Brush Grafted Membranes for Protein Immobilization

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I would like to thank both my supervisors Dr. Jenny Lawler and Dr. Brendan O'Connor for their guidance and direction throughout this project and to the DCU School of Chemistry staff who have always been very accommodating. Thank you to my family and loved ones for being my rock. Last but not least, I would like to recognise my mental health, that has helped me push past all barriers to accomplish my dreams.

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ABSTRACT

In this study two types of membranes were modified to selectively bind protein; polysulfone membranes were used to purify Green Fluorescent Protein (GFP) from a complex mixture of proteins, whereas cellulose membranes were selected to bind the enzyme laccase, which was examined for the breakdown of the anti-inflammatory drug Diclofenac.

Chloromethylated polysulfone (CMPS) ultrafiltration membrane adsorbers grafted with polyacrylic acid polymer brushes for binding of green fluorescent protein were fabricated using the non-solvent induced phase separation (NIPS) method. Polysulfone (PS) was chloromethylated using chlorotrimethylsilane and paraformaldehyde to obtain chloromethylated polysulfone, indicated by a peak at 4.5 ppm using ^{1}H-NMR spectroscopy. The introduction of chloromethyl groups to the membrane surface acted as an initiator for surface polymerization of tert-butyl acrylate (tBA) monomer via surface initiated atom transfer radical polymerization (SI-ATRP) to poly-tert butyl acrylate (pol-tBA). Selective acid hydrolysis of the pol-tBA brushes for deprotection of the tert-butyl groups yielded polyacrylic acid (PAA) brushes, with effective binding sites for immobilization of protein, confirmed using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy identified by decrease in transmittance at 1640 cm^{-1} corresponding to a decrease in hydrogen bonding interactions due to the presence of poly(AA). Increasing the length of time for ATRP led to an increase in polymer grafting, which led to an increase in hydrophilicity, demonstrated from water contact angle studies. Adsorption and flux studies were carried out to further examine the anti-fouling properties of the membranes. Scanning electronic microscope (SEM) images reveal that the grafted membranes exhibit typical features of ultrafiltration membranes, with a dense skin layer and well defined finger-like macrovoid sublayer. Green Fluorescent Protein (GFP) was successfully bound to the grafted brushes. Fluorescent microscopy identified the stable

binding of GFP. The produced membranes have strong potential for the selective separation of proteins in downstream processing.

The second part of this project used surface initiated atom transfer radical polymerization (SI-ATRP) to successfully graft hydrophilic polymer brushes onto commercial cellulose membrane surfaces, which were capable of immobilizing laccase enzyme. Initiator immobilisation was achieved through esterification of the hydroxyl groups present on the membrane surface with α-bromoisobutyryl bromide (BIBB). As above, SI-ATRP was used to introduce the poly tBA brushes and acid hydrolysis carried out to create poly (AA) brushes. Their presence on the surfaces of the hydrolysed membranes was confirmed through FTIR analysis. The stability of the bound enzymes were challenged at a range of pHs, temperatures and ionic strengths. It was found that the stability of immobilised laccase was enhanced at pH 4 and pH 5 in comparison to the free form at 10^{o}C and at pH 5 at 22 ^{o}C. There was no significant difference in activity between the two enzyme forms at pH 6 and pH 7 at 22^{o}C and pH 5 at 30^{o}C. These results suggest that the association of laccase with a support surface may not affect its structural integrity and make it suitable for use in a wide range of conditions. The enzyme bound membranes (EBMs) were capable of breaking down 96% of the anti-inflammatory drug Diclofenac (DCF) after 2 hrs without the addition of any mediator at 22^{o}C and pH 7; conditions similar to drinking water treatment, indicating their strong potential for industrial use.

1 Literature Survey

Polymer brush membranes have a 3D structure that offers multiple binding sites, enhancing the membrane binding capacity immensely. These brushes can assist in protein separation, binding the targets in the same way as conventional chromatography; through affinity, ion exchange or hydrophobic/hydrophilic interactions. Polymer brushes can also offer membranes superior characteristics such as increased hydrophilicity and protein antifouling properties. The work carried out throughout this project has two distinct strands utilizing the capabilities of membranes grafted with polymeric brushes. In the first half of the project, polymer brushes were grafted onto manually prepared Polysulfone membranes for the capture of a fluorescent protein. The protein functionality established on the basis of fluorescent activity after purification. For the second half of the project, hydrophilic polymer brushes grafted onto commercial cellulose membrane surfaces were created to immobilise laccase enzyme with the aim of degradation of the non-steroidal anti-inflammatory pharmaceutical pollutant, Diclofenac.

There is an ever increasing requirement for production of proteins in industrial and academic settings, for a variety of applications, including exploratory research; drug discovery and biopharmaceutical production (Bianchi et al., 2011). When a therapeutic protein is manufactured, there are many different glycoforms present due to batch variation. Regulatory bodies such as the FDA will insist on full glycoprofiling in the future. To date there are more than 100 therapeutic proteins approved for clinical use in the EU and USA, for example insulin which is used to treat patients with diabetes, and mecasermin which treats children that have growth failure due to growth hormone deficiency or chronic renal insufficiency (Leader, Baca, & Golan 2008). Advances in large scale production of these proteins have led to increases of cell culture/fermentation titers of up to tens of grams per liter, and there is a subsequent need to ensure all impurities are removed and that sufficient amounts of highly purified proteins are obtained for the desired application (Lowe, 2001). Escalating demands for increased protein titers have shifted the bottleneck step from production to purification, with downstream processes, including purification, representing between 45-92% of the total cost of manufacturing a recombinant protein (Saraswat et al., 2013).

A common purification method used is affinity chromatography (Figure 1), which relies on specific interactions between an immobilised ligand and a target protein (Armada, et al., 2015).

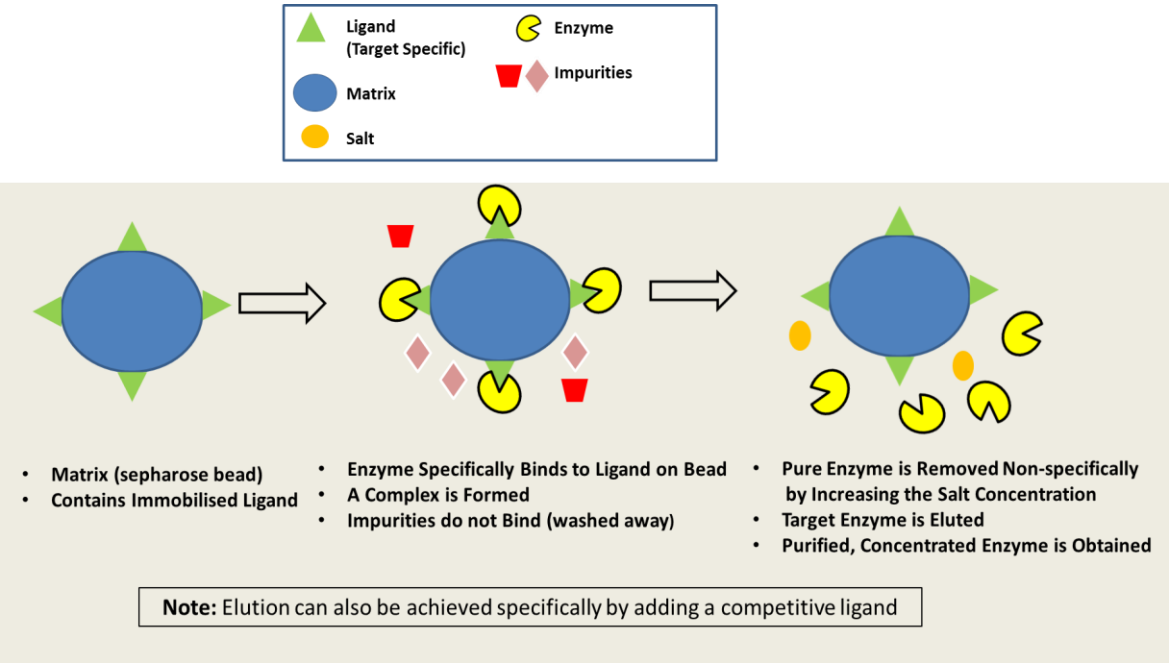


Figure 1: Affinity Chromatography

However, mechanisms of mass transfer in chromatography are typically limited by diffusion (McGuffin, et al., 1998). In contrast, with membrane chromatography, the mass transfer is typically governed by convection, which can lead to faster processing times and in situ protein separation (Ruckenstein, 1999). Membrane adsorption technology is a promising alternative to chromatography operations, and is used in the biopharma industry currently to separate proteins based on charge and size. These membranes are of great interest for bioseparation applications (J. Wang et al., 2013) due to the potential for improved process economics, efficient contaminant removal, superior large molecule capture, and high flow rates at low pressure drop, ease of scaleability and process flexibility. Efforts to develop low cost disposable chromatography columns have yielded very few commercial products. In comparison, membrane adsorber systems are inherently scalable and are frequently disposable due to the low cost of manufacture, giving them significant potential as tailored high performance separation materials (Nam et al. 2015; Atkinson, 2009). This technology is a cost effective alternative to traditional

chromatography, aimed at reducing analytical times and increasing yields of the target protein. Polymer brush grafted membranes are particularly suited for immobilization of biomolecules because they possess a well-defined structure, excellent mechanical stability and dense functional groups (Feng et al., 2014). The number of binding sites for biomolecules can be controlled by the polymer chain length (Murata et al., 2007). They also have the potential to streamLine the number of unit operations required in downstream processing. The first part of this project was aimed at developing novel polymeric brush membranes, as a pre-cursor to the development of membranes which would allow the selective separation of mixtures of glycoproteins, which has strong commercial potential.

The second objective of the project was to immobilise the enzyme Laccase onto commercially available cellulosic microfiltration membranes, for the removal of Diclofenac from aqueous solution. Diclofenac is a non-steroidal, anti- inflammatory drug, which is commonly found in pharmaceutical waste and in fresh water worldwide (Guiloski et al., 2015). It has been recently added to the EPA's watch list (US EPA, 2014), as it has been shown to have detrimental effects on wildlife. Traces of this drug found in carcasses has been linked to a rapid decline (97% in 5 years) in the vulture population across India (Taggart et al., 2007). This drug was used to treat domestic wild stock shortly before their death, which the vultures fed off. It has also been shown to impact the health of on aquatic organisms; studies show it decreased testosterone levels in *Hoplais malabaricus* male fish and caused damage to its liver, consequently impacting their metabolism (Guiloski et al., 2015). Rainbow trout that were exposed to varying concentrations of 1-500 µg Diclofenac/L for 4 weeks had organ damage to their gills, and induced kidney and renal lesions (Schwaiger et al., 2004). Diclofenac is commonly administered in both oral and topical form, and 15 % is typically excreted, while only 85% is metabolized following topical application. Breakdown of diclofenac in wastewater treatment plants (WWTPs) is not complete, with levels downstream of WWTPs found to be in the range of up to 2µg/L (Schwaiger et al., 2004).

Hazardous organic pollutants (HOPs) such as pharmaceuticals, endocrine disrupting compounds, hormones and pesticides have been found to ng/L to µg/L levels in drinking

sources around the world (Jung et al., 2015; Sanches et al., 2012). Traditional treatment options include technologies based on photocatalysis (e.g. TiO_2), ozone and UV. These technologies can be energy intensive, expensive and inefficient (Ufarté et al., 2015). Enzyme catalysed breakdown of pharmaceutical pollutants has been shown to be an environmentally friendly alternative for treatment of polluted waters (Ufarté et al., 2015).

Enzymes have been immobilised in a number of different ways, ensuring that continued use and re-use of the enzyme is possible. Laccase enzymes are used to oxidise phenol and non-phenol compounds, such as industrial dyes and pesticides (Beloqui et al., 2006), hydrolases such as esterases, are used to detoxify pesticides by cleaving ester bonds (Ufarté et al., 2015) and nitrilases are used to degrade pharmaceutical waste into harmLess substances by hydrolysing nitriles into ammonia and carboxylic acids (Gong et al., 2012; Ufarté et al., 2015). For optimum efficacy, the immobilised enzyme must retain its selectivity and stability across the region of potential operation (Singh et al., 2013). In many cases, the characteristics of an immobilised enzyme may in fact be improved over the free enzyme. Enzymes such as lipases are popular biocatalysts used in industry; however it is sensitive in organic media. Disadvantages of free enzymes include their inability to be reused and low stability to temperature shifts and organic solvents. Immobilised lipase onto co-polymer of polyvinyl alcohol (PVA) and chitosan (CH) gave excellent reusability properties and up to 4.5 times more activity in comparison to free lipases (Badgujar & Bhanage, 2015).

Membrane immobilised enzymes have attracted increasing research in recent years due to their inherent scaleability, low cost of manufacture, and ease of operation. They have played an important role in the reprocessing of waste from pharmaceutical and food industries, acting as an "environmentally friendly" approach to waste treatment, being able to operate in mild conditions. Polyvinylidene difluoride (PVDF) membranes immobilised with β-galactosidase have been applied to treat lactose waste produced through dairy processing. The enzyme β-galactosidase converts lactose into a probiotic called galacto-oligosaccharides which can be remarketed (Palai, Singh, & Bhattacharya, 2014). Penicillin(G) acylase (PGA) grafted membranes have been used for the formation of

antibiotics, and can be reused up to 16 times (C. Chen et al., 2011). For the treatment of pharmaceutical pollutants, laccase immobilised membranes have successfully degraded tetracycline (Cazes et al., 2015), Diclofenac, (Nair, Demarche, & Agathos 2013), steroidal hormones (Auriol et al., 2008) and more. These enzymes can maintain activity for up to 5 months, when stored at 5 ^{o}C (Saeki et al., 2013). Further information on the uses of laccases for Diclofenac breakdown can be found in section 1.3.3.

1.1 Membrane Chromatography

Chromatography is the most popular technique applied for separation and purification of proteins; conventional chromatography is achieved using a column packed with a resin of desirable properties for the targeted separation. Downstream processing faces two main challenges; isolate the targeted glycoprotein without compromising its integrity and reduce the process volume by performing a concentration step (Ghosh et al., 2014). During processing the glycoprotein often gets exposed to unfavourable conditions, such as low pH during viral inactivation, which can form aggregates and impact overall yield. Monoclonal antibody (mAb) production is the largest sector of biopharmaceutical industry, costing millions to pack a 2x2 meter column of Protein A resin (Lintern et al., 2016). This resin is at the core of downstream processing, using immobilised ligands which have strong affinity for mAbs, however the technique is limited by drawbacks such as an increased drop in pressure during processing caused by colloidal material gathering, diffusion which causes long processing times as the proteins have to travel through beads (Figure 2) and channelling; cracks in the bed result in inadequate separation, resulting in many difficulties during the scale-up process (Ghosh, 2002). Membranes are routinely used in upstream and downstream bioprocessing for ultra-filtration, microfiltration, tangential flow filtration and viral filtration processes (Saxena et al., 2009). Membrane chromatography is an evolving technology that utilises chromatography techniques to give high-throughput protein separation, overcoming restrictions of conventional methods. Unlike packed bed chromatography, binding of the macromolecules occurs primarily on the membrane surface by convection which assists in processing time, seen in Figure 2. These membranes are designed by grafting ligands onto the membrane surface that assist in the separation of targeted biomolecules through adsorption. Advantages of chromatography membranes

include being easily scalable, disposable, cost efficient (no cleaning) and ability to use at high flow rates without compromising binding efficiency (Ghosh, 2002). Higher flow rates can also be applied because separation is achieved through convection flow, this delivers product directly to the membrane surface ligands, unlike resin beads that require pore diffusion to obtain maximum binding (Muthukumar et al., 2017).

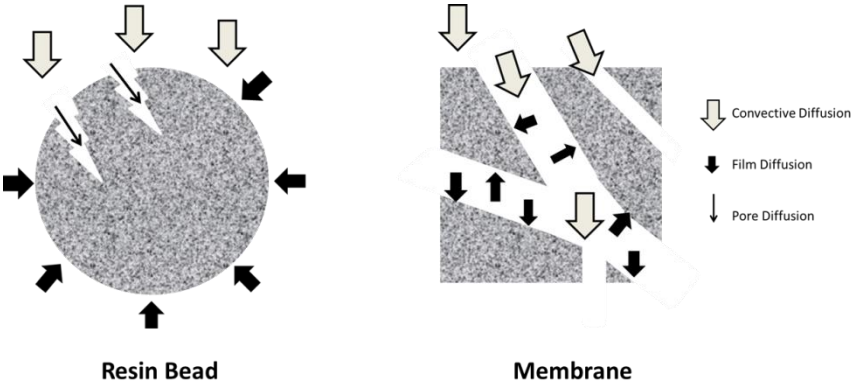


Figure 2 Transport of Solutes through Packed Bed Chromatography and Membrane Chromatography

A comparative cost study was carried out by Muthukumar et al., (2017) between purification of monoclonal Antibodies (mAbs) using a chromatography membrane and a packed column. The study accounted for costs in every step of the process, including media, buffer, and staff to operate the equipment. To produce 50g of mAb using column chromatography, it totalled to $14,711.90, in comparison, the same yield was achieved using membrane chromatography at a cost of $2,311.10; a saving of 75%. Using disposable membranes eliminate the costs that go alongside cleaning validation, and cleaning pre and post use, including the use of buffers for column regeneration and storage. Disposable technologies help reduce the risk of cross contamination between batches and give more flexibility to production of biopharma companies if they are only required for a short campaign, where lower capital investment is required. Further evidence of this is seen in a study carried out by Jacquemart et al., (2016) using Protein A chromatography membranes that captured 200 g/hr/L media of mAb which performed 15% better than a Protein A column. It was observed that using a 0.5 L chromatography membrane column over 10 days would yield 4.5 kg of mAb, which would require a 24L resin column to

achieve the same result, demonstrating that membrane chromatography not only reduces the cost, and time, but it also reduces the footprint for industry. Similarly Mora et al., (2006) noted that buffer use can be reduced by 95% using membrane adsorbers, reducing the cost of downstream materials significantly.

There are three various types of membrane used for chromatography; flat sheet, radial flow and hollow fibre; the most popular one being flat sheet membranes, followed by radial flow (Madadkar & Ghosh, 2016). Multiple stacks of flat sheet membranes are commonly used for separation processes to achieve higher separation efficiency, for example in the ultrafiltration diafiltration (UF/DF) step in downstream processing. The flow distribution throughout the different membranes can be visualised in Figure 3. The feed is only in contact with the membrane surface for the flat sheet membrane, whereas the hollow fibre and radial flow membranes have a larger surface area for binding capacity. This is an important parameter to be considered during separation process; however adsorption binding kinetics can be limited, and cannot always be improved by adjusting the transport phenomena (Ghosh, 2002). Hollow fibre membranes are used in ultrafiltration processes, they consist of numerous tubular membranes ranging in diameter between 0.25 to 2.5 mm placed inside a core shell. Radial membrane adsorbers consist of a number of flat sheet membranes spirally woven over a cylinder. A list of commercially available membrane adsorbers is presented in Table 1.

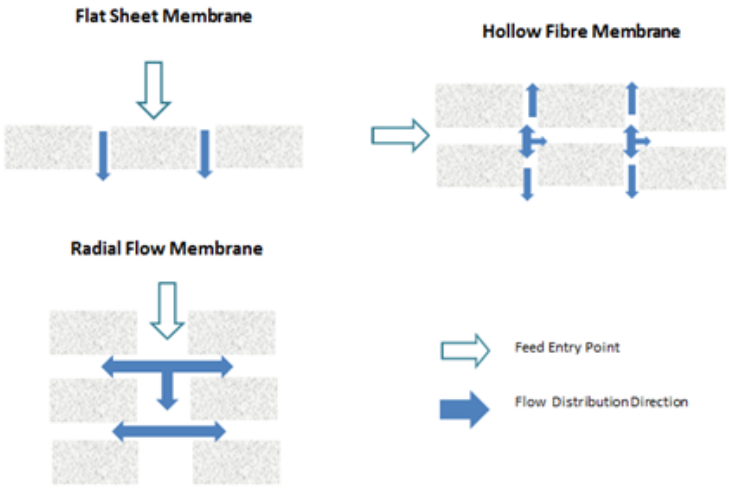


Figure 3 Flow Distribution of Different Membrane Adsorbers

Product Name	Membrane Type	Configuration	Manufacturer
Sartobind ®	Ion Exchange	Radial Flow & Flat Sheet	Sartorius
Mustang®	Ion Exchange & HIC	Radical Flow	Pall
Vivapure®	Ion Exchange	Flat Sheet	Vivascience
ReadyToProcess Adsorber	Ion Exchange & HIC	Flat Sheet	GE Healthcare
QyuSpeed D	Ion Exchange	Radical Flow	Asahi

Table 1 Commercially Available Membrane Adsorbers

Modification of membranes throughout the literature is primarily concentrated to affinity separation; this may be due to its ease of attaching ligands to the membrane's surface, followed by ion exchange chromatography (IEC) membranes, which have been used widely in microfiltration. Other techniques include hydrophobic interactions (HI) and reverse phase (RP) chromatography. The first ever FDA approved chromatography was an Anion Exchange Membrane (2001) as a DNA clean up step in downstream processing (Mora, 2006). There are predominantly four different types of ligands used in affinity chromatography; protein A/G, immunoaffinity ligands, low molar mass and other ligands (Ghosh, 2002). Ligands are used to specifically bind a target region on the protein of interest, both polyclonal and monoclonal antibodies used for membrane chromatography. Polyclonal antibodies have two different epitope recognition regions, in contrast monoclonal only have one. Attaching ligands onto the membranes surfaces has been achieved through different immobilisation strategies, where it is essential not to block the antigen binding region.

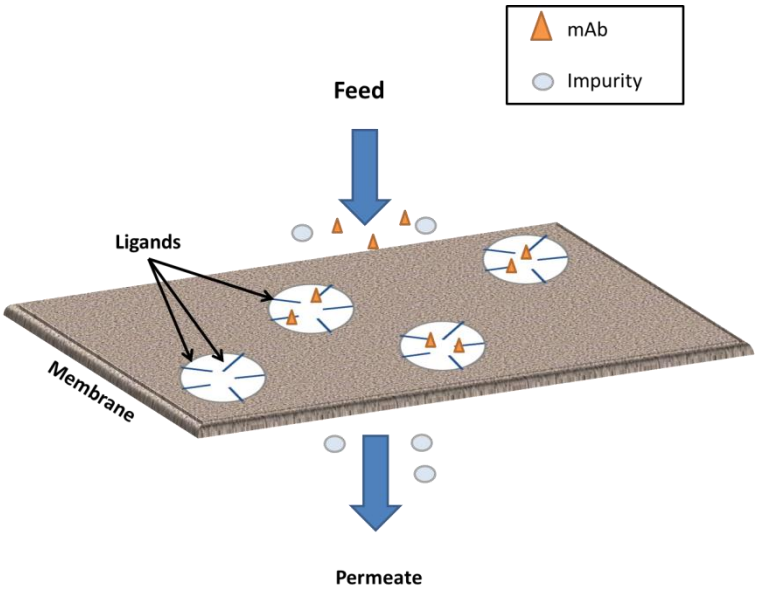


Figure 4 Principle of Affinity Membrane Chromatography to Purify Monoclonal Antibodies (mAb)

Affinity chromatography membranes have been studied as a promising substitute to Protein A resin. Almost every recombinant protein can be purified using affinity chromatography through specific antigen binding interactions, shown in Figure 4 (Saxena et al., 2009). Cellulose membranes immobilised with ligands such as protein A, human IgM, human IgG and pectinase have to been examined for the purification of IgG. The recovered product maintained 95% activity, showing that the process did not denature the protein, which is owed to the quick processing time of only 30min per cycle and buffers used at pH close to the product's isoelectric point (Zhou et al., 1999). In contrast, the same technique applied using the immobilised ligands on a resin achieved product with less than 24% activity, it was suggested this may have been caused by ligands being deactivated and no longer able to bind IgG during the length of time it took to process, due to slow intraparticle diffusion (Huse, Böhme, & Scholz, 2002). The membranes are saleable and have achieved 99% recovery yielded during a 'one step' process, enhancing their appeal to industry (Jacquemart et al., 2016), however one drawback of these membranes is the limited capacity for ligand binding on the membranes surface. Conversely, ligands immobilised onto resin beads tend to have less accessibility to their target protein, whereas membrane ligand's active site are more reachable (Murphy, Tatyana, & O'Kennedy, 2016). Recent studies carried out by Jacquemart et al., (2016), comparing a protein A affinity membrane

to a protein A resin in the purification of IgG from a crude cell lysate concluded the chromatography membrane was better at reducing total host cell protein load, and could perform separation using a variety of buffers, demonstrating its ability to reduce cell culture impurities following upstream processing. Commercially available cellulose membranes created by Sartorius Stedim Biotech have been modified as Protein A affinity membranes utilized to obtained 95% recovery from a IgG solution (Boi et al., 2008). Chitin membranes with affinity ligands (N-acetyl-D-glucosamine) have favourable characteristics over chitin beads for separation including 20 times more adsorption capacity. The membranes have achieved 99% purity of the lectin wheat germ agglutinin from a wheat germ mix (Zeng & Ruckenstein, 1999). These membranes have also successfully isolated lysozyme enzyme with 98% purity, without compromising its activity (Saxena et al., 2009).

A major drawback of radial flow and flat sheet chromatography membrane is poor flow distribution, which can hinder the binding ability. Stacked disc membranes are frequently used in processing, where circular membrane is fed from the center (Figure 5) which can lead to an uneven distribution of materials on the surface, primarily gathering at the center, causing the membrane to be compromised (fouled) sooner than it should be (Madadkar et al., 2015).

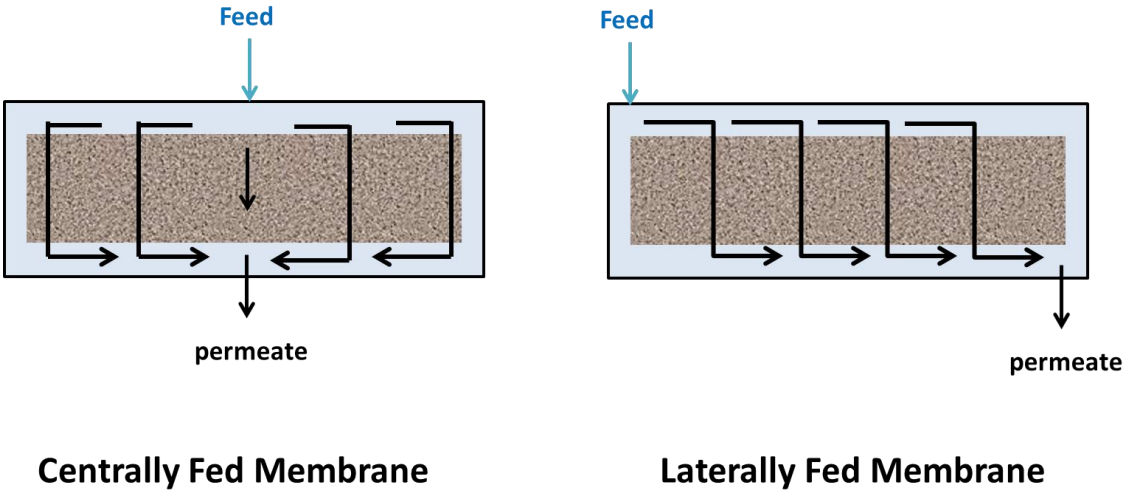


Figure 5 Flow Direction of Membranes Fed Both Centrally and Laterally

Laterally fed membrane chromatography (LFMC) has been designed for separation of complex mixes. In Figure 5 the laterally fed membrane has product pushed from one side of the membrane to the other, the eluent is captured using an additional hydraulic resistant lateral flow channel (Ghosh et al., 2016). This system helps for controlling pressure, unlike the centrally fed membrane which is more likely to become saturated and blocked. The resolution achieved has proven to be a significant improvement in comparison to the centrally fed method (Madadkar et al., 2015) and given similar results to that of a packed resin method (Madadkar & Ghosh, 2016). Anion exchange chromatography PDVF membranes were compared for flow distribution and binding abilities using novel LFMC, to the centrally fed routine method. Both methods were seen to have similar elution profiles, but the novel LFMC peak is sharper and more asymmetric, meaning it gives a superior separation. The broader peak observed from the conventional centrally fed method indicates a more dilute sample. This would result in extra cost for industry, having to add an additional concentration step to the process (Madadkar et al., 2015).

Similar results were seen by Madadkar & Ghosh, (2016) when comparing cation exchange chromatography membranes using lateral and central fed systems, where lateral flow showed a much sharper and asymmetric elution profile of 3 different proteins; lysozyme, conalbumin and ovalbumin. SDS-PAGE analysis revealed that these membranes were successful in separating a multicomponent mixture for each target. It was observed that elution happened earlier using the later flow method, suggestion that this technique not only saves time, footprint and gives greater separation, but can reduce cost of materials, as less buffer is required than traditional methods.

Although membrane chromatography is not implemented in industry currently, small scale studies have shown promising results. The membranes have been shown to be compatible with a wide range of buffers, isolating the product quicker than conventional column chromatography methods and retrieving a high yield of a pure protein (>90%). Tailor made membranes can be made based on the type of separation required. Single use technologies such as membranes provide a huge commercial appeal to industry eliminating the need for cleaning validation, which can take months to carry out and costs large amounts on materials and staffing.

1.1.1 Atom Transfer Radical Polymerization (ATRP)

ATRP is a method for controlled growth of polymeric brushes on surfaces, based on early research into free radical polymerization (RP). RP was shown to have limitations in being able to regulate the structure of polymers, their molecular weight and having high polydispersity (Matyjaszewski & Spanswick, 2005). Controlled radical polymerization (CRP) (Kato et al., 1995) overcame these limitations, allowing for the creation of bioconjugates, both organic and inorganic, with fixed adjoining polymer chains, shown in Figure 6 (Matyjaszewski & Spanswick, 2005). Some structures that can be designed include introducing site-specific functional groups (Coessens, Pintauer, & Matyjaszewski, 2001), multi-star arms and tri polymers (Siegwart, Oh, & Matyjaszewski, 2012). CRP methods include nitroxide mediated polymerization (NMP) (Hawker, Bosman, & Harth, 2001), reversible addition fragmentation chain transfer (RAFT) (Harrisson et al., 2014) and the most popular, atom transfer radical polymerization (ATRP) (Rabea & Zhu, 2015).

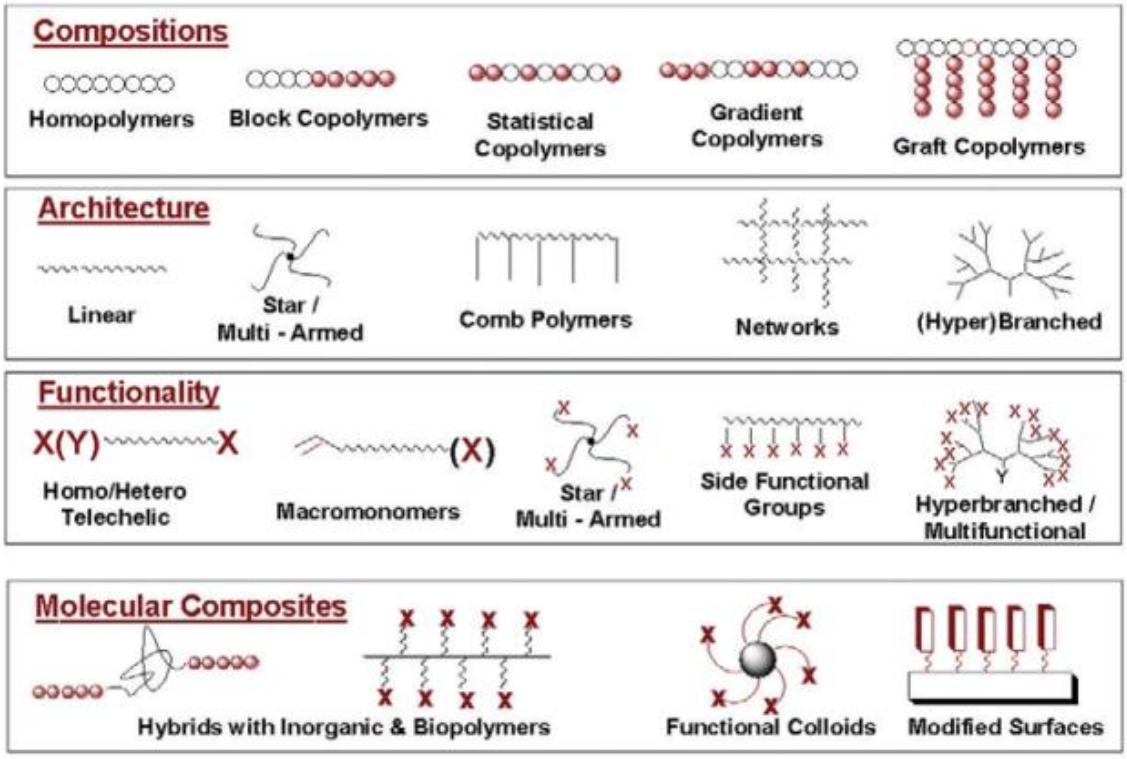


Figure 6 Potential Structure s of Polymers Created by ATRP (Matyjaszewski & Spanswick, 2005)

The primary difference between CRP and RP is that RP allows radical termination indefinitely, in contrast there is only a small fraction of chains terminated in ATRP (~1-

10%) (Matyjaszewski & Spanswick, 2005). For the remaining chains the process is reversible; the chains can be reactivated from a dormant state, therefore ATRP is known as a 'living' radical polymerization method (Goto & Fukuda, 2004). This balance is established by keeping an equilibrium between the active and dormant radicals, in ATRP, the equilibrium is on the left side of the equation (Matyjaszewski & Spanswick, 2005). Figure 7 shows the dormant and active transitions of the polymer chains, the equation is represented by:

P	Polymer
X	Stable free radical
K_{act}	Activation rate constant (transition metal (usually Copper) that drives the activation of polymerization)
K_{deact}	Deactivation rate constant
P ·	Polymer Radical
M	Monomer of choice, for this project tert-Butyl acrylate was chosen
K_p	Propagation rate constant

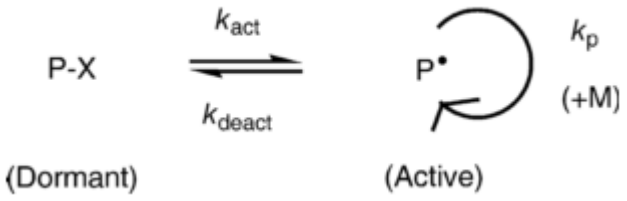


Figure 7 Reactivation of "Living" Chains Produced using ATRP

The controlled polymerization during ATRP is based on two principles; initiation should be instant and provide equal growth of the polymer chains and secondly, the equilibrium must be maintained between the dormant and active radicals, by the formation of a covalent terminal (Xue, He, & Xie, 2015; Coessens et al., 2001). The prompt initiation of polymerization helps to control the degree of polymerization onto a surface, unlike RP, where the initiator step can be unpredictable and slow. During ATRP, an atom transfer step is responsible for the constant growth of the polymer chains. Figure 8 shows the fundamentals of ATRP. To the left of the equilibrium equation, a low transition state

metal/ligand complex (Mt^n/L) reacts with an alkyl halide initiator (P_m –X). P_m-X is split producing a free radical resulting in the metal/halide complex becoming oxidised to a higher oxidation state, pushing the equilibrium to the left side of the equation (k_a), where there is a lower concentration of radicals (Coessens et al., 2001). The free radicals will join the monomer to produce polymer chains (k_p), finally, the reaction ceases following deprotonation (k_t) (Rabea & Zhu, 2015). The transition metal/ligand complex formed (X-M_t^{n+1} /L) can improve the solubility of the complex and enhance the metal's redox reactions (Xue et al., 2015).

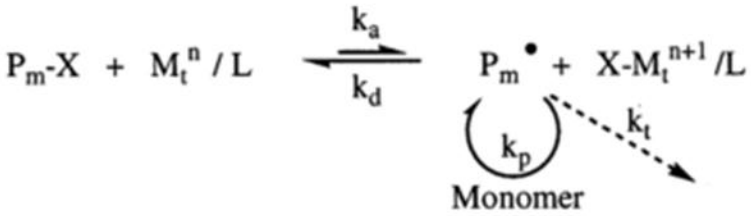


Figure 8 Schematic Overview of ATRP (Coessens et al., 2001)

There is a growing need to find a 'greener' alternative to the transition metals used for ATRP which can be toxic to the environment, including copper(i), copper (ii), iron, niobium and ruthenium (Wang & Matyjaszewski,1995). Substitutes such as cobalt have been studied, but have significantly less control over the polymerization (Hurtgen et al., 2011). ATRP allows for the development of well-structured polymers with low polydispersity and controlled molecular weight (Coessens et al., 2001), which not only helps to improve current polymers on the market, but also assists in creating novel tailor made materials to meet consumer needs. Throughout this project ATRP is utilized for the grafting of polymer brushes onto the surface of membranes used for protein immobilization.

1.1.2 Polymer Brush Grafted Membranes

Polymer brushes are polymer chains that are modified onto a surface at one end and branch out at the other end, due to interactions with its' neighbouring polymer. This feature is what makes them appealing over having a polymer layer, giving the brushes ability to extend and reach more targets which avoids overlapping (Sejoubsari et al., 2016). If solvent is present in their construction, the brushes will separate based on their affinity for the

solvent, if there is no solvent, they will stretch to make space (Milner, 1991). Immobilisation to a solid support can be achieved through a chemical bond or through adsorption. Current biotechnological uses of polymer brushes include nanoparticle drug delivery devices (Liu & Lecommandoux, 2008; Ayres, 2010), protein/enzyme interactions (Feng et al., 2014; Park et al., 2016) and cell culture, where the brushes have induced cell proliferation and differentiation (Villa-Diaz et al., 2010). Polymer brushes are also used in nature to prevent friction between cartilage on various joints (Zappone et al., 2007). Polymer brush membranes can be chemically immobilised using either a Method 1 ("grafting to") or Method 2 ("grafting from"); Method 1 being the most popular (Sejoubsari et al., 2016). Method 1 works by growing polymer bushes on the surface of the support, in contrast, Method 2 uses covalent bond initiators as anchors on the support matrix surface to grow (Ayres, 2010). Initiators improve the grafting density, as the brushes will specifically bind by chemical interactions, without initiators, the polymers may take up too much space, as a result it can block other polymers from attaching (Figure 9) and can coil randomLy (Krishnamoorthy et al., 2014). Polymer brushes can bind their targets through affinity, ion exchange or hydrophobic/hydrophilic interactions (Kawai, Saito, & Lee 2003).

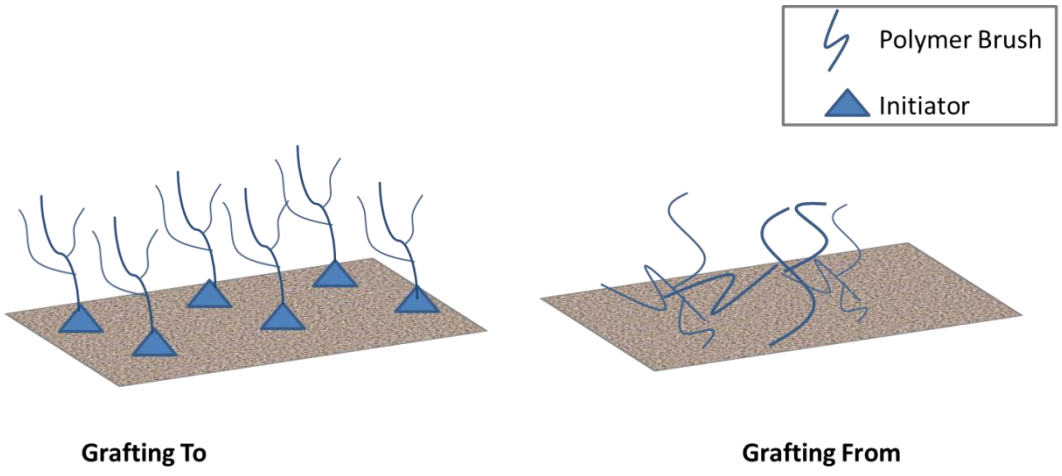


Figure 9 "Grafting to" and "Grafting From" Methods of Polymer Brush Immobilisation

A summary of the publications detailing the production of polymer brush membranes and their targeted modification over the past 10 years are listed in Table 2.

Polymer Brush	Membrane	Target	Bond	Reference
Glycidyl methacrylate	Polyethylene	Lipase Enzymes	Covalent	Goto et al., 2007
Nitrilotriacetate(NTA) poly(acrylic acid)	Silicon	Ribonuclease A	Covalent & Ion Exchange	Cullen et al., 2008
NTA poly(2-(methacryloyloxy) ethyl succinate)	Nylon	Lysozyme Enzyme	Ion Exchange	Jain et al., 2010
Poly(methyl methacrylate)	Polyvinylidene difluoride (PVDF)	Microfiltration	Hydrophilic	Y. Chen et al., 2007
Poly(2-hydroxyethyl methacrylate)	Polyethersulfone (PES)	Ultrafiltration	Hydrophilic	Zhu et al., 2014
Poly(oligo(ethylene glycol) methyl ether methacrylate)	PVDF	Resist Protein Adsorption	Ion Exchange	Chang et al., 2011
Poly(MES)	Nylon	Lysozyme Enzyme	Covalent	Anuraj et al., 2012
Poly 2-hydroxyethyl methacrylate & poly acrylic acid	Cellulose	Bovine Liver Catalase Enzyme	Covalent	Feng et al., 2014
[2-(acryloyloxy)ethyl] trimethyl ammonium chloride (AETMA)	PES	Resist Protein Adsorption	Ion Exchange	Malaisamy et al., 2010

Table 2 Polymer Brush Membranes and their Target Purification

Through affinity binding, this technique can be applied to mimic affinity chromatography. Studies have shown that immobilizing enzymes using polymer brushes can in fact improve their activity over the free form (Goto et al., 2007). One of the most useful applications of polymer brush membranes is their ability to enhance membrane chromatography, by separating targeted proteins from crude lysate. Histidine (His) tags are routinely used in affinity chromatography which has a strong attraction to a Nickel column. Polymer brushes have been utilised to mimic this separation by grafting them onto membrane surfaces. The

two different brushes poly(oligoethylene glycol methacrylate) (POEGMA) and poly(hydroxyethyl methacrylate) (PHEMA), have been successful in selectively binding His-tagged Green Fluorescent Proteins (His-GFP) using the "grafting from" method called ATRP (Gautrot et al., 2010). These polymer brushes are derivatives of Poly(ethylene glycol) which are prone to protein resistance, even after long length of exposures to crude cell lysate solutions (Ma et al., 2006). Modification achieved by introducing nitrilotriacetic acid (NTA) regions onto the brushes gave its selectivity to the His tag. Furthermore, these Green Fluorescent proteins did not lose their activity, demonstrated by fluorescent microscopy, indicating this membrane process could replace traditional Nickel column chromatography. A visual representative of the procedure is shown in Figure 10.

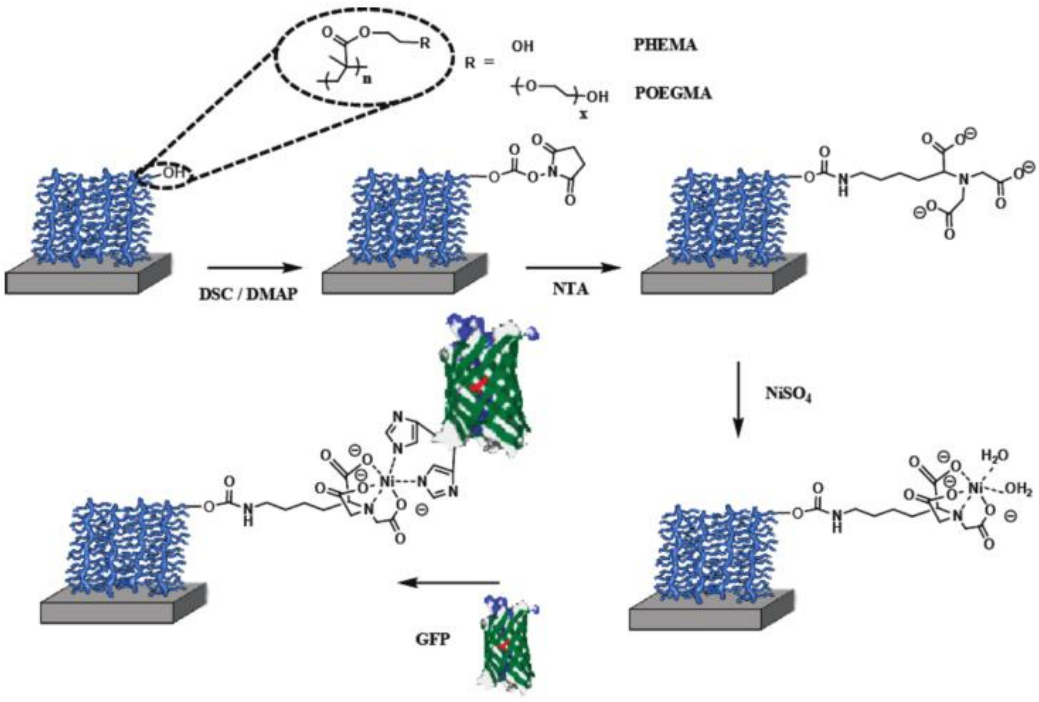


Figure 10 Immobilization of GFP using PHEMA and POEGMA Polymer Brushes (Gautrot et al., 2010)

Disuccinimidyl carbonate (DSC) and 4-(dimethylamino)pyridine (DMAP) are used to activate the polymer brushes, producing the NTA ligands, then treated with a Nickel solution resulting in a Nickel-NTA ligand that can selectively bind His-GFP. Similar studies using POEGMA brushes have been used to purify isolate lysozyme (Yuan et al., 2011) which is an important enzyme for the food preservative and medical treatment

industries (Proctor & Cunningham, 1988), heparin, a blood thinning mAb (F. J. Xu, 2005) and other mAbs (F. J. Xu et al., 2009).

This project is aimed at using polymer brushes for immobilization of Green Fluorescent Protein, which is a commonly used protein to monitor cellular responses. The 3D structure offered by the polymer brushes gives multiple attachment points for GFP. GFP, in turn, is a protein which could be further functionalized with glycoselective ligands which could be produced recombinantly, allowing for the selective separation of glycoproteins, in future work. The functionalised membranes have surface properties that prevent non-specific binding, which is an important tool for future proteomic analysis if studying cellular activity *in situ* using GFP, or in future selective work.

1.2 Membrane Preparation Techniques

There are four different procedures that can be used to prepare UF membranes; Non-Solvent Induced Phase Separation (NIPS) which involves precipitating the polymer in a non-solvent coagulation bath, the Thermally Induced Phase Separation (TIPS) method includes a cooling process, the Evaporation Induced Phase separation (EIPS) methods relies on an evaporation step and the Vapour Induced Phase Separation (VIPS) consists of a vapour induced separation process (Ulbricht, 2006). The NIPs Method was employed in this study.

1.2.1 Non-Solvent Induced Phase Separation (NIPS) Method

The non-solvent induced phase separation method (NIPS) is the most popular method used to prepare filtration membranes (Wang, et al., 2013). This method involves the formation of membranes by placing a layer of prepared polymer-solvent solution onto a solid support, such as a glass plate (Cheng et al., 1999) and immersing the plate into a coagulation bath (Karimi et al., 2015) which is illustrated in Figure 11. This technique relies on an exchange between the solvent and non-solvent in order to solidify, therefore the 2 components must be miscible (Guillen et al., 2011). The main features that contribute to membrane morphology during the NIPS preparation method will be discussed in the following sections.

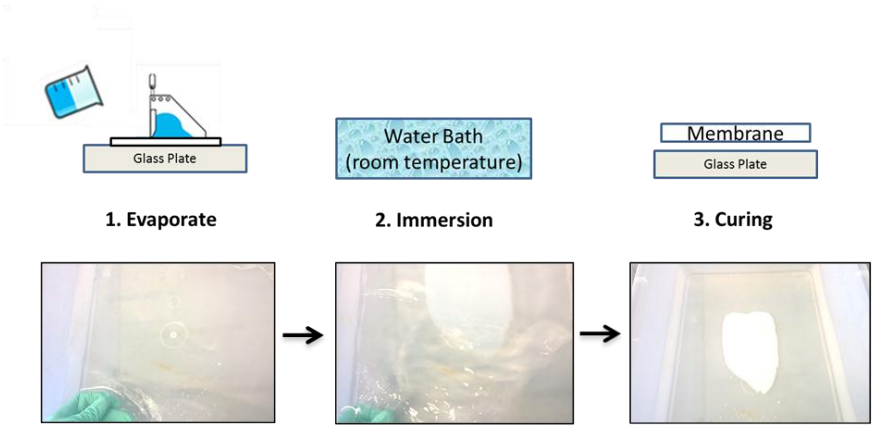


Figure 11: ***Steps involved casting membranes using the NIPS method***

1.2.2 The Polymer and the Solvent

Choosing a polymer to work with in the NIPS method will affect what solvent and non-solvents can be used during the casting procedure, the polymer should be soluble in the chosen solvent and this solvent needs to be miscible with the non-solvent used during the casting procedure (Guillen et al., 2011). Recommended solvents to be used when casting polysulfone membranes include dimethylformamide (DMF), dimethylacetamide (DMAc), dimethylsulfoxide (DMSO) and N-methylpyrrolidone (NMP) (Guillen et al., 2011). Methods developed to calculate the solubility parameters of the polymer include the Flory interaction parameter, which takes into account the molar volume of the solvent, the temperature and the volume fraction of polymers (Miquelard-Garnier et al., 2016) and the Scatchard and Hildebrand equation examines the energy of vaporization (Koenhen, 1975). A variety of different polymers are readily available for membrane casting, based on the desired material properties. Choosing the right material is important because these polymers will influence properties of the membrane such as its separation capabilities, its' mechanical features and its structure (Guillen et al., 2011). Membranes used for gas separation include polysulfone (Rafiq et al., 2011), Polyetherketone (Ying & Yang, 2002), polyurethane/poly(ether-block-amide) (Mozaffari et al., 2017) and Polybenzoxazole (Kushwaha et al., 2017). In ultrafiltration (UF) systems polymer membranes made of poly(vinylidene fluoride) (PVDF) (Nguyen et al., 2013), polyethersulfone (PES) (Zhu et al., 2014), polyimide (Wienk et al., 1996) and polysulfone (Nguyen et al., 2016a) are utilised. Furthermore for protein separations, polymers such as cellulose (Liu, et al., 2014),

polyethylene (Goto et al., 2007), PVFD, (Chang et al., 2011) and PES (Malaisamy et al., 2010) are commonly chosen. Polysulfone is the most popular polymer used for the NIPS method, due to ease in accessibility, simplicity to process and desirable stability properties (Guillen et al., 2011). The right concentration of polymer is essential in membrane preparation, as too high a concentration can negatively impact the membrane performance by creating membranes with little or no pores, resulting in a dramatic decrease in flux, sometimes to zero (Wijmans et al., 1985). Cellulose polymers are also common in membrane preparation because of their attractive features such as pore forming abilities, low fouling capabilities, high salt rejection properties and are inexpensive and easily accessible (Worthley et al., 2011). Polymers are often mixed with others to create a polymer blend that will enhance the membrane characteristics such as thickness and density, some blends include Chitosan/Carboxymethylcellulose (Chen, 2005), polysulfone (PSf)/poly(ethylene glycol) (PEG) (Park, et al., 2006) and polysulfone/polyimide (Rafiq et al., 2011).

1.2.3 The Composition of the Coagulation Bath

The membrane structure is influenced by the components of the coagulation bath during the NIPS casting method. The miscibility between the solvent and non-solvent affects the precipitation of the membrane and contributes to its' morphology and is therefore an important parameter to consider for optimizing the preparation method (Rafiq et al., 2011). Often water is used as the non-solvent. Rafiq et al., (2011) created a blend of asymmetric polysulfone/polyimide (PSF/PI) membranes by immersing membranes with different ratios (80:20, 50:50 and 20:80) of N-methyl-2-pyrrolidone/dichloromethane (NMP/DCM) (solvent) and ethanol (non-solvent). The impact to the membrane's physical characteristics was assessed using Scanning Electron Microscopy (SEM). SEM images revealed all membranes had a homogenous surface which was non porous, while the cross sections pictures revealed a porous infrastructure. The 20:80 NMP/DCM blend membrane had a thin skin with smaller pores throughout, in comparison to the other two forms. The solubility parameters of the membrane mixture in the coagulation bath were also affected by the different NMP/DCM mixtures. The 20:80 NMP/DCM had the highest solubility parameter, next to 50:50, then 80:20, meaning the solvents are miscible and less energy is required to evaporate the solvent, making the casting procedure less time consuming. Chun

et al., (2000) observed how pore formation was affected in asymmetric polyimide membranes by alternating the amount of N,N'-Dimethylacetamid (DMAc) solvent and non-solvent (water) to the coagulation bath, using the NIPS casting method. Scanning Electron Microscopy (SEM) revealed that an increase in solvent to non-solvent ratio created less porous sub-structures forming on the membrane. This was explained as being caused by diffusion exchange between the solvent and non-solvent in the precipitation bath, which impacts the polymer solution's phase inversion, therefore the resulting membrane's physical features. A relationship between the pore formation and coagulation was determined when varying the amount of solvent in non-solvent. The results reveal as pore formation increases, the coagulation value decreases, suggesting that there is an affinity between the solvent and non-solvent. A higher porosity is achieved when the coagulant is easier to infiltrate the polymer solution (Chun et al., 2000).

1.2.4 Additives

During membrane preparation, important consideration should be put into what additives are required to obtain the desired structure. Additives can positively influence properties such as the porosity, pore connectivity, hydrophobicity and create sponge like morphology structures (Guillen et al., 2011). The most popular additive used is polyvinylpyrrolidone (PVP), which promotes a sponge like morphology, preventing macrovoids (Smolders, et al., 1992), increases porosity and provides well interconnected pores explained as being due to PVP walls that breakdown after the membranes are dried (Wienk et al., 1996) . This is a water soluble hydrophilic additive often used for preparing polyethersulfone (PES), polysulfone (PSF) or polyetherimide (PEI) membranes (Wienk et al., 1996). Hyun et al., (2004) examined the effects on how PVP of different molecular weights impacted the polyimide (PI) membrane morphology. Without the PVP additive, a macrovoid "finger like" structure was acquired; in contrast the addition of PVP caused a sponge like structure to be obtained. The amount of PVP added to the system, directly impacted the amount of macrovoid structure. Scanning Electron Microscopy results reveal the macrovoid appearance decrease as the concentration of PVP increased; furthermore the higher molecular weight PVP (360kDa) membranes resulted in a decrease in macrovoid formation in comparison to the lower PVP value (40kDa), and the pores were more interconnected. The change was explained as being a consequence of the solution viscosity which was

enhanced by the addition of PVP. These were important studies in membrane research because macrovoid structures can cause the membrane to collapse under high flux and makes them unsuitable for some separation procedures, however they are suitable for ultrafiltration processes, acting as structural support (Hyun et al., 2004) . Other studies examining the effects of PVP on morphology of polysulfone membranes revealed that this additive also affected the membrane's flux; the higher the molecular weight of PVP added, the higher the porosity and pore area, resulting in a higher flux (Chakrabarty, et al., 2008). Other examples of additives include Polyethylene glycol (PEG) which promotes pore formation and improves hydrophilic properties and propionic acid (PI) which enhances the membranes permeability when used in gas separation up to ten fold (Guillen et al., 2011). By carefully considering the right polymer, solvent, non-solvents and immersion bath additives, membranes can be tailored to specific morphologies and enhanced stability to achieve the best results when used in filtration systems.

1.3 Enzyme Immobilised Membranes

Enzymes are derived from living organisms and used to carry out metabolic reactions within the cell. They are biological catalysts that speed up a chemical reaction without being used up themselves and their ability to carry out specific chemical transformations has made them popular for use in a variety of industrial applications in areas such as food, textile, paper, therapy, cosmetic and diagnostics (Li et al., 2012). The global industrial enzyme market for the food and beverage sector was worth $1.3 billion in 2011, which is estimated to grow to $2.1 billion by 2016, technical enzymes were valued at $1.2 billion in 2011, which is projected to rise to $2.2 billion by the end of 2016, these two sectors take up the majority of the market, with other enzyme sectors being valued at $1.5 billion in 2011 and expected to rise to $2.2 billion by 2011 (Prakash et al., 2013). Other favourable properties of enzymes include their ability to work rapidly in mild temperature and pH ranges even at a low concentration, they have low toxicity and are relatively easy to terminate reactions (Jegannathan & Nielsen, 2013). However drawbacks in applications include their limitability of activity in a wide range of pH and temperature ranges that industrial process commonly used and retrieving the enzyme at the end of a chemical

reaction is problematic if it is not immobilised onto a solid support. Being able to reuse the enzyme saves on cost, these immobilised enzymes hold other advantages such as creating an enzyme free product, reducing the need to carry out an additional separation step; they are also suitable for continuous processes and can improve the enzyme stability making immobilised enzymes preferable over the free form equivalents. Various approaches used for enzyme immobilisation include physical methods such as entrapment where the enzyme is trapped within a porous solid matrix such as gelatine, and encapsulation technique where the enzyme is surrounded by a porous insoluble material such as a calcium alginate (Sánchez & Demain, 2011). Immobilization using chemical methods include adsorption, which relies on ion interactions and other attractions between the enzyme and support material; covalent binding, which is used when the enzyme's functional groups (nucleophiles) bind to reactive groups on the support's surface; and self-immobilization, which is a technique in which the enzymes bind to each other forming covalent bonds (Fernández-Fernández, Sanromán, & Moldes, 2013)(Figure 12).

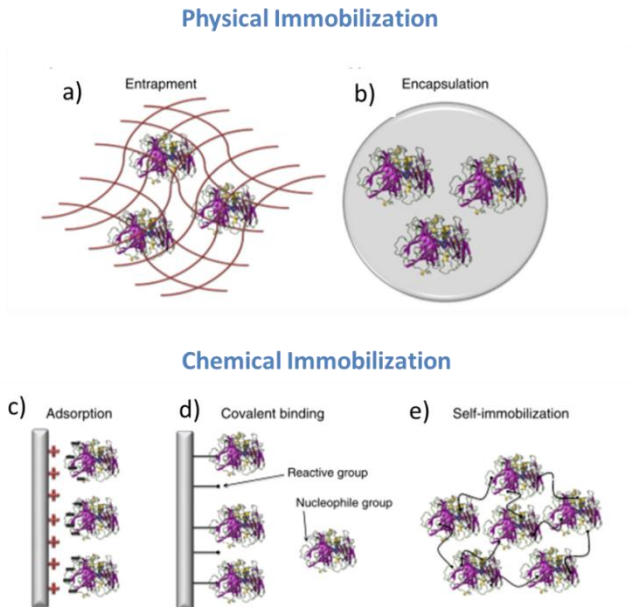


Figure 12 Enzyme Immobilization Methods; Physical and Chemical (Fernández-Fernández et al., 2013)

Enzyme immobilised membranes play an important role in environmental applications for removal of micro pollutants. Effort is being invested by researchers to create such membranes as pre-treatment mechanisms for water treatment plants as an alternative to

current chemical catalyst strategies, due to their high specificity and ability to operate in a range of temperatures, pHs, and salinity, even in toxic compounds (Escalona et al., 2014). Both nano-filtration (NF) and ultra-filtration (UF) membranes have the potential to be used for water treatment due to their ability to separate based on size, adsorption and electrostatic repulsion (Taheran et al., 2016). UF membranes have been shown to retain 100% of biomass, demonstrating that it can retain suspended components, whereas NF membranes will reject recalcitrant organics and allow salts and other biological degradable substances to pass, which have been redirected into an activated sludge bioreactor allowing for further decomposition of the waste material (Venugopal & Dharmalingam, 2012).

Bisphenol A (BPA) an endocrine disrupting compound, is a micro-pollutant that is commonly found in industrial waste water and therefore demands a fast and effective detection method (Fauzan, Omar, Ahmad, & Zaharin, 2016). It is a prototypical non-steroidal estrogen that interferes with estrogen and thyroid hormones production (Ghisari & Bonefeld-jorgensen, 2005), affecting the central nervous system. This was demonstrated in studies where mice exposed to BPA throughout their embryonic life had underdeveloped brains, testes and kidneys due to induced oxidative stress (Kabuto, Amakawa, & Shishibori, 2004) and also affects the immune system by augmenting T cells (Alizadeh et al., 2006). BPA removal from an aqueous system was investigated using the encapsulation of laccase enzyme onto electrospun fibrous membranes. The immobilised enzyme was shown to have enhanced stability and 85% higher activity recovery in comparison to the free enzyme form. Furthermore, the immobilised enzyme was successful in removing BPA over a wide range of temperatures and pHs; 35 ^{o}C to 45 ^{o}C was the optimal range for free laccase, with no activity observed at 5 ^{o}C, however the immobilised enzyme was capable of degrading 70% BPA at this temperature and had a high removal rate at pH 4-7, in comparison to the free form, with highest degradation at pH 5 (Dai et al., 2016).

Tyrosine enzymes immobilised onto membranes have shown promising results in the removal of phenolic compounds. This enzyme was immobilised onto polyethersulphone capillary membranes by crosslinking with glutaraldehyde as a matrix and examined for breakdown of p-cresol in waste water. P-Cresol is an organic compound commonly found in waste water from industrial plants (Balachandran et al., 2016). The concentration of p-

cresol was decreased using the polyethersulphone capillary membranes with immobilised tyrosine in a bioreactor system making it a possible treatment strategy for the breakdown of water polluted with phenols. Burton et al., (1998) immobilised tyrosine onto nylon membranes using covalent bonding and polysulfone capillary membranes by adsorption. These high flux membranes were placed into bioreactors and tested for the breakdown of phenols including p-cresols and methoxyphenol. HPLC analysis revealed that immobilization of enzymes onto the nylon membranes results in faster breakdown of phenol contaminants in comparison to the free enzyme; furthermore the polyethersulphone capillary membranes resulted in increased breakdown of phenol compounds before deactivation demonstrating that the enzyme's support enhanced its stability demonstrating that immobilization of enzymes have many benefits when being used for application such as water treatment (Burton et al., 1998).

1.3.1 Diclofenac

Diclofenac (DCF) is a non-steroidal anti-inflammatory drug (NSAID) that is commonly prescribed in veterinary medicine and used to treat inflammatory and pain diseases such as arthritis, gout and dysmenstruation (Roh et al., 2010; J.-B. Chen et al., 2014). It is considered one of the most dangerous environmental contaminants, due to its detrimental effects in the food chain to non-targeted organisms (Sathishkumar et al., 2012). Diclofenac has been found in surface water, drinking water and ground water in ng/L to µg/L worldwide (Chen et al., 2014). The annual global consumption of this drug is 940 tonnes, some of this drug is not metabolised fully and excreted as unchanged compounds, which is discarded to waste water treatment plants (WWTPs) (Zhang et al., 2008). The increased production and consumption of these pharmaceuticals is a worrying concern for environmental pollution. There are many ways which drugs such as diclofenac can enter the environment such as hospitals, unused medicine and excretion, however the most common source is waste water treatment plant discharges (Dular et al., 2015).

The inefficient treatment of pharmaceutical waste has led to micropollutants such as Diclofenac to contaminate water supplies (Bonvin et al, 2015). Swiss authorities have recently introduced new regulations to upgrade waste water treatment plants that require extra downstream processing steps aimed at reducing the discharge of micro pollutants

(MP) to aquatic environments (Eggen et al, 2014). This has set an example for other countries to follow suit, creating a demand for ecological and cost effective strategies to remove MPs and improve water quality. A promising approach has been tested in large scale and can remove up to 80% of MPs using powdered activated carbon (PAC) and sand or ultra-filtration (Margot et al., 2016). This process is desirable due to its high percentage removal capability, without the production of potentially toxic by-products, which has been seen in other techniques. However, it is expensive due to the amount of raw materials and energy required to carry out the procedure, leading to the need to discover other cost-effective technologies. In 2001 the European Commission added three new pharmaceuticals to the EU Water Framework Directive watch list including Diclofenac, 17a-ethinylestradiol and 17b-estradiol. Diclofenac is resistant to current strategies for drinking and wastewater treatment, consequently there is a need to develop new strategies for its complete removal in waste water treatment plants (Dular et al., 2015).

1.3.2 Current Strategies for Diclofenac Removal

A major drawback in drinking and wastewater treatment strategies is the wide range in efficiencies observed in removal of pharmaceuticals (Nguyen et al., 2016). Adsorption by activated carbon (AC) is a commonly employed method that has been studied for the removal of pharmaceuticals (Calo, et al., 2004). Activated carbon (AC) production requires two steps; pyrolysis, in which the carbon material is converted to carbonized material (Yusof et al., 2012) and an activation process which increases its surface area (Jodeh, et al., 2015). The adsorption ability of activated carbon (AC) is due to its pore size and carbon source; its adsorption ability is high when its pore size matches or exceeds the size of its target (Huang, et al., 2014). Removal of diclofenac by AC in single component studies has been shown to be effective (e.g. Figure 13), however removal from real drinking and wastewater treatment sources can be hampered by competitive adsorption (Matsui et al., 2012; Sch et al., 2002).

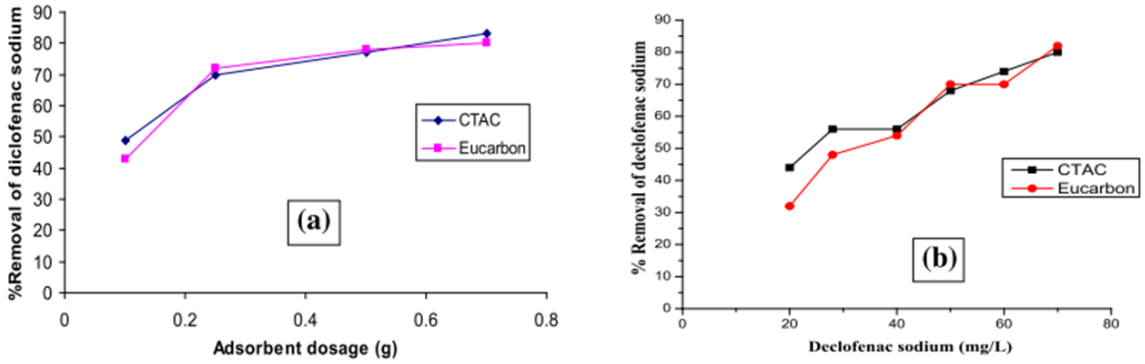


Figure 13: Effect of AC from *Cyclamen persicum* tubers and Eucarbon on the removal of diclofenac at 25°C, pH4; (a) adsorbent quantity (b) diclofenac sodium of varying concentrations (Jodeh et al., 2015)

Other novel approaches have been investigated, including the use of techniques such as hydrodynamic cavitation. Studies reveal that up to 79% of Diclofenac was removed from wastewater effluent after 30 min at 50 ^{o}C using 3.4 g/L Hydrogen peroxide (H_2O_2), alongside the removal of other pharmaceuticals (Table 3). Although effective, this method requires the addition of a toxic oxidizing agent Hydrogen peroxide (H_2O_2) which will need to be removed from the water in another treatment process, adding extra expenses to the procedure, along with the energy costs of carrying out the procedure at 50 ^{o}C.

Table 3 Removal of pharmaceuticals from waste water effluent using hydrodynamic cavitation at varying concentrations of H_2O_2 at the optimal operational temperature of 50°C (Adapted from Dular et al., 2015)

Time (min)	15	15	30
Hydrogen peroxide (g/L)	**0.34**	**3.4**	**3.4**
		% Removal	
Diclofenac	26	45	79
Naproxen	40	44	74
Carbamazepine	1	24	62
Ketoprofen	2	24	55
Ibuprofen	0	28	54
Clofibric Acid	4	15	37

A biological alternative to eliminate specific micro pollutants is the use of enzymes. Advantages they hold over other common treatments include their ability to operate at a range of temperatures, pH and salinity (Durán & Esposito, 2000).

1.3.3 Laccase Enzymes for Breakdown of Pharmaceutical Waste

Laccase enzymes are multi-copper oxidases that oxidise a range of aromatic compounds such as phenols, reducing oxygen to water (Ramírez-Cavazosa et al., 2014) making them suitable for diclofenac degradation. The oxidation of phenolic compounds by laccase is illustrated in Figure 14, these enzymes are well studied in industrial applications and have been used for the removal of dyes and textile effluent (Zapata-Castillo, 2012), baking to improve dough quality by crosslinking biopolymers (Renzetti, et al., 2010), removing oxygen at the end of beer processing to increase shelf life and for improving the flavour of oils such as soybean, by means of deoxygenation (Osma, Toca-Herrera, & Rodríguez-Couto, 2010). The major setback of laccase applications is industry is the large amounts

required to meet industrial needs, only in 1996 was the first industrial preparation of laccase introduced – DeniLite® by Novaenzyme (Alessandra, 2010).

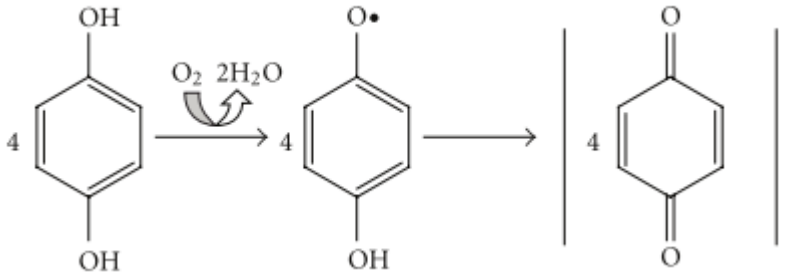


Figure 14: Laccase oxidation of phenolic compounds (Osma et al., 2010)

Laccase can oxidize a range of phenols with higher E_o rate then it's own, but some kinetics are unfavorable for laccase to carry out oxidation (Xu et al., 2000), therefore a redox mediator is often applied to broaden the substrate of laccase which facilitates as an electron carrier. An example of a synthetic mediator is 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and a natural one is P-coumaric acid (Nguyen, et al., 2016). However, studies reveal that mediators such as syringaldehyde (SA) which encase laccase activity may also have toxic effects; this should be monitored and taken into consideration during experimental design. There is a growing interest in its application towards the biodegradation of trace organic contaminants (TrOCs) such as Diclofenac in recent years with much success (Nguyen et al., 2013).

Free laccase has been tested for the degradation of anti-inflammatory drugs such as diclofenac, naproxen and ketoprofen collected from two water samples in Turin municipal waste water treatment plant Italy, at different times of the year (Spina et al., 2013). Laccases of *T. pubescens* MUT 2400 was capable of degrading over 70% of diclofenac and all other anti- inflammatory drugs tested (Figure 15). However, the enzymes activity decreased dramatically by 60% after 24hrs. The real water samples are said to have many factors that can interfere with the enzymes activity, such as reducing anions, salts, temperature, organic solvents and proteolytic enzymes (Cabana et al., 2007; Spina et al., 2013). Further strategies are needed to improve the robustness of enzymes, such as immobilisation onto a solid support. Other studies such as Tran et al., (2010) degraded

100% of diclofenac using crude and commercial laccase after 48hrs. Their studies revealed that increasing laccase activity lead to an increase in detrition of pharmaceutical compounds.

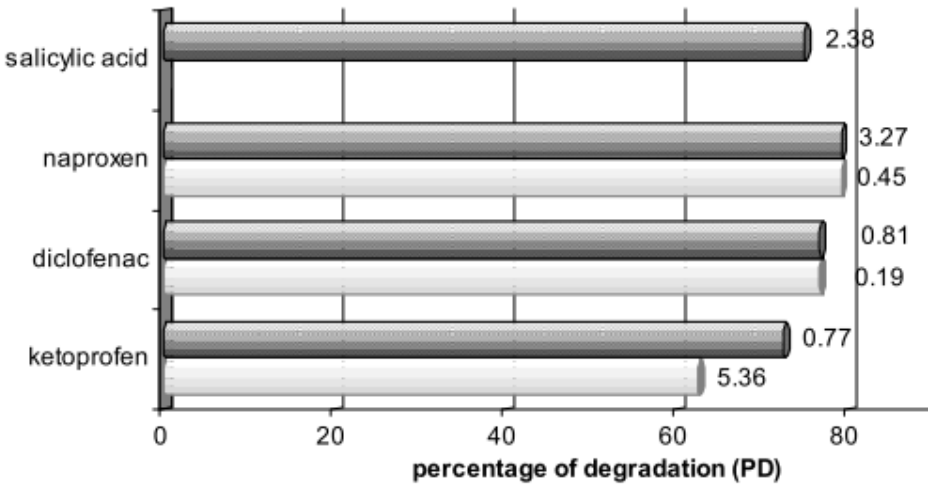


Figure 15: The Percentage of Degradation (PD) of a range of anti-inflammatory drugs after treatment with laccase at 100U/L in two different water samples (white and grey) (Spina et al., 2013)

Immobilised enzymes have distinct advantages over the free form such as being retrievable from the reaction, making the enzymes reusable, reducing the need for an additional purification step; also making them economical. Immobilised enzymes generally have better thermal, pH and operational stability (Georgiou et al., 2016; Sathishkumar et al., 2014) . Furthermore they are less probable to denature in comparison to its free state. Immobilised laccase has been used for applications such as degrading dyes (Sathishkumar et al., 2014; Ramírez-Montoy, et al., 2015), baker's yeast effluents and simulated molasses wastewaters (Georgiou et al., 2016), removing 96% of the micro pollutants (MP) Carbamazepine (CBZ) from wastewater effluents (Ji, et al., 2016).

Only a small number of studies have examined the immobilisation of laccase onto membrane supports (Escalona, et al., 2014; Ba, et al., 2014). Enzymatic membrane reactor (EMR) is the name given to bioreactors in which membrane immobilised enzymes are applied. Laccase was immobilised onto polyacrylonitrile membranes, with an activity of 180$\mu M_{(DMP)}$/ min in a bioreactor with a flux value of 1.1 L/m^2/Hr, then examined against a wide range of trace organic contaminants (TrOCs) as seen in Figure 16. The results show

that the addition of 10µM syringaldehyde (SA) to the EMR reaction increased the degradation of Diclofenac (5 µg/L) by 20-35% alongside other trace organic contaminants (TrOCs) such as metronidazole and gemfibrozil (Nguyen, et al., 2016).

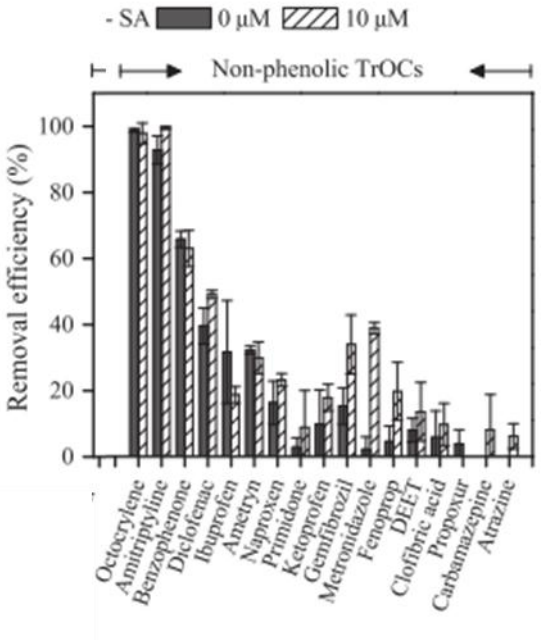


Figure 16: The removal of trace organic contaminants TrOCs (5µg/L) using membrane immobilised laccase at 0 µM and 10µM SA (Nguyen, et al., 2016)

Further tests increased the concentration of syringaldehyde (SA) mediator from 50µg to 100µg, which in some cases decreased the removal of trace organic contaminants TrOCs (100µg/L) from the EMR e.g. Benzophenone and Ametryn (Figure 17). This was explained as being due to the mediator concentration reaching a saturation point where no more compound removal can be achieved and is the first time this situation has appeared in EMR in the literature (Nguyen, et al., 2016).

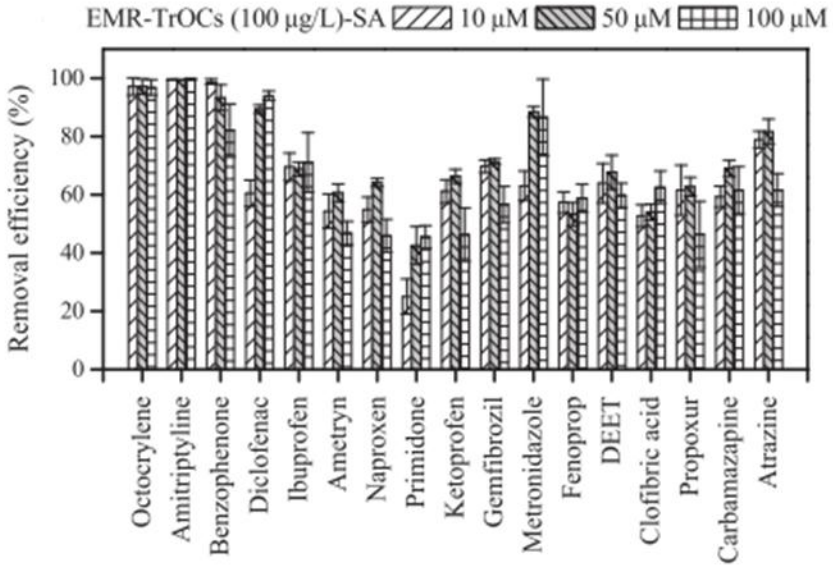


Figure 17: Effects of trace organic contaminants (TrOCs) (100µg/mL) removal by varying syringaldehyde (SA) concentration (Nguyen, et al., 2016)

The toxicity effect on using different concentration of syringaldehyde (SA) in the influent and effluent was also examined, which is crucial to test if this technique is to be applied in waste water treatment plants because toxicity levels need to be monitored and eliminated before the water is released back into the environment. An increased concentration of syringaldehyde (SA) decreased the toxicity of the influent in comparison for the 10 µM and 50µM samples, using the EMR system developed. There was however an increase in effluent toxicity in comparison to the influent after the addition of 100µM syringaldehyde (SA). This is due to the excess mediator and reactive radicals created after laccase oxidation that permeate through the membrane, increasing the toxicity levels (Nguyen, et al., 2016). Figure 18 illustrates the least toxic conditions for the removal of trace organic contaminant (TrOCs) such as Diclofenac (100µg/L), which is achieved by using 10µM syringaldehyde (SA) to obtain ~60% removal. An increase in syringaldehyde (SA) to 50 µM removes ~90% trace organic contaminant, with a low toxicity consequence (7 rTu). The highest concentration of syringaldehyde (SA) (100 µM) caused the maximum toxicity level of 20 rTu. Ideally, the least toxic effects (if any) would be the most desirable if this was to be applied in drinking or wastewater treatment plants.

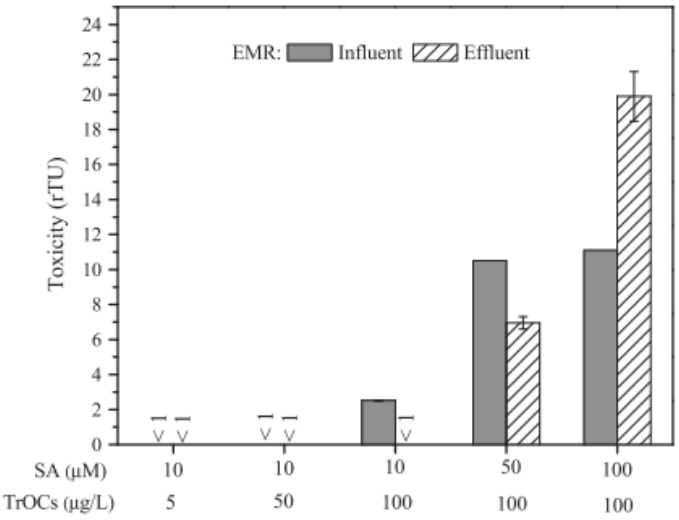


Figure 18: Toxicity effects on EMR by altering concentrations of syringaldehyde (SA) and TrOCs (Nguyen, et al., 2016)

Laccase from *Trametes versicolor* was immobilised onto nylon membranes and applied in a non-isothermal bioreactor, to treat olive oil mill wastewater (OMW) effluent, composed of ~10% toxic organic matter, containing phenol groups (Attanasio et al., 2005). OMW has a negative effect on the soil characteristics, such as the porosity and humus reduction, which is responsible for contributing nutrients to the soil (Attanasio et al., 2005). Glycidyl methacrylate (GMA) monomer was grafted onto the surface, followed by 7.1 $x10^3$ mg of laccase per mg of nylon membrane (Attanasio et al., 2005). It was found that the immobilised form maintained much of its activity in acid pH and high temperatures above 60 ^oC. In contrast, the soluble form had little or no activity during these harsh conditions, making it a sustainable approach for the treatment of oil mill wastewater treatment plants. Eight different batches of olive oil waste water were treated with immobilised *Lentinula edodes* laccase onto Eupergit C, a non-porus hydrophilic acrylic bead by D'Annibale et al., (2000). The immobilised laccase was found to be highly effective, removing up to 70% of the phenol groups (Figure 19). In comparison to studies of soluble laccase, the immobilised form had a higher pH, thermal and proteolytic stability, and most importantly, it's optimum pH activity was at 7, which is similar to the natural pH of water, making this laccase a very attractive approach for the treatment of phenols in waste water plants (D'Annibale et al., 2000). Similar studies carried out by Casa et al., (2003) obtained 65% and 85% reduction in total phenol when treating OMW with soluble laccase.

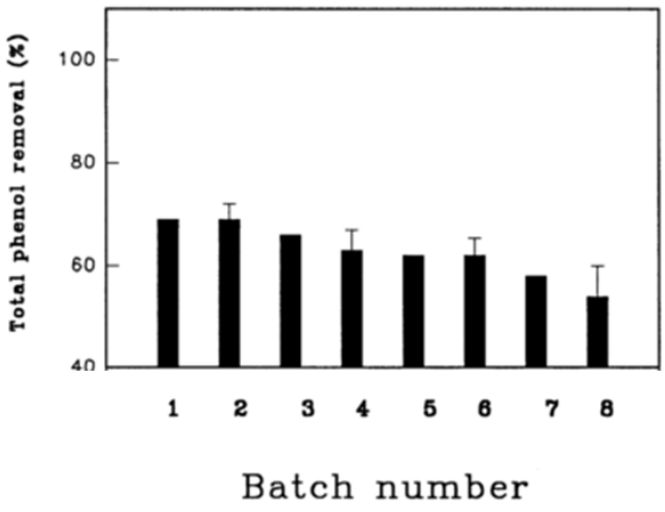


Figure 19: Removal of total phenols from eight batches of oil mill wastewater using immobilised laccase on Eupergit® (D'Annibale et al., 2000)

In recent years Laccase immobilisation studies have demonstrated that it has an enhanced stability in comparison to the soluble form, throughout a wide range of pH and temperatures. The activity of laccase as a function of pH and temperature was examined, following immobilisation of laccase; its optimum pH range (activity over 90%) has doubled in comparison to its soluble form (Figure 20). The soluble form has a pH optimum of 3.7-4.3, in contrast the insoluble form has a pH optimum has extended is range of being between 4.3-5.6 (Figure 20a). Furthermore, a significant reduction in activity is seen at pH 3 with the free laccase only having 15 % activity, in comparison to the catalytic membrane maintaining 65 % laccase activity. Upon examination of the isothermal characterisation (Figure 20b), the optimum range of the soluble form is 50 ^{o}C, while the insoluble form has a higher temperature optimum of 60 ^{o}C before any reduction in activity is observed, this is due to its support material providing a protective barrier and maintaining the enzyme structure; an advantage immobilised enzymes hold over their free form (Attanasio et al., 2005). These results suggest that the catalytic membranes prepared would have to be used under high temperature conditions in a non-isothermal bioreactor.

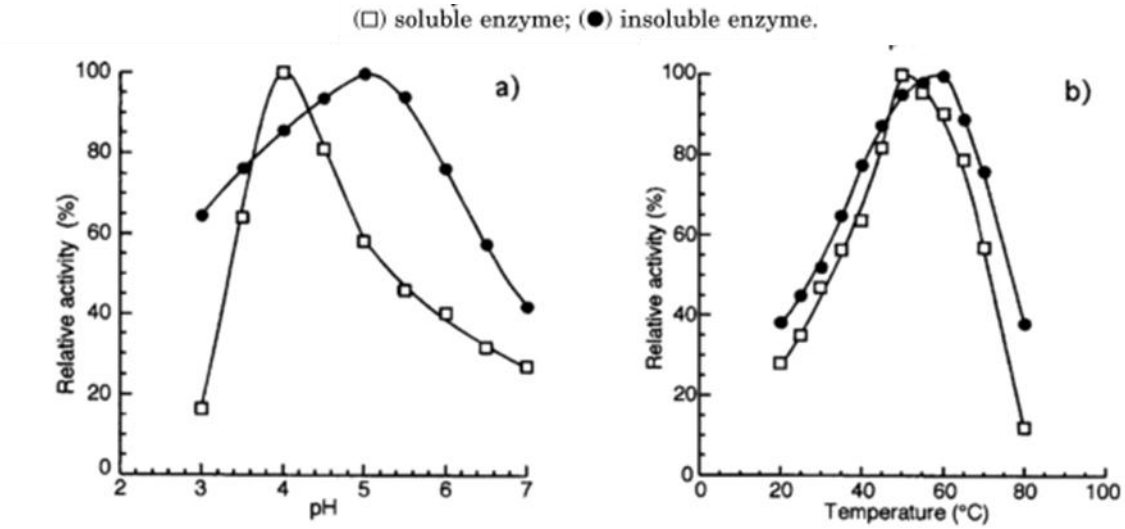


Figure 20: Effect of a) pH at 25oC and b) Temperature on Laccase Activity Immobilised and Free (Attanasio et al., 2005)

Two different sources of Laccase (*M. thermophila* and *T. hirsute*) were immobilised onto mesoporous silicate (MPS) particles, which are spherical shaped porous inorganic solids by Forde et al. (2010). Thermal profiles revealed that both enzymes could withstand temperatures of up to 75 ^{o}C before inactivation; furthermore, *T. hirsute* laccase had doubled its activity at 75 ^{o}C in comparison to how it performed at 25 ^{o}C. Treatment of citraconic anhydride to immobilise M. *thermophila* laccase resulted in a 10 fold increase in its half-life in comparison to the unmodified soluble form, moreover treatment with bio-functional reagents ethylene-glycol-N-hydroxy succinimide (EGNHS) and glutaraldehyde improved the immobilised laccase stability 6 and 8.7 fold (Forde et al., 2010). Other studies have invested efforts into immobilizing laccase from *Coriolopsis gallica* onto mesoporous silicate (MPS) such as Nair et al., (2013). The thermal stability of this insoluble enzyme was examined against the soluble form, and as seen before, its' stability increased following immobilization (Figure 21). At the higher temperature ranges of 65-75 ^{o}C the native laccase has half-lives of 2hrs and 0.6hrs, in contrast the immobilised form having 8.3 and 3.9 correspondingly (Figure 21). This is an important feature because the improved stability will contribute to a reduction in industry costs if the enzyme activity is improved, in addition, high temperatures are often used in bioreactors to improve the productivity (Liang et al., 2012).

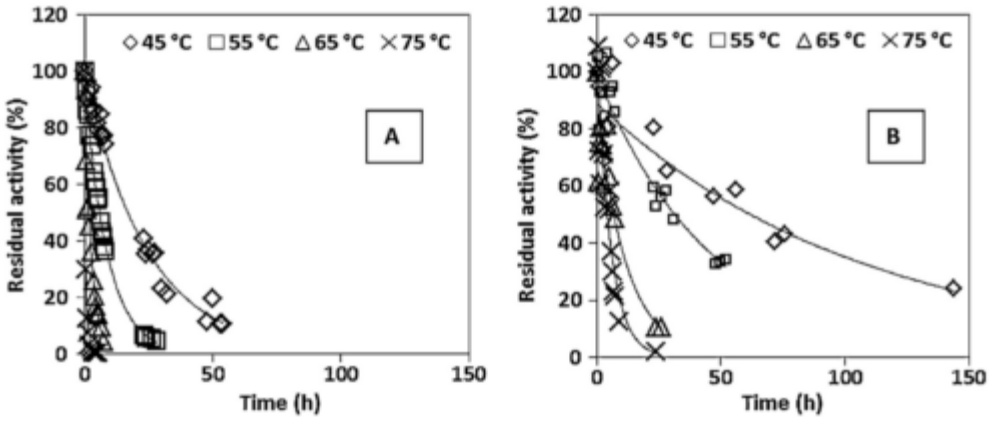


Figure 21: Thermal profiles of a) free and b) immobilised laccase at various temperatures (Nair et al., 2013)

Laccase immobilised mesoporous silicate (MPS) was tested in a membrane bioreactor (MBR) using real waste water containing Diclofenac (DCF), 17-a-ethinylestradiol (EE2) and bisphenol A (BPA). 85% of the EE2 and BPA were degraded, alongside 30% of the DCF (Figure 22). The lower rate of DCF removal was explained as being due to various factors such as the presence of organic matter in the sample and the fact that this drug is known to degrade best at pH 7, whereas pH 4.5 was used in this case (Nair et al., 2013). As mentioned previously, there are many contributing factors that may affect this breakdown using real water samples. Almost no activity was lost throughout all reactor conditions, in comparison to work carried out by Auriol et al. (2008) which resulted in a loss of 97-98% activity treating municipal wastewater, making this method a feasible one to be applied for waste water treatment applications.

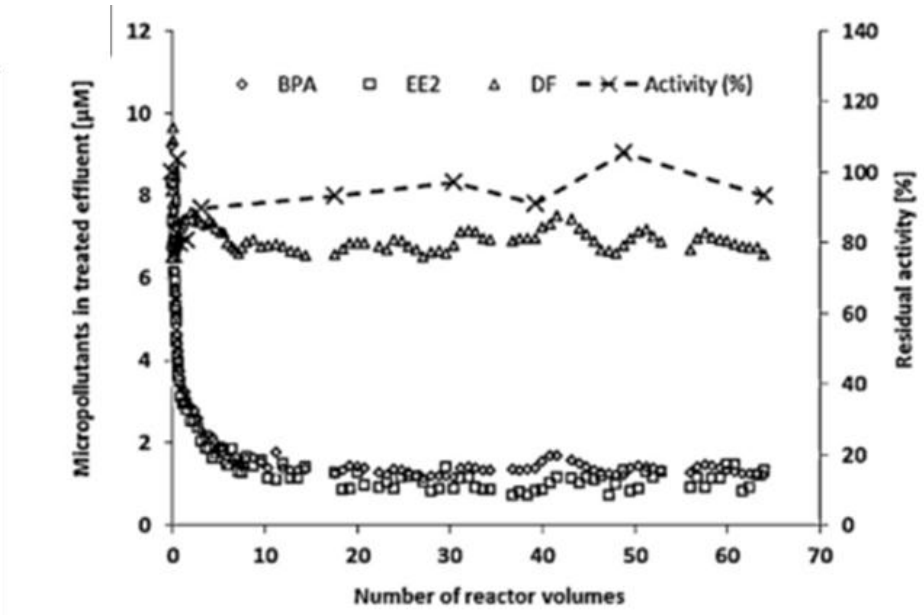


Figure 22: Time course experiment of Pharmaceutical compound degradation after laccase treatment in secondary water effluent (Nair et al., 2013)

Breakdown of Diclofenac using Laccase from *T. versicolor* pellets exhibited immediate effect upon laccase application, with no DCF detected after 30 min indicted by a decreased peak using HPLC. The removal of diclofenac from the control samples was explained as being due primarily to sorption (Marco-Urrea et al., 2010). Almost complete removal (94%) was obtained after the first hour, this high speed effective removal had not been observed before and was an improved strategy compared to other studies (e.g. Ernest et al. (2007)). As there are low concentrations of pharmaceutical drugs found in waste water (ng-µg/L), this was accounted for using a low concentration of 40µg/L to mimic this circumstance in (Figure 23b). Once again, Diclofenac degradation began immediately in the presence of laccase; most of this activity took place in the first 30 min, with 95 % being broken down within 4.5Hrs (Marco-Urrea et al., 2010). Further examination of the metabolites produced using a Microtox bioassay revealed that this reduced the ecotoxicity effects, thus revealing this strategy as an attractive approach for treating pharmaceutical waste waters.

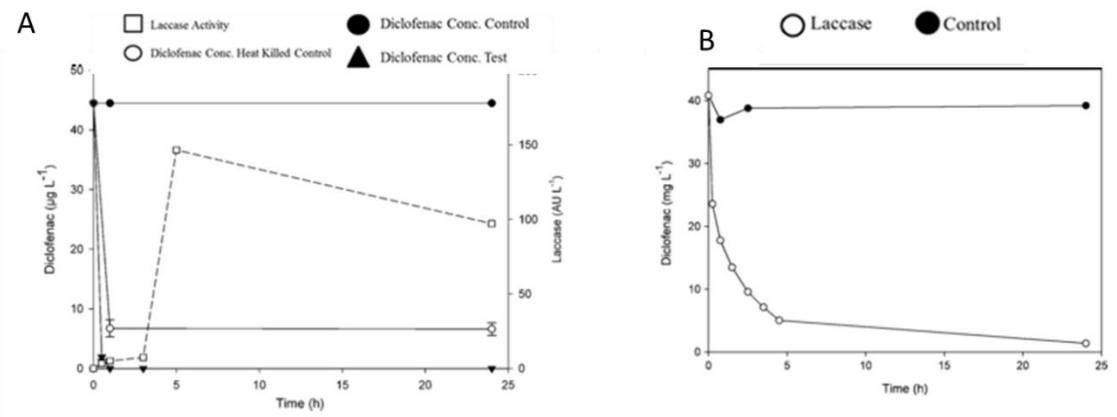


Figure 23: Time course experiment of a) Diclofenac 10 mg/L b) diclofenac (40 µg/L) degradation using laccase (2,000 U/L) and controls (Marco-Urrea et al., 2010)

2 Materials and Methods

2.1 Materials

Whatman® regenerated cellulose microfiltration membranes (25 mm), paraformaldehyde, Laccase from *Trametes versicolor*, 2,2′-Bipyridyl, α-Bromoisobutyryl bromide (BIBB), Copper Bromide (CuBr), tert-Butyl acrylate (tBa), 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Tin (IV) Chloride and 1,4-Dioxane, were purchased from Sigma- Aldrich. Pierce™ BCA protein assay kit, acetonitrile, triethylamine (TMA), chloroform, tetrahydrofuran (THF), Whatman® Cellulose Filter Paper, hydrochloric acid (HCl), ethanol and dichloromethane (DCM) were purchased form Fisher Scientific. HPLC analytical column was a C18 with 3.5µm, 4.6 mm x 150 mm from SunFire™. Buffers and coomassie stain used were from stock pre made in the lab. The protein marker was from New England Bio Labs (2-112 kDa). Polysulfone P-3500 was supplied by Solvay Speciality Polymers, Belgium. Flux studies were carried out using the Amicon stirred cell model 8010, from Millipore. NMR was performed using a Bruker Avance 400 (400 MHz) spectrometer, ATR-FTIR was performed using a Perkin-Elmer Spectrum 100, water contact angle was measured on FTÅ 200 contact angle analyser (First Ten Angstroms, Inc., USA), Surface Electron Microscopy (SEM) was conducted with a Hitachi S3400 and Florescent microscope (IX81, Olympus Co, Japan).

2.2 CMPSf Membrane Preparation

2.2.1 Preparation of Chloromethylated Polysulfone (CMPSf)

5 g polysulfone was weighed out and left to dry at 60 ^{o}C overnight. This was dissolved in 250 mL of chloroform, stirring at 300 rpm with a magnetic stirring bar in a 500 mL round bottom flask. To this mixture, 3.4 g paraformaldehyde was added a small bit at a time, then 14 mL of chlorotrimethylsilane, which was measured in a glass cylinder. Tin (IV) chloride (0.4mL) in 10mL of chloroform was added drop wise at 55 ^{o}C, before setting up a water condenser. The reaction was left stirring for 72 hrs. After this time, the reaction was allowed to cool to room temperature by turning off the heat. The solution was precipitated out by slowly adding it to a 1 L beaker containing 500 mL methanol, stirring at 300 rpm. The polymer was filtered using a buncher funnel containing Whatman® Cellulose Filter

Paper, attached to a 2 L buncher flask with a vacuum. This was left to dry for 15 min. The polymer was dissolved in 100 mL of chloroform, stirring in a 500 mL beaker and covered with a petri dish. Once more the polymer was precipitated out by slowly adding it to a beaker of 250 mL methanol, this was covered with parafilm. A weight put on top and left in the dark for 24 hrs. The mixture was filtered using a vacuum and buncher funnel and then dried for 15 min. Finally, the polymer was dried at 40 ^{o}C for 1 hr and stored in an air tight bag. An NMR spectrum was taken to examine the molecular structure.^{1}H NMR (400 MHz, deuterated dichloromethane (CCl_3D)) σ 4.5 (CH_2Cl).

2.2.2 Membrane Casting

Membranes were cast using the NIPS method. 8 mL NMP was pipetted into a 25 mL beaker, this was heated to 50 ^{o}C then 0.7 g of PVP was added slowly while stirring and allowed to dissolve. Gradually 1.5 g of CMPSf was added, the solution was covered and left stirring for 1 hr. Subsequently, the heat was turned off, the beaker was covered in parafilm and left stirring for 20 hrs. Following this, the mixture was sonicated for 1 hr to remove any air bubbles, then immediately poured onto a glass plate, where an elcometer set at 250 μm was used to evenly cast the polymer solution. After 30 sec the glass plate was immersed in water at room temperature, where the polymer underwent precipitation. Once the membrane detached from the plate, it was instantly placed into a new water bath at room temperature (RT) and kept overnight. The following day the membrane was placed between two sheets of A4 printing paper and left to dry at RT for 24 hrs. After this time, the membrane was placed in a 40 ^{o}C oven for 1 hr, and then stored in a plastic sheet until further use.

2.3 CMPSf Membrane Functionalization

2.3.1 Atom Transfer Radical Polymerisation (ATRP) - General Method

Membranes of 25 mm diameter circles were dried in a vacuum oven before noting the ‘before ATRP’ weight, then placed into a 100 mL round bottom flask, Copper (I) was added before sealing with an adaptor and septum. The flask was backfilled with Argon (2,000psi) 3 times at 1 min intervals to create an oxygen free environment. A mixture of 10 mL acetonitrile, 4.4 mL tert-Butyl acrylate (monomer) and 40 μl 2,2-bipyridyl (ligand)

were added to a vial and sealed with a septum. This mixture was purged for 30 min before being transferred to the membrane flask via a syringe. Argon was applied for an additional 5 min, before allowing the reaction to commence. Following ATRP the membrane were washed. The following day, the membrane was put into a beaker of deionised water (dH_2O) for 3 hrs. Next the membrane was placed between filter paper and let dry in a vacuum oven for 12 hrs. The 'after ATRP' weight was measured and used to calculate the degree of grafting using Equation 1.

$$DG = \frac{(M_1 - M_0)}{A} \qquad \text{Equation 1}$$

Where M_o is the weight (µg) before ATRP, M_1 is the weight (µg) after ATRP and A represents the surface area of the membrane (4.91 cm^2)

**For CMPSf membranes the flat sheets were cut in 25 mm circles, 36.3 mg Cu(I) was used and ATRP reaction carried out for 1 hr, 2 hr and 3 hr time intervals, before exposing the flask to the atmosphere and ceases the reaction, wash steps were carried out by placing membrane in a beaker with methanol, covered overnight. The following day, the membrane was put into a beaker of deionised water (dH_2O) for 3 hrs.*

**For the Cellulose membranes 71mg of CuBr was used. ATRP was carried out for 1 hr and wash steps were carried out in DCM followed by ethanol for 10 min each.*

2.3.2 Acid Hydrolysis of Poly(tBa) Brushes

Membranes were placed in a round bottom flash with 10 mL of 0.1 M HCl, containing 1 mL 1,4-Dioxane before being sealed and left to incubated at 50 ^oC for 24 hrs. The following day, the membrane s were put into a 50 mL beaker with enough methanol to cover the surface and left for 1 hr. Membranes were put into deionised water for 2 hours, then dried in 40 ^oC oven for 12 hr. Fourier Transform Infrared Spectroscopy (FTIR) was carried out to examine the chemical properties of the membrane surface before and after acid hydrolysis. Membranes were placed on a stage and clamped before analysis.

2.3.3 Binding of Green Fluorescent Protein – Shaker Flask Method

Functionalised CMPSf membranes were placed face down into a 10 mL beaker and immersed in a 2 mL solution of Green Fluorescent Protein (GFP) with a concentration of

342 µg/mL. The beakers were wrapped in parafilm to prevent splashing and left on a shaker at 100 rpm for one hour. The membranes were washed twice in 2 mL deionised water (dH_2O) for 30 mins each wash. Samples analysed using a Bicinchoninic Acid Kit (BCA) assay included initial solution, end reaction solution and wash steps, to allow mass balance calculations. The amount of protein bound was calculated using Equation 2:

$$Q_I = \frac{(C_{I0} - C_{I1}) \times V}{M_d} \qquad \text{Equation 2}$$

where Q_I is the amount of protein immobilised per unit mass of the membrane (µg/ cm^2). C_{I0} is the initial protein concentration (µg/mL), C_{I1} is the protein concentration following immobilisation (µg/mL), V_I is the volume of protein solution used and M_d is the area of the membrane (4.91cm^2).

2.4 Cellulose Membrane Functionalization

Commercially available regenerated cellulose membranes with pore size 0.25 µm (Whatmann) were used to graft poly(AA) brushes using ATRP.

2.4.1 Initiator Immobilisation onto Cellulose Membranes

Two different concentrations of BIBB and TEA were used to test which was the best option to proceed with, previously developed by Liu Pingsheng et al., (2014) and Feng et al., (2014).

2.4.2 High Initiator Concentration:

Membranes were placed in a 100 mL round bottom flask and immersed with 20 mL THF for 10 min. THF was replaced with a solution of 4.44 g TMA in 50 mL THF to which 9 g (39 mM) of BIBB was added dropwise. The flask was sealed with a septum and incubated at 35 ^oC for 3 hrs. Membranes were rinsed in DCM then ethanol for 10 min each, before drying in a vacuum oven overnight.

2.4.3 Low Initiator Concentration

Membranes were placed in a 100mL round bottom flask, to which 20 mL of THF was added and left for 10 min. THF was removed and a solution of 10 mM BIBB (made up in THF) and 10 mM TEA (prepared in dH_2O) in 50 mL THF was added. The flask was sealed

and incubated at 35 ^{o}C for 3 hrs before washing with DCM and ethanol for 10 min each, and left to dry overnight in a vacuum oven.

2.4.4 Laccase Immobilisation

Following ATRP of the cellulose membranes, modified and unmodified membranes were placed into 100 mL conical flasks containing 10 mL of laccase enzyme (0.15 mg/mL) and agitated at 100 rpm for 6 hrs. A sample was taken every hour and a BCA assay was used to calculate the concentration of laccase at each time point. The amount of laccase adsorbed onto the membrane was determined using Equation 2.

2.4.5 Statistical Analysis

P values were calculated using the one-way analysis of variance (ANOVA) in excel data analysis.

2.5 Membrane Characterisation Methods

2.5.1 Water Contact Angle Measurement

Membranes were cut in half and stuck to a glass slide using double sided tape before placing on the stage. A single droplet was placed onto the membrane and image captured after 2 seconds. Image J software was used to analyse the droplet area and the mean calculated after taking 3 readings at random locations.

2.5.2 Fluorescent Microscopy

Membranes were cut in half and placed on a plastic petri dish, then examined under a blue filter. The images were analysed using CellR software and captured at 4x magnification.

2.5.3 Scanning Electron Microscopy (SEM)

Membranes were folded in half and fractured in liquid nitrogen. After loading the sample onto a SEM stub, they were sputtered in gold before analysis was performed on a Hitachi S3400.

2.6 Green Fluorescent Protein Preparation

2.6.1 Creating Chemically Competent Cells – The TB Method

10 µL of JM109 *E. coli* stock was added to 2 mL of Super Optimal Broth (SOB) media. The following day 2 mL of this overnight culture was inoculated in a sterile 1 L flask with 200 mL of SOB Broth and incubated in a shaker at 37^{o}C. The absorbance was read at OD_{600} (optical density) each hour until it got to 0.300 OD, then it was observed every 15 min until it reached 0.400 OD, which signifies the cells are at their early exponential phase of growth. The flask was placed in ice to stop the cell growth and transferred to a sterile centrifuge tube, which was spun at 4,500 rpm for 5 min. The supernatant was disposed of and the pellet was gently re-suspended by pipetting using 80 mL of chilled TB buffer. This was left on ice for 10 mins and spun again at 5,500 rpm for 5 mins to collect the cells. The supernatant was removed and the pellet was re-suspended slowly in 15 mL of chilled TB Buffer. 7% (v/v) Dimethyl Sulfoxide (DMSO) was added 100 µl at a time to the suspension in the cold room, and left on ice for 10 mins before making 400 µl aliquots in 1.5 mL sterile Eppendorfs and flash freezing by using a metal block before storing in -80^{o}C.

2.6.2 Preparing LB Ampicillin Agar Plates

Add 15 g/L of agar to a chosen volume of LB into a conical flask. Wrap top of flask in tinfoil and autoclave. Allow to cool before aseptically adding Ampicillin at a final concentration of 100 µg/mL. Pour into petri dishes over a flame and allow to harder before use. Store the plates upside down at 4 ^{o}C and wrap in cling film.

2.6.3 Transformation

For each transformation, 200 µL of chemically competent JM109 *E. coli* cells were thawed on ice, before gently mixing 2.5 µg (3 µL) of plasmid DNA into a sterile Eppendorf tube. The cells were left on ice for 30 mins which allows the plasmid DNA to bind to the outside of the cells. The cells were then heat shocked at 42 ^{o}C for 30 secs to allow the plasmid DNA to enter the cells, and placed back on ice for 2 mins. Next 800 µL of sterile LB was added to the cells and incubated at 37^{o}C for 1 hr. From the tube, 200 µL of the cell suspension was plated on LB Ampicillin plates and incubated overnight at 37^{o}C. Ampicillin (Amp) serves as a selective marker for the growth of cells.

2.6.4 Protein Expression

To inoculate *E. coli*, 2 mL of stock was added to a conical flask containing 200 µL of Amp in 200 mL of TB and shaken at 37 ^{o}C until an OD_{600} of 0.4-0.6 was obtained (approximately 2 hrs). 50 µM IPTG was added, followed by agitation overnight at 30 ^{o}C. The culture was added to a sterile centrifuge tube and spun down at 4,000 rpm for 10 min. The supernatant was discarded and the pellet stored at -20^{o}C until time of use. The pellet was resuspended in 100 mL of 20 mM imidazole lysis buffer with 0.01 % anti-foam. The solution was run through a cell disruptor twice to ensure sufficient lysis. The lysate was then added to a high speed centrifuge tube and spun at 13,000 rpm for 40 min to pellet the insoluble material. The protein of interest is found in the lysate. This was filtered through a Whatman filter and ready for purification.

2.6.5 Protein Purification

Green fluorescent protein (GFP) is His tagged; therefore a Nickel based Metal Affinity Chromatography (IMAC) column was used for purification. The columns were equilibrated with 10 column volumes (CV) of 20 mM Imidazole. Around 50 mL of the filtered lysate is added, the flow through was collected and labelled as unbound. 20 mL of 20 mM Imidazole was added, followed by 10 mL of 100mM Imidazole. To elute the protein 10 mL of 250 mM Imidazole was added and fractions collected. The samples were stored at 4^{o}C.

2.6.6 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Protein samples were run on an SDS PAGE gel to examine the sample purity and size of the protein. Preparation was carried out by adding 18 µl of sample to 2 µl of 10X loading dye, followed by boiling for 5 min. A total of 12 µl sample was loaded onto the gel, along with 12 µl of ladder. This ran at 30 mA until sample leaves stacking gel, then adjusted to 65 mA until dye runs off gel. The gel was removed from the glass and rinsed in deionised water (dH_2O) before covering it in Coomassie Stain and left agitated for 1 hr. This was followed by rinsing it twice more in dH_2O and leaving it in a Destain solution gently rocking overnight, the following day the bands were analysed.

2.7 Membrane Performance Characterisation Methods

2.7.1 Flux Measurement

A stirred dead-end ultrafiltration Amicon 8200 cell (Millipore Co., USA) connected to a 1 L capacity reservoir was used to measure the pure water flux of the membranes at 1 bar. The pressure during the measurements was controlled by supplying N_2 gas. A circular piece of hybrid membrane with diameter 25 mm was fixed initially at the bottom of the stirred cell and then 700 mL deionised (DI) water was passed through each membrane at 2 bar to attain a stable pure water flux. DI water was further permeated through the membranes for an additional 30 min at 1 bar and permeate was collected in a glass beaker. The weight of permeated water was measured on a digital balance (Ohaus Adventurer™ Pro Balance, UK). The pure water flux (J_w; L m^{-2} h^{-1}) was calculated using Equation 3

$$J_w = \frac{V}{A \times t} \qquad \text{Equation 3}$$

where V is the volume of the permeate (L), t is the filtration time (h), and A is the effective membrane area (m^2).

2.7.2 Adsorption of Protein to Membranes

For cellulose membranes, a known concentration of BSA was prepared in pH 3 (795 µg/mL) and pH 7 (707 µg/mL). The amount adsorbed onto the control cellulose membrane surface was calculated using a BCA assay.

2.7.3 Protein Fouling Analysis

Protein fouled onto control membranes was examined using Bovine Serum Albumin (BSA) in the stirred cell unit. BSA was made up to a concentration of 4 mg/mL (CMPSf membranes) and 4 mg/mL (Cellulose membranes) in pH 3 and pH 7 then passed through the membrane at a feed pressure of 0.25 bar. A sample was taken every 30 seconds and the amount of protein adsorbed onto the membrane surface was calculated by reading the absorbance at 280 nm using Equation 4

$$A = \varepsilon C l \qquad \text{Equation 4}$$

where A is the absorbance, ε is the excitation coefficient (cm^{-1} M^{-1}), C is concentration in moles and l is the path length (cm). The excitation coefficient of BSA is 6.67 cm^{-1} M^{-1}. The pure water flux was measured before and after protein adsorption to see how it affected the membrane performance.

2.8 Free and Immobilised Laccase Characterisation Studies

2.8.1 Enzyme activity

An enzyme assay was carried out to monitor laccase activity (Forde et al., 2010). The average of five samples was reported. 1.58 mL pH 4.5 buffer and 200 µL 1 mM ABTS was added as substrate to a 1 mL cuvette and left to incubate for 10 min at 25 ^{o}C before adding 20 µL of enzyme. The absorbance was read immediately and after incubating for 2 min at 25^{o}C. Enzyme activity was expressed in units; 1 unit of enzyme oxidises 1 µmol of ABTS (ε 420nm = 36,000M^{-1} cm^{-1}) in 1 min at 25^{o}C and calculated using Equation 4.

2.8.2 Effect of pH on Activity and Stability

Citrate buffers were prepared for the pH range 3-5 and phosphate buffers for pH 6-8. 10 mL of buffer was added to a tube. For the free enzyme tests, 1 mL of laccase at 55 U/L was added and for the immobilised enzyme test, membranes were cut in quarters and added to the flask. Samples were incubated at 35 ^{o}C for 10 min before adding 1 mL 1 mM ABTS. The absorbance was read immediately at 420 nm and again after the samples were incubated at 35^{o}C for 1 hr (Ramírez-Cavazos et al., (2014)). The amount of ABTS oxidised was calculated by increased absorbance at 420nm (Zapata-Castillo, 2012) with the molar excitation coefficient (ε) of 36,000 M^{-1} cm^{-1}. The pH sample with the most oxidation was defined as 100% and every other sample was taken in relation to this value. Tests were carried out in triplicate.

2.8.3 Effect of Temperature of Laccase Activity

The thermal stability of laccase was tested over a range of temperatures (20-70 ^{o}C) to determine optimum activity (Chefetz & Chen, 1998). For the free laccase test 10 mL of a chosen pH buffer and either 1 mL of free laccase or a laccase immobilised membrane was added to a 100 mL conical flask. The flask was incubated for 10 min at the desired

temperature before adding 1 mL of 1 mM ABTS as substrate. The absorbance was read immediately and every 10 min after for 1 hr. All tests were carried out in triplicate.

2.9 Diclofenac Removal Analysis

An initial concentration of 43 ppm Diclofenac (DCF) was prepared. Functionalized Cellulose membranes were placed into amber (light sensitive) beakers, 5 mL of DCF solution was added and the lid was tightened. The beaker was rocked at 125 rpm for 2 hrs at room temperature (22 ^{o}C) before the reaction was stopped by removing the membranes. Tests were carried out in duplicate and a control was used, which contained the DCF solution without a membrane. Samples tested for analysis were the initial solution of DCF, the test and the control beaker after 2 hrs (Sathishkumar et al., 2012).

2.9.1 High Performance Liquid Chromatography (HPLC) of DCF

Liquid chromatography equipped with a SunFire^{TM} C18 capillary column, particle size 3.5 µM was used to determine diclofenac transformation. The mobile phase was a mixture of methanol and 0.1 % aqueous formic acid pH 2.5 (80:20, v/v), the wash step was methanol 80 % and the flow rate was 1 mL/min. The wavelength used in the detector was 278 nm.

3 Results and Discussion

3.1 CMPSf Membranes for Protein Immobilization

The membranes outlined in this work are intended as pre-cursor developmental membranes towards the eventual fabrication of membrane adsorber systems for selective separation of glycoproteins. This study outlines a proof of concept work, where the immobilization of a recombinant protein on polymer brushes grafted onto membranes is achieved.

Chlormethylated polysulfone membranes prepared using a series of CMPSf and Polyvinylpyrrolidone (PVP) blends were tested when casting the membranes to examine the impact on the flux; ratios were chosen based on previous studies in the literature (Shi et al., 2013; Fan et al., 2012; Meng et al., 2012). A schematic for the reaction route is presented in Figure 24.

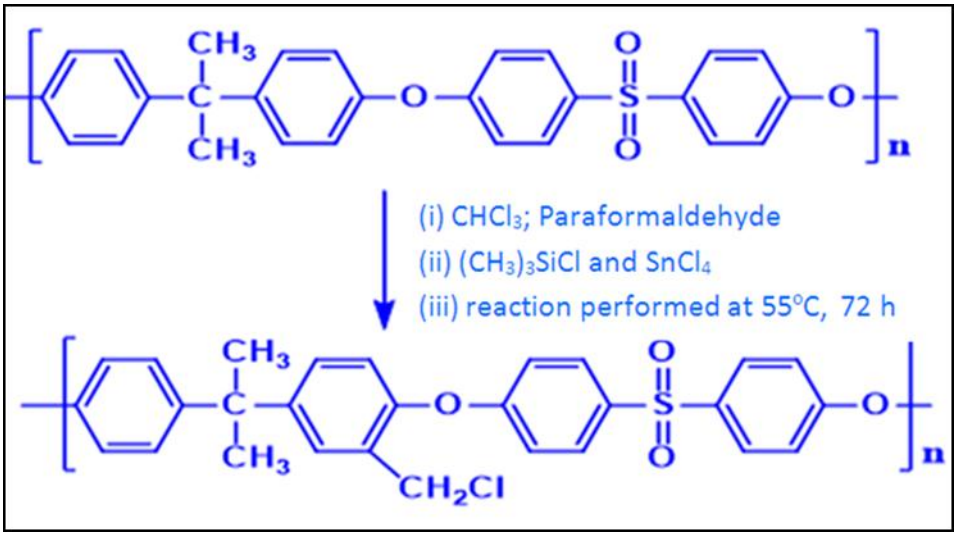


Figure 24: Chloromethylation of Polysulfone.

3.1.1 Membrane Flux

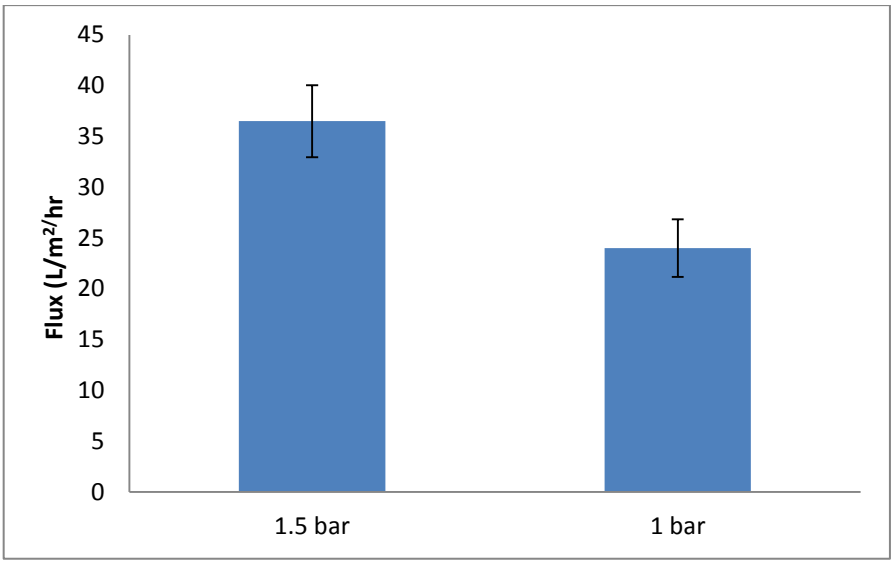


Figure 25: Flux of unmodified CMPSf membrane (4% PVP)

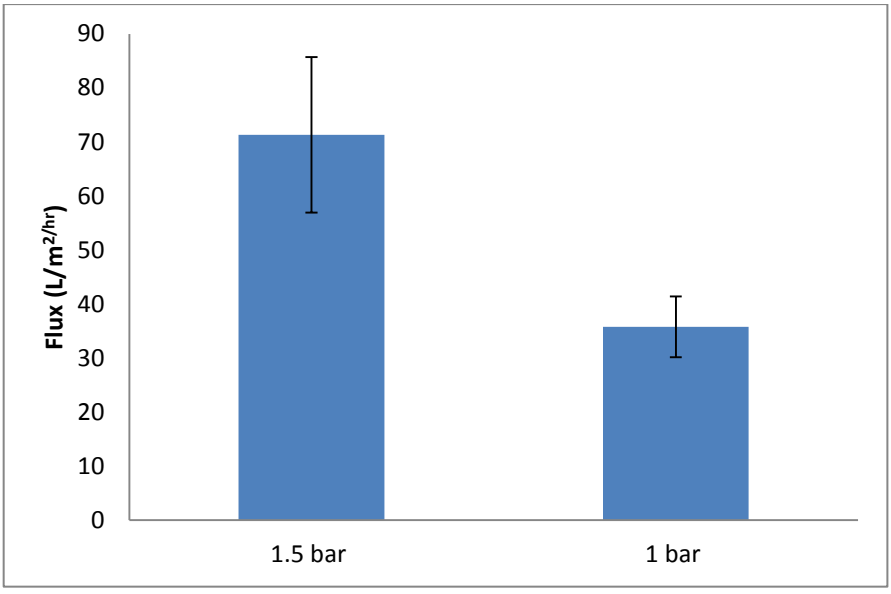


Figure 26: Flux of unmodified CMPSf membrane (7% PVP)

The 4% PVP membranes gave a flux value of 37 L/m^2/hr at 1.5 bar and 24 L/m^2/hr at 1 bar (Figure 25), whereas the 7% PVP membranes had a higher flux at both pressures,

displaying 71 L/m^2/hr at 1.5 bar and 36 L/m^2/hr (Figure 26), this is because an increase in PVP concentration increases the porosity of the membranes allowing for more liquid to pass through in comparison to a lower PVP concentrated membrane (Kumar, 2010). A highly permeable membrane is preferred (while maintaining selectivity, where required) because it would potentially be able to filter production batches of proteins in a shorter amount of time in industry. Therefore, the 7% PVP concentrated membranes were selected to move forward for functionalization. As the purpose of the prepared membranes is for membrane chromatography via adsorption, the retention of proteins via the membrane itself is not as important as the ability to deliver a high throughput membrane with binding sites for protein adsorption.

3.1.2 Chloromethylation of Polysulfone

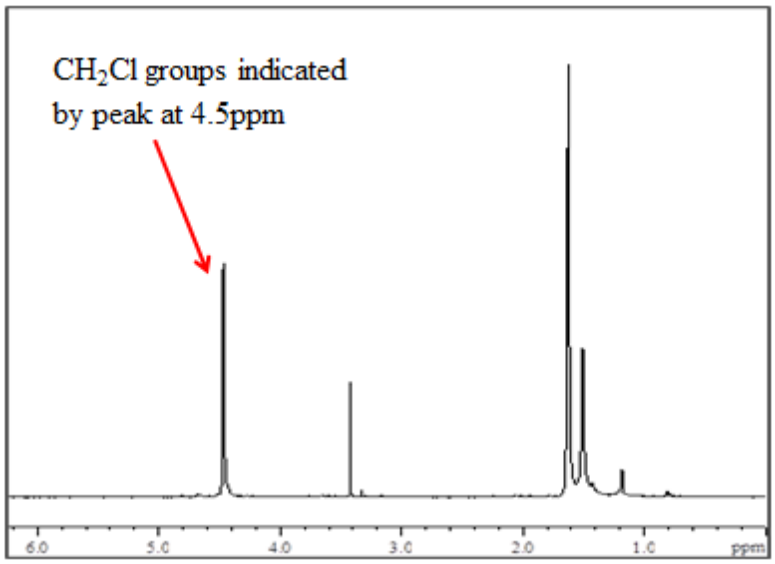


Figure 27: ^{1}H-NMR after chloromethylation of polysulfone

Chloromethylation of PSf provides ATRP initiator groups for ATRP on the membrane surface (Avram, et al., 2000). Nuclear magnetic resonance (NMR) spectroscopy was performed to examine the chemical properties of CMPSf (Figure 27), showing CH_2Cl groups indicated by a peak at 4.5 ppm.

3.1.3 ATRP and Acid Hydrolysis of CMPSf Membranes

Modification of the surfaces was carried out using SI-ATRP to graft the polymer (tert-Butyl Acrylate (tBa)) brushes. This is termed a "grafting from" approach because initiator

groups on the membrane surface allow for the growth of polymer chains (Krishnamoorthy, et al., 2014). A low degree of grafting can give rise to long extended brushes, where as a high degree of grafting can result in the chains becoming tangled in a mushroom shape (Figure 28). As such, ATRP was carried out at different time points to find the best grafting density to bind the most protein. If there was too high of a degree of grafting (DG), then the mushroom brush structure would coil, leading to less available binding sites.

Figure 28: Structures of Polymer Brushes Following ATRP

The degree of grafting achieved following ATRP after 1 hr, 2 hr and 3 hr is shown in Figure 29.

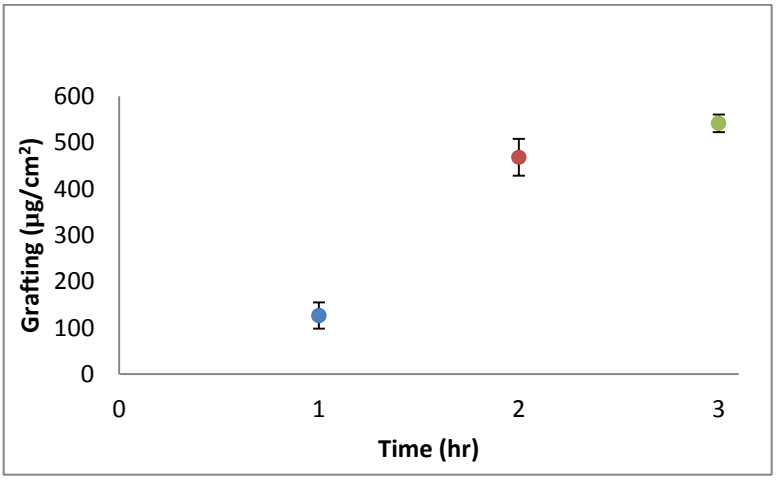


Figure 29: Degree of Grafting after ATRP (average of 5 samples)

An increase in ATRP time leads to an increase in polymer brush grafting. After 1hr a degree of grafting of 126 µg/cm^2 was achieved, after 2 hrs 468 µg/cm^2 and after 3 hrs 541 µg/cm^2 (Figure 29).

Acid hydrolysis provides reactive carboxylic acid (COO-) groups for the conjugation of the protein of interest to the brushes on the membrane's surface. This binding is achieved through coupling chemistry between the carboxyl groups of the membrane and the amino groups of ε-lysine residue of the target protein. ATR-FTIR analysis was conducted on membranes to establish if ATRP was successful. A peak between 1718 – 1738 cm^{-1} indicates that the polymer was grafted successfully (Figure 30). Acid hydrolysis was conducted for deprotection of the pol(tBA) to enable protein binding, indicated by a decrease in the peak size between 1718-1738 cm^{-1}.

Local decrease in ATR-FTIR transmission at 1718-1738 cm^{-1} (Figure 30) corresponds to a decrease in hydrogen bonding interactions, indicating that this step was successful.

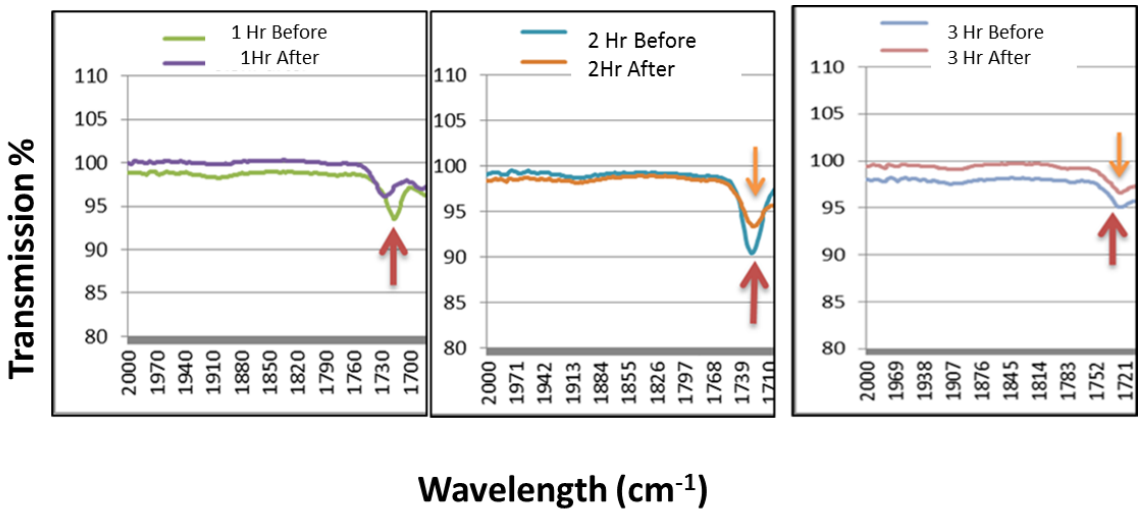


Figure 30: FTIR results following ATRP and Acid Hydrolysis

The postulated membrane structure following ATRP and acid hydrolysis is outlined in Figure 31.

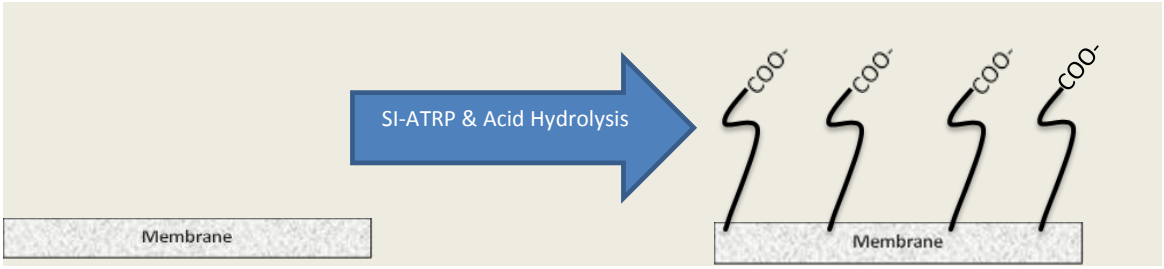


Figure 31: Membrane structure following SI-ATRP and Acid Hydrolysis

3.1.4 Functionalized Membrane Characterization

In preliminary experiments, membranes were treated with trifluoroacetic acid and acetonitrile to create the carboxylic acid groups during acid hydrolysis. It was discovered that this led to tears forming on the surface, after fluorescent microscopy revealed noticeable cracks and an abnormally high flux reading of 4,000 L/m^2/hr at 2 bar pressure. The experimental method was altered by using a low concentration of 1-4 Dioxane, which was successful in carrying out acid hydrolysis, moreover the flux of the control (un-functionalized) membrane and that following 1 hr ATRP are comparable in scale, indicating membrane integrity (Figure 32).

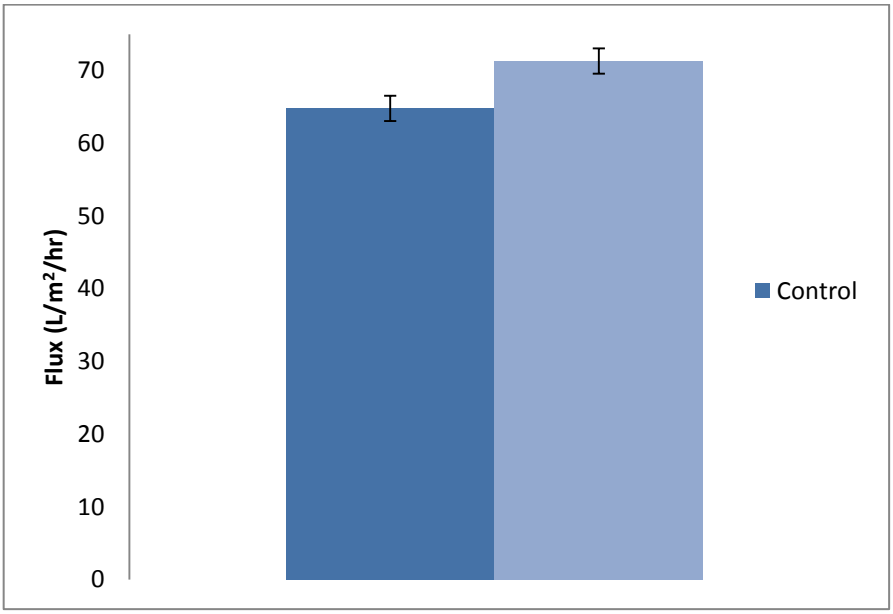


Figure 32: Flux of pure water at 1.5 bar after using a CMPSf membrane (7% PVP), after 1 hr ATPR and Acid Hydrolysis

The membrane hydrophilicity was examined using water contact angle measurements, before and after functionalization. A decrease in contact angle is observed after functionalization in comparison to the control (Figure 33).

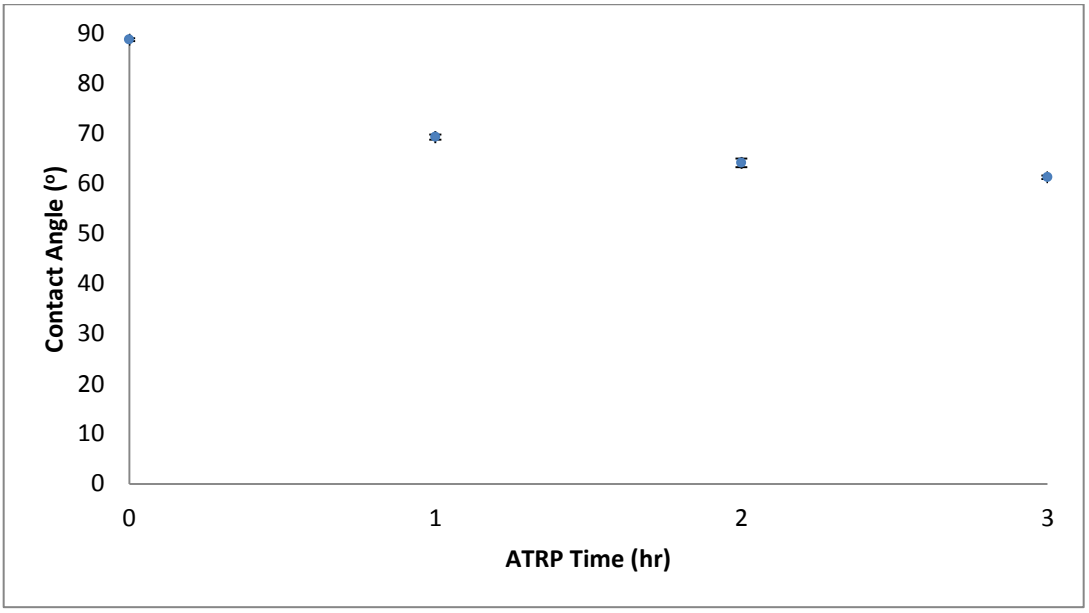


Figure 33: Water Contact Angle of CMPSf Membrane at varied ATRP times (average of samples triplicate)

The un-functionalised membrane has a contact angle θ_a =89^o, with a marked increase in membrane hydrophilicity observed with functionalization, with 1hr functionalized membrane contact angle θ_a =69^o, with further moderate decrease with increased ATRP times to an angle of θ_a =64^o for 2hr ATRP and θ_a= 61^o for 3 hr ATRP, with the increase in hydrophilicity attributed to the grafting of poly(AA) brushes onto the membranes surface (Wang et al., 2015; Liu et al., 2014; Adamson 2016). This increase in hydrophilicity can potentially explain the increase in flux observed between the functionalized and un-functionalized membranes shown in Figure 32.

SEM images presented in Figure 34 shows the membrane sublayer structure, where a finger-like macrovoid structure is observed. No distinctive effect of ATRP time on the membrane substructure is observed.

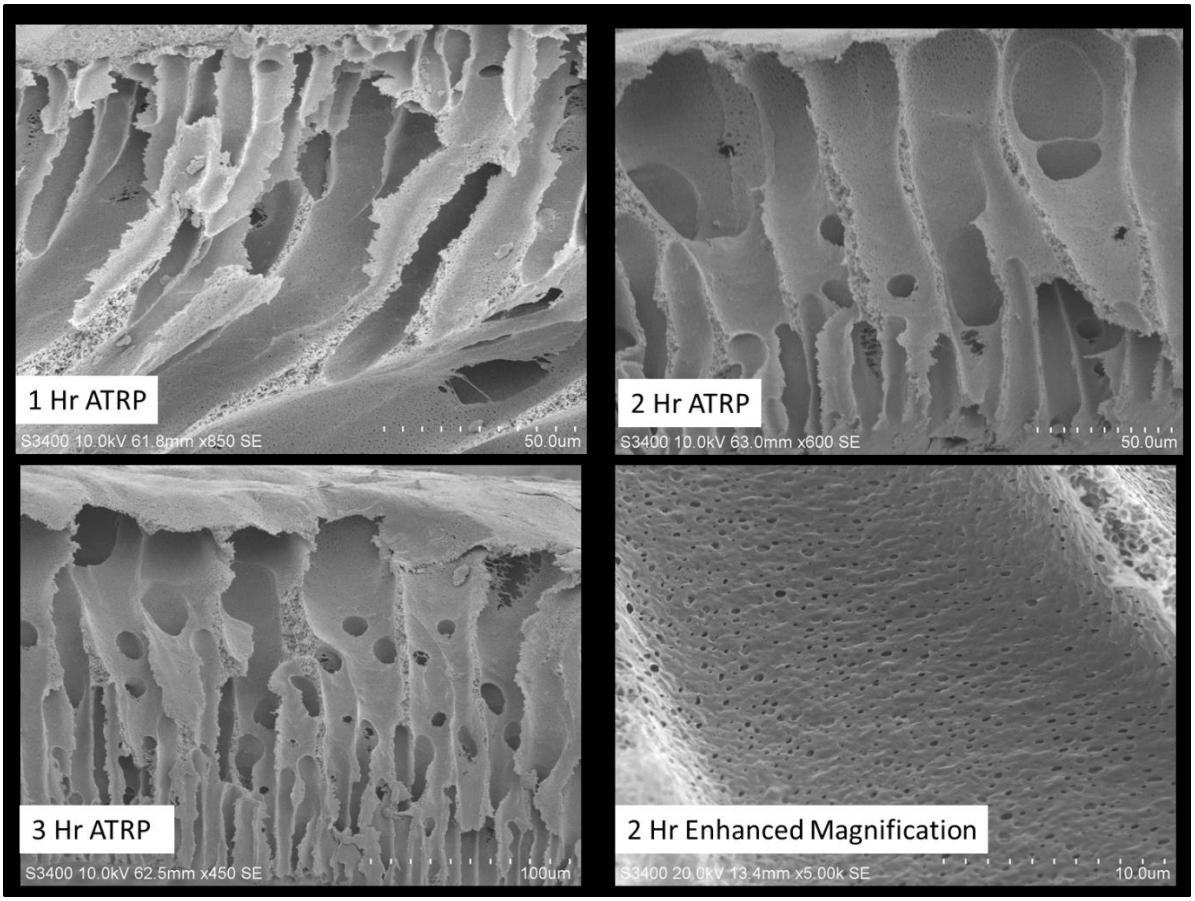


Figure 34: Cross section SEM images of CMPSf membranes at different magnifications. 1 hr at x650, 2 hr at x600 and x5,000 and the 3 hr at x450

3.1.5 Protein Purification

Green fluorescent protein (GFP) was expressed by JM109 *E. coli* cells which contained six histidine tags in the protein sequence. These His tags make the protein suitable to separate using affinity chromatography, as it has high specificity and affinity to bind to a column containing Nickel ions (Figure 35).

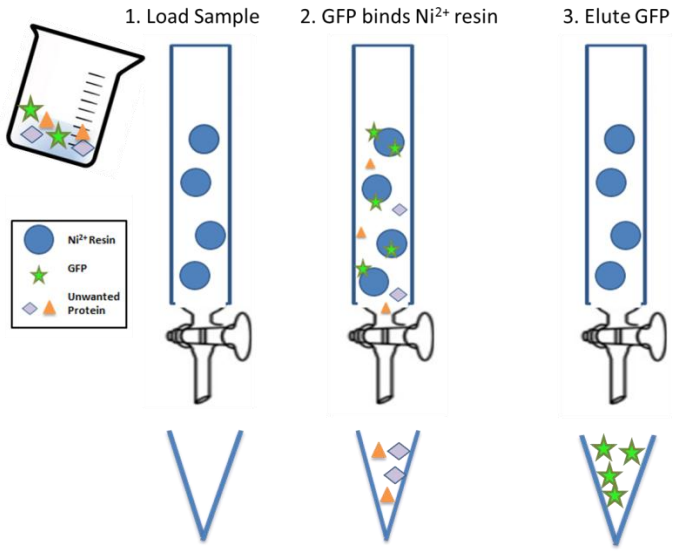
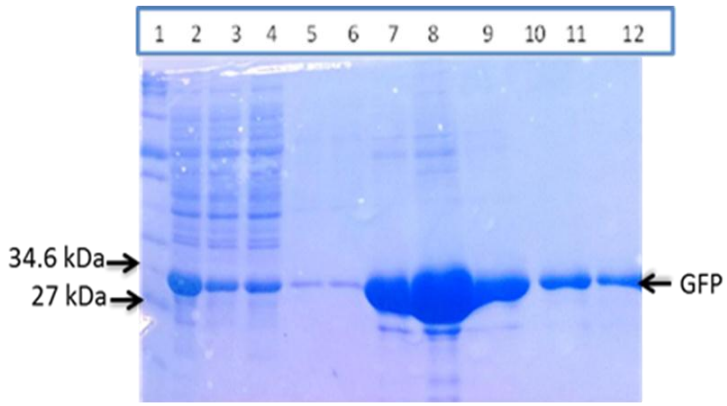


Figure 35: Nickel ions bind His tagged GFP

The other unwanted proteins get passed through the matrix during wash steps and GFP is eluted by using a higher concentration of imidazole (250 Mm) buffer.



Lane	Sample
1	Ladder*
2	Protein filtrate
3	Unbound Protein
4	Unbound Protein
5	20mM Imidazole Wash
6	100mM Imidazole Wash
7	Elution Fraction1
8	Elution Fraction 2
9	Elution Fraction 3
10	Elution Fraction 4
11	Elution Fraction 5
12	Purified Protein

Figure 36: SDS PAGE gel of purified GFP using a Nickel Column

The SDS page gel (Figure 36) signifies that each purification step was successful in eliminating any unwanted containments; Lanes 10 and 11 gave a single band at 27 kDa,

corresponding to the molecular mass of GFP. The single band signifies no other proteins were present in this sample; therefore it is the most pure and was used for further studies when testing adsorption onto the functionalised membranes.

3.1.6 Protein Binding

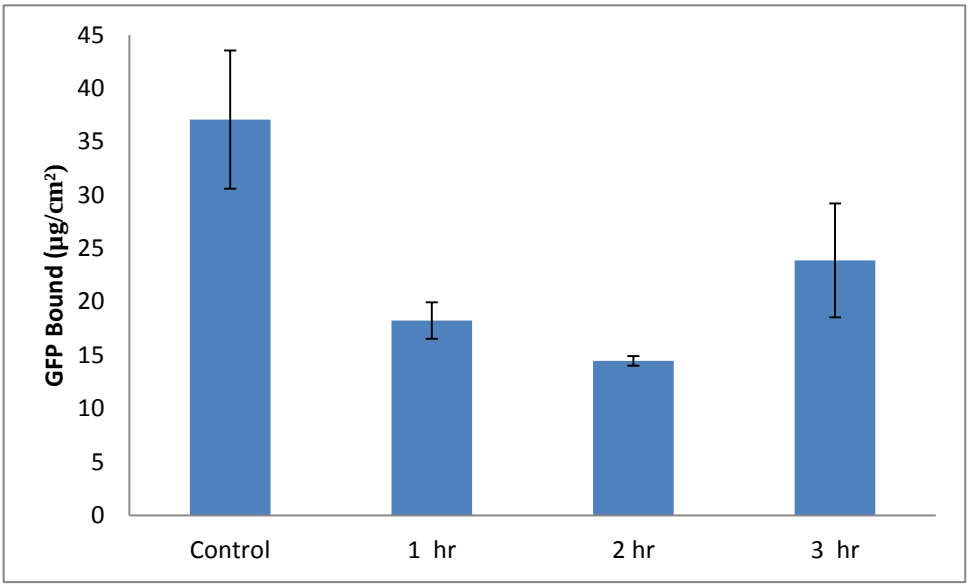


Figure 37: Adsorption of GFP

Using a shake flask method, prepared GFP was bound to membranes functionalized using ATRP over a range of up to three hours (with increase in polymer grafting). Figure 37 shows that the control binds the largest amount of protein, having 51% more than the 1hr ATRP membrane, 62% more than the 2 hr and 35% more than the 3 hr functionalised membrane. This indicates that the functionalized membranes can be used to bind GFP protein by grafting polymer brushes onto the membrane's surface. However, because there is little difference between the amount of polymer brush on the membrane between the 2 and 3 hour ATRP steps, and the amount of protein bound, more studies would need to be carried out to examine the significance of these results. The 3 hr ATRP step achieved the most GFP adsorption (24 µg/cm^2), however there was no clear trend observed. A decrease

adsorption was observed at 2 hr (14 µg/cm^2) in comparison to the 1 hr (18 µg/cm^2) functionalised membranes.

To examine if the GFP was still active after immobilisation, the membranes were examined under a blue filter using fluorescent microscopy (Figure 38).

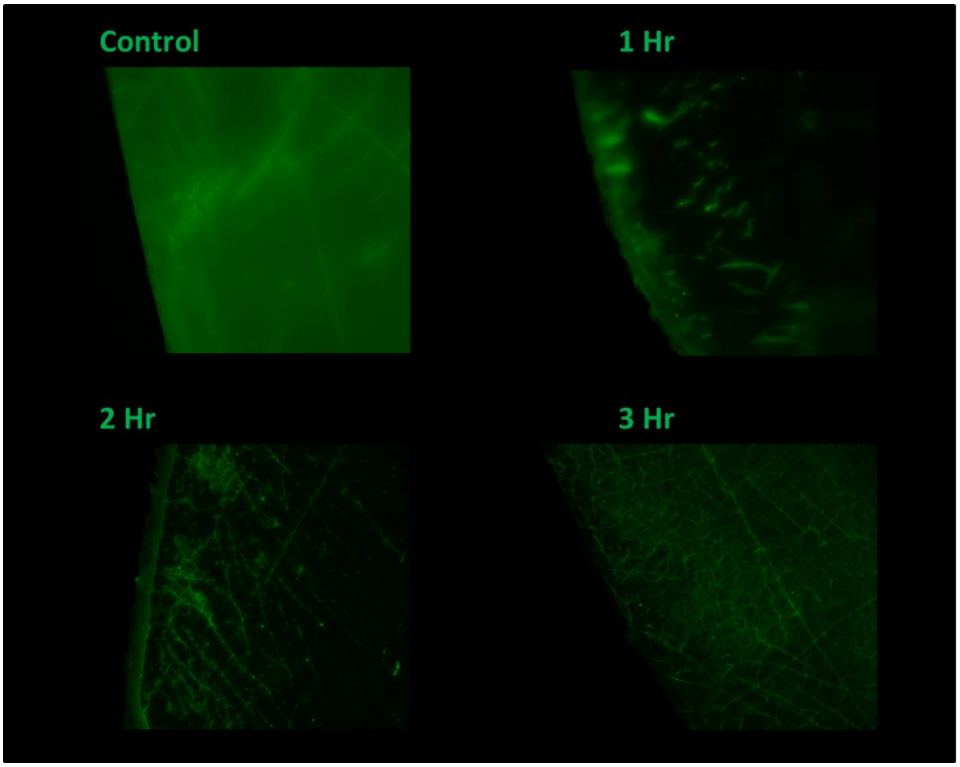


Figure 38: Fluorescent Microscopy Images of GFP adsorbed onto the membrane surfaces

The images reveal fluorescent protein in every sample; moreover the 3hr membrane is brighter than the 1 hr and 2 hr, supporting the previous results. The membranes were successful in binding GFP without inactivating its ability to fluoresce. GFP is often used as a reporter gene to detect if a plasmid or piece of DNA has been taken up by the cell and is popularly used as a fusion tag (linked to a protein of interest) to monitor the proteins fate and localization (Tsien, 1998). This has potential for a wide range of applications such as screening, micro-arraying and cell arraying (Gautrot, et al., 2010).

3.2 Enzyme Immobilised Cellulose Membranes

Cellulose is a biodegradable, renewable polymer found abundant in nature, making it an attractive source of material for industrial applications, such as membrane adsorption technology. Modification of cellulose was carried out to facilitate the immobilization of the enzyme laccase. Initiator immobilisation was achieved through esterification of the hydroxyl groups present on the surface of commercially available cellulose membranes with α-bromoisobutyryl bromide (BIBB). Surface initiated atom transfer radical polymerization (SI-ATRP) was used to successfully graft hydrophilic polymer brushes onto surfaces, which provided active sites for immobilization of laccase. Conversion of poly(tBa) to poly(AA) was carried out through selective acid hydrolysis (Figure 39). The chemical modification allowed for the stable binding of laccase onto cellulose based membrane surfaces, intended for use in the removal of Diclofenac (or other phenolic contaminants) from contaminated drinking water sources.

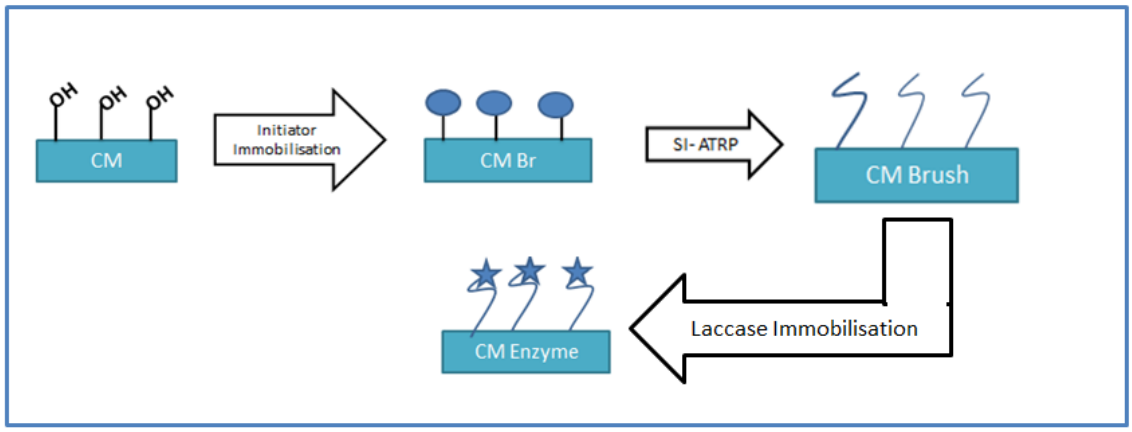


Figure 39: Modification of Cellulose Membranes

3.2.1 Unmodified Cellulose Membrane Characterisation

A sponge like morphology of the membrane subsurface is observed (Figure 40) which enables these membranes to work under high pressures (Ying & Yang, 2002).

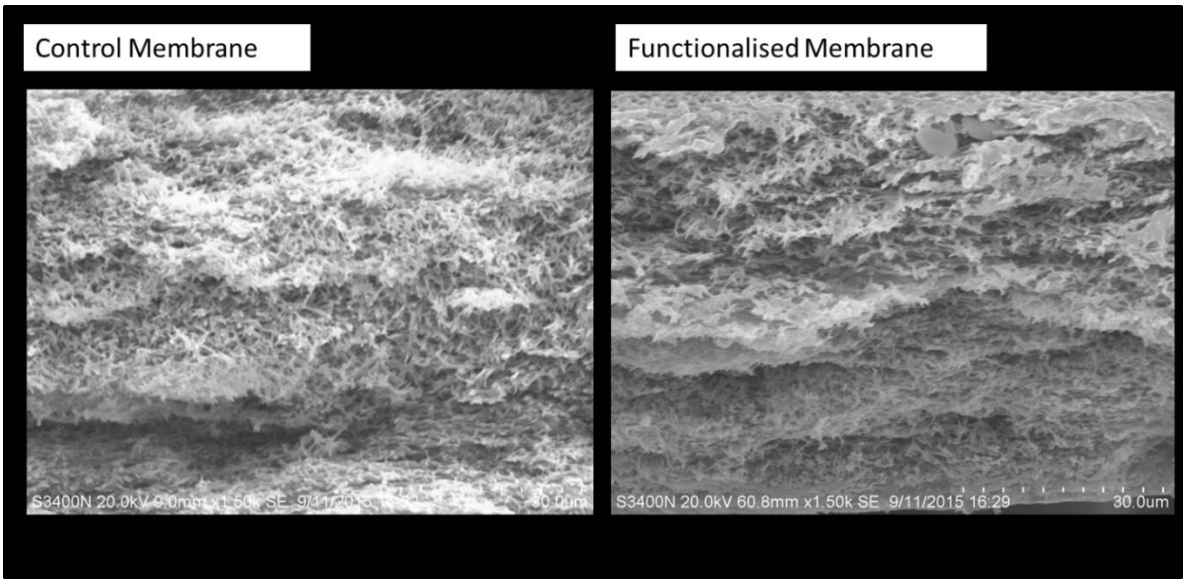


Figure 40: Cross section SEM images of the control and 1 hr ATRP modified cellulose membranes

The flux of unmodified cellulose control membranes using a range of pressures was measured (Figure 41). Error bars are representative of the standard deviation, samples were carried out in duplicate. An increase in pure water flux is achieved by an increase in pressure.

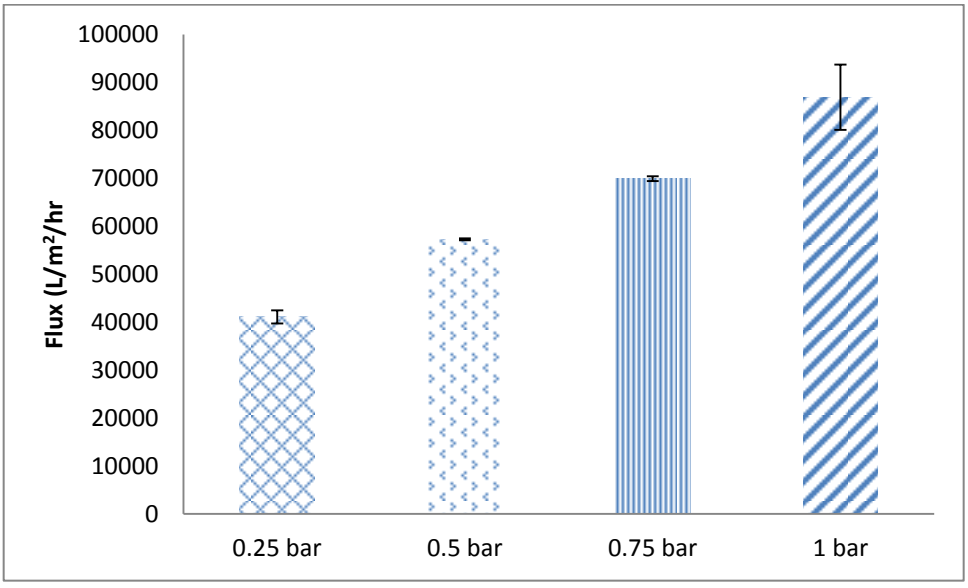


Figure 41: Pure Water Flux of control membranes across a range of pressures

A known concentration of BSA was prepared in pH 3 and pH 7. The amount adsorbed onto the control cellulose membrane surface was calculated using a BCA assay (Figure 42).

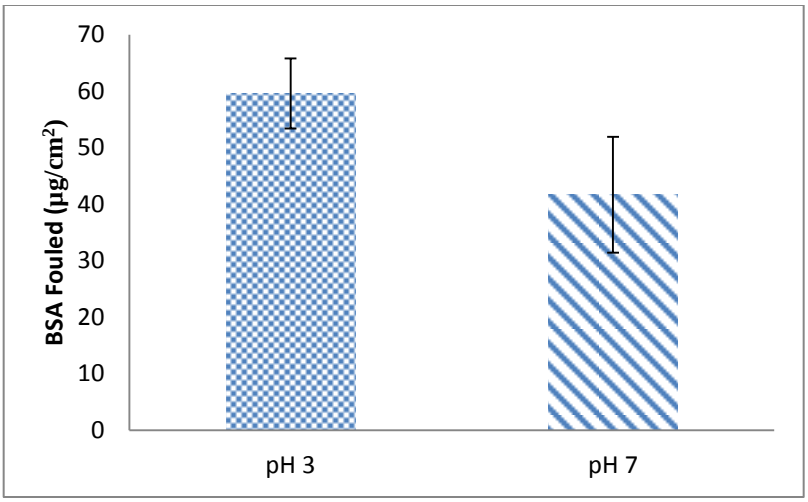


Figure 42: Protein adsorption onto unmodified membranes

Protein fouling of the unmodified membrane was examined by passing BSA through the membrane at 0.25 bar.

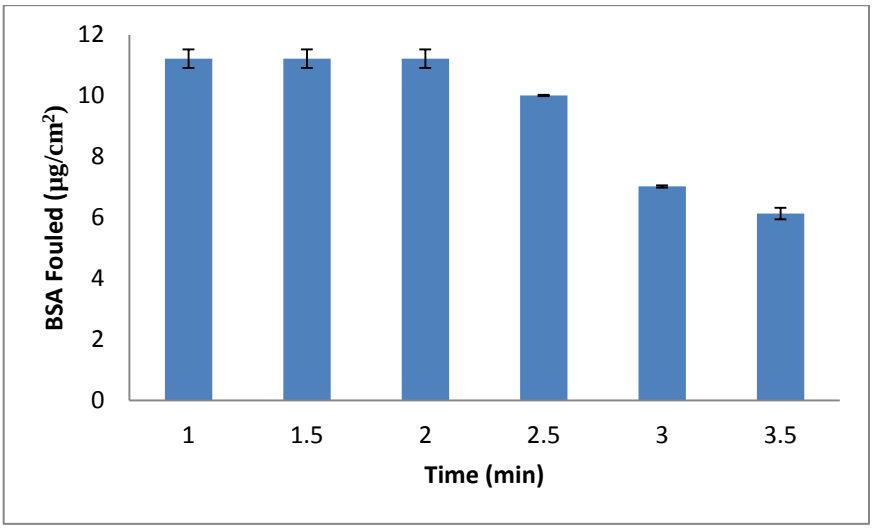


Figure 43: Protein fouling on control membrane surface

The regenerated cellulose microfiltration membranes exhibit a high flux value and very low protein binding, with some tendency for fouling during filtration of a protein solution (Figure 43).

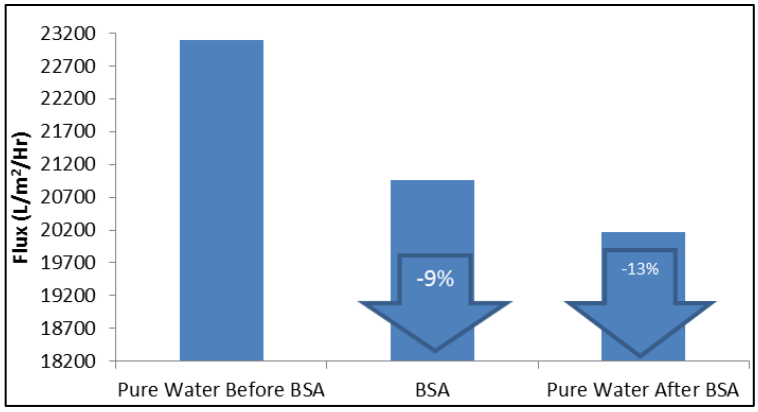


Figure 44: Effect of BSA at pH3 on cellulose membrane flux

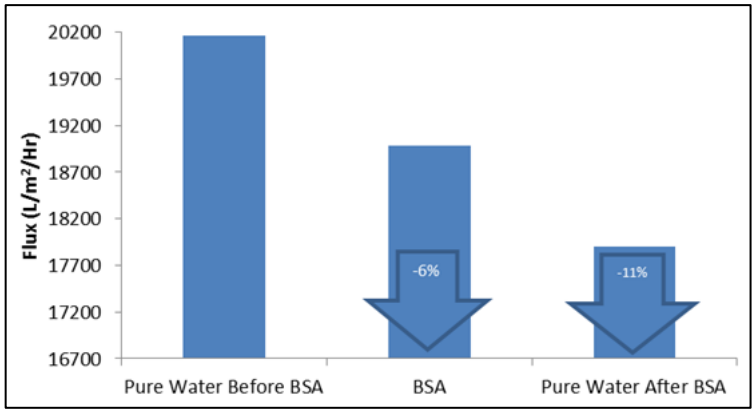


Figure 45: Effect of BSA at pH7 on cellulose membrane flux

A relatively low flux decline was observed when passing BSA through the stirred cell unit which would not impede their performance for commercial applications significantly due to the nature of their high flux value overall; 9% decline at pH 3 and 6% drop at pH 7 (Figure 44 and Figure 45). Following BSA filtration, dH_2O was passed through the membranes, which showed a decrease in flux of 11% for the pH 3 and 13% decline of the pH 7 solution, demonstrating that these membranes can be easily re-useable, which is ideal for industrial filtration procedures. A validated cleaning cycle could perhaps remove the fouled BSA off the surface and revive the flux, improving the use cycles for further applications making it another attractive feature for cost effective technologies.

3.2.2 Cellulose Membrane Modification

Two different concentrations (39 mM and 10 Mm) of BIBB initiator were immobilised onto the membrane surface. Following initiation, FTIR was carried out to examine the

membrane's surface chemical composition (Figure 46). An overlapping image between the two samples suggests that the same amount of initiator is fixed onto the membrane surface, despite the difference concentrations of BIBB used.

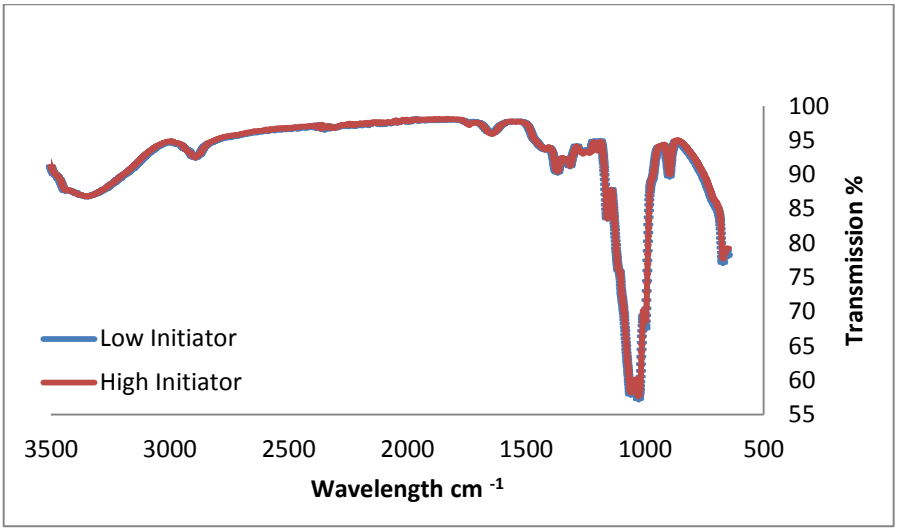


Figure 46: FTIR of membrane surface following initiator immobilisation

Characteristic cellulose peaks of symmetric C-H bonds at 2,940 cm^{-1} and asymmetric C-H bonds at 2,850 cm^{-1} are observed, followed by a stretching asymmetric C–O–C bridge represented by a peak at 1,110 cm^{-1} (Worthley et al., 2011). The introduction of the BIBB is reflected by an enhanced peak at 1,030 cm^{-1} which signifies an ester group (C-O=C) (Ali, et al.,2010) on the membrane surface, caused by esterification of the hydroxyl groups. Both sample peaks overlap at this point, showing there is an equal amount of initiator immobilisation, which led to the lower concentration method to be used going forward, as a cost effective measure.

ATRP was carried out to graft tBa poly brushes onto the membrane's surface. This reaction was allowed to proceed for one hour only and the degree of grafting was consistent at 40.73 $\mu g/cm^2$ for each of the samples (Figure 47).

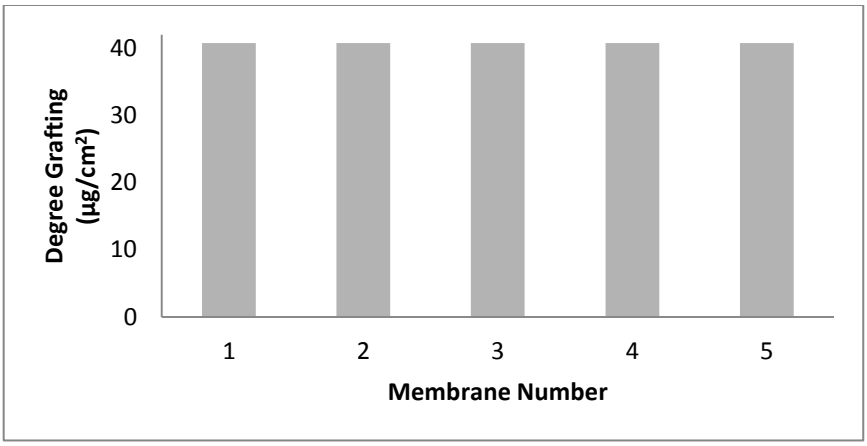


Figure 47: Degree of grafting following 1hr ATRP

The presence of poly(AA) on the surfaces of the hydrolysed membranes has been confirmed through FTIR analysis (Figure 48). The broad peak observed at 3,000 cm^{-1} shows the presence of an –OH group introduced after ATRP. This demonstrates that grafting of the hydrophilic tert-Butyl acrylate (tBa) was successful.

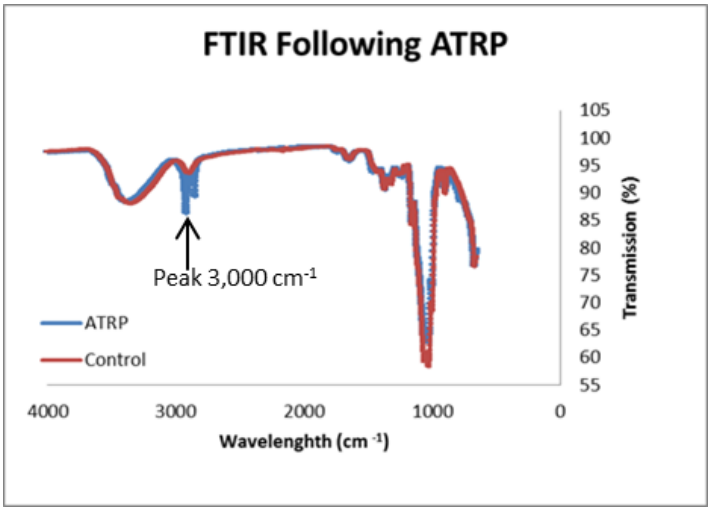


Figure 48: FTIR spectra of membrane before and after ATRP

To provide active sites for immobilization of laccase, conversion of poly (tBa) to polyacrylicacid poly(AA) brushes was carried out through selective acid hydrolysis. A local decrease in transmission at 1,640 cm^{-1} (Figure 49) corresponds to a decrease in H-O-H interactions.

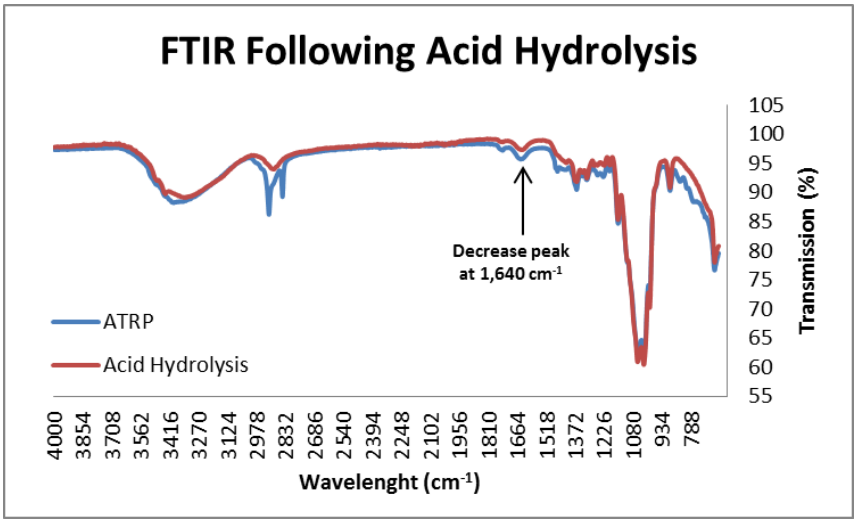


Figure 49: FTIR spectra of membrane before and after acid hydrolysis

Acid hydrolysis provides for a reaction with ester groups on the membrane surface to produce carboxylic acid (COO-) groups (Figure 50).

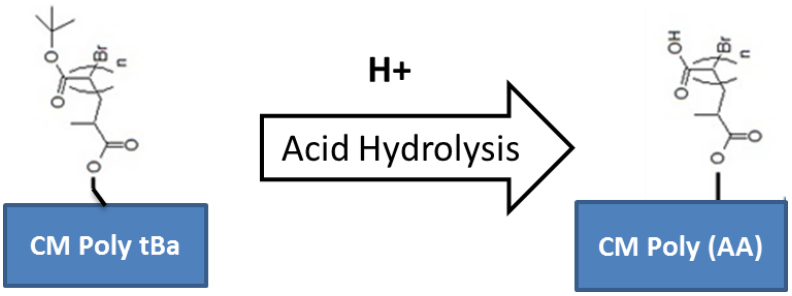


Figure 50: Conversion of poly(tBa) to poly(AA) using acid hydrolysis

3.2.3 Enzyme Immobilization

The polymeric brushes contain functional groups to which the enzyme may be bound using a physical adsorption process.

Laccase enzyme was dissolved in a pH 4 buffer, close to its pI (4.15) and a stock concentration of 1.8 mg/mL was made up to test enzyme immobilisation to both functionalised and un-functionalised membranes.

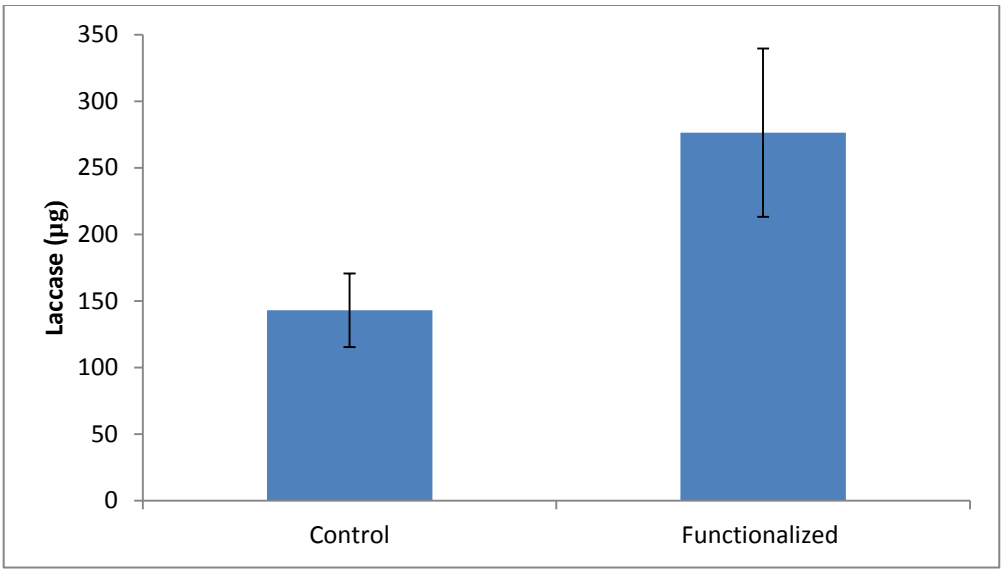


Figure 51: Laccase immobilised onto control and functionalized membranes

Following immobilisation, an average of 276 µg of enzyme bound to the functionalized membrane's surface (Figure 51).

Table 4: Enzyme Immobilization

	Average Amount of Enzyme Bound	
	Total Membrane (mg)	***Total Enzyme /membrane (mg/ mg)***
Functionalised Membrane	0.255	0.0015
Control Membrane	0.143	0.0008
p-value Comparison	0.0141	0.0139

A p-value of 0.0139 is found when comparing the amount of enzyme bound to the test membrane (0.0014 mg/mg) to the control (0.0008 mg/mg) (Table 4) indicating that there is a significant difference between the control and functionalized membranes, therefore grafting of polymer brushes enhanced the membrane enzyme immobilization ability.

The enzyme-bound-membrane (EBM) was tested against a range of pH (5, 6, 7) and temperatures (10, 22, 30 ^{o}C), and then contrasted against the free form, to see if immobilisation gave an enhanced stability. All data points were recorded after the oxidation of ABTS was allowed to proceed for one hour.

To investigate whether immobilisation improved laccase stability, the enzyme's activity was examined over a range of temperature and pH values. The activity was observed by its' ability to oxidise ABTS, the increase in absorbance at 410nm was recorded every 10 min for 1 hr. All assays were performed in duplicate.

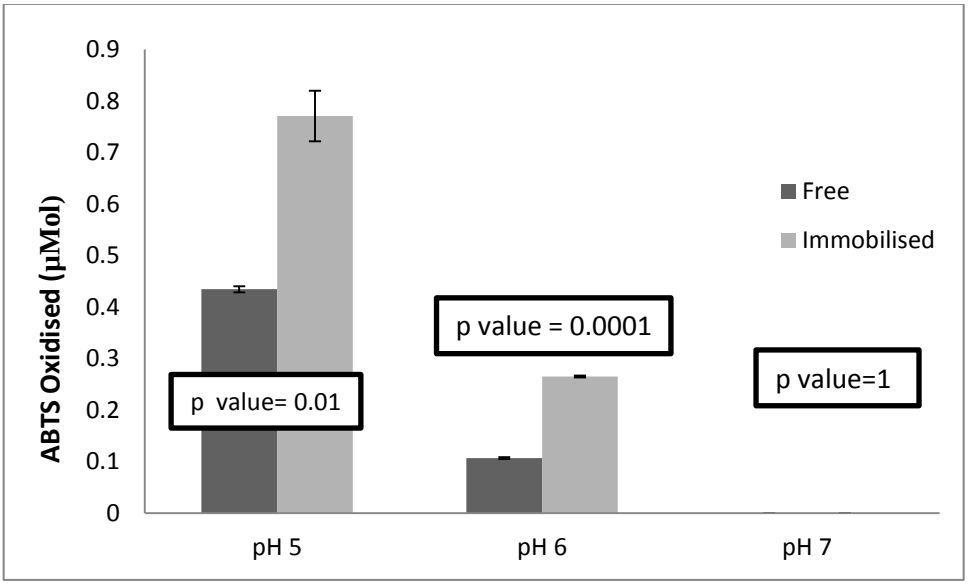


Figure 52: Oxidation of ABTS using Free and Immobilised Laccase at 10^{o}C

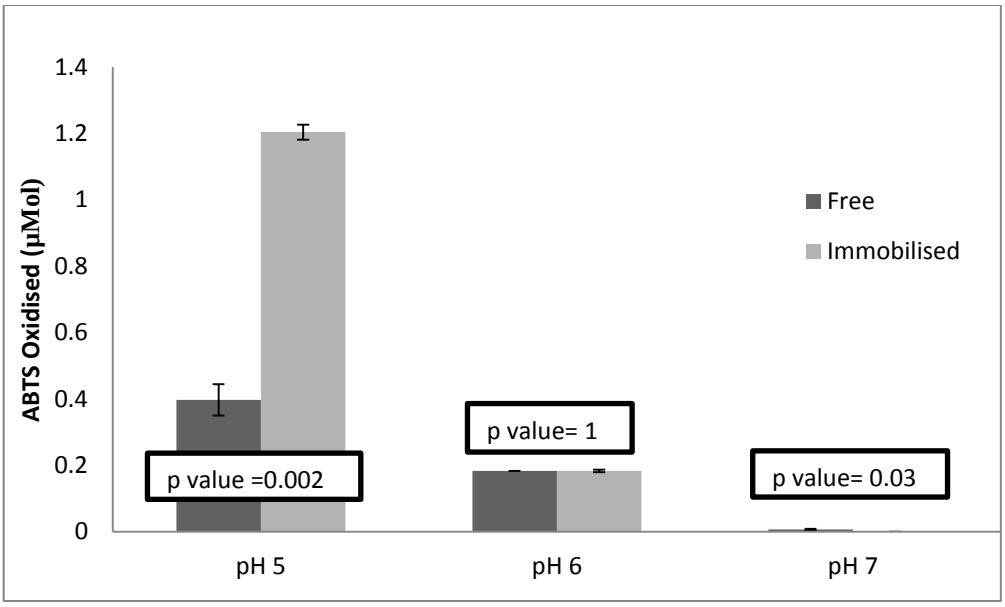


Figure 53: Oxidation of ABTS using Free and Immobilised Laccase at 22°C

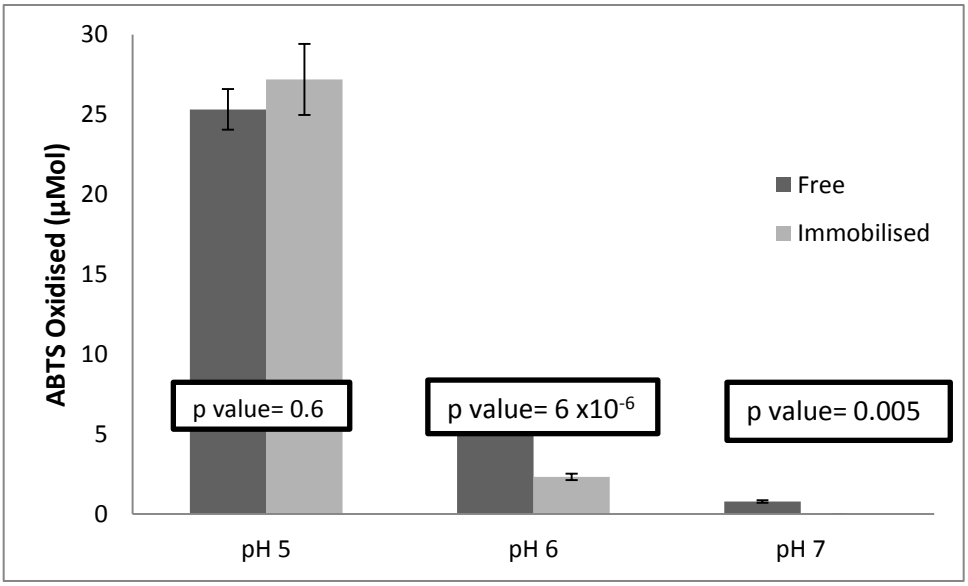


Figure 54: Oxidation of ABTS using Free and Immobilised Laccase at 30°C

At the lowest temperature examined (10 °C), the immobilised enzyme had enhanced ability to oxidise 56% more ABTS over the free form in pH 5 (p value 0.01) and 41% more at pH 6 (p value 0.0001) (Figure 52), however at pH 7 there was no activity observed from either forms of the enzyme. Other studies have revealed that laccase has little or no activity at pH

7, so this is not uncommon (Margot et al., 2013). Overall, it is clear that following immobilisation laccase has higher activity at the cool temperature of 10 ^{o}C, in comparison to the free form, consequently the membranes have enhanced the enzymes stability.

Similarly at room temperature 22 ^{o}C, the immobilised enzyme has a heightened activity (33% more) in comparison to the free form at pH 5 (Figure 53). This is a significant difference, supported by a p value of 0.002, demonstrating the enhancement of enzyme stability via immobilization to the membrane. However, upon observation at pH 6, there is no significant difference between the different enzyme forms; both their activities are equal, confirmed by p value 1. At pH 7 a small amount of activity is recorded for the free enzyme, but nothing for the EBM (enzyme bound membrane). These conditions are unfavorable for the enzyme activity.

At the highest temperature studied (30 ^{o}C), the most enzyme activity took place at pH 5 (Figure 54), in comparison to all the different pH and temperature ranges. This indicates that these are the ideal set of parameters to get the most activity when using the enzyme. Under these conditions, there was no significant difference in activity documented between the free and immobilised enzyme forms, supported by a high p value of 0.06. Immobilisation of the enzyme negatively impacted the stability of the enzyme's activity at pH 6 and pH 7 at this temperature; with the free form having more 43% more activity at pH 6 (p value 6 x10^{-6}) and 90% at pH 7 (p value 0.005) in comparison to the EBM.

A time dependent study was carried out to examine at what point the enzyme reached the most activity. Data was recorded every 10 min (Figure 55, Figure 56 and Figure 57).

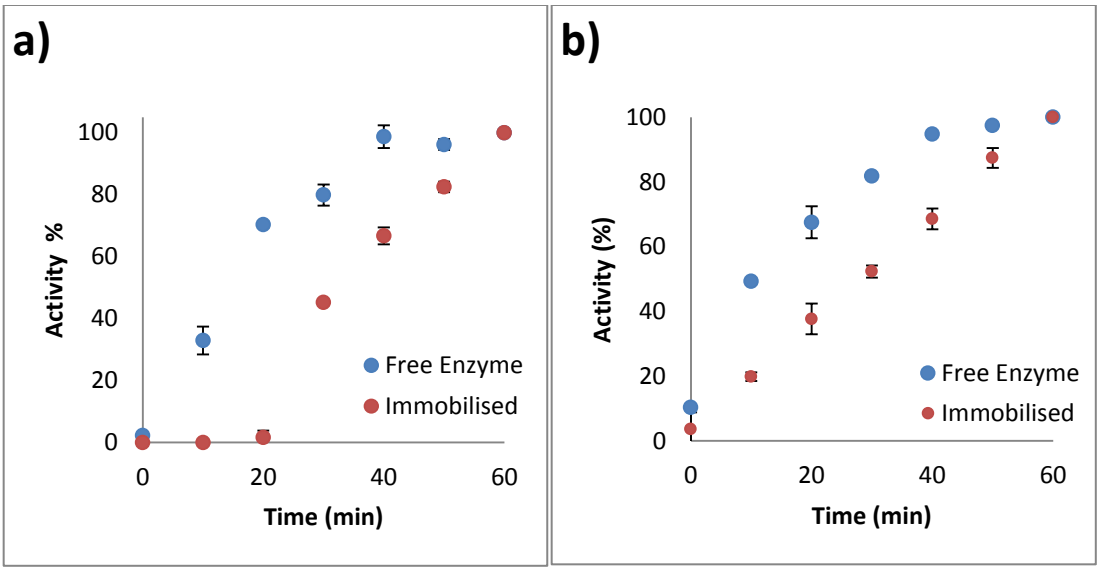
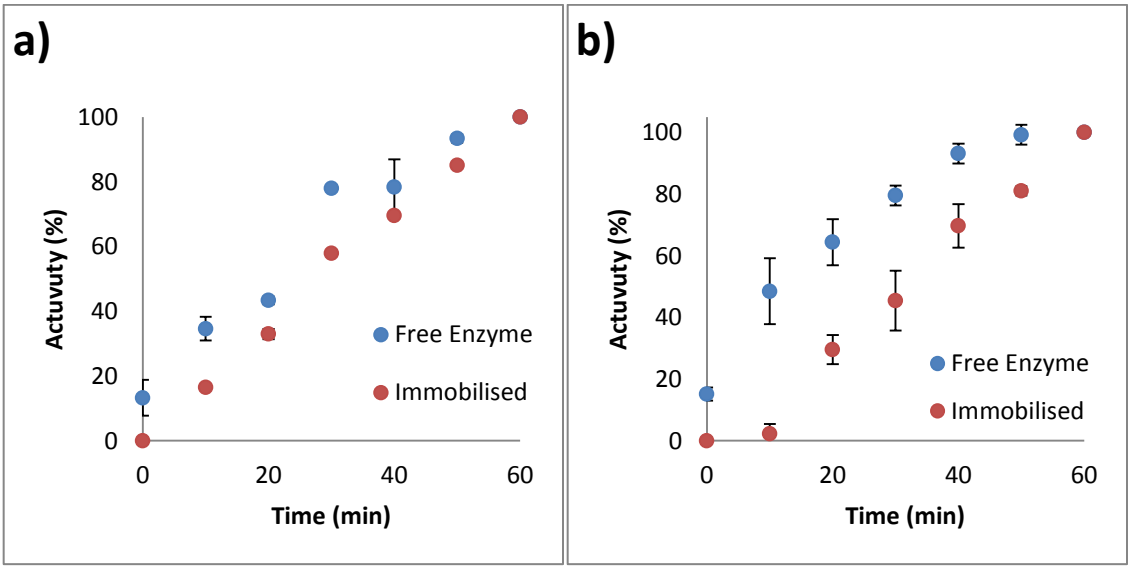


Figure 55: Laccase Activity at 10°C in pH 5 (a) and pH 6 (b) (samples in duplicate)

At 10 °C the highest activity observed in every situation (pH 5 and pH 6) occurs after 60 mins, therefore an increase in time results to an increase in enzyme activity. There was no decline in activity either, which indicates that the enzyme has not reached a saturated state and possibly would still be active if another data point was recorded. As seen before, no activity was observed at pH 7, but both forms of enzyme displayed activity at 30 °C in pH 7.



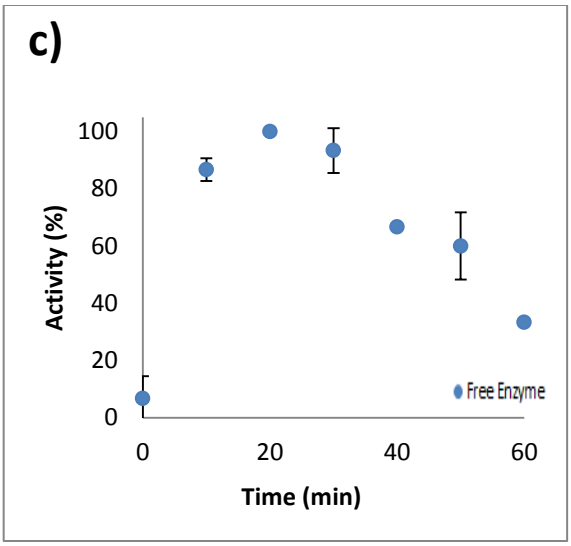
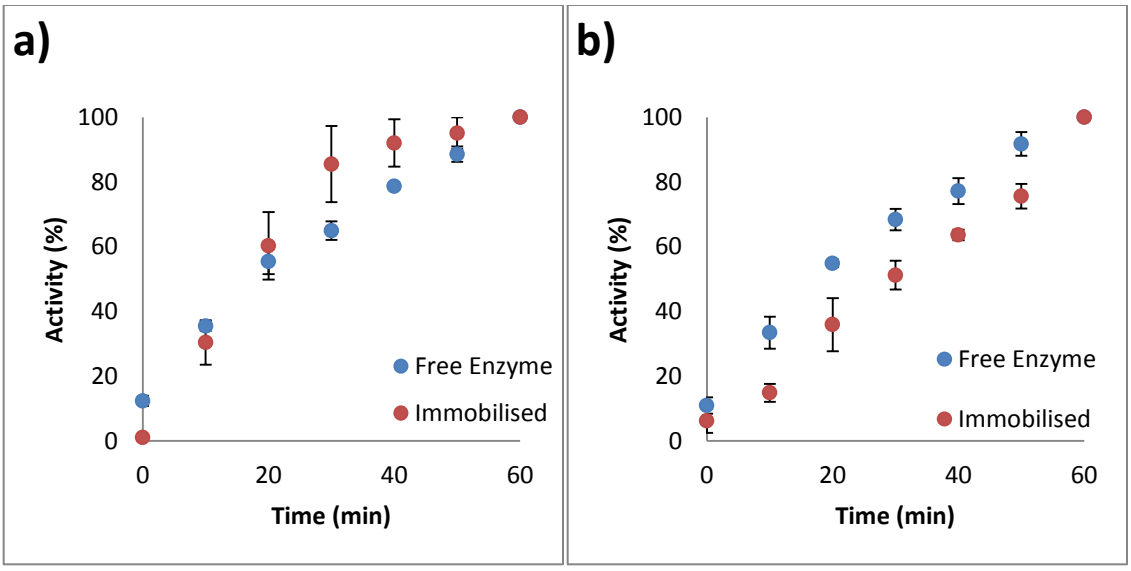


Figure 56: Laccase Activity at 22°C in pH 5 (a), pH6 (b) and pH 7 (c) (samples in duplicate)



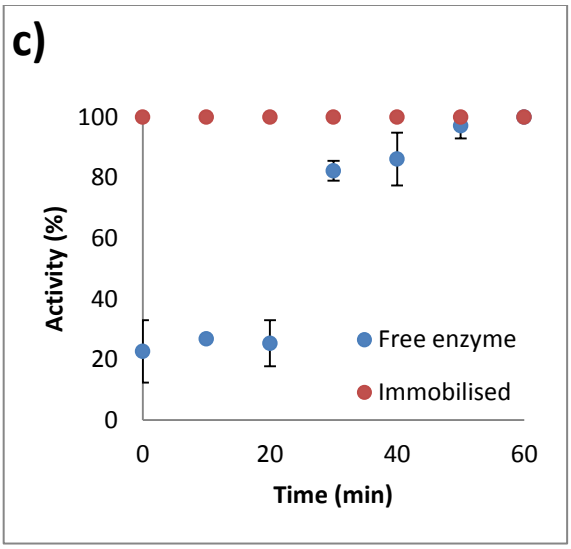


Figure 57: Laccase Activity at 30°C in pH 5 (a), pH6 (b) and pH 7 (c) (samples in duplicate)

Similar results were observed at 22 °C and 30 °C, with increased activity observed over time. There was only activity observed for the free enzyme form at pH 7 at 22 °C, which was also observed in earlier results. Upon closer examination of the enzymes ability to oxidize ABTS, the data reveals that a pH shift from 5 to 6 or 7 leads to a lower conversion of substrate to product, in all temperatures. This is because enzymes have optimal pH and temperature range, for this activity study, the most oxidation is achieved at 30 °C in pH 5. At these conditions, there is no significant difference in activity between the free and immobilised form of the enzyme, indicating that immobilization of laccase onto a membrane substrate is a promising tool.

3.2.4 Diclofenac Breakdown

Treatment of waste water may be done at various temperatures and pH levels, it is therefore important to examine a variety of conditions so that these EBM can be suitable for industrial applications. Heating the water to 30 °C or cooling to 10°C would increase the energy cost. As such, testing of Diclofenac breakdown in water was tested at normalized water conditions; pH 7 and 22°C. Although there was no activity observed during ABTS oxidation under these conditions, studies by Lloret, et al., (2010; 2013) show that the optimal pH for Diclofenac breakdown using laccase is achieved using a pH between 4 and

5. During their studies of the oxidation of ABST at pH 7, the enzyme only maintained 3% activity compared to pH 4. Similarly, a significant decrease in activity close to zero at pH 7 was observed by Ramírez-Cavazosa et al., (2014). Although the enzyme is still active at pH 7, there is a compromise between its stability and activity; an increase in pH, decreases the enzyme's activity. After testing the EBM stability, these membranes were put forward to test their ability to breakdown Diclofenac (DCF) in a spiked water source. The optimum pH and temperature were not chosen for this experiment, instead the conditions were carried out at 22 ^{o}C in pH 7 which only had 1% of the activity compared to the membranes preferred conditions. These settings were chosen because they imitate waste water treatment conditions (Margot et al., 2013) which is the aim of the application. A sample of the Diclofenac (DCF) solution was tested before and after the addition of the EBM and taken for HPLC analysis to quantify the anti-inflammatory drug.

The enzyme bound membrane's ability to break down Diclofenac was analysed using HPLC. After 2hrs, 96 % of DCF was degraded (Figure 58 and Figure 59). The test was carried out in duplicate. No more degradation was observed after 24 hrs and the control showed no breakdown of the drug.

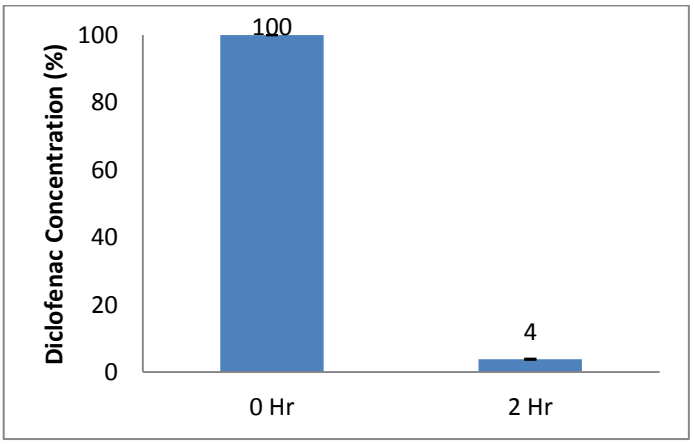


Figure 58: Diclofenac breakdown using immobilised enzyme after 2 hrs

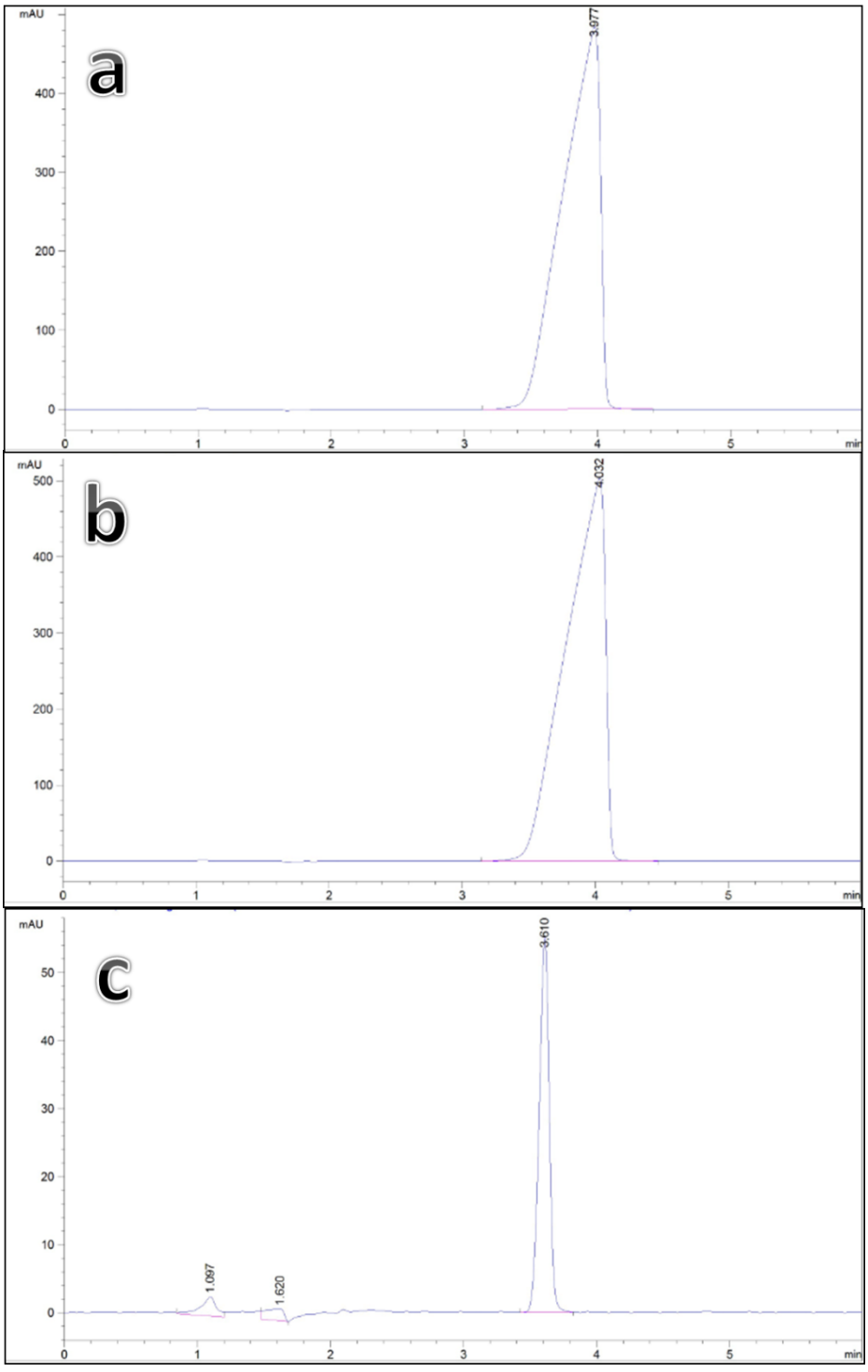


Figure 59: HPLC analysis of Diclofenac Breakdown; a) 0 hr b) 2 hr control and c) 2hr Enzyme Bound Membrane treated water

After the 2hr reaction commenced, Figure 59 shows a dramatic reduction in the DCF peak area from the initial solution (Figure 59a) in comparison with the EBM treated sample (Figure 59c). The control showed no change in peak area after 2 hrs (Figure 59b.) indicating that the breakdown of the drug was due to the EBM treatment. Overall 96% of Diclofenac was degraded, which started with an initial concentration of 43.3 ppm and decreased to 1.7 ppm (Figure 58). These results indicate that under normal drinking water treatment conditions, these novel EMBs can be used to effectively degrade the hazardous drug Diclofenac from aqueous solution.

4 Conclusions

Membrane chromatography/membrane adsorber technology is of great interest for bioseparation applications due to the potential for improved process economics, efficient contaminant removal, superior large molecule capture, high flow rates at low pressure drop, ease of scaleability and process flexibility. Polymer brush grafted membranes are particularly suited for immobilization of biomolecules because they possess a well-defined structure, excellent mechanical stability and dense functional groups.

Surface initiated atom transfer radical polymerization (SI-ATRP) was used to successfully graft hydrophilic polymer brushes onto membrane surfaces, which were capable of immobilizing GFP and laccase enzyme. Initiator immobilisation was achieved through esterification of the hydroxyl groups present on the membrane surface with α-Bromoisobutyryl bromide (BIBB). The presence of poly(AA) on the surfaces of the hydrolysed membranes has been confirmed through FTIR analysis. Conversion of poly(tBa) to poly(AA) was carried out through selective acid hydrolysis, confirmed by decrease in peak area, corresponding to a decrease in hydrogen bonding interactions, due to the presence of poly(AA).

Ultrafiltration chloromethylated polysulfone (CMPSf) membrane adsorbers grafted with polyacrylic acid polymer brushes for binding of green fluorescent proteins were fabricated using the non-solvent induced phase separation (NIPS) method. The polymer bushes enhanced the hydrophilicity of the membranes shown by an increased flux from 65 $L/m^2/hr$ to 71 $L/m^2/hr$, also confirmed by a decrease in water contact angle from 80^o to 69^o, this improved property of the membrane will assist in quicker processing times during UF. Despite the most GFP binding to the control membrane totaling to 37$\mu g/cm^2$, there was adsorption of the protein on all the polymer brush membranes of up to 24$\mu g/cm^2$. These results suggest that the protein may have bound non-specifically to the control membrane. As more protein bound after more brushes were grafted, further studies of increase grafting could increase the binding of GFP, meaning tailor made separating membranes could be a possibility. Furthermore, the stability of the protein was not affected, supported by fluorescent microscopy.

Recent studies by Audouin & Heise, (2014) have bound GFP to polyHIPEs using a chemical method here, non-chemical alternative techniques are presented, with potential for a wide range of applications such as screening, micro-arraying and cell arraying (Gautrot et al., 2010). The produced CMPSf membranes have strong potential for the selective separation of proteins in downstream processing. Novel polymeric brush membranes with immobilised glycospecific lectins can be developed using this method that would allowing the selective separation of mixtures of glycoproteins, which has strong commercial potential.

Hydrophilic polymer brushes grafted onto commercial cellulosic microfiltration membrane surfaces were capable of immobilizing laccase enzyme, following SI-ATRP. In comparison to the control membrane (143 μg/cm^2), there was significantly more laccase adsorbed following addition of polymer brushes (276 μg/cm^2). This is a due the architectural structure of the brushes, being able to branch out and bind more enzyme. In almost all cases, the laccase enzyme had enhanced stability over the free form in varying temperatures at pH conditions. Another benefit of immobilizing the enzyme onto a solid support is the ability to reuse the membrane. The optimal pH and temperature range examined where the most oxidation is achieved at 30 ^{o}C in pH 5. At these conditions, there is no significant difference in activity between the free and immobilised form of the enzyme, indicating that immobilization of laccase onto a membrane substrate did not hinder its stability and thus, can be used as a substitute to the free form, without any compromise.

Enzyme-catalyzed breakdown of pharmaceutical pollutants has been shown to be an environmentally friendly alternative for treatment of polluted waters (Ufarté et al., 2015). Laccase enzymes are used to oxidize phenol and non-phenol compounds by cleaving ester bonds, degrading pharmaceutical waste into harmLess substances. The novel laccase immobilised polymer brush membrane was successful in the removal of 96% traces of Diclofenac after only 2 hours. Other studies have shown complete deterioration of Diclofenac using conditions closer to the enzymes optimum pH and temperature (Sathishkumar et al., 2012). The conditions applied for the breakdown of Diclofenac here were a reflection on what was expected to be seen naturally at a waste water treatment

facility; pH 7 and 22^{o}C. Treatment of water using these membranes alone, provide a low cost strategy with little energy required, that is saleable and easy to use.

In conclusion brush grafted membranes for protein immobilization have been developed in a cost effective manner with enhanced properties, such as decreased protein binding, increased hydrophilicity and the potential for selective binding capabilities. Immobilization of targeted proteins and enzyme has been achieved through using a non-chemical method. Potential applications for these membranes have been discussed throughout the course of this work. Future work on optimizing the grafting density, to tailor binding abilities and testing the membranes using a crude lysate samples and genuine waste water samples will add significant value to this research.

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