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FOULING INDUCED ENZYME IMMOBILIZATION USING ULTRA FILTRATION MEMBRANE THROUGH TANGENTIAL FLOW FILTRATION: ADVANCES AND IMPLICATIONS IN BIO-PHARMACEUTICAL DOWNSTREAM PROCESSING

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ABSTRACT

The biopharmaceutical industry is one of the most scientifically demanding fields in healthcare, focused on the production of therapeutic proteins, monoclonal antibodies (mAbs), nucleic acids, and other biologically derived agents. These products are typically generated using recombinant DNA technology, cell culture, or microbial fermentation. Due to their complexity and sensitivity, maintaining high product yield, purity, and integrity during downstream processing (DSP) is essential. However, membrane fouling, especially biofouling, remains a significant challenge in DSP operations such as tangential flow filtration (TFF), ultrafiltration (UF), and diafiltration (DF), impairing filterability and membrane longevity. To address this, enzyme immobilization has emerged as a promising strategy, particularly when integrated with membrane-assisted systems. Immobilizing enzymes onto or within membranes enhances enzyme stability, supports reusability, and enables coupling of catalytic and separation functions. A novel approach gaining attention is fouling-induced immobilisation, which utilises natural fouling phenomena, such as pore blockage, cake layer formation, and surface adsorption, to entrap enzymes without requiring chemical modification. TFF is especially well-suited for such applications due to its ability to regulate shear stress and control fouling dynamics. Innovative membrane configurations, such as the ―sandwich mode,‖ where a porous support is layered above the membrane, have demonstrated improved enzyme retention and sustained flux. The choice of membrane material also plays a key role: hydrophilic regenerated cellulose supports compatibility with enzymes, while polysulphone membranes promote adsorption, albeit with greater fouling risks. TFF thus serves as both a filtration and biocatalysis platform in modern bioprocessing.

KEYWORDS: Biopharmaceuticals, Enzyme Immobilization, Tangential Flow Filtration, Membrane Fouling, Downstream Processing, Fouling-Induced Immobilization.

1. INTRODUCTION

The biopharmaceutical sector, a pivotal component of modern healthcare, focuses on the production of medicinal products derived from biological sources, such as proteins, nucleic acids, cells, or tissues (Walsh, G., 2013). Biopharmaceuticals are generally huge, intricate entities in contrast to conventional medications, and are produced using advanced biotechnology methods such as recombinant DNA technology, cell culture, and fermentation (Bhatia, S. and Goli, D., 2018). Biopharmaceutical products require meticulously regulated manufacturing settings due to their inherent sensitivity and susceptibility to deterioration or contamination, with downstream processing (DSP) being a vital step for product purification, recovery, and formulation.

Ultrafiltration/Diafiltration (UF/DF) utilizing membrane-based technology has become essential for the concentration and buffer exchange of therapeutic proteins among DSP procedures (Saxena et al., 2009). Tangential flow filtration (TFF) provides notable benefits by enabling the feed to traverse tangentially over the membrane surface, thus minimizing direct pore obstruction and prolonging membrane longevity (Musumeci et al., 2018). Nonetheless, membrane fouling and protein aggregation continue to pose significant problems that can undermine both output and product quality.

The fouling behaviour during ultrafiltration/diafiltration is affected by various parameters, including protein molecular weight, glycosylation patterns, viscosity, and intermolecular interactions. Operating parameters, including transmembrane pressure (TMP), pH, ionic

strength, and shear rate, significantly influence the degree and cause of fouling (Delechiave, G., 2024). For example, increased TMP may facilitate convective transport while concurrently augmenting surface deposition, whereas neutral pH conditions may promote protein entrapment by hydrogen bonding (Wang et al.2023). Therefore, achieving the optimal equilibrium in operational environments is essential for the effective and consistent processing of biologics.

The choice of membrane molecular weight cut-off (MWCO) is crucial, as it determines the retention of therapeutic proteins while allowing for the elimination of contaminants, salts, or solvents. An inadequate selection of MWCO or operational parameters can intensify concentration polarization, facilitate irreversible adsorption, or perhaps lead to product denaturation (Imbrogno et al., 2025). Therefore, a comprehensive understanding of protein-specific fouling tendencies is essential to optimize UF/DF methods for various categories of biopharmaceuticals.

This table highlights representative therapeutic proteins and monoclonal antibodies (mAbs), detailing their molecular size, typical molecular weight cut-off (MWCO) selection, and observed fouling behaviors during ultrafiltration/diafiltration (UF/DF) operations. This comparative analysis highlights the necessity of customizing membrane and process parameters based on product-specific characteristics to attain sustainable, scalable, and economically viable biomanufacturing.

Table 1: Biopharmaceutical UF/DF Filtration and Fouling Behavior.

Sr. No.	Product	Molecular Size	Typical Membrane MWCO Used	Fouling Behaviour in UF/DF Filtration
1	Trastuzumab	145–148 kDa	30–50 kDa	High fouling (typical IgG, aggregation and concentration polarization).
2	FSH	35.5 kDa	10–30 kDa	Low fouling (small glycoprotein, some membrane adsorption possible).
3	EPO	30–34 kDa	10–30 kDa	Low fouling (small glycoprotein, but glycosylation may cause mild adsorption).
4	Rituximab	~145 kDa (full IgG, not 45 kDa)	30–50 kDa	High fouling (mAb, prone to aggregation and pore blocking).
5	Etanercept	150 kDa	30–50 kDa (sometimes 100 kDa)	Very high fouling (fusion protein, high viscosity at concentration).
6	Denosumab	147 kDa	30–50 kDa	High fouling (IgG2 structure, similar to other antibodies).
7	Bevacizumab	145–148 kDa	30–50 kDa	High fouling (VEGF antibody, aggregation- prone).
8	Romiplostim	60 kDa	30 kDa	Moderate fouling (peptibody, some aggregation, less than IgG).
9	Adalimumab	148 kDa	30–50 kDa	High fouling (fully human IgG, but similar to other mAbs).
10	Dhantumumab	148 kDa	30–50 kDa	High fouling (typical IgG).
11	Pertuzumab	148 kDa	30–50 kDa	High fouling (typical IgG).
12	Aflibercept	148 kDa	30–50 kDa	Very high fouling (fusion protein,
13	Vedolizumab	147 kDa	30–50 kDa	High fouling (antibody, similar to others).
14	Ustekinumab	148.6 kDa	30–50 kDa	High fouling (antibody, similar profile).
15	Pembrolizumab	149 kDa	30–50 kDa	High fouling (antibody, aggregation-prone).
16	Hyaluronidase	61–90 kDa	30 kDa (sometimes 50 kDa for larger glycoforms)	Moderate fouling (enzyme, less viscous but sticky).
17	Tibriribine	0.32 kDa	<1 kDa (nanofiltration/RO, not protein UF)	Minimal fouling (small molecule, passes through membranes).

2. Fouling Mechanism

Biofouling manifests through diverse mechanisms, broadly classified into particulate, organic, inorganic (scaling), and biological categories. Mechanistically, this includes pore blocking, cake layer formation, adsorption of biomolecules, and biofilm development (Gizer et al., 2023). These processes result in increased transmembrane pressure (TMP), reduced flux, and unpredictable selectivity shifts all of which compromise the consistency and reliability of GMP- compliant biomanufacturing. Despite design advances such as tangential flow geometry and hydrophilic membrane coatings, fouling remains inevitable due to the complex and variable nature of biological fluids.

One of the major bottlenecks in downstream operations is membrane fouling, particularly biofouling, a pervasive problem during filtration steps such as tangential flow

filtration (TFF), ultrafiltration, and diafiltration. While TFF is preferred over dead-end filtration for its capacity to reduce surface buildup via tangential fluid flow, the technique remains susceptible to the progressive accumulation of foulants that reduce membrane permeability, increase pressure differentials, and compromise product yield and purity.

2.1 Types and Mechanisms of Biofouling

Biofouling can be categorized into four main types, depending on the nature of the foulants and their interactions with membrane surfaces

1. Particulate Fouling: It entails the build-up of colloidal material or aggregations that may clog the pores or deposit on the membrane surface.
2. Organic Fouling: Due to proteins, lipids, nucleic acids, and polysaccharides that stick to membrane surfaces or penetrate through pores.
3. Scaling (Inorganic Fouling): It is caused by the precipitation of salts like calcium phosphate or magnesium carbonate.
4. Biological Fouling: Occurs through microbial adhesion, growth, and subsequent biofilm development. Biofouling reduces throughput, causes product entrapment, and lowers yields, leading to compromised batch recovery.

It increases costs by requiring frequent membrane replacement, more cleaning cycles (CIP/SIP), and longer processing times. It destabilizes processes by reducing selectivity and raising transmembrane pressure (TMP), which lowers separation efficiency and reproducibility. It also promotes biofilm formation, heightening the risk of microbial contamination and threatening sterility assurance levels (SAL) in aseptic processing.

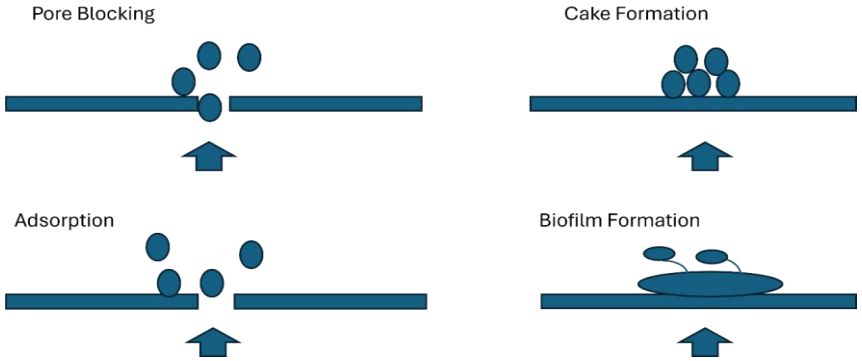

Figure 1: Schematic illustration of fouling mechanisms pore blocking, adsorption, cake formation, biofilm development.

2.2 Impact on Biopharmaceutical Operations

Biofouling has significant implications for both process performance and regulatory compliance, as it can cause product loss through reduced throughput and product entrapment, leading to lower yields and compromised batch recovery. It also drives cost escalation due to the need for frequent membrane replacement, increased cleaning cycles (CIP/SIP), and extended processing times, all of which raise operational expenditure. Furthermore, biofouling contributes to process instability by diminishing selectivity and increasing transmembrane pressure (TMP), which negatively impacts separation efficiency and reproducibility. In addition, the formation of biofilms elevates the risk of microbial contamination and can compromise sterility assurance levels (SAL), posing serious concerns in aseptic processing environments. Collectively, these factors can undermine Good Manufacturing Practices (GMP) and delay product release, highlighting the urgency of antifouling innovations.(Saxena et al., 2009).

3. Enzyme immobilization Strategies

Enzyme immobilization on or within membranes can improve enzyme stability, but it often results in reduced permeability. For instance, Sen et al. observed a decrease in ultrafiltration (UF) membrane permeability by 19–87% following the covalent immobilization of β-galactosidase enzymes onto the membranes. Similarly, Giorno et al. reported that entrapping fumarase within the spongy layer of a capillary membrane (at 0.009–0.052 mg cm^2) caused a significant permeability reduction of 43–84%, attributed to pore blockage in the membrane.

Alcohol dehydrogenase (ADH) was used as a model enzyme. Decreasing the pressure, increasing the concentration of the enzyme, and reducing the pH increased the irreversible fouling resistance and reduced the permeate flux. High pH during the immobilization led to increased permeate flux but decreases in the rate of conversions, possibly due to the low immobilization caused by strong electrostatic repulsion between the enzymes and the membrane. The results indicated that the pore blocking as a fouling process allowed increased loading of the enzymes but caused greater permeability decline, whereas cake layer formation enhanced the stability of the enzymes but led to low loading rate.

Low pH (approximate isoelectric point) supported the adsorption of enzymes on the membrane through hydrophobic and electrostatic interaction, reducing the stability of the enzymes. Neutral pH, however, favored

entrapment and association of enzymes on the membrane via hydrogen bonding, which enhanced the stability of the enzymes. The study indicates that a compromise between various fouling/immobilization processes needs to be achieved to maximize the immobilization performance both in terms of loading of the enzymes as well as the activities of the enzymes.

3.1. Strategies for Enzyme Immobilization

Enzyme immobilization is a cornerstone technique in modern biocatalysis and bio-separation, enabling the reuse of enzymes, enhancing operational stability, and integrating catalytic functions into filtration or reaction systems. In the context of membrane immobilized enzymes not only mitigate fouling by degrading deposited bio-foulants but also transform passive filtration surfaces into bio-functional interfaces. These interfaces support in situ conversion, selective bioprocessing, and self-cleaning dynamics. This section explores the physicochemical mechanisms of immobilization, outlines advanced material strategies like polydopamine (PDA), TA/APTES nanospheres, and dye-ligand affinity systems, and describes how these are utilized in modern bioreactor applications.(Luo et al., 2014)

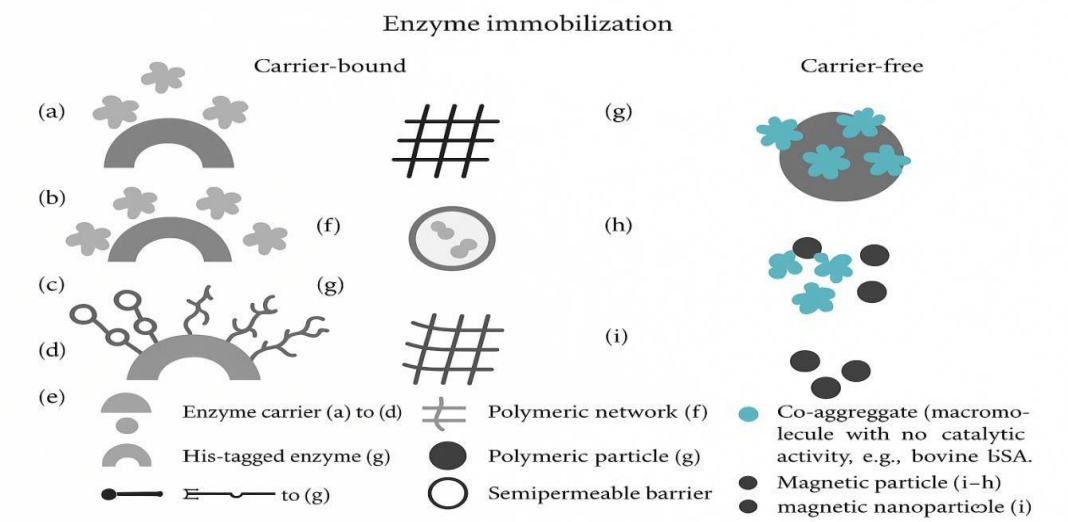

Figure 2: Schematic illustration of enzyme immobilization.

Fouling, which was once regarded solely as a limitation in membrane operations, is now being strategically utilized for beneficial purposes. Luo et al. (2014) reported that enzyme fouling can be effectively controlled by adjusting filtration parameters, enabling the immobilization of enzymes either on the membrane surface or within its pores. This process is primarily governed by four key fouling mechanisms. The first is pore blocking, where enzymes adsorb inside or near the membrane pores. The second involves aggregation and cake layer formation, in which enzymes accumulate on the membrane surface to form a deposit. The third is concentration polarization, where the build-up of enzymes near the membrane surface influences their transport and distribution. Finally, adsorptive interactions, which are significantly affected by factors such as pH, ionic strength, and the physicochemical properties of the membrane surface, also play a crucial role in the immobilization process.

3.2. Operational conditions: (pressure, pH, enzyme concentration) influence which fouling phenomenon will predominate. Low pH favors entrapment and hydrophobic interactions, for example, and increased pressure favors cake formation and convective flux.(Hassan et al., 2019)

4. TFF as an Immobilization Platform

Tangential Flow Filtration (TFF) exposes the feed solution tangentially to the membrane surface, creating a boundary layer that allows fine control over concentration polarization and fouling dynamics. Unlike dead-end filtration, TFF offers several advantages, including decreased cake resistance due to crossflow shear, improved enzyme retention through recirculation control, enhanced stability and activity during continuous operation, and precise immobilization achieved by modulating flow rate and pressure. In the study by Goulas et al. (2004), TFF was employed to facilitate the efficient synthesis of isomalt oligosaccharides (IMOs) using co-immobilized dextransucrase and dextranase. This configuration provided multiple benefits, such as the retention of high-molecular-weight enzymes, continuous removal of low-molecular-weight products, sustained enzyme activity over extended operation, and improved productivity through controlled residence times and minimized enzyme deactivation. The combination of enzymatic specificity and membrane selectivity in TFF reactors thus enables not only effective immobilization but also downstream separation, making it a critical tool for integrated bioprocessing.

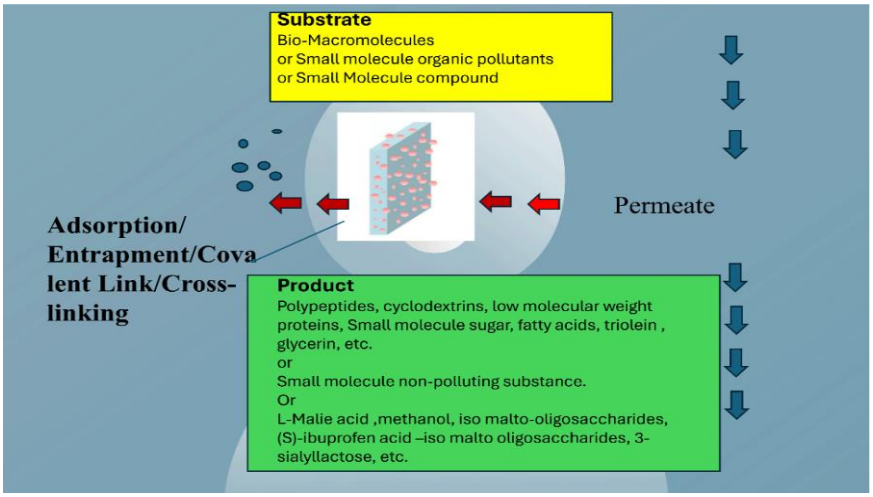

Figure 3: Schematic illustration of TFF (Tangential flow filtration) through enzyme immobilized membrane.

4.1. Enzyme Immobilization as Antifouling and Functional Strategy

To overcome these limitations, enzyme immobilization has emerged as a versatile method to both mitigate fouling and introduce catalytic functionality into membranes. Immobilized enzymes such as proteases, amylases, dextranases, or lipases can degrade foulants (e.g., proteins, EPS, polysaccharides) at the membrane interface in situ, enabling self- regenerating or self-cleaning filtration systems. This strategy enhances membrane longevity, operational stability, and process sustainability, while reducing chemical clean-in-place (CIP) frequency and its associated environmental burden.

4.1.1. Surface Chemistry Enhancements: PDA, TA/APTES, and Dye-Ligand Systems

To improve immobilization yield and functionality, various surface modification strategies have been developed to enhance enzyme–membrane interactions. Polydopamine (PDA) coatings, for instance, introduce catechol and amine groups that enable enzyme attachment through Schiff base formation and Michael addition reactions, while simultaneously increasing membrane hydrophilicity. Similarly, tannic acid/APTES nanospheres provide nanoscale porosity along with a dense array of reactive quinone groups, facilitating multi-point enzyme binding with minimal loss of catalytic activity. Another effective approach is dye-ligand affinity immobilization, which is inspired by affinity chromatography and employs triazine-based dyes or other ligands that mimic natural substrate or cofactor binding sites on the enzyme. This method not only anchors enzymes firmly to the surface but also helps orient them in an active conformation, thereby preserving and enhancing their functional performance. These methods offer biochemical specificity, mechanical stability, and tunable activity retention, depending on the nature of the enzyme and the application.

4.1.2. Fundamental Mechanisms of Enzyme Immobilization

Immobilization can be defined as the physical confinement or localization of enzymes on solid support while preserving their catalytic activity. Immobilization strategies are typically categorized based on the nature of the enzyme-support interaction.

4.1.3. Covalent Bonding

Covalent immobilization involves the formation of stable chemical bonds between functional groups on the enzyme, such as amine, carboxyl, or thiol groups, and reactive sites present on the support material. While this method provides strong and durable attachment, it may also lead to certain challenges, including rigidification of the protein structure, blockage of the enzyme's active site, and non-specific orientation that can reduce catalytic efficiency. Common coupling strategies used in covalent immobilization include carbodiimide activation (EDC/NHS chemistry), glutaraldehyde crosslinking, and Schiff base formation, each of which enables effective enzyme anchoring but requires careful optimization to balance stability with activity retention.

4.1.4. Adsorption

Physical adsorption relies on electrostatic, hydrophobic, or van der Waals interactions between enzymes and the support. It is reversible, low-cost, and preserves activity, but prone to desorption and leaching under operational stresses like pH or ionic strength changes.

4.1.5. Entrapment and Encapsulation

Here, enzymes are physically entrapped within a matrix (e.g., sol-gel, polymer beads) or porous membrane. These approaches offer diffusional shielding and biocompatibility but may limit mass transfer and are difficult to scale in flow systems.

4.1.6. Affinity-Based Immobilization

This strategy mimics biorecognition phenomena using tags or ligands to anchor enzymes via specific

interactions (e.g., metal-affinity, dye-ligand). It enables oriented binding, which preserves or enhances activity, and is ideal for membranes with surface-functional affinity groups.

4.1.7. Fouling-Induced Enzyme Immobilization

Unlike traditional strategies that focus on preventing fouling, fouling-induced immobilization intentionally utilizes the fouling process to anchor enzymes at the membrane interface. During filtration, enzymes are deposited within a ―fouling layer,‖ a process that resembles irreversible biofouling but offers distinct functional advantages. This approach eliminates the need for complex coupling chemistry, can be performed directly in-line, and allows for the creation of multi-layered enzyme assemblies on the membrane surface. To better understand this process, Luo et al. (2014) developed mechanistic models of enzyme fouling, which include complete pore blocking, standard pore blocking, and cake filtration, each describing different modes of enzyme deposition and transport behavior within membrane systems. They concluded that standard blocking and cake formation mechanisms facilitated the most effective enzyme entrapment. The study showed that ultrafiltration membranes under pressure could immobilize alcohol dehydrogenase (ADH), which retained catalytic function in converting formaldehyde to methanol.(Goulas et al., 2004).

4.1.8. Process Parameter Influence

Tangential Flow Filtration (TFF), various process parameters have a major impact on performance. Some of these major parameters are transmembrane pressure (TMP), crossflow velocity, and temperature, which have an impact on flux, membrane longevity, and product quality. Optimization entails getting proper balance among these parameters to ensure desired throughput with product integrity.

Transmembrane pressure (TMP) is a key parameter in membrane operations, as it provides the driving force for fluid flow through the membrane. TMP directly influences permeate flux and contributes to the development of a gel layer that causes surface fouling. However, excessively high TMP can lead to the formation of a thick gel layer, reducing flux and potentially compromising product quality. Thus, the optimal TMP must strike a balance between achieving sufficient flux and preventing gel layer formation, with adjustments made according to the specific application and membrane type. Another critical parameter is crossflow velocity, which refers to the movement of feed liquid across the membrane surface.

Higher crossflow velocities help minimise fouling by sweeping away accumulating particles; however, excessively high velocities may cause shear damage to sensitive products. Therefore, optimization requires balancing fouling resistance with product stability. Temperature also plays a significant role, as it affects the solubility and viscosity of the feed solution, thereby influencing membrane performance and flux. The appropriate operating temperature must be chosen according to the application and product, while also considering potential effects on membrane stability and long-term function. Other important factors include membrane selection, where both the material and pore size must ensure efficient separation and purification; buffer composition, which affects both membrane behaviour and product stability; and process integration, where TFF must align seamlessly with upstream and downstream operations. Scalability is also crucial, as parameters optimized at small scale must remain effective in large-scale processes, while proper cleaning procedures are necessary to prevent fouling and maintain consistent performance.

The immobilization process itself is highly sensitive to operational and material conditions. Applied pressure enhances fouling and entrapment but, if excessive, can denature enzymes. pH strongly influences fouling, with maximum deposition typically occurring near the enzyme's isoelectric point, where adsorption is greatest but aggregation also occurs. Ionic strength is another critical factor: moderate salt concentrations reduce electrostatic repulsion and facilitate fouling, whereas high concentrations risk denaturing enzyme structures. Finally, membrane material determines fouling characteristics, with regenerated cellulose offering resistance to protein denaturation and stable fouling layers, while hydrophobic membranes often promote irreversible adsorption. Together, these parameters govern the efficiency and stability of enzyme immobilization in TFF-based systems (Goulas et al., 2004).

5. Functional Surface Engineering for Enhanced Immobilization

Membranes are often modified with bioinspired or nanostructured surface chemistries to enhance the capacity, stability, and activity of immobilized enzymes. Polydopamine (PDA), inspired by mussel adhesive proteins, is a widely used surface functionalization agent that spontaneously forms coatings on a broad range of substrates under mild alkaline conditions. Its binding mechanism relies on catechol and quinone groups, which can undergo Michael addition or Schiff base reactions with nucleophilic residues on enzymes such as $–NH_2$ and –SH groups. Beyond enabling covalent attachment, PDA enhances surface hydrophilicity, thereby reducing nonspecific fouling and promoting a protein- friendly environment. The major advantages of PDA include the absence of toxic reagents, compatibility with aqueous and enzyme-friendly conditions, and its dual functionality as both an antifouling layer and an enzyme anchoring platform. For example, dextranase immobilized on PDA-modified membranes demonstrated altered hydrolysis behaviour, favouring exo-over endo-action, which enabled selective oligosaccharide production (Su et al., 2021).

Another versatile approach involves tannic acid (TA)/APTES nanospheres. TA, a plant-derived polyphenol, can chelate metal ions and crosslink with amines such as APTES to form nanostructured coatings characterized by high surface area, abundant phenolic and amine functionalities, and nanoscale porosity that facilitates efficient mass transfer. These nanospheres interact with enzymes through hydrogen bonding and π–π stacking while offering multiple binding sites to reduce enzyme leaching. Such systems have been applied to glucoamylase immobilization, yielding stable, high-activity membranes suitable for continuous starch hydrolysis (Konovalova et al., 2016). A schematic representation of tangential flow filtration (TFF) operating with enzyme-immobilized membranes is provided in Fig. 3.

Dye-ligand affinity immobilization represents yet another powerful strategy. Dye ligands such as Cibacron Blue mimic nucleotide or coenzyme structures, enabling highly specific binding to enzymes. This method promotes oriented attachment, thereby minimizing steric hindrance and allowing better preservation of enzyme activity. Additionally, it supports affinity regeneration, making it particularly suitable for applications in affinity filtration or chromatography. Dye-ligand immobilization is especially advantageous for NAD^+/NADH-dependent enzymes, such as dehydrogenases used in biotransformation reactors (Goulas et al., 2004; Su et al., 2021).

6. Ultrafiltration membrane Integration and Applications

Fouling-immobilized enzymes have shown promise in various membrane filtration techniques and configurations. In enzymatic membranes for oligosaccharide production, dextranases and amylases immobilized on PDA-modified membranes catalyze the breakdown of polysaccharides while simultaneously allowing size-selective filtration of products.(Su et al., 2021)

Recent research has demonstrated the ability to harness membrane fouling not just as an operational issue but as an immobilization approach for enzymes.(Goulas et al., 2004) By capitalizing on regulated fouling behavior, enzymes can be retained in membrane structures in a stable fashion through chemical and physical forces, obviating the need for complex immobilization chemistries. The extent and nature of fouling are heavily governed by operative variables like transmembrane pressure, enzyme concentration, solution pH, and the physicochemical nature of the membrane. All these variables ultimately dictate not only the membrane's permeability but also the efficiency of enzyme retention and long-term stability of catalytic function.

Experiments on alcohol dehydrogenase (ADH) as model enzyme demonstrated that at low transmembrane pressures (1–2 bar), high enzyme loads (0.2 g/L), and near-isoelectric point pH (pI 5.4–5.8), significant irreversible fouling and considerable loss of permeate flux occurred.(Luo et al., 2014). These conditions maximize hydrophobic and electrostatic adsorption of enzymes for higher loading but at the expense of stability. At neutral pH, immobilization was dominated by hydrophobic and physical entrapment, which ensured higher enzymatic stability and lower risk of enzyme leaching. Conversely, alkaline pH disrupted enzyme-membrane adhesion due to electrostatic repulsion and caused lower immobilization efficiency and conversion despite better permeate flux.

Separate fouling processes were found to impact immobilization differently. Blocking of pores enabled greater enzyme loading density at the expense of significant loss in permeability. In contrast, exterior cake layer development established a more constant microenvironment for enzyme support, enabling extended durations of action at the expense of immobilization capacity. These trade-offs highlight the necessity of balancing enzyme loading against both the function of the enzyme and membrane throughput to maximise immobilisation performance.(Goulas et al., 2004). Membrane structure and operation orientation also influenced fouling behavior. Reverse orientation of the membrane, having the more permeable support layer facing the feed, promoted enzyme penetration and localized fouling in the membrane's structural network. A polypropylene support layer placed under the skin layer in a "sandwich" structure served to reduce compression and preserve structural integrity under pressure. Through deliberate fouling profile manipulation, immobilization environments favoring high enzyme retentivity and catalytic durability can be engineered. In changing fouling from constraint to asset, it allows for enzyme immobilization via scalable and simple non-covalent, physical means. Consolidating insights from fouling proteins' research in general and ultrafiltration at isoelectric points in particular allows for better understanding of how adsorption, entrapment, and hydrodynamic forces can be utilized to create functionally active and stable enzyme films. In the end, controlled fouling immobilization presents a low-complexity tunable platform for optimizing enzyme function in various applications.

Three membranes GR61PP, GR51PP from Alfa Laval, and PLTK were selected for testing because of their distinct features and potential applications. The GR51PP membrane had a molecular weight cut-off of approximately 50 kDa and consisted of a polypropylene support with a polysulphone skin layer. Its thickness was around 300 micrometers, with an isoelectric point ranging between 4 and 5, and a permeability of about 45.2 $L/m^2 \cdot h \cdot bar$, subject to some variation.

Likewise, Alfa Laval's GR61PP was also created at a lower MWCO of 20 kDa with the same support and skin materials as the GR51PP. A bit thicker at 350

micrometers in thickness, the membrane had an isoelectric point of between 5 to 6 and was found to have a permeability of 52.1 L/m²·h·bar through measurement, although with high variation. Compared to it, the PLTK membrane supplied by Millipore had a different profile. Having MWCO of 30 kDa, it had regenerated cellulose as its skin material and used polypropylene as support material. The thinner membrane was 230 micrometers in thickness and possessed an isoelectric point of approximately 3.5. The membrane showed much higher permeability of 335.9 L/m²·h·bar with moderate variation. The specific composition and performance of each membrane suited it for various filtration conditions in process experiments. Refer to the above schematic outline in (fig,4) for the pathway of product contact with the membrane.

7. Case Study in Monoclonal Antibody (mAb) Purification

In downstream monoclonal antibody (mAb) purification, incorporating enzyme-functionalized membranes during the clarification and polishing steps is especially promising. Apart from facilitating enzyme immobilization, tangential flow filtration (TFF) is widely utilized in the biopharmaceutical industry for product recovery and purification. After upstream synthesis and enzymatic modification, TFF is applied to concentrate therapeutic proteins, conduct buffer exchange (diafiltration), and separate high-molecular-weight target molecules from smaller impurities or reaction by-products.

The ability of tangential flow filtration (TFF) to operate continuously, handle large volumes, and maintain gentle processing conditions makes it particularly well-suited for the purification of delicate biological molecules such as monoclonal antibodies (mAbs) and enzymes (Nadar et al., 2021; Liu et al., 2010). Enzymes including nucleases and glycosidases can be immobilized within these systems to perform specific functions that enhance bioprocessing efficiency. For example, nucleases can degrade host cell DNA early during clarification, reducing downstream contamination risks; glycosidases can be employed to modify glycoforms, enabling the production of biosimilars with desired glycosylation patterns; and other enzymes can facilitate improved virus or endotoxin removal prior to Protein A capture. Collectively, these applications highlight the versatility of enzyme-immobilized membranes in advancing integrated biomanufacturing.

In single-pass tangential flow filtration (SPTFF), enzyme-immobilized membranes can process high-density feedstocks without recirculation, minimizing shear damage and reducing system footprint. This aligns with trends toward continuous bioprocessing and modular, single-use platforms in the industry. Challenges: Enzyme Leaching, Activity Loss, and Resistance Accumulation.

Despite promising data, several barriers remain

1. Enzyme leakage through large pores or degraded fouling layers.
2. Activity loss due to improper orientation, pH shifts, or desorption.
3. Fouling resistance buildup, reducing overall membrane permeability over time.
4. Configuration-induced Inefficiencies, such as reverse-mode compaction or limited enzyme accessibility in asymmetric membranes.

7.1. Parameter Optimization and Process Control

Understanding and Maximizing Crossflow Filtration in Ultrafiltration/Diafiltration Processes

Crossflow filtration, particularly in ultrafiltration/diafiltration (UF/DF) applications, is a key process in separating, concentrating, and recovering proteins, as well as other biomolecules. The effectiveness of a TFF (tangential flow filtration) process depends significantly upon crossflow rate, transmembrane pressure (TMP), and protein concentration—factors that must be optimized to find a balance among flux, yield, and membrane area.(Merck Millipore, 2024).

Crossflow Rate is central to controlling high flux by minimizing concentration polarization at the membrane surface. Simply put as feed flow per square meter (L/min/m²), increased crossflow increases the sweeping action over the membrane, keeping a lower gradient of concentration, and reduces fouling. But there are trade-offs. Too high a level of crossflow exposes more product to pump shear, which can lead to degradation, and requires more substantial pumps and pipes, adding system holdup volume and potential losses. Thus, in process development, feed flow and TMP should be optimized together to achieve high flux, while minimizing holdup, as well as product degradation.

Transmembrane Pressure (TMP) serves as a force to drive permeate flow through the membrane. TMP can be calculated as

$$TMP = \frac{\text{P\_Filter Feed} + \text{P\_Retentate}}{2} - \text{P\_Filtrate.}$$

TMP and flux have a nonlinear correlation. Flux increases as a function of TMP (pressure-dependent regime), initially, until a plateau is achieved (pressure-independent regime). The "knee" of the curve indicates the optimal point, where maximum flux is attained without the risk of fouling or concentration polarization. Operating within the pressure- independent region can boost productivity but must be managed carefully to prevent protein precipitation or membrane fouling.

Filtrate control is most crucial in applications for open membranes (>100 kDa) where flow is so great that it approximates normal flow filtration (NFF), which obviates the advantage of TFF. Here, filtrate flow should be controlled by a valve or a pump to ensure proper TMP and to facilitate tangential flow to sustain membrane

effectiveness and reduce fouling.(Merck Millipore, 2024).

Diafiltration (DF), applied to buffer exchange or removal of contaminants, can be done in constant-volume or batch modes. Constant-volume DF is preferable for process reliability, though it necessitates level control. The timing in terms of protein concentration is key: DF at low protein concentrations provides higher flux but consumes more buffer, while at higher protein concentrations it conserves buffer but necessitates more membrane area. The optimum point is determined by plotting the DF Optimization Parameter (C × Jf) versus protein concentration, choosing that point which maximizes it.

Process Characterization and Scale-Up should involve generating flux versus TMP curves at various feed rates and protein concentrations, beginning with conditions that cause the least fouling. Evaluate flux, retention, yield, and mass balance during this process.

Estimate membrane area though: Area = Volume of filtrate / (Average Flux × Process Time) and always include a safety margin (usually 20%) to compensate for variations.

To address existing limitations and enable industrial-scale application, recent advances are focusing on hybrid immobilization and the development of smart membranes. Hybrid immobilization strategies combine fouling-induced enzyme deposition with covalent bonding or affinity ligands, thereby enhancing stability and control over enzyme orientation. At the same time, stimuli-responsive membranes are being designed to adjust porosity or enzyme exposure in response to changes in pH, temperature, or ionic strength, offering dynamic regulation of activity. In addition, three-dimensional nanostructured supports, such as electrospun mats and metal-organic frameworks (MOFs), provide increased surface area and improved orientation control for immobilized enzymes. To complement these advances, integrated process analytical technology (PAT) tools are being incorporated to enable real-time monitoring of enzyme activity and fouling layer evolution, ensuring both process consistency and product quality.

These innovations aim to deliver self-regulating, high-flux, long-lived biocatalytic membranes tailored to specific process needs, from glycan trimming to nucleic acid degradation or viral clearance.Biopharmaceutical Applications: Case Study in Monoclonal Antibody (mAb) Purification.(Nadar et al., 2021; Liu et al., 2010).

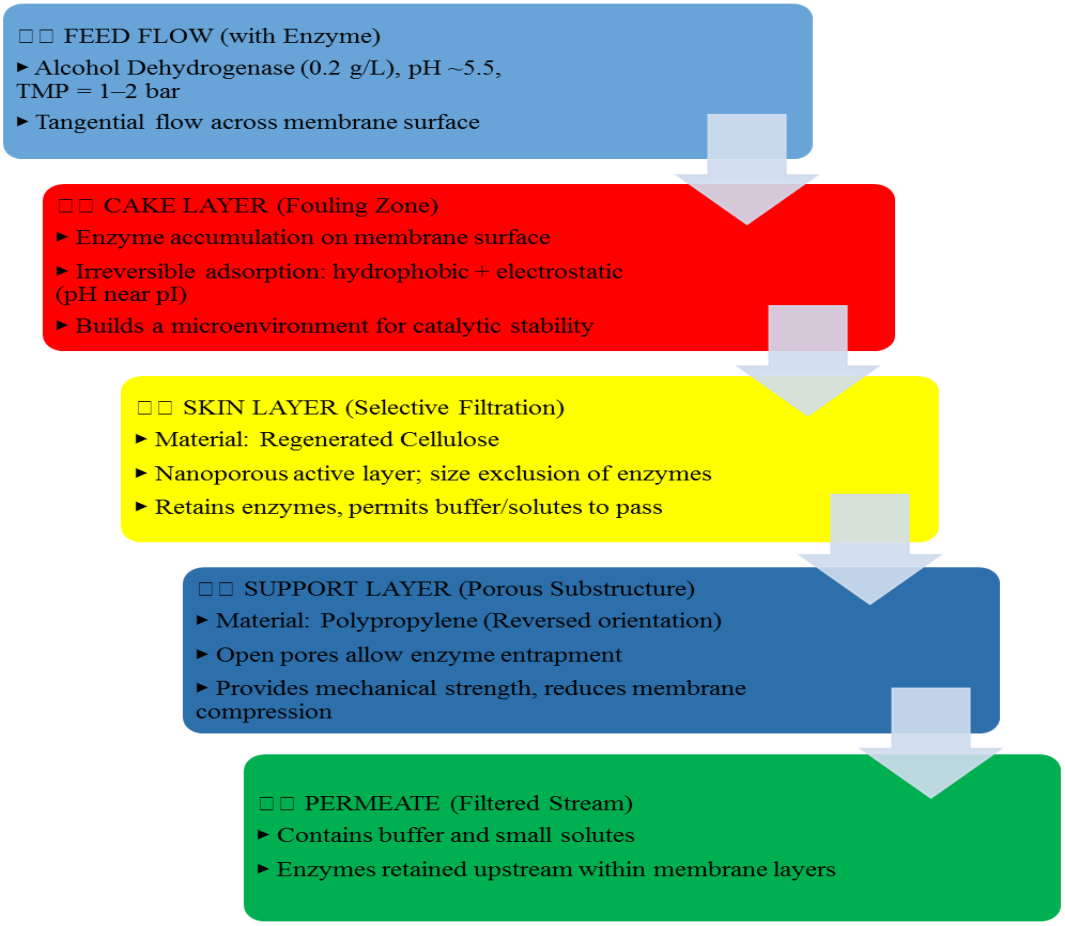

Figure 4: Schematic illustration of product contact with membrane.

Monoclonal antibody production often encounters DSP challenges due to high biomass titers and complex impurity profiles.

Tangential Flow Filtration (TFF) Enhancement Enzymes like nucleases or proteases immobilized on membranes can degrade host cell DNA or proteins in situ. Enzyme-immobilized membranes play a valuable role in downstream bioprocessing by preventing fouling of Protein A columns, thereby extending their lifespan and improving process efficiency. In clarification and polishing modules, enzyme-functional membranes integrated into single-pass TFF or virus filtration units help reduce particle loads and enhance virus removal, which supports regulatory compliance and product safety. Additionally, immobilized glycosidases or sialidases can be applied for glycan engineering during polishing, enabling the modification of glycan structures to closely match those of innovator biologics, an essential step in biosimilar production.

8. Challenges and Future Directions

While promising, immobilized enzyme systems face critical limitations:

1. Leaching and Instability: Especially in adsorption-based systems under flow and shear stress.
2. Reduced Activity: Poor orientation or multivalent attachment can occlude active sites.
3. Fouling Resistance Build-Up: While initial layers are catalytic, subsequent fouling can block activity.
4. Configurational Constraints: Hollow fibre vs. flat-sheet designs affect residence time and flow dynamics. Overcoming these requires hybrid strategies, combining fouling-induced loading with covalent anchoring or nanostructured surfaces.

8.1. Future Directions

The combination of enzyme immobilization with tangential flow filtration (TFF) systems is an exciting area for biopharmaceutical innovation. In keeping with ongoing advances in bioprocessing toward increasing productivity, continuous processing, and sustainability, enzyme-immobilized TFF systems are particularly well-positioned to meet these objectives. In the future, some principal directions for advancing this technology are: Smart membrane material development. Future research will be centered on the synthesis of novel membrane material with specially designed surface chemistries for improved enzyme compatibility, reduced denaturation, and extended operational life. Functionalized or stimulus-responsive membranes having the ability to change properties according to pH, temperature, or presence of substrates will allow dynamic control of immobilization strength and catalytic performance. Nanostructured or composite membranes can also be used to control pore geometry and surface affinity for optimal enzyme performance. (Zhang et al., 2021).

8.1.1. Integration of continuous and single-use bioprocessing

Flexible, single-use, and continuous systems demand is increasing at a rapid pace in biopharmaceutical production. TFF modules with enzyme immobilization, particularly as disposable units, can be integrated into continuous DSP platforms with ease. This would enable real-time biocatalytic conversion with product separation, minimizing downtime relating to cleaning and risk of cross-contamination significant positives for multi-product facilities and rapid deployment situations.

8.1.2. Multi-Enzyme Cascade Systems

Another promising thrust is immobilization of several enzymes in a spatially organized way in the TFF module for enabling cascade reactions. This can simplify complex bioconversions like glycosylation, peptide synthesis, or cofactor regeneration in situ in the filtration setup. High specificity and co-localization of enzymes on membrane surfaces would enable simultaneous catalysis and purification and shorten process times considerably.

8.1.3. Modelling and Process Optimization in Digitization

Advanced computational models and machine learning software will become increasingly important for optimizing enzymes' immobilization and filtration conditions. In-line monitoring of enzyme function, fouling behavior, and flux lossalong with prediction modelingcan be used to optimize process control and limit trial-and-error during scale-up. Digital twins for immobilized TFF systems can also be used in risk evaluation, life prediction, and economic viability assessment.

8.1.4. Broader Application within Product Classes

Although enzyme-immobilized TFF systems have demonstrated promise for oligosaccharide synthesis and selective protein modification, future uses may target more sophisticated biologics such as viral vectors, intermediates for gene therapy, and antibody-drug conjugates (ADCs). Immobilized enzymes can also be designed for selective host cell impurity degradation or for the activation of products to allow new processing and purification strategies.

8.1.5. Regulatory and Scalability Factors

To achieve optimum industrial uptake, regulatory clarity and sound validations protocols will be required. Reproducibility, leachability, and GMP compatibility of immobilized systems need future attention. Pilot plant demonstrations and scale-up examples are also needed to take lab-scale results to the stage of being commercially viable technologies.

9. CONCLUSION

Tangential Flow Filtration (TFF) has progressed from a traditional separating method to a flexible enzyme immobilization and integrated biocatalysis platform of great utility particularly in the demanding requirements

of biopharmaceutical downstream processing. Utilizing membrane fouling is usually regarded as a weakness as an operative tool, scientists have opened a new route for immobilizing enzymes effectively without resorting to elaborate chemical alterations. This immobilization based on fouling permits concurrent biologic purification and transformation and the resultant self-cleaning membranes and longer membrane lifetimes at lower processing costs. Control of key parameters like transmembrane pressure, pH, enzyme concentration, and membrane material has proved to have a dramatic effect on immobilization efficiency, enzyme stability, and filtration performance. Additional surface engineering utilizing bioinspired materials including polydopamine, TA/APTES nanospheres, and dye-ligand systems also enhance enzyme loading, orientation, and catalytic functionality. Case studies most notably in monoclonal antibody production illustrate the industrial and scalable utility of the systems in clarifying complex bioproducts, eliminating host cell contaminants, and facilitating continuous processing. Even with these breakthroughs, existing challenges in enzyme leaching, loss of activity, and fouling layer stability must be overcome through future directions in the form of hybrid immobilization approaches, environmental stimulus-responsive membranes, and multi-enzyme cascade systems. The application of in-line monitoring and digital process automation will also be critical for scalability and real-time optimisation. Enzyme-immobilised TFF systems eventually embody a pivotal transition toward sustainable, high-throughput, and precision biomanufacturing. Their evolution is also testimonial to the intersection of membrane engineering, enzymology, and integration in offering solutions for next-generation biologics manufacturing.

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