Development of a New Method of Template Removal for Molecularly Imprinted Polymers (MIPs) Utilising Biotechnology and Its Potential in Optical Sensing Application

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Abstract

Molecularly imprinted polymers (MIPs) are polymers synthesised with specific affinity towards a molecule of interest. Template removal is a critical step in MIP preparation as it influences the effectiveness of the MIP. This work introduces a template removal step during the synthesis of MIPs where yeast is utilised as the template remover, signifying the biotechnology aspect. Glucose imprinted polymers (GIPs) in the form of films were synthesised in this study using PVA as the polymeric membrane. This newly developed method for synthesising MIPs indicated a more environmentally friendly option as harsh chemicals were omitted and long polymerisation hours were avoided. Optimisation of the GIPs was conducted in terms of film formation and template concentration. Various plots and binding models such as the Scatchard plot, bi-Langmuir and Freundlich models were used to investigate the adsorption kinetics of the GIP. It was found that the GIP acquired a heterogeneity index of 0.5181, indicating heterogeneity and comprised mainly of two distinct binding sites. From the analysis of the binding models, the two distinct binding sites have binding affinities of 252.41 and 87.3 at densities of 1266.92 mM g\(^{-1}\) and 0.84 mM g\(^{-1}\) respectively. The presence of high densities of binding sites with higher affinity towards the glucose template molecule indicated successful imprinting within the PVA film. The GIP was studied for use as a potential optical sensor by coating it on fluorescent carbon dots (CDs). Optimisation for the coating process was performed. The binding activity of glucose molecules into the GIP was reflected through the changes of fluorescence intensity of the CDs. This demonstrated that the CDs were coated with GIPs and the fluorescence was affected by the removal and rebinding of glucose template molecules. A range of glucose concentrations were tested on the system as part of quantifying the analytical potentials of the CDs coated GIP. A Stern-Volmer plot was plotted with the \(K_{sv}\) value of 51.943 and the limit of detection for the glucose was evaluated to be 4.09×10\(^{-5}\) M. This study has successfully demonstrated a new method of template removal from MIPs using biotechnology and also the use of the synthesised GIP as a potential optical sensor.
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Declaration

I declare that this dissertation contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the examinable outcome. It is also to the best of my knowledge contains no material previously published or written by another person except where due reference is made in the text of the examinable outcome.

DIANA CHOO CHUNG YIING
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<th>Description</th>
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<tbody>
<tr>
<td>AgNPs</td>
<td>silver nanoparticles</td>
</tr>
<tr>
<td>ANS</td>
<td>3-amino-5-nitrosalicylic</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CDs</td>
<td>carbon dots</td>
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<tr>
<td>CDs@GIP</td>
<td>carbon dots coated with glucose imprinted polymer</td>
</tr>
<tr>
<td>CDs@NGIP</td>
<td>carbon dots coated with non-glucose imprinted polymer</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle's Medium</td>
</tr>
<tr>
<td>DNSA</td>
<td>3,5-Dinitrosalicylic acid</td>
</tr>
<tr>
<td>EDA</td>
<td>ethylenediamine</td>
</tr>
<tr>
<td>FETs</td>
<td>field-effects transistors</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence or Förster resonance energy transfer</td>
</tr>
<tr>
<td>GA</td>
<td>glutaraldehyde</td>
</tr>
<tr>
<td>GIP</td>
<td>glucose imprinted polymer</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>ICP</td>
<td>inductively coupled plasma</td>
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<tr>
<td>MIPs</td>
<td>molecularly imprinted polymers</td>
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<tr>
<td>MISPE</td>
<td>molecularly imprinted solid phase extraction</td>
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<td>MIT</td>
<td>molecularly imprinted technology</td>
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<td>NGIP</td>
<td>non-glucose imprinted polymer</td>
</tr>
<tr>
<td>NIPs</td>
<td>non-imprinted polymer</td>
</tr>
<tr>
<td>OSCs</td>
<td>organic solar cells</td>
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<tr>
<td>PDMS</td>
<td>polydimethyl siloxanes</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>PEI</td>
<td>polyethylenimine</td>
</tr>
<tr>
<td>PhLEDs</td>
<td>phosphor-based light-emitting diodes</td>
</tr>
<tr>
<td>PMMA</td>
<td>polymethyl methacrylate</td>
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<tr>
<td>PS</td>
<td>polystyrene</td>
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<tr>
<td>PTFE</td>
<td>polytetrafluoroethylenes</td>
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<tr>
<td>PtTFPP</td>
<td>platinum tetrakis pentrafluorophenyl porphine</td>
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<tr>
<td>PVA</td>
<td>polyvinyl alcohol</td>
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<tr>
<td>PVC</td>
<td>polyvinyl chloride</td>
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<tr>
<td>QDs</td>
<td>quantum dots</td>
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<tr>
<td>SPE</td>
<td>solid phase extraction</td>
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1. Introduction

1.1 Background

Molecular recognition represents the basis of most functions and structures involve in the biological world. In order for these living processes to be carried out, very specific interactions on a molecular level are required. Examples of these processes include the antibody-antigen interaction, the induced fit of an enzyme-substrate interaction and also the replication, transcription and translation processes of DNA (Ye & Mosbach 2001). The specific recognition properties present in these processes have inspired the birth of molecularly imprinted polymers (MIPs). Molecular imprinting allows for the creation of synthetic receptors with recognition properties akin to those of biological systems involving specific recognition sites as mentioned earlier. Generally, the molecular imprinting process involves the polymerisation of functional monomers with the presence of an analyte (template), followed by the extraction of the template which will leave cavities specific to the template. Subsequently, this forms the MIPs (Kryscio & Peppas 2012). Template removal is a critical step in the synthesis of MIPs. Therefore, this research has focused on the extraction step of the template during the synthesis of MIPs. A method of template removal is proposed via a biotechnology approach where biological agents and processes are utilised. Yeast was selected as the biological agent while glucose represents the candidate template for the MIP development, forming glucose imprinted polymer (GIP). Glucose was selected as the candidate template due to its importance in biomedical applications. For instance, glucose is the key marker for diabetes which has become prevalent in today's world. The potential of GIP for sensing was also investigated. Nearly all commercially available glucose sensors available in the market today work by the indirect detection of hydrogen peroxide produced by the enzymatic oxidation of glucose by glucose oxidase (Wu et al. 2010). However, enzyme based sensors have limitations such as the instability of the enzyme due to denaturation and the high cost for developing the glucose sensor (Pickup et al. 2005). Therefore, the synthesised GIP was coated around a fluorophore, namely carbon dots, to demonstrate the sensing potential.

The synthesis of GIP represents the initial stage for sensor development in this study. The combination of both the molecular imprinting and optical sensor technologies could produce novel detection probes with good sensitivity and
excellent selectivity (Gao et al. 2013; Mao et al. 2012). Therefore with the successful development of the GIP synthesis method utilising yeast to extract the glucose template and the incorporation of the GIP into a fluorescent optical detector, the fusion of MIP synthesis and its application as a sensor is able to be demonstrated.

1.2 Research Motivation

Templates are traditionally extracted via chemical means during MIP synthesis. Currently, there are three main approaches for the removal of templates which are the extraction with common solvents, supercritical or subcritical fluids, and physically-assisted solvent extraction (Lorenzo et al. 2011). However, these methods have shortcomings such as incomplete template removal, costly, distortion of cavities due to harsh extraction conditions, collapsing of the cavities due to possible swelling of the polymer during the extraction stage, and also the introduction of toxicity threats from the extraction solvents (Ellwanger et al. 2001; Levi & Srebnik 2009; Lorenzo et al. 2011; Yungerman & Srebnik 2006). These drawbacks have become the drive of the template removal method via biotechnological approach which is able to overcome most of the limitations.

To further enhance the potential of the developed GIP as a sensor, it will be coated around fluorescent carbon dots. A signal response has to be present for optical detection. Different responses such as fluorometry and visible spectrophotometry could be utilised for the optical detection aspect. Particularly in this study, fluorometry has been chosen as it offers advantages of being a cost-friendly measurement system, has low detection limits and also high sensitivity (Li et al. 2011; Liu et al. 2013b).

As a whole, the merging of fluorescence and MIPs will enable specific recognition of analyte via an optical method. This omits the complicated and time-consuming process of the conventional methods such as high performance liquid chromatography and gas chromatography/mass spectrophotometry that requires sample preparation before quantification can be done (Gao et al. 2013).
1.3 Research Objectives

This study includes two main parts which are the synthesis of GIP and the coating of GIP on carbon dots. Thus, the main objectives for this study are:

a) to synthesise, characterise and optimise the GIP,
b) to utilise yeast in template removal,
c) to synthesise, optimise and characterise GIP coated on carbon dots and
d) to demonstrate the effect of glucose rebinding on carbon dots coated with GIP for sensing application.

1.4 Research Direction

In order to achieve the objectives set for this study, the study directions have been determined.

For the first part, the main approach is to demonstrate a method for the removal of template during the synthesis of MIPs from a sustainable biotechnology approach. The MIPs will be synthesised in the presence of glucose templates, forming the intended GIPs. Characterisation and optimisation studies will be done for the synthesis of the GIPs. A biological agent, yeast, will be utilised in the removal of template, representing the biotechnology approach of this method. The success of the imprinting will then be tested with the rebinding study carried out in known concentrations of glucose solutions. Analysis of the binding activity for the synthesised GIPs will be carried out through binding models of Bi-Langmuir and Freundlich.

The second part involves the coating of carbon dots with the GIP. The main focus is to study the coating process of bare CDs with the synthesised GIP. The fluorescence intensity of CDs will be observed for any changes before and after coating attempts. Besides this, the effect of the presence of glucose on bare CDs and coated CDs will be studied and comparisons will be made. Based on the results, quantitative measurements of the coated CDs towards different concentrations of glucose will be carried out.
1.5 Organisation of Thesis

This thesis consists of five chapters with the following details.

Chapter 1: Introduction

The first chapter provides a brief background on the studies conducted throughout this thesis. It includes the motivation behind the research of MIPS which includes the discussion on existing systems, their shortcomings and also the proposed measure to improve the advancement in the development of MIPs. The aims to be achieved throughout this study are also stated. In order to carry out the research smoothly, the research directions must be defined and are therefore elaborated as part of the introduction.

Chapter 2: Literature Review

This chapter provides four main topics relevant to this study: MIPs, yeast, CDs and optical sensors. Under MIPs, the technology used in synthesising MIPs and the diverse usage of MIPs in various fields are covered. For yeast, a brief introduction on yeast, its metabolic pathways and the usage of yeasts are stated. For CDs, a background on CDs, different methods of CDs synthesis, benefits of using CDs and the applications of CDs are covered. Lastly, under optical sensors, a brief introduction, different types of sensor platforms available, methods of optical chemical sensor detection and also different kinds of immobilisation of sensing receptors are reviewed.

Chapter 3: Development of GIP

Chapter three covers the synthesis of GIPs utilising yeast as an agent for template removal. The materials, instruments and methods used will be listed and elaborated. The methodology includes the preparation of all solutions and reagents, optimisation of film setting temperature, water-swelling tests, viable yeast test, preparation of GIPs, effect of template glucose concentration on rebinding, and effect of glucose rebinding solution concentration of rebinding. All results obtained are discussed and followed with conclusions at the end of this chapter.
Chapter 4: Application of GIP

Chapter four covers the study on the coating of CDs with GIP. The materials, instruments and methods used are listed and elaborated. These include the synthesis of CDs, optimisation of fluorescence of CDs, effect of glucose on CDs, coating of CDs with PVA, coating of CDs with GIP and lastly the quantification of coated GIPs with glucose. All results obtained will be discussed and conclusions for this study are made at the end of this chapter.

Chapter 5: Conclusions and Further Work

Under this chapter, overall conclusions will are drawn for the entire study of this thesis. Further works which could be done to bring this study to the next level based on the results obtained are also suggested.

1.6 Summary

As a brief summary, this thesis consists of five chapters, ending with a list of references after. The first chapter gives a brief introduction to this study, the aims to be achieved and the research directions taken to achieve the aims. The following chapter consists of a review based on existing literature, covering topics which are relevant to the entire study. Chapter three represents the chapter involving the synthesis of GIPs. Existing methods of template removal for the synthesis of MIPs have multiple drawbacks. With this known, a method of template removal utilising yeast has been proposed and studied. A thorough methodology has been elaborated for each of the tests carried out for GIP synthesis. The findings are reported and discussed within this chapter. However, just synthesising a GIP is not substantial enough. Chapter four then covers the coating of fluorescent CDs with the synthesised GIP, demonstrating the application of GIP as a sensor. This enables correlation between the fluorescence of CDs and rebinding of glucose templates into the GIP. Chapter five consists of overall conclusions made for the entire study. Further works are also proposed with relation to both studies.
2. Literature Review

2.1 Molecularly Imprinted Polymers (MIPs)

2.1.1 Molecularly Imprinting Technology
Molecularly Imprinted Technology (MIT) is a technique employed to design receptors with high specificity and selectivity towards a particular analyte (Vasapollo et al. 2011). MIT has undergone substantial development in the last decade (Lorenzo et al. 2011) and has become one of the sustainable synthetic approaches to design strong molecular recognition materials which are able to mimic natural recognition processes such as biological receptors (Ye & Mosbach 2008).

2.1.2 Synthesis of MIPs
The MIT allows for the design and synthesis of materials containing specific sites of recognition for a particular analyte. The analyte (template) would be mixed with a concoction of functional monomers and their respective cross-linkers. During polymerisation, the monomers would interact with the template. After polymerisation, the template would then be extracted; leaving cavities of specific binding sites complementary in size, shape and chemical functionality to the template within the polymer (Kryscio & Peppas 2012). The presence of these cavities indicates the success in imprinting of template into the polymer, hence the formation of MIPs (Figure 2-1).
2.1.3 Approaches to Imprinting

There are different kinds of techniques for imprinting such as bulk, surface and epitope, particle or aspira partial imprinting (Lv, Tan & Svec 2013). Bulk imprinting is a conventional technique which has been used widely for the imprinting of small molecules. It represents the simplest and most direct approach for macromolecular imprinting. An example would be for protein imprinting where three-dimensional binding sites are formed for the whole protein within the polymer matrix (Hawkins, Stevenson & Reddy 2005; Kempe & Mosbach 1995; Ou et al. 2004; Tao et al. 2006). However, this kind of imprinting has its drawbacks as it requires grinding and sieving of the polymer produced. Thus, this may result in irregularly shaped particles and could also potentially destroy the binding sites via physical means (Vasapollo et al. 2011).

Surface imprinting is done by attaching a molecule to the surface of a substrate and polymerisation occurs around it, or synthesising a thin film of polymer with analyte templates in it (Kempe, Glad & Mosbach 1995; Liu et al. 2011a; Shi et al. 1999). When it comes to surface imprinting of proteins, metal chelating monomers such as Cu$^{2+}$ are used. Due to the usage of metal chelating monomers, this method of imprinting is restricted to protein templates which have exposed histidine residues. This is due to histidine being a chelator, allowing the binding to the metal ions (Kempe, Glad & Mosbach 1995).

Aspira partial or epitope imprinting involves just a small structural element of the protein as the template rather than the entire protein (Nishino, Huang & Shea 2006;
Rachkov & Minoura 2000, 2001; Tai et al. 2010; Tai et al. 2005). This opens another channel of imprinting proteins as only a short sequence is required, and the role of exposed histidine residues does not play a part.

2.1.4 Applications of MIPs

MIPs are considered a promising as they are able to recognise both chemical and biological molecules. This makes MIP applicable to a wide range of applications in diverse fields. MIP has become attractive as an affinity matrix for sensor development.

In the food industry, the detection of contaminants is vital as it determines the safety of food for consumption. These contaminants include pesticides, herbicides, harmful elements found in water, soil and packaging materials, naturally occurring toxins and also food dyes. Gao and team have managed to develop MIPs in the form of microspheres for the detection of cyhalothrin in honey (Gao et al. 2014). They incorporated allyl fluorescein as the signal provider for optical tracking. Huang and team also synthesised fluorescent MIPs in the form of microspheres for the detection of Bisphenol A (Huang et al. 2013). The magnetic particles were coated with the fluorescein isothiocyanate together with MIP for the detection of Bisphenol A. A caffeine imprinted polymer was developed by Alizadeh and team (Alizadeh et al. 2010). This MIP was then incorporated into a carbon paste electrode which aided in pre-concentrating the caffeine and also acted as the selectivity recognition receptor.

Besides the usage of MIPs in the food industry for detecting food contaminants, there is also interest in imprinting proteins. The development of protein imprinting is comparatively slower as proteins are delicate compounds to deal with (Bossi et al. 2007). There are three main complications according to Bossi et al. in protein imprinting. The solubility of protein in water that is not compatible with organic solvents for the preparation of MIPs, large number of functional groups in a protein that causes complex interactions with the functional monomers and lastly, a flexible conformation and structure which changes easily based on surrounding environment properties such as temperature. Despite this, research on imprinting proteins has been growing. There are some possible ways to overcome the abovementioned limitations and can be classified into two main ways, which is by having MIPs that recognise the shape of the protein, or to recognise part of or the entire sequence of the protein (Bossi et al. 2007). Pang and group managed to synthesise MIPs in the form of gel beads imprinted with a protein, bovine serum
albumin (BSA) (Pang et al. 2006). Another research team successfully imprinted the protein lysozyme in a polymer which was coated on carbon nanotubes (Yuan et al. 2014). Cadmium tellurium quantum dots have also been coated by MIPs for the detection of the protein cytochrome C (Zhang et al. 2011b).

MIPs have been shown to have great potential in the area of medical therapy and therapeutics (Sellergren & Allender 2005). In terms of drug delivery, there is a need for the control of drug amount released at the targeted site (Cunliffe, Kirby & Alexander 2005). A drug delivery system with molecular recognition characteristics would be the perfect solution as it will be able to specifically bind and release the encapsulated drug under optimum conditions at optimal doses. MIPs with ‘drug-like’ effects have also been studied. For instance, cholesterol selective MIP was developed to absorb cholesterol from its environment such as gastrointestinal fluids (Sellergren et al. 1998). Another example would be the development of a cholesterol lowering polymeric drug by molecularly imprinting bile acid sequestrants (Huval et al. 2001). These sequestrants bind to certain components of the bile acid, preventing them from being reabsorbed into the blood and aids in lowering cholesterol. Besides MIPs acting as drugs, uric acid imprinted membranes have also been synthesised for selective blood purification and filtration. These imprinted membranes acted as selective uric acid removers as they are able to remove uric acid from blood when there is an increase of serum uric acid values, which may lead to medical complications (Cristallini et al. 2004). Sellergen and team have suggested a few concepts to utilise MIPs in the field of drug delivery (Sellergren & Allender 2005). One of the concepts involves the containment of drug within the polymer of the MIP and when a specific release trigger binds into the MIP such as an antibody, or a specific cell surface epitope, the drug would be released. This ensures the specificity of the drug delivered as the delivery works only occurs in the presence of another compound. Another concept suggested was the internalisation of the entire MIP loaded with drug into the cell. The MIP constructed would bind to the surface of the cell and initiates the release of the drug-loaded MIP into the cell. Besides internalisation of drug-loaded MIP, the MIP would bind to the surface of the cell and trigger the release of the drug. These concepts are very interesting and open a lot of windows for exploration of MIP for specific and controlled drug delivery.

The usage of MIPs in sensors, especially those targeting biological molecules, enables for the usage of them in harsh environments as they are very stable.
sensors compared to biological receptors (Yano & Karube 1999). The fusion of MIP and biosensors is generally categorised based on how the signals of polymer recognition are converted to quantitative measurements. The different ways of obtaining these quantitative measurements involve the use of electrochemical and optical detections. A MIP imprinted with human cardiac troponin sensor was developed by Karimian and team (Karimian et al. 2013). In this study, the MIP was electro-polymerised on a gold electrode, and electrochemical methods were used to monitor the polymerisation process, template removal and also the rebinding. For fluorescent detection methods, the MIP can be constructed in three ways. When the analyte is fluorescing by itself, its binding to the MIP can be monitored directly. The second approach is when the analyte is not fluorescent so the polymer is tagged with a fluorescent reporter which will show changes in its fluorescence during rebinding. Lastly, also when the analyte is not fluorescent, a fluorescing analogue is incorporated in the MIP to compete with the analyte for the binding sites (Moreno-Bondi et al. 2008).

Another vital area of MIP usage is in the field of separation and purification science. Sample pre-treatment methods majority adopt the solid phase extraction (SPE) technique. It is normally used to concentrate compound of interest from sample and also to remove interfering compounds before analysis. MIPs can be used as the chromatographic material in SPE to allow higher specificity during SPE (Chassaing et al. 2004). The usage of MIPs in SPE is commonly referred to molecularly imprinted solid phase extraction (MISPE) (Sellergren 1994). MIPs have an advantage point of being able to selectively and specifically bind to a particular analyte in a pool of other similar structured compounds. This makes MISPE a very attractive technique for the development of trace analysis methods (Chassaing et al. 2004). The usage of MISPE is very similar to the traditional SPE, sharing the same steps of conditioning, loading, clean up and elution (Caro et al. 2006). The MIPs can be packed in small amounts into a cartridge, short high performance liquid chromatography (HPLC) column or even in a 96-well extraction plate (Baggiani, Anfossi & Giovannoli 2007). The first ever MISPE technology used in a 96-well block format was demonstrated successfully in 2004 for the determination of a pharmaceutical compound in plasma (Chassaing et al. 2004).
2.2 Yeast

2.2.1 Introduction to Yeast
Yeast (Figure 2-2), known as the *Saccharomyces* genus, is generally oval, spherical or cylindrical in shape and they undergo cell division by budding (Madigan et al. 2010). The budding process involves the formation of a new cell through gradual enlarging of an outgrowth from the parent cell, which eventually separates from the parent cell (Madigan et al. 2010). However, depending on the environment conditions, some forms of yeast such as baker's (or brewer's) yeast (*Saccharomyces cerevisiae*) are able to form filaments instead of reproducing as single cells through the budding process. The size of a single cell of *S. cerevisiae* is approximately 6 µm in diameter.

![Yeast cells as observed under 40X magnification via inverted light microscope.](image)

2.2.2 Yeast Metabolism
Glucose is a kind of sugar which represents the primary fuel for microorganisms and is the monosaccharide which is found most abundantly in nature (Johnston 1999). Yeasts thrive in conditions where sugars are present and are able to take up these sugars with and without the presence of oxygen (Madigan et al. 2010). This makes them facultative aerobes. They are able to switch between aerobic and anaerobic metabolism depending on the availability of oxygen in the surrounding environments. In the presence of high amounts of oxygen and glucose, yeast grows efficiently, producing more yeast cells and releasing carbon dioxide gas in
the process (Equation 1). However under conditions with no oxygen, yeast cells will utilise the glycolytic pathway and will switch to the fermentative metabolism. Under this metabolic pathway, they metabolise glucose and produce ethanol and carbon dioxide instead (Equation 2). In this process, new yeast cells production is reduced as opposed to its metabolism in the presence of oxygen. However, high amounts of the fermentation products carbon dioxide and ethanol are produced. This makes it very popular in the production of breads and most alcoholic beverages such as wine, whisky, brandy, beer, gin and vodka.

Glucose respiration: \[ C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 36/38 \text{ATP} \] (1)

Alcoholic fermentation: \[ C_6H_{12}O_6 \rightarrow 2CO_2 + C_2H_5OH + 2\text{ATP} \] (2)

The occurrence of an organism to ferment and respire glucose has become known as the Pasteur Effect (Madigan et al. 2010). Yeast represents a good example of demonstrating the Pasteur Effect. This is because glucose fermentation occurs at the maximum in the absence of oxygen, while the presence of oxygen would inhibit fermentation. The respiration process of yeasts in the presence of oxygen yields more energy for every glucose molecule compared to the fermentation process. As cells prefer to carry out metabolic pathways which are of most energetically beneficial to them, the respiration of glucose molecules would be preferred if oxygen is present. A good example of explaining this would be the production of wine (Madigan et al. 2010). Generally, grapes are squeezed for its juice, producing must. On the grapes, small amounts of yeast cells are present and these will be transferred to the must during the squeezing process. The yeast cells would grow by respiration due to the presence of oxygen, until the surrounding environment of becomes anoxic. The yeasts have undergone respiration in the juice as opposed to fermenting it as more energy is produced for the cell itself. Once the condition becomes anoxic however, the yeast switches from aerobic to anaerobic metabolism to produce alcohol.
2.3 Carbon Dots

2.3.1 Introduction to Carbon Dots
Generally, carbon is referred to as just a black material. They have always been shown to have low or no fluorescence and low solubility in water, however until recently, this has been proven otherwise (Esteves da Silva & Gonçalves 2011). Carbon dots (CDs) are a new class of nano-sized carbon materials which are smaller than 10 nm in size (Xu et al. 2004). CDs were discovered serendipitously during the gel electrophoresis purification step of single-walled carbon nanotubes (SWCNTs) (Xu et al. 2004).

2.3.2 Synthesis of Carbon Dots
The synthesis of CDs can generally be divided into three major steps namely, synthesis of carbon nanoparticles, passivation of the carbon nanoparticles and functionalisation of the carbon nanoparticles into CDs (Esteves da Silva & Gonçalves 2011). There are two main approaches to synthesise CDs, which are the top-down approach and the bottom-up approach (Figure 2-3) (Esteves da Silva & Gonçalves 2011). The top-down approach forms CDs through the post-treating of carbon particles which have been “fragmented off” from a larger carbon structure while the bottom-up approach forms CDs from molecular precursors (Esteves da Silva & Gonçalves 2011).

Some examples of top-down approach are the arc-discharged soot (Xu et al. 2004), laser ablation (Hu et al. 2009; Sun et al. 2006a), electrochemical shocking of carbon nanotubes (Zhou et al. 2007) and electrochemical exfoliation of graphite (Lu et al. 2009; Zheng et al. 2009).

As for the bottom-down approach, there are a few available methods such as acid dehydration of carbohydrates (Peng & Travas-Sejdic 2009), synthesis using microwaves and in aqueous solutions (Zhu et al. 2009), thermal carbonisation (Bourlinos et al. 2008; Liu et al. 2009; Wang et al. 2010) and soot obtained from candle and natural gas burner (Liu, Ye & Mao 2007; Ray et al. 2009; Tian et al. 2009).
2.3.3 The Beauty of Carbon Dots

CDs have emerged as a promising fluorophore with similar photochemical stability and photo-physical performance to that of the famously known quantum dots (QDs) (Esteves da Silva & Gonçalves 2011). QDs are generally made up of heavy metals and therefore show risk of cytotoxicity towards mammalian cells. There are also raising concerns of QDs as health and environmental hazards (Hardman 2005). CDs on the other hand have shown excellent biocompatibility towards cells. A study was conducted on the incubation of varying amounts of CDs with human colon adenocarcinoma HT-29 cells cultured in Dulbecco's Modified Eagle’s Medium (DMEM) media under standard cell culture conditions (Jaiswal, Ghosh & Chattopadhyay 2012). Low cytotoxicity was observed after 24 hours of incubation indicated by observation of cell viability of above 80% even at high CDs concentration. It was suggested that the chemical inertness of CDs could be the contributing factor of its low cytotoxicity which prevents the release of any toxic species unlike the QDs mentioned earlier (Zhao et al. 2008). Another advantage of CDs is their solubility in water allowing them to be easily used in different applications (Esteves da Silva & Gonçalves 2011). Some of the synthesis approaches for CDs are generally simple with the one-step synthesis route (Hao et al. 2014; Jaiswal, Ghosh & Chattopadhyay 2012; Wei et al. 2014).
2.3.4 Applications of Carbon Dots

QDs have been widely used in the industries for biomedical imaging (Alivisatos 2004; Chen & Gerion 2004), drug and gene delivery (F. Scherer et al. 2002; Yu & Chow 2005), electronics (Wu et al. 2004), metal ions detection in environmental and food monitoring (Wang et al. 2014) and lastly, in the detection of pathogenic bacteria (Huang et al. 2014). However as mentioned previously, QDs are mainly made up of heavy metals, therefore raising cytotoxicity concerns in the health and environment sectors (Hardman 2005). With this major drawback of QDs, CDs can be an attractive substitute for QDs.

The first study conducted on CDs for bioimaging purposes was done by Sun and team (Cao et al. 2007). They demonstrated CDs as a cell imaging tool by culturing human breast cancer MCF-7 cells and incubating them with CDs. The breast cancer cells showed fluorescence when imaged under fluorescence microscope after internalisation of the CDs. CDs prepared by Liu and co-workers using microwave assisted pyrolysis of D-Glucosamine showed strong fluorescent properties (Liu et al. 2013a). These CDs showed multicolour photoluminescence, low toxicity and good water solubility properties. They demonstrated COS-7 cells tagged with CDs emitting blue, green and red fluorescence when excited at 405 nm, 488 nm and 543 nm wavelengths. Due to the multicolour luminescence, the CDs allowed for the flexibility of using a suitable excitation wavelength for observation (Liu et al. 2013a). Another study was conducted by a separate team where CDs were obtained from commercially available Nescafe® original instant coffee (Jiang et al. 2014). The CDs produced were tested for in vitro cell imaging and also in vivo small animal imaging. For the in vitro cell imaging studies, human hepatocellular carcinoma cell line SMMC-7721 cells were used. These cells were incubated with the CDs for 24 hours and their fluorescence was captured using a fluorescent microscope to capture blue, green and red region images. It was observed that the human hepatocellular carcinoma cells incubated with CDs became bright compared to those control cells without CDs. For the in vivo small animal imaging study, the guppy fish was used. The fish was exposed to CDs via a feeding technique where the fish food was mixed with the CDs. The fish treated with CDs-food mixture showed fluorescence whereas the control fish did not. This shows that CDs can be used in the bioimaging industry for both cells and animals.

In the field of gene delivery, the use of fluorescent labels has been around for quite some time. Nanoparticles are tagged with fluorescent labels for the purpose of
gene transfection and optical intracellular trafficking (Roy et al. 2005). Besides that, nanomaterials have also been studied as a nonviral vector for nucleic acid delivery (Liu et al. 2012). Examples such as QDs (Zhang & Liu 2010), gold nanoparticles (Lee et al. 2011), nanodiamonds (Zhang et al. 2011a), graphene (Kim et al. 2011), silica nanoparticles (He et al. 2011), and carbon nanotubes (Liu et al. 2011b). CDs have been studied to be used in gene delivery as well. Liu and team synthesised CDs which were functionalised by polyethylenimine (PEI) as the passivation agent and also as a polyelectrolyte to condense DNA for gene transfection (Liu et al. 2012). The CDs were synthesised via the microwave assisted pyrolysis method, where glycerol as the starting material was mixed with PEI and then placed in a microwave to undergo pyrolysis. This was unique as the synthesis and passivation of CDs were done in one pot. The CDs formed a complex with pDNA and were then transfected into cells of COS-7 (African green monkey kidney cells) and HepG2 cells (human hepatocellular liver carcinoma line). Their study showed successful gene transfection into the two different cell groups. They were able to observe fluorescence in the cells which were transfected with the CD/pDNA complex while the negative controls showed no fluorescence.

CDs have also been demonstrated to be growing popularity in the field of electronics. A study was carried out by Kwon and team on CDs and their ability to be used in electronic devices (Kwon et al. 2013). They synthesised CDs and immobilised them in a film to act as channels of field-effects transistors (FETs). This opened a whole new range of prospects for the fabrication of CDs based electronic devices such as light-emitting devices, optical sensors and solar cells. With this known, Kwon and team then further developed the CDs. The CDs were synthesised based off a ‘water-in-oil’ emulsion, and were capped with oleylamine, where the sizes of the CDs were controlled using emulsifiers (Kwon et al. 2014). These CDs were tested for use in phosphor-based light-emitting diodes (PhLEDs) as phosphors to convert UV to visible light. The CDs were dispersed in a transparent polymer matrix to fabricate phosphor films. The dispersion of CDs in a polymer matrix has prevented solid-state quenching as well as providing a mechanical support. Films containing different CDs were developed, which produced bright green, yellow and white light. The various colours were a result of the different sized CDs fabricated into the films. Besides studying the use of CDs in PhLEDs, Kwon and team also further explored the use of CDs as photoactive materials and fabricated CDs-based organic solars (OSCs) (Kwon et al. 2014).
CDs have also been shown to be useful in the area of metal sensing. A CDs system can be developed to detect the presence of both copper (II) ions (Cu\(^{2+}\)) and L-cysteine (Zong et al. 2014). The CDs functioned as fluorescent probes with an ‘on and off’ function for the detection mechanism. The detection of Cu\(^{2+}\) was based on the quenching of fluorescence of the CDs, while the detection of L-cysteine occurs by the binding of L-cysteine towards the Cu\(^{2+}\) ions. This binding caused the Cu\(^{2+}\) to be removed from the surface of the CDs, resulting in the restoration of the fluorescence. This system was able to detect both Cu\(^{2+}\) and L-cysteine via quenching for Cu\(^{2+}\) and then fluorescence restoration by L-cysteine. Simple and label-free CDs were also demonstrated to have metal sensing abilities. Liu and team demonstrated the capabilities of CDs in detection of mercury ions (Hg\(^{2+}\)), by great quenching in fluorescence when Hg\(^{2+}\) ions were present in the solution (Liu, Liu & Zhang 2012). The probe was tested on drinking water and found that the CDs experienced quenching as well. To validate their results, the Hg\(^{2+}\) concentration measured by the CDs in the water was checked against the inductively coupled plasma (ICP) optical emission spectrometer. They found out that the CDs produced showed better accuracy compared to ICP.

2.4 Optical Chemical Sensors

2.4.1 Introduction

Interests in optical chemical sensors have been growing in the field of analytical chemistry (Samadi-Maybodi & Rezaei 2014). They are great tools for analysis and detections for wide range of applications such as pharmaceuticals, healthcare and biomedical research to environmental monitoring and to uses in the battlefield and homeland security (Narayanaswamy & Wolfbeis 2004). Chemical sensors are defined as, “Chemical sensors are miniaturised devices that can deliver real time and on-line information on the presence of specific compounds or ions in even complex samples” (Cammann et al. 1996; Wolfbeis 2004). Through this definition, a sensor is then required to be able to function in complex samples, ideally without sample pre-treatment and also small in size. Generally, a sensor system is made up of three main elements. They are the sample which contains the analyte, a transduction platform and lastly, the signal-processing stage which translates the signal obtained into concentrations of the analyte (McDonagh, Burke & MacCraith 2008). The advantages of utilising optical chemical sensors are their capability of allowing for remote sensing, immunity to electro-magnetic interference, ability to provide multiple signal detections within one device (Fan et al. 2008), cost-
effectiveness, performance in harsh environments and lastly, the possibility of miniaturisation of the optical chemical sensor (Samadi-Maybodi & Rezaei 2014). For the development of an optical chemical sensor, certain key aspects of the sensor must be determined. This includes the determination of sensor platform type, method of sensor detection and when required, the immobilisation matrix.

2.4.2 Types of Sensor Platforms

In the development of an optical chemical sensor, the kind of physical platform incorporated into the sensor plays an important role. Generally, there are two main kinds of sensing platforms available; fibre optic and planar waveguide-based sensor platforms.

Optical fibres are the most commonly used platforms in the design and development of optical chemical sensors (McDonagh, Burke & MacCraith 2008). Optical fibres are capable of transmitting light based on the principle of total internal reflection. This allows the light rays to be conducted through the fibre optic core with minimal loss to the surroundings (Marazuela & Moreno-Bondi 2002). Fibre optics also enable optical spectroscopy to be conducted over long distances, on sites where conventional spectroscopy cannot access and on spots along the fibre itself (Wolfbeis 2004). Other advantages of fibre optics are its capability to perform in harsh surrounding environments, its immunity to magnetic or electronic interference, multiplex capabilities to guide light of different wavelengths and directions and lastly, ease of scaling down to be used for in-vivo measurements (Marazuela & Moreno-Bondi 2002). The usage of optical fibres in optical sensors is classified into extrinsic and intrinsic sensors. In extrinsic sensors, the optical fibre detects the fluorescence or colour of an immobilised label, indicator or any other kind of optically detectable bioprobes which is attached to the optical fibre itself (Wolfbeis 2004). In intrinsic sensors, the optical fibre detects the fluorescence or colour of an immobilised label, indicator or any other kind of optically detectable bioprobes which is attached to the optical fibre itself (Wolfbeis 2004). Examples of developed intrinsic sensors are sensors which detect analytes such as chloride (Huber et al. 2001b), pH (Vasylevska et al. 2006) and oxygen (Valledor et al. 2009).

Planar waveguide-based chemical sensors have become an attractive option for sensor development. It allows for the integration of multiple functionalities within one sensor chip. Together with microfabrication and microfluidics technologies, lab-on-a-chip systems are born (McDonagh, Burke & MacCraith 2008). Planer
waveguide-based sensors show a distinct practical advantage over optical fibre based sensors, with the ability to be used in environments outside of the laboratory, demonstrating their robustness. It has a planar substrate as its core which can be made of plastic, silicon or glass, forming the basis of the chip and a second waveguide layer which is added onto the planar substrate. The purpose of the waveguide layer (sometimes may be the planar substrate itself), is for the propagation of light which assists in the operation of the planar platform as a sensor. This occurs via the interaction of the evanescent field of light with the sensing environment containing the analyte above the waveguide layer.

2.4.3 Methods of Optical Chemical Sensor Detection

Optical chemical sensor detection can be classified under three main groups, namely direct label-free sensors, fluorescence-based sensors and reagent-mediated sensors.

Label-free sensors detect the molecule of interest without prior labelling or changing of their natural forms. This detection is simple and cost-effective, which enables for both kinetic and quantitative measurement of molecular interaction (Fan et al. 2008). Label-free optical sensors carry out detection based on a few methods such as via ultraviolet and infrared absorption, Raman spectroscopy, refractive index of the analyte, and also via fluorescence. An example of a simple label-free sensor is one developed by Feng and team, where a label-free pH sensor was developed utilising fluorescent silicon nanodots (Feng et al. 2014). The silicon nanodots showed different fluorescence intensity at different pH values. A label-free DNA methylation biomarker detection sensor was also developed for early cancer detection and progression and also in drug treatments (Shin et al. 2013). This sensor was developed by immobilising the methylated DNA probes which are complementary to the target DNA on a silicon surface using the amine modification method.

On the other hand, fluorescence-based detection involves the labelling of the target molecule or biorecognition molecules with a fluorescent tag such as dyes or fluorescent nanoparticles. This method of detection works based on the interaction between the biorecognition and target molecule (Fan et al. 2008). An example is the attachment of a red fluorescent protein onto a copper (I) binding protein at three different sites to detect the presence of copper in an organism (Liang et al. 2014). Besides using fluorescent proteins, fluorescent nanoparticles such as QDs have been used as well. In one study, ZnO-nanoparticle coated with silica was surface
modified with biotin to tag avidin beads onto the biotinylated QDs. These biotinylated QDs were used as fluorescent probes in bioimaging systems by attaching it to nerve cells of mouse spinal cord by antibody and avidin-recognition molecules (Matsuyama et al. 2013). With this, these synthesised QDs can be used as fluorescent probes to detect the presence of specific cells of interest.

Reagent-mediated sensors are used when an analyte does not have an appropriate optical property such as luminescence or absorption which can be detected rapidly. Therefore, reagents are required to aid in exhibiting the presence of the analyte through its own optical response (McDonagh, Burke & MacCraith 2008). A common example would be the use of pH responsive dyes for the development of optical pH sensors. Responsive dyes such as methyl red and phenol red have been entrapped in a sol-gel matrix for the fast detection of pH for biomedical purposes such as the pH of the esophageous (Fabbri et al. 2011). Besides the use of chemical sensors for biomedical purposes, a wide range pH optical sensor was developed by immobilising both neutral red and thionin on an agarose film coated glass slide (Hashemi & Zarjani 2008). Besides pH optical sensors, temperature and oxygen sensors have been developed based on the reagent-mediated detection method. A composite xerogel film containing two dyes which are oxygen and temperature sensitive was developed for a dual-detection optical sensor. The oxygen sensitive dye used in this sensor was platinum tetrakis pentrafluoropheny porphine (PtTFPP) while the temperature sensitive dye was 7-amino-4-trifluoromethyl coumarin (Chu & Lin 2014).

2.4.4 Immobilisation of Sensing Receptors

When reagents or nanoparticles are used in optical chemical sensors, they require immobilisation in a solid matrix. The purpose is to encapsulate the reagent or nanoparticles for the analyte to interact with it. The matrix can be in the form of a thin film or a porous structure allowing for the interaction of analytes. There are two common categories of immobilisation matrixes used for optical chemical sensor development, a sol-gel matrix and a polymer matrix.

Sol-gel technology has been very attractive in terms of sensor development due to its versatility and simplicity (Doong & Tsai 2001; Floch & Belleville 1994; Lin et al. 2006). The sol-gel technique involves the formation of a porous silica matrix through condensation and hydrolysis of metal alkoxide solutions under either basic or acidic conditions. In chemical sensors, the resulting porous matrix would contain the analyte-sensitive component which the analytes would come in contact with
once they diffuse through the porous matrix (Avnir, Levy & Reisfeld 1984; McDonagh, Burke & MacCraith 2008). Advantages of using sol-gel is the ability to trap the sensing receptors, non-leaching of the encapsulated dye, photostability and toughness of the matrix, resistance to harsh environments, reduction of interference from external components and lastly, high loading of receptors that contribute to rapid response times which may be viewed under naked eyes (e.g. colour change of reagent) (Avnir, Levy & Reisfeld 1984; Tang et al. 2003; Yang & Saavedra 1995). Conditions during the sol-gel formation influence the porosity and polarity of the resulting matrix. Parameters such as the type of solvent used, pH, temperature, water to silica molar ratio and the catalyst all contribute to the end result of the matrix formed (McDonagh et al. 1996). The sol-gel must be porous enough for the diffusion of analyte to contact the sensing receptors, but not overly porous that allows for leaching of the sensing receptors (Samadi-Maybodi & Rezaei 2014). A newly developed optical sensor for the determination of zinc was developed using a sol-gel material on active glass slides containing 1,5-diphenylcarbazone. In this study, 1,5-diphenylcarbazone would change in its colour intensity based on the concentrations of zinc, which can be used for quantification purposes (Samadi-Maybodi & Rezaei 2014). Besides entrapping coloured dyes or reagents in sol-gel films, nanoparticles can be entrapped as well for the development of chemical sensors. A study was done based on silicate sol-gel stabilised silver nanoparticles (AgNPs) as a sensor for the detection of hydrogen peroxide, mercury ions and nitrobenzene (Rameshkumar, Viswanathan & Ramaraj 2014).

Even though sol-gel matrices have shown many desirable features for the development of optical chemical sensors, polymers have also been widely used for the same purpose. Some polymers such as polyvinyl chloride (PVC), polymethyl methacrylate (PMMA), polystyrene (PS), polytetrafluoroethylenes (PTFE), polydimethyl siloxanes (PDMS) and those of cellulose derivatives are more suitable compared to sol-gels when the end applications are of high temperatures (McDonagh, Burke & MacCraith 2008). The hydrophobicity of the matrices also plays a role depending on the end usage of the sensors. When oxygen sensors are constructed, hydrophobic matrices such as PDMS (Xue et al. 2014) and PMMA matrices are usually selected (Ramamoorthy, Dutta & Akbar 2003). For pH sensing, hydrophilic matrices like ethyl cellulose derivatives would usually be used (Posch, Leiner & Wolfbeis 1989). Fibres made from polyvinyl alcohol (PVA) hydrogel have also been shown to act as a sensor for heavy metal cations as demonstrated by
Tou and team (Tou, Koh & Chan 2014). The sensor acted based on the presence of the nickel ion that promoted the cross-linking of the PVA hydrogel which can be detected by its refractive index. A colorimetric naked-eye hydrogel sensor was also developed for a rapid determination of iron (III) ions (Fe$^{3+}$) (Ozay & Ozay 2013). The sensor was partially made up of a rhodamine group-containing polymer, N-(Rhodamine-6G) lactam-N’-acryloyl-ethylenediamine (RH6GAC), which has the ability to show the presence of Fe$^{3+}$ ions under visible light, via naked-eye detection. Besides showing colour change, fluorescence was also shown to be emitted in the presence of Fe$^{3+}$ ions. The developed sensor can be reused by submerging the hydrogel in ethylenediamine (EDA) solution, which removes the Fe$^{3+}$ ions. Besides physical fibers and hydrogels, an emulsion system stabilised by polymers made up of a hydrogel with trapped plasticiser droplets has been developed for a nitrate-selective optical sensor by Huber and team (Huber et al. 2001a). Cationic potential-sensitive fluorescent dye was placed near the surface of the plasticiser droplets. This dye is able to extract the nitrate out of sample solutions, forming a complex. Due to the lipophilicity of the formed complex, it moves into the plasticiser droplet resulting in an increase in fluorescence intensity of the dye, allowing for nitrate detection.
3. Development of MIPs Utilising Biotechnology

This chapter describes the study of MIP synthesis utilising yeast as a biological agent to remove glucose molecules that were selected as the template analyte in this study. This chapter is organised in an order of the materials, reagents and instruments, methodologies, results obtained, discussion of results and lastly the conclusions achieved from the study.

3.1 Materials & Reagents

All the chemicals and reagents used were of analytical grade or higher unless otherwise stated. All percentage measurements were of a weight to volume ratio (w/v %) unless otherwise stated. Poly (vinyl alcohol) (PVA) with hydrolysis degree of 99+ % and average molecular weight of 130 000 g/mol was purchased from Aldrich. D(+) - Glucose (monohydrate), dinitrosalicylic acid, sodium potassium tartrate, methylene blue and sodium citrate were purchased from R&M Chemicals. Glutaraldehyde (GA) solution, Grade II, 25% in H2O was purchased from Sigma-Aldrich. Commercial baker’s yeast, *S. cerevisiae* was obtained from the local supplier in Kuching, Sarawak, Malaysia. Ultrapure water was obtained from Millipore Milli-Q Advantage-A10 and Millipore Elix-5 water purification system (~18.2 Ω, 25 ºC) and used as the solvent system throughout this work.

3.2 Instrumentation

All sonication were performed using Branson B5510 Ultrasonic Cleaner. The water bath used in this work was Memmert’s Waterbath Model WNB 10. All drying steps were carried out in a controlled temperature drying oven from TUFF: OEM Lab Equipment and model TFAC-136. Microscopic images were obtained using the Nikon Eclipse Ti-S inverted light microscope. All absorbance measurements were taken using Varian Inc. Cary®50 UV-Vis Spectrophotometer (dual beam configuration) running from 800.00 to 200.00 nm and baseline was obtained using ultra-pure water. Quartz cuvette having two sides clear with a path length of 1cm was used for all absorbance studies.
3.3 Methodology

3.3.1 Preparation of Solutions and Reagents
Throughout this section, specific reagents and solutions were required. For the staining of yeast cells in the yeast viability test, methylene blue was prepared by dissolving 0.001 g of methylene blue and 0.200 g of sodium citrate in 20 mL of ultra-pure water and filtered through filter paper (Painting & Kirsop 1990). Throughout this section, stock PVA of 5.0% was prepared by dissolving 2.50 g of PVA in 50 mL of ultra-pure water under heating at 90 °C and stirring for two hours. The heating was then switched off and the PVA solution was left to stir while cooling down. For the quantification of glucose, 3, 5-Dinitrosalicylic acid (DNSA) reagent was used. The DNSA reagent was prepared by adding 2.50 g of dinitrosalicylic acid, 75 g of sodium potassium tartrate (Rochelle Salt) and 4.0 g of sodium hydroxide into 250 mL of ultrapure water (Coughlan & Moloney 1988). This mixture was gently heated up under stirring to dissolve the reagents until a clear orange solution was obtained.

3.3.2 Preparation of Glucose Imprinted Polymer Films
The GIP films were prepared based on a method reported by Figueiredo, Alves and Borges with modifications (Figueiredo, Alves & Borges 2009). A stock solution of 1.0% glucose was prepared by dissolving 0.50 g of glucose in 50 mL of ultra-pure water under stirring until all of the powder has been dissolved. GA was used as the crosslinker for PVA. The GA solution was used as it was without further dilutions. A fixed volume of 200 µL of 1.0% glucose solution was added to 4 mL of 5.0% PVA solution (polymer) in a glass vial. This solution mixture was left to sonicate for 30 minutes to ensure proper mixing. It was then spread onto a Petri dish to produce a film that was dried at 40°C for 48 hours. 0.100 g of yeast in 20 mL of pre-warmed ultrapure water (40°C) was poured onto the film to extract the glucose templates present. After incubation of four hours, the yeast solution was discarded and the film was rinsed with 20 mL of ultrapure water. The films were then dried at 70°C overnight to denature the active yeast cells which could possibly be present on the film. The dried films were then used for further studies on rebinding. Control films known as non-glucose imprinted polymer (NGIP) films were synthesised the same way without the addition of glucose.
3.3.3 Optimisation of GIP Matrix

A. Film Setting Temperature

During the synthesis of GIP, different synthesis parameters have been optimised. The first optimisation was done for the film matrix of the GIP. Different temperatures were studied to obtain a suitable temperature for the setting of PVA films. 4.0 mL of 5.0% PVA solution was poured into a glass vial and left to set in a drying oven of controlled temperature. The PVA samples were left to dry for 24 hours. The temperatures studied were 40, 60 and 70°C. Observations of the physical appearance of the films were made under the naked eye.

B. Film Setting Duration and Glutaraldehyde Content

Besides the temperature for film setting, the effects of setting duration and glutaraldehyde content of the PVA films were studied. The studies were based on the water-swelling property of the film. The degree of water-swelling would indicate the robustness of the film. A robust film would be preferred as it would prevent the collapse of cavities within the GIP film. For the water-swelling test, a series of different GA to PVA volume ratios from 0.00 to 0.10 were left to set for 24 hours at 40°C first. After that, the films were cut into strips of 2 x 2 cm² and their dry weights were taken. The film strips were then immersed into approximately 5.0 mL of ultra-pure water at 40°C for 24 hours to investigate the water uptake of the films. 40°C was chosen as it was the temperature used during the template removal step via yeast. After 24 hours, the films were taken out and their weights measured after blotting off the excess surface solution. This was repeated for films which were set at 40°C for 48 hours and immersed in ultra-pure water at 40°C. The water-swelling degree (g/g) was used as the measurement of water-swelling by the films and calculated as shown in Equation 3.1 (Varshosaz & Koopaie 2002).

Equation 3-1: The equation used for calculating water-swelling degree (g/g) of films.

\[
\frac{\text{swollen polymer weight (g)} - \text{dry polymer weight (g)}}{\text{dry polymer weight (g)}}
\]
3.3.4 Optimisation of Glucose Concentration for GIP Synthesis

The template concentration of the GIP was also optimised in this study. This was to obtain the concentration of glucose which would allow the formation of GIP with the best rebinding capabilities. The study was characterised based on the rebinding capabilities of glucose molecules into the GIP. The stock solution of 1.0% glucose was prepared as a source of the template molecule. Different volumes of the stock glucose solution from the range of 0 to 1000 µL were used to make up the incubation solutions. After the treatment of yeast and overnight drying, the GIP films were then immersed into the incubation solutions. The amount of glucose bound on the GIP was obtained via indirect measurements, where the amount of glucose left in the solution was measured. This would provide the amount of glucose bound on the GIP by subtracting the amount identified in the solution from the initial concentration of glucose used. DNSA was used as the indicator for the quantitative glucose assay. DNSA is a quantitative and qualitative indicator for reducing sugars such as glucose. During the DNSA test, a redox reaction occurs resulting in a colour change of the DNSA reagent from yellow to brown. In glucose, oxidation of the aldehyde functional group occurs to form the carboxyl group. Concurrently, the yellow DNSA reagent would be reduced to 3-amino-5-nitrosalicylic (ANS) acid which is brown in colour (Miller 1959). As the colour of ANS deepens as measured by UV-Vis, this indicates an increasing concentration of glucose present in the solution. This forms the basis of the DNSA test in this study. The NGIP film underwent the same treatment, acting as the control.

For the DNSA test, 0.50 mL of DNSA reagent was added to 1.00 mL of glucose solution. This mixture was heated at 90°C for 15 minutes. The sample vials were then left to cool in water down to room temperature to stop the reaction. After cooling down, the absorption readings of the glucose solutions were taken.

3.3.5 Viable Yeast Test

During the preparation of GIP films, the removal of glucose templates was done using yeast cells. Since yeast was tasked with removing the glucose templates, they will have to be deactivated after the removal step. This is because yeast will be able to metabolise the external glucose which will be added in for rebinding studies. In order to ensure that the yeast cells have been denatured and that they will not play an interfering role during the rebinding study, the films were dried at 70°C overnight. Therefore, a quick assay on the viability of yeast at 40°C and 70°C was done using the methylene blue stain method to determine the suitability of the
temperature for killing the yeast cells. Yeast suspensions were prepared by using a known mass of baker’s yeast pellets mixed with 10 mL of 1.0% glucose solution and later agitated to dissolve the pellets. The yeast suspensions were placed at 40°C and 70°C respectively. 1.0 mL of methylene blue solution was added to 500 µL of yeast suspension and gently swirled to ensure an even mix. This yeast sample was then placed on a glass slide and observed under 40X magnification using an inverted light microscope.

### 3.3.6 Rebinding Studies for NGIP and GIP Films

After the optimisation of the GIP films were done, rebinding studies for the GIP studies were carried out. This was to characterise the NGIP and GIP films in terms of their heterogeneity or homogeneity and their binding affinities. The GIP films were imprinted with the optimised template glucose concentration for further studies dealing with the rebinding activity of GIP films. Different glucose rebinding solution concentrations were obtained by successive dilution of the glucose stock solution (1.0%). The film was cut into strips weighing approximately 0.01 g and placed into the respective vials containing the glucose rebinding solution. DNSA was used as the indicator for the glucose assay. GNIP film was treated the same way, acting as the control.
3.4 Results & Discussion

3.4.1 Optimisation of Film Setting Temperature

As part of the optimisation study, the films were left to set at different temperatures for 24 hours (Table 3-1). The desirable attributes of the films was that they dried clear and could be easily removed from the glass vial. An additional sample was also performed by setting the film at room temperature. However, the sample was not dried after 24 hours and was therefore omitted from the Table 3-1. For the films which were dried at relatively higher temperatures such as 60 and 70°C, cloudy patches were observed to form on the films. Some parts of the films were brittle as they tore when attempts were made to remove them from the glass vial. However, the films were stuck to the bottom of the glass vials as they could not be completely removed (Figure 3-1). Therefore, films were left to set at a lower temperature of 40°C. The resulting films dried clear and were easily removed from the jar (Figure 3-2).

Table 3-1: Physical appearance of films observed when set under different film setting temperatures for 24 hours.

<table>
<thead>
<tr>
<th>Temperature for film setting (°C)</th>
<th>Appearance of Film</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>Clear</td>
</tr>
<tr>
<td>60</td>
<td>Cloudy patches</td>
</tr>
<tr>
<td>70</td>
<td>Cloudy patches</td>
</tr>
</tbody>
</table>

From the results obtained, it was observed that temperature represented one of the parameters which affected the drying of polymer solutions. At higher temperatures, the rate of water evaporation from the sample solution would be higher compared to those at lower temperatures. Water evaporation results in the concentration of the polymer chains in a closely packed arrangement, thus forming films (Keddie et al. 1995). The cloudy patches observed could be due to the rapid removal of water, causing an uneven concentration of polymer particles throughout the film. The breakage of films due to brittleness could be caused by the complete drying of the films at higher temperatures above 40°C (Jayasekara et al. 2004). Based on the determined optimum temperature for film setting of 40°C, all the PVA films were set at this temperature throughout the rest of this study.
Figure 3-1: Films set at A: 60 and B: 70°C where cloudy patches were observed along the edges and the films could not be removed from the glass vial.

Figure 3-2: Film set at 40°C dried clear and was able to be removed from the glass vial.

3.4.2 Optimisation of Film Setting Duration

PVA is a synthetic water-soluble and biocompatible polymer that is commonly casted into films. PVA films have poor stability in aqueous solutions, leading to a decrease in its end application performance (Figueiredo, Alves & Borges 2009). One common method of increasing the strengths of the PVA films is by cross-linking. GA was chosen as the cross linker for the PVA films due to the simplicity that thermal treatment was not needed for the cross-linking reaction.
PVA films were left to set at 24 and 48 hours to determine the optimum film setting duration. Figure 3-3 shows the effect of film setting duration on the water-swelling degree of PVA films. It was observed that the PVA films had a lower water-swelling degree when they were left to set for a longer period of time. During the film setting process, cross-linking occurs and water evaporates from the PVA solution. Therefore with a longer setting time, the films would contain lower amounts of water and a higher degree of cross-linking. This leads to a lower swelling capability of the film, which is more favourable as the end application is for the synthesis of GIP. The swelling of the film would distortion of the cavities within the GIP and this will contribute to low selectivity and recovery of the GIP, which represents one of the most common problems faced in MIP synthesis (Yungerman & Srebnik 2006).

![Figure 3-3: Effect of PVA film setting duration on the water-swelling degree of PVA films.](image-url)
3.4.3 Optimisation of Glutaraldehyde to PVA Volume Ratio

After determining the effect of setting duration on the water-swelling of films, the effect of GA to PVA ratio was studied.

Table 3-2 shows the effect of GA to PVA ratio on the water-swelling degree of the PVA films when immersed in water at 40°C. For each ratio, the films were left to set at two different durations. The water-swelling degree was used to deduce the cross-linking density of the PVA films. It was observed that for all ratios of GA to PVA films set at 48 hours, the films have a lower degree of water-swelling. This agrees with the previously mentioned findings where a longer setting time lead to a lower swelling capability of the films. It was generally observed that the increasing amount of GA added resulted in a decrease in the degree of water-swelling. However, further increasing the GA content to higher levels, for instance the ratio of GA over PVA of 0.05 and 0.10 have caused an increase in water-swelling degree instead. This could be due to the branching of the polymer chains rather than cross-linking in the presence of high GA concentrations (Yeom & Lee 1996). The reaction between PVA and GA results in two typical products (Figure 3-4). It consists of either the cross-linking product through an ether linkage formation or an aldehyde formation through the monofunctional reaction of GA. The addition of GA to the PVA solution at the ratio of 0.01 showed a decrease of water-swelling degree compared to PVA films which had no GA added. During the early stages of the reaction, effective cross-linking can occur as the GA molecules are able to react with the hydroxyl groups of the PVA. However as the reaction proceeds, the chain mobility in the film decreases, leading to the monofunctional reaction of GA where branching of PVA chains can occur. This branching of PVA will increase the space between the chains causing it to be less dense. Therefore, this increases the solubility of the PVA films in water, as shown by the results obtained in Table 3-2. The optimum conditions for film setting were observed to be a PVA solution containing a GA to PVA volume ratio of 0.01, dried at 40°C for 48 hours.
Table 3-2: The water-swelling degree of PVA films cross-linked with different amounts of GA.

<table>
<thead>
<tr>
<th>Surrounding Immersion Temperature (°C)</th>
<th>Duration for film to set at 40°C (hours)</th>
<th>GA / PVA volume ratio</th>
<th>Water-swelling degree (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40.00</td>
<td>24.00</td>
<td>0.00</td>
<td>218.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>59.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>Soluble</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>Soluble</td>
</tr>
<tr>
<td>48.00</td>
<td>24.00</td>
<td>0.00</td>
<td>54.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01</td>
<td>37.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>Soluble</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10</td>
<td>Soluble</td>
</tr>
</tbody>
</table>
3.4.4 Viable Yeast Test

Since yeast will be used as the template remover for the synthesis of GIP, proper measures had to be taken to dispose of the yeast after the glucose template removal step. Thus, during the synthesis of GIP, the film will be treated at 70°C overnight to deactivate the yeast. This was to ensure that the yeast was not a contributing factor in the decrease of the glucose concentration present in the solution after the incubation study.

This study utilised a high temperature to deactivate the yeast. The results showed that yeast cells at 40°C were viable while they were not viable after being exposed to a temperature of 70°C. This was derived from the methylene blue staining test. When yeast cells are viable, they contain an enzyme called dehydrogenase which

Figure 3-4: Mechanism between GA and PVA, which results in the cross-linking of PVA (A) or the branching of PVA (B). Image obtained from Figueiredo and team (Figueiredo, Alves & Borges 2009).
is able to decolourise methylene blue, while dead cells do not (Painting & Kirsop 1990). During the respiration of yeast cells, hydrogen atoms are removed from glucose by these enzymes and passed onto hydrogen acceptors, which releases energy for the cell. Methylene blue acts as an artificial hydrogen acceptor in this test. It turns colourless as it accepts hydrogen atoms in live cells. Therefore, viable yeast cells will not be stained by the methylene blue in this test. It was obvious from Figure 3-5 that dead yeast cells were present after being left at 70°C, as they were stained blue (Figure 3-5A) while for those treated at 40°C, yeast cells were colourless which indicated their viability (Figure 3-5B).

Figure 3-5: (A) Yeast cells incubated at 70°C stained blue after methylene blue test (B) Yeast cells incubated at 40°C remained colourless after methylene blue test.
3.4.5 Physical Observations of NGIP and GIP Films

Figure 3-6 show the NGIP (A) and GIP (B) films as observed under naked eyes. There were no observable differences in the appearance between the two films. This showed that both the NGIP and GIP mixtures were mixed well respectively as there were no observable differences in their appearances. Also, this justified the comparison of the two films as any difference in behaviour of the films would not be due to their appearance. Besides that, they both dried clear and were easily removed from the petri dishes. The clearness of the films could be due to PVA and glucose being colourless when in solution form, therefore drying clear when are casted into films. The clear films were of an advantage as there would not be any risk of colour leaching into samples from the films as compared to coloured films.

Figure 3-6: NGIP (A) and GIP (B) films as seen under naked eyes.

3.4.6 Determination of Optimum Template Glucose Concentration

The effect of the initial concentration of glucose templates added during the GIP synthesis was investigated by conducting a rebinding study. GIP films were synthesised with different concentrations of glucose templates, within the range of 0.00 M to $1.1 \times 10^{-2}$ M. The binding behaviour of glucose molecules into the GIPs were obtained and explained through various binding isotherms.

It was observed from Figure 3-7 that the rebinding of glucose into GIP was enhanced as the initial concentration of glucose was increased. This was due to the increase of imprinted glucose cavities in the GIP with the increase of template concentration. However, when the concentration of initial glucose templates exceeded $5.0 \times 10^{-3}$ M, a decrease in the bound amount during rebinding was observed. This could be due to an over saturation of glucose template molecules.
within the films. The presence of glucose templates in high quantities can lead to the overlapping of the templates within the film (Figure 3-8). This can result in inaccurate cavity shape formed within the GIP film, which leads to low rebinding capacity of glucose into the GIP. Therefore for the synthesis of GIP films, the optimum initial glucose template concentration was $5.0 \times 10^{-3}$ M and this was used throughout for further studies.

![Figure 3-7: The effects of glucose template concentration on the rebinding of GIP.](image-url)
3.4.7 Effect of Rebinding Solution Concentration

The adsorbent dose (concentration of rebinding solution) represents an important factor on the rebinding of glucose molecules into the cavities as it influences the binding efficiency of the synthesised GIPs. To study this, the GIP films were incubated in a series of glucose solutions ranging from a concentration of $1.12 \times 10^{-2}$ M to $5.60 \times 10^{-2}$ M. Multiple binding isotherms were used to further explain the binding behaviour of glucose molecules into the GIPs.

From Figure 3-9, as the concentration of rebinding solution increased, a decrease in percentage of rebound glucose against the total glucose was observed. As the GIP films were placed in glucose solutions, the presence of an equilibrium shift became significant. The equilibrium shifted towards the rebinding of the glucose molecules into the cavities. Thus, at low concentrations of rebinding solutions,
glucose molecules would be attracted to bind into the cavities within the GIP. However at higher concentrations of glucose solutions, a saturation of glucose molecules is present, therefore a shift against the equilibrium is observed. This is based on the Le Chatelier’s principle of equilibrium stating that when a dynamic equilibrium is disturbed by the change of conditions of its surroundings such as temperature, pressure and concentration of the reactants or products, a shift of equilibrium to counteract the changes occurs. The shift in equilibrium of the glucose molecules binding into the cavities at increasing concentrations of glucose is shown by Equation 3.2.

Equation 3-2: The equilibrium equation for binding of free glucose and cavities within the GIP.

\[
\text{Glucose}_{\text{free}} + \text{GIP} \rightleftharpoons \text{GIP-Glucose}_{\text{bound}}
\]

Therefore based on the Le Chatelier’s principle, the observed decrease in the percentage of bound glucose is due to the shift in equilibrium of the system to counteract the increase in free glucose concentration.

![Figure 3-9: Amount of glucose (%) bound to GIP after incubation in different concentration of glucose solutions.](image)
Generally for materials with surface containing recognition properties such as MIPs, their characteristics can be modelled into two categories: homogeneous and heterogeneous (Figure 3-10). For homogenous systems, all the binding sites present are assumed to have the same selectivity and affinity, symbolised by cavities of identical depths and shapes. However for heterogeneous systems, the binding sites have varying affinity and selectivity as cavities of different depths and shapes are present. MIPs generally contain binding sites with a wide range of affinities and selectivity (Umpleby II et al. 2004). The heterogeneity of the sites strongly influences the binding properties of the MIPs, which makes it highly reliant on the concentration of template presence during the rebinding stage. Therefore, in order to study the affinity of the GIPs synthesised, certain binding models such as the basic Scatchard plot and the more commonly applied discrete and continuous binding models have been employed.

![Figure 3-10: Schematic illustration for binding sites of homogeneous and heterogeneous systems.](image)

For a Scatchard plot analysis, the binding isotherms obtained experimentally would be plotted in the Bound/Free (B/F) versus Bound (B) format. For homogenous systems, the plot of B/F versus B will fall on a straight line, following the Scatchard equation shown below

**Equation 3-3: The Scatchard equation.**

\[
\frac{[B]}{[F]} = KN - KB
\]

where B represents the concentration of bound ligand to MIP and F represents the concentration of free ligand in solution. The other two components represent the binding characteristics of the system where K represents the binding affinity while N represents the density of binding sites.

The Scatchard plots obtained from the GIP and control NGIP binding study are shown in Figure 3-11. Within the concentration range studied, the plot of B/F versus
B presented a non-linear, curved relationship for both the GIPs and NGIPs. Normally, an almost horizontal line is observed for Scatchard plots of control non-imprinted polymers (NIPs) (Scorrano et al. 2011). However, there have also been studies where the NIPs demonstrate a slightly curved plot as similar to that observed in this study (Allender, Brain & Heard 1997; Byun, Yang & Cho 2013). For MIPs, the Scatchard plots obtained are usually curved due to the heterogeneity of binding sites (Allender, Brain & Heard 1997). Therefore, the presence of a curve relationship for both the NGIP and GIP indicates the presence of binding site heterogeneity. Since both the NGIP and GIP have Scatchard plots which show a curved relationship, the bi-Langmuir model has been employed for the binding analysis. This heterogeneity obtained by the curve relationship can be modelled as two straight lines which represents the graphical method of the bi-Langmuir application. Various binding parameters such as binding affinity and density of binding sites can be obtained through the bi-Langmuir model. These binding parameters can be used to compare the characteristics of the NGIP and GIP to demonstrate their differences and also the impact of template imprinting.

Based on Figure 3-12, the Scatchard plot for NGIP was modelled into two linear lines of best fit, in line with the bi-Langmuir fitting model. The steeper line obtained from the model indicates high-affinity sites while the flatter line indicates low-affinity
sites. From the Scatchard equation (Equation 3-3), the binding affinity, K and density of binding sites, N can be obtained from the Scatchard plots. The slope of the plot obtained represents the negative binding affinity while the density of binding sites is obtained through the horizontal intercept of the plot (Umpleby II et al. 2004).

Two distinct groups of binding affinity were obtained for the NGIP as observed by the difference in binding affinity and binding site density (Table 3-3). Comparing the values of the binding site density, it was observed that there was a higher density of binding sites with low binding affinity towards the glucose molecule in the NGIP compared to sites of high affinity. The amount of low affinity binding sites was 4.91 times the amount of high affinity binding sites within the NGIP. This supports the use of NGIP as a control for the study as there was a high density of binding sites with low affinity towards glucose.

![Figure 3-12: Scatchard plot for NGIP modelled as two straight lines for the bi-Langmuir model.](image-url)
The Scatchard plot for GIP was modelled into two linear lines of best fit, in line with the bi-Langmuir fitting model (Figure 3-13). The two linear trends were used for the determination of binding affinities and binding site densities.

Table 3-3: Binding characteristics obtained from the bi-Langmuir model for NGIP. Sample calculation shown in Appendix.

<table>
<thead>
<tr>
<th>Binding site</th>
<th>Binding affinity, K (mM g⁻¹)</th>
<th>Density of binding sites, N (mM g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>164.62</td>
<td>308.87</td>
</tr>
<tr>
<td>Low</td>
<td>14.93</td>
<td>1515.73</td>
</tr>
</tbody>
</table>

The presence of two distinct binding characteristics obtained indicated that the GIP consisted of two groups of binding affinity (Table 3-4). However, the GIP has a higher density of high affinity binding sites and very little amount of low affinity binding sites. This was contradictory to that obtained for the NGIP where a high density of low affinity binding sites was present instead. Based on Tables 3-3 and 3-4, the binding affinity obtained for the high affinity binding sites by the GIP was 1.53 times of that obtained by the NGIP. This difference between GIP and NGIP indicates that the imprinting process was capable of generating binding sites with higher affinity to glucose molecules. Moreover, the density of binding sites with higher binding affinity towards the glucose molecule is 1,508.24 times higher than the binding sites of low binding affinity within the GIP. This demonstrates the success of imprinting within the GIP. To further support the effect of imprinting, the density of high affinity binding sites in the GIP is 4.10 times of that present in the NGIP (Sample calculation for comparison values are shown in Appendix).
The bi-Langmuir model has successfully demonstrated the presence of two main binding affinities within the GIP. However, this model is a discrete model and therefore has shortcomings in characterising heterogeneity as the allocation of one discrete affinity to MIPs is arguable (Ng & Narayanaswamy 2011; Umpleby II et al. 2004). MIPs have a high probability of having a unimodal and continuous distribution, therefore continuous binding models are of a better fit for MIP characteristic studies (Umpleby II et al. 2004). The benefits of utilising continuous binding models are their ability to quantitatively measure the distribution and heterogeneity of the MIP and also more appropriate binding parameters are obtained. The continuous distribution model to be used is the Freundlich model.

For continuous distribution models, the Freundlich isotherm can be employed. It is based on the equation of $B = aF^m$. The parameters $B$ and $F$ represent the
concentration of bound ligand into the MIP and free ligand in the solution respectively. The other two components of the equation, $a$ and $m$, are able to provide physical binding measures. The pre-exponential factor $'a'$ is a combined measure of both the average affinity ($K$) and the capacity ($N$). However, additional steps are required to obtain $K$ and $N$ solely from $a$. Therefore, the other parameter, $m$, is preferred as a measure of physical binding instead. This parameter, $m$, can be directly obtained from the slope of the plot and is known as the heterogeneity index where the values range from zero to one. The value of one indicates the presence of homogeneity while values towards zero indicate heterogeneity within the MIP.

The Freundlich model was applied by plotting the data recorded into the format of log $B$ versus log $F$ (Figures 3-14 and 3-15).

![Figure 3-14: Freundlich model for NGIP plotted in Log B versus Log F format.](image)
Figure 3-15: Freundlich model for GIP plotted in Log B versus Log F format.

Based on Table 3-5, the m value for GIP is lower compared to the control NGIP. This indicated that the GIP was more heterogeneous compared to the NGIP. The process of molecular imprinting created cavities within the polymer which enhanced the binding affinity of the template molecule. Therefore as cavities were imprinted, the matrix of the GIP would be altered and therefore not as homogeneous as the NGIP that did not undergo the imprinting process. Moreover during the imprinting process, not all binding sites created would have the same binding affinity as demonstrated by the previous bi-Langmuir model (Table 3-4). Binding sites of different affinities would have been created, including sites which were non-specific too, similar to those present in the NGIP. This was further supported by the similar binding affinity values of the low affinity sites obtained for both the NGIP and GIP at 14.93 and 8.73 respectively (Tables 3-3 and 3-4).
With all the different binding models used and illustrated as above, it has been shown that the GIP has been successfully imprinted with cavities of high affinity towards the glucose molecule compared to that of the NGIP. Furthermore, the density of high affinity binding sites was 4.10 times higher than that was present in the NGIP, indicating a high density of imprinted cavities within the GIP. Also, the findings obtained showed that the NGIP was more homogeneous compared to the GIP. This is as expected since the NGIP does not undergo any imprinting. With the success of this imprinting, the GIP can be further used in sensing as it has a high affinity for glucose molecules compared to the NGIP.

### 3.5 Conclusion

As a conclusion of this chapter, it was determined that the optimised PVA film synthesis occurred when the films were set at 40°C for 48 hours with a GA over PVA ratio of 0.01. The ideal glucose template concentration was $5.0 \times 10^{-3}$ M for imprinting during the synthesis of the GIP. A key step during the synthesis was the denaturation of yeast cells after the removal step to ensure that the yeast cells do not consume the glucose during the rebinding step. Besides that, the synthesised GIP has physical binding parameters showing the successful effect of imprinting. The GIP was heterogeneous as it has a heterogeneity index of 0.5181 and contained two distinct groups of binding sites with different binding affinities. The binding affinities of the two distinct binding affinity groups within the GIP were 252.41 and 8.73 at densities of 1266.92 mM g$^{-1}$ and 0.84 mM g$^{-1}$ respectively. This demonstrates the successful imprinting of glucose molecules within the synthesised GIP utilising yeast as the template remover.
4. Application of MIPs for Sensing

This chapter describes the application of GlP for optical sensing. The GlP synthesised as reported in Chapter 3 was surface coated on fluorescent CDs to introduce a specific binding site towards glucose for sensing purpose. This chapter is organised in a chronological order with the following subtitles; the materials, reagents and instruments, methodologies, results achieved and discussion, and finally the conclusions derived from the study.

4.1 Materials & Reagents

All the chemicals and reagents used in this section were of analytical grade or higher unless otherwise stated. All percentage measurements (%) of solutions prepared were of a weight to volume ratio (w/v) unless otherwise stated. The GlP was prepared using the same materials as Chapter 3, where PVA represented the matrix, glucose the template and yeast as the template remover. Poly (vinyl alcohol) (PVA) with hydrolysis degree of 99+ % and average molecular weight of 130 000 g/mol was purchased from Aldrich. D(+) Glucose (monohydrate) was purchased from R&M Chemicals. Commercial baker's yeast, *S. cerevisiae* was obtained from the local supplier in Kuching, Sarawak, Malaysia. Sodium alginate was used as the carbon precursor for CDs synthesis and was purchased from Aldrich. Ultrapure water was obtained from Milipore Mili-Q Advantage-A10 and Millipore Elix-5 water purification system (~18.2 Ω, 25 ºC) and was used as the solvent system throughout this work.

4.2 Instrumentation

Different instruments were used to perform the study reported in this section, ranging from those used in the synthesis of CDs to the fluorescence scans of the CDs. The synthesis of CDs was done via a laboratory furnace; Carbolite ELF 11/14 B. Fluorescence measurements were taken using a fluorescence spectrophotometer, Varian Cary Eclipse Fluorescence Spectrophotometer. The synthesised CDs were dissolved in water at a desired concentration and then transferred into a four-side clear quartz cuvette with path length of 1 cm. The excitation and emission slits were both set to 10 nm. A commercially available domestic microwave from Panasonic with an output of 2450 MHz was used in assisting the coating of CDs.
4.3 Methodology

4.3.1 Synthesis of Carbon Dots
The GIPs will be used as recognition element in sensing, but it further requires an optical signalling component as the analytical measurable during the detection. In this study, fluorescent CDs were selected to represent this component. The CDs were synthesised via the pyrolysis method using an organic starting precursor. In brief, 1.00 g of sodium alginate was weighed out and placed in a crucible, followed by heating it up to 200°C for a period of two hours. After two hours, the crucible containing the carbonised alginate was removed from the furnace and left to cool to room temperature. The carbonised alginate was then dissolved in water to form a mass concentration of 5 mg/mL. This solution was then filtered off using a syringe filter having a pore size of 0.22 µM. This represented the stock CDs solution which was to be used for further studies.

4.3.2 Optimisation of Fluorescence of Bare Carbon Dots
After the synthesis of CDs, the optimum excitation and emission wavelengths of the CDs were to be determined. The determination of these wavelengths aided in the analysis of GIP effects on the CDs. This was because binding activity of the GIP was inferred using the changes in peak fluorescence intensities obtained after every step of glucose removal and rebinding. In order to achieve fluorescence with highest intensity, the optimum excitation and emission wavelengths were determined via 3D and excitation scans.

4.3.3 Effect of Glucose on Bare Carbon Dots
Since GIP was synthesised in the previous study as reported in Chapter 3, the synthesised CDs were tested against glucose to record the effect on the fluorescence of CDs. To achieve this, glucose solution having a concentration of 1.0% was dripped into a solution of CDs. The fluorescence intensity of the CDs was then measured using the fluorescence spectrophotometer. To form a control, the same volume of water was also added to a separate set of CDs solution of same concentration. This was to omit the effect of dilution on the fluorescence intensity of the CDs.
4.3.4 Optimisation of Coating of Bare Carbon Dots with PVA

In order to coat the CDs with GIP, a primary study was done by just using the PVA matrix to determine the effect of surface passivation on the fluorescence signal of the CDs. In order to perform this, a capped glass vial having 100 µL of CDs (from the CDs stock solution) was added to 8 mL of 5.0% PVA solution. The resulting mixture was placed into a microwave which assisted in the coating of CDs. After that, the solution was left to cool and its fluorescence measurement was taken. The control used for this study was CDs in ultrapure water, in the same volume ratio.

4.3.5 Coating of Carbon Dots with Glucose Imprinted Polymer

The coating of CDs with GIP (CDs@GIP) was prepared by adding 1 mL of 10% glucose solution, 100 µL of stock CDs solution and 7 mL of 5.0% PVA solution into a capped-glass vial. The resulting mixture was placed into a microwave to assist the coating of the CDs with the GIP. After heating, the solution was left to cool and its fluorescence measurement was taken. After that, yeast was added to the mixture at 40°C for four hours for the removal of the glucose templates. The yeast was then removed using syringe filters of pore size 0.45 µM and the fluorescence of the filtered CDs was measured. The rebinding study was performed by the addition of 200 µL of 1.0% glucose solution into the CDs@GIP. The control used for this study was CDs coated with NGIP (CDs@NGIP), where similar synthesis procedure was performed in the absence of glucose in the mixture. This was done by adding 8 mL of 5.0% PVA solution to 100 µL of stock CDs solution, followed by the same treatment as during the synthesis of CDs@GIP.

4.3.6 Quantification of CDs@GIP with Glucose

As a potential sensing probe, the binding of glucose into the GIP and its correlation to the change of fluorescence signal has been studied. The calibration curve for the analysis was carried out by adding an increasing known amount of glucose into the CDs@GIP. The fluorescence signal was then measured after a fixed incubation period. Later, the fluorescence signal change recorded was plotted against the amount of glucose added to establish the correlation for the binding and the signal change.
4.4 Results & Discussion

4.4.1 Synthesis of Carbon Dots
The CDs were synthesised via thermal decomposition of sodium alginate. The cream coloured sodium alginate powder was observed to have turned into a dark shade of brown after treatment with furnace at 200°C for two hours (Figure 4-1). The product obtained after furnace treatment was shown to be fully dissolved in water as well.

![Figure 4-1: Sodium alginate powder before (A) and after (B) pyrolysis using a furnace.](image)

The change in colour of the sodium alginate powder indicated that it has undergone thermal decomposition and carbonisation. The thermal effect on sodium alginate is known to undergo three stages, consisting of an initial dehydration process and followed by two stages of decomposition (Soares et al. 2004). The two stages of decomposition involve the degradation of sodium carbonate and the decomposition of the formed carbonised material. The first stage of thermal decomposition of sodium alginate occurs roughly at 100°C, where water loss occurs and energy is required to vaporise the water (Zhen et al. 2010). After that, the two stages are exothermic and they occur at a maximum of 250°C and 410°C respectively (Soares et al. 2004). The first exothermic stage involves the scissoring of chains within the sodium alginate compound, cyclisation, carbonisation and formation of immediate fragments of stable metal oxalates (Said & Hassan 1993). The last stage involves the formation of metal oxides due to decomposition of the intermediate metal
oxalates and also the decomposition of the carbonised products (Zhen et al. 2010). Therefore, the heating of the sodium alginate powder at 200°C results in carbonisation as this process occurs at the maximum temperature of 250°C where beyond this temperature, decomposition of carbonised products occurs instead.

The carbonised sodium alginate powder was then dispersed in water. This formed the CDs solution. The solution was a clear yellow under visible light (Figure 4-2) and it exhibited a bright blue fluorescence under UV light (Figure 4-3).

Figure 4-2: Water (A) as comparison and synthesised CDs dispersed in water (B) under visible light.
4.4.2 Optimisation of Fluorescence of Carbon Dots

To characterise the CDs, the maximum excitation and emission wavelengths were determined using a 3D-scan run by the fluorescence spectrophotometer. Based on Figure 4-4, it was determined that the optimum excitation wavelength for the CDs was at 350 nm. When the CDs were excited at wavelengths ranging from 320 to 350 nm, an increase in fluorescence intensity was seen. From excitation wavelengths of 360 until 380 nm, a decrease in fluorescence intensity was observed instead. However, the emission peaks remained at 438 nm regardless of the excitation wavelength used. This shows that the CDs exhibit a slight excitation dependence on emission character as an optimum wavelength of 350 nm was required to obtain the maximum fluorescence intensity. Excitation wavelengths before and beyond 350 nm would result in lower fluorescence intensity. The emission of fluorescence at 438 nm indicates fluorescence within the blue region of the visible spectrum. This agrees with the observation showing CDs dispersed in water emitting blue fluorescence under UV light exposure (Figure 4-3B).

The optical properties of the CDs were obtained via UV-Vis and fluorescent scans. The absorption spectrum (Figure 4-5A) obtained for the CDs showed a slight peak at 260 nm which could indicate $\pi \rightarrow \pi^*$ transitions of C=C which are the most common transitions observed in organic molecular UV-Vis (Kong et al. 2014). As the peak intensity occurred at an emission wavelength of 438 nm when the CDs were excited at 350 nm, a scan was carried out to double confirm that the excitation...
wavelength was indeed at 350 nm. Figures 4-5B and 4-5C shows the excitation and emission spectrum where both the intensities matched. This indicates that the optimum excitation wavelength of the CDs is 350 nm. This wavelength was used throughout further studies during the measurement of fluorescence emitted by the CDs.

Figure 4-4 Fluorescence emission spectrum of the synthesised CDs at different excitation wavelengths via 3D scan.
4.4.3 Effect of Glucose on Carbon Dots

As the bare CDs were going to be coated by the GIPs obtained in Chapter 3, the bare CDs were tested against glucose to see if there was any effect of glucose on the fluorescence. Fixed volumes of glucose solution were added into the CDs solution. To omit the effects of dilution as glucose solution was used; the effect of water was also studied by adding the same volumes into the bare CDs solution. Both of the fluorescence plots obtained were plotted together as a comparison (Figure 4-6). The effect of water on the fluorescence of the CDs was used as a baseline for determining the effect of glucose on the fluorescence. As observed in Figure 4-6 glucose does not have any significant effect on the fluorescence of the bare CDs. The decrease in fluorescence was due to dilution of the CDs solution as shown by the plot obtained when water was added. There was not a substantial difference between the effects of water and glucose therefore, the conclusion can be made that glucose has no effect on the fluorescence of the bare CDs. This could be due to glucose being a neutral molecule. During the carbonisation process, carbonyl groups are introduced to the surface of the CDs. This allows for interactions between CDs and analytes. Since glucose is a neutral molecule, the affinity of interaction between CDs and glucose will be low.
4.4.4 Coating of Carbon Dots

It was confirmed that the synthesised bare CDs are not affected by the presence of glucose. Thus, the coating of CDs with GIP was attempted to introduce binding sites of the CDs. PVA was selected as it represented the matrix of the synthesised GIP in Chapter 3. The coating of nanoparticles with polymers has become a common practice for various reasons ranging from the field of drug delivery to usage as nanosensors (Muthiah, Park & Cho 2013; Sundaresan et al. 2014; Wadajkar et al. 2013). Moreover, extensive research has been conducted on the coating of nanoparticles with MIPs (Ahmadi, Madrakian & Afkhami 2014; Chen et al. 2015). However, CDs are fairly new and have been shown to contribute greatly to the field of sensing. CDs are able to undergo functionalisation to perform specific sensing activities. One common method of CDs functionalisation is via coating using polymers such as polyethylene glycol (PEG) (Gonçalves et al. 2010; Mihalache et al. 2014).

Microwave irradiation has been a common method of choice in the coatings of nanoparticles with polymers (Bahadur et al. 2011; Elizabeth et al. 2012; Pal & Kanti Deb 2012). Therefore the coating of the synthesised CDs with PVA was attempted using a domestic microwave. A domestic microwave has been shown by previous studies for its ability on synthesising nanoparticles and coating them with polymers (Agarwal et al. 2012; Pal & Kanti Deb 2012). The microwave heating process

Figure 4-6: Fluorescence spectrum showing the effect of glucose on CDs.
provides the ability to work under high pressure and the high heating temperatures obtained makes reaction time faster than that of conventional heating methods (Kappe 2004). Moreover, microwave irradiation promotes heating homogeneity to a system since microwave is absorbed and spread uniformly in the solution.

The emission spectrum of the coated CDs with PVA after microwave irradiation was obtained. It was observed that at excitation wavelength of 350 nm, the PVA-coated CDs emitted fluorescence at 431 nm. This was in accordance with the fluorescence spectrum of the bare CDs as well (Figure 4-7). This suggested that the PVA coating did not restrict or alter the fluorescent properties of the CDs as PVA solution is clear. As the domestic microwave has different heat settings, the ideal heat setting to be used throughout the studies was determined. It was observed that the fluorescence of the CDs decreased as the heat intensity increased (Figure 4-8). However, the decreased in intensity was not very prominent between the different microwave heat settings.

Besides studying the effects of the microwave power on fluorescence of the CDs, the duration of the sample exposure to microwave irradiation was investigated. The sample consisting of CDs and PVA solution was exposed to microwave at time durations of 1, 2, 3 and 5 minutes. From Figure 4-9, it was observed that the microwave irradiation time did not show any effect on the fluorescence of the CDs when exposed for up to three minutes. However, when the CDs were exposed to microwave irradiation for five minutes, an increase in the fluorescence intensity up to 1.3 times of the initial fluorescence was observed.

Based on the findings (Figures 4-8 and 4-9), steps resulting in an increase of fluorescence was preferred. This was because the CDs would have undergone functionalisation in the form of surface passivation, resulting in the modification of the fluorescence of the CDs. Moreover, CDs have been known to show stronger fluorescence after passivation (Sun et al. 2006b). It has been reported that the better the surface passivation, the higher the fluorescence activity can be observed for the CDs (Dong et al. 2012). Therefore, for further studies, the CDs would undergo 5 minutes of microwave irradiation under low microwave power.
Figure 4-7: Fluorescence spectra of CDs and CDs coated with PVA

Figure 4-8: The change in fluorescence of CDs after microwave treatment at different microwave power settings as compared to CDs without exposure to microwave irradiation.
Figure 4-9: The change in fluorescence of CDs when exposed to microwave irradiation for different durations of time.

4.4.5 Coating of Carbon Dots with Glucose Imprinted Polymer

As mentioned beforehand, the coating of nanoparticles with MIP has been a common practice as the fusion of molecular imprinting and optical detection allows for good sensitivity and also excellent selectivity. (Gao et al. 2013; Mao et al. 2012). Thus with the success of PVA coating, the coating of CDs with GIPs was carried out next in this study. The CDs were also coated with NGIP as a control throughout the studies. A solution of CDs containing the PVA polymer and glucose template was treated with microwave irradiation. The fluorescence intensities after every step during the synthesis were recorded.

As demonstrated by the previous chapter, the synthesis of GIP includes removal of glucose templates using yeast. This results in the formation of cavities that are specific to glucose templates, allowing for the later rebinding of glucose molecules. Therefore, the same process was conducted for the CDs@GIP. The presence of glucose in the cavity could cause the fluorescence quenching of the CDs, while the removal of glucose by the yeast could increase the fluorescence intensity. Thus, when the glucose molecules rebind back into the cavities within the GIP, the fluorescence intensity would be quenched as illustrated by Figure 4-10.

It was observed from Figure 4-11 (Fluorescence spectra shown in Appendix) that after the treatment with yeast, the fluorescence intensity of the CDs was enhanced.
It was also observed that the fluorescence of CDs quenched when glucose molecules were introduced during the rebinding process. This was the evidence of the interaction between the CDs and glucose molecules with the GIP as interface for the binding. The mechanism for the quenching of fluorescence was suggested to be the energy transfer between the CDs and glucose molecules. Fluorescence or Förster resonance energy transfer (FRET) is a phenomenon which occurs when non-radiative energy from a photoexcited donor molecule is transferred to an acceptor molecule which is of a different species. This occurs when the acceptor molecule is brought in close vicinity to the donor molecule (Lakowicz 1999). In this study, CDs represent the donor molecule while glucose represents the acceptor molecule. During the rebinding process, glucose molecules are bound into the cavities of the CDs@GIP due to the cavities’ high affinity for glucose molecules. This results in glucose molecules being drawn near to the CDs due to the cavities present in the GIP layer, resulting in a transfer of energy from CDs to glucose molecules. The consequence of the energy transfer from CDs to glucose molecules is the observed quenching of fluorescence of the CDs. Therefore with this proposed mechanism of quenching, when the glucose molecules were extracted from the cavities in CDs@GIP via yeast, there is no energy transfer between the CDs and glucose due to the increase of distance between the two. This results in the observed enhancement of fluorescence of CDs@GIP.

To further demonstrate the use of GIP in sensing, the same sample of CDs@GIP underwent repeated cycles of template removal and rebinding (Figure 4-12, Fluorescence spectra shown in Appendix). It was observed that in both cycles, the removal of glucose via yeast resulted in an increase of fluorescence while the rebinding of glucose caused quenching. Moreover, the increment and quenching of fluorescence for both cycles were both similar, showing the stability of the CDs@GIP system. The ability of the CDs@GIP system to demonstrate repeated cycles indicates the possible reuse of CDs@GIP for sensing of glucose molecules in samples in a sustainable manner.
Figure 4-10: Scheme showing the effect on CDs fluorescence due to extraction and rebinding of glucose molecules.

Figure 4-11: Normalised fluorescence intensities of CDs after each step of GIP synthesis.
As glucose solution was added into the CDs@GIP system for rebinding, the effects of water on the fluorescence of CDs@GIP was also investigated. This was to ensure that the quenching of the CDs@GIP was due to rebinding of glucose and not due to the addition of water into the system causing dilution. When dilution occurs, the fluorescence of CDs would be reduced as demonstrated previously. Therefore, a separate sample of CDs@GIP was prepared and water was added to investigate the effect of dilution on the fluorescence. The volume of water added was the same as that of the glucose which was used previously for the rebinding step. Based on Figure 4-13, it was observed that the fluorescence of CDs@GIP was not significantly affected by the addition of water. This indicates that the quenching of fluorescence observed during the rebinding of glucose was not due to the occurrence of dilution (Figure 4-11). To further demonstrate that the quenching of CDs@GIP was indeed due to the rebinding of glucose, comparisons were made between the effect of dilution and the effect of glucose addition (Figure 4-14). A larger quenching effect was observed after the addition of glucose as compared to the addition of water. Thus, this also supports the findings that the quenching is indeed due to the rebinding of glucose molecules. Moreover, the amount of water added was very low compared to the initial volume of CDs@GIP sample. The total volume of water added was 2.44 % of the final volume; therefore it does not play a significant role in terms of CDs@GIP quenching during the rebinding of glucose.

Figure 4-12: Fluorescence intensities of CDs@GIP after each step for two cycles of glucose extraction via yeast and rebinding.
Figure 4-13: Fluorescence intensities of CDs@GIP for a control study investigating the effect of water on fluorescence.

Figure 4-14: Normalised fluorescence intensities of CDs@GIP showing the effect of water addition and glucose addition for dilution and rebinding studies respectively.
After demonstrating the success of the CDs@GIP system, certain steps were done in order to demonstrate the effectiveness of this system. To achieve this, control studies were conducted using synthesised CDs@NGIP. The fluorescence of the CDs@NGIP showed the same trend as the CDs@GIP during the conditioning stages prior to addition of glucose for rebinding. (Figure 4-15). This demonstrates the reproducibility of coating CDs with the polymer. For the rebinding step where additional glucose was added, it was observed that the fluorescence of the CDs@NGIP quenched as well.

Since quenching occurred with the CDs@NGIP, its fluorescence was then compared with that obtained by CDs@GIP (Figure 4-16). Overall, the fluorescence intensity of CDs@NGIP was lower than that obtained for CDs@GIP. Hence, the intensities had to be normalised for comparison (Figure 4-17). It was observed that the quenching obtained by CDs@GIP was larger than that of CDs@NGIP. This indicates that the quenching efficiency of CDs@GIP at rebinding was higher than that of the CDs@NGIP. This was expected as no-tailor made sites for glucose were formed within the NGIP. The quenching of CDs@NGIP might have been due to the polymer itself which showed affinity towards glucose as demonstrated in the previous chapter. Therefore, this confirmed that the CDs have been successfully coated with functioning GIPs, allowing for the optical detection of removal and rebinding activity of glucose molecules with the GIP. Additionally, the efficiency of the CDs@GIP at rebinding glucose was 1.20 times of CDs@NGIP.
Figure 4-15: Fluorescence intensities of control CDs@NGIP throughout the series of steps taken when it underwent the same steps used for the synthesis of CDs@GIP.

Figure 4-16: Fluorescence intensities of both CDs@GIP and control CDs@NGIP throughout the series of steps taken during its synthesis.
4.4.6 Quantification of Coated Carbon Dots with Glucose

After forming the CDs@GIP system and demonstrating its ability to detect glucose, the analytical performance of the system as a sensing probe was studied. The fluorescence quenching of the CDs@GIP system over a series of glucose solutions was plotted in the Stern-Volmer plot where $F_0$ and $F$ represents the fluorescence intensity of the CDs@GIPs in the presence and absence of glucose respectively and $K_{sv}$ representing the Stern-Volmer constant. From the Stern-Volmer plot, the limit of detection (LOD) and linear range of the sensing system was investigated. The LOD was determined by three times the standard deviation of a control sample divided by the slope of the standard curve obtained.

The CDs@GIP was incubated in a series of glucose solutions with increasing concentrations to obtain its effect on the quenching intensity of the system. It was observed that the CDs@GIP system demonstrated different analytical behaviour depending on the range of glucose incubation solution concentration. As the concentration of glucose incubation solution increased, the CDs@GIP became more sensitive to glucose molecules as indicated by Figure 4-18. This can be derived from the different $K_{sv}$ values obtained (Table 4-1) where a higher $K_{sv}$ indicates a higher sensitivity of the CDs@GIP towards the glucose molecules. At lower concentrations of rebinding solutions, the $K_{sv}$ was determined to be 8.2795.
while the LOD was 0.0257 mM. However, at higher concentration range of glucose rebinding solutions, the $K_{sv}$ was 51.943 while the LOD was 0.0409 mM. This behaviour suggested that the CDs@GIP had a concentration dependent quenching reaction towards glucose.

To further investigate the possible reasoning behind the initial analytical trend of the CDs@GIP, a Stern-Volmer plot of the bare CDs exposed to glucose was obtained and compared against that of the CDs@GIP (Figure 4-19). It was observed that initially at low concentrations of glucose, the CDs@GIP had similar analytical characteristics to that of the bare CDs when exposed to glucose. This indicated that at low concentrations of glucose, the quenching effect was not significant. The decrease in fluorescence for the bare CDs by glucose was due to dilution as proven earlier. At this low concentration of glucose below the LOD, the dynamic equilibrium between the CDs@GIP and glucose was not established, thus will not show any significant effect in the binding interaction. However as the concentration of glucose increases, the effect of dynamic equilibrium becomes significant and can play a role in shifting the equilibrium for the rebinding of glucose. This can be explained by Le Chatelier's principle of equilibrium where if a dynamic equilibrium is disturbed by changing conditions such as concentration, temperature and pressure, the position of equilibrium shifts to counteract the changes. Therefore, as the concentration of glucose increased, more glucose would be bound into the cavities within the CDs@GIP, causing a greater quenching effect as observed. This is due to the position of equilibrium shifting towards the binding of glucose into the CDs@GIP. As demonstrated by Equation 4.1 where, when there is an increase in free glucose, the equilibrium would be shifted towards the right hand side. This resulted in the promotion of glucose molecules binding into the cavities of the GIP coated around the CDs. Thus, greater quenching of CDs@GIP observed at higher concentrations of glucose compared to lower concentrations as demonstrated in Figure 4-18.

**Equation 4-1: The equilibrium equation for binding of free glucose and CDs@GIP.**

\[
\text{Glucose}_{\text{free}} + \text{GIP} \rightleftharpoons \text{GIP-Glucose}_{\text{bound}}
\]

A very significant difference between the quenching effects of the bare CDs and CDs@GIP was observed at higher concentrations of glucose (Figure 4-19). The Stern-Volmer plot of the CDs@GIP was steeper compared to that of the bare CDs.
This has demonstrated the successful coating of CDs with GIP. This has also allowed for glucose molecules to bind into the cavity, which resulted in the enhanced quenching of the CDs@GIP as explained before. This significant finding supported the success of both the synthesis of the GIP and also its coating on CDs.

![Figure 4-18: Stern-Volmer plot of CDs@GIP.](image)

Table 4-1: Stern-Volmer equations, $K_{sv}$ and LOD values obtained for the quantitative study of the CDs@GIP system.

<table>
<thead>
<tr>
<th>Glucose Concentration Range, mM</th>
<th>Stern-Volmer Equation</th>
<th>Stern-Volmer Constant, $K_{sv}$</th>
<th>Limit of Detection (LOD), mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 – 1.80</td>
<td>$F_0/F = 8.2795 \text{[Glucose]} + 0.9984$</td>
<td>8.2795</td>
<td>0.0257</td>
</tr>
<tr>
<td>1.80 – 2.50</td>
<td>$F_0/F = 51.943 \text{[Glucose]} + 0.9186$</td>
<td>51.943</td>
<td>0.0409</td>
</tr>
</tbody>
</table>
4.5 Conclusion

In conclusion, water-soluble CDs were successfully synthesised via thermal decomposition at 200°C for two hours from the carbon source of alginate powder. The CDs have an excitation wavelength of 350 nm and an emission wavelength in the blue region of 438 nm. The CDs were also successfully coated by GIP using a domestic microwave. Yeast was used to remove the glucose templates during the synthesis to form GIP. The coating of GIP on CDs has made the initially insensitive CDs able to interact with glucose. This indicates the ability of the CDs@GIP system to act as a sensing system for glucose. The CDs@GIP system was more sensitive towards the presence of glucose molecules at concentrations above 1.80 mM, where concentrations below that showed no significant effect. The sensitivity of the CDs@GIP towards glucose at concentrations above 1.80 mM was high as indicated by the $K_{sv}$ value of 51.943, while the LOD of the CDs@GIP system was 0.0409 mM.

Figure 4-19: Stern-Volmer plot of bare CDs and CDs@GIP with glucose.
5. Conclusions and Suggestions for Further Work

The custom-made synthetic receptors produced by MIT have been acquiring budding attention and focus due to their highly sought after properties such as their selective and specific performance, durability and also applicability in wide fields. These vast application fields range from drug delivery to separation sciences. The recent research in the field of MIPs are focused on two key areas, involving the synthesis or development of novel MIPs and the application of MIPs to solve problems associated with the recognition aspect for compounds of interest. However, even with the increasing number of achievements made, the area of developing MIPs brings about existing challenges.

One key challenging area involves the template removal step during the synthesis of MIPs. The template removal step ensures the efficiency of the MIP as the cavities available for analyte rebinding plays an important role in MIP performance. The commonly faced template removal problems include incomplete removal of templates, distortion of binding sites, collapse or rupture of cavity due to harsh extraction conditions and lastly the toxic effects of the extraction solvents (Lorenzo et al. 2011). Extensive efforts have been made in terms of template removal utilising different methods. However, these conventional methods of extraction have setbacks and therefore contribute to template removal generally being the least cost-effective and sustainable step of the entire MIP synthesis. This is due to the involvement of harsh solvents involved in extraction and long running time for the removal of template. Therefore with the awareness of the drawbacks involved in template removal steps, this dissertation aims at introducing a method of template removal which substitutes the usage of harsh solvents and the reduction of long hours for the entire synthesis of the MIPs. After achieving that milestone, the next aim was to demonstrate the potential use of the developed MIPs in the field of sensing.

This dissertation is fractioned into two major parts where the first involves the development of MIPs via a sustainable biotechnology approach. The processes used throughout the MIPs development were mild as they did not require the use of harsh and harmful solvents. The templates used for the development of the MIPs were glucose molecules, forming glucose imprinted polymers known as GIPs. The
highlight of this study was the use of a biological agent; yeast as template removers. The yeasts represented the biotechnology approach which omitted the use of harmful solvents leading to a sustainable approach of GIP development. A biocompatible polymer, PVA was used as the matrix. The GIPs were synthesised in the form of films. As an assurance step, yeast was denatured via heat treatment successfully after the removal of glucose from the polymer as to ensure that the yeast was not consuming the added glucose molecules which could lead to false positive results. Optimisation studies were carried out covering the areas of the film setting temperature and duration, PVA to glutaraldehyde (its cross-linker) ratio and glucose template concentration for GIP synthesis. The optimised GIP synthesis conditions occurred when the GIP films were left to set at 40°C for 48 hours with a PVA to glutaraldehyde ratio of 0.01. For the imprinting of GIP, the ideal glucose template concentration was 5.0 ×10⁻³ M. After the development of GIP films, various binding models such as the Scatchard plot, bi-Langmuir and Freundlich models were employed to study the affinity and binding characteristics of the developed GIP. From these binding models, it was obtained that the developed GIPs were successfully imprinted with glucose cavities. The GIP showed heterogeneity as it has a heterogeneity index of 0.5181 and this is further supported by its Scatchard plot which shows two distinct groups of binding sites with different binding affinities. The GIP had high binding sites of binding affinity of 252.41 at densities of 1266.92 mM g⁻¹ and low binding sites with binding affinity of 8.73 at densities of 0.84 mM g⁻¹. The control non-imprinted polymer, NGIP, had two binding sites as well. However, its lower affinity binding sites had a higher density compared to its higher affinity binding sites. Additionally to further support the successful effect of imprinting, the binding affinity of the high binding sites present in GIP was 1.5 times of the NGiP and was present at binding densities which were 4.10 times of that in the NGiP. From the results obtained, this study successfully demonstrated the success of utilising a biological agent instead of harmful solvents for the extraction of templates for the development of MIPs.

After demonstrating the success of synthesising MIPs utilising biological agents for the template removal step, the potential application of the synthesised GIP was studied. Currently, nearly all commercially available glucose biosensors work by the detection of hydrogen peroxide which is produced by the enzymatic oxidation of glucose by glucose oxidase (Wu et al. 2010). However, enzyme-based sensors are unstable due to the denaturation of enzyme and are also costly (Pickup et al. 2005). This leads to the second major part of this dissertation where a study on utilising
the developed GIP in the field of sensing. The drawbacks as mentioned before would be avoided as no enzymes were involved, omitting the concerns of enzyme denaturation and also high costs of development. The GIP involved in this section was also synthesised utilising yeast as the template remover. To demonstrate the potential use of the GIP in sensing, the GIP was tagged with a fluorophore. Carbon dots, CDs were used as the fluorophore for this section as they were relatively simple to produce, water-soluble and biocompatible. Water-soluble CDs were successfully produced from the pyrolysis of sodium alginate at 200 °C for two hours with bright blue fluorescence emission at 438 nm with excitation wavelength of 350 nm. These CDs were successfully coated with the GIP using a domestic microwave for five minutes on low heat setting. This was observed via the increase in fluorescence of the CDs, indicating successful surface passivation of the CDs with the GIP. Yeast was removed via syringe filtration where the sample was passed through a filter of 0.45 µM pore size as yeast cells are approximately 6 µM in diameter (Madigan et al. 2010). The GIP-coated CDs, CDs@GIP showed fluorescence changes to glucose while the bare CDs did not. This indicated the potential use of the CDs@GIP system in sensing. To further support the demonstration of the system in sensing, the removal and rebinding of glucose molecules into the CDs@GIP system was reflected in the fluorescence signals. The sensitivity of the CDs@GIP towards glucose was high as it obtained a $K_{sv}$ value of 51.943 and had an LOD of 0.0409 mM. Therefore, it can be said that the CDs@GIP system has been successfully developed with abilities of detecting glucose template removal and rebinding activities with high sensitivity.

From the findings presented in this dissertation, it can be concluded that the studied method of developing MIP with the use of a biological agent in template removing, without the use of harmful and harsh solvents is possible. Moreover, the running time and cost of the MIP development utilising biotechnology as demonstrated is efficient. These positive factors contribute to the benefits of the studied method of development, signifying its sustainability. Additionally, the developed GIP showed its potential in sensing as the removal and rebinding of glucose molecules were reflected in the changes of fluorescence and the fluorescence changes were able to be quantified for analytical purposes.

To further develop the GIP system, further studies could be carried out. Selectivity studies could be done where solutions of different types of sugars such as fructose and galactose would be used as rebinding solutions. Also, a solution containing a
mixture of sugars could be prepared and used for rebinding studies to further investigate the selectivity of the system. Additionally, real life samples such as food or environmental samples could be obtained where the CDs@GIP system could be used to quantify the amount of glucose present in the samples.

Furthermore, with the promising results obtained in this dissertation study, the room for future prospects is vast. A possible further work would be to fuse the CDs and MIP system onto a solid platform. An example would be a flow injection fluorescence sensor utilising the MIPs and CDs. As the developed system studied in this dissertation demonstrated its reusability, the CDs coated with MIPs could be packed into a flow cell which could act as the recognition element for a targeted analyte. Besides that, the system could also be developed for drug delivery where the release of drug could be signified by the change in fluorescence. This shows the applicability of the dissertation system in a large range of fields and the promising factor it has for development.
Appendix

1. **Sample Calculations for Tables 3.3 and 3-4**

Based on the Scatchard equation, the slope obtained represents the negative binding affinity, $K$ while the horizontal intercept represents the density of binding sites, $N$.

Sample calculations based on plot obtained for high binding site for NGIP.

Equation obtained from Scatchard plot:

$$
\frac{[B]}{[F]} = -164.62[B] + 0.5339
$$

**Binding affinity, $K = 164.42$**

**Density of binding sites, $N$:**

Horizontal intercept, therefore, $\frac{[B]}{[F]} = 0$

- $-164.62[B] + 0.5339 = 0$
- $-164.62[B] = -0.5339$
- $164.62[B] = 0.5339$
- $[B] = 0.00324 \text{ M} = 3.24 \text{ mM}$

Average weight of film used for rebinding study = 0.01049 g

Density of binding sites, $N$

$= 3.24 \text{ mM} \div 0.01049 \text{ g}$

$= 308.87 \text{ mM g}^{-1}$

2. **Sample calculations for comparison values of binding affinities and densities of binding sites between NGIP and GiP**

Example: Binding affinity obtained for the high affinity binding sites by the GiP was 1.5 times of that obtained by the NGIP.

Binding affinity for GiP = 252.41

Binding affinity for NGIP = 164.62

Comparison value calculations:

$252.41 \div 164.62 = 1.53$
3. **Fluorescence Spectra for Figure 4-11**

Appendix 1: Fluorescence spectra for Figure 4-11.
4. **Fluorescence Spectra for Figure 4-12**

Appendix 2: Fluorescence spectra for Figure 4-12.
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