

Current problems and future avenues in proteoliposome research

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Abstract

Membrane proteins are the gatekeepers between different biological compartments separated by lipid bilayers. Being receptors, channels, transporters, or primary pumps, they fulfill a wide variety of cellular functions and their importance is reflected in the increasing number of drugs that target membrane proteins. Functional studies of membrane proteins within a native cellular context, however, is difficult due to the innate complexity of the densely packed membranes. Over the past decades, detergent-based extraction and purification of membrane proteins and their reconstitution into lipid mimetic systems has been a very powerful tool to simplify the experimental system. In this review, we focus on proteoliposomes that have become an indispensable experimental system for enzymes with a vectorial function, including many of the here described energy transducing membrane proteins. We first address long standing questions on the difficulty of successful reconstitution and controlled orientation of membrane protein into liposomes. A special emphasis is given on coreconstitution of several membrane proteins into the same bilayer. Second, we discuss recent progress in the development of fluorescent dyes that offer sensitive detection with high temporal resolution. Finally, we briefly cover the use of giant unilamellar vesicles for the investigation of complex enzymatic cascades, a very promising experimental tool considering our increasing knowledge of the interplay of different cellular components.

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Introduction

Lipid membranes are found in all living cells and provide several vital functions. Not only do they protect the organism from a potentially hostile environment, but they also allow chemical compartmentalization, e.g. reducing and oxidizing conditions in bacterial cytoplasm and periplasm, respectively. In eukaryotes, cellular organelles such as mitochondria, endoplasmic reticulum or endosomes are also surrounded by membranes. The chemical scaffold of these membranes is a bilayer of lipid molecules, that hosts small hydrophobic components such as vitamins, quinones, and pigments, as well as very large and complex membrane proteins (MP). The mass percentage of proteins in natural membranes varies between 25% in the myelin sheath to 75% in energy transducing membranes such as the mitochondrial inner membrane.¹⁻³ This complexity makes the direct investigation of membrane protein function in native membranes difficult. To circumvent this problem, membrane proteins are purified and reinserted into membrane mimetic systems, such as protein wrapped lipid patches (nanodiscs), (supported) planar lipid bilayers or lipid vesicles.⁴ Here, we focus on the use of small to giant unilamellar vesicles (diameter ranging from 30 nm to 50 μ m), called liposomes, to study membrane proteins with vectorial functions. Bordered by a single lipid bilayer, liposomes form spontaneously from dried lipids resuspended in aqueous solution.

The advent of proteoliposome studies is tightly coupled to the experimental verification of the chemiosmotic theory brought forward by Peter Mitchell in 1961.^{5,6} This hypothesis led to a controversy amongst research groups across continents that lasted for decades.⁷ A crucial experiment, positively stimulating the debate, was the coreconstitution of purple membrane *Halobacterium salinarum* (i.e. essentially 2D-crystallized bacteriorhodopsin) and the F_1F_0 ATP synthase from mitochondria, showing ATP synthesis upon illumination, as described by Racker and Stoeckenius⁸ along with other coreconstitutions.⁹⁻¹¹ It was the simplicity of these experiments which stood out and manifested the connection between an electrochemical gradient and ATP synthesis. The use of purified components inserted into an empty lipid bilayer ensured that no unknown factors had to be considered during the interpretation of results. Racker's method was readily picked up by many researchers to advance the mechanistic understanding of various membrane proteins. A comprehensive overview of numerous pioneering experiments is given by Etemadi.¹²

In general, proteins embedded in liposomes can be investigated employing the same techniques that are also used to study enzymes in solution, such as UV/VIS and fluorescence spectroscopy. Nevertheless, the signal-to-noise ratio of optical measurements is typically decreased in proteoliposomes due to light scattering. Importantly, proteoliposomes also allow for electrometric techniques to follow charge movements across the membranes, as initially developed by Drachev^{13,14} and colleagues for light-inducible reactions and recently adapted by Fendler and colleagues for slower reactions that require mixing techniques (SSM electrophysiology).^{15,16} Proteoliposomes can also be immobilized onto many different kinds of support further increasing their experimental value.¹⁷

Besides examining transmembrane transport, liposomes have been used to investigate other membrane related biological phenomena, especially membrane fusion events. Fusion of lipid bilayers is vital during virus entry into their host cells, for instance, where it is either triggered by direct virus-membrane interaction or it is receptor-mediated. Liposome studies have successfully been used to investigate virus-target cell interactions and potential inhibitors,¹⁸ mechanistic studies of virus-membrane fusion,¹⁹⁻²² or interaction of viral protein, receptor and antibodies in HIV.²³ Furthermore, proteoliposomes have been used for NMR based structure determination of membrane proteins, wherein the membrane ensures

proper protein folding due to the near-native environment.²⁴⁻²⁶ Finally, liposomes have been long-known as promising drug delivery systems for both hydrophobic and hydrophilic drugs alike,²⁷⁻³² which is a very active field of research.

In this short review, we will highlight three different aspects of current proteoliposome research. In the first, main part, we will focus on the use of liposomes for the investigation of membrane proteins, many of them being ion-translocating enzymes involved in cellular bioenergetics. As our knowledge of the interplay of cellular components vastly expanded in the last years, experimental setups of more complex systems are of increasing interest. This also involves the coreconstitution of several membrane proteins in desired orientations into the same liposomal membrane. However, robust protocols to measure or guide orientation of reconstituted proteins or to coreconstitute membrane proteins into the same membrane are still in development. We discuss current problems during reconstitution of membrane proteins and their joining into more complex systems. In the second part, we will discuss recent progress in the development of fluorescent dyes that are suitable for proteoliposome studies. Using orthogonal chemistry and different linker techniques, such dyes can be tailored to meet specific requirements, ensuring high sensitivity and temporal resolution. Finally, we briefly describe the use of giant vesicles in the bottom-up construction of synthetic cells, highlighting recent projects, in which an impressive complexity of experimental systems was achieved.

Membrane protein reconstitution in a nutshell

In the seminal experiment of Racker and StoECKENIUS described above, native purple bacteria were mixed with cholate-extracted mitochondrial particles (containing enriched F_1F_0 ATP synthase) with asolectin lipids that had been sonicated and solubilized by the bile salt sodium cholate. This mixture was then dialyzed to remove excess detergent allowing the formation of proteoliposomes capable of light-driven ATP synthesis.⁸ Almost 50 years later, close variants of this very method are still powerful protocols to reconstitute membrane proteins. Over the years, several other approaches to functionally reconstitute purified MPs into liposomes were developed. Some employ organic solvents, or mechanical means such as sonication, freeze-thawing, or French-press.^{33,34} However, the most common methods are based on the use of detergents discussed in the next paragraphs. For an overview of different reconstitution techniques consider the pioneering reviews³³ and¹², for more recent perspectives refer to.^{4,35–37}

Generally, the process of detergent-mediated reconstitution can be divided into two main steps. First, a purified and detergent-solubilized MP is mixed with lipids. Second, the detergent is removed from the mixture, leading to the incorporation of the MP into liposomes. In the first step, the lipids are added to the solubilized MP either as fully solubilized mixed detergent-lipid micelles, or as preformed, detergent-destabilized vesicles. Detergent removal in the second step is achieved by various methods based on the critical micelle concentration (CMC) of the employed detergent.³³ For small sized micelles (< 25 kDa, high CMC; e.g. sodium cholate, octyl glucoside), rapid dilution, dialysis with an appropriately sized membrane or size exclusion chromatography can be used. For detergents forming large micelles (typically very low CMC), adsorption to polystyrene beads or complex formation with various cyclodextrins is used.^{35,38–40} Especially the use of cyclodextrins holds promise as different ring sizes are available that offer some specificity for certain detergents,³⁸ e.g. allowing the selective removal of one detergent from a detergent mixture. While the exact mechanism of reconstitution by detergent removal is still not fully understood,^{36,41} there is agreement that at a critical detergent concentration (which depends on a variety of factors), solubilized MPs cannot be kept in a soluble state any further and either precipitate or spontaneously integrate into the present phospholipid membrane.^{4,36} The efficiency of the reconstitution process (fraction of the solubilized MP that incorporates into liposomes) depends on various factors, as e.g. the MP of interest itself, the type of detergent used, the initial detergent concentration, the composition of the lipid membrane, the choice of buffer and ionic strength and importantly, the method and rate of detergent removal.^{33,36}

A special role is assigned to the lipid composition in proteoliposomes. Critical aspects for a good lipid mixture are easy liposome formation, preservation of enzyme activity and tightness of the liposomes towards leakage of protons or other ions. For many membrane proteins, a (rather crude) lecithin (phosphatidylcholine (PC)) extract of soybean has been successfully applied. In this extract, next to PC, phosphatidylethanolamine (PE), and the negative lipid phosphatidylinositol (PI) are the other main components. Although both PC and PI are not found in bacteria, the extract has been successfully used for many prokaryotic membrane proteins. In addition, natural extracts from bacteria, e.g. from *Escherichia coli* or mixtures from synthetic lipids are used. Tsai and Miller have convincingly shown that mixtures of synthetic lipids are much tighter towards proton leakage compared to a polar extract of *E. coli* lipids.⁴² Although being outside of the scope of this review, it is noteworthy that the correct lipid composition can have a direct impact on the protein activity as briefly described in the following few examples. Non-bilayer lipids such as PE were shown to stimulate the activity of secondary transporters

(e.g. Lyp1 of *Saccharomyces cerevisiae*).⁴³ Further, the head groups of lipids, especially of anionic lipids, are responsible for the topology and the regulation of conformational dynamics of transporters by interacting with the transport proteins.^{44–48} The importance of negatively charged lipids, especially cardiolipin, for the activity and stability of bacterial and mitochondrial respiratory (super)complexes has also been described.^{49–51}

The impact of lipids on the reconstitution process has been studied for some proteins, e.g. Na⁺/K⁺-ATPase.^{52–56} De Lima Santos et al. proposed that the lipids surrounding the MP, as well as the physical state of the lipid environment as e.g. its fluidity, have a stabilizing effect on MPs.⁵² Longer saturated fatty acyl chains decreased membrane fluidity leading to activity loss of the MP. Thus, phospholipid mixtures that contribute to membrane fluidity (higher fractions of short-chain or unsaturated fatty acyl chains) can reduce activity loss.^{52,53,55,57} Further excellent studies, in which many of these parameters have been described, were published for bacteriorhodopsin and ATP synthase by Paternostre, Rigaud, Levy and colleagues, as well as for LacS by Knol, Poolmann and colleagues (see Table 1 and references therein as well as Lichtenberg et al).⁵⁸ These studies show that the lipid composition can affect both the reconstitution yield and the orientation of the protein in the membrane (see below). A final complication is the natural asymmetry of lipid composition in the two leaflets of the bilayer as observed in eukaryotes and prokaryotes^{59–61} and first protocols for the generation of asymmetric liposomes have been described.^{62,63}

In contrast to natural membranes, proteoliposomes contain much fewer proteins (< 5% of lipid weight), as higher amounts of protein often negatively affect the reconstitution process. An interesting approach to tackle this problem is the GreCon method, in which the solubilized protein is placed onto a sucrose density gradient with increasing concentrations of cyclodextrin and detergent destabilized liposomes. During centrifugation, the detergent is gradually replaced by lipids, yielding liposomes with very high protein content, even triggering 2D-crystallization.⁶⁴ In the density gradient, proteoliposomes and empty liposomes migrate differently and are visible as opaque bands allowing their facile separation. Such proteoliposomes have been successfully used for electron tomography imaging using large membrane complexes⁶⁵, but have not yet been tested for transport measurements.

In recent years, peptides mimicking the properties of nanodiscs,⁶⁶ i.e. embedding the membrane protein in small lipid bilayer discs surrounded by a scaffold protein, have been described.^{67–69} While these have the advantage over traditional nanodiscs that their size can be modulated by varying the peptide to lipid ratio, they share the common drawback that they cannot be used to extract protein from native membranes, making the prior use of detergent necessary. A membrane protein extraction method not requiring any detergent is based on the use of styrene maleic acid (SMA) lipid particles (SMALPs), which has been successfully employed to purify^{70,71} and also functionally reconstitute MPs into lipid bilayers.^{72–76} Recent examples are the purification and reconstitution of cytochrome *c* oxidase from *Saccharomyces cerevisiae*⁷⁵ and of a plant sodium/proton antiporter.⁷⁶ Although not without downside (the solubilization properties of SMAs are pH-dependent),⁷⁷ these novel molecules are valuable gadgets in the toolbox of membrane protein biochemists.

The problem of protein orientation

In cells, insertion of membrane proteins in membranes is thought to happen co-translationally and the final orientation of the protein is fixed during insertion and remains static (see ⁷⁸ for a recent discussion on the topic). Unfortunately, this is not the case during membrane protein reconstitution into liposomes

as no translational machinery or chaperones are present that help to insert membrane proteins into the liposomal bilayer.^{79,80}

The relative orientation of the inserted MP strongly affects functional studies. As liposomes typically contain many copies of the MP in a random orientation, hundreds of different proteoliposome populations are formed during a single reconstitution process, causing a strong heterogeneity in the experimental system (see figure 1B). In some cases, functional unidirectionality can be imposed over random orientation of the MPs by using substrates which are unable to penetrate the membrane (e.g. ATP, NADH, cytochrome *c*), but in others, the substrate binding site is located in the hydrophobic part of the membrane and, hence, both populations are stimulated (e.g. quinone-coupled enzymes). Alternatively, membrane-impermeable inhibitors can be used, which selectively inhibit one enzyme population.⁸¹ In a worst-case scenario, the reconstitution method yields unidirectional insertion, but in the non-preferred orientation (e.g. substrate binding site on the inside), complicating functional experiments. The orientation of MPs is not only important for primary ion pumps but has to be considered as well for secondary transport proteins. While these can often catalyze transport of their substrates in both directions, the affinities for the substrate might be different on either side of the membrane, a situation that severely complicates the quantitative interpretation of experimental results. The difficulties imposed by uncontrolled orientation are even more pronounced if the coupled activity of two or more MPs coreconstituted in the same liposomal membrane is investigated (see Table 1).

The orientation of reconstituted MPs is difficult to predict and even harder to influence, and it seems to be essentially unique for each protein and specific set of reconstitution technique.³⁶ Over the past decades, however, several studies have accumulated knowledge on protein orientation (see table 1). Many reports describe the use of bacteriorhodopsin or proteorhodopsin, which are good models for monomeric membrane proteins with no soluble domain, but inadequate as models for large multi-subunit membrane proteins or membrane proteins harboring large soluble domains. A prerequisite for the investigation of MP orientation in liposomal membranes is a reliable assay to determine the ratio of the two possible populations. If feasible, orientation can be assessed via a functional assay that is able to discriminate the relative contribution of both populations. As an example, the orientation of respiratory complex I in liposomes can be determined by measuring NADH:hexaammineruthenium oxidoreductase activity that can be spectroscopically followed at 340 nm. If NADH, which is membrane-impermeable, is added to liposomes, only the population with the NADH binding site oriented towards the outside will contribute to the activity. Upon solubilization of the proteoliposomes with detergent, all complex I molecules will contribute to the activity. Setting both activities in relation allows for estimation of the orientation of complex I in the liposomes.⁸² However, care has to be taken in such approaches, as detergents often affect turnover activities of enzymes. Furthermore, if the measured activity is independent of the presence of the membrane, non-incorporated enzyme also contributes to the activity. A different method that has been used to determine orientation of proteo-/bacteriorhodopsin, is the use of proteases which will only digest MP domains accessible from the outside of the liposomes due to their inability to cross the lipid bilayer. The cleavage pattern can then be analyzed by SDS-PAGE, Western blot or mass spectrometry.⁸³⁻⁸⁵ However, this cannot be considered as a general approach, since cleavage patterns are expected to become increasingly complex with larger proteins, and proteolysis might be incomplete. A special case is the F_1F_0 ATP synthase, in which those hydrophilic F_1 head group that are located on the outside of the liposomes can be specifically stripped off using defined buffer conditions.⁸⁶ Yet another approach, often used for secondary antiporters, is based on the selective labelling of cysteine residues from the outside by a membrane impermeable thiol-

reactive compound. This is followed by complete labelling with membrane permeable biotin-maleimide that can later be detected by Western-blot analysis.⁸⁷⁻⁸⁹ Further attempts to determine orientation of MPs reconstituted into liposomes can be found in Table 1.

Over the years, it has been found that numerous parameters can affect enzyme orientation during reconstitution. De Lima Santos *et al.* report that at a slow detergent removal rate, liposome formation precedes protein incorporation into liposomes (leading to a more unidirectional incorporation of the MP), while liposome formation and protein incorporation happen simultaneously with fast detergent removal (resulting in a random orientation of MPs).⁵² Knol, Poolman and colleagues found a similar behavior for LacS, including further differences depending on the detergent used for reconstitution.⁸⁷ If unidirectional orientation is not desirable, repetitive freeze/thaw cycles were shown to randomize orientation.³⁹

In general, experiments have shown that orientation seems to be more uniform when the MPs are reconstituted into preformed, partially detergent-solubilized liposomes.^{41,90-92} A rationale behind this observation is that the most hydrophilic domain will be least efficient in crossing the bilayer, and the protein will insert with its most hydrophobic side first.⁴¹ However, experiments with cytochrome *c* oxidases show that the hydrophilic extramembraneous cytochrome *c*-binding domain (~ 25 kDa) of subunit II is not sufficient to promote more asymmetry than a 70:30 ratio.⁹³ F₁F₀ ATP synthase, on the other hand, with its soluble 350 kDa F₁ head group, has been reported to incorporate >95% with its head piece towards the outside with a similar reconstitution protocol⁸⁶. In proteins such as bR and pR, where no large soluble domain is present, the interaction of the surface charge of the proteins and liposomes seems to play a role.^{85,94} Tunuguntla *et al.* hold the asymmetry of charge distribution of pR, i.e. an overall positive charge at the C-terminus and a negative charge at the N-terminus, responsible for the lipid-charge dependent orientation of pR in liposomes. Through the use of either positively or negatively charged lipids in their liposomes, the N- or the C-terminus could be attracted towards the liposomal membrane, respectively, promoting a unidirectional orientation of pR.⁸⁵

In one of the very few attempts to actively influence orientation, Ritzmann *et al.* recently showed that fusion domains can guide orientated insertion of pR into liposomes.⁹⁵ By genetic engineering, GFP and mCherry were added to the C-terminus or to the N-terminus as fusion domains, respectively. The resulting fusion proteins pumped protons across the liposomal membrane in opposite directions upon reconstitution. This elegant method is unique in the sense that both orientations can be chosen by attaching a fusion domain on either end of the protein and is a promising approach for small proteins with no soluble domain. Whether the addition of a GFP is sufficient to orient larger membrane proteins remains to be determined. Other approaches for guided orientation employed Ni-NTA-functionalized beads to immobilize His-tagged MPs prior to reconstitution,⁹⁶⁻⁹⁸ a method also used to form planar bilayers for AFM studies.^{99,100} The liposomal membrane was formed *de novo* between the immobilized MPs either around the bead-support,⁹⁶ or the beads were suggested to force unidirectional orientation because they were too big to be incorporated into the newly formed liposomes.^{97,98} However, a more thorough characterization of these methods, e.g. regarding membrane leakiness, is required. Taken together, despite identification of several parameters that influence orientation of a MP in liposomes, there is still no general method to reconstitute MPs independently of all these parameters. The rather unpredictable effect of the lipid composition on the yield and on the orientation displays a major problem, as the effect of different lipid compositions on protein activity is a frequent aspect of research. A general method for guided orientation of membrane proteins independent of the lipid composition is highly desirable. Approaches, in which orientation is guided by steric constraints might display a

promising tool that has to be further developed in the future.^{95–98} Robust and easy to implement methods for the quantitative determination of incorporation yield and relative protein orientation are required to compare established and develop new reconstitution protocols, e.g. only a small subset of detergents have been used in reconstitution.

Coreconstitution of Membrane Proteins

The incorporation of different MPs into the same liposomal membrane is called coreconstitution and is desirable for several reasons. The small interior volume of liposomes leads to quick accumulation or depletion of the transported substrate, which does not allow for long steady-state measurements and thus complicates quantitative interpretation of the data. For instance, if a membrane potential is required for the transport process, often a potassium/valinomycin diffusion potential is used that quickly exhausts due to the rapid change of the internal K^+ concentration. Furthermore, as our understanding of biological processes increases, the interplay of different proteins at the molecular level becomes an important field of research. An impressive example of cooperating enzymes are the members of the respiratory chain which have been shown to form different supercomplexes in mitochondria.^{101,102} Functional measurements comparing kinetics and efficiencies between individual complexes or multiple complexes arranged in a supercomplex are necessary to understand the functional relevance of such supramolecular arrangements.^{103,104}

There are relatively few reports on the coreconstitution of more than one type of membrane protein in the same liposomal membrane. Most work has been published on the coreconstitution of ATP synthase together with proton pumps such as bacteriorhodopsin, cytochrome bo_3 ubiquinol oxidase and cytochrome c oxidase, which energize the liposomal membrane with an electrochemical potential. Such systems have been recently extended by the addition of peripheral membrane proteins. Biner *et al.* added trypanosomal alternative oxidase to proteoliposomes containing coreconstituted ATP synthase from *E. coli* and mitochondrial complex I to generate a minimal respiratory chain.¹⁰⁵ Furthermore, ATP synthase has been used as a constant generator of proton motive force for several proton dependent secondary transporters, such as Na^+/H^+ antiporter^{106,107} and glutamate transporter.¹⁰⁸ An alternative way to regenerate ATP from ADP and phosphate was recently shown in a synthetic metabolic network consisting of coreconstituted ArcD and OpuA as well as soluble proteins ArcA, B and C entrapped in the vesicle lumen. In this complex network, ArcA – D couple the breakdown of arginine to the regeneration of ATP which is then used by OpuA for glycine betaine transport which regulates the internal osmotic balance of the vesicles.¹⁰⁹ Non-transport related processes can also be studied by coreconstitution such as the chaperone activity of the membrane protein YidC on the folding of LacY.¹¹⁰ Further examples of coreconstitutions are given in Table 1. In all these examples, both types of integral membrane proteins have been reconstituted in parallel. However, given the individual requirements of every MP for optimal reconstitution and orientation, it has to be assumed that these coreconstitutions were far from being optimal.^{8,111,112}

One way to resolve this problem is to split the coreconstitution into two steps. First, either protein of interest is reconstituted under optimal conditions individually, followed by fusion of the two populations. Successful functional coreconstitution of two MPs by fusion was first reported using a minimal SNARE machinery that fused liposome populations containing either F_1F_0 ATP synthase or bo_3 oxidase.¹¹³ The same enzymes have also been successfully coreconstituted using fusion of oppositely charged proteoliposome populations.^{114,115} However, the latter method requires the use of non-natural positively charged lipids, limiting the free choice of the lipid composition. Alternative techniques which

have been used for “pure” liposome fusion are coiled-coil forming peptides¹¹⁶ as well as complementary DNA strands.^{117,118} However these methods have not yet been tested with MP containing liposomes.¹¹⁹

From the above considerations on orientation and coreconstitution experiments, it is obvious that the relative number of MPs reconstituted (stoichiometry) and the distribution of orientation of these MPs is of high importance for quantitative interpretations. Given the various parameters influencing reconstitution efficiency and orientation, this seems an almost insuperable obstacle. A ray of hope was provided by Raschle *et al.* with a method that ensures a 1:1 reconstitution stoichiometry of MPs. Using maleimide chemistry, complementary DNA molecules were attached to a unique cysteine of individual VDAC populations¹²⁰. Upon DNA hybridization, the two populations form a stable complex that can be purified and reconstituted. This technique should be applicable to any MP and using DNA linkers of appropriate lengths might not only ensure the correct stoichiometry, but also correct relative orientation of the reconstituted proteins. Alternatively, proteins can be genetically linked by creating a fusion construct that can be cleaved via proteases, as was used in the coreconstitution of SecYEG and YidC in a 1:1 stoichiometry.¹²¹

Rational design of fluorescent dyes to follow enzyme function

As mentioned in the introduction, many different methods have been successfully used to follow chemical reactions in proteoliposomes, ranging from fast techniques such as absorption spectroscopy or electrometry, over luminescence (ATP detection) to slow methods such as micro electrodes, NMR, or uptake/release of isotope labeled substrates.^{122–125} Probably the best combination of high sensitivity and high temporal resolution is found in fluorescent dyes which are able to detect a variety of reactions such as the change of proton or other ion concentrations or the presence of a membrane potential. Some of these dyes are membrane impermeable and have to be entrapped inside the liposomes (and the nonincorporated dye has to be removed) while others can be added from the outside, where they typically interact with the membrane. These latter, hydrophobic dyes are mainly used for the detection of proton gradients (e.g. ACMA, acridine orange)^{112,126–129} or membrane potentials (e.g. oxonol VI, TMRE, VoltageFluors)^{130–134} and do not directly report the translocated substrates.

In the following, we focus on membrane-anchored fluorescent probes that offer several advantages over entrapped dyes. A prototype of such a lipid anchored dye is obtained by the reaction of an amine-reactive carboxyfluorescein derivative with phosphatidylethanolamine, first reported in 1984 by Thelen *et al.*¹³⁵ Such lipophilic probes have been mostly used *in vivo*^{136–141} or to characterize different membrane properties.¹⁴² Recently, Kemmer *et al.* have coupled the ratiometric pH sensitive dyes pHrodo or SNARF to phosphatidylethanolamine, which in contrast to fluorescein increase fluorescence upon acidification. Notably, they found a significant increase of the apparent pK_a of the lipid coupled dyes in comparison to their soluble counterparts, an effect that is likely related to the close proximity of the dye to the membrane.^{143,144} To avoid this pK_a shift, a linker can be introduced between the fluorophore and the lipophilic moiety.¹³⁷

The main advantage of membrane-anchored sensors compared to soluble dyes is their efficient incorporation into the liposomes. The lipid moiety of the former ensures stable and efficient integration into the lipid bilayer which reduces the amount of potentially costly dyes that have to be used, as entrapment of freely soluble dye is an inherently inefficient procedure.^{145–150} Even more importantly, membrane-anchored dyes do not leak from the membrane, a problem often encountered with soluble dyes containing carboxylic acids. On the other side, an obvious drawback of lipid coupled dyes is that they are randomly distributed in both leaflets and selective removal or quenching is impossible or

connected to the use of harsh chemicals that are often incompatible with delicate membrane proteins and their catalytic activities.¹⁵¹ We have recently solved this limitation by using a DNA double strand between the lipophilic anchor (cholesterol) and fluorescent moiety. Incorporation efficiency of the dye was shown to be >90%, and the outer dye is conveniently removed by a short incubation of the liposomes with DNase I and an ultracentrifugation step. In addition, the use of a DNA linker suppressed the undesired pK_a shift, previously observed with dyes directly coupled to a lipid. The simple chemistry involved makes this method attractive for many different sensors and the use of DNA hybridization technology ensures a high versatility.¹⁵² In addition to these simple examples, more advanced membrane-anchored sensors rely on structural changes of environmentally sensitive DNA motifs, such as the pH sensitive i-Motif^{153,154}, G-quadruplexes^{141,155,156}, aptamers¹³⁸, DNAzymes¹⁵⁷, nanotweezers^{158,159} and nanoswitches¹⁶⁰. In these, the structural changes are transformed into a fluorescent read-out by attaching fluorescence quencher or FRET pairs to the DNA, in order to detect protons, metal ions and even small solutes such as ATP. Although mostly used *in vivo* so far, such complex sensors could also be useful for liposomal studies.

The development of new fluorescent dyes that are photostable as well as progress in the sensitivity of microscope cameras has further stimulated the field of single-molecule techniques. Here, instead of observing the readout of an ensemble of a large number of molecules, single enzymes are monitored by fluorescence microscopy which allows to classify them into populations with different enzymatic behaviors. While such techniques have been around for many years for soluble enzymes and membrane proteins in detergent solution,^{161–164} their application with proteoliposomes is very limited so far. It is however thanks to single molecule experiments with soluble and liposome embedded F₁F₀ ATP synthase that we have such a detailed picture of the F₁ binding change mechanism.¹⁶⁵ Apart from the ATP synthase, the groups of Jeuken and Stamou have performed single molecule proton pumping measurements with the quinol oxidase of *E. coli*¹⁶² and the plasmamembrane P-type ATPase¹⁶⁶, in which the pH change within the lumen of small unilamellar vesicles (SUVs) was followed by fluorescent pH sensors. Surprisingly, in both systems they have observed long phases of enzymatic inactivity or even passive proton leakage through the protein and have attributed their findings to a sensitive enzyme regulation by the local environment (but see Berg *et al.* for a different finding).¹⁶⁷ In case of the *bo*₃ oxidase, Jeuken and colleagues combined single protein/liposome studies with electrochemical measurements by tethering the proteoliposome to a gold electrode. By applying a voltage to the electrode, electrons were directly donated via lipid embedded ubiquinone to the *bo*₃ enzyme.¹⁶²

The challenges of fluorescent detection is to find a probe with all desired properties, such as high incorporation into vesicles, resistance to bleaching, specificity for the substrate/product, and high signal-to-noise ratio. Rational design of fluorescent dyes using click-chemistry in combination with biomolecules such as DNA allow for optimization of these properties leading to improved probes for the investigation of enzyme function in proteoliposomes.

Towards a synthetic cell

In the last section, we would like to briefly touch the potential of giant unilamellar vesicles (GUVs) to study membrane protein function. In contrast to “classical” liposomes, GUVs are much larger in size (1 – 100 μm) and can directly be observed by light microscopic techniques.¹⁶⁸ On the downside, GUVs are less robust and their preparation is less straightforward (see ^{169–178} for literature on GUV formation). Compared to SUVs, GUVs have a vastly increased surface and inner volume that allows for the encapsulation of entire protein machineries, small vesicles or even whole bacteria, in order to mimic increasingly complex functions of living cells.¹⁷⁹ Already in 2004, Noireaux and Libchaber¹⁸⁰

demonstrated the successful *in vitro* transcription and translation (IVTT) of GFP within GUVs, and recently similar systems were used to produce membrane proteins directly inside GUVs, with either spontaneous¹⁸¹ or assisted insertion¹⁸² into the membrane. Very few examples describe the use of GUVs as replacement for SUVs in traditional vectorial transport experiments with membrane proteins. A reason for this lack of transport experiments is the less than straightforward insertion of membrane proteins into the fragile GUV membrane which is an ongoing field of research.^{114,115,168,183} Levy and colleagues showed successful detergent-mediated (using very small amounts of detergent) incorporation of bacteriorhodopsin which acidified the GUV lumen upon illumination and was followed by the pH sensitive dye pyranine.¹⁸⁴ Biner *et al.* applied charged mediated fusion to insert up to three different membrane proteins reconstituted in positively charged SUV into a negatively charged GUV membrane¹¹⁹. Hansen *et al.* finally reconstituted glucose transporter GLUT1 into GUVs using hydrogel-assisted swelling. Passive uptake of glucose into the GUV lumen was detected with a glucose oxidase coupled fluorescence system that was entrapped during GUV formation.¹⁸⁵

Given their size, GUVs are also of interest in the research on synthetic or artificial cells.^{183,186,187} A recent example is the light-induced energization of IVTT in GUVs after entrapment of SUVs containing bacteriorhodopsin and ATP synthase,¹⁸⁸ mimicking a cellular organelle. Lee *et al.* used photosystem II (PSII) and proteorhodopsin coreconstituted with ATP synthase in SUVs that can individually be stimulated by red and green light, respectively. PSII generates a pH gradient by oxidation of water on the inside of the vesicles and pR dissipates the gradient by outward proton pumping. These liposomes were entrapped in GUVs and, depending on the used light, ATP synthesis is either stimulated or abolished.¹⁸⁹

Another impressive example to use GUVs as a cell-mimicking system was demonstrated by Ces and colleagues by creating a non-natural signal cascade. Passive calcium influx into the GUV lumen via α -hemolysin activates phospholipase A2 that triggered liposome embedded mechanosensitive channels. The successful signal cascade was demonstrated by the release of calcein from the liposomes which can be detected as a fluorescence increase.¹⁹⁰ Finally, Chen *et al.* demonstrated the bottom-up synthesis of pancreatic beta cells using GUVs. The uptake of glucose via the glucose transporter GLUT2 into the GUV lumen triggered a cascade that mediated controlled insulin release by fusion of insulin containing vesicles with the GUV membrane. Most impressively, the system was able to discriminate between normal and hyperglycemic glucose concentrations.¹⁹¹

As mentioned above, GUVs are not very stable and have also been shown to be susceptible for leakage.^{192,193} Cell-sized vesicles produced from synthetic block copolymers, so called polymerosomes¹⁹⁴ or hybrids thereof with liposomes are improved in this regard^{186,195} and have successfully been used to mimic compartmentalization of the eukaryotic cell¹⁹⁶ or to achieve spatial separation of otherwise incompatible multienzyme synthesis reactions.^{197,198} However, initial experiments on polymersome embedded membrane proteins have shown that enzymatic activity was decreased compared to pure lipid vesicles.^{199,200} Nevertheless, proton pumping by *bo*₃ oxidase was recently demonstrated in hybrid polymer-lipid GUVs.²⁰¹ Some of these examples discussed above are depicted in Figure 2, see legend for further details.

These cell-mimetic applications greatly demonstrate the potential of giant vesicles. Importantly, a wise choice of detection method is crucial that allows high temporal resolution combined with sufficient sensitivity. To this end, photostable fluorophores are required to minimize bleaching during observation of single vesicles. To allow for prolonged observation and addition of substrates, vesicles have to be immobilized, which might have adverse effects on membrane properties such as the tightness of the membrane. Recently, experiments using microfluidics have shown formation and entrapment of GUVs in a nano-factory on a chip,^{202,203} which in the future might allow to create automated systems. However,

many of the current approaches (single molecule measurements, microfluidics) require technical expertise and specialized equipment which limits the use of the techniques in other labs. In the future, robust methods with a broad application and high reproducibility are critical for the success of GUVs in the field of membrane protein research. Equivalently important is a detailed description of the data treatment. As microscopy experiments produce a large amount of data, user-friendly yet powerful analytical software is a necessary support during data analysis.

Perspective section

- (i) highlight the importance of the field
Proteoliposome research continues to be an indispensable tool for the mechanistic understanding of membrane proteins. With the advent of structure determination by cryo electron microscopy, functional measurements are becoming the rate-limiting step.
- (ii) a summary of the current thinking
Development of improved methods for the precise orientation and coreconstitution of MPs into membranes are required. This is especially relevant for the bottom-up construction of artificial cells. Using fluorescent dyes with precisely tailored properties will ensure high sensitivity and temporal resolution.
- (iii) a comment on future directions
Microscopy based measuring techniques applied on liposomes will further minimize the required amount of membrane proteins, enabling the investigation of eukaryotic enzymes. The use of microfluidics for the generation and manipulation of GUVs might allow a higher level of control in lab-on-a-chip type experiments.

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Figure 1:

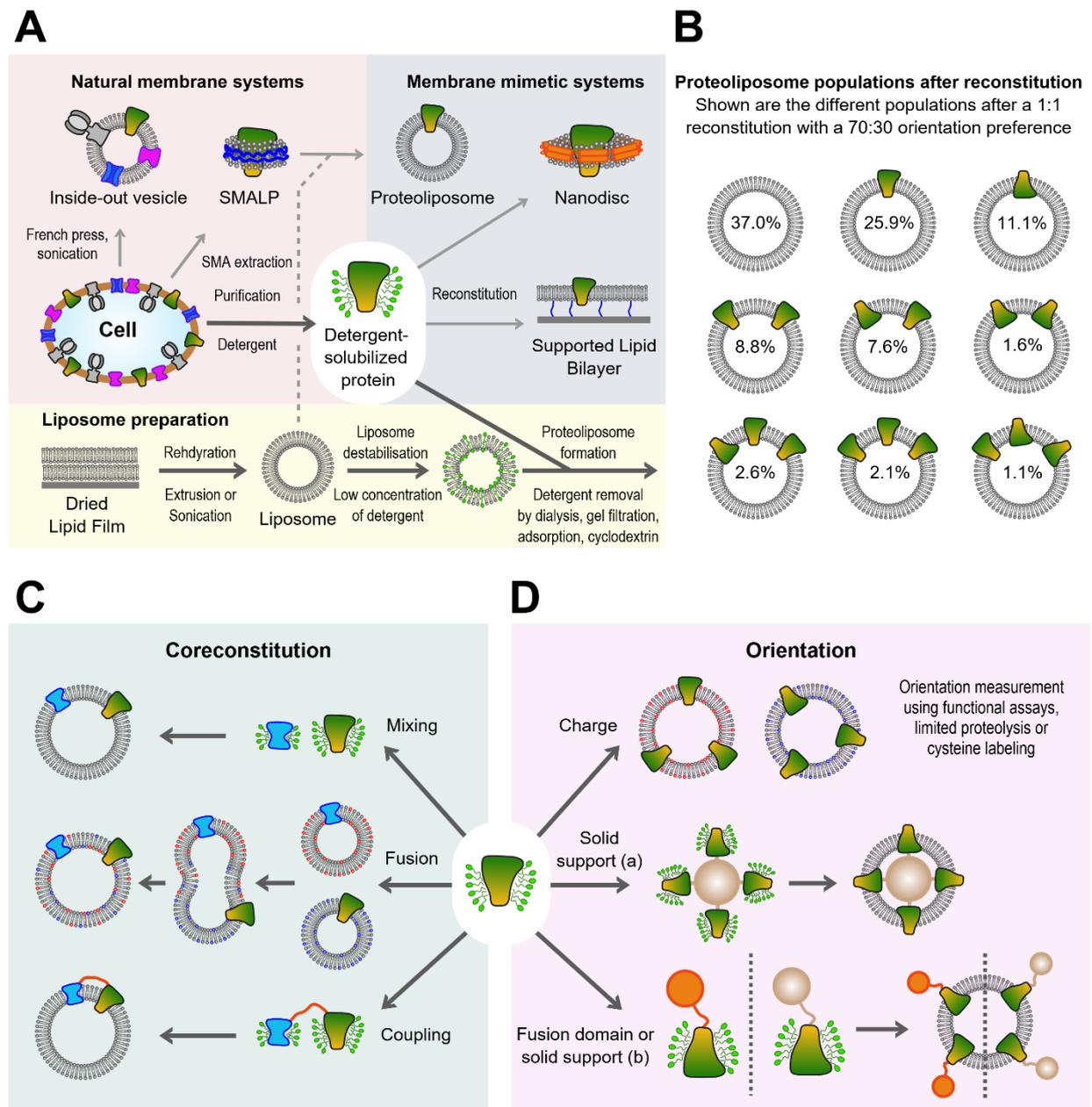


Figure 1: The ins and outs of membrane protein reconstitution

A) The general procedure for the formation of proteoliposomes is shown as well as a few alternative approaches for the *in vitro* study of membrane protein (MP) function. In a first step, MPs are extracted and purified from the native cell environment using detergents. In parallel, liposomes are formed by rehydration of a dried lipid film and subsequent extrusion or sonication of the vesicles. These liposomes are partially solubilized by the addition of detergents and mixed with the detergent-solubilized MP to form proteoliposomes after removal of the detergent by a variety of methods. Detergent-free extraction of MPs from the cell can be achieved by generating inside-out vesicles²⁰⁴ or using styrene-maleic acid (SMA) copolymers to generate SMALPs. The latter can be used for the detergent-free reconstitution of

MPs into liposomes.⁷⁰⁻⁷⁶ If a closed compartment is not needed, detergent-solubilized MPs can also be reconstituted into planer supported lipid bilayers or nanodiscs.⁴

B) The calculated distribution of proteoliposome populations after reconstitution with a 1:1 liposome to protein stoichiometry and a 70% green-side out preference in orientation. Values were calculated assuming a Poisson distribution for reconstitution and a binomial distribution for orientation. Only populations >1% are shown.

C) Coreconstitution of more than one membrane protein. Shown are coreconstitutions by a combined incubation of both MPs with liposomes, via fusion of different proteoliposome populations (e.g. charge-mediated fusion, red lipids = negatively charged, blue lipids = positively charged),^{114,115} or via covalent/transient coupling of the MPs prior to reconstitution.¹²⁰

D) Methods for the guided orientation during reconstitutions. Shown are examples of charge-controlled insertion,^{85,94} by coupling the MP to a solid support (a) that will be encapsulated by the liposome⁹⁶ or by attaching a fusion domain⁹⁵ or coupling to a solid support (b)^{97,98} that will be excluded from the vesicle interior.

Figure 2:

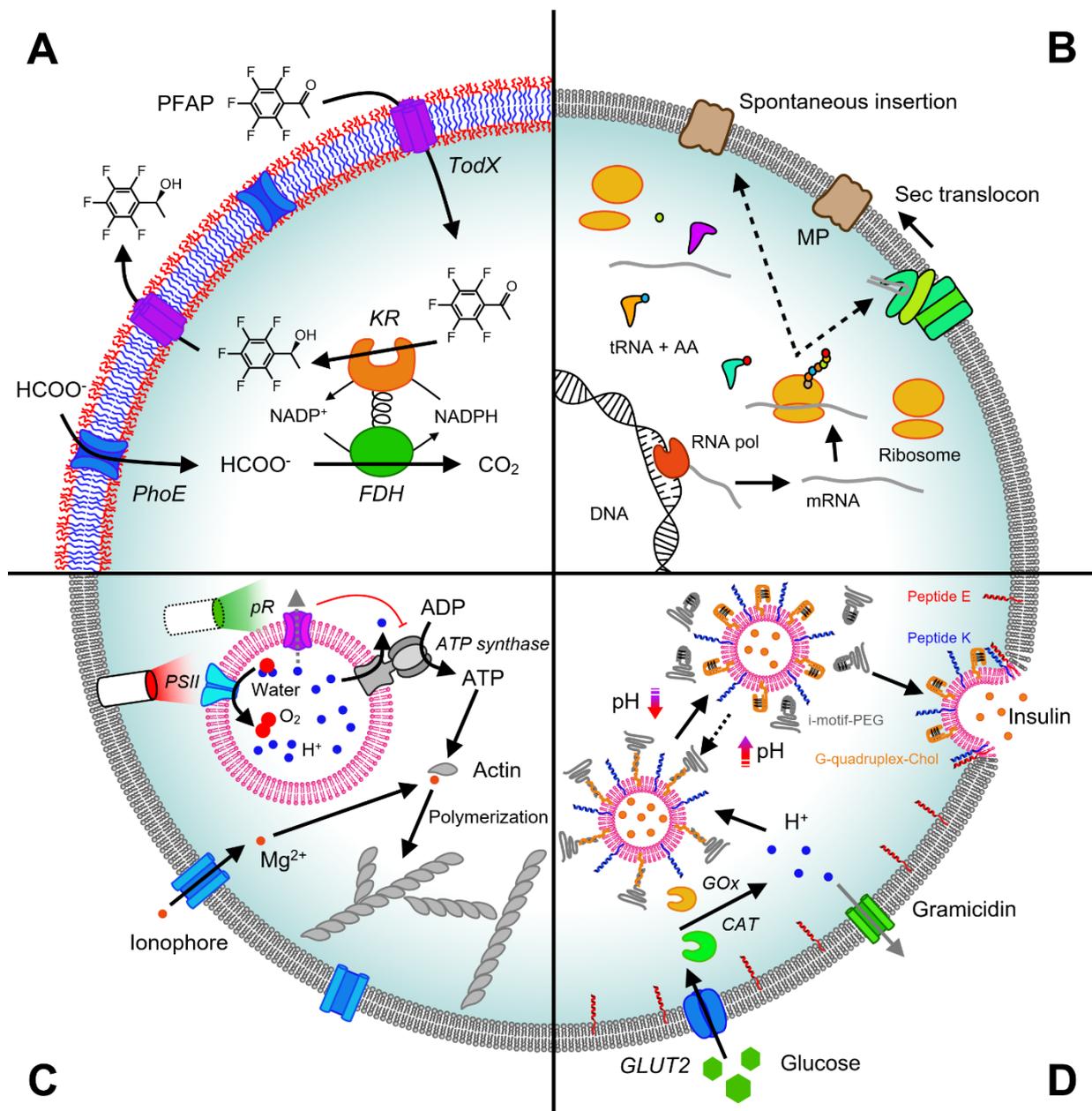


Figure 2: Applications of membrane proteins (MPs) in giant unilamellar vesicles (GUVs) and polymersomes.

A) MPs embedded in a polymersome membrane enables import and export of substrates and products from a synthetic reaction cell. In that cell, pentafluoroacetophenon (PFAP) is transformed to (S)-pentafluorophenyl ethanol ((S)-PFE) by ketoreductase (KR) inside of polymersomes with NADPH as cofactor. NADPH is regenerated by formate dehydrogenase (FDH) which is fused to KR. As PFAP, (S)-PFE and formate do not readily diffuse across the polymersome membrane, the selective channel protein TodX and the outer membrane pore PhoE were reconstituted in the membrane to alleviate the mass transport limitations.¹⁹⁸

B) Membrane protein synthesis in GUVs by *in vitro* transcription and translation. Relevant components shown for these processes are DNA, RNA polymerase (RNA pol), the produced mRNA, ribosomes, tRNAs

and amino acids (AA). After translation, the synthesized MP inserts either spontaneously¹⁸¹ into the vesicle membrane, or requires the Sec translocon²⁰⁵ for correct insertion.¹⁸²

C) Regulated ATP synthesis promoting actin polymerization in synthetic organelles. The membrane proteins F_1F_0 ATP synthase, photosystem II (PSII) and proteorhodopsin (pR) are coreconstituted in SUVs that are entrapped in GUVs. Red light stimulates PSII leading to oxidation of water and thus acidification of the SUV interior. Acidification leads to a proton gradient across the SUV membrane energizing ATP synthesis in the GUV lumen that is used to polymerize actin. On the other hand, green light stimulates proton export from the SUV lumen by proteorhodopsin, abolishing the proton gradient. Consequently, synthesis of ATP is stopped and actin polymerization is interrupted.¹⁸⁹

D) Design of minimal pancreatic beta cell. The glucose transporter GLUT2 is reconstituted into the membrane of GUVs containing SUVs with encapsulated insulin. After uptake by GLUT2, glucose is oxidized by glucose oxidase (GOx) and catalase (CAT), leading to an acidification of the GUV lumen. This pH drop leads to the dehybridization of DNA double strand anchored to the SUV outer membrane by the formation of a pH-sensitive motif, exposing the fusogenic peptide K on the outer SUV membrane. This allows fusion of the SUV with the GUV membrane via peptide E/K interaction, leading to the release of the encapsulated insulin. Gramicidin present in the GUV membrane modulates the pH response and allows to discern between normal and hyperglycemic conditions.¹⁹¹

Figures 2A-D are adapted from the respective publications.

Table 1:

Non-exhaustive list of different studies with a focus on coreconstitution of MPs or orientation of MPs. For the latter, studies are further distinguished between simply investigating orientation under one or several different conditions and actively influencing orientation by different means. Studies are grouped according to the investigated MP and the origin of the MP and the studied parameter is indicated. A short summary of the study is provided in the comments row.

| Membrane Protein | Organism | Study | Comments | References |
|---|---|---------------------------|---|-----------------------|
| aa ₃ CcO | <i>Rhodobacter sphaeroides</i> | Coreconstitution | Coreconstitution of aa ₃ CcO with F ₁ F ₀ ATP synthase (<i>E. coli</i>) or spinach ATP synthase. | 112 |
| | | Investigating orientation | 70 - 80 % CcO was found with soluble domain of subunit II carrying the cytochrome <i>c</i> -binding site towards the outside of the liposomes. Functional unidirectionality can be imposed over orientation of the MP in the liposomal membrane by providing cytochrome <i>c</i> and electrons only on one side of the membrane. | 93 |
| | <i>Paracoccus denitrificans</i> | Influencing orientation | MP was immobilized on Ni-NTA-functionalized silica nanoparticles for orientated encapsulation into liposomes (bead on outside of proteoliposome). | 97,98 |
| ArcD2 | <i>Lactococcus lactis</i> | Coreconstitution | Coreconstitution with OpuA and soluble proteins ArcA, B and C (<i>L. lactis</i>) | 109 |
| bacteriorhodopsin | <i>Halobacterium salinarum</i> | Coreconstitution | Coreconstitution with ATP synthase from bovine heart mitochondria. | 8 |
| | | Investigating orientation | Only slight preference for inside-out was detected. Orientation in proteoliposomes was shown to depend on lipid composition of the liposomes, pH value, ionic strength, & membrane curvature (in order of decreasing influence on orientation). | 206 80 |
| Ca ²⁺ -P-ATPase | rabbit sarcoplasmic reticulum | Investigating orientation | Unidirectional orientation with 80% - 100% of the cytoplasmic domain facing outwards was observed, depending on the rate of detergent removal | 207 |
| cytochrome <i>bo</i> ₃ ubiquinol oxidase | <i>E. coli</i> | Coreconstitution | coreconstitution of <i>bo</i> ₃ oxidase with F ₁ F ₀ ATP synthase (<i>E. coli</i>) via charge-mediated fusion of liposomes to GUVs coreconstitution of <i>bo</i> ₃ oxidase with F ₁ F ₀ ATP synthase (<i>E. coli</i>) or spinach ATP synthase coreconstitution of <i>bo</i> ₃ oxidase with F ₁ F ₀ ATP synthase (<i>E. coli</i>) via SNARE-mediated fusion | 114,115 112 113 |
| | | Investigating orientation | 72 - 77 % to pump protons out of liposomes Unidirectional orientation is reported using a reconstitution method based on Rigaud <i>et al.</i> ³³ However, no biochemical proof of unidirectionality is provided. | 162,167 208 |
| cytochrome <i>b</i> -563/ <i>c</i> -554 (Qbc) | <i>Synechococcus 6716</i> | Coreconstitution | Coreconstitution with H ⁺ -ATP synthase from <i>Synechococcus 6716</i> . | 111 |
| F ₁ F ₀ ATP synthase | <i>E. coli</i> | Coreconstitution | Refer to cytochrome <i>bo</i> ₃ ubiquinol oxidase. Further coreconstitutions with Na ⁺ /H ⁺ antiporters (<i>Thermus thermophilus</i> NapA and human NHA2),rat VGLUT2 as well as mitochondrial complex I from <i>Bos Taurus</i> together with alternative oxidase from <i>trypanosoma brucei brucei</i> . | 105-108 |
| | | Investigating orientation | > 97 % of F ₁ facing outwards was shown. | 86 |
| LacS | <i>Streptococcus thermophilus</i> | Investigating orientation | Different detergents & detergent concentrations were evaluated as well as different rates of detergent removal. Unidirectional inside-out orientation is reported for reconstitution with Triton X-100 and random orientation for reconstitution with n-Dodecyl β-D-maltoside. | 87,209 |
| large-conductance calcium- and voltage-activated potassium channel (BK) | <i>Homo sapiens</i> | Investigating orientation | 70% inside-out orientation of MP is reported. | 210 |
| mechanosensitive channel of small conductance (MscS) | <i>bacterial</i> (no further statement made) | Investigating orientation | Unidirectional incorporation is reported based solely on electrophysiological results; no physical evidence is provided. Two ion channel reconstitution methods based on dehydration/rehydration of liposomes in presence of MP were tested. | 211 |
| Na ⁺ /K ⁺ -P-ATPase | <i>Electrophorus electricus</i> or <i>Squalus acanthias</i> | Investigating orientation | Functional unidirectionality was imposed over orientation of MP in membrane by selective inhibition of one of the two orientation populations with ouabain (exterior) or vanadate ions (interior). | 212,213 |

| | | | | |
|--|---|---------------------------|--|--------------------|
| | dark red outer medulla of kidney of adult New Zealand white rabbits | Investigating orientation | Different protein-to-lipid ratios, different phospholipids and methods of detergent removal were investigated. | 52 |
| proteorhodopsin | uncultured <i>Gammaproteobacterium</i> EBAC31A08 | Coreconstitution | Coreconstitution with <i>Spinacia oleracea</i> PSII and <i>Bacillus pseudofirmus</i> ATP synthase | 189 |
| | | Influencing orientation | Interaction of MP with the surface of the liposomes was shown to dictate orientation. The surface charge of liposomes was modulated to prearrange orientation. MP was immobilized on Ni-NTA-functionalized silicate beads for orientated reconstitution (bead on outside of proteoliposome). Fusion domains were reported to guide the orientated insertion of proteorhodopsin into liposomes. | 85,94 214 95 |
| various (Ca ²⁺ -P-ATPase, H ⁺ -F-ATPase, LacS) | various | Investigating orientation | More uniform orientation of MPs was observed in the reconstituted liposomal bilayer when reconstituted into preformed, detergent-destabilized liposomes. | 81,87,90,91,215 |
| voltage-dependent K ⁺ channel (K _v AP) | <i>Aeropyrum pernix</i> | Influencing orientation | Ni-NTA-functionalized beads were used as membrane organization centers during bilayer reconstitution (bead in proteoliposome; bSUM). | 96 |
| YidC | <i>Escherichia Coli</i> | Coreconstitution | Coreconstitution with LacY and SecYEG as a fusion construct | 110,121 |

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