DEVELOPMENT OF A SYNTHETIC AFFINITY MEMBRANE FOR THE PURIFICATION OF RECOMBINANT MALTOSE BINDING PROTEINS

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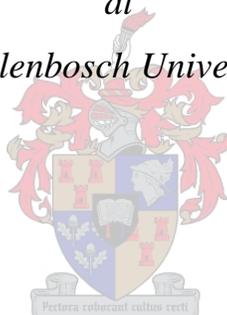
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Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the owner of the copyright thereof (unless to the extent explicitly otherwise stated) and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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SYNOPSIS

The aim of this project was to fabricate a new affinity membrane-based system that is bio-specific and biocompatible, and which could be used as an adsorption matrix for the immobilization of the recombinant protein maltose binding protein human estrogen receptor alpha ligand binding domain (MBP-hERαLBD). The viability of the affinity membrane system (AMS) for the detection of estrogenic compounds (ECs) in drinking water, using affinity principles was determined. This affinity separation was based on the interaction between the analyte 17β-estradiol (E_2) and the recombinant protein MBP-hERαLBD. The MBP-hERαLBD was immobilized on a solid matrix support. The alpha human estrogen hormone receptor (hERα) was used to test for the binding affinity of the fusion protein to a ligand, radiolabelled E_2. Each component of this bioaffinity system, from the membrane matrix to the expression/purification of the bioligand, and raising of antibodies against the purified bioligand, was studied with the aim of producing a well-characterized system with the following advantages: robust in nature, cost effective and high loading capacity.

Specifically, this study describes:

1. Expression of the bioligand maltose-binding protein (MBP) to be used as an affinity ligand for immobilization onto a solid membrane matrix.
2. Expression of MBP as a fusion protein to the human estrogen receptor alpha ligand binding domain (hERαLBD).
3. The affinity purification of biospecific bioligands (MBP and MBP-hERαLBD) using a one-step affinity purification system with amylose forming the solid phase of the affinity chromatographic column.
4. Generation of anti MBP-hERαLBD antibodies to be used for the characterization of the bioligands by means of western blotting.
5. The fabrication and characterization of a flat-sheet membrane as a model affinity-matrix.
6. Developing an affinity immobilization protocol for the immobilization of the bioligand onto the affinity membrane (AM) matrix.
7. Quantitative analysis of the immobilized bioligand present on the surface of the membrane matrix using tritiated E_2.

The recombinant protein (MBP-hERαLBD) was successfully expressed and purified to form a bio-specific ligand for its immobilization onto a cellulose acetate (CA)/amylose functionalized affinity membrane. Polyclonal antibodies were successfully raised against the purified recombinant protein. The anti-MBP-hERαLBD antibodies were subsequently used as a potential 'marker' to confirm the immobilization of the recombinant protein onto the CA/amylose functionalized membrane. Attempts to utilize the protein-coated membrane for the selective recovery of E_2 were, however, unsuccessful.

OPSOMMING

Die doel van hierdie projek was om 'n affiniteits-gebaseerde membraansisteeem te ontwikkel wat nie net biospesifiek en bioversoenbaar is nie, maar wat ook as 'n adsorpsiematriks vir die immobilisering van 'n rekombinante maltose bindingsproteïen menslike estrogeenreseptor-α-ligand bindingsdomein (MBP-hERαLBD) kan dien. Die lewensvatbaarheid van die affiniteitsmembraan sisteem (AMS) vir die deteksie van estrogeen-verwante stowwe (ECs) in drinkwater deur die gebruik van affiniteitsmetodes was bepaal. Affiniteitskeiding was gebaseer op die interaksie tussen die analiet 17β-estradiol (E$_2$) en die rekombinante proteïen MBP-hERαLBD. Die rekombinante proteïen, MBP-hERαLBD, was op 'n soliede matriks geïmmobiliseer. Menslike alfa estrogeen hormoon reseptor (hERα) was gebruik om vir die bindingsaffiniteit van die fusieproteïen aan die ligand, E$_2$ te toets. Elke komponent van hierdie studie, insluitende die bioaffiniteitssisteem, die membraan matriks sowel as die suiwering van die rekombinante proteïen en die suiwering daarvan, was bestudeer met 'n poging tot die lewering van 'n goedgekarakteriseerde sisteem met die volgende voordele: langdurigheid in gebruik, koste-effektiwiteit en hoë ladingskapasiteit.

Hierdie studie beskryf spesifiek die volgende

1. Ekspressie van die bioligand, maltose bindingsproteïen (MBP) om as affiniteitsligand te dien vir immobilisering tot 'n soliede matriks.

2. Ekspressie van 'n MBP as fusieproteïen tot die menslike estrogeen reseptor-α-ligand-bindingsdomein (hERαLBD).

3. Die affiniteitssuiwering van biospesifike bioligande (MBP en MBP-hERαLBD) deur gebruik te maak van enkel stap affiniteitssuiwering met amilose as soliede fase vir die affiniteitschromatografiekolom.

4. Opwekking van anti-MBP-hERαLBD-antiliggame wat gebruik kan word vir karakterisering van bioligande met behulp van Imunnokladtegnieke

5. Die ontwikkeling en karakterisering van 'n platvelmembraan as 'n model vir affiniteitschromatografie.

6. Die ontwikkeling van 'n affiniteitsimmobiliseringsprotokol vir die immobilisering van die bioligand op 'n affiniteitsmembraanmatriks.

7. Kwantitatiewe analisering van die geïmmobiliseerde bioligand teenwoordig op die oppervlak van die membraanmatriks deur gebruik te maak van 17β-estradiol.

Die rekombinante proteïen (MBP-hERαLBD) is suksesvol uitgedruk en gesuiwer om 'n biospesifieke ligand vir die immobilisering daarvan op selluloseasetaat (CA)-amilose funksionele affiniteitsmembraan. Poliklonale teenliggame, gerig teen die gesuiwerde rekombinante proteïen, is suksesvol opgewek. Die anti-MBP-hERαLBD-antiliggame is vervolgens as 'n potentiële merker geïdentifiseer om die immobilisering van die rekombinante proteïen op die CA-amilosemembraan te bevestig. Pogings om die proteïenbedekte membraan vir die selektiewe herwining van E_2 te gebruik was onsuksesvol.

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LISTS OF ABBREVIATIONS AND ACRONYMS

AC	Affinity chromatography
AFM	Atomic force microscopy
AM	Affinity membrane
AMS	Affinity membrane system
Amp	Ampicillin
ATP	Adenine triphosphate
CA	Cellulose acetate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	1, 4-Dithiothreitol
E_2	17β-estradiol
ECs	Estrogenic compounds
EDCs	Endocrine disrupting chemicals
EEDs	Environmental endocrine disruptors
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
ER	Estrogen receptor
FT-IR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GFC	Gel filtration chromatography
GST	Glutathione S-transferase
HIC	Hydrophobic interaction chromatography
HRP	Horseradish peroxidase
IEC	Ion-exchange chromatography

IPTG	Isopropyl-β-D-thiogalactopyranoside
LC	Liquid chromatography
MAC	Membrane affinity chromatography
MBP	Maltose binding protein
MBP-hERαLBD	Maltose binding protein human estrogen receptor alpha ligand binding domain
MCS	Multiple cloning site
MS	Mass spectrometer
NMR	Nuclear magnetic resonance spectroscopy
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffer saline
PSMF	Phenylmethanesulfonic acid
RNA	Ribonucleic acid
RPC	Reverve phase chromatography
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SPE	Solid phase extraction
TBST	Tris-buffered saline Tween-20
XPS	X-ray photoelectron spectroscopy

CHAPTER 1

INTRODUCTION

1.1   BACKGROUND TO PRESENT STUDY

There is growing evidence that certain hormone active agents in the environment can disrupt chemical messengers (hormones) of the endocrine system by sending erroneous signals or blocking legitimate signals. The putative hormone active agents, also known as endocrine disrupting compounds (EDCs), exert their profound and deleterious effects on humans and wildlife by mimicking, blocking and disrupting the physiological functions of the messengers of the endocrine system [1,2]. These chemical messengers generally exert their functions by interacting with their corresponding receptors in the cells to trigger responses and prompt normal biological functions such as growth, development, behaviour and reproduction [2]. Interferences with the activities of the chemical messengers, such as is the case with EDCs, will damage the system receiving the message. These interferences can lead to reversible or irreversible abnormal biological outcomes including stunted growth, impairment of short term memory, tubal pregnancy, low sperm count, reproductive failure and damage of the immune system [1,2]. It is clear that as researchers continue to look at the adverse effects caused by these hazardous compounds on humans and wildlife they continue to find significant, often permanent, effects at remarkably low doses.

EDCs can be categorized into three major groups according to the abnormal biological conditions they exert on humans and wildlife. The three major groups include: androgenic (compounds that mimic or block natural testosterone), thyroidal (compounds with a direct or indirect impact on the thyroid glands) and estrogenic (compounds that mimic or block natural estrogens). Despite the broad spectrum of EDCs, it is the estrogenic compounds (ECs) that are the most prominent and most studied [3]. This is in all likelihood due to the importance of ECs in cancer research [2]. ECs are found in low doses in literally thousands of products, some of which include: diethylstilbestrol, bisphenol A, polybrominated diphenyl ethers and phthalates. These compounds have been widely reported to be present in very low concentrations in the environment, but their relatively high fat solubility enables them to bioaccumulate up the food

chain, leading to significant physiological responses at these low concentrations [2]. Other relevant sources of endocrine disrupting chemicals are found in insecticides, herbicides, fungicides, plasticizers, plastics, resins and industrial chemicals such as detergents. The hydrophobicity of ECs, coupled with other chemical properties, has created unique challenges to environmental analytical chemists in developing techniques required for detecting and screening of these compounds. Several analytical techniques have been used to detect these compounds in the environment [2,3-5]. Analytical methods frequently used for detecting these compounds include: high performance liquid chromatography (HPLC) [6,7], liquid chromatography/mass spectrometer (LC/MS) [8,9], gas chromatography/mass spectrometer (GC/MS) [10,11], and solid phase extraction (SPE) [12,13]. These techniques are, however, generally unsuitable for large scale detection. Their use is further limited due to costs, intensive labour and the relatively poor sensitivity of the techniques, since they are specific only for one analyte or a limited class of structurally related compounds. However, affinity chromatography (AC) is considered to be an effective method for analytical detection of ECs in the environment [14].

AC systems are powerful techniques in which biospecific and reversible interactions are used for the selective separation and purification of biological molecules from complex biological matrices [15-17]. These systems are increasingly being used in the field of biotechnology due to the specific interaction between the immobilized ligands (usually grafted or linked to the matrix via the use of a spacer arm) and the bio molecules to be separated. These affinity systems consist of two distinct parts: the mobile phase, which carries the biological molecule to be separated and the solid phase, which is usually modified to carry the affinity ligands. Not withstanding the fact that these systems are widely used, they still have some shortcomings, including the requirements for a large column set-up and a longer diffusion path length, which in turn leads to a significant increase in the time required for the entire downstream processing from the introduction of the crude extract to the final purified product. Membrane affinity chromatography (MAC) was introduced to overcome some of the shortcomings of column affinity chromatography. Its introduction has significantly reduced the number of steps needed to obtain a pure product due to the specificity of the interaction between the stationary phase and the target biomolecule, not withholding the larger surface area and shorter diffusion path length that the system offers [18]. With the above-mentioned advantages, affinity membrane system (AMS) should serve as a powerful technique for analytical detection and removal processes of ECs from the environment.

ECs mimic or block the endogenous estrogens by binding to the ligand binding domain of the estrogen receptors (ERs) of the endocrine system. Exploiting the interaction existing between the estrogen and its receptors, and using the chemical information obtained from this interaction, a more reliable and more specific analytical method (i.e. a composite CA/amylose functionalized affinity membrane) than the HPLC, GC, MS and LCMS techniques can be introduced for detecting and concentrating ECs in the environment.

1.2   OBJECTIVES

This project aims at proving that synthetic CA/amylose functionalized AM can be used as affinity adsorption matrices for the immobilization of recombinant protein (MBP-hERαLBD). The AMS could be used to design other models that will provide a means of capturing molecules such as large proteins, antibodies, enzymes and other biological molecules from their respective environments. Each component of this bioaffinity system, starting from the membrane matrix to the expression/purification of the recombinant protein, the generation of antibodies against the purified protein, and the immobilization of the protein on the membrane is to be studied with the aim of producing a well characterized AMS with the following advantages: robustness, cost effectiveness and regeneration properties.

The objectives of this study can be classified into two main groups;

1.   The fabrication of a composite CA/amylose functionalized affinity membrane for the immobilization of the affinity ligand (MBP-hERαLBD).
2.   Application of the MBP-hERαLBD-immobilized hybrid membrane for the selective recovery of specific biomolecules, in this case ECs.

1.3   METHODOLOGY

The affinity membrane process developed in this project will involve the immobilization of the carrier MBP-hERαLBD fusion protein onto a CA/amylose hybrid membrane. This hybrid membrane will be designed to increase the stability of the carrier. The MBP, with its affinity for amylose, will interact specifically with the amylose in the composite affinity membrane while the hERαLBD on the other hand will be used as a potential receptor to capture the ECs present in

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the solution.

Concisely, in order to achieve the above-mentioned objectives, the experimental rationale and attendant tasks are briefly described below, and a simplified flow diagram illustrating these experimental tasks is depicted in Figure 1.1.

1.3.1 Expression of MBP-hERαLBD

The pMalc2 vector, containing the cloned gene (hERαLBD), was transformed into a TB1cell expression system. MBP-hERαLBD expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) in the growth solution. After sonication of the cell suspension, the protein was harvested by centrifugation.

1.3.2 Purification of MBP-hERαLBD

Purification of the expressed proteins (MBP-hERαLBD) was achieved following the basic principles of conventional affinity chromatography on an amylose resin.

1.3.3 Antibody generation

The purified MBP-hERαLBD was used to elicit polyclonal antibodies in rabbits. Serum from the rabbit was collected on days 0, 28, 43 and 45 for the determination of the antibody titre. The antibodies was used to asses the ability of the new hybrid membrane to immobilise the expressed MBP and MBP-hERαLBD.

1.3.4 Membrane fabrication and characterization

Membranes were fabricated from commercially available polymers, CA and amylose, using the immersion precipitation technique. Sections of the fabricated membrane ($1cm^2$) were used as matrices for membrane surface characterization by scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FT-IR).

1.3.5 Protein immobilization

Sections of the manufactured hybrid membranes ($1cm^2$) were used for the immobilization

procedures. This was achieved by incubating the $1cm^2$ membranes in a solution containing the recombinant protein (MBP-hERαLBD).

1.3.6 Immobilized membrane quantification

Following the immobilization of the recombinant protein onto the hybrid membrane, the immobilised hERαLBD was used for capturing radio labelled 17β-estradiol (E_2) in environmental solution. The efficiency of E_2 captured by the membrane-immobilised hERαLBD was assessed by liquid scintillation spectrometery.

Expression of MBP from pMAlc2 (Control)

Expression of MBP-ERLBD from pMAlc2

Affinity purification of the expressed biospecific ligands (MBP and MBP-ERLBD) for

Immunization of a rabbit for possible antibody production against the purified biospecific ligands

Affinity membrane matrix fabrication and characterization

Immobilization of MBP onto the affinity matrix

Immobilization of MBP-ERLBD onto the affinity matrix

Qualitative assay (Western blot analysis) for positive confirmation of the immobilized biospecific ligands

Affinity interaction between the MBP-ERLBD and E_2

Quantitative (radioactive) assay for positive confirmation of membrane recovery capacity

Figure 1.1: Flow diagram illustrating the methodology used in this study.

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1.4   LAYOUT OF THESIS

A brief description of the aims and significance of each chapter of this thesis is outlined below.

In Chapter 1, a brief background on EDCs is presented. The concept of membrane affinity separation as an application process in biotechnology is also discussed. The objectives of the study within the confines of the broader aim of developing a reliable affinity membrane for protein biomolecule separation coupled with a concise lay out of the thesis are presented.

A literature review of the field of liquid chromatography and membrane technology is presented in Chapter 2. Herein, different types of liquid chromatography systems are discussed, with emphasis on affinity chromatographic systems. Membrane configuration, models/designs and membrane transport mechanism are also discussed. The structural/functional characteristics of MBP and an overview on EDCs are presented.

The expression and purification of MBP and the recombinant MBP-hERαLBD fusion proteins are discussed in Chapter 3. The pMalC2 expression vectors harbouring the proteins of interest were used for expression in a TB1 cell expression host system. Soluble products obtained from the expression were purified using a one-step purification system.

Immulogical studies on MBP-hERLBD are described in Chapter 4. This chapter deals with the preparation of antibodies against the purified MBP-hERLBD and the application of these antibodies for the specific detection of MBP and MBP-hERαLBD fusion proteins.

The fabrication of a functionalized composite flat-sheet membrane using the immersion precipitation method is described in Chapter 5. Membrane characterization using SEM and FT-IR are also discussed in the same chapter. Qualitative and quantitative analyses were performed on the fabricated membranes, and the results obtained are documented in Chapter 6.

In Chapter 7, the findings obtained from this experimental work are summarised.

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CHAPTER 2
LITERATURE REVIEW

2.1   INTRODUCTION

The selective recovery, purification and characterization of biomolecules is a very important phenomenon in biotechnology. The high demands for ultra-high purity and yield of these biomolecules coupled with the rapid progress made and achievements attained in the fields of medical and biopharmaceutical applications have led to the development of methods for generating large sets of genomic and proteomic biomolecules. Some of these biomolecules (e.g. DNA, interferons, vaccines, antibodies, therapeutic proteins, polypeptides, hormones, polynucleotides, insulin, erythro proteins, tissue plasminogen activator [1,2]) are extremely sensitive and recovery of these molecules from their biological host environment requires great attention to their unique characteristics [3].

Some industries and commercial sectors are known for producing and discharging large quantities of heavy metals into the environment [4]. These heavy metals include nickel, lead, mercury, silver, selenium, zinc, copper, arsenic and cadmium, all of which are known to pose a great threat to human health when released in any quantities into the environment. As previously mentioned in Section 1.1, several endocrine modifying chemicals are constantly being released into the environment [5,9]. These environmental endocrine disruptors (EED) are usually byproducts of industrial wastes or from agricultural run-offs. EED are a broad group of compounds known to interfere with the normal functioning of the endocrine system, leading to abnormal biological conditions in humans and wildlife. Creating a downstream process for the selective recovery of these metals/chemicals from the environment as soon as they are discharged is therefore very important.

Considering the increasing need for biomolecules by pharmaceutical companies, coupled with the large number of hazardous chemicals constantly released into the surroundings, there is an increasing need for methods that will facilitate rapid, reliable and efficient screening and recovery of these biomolecules and chemicals. Various downstream separation techniques have been used in the field of biomolecules separation [10-15], amongst which column chromatography has been the most successful. Within the frame of liquid column chromatography, affinity column chromatography has exhibited the best performance in terms of product purity with respect to its high specificity [16-21]. However, due to the high costs and

tedious input required associated with conventional column chromatography, researchers' efforts have shifted towards the use of membranes as affinity matrices for high-resolution separation of biomolecules. An example of such a matrix has been illustrated with bioreactors [22,23] in which the membrane processes have shown greater scope than conventional support matrices such as polymeric beads and agarose gel [24,25].

2.2   CHROMATOGRAPHY

Among the many techniques used in biotechnology for product recovery purposes over the past few decades, chromatography has been the most successful. The term chromatography was first coined by Tswett in 1906 where he referred to chromatography as any separation technique involving components distributed between a stationary and a mobile phase. Separation of a sample is necessitated by the fact that the sample components have different affinities for both the stationary and mobile phases, and therefore the compounds migrate at different rates along the column. Chromatographic separations can be carried out using a variety of supports, including immobilized silica on glass plates (thin layer chromatography), volatile gases (gas chromatography), paper (paper chromatography), and liquids, which may incorporate hydrophilic, insoluble molecules (liquid chromatography).[1] Liquid chromatography has been the most widely used chromatographic technique due to its high selectivity and purity of the resulting biomolecules.

2.3   LIQUID CHROMATOGRAPHY

Some examples of liquid chromatographic techniques are briefly described below.

2.3.1   Ion-exchange chromatography

The use of Ion-exchange chromatography (IEC) allows molecules to be separated based upon their charge. Families of molecules (acidic, basic and neutral) can be separated by this technique. Basic proteins, which are positively charged, will bind to a support, which is negatively charged, while acidic proteins, which are negatively charged, will bind to a positive support. Elution of the desired product is usually accomplished by simply increasing the salt concentration or altering the pH of the eluting buffer.

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2.3.2 Hydrophobic interaction/Reverse phase chromatography

Generally, not all molecules to be separated are charged molecules. Some molecules contain hydrogen side-chains that are not charged and therefore cannot be separated using the ion-exchange technique. These hydrophobic molecules contain their active groups (e.g. amino acids) buried inside the molecule as the molecule folds to its native form. During hydrophobic interaction, these hydrophobic molecules will bind to a support that contains immobilized hydrophobic groups. The interaction taking place between the hydrophobic molecules and the immobilized hydrophobic support is the "clustering" effect, since no covalent and ionic interactions are involved. Elution of the desired product from the hydrophobic adsorbents can be effected by lowering the temperature, change in pH, or by adding a polyol such as ethylene glycol or a non ionic detergent to the elution buffer [3].

2.3.3 Gel-filtration chromatography

Gel-filtration chromatography (GFC) also known as size-exclusion chromatography is an isocratic system, based on the ability of molecules to move through the column of gel that has pores of clearly defined sizes. The larger molecules cannot pass through the pores, thus they pass quickly through the column and elute first. The slightly smaller molecules can enter some pores and so take longer to elute. The smallest molecules will go through most or all the pores and will thus be delayed longer in the column.

2.3.4 Affinity chromatography

The term affinity chromatography (AC) was first used in 1968 by Cuatrecasas *et al.* [26]. It is a type of adsorption chromatography in which the molecule to be separated is specifically and reversibly adsorbed by a complementary bio-specific ligand that is covalently attached to the chromatographic bed material or the matrix [16]. The complexes formed as a result of the interactions taking place in the process are often similar to complexes occurring in nature. The molecular forces and bond interactions forming these complexes are systematic, and include ionic bonds, hydrophobic interactions, hydrogen bonding, Van der Waals forces, London dispersive forces, dipole-dipole interactions and charge-transfer interactions [16].

Each of the above-mentioned separation techniques is based on a particular property of the biomolecules to be separated, i.e. how these biomolecules differ from one another and also the

interaction existing between the molecule and the stationary phase of the column. Some of the properties that are used as basis for separation are:

- charge;
- hydrophobicity;
- affinity (biological affinity for another molecule);
- solubility or stability (sensitivity to the effects of environmental conditions, such as heat); and
- molecular mass.

Among the above-described chromatographic techniques, AC occupies a unique place in separation technology since it is the only technique that enables the separation of almost any biomolecule on the basis of its biological or chemical structure. Comparisons between the different types of liquid column chromatographic techniques are listed in Table 2.1.

Table 2.1: Comparisons between different types of liquid column chromatographic techniques [27]

Property	Affinity		Ion-exchange	Reverse phase/ hydrophobic interaction
	Group specific	Bio-specific		
Adsorption capacity	Medium-high	Low	High	Medium-high
Selectivity	Medium-high	High	Low-medium	Low-medium
Recovery	High	Medium	High	Medium
Loading phase	Mild	Mild	Mild	Usually harsh
Elution phase	Mild	Harsh	Mild	Mild
Regeneration	Complete	Incomplete	Complete	Incomplete
Cost	Low	High	Low	Low

The understanding and quantification of the interactions taking place within the solid-liquid interface during AC have recently become important areas of study since the introduction of highly sensitive and non-destructive analytical techniques such as, scanning probe microscopy, ellipsometry and surface plasmon resonance spectroscopy [28].

2.4   SALIENT CHARACTERISTICS OF AFFINITY MATRICES

When designing an affinity chromatographic system, the solid support should be the first, and most important point of consideration, since it comprises the greater part of the column and it is also the domain onto which the biomolecules couple. Designing such a domain must be carefully done to favour the interaction between the support and the molecule of interest. Affinity matrices are usually designed to make use of the physical and chemical properties of the molecule of interest. In designing such a matrix, certain salient characteristics need to be taken into consideration [27, 29]:

- it must be insoluble in the buffers used;
- it must possess good flow characteristics;
- it must have mechanical and chemical stability; and
- it must have sufficient surface area available for ligand accessibility.

Generally, AC matrices are usually classified into two groups: first, the predominantly single-composition matrices (e.g. agarose, collagen, cellulose, and controlled pore glass), and second, the dual composition and/or chemically modified matrices (e.g. agarose coated polyacrylamide, cross-linked agarose and acrylic coated iron particles [30]). One of the most widely used matrices is the sepharose beads. This bead-formed agarose gel displays virtually all the features required of a successful matrix for the immobilization of biologically active molecules. The sepharose beads contain hydroxyl groups that can be easily derivatized for covalent attachment of the ligand.

2.4.1   Ligands for affinity chromatography

As previously mentioned, AC involves the immobilization of bio-specific ligands (biological and synthetic) onto a solid support [31]. The correct choice of a ligand complementary to the biomolecule to be separated still poses one of the most challenging problems in the use of this separation technique [32]. Selecting a biological ligand for an affinity chromatographic system is thus influenced by two major factors. First, the bio-ligand to be immobilized should exhibit specific and reversible binding affinity for the substance to be purified and, second, it should have chemically modifiable groups that allow it to be attached to the solid support (matrix) without destroying the binding activity of the ligand. Selecting synthetic ligands for AC can be categorized into three main groups: rational, combinatorial and the combined [33]. A brief

comparison between the biological and synthetic ligands used in AC systems is presented in Table 2.2 below.

Table 2.2: Comparisons between biological and synthetic ligands [33]

Ligand category	Factors under consideration				
	Selectivity	Stability	Capacity	Cost	Toxicity
Synthetic	Medium to high	High	High	Low to medium	Medium
Biological	Very high	Low to medium	Low to medium	Medium to high	Low

During binding of the bio-ligand to the solid support, it is important to take into consideration which region of the ligand interacts with the support. If the ligand contains several coupling groups, the group with the least binding affinity for the biomolecule to be separated should be used in forming the complex with the solid support. Some commonly used ligands or ligates used in AC include amino acids, dye ligands, metal chelating ligands, ion-exchange ligands, immunoaffinity (antibodies and antigens) ligands.

The development of recombinant protein technology [33] and the use of pseudobiospecific ligands (e.g. reactive dyes, metal ions, L-histidine) [34] has been very useful in solving some of the problems regarding ligand choice in AC. Several epitope peptides and proteins have been developed recently to over-produce recombinant proteins [35].

Despite the enormous efforts invested in the field of AC, a few aspects regarding the AC system remain unresolved. These include insufficient ligand binding capacity of the support, lack of bio-specificity of the affinity ligand, different coupling methods, high pressure packing, pressure drop in the column, slow intra-bead diffusion of solutes, physical and chemical stability of the system, and the cost involved [36, 37]. These shortcomings of the system have prompted researchers to develop affinity membranes or modify traditional membranes to affinity matrices as alternative AC matrices [1,3].

2.5   MEMBRANE DEVELOPMENT

2.5.1   Introduction

Membrane filtration systems are finding an increasing number of applications worldwide, in the purification of potable and industrial water, water desalination, as well as in the removal of heavy metal ions, nitrates, phosphates, pesticides and phenol [38,39]. These membrane systems are not only used in water reclamation, but have also resulted in numerous applications in fields of pharmaceutical and biomedical applications [40]. Their design and use is set to grow considerably in years to come due to their importance in these applications. With the recent development of membrane technology, new knowledge has been applied for the production of more complicated and efficient synthetic membranes with the ability to compartmentalize processes. Membrane filtration systems are currently the best available technology for water and wastewater treatment [41-44].

The extensive use of such membrane filtration systems has led to the development of different types of membranes filters (hollow fibre and flat sheet forms), classified according to the size of the particles that can pass through the membrane pores. In descending order, by pore size, the different types of membrane technology include: microfiltration (MF), which removes particles down to 0.1 microns; ultrafiltration (UF), which removes particles from 0.01 to 0.1 microns; nanofiltration (NF), which removes most organic compounds; and reverse osmosis (RO), which removes dissolved salts and metal ions [41,43,44].

Subsequent to the recent advances in membrane material technology, various membrane separation processes have been investigated as alternative techniques to solid phase extraction (SPE) [10-15]. Among these, applications of liquid membrane processes in the separation of metal ions have been extensively studied [38,39]. Although these membrane processes have shown feasibility to some extent, they have not attained large-scale commercial application, most probably because of the membrane instability, loss of organic solvents, expensive clean-ups, poor robustness and difficulty with regeneration of the organic carriers. In consideration of these shortcomings, a new technique for solvent extraction with an immobilized interface was raised, originally by Kiani *et al.* [45]. Although this technique did reduce the aspect of instability to some extent, the loss of organic carriers still persisted.

Application of a membrane for the adsorptive recovery of biomolecules can be categorized according to the ligand chemistry of adsorption [3]. Membranes can also be used as an affinity, ion-exchange, hydrophobic and reverse-phase matrix for biomolecular separation.

2.5.2   Affinity membrane separation

The first membrane chromatographic system, developed in 1988, was introduced to resolve most of the shortcomings experienced by the existing traditional column chromatographic techniques [46]. Membrane chromatographic systems operate in convective mode, which can significantly reduce the diffusion limitation commonly encountered in column chromatography [1]. A recent variation of membrane filtration, membrane affinity chromatography (MAC), was introduced to increase the sensitivity of membranes towards targeted biomolecules [47]. MAC, just like the conventional affinity chromatography, exploits the molecular recognition between an immobilized ligand and the target molecule. In the MAC system the ligands routinely used are the pseudo-specific ligands[2] (e.g. dyes, amino acids, chelated metal ions) rather than biospecific ligands such as proteins, antibodies, bacterial proteins, receptors and lectins used in the earlier systems [34]. The use of pseudo-specific ligands in the MAC system is most probably encouraged by the inherent instability of the biospecific ligands during the cleaning-in-place and the sanitary-in-place procedures applied in cleaning the membrane system. The absorption of biospecific ligands to solid supports is a very complex process. It is determined by the chemical structure, surface roughness, the degree of hydrophobicity of the surface, the electrostatic interactions of the biospecific molecules with each other and with the surface and the structural stability of the biospecific ligand. Most biospecific molecules, when immobilized to a polymer membrane, undergo a conformational change that can greatly affect the integrity of the ligand [48].

The membrane chromatography systems have proven to be generally superior to the conventional affinity chromatography in certain aspects; in the case of the former system there is a convective flow of the solute through the membrane, no intra-bead diffusion (Figure 2.1) and small thickness of the membrane can result in a low or negligible pressure drop, which in turn causes a high flow-rate within the system. The membrane system experiences no bed compaction and can be scaled up easily. Additionally, in order to serve as affinity adsorbents,

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[2] Ligands that have a structural similarity to the natural ligand of an enzyme or a receptor.

these membranes can be designed or modified as ion-exchange, hydrophobic interaction and filtration membranes. Herein, the interaction between the dissolved molecules and the active sites on the membrane occurs by convective flow through the pore, rather than in the stagnant fluid inside the pores of an adsorbent matrix [49].
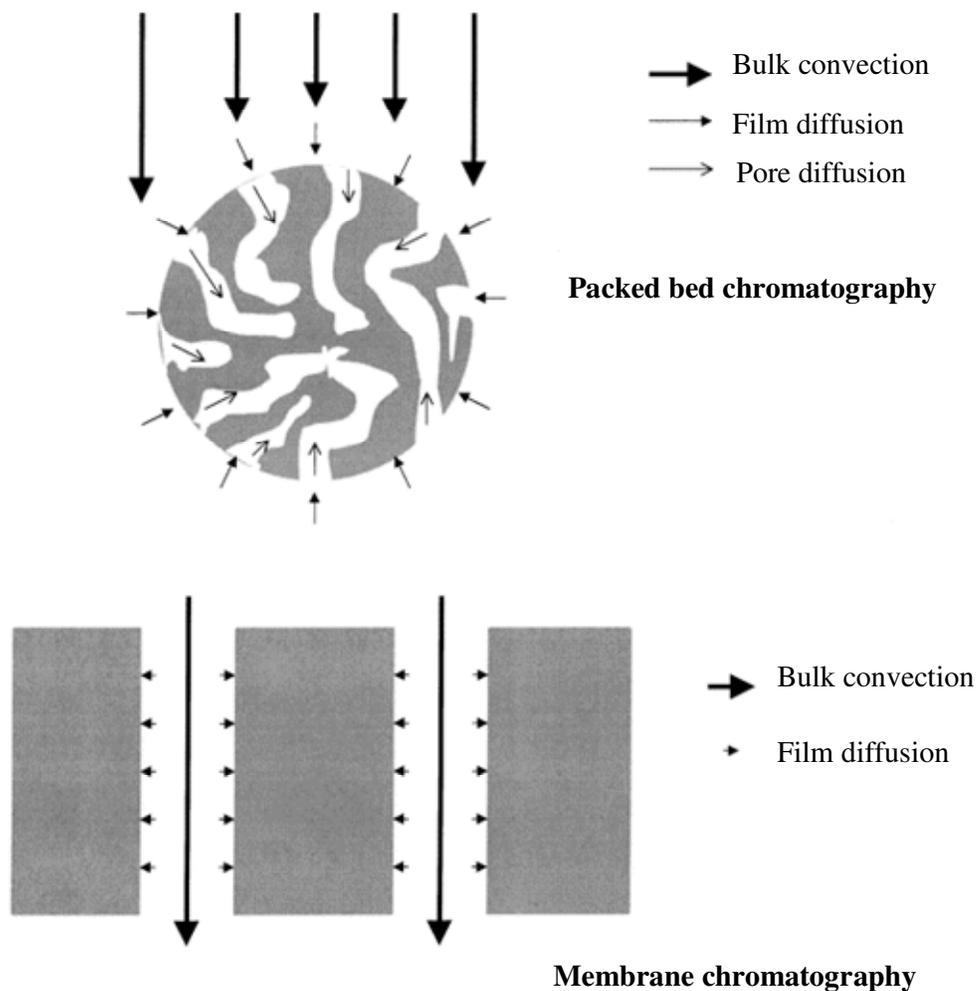
Figure 2.1: Solute transport in packed bed chromatography and membrane chromatography [50].

Although the membrane chromatography system presents a greater advantage to the packed bed column, the large diameter to length ratio of the membrane introduces the challenge of achieving uniform flow distribution across the membrane. Yuan *et al*. [51] designed a membrane system to overcome this problem. This membrane system gave a uniform distribution in numerical simulation and laboratory prototypes.

2.5.3   Membrane configuration and module designs

Membrane systems are usually designed to allow the continuous free flow of solution within the system [3].   The different module designs result in different flow-operation conditions and distinct separation efficiencies.   Various membrane shapes and module designs have been developed taking into consideration the different adsorptive membrane processes.   The membrane systems were derived from filtration modules and thus exist in a variety of configurations (i.e. stacked membranes, hollow fibre membranes, spiral-wound membranes, plate-and frame membranes) [3].   The two most commonly used shapes of membranes in appropriate module designs are the flat-sheet and the hollow-fiber membranes [1], of which the former have been most widely used [50].   Typical modules used in membrane housing and commonly used membrane configurations are illustrated in Figures 2.2 and 2.3 respectively.
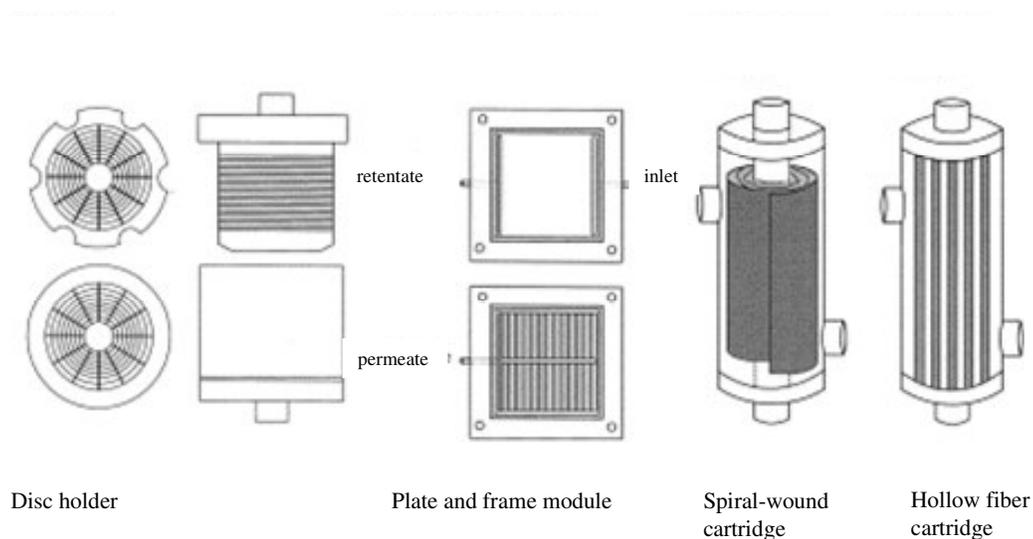
Figure 2.2: Schematic diagrams for some typical membrane modules [1].

The different module designs operate at different flow rates.   The appropriate design of a membrane chromatography module is only possible when the transport phenomena involved are well understood.   Detailed literature regarding the membrane shapes, modules, and transport phenomena of membrane chromatography has been documented [1,50].

Thin sheet   Membrane stack

Polymer rod   Spiral wound   Hollow fiber
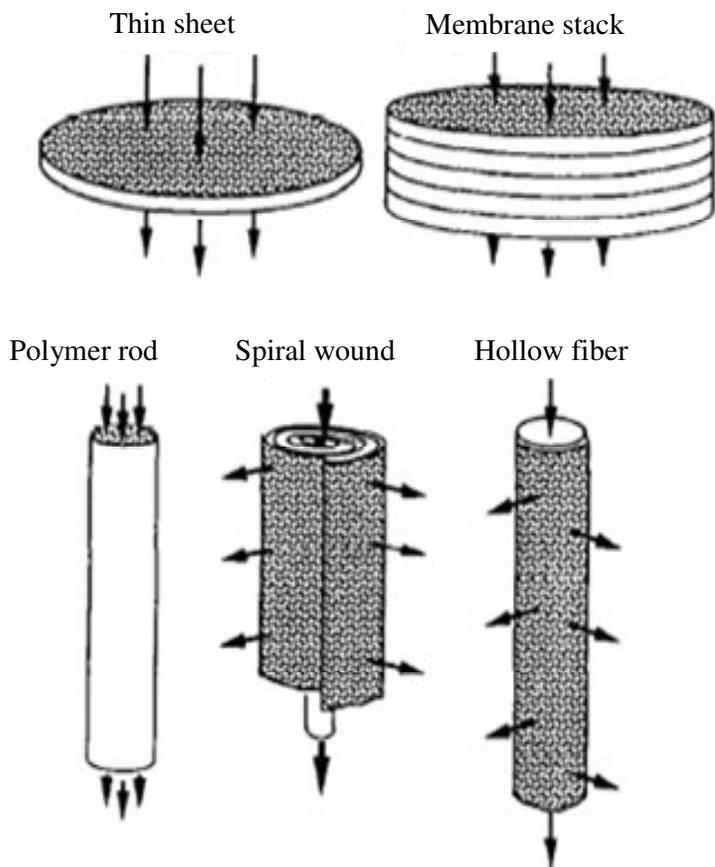
Figure 2.3: Schematic diagrams for commonly used membrane configurations, with arrows illustrating the directions of bulk flow [3].

2.5.4   Transport phenomena in membrane chromatography

The predominance of convective material transport has given membrane chromatography a great advantage over the packed-bed system. In membrane chromatography, the dissolved solute molecules are transported through an adsorptive membrane by bulk flow of the mobile phase. Within the system, the bulk flow of solvent carries the solute molecules toward the outlet at the same rate in which the solvent flows in the chromatographic system. During the transport of the adsorbate molecules the ideal equilibrium separation is affected by three main factors: Brownian thermal diffusion, dispersion and kinetic sorbate-sorbent interaction [3]. Much work has been done on the transport phenomena of membrane chromatography, as documented in several research papers [3,52-58]. Figure 2.4 shows a schematic representation of solvent movement in membrane adsorbers [50].

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Flat sheet

Hollow fibre

Radial flow
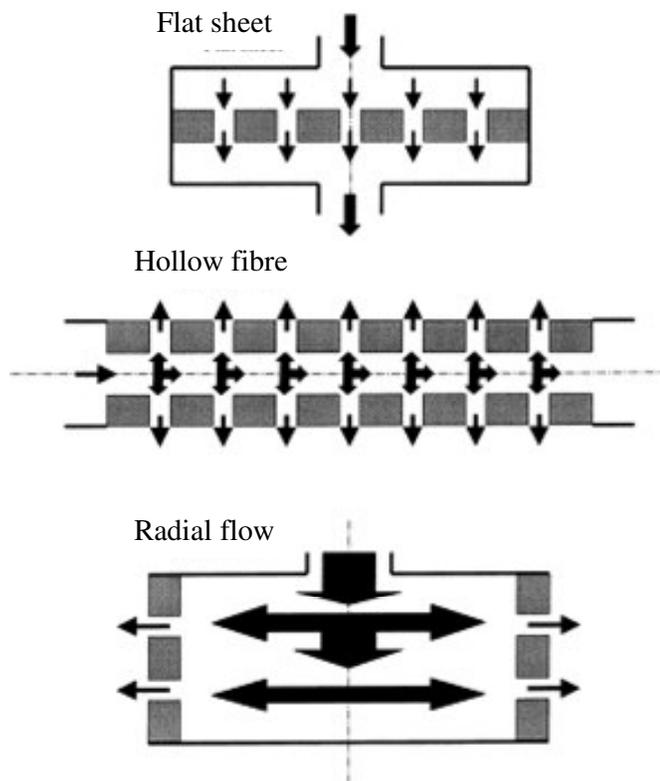
Figure 2.4: Schematic representation of flow in membrane adsorbers [50].

2.5.5   Matrices used in adsorptive membranes

In designing a system for affinity membrane separation, the first point to take into consideration should be the composition of the membrane matrix. The matrix design should take into account the biomolecules to be separated as well as the solvent required for its separation. An ideal matrix should have similar characteristics as conventional chromatographic matrices [3,59,60]. The required matrix characteristics include:

- high hydrophobicity and low non-specific adsorption;
- high specific surface area;
- fairly large pore sizes;
- high chemical, thermal, and mechanical stability; and
- sufficient surface functional groups.

In general, matrix materials can be categorized into two main groups: polymeric and inorganic materials [1,3]. The latter usually show better performance in terms of mechanical strength, thermal stability and chemical resistance than the former. On the other hand, the pore properties,

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cost, and capability for surface modification of the polymeric matrices are more competitive [1]. Some commonly used materials for matrices' fabrication include titanium, silicon dioxide glass, cellulose, regenerated cellulose, nylon, poly(glycidyl methacrylate-co-ethylene dimethacrylate), poly(glycidyl methacrylate), polyethylene, poly(styrene-co-divinylbenzene), polyvinylalcohol, polysulfone, polyethersulfone and polycarbonate [3]. The properties of these materials have been thoroughly evaluated in several review papers [61-71].

2.5.6   Selection of ligands for adsorptive immobilization

The ligand type in adsorptive membrane chromatography is generally used to categorize the affinity mode. The following selected criteria should be taken into account when choosing a ligand:

- the ligand must specifically and reversibly bind the targeted molecule and must contain groups that can be chemically modified to allow attachment to the support; and
- the chemical modification of the ligand must not impair its specific binding activity [72].

Ligands are commonly classified into two main groups, namely general or group specific ligands, such as metal ions, affinity dyes, amino acids, proteins A and G, lectin and coenzymes, and specific or bio-specific ligands, such as enzymes and substrates, antibodies and antigens. Although the bio-specific ligands have proven to offer a better selectivity than pseudo-specific ligands, the pseudo-specific or group specific ligands have gained increasing attention in the field of adsorptive membrane chromatography [1]. The various types of ligands used in affinity chromatographic systems and their corresponding ligates are summarized in Table 2.3.

Table 2.3: Ligands used in affinity chromatographic systems and their corresponding ligates [73]

Ligands		Ligates
Cibacron Blue F3GA		Enzymes, calmodulin, serum albumin, lipoprotein, interferon, thrombin, synthetase, transferase, myogubin, growth factor
Dyes	Protein Red HE3B K2BP	Enzyme, lipoprotein, cytotoxicity, carboxypeptidase G, kinase, dehydrogenase, alkaline phosphatase, polypeptide hormone
Amino Acid	Trp	Carboxypeptidase A
	Arg	Serine proteinase
	Lys	DNA, RNA
	His	Pyrogen, endotoxin, yeast proteinase
	Phe	γ-Glubulin
Protein A and G	Concanavalin A	IgA, IgG, IgM, antibody, insulin-like growth factor
	Lentil lectin	Polysaccharide, glycoproteins, membrane glycoproteins, glucolipid, enzymes and coenzymes with glycosyl
Lectin	Wheat germ lectin Peanut lectin	
Heparin		Human antithrombin polymerase, coagulation factor
Polymyxin B		Endotoxin
Metal chelates		Histidine, tryptophan, cysteine-containing proteins
Gelatin		Fibronectin
Calmodulin		Phosphodiesterase, ATPase, and calcinerin
Benzamidine		Urokinase, trypsin, thrombin, kallikrein
Hormone		Receptor
DNA, RNA, ribose		Nuclease, polymerase, nucleotide
Antibody		Antigen
Antigen		Antibody
Enzyme		Enzyme inhibitor
Enzyme inhibitor		Enzyme
Enzyme cofactor		Enzyme

Although MAC has several distinct advantages over conventional chromatography, the MAC system does have some shortcomings, for example:

- inlet flow distribution;
- low binding capacity between the ligand and the solid support;
- uneven membrane thickness; and
- pore distribution on the membrane [3]

Following the short-comings faced by the MAC system, as earlier mentioned in Section 1.3, a new membrane affinity system will be established to combat some of the drawbacks. Briefly, the affinity ligand (hERαLBD), used for the selective separation of the targeted biomolecule, will be expressed as a fusion protein to MBP. The resulting recombinant protein will, through affinity interaction, bind to a domain (amylose) that forms an integral part of the membrane matrix.

2.6 THE MALTOSE BINDING PROTEIN

2.6.1 Introduction

The malE gene that codes for MBP is part of the maltose/maltodextrin transport system of the *E. coli*. This transport system is responsible for the uptake and efficient catabolism of maltose and its higher homolog, maltodextrin [74]. It is a complex regulatory and transport system involving many proteins and protein complexes. The MBP is a soluble protein located in the periplasm of the *E. coli* [75]. In collaboration with an inner membrane-associated protein complex forming a channel (MalFGK$_2$), the MBP, through affinity interaction, actively transports the saccharides maltose and its higher homologs across the cytoplasmic membrane of the *E. coli*. The MBP and the inner membrane-associated complex both constitute the maltose transport specific system, which belongs to the large family of the ATP binding cassette, the ABC transporter [76,77]. Also, in association with the membrane chemotransducer, the MBP are involved in the chemotactic response of *E. coli* to maltose [78-80]. It should, however, be noted that the protein-dependent transport system not only recognises and tightly binds not only the disaccharide maltose, or the long linear maltodextrin but it is also involved in the transportation of even cyclodextrins with Kd value in the micromolar range [81]. The transportation of the maltose and its homologs across the cytoplasmic membrane is facilitated by the unique structure of the MBP.

2.6.2 Structure of the MBP

The MBP is the second largest binding protein in the family of binding proteins located in the periplasm of the gram-negative bacteria (*E. coli*). The periplasmic located protein, as reported by X-ray crystallography, is a protein with a relative molecular mass of approximately 40.6 kD and comprising 370 amino acids [82]. MBP is structurally composed of two main domains of approximately equal sizes, with the domains separated from each other by a deep groove (the ligand binding site) [83]. Each of the two domains is built from a secondary structural element belonging to both the amino- and carboxyl-terminal halves of the protein [82].

The ligand binding site, located in the deep groove separating the two domains is, heavily populated by polar and aromatic groups, some of which interact with the maltose and its higher homologs through hydrogen binding and van der Waals interactions. Upon binding of the ligand to the binding cleft, the MBP undergoes a conformational change from the open to the closed conformation, completely preventing the ligand from being accessed by the environmental solvent [83].
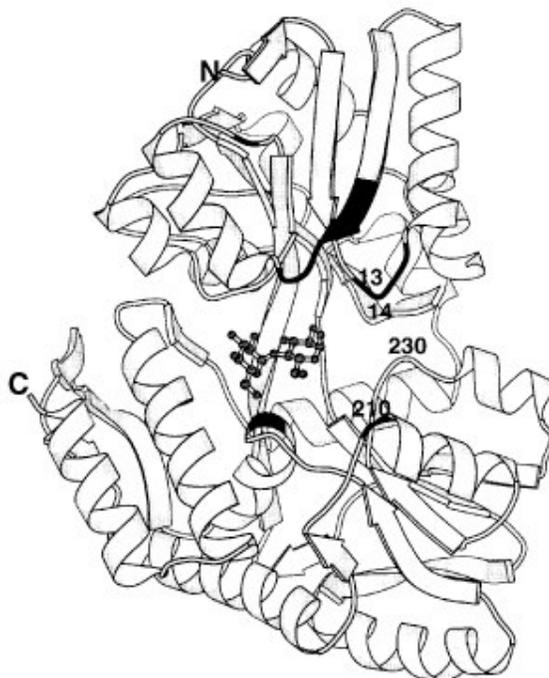
Figure 2.5: Graphic illustration showing the ribbon-style drawing of MBP [83].

The three-dimensional structure of the MBP as illustrated in Figure 2.5 shows the characteristic alpha and beta conformations of the two globular domains joined by the hinged region. The binding site for maltose and the maltodextrin is located in the deep groove found between the N

and the C domains. The beta strands and alpha helices are represented as flat arrows and coils respectively.

2.6.3 Translocation of MBP across the cytoplasmic membrane

In the periplasm of *E. coli* MBP serves as a soluble receptor for the high affinity transportation of maltose and maltodextrin [77]. The MBP or MalE of *E. coli,* just like other proteins of the eukaryotic cells [84-88], is synthesized in the cytoplasm as a precursor protein, preMalE [89]. Once the precursor protein is synthesized it is transported across the cytoplasmic membrane into the periplasm of the organism [90]. The translocation of the precursor molecule across the cytoplasmic membrane is initiated by an extension of some 15 to 30 predominantly nonpolar and hydrophobic amino acids [89]. The amino acid extension (the signal sequence) is generally heterogeneous in primary sequence and, as observed [91], many of the amino acid sequences can substitute for a natural signal sequence, at least partially. Therefore, signal sequence function does not appear to require a specific sequence of amino acids to initiate the translocation process. Once the translocation of the precursor protein across the cytoplasmic membrane is completed, the signal sequence is cleaved by the signal peptidase and the mature protein is released from the membrane into the periplasm where it folds to its native conformation [92]. The mature MBP, once in the periplasmic space of the organism, is now ready for its affinity interaction with the ligands (maltose and its higher homologs).

2.6.4 Maltose/maltodextrin transportation by the MBP

Maltose or maltodextrin will bind to the periplasmic MBP present in its open form (Figure 2.7A). This leads to a conformational change to the closed form (Figure 2.7B), and enables interaction with the transporter MalFGK2. Binding to the transporter, in turn, stimulates a conformational change back to the open form. The transporter opens at the periplasmic site with concomitant release of maltose into the transporter and the binding of ATP to the ATP-binding cassette, the MalK protein. Since the open form of MBP has a low affinity for the transporter, it dissociates, thereby triggering the closure of the transporter at the periplasmic site and opening at the cytoplasmic site in a process driven by ATP hydrolysis [95]. Figure 2.6 illustrates the mechanism by which maltose is transported from the periplasm to the cytoplasm of a gram-negative bacteria.
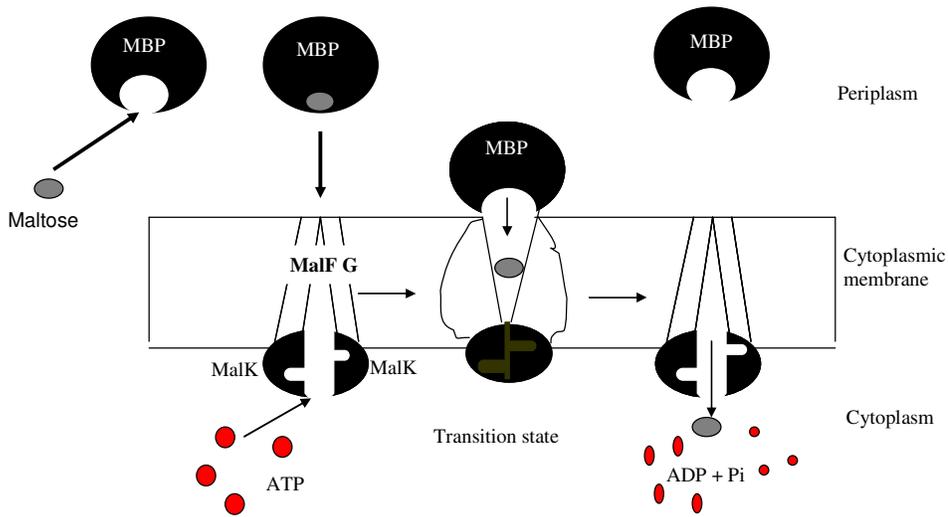
Figure 2.6: Schematic representation of the maltose uptake system of the *E. coli* [93].

The maltose (represented by black spots) is first bound to the MBP transporting unit. Upon contact with the membrane transporting system, composed of the MalF and MalG, a conformational change of the ATP binding cassette is triggered. MBP then opens up to release the maltose. Finally, the maltose is transported into the cytoplasm, a process driven by ATP hydrolysis [93].
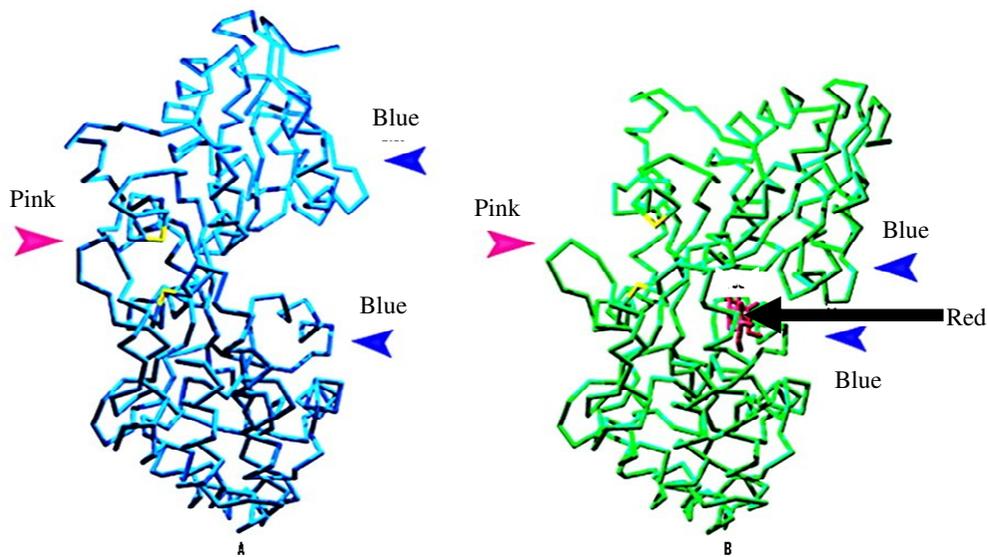
Figure 2.7: Diagrammatic representation of the open form (A) and the closed form (B) of the MBP [94].

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The ribbon (red) present in the centre of the closed form represents the bound maltose. The blue arrowheads show general regions of contact with the receptor, which are closely spaced in the closed form, but well-separated in the open form. The pink arrows indicate the interface, that is, the site located directly opposite to the binding site. Mutation of certain groups present in the interface has proven to have great influence on the MBP affinity for its ligands [95,96].

2.6.5 MBP as a solubility enhancer

Some proteins are not amenable to overproduction in the cytoplasm of *E. coli*. This is most probably because these proteins might either be toxic in the cytoplasm of the *E. coli,* or on the other hand, contain disulphide bonds. For proteins with disulphide bonds, the periplasm of the *E. coli* has the advantage of providing a more reducing environment coupled with a variety of protein disulfide isomerases [97]. Moreover, most of these proteins, when overexpressed, form aggregates and inclusion bodies. Protecting the expressed proteins from their hydrophobic environment and subsequent transportation to the periplasm of *E. coli* can be a possible solution to the problem of under-production of protein during protein expression.

The best strategy to avoid insolubility or inclusion body formation during protein expression is to fuse the protein of interest to a protein known to be expressed at substantial levels in soluble form in *E. coli.* The expression of the insoluble protein gene as a fusion partner to the protein-dependent transport system of the *E. coli* (MBP) has been an effective recombinant technique for the large scale production of soluble, active protein [98]. Additionally, not only will the MBP facilitate the transportation of the expressed proteins from the cytoplasm to the periplasm of the organism to confer its solubility, they also play an active role in a one-step purification of the expressed proteins by virtue of the MBP affinity for solid-phase amylose [98]. As mentioned in Section 1.3, the expression of the ligand (hERαLBD) as a fusion partner to MBP, will not only confer solubility to the ligand, but will provide a soluble, active affinity ligand that can be use for the capturing of EDCs.

2.7 CHARACTERIZATION OF ENDOCRINE DISRUPTING COMPOUNDS

2.7.1 Introduction

Over the past few decades there has been a significant increase in the number of chemicals in the biosphere capable of mimicking the action of endogenous hormones (such as estrogen) [99]. These chemicals, known as EDCs, have the potential of disrupting the normal functioning of the endocrine system by interfering with the transport, biosynthesis and metabolism of the endogenous hormones. Estrogenic compounds (ECs) are a class of EDCs that display estrogenic activity by mimicking the estrogen hormone. These ECs have been linked to hormone-related diseases such as breast cancer, testicular cancer, low sperm count and feminization in both humans and wildlife [99-105]. In the endocrine system, steroid hormones generally carry out their functions by binding to the ligand-binding domain (LBD) of specific receptors. Exploiting the interaction that occurs during the binding of endogenous hormones to their receptors can provide reliable and more specific methods for the detection and selective screening of potential environmentally toxic ECs.

2.7.2 Effects of estrogenic compounds and their mechanism of action

ECs, mimic the effects of estrogen hormones, resulting in abnormal responses [99-105]. These compounds, when bound to the endocrine receptors, do not only affect the reproductive system but are potential disruptors of other body organs such as the liver and heart [100,104]. The endocrine system controls and coordinates most of the basic human and animal development, differentiation and reproductive functions through hormones, secreted by ductless glands. Generally, these hormone signals tell "message receivers" in certain tissues (i.e. in reproductive organs, bones, the heart, adipose tissues), what to do. The way these hormones bind to their receptors can be visualized as a key and lock mechanism. The binding of a hormone to a specific receptor causes a conformational change in the receptor (i.e. causing the dissociation of the heat shock proteins), leading to the binding of the ligand receptor complex to the human hormone response element (HHRE) of the DNA. Binding of the receptor to the DNA leads to co-activation of the receptor-DNA complex, followed by transcription then translation (Figure 2.8). Some compounds can mimic the hormones that normally bind to receptors. Such interactions can lead to adverse effects [100-102], and hence these compounds are called endocrine disruptors.
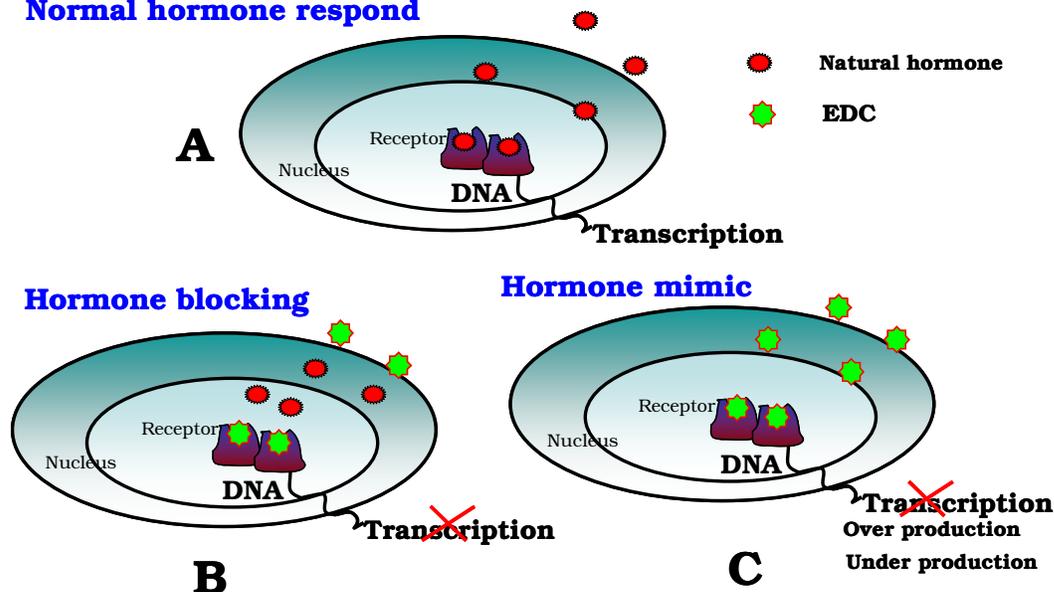
Figure 2.8: Diagrammatic illustration of the mechanism of action of the endogenous hormones and the effect of EDCs on the mechanism of action: (A) Endogenous hormones traveling to their target cell and binding to their specific receptors; (B) interference of the endogenous hormones by hormones blocker (EDCs) from getting to their target receptors; (C) EDCs mimicking the action of the endogenous hormones.

2.7.3   Structural and chemical properties of the estrogenic compounds

ECs have a variety of purposes and forms. The structures of these environmental estrogens are as varied as their functions. Apart from the fact that they almost always have aromatic rings, they have no general similarity to the normal hormone structure. Examples of these compounds include; diethylstilbestrol (DES), a pharmaceutical estrogen banned from use in the 1970s; coumestrol, a phytoestrogen that is a natural plant compound with some estrogenic tendencies; kepone (chlordecone), a synthetic pesticide banned in the United States; polychlorinated biphenyls (PCBs), used in electrical transformers and cooling systems; 4-nonylphenol (NP), a break-down product of detergents that are widely used in household products, industrial applications and plastics manufacturing; and O.p'-DDT, an isomer of the pesticide DDT. This diversity in chemical structure (Figure 2.9) makes it difficult to predict the hormonal effect of a particular compound [103,104,33]. Other factors that increase the potency of these chemicals are their high fat solubility, low molecular mass and resistance to environmental breakdown, that enable the ECs to easily reach the site where they manifest their action. Furthermore, these compounds lack visible characteristics, which make their identification and removal from the

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natural environment difficult. The occurrence of these compounds in some habitats, especially the environmental water supplies, poses a great threat to human health and wildlife.
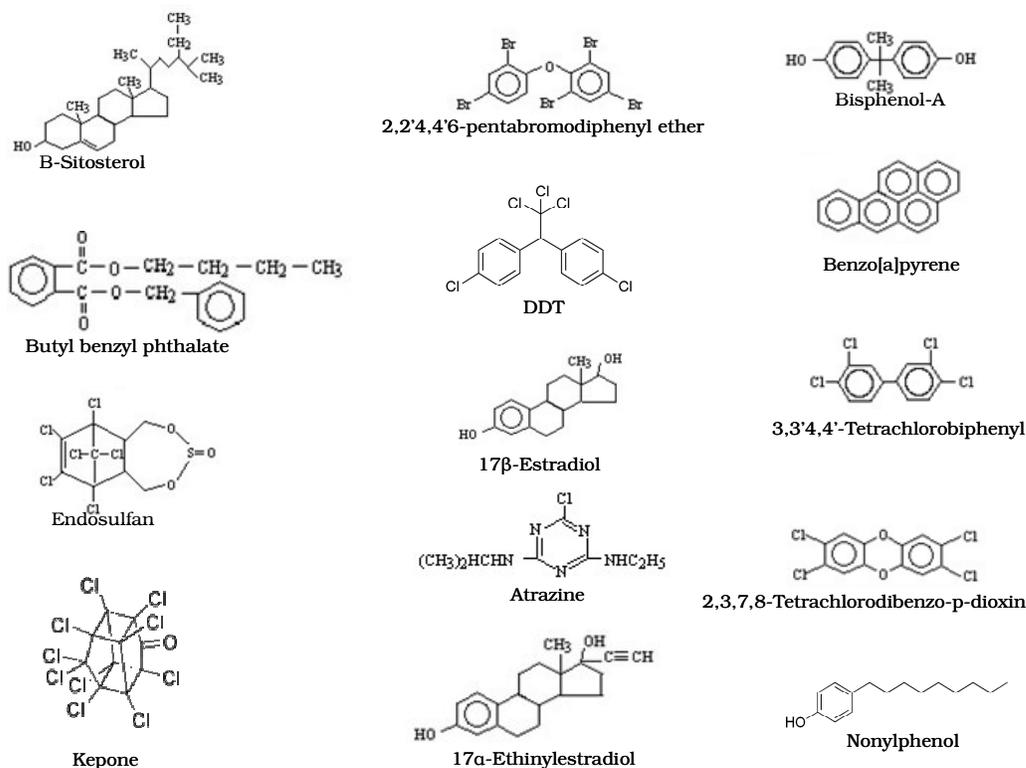
Figure 2.9: Compounds illustrating the structural diversity of chemicals in the environment reported to be estrogenic.

2.7.4 Estrogenic compounds in water supply

Scientists have in the past paid little attention to EDCs (which are less persistent) and focused their research work on persistent bioaccumulating toxic substances, which include dioxins, PCBs, and organochloride pesticides. Lately, scientists have started to focus on a number of substances not so highly persistent, but still widespread in the natural environment. These ECs can affect growth, reproduction, and development of organisms even at relatively low concentration in water [106]. There has also been some evidence that environmental disrupting chemicals exhibit synergistic effects; two weakly estrogenic compounds may combine to produce a stronger response [107]. These chemicals eventually permeate water supply through agricultural runoff since most of them are herbicides and pesticides. Futher, some of these ECs are products of industrialization and may get into the water supply through leakage from storage tanks, accidental spillages or illegal dumping.

2.7.5   Techniques used for the detection of endocrine disruptors

Much work has been carried out in recent years with the aim of improving the techniques used for the detection and quantification of ECs in the environment. Some of these models include: solid phase extraction (SPE) performance with oasis cartridges, followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS); SPE-gas chromatography-mass spectrometry (GC-MS); GC-MS/MS; high performance liquid chromatography (HPLC); HPLC-MS; HPLC-MS/MS; enzyme linked immunosorbent assay (ELISA); and ELISA-SPE [108,109]. Even though the above listed methods can be reliable, they have some potential drawbacks including the requirement of specialized workers, decreased robustness in nature, the inability to generate the solid matrix used, reduced accessibility, complex derivation, expensive clean-up and purification. The high cost, time-consuming nature and complexity of these techniques lead to limitations in their application on large-scale operations in the field of selective detection and separation.

To circumvent some of the shortcomings of the above-mentioned techniques, the concept of immobilized metal affinity chromatography was introduced, in which metals (mostly transition metals) were immobilized onto a matrix support by means of a ligand carrier through a covalent linkage [19,110-113]. Here, the metal ion attached to the carrier has one of its coordination sites open through which binding of the bio-macromolecules (proteins or enzymes) can take place. It was with this mechanism that molecules could be selectively retained and separated. This model, however, also had its shortcomings, such as the susceptibility of the metal ion to metal-catalyzed oxidative reactions, structure of the affinity matrix, susceptibility of the matrix to reducing agents, pH, ionic strength and the position of the open coordination site of the metal ion [114,115].

Since ECs exert their effects by binding to the ligand-binding domain of the estrogen receptor, affixing hERαLBD onto a matrix support will provide a viable alternative for the selective removal of these compounds from water. In this study the hERαLBD will be expressed as an MBP fusion protein. The MBP will be used to immobilize the hERαLBD on CA-amylose hybrid membranes. This system will then be tested for the binding of ECs. The cloning and expression of and MBP- hERαLBD for this purpose will be described in the next Chapter.

2.8   REFERENCES

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CHAPTER 3

EXPRESSION OF MBP AND MBP-hERαLBD FUSION PROTEIN IN *ESCHERICHIA COLI*

3.1   INTRODUCTION

The most widely used and best-studied host system for high-level expression of proteins and recombinant proteins is the gram-negative bacteria, *E. coli*. The *E. coli* expression system is a relatively simple system; it is inexpensive, has a fast high-density cultivation period, and its well-known genetics and numerous compatible tools available for biotechnology makes it a suitable system of choice for facilitated protein expression. Unlike in the case of the expression of soluble proteins, major shortcomings have been encountered when using the host system for the expression of many heterologous proteins and proteins lacking compatibility with the *E. coli* cytoplasm [1].

Most proteins (especially from eukaryotes) expressed in *E. coli* are found as insoluble aggregates in inclusion bodies [2-18] and when overexpressed most of these proteins become biologically inactive. This may be because the bacterium lacks the machinery for eukaryotic post translational modifications [19]. Formation of the aggregates or inclusion bodies may be attributed also to the formation of aggregates by the intermediates responsible for the folding of the proteins [20]. The solubility of a protein is one of the key properties determining its choice in functional and structural studies. Thus, overcoming aggregation and formation of inclusion bodies during protein expression in the cell cytoplasm is very important.

Regarding the aforementioned problems encountered during protein expression in the gram-negative bacteria expression system, it is possible to enhance the solubility and stability of some of these proteins. This can be achieve by reducing the induction temperature during expression and/or expressing the targeted protein as fusion proteins to fusion partners, such as the maltose binding protein (MBP) [21,22], glutathione S-transferase (GST) [23], hexahistidine [24,25] and thioredoxin [26]. These fusion partners are also advantageous in protecting the passenger or targeted proteins from intracellular proteolysis [27]. They may also serve as specific expression reporters [28,29]. The MBP has been cited by several authors as being a suitable fusion partner to GST, hexahistidine and thioredoxin since its fusion to proteins during expression helps to stabilize the expressed proteins, thus leading to an efficient functional expression of the proteins in the cell cytoplasm in a soluble, active form [31-40]. Not only are these fusion partners

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responsible for stability and solubility of proteins during protein expression but most of them are also exploited for specific affinity purification strategies [27,31,41,42,43].

In this study the targeted protein hERαLBD was expressed as a fusion protein to MBP. MBP, being the main supplier of carbon to gram-negative bacteria, is known to have a great affinity for maltose and, in the absence of the maltose, has a great affinity for amylose. The affinity chromatographic column used in this study was set up using amylose resin. Purification of the recombinant protein was possible, since these proteins will couple (using the MBP) to the amylose column via affinity interaction. Elution of the bound recombinant protein from the chromatographic column was done by adding maltose to the elution buffer, and since maltose has a greater affinity for MBP than amylose, it will displace the bound proteins from the column thus leading to the elution of the targeted proteins from the column.

3.2   MATERIALS AND METHODS

3.2.1   Reagents

Trypton, yeast extract, agar, phenylmethanesulfonic acid (PMSF) and Coomassie brilliant blue (G250 and R250) were obtained from Fluka Biochemika (South Africa). *E. coli* TB1 cells, pMalc2 plasmid vector, and amylose resin were purchased from New England Bio-labs (UK). 1, 4-dithiothreitol (DTT), isopropyl-β-D-thiogalactopyranoside (IPTG) and HindIII were obtained from Roche Diagnostics (South Africa). Sal I, EcoRI and BglII were purchased from Promega (South Africa). Ampicillin and ammonium persulfate was purchased from Sigma Aldrich (South Africa). Rainbow marker was obtained from Amarsham Life Science (South Africa) while ethylenediaminetetraacetic acid (EDTA) and QIAGEN Midi Kit 25 were obtained from BDH Chemicals (UK). All the other chemicals were obtained from Merck (South Africa).

3.2.2   Plasmid isolation and restriction digest assay

All clones were transformed into *E. coli* TB1 competent cells. For plasmid isolation and protein expression, 20 µl aliquots of the bacteria medium were streaked on agar plates containing ampicillin to a final concentration of 0.1 mg/ml and the plates incubated at 37°C overnight. For a starter culture, a single colony from an overnight agar plate was used to inoculate 5 ml of LB medium containing ampicillin to a final concentration of 0.1 mg/ml, and cultivated on a shaker (300 rpm) at 37°C for 9 h.

Subsequently, plasmid isolation was performed following instructions obtained from the QIAGEN Midi Kit (25). The plasmid harbouring the MBP was digested using two restriction enzymes, HindIII and BglII, while the plasmid containing the recombinant protein maltose binding protein human estrogen receptor alpha ligand binding domain (MBP-hERαLBD) was digested using EcoRI, SalI and HindIII. Figure 3.1 illustrates the ligation of the pMalc2 expression vector with the insert (hERαLBD) and Figure 3.2, the expected fractions after digestion of the plasmid with the appropriate restriction enzymes.
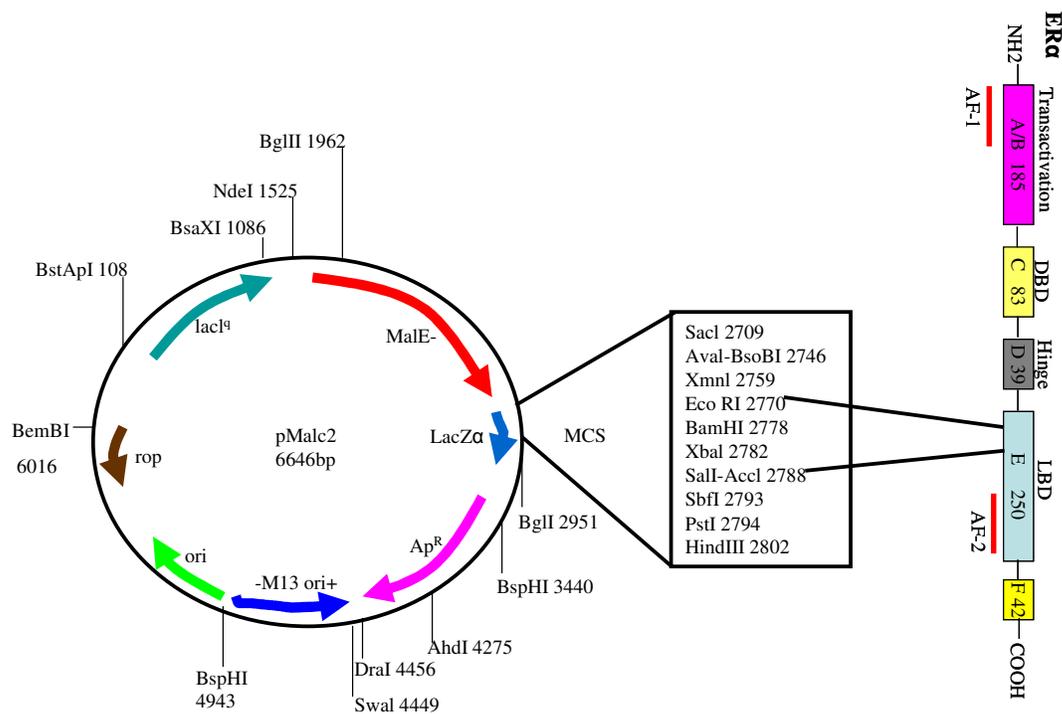
Figure 3.1: Diagrammatic representation of the pMalc2 expression vector showing the cloning of the insert hERαLBD into the multiple cloning side (MCS) using two restriction enzymes Eco RI and SalI.

The LBD gene cloned, from the alpha human estrogen receptor, is a protein consisting of 250 amino acids with a molecular mass of approximately 25 kDa. The gene was ligated into a plasmid expression vector pMalc2 and transformed into *E. coli* TB1 cells for expression. The LBD was expressed as a fusion protein to fusion partner MBP a malE gene of molecular mass 40.6 kDa and 370 amino acids.
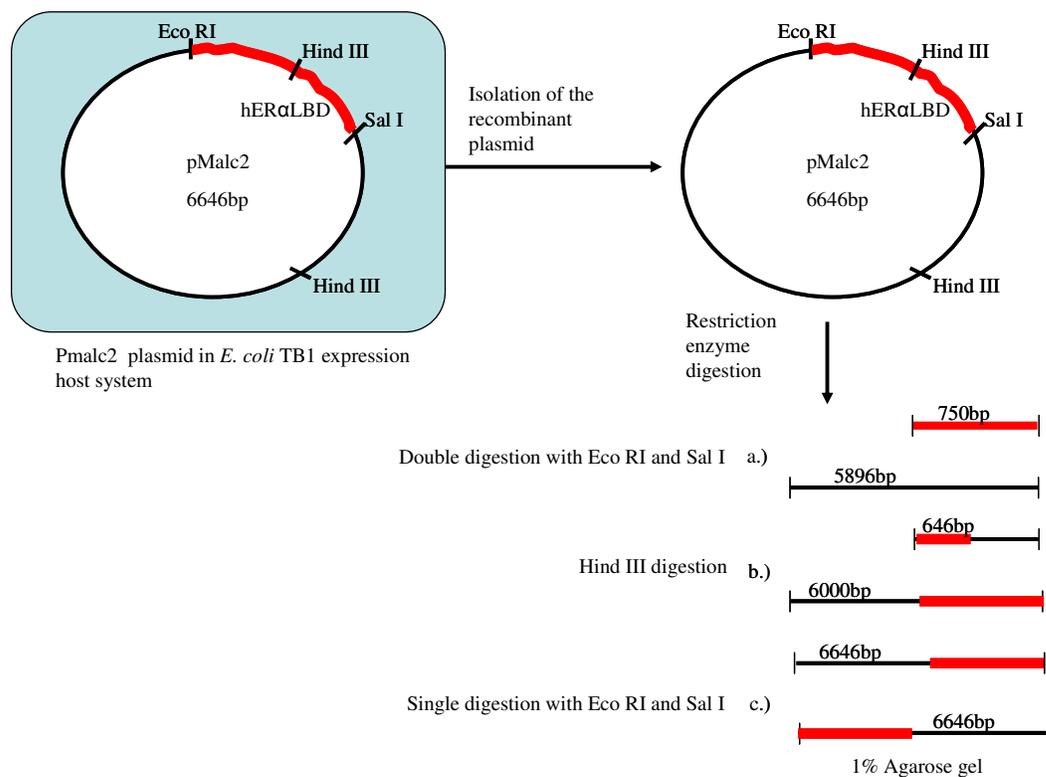
Figure 3.2: Flow diagram illustrating the plasmid isolation of the pMalc2 recombinant plasmid from TB 1 *E. coli* cells expression host system, followed by restriction digestion using Eco RI, SalI and HindIII as restriction enzymes.

3.2.3   Protein expression assay

For the expression of MBP and the recombinant MBP-hERαLBD fusion protein, cells were grown at 37°C in 100ml of LB medium (containing 100µg/ml ampicillin) to an absorbance of approximately 0.5 at 600nm with shaking at 300rpm. Prior to induction, the temperature was adjusted to 25°C and the culture medium was allowed to reach the desired temperature. Expression was induced by the addition of IPTG to a final concentration of 0.3 mM. Cells were subsequently incubated for an additional 12 h. The cells were harvested from the culture medium by centrifugation at 4000 *g* for 20 min and pellets containing the desired expressed proteins were frozen at –20°C overnight until required for further analysis.

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3.2.4 Cell disruption

Frozen pellets were resuspended in lysis buffer (10 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PSMF), 10 ml of lysis buffer per gram cells. Chilled beakers containing the resuspended pellets were placed in a slurry of ice, NaCl and ethanol. Cells were disrupted by sonication at 40 watts with 50% duty cycles for 10 min with 1 min interval using an ultrasonic cell disruptor sonicator (Branson Sonic Power Company). The resulting cell lysate was centrifuged at 9000 *g* for 20 min at 4°C, with the supernatant containing the soluble extracts.

3.2.5 Protein concentration determination (Pierce-method)

Protein determinations were carried out according to the bicincoconic acid method using a Pierce protein™ determination kit according to the manufacturer's instructions.

3.2.6 Protein analysis by SDS PAGE

Typically a 12 % acrylamide gel was prepared for SDS PAGE and cast in a Bio-Rad Mini-protein® Cell for gel electrophoresis. Prior to sample loading, all samples were treated with treatment buffer (0.125 M Tris-HCl pH 6.8, 4 % SDS, 20 % glycerol) and the resulting samples incubated at 90°C for 5 min. After the application of samples to the gel (i.e. 20 μg of protein/well), electrophoresis was carried out at 200 V. The resulting gel was stained for 1h with Coomassie blue R-250 (0.125 % Coomassie blue R-250, 50 % methanol, 10 % acetic acid). Destaining was carried out first with destaining solution I (50 % methanol, 10 % acetic acid) and then destaining solution II (7 % acetic acid, 5 % methanol).

3.2.7 Purification of the soluble extracts

The MBP and the recombinant MBP-hERαLBD expressed in *E. coli* were purified using a one-step affinity purification system. Unless stated otherwise all steps were carried out on ice. Briefly, amylose resin, with a binding capacity of 6.0 mg/ml bed volume, was used to pack a chromatography column (2.5 × 10 cm) to a height of 3 cm, corresponding to approximately 15 ml of the resin. The amylose resin column was equilibrated with 5 volumes of column buffer (20 mM Tris-HCL, 200 mM NaCl, 1 mM EDTA and 1 mM DTT) and its flow rate adjusted to 1 ml/min using an Econo system controller (Bio-Rad). Prior to sample loading, the sample was diluted 1:4 with the column buffer. The diluted sample was loaded onto the column, and non-

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specifically and weakly bound proteins were removed by washing the column with $12 \times$ column volume of the column buffer. The bound proteins were eluted with the elution buffer (20 mM Tris-HCL, 200 mM NaCl, 1 mM EDTA, 1 mM DTT and 10 mM maltose), while collecting fractions at 10 min intervals for up to 2 h. The purified samples were subjected to dialysis against a buffer (20 mM Tris-HCl pH 7.4, 50 mM NaCl and 1 mM EDTA) of low salt concentration for 24 h at 4°C. Protein concentration and the SDS PAGE analysis was performed as described in Sections 3.2.5 and 3.2.6. Figure 3.3 illustrates the steps used in the purification procedure of the fusion protein.
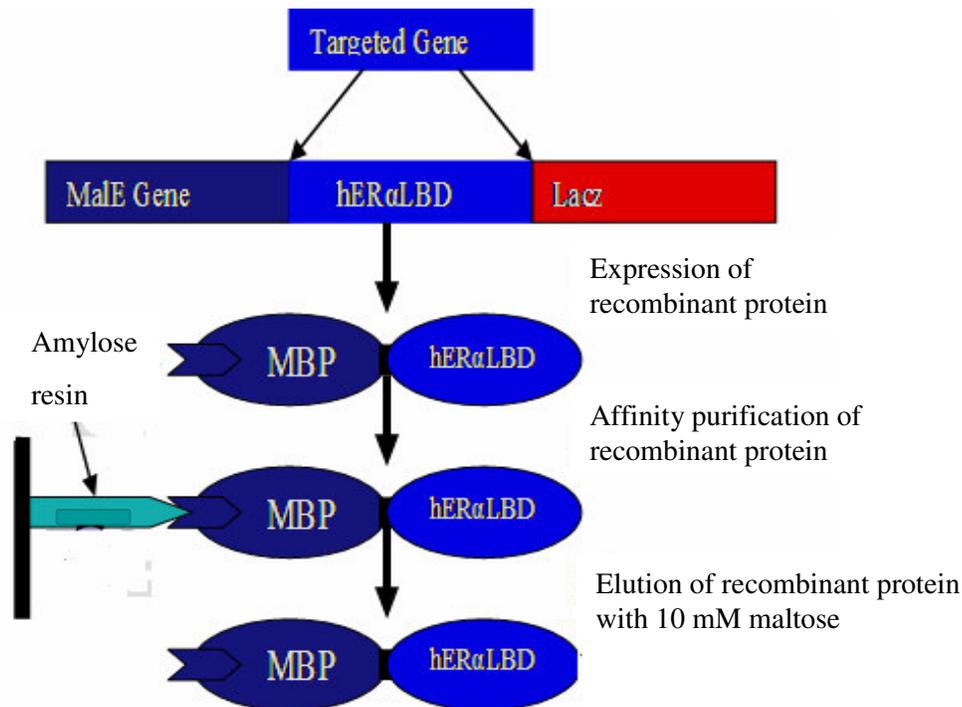
Figure 3.3: Diagrammatic illustration of the purification procedure of the MBP-hERαLBD using the affinity chromatography technique.

3.3 RESULTS AND DISCUSSION

3.3.1 Digestion of the recombinant plasmid

The pMalc2 recombinant plasmid was digested using EcoRI and SalI because these were the two restriction enzymes used in creating the sites for ligating the hERαLBD gene into the MCS of the vector. Both enzymes digested the plasmid at unique positions since the inserted gene offered no EcoRI and Sal I restriction sites. Analysing the above-mentioned restriction fragments on a 1%

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agarose gel indicated a single band at position 750 bp, corresponding to the size of the inserted gene, and another band at 6578 bp ascribed to the pMalc2 backbone (Figures 3.2 and 3.4). The Hind III confirmed that the plasmid was inserted in the correct orientation. Hind III digested the plasmid at two positions: on the pMalc2 backbone and within the inserted gene. A band corresponding to approximately 700 bp on a 1% agarose gel, indicated that the gene was inserted in the correct orientation (Figures 3.2 and 3.4).
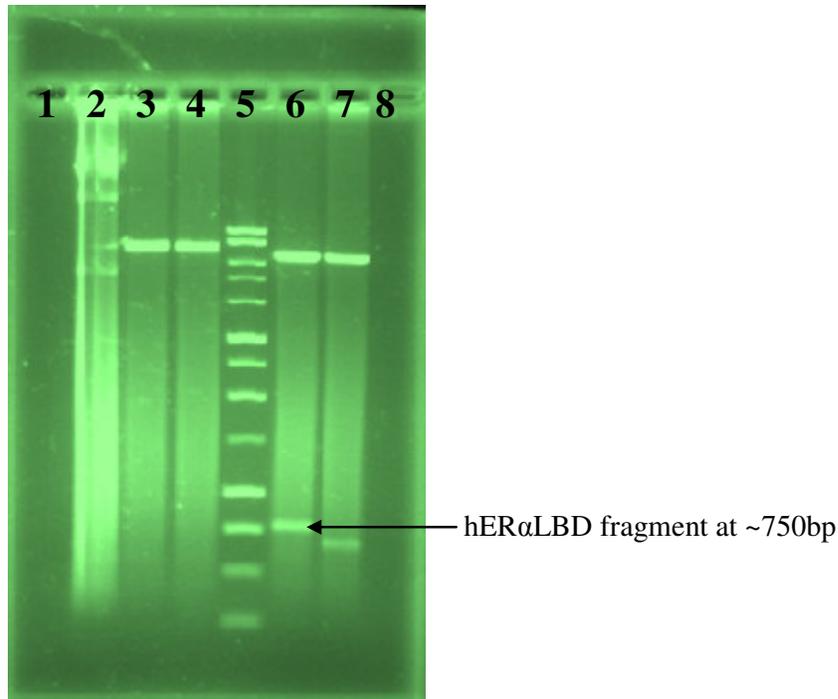
Figure 3.4: Restriction digestion of pMalc2-hERαLBD with EcoR1, SalI, and HindIII. Lane 2: pMalc2-hERαLBD uncut, lane 3: pMalc2-hERαLBD cut with EcoRI, lane 4: pMalc2-hERαLBD cut with SalI, lane 5: 1kb marker, lane 6: pMalc2-hERαLBD cut with SalI and Eco RI, and lane 7: pMalc2-hERαLBD cut with Hind III. Lanes 1 and 8 were empty.

3.3.2 Digestion of the pMalc2 plasmid

The pMalc2 vector harbours the malE gene which encodes for the MBP. In order to verify the presence of the malE gene the plasmid was digested with two restriction enzymes Bgl II and Hind III, which cut the plasmid at two positions, 1962 bp and 2803 bp, respectively. Analysis of the digest on a 1% agarose gel indicated two broad bands at positions 5806 bp and 840 bp, illustrating a positive confirmation of the presence of the malE gene in the pMalc2 vector (Figure 3.5).
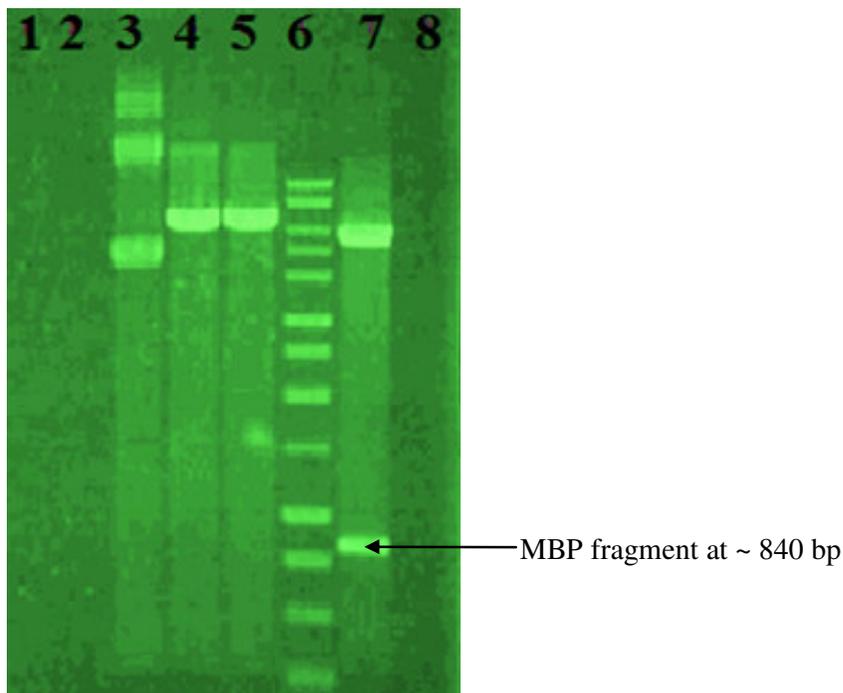
Figure 3.5: Restriction digestion of pMalc2 vector with Bgl II and Hind III. Lane 3: pMalc2 vector uncut, lane 4: pMalc2 vector cut with Bgl II, lane 5: vector cut with Hind III, lane 6: 1 kb marker and lane 7: plasmid vector cut with Bgl II and Hind III. Lanes 1,2 and 8 were empty.

3.3.3 Protein expression and purification

The concentrations of MBP and MBP-hERαLBD, *expressed in E. coli*, were 6.44 and 5.73 mg/ml respectively.

With positive results obtained from the plasmid isolation, *E. coli* TB1 cells harbouring pMalc2 vectors containing MBP-hERαLBD and MBP were cultured for the expression of the two proteins. Results obtained from SDS-PAGE analyses of the two respective cell lysates are given in Figures 3.6 and 3.7. A band corresponding to an apparent molecular mass of 66 KDa (Figure 3.6) was in good agreement with the molecular mass of MBP-hERαLBD reported in literature, while the band corresponding to an apparent molecular mass of 42 KDa (Figure 3.7) was in good agreement with the reported molecular mass of MBP.
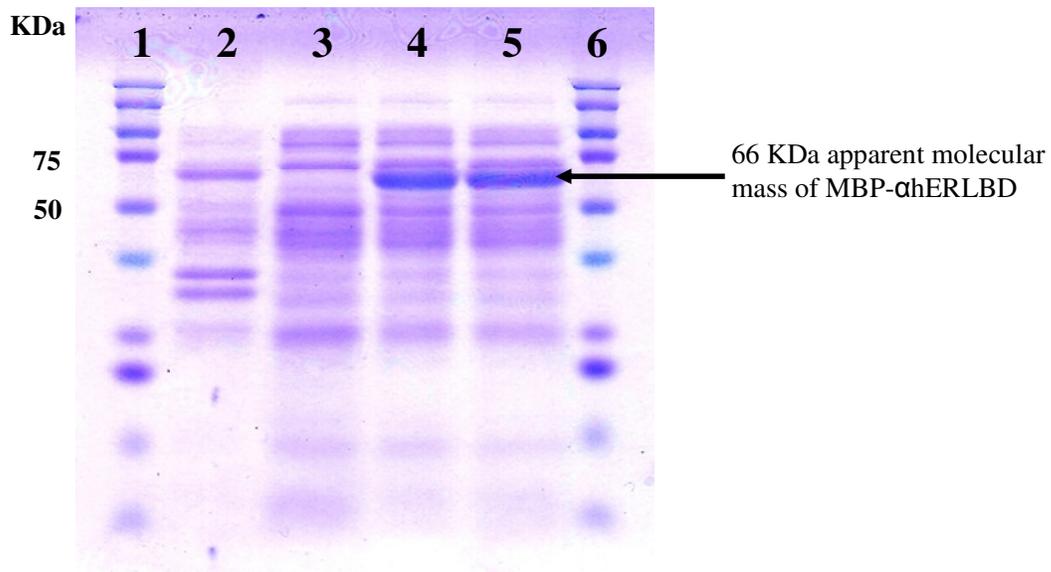
Figure 3.6: SDS-PAGE gel showing the protein molecular mass markers (lanes 1 and 6), the insoluble product of the protein lysate (lane 2), the uninduced samples (lane 3), and IPTG induced *E. coli* protein lysates (lanes 4 and 5).
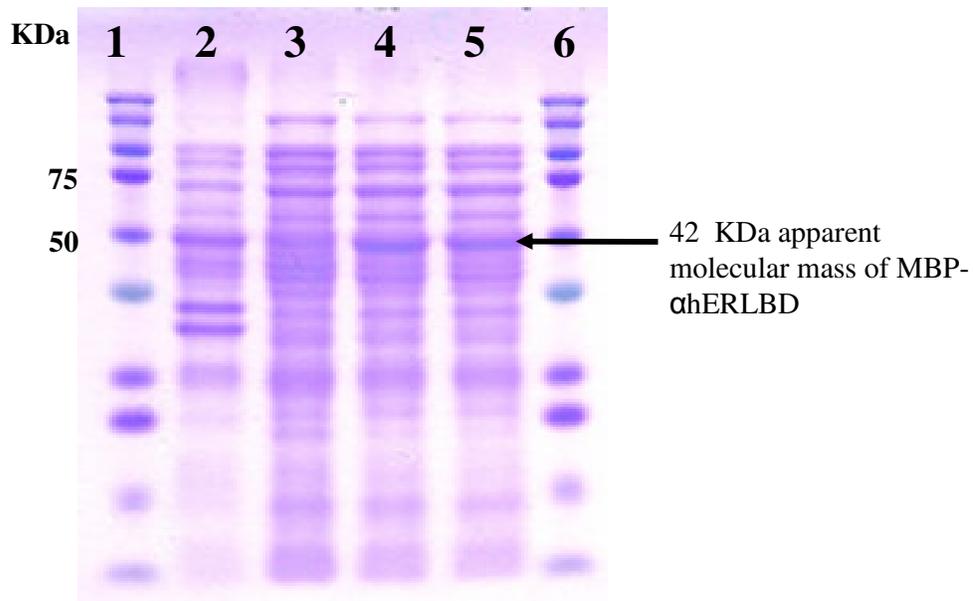
Figure 3.7: SDS-PAGE gel showing the protein molecular mass markers (lanes 1 and 6), the insoluble product of the protein lysate (lane 2), the uninduced samples (lane 3), and IPTG induced *E. coli* protein lysates (lanes 4 and 5).

The MBP-hERαLBD fusion proteins and the MBP proteins were purified on an amylose column by affinity chromatography. The coupled fusion protein was eluted from the affinity column by including 10 mM of maltose in the elution buffer. Figure 3.8 shows the loading and elution profiles of the proteins during the affinity purification procedure.

According to the SDS-PAGE analysis (Figure 3.9), MBP and the recombinant MBP-hERαLBD proteins migrated corresponding to molecular masses of 42 KDa and 66 KDa respectively. These molecular masses corresponded exactly to those seen on the expression gel for the MBP-hERαLBD protein (Figure 3.6) and the MBP protein (Figure 3.7).
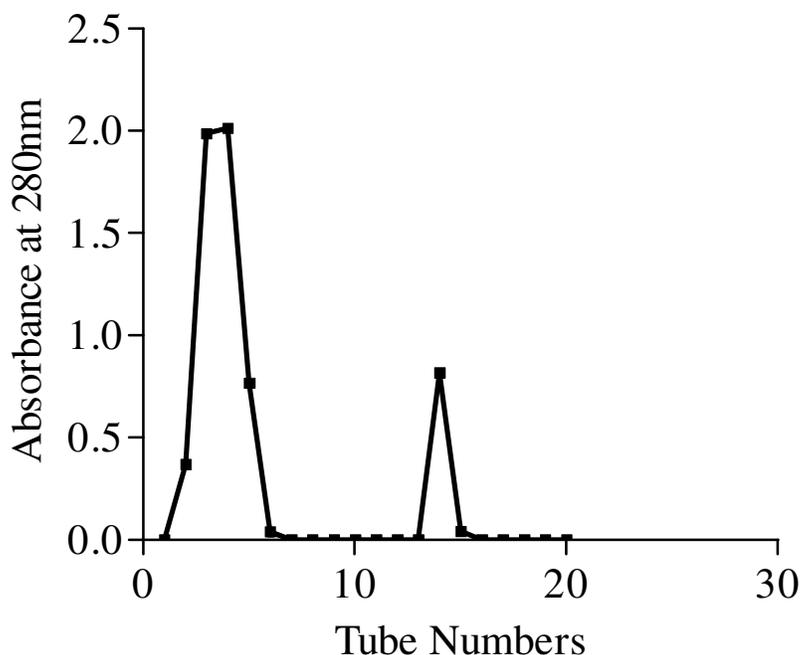
Figure 3.8: The elution profile of the MBP-hERαLBD after elution from the affinity column with a maltose buffer (20 mM Tris-HCL, 200 mM NaCl, 1 mM EDTA, 1 mM DDT, and 10 mM maltose).
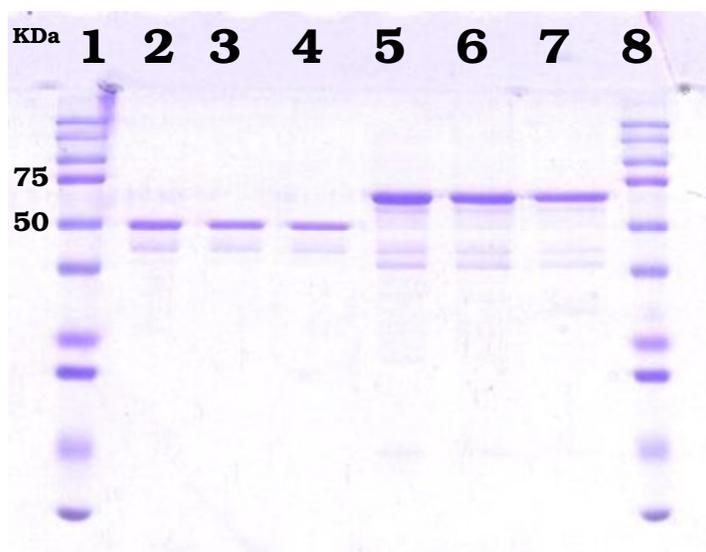
Figure 3.10: SDS-PAGE gel of the purified MBP/MBP-hERαLBD samples. Lanes 2, 3 and 4 are indicative of the purified MBP fusion protein loaded on the SDS-PAGE gel at different concentrations, while lanes 5, 6 and 7 are indicative of the purified MBP-hERαLBD fusion protein. The protein molecular mass marker in lanes 1 and 8 was used to determine the size of the proteins in the respective bands.

3.4   CONCLUSIONS

This study showed that MBP-hERαLBD ligated into the pmalc2 plasmid, and the MBP gene (malE product) from the pmalc2 plasmid can be expressed in high yield using the *E. coli* expression system. It also showed the application of a one-step affinity purification system with amylose as the solid phase for the effective purification of these proteins. These purified proteins will subsequently be used to raise antibodies, which will be used to investigate the immobilization of the expressed proteins on a modified cellulose acetate membrane, for the ultimate binding and removal of EDCs by the MBP-hERαLBD immobilized fusion protein.

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CHAPTER 4

IMMUNOLOGICAL STUDIES WITH MBP-hERαLBD

4.1 INTRODUCTION

Antibodies are specific molecules used in some biological applications for both quantitative and qualitative analyses [1]. The increased use of these biological molecules means that their production is of great importance. The process involved in the production of the antibodies is generally termed as immunisation. The antibodies produced from the immunisation process can selectively and specifically interact with other molecules generally termed as antigens [1-3]. The interactions between the antibodies and the antigens are generally required for analytical techniques such as enzyme-linked immunosorbent assays (ELISA), immunoblotting, immunoprecipitation, immunochemistry and also in studies involving the structural relationship between proteins. Generally, two types of antibodies are produced from the immunisation process: polyclonal and monoclonal antibodies. These two antibodies have proven to be very effective in a number of different applications, but there are distinct advantages and disadvantages associated with using each type of antibody for a particular study.

A problem often encountered in the production of polyclonal antibodies is that relatively large amounts of the protein are required for the immunisation process. In most cases these proteins are available in very small amounts that are insufficient for standard immunisation protocols [4]. The use of acid-treated or naked bacteria as immune carriers for proteins is an approach to specific antibody production aimed at overcoming the aforementioned problem [4]. In this approach, the natural antigenic determinants of *Salmonella minnesota* R595 are selectively stripped by mild hydrolysis generating an outer hydrophobic cell wall surface [4,5]. The hydrophobic cell wall will facilitate the interaction of hydrophobic antigen on the acid treated bacteria through hydrophobic interaction. The use of acid-treated bacteria as immune carriers has shown that they possess a strong immunostimulatory activity, making it possible to use less protein-antigen for the immunization procedure [4,5].

In this study, polyclonal antibodies will be raised against the recombinant protein (MBP-hERαLBD) by immunizing a rabbit with the MBP-hERαLBD naked bacteria complex. Upon achieving this and determining the specificity of these antibodies, they will be used to investigate the immobilization of the purified proteins (MBP and MBP-hERαLBD) on a functionalized cellulose acetate membrane.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

Monoclonal rat anti hERαLBD antibody was a gift from Z. Allie (Department of Biochemistry University of Stellenbosch). All other reagents used in the following experiment were of biological grade and obtained either from Merck or Sigma, unless otherwise stated.

4.2.2 Adsorption of MBP-hERαLBD fusion protein onto the naked bacteria

Purified MBP-hERαLBD proteins were adsorbed to the hydrophobic walls of the naked bacteria to evoke an immune response [1]. A fine suspension of 2 mg/ml of the bacteria was prepared from *Salmonella minnesota* R595, by gentle homogenization of the freeze-dried bacteria in sterilized Milli-Q® water. The purified MBP-hERαLBD was dissolved in sterilized Milli-Q® water to a final concentration of 1 mg/ml. The MBP-hERαLBD solution was added to the bacteria suspension to give a 5:1 ratio of naked bacteria-MBP-hERαLBD complex. The mixture was then dried on a rotary evaporator. The naked bacteria-MBP-hERαLBD complex thus formed was resuspended in phosphate buffer saline (PBS) to a final concentration of 200 µg of naked bacteria- MBP-hERαLBD complex/ 0.5 ml PBS, pH 7.2. Prior to immunization, a 120 µg naked bacteria- MBP-hERαLBD complex/ 0.5 ml PBS, pH 7.2 suspension was prepared and 500 µl of the suspension was used per immunisation.

4.2.3 Antibody production against MBP-hERαLBD fusion protein

Immunization of a rabbit with the naked bacteria-MBP-hERαLBD complex was affected intravenously in the marginal ear of the rabbit. The rabbit was bled before immunisation was commenced and the sera collected (day zero sera) were used to correct antibody titre for non-specific binding. After the collection of the day zero sera, the rabbit was injected with 200 µg of the protein-bacteria complex on days 0, 3, 7, 14, 17 and 22. Two millilitres of blood was drawn from the central artery of the rabbit's left ear on day 28. This was followed by a further injection of the rabbit with 200 µg of the protein-bacteria complex on days 28, 31, and 37. The rabbit was test bled on day 43 and a large volume of blood was collected on day 45. All blood samples collected were later tested for antibody activity using an ELISA assay (Figure 4.2).

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4.2.4 Enzyme-linked immunosorbent assay

The quantitative measurement of anti-MBP-hERαLBD in serum over time was performed applying the ELISA kit (Nunc Immunoplate I) according to the manufacturers' instructions. The micro titre plate wells were coated with 100 µl of the protein antigen (purified MBP-hERαLBD) diluted to an optimal concentration in carbonate buffer (pH 9.6). The micro titre plate was then incubated at 4˚C overnight. The contents of the wells were removed and the wells blocked for one hour at 37˚C by the addition of 200 µl casein buffer (10 mM Tris-HCl, 0.15 M NaCl, 0.02% thiomersal and 0.5% casein) in each well. The contents of the wells were removed and rinsed thrice with PBS-Tween 20 buffer (1.4 mM KH_2PO_4, 8 mM Na_2HPO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.3 and 0.05% Tween-20).

A dilution series of the sera (ranging from 1 in 20 to 1 in 10 240) was prepared in casein buffer and 100 μl dispensed into each well. The plate was incubated at 37˚C for 1 h. The contents of the wells were removed and the wells washed three times with PBS-Tween 20 buffer. This was followed by the addition of 100 µl of the donkey anti-rabbit secondary antibody diluted 1:10 000 times in casein buffer, and the plate was incubated at 37˚C for 1 h. After the incubation period, the secondary antibody solution was removed and loosely adherent ones were washed off as before. Subsequently, 100 µl of the enzyme substrate, 2,2'-azino-di-(3-ethylbenzenethiazoline-6-sulphonate) (ABTS) and H_2O_2 in citrate buffer (pH 5) was added to each well, and the plate was incubated at room temperature for 30 min. The absorbance of the contents of each well was measured at 405 nm on a micro titre plate reader. The colour changes observed on the plate upon addition of the substrate solution were proportional to the amount of specific rabbit anti-MBP-hERαLBD dependent enzymes antibodies. Figure 4.1 illustrates the steps involved in antibody production and characterization.
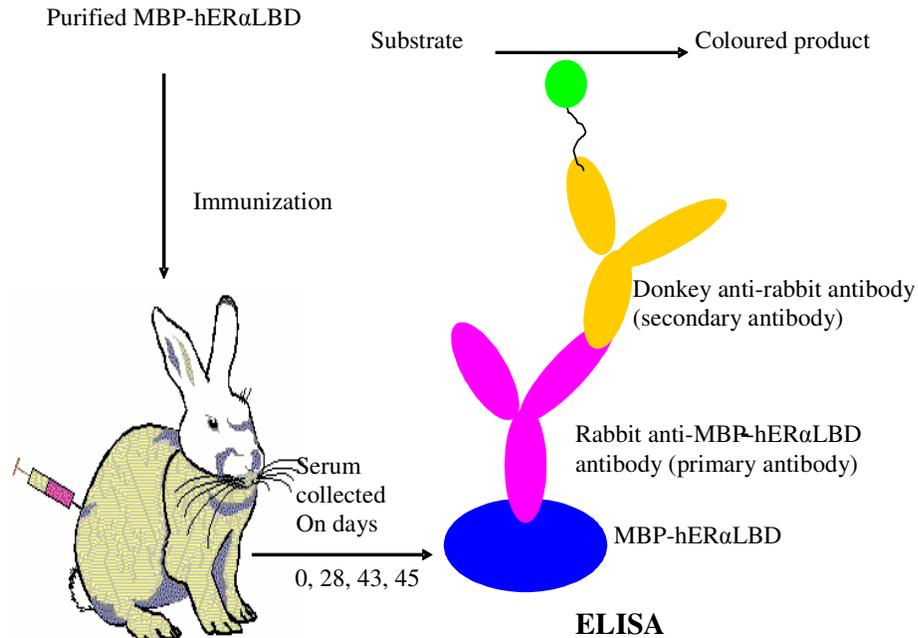
Figure 4.1: Diagram showing the production of antiMBP-hERαLBD and an ELISA assay performed using the antiMBP-hERLBD.

4.2.5   Immunoblott analysis

The purified protein fractions referred to in Chapter 3, i.e. maltose binding protein (MBP) and the MBP-hERαLBD fusion protein were subjected to a 12% SDS-PAGE analyses.   Upon completion of electrophoresis, one of the gels was stained with Coomassie brilliant Blue (R-250) to confirm the presence of the protein of interest, and the other (unstained) gel was electrophoretically transferred to a nitrocellulose membrane, using a wet transfer system (17 h at 120 mA).   The gel was carefully positioned on the nitrocellulose membrane after which the membrane and the gel were "sandwiched" between blotting papers and "Scotch guard".   The assembly was supported and secured in place by two Perspex plates, and the entire assembly was placed in a transfer chamber pre-filled with transfer buffer (25 mM Tris, 195 mM Glycine, 10% methanol, pH 8.3) in such a way that the gel was closest to the cathode and the nitrocellulose membrane closest to the anode.   The resulting nitrocellulose membrane was used for the identification of the MBP, MBP-hERαLBD antigens by immunoblotting procedure with the specific antibody raised against the MBP-hERαLBD antigen (see Section 4.2.3).

Prior to the identification of the proteins transferred to the nitrocellulose membrane with the specific ligand (rabbit anti-MBP-hERαLBD antibody), all the unoccupied binding sites on the membrane were blocked using casein buffer (10 mM Tris pH 7.6, NaCl 0.15 M, thiomersal 0.02% and casein 0.5%) to prevent any non-specific binding of protein. The blocked nitrocellulose membrane was incubated with the primary antibodies (rabbit anti-MBP-hERαLBD) at 37˚C for 1 h in casein buffer (1:10 000). The unbound antibodies were removed and the membrane was washed three times with TBST buffer (25 mM Tris, 150 mM NaCl, pH 7.6, and 0.05% Tween-20). The membrane was subsequently incubated with secondary antibodies (goat anti-rabbit-HRP) in casein buffer (1:10 000) for 1 h at 37˚C. After incubation, loosely adherent secondary antibodies were washed off the membrane as before. Antibodies binding MBP-hERαLBD were identified by enhanced chemiluminescence. Briefly, upon completion of probing, the membrane was sandwiched between two transparencies, exposed at different time frames, transferred into the developer and finally into the fixer for clear visualization of the blot (Figures 4.3[1] and 4.4).

Protein bands on the nitrocellulose membrane were also visualized, not using the chemiluminescent luminol substrate, but rather 4-chloro-1-naphtol and H_2O_2, in PBS. The reaction was terminated after 10 min by washing the nitrocellulose membrane with distilled water (Figure 4.3[2]). In this reaction, the development of the colour was due to the reaction between H_2O_2 and the peroxidase leading to the release of an oxygen radical, which in turn leads to the formation of a double bond in 4-chloro-1-naphtol.

4.3   RESULTS AND DISCUSSION

For further analytical experiments on the purified MBP-hERαLBD, polyclonal antibodies were raised against the MBP-hERαLBD. This was achieved by adsorbing the MBP-hERαLBD antigen onto acid-treated naked bacteria and subsequently injecting the complex into a rabbit for anti-MBP-hERαLBD antibody production. The immune response over time was studied using an ELISA assay (Figure 4.2). High antibody titres were observed at day 45 of the immunization (Table 4.1). The resulting anti-MBP-hERαLBD antibodies were used in immunoblotting experiments to determine if the polyclonal antibodies could recognise its antigen. Results obtained from these experiments showed that the generated polyclonal antibodies could specifically recognise the MBP-hERαLBD antigen (Figures 4.3[1] and 4.3[2]). The result also showed some degree of cross-reactivity. This cross-reactivity could be attributed to the impurities present with the antigen during the immunization procedure.

Although the polyclonal antibodies were raised against the recombinant protein (MBP-hERαLBD), it was interesting to note that the anti- MBP-hERαLBD antibodies could recognise both the recombinant form of the protein (lanes 2-5 and lanes 5-7 of Figures 4.3[1] and 4.3[2] respectively) as well as the single protein MBP (lanes 7-10 and lanes 2-4 of Figures 4.3[1] and 4.3[2] respectively). Figure 4.2 and Table 4.1 summarize the immune response over time of the rabbit injected with the fusion protein.
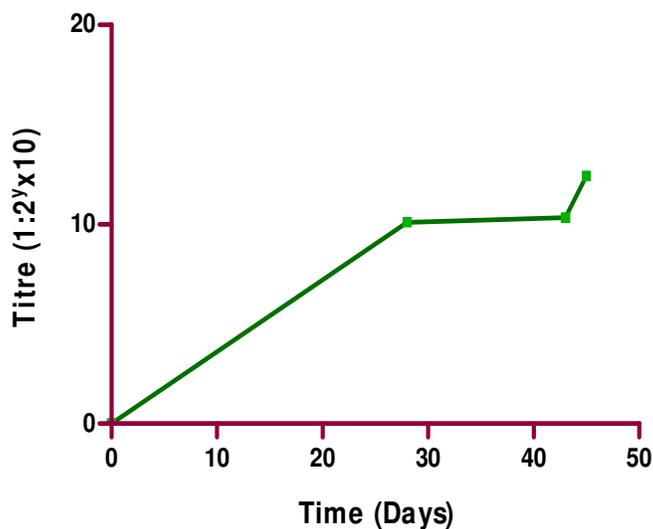
Figure 4.2: Curve illustrating the immune response of a rabbit immunized with MBP-hERαLBD fusion protein.

Table 4.1: Calculated titre values obtained from the immune response curve of a rabbit immunized with MBP-hERLBD fusion protein

Days	0	28	43	45
Titres (1: 2^yX10)	0	10.1	10.3	12.4

To confirm the presence of the MBP-hERαLBD antigen, monoclonal antibodies raised against the human estrogen receptor ligand binding domain (hERαLBD) were used as control during the immunoblotting experiment. Results obtained showed that the monoclonal antibody had recognisable functional groups (epitope) for the MBP-hERαLBD antigen (Figure 4.4[1]) but not for MBP (Figure 4.4[2]).

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1
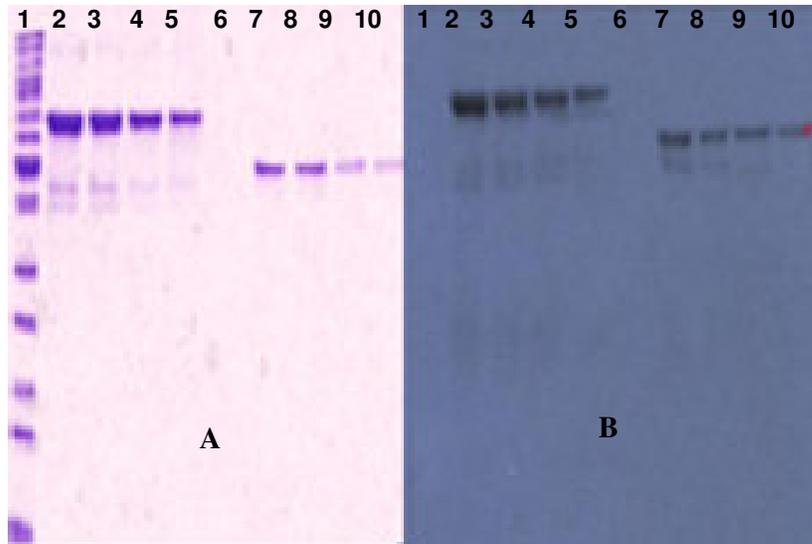
2
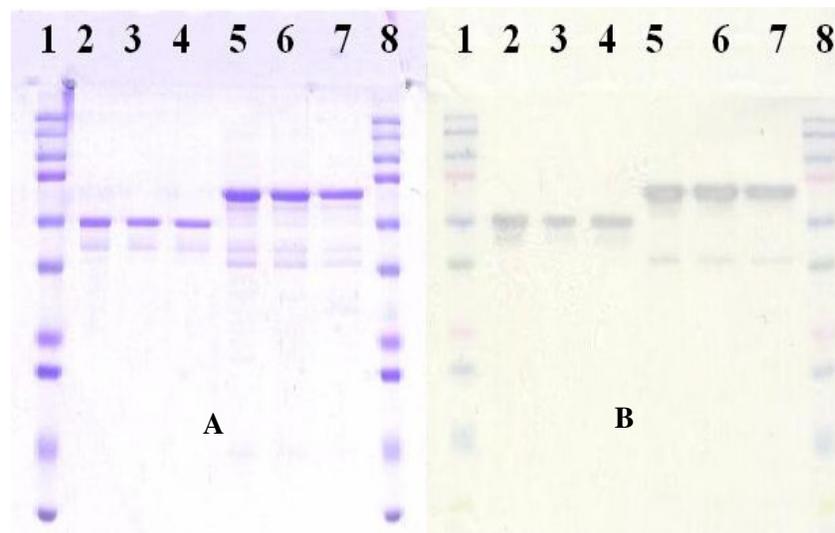
Figure 4.3: SDS-PAGE gels A with their corresponding immunoblots B. (1) represent immunoblot assays using enhanced chemiluminescence technique and (2) represent immunoblot assay using immunofixation technique. Raised polyclonal anti MBP-hERαLBD antibodies were used as the secondary antibody in both immunoblots. Lanes 2-5 of (1) represent bands of MBP-hERαLBD and 7-10 represents MBP, while lanes 2-4 and 5-7 of (2) are bands corresponding to MBP and MBP-hERαLBD respectively.
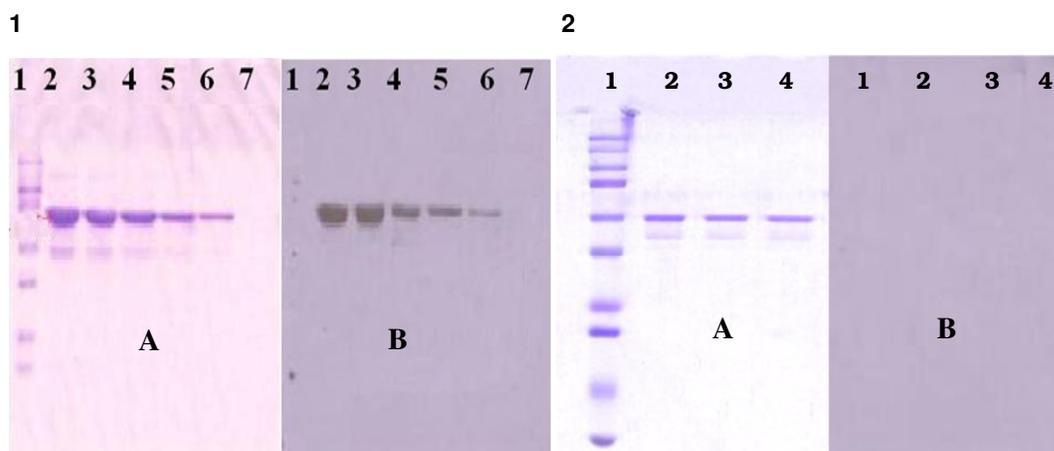
Figure 4.4: SDS-PAGE gels A with their corresponding immunoblots B. Moclonal rat anti hERαLBD antibodies were used as the secondary antibody in both immunoblots. (1) represent immunoblot assays using MBP-hERαLBD antigen (lanes 2-6) and (2) represent immunoblot assay using MBP antigen (lanes 2-4).

4.4   CONCLUSIONS

In this study, polyclonal anti MBP-hERαLBD antibody was successfully raised by immunizing a rabbit with MBP-hERαLBD acid-treated naked bacteria conjugate. Results obtained from the immune response curve depicted the highest titre value on day 45 of the immunization. The MBP protein was recognised by the polyclonal antibody, because of the MBP epitopes present on the antibody. To further confirm the viability of the raised polyclonal antibody, monoclonal rat anti hERαLBD antibody was used. Results from this experiment revealed that the rat anti-hERαLBD monoclonal antibody could recognize the hERαLBD of the purified MBP-hERαLBD fusion protein but not the MBP. However, based on the recognition of the rabbit anti MBP-hERαLBD antibody for both MBP and MBP-hERαLBD, and using MBP and MBP-hERLBD as the immobilizing ligands, the rabbit anti MBP-hERαLBD antibody was preferred to the monoclonal rat anti-LBD antibody through out this experimental work. The polyclonal rabbit anti MBP-hERαLBD antibody will be used as a potential 'marker' to investigate the immobilization of the purified proteins on a modified cellulose acetate membrane for the ultimate binding and removal of EDCs by the MBP-hERαLBD immobilized fusion protein. To support the aforementioned ideas, an analogous system has been described previously wherein LBD was utilized as a receptor to selectively capture analyte target specific for the LBD.[3]

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[3] http://www.patenstorm.us/patents/6500629/fulltext.html.

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CHAPTER 5
FABRICATION OF A CELLULOSE ACETATE MEMBRANE MODIFIED WITH AMYLOSE FOR MBP IMMOBILIZATION

5.1   INTRODUCTION

Cellulose acetate membranes have become increasingly popular in the biomedical and biotechnology fields. These membranes are used in a wide range of applications due to their good performance in permeability, mechanical stability and their versatile nature [1-13]. However, grafting of protein molecules on the surfaces of these membranes can induce decreasing water and solute permeability, and a series of biochemical reactions [14]. Other shortcomings of these membrane matrices include: lack of biospecificities of some of the affinity ligands, less resistance of ligands to harsh chemicals and temperatures, difficulty to clean up, and insufficient ligand binding capacity of the support. To reduce some of the aforementioned shortcomings, several different approaches have been used in the fabrication of the membrane matrices [15-17].

Each application dictates specific requirements on the membrane materials and structure. Selection of the correct material for membrane fabrication requires an in-depth understanding of the membrane material to be used as well as an understanding of both the biomolecule and immobilizing molecule (in case of affinity separations). Generally there are several techniques used in the fabrication of synthetic polymeric membranes. Some of the techniques used are:

- thermally induced phase separation [18];
- air-casting of a polymer solution [18,19];
- precipitation [18]; and
- immersion precipitation [18, 20, 21].

In this study, based on the interaction occurring between the membrane matrix and the immobilized ligand, a novel membrane matrix of CA modified with amylose will be fabricated using the immersion precipitation technique.

Several fundamental studies have been put in place to investigate the effects of membrane surface properties such as pore size, pore size distribution, electrokinetic characteristics, surface roughness, chemical properties and specific chemical structure [22-26]. Some of the techniques

used for membrane properties characterization include: atomic force microscopy (AFM) and scanning electron microscopy (SEM) (surface roughness, structure, topography) [27, 28], X-ray photoelectron spectroscopy (XPS) and Fourier transform infrared spectroscopy (FT-IR) (surface chemical functional groups) [29-31], nuclear magnetic resonance (NMR) spectroscopy (permeability), contact angle [32,33], Raman spectroscopy (structure) and electron spin resonance (solute mobility in membrane polymer matrix and pores) [22]. However, despite the various techniques used in characterization of the membrane surface properties, there is no single technique which can fully characterize membrane surface substructure. In this study, two analytical techniques (SEM and FT-IR) will be used for the characterization of the modified CA membrane.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

DMSO was obtained from BDH chemical (UK). The amylose resin was purchased from Fluka (South Africa), while the cellulose acetate was obtained from Prof EP Jacobs from the Polymer Department, University of Stellenbosch (South Africa).

5.2.2 Membrane fabrication

An amylose functionalized CA membrane was fabricated from a mixture containing 25% m/m CA, 2% m/v and 4% m/v amylose respectively, 90% dimethyl sulfoxide (DMSO) and 10% H_2O. The CA and the amylose were dissolved in DMSO and H_2O by stirring at 75ºC for 1 h. The CA and amylose solutions were pooled in known proportions and stirred for 30 min to form a casting mixture. The casting mixture was subsequently degassed overnight and then used to cast flat-sheet membranes by immersion precipitation using a suitable solvent (Milli-Q® water). The membranes were thoroughly washed with Milli-Q® water and stored in an aqueous 2%v/v sodium azide solution until required. The surface membrane topography, thickness and functional groups modification were determined using SEM and FT-IR respectively.

5.2.3 Membrane characterization

5.2.3.1 Scanning electron microscopy

The surface structure of the fabricated membranes was characterized using SEM. Membrane samples approximately 1 cm^2 were freeze-dried in liquid nitrogen. The resulting freeze-dried samples were gold plated, mounted onto a plate holder before loading into the LEO-1430PV SEM chamber for imaging. The images were generated as a result of the interaction between highly energetic electron beam from the instrument and the gold plated membrane surface. The characterization of the fabricated membranes using the SEM will yield information such as; the surface features of the membranes, their textures and direct relation between the features and material properties (hardness, reflectivity). The aforementioned information was used to evaluate the differences in topography between the different fabricated membranes.

5.2.3.2 Photo-acoustic Fourier transform infrared spectroscopy

The photo-acoustic Fourier transform infrared (FT-IR) system was used to characterize the surface composition of the hybrid membrane. The samples were placed in a sample cup holder and subsequently introduced into a MTEC 300 chamber, allowing as little air into the sample component as possible. The samples were later flushed with high-purity helium and the spectra obtained were recorded on a Perkin Elmer Paragon 1000 FT-IR apparatus. The membrane samples were analysed based on the interferometric detection of light intensity that is transmitted through the membrane and the Fourier transformation. The parameters used for the analyses are tabulated in Table 5.1. Information from the FT-IR was used to compare the differences between the functional groups of the different fabricated membranes.

Table 5.1: Parameters used for FT-IR analysis of the CA membrane

FT-IR parameters	Values
Mirror velocity (OPD)	0.1 cm/s
Resolution	8 cm^{-1}
Source aperture	maximum
Spectral range	400 - 4000 cm^{-1}
Number of scans	128
Sample reference	Carbon black
Detector gas atmosphere	Helium

5.3   RESULTS AND DISCUSSION

5.3.1   Scanning electron Microscopy

Quantitative information on membrane topography and morphology were investigated by SEM imaging. Figure 5.2 depict clearly the differences in surface morphology between the fabricated membranes. Figure 5.2a depict membrane formed from a casting dope of CA while Figures 5.2b and 5.2c depict membranes formed from a casting dope containing CA and amylose, with the amylose present at concentrations of either 2% m/v or 4% m/v.

The first observation shown by the SEM images is the differences in the pore size and pore size distribution. The sizes of the pores increased with an increase in amylose content. The average pore sizes were 1.58 µm and 2.61 µm for CA membrane with 2% m/v and 4% m/v amylose respectively. Some research groups suggest that pore formation in composite membranes is as result of nucleation and growth of the polymer-lean phase in the liquid-liquid delayed demixing of the polymer solution during inversion [16,17]. Based on the above, it is suggested that the increase in amylose content in polymer solution causes a delay in demixing hence leads to increased pore formation. Figure 5.2 depict membrane surface images obtained from the SEM analysis.
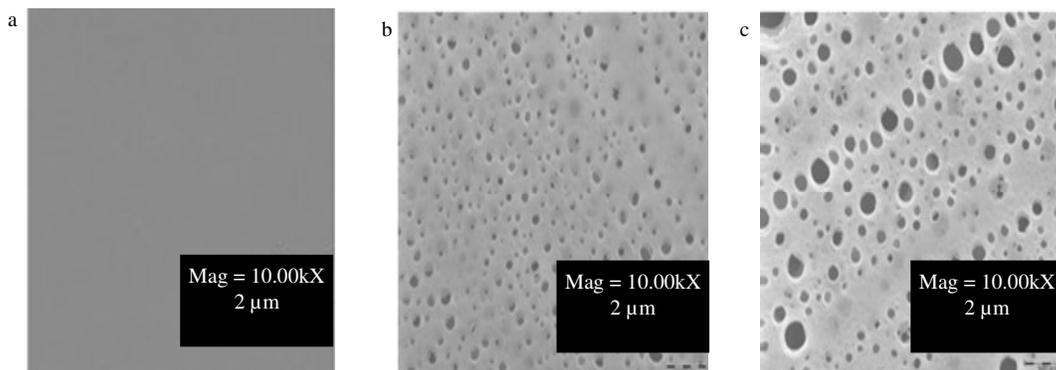
Figure 5.1: SEM images of (a) a CA membrane and (b and c) CA/amylose hybrid membranes with (a) 2% (m/v) and 4% (m/v) amylose respectively.

5.3.2   Photo-acoustic Fourier transform infrared spectroscopy

It is important to characterize the chemical functional groups on a membrane surface when membranes are to be used for a particular application [27]. The functional groups of the described membranes were characterized using photo-acoustic spectroscopy (PAS) FT-IR. This

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technique requires little or no sample preparation for high quality spectra and no alignment of the infrared incident beam was necessary during the experimental procedure. PAS FT-IR spectra of the membranes are included in the appendix. . Figures A1 to A8 in the appendix were used to contrast the differences between the CA and CA/amylose membrane surfaces, the CA and amylose powder and also to observe the most prominent changes in terms of functional groups when the percentage of amylose was increased from (10-15)% in the CA membrane.

All FT-IR spectra, apart from the intense peak observed at 1642.25 cm^{-1} (corresponding to C=O, C=C, H-O-H stretching) on the amylose powder spectrum, were similar to each other. The spectra can be categorized to three main regions with respect to the stretching vibrations occurring within these areas. Firstly, peaks observed at the region 2 850-3 650 cm^{-1} corresponded to the –CH$_3$, –CH$_2$, –CH and –O-H groups, chemical groups characterizing the cellulose and amylose backbone. Secondly, a broad peak was observed at region 2 000-2 500 cm^{-1} corresponding to the C-C-O ester stretching. This potion indicate the presence of the acetate group present in the CA since this same peak was absent in the amylose powder spectrum. Lastly, a characteristic peak was observed at the region 1700-1800 cm^{-1} corresponded to the C=O stretching. No other deductions could be made from the spectra given the similarity between cellulose and amylose (Figure 5.3).
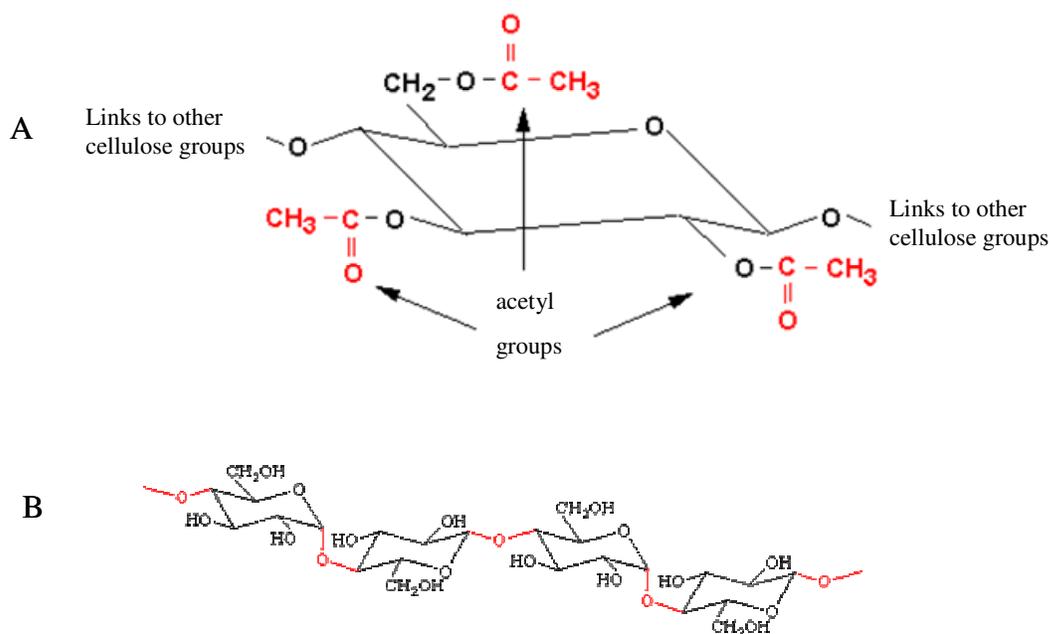

Figure 5.2: Structural representation of cellulose acetate (A) and amylose (B), with the acetate group occurring as the only difference between the two structures.

As shown from another FT-IR analysis using a CA membrane, CA/1% amylose and CA/2% amylose membranes, result revealed a notable single peak in the 1 700-1 800 cm^{-1} region for the various membranes used. As the concentration of the amylose in the membrane was increased, the intensity of the peak at 1 755 to 1 741.90 cm^{-1} decreased. This could be due to the fact that the amylose shields the C=O stretching groups of the cellulose acetate. This difference in peak intensity gives an indication of the presence of amylose in the membrane.
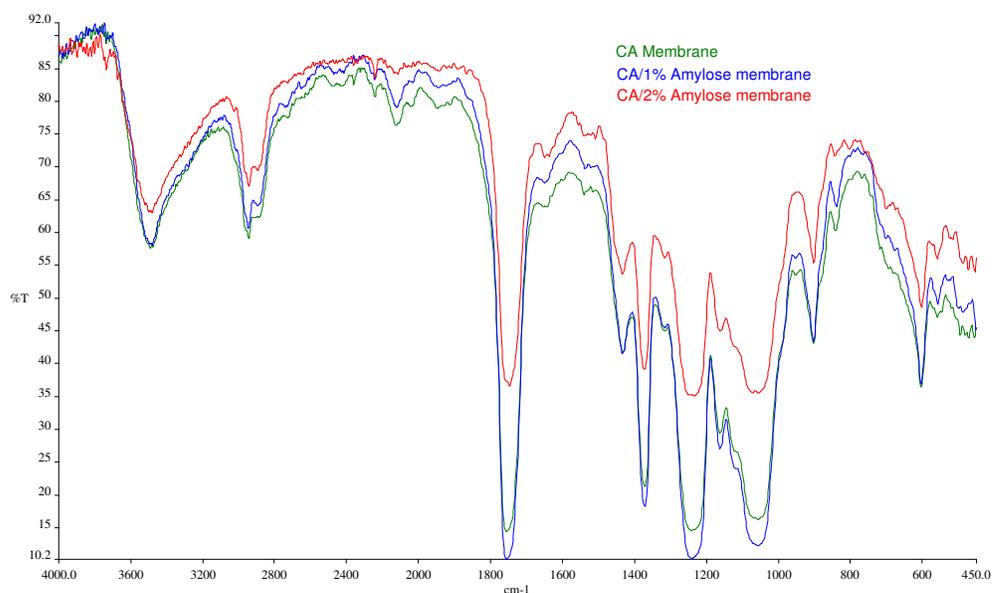
Figure 5.3: FT-IR spectra of a CA membrane, CA/1% amylose membrane and CA/2% amylose membrane.

5.4   CONCLUSIONS

In the present study, both CA and amylose powers were successfully dissolved in a 90% DMSO and 10% H$_2$O solution. The resulting mixture was successfully used as a casting dope for the fabrication of a CA/amylose functionalized membrane using the immersion precipitation technique. The membrane topography and morphology was studied with the aid of the SEM technique, while the surface chemistry of the membrane was monitored with FT-IR. Following the information obtained from the membrane morphology and surface chemistry the membrane could be used for affinity immobilization of the specific bio-ligands MBP and MBP-hERαLBD fusion proteins. Membranes containing higher percentages of amylose were ductile, fragile and therefore could not be used as solid supports for further experimental analyses. Therefore in the present study, only a 2% amylose membrane was used for the immobilization study. In future, however, higher percentages of amylose could be incorporated into the CA membrane but this will be followed by using some plasticizer to render the membrane more resilient to physical dmage and deformation [34-37].

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CHAPTER 6

ASSESSING THE VIABILITY OF THE IMMOBILIZED MBP/MBP-hERαLBD FUSION PROTEIN USING RADIOACTIVE ESTRADIOL

6.1 INTRODUCTION

Large sets of genomic and proteomic samples constantly generated has lead to a need for tools that will facilitate rapid screening and analysis. Selective screening and/or purification of these biological molecules from their natural environment poses serious problems in the field of biotechnology. This has lead to the development of techniques which are cost effective, robust and specific for the purification of these samples. The immobilization of proteins and other biological molecules on solid matrices is constantly and increasingly being used as a suitable method of this purpose. During the immobilization procedures, the proteins and/or other biological molecules are immobilized on a carrier surface either covalently by attachments to activated groups or passively through ionic interaction [1]. The immobilized ligand is usually specific for a particular application.

Immobilization technology has been widely used in many different areas of biotechnology e.g. the immobilization of molecules in ELISA assays, diagnostics, biosensors, affinity chromatography and immobilization of molecules onto nanoparticles for drug/gene delivery [2-6]. Immobilization of macromolecules on a micro porous membrane as a solid surface is constantly being used. The reason for this being that membrane surfaces offers low diffusional resistance, high compressibility and chemical stability to harsh conditions of adsorption, elution and regeneration [2]. However, the retention and structural integrity of the immobilized macromolecule still present some challenges [1]. Common to most immobilization systems is the application of one or more thermochemical/chemical steps. Reagents used in these steps may have the potential of degrading the structure and/or function of the immobilized protein [7-11]. There is a rising need to improve on or to development new immobilization techniques where the functional/ structural properties of the immobilized components are retained. Thus, designing a system to be use for immobilization, there are certain factors which must be taken into account. Some of which include [12-14]:

- the type of solid support to be used;
- the orientation of the bound protein;
- the flexibility of the bound protein;

- the concentration of the immobilized ligand;
- the solvent compatibility (in case of hybrid support); and
- the interaction between the solid interface and the immobilized ligand etc.

In this study MBP-hERαLBD fusion protein will be immobilized to an amylose functionalized membrane with the amylose present in the membrane surface offering the binding sites for the MBP-hERαLBD immobilization since MBP has an affinity for amylose. Also included herein, will be the application of the membrane system for the selective recovery of estradiol from solution.

6.2   MATERIALS AND METHODS

6.2.1   Reagents

[2,4,6,7-^{3}H (N)] 17-β estradiol (250 uCi, 100.0 Ci/mmol) was obtained from PerkinElmer (Boston MA02118 USA), β-Estradiol from Fluka chemie GmbH, progesterone was obtained from Sigma-Aldrich Chemie GmbH, NaCl, NaOH, Tris-HCl and EDTA were obtained from Merck.

6.2.2   Amylose/E$_2$ binding assay

Prior to the CA/amylose flat-sheet membrane binding assay, the activity and binding of the recombinant protein, MBP-hERαLBD, onto the resin was tested using radioactive 17-β estradiol (E$_2$). Briefly, amylose resin was equilibrated with buffer A (20 mM Tris-HCl pH 7.4, 0.1 M NaCl and 0.1 mM EDTA) to yield a working slurry. For the determination of the E$_2$-binding, 300 µl of the amylose slurry was incubated overnight at 40°C with extract containing MBP-hERαLBD, diluted to approximately 0.1 mg/ml using buffer A. [^{3}H]–Estradiol working solution was prepared by diluting 10 µl of [2, 4, 6, 7-3H (N)] 17-β-estradiol (250 uCi, 100.0 Ci/mmol) obtained from the manufacturer with 10 µl of non-radioactive 1×10^{-3} M 17-β-estradiol in 96% ethanol. The organic solvent content was reduced to approximately 1 µl using a stream of dry nitrogen gas and subsequently diluted with buffer A to yield [^{3}H]-E$_2$ working solution of 2.44×10^{-5} M. Prior to E$_2$ binding, the amylose/MBP-hERαLBD slurry was washed three times for 4 min each using chilled 50 mM Tris-HCl, pH 7.4. The washed slurry was later incubated for 3 h at room temperature with 300 µl of buffer A containing 2.44×10^{-7} M of the [3H]-E$_2$ working solution. Upon completion, the washing step was repeated and 4 ml of scintillation cocktail was

added to the slurry. The mixtures were transferred into scintillation vials and later counted using a liquid scintillation counter.
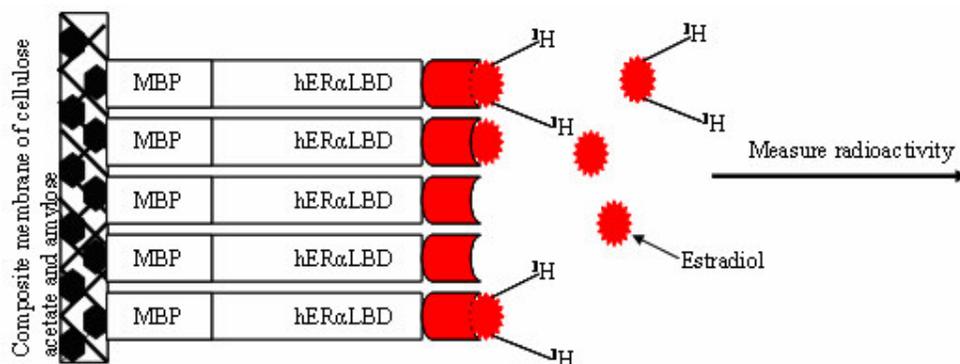

Figure 6.1: Diagrammatic representation of an amylose resin assay to determine if hERαLBD can capture estrogen from solution while immobilized on a solid matrix. Radiolebelled estradiol was used to detect binding to the hERαLBD of the fusion protein.

6.2.3 Membrane coating assay

Prior to immobilization of the adsorbates (i.e. MBP-hERαLBD and MBP) onto the 2% amylose functionalized CA membranes, the membranes were shaped such that the external surfaces were 1×1 cm^2 in diameter. The membranes were washed overnight in sterile, deionised water, followed by three further washes. This was followed by the overnight incubation of the membranes at 4°C in a buffer (10 mM Tris pH 7.6, 0.15 M NaCl and 0.02% thiomersal) containing the adsorbates (0.1 mg/ml final concentration). The overnight solution was discarded and the membrane incubated with rabbit anti-MBP-hERαLBD antibody for 2 h at 4°C. The membranes were washed with PBST buffer followed by incubation with donkey anti-rabbit antibody for 2 h at 4°C. After the final wash step, the membranes were subjected to a chemilluminescent detection method using the ECL Western Blotting Detection solutions (Amersham Biosciences) and resulting signals were analyzed by Hyper film ECL.

6.2.4 Ligand binding capacity

To investigate on the binding capacity of the bioligand to the amylose functionalized membrane, washed membrane (1×1cm^2 in size) were incubated with buffer A containing the adsorbates to a final concentration of 0.1 mg/ml. The resulting membranes were incubated at 4°C for 3 h with

samples collected very 30 min for Pierce protein determination (Figure 6.5).

6.2.5 Ligand recovery and characterization

To further characterize the binding of the affinity ligand to the composite amylose functionalized membrane, washed membranes (1×1 cm^2 in diameter) were incubated overnight at 4°C in buffer A (20 mM Tris-HCl pH 7.4, 0.1 M NaCl and 0.1 mM EDTA) containing the affinity ligands to a final concentration of 0.1 mg/ml. Subsequent to the incubation procedure, the solution was discarded and the membranes washed four times in chilled 50 mM Tris-HCl, pH 7.4. The resulting membranes were transferred into new vials and incubated for 1 h in 1 ml of buffer A to which a final concentration of 10 mM maltose was added. The resulting supernatant were analysed with SDS-PAGE and Western blot.

6.2.6 Membrane/E$_2$ binding assay

The MBP-hERαLBD immobilized membrane/ E$_2$ binding assay was performed following the same general procedure used in the amylose E$_2$ binding assay described above. E$_2$ binding was measured by incubating the MBP-hERαLBD coated membrane with 1 ml of buffer A containing 2.44×10^{-7} M of the [^{3}H]-estradiol working solution (prepared as mentioned above) for 3 h at room temperature. After incubation, the washing step was repeated and the washed membranes transferred in to scintillation vials containing 4 ml of the scintillation cocktail. Counting was done using a liquid scintillation counter.

6.3 RESULTS AND DISCUSSION

To assess the binding of the purified MBP-hERαLBD to the amylose resin and to test if the purified hERαLBD could capture estrogen while immobilized on a matrix, radio-labelled E$_2$ was used due to its affinity for LBD. MBP and BSA were included as controls in the binding assay. From the results obtained, the percentage of radioactivity remaining after incubation of the E$_2$ with the functionalized membranes gives an indication that the purified affinity ligand, MBP-hERαLBD, could capture estrogen while immobilized onto the amylose resin. However, MBP and BSA on the other hand showed little or no binding confirming that they both lack binding sites for either the resin or E$_2$ or both. The results shows that an active form of LBD will successfully capture estrogens or estrogenic compounds while immobilizes onto a solid support.
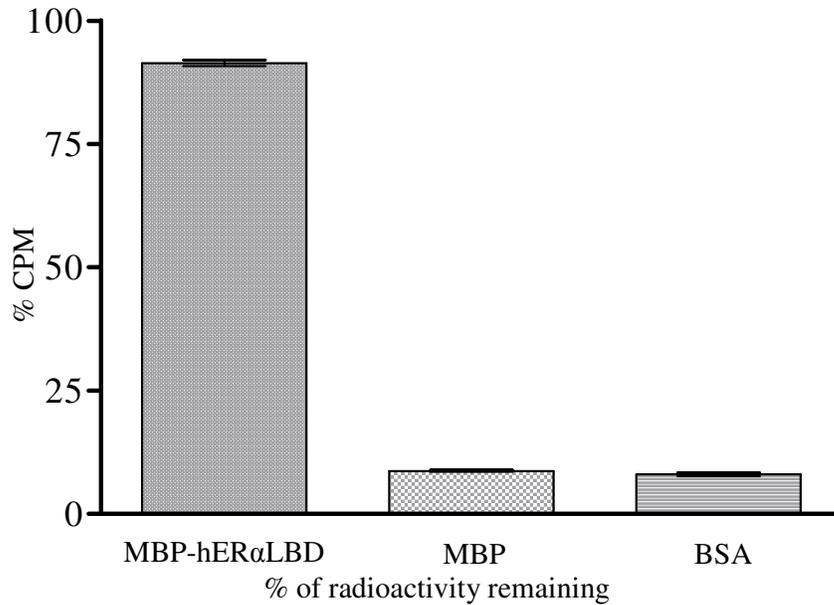
Figure 6.2: Graph illustrating a radioactive assay performed using amylose resin. This was to determine if MBP-hERαLBD fusion protein can capture estrogen from solution. The results are given as percentage of radioactivity remaining, represented as count per minute (CPM) on the resin.
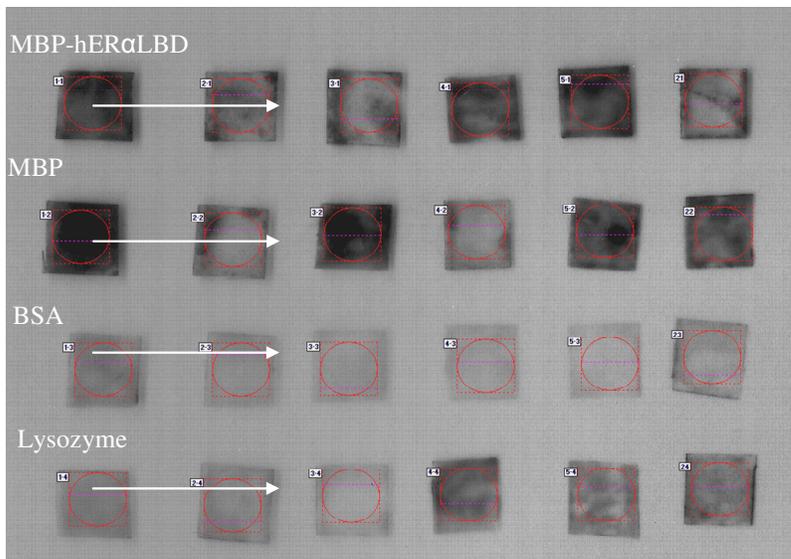

Figure 6.3: Diagrammatic representation of an immunoblot assay of MBP/MBP-hERαLBD, MBP. BSA and Lysozyme detected using the chemiluminescent approach with anti MBP-hERαLBD and donkey anti-rabbit as primary and secondary antibodies respectively.

To characterize the membranes for protein immobilization, 0.1 mg/ml of the proteins (MBP and MBP-hERαLBD fusion protein) were incubated with 1 cm^2 of the amylose functionalized membrane. The protein immobilized membranes were subsequently used for Western blot analysis (Figure 6.3). Though it was evident that the degree of binding of the proteins to the functionalized membrane was different for each protein used, it was difficult to draw a precise conclusion based on the results obtained from the Western blot analysis. In this regard, digital quantification of the black spots present on each 1 cm^2 membrane was carried out and the obtained results plotted on a bar chat (Figure 6.4). From the result obtained, MBP and MBP-hERαLBD showed greater binding compared to BSA and Lysozyme used.
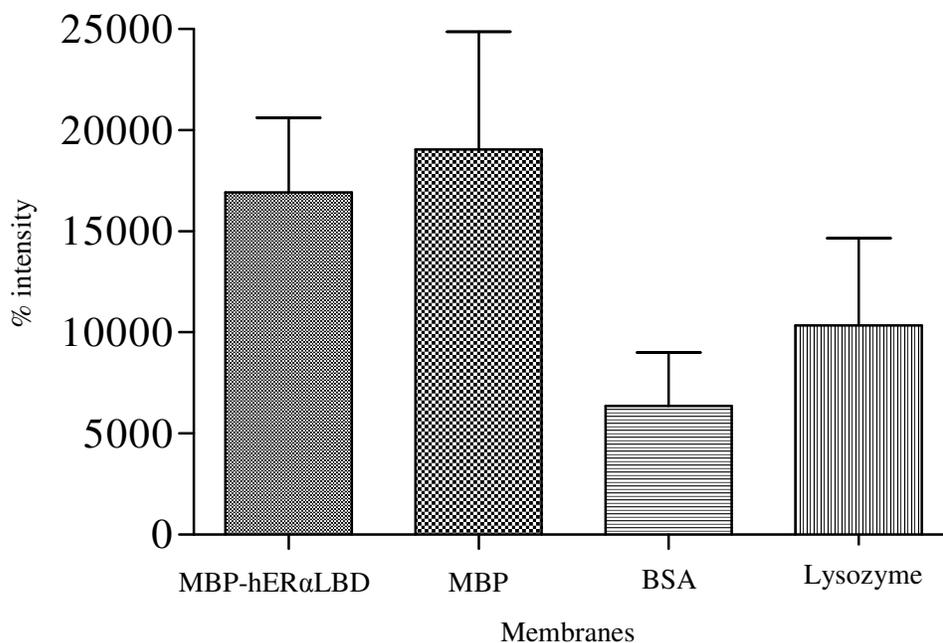

Figure 6.4: Diagram representing the digital quantification of the black spots present on each 1 cm^2 membrane of the above western blot analysis (Figure 6.3).
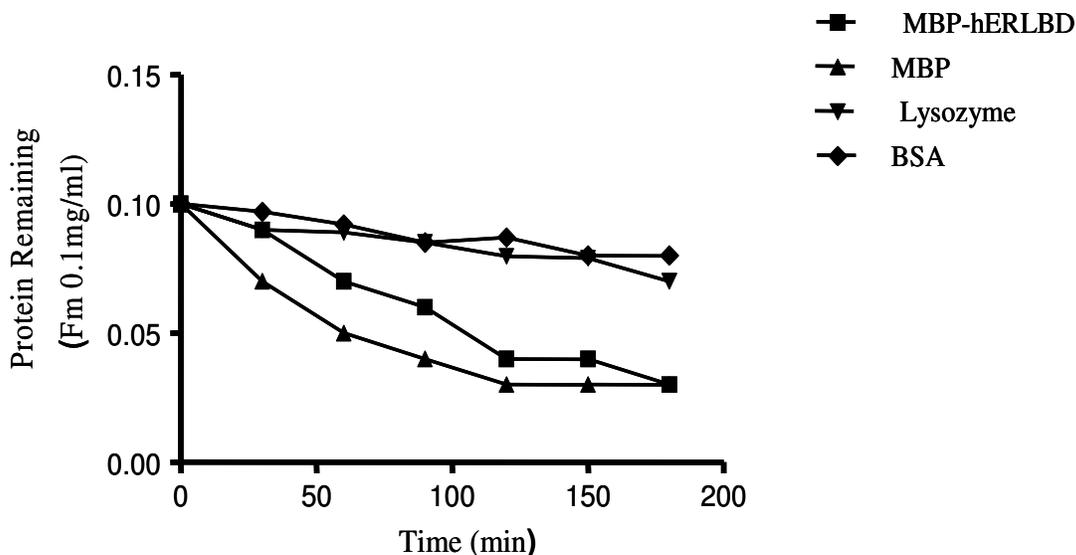
Figure 6.5: Graphical representation of the degree of binding of MBP, MBP-hERαLBD, BSA and lysozyme onto the amylose functionalized membrane over time. The proteins concentration prior to the addition of the membranes was 0.1mg/ml.

MBP and MBP-hERαLBD binding assay was performed to assess the affinity of the bio-affinity ligands to amylose present in the membrane (Figure 6.5). This result indicates that both affinity ligands had an affinity for amylose though as depicted by the result MBP had a greater affinity than MBP-hERαLBD. This could be as a result of the fused hERαLBD creating steric hindrance on the MBP fusion protein thus affecting the binding activity of this molecule. BSA and Lysozyme were included in the assay as negative controls. Comparing the binding of the proteins to the modified membrane, it can be agreed that the affinity functionalized membrane was not specific for MBP and MBP-hERαLBD binding only, since the BSA and Lysozyme bound to the membrane even though it did so partially. The latter result may be attributed to the fact that the membrane offers binding sites for BSA and Lysozyme or some degree of hydrophobicity causing the BSA and Lysozyme to adhere to the membrane.

To further verify the immobilization of the protein onto the functionalized membrane a desorption assay was performed. In this experiment the membranes containing the immobilized proteins were incubated with a solution containing maltose (final concentration of 10 mM). After the incubation of the immobilized membranes with maltose, the solution obtained was characterized using Western blot analysis. As controls, water and another sugar moiety (10 mM Sucrose) were used. This was done to verify if the MBP and MBP-hERαLBD could be displaced from the membrane using maltose through competitive binding (maltose has a greater

affinity for amylose than MBP and MBP-hERαLBD fusion protein). From the Western blot results obtained, it was evident that both sugar moieties used could displace the proteins from the membrane while a sugar free solution (Milli-Q® water) were unable to do this.
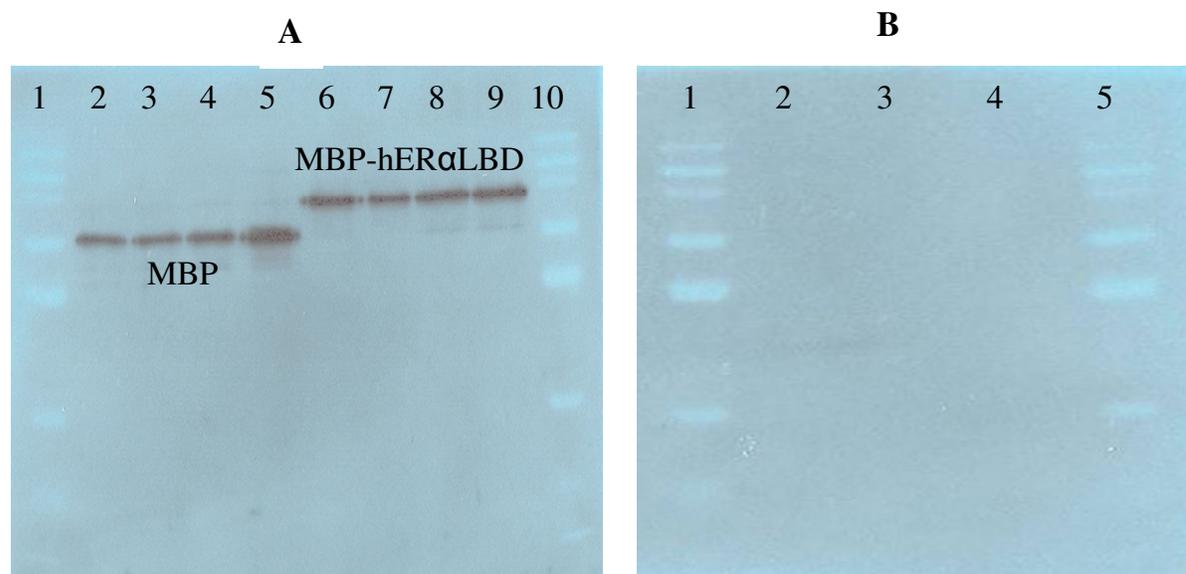

Figure 6.6: Western blot performed with the supernatant collected after desorption of MBP/ MBP-hERαLBD fusion proteins from the amylose functionalized membrane. Desorption was done using maltose and sucrose to a final concentration of 10mM. Lanes 2, 3 and 8, 9 of A represent the maltose fractions while lanes 4, 5 and 6, 7 the sucrose fractions. Lanes 2, 3 and 4 of B represents an assay performed with the supernatant after the immobilized membrane was washed with Milli-Q® water. Lanes 1 and 10 of A and 1 and 5 of B represent the molecular mass markers.

Maltose and sucrose are both disaccharides consisting of two simple sugars: maltose (glucose 1α-4 glucose) and sucrose (glucose 1α-4 fructose) with the only difference between these two sugars being the ring structure of one of the monomer unit. In terms of chemical composition, these two sugars are very much alike and the two disaccharides can therefore share a common binding site on MBP.

The above characterization confirms that the MBP fusion protein and the recombinant protein (MBP-hERαLBD) were successfully immobilized on the amylose functionalized membrane. After the verification, the immobilized membrane was used as a solid affinity matrix for the selective recovery of labelled E_2 from solution.

Considering the interaction existing between the hERαLBD and the endogenous estrogen hormone, it was expected that an analog of estrogen, such as E_2, will bind to an active hERαLBD. This binding was, however, not observed in this study as depicted by the above E_2 binding results (Tables 6.1-6.4).

Table 6.1 bolow represent an assay performed using different proteins coated membranes incubated with labelled E_2 for 1 h. From the results it was observed that there was no significant difference in the percentage binding of E_2 existing between the different protein-coated membranes. The percentage binding show that all the membranes bound E_2 to the same extent irrespective of whether the ligand was immobilized onto the membranes. Another approach was subsequently introduced considering that the above results observed could have been as a result of the hydrophobicity of the functionalized membrane (Table 6.2). Herein, prior to the immobilization of the different proteins to the flat sheet membrane, the membranes were hydrophilized with 0.1 M NaOH pH 10 for 24 h. This was to render the membrane less hydrophobic. The E_2 binding assay was repeated with the hydrophilized protein-coated membranes. The results obtained from the assay were similar to the results observed when the membranes were unhydrophilized.

To further verify the affinity binding of E_2 to the hERαLBD present on the membrane, the above experiments were repeated with the incubation time of the membrane in E_2 solution increased to 3 h (Tables 6.3 and 6.4). There was no significant change observed in the percentage binding of E_2 to the hydrophilized membranes. The only conclusion that could be drawn from the above results was that the E_2 binding to the membrane increased as the incubation time was increased. Also, the binding of E_2 to the membrane was not specific.

Several attempts to bind E_2 to the amylose functionalized protein coated membrane specifically were unsuccessful. Several factors may have attributed to this failure. Firstly, it may be that the activity of the recombinant protein could be affected by potential interaction between MBP and the amylose present in the flat-sheet membrane [15]. The binding of the recombinant protein to the membrane may have caused a conformational change within the hERαLBD thus affecting the affinity of the hERαLBD for the E_2. Another factor that could have contributed to the above shortcoming may be the orientation of the affinity ligand on the membrane. The recombinant protein may have been incorporated into the membrane in such a way that the affinity site of the hERαLBD is not properly exposed for E_2 binding.

Other contributing factors, such as the uneven distribution of the amylose within the membrane, the lower surface to bed volume ratio, steric hindrance affecting the affinity ligand, uneven membrane thickness as well as the flow distribution can also explain the inability of the membrane immobilized MBP- hERαLBD to bind E_2 [16-18]. The results of E_2 binding obtained from this bio-assay are represented in Tables 6.1-6.4 below as percentage binding of E_2 onto the membrane.

Table 6.1: Percentage binding of E_2 on an unhydrophilized protein coated membranes. The E_2 binding assay was carried out at room temperature for 1 h

Membrane Type	Membrane CPM	Total Supernatant CPM	Total CPM Used	% Binding of E_2 on membrane
Uncoated membrane	1510	2451	3960	38.1
MBP-hERαLBD coated membrane	1559	2276	3835	40.7
MBP coated membrane	1899	1879	3779	50.3
Lysozyme coated membrane	1653	2119	3772	43.8
BSA coated membrane	1657	2194	3851	43.0

Table 6.2: Percentage binding of E_2 on a 24 h hydrophilized protein coated membranes. Hydropilization was done using 0.1 M NaOH pH 10. The E_2 binding assay was carried out at room temperature for 1 h

Membrane Type	Membrane CPM	Total Supernatant CPM	Total CPM Used	% Binding of E_2 on membrane
Uncoated membrane	1549	2179	3728	41.6
MBP-hERαLBD coated membrane	1551	2154	3705	41.9
MBP coated membrane	2010	1942	3951	50.9
Lysozyme coated membrane	1657	2173	3831	43.3
BSA coated membrane	1488	2269	3757	39.6

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Table 6.3: Percentage binding of E_2 on an unhydrophilized protein coated membrane. The E2 binding assay was carried out at room temperature for 3 h

Membrane Type	Membrane CPM	Total Supernatant CPM	Total CPM Used	% Binding of E_2 on membrane
Uncoated membrane	2570	1372	3942	65.2
MBP-hERαLBD coated membrane	2384	1453	3836	62.1
MBP coated membrane	2516	1477	3994	63.0
Lysozyme coated membrane	935	624	1559	60.0
BSA coated membrane	864	548	1412	61.2

Table 6.4: Percentage binding of E_2 on a 24 h hydrophilized protein coated membranes. Hydropilization was done using 0.1 M NaOH pH 10. The E_2 binding assay was carried out at room temperature for 3 h

Membrane Type	Membrane CPM	Total Supernatant CPM	Total CPM Used	% Binding of E_2 on membrane
Uncoated membrane	2377	1125	3502	67.9
MBP-hERαLBD coated membrane	2269	1623	3892	58.3
MBP coated membrane	2667	1356	4024	66.3
Lysozyme coated membrane	2733	1350	4083	66.9
BSA coated membrane	2418	1493	3911	61.8

From Section 6.3, Figure 6.5, approximately 0.063mg of the recombinant protein was successfully bound to a 1 cm^2 membrane. This implies that a final concentration of approximately 1nmol of the MBP-hERαLBD was present on the 1 cm^2 membrane given the molecular mass of the recombinant protein (66KDa) as depicted in Figure 3.6. Theoretically, one molecule of LBD can only bind one molecule of E_2, therefore with 1nmol LBD present on the 1 cm^2 membrane, an equal amount of E_2 (1nmol) can bind to the binding sites of the recombinant protein. Relating the aforementioned result in terms of CPM, a total of 30000CPM and cold E_2, to a final concentration of 10nmol was used in the binding assay. Therefore the coated membrane was expected to have approximately 3000CPM. However, this was not the case as both the uncoated and coated membranes bound equal quantities of radio-activity (2000 CPM), indicating none-specific binding of the E_2 onto the membrane. It was expected that if the recombinant protein was probably immobilized onto the 1 cm^2 membrane, exposing its binding

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sites to freely bind E_2, there should have been a significant difference in the binding of the E_2 by the LBD irrespective of the nature of the membrane.

6.4   CONCLUSIONS

The results obtained from the work described in this chapter have clearly shown that the recombinant protein and the MBP fusion proteins have been immobilized onto the amylose functionalized membrane**.** All attempts, however, to utilize the protein coated membrane for the selective binding of E_2 were unsuccessful. This result can be attributed to the fact that, the LBD was incorporated into the 1 cm^2 membrane in such a manner that the binding sites of the affinity ligand wan not well exposed to bind E_2.

6.5 REFERENCES

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CHAPTER 7
SUMMARY AND RECOMMENDATIOMS

7.1   SUMMARY

The constant release of endocrine disrupting compounds (EDCs) into water has led to the development of several techniques for their detection and recovery. Among them, affinity techniques have proven to be most effective. In this study, a novel affinity CA modified membrane was fabricated based on the interaction occurring between the membrane matrix and its immobilized ligands. The novel membrane was applied as a detection system for the recovery of EDCs from environmental water through affinity coupling.

A basic membrane fabrication technique (immersion precipitation) was successfully implemented in the fabrication of the affinity CA/amylose functionalized membrane. The membrane surface roughness and surface chemical functional groups were characterized successfully, using SEM and FT-IR, respectively. The results from the SEM and FT-IR were used to gain information regarding amylose availability in the membrane. Changes were depicted from the membrane appearance, structure and composition as the concentration of amylose in the CA membrane was gradually being inceased.

 Once the membrane was constructed, human estrogen alpha ligand binding domain (hERαLBD) affinity ligand was immobilized onto the CA/amylose functionalized affinity membrane to create appropriate binding sites for EDCs. With the CA/amylose functionalized membrane lacking in hERαLBD binding sites, hERαLBD was successfully expressed as a fusion protein to maltose binding protein (MBP) using *E. coli* TB1 cells as the host system. Purification of the recombinant protein (MBP-hERαLBD) was achieved by using an affinity chromatography column with amylose as the affinity matrix. The purification results were confirmed by immunoblot analysis using commercial monoclonal rat anti-hERαLBD antibodies. They were also confirmed by using rabbit anti-MBP-hERαLBD polyclonal antibodies, generated by immunizing a rabbit with MBP-hERαLBD acid-treated naked bacteria conjugates. The resulting polyclonal anti-MBP-hERαLBD antibodies, as shown by the western blot analyses, did recognize both MBP-hERαLBD and MBP.

Applying the basic principles of column affinity chromatography, the activity of the ligand binding domain (LBD) of the recombinant protein, as well as the interaction between the

recombinant protein and the amylose, was investigated. The binding assay proved to be very successful since the recombinant protein could successfully bind E_2 while immobilized onto an amylose support present in a chromatographic column. According to the above assay, if amylose is successfully incorporated into the membrane matrix in its correct orientation and concentration, there is a high probability that it will successfully bind the recombinant ligand.

Basic techniques such as the western blot assay, E_2 binding assay and a desorbtion assay, were successfully applied in the characterization of the immobilized proteins on the membrane. The results of this study showed that when incubated with the affinity membrane, the MBP-hERαLBD will successfully bind the amylose in the membrane. It was also observed (using the desorption assay) that a solution containing maltose and sucrose will successfully displace the proteins adsorbed on the membrane, giving an indication of ligand immobilization.

Once the composite affinity membrane was modified successfully, the membrane was applied as an affinity matrix for E_2 recovery. Several attempts to get the affinity ligand (LBD) to bind E_2 specifically in solution were unsuccessful. The results obtained may be attributable to a number of factors, including:

- uneven distribution of the amylose within the membrane;
- hydrophobic nature of the membrane matrix;
- the lower surface to bed volume ratio;
- steric hindrance affecting the affinity ligand;
- uneven membrane thickness; and
- flow distribution within membrane.

Resolving the above parameters is a good starting point in solving the abovementioned problems encountered by the affinity membrane.

7.2   RECOMMENDATIONS

MBP-hERLBD expression and membrane technology are separate fields of study and therefore present a plethora of opportunities for future research work. Following the shortcomings of the membrane system used in this study, the following areas should receive further attention.

7.2.1   The membrane matrix

The following parameters should be taken into consideration when developing the membrane matrix:

- more compatible solution(s) should be used in dissolving the polymers to prevent demixing of the polymers;
- changing the orientation of amylose in the membrane to offer better binding to the affinity ligand. This can be achieve by using different casting techniques for the membrane production; and
- with the membrane matrix binding more of the E_2 than it should have, using other polymeric materials as the matrix support of lower hydrophobicity or reducing the hydrophobic nature of the already formed CA membrane.

7.2.2   Membrane affinity ligand

Another point of interest that should be taken into consideration is the adsorption of the affinity ligand to the membrane;

- The affinity ligand should be modified in such a way to minimize steric hindrance as much as possible.

APPENDIX

The molecular organization and molecular dynamics of polymeric materials are analyzed using the combination of techniques. The FT-IR has been used successfully to characterize the structural changes occurring during the fabrication of composite polymeric membranes. Figures A1 to A8 depicts FT-IR diagrams of amylose powder, CA powder, a CA membrane surface, a CA membrane, a CA/10% amylose membrane, a CA/15% amylose membrane, a CA/10% amylose membrane surface and a CA/15% amylose membrane surface respectively.
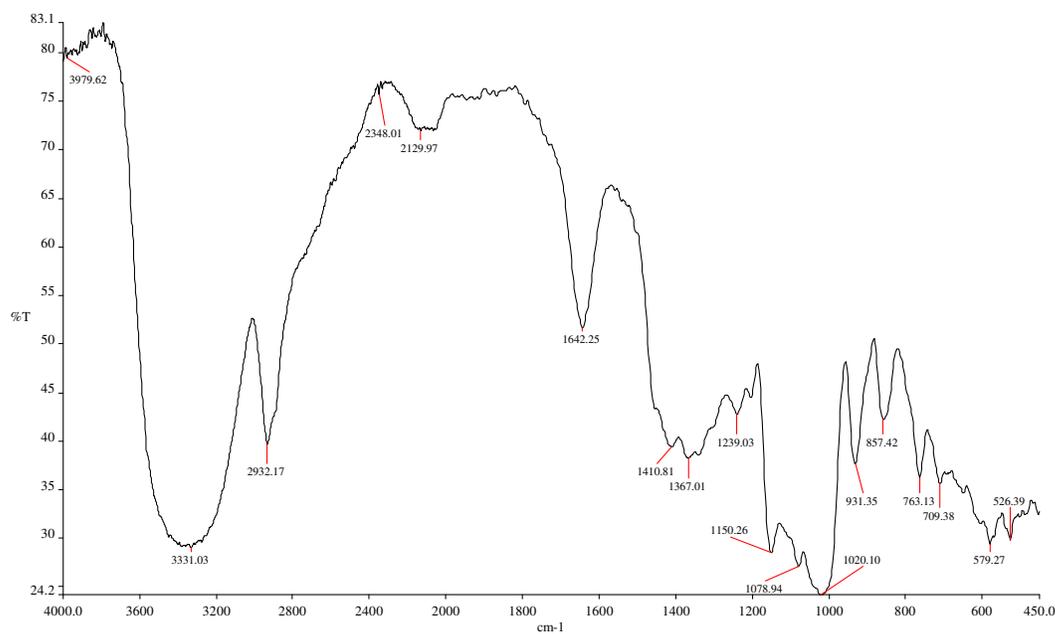
Figure A1: FT-IR spectrum of amylose powder.

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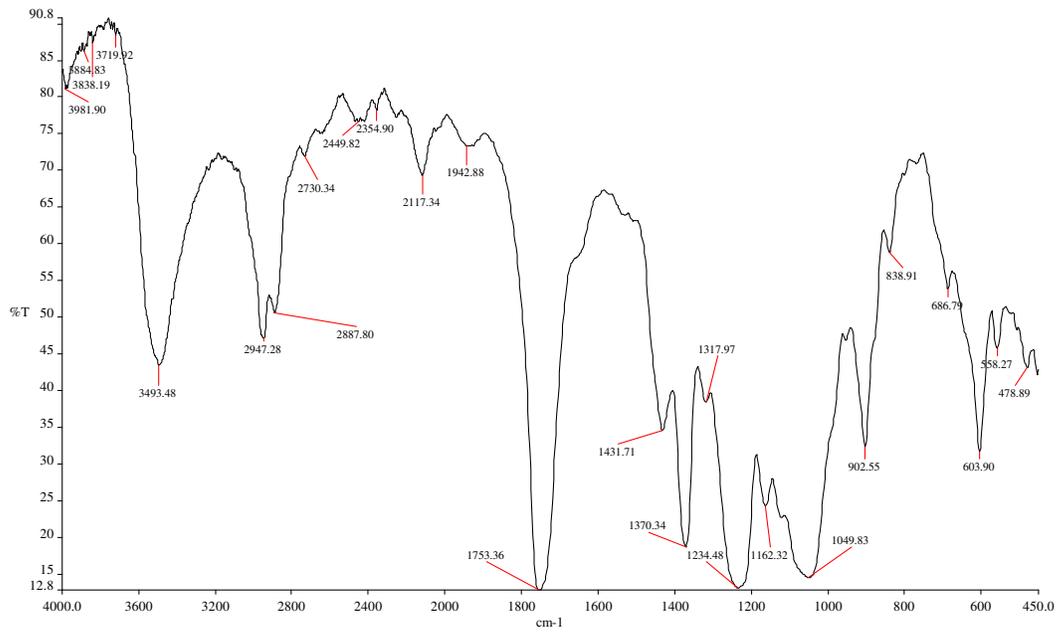
Figure A2: FT-IR spectrum of CA powder.
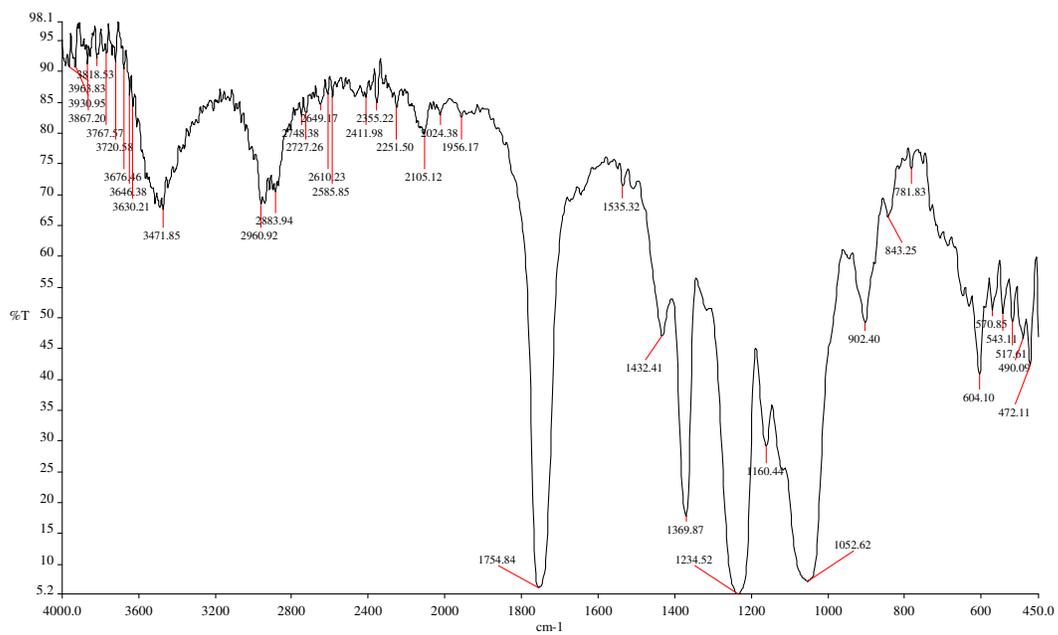
Figure A3: FT-IR spectrum of CA membrane surface.
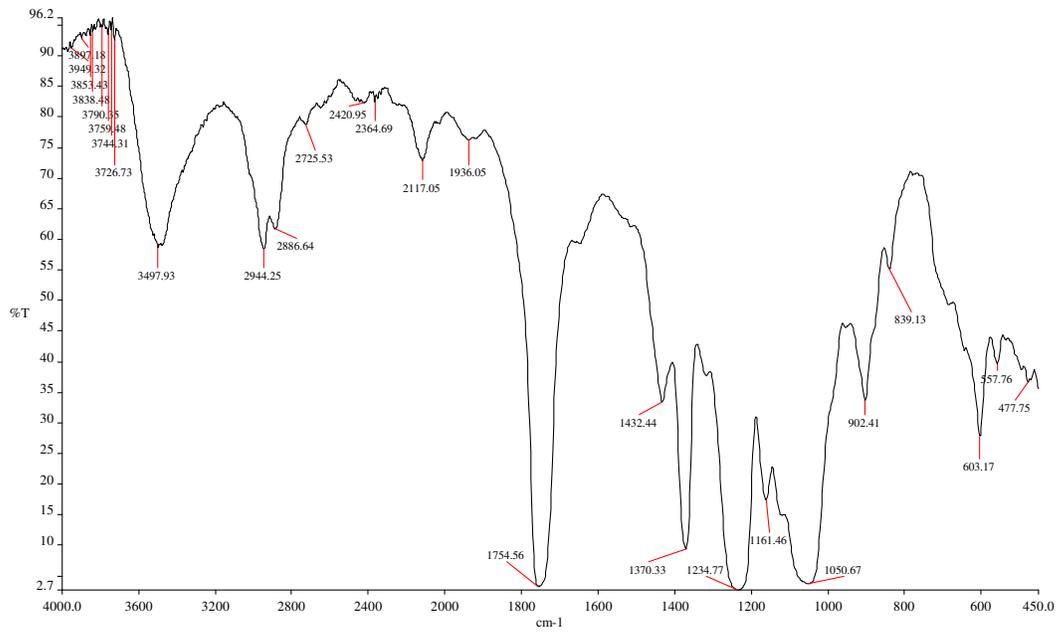
Figure A4: FT-IR spectrum of CA membrane.
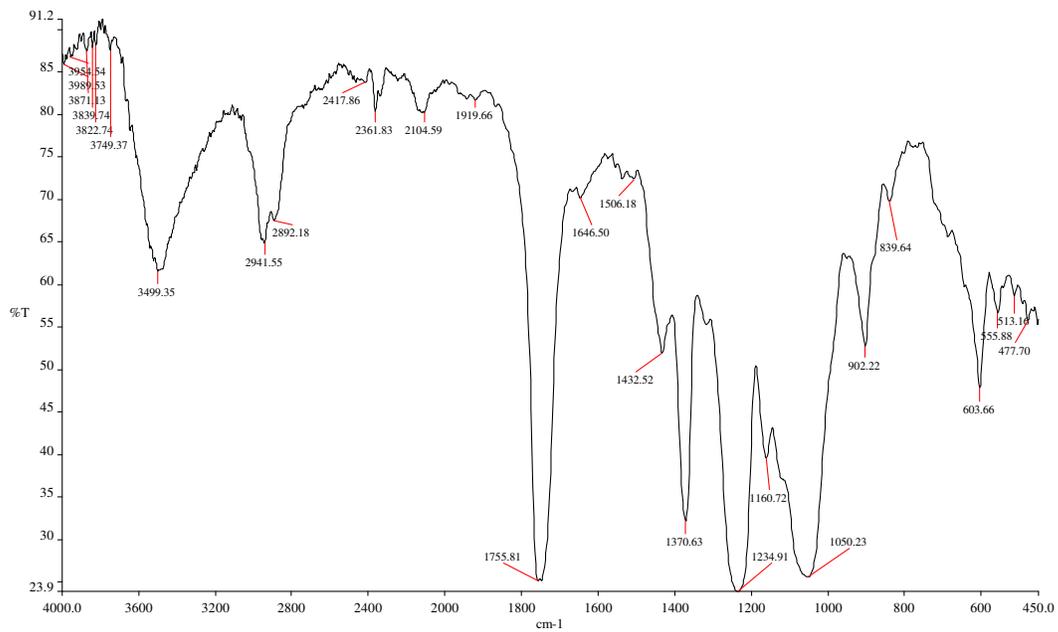
Figure A5: FT-IR spectrum of CA/10% amylose membrane.
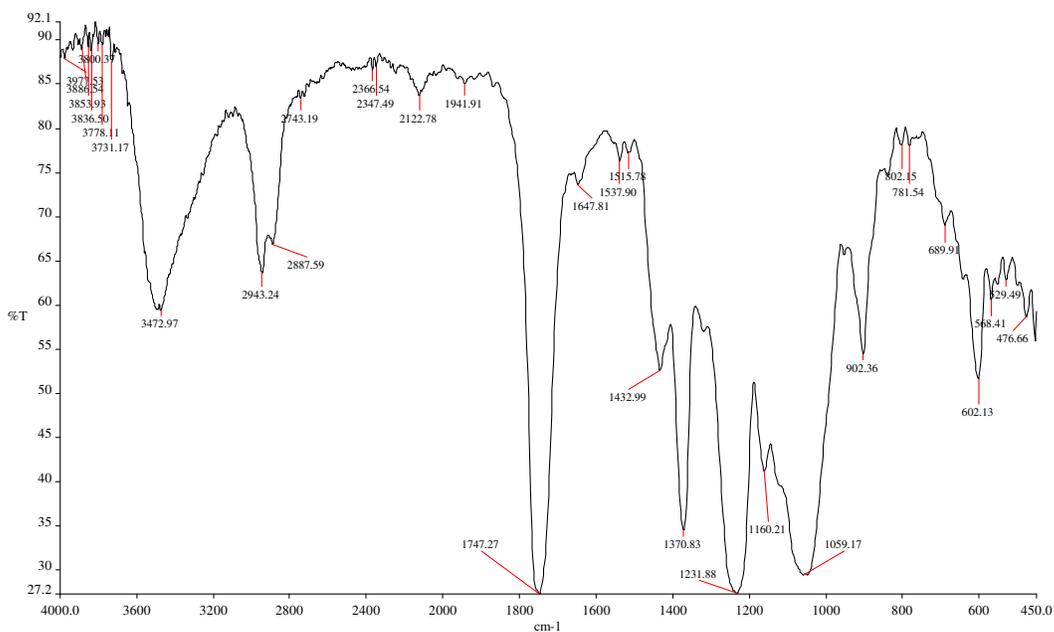
Figure A6: FT-IR spectrum of CA/15% amylose membrane.
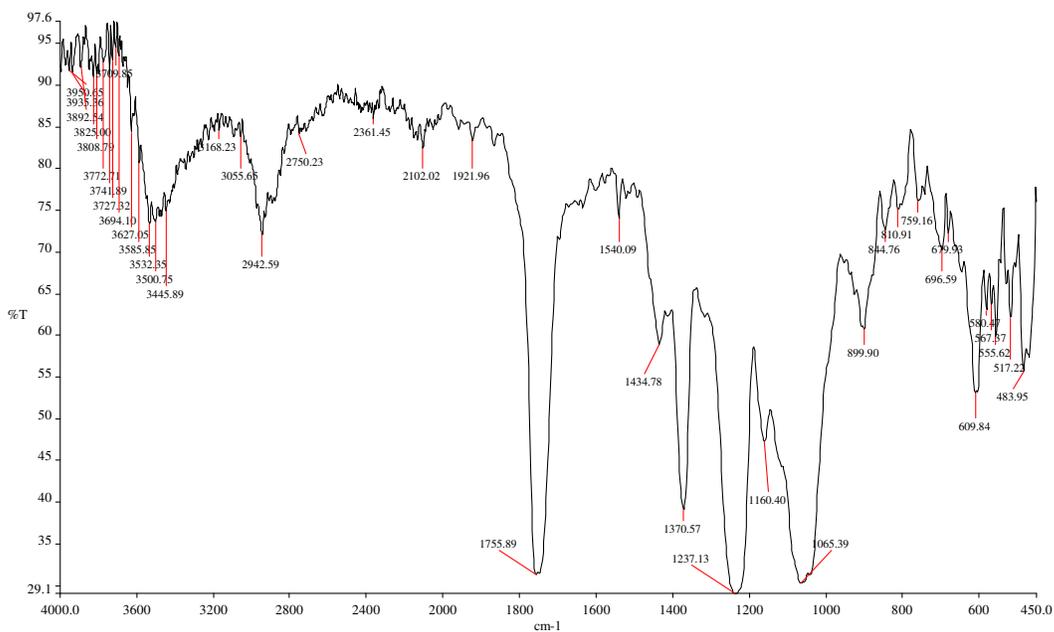
Figure A7: FT-IR spectrum of CA/10%amylose membrane surface.

94
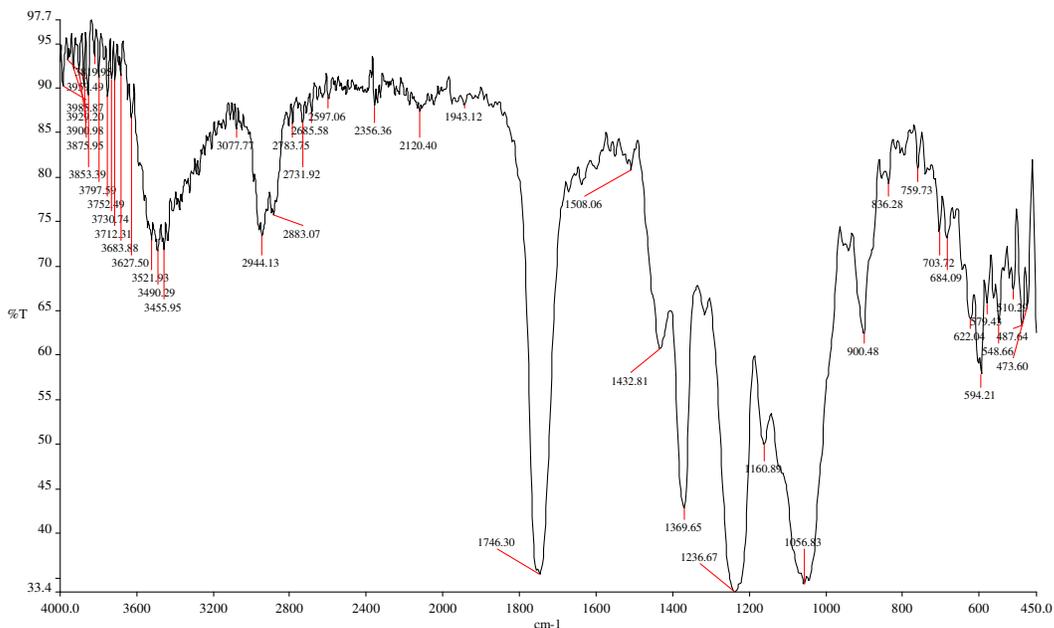
Figure A8: FT-IR spectrum of CA/15% amylose membrane surface.
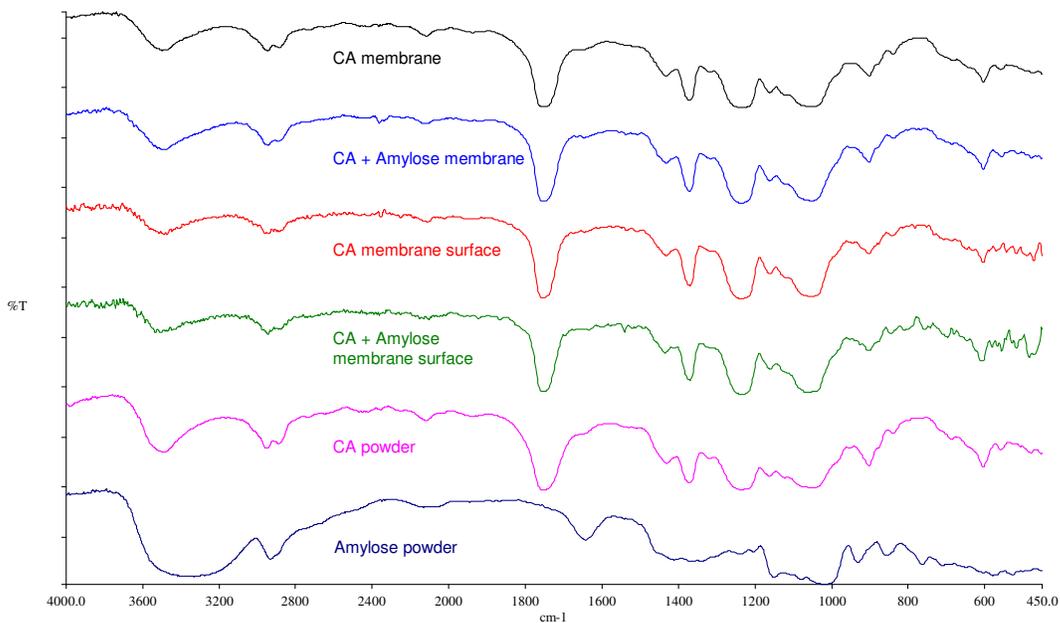
Figure A9: FT-IR spectra Comparing CA and amylose powders, CA/amylose membrane surface, CA membrane surface, CA/amylose membrane and CA membrane.