DEVELOPMENT OF TECHNOLOGY FOR THE SELECTIVE REMOVAL OF BIOACTIVE POLLUTANTS BY LIGANDS, NON-COVALENTLY IMMOBILISED ON MEMBRANES

Report to the

WATER RESEARCH COMMISSION

by

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WRC Report No 1165/1/06

ISBN No 1-77005-312-3

FEBRUARY 2006

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EXECUTIVE SUMMARY

The presence of biologically active organic species in water is of international concern. These chemicals are known as endocrine disruptive chemicals (EDCs) and they interfere with the endocrine systems of living species that control a large number of physiological processes. EDCs act as pseudo hormones, estrogen-mimicking agents, and have been held responsible for the global reduction in male fertility, for example. Their concentration levels in water are extremely low, which makes their detection, isolation and characterisation a very difficult and costly exercise.

This project concerned the development of a technique by which biologically active species could be separated from water by way of a recognition system, which is very specific for the species targeted, for example EDCs that mimic estrogen, a female hormone. Affinity separation, the technology referred to here, is commonly practised in many biochemistry laboratories. It is based on the recognition of biological species, such as hormones, by receptor sites and forms the basis of specificity in the biochemistry of any living species, a phenomenon without which life, as we know it today, would not be possible.

The principle of affinity separation is based on a recognition system where the chemistry and molecular architecture of both the ligand (immobilising agent) and ligate (targeted species) are important. Specificity and recognition of targeted species and their attachment to the ligand is based on complementary intermolecular forces and molecular architecture. However, by changing the aqueous environment, pH, ionic strength, temperature, etc, the ligand and ligate molecules are distorted, the intermolecular forces ruptured, allowing the ligate to be eluted from the system. Figure 1 depicts the basics of the affinity separation approach, showing the selective removal of a specific bioactive molecule form a mixture containing various other molecules.

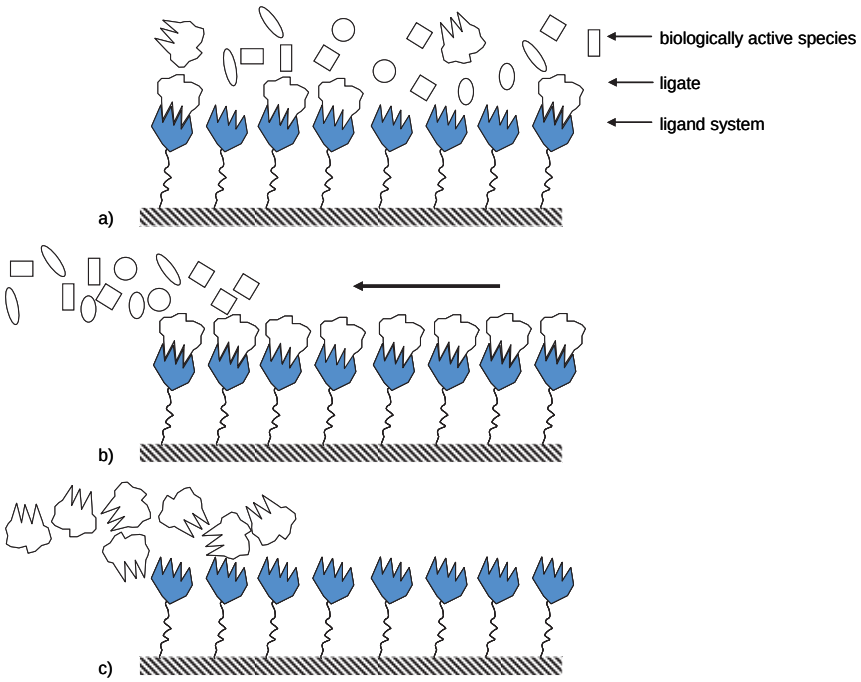

Figure 1: **Sequence of events in affinity separation: a) feeding the ligand system with a mixture of biologically active species and the targeted ligate, b) rinsing the system to remove untargeted species, and c) elution of the concentrated targeted species from the affinity system**

This study made use of membranes as the immobilisation matrix. The use of membranes holds several advantages over the conventional agarose and polymer based matrices normally used in affinity separation. It is known that membrane based matrices deliver high flow rates at relatively low pressures, apart from the fact that these matrices are not subject to channelling, bed shift or bed collapse and the generation of fines. In addition, membranes lend themselves ideally for scaled up.

The affinity system studied during the course of the project differs from conventional affinity chromatography in the manner in which the ligand is immobilised on the support surface. In this work the ligand is first attached to a carrier, after which said carrier is affixed to a substrate matrix not by intramolecular or covalent bonding, but rather through adsorption or intermolecular bonding. A water-soluble tri-block copolymer, called Pluronic® F108 (BASF), was used as the carrier for ligands in this investigation.

The non-covalent ligand immobilisation concept studied is explained by way of Figure 2. The conventional affinity separation practise is depicted in Figure 2a. The figure shows the tether by which the ligand is covalently attached to the support matrix. The molecular formula of the Pluronic (Figure 2b) shows that the tri-block copolymer is made up of two blocks of poly(ethylene oxide) (PEO) that flank a block of poly(propylene oxide) (PPO). The *n* and *m* subscripts denote the number of repeat units in each block segment. The hydroxyl (–OH) groups at the ends of the polymer provide for a chemical route for functionalisation and ligand attachment. A number of techniques were developed and tested to achieve this.

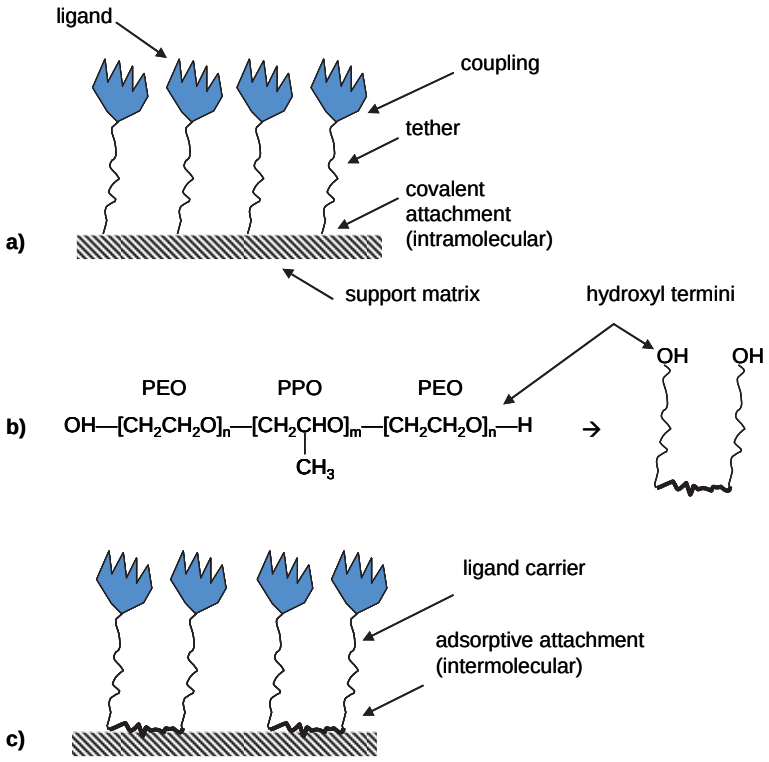

Figure 2: Affinity separation ligand carrier arrangement: a) conventional covalently attached ligand system, b) chemistry of the chosen ligand carrier and c) the alternative adsorbed ligand-carrier system

It is a requirement of this approach that the carrier molecule must have a hydrophobic segment. This will facilitate its immobilisation onto a hydrophobic surface through hydrophobic-hydrophobic interaction (Figure 2c), essentially Van der Waals forces.

The initial membrane of choice was polysulphone. This material was chosen for its hydrophobic character and also because it was known, through Water Research Commission project K5/1035, that Pluronic over coated polysulphone in a manner that reduced the tendency for foulants to adsorb onto the membrane. Of importance in this study was that the substrate surface be coated homogeneously and that non-specific adsorption should be minimised.

Various other polymers were also used as support matrix. These materials have different surface and chemistry properties and included poly(ether imide) and poly(vinylidene fluoride), know membrane-forming materials. Polystyrene was another hydrophobic polymer surface studied.

It was important to establish the maximum loading capacity of the Pluronic material on the different surfaces investigated. With the help of such information it was possible to develop protocol to monitor the loading concentration of the ligand carriers on specific substrates. The maximum loading capacity of the ligand-carrier was initially quantified by means of Langmuir adsorption isotherms, but that did not give information on ligand loading density. Later in the project it became possible to correlate the Langmuir adsorption data through spectroscopic studies of surfaces coated with Pluronic conjugates carrying a Ni-complexing ligand. This provided the sought after ligand loading-density information by which the minimum loading capacity of an affinity system could be determined.

Various physico-chemical routes exist by which ligands are attached to tethers in conventional affinity separation. This is normally done by liquid-solid phase reaction schemes. One of the challenges faced was to devise mechanisms and synthetic routes by which to couple different ligands to the PEO end-groups of the Pluronic material. Most of the initial research was focussed on this problem, keeping in mind that the reactive hydroxyl end-group of the Pluronic material is very dilute in comparison with the bulk of the tri-block copolymer. A number of techniques were researched and successfully applied in the ligand-carrier approach to affinity separation.

Primary amines provided one approach by which to link carboxylic moieties to the Pluronic carrier. To achieve this, the hydroxyl termini on the Pluronic were substituted with a primary amine. The first synthetic route developed involved a three-step reaction protocol that was later successfully adapted to a two-step reaction scheme. In this case the hydroxyl termini were substituted with hydrazine, an alternative functional group to the primary amine. The hydrazine is also reactive towards carboxylic acids and provided an alternative route by which a protein, such as an enzyme, could be attached to the Pluronic.

Biotin interacts strongly with avidin, the strength of the intermolecular attachment being close to that of covalent bonding. This route to binding a ligand to the Pluronic carrier was also research and developed successfully, the first step being the development of synthetic routes to substitute the hydroxyl groups on Pluronic with the biotin.

Ligands can also be immobilised through complexation with metals and Ni is often used for this purpose. However, Ni must first be coordinated onto the Pluronic carrier. By adapting the chemistry of the well-know metal chelating agent, EDTA, a ligand was prepared that allowed Ni to be bound to Pluronic through coordination chemistry. This metal complex was later used in spectroscopic studies to characterise and confirm the extent of Pluronic adsorption, but more importantly, it provided the necessary means to immobilise histidine-tagged proteins on membranes coated with the Ni-pluronic adduct.

Quantification of the modified adsorbed Pluronic was a further problem to solve. In this study a simple procedure for bi-phasic extraction and colorimetric detection of Pluronic adsorbed onto dense skinned membranes was devised. The extraction and detection method described were also used to study the interfacial adsorption onto capillary membranes and similar curved non-porous surfaces. After extraction of the adsorbed Pluronic or Pluronic derivatives, the extracted Pluronic was reacted with NH_4FeSCN to form a colour complex. A pink to purple colour development occurs rapidly after mixing at room temperature with λ_{max} at 505 to 510 nm. A linear response over a concentration range of 3 μg/mL to 130 μg/mL of Pluronic was obtained.

Was there a simple way to establish whether a certain ligand would bind a targeted ligate? The technique ultimately devised and tested was quite simple, yet sensitive and relied on the avidin-biotin technology earlier mentioned. Biotin was coupled to the modified hydroxyl terminal of Pluronic. The biotinylated Pluronic was subsequently coated onto polysulphone and polystyrene hydrophobic surfaces. An enzyme, horseradish peroxidase, coupled to avidin, was added to the immobilised biotin. The enzyme, now bound to the ligand-carrier, was assayed spectrophotometrically through its conversion of a colourless substrate to a coloured product. The intensity of the developed colour is a direct indication of the number of enzyme molecules immobilised and hence the success of the ligand-carrier affinity separation technique developed.

Experimental proof was found in the course of the investigation that ligates would couple to ligands attached to the ligand-carrier, that the ligand-carrier did adsorb fairly evenly on the receiving surfaces evaluated, that the ligand-carrier could be displaced by means of competitive adsorption, and that this approach for affinity separation was sound.

In this study the foundation was laid for the non-covalent immobilization of different ligand binding moieties on hydrophobic synthetic membranes. An enzyme (horseradish peroxidaze) was successfully immobilized using Pluronic-biotin-avidin technology and it was shown that a metal-binding Pluronic derivative could be used to bind Ni as well as histidine-tagged proteins.

The separation of estrogen mimicking agents (endocrine disruptive chemicals) from water was not demonstrated at the time this report was documented. The main stumbling block concerned expressing of the ligand-binding domain of the human receptor for estrogen, which is not a trivial task. However the soundness of the approach to affinity separation investigated was demonstrated in a number of ways. The progress to date has presented us with tested avenues by which to immobilise the ligand-binding domain of the human estrogen receptor, which lies at the heart of the EDC detection system envisaged.

A good understanding of the affinity separation technology originally envisaged was generated. This will help to expedite the development of an endocrine disruptive chemical (EDC) recognition tool, once the ligand-binding domain of the estrogen receptor has been expressed in large quantity by fermentation. All the biochemical problems associated with the expression of the estrogen receptor-binding domain have largely been overcome.

It is a recommendation of this project that the work be continued and a concerted effort made to develop a tool by which the presence of any species with EDC properties could be detected in water.

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ACKNOWLEDGEMENTS

The research documented emanated from a project funded by the Water Research Commission entitled:

Effluent harvesting and detection of steroidogenic agents by affinity separation

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- The stimulating and supportive contributions of the Steering Committee are acknowledged.
- Support for the project and the financial support of the Water Research Commission and contributions of the Steering Committee is acknowledged greatfully.
- Funding by the NRF and Sub Committee B of the University of Stellenbosch for research on the EDC detection system is acknowledged with grattitude.

Various individuals and institutions made contributions and the authors would like to express their sincere thanks to the following:

- Dr BS Katzenellenbogen, Dept of Physiology & Biophysics, University of Illinois, Urbana-Champaign for generous gift of the pET15b plasmid containing the ligand binding domain of the human estrogen receptor;
- Dr E, Strauss, Department of Chemistry and Polymer Science, University of Stellenbosch, for providing recombinant *Escerichia coli* pantothenate kinase;
- The Depts of Biochemistry and Chemistry & Polymer Science, especially Dr AC Swart with respect to recombinant DNA experiments, and also Dr Haneli Adendorff for her involvement with the metal ligand development work;
- Mr Deon Koen, Institute of Polymer Science, University of Stellenbosch; and
- Themba Laboratories, Materials Research Group, especially Dr Woijech Przybylowicz and Dr Chris Theron

A special word of thanks to my colleages, Prof P Swart and Dr MW Bredenkamp, and all the students associated with the project.

LIST OF ABBREVIATIONS AND GLOSSARY OF TERMS

A	poly(ethylene oxide), hydrophilic moiety of Pluronic
agonist	An agonist is a substance that binds to a receptor and triggers a response by the cell. An agonist is the opposite of an antogonist in the sense that while an antagonist also binds to the receptor, it fails to activate the receptor. A partial agonist activates a receptor but does not cause as much of a physiological change as does a full agonist. The receptors of the human body work by being stimulated or inhibited by natural (such as hormones and neurotransmitters) or synthetic (such as drugs) agonists and antagonists.
antagonist	a substance that inhibits the normal physiological function of a receptor. Many drugs work by blocking the action of endogenous receptor agonists such as hormones and neurotransmitters. Antagonists that compete with an agonist for a receptor are competitive antagonists.
antibody	An antibody is a protein complex used by the immune system to identify and neutralize foreign objects like bacteria and viruses. Each antibody recognizes a specific antigen unique to its target
antigen	any substance that the body sees as being foreign. This triggers an immune response from the body's immune system.
AP	Amino-pluronic (terminal OH of Plu substituted with an amine group)
B	poly(propylene oxide), hydrophobic moiety of Pluronic
Beer Lambert Law:	the law basically states that absorbance is proportional to the concentration of light-absorbing molecules in the sample analysed
bp	base pairs: In genetics, two nucleotides on opposite complementary DNA or RNA strands that are connected via hydrogen bonds are called a **base pair**. As DNA is usually double-stranded, the number of base pairs in the dsDNA strand equals the number of nucleotides in one of the strands. In DNA, adenine and thymine, as well as guanine and cytosine, can be a base pair. In RNA, thymine is replaced by uracil.
BSA	bovine serum albumin (protein with many accessible COOH groups; used as a positive control for carbodiimide chemistry)
cmc	critical micelle concentration: Depending on the relative strengths of the hydrophobic repulsion and the hydrophobic attraction and the temperature, this is the minimum concentration (number of molecules for unit volume) needed for the resultant micelle to be stable.
DNA	Deoxyribonucleic acid (DNA) is a nucleic acid that carries genetic instructions for the biological development of all cellular forms of life and many viruses. DNA is sometimes referred to as the molecule of heredity as it is inherited and used to propagate traits. During reproduction, it is replicated and transmitted to offspring. In bacteria and other simple cell organisms, DNA is distributed more or less throughout the cell. In the complex cells that make up plants, animals and in other multi-celled organisms, most of the DNA is found in the chromosomes, which are located in the cell nucleus. The energy-generating organelles known as chloroplasts and mitochondria also carry DNA, as do many viruses.

DNA	**DNA** in all uppercase refers to deoxyribonucleic acid. All-in-lower-case ***dna*** AND italicized refers to the genes that encode enzymes involved in DNA replication. **Dna** with the first letter capitalized refers to the enzymes involved in DNA replication.
DNA sequence	A DNA sequence (sometimes genetic sequence) is a succession of letters representing the primary structure of a real or hypothetical DNA molecule or strand. The possible letters are **A**, **C**, **G**, and **T**, representing the four nucleotide subunits of a DNA strand (adenine, cytosine, guanine, thymine, and typically these are printed abutting one another without gaps, as in the sequence AAAGTCTGAC. This coded sequence is sometimes referred to as genetic information. A succession of any number of nucleotides greater than four is liable to be called a sequence. With regard to its biological function, which may depend on context, a sequence may be sense or anti-sense, and either coding or noncoding. DNA sequences can also contain "junk DNA".
EDAC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (a water-soluble derivative of carbodiimide)
EDC	endocrine disruptive chemical
ELISA	enzyme linked immunosorbent assay
ER	estrogen receptor
HP	hydrazide-pluronic (terminal OH of Pluronic substituted with a hydrazine group)
HRP	horseradish peroxidase
LBD	ligand binding domain
PEI	polyetherimide
plasmid	a structure consisting of DNA (just a few genes) that exists outside the chromosome in a procaryotic cell and is able to replicate independently. Plasmids enable the cell to resist antibiotics, metabolise special compounds as nutrients and perform other special functions
Pluronic® F108	Pluronic is a range of surfactant products from BASF. It is a tri-block copolymer containing two end blocks consisting of poly(ethylene oxide) and a middle segment consisting of poly(propylene oxide). F108 is one product from that range of surfactants.
Pluronic	abbreviation used throughout the text when referring to Pluronic® F108, an amphiphilic tri-block copolymer of BASF
PS	polystyrene
PSU	polysulphone
PVDF	poly(vinylidene fluoride)
RBS	Rutherford backscattering spectroscopy
RNA	**Ribonucleic acid** (**RNA**) is a nucleic acid consisting of a string of covalently-bound nucleotides. It is biochemically distinguished from DNA by the presence of an additional hydroxyl group, attached to each pentose ring; as well as by the use of uracil, instead of thymine. RNA transmits genetic information from DNA (via transcription into proteins (by translation).
SBTI	soya bean trypsin inhibitor
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis trypsin
transcription	transcription is the process of copying DNA to RNA by an enzyme called RNA polymerase

1. Introduction

The presence of biologically active organic species in water is of international concern. These chemicals, known as endocrine disruptive chemicals (EDCs), they interfere with the endocrine systems of living species that control a large number of physiological processes. EDCs act as pseudo hormones, estrogen-mimicking agents, and have been held responsible for the global reduction in male fertility, for example. Their concentration levels in water are extremely low, which makes their detection, isolation and characterisation a very difficult and costly exercise.

This project concerned the development of a technique by which biologically active species could be separated from water by way of a recognition system, which is very specific for the species targeted (e.e. EDCs that mimic estrogen, a female hormone). Affinity separation, the technology referred to here, is commonly practised in many biochemistry laboratories. It is based on the recognition of biological species, such as hormones, by receptor sites and forms the basis of specificity in the biochemistry of any living species, a phenomenon without which life, as we know it today, would not be possible.

Separation is a fundamental requirement in most biotechnological processes and affinity chromatography (separation) is one of the more important. Many technologies under development use recombinant proteins or require high-purity enzymes for immobilization, encapsulation or for use in organic synthesis. Separation methods based on affinity interaction of biological compounds are often regarded as amongst the most effective of the protein purification techniques.[1,2,3] The large-scale application of these techniques may be restricted because of:

- the economic burden (high cost of support matrices and ligands);
- the practical limitations of the chromatographic column size and throughput; and
- effort of operation.

These chromatographic techniques are very time-consuming and have a limited industrial application because of the low binding capacity of the current affinity matrices. Since the development of the first synthetic membrane, membrane technology made considerable advances in process industry, especially within the water sector.[4,5,6,7] Compared with traditional industrial techniques, membrane filtration processes constitute a more cost-effective and environmentally friendly approach to separation than other processes.

1 MR Buchmeiser, New synthetic ways for the preparation of high-performance liquid chromatography supports, J. Chromatogr. A, 918(2001) 233-266.

2 BT Croll, Membrane technology: the way forward?, J IWEM, 6(1992)121-129.

3 CP Woodbury Jr., DL Venton, Methods of screening combinatorial libraries using immobilized or restrained receptors, J. Chromatogr. B, 725(1999)113-137.

4 AJM Arnal, SM Fernández, MG Verdú, LJ García, Design of a membrane facility for water potabilization and its application to Third World countries, Desalination, 137(1-3)(2001) 63-69.

5 H Kurama, J Poetzschke, R Haseneder, The application of membrane filtration for the removal of ammonium ions from potable water, Water Research, 36(11) (2002) 2905-2909.

6 H Kurama, J Poetzschke, R Haseneder, The application of membrane filtration for the removal of ammonium ions from potable water", Water Research, 36(11) (2002) 2905-2909.

7 H-H Yeh, I-C Tseng, S-J Kao, W-L Lai, J-J Chen, GT Wang, S-H Lin, Comparison of the finished water quality among an integrated membrane process, conventional and other advanced treatment processes, *Desalination*, 131(1-3) (2000) 237-244.

The rapid developments in biotechnology and the potential of biomolecules in the pharmaceutical industry are fuelling the demand for reliable and efficient methods to purify preparative mixtures of proteins, lipoproteins, peptides and nucleic acids. Affinity separation has grown with this demand and there has been a similar increase in the number of affinity adsorbents available for laboratory scale purifications. However, despite the obvious advantages that affinity separation offers, the technique has only recently begun to find favour for the purification of high-value products in process-scale purifications. This is most likely because of the inherent cost to immobilise ligands on supports as well as problems posed by ligand leakage, the complicated validation procedures, and the need for adsorbents which can withstand cleaning and/or sterilization regimes.[8]

Most affinity processes are typically performed using packed beds, which have several major limitations. The pressure drop across a packed bed is generally high and tends to increase during a process due to the combined effects of bed consolidation (media deformation) and column blinding (accumulated colloidal material). Other major limitations are channelling (formation of flow patterns due to bed cracking) and the dependence on intra-particle diffusion for the transport of solute molecules to their binding sites within the pores of such media. The latter increases the process time since transport of macromolecules is slow. Consequently the recovery liquid volume needed for elution also increases. Some of these factors and the fact that the transport phenomenon is complicated make scale-up of packed-bed chromatographic processes difficult.[9]

In adsorptive membrane chromatography systems, the adsorptive packing consists of one or more microporous membranes, each derivatized with adsorptive moieties. Membranes can be configured in tangential flow or dead-end filtration geometries. The major advantage of affinity membranes lies in the predominance of convective material transport. Generally, three types of membrane supports are used, namely flat-sheets, hollow fibres and radial flow adsorbers. The use of affinity membranes, in the scope of this work, is intended to increase the selectivity of membrane separations based on affinity ligands, which are coupled to nano particles such as a Pluronic surfactant (a tri-block copolymer) in order to selectively bind proteins (or other molecules of interest), while at the same time preventing or sterically hindering non-specific adsorption.

The ability to characterise adsorption equilibriums accurately is important in all chromatography processes, including affinity separation. No prediction of column performance could be achieved without first measuring or estimating the adsorption isotherms of these components under the conditions of interest, and the assurance of the prediction is strongly dependent on the accuracy of these data.[10] The understanding of the interfacial properties of Pluronic surfactants with respect to steric stabilisation and suppression of protein adsorption is also of interest in the use of Pluronic or modified Pluronic as an affinity linker to polymer membranes for affinity separation.

8 DK Roper, EN Lightfoot, Separation of biomolecules using adsorptive membranes, J. Chromatography A. 702(1995) 3-26.
9 R Ghosh, Protein separation using membrane chromatography: opportunities and challenges J. Chromatography A, 952(2002)13–27.
10 Q Lan, AS Bassi, J-X Zhu, A Margaritis, A modified Langmuir model for the prediction of the effects of ionic strength on the equilibrium characteristics of protein adsorption onto ion exchange/affinity adsorbentsChemical Engineering Journal, 81(2001)179-186.

Affinity interactions are among the most useful bio-separation tools because of their high specificity. Our approach is a paradigm shift away from the current analytical methods and a positive move towards a more preventative/regulatory approach that could address the problem with onsite monitoring (quantitative analysis) of EDCs where a potential discharge risk is suspected.

The efficiency of the affinity membrane depends on the preparation method, i.e. the activator or activation process used for ligand immobilization and use. The immobilization method is particularly important when biomolecules such as proteins or enzymes act as ligands. This is because the activity of the ligand can be affected by denaturation during the immobilization process.

The adsorption of the amphiphilic tri-block copolymer, Pluronic® F108 (Pluronic) onto hydrophobic membranes is well-studied. Pluronic is a tri-block copolymer (A-B-A) consisting of a hydrophobic core (B) flanked by two hydrophilic blocks (A). The B-block forms a strong and stable hydrophobic link to hydrophobic surfaces and can be immobilized by the simple process of adsorption. After immobilization, the A-blocks are facing the aqueous solution and have an efficient hydrocarbon length to prevent steric hindrance when interacting with biomolecules.

Traditional linkers or spacer arms must generally be synthesized to an efficient length of hydrocarbon backbone and this is usually an expensive method, especially during coupling reactions. Furthermore, by using this hydrophilic tether, it allows that even larger proteins are presented at the surface in a biological active form (conventional spacer arms generally consist of 4 to 10 carbon atoms, which is only effective for small ligands and target solutes, but not for macromolecules or biological complexes). Pluronic is effective during coupling reactions because the terminal –OH groups can be converted into amino groups for reactions with specific conjugating chemicals and biomolecules.[11,12,13]

In the present study, affinity principles to detect and monitor for potential estrogenic chemicals (known as endocrine disrupting chemicals) in drinking water are under study. Derivatized Pluronic was used as the vehicle to link the ligand(s) onto the membrane through hydrophobic interactions. The end-groups of the tri-block copolymer were modified specifically, so that a hormone receptor (i.e. estrogen receptor) could be attached to it and serve as an affinity linker for the interactions between hormone receptor and the hormone.

The affinity separation technique studied relied on the hydrophobic-hydrophobic interaction between the hydrophobic moiety of the affinity linker (Pluronic) and the support matrix, in this case a synthetic membrane. The description of the research could be divided into different phases, and in this document, attention was given to:

11 C Nardin, W Meier, Hybrid materials from amphiphilic block copolymers and membrane proteins. Rev. Mol. Biotechnol., 90 (2002) 17-26.

12 JC Neal, S Stolnik, E Schacht, ER Kenawy, MC Garnett, SS Davis, L Illum, In vitro displacement by rat serum of adsorbed radiolabelled poloxamer and poloxamine copolymers form model and biodegradable nanospheres, J. Pharma. Sci., 87(10) (1998) 1242-1248.

13 N Tirelli, MP Lutolf, A Napoli, JA Hubbell, Poly(ethylene glycol) block copolymers. Rev. Mol. Biotechnol., 90(2002) 3-15.

- surface analysis of Pluronic immobilised on different membranes;
- the regeneration of coated membrane surfaces;
- synthesis of ligands and ligand coupling mechanisms;
- production of recombinant polyhistidine tagged proteins;
- immobilised metal affinity chromatography; and.
- production of recombinant ligands.

As indicated above, one of the aims of the study was to develop a technique to detect EDCs in wastewater and water courses by means of affinity membrane separation. This study tested the viability of what we think could be a rapid, simple and inexpensive detection method for EDCs with a view to its use in the field to assess the compliance of drinking/purified water to norms.

2. Pluronic adsorption and desorption studies

2.1 Introduction

The advent of membrane technology in the last two decades has resulted in numerous applications in the fields of biotechnology, waste water treatment, pharmaceutical and bio-medical applications. The efficiency of membrane technology is highly dependent on its ability to resist the non-specific adsorption of organic foulants due to protein and cell adhesion.[14] This often unwanted adsorption leads to decreased flux in water treatment,[15] cytotoxicity in drug delivery and increased thrombogenicity in biomaterial and cardiovascular therapeutics.[16]

Poly(ethylene oxide)$_m$-poly(propylene oxide)$_n$-poly(ethylene oxide)$_m$ (PEO_m—PPO_n—PEO_m), tri-block copolymers (Pluronic®, BASF, Co) are water-soluble, non-ionic amphiphilic surfactants. These compounds are surface active and form micelles and lyotropic crystalline phases[17] and have a high chemical and thermal stability. These block copolymers of PEO and PPO are approved as thermoviscofying agents by the food and drug administration and the environmental protection agency as direct and indirect food additives, pharmaceutical ingredients, agricultural products and in biotechnological applications.[18]

The interfacial adsorption behaviour of Pluronic is receiving increasing attention due to its amphiphilic nature, which is attributed to the difference in solubility of the hydrophobic PPO and the hydrophilic PEO segments in water. This feature has contributed to its emergence in the fields of polymer adsorption and steric stabilisation.[19] It has also become a popular coating material because of its ability to non-covalently attach to hydrophobic surfaces and shield said surfaces from the non-specific adsorption of macromolecules from the surrounding bulk phase.[1,3,4,5] This is a particularly attractive feature in applications using synthetic polymer membranes.

Pre-treatment of membranes is an important strategy for the reduction of protein and cell adsorption. It is therefore important to understand the complex multi-parameter process of polymer adsorption onto membranes. Pluronic pre-treatment is achieved by passive adsorption of the hydrophobic PPO centre block of the tri-block copolymer onto the membrane surface, while the pendant hydrophilic PEO groups protrude out into solution forming a secondary hydrophilic layer that sterically hinders the adsorption of potential foulants.

14 J-T Li, J Carlsson, S-C Huang, KD Caldwell, Adsorption of poly(ethylene oxide)-containing block copolymers: a route to protein resistance Advances Chemistry Series. 248, 67 (1996).

15 JA Howell, V Sanchez, RW Field, Membranes in Bioprocessing: Theory and applications. (1993).p. 203.

16 F Ahmed, P Alexandridis, S Neelamegham, Synthesis and application of fluorescein-labelled Pluronic block copolymers to the study of Polymer-surface interactions, Langmuir, 17, (2001) 537.

17 CRE Mansur, MP Barboza, G Gonzalez, EP Lucas, PLURONIC × TETRONIC polyols: study of their properties and performance in the destabilization of emulsions formed in the petroleum industry J. Colloid Int. Sci., 271 (2004) 232-240.

18 S Stolnik, B Daudali, B Arien, J Whetsone, CR Heald, MC Garnett, SS Davis, L Illum, The effect of surface coverage and conformation of poly(ethylene oxide) (PEO) chains of poloxamer 407 on the biological fate of model colloidal drug carriers Biochimica Biophysica Acta. 1514 (2001) 261.

19 M Bohner, TA Ring, KD Caldwell, Macromolecules. Studies on the effect of particle size copolymer polydispersity on the adsorption of a PEO/PPO/PEO copolymer on PS latex particles Macromolecules, 35 (2002) 6724.

A wide range of techniques such as small angle X-ray spectroscopy, sedimentation field flow fractionation,[6] photon correlation spectroscopy,[20] surface plasmon resonance spectroscopy,[21] atomic force microscopy, ellipsometry, neutron scattering, and X-ray photoelectron spectroscopy[22] have been used to study the adsorption of Pluronic onto polymeric surfaces. It is to a large extent the physical and chemical properties of the adsorption matrix that determines the suitability of the attendant analytical methodology. Since most direct and indirect analytical instruments are not entirely suitable or routinely available for quantifying bound Pluronic, this study focused on a simple, inexpensive and readily available protocol for Pluronic extraction and detection on synthetic membranes.

This work was aimed at developing a robust extraction and detection system for membrane-adsorbed Pluronic in order to investigate the interfacial properties of Pluronic and modified Pluronic, adsorbed onto flat-sheet or planar membranes and capillary membrane. Planar macroscopic surfaces such as membranes are ideal for fluid-solid systems since many experimental techniques are designed primarily for flat surfaces.[23] Polysulphone (PSU), poly(ether imide) (PEI) and poly(vinylidene fluoride) (PVDF) were cast as dense skinned or symmetrically homogenous planar membranes, which resulted in three matrices that differed in their hydrophobicity, such that they could adsorb the amphiphilic Pluronic. Non-porous curved capillary membranes were also fabricated in order to investigate the possible contributions of interfacial curvature to Pluronic adsorption. We decided to base our assay on the phospholipid detection protocol described by Stewart,[24]and the solvent extraction combination used for Pluronic extraction was hexane-isopropanol (3:2 v/v).

In this study we report a simple procedure for bi-phasic extraction and colorimetric detection of Pluronic adsorbed onto dense skinned membranes. The extraction and detection method described were used to study the interfacial adsorption onto capillary membranes and similar curved non-porous surfaces.

2.2 Colorimetric estimation of Pluronic using the biphasic assay system

Most of the existing colorimetric methods for measuring PEO based surfactants in biological samples require the removal of proteins, lipids and sugars by precipitation and or filtration.[25] Colorimetric estimation of PEO based surfactants and Pluronic have been based on the formation of a complex with cobalt thiocyanate,[26] the Wickbold method,[27] or titration with tetrakis(4-halophenyl) borate.[28] The cobalt thiocyanate assay required repeated

20 SM O'Connor, SH Gehrke, GS Retzinger, Ordering of PEO/PPO/PEO triblock copolymers in condensed films, Langmuir, 15, 2580 (1999).

21 RJ Green, S Tasker, J Davies, MC Davies, CJ Roberts, SJB Tendler, Adsorption of PEO-PPO-PEO triblock copolymers at the solid/liquid interface: A SPR study, Langmuir, **13** (1997) 6510.

22 K Eskilsson, LM Grant, P Hansson, F Tiberg, Self-aggregation of triblock copolyerms at the solid silica-water interface, Langmuir, 15 (1999) 5150.

23 GJ Fleer, NA Cohen-Stuart, JMHM Scheutjens, T Cosgrove and B Vincent, In Polymers at Interfaces. Chapman and Hall (1993) p. 45.

24 JCM Stewart, Colorimetric determination of phospholipids with ammonium ferrothiocyante, Anal. Biochem., 104, 10 (1980).

25 A Nag, G Mitra and PC Ghosh, A colorimetric assay for estimation of polyethylene glycol and polyethylene glycolated protein using ammonium ferrothiocyanate, Anal. Biochem., 237, 224 (1996).

26 AM Tercyak, TE Felker, Colorimetric assay for Pluronic F68 as measured in isolated rat liver perfusion systems, Anal. Biochem., 187, 54 (1990)

27 H Ghebeh, A Handa-Corrigan, and M Butler, Development of an assay for the measurement of the surfactant Pluornic F68 in mammalian celll culture medium, Anal. Biochem., 262, 39 (1998).

28 M Tsubouchi, N Yamasaki, K Yanagisawa, Hydrophobic indicators for two-phase titrations and their application to the determination of silver and pentachlorophenol, Anal. Chem., 57, 783 (1985).

centrifugation steps while the Wickbold method involved the use of a specifically designed apparatus for concentrating the colour complex.

In this study the extracted Pluronic formed a colour complex with NH_4FeSCN. This is based on partitioning of a chromophore present in NH_4FeSCN from an aqueous phase to a $CHCl_3$ phase in the presence of Pluronic. A pink to purple colour development occurs rapidly after mixing at room temperature with λ_{max} at 505 to 510nm. The $Fe(SCN)_3$ chromophore on its own does not partition into the chloroform layer in the biphasic assay system, so it is highly likely that the amphiphilic Pluronic solvates the $Fe(SCN)_3$ complex due to its solubility in both water and $CHCl_3$.

A linear response over a concentration range of 3 μg/mL to 130 μg/mL is obtained (Figure 3). Beyond 130 μg/mL Beer-Lambert's Law is not obeyed and the concentration curve deviates progressively from linearity. The robustness of this assay was tested with a halogenated Pluronic derivative, which had been used in another study for solid-state detection of Pluronic on membranes (results not shown). Pluronic-Iodide was characterised with ^{13}C NMR (Table 1) and showed a similar linearity at 510nm (Figure 3) and the linear response was also within the dilution range of 3 to 130 μg/mL. In comparison to the systems described in,[14 – 17] both the protocols for Pluronic desorption and detection are relatively simple, sensitive, inexpensive, quick and reproducible over a wide range of Pluronic coating concentrations and membrane surface chemistries.

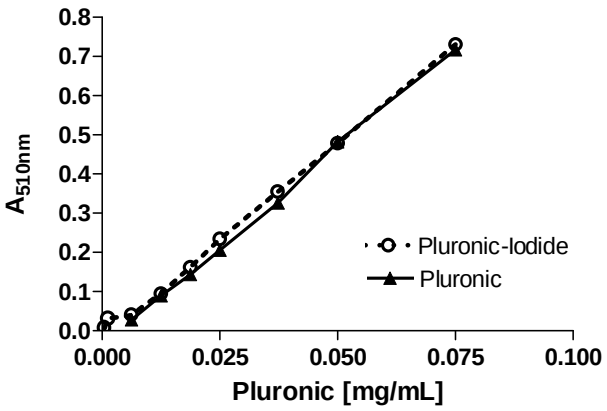


Figure 3: Typical standard curves for Pluronic® F108 (r^2 = 0.9985) and for Pluronic® F108-Iodide (r^2 = 0.9966)

In an attempt to further test the versatility of this method of Pluronic estimation in the presence of common biological material such as plasma, sugars, proteins and vitamins etc., the effect of these additives on the recovery of Pluronic was investigated. The results in Table 2 show the effect of bio-additives on the quantification of Pluronic. Biotin, BSA and dextran had no effect on Pluronic recovery while the presence of human plasma had a minor impact on the assay. SDS and lysozyme, however, were incompatible with the assay.

Table 1: ^{13}C-NMR chemical shifts (δ) of Pluronic-I in $CDCl_3$ at 25°C

Carbon	Pluronic-I
$-CH_2-CH(CH_3)-O$	17.05; 17.18
$-CH_2I$	29.44
$-CH_2-CH_2-I$	70.46
$-CH_2-CH(CH_3)-O$	72.74; 72.78; 72.82; 72.87; 73.28
$-CH_2-CH(CH_3)-O$	75.03; 75.24; 75.27; 75.44
o-Ts	-
m-Ts	-
p-Ts	-
Ts-CH3	-

Table 2: Effects of bio-additives on the NH_4FeSCN-$CHCl_3$ assay for Pluronic

Additive	Concentration	Change in OD
none	-	0
human plasma	1.5 mg/L	<2 to 5%
biotin	1.0 mg/L	0
bovine serum albumin	1.0 mg/L	0
dextran	1.0 mg/L	0
sodium dodecyl sulphate	34 mM	>70%
lysozyme	1.0 mg/L	>90%

2.2 Efficacy of hexane:isopropanol extraction of Pluronic

Both hexane and isopropanol have been used for lipid extraction from plant tissue with an efficacy of 52 and 45% respectively.[29] Due to its proven efficacy with lipids and oils,[30] a binary solvent system consisting of hexane:isopropanol was used to desorb Pluronic from polymer membranes in Pluronic determinations.

The atomic force micrographs in

Figure 4 serve as a macroscopic indicator of the extent of Pluronic desorption with the hexane:isopropanol solvent system. A typical native, un-treated PSU planar membrane is shown in

Figure 4a, while

Figure 4 shows a Pluronic coated membranes with the bright clusters suggesting micelle formation.[13] AFM analysis indicated uniform adsorption of Pluronic onto flat sheet PSU membranes, while native membranes were characterised by rough and very heterogeneous surfaces, irrespective of the polymer used in its preparation. Typical membrane treatment with hexane:isopropanol for Pluronic desorption is shown in

Figure 4c and

Figure 4d.
Figure 4c and d are micrographs showing hexane-isopropanol extraction below the melting point of Pluronic (55°C) and at ~70°C respectively. The colour intensity shows the vertical

29 A Hara, NS Radin, Lipid extraction of tissues with a low-toxicity solvent, Anal. Biochem., 90, 420 (1978).
30 K Schafer, Accelerated solvent extraction of lipids for determining the fatty acid composition of biological material, Analytica Chimica Acta, 358, (1998) 69.

profile of the membrane surface, with light regions indicating the highest points (micelles), the dark regions being the depressions inherent to the fabrication process, while the intermediate region is the membrane surface. The variation in light intensity, or 'brightness', of the adsorbed Pluronic suggests that Pluronic aggregation occurred on the membrane surface. A similar profile for native, Pluronic-coated and hexane-isopropanol treated membranes were observed with an environmental scanning electron microscope (results not shown).

Since the extraction kinetics and the extraction efficiency of a suitable solvent are positively influenced by temperature, methods based on extraction at elevated temperatures are in common use. This is also applicable to hexane-isopropanol extraction of membrane adsorbed Pluronic. Bi-solvent extraction at 65 to 70°C (above the melting point of Pluronic) was comparatively much more effective in desorbing the surfactant coating (

Figure 4d) than treatment at lower temperatures (
Figure 4c). The robust membrane and the low melting point Pluronic are an ideal adsorbent and adsorbate system for this binary solvent extraction protocol.

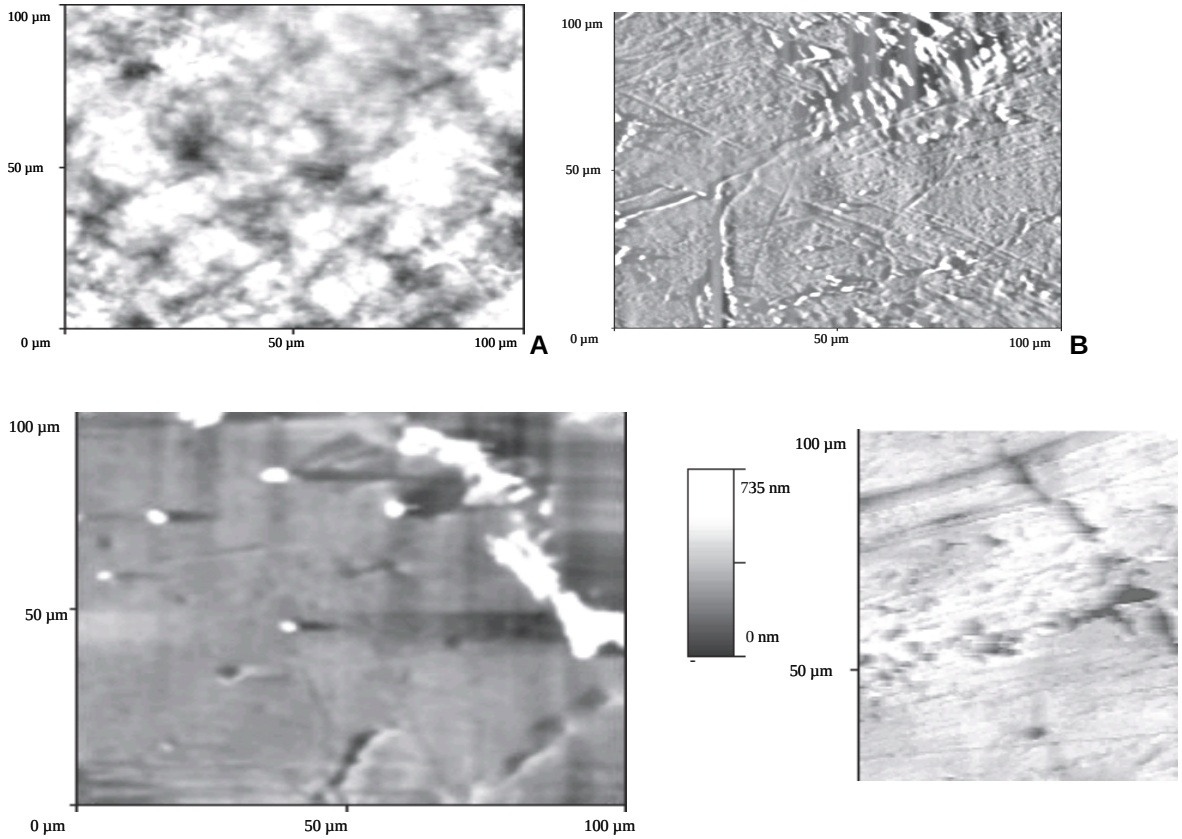


Figure 4: a) Atomic force micrographs showing a native PSU flat sheet membrane, b) Pluronic coated flat sheet PSU membrane, c and d) typical planar Pluronic coated PSU membranes that were treated with hexane:isopropanol at 50 and ~70°C respectively (the inset in
Figure 4d correlates the surface-peak differences on the hexane-isopropanol treated membranes with respect to colour intensity)

2.3 Interfacial analysis of Pluronic adsorption on planar membranes

The saturation curves for the adsorption of Pluronic onto PSU, PVDF and PEI are shown in Figure 5. The results presented in Figure 5 were analysed in terms of the Langmuir isotherm. The adsorption/desorption data of Pluronic on dense skin planar membranes was fitted to the Langmuir isotherm:

$$Q = Q_{max} KC (1 + KC)^{-1} \quad [1]$$

where Q and Q_{max} are the equilibrium amount and adsorption capacity of Pluronic adsorbed per $1cm^2$ of adsorbent respectively. C is the liquid phase adsorbate concentration at equilibrium and K is the binding constant.

The isotherms obtained followed a Langmuir type profile as characterised by a steep initial slope at low copolymer equilibrium concentration (< 1 mg/mL) and an adsorption plateau was reached above a bulk Pluronic coating concentration of 5 mg/mL. Since the adsorption capacity of the different membrane substrates differ due to their inherent surface chemistries, the equilibrium adsorption concentration also differed for all the membrane matrices under investigation. The critical micelle concentration of the Pluronic used in this study was calculated from surface tension measurements to be 7 mg/mL,[31] so the plateau does deviate from linearity for coating solutions greater than 5 mg/mL Pluronic, due to the formation of micelles in the bulk coating solution.

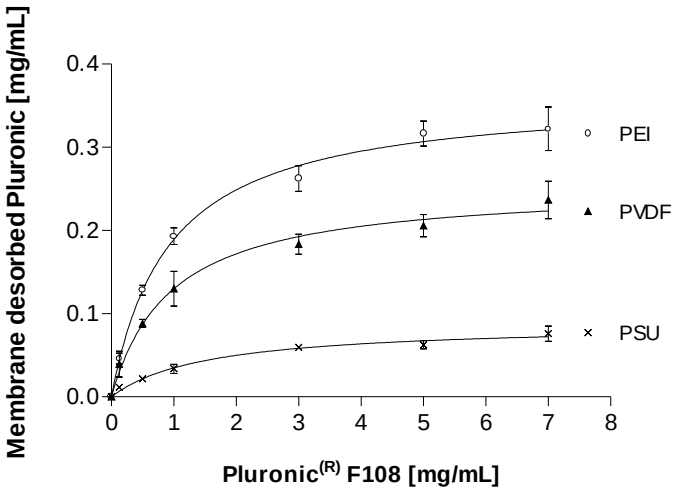


Figure 5: Saturation curves for Pluronic adsorbed on candidate planar membranes

From the saturation curves in Figure 5, the curve for Pluronic adsorption onto PEI lies above the isotherm plots for both those of PVDF and PSU. This suggests that Pluronic adsorbs more strongly to PEI than it does to PVDF and PSU. The slopes of the isotherms for PVDF and PSU appear steeper than that for PEI, which indicates that the adsorptive capacity of PVDF and PSU increases at higher equilibrium solute concentrations.

Contact angle measurements for both native and Pluronic coated flat sheet membranes are shown in Table 3. Static contact angle measurements show that native PEI membranes are relatively hydrophilic and Pluronic adsorption could result in a reversal of the surface properties to that of a relatively hydrophobic membrane. This could be due to the self-

31 C Yanic, MW Bredenkamp, EP Jacobs, P Swart, Bioorganic and Medicinal Chemistry Letters. 13 (2003) 1381.

assembly of the hydrophilic PEO segments as trains in contact with the membrane surface with the PPO loops sticking out. This scenario differs markedly from the hydrophilic brushes formed by PEO chains on the hydrophobic PSU and PVDF membranes, which form a hydrophilic layer that can potentially sterically hinder the adsorption of proteins and cells.[3,5]

Table 3: Effect of Pluronic coating on contact angle

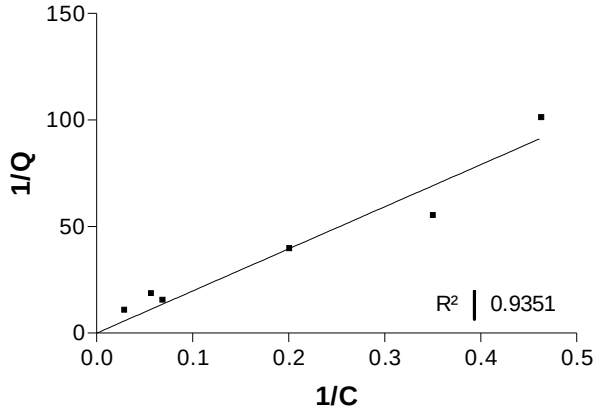
	Virgin membranes			Pluronic-coated membranes		
Polymer type	Contact angle	N	Sdev	Contact angle	N	Sdev
PEI	51.38	6	2.82	61.84	6	1.85
PSU	57.74	6	1.81	40.3	6	1.69
PVDF	66.61	6	2.35	51.34	6	2.84

Langmuir isotherms for planar PSU, PVDF and PEI non-porous membranes are described (Figure 6) and the corresponding adsorption isotherms (Figure 6a and c) obey the Langmuir equation. The isotherms generated on PEI membranes (Figure 6b) showed the greatest deviations from linearity. This could be attributed to the conformational self-assembly of the PEO groups on the surface such that the PPO centre block served as a matrix for multi-layer Pluronic adsorption. This was undesirable from a membrane pre-treatment perspective since the Pluronic adsorption process would be more complex. This also reduces the sterically repulsive forces provided by the highly hydrated PEO layer at high surface coverage that allows the stabilization of colloidal dispersions, which enable resistance to fouling. The linear fit of the Langmuir isotherms for PSU and PVDF suggests that the adsorption of Pluronic onto these membranes is due to monolayer formation since there may be minimal Pluronic-Pluronic interaction.

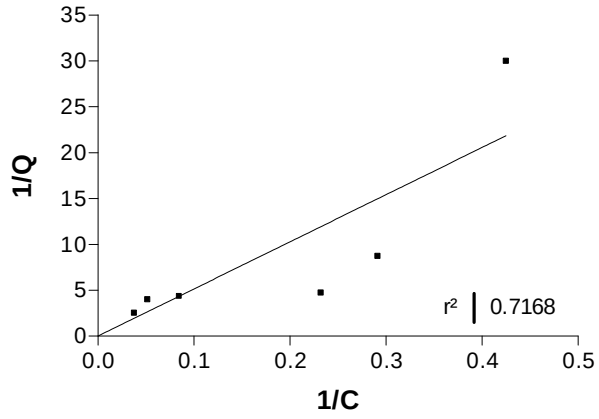
2.4 Influence of interfacial curvature on Pluronic adsorption

Curved capillaries with their defined radii can be easily used for studying adsorption under controlled flow conditions and as models for adsorption on porous substrates. When previous studies[18] were conducted with flat-sheets it was assumed that the radius of curvature was large with respect to the thickness of the adsorbed layer. However according to Fleer et al., 1993,[10] in order to describe the adsorption of polymers on small spherical particles or in pores, or to model the self-assembly of surfactants or block copolymers into spherical or cylindrical micelles, a flat lattice geometry is inadequate. According to theoretical studies by Wijmans et al., 1988,[32] curvature effects become important when the radius of curvature of the substrate is of the same order of magnitude as the adsorbed layer thickness. These authors predicted an increase of the adsorbed amount of di-block copolymer and the hydrodynamic layer thickness with increasing curvature. Baker et al. 1988,[33] measured the layer thickness for various Pluronic surfactants on polystyrene particles and concluded that the specific adsorption was independent of particle size while the adlayer thickness decreased with an increase in particle radius. Considering these and many other conflicting reports on Pluronic adsorption on curved polymeric surfaces an experimental study of Pluronic adsorption on capillary membranes was undertaken.

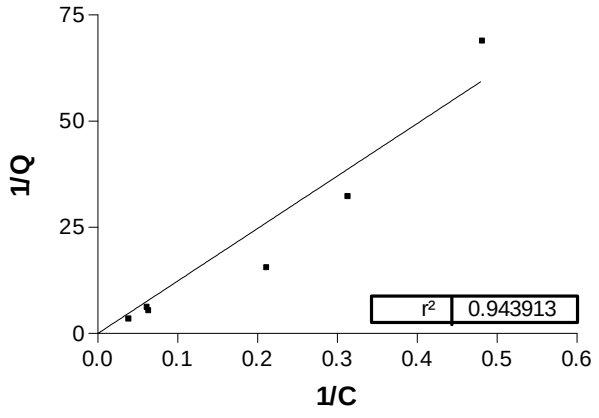
32 CM Wijmans, FAM Leermakers, GJ Fleer, Multiblock Copolymers and Colloidal Stability, Langmuir. 10 (1994) 1331.
33 JA Baker, JC Berg, Influence of particle curvature on polymer adsorption layer thickness, Langmuir. 4 (1988) 1055.



a)



b)



c)

Figure 6: **Typical Langmuir isotherm for a) PSU flat sheet membranes at 25ºC, b) PEI flat-sheet membranes at 25°C and c) PVDF flat sheet membranes at 25°C. The interfacial adsorbed amount of Pluronic (Q) and the liquid phase equilibrium concentration of Pluronic (C) were used to construct the Langmuir isotherms**

Measurements of the adsorbed amount of Pluronic on each of the model membrane matrices are summarised in
Table 4. The results show an increase in Pluronic adsorption (0.055 to 0.14 mg/cm^2) with an increase in membrane radius (0.9 to 1.88 mm). The capillaries with a larger lumen diameter, hence less convex than the HFF showed greater Pluronic adsorption. This correlates with the trend seen with Pluronic adsorbed onto polystyrene lattices of varying thickness.[20] However, reported results in literature appear to be plagued with inconsistencies,[5-9] and this is due to a

combination of the instability of the adsorption matrix used and the constraints imposed by the analytical instrumentation or technique.[5] The reliable hexane:isopropanol protocol and the sensitive biphasic $NH_4FeSCN/CHCl_3$ assay system is well-suited to Pluronic analysis on synthetic polymer membranes which limits the possibility of drawing convoluted conclusions from the interfacial curvature data.

The larger interfacial curvature of HFF membranes causes additional lateral crowding in the adsorbed layer, which might sterically hinder the adsorption of further PPO chains. The capillary membrane has a larger radius of curvature thus limiting lateral crowding and this is reflected in the much greater amounts of Pluronic adsorbed per cm^2 of membrane. On theoretical grounds, it can be expected that an increase in particle size would provide an increase in the adsorbed layer thickness up to the point where the curvature of the surface was essentially the same as a planar surface. The progression from capillary to flat sheet did not show a similar increase in Pluronic adsorption. This was most likely due in large part to the surface physico-chemical properties that arose due to the differing membrane fabrication protocols that are inherent to the manufacture of capillary and planar membranes.

2.5 Summary

The biphasic colorimetric quantification using NH_4FeSCN and $CHCl_3$ was sensitive to Pluronic [3 to 130 μg/mL] and insensitive to dextran, biotin, human plasma and halogenated Pluronic derivatives. Modification of the hydroxyl end groups of Pluronic to Pluronic-iodide showed a similar linearity within the described dilution range.

Table 4: Data for Pluronic desorption from 1 cm^2 curved surfaces

PSU membrane type adsorbed	Replicates	Average amount of Pluronic desorbed (mg/cm^2)	SD
capillary	3	0.14	0.0025
hollow-fine fibre	3	0.055	0.0010

The bisolvent extraction of Pluronic using hexane:isopropanol treatment at ~70˚C, was highly reproducible for a wide range of Pluronic coating concentrations and surface chemistries in addition to being relatively effective as a desorbent based on AFM and electron microscopy analysis. The saturation curves followed Langmuir type adsorption and the corresponding Langmuir isotherms correlated to the current understanding of the adsorption of Pluronic onto hydrophobic surfaces such as the candidate membranes.

The specific spectrophotometric detection of Pluronic in the presence of some common bio-additives such as human plasma, serum albumin and dextran, suggest that this assay system could also be used to quantify Pluronic and poly(ethylene oxide) based surfactants in biomaterial studies and drug delivery devices. The methods described in this study for the extraction and detection of Pluronic were also successfully used for studying interfacial curvature and the results were in good agreement with many other reports concerning surfactant adsorption onto curved polymer surfaces.

3. Membrane regeneration

3.1 Introduction

An interface that is formed between two different bulk phases usually has a higher standard free energy than the bulk phase.[34] As a result the interface is apt to be thermodynamically stabilised by adsorbing any substances that are different from the solvent molecules. The substances are more or less varied in their structures upon adsorption on the surface and sometimes change their functions. In particular, adsorption of proteins on a solid surface is a generally observed phenomenon in various fields and the changes in their structure, function and adsorbed amounts can have an important consequence on a bio-processes.[35]

There has been much work reported on minimizing non-specific protein adsorption, which is important in bio-process applications involving chromatographic supports, blood contacting devices and membrane filtration. One of the most effective polymers for protein resistant surfaces is PEO.[36] The protein resistant character of PEO is probably caused by a steric stabilization effect. Since recent, Pluronic copolymers have been used increasingly as coating materials in pharmaceutical, biomedical and filtration applications.[37,38,39]

In protein-surface interactions, the governing factors are determined by both the physical state of the membrane matrix and protein surface and the solution environment. Factors include bound ions, surface charge, charge roughness, surface elemental composition and surface energetics. In an effort to produce a robust membrane based affinity separation system that can compete with commercial columns, the membrane matrix must be capable of being efficiently regenerated for reliable re-use. As with any chromatographic bioprocess, there will be an accumulation of proteins and other bio-molecules at the interface. Membranes are no exception since all proteins will eventually adsorb on surfaces whether it be hydrophilic or hydrophobic.[40]

The interactions of polymers and surfactants, mainly in oppositely charged polymer-surfactant systems have received much attention.[41] This study focuses on the interaction of Pluronic (a non-ionic surfactant) and sodium dodecyl sulphate (SDS) (anionic surfactant) since they are much studied and from a process application point of view they are amongst the few thermo-viscofying materials approved by FDA and EPA as direct and indirect food additives, pharmaceutical ingredients and agricultural products (BASF, 1993). It has been reported that the phase behaviour and microstructure of Pluronic block copolymers are affected by SDS micelles (Li et al., 2001). The ability of Pluronic to reduce adsorption of

34 K Nakashini, T Sakiyama, K Imamura, Review: On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon, J. Biosci. Bioeng. 91(3) (2001) 233-244.

35 J-T Li, J Carlsson, S-C Huang, KD Caldwell, Adsorption of poly(ethylene oxide)-containing block copolymers, Advances in Chemistry Series. 248 (1996) 61-78.

36 NV Efremova, SR Sheth, DE Leckband, Protein induced changes in poly(ethylene glycol) brushes, Langmuir. 17 (2001) 7628-7636.

37 A Higuchi, K Sugiyama, T Shirai, Serum protein adsorption snd platelet adhesion on Pluronic adsorbed polysulfone membranes, Biomaterials, 24 (2003) 3235-3245.

38 M Malmsten, JM Van Alstine, Adsorption of poly(ethylene glycol) amphiphiles to form coatings which inhibit protein adsorption, J. Coll. Int. Sci. 177 (1996) 502-512.

39 RJ Green, S Tasker, SJB Tendler, Adsorption of PEO-PPO-PEO triblock copolymers at the solid/liquid interface, Langmuir, 13 (1997) 6510-6515.

40 JJ Ramsden, Puzzles and paradoxes in protein adsorption, Chemical Society Reviews, 73-78 (1995).

41 T Cosgrove, SJ Mears, T Obey, L Thompson, RD Wesley, Polymer, particle, surfactant interactions. Coll. Surf. A. 149 (1999) 329-338.

high levels of protein from solution and the ability of SDS to desorb both bound Pluronic and proteins from PSU, PEI and PVDF membranes will be discussed.

At surfaces, additional interactions between the adsorbed molecules and the surface come into play. These interactions are both hydrophobic and electrostatic in nature and the interactions between the protein, surfactant and copolymer, are expected to be altered by the presence of the surface. Furthermore, the relation between the properties of the complex will determine whether the surfactant will be able to desorb the protein.[42]

In this study we report on a well-established procedure for Pluronic pre-treatment of membranes to inhibit non-specific protein adsorption and attempts to regenerate said membranes with SDS. The results obtained in this study will contribute to the understanding of SDS displacement of Pluronic and a method is described to separate SDS desorbed Pluronic for colorimetric quantification using a biphasic assay.

3.2 Results and discussion

Figure 7 shows the typical Pluronic displacement trends observed with hexane:isopropanol and SDS respectively. Of importance it was noted that SDS was efficiently separated from the Pluronic prior to biphasic colorimetric analysis, where SDS was found to interfere with the assay.

In general SDS was not as effective as high temperature bi-solvent extraction of Pluronic, but SDS treatment was initially far from optimised with respect to incubation time, shaking and temperature. SDS did however show potential for removing organic protein foulants from Pluronic coated membranes (
Table 5), while recent work has also shown that the protein repellent properties of the membrane were still preserved (results not shown).

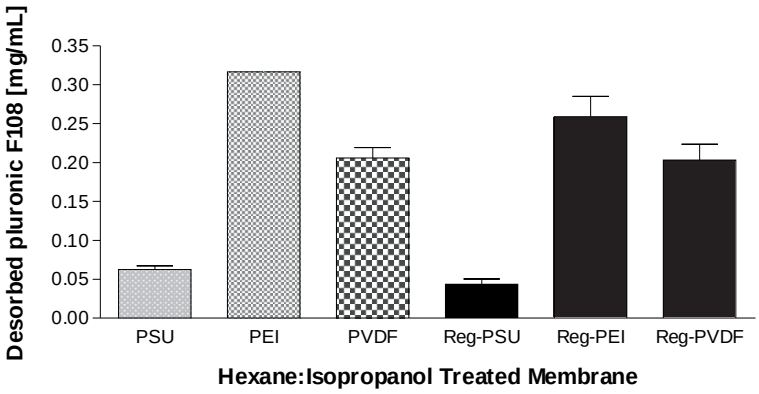


Figure 7: Typical hexane-isopropanol desorption of Pluronic from native flat-sheet membranes and membranes that were regenerated with 5mM SDS

Pluronic treated membranes were useful in reducing the amount of protein adsorbing onto the membrane from a controlled solution. Typically, the higher the bulk concentration of the protein the greater the adsorbed amount at the membrane surface. Since PVDF is more hydrophobic than PSU, it adsorbed Pluronic more efficiently and was more effective in

42 JC Froberg, E Blomberg, PM Claesson, Description of lysozyme layers by SDS studies with the surface force technique, Langmuir, 15(1999)1410-1417.

preventing both BSA and Lysozyme adsorption from solution. PEI is fairly hydrophilic and adsorbs Pluronic just as effectively as PVDF. Pluronic treated PEI membranes were, however, not very effective in reducing protein adsorption. This was most likely due to PEO blocks of Pluronic self-assembling onto the PEI surface such that the centre PPO block was exposed to the liquid phase. This reduced the steric hindrance offered by PEO and encouraged hydrophobic interaction with both proteins.

The interactions between proteins and surfactants are generally described as arising from both electrostatic and hydrophobic interactions.[42] Between proteins such as lysozyme (+) and BSA (-) or polyelectrolytes and an oppositely charged surfactant, the attractive interaction is in general very strong, and the initial binding is more site specific than in the case of inert (uncharged) polymers such as Pluronic and the candidate native membranes used in this study.

From the data in
Table 5, it is evident that Pluronic treated membranes offer close to 100% resistance to lysozyme adsorption and almost 80% resistance to BSA adsorption. SDS stripping of the Pluronic treated membranes suggests that the depletion method is an accurate indicator of protein adsorption onto flat sheet membranes and that water-soluble SDS has the potential to be a useful reagent for the regeneration of the dense skinned membranes used in this study.

Table 5: Lysozyme and Bovine serum albumin adsorption onto flat-sheet membranes based on the depletion method to estimate protein adsorption

System	PSU (µg/mL)	SDev	PEI (µg/mL)	SDev	PVDF (µg/mL)	SDev
Virgin + LYS	109.46	5.57	121.46	4.433	113.57	6.705
Pluronic + LYS	6.800	7.183	5.761	2.040	1.865	0.329
SDS stripping of LYS from Pluronic-coated membranes	1.083	1.003	6.063	1.246	0.512	0.887
Virgin + BSA	67.87	11.894	66.44	0.017	54.152	17.086
Pluronic + BSA	57.50	1.750	65.14	1.921	51.78	5.818
SDS stripping of BSA from Pluronic coated membranes	11.67	1.470	11.96	1.063	11.142	2.200

LYS: lysozyme;
SDS: sodium dodecyl sulphate
BSA: bovine serum albumin
Stripping experiments performed in triplicate

At low SDS concentrations, below critical micelle concentration (cmc), the binding takes place via electrostatic interactions between the charged head group of the surfactant and the oppositely charged residues in the protein molecule. As a result of this initial binding, the protein-surfactant complex becomes less charged and more hydrophobic than the protein itself, which may lead to aggregation and precipitation. At higher surfactant concentrations (above cmc), binding most likely occurs via hydrophobic interactions, with the SDS headgroups pointing out from the protein surface.[43] At this stage the hydrophobicity of the protein-surfactant complex decreases,[42] and it becomes more hydrophilic and redissolves, eventually acquiring a negative charge like the SDS molecule.

43 MN Jones, P Manley, PJW Midgeley, Adsorption maxima in protein surface solution, J. Colloid and Interface Science. 82(1)(1981)257–259.

Figure 8 indicates that 5 mM SDS was more effective in displacing Pluronic from the membrane surface than at concentrations at or above the cmc. Results were comparable with trends observed with hexane:isopropanol extraction. These results (Figure 8) were similar to findings by Cosgrove et al.[41] where the same trend was observed with SDS and poly(ethylene glycol) (PEG) adsorbed on polystyrene beads. In said paper, it was found that the PEO acts as a nucleation centre for the formation of micelles at SDS concentrations above the cmc of the surfactant. These authors concluded that the driving force for SDS displacement of PEG from solid supports was due to polymer-surfactant interactions and not competitive adsorption for sites. It can be argued that membrane Pluronic would behave differently than the very hydrophilic PEG with the SDS interaction forming a solution complex with the PPO centre block of Pluronic at high SDS concentrations.

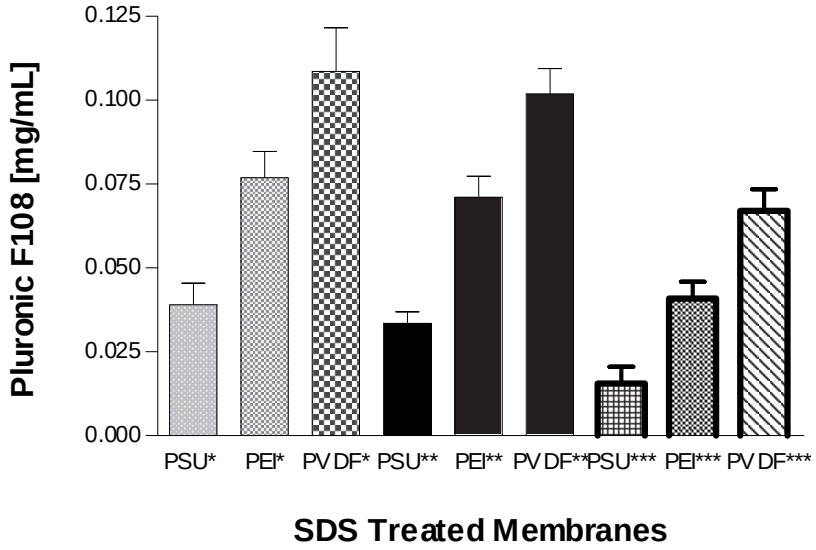


Figure 8: Influence of SDS concentration on Pluronic desorption. (* 5 mM, ** 8 mM and * 34 mM). Desorption at 25°C for 2h with gentle shaking**

A contradiction to the observed displacement of the preadsorbed Pluronic layer on all membrane types, was noted in a study by Ma and Li, 1993.[44] These authors showed a reverse trend, where SDS above its cmc was more effective in displacing a preadsorbed layer of PVP while the layer thickened at low SDS concentrations below its cmc. It is believed that increasing the SDS concentration close to and above the cmc, results in micellisation of SDS and the micelle association number decreases until pure SDS micelles and individual Pluronic macromolecules saturated with SDS are present.[45]

Stigter,[46] showed that for different head-groups on the same alkyl chain, it is usually found that the binding again follows the micellization, where the higher the tendency to form micelles the stronger the interaction with proteins. This could explain the reason for greater Pluronic displacement of proteins at 34mM, which was higher than the cmc of SDS (8 mM). Further work could involve optimising this SDS stripping strategy by increasing the SDS concentrations above 34 mM to remove organic foulants, washing the membranes and then treating it with a more dilute SDS solution to displace Pluronic.

44 C Ma, J Li, Interaction between PVDP and SDS at solid/liquid interfaces, J. Coll. Int. Sci. 131 (1989) 485-492.
45 R Ivanova, P Alexandridis, B Lindman, Interaction of poloxamer block copolymers with cosolvents and surfactants, Coll. Surf. A. 183-185 (2001) 41-53.
46 D Stigter, Micelle formation by ionic surfactants. J. Coll. Int. Sci., 47(2) (1974) 473-482.

Figure 9 shows the time dependent nature of SDS displacement of Pluronic. Initially experiments were performed under empirical conditions with 2h incubation at room temperature. Much more Pluronic was found to be displaced after a 20 to 40h incubation period, but the desorbed amount from all 3 candidate membranes was still not equivalent to the amount of Pluronic that can be desorbed by hexane-isopropanol extraction at temperatures above the melting point of Pluronic. Results have shown that this bi-solvent extraction protocol is temperature dependent,[47] such that incomplete desorption is observed at temperatures below 40°C. Much work is still required to optimise this promising approach of SDS displacement of both protein foulants and Pluronic using SDS. Future work could involve using varying SDS concentrations at different temperatures with a range of incubation times with the membrane under shear stress.

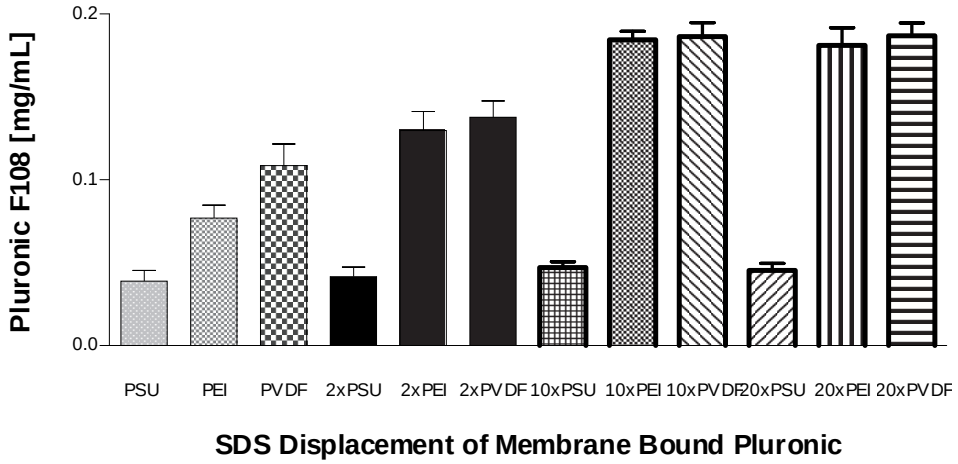


Figure 9: **Time dependent stripping of membranes using 1.0% (w/v) SDS. Initial experiments involved 2h incubations with SDS and this incubation period was increases by 2x, 10x and 20x to 4, 20 and 40h periods respectively**

47 S Govender, EP Jacobs, MW Bredenkamp, P Swart, A robust approach to the study of Pluronic F108 adsorbed onto non-porous membranes, J. Colloid. Interface Science. *In press*.

4. Modification of Pluronic termini

Non-covalent bonding onto the surface of membranes is the primary contribution of this technology, allowing for adsorbent preparation away from the membrane as well as membrane regeneration by removal and replacement of the absorbent. The focus of this section is the harnessing of the absorbent termini for attachment of ligands or ligates, enabling the securing of a variety of species for diverse membrane-based separation applications.

4.1 Introduction

The terminal polymer blocks of Pluronic are PEG (polyethylene glycol) with an alcohol group as terminal functional group. This presents a handle for the attachment of the required functional groups or modification to another functional group that may be required for ligand/ligate attachment. The rest of the polymer is inert to most reactions because it is comprised of ether groups which are stable.

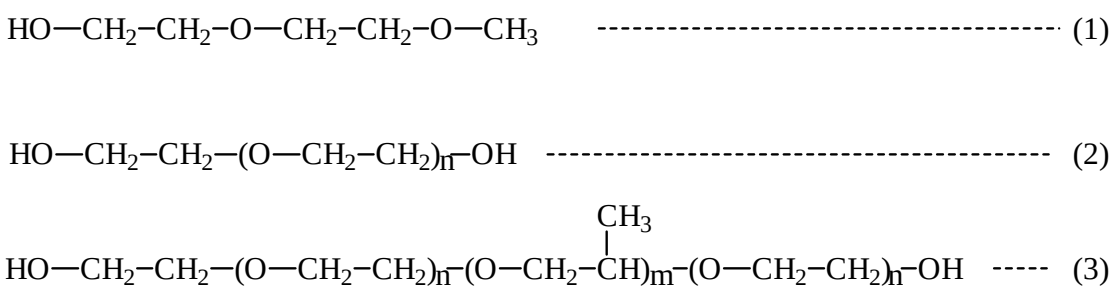
Modification of the alcohol group to a primary amine was the first process envisaged (Section 4.2.1). Amines are important for the attachment of biological ligands and ligates. Initially amine termini were also considered for EDTA-based tetradentate ligands for IMAC. A more direct route was later developed where EDTA was attached to unmodified Pluronic.

Pluronic is a large molecule, posing difficulties in its chemistry, work-up, purification and characterisation. For this reason use was made of model compounds to develop the technology in all its aspects and gain an understanding of the chemical and physical characteristics of the termini of Pluronic. Two stages of compound modelling was made use of in most of the development, *viz* diethylene glycol monomethyl ether (1) (DEG) and polyethylene glycol (2) (PEG) (M_n = 600). DEG represents the first two nodes of Pluronic (3) but has the advantage of 5-carbon simplicity and lends itself to standard purification techniques as practiced in synthetic organic chemistry. PEG bridged the gap between the simplicity of DEG and the tri-block-polymer complexity of Pluronic, allowing application of the technology on a simple and homogeneous polymer. The work-up procedures and challenges of polymer modification were encountered and developed for application to the more complex Pluronic. An example of how the model compounds were used to develop the technology may be seen in the use of NMR to characterise the modified Pluronic and all the intermediates. A paper was published in this regard.[48]

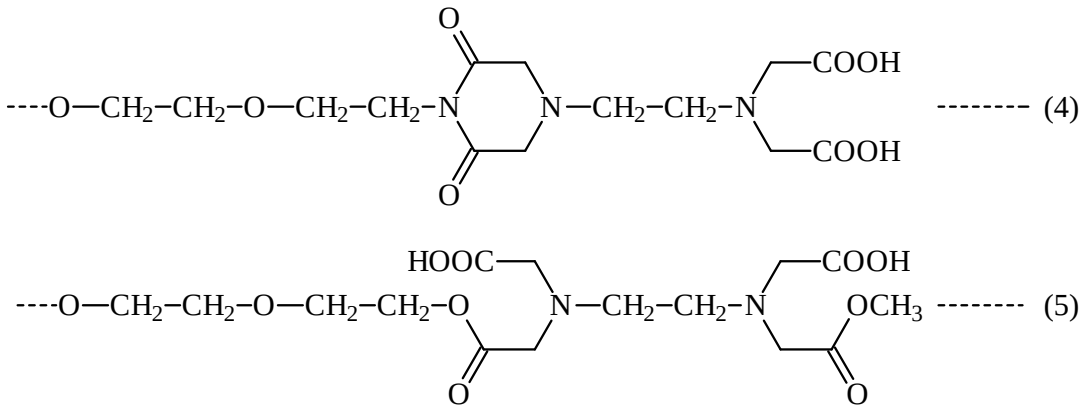
The complexity of the three-step process of converting the hydroxy termini of Pluronic to amines, developed in this project, led to a search for alternatives. A simpler, two-step process for the incorporation of hydrazide termini was found in the literature,[49] an alternative functional group that presents properties sufficiently equivalent to an amine to use in the required biochemical technology.

48 C Yaniç, MW Bredenkamp, EP Jacobs, HSC Spies, P Swart, NMR Spectroscopy – A basis for the characterisation of Pluronic®F108 and its derivatives, Journal of Applied Polymer Science, 2000, 78, 109-117.

49 J Li, J Carlsson, J Lin, KD Caldwell, Bioconjugate Chem., 7, 1996, 592-599.



The difficulties experienced in attaching EDTA to an amine to form an imide (4), led to the conceptualization of a different type of tetradentate EDTA derivative (5), which was compatible for attachment to unmodified Pluronic, simplifying the whole procedure (Section 4.3).



4.2 Functional group modification

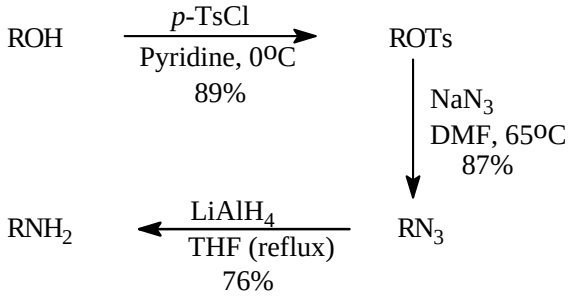
The most important functional group modification of Pluronic was the conversion of the hydroxy termini to the primary amine, and amine-like functionality (Section 4.2.1). This was necessary for the attachment of species that have a functional group that is designed to attach to amines and not able to attach to hydroxy groups. This includes Cibacron Blue that was used in the initial investigation of affinity separation concept by Yaniç[50,51] to serve as ligate to bind bovine serum albumin in an affinity separation process. It was also required for the attachment of biotin which serves as ligate for avidin and streptavidin (Section 4.2.2). Furthermore it was considered for immobilising EDTA as a tetradentate ligand for IMAC. The hydrazide terminated Pluronic was easier to produce and as effective in application.

Other functional groups that were incorporated as termini for Pluronic were ions (Section 4.2.3) for a more hydrophilic coating on the membrane. Quaternary ammonium salts were incorporated for cations. Efforts to produce sulphonic acid termini to perform the role of anionic termini was successfully performed on the model compounds but failed on Pluronic.

50 C Yaniç Polymer end-group modification, PhD thesis, University of Stellenbosch, 1999.
51 C Yanic, MW Bredenkamp, EP Jacobs, P Swart, Modification of Ethylene Glycol Polymers. *Bioorganic and Medicinal Chemistry Letters*, 2003, 13, 1381-1384.

4.2.1 Amine terminated Pluronic

There are no high-yielding direct routes for the conversion of primary alcohols to primary amines. Primary alcohols are very stable and need to be activated as leaving groups before they can be successfully replaced by other functional groups. On the other hand, ammonia or its amide anion, that are the necessary reagents for direct incorporation of a primary amine, are too reactive for high-yielding processes because of their strong basic character that competes in elimination processes yielding alkenes. It was therefore necessary to incorporate an amine precursor that is strongly nucleophilic and not basic. The conversion process was thus a three-step process *viz.* i) activation of the alcohol as a leaving group, ii) substitution of the activated oxygen atom with a nucleophilic nitrogen-containing group and iii) conversion of that group into a primary amine. The alcohol group was activated by tosylation and substituted by azide (Scheme 1). The azide-terminated Pluronic was then reduced with lithium aluminiumhydride to yield the desired amine terminated Pluronic.



DEG R = $CH_3[O(CH_2)_2]_2$—
PEG R = —$[(CH_2)_2O]_n(CH_2)_2$—
Pluronic R = —$[(CH_2)_2O]_n[CH_2CH(CH_3)O]_m[(CH_2)_2O]_n(CH_2)_2$—

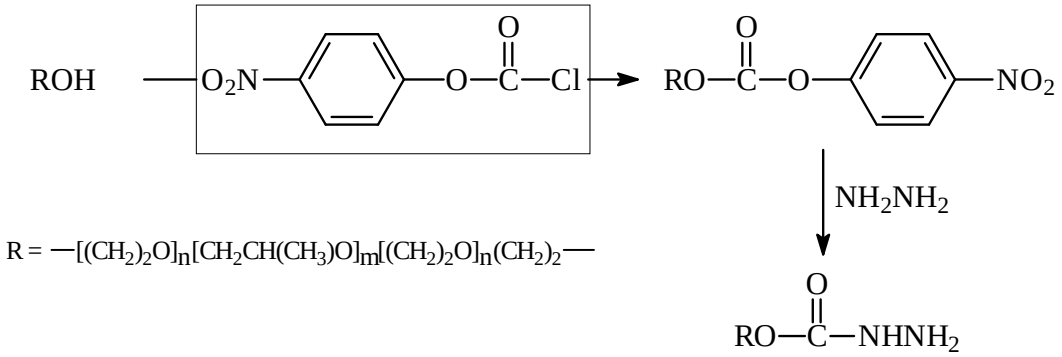
Scheme 1: Chemical procedure for the conversion of the termini of the model compounds and Pluronic to the primary amine. The yields indicated are with respect to Pluronic

Problems with this synthesis was i) the time some of these reactions took and ii) difficulties with the reaction work-up. In particular, the tosylation reaction took 7 d, and the work-up of the reduction of the Pluronic azide resulted in emulsions that required all sorts of procedures including continuous extraction. If this process were to be scaled up it would pose enormous challenges. For that reason it became necessary to find an alternate route to synthesize amine terminated Pluronic.

The work by Caldwell[52] offered a rapid, two steps in one pot, high yielding and efficient alternative. It is the preparation of a hydrazide terminated polymer that chemically presents itself very similar to a terminal amine (Scheme 2). The hydroxy terminus is augmented by reaction with *p*-nitrophenoxycarbonyl chloride, yielding a carbonyl which is very electrophilic because of the nitrophenoxy group which is a good leaving group. Reaction with hydrazine substitutes the nitrophenoxy group, forming a carbamate. The nitrogen of carbamates is not a good nucleophile and fails to substitute a terminal amine. Hydrazine, however, has a second nitrogen group that is not involved with the conjugating carbonyl group of the carbamate and is a good replacement for the amine group of amino Pluronic.

52 J Li, J Carlsson, J Lin, KD Caldwell, Bioconjugate Chem., 7 (1996) 592-599.

Work-up after the one-pot process essentially involved precipitating the product out of solution with ether. This derivative of Pluronic was used in most of the processes that required an amino end-group.



Scheme 2: The two-step procedure for the preparation of Pluronic hydrazide, a useful alternative to amino Pluronic

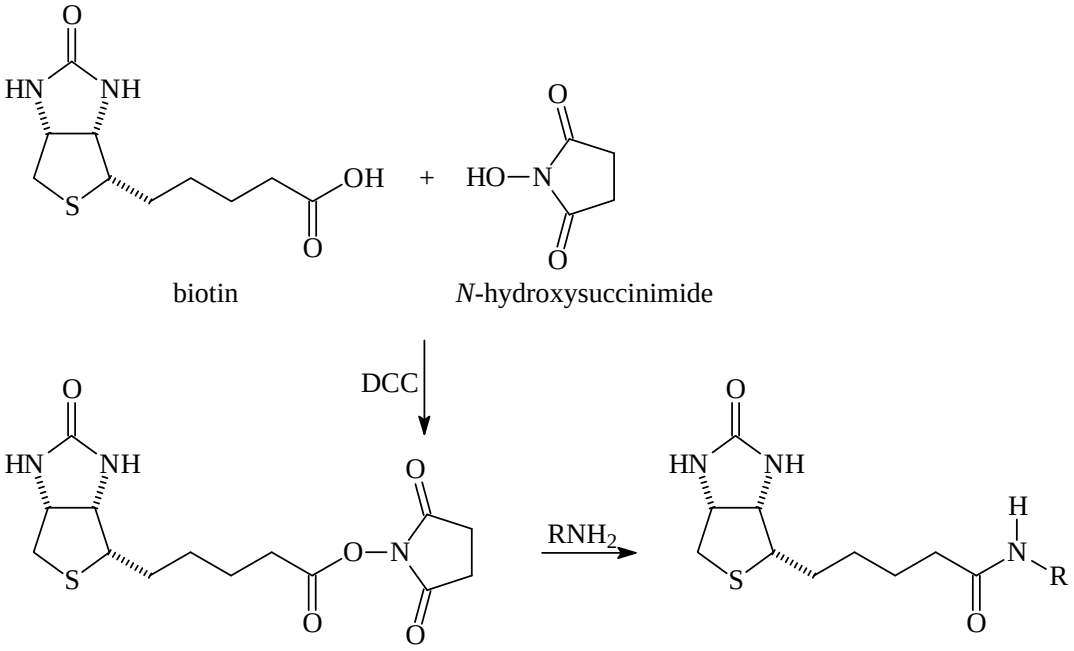
4.2.2 Biotinylated Pluronic

Biotin is a ligate that bonds strongly and selectively to the protein avidin as ligand. This is the strongest non-covalent chemical coupling known. For this reason it is exploited in many biochemical processes. It is often used in the immobilisation of immuno-globulins with all the applications associated with this process. Biotin itself is immobilised by binding its carboxylic acid group to a tethered primary amine. One of the reasons amine terminated Pluronic was prepared was for the immobilisation of biotin.

Even though biotinylation technology is well established, it was necessary for us to familiarise ourselves with it in our laboratory, especially with respect to the characterisation of the coupling process to Pluronic with NMR, developed in our laboratory. Model compound DEG was therefore used. The coupling process (Scheme 3) entailed the activation of biotin by esterification to *N*-hydroxysuccinimide (NHS). Exposure of a DMF solution of NHS-biotin to a primary amine, had the amine displace the NHS group, yielding the desired biotin conjugate. Both amino Pluronic as well as Pluronic azide was used with success.

4.2.3 Ion terminated Pluronic

The incorporation of ions on the termini of Pluronic could serve several purposes. It would make the terminal PEG blocks more hydrophilic. Membrane foulants are generally anionic by nature, therefore an anionic terminus would repel those foulants. A water purification membrane preferentially “fouled” with an anionic terminated Pluronic would thus have prolonged cycles before the need for membrane cleaning. Ion exchange could also be performed on membranes coated with ion terminated Pluronic.

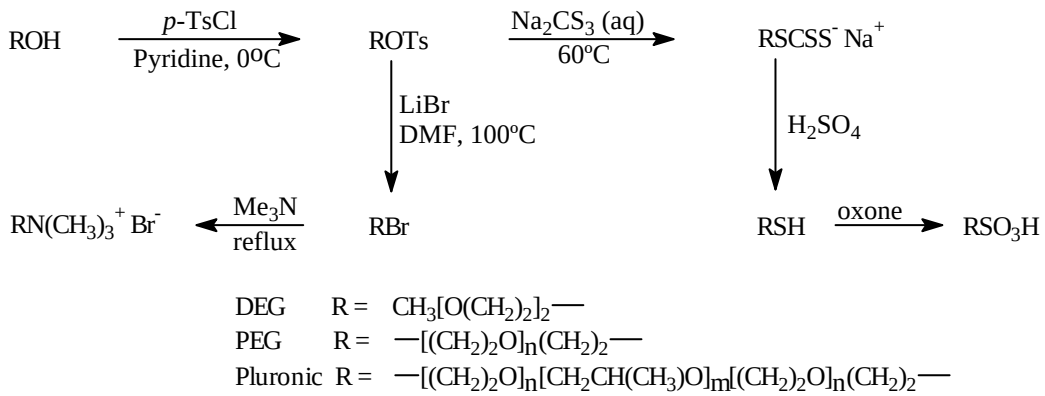


Scheme 3: Coupling procedure for attaching biotin to two forms of amine terminated Pluronic and DEG as model compound

For a cation terminated Pluronic, a quaternary ammonium salt was envisaged. The simplest route would be to use trimethylamine to nucleophilically displace a good leaving group. The Pluronic tosylate, already used for the preparation of amino Pluronic (Section 4.2.1) proved to not be a sufficiently good leaving group for displacement by trimethyl amine. It therefore became necessary to displace the tosyl group by a halogen, which in its turn was a good leaving group for displacement by trimethylamine. As was the case with amino Pluronic, this technology was developed on DEG and PEG before application to Pluronic (Scheme 4).

Sulphonic acid terminated Pluronic was envisaged for anionic terminated Pluronic. The process developed on the model compounds was (Scheme 4) displacing the oxygen group of the Pluronic by treating Pluronic tosylate with sodium trithiocarbonate. The alkyl trithiocarbonate eliminated carbon disulphide on acidification and the resultant mercaptan was oxidised by oxone furnishing the desired sulphone. With Pluronic every step but the last could be done. Careful analysis of the final product indicated that over oxidation cleaved the carbon-sulphur bond, yielding Pluronic again with an alcohol terminus.

The problem in producing the anionic terminated Pluronic should be overcome by using less oxone in the final step. Alternatively use of propane sultone directly on the alkoxide of Pluronic should yield a sulphonate terminated Pluronic where the last node of the polyether will be three carbons long, terminating in the sulphonate. This section on ion terminated Pluronic was included in the articles on the synthesis and NMR of amino Pluronic.



Scheme 4: Synthetic route for the preparation of cationic and anionic terminated Pluronic and the model compounds

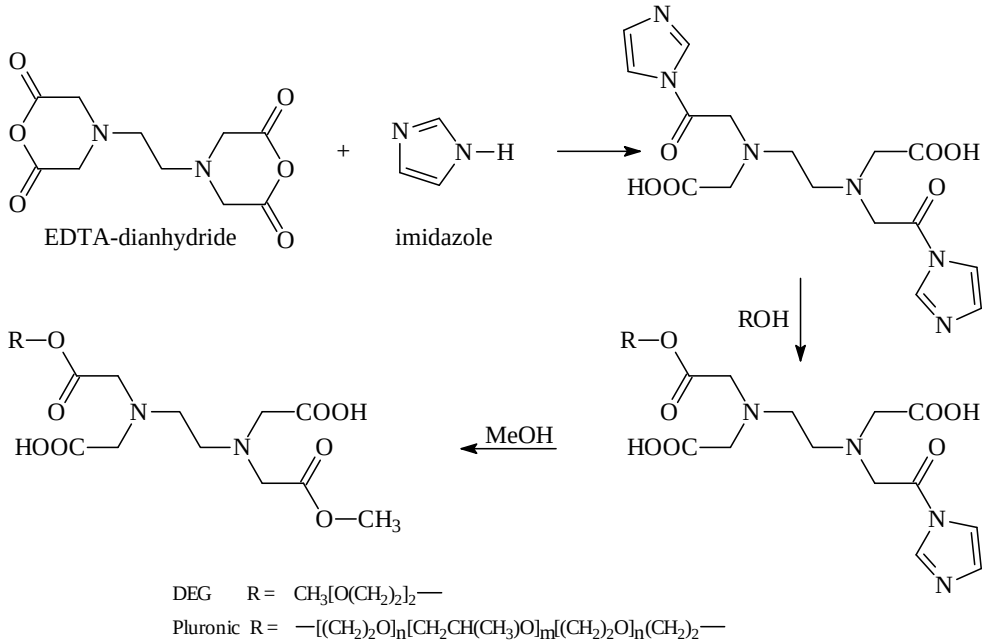
4.3 Direct chemical attachment

The possibility of direct chemical attachment is always considered first because every step in a synthesis is cumbersome and leads to loss of compound, even if the steps are high yielding. The problems are multiplied when working with polymers because they are large molecules that are not soluble in a variety of solvents. The unreacted precursors and by-products are difficult to get rid of, often leading to products that are comprised of a mixture of functionality. Attachment of EDTA (ethylenediaminetetraacetic acid) to Pluronic for IMAC (immobilised metal affinity chromatography) is an example of direct chemical attachment.

To perform IMAC successfully an immobilised ligand is required that uses enough positions in the metals co-ordination sphere to secure the metal, but leaves open sufficient positions to allow for the binding ligand of the substrate to be isolated or secured to bind to the metal, complementing its co-ordination sphere. Ligands usually used are nitrilotriacetic acid (NTA) and tris(carboxymethyl)ethylenediamine (TED). A cheaper equivalent was sought because for water purification large volumes of these compounds are required. EDTA is a common and relatively inexpensive compound designed to very efficiently complex a large variety of metal cations. It is hexadentate and fills the co-ordination sphere of any metal in the octahedral co-ordination system. It was envisaged to use one of the acid groups to bind to Pluronic by esterification removing it from co-ordination and also another by simply esterifying with a short alcohol. This would render a tetradentate ligand, leaving two co-ordination sites on the octahedral system open for the ligand to attach. It would be advantages if these two open sites were adjacent and not antipodal.

The synthetic technology was developed using DEG as model. Methanol was used to form the second ester. The reactive precursor derivative of EDTA used was EDTA dianhydride. This allowed for the formation of two esters. The successful process (Scheme 5) entailed dissolution in DMF of 10 molar equivalents of EDTA-dianhydride and 20 molar equivalents of imidazole to activate and solubilize the EDTA-dianhydride. The Pluronic dissolved in DMF was added slowly and the mixture left overnight at 40°C. Methanol was added and the mixture refluxed for 8 h. The solvents were removed and the excess EDTA-anhydride that

had reacted with two moles of methanol was precipitated by dissolving the residue in toluene.[53]



Scheme 5: Synthesis of IMAC ligand tethered to Pluronic and DEG as model compound

53 L van Kralingen, Ligand modification of Pluronic F108 for use in immobilized metal affinity separation of bio-molecules MSc thesis, University of Stellenbosch, (2002).

5. Immobilisation and detection studies

The first question that comes to mind is whether the ligand-carrier approach is amenable to affinity separation. Earlier work on serum albumin separation by means of immobilised Cibacron Blue was the first evidence that it was possible to effect affinity separation in a system where the ligand was covalently bound to the Pluronic carrier that was in turn immobilised on a support matrix through adsorption. However, that work did not provide insight into the robustness and broad applicability of the system under study then. So, further evidence and verification of the technology under development was necessary, making use of different ligands and ligates, to prove the original hypothesis on which the Cibacron Blue and serum albumin affinity system was based, beyond doubt.

5.1 Biotin-avidin affinity separation

Biotin is a water-soluble vitamin and avidin is a protein that can be found in the egg white of avian eggs and invertebrate egg jelly. What they have in common is their extraordinary strength of interaction. The binding of avidin and biotin is characterized by a dissociation constant of 10^{-15}M.[54] This high binding constant makes the avidin-biotin interaction essentially irreversible. This interaction forms the basis of avidin-biotin technology.

5.1.1 Biotin

Biotin, the water-soluble vitamin, has a role in enzymatic carboxylation reactions, fatty acid synthesis and carbohydrate metabolism. It has a double ring structure of an imidazole ring cis-fused to a tetrahydrothiopene ring substituted at position 2 by a valeric acid (Figure 10).

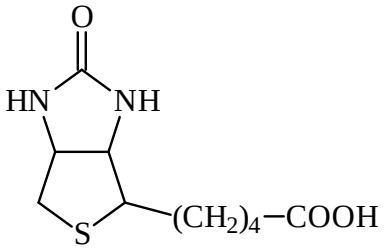


Figure 10: The double ring structure of biotin with valeric acid substituted at position 2.[54]

Biotin and its derivatives can couple to proteins and other compounds. This is a feature of its biological function. Biotin acts as a mobile carboxyl group carrier in biotin-dependent carboxylation reactions. It is covalently linked by its valeric side chain to a ε-amino group of a lysine residue of the carboxylase enzyme. This forms the coenzyme biocytin (Figure 11). The biotin ring system is tethered to the protein leaving it available to perform its function during the enzymatic reaction. By this mechanism proteins can be labelled with a biotin moiety without affecting their properties much.

The valeric acid side chain can be derivatized to different functional groups that will increase the types of compounds that can be labelled with a biotin moiety. Examples are the amines of proteins, aldehydes of carbohydrates, polysaccharides and nucleic acids.[54]

54 RH Garrett, CM Grisham, Metabolism – An overview, 2nd, 18 (1999)600-601.

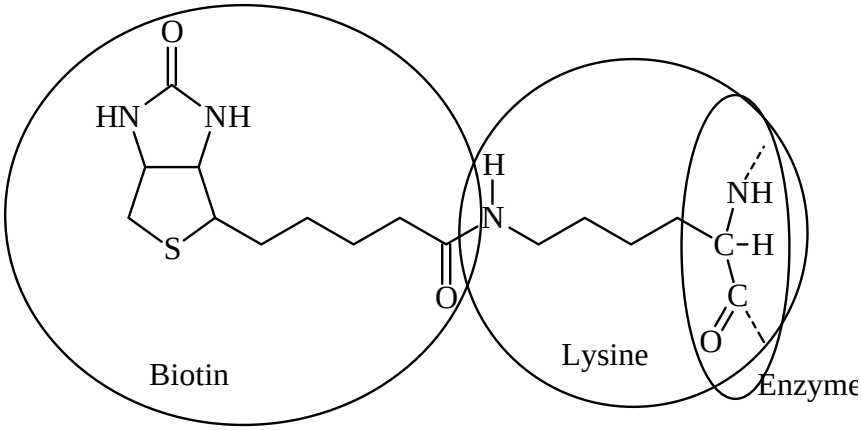


Figure 11: Biocytin is a biotin moiety covalently linked to a carboxylase enzyme via the primary amino group of a lysine and the valeric acid side chain of biotin.[54]

Biotin N-hydroxysuccinimide ester (Figure 12) is a biotin derivative that is commonly used to biotinylate amine groups of proteins via their lysine residues. Lysine residues are numerous in most proteins and characteristically occupy exposed positions on the proteins.[55] In this project, Pluronic hydroxyl termini were derivatized to amine hydrazine groups, which could be biotinylated.[56]

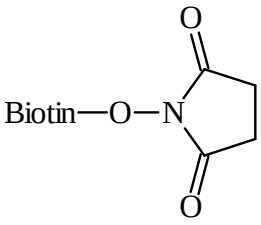


Figure 12: N-hydroxysuccinimide ester of biotin is commonly used to biotinylate proteins via their amine groups.[57]

Avidin is a basic glycoprotein that is found in egg white of birds and egg jelly of invertebrates. It inhibits bacterial growth in egg white by scavenging biotin from the tissue. Streptavidin is a close relative of avidin that is produced by the bacterium *Streptomyces avidinii*. Its structure is remarkably similar to avidin, but it is not a glycoprotein and it does not have a basic isoelectric point. Its properties do not differ much from that of avidin.[58] Streptavidin is less expensive and easier to isolate than avidin making it more widely used.
Avidin is a tetramer with 2-fold symmetry with the binding sites arranged in two pairs on opposite faces of the molecule (Figure 13:). This means that four biotin molecules can bind to each avidin molecule.[59]

Avidin has a high affinity for biotin by binding to the double-ring structure of biotin. With the dissociation constant of 10^{-15}M, the non-covalent association between biotin and avidin is essentially irreversible. This high affinity means that avidin can still bind biotin even though biotin may be coupled to a protein or other macromolecule. Cross-linking between different biotinylated molecules is possible using avidin as a bridging molecule.

55 EA Bayer, E Wilchek, Protein Biotinylation. Methods in Enzymol, 184 (1990) 138-162.
56 E Wilchek, EA Bayer, Introduction to avidin-biotin technology, Methods in Enxymol. 184(1990)5-11.
57 LE Liebenberg, Non-covalent immobilisation of a ligand system: A new approach to affinity separation, MSc thesis, University of Stellenbosch (2002)
58 EA Bayer, H Ben-Hur, E Wilchek, Isolation and Properties of Streptavidin. Methods in Enzymol., 184 (1990) 80-89.
59 MN Green, Avidin and Streptavidin. Methods in Enzymol., 184 (1990) 51-67.

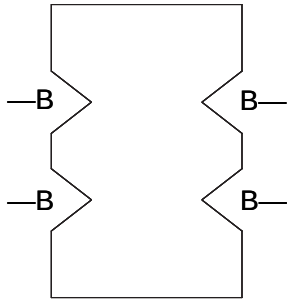


Figure 13: Avidin is a tetramer with a 2-fold symmetry which has four binding sites for biotin.[6]

5.1.2 Avidin-biotin cross-linking

Avidin-biotin interactions establish a cross linking system to link a biotinylated protein, or other macromolecule, to a synthetic membrane via biotinylated amino or hydrazine Pluronic as described earlier. Avidin forms the bridge between the biotinylated Pluronic and a biotinylated macromolecule. To prove that this concept works a reporter enzyme, horseradish peroxidase, conjugated to streptavidin, was used to detect biotinylated amino Pluronic on two hydrophobic surfaces, namely polystyrene and polysulphone membranes (Figure 14).

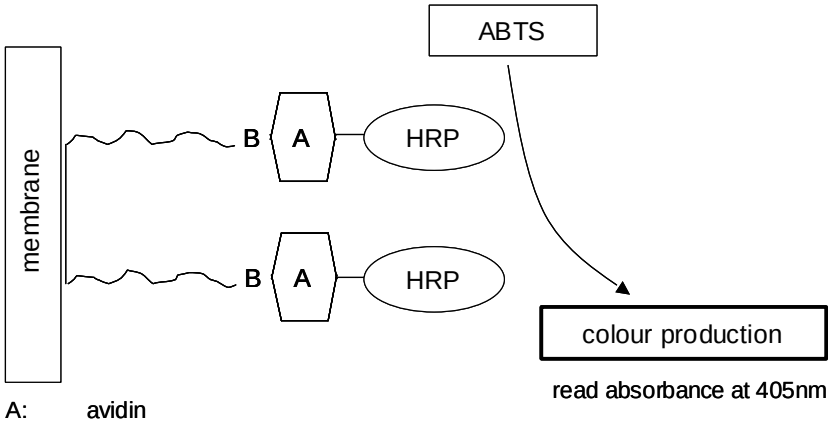
5.1.3 Use of a tether for ligand immobilisation

It has previously been found that enzymes lose their catalytic activity when they interact with a hydrophobic surface, because they lose their structure (denaturation) when exposing their hydrophobic interior to the surface. The HRP enzyme was investigated in this study as previous investigations showed that an appreciable loss of activity occurred when the native enzyme adsorbed onto a hydrophobic surface.[60] The solid support also sterically hinders the ligate from binding the ligand. To allow the ligand to enter into, and interact with the binding site of the ligate, a flexible spacer must be attached between the solid support and the ligand. The spacer must be long enough to reach the active site of the ligate. It must, however, not be too long, as sites for non-specific binding may be created and the spacer could also fold back on itself, making the effective length of the spacer much shorter.[61]. It was also found that the avidin-biotin interactions on a solid phase are several magnitudes slower than similar reactions in solution. This might be because the researcher, in that particular study, did not make use of a tether between the solid support and the avidin. A tether between the avidin and the solid support would make the immobilised avidin act as if in solution and would subsequently bind with much less sterical hindrance to the biotin moiety. Gretch et al.[62] also stated that the ideal situation should allow the antibody (ligand) to be free enough to react with the antigen (ligate) in the liquid phase. These considerations are all valid for the Pluronic system under study.

60 RK Sandwick, KJ Schray, The inactivation of enzymes upon interaction with a hydrophobic latex surface. J. Coll. Int. Sci. 115(1)(1986) 130-138.

61 WH Scouten, Affinity chromatography: Bioselective adsorption on inert matrices. John Wiley & Sons, New York, (1981) pp: 38-39

62 DR Gretch, M Suter, MF Stinski, The use of biotinylated monoclonal antibodies and streptavidin affinity chromatography to isolate herpesvirus hydrophobic proteins or glycoproteins. Anal. Biochem. 163(1986) 270-277



A: avidin
B: biotin
HRP: horse radish peroxidase
ABTS: 2,2'-azino-bis(3-ethyl benzthiazoline-6-sulphonic acid)

Figure 14: Schematic representation of the Avidin-HRP assay experiment to prove that the avidin-biotin cross-linking can be utilised to immobilize an enzyme on a hydrophobic surface using biotinylated Pluronic.[63]

5.1.4 Immobilization of streptavidin horseradish peroxidase

Biotinylated Pluronic was immobilized onto two hydrophobic surfaces, polysulphone membranes and polystyrene. The polystyrene surface was in the form of the Nunc™ micro titer plates used. The polysulphone surface was in the form of small tubes (4mm x 1cm) (**Error! Reference source not found.**). For adsorption onto the Nunc™ and polysulphone tubes a 5mg/mL, biotin-Pluronic and Pluronic solutions were incubated overnight at 4°C. (A more detailed description of the protocol is given in Appendix 2). Deionized water was also used as a control. The solutions were decanted and washed with deionized water. A dilution series of streptavidin horseradish peroxidase (streptavidin-HRP) was prepared in the wells of the Nunc™ micro titer plate and a separate micro titer plate was prepared for the polysulphone tubes. The plates were incubated at 37°C for 60 min. The streptavidin-HRP solution was decanted and the wells where washed with PBS to prevent non-specific binding. The substrate solution, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), was added to each of the wells and incubated at 37°C for 30 min. HRP converts the colourless ABTS solution to a coloured product. This colour development was measured at 405nm on a micro titre plate reader.[64]

5.1.5 Results and discussion

A photograph, which shows the final colour development by the peroxidase reactions in the different wells of micro titre plate A, is shown in
Figure 15. The enzyme was active as can be seen from the apparent colour development.

63 EA Bayer, E Wilchek, Protein Biotinylation. Methods in Enzymol, 184 (1990) 138-162.
64 EA Bayer, E Wilchek, Protein Biotinylation. Methods in Enzymol, 184 (1990) 138-162.

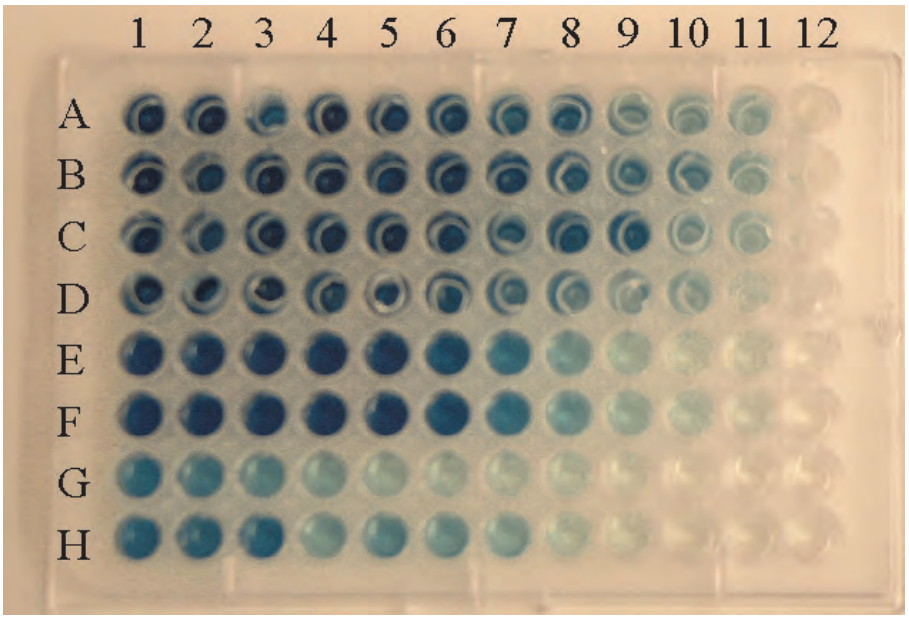


Figure 15: **Plate A: Results of avidin-HRP interaction with Pluronic immobilised biotin. Rows A and B, contained membranes coated with the biotin-Pluronic solution. Row C contained membranes coated with Pluronic. Row D contained uncoated membranes. Rows E and F were coated with the biotin-Pluronic solution. Row G was coated with Pluronic. Row H was not coated. Column 1-11: Avidin-HRP dilution series starting with 25 µg/mL to 0.024 µg/mL. Column 12: No Avidin-HRP**

The coating efficiency of the biotinylated Pluronic onto the polysulphone membranes and polystyrene surface of the micro titre plate is difficult to determine. It was therefore not possible to estimate in advance which concentration of avidin-HRP had to be used to get an optimal reading that would allow a valid comparison between surfaces that contained immobilised biotin and those that did not. This is a problem often encountered in pharmacology and immunology where the exact dose of a drug or the titre of an antibody has to be determined. Consequently, a very broad concentration range is initially used to find a "response" that falls within the measuring range of the technique used to determine the interaction. In this study the absorbance at 405 nm was the "response" and an absorbance value of 1 would fall within the range where the Beer Lambert law would be obeyed. The avidin-HRP was subsequently diluted over a wide concentration range from 25 µg/mL to 0.024 µg/mL and the colour development at 405 nm, which is a direct indication of bound HRP, was measured.

To plot the data, the log of the dilution is plotted on the X-axis while the absorbance is plotted on the Y-axis. The resulting curve typically has a sigmoidal or inverted sigmoidal shape. As the starting concentration is known any value on the X-axis can be readily determined. The log dilution response curves for the interaction of avidin-HRP with biotin-Pluronic coated polystyrene wells and polysulphone membranes are shown in Figure 16. In Figure 16b the log dilution response curves for the same interactions of avidin-HRP, but with Pluronic-coated polystyrene wells and polysulphone membrane tubes are shown. In Figure 16c the log dilution response curves for the interactions of avidin-HRP with uncoated polystyrene wells and polysulphone membrane tubes are shown. Data manipulation was carried out using the GraphPad Prism software, version 3.0 from GraphPad Software, Inc.

The aim here was not to compare the two solid surfaces with each other, but rather the different interactions of avidin-HRP with the differently coated surfaces. On the polystyrene surfaces the interactions of the avidin-HRP with the underivatised Pluronic were highly unspecific and notably less than with the biotin-Pluronic. The absence of a typical inverted sigmoidal curve ($R^2 < 0.95$) indicated non-specific interaction of the avidin-HRP with the uncoated polystyrene surface. A typical inverted sigmoidal curve ($R^2 > 0.95$) was obtained for the biotin-Pluronic coated polystyrene surface indicating specific concentration dependent interaction between die biotin and avidin derivatives. A comparison of the interaction of avidin-HRP with the biotin-Pluronic coated and uncoated polystyrene surface indicate that, for the biotin-Pluronic coated surface, a 48-fold dilution of biotin-Pluronic was needed to yield a response of 1 absorbance unit while a 4-fold dilution was required in the case of the uncoated polystyrene surface (The antilog of the values corresponding to a response of 1 absorbance unit were taken to calculate the fold-dilution required to yield an absorbance value of 1.) It can be deduced from these results that the avidin-HRP interacted specifically with biotin-Pluronic immobilised on the polystyrene solid surface.

On the polysulphone surface the interactions with the underivatised Pluronic were highly unspecific. The absence of typical inverted sigmoidal curves ($R^2 < 0.95$) indicated non-specific interaction of the avidin-HRP with the uncoated as well as the Pluronic coated polysulphone surface. A typical inverted sigmoidal curve ($R^2 > 0.95$) was obtained for the biotin-Pluronic coated polysulphone surface, indicating specific concentration dependent interaction between the biotin and avidin derivatives. A comparison of the interaction of avidin-HRP with the biotin-Pluronic coated and uncoated polysulphone surface indicate that, for the biotin-Pluronic coated surface, a 121-fold dilution of avidin-HRP was needed to yield a response of 1 absorbance unit while a 36-fold dilution was required in the case of the polystyrene surface. It can be deduced from these results that the avidin-HRP interacted specifically with biotin-Pluronic immobilised on the polysulphone membranes.

5.1.6 Conclusions

The results summarised in Figure 16 indicate that coating the polysulphone and polystyrene surfaces with biotinylated Pluronic enhanced specific interaction with the avidin-HRP. The immobilisation of biotin to obtain affinity onto both the hydrophobic solid surfaces studied was therefore successful. This suggests that the biotin was indeed available for interaction with the avidin. The Pluronic served as a tether making the biotin-moiety act as if in solution and therefore more accessible for interaction with the avidin. Even after the several washing steps, affinity was still retained onto the surface. The interactions between the hydrophobic surfaces, biotin-Pluronic and between the biotin moiety and avidin were strong enough to withstand the washing steps. This indicates the feasibility for use of this system in affinity chromatography as well as bioreactors.

The polysulphone surface showed very high non-specific interaction with avidin that could explain the high intensity colour observed. This non-specific interaction plays a role when working with the polysulphone surface and the reduction of this type of interaction will have to be addressed in future studies. The use of the biotinylated Pluronic on the two adsorbing surfaces studied will contribute to the efficiency and specificity of ligand loading onto hydrophobic surfaces in future studies.

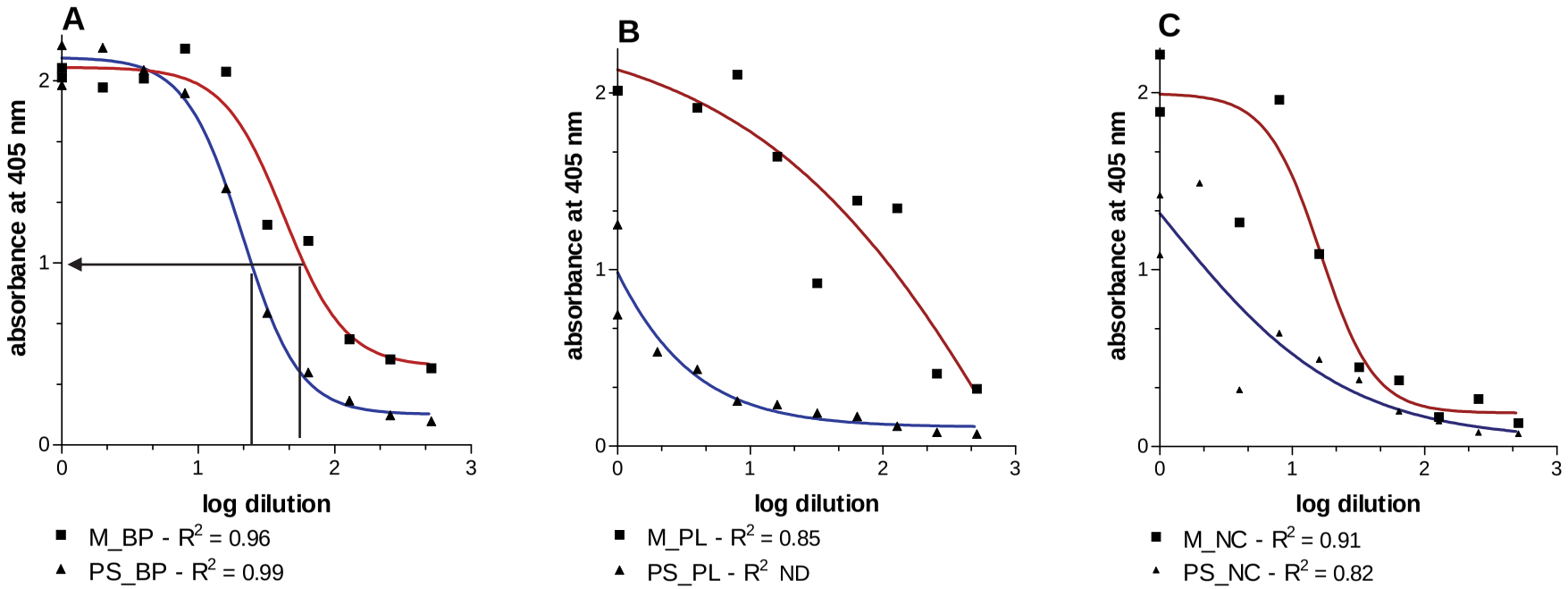


Figure 16: **Log dilution curves of avidin-HRP reacting with polysulphone and polystyrene coated with biotinylated Pluronic. All data points are the average of two determinations. (M_BP: polysulphone membranes coated with biotinylated Pluronic, PS_BP: polystyrene coated with biotinylated Pluronic, M_PL polysulphone membranes coated with Pluronic, PS_PL: polystyrene coated with Pluronic, M_NC: uncoated polysulphone membranes, PS_NC: uncoated polystyrene**

With reference to Figure 16:

- **A]** Typical inverted sigmoidal curve ($R^2 > 0.95$) was obtained for both experiments indicating specific concentration dependent interaction between the biotin and avidin derivatives. The arrows indicate the dilution of avidin-HRP required to yield an absorbance of 1.
- **B]** Log dilution curve of avidin-HRP reacting with polysulphone and polystyrene coated with underivatised Pluronic. It is evident that the interactions were highly unspecific and that the Pluronic alone could not influence the association between the biotin and avidin derivatives in a concentration dependent manner. The non-specific binding of the avidin-HRP to the Pluronic coated polysulphone membrane was high while notably less interaction was seen with the Pluronic-coated polystyrene surface.
- **C]** Log dilution curve of avidin-HRP reacting with uncoated polysulphone and polystyrene surfaces. The absence of typical inverted sigmoidal curves ($R^2 < 0.95$) indicated non-specific interaction of the avidin -HRP with the two surfaces with the polysulphone surface again showing the highest non-specific interaction.

5.2 Immobilised metal affinity separation

5.2.1 Introduction

There is an increasing trend towards the study and development of functionalised materials by using the principles of molecular recognition and molecular imprinting for selective binding of targeted molecules and molecular assemblies.[65] This, in particular, has led to the development of membrane-associated metal chelating ligands that are frequently used in the separation of biomolecules. Immobilised metal-affinity chromatography (IMAC) has proven to be a useful and versatile technique for the one-step isolation and purification of tagged proteins.

As ligands for affinity separations, metal ion complexes offer important advantages over biological affinity agents such as inhibitors and antibodies. Small, inexpensive metal complexes are stable under a wide range of conditions, can be recycled many times without loss of activity and can be formulated into very high capacity chromatographic supports. Elution can be effected under relatively mild conditions, and the columns can be cleaned and regenerated easily, without reduction in protein binding capacity. The selectivity of the separation can be tailored through the choice of metal ion, solvent conditions or by modification of the target protein (e.g., the addition of histidine-rich affinity tags).

Rapidly reversible interactions with metal ions immobilised on a hydrophilic chromatographic support (e.g., Ni^{2+} by an iminodiacetate-derivitised resin) result in the retention of proteins with metal coordinating ligands on their surfaces. In sorption of proteins by metal affinity, the exposed electron-donating amino acid residues, such as the imidazole group of histidine, the thiol group of cysteine and the indoyl group tryptophan, will contribute to the binding of the immobilised metal. The binding of ligands to biopolymers involves a complex set of Van der Waals hydrophobic and electrostatic forces as well as metal chelation. Proteins can be released from their bound complexes by any mode which reduces the affinity constant between the immobilised metal ion and the protein such as changing the ionic strength, pH or displacement by a competitive agent which is similar in structure as the amino acid residues involved in binding. Imidazole and histidine are two competitive agents used in IMAC to release bound proteins. A stepwise or gradient elution scheme in one of these desorption methods can be used to selectively recover and purify proteins. In most cases proteins still retain their biological activity after elution from an IMAC column.[67]

In aqueous environments, metal ions are solvated by surrounding water molecules. The metal ion serves as a Lewis acid and water as a Lewis base. However, when water, is replaced by a stronger base, a coordination complex is formed. A molecule with a single donor atom will form a mono-dentate ligand, resulting in a metal complex. A polydentate ligand formed by two or more atoms from the same molecule with a metal ion results in a metal chelate. The binding of a metal ion to a ligand to a metal chelate is much stronger in a metal chelate than in a metal complex,

65 A Singh, D Puranik, Y Ghao, L Chang, Towards the selectivity in metal ion binding by fixing ligand-chelator complex geometry in polymers, React. Functional Polymers, 44 (2000) 79-89.

due largely to the greater stability arising from the loss of free energy produced by ring formation from the polydentate ligand.

The most commonly used commercial chelating ligand is nitrilotriacetic acid (NTA). This tetradentate ligand occupies 4 of the 6 ligand-binding sites in the coordination sphere of Ni^{2+}. Conventionally the NTA is covalently coupled to Sepharose® CL-6B and is chelated to Ni^{2+} by perfusing an NTA-Sepharose® column with a metal ion solution until equilibrium is reached between the metal chelated to the stationary phase and the metal ion in solution.

In sorption of proteins by metal affinity, the exposed electron-donating amino acid residues, such as histidine, the thiol group of cysteine and the indoyl group of tryptophan will contribute to immobilized metal binding. The binding of ligands to biopolymers involves a complex set of Van der Waal's, hydrophobic and electrostatic forces as well as metal chelation.[66] Proteins can be released from their bound complexes by any mode which reduces the affinity constant between the immobilized metal ion and the protein such as changing the ionic strength, pH or displacement by a competitive agent which is similar in structure as the amino acid residues involved in binding. There are many advantages of membrane and IMAC over typical methods in affinity separation.[67] Different metal ions can be immobilized on the chelator and can be removed easily for regeneration by a stronger chelating agent. The chelating membrane is stable and the eluted proteins still retain the biological activity.

In the work by van Kralingen,[68] the linear tetradentate EDTA type ligands; diethyl and dimethyl N,N'-dicarboxymethyl-3,6-diazaoctanedioate (DEDDO and DMDDO) were investigated as model ligands where two of the acid groups were converted to ethyl and methyl esters respectively. In this study we showed, using solid-state analysis, the specific chelation of Ni to the ligand-modified membranes for specific binding to a histidine tagged protein. Preliminary work was also initiated in an attempt to study desorption of metal ions from the ligands.

5.2.2 Particle induced X-ray emission

In order to confirm the specific chelation of Ni^{2+} ions to Pluronic-*N,N*-dicarboxymethyl-3,6-diazaoctanedioate (Pluronic DMDDO) adsorbed onto the membrane surface, micro-particle induced X-ray emission (PIXE) analysis was conducted by focusing and scanning a proton beam along 1 mm^2 of the membrane surface. For consistency in analysis, the beam was focused as close to the center of membrane surface, where the highest coating homogeneity was expected. As with Br detection on membrane surfaces using PIXE, the charged particles on the membrane surface did not lose significant energy, which suggests that there were no matrix effects due to proton beam analysis of the membranes. Typical PIXE spectra are depicted in Figure 17 to Figure 19, where distinct Ni K_α and K_β peaks can be seen.

66 JW Wong, RL Albright, N-HL Wang, Immobilised metal ion affinity chromatography (IMAC) chemistry and bioseparation applications, Sep. Purif. Methods. 20(1) (1991) 49-106.
67 J Porath, Trends in Analytical Chemistry. 7(7) (1988) 254-266.
68 L van Kralingen. Ligand modification of Pluronic F108 for use in immobilized metal affinity separation of bio-molecules. MSc Thesis, University of Stellenbosch. (2002) Stellenbosch, South Africa.

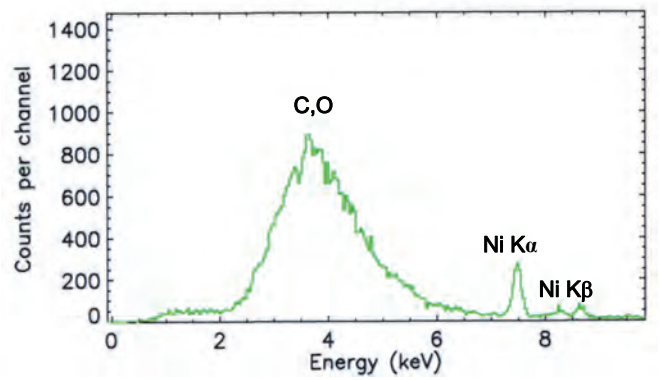


Figure 17: PIXE spectrum of a non-derivatised Pluronic coated PVDF membrane chelated with $NiCl_2$.

Figure 17 is a negative control, where a PVDF membrane was coated with non-derivatized Pluronic and then chelated with $NiCl_2$. PIXE is a very sensitive surface analysis technique and even trace amounts of surface elements can be detected and quantified. Ni^{2+} can adsorb passively and non-specifically to a membrane surface and even to Pluronic modified membranes (Figure 17). Between 777.16 and 650,7 ng Ni/cm^2 was detected on PVDF and Pluronic coated PVDF membranes respectively (Table 6). However, when a PVDF membrane was modified with DMDDO coupled Pluronic, a much greater amount of Ni was observed (Figure 18). The distinct Ni K_α and K_β peaks in Figure 18 correspond to 4975.2 ng/cm^2. Figure 19 and Figure 20 are RBS confirmation of PIXE.

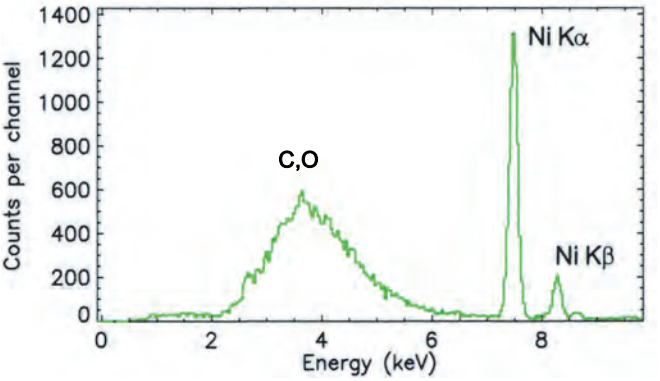


Figure 18: PIXE spectrum showing Ni chelation to a PVDF membrane modified with Pluronic-DMDDO

5.2.3 Desorption and repeated use

One of the most important characteristics of chelating copolymers is their ability to be regenerated and reused.[69] The possibility of the repeated application of the chelating copolymer can be estimated from the results obtained in several sorption/desorption cycles. Literature data regarding the regeneration of membrane associated metal chelating ligands make use of either the relatively non-specific monochlorotriazinyl dye cibacron blue F3GA[70] in conjunction with strong acids (2M HCl) or conventional ligands like IDA.[71] Desorption of metal ions from Pluronic-DMDDO can be

69 F Svec, F Angew. Chem. Int. Ed., 144 (1986) 39-43.
70 A Denizli,, R Say, Y Arica, Removal of heavy metal ions fron aquatic solutions by membrane chromatography, Separation and Purification Technology. 21 (2000) 181–190.
71 Y-C Liu, J. Chromat. B, 794 (2003) 67 – 76.

accomplished very effectively by changing the pH with a mild buffer, rather than a corrosive acid. Since the metal chelating ligand is also stable at higher pH, histidine protein elution can be accomplished under non-denaturing conditions with a competing agent like imidazole.

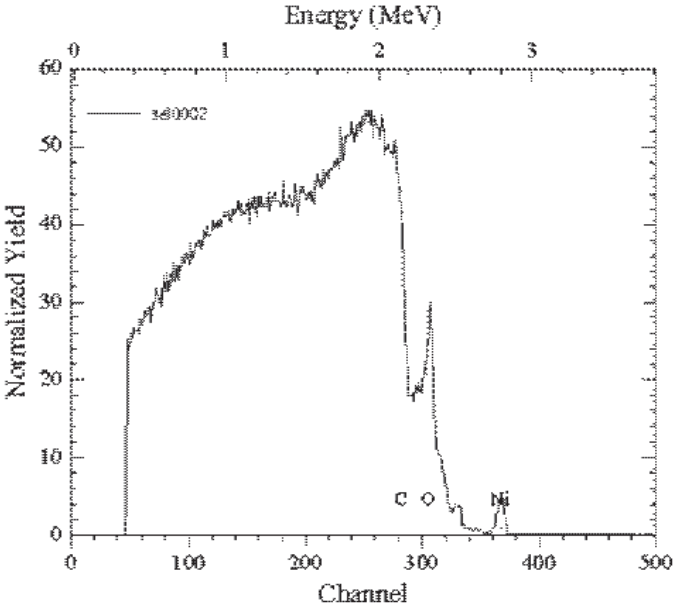


Figure 19: RBS spectrum of a ligand (DMDDO) derivatised Pluronic coated PVDF membrane that was chelated with $NiCl_2$

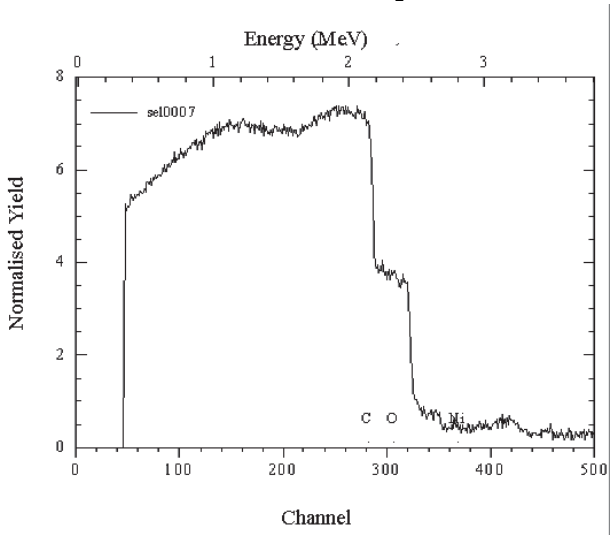


Figure 20: RBS spectrum of a non-derivatised Pluronic modified PVDF membrane chelated with $NiCl_2$

The sorption rate and capacity of the chelating polymers towards metal ions depend on several parameters such as shear stress (or flow rate), structural properties of the sorbent, metal ion properties (hydrated ionic radius), metal ion concentration, pH, chelate formation rate, and the presence of ions competing for the active sites.[72] However, the published results regarding metal sorption on chelating polymers have been obtained under different experimental conditions, so it's difficult to compare the results.

72 A. Nastasovic, S. Jovanovic, D. Dordevic, A. Onija, D. Jakovlejevic, T. Novakovic, Metal sorption on macroporous poly(GMA-co-EGDMA) modified with ethylene diamine. Reactive and Functional Polymers' 58 (2003) 139 – 147.

Preliminary ICP analysis of Ni chelated membranes that were stripped with sodium acetate buffers (pH 2.5 to 4.5) suggested that this process was indeed pH dependent. Although ICP analysis gave information about bulk equilibrium concentrations of metal ions remaining in solution, either after membrane sorption or desorption, the ligand modified membranes used in the desorption analysis were estimated to bind approximately 3µg of Ni, which suggests that not all the metal ions were stripped from the chelated membrane. The empirically chosen parameters for Ni desorption, however, needs refinement to improve the regeneration capabilities of the system.

Table 6: ICP data for desorption from Ni chelated membranes at different pH using 0.1M sodium acetate

pH of stripping solution	Ni desorbed from membrane (µg)
pH 2.5	2.59
pH 3.5	1.74
pH 4.5	1.66

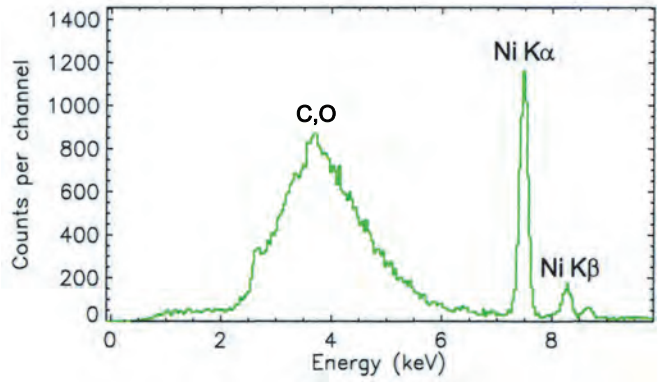


Figure 21: PIXE spectrum of a PVDF-F108-DMDDO membrane that was regenerated with 0.1M sodium acetate, pH 2.5 and chelated with $NiCl_2$ for a second time

Table 7: Summary of PIXE analysis on PVDF membranes that were chelated with $NiCl_2$ in 7mM NaOH

System	$[Ni^{2+}]$ (ng/cm^2)	SDev	N
Native PVDF surface	14.65	14.65	2
PVDF + $NiCl_2.6H_2O$	777.15	1.343	2
PVDF˜Pluronic + $NiCl_2.6H_2O$	650.7	32.67	2
PVDF˜Pluronic-DMDDO + $NiCl_2.6H_2O$	4 975.2	83.01	2
Regen. PVDF˜Pluronic-DMDDO + $NiCl_2.6H_2O$	2 802.0	81.32	2

5.2.4 Affinity binding of histidine tagged proteins

A preliminary study to confirm the ability of a Ni-chelated ligand modified membrane to remove a histidine tagged protein from solution was performed using a histidine tagged pantothenate kinase solution in phosphate buffer, pH 7.4. The hypothesis of this study was that if the Ni-chelated DMDDO-Pluronic modified membrane could specifically bind the histidine tagged enzyme, then the typical activity profile would change. The enzyme assay is dependent on the decrease in NADH concentration with time and when the enzyme is limiting, the activity would show a lag, followed by a flatter curve.

Figure 22a is a typical NADH depletion curve due to the action of pantothenase kinase in the presence of the substrate sodium pantothenate. The standard conditions[73] described in the figure caption are different from the histidine tag protein binding conditions where the enzyme solution is incubated with membranes for 30 min at 25°C. It is possible that enzyme activity could be lost during this time due to degradation or shear induce denaturation of the protein, but the curves and NADH concentrations in Figure 22a and b are fairly similar, which suggest that the Pluronic modified PVDF membrane did not bind much protein. A Pierce protein assay confirmed the protein repellent properties of the Pluronic-treated membrane.

However, Figure 22 represents an activity profile of the bulk equilibrium solution remaining, after a chelating ligand modified membrane was removed, following incubation with the enzyme solution. - The rate of the reaction (0.01045 U/min) was lower than that observed when a Pluronic coated membrane (Figure 22b, 0.01725 U/min) was incubated with the enzyme solution. The equilibrium protein concentration remaining after membrane removal was 0.167 mg/mL for unmodified Pluronic coated PVDF and 0.1146 mg/mL for ligand modified PVDF. This suggested increased histidine tagged protein removal from solution, which can be attributed to the presence of the Ni^{2+} ions that coordinated the surface-exposed imidazole moieties of the protein. Sequential treatment of a 0.2 mg/mL enzyme solution with three liagnd modified PVDF membranes resulted in virtually no residual enzyme activity as depicted in Figure 22d where the apparent reaction rate was 0.00128 U/min. The low bulk equilibrium concentration of $(his)_6$CoaA (0.0781 mg/mL), indicated that the low enzyme activity was due to membrane removal of enzyme from solution. Further work needs to be done to ascertain the histidine tagged protein-binding capacity of the Ni-chelated Pluronic-modified membrane and if the histidine tagged protein can be eluted from the membrane with an appropriate displacement agent.

73 E Strausss, TP Begley, The antibiotic activity of N-Pentylpantothenaimde results from its conversion to ethyldethia-coenzyme A, J. Biol. Chem. 277(50) (2002) 48205-48209.

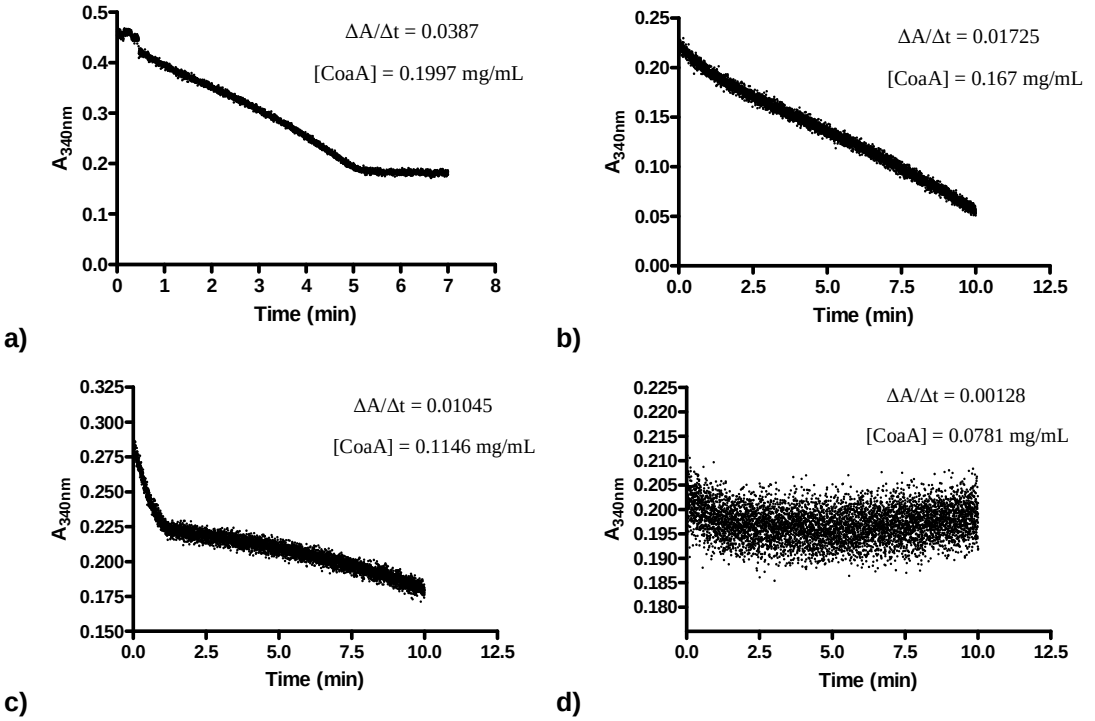


Figure 22: Pantothenate Kinase (CoaA) activity, a) Typical CoaA assay under standard conditions, Histidine tagged CoaA incubated with b) Pluronic coated PVDF membrane, c) Ni chelated ligand modified Pluronic-PVDF, d) three (3x) Ni chelated ligand modified Pluronic-PVDF membranes. The change in absorbance over time for each assay is depicted as ΔA/Δt, while the enzyme concentrations were calculated from the bulk equilibrium protein remaining after removal of membranes from the incubation vessel. The initial enzyme concentration prior to addition of membranes was ~0.2 mg/mL

5.2.5 Conclusions

From the data presented in this chapter it is evident that the immobilisation of enzymes via the Pluronic biotin-avidin system as well as the Ni-chelating Pluronic derivative was successful. In addition the characterisation of Pluronic adsorption and desorption onto hydrophobic membrane surfaces was successfully completed. From the data obtained further development of affinity based detection and purification systems will be facilitated, in particular the final development of an EDC-detection system for drinking water and related applications. The results presented show that both the biotin-avidin as well as the metal affinity route can be used to immobilise a wide range of proteins and ligands.

6.0 Preparation of protein ligates

The following section covers the molecular biology aspects necessary for the preparation of the affinity ligand for estrogenic compounds (ECs). This is an important facet in the membrane assisted affinity separation of ECs in drinking water.

The first three subsections cover the basic background principles of affinity separation as well as the effects and interactions of endocrine disrupting chemicals (EDCs) on human health. Since the estrogen receptor (ER) is the major target of ECs, emphasis was on the structure of this receptor. Given that the techniques used in molecular biology are usually specialized, the detail on experimental procedures followed is given in Appendix 3 and Appendix 4.

6.1 Introduction

Separation is a fundamental requirement in most biotechnological processes with affinity chromatography, an established separation process, being one of the more important. Many technologies under development use recombinant proteins or require high-purity enzymes for immobilization, encapsulation or in organic synthesis. Separation methods based on affinity interaction of biological compounds are often regarded as among the most effective of the protein purification techniques.[74,75,76] The large scale application of these techniques may be restricted because of the following:

- the economic burden (high cost of support matrices and ligands);
- the practical limitations of the chromatographic column size and throughput; and
- effort of operation.

Since the development of the first synthetic membrane, membrane technology made considerable advances in process industry, especially within the water sector.[77,78,79,80] Compared with traditional industrial techniques, membrane filtration processes constitute a more cost-effective and environmentally friendly separation approach than other processes.[81,82,83,84,85]

74 MR Buchmeiser, New synthetic ways for the preparation of high-performance liquid chromatography supports, J. Chromatogr. A, 918 (2001) 233-266

75 BT Croll, Membrane technology: the way forward, J. IWEM, 6 (1992) 121-129.

76 CP Woodbury Jr. and DL Venton, Methods of screening combinatorial libraries using immobilized or restrained receptors, J. Chromatogr. B, 725 (1999) 113-137.

77 AJM Arnal, SM Fernández, MG Verdú, LJ García, Design of a membrane facility for water potabilization and its application to Third World countries, Desalination, 137(1-3) (2001) 63-69.

78 H Kurama, J Poetzschke, R Haseneder, The application of membrane filtration for the removal of ammonium ions from potable water, Water Research, 36(11) (2002) 2905-2909.

79 R Rautenbach, K Voßenkaul, Pressure driven membrane processes — the answer to the need of a growing world population for quality water supply and waste water disposal, Sep. Purific. Technol., 22 (2001) 193-208.

80 H-H Yeh, I-C Tseng, S-J Kao, W-L Lai, J-J Chen, GT Wang, S-H Lin, Comparison of the finished water quality among an integrated membrane process, conventional and other advanced treatment processes, Desalination, 131(1-3) (2000) 237-244.

81 S Bouguecha, M Dhahbi, The role of membrane technologies in supplying drinking and industrial water in Tunisia: conventional process and new trends, Desalination, 151 (2002) 75-86.

82 M Gander, B Jefferson, S Judd, Aerobic MBRs for domestic wastewater treatment: a review with cost considerations, Sep. Purific. Technol., 18 (2000) 119-130.

83 B Goers, M Forstmeier, B Wendler, G Wozny, A systematic approach for water network optimisation with membrane processes, Res. Conserv. Recycl., 37 (2003) 217-226.

84 S Han, FC Ferreira, A Livingston, Membrane aromatic recovery system (MARS) — a new membrane process for the recovery of phenols from wastewaters, J. Membr. Sci., 188(2) (2001) 219-233.

Affinity interactions are among the most useful bioseparation tools because of their high specificity. This study will employ membrane filtration, an established separation process in various industries, as a tool towards the development of a membrane-based affinity separation process. Our approach is a paradigm shift away from the current analytical methods and a positive move towards a more preventative/regulatory approach that addresses the onsite monitoring (quantitative analysis) of ECs where a potential risky area or discharge is suspected.

Environmental testing arose out of concern about hazardous wastes present in the environment. High quality tap water is necessary for good health and previous studies showed the presence of estrogenic chemicals as potential hazardous substances in recycled water. Surface water contains quantities of xenoestrogens or EDCs as a result of industrial wastewater and domestic sewage effluent discharge. The adverse effects of EDCs are known, but the analytical methods to test for these chemicals are not readily available and expensive. Testing for the presence of these chemicals with state-of-the-art analytical equipment is furthermore a subject of specialization and not trivial. Conventional wastewater treatment processes have proved ineffective to remove these compounds from wastewater. This is because of the stable nature of many EDCs and their resulting poor biodegradability.[86,87,88]

he efficiency of the affinity membrane depends on the preparation method i.e. the activator or activation process used for ligand immobilization and use. The immobilization method is particularly important when biomolecules such as proteins or enzymes act as ligands. This is because the activity of the ligand can be affected by denaturation during the immobilization process. The adsorption of the amphiphilic tri-block copolymer, Pluronic, onto hydrophobic membranes is already an accepted phenomenon in membrane separation. The most exploited feature of Pluronic in the field of affinity chemistry is its structural characteristic as previously described.[89,90,91]
In the present study, affinity principles will be employed for the detection and monitoring of potential ECs in drinking water and hydrophobic polysulphone membranes will provide the matrices for the affinity system. Pluronic will be used as the vehicle to immobilize the ligand(s) onto the membrane through hydrophobic interactions. To achieve this, the end-groups of the tri-block copolymer was specifically modified so that a hormone receptor (i.e. estrogen receptor) could be attached to it and serve as an affinity linker for the interactions between hormone receptor and hormone (i.e. estrogenic chemical).

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86 R Espejo, K Valter, M Simona, Y Janin, P Arrizabalaga, Determination of nineteen 4-alkylphenol endocrine disruptors in Geneva municipal sewage water, J. Chromatogr. A, 976 (2002) 335-343.

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91 N Tirelli, MP Lutolf, A Napoli, JA Hubbell, Poly(ethylene glycol) block copolymers, Rev. Mol. Biotechnol., 90 (2002) 3-15.

6.1.1 Endocrine disrupting chemicals

Considerable scientific evidence indicates that a magnitude of environmental contaminants (synthetic and naturally occurring) can disrupt the endocrine system by modulating the actions of steroid hormones and produce agonistic or antagonistic biological responses. Reported increased incidences of human cancers in organs of the reproductive and hormonal system (i.e. breast, testes, prostate) have led to speculation that this could be related to environmental endocrine disruption.[92,93,94] These chemicals, known as endocrine disrupting chemicals (EDCs), are defined as exogenous compounds interfering with the biosynthesis, transport, binding, action or metabolism of endogenous hormones and hence, have the ability to disrupt the normal function of the endocrine system. To date, little is known about how these EDCs alter the function of the hormone receptor at the molecular level.

The endocrine system is a complex, multi-component system which evolved to coordinate and regulate complex mechanisms such as reproduction, growth and development of species. The inherent specificity in biological regulatory systems can be attributed to the specific recognition of a hormone by its cognate cellular receptor. For example, the steroid hormone, estrogen, mediates its activity by interaction and activation of a specific receptor protein (i.e. estrogen receptor, ER). Therefore, any substance that interferes with the normal communication between the messenger and the receptor in the cell will disrupt the endocrine system. EDCs are thought to interfere or disrupt the balance of normal hormone function of the endocrine system by binding to the hormone receptors as an agonist or as an antagonist. EDCs that interfere with the normal function of the ER are defined as estrogenic chemicals (ECs). The ER is a member of the nuclear receptor super-family of ligand-regulated (ligand-induced) transcription factors that regulate gene activation and repression by binding to their regulatory regions as a receptor-ligand complex. The estrogen ligand binds specific to a domain on the ER known as the ligand binding domain to impose a conformational change for the activation or repression of gene expression.

Various synthetic and naturally occurring chemicals found in our environment are capable of mimicking the inherent actions of endogenous hormones (such as estrogens) thereby disrupting the normal function of the endocrine system (Figure 23).[95,96,97]

ECs can be hormone mimics where these chemicals attach to normal receptors but elicit an unnatural cellular response (e.g. a decrease or an increase in cellular response) or they can occupy the receptor thereby blocking the normal hormone-receptor action and stimulate no response, giving rise to serious consequences from

92 J Matthews, T Celius, R Halgren, T Zacharewski, Differential estrogen binding of estrogenic substances: a species comparison, J Steroid Biochem. Mol. Biology, 74 (2000) 223-234.

93 F Regan, A Moran, B Fogarty, E Dempsey, Development of comparative methods using gas chromatography-mass spectrometry and capillary electrophoresis for determination of endocrine disrupting chemicals in bio-solids, J Chromatogr. B, 770 (2002) 243-253.

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95 P Cotton, Environmental estrogenic agents area of concern, JAMA, 271(6) (1994) 414.

96 GG Fein, JL Jacobson, SW Jacobson, PM Schwartz, JK Dowler, Prenatal exposure to polychlorinated biphenyls: Effects on birth size and gestational age, J. Pediatrics, 105 (1984) 315-320.

97 S Gimeno, A Gerritsen, T Bowner, H Komen, Feminization of male carp, Nature, 384 (1996) 221-222.

relative low dosage.[98,99,100] There has also been evidence that environmental disrupting chemicals exhibit synergistic effects. Two weakly estrogenic compounds, for example, may produce a stronger response if they are combined in drinking water and present in the body.[101,102,103,104] These include substances found in industrial and municipal effluents and in agricultural runoff, natural estrogens in plants (phyto-estrogens) and specific chemicals such as alkylphenols. This affinity (or binding ability) of ECs towards the ER is an important factor for consideration in the screening or testing of their potential environmental toxicity and also offers a route for their isolation.

The most common characteristics of EDCs are their solubility in fats and near insolubility in water. As a result, these chemicals have very high bioaccumulation potential (persistence in the environment and in the fatty tissue of organisms) and are subject to food chain magnification. Urban and industrial wastewaters are the main source of EDCs and their distribution into the environment, especially in surface and ground waters. Major research emphasis in recent years was to identify and analyze for chemicals with estrogenic activity in the environment. However, existing analytical methods such as gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography coupled to MS and high performance liquid chromatography (HPLC) are costly and time-consuming.[105,106,107] Routine analyses, however, do not detect EDCs, which have no visible characteristics, smell or taste. Also, these chemicals are present, and show effects on life, at very low concentration levels. Furthermore, since these traditional analytical techniques cannot be applied on the spot, the continuous monitoring of a potential risky area is very difficult if not virtually impossible because of expense.[108,109,110,111,112]

98 R Bolger, TE Wiese, K Ervin, S Nestich, W Checovich, Rapid screening for environmental chemicals for estrogen receptor binding capacity, Environ. Sci. Technol., 106(9) (1998) 551-557.

99 V Granek, J Rishpon, Detecting environmental-disrupting chemicals by fast impedance measurements, Environ. Sci. Technol., 36(7) (2000) 1574-1578.

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101 SF Arnold, DM Koltz, BM Collins, PM Vonier, LJ Guillette Jr., JA McLachlan, Synergistic activity of estrogen receptor with combinations of environmental chemicals, Science 272 (1996) 1789-1492.

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103 I Nukatsuka, S Nakamura, K Watanabe and K Ohzeki, Determination of phenol in tap water and river water samples by solid-phase spectrophotometry, Anal. Sciences 16 (2000) 269-273.

104 K Ramamoorthy, F Wang, I Chen, JD Norris, DP McDonnell, KW Gaido, WP Bocchinfuso, KS Korach and S Safe, Potency of combined estrogenic pesticides, Science, 275 (1997) 405.

105 A Higuchi, BO Yoon, T Asano, K Nakaegawa, S Miki, M Hara, Z He, I Pinnua, Separation of endocrine disruptors from aqueous solutions by pervaporation, J Membr. Sci., 198 (2002) 311-320.

106 NE Skakkebaek, H Leffers, E Rajpert-de Meyts, E Carlson, KM Grigor, Should we watch what we eat and drink, TEM, 11(7) (2000) 291-293.

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108 I Coille, S Reder, S Bucher, G Gauglitz, Comparison of two fluorescence immunoassay methods for the detection of endocrine disrupting chemicals in water, Biomol. Eng., 18 (2002) 273-280.

109 MA Dalvie, E Cairncross, A Solomon, L London, Contamination of rural surface and ground water by endosulfan in farming areas of the Western Cape, South Africa", Environ. Health: A Global Access Science Source, 2 (2003) 1.

110 B Erickson, Screening and testing for endocrine disrupters, Anal. Chem., Aug. (1998) 528A-532A.

111 DF Grobler, A note on PCBs and chlorinated hydrocarbon pesticide residues in water, fish and sediment from the Olifants River, Eastern Transvaal, South Africa, Water SA, 20(3) (1994) 187-194.

112 M Takeuchi, K Mizuishi, T Hobo, Determination of organotin compounds in environmental samples, Anal. Sciences, 16 (2000) 349.

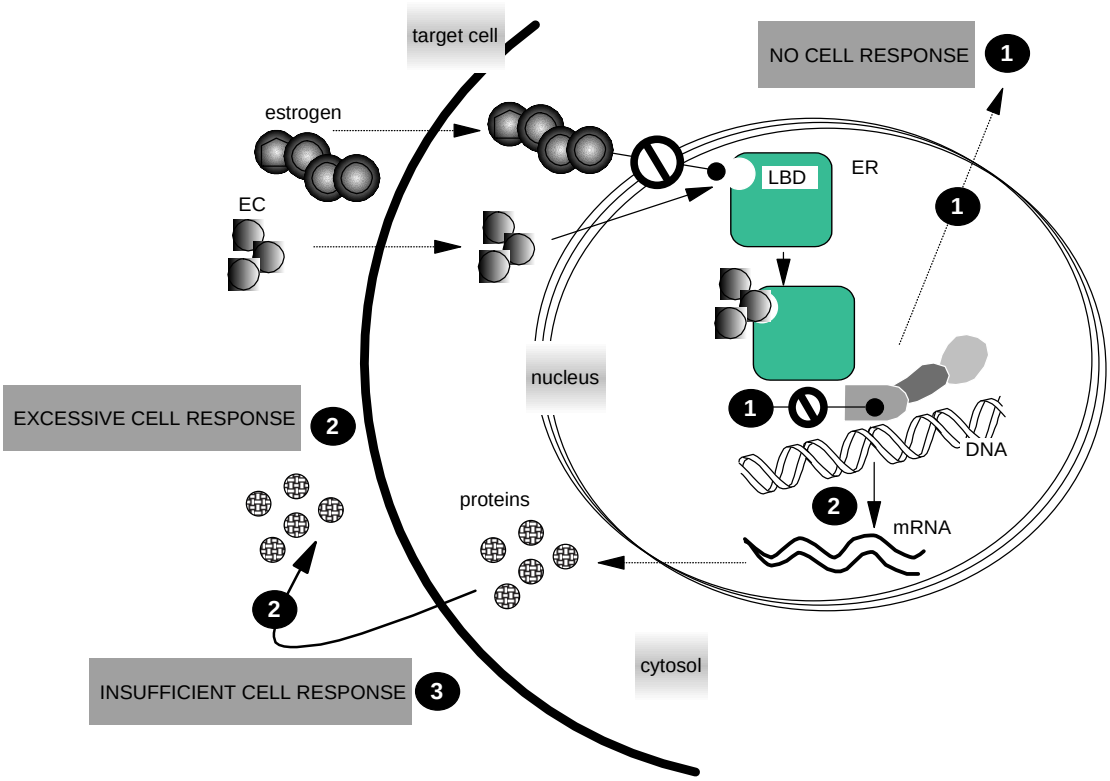

Figure 23: The mode of action of an estrogenic compound (EC). (1) The EC binds to and blocks the binding of estrogen, and hence, no cellular response is initiated. (2 & 3) The EC binds to and initiates gene expression leading to unnatural cellular responses

The effect of an EDC can be classified according the specific hormone receptor it targets; chemicals with androgenic effects target the androgen receptor while those that elicit an estrogenic effect target the ER. The environmental assay employed in this study, will be designed to target ECs. Previous studies showed that chemicals with estrogenic activity may potentially cause adverse effects on wildlife and human health.[113,114,115] In addition to potential human health effects, correlation evidence from many areas indicates that some population of fish, birds, reptiles and mammals have been harmed by environmental contaminants affecting their endocrine systems.[116,117,118] These studies on chemicals with endocrine disrupting potential suggest the high impact of estrogens and ECs on human and wildlife. Chemicals such as pesticides, alkylphenols, phtalates, by-products of conventional water purification processes and paper and pulp production, are found in packaged foods, drinking

113 T Colborn, FS vom Saal, AM Soto, Developmental effects of endocrine-disrupting chemicals in wildlife and humans, Environ. Health Perspect., 101 (1993) 378-384

114 A Higuchi, K Sugiyama, B Yoon, M Sakurai, M Hara, M Sumita, S-I Sugawara, T Shirai, Serum protein adsorption and platelet adhesion on pluronic-adsorbed polysulfone membranes, Biomaterials, 24 (2003) 3235–3245.

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117 LYT Li, DA Campbell, PK Bennett, J Henion, Acceptance criteria for ultratrace HPCL-tandem mass spectrometry: Quantitative and qualitative determination of sulfonylurea herbicide in soil, Anal. Chem., 68 (1996) 3397-3404.

118 I Lutz, W Kloas, Amphibians as a model to study endocrine disruptors: I. Environmental pollution and estrogen receptor binding, Sci. Total Environ., 225 (1999) 49-57.

water, rivers, lakes and oceans.[119,120] Xenoestrogens or estrogenic compounds found in industrial wastewater and domestic sewage either enter the environment directly or undergo wastewater treatment, which are often not designed or capable of removing these contaminants. From this a need for suitable environmental monitoring tools arose. Many of these tools are expensive or not readily available. Many of these tools also need specialized personnel to operate them. To fill the niche of a broad-based analytical tool that can be used as an initial onsite tool to measure whether ECs are present in a body of water, we aim to develop an easy monitoring tool that can do this. Estrogenic compounds bind to a functional domain of the ER, the ligand binding domain (LBD). This interaction is exploited by immobilizing the human ER alpha LBD (hERαLBD) onto a polysulphone membrane, and separating and monitoring ECs by hormone-receptor affinity interactions.

6.1.2 The structure of the estrogen receptor

The ER belongs to the steroid receptor family of nuclear hormone receptors. These receptors are ligand-induced regulators of gene expression. They do this by binding specific DNA sequences that control the expression of an estrogen responsive gene. The ligand for the ER is estrogen, which is responsible for physiological effects that include:

- initiation and maintenance of female sex differentiation patterns;
- reproductive cycle and pregnancy;
- liver, fat and bone cell metabolism;
- cardiovascular and neuronal activity; and
- embryonic and fetal development.

The estrogen receptor has a conserved structure that consists of six regions (Figure 24).

The N-terminal is a variable region (A/B) that contains the hormone independent *trans*-activation function (AF1). The DNA binding domain (C) is highly conserved. This region is responsible for the binding of the receptor to the estrogen response elements of an estrogen responsive gene. The hinge region (D) (of which the function is unknown) is found between the DNA binding domain and the ligand-binding domain. The ligand-binding domain (E) is the region where specific ligands (e.g. hormones) bind to specific receptors. This region also contains the hormone-dependent *trans*-activation function (AF2). The AF2 region binds co-factors that aid in the activation of gene expression. The function of region F is unknown.[121,122,123]

119 ES Quabius, DT Nolan, CJ Allin, SEW Bonga, Influence of dietary exposure to polychlorinated biphenyls and nutritional state on stress response in tilapia (Oreochromis mossambicus) and rainbow trout (Oncorhynchus mykiss), Environ. Toxicol. Chem., 19(12) (2000) 1892-2899.

120 M Rodriquez, DB Orescan, Confirmation and quantitation of selected sulfonylurea, imidazolinone and sulfonamide herbicides in surface water using electrospray LC/MS, Anal. Chem,. 70 (1998) 2710-2717.

121 PS Daneilian, R White, JA Lees, MG Parker, Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors, The EMBO Journal, 11 (1992) (3) 1025-1033.

122 S Folkersma, P van Noort, J van Durme, H Joosten, E Bettler, W Fleuren, L Oliveira, F Horn, J de Vlieg, G Vriend, A family-based approach reveals the function of residues in the nuclear receptor Ligand Binding Domain, J. Mol. Biol., 341 (2004) 321-335.

123 R Kumar, EB Thompson, The Structure of the Nuclear Hormone Receptors, Steroids, 64 (1999) 310-319.

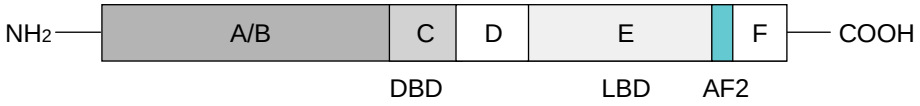


Figure 24 **The structural organization of the estrogen receptor. Region A/B is the variable region. Region C is the DNA binding domain (DBD). Region D is the hinge region. E is the ligand-binding domain (LBD) that contains the hormone dependent activation function (AF2)**

Steroid hormones, like estrogen, act by binding to the receptor that is located in the cytoplasm of the target cell. Here in its pre-ligand bound form (apo-form) the ER is in complex with the heat shock protein 90 (HSP 90). This complex inactivates the estrogen receptor and keeps it from migrating to the nucleus. This means that only when a ligate binds will the receptor actually have an effect on gene expression. Once ligand binding has occurred the receptor will dissociate from the HSP 90 proteins and associate with another ER to form a dimer in a process known as dimerization. Depending on the cell type and ligand bound, the ER will move to the nucleus where it will promote or inhibit gene expression.

6.1.3 The ligand binding domain[124]

Key to this project is the preparation and purification of the ligand-binding domain, which will be used as a receptor in preparations for the affinity ligand (i.e. EC).

The LBD is the domain where ligands for the estrogen receptor, or any compound that has an affinity for the receptor, binds. It contains several key residues that are required for ligand binding, dimerization and co-factor binding. It consists of about 250 amino acids (approximate molar mass of 30 kDa). It is much less conserved than the DNA binding domain amongst nuclear hormone receptors. This is because the receptors bind different ligands. The LBD folds into a three-dimensional formation consisting of twelve helices. These helices organize in a helical sandwich (Figure 25). The ligand-binding pocket is highly hydrophobic and formed mainly by helices H3, H5, H6 and H11. Helix 12 (H12) contains the hormone-dependent activation function (AF2).

On hormone binding the LBD undergoes a change in conformation that results in the release of the receptor from its HSP90 complex and movement towards the nucleus. Recruitment of transcription factors is also the result of the LBD's conformational change. Central to this change is H12. The position of helix 12 is flexible. Its spatial displacement is important for the binding of the transcription factors of the receptor. If the agonist binds to the receptor the helix 12 is displaced from its initial position. This makes it possible for transcription factors to bind to the receptor as well an estrogen dimer to form. Importantly, the ligand binding to the receptor causes it to dissociate from the heat shock 90 proteins. When the receptor is bound to these proteins it is inactive. When an antagonist binds to the receptor the helix 12 displaces to an alternate position blocking transcription factors from accessing the receptor. This inhibits the activation of estrogen responsive genes.

124 M Ruff, M Gangloff, JM Wurtz, D Moras, Estrogen receptor transcription and transactivation: Structure-function relationship in DNA- and ligand binding domains of estrogen receptors, Breast Cancer Res., 2 (2000) 353-359.

In experiments with recombinant ligand binding domains it has been found that they have the ability to function excised from the full-length estrogen receptor.[125] A separated estrogen LBD can still bind and activate an unrelated gene with estrogen as it's ligate and it still has the same binding affinities as the full-length receptor. This has also been found true for other nuclear hormone LBD, for example the androgen receptor.

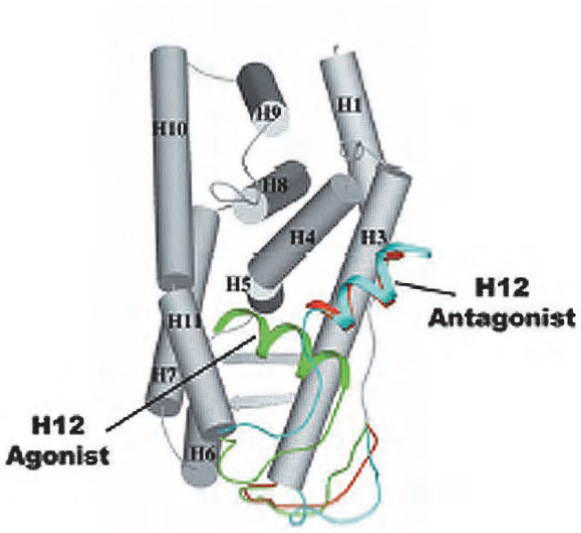


Figure 25: The three-dimensional structure of the estrogen receptor alpha showing the different conformations of helix 12 with estradiol (agonist conformation); raloxifen and tamoxifen (antagonist conformation).[51]

Important to this project is that estrogen is not the only ligand for the ER. It can, with a different affinity, bind other so-called estrogen mimicking compounds that can be both natural and synthetic. These compounds can either activate estrogen responsive genes (agonist) or inhibit these genes (antagonist). Since the body has no control over the amounts or distribution of these chemicals the results may be entirely undesirable.

6.1.4 Estrogenic compound detection with LBD of estrogen receptors

The task to identify chemicals, leave alone their breakdown products, for endocrine disruptive properties is virtually impossible. Literally thousands of chemicals and combinations of chemicals have to be analyzed to determine their effect on the endocrine system and whether this disruption is statistically significant for them to be removed from circulation. The task also includes removing already present and proven endocrine disruptors.

The research documented in this report aims towards the development of a detection system that will aid in this task and **Error! Reference source not found.**the approach followed is based on the fact that ECs target the ER. By immobilizing the ER on a membrane or other solid supports, the EC can be captured, concentrated and detected. By making use of only the LBD, a smaller protein can be immobilized on the membrane instead of the whole ER.

125 ME Brandt, LE Vickery, Cooperativity and Dimerization of Recombinant Human Estrogen Receptor Hormone Binding Domain, J. Biol. Chem., 272 (1997) 4843-4849.

The LBD protein is biotinylated and linked *via* avidin-biotin coupling to a biotinylated Pluronic derivative. The usefulness of the biotin-avidin-biotin coupling route was already demonstrated earlier in the project. The interaction between biotin and avidin is essentially irreversible with an affinity of 10^{-15}M. Using this strong association between four biotin molecules to one avidin molecule, a cross-linking system can be used to immobilize a protein onto a solid support.
The membrane will concentrate the ECs to quantities where they can be detected using monoclonal antibodies. These antibodies differentiate between bound and unbound LBDs, therefore the presence or absence of estrogenic compounds in a body of water.

6.2 Results

In this section the results will be presented obtained during the molecular biology experiments. This approach was followed for the preparation of ligands (i.e. hERαLBD) that have specificity for ECs or ligates. This affinity ligand (hERαLBD) needed for the membrane assisted affinity separation of ECs from drinking water *via* receptor-ligand interactions.

6.2.1 Restriction enzyme digestion and gel analysis: Non-expression host organism

The non-expression host organism, *E. coli* JM109, was used for the transformation, amplification and isolation of the plasmid containing the hERαLBD. Restriction enzyme digestion, agarose gel analysis and DNA sequencing were used to confirm the presence of the DNA insert (hERαLBD).

Restriction enzyme digestion is important to confirm the DNA insert as the hERαLBD and to determine the correct orientation of the DNA (i.e. the correct reading frame for the translation process). The sizes (number of basepairs) of both the plasmid (5 708 bp) and the DNA insert (756 bp) are known as well as their specific restriction sites. Therefore, the relative size of the resultant DNA fragments, after digestion with specific enzymes, would reflect the presence of the DNA insert. The correct orientation of the DNA insert is important for the expression of a functional protein. If the orientation of the DNA insert is wrong, it could result in the expression of a non-functional protein. After specific restriction enzyme digestions, the resultant DNA fragments were analyzed with the use of agarose gel electrophoresis, which is specific for the detection of nucleic acid molecules. Agarose gel electrophoresis is a technique that separates DNA molecules according to size where the smallest molecules will move the furthest through the gel. The DNA sequence of the hERαLBD was confirmed by DNA sequence analysis, which determines the exact order of the DNA chemical building blocks.

Figure 26 shows the results obtained after restriction enzyme digestion with Hind III and Xba I alone.

Restriction digestion with HinD III resulted in two fragments; one smaller fragment just below the 1 033 bp molecular marker, which could account for the expected 873

bp fragment and the larger fragment which could be ascribed to the expected fragment of 5591 bp. The Xba I digestion resulted in two fragments; the smaller fragment between the 453 bp and 394 bp molecular markers, which could account for the expected 400 bp fragment and the expected larger fragment of 6 064 bp.

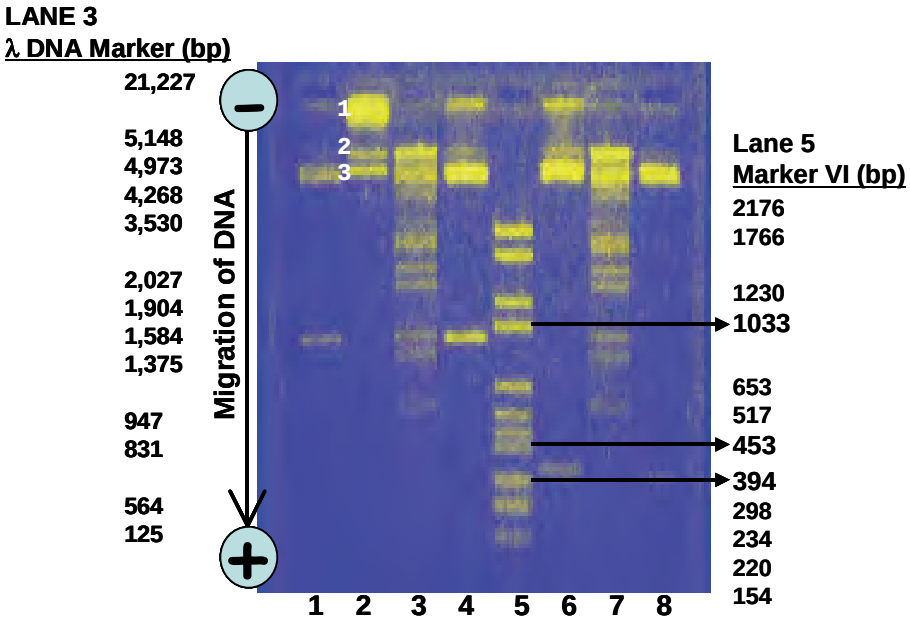


Figure 26: **Gel analysis (2 % agarose) after restriction enzyme digestion with Hind III alone (lanes 1 & 4) and Xba I alone (lanes 6 & 8). Lane 2, undigested plasmid; Lanes 3 & 7, lambda DNA Marker; Lane 5, DNA Marker VI. The bands observed in lanes 1 & 4 and lanes 6 & 8 indicate the correct orientation of the hERαLBD insert**

From these results (
Table 8) it was confirmed that the target DNA insert was in the correct reading frame for the expression of a functional protein.

Table 8: **Expected fragments after restriction enzyme digestion**

Restriction digestion	Expected fragments (bp) for correct reading frame	Expected fragments (bp) for wrong reading frame
Hind III	873 & 5 591	463 & 6 001
Xba I	400 & 6 064	574 & 5 890

6.2.2 DNA sequence analysis

Once the correct reading frame was determined, the target DNA sequence, hERαLBD, was verified with DNA sequence analysis (Figure 27). By comparing the annotation to those on the various online databases, no discrepancies in the annotation were found.

The results obtained in the non-expression host organism confirmed the presence of the hERαLBD insert. The next step was the expression of the hERαLBD in an expression host organism to obtain the desired protein.

6.2.3 Expression of target protein

The recombinant plasmid was transformed into two expression host organisms, *E. coli* BL21(DE3) and *E. coli* BL21(DE3)pLysS, amplified and the DNA insert was verified with restriction enzyme digestion followed by agarose gel analysis (Figure 28). Restriction digestion with BamH I alone resulted in two fragments (as expected) with the smaller fragment corresponding to the 756 bp DNA insert. This fragment was above the 653 bp marker DNA.

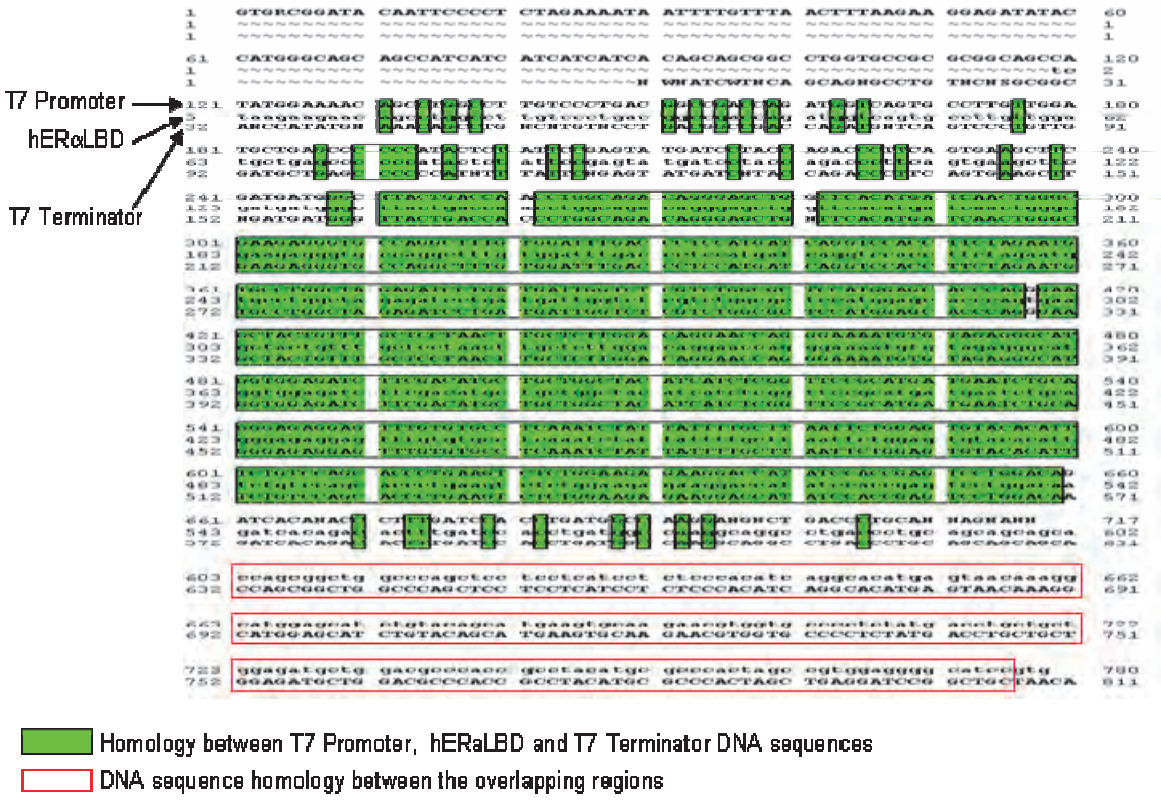


Figure 27: DNA sequence homology of the target DNA with the hERαLBD annotation obtained from online databases

The expression of the target protein (hERαLBD) was performed via IPTG induction on all positive clones (cells that contain the recombinant plasmid). Results from experiments with the *E. coli* BL21(DE3) cells were inconclusive after expression. There was no visible difference (SDS-PAGE) analysis between uninduced and induced samples (data not shown). However, SDS PAGE analysis showed a notable difference between protein levels in uninduced and induced samples obtained after expression in the *E. coli* BL21(DE3)pLysS cells. These results were confirmed by Western blot analysis (Figure 29), which showed a distinct band at 30 kDa, corresponding to the molar mass of the hERαLBD. The band observed in the lanes of the protein markers represents the 30 kDa carbonic anhydrase. It should be noted that no bands were visualized after Western blot analysis, except the carbonic anhydrase, which interact during Western blot analyses because of its five surface histidine molecules. The molar mass markers were redrawn onto the photo (Figure 29a) for visual aid. Lane 7 (Figure 29a) indicates the histidine-tag probe used as a control during Western blot analysis. No notable difference in protein levels was observed between the different induction times (4 h, 6 h and overnight). Large-scale expression of hERαLBD would thus be performed at the minimum time of 4 h.

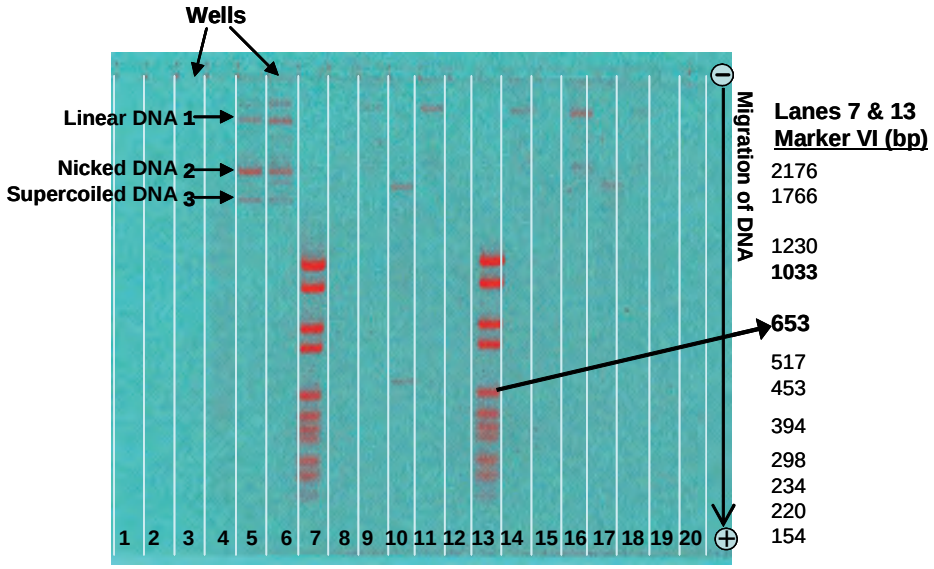


Figure 28: **Gel analysis (0.8% agarose) of restriction enzyme digestion with BamH I (Lane 10). Lanes 5 & 6, undigested plasmid; Lanes 7 & 13, DNA Marker VI**

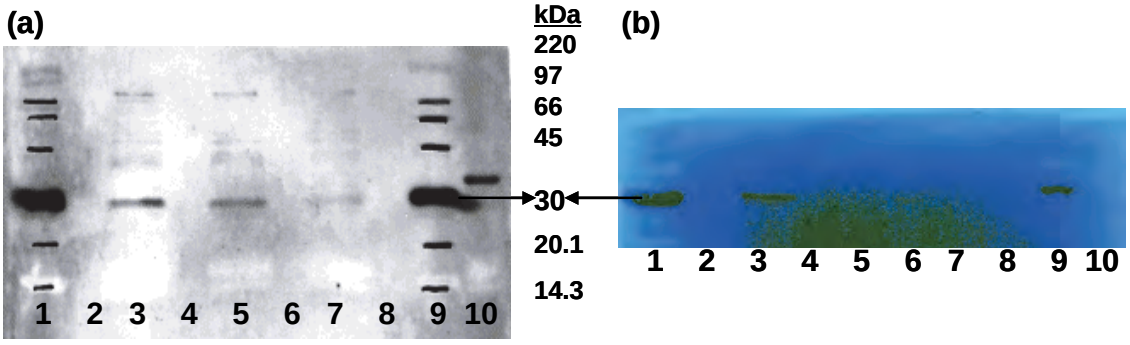


Figure 29: **Western immunoblot analysis of the expression of the hERαLBD synthesized in *E. coli* BL21(DE3)pLysS cells. (a) Lanes 1 & 9, protein molar mass marker; lanes 2, 4 & 8, empty; lane 3, induced (4 h); lane 5, induced (6 h), lane 7, induced (overnight); lane 10, histidine probe (positive control). (b) Lanes 1 & 9, protein molar mass marker; lanes 2 , 4 & 8, uninduced crude extracts**

7. Trypsin affinity ligand construction

Trypsin affinity ligand construction and the separation of trypsin inhibitor from soymilk were attempted as a model for endocrine disrupting chemical chromatography.

7.1 Introduction

Soyabeans (*Glycine max*) are widely used as an inexpensive source of high quality protein. The whole soyabean contains on a dry basis about 40% protein and 20% oil. Compared with indirect conversion of vegetable oil to human protein, direct use of soyabeans into human diets offers a more efficient and inexpensive way to alleviate malnutrition especially in low-income countries. Soyabean protein is a good substrate for the production of soyabean products such as protein hydrolysates and soymilk. Soymilk, essentially the water extract of soyabeans, is traditionally produced by soaking the beans, followed by wet grinding, filtering and cooking. Aqueous extraction of soluble components is generally the basis of several industrial processes to obtain products, such as soymilk, protein isolate and concentrate.

Soyabeans have to be processed before inclusion in diets due to the presence of certain bioactive or antinutritional compounds. Trypsin inhibitors are considered the main bioactive or antinutritional factors in soyabeans. Extreme heat processing is a requirement to denature or inactivate the heat-stable soyabean trypsin inhibitor (SBTI), which blocks the digestion of proteins (SBTI inhibits trypsin, a proteolytic enzyme). Heat treatment may destroy essential amino acids and therefore decreases the nutritional value of soymilk. This processing increase costs and reduces the economic viability of soyabean products.[126,127,128]

Separations are a fundamental requirement for most biotechnological processes. Affinity interactions are among the most useful bioseparation tools as a result of their high specificity. Affinity chromatography is an established separation process in the biotechnology industry. However, these separation techniques are expensive and cumbersome compared to traditional separation techniques. This is because little research has been devoted to industrial scale applications. In addition, the industrial application of affinity separations is limited by the low binding capacity of affinity matrixes and expensive ligands. The current study employs membrane filtration, an established separation process in various industries, as a membrane-based affinity separation process for the isolation of SBTI from soymilk.[129,130,131,132]

126 Y Han, CM Parsons, T Hymowitz, Nutritional evaluation of soybeans varying in trypsin inhibitor content, Poultry Science, 70 (1991) 896-906

127 WD Savage, LS Wei, JW Sutherland, SJ Schmidt, Biologically active components inactivation and protein insolubilization during heat processing of soy beans, J. Food Sci., 60(1) (1995) 164-168 & 180.

128 K-C Kwok, K Niranjan, Effect of thermal processing on soymilk International, Int. J. Food Sci. Technol., 30 (1995) 263-295.

129 GW Niven, PG Scurlock, A method for the continuous purification of proteins by affinity adsorption, J. Biotechnol., 31(2) (1993) 179-190.

130 AD Butterfield, D Bhattacharyya, S Daunert, L Bachas, Catalytic biofunctional membranes containing site-specifically immobilized enzyme arrays: a review, J. Membr. Sci., 181 (2001) 29-37.

131 W Guo, E Ruckenstein, A new matrix for membrane affinity chromatography and its application to the purification of concanavalin A, J. Membr. Sci., 182 (2001) 227-234.

132 FH Isgrove, RJH Williams, GW Niven, AT Andrews, Enzyme immobilization on nylon–optimization and the steps used to prevent enzyme leakage from the support, Enzyme Microb. Technol., 28 (2001) 225-232.

The surface chemistry of the material must first be modified to obtain beneficial interactions with the desired molecules.[133,134,135,136] Pluronic is effective during coupling reactions because the terminal –OH groups can be converted to amino groups for reactions with specific conjugating chemicals and biomolecules. The current study uses AP and HP as the added amines to form amide (peptide) bonds with the carboxyl (–COOH) groups of trypsin after activation with EDAC. Serine proteases such as trypsin tend to shield their –COOH groups because of the serine-histidine-aspartate/glutamate (aspartate and glutamate have –COOH as functional groups) catalytic triad. BSA, therefore, was used as a control during the coupling reactions because this molecule has many –COOH groups available on its surface.

7.2 Chemical cross-linking (coupling reaction)

Previously modified Pluronic molecules, namely aminopluronic (AP) and hydrazine-aminopluronic (HP), were used for the carbodiimide coupling reactions (Figure 30).[137]

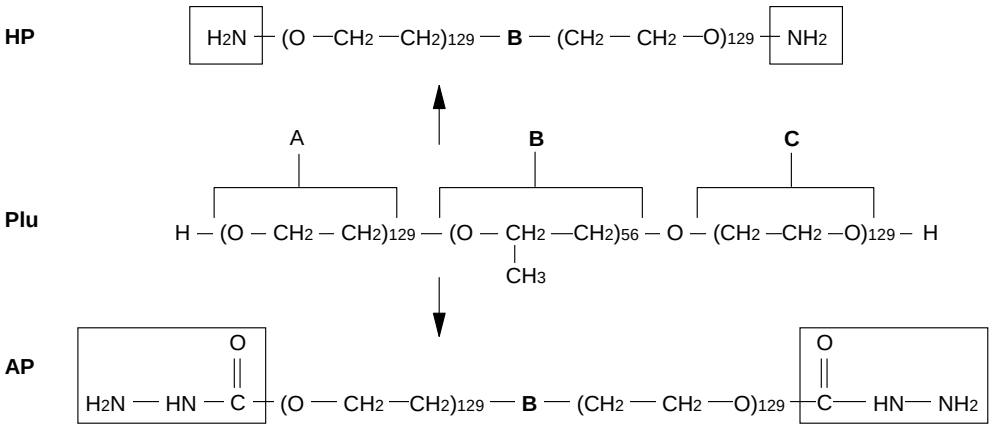


Figure 30: Reaction scheme for the modification of the terminal OH groups of Pluronic to form aminopluronic (AP) and hydrazine-pluronic (HP), respectively

Cross-linkers are chemical reagents used to conjugate molecules together by a covalent bond. In this chemistry, the cross-linking reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) is used as a water-soluble derivative of carbodiimide and is not incorporated into the final product. EDAC catalyzes the formation of amide bonds by activating carboxyl acids (–COOH groups of trypsin) to form an O-urea derivative (this derivative reacts readily with nucleophiles). The activated –COOH groups are then reacted with an added amine (primary $–NH^{3+}$ of AP and HP) to give amidated –COOH groups and the urea derivative of the carbodiimide.[138,139]

133 JS Tan, DE Butterfield, CL Voycheck, KD Caldwell, JT Li, Surface modification of nanoparticles by PEO/PPO block copolymers to minimize interactions with blood components and prolong blood circulation in rats, Biomaterials, 14(11) (1993) 823-833.

134 JC Neal, S Stolnik, E Schacht, ER Kenawy, MC Garnett, SS Davis, LJ Illum, In vitro displacement by rat serum of adsorbed radiolabeled poloxamer and poloxamine copolymers from model and biodegradable nanospheres, J. Pharmaceut. Sci., 87(10) (1998) 1242-1248.

135 JA Neff, PA Tresco, KD Caldwell, Surface modification for controlled studies of cell-ligand interactions, Biomaterials, 20 (1999) 2377-2393.

136 E Klein, Affinity membranes: a 10-year review, J. Membr. Sci., 179 (2000) 1-27.

137 C Yanic, MW Bredenkamp, EP Jacobs, HSC Spies, P Swart, NMR spectroscopy as basis for characterization of Pluronic® F108 and its derivatives, J. Appl. Pol. Sci., 78 (1999) 109-117.

138 R Timkovich, Detection of the stable addition of carbodiimide to proteins, Anal. Biochem., 79 (1997) 135-143.

139 V Arasaratman, YI Galaev, GB Mattiasson, Reversibly soluble biocatalyst: optimization of trypsin coupling to Eudragit S-100 and biocatalyst activity in soluble and precipitated forms, Enzyme Microbiol. Technol., 27 (2000) 254-263.

A 2-step reaction at different pH-values was followed to ensure efficient coupling (Figure 31**Error! Reference source not found.**). The reaction between the carboxyl groups of trypsin (i.e. aspartate and glutamate) and EDAC should be at as low a pH value as possible: pH 4.5 or less (reaction 1, Figure 2). At these pH values you are relying on some (at least) of the carboxyl groups being in the non-ionized form (i.e. –COOH). In addition, EDAC is most reactive at acidic pH (pH 4 to 5). For reaction with the primary amino groups of AP and HP, the pH should be above 7 (preferably 8 or above) to ensure the formation of $–NH_2$ groups and not $–NH^{3+}$ (reaction 2, Figure 31).

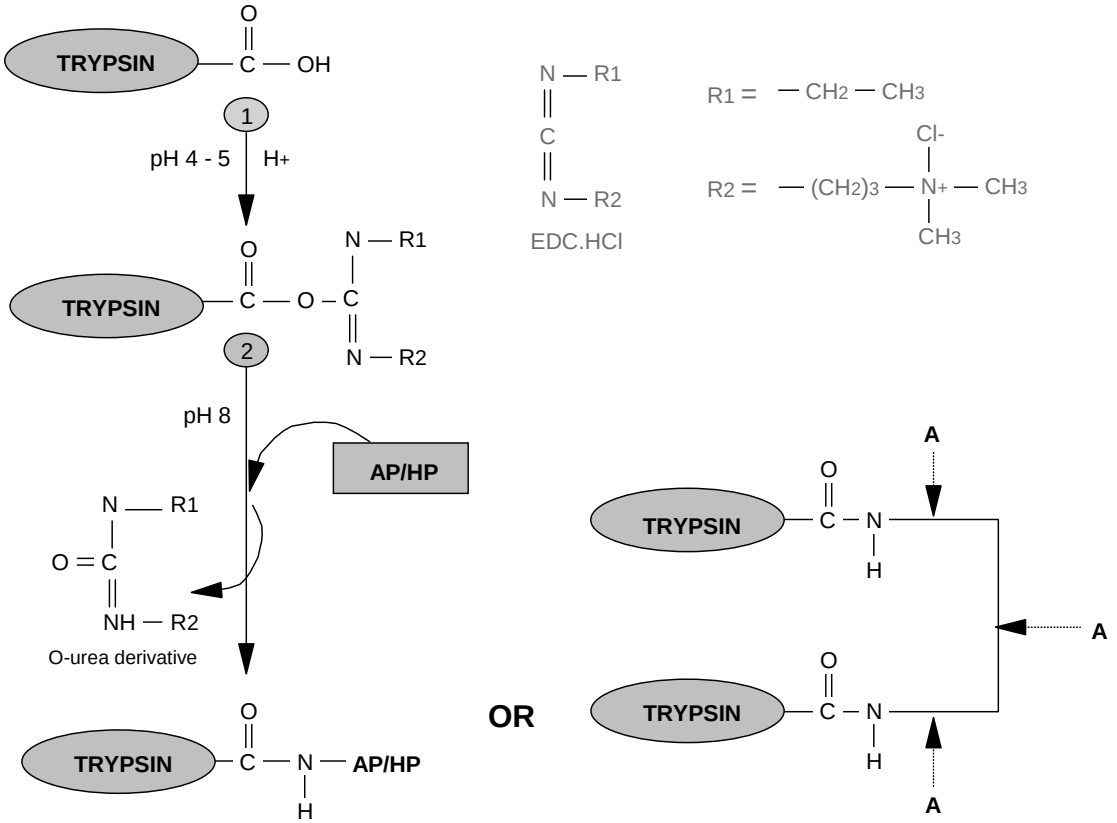


Figure 31: Reaction scheme for the conjugation process of aminopluronic (AP) or hydrazine-pluronic (HP) to trypsin via the carbodiimide process

Trypsin autolyzes (self-digests) at alkaline pH, and 1 mM HCl was used as stock solution (trypsin withstands 1 mM HCl; often used as a stock solution to prevent self-digestion at higher pH values). The overall coupling therefore needs to be carried out as two half-reactions.

The resultant AP-trypsin and HP-trypsin (AP-T) complexes will then be adsorbed onto the polysulphone membrane. This adsorption mechanism is because of the hydrophobic interactions between the hydrophobic core of the Pluronic moiety of the complex and the hydrophobic membrane surface.[140] The surface chemistry of the polysulphone membrane is subsequently altered; advantageous for the separation of the trypsin inhibitor found in soymilk (Figure 32).

140 F Ahmed, P Alexandridis , S Neelamegham, Synthesis and application of fluorescein-labeled Pluronic block copolymers to the study of polymer-surface interactions, Langmuir, 17 (2001) 537-546.

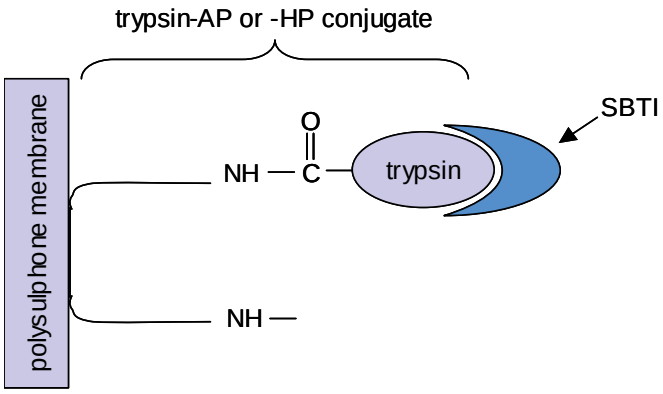


Figure 32: The immobilization of trypsin affinity ligand onto polysulphone membranes for the separation of soyabean trypsin inhibitor (SBTI) from soymilk

7.3 Results

Following, are the results obtained for the conjugation experiments of trypsin to AP and HP with the use of a water-soluble carbodiimide. The trypsin, conjugated to Pluronic, acts as an affinity ligand for the separation of SBTI from soymilk.
During all SDS PAGE experiments, untreated trypsin and untreated BSA were included as controls. Different combinations of trypsin and EDAC concentrations were used during coupling reactions.

7.3.1 Coupling reactions

BSA was used as a positive control during the carbodiimide coupling reaction (10x excess EDAC). These results showed the effectiveness of the coupling method followed; gel analysis showed an increase in molar mass after coupling (Figure 33**Error! Reference source not found.**).
However, results obtained during the coupling reactions of trypsin to AP and HP, with the use of 10x and 100x excess EDAC, were inconclusive. No increase in molar mass was observed after Coomassie blue staining (Figure 34). If trypsin was successfully coupled to AP or HP, an increase in molar mass was expected.

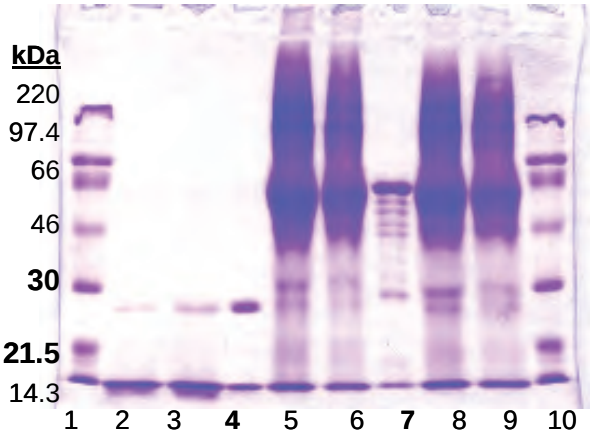


Figure 33: Coomassie blue staining: SDS PAGE (10 %) analysis after different time periods carbodiimide coupling of trypsin to aminopluronic (AP) and hydrazine-pluronic (HP). Lanes 1 and 10, molar mass marker; lanes 2 and 3, AP coupled to trypsin for 7 h and 21 h, respectively; lane 4, untreated trypsin (20 μg); lanes 5 and 6, AP coupled to BSA for 7h and 21 h, respectively; lane 7, untreated BSA (20 μg); lanes 8 and 9, HP coupled to BSA for 7h and 21 h, respectively

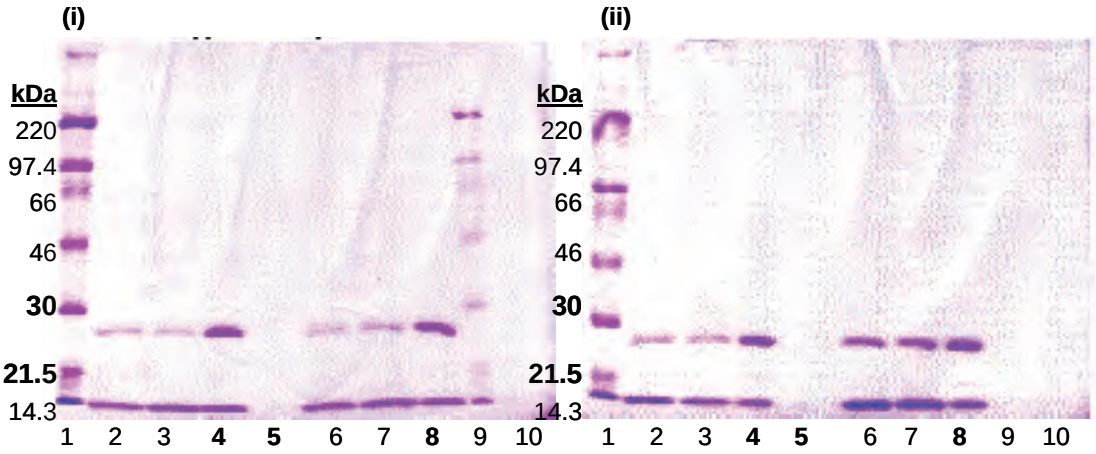


Figure 34: **Coomassie blue staining: SDS PAGE (10 %) analysis after a 7 h carbodiimide coupling reaction of trypsin to (i) aminopluronic (AP) and (ii) hydrazine-pluronic (HP). (i) Lanes 1 and 9, molar mass marker; lanes 4 and 5, trypsin (10 μg and 20 μg, respectively); lane 5, AP. (ii) Lane 1, molar mass marker; lanes 4 and 8, trypsin (10 μg and 20 μg, respectively). Refer to Table 9 for loading of other samples**

Table 9: **Coomassie blue staining: Loading of samples during SDS PAGE analysis for AP and HP carbodiimide coupling to trypsin (10x and 100x excess EDAC)**

Lane	Description (10x & 100x EDAC)	
	(i) Aminopluronic	(ii) Hydrazine-pluronic
1	Marker	Marker
2	T_{10}-E10x	T_{10}-E10x
3	T_{10}-E100x	T_{10}-E100x
4	**T (10 μg)**	**T (10 μg)**
5	**AP**	**HP**
6	T_5-E10x	T_5-E10x
7	T_5-E100x	T_5-E100x
8	**T (20 μg)**	**T (20 μg)**
9	Marker	None
10	None	None

T_5 & T_{10} = 5 mg/mL and 10 mg/mL trypsin, respectively; E10x & E100x = 10x & 100x excess EDAC, respectively; AP = aminopluronic; HP = hydrazine-pluronic; e.g. T_{10}-E10x – 10 mg/mL trypsin was used for coupling with 10x excess EDAC

The higher EDAC concentration (200x and 400x excess) (Figure 35) was used after inconclusive results were obtained with the use of 10x and 100x excess EDAC.

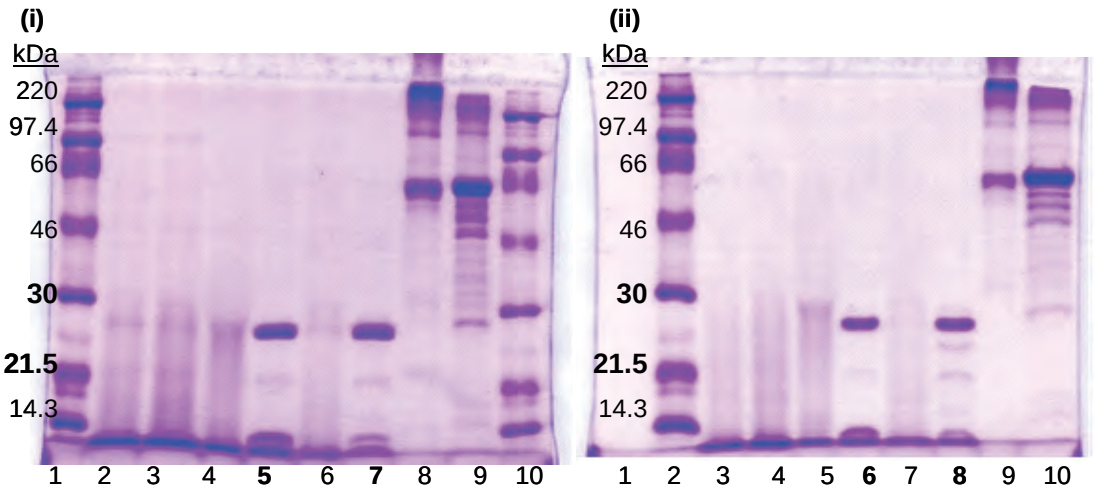


Figure 35: **Coomassie blue staining: SDS PAGE (10 %) analysis after a 7 h carbodiimide coupling reaction of trypsin with (i) 200x excess EDAC and (ii) 400x excess EDAC. (i) Lanes 1 and 10, molar mass marker; lanes 5 and 7, untreated trypsin (10 μg); lane 9, untreated BSA (10 μg). (ii) Lane 2, molar mass marker; lanes 6 and 8, untreated trypsin (10 μg); lane 10, untreated BSA (10 μg). Refer to Table 10 for loading of other samples**

Table 10: **Coomassie blue staining: Loading of samples during SDS PAGE analysis for AP and HP carbodiimide coupling to trypsin (200x and 400x excess EDAC)**

Lane	Figure 34		Figure 35	
	(i) 200x EDAC	(ii) 400x EDAC	(i) 200x EDAC	(ii) 400x EDAC
1	Marker	None	Marker	Marker
2	T_5-AP	Marker	T_5-AP-S	T_5-AP-S
3	T_5-HP	T_5-AP	T_5-AP-P	T_5-AP-P
4	T_c-HP	T_5-HP	T_5-HP-S	T_5-HP-S
5	**T (10 μg)**	T_c-HP	T_5-HP-P	T_5-HP-P
6	T_{h5}-HP	**T (10 μg)**	**T (5 μg)**	**T (5 μg)**
7	**T_h (10 μg)**	T_{h5}-HP	T_c-HP-S	T_c-HP-S
8	B-HP	**T_h (10 μg)**	T_c-HP-P	T_c-HP-P
9	**B (10 μg)**	B-HP	B-HP-S	B-HP-P
10	Marker	**B (10 μg)**	B-HP-P	B-HP-S

T_{h5} – 5 mg/mL of trypsin with the higher activity; B – BSA; AP – aminopluronic; HP – hydrazine-pluronic; S and P – supernatant and pellet of given sample; e.g. T_5-AP – 5 mg/mL trypsin was used for coupling with aminopluronic

Coupling reactions performed with 200x and 400x excess EDAC led to the precipitation of the coupled AP-T and HP-T complexes. These insoluble coupled complexes were centrifuged for 15 min (13,000 x g) and the supernatant was kept. The pellets were washed with water and redissolved in a minimum volume of SDS (10%) and β-mercaptoethanol. These denatured samples were analyzed further (Figure 36 and Figure 37).

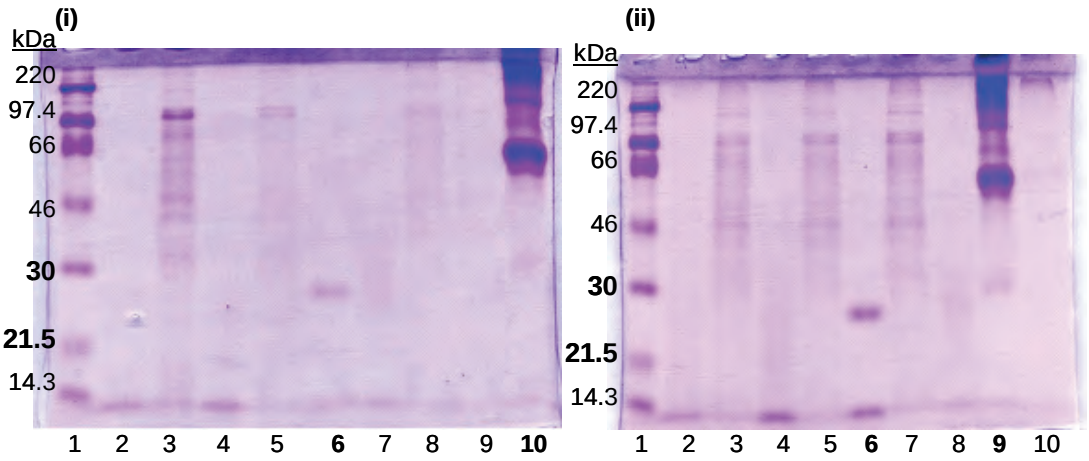


Figure 36 **Coomassie blue staining: SDS PAGE (10 %) analysis of insoluble complexes after carbodiimide coupling reaction with (i) 200x excess EDAC and (ii) 400x excess EDAC. (i) Lane 1, molar mass marker; lanes 2, 4, 7 and 9, supernatant of given sample; lanes 3, 5, 8 and 10, pellet of given sample; lane 6, untreated trypsin (5 μg). (ii) Lane 1, molar mass marker; lanes 2, 4, 8 and 10, supernatant of given sample; lanes 3, 5, 7 and 9, pellet of given sample; lane 6, untreated trypsin (10 μg). Refer to Table 11 for loading of other samples**

After different concentrations of EDAC were used, gel analysis with Coomassie blue staining was not sensitive enough to display any clear bands with a higher molar mass. Silver staining was performed because it has a detection limit of 2 to 5 ng protein/band and it is at least 100-fold more sensitive than Coomassie blue staining. Gel analysis with silver staining showed an increase in molar mass, compared to the untreated trypsin, after coupling reactions (Figure 37).

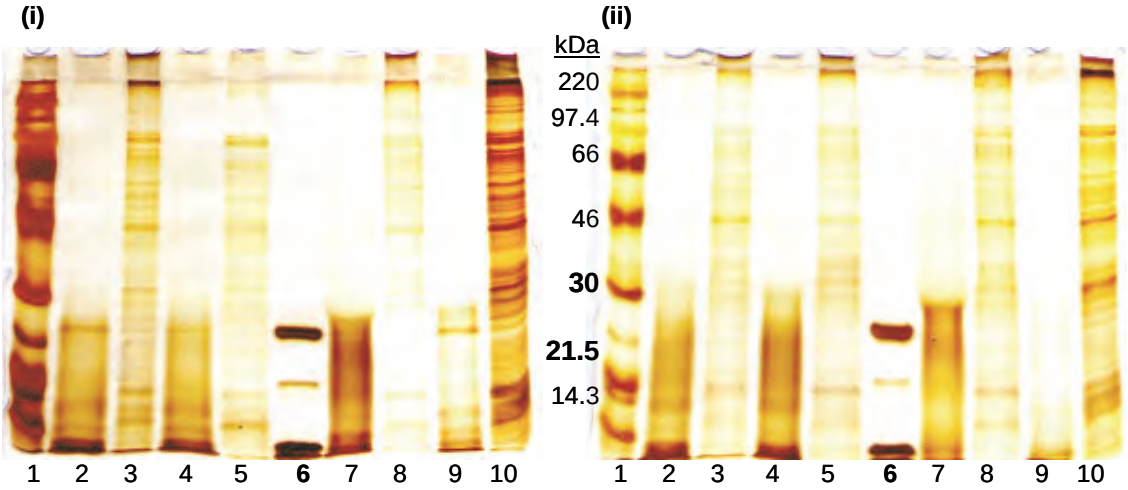


Figure 37: **Silver staining: SDS PAGE (10 %) analysis of insoluble complexes after carbodiimide coupling reaction with (i) 200x excess EDAC and (ii) 400x excess EDAC. (i) Lane 1, molar mass marker; lanes 3, 5, 8 and 10, pellet of given sample; lanes 2, 4, 7 and 9, supernatant of given sample; lane 6, untreated trypsin (5 μg) (ii) Lane 1, molar mass marker; lanes 3, 5, 7 and 9, pellet of given sample; lanes 2, 4, 8 and 10, supenatant of given sample; lane 6, untreated trypsin (5 μg). Refer to Table 11 for loading of other samples**

Table 11: Loading of samples (supernatants and pellets) for AP and HP carbodiimide coupling with trypsin

Lane	Description	
	(i) 200x EDAC	(ii) 400x EDAC
1	Marker	Marker
2	T_5-AP-S	T_5-AP-S
3	T_5-AP-P	T_5-AP-P
4	T_5-HP-S	T_5-HP-S
5	T_5-HP-P	T_5-HP-P
6	**T (5 μg)**	**T (5 μg)**
7	T_c-HP-S	T_c-HP-P
8	T_c-HP-P	T_c-HP-S
9	T_{h5}-HP-S	T_{h5}-HP-P
10	T_{h5}-HP-P	T_{h5}-HP-S

T_{h5} – 5 mg/mL of trypsin with the higher activity; AP – amino-Pluronic; HP – hydrazine-Plu; S & P – supernatant and pellet of given sample; e.g. T_5-AP – 5 mg/mL trypsin was used for coupling with amino-Pluronic

7.4 Discussion

SDS PAGE separates proteins based on their molar mass and is a good analytical technique under denaturing conditions. After coupling reactions, conjugated samples can be identified based on the difference in their molar mass. The conjugated samples should have a higher molar mass than the untreated trypsin (molar mass, 23.8 kDa).
The coupling reactions using a trypsin:EDAC molar ratio of 1:10 and 1:100 show no significant visualization of trypsin-conjugated samples with Coomassie blue staining. The absence of bands may be due to the fact that Coomassie blue tends to be attracted to positively charged groups (lysine and arginine). Thus, basic proteins tend to stain more strongly and some acid proteins have escaped detection. It could also be that Coomassie blue staining is not sensitive enough; the amount of protein is too small to be detected. Samples with untreated AP and HP show no bands because these are synthetic polymers and do not react with the Coomassie Blue (interacts with amino acids of proteins).

When the molar ratio of trypsin:EDAC is raised to 1:200 and 1:400, small bands are detected with Coomassie Blue staining for all trypsin-AP/HP conjugates. The coupling reaction with concentration ratio of 1:1 (Tc:EDAC) (molar ratio of 1:124, Tc:EDAC), serves as a control against the molar ratio experiments. Coupling reactions with BSA shows a difference in molar mass of BSA before and after coupling. Darker and broader bands are also observed for BSA coupled to AP and HP.

After coupling with 200x and 400x excess EDAC, mixtures formed precipitates and were centrifuged (supernatant was kept for SDS PAGE analysis). The pellet was washed, denatured (with SDS and β-mercaptoethanol) and prepared for SDS PAGE analysis.

Analysis by SDS PAGE (Coomassie blue and silver staining) of supernatants and pellets gives distinct characteristics on the coupling reaction. The absence of significant bands in the supernatant lanes and bands visible in the pellet lanes, indicate the presence of coupled complexes only in the pellet (precipitate). These results are further emphasized with silver staining which has a detection limit of 2 to 5 ng protein /band. After silver staining, a considerable number of bands are visible above the

bands represented by the trypsin control (untreated trypsin). These bands show the formation of trypsin-AP/HP complexes, which have higher molar masses than the untreated trypsin. The wide variety of bands is due to the attachment of several AP/HP to activated –COOH groups on one trypsin molecule (multipoint attachment of one molecule of polymer to several available groups from the same polypeptide chain). Intramolecular cross-linking for the conjugated complexes may also occur. In addition, AP and HP have two tethers where one or both ends can be conjugated to trypsin. Coupling is also determined by the activity of the enzyme. Compared to the trypsin with the lower activity (T), significant more complexes (trypsin-AP/HP conjugates) are obtained when trypsin is used with a higher activity (T_h).

7.5 Step forward

In a quest to simplify synthetic routes, an alternative coupling method is currently being tested where the terminal hydroxyl (-OH) groups of Pluronic is reacted with primary amine groups of trypsin with the help of disuccinimidyl carbonate (DSC). No modification of the terminal –OH groups of Pluronic is necessary and Pluronic is commercially available, whereas AP and HP are not. The –OH groups of Pluronic are activated with DSC. The resultant carbonate derivative (Pluronic-NHSC complex) is coupled to the amino groups of trypsin instead of the less accessible –COOH groups (lysine residues are more accessible than the –COOH groups).[141]

After standardization of the coupling reactions (purification of specific conjugates, electron spray mass spectrophotometry (ES-MS) analysis of purified samples, determination of the activity of trypsin before and after coupling), the affinity ligand complex will be adsorbed onto flat-sheet polysulphone membranes (Figure 38). This is followed with the determination of the efficiency of enzyme immobilization and separation of SBTI from soymilk.

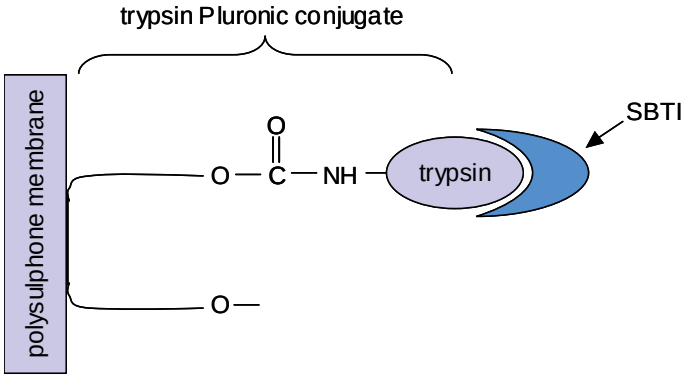
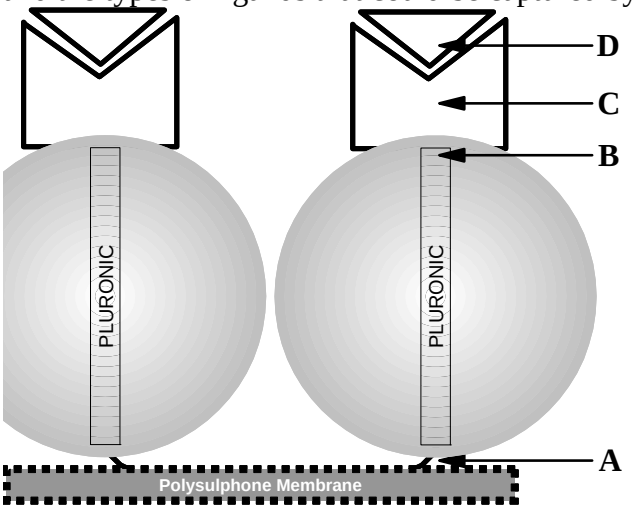


Figure 38: An alternative method for the immobilization of trypsin affinity ligand onto polysulphone membranes for the separation of soyabean trypsin inhibitor (SBTI) from soymilk

141 M Morpurgo, EA Bayer, MJ Wilchek, N-hydroxysuccinimide carbonates and carbamates are useful reactive reagents for coupling ligands to lysines on proteins, J. Biochem. Biophys. Methods, 38(1) (1999) 17-28.

8. Conclusions

The work described in this report focused on the exploitation of the unique interaction between the triblock copolymer, Pluronic, and hydrophobic membrane surfaces, which allowed for the successful development of a non-covalently immobilized affinity linker. Previous studies showed that the hydroxyl groups of the Pluronic molecules could be modified chemically into reactive functional groups (B) which could react with ligands or binding molecules (C) while the hydrophobic part of the modified Pluronic (A) immobilized the molecule on the membrane surface, as shown in the accompanying figure. This new approach to membrane based affinity separations had to be characterized with respect to the non-covalent association of the Pluronic with the membrane (A), the modification of the Pluronic hydroxyl groups (B), the types of ligands or bonding molecules that could be coupled to Pluronic (C) and the types of ligands that could be captured by the system (D).



The interaction of the Pluronic with hydrophobic membranes (A) was characterized and isotherms were obtained that facilitated the calculation of the amount of Pluronic that would be needed to cover a given surface of the membrane. In addition the influence of curvatures on the interaction of the Pluronic with hydrophobic surfaces was also determined. From these results it was concluded that the capacity of hydrophobic membranes to bind Pluronic was sufficient to allow for the "loading" or immobilization of significant quantities of modified Pluronic that would make this a viable system for practical applications in bio censoring or bio separations.

The modification of the Pluronic hydroxyl groups (B) was successful and different functionalities were introduced that allowed the synthesis of a metal chelating Pluronic as well as biotynilated Pluronic. Results obtained with the metal chelating as well as the biotynilated derivatives (C) showed that enzymes as well as metal ions can be bound specifically and that the system could be regenerated for further use. These results clearly indicate the viability of the non-covalent immobilization strategy and opens up a wide variety of applications for this technology.

One specific application investigated in this study was the immobilization of the ligand-binding domain of the estrogen receptor (C in the figure). Once immobilized the system could be used for the binding, and indication via ELISA, of potential EDC's in drinking water. To this end the ligand binding domain of the estrogen receptor had to be produced in our laboratories (cloned and expressed in

microorganisms) as this component of the system cannot be obtained commercially. We have made good progress in the cloning and expression of the ligand-biding domain and in a follow up project the technology developed during the current study will be applied in the design and manufacture of a membrane "strip test" for EDC's in drinking water.

To summarize: A good understanding of the affinity separation technology, originally envisaged, was generated. This knowledge will certainly expedite the development of applications such as the EDC test described above as well as the possible harvesting of precious metal ions from effluents and the immobilization of enzymes in special bioreactors.

9. Recommendations

EDC detection

A small number of tasks still remain towards the development of an EDC detection system and it is recommended that this work is continued to its logical conclusion. Successful expression of the LBD is one of the tasks. After establishing optimal expression, the culture containing the pET15b-hERαLBD construct, will be used for large-scale production of the protein. Affinity chromatography, with the use of a nickel-agarose affinity resin, will be used to purify the histidine-tagged hERαLBD protein.

Two routes by which to couple the protein to the membrane are now available for further investigation. We have shown that the Pluronic biotin-avidin system is a feasible method for the immobilisation of proteins on hydrophobic membranes. In addition, the results obtained with the Ni-binding Pluronic indicate another possible route using the His-tag already present on the expressed ligand-binding domain.

The recombinant ligand-binding domain will also be tested for estrogen binding and receptor activation through temperature increase. Monoclonal antibodies against the activated hERαLBD will subsequently be generated and used to detect estrogen binding by the ligand-binding domain immobilised on the hydrophobic membrane (Figure 39). The last part of the initial research will involve affinity membrane preparations and bench scale analysis of drinking water with known ECs as standards.

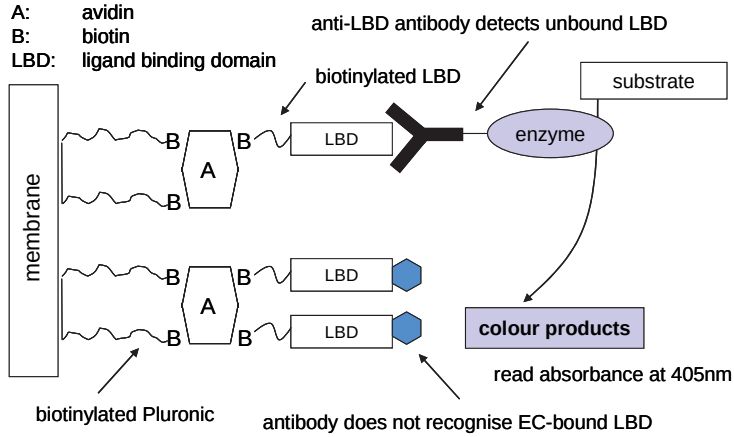


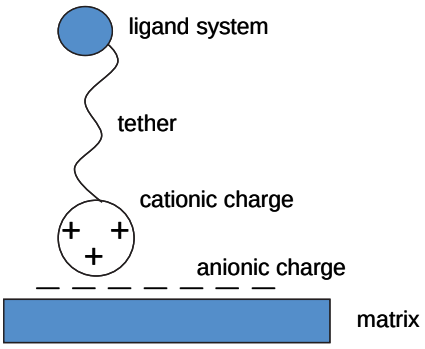
Figure 39: Diagram showing the process of membrane-assisted affinity separation of estrogenic compounds. Immobilization of the LBD protein onto a polysulphone membrane is achieved using biotin, avidin and Pluronic

Ligand loading density

Van der Waals forces such as dipole-induced dipole and London dispersion forces are exploited in the current approach to immobilize the ligand-carrier on the surface of the support matrix. These intermolecular forces of attraction are relatively weak and rely on the size of the PPO segment of Pluronic to immobilize the ligand carrier, i.e. the Pluronic is immobilized by the cumulative effect of the weak intermolecular forces of attraction between the PPO segment and the receiving surface. The larger the PPO moiety, the stronger will Pluronic adsorb onto a receiving surface. However, larger PPO segments (footprint) will

have an obvious result on the maximum loading capacity of the related ligand carrier. On the other hand, by reducing the PPO footprint to increase the loading capacity, the permanency of the adsorptive attachment could be compromised.

Ion-dipole attractive forces are much greater than the above-mentioned and ionic (anion-cation) forces are even greater. Printing technology exploits all of the above intermolecular forces to keep particulate pigments in permanent suspension (solution). Adsorbents that associate very strongly with the solvent environment, on the one hand and the pigment particle on the other, are engineered to keep a specific pigment in solution. The suggestion is that a similar approach is considered to increase the loading density of a hydrophilic tether, such as PEO, onto a surface.



The diagram shows a simplification of such an envisioned system. The matrix could be a cation exchange resin or membrane, or a carboxylated polystyrene film. The matrix could also constitute a conducting material such as carbon, connected to an electrical device to generate a non-permanent negative (or positive) surface charge on the receiving carbon body. The anchoring part of the ligand carrier would contain moieties of opposite charge, but of great enough density to counter the combined competing effect of water molecules to disassociate the carrier from the matrix.

Biotechnology

Membrane bioreactors, where enzymes are immobilized on the surface of supports are used commercially. The concept entails the immobilization of enzymes to a surface. A biological mixture can be passed over the immobilized enzymes, which performs its function, producing the desired effect to the mixture. The advantage is that the enzyme is not lost in the biomixture but is retained for further processes, or used in a continuous process.

The efficacy of such reactors will be enhanced if the enzymes were immobilized on a tether, thus making more of the active part of the enzyme available to catalyze conversion reactions. At present, the enzymes are immobilized by direct adsorption. This reduces the net activity of the enzymes because of partial denaturation and obscuring of the active site because of non-specific adsorption.

However, it would be very costly to immobilize enzymes in such reactors making use of tethers that are covalently attached to the support structure. A less expensive alternative is to exploit the carrier-concept into membrane bioreactors and biotechnology. This theory may be explained with rennin, an enzyme found in the stomachs of mammalian infants to aid in the digestion of milk. Rennin is used to curd milk in cheese production. Adding a polyhistidine

tag to its inactive end may for instance modify this enzyme. The tagged enzyme may then be immoblised in an IMAC system and milk passed over the surface onto which the rennin is immobilized, producing curd and whey on a continuous basis.

Appendix 1

MEMBRANE FORMATION

Most commercial membranes are fabricated by means of the wet-phase inversion process. Membranes are generally supported, that is they are cast onto a non-woven fabric carrier to impart some mechanical integrity to the otherwise fragile membrane, or they are unsupported. Capillary or hollow fibre membranes are of the latter kind. By virtue of their small diameters, these membranes can be pressurized internally or externally without inflation or collapse. Most of the work, however, was conducted with flat-sheet membranes, cast from either polysulphone (PSU), polyetherimide (PEI) or poly(vinylidene fluoride), although capillary membranes and larger bore tubular membranes were also used.

In our study we were more interested in the surface properties of native materials, and more specifically, those materials from which microfiltration membranes could be made. Because we were more interested in the material itself and the way the ligand carrier would adsorb onto it, and also which of the materials performed better as receiving surfaces, the 3 materials experimented with was chosen for their know hydrophobic properties. The membrane materials experimented with were polysulphone (PSU), polyetherimide (PEI) and poly(vinylidene fluoride) (PVDF).

The membranes were never characterised for flux or their morphologies developed to give ultra porous structures. No additives were used to improve surface porosity either. The membranes were cast from a solution that only contained a good solvent for the polymer. The membranes were cast by the wet-phase inversion technique and only distilled water was used as the non-solvent. Generally this approach leads to a membrane with a very thin skin layer, which, when allowed to dry out, may be regarded as dense.

A1.1 Flat-sheet membrane formation

Flat sheet membranes can be cast on a support fabric or they can be prepared without a support. The latter membranes are referred to as unsupported.

A1.1.1 Unsupported flat-sheet membranes

The simplest way to produce a membrane is to cast it on plate glass, making use of a blade to scrape the casting solution to the required thickness. Typical membrane-forming polymer concentrations used for this type of membrane were 15 to 20% by mass, and the thickness of the membrane was typically 150 μm. The membrane-forming polymer is dissolved in an appropriate solvent such as N, methyl, 2-pyrrolidone (NMP) until the solution is completely homogeneous. The solution is filtered, degassed and spread out on a glass plate by means of a casting knife of which Figure 40 is a simple and inexpensive example.

The cast membrane is immersed into distilled water containing a small amount of NMP, typically 2%m/m. The membrane peels loose from the glass plate after a while and is placed in distilled water for the remainder of solvent to leach from the film.

The membrane is cut into test specimen size and stored in distilled water containing a small amount of an agent to prevent bacterial activity development.

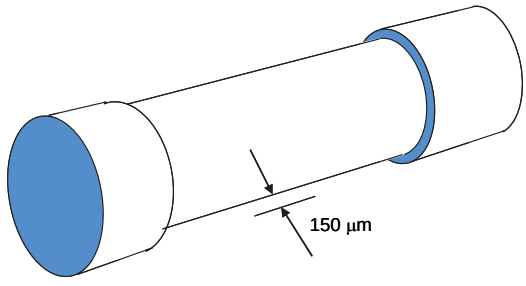


Figure 40: Simple casting bar machined to appropriate dimensions

A1.1.2 Supported flat-sheet membranes

Supported flat-sheet membranes were cast on a non-woven spun-bonded fabric. This fabric was from polyester and is not affected by the polymer solvent. The equipment used to make the membranes is shown in Figure 41.

A constant speed take-up device pulls the fabric from the tensioned dispensing roller underneath the membrane coater that scrapes the membrane solution to the required thickness. After a short air exposure period the nascent membrane enters into the coagulation and primary leach bath. The membrane-length is slit from the take-up roller and allowed to leach to completion in distilled water.

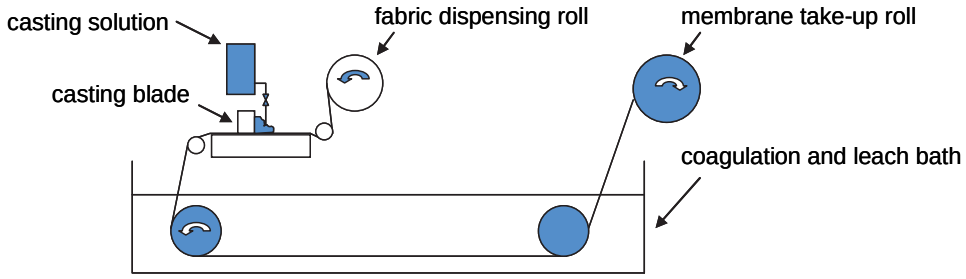


Figure 41: Supported flat-sheet membrane casting machine

A1.2 Tubular-type membranes

Tubule and tubular membranes (OD 8 to 25mm) are normally cast inside non-woven fabric supports to ease handling and protect the membranes. Capillary membranes are of such small OD (typically 0.8 to 3 mm) that they can withstand operating pressures in excess of 1 bar. These membranes are therefore not fabric supported.

A1.2.1 Unsupported tubular membranes

In some instances it was necessary to make small diameter tubular membranes to fit into the vials of a microtiter plate. A very simple technique was used to make these small diameter (OD 8mm) tubular membranes.

A glass tube of appropriate inside diameter was filled with casting solution and the solution was allowed to drain. The tube was subsequently immersed evenly into distilled water to allow the membrane to coagulate and initial leaching to take effect.

During this process the membrane would shrink away from the forming body and slip out.

A refinement of the technique was to drop a steel ball into the forming body, the ID of the forming body and OD of the steel ball would determine the thickness of the nascent membrane. A membrane of greater evenness in wall thickness could be prepared by means of this technique.

A1.2.2 Capillary membranes

Capillary membranes were spun by means of a spinneret of tube-within-tube design (Figure 42). Spinning dope is metered under pressure through the annular die and exist the spinneret in a fluid state. Lumen coagulant is metered through the centre needle. On contact with the spinning dope the inner skin layer of the membrane is formed. This provides sufficient strength to the nascent membrane to allow it to pass for a short distance in air, before it traverse the first roller and continue in the leach tank where solvent-solvent exchange is completed before take-up. The membranes are removed for further leaching and processing for use.

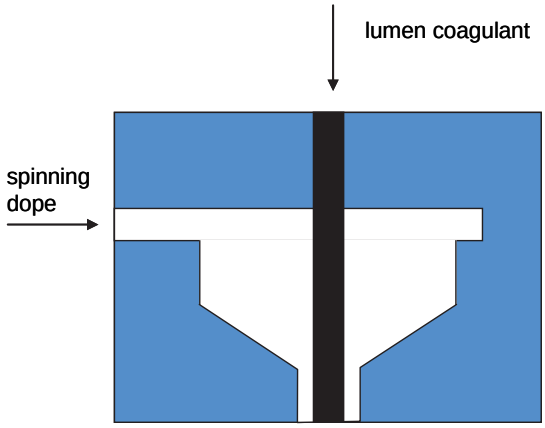


Figure 42: Tube-within-tube spinneret used for capillary membrane spinning

The diameter and wall thickness of the membrane depends on the relative sizes of the annulus. Membranes with outside diameters of 600µm and 1.8 mm OD were prepared.

Appendix 2

EXPERIMENTAL PROCEDURE:AVIDIN-HRP DILUTION PROTOCOL

Appropriate Avidin-HRP (horseradish peroxidase) dilution and testing the hydrophobic surfaces for affinity

The wells of the micro titre plate is laid out in 8 rows, marked A to H, and 12 columns, marked 1to 12. This makes identification of the wells and planning of the experiment more convenient.

In this experiment rows A to D were used for the membrane-contact experiments done with the membranes and rows E to H were used for the experiments where the micro titre plate itself was used as a solid polystyrene adsorbing surface.

Polysulphone tubes (**Error! Reference source not found.**) were cut into ca. 1 cm pieces and incubated overnight in separate glass beakers in solutions of Pluronic-biotin (5 mg/mL), Pluronic (5 mg/mL) and deionised water respectively. Rows E and F of the wells of the micro titre plate wells (plate A) were coated overnight with 5 mg/mL Pluronic-biotin solution. Row G was coated with 5mg/mL Pluronic and row H contained only deionised water (Figure 43). All the coating steps were done at room temperature. After incubation the wells and membranes were washed three times with deionised water.

The avidin-peroxidase dilution series was subsequently carried out in two separate micro titre plates. In plate A the dilution series was made from row E to H which already contained the aforementioned coating solutions. In a new plate (plate B), the dilution was carried out in rows A to D and the coated membrane tubes were subsequently placed in this plate.

The dilution series were prepared as follows: A 25 μg/mL avidin-peroxidase solution was prepared in PBS. PBS (150μL) was pipetted into all the wells of columns 2 to12 and in the specific rows as discribed above, while 300 μL of the avidin-peroxidase solution was pipetted into the wells of column 1. Of the solution in the wells of column 1, 150 μL was transferred into the wells of the second column (where 150μL PBS had already been pipetted into) and mixed thoroughly. As a result, a 2x dilution of the solution in the wells of column 1 was obtained in the wells of column 2. Now 150 μL of the diluted solution in the wells of column 2 was pipetted and mixed with 150 μL PBS already present in the wells of column 3 to yield a 4x dilution. This process was repeated for all the columns except the last column, column 12. As a result the wells of columns 1 to11 contained a concentration dilution series of avidin-peroxidase with the most concentrated solution being in the wells of column 1 and the most diluted solution in the wells of column 11 (102 400x dilution). The last column was not filled with avidin-peroxidase solution and served as a negative control.

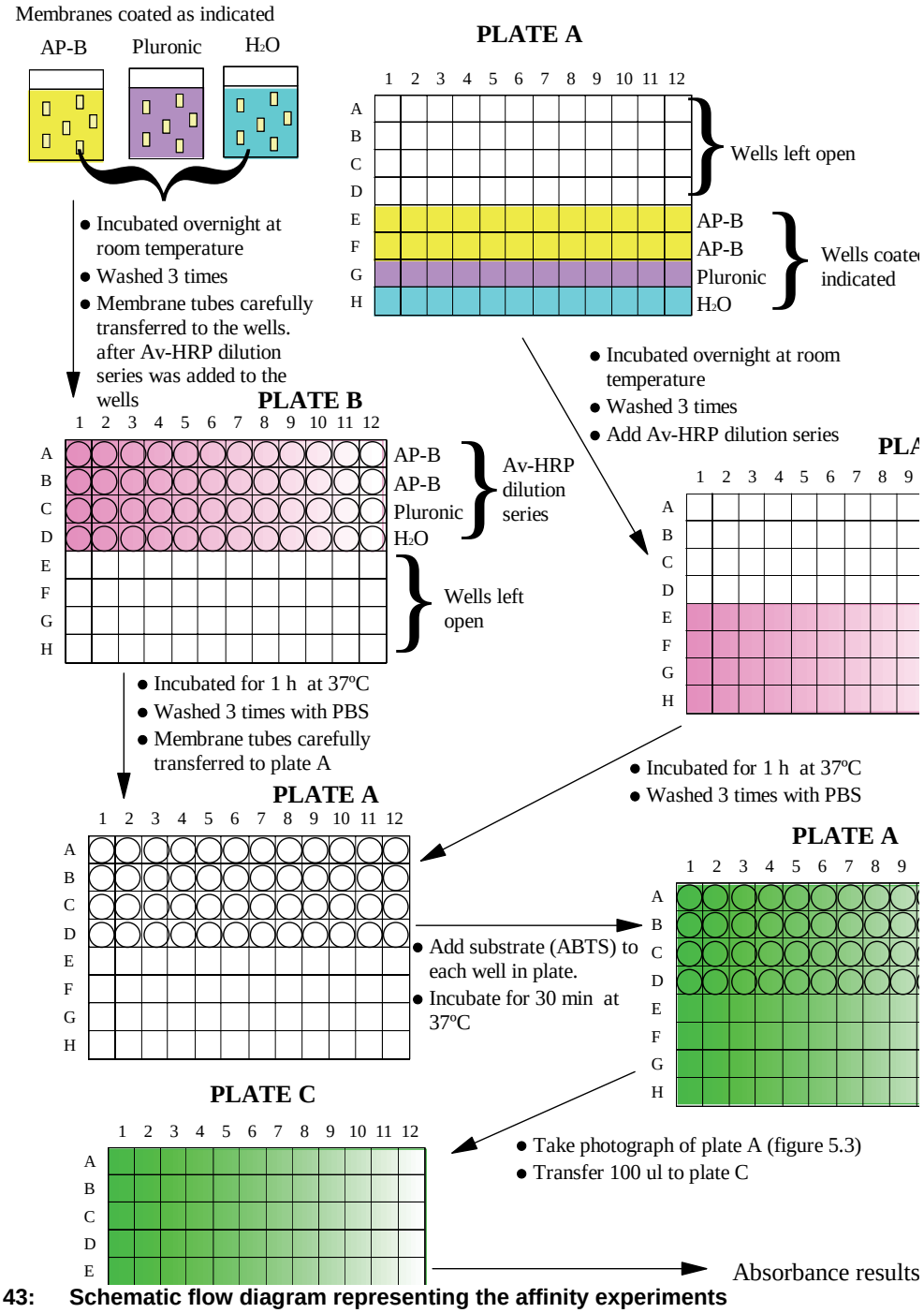


Figure 43: Schematic flow diagram representing the affinity experiments

Both micro titre plates were covered with cling-wrap and incubated at 37°C for 1 h to allow interaction between the immobilised biotin and the avidin-HRP. After incubation the wrap was removed and the solutions decanted from all the wells in both plates. All the wells were washed three times with PBS to remove all excess and unbound avidin-peroxidase.

The coated membranes, which were incubated with the avidin-HRP in the wells of rows A to D of plate B, were subsequently carefully transferred to the corresponding vacant wells of rows A to D in plate A. This step was necessary to ensure that the

activity of the immobilised HRP measured with the colour reaction came exclusively from avidin-HRP immobilised onto the membrane surfaces by biotin-Pluronic. Non-specific interaction of avidin-HRP with the wells was therefore excluded.

The substrate solution (200 µL) was subsequently added to all the wells of plate A. The plate was again covered with cling wrap and incubated at 37°C for 30 min. After 30 min the plate was uncovered and photographed (
Figure 15). From each well in plate A, 100 µL was transferred to a new micro titre plate (plate C) for the absorbance reading at 405 nm on a micro titre plate reader.

Appendix 3

EXPERIMENTAL PROCEDURE: CLONING

A3.1 Cloning

Plasmids are extrachromosomal genetic elements in microorganisms that replicate independently of chromosomal DNA. A plasmid is a small double-stranded, circular DNA molecule and is used for cloning vectors and for protein over-expression. An expression vector must have the following (an example is shown in Figure 44):

- a promoter (strong or weak) – induces the expression of the target protein;
- a terminator;
- a ribosome binding site (Shine Delgarno) – to initiate translation of the target protein; and
- control elements – it should be inducible.

Foreign DNA can be cut with a restriction enzyme, inserted into the plasmid and bacteria can take up the resultant recombinant plasmid. If the plasmid contains a selection marker (e.g. antibiotic resistance gene) only bacteria that contain the recombinant plasmid will grow in selective media. For example, the pET15b vector contains an ampicillin resistance marker, suggesting that only bacteria with the transformed plasmid will grow on media containing the ampicillin antibiotica. This procedure allows proliferation of target DNA in bacteria. The plasmid is recovered from the bacteria by a plasmid isolation procedure.

The vector construct used during these experiments, pET15b-hERαLBD, was a gift from Prof. B.S. Katzenellenbogen, University of Illinois at Urbana-Champaign. The target DNA, hERαLBD, was subcloned into the histidine-tagged expression vector, pET15b.

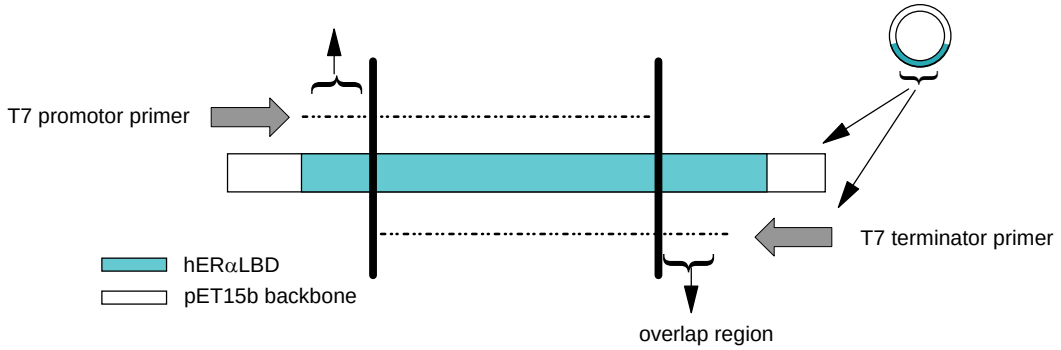


Figure 44: Characteristics of the expression vector, pET15b, which uses a promoter from phage T7

Protein expression is induced by the addition of the proper inducer or by changing the growth conditions. From this point on the cells will use most of their resources for the production of the target protein and will not grow much further.

The pET system is one of the most powerful systems developed for the cloning and expression of recombinant proteins in *E. coli*. The pET system involves not only an

expression vector, but also a genetically engineered host bacterium, typically *E. coli* strain BL(DE3). This strain has integrated into its chromosome the gene for T7 RNA polymerase. The T7 RNA polymerase in the host genome is constructed such that it is under the control of a lac promoter and operator. Thus, induction by the lactose analogue, isopropylthiogalactoside (IPTG), causes the host to produce T7 RNA polymerase. However, the *E. coli* host genome also carries the lacI (repressor) gene, which expresses the repressor protein. The repressor binds to the operator and blocks the binding of RNA polymerase. In the presence of IPTG, the repressor forms a complex with IPTG and does not bind to the operator thus, allowing the binding of RNA polymerase. Therefore, induction by IPTG, results in the following:

- derepression of T7 RNA Polymerase gene on host chromosome with subsequent production of this polymerase;
- derepression of target gene under lac O regulation; and
- transcription of target gene by T7 RNA Polymerase.

Protein expression is induced by the addition of the proper inducer (such as IPTG). These cells are incubated and then induced with IPTG, which binds to the repressor thereby blocking expression. This repressor blocking allows the T7 RNA Polymerase, synthesized from the DE3 phage DNA, to bind to the promoter for the hERαLBD gene and the target gene is synthesized. Thus, the pET15b-ERαLBD vector was allowed to express a histidine-tag fused with the hERαLBD in *E. coli* BL21(DE3) cells using the T7 expression system.

A3.2 Restriction enzyme digestion

Restriction enzymes are endonucleases that cut DNA at specific recognition sequences called “restriction sites”. Restriction enzymes recognize specific 4 to 8 base regions of DNA. For example, one restriction enzyme, Eco RI, recognizes the following six base sequences:

5' . . . G-A-A-T-T-C . . . 3'
3' . . . C-T-T-A-A-G . . . 5'

A piece of DNA incubated with Eco RI in the proper buffer conditions will be cut wherever this sequence appears. There are hundreds of restriction enzymes that have been isolated and each one recognizes its own specific nucleotide sequence. Sites for each restriction enzyme are distributed randomly throughout a particular DNA stretch. Digestion of DNA by restriction enzymes is very reproducible; every time a specific enzyme cuts a specific piece of DNA, the same pattern of digestion will occur.

Restriction enzymes are commercially available and their use has made manipulation and identification of DNA very easy. Restriction enzyme digestion involves digesting DNA with a series of restriction enzymes and then separating the resultant DNA fragments by agarose gel electrophoresis. The distance between restriction enzyme sites can be determined by the patterns of fragments that are produced by the restriction enzyme digestion. In this way, information about the structure of an unknown piece of DNA can be obtained by a specific “fingerprint” of the particular DNA being digested with specific restriction enzymes.

A3.3 Western blot analysis

After protein separation by electrophoresis, using an antibody that is specific to that protein, individual protein bands can often identify the protein. However, to be accessible to antibodies the proteins in the gel must first be transferred from the gel to a membrane. This process is called Western blotting (after E. M. Southern) and consists of the following steps (Figure 45):

Gel electrophoresis – The proteins of the sample are separated according to size on a gel using SDS PAGE. The gel has several lanes so that several samples can be tested simultaneously and each lane contains one or more protein bands.

Nitrocellulose transfer – The proteins in the SDS PAGE gel are then transferred onto a solid membrane made of nitrocellulose or PVDF, by pressure or by applying a current. This is the actual blotting process necessary to expose the proteins for non-specific binding with the membrane (membrane binds all proteins equally well). For this procedure, an electric current is applied to the gel so that the separated proteins transfer through the SDS PAGE gel and onto the membrane in the same pattern as they separate on the SDS PAGE. Although the types of bonds that hold proteins to nitrocellulose are not known, oils or other proteins block the binding. All sites on the membrane that do not contain blotted protein (known as the antigen) from the gel can then be non-specifically blocked so that the antibody will not non-specifically bind to them, causing a false positive result.

Blocking – The membrane is then *blocked* by a solution of BSA or dry milk thereby suppressing non-specific protein interactions. Without this blocking step, the antibody to be applied in the next step would bind to the nitrocellulose.

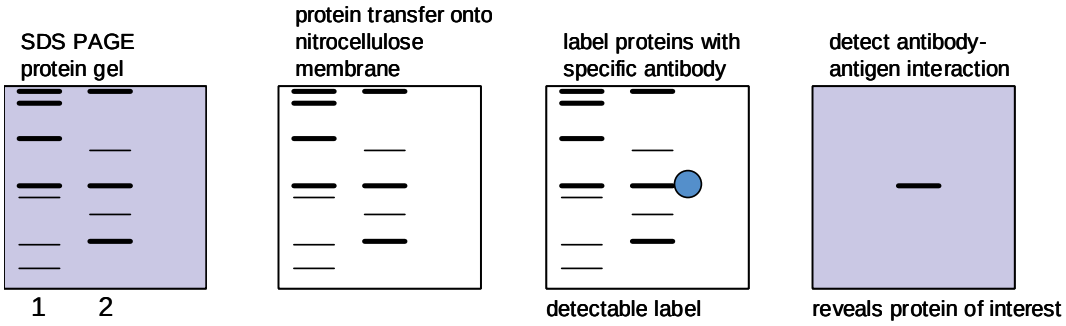


Figure 45: Schematic diagram depicting the steps during Western blot analysis

The *first antibody* is applied that recognizes only the protein of interest and will not bind any of the other proteins on the membrane. It is obtained by immunizing an animal (usually a rabbit or goat) with the protein of interest (i.e. injecting the protein into the animal's blood) and collecting the antibodies the animal produces against that protein. The *second antibody* is applied which binds to the first antibody and is usually produced by a different animal (e.g. goat anti-rabbit antibody might be used if the first antibody was produced by rabbits). This second antibody is linked to a chemical signal that can visually identify where on the membrane it has bound. Similar to the ELISA procedure, this chemical signal is often an enzyme (e.g. HRP) which can produce fluorescence in its substrate. Free antibody is washed away and a substance is added to the membrane so that the second antibody will become visible. Since the first antibody only recognizes the protein of interest and the second

antibody only recognizes the first antibody, then, if there is stain present on the membrane, the protein of interest must also be present on the membrane. Thus, the protein bands on the membrane that are stained contain the protein that was to be detected, the other bands do not. Size approximations can be done by comparing the stained bands to that of a pre-stained protein marker.

Appendix 4

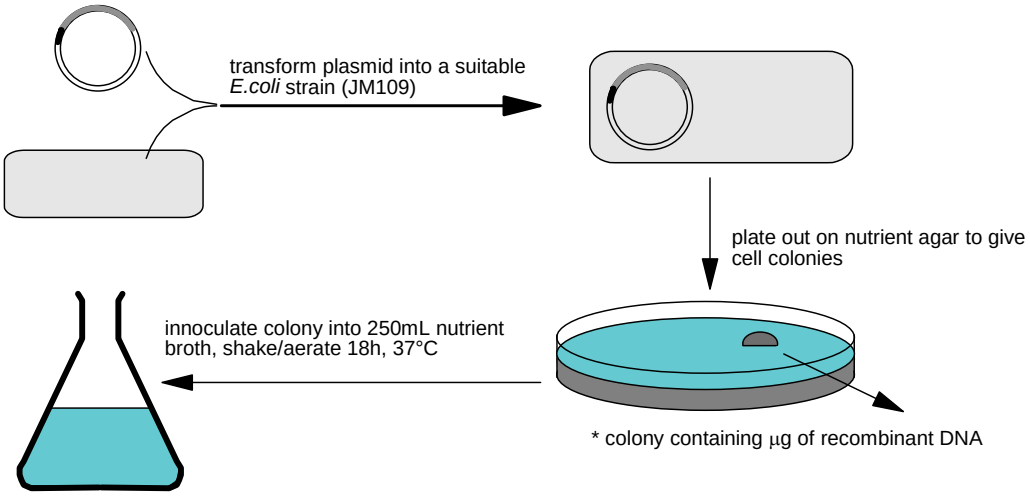
EXPERIMENTAL PROCEDURES: EXPRESSION OF TARGET PROTEIN

All chemicals used were analytical grade and were purchased from Sigma Chemical Co., Merck (Darmstadt, Germany), Saarchem or Pierce Chemical Co. (Rockford, IL). Restriction enzymes were obtained from Sigma Chemical Co. and Promega. DNA-modifying enzymes (DNase I, RNase A) were obtained from Amersham (Buckinghamshire, UK). The plasmid pET15b containing the ligand binding domain of human ER was a generous gift from Dr. B.S Katzenellenbogen (Department of Physiology & Biophysics, University of Illinois, Urbana-Champaign, IL).

A4.1 Amplification of target DNA (hERαLBD): Non-expression host organism

The pET15b vector, containing the hERαLBD, was transformed in a non-expression host organism, *E. coli* JM109. This organism will only amplify the target plasmid and not initiate expression of the target protein.

hERαLBD was subcloned into the plasmid, pET15b
↓
transformation into a non-expression host organism, *E. coli* JM109
↓
identification of cells containing pET-hERαLBD
↓
isolation of plasmid DNA (pET-hERαLBD)
↓
verification of target DNA



* provides several mg recombinant DNA - can be isolated using a plasmid preparation

Figure 46: Experimental procedure for the amplification and selection of plasmid containing the target DNA in a non-expression host organism

A4.2 Verification of target DNA (hERαLBD): Non-expression host organism

The hERαLBD DNA insert was verified with the use of various restriction enzyme digestions, gel analyses and DNA sequence analyses.

A4.2.1 Restriction enzyme digestion

The hERαLBD was previously subcloned into the pET15b plasmid with the use of BamH I. Different restriction enzymes (EcoR I, Nco I, HinD III and Xba I) were used on the pET15b-hERαLBD construct, which cut the plasmid as well as the insert only once (Figure 47).

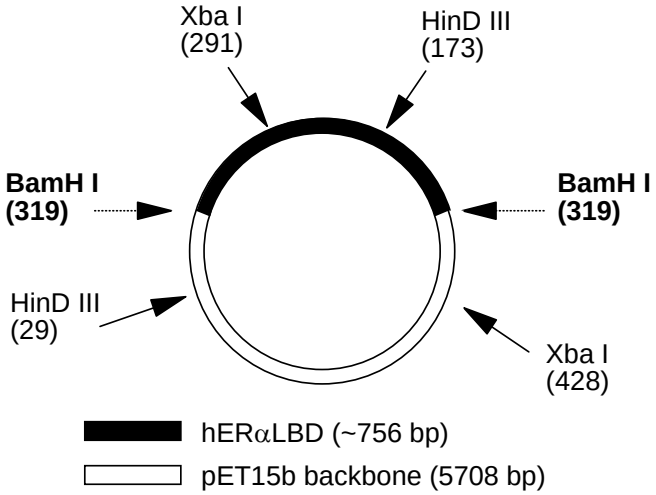


Figure 47: Some of the restriction enzyme sites of pET15b and the hERαLBD

A4.2.2 Gel analysis

Electrophoretic separation is based upon the different size of each DNA fragment where the smallest molecules will move the farthest from the well.

Agarose gel analysis (1.2% agarose, separates 400-7 000 bp) was performed to determine the size of DNA fragments after restriction enzyme digestion. The DNA insert can thus be confirmed from the known sizes of the pET15b vector and hERαLBD, which are approximately 5 708 bp and 756 bp, respectively. As a control, the undigested plasmid is also included, which has three distinct bands, that depict the following:

- closed circular DNA (form-I); typically supercoiled;
- nicked circular (form-II); and
- linear DNA (form-III)

A4.3 DNA sequence analysis

DNA sequencing is the process of determining the exact order of the chemical building blocks (bases A, T, C, and G) of a desired DNA fragment. By comparing the annotation to known DNA sequences, the target DNA insert can be verified. During

this process two short DNA fragments (known as primers) adhere to sites flanking the DNA segment of interest (i.e. hERαLBD insert in the pET15b plasmid). The enzyme DNA polymerase amplifies the DNA between the primers, resulting in the generation of the target DNA (Figure 48). The primers used were the T7 promoter primer and the T7 terminator primer (Whitehead Scientific), which had the following sequences:

T7 Promoter TAATACGACTCACTATAGGG
T7 Terminator GCTAGTTATTGCTCAGCGG

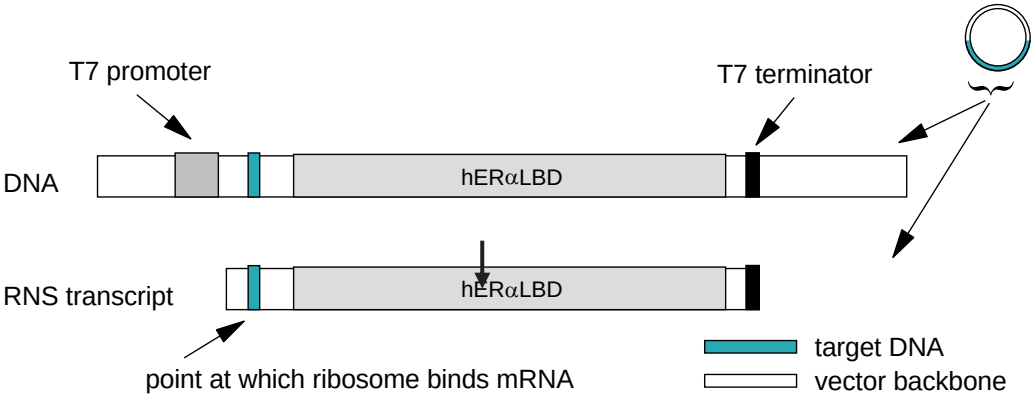


Figure 48: Experimental procedure for the sequencing of the target DNA

A4.4 Induction and expression of target protein

After positive identification of the target DNA, the positive clones were transformed into expression host organisms, *Escherichia coli* BL21(DE3) and BL21(DE3)pLysS (Novagen, USA) (Figure 49).

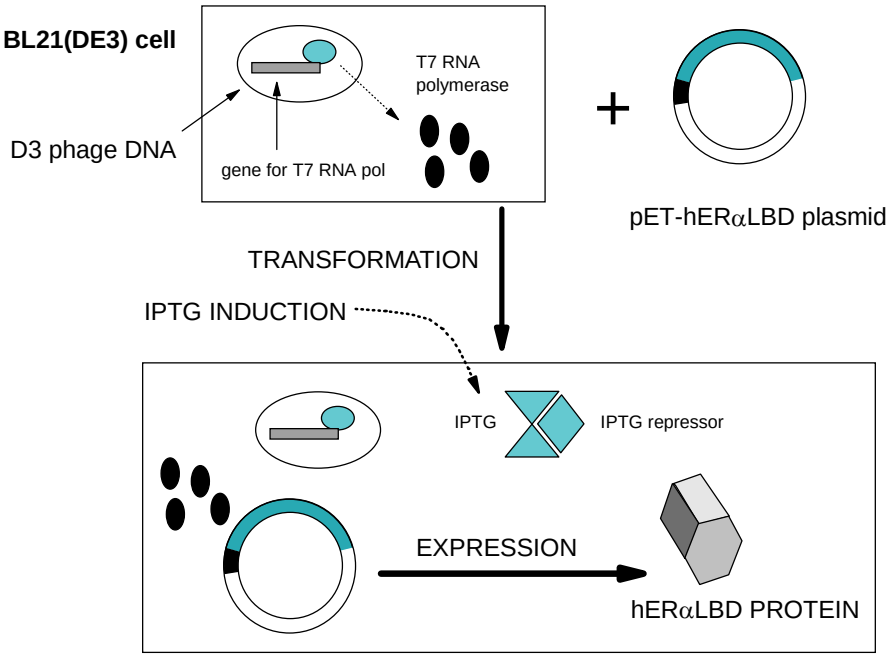


Figure 49: Transformation of the positive clones and induction of the hERαLBD protein in an expression host organism, *E. coli* BL21(DE3) strain

Plasmid pET15b-hERαLBD, the bacterial expression vector containing the human ER ligand binding domain (hERαLBD, amino acids 302 to 553) gene, fused with the N-terminal polyhistidine sequence, has been constructed previously. This vector was expressed in the hERαLBD in *E. coli* BL21(DE3 and BL21(DE3)pLysS cells using the T7 expression system. *E. coli* cells containing pET15b-hERαLBD were grown overnight at 37°C in 4 mL of Luria Broth supplemented with 200 μg/mL ampicillin and 34 μg/mL chloramphenicol. After overnight growth, the cells were diluted 10-fold in fresh medium containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol. The outgrowth was allowed to proceed to an optical density of 1.0 at 600 nm. Recombinant protein production was initiated by the addition of isopropyl thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubation was continued for 4 h, 6 h and overnight. The cells were collected by centrifugation (4,000 x g) for 20 min at 4°C. The pellet was resuspended in 8 mL of cold lysis buffer (50 mM NaH_2PO_4, NaOH (pH 8.0), 5 mM β-mercaptoethanol, 0.1 mM PMSF, 5 mM NaMBS). The resulting suspension was frozen at 80°C and then thawed at 37°C. Following sonication on ice (15 s bursts, 200 W), DNase I and RNase A were added to a final concentration of 5 mg/mL and 1 mg/mL, respectively. The mixture was incubated for 30 min at 4°C. Triton X100 was added to the final concentration of 0.1 % and NaCl to a final concentration of 300 mM. Cell fragments and insoluble material were removed by centrifuging (20,000 x g) for 20 minute at 4°C. The supernatants (soluble material) were kept at 4°C for further analysis. The same procedures were followed for uninduced samples (no addition of IPTG), which were used as controls during SDS PAGE and Western blot analysis.

A4.5 Western blot analysis

Evaluation of expression was accomplished by SDS PAGE and Western blot analysis. The protein content of the samples was measured using the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL) with bovine serum albumin as a standard. SDS PAGE (15 % gel) was used for the separation of proteins (20 μg) according to size. The gels were either stained with Coomassie Brilliant Blue or transferred to a nitrocellulose membrane in a semidry blotting apparatus for Western blot analysis using the INDIA™ HisProbe-HRP (Pierce) immunoblotting protocol. After electrotransfer of proteins from the SDS PAGE gel to the nitrocellulose membrane for Western blotting, the filter was blocked and incubated with the anti-histidine horseradish peroxidase conjugate. The bound antibody was detected with SuperSignal® West Pico Chemiluminescent reagents (Pierce) using a molecular light imager.

Appendix 5

MEMBRANE SURFACE CHARACTERISATION

A5.1 Introduction

Polymeric membranes are widely used in chromatographic areas for separation and purification of proteins from biological fluids. The performance of an affinity membrane depends on its surface properties. The surface properties of an affinity membrane may affect the adsorption capacity and its behaviour by controlling adsorption of proteins present in the liquid phase.[142,143] In particular, non-specific protein adsorption and bio-specific affinity adsorption is dependent on the surface composition and morphology of the affinity membrane.

There are numerous reports that protein and cell adsorption on membrane surfaces are quantitatively changed, depending on the type of ligands immobilised.[144,145] The adsorption of proteins is a complex and poorly understood process, which can be determined by several factors. Among these the chemical structure, surface roughness, degree of surface hydrophobicity, electrostatic interactions of the protein molecules with each other and with the surface and the structural stability of protein molecules are the most important.[146]

Therefore, the surface properties of a membrane, which is being used as an affinity matrix must be characterised to explain its interactions with proteins and its suitability for a particular application. Characterisation methods can be conveniently divided into two areas, for porous and non-porous membranes. Typically would a porous membrane be characterised for its filtration properties while non-porous membranes are of dense polymeric composition, without pores and separation is thus dependent upon the material properties and morphology.

Non-porous membranes (such as those used in this study) are surface functionalised with ligands, and in many cases the chemical nature of this functional layer or surface is not precisely known and surface analysis is normally required. In general, the field of surface analysis is vast and rapidly growing, requiring specialised equipment and skilled operators. It is largely the surface matrix under investigation and the information required that determines the surface analysis technique. These techniques include proximal probes, electron microscopy, electron spectroscopy, ion beam analysis, X-ray techniques and optical analysis. This has presented an enormous challenge since locally we either lack the instrumentation and or the skilled operators to adapt existing instruments for specific applications.

142 MY Arica, G Bayramoglu, Affinity mebrane chromatography, J. CHROMAT. B. 805 (2004) 315-323.
143 G. Bayramoglu, M.Y. Arica, Evaluation of lysozyme behaviour in related to the surface energy and its components in chromatographic fields, COLL. SURF. A. 243 (2004) 11-21.
144 S. Belfer, Y. Purinson, O. Kedem, Surface characterization by ATR-FTIR of PES membranes, J. MEM. SCI. 172 (2000) 113- 124.
145 C. Zhao, X. Liu, N. Nishi, Surface characterization of PSU membranes by DNA immobilization, J. MEM. SCI. 214 (2003) 179-189.
146 K. Nakanishi, T. Sakiyama, K. Imamura, On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon, J. BIOSCI. BIOENG. 91(1) (2001) 233-244.

The surface analysis of ligand modified and unmodified non-porous membranes in this study attempts to use locally available solid state techniques to generate information that can contribute to the understanding of both the native membrane surface and the chemical state of the ligand termini that in practise will be present in a hydrophilic brush layer on the membrane surface.

Ligand modified Pluronic involves the conversion of the hydroxyl end groups to primary amine equivalents for the covalent attachment of the affinity ligand. Synthesis of ligand terminated Pluronic in the lab frequently involves the activation of the oxygen functionality to enhance its capacity as a leaving group. Amine terminated Pluronic at the outset of this study was a time consuming process, due to the large quantities required for adsorption and membrane characterisation experiments. The amino terminus was also not a suitable chemical constituent for solid-state analysis due to its low backscattering potential and proximity to O and C in both the membrane and Pluronic. In order to model a covalently attached affinity ligand a halogen derivative was considered for both adsorption experiments and ion beam and x-ray analysis.

In this study, scanning electron microscopy and atomic force microscopy were used to generate electron and force images of native and Pluronic modified membranes. A halogenated Pluonic derivative (Pluronic-bromide) was synthesized to model an affinity ligand, using a synthesis scheme that was very similar to many of the derivatives that will be described in other parts of this report. The three candidate membranes (PSU, PVDF and PEI) were non-covalently modified with the halogenated derivative and ion-beam and x-ray analysis was then performed on these surfaces so that accurate solid state measurements could be performed that would generate reliable and accurate data. Of particular interest was the coating homogeneity of the Pluronic and the potential number of ligand binding sites.

A5.2 Results and discussion

A5.2.1 Brominated Pluronic

In order to verify these data for PSU and to actually generate data with PVDF and PEI, it was decided to coat the membranes with derivatised Pluronic carrying a ‘heavy’ element such as Br covalently coupled to the hydroxyl terminus of PEO. (The coupling of Br to Pluronic is detailed in the next section under synthesis of derivatised Pluronic). This should theoretically give a high signal to background ratio. Another advantage would be that since the Br is coupled to the hydroxyl group, which is the functional group that is modified to accept a ligand in affinity chromatography; RBS would be able to accurately determine the number of ligand binding sites on a saturated 1 cm^2 of membrane by measuring the energy loss of the Br.

NMR Results

^{13}C-NMR ($CDCl_3$): δ 17.05 and 17.18 [2q, $(CH_2\text{-}CH(CH_3)\text{-}O)_n$]; 29.44 (t, $\text{-}CH_2\text{-}Br$); 70,46 (major signal); (t, $\text{-}CH_2\text{-}CH_2\text{-}Br$); 72.74, 72.78, 72.82, 72.87 (smaller signals); 73.28 (major signal) [5t, (CH_2-$CH(CH_3)$-O)n]; 75.03, 75.24, 75.27 and 75.44 [4d, $(CH_2\text{-}CH(CH_3)\text{-}O)_n$]

Electron Microscopy

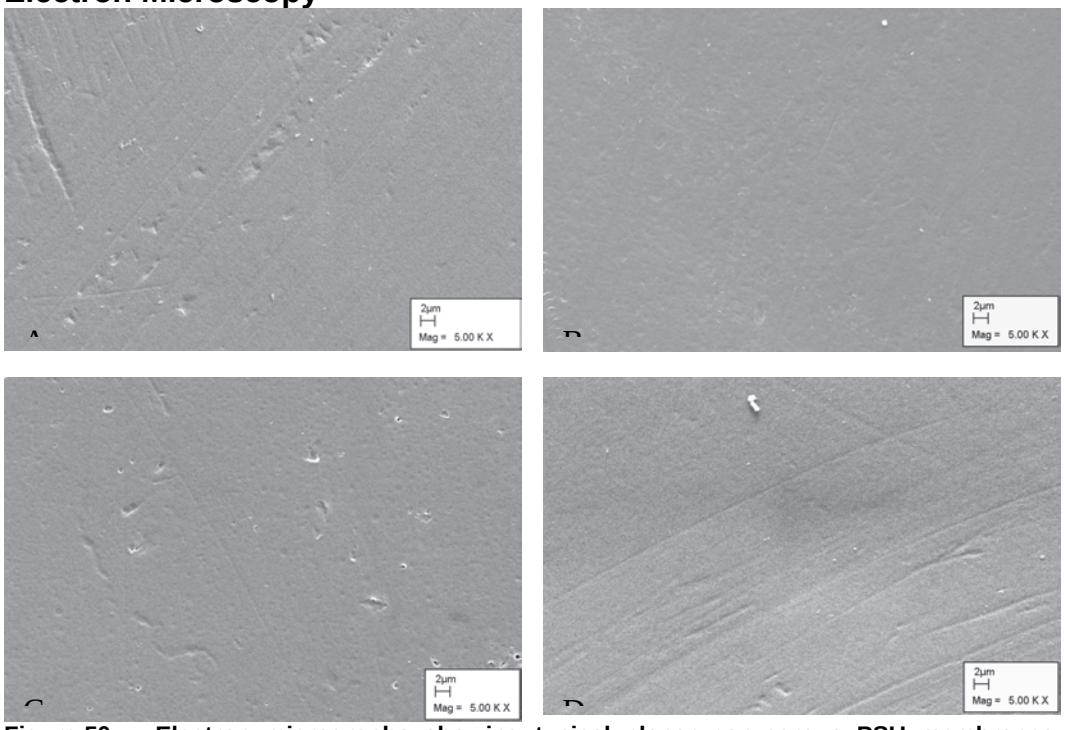


Figure 50: Electron micrographs showing typical planar non-porous PSU membranes that were used in Pluronic coating and desorption. a) Native PSU membrane; b) Pluronic coated PSU, c) SDS displacement of Pluronic treated membranes, and d) Hexane-isopropanol treated membranes modified with Pluronic. Manification = 5000X

The electron micrographs in Figure 50 show the membrane surface of PSU membranes. The native PSU membrane in Figure 50a is typical of the planar non-porous membranes prepared in this study. The surfaces are generally very rough compared with the polished silica surfaces that are conventionally used for force and ion beam analysis. This is unfortunately inherent to the fabrication protocol, but is nevertheless not an undesirable factor for membrane adsorption.

Figure 50b suggests that Pluronic coating of the PSU surface changes the microscopic appearance of the membrane surface and the coating at 5mg/mL appeared uniform over the surface. SDS displacement of Pluronic appeared very effective, as the native surface could once again be observed. The hexane-isopropanol extraction of Pluronic depicted in Figure 50d seemed much more effective. Figure 50d lacked the electron dense structures in Figure 50c, which suggests both efficient Pluronic removal and lack of surface deposits such as SDS micelles.

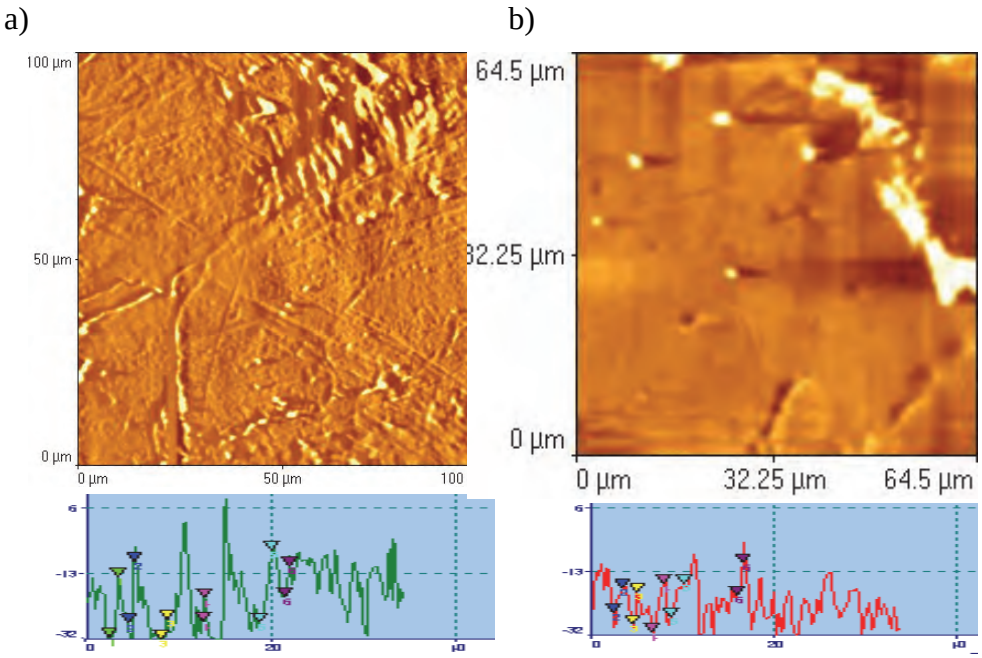


Figure 51: Atomic force micrographs showing a) Native PSU membrane and b) Pluronic modified PSU surface.

The atomic force micrographs in Figure 51 and the attendant force curves suggest that the average surface roughness decreases with Pluronic modification of the surface. A similar trend was observed with PVDF and PEI membranes (results not shown).

Rutherford backscattering spectroscopic analysis

Samples need to be conductive to generate RBS spectra, so a thin layer of Ni was coated onto flat sheet membranes fouled with Pluronic as previously described. Since the chemical composition of Pluronic and the membranes were so similar, this posed a problem for measuring the adsorbed layer thickness. The only elemental difference for PSU, PVDF, and PEI with respect to Pluronic was S, F and N respectively. Unfortunately the elemental composition of Pluronic (C, H, O) prevented depth measurement using conventional RBS methodology. In an attempt at measuring the layer thickness of Pluronic it was decided to coat a very thin but uniform layer of Ni (not more than 50 Å onto the samples. Backscattering spectra of Ni was measured in relation to the S, F and N in the three types of membranes.

All experiments were conducted in triplicate and an unfouled membrane was used as a control. Differences in eV readings could be used to measure the adsorbed layer thickness of Pluronic. Other information hoped to be retrieved using this modified approach was the coating homogeneity of the fouling process. From an analytical point of view, RBS compatible materials are heavier thin-films on lighter substrate materials. Heavier thin films give high intensity RBS signals free of background. RBS measurements of 'lighter' elements such as the C and O in Pluronic and the S (PSU), F (PVDF) and N (PEI) in the respective membranes are more difficult due to low signal – to background ratio.

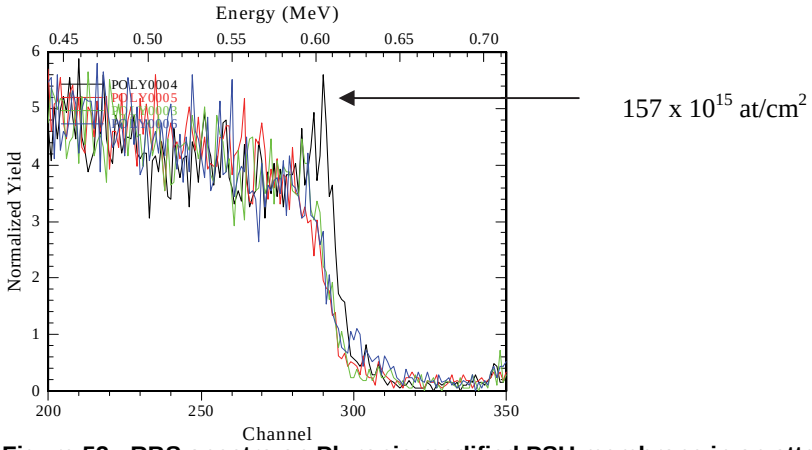


Figure 52: RBS spectra on Pluronic modified PSU membrane in an attempt to measure adsorbed layer thickness

Figure 52 represents an RBS spectrum of a Ti coated Pluronic modified PSU membrane. Computer simulations using a Rutherford universal modification program (RUMP) which is a standard program specifically designed for analysis and simulation of RBS data, was used to calculate the optimum coating for soft, non-homogenous non-porous membranes. Titanium was found to give the best simulation with a thickness of 90 nm. Accurate coating was done with a sputter coater coupled to an electronic thickness monitor.

RBS could not generate reliable data to measure the layer thickness on PVDF and PEI membranes due in large part to the difficulty in discriminating the F and N peaks from the C and O background in the Pluronic. However, if we assumed that Pluronic formed a monolayer after adsorption and washing in deionised water, the thickness would have to be in the order of μm to be reliably measured. RBS is generally not suited to measuring depths in the nm range. Coating homogeneity data was only generated for PSU since the S was able to give an appreciable backscattering yield compared with the lighter elements F and N where no significant energy loss was detected. To explain the 157.07 keV difference (612.6-606.4 keV), between PSU poly 004 (control) and the Pluronic coated PSU membranes, requires a layer with an assumed average composition of (C:H:O = 7:16:5) to be 157 x 10^{15} at/cm^2 thick. Thus for an assumed densely packed monolayer, this would suggest PPO strands with a nearest neighbour distance of about 4.7 nm/cm^2.

In order to develop a method for the solid state RBS quantification of ligand binding sites on derivatised Pluronic and or a metal chelating Pluronic, a halogenated derivative of Pluronic was synthesised. Halogenation of Pluronic was accomplished by covalently coupling bromine to the hydroxyl group of Pluronic. Although there are several methods for the direct conversion of the hydroxyl group with a halogen, an indirect approach using the 'established' method of tosylation reaction (Yanic *et al.*, 2000) was opted for.

Preliminary results depicted in Figure 53 show solid-state detection of Pluronic-Br corresponding to approximately 1 to 5 x 10^{14} Br atoms/cm^2.

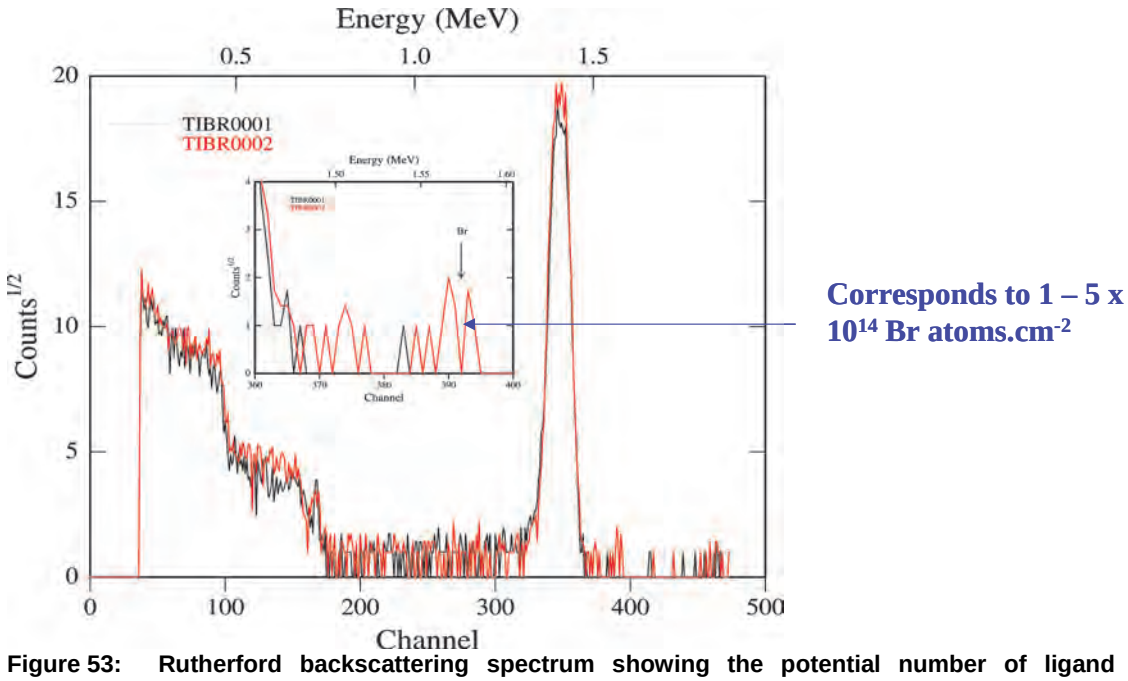


Figure 53: Rutherford backscattering spectrum showing the potential number of ligand binding sites on derivatised Pluronic using the model Pluronic-Br

The protocols developed here were based on initial experiments performed under emperical conditions. Generally counts per channel were too low to give statistically reliable data. The best fit in Figure 53, was 1 – 5 x 10^{14} Br atoms per cm^2 using backscattered αHe particles. This equates to a nearest neighbour Br distance of 0.8 nm under optimised conditions.

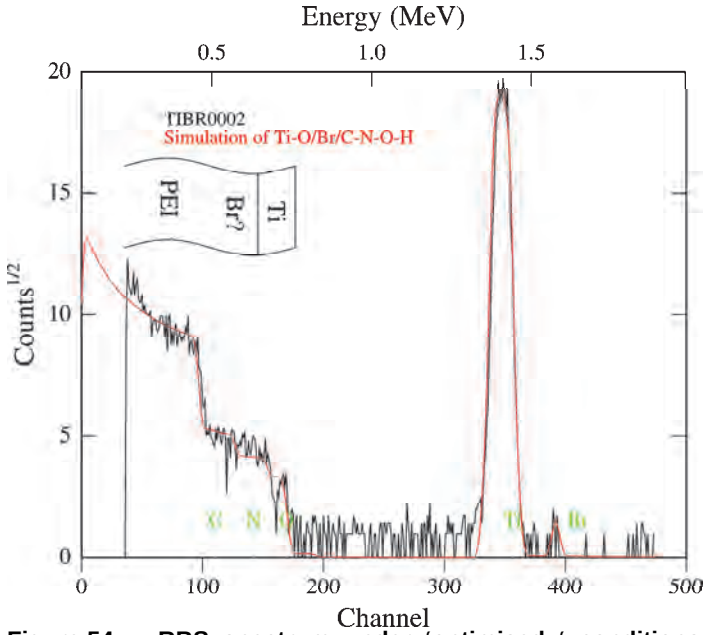


Figure 54: RBS spectrum under 'optimised ' conditions of a PSU membrane coated with Pluronic-Br (5.0 mg/mL)

Figure 54 represents an RBS sprectrum performed under the optimised conditions calculated from data generated with Figure 53. The conditions used to generate data with up to 90% confidence levels was achieved using a current of 2 nA at 20 000 μC and 1 MeV. Higher currents caused overheating of the polymer so the reduced current extended the analysis time to 167 min per sample. This was very labour

intensive and could be viewed as a practical difficulty with RBS analysis of Pluronic modified polymers.

A PIXE analysis of the sample then showed a Br concentration of 1199.2 ng/cm^2 (Table 12).

Table 12: PIXE analysis of PSU membranes modified with Pluronic-Br

	Element	Concentration (ng/cm^2)	% Stat
Negative control	^{35}Br	23.8	37.18
Membrane-bound Pluronic-Br (1mg/mL)	^{35}Br	519.1	3.64
Membrane-bound Pluronic-Br (5 mg/mL)	^{35}Br	1199.2	1.79

X-Ray Photoelectron Spectroscopy

By fitting C1s spectra, the ratio of ether carbons (C-O) to total carbon atoms (C-O + C-H + C-C) can be calculated for PVDF, which has no surface ether or ester groups. The activity of this membrane for different interfacial layers such as Pluronic, brominated Pluronic and or a metal chelating Pluronic can be estimated. XPS in this regard is being used as a complimentary technique for RBS and PIXE.

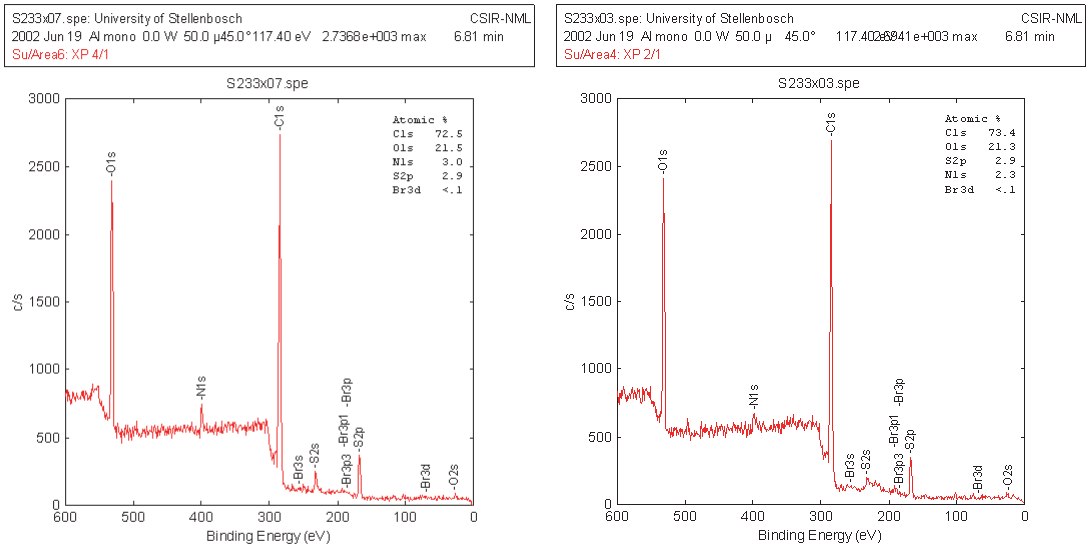


Figure 55: X-Ray analysis of PSU membranes modified with a) Pluronic and b) Pluronic-Br

XPS was used to quantify the O1s/C1s ratios, which indicate the C-O bonds in the sample. With respect to Pluronic-Br adsorbed onto PVDF, the C-O bonds could be attributed to C-OH and C-O-C (i.e. the hydroxyl carbons and ether carbons respectively). Changes in O1s/C1s ratios would then indicate the amount of -OH groups that were derivatised with the Br terminus.

The O1s/C1s ratios indicate C-O bonds. This is due to C-OH; C-O-C. By subtracting Figure 55b from Figure 55a (negative control with most of the background ether groups). This will then give C-O and C-O-O contribution by the adsorbed Pluronic. The levels of Br in the sample however fell below the detection limits of XPS.

Appendix 6

EXPERIMENTAL PROCEDURES:ANALYTICAL AND SYNTHESIS

A6.1 Reagents and Chemicals

Pluronic® F108 was obtained from BASF (Germany). Unless otherwise stated all chemicals and reagents were purchased from Merck NT laboratory suppliers, SA. The ammonium ferrothiocyanate assay solution was prepared by dissolving 30.4g NH_4SCN from Protea Holdings Ltd., SA and 27.03g $FeCl_3.6H_2O$ (Sigma-Aldrich Chemical Company, SA) in deionised water made up to 1L. The deionised water was purified with a Milli-Q water purification system from Millipore. Unless otherwise stated all reagents were purchased from Sigma-Aldrich Chemical Company, SA.

Bovine serum albumin (BSA) and lysozyme (Roche) were used as model protein adsorbates and were reconstituted in 0.25 mg/mL solutions in 0.1 M phosphate buffer, pH 7.4. The pI values of BSA and lysozyme are 4.8 and 10.45 respectively, and after correcting the pH to 7.4, a negative charge was induced to BSA while lysozyme was rendered positive. SDS (Merck, Darmstad, Germany) was used as a stripping agent.

A6.2 Analysis and spectroscopy

A6.2.1 Inductively coupled plasma (ICP)

Analysis was performed on a Liberty Series II radial emission ICP-atomic emission spectrometer. All reagents used in this work were of commercial high purity grade. The water used was Milli-Q Water, prepared by further purification of de-ionised water by a Milli-Q system. Samples were dissolved in Milli-Q water (min. 10 mL).

A6.2.2 Proton induced X-ray emission

Proton induced X-ray emission (PIXE) was used to quantify the number of Br atoms on the surface of F108-Br adsorbed membranes. The micro-PIXE beam line at the van de Graaf facility was used. A mass 2.0 MeV H^+ beam was focused and collimated to a 100 um beam spot. An ORTEC intrinsic high purity Ge detector was used to detect the X-rays at 135º to the beam direction.

A6.2.3 Nuclear magnetic resonance (NMR) spectroscopy

NMR analysis was done using a Varian VXR 300 NMR spectrometer. 40.2 mg Pluronic-iodide was dissolved in deuterated chloroform and subjected to ^{13}C analysis.

A6.2.3 Ion beam analysis

RBS

A Varian sputter coater with a high vacuum evaporator system and a control unit for online thickness monitoring was used to coat the samples with 50 Å Ni at a rate of 4.6 Å/s with a current strength of 100 mA. The beam energy used for RBS was 1 MeV with the He^{2+} backscattering measured at an incident angle set at 0° with the

backscattering angle at 15°. SiO_2 and Pt/Si were used as reference standards. The RUMP source code was used to analyse the RBS data.

PIXE

Proton induced X-ray emission (PIXE) was used to quantify the number of Br atoms on the surface of F108-Br adsorbed membranes. The micro-PIXE beam line at the van de Graaf facility was used. A mass 2.0 MeV H^+ beam was focussed and collimated to a 100 μm beam spot. An ORTEC intrinsic high purity Ge detector was used to detect the X-rays at 135° to the beam direction.

X-Ray Analysis

The XPS instrument used in this study was a PH1 5300. An Al Kα with a radiation of 1 486.6 eV was used as primary X-ray source, which was obtained by an acceleration voltage of 15 keV and an emission current of 20 mA. Two different angles (20 and 70°) between the sample surface and the position of the analyser were used to measure the photoelectrons. At low angles, the analyser only detects the photoelectrons coming from the outermost surface, while at larger angles (70°) information from those electrons that are coming from regions deeper inside the surface layer, is also included. In this way, no sputtering is required to obtain depth information and thus eliminates the possibility of sputter-induced modification of the surface layer.

Atomic force microscopy

Atomic force microscopy (AFM) has already been applied extensively for the characterisation of surface adsorbed Pluronic[147] and of microfiltration, ultrafiltration, nanofiltration and gas permeable membranes. In the tapping mode, the cantilever is oscillated vertically at high frequency during raster scanning. A topographical map was obtained by scanning a silicon nitride tip attached to a cantilever over the air-dried membrane surface, while maintaining a constant force between the tip and the sample. The deflection of the tip and cantilever, was measured optically by a reflected laser light beam off the back-face of the cantilever (AFM, TMX 2000 Explorer, Topometrix, Santa Barbara, CA).

Electron microscopy

A Leo 1430VP scanning electron microscope (SEM), fitted with backscatter, cathodoluminescence, variable pressure and energy dispersive detectors, as well as a Link EDS system and software for microanalysis and qualitative work was used to generate images of the membrane surfaces.

A6.3 Contact angle analysis

Contact angles for the planar membranes were calculated using the sessile drop technique.

A6.4 Protein determination

Protein concentration was measured with the Pierce protein assay kit, using bovine serum albumin as standard.

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147 K Eskilsson, LM Grant, F Tiberg, LANGMUIR. 15 (1999) 5150-5156.

A6.5 Cleaning regime

Flat sheet and capillary membranes (1 cm^2 external surface area) were stored in 0.04 M sodium azide to prevent microbial growth. Prior to adsorption, membranes were washed overnight in sterile, deionised water, followed by three further washes with deionised water. Membranes were then sonicated three times in sterile deionised water in an ultrasonic bath for 5 min followed by drying in a laminar flow cupboard.

A6.6 Synthesis of Pluronic derivatives

A6.6.1 Synthesis of Pluronic-iodide

p-Toluene sulphonyl chloride (19.06 g) was added to a solution of Pluronic® F108 (14.6 g) in dry pyridine (50 mL) at ambient temperature. The reaction mixture was cooled to 4°C and retained for 7 d. The reaction mixture was slowly poured into rapidly stirring ice and water (150 mL). The mixture was then extracted with chloroform (4 x 100 mL). The combined chloroform ($CHCl_3$) extracts were then washed with hydrochloric acid (6M, 150 mL) then deionised water (100 mL), dried over K_2CO_3-Na_2SO_4, evaporated under high vacuum (0.04 mmHg) at ambient temperature for 2 h to give Pluronic – tosylate. Lithium iodide (2.658 g) was added to a solution of tosylated Pluronic (2.908 g) in dry DMF (20 mL) and heated to 100°C under an Ar atmosphere for 3 h. The reaction mixture was treated with HCl (3M; 50 mL) and extracted with chloroform (3 x 100 mL). The combined chloroform extracts were evaporated overnight in a freeze dryer to give polyoxyethylene (di-iodide)-block-polyoxypropylene (Pluronic-iodide). The structure of Pluronic-iodide was confirmed by ^{13}C nuclear magnetic resonance (NMR) spectroscopy using a Varian VXR 300 NMR spectrometer.

A6.6.2 Brominated Pluronic

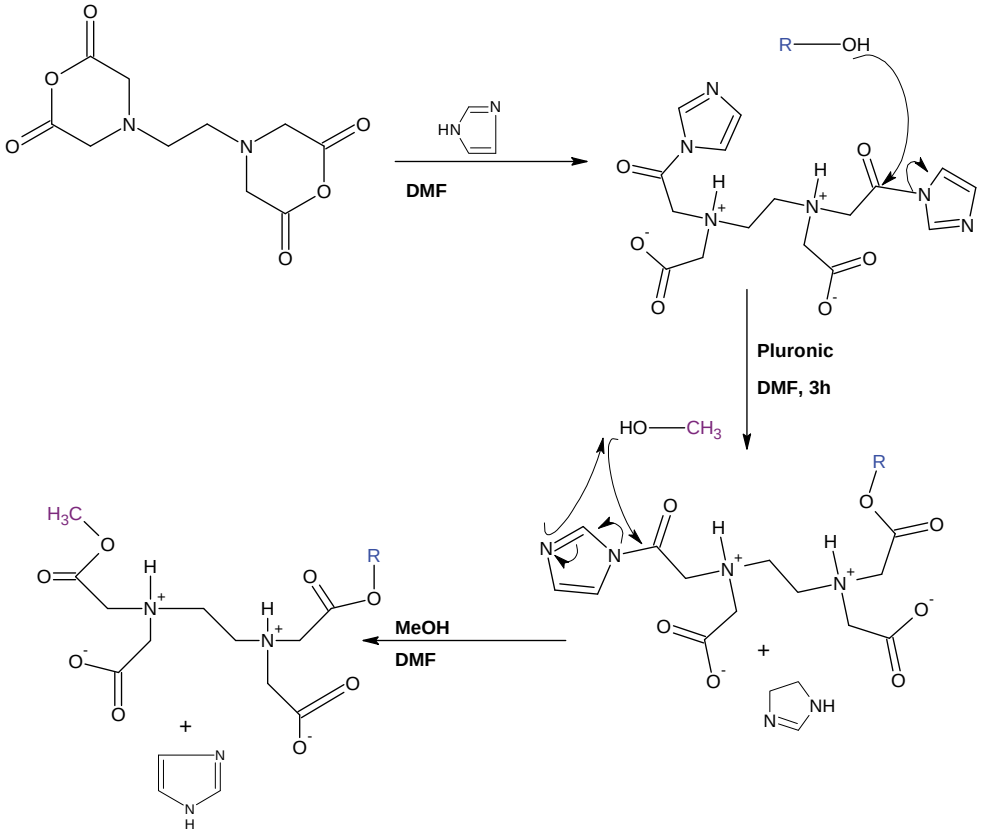
p-Toluene sulphonyl chloride (19.06 g) was added to a solution of Pluronic (14.6 g) in dry pyridine (50 mL) at ambient temperature. The reaction mixture was cooled to 4 °C and retained for 7 d. The reaction mixture was slowly poured into rapidly stirring ice and water (150 mL). The mixture was then extracted with chloroform (4 x 100 mL). The combined chloroform extracts were then washed with hydrochloric acid (6M, 150 mL) then deionised water (100 mL), dried over K_2CO_3-Na_2SO_4, evaporated under high vacuum (0.04 mmHg) at ambient temperature for 2 h to give Pluronic – tosylate.

Lithium bromide (2.658 g) was then added to a solution of tosylated Pluronic (2.908 g) in dry DMF (20 mL) and heated to 100°C under an Ar athmosphere for 3 h. The reaction mixture was treated with HCl (3M; 50 mL) and extracted with chloroform (3 x 100 mL). The combined chloroform extracts were evaporated overnight in a freeze dryer to give Pluronic-bromide.

NMR analysis was done using a Varian VXR 300 NMR spectrometer. 40.2 mg Pluronic-bromide was dissolved in deuterated chloroform and subjected to ^{13}C and ^{+}H analysis.

A6.6.3 Synthesis of a chelating-ligand modified Pluronic™ F108

The terminal hydroxy groups of Pluronic were modified in a two-step reaction to yield the tetradentate DMDDO type ligand at the end of Pluronic^{TM}. This reaction was carried out by dissolving a ten-fold excess of the dianhydride and imidazole in distilled DMF after which the Pluronic was added and reacted for 8 h at 40°C. Methanol was then added and reacted for another 8 h after which the DMF was removed *in vacuo*. It was decided to use longer reaction times due to the size of Pluronic. The residue was treated with toluene to selectively dissolve ligand-modified-Pluronic from the DMDDO by-product. The final product was characterised mainly by NMR with the aid of the model ligands.[148]



Scheme: Reaction scheme for the synthesis of Pluronic – N,N-Dicarboxymethyl-3,6-Diazaoctanedioate

A6.7 Pluronic adsorption onto non-porous membranes

A dilution series of Pluronic in deionised water was made, starting at 7 mg/mL Pluronic down to 0.125 mg/mL. Membrane sections (1cm^2) were incubated in each solution for 8 to 12 h at room temperature to ensure that the adsorption reaction reached equilibrium. After incubation the membranes were washed three times with deionised water and allowed to air-dry for 30 min.

A6.8 Hexane:Isopropanol extraction of Pluronic

Dried Pluronic-coated membranes were then submerged in 25 mL hexane-isopropanol (3:2 v/v) mixture and incubated for 1 h at room temperature. The mixture containing

148 L van Kralingen. Ligand modification of Pluronic F108 for use in immobilized metal affinity separation of bio-molecules. MSc Thesis, University of Stellenbosch. (2002) Stellenbosch, South Africa.

the membranes was subsequently boiled for 15 min. and washed twice with 10 mL pre-heated hexane-isopropanol (3:2 v/v). The mixture was then filtered through Whatman no. 1 filter paper. The hexane-isopropanol filtrate was subsequently evaporated under N_2 gas and slight heat, until complete dryness. The extracted Pluronic was re-dissolved in 10 mL $CHCl_3$. Native or uncoated membranes were used as controls.

A6.9 Colorimetric assay procedure

A dilution series of Pluronic in $CHCl_3$ was prepared and an assay was performed on each dilution in order to generate a standard curve. The Pluronic-$CHCl_3$ standard solution (3 mL) was added to NH_4FeSCN solution (3 mL) and the mixture was vortexed thoroughly for 2 min. The resultant two phases, were then allowed to separate. The bottom chloroform phase was pipetted out and the absorption was measured spectrometrically at 510 nm using a 1 cm beam cuvette in a CARY 100 spectrophotometer. The average optical density (OD) was plotted for Pluronic® F108 and its halogen derivative. A curve of absorption at 510nm *versus* Pluronic concentration was determined.

The robustness of this assay was experimentally verified by adding human plasma (1.5 mg/mL), biotin from Sigma Aldrich Chemical Company, SA (1mg/mL), bovine serum albumin from Sigma Aldrich (1mg/mL), dextran (1mg/mL), sodium dodecyl sulphate (SDS) (34mM) and lysozyme from Sigma Aldrich (1mg/mL) directly into the cuvette used for the Pluronic assay. Any interference or change in the stable OD reading at 510nm would be attributable to the presence of the attendant additive.

A6.10 Pluronic adsorption at curved interfaces

Studies were conducted on PSU capillaries and hollow fine fibres (HFF), by adsorbing 5.0 mg/mL Pluronic onto the convex external surface of the membranes and measuring the adsorbed fraction using the hexane:isopropanol - desorption approach previously discussed. The effect of the concave inner surface of the fibres was not considered at this stage, due largely to the difficulty in accurately extracting the adsorbed Pluronic layer from the lumen of the membrane. The dimensions of the capillary and HFF were measured using an optical light microscope with a vernier scale and these measurements were verified via scanning electron microscopy (SEM). In order to compare the adsorption properties of the fibres with the 1 cm^2 flat-sheets used in previous experiments the lengths of the fibres were prepared such that the external surface area was equivalent to 1 cm^2.

A6.11 Protein adsorption assay on the membranes

BSA (Roche) and Lysozyme (Sigma) solutions [0.25 mg/mL] in phosphate buffer, pH 7.4 were prepared and stored at 4°C. Membranes were statically incubated for 120 min at 20°C in 10 mL of the respective protein solutions. The membranes were then rinsed three times with PB buffer and then inserted into a vial containing 10 mL of 1.0 % (w/v) SDS. These vials were then shaken for 120 min on a belly dancer shaker. The amount of proteins adsorbed on the membrane surface was calculated from the

concentration of proteins in the SDS solution using a Pierce™ protein assay reagent kit. Pluronic was assayed for as described earlier.

In a conventional adsorption experiment,[149] also called the depletion method, the amount of adsorbed proteins is determined based on the decrease in protein concentration in the solution after contacting with the solid surface. In general this method requires a large surface area to be sufficiently accurate.

A6.12 Regeneration of the membranes

The candidate membranes were statically incubated with Pluronic F108 [5 mg/mL] as described in section 2. The membranes were then stripped of the Pluronic using a a 34 mM SDS solution in Milli-Q water. This was initially accomplished by statically equilibrating the Pluronic-coated membranes in 10 mL of the SDS solution for 1 h and then transferring said vial to a belly dancer for 2h of shaking. After SDS desorption of Pluronic-coated membranes. In an attempt to ascertain if this was a time dependent process, the shaking incubation period was increased from 2h to 4h, 20h and49h. A concentration range of SDS 95 mM, 8mM and 34 mM) was also investigated in order to ascertain if SDS micelles encouraged Pluronic desorption.
After SDS stripping the membranes were washed 5x with Milli-Q water and then air-dried, prior to adsorption with 5.0 mg/mL Pluronic as described previously. After adsorption was complete, the membranes were washed and the Pluronic was extracted using hexane-isopropanol as described in section 2. The desorbed Pluronic was then quantified and compared to previous results on native Pluronic-treated membranes, in an attempt at evaluating the membrane regeneration process.

A6.13 Metal coordination of PEO-PPO-DMDDO

0.2575g Pluronic-DMDDO, 0.14g NaOH and 0.00685g $NiCl_2.6H_2O$ were added to 10 mL milli-Q water at 85ºC with stirring for 120 min. The viscous mixture was then cooled to room temperature, filtered (Whatman 1) and diluted to 10 mL with milli-Q H_20.

0.2596g Pluronic-DMDDO and 0.0710g $CuCO_3.Cu(OH)_2$ was added to 10 mL milli-Q water at 70ºC with stirring for 120 min. The viscous mixture was then cooled to room temperature, filtered (Whatman 1) and diluted to 10 mL with milli-Q H_20.

A6.14 Batch chelation using ligand modified membranes

For batch chelation with divalent metal ions, 1 cm^2 pieces of planar non-porous membranes (non-covalently modified with DMDDO) were incubated in 10 mL of 0.05 M solutions of $NiCl_2.6H_2O$, $Cu(NO_3)_2.xH_2O$) and $Zn(NO_3)_2.6H_2O$ in 0.34 M NaOH. The incubation was carried out at room temperature for 60 min. Membranes were then removed, washed in 9 mL dH_2O, air-dried and stored under inert atmosphere for further analysis. Metal ions in the bulk equilibrium solution were quantified by ICP analysis.

149 K Nakanishi, T Sakiyama, K Imahura, On the adsorption of proteins on solid surfaces, a common but very complicated phenomenon, J. BIOSCI. BIOENG. 91(3) (2001) 233-244.

A6.15 Separation of histidine-tagged proteins

Recombinant *Escerichia coli* pantothenate kinase (a kind gift from Dr. E. Strauss, Department of Chemistry and Polymer Science, University of Stellenbosch), was purified on an Amersham AKTA Prime using a Ni-NTA resin and was eluted with an imidazole gradient.

Pantothenate kinase (CoaA) activity[150] was based on the decrease in absorbance at 340 nm. An extinction coefficient of 6220 M-1.cm-1 was used for NADH. Reactions were performed at 25°C in a CARY 110 UV-Vis spectrophotometer. CoaA activity was determined using a continous spectrophotometric assay that coupled the production of ADP to the consumption of NADH. Each 500 µl reaction mixture contained ATP (1.5 mM), Tris-Cl, pH 7.6 (50 mM), $MgCl_2$ (10 mM), KCl (20 mM), NADH (0.3 mM), phospho enol pyruvate (0.5 mM, pyruvate kinase (5 units), lactic dehydrogenase (5 units), pantothenate kinase (5 µg) and sodium pantothenate (0.5 mM). The reaction was initiated by the addition of the pantothenic acid substrate.

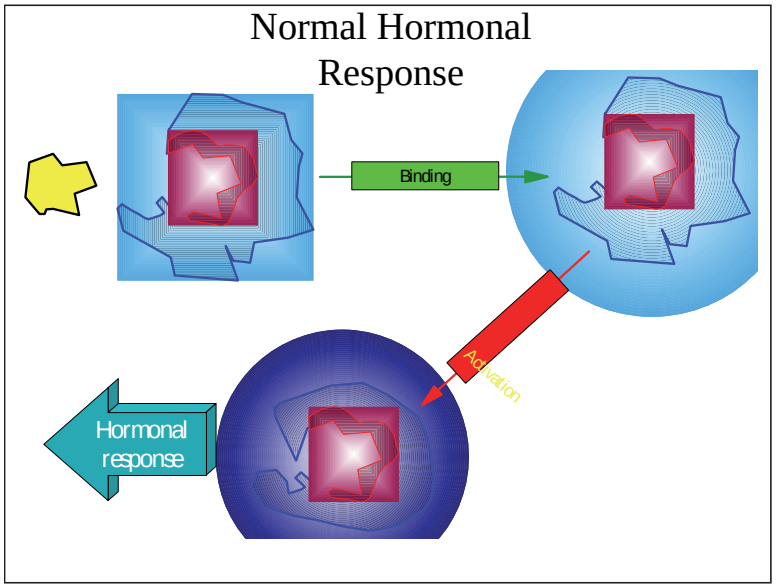
A6.16 Binding of his_6(CoaA) to DMDDO-Ni^{2+}

PVDF membranes were coated in a solution of Pluronic-DMDDO for 8 h at 25°C. These ligand modified membranes were then chelated with $NiCl_2.6H_20$ as described in A.13. The membranes were washed 3 times in 9 mL dH_20, air-dried and then immersed into a solution of his_6(CoaA) at a bulk initial concentration of 0.2 mg/mL. A non-derivatised Pluronic coated PVDF membrane was used as a negative control. After 30 min incubation with gentle shaking at room temperature the enzyme assays were performed on the bulk equilibrium enzyme solution and the protein concentration was measured.

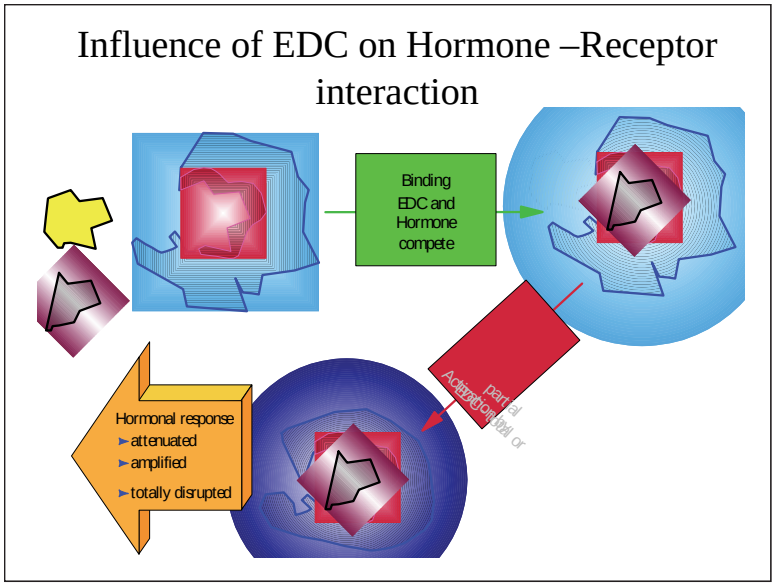
[150] E. Strauss, T.D. Begley, The antibiotic activity of N-pentylpantothenamide results from its conversion to ethyldethia-coenzyme A. J. BIOL. CHEM. 277(50) (2002) 48205-48209.

Appendix 7

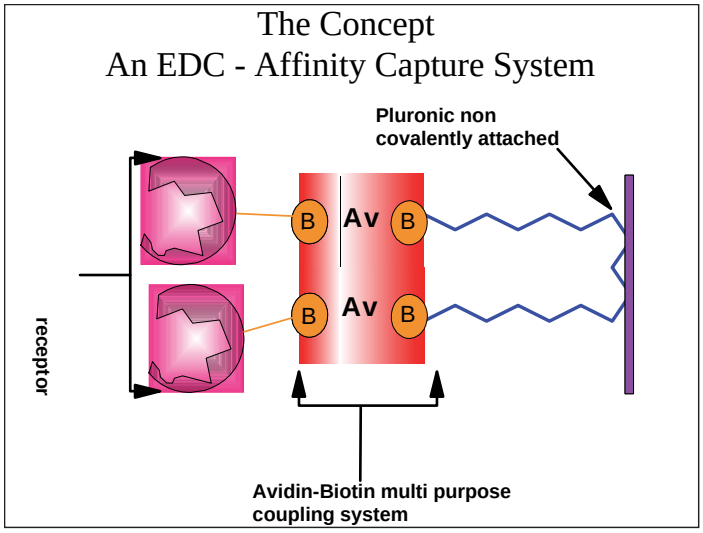
HORMONE DETECTION SYSTEM ENVISAGED



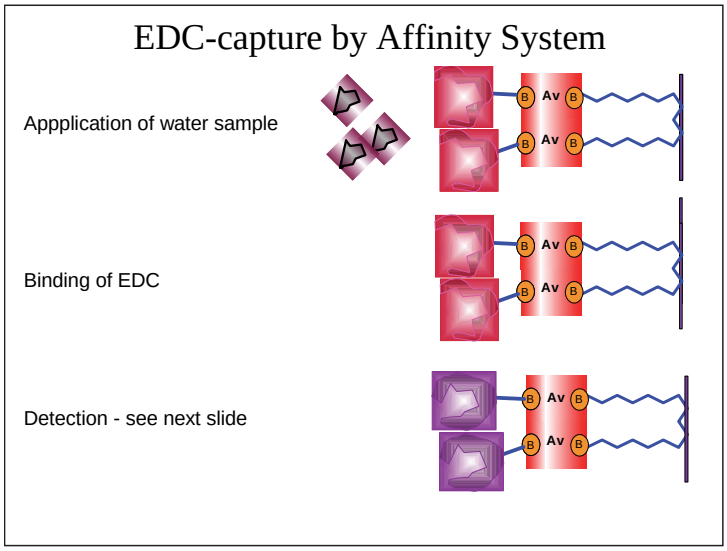
- The Hormone or ligand (yellow) binds the receptor (blue with ligand binding area in red) to yield a hormone receptor complex receptor.
- The hormone receptor complex undergoes a conformational change during activation and elicits the "hormonal response"
- This is how steroid and thyroid hormones (for instance) work.



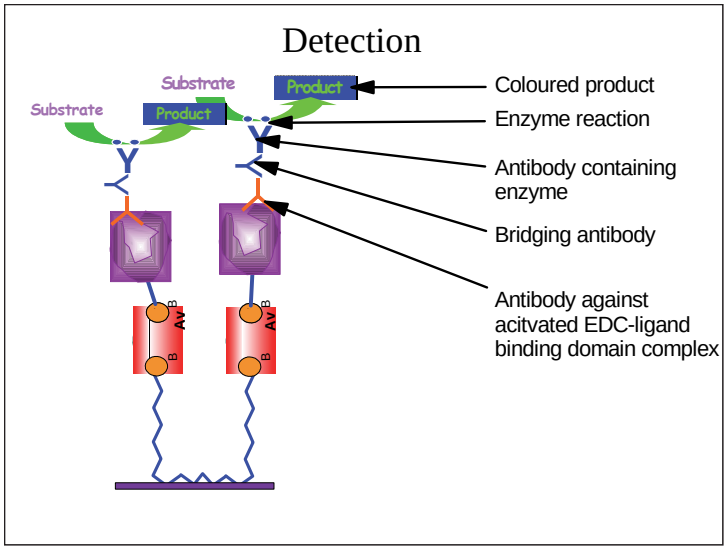
- EDC (purple) and Hormone (yellow) compete for binding to receptor (blue).
- If in high enough concentration or higher affinity EDC binds preferentially and competes Hormone off.
- EDC can elicit endocrine disrupting response as illustrated.



1. The ligand binding domain of the receptor is non-covalently immobilized on a membrane contactor via the avidin-biotin system
2. Advantages:
   - more than one receptor can be immobilized on one contactor strip
   - success of coupling, therefore quality control is much easier
   - new receptors can be readily added if needed



- Water is applied to the membrane immobilized ligand binding domain of the receptor (the whole receptor is not needed). If the concentration of EDC's are low one can expose for prolonged periods as the affinity system will concentrate the ligands.
- Binding takes place followed by a conformational change due to activation
- Detection of the ligated EDC through a colour reaction.



- The membrane is treated with a solution containing an antibody specific against the receptor-EDC complex (this antibody we make)
- A bridging antibody is applied (this antibody we buy)
- An enzyme labeled antibody is added (this we also buy)
- The substrate solution is subsequently added and a color develops which can be observed, only if EDCs are bound to the receptor.

Appendix 8

EXPERIMENTAL PROCEDURES

A8.1 Chemicals

The protein content of the samples was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA) as a standard. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) was obtained from Sigma, bovine serum albumin (BSA), fraction V from Roche, high molar mass rainbow markers from AEC Amersham and the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) gel system obtained from Gibco BRL. Lämmli sample buffer, obtained from Fluka, is 2x concentrated and contains 4% SDS, 20 % glycerol, 10 % β-mercaptoethanol and 0.004 % bromophenol blue in 0.125 M Tris HCl, pH~6.8. Unless otherwise stated, all chemicals used for the Coomassie Blue staining and the silver staining of gels were obtained form Merck. All chemicals used were of analytical grade.

Table 13: Characteristics of trypsin enzymes used

Trypsin	Type (Company)	Molar ratio (X Trypsin : 1 AP/HP) used during experiments
TX	Bovine pancreas, activity of 191 U/mg protein (Separations)	X
TC	Bovine pancreas, activity of 191 U/mg protein (Separations)	c, [Trypsin] = [AP] or [HP], (w/v)
ThX	Bovine pancreas, activity of 11,600 U/mg protein (Sigma)	X

e.g. X=2 and X=1 will be a 2:1 and 1:1 molar ratio, respectively
T_h – trypsin with a higher activity; AP – Aminopluronic; HP – hydrazine-Plu

A8.2 Coupling reaction

Water-soluble carbodiimide (EDAC) was used for the coupling reactions of trypsin to AP and HP.

Excess EDAC (10x, 100x, 200x and 400x excess) dissolved in 250 mL water was added to trypsin dissolved in 25 mM phosphate buffer (pH 5.0) with continuous stirring. The pH of the mixture was continuously monitored and titrated back to pH 5.0 with 1 mM HCl, if necessary. After stabilization of 15 min, activated trypsin mixture was added dropwise to 1.5 mL amine (AP and HP) in 0.2 M phosphate buffer (pH 8.0). The mixture was gently shaken for a minimum period of 3 h and a maximum period of 24 h. BSA (68 kDa) was included as a control for the coupling reactions. The molar ratio used for trypsin to AP and HP was 2:1 (during 10x and 100x excess EDAC experiments) and 1:1 (during 200x and 400x excess EDAC experiments).

A8.3 Analytical procedures: gel electrophoresis and gel staining

SDS PAGE is used as an analytical tool to examine the effectiveness of the coupling process by comparing the difference in molar mass of samples (Table 14).
After coupling reactions, untreated and treated samples were analyzed by SDS PAGE in a discontinuous, denaturing gel with a 3.9% (w/v) stacking gel and a 10% (w/v) resolving gel (18 to 75 kDa separating resolution) of acrylamide. For the SDS PAGE analysis, samples were diluted in sample buffer and heated at 100°C for 2 to 10 min. Rainbow proteins markers were included as standard molar markers during gel analysis. After electrophoresis, the protein bands were stained with Coomassie brilliant blue R250 and with silver staining.

Table 14: Molar mass of samples used during carbodiimide coupling reactions

Molecule	Molar mass (kDa)
Trypsin (T & T_h)	23,8
BSA	68,0
AP & HP	~16,0

T_h – trypsin with a higher activity