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REVIEW

Making the Most of Fusion Tags Technology in Structural Characterization of Membrane Proteins

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Abstract Membrane proteins can be investigated at various structural levels, including the topological structure, the high-resolution three-dimensional structure, and the organization and assembly of membrane protein complexes. Gene fusion technology makes it possible to insert a polynucleotide encoding a protein or polypeptide tag into the gene encoding a membrane protein of interest. Resultant recombinant proteins may possess the functions of the original membrane proteins, together with the biochemical properties of the imported fusion tag, greatly enhancing functional and structural studies of membrane proteins. In this article, the latest literature is reviewed in relation to types, applications, strategies, and approaches to fusion tag technology for structural investigations of membrane proteins.

Keywords Membrane proteins · Fusion tags · Topological structure · 3D structure · Membrane protein complexes

Introduction

Integral membrane proteins (IMPs) represent a specific class of proteins that require lipid membranes to maintain structure and function. IMPs can be single gene products or protein complexes and are involved in many physiological processes such as those mediating the flow of materials and information between cytosol and the extracellular environment, or those involved in energy generation and transformation. Analyses of available genome-wide sequences suggest that IMPs comprise 20–30% of all encoded proteins [1, 2]. However, structural investigations of IMPs are very challenging, due to the low expression levels and hydrophobic nature of membrane proteins.

To date, many techniques, including gene fusion technology, have been developed to facilitate structural and functional investigations of IMPs. Using gene fusion technology, fusion tags such as short peptides, protein domains, or entire functional proteins can be engineered into target proteins at the genetic level. The resultant recombinant proteins may possess the biochemical properties of the imported fusion tags. Therefore, it is possible to take advantage of fusion tags to improve and evaluate protein expression, to detect and track protein targets, and to purify and characterize proteins. Fusion tags vary in their sizes, sources, and biochemical properties [3, 4]. Depending on their applications and biochemical properties, fusion tags are categorized into a number of groups (which may overlap with each other), including affinity fusion tags, fluorescence protein tags, and epitope tags. These fusion tags are widely used in the structural characterization of membrane proteins (Table 1). Two fusion strategies are used, according to the site in the target protein where the protein or peptide tags are to be attached. The insertional gene fusion strategy can be used to insert fusion tags into intact proteins for analyzing membrane protein topology, displaying randomized protein libraries, and designing biosensor proteins [5]. Alternatively, fusion tags are placed at either the amino or the carboxyl terminus

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Table 1 Types and applications of fusion tags cited in this paper

Fusion tag types	Applications in structural characterization of membrane proteins	Comments	Fusion tag examples
Enzyme tag	Topology identification	Large size, which may interfere with protein structure and functions	β-galactosidase (LacZ) [6, 7], alkaline phosphatase (Pho) [6, 8, 9], β-lactamase [9, 10], chloramphenicol acetyltransferase (CAT) [10]
Fluorescent protein tag	Topology identification, displaying protein interactions	Large size, which may interfere with protein structure and functions	GFP[7, 11–13], CFP and YFP[14]
Epitope tag	Topological identification, hydrophilic surface enhancement, visualization of protein subunit	Small size, with lower probability of interfering with protein structure and functions	Myc tag [15], M2 g [16], FLAG [16, 17], V5 [18]
Affinity tag	Affinity purification, visualization of protein subunit, expression partner	Variable size, which may interfere with protein structure and functions	His tag [19–22], MBP [23], GST [24], Strep tag [25]
Protein tag	Expression partner, protein stabilization	Large size	Ubiquitin [19], cytochrome b562 [26], Protein Z [27], thioredoxin [28], Bcl-XL [29]
Peptide tag	Detecting proteins, topology identification, displaying protein interactions;	Small size	S tag [30], BTX tag [31], SBP tag [31], tetracysteine [32]

("end to end" gene fusion strategy) to minimize any potential disruption to the tertiary structure and function of the protein [5].

Structural Investigations of Membrane Proteins

Protein structure is organized hierarchically into primary structure, secondary structure, tertiary structure, and quaternary structure. Similarly, advanced membrane protein structures are characterized at different structural levels, including topological structure, high-resolution three-dimensional structure, and protein–protein interactions within protein complexes.

The topology of membrane proteins refers to the number of transmembrane segments and their orientation in lipid membranes. The distribution of hydrophobic transmembrane helices and hydrophilic connection loops described by membrane protein topology can be verified via a variety of biochemical and molecular techniques. Hydrophilic loops contain characteristic sites such as N-glycosylation sites, cysteine residues, iodinatable sites, antibody epitopes, and proteolytic sites. These sites can be either naturally occurring or generated by means of molecular techniques such as site-directed mutagenesis. Due to the impermeability of lipid membranes to hydrophilic molecules, sidedness of membrane proteins can be determined by testing accessibility of these sites to various agents [33]. Using fusion tag technology, report fusion tags such as fluorescence proteins, enzymes, or epitope tags can be attached to hydrophilic loops or the terminus of a membrane protein as a topologically informative reporter. The subcellular location of reporters is deduced by appropriate biochemical or immune-fluorescent assays and positions of hydrophilic loops are subsequently assigned [33].

The high-resolution three-dimensional structure of membrane proteins can be elucidated by means of NMR spectroscopy [34], X-ray crystallography [35], cryo-electron microscopy [36], or atomic force microscopy (AFM) [37]. Although nearly one third of all open reading frames in fully sequenced genomes are predicted to encode membrane proteins [1, 2], it is extremely difficult to obtain high-resolution three-dimensional structures of membrane proteins. The major obstacles are the naturally low expression levels and poor stabilities of membrane proteins in hydrophilic (lipid-free) environments. Using fusion tags as expression partners, one can not only enhance the expression levels of membrane proteins but also improve the stability of membrane proteins in hydrophilic environments. For characterizing membrane protein structures via X-ray crystallography or cryo-electron microscopy, scientists need to use 2D or 3D protein crystals. IMPs consist of two types of surface areas: hydrophobic areas buried within lipid membranes and hydrophilic areas exposed outside lipid membranes. Polar–polar interactions are crucial for forming protein crystals. If the hydrophilic surface areas of membrane proteins are too small to contribute sufficiently to polar–polar protein interactions, it will be difficult to obtain high-quality protein crystals. These problems may be solved by using large hydrophilic fusion partners to enlarge the hydrophilic portion of membrane proteins [20, 21].

Membrane proteins exist not only in the form of a single gene product, but also function as protein complexes. Understanding the interactions and assembly of protein subunits within protein complexes is important in biochemistry and cell biology. Fusion tag technology provides a means to examine and display the contact points and spatial location of protein subunits. Different types of fusion partner such as affinity tags, fluorescence tags, and epitope tags have been widely used in the structural investigation of membrane protein complexes.

The remainder of this paper will give more information regarding how fusion tag technology is applied in investigating topological structures, 3D structures, and protein complexes of IMPs.

Use of Fusion Tags in Structural Characterization

General Considerations

Although various fusion tags are available for membrane protein structural characterization, there are some general considerations for making fusion tags more effective and avoiding disruption of the native structure and function of proteins.

The first consideration is the size and type of fusion tags. In general, large fusion tags are more likely to interfere with the structure and function of target proteins than small tags. Scientists also have to evaluate the possibility that fusion tags may perturb subsequent purification steps and functional assays. For example, affinity purification of maltose binding protein (MBP)-tagged membrane proteins relies on interactions between the MBP tag and maltose. An essential step prior to affinity chromatography is to solubilize membrane proteins with appropriate detergents. Some commonly used detergents such as dodecyl-β-D-maltoside (DDM) are analogs of maltose. These detergents will compete for the maltose binding site on MBP and affect subsequent affinity purification. In this situation, scientists have to change either the MBP tag or the detergent.

The second consideration is the topology of membrane proteins. In IMPs, transmembrane alpha helices consist of a string of 18–30, predominantly polar, amino acids with a high overall hydrophobicity. Hydrophilic loops that connect transmembrane segments obey the "positive inside" rule, which states that positively charged residues more likely distribute in nontranslocated loops than translocated loops [38]. Figure 1 shows the topological prediction of MrpA, a membrane protein of *Bacillus subtilis*, generated by the webware package Toppred [39]. When introducing fusion tags into a membrane protein, scientists need to consider both the "hydrophobic" rule and "positive inside" rule, i.e., a polypeptide tag should not be fused to the hydrophobic portions, and polypeptide tags with highly positive charged residues can only be fused to intracellular hydrophilic loops or ends. For example, the His tag is highly positively charged, while the Strep tag [40] is nearly neutral. If a membrane protein has an N_{in} topology as shown in the top panel of Fig. 1, both tags can be used for constructing an N-terminal fused protein. If a membrane protein has an N_{out} topology as shown in the bottom panel of Fig. 1, only the Strep tag can be used to make N-terminal fusions. Scientists also have to consider including an N-terminal signal peptide to facilitate exporting the N-terminus of the recombinant protein.

The third consideration is the linker sequence between the fusion tag and the target protein. The function of the linker sequence is to improve the spatial flexibility of fusion tags. Many scientists prefer artificial glycine-rich linkers, due to the simplicity of the side chain of glycine residues. Kavoosi et al. [41] compared naturally occurring linkers (PT)xP, an artificial linker S3N10, designed to be resistant to proteolysis, and traditional poly-glycine linkers, poly(G). They found that (PT)xP and S3N10 linkers were significantly more stable than the other linkers and that Factor Xa processing of fusion proteins depended strongly on linker chemistry. Luminescence resonance energy transfer studies revealed that the separation distance increased with increasing linker length. This increase was particularly large for poly(G) linkers, suggesting that this linker chemistry adopts a hydrated, extended configuration that makes it particularly susceptible to proteolysis.

The fourth consideration is the potential use of tandem copies of fusion tags or multiple tags instead of just one. Sensitivity is an important issue in epitope tagging. Use of tandem tags significantly improves the signal strength and signal-to-noise ratio [42, 43]. Examples of multiple epitope tags include multiple copies of the FLAG [44, 45], c-myc [46, 47], and HA tags [48]. In affinity purification experiments, use of multiple tags or tandem tags is also popular in providing additional mechanisms to remove contaminant proteins. Tandem affinity purification (TAP) is one such example. The TAP tag consists of two types of affinity tags: an IgG binding domain (protein A) and a calmodulin-binding peptide (CBP), separated by a cleavage site for the tobacco etch virus (TEV) protease [49, 50]. TAP-tagged proteins are initially purified by protein A affinity and further purified by calmodulin affinity. After these two consecutive affinity chromatography steps, purification of the target protein to homogeneity is achieved. Use of two or more different tags also makes it possible to combine the desired features of these tags. For example, a combination of epitope tagging and affinity tagging allows for both the detection of fusion proteins with specific antibodies and their purification by affinity chromatography [51, 52].

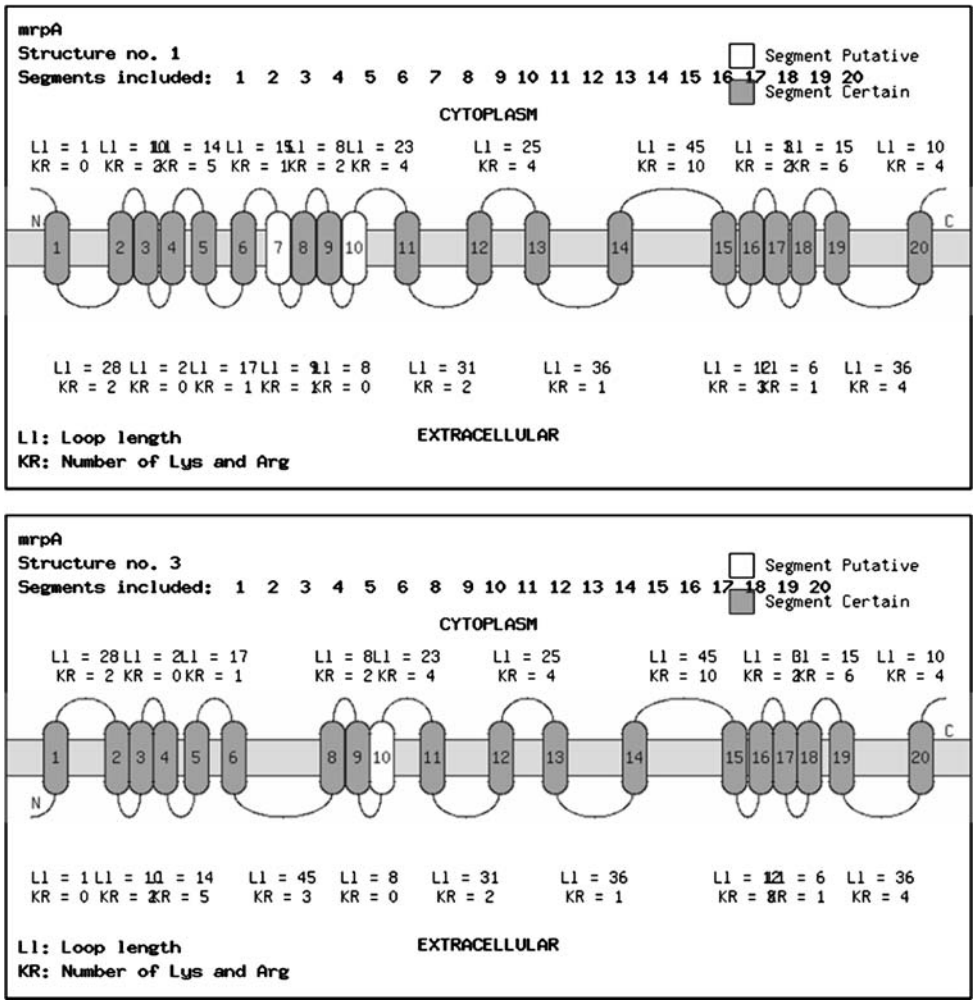

Fig. 1 Two different topological models predicted by Toppred, based on the amino acid sequence of MrpA, an integral membrane protein of *Bacillus subtilis* [39]

Enzyme Tags

Alkaline phosphatase (PhoA), β-galactosidase (LacZ), β-lactamase, and chloramphenicol acetyltransferase (CAT) are commonly used enzyme tags in topological analysis of membrane proteins. PhoA is a periplasmic enzyme that does not have any activity in the cytoplasm because it's folding and assembly occurs only after export to the periplasmic space [53]. In contrast, LacZ exhibits its enzymatic activity in the cytoplasm. When attached downstream of an export signal, LacZ remains trapped in the membrane and fails to fold into the proper conformation [54]. The β-lactamase tag is used as an alternative to PhoA because β-lactamase fusion proteins can provide *E. coli* with ampicillin resistance only if the β-lactamase moiety is translocated to the periplasm [18]. CAT is used as an alternative to LacZ because CAT fusion proteins can provide *E. coli* with chloramphenicol resistance only if CAT is present in the cytosol [18]. The subcellular locations of these enzymes can therefore be determined easily on indicator plates containing appropriate substrates or antibiotics.

Using these enzymes as N-terminal fusion tags, various topology models have been tested. For studying the topology of integral monotopic membrane proteins with only one transmembrane segment, scientists can introduce an enzyme tag into the N-terminus of membrane proteins with little effect on the structure and function of the target protein. For the topological analysis of bitopic or polytopic membrane proteins with two or more transmembrane segments, scientists must construct a series of "end to end" recombinant proteins with N-terminal fusion tags by breaking down the membrane protein at appropriate positions in hydrophilic connecting loops between transmembrane segments. An example is the topological characterization of the DrrB protein, a doxorubicin transporter of *Streptomyces peucetius* [6]. Based on the predicted membrane topology of DrrB, scientists constructed and expressed a series of fusion proteins with N-terminal tags of LacZ or PhoA, or a C-terminal green fluorescence protein (GFP) tag. Analysis of enzymatic activity or fluorescence of these fusion tags suggested that DrrB contains a long N-terminal cytoplasmic tail, three

cytoplasmic loops and a short cytoplasmic C-terminal tail. Other examples include topological investigation on the membrane proteins NorM of *Vibrio cholerae* and AspT of *Tetragenococcus halophilus* [9, 10].

The obvious disadvantage of the "end to end" fusion strategy for modifying bitopic or polytopic membrane proteins is the potential for disruption of the natural structure of these proteins, which may lead to interference in expression and activity. An alternative approach is to take advantages of the insertional fusion strategy, in which a reporter tag is inserted in a connecting hydrophilic loop between two transmembrane segments, resulting in little or no affect on the structure or function of the membrane proteins of interest. The reporter fusion tags are located by appropriate biochemical methods and the positions of the hydrophilic loops can then be assigned. For example, to investigate the membrane topology of AroP, the general aromatic amino acid permease of *Escherichia coli*, Cosgriff et al. [8] inserted the PhoA tag into the hydrophilic loops of AroP and generated a series of AroP-PhoA sandwich fusions. Based on alkaline phosphatase assays and other relevant analyses, they verified and revised the proposed topological model of AroP.

Fluorescent Tags

Aromatic amino acid residues such as tryptophan, tyrosine, and phenylalanine may contribute to the intrinsic fluorescence of certain proteins. A special case of fluorescence occurs in GFP, where the fluorophore originates from an internal serine-tyrosine-glycine sequence which is post-translationally modified to a 4-(*p*-hydroxybenzylidene)-imidazolidin-5-one structure. Fluorescent proteins can be fused to the protein of interest as a marker for gene expression and protein localization [55]. Applications of fluorescent proteins include visualization and determination of cellular protein localization, identification of protein interactions, and determination of the subunit structure of protein complexes. Using GFP and its variants, scientists have obtained structural information on membrane proteins in terms of topological structure and protein–protein interactions.

Using GFP fusions, the topology of membrane proteins localized to the cytoplasmic membrane can be determined. For example, Drew et al. [7] established the topology of 12 inner-membrane proteins from *E. coli* using GFP as a C-terminal tag and PhoA as an N-terminal tag. Lorenz et al. [11–13] developed a GFP-based fluorescence protease protection (FPP) assay for assessing the topology of membrane proteins localized to a wide range of organelles, including the endoplasmic reticulum (ER), Golgi apparatus, mitochondria, peroxisomes and autophagosomes. In this assay, the GFP-tagged membrane proteins are expressed in certain organelles and visualized with fluorescence microscopy. The cells are then treated with the cholesterol-binding drug digitonin to selectively permeabilize the plasma membrane. This allows the cytosolic contents to diffuse across the plasma membrane, while intracellular organelles and the cytoskeletal system are retained. After digitonin treatment, a protease, either trypsin or proteinase K, is added. This readily enters the cytoplasm from the extracellular environment but cannot cross the intact membranes of intracellular organelles. Any GFP molecule attached to a protein of interest that is facing the cytosol will be destroyed and lose its fluorescence. It thus becomes possible to determine whether an organellar protein is membrane associated or luminal and which portions of a membrane protein face the lumen (cell exterior) or cytoplasm. The FPP assay, which was adapted from biochemical protease protection assays, thus uses GFP fusion proteins as noninvasive tools to obtain details of protein topology and localization within living cells in a rapid and straightforward manner.

GFP variants such as blue fluorescent protein (BFP), cyan fluorescent protein (CFP), and yellow fluorescent protein (YFP) exhibit shifted and enhanced excitation/emission characteristics. Using GFP and its derivatives, scientists developed the fluorescence resonance energy transfer (FRET) technique, which makes it possible to investigate interactions between proteins containing fluorescent tags. FRET is based on the principle of nonradiation transfer of photon energy from an excited donor fluorophore to another acceptor fluorophore when both are located within close proximity (1–10 nm). FRET systems frequently used for examining protein–protein interactions are the CFP/YFP tag system, and the GFP/BFP tag system [56]. An example is the investigation of the transient receptor potential channel (TRPC), which is assumed to be composed of multiple TRPC proteins, from subunits TRPC1 to TRPC7. To demonstrate the direct protein–protein interaction of TRPC subunits in living cells, Hofmann et al. [14] generated C-terminal fusion constructs of TRPC subunits with CFP or YFP tags and assessed the proximity of co-expressed TRPC subunits, differentially tagged on their C-termini, by FRET. They found that each TRPC-CFP channel consistently displayed FRET when tested against its TRPC-YFP counterpart, indicating that each of the TRPCs investigated is capable of forming homo-multimers. When CFP and YFP fusion constructs of distinct TRPC channels were systematically co-expressed, a FRET signal significantly higher than the controls was observed in the following combinations: TRPC1/4, TRPC1/5, TRPC4/5, TRPC3/6, TRPC6/7 and TRPC3/7. These results are helpful in defining the mechanisms of TRPC channel formation and offer a conceptual framework to assess the physiological role of distinct TRPC proteins in living cells.

The major limitations of tagging proteins with GFP or its variants are (i) that the relatively large size of GFP can perturb the structure and function of tagged proteins; (ii) that it is not easy to distinguish intracellular from extracellular pools of GFP-tagged membrane proteins in live cells; (iii) multicolor experiments are required to separate DNA constructs for each variant [31]. One way to overcome these problems is to use epitope tags.

Epitope Tags

In 1984, Munro and Pelham [57] described epitope tagging technology. This technology makes use of artificial epitope tags that are engineered into a protein sequence. The epitopes in the expressed tagged proteins are then recognized by existing antibodies. To date, many epitopes with high detection sensitivity have been developed, such as the OLLAS tag, a 14-amino acid sequence consisting of sequences from both the OmpF derived linker and mouse Langerin [58]. OLLAS-tagged proteins are detected by anti-OLLAS mAb with much higher sensitivity than conventional epitope tags and other anti-tag mAbs.

Epitope tags can be used for visualizing proteins and protein domains in a similar way to fluorescent proteins. However, epitope tags are much smaller (typically 6–30 amino acids residues) than fluorescent proteins or enzyme tags and this reduces the possibility of interfering with the structure and function of target proteins. This advantage makes epitope tagging a powerful tool for investigating membrane proteins at different structural levels.

Epitope tagging is an effective tool for topological studies of membrane proteins. Epitope tags can be placed anywhere in the target membrane proteins. The subcellular locations of topologically informative epitope tags can then be examined with appropriate antibodies. For example, STRA6 is a polytopic membrane protein that functions as the high-affinity receptor for plasma retinol binding protein (RBP) and mediates cellular uptake of vitamin A from the vitamin A–RBP complex. By inserting an epitope tag into all possible extracellular and intracellular domains of STRA6, Kawaguchi et al. [15] systematically analyzed the accessibility of each tag on the surface of live cells, the accessibility of each tag in permeabilized cells, and the effect of each tag on RBP binding and STRA6-mediated vitamin A uptake. These studies and other relevant analyses not only revealed STRA6's extracellular, transmembrane, and intracellular domains but also implicated extracellular regions of STRA6 in RBP binding. Similarly, Paterson et al. [16] examined the topology of BM2, an integral monotopic membrane protein of the influenza B virus, using "end to end" fusion proteins. They constructed and expressed two forms of recombinant BM2 proteins with an N-terminal M2g tag or a C-terminal FLAG tag. Biochemical analyses indicated that the BM2 protein adopts an $N_{out}C_{in}$ orientation in membranes.

Epitope tagging is also an important tool for investigating membrane protein complexes. By using small epitope tags and an appropriate monoclonal antibody, one can precisely locate the position of a protein subunit within an intact complex. For example, Barrera et al. [18] determined the subunit stoichiometry of the transient receptor potential C1 (TRPC1) channel, fused with affinity tags and epitope tags, by imaging isolated channels with AFM and monoclonal antibodies. AFM gives a simple, rapid and convenient way to image the shape of protein specimens [37]. By measuring and calculating the molecular volumes of individual channel particles, they found that TRPC1 exists both in monomeric and tetrameric forms. Based on observations of complexes formed between TRPC1 channels and antibodies against a V5 epitope tag present on each subunit, they found the angles between pairs of bound antibodies had peaks at either 88° or 178°, indicating that the channel assembles as a tetramer.

Membrane proteins that contain large hydrophilic surfaces form stable polar–polar interactions relatively easily. This is essential for building high-quality protein crystals for 3D structural determination by techniques such as X-ray crystallography. However, expansion of the polar surface of a membrane protein by direct fusion may disrupt the structure and function of target protein. An alternative strategy is to make use of antibody fragments to increase the 'soluble' portion of the membrane protein [59]. However, due to the time and expense associated with producing monoclonal antibodies to the target protein, this approach is limited in the era of proteomics, which requires high-throughput technology for structural establishment. Roosild et al. [17] showed that this problem may be solved by introducing an epitope tag to the target membrane protein. They engineered a FLAG epitope tag into a known loop region of the K^+ channel protein, KvPae. After functional expression and purification, this recombinant KvPae was co-crystallized with anti-FLAG M2 monoclonal antibodies. They confirmed the interaction between the fusion FLAG tag and the anti-FLAG M2 Fab domain at 1.86 A resolution. The results suggest the use of antibody fragments for improving the stability of target proteins can be applied to the study of membrane protein structures by placing a short epitope tag within a predicted peripheral loop of the protein and utilizing antibody fragments.

Limitations exist for currently used epitope tags, such as the large size of detection molecules (antibodies), poor availability of detection molecules, and unsuitability for use in living animals. To circumvent these limitations, scientists developed new reporter proteins as alternatives to antibodies to detect peptide tags such as the S tag, BTX tag, and the SBP tag. The S tag is a domain derived from

pancreatic ribonuclease A that is recognized by a polypeptide representing a different portion of the ribonuclease protein [30]. The reporter protein of the BTX tag is α-bungarotoxin, and the reporter protein for the SBP tag is streptavidin. Both reporter proteins can be conjugated with fluorophores. McCann et al. [31] demonstrated that both the BTX tag and the SBP tag can be used for labeling membrane proteins in live cells. Another epitope-like approach for labeling membrane proteins is a strategy based on fluorescein arsenical hairpin binder (FlAsH) [32]. FlAsH is a small, membrane-permeant fluorescein derivative, targeted to a short tetracysteine sequence. Hoffmann et al. [32] showed that FRET from CFP to FlAsH reported G protein coupled receptor (GPCR) activation in living cells without disturbing receptor function. This shows that the small size of the tetracysteine-biarsenical tag can be extremely advantageous.

Affinity Tags

An affinity tag is a short peptide or an entire protein that has been engineered into a target protein, allowing purification by affinity chromatography. Affinity purification relies on specific interactions between affinity fusion tags and immobilized ligands such as immobilized metal affinity chromatography matrices for purification of polyhistidine tagged proteins. Using affinity tags, the purification of many membrane proteins can achieved to a high purity prior to structural studies. Examples include affinity purification of the His-tagged lactose transporter and glycerol-3-phosphate transporter that are used for growing 3D crystals for X-ray crystallography studies [60, 61]. Another example is the putative tetraspan integral membrane protein, peripheral myelin protein 22 (PMP22). Mobley et al. [20] constructed a recombinant version of the PMP22 protein incorporating a His tag. After expression and purification of the recombinant fusion protein and cleavage of the fusion partner, they confirmed that PMP22 is highly helical and shows evidence of a having a stable tertiary structure.

In addition to purification, affinity fusion tags can be used for investigating the composition of membrane protein complexes and interactions between subunits, via co-purification of interacting components. For example, the membrane-embedded F0 complex of ATP synthase in *Escherichia coli* consists of subunits *a*, *b*, and *c*. Stalz et al. [21] fused the subunit *a* with an N-terminal His tag and were able to purify an *ab*2 subcomplex rather than just the single subunit *a* carrying the His tag, whereas subunit *c* was completely removed during the washing steps prior to elution of the protein. They also confirmed that the interaction between subunits *a* and *b* is stoichiometric and functional. Affinity between the His tag and bound molecules can also be used for imaging tagged subunits within a membrane protein complex. For example, Büchel et al. [22] constructed an N-terminal His-tagged version of PsbH protein, a transmembrane subunit within the photosystem II complex. For investigating the location of PsbH within the photosystem II complex, the His-tagged PsbH protein was labeled with a Ni^{2+}-NTA gold cluster and subjected to electron microscopy and image analysis. The data indicated that the N-terminus of PsbH is close to the two transmembrane helices of cytochrome b559.

Protein Tags with a High Solubility

Use of large affinity tags, such as MBP, may improve the solubility of fusion proteins. Scientists suggest that MBP has chaperon-like features and facilitates correct protein folding [62–64]. Thus MBP may provide a protein scaffold that prevents the membrane protein portion of the fusion from aggregating. For example, Korepanova et al. [23] successfully expressed 16 small IMPs as fusions to the C-terminus of MBP. These proteins did not show any expression while fused with an N-terminal His tag in a previous study [22]. Fusing recombinant proteins to highly soluble partners is a strategy that is frequently used to prevent aggregation of recombinant proteins in *Escherichia coli*. Moreover, co-overexpression of prokaryotic chaperones can increase the amount of properly folded recombinant proteins. To understand the solubility enhancement of fusion proteins, Douette et al. [65] designed two recombinant proteins composed of uncoupling protein 1 (UCP1), which is a mitochondrial membrane protein, in fusion with MBP or NusA. They were able to express soluble forms of MBP-UCP1 and NusA-UCP1 despite the high hydrophobicity of UCP1. The yield of soluble fusion proteins depended on co-overexpression of GroEL, which catalyzes folding of polypeptides. Their findings suggest that MBP and NusA act as solubilizing agents by forcing the recombinant protein to pass through the bacterial chaperone pathway as a complete fusion protein. Wittlich et al. [19] also showed soluble expression of a membrane protein fused with an N-terminal ubiquitin.

Although use of protein tags can enhance protein expression, many membrane protein fusions are expressed in inclusion bodies rather than in a soluble form or in lipid membranes. These aggregated proteins can still be purified and applied for structural analysis. There has already been reports of the purification of small ubiquitin-related modifier (SUMO) or Bcl-XL (a mutant form of the anti-apoptotic Bcl-2 family protein) fused membrane proteins, expressed as inclusion bodies [29, 66]. Fusing membrane proteins with highly soluble tags can also be used in cell-free expression systems, which is an alternative strategy to solve problems such as the formation of insoluble

aggregates in *Escherichia coli* and other cell-based expression systems. In work using cell-free protein expression of GPCRs with an *E. coli* S30 extract, Ishihara et al. [28] found that a thioredoxin (Trx)-fusion vector induced higher protein expression levels than nonfusion and hexa-histidine-tagged proteins. The unpurified, reconstituted thioredoxin-fused receptor proteins exhibited functional activity.

Soluble proteins having a large hydrophilic surface may contribute to polar–polar protein contacts of tagged membrane proteins, which will benefit subsequent 2D or 3D crystallization experiments. For example, Zhuang et al. used cytochrome b 562 as an insertional fusion tag by fusing it in the middle cytoplasmic loop of lactose permease LacY [20]. After expression and purification, the recombinant protein was reconstituted in the presence of phospholipids, yielding densely packed vesicles and well-ordered 2D crystals from which structural information of LacY permease was obtained. Another example is cytochrome bo3 ubiquinol oxidase, whose structural elucidation is limited by the quality of the crystals due to weak protein–protein contacts within the crystal lattice. To improve these contacts, Byrne et al. introduced protein Z with high solubility and stability as a C-terminal fusion tag to subunit IV of cytochrome bo3 [21]. The results showed that the fusion tag protein Z improved the quality of protein crystals.

Tag Removal

Tag removal is not usually necessary when simply detecting or visualizing proteins. However, it is necessary to eliminate any influence of the fusion tag in structural characterization experiments. Scientists must therefore remove fusion tags prior to structural and functional analyses when fusion tags are suspected of interfering with the biological activity of a protein, impeding its crystallization, or otherwise influencing its behavior. The fusion tag can be removed by either harsh chemical treatment or mild enzymatic cleavage [67]. The prerequisite for chemical methods is to place a unique methionine residue at the junction between the fusion partner and the target protein [68, 69]. Tag-free proteins can then be produced by treatment with either cyanogen bromide or hydroxylamine. Chemical methods are rather nonspecific and may lead to unnecessary denaturation or modification of the target protein.

Enzymatic methods are more specific and tag removal is usually possible under mild conditions. Two types of proteases are available for enzymatic cleavage: endoproteases and exopeptidases. Endoprotease-mediated tag removal requires an enzymatic cleavage site to be placed between the fusion tag and the target protein. Thrombin, factor Xa, enterokinase, TEV protease, and PreScission protease are the most widely used endoproteases [19, 23, 70, 71]. For example, Yeliseev et al. [71] expressed the human peripheral-type cannabinoid receptor (CB2) in *Escherichia coli* as a fusion with the MBP, thioredoxin, and a deca-histidine tag. After affinity purification, they removed all fused tags with TEV protease digestion and obtained the CB2 protein with a high purity for structural and functional analysis.

The use of exopeptidases is an alternative enzymatic approach for tag removal. Several aminopeptidases and carboxypeptidases are available, including DAPase, aminopeptidase M (APM) and carboxypeptidase A and B (CPA and CPB) [72–74]. APM, CPA, and CPB release a single amino acid sequentially from the N- or C-terminus of a protein with certain amino acids being released either slowly or not at all. Thus scientists can design a stop site and control reaction conditions for tag removal. DAPase is a recombinant rat dipeptidyl aminopeptidase I (DPPI) that is part of the TAGZyme system, with lysine, arginine, praline, or glutamine used as stop positions. The TAGZyme system provides highly specific and sequential exoproteolytic cleavage of N-terminal amino acids for the removal of small affinity tags from proteins. Block et al. [75] described an efficient strategy to produce high-quality proteins by using a single, large IMAC chromatography column and enzymatic His tag removal via the TAGZyme system. They performed numerous quality assays to demonstrate the high purity and quality of the final protein product. The results show that the applied workflow is suitable for industrial production of protein-based biopharmaceuticals.

As well as protease-mediated tag removal, scientists have developed self-cleaving tags. Mee et al. [76] described the use of self-splicing inteins to generate a protein fused with a self-cleaving aggregation tag for chromatography-free protein purification. They constructed a recombinant protein with the target protein located downstream of an aggregation tag and intein. The aggregation-tagged protein could be purified by simple centrifugation after cell lysis. The cleaving reaction was then initiated by either changing the pH or adding thio reagents. The released target protein and aggregation tag was then be separated by another round of centrifugation.

Conclusions

Structural characterization of membrane proteins is a difficult challenge in structural and functional proteomics. In addition to the traditional techniques used for structural characterization such as X-ray crystallography and NMR, the use of various fusion tags such as epitope tags and

fluorescence proteins provides alternative methods. Use of fusion tag technology for membrane proteins relies on predicting and verifying the membrane topology of the protein of interest. For successful expression and structural characterization of tag-fused membrane proteins, scientists need to select fusion tags and design fusion strategies based on specific research purposes and predicted topological models, paying particular attention to the orientation of the N- and C- termini of the membrane protein of interest. In some applications, tag removal and the linker sequence between the fusion tag and the target protein are also important factors to be considered.

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References

1. Wallin, E., & von Heijne, G. (1998). Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Science, 7*, 1029–1038.
2. Ahram, M., Litou, Z. I., Fang, R., et al. (2006). Estimation of membrane proteins in the human proteome. *In Silico Biology, 6*, 379–386.
3. Waugh, D. S. (2005). Making the most of affinity tags. *Trends in Biotechnology, 23*(6), 316–320. doi:10.1016/j.tibtech.2005.03.012.
4. Terpe, K. (2003). Overview of tag protein fusions: from molecular and biochemical fundamentals to commercial systems. *Applied Microbiology and Biotechnology, 60*(5), 523–533.
5. Doi, N., & Yanagawa, H. (1999). Insertional gene fusion technology. *FEBS Letters, 457*(1), 1–4. doi:10.1016/S0014-5793(99)00991-6.
6. Gandlur, S. M., Wei, L., Levine, J., et al. (2004). Membrane topology of the DrrB protein of the doxorubicin transporter of *Streptomyces peucetius*. *The Journal of Biological Chemistry, 279*(26), 27799–27806. doi:10.1074/jbc.M402898200.
7. Drew, D., Sjostrand, D., Nilsson, J., et al. (2002). Rapid topology mapping of *Escherichia coli* inner-membrane proteins by prediction and PhoA-GFP fusion analysis. *Proceedings of the National Academy of Sciences of the United States of America, 99*(5), 2690–2695. doi:10.1073/pnas.052018199.
8. Cosgriff, A. J., & Pittard, A. J. (1997). A topological model for the general aromatic amino acid permease, AroP, of *Escherichia coli*. *Journal of Bacteriology, 179*, 3317–3323.
9. Nanatani, K., Ohonishi, F., Yoneyama, H., et al. (2005). Membrane topology of the electrogenic aspartate-alanine antiporter AspT of *Tetragenococcus halophilus*. *Biochemical and Biophysical Research Communications, 328*, 20–26. doi:10.1016/j.bbrc.2004.12.133.
10. Singh, A. K., Haldar, R., Mandal, D., et al. (2006). Analysis of the topology of *Vibrio cholerae* NorM and identification of amino acid residues involved in norfloxacin resistance. *Antimicrobial Agents and Chemotherapy, 50*(11), 3717–3723. doi:10.1128/AAC.00460-06.
11. Lorenz, H., Hailey, D. W., & Lippincott-Schwartz, J. (2006). Fluorescence protease protection of GFP chimeras to reveal protein topology and subcellular localization. *Nature Methods, 3*, 205–210. doi:10.1038/nmeth857.
12. Lorenz, H., Hailey, D. W., Wunder, C., et al. (2006). The fluorescence protease protection (FPP) assay to determine protein localization and membrane topology. *Nature Prot, 1*, 276–279. doi:10.1038/nprot.2006.42.
13. Lorenz, H., Hailey, D. W., & Lippincott-Schwartz, J. (2008). Addressing membrane protein topology using the fluorescence protease protection (FPP) assay. *Methods in Molecular Biology (Clifton, N.J.), 440*, 227–233. doi:10.1007/978-1-59745-178-9_17.
14. Hofmann, T., Schaefer, M., Schultz, G., et al. (2002). Subunit composition of mammalian transient receptor potential channels in living cells. *Proceedings of the National Academy of Sciences of the United States of America, 99*(11), 7461–7466. doi:10.1073/pnas.102596199.
15. Kawaguchi, R., Yu, J., Wiita, P., et al. (2008). Mapping the membrane topology and extracellular ligand binding domains of the retinol binding protein receptor. *Biochemistry, 47*(19), 5387–5395. doi:10.1021/bi8002082.
16. Paterson, R. G., Takeda, M., Ohigashi, Y., et al. (2003). Influenza B virus BM2 protein is an oligomeric integral membrane protein expressed at the cell surface. *Virology, 306*(1), 7–17. doi:10.1016/S0042-6822(02)00083-1.
17. Roosild, T. P., Castronovo, S., & Choe, S. (2006). Structure of anti-FLAG M2 Fab domain and its use in the stabilization of engineered membrane proteins. *Acta Crystallographica, F62*, 835–839.
18. Barrera, N. P., Shaifta, Y., McFadzean, I., et al. (2007). AFM imaging reveals the tetrameric structure of the TRPC1 channel. *Biochemical and Biophysical Research Communications, 358*, 1086–1090. doi:10.1016/j.bbrc.2007.05.039.
19. Wittlich, M., Wiesehan, K., Koenig, B. W., et al. (2007). Expression, purification and membrane reconstitution of a CD4 fragment comprising the transmembrane and cytoplasmic domains of the receptor. *Protein Expression and Purification, 55*, 198–207. doi:10.1016/j.pep.2007.05.007.
20. Mobley, C. K., Myers, J. K., Hadziselimovic, A., et al. (2007). Purification and initiation of structural characterization of human peripheral myelin protein 22, an integral membrane protein linked to peripheral neuropathies. *Biochemistry, 46*, 11185–11195. doi:10.1021/bi700855j.
21. Stalz, W. D., Greie, J.-C., Deckers-Hebestreit, G., et al. (2003). Direct interaction of subunits a and b of the F0 complex of *Escherichia coli* ATP synthase by forming an ab2 subcomplex. *The Journal of Biological Chemistry, 278*(29), 27068–27071. doi:10.1074/jbc.M302027200.
22. Büchel, C., Morris, E., Orlova, E., et al. (2001). Localisation of the PsbH subunit in photosystem II: a new approach using labelling of His-tags with a Ni(2+)-NTA gold cluster and single particle analysis. *Journal of Molecular Biology, 312*(2), 371–379. doi:10.1006/jmbi.2001.4951.
23. Korepanova, A., Moore, J. D., Nguyen, H. B., et al. (2007). Expression of membrane proteins from *Mycobacterium tuberculosis* in *Escherichia coli* as fusions with maltose binding protein. *Protein Expression and Purification, 53*(1), 24–30. doi:10.1016/j.pep.2006.11.022.
24. Huber, W. J., & Backes, W. L. (2007). Expression and characterization of full-length human heme oxygenase-1: The presence of intact membrane-binding region leads to increased binding affinity for NADPH cytochrome P450 reductase. *Biochemistry, 46*, 12212–12219. doi:10.1021/bi701496z.
25. Schmidt, T. G. M., & Skerra, A. (2007). The strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nature Protocols, 2*(6), 1528–1535. doi:10.1038/nprot.2007.209.
26. Zhuang, J. P., Prive, G. G., Werner, G. E., et al. (1999). Two-dimensional crystallization of *Escherichia coli* lactose permease. *Journal of Structural Biology, 125*, 63–75. doi:10.1006/jsbi.1998.4059.

27. Byrne, B., Abramson, J., Jannson, M., et al. (2000). Fusion protein approach to improve the crystal quality of cytochrome bo3 ubiquinol oxidase from *Escherichia coli*. *Biochimica et Biophysica Acta, 1459*, 449–455. doi:10.1016/S0005-2728(00)00183-3.
28. Ishihara, G., Goto, M., Saeki, M., et al. (2005). Expression of G protein coupled receptors in a cell-free translational system using detergents and thioredoxin-fusion vectors. *Protein Expression and Purification, 41*(1), 27–37. doi:10.1016/j.pep.2005.01.013.
29. Thai, K., Choi, J., Franzin, C. M., et al. (2005). Bcl-XL as a fusion protein for the high-level expression of membrane-associated proteins. *Protein Science, 14*, 1–8. doi:10.1110/ps.041244305.
30. Therien, A. G., Glibowicka, M., & Deber, C. M. (2002). Expression and purification of two hydrophobic double-spanning membrane proteins derived from the cystic fibrosis transmembrane conductance regulator. *Protein Expression and Purification, 25*(1), 81–86. doi:10.1006/prep.2001.1612.
31. McCann, C. M., Bareyre, F. M., Lichtman, J. W., et al. (2005). Peptide tags for labeling membrane proteins in live cells with multiple fluorophores. *BioTechniques, 38*(6), 945–952. doi:10.2144/05386IT02.
32. Hoffmann, C., Gaietta, G., Bünemann, M., et al. (2005). A FlAsH-based FRET approach to determine G protein-coupled receptor activation in living cells. *Nature Methods, 2*(3), 171–176. doi:10.1038/nmeth742.
33. Geest, M. V., & Lolkema, J. S. (2000). Membrane topology and insertion of membrane proteins: Search for topogenic signals. *Microbiology and Molecular Biology Reviews, 64*(1), 13–33. doi:10.1128/MMBR.64.1.13-33.2000.
34. Nielsen, N., Malmendal, A., & Vosegaard, T. (2004). Techniques and applications of NMR to membrane proteins. *Molecular Membrane Biology, 21*(3), 129–141. doi:10.1080/09687680410001693679.
35. Caffrey, M. (2003). Membrane protein crystallization. *Journal of Structural Biology, 142*, 108–132. doi:10.1016/S1047-8477(03)00043-1.
36. Tate, C. G., & Rubinstein, J. L. (2008). Membrane protein structure determination by electron cryo-microscopy. In E. Pebay-Peyroula (Ed.), *Biophysical analysis of membrane proteins: Investigating structure and function*. Weinheim: Wiley-VCH.
37. Goncalves, R. P., & Scheuring, S. (2006). Manipulating and imaging individual membrane proteins by AFM. *Surface and Interface Analysis, 38*, 1413–1418. doi:10.1002/sia.2350.
38. von Heijne, G. (2006). Membrane-protein topology. *Nature Reviews. Molecular Cell Biology, 7*, 909–918. doi:10.1038/nrm2063.
39. http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py?form=toppred.
40. Bungert, S., Krafft, B., Schlesinger, R., et al. (1999). One-step purification of the NADH dehydrogenase fragment of the *Escherichia coli* complex I by means of Strep-tag affinity chromatography. *FEBS Letters, 460*, 207–211. doi:10.1016/S0014-5793(99)01341-1.
41. Kavoosi, K., Creagh, A. L., & Kilburn, D. G. (2007). Strategy for selecting and characterizing linker peptides for CBM9-tagged fusion proteins expressed in *Escherichia coli*. *Biotechnology and Bioengineering, 98*, 599–610. doi:10.1002/bit.21396.
42. Jarvik, J. W., & Telmer, C. A. (1998). Epitope tagging. *Annual Review of Genetics, 32*, 601–618. doi:10.1146/annurev.genet.32.1.601.
43. Brizzard, B. (2008). Epitope tagging. *BioTechniques, 44*(5), S693–S695. doi:10.2144/000112841.
44. Hernan, R., Heuermann, K., & Brizzard, B. (2000). Multiple epitope tagging of expressed proteins for enhanced detection. *BioTechniques, 28*, 789–793.
45. Zhang, L., Hernan, R., & Brizzard, B. (2001). Multiple tandem epitope tagging for enhanced detection of protein expressed in mammalian cells. *Molecular Biotechnology, 19*(3), 313–321. doi:10.1385/MB:19:3:313.
46. Sharrock, R. A., & Clack, T. (2004). Heterodimerization of type II phytochromes in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 11500–11505. doi:10.1073/pnas.0404286101.
47. Graumann, J., Dunipace, L. A., Seol, J. H., et al. (2004). Applicability of tandem affinity purification MudPIT to pathway proteomics in yeast. *Molecular and Cellular Proteomics, 3*, 226–237. doi:10.1074/mcp.M300099-MCP200.
48. Ross-Macdonald, P., Sheehan, A., Roeder, G. S., et al. (1997). A multipurpose transposon system for analyzing protein production, localization, and function in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America, 94*, 190–195. doi:10.1073/pnas.94.1.190.
49. Veraksa, A., Bauer, A., & Artavanis-Tsakonas, S. (2005). Analyzing protein complexes in *Drosophila* with tandem affinity purification-mass spectrometry. *Developmental Dynamics, 232*, 827–834. doi:10.1002/dvdy.20272.
50. Li, Q., Dai, X.-Q., Shen, P. Y., et al. (2004). A modified mammalian tandem affinity purification procedure to prepare functional polycystin-2 channel. *FEBS Letters, 576*, 231–236. doi:10.1016/j.febslet.2004.09.017.
51. DiCiommo, D. P., Duckett, A., Burcescu, I., et al. (2004). Retinoblastoma protein purification and transduction of retina and retinoblastoma cells using improved alphavirus vectors. *Investigative Ophthalmology and Visual Science, 45*, 3320–3329. doi:10.1167/iovs.04-0140.
52. Gloeckner, C. J., Boldt, K., Schumacher, A., et al. (2007). A novel tandem affinity purification strategy for the efficient isolation of native protein complexes. *Proteomics, 7*, 4228–4234. doi:10.1002/pmic.200700038.
53. Akiyama, Y., & Ito, K. (1993). Folding and assembly of bacterial alkaline phosphatase *in vitro* and *in vivo*. *The Journal of Biological Chemistry, 268*, 8146–8150.
54. Silhavy, T. J., Shuman, H. A., Beckwith, J., et al. (1977). Use of gene fusions to study outer membrane protein localization in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America, 74*, 5411–5415. doi:10.1073/pnas.74.12.5411.
55. Tsien, R. Y. (1998). The green fluorescent protein. *Annual Review of Biochemistry, 67*, 509–544. doi:10.1146/annurev.biochem.67.1.509.
56. Truong, K., & Ikura, M. (2001). The use of FRET imaging microscopy to detect protein-protein interactions and protein conformational changes in vivo. *Current Opinion in Structural Biology, 11*, 573–578. doi:10.1016/S0959-440X(00)00249-9.
57. Munro, S., & Pelham, H. R. B. (1984). Use of peptide tagging to detect proteins expressed from cloned genes: Deletion mapping functional domains of *Drosophila* hsp70. *The EMBO Journal, 3*(13), 3087–3093.
58. Park, S. H., Cheong, C., Idoyaga, J., et al. (2008). Generation and application of new rat monoclonal antibodies against synthetic FLAG and OLLAS tags for improved immunodetection. *Journal of Immunological Methods, 331*(1–2), 27–38. doi:10.1016/j.jim.2007.10.012.
59. Hunte, C., & Michel, H. (2002). Crystallisation of membrane proteins mediated by antibody fragments. *Current Opinion in Structural Biology, 12*, 503–508. doi:10.1016/S0959-440X(02)00354-8.
60. Abramson, J., Smirnova, I., Kasho, V., et al. (2003). Structure and mechanism of the lactose permease of *Escherichia coli*. *Science, 301*, 610–615. doi:10.1126/science.1088196.
61. Huang, Y., Lemieux, M. J., Song, J. M., et al. (2003). Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*. *Science, 301*, 616–620. doi:10.1126/science.1087619.
62. Kapust, R. B., & Waugh, D. S. (1999). *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the

solubility of polypeptides to which it is fused. *Protein Science, 8*, 1668–1674.
63. Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology, 10*, 411–421. doi:10.1016/S0958-1669(99)00003-8.
64. Sachdev, D., & Chirgwin, J. M. (1998). Solubility of proteins isolated from inclusion bodies is enhanced by fusion to maltose-binding protein or thioredoxin. *Protein Expression and Purification, 12*, 122–132. doi:10.1006/prep.1997.0826.
65. Douette, P., Navet, R., Gerkens, P., et al. (2005). *Escherichia coli* fusion carrier proteins act as solubilizing agents for recombinant uncoupling protein 1 through interactions with GroEL. *Biochemical and Biophysical Research Communications, 333*(3), 686–693. doi:10.1016/j.bbrc.2005.05.164.
66. Zuo, X., Li, S., Hall, J., et al. (2005). Enhanced expression and purification of membrane proteins by SUMO fusion in *Escherichia coli*. *Journal of Structural and Functional Genomics, 6*, 103–111. doi:10.1007/s10969-005-2664-4.
67. Arnau, J., Lauritzen, C., Petersen, G. E., et al. (2006). Current strategies for the use of affinity tags and tag removal for the purification of recombinant proteins. *Protein Expression and Purification, 48*, 1–13. doi:10.1016/j.pep.2005.12.002.
68. Fairlie, W. D., Uboldi, A. D., De Souza, D. P., et al. (2002). A fusion protein system for the recombinant production of short disulfide-containing peptides. *Protein Expression and Purification, 26*, 171–178. doi:10.1016/S1046-5928(02)00521-1.
69. Rais-Beghdadi, C., Roggero, M. A., Fasel, N., et al. (1998). Purification of recombinant proteins by chemical removal of the affinity tag. *Applied Biochemistry and Biotechnology, 74*, 95–103. doi:10.1007/BF02787176.
70. Mannelli, L. D. C., Pacini, A., Toscano, A., et al. (2006). Gi/o proteins: Expression for direct activation enquiry. *Protein Expression and Purification, 47*(1), 303–310. doi:10.1016/j.pep.2005.11.005.
71. Yeliseev, A., Zoubak, L., & Gawrisch, K. (2007). Use of dual affinity tags for expression and purification of functional peripheral cannabinoid receptor. *Protein Expression and Purification, 53*(1), 153–163. doi:10.1016/j.pep.2006.12.003.
72. Abdullah, N., & Chase, H. A. (2005). Removal of poly-histidine fusion tags from recombinant proteins purified by expanded bed adsorption. *Biotechnology and Bioengineering, 92*, 501–513. doi:10.1002/bit.20633.
73. Pedersen, J., Lauritzen, C., Madsen, M. T., et al. (1999). Removal of N terminal polyhistidine tags from recombinant proteins using engineered aminopeptidases. *Protein Expression and Purification, 15*, 389–400. doi:10.1006/prep.1999.1038.
74. Kenig, M., Peternel, S., Gaberc-Porekar, V., et al. (2006). Influence of the protein oligomericity on final yield after affinity tag removal in purification of recombinant proteins. *Journal of Chromatography. A, 1101*(1–2), 293–306. doi:10.1016/j.chroma.2005.09.089.
75. Block, H., Kubicek, J., Labahn, J., et al. (2008). Production and comprehensive quality control of recombinant human Interleukin-1β: A case study for a process development strategy. *Protein Expression and Purification, 57*(2), 244–254. doi:10.1016/j.pep.2007.09.019.
76. Mee, C., Banki, M. R., & Wood, D. W. (2008). Towards the elimination of chromatography in protein purification: Expressing proteins engineered to purify themselves. *Chemical Engineering Journal, 135*, 56–62. doi:10.1016/j.cej.2007.04.021.