

Sulfonated Amphipols: Synthesis, Properties, and Applications

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ABSTRACT:

Amphipols (APols) are amphiphatic polymers that keep membrane proteins (MPs) water-soluble. The best characterized and most widely used APol to date, A8-35, comprises a polyacrylate backbone grafted with octyl- and isopropylamine side chains. The nature of its hydrophilic moieties prevents its use at the slightly acidic pH that is desirable to slow down the rate of amide proton exchange in solution NMR studies. We describe here the synthesis and properties of pH-insensitive APols obtained by replacing isopropyles with taurine. Sulfonated APols (SAPols) can be used to trap MPs in the form of small complexes, to stabilize them, and to keep them water-soluble even at low pH. [^{15}N , ^1H]-transverse relaxation-optimized spectroscopy NMR spectra obtained at pH 6.8 of a bacterial outer MP folded in SAPols show that the protein is correctly folded. The spectra have a resolution similar to that achieved with A8-35 and reveal water-exposed amide and indole protons whose resonance peaks are absent at pH 8.0. © 2011 Wiley Periodicals, Inc.

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INTRODUCTION

Integral membrane proteins (MPs) are key protagonists in such essential cell functions as growth control, regulation of physiological functions, signaling, exchanges of solutes, and recognition by pathogens. As a result, their role as pharmacological targets is primordial. In vitro studies of MPs are complicated by their insolubility in aqueous solutions, a consequence of the highly hydrophobic nature of their transmembrane surface. MPs, therefore, are most often handled in aqueous solutions of detergents.^{1,2} Detergents, however, tend to inactivate MPs.^{3–5} This has prompted the development of alternative surfactants, such as fluorinated surfactants,^{6,7} lipopeptides,⁸ nanodiscs,^{9,10} and amphipols (APols)¹¹ (for review, see Refs. 4 and 5). APols are short amphiphatic polymers that adsorb onto the transmembrane region of MPs,^{12,13} thus providing them with a non-denaturing solubilizing environment (reviewed in Refs. 5 and 14–16). The most intensively studied APol to date, A8-35, is composed of a polyacrylate backbone grafted with octyl- and isopropylamine side chains (Figure 1A).^{11,17,18} A8-35-trapped MPs are amenable to structural and functional studies by such methods as absorption or fluorescence spectroscopy,^{19–22} circular dichroism,^{23,24} surface plasmon resonance, and other approaches to ligand-binding studies,²² as well as electron microscopy,^{21,25,26} and solution NMR spectroscopy (see below). A8-35 can also be used to fold to their native state MPs that have been either denatured or expressed under an inactive form^{23,27} and to present them to the immune system.²⁸

Because the solubility of A8-35 is due to the presence of carboxylate functions, it is sensitive both to the presence of calcium ions and to lowering the pH of the solutions below pH \approx 7, two types of conditions that induce the aggregation

Additional Supporting Information may be found in the online version of this article.

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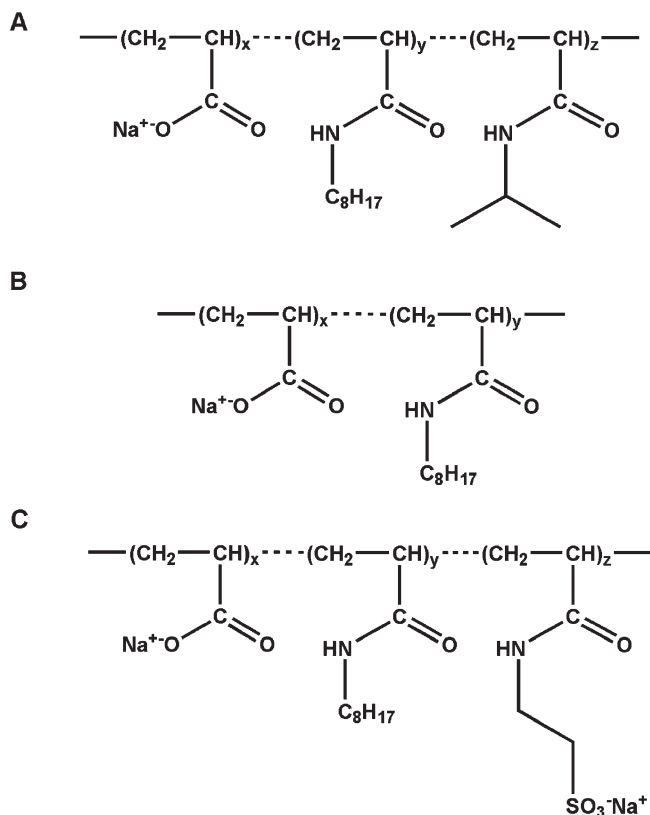


FIGURE 1 Chemical structure of various amphipols. A: A8-35: $x \approx 0.35$, $y \approx 0.25$, and $z \approx 0.40$ (from Ref. 11). B: A8-75: $x \approx 0.75$ and $y \approx 0.25$ (from Ref. 11). C: SAPols: $x \approx 0.35$, $y \approx 0.25$, and $z \approx 0.40$ (this work; see Table I, for exact composition of each batch).

of A8-35 particles and MP/A8-35 complexes.^{18,21,29,30} This can represent a limitation when handling or folding MPs whose stability requires an acidic pH or the presence of multivalent cations, as well as under certain experimental circumstances (see below). This constraint has prompted the development of alternative structures, such as APols carrying zwitterionic groups^{30,31} or nonionic APols^{32–34} (reviewed in Refs. 5, 14 and 15), and has been a major incentive for the present work.

MP/A8-35 complexes can be studied by solution NMR.^{12,13,35–37} Because these complexes are slightly larger than the smallest MP/detergent ones, tumbling is slightly slower, and transverse relaxation-optimized spectroscopy (TROSY) spectra are slightly less resolved. For MPs that do not resist the harsh conditions of temperature and detergent concentration imposed by NMR, however, this is more than compensated for by the higher stability of APol-trapped MPs and the possible recourse to higher working temperatures. Many NMR experiments can be carried out above pH 7 (see, e.g., Refs. 35 and 36), but the insolubility of A8-35 in acidic solutions is a drawback when attempting to solve MP structures by solution NMR. Indeed, the observation of the

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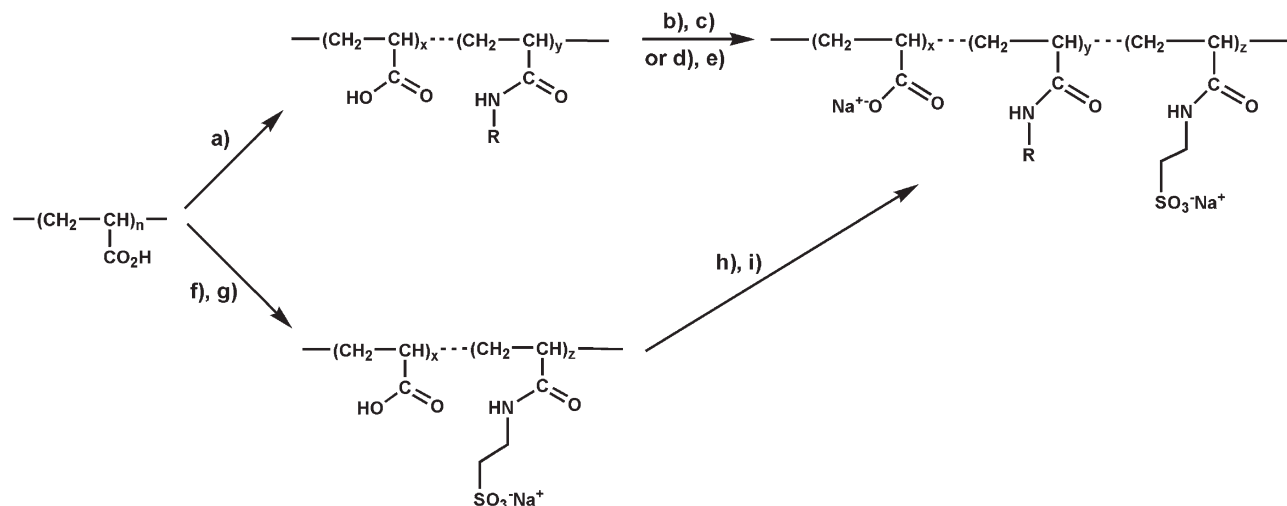
resonance peaks of amide protons, a major tool in the de novo resolution of a protein structure by solution NMR, depends on the exchange of these protons with the solution being sufficiently slow.³⁸ For protons that are freely exposed to the solution, this is best achieved by working at a slightly acidic pH.³⁸ We have therefore explored the possibility to develop APols whose structure would remain as close as possible to that of A8-35, while eliminating the latter's sensitivity to low pH and to calcium ions. This could conceivably be achieved by endowing them with sulfonate groups.

The first reported syntheses of sulfonated amphiphilic polymers resorted to copolymerization of a sulfonated monomer, 2-acrylamido-2-methyl-propanesulfonic acid (AMPS), with such hydrophobic monomers as styrene³⁹ or methacrylamide bearing either bulky hydrophobic substituents⁴⁰ or an alkyl chain.⁴¹ Similar polymers were subsequently obtained by partial hydrophobization of a hydrophilic poly(acrylate-*co*-sulfonate) precursor.⁴² All these polymers exhibited a high solubility in aqueous solutions, even for low percentages of AMPS, as well as a high tolerance toward low pH, high ionic strength, or the presence of divalent cations. None of them has been used for MP trapping. In the present work, we have sought to develop sulfonated polymers whose structure would remain close to that of already validated APols, while widening the accessible pH range.

The chemical structure of A8-35 comprises $\sim 35\%$ of free carboxylates, $\sim 25\%$ of octylamide moieties, and $\sim 40\%$ of isopropylamide ones (Figure 1A).¹¹ Early studies have shown that a sister structure dubbed A8-75 was as efficient as A8-35 in terms of its ability at keeping MPs soluble in the absence of detergent.^{11,43} A8-75, an intermediate in the synthesis of A8-35, differs from it only in the fact that no isopropylamide groups have been grafted (Figure 1B). The charge density along the chain, therefore, is much higher than in A8-35, because $\sim 75\%$ rather than $\sim 35\%$ of the carboxylates have been left free. In the present work, we have developed the synthesis and studied the properties of a new family of APols, sulfonated APols (SAPols). SAPols are derived from A8-75 by grafting $\sim 40\%$ of the carboxylates with taurine (Figure 1C). As a result, their charge density is identical to that of A8-75, but with the essential difference that the sulfonate groups thus grafted do not protonate even at pH 0. We report here on the synthesis and solution properties of SAPols, on their use to maintain MPs water-soluble and to fold one of them, and on their application to solution NMR.

RESULTS

Several synthesis routes were tested, yielding sulfonated polymers with similar or slightly different structures. The resulting preparations were examined with respect to their compo-



SCHEME 1 Synthesis routes. A: Synthesis of SAPol-1: (a) 25% molar octylamine, DCC/NMP, 60°C, 1 h, RT, 2 h; (b) NaOH; (c) aqueous solution, taurine 40% molar, EDC · HCl, 2 h. B: Synthesis of SAPol-3 and SAPol-4: (a) 25% molar octylamine (SAPol-3) or dodecylamine (SAPol-4), DCC/NMP, 60°C, 1 h, RT, 2 h; (d) 40% molar taurine sodium salt, DCC/NMP, 60°C, 1 h, RT, 2 h; (e) NaOH. C: Synthesis of SAPol-2: (f) aqueous solution pH = 7, 40% molar taurine, EDC · HCl; (g) Dowex-H⁺, NaOH (1 equivalent per mol of sulfonic acid functions); (h) 25% molar octylamine in NMP/H₂O 15/1, DCC, 60°C, 1 h, RT, 2 h; (i) NaOH.

sition and yield of synthesis, their solution behavior, and their ability to trap membrane MPs. The most promising of them was used to explore the applications of SAPols in MP biochemistry and biophysics, namely MP stabilization and folding and solution NMR studies.

Synthesis

Three synthetic pathways were explored (Scheme 1), with the aim of optimizing reaction efficiency, purification yields, and the solution behavior of the polymers:

- Octyl chains were first grafted onto polyacrylic acid (PAA) in organic medium, followed by grafting of taurine groups in aqueous solution (SAPol-1). A batch of SAPol-1 further purified by aqueous size exclusion chromatography (SEC) is denoted SAPol-1'.
- PAA was grafted with taurine in water, followed by grafting octylamine in organic medium (SAPol-2).
- Octylamine and the sodium salt of taurine were grafted successively onto PAA in organic medium (SAPol-3). SAPol-4 was synthesized in the same way except for the substitution of dodecylamine to octylamine.

The composition and synthesis yield of each batch of SAPol are summarized in Table I. The three pathways led to similar polymers, with the expected composition. However, the yield, the level of purity, and the solution behavior of the polymers were different. In all cases, PAA was entirely converted into SAPol, but the purification was far from quantitative, leading to overall yields ranging from 27 to ~70%. Indeed, because

SAPols were designed to be soluble in aqueous solutions at low pH, their purification cannot be carried out as that of A8-35, which relies on cycles of precipitation at acidic pH and redissolution at alkaline one.^{17,18} After an initial purification by dialysis, SAPol-1 was further purified by SEC on a preparative Superose column, so as to remove a persistent impurity (see Materials and Methods section), yielding SAPol-1' (see Supporting Information). SAPol-2 was purified by dialysis in order to remove *N*-methylpyrrolidone (NMP), but a large fraction of the material was lost at this stage. SAPol-3 was purified by precipitation in organic medium, but a fraction of the polymer was lost in the filtrate. The purification of SAPol-4 was more easily achieved, because most of the dicyclohexylurea (DCU) contaminant was removed upon extracting the polymer in organic medium (biphasic *n*-hexane/methanol/NMP mixture). Furthermore, at variance with *N*-ethyl-*N'*-(3-dimethylaminopro-

Table I Composition of the Four Batches of SAPols Used in the Present Work

Batch ^a	x (CO ₂ ⁻)	y (C ₈)	z (SO ₃ ⁻)	Yield (%) ^b
SAPol-1'	0.33	0.25	0.42	27
SAPol-2	0.35	0.25	0.40	43
SAPol-3	0.35	0.25	0.40	64
SAPol-4	0.42	0.25	0.33	71

^a SAPol-1' is a fraction SAPol-1 purified by SEC (see Experimental section).

^b Yield of final, purified polymer with respect to the amount of PAA used as the starting material.

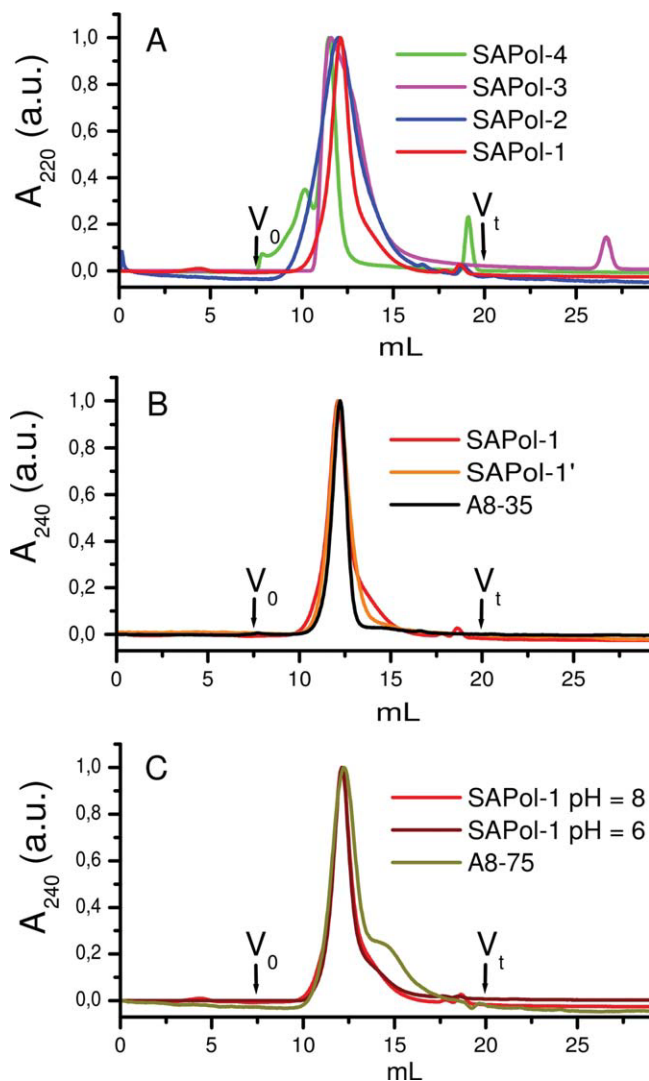


FIGURE 2 Size exclusion chromatography of amphipols A8-35, A8-75, and various batches of SAPols. Analyses were performed on a Superose 12 10/300GL column. Elution was carried out either with Tris buffer (pH = 8.0) or with NaPi_4 buffer (pH = 6.0). A: SAPol-1, SAPol-2, SAPol-3, and SAPol-4. Elution at pH 8, detection at 220 nm. B: SAPol-1, SAPol-1', and A8-35. Elution at pH 8, detection at 240 nm. C: SAPol-1 at pH 6 and 8 and A8-75 at pH 8. Detection at 240 nm. Chromatograms were normalized to the maximal absorbance (a.u.: arbitrary units).

pyl)carbodiimide (EDC), both the dicyclohexylcarbodiimide (DCI) coupling reagent that was used for this synthesis and the resulting by-product (DCU) are hydrophobic. In consequence, they precipitated when SAPol-4 was dissolved in aqueous solution. After filtration, dialysis, and freeze-drying, ^1H NMR spectroscopy of a sample redissolved in water confirmed that SAPol-4 was free of contaminants.

Solution Properties of SAPols

Effect of the Density of Sulfonate Groups on the Solubility at Low pH or in the Presence of Calcium Ions. In prelimi-

nary experiments (results not shown), we observed that when <30% of the acrylate units were grafted with taurine, the resulting polymers exhibited a solution behavior intermediate between that of A8-35 and that of the SAPols described here. Namely, (i) they precipitated at low pH, but only under very acidic conditions (pH < 2), and (ii) they were soluble at pH 2–7, but, according to SEC experiments performed at pH 6, they formed heterogeneous populations of particles (not shown). They were also highly sensitive to the addition of millimolar concentrations of calcium. By contrast, above ~30% of taurine, neither aggregation nor precipitation was observed whatever the pH (down to pH –1), nor any sensitivity to the presence of divalent cations.²⁹

Influence of the Synthetic Pathway on Self-Assembly. The self-assembly properties of SAPols in aqueous solutions were characterized by SEC. Despite their similar global composition, the four SAPols behaved somewhat differently (Figure 2A and Table II). SAPol-1 was the only one to form small and essentially monodisperse particles. As discussed below, this is presumably due to the fact that SAPol-1 is obtained by grafting with taurine pre-existing particles of A8-75 in aqueous solution, while the other synthesis pathways involve grafting of dispersed molecules in organic solution (see Discussion section). Monodispersity was improved after fractionation of SAPol-1 by preparative SEC (yielding SAPol-1') (Figure 2B). Among the various preparations tested, the size and dispersity of particles of SAPol-1 and SAPol-1' are most similar to those of A8-35 (Figure 2B and Table II). The behavior of SAPol-1 upon SEC was similar to that of its nonsulfonated precursor A8-75 and did not vary when the pH was lowered from 8 to 6 (Figure 2C).

Trapping MPs with SAPols

The ability of SAPols to keep membrane proteins (MPs) soluble in aqueous solutions was tested using two model MPs,

Table II Elution Volume, Stokes Radius (R_s), and Peak Width at Half Height for SAPol and A8-35 Particles Eluted in Tris Buffer on a Calibrated SEC Superose 12 HR 10–30 Column

Sample	Elution Volume (ml)	HHW (ml)	R_s (nm)
A8-35	12.2	0.90	3.2
SAPol-1	12.1	1.11	3.4
SAPol-1'	12.2	1.20	3.3
SAPol-2	12.0	2.20	3.6
SAPol-3	11.6	2.35	4.5
SAPol-4 ^a	7.8	—	9.0
	10.2	—	6.3
	11.5	0.87	4.8

^a The chromatogram of SAPol-4 showed three main peaks (cf. Figure 2A).

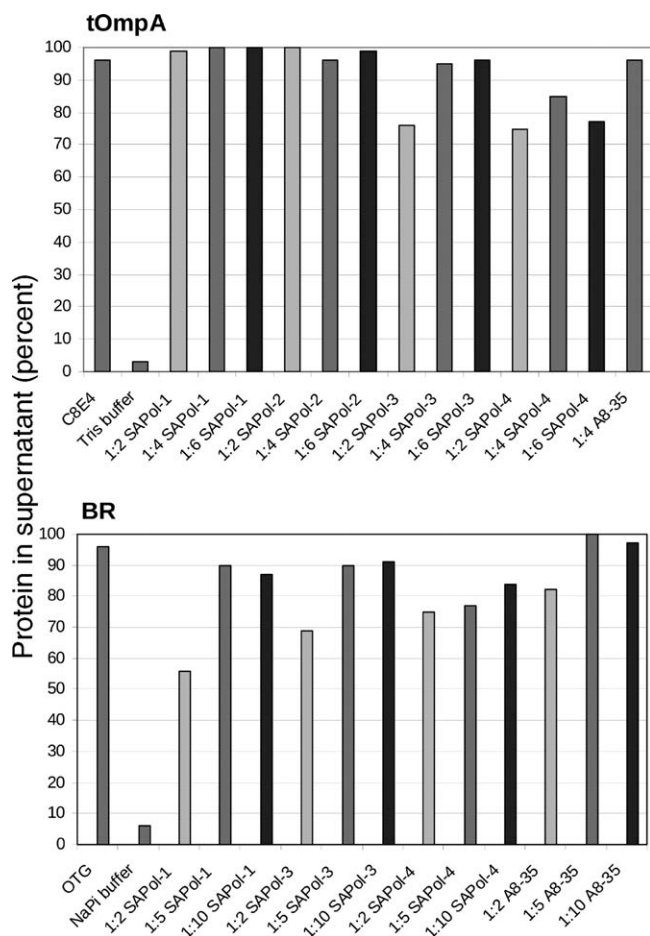


FIGURE 3 Trapping of bacteriorhodopsin (BR) and tOmpA by sulfonated amphipols. The proteins in detergent solution (18 mM OTG in NaPi₂ for BR and 19.6 mM C₈E₄ in Tris buffer for tOmpA) were supplemented with SAPols at the MP/APol mass ratios indicated, diluted 3× (BR) or 10× (tOmpA) with surfactant-free buffer, bringing the detergent concentration 1.5× and 4× below the cmc of OTG and C₈E₄, respectively, and centrifuged for 20 min at 80,000 rpm (200,000 × g). The concentration of protein in the solutions before and after centrifugation was determined from their absorbance at 554 nm (BR) or 280 nm (tOmpA). The percentage of protein retained in the supernatant is given by reference to the concentration before centrifugation. Controls included dilution with detergent-containing or detergent-free buffer in the absence of APols (first two bars) and trapping with A8-35 (last bars).

bacteriorhodopsin (BR), an α -helical MP from *Halobacterium salinarum*, and the transmembrane β -barrel domain (tOmpA), of *Escherichia coli*'s outer MPA (cf. Refs. 20 and 21). After adding various amounts of SAPols to aliquots of the proteins in detergent solution—octylthioglucoside (OTG) in the case of BR, tetraethylene glycol mono-octyl ether (C₈E₄) in that of tOmpA—the samples were diluted below the critical micellar concentration (cmc) of the detergent using surfactant-free buffer and centrifuged at 80,000 rpm (200,000 × g) for 20 min. The concentration of protein in the supernatant

was determined from its absorbance at 554 nm for BR and at 280 nm for tOmpA. Controls included samples without APols, samples supplemented with APol A8-35 instead of SAPols, and APol-free samples diluted with detergent-containing solutions. In the absence of polymer, dilution under the cmc of the detergent led to the aggregation and subsequent precipitation of most of the protein, whereas ~95% of the protein diluted in a detergent-containing buffer remained in solution. The four SAPol preparations efficiently kept MPs soluble in the absence of micellar detergent, SAPol-4, however, being somewhat less efficient (see Figure 3). The polymer-to-protein mass ratio required to keep most of BR or tOmpA in solution (~4 g/g) is similar for SAPols and for A8-35 (see Figure 3).

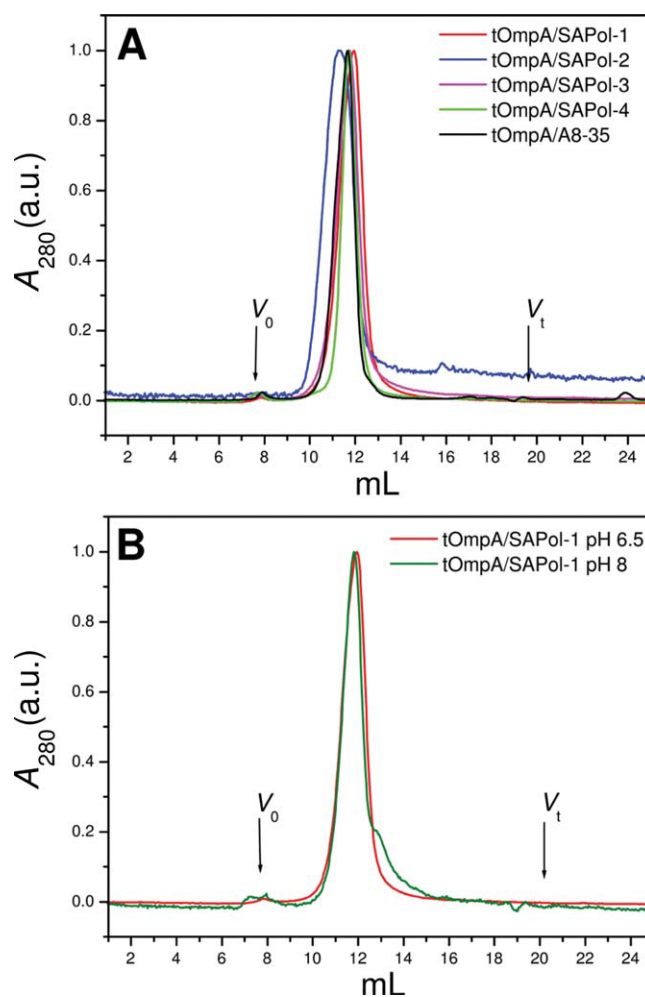


FIGURE 4 Size exclusion chromatography of tOmpA/SAPol and tOmpA/A8-35 complexes. The protein was trapped at a tOmpA/APol mass ratio of 1:4. Analyses were performed at room temperature on a Superose 12 10/300GL column (Amersham). A: Comparison of complexes formed with either of the four SAPols (NaPi₁ buffer, pH 6.5) or with A8-35 (Tris buffer, pH 8.0). B: Comparison of the behavior of tOmpA/SAPol-1 complexes at pH 6.5 and pH 8.

Table III Elution Volume, Stokes Radius (R_s), and Peak Width at Half Height (HHW) for tOmpA/SAPols and tOmpA/A8-35 Complexes Eluted, respectively, in NaPi_1 and in Tris Buffer on a Calibrated Superose 12 HR 10-30 Column

Sample	tOmpA/APol ratio (w/w) ^a	Elution Volume (ml)	HHW (ml)	R_s (nm)
tOmpA/SAPol-1	1:4	11.9	1.15	4.6
	1:8	11.9	1.06	
tOmpA/SAPol-2	1:4	11.3	1.45	5.2
tOmpA/SAPol-3	1:4	11.7	1.06	4.8
	1:8	11.8	1.04	
tOmpA/SAPol-4	1:4	11.7	0.72	4.8
	1:8	11.7	0.73	
tOmpA/A8-35	1:4	11.7	0.95	4.9

^a tOmpA/APol mass ratio used for trapping.

Homogeneity and Size of MP/SAPol Complexes

The size and homogeneity of tOmpA/SAPol complexes were examined by SEC (see Figure 4). tOmpA/SAPols complexes have typically a Stokes radius $R_s = 4.6$ – 4.8 nm, slightly smaller than that of tOmpA/A8-35 complexes (4.9 nm).²⁰ The polydispersity of tOmpA/SAPol-1 and tOmpA/SAPol-3 complexes is comparable to that of tOmpA/A8-35 ones (Table III), whereas tOmpA/SAPol-2 particles are somewhat more polydisperse and tOmpA/SAPol-4 ones somewhat less so. The Stokes radius of tOmpA/SAPol-1 complexes is the smallest (4.6 nm). The fair monodispersity of tOmpA/SAPol-4 complexes was unexpected, given the polydispersity of SAPol-4 particles (Figure 2A): as a rule, APols that do not form monodisperse particles by themselves tend to yield polydisperse MP/APol complexes (see, e.g., Ref. 21). tOmpA/SAPol-4 complexes actually appeared less polydisperse than tOmpA/SAPol-1 and tOmpA/SAPol-3 ones (~30% decrease of the HHW, see Table III), suggesting that the length of the fatty chains may influence the structure of the complexes. The elution profiles of tOmpA/SAPol complexes did not depend significantly on either the protein/APol mass ratio (1:4–1:8) or the pH (6.5–8.0) (Figure 4B).

SAPol-1, whose particles appear more homogeneous and which forms small complexes with tOmpA, was selected for further examination of the properties of MP/SAPol complexes and of their applications. Unless otherwise specified, experiments were carried out with SAPol-1, whose preparation does not require the lengthy purification procedure involved in that of SAPol-1'.

Biochemical and Colloidal Stability of SAPol-Trapped BR

As previously reported,^{11,21} the biochemical stability of bacteriorhodopsin (BR) is greatly improved following its transfer

from OTG to A8-35 (see Figure 5). The stability of APol-trapped BR was tested in the presence of either 0 or 100 mM NaCl. Although ionic strength had little or no effect on the stability of A8-35-trapped BR, it markedly affected that of SAPol-trapped BR (see Figure 5). At low NaCl concentration, BR denaturation was observed both upon trapping and during extended storage at 4°C in the dark. In the presence of 100 mM NaCl, on the other hand, the stability of BR was comparable following complexation by one or the other type of APol.

SEC analysis indicated that tOmpA/SAPol-1 complexes remain monodisperse in 100 and 200 mM NaCl, but show signs of aggregation at 300 mM (data not shown), in keeping

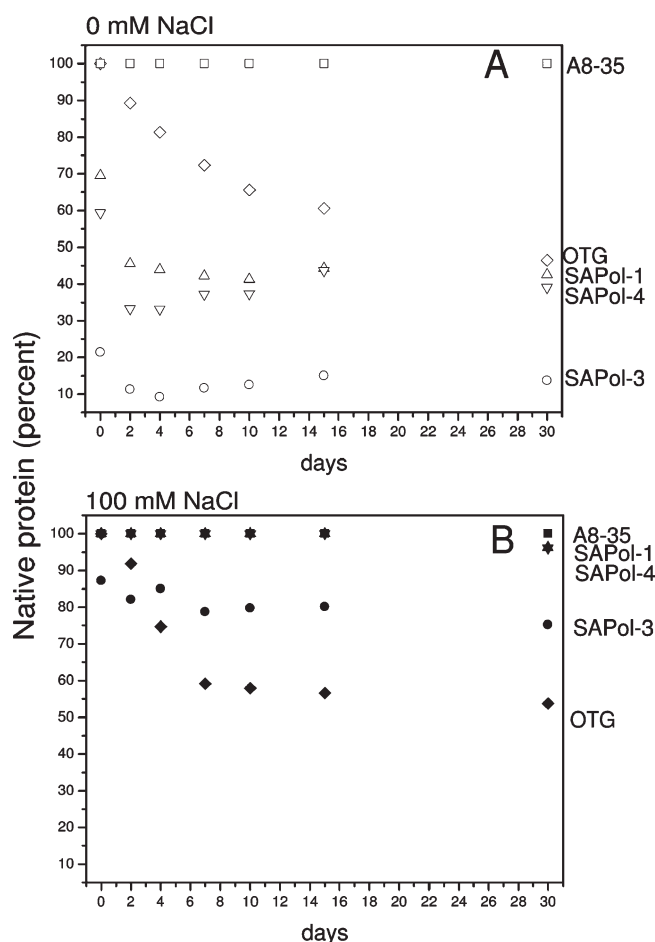


FIGURE 5 Stability of BR/surfactant complexes stored at 4°C in the dark. BR was trapped in either SAPols or A8-35 at a 1:10 mass ratio, following the same protocol as described in the legend to Figure 3. Similar results were obtained at 1:5 w/w. The buffer (20 mM NaPi and pH 7.0) comprised either no salt (NaPi_3) or 100 mM NaCl (NaPi_2). UV-vis absorption spectra were recorded at various times after trapping and A_{554} taken as a measure of the concentration of native holoprotein. Data are plotted as the percentage of native protein (determined using the A_{554}/A_{280} ratio). As a control, BR was stored under the same conditions in 18 mM OTG in the same buffers.

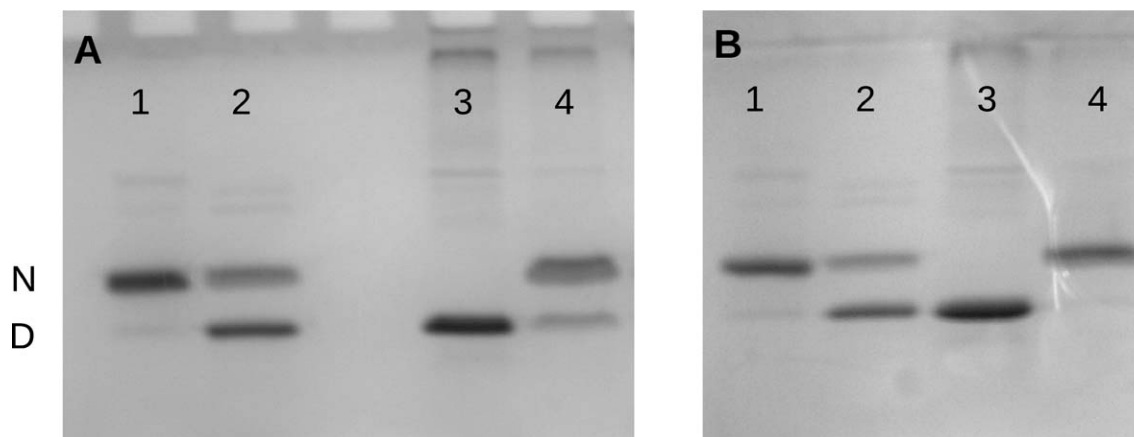


FIGURE 6 SAPol-assisted folding of tOmpA. Inclusion bodies containing tOmpA were solubilized in urea and the protein folded by transfer to SAPol-1 (see Experimental section). Analysis by SDS-PAGE on a 12% polyacrylamide gel shows the shift to higher apparent masses characteristic of folded tOmpA. A: Analysis performed after slow dilution of urea-denatured tOmpA into a urea-free buffer containing 2.3 g L^{-1} SAPol-1 (final concentration of tOmpA, 0.57 g L^{-1}). B: Analysis of the same samples after 2 days of incubation at room temperature. Lane 1: Native tOmpA in C_8E_4 ; Lane 2: The same sample denatured by boiling in SDS buffer; Lane 3: Inclusion bodies solubilized in 8 M urea; Lane 4: tOmpA folded in SAPol-1. Except for sample 2, samples were dissolved in SDS-loading buffer at room temperature. N: native; D: denatured.

with radiation scattering observations on BR/A8-35²¹ and cytochrome *b*_c₁/A8-35 complexes.¹⁴

SAPol-Assisted Folding of tOmpA

A8-35 has proven remarkably efficient at promoting the folding of denatured MPs, whether their transmembrane region is formed of a β -barrel or of a bundle of α -helices.^{23,27,44} Similarly, SAPol-1 proved a good medium in which to fold tOmpA, obtained from inclusion bodies, and solubilized in a denatured form in 8M urea. The formation of native tOmpA following dilution of the preparation into a urea-free buffer containing SAPol-1 in a 1:4 tOmpA/SAPol mass ratio was followed by SDS-PAGE. After transfer of tOmpA to SAPol-1, a shift on the gel is observed, reflecting the folding of tOmpA to its native 3D structure⁴⁵ (Figure 6A). Folding is complete after 2 days at room temperature under continuous stirring (Figure 6B).

Solution NMR Analysis of tOmpA/SAPol Complexes

The structure of tOmpA folded in SAPol-1 was examined by solution NMR. The 3D structure of detergent-solubilized tOmpA has been solved both crystallographically⁴⁶ and by solution NMR.^{47,48} Previous studies have shown that the two-dimensional (2D) [¹H,¹⁵N]-TROSY spectrum^{49,50} of A8-35-trapped tOmpA features very similar chemical shifts to those observed in detergent solution, indicating that the protein is in its native state.¹² A very similar spectrum was observed for tOmpA folded in SAPols, diagnostic of correct folding (see Figure 7).

At variance with MP/A8-35 complexes,²¹ MP/SAPol ones remain soluble and monodisperse at low pH (Figure 4B). This makes it possible to observe solvent-accessible amide protons, whose exchange with the solution is too rapid at the slightly basic pH (8.0) used in NMR studies of tOmpA/A8-35 complexes.¹² At pH 6.8, many correlations peaks belonging to loop residues (upfield in the ¹H dimension, i.e., between 7.5 and 8.5 ppm) indeed become observable. Downfield in the ¹H dimension (~ 10.1 ppm), the five indole protons are now visible, when compared with only one in A8-35 at pH 8.0.¹² Also, correlation peaks in the center of the spectrum that are enlarged at pH 8.0 due to unfavorable fast chemical exchange become much sharper at pH 6.8 (see Figure 7). tOmpA/A8-35 and tOmpA/SAPol TROSY spectra feature similar line widths, in keeping with the similar particle sizes observed by SEC (Figure 4 and Table III).

The stability of tOmpA/SAPol complexes was tested by recording 2D [¹⁵N,¹H]-TROSY spectra at 30, 50, and 70°C (see Figure 8). The very similar and well-dispersed correlation peaks indicate that SAPol-trapped tOmpA retains its native 3D structure for days even at high temperature. As expected, the line shape and intensity of signals originating from loop residues change more with temperature than those from β -sheet residues, due to faster conformational exchange in the loops.

DISCUSSION

Although conceptually simple, the preparation of SAPols turned out to be quite difficult to develop. There are two

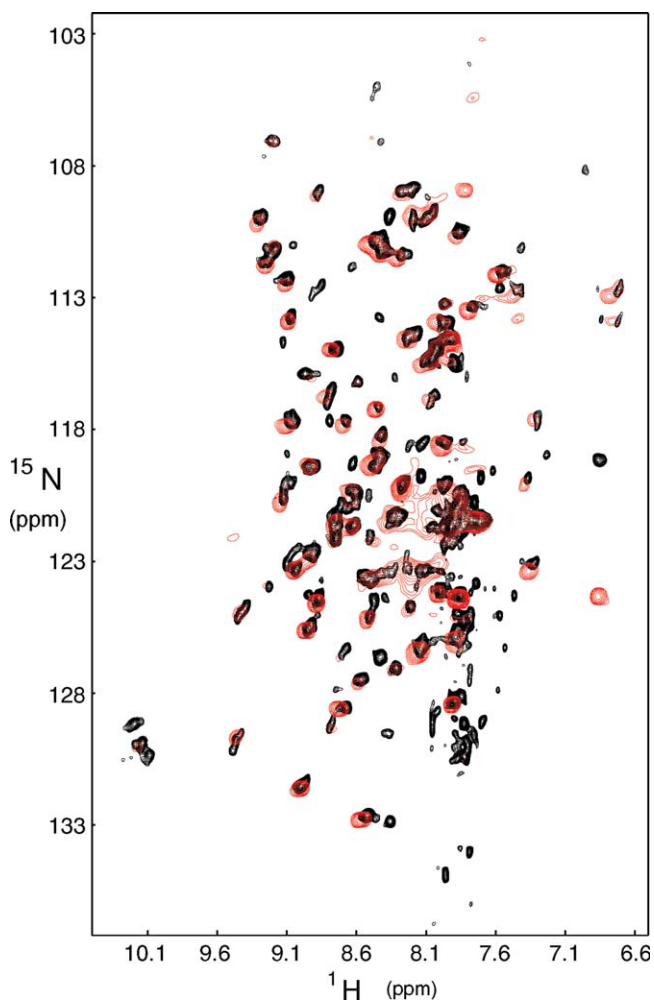


FIGURE 7 Superimposed 2D $[^{15}\text{N}, ^1\text{H}]$ -TROSY spectra of $[\text{u-}^2\text{H}, ^{15}\text{N}]$ tOmpA trapped either with SAPol-1 (in black; 20 mM NaPi buffer, pH 6.8, and 100 mM NaCl) or with A8-35 (in red; 20 mM Tris buffer, pH 8.0, and 100 mM NaCl). The experiments were carried out at 30°C. Note the extra peaks that can be observed at pH 6.8, for example, indole protons at ~ 10 ppm in the ^1H dimension and amide protons belonging to exposed parts of extracellular loops, mostly between 7.5 and 8.0 ppm.

levels of difficulties, those relative to the synthesis itself and those involved in the purification of the final product, the latter tending to be the hardest to optimize. The three protocols we explored (Scheme 2) comprise the following steps:

- Protocol *i*: Octylamine is grafted onto PAA in organic medium; the resulting APol (A8-75; see Ref. 11) is purified and grafted with taurine in aqueous solution.
- Protocol *ii*: Taurine is grafted onto PAA in water. The resulting sulfonated, hydrophilic polymer is purified and grafted with octylamine in organic solution.
- Protocol *iii*: PAA in organic solution is grafted successively with octylamine and with the sodium salt of taurine.

All three approaches yielded the desired polymers, but with variable efficiencies. On the basis of the SEC behavior of the resulting SAPol particles, route *i* was selected as the best. It is to be noted that, while routes *ii* and *iii*, as well as the synthesis protocol for A8-35, aim at generating as random a distribution of grafts along the PAA backbone as can be obtained, route *i* does not. The first step, carried out in organic solution, is expected to yield a largely random distribution of octyl chains. The second step, however, is carried in aqueous solution, that is, by grafting taurine onto self-assembled A8-75 particles, in which individual chains are presumed to expose to the solution their most hydrophilic regions. The end result is that the surface of these particles will be decorated with sulfonate groups, which substitute to part of the original carboxylates. Perhaps for this reason, SAPols obtained by this route presented the most satisfactory behavior upon SEC in aqueous buffers, yielding monodisperse populations of small particles. Monodispersity and purity could be further improved by preparative SEC. Extensive studies of A8-35 particles have shown that SEC data are in excellent agreement with radiation scattering and ultracentrifugation studies.^{17,18} On the basis of SEC analyses, SAPols obtained according to route *i* were therefore selected for biochemical and biophysical studies.

Two test MPs, BR and tOmpA, were trapped in SAPols using the conventional protocols of supplementing the protein in detergent solution with APols and either diluting the samples below the cmc of the detergent¹¹ or adsorbing the latter onto polystyrene beads.¹² The MP/SAPol mass ratios at which MPs were quantitatively retained in solution as water-soluble, monodisperse complexes were comparable to those determined earlier for A8-35 (see, e.g., Refs. 11, 20 and 21). The Stokes radius of tOmpA/SAPol particles, $R_s = 4.5\text{--}5$ nm, was also quite comparable to that of tOmpA/A8-35 ones (Ref. 20 and present work), suggesting that the complexes are similar, which is consistent with the comparable resolution of the solution NMR spectra.

A primary incentive in developing SAPols was to eliminate the aggregation that A8-35 particles and MP/A8-35 complexes undergo at $\text{pH} < 7$,^{18,21} which is a limitation in certain experimental circumstances. As expected, both SAPol particles and tOmpA/SAPol complexes remained soluble and monodisperse at acidic pH, down to the lowest pH tested, namely pH -1 (HCl 12 N) as regards the solubility of SAPols alone, pH 6.5 as regards the dispersity of MP/SAPol complexes. SAPols are also insensitive to the presence of Ca^{2+} ions.²⁹

The stability over time of SAPol-trapped BR was markedly dependent on the presence of salt, a phenomenon that is not observed with the less highly charged A8-35. The underlying

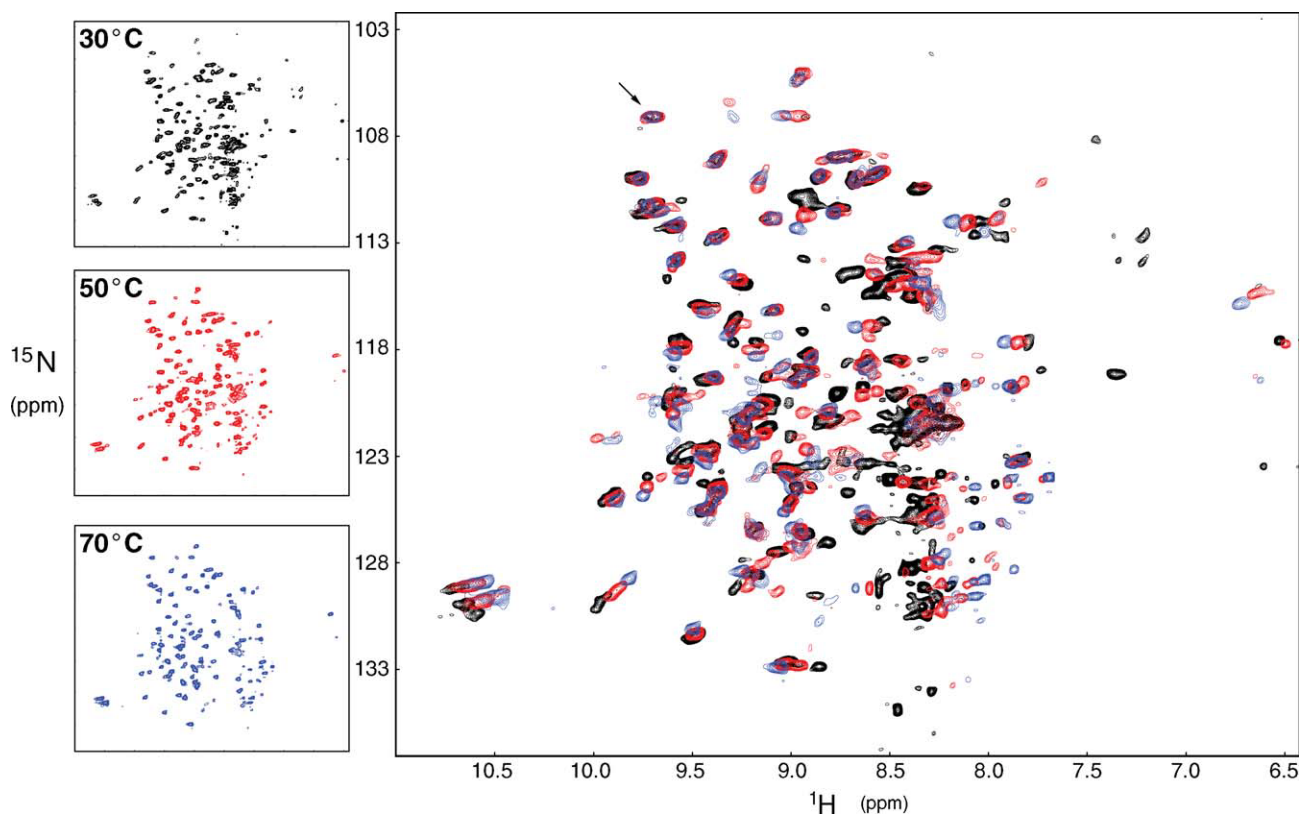


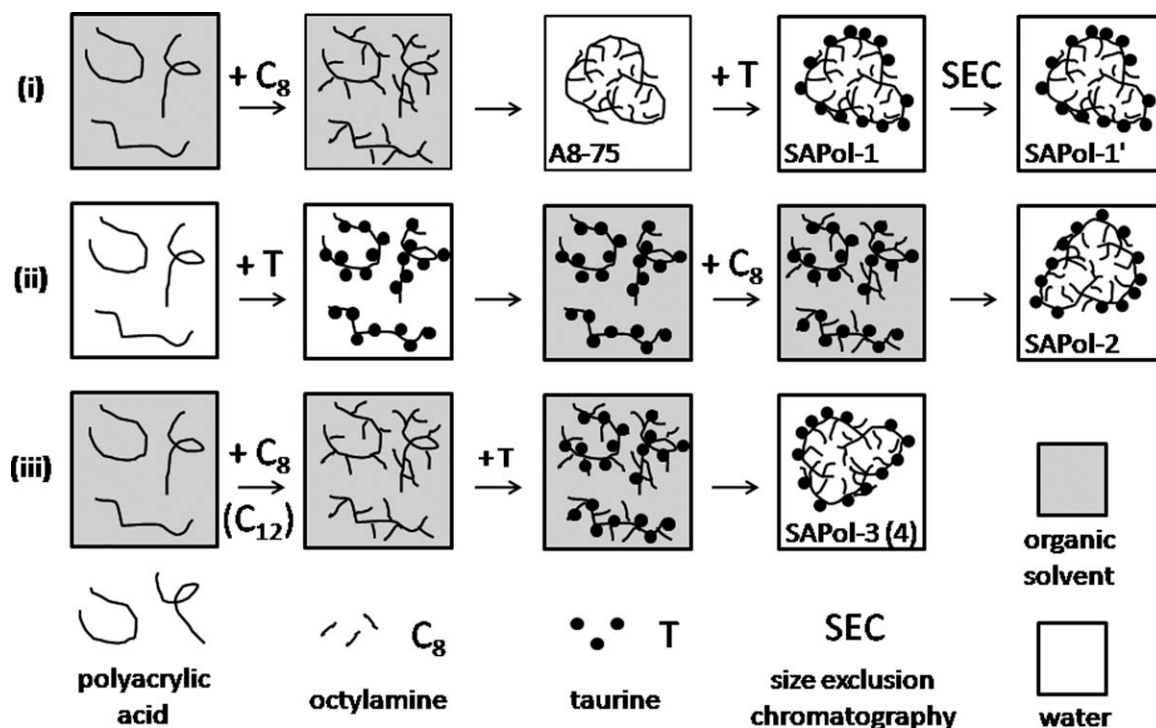
FIGURE 8 Comparison of 2D [$^{15}\text{N}, ^1\text{H}$]-TROSY spectra of SAPol-trapped [$u\text{-}^2\text{H}, ^{15}\text{N}$]tOmpA acquired at 30°C (in *black*), 50°C (in *red*), and 70°C (in *blue*). In the panel to the right, the spectra recorded at 50 and 70°C have been shifted so as to superimpose the G41 peak (arrow) to that observed at 30°C in order to facilitate the comparison of the chemical shifts of residues participating to the β -barrel structure (downfield in the ^1H dimension).

mechanism is, very likely, that at low ionic strength, MP-adsorbed SAPol molecules have a greater tendency to repulse each other, which favors unfolding of the protein. In the presence of 100 mM NaCl, the stability of BR appeared comparable in the two APols. It is worth noting that, in previous experiments carried out in the presence of 100 mM KCl, SAPols were found to stabilize the calcium-free sarcoplasmic calcium ATPase (SERCA1a), a particularly fragile MP, less efficiently than A8-35.²⁹ In the same series of experiments, it was noted that SAPols inhibited the enzymatic cycle of the ATPase to a lesser extent than A8-35 does. Inhibition and stabilization of SERCA1a by APols with respect to detergent solutions have been tentatively ascribed to a common cause dubbed the “Gulliver effect.”^{5,14,16,29} It is postulated that the relatively large-scale (nm) rearrangements of the protein’s transmembrane surface that accompany both the enzymatic cycle of SERCA1a^{51,52} and the opening of the transmembrane helix bundle that initiates its denaturation⁵³ entail a conformational adjustment of the APol backbone. This would increase the free energy of activation, slowing down both processes. It is possible that backbone rearrangements are facilitated in SAPols by

electrostatic repulsion, which would explain why SERCA1a is both more active and less stable after trapping with SAPols rather than with several less densely charged APols.²⁹

It has been shown previously that A8-35 is a remarkable medium in which to fold MPs from a denatured state to their native structure.^{23,27} This application was tested with SAPols using tOmpA as a model. Dilution of urea-denatured tOmpA into a urea-free SAPol solution led to quantitative reformation of the eight-stranded β -barrel characteristic of the native state, as deduced from the migration of the protein upon SDS-PAGE. The accuracy of the refolding was examined by comparing TROSY spectra of ^1H , ^{15}N -labeled tOmpA to that of the native form in detergent solution or A8-35. Chemical shifts were essentially indistinguishable, a direct proof that the protein folded in SAPols had acquired its correct 3D structure.

Extending the field of structural studies of MPs by solution NMR has been a major reason for developing SAPols. The resolution of TROSY spectra of ^1H , ^{15}N -labeled tOmpA folded in SAPols was found to be comparable to that observed for A8-35-trapped tOmpA. At variance with the latter, however, the



SCHEME 2 A schematic rendition of the synthesis routes to the various batches of SAPols studied in the present work.

pH could be lowered to 6.8 without inducing any aggregation. This made it possible to observe solvent-exposed, rapidly exchanging protons such as the amide protons of loop residues and the indole protons of tryptophane side chains, most of which are not seen at pH 8. When the temperature was raised from 30°C to 50°C or 70°C, the protein retained its structure. Working at high temperature, which APols make distinctly easier than detergents (see, e.g., Refs. 5, 16 and 27), increases both the sensitivity and resolution of NMR spectra, which is very helpful in 3D NMR experiments aimed at collecting information on chemical shifts assignments and structural constraints. For instance, the NMR structures of tOmpA and PagP were determined, respectively, at 50°C and 40°C,^{47,54} that is, under conditions that very few MPs resist in detergent solution. When compared with A8-35, the main advantage of SAPols for solution NMR studies is the larger pH range that becomes accessible. When compared with other pH-insensitive APols that have been described (see, e.g., Refs. 30 and 34), SAPols have the advantages of being more similar to A8-35, whose properties have been extensively studied, and of being easier to deuterate, an important advantage in many NMR experiments (see, e.g., Refs. 12, 13 and 35). Indeed, deuteration of SAPols, should it be required, could be achieved following the same route as used previously for A8-35.¹⁷

Two drawbacks that must be considered are that the current protocol for obtaining highly purified SAPols involves

time-consuming SEC purification steps, which limit the amounts of material that can be conveniently produced, and that their higher charge density is likely to make them less easily tolerated than A8-35 by particularly fragile MPs (cf. above). The synthesis and purification could conceivably be simplified by resorting to a procedure that does not involve any coupling agent, such as the use of microwaves (see, e.g., Ref. 55). Milder SAPols could likely be obtained by replacing most of the remaining free carboxylates with isopropylamide, so as to generate a sulfonated equivalent of A8-35 rather than of A8-75. Grafting all the carboxylates of PAA is not practically feasible (C. Prata & C. Tribet, personal communication; F.G., unpublished data), but alternative synthetic routes could be devised.

In summary, the present work shows that it is possible to synthesize sulfonated APols that efficiently trap MPs and keep them soluble even at acidic pH. In the presence of 100 mM salt, they stabilize BR against denaturation as efficiently as does the prototypical APol A8-35. SAPols can be used to fold tOmpA to its native form. They therefore present the potential to be used to fold and study MPs that are most stable at acidic pH. They also make it possible to study MP structures by solution NMR at any desirable pH, generating more complete sets of data than can be obtained using A8-35. Finally, it is worth noting that APols are starting to be used to deliver MPs or hydrophobic peptides to cells or

whole organisms, for example for therapeutic purposes.^{16,28} It is likely that the fate of the carrier APols upon their internalization in acidic endocytotic compartments, and, therefore, their effects on cellular trafficking will depend on both their chemical composition and their sensitivity to pH changes. From this point of view as well, having access to a range of APols with distinct chemical structures and physical chemical properties is likely to prove useful.

MATERIALS AND METHODS

Materials and Buffers

All organic solvents were from SDS (Vitry, France). Peptone LP0037 was from Oxoid (Dardilly, France), urea ultrapure grade from USB/Affymetrix (Cleveland, Ohio), and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) from Cortecnet (Paris, France). PAA and taurine were from Acros (Halluin, France). OTG was from Anatrax/Affymetrix and C₈E₄ was from Bachem (Philadelphia, PA). All other reagents were from Aldrich (Saint Quentin Fallavier, France). The average molecular weight of PAA was estimated by gel permeation chromatography¹⁷ to be ~5 kDa. All aqueous solutions were made in water purified on a MilliQ-academic system equipped with a Q-Gard1 cartridge and two Organex cartridges (Millipore, Saint-Quentin-en-Yvelines, France) ("mQ-water"). Tris buffer: Tris/HCl 20 mM, pH 8, 100 mM NaCl. Sodium phosphate (NaPi) buffers: NaPi₁: 20 mM NaPi, pH 6.5, and 100 mM NaCl; NaPi₂: 20 mM NaPi, pH 7, and 100 mM NaCl; NaPi₃: 20 mM NaPi and pH 7; NaPi₄: 20 mM NaPi, pH = 6.0, and 100 mM NaCl). Urea buffer: 20 mM NaPi, pH 6.5, 100 mM NaCl, and 8 M urea. SDS buffer: 4 mM Tris/HCl, pH 8.0, 20 mM NaCl, and SDS 2% w/v.

Synthesis of A8-35

A8-35 (batch FGH20) was synthesized as described in Ref. 18. Briefly, the PAA precursor was hydrophobically modified in two steps, first with octylamine, then with isopropylamine, in NMP, in the presence of the coupling reagent DCI. After purification and neutralization (both performed in aqueous medium), the solution of A8-35 was dialyzed and freeze-dried (yield 90%).

Synthesis of SAPol-1 and SAPol-1'

The PAA precursor (1 g and 13.9 mmol of carboxylic acid functions) was first grafted with octylamine (0.45 g and 3.5 mmol) in NMP, using DCI as the coupling reagent,¹⁸ yielding A8-75. Following purification,¹⁸ A8-75 (1.6 g) was dissolved in 100-ml mQ-water along with taurine (0.7 g and 5.6 mmol). EDC·HCl (pH 7) was added (1.074 g and 5.6 mmol), and the solution was stirred for 12 h at room temperature. Two milliliters of NaOH 10N were then added, and the solution of polymer was dialyzed against pure water for 1 week. It was then lyophilized to yield 2.05 g of SAPol-1 (batch FGH58) which is still contaminated with the EDC urea derivative formed during the coupling of taurine. To remove it, 1.5 g of the polymer was fractionated in Tris buffer on an XK-26/100 Superose column,¹⁸ brought to pH 11 with NaOH, concentrated, dialyzed for 4 h against mQ-water, and lyophilized, yielding the pure SAPol (SAPol-1', batch FGH58_{frac}) (0.4 g and 27%).

Synthesis of SAPol-2

In brief, PAA was first modified with taurine in mQ-water in the presence of EDC/HCl, as described above, yielding, after precipitation in ethanol, the sodium salt of poly(acrylate-*co*-*N*-(2-ethanesulfonate) acrylamide). This precursor was grafted with octylamine in the presence of DCI in a 15:1 vol/vol NMP/water mixture heated at 60°C for 14 h. Purification by precipitation and dialysis against mQ-water yielded SAPol-2 (batch FGH57, 43%; see Supporting Information 1.1 for the detailed procedure).

Synthesis of SAPol-3

PAA was successively grafted in organic solution with octylamine and with the sodium salt of taurine in the presence of DCI and 1-hydroxybenzotriazole, according to a procedure similar to that described in Ref. 18. Purification yielded SAPol-3 (batch FGH523, 64% yield; see Supporting Information 1.2 for the detailed procedure).

Synthesis of SAPol-4

One gram PAA was modified as described for SAPol-3, except for substituting octylamine with dodecylamine. Purification yielded SAPol-4 (batch FGH520, 71% yield; see Supporting Information 1.3 for the detailed procedure).

Chemical Analysis of SAPols

The chemical composition of the four SAPols was determined by ¹H and ¹³C NMR spectroscopy analysis performed in deuterated methanol, as reported in Supporting Information 2. The results are summarized in Table I.

Purification of Purple Membrane and Solubilization of BR

Halobacterium salinarum cells (S9 strain, a gift of G. Zaccai, IBS, Grenoble) were grown under illumination at 37°C in a liquid growth medium-containing 12 g L⁻¹ peptone (LP0037, Oxoid).⁵⁶ PM was isolated as described⁵⁷ and stored at -80°C. The next steps were performed at 4°C in the dark. PM, containing 4–6 g L⁻¹ BR, was incubated for 40 h under constant magnetic stirring with 100 mM OTG (final concentration) in either NaPi₂ or NaPi₃ buffer. The solubilized PM was briefly ultracentrifuged (200,000 × *g* for 20 min). The concentration of BR in the supernatant was estimated using $\epsilon_{554} = 43 \text{ mM}^{-1} \text{ cm}^{-1}$.⁵⁸

Overexpression, Folding, and Purification of tOmpA

The transmembrane region of *Escherichia coli*'s outer MP A (tOmpA) was expressed from plasmid pET3b-OmpA171, encompassing the coding region for tOmpA,⁴⁵ to which an eight-histidine tag was added at the *N*-terminus.²⁰ tOmpA was overexpressed as inclusion bodies and folded either in detergent or in SAPol. For detergent-assisted folding, tOmpA was folded and purified as described.⁴⁵ Protein purification in C₈E₄ was performed by immobilized metal affinity chromatography.⁵⁹ The final buffer contained 20 mM Tris/HCl, pH 8, and 0.6% (19.6 mM) C₈E₄. For SAPol-assisted folding, inclusion bodies were solubilized in urea buffer, and the solution was slowly injected (typically over 2–3 h) into urea-free NaPi₁

buffer containing SAPol (to a final ratio tOmpA/SAPol 1:4 w/w and final concentration of urea 0.88M). Refolded tOmpA in SAPol-1 was concentrated by centrifugation (Centricon from Millipore, 10 kDa cut-off) and transferred during the concentration step to NaPi₁ buffer-containing 5% D₂O.

Preparation and Stability of BR/APol and tOmpA/APol Complexes

APol, as a 10% stock solution in water, was added in a 5:1 APol/BR mass ratio to solubilized BR in OTG 18 mM. After 25 min, the samples were diluted 3× into detergent-free NaPi₂ or NaPi₃ buffer, pH 7, thus bringing the concentration of OTG below its cmc. The stability of APol-trapped BR was followed by monitoring the absorbance at 554 nm of the native holoprotein. To trap tOmpA, the same APol stock solution was added in a 4:1 APol/tOmpA mass ratio to purified tOmpA in C₈E₄ 19.6 mM. After 25 min of incubation, the samples were diluted 10× into detergent-free Tris buffer (A8-35) or in NaPi₁ buffer (SAPols).

SEC Analysis

All size exclusion chromatography (SEC) experiments were performed on an Äkta Explorer 100 (Pharmacia) FPLC system equipped with a Superose 12 HR 10/30 column (Pharmacia). The column was calibrated as described¹⁷ in either Tris buffer pH 8 or NaPi₁ buffer. Elution was monitored by UV-vis detection. For each polymer, 60 μL of a 10 g L⁻¹ buffered solution was loaded and eluted on the equilibrated column with either Tris buffer (for A8-35 and all SAPols) or NaPi₁ buffer (for SAPol-1 at pH 6.5). The samples of tOmpA/SAPol and tOmpA/A8-35 complexes to be analyzed by SEC were prepared as described above, except that, following the addition of APols, the detergent was removed by adsorption onto polystyrene beads (Bio-Beads SM2) for 2 h at room temperature (1:10 detergent/beads weight ratio), in order not to dilute the protein, and 80 μL of sample were loaded onto the column and eluted with either Tris buffer (tOmpA/A8-35 complexes) or NaPi₁ (tOmpA/SAPol complexes). Stokes radii (R_s) and the half-height width of the peaks (HHW) are listed in Tables II and III.

NMR Spectroscopy of tOmpA/SAPol Complexes

NMR experiments were carried out on a Bruker DRX700 Avance II spectrometer. 2D [¹H, ¹⁵N] TROSY experiments^{49,50} were performed with 64 transients per increment and a time domain data size of 128 × 1024 complex point [$t_{1\max}$ (¹⁵N) = 56 ms and $t_{2\max}$ (¹H) = 244 ms], as described in Ref. 36. Data were processed with NMRPipe⁶⁰ and visualized with NMRView⁶¹ softwares.

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