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Nonionic Homopolymeric Amphipols: Application to Membrane Protein Folding, Cell-Free Synthesis, and Solution Nuclear Magnetic Resonance

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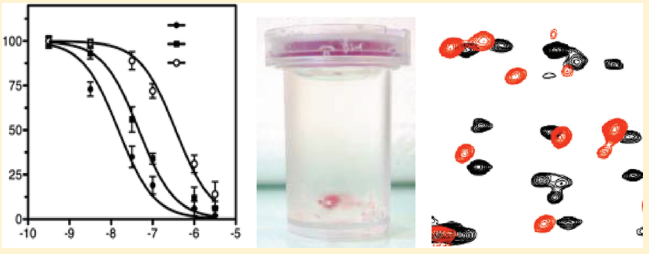
ABSTRACT: Nonionic amphipols (NAPols) synthesized by homotelomerization of an amphiphatic monomer are able to keep membrane proteins (MPs) stable and functional in the absence of detergent. Some of their biochemical and biophysical properties and applications have been examined, with particular attention being paid to their complementarity with the classical polyacrylate-based amphipol A8-35. Bacteriorhodopsin (BR) from *Halobacterium salinarum* and the cytochrome b_6f complex from *Chlamydomonas reinhardtii* were found to be in their native state and highly stable following complexation with NAPols. NAPol-trapped BR was shown to undergo its complete photocycle. Because of the pH insensitivity of NAPols, solution nuclear magnetic resonance (NMR) two-dimensional $^{1}H-^{15}N$ heteronuclear single-quantum coherence spectra of NAPol-trapped outer MP X from *Escherichia coli* (OmpX) could be recorded at pH 6.8. They present a resolution similar to that of the spectra of OmpX/A8-35 complexes recorded at pH 8.0 and give access to signals from solvent-exposed rapidly exchanging amide protons. Like A8-35, NAPols can be used to fold MPs to their native state as demonstrated here with BR and with the ghrelin G protein-coupled receptor GHS-R1a, thus extending the range of accessible folding conditions. Following NAPol-assisted folding, GHS-R1a bound four of its specific ligands, recruited arrestin-2, and activated binding of GTPγS by the $G_{\alpha q}$ protein. Finally, cell-free synthesis of MPs, which is inhibited by A8-35 and sulfonated amphipols, was found to be very efficient in the presence of NAPols. These results open broad new perspectives on the use of amphipols for MP studies.



Specially designed amphipathic polymers called amphipols (APols) were introduced 15 years ago as milder substitutes for detergents for handling and studying membrane proteins (MPs) in vitro.[1] All MPs tested to date can be kept soluble in detergent-free solutions as complexes with APols, and most of them are strongly stabilized compared to detergent solutions (reviewed in refs 2 and 3). Applications of APols that have been validated to date include folding MPs to their native state, immobilizing them onto solid supports, studying them by nuclear magnetic resonance (NMR) and by electron microscopy, and delivering them to lipid vesicles, black lipid films, or living cells (for a general review, see ref 3), as well as formulating them for vaccination purposes.[4] Most of these studies were conducted using a specific APol, called A8-35 (Figure 1A); its solution properties have been studied in detail by a broad range of biophysical techniques (reviewed in ref 3) and modeled by molecular dynamics simulation.[5]

While it is by far the best-studied and most widely used APol, A8-35 suffers from limitations caused by the fact that (i) it is charged and (ii) its aqueous solubility depends on its carboxylate moieties being deprotonated. Upon protonation, which starts at or just below pH 7,[6,7] or in the presence of multivalent cations such as Ca^{2+},[8,9] A8-35 particles and MP/A8-35 complexes become more hydrophobic, aggregate, and may precipitate. In solution NMR studies, this behavior prevents access to the mildly acidic conditions that are required for slowing the exchange of solvent-exposed amide protons with the solution.[10] It also limits the range of conditions that can be explored when optimizing the yield of APol-assisted MP folding and, more generally, stabilization conditions. Presumably because it is charged, A8-35 cannot be used as an accepting

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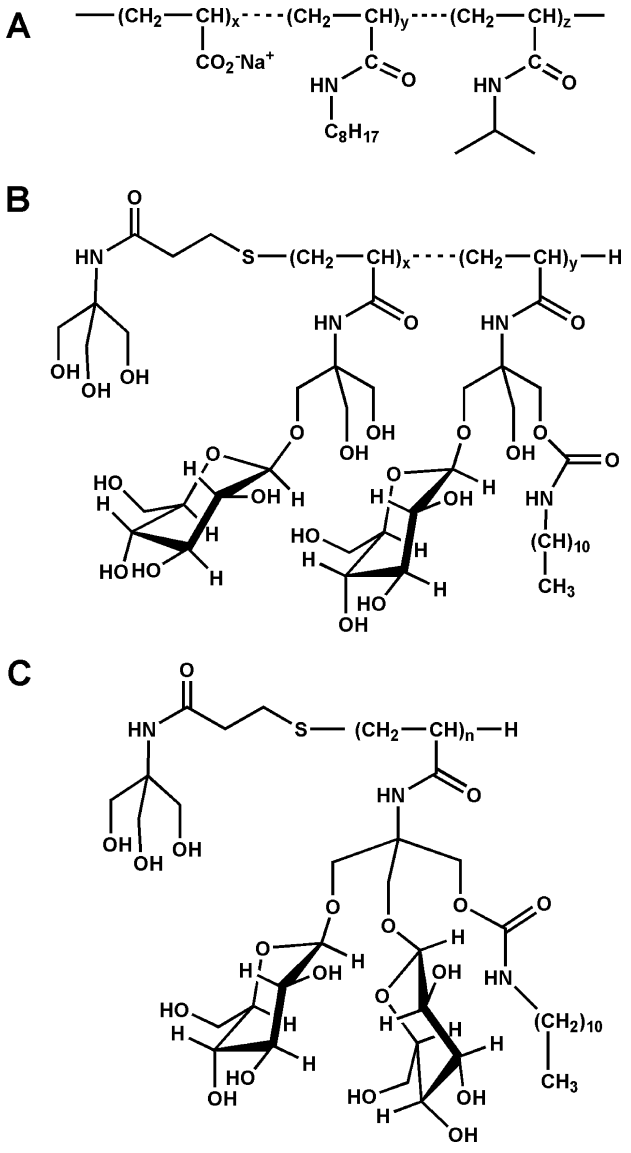

Figure 1. Chemical structures of various amphipols. (A) Amphipol A8-35.[1] (B) Heteropolymeric, glucosylated, nonionic amphipols obtained either by cotelomerization[15] or by grafting of a homopolymer.[16] (C) Homopolymeric, glucosylated, nonionic amphipols obtained by telomerization of an amphipathic monomer.[17,18]

medium for cell-free MP synthesis, which it inhibits.[11] Finally, the fact that A8-35 carries a net charge hampers the separation of MP/A8-35 complexes by electrofocusing or ion-exchange chromatography, and it is far from an optimal property for MP stabilization and crystallization. These and other considerations have prompted the design and validation of APols with alternative structures (reviewed in ref 3), among which are zwitterionic[9,12] or sulfonated APols (SAPols).[13] Of particular interest, however, would be the development of totally nonionic APols. The feasibility of such an approach was demonstrated 10 years ago,[14] but the solubility of the polymers developed in this first stage, which relied on the presence of multiple hydroxyl groups, proved to be insufficient for routine use.

A much higher solubility can be achieved with glycosylated APols, the first of which were obtained either by cotelomerization of hydrophobic and hydrophilic monomers[15] or by randomly grafting hydrophobic chains onto a glycosylated homotelomer,[16] yielding heteropolymers (Figure 1B). A recent study established that equivalent results can be obtained with simpler NAPols, obtained by homotelomerization of a monomer carrying two glucose moieties and a single alkyl chain.[17] These new polymers (Figure 1C), which are the first homopolymeric APols to be validated, are easier to synthesize in a batch-to-batch reproducible manner, and they are less heterogeneous at the molecular level, because of the absence of the group distribution variability that is inherent both to copolymerization and to random grafting. We describe elsewhere the synthesis and solution properties of homopolymeric NAPols and demonstrate their ability to keep MPs water-soluble in the absence of detergent.[17] Analytical ultracentrifugation (AUC), small angle neutron scattering (SANS), and size exclusion chromatography (SEC) studies show that homopolymeric NAPols spontaneously organize into small, micelle-like particles, similar in size to those formed by A8-35.[17] They efficiently trap two model MPs, bacteriorhodopsin (BR) from *Halobacterium salinarum* and outer membrane protein X (OmpX) from *Escherichia coli*. MP/NAPol complexes are well-defined, and their size is comparable to that of MP/A8-35 complexes.[17] In this work, we characterize the stability and functionality of several MPs trapped with or folded in homopolymeric NAPols (hereafter simply designated NAPols) and examine some of the applications of these new molecules, with a particular emphasis on their complementarity with those of A8-35.

EXPERIMENTAL PROCEDURES

Buffers. SDS buffer consisted of 5% SDS (Bio-Rad), 50 mM sodium phosphate, and 100 mM NaCl (pH 7.0). K buffer consisted of 30 mM potassium phosphate and 150 mM KCl (pH 7.0). KP buffer consisted of 50 mM potassium phosphate and 150 mM KCl (pH 7.5). Tris buffer consisted of 20 mM Tris-HCl and 100 mM NaCl (pH 8.0). Phosphate buffer consisted of 100 mM NaCl and 20 mM sodium phosphate (pH 7.0). NMR buffer consisted of 100 mM NaCl, 20 mM phosphate buffer, and 0.05% NaN_3 (pH 6.8) in 10% D_2O. Triton X-100/phosphate saline buffer (TX/PBS) consisted of 50 mM Na_2HPO_4, 300 mM NaCl, and 0.2% Triton X-100 (pH 7.5).

Amphipol Synthesis. A8-35 (batch CLBH4) was synthesized as described in refs 6 and 7. NAPols were synthesized as described in refs 17 and 18. Briefly, the synthesis is based on free radical homopolymerization of a diglucosylated amphiphilic monomer[19] in the presence of a thiol-based transfer agent.[15] The number-average molecular weights ($\langle M \rangle$) and number-average degrees of polymerization (DPn) of the three batches of NAPols used here are listed in Table 1 (from ref 17).

Table 1. Characteristics of the Three Batches of Homopolymeric NAPols Used in This Study

batch number (sample code)	DPn[a]	$\langle M \rangle^b$ (kDa)
SS174 (NA10)	15	10
SS298 (NA29)	43	29.5
SS325 (NA11)	17	11.3

[a]The degree of polymerization is the number of monomer units + 1 (cf. ref 17). [b]Number-average molecular mass. Data and codes from ref 17.

Production of OmpX. Overexpression of uniformly ^2H-, ^{13}C-, and ^{15}N-labeled OmpX ([U-^2H,^{13}C,^{15}N]OmpX) in *E. coli*

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using D_2O-based minimal growth medium (>99% ^2H) with 2 g/L [^2H,^{13}C]-D-glucose (>97% ^2H and >98% ^{13}C) and 1 g/L $^{15}NH_4Cl$ (>99% ^{15}N) and purification of inclusion bodies were similar to procedures described previously.[20,21] Inclusion bodies were solubilized in 6 M urea, 20 mM Tris-HCl, and 5 mM EDTA (pH 8.5). OmpX was refolded by slow dilution[21] into a detergent solution of dihexanoylphosphatidylcholine (DHPC, Avanti Polar Lipids) at 4 °C. The final protein concentration was 0.46 g/L in 2% (w/v) DHPC.

Production of Bacteriorhodopsin. *H. salinarum* (S9 strain, a gift of G. Zaccaï, IBS Grenoble, Grenoble, France) was grown as described in ref 22. Purple membrane (PM) was isolated as described in ref 23 and stored at −80 °C. Solubilization with octyl thioglucoside (OTG; cmc ≈ 9 mM) was conducted at 4 °C in the dark as described in ref 24.

Expression of the Ghrelin Receptor in Inclusion Bodies. The sequence encoding the human ghrelin receptor GHS-R1a was cloned downstream of that encoding the α_5 integrin fragment[25] and a thrombin cleavage site. Receptor expression and purification under denaturing conditions were conducted as described in ref 26.

Purification of Cytochrome b_6f from *Chamydomonas reinhardtii*. Cytochrome b_6f was purified in 0.2 mM *n*-dodecyl β-D-maltoside (DDM) using the method described in ref 27. Plastoquinol:plastocyanin electron transfer activity was measured in 0.2 mM DDM as described in ref 28. The cytochrome b_6f concentration in the samples was 1.6 μM. A remark about apparent differences between results obtained here and in ref 1 is in order. The b_6f complex used in ref 1 was solubilized in a mixture of Hecameg and lipids,[28] whereas that used here was in very low concentrations of dodecyl maltoside (DDM) not supplemented with lipids.[27] When the complex is transferred to A8-35 from a Hecameg/lipid solution, its stability remains roughly unchanged.[1] When transferred to A8-35 from a DDM solution, it becomes markedly less stable (see Results). The origin of this difference has not been explored in detail. It may be related to a dearth of lipids in the b_6f/A8-35 complexes formed by transfer from DDM.

Trapping of BR with NAPols. This was conducted as described in ref 16.

Preparation of Cytochrome b_6f/APol Complexes. Cytochrome b_6f preparations were incubated for 20 min at 4 °C with aliquots of NAPol, SAPol, and A8-35 stock solutions (100 g/L in water) to produce the final indicated protein:polymer mass ratio. DDM was removed by overnight incubation with polystyrene beads (Bio-Beads SM2, Bio-Rad). After centrifugation at 100000*g* for 20 min, the concentration of b_6f was determined spectroscopically.

Preparation of OmpX/NAPol Samples for NMR Spectroscopy. [U-^2H,^{13}C,^{15}N]OmpX/NAPol complexes were prepared as reported previously for OmpX/A8-35 complexes,[29,30] using a 1:4 OmpX:NAPol ratio (w/w). After incubation with NAPols for 30 min, DHPC was removed by adsorption onto Bio-Beads (10:1 bead:detergent mass ratio). The beads were removed by centrifugation after incubation at room temperature for 3 h. To remove any traces of DHPC, 10 cycles of dilution and concentration with NMR buffer were performed using a centrifugal filter unit (10 kDa cutoff, Amicon, Millipore). The final NMR sample contained ∼1.3 mM [U-^2H,^{13}C,^{15}N]OmpX.

NMR Spectroscopy. Two-dimensional (2D) NMR $^{15}N-^1H$ heteronuclear single-quantum coherence (HSQC) experiments were conducted at 30 °C on a Bruker Avance II 700 spectrometer equipped with a 5 mm triple-resonance (TXI) gradient probe as described previously for OmpX/A8-35 complexes.[30] The following parameters were used: 256 (t_1) × 1024 (t_2) complex points, $t_{1max}(^{15}N)$ = 281 ms, $t_{2max}(^1H)$ = 341 ms, and eight transients per increment. Samples of OmpX in DHPC at both pH 6.8 and 8.0 were used to assign peaks in the HSQC spectra of OmpX/A8-35 and OmpX/NAPol complexes (chemical shift assignments are those available for OmpX/DHPC complexes in the Biological Magnetic Resonance Data Bank, BMRB entry 4936). Data processing was performed with TOPSPIN.

Renaturation of Bacteriorhodopsin. BR renaturation was conducted by precipitating dodecyl sulfate as its potassium salt (PDS), as described in ref 31. NAPols were added to SDS-solubilized PM. After a 15 min incubation at room temperature, the solutions were supplemented, under vigorous stirring, with enough KCl (4 M) to bring the nominal concentration of free K^+ ions to 150 mM.[32] After 30 min, the PDS precipitate was removed by two 5 min centrifugations at 6000 rpm (∼6000*g*) in a Hettich Mikro 12-24 centrifuge. The supernatant was dialyzed twice against 100 volumes of K buffer [100 mM NaCl, 150 mM KCl, and 30 mM potassium phosphate (pH 7)] for a total of 48 h at room temperature, to remove residual dodecyl sulfate. The final concentration of renatured BR was in the range of 0.3−0.4 g/L.

Folding of the Ghrelin Receptor. NAPols were added at a ratio of 10 g of NaPol per gram of SDS-unfolded GHS-R1a receptor in the presence of asolectin at a 0.2:1 (w/w) lipid:amphipol ratio, with cholesteryl hemisuccinate (0.02%, w/v). After incubation at room temperature for 30 min, folding was initiated by precipitating dodecyl sulfate with KCl. After 30 min, the PDS precipitate was removed by two 10 min centrifugation runs at 16100*g*. The supernatant was dialyzed against KP buffer.

Alexa Fluor 350 Labeling of the Ghrelin Receptor. The ghrelin receptor in KP buffer was incubated with Alexa Fluor 350 carboxylic acid succinimidyl ester (10:1 dye:protein molar ratio) for 2 h at room temperature under constant stirring. The conjugate was separated from unreacted labeling reagent by dialysis against KP buffer. The relative efficiency of the labeling reaction was determined by measuring the absorbance of the protein at 276 nm and that of the dye at its maximal absorbance (346 nm).

Ghrelin Receptor Functional Assays. Ligand binding assays were performed using fluorescence energy transfer with the purified receptor labeled with Alexa Fluor 350 and a ghrelin peptide, JMV4946, labeled with fluorescein isothiocyanate-1 (FITC). Competition experiments were conducted by adding increasing concentrations of the competing compound to a receptor/JMV4946 mixture (100 nM concentration range). Fluorescence emission spectra were recorded at 20 °C between 400 and 600 nm on a Cary Eclipse spectrofluorimeter (Varian) with excitation at 346 or 488 nm. Buffer contributions were systematically subtracted. The FRET ratio corresponds to the ratio of the acceptor-emitted fluorescence at 520 nm upon excitation at 346 nm versus that at 488 nm (for details, see ref 33). Binding data were analyzed using GraphPad Prism (version 4.0). GTPγS and bimane recruitment assays were conducted as described in ref 33.

Cell-Free Synthesis of BR. The bacterio-opsin coding region was amplified by polymerase chain reaction (PCR) from the chromosomal DNA of *H. salinarum* and subcloned into the pIVEX-2.4b plasmid (Roche) carrying an N-terminal poly-

histidine tag. Small- and large-scale syntheses were performed with 0.5 and 15 μg of plasmid in 50 μL of RTS 100 HY and 1 mL of RTS 500 HY lysate (5Prime) for 6 and 20 h, respectively. A8-35, SAPols, or NAPols were added to the synthesis medium at concentrations ranging from 1 to 10 g/L. DDM was used as a control at a concentration of 2 mM. Incubation was conducted at 600 rpm at 30 °C in an Eppendorf Thermomixer. To purify the recombinant protein, the lysate was incubated overnight at 4 °C with 1 mL of Ni-NTA agarose beads (Qiagen) and equilibrated in 20 mM $NaPO_4$ (protein buffer) containing 10 mM imidazole. The column was then packed and washed with protein buffer and 20 mM imidazole. Bound proteins were eluted in four steps with 200 μL of protein buffer and 250 mM imidazole. After purification, protein samples were analyzed via sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) on 15% polyacrylamide gels. Concentrations were determined using the bicinchoninic acid method (Pierce Chemical Co.). For Western blot analysis, the samples were centrifuged at 16100*g* for 10 min at 4 °C to separate aggregated protein from soluble protein. The pellet and supernatant were analyzed by electrophoresis on SDS−urea−12−18% polyacrylamide gels and detected by immunoblotting using an anti-$(His)_6$ antibody (Sigma). The stability of the purified BR was tested spectroscopically by monitoring the A_{554}/A_{280} ratio.

Analysis of the Photocycle of BR by Time-Resolved Absorption Spectroscopy. Photocycle measurements were performed with a pump−probe spectrophotometer[34] in which the probe and pump flashes were provided by an Optical Parametric Oscillator (Panther, Continuum) and a frequency-doubled Nd:Yag laser (Brilliant, Quantel), respectively. The analysis was conducted as described in ref 31.

RESULTS

In the following, we characterize the functionality and stability of several NAPol-trapped MPs and present a first investigation of some applications for which the physical and chemical properties of NAPols appear to present practical advantages over those of A8-35 or SAPols. Three NAPol samples were used in this work. Their properties are summarized in Table 1. Although their average mass was set to spread the range from ∼10 kDa (batches SS174 and SS325) to ∼30 kDa (batch SS298), they behaved very similarly: all samples form free APol particles of the same size, and they trap and stabilize MPs equally efficiently.[17] In this work, no differences were found among these three batches (see Figures 2, 4, and 6).

Functionality of NAPol-Trapped Bacteriorhodopsin. Bacteriorhodopsin (BR) is a light-activated proton pump that accumulates in the plasma membrane of the archaebacterium *H. salinarum*, where it forms so-called purple membrane (PM) patches. When BR is illuminated, it undergoes a complex photocycle, as a result of which the absorption of a photon drives the transport of a proton from the cytoplasm to the extracellular medium. Transitions between photocycle states are accompanied by spectral changes, because of the sensitivity of BR's chromophore, retinal, to its protein environment (for a review, see ref 35). The spectral state designated M corresponds to two conformationally different but spectroscopically indistinguishable states, M_1 and M_2. The kinetics of the spectroscopic transition to the M state therefore results from a rapid transition from L to M_1, followed by slower equilibration among the L, M_1, and M_2 states. In the two M states, the Schiff base that covalently binds retinal to a lysine residue is deprotonated, which gives rise to a large hypsochromic shift, the absorption maximum of retinal being shifted to the blue by ∼150 nm. The whole cycle takes a few tens of milliseconds at room temperature and can be conveniently followed by time-resolved absorption spectroscopy.[35]

BR was solubilized from PM, along with PM lipids, using OTG.[24] It was transferred to either NAPols or A8-35 by addition of the polymers to the detergent solution and adsorption of OTG onto polystyrene beads.[17,24] APol-trapped BR remains in the supernatant upon ultracentrifugation of the detergent-depleted samples, while it precipitates in the absence of APols.[17,24] The kinetics of the buildup and decay of the M states by NAPol-trapped BR following an actinic laser flash was followed over a time scale of 5 ns to 500 ms, as described in ref 31, and compared to that of BR in PM, its natural environment, in OTG, and after being trapped with A8-35 (Figure 2).

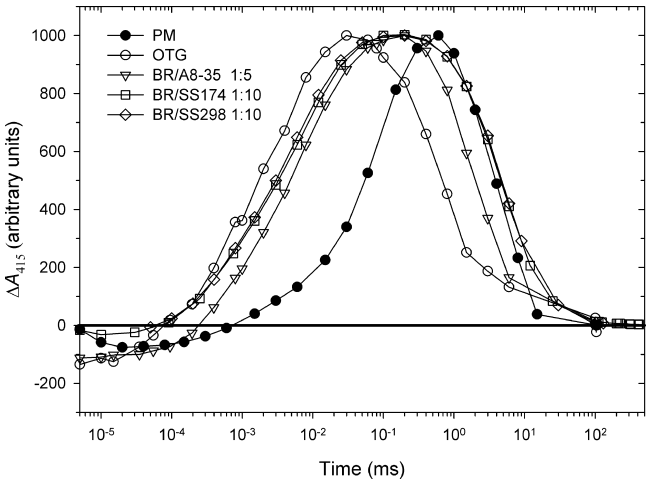

Figure 2. Kinetics of flash-induced absorption changes of NAPol-trapped BR. The photocycle was triggered by a saturating laser flash (532 nm, 5 ns full width at half-maximum, 5 mJ/cm^2) and the rise and decay of the M states followed by measuring time-resolved absorption changes at 415 nm.[31] For the sake of readability, the profiles obtained with five distinct samples have been normalized to the same maximum. BR was either in its native purple membrane (●), solubilized in 18 mM OTG (○), or trapped in either NAPol SS174 (□), NAPol SS298 (◇), or A8-35 (▽) at the indicated protein:APol mass ratio.

As is classically observed whenever BR is solubilized,[36] the accumulation of the M states is strongly accelerated in NAPol-trapped BR, as is also the case both in OTG solution and after transfer to A8-35.[24] This phenomenon is attributed to a conformational relaxation that would bring the side chain of Asp-85, the residue that accepts the Schiff base's proton, closer to the latter.[36] The rate of buildup of M in NAPols is intermediate between those observed in OTG and A8-35, with a suggestion that it is somewhat less homogeneous, the full transition to M being spread over a slightly larger time scale (Figure 2).

Solubilization of BR by OTG leads to a polyphasic decay of M, with, as compared to PM, a major phase that is strongly accelerated and a minor one that is decelerated (Figure 2).[31] A more detailed deconvolution of multiwavelength spectral data reveals that this is due to a transient population of state N that lasts longer in OTG solution than in PM (see ref 24). When the sample is transferred to A8-35, the kinetics of M decay goes

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back to nearly, although not fully, that observed in PM[24,31] (Figure 2). Following refolding of SDS-denatured BR in A8-35 in the presence of PM lipids, the same decay rate is observed as upon trapping native BR,[31] while the rate of decay of BR folded in A8-35 in the absence of lipids resembles that in detergent solution.[37] This observation has led to the suggestion that the rebinding of PM lipids to specific sites on the transmembrane surface of BR may be responsible for the recovery of native-like decay kinetics upon the transfer from OTG to A8-35.[37] When native, OTG-solubilized BR is transferred to NAPols, the kinetics of M decay becomes essentially indistinguishable from that in PM, but for a small late phase (Figure 2). This could mean that the rebinding of PM lipids to BR is even more extensive in NAPols than it is in A8-35.

Stability of NAPol-Trapped Cytochrome b_6f and BR. The photosynthetic b_6f complex extracted from thylakoid membranes of the chloroplast of the unicellular green alga *C. reinhardtii* is a superdimer, each monomer comprising eight transmembrane subunits and numerous cofactors, including a molecule of chlorophyll *a* and many lipids.[27,28] The b_6f complex is a useful model for testing novel surfactants, because of its exquisite sensitivity to detergent-induced inactivation.[28,38] Inactivation, which is accelerated under delipidating conditions, is accompanied by the loss of two of the subunits, monomerization, and a bathochromic shift of the main visible absorption peak of chlorophyll *a* from ~667 to ~670 nm.[38,39] This spectral shift, which is due to the cholorophyll being released from its binding site, offers a convenient way to follow the evolution of the complex over time (Figure 3). The released chlorophyll is unstable and bleaches rapidly, which is also diagnostic of the inactivation of the complex.[39]

The purified b_6f complex in a 0.2 mM DDM solution was supplemented with NAPols, SAPols, or A8-35 and DDM removed by overnight incubation with Bio-Beads. The samples were stored at 4 °C, and the evolution of their absorption spectrum was followed over time. Control samples were kept in DDM. After 20 days, the visible absorption spectrum of chlorophyll *a* was found to peak at 667 nm, which is characteristic of the native state, when cytochrome b_6f was either kept in 0.2 mM DDM or transferred to NAPols (Figure 3A). The peak shifted to 670 nm, on the other hand, when it was transferred to either A8-35 or SAPols (Figure 3A). This shift indicates that the complex had become inactivated, which was confirmed by electron transfer measurements performed according to ref 28 (not shown). When measured shortly after the transfer to NAPols, the ratio of the absorption peaks of chlorophyll *a* and reduced cytochrome *f* was identical to that in DDM (Figure 3B) and typical of that of freshly prepared, native complexes.[28,39] After the samples had been stored at 4 °C in the dark for 20 days, this ratio had dropped by ~66% in DDM solution, versus only ~20% in NAPols (Figure 3B), indicating that the corresponding fraction of chlorophyll had dissociated and become bleached. The b_6f complex, therefore, is markedly more stable after being trapped by NAPols than it is in even very low concentrations of DDM.

Stabilization upon the transfer to NAPols was also observed with BR. Over a period of 13 days at 4 °C in the dark in phosphate buffer, OTG-solubilized BR was inactivated by almost 50%, while NAPol- and A8-35-trapped BR remained perfectly stable (Figure 4). As shown below, BR synthesized in vitro in the presence of NAPols was stable for months under the same conditions.

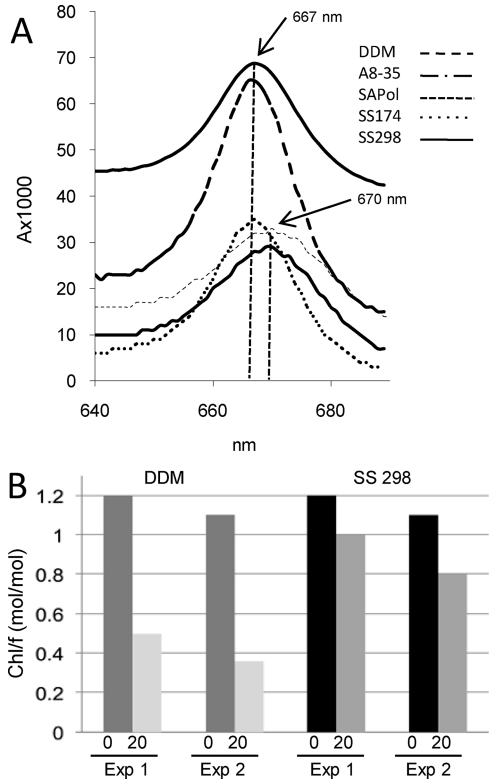

Figure 3. Biochemical stability of NAPol-trapped cytochrome b_6f. (A) Absorption spectrum of cytochrome b_6f purified in DDM and subsequently transferred to either A8-35, NAPol SS174, NAPol SS298, or SAPols. Spectra, recorded after the detergent had been removed from the b_6f/APol samples by overnight incubation with Bio-Beads, have been displaced vertically for the sake of readability. (B) Evolution over time of the relative absorbance of chlorophyll *a* (667 nm) vs that of reduced cytochrome *f* (554 nm). Visible spectra of ascorbate-reduced preparations were recorded either immediately after purification and trapping or after incubation at 4 °C in the dark for 20 days. Two independent experiments with two different b_6f preparations, transferred to NAPol SS298 at a 1:3 (w/w) protein:APol ratio.

These data show that NAPols can be used to stabilize MPs while keeping them functional. In the following, we examine some of their applications, with an emphasis on their complementarity with other APols.

Solution NMR HSQC Spectrum of NAPol-Trapped OmpX. 2D $^1H-^{15}N$ HSQC spectra of OmpX/NAPol complexes feature a high resolution and a wide spectral dispersion in both dimensions equivalent to those observed with OmpX/A8-35[29,30] and tOmpA/SAPol[13] complexes (Figure 5A,B). The small size and monodispersity of MP/NAPol complexes, which are comparable to MP/A8-35 complexes,[17] give rise to a good sensitivity and resolution of the 2D HSQC spectrum at a ^1H Larmor frequency of 700 MHz, without the need for transverse relaxation-optimized spectroscopy. Both amide ^1H and ^{15}N chemical shifts are very similar in the spectra of the NAPol- and A8-35-trapped samples recorded at pH 6.8 and 8.0, respectively (Figure 5C). On the basis of the assumption that the closest peaks observed in NAPols and A8-35 versus those in DHPC correspond to the same residue, and if one excepts residue Ala-10 (located toward the end of the first transmembrane β-strand), the ^1H and ^{15}N weight-average chemical shift differences[40] between NAPol- and A8-35-complexed OmpX lie between 0.001 and 0.20 ppm,

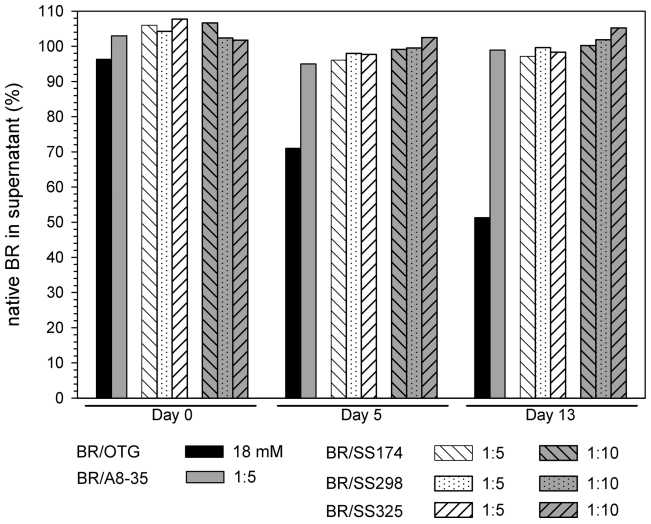

Figure 4. Stability over time of NAPol-trapped BR. The complexes were prepared at the final protein:polymer mass ratio indicated and stored at 4 °C in the dark for up to 13 days. The evolution of the concentration of native BR over time was determined from the absorbance of the solutions at 554 nm. On day 0 the concentration of native BR in the supernatant is given by reference to that following the removal of detergent, before centrifugation (100000*g* for 20 min at 4 °C), and on days 5 and 13 by reference to that in the supernatant on day 0. Errors are approximately ±5%. As a control, BR was kept in 18 mM OTG.

with an average of 0.04 ppm (Figure 5C). If we keep in mind the fact that the chemical shifts of OmpX/A8-35 samples are themselves very close to those observed in DHPC,[30] in which the NMR structure was obtained,[21] and the fact that the NMR structure is similar to the X-ray structure,[20] this indicates that NAPol-trapped OmpX also is properly folded.

For non-solvent-exposed residues, the line width of the peaks in the 2D spectrum of OmpX/NAPol complexes is very similar to that for OmpX/A8-35 complexes [see, e.g., residue G81 (Figure 5B, left)]. On the other hand, peak intensities are substantially higher at pH 6.8 for solvent-exposed residues, such as those located in the loops or turns, whose ^{1}H chemical shifts lie roughly between 7 and 8.5 ppm (see, e.g., residue G22 in Figure 5B, right). As a result, the spectrum of the OmpX/NAPol sample at pH 6.8 shows additional cross-peaks in this region (Figure 5A).

NAPol-Assisted Renaturation of Bacteriorhodopsin and a G Protein-Coupled Receptor. Charged APols have proven to be a highly efficient medium in which to fold denatured MPs to their native state.[13,25,31,41] NAPols, which appear to provide MPs with a particularly mild environment, could potentially provide an interesting alternative, while giving access to a broader range of folding conditions. This was first tested using BR as a convenient model protein. Assessing the (re)folding of BR indeed is particularly simple. Upon denaturation, BR releases its cofactor, retinal, whose main absorption peak shifts from ∼554 nm (dark-adapted solubilized BR) to ∼380 nm (free retinal). Upon renaturation, retinal spontaneously rebinds to the refolded apoprotein, regenerating the characteristic purple color of the holoprotein.[42] The denatured protein can be conveniently obtained by dissolving PM in SDS, which yields a mixture of PM lipids, denatured bacterio-opsin and retinal in a 1:1 protein:retinal molar ratio. Native BR has previously been renatured from this mixture by being transferred to detergent/lipid mixed micelles,[42] to pure lipids,[32] or to APol A8-35.[31]

SDS-solubilized PM was supplemented with NAPols at final protein:APol mass ratios of 1:5, 1:10, and 1:25, chosen on the basis of the experience gained when folding BR with A8-35.[31] A control sample was supplemented with A8-35 at a 1:5 BR:A8-35 mass ratio. Under these conditions, BR is known to refold quantitatively.[31] After incubation with the mixture of surfactants for 30 min, folding was initiated by precipitating dodecyl sulfate as its potassium salt (PDS).[31,32] The PDS crystals were removed by centrifugation, and the residual dodecyl sulfate was eliminated by dialysis for 48 h. The purple color of BR started to develop within minutes after the centrifugation step, its intensity being comparable to that of the sample renatured in A8-35 (Figure 6A). Quantitative analysis by UV−visible absorption spectroscopy indicated that ≥90% of BR had become renatured, whichever APol was used. Upon SEC, BR refolded in NAPols migrated as homogeneous particles with an apparent R_H smaller than that of BR/A8-35 complexes (Figure 6B,C), as classically observed when comparing MP/A8-35 and MP/NAPol complexes, an effect that does not result from a difference in size but from different interactions with the chromatographic support.[17]

APol A8-35 has been used successfully to fold GPCRs to their native state.[25,41] In the case of the ghrelin receptor GHS-R1a, however, the nature of the ligand makes it difficult to assess folding in the presence of A8-35. Indeed, ghrelin, in addition to being amphipathic, carries a high density of positive charges (four lysines and three arginines in this 28-residue peptide). As a consequence, overwhelming nonspecific binding to A8-35 interferes with ligand binding studies. The use of NAPols to fold GHS-R1a to its native state was therefore investigated. The receptor was expressed in *E. coli* inclusion bodies using an integrin fusion partner[26] and purified under denaturing conditions in the presence of SDS. The strategy used to fold GHS-R1a from its SDS-solubilized state was similar to that described for A8-35-assisted folding of other GPCRs.[41] NAPol was added at a 1:10 protein:APol mass ratio in the presence of lipids [0.2:1 (w/w) asolectin:NAPol mass ratio, 0.2% (w/v) cholesteryl hemisuccinate] and SDS removed by KCl precipitation followed by dialysis. Folding efficiency was then assessed by measuring the fraction of ligand-competent receptor. A folding yield in the ∼40% range was achieved (Figure 7A). This is remarkably high for a receptor that displays per se a high constitutive activity and is therefore likely to adopt an unstable active conformation. To remove the misfolded receptor, the preparation was run onto an affinity chromatography column carrying an immobilized ghrelin peptide grafted onto a streptavidin column through a C-terminal biotin. The protein fraction obtained after elution with a low-affinity antagonist of GHS-R1a and subsequent dialysis to remove the bound ligand was ∼97% active (Figure 7A).

To probe the quality of folding, the binding properties of the NAPol-folded GHS-R1a receptor were tested by monitoring the decrease in the FRET ratio between the receptor, labeled with Alexa Fluor 350, and a ghrelin peptide labeled with FITC, upon addition of increasing concentrations of various GHS-R1a specific ligands, namely, two synthetic antagonists, JMV3002 and JMV3018, and the inverse agonist [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]Substance P (SPA). The K_i values inferred from the competition profiles in Figure 7B (12, 23, and 300 nM for JMV3002, JMV3018, and SPA, respectively) are within the same range as those inferred from radioactive and TagLite-

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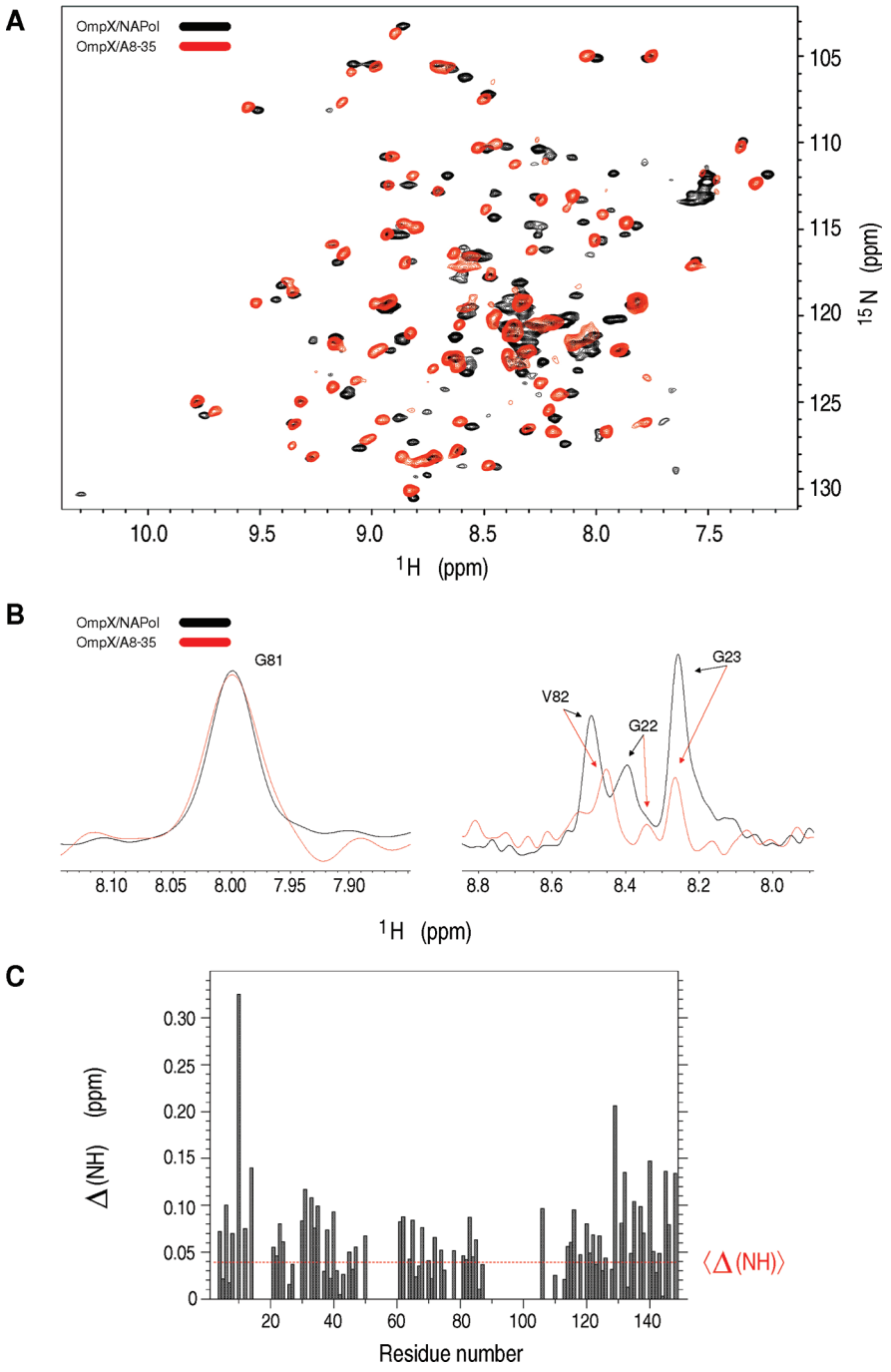

Figure 5. Solution NMR spectra of OmpX stabilized by either A8-35 or NAPols. (A) Superimposed NMR 2D $^{15}N-^{1}H$ HSQC spectra of [U-^{2}H,^{13}C,^{15}N]OmpX complexed by either APol A8-35 (pH 8.0, red) or NAPol (batch SS325; pH 6.8, black). (B) Comparative extracted rows at the same signal:noise ratio at the ^{15}N Larmor frequencies for G81 (left) and G23 (right) (from spectra in panel A). G81, located in the middle of the transmembrane β-barrel, is one of the least solvent-exposed residues in A8-35-trapped OmpX, whereas G23 is very accessible.[30] The peaks for G22 and V82, which have ^{15}N chemical shifts similar to those of G23, are also visible. In the left panel, to allow for a better comparison of the intensity and line width of the G81 resonance, the two peaks have been superimposed at the proton chemical shift observed for OmpX/NAPol complexes. (C) Weight-average chemical shift differences $[(\Delta(NH) = \{\Delta(^{1}H^{N})^{2} + [\Delta(^{15}N)/5]^{2}\}/2)^{1/2}]$ measured from the spectra in panel A, where $\Delta(^{1}H^{N})$ and $\Delta(^{15}N)$ represent the differences in chemical shifts measured in the proton and nitrogen dimensions, respectively, for each correlation peak observed with OmpX/NAPol complexes vs the closest peak observed with OmpX/A8-35 complexes.[40] Gaps in the sequence correspond to proline residues, to residues for which $^{1}H^{N}$ and ^{15}N chemical shifts are not known, to residues that could not be assigned unambiguously, to overcrowding of the lines, or to residues with indistinguishable $^{1}H^{N}$ and ^{15}N chemical shifts.

based measurements of HEK293 cells transiently expressing GHS-R1a.[43] This strongly suggests that upon folding in NAPols the ghrelin receptor adopts a conformation closely related to that in the plasma membrane.

The ability of GHS-R1a folded in NAPols to activate its cognate G protein partner was tested by measuring ghrelin-induced binding of GTP to $G_{\alpha q}$. GHS-R1a has been shown to display a significantly high constitutive activity (~50% of the maximal ghrelin-induced effect) toward $G_{\alpha q}$ both in heterolo-

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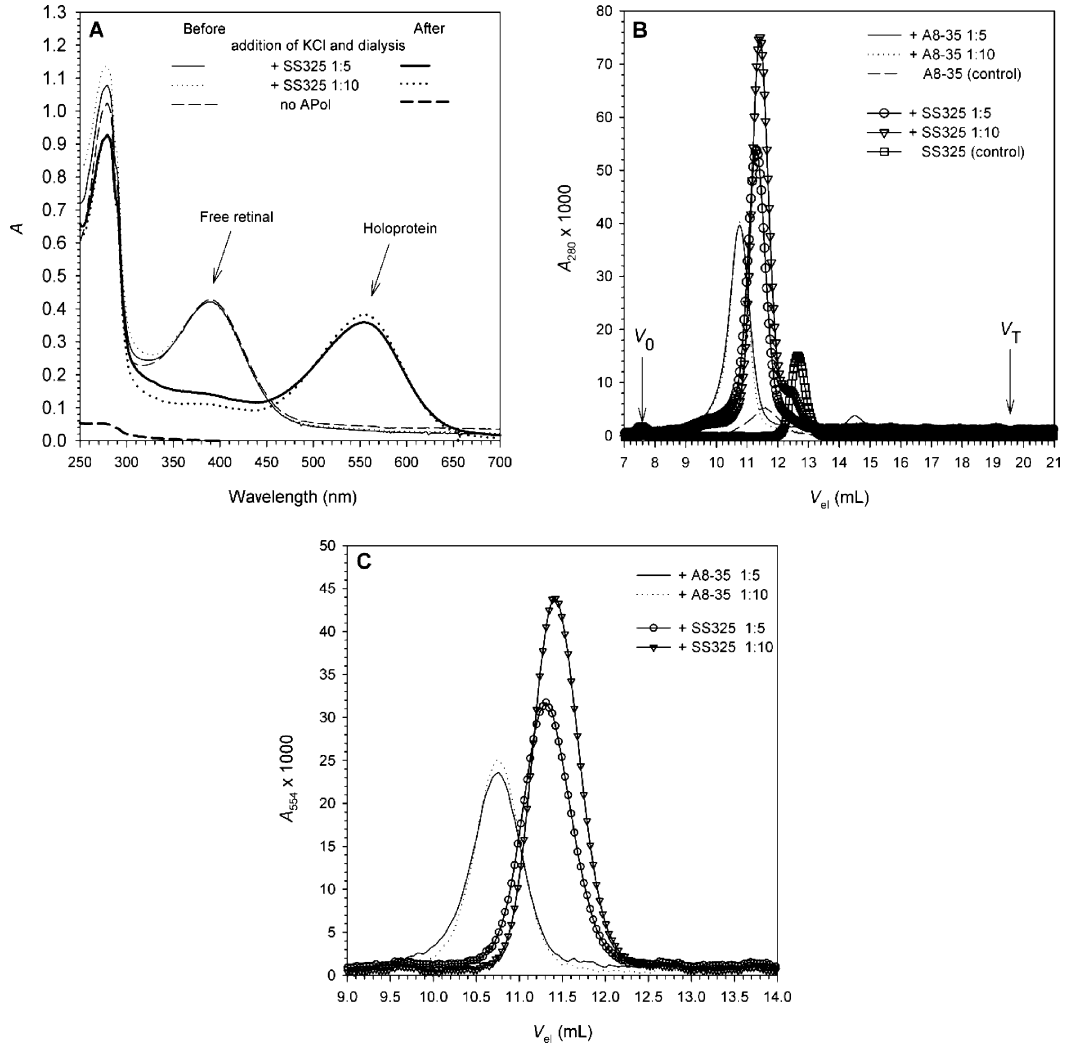

Figure 6. NAPol-assisted refolding of BR. (A) UV−visible spectra of SDS-solubilized PM before and after the addition of KCl (precipitation of PDS crystals) and dialysis (removal of residual detergent) in the presence or absence of NAPol (batch SS325) at the indicated BR:NAPol mass ratio. The protein precipitated when dodecyl sulfate was removed in the absence of APol. (B) SEC elution profiles at 280 nm of BR refolded in either A8-35 or NAPol at two BR:APol mass ratios (1:5 and 1:10). The chromatograms of the polymers alone are included ("control"). V_0 and V_T stand for the exclusion volume and the total volume, respectively. (C) Close-up of the elution profiles at 554 nm of BR refolded in the presence of either A8-35 or NAPols at two BR:APol mass ratios (1:5 and 1:10).

gous expression systems[44] and in vivo.[45] As shown in Figure 8A, the purified receptor folded in NAPol triggered binding of GTPγS to $G_{\alpha q}$ in the absence of agonist. This constitutive activity closely approximated that measured for GHS-R1a in heterologous systems such as HEK cells.[44] Adding ghrelin further increased the extent of receptor-catalyzed GTPγS binding, whereas the constitutive activity was significantly reduced upon binding the inverse agonist SPA (Figure 8A). NAPol-assisted folding of denatured GHS-R1a therefore confers upon it both the ability to activate G proteins in the absence of agonist and the ability to undergo further activation in the presence of ghrelin.

GHS-R1 has been shown to recruit arrestin-2 and arrestin-3 in an agonist-dependent manner.[46] To assess whether ghrelin-induced activation of the purified NAPol-folded receptor triggers arrestin-2 recruitment, its interaction with monobromobimane-labeled arrestin-2 was examined by fluorescence measurements. Bimane is a small fluorophore with a high sensitivity to the polarity of its molecular environment. It can be used as a sensor to detect interactions between arrestin and its protein partners, as previously reported for visual arrestin and rhodopsin.[47] As shown in Figure 8B, no significant interaction between GHS-R1a and arrestin-2 was observed in the absence of ligand. In contrast, adding ghrelin triggered arrestin recruitment, as evidenced by the change in bimane emission intensity. Therefore, as is the case for G protein activation, folding the ghrelin receptor to its native state using NAPols confers upon it the ability to interact with arrestin in an agonist-dependent manner.

Cell-Free Synthesis of Bacteriorhodopsin in the Presence of NAPols. Cell-free synthesis (CFS) relies on providing a cell lysate that contains the machinery for transcripting and translating DNA with a gene encoding the target protein, cloned in an appropriate plasmid.[48] In the case of MPs, synthesis is conducted in the presence of either detergent or lipid vesicles, or in the absence of any surfactant, in which case the protein precipitates and is later solubilized with a detergent.[49−53] Achieving functional expression, however, is not warranted, which is hardly surprising, given that detergents tend to denature MPs rather than favor their folding and that

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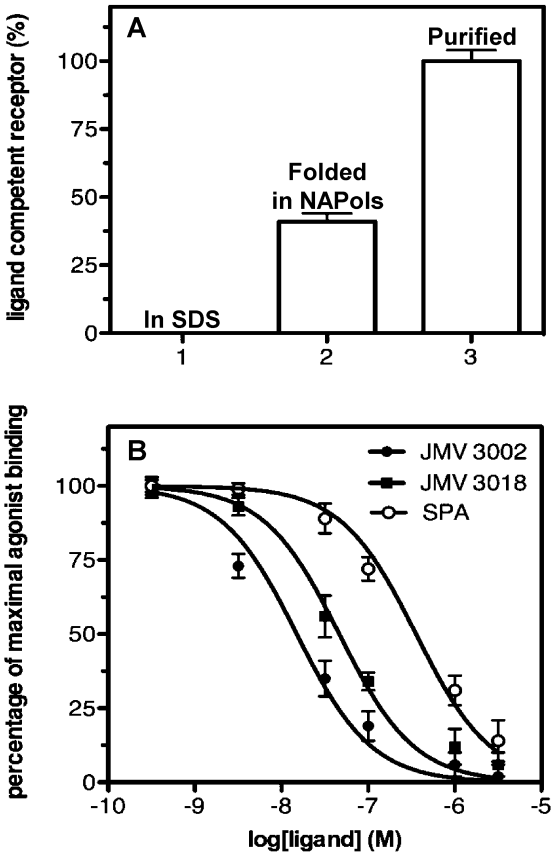

Figure 7. NAPol-assisted folding of the ghrelin receptor GHS-R1a. (A) Fraction of ligand-competent receptor before (1) and after (2) folding SDS-solubilized GHS-R1a in NAPol in the presence of asolectin, and (3) after affinity chromatography purification of the NAPol-folded receptor. The fraction of ligand-competent receptor was calculated as the amount of receptor able to bind a ghrelin-derived peptide divided by the amount of unfolded receptor used in the folding assay (1 and 2) or by the total amount of receptor in the purified fraction (3). (B) FRET-monitored competition between a ghrelin peptide labeled with FITC and either antagonist JMV3002 or JMV3018 or inverse agonist SPA. In both panel A and B, error bars correspond to the standard deviation from the mean calculated from three independent experiments.

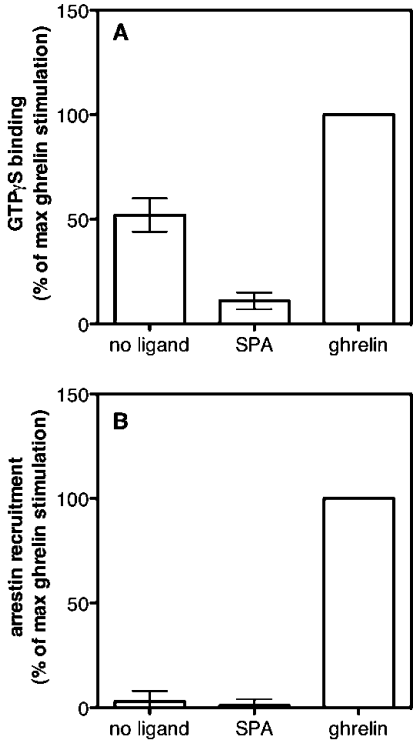

Figure 8. G protein activation and arrestin recruitment by the NAPol-folded ghrelin receptor. (A) BODIPY FL GTPγS binding to the $G_{\alpha q}$ protein induced by GHS-R1a in the absence of ligand, in the presence of 5 μM SPA, or in the presence of 5 μM ghrelin. Data are presented as the percentage of maximal BODIPY FL fluorescence change measured in the presence of ghrelin. (B) Changes in the emission intensity of bimane-labeled arrestin-2 induced by GHS-R1a in the absence of ligand or in the presence of either 5 μM SPA or 5 μM ghrelin. Data are presented as the percentage of maximal bimane fluorescence change measured in the presence of ghrelin. In panels A and B, the data represent the mean value ± the standard deviation from three independent experiments.

not all MPs spontaneously integrate into preformed bilayers without denaturing. This has prompted an active search for milder surfactants that could be used as accepting media (see, e.g., refs 11 and 54−57). APols could appear a priori as attractive candidates, because of their mildness and ability to assist MP folding. Unfortunately, neither A8-35 nor SAPols can support in vitro MP synthesis.[3,11] This may be related to the observation that charged detergents tend to block MP CFS.[58] There was therefore a strong incentive to examine whether NAPols could provide a more favorable CFS medium.

Small-scale CFS (50 μL batches) of polyhistidine-tagged BR was tested at different concentrations of NAPols (0.5−4 g/L), in the presence or absence of retinal. SDS−PAGE analysis followed by immunoblotting and revelation with an anti-His tag antibody indicated that synthesis took place in all cases.[59] BR expressed in the presence of NAPols and retinal folded to a greater extent than in the presence of DDM, as shown by the disappearance of the 380 nm absorption peak of free retinal and the appearance of the visible peak at 554 nm characteristic of native BR in its soluble state.[59] The samples were centrifuged, and the distribution of BR between the pellet and supernatant was determined, to establish the optimal concentration of polymer to be used. The best results were obtained at 3 g/L NAPols in the presence of 70 μM retinal, under which conditions the majority of BR was correctly folded and in a soluble form.[3,59] CFS was then scaled up to 1 mL batches (Figure 9A), which yielded, after nickel affinity purification, ∼0.4 mg of pure BR, a majority of which (∼90%) was present in the form of soluble BR/NAPol complexes (Figure 9B). As observed for native BR trapped with NAPols in the presence of PM lipids (refs 3 and 59 and this work), BR synthesized in vitro in the presence of NAPols and in the absence of lipids was highly stable, being inactivated only very partially after being stored at 4 °C for more than 6 months (Figure 9C). This stability is comparable to that observed after trapping BR in A8-35 in the presence of PM lipids.[24]

DISCUSSION

Stability of NAPol-Trapped MPs. Homopolymeric non-ionic polymers (NAPols) can be used to trap BR and OmpX under the form of small water-soluble complexes, and BR thus trapped is in its native state, which validates these polymers as bona fide APols.[17] This work establishes that NAPol-trapped OmpX also has a native-like three-dimensional (3D) structure, and it extends these observations to two less resilient MPs,

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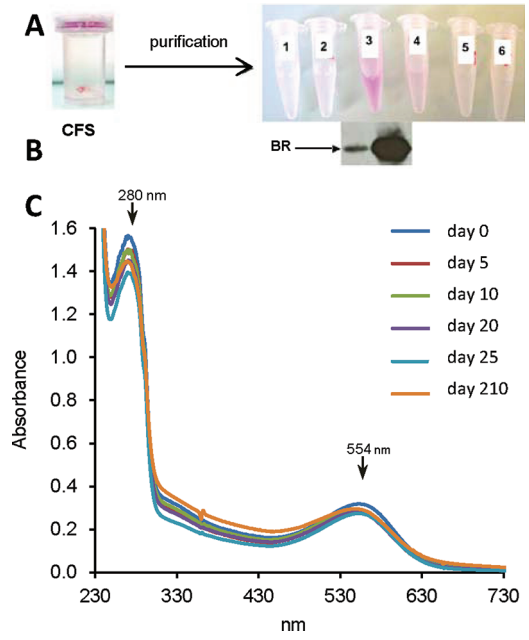

Figure 9. Cell-free synthesis of histidine-tagged bacteriorhodopsin in the presence of NAPols. The lysate was supplemented with 70 μM retinal and 3 g/L NAPol SS325. (A) After synthesis for 20 h and a one-step purification on a nickel affinity column (fractions 1–6), one fraction (3) was found to contain most of the protein; the purple color indicated that it had folded correctly and bound its cofactor. (B) Fraction 3 was centrifuged, and the pellet (P) and supernatant (S) were analyzed by SDS–PAGE; BR was detected by immunoblotting using an anti-His tag antibody. (C) Stability of BR synthesized in the presence of NAPols evaluated spectroscopically. Fraction 3 was stored at 4 °C in the dark, and its UV–visible absorbance spectrum was monitored as a function of time.

cytochrome b_6f and the ghrelin G protein-coupled receptor. The stabilities of A8-35- and NAPol-trapped BR appear to be comparable, whereas cytochrome b_6f is much more stable in NAPols. Taken together with other observations,[3,8,13] this suggests that the stabilizing efficiency of APols increases in the following order: SAPols < A8-35 < NAPols. This appears to inversely correlate with the charge density of the polymers, which decreases in the following order: SAPols (~75% of charged units) > A8-35 (~35%) > NAPols (0%). A destabilizing effect of electrostatic repulsion between net charges is evidenced by the fact that BR complexed by SAPols is markedly more stable in the presence of moderate concentrations of salt (100 mM NaCl) than in their absence.[13] This phenomenon is reminiscent of the fact that detergents carrying a net charge are notoriously more aggressive toward MPs than neutral detergents.[60] A plausible interpretation is that, in both cases, electrostatic repulsion tends to disrupt large MP/surfactant complexes into smaller units, which would favor the dissociation of oligomers into subunits and the unfolding of individual subunits. One of the most promising avenues opened by APols in general is capturing fragile MP complexes and supercomplexes that exist in the membrane but cannot be easily purified in detergent solutions because they are insufficiently stable. A8-35 has thus been used recently to purify the mitochondrial supercomplex $I_1III_2IV_1$ and determine its three-dimensional organization by single-particle cryoelectron microscopy.[61] NAPols represent new, potentially even milder tools for conducting such studies.

Functionality of NAPol-Trapped MPs. The two MPs whose function as NAPol-trapped complexes has been studied to date, BR and the ghrelin receptor GHS-R1a, were both found to be functional. As is the case in A8-35,[24] NAPol-trapped BR undergoes its full photocycle. At variance with what is observed in OTG, the kinetics of decay of the M states is very close to that observed in native PM. Examination of the late phase of the cycle suggests that rebinding of PM lipids after they had been transferred from OTG to NAPols may be even more complete than that thought to occur in A8-35,[37] which could be an additional factor contributing to MP stabilization. NAPol-trapped GHS-R1a binds its specific ligands, activates its cognate G protein, and recruits arrestin (this work), the two recruiting processes being more rapid than in A8-35 (J.-L. Banères, unpublished observations). It is particularly remarkable that the level of basal activity of GHS-R1a, relative to that induced by ghrelin, is comparable to that in the membrane-bound state,[44] suggesting that transfer to NAPols does not significantly alter conformational equilibria.

Table 2. A Summary of Proven or Putative Advantages and Disadvantages of Ionic and Nonionic Amphipols for Various Applications

application[a]	A8-35	SAPols	NAPols	comments
MP stabilization	++	+?	+++?	insufficient data to date to form a general picture; however, it seems likely that the stabilizing character of these three types of APols decreases in the following order: NAPols > A8-35 > SAPols
NMR	+	+	+	a balance of advantages and disadvantages: SAPols and NAPols can be used at low pH, but A8-35 cannot; A8-35 and SAPols are relatively easy to deuterate, but NAPols are not; all MP/APol complexes are of comparable size (for stability issues, see above)
renaturation	++	+?	+++?	SAPols and NAPols extend the range of accessible folding conditions (low pH, multivalent cations); efficiency at folding fragile MPs may follow stabilizing power
isoelectric focusing, ion-exchange chromatography	–	–	+	charges carried by the APol will perturb the behavior of MP/A8-35 and MP/SAPol complexes
cell-free synthesis	–	–	+	possible only with NAPols
binding studies	+	+	++	for some ligands, background lower with NAPols; G protein activation more efficient
crystallization	±	?	?	only A8-35 has been tested to date, with mitigated success;[3] NAPols would prevent electrostatic repulsion between complexes and may facilitate the use of extreme ionic strengths and multivalent cations

[a]Only those applications whose success has been shown (or can be expected) to depend on the choice of the APol are listed. Plus and minus signs are meant to compare the relative advantages and disadvantages of A8-35, SAPols, and NAPols for a given application, not from one application to another. Question marks indicate that the assessment proposed is based on reasonable expectations and/or fragmentary data, not on extensive studies. For a broader survey, see refs 2 and 3.

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A first examination of the usefulness of NAPols in MP biophysics and biochemistry reveals interesting potentialities. We first comment on each of the three applications tested here, NMR, MP folding, and cell-free synthesis, and then attempt to delineate the complementarity of NAPols with A8-35 and SAPols (Table 2).

Application to Solution NMR. The first APol to be developed, A8-35, has long been validated for MP solution-state NMR studies.[29,30,62−65] However, because of the polyacrylic nature of its backbone, it must be used at pH >7, to prevent aggregation. This can hamper certain NMR studies, e.g., when studying MPs that are unstable or inactive in basic solutions or when one needs to record signals that disappear under such conditions. To circumvent this limitation, pH-insensitive APols have been developed, such as SAPols.[13] As shown with the transmembrane domain of *E. coli*'s outer membrane protein A (tOmpA), MP/SAPol complexes remain soluble, resistant to high temperatures, and monodisperse in acidic solutions, giving rise to well-resolved NMR spectra.[13] On the down side, the current protocol for preparing SAPols requires a lengthy and demanding purification. Furthermore, their higher charge density, compared to those of A8-35 and NAPols, may negatively affect the stability of fragile MPs (see above). It was therefore of great interest to examine whether NAPols could be used for MP solution NMR studies.

The spectral dispersions observed for OmpX/NAPol complexes are equivalent to those observed with OmpX/DHPC or OmpX/A8-35 samples,[30] and the amide ^{1}H and ^{15}N chemical shifts are sufficiently similar to attest that NAPol-trapped OmpX retains its native 3D structure. Line widths are similar for OmpX/NAPols and OmpX/A8-35 complexes, suggesting that, as shown for BR/A8-35 versus BR/NAPol complexes in SANS and AUC studies,[17] their hydrodynamic radii are comparable. OmpX/NAPol complexes migrate, upon SEC, as if they were smaller than OmpX/A8-35 complexes,[17] but this most likely is due to the latter interacting with the resin (for a discussion, see ref 17). Rather, differences between OmpX/NAPols and OmpX/A8-35 NMR spectra appear to be related to the lower pH (6.8 vs 8) at which OmpX/NAPol complexes could be studied. The improvement indeed mostly affects solvent-exposed residues located in the loops or turns, leading to the observation of additional cross-peaks in the corresponding region of the spectrum.

The high quality of NMR signals observed with OmpX/NAPol complexes opens perspectives of complete structural determination of MP/NAPol complexes, provided the size of the MP of interest is compatible with solution NMR studies. APols thus join bicelles[66,67] or nanodiscs[68,69] as useful alternatives to detergents. MP/APol complexes are slightly larger than the smallest MP/detergent complexes.[24] However, the search for the fastest tumbling times for solution-state NMR studies, which was justified a decade ago, appears not to be so critical today, with the progress of NMR equipment and methodology, and with the help of improved isotopic labeling strategies.[70] Depending on the MP being studied, a slight loss of resolution might be more than offset by the improvement in stability, which allows longer collection times. Futhermore, a higher stability may also make it possible to improve the resolution by increasing the temperature. Media milder than detergents, whether they provide a bilayer-like environment, like bicelles and nanodiscs, or favor the retention of MP-bound lipids, as APols seem to, are likely to better preserve native-like structural features. This is suggested by differences in protein signal chemical shifts between MP/detergent and either MP/bicelle[71,72] or MP/nanodisc[73−75] complexes. It will be interesting to examine how well APols fare in this respect. A notable advantage of APols is the simplicity of preparation and handling of MP/APol complexes. MP/bicelle complexes indeed require a strict control of the ratio of long chain versus short chain lipids or detergents all along the preparation and data collection to ensure the presence of small and monodisperse bicellar particles.[76,77] This control is far from trivial when buffer-exchange or protein concentration steps are required. MP/nanodisc complexes are simpler to handle once they have been formed; however, their preparation involves the production and purification of substantial amounts of lipoproteins, especially if one aims to capture a single MP per nanodisc, and some purification work is required to obtain monodisperse preparations (see, e.g., ref 78). From a practical point of view, another marked advantage of A8-35 or SAPols is that they are relatively easy to deuterate (refs 6 and 7 and unpublished data), which is a great asset for certain NMR studies (see, e.g., refs 62, 63, and 65). They would have to be preferred over NAPols should it be necessary to work with unprotonated APols, because perdeuteration of glucosylated NAPols would be difficult and costly. On the other hand, as already mentioned, NAPols are likely to be milder.

NAPol-Assisted Folding of Membrane Proteins. A8-35 has proven to be an excellent environment in which to fold or refold to their native structure MPs that either have been denatured from their native state or were overexpressed in an inactive form, e.g., in inclusion bodies. To date, A8-35 has been used to fold or refold BR,[31] two β-barrel MPs, OmpA and FomA,[31] and six GPCRs.[25,41] SAPols have been used to fold tOmpA.[13] Depending on the protein considered and the experimental conditions, yields vary between 40 and ∼100%. We show here that BR refolds in NAPols as efficiently as it does in A8-35, i.e., essentially quantitatively, and that NAPols can also be used to fold a GPCR, ghrelin receptor GHS-R1a, whose binding properties are difficult if not impossible to study in the presence of A8-35, because of nonspecific binding. As compared to A8-35, NAPols and SAPols extend the range of folding conditions that can be explored, e.g., to acidic buffers or to solutions containing multivalent cations. This may be useful for folding MPs whose stability depends on these parameters. Furthermore, if the differential sensitivity of cytochrome b_6f to A8-35 and NAPols is any guide, some MPs may be more stable in NAPols than in charged APols, which, a priori, ought to facilitate their folding.

The choice of the APol to be used for folding a target MP depends on how the folded MP will be used. As discussed above, many forms of NMR can be readily carried out in A8-35. SAPols or NAPols will have to be used, however, if amide protons that are fully exposed to the solution are to be studied. Deuterated A8-35 or SAPols could be preferable for studying ligands by NMR.[63] With regard to binding studies, certain types of ligands, such as leukotriene B_4 and some of its analogues, which have been used in the study of A8-35-folded BLT1 and BLT2 receptors, do not exhibit any important nonspecific binding to A8-35.[25,41,63] Such is also the case for ligands of the CB1 cannabinoid receptor and the 5-$HT_{4(a)}$ serotonin receptor.[41] For cationic, amphipathic ligands such as ghrelin, diprenorphine, or cytokines, however, heavy partitioning into A8-35 (and, presumably, SAPols) makes binding measurements difficult, if not impossible (ref 59 and unpublished data of J.-L. Banères and F. Fieschi). In such cases, the recourse to NAPols

presents compelling advantages (this work and personal communication with F. Fieschi). Furthermore, it has been observed, as mentioned above, that the interactions of GPCRs with G proteins and arrestins are more efficient in the presence of NAPols than in the presence of A8-35, presumably because of the absence of electrostatic repulsion (J.-L. Banères, unpublished data).

Use of NAPols for MP Cell-Free Synthesis. CFS is one of the most promising techniques for producing MPs whose toxicity prevents their expression in vivo in a functional form and that cannot be easily folded from the inactive state that accumulates in inclusion bodies. CFS also provides an attractive route for inserting specific isotopically labeled or unnatural residues into proteins. One of the intrinsic difficulties of the application of CFS to MPs, however, is the fact that detergents are not a particularly favorable medium for folding of MPs (they rather tend to inactivate them), while few MPs insert into lipid vesicles in their native state in the absence of the translocon machinery. There is therefore much interest in examining whether milder surfactants are compatible with CFS and can act as an accepting medium.[3,11,54,57,59] Unfortunately, neither A8-35 nor SAPols can support in vitro MP production. Indeed, both types of APols inhibit the synthesis of several test MPs,[3,11] without affecting that of a soluble control protein, the green fluorescent protein.[11] An inhibitory effect has also been reported recently for APol PMAL-B-100, while an alkylated polysaccharide was observed to be permissive.[57] A8-35 has been shown previously to interact with lysozyme, a soluble basic protein.[79] It seems therefore plausible that the charges conveyed by A8-35 and SAPols may lead them to interact with basic patches at the surface of the ribosome, which would block translation (for a discussion, see ref 11). Whatever the inhibition mechanism, it is remarkable that it does not come into play when using NAPols. The synthesis and folding of BR are highly efficient, and the protein is in its native state and highly stable. NAPol-assisted CFS is probably a general approach, because similar conditions have been successfully applied to synthesizing several GPCRs, as well as a trimeric β-barrel bacterial outer membrane protein (E. Billon-Denis and F. Zito, unpublished data).

Studying Membrane Proteins as MP/NAPol Complexes or Transferring Them to Other Environments. Once a MP has been successfully folded or synthesized using a given APol, it can be studied in the same medium or transferred to a more convenient one (reviewed in ref 3). We have noted above that NAPols provide a particularly favorable environment for specific experiments, such as binding studies with certain types of ligands or NMR studies of exposed amide protons. Other approaches that have been validated using A8-35 and its derivatives should be readily extendable to NAPols. Such should be the case, for instance, for binding studies that resort to APol-mediated immobilization of the target MP onto appropriate solid supports. The feasibility and generality of this approach have been established using biotinylated A8-35.[80] This functionalized APol can be used to fold GPCRs with the same efficacy as unmodified A8-35 (J.-L. Banères, unpublished data). The mode of synthesis of NAPols would make grafting them with functional groups extremely easy.[17] It can be readily conceived, therefore, to fold or express in vitro a target MP in the presence of a tagged NAPol and to use the latter for immobilization. Alternatively, in the absence of appropriate derivatives, or if NAPols for some reason interfere with the experiments to be conducted, exchange for another type of functionalized APol could likely be conducted. Although exchange of APols at the surface of MPs has been demonstrated thus far only between differentially labeled forms of A8-35,[81] it is likely to extend to that between charged and uncharged APols. Similarly, and by analogy with observations with charged APols, MPs expressed or folded in the presence of NAPols could likely be transferred to lipid vesicles, black lipid films, a detergent solution, the plasma membrane of living cells, or, conceivably, lipid cubic phases (reviewed in ref 3).

Conclusion. In addition to the applications discussed above, NAPols present other conceivable advantages over existing APols. For instance, NAPols make it possible to resort to charge-based separation techniques, such as isoelectric focusing (P. Bazzacco, E. Point, and F. Zito, unpublished data) and ion-exchange chromatography, which the net charges carried by other APols render problematic if not impossible. Both A8-35[29,82] and NAPols[82] are compatible with mass spectrometric studies of the proteins, lipids, and cofactors present in MP/APol complexes, which opens another interesting route to exploring the composition of fragile MP assemblies.

Particularly intriguing is the application of NAPols to the growth of 3D MP crystals, a field in which the use of A8-35 has yielded only limited results to date.[3] Among the obvious drawbacks of using A8-35 for such attempts are the electrostatic repulsion between MP-bound APol layers (cf. ref 83) and the fact that many putative crystallization conditions (low pH, presence of multivalent cations, and high ionic strength) are inaccessible or problematical with this particular APol. Resorting to NAPols would not necessarily solve all of the difficulties involved in such attempts, but it would eliminate or mitigate many of them. Most obviously, the absence of net charges carried by the APol belt is likely to represent a great benefit.

Our current view of the place that NAPols may take besides A8-35 and SAPols in the field of APol-assisted MP studies is summarized in Table 2.

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Notes

The authors declare the following competing financial interest(s): B.P., J.-L.P., K.S.S., P.B., G.D., and F. Giusti hold a patent on homopolymeric NAPols.[18]

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■ ABBREVIATIONS

A8-35, specific type of polyacrylate-based amphipol; APol, amphipol; AUC, analytical ultracentrifugation; BR, bacteriorhodopsin; CFS, cell-free synthesis; cmc, critical micelle concentration; DDM, *n*-dodecyl β-D-maltoside; DHPC, dihexanoylphosphatidylcholine; FITC, fluorescein isothiocyanate-1; GHS-R1a, growth hormone secretagogue receptor type 1a (ghrelin receptor); GPCR, G protein-coupled receptor; MP, membrane protein; *M*, molecular mass; NAPol, nonionic amphipol; OmpX, outer membrane protein X from *E. coli*; OTG, *n*-octyl β-D-thioglucopyranoside; PDS, potassium dodecyl sulfate; SANS, small angle neutron scattering; SAPol, sulfonated amphipol; SEC, size exclusion chromatography; SPA, [D-Arg^1,D-Phe^5,D-Trp^{7,9},Leu^{11}]Substance P; tOmpA, transmembrane domain of *E. coli*'s outer membrane protein A.

■ REFERENCES

(1) Tribet, C., Audebert, R., and Popot, J.-L. (1996) Amphipols: Polymers that keep membrane proteins soluble in aqueous solutions. *Proc. Natl. Acad. Sci. U.S.A. 93*, 15047−15050.

(2) Popot, J.-L. (2010) Amphipols, nanodiscs, and fluorinated surfactants: Three non-conventional approaches to studying membrane proteins in aqueous solutions. *Annu. Rev. Biochem. 79*, 737−775.

(3) Popot, J.-L., Althoff, T., Bagnard, D., Banères, J.-L., Bazzacco, P., Billon-Denis, E., Catoire, L. J., Champeil, P., Charvolin, D., Cocco, M. J., Crémel, G., Dahmane, T., de la Maza, L. M., Ebel, C., Gabel, F., Giusti, F., Gohon, Y., Goormaghtigh, E., Guittet, E., Kleinschmidt, J. H., Kühlbrandt, W., Le Bon, C., Martinez, K. L., Picard, M., Pucci, B., Rappaport, F., Sachs, J. N., Tribet, C., van Heijenoort, C., Wien, F., Zito, F., and Zoonens, M. (2011) Amphipols from A to Z. *Annu. Rev. Biophys. 40*, 379−408.

(4) Tifrea, D. F., Sun, G., Pal, S., Zardeneta, G., Cocco, M. J., Popot, J.-L., and de la Maza, L. M. (2011) Amphipols stabilize the *Chlamydia* major outer membrane protein and enhance its protective ability as a vaccine. *Vaccine 29*, 4623−4631.

(5) Perlmutter, J. D., Drasler, W. J., Xie, W., Gao, J., Popot, J.-L., and Sachs, J. N. (2011) All-atom and coarse-grained molecular dynamics simulations of a membrane protein stabilizing polymer. *Langmuir 27*, 10523−10537.

(6) Gohon, Y., Pavlov, G., Timmins, P., Tribet, C., Popot, J.-L., and Ebel, C. (2004) Partial specific volume and solvent interactions of amphipol A8-35. *Anal. Biochem. 334*, 318−334.

(7) Gohon, Y., Giusti, F., Prata, C., Charvolin, D., Timmins, P., Ebel, C., Tribet, C., and Popot, J.-L. (2006) Well-defined nanoparticles formed by hydrophobic assembly of a short and polydisperse random terpolymer, amphipol A8-35. *Langmuir 22*, 1281−1290.

(8) Picard, M., Dahmane, T., Garrigos, M., Gauron, C., Giusti, F., le Maire, M., Popot, J.-L., and Champeil, P. (2006) Protective and inhibitory effects of various types of amphipols on the Ca^{2+}-ATPase from sarcoplasmic reticulum: A comparative study. *Biochemistry 45*, 1861−1869.

(9) Diab, C., Tribet, C., Gohon, Y., Popot, J.-L., and Winnik, F. M. (2007) Complexation of integral membrane proteins by phosphorylcholine-based amphipols. *Biochim. Biophys. Acta 1768*, 2737−2747.

(10) Wüthrich, K. (1986) *NMR of proteins and nucleic acids*, Wiley, New York.

(11) Park, K.-H., Billon-Denis, E., Dahmane, T., Lebaupain, F., Pucci, B., Breyton, C., and Zito, F. (2011) In the cauldron of cell-free synthesis of membrane proteins: Playing with new surfactants. *New Biotechnol. 28*, 255−261.

(12) Nagy, J. K., Kuhn Hoffmann, A., Keyes, M. H., Gray, D. N., Oxenoid, K., and Sanders, C. R. (2001) Use of amphipathic polymers to deliver a membrane protein to lipid bilayers. *FEBS Lett. 501*, 115−120.

(13) Dahmane, T., Giusti, F., Catoire, L. J., and Popot, J.-L. (2011) Sulfonated amphipols: Synthesis, properties and applications. *Biopolymers 95*, 811−823.

(14) Prata, C., Giusti, F., Gohon, Y., Pucci, B., Popot, J.-L., and Tribet, C. (2001) Non-ionic amphiphilic polymers derived from *tris*(hydroxymethyl)-acrylamidomethane keep membrane proteins soluble and native in the absence of detergent. *Biopolymers 56*, 77−84.

(15) Sharma, K. S., Durand, G., Giusti, F., Olivier, B., Fabiano, A.-S., Bazzacco, P., Dahmane, T., Ebel, C., Popot, J.-L., and Pucci, B. (2008) Glucose-based amphiphilic telomers designed to keep membrane proteins soluble in aqueous solutions: Synthesis and physicochemical characterization. *Langmuir 24*, 13581−13590.

(16) Bazzacco, P., Sharma, K. S., Durand, G., Giusti, F., Ebel, C., Popot, J.-L., and Pucci, B. (2009) Trapping and stabilization of integral membrane proteins by hydrophobically grafted glucose-based telomers. *Biomacromolecules 10*, 3317−3326.

(17) Sharma, K. S., Durand, G., Gabel, F., Bazzacco, P., Le Bon, C., Billon-Denis, E., Catoire, L. J., Popot, J.-L., Ebel, C., and Pucci, B. (2012) Non-ionic amphiphilic homo-polymers: Synthesis, solution properties, and biochemical validation. *Langmuir*, DOI: 10.1021/la205026r.

(18) Pucci, B., Popot, J.-L., Sharma, K. S., Bazzacco, P., Durand, G., Giusti, F. (2009) Polymères comprenant une majorité de monomères amphiphiles destinés au piégeage et à la manipulation de protéines membranaires. Patent FR 2,952,642.

(19) Sharma, K. S., Durand, G., and Pucci, B. (2011) Synthesis and determination of polymerization rate constants of glucose-based monomers. *Des. Monomers Polym. 14*, 499−513.

(20) Vogt, J., and Schulz, G. E. (1999) The structure of the outer membrane protein OmpX from *Escherichia coli* reveals possible mechanisms of virulence. *Structure 7*, 1301−1309.

(21) Fernández, C., Adeishvili, K., and Wüthrich, K. (2001) Transverse relaxation-optimized NMR spectroscopy with the outer membrane protein OmpX in dihexanoyl phosphatidylcholine micelles. *Proc. Natl. Acad. Sci. U.S.A. 98*, 2358−2363.

(22) Oesterhelt, D., and Stoeckenius, W. (1974) Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol. 31*, 667−678.

(23) Lobasso, S., Lopalco, P., Lattanzio, V. M., and Corcelli, A. (2003) Osmotic shock induces the presence of glycocardiolipin in the purple membrane of *Halobacterium salinarum*. *J. Lipid Res. 44*, 2120−2126.

(24) Gohon, Y., Dahmane, T., Ruigrok, R., Schuck, P., Charvolin, D., Rappaport, F., Timmins, P., Engelman, D. M., Tribet, C., Popot, J.-L., and Ebel, C. (2008) Bacteriorhodopsin/amphipol complexes: Structural and functional properties. *Biophys. J. 94*, 3523−3537.

(25) Banères, J.-L., Popot, J.-L., and Mouillac, B. (2011) New advances in production and functional folding of G protein-coupled receptors. *Trends Biotechnol. 29*, 314−322.

(26) Arcemisbéhère, L., Sen, T., Boudier, L., Balestre, M.-N., Gaibelet, G., Detouillon, E., Orcel, H., Mendre, C., Rahmeh, R., Granier, S., Vivès, C., Fieschi, F., Damian, M., Durroux, T., Banères, J.-L., and Mouillac, B. (2010) Leukotriene BLT2 receptor monomers activate the $G_{(i2)}$ GTP-binding protein more efficiently than dimers. *J. Biol. Chem. 285*, 6337−6347.

(27) Stroebel, D., Choquet, Y., Popot, J.-L., and Picot, D. (2003) An atypical haem in the cytochrome b_6f complex. *Nature 426*, 413−418.

(28) Pierre, Y., Breyton, C., Kramer, D., and Popot, J.-L. (1995) Purification and characterization of the cytochrome b_6f complex from *Chlamydomonas reinhardtii*. *J. Biol. Chem. 270*, 29342−29349.
(29) Catoire, L. J., Zoonens, M., van Heijenoort, C., Giusti, F., Popot, J.-L., and Guittet, E. (2009) Inter- and intramolecular contacts in a membrane protein/surfactant complex observed by heteronuclear dipole-to-dipole cross-relaxation. *J. Magn. Reson. 197*, 91−95.
(30) Catoire, L. J., Zoonens, M., van Heijenoort, C., Giusti, F., Guittet, E., and Popot, J.-L. (2010) Solution NMR mapping of water-accessible residues in the transmembrane β-barrel of OmpX. *Eur. Biophys. J. 39*, 623−630.
(31) Pocanschi, C. L., Dahmane, T., Gohon, Y., Rappaport, F., Apell, H.-J., Kleinschmidt, J. H., and Popot, J.-L. (2006) Amphipathic polymers: Tools to fold integral membrane proteins to their active form. *Biochemistry 45*, 13954−13961.
(32) Popot, J.-L., Gerchman, S.-E., and Engelman, D. M. (1987) Refolding of bacteriorhodopsin in lipid bilayers: A thermodynamically controlled two-stage process. *J. Mol. Biol. 198*, 655−676.
(33) Damian, M., Marie, J., Leyris, J.-P., Fehrentz, J. A., Verdié, P., Martinez, J., Banères, J.-L., and Mary, S. (2012) High constitutive activity is an intrinsic feature of the ghrelin receptor protein. *J. Biol. Chem.*, DOI: 10.1074/jbc.M111.288324.
(34) Béal, D., Rappaport, F., and Joliot, P. (1999) A new high-sensitivity 10-ns time-resolution spectrophotometric technique adapted to *in vivo* analysis of the photosynthetic apparatus. *Rev. Sci. Instrum. 70*, 202−207.
(35) Haupts, U., Tittor, J., and Oesterhelt, D. (1999) Closing in on bacteriorhodopsin: Progress in understanding the molecule. *Annu. Rev. Biophys. Biomol. Struct. 28*, 367−399.
(36) Milder, S. J., Thorgeirsson, T. E., Miercke, L. J., Stroud, R. M., and Kliger, D. S. (1991) Effects of detergent environments on the photocycle of purified monomeric bacteriorhodopsin. *Biochemistry 30*, 1751−1761.
(37) Dahmane, T. (2007) Protéines membranaires et amphipols: Stabilisation, fonction, renaturation, et développement d'amphipols sulfonatés pour la RMN des solutions. Ph.D. Dissertation, Université Paris-7, Paris.
(38) Breyton, C., Tribet, C., Olive, J., Dubacq, J.-P., and Popot, J.-L. (1997) Dimer to monomer conversion of the cytochrome b_6f complex: Causes and consequences. *J. Biol. Chem. 272*, 21892−21900.
(39) Pierre, Y., Breyton, C., Lemoine, Y., Robert, B., Vernotte, C., and Popot, J.-L. (1997) On the presence and role of a molecule of chlorophyll *a* in the cytochrome b_6f complex. *J. Biol. Chem. 272*, 21901−21908.
(40) Foster, M. P., Wuttke, D. S., Clemens, K. R., Jahnke, W., Radhakrishnan, I., Tennant, L., Reymond, M., Chung, J., and Wright, P. E. (1998) Chemical shift as a probe of molecular interfaces: NMR studies of DNA binding by the three amino-terminal zinc finger domains from transcription factor IIIA. *J. Biomol. NMR 12*, 51−71.
(41) Dahmane, T., Damian, M., Mary, S., Popot, J.-L., and Banères, J.-L. (2009) Amphipol-assisted *in vitro* folding of G protein-coupled receptors. *Biochemistry 48*, 6516−6521.
(42) Huang, K.-S., Bayley, H., Liao, M.-J., London, E., and Khorana, H. G. (1981) Refolding of an integral membrane protein. Denaturation, renaturation, and reconstitution of intact bacteriorhodopsin and two proteolytic fragments. *J. Biol. Chem. 256*, 3802−3809.
(43) Leyris, J.-P., Roux, T., Trinquet, E., Verdié, P., Fehrentz, J. A., Oueslati, N., Douzon, S., Bourrier, E., Lamarque, L., Gagne, D., Galleyrand, J.-C., M'kadmi, C., Martinez, J., Mary, S., Banères, J.-L., and Marie, J. (2011) Homogeneous time-resolved fluorescence-based assay to screen for ligands targeting the growth hormone secretagogue receptor type 1a. *Anal. Biochem. 408*, 253−262.
(44) Holst, B., and Schwartz, T. W. (2004) Constitutive ghrelin receptor activity as a signaling set-point in appetite regulation. *Trends Pharmacol. Sci. 25*, 113−117.
(45) Petersen, P. S., Woldbye, D. P., Madsen, A. N., Egerod, K. L., Jin, C., Lang, M., Rasmussen, M., Beck-Sickinger, A. G., and Holst, B. (2009) *In vivo* characterization of high basal signaling from the ghrelin receptor. *Endocrinology 150*, 4920−4930.
(46) Holliday, N. D., Holst, B., Rodionova, E. A., Schwartz, T. W., and Cox, H. M. (2007) Importance of constitutive activity and arrestin-independent mechanisms for intracellular trafficking of the ghrelin receptor. *Mol. Endocrinol. 21*, 3100−3112.
(47) Sommer, M. E., Smith, W. C., and Farrens, D. L. (2005) Dynamics of arrestin-rhodopsin interactions: Arrestin and retinal release are directly linked events. *J. Biol. Chem. 280*, 6861−6871.
(48) Zubay, G. (1973) *In vitro* synthesis of protein in microbial systems. *Annu. Rev. Genet. 7*, 267−287.
(49) Schwarz, D., Dötsch, V., and Bernhard, F. (2008) Production of membrane protein using cell-free expression system. *Proteomics 8*, 3933−3946.
(50) Katzen, F., Peterson, T. C., and Kudlicki, W. (2009) Membrane protein expression: No cells required. *Trends Biotechnol. 27*, 455−460.
(51) Junge, F., Haberstock, S., Roos, C., Stefer, S., Proverbio, D., Dötsch, V., and Bernhard, F. (2011) Advances in cell-free protein synthesis for the functional and structural analysis of membrane proteins. *New Biotechnol. 28*, 262−271.
(52) Ma, Y., Münch, D., Schneider, T., Sahl, H. G., Bouhss, A., Ghoshdastider, U., Wang, J., Dötsch, V., Wang, X., and Bernhard, F. (2011) Preparative scale cell-free production and quality optimization of MraY homologues in different expression modes. *J. Biol. Chem. 286*, 38844−38853.
(53) Matthies, D., Haberstock, S., Joos, F., Dötsch, V., Vonck, J., Bernhard, F., and Meier, T. (2011) Cell-free expression and assembly of ATP synthase. *J. Mol. Biol. 413*, 593−603.
(54) Park, K.-H., Berrier, C., Lebaupain, F., Pucci, B., Popot, J.-L., Ghazi, A., and Zito, F. (2007) Fluorinated and hemifluorinated surfactants as alternatives to detergents for membrane protein cell-free synthesis. *Biochem. J. 403*, 183−187.
(55) Katzen, F., Fletcher, J. E., Yang, J. P., Vasu, S., Peterson, T., and Kudlicki, W. (2008) Cell-free protein expression of membrane proteins using nanolipoprotein particles. *BioTechniques 45*, 469.
(56) Rajesh, S., Knowles, T. J., and Overduin, M. (2011) Production of membrane proteins without cells or detergents. *New Biotechnol. 28*, 250−254.
(57) Klammt, C., Perrin, M.-H., Maslennikov, I., Renault, L., Krupa, M., Kwiatkowski, W., Stahlberg, H., Vale, W., and Choe, S. (2011) Polymer-based cell-free expression of ligand-binding family B G-protein coupled receptors without detergents. *Protein Sci. 20*, 1030−1041.
(58) Klammt, C., Schwarz, D., Fendler, K., Haase, W., Dötsch, V., and Bernhard, F. (2005) Evaluation of detergents for the soluble expression of α-helical and β-barrel-type integral membrane proteins by a preparative scale individual cell-free expression system. *FEBS J. 272*, 6024−6038.
(59) Bazzacco, P. (2009) Non-ionic amphipols: New tools for *in vitro* studies of membrane proteins. Validation and development of biochemical and biophysical applications. Ph.D. Dissertation, Université Paris-7, Paris.
(60) Møller, J. V., le Maire, M., and Andersen, J. P. (1986) Uses of non-ionic and bile salt detergents in the study of membrane proteins. In *Progress in protein-lipid interactions* (Watts, A., and De Pont, J. J. H. H. M., Eds.) pp 147−196, Elsevier Science Publishers, Amsterdam.
(61) Althoff, T., Mills, D. J., Popot, J.-L., and Kühlbrandt, W. (2011) Assembly of electron transport chain components in bovine mitochondrial supercomplex $I_1III_2IV_1$. *EMBO J. 30*, 4652−4664.
(62) Zoonens, M., Catoire, L. J., Giusti, F., and Popot, J.-L. (2005) NMR study of a membrane protein in detergent-free aqueous solution. *Proc. Natl. Acad. Sci. U.S.A. 102*, 8893−8898.
(63) Catoire, L. J., Damian, M., Giusti, F., Martin, A., van Heijenoort, C., Popot, J.-L., Guittet, E., and Banères, J.-L. (2010) Structure of a GPCR ligand in its receptor-bound state: leukotriene B_4 adopts a highly constrained conformation when associated to human BLT2. *J. Am. Chem. Soc. 132*, 9049−9057.
(64) Raschle, T., Hiller, S., Etzkorn, M., and Wagner, G. (2010) Nonmicellar systems for solution NMR spectroscopy of membrane proteins. *Curr. Opin. Struct. Biol. 20*, 471−479.

(65) Catoire, L. J., Damian, M., Baaden, M., Guittet, E., and Banères, J.-L. (2011) Electrostatically-driven fast association and perdeuteration allow detection of transferred cross-relaxation for G protein-coupled receptor ligands with equilibrium dissociation constants in the high-to-low nanomolar range. *J. Biomol. NMR 50*, 191−195.
(66) Vold, R. R., and Prosser, R. S. (1996) Magnetically oriented phospholipid bilayered micelles for structural studies of polypeptides. Does the ideal bicelle exist? *J. Magn. Reson., Ser. B 113*, 267−271.
(67) Sanders, C. R., and Prosser, R. S. (1998) Bicelles: A model membrane system for all seasons? *Structure 6*, 1227−1234.
(68) Bayburt, T. H., Grinkova, Y. V., and Sligar, S. G. (2002) Self-assembly of discoidal phospholipid bilayer nanoparticles with membrane scaffold proteins. *Nano Lett. 2*, 853−856.
(69) Denisov, I. G., Grinkova, Y. V., Lazarides, A. A., and Sligar, S. G. (2004) Directed self-assembly of monodisperse phospholipid bilayer Nanodiscs with controlled size. *J. Am. Chem. Soc. 126*, 3477−3487.
(70) Tugarinov, V., Kanelis, V., and Kay, L. E. (2006) Isotope labeling strategies for the study of high-molecular-weight proteins by solution NMR spectroscopy. *Nat. Protoc. 1*, 749−754.
(71) Chou, J. J., Kaufman, J. D., Stahl, S. J., Wingfield, P. T., and Bax, A. (2002) Micelle-induced curvature in a water-insoluble HIV-1 Env peptide revealed by NMR dipolar coupling measurement in stretched polyacrylamide gel. *J. Am. Chem. Soc. 124*, 2450−2451.
(72) Lee, D., Walter, K. F. A., Brückner, A. K., Hilty, C., Becker, S., and Griesinger, C. (2008) Bilayer in small bicelles revealed by lipid-protein interactions using NMR spectroscopy. *J. Am. Chem. Soc. 130*, 13822−13823.
(73) Glück, J. M., Wittlich, M., Feuerstein, S., Hoffmann, S., Willbold, D., and Koenig, B. W. (2009) Integral membrane proteins in nanodiscs can be studied by solution NMR spectroscopy. *J. Am. Chem. Soc. 131*, 12060−12061.
(74) Raschle, T., Hiller, S., Yu, T. Y., Rice, A. J., Walz, T., and Wagner, G. (2009) Structural and functional characterization of the integral membrane protein VDAC-1 in lipid bilayer nanodiscs. *J. Am. Chem. Soc. 131*, 17777−17779.
(75) Shenkarev, Z. O., Lyukmanova, E. N., Paramonov, A. S., Shingarova, L. N., Chupin, V. V., Kirpichnikov, M. P., Blommers, M. J., and Arseniev, A. S. (2010) Lipid-protein nanodiscs as reference medium in detergent screening for high-resolution NMR studies of integral membrane proteins. *J. Am. Chem. Soc. 132*, 5628−5629.
(76) Triba, M. N., Warschawski, D. E., and Devaux, P. F. (2005) Reinvestigation by phosphorus NMR of lipid distribution in bicelles. *Biophys. J. 88*, 1887−1901.
(77) Sanders, C. R., and Sönnichsen, F. (2006) Solution NMR of membrane proteins: Practice and challenges. *Magn. Reson. Chem. 44*, S24−S40.
(78) Ritchie, T. K., Grinkova, Y. V., Bayburt, T. H., Denisov, I. G., Zolnerciks, J. K., Atkins, W. M., and Sligar, S. G. (2009) Reconstitution of membrane proteins in phospholipid bilayer nanodiscs. *Methods Enzymol. 464*, 211−231.
(79) Champeil, P., Menguy, T., Tribet, C., Popot, J.-L., and le Maire, M. (2000) Interaction of amphipols with the sarcoplasmic reticulum Ca^{2+}-ATPase. *J. Biol. Chem. 275*, 18623−18637.
(80) Charvolin, D., Perez, J.-B., Rouvière, F., Giusti, F., Bazzacco, P., Abdine, A., Rappaport, F., Martinez, K. L., and Popot, J.-L. (2009) The use of amphipols as universal molecular adapters to immobilize membrane proteins onto solid supports. *Proc. Natl. Acad. Sci. U.S.A. 106*, 405−410.
(81) Zoonens, M., Giusti, F., Zito, F., and Popot, J.-L. (2007) Dynamics of membrane protein/amphipol association studied by Förster resonance energy transfer. Implications for *in vitro* studies of amphipol-stabilized membrane proteins. *Biochemistry 46*, 10392−10404.
(82) Bechara, C., Bolbach, G., Bazzacco, P., Sharma, S. K., Durand, G., Popot, J.-L., Zito, F., and Sagan, S. (2012) MALDI mass spectrometry analysis of membrane protein/amphipol complexes. *Submitted for publication,*.
(83) Popot, J.-L., Berry, E. A., Charvolin, D., Creuzenet, C., Ebel, C., Engelman, D. M., Flötenmeyer, M., Giusti, F., Gohon, Y., Hervé, P., Hong, Q., Lakey, J. H., Leonard, K., Shuman, H. A., Timmins, P., Warschawski, D. E., Zito, F., Zoonens, M., Pucci, B., and Tribet, C. (2003) Amphipols: Polymeric surfactants for membrane biology research. *Cell. Mol. Life Sci. 60*, 1559−1574.