

EXECUTIVE SUMMARY

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

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

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

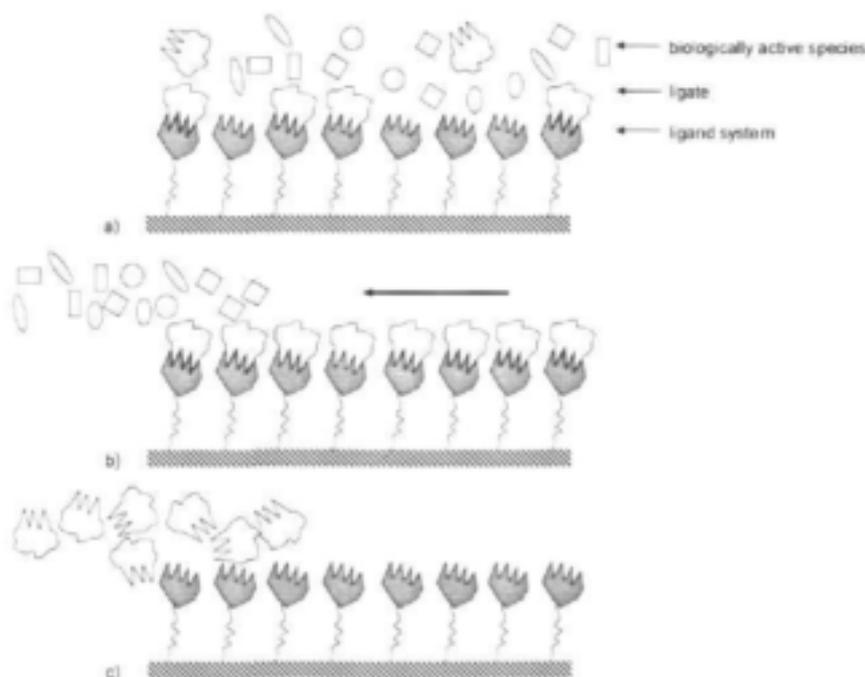


Figure 1: Sequence of events in affinity separation: a) feeding the ligand system with a mixture of biologically active species and the targeted ligate, b) rinsing the system to remove untargeted species, and c) elution of the concentrated targeted species from the affinity system

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

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

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

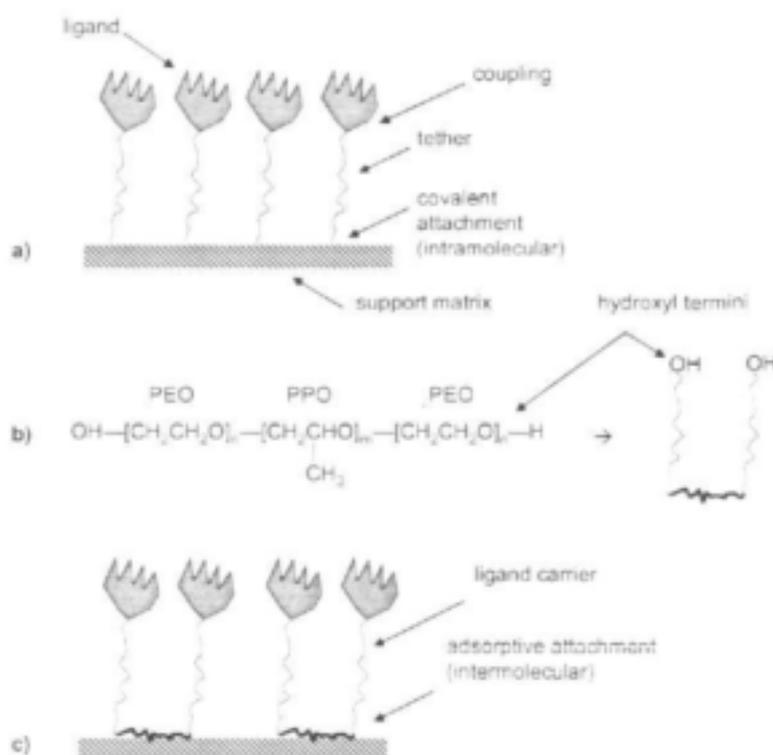


Figure 2: Affinity separation ligand carrier arrangement: a) conventional covalently attached ligand system, b) chemistry of the chosen ligand carrier and c) the alternative adsorbed ligand-carrier system

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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