Bifunctional DNA duplexes permit efficient incorporation of pH probes into liposomes

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Abstract

Enzyme-mediated proton transport across biological membranes is critical for many vital cellular processes. pH sensitive fluorescent dyes are an indispensable tool for investigating the molecular mechanism of proton-translocating enzymes. Here, we present a novel strategy to entrap pH-sensitive probes into the lumen of liposomes that has several advantages over soluble or lipid-coupled probes. In our approach, the pH sensor is linked to a DNA oligomer with a sequence complementary to a second oligomer modified with a lipophilic moiety that anchors the DNA conjugate to the inner and outer leaflet of the lipid bilayer. The use of DNA as a scaffold allows subsequent selective enzymatic removal of the probe in the outer bilayer. The method shows a high yield of insertion and is compatible with reconstitution of membrane proteins via different methods. Using the two large membrane protein complexes the usefulness of the conjugate for time-resolved proton pumping measurements is demonstrated.

Introduction

Protons are the smallest chemical molecules, but they are highly relevant in various aspects of biological life. The concentration of free protons in an aqueous solution determines its pH and regulates the enzymatic activity and stability of all proteins. In addition, protons play important roles in transmembrane transport processes. Not only are proton pumps used to establish and maintain the essential electrochemical gradient, but protons also serve as coupling ion in secondary transporters that use the electrochemical gradient to e.g. enrich important substrates in biological compartments [1], [2]. An impressive example of a proton coupled system are the members of the respiratory chain and the F_1F_0 ATP synthase found in the inner membrane of mitochondria or the cytoplasmic membrane of bacteria. These large multi-subunit complexes are responsible for the energy conversion of food-derived reduction equivalents (e.g. NADH) into the universal energy currency of the cell, adenosine triphosphate (ATP), [3] that energizes a wide variety of cellular reactions including nutrient uptake, nerve conduction and muscular motion. During this process termed oxidative phosphorylation, respiratory chain complexes I to IV transfer electrons from NADH and succinate to oxygen in a stepwise manner [4]. These exergonic processes are coupled to transmembrane proton pumping that establishes and maintains an electrochemical proton gradient (proton motive force, *pmf*) that is used by the rotating F_1F_0 ATP synthase to regenerate ATP from ADP and inorganic phosphate [5].

Not surprisingly, measurement of these transmembrane proton movements are key experiments to understand the molecular mechanism of these and many other membrane proteins. Typically, after purification in detergent solution, membrane proteins are reconstituted into liposomes, imitating their native environment for functional analysis. Proton transport activities are measured with soluble pH sensitive dyes that are incorporated into the liposomes and detected either via absorbance or fluorescence measurements [6]–[8]. After reconstitution, the non-incorporated dye has to be removed by gel filtration or ultracentrifugation or a combination of both. While convenient, the method suffers from poor incorporation yield (<5%) of the dye which is not desirable if precious dyes are used. Furthermore, many dyes (e.g. containing carboxylic acids) tend to cross the membrane over time [9], [10], rendering this technique somewhat inefficient. Recently, pH-sensitive fluorophores such as Oregon Green 488 or pHrodo coupled to a phosphatidylethanolamine lipid have been used to follow the activity of ATPases [9]–[11]. In contrast to freely soluble dyes, lipid-coupled dyes incorporate into the membrane of the liposome with very high yield. However, as lipid orientation is random during liposome formation, the dye will be evenly distributed in both membrane leaflets. As only the pH of the inside of the liposome changes during proton transport (the outside is buffered), the constant signal of the dye in the outer leaflet is expected to affect the overall signal to noise ratio of the proton transport signal [10].

Here, we present a method using commercially available tools to circumvent this drawback. Using cholesterol modified DNA-oligomers and complementary DNA-carboxyfluorescein conjugates, the dye is incorporated in both leaflets during reconstitution. In a follow-up step, the DNA embedded in the outer leaflet is digested by DNase I and liposomes containing dye solely on the inner leaflet can be obtained by ultracentrifugation (Figure 1A). Using two large multi-subunit membrane protein complexes from bacterial respiratory chains, F_1F_0 ATP synthase and cytochrome *c* oxidase, we show that transmembrane proton pumping can be followed into either direction and that the signal-to-noise ratio is improved in the DNase I treated samples. Additionally, a probe with a different pH-sensitive dye, namely CypHer5E, was synthesized showing the versatility of the presented approach.

Results and Discussion

Design of the probe

In the past, lipophilic oligonucleotides (LON's) have been used in a variety of studies. Examples are immobilization of lipid vesicles to solid surfaces or supported bilayers, tethering of liposomes to induce membrane fusion or functionalizing of vesicles with different nanostructures [12]–[15]. LON's have also been used to immobilize biosensors for ions or small molecules [16]–[19]. Being commercially available, DNA modified with cholesterol has been used in many of these studies and the influence of using one or two cholesterol moieties for membrane anchoring has been compared [20].

Here, the following commercially available DNA oligomers have been used. A 30 nt long oligo, either unmodified (ON*1*) or modified with cholesterol at the 3'-end (ON*2*) has been used as the scaffold for the lipophilic probe. The pH sensitive dye 5(6)-carboxyfluorescein was coupled to the 3'-end of a 18 nt oligo (ON*3*) that is complementary in sequence to the 5'-end of oligos 1 and 2 (apparent melting temperature 37.8°C of the pairing region). Additionally, a cholesterol modified oligo at the 5'- end (ON*4*) that is complementary to the 3' region of oligos ON*1* and ON*2* was used to build the lipophilic probe with two cholesterol moieties (apparent melting temperature of 40°C). An overview of the different probes is given in Supplementary Figure S1. The respective complementary oligos were allowed to hybridize for 10 min at room temperature and hybridization was confirmed using TBE 20% polyacrylamide gel electrophoresis (Figure S2).

Incorporation of DNA into LUVs

In a first series of experiments, the incorporation efficiency of the different lipophilic probes into liposomes was compared to free and lipid anchored carboxyfluorescein (CF). The variants comprised of free CF, CF coupled to a lipid (18:1 PE CF), CF coupled to DNA without (ON-CF), with one (LON-CF) or with two cholesterol-moieties (L_2ON-CF) and were incorporated into unilamellar 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) vesicles. In addition, liposomes also contained 0.03 mol% TexasRed™-labeled lipids (TR-DHPE) that was used to normalize for lipid loss or dilutions during the procedure. Schematic representations of the different variants can be seen in Figure 1A. The lipid-coupled dyes (18:1 PE CF and TR-DHPE) were mixed with DOPC in chloroform prior to lipid drying, while all other variants were added in the rehydration buffer used to form the liposomes, as described in the Methods section. Addition of the DNA containing variants to the lipids prior to drying using an organic solvent mixture (chloroform: methanol: water = 6.25:6.25:1, v/v/v) was abandoned after initial trials, as no improvement over addition to the rehydration buffer was observed. However, we note that it is

also possible to add the DNA conjugates at an earlier step prior to drying, which might be useful for other applications or LON-probes. After rehydration, 100 nm unilamellar liposomes were produced using the freeze-thaw and extrusion method, split into two fractions to which either DNase I or water was added and incubated for 20 min at 37°C (Figure 1B). Subsequently, free dye was removed by ultracentrifugation and pelleted liposomes were resuspended, mixed with measuring buffer and carboxyfluorescein and Texas Red fluorescence was measured. Relative recovery of CF was calculated as the intensity ratio of the sample after and before the centrifugation. The liposome recovery was calculated similarly using the TR-DHPE intensities after and before the centrifugation. Finally, incorporation yield of CF into liposomes was determined as the ratio of CF and liposome recovery (Table 1).

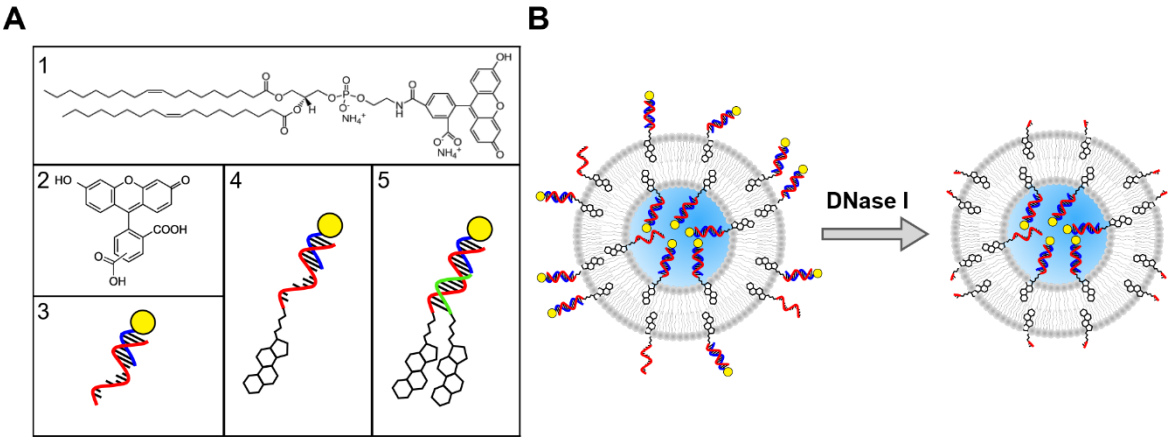

Figure 1: Scheme of the different CF variants and of the dye removal by DNase I. A) Schematic representations of the different CF variants added to the liposomes. 1 = 18:1 PE CF, 2 = CF, 3 = ON-CF, 4 = LON-CF, 5 = L_2ON-CF. For the LON-probes, the DNA strands are shown in red, blue and green, CF is depicted as a yellow dot. B) Scheme of the dye removal by DNase I treatment of vesicle incorporated LON-CF.

No.	Dye variant	Without DNase I	With DNase I
1	18:1 PE CF	103.5 ± 11.5 %	102.0 ± 10.8 %
2	CF	3.4 ± 0.3 %	3.6 ± 0.3 %
3	ON-CF	16.6 ± 1.6 %	4.5 ± 0.4 %
4	LON-CF	81.7 ± 8.8 %	43.0 ± 5.9 %
5	L_2ON-CF	84.7 ± 11.8 %	37.8 ± 4.3 %

Table 1: Incorporation yields of different CF variants into liposomes. The incorporation was calculated as detailed out in the text. Each sample (extruded liposomes, liposomes with DNase I treatment,

liposomes without DNase I treatment) was measured three times and three separate experiments were performed.

The results are shown in Table 1. Not surprisingly, lipid-coupled CF is fully incorporated into the vesicles while only very little free CF is incorporated (~3.5%), and both values were unaffected by DNase I treatment. Surprisingly, ~17% of fluorophore coupled to DNA (ON-CF) was found in the preparation without DNase I treatment, while only ~4.5% was left after the nuclease treatment, indicating an unspecific interaction of the DNA with the zwitterionic membrane as observed earlier [21]. Finally, the assembled lipophilic probes (LON- and L_2ON-CF) were incorporated to >80% before and 40% after DNase I treatment, respectively. These values represent good incorporation of the dye via the cholesterol moiety and successful DNA cleavage after treatment. The non-incorporated 15-20% percent indicate that free DNA-fluorophore-conjugate is present or that some of the DNA-pair contains no cholesterol moiety (the DNA conjugates were used as obtained by the company). Alternatively, a small portion of the DNA-pair might have partitioned into the aqueous phase due to the hydrophilic nature of the DNA moiety.

It an earlier study [20] a DNA trimer containing two cholesterol moieties was found to yield a more stable incorporation of the DNA in the membrane than a single cholesterol conjugate, but in our experiments, no significant difference between the two corresponding constructs LON-CF and L_2ON-CF was observed. In the original article, incorporation into planar, supported lipid bilayers was investigated, while spherical vesicles were used here. As no significant difference was seen between the two conjugates, LON-CF was used in the remainder of the experiments.

pH dependency of DNA-cholesterol coupled carboxyfluorescein

It has been reported that pH sensitive groups coupled to lipid head groups change their apparent pK_a (up to several pH units) when incorporated into liposomes, likely due to the influence of the nearby membrane surface [9], [10]. In the worst case, this effect shifts the detectable pH range outside the experimental conditions. In our scenario, the dye is expected to be several nanometers away from the membrane surface and thus to be unaffected by it. Nevertheless, in the next series of experiments, the useful pH range of the reconstituted DNA-carboxyfluorescein dye was determined. To this end, we reproduced the elegant experiment with lipid coupled Oregon Green reported by Schwamborn *et al.* [10]. CF and Oregon Green have very similar characteristics, exhibiting high fluorescence at neutral to alkaliphilic pH and a low fluorescence at acidic pH. In this experiment, liposomes containing DNA coupled dye, either untreated or treated with DNase I, where incubated at different pH values ranging

from 5.5 to 10.3 at 4°C overnight, allowing to equilibrate the luminal pH value with the bulk pH. The next day, liposomes were diluted into buffer with a pH of 6.9 and fluorescence emission was measured. This situation mimics an experiment, where protons have either been pumped to the inside or to the outside of the liposome (luminal pH changes between pH 5.5 and 10.3), while the bulk pH remains constant at pH 6.9 (Figure 2A, red traces). As shown in Figure 2 for the DNase I treated sample (triangles), fluorescence was maximal at pH > 8, but decreased when the pH was lowered, and at pH 5.5, essentially no fluorescence was observed. In the untreated sample (squares), the fluorescence also decreased at higher pH values but remained at ~50% at pH 5.5. The remaining fluorescence at low luminal pH values stems from the dye on the outside of the liposome, where the pH is unchanged (pH 6.9), similar to what has been observed with the lipid coupled Oregon 488 dye [10]. As a control, liposomes were also measured in the same buffer as incubated overnight (luminal pH = bulk pH, black dotted traces), yielding a very similar dependence to the DNase I treated sample. A sigmoidal dose-response curve was used as a fit and apparent pKa values in the range of ~6.9 were obtained. The pKa of the membrane embedded conjugate is thus slightly higher than free carboxyfluorescein with a reported pKa of 6.5 [22], but a similar shift from free to DNA-conjugated fluorescein has been reported in the literature [23]. These data show that the apparent pKa of the DNA-coupled dye is therefore not influenced by the membrane and is useful for pH measurements in the physiologically important range between pH 6 and pH 8. Furthermore, DNase I experiments show that removal of the dye from the outer leaflet increases the available signal range in these experiments.

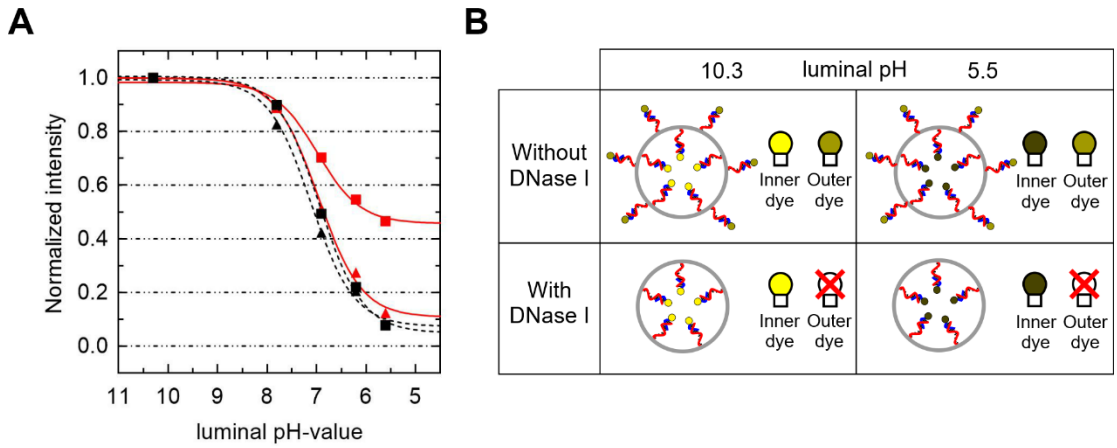

Figure 2: pKa and signal range determination of vesicle incorporated LON-CF. A) Fluorescence intensity of LON-CF in LUVs measured at different pH values. LUVs were either measured at the same luminal and bulk pH (black dotted traces) or at a constant bulk pH with varying luminal pH (red traces). A sigmoidal curve was used to fit the points. Data were normalized to the fluorescence at pH 10.3. B) Schematic representation of liposomes containing LON-probe treated with or without DNase I at a constant bulk pH of 6.9 with a luminal pH of 10.3 (left) and 5.5 (right). Next to the liposomes, two light

bulbs represent the inner and outer fluorophores, respectively. For both treated and untreated liposomes, fluorescence of the inner CF is strong at a luminal pH of 10.3 and weak at a luminal pH of 5.5. For the untreated liposomes, outer CF has a medium fluorescence both at luminal pH 10.3 and 5.5 due to the constant bulk pH of 6.9. For the treated liposomes, the additional medium fluorescence of the outer CF is removed.

Measuring proton translocation with respiratory enzymes

Next, the conjugate was used to monitor ATP driven H^+-pumping by the ATP synthase of *Escherichia coli*. In this experiment, F_1F_0 ATP synthase was reconstituted into liposomes containing LON-CF using two different established methods. In the first, ATP synthase was mixed with liposomes in the presence of 0.8% *n*-octyl-β-d-glucoside (OG), incubated on ice followed by detergent removal using SM-2 BioBeads (Figure 3). In the second reconstitution method, sodium cholate was used as a detergent that was subsequently removed by gel filtration chromatography (Supplementary Figure S3). After reconstitution, half of the sample was treated with DNase I and liposomes from both samples were collected by ultracentrifugation and resuspended in buffer. The optimal amount of DNase I was determined by titrating the amount of DNase I using a liposome acidification assay to detect digestion of the DNA as described in Supplementary materials (Figure S4+S5).

The experimental setup is shown in Figure 3A. Proton pumping into the vesicle is started by addition of ATP binding to the catalytic site of the F_1 part of the ATP synthase that is exposed to the outside of the liposomes [24]. The acidification of the liposome interior was then followed using time resolved fluorescence spectroscopy. As depicted in Figure 3B (raw data) and Figure 3C (normalized to initial fluorescence), addition of ATP lead to a rapid decrease of the CF signal in the DNase I treated (red) and untreated (blue) sample, indicating ATP driven proton influx. Addition of the protonophore carbonyl cyanide m-chlorophenyl hydrazine (CCCP) dissipated the proton gradient and the fluorescence returned to its initial value. As expected from the experiments described above, the removal of the outer dye led to an increased signal in the normalized data (Figure 3B). No change in fluorescence was observed in liposomes containing no ATP synthase (yellow).

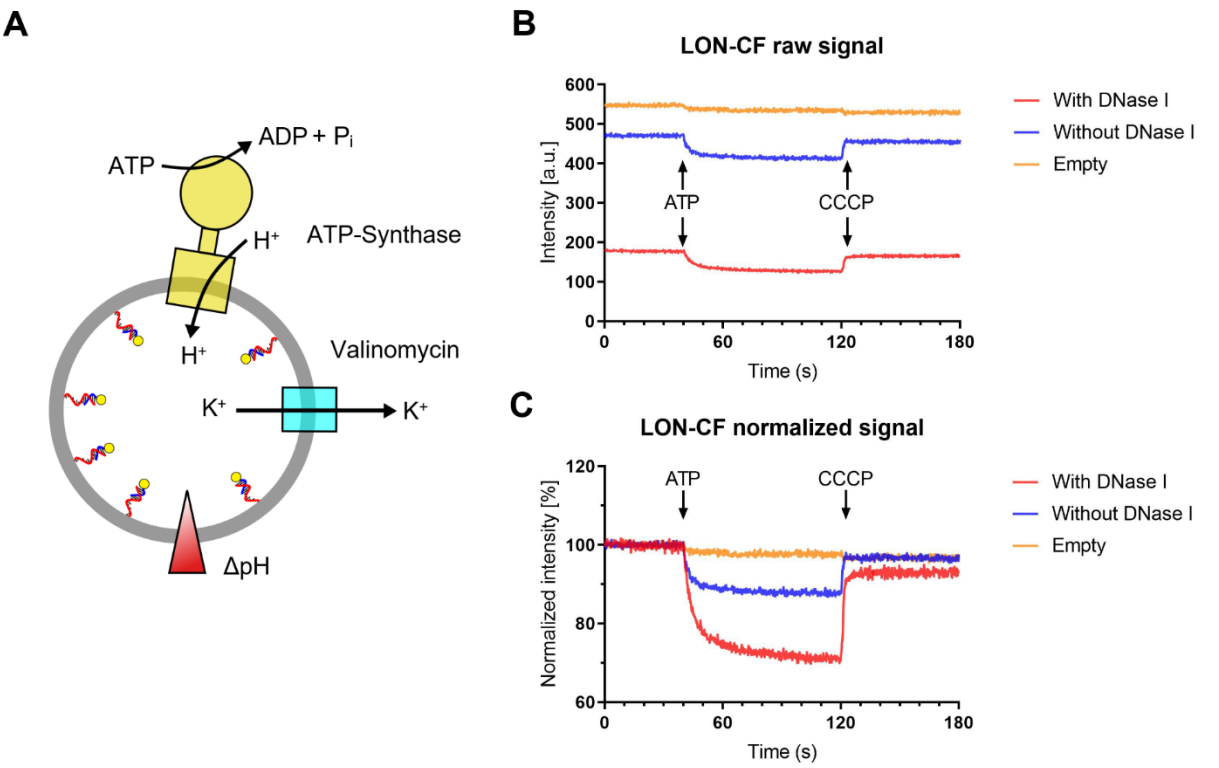

Figure 3: ATP hydrolysis driven proton pumping monitored by LON-CF fluorescence. A) Cartoon representation of the experiment. Proteoliposomes containing F_1F_0 ATP synthase were treated with (red trace) or without DNase I (blue trace). As a negative control, empty liposomes (yellow trace) were measured. Proton translocation is initiated by the addition of 1 mM ATP and the proton gradient is dissipated by addition of 5 µM CCCP. B) Raw data of the different proteoliposome preparations. C) Same as 3B, but traces were normalized to the averaged intensity before addition of ATP.

LON-CF was also used to follow proton transfer from the inside to the outside of the liposomes. To this end, purified bacterial cytochrome *c* oxidase from *Rhodobacter sphaeroides* and reconstituted into liposomes. This enzyme catalyzes the final step of oxidative phosphorylation and uses the free energy released during oxygen reduction (to H_2O) to pump protons across the membrane and generate a proton motive force (Figure 4A). Cytochrome *c* oxidase of *R. sphaeroides* contains four redox centers, Cu_A, heme *a* and the binuclear site consisting of heme a_3 and Cu_B that binds oxygen. Electron transport to the enzyme is mediated via reduced cytochrome *c* (cyt *c*), a small soluble protein that is unable to penetrate the membrane. If added to liposomes containing reconstituted cytochrome *c* oxidase, cyt *c* docks to its binding site and donates electrons via Cu_A and heme *a* to the binuclear site, where oxygen is reduced to water [25], [26]. As a consequence, protons required for the reaction of O_2 to H_2O and those pumped across the membrane are taken up from the inside of the liposome, leading to an alkalization of the liposome lumen. Using CF as the probe, in contrast to the inwardly pumping ATP synthase, an increase in fluorescence signal should be observed. Proteoliposomes were mixed with

oxidized cyt *c* and a baseline was recorded (Figure 4B) before 2 mM sodium ascorbate was added. Ascorbate is used as electron source that reduces cyt *c* in solution within milliseconds. This ensures a continuous steady state reaction after cyt *c* has donated its electron to cytochrome *c* oxidase and is released from the enzyme in its oxidized form. Upon addition of ascorbate, a rapid increase in the fluorescence signal is observed, indicating proton efflux from the liposomes. As already observed for the ATP synthase in the experiment above, removal of the dye from the outer leaflet increased the relative signal increase substantially (red trace compared to blue trace). Addition of CCCP dissipated the proton gradient and signal returned to the value before ascorbate addition. Only a very small increase in signal was observed in liposomes containing no protein.

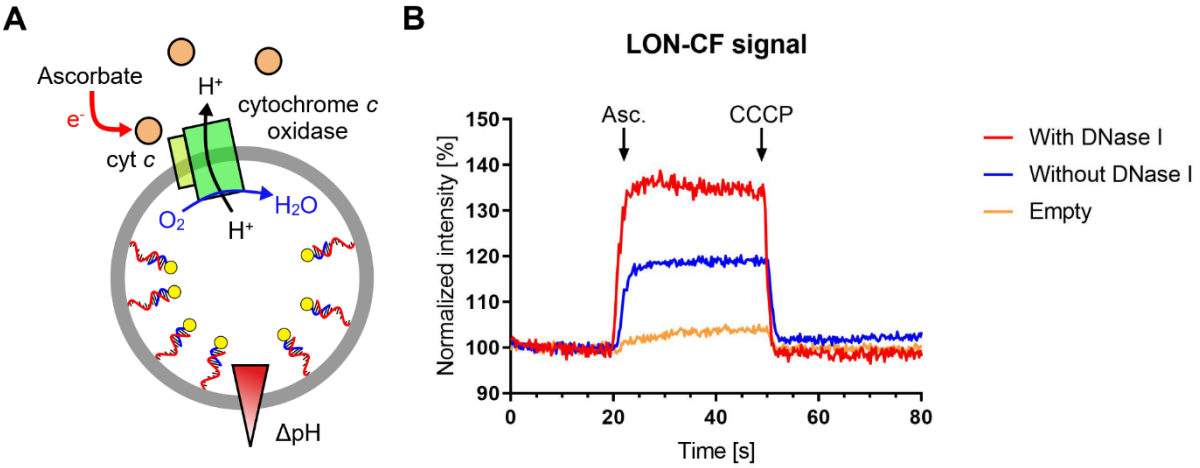

Figure 4: **Redox-driven proton pumping in cytochrome *c* oxidase following LON-CF fluorescence.** A) Cartoon representation of the experiment. Proteoliposomes containing *R. sphaeroides* cytochrome *c* oxidase were treated with (red trace) or without DNase I (blue trace). As a negative control, empty liposomes (yellow trace) were measured. A baseline is recorded after addition of 10 µM cyt *c* and proton translocation is initiated by the addition of 2 mM Na-ascorbate (Asc.). The proton gradient is dissipated by the addition of 5 µM CCCP. B) Proton translocation of proteoliposomes treated with and without DNase I and empty vesicles treated without DNase I normalized to the averaged intensity before addition of ascorbate.

Discussion:

Here we present a novel tool to incorporate fluorescent dyes into liposomes to follow the function of membrane proteins. An oligonucleotide with a lipophilic moiety, in this case cholesterol, was hybridized with a second oligonucleotide labeled with a pH-sensitive fluorophore and the conjugate was incorporated into the vesicle membrane during liposome formation. Compared to soluble fluorophores, a high incorporation was reached with the lON-probe, comparable to the yield with the lipid-coupled fluorophore. Treatment with DNase I and subsequent ultracentrifugation led to a

selective removal of the dye present in the outer leaflet of the vesicle, yielding a clean labeling of the inner leaflet. Thereby, the loss of the dye is limited to ~50% compared to typical losses of >95% with soluble dyes, yielding an at least 10-times incorporation efficiency. Previously, several methods that have been described to achieve selective labeling of the inner leaflet involved either chemical bleaching or photophysical quenching of the dye in the outer leaflet, for example by the addition of sodium dithionite, nitrobenzoxadiozole or iodide, or involved physical removal of the labeled lipids using either BSA, cyclodextrins or unlabeled acceptor vesicles[27]. While the reagents needed for the first approach will likely interfere with the function of the MP, especially for proteins involved in redox reactions, the second approach is unspecific and might affect the stability and tightness of the liposomes that is crucial to ensure in proton transporting experiment. Our enzyme-mediated method for the removal of the outer leaflet-embedded dye is specific and compatible with the presence of delicate multi-subunit protein complexes and redox reactions. The tight anchoring of the lipid dye will further suppress passive leakage of the dye across the membrane and the hydrophilic nature of the DNA backbone makes flipping from the inner to the outer leaflet highly unlikely. Taken together, we are convinced that our method is a valuable addition to the set of existing tools.

Our measurements with the respiratory enzymes F_1F_0 ATP synthase and cytochrome *c* oxidase show that the presence of the dye does not affect protein function, neither before nor after treatment with DNase I. The simple incorporation procedure during rehydration also ensures that the technique is compatible with a variety of reconstitution techniques. While not tested here, we envisage that the conjugates could also be added together with the enzyme during the reconstitution process, further simplifying the experimental process.

Certainly, the major advantage of the presented probe design is its versatility and flexibility based on the self-assembly of the complementary DNA strands. Here, we have chosen a commercially available carboxyfluorescein DNA modification to establish the principle. During the course of the experiment, the oligo ON*1* was also customized with another pH sensitive dye CypHer5E to yield ON*5*. The commercially available succinimidyl ester of Cypher5E was coupled to oligo ON*1* containing a C6-linker with a primary amine-group on the 3' end and HPLC purified (performed at Microsynth, Balgach, Switzerland). In contrast to CF, CypHer5E fluorescence increases with decreasing pH values, making it an interesting complementary tool to CF-based sensors[28]. We have used the dye in ATP synthase measurements and while not delivering an increased signal-to-noise ratio compared to the CF conjugate, the experiment shows that selective removal of the outer leaflet probe did strongly enhance the apparent sensitivity of the dye (Supplementary Figure S6, blue and red trace). Given the simple coupling reaction, i.e. NHS-ester to primary amine on oligonucleotide, essentially any dye or probe can be selectively coupled to the inner leaflet. We also envisage that such probes have applications for

microscopic approaches. Recently, efforts have been made to follow the function of membrane proteins in giant unilamellar vesicles[10], which in terms of their size (1-100 µm) are very similar to eukaryotic cells and thus provide an interesting experimental model system. There, by the cleavage of the probe, unwanted background signals could be removed.

Finally, the use of DNA strands opens an interesting opportunity to control the distance of the dye to the surface of the membrane. In our experiments, the DNA linker ensured sufficient distance of the dye from the membrane to keep its protonation properties unchanged, a drawback that is encountered by direct coupling of e.g. pH-sensitive dyes to a lipid headgroup such as phosphatidylethanolamine. Use of different linker lengths should allow to separate the inside of a giant vesicle into different zones, e.g. to monitor membrane binding or release processes.

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Material and Methods:

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-carboxyfluorescein (18:1 PE CF) were obtained from Avanti Polar Lipids (Alabaster, USA), Texas Red™ 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine (TR-DHPE) from ThermoFisher Scientific (Waltham, Massachusetts, USA), DNase I from bovine pancreas, grade II from Roche (Basel, Switzerland), CypHer5E NHS Ester from Sigma (St. Louis, Missouri, USA) and 5(6)-carboxyfluorescein from Molecular Probes (Eugene, Oregon, USA). Oligonoucleotides ON*1* to ON*5* 5'-TGG ACA TCA GAA ATA AGG CAC GAC GGA CCC-3' (ON*1*), 5'-TGG ACA TCA GAA ATA AGG CAC GAC GGA CCC-3'-cholesterol (ON*2*), 5'-TAT TTC TGA TGT CCA CCC-3'-carboxyfluorescein (ON*3*), cholesterol-5'-CCC TCC GTC GTG CCT-3' (ON*4*), 5'-TAT TTC TGA TGT CCA CCC-3'-C6-CypHer5E (ON*5*) were obtained from Microsynth (Balgach, Switzerland).

Liposome formation with LON-probes

Lipids dissolved in chloroform were mixed in the desired ratio in a 25-mL round-bottom flask and chloroform was evaporated under a constant stream of N_2 while rotating the flask. The thin lipid film was further dried for ≥ 2h under high vacuum. Single stranded DNA (ssDNA) molecules were mixed in the desired amounts (See supplementary methods for details) in rehydration buffer and self-hybridized to form the LON-probes. The dried lipid film was resuspended at 5 mg mL^{-1} using the buffer containing the LON-probe. Unilamellar liposomes were subsequently formed by seven freeze–thaw cycles and extruded through a Whatman polycarbonate membrane (Little Chalfont, UK) with a pore size of 100 nm.

DNase I treatment

Lyophilized powder of DNase I was dissolved in Milli-Q water at 10 mg mL^{-1} and stored for maximally 1 week at 4°C. Prior to experiments, the stock was diluted to 0.5 mg mL^{-1} and 5 µL was added per 100 µL of liposomes (final concentration 24µg mL^{-1}). For untreated samples, 5 µL of Milli-Q water was added instead. The liposomes were next incubated at 37° C and 500 rpm for 20 min, cooled on ice, diluted 5-fold and subsequently centrifuged at 200'000 x g for 90 min to remove the unbound or cleaved dye and DNase I. The supernatant was discarded, and the liposome pellets were carefully resuspended in the desired volume.

Incorporation of conjugates during rehydration

DOPC and TR-DHPE liposomes were formed as described above in rehydration buffer (10 mM MOPS pH 7.4, 50 mM KCl, 2.5 mM $MgCl_2$) containing different variations of carboxyfluorescein. The final

concentration of the lipids in the liposomes was 6.7 mM for DOPC and 1.8 µM for TR-DHPE. The concentrations of ssDNA molecules in the rehydration buffer are shown in Table 2.

	ON-CF	LON-CF	L_2ON-CF
ON*1*	10.2µM	-	-
ON*2*	-	10.2µM	10.2µM
ON*3*	8µM	8µM	8µM
ON*4*	-	-	12µM

Table 2: Final concentrations of oligonucleotides ON*1*-ON*4* used for the formation of liposomes

Free CF was used at 8.0 µM in the rehydration buffer. For the lipid-coupled CF, 18:1 PE CF was dissolved in chloroform and mixed with the lipids in the round-bottom flask to get a final concentration of 8.1 µM in liposomes. Therefore, all CF variants were added to yield 0.12 mol% CF in liposomes while TR-DHPE was used at 0.03 mol%. DNase I treatment was performed using 200 µL liposomes as described above and 70 µL of liposomes before and after treatment were mixed with 700 µL measuring buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$ and 1% (v/v) Triton). For measurements, 150 µL of diluted liposomes were added to a 150 µL quartz cuvette and fluorescence was measured using 589 nm and 615 nm as excitation and emission wavelengths respectively for TR-DHPE. For CF fluorescence, excitation and emission wavelengths of 495 nm and 520 nm were used. Each sample was measured three times and three separate experiments were performed. To calculate the incorporation of CF, the intensities of the three experiments were averaged and standard deviations of the measured CF and TR-DHPE fluorescence were calculated. Next, signals of both dyes were normalized to the liposomes before treatment to obtain the percentage of signal in the treated liposomes compared to the total signal for each dye. Finally, the normalized CF signal was divided by the normalized TR-DHPE signal to account for vesicle loss or dilution. The error was calculated by propagation of the standard deviation.

pH-dependency

Liposomes were formed as described with DOPC and TR-DHPE in rehydration buffer (5 mM MES, 5 mM Bistris propane, 5 mM CAPS, adjusted to pH 6.9, 50 mM KCl, 2.5 mM $MgCl_2$) containing 10.2 µM ON*2* and 8.0 µM ON*3* for LON-CF. The final concentration of the lipids in the liposomes was 6.7 mM for DOPC and 1.4 µM for TR-DHPE. Therefore, LON-CF was added to yield 0.12 mol% CF in liposomes and TR-DHPE was used at 0.02 mol%. DNase I treatment was performed as described with the following modification. A suspension of 1.1 mL liposomes was treated with either water or DNase I and split to

5x 200 µL before ultracentrifugation. The pellets were resuspended in buffers (10 mM MES, 10 mM Bis-Tris propane, 10 mM CAPSs, 50 mM KCl, 2.5 mM $MgCl_2$) with pH adjusted to 10.3, 7.8, 6.9, 6.2 and 5.5 and incubated overnight to equilibrate pH values on the inside and outside. Fluorescence was measured in a 150 µL quartz cuvette by mixing 20 µL liposomes and 200 µL buffer and emission spectra were recorded from 510 nm to 600 nm with 495 nm excitation for CF and from 615 nm to 670 nm using an excitation of 589 nm for TR-DHPE.

Purification of membrane proteins

F_1F_0 ATP synthase from *E. coli* and cytochrome *c* oxidase from *R. sphaeroides* were purified according to published protocols [29], [30].

Reconstitution of membrane proteins with BioBeads

Purified *E. coli* F_1F_0 ATP-synthase was reconstituted using detergent removal by SM-2 BioBeads. 200 µL liposomes (5 mg mL^{-1}) containing LON-probe in reconstitution buffer (10 mM MOPS pH 7.4, 50 mM KCl, 2.5 mM $MgCl_2$) were mixed with 7.5 µL 20% *n*-octyl-β-d-glucoside before 10 µL 6.5 µM purified ATP synthase was added. The sample was incubated for 30 min with gentle shaking every 5 – 10 min. A total of 180 mg SM2 BioBeads, prewashed with isopropanol and water and equilibrated with reconstitution buffer, was added in three steps of 30 mg, 60 mg and 90 mg with incubation of 30 min at 4°C for the first two steps and 50 min after the last addition of beads. Beads were removed from the suspension prior to DNase I treatment.

Reconstitution of membrane proteins using gel filtration

R. sphaeroides cytochrome *c* oxidase and *E. coli* F_1F_0 ATP-synthase were reconstituted using detergent removal by gel filtration [24]. 200 µL liposomes (5 mg mL^{-1}) containing LON-CF in reconstitution buffer were mixed with 5 µL 10% Na-cholate, briefly incubated and 10 µL of either purified ATP synthase (6.5 µM) or 15µL cytochrome *c* oxidase (44 µM) was added. The sample was incubated for 30 min at room temperature with gentle shaking every 5 – 10 min. The sample was run on a P10 column equilibrated with 25 mL buffer. Briefly, sample and 750 µL buffer were loaded on the column and eluted with 1.2 mL buffer. The eluted sample was centrifuged at 200'000 x g for 90 min. The liposome pellet was resuspended in 200 µL reconstitution buffer and DNase I treatment was performed as described.

Proton translocation

Twenty µL proteoliposomes were mixed with 980 µL buffer containing 10 mM MOPS pH 7.4, 50 mM KCl, 2.5 mM $MgCl_2$ in a 1.5 mL quartz cuvette, equipped with a magnetic stir bar. CF fluorescence was followed at excitation and emission wavelengths of 495 nm and 520 nm, respectively. In all measurements, slit widths of 5 nm were used. All measurements were performed in the presence of

50 nM valinomycin. Proton translocation with the ATP synthase was started by addition of 5 µL of 200 mM ATP, and after a few minutes, 5 µL of 1 mM CCCP was added to dissipate the proton gradient. Proton translocation with the cytochrome *c*-oxidase was monitored in the presence of 10 µM oxidized cyt *c* and was started by addition of 2 µL 1 M ascorbate. After a few minutes, 5 µL of 1 mM CCCP was added to dissipate the proton gradient.