



Engineered-membranes and Engineered-micelles as Efficient Tools for Purification of Membrane Proteins

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**Engineered-membranes and Engineered-micelles as Efficient Tools for Purification
of Halorhodopsin and Bacteriorhodopsin**

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Abstract

We describe two alternative and complementary purification methods for **halorhodopsin and bacteriorhodopsin**. The first relies on a unique form of detergent micelles which we have called *engineered-micelles*. These are specifically conjugated in the presence of [hydrophobic chelator : Fe²⁺] complexes and form detergent aggregates into which membrane proteins partition, but hydrophilic water-soluble proteins do not. The approach was tested on the membrane protein, bacteriorhodopsin (bR), with five non-ionic detergents (OG, OTG, NG, DM, DDM), commonly used in purification and crystallization of membrane proteins, in combination with the commercially available bathophenanthroline or with one of the three synthesized phenanthroline derivatives (Phen-C10, Phen-C8 and Phen-C6). Our results show that bR is extracted efficiently (60-86%) and directly from its native membrane into diverse detergent aggregates with preservation of its native conformation, while 90-95% of an artificial contaminating background is excluded. For implementation of the second method, based on *engineered-membranes*, the use of detergents, which in some cases may produce protein denaturation, is not required at all. Protein-containing membranes are conjugated *via* the same hydrophobic [chelator:metal ion] complexes but maintain the membrane protein in its native bilayer environment throughout the process. This method is demonstrated on the membrane protein halorhodopsin from *Natronomonas pharaonis* (phR) and leads to good recovery yields (74-89%) and removal of >85% of artificial background impurities while preserving the native state of phR. The detailed structure of the hydrophobic chelator used has been found to have a marked effect on the purity and yield of both methods.

Introduction

Isolation of membrane proteins (MPs) in a pure, concentrated and functional state is a precondition for structural determination by X-ray crystallography, electron microscopy or NMR. To obtain sufficient material, MPs are generally expressed in diverse host organisms and once expression levels are satisfactory, purification is initiated. Ideally, the expressed MP is embedded in the membrane of the host cell and its extraction from the membrane is achieved by addition of detergents above their critical micellar concentration (cmc).¹ Under these conditions, the detergent disrupts the membrane and, in parallel, surrounds and covers the hydrophobic domains of the protein, leading to water-soluble [detergent-MP-lipid] ternary complexes. Purification is accomplished either *via* classical chromatographic methods (e.g. ion exchange chromatography) or by genetically engineered affinity-tags (e.g. His-tag) which can lead to highly pure protein preparations.² Clearly, exclusion of most of the host proteins by non-chromatographic means prior to the chromatographic step, would simplify purification and potentially lead to higher recovery yields and overall greater purity.

In 1981, Bordier demonstrated that MPs (being hydrophobic) partition efficiently into detergent rich phases composed of the non-ionic detergent Triton X-114, whereas water-soluble proteins do not.³ This partitioning process, called *cloud point extraction*, relied on the ability of Triton X-114 to undergo phase separation at ~22°C into detergent rich and poor phases.⁴ Although Triton X-114 provided working conditions that could preserve the functionality of many MPs, the approach was limited by the fact that numerous other detergents, commonly used in the purification of membrane proteins, only reach the cloud point at elevated temperatures that would denature most proteins. Successful attempts to lower the cloud point temperature in the presence of high concentrations of water-soluble polymers (e.g. dextran, PEG-40000⁵⁻⁷) or precipitants (e.g. ammonium sulfate⁸⁻⁹) have been reported. However, it is clear that purification would be greatly simplified if neither polymers nor precipitants were required.

To overcome these limitations and produce detergent rich phases under mild conditions regardless of (a) the cloud point temperature of the detergent used, (b) at low detergent concentration, and (c) without polymers or precipitants, we have developed a new class of micelles which we call *engineered-micelles*.¹⁰ These micelles can be specifically conjugated *via* strong complexation between hydrophobic

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3 metal chelators (embedded within the micelle) and divalent metal ions, particularly
4 Fe^{2+} , which serve as mediators in the aqueous phase (Figure 1, A). We recently
5 demonstrated that conjugated *engineered-micelles* composed of: [octylglucoside
6 (OG) / the hydrophobic chelator, bathophenanthroline / Fe^{2+}] can purify the light
7 driven proton pump, bacteriorhodopsin (bR) from added *E. coli* lysate.¹¹ Here we
8 show that four additional non-ionic detergents commonly used in the purification of
9 MPs are also able to produce high levels of bR purification.
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16 Since in some cases membrane disruption by detergents may be accompanied by
17 the denaturation of the membrane-bound proteins, we have in addition developed a
18 complementary approach that relies on conjugation of membranes containing the
19 light driven chloride pump: halorhodopsin (phR) (rather than micelles), using the
20 same [chelator:metal] complexes and thereby maintaining the membrane protein
21 within its native environment and circumventing the need for detergents. Synthesized
22 phenanthroline derivatives are shown to be able to produce high levels of both bR
23 and phR purification.
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Results

Conjugation of *engineered-micelles*

Aqueous suspensions containing 200mM NaCl and the non-ionic detergent OG (22mM) were incubated at room temperature with either the commercially available hydrophobic chelator bathophenanthroline (1mM) or each of the three synthesized phenanthroline derivatives (Phen-C10, Phen-C-8 and Phen-C6). Following the addition of Fe²⁺ (0.5 mM) red oily globules appeared in the light microscope except in the case of Phen-C6 (Figure 1, B). The color of the globules indicates that they contain the red [(phenanthroline)₃:Fe²⁺] hydrophobic complex. No globules appeared in the absence of the chelators, the metal or both (not shown).

These findings are consistent with our previous studies demonstrating that conjugation of detergent micelles is a rapid process, occurring within seconds after Fe²⁺ addition.^[10] Similar short times were found within the complete temperature range of interest, *i.e.* 4-23 °C.^[10] We attribute this temperature independence to the strong binding affinity between Fe²⁺ and the chelators used, all of which are derivatives of 1,10-phenanthroline, known to generate very strong complexes with Fe²⁺.^[12] Cryo-TEM analysis of the cylindrical micelles, stacked-membranes or multilamellar vesicles, formed within seconds following addition of Fe²⁺ to engineered-micelles, provides strong support for this conclusion.^[13] Finally, phase separation is accomplished with detergent concentration in the vicinity of the cmc and in the absence of precipitant.

Purification of bacteriorhodopsin (bR) with conjugated *engineered-micelles*

Purification of bR, embedded within purple membranes, from the *E. coli* lysate added as an artificial background, was accomplished with bathophenanthroline, Phen-C10 or Phen-C8 in combination with each of the non-ionic detergents: OG, OTG, NG, DM or DDM. The procedure was performed in three steps. In the first step, conjugated *engineered-micelles*, containing one of the above-listed detergents and one of the hydrophobic chelators, were prepared. Conjugation was induced by addition of Fe²⁺ (at 4°C) and resulted in the formation of a distinct (red) detergent aggregate. Immediately thereafter, a mixture of bR and the *E. coli* lysate was added. Gentle agitation at 4°C in the dark followed by a short (2 min.) low spin centrifugation

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3 generated red pellets for all [detergent/chelator/ Fe^{2+}] combinations and a clear
4 colorless supernatant.
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8 SDS-PAGE analysis of the pellets showed that they all contained bR. However, yield
9 and purity differed among the [detergent:chelator] combinations (Figure 2, A).
10 Highest recovery yields (by densitometry) were obtained with the two synthesized
11 phenanthroline derivatives: Phen-C10 (45-86%) and Phen-C8 (60-85%), whereas the
12 commercial chelator bathophenanthroline was less efficient (27-75%) (Figure 2, A-C).
13 More than 90% of the contaminating background was removed by all [detergent :
14 chelator] combinations as determined by densitometry. The highest purity, on
15 average, was obtained with Phen-C10 and the least with bathophenanthroline
16 (Figure 2, A-C). Analysis of detergent aggregates devoid of protein demonstrated
17 that they migrate at the front of the gels and are also stained with coomassie brilliant
18 blue (see red arrows in Figure 2, A-C, lanes 3-8).
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28 **Absorption spectrum of bR after partitioning into conjugated *engineered-*** 29 ***micelles* and pellet dissolution** 30 31

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33 The absorption spectrum of bR was measured after partitioning into different
34 conjugated *engineered-micelles* and following pellet dissolution (Figure 3, A). Micellar
35 conjugation was carried out with the [(Phen-C10)₃ : Zn^{2+}] complex to prevent the
36 overlapping absorption of the [(phenanthroline)₃ : Fe^{2+}] red complex. Efficient pellet
37 dissolution was observed in the presence of EDTA (50mM) and imidazole (200mM).
38 Characteristic absorption (at 550-565nm) of the native bR was observed with all five
39 non-ionic detergents (Figure 3, A).
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49 **Purification of halorhodopsin from *Natronomonas pharaonis* (phR) with** 50 **conjugated *engineered-membranes*** 51

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53 Purification of phR proteins embedded within their native membranes was obtained
54 with three hydrophobic chelators (bathophenanthroline, Phen-C10 or Phen-C8) in the
55 presence of ZnCl_2 without any added detergent (Fig. 4A). We found that phR-
56 containing membranes could be efficiently precipitated after a brief incubation (2 min)
57 with one of the above hydrophobic chelators followed by the addition of Zn^{2+} . SDS-
58 PAGE analysis of the resulting purple pellets showed that most of the impurities due
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3 to the *E.coli* lysate background (>85% by densitometry) were removed by all three
4 chelators tested. However, the two synthesized chelators (Phen-C10, Phen-C8) were
5 found to lead to purer samples than the commercially available bathophenanthroline
6 (Figure 4B, lanes 4-5 vs. 3). The presence of EDTA (10-50mM) after the addition of
7 Zn^{2+} and during the washing step of the pellet was found to substantially increase
8 pHr purity and therefore was used in all experiments. Similar results were also
9 observed with $FeSO_4$ under identical conditions (not shown). Recovery yields of pHr
10 with bathophenanthroline, Phen-C10 and Phen-C8 were: 82-89%, 74-79% and 31-
11 38%, respectively (by densitometry) (Figure 4B, lanes 3-5).
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21 **Absorption spectrum of pHr following pellet dissolution**

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23 The absorption spectrum of pHr was measured in *engineered-membranes*, conjugated with
24 the [(bathophenanthroline)₃:Zn²⁺] complex, and following pellet dissolution by each of 4
25 different nonionic detergents (Figure 5, A-D). Characteristic absorption of the native pHr at
26 578nm and the absorption of the carotenoid chromophore including its fine structure, were
27 essentially preserved with all four non-ionic detergents tested (Figure 5, A-D). The purity of
28 bR or pHr could not be assessed by measurement of the 280:568nm or 280:578nm
29 ratios respectively, since all detergent aggregates contain hydrophobic chelators that
30 absorb at 280nm.
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Discussion

The ability of conjugated *engineered-micelles* to phase separate at low temperature (4°C) and under mild conditions that do not require high detergent concentration, water-soluble polymers (e.g. PEG) nor precipitants (e.g. ammonium sulfate), motivated us to assess the generality of such a procedure for the purification of bR (Figure 1, A).

Micelles composed of five non-ionic detergents, commonly used in the purification of membrane proteins (OG, OTG, NG, DM and DDM), were studied. These were first transformed into the corresponding *engineered-micelles* by brief incubation with one of four hydrophobic metal chelators (bathophenanthroline, Phen-C10, Phen-C8 and Phen-C6), followed by specific conjugation with Fe²⁺. Light microscopy images resulting from such an experiment with OG micelles reveal red oily globules that were observed with all chelators except for Phen-C6 (Figure 1, B). Apparently the alkyl tail of Phen-C6 is too short to support efficient partitioning into OG micelles. Therefore, further investigations did not include Phen-C6. Similar images were observed with the other four detergents (data not shown).

The hydrophobic nature of the red oily globules suggested that bR, being a MP, would partition into them whereas water soluble proteins would not. Moreover, we hypothesized that partitioning could take place while bR is embedded within its native lipid bilayer. That would constitute a single-step method to direct bR from its membrane environment into diverse detergent aggregates. Such a scenario would provide a distinct advantage over current phase separation methods (*i.e. cloud point extraction*), which rely on two sequential steps: (i) extraction of MPs from the membrane with appropriate detergents and the formation of stable protein detergent complexes (PDCs), followed by (ii) aggregation of PDCs and formation of a detergent rich phase.¹⁴ To test the above hypothesis, conjugated *engineered-micelles* were incubated with *E. coli* lysate (serving as an artificial contamination background) and purple membranes containing the membrane protein, bacteriorhodopsin (bR). bR is an intrinsic small (26kDa) MP, that functions as a light driven proton pump in *Halobacterium salinarum*.¹⁵ It is by far the major membrane protein in purple membranes (Figure 2, A-C lanes 2) and the artificial impurity background was required to demonstrate process efficiency (*i.e.* removal of hydrophilic proteins).

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3 We found that gentle agitation at 4°C of bR-containing purple membranes with
4 different [detergent : chelator : Fe²⁺] combinations, generated red pellets after a short
5 low-spin centrifugation step (985xg) and that these contained the target membrane
6 protein bR as determined by SDS-PAGE analysis (Figure 2, A-C, lanes 3-7). In
7 addition, most water-soluble proteins (>90%, by densitometry) were excluded from
8 the pellets, regardless of the combination used. These results show that bR partitions
9 directly from its native membrane into conjugated *engineered-micelles* whereas the
10 large majority of soluble proteins present in the lysate, do not. Surprisingly, the
11 chemical structure of the hydrophobic chelator was found to have a marked effect on
12 the efficiency of the purification process. The lowest yields and purity were obtained
13 with the commercially available bathophenanthroline, whereas the two synthesized
14 derivatives, Phen-C10 and Phen-C8, led to higher recovery yields and purity (Figure
15 2, A-D). In particular, Phen-C10 excluded more than 95% of the impurities (by
16 densitometry) and also appeared to be superior to Phen-C8 with respect to purity
17 (Figure 2, B vs. C). These results point to the pivotal role of the hydrophobic anchor
18 of the chelator moiety (1,10-phenanthroline). The hydrophobic anchor affects both
19 purity and yield and single aliphatic tails containing 8 or 10 carbons are favored over
20 the two phenyl groups present in bathophenanthroline (Figure 1, B). Therefore,
21 further process optimization may require defining the ideal chain length, regardless of
22 the chelating moiety used.
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37 Phen-C10 was chosen as the preferred chelator since it led to the highest purity
38 (Figure 2, B) and moderate recovery yields (Figure 2, D). Even though micellar
39 conjugation was accomplished near the cmc values of the detergents studied, upon
40 conjugation the detergent concentration within the aggregates increases significantly.
41 It was thus essential to assess whether bR had experienced a denaturation process,
42 and therefore the absorption spectra of bR were recorded following the purification
43 process. In this case, conjugation was induced by Zn²⁺ so as to circumvent the
44 overlapping absorption of the [(Phen-C10)₃ : Fe²⁺] red complex (Figure 3, B), with that
45 of bR. The results show that the native state of bR was not significantly perturbed
46 during purification and only minor deviations from the absorption maximum (568nm)
47 in the native membranes were observed (Figure 3, A). These may likely be attributed
48 to the presence of detergents as has been found for example with: OG¹⁶, OTG¹⁷ and
49 NG.¹⁸ Interestingly, efficient pellet dissolution also required the presence of EDTA
50 (50mM) and imidazole (200mM), presumably because these can compete with the
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3 hydrophobic chelator for the chelated Zn^{2+} and thus promote more efficient
4 disintegration of engineered-micelles.
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9 Current findings demonstrate that the detergent-based membrane protein purification
10 method presented here benefits from the following features. **(a)** Micellar aggregates
11 are formed at low detergent concentrations, close to the cmc values of the detergent
12 used. **(b)** The utilization of a single detergent and the absence of polymers or
13 precipitants throughout the process, simplifies protein isolation. **(c)** Purification is
14 conducted at low temperature (4°C) thus making the approach applicable to heat-
15 sensitive membrane proteins. **(d)** Exclusion of most impurities (90-95%) in a single
16 step is an advantageous starting point for any additional chromatography. **(e)** The
17 red color of the [(phenanthroline)₃ : Fe²⁺] complex serves as an indicator for the
18 process efficiency, and its presence in the supernatant serves as a marker for
19 *engineered-micelles* that did not precipitate. Loss of protein after washing the pellet
20 can be determined accordingly. **(f)** bR partitions directly from its native membrane
21 into the detergent aggregate, thus circumventing an intermediate step required in
22 different phase separation approaches. MPs are first extracted from the membrane
23 with detergents and only then PDCs are induced to aggregate into a detergent rich
24 phase. **(g)** The process is rapid (~15-30min) and requires only simple
25 instrumentation (vortex + table centrifuge). Finally, removal of the hydrophobic
26 chelator from the protein may be accomplished in a similar manner to the way
27 detergents are exchanged for other detergents while the target protein is bound to
28 the chromatographic media.
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43 A significant drawback inherent to the conjugated-micelle method of bR purification is
44 the locally high detergent concentration within the aggregate which can induce
45 irreversible denaturation of some membrane proteins. To overcome this problem, we
46 developed a complementary strategy that does not include detergent and maintains
47 the target membrane protein within its native bilayer throughout the purification
48 process. This strategy relies on our previous observations that purple membranes
49 can be conjugated in the presence of diverse metals and hydrophobic chelators,
50 while bR remains in its native functional state.¹⁹ We therefore applied similar
51 conjugation conditions to aqueous suspensions of native, pH_R-containing
52 membranes in a background of *E. coli* lysate. pH_R has shown itself to be sensitive to
53 high detergent concentration (not shown).
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3 The phR used in this study derives from *Natronobacterium pharaonis* and is an
4 inward-directed light driven chloride pump.²⁰ It belongs to a subfamily of 7
5 transmembrane integral membrane proteins (archaeal rhodopsins) and has
6 sequence homology to bR as well as to sensory rhodopsins. phR binds retinal *via*
7 K242 on helix G as a protonated Schiff base and absorbs green light with a
8 maximum wavelength of 578nm.²⁰ Photon absorption triggers its photocycle by
9 inducing isomerization of the retinal *all-trans* isomer to the 13-cis. It was expected
10 that partitioning of the highly hydrophobic chelators: bathophenanthroline, Phen-C10
11 or Phen-C8 into membranes containing phR would transform the latter into the
12 corresponding *engineered-membranes* and allow their conjugation with an
13 appropriate metal (Figure 4, A).
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22 Indeed, SDS-PAGE analysis of pellets comprised of conjugated *engineered-*
23 *membranes* were found to contain the target phR whereas most of the *E. coli*
24 impurities (>85%, by densitometry) were excluded (Figure 4, B). These results
25 demonstrate that separation of a membrane protein from soluble proteins can be
26 achieved in the absence of detergent thereby preventing potential denaturation of the
27 target membrane protein. Whereas the commercially available bathophenanthroline
28 led to higher recovery yields (82-89%, by densitometry) the two synthesized
29 chelators, Phen-C10 and Phen-C8, led to 74-79% and 31-38% recovery,
30 respectively, but provided greater purity (Figure 4, B lanes 3-5). The greater purity of
31 the synthesized derivatives is consistent with the results obtained with *engineered-*
32 *micelles* and shows that the structure of the chelator used is a major factor in the
33 purification process affecting both purity and yield. We found that the pellets of the
34 conjugated phR-containing membranes could be readily dissolved with relatively low
35 concentrations of several non-ionic detergents (e.g. 8mM DDM, 14mM DM, 18mM
36 OTG and 46mM OG) while maintaining phR in its native state (Figure 5, A-D).
37 Measurement of the absorption spectra of the dissolved pellets showed that the
38 characteristic absorption of the retinal moiety at 578 nm is preserved as well as the
39 ratio of the beta-carotene and the retinal chromophores (2.2 :1, respectively).
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52 Finally, the finding that precipitation of the membrane proteins embedded within their
53 bilayers takes only a few minutes under mild conditions, appears to be a valuable
54 asset for proteomic studies: samples containing both membrane and soluble proteins
55 could be rapidly and efficiently separated prior to 2D gel electrophoresis, thus
56 reducing the number of spots per gel and simplifying the analysis.
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Experimental

Materials

Bathophenanthroline, octyl β -D-glucoside (OG), octyl β -D-1-thiogluconate (OTG), nonyl- β -D-glucoside (NG), decyl β -D-maltoside (DM), dodecyl β -D-maltoside (DDM), Sodium dodecyl sulfate (SDS), NaCl, FeSO₄, were obtained from Sigma-Aldrich (St. Louis, MO).

Synthesis of hydrophobic phenanthroline derivatives (Phen-C6, Phen-C8 and Phen C-10).

To a vigorously stirred saturated aqueous solution of NaHCO₃ (18 mL) at 0 °C was added 1,10-phenanthroline-5-amine (400 mg, 2.05 mmol) followed by hexanoyl chloride (0.350 mL, 337 mg, 2.5 mmol) or octanoyl chloride (0.432 mL, 407 mg, 2.5 mmol) or decanoyl chloride (0.515 mL, 477 mg, 2.5 mmol). After continued stirring till the completion of the reaction (3 h, monitored by TLC), the mixture was extracted with ethyl acetate (3 × 10 mL). The organic layer was then treated with a few drops of pyridine and washed successively with 5% aqueous HCl (10 mL), 5% NaHCO₃ (10 mL) and water until neutral pH was achieved. The organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo* to provide the crude amide which was purified by silica gel column chromatography.

Characterization of phenanthroline derivatives

N-(1,10-Phenanthroline-5-yl)hexanamide (Phen-C6): off-white solid; yield 490 mg, 82%; mp 72-74 °C; IR (KBr, cm⁻¹) 3261 (vs), 2954 (s), 2926 (vs), 2851 (m), 1660 (s), 1535 (vs), 1424 (m), 1318 (w), 1235 (w), 1190 (w), 1105 (w), 878 (w), 802 (w), 739 (s); ¹H NMR (CDCl₃, 500 MHz) δ 9.05 (d, *J* = 4.3 Hz, 1H), 8.99 (d, *J* = 4.3 Hz, 1H), 8.48 (d, *J* = 8.4 Hz, 1H), 8.32 (d, *J* = 8.0 Hz, 1H), 7.98 (s, 1H), 7.78 (dd, *J* = 8.4, 4.3 Hz, 1H), 7.69 (dd, *J* = 8.0, 4.3 Hz, 1H), 2.60 (t, *J* = 7.5 Hz, 2H), 1.79 (quint, *J* = 7.5 Hz, 2H), 1.45 (m, 4H), 0.98 (t, *J* = 6.9 Hz, 3H); ¹³C NMR (CDCl₃, 125 MHz) δ 176.2, 151.0, 150.7, 147.1, 145.3, 137.7, 133.1, 133.0, 129.9, 126.8, 125.1, 124.5, 123.0, 37.6, 32.8, 26.8, 23.7, 14.5; MS (ES⁺, Ar) *m/z* (rel intensity) 295 ([MH+1]⁺, 28), 294 (MH⁺, 100); HRMS (ES⁺, Ar) calcd for C₁₈H₂₀N₃O (MH⁺) 294.1606, found 294.1615.

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3 **N-(1,10-Phenanthroline-5-yl)octanamide (Phen-C8):** off-white solid; yield 505 mg,
4 77%; mp 83-85 °C; IR (KBr, cm⁻¹) 3259 (vs), 2954 (m), 2925 (s), 2851 (m), 1659 (vs),
5 1535 (vs), 1424 (m), 1312 (w), 1275 (w), 1236 (w), 1190 (w), 1107 (w), 972 (w), 878
6 (w), 802 (w), 739 (m); ¹H NMR (CDCl₃, 400 MHz) δ 8.94 (d, *J* = 4.3 Hz, 1H), 8.87 (d,
7 *J* = 4.3 Hz, 1H), 8.35 (d, *J* = 8.1 Hz, 1H), 8.11 (d, *J* = 7.9 Hz, 1H), 7.77 (s, 1H), 7.61
8 (dd, *J* = 8.1, 4.3 Hz, 1H), 7.54 (dd, *J* = 7.9, 4.3 Hz, 1H), 2.55 (t, *J* = 7.4 Hz, 2H),
9 1.76-1.71 (m, 2H), 1.29-1.40 (m, 8H), 0.90 (t, *J* = 5.9 Hz, 3H); ¹³C NMR (CDCl₃, 100
10 MHz) δ 176.0, 150.7, 150.4, 146.7, 144.8, 137.4, 132.8, 132.6, 129.5, 126.4, 124.8,
11 124.2, 122.6, 37.6, 33.0, 30.5, 30.2, 27.0, 23.8, 14.6; MS (ES⁺, Ar) *m/z* (rel intensity)
12 323 ([MH+1]⁺, 30), 322 (MH⁺, 100); HRMS (ES⁺, Ar) calcd for C₂₀H₂₄N₃O (MH⁺)
13 322.1919, found 322.1907.
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24 **N-(1,10-Phenanthroline-5-yl)decanamide (Phen-C10):** was characterized
25 previously.¹⁰
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31 Preparation of conjugated *engineered-micelles* (general protocol)

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33 *Engineered-micelles* composed of non-ionic detergents were prepared by addition of
34 5μl aliquots of freshly prepared 20mM bathophenanthroline (in methanol) into
35 aqueous solutions containing either: 11μl (200mM OG), 10μl (100mM OTG), 10μl
36 (80mM NG), 12.5μl (20mM DM), 10μl (10mM DDM) with vigorous vortexing. This
37 was followed by the addition of double distilled water (DDW) to a final volume of 35μl.
38 Conjugation of *engineered-micelles* was achieved by further addition of 50μl of
39 FeSO₄ (1mM) in NaCl (400mM).
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49 Purification of bR with conjugated *engineered-micelles* (general protocol)

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51 Into 85μl of freshly prepared conjugated *engineered-micelles* (85μl), *E. coli* lysate (7-
52 15μl) and purple membranes (1-2μl) containing bR at 7.5 mg/ml were added without
53 vortexing. The system was gently agitated with an orbital shaker (300 rounds per
54 minute) for 15-30 minutes at 4°C in the dark. Samples were then briefly centrifuged
55 for 2 minutes at 985xg; the majority of the supernatant (100 μl) was carefully
56 discarded; and the resulting pellet was briefly washed with cold 200mM NaCl (90μl).
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3 An additional spin (1 min. at 3935xg) was applied; the supernatant was removed; and
4 the pellet composition was analyzed by SDS-PAGE.
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10 **Purification of bR with conjugated *engineered-micelles* containing synthesized** 11 **phenanthroline derivatives (Phen-C8 and Phen-C10)** 12

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14 Purification was identical to the general protocol described above but Phen-C8 or
15 Phen-C10 (in MeOH) was present rather than bathophenanthroline.
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20 **Pellet dissolution** 21

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23 Pellets were dissolved by vortexing with 20 μ l imidazole (2M, pH = 7.5) and 20 μ l
24 EDTA (0.5M, pH=7.5) for 30 seconds. This was followed by further vortexing for 60
25 seconds with either 50mM OG, 50mM OTG, 20mM NG, 10mM DM, or 20mM DDM in
26 a final volume of 200 μ l. Samples were subjected to a short (3 minutes), low-spin
27 (2236xg) step which led to a purple supernatant and a very small, off-white
28 precipitate. Essentially, total dissolution was observed with pellets containing OG,
29 OTG, NG and DM, whereas pellets containing DDM led to partial dissolution.
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38 **Purification of Halorhodopsin (phR) with conjugated *engineered-membranes*** 39

40 Into an aqueous mixture containing: DDW (60 μ l), 0.8M NaCl (50 μ l), *E. coli lysate*
41 (20 μ l), hR (30 μ l of 0.2mg/ml), 10 μ l of either: bathophenanthroline, Phen-C10 or
42 Phen-C8 (all at 20mM in MeOH) were added with constant vigorous vortexing. After
43 a short incubation (2min.) at room temperature (or 4°C), 10 μ l of 50mM ZnCl₂ and
44 10 μ l of 0.5M EDTA (pH=7.5) were added sequentially with constant vortexing.
45 Samples were centrifuged for 3 minutes at 1537xg and the majority of the
46 supernatant (160 μ l) was discarded. Pellets were resuspended by addition of 160 μ l of
47 50mM EDTA (pH=7.5), centrifuged at 6150xg for 2min. and the supernatant
48 discarded. Washed pellets were then dissolved with sample buffer, by vortexing or by
49 crushing with a pipette tip, and analyzed by SDS-PAGE. For spectral analysis, pellets
50 were dissolved with 50-200 μ l of either: 14mM DM, 8mM DDM, 46mM OG or 18mM
51 OTG in DDW.
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Preparation of purple membranes

Halobacterium salinarum was grown from the S9 strain and purple membranes containing bacteriorhodopsin (bR) were isolated as previously described.²¹

Preparation of Halorhodopsin (pHR)

Halorhodopsin was produced from the mutant KM-1 strain of *Natronomonas pharaonis* as previously described by Ihara *et al.*²² In brief, the cells were grown under illumination for two weeks in a culture medium at pH 9 containing 1 g KH₂PO₄, 1 g KCl, 1 g NH₄Cl, 200 mg MgSO₄·7H₂O, 200 g NaCl, 1 g monosodium glutamate, 5 g casamino acids, 5 g yeast extract, 15 g Na₂CO₃, and trace metals (1 µL HCl (32%), 0.2 mg FeCl₂·4H₂O, 0.025 mg CoCl₂·6H₂O, 0.01 mg MnCl₂·4H₂O, 7 µg ZnCl₂, 0.6 µg H₃BO₃, 4 µg Na₂MoO₄·2H₂O, 7 µg NiCl₂·6H₂O, 0.2 µg CuCl₂·2H₂O, 2.5 µg AlCl₃, 0.6 µg Na₂WO₄·2H₂O, 2.5 µg AlCl₃, 0.6 µg Na₂WO₄·2H₂O). The cell pellets were obtained by centrifugation, suspended in basal salt medium and frozen overnight. The next day after thawing it was then treated with DNase at room temperature for few hours followed by dialysis for two days. Next the cell debris was discarded by fast centrifugation. The supernatant was collected and washed with 100 mM NaCl several times to remove the excess bacterioruberin. Finally the hR membranes were suspended in 100mM NaCl.

UV spectroscopy

Absorption measurements were performed using the HP 8453 UV-Vis spectrophotometer.

Light microscopy

Light microscope images were obtained using an Olympus CX40 microscope.

Densitometry

The density of bands in the gels was quantified using the ImageJ (NIH) image analysis program.

Conclusions

Following conjugation, *engineered-micelles* composed of non-ionic detergents, hydrophobic chelators and divalent cations were found to efficiently extract bacteriorhodopsin from the purple membrane while preserving its native state and functionality; the majority of hydrophilic macromolecules in the contaminating background were excluded. As an alternative procedure, the conjugation of membranes containing halorhodopsin succeeded in purifying the protein while avoiding exposure to high detergent concentrations and potential denaturation. Therefore, depending on the particular membrane protein, either *engineered-micelles* or *engineered-membranes* provide a practical route for purification: no sophisticated instrumentation is required (only vortex + table centrifuge); the process is relatively rapid, is performed under mild conditions (4°C) and is capable of removing the large majority of hydrophilic impurities prior to a final chromatographic step. Further improvements in process efficiency, purity and yield, may come from metal chelators, other than hydrophobic 1,10-phenanthroline derivatives (e.g. 8-hydroxyquinoline, catechol), and these are currently being explored.

Acknowledgments

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Graphical abstracts

Figure 1: (A) Schematic illustration of the purification process with engineered-micelles. Step I: Transformation of micelles composed of non-ionic detergents into *engineered-micelles* upon addition of hydrophobic chelators. Step II: Conjugation of *engineered-micelles* to produce detergent aggregates in the presence of Fe^{2+} but in the absence of precipitant or high salt concentration. Step III: Partitioning of membrane proteins, but not water soluble proteins, into the detergent aggregate. All three steps are performed at 4°C . **(B) Light microscope images of detergent aggregates formed in the presence of indicated metal chelators, OG micelles and Fe^{2+} .** Scale bar represents 0.1mm.

Figure 2: (A-C) SDS-PAGE analysis of detergent aggregate pellets containing the [detergent : chelator] combinations indicated. Lane 1: *E. coli* lysate; lane 2: bR within purple membranes; lanes 3-7: pellet composition; lane 8: pellet composition in the absence of the *E. coli* lysate / bR mixture. Red arrows point to stained detergent aggregates devoid of protein. Gels are coomassie stained. **(D) Average recovery yield of bR with different [detergent : chelator] combinations** (based on densitometry of at least 5 experiments).

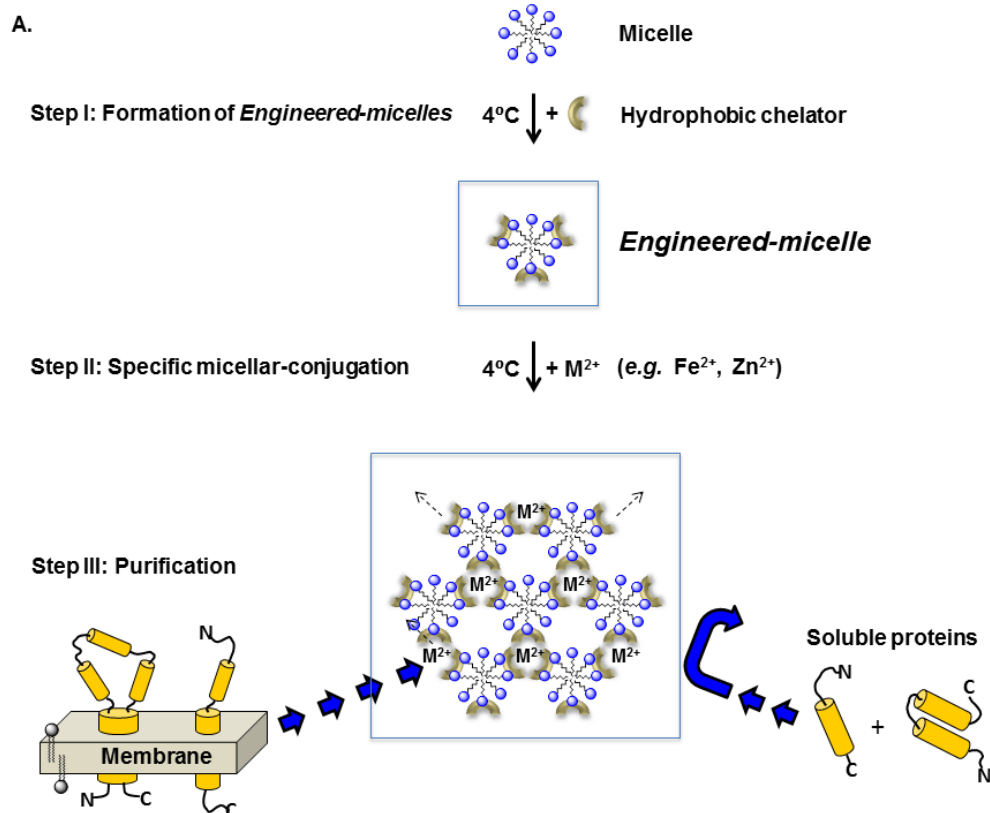
Figure 3: (A) Absorption spectra of bR after purification with conjugated *engineered-micelles* and following pellet dissolution (as described in the *Experimental* section). bR was purified with five different non-ionic detergents in the presence of the $[(\text{Phen-C10})_3 : \text{Zn}^{2+}]$ complex. **(B) Images of pellets.** Pellets composed of: [bR\ *E. coli*. mixture : OG : Phen-C10 : Fe^{2+}] (left) or Zn^{2+} (right) after low-spin centrifugation.

Figure 4: Schematic illustration of the purification process with engineered-membranes. **(A)** Spontaneous partitioning of hydrophobic chelators transform membranes containing an expressed membrane protein into engineered-membranes which are conjugated with metal ions (e.g. Zn^{2+} , Fe^{2+}). **(B) Purification of pHr with engineered-membranes.** Lane 1: *E. coli* lysate; lane 2: hR; lanes 3-5 pellet composition generated in the presence of hydrophobic chelators and Zn^{2+} . The red arrow points to pHr present within the membrane aggregate. Gels are coomassie stained.

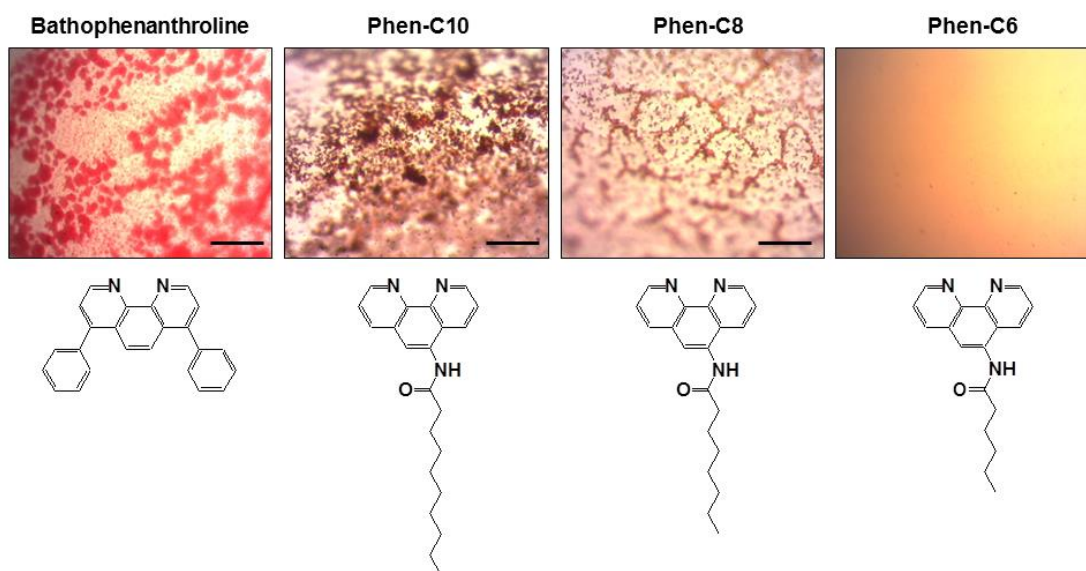
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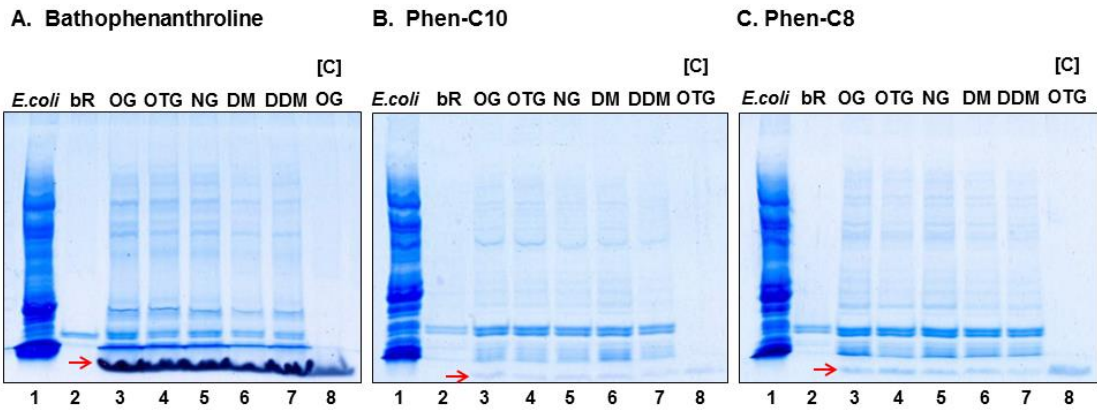
Figure 5: Absorption spectra of phR after purification with conjugated engineered-membranes and following dissolution (as described in the *Experimental* section). Red line - Native phR. Black line - phR after precipitation with the [(bathophenanthroline)₃ : Zn²⁺] complex and dissolution with: 14mM DM **(A)**, 8mM DDM **(B)**, 46mM OG **(C)** or 18mM OTG **(D)**.

Figures



B.





D.

Chelator \ Detergent	OG	OTG	NG	DM	DDM
Bathophenanthroline	70-75%	47-53%	67-71%	27-34%	35-41%
Phen-C10	79-86%	75-84%	71-79%	70-78%	45-57%
Phen-C8	78-85%	72-80%	78-85%	61-68%	60-67%

Figure 2

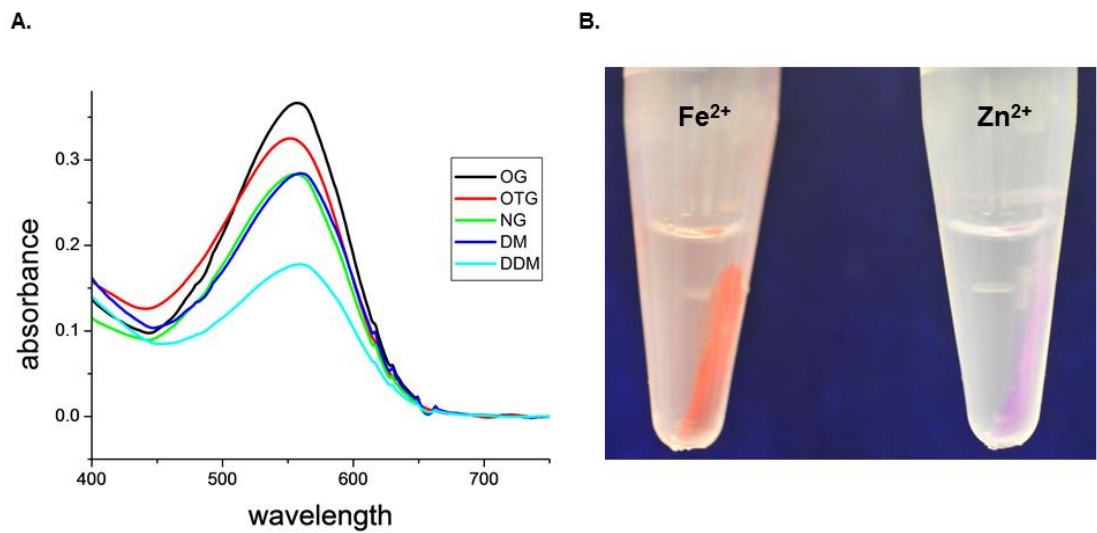


Figure 3

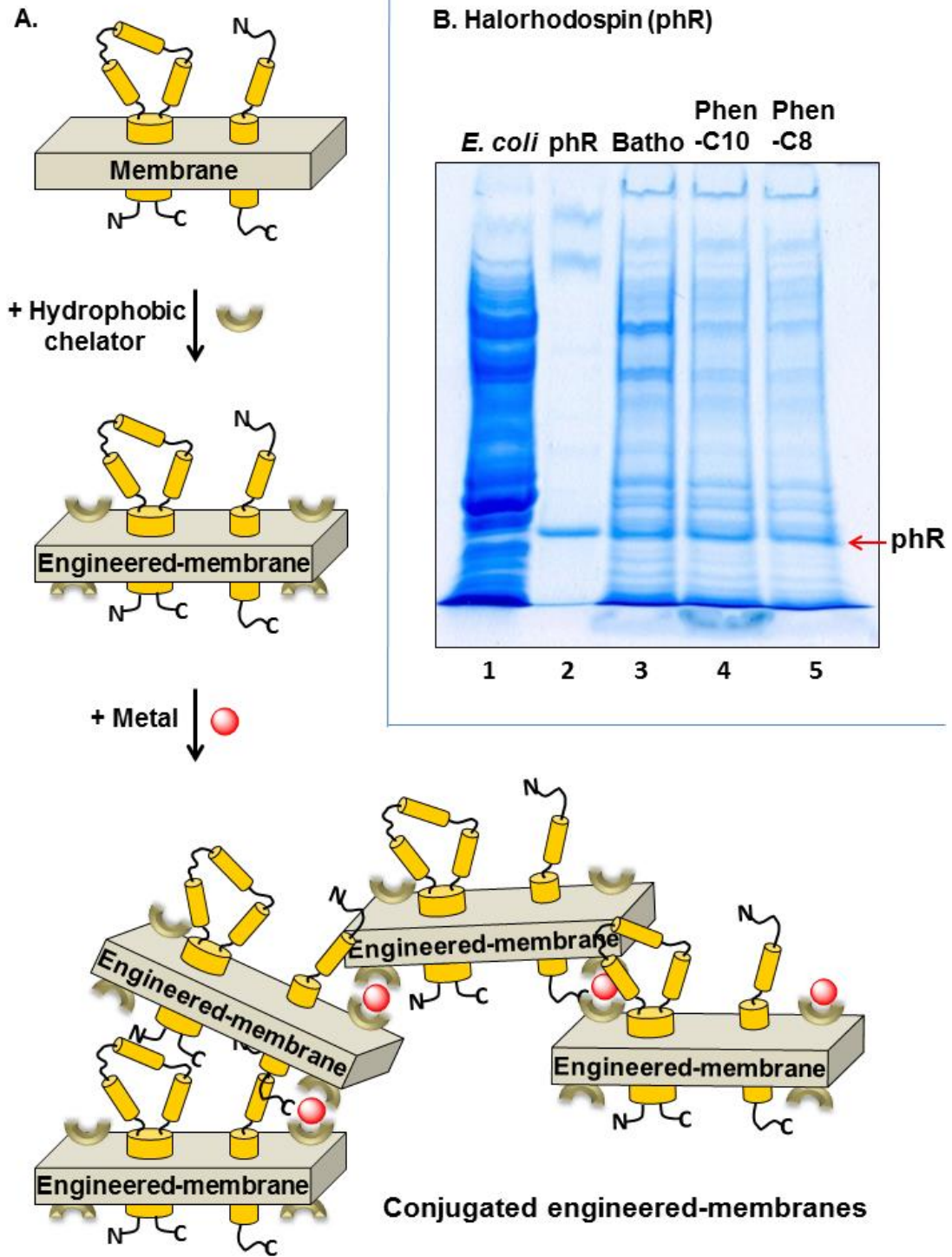


Figure 4

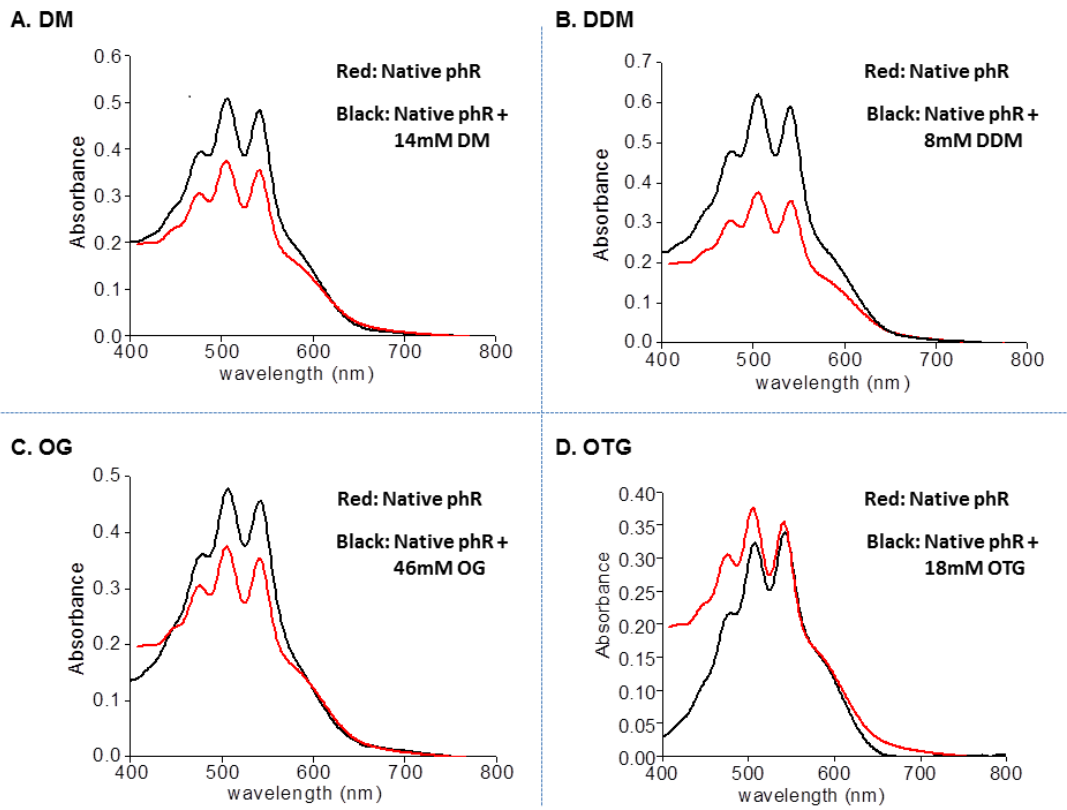


Figure 5

Bibliographic references and notes

- 1) R. M. Garavito and S. Ferguson-Miller, 2001, *J. Biol. Chem.*, **276**, 32403-32406.
- 2) P. J. Loll, 2003, *J. Struct Biol.*, **142**, 144-153.
- 3) C. Bordier, 1981, *J. Biol. Chem.*, **256**, 1604-1607.
- 4) W. L. Hinze and E. Pramauro, 1993, *Crit. Rev. Anal. Chem.*, **24**, 133-177.
- 5) P. A. Albertsson, 1973, *Biochemistry.*, **12**, 2525-2530.
- 6) M. A. Lukyanova and N. M. Petukhova, 1976, *Biochemistry (Mosc.)*, **41**, 1468-1475.
- 7) U. Sivars and F. Tjerneld, 2000, *Biochim. Biophys. Acta.*, **1474**, 133-146.
- 8) B. Fricke, 1993, *Anal. Biochem.*, **212**, 154-159.
- 9) C. R. Parish, B. J. Classon, J. Tsaqaratos, I. D. Walker, L. Kirszbaum and I. F. McKenzie, 1986, *Anal. Biochem.*, **156**, 495-502.
- 10) G. Patchornik, I. N. Namboothiri, D. K. Nair, R. Persky, 2012, *Soft Matter*, **8**, 8456-8463.
- 11) G. Patchornik, G. D. Danino, E. Kesselman, E. Wachtel, N. Friedman and M. Sheves, 2013, *Bioconj Chem.* **24**, 1270-1275.
- 12) R. M. Smith and A. E. Martell, in *Critical Stability Constants*, Plenum Press, New York and London, 1990, vol. 2, pp. 251–253.
- 13) G. Patchornik, E. Wachtel, E. Kesselman and D. Danino, 2014, *Soft matter* **10**, 4922-4928.
- 14) T. Arnold and D. Linke, 2007, *Biotechniques*, **43**, 427-440.
- 15) H. G. Khorana, 1993, *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 1166-1171.
- 16) N. A. Dencher and M. P. Heyn, 1978, *FEBS Lett.* **96**, 322-326.

1
2
3 17) Y. Gohon, T. Dahmane, R. W. Ruiqrok, P. Schuck, D. Charvolin, F. Rappaport,
4 P. Timmins, D. M. Engelman, C. Tribet, J. L. Popot and C. Ebel, 2008, Biophys
5 J. **94**, 3523-3537.
6
7

8
9 18) G. Q. Chen and E. Gouaux, 1999, Biochemistry, **38**, 15380-15387.
10

11 19) G. Patchornik, I. N. Namboothiri, D. K. Nair, E. Wachtel, S. R. Cohen, N.
12 Friedman and M. Sheves, 2012, J. Colloid Interface Sci. **388**, 300-305.
13
14

15
16
17 20) M. Kolbe, H. Besir, L. O. Essen and D. Oesterhelt, 2000, Science, **288**, 1390-
18 1396.
19

20
21 21) D. Oesterhelt, and D. Stoeckenius, 1974, Methods Enzymol. **31**, 667-678.
22
23

24
25 22) K. Ihara, A. Narusawa, K. Maruyama, M. Takeguchi and T. Kouyama, 2008,
26 FEBS Lett. **582**, 2931-2936.
27
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Abbreviations

NMR - nuclear magnetic resonance, MP - membrane protein; PDC - protein detergent complex, batho - bathophenanthroline; bR - bacteriorhodopsin, phR – halorhodopsin; cmc - critical micelle concentration; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Graphical Abstract

Table of Contents Graphic

