Monitoring of endocrine disrupting chemicals in surface water

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Abstract
Environmentally prevalent endocrine disrupting chemicals (EDCs) may interfere with the bodies' complex and carefully regulated hormonal messenger system (endocrine system). They can mimic or block chemicals naturally found in the body, alter hormonal levels, and thus, affect functions that these hormones control. Potential EDCs can be found among pesticides, plasticizers, pharmaceuticals, cosmetic ingredients, household products and industrial chemicals. Surface waters are the main sink of said EDCs. Accurate EDC detection is usually via time consuming and costly ex situ LC-MS and GC-MS analysis. An important class of biosensors include those that respond to the presence of a specific substance based on the specific recognition of a biomolecule. Biosensor development is thus described with a focus on surface modification to create a biocompatible matrix. Thus the aim of this paper is to show the development of solid phase polymeric extraction and detection systems for estrogen mimicking agents in aqueous solutions. Specifically this study decscribes: 1) Development of a recombinant human estrogen receptor; 2) Development of a colloidal-poly(vinyl diene) affinity membrane matrix system; 3) Characterisation of the distribution of binding sites on the membrane; 4) Specific binding of EDCs and steroids such as 17-β estradiol.

Keywords
EDCs; membranes; endocrine system; environmental biosensor; estrogen;

INTRODUCTION

Estrogenic compounds are non-steroidal by-products of industry, agriculture and pharmaceuticals. They can bind the estrogen receptor, which can lead to the expression of estrogen sensitive genes. The compounds induce the receptor to change conformation, to dimerize, and recruit transcription co-factors necessary for expressing the genes. The expression of estrogen sensitive genes in turn leads to various uncontrolled physiological effects, such as hypospadia, cryochidism and cancer [1]. Since estrogenic compound's physiological target is the estrogen receptor, assaying for binding to this receptor may be useful for detecting these estrogenic compounds. By immobilizing the estrogen receptor on a membrane or other solid supports the estrogenic compound can be captured, concentrated and detected in an artificial system.

Comparing DNA sequences of nuclear hormone receptors, the family of receptors to which the estrogen receptor belongs, two regions were found that are conserved amongst the receptors. If a DNA sequence is conserved between related members of a receptor family, then this section of the protein has an important function. In the case of nuclear hormone receptors, two regions identified are the DNA-binding domain (DBD) and the ligand binding domain (LBD). The DBD is found near the N-terminal end of the protein. It is responsible for binding specific DNA sequences located adjacent to an estrogen responsive gene. The DNA sequences are called estrogen response elements (ERE). The LBD is found near the C-terminal of the receptor. It is a multifunctional domain that contains the ligand-binding pocket, the ligand inducible gene activation function (AF2) and provides the surface for dimerization of the receptor.

The conventional approach to the monitoring of EDCs such as estrogenic compounds in surface waters or the environment is usually 2 fold. Firstly, the observation of biomarkers such as fish and frogs over a period of time, and or sample analysis involving solid phase extraction followed by costly chromatographic and mass spectrophotometric analysis. There is therefore a need for a rapid, economical and sensitive detection system that can enhance and support monitoring systems worldwide. This has created an interest in modified polymers as environmental biosensors.

Typically, biosensors are hydrophobic surfaces, active to the adsorption of a wide range of polymeric material such as biomolecules with hydrophobic components, proteins and lipids. At the molecular level, these diverse bio-adhesives condition the surface for the subsequent adhesion of other polymers [3]. As with conventional affinity membrane processes, much effort is being devoted to the development of protein shielding and biocompatible surfaces [2-4]. Such surface modified materials reduce non-specific and uncontrolled polymer adsorption, thereby increasing the signal to noise ratio for biosensors while maintaining the ligand capacity of affinity membranes [2].

A number of surface modification techniques can be applied, and most methods rely on post-treatments of an existing polymeric membrane [5]. These techniques include chemical reactions [6], grafting [4], crosslinking [7], plasma treatment, *in situ* polymerisation [8] and adsorption [9]. Chemical reactions introduce hydrophilic groups and ionic groups such as sulphonic and quaternary ammonium groups, whereas plasma treatment functionalises surfaces. Both techniques involve structural modification of surfaces while plasma treatment is particularly irreproducible [5,8].

Alternatively, membranes can also be non-permanently pre-treated with polymeric surfactants by physical adsorption of the polymer, either from a solution onto the membrane or by convective adsorption during filtration of the adsorbent [5,9,10]. The use of PEG based non-ionic surfactants such as the Pluronic tri-block copolymers for surface modification is also becoming popular [2,4,10]. Pluronic® (BASF, Corp) are a group of PEO-PPO-PEO surfactants that are being widely studied for applications in the pharmaceutical and medical industry because of their unique surfactant abilities and their low toxicity and immunogenic behaviour [2]. Pluronic copolymers are approved by the Food and Drug Administration (FDA) and the Environmental Protection Agency (EPA) as thermoviscofying additives in food and agricultural products. Moreover, they also have the ability to suppress protein adsorption and platelet adhesion due to their PEO segments [2,4].

This study describes the fabrication of a polymeric membrane matrix and a novel bio-ligand for the development of an environmental biosensor. These biosensors are specific for the binding and hence the detection of estrogen mimicking compounds in water.

EXPERIMENTAL

Reagents and Chemicals

Proteins such as bovine serum albumin (BSA), lysozyme and enzyme solutions were obtained from (Roche, Penzberg Germany) and were reconstituted as 0.25 $mg.ml^{-1}$ solutions in 0.1 M phosphate buffer, pH 7.4. SDS (Merck, Darmstadt, Germany) was used as a desorption agent. Pluronic® F108 (14 600 $g.mol^{-1}$) was obtained from BASF corporation (New Jersey, USA) and biotinamidohexanoic acid *N*-hydroxysuccinimide ester (NHS-Biotin) from Sigma chemical company, South Africa. Conjugated streptavidin-peroxidase (Av-P) and 2,2´-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) were purchased from Roche. Unless otherwise stated, all other reagents were purchased from Merck (Darmstadt, Germany).

Polymer fabrication

Planar nonporous immersion precipitation membranes were cast from a solution containing 27 % [m/m] PVDF and 73 % (m/m) *N*,*N*-Dimethylacetamide (DMAc). The PVDF solution required sonication in an ultrasonic water bath for 30 min and further heat treatment at 55°C for 48 h to dissolve. The solutions were subsequently degassed before being used to cast the 200 µm planar membranes. These membranes were cut into 1 cm^2 sections and were stored in 0.04 M sodium azide to prevent microbial growth. Prior to adsorption, membranes were washed overnight in sterile, deionised water, followed by three further washes with deionised water. Prior to noncovalent surface modification, the membranes were sonicated three times in sterile deionised water in an ultrasonic bath for 5 min followed by drying in a laminar flow cupboard.

Spectrophotometric Analysis

A biphasic colorimetric assay for Pluronic quantification was performed as described by Govender *et al.* [10]. A plot of absorbance at 510 nm *versus* Pluronic concentration yielded a linear standard curve. Protein concentration was measured using a bicinchoninic acid protein assay kit from Pierce™, (Rockford, USA), with bovine serum albumin as a protein standard. Pantothenate kinase (PK) activity was based on the measurement of the decrease in the absorbance at 340 nm, where an extinction coefficient of 6220 $M^{-1}.cm^{-1}$ was used for the calculation of NADH concentrations. Reactions were monitored at 25°C in a CARY 110 UV-Vis spectrophotometer. A Beckman spectrophotometric plate reader was used to measure the activity of avidin conjugated peroxidase (Av-P). In the presence of sufficient affinity immobilized Av-P, the ABTS solution (Roche) undergoes a distinct colour change from yellow/green to dark green/blue indicating Av-P activity. The log of Av-P dilution was plotted against absorbance to illustrate specific binding of Av-P to membrane bound biotinylated Pluronic.

Fouling Reduction and surface Regenration

Pluronic modified membranes were stripped of adsorbed Pluronic using an aqueous SDS solution. These membranes were initially statically equilibrated in 10 ml of the SDS solution for 1 h and then transferred to a Stoval Belly Dancer™ shaker for 20 h of vigorous shaking. After incubation in SDS, the membranes were washed in a solution of 100 ml dH_20 for 12 h and finally rinsed three times in dH_2O. Pluronic was separated from SDS after solvent evaporation, followed by the addition of 10 ml $CHCl_3$. SDS is insoluble in $CHCl_3$ and can be separated from Pluronic by filtration through Whatman filter paper. To demonstrate competitive Av-P binding to biotinylated membranes, studies were also performed with 0.2 $mg.ml^{-1}$ of model protein contaminants BSA and lysozyme. Affinity bound Av-P was eluted using 6mM d-biotin in PBS. The bioactivity of the eluted Av-P was confirmed using the ABTS based spectrophotometric assay.

Production of the Biological Hormone receptor

The ER LBD is a hydrophobic protein which makes protein purification complicated. However, the LBD can be successfully purified using fusion protein constructs. ER-LBD fused with maltose binding protein (MBP) confers solubility to the LBD. The fusion protein is traditionally isolated from the soluble fraction of cells and purified by affinity purification using any amylose resin. Sub-cloning involves inserting a DNA sequence into a target plasmid for further protein expression or storage of that sequence.

The human ER^{α} ligand binding domain (LBD) from the histidine tagged expression plasmid pET15b-LBD (Fig. 1) was also placed into a pMalc2 expression vector, allowing for expression of the ER as a MBP-fusion protein (Fig. 2). Purification studies and radiolabelled binding studies were performed to confirm its biological activity. Polyclonal antibodies were also raised against both this fusion protein and the native ER which can be used for detection studies.

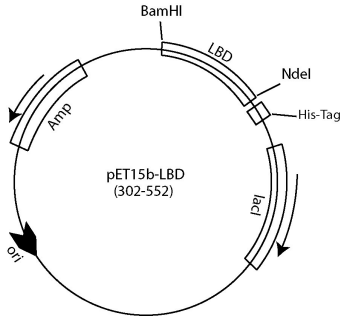

Figure 1. The pET15b-LBD plasmid with the LBD sequence directionally cloned into the plasmid with *NdeI* and *BamHI* restriction enzyme sites. Allows for expression of a histidine tagged ER (LBD).

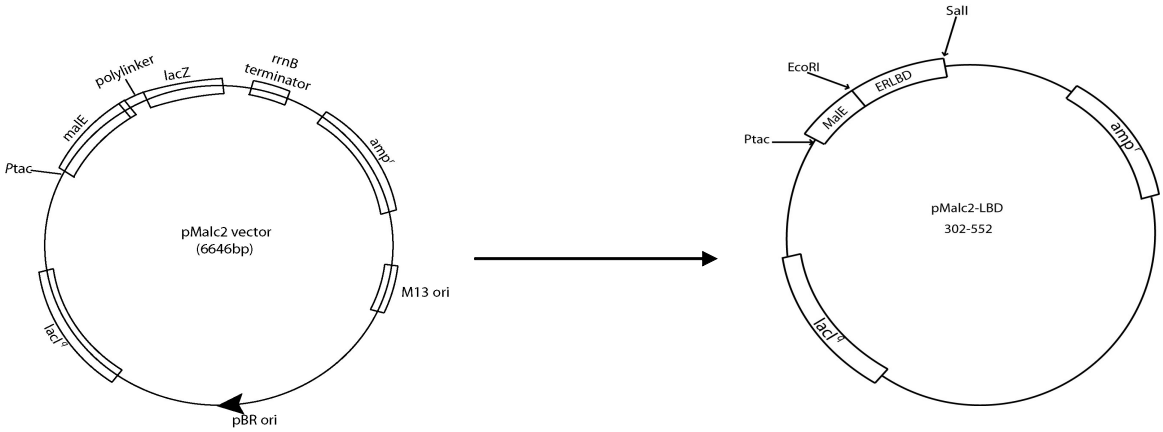

Figure 2. The pMalc expression plasmid containing the MBP-ER fusion protein.

RESULTS AND DISCUSSION

Pluronic Analysis

The saturation curves for the adsorption of Pluronic® F108 onto PSU, PVDF and PEI are shown in Fig. 3. The adsorption/desorption data of Pluronic F108 on dense skin planar membranes was fitted to the Langmuir isotherm: $Q = Q_{max} \cdot K \cdot C \cdot (1 + K \cdot C)^{-1}$ [1] where Q and Q_{max} are the equilibrium amount and adsorption capacity of Pluronic F108 adsorbed per $1cm^2$ of adsorbent respectively. C is the liquid phase adsorbate concentration at equilibrium and K is the binding constant. The isotherms obtained followed a Langmuir type profile as characterised by a steep initial slope at low copolymer equilibrium concentration (< 1 $mg.ml^{-1}$) and an adsorption plateau was reached above a bulk Pluronic coating concentration of 5 $mg.ml^{-1}$. Since the adsorption capacity of the different membrane substrates differ due to their inherent surface chemistries, the equilibrium adsorption concentration also differed for all the membrane matrices under investigation. The critical micelle concentration of the Pluronic® F108 used in this study was calculated from surface tension measurements to be 7 $mg.ml^{-1}$ [20], so the plateau does deviate from linearity for coating solutions greater than 5 $mg.ml^{-1}$ Pluronic, due to the formation of micelles in the bulk coating solution. From the saturation curves in Fig. 3, the curve for Pluronic adsorption onto PEI lies above the isotherm plots for both those of PVDF and PSU. This suggests that Pluronic adsorbs more strongly to PEI than it does to PVDF and PSU. The slopes of the isotherms for PVDF and PSU appear steeper than that for PEI, which indicates that the adsorptive capacity of PVDF and PSU increases at higher equilibrium solute concentrations.

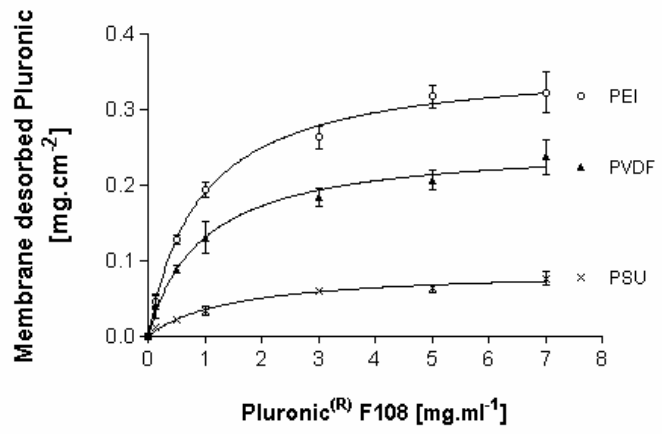

Figure 3. Saturation curves for Pluronic on candidate polymeric membrane surfaces.

Solid Phase Extraction and Detection

A schematic illustration of the process from membrane surface modification and Av-P immobilisation to regeneration and biosensor applications is illustrated in Fig. 4a, while a biosensor that is histidine tagged based is illustrated in Fig. 4b.

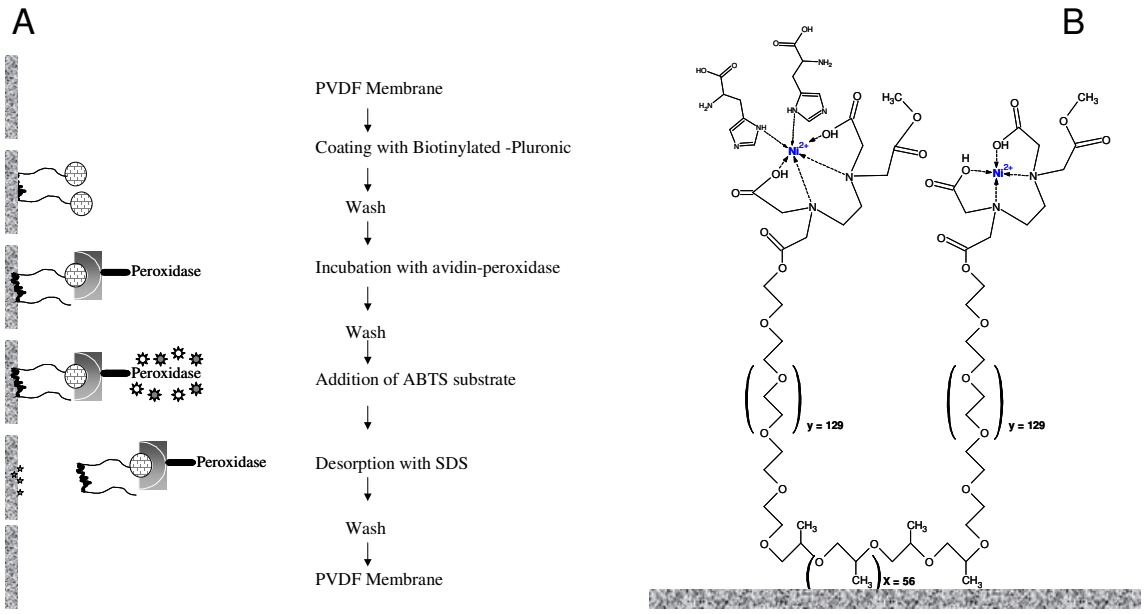

Figure 4. Multifunctional illustrations of modified Pluronic surfactants and their non-covalent binding to PVDF surfaces for potential biosensor matrix development. A) Detailed description of the process of ligand binding using the biotin-avidin peroxidase system as an example, with solid state colorimetric/photometric determination possible. (B) A chelating EDTA derived ligand for the binding of histidine tagged proteins.

The attachment of the widely used and relatively inexpensive EDTA chelator to Pluronic is an example of direct chemical coupling (Fig. 4b). EDTA is hexadentate, capable of filling the octahedral coordination system and can thus complex a number of metal cations. The coupling chemistry was based on using one of the acids of EDTA dianhydride, the reactive precursor derivative of EDTA to bind to Pluronic by esterification (thus removing it from coordination) and another acid by esterification with methanol. This yielded a tetradentate ligand with coordination sites on the octahedral system open for ligand attachment and a nonpolar centre

block available for hydrophobic surface interaction. The ligand Pluronic-DMDDO was characterised using ^{13}C NMR spectroscopy and is soluble in water or organic solvents and can be stored indefinitely, both in solution or as a desiccate [11].

Protein Expression

Isopropyl-β-D-thiogalactopyranoside (IPTG) induced expression of the fusion protein in the *E. coli* cells. These cells were disrupted by sonication and the MBP-LBD fusion protein was separated from the crude cell extract by affinity chromatography using amylose resin. The MBP protein has an affinity for amylose. Once the fusion protein binds to the amylose resin it can be eluted with maltose. The protein was isolated by affinity chromatography using amylose resin that has an affinity for the maltose binding protein.

The protein was eluted with maltose; the fraction in which this was tracked is shown in the graph below. The protein fractions were visualised on an SDS-page gel (Fig. 5). The protein fractions loaded on the SDS-PAGE gel confirms a protein of 66 kDa was expressed. This correlates to the expected size of the maltose binding protein and LBD fusion protein.

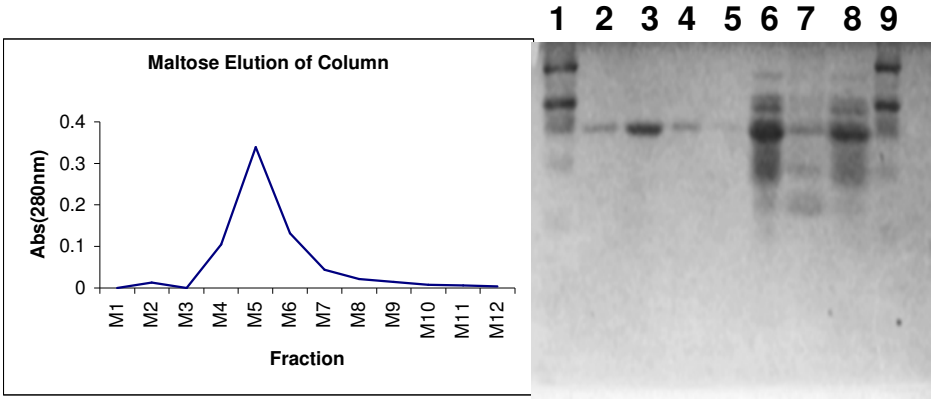

Figure 5. UV analyses of the eluted protein fractions at 280nm. The fractions that contained the fusion protein peaked by fraction 6 (left). The SDS-page of the fractions collected. Lane 1 protein marker, lanes 2-5 are the purified protein fractions, lane 6 affinity column flow through, lane 7 insoluble cell extract and lane 8 crude extract.

Ligand Binding Studies

The standard method to measure hormone binding to nuclear hormone receptors is to label the hormones with radioactive moiety. We adapted the method to test if the LBD purified could capture estrogen while immobilised on a matrix. The fusion protein was immobilised onto amylose resin, which acted as the matrix. A set concentration of radiolabelled 17β-estradiol was added to bind to the LBD of the fusion protein (Fig. 6).

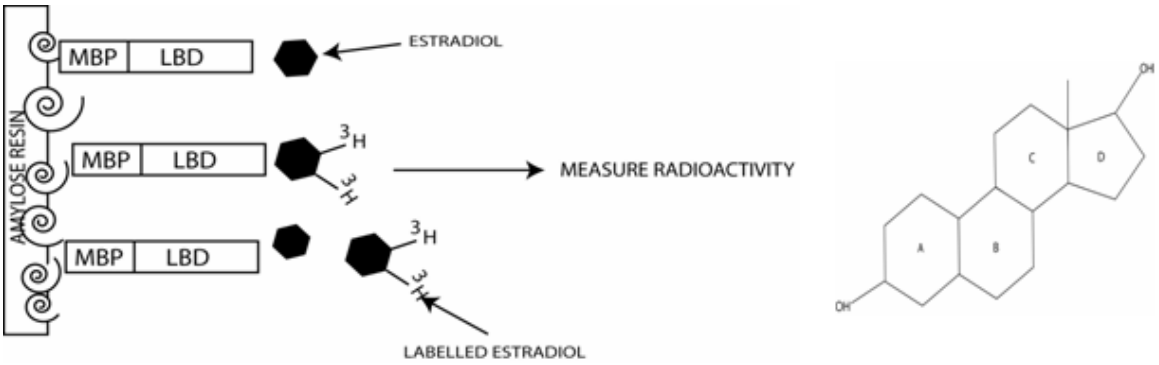

Figure 6. The amylose resin assay to determine if the LBD can capture estrogen from a solution while immobilised on a matrix. Radiolabelled 17β-estradiol was used to detect binding to the LBD of the fusion protein.

The results in Fig. 7 suggest that the cloned and expressed recombinant human ER LBD will be able to bind to estrogenic chemicals, despite being immobilised onto a solid support. This means that its activity remains intact and is a viable candidate for further EDC biosensor development.

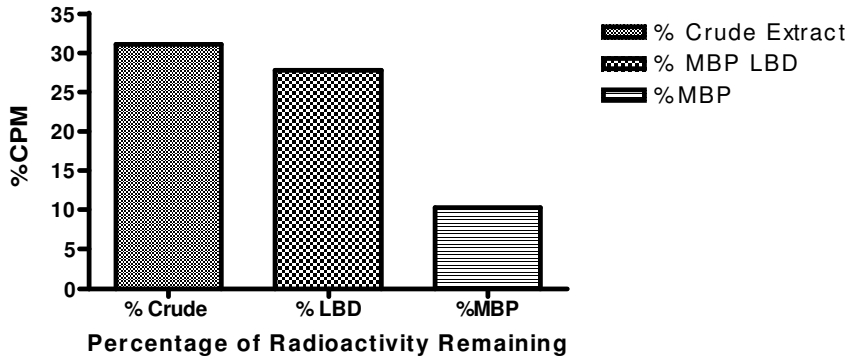

Figure 7. Graphical representation of the results depicted in the experiment illustrated in Fig. 6 to test if the amylose based radioactive assay to determine if the immobilised ER-LBD can capture estrogen from solution.

CONCLUSIONS

When used as an affinity linker, the amphiphilic surfactant Pluronic F108 provides a method to: 1) hydrophilise a hydrophobic affinity membrane matrix, 2) serve as a spacer molecule and 3) allows for the covalent modification of the terminal hydroxyl groups of the PEO chain for the direct coupling of a novel DMDDO ligand. The synthesised nonporous affinity matrices and ligand were stable and well characterised using direct solid-state PIXE analysis. Non-covalent coupling of the chelating ligand to PVDF membranes followed Langmuir type adsorption with maximum monolayer coverage at 5 $mg.ml^{-1}$.

Said derivatives exhibited typical Langmuir type adsorption on hydrophobic PVDF and PSU surfaces, while the saturation curves were similar to the unmodified parent compound [10].

Furthermore F108 surfactants self-assembled onto hydrophobic polymers via the hydrophobic PPO centre block while the PEO chains formed a brush layer that hydrophilised the surface. The chelated Pluronic-DMDDO ligand can be used for affinity purification of histidine tagged proteins. A regeneration formulation based on anionic SDS detergent desorbed pluronic modified polymeric membranes and the possibility of re-usability increases the process lifetime of such affinity matrices.

Production of an estrogen receptor, was possible using standard recombinant DNA technology, where both a histidine tagged fusion ER and a maltose fused ER were expressed and purified. Radiolabelled binding studies conclusively showed that the recombinant ER was active and receptive towards 17-β estradiol. Biosensor development was further demonstrated with the binding studies of HRP on Pluronic-biotin surfaces and the metal chelating DMDDO-PVDF system was another option for using a histinde tagged ER as the ligand. Future work involves incorporating the ER bound PVDF-Pluronic system and application with an environmental sample. This will form the basis of an innovative environmental biosensor for EDC and estrogen detection and monitoring in surface water.

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