

# Towards a Synthetic Mitochondrion

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**Abstract:** Our group at the University of Bern uses biochemical and biophysical techniques to unravel details of the molecular mechanism of membrane proteins. Of special interest are the large multi-subunit complexes of the universally conserved respiratory chain and the ATP synthase that are found in mitochondria and aerobic bacteria. In a bottom-up approach using purified membrane proteins and synthetic lipids, we aim to mimic the basic processes of oxidative phosphorylation. We further develop methodologies to increase the complexity of such artificial systems, paving the way for a synthetic mitochondrion. In this minireview, we summarize recent efforts of our groups and others towards a synthetic respiratory chain.

**Keywords:** ATP synthesis · Cellular energy · Liposome · Membrane protein reconstitution · Synthetic biology



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was promoted 2017 to associate professor. His group focuses on understanding the molecular mechanism of membrane proteins and on the development of novel techniques for membrane protein investigation.

## 1. Mitochondria – The Powerhouse of the Cell

Biological life requires energy that is ultimately supplied by either the sun or chemical nutrients. In all cells, the majority of the energy for chemical work is made available through the universal energy currency adenosine triphosphate (ATP) that is continuously regenerated from its precursors ADP and inorganic phosphate. ATP drives a large number of energy-consuming processes such as macromolecule biosynthesis, mechanical motility, membrane transport of small molecules and proteins, regulatory networks, and nerve conduction.<sup>[1]</sup> In humans or eukaryotic organisms in general, the vast majority of ATP is produced in mitochondria by a process called oxidative phosphorylation. In the first step of this process, energy-rich nutrients such as sugars, proteins and fatty acids are oxidized to acetyl-Coenzyme A that is converted to carbon dioxide in the citric acid cycle, in which the cellular reduction equivalents NADH and succinate are formed. In the second step, NADH and succinate are oxidized to NAD<sup>+</sup> and fumarate, respectively, and their electrons are fed into the electron transfer chain (ETC) to finally reduce oxygen to water. The energy of this highly exergonic reaction is released stepwise by components of the ETC and converted to an electrochemical proton gradient (proton motive force) that is used by the ATP synthase to synthesize ATP by a rotary mechanism. The overall process is highly conserved in all aerobic cells. The redox reactions of the ETC are catalyzed

by a series of membrane proteins (MPs) called complex I to IV according to their appearance in the ETC (Fig. 1). Each respiratory enzyme is a multi-subunit complex containing redox active cofactors such as flavins, hemes, copper ions and Fe/S clusters. The components of the ETC have been studied in great detail over the last decades, including their high-resolution molecular structure.<sup>[2]</sup> More recently, these complexes have been found to interact to form a higher level of organization called supercomplexes,<sup>[3]</sup> but their functional significance is under current debate. The ATP synthase, the ultimate consumer of the proton motive force, is not part of these supercomplexes but also forms a superstructure in form of dimer rows along the cristae edges in mitochondria.<sup>[4]</sup> Our research group is interested to connect these two separate research fields by studying the functional interplay of respiratory chain enzymes and the ATP synthase. Of special interest is the influence of the proton-impermeable inner mitochondrial membrane that works as a capacitor storing the redox energy of the ETC as proton motive force. In our experiments, we typically purify the different protein complexes to homogeneity and put them back into a membranous environment, a process called MP reconstitution. Using this *in vitro* approach with a minimal number of components facilitates the assignment of the observed effects to a specific component. Varying these components (*e.g.* mutant protein variant, lipid composition) further allows to mimic physiological and pathological conditions. Finally, we aim to assemble a complete respiratory chain from purified proteins *in vitro* which would hallmark an important step in the field of synthetic biology towards a synthetic mitochondrion. In the following paragraphs, we will introduce the reader to general concepts of MP reconstitution methods and will describe our recent efforts.

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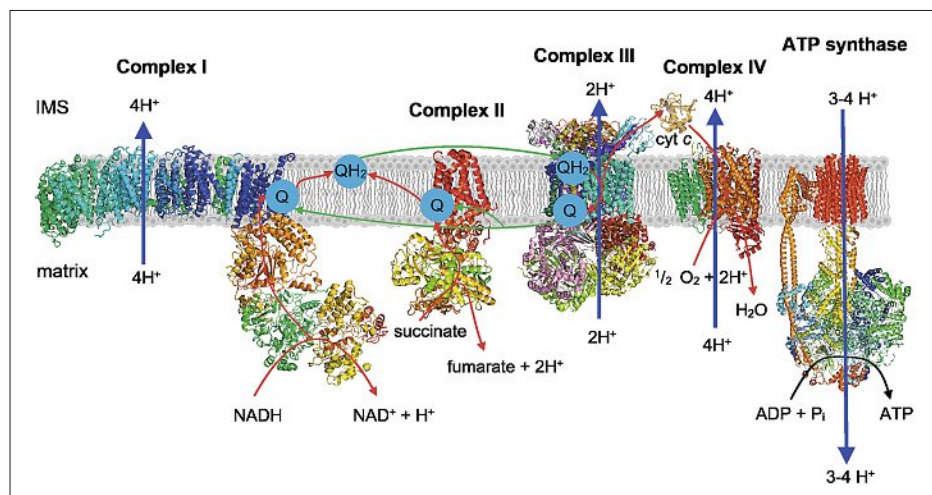


Fig. 1. Schematic representation of the respiratory chain in a mitochondrion. Complex I (PDB: 4WZ7) oxidizes NADH to NAD<sup>+</sup> to reduce ubiquinone (Q) to ubiquinol (QH<sub>2</sub>). The electron transfer is coupled to the transport of four protons from the mitochondrial matrix to the inner membrane space (IMS). Complex II (PDB: 2WDV) oxidizes succinate to fumarate and reduces Q without proton translocation. The electrons are then transferred from QH<sub>2</sub> to cytochrome c via complex III accompanied by the translocation of two protons. Finally, four molecules of cytochrome c are used to reduce oxygen to water in complex IV coupled to the transport of four protons. Overall, a total of ten protons are translocated across the mitochondrial membrane per NADH oxidized. The electrochemical proton gradient is dissipated by the ATP synthase to generate ATP. Electron and proton transfer processes are depicted with red and blue arrows, respectively.

## 1.1 Model Membrane Systems

*In vivo* studies of MP function are often hampered by the high complexity of the natural membrane and the high diversity and number of MPs in the membrane. This is particularly true for enzymes that are influenced by the proton motive force, which is the energy source for numerous physiological processes unrelated to ATP synthesis. A common strategy is to produce membrane proteins for *in vitro* studies by homologous or heterologous overexpression, followed by chromatographic purification in the presence of detergents. These amphiphilic molecules shield the hydrophobic surface of the MP from direct contact with water and keep MPs solubilized in aqueous solutions. Some functional measurements can be performed with the enzyme in detergent solution, but processes that require the presence of a membrane can only be investigated after reconstitution of the MP into a model membrane system.

Beside liposomes,<sup>[5]</sup> which are discussed in more detail below, respiratory chain enzymes have been successfully reconstituted into a variety of model membrane systems such as supported lipid bilayers,<sup>[6]</sup> nanodiscs,<sup>[7]</sup> styrene maleic acid co-polymers<sup>[8]</sup> or polymersomes,<sup>[9]</sup> each having its assets and drawbacks. In supported lipid bilayer experiments, the membrane is floating in aqueous solution and is attached to a solid support (typically functionalized glass) *via* a linker molecule of variable length increasing the durability and mechanical stability of the bilayer. This setup is well suited to follow electron transport *via* electrodes or conformational

changes but of limited value to investigate transmembrane transport processes that require a sealed compartment. Furthermore, the close proximity of the membrane to the glass support can impair protein function.<sup>[10]</sup> Nanodiscs are small circular lipid bilayer patches (containing a few hundred lipid molecules per disc), which are stabilized by a dimer of membrane scaffold proteins that forms a belt-like structure around the lipid patch. In comparison to liposomes, nanodiscs contain much less ‘free’ lipid per protein, greatly enhancing the quality of spectroscopic measurements due to minimal light scattering. Analogously to supported lipid bilayers, the drawback of nanodiscs is that no continuous lipid bilayer is formed, preventing measurements of membrane transport processes. The most commonly used model membrane systems are liposomes, which are globular vesicles that spontaneously form if a suitable lipid mixture is suspended and homogenized in an aqueous solution (Fig. 2). Liposomes have an inner aqueous volume that is separated from the outside environment by an impermeable membrane, allowing to study transport processes across this membrane. Liposomes are sub-classified according to their diameter into small (SUVs, <100 nm), large (LUVs, 100–800 nm) or giant unilamellar vesicles (GUVs, >800 nm). SUVs and LUVs are readily formed in the laboratory from various lipid mixtures and are relatively stable vesicles possessing a tight membrane. Nevertheless, their small inner volume can be limiting for kinetic measurements, as substrates in the inner volume exhaust or saturate quickly. This

limitation is overcome with GUVs that are often >20 μm in diameter and thus possess the size of a eukaryotic cell. The main advantage of GUVs is the possibility to directly observe them by optical microscopy, enabling single vesicle studies instead of spectroscopic ensemble measurements that are used with SUVs and LUVs. On the other side, GUVs show a decreased membrane stability and tightness compared to smaller liposomes. A general limitation for the use of GUV in MP studies is the lack of convenient and reproducible protocols for MP reconstitution, which is an active field of research while reconstitution into small liposomes is well established.<sup>[5]</sup> The stability of cell-sized vesicles can be improved by the use of synthetic polymers (partially) replacing natural lipids, yielding so-called polymersomes, which exhibit increased stability and reduced permeability compared to liposomes.<sup>[9,11]</sup>

### 1.1.1 MP Reconstitution into Unilamellar Liposomes

Liposomes were first described as a membrane mimetic system in the 1960s<sup>[12]</sup> and have become an invaluable tool to investigate MP's function. Since the first introduction of detergents to solubilize MPs by Helenius and Simons,<sup>[13]</sup> successful MP reconstitutions were achieved using multiple strategies. Some MPs have been reported to integrate spontaneously into liposomes,<sup>[14]</sup> while others were dissolved in organic solvents together with lipids, vacuum dried and reconstituted during liposome formation.<sup>[5,15]</sup> These strategies only work for a few MPs and the nowadays most successful technique was established by Rigaud and others,<sup>[5]</sup> in which the MP is added to a fully or partially detergent-solubilized liposome suspension. The liposome is thought to form a ternary complex with the enzyme and its surrounding detergent, yielding a MP-containing liposome after removal of the detergent by either gel-filtration, dialysis or addition of detergent-absorbing polymers.<sup>[5]</sup> Typically, this process has to be optimized for each single MP by testing different detergents, detergent removal techniques, lipid compositions and lipid/protein ratios.

## 2. Co-reconstitution of Membrane Proteins Using Liposome Fusion

Given the individuality of MP reconstitution described above, it is not surprising that only a couple of functional MP co-reconstitutions have been described, pioneered by Racker and colleagues in 1974 using bacteriorhodopsin and ATP synthase to strongly support Mitchell's chemiosmotic hypothesis.<sup>[9,16]</sup> In these reports, co-reconstitutions were limited to two MPs



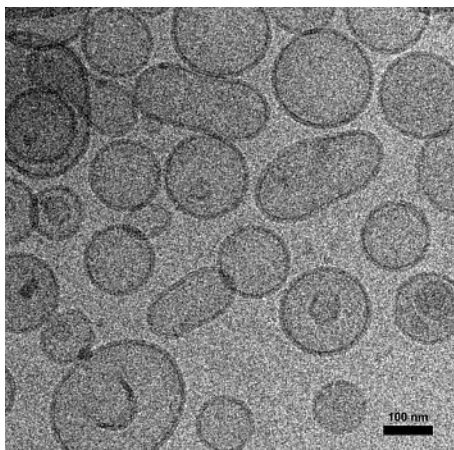


Fig. 2. Cryogenic transmission electron microscopy image of liposomes. Depicted is a typical cryo-TEM image of synthetic unilamellar liposomes, which were extruded through a 100 nm membrane. The vesicles have an average size of about 100 nm, however, smaller and larger liposomes are also found in the sample.

while a complete synthetic aerobic respiratory chain would require the co-reconstitution of at least four MPs (Complex I, III, IV and ATP synthase). Two major obstacles become evident if more than one MP is to be reconstituted. First, as every single MP requires its own optimized reconstitution procedure, a common recipe for the incorporation of four MPs seems unlikely and second, the orientation of MP insertion into liposome cannot be controlled easily and is influenced by physical properties of the MP such as size and surface charge. Consequently, a mixture of four MPs would yield a large number of liposome populations with different enzyme orientations making interpretation of quantitative data impossible. A few years ago, we envisioned to tackle the first problem by a stepwise reconstitution of two MPs.<sup>[17]</sup> In

a first step, the reconstitution of each MP into separate liposomes is optimized and in a second step the different proteoliposome populations are mixed to combine their membranes in a controlled fusion process (Fig. 3A).

Two main strategies have been described to fuse liposomes. In unspecific methods, fusion is either mediated by divalent metal ions (*e.g.*  $\text{Ca}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Zn}^{2+}$ ),<sup>[18]</sup> viral fusion proteins<sup>[19]</sup> or synthetic peptides,<sup>[20]</sup> but has been shown to induce several rounds of fusion yielding heterogeneous liposome populations that are often prone to aggregation and leakiness.<sup>[18c]</sup> Secondly, specific fusion methods that stop after one round of fusion include the use of the cellular fusion proteins, called SNARE proteins.<sup>[21]</sup> The minimal machinery for liposome fusion *in vitro* consists of two SNARE complexes that reside in the opposite membranes and interact to bring the membranes together for fusion. The complexes are called the vesicle SNARE (v-SNARE) and the target membrane SNARE (t-SNARE) complex.<sup>[22]</sup> SNARE proteins contain a single transmembrane helix and incorporate easily into the liposomal membrane by common reconstitution methods.<sup>[23]</sup> In our study, we have used purified v- and t-SNAREs to co-reconstitute the *E. coli* respiratory proteins ubiquinol *bo*<sub>3</sub> oxidase and ATP synthase, both multi-subunit complexes that possess >20 transmembrane helices. In brief, liposomes containing *bo*<sub>3</sub> oxidase/v-SNARE were fused with vesicles containing ATP synthase/t-SNARE, yielding a minimal ATP producing unit after 20 min fusion time.<sup>[17]</sup> SNARE-mediated fusion stops after one fusion event because the two SNARE proteins form a stable four-helix bundle that cannot be regenerated *in vitro*. In the past decade, several SNARE-

mimicking methods were described (Fig. 3B), either using short peptides,<sup>[24]</sup> DNA oligomers<sup>[25]</sup> or peptide nucleotide acids (PNA).<sup>[26]</sup> All these approaches mimic the SNARE machinery by bringing the adjacent membranes into close proximity, overcoming the hydration shell and electrostatic repulsion between the lipid bilayers leading to spontaneous membrane fusion. These approaches are rather slow (20–60 min) and a substantial fraction remains in a hemifusion state, in which only the outer lipid leaflets of the liposomes but not the inner leaflets are fused.

## 2.1 Charge-mediated Fusion of Proteoliposomes

A different strategy exploits the use of cationic lipids, which do not occur in natural membranes.<sup>[27]</sup> Cationic lipids can be mixed with neutral lipids to form liposomes with an overall positive surface charge that spontaneously fuse with overall negatively charged liposomes within a few minutes, mainly depending on the amount of charged lipid.<sup>[27a]</sup> As depicted in the FRET based lipid mixing experiment of Fig. 3C, fusion with oppositely charged liposomes (30% charged lipids per population) was ~10-times faster than SNARE-mediated fusions. If roughly the same amounts of positively and negatively charged lipids are used in either liposome population, it can be assumed that a 1:1 fusion is enforced as the resulting liposomes have not enough net charge for a further fusion event. Different synthetic cationic lipids such as 1,2-dioleoyl-3-trimethylammoniumpropane (DOTAP) and 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (EDOPC) were described to undergo fusion with natural biomembranes containing negatively charged lipids like cardiolipin, phosphatidylserine or phosphatidylglycer-

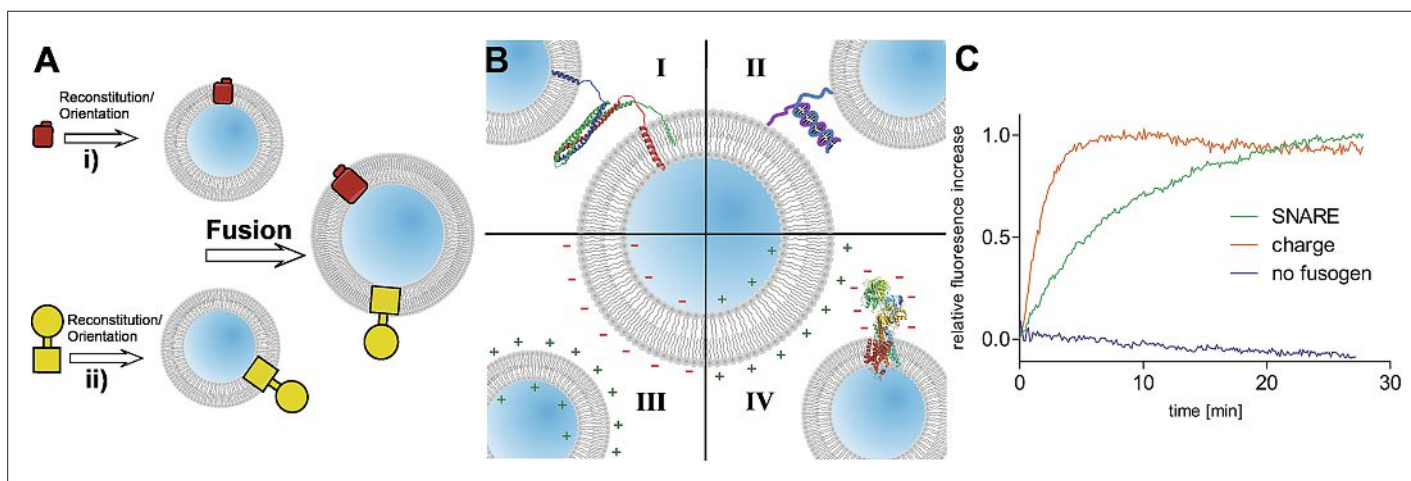


Fig. 3. (A) Co-reconstitution of two membrane proteins by liposome fusion. First, the conditions for the reconstitution and orientation of each MP (i) MP in red; ii) MP in yellow) in liposomes are optimized, followed by fusion of the proteoliposomes. Fusion leads to co-reconstitution of the two MPs in a single lipid bilayer. (B) Different approaches to specifically fuse liposomes. Liposome fusion can be mediated either by SNARE proteins or SNARE mimicking peptides (I), DNA or PNA oligomers (II), oppositely charged lipids (III), or charged proteins (IV). (C) Comparison of lipid mixing kinetics of SNARE and charge-mediated fusion methods. Details of the FRET-based assay can be found in ref. [28].

ol or with liposomes containing synthetic anionic lipids like 1,2-dioleoyl-sn-glycero-3-phospho-1'-(rac-glycerol) (DOPG) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (POPA) (Fig. 4A). However, charge-mediated fusion has not been exploited to fuse liposomes containing membrane proteins before we<sup>[28]</sup> and others<sup>[29]</sup> explored the possibility to use the method as an alternative for SNARE mediated MP co-reconstitution. In brief, liposomes containing zwitterionic phosphatidylcholine were charged with either 10–40% cationic lipids or negatively charged lipids. Complex IV from *E. coli* ( $bo_3$  oxidase) or *Rhodobacter sphaeroides* ( $aa_3$  oxidase) was then incorporated into cationic liposomes and mixed with anionic proteoliposomes containing ATP synthase. Functional co-reconstitution of both enzymes was observed within five minutes.<sup>[28,29]</sup> The process critically depended on the molar content of charged lipids, the temperature and the liposome size.<sup>[28]</sup> We found that smaller vesicles fuse faster than larger ones, an effect that can be explained by the increased curvature stress of small liposomes. Release of curvature stress during membrane fusion is thought to be a fusion-promoting event. A limitation of the method is the non-natural origin of positively charged lipids, making it necessary to test the activity of a MP in cationic liposomes. Furthermore, a 1:1 fusion of oppositely charged liposomes yields the surface of the fused membrane roughly neutral, while biological membranes have a net negative charge.

Recently, we investigated the use of pH-sensitive lipids that are only positively charged below their  $pK_a$  (4.5 to 6.5) to overcome this limitation. In other words,

while fusion occurs only at pH values below the  $pK_a$ , the lipids would be neutral at physiological pH values (pH 7 to 8), yielding a fused membrane carrying a net negative surface charge. In our initial studies, we have used the pH-sensitive lipid 1,2-dioleoyl-3-dimethylammonium-propane (DODAP) that contains two methyl groups instead of three at the nitrogen atom (Fig. 4A). The  $pK_a$  of DODAP was determined to be  $\sim 6.5$  and efficient lipid mixing has been reported at pH 4.<sup>[30]</sup> In our experiments, we compared pH-dependent lipid and content mixing between vesicles containing either DODAP or DOTAP and vesicles containing an equimolar amount of DOPG (Fig. 4B). Consistent with the  $pK_a$  value of DODAP, fast and complete lipid mixing was observed below pH 6.5, whereas DOTAP mediated fusion was unaffected by pH (Fig. 4B). However, only vesicles containing DOTAP led to efficient co-reconstitution of the *E. coli*  $bo_3$  oxidase and ATP synthase, indicating that DODAP-mediated fusion probably stopped in the hemi-fusion state (Fig. 4C). Earlier, we had shown that complete fusion requires the presence of positively charged lipids in both leaflets of the membrane.<sup>[28]</sup> Thus, DODAP is of limited value to overcome the ‘charge problem’ and other pH sensitive lipids will be tested in the future.

## 2.2 Insertion of MPs into GUVs

As mentioned above, GUVs have the unique advantage that they can be directly observed using optical microscopy. Furthermore, in contrast to smaller liposomes, the surface of GUVs is nearly planar without curvature stress, mimicking the physiological situation in eukaryotic

cells. Typically, GUVs are either formed using electroformation techniques,<sup>[31]</sup> a technique that is related to small liposome formation, or in so-called inverted emulsion formation experiments.<sup>[32]</sup> During electroformation, lipids dissolved in organic solvents are dried, forming a thin layer on indium tin oxide coated glass slides or on platinum wires. The dried lipid film is subsequently rehydrated in an aqueous solution while an AC-electric field is applied that supports swelling and formation of the giant vesicles. A completely different approach is taken in the inverted emulsion technique. There, an aqueous solution is emulsified in a lipid-saturated oil and it is envisioned that a lipid monolayer forms around the aqueous droplets, effectively shielding water from oil. This emulsion (or oil containing monolayered vesicles) is carefully placed on top of an aqueous solution and GUVs can be formed by forcing the vesicles from the emulsion through the oil-water interface, where a lipid monolayer has formed.<sup>[33]</sup> Several strategies for MP incorporation into GUVs have been described,<sup>[34]</sup> but many of them require a partial protein dehydration step, which is incompatible with delicate multi-subunit protein complexes. Dezi *et al.*<sup>[34a]</sup> reconstituted MPs into GUVs avoiding this limitation by detergent-mediated MP insertion. While this is a generally applicable method, it requires a very delicate titration of detergents to ensure that GUVs do not solubilize during the reconstitution process.

From our experience with charge-mediated liposome fusion, we envisioned that the same technique could be used to incorporate MP from SUVs into GUVs in a detergent-free approach (Fig. 5). In contrast

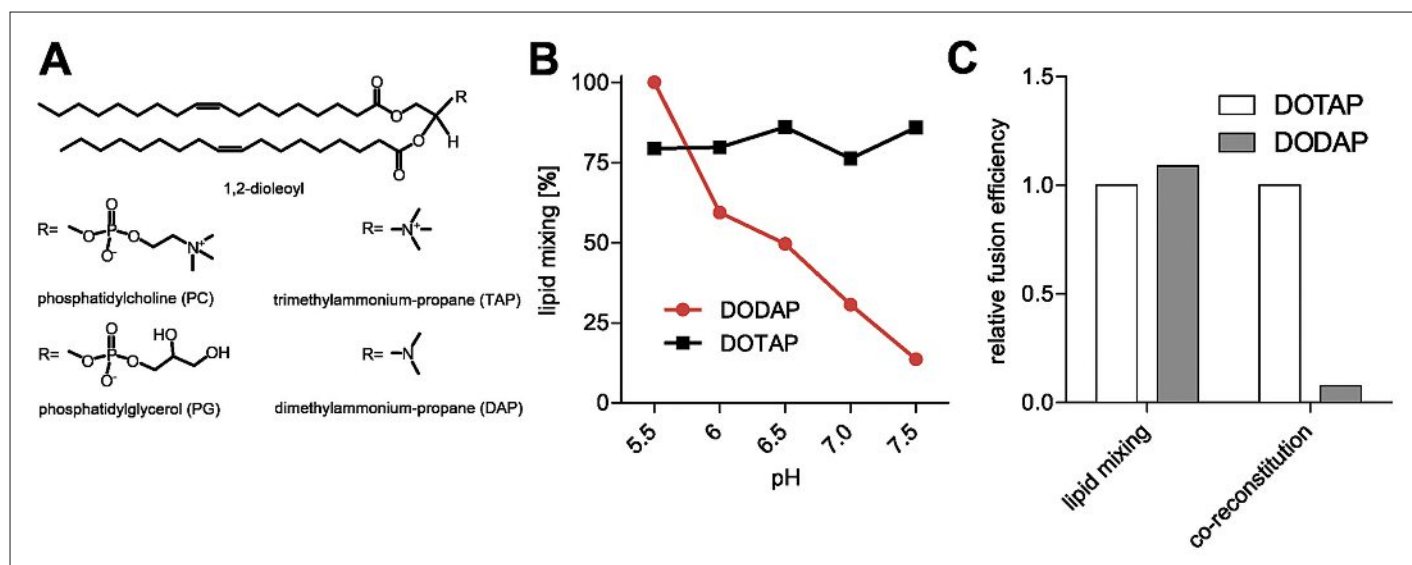


Fig. 4. (A) Chemical structure of lipids used for charge-mediated liposome fusion. (B) Comparison of pH dependency between lipid mixing experiments performed with DODAP and DOTAP. To characterize pH dependency of DODAP-mediated liposome fusion lipid mixing experiments were performed by varying the pH from 5.5 to 7.5 as described elsewhere.<sup>[28]</sup> (C) Comparison of lipid mixing and content mixing assays with DODAP- and DOTAP-mediated fusion. Co-reconstitution was measured as the coupled activity of the *E. coli*  $bo_3$  oxidase and ATP synthase using a luciferase-based assay as described before.<sup>[16d]</sup>



to SUVs, GUVs have essentially no curvature stress which is expected to decelerate the fusion process. We therefore used very small proteoliposomes (~30 nm) and found that using 30 mol% of oppositely charged lipids in either vesicle population promoted efficient GUV-SUV fusion within 20 min.<sup>[28]</sup> As the surface of a 20  $\mu\text{m}$  GUV is ~160'000 times larger than the surface of a 50 nm SUV, thousands of fusion events can take place on a single GUV. In other words, using appropriate molar ratios of GUVs and SUVs which contain different MPs, a highly complex and still negatively charged membrane containing various MPs can be obtained after the fusion process.

We have tested this hypothesis and fused the light-driven proton pump proteorhodopsin,  $bo_3$  oxidase, ATP synthase and the sodium/proton antiporter NapA into the same GUV membrane, as confirmed by fluorescence microscopy.<sup>[28]</sup> The enzymes were shown to be functional and ATP synthesis energized by a proton gradient established by  $bo_3$  oxidase was measured directly in GUVs, indicating that fusion of SUVs with GUVs yields proton-tight vesicles that are able to maintain an electrochemical gradient and that the fusion process does not change the enzyme orientation.<sup>[28]</sup> Similar experiments, in which ATP synthase and  $bo_3$  oxidase were

reconstituted into GUVs using slightly different lipid compositions were performed by Ishmukhametov *et al.*<sup>[29]</sup> and published shortly after our work.

Yet another approach using oppositely charged molecules was reported a few months later by Bian *et al.*,<sup>[35]</sup> where they incorporated the negatively charged sarco/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) into SUVs and fused them to oppositely charged GUVs.

### 3. Conclusions and Outlook

In this review, we have described our recent efforts to investigate the functional interplay of respiratory enzymes using *in vitro* reconstitution methods. Our interest in the development of these techniques is twofold. First, we aim to contribute to the understanding of the molecular mechanism of these fascinating molecular machines. Many details about their mechanisms have been described in the last decade based on studies using a single enzyme complex. Less is known about their functional interdependence, which is crucial for their physiological role. A prominent example is the hypothesis of local proton coupling between a proton pump and the ATP synthase.<sup>[36]</sup> In this theory, protons that are ejected by respiratory enzymes to generate an electrochemical gradient do not immediately equilibrate with the bulk solution, but are kinetically trapped for a certain time in the proximity of the membrane. During this trapped state, protons are assumed to travel quickly along the membrane surface creating a local pH drop near the membrane surface that acts as a driving force for ATP synthesis. The importance for such a scenario is best illustrated in alkaliphilic bacteria, which have an inverted pH gradient compared to mitochondria, making ATP synthesis thermodynamically unfavourable. Using our *in vitro* reconstituted systems, we aim to contribute experimental evidence for such a mechanism with a special emphasis on the role of the lipid composition of the membrane.<sup>[16d,37]</sup> Our second interest is to create complex biological systems using a bottom-up approach. We currently use bacterial respiratory enzymes as our model proteins, but the technique is applicable for every type of MP. The use of GUVs as a model membrane system further minimizes the amount of required enzyme for reconstitution, a critical factor for the functional investigation of purified eukaryotic MPs, which are often obtained in very small amounts. An important limitation in the quantitative evaluation of such experiments is the poor control over the orientation of the inserted protein. While most MPs orient in a mixed orientation, we found that the ATP synthase can

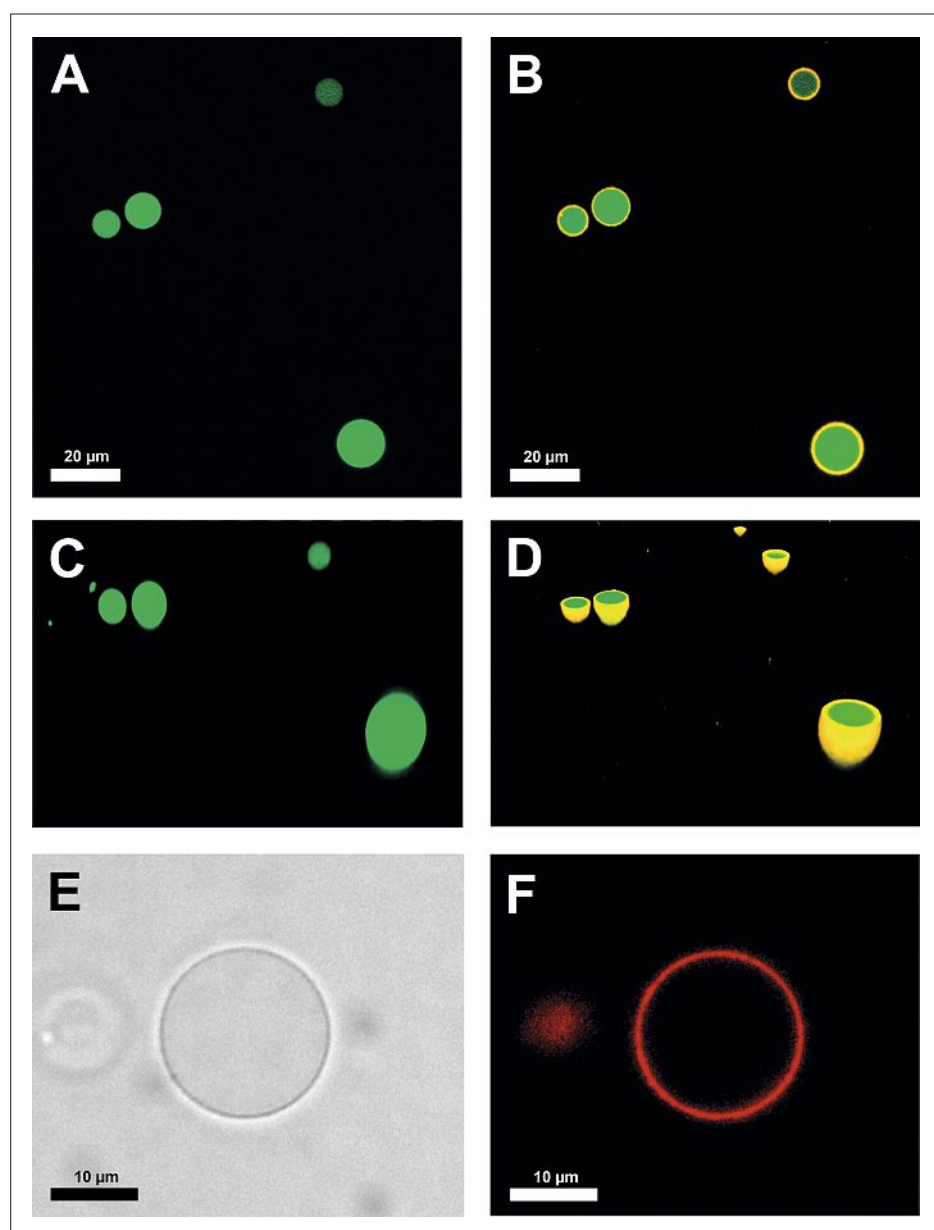


Fig. 5 (A) GUVs enfolding pyranine. Negatively charged GUVs containing the soluble pH-sensitive fluorophore pyranine (green). (B) Fusion of SUVs to GUVs. Positively charged small liposomes bearing Lissamine Rhodamine-DHPE (yellow) were fused to oppositely charged GUVs. (C) Tilted view of Fig. 5A showing the globular shape of GUVs. (D) Intersected 3D view of GUVs after fusion to fluorescent SUVs shown in Fig. 5B. (E) Brightfield microscopy image of a negatively charged GUV prior to fusion. (F) Proteo-GUV. Negatively charged GUV after fusion with SUVs containing DY647-labeled  $bo_3$ -oxidase (red) from *E. coli*.

be oriented unidirectionally using a detergent-mediated reconstitution, possibly due to its large hydrophilic moiety that cannot pass the membrane of partially solubilized liposomes.<sup>[38]</sup> Another focus in our lab is to mimic this behaviour by attaching reversibly large soluble moieties to any membrane protein prior to reconstitution.

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