Membrane Proteins: Challenging Biotherapeutic Targets

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Abstract

Membrane proteins are about 30% of the human proteome and serve important physiological roles, including chemical transport, cell signaling, and energy transduction.[1,2] Consequently, they represent about half of all drug targets.[2–5] Small molecule drugs have been most commonly used to target membrane proteins, particularly G-protein coupled receptors and ion channels. However, these small molecules can lack selectivity and produce side effects. Biotherapeutics offer the potential to target specific conformations of proteins, thereby improving potency and selectivity.[6] However, membrane proteins present significant challenges for biotherapeutic development, especially from their instability, insolubility, and limited expression levels.[6–8] In this chapter, we explore biotherapeutic targeting of different families of membrane proteins, strategies to solubilize and stabilize membrane proteins for analysis, and mass spectrometry (MS) approaches to study their structure and interactions. Our focus will be primarily on biotherapeutic applications, but we will draw on promising emerging technologies that have been used in structural biology.

Membrane Proteins as Challenging Biotherapeutic Targets

Families of Membrane Proteins

Nearly all physiological processes intersect the membrane. As the primary conduits of material and information across the lipid bilayer, membrane proteins thus play critical biochemical roles. They account for about 30% of the proteome and roughly half of drug targets. [2–5] However, membrane proteins are challenging targets for biotherapeutic development for several reasons.[6–8] First, membrane proteins tend to have low expression levels, which creates an obstacle to gaining enough purified protein for antibody development.[9,10] Second, they have low stability outside membranes, so it is challenging to deliver these proteins as immunogens.[11] For example, using detergents to solubilize membrane proteins can interfere with the exposure of extracellular epitopes and may be incompatible with *in vivo* antibody generation.[11] Finally, post-translational modification, especially glycosylation, can lead to antigenic heterogeneity and are difficult to correctly reproduce in non-human expression systems.[12,13] In this section, we will begin our discussion by highlighting the most significant membrane protein families targeted by biotherapeutics and consider throughout the chapter how these challenges are addressed.[6,14]

Receptor Tyrosine Kinases

Receptor tyrosine kinases (RTKs) play roles in diverse cellular processes, including growth, differentiation, cell proliferation, metabolism, migration, and vascular function. They are also involved in oncogenesis, making them important drug targets.[15–17] The extracellular ligand binding

domain of RTKs generally interacts with protein ligands (**Figure 1A**), including growth factors, hormones, and cytokines. When a ligand binds to the extracellular domain, RTKs dimerize to promote downstream signaling through the intracellular kinase domain.[15,18]

Humans have 58 known RTKs, and they are classified based on their interactions and the structure of their extracellular domains into 20 subfamilies, including epidermal growth factor receptors (EGFRs, class I), insulin receptors (IRs, class II), platelet-derived growth factor receptors (PDGFRs, class III), vascular endothelial growth factor receptors (VEGFR, class IV), fibroblast growth factor receptors (FGFRs, class V), cholecystokinin receptor family (CCK, class VI), and others.[18–20]

Because they are easily accessible on the surface of the cell, targeting the extracellular ligand binding domain of RTKs has been an effective strategy for biotherapeutics, especially for cancer. The advantage of this approach is that a truncated, soluble ectodomain can be used for antibody generation, which avoids issues of membrane protein expression and solubilization.[21] With this approach, monoclonal antibodies (mAbs) can be developed to inhibit ligand binding to receptors or to inhibit receptor dimerization/activation after ligand binding (**Table 1**).[15] For example,

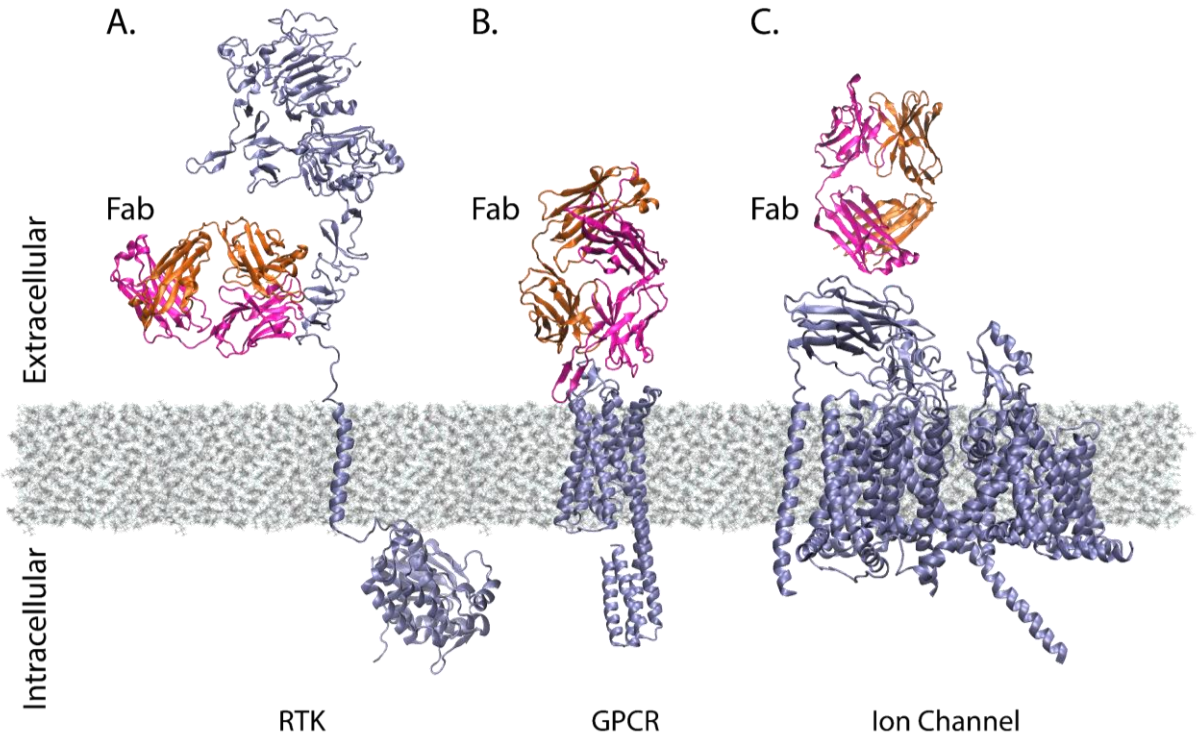

Figure 1: Examples of Fab binding to different membrane protein types: (A) an example RTK, HER2 (PDB: 1N87 for trastuzumab bound extracellular region, 2KS1 for transmembrane domain, 3PP0 for intracellular kinase domain); (B) an example GPCR, a serotonin receptor (PDB: 5TUD); (C) an example ion channel, $Na_v1.4$ (PDB: 5XSY). Fabs (colored in orange and magenta) are antigen-binding fragment of antibodies. The ice blue color represents the membrane proteins.

trastuzumab, pertuzumab, cetuximab, panitumumab, and nimotuzumab target EGFRs to inhibit cell proliferation, angiogenesis, and cell survival.[15,22,23] Initially approved as Herceptin, trastuzumab was the first antibody to target an RTK and was a breakthrough in targeted cancer therapeutics.[15]

Beyond the EGFR family, the PDGFR family has also shown growing interest as a target for several cancer types. For example, olaratumab and tovetumab are directed against PDGFRs for treating solid tumors.[15] The VGFR family has also been a target in cancer therapy due to their role in angiogenesis. For example, icrucumab inhibits tumor activation and angiogenesis, ramucirumab blocks neo-angiogenesis, and tanibirumab shows anti-tumor activity against lung, breast, colorectal, and glioblastoma cancer models.[15,19,24–26]

Although many mAbs targeting RTKs have been successful, several drug candidates have failed in clinical trials. For example, onartuzumab, which targeted hepatocyte growth factor receptor, and cixutumumab, which targeted insulin receptors, failed due to lack of efficacy in cancer trials.[15,27,28] Other mAb therapies were abandoned because tumors developed resistance. For antibodies targeting EGFR and HER2, developing resistance within a year is common through different types of mechanisms including mutations, upregulation or downregulation of signaling, and activation of alternative pathways.[15,29]

To address challenges with resistance and efficacy, more advanced biotherapeutic strategies can be used. To improve anti-tumor efficacy and limit resistance, bispecific antibodies can be used to target two receptors simultaneously.[15,30–32] Similarly, combination therapies, such as trastuzumab and pertuzumab, can enhance efficacy through synergistic effects.[15,33] Finally, antibody-drug conjugates can be used to deliver cytotoxic agents to tumor cells, enhancing treatment efficacy and simultaneously minimizing side effects.[34] For example, Ado-trastuzumab emtansine is a notable antibody-drug conjugate for HER2-positive breast cancer.[15] As these advanced biotherapeutic modalities mature, RTKs will continue to be an important membrane protein target for biotherapeutics.

G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are the largest family of membrane proteins in number and one of the most important classes of proteins for drug discovery.[11] They are involved in diverse cellular processes, including cell growth, metabolism, sensory perception, immune response, and neurotransmission.[9,35,36] These diverse functions come from binding diverse ligands, ranging from small molecules to larger peptides and proteins.[37,38]

There are around 800 GPCRs, but more than half are olfactory or sensory receptors, leaving roughly 370 GPCRs that are considered potential drug targets. GPCRs are grouped into four families: family A (the rhodopsin family), family B (the secretin and adhesion subfamilies), family C (the metabotropic glutamate family), and family D (the frizzled family).[9,11] The rhodopsin family represents the largest and most diverse group of receptors. They are a major focus for drug development with receptors for histamine, dopamine, glycoprotein hormones, adrenergic agents, neuropeptides, and chemokines.[11,37] Family B GPCRs are split into two subfamilies where secretin binds large peptide ligands, but so far lacks small-molecule drug targets. Most of the adhesion subfamily ligands have not been identified yet. Family B of GPCRs is well known for their metabolic function, regulating homeostasis, endocrine functions, and neuronal activity.[37] The metabotropic glutamate family binds small metabolites like glutamate, and play important roles in neuronal and calcium homeostasis. The frizzled family is a promising target because they are involved in developmental biology and tissue homeostasis. [11,39,40]

Although GPCRs are important therapeutic targets, they have largely been limited to small molecule or peptide therapeutics.[6,41] Developing antibodies for GPCRs has been especially challenging for several reasons. First, unlike RTKs, GPCRs generally do not have large soluble domains that can be detached for antibody development (**Figure 1B**). Thus, the immunogenic regions are often limited to the N-terminal domain and extracellular loops. Second, GPCRs are conformationally dynamic and difficult to stabilize outside the membrane.[37] Combined with their low expression levels, these challenges mean that it is difficult to express, purify, and stabilize GPCRs for antibody development. Finally, the development of GPCR biotherapeutics is also limited by biased signaling, where different ligands binding the same receptor can induce different downstream signaling responses. [37,42,43] It can be challenging to develop antibodies that target these biases.

Due to these challenges, only three FDA-approved antibodies are directed toward GPCRs (**Table 1**). Mogamulizumab is a humanized IgG1 antibody directed against C-C chemokine receptor type 4 (CCR4) to induce antibody-dependent cellular cytotoxicity for treatment of blood cancers.[44] Erenumab and eptinezumab are antagonists for calcitonin gene-related peptide (CGRP) and are used to treat migraine.[37]

There are several GPCR-targeted antibodies currently in clinical trials, but several recent attempts have failed. For example, plozalizumab failed in the phase 2 trial due to a lack of efficacy for treating rheumatoid arthritis.[11] Here, one challenge was the low homology between human chemokine receptor type 2 (CCR2) and other species, which limited preclinical studies in nonhuman models.[37]

Despite these challenges, innovative new technologies are being employed for GPCR biotherapeutics. For example, protein mutations are widely used to improve the expression and stability of GPCRs for large-scale purification.[11,42,45] Similar engineering approaches can also be used to bias the conformation. Another approach to stabilize the protein for antibody development is to use different membrane mimetics (discussed below), and a range of these different platforms have been used for antigen presentation.[37,46] Using these novel approaches, it is likely that future biotherapeutics for GPCR targets will help to treat a variety of diseases.

Ion Channels

Ion channels are the second most abundant class of membrane proteins after GPCRs, comprising almost 400 members. Ion channels transport ions across membranes and play crucial roles in maintaining ion homeostasis.[47,48] Based on their gating mechanisms, ion channels are broadly classified into three main groups: voltage-gated ion channels, ligand-gated ion channels, and mechanosensitive ion channels.[47]

Despite their importance in diseases like cancer, glaucoma, infectious disease, inflammation, and migraine, ion channels are challenging for the development of biotherapeutics for many of the same reasons as GPCRs. First, they have smaller extracellular loops that limit the potential immunogenic regions (**Figure 1C**). They can also be poorly expressing and challenging to stabilize and purify. Finally, ion channels often have highly conserved sequences between species, so they are not immunogenic enough to elicit strong antibody responses in mammals used for antibody development. [47,49]

Due to these challenges, ion channels are less explored for biotherapeutics than GPCRs, and there are currently no approved biotherapeutic drugs for these targets.[50] Several companies have attempted antibody drug development for ion channels. For example, Visterra Inc. developed mAbs that targeted voltage-gated sodium channels (Na_v) for treatment of pain. Here, they used several of the strategies described above for GPCRs. For example, to improve stability, they engineered a chimeric construct fused with the more stable prokaryotic form and reconstituted the protein in nanodisc for efficient immunization.[47] Amgen also explored developing antibodies towards transient receptor potential ankyrin 1 (TRPA1) by using DNA transfection rather than protein immunization.[47,51] Unfortunately, none of these mAbs appear to be under ongoing development.

Although there has been considerable focus on Na_v channels, other ion channels have been explored as well. For example, the ligand-gated purinergic channel (P2X) family shows larger extracellular regions compared to other ion channels, so it is easier to target using biotherapeutics. A polyclonal antibody that targets nfP2X7, a non-functional form of P2X7, is in phase 1 clinical trials to treat basal cell carcinoma.[47,52] Also, a mAb targeting the potassium channel, $K_v1.3$, is in preclinical trials for treatment of autoimmune and inflammatory diseases.[53,54]

Overall, ion channels present challenging but promising targets for biotherapeutic development. For ion channels and GPCRs, continued advances in molecular biology, protein engineering, and membrane mimetics will help improve platforms for drug development.[11,15,37,47] For the more mature field of RTK therapeutics, new modalities to combat resistance will lead to more effective therapies. The next section will discuss advances in detergents and membrane mimetics to solve challenges in purification and presentation of membrane proteins. The final section will then discuss the unique considerations and opportunities for MS analysis of membrane proteins in a biotherapeutic context.

Table 1. List of FDA-approved mAbs for membrane proteins.[11,15,37,55]

	Name of drug	**Company**	**Year of Approval**	**Target Protein**
RTK	Trastuzumab	Genentech	September 1998	Human epidermal growth factor receptor 2 (HER2)
	Pertuzumab	Genentech	June 2012	HER2
	Cetuximab	ImClone	February 2004	Epidermal growth factor receptor (EGFR)
	Panitumumab	Amgen Inc	September 2006	EGFR
	Necitumumab	Eli Lilly Co	November 2015	EGFR
	Margetuximab	Macrogenics Inc	December 2020	HER2

	Teprotumumab	Horizon Therapeutics Ireland	January 2020	Insulin-like growth factor type I receptor (IGF-IR)
	Ramucirumab	Eli Lilly Co	April 2014	Vascular endothelial growth factor (VEGF)
	Olaratumab	Eli Lilly Co	October 2016	platelet-derived growth factor receptor alpha (PDGFRα)
	Amivantamab	Janssen Biotech	May 2021	Bispecific EGFR and mesenchymal-epithelial transition (MET) receptor
	Bevacizumab	Genentech	February 2004	VEGF-A
	Brolucizumab	Novartis Pharms Corp	October 2019	VEGF-A
	Burosumab	Kyowa Kirin	April 2018	Fibroblast growth factor 23 (FGF23)
GPCR	Mogamulizumab	Kyowa Hakko Kirin	August 2018	C-C chemokine receptor type 4 (CCR4)
	Erenumab	Amgen Inc	May 2018	Calcitonin gene-related peptide (CGRP)
	Eptinezumab	Lundbeck Seattle BioPharmaceuticals, Inc.	February 2020	CGRP

Membrane Protein Solubilization Methods

Many applications of membrane proteins in biotherapeutics require the extraction of membrane proteins from their natural environments (**Figure 2A**) into membrane mimetics.[56,57] However, different membrane mimetics vary in their properties and require different considerations for analysis.[58–60] Here, we will give an overview of different membrane protein solubilization techniques and their applications for membrane protein biotherapeutics.

Detergent Micelles

Detergents are the most widely used agents to solubilize membrane proteins (**Figure 2b**).[61–64] Detergents are amphiphilic small molecules with a hydrophilic head group and a hydrophobic tail. The amphiphilic nature of detergents enables them to protect the hydrophobic regions of membrane proteins inside micelles. There are variety of different types of detergents, and it is important to choose the right detergent for the target protein and analysis performed.[63,65,66] In particular, there are trade-offs between effective solubilization and preservation of protein structure.

Detergents can be broadly classified based on the charge they carry on their hydrophilic head groups.[59,60,65,66] Ionic detergents carry a net charge, such as sodium dodecyl sulfate (SDS).[60,67] These detergents are generally very effective at solubilization but can be too harsh, causing denaturing of proteins. Therefore, SDS is typically used for solubilization under denaturing conditions.

In contrast, non-ionic detergents contain neutral hydrophilic headgroups and offer relatively mild solubilizing properties. Examples include Triton X-100, *n*-dodecyl-ß-D-maltoside (DDM), and *n*-octyl-ß-D-glucoside (OG). Because these non-ionic detergents are less likely to disrupt membrane protein structure and function during the purification process, they are commonly used for membrane protein extraction and analysis under non-denaturing conditions.[64,67]

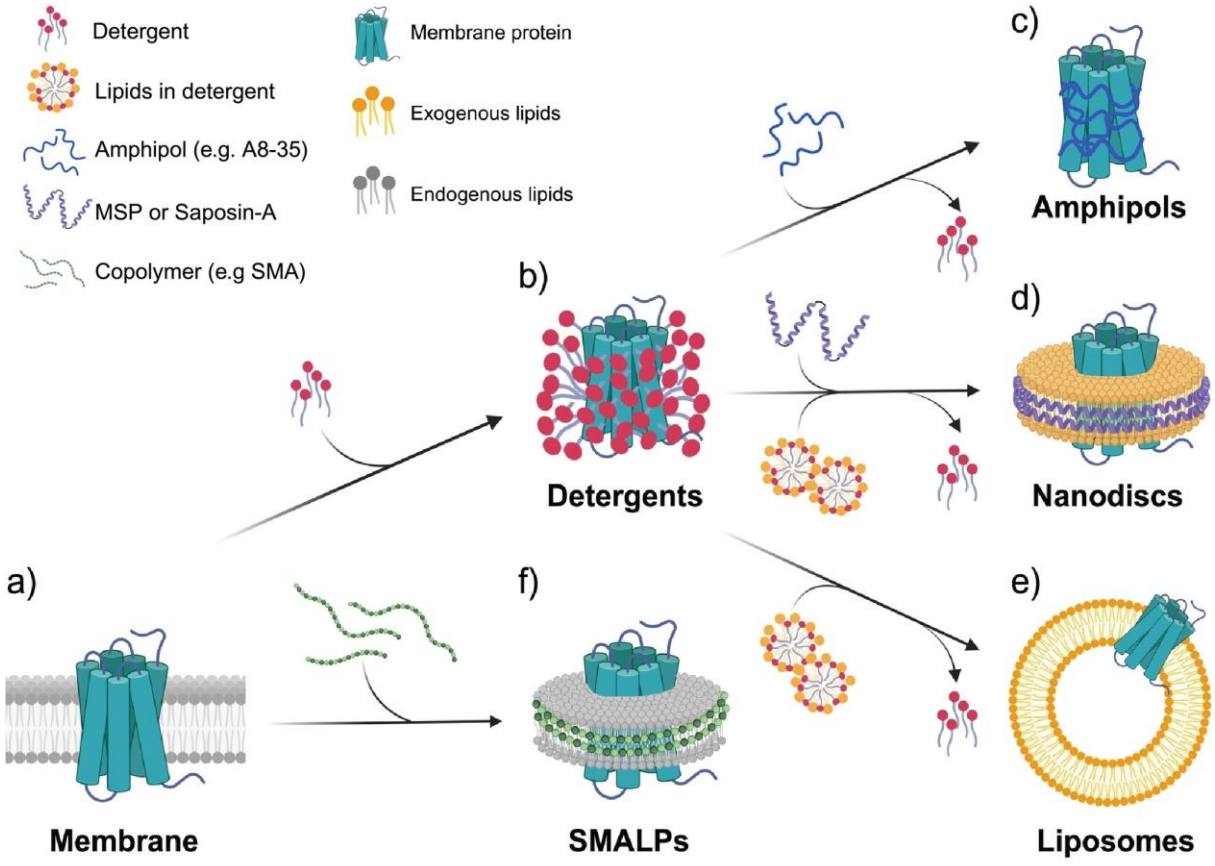

Figure 2. Illustration of different approaches used to solubilize membrane proteins. Membrane proteins are naturally present in (**a**) lipid bilayer membranes. Proteins can be extracted from the membrane into (**b**) detergent micelles. From here, it can be reconstituted into (**c**) amphiphols, (**d**) nanodiscs, or (**e**) liposomes. Alternatively, the protein can be extracted from the membrane directly into (**f**) SMALPs. Adapted with permission from Woubshete *et al.*, 2024. https://doi.org/10.1002/cplu.202300678.

Due to their importance in structural biology applications, recent research has developed novel non-ionic detergents with modifications that improve solubilization efficacy, protein stability, and analytical compatibility.[68,69] Examples of these newer classes of detergents include oligo-glycerol

detergents,[70,71] neopentyl glycols (such as LMNG),[72,73] and glyco-diosgenin (GDN) [74]. An important feature of newer detergents like LMNG and GDN is their low critical micelle concentration (CMC) values, which allow them to be used in dilute solutions like those used in cryo-EM.[68]

Zwitterionic detergents have both positively and negatively charged groups and fall in between ionic and non-ionic detergents.[60,75] Some zwitterionic detergents, such as fos-cholines, can be harsher and behave more like ionic detergents.[76] Others, like 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and *n*-dodecyl-*N,N*-dimethylamine-*N*-oxide (LDAO), tend to be gentler and have been broadly used in structural biology applications.[75,77,78]

There are several important properties to consider in detergents, including the CMC, aggregation number, and hydrophilic-lipophilic balance (HLB).[63,69,79] At lower concentrations, detergents remain as individual molecules in solution. As the concentration increases, they assemble into micellar structures. The minimum concentration required for detergent molecules to form micelles is called the CMC, and the average number of detergent molecules per micelle is the aggregation number.[60,67,75] Both properties are influenced by the length of the detergent's alkyl chain, where longer chains tend to decrease the CMC and increase the aggregation number.[80] HLB describes the surface activity based on the hydrophilic and hydrophobic properties of a detergent. Typically, detergents with HLB values from 12–15 and at concentrations above the CMC are recommended for membrane protein extraction.[71,80]

In a recent example [81], Urner and colleagues created a library of detergents with diverse properties, such as HLB and packing parameter (a structural property that relates the head group area with the tail volume [82]). They then solubilized proteins from bacterial inner membranes and used native MS to measure how each protein co-purified with phospholipids and lipopolysaccharide (LPS) in each unique detergent condition (**Figure 3**). By adjusting detergent HLB and packing density (**Figure 3a**), they enhanced phospholipid retention during extraction and purification of membrane proteins (**Figure 3b–c**). This study demonstrated how detergents can be designed to optimize membrane protein solubilization and interactions.

Although detergents are useful for solubilizing membrane proteins, they are not natural lipid bilayers and may not be effective at preserving membrane protein activity and interactions.[63,83–85] In membrane protein biotherapeutics, identifying optimal detergent conditions to stabilize membrane proteins is a critical step. Often, a range of detergents need to be screened to find the optimal type. For example, Kotov and coworkers developed a high-throughput screen that tested 94 detergents to find which best stabilized different membrane proteins.[86] Although each protein had a unique profile of optimal detergents, LMNG and DDM tended to be stabilizing, and fos-choline tended to be destabilizing. Overall, careful selection of the detergent is essential for studying membrane proteins.

Alternative Membrane Mimetics

To address challenges in using detergents to solubilize membrane proteins, alternative membrane mimetics have been developed. Although detergents may be required to initially extract proteins from the membrane, they can be reconstituted into various membrane mimetics for characterization in defined lipid bilayers (**Figure 2**). Other mimetics enable direct reconstitution without intermediate detergent, such as styrene maleic acid lipid particles (SMALPs) or some forms of liposomes. In this

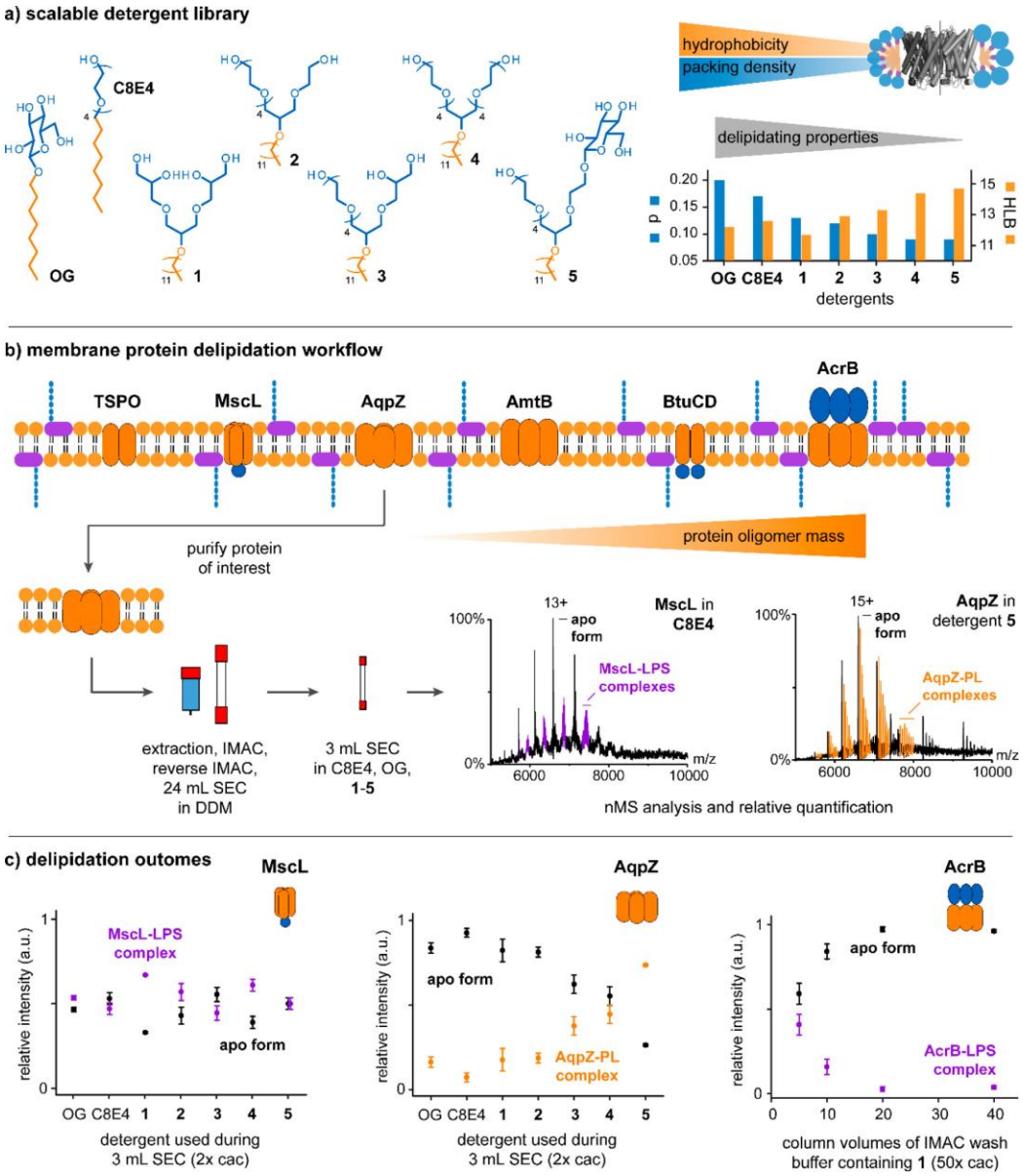

Figure 3. (**a**) Structures of detergents with the different HLB and packing parameters. (**b**) Schematic of solubilizing different inner membrane proteins from bacteria and using native MS to quantify delipidation outcomes. (**c**) Relative intensities of the apo state, protein-phospholipid complexes, and protein-LPS complexes acquired using native MS. Reprinted with permission from Urner *et al.*, 2024. https://pubs.acs.org/doi/full/10.1021/jacs.3c14358

section, we will provide an overview of some of the key membrane mimetic systems and their applications in biotherapeutic development.

Amphipols

Amphipols are amphipathic polymers used to solubilize membrane proteins (**Figure 2c**).[87–90] Different structures of amphipols have been developed,[91,92] with A8-35 being the most extensively used.[92–94] To incorporate membrane proteins into amphipols, detergent-extracted membrane proteins are mixed with the amphipol at a predefined ratio and incubated. The amphipol molecules interact with the membrane proteins, and the detergent is then removed using polystyrene beads, a detergent removal column, or dialysis.[94,95]

Unlike detergent micelles, which must be above their CMC, the primary advantage of amphipols is that they bind tightly to membrane proteins and can work at dilute concentrations. However, like detergents, they do not provide a lipid bilayer environment and thus may not preserve the natural structure and interactions found in lipid membranes.[83] Overall, although amphipols have been effectively used for the structural and biophysical characterization of membrane proteins [96–99] and have been used to study antibody binding to membrane proteins,[100,101] their application in biotherapeutics has been relatively limited.

Nanodiscs

Nanodiscs are nanoscale discoidal lipid bilayers encircled by two amphipathic membrane scaffold proteins (MSPs), which are derived from human ApoA1 (**Figure 2d**).[56,102–105] To reconstitute membrane proteins into nanodiscs, MSP and lipids dissolved in detergent are mixed with the membrane protein. Then, porous polystyrene beads are added to the mixture to remove the detergent and drive self-assembly.

In nanodiscs, the hydrophilic regions of the MSP are exposed to the surrounding solution, and the hydrophobic regions interact with the lipids in the core.[105–107] The size of the nanodisc can be adjusted by altering the length of the MSP, and the thickness can be customized by modifying the lipid composition.[56,84,107,108] Recently, circularized nanodiscs have been engineered by covalently linking the N- terminus to the C-terminus, which has further improved their homogeneity, stability, and size range.[109,110]

Due to their stability and biocompatibility, nanodiscs have become a useful platform for a range of biotherapeutic applications.[105,111,112] To develop therapeutic antibodies targeting membrane proteins, nanodiscs have been used to present antigens in more natural conformations. One example developed a single-domain antibody targeting the human apelin receptor (APJ), which is a GPCR and a target for treating chronic heart failure.[113] Here, Zhang and coworkers reconstituted APJ into nanodiscs that were then injected into camels as immunogens. Other approaches, such as by Kossiakoff and coworkers, have used nanodiscs with phage display technology to generate antibodies against membrane proteins.[114] Finally, it is possible to use nanodiscs for vaccines. In one study, injecting nanodiscs with embedded influenza hemagglutinin generated a robust and protective antibody response.[115] However, due to cost and stability limitations, nanodiscs are more likely to be used in antibody development rather than direct vaccine delivery.

In addition to presenting membrane proteins for vaccines or antibody development, nanodiscs are also useful as drug delivery vehicles, especially in delivery of hydrophobic drugs.[108,111] For example, one study used nanodisc to deliver the anticancer drug, cabazitaxel, where they found a sustained release profile and higher drug efficacy.[116] Moreover, both the MSP belt and the lipids can be engineered to attached chemical functionality and enable targeted delivery. For example, another study attached an antibody that targeted carcinoembryonic antigen to a PEGylated lipid in nanodiscs.[117] Further conjugation of a radioactive Cu chelator allowed PET imaging of these targeted nanodiscs and demonstrated localization to the tumor. Future research using nanodiscs as a drug delivery vehicle holds the potential to develop unique biotherapeutics capable of targeted delivery of lipids and hydrophobic drugs.

Peptide Nanodiscs

Alongside nanodiscs encircled by protein belts, there are peptide-based nanodiscs that also serve as membrane mimetics. For example, saposin-lipid nanoparticles (SapNPs) use a small protein, saposin A, as the belt.[118,119] Unlike MSP, SapA covers both leaflets of the bilayer with a single monomer without encircling the particle. This modular assembly enables the incorporation of varying numbers of SapA molecules per complex, making SapNPs adaptable in size and composition.[120] SapNPs have been used in a variety of structural and biophysical studies[121] and have been used to present membrane proteins for antibody development.[112]

Another novel peptide nanodisc system is the peptidisc.[122] In peptidisc assembly, hydrophobic regions of membrane proteins are protected by an amphipathic bi-helical peptide. Unlike nanodiscs, peptidiscs eliminate the need for additional lipids, incorporating only the copurified annular lipids with the membrane protein.[122,123] The peptidisc system has been useful in determining structures of membrane proteins and profiling membrane proteomes.[123–127]

Several recent studies have showcased the use of peptide nanodiscs as vaccines.[128] Here, Moon and coworkers used a synthetic peptide from ApoA1 called 22A to create peptide nanodiscs and deliver antigens and adjuvants to lymphoid organs as a vaccine. Administration of these peptide nanodiscs resulted in more efficient and prolonged antigen presentation on major histocompatibility complex molecules, leading to stronger T-cell responses. These studies have since been extended to use 22A nanodiscs in vaccination for a variety of different types of cancer.[129,130] These applications of peptide nanodiscs demonstrate a range of potential uses in biotherapeutic development.

SMALPs

Although the mimetics discussed above provide lipid environments for studying membrane proteins, most of those mimetics still require the use of detergents for the initial solubilization of the proteins. In contrast, SMALPs are a detergent-free membrane mimetic that allows the extraction of membrane proteins directly from their natural lipid bilayers.[62,131] SMALPs are created from lipids and poly-styrene-co-maleic acid (SMA), which is an amphipathic copolymer comprising hydrophobic styrene and hydrophilic maleic acid moieties.[132,133] When added to membranes, SMA inserts into the lipid bilayer,[132,134] allowing the SMA to extract the protein from lipid membranes.[135,136] SMALPs are compatible with a wide range of targets, including integral and peripheral membrane proteins.[84,136]

Early SMALP technology had several limitations, most notably that they required a higher pH and were sensitive to divalent cations, making them challenging to use in systems that require calcium and magnesium.[136] There were also limited in size and could not accommodate very large membrane protein complexes.[136–138] To address these challenges, researchers have developed a variety of new polymers, including diisobutylene maleic acid (DIBMA), styrene maleic imide (SMI), and styrene maleimide quaternary ammonium (SMA-QA).[137,139] These new polymers have different optimal pH levels, sizes, and tolerances to divalent cations, enhancing their applicability in diverse applications.[137,139]

The unique ability of SMALPs to disrupt membranes directly to extract membrane proteins facilitates higher yields of membrane proteins from low-expressing cell lines, which enables drug development with more challenging membrane protein targets.[140] For example, SMALPs can be used to study binding of ligands and antibodies to GPCRs.[138,141] Moreover, SMALPs can be used in antibody development, vaccines, and drug delivery in similar ways to the peptide and protein nanodiscs described above.[112] Although each system has unique properties and distinct advantages, nanoscale lipid bilayer particles enable diverse applications in biotherapeutics for membrane proteins.

Liposomes and Bicelles

Finally, we will introduce membrane mimetics composed primarily of lipids, which can take different structural forms. Among the different mimetic systems discussed above, liposomes can be the simplest membrane mimetic in composition, requiring only a phospholipid bilayer vesicle.[83,90] However, liposomes can also be customized for different target membrane systems by changing the lipid composition, size, and protein-to-lipid ratio.[142,143] In conventional assembly of liposomes, purified membrane proteins in detergents are reconstituted into preformed liposomes, with the detergent being removed during the assembly process.[144–146] However, it is also possible to create liposomes directly from cell membranes, such as with extracellular vesicles.[147,148]

Liposomes have numerous biotherapeutic applications.[149–151] In one example, Lenormand and coworkers used liposomes to deliver two pro-apoptotic membrane proteins, voltage-dependent anion channel (VDAC) and Bcl-2 homologous antagonist/killer (Bak), to cells.[149] This study demonstrated that functional VDAC and Bak proteins were successfully delivered into cancer cells, where they triggered apoptosis. Thus, liposomes can be used to deliver membrane proteins as therapeutic agents.

Bicelles are similar to liposomes but have a mixture of two different lipid-like molecules.[152] Typically, one lipid forms a bilayer while the other has more detergent-like properties and caps the edges of the bilayer. Bicelles can form a variety of structures, including discoidal lipid nanoparticles like nanodiscs. Bicelles have been broadly used for membrane protein analysis, especially by NMR, and have been applied for drug delivery.[153]

In summary, mimetics do not always eliminate the need for detergents, but they provide novel strategies to study membrane proteins within bilayer environments. They provide versatile platforms for membrane protein biotherapeutic development and drug delivery. Continued development and optimization of these approaches will enhance our understanding of membrane proteins and advance biotherapeutic development.

MS Analysis of Membrane Proteins

Mass spectrometry has increasingly become an indispensable tool in biotherapeutics. In this section, we will explore how various structural MS techniques are used, both independently and in combination, to address critical biological questions related to membrane proteins in biotherapeutic applications. Drawing on the prior section, we will consider how different membrane mimetics are used.

Proteoform Characterization

One of the main questions in MS of biotherapeutics is proteoform characterization. Proteins synthesized from a single gene can have a high level of heterogeneity due to post-translational modifications (PTMs), genetic variations, and splice variants.[154–156] Each unique variant of the protein is called a proteoform. For membrane proteins, the proteoform landscape is particularly complex due to frequent glycosylation of proteins on the cell surface.[157,158] Characterizing this proteoform landscape is important to ensure consistent biotherapeutic manufacturing and generation of antibodies that hit the correct target.[139]

Recent advancements in MS have enhanced the ability to quantitatively analyze PTMs at a variety of different levels. Intact mass analysis directly profiles the mass distribution, revealing the ratios of each proteoform that has a distinct mass. Often, intact mass analysis is performed on denatured proteins to reveal the mass distribution of the protein alone.[159–161] However, it is also possible to perform native MS by using non-denaturing ionization conditions, which help maintain non-covalent interactions.[56,162] Native MS typically requires careful consideration of the membrane mimetic system to keep the protein soluble during analysis, as discussed below.

Complementing these intact MS measurements, more detailed sequence information can be gained from bottom-up and top-down proteomics approaches. Bottom-up analysis uses enzymes to digest proteins to peptides, which are then sequenced to identify sites of PTMs.[163] Top-down approaches perform intact mass analysis first and then use fragmentation within the mass spectrometer to sequence the protein. Top-down proteomics enables detailed characterization of the position of the PTMs and disulfide bridge patterns [164–166] Increasingly, proteomic approaches are being combined with native MS to characterize PTMs in protein complexes.[167–169] For bottom-up and denatured intact analysis methods, there are fewer special considerations for membrane proteins because the protein structure does not need to be preserved.

MS analysis of all different flavors has been applied to characterize proteoforms in a range of applications. Native MS has been used to characterize membrane protein proteoforms in detergents[170] and nanodiscs.[171] As discussed in prior reviews, detergents have dominated native MS analysis, but new membrane mimetics have enable unique new types of experiments.[56,83,172,173] However, there are not many applications characterizing PTMs of membrane proteins for biotherapeutic applications.

To address this gap, advanced native MS methods have recently been developed for highly heterogeneous biotherapeutics with multiple glycosylation sites.[174] Building on these studies, future research will dive deeper into characterizing proteoforms for biotherapeutic development of membrane proteins. For example, developing therapeutics that target particular glycosylation sites

could be a powerful application where MS methods would be essential in defining the composition of the target.

Complex Architecture

Another key question in MS of biotherapeutics is how proteins assemble into complexes. Many membrane proteins either oligomerize or form macromolecular assemblies to perform their biological roles.[175,176] Understanding the architecture of membrane protein complexes is important to develop effective biotherapeutics to target the correct form of the complex.

Native MS (introduced above) is commonly used to study complex architecture.[162] Although the direct applications to membrane protein biotherapeutics are limited, a number of studies have explored the architecture of therapeutically important membrane protein targets.[177] Early examples used native MS to study protein complex architecture of membrane proteins in detergents, with a striking example being ATPase complexes.[178] These studies primarily relied on common nonionic detergents, such as DDM. However, more challenging targets have required advances in detergent selection.[70,71,84] For example, Robinson and coworkers have advanced native MS to study GPCR targets, which have relied on careful detergent selection and design.[179–181]

Alternative membrane mimetics have also been used to study membrane protein complex architecture.[56,83,172,173] In one example, Townsend *et al*. embedded the influenza A M2 protein in lipid nanodiscs with different lipids.[182] They discovered different stoichiometries of complex formation in different lipid environments and observed drug binding to the tetramer species. To capture interactions in more natural environments, Robinson and coworkers used sonicated lipid vesicles prepared directly from cell membranes.[183–185] Together these methods demonstrate the potential for native MS to study membrane protein complex architecture in complex environments.

Another technique to characterize complex architecture is cross-linking (XL)-MS. XL-MS measures the proximity of amino acid residues through covalent crosslinking with bifunctional linkers.[186,187] These linkers typically target specific amino acids, such as the primary amine groups on lysine side chains, with the spacer arm defining the distance constraints between the cross-linked residues. After digesting the crosslinked protein, analyzing the resulting peptides using bottom-up proteomics reveals the spatial relationships between residues.

Complementing native MS studies, XL-MS provides unique information on which subunits are close together in the complex. For example, Schmidt and coworkers used XL-MS to further characterize the ATPase complex, first in detergents [188] and recently in synaptic vesicles.[189] This example highlights the ability of XL-MS to study protein complex architecture *in situ*, which we will also discuss below. Overall, these MS methods help define how membrane protein complexes are assembled.

Interface Mapping

Interface mapping is important for biotherapeutic applications to identify binding epitopes for antigen-antibody complexes. Mapping membrane protein interfaces can be achieved using various structural MS techniques. Each technique provides complementary information on binding surfaces by measuring unique properties such as solvent accessibility, distant restraints, and structural dynamics.

Hydrogen-deuterium exchange (HDX)-MS is widely used in biotherapeutic applications to map protein-protein binding interfaces.[190–193] In HDX-MS, labile hydrogens on the protein backbone exchange with deuterium from the solvent. This exchange is possible when hydrogen atoms are solvent-accessible and not involved in intra- or inter-molecular bonding.[191,194] After quenching the exchange, the protein is digested, and the resulting peptides are analyzed using MS.

HDX-MS has been used for a range of biotherapeutic applications, especially epitope mapping.[192,195] In one example with membrane proteins, Kim *et al.*[196] used HDX-MS to perform epitope mapping on broadly neutralizing antibodies for HIV that targeted the membrane-proximal external region (MPER) of the HIV GP41 envelope protein. Comparing the MPER peptide presented in free solution and bound to liposomes, they discovered that significantly less exchange occurred when the antibody bound the MPER in liposomes, revealing a different presentation when the antigen is bound to a lipid bilayer. This study demonstrated the use of HDX to perform epitope mapping on membrane proteins presented in different environments.

Due to the complexity of natural membranes, HDX-MS requires purified proteins solubilized in membrane mimetics. With detergents, no additional sample preparation may be needed. For example, Chung *et al.* investigated the formation of a complex between the β2 adrenergic receptor (β_2AR), a GPCR, and Gs, a stimulatory G protein, in a neopentyl glycol detergent without any special cleanup steps.[197] However, when membrane mimetics with lipids are used, additional sample preparation is often needed to protect the reverse phase column used to separate peptides. Because the labeling is reversible, these sample clean-up steps must be performed quickly. For example, in the liposome study of MPER described above, a second guard column was used to trap lipids.[196] Recent approaches have used zirconium oxide beads to remove lipids.[198–201] Sequence coverage can also be improved by using urea to denature membrane proteins prior to digestion.[202] Using these techniques, HDX has been applied to a variety of different membrane mimetic systems.

Covalent labelling (CL)-MS is another bottom-up method used to characterize protein structures by covalently modifying solvent-accessible amino acids.[203,204] These labeling reagents can either target specific amino acids (such as using N-ethylmaleimide to target cystine) or can non-specifically label multiple residue types on the protein surface (such as with hydroxyl radicals and carbenes).[205–207] The type of labeling reagent used in CL-MS determines the locations on the protein structure that can be probed. For example, nonspecific hydroxyl radicals provide broader structural coverage compared to labels that target specific amino acid residue types.[204] A few factors also influence how the protein structure gets labeled using covalent labeling, such as solvent accessibility, the inherent reactivity of amino acids, and the changes in microenvironment introduced by the binding of ligands.[208] After the protein is labeled, the labeled protein is digested and analyzed by LC-MS/MS to identify the relative amount of labeling at specific amino acid residues.[208]

CL-MS has also been used in biotherapeutics to characterize binding interfaces of membrane proteins, mostly on monoclonal antibodies to determine antigen interactions.[209] With purified samples, fast photochemical oxidation of proteins (FPOP) with hydroxyl radicals has been used for epitope mapping on protein ectodomains from RTKs.[210,211] FPOP has also been applied in detergents, nanodiscs,[212] liposomes,[213] and cells.[214] Unlike HDX, the irreversible labeling in

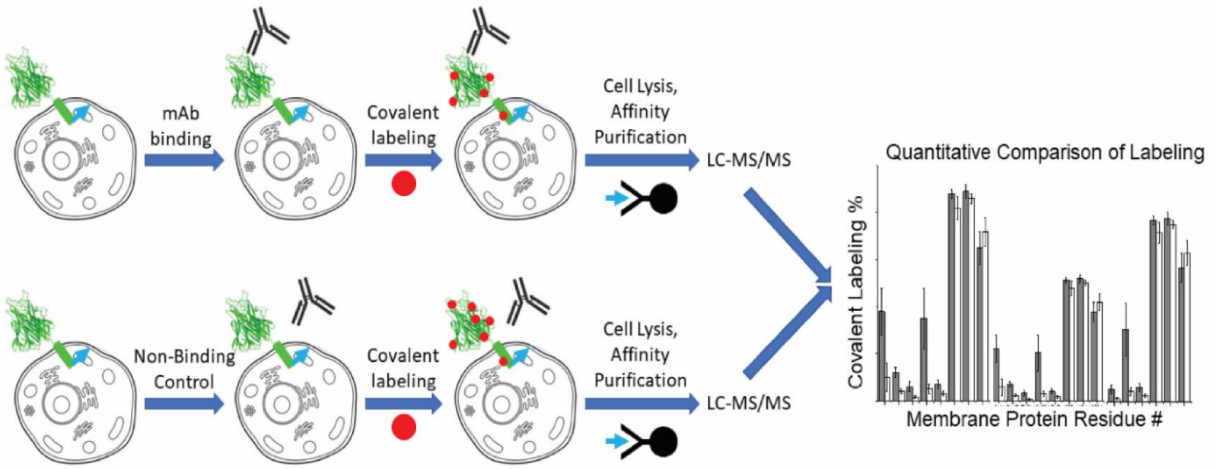

Figure 4. Workflow for in-cell labeling of mTNFα. mTNFα expressed in HEK293T cells was incubated with a binding or nonbinding mAb and then labeled by DEPC. After the DEPC reaction, the cells were lysed, and the protein was purified from the cell lysate using a C-terminal EPEA affinity tag. LC–MS/MS was then used to compare the labeling extents between the two conditions. Reprinted with permission from Kirsh *et al.*, 2023. https://pubs.acs.org/doi/10.1021/acs.analchem.2c05616. Copyright 2023 American Chemical Society.

CL-MS enables a range of downstream sample preparation strategies, such as protein precipitation [212], to remove lipids and clean up the sample prior to MS analysis.

In one example of using CL-MS to map membrane protein interfaces with biotherapeutics, Vachet and coworkers used CL-MS to explore the binding interactions of three monoclonal antibodies with membrane-bound tumor necrosis factor α (mTNFα), in living HEK293T cells (**Figure 4**).[207] They first transfected HEK293T cells with a plasmid encoding mTNFα with a C-terminal purification tag. Next, they mixed the cells with the monoclonal antibodies for binding and performed labeling using diethylpyrocarbonate (DEPC) as the covalent labeling reagent to modify solvent-accessible amino acid residues on proteins while still *in situ*. After labelling, they quenched the reaction and isolated the membrane protein using MS-compatible buffers with DDM detergent. Finally, they digested the proteins and conducted LC-MS/MS analysis. The authors observed decreased DEPC labeling in residues buried in the epitope upon antibody binding. Additionally, they observed changes in labeling away from the epitope, suggesting alterations in mTNFα homotrimer packing or conformational changes. Importantly, because CL-MS methods can be performed in cells (unlike HDX), they do not require any membrane mimetics during the actual experiment, allowing the membrane proteins to be probed in their native environment. Thus, CL-MS provides a powerful method for detecting binding interfaces of membrane proteins in living cells.

XL-MS can also be performed in cells or organelles, providing the most natural environment for studying membrane protein interaction interfaces.[189,215,216] For example, Bruce and coworkers developed an innovative XL-MS workflow to study a peptide interacting with mitochondrial proteins (**Figure 5**).[217] Here, intact mitochondria were isolated and treated with a peptide therapeutic, SS-31. After crosslinking SS-31 with mitochondrial proteins, the crosslinks were enriched through a biotin tag on the SS-31. Twelve inner mitochondrial membrane proteins were identified as interacting

with the protein, helping to reveal the targets of the therapeutic and interfaces on these proteins where SS-31 bound. The ability to expand these measurements to proteome scales enables membrane protein interaction networks and interfaces to be uncovered.[189]

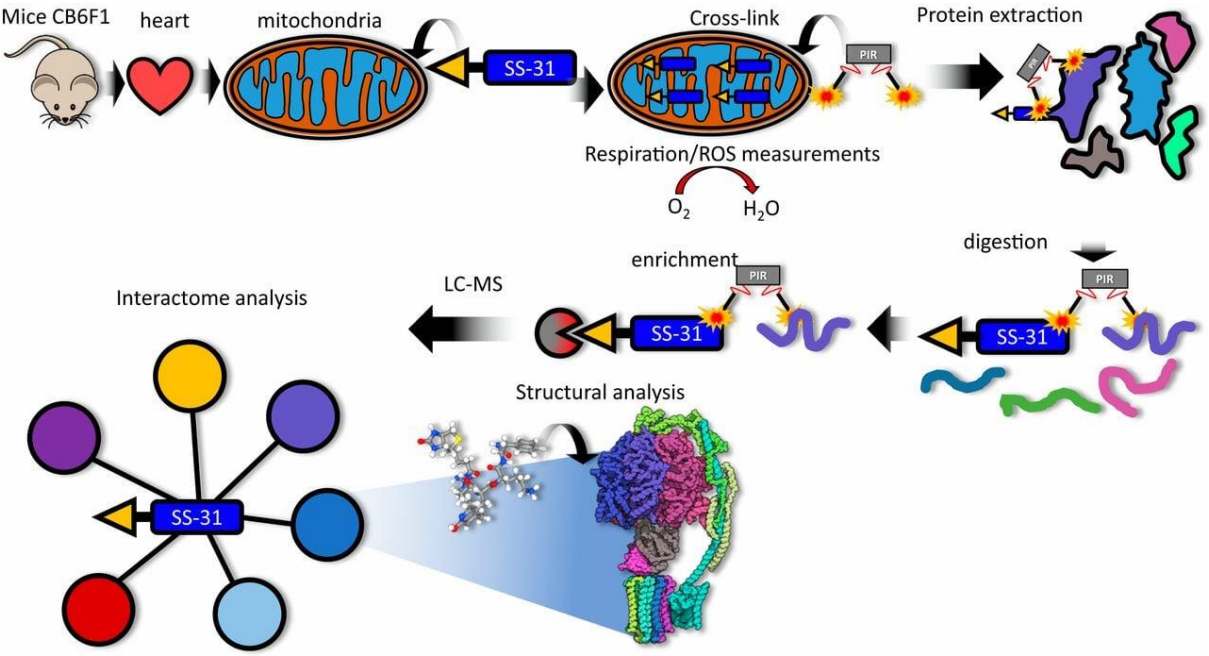

Figure 5: Mitochondria isolated from mouse heart tissue were treated with the SS-31 peptide labelled with a biotin tag (indicated by a triangle). Addition of a protein interaction reporter (PIR) crosslinker crosslinked the SS-31 peptide to interacting proteins. The peptide was then extracted with crosslinked proteins. After digestion and enrichment of crosslinked peptides, LC-MS/MS analysis revealed the interacting proteins and interaction interfaces. Reprinted from Chavez *et al.*, 2020. https://www.pnas.org/doi/full/10.1073/pnas.2002250117.

Finally, these structural MS methods can be used in combination for more complex characterization of interaction interfaces. For example, Li *et al*. used an integrative MS-based approach for epitope mapping and structural characterization.[218] Here, the authors used a combination of HDX, FPOP, and site-specific carboxyl group footprinting to investigate the binding of the soluble ectodomain of human interleukin-6 receptor (IL-6R) to adnectin protein therapeutics. They found that the conserved epitope for both adnectins is a flexible loop connecting two β-strands in the cytokine-binding domain of IL-6R. These findings revealed the value of combining information from multiple techniques to characterize interfaces of protein-protein complexes. Overall, MS methods can provide unique insights that address key biological questions surrounding membrane protein biotherapeutics.

Conclusion

In this chapter, we have discussed various families of membrane proteins that are of biotherapeutic interest, including receptor tyrosine kinases, G-protein coupled receptors, and ion channels. Next, we explored the challenges associated with the solubilization of membrane proteins from their native lipid bilayers into aqueous solutions. Finally, we discussed various structural MS techniques used to address challenging biological questions in the field of biopharmaceuticals for membrane protein targets.

In conclusion, although significant progress has been made in the characterization and therapeutic targeting of membrane proteins, ongoing research and innovative approaches are important to overcome existing challenges. The integration of advanced solubilization techniques and structural MS approaches holds promise for the future of membrane protein biotherapeutics, potentially leading to the development of new treatments for a variety of diseases.

Acknowledgements

This work was supported by the National Institutes of Health/National Institute of General Medical Sciences under award number R35 GM128624 to MTM. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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