

Solubilization and Stabilization of Membrane Proteins by Cycloalkane-Modified Amphiphilic Polymers

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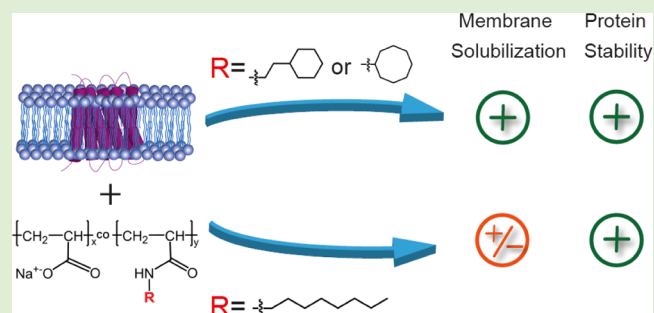
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ABSTRACT: Membrane proteins (MPs) need to be extracted from biological membranes and purified in their native state for most structural and functional *in vitro* investigations. Amphiphilic copolymers, such as amphipols (APols), have emerged as very useful alternatives to detergents for keeping MPs water-soluble under their native form. However, classical APols, such as poly(acrylic acid) (PAA) derivatives, seldom enable direct MP extraction. Poly(styrene maleic anhydride) copolymers (SMAs), which bear aromatic rings as hydrophobic side groups, have been reported to be more effective extracting agents. In order to test the hypothesis of the role of cyclic hydrophobic moieties in membrane solubilization by copolymers, we have prepared PAA derivatives comprising cyclic rather than linear aliphatic side groups (CyclAPols). As references, APol A8-35, SMAs, and diisobutylene maleic acid (DIBMA) were compared with CyclAPols. Using as models the plasma membrane of *Escherichia coli* and the extraction-resistant purple membrane from *Halobacterium salinarum*, we show that CyclAPols combine the extraction efficiency of SMAs with the stabilization afforded to MPs by classical APols such as A8-35.



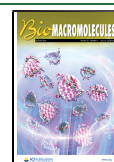
1. INTRODUCTION

As gatekeepers of all membrane compartments in cells, membrane proteins (MPs) fulfill essential biological functions and are key targets for drugs.^{1,2} Their importance motivates structural and biophysical studies, which, however, critically depend on extraction/purification procedures that keep MPs in their native state. Detergents can disrupt biological membranes and keep MPs water-soluble, but they often entail MP inactivation, in part due to their propensity to detach from MP endogenous lipids or cofactors needed for activity. This has prompted the development of macromolecular amphiphiles such as amphipols (APols),^{3–5} which were expected to be less aggressive dispersing agents and less prone to reach hidden hydrophobic regions in the protein. The prototypical APol, named A8-35, is a poly(acrylic acid) (PAA) polymer randomly modified with octylamine and isopropylamine side chains (Figure 1A). The mildness of APol association with MPs results in an increased lifetime of the native state of APol-complexed MPs.⁵ Another advantage of APols is the improvement of images in the fast-growing field of single-particle electron cryo-microscopy (cryo-EM) reconstitution.^{5,6} Conventional APols are however poor solubilizing agents for biological membranes, by far less efficient than most detergents.^{7,8} They can break liposomes (lacking MPs),^{9–11} but in practice, MP/APol complexes can rarely be directly obtained from biological membranes and must be prepared by

transferring the protein from detergent-solubilized extracts.^{5,12} Styrene maleic acid (SMA) copolymers (Figure 1B) are APol variants with the distinctive property of enabling direct dispersion of proteolipidic membranes with no need for detergent.^{13,14} They form lipid–protein–polymer mixed nanodiscs, called SMALPs. Spontaneous formation of lipid nanodiscs is not specific to SMAs as it has been observed with lipids and diisobutylene/maleic acid (DIBMA) copolymers (Figure 1C)¹⁵ and with copolymers similar to APols.¹⁶ Despite several limitations such as high UV absorbance, due to the presence of aromatic groups, and polydispersity of the discs,¹⁷ the use of SMAs has developed in the field of MPs, thanks to their solubilizing property. However, in the field of structural biology, the frequent need to use harsh extraction conditions may explain the low number of high-resolution cryo-EM structures established using SMAs.^{6,18} To date, only a small number of single-particle cryo-EM structures of SMALPs have been obtained.⁶ In comparison, despite the need for surfactant exchange, classical APols (mainly A8-35 and PMAL-C8)^{3,19,20}

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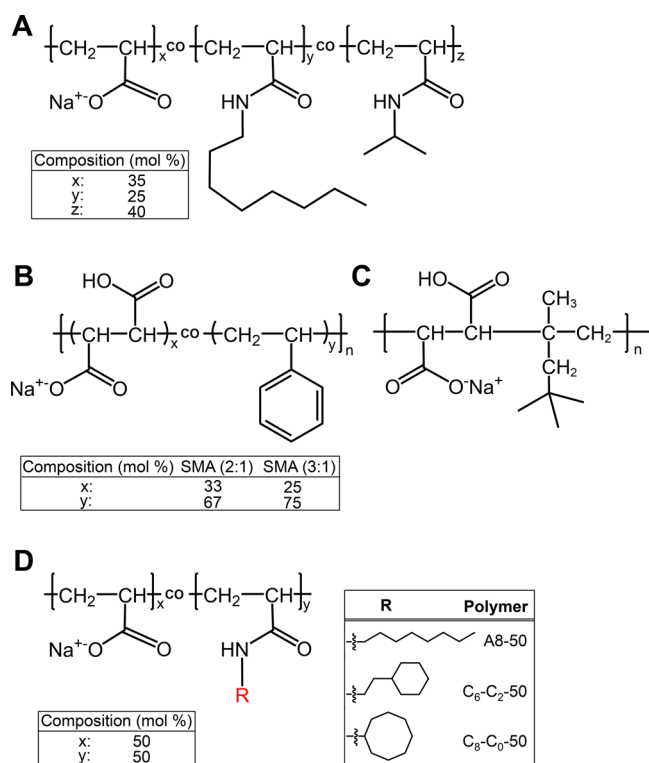


Figure 1. Chemical structures of amphipathic polymers used in this study. (A) A8-35; molar percentages of each unit are from ref 3. (B) SMA copolymers, with molar ratios of styrene to maleic acid units of 2:1 or 3:1 ($n \approx 9$).¹⁵ (C) DIBMA copolymer ($n \approx 37$).¹⁵ (D) PAA modified with either linear or cyclic hydrocarbon groups (R) of eight carbon atoms. The \bar{M}_n values for C₆-C₂-50, C₈-C₀-50, and A8-50 (sodium salt form) were calculated using $\bar{X}_n \approx 35$ for the parent PAA²¹ and the degree of grafting, y , determined by ¹³C and ¹H NMR spectroscopy and potentiometric titration ($\sim 53 \pm 1$, $\sim 51 \pm 3$, and $\sim 56 \pm 4$ mol % for C₆-C₂-50, C₈-C₀-50, and A8-50, respectively; see Figures S1–S7 in the Supporting Information and Table 1).

represent the most frequently used polymers.⁶ To circumvent the need for surfactant exchange, improving the direct solubilization of membranes by APol-like polymers under mild experimental conditions would be highly desirable.

We hypothesized that replacing the flexible *n*-alkyl side groups of classical APols by cyclic hydrophobic groups might possibly mimic the role of SMA's aromatic side groups. Of note, introducing cyclic groups in the aliphatic tail of dodecylmaltoside results in MP-stabilizing analogs.²² We therefore examined whether cyclic hydrocarbon groups grafted onto PAA might not (i) improve the polymers' ability to break up MP-containing membranes while (ii) preserving the long-term stability of MPs. To this end, we prepared copolymers, named CyclAPols, in which the linear octyl groups of A8-35-like APols were replaced by cyclic ones having the same

number of carbon atoms (either cyclohexylethylamine or cyclooctylamine, yielding the polymers C₆-C₂-50 and C₈-C₀-50, respectively, Figure 1D). Cycloalkanes were preferred over aromatic cycles in order to keep the CyclAPols UV-transparent. As references, we compared CyclAPols with SMA (2:1) and (3:1) of relatively short length,^{14,23,24} DIBMA, and polyacrylate-based APols with linear side groups, namely, A8-35 and A8-50 [an APol possessing a density of hydrophobic moieties of $\sim 50\%$, i.e., equal to that of SMA (2:1) and CyclAPols].

We studied the membrane-solubilizing properties of CyclAPols using as a model the plasma membrane of *Escherichia coli*, a host cell widely used for producing recombinant MPs.^{25,26} We expressed two different MPs, YidC of *E. coli* fused to a green fluorescent protein (GFP) and the bacteriorhodopsin (BR) of *Haloquadratum walsbyi* (HwBR). To further examine the solubilizing properties of CyclAPols, we used the native purple membrane from *Halobacterium salinarum*, which is well known for its resistance to solubilization by polymers, be they SMAs^{13,27} or classical APols.⁵ Finally, the thermal stability of BR from *Hb. salinarum* (HsBR) extracted with various APols was evaluated.

2. EXPERIMENTAL SECTION

2.1. Chemicals. DIBMA and *n*-dodecyl β -D-maltoside (DDM) were supplied by Anatrace. As indicated by the supplier, DIBMA was a purified version of Sokalan CP9 from BASF. SMA (2:1) and SMA (3:1) were obtained from ProFoldin (SMA21S) and Polyscope (Xiran SL25010 P20), respectively. The number-average molecular masses (\bar{M}_n) of the DIBMA and SMA (3:1) copolymers were ~ 8400 and ~ 4000 g·mol⁻¹, respectively.¹⁵ The lipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids.

2.2. Synthesis of APols and Composition Determination. The synthesis of A8-35 was performed as previously reported.³ APol derivatives carrying either octylamine, cyclohexylethylamine, or cyclooctylamine were obtained after a single step of PAA modification. Briefly, the hydrophobic side chains were randomly grafted onto PAA of $\bar{M}_w \sim 5.5$ kDa in *N*-methylpyrrolidone in the presence of dicyclohexylcarbodiimide. In each case, the resulting polymer obtained under the acidic form was purified by four cycles of precipitation/solubilization in water, performed at acidic and basic pH, respectively. The final polymer solution adjusted at pH 8–9 was dialyzed against deionized water and freeze-dried, yielding the expected APol. After synthesis, the final yields of APols were 98 and 96% for the CyclAPols C₆-C₂-50 and C₈-C₀-50, respectively, and 86 and 52% for the APols of reference A8-35 and A8-50, respectively.

The grafting ratio of hydrophobic side chains was determined by ¹³C and ¹H NMR spectroscopy (Figures S1–S6) and potentiometric titration in a water/ethanol mixture (20:80, v/v) (Figure S7). The ¹³C NMR spectra of polymers, at 100 g·L⁻¹ in deuterated methanol, were obtained after a long acquisition time (48–60 h) due to the long delay between each frequency pulse required for a complete relaxation of all carbon nuclei. The average degree of polymerization (\bar{X}_n) of the commercial PAA used in this study for synthesizing A8-35 and the

Table 1. Chemical Compositions and Number-Average Molecular Masses of Polyacrylate-Based APols^a

APol	CO ₂ ⁻ (x_1/x_2 , %)	hydrophobic groups (y_1/y_2 , %)	x (%)	y (%)	\bar{M}_n (g·mol ⁻¹)
C ₆ -C ₂ -50	47/46	53/54	46.5 ± 0.5	53.5 ± 0.5	4919
C ₈ -C ₀ -50	52/46	48/54	49 ± 3	51 ± 3	4843
A8-50	47/40	53/60	43.5 ± 3.5	56.5 ± 3.5	5050

^aThe percentages of grafts and free carboxylates were deduced from an average of ¹H and ¹³C NMR analysis (yielding y_1 and x_1) and from acid–base titrations (yielding y_2 and x_2) for each APol. The values were averaged, giving the final x and y ratios. For the APol of reference A8-35, the averaged final ratios were $x = 34\%$, $y = 23\%$, and $z = 43\%$, and the \bar{M}_n value was 4276 g·mol⁻¹.

APol derivatives was previously determined to be close to ~ 35 by size exclusion chromatography (SEC) analysis in an organic solvent.²¹ The number-average molar masses (\bar{M}_n) of sodium salts of APol derivatives were calculated with the \bar{X}_n value and the grafting ratio (γ) averaged from ^{13}C and ^1H NMR spectroscopy and pH-titration determination (Table 1).

2.3. Expression of YidC-GFP of *E. coli* and BR of *Hq. walsbyi* in the *E. coli* Strain and Quantification of Protein Extraction Using Polymers. The expression vectors encoding for YidC of *E. coli* fused at its C-terminus to GFP (YidC-GFP) and BR of *Hq. walsbyi* (*HwBR*) were kindly provided by the laboratories of Jan-Willem de Gier (Stockholm University) and Anant Menon (Weill Cornell Medical College), respectively. The strains *E. coli* C41 and C43 (DE3) were transformed with each vector, respectively, and growth at 37 °C in Luria Bertani broth in the presence of either ampicillin or kanamycin. When the optical density at 600 nm reached 0.6–0.8, the cultures were supplemented with isopropyl β -D-1-thiogalactopyranoside. In the case of *HwBR* production, 5 μM all-trans retinal (Sigma) was also added. After induction, the cells were harvested. The pellet of each culture was resuspended with a buffer containing 20 mM Tris–HCl, 150 mM NaCl, pH 8.0, and cell lysis was performed with a cell disruptor (Constant Systems). Unbroken cells and cellular debris were removed by centrifugation (20 min at 10,000 $\times g$). The membranes were harvested from supernatants by high-speed centrifugation (1 h at 100,000 $\times g$). The concentration of total MPs present in the membrane extracts was assessed with a calorimetric assay.

The solubilization of YidC-GFP from *E. coli* membranes was performed at a total MP concentration of 2 $\text{g}\cdot\text{L}^{-1}$ with either 1% w/v DDM, used as a reference condition, or 2 $\text{g}\cdot\text{L}^{-1}$ polymers, corresponding to a total MP/polymer ratio of 1:1 (w/w). After 1 h incubation at 4 °C, the samples were centrifuged for 30 min at 100,000 $\times g$. The supernatants and the pellets, resuspended in the initial volume of solubilization with a buffer containing 20 mM Tris–HCl, 150 mM NaCl, pH 8.0 and 5% sodium dodecyl sulfate (SDS), were loaded on 12% acrylamide gels. After migration, the gels were washed with water, and the fluorescence of GFP within the gel was detected with a Typhoon TLA 9500 (GE healthcare). The excitation and emission wavelengths were those of Alexa Fluor 488, that is, 495 and 519 nm, respectively. The fluorescence of the band corresponding to YidC-GFP visible on the acrylamide gels was quantified using ImageJ software. The fluorescence of YidC-GFP measured for each supernatant was normalized with the fluorescence quantified for the 1%-DDM supernatant, which was defined as the maximum of solubilization. For the kinetics of YidC-GFP extraction, the solubilization was performed under the same experimental conditions except that aliquots taken off from samples were centrifuged for 10 min at 250,000 $\times g$.

In the case of *HwBR*, the solubilization was carried out with both pure *E. coli* membranes and *E. coli* membranes after fusion with 100 nm extruded DMPC liposomes, prepared in 20 mM Tris–HCl, 150 mM NaCl, pH 8.0, by sonication in the dark for 30 min using a bath sonicator (VWR). The total MP/DMPC ratio was 1:0.5 (w/w), as previously reported.²⁸ The membranes were then supplemented with either 0.2% w/v DDM or polymers at a final concentration of 2 $\text{g}\cdot\text{L}^{-1}$, corresponding to a total MP/polymer ratio of 1:1 (w/w). After incubation for 4 h at room temperature, the samples were centrifuged for 30 min at 100,000 $\times g$, and *HwBR* present in the supernatants was assessed with absorbance at 554 nm.

2.4. Quantification of Native BR Extracted from the DMPC-Fused Purple Membrane by Polymers. The purple membrane was purified from *Hb. salinarum* as described.²⁹ The concentration of native BR of *Hb. salinarum* (*HsBR*) was adjusted to 5 $\text{g}\cdot\text{L}^{-1}$ with the buffer 20 mM sodium phosphate, 100 mM NaCl, pH 7.0, and the membrane preparation was stored at -80 °C until usage.

Fusion of the native purple membrane with DMPC liposomes was performed as reported.^{15,27} Briefly, an aliquot of the purple membrane was mixed with 100 nm extruded DMPC liposomes to reach a final *HsBR*/DMPC ratio varying from 1:3 to 1:6 (w/w). The samples, at a *HsBR* concentration of 0.5 $\text{g}\cdot\text{L}^{-1}$, were sonicated in the dark for 30

min using a bath sonicator (VWR) and then stored at -80 °C until usage.

Solubilization was initiated by mixing at a 1:1 volume ratio DMPC-fused purple membrane with polymer solutions to reach a final *HsBR*/polymer ratio varying from 1:3 to 1:9 (w/w). The samples were incubated overnight in the dark at 25 °C under shaking in a thermomixer. After an overnight incubation, all the samples were centrifuged at 4 °C for 20 min at 200,000 $\times g$. The UV–visible spectra of supernatants were recorded with a spectrophotometer (Hewlett Packard). The quantification of extracted *HsBR* was evaluated with the ratio of absorbance values at 554 nm measured before and after centrifugation.

Because APols and SMAs are polydisperse polymers, the representation of the molar ratios of *HsBR*/macromolecular chains depends on the weighted average of the polymer mass. Here, we used the number-averaged experimental values of \bar{M}_n . For *HsBR*/polymer weight ratios of 1:3 and 1:9 (w/w), the corresponding molar ratios of *HsBR*/ $\text{C}_8\text{--C}_0\text{-50}$ are $\sim 1:16.7$ and $\sim 1:50.3$, respectively. For SMA (3:1), the average molar ratios of *HsBR*/SMA correspond to $\sim 1:20.3$ and $\sim 1:60.8$, respectively. At the (w/w) *HsBR*/DMPC ratio fixed to 1:5, the (w/w) *HsBR*/polymer ratios of 1:3 and 1:9 correspond in addition to molar ratios of DMPC/cycloalkane (from $\text{C}_8\text{--C}_0\text{-50}$) of $\sim 1:1.6$ and $\sim 1:4.9$, respectively. For SMA (3:1), the molar ratios of DMPC/phenyl rings were similarly $\sim 1:2.8$ and $\sim 1:8.3$, respectively.

2.5. Dynamic Light Scattering. The scattering intensity was measured at 25 °C and an angle of 90° in a BI-200SM Brookhaven Instrument system equipped with a 30 mW 637 nm laser and photomultiplier detection. Size distributions from all samples showed a monomodal distribution as analyzed by CONTIN. Hydrodynamic diameters (D_H) were calculated by fitting the correlation functions by the cumulant analysis method at a quadratic order. For the kinetics of solubilization, a sample of DMPC-fused purple membrane (at 0.2 $\text{g}\cdot\text{L}^{-1}$ *HsBR*) was mixed with a polymer solution (1:1 v/v) to reach a final *HsBR*/polymer ratio of 1:12.5 (w/w).

2.6. Size Exclusion Chromatography. The solution dispersity of *HsBR*/DMPC/polymer complexes was characterized by SEC. After *HsBR* extraction, the samples were injected onto a Superose 12 10/300 GL column connected to an Äkta purifier-10 system (GE Healthcare). The sample and elution buffers contained 20 mM sodium phosphate, 100 mM NaCl, pH 7.0. Elution was carried out at room temperature and UV absorbance monitored at three wavelengths (220, 280, and 554 nm).

2.7. Stability Assay. Samples of *HsBR* (extracted directly from the DMPC-fused purple membrane with polymers) were incubated at 50 °C for 6 h in the dark in a thermomixer. UV–visible spectra of samples were recorded each hour. The decrease of the absorbance value at 554 nm reflects the loss of retinal and a change in the native conformation of the protein. The absorbance values were normalized with the initial one measured at 4 °C.

3. RESULTS

3.1. Synthesis of CyclAPols. The synthesis of the CyclAPol derivatives consists in the random grafting on a PAA backbone of cycloalkane moieties via the formation of amide bonds. The average degree of grafting was controlled quantitatively by the introduction of ad hoc percentages of cycloalkylamine reactants to reach values similar to the hydrophobic moieties in SMA (2:1), that is, 50 mol % (within the experimental error). The average percentages of grafting of cyclohexylethylamine and cyclooctylamine, yielding $\text{C}_6\text{--C}_2\text{-50}$ and $\text{C}_8\text{--C}_0\text{-50}$, respectively, were then determined by both ^{13}C and ^1H NMR spectroscopy and pH titration (Table 1). The resulting CyclAPols were highly soluble in aqueous solutions and UV-transparent (Figure S8).

The solubilizing properties of CyclAPols, assessed with biological membranes from two different origins, were compared with those of a set of five other polymers used as references: (i) the commercially available polymers SMA (2:1)

and SMA (3:1), which both carry cyclic aromatic rings as hydrophobic moieties with a density of 50 and 67 mol %, respectively, (ii) the commercially available polymer DIBMA, which carries short-length branched acyclic side groups present along the polymer at 33 mol %, and (iii) the polyacrylate-based APols A8-35 and A8-50, which carry linear octyl chains grafted at 25 and 50 mol %, respectively. In contrast to A8-50, the archetypical APol A8-35 also comprises isopropyl moieties at 40 mol %, whose role is to reduce its charge density. The number-average molar masses (\bar{M}_n) of CyclAPol derivatives, A8-50, A8-35, and SMA (3:1) are comparable. In terms of dispersity, CyclAPols, A8-50, and A8-35 were derived from the same PAA parent chain and, as a result, have the same polydispersity ($\bar{D} \approx 2.0$), whereas the polydispersity of SMAs is slightly broader (typically 2.0–2.5).²³ Regarding DIBMA, its \bar{M}_n value is larger, and its polydispersity is slightly narrower ($\bar{D} \approx 1.8$)³⁰ than that of the other copolymers used in this study.

3.2. Solubilization by CyclAPols of *E. coli* Plasma Membrane Fragments. The solubilizing properties of CyclAPols were tested with membrane fragments from *E. coli*. Two different MPs were expressed and used as target MPs to estimate the extraction efficiency of each polymer. The first protein was YidC of *E. coli*, an insertase whose transmembrane domain comprises six α -helices.³¹ A version of YidC fused at its C-terminus with GFP (YidC-GFP) was used as a reporter to quantify the solubilization yields. In Figure 2 are reported the amounts of polymer-extracted YidC-GFP recovered in the supernatants after 1 h of incubation at 4 °C at a fixed total MP/polymer ratio of 1:1 (w/w). Under these experimental conditions, the yields of YidC-GFP extracted with CyclAPols were the highest as compared to the references, which are A8-35, A8-50, the two SMAs, and DIBMA (Figure 2A). The amounts of CyclAPol-extracted YidC-GFP were almost twice that obtained with A8-50 (~70–75 vs ~40%). Monitoring the solubilization over 2 h incubation of the membranes with CyclAPols revealed a rapid increase of YidC-GFP fluorescence in supernatants before reaching a plateau after ~45 min (Figure 2B). A 1 h incubation suffices to reach the maximal extraction (Figure 2A), whereas extraction by either one of the two SMAs or by A8-35 progresses more slowly (Figure 2B).

The second MP expressed in *E. coli* and used as a reporter was the archaeobacterial BR of *Hq. walsbyi* (*HwBR*) produced under its native conformation as indicated by its spectroscopic signature. This protein is a light-driven proton pump, whose transmembrane domain folds in a bundle of seven α -helices.³² The amounts of *HwBR* extracted with A8-35 and SMA (2:1) were the lowest (Figure 3). In contrast to observations with YidC-GFP, the results obtained with *HwBR* revealed significant differences between A8-35 and the two SMAs. SMA (3:1) appeared to be more efficient at extracting *HwBR* from *E. coli* membranes than either SMA (2:1) or A8-35 (Figure 3). When solubilizing *E. coli* membranes with CyclAPols, the amounts of extracted *HwBR* were 2.5× higher than with SMA (3:1). As previously reported, the solubilization efficiency of SMA (3:1) can be improved by modulating the bilayer characteristics with the addition of the synthetic phospholipid DMPC.²⁸ This treatment increased the amount of SMA (3:1)-solubilized *HwBR* (+50%), which remains nevertheless below the amount extracted by CyclAPols (Figure 3B). Intriguingly, no significant differences between CyclAPols and A8-50 were noted, in contrast to observations with YidC-GFP. The kinetics of solubilization over 4 h incubation of the *E. coli* membranes with polymers revealed that the maximal

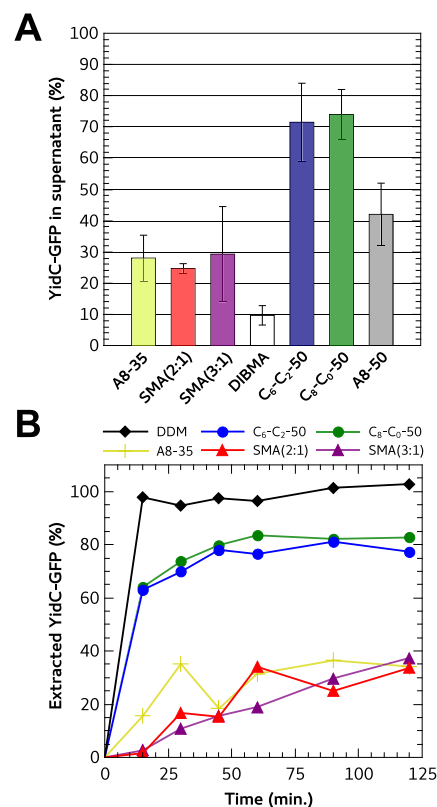


Figure 2. Fraction of YidC-GFP extracted from *E. coli* membranes upon addition of different polymers. (A) Quantification of YidC-GFP extracted from *E. coli* membranes. The membranes were supplemented with each polymer stock solution to reach a final polymer concentration of $2 \text{ g} \cdot \text{L}^{-1}$ [i.e., a total MP/polymer (w/w) ratio of 1:1] and incubated for 1 h at 4 °C. The buffer contained 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. After centrifugation (30 min at $100,000 \times g$), the protein content in the supernatants was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The in-gel detection of YidC-GFP fluorescence was quantified with ImageJ. The fluorescence intensity of polymer-extracted YidC-GFP was normalized to the fluorescence intensity for YidC-GFP solubilized with 1% w/v ($10 \text{ g} \cdot \text{L}^{-1}$) DDM, used as a reference for a complete extraction. (B) Kinetics of YidC-GFP extraction from *E. coli* membranes after addition of polymers. Over 2 h of incubation, aliquots were taken off from each sample and centrifuged (10 min at $250,000 \times g$). The fluorescence of the supernatants was quantified in gel (Figure S9).

extraction of *HwBR* in CyclAPol-containing supernatants was reached in 1 h, whereas extraction by SMA (3:1) progressed more slowly (Figure S10).

3.3. Solubilization by CyclAPols of the Purple Membrane of *Hb. salinarum*. To further investigate the membrane-solubilizing capacities of CyclAPols, we tested them on the purple membrane from *Hb. salinarum*, which represents both a simpler model than *E. coli* membrane fragments as it contains a single protein (*HsBR*) and a demanding one because of the high density and tight packing of proteins (*HsBR* is accounting for three-quarters of the membrane mass).²⁹ Indeed, purple membrane fragments proved resistant to solubilization by all of the polymers tested. Dilution of *HsBR* by fusion of the fragments with DMPC lipids was thus used to lower this resistance to solubilization.^{13,27} In order to be able to rank the polymers according to their solubilization efficiency, solubilization was assessed at various *HsBR*/DMPC ratios (Figure 4). A smaller pellet (Figure 4A) and higher

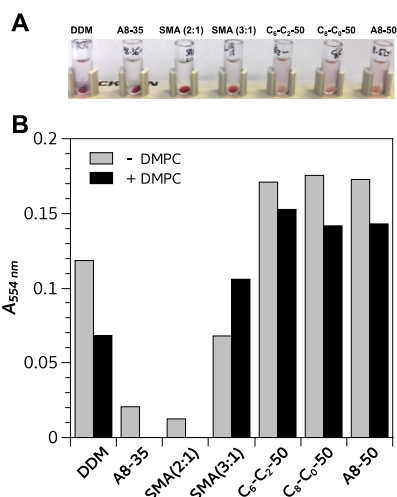


Figure 3. Extraction of *HwBR* from *E. coli* membranes using various surfactants. The solubilization of *E. coli* membranes was carried out at room temperature at a final polymer concentration of $2 \text{ g}\cdot\text{L}^{-1}$, corresponding to a total MP/polymer ratio of 1:1 (w/w). The reference condition in the presence of detergent was carried out with 0.2% w/v ($2 \text{ g}\cdot\text{L}^{-1}$) DDM. The buffer contained 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. After 4 h, the samples were centrifuged (30 min at $100,000 \times g$), and the concentration of *HwBR* in the supernatants was quantified by measuring the absorbance at 554 nm. The same experiment was performed with *E. coli* membranes fused with DMPC at a total MP/DMPC ratio of 1:0.5 (w/w) as described in ref 28. In the case of A8-35 and SMA (2:1), the absorbance values at 554 nm measured for supernatants collected after solubilization of DMPC-fused *E. coli* membranes were equal to 0.

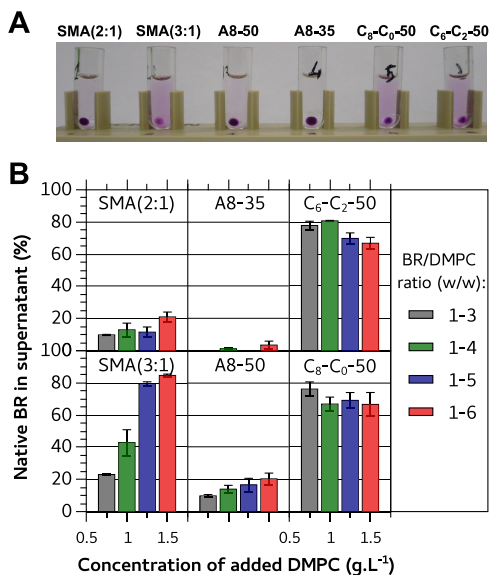


Figure 4. Solubilization by copolymers of the DMPC-fused purple membrane at an *HsBR*/polymer ratio of 1:6.25 (w/w) upon varying the amount of added DMPC. DMPC-containing proteoliposomes were prepared by fusion between DMPC liposomes and the purple membrane diluted at $0.5 \text{ g}\cdot\text{L}^{-1}$ [*HsBR*] (see the Experimental Section) before being mixed with an equal volume of the polymer solution. Samples were incubated overnight at $25 \text{ }^\circ\text{C}$ in the dark and then ultracentrifuged (20 min at $200,000 \times g$). (A) Photographs of samples after centrifugation (*HsBR*/DMPC ratio = 1:5 w/w). (B) Percentage of native *HsBR* in the supernatants as quantified by the ratios of absorbance values at 554 nm measured before and after centrifugation.

recovery of native *HsBR* in supernatants (Figure 4B) indicated that SMA (3:1) was by far more efficient at extracting *HsBR* than SMA (2:1) and DIBMA (Figure S11). Under the same experimental conditions, A8-35 was unable to extract *HsBR*, confirming its poor detergency. Even the more hydrophobic A8-50, which differs from A8-35 by a two-fold higher density of *n*-octyl chains, did not perform better than SMA (2:1). In contrast, CyclAPols, with a similar degree of grafted side groups as A8-50, were efficient solubilizers. The amounts of native *HsBR* found in CyclAPol- and SMA (3:1)-containing supernatants were comparable (~ 80 – 85%). As expected, a decrease of extraction efficacy by SMAs was observed when lowering the DMPC content in proteoliposomes. In contrast, high percentages of solubilization were obtained with CyclAPols even at the lowest DMPC ratio, i.e., in the more demanding conditions (Figure 4B).

To compare further CyclAPols and SMA (3:1), we assessed the effect of polymer concentration on the DMPC-fused purple membrane (at a fixed *HsBR*/DMPC ratio of 1:5 w/w) and measured the size of the complexes formed in supernatants. The percentage of native, solubilized *HsBR* decreased sharply at low polymer concentrations (Figure 5), indicating

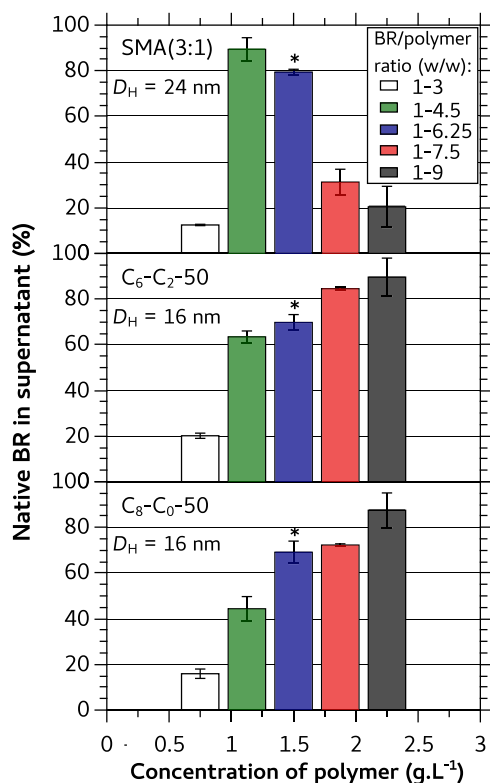


Figure 5. Fraction of native *HsBR* extracted as a function of polymer concentration. The *HsBR*/DMPC ratio was 1:5 (w/w), and the extraction procedure was as described in the legend to Figure 4. Asterisks point to samples used for D_H measurement in supernatants by DLS at $25 \text{ }^\circ\text{C}$.

that a polymer/*HsBR* ratio of 3:1 (w/w) is not sufficient to fully disperse all of the *HsBR*. All three copolymers however enabled up to 90% solubilization at higher polymer/*HsBR* ratios. Surprisingly, the amount of SMA (3:1)-solubilized *HsBR* passed through a maximum, which is confirmed by the size of the purple pellets (Figure S12). The hydrodynamic diameters (D_H) of the complexes (at a *HsBR*/polymer ratio of

1:6.25) were measured by dynamic light scattering (DLS). Compared to SMA (3:1), the D_H of complexes formed with CyclAPols were smaller. The population of solubilized objects was also analyzed by SEC, confirming that *HsBR*/DMPC/SMA (3:1) supernatants contained larger objects than those formed with CyclAPols (more material eluted in the void volume of the column; Figure S13). Therefore, whereas CyclAPols and SMA (3:1) can solubilize up to 90% *HsBR*, the complexes formed with CyclAPols appear smaller and more monodisperse.

Next, we assessed whether the copolymers solubilized the lipids along with *HsBR*. To this aim, the kinetics was recorded by DLS at a *HsBR*/polymer ratio of 1:12.5 w/w (Figure 6).

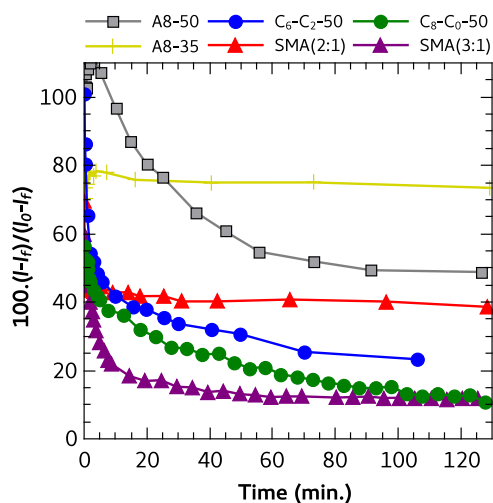


Figure 6. Kinetics of disruption of DMPC/purple membrane proteoliposomes upon addition of various polymers. At time 0, one volume of polymer solution was mixed to one volume of proteoliposomes. The kinetics was monitored at 25 °C by measuring the light scattering intensity, I , at an angle of 90° (I_0 is the initial intensity and I_f is the intensity scattered by supernatants 24 h after adding the polymers). The w/w ratios of *HsBR*/DMPC and *HsBR*/polymer were 1:5 and 1:12.5, respectively.

The scattered intensity reflects mainly evolution of proteoliposome size and concentration because the small particles (free polymer particles and MP/polymer complexes; $D_H < 40$ nm) contribute less than 6% of the intensity. The scattered intensity typically decreased immediately after supplementation of the *HsBR*/DMPC proteoliposomes with a solution containing the polymer (1:1 v/v, see the Experimental Section) and gradually reached a plateau (in <60 min in the slowest case, that of A8-50). First, this kinetics validates that the 24 h incubation used to quantify the amount of solubilized *HsBR* was sufficient to reach maximal extraction. Second, the average D_H as measured by DLS did not vary significantly with time and was about 130–200 nm (Table S1). This indicates that the proteoliposomes were the predominant scatterers during the whole solubilization. The absence of size variation, while the total intensity dropped down by up to 90%, rules out a gradual extraction of lipids or *HsBR* from all membranes and is thus incompatible with the “cookie-cutter” model proposed for SMAs.³³ In contrast, it is the number of proteoliposomes that decreases, without affecting the mean diameter of the residual ones. This probably indicates that once a liposome is destabilized (possibly due to the polymer gaining access to its internal surface), it rapidly disperses into small particles rather

than undergoing a progressive size decrease. Interestingly, the plateau measured at long times correlates with the percentage of solubilized *HsBR*, namely, the plateau reached with A8-35 was the highest, revealing that ~80% of the initial proteoliposomes were left intact. In the presence of efficient solubilizers, either SMA (3:1) or CyclAPols, the scattered intensity decreased by up to 90%, which can be compared with the 90% *HsBR* solubilization in Figures 4 and 5.

3.4. Stability of CyclAPol-Extracted *HsBR*. *HsBR* carries a retinal chromophore whose spectroscopic properties are extremely sensitive to the conformation of its binding pocket.³⁴ Accordingly, the stability of *HsBR* was assessed by measuring its absorbance at 554 nm. The kinetics of degradation was accelerated by incubating the complexes at 50 °C. As shown in Figure 7, a rapid decrease of the fraction of native *HsBR* is observed for SMA (3:1), whereas the spectral properties of *HsBR* are stable for hours in CyclAPol-solubilized samples.

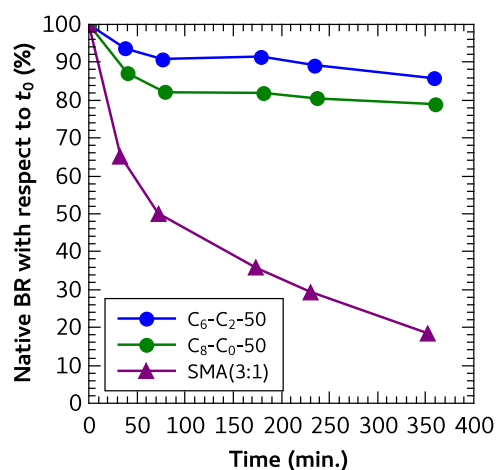


Figure 7. Kinetics of inactivation of *HsBR* incubated at 50 °C after extraction from the DMPC-fused purple membrane with polymers. The (w/w) ratios of *HsBR*/DMPC and *HsBR*/polymer were 1:5 and 1:6.25, respectively. The rate of inactivation in SMA (2:1) is similar to that in SMA (3:1), but because they bear on very small amounts, the data have not been plotted here.

5. DISCUSSION

Detergent-free extraction of MPs using amphipathic polymers presents two main interests for structural and functional investigations of MPs. First, it avoids exposing the protein to detergents, which is a frequent cause of inactivation. Second, it increases the probability that the protein will retain lipids that are important to its function and stability. Among amphipathic polymers that can substitute for detergents, classical, polyacrylate-based APols (the prototype of which is A8-35) and SMA copolymers are the most frequently used.^{4,5,14} Classical APols such as A8-35 are very mild surfactants so that MPs must generally be first extracted with detergents before being transferred to the polymer.¹² SMAs present the advantage of being more solubilizing so that direct MP extraction is often possible.¹⁴ In the present study, improving the efficiency of polyacrylate-based APols at extracting MPs directly from membranes while preserving their stabilizing effect was attempted using newly designed APols carrying cycloalkane moieties (CyclAPols).

Although pure lipid liposomes can be dispersed with a comparable efficiency in the presence of many copolymers,

such as A8-35, SMA (2:1), and CyclAPols (see Figure S14), this does not reflect their efficiency at extracting MPs. Biological membranes are indeed more complex systems than pure liposomes, and the proteins to be extracted differ from one another. In the present study, the archaeobacterial BR from two different organisms, that of *Hb. salinarum* naturally present in the purple membrane and that of *Hq. walsbyi* produced as a recombinant protein in *E. coli*, was used as a target protein to assess the efficiency of polymers at solubilizing MPs. The two BRs share 56% sequence identity; their transmembrane domain adopts the same fold, that is, a bundle of seven α -helices, and they bind the same cofactor, retinal, which, when bound to the native protein, gives BR its spectroscopic signature. By looking at the conditions required to extract the highest amounts of the two BR versions from their respective membranes, i.e., the densely packed purple membrane and the more fluid *E. coli* membranes, the solubilizing properties of the polymers appear very similar. For instance, the efficiency of A8-35, SMA (2:1), and DIBMA at extracting BR is very limited regardless of the membrane used, whereas that of SMA (3:1) improves in the presence of exogenous lipids. The predominant lipid components of the plasma membrane of *E. coli* are the zwitterionic lipid phosphatidylethanolamine (75%) and the anionic lipids phosphatidylglycerol (20%) and cardiolipin.³⁵ By fusing DMPC, a zwitterionic lipid, the overall charges of the membrane decrease, and the fluidity of the membrane is modified. These parameters presumably affect the efficiency of SMA (3:1) at extracting *Hw*BR. Under the same experimental conditions, i.e., at low polymer concentrations, CyclAPols show better capacities to extract *Hw*BR than either A8-35, SMA (2:1), or SMA (3:1). In addition, solubilization by CyclAPols is not as dependent on the addition of exogenous lipids as that by SMA (3:1) is. When the reporter protein is YidC-GFP, the protein-extraction efficiency of CyclAPols remains high, which suggests that the solubilizing properties of CyclAPols may be protein-independent. Recovering the highest amount of MPs (i) at low polymer concentrations over a short period of incubation (typically 1 h) and (ii) without the need to add exogenous lipids presents two advantages. First, it minimizes the risk of disruption of fragile interactions in MP complexes (amphipathic polymers, despite their lower dissociating character as compared to detergents, can also be disruptive when used at high concentrations).^{5,36,37} Second, the natural lipid composition in the vicinity of the MP to be extracted is not modified.

The comparison of CyclAPols with their counterpart A8-50 highlights the importance of the cyclic nature of the hydrophobic moieties. A8-50 and the two CyclAPols have the same density of hydrophilic and hydrophobic moieties, that is, ~ 50 mol %. However, A8-50, which comprises linear aliphatic (octyl) tails, allows one to recover lower amounts of both *Hs*BR from the DMPC-fused purple membrane and YidC-GFP from the *E. coli* membrane as compared to CyclAPols. The comparison of CyclAPols with DIBMA, which carries ~ 33 mol % of acyclic short-length branched diisobutyl, also shows a more efficient extraction with CyclAPols. The improvement of solubilization achieved with CyclAPols is therefore due to the cyclic nature of the hydrophobic side groups. In the case of detergents, the few available data suggest that cyclic hydrophobic groups markedly affect their solution behavior.^{38–40} In-tail cycles restrict packing possibilities, which is reflected in a lower propensity

to self-assemble (up to 8 times higher critical micelle concentration than linear counterparts, a lower aggregation number, and a more hydrated core).^{38–40} A lower stability of intrapolymer hydrophobic association in CyclAPols likely makes these groups more available to interact with lipid bilayers and thus to extract MPs. However, other molecular parameters may also play a role. Not surprisingly, a higher density of hydrophobic side groups in amphiphilic copolymers enhances their interaction with liposomes,^{9,10} which is in line with their relative efficiency at solubilizing BR [SMA (2:1) < SMA (3:1) and A8-35 < A8-50]. Overall, however, the density of side groups does not suffice to account for the solubilization efficiency. Thus, the density of hydrophobic moieties along C₈–C₀-50 (or C₆–C₂-50) is the same as that in A8-50, whereas these polymers exhibit significant differences in extraction efficiency (C₈–C₀-50 \gg A8-50). Moreover, because cycloalkyl moieties expose less surface to water than linear chains, cyclic side groups are effectively less hydrophobic than linear ones, so that CyclAPols rank below A8-50 in terms of effective hydrophobicity and could be expected to be less efficient solubilizers. A second possibly relevant parameter is the degree of ionization of the copolymer chains.¹⁰ As APols are weak acids, ionization of polymer chains is reported to lower the detergency by markedly increasing hydrophilicity. Potentiometric titrations of APols and CylAPols revealed a subtle shift in the degree of ionization, at a fixed pH, of the various polymers (Figure S15). The degree of ionization at a neutral pH, however, ranks the polymers as follows: A8-50 < C₈–C₀-50 < A8-35, which does not correlate with the extraction efficiency. It seems reasonable to conclude that the cyclic nature of the side groups, rather than the hydrophilic/hydrophobic balance, plays the key role in improving MP extraction.

The relative efficiency of polymers with respect to MP extraction varies depending on the protein under consideration. For instance, the amounts of *Hw*BR extracted from *E. coli* membranes with either A8-35 or SMA (2:1) were much lower than that obtained with SMA (3:1), whereas the amounts of YidC-GFP extracted with these three polymers were similar. A8-50 also showed some variability depending on the protein and/or the membrane used. Similarly, DIBMA showed a poor efficiency at extracting both YidC-GFP and BR, whereas it has been reported to be able to extract other MPs.^{30,41} In the present series of experiments, the two CyclAPols showed little sensitivity to the nature of the proteins. In addition, an interesting observation is that *Hs*BR is much more stable when extracted with CyclAPols than with SMAs: it retains 80–85% of its native conformation after 6 h at 50 °C versus $\sim 20\%$ in SMAs. How general this effect is and which mechanism underlies it will require further investigations.

6. CONCLUSIONS

In conclusion, we have shown that cyclic hydrophobic moieties improve the copolymer ability to solubilize membranes as compared to linear aliphatic chains. Detergent-free solubilization of *Hs*BR by CyclAPols was as efficient as with SMA (3:1), and the CyclAPol-extracted *Hs*BR was as stable as in A8-35⁵ (a stability that SMAs failed to provide). The CyclAPols can also solubilize different MPs from *E. coli* membranes over a short period of incubation (typically 1 h) and at low concentrations. Recently, CyclAPols have been validated in structural studies of MPs using cryo-EM single-particle analysis and electrospray

ionization mass spectrometry.⁴² Moreover, CyclAPols are compatible with UV–visible spectroscopic measurements (Figure S8). By ranking the various copolymers according to their detergent-like efficiency, it appears that differences between CyclAPols and classical APols correlate neither with their hydrophobicity nor with chain ionization and thus likely reflect specific properties of cycloalkane side-chain self-assemblies and/or binding to membranes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.biomac.0c00929>.

Polymer characterization by ¹H and ¹³C NMR spectroscopy and potentiometric titrations, UV–visible spectra of polymers, SDS-PAGE analysis of YidC-GFP, kinetics of HwBR extraction, photographs of tubes containing polymer-extracted HsBR after ultracentrifugation, SEC profiles, hydrodynamic diameters of HsBR/DMPC proteoliposomes upon addition of polymer solutions, solubilization of pure liposomes by polymers, and potentiometric titrations of polymers in the presence of DMPC liposomes (PDF)

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Author Contributions

M.Z. led the project. A.M., B.M., F.G., C.T., and M.Z. designed the experiments. A.M. synthesized and characterized the CyclAPols with the help of F.G. A.M. and M.Z. performed the quantification and kinetics of YidC-GFP solubilization. M.Z. carried out the HwBR extraction experiments. A.M. and B.P. assisted by M.Z. performed the HsBR extraction and stabilization experiments. B.M. investigated the effects of HsBR/DMPC and HsBR/polymer ratios. C.L.B. performed the SEC analysis. C.T. performed and analyzed the DLS experiments. M.Z. and C.T. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

APols, amphipols; CyclAPols, amphipols bearing cycloalkane side groups; DDM, *n*-dodecyl β -D-maltoside; DIBMA, diisobutylene maleic acid copolymer; DLS, dynamic light scattering; GFP, green fluorescent protein; HsBR, bacteriorhodopsin of *Halobacterium salinarum*; HwBR, bacteriorhodopsin of *Haloquadratum walsbyi*; MPs, membrane proteins; PAA, poly(acrylic acid); SEC, size exclusion chromatography; SMAs, styrene maleic acid copolymers

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