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Affinity chromatography using biocompatible and reusable biotinylated membranes

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Abstract

A novel, reusable biotinylated affinity chromatography strategy for the bio-specific binding of bioactive avidin tagged enzymes or polypeptides is reported. Using an avidin coupled peroxidase fusion protein as a test system; non-specific protein shielding and matrix regeneration were also shown. The amphiphilic surfactant Pluronic® F108 was used as an affinity linker, by non-covalent binding to membrane chromatographic matrices while the terminal hydroxyl groups of Pluronic were covalently coupled to the biological ligand biotin. Planar nonporous membranes of varying surface chemistry were synthesised to test the matrix dependent affinity binding of biotinylated Pluronic and their respective ability to resist non-specific protein adsorption. Membrane regeneration using sodium dodecyl sulphate (SDS) was capable of displacing both adsorbed proteins and Pluronic. SDS micelles (34 mM) were effective in desorbing membrane bound protein while 5 mM SDS removed up to 85% of the bound ligand after 20 h incubation at 20 °C. In this study, polyvinylidene membranes had the highest ligand binding capacity of 0.22 mg cm^{-2} and specific, competitive affinity binding of avidin-peroxidase was shown in the presence of up to 0.2 mg ml^{-1} 'contaminant' proteins. The resultant biocompatible affinity chromatographic system was regenerated and reused with no significant change in performance for up to five cycles.

Keywords: Affinity chromatography; Avidin tagged proteins; Biotinylation; Pluronic; Regeneration

1. Introduction

Synthetic polymeric membranes are becoming increasingly popular as solid chromatographic matrices in biological applications ranging from enzyme immobilisation for biosensors [1,2] to separation and filtration in downstream bio-processing [3,4]. As with all types of membranes, the interaction between the surface properties of the membrane matrix and the molecules in solution determine the extent of fouling and flux through the membrane. Bio-fouling is usually characterised by the uncontrolled, irreversible adsorption or adhesion of macromolecules such as proteins, lipids and cells [5]. The extent of biochemical diversity in different biological separations severely complicates the manufacture of the perfect chromatographic support for biological processes while non-specific protein adsorption, ligand leakage and interactions on surfaces are also of considerable technological concern [6,7].

The prevention of protein adsorption on surfaces influences the design and viability of biomaterials including membranes. Surface protection or 'shielding' is afforded by either pre-filtration of the macromolecular solution and or membrane surface modification to prevent bio-fouling. A popular approach to enhance surface bio-compatibility was to graft polymeric molecules such as poly(ethylene oxide) chains [6], betaines, phospholipids, poly(acryl amide) and polysaccharides [8].

The interactions between proteins and surfactants are generally described as arising because of both electrostatic and hydrophobic interactions [4,9]. In protein–surface interactions, the governing factors are determined by both the physical state of the adsorption matrix, protein surface and the solution environment. These factors include bound ions, surface charge,

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roughness, surface elemental composition and surface energetics [10,11]. Many reports focus on the interaction of Pluronic® F108 (a non-ionic surfactant) and SDS (an anionic surfactant) and from a process application point of view are amongst the few thermo-viscofying materials approved as direct and indirect food additives, pharmaceutical ingredients and agricultural products [2,12,13].

Pluronic surfactants are poly(ethylene oxide)$_x$–poly(propylene oxide)$_y$–poly(ethylene oxide)$_x$ (PEO_x–PPO_y–PEO_x) triblock copolymers, that are important to many bio-medical and biotechnological applications [10–12]. These commercially available, amphiphilic surfactants self-assemble onto hydrophobic surfaces via the hydrophobic PPO centre block, while the longer hydrophilic PEO chain forms a flexible tether that terminates in a functional hydroxyl moiety. This terminal hydroxyl group has also been targeted for the covalent attachment of ligands [11,12]. A ligand of particular interest is biotin which ligates strongly and specifically to the protein avidin [14,15].

The well-documented biotin and avidin interaction is one of the strongest non-covalent coupling processes in nature ($K_d = 10^{15}\ M^{-1}$), and has also been used as a model for biosensor development, usually via surface immobilisation of biotin onto a transducer [14,16]. Additionally, the avidin tag can be easily engineered onto target proteins using the numerous commercially available plasmid constructs for heterologous expression. Many biotin–avidin-based separation systems have been described [3,4,14] but there have been very few large scale or commercial applications due in part to poor scalability of conventional column chromatographic supports, fouling, mass transfer limitations and high costs related to ligand and matrix synthesis [1,3,10,12,17,18].

This study is directed towards the development of a robust and reusable membrane-based affinity chromatographic system for the specific immobilisation of a model avidin tagged enzyme using a novel biotinylated Pluronic as an affinity ligand. The use of biotinylated derivatives as ligands on reusable affinity matrices provides an attractive approach for the specific isolation of biochemical ligates such as hormones and receptors [3,14,15]. Candidate chromatographic membrane supports of varying surface chemistry were fabricated and the capability of these membrane matrices to resist non-specific protein adsorption and of being efficiently regenerated for reliable reuse was also investigated. The synthesis and binding capacity of the novel biotinylated ligand for affinity immobilisation of bio-molecules onto polyvinylidene membranes is also described and will contribute to the development of a reusable and biocompatible affinity chromatographic matrix.

2. Experimental

2.1. Reagents and chemicals

Bovine serum albumin (BSA) and lysozyme (Roche, Penzberg, Germany) were used as model protein adsorbates 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).

2.2. Analytical assays

A biphasic colorimetric assay for Pluronic quantification was performed as described by Govender et al. [12]. 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. Unless otherwise stated, all analyses were performed in triplicate.

2.3. Membrane matrix fabrication

Planar nonporous membranes were cast from solutions containing 27% (m/m) [Udel P3500 polysulphone (PSU), poly(ether imide) (PEI) and poly(vinylidene fluoride) (PVDF)], respectively and 73% (m/m) *N*,*N*-dimethylacetamide. The surface hydrophobicity of PVDF and PSU membranes was verified using static contact angle analysis, while PEI membranes were confirmed to be hydrophilic [12].

2.4. Non-specific protein shielding

Protein shielding is possible via the large hydrophilic PEO chains and this was tested using bovine serum albumin (BSA) and lysozyme as model foulants. 67 000 Da BSA and lysozyme (14 700 Da) solutions [0.25 mg ml^{-1}] in 0.1 M phosphate buffer (PB), pH 7.4 were prepared and stored at 4 °C. Membranes were non-covalently modified with Pluronic by static incubation in 5 mg ml^{-1} Pluronic at 20 °C for 8 h. Membranes (native and Pluronic coated) were statically incubated for 120 min at 20 °C in 10 ml of the respective protein solutions. The membranes were then rinsed three times in PB and then inserted into a vial containing 10 ml of 1.0% (w/v) SDS. These vials were then shaken for 120 min and the protein concentration in the SDS solution was measured using a Pierce™ protein assay reagent kit. In a conventional protein adsorption experiment [5], also called the depletion method, the amount of adsorbed proteins was determined based on the decrease in protein concentration in the solution after contacting with the solid surface.

2.5. Membrane regeneration

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 2 h of vigorous shaking. In an attempt to determine if this was a time dependent process, the shaking incubation period was increased

from 2 h to 4 h, 20 h and 48 h, respectively. A concentration range of SDS (5 mM, 8 mM and 34 mM) was also investigated in order to ascertain if SDS micelles facilitated Pluronic desorption from candidate membranes of varying surface chemistry. The critical micelle concentration of SDS is 8 mM. After incubation in SDS, the membranes were washed in a solution of 100 ml dH_2O 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.

2.6. Synthesis of biotinylated Pluronic

The terminal hydroxyl groups of Pluronic® F108 were modified in a two-step reaction to yield an amine terminated Pluronic (Fig. 1). Pluronic® F108 (2 g) [I] was dissolved in benzene (6 ml) and then added drop-wise to 4-nitrophenyl chloroformate in 6 ml of benzene. After 24 h of stirring, the reaction product [II] was precipitated with excess ether, filtered, dried under high vacuum and re-dissolved in benzene. This procedure was repeated at least three times [11]. The dried activated Pluronic (1.5 g) was then dissolved in 6 ml methanol, with slow drop-wise addition of 1 ml hydrazine (NH_2NH_2). After an 8 h reaction period, the product was precipitated with an excess of ethyl ether. Precipitation from methanol was repeated at least three times, and the final product [III] was dried under high vacuum overnight. Hydrazine Pluronic (40 mg) and NHS-biotin (12.5 mg) were then dissolved in 5 ml dry DMF. This reaction mixture was stirred for 48 h at 20 °C, followed by drying under high vacuum. The dry product [IV] was re-dissolved in dH_2O to a final concentration of 5 mg ml^{-1}.

The structure of the biotinylated Pluronic derivative was confirmed by ^{13}C nuclear magnetic resonance (NMR) spectroscopy using a Varian VXR 400 NMR spectrometer. All samples were analysed in deuterated chloroform (Sigma) at 25 °C with tetramethylsilane as the internal standard.

2.7. Affinity immobilisation of avidin-peroxidase

Membrane surfaces were modified by static adsorption for 8–12 h at 25 °C, in 5 mg ml^{-1} solution of Pluronic® F108 [12] and biotinylated Pluronic, respectively. Membranes incubated in unmodified Pluronic were used as negative controls. For affinity immobilisation of Av-P, two sets of eight membranes were then transferred to glass scintillation vials containing a dilution series of Av-P in phosphate buffered saline, pH 7.4 (serial dilution series from 1 U ml^{-1} to 0.0156 U ml^{-1}) in a total reaction volume of 2 ml for 60 min with vigorous shaking. Membranes were then washed three times in dH_2O, air dried and transferred to a 8 × 12 well NUNC™ microtitre plate. An ABTS solution (300 μl of 0.5 mg ml^{-1}) in citrate buffer, pH 5 and 1 μl ml^{-1} H_2O_2, was added to each well. Plates were immediately shaken at 37 °C for 30 min before removing 150 μl of the ABTS solution for analysis at 405 nm. In the presence of sufficient affinity immobilised Av-P, the ABTS solution undergoes a distinct colour change from yellow/green to dark green/blue indicating Av-P activity. The log of Av-P dilution was plot-

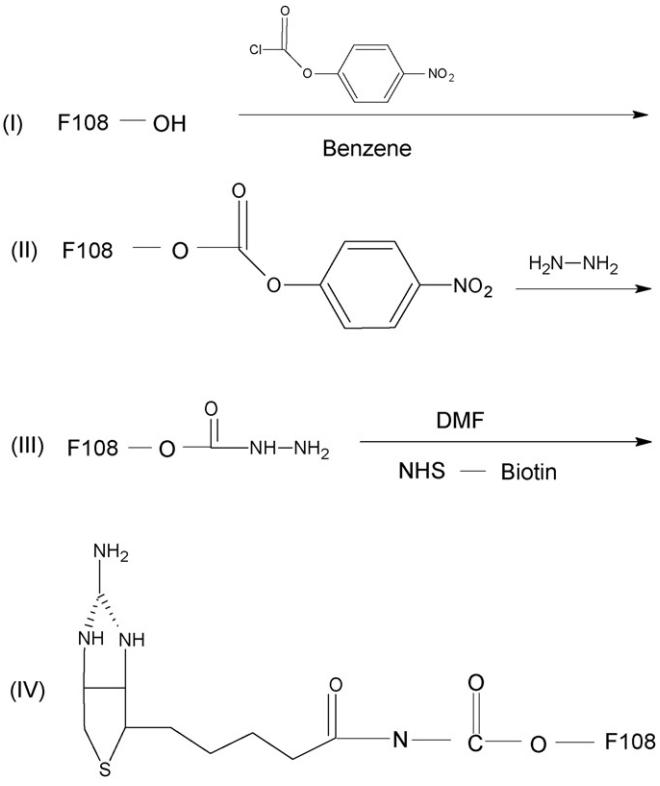

Fig. 1. Reaction schematic for the synthesis of biotinylated Pluronic.

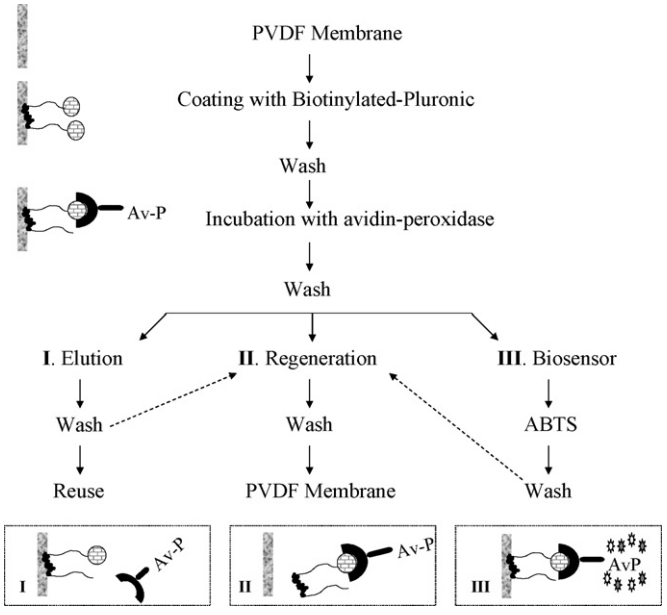

Fig. 2. A multifunctional schematic illustration of affinity immobilisation of avidin-peroxidase onto biotinylated PVDF membranes. After immobilisation of the Av-P, there are three possible routes to follow: (I) Av-P elution and reuse of the affinity chromatographic system for additional Av-P isolation from solution; (II) regeneration of the chromatographic support, followed by re-coating with biotinlyated Pluronic for Av-P immobilization and (III) solid state analysis or use of the immobilised bioactive Av-P as a biosensor by contacting with ABTS for colorimetric spectrophotometric analysis or ELISA.

ted against absorbance to illustrate specific binding of Av-P to membrane bound biotinylated Pluronic.

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 6 mM D-biotin in PBS. The bioactivity of the eluted Av-P was confirmed using the ABTS-based spectrophotometric assay. A schematic illustration of the process from membrane surface modification and Av-P immobilisation to regeneration and possible biosensor applications is illustrated in Fig. 2.

3. Results and discussion

3.1. Regeneration of Pluronic modified membranes

An important practical consideration for the implementation of membranes in affinity chromatography is its lifetime and regeneration capacity [3]. Regeneration was investigated using SDS treatment of Pluronic coated affinity membranes, because SDS is non-toxic, water-soluble, economic from a process point of view and is a known competitive displacer of adsorbed polymers that can also bind organics in solution in the micellar form [19,20]. SDS treatment was performed at room temperature (20 °C) and its efficacy was compared with that of an optimised high temperature (70 °C), method of Pluronic extraction described in another study [12]. However, this efficient biphasic solvent system (hexane–isopropanol), poses potential problems with degradation of polymers such as perspex and polyvinyl chloride (PVC) that are routinely used in laboratory scale membrane cartridge manufacture.

SDS treatment was considered as a non-solvent-based Pluronic desorption alternative. It has been reported that the phase behaviour and microstructure of Pluronic block copolymers are affected by SDS micelles [7]. When compared to untreated membranes (Fig. 3A and B), Pluronic ligand displacement using SDS appeared effective as the native membrane surface could once again be observed (Fig. 3C), however, the electron dense structures observed on the surface suggested the possible remnants of SDS deposits. The *ex situ*, energy intensive but highly efficient hexane–isopropanol extraction of Pluronic depicted in Fig. 3D appeared more effective, since it lacked the apparent electron dense structures in Fig. 3C. A 'cleaning in procedure' or a more rigorous wash process after SDS treatment was useful in removing micelles or aggregates, as SDS is water-soluble. This is important since most surfaces acquire some surface charge when exposed to ionic solutions and in such situations long-range electrostatic interactions will domi-

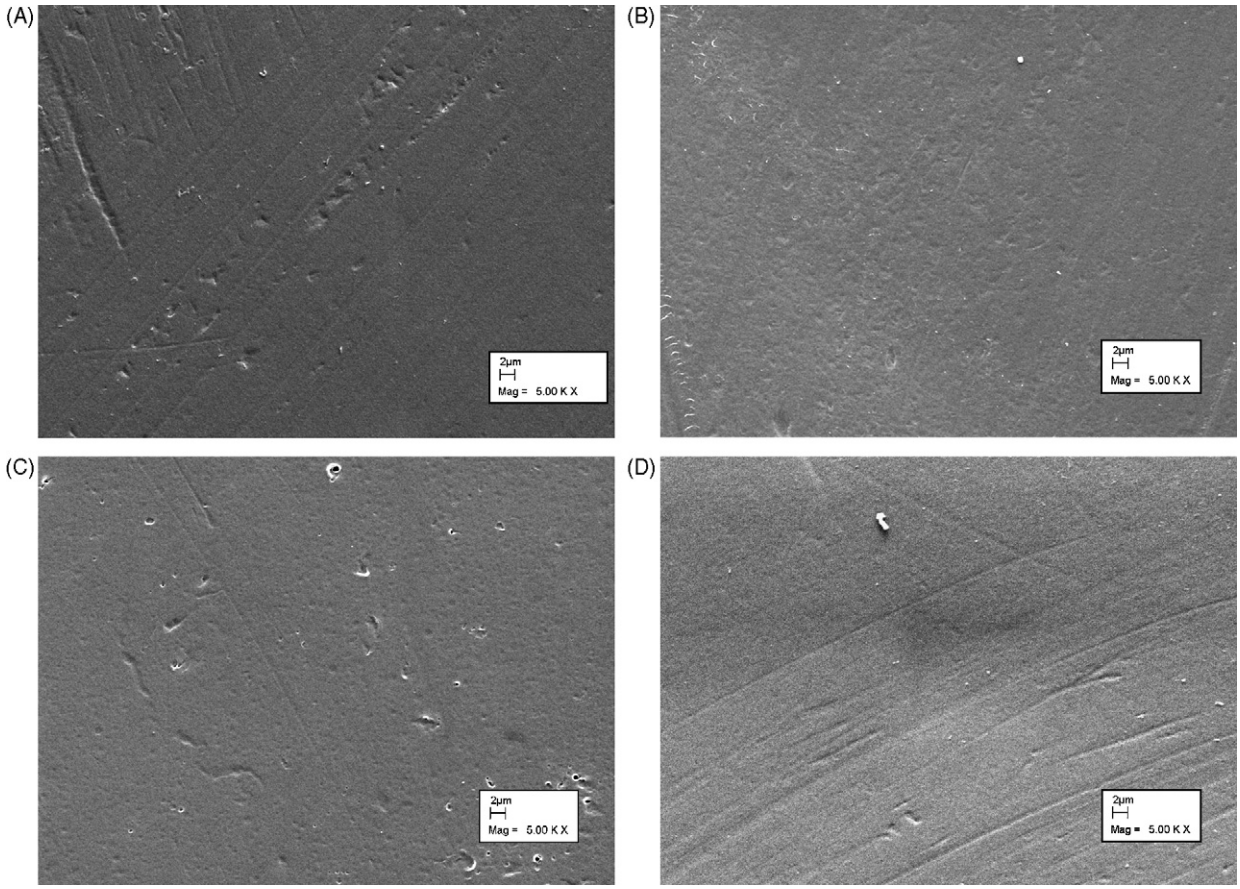

Fig. 3. Electron micrographs showing typical planar nonporous PSU membranes that were used in Pluronic coating and desorption. (A) Native or virgin PSU membrane surface; (B) pluronic coated PSU, (C) SDS displacement of Pluronic treated membranes and (D) hexane–isopropanol treated membranes modified with Pluronic. Magnification = 5000× and bar = 2 μm.

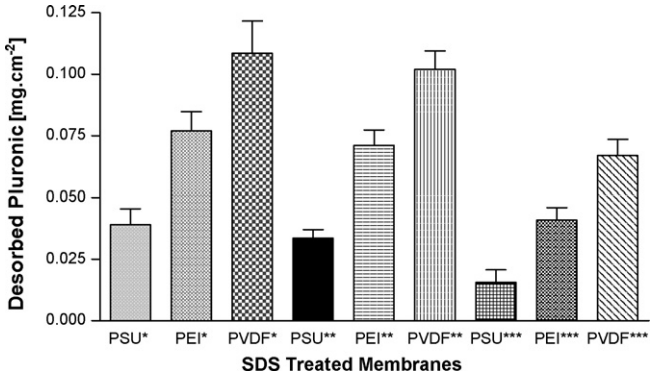

Fig. 4. Influence of SDS concentration on Pluronic desorption. (*) 5 mM, (**) 8 mM and (***) 34 mM. Pluronic desorption occurred at 20 °C for 2 h with gentle shaking. $N = 3$.

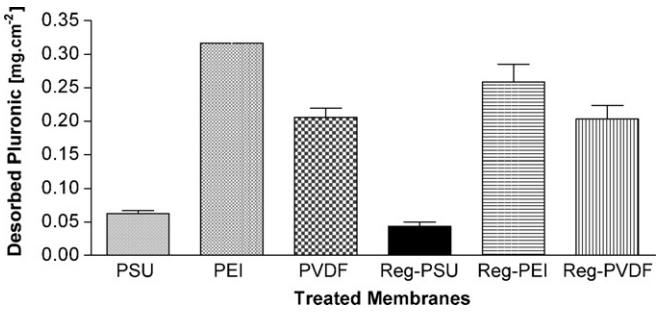

Fig. 5. A comparison between an efficient hexane–isopropanol extraction method of desorbing Pluronic from native planar membranes and membranes that were regenerated (Reg-) with 5 mM SDS. $N = 3$.

nate protein adsorption. Most physiological buffers such as PB will confer a charge to proteins (except at the isoelectric point) and this can orient the protein towards an oppositely charged surface.

The data in Fig. 4 suggest that 5 mM SDS was more effective in displacing Pluronic from the membrane surface than at concentrations at or above the 8 mM critical micelle concentration (cmc). These results were similar to findings by Cosgrove et al. [19], where the same trend was observed with SDS and poly(ethylene glycol) (PEG) adsorbed on polystyrene beads. In said paper, it was found that PEG served as a nucleation centre for the formation of micelles at SDS concentrations above the cmc of the surfactant. These authors concluded that the driving force for SDS displacement of PEG from solid supports was due to polymer–surfactant interactions and not competitive adsorption for sites. It can be argued that membrane bound Pluronic ligand would behave differently than the extremely hydrophilic PEG with the SDS micelles forming a solution complex with the PPO centre block of Pluronic at high SDS concentrations (34 mM).

Table 1 shows the time dependent nature of SDS displacement of Pluronic. Initially experiments were performed under empirical conditions with 2 h incubation at room temperature, but much more Pluronic was displaced after a 20–40 h incubation period. In comparison with optimised solvent extraction at 70 °C [12], up to 85% of bound Pluronic ligand (0.19 mg cm^{-2}) was desorbed using 5 mM SDS treatment for 20 h at 20 °C. The time dependence of SDS desorption was most significant for hydrophilic PEI membranes rather than hydrophobic PSU and PVDF. It is possible that the multi-layer formation of Pluronic at the PEI interface compared to the Langmuir type monolayer coverage observed with PSU and PVDF in another study [12] complicated the association of SDS micelles with the PPO blocks of Pluronic.

Fig. 5 illustrates the typical Pluronic displacement trends observed with hexane–isopropanol and SDS, respectively. Regeneration of hydrophobic PVDF membranes using 5 mM SDS, typically removed 0.19 mg cm^{-2} Pluronic, while up to 0.22 mg cm^{-2} can be removed using the bi-solvent method. The results were similar for both the hydrophobic PSU and the hydrophilic PEI membranes. However, the empirically selected SDS desorption parameters were far from optimised with respect to incubation time, shaking and temperature. Furthermore, studies have shown that even the bi-solvent extraction protocol is temperature dependent [5,12], such that incomplete desorption is observed below 65 °C. Although the empirically selected conditions for SDS displacement are far from optimised, this remains a promising approach for Pluronic displacement from membranes and related polymers.

3.2. Desorption of model protein foulants

At membrane surfaces, additional interactions between the adsorbed molecules and the surface come into play. These interactions are both hydrophobic and electrostatic in nature and the interactions between the protein, surfactant and copolymer, can be affected by the presence of the surface. Furthermore the relation between the properties of the complex will determine whether the surfactant will be able to displace the protein [13]. The ability of SDS to displace membrane-adsorbed proteins is depicted in Table 2.

Table 1
Time dependent displacement of Pluronic coated membranes at 20 °C using 5 mM SDS

	Incubation time in 5 mM SDS							
	2 h		4 h		20 h		40 h	
	mg cm^{-2}	S.D.	mg cm^{-2}	S.D.	mg cm^{-2}	S.D.	mg cm^{-2}	S.D.
PEI	0.077	0.013	0.13	0.020	0.18	0.009	0.181	0.018
PSU	0.039	0.011	0.042	0.010	0.047	0.0059	0.045	0.0071
PVDF	0.11	0.022	0.14	0.017	0.19	0.014	0.19	0.013

The amount of SDS displaced Pluronic (mg cm^{-2}) was measured after varying the incubation period from 2 h to 40 h. $N = 3$.

Table 2
SDS displacement of pre-adsorbed polymers on native and Pluronic modified membrane surfaces after 20 h of incubation at 20 °C

	Lysozyme		Bovine serum albumin		Pluronic® F108		
	μg cm^{-2}	S.D.	μg cm^{-2}	S.D.	mg cm^{-2}	S.D.	*N*
PEI	9.30	1.98	9.55	1.12			3
PSU	8.23	1.54	8.56	0.98			3
PVDF	7.18	1.27	6.33	1.00			3
PEI–Pluronic	1.081	1.25	11.96	1.06	0.26	0.05	3
PSU–Pluronic	6.063	1.00	11.67	1.47	0.04	0.01	3
PVDF–Pluronic	0.512	0.09	11.14	2.20	0.20	0.04	3

The physical displacement of proteins is most likely due to the conformational change in the protein structure after denaturation by SDS micelles. Stigter [21] showed that for different headgroups on the same surfactant alkyl chain, it is usually found that the binding again follows the micellisation, where the higher the tendency to form micelles the stronger the interaction with proteins. Recent work has also verified that the protein repellent properties of the membrane were still retained after re-coating with Pluronic. Non-specific protein shielding on the Pluronic modified membranes was thus determined to be of the order PVDF > PSU > PEI.

At low SDS concentrations (5 mM), binding is thought to take place via electrostatic interactions between the charged headgroup of the anionic surfactant and the oppositely charged residues in the protein molecule. As a result of this initial binding, the protein–surfactant complex becomes less charged and more hydrophobic than the protein itself, which may lead to aggregation and precipitation. At higher surfactant concentrations (>8 mM), binding most likely occurs via hydrophobic interactions, with the SDS headgroups pointing out from the protein surface [22]. At this stage the hydrophobicity of the protein–surfactant complex decreases and it becomes more hydrophilic and re-dissolves into the polar bulk equilibrium solution, eventually acquiring a negative charge like the SDS molecule [13].

3.3. Biotinylated affinity membranes

In an attempt to demonstrate a reusable, membrane-based bioaffinity chromatographic separation technology, the biotin–avidin system was used as a model of biological (ligand–receptor) interaction. However, the strength of the non-covalent interaction between biotin and avidin can pose problems for ligate retrieval. In this study a weaker binding biotin ligand (the *N*-hydroxysuccinimido ester of (iminobiotinylamido)hexanoic acid) was used, that is displaceable by biotin, thus ensuring elution with a biotin containing buffer. Coupling was achieved by displacement of the NHS group in the conjugated biotin by the amine group in hydrazide Pluronic in a DMF solution (Fig. 1).

The structure of biotinylated Pluronic was confirmed with ^{13}C NMR. The similarities in the saturation curves in Fig. 6 indicate that biotin coupling to the hydroxyl terminus of Pluronic did not affect its adsorption affinity for hydrophobic surfaces via the unmodified PPO moiety. Protein adsorption isotherms using lysozyme and BSA (results not shown) also indicate that the covalently modified biotinylated Pluronic retains the protein shielding ability described in this study. The adsorption of biotinylated Pluronic (Fig. 6) showed a typical Langmuir type adsorption profile at 25 °C with a plateau at ~5 mg ml^{-1}. The ligand binding capacity of the PVDF membrane was approximately 0.22 mg cm^{-2} at an initial coating concentration of 5 mg ml^{-1}.

3.4. Affinity immobilisation

PVDF was selected as the substrate affinity membrane in this study because of its high hydrophobicity, protein shielding ability, mass transfer properties as hollow fibers [23] and because it is a popular piezoelectric and electro-active polymer in biosensor development [10]. Dose–response curves were obtained that related the solid phase biotinylated Pluronic to concentration of Av-P (Fig. 7). The normalised curves follow a typical inverted sigmoidal shape, reaching a plateau when all the available binding sites on the membrane are occupied by the enzyme conjugate.

The typical dose–response effect in Fig. 7A suggests that there is a specific correlation between Av-P binding to surface immobilised Pluronic–biotin and the response is proportional to the concentration of Av-P used in the binding assay. The curves

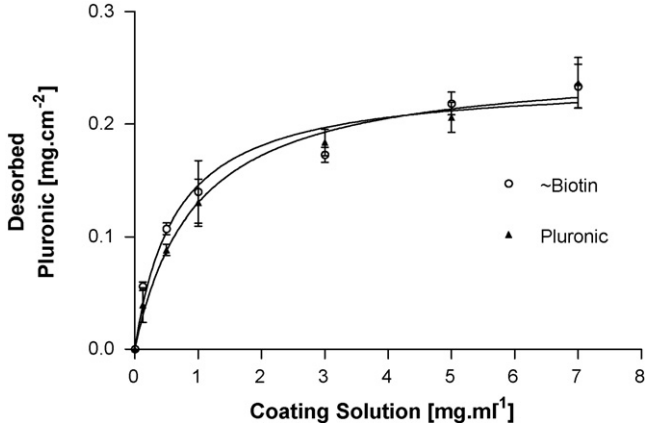

Fig. 6. Saturation curves for biotinylated Pluronic (r^2 = 0.9162) and unmodified Pluronic® F108 (r^2 = 0.9307) on planar PVDF membranes. The coating solutions contained the ligands biotinylated-Pluronic and unmodified Pluronic® F108, respectively. Both Pluronic® F108 and biotinylated-Pluronic were desorbed from PVDF surfaces using the optimised hexane–isopropanol extraction method [12].

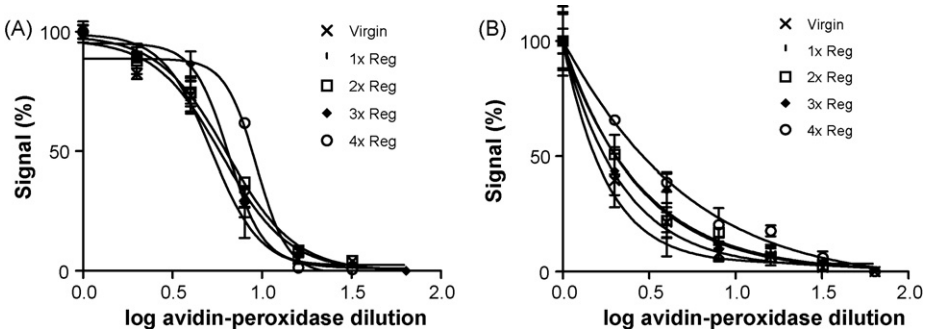

Fig. 7. Dose–response obtained with (A) biotinylated membranes and (B) unmodified Pluronic treated membranes incubated with a serial dilution of avidin-peroxidase. Avidin-peroxidase was serially diluted from 1 U ml^{-1} to 0.0156 U ml^{-1}. Virgin biotin modified membranes and Pluronic coated membranes were subjected to four regeneration cycles and each experiment was performed in triplicate. EC_{50} data for the virgin biotinylated PVDF membrane through to the four regenerated cycles described in part (A) are 5.95 μM, 5.923 μM, 6.358 μM, 6.506 μM and 9.231 μM, respectively. To make comparisons possible, all curves have been normalised so that their highest signal corresponds to 100%. Baselines are less than 10% of the maximum and were not subtracted when calculating EC_{50} values using GraphPad Prism® for the biotin–avidin-peroxidase interaction.

in Fig. 7B however, are of the interaction between Pluronic and Av-P. Interactions producing a signal in Fig. 7B are due to non-specific binding of protein to Pluronic. The highest response is at the highest enzyme concentration (1 U ml^{-1}) where protein saturation of the surface at high concentration occurred. The subsequent dilution steps yielded a much lower signal or response suggesting that there was comparatively low non-specific binding. Bioactive Av-P was eluted using a 6 mM biotin buffer and the activity was confirmed using an ABTS-based ELISA technique.

3.5. Membrane regeneration

Ligand coated membranes that were repeatedly regenerated only appeared to lose their protein shielding properties after the fourth regeneration cycle as observed by the increase in the signal intensity shown in Fig. 7A. SDS displacement of Pluronic is not 100% efficient as depicted in Figs. 3 and 4, and if Av-P remained on the surface after regeneration, then there could be an increased tendency after repeated cycles for the conjugated avidin to non-specifically bind more protein from solution via hydrophobic interactions, eventually forming protein multilayers at the interface.

However, regenerated membranes treated with biotinylated Pluronic (Fig. 7A), still produced characteristic dose–response curves, but EC_{50} analysis revealed that with increasing regeneration cycles, the concentration of Av-P required to induce a response halfway between the maxima and the baseline decreased. EC_{50} data for the virgin PVDF through to the four regenerated cycles described in Fig. 7A are 5.95 μM, 5.923 μM, 6.358 μM, 6.506 μM and 9.231 μM, respectively. This is most likely due to incomplete regeneration of the membranes with SDS-treatment, where protein–protein interactions progressively increased resulting in higher EC_{50} values. However, the colorimetric assay used to measure Av-P is extremely sensitive, and the consistent shapes of the binding curves warrant further investigation of SDS treatment of affinity membranes to increase their capacity and performance.

The specificity of Av-P binding to membrane immobilised biotin and the protein shielding ability of the ligand-modified Pluronic, were tested with a competitive binding assay where 0.2 mg ml^{-1} of model protein contaminants were incubated with each of the serially diluted Av-P containing vials. In Fig. 8 the presence of 0.2 mg ml^{-1} of lysozyme did not cause a significant change to the typical dose response effect (EC_{50} = 5.414 μM) suggesting that the hydrophilic PEO tether of Pluronic F108 shielded the membrane surface from non-specific lysozyme adsorption, whilst freeing the biotinylated ligand binding sites to recognise Av-P.

However, a mixed solution of BSA and lysozyme (0.2 mg ml^{-1}) did not yield a similar trend with a dramatic shift in the EC_{50} from 5.414 μM to 0.88 μM. It is postulated that since BSA adsorbs on both hydrophobic and hydrophilic surfaces [5], such as Pluronic® F108 coated membranes (Table 2), BSA blocked many of the avidin binding sites on the biotinylated PVDF membrane. This would cause a reduction of the Av-P binding capacity of the membrane resulting in lower sig-

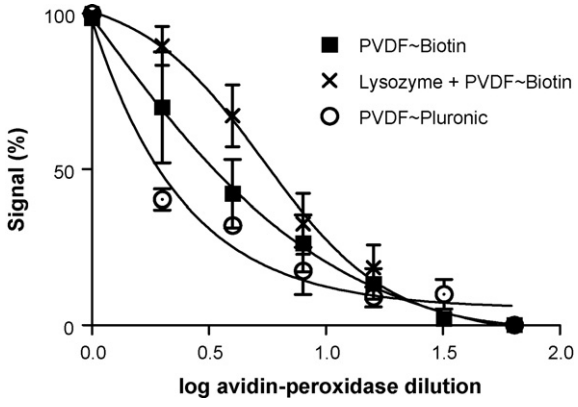

Fig. 8. Normalised competitive binding assay for avidin-peroxidase in the presence of 0.2 mg ml^{-1} model protein contaminants. Symbol ■ represents biotinylated PVDF membranes that were incubated with 0.1 mg ml^{-1} BSA and 0.1 mg ml^{-1} lysozyme (EC_{50} = 0.88 μM), symbol × represents biotinylated PVDF membranes incubated with 0.2 mg ml^{-1} lysozyme (EC_{50} = 5.414 μM) while symbol ◯ represents a non-derivatised Pluronic coated membrane with 0.1 mg ml^{-1} BSA and 0.1 mg ml^{-1} of lysozyme. N = 3.

nal intensity. Furthermore, the adsorbed BSA could also serve as a nucleation centre for further protein adsorption if the reaction incubation times were extended.

4. Conclusions

Both the amphiphilic surfactant Pluronic® F108 and the attendant covalently modified biotin derivative, coupled to synthetic polymeric membranes via predominantly hydrophobic interactions. The protein shielding ability of membrane bound ligand was dependent on its adsorption capacity on the membranes, which was influenced by the surface hydrophobicity conferred by the fabrication polymer. PVDF membranes showed the best compromise between ligand binding and protein shielding. These properties in addition to its ability to be displaced by SDS make this affinity membrane technology possible to regenerate, thus improving its process lifespan and capacity. Additionally, the regeneration protocol developed in this study can be incorporated into both *in situ* and *ex situ* membrane systems by utilising existing equipment for re-circulation of the various system components.

This study has also shown specific affinity immobilisation of Av-P onto biotinylated PVDF membranes using a novel ligand (Pluronic–biotin). Biotin coupling to the hydroxyl terminus of the PEO moiety of Pluronic produced a characteristic Langmuir type adsorption profile on the electro-active polymer PVDF. The dose–response curves for regenerated biotinylated membranes followed the same profile as that of the virgin biotinylated membrane, with a significant EC_{50} increase only after more than four cycles of regeneration and reuse. Furthermore, the stable and active affinity bound Av-P could potentially be used as a bridging molecule to bind biotinylated proteins, thus also making it an attractive option for use in biosensor development. Competitive binding assays also suggest that this specific binding is not influenced by up to 0.2 mg ml^{-1} of lysozyme, but can be susceptible to large amounts of globular BSA. Pre-filtration of globular proteins is recommended to prevent the subsequent blocking of affinity binding sites for Av-P.

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