactive residues such as lysine, histidine, serine and glutamic acid, which are different from the commonly used immobilization supports, therefore, wool’s suitability and limitation in immobilization should be identified.

The most immediate application of a fabric or cloth with immobilized enzymes is in self-cleaning or enhanced cleaning of this fabric. It has been established that oily stains on cloth are quite difficult to remove using only detergents [15]. In laundry washing ingredients, most of the capacities for fatty stain removal come from alkaline additives. The fatty acid component of fatty stain forms water-soluble soaps in the alkaline solution, which can easily be removed during the washing process. But this cleaning ability is limited to the fatty acid component of the dirt and most of the triglycerides still remain on the cloth due to its hydrophobicity. The enzyme additive in laundry ingredients can promote the cleaning process. However, it was found the lipase possesses only limited cleaning
efficiency in the water solution because of the mass transfer barriers between free enzymes and the stains on the cloth [16]. It has been suggested that if the lipase was adsorbed onto the cloth surface and the lipid stain hydrolysis could either occur during storage [17] and/or cleaning efficiency in water would be enhanced by removing the aforementioned mass transfer resistances. But, these adsorbed lipases are not stable on woollen cloth. This enhancement in cleaning efficiency will therefore disappear when these adsorbed lipase leave the cloth. Thus, a self-cleaning woollen cloth produced by a stable enzyme immobilization technique is needed to overcome the above-mentioned disadvantages of the current cleaning technique.

In light of the above description, in this project, we were trying to develop a cheap and effective method to immobilize lipase on woollen cloth to investigate its potential in oily soil removal on woollen cloth. As a model lipase, the immobilization on lipase from *pseudomonas fluorescens* was adopted. However, the immobilization method developed in here was not only limited to lipase, and another enzyme has been used to demonstrate the wider application of the immobilization methods developed and the enzyme-immobilized woollen products.

1.2 Objectives of this Research

Consequently, the overall objective of the current research is to determine the feasibility of using woollen cloth as support matrix for enzyme immobilization and to optimise the immobilization of these enzymes in terms of amount of enzymes retained on the wool, the immobilized activity and kinetics of these enzymes and the stability of this immobilized enzyme system in storage and reuse. To meet these objectives, this project is separated into several tasks:

Task 1: To develop a benchmark method to immobilize enzyme on woollen cloth that will provide good stability, immobilized activity and enzyme loading and determine the operating factors affecting immobilization and characterize the immobilized enzyme. Here, lipase was selected as the model enzyme.

Task 2: To investigate the effectiveness of this immobilized lipase preparation in removing oily stains from a woollen cloth compared to a bare woollen cloth.
Task 3: To determine the robustness of the immobilization methods developed, the benchmark method was used to immobilize enzymes other than lipase onto wool and the immobilized activity, and operational stability was determined.

Task 4: To determine the effect of the immobilization on the enzymes by comparing the kinetics of the free enzymes to that of the enzymes immobilized by the benchmark method.

Task 5: The immobilization method developed in Task 1 was further varied to increase the maximum amount of enzyme immobilized and overcome any limitations.

Task 6: The immobilization protocol was further varied by immobilizing lipases with another enzyme (transglutaminase), and the stability (both operational stability and storage stability) was examined.

1.3 **Outline of the Thesis**

To meet these objectives, this thesis is developed as follows: Chapter 2 is the literature review, where the relevant background knowledge as well as recent progress on enzyme immobilization is introduced and contextualised in terms of this project. The gaps in the literature that this work fills are also outlined. Chapter 3 covers the immobilization protocols and assay methods used or developed in the current study. Several different immobilization protocols were tested and developed in this project. So, in order to group the relevant experimental methods with the relevant results, the description of immobilization protocols are separated into Chapter 4 dealing with the benchmark immobilization method, and Chapter 6, where various chemicals and immobilization conditions of the benchmark method are tried to improve the enzyme loading and activity. Galactosidase immobilization on woollen cloth by the benchmark method is also described in Chapter 4. The characteristics of the immobilized lipase and its effectiveness in the removal of oily stain as well as the kinetics of the free and immobilized lipase are discussed in Chapter 5. In Chapter 7, the investigation of lipase immobilization on woollen cloth in the presence of the second enzyme: transglutaminase is detailed. Finally, the overall conclusions and recommendations are made in Chapter 8. This is followed by references and appendices.
2. Literature Review

2.1 Wool

2.1.1 Wool Structure and Protein Composition

Wool (like human and other mammalian hair) belongs to a group of proteins known as keratin. These proteins have high sulphur content, mainly present in the form of cysteine. Wool is composed of about 97% protein, 2% lipids, and 1% minerals. [18] As shown in Figure 2-1, wool fibre has a so-called skin-core structure. In this structure, the inner cortex is hydrophilic, due to the existence of large number of the polar groups it contains. The outer surface is the hydrophobic cuticle. This cuticle cell is about 0.3-0.6 μm thick and approximately 30 μm long. The cuticle is 10% of the weight of wool, and the cortex is 90%. The fibre is surrounded by cuticle cells that cover it in one direction and consist of four layers: the epicuticle, the A and B layer of the exocuticle and the endocuticle. The cuticle and cortical cells are separated by a cell-membrane complex comprising of internal lipids and proteins [19]. This hydrophobic feature could either bring benefits or problems for enzyme immobilization. Enzymes can be easily immobilized onto wool via a hydrophobic interaction [20]. However, this layer could also become a barrier for chemical immobilization of the enzyme, as most of the chemical reagents used for immobilization are hydrophilic [21].
For enzyme immobilization via a chemical approach (see Section 2.3.2), the most important amino acid residues are those with amine and carboxylic groups, such as aspartic acid, glutaminic acid, lysine and histidine, which can be easily activated by reactions such as diazo coupling [23], reactions with acid anhydride, reactions with isocyanate and its derivatives [24], epoxide reactions [25], Schiff’s base reaction [26] and carbodiimide reactions [27]. The typical amino acid residue content on wool protein is listed in Table 2-1. From this table, it can be found that there are more lysine residues existing in proteins with low sulphur content, which is mainly located in inner part of wool structure. In contrast, high sulphur content proteins are located on the wool surface. In terms of lysine content, it seems that inner part of wool is more favourable for enzyme immobilization.

Table 2-1 Wool and its dissolved protein composition [28]

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Whole wool</th>
<th>Low sulphur protein</th>
<th>High sulphur protein</th>
<th>Ultra-high sulphur protein</th>
<th>High-tyrosine proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>5.2</td>
<td>7.7</td>
<td>2.9</td>
<td>2.0</td>
<td>3.1</td>
</tr>
<tr>
<td>Arginine</td>
<td>6.2</td>
<td>7.8</td>
<td>6.2</td>
<td>6.9</td>
<td>3.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>5.9</td>
<td>9.6</td>
<td>2.3</td>
<td>0.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Cysteine (half)</td>
<td>13.1</td>
<td>6.0</td>
<td>22.1</td>
<td>29.9</td>
<td>6.4</td>
</tr>
<tr>
<td>Glutaminic acid</td>
<td>11.1</td>
<td>16.9</td>
<td>7.9</td>
<td>7.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>8.6</td>
<td>5.2</td>
<td>6.2</td>
<td>4.2</td>
<td>26.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.8</td>
<td>0.6</td>
<td>0.7</td>
<td>1.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.0</td>
<td>3.8</td>
<td>2.6</td>
<td>1.7</td>
<td>0.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>7.2</td>
<td>10.2</td>
<td>3.4</td>
<td>1.4</td>
<td>3.5</td>
</tr>
<tr>
<td>Lysine</td>
<td>2.7</td>
<td>4.1</td>
<td>0.6</td>
<td>0.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.5</td>
<td>0.6</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.5</td>
<td>2.0</td>
<td>1.6</td>
<td>0.5</td>
<td>9.6</td>
</tr>
<tr>
<td>Proline</td>
<td>6.6</td>
<td>3.3</td>
<td>12.6</td>
<td>12.8</td>
<td>6.7</td>
</tr>
<tr>
<td>Serine</td>
<td>10.8</td>
<td>8.1</td>
<td>13.2</td>
<td>12.7</td>
<td>12.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.5</td>
<td>4.8</td>
<td>10.2</td>
<td>11.1</td>
<td>3.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.8</td>
<td>2.7</td>
<td>2.1</td>
<td>1.8</td>
<td>18.0</td>
</tr>
<tr>
<td>Valine</td>
<td>5.7</td>
<td>6.4</td>
<td>5.3</td>
<td>4.3</td>
<td>4.7</td>
</tr>
</tbody>
</table>

2.1.2 Treatment of raw wool: chlorination and polymer adsorption on wool surface

Most raw wool has to be treated before being used to produce a woollen garment, as in its natural state the wool is stiff and tends to shrink after washing. There are two aims for woollen surface treatment. The first aim is to smooth the scale structure on the surface. Cuticle cells are arranged like
roof tiles, which lead to the friction coefficient in the root-to-tip direction being different from the one in the tip-to-root direction. As a result, wool fibres tend to shrink after washing and felt together to form a solid structure under heat and pressure. After oxidation, parts of scales are dissolved and the disulfide bond formed between two cysteine residues (Cys-s-s-Cys) are converted into cysteic acid residues. These cysteic acid residues are believed to be responsible for the soft scale characteristic of wool [19]. These cysteic residues also produce a large excess of anions and consequently a more negative surface charge on the wool fibres, thereby resulting in an improvement in the cationic resin adsorption as well [29]. This treatment not only improves shrinkage resistance, but also prevents the development of yellowness, improves whiteness, all with minimum chemical and physical damage to the wool [30]. The second aim is to remove the surface lipid layer to facilitate the resin application. Untreated wool is quite water repellent. Scientific evidence suggested that the lipids are not only physically adsorbed but connected to the fibre surface through a thioester linkage as well [31]. Alkali, chlorination and amine treatments are capable of removing some of the lipids [19]. The lipid removal treatment alters the surface properties of the fibre by reducing its hydrophobic nature and enhances the resin coating. It was reported that cationic PAE resin (Polyamideamine-epichlorohydrin resin) tends to spread as a film over the whole surface after chlorination, rather than polymerising as individual globules as it does with untreated wool [19].

The most widely used process is known as the chlorination/ Hercosett process. Two processing steps are normally adopted in this wool shrinkage-proof treatment. The fabrics are firstly treated with an oxidative agent, namely chlorine, and then treated in a reductive solution to remove the chlorine, subsequently with a dip in the reactive cationic resin of PAE (Polyamideamine-epichlorohydrin resin) to complete the treatment. Several chlorine agents were used for wool surface treatment, including sodium hypochlorite, potassium permanganate, dichlorocyanuric acid (DCCA) and chloramine T [30, 32]. Hypochlorite has been the most widely used of these. However it is a strong oxidation agent and can lead to excessive removal of wool protein. In order to reduce the damage to the wool structure from such rapid oxidation, the activity of chlorine can be slowed using chlorine-retarding agents, such as urea formaldehyde or melamine formaldehyde precondensate resins. An alternative is to use DCCA, which is able to form hypochlorous acid in a water solution, which can slowly release chlorine and act as an oxidizing agent. Consequently, DCCA has gradually replaced hypochlorite as the major chlorination reagent used in the wool industry. The commercial anhydrous sodium dichloroisocyanurate (DCCA-Na) contains 63% active chlorine.
As the protective resin adsorption on wool is closely related to lipid removal, the processing parameters were studied in more detail. It was documented that the release of fatty acid from untreated woolen surface was related to the concentration of DCCA used [30]. Fatty acid 18-MEA is a constituent of the fatty acid layer (f-layer) on the wool surface, which is covalently bound to the cuticle via a thioester bond. Its release from wool fibres treated with 10 % owf (weight of cloth) of DCCA applied at 30°C for 60 minutes was reported, but not from those treated with 5% DCCA.

In summary, wool contains a number of reactive groups, such as lysine, aspartic acid and glutaminic acid, which can be activated by chemical reagents, namely carbodiimide, glutaraldehyde and epoxide. Thus wool has the potential to be a support matrix for enzyme immobilization. However attention must be paid to the hydrophobicity of the wool surface, which might become a barrier for surface activation. Chlorination and alkaline hydrolysis was reported to be able to remove the covalent bound lipid and facilitate the cationic resin adherence. This knowledge is very useful for surface activation of wool as an immobilization support matrix, especially when a spacer arm is needed.

### 2.2 Enzymes: Reactions and Stability

An enzyme is kind of protein with a well-defined tertiary and quaternary structure, which incorporates other structures, such as carbohydrate, lipids or metal ions. Enzymes are biocatalysts
existing in a biological cell, which are used for catalysing chemical reactions in order to achieve more rapid reaction rates and higher selectivity under milder conditions. Enzymes are derived from a wide range of different organisms, such as bacteria [34], fungi [35], mammalian cells [36] and plants [37]. The growth conditions of the enzymes in the organisms to a large extent determine the optimal operating conditions that can be used for this enzyme.

The relationship between enzyme and its substrate has been described as an analogue to the “key and lock”, as illustrated in Figure 2-3, i.e. one type of enzyme can normally work on only one type of substrate [1]. This model has also been extended to ‘induced fit’ model accounting for the fact that enzymes are flexible. In this model, the active site of enzyme is not regarded as the same all the time; instead its shape is continuously changed with the interaction with its substrate when substrate is close to enzymes, until the substrate is fully bound [38]. The enzymatic reaction is generally restricted in water solution at a neutral pH, ambient temperature and a low substrate concentration [1]. Consequently, a quantification of the limits of reaction, immobilization and enzyme stability with respect to pH and temperature are highly important in studies of enzyme and their reactions.

As mentioned in Section 1, the enzyme stability is an important factor to consider for enzyme applications, which is normally expressed as half-life, i.e. the time span of the enzyme losing 50% of its original activity. Normally enzymes exhibit better stability when stored in low temperatures and biological pHs where an enzyme is derived. Some researchers have also found that some enzymes demonstrate better stability in organic solvents, due to the rigidity of the structures of the enzyme in
a low water medium [39]. By the same token, immobilization tends to improve the enzyme stability, as immobilization confines enzyme movement and reduces the chance of structure changes [40]. The disadvantage that comes with this, however is that the enzyme might lose its activity during immobilization.

The relationship between enzyme activity and pH in aqueous solution normally shows a bell-shaped curve [41], as shown in Figure 2-4. This is due to the polyionic properties of enzymes. Its three dimensional structure and active site-substrate interaction are all related to solution pH. The optimal pH of enzyme closely matches the pH of the biological environment where enzyme was derived. Moreover, the solution pH affects enzyme stability as well. The relationship between enzyme stability and solution pH is also a bell shape as the pH and activity relationship, but span a wider pH range [41].

![Figure 2-4 Typical bell-shape curve of the relationship between enzyme activity and pH [41]](image)

Just like a non-biological chemical reaction, the reaction rate catalysed by enzymes also increases with temperature according to the Arrhenius law. However, for enzymes, this increase in temperature also increases the chance of enzyme deactivation. Thus, the optimal operational temperature for a free enzyme is practically selected slightly above the temperature of minimum growth tolerance of the organism from which the enzyme was derived [1].

Three types of enzymes are used in the current study: lipase from *pseudomonas fluorescens*, β-galactosidase from *Kluyveromyces lactis* and microbial transglutaminase (mTGase) isolated from *Streptomyces mobaraense*. Lipase from *pseudomonas fluorescens* and β-galactosidase from *Kluyveromyces lactis* are the enzymes to be immobilized. The reason to choose these two enzymes
is that their immobilization on other support matrixes is well studied and the characteristics are properly defined. While, mTGase from *Streptomyces mobaraense* is used to immobilize lipase. Background on each of these three is addressed in more detail in Section 2.2.1, Section 2.2.2 and Section 2.2.3 respectively.

### 2.2.1 Lipase from *Pseudomonas fluorescens*

#### 2.2.1.1 Overview of Lipases

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are the enzymes that are capable of catalysing the hydrolysis of triacylglycerols into monoglycerides, diglycerides, free fatty acids and glycerol. When in low water conditions (such as in organic solvents), they are able to catalyse the reverse reactions to these [42].

Lipases have a very special tertiary structure. Around the active site, there is piece of hydrophobic peptide chain present, a so-called “lid”, as shown in Figure 2-5. In aqueous solutions, lipases exist in a closed and inactive conformation, since the active site is completely covered with this hydrophobic lid. However, the lipase can be converted into an active form in a hydrophobic environment, where the “lid” is removed and the lipase substrate gains access to the active site. This phenomenon is also termed as “interfacial activation”, i.e. interfacially activated lipases often demonstrate extremely high activity, which is even higher than free lipases in aqueous solution [43]. Due to this interaction, lipase adsorption on hydrophobic surface is very strong. It was reported that the lipase from *pseudomonas fluorescens* adsorbed on octyl agarose could not be released to the medium even after extensive washing with deionised (DI) water, 50% glycerol or polyethylene glycol and 10% acetonitrile. Desorption happens only under high concentration of detergent, urea or guanidine [44]. This adsorption via interfacial activation has already become a very attractive immobilization method. Through conventional immobilization techniques, the free enzymes normally show activity loss during immobilization. However, an activity increase was reported for those lipases immobilized via hydrophobic immobilization [45].
2.2.1.2 Characteristics of Lipase from Pseudomonas fluorescens

Lipase from *Pseudomonas fluorescens* (PFL) appears to be a single-chain protein and contains neither sugar nor lipids. The molecular weight of triacylglycerol lipase (EC 3.1.1.3) from *Pseudomonas fluorescens* is estimated to be approximately 33,000 Da by sodium dodecyl sulphate electrophoresis and Sephadex G-75 gel filtration [47]. The structure of PFL is shown in Figure 2-6, indicating the active site pocket and the lid of free PFL used for the lipase “open-closed” structure as detailed in Section 2.2.1.1. This shows that the lysine residue is at the opposite side to the active centre, so that there is no lysine residue near the active site. This lipase is quite a hydrophilic protein; the hydrophobic part is the area around the catalytic site. This structure enables the lipase’s active site to remain relatively unaffected during chemical immobilization, as only the lysine residue in the back of active site attending the immobilization reaction and the active site is forced to orientate toward the medium [46].

PFL can be used for a range of different reactions. Broad substrate specificity for the 1-, 2- and 3-positions of triglyceride (short to long fatty acids) was found. The optimum pH is between 5.0 and 9.0, and the optimum temperature is between 40 and 65°C. The enzyme can operate stably for adequate reaction times: for example this lipase was stable up to 40 °C under the condition of pH 7.0 for 30 min and had more than 80% of the remaining activity between pH 5.0-11.0 at 37 °C for 60 min [48]. Of relevance to New Zealand, PFL is used extensively in the dairy industry for the hydrolysis of milk fat. Milk spoilage is predominantly caused by this enzyme, which produces short-medium-chain (C4–C12), even-carbon number, fatty acids milk triacylglycerol [49].
This lipase is inhibited by several chemical species, which limits its applicability [50]. It has been found that the enzyme is partially inhibited by FeCl$_3$, and 30% inhibition was observed with ZnCl$_2$, and HgCl$_2$. Iodine completely inactivates the enzyme and partial inactivation was observed with N-bromosuccinimide. However, the inhibitory effect of iodine on the lipase disappeared in the presence of the substrate. On the other hand, BaCl$_2$, and CaCl$_2$, increased the activity by 20% [50]. Calcium ions can also help PFL resist denaturation by guanidine hydrochloride and enzyme activity from polar cosolvent like dimethyl sulfoxide (DMSO) and trifluoroethanol, suggesting that Ca$^{2+}$ plays an important role in inducing conformational changes and consequently in maintaining enzyme structural stability [49]. Between these two ions, Ca$^{2+}$ is more effective bivalent ion in stabilization of the native structure of PFL [51]. Thus normally calcium ion is added to the Tris buffer solution used in lipase from *Pseudomonas fluorescens* as an enzyme stabilizer [52, 53]. The other chemical species detailed above will be avoided where possible to minimise lipase inhibition.

It is well known that enzymes suffer activity losses after immobilization, especially when a chemical method is used (Section 2.3.2). However, the sensitivity of an individual enzyme toward a denaturant strongly relies on its properties such as microbial source, molecular weight and relative lysine content. It was reported [52] that lipases with a large average molecular weight of 60 kDa were more sensitive to cross-linking agents than those lipases with an average weight of 25 to 35 kDa. The position of a particular lysine in a lipase and its reactivity as a nucleophile also determine
the stability of a lipase towards a coupling agent [52]. As the molecular weight of lipase from *Pseudomonas fluorescens* (PFL) is only 33,000 Da and there is no lysine residue near the active site, the lipase selected for this thesis can be deduced to be relatively stable in the presence of a chemical denaturant.

### 2.2.1.3 Formation of a PFL Aggregate

The character of PFL is different at high and low lipase concentration due to existence of a bimolecular aggregate. Consequently, for this project this needs to be taken into account for both reactions with free enzyme and for immobilization. These aggregates are able to form even at moderate enzyme concentrations [54]. It was reported that the bimolecular aggregate formed at a lipase concentration of 44 μg ml⁻¹ [55]. The monomolecular form was found as unique only at low lipase concentration and in the presence of detergents. The formation of the aggregate is due to the special lipase structure, the so-called “closed-open” structure as mentioned in Section 2.2.1.1. As shown in Figure 2-6, there is a hydrophobic “lid” around the catalytic site on the surface of PFL. This “lid” presents an exceptionally high affinity for hydrophobic interfaces, including those generated on air-aqueous media, on solvent-aqueous media and solid surface coated with hydrophobic moiety, even lipase itself. This sort of aggregate is also found for other lipase species, including lipase from *B. thermocatenulatus*, lipase from *R. miehei* and lipase from *R. oryzae*. However, lipase from *C. antarctica* (fraction B) does not belong to this category, as this lipase only possesses a very small hydrophobic area. [46]

The biomolecular and monomolecular lipases from *pseudomonas fluorescens* display different functional properties. Firstly, the bimolecular structure is much more stable than the monomolecular species (the bimolecular structure maintained over 80% of initial activity after 72 hours at 45°C, while the unimolecular structure retained only around 30% of initial activity after 4 hours of incubation under the same experimental conditions [56]; and the bimolecular form presented a higher optimal temperature). Secondly, the monomolecular form showed a much lower Michaelis–Menten parameter of $K_M$ (Michaelis constant) for its substrate, such as ethyl butyrate, than the bimolecular form, indicating higher mass transfer barrier exists for the biomolecular structure. [56]

Of most importance to the immobilization techniques developed in this thesis is the fact that this bimolecular aggregate does not only occur in solution, but also happens on a solid surface. Palomo
et al. [46] described a lipase-lipase aggregate formation from lipase from *Pseudomonas Fluorescens* on agarose. As shown in Figure 2-7, after this lipase was covalently immobilized onto glyoxyl-agarose, it was found that this immobilized lipase could further adsorb more PFL lipase.

![Diagram of lipase-lipase aggregate formation](image)

**Figure 2-7 Illustration of formation of lipase-lipase aggregate formed by the lipase from pseudomonas fluorescens, when this lipase was immobilized on glyoxyl-agarose, from reference [46].**

For 1 g of glyoxyl agarose in 5 ml of lipase solution containing 1 mg ml⁻¹ lipase, about 40% of enzyme was adsorbed within one hour. SDS-PAGE assay shows a single band, indicating that only lipase adsorbed, not the contaminant protein. This lipase can be desorbed by incubation in 0.5% triton X-100 solution. Palomo tried to display the potential to use aggregate formation to purify PFL from other impurities. However, this type of aggregate could be used to potentially enhance the stability and activity of the immobilized lipase in this thesis: it is hypothesised that a high immobilized-activity might present for immobilization performed under a high PFL concentration, as the PFL aggregate formed is more stable under chemical attack than monomolecular PFL. Moreover, the chance of the lipase contacting the immobilization site on immobilization support might increase, as more enzymes should be adsorbed to immobilization matrix surface.

### 2.2.1.4 Applications of Lipase: Laundry washing

The most immediate application of a fabric or cloth with immobilized lipases is in laundry washing to produce a self-cleaning or enhanced cleaning fabric. Consequently, this is the main application that the enzyme immobilization in this thesis is aimed at. The background knowledge in this field is introduced in this section.
A. Lipase in laundry washing

The detergents industry is one of the biggest end users of industrial enzymes. Due to a change of washing habits from hot-water washing from between 50 and 60° C to cold-water washing and strict environment regulations, more enzyme ingredients now appear in laundry washing formula [57]. As phosphate builder and bleaching agents also appear in laundry ingredients, the enzymes used must be stable at alkaline pH and as well as being temperature resistant. Unfortunately, most of natural enzymes used nowadays prefer neutral and slight acidic environments. Genetic engineering therefore has to be employed to produce laundry grade enzymes, i.e. enzymes that are stable in alkaline environment [58]. However, since the use of alkaline builders and hot temperatures are incompatible with the laundry washing of woollen garments, lipase originating from natural bacteria can be used.

A number of lipases originating from different sources have been investigated as potential detergent lipases, notably lipases from *Aspergillus* [59] (the first industrial Lipolase™ was made merely from the transformation of the fungal lipase gene into an strain of *Aspergillus*), lipases from *Pseudomonas* [60, 61] (these have been already industrialized by Gist-Brocades under the trade name of lipomax™), and lipases from *Candida cylindracea* [62, 63]. Among them, the lipase from *pseudomonas* is considered a good candidate for application in detergents industry, as it is thermoresistant and active in alkaline conditions.

B. Limitation of the current cleaning techniques

It has been established that oily stains on cloth are quite difficult to remove using detergents only [15], as most of the capacity for oily stain removal derives from the alkaline additives in washing powders rather than the detergent. This is because the fatty acid component of oily stain forms water-soluble soaps in the alkaline solution, which can easily be removed during the washing process. But this cleaning ability is only limited to the fatty acid component of the dirt and most of the triglycerides still remain on the cloth due to its hydrophobicity. Another important factor resulting in low cleaning efficiency is water hardness: the calcium ion and magnesium ion in water are able to form low water-soluble soap on the cloth surface and make triglyceride even harder to remove.
In theory, the addition of lipase in the laundry ingredients can promote the hydrolysis of lipid and generate more fatty acid. However, the mass transfer barriers between free lipase and the stains on the cloth hamper the cleaning efficiency from enzyme addition [16]. Using an immobilized enzyme would potentially overcome this problem by reducing the mass transfer barriers. However, the capacity of using enzymes to enhance cleaning of woollen fabrics has not been fully realised by the current techniques.

C. Previous studies of immobilized enzymes on fabrics for cleaning

Previous study on lipase adsorption on cloth for stain removal has addressed the issue of immobilized enzymes on cloth for enhanced cleaning [17]. It was found that if the lipase was adsorbed onto cloth surface, the lipid stain hydrolysis could occur during storage and cleaning efficiency in water would be enhanced by removing the aforementioned mass transfer resistances [17]. In terms of efficiency on the triglyceride stain removal in this system, it is necessary to use a “multi-cycle wash” to obtain the best cleaning results. The “multi-cycle wash” here means that the oily stain cannot be effectively removed through one washing cycle. As the lipase was only physically adsorbed onto cloth, the lipase activity that existed is limited due to the deactivation and restricted holding ability of the cloth. However, one problem identified is that the lipolysis product formed during dry hydrolysis caused an off-odour. Thus it would be of interest to have the fatty stain removed just in one wash if more lipase was adsorbed.

In light of above technical shortage, a two-stage washing model was suggested by Flipsen [64]. Calcium independent lipase, such as cutinase, was recommended to replace the calcium dependent lipase used in the cleaning industry, such as Lipolase™. As it is unnecessary to maintain a high calcium concentration in washing solution to activate the lipases, the calcium concentration could be reduced by chelators and the difficulty in removing the water insoluble calcium soap was avoided. In this model, firstly the cloth containing oily soil was hydrolysed in the optimum pH of cutinase (pH 9), and then hydrolysis product was removed at elevated pH (pH >10.5), as the oleic acid formed from the hydrolysis almost fully partitioned in water when pH > 10.5. The stain removal is illustrated in Figure 2-8.
Figure 2-8 Fatty acid removal in the sequential washing system with Lipolase™ and cutinase, from reference [64].

The black column shows the dirt removal via an immersion of 1.5% (w/w) soiled brominated olive oil on polyester/cotton cloth in 1.3µg ml⁻¹ enzymes in Davies buffer at pH 9.0 at 30°C for 30 min; and the grey column show that the cloth was then incubated in 5 g. L⁻¹ detergent solution for another 30 min after immersion in enzyme solution.

In summary, in the prior studies, the advantage from adsorbed lipase to laundry washing has also been described. In Flipsen’s washing model, factors for an effective cleaning in lipase contained washing liquor were addressed, i.e. calcium-free washing solution should be used and higher pH would benefit oily stain removal. However in these studies, lipases have to be added to washing liquor every time. To overcome this shortage, in this thesis the potential of using chemically immobilized lipase in laundry washing is attempted.

### 2.2.2 Transglutaminase

Transglutaminase (TGase, EC 2.3.2.13) are acyl transferases that catalyse formation of amide bonds between the γ-carboxyl group of glutaminyl residues and a primary amino group, this is usually the ε-amino group of a lysine residue [65, 66].

#### 2.2.2.1 The Characteristics of TGase

Two types of transglutaminase are available in the market: microbial transglutaminase (mTGase) and mammalian TGase. mTGase is distinct from its mammalian counterpart in that it is smaller,
more stable, and calcium-independent; furthermore its use in food systems has become of great interest [67]. This microbial transglutaminase (mTGase) is typically isolated from *Streptomyces mobaraense* and has already been commercialised at industrial scale and available is at a reasonable price. The most popular mammalian transglutaminase is TGase from guinea pig liver. As mammalian TGase is currently only available at a higher price than mTGase, its main application is currently mainly in small quantities as part of academic research.

mTGase expresses its activity over a wide pH range. The optimum pH of mTGase is around 5 to 8. However, even at pH 4 or 9, mTGase still expresses some enzymatic activity. Its optimum temperature is 50 °C: mTGase has fully sustained its activity even at 50 °C for 10 min. At higher temperatures it is not as stable however: it loses its activity within a few minutes on heating to 70 °C. mTGase can maintain its activity under low temperatures - as low as the freezing-point [68].

mTGase prefers proteins with good accessibility to its active site as substrates, i.e. those proteins with less tertiary structure [69]. For example, casein (except for alfa-casein) is an excellent substrate for TGase, due to its flexible structure and the absence of disulfide bonds [70]. In contrast, globular proteins such as γ-lactalbumin, α-lactoglobulin, and bovine serum albumin cannot be completely attacked by TGase in its native state. Consequently, these substrates are not used as substrates for TGase. While if substrate is not available, TGase will take water as nucleophilic acceptor to deamidize the polymer formed [71].

With casein as a substrate, it was found that some indigenous TGase inhibitors exist in bovine, goat, sheep and human milk. The detail of the chemistry is still unknown however. What is known is that this inhibitor reacts sensitively to heat treatment. Heating at 85°C for 60 min increased the cross-linking reaction catalysed by TGase [72]. However the heat treatment for sodium caseinate is not necessary [73]. The breaking strength of casein gel formed in the presence of mTGase is related to enzyme concentration, incubation time, incubation temperature and incubation pH [74]. The break strength increased sharply with the increase in mTGase concentration until reaching maximum value. Further increase will lead to formation of soft and fragile protein gel. The gel strength improved when extending the incubation time. For sodium caseinate 120min to 250 min was required to obtain maximum gel breaking strength. After that the extent of increase in strength became smaller. And the best incubation temperature and pH for casein gel was found at 50 °C and pH 9.
Enzymatic cross-linking of TGase can be affected by inhibitory compounds. Metal ions like \( \text{Cu}^{2+} \), \( \text{Zn}^{2+} \), \( \text{Pb}^{2+} \) and \( \text{Li}^{2+} \) inhibit the mTGase by blocking the thiol group of its single cysteine residue. Several substances like N-ethylmaleimide (NEM), monoiodoacetate (MIA) and parachloromercuribenzoic acid (PCMB) are well known inhibitors of TGase activity. These substances block the active site of TGase by reacting with the thiol group. [72]

Note that in order to upgrade the globular proteins accessibility to TGase, genetic engineering can be used. For instance, \( \beta \)-galactosidase was converted into TGase substrate after a protein tag was attached to this enzyme by the means of a genetic technique [66]. As this is not the subject of this work, no further details will be given however.

### 2.2.2.2 Application of mTGase

mTGase has been used to form cross-linked protein gels, widely applied in the food industry and enzyme immobilization. For example, mTGase has been used to modify functional properties of food proteins, namely, protecting lysine from chemical reactions, modifying functional properties, and producing food proteins with higher nutritive values through cross-linking different proteins containing complementary or essential amino acids [75]. The properties of food protein that are improved after mTGase treatment include: increased solubility, increased water-holding capacity and thermal stability [68].

As a transferase enzyme, its application in enzyme immobilization has been studied. The simplest way of using mTGase in immobilization is through enzyme fixation by entrapment. The following details studies that have been used as a basis of the work performed in this thesis:

One method of enzyme immobilization with mTGase was studied by Motoki, Masao et al. [76] using \( \beta \)-glucosidase, \( \alpha \)-mannosidase, \( \beta \)-galactosidase, and glucose oxidase entrapped in \( \alpha s1 \)-casein film. The enzyme to be immobilized was added to a mixture of 5% \( \alpha s1 \)-casein and mTGase. Before gelation, casein/enzyme mixture was quickly spread on a horizontal plate. After air-drying, this film was removed from the plate and the activity was tested. It was reported that none of the immobilized enzymes lost activity on repeated usage. Alternatively, the direct connection between the support matrix and enzyme by the help of mTGase was also attempted [77]. However, until now, no
successful research has been reported, since mTGase has limited ability of taking globular enzymes as its substrate.

Immobilization of enzymes using mTGase also improves the properties of these enzymes. For instance, improvements in heat stability improvement may have been found recently. Noriho Kamiya et al. [77] studied the performance of crude lipase from *Rhizopus* sp. after *Streptoverticillium* transglutaminase (mTGase) treatment. After incubation of this lipase for 1 hour at 25°C in a solution containing mTGase from *Streptoverticillium*, it was found that its half-life was increased more than 10-fold compared to that of an untreated one. However, these researchers were not confident that this was the result of mTGase cross-linking the lipase, and instead they ascribed it to the cross-linking of some impurity in the crude lipase, which in turn affected the apparent lipase thermostability [77]. Unfortunately this improvement of thermal stability was not observed in this project, in the case of lipase from *pseudomonas fluorescens* (data was not shown), and so further experiments are needed to prove this.

### 2.2.3 Galactosidase

The lactose hydrolysis catalysed by β-galactosidase, is industrially important both to produce low lactose milk products for lactose intolerant people, and to make use of the whey product from the cheese making industry [78]. This research is especially important to New Zealand, due to the economic importance of the diary industry. Thus, in this review, background information on the use of β-galactosidase is also included.

Lactose is the main sugar in mammal milk, constituting about 3-8% (w/v) of milk by dry weight [79]. However, some people are intolerant to this sugar. Lactase (β-galactosidase or β-D-galactoside galactohydrolase EC 3.2.1.23) hydrolyses lactose into glucose and galactose, and galactooligosaccharides, which are sweeter, more digestible and more soluble than lactose. These hydrolysed milk products can be consumed by the 'lactose-intolerance' people [79]. Lactose is also a waste from cheese making industry, which has consequently become an environmental and economic concern. Moreover, there has been a steady 3% annual increase in cheese production [80], making the already problematic use of lactose a greater concern for the dairy industry. By the means of β-galactosidase-catalysed hydrolysis, lactose can be converted into galactooligosaccharides (GOS), a prebiotic functional food ingredient that selectively stimulates the growth of bifidobacteria
in the lower part of the human intestine and a yeast growth medium as well. A number of studies have been conducted using free and immobilized β-galactosidase to hydrolyse this lactose. The relevant studies to the work conducted in this thesis are detailed below:

Commercial and large-scale processes have been reported. For example, immobilized enzyme reactors for continuous hydrolysis of lactose on dairy products were developed first by SNAMProgetti in Italy and by Sumitomo Chemicals in Japan in the 1970s. Bigelis reported a commercial process using lactase immobilized on porous glass beads for the hydrolysis of lactose in whey [81]. Ceriesi presented another plant process to hydrolyse lactose in milk using beta-galactosidase immobilized on methacrylamide copolymer [81]. In general, reports about large-scale applications of immobilized enzymes are rare due to the costly process with immobilized enzyme. However, recently a number of interesting publications appeared to bridge this difficulty.

N. Albayrak and Shang-Tian Yang [80] developed a method for the production of galacto-oligosaccharides (GOS) from lactose by immobilization of β-galactosidase from Aspergillus oryzae on cotton cloth. They claimed that this immobilization involving PEI-enzyme aggregate formation and growth of aggregates on individual fibrils of cotton cloth leading to multilayer immobilization. A large amount of enzyme was immobilized (250 mg/g support) with about 90-95% efficiency. A maximum GOS production of 25-26% (w/w) was achieved at near 50% lactose conversion from 400 g/L of lactose at pH 4.5 and 40°C.

Quinn Z.K. Zhou and Xiao Dong Chen [79] used a different approach to immobilize galactosidase. β-galactosidase from Kluyveromyces lactis was immobilized on pre-activated graphite surfaces. BSA and glutaraldehyde were applied here as a matrix for enzyme immobilization. The graphite surface was immersed in the enzyme solution containing BSA and GA, and left at the ambient temperature overnight to complete the reaction. The specific activity yield of 17% and 25%, while the enzyme loading was 1.8 and 1.1 U/cm² of the graphite external surface area, respectively. Lactose hydrolysis at a concentration of 5% (w/v) by the immobilized enzyme was also investigated. The degree of lactose hydrolysis was approximately 70% at 37°C over a period of 3 hours. The immobilized enzyme showed good storage and operational stability.

Based on this previous work, this thesis will aim to produce a new method for immobilization of β-galactosidase on woollen cloth, which would potentially improve this past work. To do this, the
optimal enzyme immobilization technique for lipase on wool is to be determined first, and then this technique will be examined if this is a suitable method for $\beta$-galactosidase immobilization.

2.3 Enzyme Immobilization

Enzyme immobilization is a natural phenomenon. In the living world, immobilization of enzymes in membranes is nature’s way of obtaining stability, orientation and efficient mass transfer. Consequently, the artificial immobilization of enzymes aims to recreate these benefits and advantages for enzymes that cannot naturally immobilize under the conditions that they are applied in.

Enzyme immobilization (as defined by Katchalski-Katzir [82] at the first Enzyme Engineering Conference, held at Henniker, NH, USA, in 1971), is an “enzyme physically or chemically confined or localized in a certain defined region of space with retention to their catalytic activities, and which can be used repeatedly and continuously”. The classification of different types of enzyme immobilization techniques is varied. As described by E. Katchalski-Katzir [82], the methods used can be grouped into six categories:

(a) Cross-linking,
(b) Adsorption to a solid support matrix,
(c) Adsorption to a solid support material with subsequent cross-linking,
(d) Covalent binding to a solid support material,
(e) Enzyme crystallization, with or without cross-linking.
(f) Other entrapment techniques, such as entrapment into fibres, microcapsules or membranes, adsorption to membranes.

The specific techniques used in enzyme immobilization are not well understood and have not been fully and rigorously defined. Consequently they are currently considered as more an art rather than science [83]. The goals of immobilization are clear however - the ideal immobilization method would be: high immobilization yield, a large quantity of enzyme to be immobilized, a chemically and mechanically stable preparation, and with a minimal mass transfer barrier [84]. Practically the temperature used for immobilization is usually kept low (often at 4 °C or ice bath at 0 ° C) to minimize protein denaturation. [1]
Despite the above detailed groupings, for simplicity in this review, the immobilization methods are classified into two major subgroups: chemical immobilization methods and physical immobilization methods. Chemical methods, will be further divided into covalent binding immobilizations and cross-linking immobilizations, which differentiates between whether a chemical reaction and/or link is used to create the bond between enzyme to support (covalent binding) or if it is an enzyme to enzyme bond (cross-linking). Those immobilization involving physical forces only, such as adsorption, encapsulation, entrapment and membrane immobilization, are categorized as physical immobilization. These methods are addressed in more details in the following sections.

### 2.3.1 Immobilization by physical method

The physical immobilization method can be further separated into two categories: (1) immobilization by adsorption, (2) immobilization by encapsulation, microencapsulation and membrane entrapment.

1. **Immobilization by adsorption.** Immobilization by adsorption is the simplest and most commercially popular protocol. In fact, the first industrial enzyme-immobilized process was amino acalase adsorbed onto an ionic exchange resin to produce D,L-amino acid [85]. Only physical forces, namely Van der Waals forces, hydrogen forces or electrostatic forces, are used when enzymes are immobilized on porous structure [86], hydrophobic surface [20] and/or electrical charged surface [87]. As no chemical reaction is involved, the activity loss that is typically seen with chemical immobilization methods is avoided. Another advantage is that the enzyme support can be recycled. In contrast, for those immobilizations via chemical reactions, when the life span of the immobilized enzyme is over, both the enzymes and the support have to be eliminated as wastes. The cost is increased, as some support matrices are quite expensive with a cost even higher than enzyme itself [88]. However, the key disadvantage of immobilization by adsorption is the lack of stability, as it is not easy to prepare a stable immobilized enzyme by a physical method only [89].

2. **Immobilization by encapsulation, microencapsulation and membrane entrapment.** By these methods, an enzyme is confined within a constraining structure tight enough to prevent proteins from diffusing into the bulk solution, whilst not limiting the accessibility of substrate through the membrane. The three methods differ only slightly: for the entrapment strategy, enzymes are embedded into a polymer network; for microencapsulation, enzymes are confined within
microencapsulation prepared from organic and/or natural polymers; and for membrane entrapment, the enzyme solution is retained by a membrane permeable only to the substrate and product.

The major advantages of this method again lie in the fact that there is no chemical modification involved and the intrinsic property of the enzyme is therefore not affected by the immobilization method. However, despite the fact that there is negligible enzyme activity loss, it does not mean a high expressed immobilized activity will be created by these methods. This is because a considerable mass transfer barrier is created by the encapsulation and/or entrapment, which lower the effective reaction rate and concentration of substrate seen by the enzyme: i.e., the “effectiveness factors” are quite small. Moreover, those methods are not applicable when the size of substrate is similar to that of the enzyme. Another problem is enzyme leakage. In contrast to the chemical methods, the physical forces involved in immobilization are too weak to keep enzyme stably retained within the immobilization matrix. Subsequently this kind of leakage is unavoidable. However, cross-linking and covalent treatments after immobilization could ease this problem [90].

In this thesis, physical immobilization techniques, including physical adsorption (enzyme adsorbed onto PEI resin) and entrapment (lipase entrapped in casein gel formed in the presence of TGase), are applied. The detailed description is presented in Sections 4.1 and 7 respectively.

### 2.3.2 Immobilization by Chemical Reactions

#### 2.3.2.1 Chemical Reactions used for Immobilization

The chemical immobilization methods can be divided into the covalent binding method and cross-linking method. Both methods are described in detail in the sections that follow. The chemical reactions used in immobilization are also described.

Various chemical reactions have been utilized in enzyme immobilization. Although the reaction mechanisms are varied, most of them select amine groups in enzymes, including NH$_2$ of lysine residue and the NH$_2$ terminal, as the reaction target. The reason is, as described by Jose M. Guisan [21]: “first most of the proteins show many lysine residues that are usually not involved in catalytic site. Secondly amine groups are polar and they are usually exposed to the medium on the protein surface. Finally, amino groups, when they are unprotonated, are very reactive, without previous activation.”
There are two types of amine groups available in proteins for chemical modification: ε-amine moiety of lysine residues and terminal amino groups. The most abundant ones are the ε-amine moiety of lysine residues. For external and exposed lysine residue, the pKa is close to that of the free amino acid, around 10.5 to 10.7. Of course, some internal lysine residues may alter the pKa value, due to the influence from the microenvironments around internal pockets. But these lysine residues are less important for immobilization, as the steric hindrances impede the reaction between the support and these internal groups. For terminal amine groups, the pKa is around 7 to 8. It was reported that the pH used in immobilization might decide what kind of amine would take part in the reaction: at alkaline pH values, the reactivity of Lys residues may be more suitable for the reaction, as it was unprotonized in that pH range. In this way, proteins should be mainly immobilized via the amino terminal group when using these supports at neutral pH values [91]. However, in some literature, it was also stressed that the pH used for reductive glutaraldehydration to the terminal amino groups needed to go to slightly acidic [92, 93].

A number of reactions are used in enzyme immobilization. The major reactions used include: diazo-coupling reactions, acid anhydride reactions, reactions of isocyanate and its derivatives, and epoxide and Schiff’s base reaction. The application of irradiation in enzyme immobilization has also been reported. This method is non-specific, but leads to low immobilization yield and high activity loss arising from radiation damage. For further details please refer to Table 2-2. Among these reactions, oxirane alkylation [25] and Schiff’s base reaction [94] are the most important, as they have low toxicity, high water solubility, and are the most often used in academic work. The reactivity of epoxide (oxirane) and its derivatives toward nucleophilic groups is in the order of thiol>amino>hydroxyl. The advantages of this reaction are that the linkages formed are extremely stable and no charged group is generated, thus avoiding non-specific adsorption. Schiff’s base linkage between the carbonyl group on the support and free amine group on the protein is the most widely used technique nowadays. This technique is fast and causes mild enzyme activity loss, the aldehyde reagent used, such as glutaraldehyde, has low toxicity. One disadvantage is the product formed might be reversible; sometimes reduction with a hydrogenation reagent is therefore necessary [95].
Table 2-2 The major reactions used in immobilization by the chemical method [95]

<table>
<thead>
<tr>
<th>Coupling reaction</th>
<th>Reaction group on support</th>
<th>Reaction group of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylation</td>
<td>Oxirane</td>
<td>Hydroxyl, thiol</td>
</tr>
<tr>
<td>Diazotization</td>
<td>Diazonium salt</td>
<td>Amine, thiol, hydroxyl</td>
</tr>
<tr>
<td>Peptide bond formation</td>
<td>Acid anhydride</td>
<td>Amine, thiol</td>
</tr>
<tr>
<td></td>
<td>Isocyanate</td>
<td>Amine</td>
</tr>
<tr>
<td></td>
<td>Imidocarbonate</td>
<td>Amine</td>
</tr>
<tr>
<td>Schiff’s base formation</td>
<td>Aldehyde</td>
<td>Amine</td>
</tr>
</tbody>
</table>

More examples of enzyme immobilization by chemical methods are listed in Table 10-2 in Appendix 10.1.

### 2.3.2.2 Immobilization by Covalent Binding

As illustrated in Figure 2-9, covalent binding method is based on covalent bonding of enzymes to a water-insoluble support. The support should have electrophilic groups, including hydroxylic, carboxylic or amino groups, which can be activated by the chemical reactions mentioned in Section 2.3.2.1. The most used active groups for covalent immobilization include the aldehyde group, the epoxy group, cyanogens bromide, and the tosyl group. Then, the nucleophilic group of the enzyme (e.g. primary amines for lysine, carboxylic function group for aspartic and lutamic acids, hydroxyl groups for tyrosine, erine and threonine, thiol for cysteine) are coupled to this activated support to complete the immobilization.

![Figure 2-9. Illustration of covalent binding immobilization](image)

Since a chemical reaction is involved, a very stable coupling can be achieved. However, the drawback is that this modification generally leads to a considerable loss of the initial activity of the
enzyme, as the three-dimensional structure of the protein is considerably modified [96]. Covalent binding is the most investigated and widespread approach in laboratory studies for enzyme immobilization. But due to the difficulty of controlling the process, this technique is not as widely accepted as the physical adsorption method at a commercial scale [97].

A. Determinant Factors for a Successful Covalent Binding Immobilization

The factors affecting immobilization differ between the different techniques that are adopted. But generally speaking, pH and temperature are the most important factors to be considered.

1. pH

pH is very important for immobilization. Due to the poly-ion characteristics of enzymes, the performance of immobilized enzymes is directly related to the solution pH. For immobilization, the amount of protein immobilized is linearly dependent on the degree of surface activation, which is related to pH. For example, glutaraldehyde and cyanogens bromide (the two most commonly used surface activation agents in enzyme immobilization), are able to yield stable enzyme–support binding only at neutral pH range. It is unsuitable for use in an alkaline pH range, as their high reactivity might cause excessive enzyme denaturation. It was reported [98] that the lysine residue in enzymes would only participate in the immobilization in alkaline solutions, as the pKa for lysine residue is 10.5 to 10.7. Only the terminal amine could participate the covalent binding reaction at neutral pH range or a slightly acidic neutral pH, as its pKa is around 7 to 8. The immobilization under high pH might lead to “multi-point attachment”, as more lysine residues exist in enzymes than terminal amino groups.

Consequently, the effect of pH on enzyme immobilization and activity is determined in this thesis.

2. Temperature

To minimize protein denaturation from the chemical reactions occurring during immobilization, it is optimal to keep the temperature low (often a cold room temperature at 4 °C or an ice bath at 0 °C) [99]. Low temperature also affects the level of contamination from microorganism, as live organisms growth rate is often insufficient to cause serious problem under low temperature.
B. Approaches for Covalent Binding Immobilization Enhancement

Besides optimising immobilization via variations in temperature and pH, to improve the performance of immobilized enzymes, other techniques, such as blocking agents, spacer arm and multipoint binding, are also often used by the enzymologist. These options are described in more detail below.

1. Blocking agents

As the presence of free aldehyde and epoxy rings group on the immobilization support can decrease the immobilized enzyme thermostability [100], blocking agents are normally used to quench them. The blocking agent can be classified into two categories: small molecules (e.g. amino acids or other amine), and macromolecules (e.g. bovine serum albumin, gelatine, PEI, and aminated polyethylene glycol) [83]. For macromolecule blocking agents, a stabilizing hydrophilic microenvironment can be created. For example, consecutive modification of penicillin V acylase from Streptomyces lavendulae immobilized on Eupergit C with bovine serum albumin led to the formation of a new biocatalyst not only with enhanced activity (1.5-fold) in the hydrolysis of penicillin V, but also enhanced stability [101]. Remarkably, this biocatalyst could be recycled for at least 50 consecutive batch reactions without loss of catalytic activity. However, as a more efficient reacting reagent, small quenching agents are therefore more preferred in this circumstance [4]. In this thesis, Tris buffer (containing amino group) is used as storage buffer to quench the excessive aldehyde group on immobilization supports. Further discussion is detailed in Section 5.2.

2. Spacer

The introduction of a hydrophilic spacer could increase the immobilized enzyme’s stability. For example, in immobilization of Bacillus stearothermophilus lipase (BSL) to silica, the polyethyleneimine (PEI) was used as a spacer [11]. BSL was covalently bound to PEI coated silica activated by GA. The presence of a PEI spacer displayed improved storage and operational stability, compared with the same lipase immobilized to glutaraldehyde-activated silanized support [11]. Most likely, the presence of PEI created a favourable microenvironment for lipase.

The other purpose of using a spacer is to enable the immobilized enzyme to be in a less tight spatial confirmation, i.e. providing more space between the cross-linked enzyme molecules and the surface of the support matrix to let the said molecules maintain their predetermined spatial arrangement for
proper enzyme function. Moreover, the presence of an amino containing spacer, such as polyethyleneimine, will increase the binding points on the carrier resulting in enhancement in immobilized activity [4]. Otherwise, the enzymes will be cross-linked directly by a relatively short chain agent, such as GA, causing them to closely contact each other, leading to improper folding and more enzyme deactivation [102].

The use of a PEI spacer is the key to the covalent binding technique developed in the current work. For further details, see Section 2.4 below.

3. Multipoint attachment

In covalent immobilization, superior stability for immobilized enzymes may be achieved by multipoint attachment [82, 103]. For multi-point attachment, enzymes are fixed to a support through more than one chemical connection for one enzyme molecule. These more rigid structures are more resistant to the conformational changes from the external denaturant, such as heat and organic solvent etc, thus are more stable than their mono-point binding counterparts. However, the disadvantage is a considerable loss of enzyme activity during the intensive multipoint attachment. To form multi-point binding, high-density activity groups on the support surface must be present. So far, the most successful example of “multi-point-attachment” is from using the commercial immobilization support Eupergit C (epoxy-activated support) [88]. A very high stability is reported: for instance, only 2 % activity loss after 200 days continuous running was found for porcine pancreas immobilization on Eupergit C [88]. For lipase from *aspergillus niger* in hydrolysis of dephamosporin C and cephalotin, no activity loss was reported for a two month batch operation [88].

The considerable stability increment from “multipoint attachment” makes it become a very attractive technique and is therefore a worthwhile method to be explored in this project. The detail discussions and analysis of this are given in Section 6.5.

2.3.2.3 *Immobilization by Cross-linking*

Immobilization by the cross-linking method is based on the formation of covalent bonds between enzymes in the presence of a bifunctional or multifunctional cross-linker. This cross-linking normally creates water-insoluble protein aggregates. Originally, the cross-linking technique involved adding a cross-linker, such as glutaraldehyde (abbreviated to “GA” hereinafter), to a concentrated enzyme solution to form water insoluble enzyme aggregates. These are known as
cross-linked enzymes (CLE). Alternatively, CLE can also form at a low enzyme concentration, by adding a lysine rich inert protein (e.g. BSA) to facilitate GA cross-linking [79, 104]. The major advantage of using cross-linking is that it often results in a greatly increased resistance to various denaturation factors, such as extreme pH and temperature, high ionic strength, and the presence of a denaturant such as protease. The stabilization effect from enzyme cross-linking can be better explained in Figure 2-10 [40]. As shown, the restriction on conformational flexibility caused by multiple cross-linking of enzyme is the reason for dramatic increase the stability against denaturation.

Although enzyme cross-linking can increase enzyme stability, there are several major disadvantages remaining, including lower activity retention (50% less than that of the native enzymes), poor reproducibility and low mechanical stability [40]. Because of the considerable decrease in enzyme activity caused by CLE, it has been gradually displaced by new and more moderate techniques (which therefore do not deactivate the enzymes as much), including cross-linked enzyme aggregation (CLEA) [3] and cross-linked enzyme crystallisation (CLEC) [105]. Here CLEC refers to the cross-linker coupling which occurs to a crystalline enzyme rather than a soluble enzyme; while for CLEA, an enzyme aggregate is induced by the addition of salts, organic solvents, or non-ionic polymers to proteins prior to cross-linker coupling. These methods do not reduce enzyme activity as much as CLE, as the enzyme tertiary structure is protected by the physical interactions formed during protein crystallization and precipitation, allowing a high rate of enzyme activity to be retained after cross-linker treatment.

Enzyme immobilization by the cross-linking method is not only suitable for aqueous preparations and also can be applied to solid surfaces. In fact, the formation of an enzyme aggregate in the pore of porous material can “anchor” the enzyme into the matrix. When a support has few binding points, enhancement of enzyme load on the support can be achieved through immobilizing a few proteins onto support surface first, followed by enzyme cross-linking (aggregation) with that protein by GA treatment after [106].
Since CLEC & CLEA are kinds of insoluble protein precipitate already, immobilization carriers for CLEA and CLEC are unnecessary. Considering that the purpose of the current research is that wool must be used as the immobilization support, CLEC and CLEA thus are not applicable. However, concerning the self-aggregating property of the lipase used in this research (see Section 2.2.1.3), it will be of interest to investigate the cross-linking behaviour of PFL aggregate formed in concentrated lipase solution on wool surface.
2.3.2.4 Cross-linking when enzymes are already immobilized on a support

When enzymes are immobilized onto a support matrix, an additional cross-linking can be used to take advantage of the enhanced stability that cross-linking of enzymes produces. The improved operational stability is however achieved at the expense of reduction of the specific activity of the enzyme immobilized, as enzyme deactivation occurs [82]. There are several other advantages (outlined below), which therefore motivated the use of this method in the work of this thesis.

**Immobilization by physical methods followed by cross-linking:** For immobilization on a water insoluble matrix, by “adsorption to a solid support material with subsequent cross-linking” [82], the cross-linking reaction rate is accelerated, as the initial adsorption of protein to the support helps to form intimate protein contact with the surface. Another benefit obtained from combination of physical adsorption and cross-linking is that the molecular weight of the immobilized enzyme is increased accompanied by the improved enzyme stability, avoiding drawbacks from physical adsorption (as described in Section 2.3.1). For instance, Lopez-Gallego et al. [107] immobilized d-amino acid oxidase (DAAO) on sepabeads through a combination of ionic adsorption on different supports, plus covalent cross-linking with GA. The highest stability was achieved by the treatment with the GA on the DAAO adsorbed on Sepabeads EA (a commercial laminated support having ethylenediamine groups). This derivative was reported to be six times more stable than the enzyme adsorbed only by ionic interaction and much more stable than the soluble enzyme.

**Immobilization by chemical reaction followed by cross-linking:** Similar stability improvement could also be obtained from an immobilization protocol combining covalent binding and cross-linking. However, direct cross-linking treatment after covalent binding immobilization would not lead to additional benefit; consequently a special protocol must be followed instead. In fact, although a single point modification by cross-linking agents to enzymes which have already been fixed on a support by covalent bond is a rapid reaction, a further cross-linking between these enzymes requires the correct alignment between two reactive groups in these enzymes whose structure are quite rigid already due to the covalent connection to the support. Thus, when a large excess of bifunctional reagent is present, it is expected that most of the amine reactive groups on these enzymes will be consumed by this “single point modification”, thereby making further cross-linking reactions impossible. To establish this kind of “intramolecular cross-linking”, R Fernande-Lafuente et al. [108] proposed a new strategy as shown in Figure 2-11. That is, after chemical modification (“activation”) of the protein surface by the cross-linking reagent, the enzyme
derivatives were washed and the excess reagent eliminated. Then, the “activated” enzyme is incubated for a long time in a solution with a proper pH to obtain cross-linking without any risk of new chemical modification”. It was reported [108] that, for penicillin G acylase immobilization on glyoxyl agarose gel surface, via the above strategy, considerable stability improvement was achieved at the expense of limited activity loss. However, in the preliminary experiment in the current project, it was found that this strategy could not improve the immobilized activity for lipase immobilization on wool, probably due to the fact that the surface chemical characteristics are different from the above examples.

![Diagram](image)

**Figure 2-11** Comparison of a new strategy and a conventional one to obtain protein cross-linking. P: protein surface; 0-O: cross-linking agent, from reference [108].

### 2.4 Important Chemicals used for Enzyme Immobilization

In this project, a number of chemicals were used. Among them, PEI (spacer arm) and glutaraldehyde (cross-linker) were the key components in the immobilization ingredients, thus are described in more detail below.

#### 2.4.1 Polyethyleneimine (PEI)

##### 2.4.1.1 Characteristics of PEI

As a versatile figure in biotechnology, PEI is widely used in a range of different applications, including protein separation [109], ion exchange resin preparation, nucleic acid and acidic protein purification [110], cell debris flocculation [111], chelating agent in water treatment and immobilization aid in enzymology [80]. While in the project, it was used as spacer arm bridging woollen support and immobilized enzymes. PEI is commercially available from the BASF Corporation under the trade name Lupasol.RTM (and is also sold as Polymin.RTM.).
Figure 2-12 Schematic view of molecule structure of branch Polyethyleneimine, from reference [112]

PEI can be linear or branched. Linear PEI is water insoluble and branched PEI is water-soluble. Branched PEI is therefore more interesting for applications in biotechnology. Thus hereinafter, branched PEI is short for “PEI”. The molecular structure of the branched PEI is shown in Figure 2-12. The amino nitrogen of PEI can be modified easily by alkylation, acylation or imine formation. The amine groups of PEI exist mainly as a mixture of primary, secondary and tertiary groups in the ratio of about 1:1:1 to about 1:2:1 with branching every 3 to 3.5 nitrogen atoms along a chain segment. Because of the presence of amine groups, PEI can be protonated by acids to form a PEI salt resulting in a product that is partially or fully ionized depending on pH. The majority of amino groups are unprotonated when the solution pH is close to 10 [112], whilst chemical modification of the amine groups is favoured at an alkaline pH when amino groups are unprotonated [110]. Thus the preferable pH for PEI spacers in enzyme immobilization is alkaline pH [112].

The protonation of PEI is different from smaller molecular amines that normally contain an invariable ionization constant. Junghun Sun et al. [112] calculated the apparent ionization constant of PEI at different pH, as shown in Table 2-3. The apparent ionization constant decreases linearly as pH is reduced. This maybe due to the fact that the more positive charge on PEI at lower pH leads to greater electrostatic suppression of the protonization. The degree of PEI protonization is also decreased at a higher concentration, due to the change of ionic strength. Therefore, practically the
PEI concentration used is normally lower than 0.05 resm (concentration of polymer was expressed in terms of the monomer residue molar concentration).

Table 2-3 Apparent ionization constant of polyamine at various pH [112]

<table>
<thead>
<tr>
<th>pH</th>
<th>PEI (0.01 resm)</th>
<th>PEI (0.05 resm)</th>
<th>L10PEI (0.0115 resm)</th>
<th>PAA (0.01 resm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3.66 (18%)</td>
<td>3.30 (33%)</td>
<td>3.05 (47%)</td>
<td>3.08 (45%)</td>
</tr>
<tr>
<td>4</td>
<td>4.31 (33%)</td>
<td>4.10 (45%)</td>
<td>3.72 (66%)</td>
<td>3.86 (58%)</td>
</tr>
<tr>
<td>5</td>
<td>4.96 (52%)</td>
<td>4.89 (56%)</td>
<td>4.38 (81%)</td>
<td>4.64 (70%)</td>
</tr>
<tr>
<td>6</td>
<td>5.61 (71%)</td>
<td>5.69 (67%)</td>
<td>5.05 (90%)</td>
<td>5.42 (79%)</td>
</tr>
<tr>
<td>6.5</td>
<td>5.93 (79%)</td>
<td>6.08 (72%)</td>
<td>5.39 (93%)</td>
<td>5.81 (83%)</td>
</tr>
<tr>
<td>7</td>
<td>6.25 (85%)</td>
<td>6.48 (77%)</td>
<td>5.72 (95%)</td>
<td>6.20 (86%)</td>
</tr>
<tr>
<td>7.5</td>
<td>6.58 (89%)</td>
<td>6.88 (81%)</td>
<td>6.05 (97%)</td>
<td>6.59 (89%)</td>
</tr>
<tr>
<td>8</td>
<td>6.90 (93%)</td>
<td>7.28 (84%)</td>
<td>6.39 (98%)</td>
<td>6.97 (91%)</td>
</tr>
<tr>
<td>8.5</td>
<td>7.22 (95%)</td>
<td>7.57 (87%)</td>
<td>6.72 (98%)</td>
<td>7.36 (93%)</td>
</tr>
<tr>
<td>9</td>
<td>7.55 (97%)</td>
<td>8.07 (89%)</td>
<td>7.06 (99%)</td>
<td>7.75 (95%)</td>
</tr>
<tr>
<td>10</td>
<td>8.20 (98%)</td>
<td>8.87 (93%)</td>
<td>7.72 (99%)</td>
<td>8.53 (97%)</td>
</tr>
</tbody>
</table>

Note: Lau10 PEI is PEI derivative with 10% amino group laurylated; PAA is short for poly (allyamine), a linear polymer consisting of primary amine only; Resm is the concentration of the polymer expressed in the terms of the monomer residue molar concentration. Numbers in brackets are the fraction of the unprotonated nitrogen in the polymer at respective pH.

Proteins have been found to be highly stable in the PEI solutions [113]. This stabilization may be from a change in the microenvironment around the enzyme due to the presence of PEI. This hypothesis was confirmed by circular dichroism (CD) measurement of the secondary structure of the enzyme in the UV range of the spectrum. Alternatively, the stabilization may also come from metal ion chelation by the PEI, which could be used for catalyzing chemical modification of a protein molecule at ambient or higher temperatures.

**2.4.1.2 PEI in Enzyme Immobilization**

PEI is extensively used in enzyme immobilization. The protonated PEI is able to produce strong physical adsorption toward a negatively charged enzyme. As the enzyme is enriched around PEI, the immobilization rate can be considerably increased. Moreover, the enzyme’s tertiary conformation is distorted less, since the electrical adsorption can also retain enzyme’s structure. The immobilization protocols involved in PEI that have previously published in literature can be generally grouped into three categories, and some of these methods had been tried in enzyme immobilization on wool in this thesis.
1. Reversible enzyme immobilization on PEI coated surface

Enzyme is strongly electrostatically adsorbed onto PEI coated support such as agarose and silica. As the adsorbed enzymes can leak out of this support, this approach is also called “reversible enzyme immobilization”. This approach is well documented in the literature [114, 115]. For example, for galactosidase from A. orizae and lipase from C. rugosa, 70-80% of protein contained in crude extract could be retained on a PEI coated support even under high ionic strengths. Around 50-60 mg of protein could immobilized per ml of PEI-agarose and 90-100 mg protein could be immobilized per ml of PEI-sepabeads, compared to only 50% of protein retention for conventional DEAE-agarose, since a high charge density (1000 μmol per ml support) is present on the PEI-support [114]. The reversible immobilized enzyme also exhibits better stability than its soluble counterpart. When glutaryl acylase adsorbed onto PEI-Sepabeads, the half-life of the immobilized enzyme is 26 fold longer than the free enzymes at 45 º C. For reversible immobilized glutaryl acylase, the stability is even higher than the covalent derivative prepared from cyanogens bromide agarose [116]. However, the instability feature of the “reversible enzyme immobilization” makes it less desirable for industrial application, thus the immobilization method via cross-linking agent treatment on the bases of physical adsorption is then developed. The content is detailed in the coming paragraphs.

2. Enzyme adsorbed onto PEI coated fabric cloth followed by GA cross-linking

A number of publications have been written on this topic, especially for enzyme immobilization on cotton cloth. Enzymes with low isoelectric point, such as glucoamylase [11], invertase [117], glucose oxidase [118], and urease [119] etc have been shown to strongly bind to PEI-coated cloth surfaces. Adsorbed enzyme was not eluted under low ionic strength, but desorbed at high ionic concentration. Cross-linking with glutaraldehyde could prevent this occurring. The presence of substrate could reduce the enzyme sensitivity to glutaraldehyde inactivation [11]. Studies that are relevant to the protocols developed in this study are detailed below:

Yamazaki et al. [117] immobilized yeast invertase onto PEI coated cotton cloth. The PEI-cloth was prepared by soaking cotton flannel in 0.1% of PEI solution (pH adjusted to 7 with HCl) for 30 minutes. Then this PEI-cotton cloth was extensively washed under running distilled water to remove excess PEI. The washed cloth was air-dried and stored at 4 º C. To immobilized invertase, this PEI-cloth was soaked in 0.1% of enzyme solution for 16 hour at room temperature. After that, this enzyme-adsorbed cotton was cross-linked in 2% GA for 2 hour at room temperature. Finally, the
invertase-immobilized cloth was washed with water, blotted dry with paper towel, and stored at 4 °C. Over 3 months continuous operation for sucrose hydrolysis was reported.

By the similar method, glucoamylase was immobilized on polyethyleneimine (PEI)-coated cotton cloth through enzyme adsorption followed by cross-linking with 0.2% glutaraldehyde in the presence of starch [11]. The PEI concentration used for adsorption was found to influence the immobilization tremendously. An increase in the PEI concentration used for cotton cloth adsorption was shown to result in the decrease in the amount of enzyme binding, which was attributed to the enhancement in the hydrophobicity of the cotton surface. Optimum adsorption was seen at 2% PEI. Further increase in PEI concentration led to a considerable decrease in the enzyme binding. Optimal adsorption of the enzyme was found when PEI coated cotton cloth was soaked in the enzyme solution for 50 min. pH and temperature were not found to affect the immobilization substantially. This bound enzyme exhibited an excellent operational stability. The reactor had been continuously operated at 45 °C for over 21 days with only 30% activity reduction.

A more encouraging protocol on enzyme immobilization on cloth was addressed by Nedim Albayrak and Shang-Tian Yang [80] in their novel technique for β-Galactosidase immobilization on cotton cloth. The aggregate of β-Galactosidase from *Aspergillus oryzae* was formed in the presence of PEI solution, which was regarded as the “driving force” for this immobilization technique. The formation of PEI-galactosidase aggregate is, as described by Albayrak: “When a clear enzyme solution in distilled water was mixed with a PEI solution, cloudy, turbid or milky slurry of PEI-enzyme aggregate was formed instantaneously”. The PEI-enzyme ratio, buffer using was found to greatly affect the precipitate formation. This enzyme multi-layer structure was fixed by 0.2% of GA solution at pH 7.0. The author claimed that a large amount of enzyme was immobilized (250 mg/g support) with about 90% to 95% efficiency. The half-life for the immobilized enzyme on cotton cloth was close to 1 year at 40 °C and 21 days at 50 °C. Three days stable and continuous operation in a plug-flow reactor was achieved.

Their result was so promising that Matella et al. [120] tried to repeat their experiment. Unfortunately, opposite results were reported. Two enzymes (β-Galactosidase Sigma-Aldrich and pure Bio-cat enzyme) were used in Matella’s study. Less than 0.2% of Sigma galactosidase was attached to cotton by the method given by Nedim Albayrak [80]. Due to presence of dextran in this enzyme preparation as protecting agent, most of the enzyme activity was found in PEI residue in water and
GA solution, which suggested that enzyme from Sigma-Aldrich did not adhere to PEI coated cloth at all. However, for β-galactosidase from Bio-cat, after immobilization by the same method, only 8% activity was found on cotton fraction, while other fraction contain only little or no active enzyme. These results complied with the data published by Mateo et al. [121] that GA cross-linking in the presence of PEI showed activity loss ranging from 50% to 100% depending on type of enzymes.

In summary, GA cross-linking after enzymes are adsorbed onto PEI coated support matrix is a promising method, which has been approved by a number of successful immobilization instances. Based on this principle, a protocol was developed in this research and the detail is described in Section 6.1.

3. PEI coated support activated with GA first, followed by enzyme adsorption

For this protocol, the support matrix is covered with PEI (which attaches by adsorption) and then derived with glutaraldehyde. Coupling the enzyme to this modified support is completed by adsorption of the enzymes from solution. As PEI is activated with glutaraldehyde first, the deactivation from direct GA contact to the enzymes in solution is avoided. Thus, this method presents a low impact on the enzyme activity and greatly improves the enzyme stability as well. Baojiao Gao and his co-workers [122] explored a penicillin G acylase immobilization approach on GA modified PEI-silica gel. This PEI-silica gel was firstly mixed with glutaraldehyde solution at room temperature for 1 hour, and then soaked in Penicilline G acylase solution at pH 7.92 for 14 hours. Satisfactory continuous operating stability was achieved: The hydrolysis reaction was carried out by agitating the immobilized enzyme in penicillin G solution (4%) for 10 minutes. 87.5% of immobilized activity was retained after continuously running for 15 operational cycles. P. Lozano [123] reported another approach for Candida antarctica lipase immobilization on PEI coated surface. A dynamic membrane by the cross-flow filtration of gelatin and PEI was formed on α-alumina ceramic surface. This membrane was preactivated with GA solution at 25 °C, followed by covalent attachment of lipase from Candida antarctica lipase B. For continuous hydrolysis of p-nitrophenyl palmitate (pNPP), the gelatin–PEI active membrane demonstrated 93% coupling efficiency and 22 hour half-life time. More examples of the application of PEI in enzyme immobilization were list in Appendix 10.2.

Inspired by these achievements from the past research, another lipase immobilization protocol (CVB protocol) was developed, and the detail is given in Section 4.
2.4.1.3 Factors affecting Enzyme Immobilization on PEI and Features of its Preparation

Generally speaking, for enzyme immobilization with PEI as a spacer, pH, GA concentration, molecular weight of the PEI and treatment time in GA solution are the main factors affecting the immobilization. Key precedents in the prior literature relevant to this project and illustrating the effect of these factors are therefore presented below:

Immobilized activity is found to be closely related to GA pH, GA concentration and treatment time in GA solution, when PEI was involved. For instance, Baojiao Gao and his co-workers [122] found that for penicillin G acylase immobilization on PEI grafted silica gel in the presence of GA cross-linker, optimal immobilization happened at pH 7.92. Selection of this pH is a compromise between enzyme isoelectric point and the zeta potential of the support. The immobilized activity increased with the soaking time in GA solution, until reaching to its maximum value at 48 hours. The amount of GA used showed great influence to the immobilized activity, as illustrated in Figure 2-13. At a low GA concentration, the immobilized activity increased with GA concentration. However, when the amount of GA used was over the optimum value, the activity decreased sharply. The author hypothesised that this might come from excessive covalent linkage between enzyme and support, resulting in severe enzyme conformation distortion.

The molecular weight of PEI grafted on the immobilization support was also found to have influence over immobilization. For PEI grafted agarose, it was reported the protein adsorption strength increased with molecule weight of PEI ranging from 700 to 25,000 Da. Moreover, the more PEI that was coated, the more enzyme that was adsorbed [114]. Likewise, when D-amino acid oxidase (DAAO) was covalent attached by GA onto PEI coated sepabeads, the thermal stability of DAAO derivative with 25 kDa polyethyleneimine was higher than when other polyethyleneimine sizes were used (700 Da, 60,000Da). This higher thermal stability for large Mw PEI could due to masking of the sepabead hydrophobicity, because the excessive hydrophobic contact that occurred to the covalently immobilized enzyme could have distorted the enzyme structure under high temperature and result in low thermal stability. PEI with smaller Mw (0.7 kDa) can not fully cover the surface to avoid the negative effect from the support hydrophobicity as much as the PEI with bigger Mw (25kDa, 60kDa) [107]. Similar results were reported by Alonso et al. [124]: the coating of the support with PEI of 70–600 kDa increased the stability of the immobilized preparation four to five-fold in comparison to the preparation on a support coated with PEI of 0.7 kDa [124].
Immobilization of an enzyme on a PEI coated support might lead to a shift of its optimum activity with pH and optimum activity with temperature compared to the free enzyme. This is due to an increase in the pH around the microenvironment of the enzyme as compared to the bulk liquid. For example, Baojiao Gao [122] reported that for a penicillin G acylase immobilized on a GA modified PEI grafted silica gel, the optimum pH for the immobilized enzyme shifted from 9.32 to 7.34 compared to the free enzyme, as shown in Figure 2-14. The optimal activity temperature changes from 50°C for the free enzyme to around 40°C for the immobilized enzyme (Figure 2-14).

The change in optimal activity with pH and temperature were therefore two important parameters that were quantified in work for this thesis.

Figure 2-14. Comparison of pH and temperature profile of immobilized penicillin G acylase immobilization on GA modified PEI grafted silica gel. Red line is free enzyme and black line is immobilized enzyme, from reference [122].
2.4.2 GA and GA Derivatives in Enzyme Immobilization

Many bi-functional compounds have been used in protein modification, including dialdehyde, diimidostester, di-isocyanates, and bis-diazonium salts. However, glutaraldehyde gives the most satisfactory and reproducible results, as well as reacting with free amine groups on proteins under mild conditions [125].

2.4.2.1 Glutaraldehyde (GA)

Although the reaction mechanism of GA cross-linking is still to be determined, it is one of the most widely used reagents in biotechnology, notably in the leather tanning industry [126], protein crystalline [127] and chemical sterilization [128]. It is even accepted by the Food and Drug Administration (FDA) to be used in food processing [129]. GA shows good activity toward the amino acid side chain of proteins, including those of lysine, cysteine, histidine and tyrosine. However, considering the relative concentration, hydrophobicity and reactivity, lysine is the most likely accepted amine groups to participate the reaction with GA. It is usually sufficient to complete the cross-linking for a protein by incubating in 1 to 3 mM (pH 6-8) of GA solution for 20 min at room temperature.

![Figure 2-15 The molecular structure of glutaraldehyde](image)

The pH of aqueous GA solution is 3.1, since part of the aldehyde groups oxidise to carboxylic acid in water. The chemical composition in GA aqueous solutions are complex, and include the free GA, dehydrates, cyclic hemiacetal and oligomers. Their composition varies with pH, temperature and GA concentration. At an alkaline pH, the fraction of oligomeric aldehydes is increased [130]; at a high temperature more cyclic hydrates are converted to the free GA; in a more concentrated solution or acidic solution, cyclic hemihydrate multimers are formed; in dilute solution, all these condensation products are reversed to monomers.

The most widely accepted theory on the reaction mechanism of GA in enzyme immobilization is that the aldehyde groups in GA react with the lysine residues in the protein through a Schiff’s base
reaction [131]. However in aqueous solutions, the product of Schiff’s base reaction is labile and would be expected to undergo the reverse reaction. Therefore the routine procedure after GA cross-linking is treating the initial Schiff’s base product with reductive agents, such as NaBH₄ or Na(CN)H₃ to convert the labile Schiff’s base into more stable amine residues. But it is found that the protein cross-linked with GA is stable enough, even without treatment with reductive agents. It is therefore likely that Schiff’s base formation is not the right mechanism. Walt [130] reported that this reaction could probably be carried out via several different reaction mechanisms. Under acidic conditions (Figure 2-16), monomers and oligomers allow GA to react with protein via a Michael addition. Under basic conditions (Figure 2-17), GA firstly undergoes intermolecular aldol condensation resulting in α-, β- unsaturated polyaldehydes (polyGA). The polyGA product then reacts with proteins either via a stabilized Schiff’s base reaction or a Michael adduct formation. Because of the existence of resonance, the Schiff’s base product formed is stable. Compared to free GA, polyGA is a more efficient cross-linking agent [132].

To stabilize the Schiff’s base product, reductive reagent treatment was reported to be necessary. Upon reduction with NaBH₄, a stable secondary amine is formed. However, under excess of amine, a secondary condensation reaction between secondary amine and GA may occur. Fernandez-Lafuente et al. [108] claimed that reduction with sodium borohydride is absolutely necessary to obtain stable cross-linked penicillin G acylase structure on the surface of highly derivated glyoxyl agarose gel. They came to the conclusion that the reduction is a suitable and necessary step in protein immobilization on glyoxyl or aminated support activated with glutaraldehyde [108]. Guisan and Blanco supported this idea [133]. They found that for trypsin immobilized on glyoxyl agarose support, borohydride reduction resulted in approximately two orders of magnitude stability improvement compared to the unreduced one. Nevertheless, the drawback for the reduction was a loss in activity. Under the most drastic experiment conditions, the immobilized derivatives lose 30-40% of their activity. Moreover, the disulfide bond in trypsin was also split, even in mild conditions (4 mg ml⁻¹ borohydride, pH 9.6, 0 ° C). The optimal conditions used for sodium borohydride reduction was: 1 mg ml⁻¹ borohydride; 50 mm borate or bicarbonate buffer, pH at 10; temperature, 25 ° C; reduction time, 30 min; and the presence of 3 mM benzamidine (the trypsin inhibitor). However for enzymes immobilized by GA, it is still under discussion if reductive reagent treatment is necessary [16]. There is little evidence showing that such reduction enhances the stability of the immobilized protein, even though this procedure operates routinely nowadays. In fact, proteins immobilized with GA are found to be sufficiently stable without any reductive reagent treatment.
Therefore the utilization of GA in biochemistry is still an art rather than science, despite of its wide application.

Figure 2-16 Reaction of GA with enzymes under acidic and neutral pH [16]. I, linear GA monomer; IV, cyclic hemiacetal; V, GA cyclic hemihydrate multimer.

In spite of the immaturity of the reaction mechanism, considering the mild reaction condition and less aggressive affect on the enzyme tertiary structure, GA was chosen as the main cross-linker in this project. However, to further reduce its toxicity to enzymes, some techniques were also developed. The detail is addressed in the following section.
2.4.2.2 Methods to overcome GA toxicity to Enzymes in Immobilization

GA is toxic to enzymes and so will substantially decrease the activity of the enzyme if no proper precaution is taken. Generally speaking, three different technical routes have been developed to overcome this problem:

**Route 1.** The first and least preferable route is to use another compound, such as polyaldehyde, instead of GA. As mentioned in Section 2.3.2, chemical reactions are able to distort enzyme’s tertiary structure resulting in deactivation. However, the degree of deactivation is related to various factors including type of toxin, toxin concentration, time in denaturant and pH. Therefore, although GA is a sort of mild cross-linker for enzymes, excessive exposure would still cause serious loss in enzyme activity [3]. Due to the large molecular size, polyaldehyde, which is formed via partial oxidation of polysaccharide [121] or excessive GA treatment of polyamine[1], cannot enter the
enzyme active site effectively. Thus, excessive conformation changes can be avoided. For example, Cesar Mateo et al. [121] use dextran polyaldehyde to prepare CLEA. In their experiment, dextran polyaldehyde was prepared by treating dextran in aqueous solution with sodium metaperiodate at room temperature for 1 hour. CLEA of penicillin G acylase, hydroxynitrile lyase, alcohol dehydrogenase, and two different nitrilases were prepared by precipitating these enzymes from their aqueous solution by adding salt or poly (ethylene glycol), following by dextran polyaldehyde treatment. As shown in Table 2-4 below, dextran polyaldehyde is a much milder cross-linker than GA, and is able to substantially reduce the activity loss of the enzyme.

Table 2-4 Effect of the cross-linker on recovered activity of CLEA

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Recovered activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glutaraldehyde</td>
</tr>
<tr>
<td>Oxynitrilase (P. amygdalus)</td>
<td>10</td>
</tr>
<tr>
<td>Nitrilase (Ps. fluorescens)</td>
<td>0</td>
</tr>
<tr>
<td>Nitrilase (BioCatalytics 1004)</td>
<td>0</td>
</tr>
<tr>
<td>ADH (L. brevis)</td>
<td>0</td>
</tr>
<tr>
<td>Penicillin G acylase (E. coli)</td>
<td>48</td>
</tr>
</tbody>
</table>

**Route 2:** The second technical route to reduce GA toxicity is to modify GA with other multifunctional chemicals to form GA polymer. One patent [134] describes a method to modify GA with diamines, such as ethylenediamine and spermidine. This cross-linker was used in a CLEA of hog pancreas lipase. It was claimed that high enzyme retention had been achieved.

**Route 3:** The third technical route is the application of GA oligomer. GA oligomer is more reactive than the monomer and also perhaps follows different reaction mechanisms from GA monomer. Its application in biotechnology has been explored previously [135-137]. GA is able to form oligomers in alkaline pH solutions and high GA concentrations. Terumichi et al. [135] prepared GA oligomer using 10% GA solution heating in pH 8.5 borate buffer solution at 60°C. The absorbance change in the ultraviolet spectrum at 235 nm and 280 nm can be used to confirm the formation of aldol condensate of the GA monomer. The cross-linking ability of these oligomers was examined in enzyme immobilization of alcohol dehydrogenase (ADH) and glutamate dehydrogenase (GLDH). The immobilized activities of ADH and GLDH were 4-fold and 13-fold higher respectively than those used untreated GA solution, but the total amount of enzyme immobilized was not changed. Furthermore, Park Seung Won [138] studied the four main parameters controlling formation of GA oligomer, including heating temperature, heating time, pH and GA concentration. For
immobilization of glutaryl-7-aminocephalosporanic acid (GL-7-ACA) acylase, the optimal parameters were found to be: pH, 8.0; temperature, 62°C; treatment time of GA solution, 25 min; GA concentration, 2%. The optimal activity recovery is 36.5%, which is 2.2 fold better than free GA solution.

Comparing all these three routes, route one looks more promising (85%-90% enzyme activity recovery) and received more research acceptance [121, 137-140]. Route three has also been tried in a number of publications [135, 138]. Therefore these routes were used in this project. The detailed content is discussed in Sections 6.2 and 6.4.

### 2.5 Immobilization Supports

The true novelty of this project is the use of wool as an immobilization support and the immobilization methods that have been developed using the precedents described in the literature above (and in the following section) to enable this. In this section, the recent progresses in proteinous materials as immobilization support and other relevant information are described.

#### 2.5.1 Introduction

The selection of supports is the one of the most important decisions to make in the technique of enzyme immobilization. Various materials have been utilised. The commonly used immobilized supports, include synthetic supports (such as acrylamide based polymers [141], maleic anhydride based polymers [142], styrene based polymers [143]), polysaccharide supports (e.g. agarose [96], cellulose [96], and dextran [96]), inorganic supports (e.g. glass beads [144] and silica [144]), and protein supports (e.g. collagen [145], keratin[14]). Nowadays, researchers have even developed support-free techniques in enzyme immobilization, such as the aforementioned CLEA [94] and also CLEC [146], where enzymes are recycled in their water-insoluble form to reduce the mass transfer problems introduced by the immobilization matrix.

In the review written by Mateo et al. [147], the principles for selecting the immobilization support were summarised as the following:

- The support should present large internal surface to have good geometrical congruence with the enzyme surface.
- The support should present a high superficial density of reactive groups.
The reactive groups in the protein and the support should present minimal steric hindrances in the reaction,

- The reactive groups in the support should react with groups frequently placed in the enzyme surface.
- The reactive groups involved in the immobilization should be stable enough to permit long enzyme–support reaction periods.
- It should be easy to get a final inert surface in the support after immobilization, by destroying or blocking the remaining reactive groups in the support without affecting the enzyme.

The immobilization matrix does not only provide the immobilization support, it also changes the microenvironment around the enzyme. For example, the charged support might alter the local substrate concentration around the immobilized enzyme, and then the pH-activity relationship for the free and immobilized enzymes become different [122].

The adoption of a support matrix introduces additional factors which need to be accounted for and optimised to give the highest overall enzyme activity. This means that many factors must be compromised, such as the binding capacity, substrate accessibility and support rigidity. Porous materials have high absorption ability, but the substrate transfer resistance is a limitation. Therefore if products formed rapidly, porous materials would not be applicable on this occasion. On the other hand, many support materials (especially organic gels) would be deformed and even broken into debris under high flow rates, so rigid, large and dense materials are needed in this circumstance. Membranes and packed-bed reactors (see Section 2.3.1) in particular build fouling easily, while sheets and film do not result in such problems, although the latter usually demonstrate limited expressed immobilized activity. Practically, fine particles and membranes are appropriate for analytical applications where a small amount of enzymes and substrates are needed. Soft gels are suitable for academic study when complicated reactions are involved and the limitation of flow is not a serious problem. For industrial applications, good stability and resistance to compression deformation is so essential that less consideration is given to the activity of immobilized enzymes.

### 2.5.2 Enzyme Immobilization on proteinous Fabrics: Wool and Silk

Recently, the application of fibrous materials as immobilization support matrix has attracted the attention of enzyme immobilization researchers [11], due to its cheap price, bulk availability,
superior mechanical properties, large surface area and easy surface activation. The most commonly used natural fabrics in enzyme immobilization is cotton [117]. The adoption of protein cloth in this field, including wool and silk, has also been reported, but is not as widely used as cotton, probably because they are more expensive, have a relatively high surface electrical charge, have less physical strength, and are susceptible to proteolysis by extraneous enzymes.

Several different enzyme immobilization reactions were used with wool and silk, namely carbodi-imide [148], diazo [149] and azide coupling [150]. Among them, activation with glutaraldehyde has received special attention [23, 151]. These different enzyme immobilizations on proteinous fabrics, including wool and silk, are addressed in more detail in the following paragraphs. Details of the preparation methods are included, as this thesis develops new and improved techniques that build upon these established techniques.

2.5.2.1 Wool in Enzyme Immobilization

The earliest report on wool as an enzyme immobilization support was given by Masri et al. in 1975 [152]. In their study, lactase was immobilized onto woollen cloth. In order to produce coupling sites, the woollen cloth was treated with POCl₃ and dimethylformamide (DMF). However, it was found that lactase could not couple with the diazotized derivatives of wool and silk properly.

Due to the complexity of creating binding sites on wool via treatment with POCl₃ and dimethylformamide (DMF), surface activation through GA was also used in enzyme immobilization on woollen fabrics. Naringin is the substance responsible for the bitter taste of grapefruit and some orange juices. Consequently, the immobilization of naringinase from *Penicillium decumbens* onto sheep wool as a debittering agent has been studied [153]. Sheep wool, were treated with petroleum ether in a Soxhlet for four hours and then washed with abundant buffer. The supports were activated with 2.5% glutaraldehyde in 50mM KH₂PO₄-NaOH buffer at pH 7.0 for 3 hours at room temperature. They were thoroughly washed with water and 0.1 M K phthalate/HCl buffer pH 3.5. The activated supports were incubated with 20 ml of 4 mg/ml enzyme solution in the same buffer for 22 hours at room temperature with stirring (100 RPM). Then the material was washed with the same buffer until protein was absent from the washing water. It was reported that enzyme retention on wool was appreciably increased by treating the support with papain, which might due to creating
more binding points. The amount of enzyme immobilized was found at 0.90 IU / g wool (papain treated wool).

Krastanov [154] studied the immobilization of yeast cells on wool in sucrose hydrolysis. Due to a more compact structure, wool fabrics demonstrate a higher flow rate compared to cotton cloth. Glutaraldehyde (GA) was used as a cross-linking agent in this study. Krastanov found that yeast cells appeared to strongly adhere to wool, even without GA treatment. The mechanism for this was unclear however. A possible explanation is that since wool itself also consists of proteins, their structural similarity makes it much easier for yeast to be adsorbed onto wool. Thus, the immobilization was believed to be a combination of physical absorption of wool/yeast and chemical binding between GA and lysine residues on proteins. Yeast cells were immobilized onto wool by treating either the yeast cells or wool or both with GA. The best result was obtained when yeast cells were initially treated with GA before immobilizing onto the wool cloth, where the cloth did not receive any GA treatments.

Previous work has also shown that not only free amine residues on wool, including lysine residue, histidine, terminal amino group, are able to take part in the cross-linking reaction with GA, but also the sulfhydryl group of cysteine can participate in this reaction [155, 156]. As previously covered in Section 2.1, protein from wool, hair and feathers belongs to a family of protein called keratin, which is unique among the natural polymeric materials. It has a high content of sulfur-containing amino acid cysteine and is important in the cross-linking provided by the cysteine S-S bond in maintaining protein structural integrity. Excellent immobilization was reported on this keratin protein in the presence of GA [151]. β-galactosidase from Aspergillus Niger was selected as the model enzyme. Keratin granules extracted from feather, both in reduced and oxidised form, were used as the immobilization matrix. When the feathers were treated with mercaptoethanol in 50% ethanol solution, 35 to 60% of the feather is dissolved. Keratin granules were then obtained from the recovered feather protein. β-galactosidase in deionized water was added in a swirling manner to this granule, followed by the addition of glutaraldehyde. The mixture was allowed to stand at 4°C overnight to complete the fixation. Apparent half-lives from 50 to 100 days was exhibited for this immobilization in a packed column reactor.

Besides amine groups, the carboxyl residues on enzyme can also be used for immobilization. β-galactosidase immobilization onto polyacrylic acid grafted woollen cloth has been reported [148]. The wool samples were gently degreased by Soxhlet extraction for 48 h with ethanol. Poly acrylic
acid was then photocured to this cloth. Then this cloth was immersed in β-galactosidase solution in 0.1M phosphate buffer (pH=6.0). The mixture was cooled to 4°C and cyclohexyl-3 (2-morpholinoethyl) carbodiimide metho-p-toluene sulphonate (CMC) was added and shaken overnight at 4°C. This enzyme coupling gave a product with an activity of 57 U/g, which is equivalent to 0.19 mg of pure enzyme per gram of copolymer. However, after 48 hours of storage, the β-galactosidase activity had fallen to 25 U/g.

Overall, the literature shows that wool has not yet been widely used as an immobilization support. Among the limited articles published, several surface activation methods were addressed:

- Amine residues was directly activated by POCl₃ and dimethylformamide treatment [152], or glutaraldehyde [153, 154];
- Sulfhydryl group of cysteine residue was activated by GA [151];
- Wool cloth was coated by functionalised polyacrylic acid resin followed by carbodiimide activation.

However, all the activation methods either involved toxic organic reagents (POCl₃ and dimethylformamide [152]), or were not stable enough after only 48 hour storage, more than 50% of the activity was lost [148]. Direct activation with GA to the cysteine residue show a promising result (50-100 days half-life for β-galactosidase was achieved), but the charged feature of proteinous support makes it only suitable for the immobilization of oppositely electrically charged enzymes (most of enzymes available nowadays have acidic isoelectric point, which is similar to wool protein [19]). Therefore, to overcome these shortcomings, enzyme immobilization on woollen fabrics through a spacer arm of PEI was developed in this project, which was much easier applied and more widely suitable for enzymes with an acidic isoelectric point. This is because PEI carries positive charge in the active pH range of enzymes, and thus bridges the negative-charged woollen surface with negative-charged enzymes.

2.5.2.2 Silk in Enzyme Immobilization

As can be seen from Section 2.5.2.1, very few studies have been conducted on the immobilization of enzymes on wool. Some more relevant studies for the work in this thesis can be derived from the immobilization of enzymes on similar proteinous fabrics. Silk, is one of such fabrics. The advantages of silk include its chemical stability, resistance to microbial contamination, and good
mechanical strength. Also, silk is rich in varied free chemical groups, such as tyrosyl and carboxylic groups, which are readily activated for immobilization. However, the silk has few free amino groups. To generate amino groups, it must be pretreated in order to cleave some of its peptide bonds, which is normally done by immersing the silk fibre in 0.2 M HCl under reflux for a few minutes. By this philosophy, alkaline phosphatase immobilization onto this sort of hydrolyzed woven silk material was studied [23]. The partially hydrolyzed silk was treated with GA diluted in 0.2 M sodium bicarbonate buffer (pH 9.0) for 3 hours at 20°C. This activated support was then placed in alkaline phosphatase solution for 30 min with continuous stirring at 30°C. The excessive aldehyde group was quenched by a soaking in NaBH₄ solution. The immobilized enzyme was then stored in 0.1M NaCl at 4°C. The amount of active fixed enzyme was determined by comparison of the activity of silk coupled with alkaline phosphatase at optimum pH, with the value obtained with free enzyme under the same conditions. For this immobilization, enzyme was found to be immobilized at 2.16 μg/g silk. The aminotransferase and alkaline phosphatase immobilization onto woven silk was also reported by Grasset and Corder through diazo coupling, absorption, glutaraldehyde cross-linking and the azide coupling method [150].

Direct activation with GA can only create limited active sites for enzyme immobilization, thus an spacer arm was used to further enhance the immobilization [157]. For alkaline phosphatase immobilization on Bombyx mori silk fabrics, the fibre was chlorinated first and followed by consecutive immersion in 2-aminoethanethiol (AET) or polyethyleneimine (PEI) solution before GA cross-linking. The amount of enzyme immobilized was up to approximately 10 mg/g fabric and the activity of the immobilized enzyme was less than 5% of that of the free enzyme. The immobilized enzyme was able to maintain 35% of its initial activity after 29 days of storage. The kinetic study, giving apparent Michaelis constants for the immobilized alkaline phosphatases demonstrated that the accessibility to the enzyme was lowered after immobilization as compared with that of the free enzyme. The thermal stability of alkaline phosphatase immobilized onto PEI-treated fabrics was improved.

Lipases immobilized on silk have drawn special research interests. The catalysed hydrolysis of olive oil by the immobilized lipase on silk was studied by Cao et al. [149]. The activity of the immobilized lipase was found peaking at 55 °C and pH 8.2. The hydrolysis rate of olive oil decreased from 96.5% to 53.0% after using the immobilized lipase 10 times repeatedly. The half-life of the immobilized lipase was about 260 h. Gao et al. [158] also studied the operational stability of the immobilized lipase on silk by the diazo method. After treating with p-b-
sulfatoethylsulfonylaniline (SESA) at pH 10.8, the p-aminobenzent sulfanilyl Et silk (ABSE-silk) was diazotized and linked with lipase at pH 7.5. The linking time was more than 10 hours and the immobilization yield was more than 52%. The half-life was reported to be 250 hours.

Generally speaking, silk and wool are similar in structure, but there is still bit of difference between these two proteins. In summary, both wool and silk have ionic amino groups and hydroxyl groups that can create hydrogen bonds, but the density of ionic groups in silk is much lower than wool [159]. Moreover, as there is low amino group density on silk, it has to be hydrolysed in acid solution to generate enough amino groups before GA activation[23]; whilst the direct GA activation on wool has been proved to be usable in enzyme immobilization, despite its low efficiency [153].

Needless to say, these two proteins still present a great deal of similarities in features, and it is thus possible to cross-reference immobilization experiences between them. Interestingly, the adoption of spacer arm, such as polyethyleneimine (PEI), results in more than a 400 fold increase in the amount of alkaline phosphatase immobilized compared to direct activation of silk, as more amine groups exist on the PEI spacers [23, 157]. Consequently, this gives further support to adopting the PEI spacer in this research. Its influence to enzyme immobilization on wool is addressed in detail in Section 4 and Section 6.

2.6 Modelling Enzyme Reactions: Enzyme Kinetics

Enzyme kinetics is the study of the biochemical reactions that are catalyzed by enzymes, with a focus on their reaction rates, which incorporates the various factors affecting the enzymatic reaction rate such as substrate, inhibitor, and enzyme concentration. From a kinetic study, we can also obtain the information on the enzymatic reaction mechanism. In enzymatic studies, kinetic parameters are often used to compare enzyme activity and its substrate affinity [79]. More importantly, this rate equation can be applied to calculate the reaction time, yield and optimum conditions, which are key parameters used in reactor design and scale-up.

The Michaelis-Menten (M-M) equation is the basis for most single-substrate enzyme kinetics [160]. This equation is derived on two crucial assumptions: the first assumption is the so called pseudo-steady-state hypothesis, namely that the concentration of the substrate-bound enzyme (and hence also the unbound enzyme) changes much more slowly than those of the product and substrate, and thus the change over time of the complex can be set to zero. The second assumption is that the total
enzyme concentration does not change over time. Thus the product formation rate depends on the enzyme concentration as well as on the substrate concentration.

The reaction involves the single substrate and single product reaction can be described in the following general reaction scheme:

Here, “S” stands for enzyme substrate, “E” for enzyme, “ES” for intermediate product of the reaction between enzyme and its substrate, “P” is for the product. From this, the M-M equation is derived as:

\[ v_0 = \frac{v_{\text{max}} [S]}{[S] + K_M} \]

Equation 1 Michaelis-Menten equation

Here:

\[ v_{\text{max}} = K_2 [E]_0 \]

\[ K_M = \frac{K_1 + K_2}{K_{-1}} \]

Where \( v \) is the reaction rate, \([S]\) the concentration of the substrate, \([E]\) the concentration of enzyme, \(K_M\) is the substrate concentration which gives half of the maximum reaction rate, \(K_f\) is the forward reaction rate constant of first stage reaction, \(K_r\) is the backward reaction rate constant of the first stage reaction, \(K_2\) is the reaction rate of second stage reaction, and \(v_{\text{max}}\) is the maximum reaction rate for the reaction or the reaction rate in infinite substrate concentration. When \(K_2\) is far smaller than \(K_r\), \(K_M\) stands for the affinity between the substrate and enzyme [161], i.e. the higher the value, the lower the affinity becomes. However, often, \(K_2\) is bigger than \(K_r\) or both of them are comparable, so normally \(K_M\) not only represents the substrate affinity, but \(K_2\) has a considerable contribution as well. The M-M equation indicates that under a low substrate concentration, the reaction rate is linear with the substrate concentration; whilst under a high concentration of substrate, i.e. \([S] \gg K_M\), the reaction kinetics follows zero order, and it is independent of the substrate concentration.

The M-M equation can be extended to include competitive product inhibition, uncompetitive product inhibition and non-competitive product inhibition, as listed in Table 2-5. However, the conventional model is still used extensively in many studies. For example, Zhou et al. [160] found
that the M-M model without inhibition is sufficient to determine the kinetic parameters at low concentration of lactose (3% (w/v)) as enzymatic hydrolysis is not influenced by inhibition behaviour from either reactant or product species.

In scientific articles, it is often assumed that the immobilized enzyme also follows M-M kinetics, and therefore comparison of the M-M parameters between the free and immobilized enzymes are made, aiming at obtaining a good insight into enzymes after immobilization and assess the effect of immobilization on performance of the enzymes. An increase $K_M$ of immobilized enzyme versus free enzymes normally suggests a decreased substrate affinity to the enzyme. This is often caused by restricted substrate access to the enzyme’s active site resulting from immobilization or structural changes in the enzyme induced by the applied immobilization procedure. A decrease in maximum velocity $v_{max}$ of immobilized enzymes is often attributable to the deactivation from immobilization and vice versa.

In terms of the enzymes used in this thesis work, the kinetics of lipase from *pseudomonas fluorescens* (PFL) were found to follow the M-M relationship [162-164]. For example, when this lipase was immobilized onto polysiloxane–polyvinyl alcohol matrix, the immobilized derivative on POS–PVA activated with epichlorohydrin was shown to have a Michaelis constant value ($K_M$) of 293 mM for the hydrolysis of olive oil [163]. Furthermore, it was found [164] that the kinetic parameters were different for unimolecular and biomolecular forms of *P.fluorescens* lipase. As a self-assembly protein, the formation of bimolecular aggregates of *P.fluorescens* lipase have been well addressed (see Section 2.2.1.3 for more detail). Experiments have shown that the $K_M$ of the dilute *P.fluorescens* lipase preparation (lipase concentration of 5 μg /ml, a product made from Amano Enzyme Inc) was much lower than that of the concentrated one (lipase concentration 160 μg /ml), suggesting that the substrate had some problems in reaching the active site in the concentrated lipase solution [164].

The M-M equation is not a first and second order rate equation, so consequently the calculation of the kinetic parameters (Michaelis constant $K_M$ and maximum velocity $v_{max}$) is not straightforward. Several graphical plots are available for this, where only the initial reaction rate and corresponding initial substrate concentration are used. As the initial experimental conditions are always known, these methods allow experimental errors affecting the kinetics to be reduced. The most commonly used plots are:
### Table 2-5 Enzymatic kinetic models based on the Michaelis-Menten equation [165].

<table>
<thead>
<tr>
<th>Kinetic Model</th>
<th>Enzymatic Mechanism</th>
<th>Rate Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-M with competitive inhibition by product</td>
<td>$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P \xleftarrow{k_{-1}}$</td>
<td>$v = \frac{v_{max} [S]}{K_m \left(1 + \frac{[P]}{K_i}\right) + [S]}$</td>
</tr>
<tr>
<td></td>
<td>$E + P \xrightarrow{k_3} EP \xleftarrow{k_{-3}}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$ES + P \xrightarrow{k_4} ESP \xleftarrow{k_{-4}}$</td>
<td></td>
</tr>
<tr>
<td>M-M with uncompetitive inhibition by product</td>
<td>$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P \xleftarrow{k_{-1}}$</td>
<td>$v = \frac{v_{max} [S]}{K_m + [S] \left(1 + \frac{[P]}{K_i}\right)}$</td>
</tr>
<tr>
<td></td>
<td>$ES + P \xrightarrow{k_4} ESP \xleftarrow{k_{-4}}$</td>
<td></td>
</tr>
<tr>
<td>M-M with non-competitive inhibition by product</td>
<td>$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P \xleftarrow{k_{-1}}$</td>
<td>$v = \frac{v_{max} [S]}{K_m \left(1 + \frac{[P]}{K_{i1}}\right) + [S] \left(1 + \frac{[P]}{K_{i2}}\right)}$</td>
</tr>
<tr>
<td></td>
<td>$E + P \xrightarrow{k_3} EP \xleftarrow{k_{-3}}$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>$ES + P \xrightarrow{k_4} ESP \xleftarrow{k_{-4}}$</td>
<td></td>
</tr>
</tbody>
</table>

### A. The Lineweaver-Burk Plot

This method uses a linearization procedure to give a straight-line graph. The M-M equation is inverted to the following form:

$$\frac{1}{v} = \frac{K_M}{v_{max}} \frac{1}{[S]} + \frac{1}{v_{max}}$$

**Equation 2 Lineweaver-Burk plot equation**

So a plot of $1/v$ versus $1/[S]$ will give a straight line (if M-M kinetics apply), and the M-M parameter can be calculated from the slope and intercept. This plot is frequently used in literature. The Double-reciprocal Plot Lineweaver Burk plot is more often employed, because it shows the relationship between the independent variable $[S]$ and $v$. However, $1/v$ approaches infinity as $[S]$ decrease, which gives undue weight to often more inaccurate measurements made at low substrate concentrations and insufficient weight to the often more accurate results at high substrate concentration.
Figure 2-18 Example of Lineweaver-Burk plot for the free and immobilized β-galactosidase on graphite surface, taken from reference [79].

B . The Eadie-Hofstee Plot

If the equation for Lineweaver-Burk plot is multiplied by:

\[
\frac{V_{\text{max}}}{K_M}
\]

And then arranged, so that the equation of Eadie-Hofstee is obtained:

\[
\frac{V}{[S]} = \frac{V_{\text{max}}}{K_M} - \frac{V}{K_M}
\]

Equation 3 Eadie-Hofstee plot equation

Using this, a plot of \(v/[S]\) versus \(v\) gives a straight line. From the slope and intercept, \(K_M\) and \(V_{\text{max}}\) can be calculated. Eadie-Hofstee plot gives slight better weight of data than Lineweaver Burk plot. A disadvantage of this plot is that the rate of reaction appears in both coordinate, while it was usually regarded as an independent variable. Can be subject to large errors since both coordinates contain \(v\), but there is less bias at low \([S]\).
C. The Langmuir Plot

The Langmuir plot is produced by multiplying the equation of Lineweaver-Burk plot by \([S]\). This linearization however minimizes the experimental error[161]. Among above three linearization plots, Langmuir plot give the most satisfactory, since the plot is equally spaced.

\[
\frac{[S]}{v} = \frac{K_M}{v_{\text{max}}} + \frac{[S]}{v_{\text{max}}}
\]

Equation 4 Langmuir plot equation

D Nonlinearization method

Another approach for the determination of the kinetics parameter is to use a non-linear regression method, which produces weighted least squares estimates of the parameters of the nonlinear M-M model. The advantages of this technique are: (1) It does not require linearization of M-M equation, (2) the estimation of parameters are reliable, as it produces weighted least squares estimates.

Before the ready availability of the computer software for nonlinear curve fitting, the nonlinearity of Michaelis-Menten plot could make it difficult to estimate \(K_M\) and \(v_{\text{max}}\) accurately, necessitating the above linearizations of the Michaelis-Menten equation. However, as the computer software is readily available nowadays, it is simple to get more accurate determinations by nonlinear regression methods without the previously covered equation transformations.

In this study, the kinetics parameters estimated from the above plots was compared, and results were chosen from those leading to the best curve fitting.

2.7 Summary of Literature Review

Overall speaking, enzyme immobilization nowadays is regarded as more an art than a science. It is therefore still a challenging job to develop a commercially feasible immobilization protocol.

Among the methods used in immobilization, a chemical protocol exhibits advantages over a physical one, as the superior stability is highly desired in industrial production, despite of its drawback, such as substantial enzyme deactivation from chemical modification of enzyme structure. A number of techniques could be used to enhance chemical immobilization, such as blocking agents, spacer arms
and multipoint binding. However, the mechanism on how to protect the enzyme activity to avoid chemical attack during immobilization is still not clear, and so more research is needed in this field.

In chemical immobilization, GA has been widely used as a cross-linking agent in enzyme immobilizations due to its fast reaction and low toxicity to enzymes [166-168]. The most widely accepted theory on the GA cross-linking mechanism is that the aldehyde groups in GA react with the lysine residues in protein through a Schiff’s base reaction [131]. To stabilize the product, treatment with reductive agents, such as sodium borohydrid, is required after GA coupling. However, there are some disagreements on the Schiff’s base mechanism, stating that some other reactions (Michael adduct formation) might also involved, making the reduction step unnecessary [16]. Another broadly used chemical in chemical immobilization is PEI. As a highly positively charged water-soluble polycation, PEI is able to interact with the negatively charged enzymes, giving them more protection. Moreover, the pending primary amine group of PEI could react with enzyme in the presence of a cross-linking agent, forming immobilization through a stable chemical bond [169].

Fabrics used as support for enzyme immobilization are producing a great deal of research interest, as they have good mechanical stability, cheap prices and wide availability. The application of fabrics, including cotton, silk, nylon, are well described in literature. However, wool, despite its similar chemical structure to silk and nylon, has rarely featured in academic research. To fill this gap, the feasibility and limitation of wool as immobilization support material is thus discussed in this thesis.

Alternatively, to reduce the enzyme deactivation from GA attack, cross-linkers other than GA have been developed. Researchers found that enzymes can be immobilized by using another enzyme: Transglutaminase (mTGase) [170, 171]. Due to the compact globular structure of enzymes, direct modification of enzymes by mTGase is impossible. Entrapment of enzyme inside casein gel formed in the presence of mTGase has been reported however [76]. Therefore in this thesis, immobilizing lipase by the means of mTGase was investigated. In contrast to the literature published, the casein gel will be formed on the surface of woollen cloth. The gel adherence and stability of the immobilized lipase can then studied.
3. Materials and Methods

3.1 Materials

3.1.1 Enzymes

The lipase used in this study is Amano lipase derived from *pseudomonas fluoresces*, purchased from Sigma-Aldrich (product code 53473-50G) and produced by Amano enzyme Inc, Japan. Microbial transglutaminase (mTGase) isolated from *Streptomyces mobaraense* was kindly donated by Yiming Biological Products Co. Ltd (China). Galactosidase from *Kluyveomyces lactis* was purchased from Sigma-Aldrich.

3.1.2 Other Chemicals

Polyethyleneimine (PEI) was bought from Aldrich (product code 25987-06-8, Mw 600,000). p-nitrophenyl palmitate (pNPP), the artificial substrate used for testing the activity of the lipase, was bought from Sigma (N-2752). Triton X-100 (which was used as a surfactant in the preparation of pNPP assay solution), was bought from BDH (product code 306324N). The buffer used in this study was prepared with Trizma-hydrochloride (Sigma, T-2253) and di-sodium tetraborate (BDH, product code 102674E) respectively. Dichlorocyanuric acid (DCCA) was purchased from the local market (as swimming pool bleaching agent). 100% woollen cloth without bleaching was kindly provided by AgResearch Limited (NZ). QuantiPro BCA Assay Kit (Bicinchoninic acid) protein assay kits were purchased from Sigma-Aldrich (product code QPBCA-1KT).

All aqueous solutions were prepared with Deionised (DI) water. All other chemicals used were of analytical grade.

3.2 Experimental techniques

3.2.1 Woollen Cloth Chlorination

Wool fibres are hydrophobic and chemical resistant, due to the aforementioned (Section 2.1) covalent bound lipid layer and sulfur-rich cortex layer on the surface [19]. Thus, as previously mentioned (Section 2.1) surface modification is necessary before immobilization. In this study, DCCA chlorination was adopted. This reagent is able to release chorine slowly and reduce the weight loss of wool, which is widely accepted in wool industry. The method described in references
[30, 172, 173] was used in the current research with minor modification. In the wool industry, chlorine concentration is often quantified by owf, i.e. weight of cloth (% chlorine equivalent to the weight of the cloth used). For example, 5% owf chlorine solution means the amount of chlorine used is 5% of cloth weight. In this research, two types of chlorination processes were adopted:

**Low pH process:** In the first part of thesis, the procedure in reference [173] was used for wool chlorination with some modifications: Cloth was treated for 30 second to 3 minutes in a beaker containing 2.5% owf of DCCA. The solution pH was adjusted to 1.5-2.0 by using solutions of sulphuric acid and acetic acid. Then the cloth was soaked in neutralization solution containing 10 g.l⁻¹ sodium carbonate and 4 g.l⁻¹ sodium sulfite for 30 sec, then rinse with water and dry.

**High pH process:** However it was found the woollen cloth could not be evenly chlorinated by the above procedure, thus the more moderate chlorination steps described in reference [30] was used later in this thesis, the detail discussion is given in Section 6.3. The chlorination steps were as follows: Bare woollen cloth was treated in 5% owf (weight of cloth) solution in 0.1 M citric acid monohydrate/0.15 M sodium hydroxide buffer, pH 4.1 for 60 minutes at room temperature (25 °C) at a liquor /cloth ratio of 20:1. After chlorination, the cloth was treated in 5% owf Na₂SO₃ in Na₂HPO₄/NaOH (pH 8.5), liquor /cloth ratio 20:1, for 30 minutes at 50°C. Thereafter, the cloth was rinsed with DI water and soaked in 2% PEI solution at pH 8.5 until used.

### 3.2.2 Buffer selection and preparation

Several buffers were used in this project, namely Tris buffer, phosphate buffer, acetate buffer, and carbonate buffer.

The buffers used in immobilization step must be inert in the presence of aldehyde group. However it was found in the experimental work that amine-contained buffers, such as ammonia buffer and Tris buffer, are able to react with GA, as observed that the pH in GA solution kept on dropping, and solution colour turn to yellow very fast when amine- contained buffers are used. This observation corresponds to the conclusion made by M. A. Hayat [174]: “The Tris buffer, like other primary amines, appears to react with glutaraldehyde and therefore should be avoided except when no alternative is available”. Therefore in this project, 0.1 M phosphate buffer was selected as the main immobilization buffer.
However, Tris buffer is an excellent storage buffer, which can quench the excessive aldehyde groups on the immobilized support surface and protect the immobilized lipase activity. The importance of using blocking agents to remove excessive aldehyde group on immobilization support have been addressed in review section 2.3.2.2 clearly. The optimal pH for lipase from *pseudomonas fluorescens* was found in the experimental work to be 8.5 (see the results in Section 5.1.1), thus this value was selected for the storage buffer.

Buffers were prepared by the methods described in the text: “Buffers for pH and metal ion control” [175]. Tris (hydroxymethyl) aminomethane–HCl buffer at pH 8.5 (Tris buffer) was prepared by mixing 50 ml of 0.1 M Tris and 14.7 ml of 0.1 M HCl solution together, and then diluting to 100 ml. Phosphate buffer (pH 6-8) was prepared by mixing Na₂HPO₄ and NaH₂PO₄ solution in the relevant proportions. Acetate buffer at pH 5 was prepared by mixing 14.8 ml of 0.2 M acetic acid and 35.2 ml of 0.2 M sodium acetate solution together, and then diluting to 100 ml. Borax buffer, Na₂CO₃/NaHCO₃ buffer were used to prepare solution ranging from pH 9 to 10, following the procedure given in [175].

### 3.2.3 Lipase Activity Assay

#### 3.2.3.1 pNPP assay Solution Preparation

The lipase-catalysed conversion of pNPP to p-nitrophenol (pNP) was used as the activity assay due to its convenience and rapid response. The assay solution was prepared by the method used by Martin *et al.* [176] with some modifications. In experiment, it was found that the colour of pNPP solution turned yellow within couple of hours in 0.4 mM pNPP solution even without lipase addition. The yellow colour developed is ascribed to the pNPP hydrolysis in alkaline solution, which will accelerate with increased pNPP concentration, pH and temperature. This process will slow down in neutral /acidic solution and at a low temperature. Therefore, pNPP stock solution is prepared in DI water and stored in a fridge (at 4 °C), and diluted to the right concentration with the right buffer solution before use. The detail of the preparation steps is as follows:

**1. Preparation of the stock solution**

The pNPP concentration in the stock solution was 0.8 mM. pNPP was firstly dissolved in 3% (wt %) of isopropanol and 1% (wt %) of Triton X-100. After pNPP was dissolved thoroughly, this solution was diluted with DI water to the pre-determined concentration. This stock solution was stable in
room for up to two days in a fridge (at 4°C), after which it gradually increased in turbidity. However, the solution could be recovered, as the solution could be made clear again, by heating to 70-80 °C until the turbidity disappeared.

2. Preparation of the working solution

Before use, this stock solution was diluted with 0.1 M Tris buffer at pH 8.5. The accuracy and freshness of the dilute solution is readily apparent at this stage: if the stock solution was stored for too long, it would turn a yellow colour directly after dilution. If this occurred, the stock solution had to be discarded and replaced with fresh pNPP solution.

3.2.3.2 Lipase Activity Assay

Lipase activity was determined by an assay using pNPP as the substrate. The pNPP solution was prepared by the method outlined in Section 3.2.3.1. The methods used to obtain concentration readings using the UV spectrometer are outlined in Section 3.2.3.4.

In the pNPP assay, UV adsorption from the yellow colour was recorded at 410 nm. This yellow colour is generated by nitrophenol, which is hydrolysed from nitrophenyl palmitate by lipase. To calculate the lipase activity, the UV absorbance has to be converted into μmol of nitrophenol produced by the means of a standard curve of nitrophenol concentration versus UV adsorption, which was prepared from ultrapure nitrophenol under testing condition, as shown in Figure 10-2 in Appendix 10.3. Three independent replicates were taken for every measurement and the results were the mean of these three repeats, and the standard deviation was also calculated.

A. Activity of free lipase

Lipase solution (0.1 ml) was mixed with 4.9 ml of 0.4 mM pNPP solution. After hand shaking for 10 seconds, its colour change in 410 nm was recorded by UV spectrophotometer (Agilent 8453) for 30 seconds. One unit of free enzyme is defined as the amount of enzyme, which catalyse one μmol of pNPP to p-nitrophenol per minute at the testing condition (25 °C, at 0.05 M pH 8.5 Tris buffer). The molar absorbance coefficient of pNP at 0.05 M, pH 8.5 Tris buffer was determined to be 8.281 10⁻³ M⁻¹. cm⁻¹ from the pNP standard curve shown in Figure 10-2 in Appendix 10.5. Thus, according to the definition in [177], the lipase specific activity was expressed as how many μmol of pNPP could be converted into p-nitrophenol per minute by one mg of lipase (μmol pNPP.min⁻¹.(mg lipase)⁻¹).
B. Activity of immobilized lipase

The pNPP (0.4 mM) assay was also utilized for the immobilized lipase. Lipase immobilized cloth (0.1 gram) was shaken in 5 ml of 0.4 mm pNPP assay solution over a recorded time period. The UV absorbance change at 410 nm in that period of time was recorded by UV spectrophotometer (Agilent 8453). Similarly to the free lipase, one unit of immobilized lipase is defined as the amount of immobilized lipases, which catalyses one μmol of pNPP to p-nitrophenol (pNP) per minute at the testing condition (25 °C, at 0.05 M pH 8.5 Tris buffer). According to reference [178], the specific immobilized activity was expressed as amount of pNPP (μmol) converted into p-nitrophenol per minute per gram of lipase-immobilized cloth (μmol pNPP.min⁻¹.(g cloth)⁻¹).

C. Activity of lipase leaked from immobilization

For lipases that are chemically immobilized on immobilization carrier, in theory, no enzyme leakage (loss of unbound enzymes from the immobilization support) should be observed. However, practically this sort of leakage is unavoidable, due to reversible cross-linking reaction, formation of enzyme aggregate, particularly in the case of lipase from pseudomonas fluorescens (see Section 2.2.1.3). Consequently, besides the two types of lipase activity defined above, which often appear in publications, another new type of lipase activity is defined in this research: leaked activity. This “leaked” activity is the activity of the lipases leaked out of cloth during activity determination. The reasons for measuring this and assay procedure are covered in Section 3.2.3.3. One unit of leaked activity is defined as the amount of lipase that produce one μmol of pNPP to p-nitrophenol per minute at the testing condition (25 °C, at 0.05 M pH 8.5 Tris buffer) and the specific leaked activity is expressed as the amount of pNPP (μmol) converted into pNP per minute caused by the lipase leaked from per gram of lipase-immobilized cloth (μmol pNPP.min⁻¹.(g cloth)⁻¹).

3.2.3.3 Determination of Immobilized Activity using UV spectroscopy

As a fast and convenient assay, spectrophotometry is widely used in biochemical research. The light used in spectrophotometric assay is visible light (wave length between 400-750 nm) and ultraviolet light (wavelength between 200-400 nm). It is known that the intensity of light is reduced when light is transmitted through a solution. The reduction is heightened when chromophore chemicals are
presented. This phenomenon is governed by the Beer-Lambert Law. In the Beer-Lambert law, the reduction is defined as absorbance, expressed in the following relationship:

\[ A = -\log_{10}\left(\frac{I}{I_0}\right) \]

Where, \( A \) is the absorbance, often expressed as “absorbance unit” or simply “AU”; \( I \) is the light intensity transmitted through a solution containing the chromophore, and \( I_0 \) is the light intensity transmitted through a solution without the chromophore.

In the absence of unusual complicating factors (such as the association of the chromophore into polymers, and in a relative low concentration), absorbance is proportional to the concentration, as described below:

\[ A = \varepsilon cl \]

Equation 5 Beer-Lambert law

Where, “\( \varepsilon \)” is absorbance coefficient, whose unit is M\(^{-1}\). cm\(^{-1}\); “\( c \)” is concentration of chromophore; and “\( l \)” is the length of cuvette, normally the length of standard cuvette is 1 cm. In theory, absorbance coefficient is the fundamental molecular property of the chromophore in a given solvent, at a particular temperature and pressure. But practically, the UV absorbance readout is related to individual instrument, as the instrument might have specific limitations, especially for the cheap and simple UV spectrophotometer. This has been considered in this work.

By the means of this relationship, the solute concentration can be determined by measuring the UV absorbance.

To obtain the reading of immobilized activity, the physically adsorbed and loss associated enzymes have to be removed thoroughly. For example, for the lipase immobilizations, the lipase-immobilized cloth is firstly washed with buffer to remove physical adsorbed enzymes, and then this cloth is tested with pNPP assay solution repeatedly, until the test result is stable enough. This result is regarded as the reading of immobilized activity. When the sample is prepared properly, the immobilized activity reading during the consecutive activity assay showed almost no change. This assay method for the immobilized activity is different from the testing procedure used by Mendes et al. [179], where all the enzyme adsorbed onto matrix was taken as the immobilized enzyme, and the activity reduction due to wash-off of the physically adsorbed enzymes was reckon to be from deactivation. The
stability of the immobilized lipase prepared in the current research is robust enough to withstand the multiple runs.

For the sake of convenience, three types of activity were defined in this research: free enzyme activity, immobilized activity and leaked activity (Section 3.2.3.2). A typical activity determination process is illustrated in Figure 3-1. In the first couple of activity determinations, the immobilized activity reading drops abruptly; this is mainly ascribed to the wash-off of the physically adsorbed enzyme (However, some of the activity drop might also be due to the lipase deactivation from GA and other denaturants, such as nitrophenol). When this drop in the immobilized activity slows down, eventually settling and stabilising within a narrow range (considering the experimental error), then this figure is taken as the immobilized activity. The same phenomenon occurs when determining the leaked activity.

![Figure 3-1 A typical change of the immobilized lipase activity during consecutive activity assay](image)

Immobilized activity and leaked activity can be obtained from just one sample measurement on the UV spectrophotometer (Agilent 8453) used in this work. The typical curve of UV absorbance versus time is shown in Figure 3-2. The X-axis is the time used for the UV measurement, which was normally 1 minute; and the Y-axis is UV absorbance measured, in arbitrary units of AU (absorbance unit). The concentration, time and volume of the pNPP solution used are selected deliberately so as that the adsorption curve is as close to a straight line as possible (to ensure the UV absorbance
measurement occurs in the optimal operational range of UV spectrophotometer). The UV adsorption at zero minutes represents the colour change from all of the lipase initially present on the cloth and is therefore the immobilized activity. In contrast, the slope of the straight line represents the colour change from these lipases leaking out of the cloth, and is therefore the leaked activity. To minimize the experimental error, the average slope in first 30 seconds was used for the leaked activity.

Figure 3-2 Typical curve of the UV absorbance change versus time used to determine the immobilized and leaked activity in the immobilized lipase assays, the slope stands for the leaked activity, intercept stands for the immobilized activity.

The reason for introducing the leaked activity is to assisting in the judgement of determining the immobilized activity. As shown in Figure 3-1, as the activity of the leaked enzymes reaches near to zero, this means that the leakages of the immobilized enzyme are minimal. Therefore we can be sure that the activity from the immobilized enzymes represents that from the chemically bound enzymes, instead of having to test the immobilized cloth 10 to 20 times before being certain of the measurement. For a good immobilization, it is desirable to obtain a high-immobilized activity and a low leaked activity. Accordingly, if the immobilized activity maintains at a constant absorbance reading and the leaked activity remains low enough compared to the immobilized activity during consecutive activity assays-this preparation can be recognized as a stable immobilization.
3.2.4 Galactosidase Activity Assay

2-nitrophenyl-β-galactopyranoside (ONPG) was the substrate used in the galactosidase activity assay. 10 mM of ONPG solution was dissolved in DI water used as stock solution. Three independent replicates were taken for every measurement and the results were the mean of these three repeats, and the standard deviation was also calculated.

A. Activity of free galactosidase

To determine the activity of the free galactosidase, 0.1 ml enzyme solution (1mg/ml) was added into 4.9 ml of 1 mM ONPG assay solution, the yellow colour released was monitored by spectrophotometer and the reading change at 410 nm per minute was recorded as the enzyme activity. One unit of free galactosidase is defined as the amount of galactosidase causing conversion of 1 μmol ONPG to o-nitrophenol per min under the test conditions.

B. Activity of immobilized galactosidase

Similarly, for the immobilized galactosidase, 0.1 gram of enzyme immobilized cloth was tested in 5.0 ml of 1 mM ONPG solution prepared in 0.1 M phosphate buffer, pH 6.6 containing 1.5 mM magnesium chloride. The colour change of the assay solution was monitored at 410 nm by UV spectrophotometer. One unit of immobilized activity was defined as the amount of galactosidase immobilized on cloth, which cause conversion of 1 μmol ONPG to o-nitrophenol per min at testing condition. The absorbance coefficient ε for o-nitrophenol under the testing conditions was reported to be 3.15 mM⁻¹. cm⁻¹[79].

3.2.5 Immobilization Methods

Several different immobilization methods had been developed in this work, based on the precedents in the literature outlined in Section 2.3. These methods are outlined below.

3.2.5.1 Lipase Immobilization by Covalent Binding Protocol (CVB)

For the past literature on this type of method, see Section 2.3.2.2 for detail. In this method, the support matrix was pre-activated by GA, and then it was adsorbed in lipase solution. Part of the adsorbed lipase was reacted with the active sites on woollen cloth to complete the chemical
immobilization. To reduce the tendency of GA oligomer formation (to make sure that experiment was carried out in the same condition), 2.5% of GA stock solution was prepared by diluting concentrated GA solution (25%) with deionized water, and it was stored in fridge at 4 °C before use. The details of the steps used in this method are as follows:

**Step 1: PEI adsorption**

Chlorinated woollen cloth (right after completion, low pH process was used here) was soaked in PEI solution (pH 8.5, 2%) for 2 hours at room temperature.

**Step 2: GA cross-linking**

The PEI fixed cloth was soaked in 0.1% GA solution (freshly prepared in a phosphate buffer at pH 9, see Section 3.2.2 for buffer preparation), and then the cloth was washed with tap water followed by pH 9.0 phosphate buffer.

**Step 3: Lipase adsorption and covalent binding**

The GA treated cloth (0.5 gram) was then shaken in 1.0 mg ml⁻¹ lipase solution (20 ml) for overnight in phosphate buffer solution at pH 8-9 by the means of incubator with temperature controlled at 25 °C (150 rpm).

After completing the immobilization, the lipase-immobilized cloth was stored in Tris buffer at pH 8.5 containing 2 mM calcium chloride in fridge until used for activity test (see Section 3.2.3) and/or stain removal (see Section 3.2.9).

---

1 Note that the reaction mechanism for GA cross-linking is still not clear [132]. The most accepted theory is the Schiff base reaction (see Section 2.4.2.1 for further details). Due to the instability of Schiff reaction, the products normally have to be treated with reducing agent, such as sodium borohydride. Some authors have stressed that treatment with sodium borohydride is definitely needed [21,133]. Therefore, in this project, a reduction protocol recommended by Guisan, Jose M and his research group [21,176, 177] was used: “After adding sufficient 0.1 M sodium bicarbonate at pH 10.05 to give a gel volume-to-total volume ratio of 1: 10, sodium borohydride was added to give a final concentration of 1 mg/ml and the suspension was stirred for 30 min at room temperature. The reduced esterase derivatives were washed successively with an excess of 0.1 M sodium phosphate buffer at pH 7 and distilled water” [178]. However, after initial experiments were conducted (results not presented) it was found that little activity and stability improvement was obtained when Guisan’s reduction protocol was applied, thus the sodium borohydride reduction step was omitted.
3.2.5.2 Lipase Immobilization by Cross-Linked Protocol (CRL)

For past literature on this type of method, see Section 2.3.2.3 and Section 2.3.2.4. This method is different from the CVB protocol, since lipase was adsorbed onto PEI coated cloth first, followed by GA treatment. Similarly to CVB protocol, to reduce the tendency of GA oligomer formation, 2.5% of GA stock solution was prepared by diluting concentrated GA solution (25%) with deionized water, and stored in fridge at 4 °C before use.

Chlorinated woollen cloth (low pH process) was first soaked in 2% PEI (prepared in DI water at pH 8.5) for 2 hours. Then this cloth was directly soaked in 2.5 mg ml\(^{-1}\) lipase solution in phosphate buffer at pH 8.0. This lipase-adsorbed cloth was finally soaked in 0.1% GA solution freshly diluted in pH 6.0 phosphates buffer (0.1M) for five minutes to complete the immobilization.

3.2.5.3 Galactosidase Immobilization on Woollen Cloth

The past literature on this enzyme is introduced in Section 2.2.3. The CVB immobilization approach was used for galactosidase immobilization. Chlorinated woollen cloth (treated by “low pH process”) was soaked in 2% PEI solution (pH 8.5) for 2 hours, then treated in 0.1% GA solution, pH 8.0 for 5 min. After the reaction completed, this cloth was rinsed with deionized (DI) water, followed by rinsing with 0.1 M phosphate buffer at pH 6.0 (see Section 3.2.2 for buffer preparation), containing 1 mM magnesium chloride. Finally this cloth was shaken in galactosidase solution (at a 20 fold dilution\(^2\)) for 1 hour to complete this immobilization. Finally, this galactosidase-immobilized cloth was stored in the same buffer in a refrigerator until the activity was tested (see Section 3.2.4)

3.2.5.4 Lipase immobilized via PEI-polyaldehyde

The PEI-polyaldehyde solution used was prepared by adding GA solution (final concentration 0.5% by weight) into 0.5 % PEI solution (pH 8.5). After storing for 3 days in room at 25 °C, the change of solution colour became stable and then this solution was used in following lipase immobilization. Chlorinated woollen cloth was soaked in this PEI-polyaldehyde solution for 24 hours, and then soaked in lipase solution (2 mg ml\(^{-1}\), pH 6.0) for 15 min to complete the immobilization.

3.2.5.5 Lipase encapsulated in Casein gel formed in the presence of TGase

\(^2\) As the commercially available galactosidase was in liquid form, this enzyme solution had to be diluted to the right concentration with 0.1 M phosphate buffer solution, pH 6.0 containing 1 mM magnesium chloride before it was used.
The past literature on this type of method is described in Section 2.3.1. In this method, after chlorination (see Section 3.2.1, by “high pH process”), bare woollen cloth was soaked in 2% PEI solution for 2 hours, and treated in 0.1% GA solution at pH 8.0 for 5 min.

Lipase powder (0.07 gram) mixed with 0.2 gram of mTGase was dissolved in 1.0 gram of 0.1M carbonate buffer solution, pH 9.0. Then this mixture was mixed with 3 grams of sodium caseinate (20%) solution. One gram of the woollen cloth (as pre-treated above) was then coated with this solution and heated up in water bath at 50 °C. After 100 minutes, a solid caseinate coating entrapped with lipase was formed on cloth surface. The lipase-immobilized cloth was stored in air at room temperature before use.

3.2.6 Preparation of GA oligomer

The preparation of GA oligomer was followed up the procedure published by Seung Won Park et al. [138] with some modifications. GA solution (1%) was diluted from 25% GA solution (commercial product) with various buffer solutions (pH 7, 8, 9, 15, 9.65, 10.45). This GA solution was heated up in water bath at 60 °C for 30 min, and then this solution was cooled down to room temperature and used in lipase immobilization.

3.2.7 Determination of mTGase concentration used in casein gel formation

Because the strength of casein gel is related to the mTGase concentration used (reported by Hiroko Sakamoto et al.[74]), thus the amount of enzyme required to obtain a firm gel is then determined through the comparison of the gel strength.

Various amounts of mTGase were dissolved in 1 gram of 0.1 M sodium carbonate buffer solution at pH 9. After mixing well, 3 gram of 20% sodium caseinate solution was added in. This casein solution was heated in water bath at 50 °C for different time, and the resulting gel strength was compared. The mTGase concentration was then known.

This comparison was carried out by the means of the relative strength between those gel formed. The gel obtained in 0.1 gram of mTGase solution and treated for 10 min in water bath at 50 °C had lowest strength, whose gel strength was defined as Grade 0. While the gel formed in 0.2 gram of mTGase and treated for 45 min in water bath had highest strength, whose gel strength was defined
as Grade 6. The others were defined between Grade 0-Grade 6, based on its relative strength, the result is shown in Table 7-1.

**3.2.8 Protein Load Determination**

**A. Protein concentration determination**

Protein concentration of lipase was determined in order to calculate the amount of lipase immobilized on cloth and the lipase purity. The protein concentration in solution was determined by the Micro BCA method [180]. The principle is based on the alkaline reduction of Cu$^{2+}$ to Cu$^{1+}$ by proteins, and the formation of a bicinchoninic acid: Cu$^{1+}$ complex having a maximum absorbance at 562 nm. The experimental procedure is as follows:

The protein sample measured was diluted to the proper concentration and tested using a QuantiPro BCA Assay Kit purchased from Sigma. The protein concentration measurement limit of this kit is from 0.5 to 30 µg ml$^{-1}$. This assay kit is composed of QA, QB and QC solution (QA is buffer at pH 11.25, QB is 4% (w/v) bicinchoninic acid solution, pH 8.5, and QC is 4% (w/v) copper (II) sulfate, solution). According to the operational procedure in the Sigma technical bulletin [180], QuantiPro Working reagent was prepared by mixing 25 parts of QA solution, 25 parts of QB solution and 1 part of QC solution. After mixing well, one part of QuantiPro Working reagent was mixed with one part of the unknown protein solution. This mixture was heated up using a water bath at 60 º C for 1 hour, and then cooled down to room temperature in air. The UV absorbance at 562 nm was recorded and the protein concentration was determined by comparison to a standard curve. This standard curve was prepared by the protein standard solution (1 mg ml$^{-1}$) included in the QuantiPro BCA Assay Kit. This protein standard was diluted into series of different concentration protein solutions from 0.5 µg ml$^{-1}$ to 30 µg ml$^{-1}$. Their UV absorbance was plotted versus protein concentration as shown in Figure 10-1 (Values are the mean of three independent replicates, and average error is less than 5%) of Appendix 10.3.

**B determination of protein load on the lipase immobilized cloth**

Normally, the amount of enzyme adsorbed on immobilization matrix can be estimated via the comparison of protein concentration between the immobilization broth before and after immobilization through protein concentration assay kit.
However, the amount of lipase adsorbed could not be taken as the “protein load” directly in the current immobilization method used for the lipase from *pseudomonas fluorescens* (see Section 3.2.3.3), since leaked activity was always observed throughout the activity determination (the figure of immobilized activity is taken when leaked activity close enough to zero. This measurement often takes 5 or even more activity determinations with pNPP assay solution). It was too complicated to obtain the exact figure of protein loads by deducting the amount of lipase leaked from the amount of lipase adsorbed. As shown in Figure 3-1, the immobilized lipase normally has a huge leaked activity in the first activity determination, then the leaked activity decreases during consecutive assay. Thus, the leaked activity from the first activity determination could be approximately used to calculate the amount of lipase leaked out.

In this research, two methods were used for protein loads estimation: pNPP activity assay and protein concentration assay kit. As the activity measurement by pNPP assay is more rapid than the procedure used for protein concentration determination. So the amount of lipase adsorbed onto cloth was first estimated from the lipase activity difference in the free lipase solution before and after cloth adsorption. The amount of lipase leak-out was estimated from the leaked activity tested by pNPP assay. Thus protein load can be calculated by Equation 6:

\[
\text{Lipase adsorbed} = \frac{(\text{Activity before} - \text{Activity after})}{(\text{specific activity} \times \text{cloth weight})} \\
\text{Lipase leaked} = \frac{\text{Leaked activity}}{\text{(cloth weight} \times \text{specific activity})} \\
\text{Protein load} = \text{Lipase adsorbed} - \text{Lipase leaked}
\]

**Equation 6 Calculation of protein load on the lipase-immobilized woollen cloth**

In Equation 6, “lipase adsorbed” stands for the amount of lipase (mg/g cloth) adsorbed onto cloth during immobilization. “Activity before” is the free lipase activity in the lipase solution without any cloth soaking. “Activity after” is the free lipase activity in the lipase solution after cloth soaking. “Specific activity” is the free lipase activity in one mg of lipase at the testing condition. Cloth weight is the weight of woollen cloth used in immobilization. “Leaked activity” is the leaked activity in the first activity determination with the lipase-immobilized cloth. The amount of lipase remained on the cloth (protein load) is then can be calculated by deducting the amount of adsorbed lipase from the amount of leaked lipase. As some lipase adsorbed might be deactivated by the denaturants, such as GA. To further confirm the result obtained from pNPP assay, protein concentration before and after cloth adsorption was also determined from QuantiPro BCA Assay Kit.
3.2.9 Oily Stain Removal of Lipase Immobilized Cloth

The most immediate use of lipase-immobilized cloth is for laundry washing, as outlined in Section 2.2.1.4. To determine the potential improvement in washing performance, the stain removal capacity of lipase immobilized woollen cloths were compared to bare woollen cloths in a washing test. The washing test is similar to the method described by C. Hemachander [15] and J.A.C. Flipsen [64] with some modifications. The test was conducted as follows:

1. Preparation of fabric strips

The lipase-immobilized cloth was soiled with olive oil, and then dried with paper towels to remove the excess olive oil. Immediately after drying with silica gel for 4 hours, the cloth weight before and after soiling was measured and recorded as “cloth weight” and “soiled cloth weight”.

2. Cloth washing

After storing for 1 day in air, the soiled cloth strips were washed with pH 8.5 Tris buffer in an incubator at 100 rpm for 30 min at ambient temperature. Immediately after drying with silica gel for 4 hours, the weight of the cloth strip was measured and recorded as “washed cloth weight”. As a comparison, the washing data of the untreated woollen cloth with pH 8.5 Tris buffer were also measured.

3. Determination of cleaning efficiency

The washing effect can be examined from the cleaning efficiency, as defined below:

\[
\text{Cleaning efficiency (percentage of oil remaining on cloth)} = \frac{(\text{Soiled cloth weight} - \text{washed cloth weight})}{(\text{cloth weight})}
\]

Equation 7 cleaning efficiency of oily stain removal of lipase immobilized cloth

Values are the mean of 3 independent replicates. Error bars represent ± one standard deviation.
3.2.10 Determination of Kinetic Parameters

The kinetic parameters of the free and immobilized lipase were determined in this work using pNPP as substrate. Its concentration was varied from 0.2 to 4 mM and 0.2 to 2.5 mM for the free and immobilized lipase, respectively. The relationship between the enzyme activity and substrate concentration is well described by the Michaelis-Menten equation, as shown in Equation 1 (Section 2.6). Despite its advantages of other plots (as outlined in Section 2.6), Lineweaver-Burk plot is still used in this research, as it is widely used in academic literature and makes the comparison with published data easier.

To do this, Equation 2 is used, where the slope of Lineweaver-Burk plots, \( \frac{K_m}{V_{\text{max}}} \), and the intercept, \( \frac{1}{V_{\text{max}}} \), is determined. From the comparison between the slope and intercept, \( K_M \) and \( v_{\text{max}} \) are calculated.

Besides Lineweaver-Burk plot, other linear and none linear methods were also used to determine the kinetics parameters, in order to eliminate the drawback of Lineweaver-Burk plot. The detail content is shown in Section 5.4.

3.2.11 SEM and ESEM

Both scanning electron microscope (SEM) and environmental scanning electron microscope (ESEM) technique were used to obtain images of enzyme-immobilized supports. These two techniques are slightly different. SEM is normally used to view dehydrated samples. Standard SEM operational procedures for biological samples involve chemical fixation, drying/dehydration, mounting on a stub and coating with a metal (e.g. chromium, gold, platinum) [181]. But SEM is not suitable for samples containing water, particularly for biological samples, where water plays an essential role to maintain the structure. As an alternative, more recently environmental SEM microscope (ESEM) has been developed, where water can be present in the samples, which is very useful for examining the native structure of biological samples.

As ESEM can visualize a sample with its original water content, thus increase the imaging capabilities with high resolution under high, low and extended low vacuum. Equipped with heating and cooling facilities, ESEM is able to view dry sample, i.e. raising the temperature of samples in a
controlled manner with a reduced pressure; or wet samples, i.e. when the temperature is decreased to maintain sample’s relative humidity also under a vacuum [182]. However the inherent limitations associated with ESEM include: reduced field of view and depth of focus, and in many cases, reduced resolution and stability at high magnification, since very often a sample is viewed without coating with a noble metal such as gold or platinum [182].

In this project, both SEM (Philips XL30S-FEG) and ESEM were used. As enzymes are a biological protein and therefore normally function in a liquid (and hydrated) environment, it was found that ESEM was more suitable. The specimen preparation procedure and setup of ESEM was as follows:

A thin layer of gold was sputtered onto the samples prior to imaging. These samples were mounted on aluminium stubs and powder coated with platinum (10 nm coating thickness) for 7 min at 10 mA using a high resolution Polaron SC7640 sputter coater. Images were taken using a FEI QUANTA 200 FEG SEM (FEI Ltd, Eindhoven, Netherlands), operating at 0.8 Torr with accelerating voltage of 5 kV.
4. Lipase and Galactosidase Immobilization by Covalent Binding Method (CVB)

The aim of this chapter was to develop a new improved protocol for lipase immobilized on wool. Due to the lack of prior literature on the effect of various parameters on lipase immobilization on wool (see Section 2.5.2.1), the effect of wool chlorination, concentration of GA, pH and incubation time in GA solution, lipase concentration and incubation time in lipase solution were evaluated.

As one of the most studied enzymes, the characteristics of lipase and the methods used in immobilization have been already well addressed (see Section 2.2.1 and Section 2.3 of the literature review and reference [183]). Lipase from *pseudomonas fluorescens* is secreted from thermophilic bacterial species and previous research have shown that it possess high stability even under chemical reagent treatment [184]. Moreover, this enzyme is readily available in the market in a cheap price, thus in this study lipase from *pseudomonas fluorescens* was chosen as the model enzyme.

4.1 **Immobilization via physical Adsorption on bare Woollen Cloth**

As described in Section 2.1, the surface of bare woollen cloth is hydrophobic, due to presence of a layer of covalent bound lipid. For lipase from *pseudomonas fluorescens*, the hyper-activation from interfacial adsorption onto hydrophobic surface has been well addressed in previous literature [185]. Hence, lipase was firstly adsorbed on bare wool to investigate if it is possible to take advantage of hydrophobicity of the bare woollen cloth and immobilize the lipase by physical adsorption via hydrophobic interaction (see Section 2.3.1 in the literature review for further details).

The bare woollen cloth (0.1 g) was soaked in lipase solution, prepared in 0.05 M Tris buffer, pH 8.5 for 10 min, and then rinsed with DI water. The immobilized activity change was measured by the pNPP assay as described in Section 3.2.3.2. The results are shown in Figure 4-1. It can be observed that the initial activity is very high, however, most of it is only weakly adsorbed and after 4 runs, the activity is decreased by more than 95%. The SEM images of bare and lipase-immobilized cloth are shown in Figure 4-2 and Figure 4-3. It can be observed that there was no difference for the SEM image between the bare and lipase immobilized cloth, further showing that very little enzyme has adsorbed on to the wool surface. Although the hyper-stability for hydrophobic immobilization has
been well addressed in previous literature [44, 186], this stability enhancement did not appear to occur in this case.

Consequently, this immobilization on bare cloth using physical adsorption method cannot meet this project’s research target. The woollen cloth therefore needs to be treated prior to being used in immobilization.

![Figure 4-1 Lipase adsorbed on untreated woollen cloth, activity drop during consecutive activity assay.](image)

### 4.2 The Effect of Chlorination on the Wool

In the wool industry, wool has to be treated to remove the surface hydrophobic layer and the stiff scale on surface to enable wool to be more water washable. As mentioned in Section 2.1, the most used technique in wool industry to convert the hydrophobic woollen surface into a hydrophilic one is chlorination/Hercoset process, where raw wool is treated in chlorine solution in acidic pH, and after is reduced with sodium bisulphite solution, and a reactive cationic resin PAE (Polyamideamine-epichlorohydrin resin) is applied.
Considering one of the potential applications for the immobilized lipase is in laundry washing, the commercial chlorination process is therefore followed. One of the possible benefits from chlorination to enzyme immobilization lies in the fact that not only the covalent-bound lipid layer is
removed, but the disulfide bonds formed between the two cysteine (Cys) residues (Cys-S-S-Cys) are oxidized to cysteic acid (Cys-SOOH) residues as well. Thus the wool surface becomes more negative charged which more easily adsorbs positively charged resin, such as PEI resin.

To facilitate the modification of wool protein, cloth was chlorinated according the method described in Section 3.2.1 (Low pH process). The SEM image of untreated woollen cloth is shown in Figure 4-4. Compared to the SEM image of chlorinated woollen cloth shown in Figure 4-5, it can be seen that the scale has been eliminated and the surface becomes smoother. However, the chlorination condition used in the experiment is quite aggressive and non-uniform:

- Almost all the scales on the wool fibre located on the surface layer had been removed.
- Furthermore, the degree of chlorination across the entire cloth is not even, where it can be seen that the surface layer received more chlorine attack than the inner layer, which suggested that more reaction time was needed for the chlorine agent to distribute into the inner layer.

But extending the reaction time would cause excessive etching on the surface layer. Thus, more moderate chlorination conditions are needed – this issue will be addressed in more detail in Section 6.3.

After chlorination, lipase was then immobilized onto this surface using the CVB technique (see the contents in Section 3.2.5.1), the factors influencing immobilization were addressed in the following sections.
Figure 4-4 ESEM picture of untreated woollen cloth, magnifications: upper 1000 x, lower 5000 x.
Figure 4-5 ESEM picture woollen cloth after DCCA chlorination and sodium bisulphite reduction, magnifications: upper 1000×, lower 5000 ×.

4.3 Effects of different parameters on immobilization activity

As mentioned in Section 3.2.5.1, lipase immobilization via the CVB method was split into three steps: (i) immersion in PEI solution, (ii) soaking in GA solution (which is affected by GA
concentration, reaction/immersion time and GA solution pH), and (iii) soaking in lipase solution. So the choices of chemicals and the effect of changing parameters on the immobilized lipase activity in these three steps are covered here:

4.3.1 Step 1: Immersion in PEI Solution

In this section, the first step of the CVB method is examined, looking at the choice of PEI as the spacer arm and justifying the use of the CVB method when using lipase, PEI and wool.

4.3.1.1 Choice of the PEI Spacer Arm

The isoelectric point of lipase from *pseudomonas fluorescens* (PFL) is 4.46 [47] and the isoelectric point of wool is approximately 2.5-3.5 [19]. The optimum pH for this lipase is reported to be between pH 5 to pH 9 [187]. To directly apply lipase to wool, the immobilization pH has to be between 3.5 and 4.46, which is not conducive for the lipase to keep its stability. Thus a positive charge spacer between wool and lipase was imperative in order to conduct this immobilization.

At first glance, PAE resin used for the chlorination/Hercoset process was thought to be a candidate. As a positively charged polymer, its connection to wool is not a problem. Consequently, a preliminary experiment was conducted to investigate the possibility of using the PAE resin as a spacer arm. For this, lipase prepared in Tris buffer was mixed with 2% of PAE resin at pH 9.0. This mixture was adsorbed onto chlorinated woollen cloth. This preparation was dried in at room temperature and atmospheric pressure for 1 week and the activity was tested by pNPP assay as described in Section 3.2.3.2. Results are not presented, as it was found that most of lipase was deactivated by this immobilization method. This may be because the cross-linking structure between PAE and wool is either too tight and no space is available for enzyme’s substrate to access, or a direct reaction occurred between the PAE resin, distorting the enzyme structure and hence deactivating it. Only the enzyme adsorbed on surface could be tested by the pNPP assay, however it was quickly washed away in the continuous activity assay.

The failure of immobilization on PAE resin showed that the reaction selected for immobilization could not be too aggressive and those reactions directly attacking the active site should be avoided. As described in Section 2.4, PEI is a versatile reagent in biotechnology, which is widely used in enzyme immobilization, separation, and even fabrication of artificial enzyme. PEI can stabilize
protein structure. This may come from the microenvironment change or the chelating metal ion, which can be used for protein deactivation catalysis. Moreover, PEI is a charged water soluble polymer, possessing a primary, secondary and tertiary structure, which is in the ratio of 1:1:1. This charged character and amine content make PEI a very useful and actually the most welcomed spacer arm in enzyme immobilization. Thus, in this research, PEI was selected as the spacer arm.

4.3.1.2 Justification of the Selection Covalent Binding Immobilization with PEI

Although immobilization by physical methods produces less structure distortion to the immobilized enzymes, it also produces the intrinsic disadvantage of instability. For industrial applications, stable operation is a more important consideration than the enzyme activity and yield [188]. Due to this, simple physical immobilizations are not industrially feasible. In contrast, chemical immobilizations are more stable, but produce a high activity loss. However, this is not always true. If proper precautions are taken, excessive activity loss can be avoided (see Section 2.3.2.3 for details of the CLEC and CLEA techniques).

As a protein, wool is highly electrostatically charged in aqueous solution. In this project, a novel protocol was developed based on this feature to facilitate immobilization. As addressed in Section 4.3.1.1, the isoelectric point of wool and lipase from *pseudomonas fluorescens* are approximately pH 2.5-3.5 and pH 4.46 respectively, while the pKa of PEI is around 10.5. At the right pH, PEI can be adsorbed onto wool, and lipase also adheres to PEI through electrostatic interactions. Then this structure was fixed by the chemical reaction with GA. Due to enrichment of lipases on the polymer surface, lipase concentration on PEI should be much higher than the one in bulk solution. This combination protocol of physical adsorption and covalent binding should hypothetically reduce the activation energy and result in a high immobilization speed.

Superior immobilization from covalent binding immobilization using PEI as spacer arm has also been reported in the available literature [122, 123].

4.3.2 Step 2: Soaking in GA solution – Investigation of the Cross-linker

4.3.2.1 Effect of GA Concentration on immobilization Activity

In this section, the second step of the CVB method is examined, looking at the effect of GA concentration on the immobilized lipase activity.
As a cross-linker in immobilization, GA has been reported as being very important to enzyme activity (see Section 2.4.2). To evaluate the effect of GA on the immobilized lipase activity via a chemical cross-linker immobilization method, GA concentration was varied between 0.1% and 12% as shown in Figure 4-6. Chlorinated cloth (treated by the “low pH process” of the chlorination method, outlined in Section 3.2.1) was firstly soaked in 2% PEI solution for 2 hours, then treated in GA solution (pH 8) for 10 min, and finally shaken in lipase solution (1 mg ml\(^{-1}\)) overnight.

![Figure 4-6 GA concentrations to immobilized activity](image)

From Figure 4-6, it can be seen that an increase in GA concentration does not improve the activity of the immobilized enzymes substantially. Thus, it is unnecessary to use a high concentration of GA solution in immobilization. Therefore a GA concentration of 0.1% (weight by weight) was used in following experiments.

### 4.3.2.2 Effect of the pH of the GA Solution and Reaction Time in the GA solution on immobilized Activity

In this section, the second step of the CVB method is again examined, this time looking at the effect of the pH of the lipase solution and the reaction time in the GA solution on the immobilized lipase activity.
Previous literature has shown an increase of immobilized enzyme activity in GA solutions with a high pH [131]. To prove this, lipase was thus immobilized using the method used above (see Section 4.3.2.1) with varied GA pH and treatment time in GA solution. The pH of the GA solution was varied between 6 and 11 and the time in the GA solution was varied between 1 and 15 minutes. The experimental results are illustrated in Figure 4-7.

From Figure 4-7, it can be seen that immobilized activity was mainly influenced by the pH of the GA solution. The relationship between lipase activity and pH in enzyme solution is a bell-shaped curve. The optimal point is around pH 9.0. This result collaborates with the content described in the Section 2.2, which the bell-shape curve is quite common for a pH versus enzyme activity relationship [41] (See section 2.2).
The optimal pH of 9.0 is most likely the result of two opposite factors that influence the immobilization process:

- PEI polymer must be first physically adsorbed onto woollen cloth through electrical interaction, before fixing to wool via GA cross-linking. As the majority amino groups of PEI are in protonated state when solution pH is smaller than 10 (see Section 2.4.1.1), the degree of interaction between wool and PEI will reduce with increased pH.
- 2 GA shows better reactivity in alkaline solutions, which might result in more reactive binding sites present on the cloth surface and subsequently lead to a better immobilized-activity at a higher pH [16].

Considering these two opposite factors, an optimal pH of around 9.0 seems reasonable.

Furthermore, by comparing Figure 4-7 A to D, it can be observed that the reaction time in the GA solution does not affect the immobilized activity too much. This result is comparable to the conclusion made in Section 4.3.2.1, where immobilized activity is independent of GA concentration. Based on this, it can be deduced that the reaction between PEI and GA is a fast process, suggesting that one or two minutes is enough to complete the reaction. Hence, in the following experiments, the following immobilization conditions for CVB protocol was used, unless stated otherwise: GA pH at 9.0, 5 minutes of incubation time and 0.1 % of GA concentration.

The independency of the immobilized activity to increase in reaction time in GA solution and the strong dependency on GA solution pH corresponds well to the results from Nedim Albayrak and Shang-Tian Yang [80] on *Aspergillus oryzae* galactosidase immobilization on PEI coated cotton cloth using GA as cross-linking agent.

Overall, the findings that increase in GA concentration and reaction time in GA solution does not improve the activity of the immobilized lipase too much, indicates that the aldehyde density on wool surface may not be related to the amount of GA present in aqueous solution. This result differs from other similar studies [122, 123, 189], where the change in GA concentration either increases or decreases the immobilized activity. However, the immobilization support used in above literature was silica and alumina ceramic, instead of wool. The biggest difference between wool and those inorganic supports is that no GA active groups are present on those inorganic support, they must be created before reacting with GA. For wool, the situation is different: a number of GA reactive
groups, such as lysine residues, cystein residues and terminal amino groups, are present. Thus, the finding that the increase in the GA concentration and reaction time in GA solution does not improve the immobilized lipase activity too much, might be related to the surface chemistry of wool.

### 4.3.3 Step 3: Lipase Addition - the Effect of Time in Lipase Solution on Immobilized Activity

In this section, the third step of the CVB method was examined, looking at the effect of lipase adsorption on the immobilized lipase activity. It needs to be stressed that the lipase used here is not pure with just enzymes; the protein concentration is only 18.5% (See Appendix 10.3), with the other components are likely to be the preservative and other stabilization compounds. In theory, the protein impurity could also occupy the aldehyde sites on PEI backbone and affect lipase immobilization. Protein purification methods, such as ionic chromatography, precipitation, could be used to increase the lipase purity. But due to time and equipment restrict, the purification step could not be carried out in the current project and has to be left in the future research.

The immobilization condition used was as follows: After chlorination, 0.5 g woollen cloth was treated in 0.1% GA solution at pH 9.0 for 5 min, and then the cloth was treated in 1 mg.ml⁻¹ lipase solution for varied time. It can be observed from Figure 4-8 that immobilized activity increased with time spent in the lipase solution until reaching a limit. Further extending the time in lipase solution does not improve the immobilized activity too much.

The dependence of immobilized activity on the immersion time in lipase solution could be ascribed to the lipase adsorption on the PEI-coated cloth. It is noted that PEI was used as spacer arm in this immobilization. In the pH range used in immobilization, PEI was able to adsorb lipase. GA modification did not change its adsorption ability to lipases too much, if the GA concentration used and treatment time in GA is taken into account. Thus, lipase was firstly adsorbed onto PEI, followed by reaction with the aldehyde groups existing on PEI. The more lipase adsorbed, the greater the chance that the lipase can be chemically immobilized until all the available surface of PEI is occupied. When cloth is only treated in lipase solution for a short time, this PEI coated cloth is not saturated with lipases. Enhancement of adsorption, such as extending soaking time and increasing enzyme concentration, can improve the immobilized activity. However, when the adsorption reaches
equilibrium, the immobilization becomes independent of these factors. Also note that not all the adsorbed lipases can participate in the covalent binding immobilization as some of lipase was just physically adsorbed.

![Graph showing specific immobilised activity vs. soaking time in lipase solution](image)

Figure 4-8 Treatment time in lipase solution to immobilized activity (Values are the mean of 3 independent replicates. Error bars represent ± one standard deviation).

The results above indicate that lipase adsorption is the rate-determining step in this lipase immobilization.

4.4 Protein Load on Immobilized Support

Lipases have been immobilized by the CVB protocol described in this chapter. However it is necessary to compare this immobilization with the one published in literature in order to rank the level of this protocol.

The quantity of enzyme is often expressed by the activity unit (see Section 3.2.3.3 for definitions used in this work), rather than mass unit of gram or mg. The reason is that not all the enzymes exist in their active form. So practically “enzyme activity” is more often used to express the enzyme quantity rather than its protein concentration. However, it is meaningless to compare the activity between different enzymes, as the assay substrates, assay conditions even reaction mechanisms are
always different. In scientific articles, protein load [190], which is defined as the amount of enzyme immobilized in terms of mass unit, i.e. mg or μg of enzymes on per gram of immobilization carrier, is used to do this comparison.

The protein load on various immobilization matrices reported from literature is listed in Table 4-1. It is often challenging to meaningfully compare the protein load between different studies, as they differ considerably, due to the wide variation in the immobilization methods and type of enzymes that are used. However from Table 4-1, the expected range of protein load can be roughly estimated. It was noticed that researchers tend to use overall amount of protein immobilized to express the protein load, rather than the fraction of enzyme in active form. Based on this, immobilization by adsorption normally has higher protein load - ranging from 200 mg.g⁻¹ support to 8 mg.g⁻¹ support (see Table 4-1). For chemical immobilization, the variation of protein load data is even larger - from 250 mg /g support to only 0.109 mg/g support.

However, the concept of protein load is often misleading or even confusing, especially for enzyme immobilization via the chemical method. Normally there is a great difference between protein load when measuring the enzyme in terms of overall protein mass content and the amount of enzyme in active form. For example, when Galactosidase from A.oryzae was immobilized on PEI coated cotton fabrics via GA cross-linking method, 250 mg enzyme/g cloth was claimed by the author [80], which is large compared to the protein load obtained through physical adsorption. However, Matella et al. [120] found only 0.1% to 8% of activity existing in this immobilization by the means of activity assay tool. Therefore, the amount of enzyme in active form must be considered if the protein load is to be compared between different immobilization methods and enzyme species.

The protein load on the lipase-immobilized woollen cloth in the current research was estimated by the method outlined in Section 3.2.8. The calculation steps are detailed in Appendix 10.4. From this result, the amount of lipase immobilized onto cloth was found to be 4.29 mg /(g cloth). Comparing this to the protein load from the other studies (Table 6), it can be seen that the amount of enzyme immobilized is low. This is might due to the feature of wool is different from the matrices used in these studies. Wool possesses a number of reactive groups on surface, such as lysine residue, cysteine residue, and terminal amino group. In contrast, most of the matrices in Table 6 are inert to GA (activation steps are necessary for some of the supports, such as silk, silica) or less reactive (cotton, for example). In addition, wool is more electrostatic charged than these support matrices [159]. This means that many of the reactions occurring during enzyme immobilization on woollen
cloth are different from the support matrices listed in Table 4-1. To improve the protein load, a number of approaches are described in Section 6.

Table 4-1 Protein load on immobilization support from previous publications.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Immobilization method</th>
<th>Protein load on support</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase</td>
<td>Absorbed in pores of PTFE membrane</td>
<td>0.020 +/- 0.002 mg protein/cm²</td>
<td>[191]</td>
</tr>
<tr>
<td><em>Candida antarctica B</em></td>
<td>Hydrophobic adsorption on mesoporous silica</td>
<td>200 mg protein/g of silica</td>
<td>[190]</td>
</tr>
<tr>
<td>Lipase from <em>Candida rugosa</em></td>
<td>Adsorption onto a macroporous copolymer</td>
<td>15.4 mg/g</td>
<td>[167]</td>
</tr>
<tr>
<td><em>C. cylindracea</em> Lipase</td>
<td>Hydrophobic adsorption on zeolite type Y</td>
<td>8.2 mg/ g support</td>
<td>[192]</td>
</tr>
<tr>
<td>Invertase <em>S. cerevisiae</em></td>
<td>Adsorbed on sepadbead EC-EP3-PEI</td>
<td>30 mg/g support</td>
<td>[89]</td>
</tr>
<tr>
<td>Lipase from <em>C. rugosa,</em> <em>H. lanuginosa,</em> and <em>Mucor miehei</em></td>
<td>Bimolecular formed of lipases adsorbed onto PEI coated Sepabead</td>
<td>3.7 mg ml⁻¹ support</td>
<td>[193]</td>
</tr>
<tr>
<td>Galactosidase from <em>A. oryzae</em></td>
<td>Chemically immobilized on PEI coated cotton activated by GA</td>
<td>250 mg enzyme/g cloth</td>
<td>[80]</td>
</tr>
<tr>
<td>D-lactate dehydrogenase &amp; <em>C. boidinii</em></td>
<td>PEI-enzyme-cell aggregates adsorbed on cotton</td>
<td>0.5 g cell and 8 mg LDH / g cotton</td>
<td>[194]</td>
</tr>
<tr>
<td>Porcine pancreas</td>
<td>Lipase adsorbed in PEI coated agarose followed by 1% GA treatment</td>
<td>1 mg ml⁻¹</td>
<td>[195]</td>
</tr>
<tr>
<td><em>C. Rugasa</em> lipase</td>
<td>Entrap in membrane, followed by GA cross-linking</td>
<td>0.098 mg/cm²</td>
<td>[196]</td>
</tr>
<tr>
<td>Protease, Flavourzyme</td>
<td>Covalent binding on Lewatit R258-K with GA.</td>
<td>1.8 mg enzyme ml⁻¹</td>
<td>[197]</td>
</tr>
<tr>
<td>Papain</td>
<td>Covalent bind to SiO₂ particles contg. amine by GA</td>
<td>15 mg/g support,</td>
<td>[198]</td>
</tr>
<tr>
<td>Glutaryl-7-aminocephalosporanic acid acylase</td>
<td>Covalent onto amino-epoxy Sepabeads</td>
<td>62 mg of protein per g of support</td>
<td>[199]</td>
</tr>
<tr>
<td>Mucor javanicus lipase</td>
<td>Silica nanoparticles bears a reactive epoxide group</td>
<td>60.9 mg/ g support</td>
<td>[5]</td>
</tr>
<tr>
<td>Lipase from <em>Candida rugosa</em></td>
<td>Chitosan nanofibrous membrane with (GA) as coupling reagent.</td>
<td>63.6 mg/g</td>
<td>[200]</td>
</tr>
<tr>
<td><em>Candida rugosa</em> lipase</td>
<td>Covalently bound to chitosan activated by using carbodiimide</td>
<td>0.109 mg/g chitosan</td>
<td>[201]</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Direct covalent bind to hydrolyzed silk by GA</td>
<td>0.1 mg enzyme/g fabric</td>
<td>[23]</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Covalent binding to PEI coated silk fabrics via GA treatment</td>
<td>10.4 mg enzyme/ g fabric</td>
<td>[157]</td>
</tr>
</tbody>
</table>

A comparison of the immobilization techniques outlined in Table 4-1 indicates that the introduction of PEI spacer can increase the protein-load considerably. For example, for alkaline phosphatase immobilization on silk fabrics, only 0.1 mg/ g fabrics was immobilized when silk was directly
hydrolysed in alkaline solution followed by a GA activation [23], while 10.4 mg/g fabrics was immobilized for PEI coated silk fabrics after GA activation [157]. This literature therefore provides another positive reason for the selection of PEI as a spacer arm in this study.

4.5 ESEM Images of Immobilized Lipase

The presence of trace amount of water is of vital importance for enzymes to maintain their tertiary structure. ESEM imaging techniques instead of SEM were thus used to visualize the immobilized enzymes with a trace amount of water present. The ESEM image of bare woollen cloth, chlorinated woollen cloth and lipase-immobilized cloth by the CVB protocol are illustrated here from Figure 4-9 to Figure 4-10. The chlorination steps were the same as the one used for the wool fibres in Figure 4-4 and Figure 4-5, and experimental conditions used for this immobilization were: temperature 25 °C, cloth was first treated in pH 9.0 phosphate buffer for 10 min, followed by soaking in 1 mg ml⁻¹ lipase solution prepared in 0.1 M pH 8 phosphate buffer for 1 day. These figures are corresponding to the experimental results in Section 4.3.2.1 and Section 4.3.2.2.

Figure 4-9 ESEM image of the immobilized lipase on woollen cloth by the CVB protocol (1000 x magnification), the immobilization condition adopted was as follows: temperature 25 °C, cloth was first treated in pH 9.0 phosphate buffer for 10 min, followed by soaking in 1 mg ml⁻¹ lipase solution prepared in 0.1 M pH 8 phosphate buffer for 1 day. The agglomerations of immobilized chemicals, which are suspected to contain the lipase attached to the fibres, can be observed on the wool fibre. It can also been seen that distribution of this aggregate is not even across the fibre surface. Instead, they concentrate on certain area of the fibre surface. In addition, the fibres in deep layer of the cloth seem have no aggregate coverage.
Firstly, from Figure 4-9 and Figure 4-10, it can be seen that the immobilization on chlorinated wool fibre was successful – there appears to be agglomerations of immobilized chemicals, which are suspected to contain the lipase attached to the fibres, since the images differ from the unchlorinated wool fibre in Figure 4-4 and Figure 4-5. However, one cannot definitely determine this aggregate is from the immobilized lipase. This might be also from the protein impurity, as the protein concentration for the lipase we used is only 18.5%. But definitely, the presence of aggregate is related to the lipase immobilization process and could be used as a reference to visualize the immobilization.

![Figure 4-10: A further magnified ESEM picture of the immobilized lipase on woollen cloth by CVB method (5000 × magnification). The immobilization condition adopted is as follows: temperature 25 °C, cloth was first treated in pH 9.0 phosphate buffer for 10 min, followed by soaking in 1 mg ml⁻¹ lipase solution prepared in 0.1 M pH 8 phosphate buffer for 1 day. The appearance of aggregate can be seen more clearly in this image.](image)

Moreover, the presence of the lipase suspect (or at least the immobilised substances) was found not to be evenly distributed throughout the cloth (Figure 4-9). Instead, this suspect of the immobilized lipase was concentrated on certain area on the fibres. Additionally, the coverage did not seem to be related to the intensity of PEI appearance. For example, presence of extensive cross-linked PEI can be clearly seen in Figure 4-11, but less presence of the immobilized lipase suspect is observed. These images give the impression that the immobilization tends to happen on the surfaces where PEI is less well coated, rather than on surfaces where the PEI is well cross-linked. This impression corresponds to the experimental results in Section 4.3.2.1 and Section 4.3.2.2 well. Nevertheless, the
uneven distribution of lipase suspect might come from other factors, such as washing out of physically adsorbed lipase, and/or dissolution of the wool protein in Tris buffer. More experimental evidence is needed to support this hypothesis however, which cannot be carried out in the current thesis work due to time limitations.

Figure 4-11 Magnified view of ESEM picture of the immobilized lipase on woollen cloth by CVB method (2500 × magnification), the immobilization condition adopted is as follows: temperature 25 °C, cloth was first treated in pH 9.0 phosphate buffer for 10 min, followed by soaking in 1 mg ml⁻¹ lipase solution prepared in 0.1 M pH 8 phosphate buffer for 1 day. The coating on fibre surface can be clearly seen, which is most likely from PEI cross-linking. Less agglomeration material is present in this area.

4.6 Illustration of CVB Immobilization Mechanism

Based on the findings above, a detailed mechanism for the CVB lipase immobilization can be proposed. The steps for the CVB immobilization protocol are illustrated in more detail by the diagrams in Figure 4-12.

(i). PEI adsorption

The first step of immobilization is PEI adsorption onto the wool surface: the positively charged PEI polymer is strongly adsorbed to wool surface under suitable pH, as illustrated in Figure 4-12 below.

(ii). Functionalization of the cloth surface
In this step (step ii in Figure 4-12 below), the amine functional group on the woollen cloth was further treated in GA solution. There were two reactions involved here: the reaction between PEI and wool (the amine groups on PEI and wool were chemically cross-linked together by GA), and the reaction between GA and amine group on PEI, where free aldehyde functional groups were produced. This free aldehyde group created on the PEI surface was used for lipase fixation in the next step. In step ii, the physically adsorbed PEI resin was chemically adhered to wool surface through a Schiff’s base formation. As the electrostatic adsorption between PEI and wool protein could accelerate the linkage formation, it can be deduced that this reaction is rapid. This corresponds to the experimental results in Section 4.3.2.1 and 4.3.2.2, where a five minute reaction time was enough to functionalise the cloth surface.

Figure 4-12 Explanation of the mechanism for lipase immobilization on woollen cloth via covalent binding method using PEI as spacer arm

(iii) Lipase immobilized on woollen cloth
In this step, the lipases in solution were firstly adsorbed onto a PEI spacer, which now are chemically fixed onto the cloth surface. Some adsorbed lipase might contact with the free aldehyde group and become chemically immobilized. However, some lipases are either electrostatically adsorbed onto the cloth surface, or form lipase-lipase aggregates, which are easily washed out during activity determination. Furthermore, due to the reversible character of the Schiff’s reaction, some of the chemically immobilized lipase may also convert back to their free form and leak out of the support matrix, becoming free (leaked) enzymes.

This ion interchange followed by covalent reaction mechanism is corroborated by Lorena Betancor [202].

4.7 Immobilization of β-galactosidase via the CVB Protocol

As described in this thesis (see the relevant content in Section 4 and Section 5), lipase was immobilized onto woollen cloth via the CVB protocol, and satisfactory results were achieved. In this section, an attempt was carried out to immobilize enzymes other than lipase by the method developed in the earlier part of Section 4. The factors affecting the immobilization were studied as well. Originally, organophosphate hydrolase (OPH) was selected as the model enzyme. However, it was nearly impossible to obtain this enzyme for research purposes, so consequently β-galactosidase and lactose hydrolysis reactions were used instead.

Like lipase, β-galactosidase is a well-studied enzyme. As introduced in the literature review (see Section 2.2.3), this enzyme possesses industrial importance in diary industry, both for production of low lactose milk products for lactose intolerant people, and to make use of the by-product of whey hydrolysis. Research is ongoing to develop an economical way to immobilize this enzyme, with a number of different support materials being tried. Because of its abundance and cheaper price, cotton material has drawn the most attention as a support material for β-galactosidase immobilization [80]. Wool - which is abundant and important to the New Zealand economy - is a natural extension of this work.

The CVB protocol outlined in Section 3.2.5.1 of this thesis was used to immobilize β-galactosidase onto woollen cloth. After completing immobilization, β-galactosidase-immobilized cloth was stored in fridge at 4 °C in 0.1 M phosphate buffer solution, pH 6.0 containing 1 mM magnesium chloride.
The storage stability was determined first and the result is illustrated in Figure 4-13. From the result here, it seems the immobilized \( \beta \)-galactosidase is not very stable under storage, where the immobilized activity continuously drops from 12 to only 4 \( \mu \)mol o-nitrophenol per min per gram of cloth within 5 days.

![Image](image.png)

**Figure 4-13** Storage stability of \( \beta \)-galactosidase immobilization on woollen cloth, value is the mean of three independent result, error bar represents mean ± one standard deviation

The activity of the immobilized \( \beta \)-galactosidase as a function of number of tests was examined as well. Figure 4-14 shows that the immobilized activity of \( \beta \)-galactosidase had almost no change after 9 rounds of activity assays (it took two minutes to complete every measurement and the whole assay was finished within one hour), which probably suggests that this enzyme had been immobilized successfully. Moreover, it was found that the stability of the immobilized enzymes (as measured by the change of immobilized activity versus number of activity determinations) was different from that of the immobilized lipase from *pseudomonas fluorescens* as shown in Figure 3-1, where several rounds of activity assay are needed to get a stable activity reading. Instead, almost all the physically adsorbed \( \beta \)-galactosidase were washed off just by one round of activity assay. This difference is reasonable, because \( \beta \)-galactosidase cannot form bimolecular aggregate as the lipase from *pseudomonas fluorescens* under the experimental conditions, it is much easier to remove the physically adsorbed enzyme, and the enzymes remained are chemically immobilized.
Figure 4-14 Activity change of immobilized Galactosidase versus measurement time (It took two minutes to complete every measurement, and the whole assay finished within one hour).

The ESEM image of the galactosidase immobilized woollen cloth was taken and shown in Figure 4-15. As expected, the galactosidase coverage over the cloth surface is limited, which corresponds to the result obtained from lipase immobilization.

The low stability for β-galactosidase immobilized by the CVB protocol is different from the result published in literature [203], where β-galactosidase was successfully immobilized onto PEI coated cotton fabrics via GA activation and two weeks continuous operation in a plug flow reaction was reported. More experiments needed to verify the reasons, which could not be completed for this thesis due to time constraints. This is therefore the subject of future work - see Section 8.2 for further details.
Figure 4-15 ESEM image of immobilized galactosidase on woollen cloth. It can be found that the spotty cluster showed in this image most likely represents the appearance of the immobilized galactosidases, if this image is compared to the bare woollen cloth (Figure 4-4 and Figure 4-5).

4.8 Conclusions

In summary, the following conclusions can be made from the experiments described in this chapter:

- Lipase could be immobilized to the wool treated by chlorination/HercoSet process followed by the covalent binding protocol developed in this chapter.

- The immobilized activity via the CVB protocol developed in this chapter was independent of GA concentration and treatment time in GA solution under the experimental conditions.

- The optimum immobilized activity was found at pH 9.0.
- For the relationship between soaking time in lipase solution and immobilized activity, the immobilized activity increased steadily in the beginning with the soaking time in lipase solution, and then the increase became slow and levels off. This may indicate that the lipase adsorption step in the CVB protocol was the rate-determining step in this immobilization protocol.

- The protein loads of lipase immobilized on woollen cloth was approximately 4.29 mg/g support.

- In the ESEM images of lipase immobilized woollen cloth, show that the distribution of immobilization chemicals (including the lipase) are clusters and are not evenly distributed over the wool fibre. This therefore means the protein load is limited and can be improved.

- Galactosidase could be immobilized onto woollen cloth via the CVB protocol developed. This immobilization could be proven by the stability of immobilized galactosidase activity during consecutive activity assay (Figure 4-14). The specific immobilized activity was found to be 10 mol o-nitrophenol per min per gram of cloth. However, the storage stability of this immobilized preparation was lower than the data published in literature [203]. After storing in 0.1 M phosphate buffer solution (pH 6.0 containing 1 mM magnesium chloride) at 4 °C for 5 days, the immobilize activity dropped from 12 μmol o-nitrophenol per min per gram of cloth to only 4 μmol o-nitrophenol per min per gram of cloth. More experiments are needed to verify the reasons for this.

This work has set the benchmark from which enzyme immobilization onto wool can be improved. Work in following chapters will focus on the improvement on this immobilization technique to increase enzyme loading/ activity, and investigating the stability, pH profile of the immobilized lipase as well as its usage in oily stain removal.
5. Characterising the Immobilized Lipase

In this chapter, the properties of the immobilized lipase produced in Chapter 4 are quantified to determine the lipase-immobilised cloth’s suitability as a self-cleaning or enhanced cleaning woollen cloth. The properties quantified to do this include:

- The activity change with pH for both the free and immobilized lipase.
- The storage stability in air and buffer solution.
- The true test of the immobilization’s potential as self-cleaning or enhanced cleaning woollen cloth is also completed by demonstrating and comparing the immobilized lipases ability to remove oily stains from the woollen support compared to a bare woollen cloth.
- Kinetics of the free and immobilized lipase.

5.1 Change in Activity of the Lipase with pH

pH has a considerable effect on the activity of enzymes, as discussed in Section 2.2 of the literature review. In order to ensure the lipase-immobilized cloths work at the optimal pH, pH and lipase activity relationship must be determined. Firstly the pH profile of the free lipase needs to be determined, so that it can be compared to that of the immobilized lipase. Any differences between pH profile of the free and immobilized lipase may indicate some changes occur in this enzyme due to the immobilization.

5.1.1 pH Profile of free Lipase

The effect of pH on the activity of the free lipase was measured in 0.1 M buffer, set at different pHs. To accomplish this, pHs between 6 and 8.0 were prepared with a phosphate buffer and pHs between 8 and 10.5 were prepared with an ammonia/ammonium sulphate buffer. The activity of 1 mg.ml⁻¹ lipase prepared in the different buffers was tested using the free lipase activity assay (see Section 3.2.3 for details). Figure 5-1 shows that the pH-activity relationship is a classical bell-shaped curve, like the one shown in Section 2.2. The optimal pH for maximum activity of the free lipase was estimated to be around pH 8.5. This result compares well (considering the experimental error) to the product data from the supplier [204], where the optimal pH is reported to be 8.0.
Figure 5-1  pH profile for the free lipases. The values are the mean of three independent measurements, and the error bar represents mean ± one standard deviation

5.1.2 pH Profile of immobilized Lipase

The effect of pH on the activity of the immobilized lipase was determined using the same buffers as those used with the free lipase. The activity of 0.1 g of lipase immobilized woollen cloth was tested in 5 ml of 0.4 mM pNPP assay solution (see Section 3.2.3 for details), in which the assay solution was prepared with different pH buffers. The stability of the immobilized lipase was found to be sufficient enough for this assay - there was negligible activity loss throughout the whole assay for the immobilized samples. The results are shown in Figure 5-2. This shows that the optimum pH for the both free and immobilized enzyme is approximately the same at somewhere around a pH of 8.5. This may indicate that the tertiary structure of lipase underwent minimal distortion during immobilization and the microenvironment between the free and immobilized lipase did not have a considerable change.

Note that the data is unfortunately incomplete, because the colour change of pNPP in alkaline solution is very fast for assays of solutions with a pH higher than 10, increasing the experimental error in these readings. Consequently, this data has to be omitted. Future work should focus on developing a more accurate assay for this work. One suggestion is to use another substrate (other than pNPP), such as an olive oil emulsion, which will have a slower reaction rate at higher pHs.
This indicates that the optimal lipase activity will occur somewhere in the region of pH 8.5. Therefore this pH was used in all further experiments, unless otherwise stated.

### 5.2 Storage Stability of free and immobilized Lipase

As addressed in literature review (see Section 2.3), stability is a very important criterion to judge the success of an immobilization, thus in this part, the stability of the immobilized lipase prepared by the protocol addressed in Section 4 is discussed in more detail. Two types of stability are often used in literature: storage stability and operational stability. Operational stability means the ability of the immobilized enzyme to retain activity after repeated substrate exposure. Storage stability is the activity change during enzyme storage, i.e. the storage efficiency defined as the ratio of free or immobilized enzyme activity after storage to their initial activity. Since one of the application of immobilized lipase in this project is for laundry cleaning, the storage stability is more meaningful, thus is described in the coming paragraphs in this section.

#### 5.2.1 Storage stability of free lipase stored in buffer

The activity change of 1 mg/ml of lipase solution stored in pH 8.5 buffer at 25 °C was monitored continuously for nearly one month and the result is shown in Figure 5-3.
Figure 5-3 Specific activity change of free lipase stored in Tris buffer pH 8.5 at 25 °C

Figure 5-3 shows that about 40% activity was lost during almost one-month storage in Tris buffer at room temperature (25°C), if the experimental error (data scatter) was considered. In previous studies, the storage stability of free Amano lipase from *Pseudomonas fluorescens* (AK PFL, same lipase as the one used in this research) was reported, but the experimental conditions are slightly different from the one used in the current research. Koki Itoyama et al. [205] reported that free AK PFL lost more than 50% of its initial activity after storing in 0.05 M phosphate buffer (pH 7.4) at 4°C within half a year. Gloria Fern [164] found that 80% of AK PFL activity was maintained after heat treatment at 45°C in pH 7.0 for 3 days. If different experimental conditions are taken into account, the storage stability data obtained in this study is acceptable.

### 5.2.2 Storage Stability of immobilized Lipase in Buffer

To investigate the stability of this immobilized preparation, lipase immobilized cloth was prepared via the CVB protocol as outlined in Section 3.2.5.1. This cloth was stored in pH 8.5, 50 mM Tris buffer at 4°C, and the activity change of the same samples were monitored periodically over 70 days. The results are illustrated in Figure 5-4.
Figure 5-4 Stability of the immobilized lipase, the samples were stored in 0.05 M Tris buffer, pH 8.5 at 4 °C. The value is the mean of three replicates; the error bar represents mean ± one standard deviation.

From Figure 5-4, it can be seen that the activity of the immobilized lipase did not drop considerably compared to the spread of the data. Consequently, it can be concluded that the lipase immobilized on wool is stable when stored in Tris buffer and at low temperature (4°C).

5.2.3 Storage Stability of immobilized Lipase in Air

Woollen garments, carpets and other items are obviously not going to be stored in a buffer solution. Consequently, to model the real situation that they will be used in, if the immobilized lipase cloth is used in woollen products, the storage stability of immobilized lipase in air was also tested. The result is illustrated in Figure 5-5. The activity of immobilized lipase decreased considerably over a period of 2 weeks. This drop is much greater than the immobilized samples stored in Tris buffer in 4°C. As enzymes normally lose their activity quickly in unfavourable environments, it is reasonable to assume that the immobilized lipase deactivated rapidly in dry air and at temperature of 25 °C. In solution, the Tris buffer might quench the free aldehyde group remaining on the cloth surface after immobilization and slow the enzyme deactivation giving the stability shown in Figure 5-4. In dry air, the presence of free aldehyde on the surface of woollen cloth might be another reason to cause the enzyme activity loss.
5.2.4 Discussion

It was found the lipase immobilized cloth shows better stability in Tris buffer at 4°C than in air at room temperature. The immobilized lipase maintained 80% of its activity when stored in Tris buffer at 4°C for more than 70 days while almost 80% activity was lost when stored in air at room temperature only within 15 days. This may be due to two reasons: firstly, enzymes normally demonstrate higher stability in their optimal pH and at low temperature, while the immobilized lipase stored in air cannot maintain a favourable pH. Instead, the pH surrounding the immobilized lipase will change gradually during the dehydration of the cloth. Secondly, the free aldehyde groups, which still remained on cloth when stored in air, might cause excessive activity loss. When the immobilized lipases is stored in Tris buffer solution, the active aldehyde groups on woollen cloth could be quenched by the amine groups contained in Tris buffer (see Section 2.3.2.2).

Stability enhancement of the immobilized enzyme from chemical immobilization have been well addressed in the past literature [138, 147]. It is however complicated to compare the stability between the free and immobilized lipase by the CVB protocol, since the lipase from Pseudomonas fluorescens (PFL) has a strong tendency to form bi-molecular aggregate, meaning that its stability is different at low and high lipase concentration (see[54] and Section 2.2.1.3). It was revealed that the
bimolecular PFL was much more stable than the unimolecular one, with the bimolecular PEL maintaining over 80% of the initial activity after 72 hours at 45°C, while the unimolecular structure retained only around 30% of initial activity after 4 hours of incubation under the same experimental conditions [164]. It was also reported that the PFL biomolecular structure appeared when the enzyme concentration was higher than 160 μ g ml⁻¹ in the absence of Triton X-100 (lipase AK from Amano enzyme Inc, purified lipase was applied) [164]. The lipase concentration used in this research is mostly higher than 1 mg ml⁻¹. Furthermore, considering that the purity of lipase AK used in this research is 18.5% (see Appendix 10.3, the others is the protein impurity and preservatives), the free lipase used here should be in the bimolecular form.

The immobilized lipase may also be either unimolecular or bimolecular. Triton X-100 was reported to be a retardant for biomolecular aggregate formation [164]. Consequently, no biomolecular aggregate of lipase can form in the presence of Triton X-100. This was evident from the experiments where the immobilized lipases on woollen cloth had been repeatedly tested by the pNPP assay solution containing Triton X-100. The bimolecular form of lipase on cloth must have been converted into monomolecular status in the presence of Triton X-100 surfactant. Compared to the free lipase stability in unimolecular structure reported in literature (70% of activity loss within 4 hours [164] at 45°C ), enhanced stability was present for the immobilized lipase by the CVB protocol (more than 80% of the immobilized lipase activity was maintained after storing for more than 70 days as shown in Figure 5-4).

It is bit difficult to compare the storage stability of lipase powder in air (lipase powder stored in air) with the immobilized lipase. The surrounding environment around the lipase powder and the immobilized lipase is considerably different, as stabiliser is contained inside the lipase powder. Also, it has no practical significance for the purpose in the current research. So this comparison was omitted.

Therefore, this lipase-immobilized preparation demonstrates excellent storage stability in Tris buffer at 4 °C, while is less stable in air in room temperature. Compared to the free counterpart, the immobilized lipase presents enhanced stability in aqueous solution.
5.3 The oily stain removal capacity of the immobilized lipase

One of the research objectives for this project is to demonstrate the potential of lipase-immobilized woollen cloth in laundry washing. Thus its performance in oily stain removal is quantified in this section. As introduced in Section 2.2.1.4, the detergent used in laundry ingredient cannot effectively remove the oily stain on fabrics, especially for woollen products, where alkaline builder and aggressive detergents cannot be used, because the existence of alkaline chemicals would lead to structure damage of woollen fabrics.

To study the oil cleaning capacity of lipase immobilized woollen cloth, the cloth was stained with olive oil and stored at room temperature for 1 day, then washed in pH 8.5 Tris buffer solution. Observations of the cleaning showed that the woollen cloth with lipase had enhanced cleaning ability: when the stained lipase immobilized woollen cloth was washed with buffer, a milky white emulsion very quickly appeared in the buffer solution and the stained cloth became clean. In comparison, the bare untreated woollen cloth was still heavily stained with oil.

This cleaning effect was quantified using the weight difference before and after buffer cleaning, and the cleaning test was repeated periodically within one month when the lipase-immobilized cloth was stored in air, as illustrated in Figure 5-6. The percentage of oil remained in the cloth was termed here as oil cleaning efficiency as defined in Section 3.2, and used as a standard to examine stain removal ability.

The lipase-immobilized cloth demonstrates excellent stain removal capacity, even without any detergent addition compared to bare woollen cloth without any lipase immobilized, as shown in Figure 5-6. Furthermore, this lipase-immobilized cloth demonstrated enough stability after one-month storage in air, which differs from the result in Section 5.2.3 that presents a decreased stability of the immobilized lipase in air. The difference is most likely because the enzymes are more stable to be repeated use in olive oil substrate than in the pNPP assay tested in Section 5.2.3. However, it might also indicate that pNPP could cause lipase deactivation. Further experimental work is needed to verify this. It can also be observed that oil content on the bare woollen cloth decreased slightly within one month. However, this sort of deduction is mainly due to the leach-out of oil droplet from cloth, rather than being cleaned by the bare woollen cloth.
Consequently, it can be concluded that the lipase-immobilized woollen cloth exhibits excellent oily stain removal ability: after being stained with olive oil and stored for one day in air at room temperature, the oily stain could be easily removed by 0.05 M pH 8.5 Tris buffer without any detergent addition. This enhanced cleaning was stable also, since after cloth was stored in air for almost one month, similar cleaning performance was observed.

### 5.4 Kinetic Model for Free and Immobilized Lipase

As addressed in the literature review (Section 2.6), the Michaelis-Menten (M-M) equation is the basic kinetic expression used for most enzymes with single substrate (Equation 1). The M-M equation assumes that both of the substrate and the enzyme are water-soluble and well mixed. If other factors are considered, including competitive product inhibition, non-competitive product inhibition, the M-M relationship can be further complicated, as listed in Table 2-5 (Section 2.6). Lipase from *pseudomonas fluorescens* (Amano lipase) had been reported to follow the M-M model for both of the free and immobilized lipase (immobilized on polysiloxane–polyvinyl alcohol matrix activated by GA) when olive oil emulsion was used as substrate [206]. M-M style kinetics was also

![Figure 5-6 The capacity of the lipase-immobilized cloth in olive oil stain removal (in % mass of olive oil remaining) compared to the bare woollen cloth at various points during the cloths storage over a month. Values are the mean of 3 independent replicates. Error bars represent ± one standard deviation.](image-url)
reported when enzyme was immobilized on the surface of fabric cloth. In the kinetic study done by Zhou et al. [207] on immobilized β-galactosidase from Kluyveromyces lactis, it was addressed that the M-M model without inhibition is sufficient to determine the kinetic parameters at low concentration of ONPG (o-nitrophenyl-β-D-galactopyranoside), as enzymatic hydrolysis is not influenced by inhibition behaviours from either reactant or product species. As only 0.4-4 mM pNPP was used in the current research, the conventional M-M model is assumed to be adequate enough to describe the kinetics. Hence the M-M model without inhibition was chosen to determine the kinetic parameters in the present study.

5.4.1 Kinetics of the free lipase

The determination of the kinetic parameters of free lipases was conducted in the methods outlined in Section 3.

Normally, the lipase activity of a commercial product is calculated based on the mass of enzymes, not the pure protein content contained in the enzymes. For example, in the Sigma-Aldrich website [208], the lipase unit of lipase from pseudomonas fluorescens is defined as the amount of lipase liberate 1 μg of oleic acid per minute at 40 °C (the activity of lipase from pseudomonas fluorescens is 20 U/ mg lipase). Therefore, the unit of reaction rate used in the current kinetic model is also defined as μmol pNPP.min⁻¹ (mg lipase)⁻¹ (weighted on mg of the commercial lipase powder).

The rate of lipase hydrolysis of pNPP, can be expressed by Michaelis-Menten equation. For comparison purpose, we used the standard experiments to measure the kinetic parameters (Michaelis constant \( K_M \) and maximum velocity \( v_{max} \)) of the free and immobilized lipase, using pNPP as the substrate (Equation 1).

Michaelis-Menten equation is not a first and second order rate equation, and so the calculation of the kinetic parameters are not straightforward. To extract the kinetic parameters, the calculation methods outlined in Section 2.6 were employed. The kinetics parameters obtained from linearization and nonlinearization methods are compared in Table 5-1. Among them, “Lsqcurvefit”, a nonlinear data-fitting function in Matlab 7.0, was used to do the non-linearization calculation, where the parameters that best fit the Michaelis-Menten equation to the experimental data in the least-squares sense was obtained. The linearization transformation of Michaelis-Menten equation described in Section 2.6 was also employed.
The goodness of the fit from the kinetic parameters for lipase from *Pseudomonas fluorescens* obtained from Table 5-1 was then assessed. This was done by comparing the experimental data to the calculated data using Michaelis-Menten equation with these parameters listed in Table 5-1, and the results are shown in Figure 5-7. All these plots predict the rate reasonably well within the range of concentration from which the parameters were estimated. However, the rate predicted from the Lineweaver-Burk plot fits the data accurately only when the substrate concentration is lowest and deviate when the concentration increases. This is because Lineweaver-Burk plot gives undue weight to the low substrate concentration as mentioned in the literature review. The rate predicted from Eadie-Hofstee plot, Langmuir plot and nonlinear regression method give a better fitting to the experimental data. Among these three methods, the results from the linearization data fitting are slightly different from the one by least square nonlinear regression. This is because the linear transformation in Eadie-Hofstee plot and Langmuir plot still amplify the experimental error and introduce more deviation into the calculation. While, nonlinear-regression method produce weighted least squares estimation of the parameters from the experimental data, so this estimation is optimal and more reliable, thus was adopted as the final result.

<table>
<thead>
<tr>
<th>Plots</th>
<th>( v_{\text{max}} )</th>
<th>( K_M )</th>
<th>( R^2 )</th>
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</thead>
<tbody>
<tr>
<td>Lineweaver Burk</td>
<td>100</td>
<td>1.12</td>
<td>0.971</td>
</tr>
<tr>
<td>Eadie Hofstee</td>
<td>138.7</td>
<td>1.933</td>
<td>0.736</td>
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<tr>
<td>Langmuir</td>
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<td>0.915</td>
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<tr>
<td>Non-linear regression</td>
<td>153.76</td>
<td>2.40</td>
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</tbody>
</table>
5.4.2 Kinetics of the immobilized lipase

The immobilized lipase was prepared by the CVB method as outlined in Section 3.2.5. The activity of immobilized preparation was tested in 0.4 mM pNPP assay solution enough times to make sure that all of the physically attached enzymes (i.e. those that will leak) left before determining the kinetic parameters. The substrate concentration was varied from 0.2 mM to 4 mM. Three independent replicates were conducted for each concentration. The UV absorbance changes for each test were recorded and converted to the relevant nitrophenol concentration by the standard curve given in Figure 10-2 in Appendix 10.3. Similarly to the free lipase, the kinetics parameters were calculated from various plots and the results are compared in Table 5-2. It has to be stressed that for the convenience of comparison, the units of \( v_{\text{max}} \) for the immobilized lipase are \( \mu\text{mol pNPP.min}^{-1}.(\text{mg lipase})^{-1} \) instead of the unit used for the specific immobilized activity (\( \mu\text{mol pNPP.min}^{-1}.(\text{g cloth})^{-1} \)). The protein load (amount of protein immobilized) was calculated in Appendix 10.4. For CVB immobilization, it was found to be 4.29 mg/g cloth.
Table 5-2 M-M kinetics parameters of the immobilized lipase calculated from various methods

<table>
<thead>
<tr>
<th>plot</th>
<th>( v_{\text{max}} )</th>
<th>( K_M^* )</th>
<th>( R^2 )</th>
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<tr>
<td>Lineweaver Burk plot</td>
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<td>Eadie-Hofstee Plot</td>
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<tr>
<td>Non-linear regression</td>
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<td>3.16</td>
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</table>

*Unit of the Michaelis-Menten constant \( K_M \) is mM and \( v_{\text{max}} \) is \( \mu \text{mol pNPP.min}^{-1} \text{(mg lipase)}^{-1} \).

The M-M model predicted value calculated from the kinetic parameter obtained from the above plots are compared to the experimental data and shown in Figure 5-8.

![Figure 5-8 Kinetics of the immobilized lipase, the comparison between experiment value and data calculated from Michaelis-Menten equation](image)

Similar to the free lipase, the parameters obtained from Lineweaver-Burk plot produce the worst data fitting. Eadie Hofstee and Langmuir plot create similar result as the non-linear regression method, despite some deviations exist. The parameters calculated from non-linear regression method were adopted as the final result as was done for the free lipase.

### 5.4.3 Results and Discussion

In the M-M model, \( K_M \) represents the substrate concentration when the reaction rate reaches half of \( v_{\text{max}} \). From the results obtained in Table 5-2, the apparent \( K_M \) value of the immobilized lipase (3.16
(2.4 mM). The parameter $v_{\text{max}}$ stands for the maximum reaction rate in infinite substrate concentration. It was found that $v_{\text{max}}$ for immobilized lipase was much smaller than the free lipase (5.16 $\mu$mol pNPP.min$^{-1}$. (mg lipase)$^{-1}$ for the immobilized lipase compared to 153.76 $\mu$mol pNP min$^{-1}$. (mg lipase)$^{-1}$ for the free lipase), if the enzyme loads of 4.29 mg/g cloth is considered (see Section 4.4). There are several reasons why different $v_{\text{max}}$ was observed with an enzyme immobilized onto the PEI coated surface via GA activation relative to the free enzyme. Firstly, the presence of GA could cause some conformational changes in the enzyme molecules. Secondly, the immobilized enzyme is located in an environment different from that when it is in the aqueous solution. As discussed in the literature review (Section 2.2.1.3), lipase from *pseudomonas fluorescens* has a strong tendency to be interfacially activated. In the aqueous solution containing pNPP microemulsion, lipase can be easily activated through absorption on the surface of emulsion droplet. However, the chance of immobilized lipases on the surface of woollen cloth to contact the hydrophobic surface of pNPP emulsion is less. Therefore it is possible that most of the immobilized lipase is in an inactive status, resulting in representing a reduced $v_{\text{max}}$. Thirdly, there is a partitioning of substrate between the solution and support. The substrate concentration in the neighbourhood of the immobilized enzyme may be tremendously different from the bulk solution because the substrate droplets have to reach the cloth surface before the immobilized lipase can use them, while the free lipase have more access to the substrate in substrate droplet where the lipases have been already adsorbed on[209]. The high $K_M$ for the immobilized lipase might also be related to such a substrate partition, as the free lipases have more access to the substrate than the immobilized one.

The $K_M$ and $v_{\text{max}}$ values reported in the literature are listed in Table 5-3. It is noticed that the kinetic parameters even for the same type of lipase, varies with substrate and immobilization protocol used during the kinetic study. Generally speaking, for enzyme immobilization by physical methods, the difference of the kinetic parameters between the free and immobilized enzyme is not large, as shown in references [167, 210]. However, for enzyme immobilization via chemical methods, the values of $v_{\text{max}}$ of immobilized enzyme decreased compared to the free one as observed in the Table 7 due to the chemical reaction involved, as in reference [211, 212]. For enzyme immobilization occurring on a surface, the mass transfer hindrance is not important, the $K_M$ change between the free and immobilized enzyme is not obvious, as shown in the results from references [211, 212]. Therefore, the decrease in $v_{\text{max}}$ and similar value in $K_M$ for the immobilized derivative prepared in the current research are in keeping with these trends observed from literature.
### Table 5-3 Comparison of kinetic parameters of immobilized lipase from literature

<table>
<thead>
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<th>Lipase name</th>
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<th>Immobilized lipase</th>
<th>Ref</th>
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<td></td>
<td>$K_M$</td>
<td>$v_{max}$</td>
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<tr>
<td>C. Rugosa</td>
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<td>1469</td>
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<td></td>
<td></td>
<td></td>
<td>1.8</td>
<td>325\textsuperscript{1}</td>
<td></td>
</tr>
<tr>
<td>C. Rugosa</td>
<td>Adsorbed onto PP membrane</td>
<td>p-nitrophenyl acetate</td>
<td>2.7</td>
<td>39.2</td>
<td>2.6</td>
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<tr>
<td>C. Rugosa</td>
<td>Adsorption onto porous support</td>
<td>Palm oil</td>
<td>8.2</td>
<td>1.3</td>
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<td></td>
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<td>8.3</td>
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<tr>
<td>C. Rugosa</td>
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<td>pNPP</td>
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<td>18.0</td>
<td>96.1</td>
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<tr>
<td>C. Rugosa</td>
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<td>pNPP</td>
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<td>4</td>
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<td>C. cylindracea</td>
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<td>1.8</td>
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<td>1.1</td>
<td>0.083</td>
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<tr>
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<td>Olive oil</td>
<td>4.8</td>
<td>0.373</td>
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<td>pNPP</td>
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<td>C. Rugosa</td>
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<tr>
<td>P. fluorescens</td>
<td>CVB on to PPA\textsuperscript{8}</td>
<td>Olive oil</td>
<td>329±99</td>
<td>26965±2343</td>
<td>401±14</td>
</tr>
<tr>
<td></td>
<td>activated by GA</td>
<td></td>
<td>401±14</td>
<td>633±68 7</td>
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<td>P. fluorescens</td>
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<td>293±58</td>
<td>1169±627</td>
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</table>

Unless mentioned, the unit for the free and immobilized lipases: $K_M$ is mM and $v_{max}$ is µmol substrate (mg protein. min)$^{-1}$(for the immobilized lipase, the $v_{max}$ is based on its protein load).

Note 1: $v_{max}$ unit for immobilized lipase is µmol pNPP (mg support. min)$^{-1}$

Note 2: $v_{max}$ unit for the free and immobilized lipase are mmol.L$^{-1}$.h$^{-1}$

Note 3: unit of $v_{max}$ for the free and immobilized lipase are µmol. Min$^{-1}$.L$^{-1}$

Note 4: unit of $v_{max}$ for the free and immobilized lipase are U.mg$^{-1}$

Note 5: unit of $v_{max}$ for the free and immobilized lipase are U.mg$^{-1}$

Note 6 unit is µmol pNPP.min$^{-1}$.g chitosan$^{-1}$

Note 7: unit of $v_{max}$ for the free and immobilized lipase are U/ mg lipase and U/mg support respectively.

Note 8: PPA refer to polysiloxane–polyvinyl alcohol.
5.5 Conclusions

Overall, the findings of this chapter can be summarized as:

- The pH profile for the free and immobilized lipase was almost the same, suggesting that microenvironment between the free and immobilized lipase was not considerably different.

- The lipase-immobilized woollen cloth exhibited excellent oily stain removal ability: after being stained with olive oil and stored for one day in air at room temperature, the oily stain could be easily removed by 0.05 M pH 8.5 Tris buffer without any detergent addition. This enhanced cleaning was stable also, since after the cloth was stored in air for almost one month, similar cleaning performance was observed.

- The immobilized lipase was more stable in Tris buffer than in the air. However compared to free lipase counterpart, enhanced stability was noticed for the immobilized lipase.

- The kinetic behaviour of both the free and the immobilized lipase follows the M-M model. The $K_m$ of the immobilized lipase is almost as same as that of the free lipase. The maximum velocity ($v_{max}$) changes from 153.76 $\mu$mol pNP min$^{-1}$ (mg lipase)$^{-1}$ for the free lipase to 5.16 $\mu$mol pNPP.min$^{-1}$.g cloth$^{-1}$, suggesting that the activity of the immobilized lipase decreases due to immobilization. However this could be attributed to the change in the surrounding environment for the immobilized lipases. Further work is needed to establish this.
6. Improvement of CVB Protocol for Lipase Immobilization

Lipases have been successfully immobilized onto woollen cloth by the benchmark CVB protocol (Section 4 and Section 5). However, this protocol has room to be improved since:

- The protein loads were only 4.29 mg/(g cloth) (see Section 4.4). Compared to Table 4-1, this value is only in the low end for enzyme immobilization via chemical methods.

- ESEM images showed that this was because there was incomplete coverage of the wool fibres and the PEI /enzymes were present in clusters across the fibres, rather than a continuous coating. Therefore immobilization protocols that can produce a more complete and continuous coverage need to be found.

Thus in this chapter, various methods were developed to improve the immobilized activity and try to identify the bottleneck which had previously prevented an increase in protein loads. These methods are:

- Lipase immobilized by cross-linking method (Section 6.1)
- Lipase immobilization by GA oligomer (Section 6.2)
- Adoption of a mild chlorination process in immobilization (Section 6.3)
- Application of PEI-polyaldehyde in lipase immobilization (Section 6.4)

6.1 CVB protocol variations: Lipase Immobilization by Cross-linking Methods (CRL)

The cross-linking method (CRL) and covalent binding method (CVB) for enzyme immobilization are very similar (see Section 2.3). The difference between these two approaches lies in the variation in treatment sequence used. If PEI is chemically fixed onto woollen cloth by GA before the addition of lipase, this method is called “covalent binding”. However, if PEI and lipase is adsorbed onto woollen cloth first then followed by GA cross-linking, this method is defined as “cross-linking” (CRL). In this section, the factors that affect lipase immobilization by the “cross-linking” method
are investigated, in order to identify if this protocol can produce a more complete and continuous coverage of PEI and enzymes over the wool fibres and achieve a higher immobilized activity.

**6.1.1 Is Free Enzyme Stabilised by PEI in GA solution?**

According to a previous comparison between the CVB and CRL protocol [216], CRL immobilization may result in a more complete enzyme coverage on immobilization support than CVB immobilization, but a higher immobilized activity may be a challenge. It was due to the fact that the CVB immobilization restricted the chance of chemical modification of enzyme only to those groups involved in immobilization and only the fraction of lipases in solution which had the chance to contact the aldehyde sites on wool could be affected, since the GA was used before the enzyme was added. This resulted in less enzyme deactivation. In contrast, for the CRL protocol, all of the enzymes that are adsorbed on the PEI may be affected, because they are all exposed to the GA solution. This might lead to greater enzyme deactivation than in CVB. To verify the previous conclusions, prior to discussion of immobilization via cross-linking protocol, the deactivation of lipase on PEI in aqueous solution containing GA was investigated.

Lipase (2.5 mg.ml\(^{-1}\)) was added into 3 ml of 2% PEI solution containing 0.25% fresh GA, the activity of lipase in solution was tested by pNPP assay (Section 3.2.3). As a comparison, lipase (2mg. ml\(^{-1}\)) was added into 0.25% GA solution at pH 8.5 without any PEI addition, and the residual activity of lipase in this solution was tested periodically using the pNPP assay. Refer to Figure 6-1 for the experimental results.

Figure 6-1 shows that, as expected, the lipase lost activity very rapidly in GA solution. Within 20 min, more than 90% of lipase activity was lost. The GA clearly deactivated the enzyme. This result is confirmed in the literature [3]. However, in the presence of PEI in the GA solution, the drop in lipase activity was substantially decreased. In other words, PEI can act as a protecting agent, reducing lipase deactivation in the presence of GA.

The protecting ability of PEI to enzyme activity has been discussed in the literature review (see Section 2.4.1.2). The work in reference [113], explained this is due to the electrostatic adsorption of enzyme on charged PEI. However, the results in Figure 6-1 perhaps also suggest that PEI reacts with the GA, and therefore could decrease the toxicity of the GA to lipase, reducing the amount of enzyme deactivation. In enzyme cross-linking, auxiliary proteins, such as BSA (bovine serum
albumin), are often used to protect enzyme against cross-linker attack as a sacrifice protein [79, 104]. Similarly, PEI on this occasion may play the same role in lipase cross-linking, avoiding excessive enzyme activity loss. As the primary amine content in PEI is 25%, which is much higher than the amine content on enzymes, the reaction between GA and PEI should be much faster than that between GA and enzymes. Consequently, PEI-polyaldehyde (product formed between PEI and GA, see the definition Section 6.4.1) was formed first. When excessive PEI is present, it is possible that PEI consumes GA before it reaches the enzymes. In this circumstance, the aldehyde groups exist in the form of PEI-polyaldehyde, rather than in a free form in aqueous solution. As a macromolecule, the toxicity of PEI polyaldehyde to enzyme should be much weaker than the free GA, since polyaldehyde is only able to attack enzyme under certain spatial position due to its electrostatic charges.

![Graph](image)

**Figure 6-1** free lipase activity changes in PEI solution containing GA (0.25 % GA and 2% PEI) represent in “black diamond”, compared to the activity change of lipase solution containing GA only (0.25%) represent in “ red triangle”, unit of specific activity is $\mu$ mol pNP. min$^{-1}$ (mg lipase)$^{-1}$. The error bar here is the mean ± one standard deviation.

This hypothesis is confirmed by the fact that the protection ability of PEI to lipase was related to GA concentration as shown in Figure 6-2. The more GA is added, the lower is the lipase activity retained. However, the results in Figure 6-1 and Figure 6-2 also suggest that, after a substantial
activity drop caused by GA addition, the lipase is reasonably stable in the solution containing GA that only slightly activity loss exhibits during storage period at room temperature.

![Graph showing specific activity of lipase in various GA concentrations](image)

**Figure 6-2** Change of free lipase activity in various concentrations of GA solutions containing PEI additive, unit of specific activity is μmol pNPP.min⁻¹. (mg lipase)⁻¹. The value here is the mean of 3 independent test results and error bar represent mean ± standard deviation.

In summary, a decrease in lipase deactivation in the presence of PEI, when GA was added into lipase solution, is highlighted in this section. Based on this feature, it is possible to adsorb PEI and lipase onto the cloth first, followed by GA treatment to attain this structure. In the following sections, the attempt to immobilize lipase through cross-linking protocol and the factors affecting this protocol are discussed.

### 6.1.2 Treatment Time and pH in GA Solution to CRL

Lipase immobilization was carried out by the protocol described in Section 3.2.3 and the detail is as follows: PEI adsorbed woollen cloth was soaked in lipase solution (2 mg.ml⁻¹) overnight. Then lipases were immobilized by soaking in 0.1% of GA solution for various length of time. Variation of immobilized activity at pH 6 and pH 7 was compared in Figure 6-3.
Figure 6-3 Treatment time in GA to immobilized activity by CRL method (value are means of three independent replicates. Error bar represent mean ± standard deviation), unit of specific immobilized activity is μmol pNPP.min⁻¹ . (g cloth)⁻¹. Error bar represents mean ± standard deviation.

To further illustrate the effect of pH to CRL immobilized activity, the cloth prepared above was treated in 0.1% of GA solution diluted with various buffer solutions for 5 minutes. The immobilized activity is illustrated in Figure 6-4.

Figure 6-3 and Figure 6-4 show that the treatment time and pH in GA solution strongly affect the immobilized lipase activity. Cross-linking is a fast reaction, considering the time needed for mass transfer, and so 1- 5 minutes is enough to complete the fixation. Further extending the soaking time in GA solution will cause unnecessary activity loss. Figure 6-4 suggests that a higher immobilized activity is obtained when the immobilization is carried out in buffer solutions at a lower pH. The immobilized activity is reduced when the pH used for immobilization is increased from pH 6 to 8. However, further increase in the pH (up to pH of 10) will not result in more decrease in immobilized activity.

GA is known to be a toxin for enzymes (see Section 2.4.2). Higher pH and excessive treatment in GA solution would deactivate the immobilized enzyme and result in a lower immobilized activity. As GA demonstrates higher activity in alkaline pH [16], a higher rate of lipase deactivation occurs
when immobilization is carried out in alkaline GA solution. On the other hand, if the pH used for immobilization was lower than or close to the isoelectric point of lipase, the interaction between enzymes, PEI and woollen cloth would be affected. For this, the charge carried by lipase will be the same as PEI or the lipase does not have enough opposite charge; less lipase would be adsorbed onto the PEI spacer, resulting in poor immobilization. The isoelectric point for lipase from *Pseudomonas fluorescens* and wool is 4.46 [47] and 2.5-3.5 [19] respectively. It can be deduced that this reduction in lipase adsorption will become worse when the pH is close to 5. Thus a pH around 6 was adopted in CRL immobilization.

![Figure 6-4](image.png)

Figure 6-4 Effect of GA pH to the immobilized lipase activity by the CRL protocol, $\mu$mol pNP. min$^{-1}$. (g cloth)$^{-1}$

It was noticed that the GA cross-linking is a fast reaction and extending the reaction time would not increase the immobilized activity. Due to the toxicity of GA to the enzymes, this may deactivate the enzyme. This lipase deactivation from excessive treatment in GA solution can also be observed in the results presented in Figure 6-2, where it is shown that residual lipase activity in PEI/GA mixture decreased with an increase in GA concentration. This result suggests that the protection function of PEI to lipase is related to GA concentration. However, since excess GA remains in solution during the immobilization via the CRL protocol, extending the treatment time in GA solution will therefore lead to more enzyme deactivation. Similar pH and treatment time dependency in GA solution for
immobilized activity was also reported by Nedim Albayrak and Shang-Tian Yang [203] for galactosidase immobilization on PEI/GA treated cotton cloth.

Consequently, for all subsequent preparations, a GA solution at pH 6 was used and the reaction time in the GA solution was 5 minutes. Unfortunately, compared to the benchmark CVB protocol, there is no improvement for immobilized activity: the optimal activity for CRL is around 4 µmol pNPP.min⁻¹.(g cloth)⁻¹ (Figure 6-3), but the optimal value for CVB is around 5 µmol pNPP.min⁻¹.(g cloth)⁻¹ (Section 4.3.2.2).

6.1.3 Evaluation of the Immobilization Procedure: Visual Analysis of the CRL immobilized Lipase

The sample used for ESEM image is prepared by the method outlined in Section 2.3.2.3. The activity of the lipase-immobilized cloth was continuously tested by pNPP assay solution until the reading of immobilized activity became stable (i.e. all but the chemically attached enzymes remained, and the leaked activity was close to zero). The ESEM images of this lipase-immobilized woollen cloth were taken.

The ESEM images for the lipase-immobilized area are illustrated in Figure 6-5 and Figure 6-6. In these images, the immobilized lipase suspect together with its associated PEI suspect can be clearly seen, which confirmed the results obtained from pNPP activity assay: lipases have been immobilized onto woollen cloth successfully. The suspect of CRL immobilized lipases was found to be concentrated only in certain area, similarly to those taken from CVB immobilized-cloth. But the appearance of the CRL-immobilized lipases is dissimilar to those immobilized by the CVB protocol (comparing Figure 4-9 with Figure 6-5 and Figure 6-6). As shown in Figure 6-6, the strong tendency of cross-linking between PEI resins that was present in the ESEM image from CVB protocol shown in Figure 4-9 is reduced. Instead, some of the cross-linked PEI suspect together with the bound lipase suspect can be found in a grainy and stringy appearance on cloth surface, as well as the nodded clusters or polymer concentrated in certain area on the cloth. This probably can be ascribed to the variation in the immobilization sequence: PEI is reacted with lipases first prior to wool fibre in CRL protocol, whereas PEI cross-linking to wool fibre is in priority in CVB protocol.

The bare wool surface can be clearly viewed in these ESEM images, especially in view C & D in Figure 6-5. Some structures were just loosely attached to the wool surface, the cross-linked coating (probably from PEI) only existing in part of the cloth surface (see Figure 6-7). This might be due to
some of PEI with its associated lipase being washed away during the immobilization steps. This kind of wash off was also addressed by Albayrak and Shan-Tian Yang on galactosidase immobilization on cotton [80]. They recommended that the consecutive washing of PEI coated cloth would lead to removal of loosely bound PEI resin and form a monolayer of immobilized enzymes, resulting in low immobilization yield. However, in this study it was found that not only the washing step after PEI adsorption, but enzyme soaking as well as GA cross-linking might all accelerate the PEI removal and result in low immobilized activity. The continuous layer of PEI coating, which is similar to the one shown in the ESEM image of the CVB protocol, can also be observed as shown in Figure 6-6. But the cluster structure covered on this PEI coating that appeared in ESEM images from the CVB protocol cannot be found in the image from the wool treated using the CRL protocol.

From the ESEM images of wool treated by the CRL protocol, it was also noticed that PEI tended to cluster in the areas that was badly etched during chlorination. As shown in Figure 6-6, a cluster of stringy materials, which are most likely to be a sign of cross-linked PEI and the bound lipases, agglomerate around one piece of fibre. This fibre looks to be badly etched. On the other fibres around, whose structures seem not to be as badly damaged as this, only few or even no stringy clusters are present. The enrichment of PEI and enzyme complex at defective sites on the fibre had also been reported by Yudanova et al. [217] on immobilization of protease C through GA and PEI treatment on fibre material of varied chemical nature: cellulose, polyester and polycaproamide. This phenomenon could be due to the easier accessibility of PEI/lipase complex on this defective area of woollen cloth. Compared to the cortex inner layer, the outer layer of wool is more cross-linked and much more resistant to chemical attack. As shown in Figure 6-6, the scale structure normally present in bare wool fibre has disappeared on this lipase-enriched part, suggesting that the outer surface of this fibre might have been removed entirely and PEI/lipase complex was fixed on the cortex of the wool. This ESEM image observation indicates that the wool surface layer is unsuitable for enzyme immobilization by the CRL protocol. If this layer was either removed or modified, a better CRL immobilization would be achieved.

Although the coverage of PEI and enzymes is less than CVB as shown in the ESEM images, it is noticed that both of the protocols produce similar levels of immobilized activity (see Section 6.1.2 and Section 4.3.2.2). This could be related to the protection function of PEI to lipase activity, which has been addressed in Section 6.1.1 previously, that the presence of free PEI in aqueous solution could minimize the toxicity from GA to lipase.
Figure 6-5 ESEM image of lipase-immobilized area on woollen cloth prepared by the CRL protocol. The original magnifications from view A to view F are 500×, 2000×, 8000×, 16000×, 40000× and 80000× respectively.

Immobilization condition: Chlorinated woollen cloth, which was stored in 2% PEI solution at pH 8, was soaked in 2 mg ml⁻¹ lipase solution pH 8.0 for 1 day, then cross-linked in 0.1% of GA solution prepared in 0.1 M phosphate buffer at pH 6.0 for 5 min. The images were taken after consecutive activity assay and all loss-associated lipases were washed off. View A is a general view of cloth fibre. In view B, a stingy structure can be observed on one of the cloth fibre. This structure is further enlarged in the view of C, D E, and F. In the high magnification image of view E and F, it can be found that the stringy structure is further composed of some fine grainy components, which most likely could be the sign of immobilized lipases.
Figure 6-6 ESEM image of immobilized lipase concentrated in defect area on wool fibre, the original magnifications from view A to view D are 500×, 1000×, 5000× and 10000× respectively. Immobilization condition: Chlorinated woollen cloth, which was stored in 2% PEI solution at pH 8, was soaked in 2 mg ml⁻¹ lipase solution pH 8.0 for 1 day, then cross-linked in 0.1% of GA solution prepared in 0.1 M phosphate buffer at pH 6.0 for 5 min. The images were taken after consecutive activity assay and all loss-associated lipases were washed off. Similarly to images above, view A is the general view of the cloth surface. In view B, one of badly etched wool fibre is highlighted, which is covered with some nodded structures. These structures are more closely viewed in the high magnification views of C and D.
Figure 6-7 ESEM image of non-lipase immobilized area on lipase immobilized woollen cloth prepared by the CRL protocol. The original magnifications from view A to view D are 500×, 2500×, 5000× and 20000× respectively. Immobilization condition: Chlorinated woollen cloth, which was stored in 2% PEI solution at pH 8, was soaked in 2 mg ml⁻¹ lipase solution pH 8.0 for 1 day, then cross-linked in 0.1% of GA solution prepared in 0.1 M phosphate buffer at pH 6.0 for 5 min. The images were taken after consecutive activity assay and all loss-associated lipases were washed off. View A is the general view of the cloth surface. In view B, one of the fibre covered with a layer of sticky coating is highlighted. This film of coating is further detailed in the high magnification image of view C and view D.

6.1.4 Proposed CRL Immobilization Mechanism

As discussed above, the CRL protocol produces similar immobilized activity as the CVB protocol. Previously, Albayrak and Shan-Tian Yang [80] had suggested a CRL immobilization mechanism for galactosidase immobilization onto cotton cloth with PEI as a spacer arm. Combining this mechanism with the result in the current work, the following mechanism for CRL immobilization is proposed: lipase is initially adsorbed onto PEI covered woollen cloth, then cross-linked with PEI and active
amine residue on the surface of woollen cloth in the presence of GA solution. This process is split into the following steps as shown in Figure 6-8:

(1) PEI adsorption

Similar to CVB, positively charged PEI polymer is physically adsorbed onto wool surface through electrostatic forces.

(2) Lipase adsorption

In the next step, enzyme is adsorbed onto PEI coated woollen cloth from solution. PEI and enzyme are all physically associated with the cloth. In this step, the enzyme solution may wash off some of the adsorbed PEI from the wool surface, and is one of the contributing factors resulting in the poor coverage of PEI and enzyme in the ESEM images.

(3) Cross-linking

In the final step, physically adsorbed PEI and enzyme are chemically fixed onto woollen cloth by GA to complete the immobilization. Also, the cross-linking between PEI-PEI and lipase-lipase might exist as shown in Figure 6-8. From the ESEM images (Figure 6-5, Figure 6-6 and Figure 6-7), it can be seen that only a portion of the PEI and GA can be effectively linked onto woollen cloth and become chemically immobilized. The others could be washed off from the wool resulting in poor coverage of the chemically bound enzymes, as the adsorbed lipases would reduce the electrical interaction between wool and PEI.
6.1.5 Conclusions

In summary, the CRL method of immobilization is inferior to the previously developed CVB method (Chapters 4 and 5). This was demonstrated by:

- Similar immobilization activity being achieved when comparing the immobilized activity between the CRL and CVB protocol, as shown in Section 4.3.2.1 and 6.1.2.
- Poor retention and coverage of enzymes on the wool as shown in Section 6.1.3.
- It was shown that lipase deactivation can be reduced when GA was added into lipase solution in the presence of PEI.

6.2 CVB protocol variation: GA Oligomer

GA is the most popular cross-linker in much biotechnology work, despite the great number of disadvantages it presents (as has been further demonstrated in the preceding work). It was found that
its cross-linking reaction changes with different pH and GA concentrations (as demonstrated in Section 4.3.2.2 in this work and the following references [79, 130, 218]). This is because aqueous GA has multiple components present: monomer, cyclic hemiacetal and oligomers, as addressed in Section 2.4.2.1 of the literature review. The reaction mechanism for monomers and oligomers is thought to be different: the monomer is thought to follow a Michael addition and the oligomer is thought to follow stabilized Schiff’s base reaction or a Michael adduction (See Section 2.4.2.1), as the existence of resonance in GA oligomer stabilizes the Schiff’s base product. In fact, GA oligomer, which is formed under high GA concentration and alkaline pH [131], is regarded to be a far more effective cross-linker than monomer GA. GA oligomer formation can be accelerated via variation of the temperature, pH and treatment time in GA solution [138, 219].

In the current study, it was noticed that the pH in GA solution showed strong influence over the immobilized activity (see Section 4.3.2). This might be related to formation of GA oligomer in an alkaline pH, considering the accelerated formation of GA oligomer in that pH range. Thus, in this section, the influence of different forms of GA on immobilization is investigated. The UV spectrophotometer is a simple but useful tool to study the variation of GA components in aqueous solution, so the UV spectra of various form of GA was examined and the result is shown in Appendix 10.6.

### 6.2.1 pH on GA Oligomer Formation and its Affect on lipase Immobilization

GA oligomer was prepared by the method outlined in Section 3.2.6. The change of UV spectra during the preparation of the GA oligomer is shown in Appendix 10.6. The lipase was immobilized onto wool using the benchmark CVB protocol described in Chapter 4, GA oligomer was used instead of freshly prepared GA solution. The activity of these immobilized preparation was then tested using the pNPP assay. The resulting relationship between immobilized activity and pH of the GA oligomer solution is present in Figure 6-9. This shows that the activity of the immobilized lipase prepared from GA oligomer solution at pHs of 9.0, 9.65 and 10.45 are higher than at pH 8 and 7 (1.6 fold for pH 11, 1.67 fold for pH 9.65 and 1.74 fold increase for pH 9.0 over the activity obtained in pH 8 buffer solution). Similar to the trend of the A235/A280 shift at various pHs (shown in Figure 10-6 of Appendix 10.6), the immobilized activity enhancement occurred in a more alkaline buffer. Combining the results in Appendix 10.6, the conclusion is that this immobilized activity increase in alkaline pH is related to the formation of GA oligomer. By the means of GA oligomer, higher immobilized activity is expected. Compared to the optimal value of lipase immobilized by the CVB benchmark protocol described in Section 4.3.2, about 40% of activity increase is observed.
(As shown in Figure 4-7, optimal activity was found at 5 µmol pNPP. min$^{-1}$. (g cloth)$^{-1}$, however the best activity for lipase immobilized cloth via the GA oligomer protocol occurred at 7 µmol pNPP. min$^{-1}$. (g cloth)$^{-1}$).

This optimal activity at pH 9 (Figure 4-7) that occurred in the benchmark CVB protocol might also be related to the formation of GA oligomer. From the results shown in Figure 10-4 of Appendix 10.6, it can be found that the enhancement at A 235, which is related to the formation of GA oligomer, appeared only after 10 minutes mixing of GA in a buffer solution at pH 9. In other word, GA oligomer can be produced in a buffer solution at an alkaline pH even in a short time. The heating and extended treatment time will strengthen the formation (Appendix 10.6). While the reason for activity reduction at more alkaline buffer (higher than pH 9) could be due to another factor in enzyme immobilization via PEI as spacer arm: the electrostatic charge carried on PEI is reduced at high alkaline solution, thus less PEI would be adsorbed onto cloth resulting in relatively low immobilized activity at that pH.

![Figure 6-9 pH of preparation of GA oligomer to immobilized activity; value showed is the mean of three replicates. The error bar represents the mean ± standard deviation.](image)

The ESEM images of this lipase-immobilized cloth shown in Figure 6-9 (prepared at pH 9.0 buffer solution) are shown in Figure 6-10.
Figure 6-10 ESEM image of lipase immobilization on woollen cloth via the GA oligomer protocol. Top: CVB immobilized cloth using GA oligomer prepared in the pH 9 buffer solution shown in Figure 6-9, magnification 1000x. Bottom: enlarged view of the “top” image, magnification 4000x. ESEM setup: HV=5.0 kv, Spot=3.0, WD=5.8 mm, pressure=0.6 mbar.
6.2.2 Effect of Treatment Time in GA Oligomer Solution on Immobilized Activity

In the benchmark CVB protocol, where freshly prepared GA solution (Section 4.3.2) was used, it was found that there was little improvement of the immobilized activity when the treatment time in GA solution was extended from 1 min to 15 min. As discussed in Appendix 10.6 (Figure 10-4), GA oligomer can be formed at an alkaline pH at room temperature. To investigate further, GA oligomer was prepared by adjusting the pH of 12.5% GA solution (fresh solution) to 9 with sodium carbonate and aging at 25°C for 1 day. Lipase was immobilized by the benchmark CVB protocol described in Chapter 4, except using GA oligomer rather than the freshly prepared GA solution. The time that the wool with adsorbed PEI spent in this GA oligomer solution (referred to as ‘treatment time’) was varied from 1 to 15 minutes. The resulting relationship between the treatment time in GA oligomer solution and the activity of the immobilized lipase is given in Figure 6-11.

![Figure 6-11 Treatment time of lipase immobilized cloth in GA oligomer solution to immobilized activity](image)

As illustrated in Figure 6-11, higher activities can be achieved with longer treatment time in GA oligomer solution. Specific activity reached its maximum in 10 minutes after which the activity plateaus. This differs
slightly to the results obtained using freshly prepared GA solution (Figure 4-7), where the immobilized lipase activity was found to be independent from the treatment time in GA solution (see Section 4.3.2.2).

### 6.2.3 Immobilization Reaction Mechanism of CVB with GA oligomer

Similarly to the CVB immobilization protocol illustrated in Figure 4-12, the proposed mechanism on lipase immobilization via the CVB protocol by the means of GA oligomer is illustrated in Figure 6-12. This immobilization is split into the following steps:

1. **CHO–CHO + CHO–CHO → CHO CHO CHO**
2. **Wool +PEI → Wool**
3. **Wool + CHO CHO CHO → Wool**
4. **Wool + Enz → Wool**

**Figure 6-12 Proposed immobilization mechanism for CVB with GA oligomer**
(1) Preparation of GA oligomer

GA oligomer was prepared according to the methods outlined in Section 3.2.6.

(2) PEI adsorption to woollen cloth

PEI was adsorbed onto negatively charged woollen surface.

(3) Functionalization of the cloth surface

Instead of freshly dilute GA solution, GA oligomer was used for PEI cross-linking onto wool surface, as well as PEI surface activation. Since GA oligomer contains multi-aldehyde residues, the aldehyde density on PEI should be more than the one treated by GA fresh dilution.

(4) Lipase immobilized on woollen cloth

In this step, same as the CV B mechanism outlined in Section 4.6, GA activated cloth was soaked in lipase solution. These lipases were firstly adsorbed onto a cloth surface, and then reacted with the aldehyde residues on the cloth surface to complete the immobilization. As more aldehyde residues are present on cloth surface, the immobilized activity should also increase. However, since GA oligomer is a stronger cross-linker than GA monomer, which could cause a greater reduction in lipase activity (GA oligomer reacts in a different mechanism from GA monomer: GA oligomer following a stabilized Schiff’s base reaction, whilst GA monomer follows a Michael addition mechanism [16]). In order to obtain the maximum activity improvement, conditions on the immobilization and GA oligomer preparation should be optimised.

6.2.4 Conclusions

A better immobilized activity than the benchmark method was obtained when more GA oligomer was present in GA solution compared to immobilization with freshly prepared GA solution, as GA oligomer is a more efficient cross-linker. The optimum immobilization activity for lipase immobilization was at 1% GA oligomer solution prepared in alkaline buffer of pH 9.0 treated in a water bath at 60 °C for 30 min, which show 40% activity increase compared to the optimal immobilized activity achieved by the CVB protocol using freshly prepared GA solution (from 5
µmol pNPP. min⁻¹. (g cloth)⁻¹ in the CVB protocol to 7 µmol pNPP. min⁻¹. (g cloth)⁻¹ in the GA oligomer protocol).

6.3 Variation of Chlorination Method for Immobilization

As addressed in Section 4, the chlorination step used is too aggressive, resulting in an uneven wool fibre etch due to the low pH (pH 1-2) in chlorination solution (The chlorination reaction is accelerated at a low pH, probably due to more chorines created). Thus a more moderate and slow chlorination procedure (“high pH process” as mentioned in Section 3.2.1) was adopted in this section. After chlorination was completed, lipase was immobilized via the CVB protocol and the ESEM images were taken and shown in Figure 6-13.

![ESEM image of lipase immobilization via the CVB protocol by “high pH process” of chlorination.](image)

Figure 6-13 ESEM image of lipase immobilization via the CVB protocol by “high pH process” of chlorination. The magnification of image A, B, C, D are 500 ×, 2500 ×, 5000 × and 10,000 × respectively. Image A is the overall view of the chlorinated wool fibre, and image B, C and D focus on one single wool fibre to demonstrate the detail of fibre etching from the chlorination process.
Compared to the wool chlorination (“low pH process”) shown in Figure 4-5, there is less wool structure damage from the chlorination present in Figure 6-13. As addressed in Section 3.2.1, the amount of chlorine in the wool industry used is scaled on the basis of weight of woollen fibre (owf), instead of the normal concept of solution concentration. Hence, the DCCA concentration was changed between 5 % owf and 50 % owf. The influence of the chlorination on the immobilized lipase activity is illustrated in Figure 6-14.

![Figure 6-14](image.png)

Figure 6-14 Average trend line of degree of chlorination to specific immobilized activity. Each point is the mean of three replicates, error bar represent the mean ± standard deviation.

As shown in Figure 6-14, a U-shape trend line was observed between the DCCA concentration and immobilized activity. At a low chlorine concentration, the immobilized lipase decreased with the increase in the chlorine concentration, until reaching 30% owf (weight of fabrics). Further increasing chlorine concentration, the immobilized activity improved again, but the wool structure suffered severe damage. In the extreme case, the wool was totally ruined and lost its property as a fibrous material (results not presented). Therefore, excessive chlorination should be avoided. Chlorination in 5 %
owf (weight of fabric) DCCA for 30 min is enough to activate the wool. Higher concentration and longer treatment time even result in decreased immobilized activity.

From the above results, it can be seen that it is necessary to use a mild chlorination process to retain some of the cuticle layer. The purpose of chlorination in immobilization is just to reduce the hydrophobicity and improve PEI adherence. In immobilization, most likely the cuticle layer serves two functions: firstly, the existence of a thioester link makes the wool resistant against chemical attack and reduces alkaline solubility (the cortex layer of wool is more soluble in alkaline solution [19]); secondly, the cystein residue is able to react with glutaraldehyde and enhance the linkage with PEI and wool [155, 156]. Therefore maintaining the integrity of cuticle layer would benefit the immobilization to some extent. The reduction in immobilized activity from dissolved wool protein can be lowered. Thus, for the chlorination step in enzyme immobilization, it is better to confine the effective treatment to the surface layer of the fibre, meaning that excessive chlorination must be avoided.

Consequently, it can concluded that 5% owf of DCCA is enough to generate a good immobilization. The chlorination under “high pH” is able to reduce the excessive chlorine-etching present in “low pH process” of chlorination and benefit the integrity of the woollen cloth. This new chlorination protocol was then used in the following experiment.

6.4 CVB protocol variation: PEI-Polyaldehyde

In this section, the CVB treatment sequence was further altered, by first reacting PEI and GA together in aqueous solution to produce a product called “glutaraldehyde-reacted polyethyleneimine”[220]. However, the nomenclature given in reference [220] does not describe this product properly, thus in this thesis, it was renamed as “PEI-polyaldehyde”. The advantage of using this PEI-polyaldehyde in immobilization is that some of free aldehyde groups still exist on the PEI backbone after PEI is reacted with GA to form a Schiff’s base product, rather than consumed entirely by the intermolecular interaction between amine groups on the PEI molecule and GA [220]. Moreover, the reactivity of this aldehyde residue is weaker than GA in its free form [217]. Thus, when this polyaldehyde is adsorbed onto woollen cloth, following up a soaking in lipase solution to complete the immobilization, it may result in a reduction of the lipase activity loss. Furthermore, the excessive cross-linking between PEI and PEI in the presence of GA, resulting in few free aldehyde
residues on PEI backbone available for enzyme immobilization appeared in the CVB protocol could be avoided. The details are discussed in the following sections.

6.4.1 Lipase Immobilization by PEI-Polyaldehyde

It has been established that PEI-polyaldehyde (see Appendix 10.6) contained free aldehyde residues. Therefore, when used in the CVB protocol, in theory it should reduce the internal PEI-PEI cross-linking and increase the activity of the resulting immobilized lipases. In this section, this sort of lipase immobilization via PEI-polyaldehyde is investigated. A range of factors, including PEI and GA solution concentration, solution pH, enzyme concentration and PEI aldehyde and treatment time, were tested.

Experiments showed that lipase was able to form aggregates with the PEI-polyaldehyde. For PEI polyaldehyde, if the concentration of GA used in PEI polyaldehyde was high enough (i.e. higher than 0.5 % GA) and stored long enough (for more than one day), concentrated lipase solution (e.g. 10 mg ml⁻¹ lipase solution) prepared in pH 8.0 phosphate buffer was able to precipitate this polyaldehyde solution out. It should be noted that this would never happen in pure PEI solution. GA concentration and treatment time in GA solution during PEI aldehyde formation was found to be important factors for producing lipase –polyaldehyde aggregate. It was hard to obtain this precipitate in a lower GA concentration solution, (e.g. 0.1% GA solution). While precipitate appeared when the GA concentration used for PEI-polyaldehyde preparation was higher than 1%. Moreover, lipase-polyaldehyde aggregate can only form when this polyaldehyde solution had aged for more than one day. Unfortunately no lipase activity was observed in this precipitate. In addition, this precipitate could not be redissolved in DI water. The formation of this aggregate suggested that the electrostatic charge distribution in this PEI-polyaldehyde had changed and was different from free PEI due to GA modification. As mentioned in Section 2.4.1.1, pure PEI is water soluble, only strongly negatively charged proteins and polyelectrolytes can neutralize the charge on PEI and settle it down from solution. As a weak polyelectrolyte, lipase from *pseudomonas fluorescens* cannot form precipitates in PEI solution. However, GA modification reduces its charge density, consequently precipitation occurred when lipase was added to this GA modified PEI solution. This experimental observation is comparable to the study done by Golander and Eriksson [220] that PEI –polyaldehyde was a more hydrophobic cationic polymer than PEI.
Lipase was immobilized by the PEI-polyaldehyde protocol (Section 3.2.5.4). As a comparison, lipase immobilized cloth prepared by the CVB protocol was also tested using the same reagent concentration and pH. The immobilized activity change and leaked activity change versus measurement time are given in Figure 6-15 and Figure 6-16 respectively. The ESEM images of lipase immobilized via the PEI-polyaldehyde protocol are shown in Figure 6-17.

![Comparison of the immobilized activity for lipase immobilized on woollen cloth by (i) the PEI-polyaldehyde method, and (ii) the benchmark CVB.](image)
Figure 6-16 Comparison of the leaked activity for lipase immobilized on woollen cloth by (i) the PEI-polyaldehyde method, and (ii) the benchmark CVB method.

Figure 6-17 ESEM image of PEI-polyaldehyde immobilized lipase on woollen cloth. Left image, 10000× magnification; right image 5000× magnification.

Figure 6-15 and Figure 6-16 show that the immobilized activity from the sample prepared by the PEI-polyaldehyde protocol is higher than that prepared by the CVB benchmark protocol in the beginning of consecutive activity assays. In Figure 6-15, the activity drops continuously in the first six rounds of consecutive activity assays for the immobilized enzymes from both methods; however beyond this, the immobilized activity of the sample from the CVB method becomes stable, indicating that the physically adsorbed enzymes have leaked out (have been washed off). This is confirmed in the changes in leaked activity of the CVB protocol as presented in Figure 6-16, which
shows that the leaked activity stabilises after 6 repeats. But in Figure 6-15, the immobilized activity from the “PEI-polyaldehyde” protocol reduces continuously. The instability of the immobilized activity by PEI-polyaldehyde method is also well illustrated by the change of leaked activity in Figure 6-16 – it starts much higher than the CVB protocol, and continues to decrease, showing that a considerable portion of the immobilize enzymes have detached. All these results suggest that the reason for the activity loss from the samples from the CVB protocol and PEI-polyaldehyde protocol is different: the activity loss in CVB method is due to the leaking out of the physically adsorbed lipase; in the PEI-polyaldehyde protocol, reasons other than lipase leaking out might be involved. Noticing that PEI-polyaldehyde is prepared from GA modification of PEI, the characteristics of PEI-polyaldehyde are considerably different from PEI. For example, the hydrophobicity of PEI-polyaldehyde increases in a great deal (Appendix 10.7). The tendency of PEI-polyaldehyde and wool forming stable chemical bonds between each other must be considerably reduced compared to the one between unmodified PEI and GA, considering the increased hydrophobicity and molecular weight of PEI-polyaldehyde. Thus, the leaking out of PEI-polyaldehyde might provide a large contribution to this activity reduction. This instability was confirmed by the ESEM image of the wool that has had lipase immobilization via the PEI-polyaldehyde protocol as shown in Figure 6-17, where only limited amount of the aggregated materials can be viewed on the surface of the wool fibre, which is supposed to be related to the immobilised lipase and PEI-polyaldehyde. The majority might have been washed out during the activity assay.

Thus the low immobilized activity achieved by the “PEI-polyaldehyde” protocol, compared to the CVB protocol, is caused by a loss of PEI-polyaldehyde together with the bound lipases from the wool. This is most likely due to the weaker electrostatic binding between the wool and PEI-polyaldehyde ascribed to the fact that PEI-polyaldehyde is more hydrophobic than PEI (see Appendix 10.6).

6.4.2 Mechanism of PEI-polyaldehyde immobilization

Combining the mechanism for PEI-polyaldehyde formation from reference [221] and the CVB immobilization described in Section 4.6, the steps for lipase immobilization by the PEI-polyaldehyde protocol are described in detail as follows and illustrated in Figure 6-18.

(1) Formation of PEI-polyaldehyde
As GA is able to form oligomer in alkaline pH solutions as discussed in Section 6.2, GA oligomer was firstly generated in PEI solution (pH 8.5). This oligomer formed was further reacted with the primary amine in PEI polymer to produce PEI-polyaldehyde.

(2) Attachment of PEI-polyaldehyde to woollen cloth

As the reactivity of aldehyde residues in this PEI-polyaldehyde can last for several months [221], when woollen cloth was soaked in this solution, some aldehyde groups on PEI-polyaldehyde would react with the amine groups on woollen cloth and chemically bind this polymer onto the cloth. Note that in the current experiments, this process was found to be difficult, as the molecular weight of PEI resin used in this experiment was around 600,000 Da (see Section 3.1.1), therefore it was not easy for the aldehyde group to reach onto the surface of the woollen cloth. Moreover, the charge density on PEI-polyaldehyde was reduced due to GA modification, so the electrostatic interaction between PEI and wool was also reduced. Only part of this polymer can be fixed chemically, most of them were merely physically adsorbed and were washed off of the cloth during usage.

(3) Lipase immobilization onto PEI-polyaldehyde coated woollen cloth

In the last step, lipase was reacted with the free aldehyde groups functioned on woollen cloth to compete the immobilization.
6.4.3 Conclusions

In conclusion, the lipase can be immobilized by the PEI-polyaldehyde protocol described in this section. However compared to benchmark CVB approach outlined in Section 3.2.5.1, no activity improvement is observed. A possible reason for this could be that the reaction between PEI-polyaldehyde and wool is weakened due to the increase in hydrophobicity of PEI-polyaldehyde, resulting in detachment of the PEI-polyaldehyde together with the immobilized lipase.

6.5 Comparison of the immobilization protocols studied in this and previous chapters

6.5.1 Activity Drop of immobilized Lipase
The aim of enzyme immobilization through a chemical method is to obtain a stable preparation. However despite varying the immobilization protocols, it was found that activity drop for the immobilized lipase was unavoidable. Some activity losses are due to poor immobilization that can be improved via better immobilization approach, whilst some is from enzyme deactivation, which is the intrinsic feature. In this section, the reasons leading to immobilized activity drop are discussed in more detail.

Based on the experimental results in prior sections, the process of the activity drop in the immobilized lipases can be split into 3 different stages: 1st stage-leaching out of the physically adsorbed lipase, 2nd stage- detach of the chemically immobilized lipase and PEI desorption, 3rd stage -lipase denaturation during storage and application.

*Stage 1- leaching of the physically adsorbed lipases*

In the first stage, most of the physically adsorbed lipases, which could be either from electrical or hydrophobic adsorption, are washed off. This produces a large activity reduction: the immobilized activity usually reduces from 10 $\mu$mol pNPP.min$^{-1}$. (g cloth)$^{-1}$ to 6 $\mu$mol pNPP. min$^{-1}$. (g cloth)$^{-1}$. This indicates that some of the lipase on cloth is just physically immobilized rather than chemically immobilized. Probably some of these leaked lipases are adsorbed to the woollen cloth through an ionic interaction with PEI [222], which would leave the cloth after two or more repeated test runs by the assay solution, as only weak electric forces are involved. However, some of these physically adsorbed lipases could be associated with the lipases already chemically immobilized on PEI through hydrophobic interaction, which is more strongly bound and therefore not as easy to wash out (see Section 2.2.1.3). In experiments, it was observed that there was still a high leaked activity exiting from the immobilized cloth after the lipase-immobilized cloth was washed extensively with buffer, DI water and even with the pNPP assay solution (the Triton X-100 surfactant in this assay solution is able to release the hydrophobically associated lipase-lipase aggregate). This phenomenon is corroborated by previous studies, which shows that lipases were able to be so strongly associated together that extensive washing with DI water, 50% glycerol or polyethylene glycol and 10% acetonitrile, cannot release them. The best way to desorb them is therefore to use concentrated detergent, urea or guanidine solution [44].

It has to be noticed that the purpose of introducing the concept of “leaked activity” in this thesis is trying to indicate the amount of lipase leaching out of the woollen cloth during consecutive activity
assay. The activity change of the physically adsorbed lipase either through electrical adsorption or hydrophobic adsorption was ignored. However this approximation might cause experimental error, as some change in enzyme activity would occur even during physical adsorption. As this was not the main research target, due to the time restrain, further experiments were not carried out. But it would be an interesting topic to examine the activity change during lipase desorption from woollen cloth, which therefore is future work.

**Stage 2- further detachments during usage**

After abrupt activity drops in the first stage, the rate of activity decrease becomes slower but steadily goes down, the activity drop then enters the second stage, as shown in Figure 3-1. In this stage, the main activity loss comes from the free lipase that is detached from PEI backbone due to the reversible GA cross-linking reaction or from the loosely bound PEI leaking out. If this test was continuously carried on within limited period of time, say one day, the immobilized activity stabilised at one reading, this reading is taken as the actual immobilized activity (see Section 3.2.3.3 for details).

Several factors result in the activity loss in the second stage. Some physically attached PEI polymer will leave cloth in this stage. This kind of activity drop is slow, but steady. The most notable example of the activity drop in stage two is the lipase immobilized by the PEI-polyaldehyde protocol (see Section 6.4), where the leaked activity was much higher than the CVB immobilization, which can be attributed to the fact that the PEI polyaldehyde is unable to form a stable connection to woollen surface due to its molecular weight and its hydrophobicity. This is likely because the mobility of aldehyde residues on PEI-polyaldehyde has been restricted by the neighbour functional groups. The mass transfer resistance between PEI-polyaldehyde (Mw of PEI is 600,000) and wool is most likely higher than that between GA and wool. Furthermore, as PEI-polyaldehyde is more hydrophobic than PEI (see Section 6.4.1), the physical adsorption between chlorinated wool and PEI-polyaldehyde is also reduced, resulting in poor chemical binding.

Too much chlorination can also lead to the activity loss in the second stage. As outlined in Section 2.1, bare woollen cloth is chemically resistant due to existence of a high-sulphur content cuticle layer. With the destruction of this cortical layer during the chlorination process, this protective layer is removed. This cortex layer-exposed wool is easier to hydrolyse in an alkaline solution than the bare wool. As most solutions used in immobilization were slightly alkaline, it is possible that parts
of wool protein were dissolved in this solution, resulting in the immobilized PEI together with the associated lipases leaving the wool surface and being leaked from the cloth. This is part of the reason why excessive chlorination should be avoided in lipase immobilization. Section 6.3 and 4.2 cover this issue in detail.

**Stage 3- lipase denaturation during storage**

The immobilized activity could also still possibly decrease during storage. This is the third stage of the immobilized activity drop. This activity drop is due to enzyme denaturation, which belongs to the intrinsic property of enzymes and is out of the range of this thesis.

### 6.5.2 The Effect of Wool as an Immobilization Support on the Reactions occurring during Immobilization

#### 6.5.2.1 Comparison of Immobilization on Wool with other Supports

Various protocols based on the benchmark CVB immobilization protocol using PEI and GA were evaluated with the aim of enhancing the immobilized activity and enzyme loading on the woollen fibres. Although many of these did not completely meet the aims, they however provided some additional insight on the immobilization mechanism.

In the various protocol variations used, increasing the GA concentration and incubation time in GA solution (i.e. fresh GA dilution, which is assumed to contain more GA monomer than oligomer) did not improve the immobilized activity. This however contradicts the results from the other studies (Table 6-1), where an improvement of immobilized activity was observed as either GA concentration or treatment time in GA solution was changed. In addition, altering the treatment sequence also did not improve the immobilized activity (see the CVB, CRL and PEI-polyaldehyde protocols). These results suggest that immobilization on woollen cloth follows a different mechanism from the immobilization on other supports (Table 6-1).
Table 6-1 GA concentration and treatment time in GA solution to immobilized activity on various supports

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Carrier</th>
<th>Optimal GA pH</th>
<th>GA Conc. to Immo. Activity</th>
<th>Time in GA to Immo. Activity</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>Aminated Porous silica</td>
<td>8.6</td>
<td>Increase with GA conc, then level off</td>
<td>No effect</td>
<td>[189]</td>
</tr>
<tr>
<td>Penicillin G acylase</td>
<td>PEI grafted silica</td>
<td>7.92</td>
<td>Maxi activity at 1.2 mmol/g GA solution</td>
<td>Maximal activity at 14 hour in GA solution</td>
<td>[122]</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>PEI coated silk fabrics</td>
<td>N/A</td>
<td>N/A</td>
<td>Increase with time in GA, then level off</td>
<td>[157]</td>
</tr>
<tr>
<td>β-glucosidase</td>
<td>PEI coated nylon</td>
<td>No</td>
<td>Increase with GA conc., then level off</td>
<td>Increase with incubation time in GA then level off</td>
<td>[4]</td>
</tr>
<tr>
<td>Invertase</td>
<td>Diaminohexane coated nylon tube</td>
<td>9</td>
<td>N/A</td>
<td>N/A</td>
<td>[168]</td>
</tr>
<tr>
<td>Glutaryl acylase</td>
<td>Aminated sepabeads</td>
<td>N/A</td>
<td>Increase with GA conc., then level off</td>
<td>N/A</td>
<td>[124]</td>
</tr>
<tr>
<td>Lipase from C. rugosa</td>
<td>Chitosan/PVA nanofibrous membrane</td>
<td>N/A</td>
<td>Increase with GA conc., then level off</td>
<td>N/A</td>
<td>[200]</td>
</tr>
</tbody>
</table>

The differences in mechanism caused by the use of wool is because woollen surfaces contain a number of active groups that are not always present on other support materials, including lysine residue, terminal amine group, cystein residue and histidine residue, which are able to react with GA. It is different from the support carriers listed in Table 6-1. In these carriers, amine groups have to be created via hydrolysis (silk, nylon) or grafting (silica, sepabead). The density of GA-active group is therefore much less than that of wool. Moreover, most of the carriers used in Table 6-1 are either not charged (silica, nylon) [4, 223, 224] or less charged (silk, chitosan) [224] than wool.

6.5.2.2 Implications of this to Immobilization Mechanism and Limited Lipase Activity and Protein Loads in Chapters 4, 5 and 6

For lipase immobilization on woollen cloth using GA as cross-linking agent and PEI as spacer arm, most likely three reactions exist:
1. Wool and PEI linked together via GA cross-linking
2. Lipase linked to PEI via GA activation
3. PEI linked to PEI via GA cross-linking

Considering these reactions, the detailed mechanism of lipase immobilization on woollen cloth can be deduced. The reactions 1 and 2 are the main reactions occurring and resulting in the features seen from the immobilized cloths in Section 4, 5 and 6, as PEI was fixed to woollen cloth and lipase was attached to PEI. Reaction 3 is a side reaction and needs to be suppressed in order to maintain the availability of aldehyde group on PEI resin for lipase cross-linking. Unfortunately, due to the existence of a high proportion of amine groups in PEI (see Section 2.4.1), reaction 3 is likely to be dominant, and would result in a majority of lipases being only physically adsorbed to positive charged PEI surface rather than chemically cross-linked. This is seen from the substantial leakage activity during successive activity tests as demonstrated in Section 4 and Section 6 above. In aqueous solution, PEI cannot easily bond with its amine groups when attacked by GA, as the same electrical charge repulses each other and prevents the reaction from occurring, which can be proven from the fact that the reactivity of aldehyde on PEI-polyaldehyde can last several months (see discussion in Appendix 10.6 and reference [221]). However when PEI was adsorbed onto the woollen cloth surface, the chance of forming PEI internal cross-linking increases, as the charge carried by PEI could be neutralized by the opposite charge on the wool protein. Thus, this could result in the acceleration of the rate of inter-molecular cross-linking between PEI to PEI. Moreover, a number of residues in the wool protein are able to react with GA as mentioned before (see Section 2.1). As wool and PEI polymer are most likely closely associated with each other through electrostatic linkages, it is also possible that these aldehyde groups on PEI are reacted with the amine residues on the wool before lipases are added. Therefore, it is easy to understand that altering the treatment sequence (e.g. CRL, CVB and PEI-polyaldehyde protocol) cannot improve the immobilization, but adoption of GA oligomer can enhance the immobilized activity (the density of aldehyde on PEI backbone is increased). These conclusions are also helpful to explain the ESEM images of immobilized lipases in Section 4 and Section 6, where a layer of polymer coating covered most of the cloth surface, which should have come from the intermolecular cross-linking of PEI, but lipase were found to be only clustered in few areas.

The reasons for the limited protein loads and immobilized activity are not only attributable to the wool (the immobilization support), however the lipase used also may be part of the causes for this problem. The lipase from *pseudomonas fluorescens* only contains three lysine residues that are
capable of participating in the immobilization reactions [46]. This enzyme must have the right spatial configuration to enable lysine residues to contact the aldehyde active sites in order to be immobilized, which is quite difficult if the size of this lipase (33,000 Da [47]) is taken into account. This situation becomes even worse on a solid surface, due to the spatial hindrance caused by the woollen support. This means that only a small fraction of the lipases that are in the correct spatial and steric orientation have a probability to immobilize chemically. The others are instead just physically adsorbed either by electrostatic or hydrophobic adsorption, since their spatial and steric orientation prevented them from participating in the cross-linking reaction.

6.6 Conclusions

Overall, the results in this chapter lead to the following conclusions:

- Changing the immobilization protocol to CRL protocol and PEI-polyaldehyde protocol, had little effect on the immobilized activity and enzyme loading compared to the benchmark CVB protocol from Section 4.

- The protection function of PEI in lipase immobilization was suggested: PEI can protect the lipase activity to some extent in aqueous solution in the presence of GA.

- A 40% increase in the optimal immobilized activity was achieved when the GA oligomer was used instead of freshly prepared GA solution (from 5 µmol pNPP.min⁻¹.(g cloth)⁻¹ in the CVB protocol to 7 µmol pNPP. min⁻¹.(g cloth)⁻¹ in the GA oligomer protocol).

- Immobilized activity was found to be related to the degree of wool chlorination, because chlorination can reduce the wool hydrophobicity and increase PEI adherence. But excessive chlorination is unnecessary, as it may ruin the property of wool as a fibrous material and present no obvious improvement on the immobilized activity.

- Based on the results in this chapter, three reactions that occur during CVB and CRL type of immobilization are proposed:
  
  - 1: PEI-PEI cross-linked by GA
- 2: PEI-lipase cross-linked by GA
- 3: PEI-wool cross-linked by GA

The PEI-lipase cross-linking is the least activated, since the lipase only contains three lysine residues on the outer surface, so to activate this reaction lipases have to be stay in a right position in order to contact the aldehyde group on the cloth surface. This hypothesis explains why the immobilized activity is independent from the GA concentration and the reaction time in GA solution in the CVB protocol (Section 4.3.2).

Overall this chapter shows that:

- Considering the surface characteristics of wool (strong electrostatically charged and consisting of multiple GA reactive groups), it is not a favourable immobilization support compared to the other fabric materials, such as cotton, nylon and silk. A further surface treatment is therefore required to increase the immobilization loading and enzyme activity when wool is used as an enzyme immobilization support.
7. Bi–enzyme System for Enzyme Immobilization

The methods used in enzyme immobilization in previous section are via chemical reaction. As a result, a portion of the enzymes used are deactivated during the chemical attack, mainly due to the use of GA. Therefore, immobilization via a less aggressive protocol is attempted in the following section.

Most of the immobilization techniques involve chemical and physical forces to adhere enzymes to a solid surface. However, recently researchers found that Transglutaminase (mTGase) could be used to immobilize other enzymes [170, 171]. For example, β-glucosidase, α-mannosidase, β-galactosidase, and glucose oxidase were reported to be immobilized in casein gel matrix formed in the presence of TGase [76]. Moreover, it was reported that mTGase could even cross-link the functional groups in wool protein, and possibly become an alternative for the chlorination/ Hercoset process used in shrinkage proof treatment on woollen fabrics [225]. Considering the benefit from mTGase to wool proteins, and the potential of replacing GA as a cross-linker, an immobilization protocol was developed via lipase encapsulation in casein gel in the presence of mTGase.

7.1 Casein Gel Formation in the presence of TGase

As described in Section 2.2.2, Transglutaminase prefers those proteins with good accessibility to mTGase active site as its substrate, i.e. those proteins with less tertiary structure, such as casein (flexible structure and absence of disulfide bonds) [69]. In contrast, globular proteins such as γ-lactalbumin, α-lactoglobulin, bovine serum albumin, and enzymes, cannot be directly attacked by mTGase. Some researches have tried to increase the mTGase accessibility through adding a protein tag to enzyme by the means of protein engineering [66]. However, this is beyond the scope of the current research. As mTGase cannot be used on direct cross-linking of enzymes, thus the best approach for this project to immobilized enzyme through the cross-linking function of mTGase is entrapment of enzyme inside the gel formed in the presence of mTGase. Casein was selected as the mTGase substrate, since it is a good substrate to mTGase, and therefore casein functioned as the gel forming protein in the immobilization protocol developed in this section.
Both casein and sodium caseinate were tried in the preliminary study. Sodium caseinate was selected and used in the following studies. This is because other forms of casein, such as the casein that originates from bovine, goat, sheep and human milk contain an indigenous mTGase inhibitor necessitating treatment at 85°C for 60 min to eliminate it \[72\]. Heat treatment of sodium caseinate is not necessary \[73\].

In order to immobilize lipase via casein gel encapsulation on woollen cloth, the gel must have some strength to withstand the continuous application and testing. Hiroko Sakamoto et al.\[74\] reported the factors affecting the strength of sodium caseinate gel formed in the presence of mTGase. Hiroko found that the optimal gelling conditions were at: pH 9.0, heat treatment at 50 °C for 60 to 120 min. However, as the commercial mTGase was used in the current study and the purity was unknown, unfortunately the optimal enzyme concentration used by Hiroko cannot be directly applied here. Therefore, a series of experiments was arranged to determine the appropriate mTGase concentration for the current application (method is detailed in Section 3.2.7) and the result is shown in Table 7-1.

**Table 7-1 The gel strength of casein gel formed in various concentration of mTGase solution**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>0.1</th>
<th>0.2</th>
<th>0.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Grade 0</td>
<td>Grade 2</td>
<td>Grade 3</td>
</tr>
<tr>
<td>20</td>
<td>Grade 1</td>
<td>Grade 4</td>
<td>Grade 4</td>
</tr>
<tr>
<td>30</td>
<td>Grade 1</td>
<td>Grade 5</td>
<td>Grade 5</td>
</tr>
<tr>
<td>45</td>
<td>Grade 3</td>
<td>Grade 6</td>
<td>Grade 4</td>
</tr>
</tbody>
</table>

It can be seen that the sample prepared in 0.2 gram of mTGase showed the best gel strength, at lower and higher mTGase concentration, the gel strength was reduced. This conclusion corresponds to Hiroko Sakamoto’s conclusion that gel strength increased sharply with increase in mTGase concentration until reaching an optimum. Further increase in the mTGase concentration resulted in a soft and fragile gel. Therefore, 0.2 gram of mTGase in 4 gram of sodium caseinate solution was used in the following study.

Also during experiment, it was also noticed that casein gel was expandable in water solution, as illustrated in Figure 7-1. After soaking in DI water for overnight, the size of this casein gel expanded to almost three times the original size. However, this expansion could be reduced by soaking in the tap water, the ions contained in the tap water may have formed water insoluble product with casein \[226\] and reduced the shrinkage in the structure of the casein gel.
7.2 **Reusability of the Lipase Immobilized by the TGase protocol**

The method described in methodology Section 3.2.7 is used for lipase encapsulation in a casein gel coating formed on the surface of the woollen cloth. Figure 7-2 shows photos of the PEI coated woollen cloth and casein gel coated woollen cloth.

It was observed that a good immobilization of lipase in casein gel could not be achieved on bare woollen cloth. The gel layer was very easily peeled off from woollen cloth after consecutive activity tests using the pNPP assay, when formed on the unmodified woollen surface. This is probably due to the fact that casein and wool protein both have an acidic isoelectric point, and both of them carry the same charge, resulting in poor adhesion between the lipase immobilized casein layer and wool. Therefore, to improve the stability of the resulting casein gel layer, a layer of polyethyleneimine (PEI) was coated onto the wool and fixed by GA, using the method described in Section 3.2.5.5. It was found that the tendency of casein gel peeling off was eased after PEI coating on the wool surface. Therefore, this layer of PEI coating prior to the application of casein gel was used in the following study.
The purpose of immobilizing enzymes is to enable it to be more easily recovered and reused compared to free enzymes. Therefore, the reusability of the lipase-immobilized cloth via mTGase protocol was tested. Lipase was encapsulated in the casein gel formed on wool surface through the method described in Section 3.2.5.5. The enzyme assay protocol taken by Motoki et al. [76] was used here: after the cloth was taken out of the assay solution and washed with 0.5 mM Tris buffer.
(pH 8.5), and allowed to dry at room temperature after each measurement. After removing the loosely bound lipase, the immobilized lipase activity was tested by the procedure outlined in Section 3.2.3 and results are illustrated in Figure 7-3.

Figure 7-3 shows that after more than 20 activity determinations (occurred within 2 hours), there is still a relatively high amount of immobilized lipase activity present. However, there is also a slight decrease in enzyme activity observed. The reduction in activity is due to enzyme leaching from the casein gel and wool surface rather than deactivation in situ. This is because this activity assay occurred within 2 hours and deactivation of immobilized lipase is not obvious within that time span as shown in Figure 7-5. However this sort of leakage is unavoidable for immobilizations via physical methods.

These results were comparable to those published by Motoki et al. [76]. In Motoki’s assay method, the lipase-immobilized cloth must be dried after each activity test, which is not practical in real applications. Thus, the change of immobilized activity after consecutive assays without drying was determined; the result is illustrated in the “baseline” data points in Figure 7-4. This curve shows that the immobilized lipase activity continuously drops after consecutive activity assays, but the reduction of the immobilized activity plateaus when the activity is reduced to 5 μmol pNP. min⁻¹.(g cloth)⁻¹.
In the literature review (see Section 2.3.1), it was shown that for enzyme immobilization via entrapment, a drop in lipase activity is unavoidable. Some of the decrease might be due to the enzyme leakage and some might be from enzyme deactivation. To reduce the leakage, one alternative solution is to coat another layer of polymer on the gel surface to enhance the curing [227]. Thus in this study, after lipase is entrapped in casein gel in the presence of mTGase, the cloth was further soaked in 2% PEI solution at pH 8.0 for 30 min. In order to strengthen the connection between PEI and casein, this derived cloth was then cross-linked in 0.1% GA solution at pH 8.0 for 5 min. The activity was tested by pNPP assay and the result is illustrated by the “PEI” data points in Figure 7-4. As shown, the presence of second PEI coating reduces the drop in activity and therefore by implication the lipase leakage to some extent. However, the immobilized activity still decreases gradually as the result of lipase leakage. The overall immobilized activity was considerably lower, due to the increased mass transfer resistance caused by this additional coating. The second PEI coating does not improve the immobilization substantially.
Interestingly, in Figure 7-4, both of the curves level off at around 5 activity units. This figure probably was the true reflection of the amount of lipase stably entrapped inside the casein gel. By the means of the casein gel method, some lipase were likely adhered or adsorbed onto casein gel and therefore could be easily washed out by the assay solution. However, with the coating by the second layer of PEI in the presence of GA, these lipases on the surface layer were deactivated by the GA cross-linking reaction, so the apparent immobilized activity in the first couple of runs were lower than “casein gel method” and the reading by PNPP assay was closer to the “true value” of immobilized activity.

### 7.3 Stability of the Immobilized Lipase

Another desirable property of any immobilized lipase is that it is stable to storage in air. Stable immobilizations which retain their activity during long term storage and transportation are one of the major factors currently limiting the industrial application of immobilized enzymes [228]. Therefore, the storage stability of the immobilized cloth was tested and shown in Figure 7-5.

![Figure 7-5 Storage stability of the immobilized lipase in air](image)

The lipase immobilized by casein on woollen cloth was simply stored in air at room temperature, and the activity was measured daily. The activity was tested over 15 days, and considering the
scatter in the data, it can be concluded that there was a negligible activity change observed for the immobilized lipase. This immobilized lipase demonstrates better stability than the free lipase stored in Tris buffer at 25°C as illustrated in Figure 5-3 and the stability of the immobilized lipase via CVB protocol stored in air at room temperature (Figure 5-5). This stability during storage in air is also superior to other immobilization techniques using the same lipase, which also shows a clear drop in activity [229]. The reason for this excellent storage stability is due to the lipase being entrapped in a casein gel formed on woollen cloth. Consequently, although stored in air, this enzyme was not stored in a dry environment: the gel matrix provides a water containing surroundings keeping the enzyme hydrated and therefore stable over the period studied. Further studies are required to determine the ultimate length of time that this activity will remain (which will most likely directly correlate to the length of time the casein gel will remain hydrated and unspoilt).

Note that although the hydrating casein gel entrapping the enzyme has this additional benefit, it also confers some disadvantages: the gel creates an additional mass transfer resistance for substrates to be catalysed by the lipase. And the casein gel formed by mTGase is able to expand and peel off in aqueous solution, leading to a further activity deduction. Additional work needs to be done to minimise this drawback. But, this might not be a problem for lipase catalysed biotransformation occurred in organic solvent.

7.4 Conclusions

A new method of immobilizing a lipase by entrapment in casein gel formed in the presence of mTGase was developed. To facilitate the mechanical stability and handleability of this immobilized preparation, the gel was coated on woollen cloth.

The lipase was immobilized onto woollen cloth by this entrapment method. The activity of the immobilized lipase remained relatively stable at approximately 5-6 μmol pNP. min⁻¹.(g cloth)⁻¹ after more than 20 consecutive activity tests, indicating that it was suitable for reuse despite some enzyme leaching. This small amount of enzyme leaching was unavoidable, as this enzyme was only physically immobilized. The immobilized lipase also demonstrated reasonable storage stability, where there is only limited activity change (approximately stable at 5-6 μmol pNP. min⁻¹.(g cloth)⁻¹) when stored in air at room temperature for fifteen days. It is hypothesized that the water contained in casein gel matrix keeps the enzyme hydrated and therefore stable over the period studied.
As the immobilization is physical encapsulation, there is some unavoidable enzyme leakage. Compared to enzyme immobilized by CVB. This approach is not very promising, when the immobilization efficiency (amount of enzyme used in comparison to the level of immobilized activity present) and the stability of the immobilized lipase is considered.
8. Overall Conclusions and Recommendations

8.1 Overall Conclusions

- Lipases have been successfully immobilized by the chemical immobilization method developed in the current study. Covalent binding protocol (CVB protocol) was taken as the benchmark method, the immobilization parameters were optimised to find the best immobilization conditions: the immobilized activity was found to be independent from GA concentration and treatment time in GA solution; the optimal pH of GA solution used was determined to be 9.0; the immobilized activity increased steadily within 5-10 hours of soaking in lipase solution, after that, the rate of increase was slower and finally levelled off; immobilized activity was found to be related to the degree of wool chlorination. Chlorination in 5% owf (weight of cloth) DCCA (Dichlorocyanuric acid) solution for 1 hour was enough at pH 4.01. Excessive chlorination would damage the cloth and thus was unnecessary.

- The characteristics of the free and immobilized lipase by the CVB protocol was compared: The pH profile for the free and immobilized lipase was almost the same. The immobilized lipase was more stable in Tris buffer than in the air. When stored for more than 80 days in Tris buffer (pH 8.5) at 4 ºC, more than 80% of the initial lipase activity remained. Whilst, almost 80% of activity was lost within 2 weeks in air. For the free and immobilized lipases, their kinetic behaviour follows the M-M model. The K_M of the free and immobilized lipase was found to be 2.40, 3.16 mM respectively. The maximum velocity (v_max) changes from 153.76 μmol pNP min⁻¹. (mg lipase)⁻¹ for the free lipase to 5.16 μmol pNPP.min⁻¹.(g cloth)⁻¹ for the immobilized lipase, suggesting that the activity of the immobilized lipase decreased during the course of immobilization. This lipase-immobilized cloth exhibits excellent oily stain removal ability- after cloth was stored in air for almost one month, similar cleaning performance was still observed. The protein loads of the lipase immobilized on woollen cloth was approximately only 4.29 mg/g support due to poor protein coverage on the cloth as shown in ESEM images.

- Galactosidase could be immobilized onto woollen cloth via the CVB protocol. The immobilization could be proven by the stability of immobilized galactosidase activity during
consecutive activity assay (Figure 4-14). The specific immobilized activity was found to be 12 $\mu$mol o-nitrophenol per min per gram of cloth. However, the storage stability of this immobilized preparation was lower than the data published in literature [203]. After storing in 0.1 M phosphate buffer solution (pH 6.0 containing 1 mM magnesium chloride) at 4°C for 5 days, the immobilized activity dropped from 12 $\mu$mol o-nitrophenol per min per gram of cloth to only 4 $\mu$mol o-nitrophenol per min per gram of cloth. More experiments are needed to verify the reasons for this.

➢ To further improve the immobilized activity, understand the mechanism of this method and explore the limitation of wool as immobilization support, a number of immobilization methods based on the CVB protocol was developed:

- Changing the immobilization protocol to CRL protocol and PEI-polyaldehyde protocol, had little effect on the immobilized activity and enzyme loading compared to the benchmark CVB protocol from Section 4.

- A 40% increase in the optimal immobilized activity was achieved when the GA oligomer was used instead of freshly prepared GA solution (from 5 $\mu$mol pNPP. min$^{-1}$. (g cloth)$^{-1}$ in the CVB protocol to 7 $\mu$mol pNPP. min$^{-1}$. (g cloth)$^{-1}$ in the GA oligomer protocol).

➢ Three reactions that occur during the CVB and CRL type immobilizations are proposed based on this work:

1. PEI-PEI cross-linked by GA,
2. PEI-lipase cross-linked by GA,
3. PEI-wool cross-linked by GA.

The PEI-lipase cross-linking is the least activated, since the lipase only contains three lysine residues on the outer surface, so to activate this reaction lipases have to be stay in right position in order to contact the aldehyde group on the cloth surface. This hypothesis explains why the immobilized activity is independent from the GA concentration and the reaction time in GA solution in the protocols.
The function of PEI in lipase immobilization was highlighted: PEI was used as a spacer arm, which was able to enrich lipase concentration on the wool surface and accelerate the covalent binding reaction. The other benefit from PEI spacer arm is that it can protect the lipase activity to some extent in aqueous solution in the presence of GA.

A new method of immobilizing a lipase by entrapment in casein gel formed in the presence of mTGase was carried out. To facilitate the mechanical stability and handleability of this immobilization, the gel was coated on woollen cloth. The lipase was immobilized onto woollen cloth by this entrapment method. The activity of the immobilized lipase remained relatively stable at approximately 5-6 μmol pNP. min⁻¹.g cloth⁻¹ after more than 20 consecutive activity tests with some enzyme leaching. This small amount of enzyme leaching was unavoidable, as this enzyme was only physically immobilized. The immobilized lipase also demonstrated excellent storage stability, where there is almost no activity change from approximately 5-6 μmol pNP. min⁻¹.g cloth⁻¹, when stored in air at room temperature for fifteen days. It is hypothesized that the water contained in casein gel matrix keeps the enzyme hydrated and therefore stable over the period studied.

Overall this work has shown that wool is not a favourable immobilization support for the immobilisation methods tested as other fabric materials, such as cotton, nylon and silk due to its surface characteristics: strong static electrical charges and consisting of multiple GA reactive groups. A surface modification is therefore needed to increase the protein load and immobilized activity.
8.2 Recommendations and Future Work

To extend the work done in this thesis, the following recommendations and future work should be considered:

1. Immobilization of enzymes onto polyamideamine epichlorohydrin (PAE) resin was found not to work, due to the poor compatibility of the lipase to the PAE resin. PAE is however one of the common resins used for wool surface treatment. Commercially, PAE resin is prepared from adipic acid, diethylenetriamine and epichlorohydrin. To improve the storage stability, the epichlorohydrin residue has to be hydrolysed. However, this residue will provide an excellent active group for enzyme immobilization. Fresh PAE resin prepared without epichlorohydrin hydrolysis has potential to become a good immobilization carrier for enzyme immobilization on wool surface. Studies looking into using this possibility should be conducted.

2. In this project, the target application of the immobilized woollen cloths was for laundry washing, to produce a self-cleaning or enhanced cleaning fabric. The immobilized enzymes and the techniques developed and evaluated in this thesis potentially have a wider application and could be applied to biotransformation, fatty acid manufacturing, and even biodiesel production. Thus, future work should also concentrate on the application of immobilized enzyme to more industrial type reaction systems.

3. The method developed in this project is based on woollen fabrics. This method is most likely also suitable to other fabric materials, such as cotton, silk, nylon. Therefore an interesting investigation could be in studying the application of the current immobilization techniques to other immobilisation supports.

4. Only two enzymes have been immobilized by the CVB benchmark protocol developed in this study. However other enzymes that have an isoelectric point in the acidic pH range, have the possibility of being immobilized onto woollen cloth using this type of protocol.
5. In the preliminary study of the current project, the co-immobilization of lipase and protease onto woollen cloth was considered. However due to the poor compatibility of protease to wool, eventually this attempt was given up. But it would be worthwhile to try this on fabrics other than wool, such as cotton and nylon.

6. When using the CVB protocol in galactosidase immobilization, the immobilized activity was found to reduce continuously during storage. This result is different from comparable results from other researchers. The reasons for the differences therefore need to be verified.

7. Lipase entrapment in the casein gel formed in the presence of mTGase looks less promising when using in the aqueous solution as the gel is water expandable. However, this should not be a problem in organic solvent. Thus, the potential application of this technique in biotransformations in organic solvents might be an interesting research topic.

8. The purpose of using SEM and ESEM images in the current research is to visualize the immobilized enzymes. However, it is difficult to distinguish between the cross-linked PEI, enzymes and enzyme impurity. To obtain better images, an appropriate staining technology might help to improve the picture quality.
9. References


[84] N. Albayrak, Production of galacto-oligosaccharide from lactose by Aspergillus oryzae immobilized on cotton cloth, in, School of the Ohio State University, 2001.


[139] F. B. WEAKLEY, C.L. MEHTRETTER, Binding of Papain to Dialdehyde Starch, Biotechnology and Bioengineering, XV (1973) 1189-1192.


[152] M.S. Masri, V.G. Randall, Stanley, Chemical modification of insoluble polymers for certain end uses, Polymer Preprints (American Chemical Society, Division of Polymer Chemistry), 16 (1975) 70-75.


[187] Lipase AK "Amano", in, Amano Enzyme Inc.


[208] Sigma-aldrich, 534730, Lipase from Pseudomonas Fluorescens, in.


[233] D. Amotz; Shmuel (Malov, Rugh; Susanne (Rungsted Kyst, DK), Markussen; Erik K. (Vaerlose, DK), Thomsen; Kurt (Allerod, DK), Carrier for immobilizing enzymes in, Novo Industri A/S (DK) 1986.


## 10. Appendices

### 10.1 Summary of Enzyme Immobilization by Chemical Methods

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Immobilization procedure</th>
<th>Property</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chymotrypsin (Direct CLEs)</td>
<td>500 mg enzyme powder in 5 ml of 0.2 M acetate buffer adjusted to pH 6 (alternatively, 0.1 M phosphate buffer at pH 6.8), 0.3 to 0.6% GA, left at room without stirring, gel appears after 10-30 min.</td>
<td>20-40% activity retained</td>
<td>[104]</td>
</tr>
<tr>
<td>Glucose oxidase gel formed with BSA</td>
<td>100 mg enzyme and 400 mg BSA are dissolved in 100 ml of 0.2 M acetate buffer at pH 5, adding 0.3 to 0.6% GA. Left at bench temperature without stirring for 1-3 hr. 1% sodium azide added</td>
<td></td>
<td>[104]</td>
</tr>
<tr>
<td>Glucose oxidase mixed with BSA, membrane formed</td>
<td>Enzyme (400 IU) added to BSA solution at pH 6.8, with 0.7% GA (final conc.) for couple of hours in 20 deg or overnight in fridge. Solution was spread as membrane. After rinsing, 1% sodium azide added</td>
<td></td>
<td>[104]</td>
</tr>
<tr>
<td>β-galactosidase from E.coli</td>
<td>At pH 6.8 of 0.02 M phosphate buffer, 5000 IU of β-galactosidase are mixed with BSA (60 mg ml⁻¹) and GA (2 mg ml⁻¹). Frozen after stirring at -30 °, and then slowly warmed in a refrigerator (~4°) for 4 hr. a sponge like insoluble aggregate enzyme formed. Finally it was lyophilized and ground</td>
<td>20% of activity recovered No activity loss in fridge 1 month</td>
<td>[104]</td>
</tr>
<tr>
<td>Glucose oxidase in cellophone pore (adsorption +CLEs)</td>
<td>Cellophone sheet adsorbed in enzyme solution, dry in refrigerator, impregnated with 2.5% GA in phosphate buffer, pH 6.8. then rinse with water and buffer</td>
<td>80% of activity retained</td>
<td>[104]</td>
</tr>
<tr>
<td>Urease entrapped in polyacrylamide-agarose gel</td>
<td>Urease aggregate directly formed in 1.25 % GA in 0.02 M phosphate buffer, pH 6.8. Aggregation proceeds at 4 ° for 10 hr, stopped in 0.1 M glycine. This aggregate encapsulated in polyacrylamide-agarose gel</td>
<td>Activity retained for several weeks at 4 °C. Without aggregation, the enzymes leaked quickly</td>
<td>[104]</td>
</tr>
<tr>
<td>Gluco-isomerase</td>
<td>1 kg of enzyme sludge is added 40 ml of a 50% glutaraldehyde solution. After 1 hr the mixture has gelled into a coherent mass. The gel is broken up mechanically, sieved, dry and ground</td>
<td>Activity recovery 50-60% after 40 bates, new enzyme addition needed.</td>
<td>[230]</td>
</tr>
<tr>
<td>Invertase</td>
<td>Enzyme was immobilized on water swellable acrylate polymer with amine residue. Invertase solutions in (pH 7.0) shaking with carriers, at 50 °C for 15 min, and then cooled down. Then GA in pH 7.0 added reacting for 15 min. then rinsed stored in fridge at 4 °C in Tris buffer</td>
<td>20% activity retained (immobilization yield). After 1 month storage 30-60% of activity remained</td>
<td>[231]</td>
</tr>
<tr>
<td>α-amylase</td>
<td>Pressing the enzyme soln. through the membrane (silica-modified polyvinylchloride polymer), then cross-linking with glutardialdehyde</td>
<td>Maltose formed 2.68 mg/min/cm² versus 0.36, 0.43, and 0.12 mg/min/cm² by other method</td>
<td>[232]</td>
</tr>
</tbody>
</table>
Continued to Table 10-1

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Preparation Method</th>
<th>Activity Retention</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>glucose isomerase (patent)</strong></td>
<td>Support matrix prepared with gelatin/alginate coating on diatomaceous earth followed by GA treatment. Enzyme spray onto this particles prepared above followed by another 0.1% GA treatment. The immobilized enzyme was either in dry form or wet form.</td>
<td>Dry particle, 48% activity recovery, and wet particle, 70% of activity retained</td>
<td>[233]</td>
</tr>
<tr>
<td><strong>β-glucosidase and glucose oxidase (1)</strong></td>
<td>(1) Dissolved in 0.2 M K₂HPO₄/NaOH, pH 7.5, 1.8 ml, 0.2 ml 4% GA added to above solution for 8 hrs at 0-4 °C, then stopped by 0.2 ml saturated sodium bisulphite solution.</td>
<td>Soluble aggregate maybe used for multi step reaction</td>
<td>[234]</td>
</tr>
<tr>
<td><strong>β-glucosidase from Aspergillus niger</strong></td>
<td>Sodium alginate (Farmus) was stirred with GA 25%, and the enzyme preparation added at 4°C. After stored for 48 h at 4°C, the slurry was dropped into a beaker containing a calcium chloride solution resting in an ice bath, and left overnight at 4°C for complete polymerization.</td>
<td>Only 10% activity recovery. 10% activity loss at 50 deg for 24 hours verse 73% loss without cross-linking</td>
<td>[235]</td>
</tr>
<tr>
<td><strong>amyloglucosidase +gelatin + bentonite</strong></td>
<td>Gelatin was boiled for few second to dissolved, then 50-500 mg enzyme was added, followed by 100 mg of inert carrier (bentonite, alumina, silica gel or celite). The suspension was stirred for 15 min, 0.5 ml, 25% aqueous GA was added with stirring and the resulting gel was broken by sonication for a few seconds</td>
<td>70% activity retained</td>
<td>[236]</td>
</tr>
<tr>
<td><strong>amyloglucosidase +gelatin</strong></td>
<td>Same as above</td>
<td>50% activity retention</td>
<td>[236]</td>
</tr>
<tr>
<td><strong>amyloglucosidase +gelatin+cellite</strong></td>
<td>Same as above</td>
<td>40% activity retention</td>
<td>[236]</td>
</tr>
<tr>
<td><strong>Amyloglucosidase+ casein</strong></td>
<td>Same as above</td>
<td>5% activity retention</td>
<td>[236]</td>
</tr>
<tr>
<td><strong>Amyloglucosidase+BSA</strong></td>
<td>Same as above It was also found the enzyme/protein ratio and pH also affect the retention yield</td>
<td>25% activity retention</td>
<td>[236]</td>
</tr>
</tbody>
</table>
# Summary of Literature on PEI Spacer arm in Immobilization

Table 10-2 Summary of enzyme immobilization via PEI spacer arm

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Immobilization procedure</th>
<th>Property</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Invertase from S. cerevisiae</td>
<td>Adsorbed in pH 8.5 buffer on sepabead EC-EP3-PEI, desorb in 3 M NaCl/ pH 1.5-2/ guanidine in high temp</td>
<td>Stronger than ionic adsorption, stable for 3 month at 4°C, protein loads 30 mg/g support</td>
<td>[89]</td>
</tr>
<tr>
<td>C. Antarctica lipase</td>
<td>Adsorbed on PEI-agarose (different temps and pH ranging from pH 5.0 to 9.0).</td>
<td>Best enantioselectivity was achieved with lipase adsorbed at pH 9.0 and 4 °C,</td>
<td>[169]</td>
</tr>
<tr>
<td>D-lactate dehydrogenase &amp; C. boidinii</td>
<td>PEI-enzyme-cell aggregates adsorbed on cotton</td>
<td>0.5 g cell and 8 mg LDH / g cotton , slightly activity loss in 3rd batch</td>
<td>[194]</td>
</tr>
<tr>
<td>Lactase A. oryzae and lipase from Candida rug</td>
<td>high-porosity mullite advanced ceramic material (ACM) were functionalized with polyethyleneimine (PEI)</td>
<td></td>
<td>[237]</td>
</tr>
<tr>
<td>Lipase from C. rugosa, H. lanuginosa, and Mucor miehei</td>
<td>Bimolecular formed of lipases adsorbed onto PEI coated Sepabead, at pH 7 and 25 ° C, immobilization yields being near 100%.</td>
<td>protein loads 3.7 mg ml⁻¹ support</td>
<td>[193]</td>
</tr>
<tr>
<td>Lipase from Alcaligenes sp. and C. Antarctica (B)</td>
<td>CLEA was prepared with lipase/PEI and lipase/PEI/DS mixture in the presence of precipitant ( PEG 600) and GA at pH 7.0 in ice bath of 2 ° C.</td>
<td>The lipase enantio-selectivity and activity was improved depending on enzyme species and polymer used</td>
<td>[238]</td>
</tr>
<tr>
<td>Glutaryl acylase</td>
<td>Co aggregate of PEI/PEG with glutaryl acylase in the presence of GA. Stability of CLEA improved due to addition of PEI</td>
<td>Keep 60% activity after 72 hr at 45 deg, while free enzyme dead after 2.5 hr.</td>
<td>[239]</td>
</tr>
<tr>
<td>Penicillin G acylase</td>
<td>Covalent immobilized on PEI coated silica activated with GA</td>
<td>87.5% activity remain after 15 times repeatedly using</td>
<td>[122]</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Polyethyleneimine-cellulose plate treated in 2.5% GA at pH 9.4 and room temp for 15 min. After rinse, soaked in enzyme ( 30 u per strip) at pH 8.0 for 2,3,6,8 hr</td>
<td>4 consecutive assay carried on and no activity drop observed</td>
<td>[240]</td>
</tr>
<tr>
<td>Glutaryl-7-aminocephalosporanic acid acylase( GAC)</td>
<td>Five grams of Sepabeads-PEI were added to 5mL of GAC (16 IU ml⁻¹) in 5mM potassium phosphate buffer at pH 7 for 3 hr at 25 ° C; then mild stirring in GA solution for 1 hr, rinse stored in cold</td>
<td>Stability 250 time higher than the free enzyme, 90% of the free activity remained in the immobilized preparation</td>
<td>[124]</td>
</tr>
<tr>
<td>glucose isomerase</td>
<td>Enzyme adsorbed in porous silicon dioxide, then crosslinked with 1% GA. Or enzyme mixed with PEI after adsorption in support followed by GA</td>
<td>GA cross-linked enzyme with PEI show better stability ( patent)</td>
<td>[106]</td>
</tr>
</tbody>
</table>
**10.3 Purity of the lipase used in this research**

The protein purity was tested with QuantiPro BCA Assay Kit purchased from Sigma-Aldrich company. The protein standard curve was prepared by the standard protein solution contained in this assay kit, as shown in Figure 10-1. The calculation chart is also attached in Table 10-3.

![Graph showing protein standard curve between A 562 and protein concentration, values are the mean of three independent replicates.](image)

**Table 10-3 Data for protein standard curve**

<table>
<thead>
<tr>
<th></th>
<th>5 µg/ml</th>
<th>10 µg/ml</th>
<th>15 µg/ml</th>
<th>20 µg/ml</th>
<th>25 µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated Conc</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample 1 A562</td>
<td>0.2573</td>
<td>0.5703</td>
<td>0.642</td>
<td>0.848</td>
<td>1.131</td>
</tr>
<tr>
<td>Sample 2 A562</td>
<td>0.2556</td>
<td>0.5126</td>
<td>0.6632</td>
<td>0.828</td>
<td>1.146</td>
</tr>
<tr>
<td>Sample 3 A562</td>
<td>0.2711</td>
<td>0.4922</td>
<td>0.6609</td>
<td>0.8296</td>
<td>1.124</td>
</tr>
<tr>
<td>Average A562</td>
<td>0.261</td>
<td>0.525</td>
<td>0.655</td>
<td>0.835</td>
<td>1.134</td>
</tr>
<tr>
<td>A562 Stddev</td>
<td>0.009</td>
<td>0.041</td>
<td>0.012</td>
<td>0.011</td>
<td>0.011</td>
</tr>
</tbody>
</table>
As some impurities are contained in the commercial lipase product, the protein purity of commercial lipase from pseudomonas fluorescens was tested and the result is shown in Table 10-4. In order to determine the protein concentration, lipase (79.2 mg) was dissolved in 40.42 gram phosphate buffer (pH 7.0, 0.1 M), lipase concentration (unpure) is then calculated as 1.97 mg lipase /ml. As the protein assay kit can only determine the protein concentration below 25 µg/ml, so the above lipase solution has to be diluted. To reduce the experiment error, the solution was diluted into series dilutions: 5 fold dilution, 10 fold dilution, 15 fold dilution and 20 fold dilution, as listed in Table 10-4.

**Table 10-4 Chart for calculation of protein purity**

<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>5 fold dil.</th>
<th>10 fold dil</th>
<th>15 fold dil</th>
<th>20 fold dil</th>
</tr>
</thead>
<tbody>
<tr>
<td>A562 of sample 1</td>
<td>2.736</td>
<td>1.786</td>
<td>1.025</td>
<td>0.811</td>
</tr>
<tr>
<td>A562 of sample 2</td>
<td>2.693</td>
<td>1.611</td>
<td>1.057</td>
<td>0.8722</td>
</tr>
<tr>
<td>A562 of sample 3</td>
<td>2.6998</td>
<td>1.57</td>
<td>1.065</td>
<td>0.8185</td>
</tr>
<tr>
<td>Average A562</td>
<td>2.71</td>
<td>1.656</td>
<td>1.049</td>
<td>0.834</td>
</tr>
<tr>
<td>Protein conc</td>
<td>61.33</td>
<td>37.189</td>
<td>23.292</td>
<td>18.365</td>
</tr>
<tr>
<td>Cal Conc in sol</td>
<td>307.263</td>
<td>373.001</td>
<td>349.558</td>
<td>368.704</td>
</tr>
<tr>
<td>Average Conc</td>
<td></td>
<td></td>
<td></td>
<td>363.754</td>
</tr>
</tbody>
</table>

Note: the unit of protein concentration used is in Table 10-4 is µg/ml

In Table 10-4, the protein concentration determined from 5-fold dilution is different from the others. The reason is that A562 for 5-fold dilution is 2.7, which has reached to the detection limit of UV spectrophotometer. The point thus is not reliable and has to be omitted. The average protein concentration in this solution is 363.75µg/ml, and the standard deviation is 12.48 µg/ml. The purity of protein in the commercial powder can be calculated from the protein concentration over the lipase concentration, the result is 18.46%.

### 10.4 Protein load of lipase immobilized cloth Via CVB protocol

Protein load of lipase immobilize cloth was estimated by two methods. In the first method, the amount of lipase adsorbed onto cloth was calculated from the activity difference in the lipase solution before & after the GA-treated woollen cloth was adsorbed in. Since some of the adsorbed lipase on woollen cloth will leave cloth after consecutive activity assay, thus this amount of lipase was tested from the leaked activity by pNPP assay. The results are shown in Table 10-5. As the presence of GA on woollen cloth could cause lipase deactivation, resulting in error in the determination of protein loads by pNPP assay, the amount of lipase adsorbed on cloth was further
tested by QuantiPro BCA Assay Kit, the results is shown in Table 10-6. Comparable results were obtained between these two methods. This might be due to the fact that most of the GA on cloth either participates the cross-linking reaction of PEI or is washed away from the cloth during the preparation steps. From Table 10-5, the protein loads was estimated to be 4.29 mg /g cloth. In Table 10-5, the unit of free lipase activity was expressed by the UV absorbance change per min (AU/min).

Table 10-5 Protein loads of lipase-immobilized cloth via the CVB protocol, estimated from pNPP assay

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>#1</th>
<th>#2</th>
<th>#3</th>
<th>#4</th>
<th>#5</th>
<th>#6</th>
<th>#7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase Conc mg/ml</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.97</td>
<td>2.17</td>
<td>1.95</td>
</tr>
<tr>
<td>Vol of lipase solution ml</td>
<td>44.80</td>
<td>45.73</td>
<td>46.16</td>
<td>46.24</td>
<td>41.15</td>
<td>42.33</td>
<td>41.73</td>
</tr>
<tr>
<td>Cloth weight (g)</td>
<td>0.89</td>
<td>1.02</td>
<td>0.95</td>
<td>0.88</td>
<td>1.06</td>
<td>0.93</td>
<td>1.12</td>
</tr>
<tr>
<td>Lipase activity in solution before CVB treated cloth adsorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A410 1st test (AU/min)</td>
<td>1.02</td>
<td>0.98</td>
<td>1.09</td>
<td>1.00</td>
<td>1.58</td>
<td>1.48</td>
<td>1.50</td>
</tr>
<tr>
<td>A410 2nd test (AU/min)</td>
<td>1.03</td>
<td>1.07</td>
<td>1.03</td>
<td>0.98</td>
<td>1.68</td>
<td>1.46</td>
<td>1.35</td>
</tr>
<tr>
<td>A410 3rd test (AU/min)</td>
<td>1.00</td>
<td>1.13</td>
<td>1.08</td>
<td>0.99</td>
<td>1.62</td>
<td>1.48</td>
<td>1.39</td>
</tr>
<tr>
<td>A410 Average (AU/min)</td>
<td>1.02</td>
<td>1.06</td>
<td>1.06</td>
<td>0.99</td>
<td>1.63</td>
<td>1.48</td>
<td>1.41</td>
</tr>
<tr>
<td>Lipase activity in solution after CVB treated cloth adsorption</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A410 1st test (AU/min)</td>
<td>0.72</td>
<td>0.73</td>
<td>0.65</td>
<td>0.67</td>
<td>1.17</td>
<td>1.17</td>
<td>1.02</td>
</tr>
<tr>
<td>A410 2nd test (AU/min)</td>
<td>0.73</td>
<td>0.73</td>
<td>0.66</td>
<td>0.71</td>
<td>1.15</td>
<td>1.23</td>
<td>1.11</td>
</tr>
<tr>
<td>A410 3rd test (AU/min)</td>
<td>0.73</td>
<td>0.72</td>
<td>0.59</td>
<td>0.66</td>
<td>1.18</td>
<td>1.22</td>
<td></td>
</tr>
<tr>
<td>A410 Average (AU/min)</td>
<td>0.73</td>
<td>0.73</td>
<td>0.63</td>
<td>0.68</td>
<td>1.17</td>
<td>1.21</td>
<td>1.06</td>
</tr>
<tr>
<td>Lipase adsorbed mg/g cloth</td>
<td>14.32</td>
<td>14.04</td>
<td>19.55</td>
<td>16.26</td>
<td>21.75</td>
<td>18.18</td>
<td>18.14</td>
</tr>
<tr>
<td>Amount of lipase leaked during activity assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A410 1st test (AU/min)</td>
<td>1.01</td>
<td>1.05</td>
<td>1.52</td>
<td>1.02</td>
<td>0.90</td>
<td>0.70</td>
<td>0.65</td>
</tr>
<tr>
<td>A410 2nd test (AU/min)</td>
<td>0.98</td>
<td>1.02</td>
<td>1.55</td>
<td>1.07</td>
<td>0.92</td>
<td>0.66</td>
<td>0.62</td>
</tr>
<tr>
<td>A410 3rd test (AU/min)</td>
<td>1.04</td>
<td>0.96</td>
<td>1.60</td>
<td>1.12</td>
<td>0.88</td>
<td>0.73</td>
<td>0.71</td>
</tr>
<tr>
<td>A410 Average (AU/min)</td>
<td>1.01</td>
<td>1.01</td>
<td>1.56</td>
<td>1.07</td>
<td>0.90</td>
<td>0.70</td>
<td>0.66</td>
</tr>
<tr>
<td>Vol of pNPP assay (ml)</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Amount of lipase left (mg/g cloth)</td>
<td>10.10</td>
<td>10.10</td>
<td>15.57</td>
<td>10.70</td>
<td>17.73</td>
<td>15.12</td>
<td>12.87</td>
</tr>
<tr>
<td>Amount of lipase remained (mg/g cloth)</td>
<td>4.22</td>
<td>3.94</td>
<td>3.99</td>
<td>5.56</td>
<td>4.02</td>
<td>3.07</td>
<td>5.27</td>
</tr>
<tr>
<td>Average protein loads</td>
<td>4.29</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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### Table 10-6 Amount of lipase adsorbed onto CVB immobilized woollen cloth, determined by protein assay kit

<table>
<thead>
<tr>
<th></th>
<th>A 562 before cloth adsorption</th>
<th>A 562 after adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample ID</strong></td>
<td>#1-1</td>
<td>#1-2</td>
</tr>
<tr>
<td><strong>Dilution rate</strong></td>
<td>14.96</td>
<td>20.00</td>
</tr>
<tr>
<td><strong>A562 1st test (AU)</strong></td>
<td>1.06</td>
<td>0.85</td>
</tr>
<tr>
<td><strong>A562 2nd test (AU)</strong></td>
<td>1.05</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>A562 3rd test (AU)</strong></td>
<td>1.07</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>A562 average (AU)</strong></td>
<td>1.06</td>
<td>0.83</td>
</tr>
<tr>
<td><strong>Lipase concentration in dilution (µg/ml)</strong></td>
<td>20.69</td>
<td>15.93</td>
</tr>
<tr>
<td><strong>Lipase concentration in original sol (µg/ml)</strong></td>
<td>309.55</td>
<td>318.68</td>
</tr>
<tr>
<td><strong>Average lipase concentration in sol (µg/ml)</strong></td>
<td>351.00</td>
<td>328.00</td>
</tr>
<tr>
<td><strong>Volume of lipase solution (ml)</strong></td>
<td>36.01</td>
<td></td>
</tr>
<tr>
<td><strong>Cloth weight (g)</strong></td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td><strong>Lipase purity (%)</strong></td>
<td>18.46</td>
<td></td>
</tr>
<tr>
<td><strong>Amount lipase adsorbed (mg/g cloth)</strong></td>
<td>17.33</td>
<td></td>
</tr>
</tbody>
</table>
10.5 Standard curve of nitrophenol concentration versus UV absorbance

![Graph showing the standard curve of nitrophenol concentration versus UV absorbance]

\[ y = 8.2813x \]
\[ R^2 = 0.965 \]

Figure 10-2 Standard curve of nitrophenol concentration versus UV absorbance (A410)
10.6 UV spectra of GA Solution

The UV spectrum of GA solution (1% in DI water, freshly prepared) was examined by Agilent 8453 spectrophotometer, and the result is shown in Figure 10-3. This GA solution was prepared by directly adding DI water to 25% of GA solution, which was stored in fridge at 4 °C. As shown, GA water solution exhibited strong adsorption at 235 nm and 280 nm, which corresponds to the studies by Terumichi [241] and Shlomo et al. [242]. The adsorption band at 280 nm could be assigned to n-π* transition of the C=O bond in the saturated aldehyde group, i.e. related to the free GA or pure GA. The adsorption at 235 nm could be due to π-π* transition of the C=C bond, which can be explained from the presence of α, β unsaturated aldehyde formed by di or polymerisation of GA. The UV spectra also prove the conclusion made by David et al. [16] that the commercial glutaraldehyde solution is not a pure GA monomer, rather a multicomponent glutaraldehyde mixture, comprising of GA monomer, GA oligomer and other impurities.

![Figure 10-3 UV adsorption of 1% GA solution (25% of GA solution diluted in DI water)](image)

However, when the GA solution is prepared with alkaline buffer solution instead of DI water, this UV spectrum changed as present in Figure 10-4. The adsorption at 280 nm (A280) has not changed too much compared to the GA solution prepared in DI water. But the adsorption at 235 nm (A235) was much stronger than the GA solution prepared in DI water, especially when this GA solution was
prepared with a more alkaline buffer solution. A235 for the GA solution prepared in buffer solution at pH 10.45 is beyond the detection limit of UV spectrophotometer. This enhancement at A235 is a fast process, occurring only after dilution within 10 minutes.

It was reported that this GA intermolecular aldol condensation is not only increased in alkaline solution, but also improved at elevated temperatures [241]. Thus above GA solutions prepared in various buffers were heated in water bath at 60 °C for 45 min. The UV spectra in above solution are compared in Figure 10-5 and Figure 10-6. In Figure 10-5, UV spectra of these GA solutions (1%) are present. As shown, A280 can be clearly seen, but most of the A235 are beyond the limit of the UV spectrophotometer. Thus the GA solutions were further diluted from 1% to 0.05% with DI water in order to obtain better view of A235, and their UV spectra are illustrated in Figure 10-6.

In Figure 10-6, as expected, A235 is enhanced compared to the GA spectra prepared in alkaline buffer solution without heat-up. The higher the pH is, more enhancement of UV absorbance exhibits. A280 also changes with the pH, as present in Figure 10-5. A280 still exists in the solution at pH of 7 and 8. However for the GA solution prepared in more alkaline buffer solution, the adsorption peaks have undergone a red shift to around 310 nm. This shift might come from C=O conjugation.
with C=C bond as the GA oligomer formed in alkaline solution bear an unsaturated aldehyde group in their molecules[241].

Figure 10-5 Comparison of UV spectra of 1% GA solution (prepared in various buffer solutions) after heating up in water bath at 60 ºC for 45 min

Figure 10-6 Comparison of UV spectra of 1% GA solution (prepared in various buffer solution) after heating up in water bath at 60 ºC for 45 min (solution in Figure 10-5 was diluted 20 fold with DI water)
As discussed in the earlier part of this section, A235 is related to the concentration of GA oligomer in solution and A280 stands for the concentration of GA monomer. The results obtained here suggest that the formation of GA oligomer is accelerated in a more alkaline buffer solution and at a higher temperature. The experimental results are in agreement with the previous literature: GA oligomerization increases with alkalinity and temperature [241]. As GA oligomer is regarded as a more efficient cross-linker than the GA monomer, thus when lipase immobilization was performed in a GA solution containing more GA oligomer, higher immobilized activity would be expected.
10.7 UV study of preparation of PEI-polyaldehyde

There is limited information regarding the reaction between PEI and GA in aqueous solution from the available literature [217, 220, 221]. Due to this limitation and the importance of PEI in enzyme immobilization, it would be beneficial to study this in more detail. To fill this research gap, the reaction between PEI and GA in water solution was examined in the section by the means of UV spectrophotometer.

UV spectra of PEI, GA and their mixture in water solution in ultraviolet range are shown in Figure 10-7. As shown, PEI (2% solution prepared in DI water, pH adjusted to 8) has no obvious adsorption peak in this range, which corresponds with the results published in literature [243]. UV spectra for GA solution (1% DI dilution) have two peaks, representing the pure GA component (peak at 280 nm A280) and GA polymer and oligomer (peak at 235 nm A235) respectively. However, when PEI and GA were mixed together (2% PEI in 1% GA, 100 fold dilution, solution stored at room temperature overnight), changes happen to the UV spectra in this mixture. As illustrated in Figure 10-7, A280 peak disappeared, while the A235 peak increased in size.

![Figure 10-7 UV adsorption spectra of GA solution (1% dilution in DI water), PEI solution (2% dilution in DI, pH 8.0) and PEI-GA mixture (2 %PEI mixed with 1% GA, 100 fold dilution before tested by UV)](image-url)
The UV spectra variation of PEI/GA mixture in A280 and A235 is in agreement with the UV spectra published in reference [217], indicating that PEI has been modified by GA in aqueous solution. This reaction has also been proven by the successful enzyme immobilization via the CVB and CRL protocol as described in previous sections (See Section 4, 5 and Section 6.1), where free aldehyde groups exist on PEI backbone after GA modification. As termed in Section 6.4, the newly formed product is named as “PEI-polyaldehyde”. The rate of reaction between PEI and GA was further viewed through successively monitoring the change of UV spectra for PEI and GA mixture within 2 minutes. It was found this initial reaction rate between PEI and GA is very rapidly. In order to demonstrate the reaction process, very dilute PEI (0.03 gram of 2% PEI solution) was added into 3 gram of GA solution (1%). As illustrated in Figure 10-8, within 2 minutes, A235 of this mixture increased from 1.4 to over 3 AU (absorbance unit). In the mean time, A280 disappeared.

![Figure 10-8 Change of UV spectra with time after PEI solution added into GA solution](image)

Furthermore, a colour change was observed in this PEI/GA mixture. Before mixing, the solution of PEI and GA is colourless. Right after mixing, the colour changed to a light yellow. This colour is gradually turned to pink, red and eventually into wine red colour. This process lasted from two hours up to several days. The degree of colour change was also found to be related to the ratio of GA to PEI. The higher the GA/PEI ratio, the deeper the colour became. After storing at room temperature and atmospheric pressure for one day, a picture was taken for the solutions prepared
with various concentrations of GA and PEI, and shown in Figure 10-9. As can be seen, in 0.1% GA solution containing 2% PEI, colour of the PEI-polyaldehyde solution is light yellow, whilst the colour turned darker with the increase in GA concentration. In 10% GA containing 2% PEI, the colour became wine red.

![Figure 10-9 Colours of PE-GA solution. All the solution was prepared in 2% PEI solution at pH 8.5, but the GA concentration was varied from 0.1%, 0.5%, 1%, 2%, 5% and 10%, as marked in picture.](image)

This colour change might be related to the formation of PEI-polyaldehyde in aqueous solution, which could be a useful indication for estimating the reaction progress between PEI and GA in aqueous solution. A full range of UV scanning released that a new peak at 540 nm (A540) appeared after GA is added into PEI solution, whilst there was an enhancement at A235 and decrease in A280. As discussed above, formation of PEI-polyaldehyde can be characterized by the variation of A280 and A 235. The peak associated with A540 could be also used for PEI-polyaldehyde characterization. Thus UV adsorption of these three absorbance peaks was compared in various concentration of PEI-GA mixture. As shown in Figure 10-11, a similar trend was observed for A 235, A280 and A540, when PEI solution (0.5%) was added in range of GA solutions. This result suggested that variation of A235, A280 and A540 were all related to the formation of PEI-polyaldehyde and could be used to characterize this reaction.
The variation of UV absorbance found in this study concur with the reaction mechanism proposed by Gutowiki et al., as illustrated in Figure 10-10 for PEI and GA reaction[221]. Although the reaction mechanism of GA in protein cross-linking has not yet been definitively proven, the formation of Schiff’s base production during the reaction between GA and PEI has been confirmed [217, 220, 221]. It is explained that the GA in PEI solution is not in monomer but the oligomer form, because of the alkaline nature of PEI itself. This GA oligomer was then reacted with the primary amine on PEI to ultimately yield the PEI-polyaldehyde. The cross-linking between PEI molecules could also occur. The presence of GA oligomer is confirmed by the UV spectra obtained in this study. The results in Figure 10-8 show that A280 is reduced rapidly while A235 increased considerably after GA addition into PEI. The enhancement in A 235 in PEI-GA mixture is quite similar to that in GA oligomer formed in heated alkaline solution as shown in Figure 10-5. This similarity suggests that GA oligomer does exist in the PEI-GA mixture.

![Figure 10-10 Mechanism of GA cross-linking reaction with PEI [221]](image)

The colour change for PEI-GA mixture associated with A540 might due to imine formation, as illustrated in the above mechanism. The appearance of yellow and red colour does not only happen in PEI-GA mixture, but also occurred in the reaction between protein (BSA, for example) and GA mixtures [244]. As both A540 and A235 reflect the formation of PEI-polyaldehyde, either of them can be used as indication of reaction process. However it is not convenient to utilize A235 and A280 (dilution is needed), so A540 was used in the following experiment instead.
The reaction between PEI and GA in aqueous solution was monitored by A540, and its change with time is illustrated in Figure 10-12 and Figure 10-13. In Figure 10-12, the change of A540 within the first 2 hours was recorded (2% PEI solution added in 1% GA solution). It can be seen that the change of A540 in the first 20 minutes is rapid, then the rate decreased, however, the colour continued developing steadily. Therefore, time span for A540 monitoring extended from hours to days. To reduce the experimental error, A540 for 0.5% of PEI in various GA solutions was monitored, and illustrated in Figure 10-13. The results show that the colour developed steadily in first three days, after which the rate of colour change was decreased. Considering the experimental error, readings taken after storage for four days therefore can be regarded as the same. Therefore, it can be concluded that the formation of PEI polyaldehyde is fast in the beginning, and then the reaction rate slows down. However, to complete this reaction, at least three days are required. It is also observed that the maximum A540 happened in the solution composed of equal amount of PEI and GA, which corresponds to the conclusion made by Carl-Gustaf and Jan Charister [220]. This probably can be ascribed to the fact that the ratio between PEI and GA is more meaningful rather than increase in the GA concentration alone in order to produce more PEI-polyaldehyde.
Figure 10-12 Monitoring A 540 change of PEI-GA mixture (2% PEI DI solution pH 8.0 added into 1% GA solution) within 2 hours.

Figure 10-13 Monitoring A 540 change of PEI-polyaldehyde within 11 days, solution used: 0.5% PEI solution (DI water dilution, pH 8) added in various GA solution.

It was reported in the prior literature [221] that the residue aldehyde activity in this PEI-polyaldehyde can last for three months or even longer. Thus in Section 6.4, the possibility of using this PEI-GA premixed product as an immobilization spacer arm was studied.