NON-COVALENT IMMOBILISATION OF A LIGAND SYSTEM: A NEW APPROACH TO AFFINITY SEPARATION

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted at any university for a degree.

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SUMMARY

Advances in pharmacology, biochemistry and biotechnology are increasingly dependant upon affinity chromatography as a preferred separation technique for the purification and characterisation of specific biomolecules.

In the past few years avidin-biotin technology has been widely and successfully used in the fields of medicine, pharmacy, biology and biochemistry. The avidin-biotin complex (ABC) has been used as a mediator for affinity chromatography, affinity cytochemistry, immunoassay, histopathology, bioaffinity sensors, crosslinking and immobilisation studies.

The main reason for the popularity of the ABC and its growing usefulness in biotechnology is the exceptionally high affinity (10^{15} M^{-1}) and stability of the noncovalent interaction between avidin and biotin. The use of the ABC is broadening as different biotin derivatives and avidin-containing conjugates are becoming commercially available.

The aim of this work was to evaluate the usefulness of a pluronic® F108 and the ABC conjugate to effect affinity separation. Towards this aim, the adsorption of pluronic® F108 onto hydrophobic polysulphone membrane surfaces was studied. This information was used to determine the theoretical maximum amount of pluronic® F108 that will adsorb onto a unit surface area of the membrane. It is known that the polypropylene oxide (PPO) centre block of the pluronic® F108 surfactant molecule governs the concentration of pluronic® F108 molecules that will adsorb onto a given hydrophobic surface. If the maximum coating concentration of pluronic® F108 is known, one can assume that the maximum coating concentration of any pluronic derivative, with the same PPO centre block size, will be the same. Adsorption studies were carried out, the Langmuir adsorption isotherm was determined, and subsequently the fractional coating was calculated.

The end-groups of pluronic® F108 were modified as follows and the substituted pluronic was adsorbed onto a membrane that was to act as the solid support matrix in the development of an affinity system: Amino pluronic was synthesised by first tosylating pluronic® F108, followed by

azidation with NaN_3 then reduction with $LiAlH_4$. The synthesised amino pluronic was then biotinylated using *N*-hydroxysuccinimide biotin ester. The suitability of this synthetic route was first assessed on a model compound, 2-methoxyethylamine, and validated by NMR (Nuclear Magnetic Resonance) spectroscopy. The synthetic protocol was then used to derivatise the larger pluronic molecule.

The affinity system was tested on two different hydrophobic surfaces: polystyrene and polysulphone membranes (PSMs). Avidin-conjugated horseradish peroxidase was obtained and used to interact with the immobilised biotin. The enzymatic reaction of the coupled peroxidase converted the substrate, 2, 2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) (ABTS) to a coloured product. The colour developed is proportional to the amount of biotin that was immobilised on the hydrophobic surfaces studied.

Non-covalent immobilisation of the synthesised biotin-pluronic molecule was successfully obtained onto the hydrophobic polystyrene as well as the polysulphone membrane surfaces.

OPSOMMING

Vooruitgang in die farmakologie, biochemie en biotegnologie word al meer afhanklik van affiniteits chromatografie as die verkose tegniek vir die suiwering en karaterisering van spesifieke biomolekules.

Oor die afgelope jare het die avidien-biotien tegnologie homself as baie bruikbaar bewys in die mediese, farmakologiese, biologiese en biochemiese velde. Toepassings waar die avidien-biotien kompleks betrokke was sluit in die toepassing as 'n mediator vir affiniteits chromatografie, affiniteits sitologie, immuno bepalings, histopatologie, bioaffiniteits sensors sowel as kruisbinding en immobiliserings studies en vele meer.

Die hoofrede vir die gewildheid van die avidien-biotien kompleks en die groeiende bruikbaarheid in die biotegnologie is die buitengewone hoë affiniteit (10^{15} M^{-1}) en stabiliteit van die nie-kovalente interaksie tussen avidien en biotien. Die toepassingsveld van die avidien-biotien kompleks word wyer met die verskeidenheid biotien derivate en avidien-bevattende konjugate wat kommersiëel beskikbaar is.

Die doel van die werk wat hier gedokumenteer word is om die bruikbaarheid van Pluronic® F108 en die avidien-biotien kompleks, vir gebruik in 'n affiniteits chromatografie sisteem, te evalueer. Om hierdie doel te bereik is die adsorpsie van Pluronic® F108 aan hidrofobiese polisulfoon membraan oppervlaktes bestudeer. Die eksperimentele data wat gegenireer is, is gebruik om die teoretiese maksimum hoeveelheid Pluronic wat per eenheids oppervlakte membraan adsorbeer te bepaal. Dit is reeds bekend dat die polipropileen (PPO) middel blok van die Pluronic emulgant die konsentrasie van die geadsorbeerde Pluronic molekules op 'n gegewe hidrofobiese oppervlakte bepaal. Indien die maksimum bedekkingskonsentrasie vir maksimum oppervlakbedekking van Pluronic® F108 bekend is, kan teoreties aanvaar word dat die bedekkingskonsentrasie vir enige Pluronic derivaat met dieselfde grootte PPO blok dieselfde sal wees. Adsorpsiestudies was uitgevoer om die Langmuir adsorpsie isoterm te bepaal. Daaropvolgend was die fraksionele bedekking bereken.

Amino-pluronic was gesintetiseer deur die eindpunte van Pluronic te derivatiseer. Hierdie Pluronic derivaat was gevolglik geadsorbeer aan 'n membraan wat gedien het as die soliede oppervlakte vir die ontwikkeling van 'n affiniteits chromatografie sisteem.

Amino-pluronic was gesintetiseer deur Pluronic eers te tosileer en daarna te asideer met NaN_3 en laastens te reduseer met $LiAlH_4$. Die produk was gebiotinileer deur gebruik te maak van *N*-hidroksisuksinimied-biotien-ester. Die bruikbaarheid van hierdie sintetiese roete is eers bepaal deur van 'n model verbinding, 2-metoksiëtielamien, gebruik te maak en dit met behulp van KMR (Kern Magnetiese Resonans) spektroskopie te karakteriseer.

Die affiniteits sisteem is getoets op twee verskillende hidrofobiese oppervlaktes naamlik polistireen en polisulfoon membraan oppervlaktes. Avidien gekonjugeerd met 'n peroksiedase ensiem is gebruik om met die geïmmobiliseerde biotien te assosieer. Die ensiematiese reaksie van die gekoppelde peroksiedase het die substraat 2, 2'-azino-di-(3-etiel-benzthiazolien-6-sulfoonsuur) (ABTS) omgesit na 'n gekleurde produk, waar dit teenwoordig is. 'n Reeks wasstappe is gebruik om die gemodifiseerde peroksidase ensiem wat nie aan die hidrofobiese oppervlakte gekoppel nie, weg te spoel. Hierdeur is die mate van binding aan die hirofobiese oppervlakte gekwantifiseer deur die kleur te kwantifiseer wat ontwikkel omdat die kleurontwikkeling direk proporsioneel is aan die hoeveelheid peroksidase wat nog aan die membraan gekoppel is.

Nie-kovalente immobilisasie van die gesintetiseerde biotien-pluronic molekule is suksesvol op beide die hidrofobiese polistireen oppervlakte sowel as die polisulfoon membraan verkry.

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CHAPTER 1

INTRODUCTION

CHAPTER 2

AVIDIN-BIOTIN TECHNOLOGY

ABBREVIATIONS

ABC	Avidin-biotin Complex
Abs	Antibodies
ABTS	2, 2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid)
ADP	Adenosine diphosphate
Ag	Antigen
AP	Amino-pluronic
AP-B	Biotinylated amino-pluronic
ATP	Adenosine triphosphate
Av	Avidin
Av-HRP	Avidin-conjugated horseradish peroxidase
B	Biotin
BBP	Biotin binding protein
BET	Brunauer-Emmett-Teller
BNP	Biotinyl-*p*-nitrophenyl
BSA	Bovine Serum Albumin
C_0	feed concentration
CCP	Carboxyl carrier protein
CIP	Cleaning-in-place
CNBr	Cyanogen bromide
ΔH_{tr}	Change in enthalpy
d	Doublet
dd	Doublet of doublets
ddd	Doublet of doublets of doublets
ΔG	Change of in Gibbs free energy
DMAc	*N*,*N*-Dimethylacetamide
DMF	Dimethyl formamide
ΔS	Change in entropy
EDDA	Ethylenediamine-*N*,*N'*-diacetic acid
EDTA	Ethelenediaminetetraacetic acid

ELISA	Enzyme Linked Immunosorbent Assay
ES-MS	Electrospray mass spectrometry
Fc	Constant region
HEDT	*N*-(2-hydroxyethyl)ethylenediaminetriacetic acid
HRP	Horse radish peroxidase
IDA	Iminodiacetic acid
IgG	Immunoglobulin G
IMAC	Immobilised metal ion affinity chromatography
J	Coupling constant
LC	Long chain
m	Multiplet
MEA	Metoxy ethylamine
NHS-B	*N*-hydroxysuccinimide biotin ester
NMR	Nuclear magnetic resonance spectroscopy
OAA	Oxalo acetate
PBS	Physiological buffer solution
PEO	Polyethylene oxide
PPO	Polypropylene oxide
PS	Polystyrene
PSM	Polysulphone membrane
R_{eq}	Separation factor
RO	Reverse osmosis
s	Singlet
SDS	sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis
SIP	Sanitation-in-place
StAv	Streptavidin
t	Triplet
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TREN	Ttris(2-aminoethyl)amine

T_{tr}	transition temperature
v/v	Volume per volume
ω	Infinity

CHAPTER 1

INTRODUCTION

The industrial explosion, increasing population growth and a consistent demand for an improvement in living standards, places a high demand on technology. Food technology, wastewater purification and drug production have to show significant progress to keep up with the increasing demands generated by the expanding world population. These industries are all dependent upon biochemical and biotechnological techniques to live up to the demands that modern society places on them. Large-scale production of pharmaceutical products, processing of foods and pollution control are imperative to provide for the world population in their basic needs. As a result, advances in pharmacology, biochemistry and biotechnology are increasingly dependent on modern but effective separation technologies, such as affinity chromatography, for the purification and characterisation of specific biomolecules [Anspach *et al.* 1989; Turková, 1993].

1.1 Affinity Chromatography

Chromatography is a well-known technique to separate and purify organic and biomolecules. The specific chromatographic method used depends on the properties of the molecules to be separated. Gel filtration chromatography and SDS-PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis) allow for the separation of molecules according to their molecular mass, whereas ion exchange chromatography separate molecules according to their net charge. These techniques, although extensively used, are often complex and time consuming and the purified molecules are often inactivated due to denaturation. Affinity chromatography, on the other hand, relies on very specific interactions between molecules and ligands, which are immobilised on a suitable medium for separation. These specific interactions are similar to those, for example, that exist between hormone and receptor, antibody and antigen, and complementary nucleic acids, to name but a few [Turková, 1993].

Chromatography comprises only a limited area in separation science. Affinity chromatography, a rapidly developing field within chromatography [Turková, 1978; Porath, 1981; Turková, 1993],

involves an inert solid support onto which naturally occurring substances, or closely related compounds of high selectivity to other natural compounds, are attached. These compounds act as adsorption centers. The interactions involved in the adsorption process are similar to those occurring in nature. The immobilised affinity molecule is called a ligand or adsorption center and its soluble complement is referred to as a ligate. The ligand-ligate complex is known as an adsorption complex. Besides using the affinity method to purify biomolecules, it is also a tool used to study molecular interactions [Porath, 1981].

1.2 Non-covalent interactions and their role in affinity chromatography

In an affinity system the ligand is immobilised onto a solid support where it interacts specifically with the ligate. These interactions are intermolecular forces also known as non-covalent interactions and include van der Waals forces, electrostatic attractions, hydrogen bonding [Streitweiser, Heathcock & Kosower, 1992] and hydrophobic interactions [Zubay, 1993].

The van der Waals attraction results from an electron correlation effect also called the London or dispersion force. It arises from correlated motion of the electrons of two molecules and requires close contact [Streitweiser, Heathcock & Kosower, 1992]. There are two types of van der Waals forces namely: attractive and repulsive. The attractive van der Waals forces involve interactions between induced instantaneous dipole moments that arise from fluctuations in the electron charge densities of the neighbouring atoms. The repulsive van der Waals forces occur when two atoms/molecules are so close that electron-electron repulsion arise when the charge clouds begin to overlap. The average separation of two molecules exclusively held together by van der Waals forces are goverened by a balance between the attractive and repulsive forces. This distance is known as the Van der Waals separation [Zubay, 1993].

There are three types of electrostatic forces namely: charge-charge, charge-dipole and dipole-dipole interactions [Zubay, 1993]. Dipole-dipole interactions take place when polar substances associate with each other in such a manner that the positive end of one dipole is directed to the negative end of another dipole. When an electronic charge produces a dipole with a charge separation it is known as an instantaneous dipole. An instantaneous dipole can induce a

charge separation in a neighbouring molecule and this phenomenon results in a dipole-dipole attraction [Petrucci & Harwood, 1993].

Hydrogen bonds are rather strong intermolecular forces, with energies in the order of 15 to 40 kJ/mole. A hydrogen bond is formed when an H-atom is covalently bound to a highly electronegative atom and the H-atom is simultaneously attracted to a small highly electronegative atom of a neighbouring molecule. Hydrogen bonding can only occur with H-atoms because all other atoms have inner shell electrons to shield their nuclei [Petrucci & Harwood, 1993].

Neutral hydrocarbon chains do not contain significant dipoles and do not have the capacity to form hydrogen bonds. They have nothing to gain by interacting with water. Hydrophobic interactions play an important role in the contribution to the stability of a molecule containing hydrocarbon chains in an aqueous solution. Hydrophobic interactions relate primarily to entropic factors and the entropic factor mainly concern the solvent and not the solute [Mathews & van Holde, 1991; Streitweiser, Heathcock & Kosower, 1992; Zubay, 1993].

In aqueous solution, the hydrophobic chain interferes with the hydrogen-bonded structure of water molecules and leads to an increased order (structure) of the surrounding molecules. This ordering corresponds to a loss of randomness in the system and the entropy is decreased. If hydrophobic chains associate with each other it causes the ordered water molecules to be released and gain freedom of movement. Thus association of hydrophobic molecules increase the randomness of the whole system and therefore yield an entropy increase. This will make a negative contribution to the free energy of the system and increase the stability of the associated hydrophobic molecules. The decrease in entropy is not compensated for by the van der Waals attraction between the water and the hydrocarbon chain, leading to association of the hydrocarbon chains. It is not that bonds are formed between hydrophobic groups, but rather that the overall structure is stabilised by the entropy effect. This source of stabilisation is referred to as the hydrophobic effect [Mathews & van Holde, 1991; Streitweiser, Heathcock & Kosower, 1992; Zubay, 1993].

1.3 Solid supports

The practical application of affinity chromatography depends very much on the solid support (matrix) onto which the ligand is attached, as well as the methods chosen for the attachment of the ligands. The solid support represents the largest volume of the affinity adsorbent [Scouten, 1981]. Affinity ligands are usually covalently coupled to a solid support [Porath, 1981] by activation of the solid support with an activating agent such as cyanogen bromide (CNBr), for example [Axen, Porath & Emback, 1967; Porath, Axen & Emback, 1967].

Certain essential characteristics of the solid support are:

1. Insolubility in the solvents or buffers that may be used;
2. Mechanical and chemical stability under the conditions required for attachment, adsorption, desorption and regeneration of the active sites;
3. Ease of coupling the ligand or spacer arm onto which the ligand may be attached to the support;
4. Large surface area accessible to the biomolecules that are being purified;
5. Good hydrodynamic flow characteristics; and
6. Resistance towards microbial and enzymatic attack [Porath, 1974; Turková, 1978; Scouten, 1981].

In this study a hydrophobic ultrafiltration polysulphone membrane was used as a solid support. This results in a more robust and cost-effective solid support than previously used [Turková, 1978]. The use of membranes as solid supports for the immobilisation of affinity ligands is a relatively new technology. Promising performance has been obtained with such systems and their use will become more common in future affinity chromatographic applications [Bueno, Haupt & Vijayalakshmi, 1995; Bueno *et al.* 2000]. The use of membranes as solid supports will be discussed in more detail in chapter 5.

1.4 The aim and outline of this study

The aim of this study was to render affinity properties, making use of the avidin-biotin technology, to a hydrophobic polysulphone membrane by cross-linking with a tether, which is attached to the membrane by adsorption.

The approach followed is shown schematically in figure 1.1. The steps shown in the figure also serve as an outline of the work reported in this thesis. Firstly the hydroxyl termini of a tri-block copolymer, pluronic® F108 (hereafter referred to as pluronic), were derivatised to facilitate the covalent attachment of a ligand system onto the end-groups of the surfactant. Avidin-biotin was used as the ligand system. The modified ligand-carrier was subsequently adsorbed onto a polysulphone membrane in order to render affinity properties to the membrane.

In chapter 2 a theoretical background on the avidin-biotin technology is provided. A sound knowledge of avidin, biotin and biotinylation reactions was neccessary in order to make informed decisions on certain reagents and reaction conditions.

In chapter 3 a theoretical background is given for pluronic (the adsorbing ligand carrier) and its adsorption behaviour was investigated. This was done to determine the concentration and coating conditions of the unmodified carrier before the more expensive and difficult-to-synthesise derivatives were used. From the information in this chapter a good indication was obtained of what the coating conditions for this particular copolymer onto the solid support should be.

In chapter 4 the synthesis and characterisation of the pluronic derivatives are discussed. Pluronic end-groups were first tosylated with *p*-toluenesulphonyl chloride and converted to azide with NaN_3. The azide end-groups were reduced to amines with $LiAlH_4$ to obtain an amino-terminated pluronic (amino-pluronic). Up to this point the reactions were duplicated from work published by Yaniç *et al.* (2000). The method for the synthesis of the biotinylating reagent was also found in previously published studies [Becker, Wilchek & Kachalski, 1971; Bayer & Wilchek, 1974]. Also reported in chapter 4 are the syntheses of biotinylated amino-pluronic (biotin-pluronic) with the activated biotinylating reagent. The synthesis and characterisation of biotin-pluronic was preceded by model compound studies to simplify purification and characterisation of the reaction products, and to establish synthetic routes.

The synthesised biotin-pluronic was tested on two different hydrophobic surfaces to determine if it could render affinity properties to the chosen hydrophobic solid supports. This work is reported in chapter 5. Conclusions, together with recommendations for possible future studies, are discussed in chapter 6.

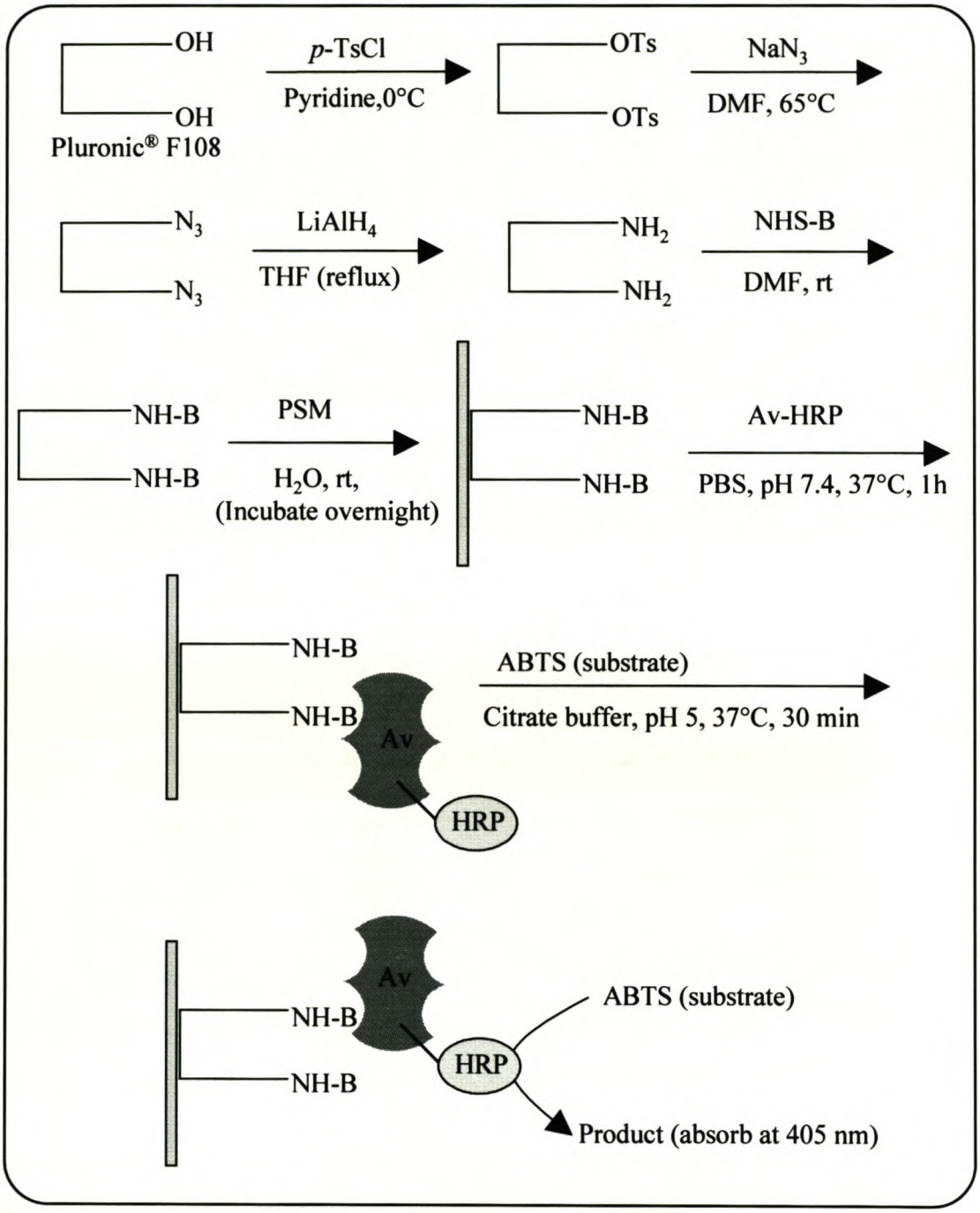

Figure 1.1: Schematic diagram of the work covered in this thesis.

1.5 Tasks

For the purpose of documenting the results, the study was divided into the tasks shown below:

1. Determination of the Langmuir adsorption isotherm of pluronic onto polysulphone flat-sheet membranes to determine the appropriate coating conditions (chapter 3).
2. Studying the effects of certain parameters on the adsorption and desorption behaviour of pluronic onto PSMs (chapter 3).
3. Syntheses and characterisation of pluronic derivatives, an activated biotinylation reagent, a biotinylated model compound and biotin-pluronic to obtain an affinity property for the pluronic end-groups (chapter 4).
4. Test the biotinylated pluronic (biotin-pluronic) on two different hydrophobic surfaces for affinity properties and determine whether the affinity system works (chapter 5).

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CHAPTER 2

AVIDIN-BIOTIN TECHNOLOGY

2.1 Introduction

Avidin-biotin technology has been widely used in various fields such as medicine, pharmacy, biology and biochemistry. Over the past years avidin-biotin technology has proved itself to be very useful. Uses where the avidin-biotin complex (ABC) is involved include applications such as a mediator for affinity chromatography, affinity cytochemistry, blotting technology, immunoassay, histopathology and even gene probes. Further uses include hybridoma technology, bioaffinity sensors, affinity targeting, drug delivery as well as crosslinking and immobilisation studies [Wilchek & Bayer, 1988]. The complex has also been used for specific staining of biological membranes in electron microscopy [Heitzmann & Richards, 1974].

The main reason for the wide popularity of ABC and its growing usefulness in biotechnology is the exceptionally high affinity (10^{15} M^{-1}) of biotin for avidin and the high stability of its non-covalent interaction [Heitzmann & Richards, 1974; Wilchek & Bayer, 1988]. The application field of the ABC becomes even broader with the several different biotin derivatives and avidin-containing conjugates that are commercially available.

2.2 Biotin

Biotin, also known as vitamin H, is found in minute amounts in almost every living cell. The main sources are the liver, kidney and pancreas, and in products such as yeast, milk and egg-white. The biotin structure (figure 2.1) is an imidazole ring *cis*-fused to a tetrahydrothiopene ring substituted at position 2 by a valeric acid [Zubay, 1993; De Clercq, 1997].

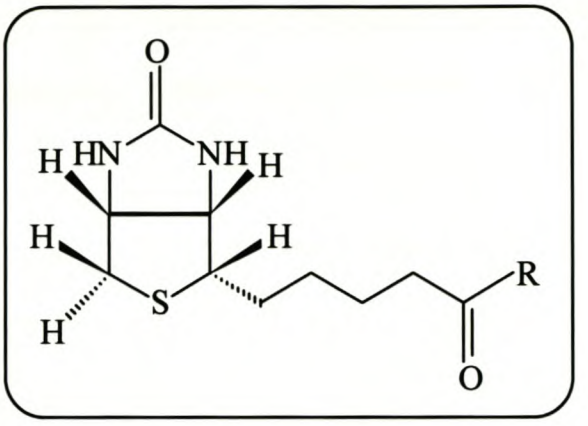

Figure 2.1: The structure of biotin, where R = OH [Zubay, 1993; De Clercq, 1997].

Biotin serves as a co-factor for enzymes involved in carboxylation reactions involving bicarbonate as the carboxylating reagent [Diamandis & Christopoulos, 1991; Zubay, 1993]. Biotin is covalently bound to carboxylase enzymes by an amide linkage between its carboxyl group and a lysyl-ε-NH_2 group in the polypeptide chain [Zubay, 1993]. One example of such an enzyme is pyruvate carboxylase, which catalyses the condensation reaction of pyruvate and CO_2 to form oxaloacetate. During the ATP-dependent carboxylation of biotin by bicarbonate, the CO_2 is first fixed to the imino group via an active carbonic-phosphoric anhydride to form carboxy-biotin. In the second step the CO_2 is passed to the acceptor substrate, e. g. pyruvate [Diamandis & Christopoulos, 1991; Zubay, 1993] (figure 2.2). More studies are focused on the application rather than on the biological function of biotin, explaining the various biotin derivatives currently available [Wilchek & Bayer, 1988; Diamandis & Christopoulos, 1991].

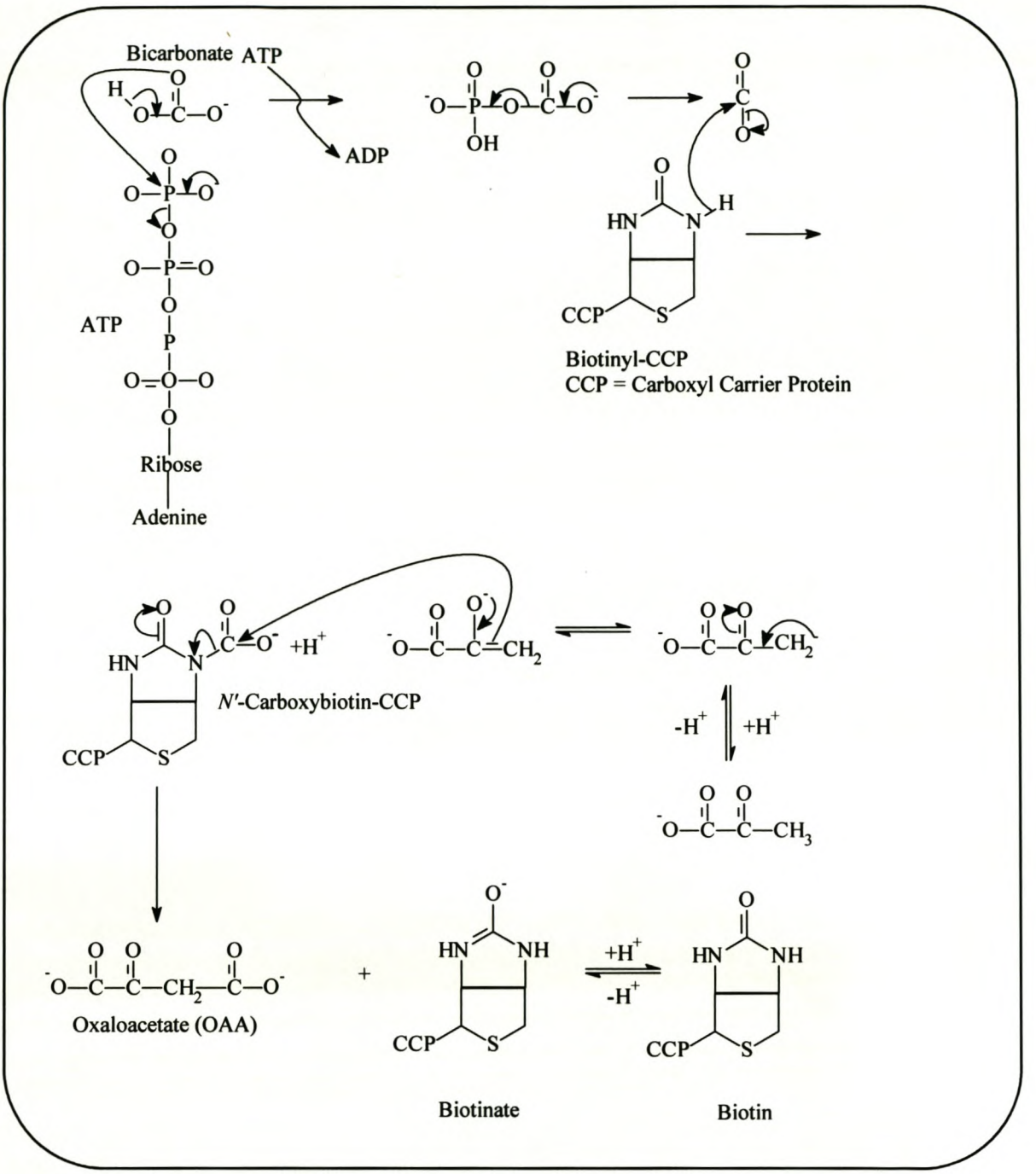

Figure 2.2: Reaction mechanism of biotin mediated carboxylation of pyruvate to form oxaloacetate [Zubay, 1993].

2.3 Biotin derivatives

Numerous biotin derivatives are commercially available. These have different and specific functional groups, aside from the bicyclic biotin ring that extend the usefulness and give rise to

diverse application of the avidin-biotin technology. Several different biotinylation reagents were developed to biotinylate diverse classes of compounds. These reagents are also used for other more specific applications, which will be discussed in a later section [Diamandis & Christopoulos, 1991].

To take advantage of the binding of biotin in the biotin-binding cleft of avidin, spacers have been introduced into many biotin derivatives. A biotin molecule can then be attached to an effector via a spacer arm. The receptor then binds the effector. In cases where the binding between the effector and receptor is too strong to elute normally, cleavable spacers of biotin-containing derivatives are used [Wilchek & Bayer, 1988].

Different functional groups require different biotinylating reagents and the biotinylation of molecules can be obtained by means of numerous methods. Biocytin, an adduct of biotin and lysine, is found naturally, but can also be synthesised. The reagent, *p*-diazobenzoyl-biocytin is specific for biotinylating tyrosyl and histidyl amino acid side chains. Proteins that contain –SH groups as a result of thiolation, reduction of S-S groups or which naturally have free –SH groups, are biotinylated by 3-(*N*-maleimidopropionyl)biocytin or iodoacetyl-long chain (LC)-biotin. Biocytin hydrazide, in a water-soluble carbodiimide reaction, can be used to biotinylate carboxyl groups of proteins while biotinylation of glycoproteins is achieved through their sugar moieties by using biotin hydrazide or biotin-LC-hydrazide. A photo-activatable analogue of biotin, photobiotin, has been synthesised and used to synthesise non-radioactive biotin labelled DNA and RNA hybridisation probes [Forster *et al.* 1985]. The active biotin esters of *p*-nitrophenyl and *N*-hydroxysuccinimide are most commonly used to biotinylate NH_2-groups [Diamandis & Christopoulos, 1991]. The chemical structures of some of the biotin derivatives and biotinylating reagents mentioned are given in figure 2.3.

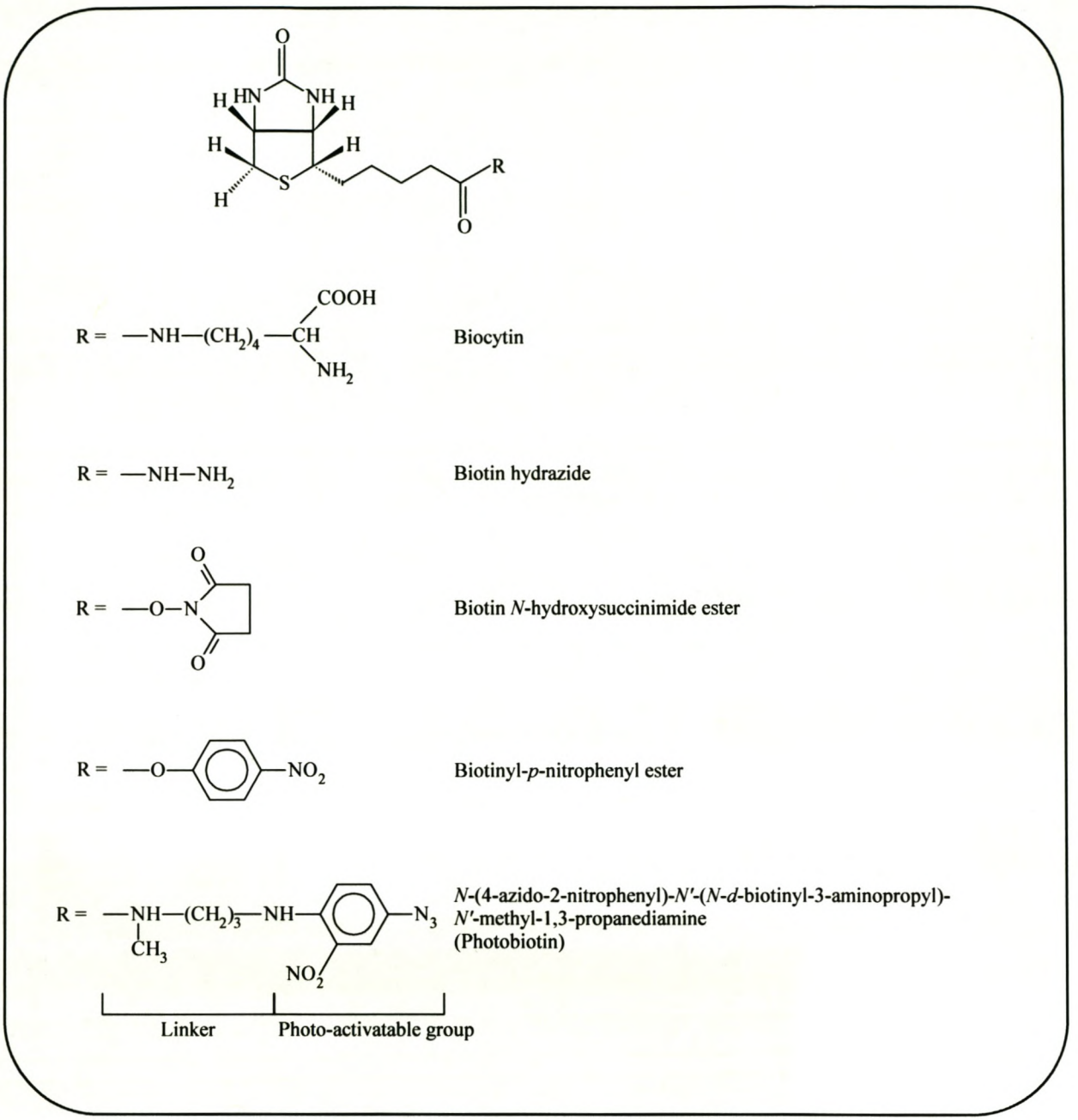

Figure 2.3: Structures of different biotinyl derivatives [Becker, Wilchek & Katchalski, 1971; Forster, *et al.* 1985; Reisfeld *et al.* 1987; Parameswaran, 1990; Diamandis & Christopoulos, 1991].

2.4 Biotinylation reactions

In the avidin-biotin system there is usually one moiety that needs to be biotinylated before the system can work [Diamandis & Christopoulos, 1991]. A desired binder molecule can be biotinylated by any of the reagents or their like, depending on what functional groups the binder molecule possesses [Wilchek & Bayer, 1988]. Components that can be biotinylated include proteins, polysaccharides, nucleic acids or low molecular mass substances, which contain the required functional groups [Diamandis & Christopoulos, 1991]. Specific functional groups such as cysteine, cystine, histidine and tyrosine may be labelled specifically by using the correct biotinylation derivative. Binding proteins with primary amine groups have, for example, been prepared by direct biotinylation, either with biotinyl-*N*-hydroxysuccinimide ester or with longer chain homologues. Many synthesised derivatives of biotin have been used to label amines (lysine), imidazoles (histidine), phenols (tyrosine) and sulfhydryls (cysteine) in proteins. Aldehydes (carbohydrates) as well as functional groups on DNA and RNA can also be labelled [Wilchek & Bayer, 1988].

The biotinylation of carbohydrates is achieved by first oxidatively cleaving the vicinal hydroxyl groups with $NaIO_4$ to yield aldehyde groups, and then allowing these groups to react with biotin hydrazide, biotin-LC-hydrazide or biocytin hydrazide [Diamandis & Christopoulos, 1991].

2.5 Specific avidin-biotin interactions

The bicyclic ring or the ureido portion of biotin is principally involved in the interaction with avidin [Honzatko & Williams, 1982; Wilchek & Bayer, 1988]. The carboxyl group is thus left open for any covalent modification to design reactive biotinyl derivatives [Wilchek & Bayer, 1988].

The unusually high affinity of avidin for biotin reflects the participation of many factors. Binding of biotin to avidin involves a highly stabilised network of polar and hydrophobic interactions [Livnah *et al.* 1993]. The primary contribution to the strong interaction between biotin and avidin is the stabilisation of the biotin resonance forms, which polarise the ureido group so that the negative charge is localised on the ureido oxygen and allows the formation of strong hydrogen bonds. The biotin molecule, in its dipolar form, is arranged inside the binding site of avidin in

such a way that its valeryl carboxylate moiety orients towards the amino group of the lysine residue present in the binding site of avidin, forming an ion-pair interaction [Torreggiani, Fagnano & Fini, 1997].

The imidazolidinone nitrogens in the biotin molecule are hydrogen-bonded to certain amino acid residues of the avidin binding site. The imidazolidinone rings also contribute to complex stabilisation by means of hydrophobic and van der Waals interactions [Torreggiani, Fagnano & Fini, 1997]. Once bound, biotin is almost completely buried in the protein core of avidin. Trp 70, Trp 97 and Phe 79 are in close contact with biotin. The binding pocket is partly closed on its outer rim by residue Trp 110 of a neighbouring subunit [Pugliese *et al.* 1993].

2.6 Avidin

Avidin is mainly found in the white of chicken eggs. It was suggested that a possible natural function of avidin is that of an antibiotic protein, inhibiting bacterial growth because of its ability to bind up to four biotin molecules non-cooperatively [Tausig & Wolf, 1964; Pugliese *et al.* 1993].

2.6.1 The chemical and physical properties of avidin

Avidin (Mr = 62 400) is a basic glycoprotein made up of four identical subunits of 128 amino acid residues each [Gatti *et al.* 1984; Diamandis & Christopoulos, 1991; Pugliese *et al.* 1993]. Avidin has 10 ± 5 % and 55 ± 4 % of its residues in helical and β-strand conformations respectively [Honzatko & Williams, 1982].

Each monomer of avidin is organised in an eight-stranded antiparallel orthogonal β-barrel with simple up-down topology and extended loop regions (figure 2.4) [Hendrickson *et al.* 1989; Livnah *et al.* 1993; Pugliese *et al.* 1993]. Each avidin polypeptide chain contains a glycosylation site at the Asn 17 residue [Gatti *et al.* 1984; Pugliese *et al.* 1993]. The structural characterisation of the avidin carbohydrate moiety was reported by Bruch and White (1982).

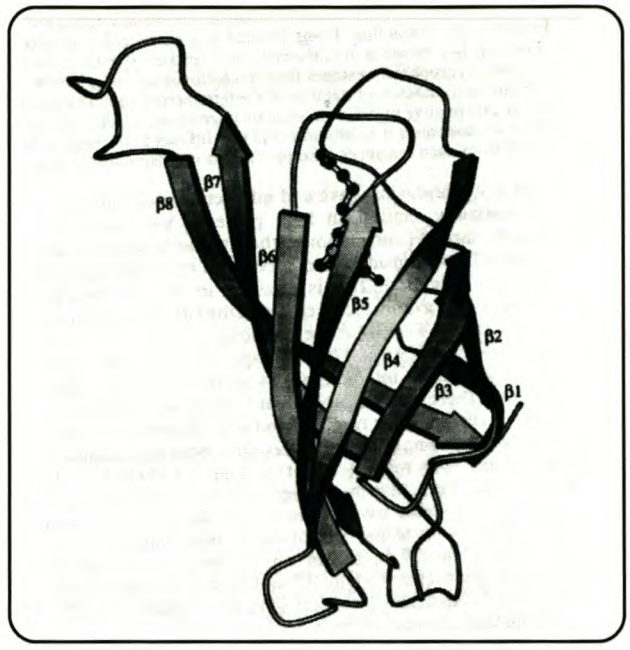

Figure 2.3: Ribbon diagram to illustrate the ß-barrel shape of the avidin-biotin monomer [Livnah *et al.* 1993].

The biotin binding site is located in a deep pocket at the center of the β-barrel, which displays both hydrophobic and polar residues for the recognition of biotin [Pugliese *et al.* 1993]. Biotin-depleted avidin is fairly thermostable with a transition temperature (T_{tr}) for the folded to unfolded form of 85°C in a pH range of 7 to 9 [Pugliese *et al.* 1993]. Donovan and Ross (1973) found that large increases in transition temperature (T_{tr}) resulted when avidin binds to biotin. The T_{tr} of avidin increased from 85°C to 135°C when bound to biotin. This, they concluded, was due to better stability and subsequently irreversible denaturation of the protein when bound to biotin [Donovan & Ross, 1973].

Biotin stabilisation was explained by Bayer, Ehrlich-Rogozinski & Wilchek (1996) through three different, but related processes. First, the network of hydrogen bonds is reinforced by the binding of biotin. Second, a tryptophan residue (contributed to the binding site by a neighbouring monomer) donates an additional inter-monomer hydrophobic interaction. Finally, the binding of biotin involves the stabilisation of a flexible loop. The valeryl sidechain carboxylate-group hydrogen binds to residues Ala 39, Thr 40 and Ser 75 and triggers the formation of a network of hydrogen bonded water molecules [Bayer, Ehrlich-Rogozinski & Wilchek, 1996].

2.6.2 Binding properties of avidin

Avidin binds non-cooperatively up to four molecules of biotin with exceptionally high affinity [Pugliese *et al.* 1993]. Iminobiotin, however, binds strongly to avidin at a high pH, although with about 100-fold less affinity than biotin, while at a low pH iminobiotin does not bind to avidin at all [Diamandis & Christopoulos, 1991].

High levels of avidin binding onto mast cells have been observed and on this basis a technique was developed to specifically identify this particular cell type microscopically. Binding appeared to be mainly the result of electrostatic interactions because changes in pH and ionic strength appeared to affect avidin-induced labelling on mast cells [Wilchek & Bayer, 1988].

Hiller *et al.* (1987) found that the oligosaccharide moiety is not essential for the binding activity of avidin.

2.6.3 Avidin derivatives and conjugates

Alternative derivatives and conjugates of avidin have been developed to minimise non-specific binding and to overcome certain problems which may occur when working with avidin. The avidin-containing derivatives or probes are mostly prepared by covalent coupling of the molecule such as a reporter group onto avidin [Wilchek & Bayer, 1988].

Succinylavidin have been synthesised to eliminate non-specific binding which occurs with avidin [Finn *et al.* 1980; Bayer & Wilcheck, 1989]. This avidin derivative is unfortunately less effective than the natural avidin [Finn *et al.* 1980].

Because avidin is positively charged (pI $\simeq$ 10) it aggregates extensively when mixed at ambient temperatures with anionic detergents at pH > 10, such as sodium dodecyl sulphate (SDS). The resultant aggregate fails to penetrate the stacking gel, making SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) analyses of the protein difficult. Bayer, Ehrlich-Rogozinski & Wilchek (1996) acetylated the lysine groups of the avidin by using acetyl *N*-hydroxysuccinimide ester, thus reducing the pI of the protein.

2.6.4 Purification of avidin

In theory avidin could be purified by a biotinylated matrix, but the strong bond between avidin and biotin makes it problematic to elute the avidin from the biotin matrix [Diamandis & Christopoulos, 1991]. Previous studies showed that the use of a biotin derivative, iminobiotin, could overcome this problem. Iminobiotin differs from biotin in that it contains a guanidino group where biotin contains an ureido group (figure 2.5). At a high pH the iminobiotin is not protonated and an almost stoichiometric binding of the iminobiotin to avidin takes place. As the pH was lowered, the dissociation constant of the avidin-iminobiotin complex was found to increase. Because the charge of the iminobiotin can be changed by simply changing the pH, avidin can specifically be retained by an iminobiotin covered matrix at pH 11 and eluted by lowering the pH to 4 [Green, 1966].

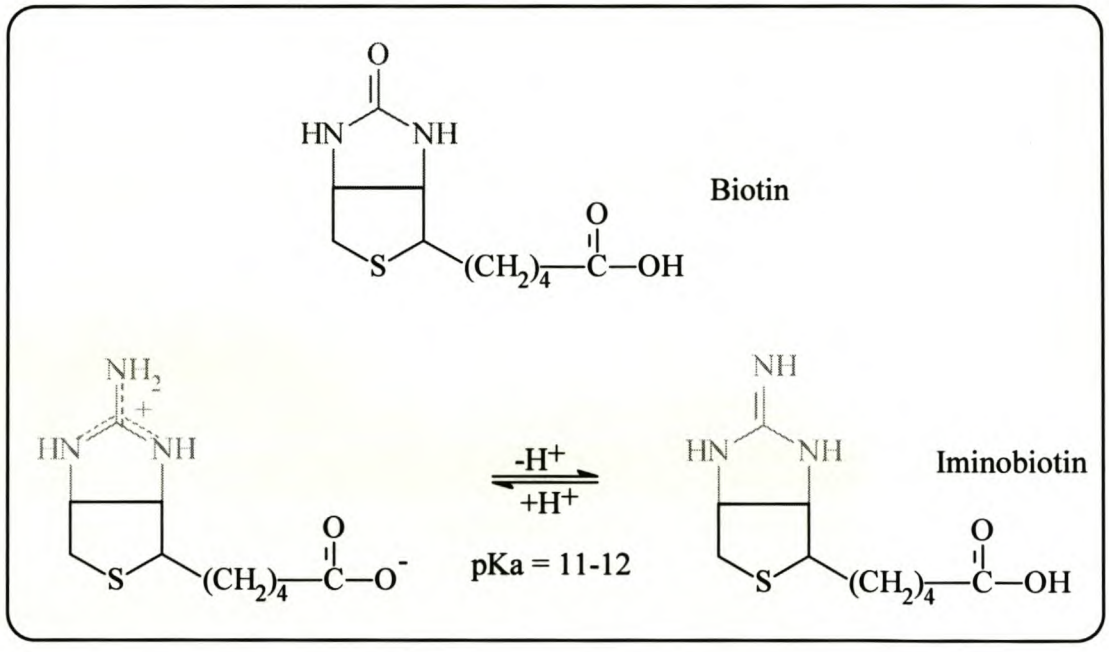

Figure 2.4: Schematic diagram of the structural differences between biotin and iminobiotin [Finn & Hofmann, 1985; Orr, Heney & Zeheb, 1986].

2.7 Applications of avidin

Avidin is used either as the native protein or in a derivatised form containing any reporter group such as fluorescent groups, electron-dense markers, enzymes or immobilising matrices [Wilchek & Bayer, 1988].

High levels of non-specific binding have been characteristically found when an avidin-containing probe has been introduced into a system. Because of the basicity of the avidin molecule, high levels of non-specific binding may occur which could be a problem when accurate results are required. Non-specific binding of egg-white avidin may also take place because of its carbohydrate chains which can bind to sugar-binding proteins.

The above-mentioned limitations can be overcome if streptavidin (StAv), which is an avidin-like protein produced by the bacteria *Streptomyces avidinii*, is used instead of egg-white avidin. Streptavidin has less non-specific binding properties because it is a neutral non-glycosylated protein.

Other ways to overcome the aforementioned problems have also been accomplished by different modifications of avidin. These modifications include acetylation or succinylation of the lysine groups which may eliminate or counter-charge residues. It was suggested that the complete blocking of lysine residues should be avoided though, since they could be required for coupling of markers. Sugar residues can also be removed enzymatically from egg-white avidin using *N*-glycosidase. Susceptible sites have also been blocked through the use of high-ionic strength buffers, milk proteins or positively charged lysozymes [Wilchek & Bayer, 1988].

2.8 Avidin-biotin polymers

Green *et al.* (1971) synthesised bisbiotinyl diamines that contained between 9 and 25 bonds between the carboxyl groups of the two biotin groups. Compounds with more than 12 residues behaved in a bifunctional manner and gave rise to linear polymers of avidin. The polymers became shorter when the chain-length of the reagent increased to 23 bonds, which suggested that the reagent was able to bind intramolecularly to two subunits of the same avidin. When the two biotin moieties were separated by between 12 to 14 bonds, polimerisation was possible and reversible. The addition of one more methylene group resulted in a slowly reversible polymerisation. The polymers with the longer chain bisbiotinyl diamines were found to be stable [Green *et al.* 1971; Green, 1989].

2.9 The principle of avidin-biotin technology

The biotin-avidin interaction has some unique characteristics that make it ideal as a bridge system [Diamandis & Christopoulos, 1991]. The non-covalent interaction of biotin with avidin has a formation (affinity) constant of 10^{15} L. mol^{-1}, which is even higher than for interaction of antigens with their specific antibodies [Wilcheck & Bayer, 1990; Diamandis & Christopoulos, 1991]. The high affinity ensures that the bond is not easily disturbed by changes in pH, the presence of chaotropes or multiple washings when the complex is immobilised.

Because biotin is such a small molecule (244.31 Da), it usually does not alter or change the biological activity of other molecules to which it is attached, e.g. biotinylated antibodies. Even if biotin is used to derivitise smaller molecules, such as mononucleotides or steroid hormones, the biotinylated moieties are still capable of acting as enzyme substrates or of binding to their specific antibodies [Wilcheck & Bayer, 1990; Diamandis & Christopoulos, 1991]. The avidin-biotin interaction is strong enough that biotin can still be available for binding by avidin even when it is coupled to proteins. Biologically active compounds are commonly chemically modified with biotin through its valeric acid side chain [Wilcheck & Bayer, 1990].

Biotin coupled to a low molecular mass molecule can still be specifically recognised by avidin, streptavidin or their derivatives [Wilchek & Bayer, 1988; Wilcheck & Bayer, 1990]. Because avidin has four binding sites for biotin, it makes cross-linking between different biotin-containing molecules possible, which adds another dimension to the avidin-biotin system [Wilcheck & Bayer, 1990].

2.10 Dissociating the avidin-biotin complex

Because of the strong bond between avidin and biotin, it would be impossible to dissociate the complex, if so required, within an chromatographical system. Fortunately, however, several solutions have been developed to overcome this limitation.

It has been suggested that digestion with proteinase K could be used to remove the avidin from a biotinylated DNA complex. The most common method used to dissociate the avidin-biotin

complex in solution is by incubating it in solutions of guanidine-HCl, pH 1.5. A milder option is to use biotin-derivatives containing a cleavable arm that can be displaced from an avidin-probe complex by simply cleaving the built-in cleavable bond [Wilchek & Bayer, 1988].

Even though dissociation is possible, the method may interfere with other components of a chromatography system (e.g. coupled proteins, enzymes and antibodies). It is therefore safer to rather use the ABC system as a cross-linking or targetting tool in an experimental set-up where dissociation is not required.

2.11 Strategies in the application of the avidin-biotin technology

Numerous strategies are available for applying biotin-avidin technology in a given experimental system (Table 2.1). One of these is to biotinylate a molecule directly. The biotinylated molecule is then directly analysed, either by direct application of an avidin conjugated probe, or by application of native avidin followed by the application of a biotinylated probe. Biotinylation of binding molecules makes this technology even more useful. The binder is defined as any molecule, usually a macromolecule or portion thereof, which possesses an inherent affinity for a specific target molecule(s) in a given experimental system. The 'affinity' can have a biochemical, chemical or physical nature and the binder molecule can be biotinylated [Wilchek & Bayer, 1988].

Strategy II (a), as can be seen in Table 2.1, uses preformed complexes in the set-up. The binder and the immobilised matrix are biotinylated before the avidin is introduced to act as the bridge between the two. These two complexes are then coupled with avidin acting as a bridge to obtain immobilised binder molecules, such as antibodies, onto the solid matrix. These immobilised binder antibodies can bind the target molecules specifically and can subsequently be used in affinity separation of the target molecules.

Table 2.1: Summary of some biotin-avidin application strategies

Strategy I: Direct biotinylation of target

a. Target-**B : Av**-Probe

b. Target-**B : Av : B**-Probe

Strategy II: Biotinylation of binder and/or probe

a. Target : Binder-**B : Av**-Probe

b. Target : Binder-**B : Av : B**-Probe

c. Target : Binder-**Av : B**-Probe

Strategy III: Use of preformed complexes

a. Target : Binder-**B :** [**Av : B**- Probe]

b. Target : [Binder-**B : Av**] **: B**- Probe

c. Target : [Binder-**B : Av : B**- Probe]

Strategy IV: Multiple binders

a. Target : $Binder_1$: $Binder_2$-**B : Av**-Probe

b. Target : $Binder_1$: $Binder_2$-**B : Av : B**- Probe

c. Target : $Binder_1$: $Binder_2$-**Av : B**-Probe

2.12 Other biotin-binding proteins

Avidin and streptavidin are not the only biotin-binding proteins known. Meslar, Camper & White (1978) purified a biotin-binding protein (BBP) from egg yolk, known as the egg-yolk BBP. The molecular mass (73 300, as opposed to 62 400 for avidin), and pI value (pH 4.6 as opposed to pH 10 for avidin) for egg-yolk BBP differ from avidin. The yolk protein contains less lysine, threonine, isoleucine and aspartic acid residues. In addition the *N*-terminal amino acid of the yolk BBP is leucine as opposed to threonine and tryptophan of avidin. Biotin release is 1 000-fold faster for egg-yolk BBP and neither protein cross-reacts with antibodies directed against the other [Meslar, Camper & White, 1978].

Despite the various differences, important similarities are that both BBPs are tetrameric with identical monomers, binding one biotin molecule each [Meslar, Camper & White, 1978; Meslar & White, 1979; Bayer & Wilcheck, 1989]. Unlike the egg-white avidin, the egg-yolk BBP is saturated with biotin, which suggests that it may play a biotin storage role and regulate the release of biotin [Bayer & Wilcheck, 1989].

Antibodies against the biotinyl group itself were raised by Berger in 1979 by preparing a highly immunogenic conjugate of biotin with bovine serum albumin [Berger, 1975; Berger, 1979; Bayer & Wilcheck, 1989; Kohen *et al.* 1997]. Disadvantages of biotin-binding antibodies are their high molecular mass (about twice that of avidin) and the fact that they only possess two binding sites for biotin (versus four for avidin). In addition, they do not have as high affinity constants for biotin as avidin does. The constant region (Fc) of the antibodies also provides an extra interference, because it is recognised by certain cell types (e.g. macrophages and lymphocytes), which have receptors for it. [Bayer & Wilcheck, 1989]. Nevertheless, the antibodies also are BBPs.

2.13 Applications of biotin-avidin technology

The Avidin-Biotin system in the form of columns was used to isolate receptors and DNA to name but a few, more applications of the avidin-biotin system will be discussed in detail in the following paragraphs.

2.13.1 Affinity chromatography

Immobilised carriers containing either biotin or avidin have been used for isolation purposes. Biotin columns were used to immobilise avidin for the subsequent isolation of receptors via biotinylated proteins [Wilchek & Bayer, 1988].

Avidin containing columns are used for the isolation of biotinylated proteins and subsequently their receptors. The complex between effector and receptor is more easily dissociated than the strong interaction between avidin and biotin, thus making this technology useful to isolate the receptors [Wilchek & Bayer, 1988].

The technology has also been used for gene enrichment and DNA isolation. This is achieved when immobilised avidin, complexed with biotinylated single-stranded DNA, is used to purify complementary single-stranded DNA [Wilchek & Bayer, 1988]. In another application, biotin was used to cap unreacted amino groups remaining after coupling of an equimolar amino acid mixture. The *N*-biotinylated sequences were removed using an avidin-coupled agarose column [Quesnel, Delmas & Trudelle, 1995]. More affinity applications involving this technology are discussed in chapter 5, section 5.2.4.

2.13.2 Localisation: affinity cytochemistry

Specific cell membrane components, such as sialic acids or amino acid side chains, can be identified topologically by selectively biotinylating it with group-specific biotinylating reagents. Avidin coupled to an electron-dense marker, such as ferritin, can then be used to 'see' the cell membranes using electron microscopy. Biotinylated effectors such as hormones, toxins and neurotoxins have been used to localise the corresponding receptor directly with the aid of an avidin-conjugated marker [Wilchek & Bayer, 1988].

The immunochemical approach involves a biotinylated antibody, which recognises the effector as its antigen, localising it without sterical hindrance. This approach is also called the multiple binder approach (Table 2.1, strategy IV). Cell membrane antigens can be detected with biotinylated antibodies, receptors with biotinylated effectors and sugars with biotinylated lectins. [Wilchek & Bayer, 1988].

Different reactive biotinyl derivatives can be used to stain proteins, glycoproteins and nucleic acids on blots as replacements for Coomassie blue or general stains currently used on gels. Avidin-conjugated horseradish peroxidase (Avidin-HRP) is commonly used as a colour-producing compound. This method is much more specific and results in a much higher sensitivity level than when applying direct methods of gel-staining [Wilchek & Bayer, 1988].

2.13.3 Diagnostics: immunoassays and gene probes

Probes are commonly labelled with radioisotopes, but problems such as stability, safety and difficult detection prompted the development of non-radioactive probes [Forster *et al.* 1985]. Avidin-biotin technology has been used to enhance the signal in immunoassays. The signal is achieved by an avidin conjugated with a reporter group or by native avidin followed by a biotinylated marker [Wilchek & Bayer, 1988].

Biotinylated analogues of dUTP and UTP have been synthesised and incorporated into DNA or RNA strands with RNA or DNA polymerases to produce affinity probes [Langer, Waldrop & Ward, 1981]. Biotin hydrazide reacts with the cytidine residues in DNA to produce biotin-labeled DNA in a one-step reaction [Reisfield *et al.* 1987].

Photobiotin has been used to biotin-label double-stranded or single-stranded DNA and RNA hybridisation probes respectively. Sensitivity levels of this approach were found to be equivalent to ^{32}P-labelled DNA probes. Labelling with photobiotin is obtained through chemical bonding and can be visualised because photobiotin-labelled nucleic acid is red [Forster *et al.* 1985; Diamandis & Christopoulos, 1991]. Single-stranded DNA is not degraded or cross-linked as a result of the photobiotin labelling procedure, and the recognition of the complementary strand is unlikely to be impaired. The biotin-labelled DNA products were found to be stable for at least

five months and are able to give reproducible results under normal hybridisation conditions [Forster *et al.* 1985].

Biotinylation of DNA results in increased sensitivity, and is much less hazardous than radioactive probes, leading to its increased use [Wilchek & Bayer, 1988].

2.14 Advantages of the avidin-biotin system

The greatest advantage of the avidin-biotin system is undoutedly the exceptionally high affinity that exists between avidin and biotin and the stability of the resulting complex. Biotin can be attached to most binders and probes using any of the vast number of biotinylation reagents available. Biotinylation of molecules normally does not disrupt the biological activity and physical characteristics of these molecules. Multiple biotin groups per binder combined with the tetrameric structure of avidin leads to an amplification of the subsequent signal. The system is also very versatile. A wide spectrum of different biotinylating reagents, biotinylated binders and avidin-containing probes are commercially available [Wilcheck & Bayer, 1990].

2.15 Conclusions

A number of important factors that have direct bearing on the experimental work of this project were obtained from the background given on biotin-avidin technology in this chapter. These may be summarised as follows:

1. Avidin binds best to biotin under physiological conditions, PBS buffer, pH 7.4, 37°C [Green, 1989]. This information could be used to ensure maximum avidin binding to the immobilised ligand (biotin).

2. NHS-biotin is the most common reagent used to biotinylate primary amine groups [Wilchek & Bayer, 1988]. It is easy and relatively inexpensive to synthesise and characterise, making the affinity system more cost-effective.

3. Avidin is stabilised when bound to biotin [Bayer, Ehrlich-Rogozinski & Wilchek, 1996]. This fact is very useful in an affinity system where the stability of the complex is of high importance.

4. The avidin-biotin interaction is the strongest natural interaction known and is thus very useful as a cross linker in an affinity system [Wilchek & Bayer, 1988].

The avidin-biotin technology is very versatile and allows flexibility in its application in various fields as discussed in this chapter. The information given in this chapter was implemented in the experimental work presented in chapters 4 and 5, where biotin-pluronic synthesis and the affinity system will be discussed.

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CHAPTER 3

ADSORPTION OF PLURONIC® F108 ONTO POLYSULPHONE FLAT-SHEET MEMBRANES

3.1 Introduction

An essential part of the study, as indicated earlier, was to immobilise an avidin-biotin complex onto a membrane by means of pluronic mediation. The aim of the work presented in this chapter was to investigate the adsorption of pluronic onto hydrophobic polysulphone membrane surfaces to gain an understanding of the adsorption density and desorption phenomena governing the interaction of the pluronic with the PSMs. This information is essential to the development of the ligand carrier/membrane system under study. The information obtained from these studies can be used to determine the theoretical maximum amount of pluronic that would adsorb onto a unit surface area of a PSM. Although the adsorption of pluronic from solution has potential in the pre-treatment of ultrafiltration membranes, this process has not yet been adequately investigated and characterised [Ruthven, 1984]. Li, Caldwell and Rapoport (1994) established that the poly(propylene oxide) (PPO) centre block of the pluronic surfactant governs the concentration of pluronic molecules that will adsorb onto a given hydrophobic surface. If the maximal coating concentration of pluronic is known, one could assume that the maximal coating concentration of any pluronic derivative, with the same PPO centre block size, will be similar.

To establish the maximal coating concentration of derivatised pluronic, it was essential to study the adsorption behaviour of underivatised pluronic onto a PSM surface of given area first. From the results of these adsorption studies, the Langmuir adsorption isotherm was determined and subsequently the fractional coating could be calculated.

In this chapter the physical and chemical properties of pluronic is discussed, followed by a discussion of the process of adsorption and the relevance of adsorption isotherms.

3.1.1 Applications of adsorption onto membranes

Adsorption is important for the successful implementation of several membrane processes and a number of applications rely on adsorption to function. A few of these are mentioned below.

In certain applications of membrane bioreactors whole cells are immobilised by adsorption onto the outside of a hollow fibre membrane with nutrients being fed to the biomass from the inside of the hollow fibre. Oxygen and nutrients can also be fed to the cell cultures through different fibres [Howell, Sanchez & Field, 1993].

Enzymes are also immobilised and used to catalyse the continuous bioformation of very specific products. In such a system it is possible to regenerate and reuse the coenzymes. Although such bioreactor configurations show potential, they have not yet been applied on large scale [Howell, Sanchez & Field, 1993c].

Adsorption onto membranes can also be exploited to produce biosensors [Howell, Sanchez & Field, 1993c].

3.2 Properties of pluronic and its general applications

Pluronic is a tri-block copolymer surfactant that consists of PEO and PPO blocks (figure 3.1). The centre block comprises a known number of PPO repeat units, flanked by two PEO blocks, also with a known number of repeat units. Various pluronic surfactants are commercially available. These materials not only differ with respect to the molecular mass of their hydrophilic and hydrophobic entities, but also in the molecular mass ratio of the hydrophobic to hydrophilic segments.

The pluronic PEO-PPO-PEO block copolymers are used in antifreeze, cutting and grinding fluids, industrial processing, latex paints and in spray cleaners. Pluronic were previously used to pre-treat ultrafiltration membranes to prevent fouling during effluent purification [Lee & Kopecek, 1989]. They are also formulated into consumer and commercial automatic dishwashing formulations because of their excellent protein-soil defoaming action, good sheeting action and low toxicity (The latter is of particular interest in our application of the polymer). The block

copolymers facilitate wetting and leave no film. Their wetting and detergent properties provide for good cleaning properties, while their ease of removal eliminates problems in subsequent painting, coating and electroplating operations. These block copolymers are also used as defoamers in water treatment as they possess good chemical and thermal stability and have a low environmental impact factor [BASF consumer manual, 2000].

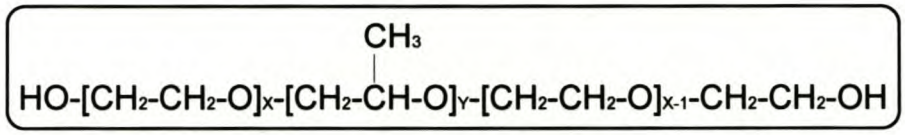

Figure 3.1: Schematic structure of a pluronic molecule.

3.3 Adsorption

3.3.1 Definition and nomenclature

Adsorption can be described as the accumulation and concentration of an adsorbate molecule (in solution) on an adsorbent surface (solid) where the interactions mainly consist of van der Waal's forces [Crittenden, 1998; Shaw, 1970].

Adsorption therefore involves competition between components in a liquid mixture for adsorption sites on a solid surface. Depending on the chemical and physical nature of the solid and the solution components, the forces involved in the adsorption process may be non-specific (van der Waals) forces or stronger more specific forces. Adsorption was very simply defined by Howell, Sanchez and Field (1993a) to be an interaction between the solute and the solid membrane.

Adsorption can also be described differently. When a drop of liquid spreads across a solid surface to form a film, it is said that the liquid wets the surface and adsorption therefore takes place. When no adsorption takes place, it is referred to as a uniform distribution of the liquid even though the molecules in the liquid are present right up to the solid surface. If at any moment the concentration of one of the components in the mixture is greater at the contacting surface compared to its proportion in bulk, the component is said to be positively adsorbed. The other components in the solution, if any, are then negatively adsorbed [Shaw, 1970].

3.3.2 The principle and process of adsorption

The mechanism of adsorption is influenced by chemical and physical factors. Adsorption mechanisms arise from physico-chemical forces acting between solute molecules and, in this case, the membrane. If the adsorbent and the adsorbate are brought together, the sum of the involved forces determines whether an attachment will be effected between a molecule in solution and the membrane. The forces involved in physical adsorption include van der Waals forces (dispersion-repulsion) as well as electrostatic forces comprising polarisation and dipole interactions. Although the van der Waals forces are always present, the electrostatic forces have a greater contribution where adsorbents, such as zeolites, are investigated [Ruthven, 1984].

During the initial stages of adsorption the concentration of an adsorbate in the proximity of a solid surface rises because the surface adsorption sites of the adsorbent are unsaturated and forces exist to attract the adsorbate molecules to these available sites. When adsorption occurs, it is said that both the repulsive and attractive forces between the adsorbent and adsorbate become balanced [Crittenden & Thomas, 1998].

Non-specific (van der Waals) forces are predominantly responsible for physical adsorption and the stronger more specific forces (e.g. ionic interaction) for chemisorption. Physical adsorption in a solution is more common than chemisorption [Shaw, 1970]. Adsorption from a solution always involves competition between the solute and the solvent or between components present in the liquid mixture for adsorption sites on the adsorbent. Adsorption from a solution can often be predicted, depending on the nature of the adsorbent and the components of the solution. For example, if the adsorbent is non-polar and the solvent is polar, fatty acids will be adsorbed stronger than the solvent. The amount of fatty acid adsorbing onto a hydrophobic surface at a given concentration is found to increase with increasing length of the non-polar hydrocarbon chain, i.e. butyric > propionic > acetic [Shaw, 1970].

In this study the PSM acted as a hydrophobic solid phase. The adsorbate, pluronic, has a centre block with hydrophobic properties and the solvent is deionised water. A positive adsorption of the pluronic molecules onto the PSM can therefore be expected because the pluronic molecules

have stronger interaction with the membrane and the adsorption will be stronger than that of the deionised water.

The initial slope of the adsorption isotherm is usually steeper than the rest of the curve, as predicted by the Langmuir equation, because more active sites are available at the start of the adsorption process. Because of steric hindrance, subsequent adsorption becomes more and more difficult and monolayer coverage is not as readily achieved as predicted by the Langmuir equation [Shaw, 1970].

Hsu, Chang and Lin (1997) proposed a theory on how the adsorption process takes place. They proposed that adsorption progresses in three consecutive steps when a soluble surface active material adsorbs or desorbs into or out of a liquid interface. First, the surfactant molecules diffuse and/or convect between a deeper layer of solution and the subsurface layer directly adjacent to the fluid interface. Second, adsorption or desorption of the surfactant molecules takes place between the subsurface layer and the fluid interface. Last, the adsorbed surfactant molecules rearrange at the fluid interface. The diffusion takes place because of the concentration gradient, and adsorption or desorption is driven by the chemical potential of the molecules involved. Rearrangement may be caused by reorientation, complex formation, phase transition, or formation of a two-dimensional micelle structure. Adsorbing polymeric molecules usually rearrange, but it may be a slow process compared to non-polymeric molecules [Hsu, Chang & Lin, 1997].

3.3.3 Characterisation of adsorption

When a small drop of liquid is placed on a solid surface, it may either spread across the surface or stand up and retain a spherical shape. A water droplet will typically stand up and retain its spherical shape on a hydrophobic surface. The angle between the surface and the tangent of the edge of the drop is called the contact angle (figure 3.2). The relative hydrophobicity of a solid surface can be described by the contact angle (θ) of a polar liquid, such as water, or by its critical surface tension. The critical surface tension is defined as the surface tension at which the contact angle approaches zero [Howell, Sanchez & Field, 1993b]. When a surfactant adsorbs onto a hydrophobic membrane, it is said that it wets the membrane.

When a polar liquid, such as water, wets a hydrophilic surface, it spreads across the surface, yielding a contact angle between zero and 90°. The more hydrophilic the surface, the smaller the contact angle, and vice versa. When a contact angle of 0° is measured, water wets the surface and the surface is regarded hydrophilic. When water spreads across the surface so that the contact angle is between 90° and 180°, the surface is regarded more hydrophobic and a droplet of a polar fluid will stand proud on such a surface (figure 3.2 c). Water does not wet the surface at all and many hydrophobic polymers in contact with water will show such behaviour. [Howell, Sanchez & Field, 1993b].

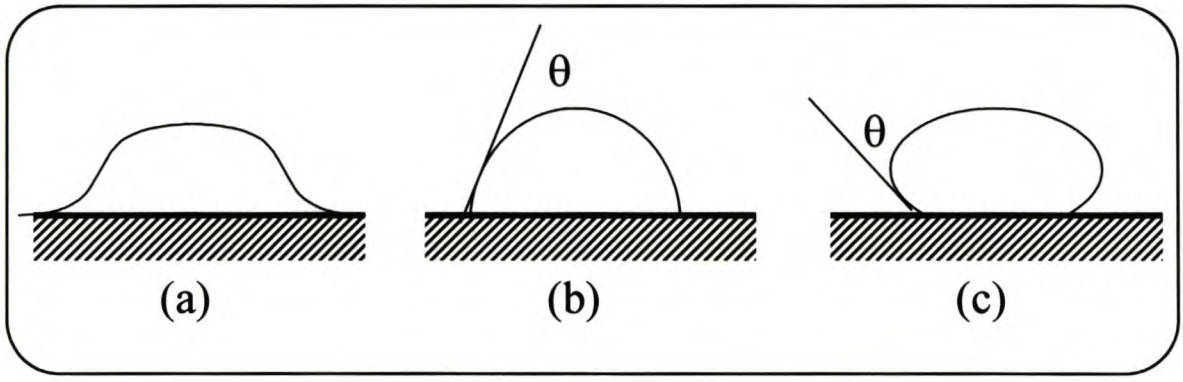

Figure 3.2: Contact angles of a liquid droplet on a solid (non-porous) material: (a) adsorption of water when the contact angle is approaching zero. (b) the contact angle is between 0° and 90°. (c) the contact angle is between 90° and 180° [Howell, Sanchez & Field, 1993b].

3.3.4 Physical adsorption and chemisorption

Generally there are two types of adsorption, namely physical adsorption and chemisorption. As mentioned earlier, physical adsorption involves relatively weak intermolecular forces. Chemisorption involves the formation of a chemical bond between the adsorbent and adsorbate. Intermediate cases are also possible. General features which distinguish between the two adsorption processes mentioned are given in Table 3.1 [Ruthven, 1984].

Table 3.1: Comparison between physical and chemical adsorption [Ruthven, 1984].

Physical adsorption	Chemisorption
No chemical bonds are involed and no electron transfer takes place. Polarization of the adsorbate may occur.	Chemical bonds between the sorbate and the surface are formed and electron transfer is inevitable.
The heat of adsorption is low relative to chemisorption.	The heat of adsorption is higher.
Non-specific.	Highly specific.
Monolayers and multilayers can be formed.	Only monolayers are possible.
Only significant at relatively low temperatures.	Adsorption is possible over a wide range of temperatures.
Does not require activation.	Requires activation.
Is rapid and reversible.	May be slow and irreversible.

3.3.5 The thermodynamics of adsorption

Adsorption is favoured at the more active sites, and therefore the heat of monolayer adsorption might be expected to become significantly less exothermic as the surface coverage increases. The heat of adsorption also becomes less exothermic as monolayer coverage is approached [Shaw, 1970]. It is well known that where any spontaneous process occurs, there is a decrease in Gibbs free energy ($\Delta G < 0$). Furthermore, during an adsorption process there must be a decrease in the entropy of the liquid because part of the molecule becomes restricted from free movement when adsorbed onto a solid surface, and $\Delta S = S_{ads} - S_{liquid}$ [Crittenden & Thomas, 1998]. From the thermodynamic equation, $\Delta G = \Delta H - T\Delta S$, it follows that ΔH also decreases and heat is released. It can therefore be said that physical adsorption is an exothermic process and as a consequence heat will always be released when adsorption occurs [Crittenden & Thomas, 1998].

3.3.6 Influence of solute adsorption onto membranes

The main consequence of molecules adsorbing onto membranes used for filtration, is the reduction in filtration rates. A change in the porous structure of the membrane was also reported [Howell, Sanchez & Field, 1993a]. In this study the "fouling potential" of a PSM was exploited to achieve affinity separation. By exploiting the adsorptive properties of the PPO centre block of

pluronic the membrane could be coated with pluronic that had its end-groups modified to carry a specific affinity ligand to effect an affinity separation.

3.3.7 Adsorption properties of pluronic

The adsorption behaviour of pluronic has not yet been discussed in detail. Li, Caldwell and Rapoport (1994) did an extensive study of the adsorption properties of pluronic, and other surfactants closely related to pluronic, onto polystyrene (PS) latex spheres. The PEO tethers of the polymer molecules were found to exhibit restricted, relatively slower motion on low curvature spheres and higher motion on smaller spheres. The freedom of movement of the PEO tethers depends on the PEO chain length: the longer the chain, the greater the motion. The PEO blocks are only weakly, if at all, involved in the actual attachment of the surfactant onto the surface. It was found that smaller PS particles take up fewer polymer molecules per unit area than the larger particles, resulting in the formation of thinner adsorption layers. However, the packing density allows the PEO chains greater freedom of movement. A longer PEO chain implies a thicker adsorption layer of the polymer as a whole, causing greater dynamics of the PEO tether ends. The same researchers found that the surface concentration of the polymers was more dependent on the molecular mass of the hydrophobic PPO centre block than on that of the flanking PEO chains.

It was concluded that pluronics with similar PPO block sizes show comparable surface concentrations regardless of the PEO chain length. A high polymer surface concentration forces an extended PEO chain conformation, the so-called 'brush' conformation, and a lower surface concentration allows a 'mushroom-like' behaviour [Li, Caldwell & Rapoport, 1994].

3.4 Adsorption isotherms

The adsorption isotherm can be described as the relationship, at a given temperature, between the equilibrium amount of molecules adsorbed onto an adsorbent surface and the concentration of the same molecules in the coating solution [Shaw, 1970]. In other words, the adsorption isotherms relate the bulk solute concentration to the solute concentration on the surface of the adsorbent [Blanch & Clark, 1997].

Many adsorption systems are characterised by the familiar hyperbolic Langmuir isotherm. Alternatively, many interactions by biological compounds, such as antibiotics, steroids and hormones, are characterised by the Freundlich isotherm. It is also known that experimental data are usually expressed in terms of an apparent adsorption isotherm. To obtain an apparent adsorption isotherm, the amount of solute adsorbed at a given temperature per unit solid surface area is plotted against the coating concentration [Shaw, 1970].

A more general classification scheme for equilibrium isotherms is based on the values of the separation factor, R_{eq}, defined as $R_{eq} = 1/(1 + K_L c_0)$, where c_0 is the feed concentration of the solute and K_L is the Langmuir constant. If $R_{eq} = 0$, the isotherm is said to be irreversible. If $0 < R_{eq} < 1$, the isotherm is favourable. If $R_{eq} = 1$ it is linear, and when $R_{eq} > 1$, the isotherm is unfavourable. A favourable isotherm appears concave because significant adsorption takes place at low coating concentrations, and an unfavourable isotherm appears convex [Blanch & Clark, 1997]. In the following paragraphs each of the isotherms will be discussed in detail.

3.4.1 Experimental adsorption isotherms

In studies on gas adsorption by Crittenden and Thomas (1998), five different isotherm types with distinctive forms were identified. This classification of the isotherms are called the Brunauer classification of isotherms and are shown in figure 3.3. [Crittenden & Thomas, 1998].

Type I isotherms have an inherent property by which adsorption is limited to the completion of a single monolayer of adsorbate onto the adsorbent surface. Type II isotherms do not reach a saturation area, but rather an inflection point where the monolayer is formed. More molecules adsorb to form successive layers, rendering a rising isotherm with an inflection point at the saturation stage of every consecutive layer. Adsorbents with a wide range of pore sizes are prone to form type II isotherms because of the condensation inside the larger pores where gaseous adsorption was investigated [Crittenden & Thomas, 1998].

Type III isotherms are continuously convex and show a steady state of increase in adsorption capacity as the pressure of the gas rises. Type IV isotherms are similar to type II isotherms except that the adsorption terminates near the relative pressure of unity. Type V isotherms are similar to

type III except that a point of inflection is reached and a saturation limit is approached as the relative pressure rises [Crittenden & Thomas, 1998]. The isotherms determined for gaseous adsorbates can be compared to adsorption from a liquid phase if the gas pressure is replaced by the concentration of the solution.

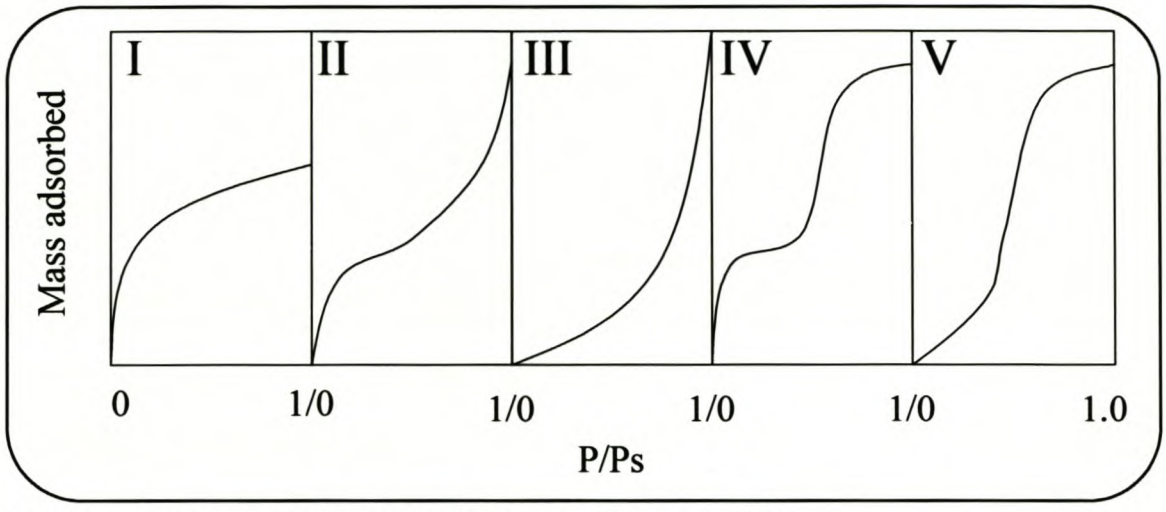

Figure 3.3: Brunauer classification of isotherms [Ruthven, 1984].

3.4.2 Langmuir adsorption isotherm

The Langmuir adsorption isotherm was originally proposed in 1918. It describes the adsorbate-adsorbent systems in which the extent of adsorbate coverage onto the solid surface does not exceed a molecular monolayer [Crittenden & Thomas, 1998].

The Langmuir and Freundlich equations are frequently used to present adsorption from solution data, for which they take the following different forms:

$$X/m = abc/(1 + ac) \qquad \text{(Langmuir)}$$

and $$X/m = kc^{1/n} \qquad \text{(Freundlich)}$$

where

X: amount of adsorbed material (mg)

m: mass of adsorbent (mg)

c: equilibrium solution concentration (mg/ml)

a, b, n and k: constants [Benefield, Judkins & Weand, 1982].

The Langmuir adsorption isotherm is based on the following assumptions:

1. only monomolecular layers are formed during adsorption and each adsorbate molecule occupies only one adsorption site;
2. adsorption is localised (molecules remain at the site of adsorption until desorbed); and
3. the heat of adsorption is constant and independent of surface coverage (a consequence of no lateral interaction between adsorbed molecules) [Shaw, 1970, Crittenden & Thomas, 1998].

The rate of adsorption depends on:

1. the rate at which the molecules collide with the solid surface;
2. the probability of striking a site on the solid surface which is accessible to adsorption; and
3. the activation energy for adsorption [Shaw, 1970].

The rate of desorption depends on:

1. the fraction of the surface which is covered; and
2. the activation energy for desorption [Shaw, 1970].

3.4.3 Freundlich adsorption isotherm

In 1935 Zeldowitch made the assumption that for many systems the heat of adsorption decreases with increasing adsorption. If the decline in adsorption is logarithmic, it implies that adsorption sites are distributed exponentially with respect to an adsorption energy which differs between groups of adsorption sites [Crittenden & Thomas, 1998]. This isotherm relates heat of adsorption to coverage.

The equation of the Freundlich adsorption isotherm is as follows:

$$\theta = k\, c^{1/n}$$

Where

θ: the fractional surface coverage

c: equilibrium solution concentration (mg/ml)

and k and n are constants, valid for $n > 1$

Unlike the Langmuir adsorption isotherm it does not indicate an adsorption limit when coverage is sufficient to fill a monolayer (θ = 1). The isotherm may be regarded as a convenient representation of the Langmuir equation at intermediate coverages ($0 < \theta < 1$).

3.4.4 Brunauer-Emmett-Teller equation

According to the theory proposed by Brunauer-Emmett-Teller, the rate of adsorption onto a surface equals the rate of desorption from that surface at equilibrium. This equation provides for multilayer adsorption.

The assumptions made in the BET theory are:

1. there are no interactions between neighbouring adsorbed molecules; and
2. the heat evolved during the adsorption of second and subsequent layers of molecules equals the heat of liquifaction [Crittenden & Thomas, 1998].

3.4.5 Interpreting isotherms

From the isotherms the performances of the different adsorbents can be compared. If the plot of the isotherm of adsorbent A lies above that of adsorbent B, the former adsorbent associates more strongly with the adsorbate than the latter. If the isotherm is steep, it indicates that the adsorptive capacity of the adsorbent increases at higher equilibrium solute concentrations. If the slope is less

steep, the adsorbent has more adsorptive capacity at lower equilibrium concentrations [Benefield, Judkins & Weand, 1982].

From the three different adsorption equations the Langmuir equation was considered the best choice for use in this study. The BET theory makes the assumption that no interaction occurs between adsorbed molecules and in this study this cannot be guaranteed. The Langmuir equation is based on the assumption that only a monolayer is formed on the solid surface. In this study the same assumption will be made because a mulilayer cannot be guaranteed at saturation.

3.5 Experimental

In the following paragraphs the materials used as well as the adsorption, desorption and related experiments will be described.

3.5.1 Materials

Pluronic was obtained from BASF S. A., hexane from Unilab, S. A. And propanol-2 from MERCK, Darmstadt. Chloroform was obtained from MERCK NT Laboratory Supplies, S. A. Deionised water was purified with a Milli-Q water purification system from Millipore. All spectrophotometric measurements were done in triplicate on a Cary 100 UV-visible spectrophotometer.

The ammonium ferrothiocyanate assay solution was prepared by adding 30.4 g NH_4SCN to 18.64 ml of $FeCl_3.6H_2O$ solution (density of 1.45 g/ml). The resulting solution was made up to a final volume of 1 ℓ with deionised water.

Flat-sheet membranes were cast from a solution containing 27 % (m/m) polysulphone (Udel P3 500) and 73 % (m/m) *N*,*N*-dimethylacetamide (DMAc). 27 g polysulphone was dissolved in 73 g DMAc by rotating the solution container for more than 48 h to obtain a homogeneous solution. The solution was degassed before it was used to cast the flat-sheet membrane of 200 μm thickness (figure 3.4). The purpose of the reverse osmosis water is to disolve the solvent to let polimerization of the polysulphone take place.

All spectrophotometric determinations were done in triplicate and all the graphs were drawn with Graphpad Prism® version 3.0. The standard error of the mean was calculated using the statistical functions of the software.

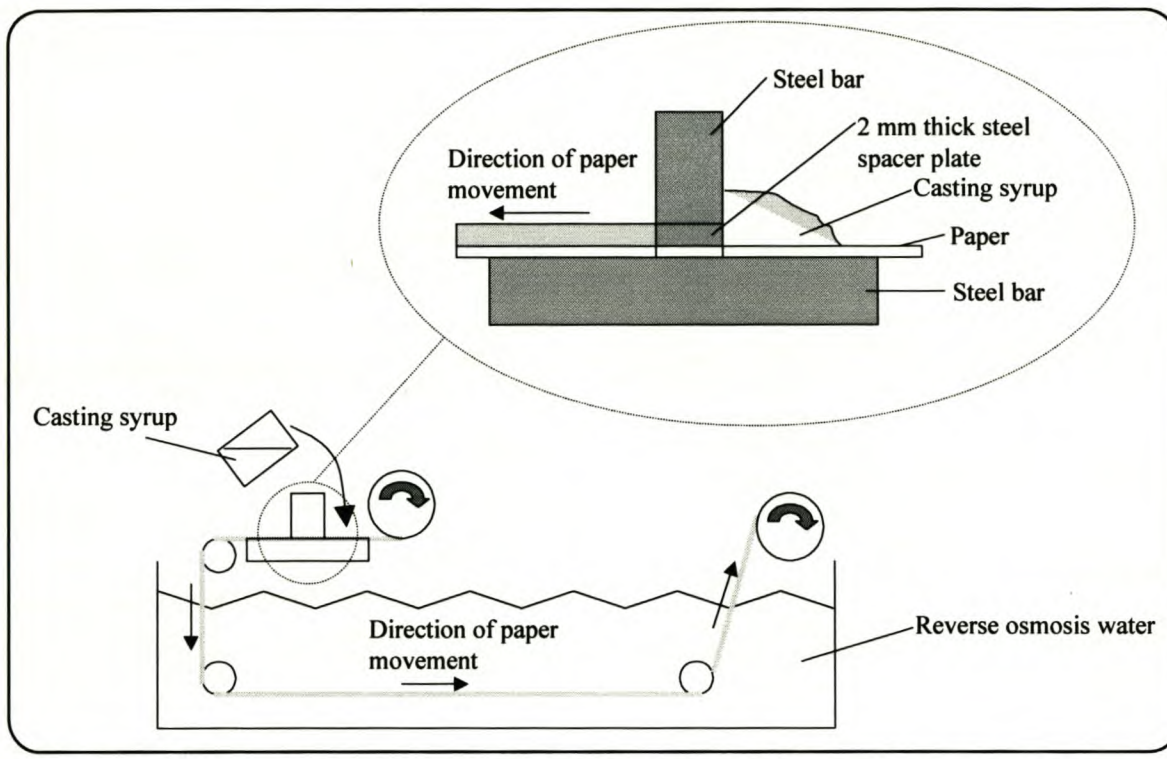

Figure 3.4: Schematic diagram of how the flat-sheet polysulphone membranes were cast.

3.5.2 Assay for pluronic

A dilution series of 0.125 to 0.3 mg of pluronic per ml was prepared in chloroform and an assay was subsequently performed in triplicate on each dilution. Three samples of 2 ml each from the chloroform standard solutions were added to test tubes and 2 ml ammonium ferrothiocyanide were added to each of these samples. The mixtures were vortexed thoroughly and the two phases were allowed to separate. The bottom chloroform phases were pipetted out and the absorption was measured at 488 nm [Stewart, 1980]. A standard curve of absorption versus pluronic concentration yielded a straight line over the concentration series used.

3.5.3 Adsorption of pluronic onto PSM

A dilution series from 5 mg/ml pluronic to 0.125 mg/ml was prepared in deionised water. Polysulphone flat-sheet membrane sections (25 cm^2) were cut and incubated in each pluronic

solution (0.125 mg/ml to 5 mg/ml) for 12 h at room temperature to ensure that the adsorption reaction reached equilibrium. One membrane section was incubated in deionised water only to serve as a control for the experiment. It was assumed that the rates of adsorption and desorption were equal after 12 h of incubation. After incubation the membranes were washed three times with deionised water and allowed to dry for 30 min. The membranes were cut into smaller pieces of ~1 cm^2, added to a 25 ml hexane-propanol-2 (3:2, v/v) mixture and incubated for 1 h at room temperature in order to extract the adsorbed pluronic from the membranes. The mixture containing the membranes was boiled gently for 15 min and subsequently filtered through Whatman no. 1 filter paper. The filter paper was washed twice with 10 ml pre-heated hexane-propanol-2 (3:2, v/v). The hexane-propanol-2 filtrate was subsequently evaporated under N_2 at slightly elevated temperature, until it was completely dry. The extracted pluronic was re-dissolved in 10 ml chloroform and the aforementioned assay was used to determine the amount of pluronic that had adsorbed onto the membrane samples during the different adsorption experiments.

3.5.4 The effect of temperature on the adsorption of pluronic onto PSMs

The same adsorption experiment was repeated at two different temperatures: 4°C and 40°C. The membranes were analysed, as previously described (sections 3.5.2 and 3.5.3).

3.5.5 The effect of time on the adsorption of pluronic onto PSMs

Eight PSM squares (25 cm^2) were cut and incubated separately in 5 mg/ml pluronic solutions. Every hour, for 8 h, one of the membrane squares was removed from the coating solution. The membrane was washed three times with deionised water and the pluronic extracted and assayed as previously described (sections 3.5.2 and 3.5.3).

3.5.6 Desorption behaviour of the pluronic from the adsorbed PSMs

Membrane squares (25 cm^2) were coated overnight at 40°C with a 5 mg/ml pluronic solution. All the membranes were washed three times with deionised water. One membrane was immediately analysed and the other membranes were incubated in deionised water at room temperature, 4°C and 40°C. After 2, 24 and 48 h one membrane from each temperature group was analysed as previously described (sections 3.5.2 and 3.5.3).

3.5.7 Calculations

The manipulations on the Langmuir isotherm and all other calculations are described in Appendix 1. The Langmuir isotherm was fitted from the Langmuir equation given below:

$$X/m = abc/(1+ac) \qquad (1)$$

3.6 Results and discussion

The following paragraphs contain a discussion of the results presented in graphical format.

3.6.1 Assay for pluronic and the standard curve for its determination

A method had to be established by which the concentration of pluronic could be determined before any adsorption experiment could be performed. A standard curve (figure 3.5.) of absorption versus pluronic concentration yielded a straight line for the concentration series used. The chromophore obeyed Beer's law over the concentration range and a straight line was obtained for the concentration range 0 to 0.2 mg/ml pluronic.

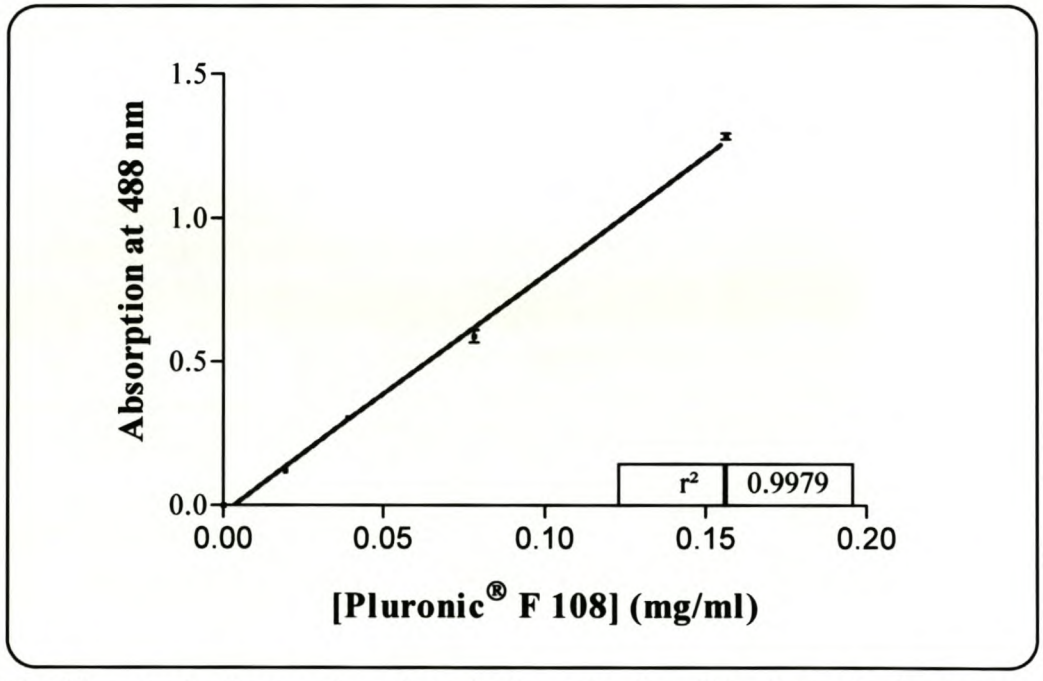

Figure 3.5: The standard curve for pluronic determination. The determinations were done in triplicate. The standard error of the mean (S. E. M.) was calculated using the statistical functions of the software.

The equation representing this straight line is y = mx + c, where m is the slope of the line, c is the y-intercept, y is the adsorption and x the pluronic concentration. From the slope and y-intercept of the standard curve, an equation for the straight line was obtained which was used to calculate any concentration of pluronic assayed within the same concentration range.

3.6.2 Adsorption of pluronic onto PSM

The saturation adsorption of pluronic onto PSM is shown in figure 3.6. Saturation was reached at a pluronic concentration of 5 mg/ml. The experimental data suggests that no more than 5 mg/ml pluronic was necessary to saturate the (25 cm^2) membrane surface under study.

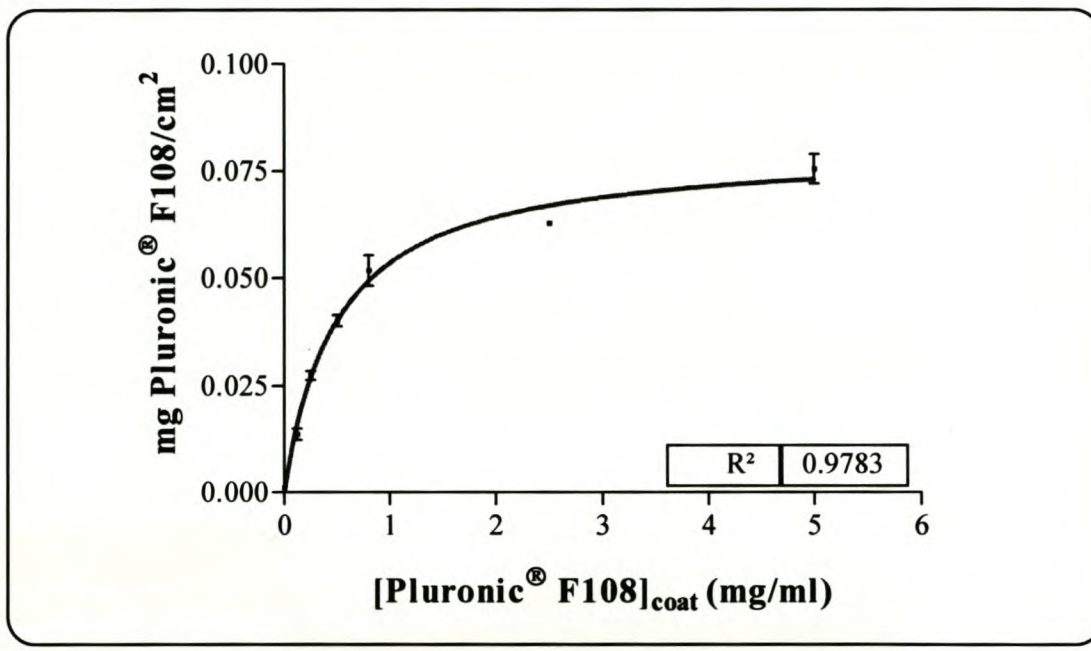

Figure 3.6: Saturation curve of pluronic adsorbed onto PSM at room temperature (Each point represents the mean of at least three determinations ±S.E.M. The standard error of the mean was calculated using the statistical functions of the software)

The next step was to calculate the Langmuir isotherm to determine the theoretical maximum amount of pluronic that could adsorb onto 1 cm^2 PSM. A plot of the reciprocal of the amount of pluronic extracted per 1 cm^2 membrane versus the reciprocal of the pluronic concentration remaining in the coating solution, yielded the adsorption curve as shown in figure 3.7. This linearised form of the isotherm was used to determine the constants as described in Appendix 1.

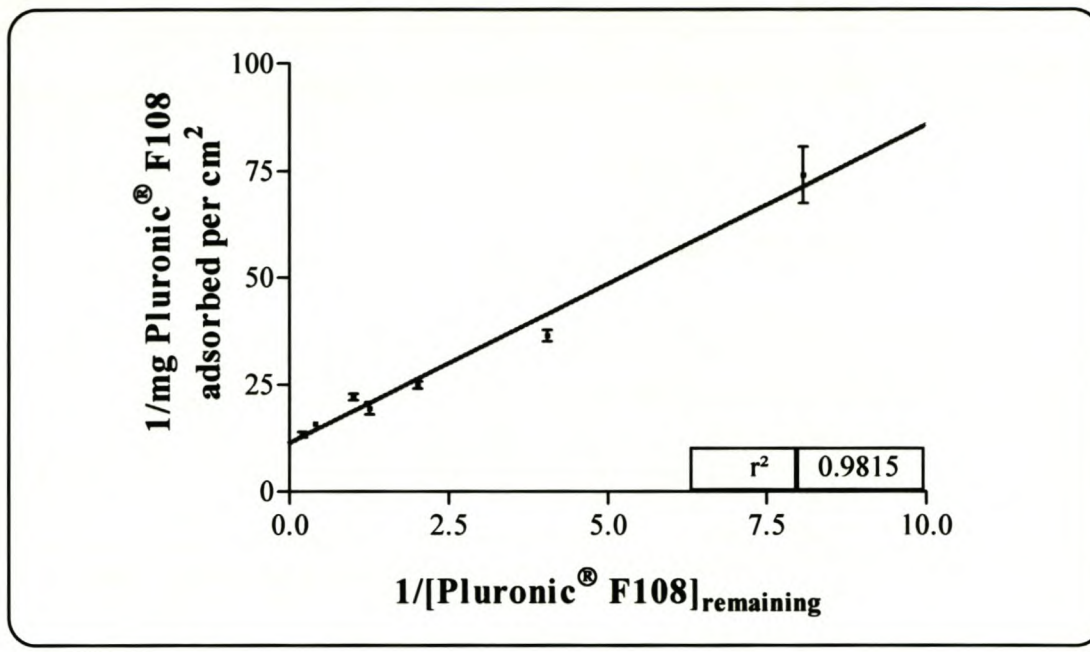

Figure 3.7: Double reciprocal plot of the concentration of pluronic remaining in solution after adsorption had taken place and pluronic that had adsorbed onto the membrane. (Each point represents the mean of at least three determinations ±S.E.M. The standard error of the mean was calculated using the statistical functions of the software.)

Slope: $1/ab = 7.429$

Y-intercept: $1/b = 11.43$

thus, $b = 0.088$

and, $a = 1.538$

From equation 4 (Appendix 1) X was determined as 0.088 mg, the amount of pluronic adsorbed onto 1 cm^2 if $c \rightarrow \infty$. The coating concentration could subsequently be determined for any required level of adsorption. The fractional coating of PSM by pluronic as a function of coating concentration is shown in figure 3.8.

At 90 % of the maximum achievable level of adsorption, 0.08 mg pluronic will have adsorbed onto 1 cm^2 of membrane area. The coating concentration to achieve this can be determined by

substituting the value for X and the constants into equation 10, which then yields a coating concentration of 5.89 mg/ml.

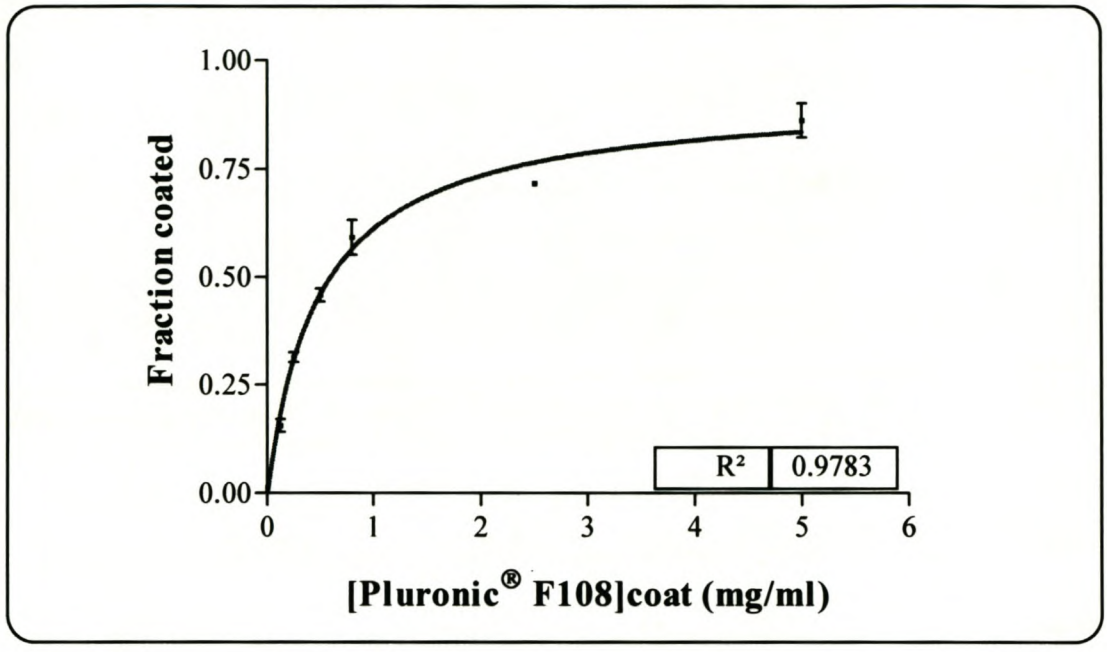

Figure 3.8: Fractional coating of PSM by pluronic as function of coating concentration. (Each point represents the mean of at least three determinations ±S.E.M. The standard error of the mean was calculated using the statistical functions of the software.)

It was also necessary to determine how long it took for the PSM/pluronic system to reach equilibrium saturation. From this study (figure 3.9), it was concluded that adsorption reached equilibrium after 6 h. An incubation period of between 6 and 8 h was therefore regarded as sufficient to achieve maximum membrane coating.

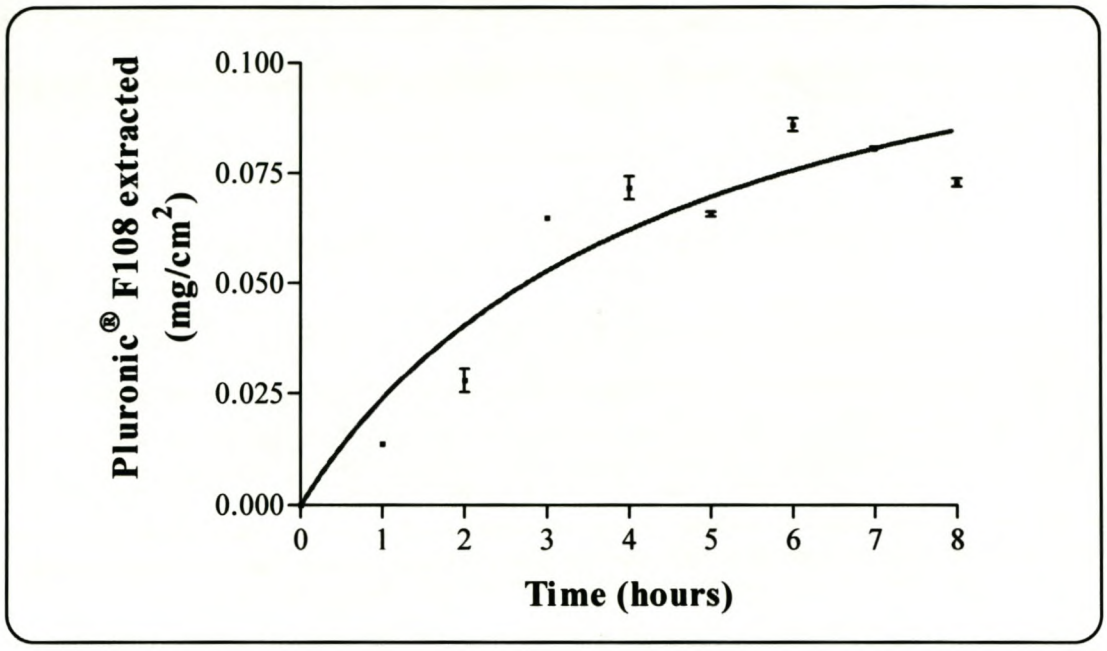

Figure 3.9: Adsorption of pluronic as a function of time. (Each point represents the mean of at least three determinations ±S.E.M. The standard error of the mean was calculated using the statistical functions of the software.)

3.6.3 The effect of temperature on adsorption

The effect of temperature on the adsorption of pluronic onto PSM was also investigated. The studies indicated (figure 3.10) that more adsorption occurred at higher temperatures. This could be ascribed to increased rates of diffusion at higher temperatures. The chance of contact between

the PPO block and the membrane surface increased at a higher temperature. Furthermore, the activation energy of adsorption could also be lowered at higher temperatures.

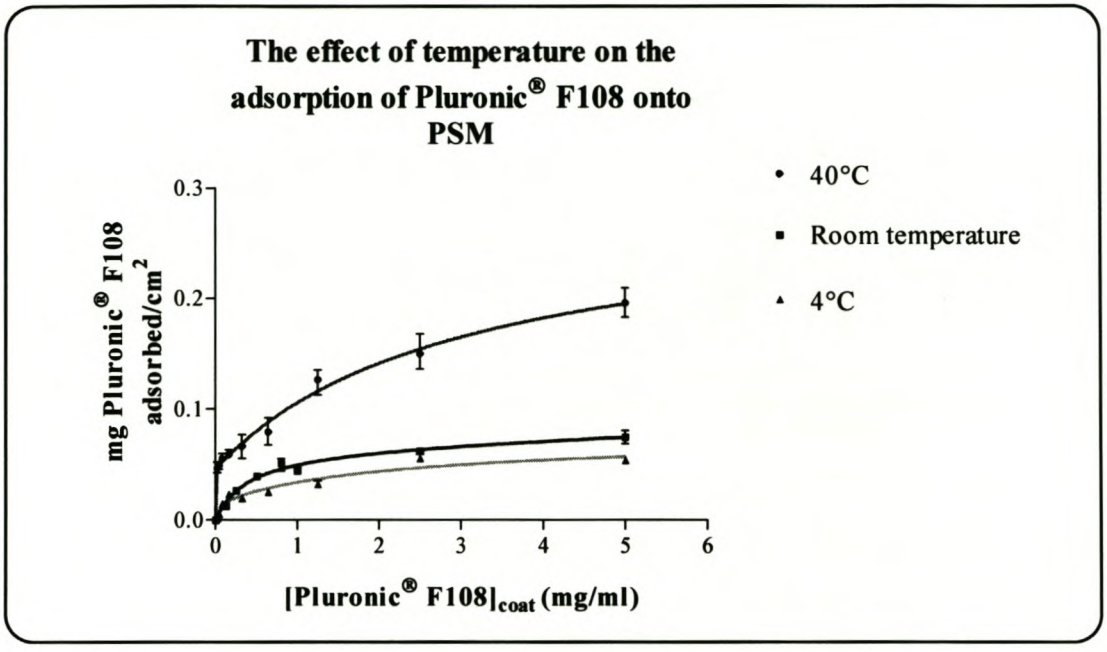

Figure 3.10: Effect of temperature on pluronic adsorption onto PSM. (Each point represents the mean of at least three determinations ±S.E.M. The standard error of the mean was calculated using the statistical functions of the software.)

3.6.4 Investigation of the desorption behaviour

The reversibility of the pluronic adsorption was also assessed. The results shown in figure 3.11 confirm that the adsorption of pluronic onto PSMs is reversible and that desorption does occur. The results further indicate that desorption is a function of both temperature and time.

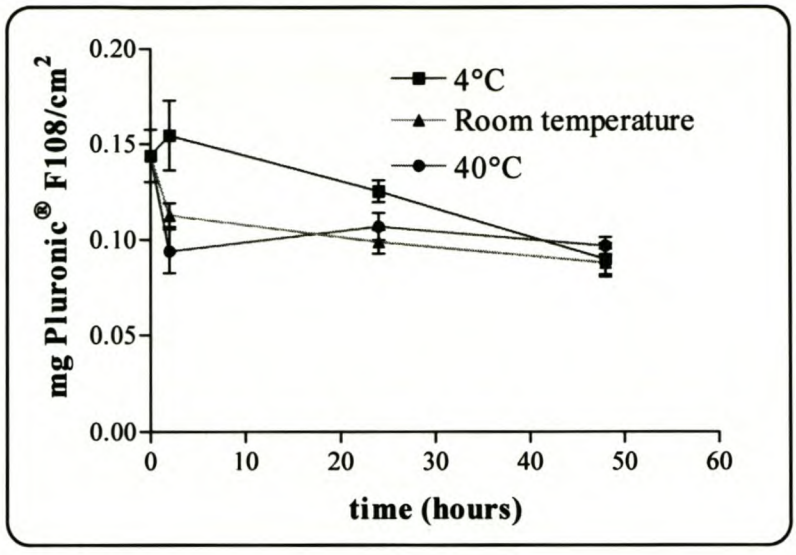

Figure 3.11: Desorption of pluronic from polysulphone membranes after 2, 24 and 48h and at 4°C, ambient temperature and 40°C respectively. (Each point represents the mean of at least three determinations ±S.E.M. The standard error of the mean was calculated using the statistical functions of the software.)

3.6.5 Calculation of the Langmuir adsorption isotherm

The next step was to calculate the Langmuir isotherm and to determine the theoretical maximum amount of pluronic that could adsorb onto a unit PSM area. The Langmuir adsorption isotherm for the pluronic/PSM system is shown in figure 3.7.

From this curve the theoretical maximum quantity of pluronic that would adsorb onto a 1 cm^2 PSM was calculated. (The calculations are shown in Appendix 1). The amount of pluronic that adsorbed onto 1 cm^2 membrane at each of the different coating concentrations was calculated as a fraction of the theoretical maximum amount per square cm. The adsorption fractions were then plotted against the coating concentrations to determine the maximal pluronic coating concentrations (figure 3.8).

3.7 Conclusions

From the preceding study the following conclusions could be reached:

1. It was possible to determine the adsorption isotherm of pluronic experimentally.
2. The effect of temperature on the adsorption could be observed from the isotherms. More adsorption takes place at higher temperatures.
3. From the isotherm it was possible to calculate what the coating concentration for pluronic must be to achieve a specific required level of coating. This implies that specific amounts of pluronic or its derivatives can be used to obtain the desired membrane surface coating.
4. The rate of desorption from the membranes could be determined. At 4°C desorption occurred at a slower rate than the higher temperatures.
5. The effect of temperature on the desorption could be determined. Initially more desorption occurred at a high temperature, but after 50 hours the desorption for all the solurions under the different temperatures were more or less the same.

These findings imply that no unnecessary or excessive pluronic or its derivatives need to be used to get the desired level of membrane coverage. This information is important. Amino-pluronic (AP) and biotinylated amino-pluronic (AP-B) takes time to synthesise and is therefore much more expensive than pluronic. For the purpose of this study, it was assumed a priori that the adsorption behaviour of AP and AP-B are similar to that of the pluronic base material studied.

In the following chapters the synthesis of AP and AP-B are described and the information obtained in this chapter would ensure that the application of these derivatives onto PSMs were closer to optimum.

3.8 References

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CHAPTER 4

THE SYNTHESIS AND CHARACTERISATION OF BIOTINYLATED AMINO-PLURONIC

4.1 Introduction

As mentioned earlier in section 3.2, pluronic were previously used to pre-treat ultrafiltration membranes to prevent fouling during effluent purification [Lee & Kopecek, 1989]. As discussed in chapter 2, the biotin-avidin complex can be used to link a biological molecule that could be used in an affinity application onto a solid support.

In this study the adsorptive properties of pluronic was used to anchor an affinity ligand onto a PSM. To achieve this goal the terminal hydroxyl groups of pluronic had to be modified to a more reactive functional group, which would facilitate chemical coupling of the affinity ligand to the PEO-PPO-PEO block copolymer. Amino-pluronic was therefore synthesised, and in turn biotinylated, using *N*-hydroxysuccinimide biotin ester, an activated form of biotin.

The suitability of this synthetic route was first assessed on a model compound, 2-methoxyethylamine, and validated by NMR spectroscopy (it was simpler to work with a smaller molecule for NMR verification purposes). The same synthetic protocol was then used to derivatise the larger pluronic molecule.

4.2 Materials and methods

4.2.1 Materials

Biotin, *N*-hydroxysuccinimide and $LiAlH_4$ were obtained from Sigma, S. A. DMF, chloroform, pyridine, 2-propanol, dicyclohexylcarbodiimide, *p*-toluenesulphonyl chloride, $MgSO_4$ and methanol was obtained from MERCK, Darmstadt. 2-Methoxyethylamine was obtained from Aldrich, S. A. Pluronic was obtained from BASF, S. A. NaN_3 was obtained from Riedel-de Haën. TLC were performed using MERCK Kieselgel 60 F-254 glass backed silica gel plates. Spots were visualised by spraying with chromic acid and gently charring over a micro bunsen

burner. Chromic acid spray reagent stock solution was prepared by dissolving 5 g $Na_2Cr_2O_7$ in 5 ml H_2O and 100 ml H_2SO_4. This stock solution was diluted 4 times prior to use. Kieselgel 60 was obtained from MERCK, article 9385, 230-400 mesh. Tetrahydrofuran (THF) was predried on anhydrous K_2CO_3 and distilled from a liquid K:Na (5:1) alloy. DMF was dried by predrying 500 ml DMF over anhydrous KOH and then distilling. The first ± 100 ml was discarded and the rest was used.

All the NMR analyses were conducted on a Varian VXR 300 NMR spectrometer.

4.2.2 Preparation of *N*-hydroxysuccinimide biotin ester

Biotin (1 g) was dissolved in 10 ml warm, dry DMF and allowed to cool to room temperature. 0.6 g *N*-hydroxysuccinimide was added while the suspension was stirred. In a separate flask 1.6 g dicyclohexyl carbodiimide was dissolved in 4 ml dry DMF. The dicyclohexyl carbodiimide solution was added to the other compounds and the resulting solution was stirred for 48 h. The urea precipitate was filtered off and the filtrate was concentrated by applying reduced pressure to the solution on a rotary evaporator coupled to a vacuum pump (0.1 mm Hg). About 30 ml ether was added to the residue and the white crystals that formed were filtered off. The crystals were washed thoroughly with the same amount of 2-propanol. The crystals were then dried *in vacuo* for 5 h at room temperature. The melting point was determined with a Gallenkamp melting point apparatus as 202.7°C (Litt: 196-210°C). [Wilchek & Katchalski, 1971; Bayer & Wilchek, 1974]. The resultant crystals were analysed with ^{1}H and ^{13}C NMR spectroscopy.

4.2.3 Coupling of biotin to 2-methoxyethylamine via NHS-biotin

The model compound used was 2-methoxyethylamine. 0.1 g NHS-biotin was dissolved in a minimum volume of DMF by heating it slowly to ± 80°C. 2-Methoxyethylamine (50 µl) was added and the mixture was stirred at room temperature for 48 h. The resulting white compound was degassed for ± 3 h under vacuum, yielding crude white crystals.

4.2.4 Purification of the biotin-coupled model compound

After synthesis and drying, the biotin analog was dissolved in a minimum volume of the eluant (8:1 chloroform-methanol). The column had a 1 cm internal diameter and was packed up to

± 15 cm with Kieselgel 60. The column was packed dry with the gel and then the eluant was allowed to run through by gravitation. Air bubbles were removed by pressurising the column with Ar gas. The sample solution was quantitatively loaded onto the column and then run by means of constant Ar pressure. The volume of the first fraction was 10 ml, and subsequent fractions were 5 ml. 22 fractions were collected in total and analysed with TLC.

4.2.5 TLC analysis of NHS- and model compound coupled biotin

The analysis was performed on glass TLC plates. The eluant was 8:1 chloroform and methanol. The dots were visualised by spraying the plates with chromium acid and then heating them slightly over a flame, so that the dots showed up brown in colour.

4.2.6 The synthesis of amino-pluronic

Amino-pluronic was synthesised in three steps. The hydroxyl end groups of the pluronic were first tosylated. The next reaction was the azidation of the tosylated pluronic and lastly, the azide derivatives were reduced to amine groups to produce amino-pluronic [Yaniç *et al.* 2000].

4.2.6.1 Tosylation of the hydroxyl end groups of pluronic

Pluronic (10 g) was dissolved in dry pyridine. *p*-Toluenesulphonyl chloride (20 g) was added to the solution at room temperature. The solution was cooled down to 0°C and incubated at 0°C for not less than 7 d. The resultant mixture was poured onto ± 200 ml of ice and water in a beaker. The solution remaining in the flask was rinsed out with water. The mixture was then extracted with chloroform (4 x 100 ml). The combined chloroform extracts were washed with 200 ml of 6M HCl. The resultant extract was carefully washed with 250 ml water. The chloroform extract was evaporated under reduced pressure at room temperature. The resultant crystals were analysed with NMR [Yaniç *et. al.* 2000].

4.2.6.2 Azidation of the pluronic-tosylate

Pluronic-tosylate (9 g) was dissolved in ± 200 ml dry DMF. NaN_3 (40 g) was added to the solution and stirred under an Ar atmosphere at 65°C for 18 to 20 h. The solution was cooled to room temperature and poured onto ± 100 ml of ice and water. The mixture was extracted three times with chloroform and the combined chloroform extracts were washed with 200 ml 2.5 M

HCl to remove the DMF and then with 250 ml distilled water. The resultant mixture was dried over anhydrous $MgSO_4$. The $MgSO_4$ was filtered off and the solvent was evaporated at reduced pressure and then under high vacuum at 50°C [Yaniç *et al.* 2000].

4.2.6.3 Reduction of the pluronic-azide derivatives

Pluronic azide (± 7 g) was dissolved in ± 150 ml freshly dried THF after which 9 g $LiAlH_4$ was carefully added to the solution. The reaction mixture was stirred under reflux at 70 to 75°C for 5 h and 40 min and cooled down to ambient temperature. The reaction mixture was carefully quenched with distilled water until the mixture had a white gel-like appearance and then continuously extracted with chloroform. The extraction process was not efficient. It therefore became necessary to modify the procedure adopted by Yaniç *et al.* (2000).

The $LiAlH_4$ did not dissolve in an alkaline medium, but resulted in the formation of a thick gel-like solution. Therefore, half the mixture was acidified with HCl to a pH of less than 1 and sonicated until the solution was less viscous. The more liquid gel was then continuously extracted with chloroform. After extraction the chloroform solution was evaporated at reduced pressure and analysed with ^{1}H and ^{13}C NMR spectroscopy [Yaniç *et. al.* 2000]. The yield was 2.1 g AP.

4.2.7 Biotinylation of amino-pluronic

NHS-biotin (30 mg) was dissolved in a minimum volume of dry DMF. Amino-pluronic (100 mg) was dissolved in dry DMF and was added to the NHS-biotin solution and stirred at room temperature for 48 h. The resultant compound was dried under vacuum and analysed with ^{1}H and ^{13}C NMR spectroscopy.

4.3 Results

As already mentioned, ^{1}H and ^{13}C NMR spectroscopy were used to determine the structures of the molecules synthesised. By comparing the NMR data found to NMR data of the same molecules in published literature the success of each synthesis were assessed. The schematic structure of NHS-biotin and the numbering of the atoms of the structure are given in figure 4.1.

The structure of biotinylated 2-methoxyethylamine with atoms numbered is given in figure 4.2. The structure of biotin-pluronic with relevant atoms numbered are given in figure 4.3.

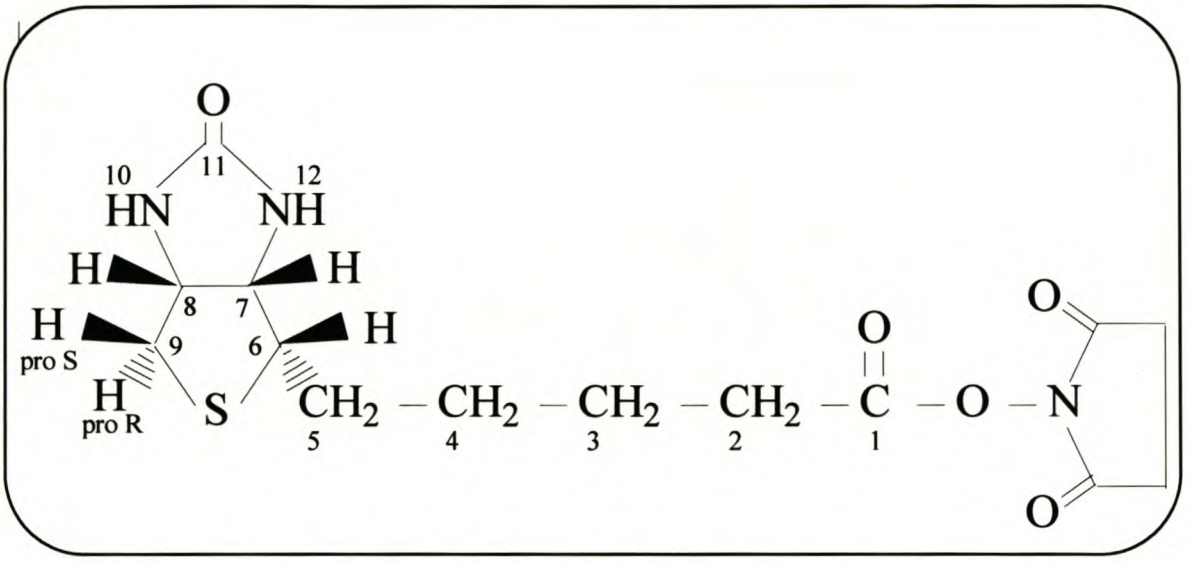

Figure 4.1: The schematic structure of NHS-biotin.

4.3.1 The ^{1}H NMR spectrum of *N*-hydroxysuccinimide-biotin ester (NHS-biotin)

δ_H (300 MHz, DMSO–d_6) 1.36-1.72 (m, 6H, (C**H**$_2$)$_3$–CH$_2$–COO–), 2.59 (d, H, $^{2}J_{H9proR,H9proS}$ = 12.3 Hz, H9$_{proR}$), 2.68 (t, 2H, $^{3}J_{H2,H3}$ =7.4 Hz, H2), 2.82 (s, 4H, –CO–C**H**$_2$–C**H**$_2$–CO–), 2.84 (dd, H, $^{2}J_{H9proS,H9proR}$ = 12.7 and $^{3}J_{H9proS,H8}$ = 5.1 Hz, H9$_{proS}$), 3.12 (ddd, H, $^{3}J_{H6,H5}$ = 7.7 and 6.5 and $^{3}J_{H6,H7}$ = 4.5 Hz, H6), 4.17 (ddd, H, $^{3}J_{H8,H7}$ = 7.6, $^{3}J_{H8,H9proS}$ = 4.6 and $^{3}J_{H8,H9proR}$ = 1.6 Hz, H8), 4.33 (dd, H, $^{3}J_{H7,H8}$ = 8.1 and $^{3}J_{H7,H6}$ = 4.9 Hz, H7) and 6.39 and 6.45 (2 × s, 2 × H, 2 × NH).

4.3.2 The ^{13}C NMR spectrum of *N*-hydroxysuccinimide-biotin ester (NHS-biotin)

δ_C (75 MHz, DMSO–d_6) 24.27 (t, –CO–**C**H$_2$–**C**H$_2$–CO–), 27.56 and 27.82 (2 × t, C3 and C5), 30.01 (t, C2), 39.93 (t, C9), 55.30 (d, C6), 59.28 (d, C8), 61.09 (d, C7), 163.1 (s, C11), 169.37 (s, C1) and 170.7 [s, –N–(**C**O)$_2$–].

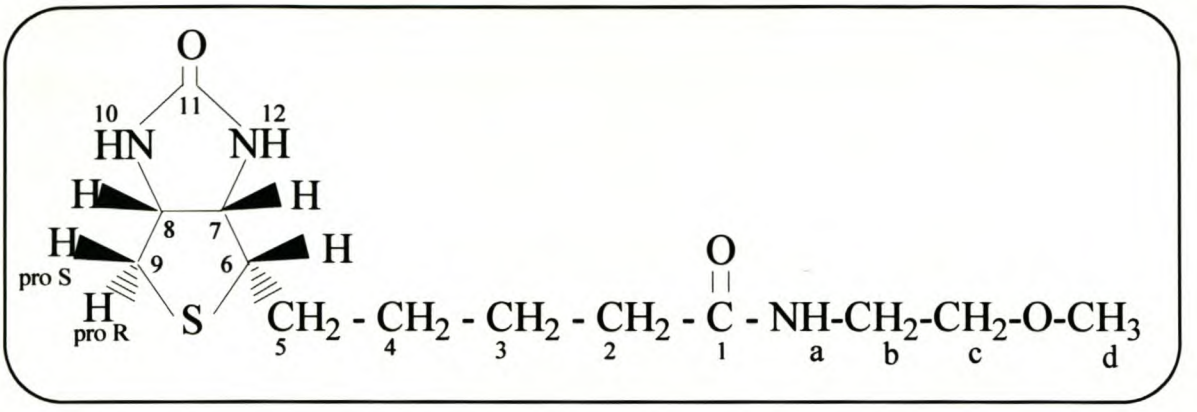

Figure 4.2: The structure of biotinylated 2-methoxyethylamine.

4.3.3 MEA-biotin ^{1}H NMR spectrum

δ_H (300 MHz, DMSO–d_6) 1.22-1.70 (m, 6H, –(C**H**$_2$)$_3$–CH$_2$–CONH–), 2.15 (t, 2H, $^3J_{H2,H3}$ = 7.4 Hz, H2), 2.66 (d, H, $^2J_{H9proR,H9proS}$ = 12.7 Hz, H9$_{proR}$), 2.91 (dd, H, $^2J_{H9proS,H9proR}$ = 12.4 and $^3J_{H9proS,H8}$ = 5.2 Hz, H9$_{proS}$), 3.19 (dt, H, $^3J_{H6,H5}$ = 8.5 and 6.1 and $^3J_{H6,H7}$ = 4.7 Hz, H6), 3.28 (q, 2H, $^3J_{Hb,Ha}$ ~ $^3J_{Hb,Hc}$ ~ 5.7 Hz, –CONH–C**H**$_2$–), 3.33 (s, 3H, –OCH$_3$), 3.41 (t, 2H, $^3J_{Hc,Hb}$ = 5.7 Hz, –CONH–CH$_2$–C**H**$_2$O–CH$_3$), 4.22 (ddd, H, 3$J_{H8,H7}$ = 7.3, $^3J_{H8,H9proS}$ = 4.5 and $^3J_{H8,H9proR}$ = 1.5 Hz, H8), 4.40 (dd, H, $^3J_{H7,H8}$ = 7.5 and $^3J_{H7,H6}$ = 5.4 Hz, H7), 6.45 and 6.51 (2 × s, 2 × H, NH-CO-NH) and 7.94 (t, H, $^3J_{Ha,Hb}$ = 5.4 Hz, –CO–N**H**–CH$_2$–).

4.3.4 MEA-biotin ^{13}C NMR spectrum

δ_C (75 MHz, DMSO–d_6) 25.21 (t, C4), 28.13 and 27.98 (2 × t, C3 and C5), 35.05 (t, C2), 38.23 (t, Cb), 39.83 (t, C9), 55.47 (d, C6), 57.94 (q, Cd), 59.24 (d, C8), 61.10 (d, C7), 70.83 (t, Cc), 163.11 (s, C11), 172.54 (s, C1).

4.3.5 Pluronic-tosylate ^{1}H NMR spectrum

δ_H (300 MHz, $CDCl_3$–d) 1.14 (m, 3nH, –CH$_2$–CH(C**H**$_3$)–O–), 2.46 (s, 3H, Ts–C**H**$_3$), 3.38-3.45 (m, nH, –O–CH**H**CH(CH$_3$)–O–), 3.50-3.59 (m, 2 × nH, –O–C**H**HC**H**(CH$_3$)–O–), 3.59-3.61 (m, 4mH, –C**H**$_2$–C**H**$_2$–O–), 3.66 (s, 2H, –C**H**$_2$CH$_2$–OTs), 4.18 (t, 2H, –C**H**$_2$–OTs), 7.33 (d, H, *m*–Ts–**H**), 7.84 (d, H, *o*–Ts–**H**).

4.3.6 Pluronic-azide ^{1}H NMR spectrum

δ_H (300 MHz, $CDCl_3$–*d*) 1.14 (m, 3nH, –O–CHHCH(C**H**$_3$)–O–), 3.39-3.44 (m, nH, –O–CH**H**CH(CH_3)–O–), 3.51-3.58 (m, 2 × nH, –O–C**H**HC**H**(CH_3)–O–), 3.66-3.68 (s, 4mH, –C**H**$_2$–C**H**$_2$–O–).

4.3.7 Pluronic-azide ^{13}C NMR spectrum

δ_C (75 MHz, $CDCl_3$–d_6) 17.08, 17.21 (2 × s, –O–CHHCH(**C**H_3)–O–), 50.53 (s, **C**H_2–N_3), 72.77; 72.82, 72.85, 72.89, 73.30 (5 × t, –O–**C**HHCH(CH_3)–O–), 75.06, 75.25, 75.29, 75.45 (4 × d, –O–CHH**C**H(CH_3)–O–).

4.3.8 Amino-pluronic ^{1}H NMR spectrum

δ_H (300 MHz, $CDCl_3$–*d*) 1.145, 1.125 (2 × d, 3nH, –O–CHHCH(C**H**$_3$)–O–), 1.27 (s, 2H, N**H**$_2$), 2.79 (s, 2H, –C**H**$_2$–NH_2), 3.40-3.43 (m, nH, –O–CH**H**CH(CH_3)–O–), 3.49-3.58 (m, 2 × nH, –O–C**H**HC**H**(CH_3)–O–), 3.65-3.7 (s, 4mH, –C**H**$_2$–C**H**$_2$–O–).

4.3.9 Amino-pluronic ^{13}C NMR spectrum

δ_C (75 MHz, $CDCl_3$–d_6) 17.29; 17.42 (2 × t, (–CHHCH(**C**H_3)–O–), 42.43 (t, –**C**H_2–NH_2), 70.71 (t, –**C**H_2–**C**H_2–O–), 72.98, 73.03, 73.08, 73.11 (m, –O–**C**HHCH(CH_3)–O–), 75.32, 75.50, 75.53, 75.71 (4 × d, –O–CHH**C**H(CH_3)–O–).

4.3.10 The biotin-pluronic (AP-B) ^{1}H NMR spectrum

δ_H (300 MHz, $CDCl_3$–*d*) 0.88 (m, 4H, H3 and H4), 1.14 (m, 3nH, –CH_2–CH(C**H**$_3$)–O–), 1.26 (s, 2H, H5), 1.82 (m, 2H, H2), 2.71 (s, H, $H9_{proR}$), 2.86 (s, H, $H9_{proS}$), 3.18 (m, H, H6), 3.42 (m, nH, –C**H**H–CH(CH_3)–O–), 3.57 (m, 2 × nH, –CH**H**–C**H**(CH_3)–O–), 3.66 (s, 4mH, –C**H**$_2$–C**H**$_2$–O–), 3.89 (s, 2H, Hb), 4.39 (s, H, H8), 4.55 (s, H, H7), 5.25 (s, H, H12), 5.59 (s, H, H10).

4.3.11 The biotin-pluronic (AP-B) ^{13}C NMR spectrum

δ_C (75 MHz, $CDCl_3$–d_6) 17.20 (q, –CH_2CH(**C**H_3)–O–), 27.65 (t, C5), 27.75 (t, C3), 29.48 (t, C2), 40.29 (t, C9), 55.11 (d, C6), 60.19 (d, C8), 61.75 (d, C7), 70.51 (t, –**C**H_2–**C**H_2–O–), 72.83 and 73.33 (2 × t, –**C**H_2–CH(CH_3)–O–), 75.34 (d, –CH_2–**C**H(CH_3)–O–).

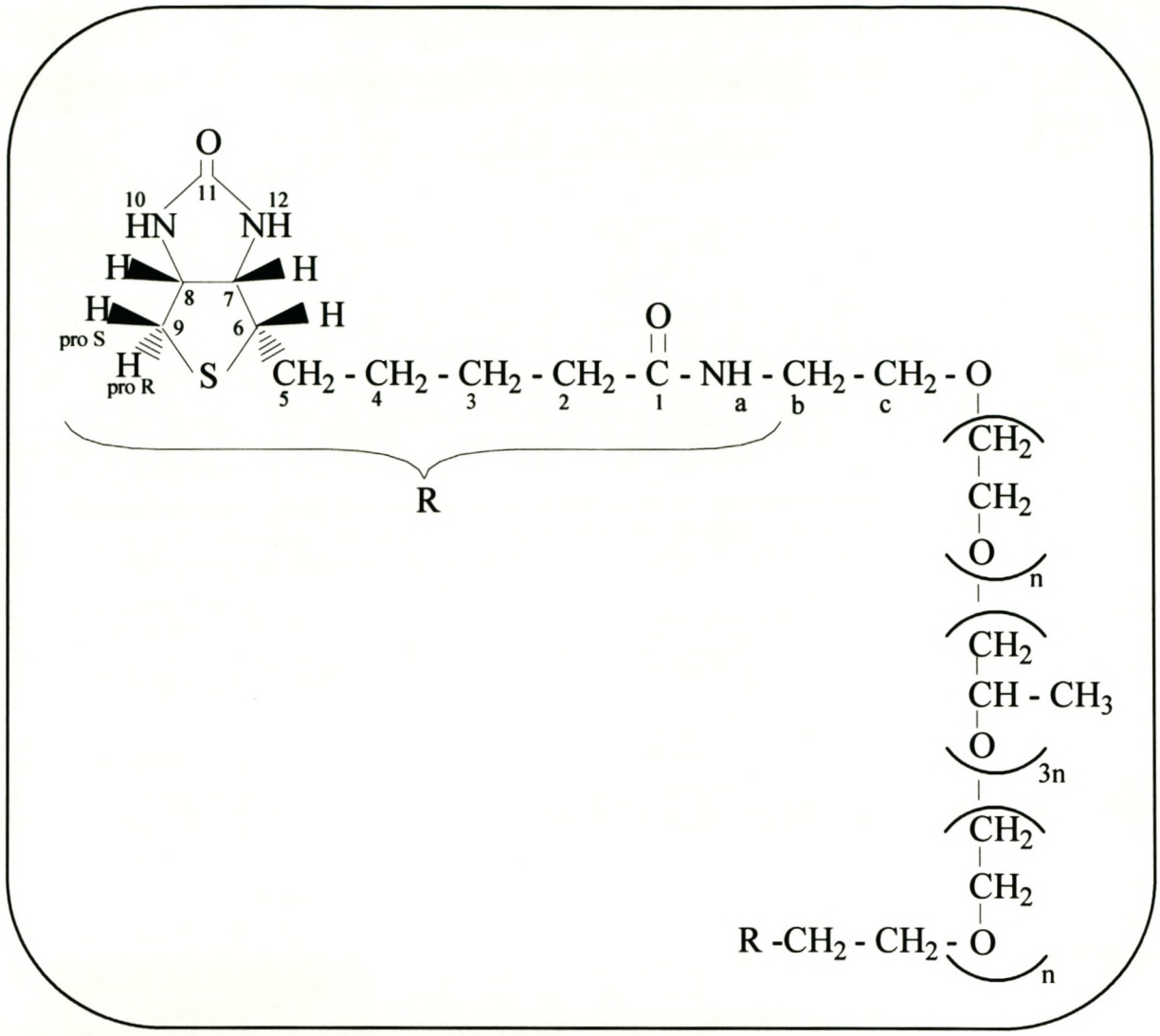

Figure 4.3: Schematic structure of biotin-pluronic.

4.3.12 The ES-MS analysis

The ES-MS results (figure 4.4) indicated the existence of a certain fraction of biotin-pluronic that could only exist if the biotin was coupled to the pluronic. This fraction is represented by the peak at 270. Most of the other peaks could be explained by the structure of biotin-pluronic. The structures of some of the ionic fragments are shown in figure 4.5.

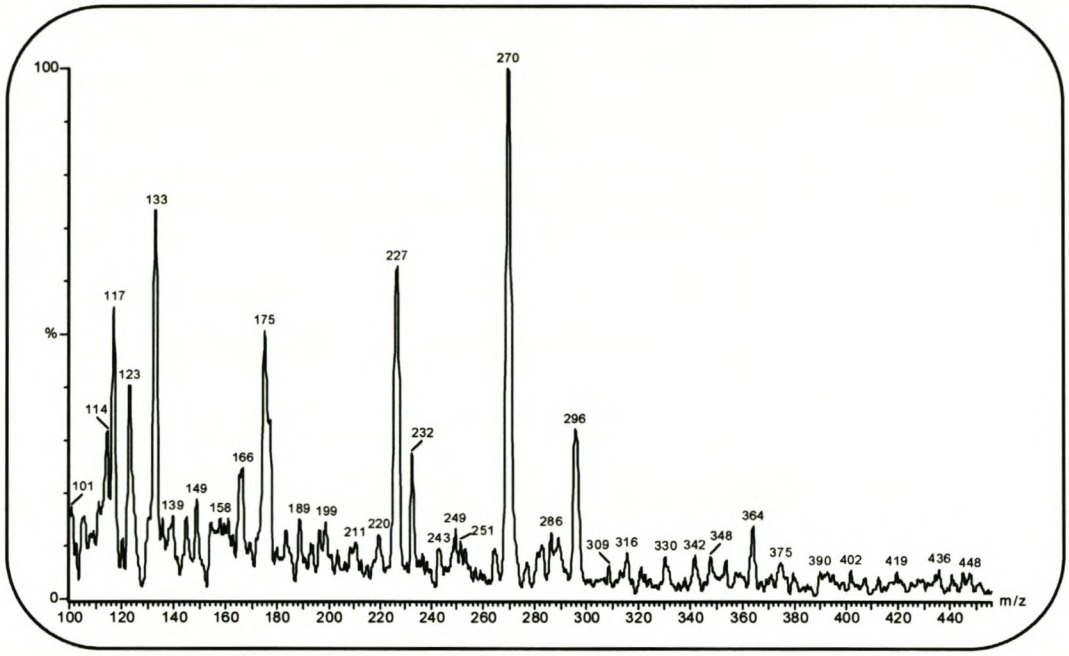

Figure 4.4: ES-MS spectrum of Biotin-pluronic.

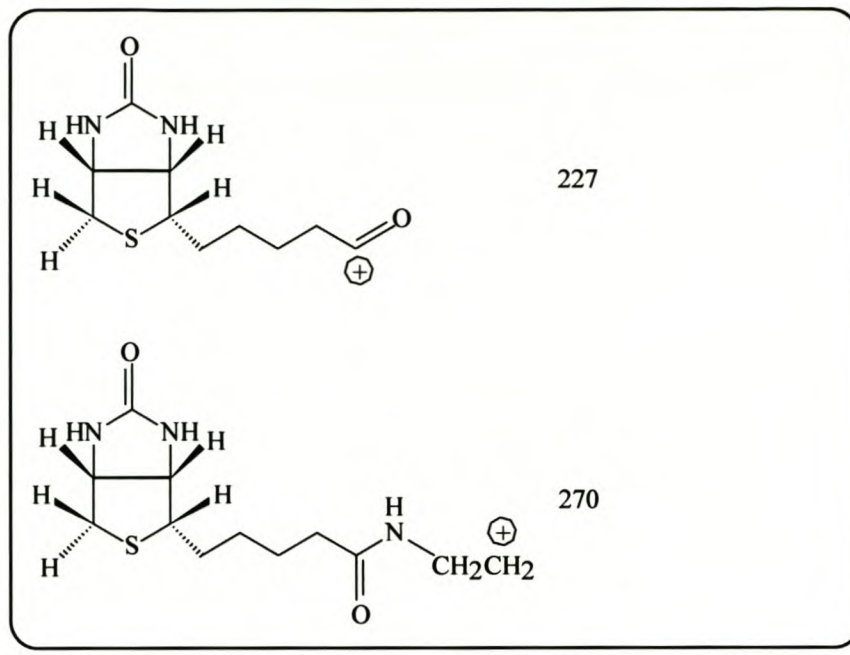

Figure 4.5: The structures of some of the ion fragments seen in the ES-MS spectrum.

4.4 Discussion

The NHS-biotin ^{1}H NMR spectrum compares very well with the NMR spectrum obtained from the literature [Parameswaran, 1990]. The NMR spectra of pluronic-tosylate, -azide and amino-pluronic all correlated very well with published work [Yaniç *et al.* 2000]. No NMR spectrum of the MEA-biotin could be found in the literature, but it could still be analysed by comparing it with the NHS-biotin. The ES-MS analysis confirmed the biotin-pluronic structure. The results in this chapter confirms that the biotin was indeed bound to the pluronic. Even though it was possible to synthesise amino-pluronic, the synthesis was very time-consuming and difficult to perform.

It was still neccessary to proof that the biotin would still be biologically active when immobilised onto a solid surface via the pluronic hydrophobic block. To test whether the biotin-pluronic was able to impart affinity properties to a hydrophobic surface, an experiment was designed to determine it. This experiment will be discussed in the next chapter.

4.5 Conclusions

The following conclusions may be made from this chapter:

1. It was possible to synthesise and characterise an activated biotin (NHS-biotin) for use in the biotinylating reactions.
2. It was possible to synthesise and characterise biotinylated 2-methoxyethylamine as model compound.
3. Pluronic could be converted to amino-pluronic.
4. The amino-pluronic could be biotinylated with the NHS-biotin.
5. During further literature surveys, better methods to synthesise pluronic derivatives were found for future use.

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CHAPTER 5

THE PLURONIC-BIOTIN-AVIDIN MEMBRANE AFFINITY SYSTEM

5.1 Introduction

The aim of the work presented in this chapter was to determine if the biotin-pluronic conjugate could interact with a hydrophobic membrane and with avidin via its biotin moiety, while being associated with the membrane. To determine whether the immobilised biotin-pluronic will interact with avidin, an avidin-horse radish peroxidase (HRP) conjugate was used to bind to the biotin-pluronic and then catalyse a colour reaction. HRP is an enzyme which catalyse the conversion of a substrate molecule, ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)], to a coloured product with a maximum absorption at a wavelength of 405 nm. The colour development would be directly proportional to the amount of enzyme immobilised onto the hydrophobic surface via the pluronic. A schematic diagram of the underlying principle of the experiments carried out with the avidin-HRP complex is given in figure 5.1. These experiments will subsequently be described and discussed in more detail.

Two different hydrophobic surfaces were used in these determinations: polystyrene and PSM. Because very little of the biotin-pluronic conjugate was available, the experiments had to be conducted on a very small scale. The hydrophobic surface of the polystyrene micro titre plate was used as an additional control.

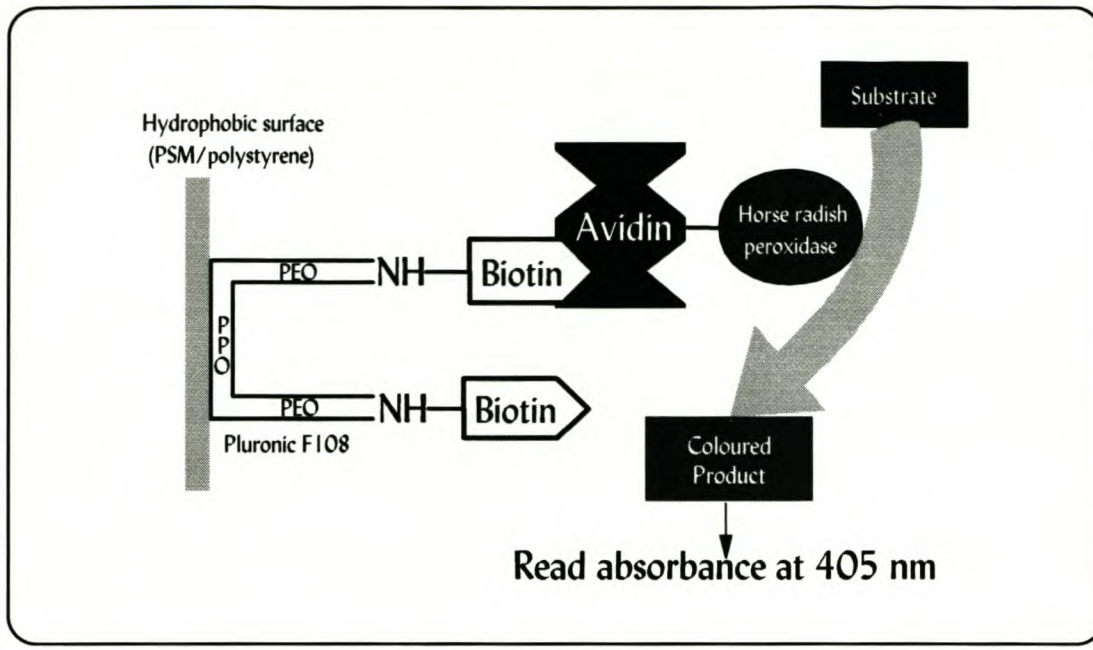

Figure 5.1: Schematic diagram of the affinity assay performed on modified membranes and polystyrene surfaces.

5.2 Theoretical background

5.2.1 Previous methods for immobilisation of proteins onto solid supports

Different solid supports and intermolecular interactions involved in affinity chromatography have already been discussed (Chapter 1). Another aspect, the immobilisation of the ligand onto the solid support can, however, not be excluded from any discussion on affinity chromatography. The activation of solid supports with CNBr is the most common method of immobilisation of ligands onto solid supports. [Axén, Porath & Ernback, 1967; Porath, Axén & Ernback, 1967].

5.2.2 The use of a tether when immobilising a ligand

It has been found previously that enzymes lose their catalytic activity when they interact with a hydrophobic surface, because they lose their structure when exposing their hydrophobic interior to the surface. A significant loss of activity was found when HRP was adsorbed onto a hydrophobic surface [Sandwick & Schray, 1986]. This is because of partial denaturation and because the solid support sterically hinders the ligate from binding to 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 for the

ligand 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, reducing the effective length of the spacer [Scouten, 1981b]. Haung *et al.* (1996) found that the avidin-biotin interactions on a solid phase are several magnitudes slower than the 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 significantly less sterical hindrance to the biotin moiety. Gretch, Suter and Stinski (1986) 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.

5.2.3 Using membranes as the solid support for affinity separation

The factors influencing the choice of a solid support, commercially available as beads and soft gels, were already discussed in Chapter 1. Some drawbacks are, however, associated with these supports when they are used in large scale applications. Compression of the gels, which results in limited flow-rates and thus lower performance [Langlotz & Kroner, 1992; Bueno, Haupt & Vijayalakshmi, 1995] is but one of the drawbacks. Smaller and more rigid materials, such as synthetic polymers and silica-based particles, have been developed, but these supports require high-pressure equipment. Silica-based particles are not stable at pH values above 8 [Langlotz & Kroner, 1992].

Good hydrodynamic flow characteristics were mentioned in Chapter 1 as one of the prerequisites for a good solid support [Porath, 1974; Turková, 1978; Scouten, 1981]. Here, membranes can offer good hydrodynamic characteristics. Membranes are rigid, pore diffusion is negligible and no high-pressure equipment is neccessary [Langlotz & Kroner, 1992]. Another advantage of using membranes as solid supports for affinity chromatography is that affinity membranes can be used on a larger scale and are not difficult to operate [Higuchi, Mishama & Nakagawa, 1991].

Petsch *et al.* (1998) compared the performances of flat-sheet and hollow fiber membranes and Sepharose 4B as solid supports, when they covalently immobilised an adsorbant, poly(ethyleneimine) which adsorbs endotoxins, onto the surfaces. The hollow fiber systems are

known as the convective systems and Sepharose as a particulate system. The system which adsorbed the endotoxin best, was the poly(ethyleneimine) immobilised onto flat-sheets, followed by the hollow fibers and then the Sepharose 4B. Petsch *et al.* (1998) stated that the convective system is superior to the particulate system because the adsorption kinetics were much faster due to the absence of pore diffusion which is the main transport resistance in particulate systems. The use of microfiltration membranes, with pore sizes greater than 0.2 μm to allow immobilisation of polymeric ligands without the risk of pore blocking, was also recommended [Petsch *et al.* 1998].

The surfaces of membranes have previously been used to purify proteins. Langlotz and Kroner used epoxy-activated polymeric composite membranes to couple protein A and rabbit IgG. These proteins were, however, covalently coupled to the membrane [Langlotz & Kroner, 1992]. Human IgG was purified using a histidine-immobilised membrane as the affinity separation system [Legallais *et al.* 1997].

In the present study the modified pluronic was adsorbed onto the membrane by means of hydrophobic interactions and the ligand, which is covalently coupled to the amino-pluronic, was never attached to the membrane directly.

5.2.4 Previous avidin-biotin immobilisation studies

The guanidino analogue of biotin, iminobiotin, was previously immobilised onto Sepharose 4B to affinity-purify avidin and its derivatives [Heney & Orr, 1981]. Gretch, Suter and Stinski (1986) also used the avidin-biotin technology to immobilise monoclonal antibodies. Biotin was used to cap unreacted amino groups remaining after coupling of an equimolar amino acid mixture, and was removed using a avidin-coupled agarose column [Quesnel, Delmas & Trudelle, 1995]. In 1996 Haung *et al.* adsorbed avidin onto polystyrene latex particles to immobilise biotinylated DNA. In the same year Morag, Bayer and Wilchek immobilised nitro-avidin onto Sepharose using the well-known CNBr method. Streptavidin was coupled to Superdex 200, Sepharose 4B and Sephacryl S-1000 gels and used to immobilise biotinylated liposomes containing photosynthetic reaction centres (membrane proteins) [Yang *et al.* 2000].

5.2.5 The principle of the immobilised biotin assay

The principle on which the experiments presented in this chapter was based, was shown in figure 5.1. Biotin-pluronic was immobilised onto the hydrophobic membrane surface through interaction of its hydrophobic PPO centre block. The avidin-HRP conjugate subsequently interacts with the immobilised biotin as avidin has four binding sites specifically for biotin. The peroxidase, which is a haemoprotein enzyme from horseradish, catalyse a colour reaction. The substrate for the enzyme in the colour reaction is ABTS, a chromogen which is oxidised by a peroxidase-H_2O_2 complex. The oxidised form of the chromogen is green and absorbs at 405 nm [Childs & Bardsley, 1975]. The colour development is proportional to the amount of biotin-pluronic immobilised onto the hydrophobic membrane surface. The absorbance reading at 405 nm can therefore be used to compare the immobilisation experiments with the control experiments (no immobilised biotin-pluronic) to determine the extend of biotin-pluronic immobilisation.

5.3 Materials and methods

Polysulphone casting solution was prepared as previously described (section 3.5.1). Biotin-pluronic was synthesised and characterised as described in section 4.2.7. Avidin-conjugated peroxidase and the ABTS substrate were obtained from Sigma S. A.

5.3.1 Preparation of polysulphone tubes

Polysulphone membrane tubes (4 mm diameter) were prepared by drawing a 13% (m/v) polysulphone solution into a glass tube by vacuum and allowing the solution to drain. The coated glass tube was immediately inserted into distilled water. The polymerised tubes were removed with a pincette and incubated overnight in fresh distilled water. The tubes were stored in 5% NaN_3 until used.

5.3.2 Testing the hydrophobic surfaces for affinity

The wells of the micro titre plate is layed out in 8 rows, marked A to H, and 12 columns, marked 1 to 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 experiments done with the membranes and rows E to H were used for the experiments where the polystyrene micro titre plate itself was used as an adsorbing surface.

Polysulphone tubes, prepared as described in 5.3.1, were cut into ± 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 (plate A) were coated overnight with 5 mg/ml pluronic-biotin solution. Row G was coated with 5 mg/ml pluronic and row H contained only deionised water (figure 5.2). All these experiments were conducted at room temperature. After incubation the wells and membranes were washed three times with deionised water.

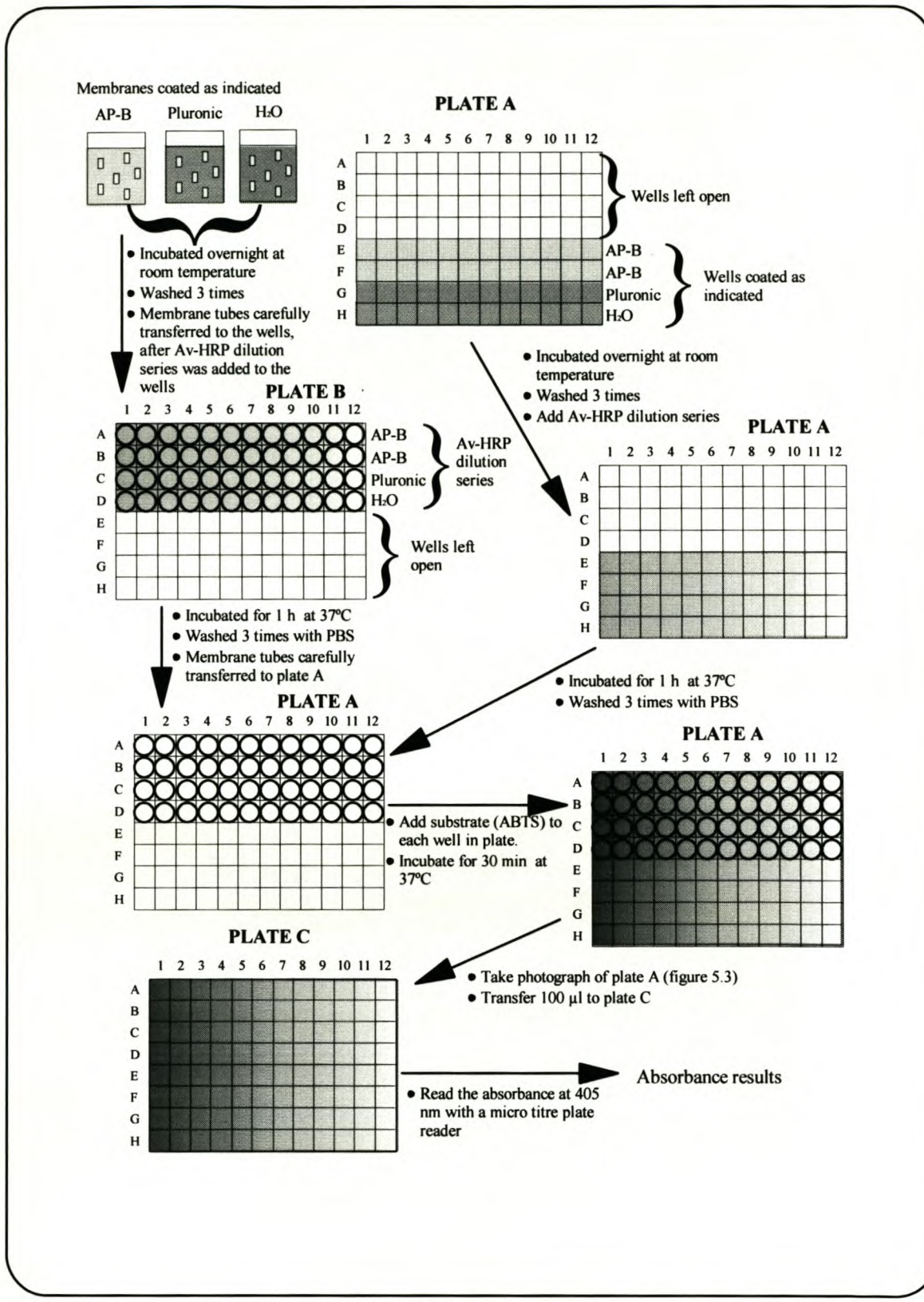

Figure 5.2: Schematic flow diagram representing the affinity experiments.

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 afore mentioned 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 (150 µl) was pipetted into all the wells of columns 2-12 and in the specific rows as described 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 150 µl PBS had already been pipetted into) and mixed thoroughly. As a result, a two times 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 four times dilution. This process was repeated for all the columns except the last column, column 12. As a result the wells of columns 1-11 contained a concentration dilution series of avidin-peroxidase with the most concentrated solution in the wells of column 1 and the most diluted solution in the wells of column 11 (102 400 × dilution). The last column was not filled with avidin-peroxidase solution and served as a negative control.

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 were 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 minutes. After 30 minutes the plate was uncovered and photographed (figure 5.2). 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.

5.4 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 5.3. The colour development is evident indicating that the enzyme was active.

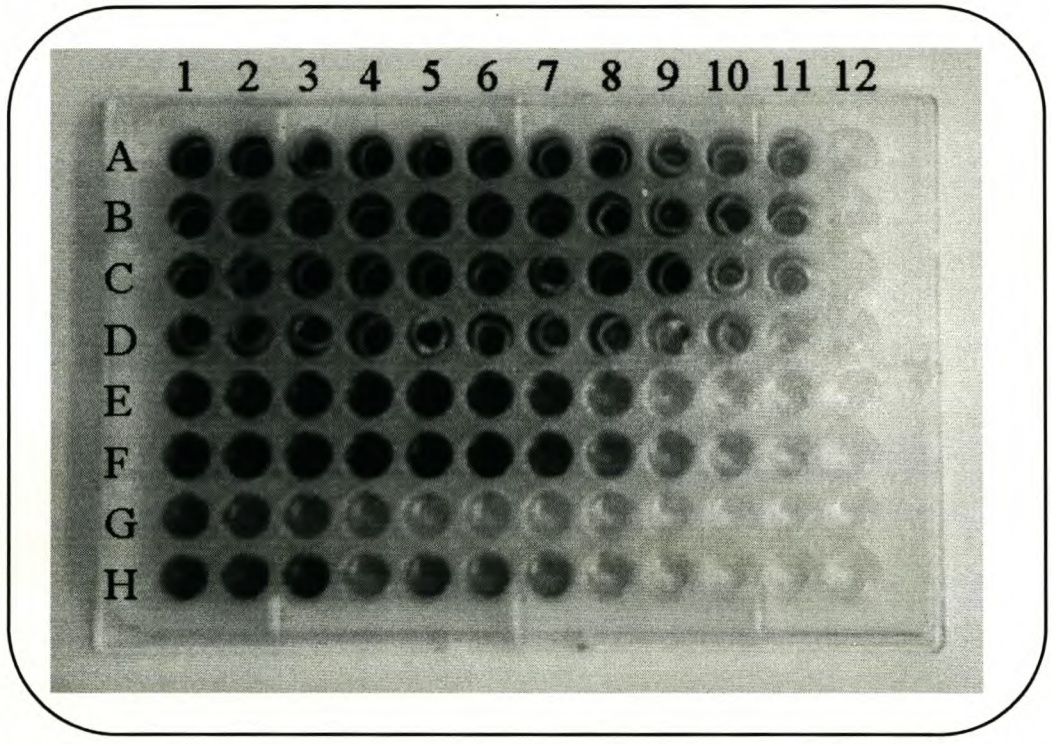

Figure 5.3: Plate A: Results of avidin-HRP interaction with pluronic immobilised biotin. Rows A and B, contained membranes coated with the biotin-pluronic solution. Row C contained membranes coated with pluronic. Row D contained uncoated membranes. Rows E and F were coated with the biotin-pluronic solution. Row G was coated with pluronic. Row H were 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 extremely 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. Here extremely wide concentration range is initially used to find a "response" that falls within the measuring range of the technique used to determine the interaction[1]. 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 graph 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. The use of log values on the X-axis allows for the plotting of a wide concentration range on one graph. 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 5.4A. In figure 5.4B 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 5.4C the log dilution response curves for the interactions of avidin-HRP with uncoated polystyrene wells and

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[1] "Dose response" curves are often used to plot data for many different types of experiments. The following is an extract from the GraphPad Prism 3.0 website, the software used to plot the data in this study: *"For example, the response might be enzyme activity, accumulation of an intracellular second messenger, membrane potential, secretion of a hormone, heart rate or contraction of a muscle. The term "dose" is often used loosely. The term "dose" strictly only applies to experiments performed with animals or people, where you administer various doses of drug. You don't know the actual concentration of drug -- you know the dose you administered. However, the term "dose-response curve" is also used more loosely to describe in vitro experiments where you apply known concentrations of drugs. The term "concentration-response curve" is a more precise label for the results of these experiments."* From **curvefit.com.** Copyright 1999 by GraphPad Software, Inc. All Rights Reserved. Http://www.curvefit.com/introduction89.htm

polysulphone membrane tubes are shown. Data manipulation was carried out using the GraphPad Prism software, version 3.0 from GraphPad Software, Inc.

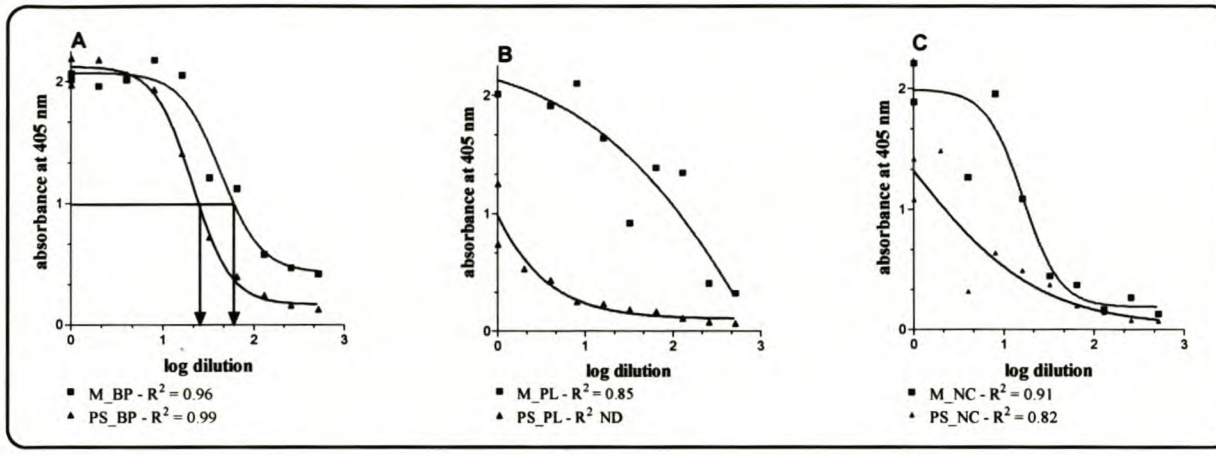

Figure 5.4: A: Log dilution curve of avidin-HRP reacting with polysulphone and polystyrene coated with biotinylated pluronic. A typical inverted sigmoidal curve ($R^2 > 0.95$) was obtained for both experiments indicating specific concentration dependent interaction between the biotin and avidin derivatives. 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 underivitised pluronic. It is clear 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 extremely high while significantly 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.
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)

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 significantly 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 the biotin and avidin derivatives. A comparison of the interaction of avidin-HRP onto the biotin-pluronic coated and uncoated polystyrene surface indicate that, for the biotin-pluronic coated surface, a 48 fold dilution of avidin-HRP 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[2]. It can be deduced from these results that the avidin-HRP interacted specifically with biotin-pluronic immobilised onto 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 onto 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 48 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 immobilised biotin-pluronic onto the polysulphone membranes.

5.5 Conclusions

The results summarised in figure 5.4 clearly indicate that coating the polysulphone and polystyrene surfaces with biotinylated pluronic significantly 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 several washing

[2] 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.

steps, affinity was still retained on the surface. The interactions between the hydrophobic surfaces, biotin-pluronic and between the biotin moiety and avidin was strong enough to withstand the washing steps. This indicates the feasibility for the use of this system in affinity chromatography as well as bioreactors.

The polysulphone surface showed very high non-specific interaction with avidin which could explain the high intensity colour observed. This non-specific interaction plays a significant 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 onto the two pluronic adsorbing surfaces will contribute significantly to the efficiency and specificity of ligand loading onto hydrophobic surfaces in future studies.

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CHAPTER 6

DISCUSSION

6.1 Introduction

The aim of this study was to render affinity properties to hydrophobic surfaces, making use of the avidin-biotin technology, to tether a ligand to the surface by adsorption of a mediating block copolymer. Polysulphone membranes and a polystyrene micro titre plate were the model hydrophobic surfaces under study in this thesis. Pluronic was used as a tether between the solid surface and the immobilised ligand. The pluronic was attached to the hydrophobic surface by adsorption of its PPO centre block. The molecules to be immobilised (biotin) were covalently attached to the modified ends of the pluronic. The affinity system was tested by the interaction with avidin-HRP, which catalyses a colour reaction.

Three objectives were set for the study. Firstly, a study of the adsorption behaviour of pluronic onto polysulphone flat-sheet membranes. Secondly, pluronic derivatives, an activated biotinylation reagent and biotin-pluronic were synthesised and characterised. Finally, the biotin-pluronic system was tested for affinity properties on two different hydrophobic surfaces to determine whether the affinity system worked.

The Langmuir adsorption isotherm of pluronic and polysulphone flat-sheet membranes was determined to establish appropriate coating conditions. The effects that certain parameters have on the adsorption and desorption behaviour of pluronic onto PSMs were studied. To establish the coating concentration requirement for effective derivatised pluronic adsorption, it was neccessary to first study the adsorption behaviour of underivatised pluronic onto a PSM surface of a given area. The concentration, temperature and duration were established to determine the optimal coating conditions. A good indication was obtained of what the coating conditions for this particular copolymer onto the PSM should be, to make the coating with the more expensive biotin-pluronic more cost effective.

Pluronic derivatives, an activated biotinylation reagent and biotin-pluronic were synthesised and characterised to obtain an affinity property for the pluronic end-groups. The hydroxyl termini of pluronic were derivatised to facilitate the covalent attachment of a ligand system onto the modified end-groups of the surfactant. The modified ligand-carrier was subsequently adsorbed onto a polysulphone membrane in order to render affinity properties to the membrane.

The biotin-pluronic was tested for affinity properties on two different hydrophobic surfaces to determine whether the affinity system works. The biotin-pluronic was tested by using avidin-HRP. The optimal avidin-HRP concentration was not known. A concentration dilution series were therefore made to obtain a dose response type result.

6.2 Overview of results

A method for establishing the concentration of a pluronic solution was determined before the adsorption experiments were performed. A linear relationship was found between absorption of 488 nm and pluronic in the concentration range 0 to 0.2 mg/ml pluronic (figure 3.5). From the slope and y-intercept of the standard curve an equation was obtained, which was used to calculate any concentration of pluronic assayed within the same concentration range.

Saturation was reached at a pluronic concentration of 5 mg/ml (figure 3.6). From this figure it appeared that no more than 5 mg/ml pluronic was necessary to saturate the membrane surface.

The Langmuir isotherm was calculated and the theoretical maximum amount of pluronic that could adsorb onto 1 cm^2 PSM was determined. (See appendix 1 for an explanation of the calculations.) A plot of the reciprocal of the mg pluronic extracted per 1 cm^2 membrane versus the reciprocal of the pluronic concentration in the coating solution, yielded the adsorption curve as shown in figure 3.7. This linearised form of the isotherm was used to determine the constants as described in appendix 1.

Slope = $1/ab$ = 7.429

Y-intercept = $1/b$ = 11.43

thus b = 0.0875

and a = 1.5384

From equation 4 (see the appendix 1) X was determined as 0.0875 mg, the amount of pluronic adsorbed onto 1 cm^2 if $c \rightarrow \infty$. The coating concentration could subsequently be determined for any acquired level of adsorption. The amount of pluronic that adsorbed onto 1 cm^2 membrane at each of the different coating concentrations was calculated as a fraction of the theoretical maximum amount per square cm. The adsorption fractions were then plotted against the coating concentrations to determine the optimal pluronic coating concentrations (figure 3.8).

The time it took for the PSM to reach equilibrium saturation with pluronic was also determined. The results of this experiment are given in figure 3.9. It was concluded that adsorption reached equilibrium after 6 hours. An incubation period of between 6 and 8 hours was therefore regarded as sufficient to achieve maximum membrane coating.

The effect of temperature on the adsorption of pluronic onto PSM was also investigated. Figure 3.10 shows that more adsorption occurs at higher temperatures. From these studies the optimal coating temperature could be determined.

The reversibility of pluronic adsorption was also assessed. The results shown in figure 3.11 confirmed that the adsorption of pluronic onto PSMs is reversible and that desorption does occur. The results further indicated that desorption is a function of both temperature and time.

The synthesised biotin-pluronic molecule was characterised with NMR and ES-MS analysis. The ES-MS results indicated a certain fraction of biotin-pluronic that could only exist if the biotin was coupled to the pluronic. This fraction is the peak at e/z 270. Most of the other peaks could be explained by the structure of biotin-pluronic. These fractions are shown in figure 4.5.

Finally the affinity on the hydrophobic surfaces, caused by the adsorption of the biotin-pluronic, was tested. A photograph of the plate which shows the final colour of the peroxidase reactions in the different wells of the micro titre plate is shown in figure 5.3. From the photo it can be observed that the enzyme conjugated with the avidin was present and active.

The graphed absorption results are shown in figure 5.4. 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. The interactions of the avidin-HRP with the underivatised pluronic were also highly unspecific and significantly less than with the biotin-pluronic. A typical inverted sigmoidal curve ($R^2 > 0.95$) was obtained for the biotin-pluronic coated polystyrene surface indicating specific concentration dependent interaction between the immobilised biotin and avidin derivatives.

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 pluronic coated polysulphone surfaces. 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.

The results obtained in this work were clear and convincing that the biotin-pluronic could be synthesised and characterised successfully and then used to render affinity properties to a hydrophobic solid surface. The avidin-HRP interacted specifically with biotin-pluronic adsorbed onto both the polysulphone membranes and on the polystyrene wells of the titre plate.

6.3 Conclusions

To set up an affinity chromatography system several factors have to be kept in mind. The system must be strong enough to handle loading, washing and eluting steps. It must be possible to regenerate. It must have good hydrodynamic flow characteristics. The ligand coupled to the solid support must be stable under all the conditions during the separation process. The ligand should not detach from the solid support too easily.

The membranes used in this system provide for good hydrodynamic flow properties. No compacting can take place and it is regenerable. It has a strong structure, is chemically stable and inert. These properties make membranes very attractive as new solid supports in chromatography. In this study the hydrophobic properties of polysulphone membranes and polystyrene micro titre plates were exploited to immobilise a ligand-carrier through hydrophobic interactions.

The hydrophobic interactions were studied in more detail by determining the adsorption isotherm of the ligand-carrier (pluronic) onto the polysulphone membranes. The optimal coating concentration for maximal coating was determined. Optimal coating conditions were determined to be at higher temperatures and 6 to 8 hours coating time.

The pluronic derivatives were synthesised and characterised successfully. Characterising the ligand-carrier with the ligand is an advantage to the system compared to the older affinity systems where the ligand attachment could not be monitored [Yaniç, 1999]. However, the synthesis route was difficult and time consuming.

It was possible to render affinity properties to both the hydrophobic polysulphone membrane and polystyrene surfaces. The immobilised ligand was biotin which had an affinity for avidin. However, as with most proteins, non-specific interactions with hydrophobic surfaces are inevitable. This was clearly seen with the uncoated surfaces. Even the pluronic coated surfaces showed non-specific interactions. The reduction of non-specific interactions will have to be addressed in future studies to improve the efficacy of the process.

The pluronic PEO blocks served as a tether, making the covalently coupled biotin appear as if 'free' in solution. This promotes the affinity properties of the surface because the ligand (biotin) would interact easier with the ligate in solution.

The affinity system proved to be stable, because it could withstand all the washing steps and still was able to show affinity. This is an important factor if the system has to be scaled up. It will have to withstand much more mechanical challenges like higher pressure and flux.

6.4 Future studies

Future studies include improvement on the current work as well as other approaches to the same avidin-biotin affinity concept. Keeping the same principle of the tri-block copolymer adsorbed onto a hydrophobic membrane in mind, several articles were studied that contained information that could lead to interesting future studies in this field. Besides the scaling up of the membrane affinity system there is still room for a variety of developments within the same system. Some of them will be discussed briefly below.

6.4.1 Immobilised antibodies

Previously antibody immobilisation had several disadvantages:

1. the antibody can be altered or inactivated during coupling;
2. the antibody can leak off the solid phase;
3. Steric hindrance between the antibody-antigen interaction can occur during purification of the antigen [Gretch, Suter & Stinski, 1986].

Gretch (1986) also stated that the ideal situation should allow the antibody to be free enough to react with the antigen in the liquid phase. Using the system in this study, the PEO blocks of the pluronic, which can act as a tether, contributes to reach this ideal situation. The tether provides that the antibody experiences less steric hindrance when interacting with the antigen. As stated above (point 1), biotinylating the antibody could inactivate it. Depending on where all the ε-amino groups on the protein are situated, this situation for the antibodies can not be by-passed. All the antibodies biotinylated at their hypervariable regions (which are exclusively involved in antigen recognition) will not show as much (if any) activity against the antigen compared to the antibodies biotinylated on their Fc region.

Previous problems about the antibodies leaking from the surface should be minimised by using this system because the leaking will be dependent upon the pluronic desorption rather than on the stronger avidin-biotin interaction. The desorption experiments done in chapter 3 show that after 2 hours no significant desorption of the pluronic from the PSM takes place. Working at lower temperatures could minimise the leakage.

6.4.2 More adsorption studies

Factors such as pH and ionic strength are known to have an effect on the adsorption of molecules onto surfaces. These parameters still need to be investigated for pluronic adsorption onto PSMs. It is also known that one molecule shows different adsorption isotherms with different surfaces [Benefield, Judkins & Weand, 1982]. These surfaces may differ in topography [Li, Caldwell & Rapoport, 1994] or chemical properties [Benefield, Judkins & Weand, 1982]. (section 3.3.8.) Adsorption of pluronic onto other surfaces besides polysulphone and polystyrene must be investigated.

6.4.3 Alternative pluronic derivatives

The synthesis of the amino-pluronic had a low yield and was very time-consuming [Yaniç *et al.* 2000]. Other derivatives which have less reaction steps, are simpler and more cost-effective should be considered. One such derivative was synthesised by Li *et al.* (1996) and is shown in figure 6.1. It was synthesised in only two simple steps. [Li *et al.* 1996]. The polymer also has a primary amino group and could also react just as well as amino-pluronic to the activated NHS-biotin.

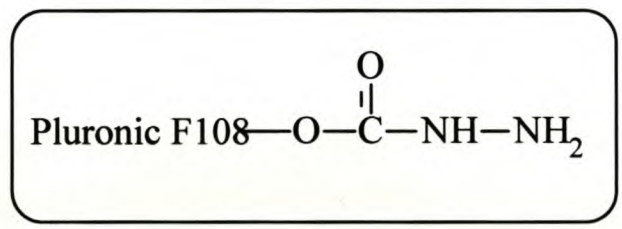

Figure 6.1: The structure of a different pluronic derivative [Li *et al.* 1996].

6.4.4 The use of nitro-avidin

The very tight interaction between biotin and avidin (Ka ~ 10^{15} M^{-1}) makes it difficult to apply in an chromatography system. This prompted researchers to study modifications on avidin to produce an avidin which has more manageable biotin-binding properties. Morag, Bayer and Wilchek (1996b) modified the tyrosine residue in avidin by using the nitration reaction developed by Sokolovsky, Riordan and Vallee (1966), rendering nitro-avidin. The nitration resulted in a lowering of the pKa of the phenol group which forms a hydrogen bond with the ureido group of biotin. At low pH values (4-5) the nitro-avidin binds biotin with a high

association constant (> 10^9 M^{-1}). By increasing the pH or by introducing excess biotin at neutral pH, the biotinylated molecule is detached [Morag, Bayer & Wilchek, 1996b]. This method allowed Morag, Bayer and Wilchek to use the nitro-avidin and nitro-streptavidin as reusable affinity matrices [Morag, Bayer & Wilchek, 1996b].

6.4.5 Avidin-biotin polymers

Green *et al.* (1971) synthesised bisbiotinyl diamines with between 9 and 25 bonds between the carboxyl groups of the two biotin groups. Compounds with longer than 12 residues behaved in a bifunctional manner and gave rise to linear polymers of avidin. The polymers became shorter when the chain length of the reagent increased to 23 bonds, which suggested that the reagent was able to bind intramolecularly to two subunits of the avidin. From 12 to 14 bonds between the two biotin moieties, polimerisation is possible and reversible. The addition of one more methylene group resulted in a slowly reversible polymerisation. The polymers with the longer chain bisbiotinyl diamines were found to be stable [Green *et al.* 1971; Green, 1989]. The phenomenon that it is possible to make cross-links between avidin proteins makes development of the system even more versatile. Cross-linking will stabilise the avidins and form a stronger matrix over the surface. If one decides to use cross-links, the correct concentration of the bisbiotinyl diamines should be determined to prevent binding of all the biotin binding sites which will inactivate the avidin.

6.4.6 The use of iminobiotin

The strong bond between avidin and biotin makes it problematic to apply in a chromatographic setup [Diamandis & Christopoulos, 1991]. Previous studies found that the use of a different biotin derivative, iminobiotin, could overcome this problem. Iminobiotin differs from biotin in that the iminobiotin contains a guanidino group where biotin contains an ureido group (figure 2.4). At a high pH (pH 11) the iminobiotin is not protonated, and almost stoichiometric binding of the iminobiotin was found. As the pH was lowered, the dissociation constant of the avidin-iminobiotin complex was found to increase. Because the charge of the iminobiotin can be changed by simply changing the pH, avidin can be dissociated from the iminobiotin by lowering the pH to 4 [Green, 1966].

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APPENDIX 1

The manipulation of the Langmuir equation as well as a explanation of how the applied calculations have been done will be described in this appendix.

The Langmuir isotherm was fitted from the Langmuir equation given below:

$$X/m = abc/(1+ac) \qquad (1)$$

m was taken as 1 because the results were calculated for 1 cm^2 of PSM.

Equation (1) then reduces as follows:

$$X = abc/(1 + ac) \qquad (2)$$

The fraction of equation (2) is divided by c at the top and bottom:

$$X = ab/(1/c + a) \qquad (3)$$

Assume that the adsorbent will adsorb at a maximum if $c \rightarrow \infty$

Thus $1/c \rightarrow 0$ and equation (3) is simplified to:

$$X = ab/a = b \quad (4)$$

If the pluronic extracted per 1 cm^2 is plotted against the pluronic in the coating solution the adsorption curve is found. If 1/extracted pluronic concentration per cm^2 is plotted against 1/concentration remaining in the solution after adsorption a straight line is obtained. From this straight line the slope and y intercept can be obtained and used to calculate the constants in equation (1).

From the straight line of the isotherm, constants were determined as follows:

Slope = $1/ab$ = 7.429

Y-intercept = $1/b$ = 11.43

thus b = 0.0875

and a = 1.5384

From equation (4) X was determined as 0.0875 mg. This is the amount of pluronic adsorbed onto 1 cm^2 if $c \rightarrow \infty$. The coating concentration can therefore be determined to acquire any level of adsorption. To make the calculations for the coating concentrations easier, equation (2) was manipulated as follows and subsequently equation (10) was used to calculate the coating concentration.

$$abc = X + Xac \qquad (5)$$

$$c = X/ab + Xac/ab \qquad (6)$$

$$c - Xac/ab = X/ab \qquad (7)$$

$$c\,(1 - Xa/ab) = X/ab \qquad (8)$$

$$c = (X/ab)/(1 - Xa/ab) \qquad (9)$$

$$c = X/a(b - X) \qquad (10)$$

At 90 % of the maximum achievable adsorption 0.0788 mg pluronic will be adsorbed onto 1 cm^2 of membrane area. Thus the coating concentration can be determined for 90 % adsorption by substituting the value for X and the constants into equation (10). At 90 % adsorption the coating concentration was calculated to be 5.8876 mg/ml.

I do not know what I may appear to the world; but to myself I seem to have been only like a boy, playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.

ISAAC NEWTON