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GENERAL REVIEW

Amphipols for Each Season

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Abstract Amphipols (APols) are short amphipathic polymers that can substitute for detergents at the transmembrane surface of membrane proteins (MPs) and, thereby, keep them soluble in detergent free aqueous solutions. APol-trapped MPs are, as a rule, more stable biochemically than their detergent-solubilized counterparts. APols have proven useful to produce MPs, most noticeably by assisting their folding from the denatured state obtained after solubilizing MP inclusion bodies in either SDS or urea. They facilitate the handling in aqueous solution of fragile MPs for the purpose of proteomics, structural and functional studies, and therapeutics. Because APols can be chemically labeled or functionalized, and they form very stable complexes with MPs, they can also be used to functionalize those indirectly, which opens onto many novel applications. Following a brief recall of the properties of APols and MP/APol complexes, an update is provided of recent progress in these various fields.

Keywords Membrane proteins · Surfactants · Stabilization · Folding · Biochemistry · Biophysics

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Abbreviations

2D	Two-dimensional
3D	Three-dimensional
A8-35	A poly(sodium acrylate) based amphipol comprising ~35 % of free carboxylates, ~25 % of octyl chains, ~40 % of isopropyl groups, whose number average molar mass is ~4.3 kDa
A8-75	A poly(sodium acrylate) based amphipol comprising ~75 % of free carboxylates, ~25 % of octyl chains, whose number average molar mass is ~4 kDa
APol	Amphipol
AUC	Analytical ultracentrifugation
BAPol	Biotinylated A8-35
BLM	Black lipid membrane
BN-PAGE	Blue native polyacrylamide gel electrophoresis
BR	Bacteriorhodopsin
CAC	Critical aggregation concentration
CD	Circular dichroism
Đ	Polydispersity index
cmc	Critical micellar concentration
DAGK	Diacylglycerol kinase
DAPol	A8-35 with deuterated octylamine and isopropylamine side chains
DHPC	Dihexanoylphosphatidylcholine
DLS	Dynamic light scattering
DPC	Dodecylphosphocholine
DPn	See $\langle X_n \rangle$
EM	Electron microscopy
EPR	Electron paramagnetic resonance
ESI	Electron spray ionization

FAPol	Fluorescently-labeled A8-35
$FAPol_{AF647}$	Alexa Fluor 647-labeled A8-35
$FAPol_{NBD}$	Nitrobenzoxadiazole-labeled A8-35
$FAPol_{rhod}$	Rhodamine-labeled A8-35
FRET	Förster resonance energy transfer
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HAPol	Hydrogenated A8-35
His_6PEG	*N*-(Penta(histidyl)histidinamide)-8-amino-3,6-dioxa-octanamide
His-tag	Hexahistidine tag
HistAPol	Hexahistidine tag-carrying A8-35
IMAC	Immobilized metal ion affinity chromatography
ImidAPol	Imidazole-carrying A8-35
IMS	Ion mobility spectrometry
INS	Inelastic neutron scattering
ITC	Isothermal titration calorimetry
KpOmpA	The transmembrane domain of outer membrane protein A from *Klebsiella pneumoniae*
LTB_4	Leukotriene B_4
$\langle M_n \rangle$ (also written $\bar{M}_n$)	Number-average molar mass
MALDI	Matrix-assisted laser desorption ionization
MOMP	The major outer membrane protein from *Chlamydia trachomatis*
MP	Membrane protein
MS	Mass spectrometry
MW	Molecular weight
nAChR	Nicotinic acetylcholine receptor
NAPol	Non-ionic APol
NBD	7-Nitrobenz-2-oxa-1,3-diazol-4-yle
ND	Nanodisc
NOE	Nuclear Overhauser effect
NTA	Nitrilotriacetic acid
ODN	Oligodeoxynucleotide
OligAPol	ODN-carrying A8-35
OmpA, OmpF, OmpX	Respectively outer membrane proteins A, F and X from *Escherichia coli*
PAA	Poly(acrylic acid)
PAGE	Polyacrylamide gel electrophoresis
PC-APol	Phosphorylcholine-based APol
perDAPol	Perdeuterated A8-35
QENS	Quasi-elastic neutron scattering
SANS	Small angle neutron scattering
SAPol	Sulfonated amphipol derived from A8-75, comprising ~40 % of taurine moieties
SAXS	Small angle X-ray scattering
SDS	Sodium dodecyl sulfate
SERCA1a	The sarcoplasmic calcium ATPase from twitch muscle
SERS	Surface-enhanced Raman scattering
SMFS	Single-molecule force spectrometry
SPR	Surface plasmon resonance
SR	Synchrotron radiation
STEM	Scanning transmission EM
Structure II	Secondary structure
SulfidAPol	Sulfide-carrying APol
TOF	Time of flight
tOmpA	The transmembrane domain of OmpA from *E. coli*
TRP	Transient receptor potential
UAPol	Universal amphipol
$UAPol\text{-}NH_2$	Amine-carrying A8-35
$\langle X_n \rangle$ (also written $\bar{X}_n$, formerly DPn)	Number-average degree of polymerization

Introduction

Amphipols (APols) were designed exactly 20 years ago, and the validation of the concept published two years later (Tribet et al. 1996). The original intent was to facilitate the study of membrane proteins (MPs) in aqueous solutions by increasing their stability compared to that achievable in detergent solutions, which is often limited. The name "amphipols" was coined to distinguish the new molecules from the many other types of amphipathic polymers used in physical chemistry and in the industry. APols are defined as "amphipathic polymers that are able to keep individual MPs soluble (in their native state) under the form of small complexes" (Popot et al. 2011). Polymers that have not been demonstrated to meet this criterium will not be considered in the present review. Other non-conventional approaches to stabilizing MPs in aqueous solutions, such as nanodiscs (NDs), fluorinated surfactants, lipopeptides, novel detergents, etc., have been discussed elsewhere (see e.g., Breyton et al. 2010; Gohon and Popot 2003; Nath et al. 2007; Popot 2010; Privé 2009).

The properties and uses of APols have been summarized in a couple of early reviews (Popot et al. 2003; Sanders et al. 2004) and two more recent ones (Popot 2010; Popot et al. 2011), which is not our purpose to supersede here. Rather, we would like to present (i) a brief reminder of the properties of APols and MP/APol complexes whose knowledge is essential to a rational use and (ii) an update on works that have been published posterior to the writing of these reviews, to which the reader is referred as regards earlier publications. In order to keep the review reasonably

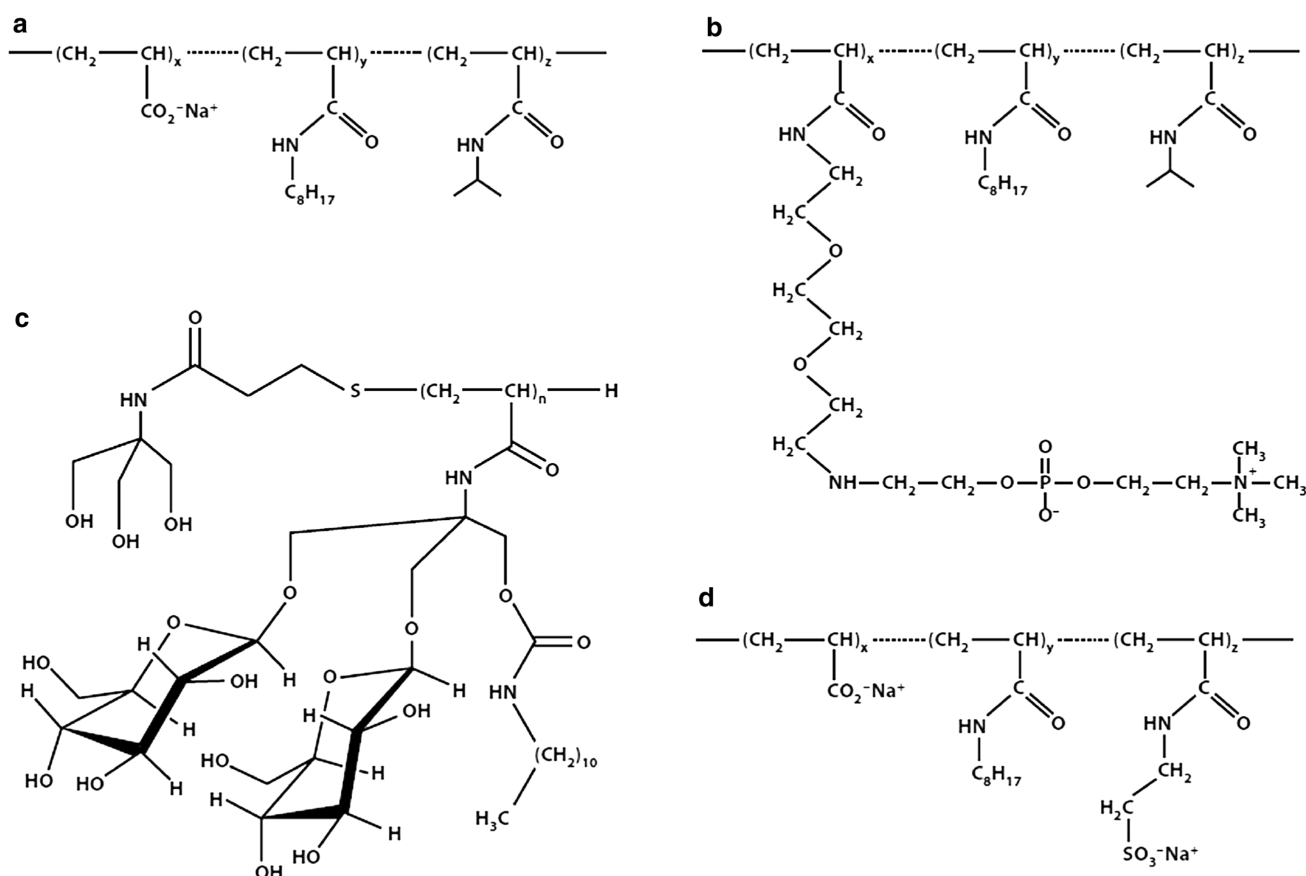

Fig. 1 Chemical structures of four types of amphipols. **a** A polyacrylate-based APol, A8-35 (Tribet et al. 1996). **b** A phosphocholine-based APol (PC-APol), C22-43 (Diab et al. 2007b). **c** A non-ionic, glucose-based APol (NAPol) (Sharma et al. 2012). **d** A sulfonated APol (SAPol) (Dahmane et al. 2011). See text, "Chemical Structure of Amphipols" section, and Table 1

short, a large fraction of the bibliography is compiled in Tables. The first section of the text recalls the fundamentals. In the second section, which presents applications, attention is focused on the most recent progress. A regularly updated compendium of publications about the properties and uses of APols is available at http://tinyurl.com/amphipolbibliography.

Basic Properties of Amphipols and Membrane Protein/Amphipol Complexes

Chemical Structure of Amphipols

APols are short amphipathic polymers designed to adsorb tightly onto the hydrophobic transmembrane surface of MPs and cover it with a thin interfacial layer of surfactant, rather than a fluffy corona, which would be disadvantageous for many applications. Achieving this goal imposes some constraints on their chemical structures, such as closely spaced hydrophobic groups (to prevent the formation of large loops), flexibility (to adapt to the small radius of curvature and irregularities of MP surface), and a high proportion of very hydrophilic moieties (to ensure a high solubility in aqueous buffers). These conditions are not always easy to reconcile, viz. the 11 years it has taken to develop non-ionic APols from proof of concept (Prata et al. 2001) to really manageable molecules (Bazzacco et al. 2012; Sharma et al. 2012). The average length of the polymers, $\langle X_n \rangle$, is usually kept short (a few tens of units), so that several molecules will be needed to keep a single MP soluble. This is meant to facilitate the homogenization of the size of the APol belt from one MP/APol complex to the next and to minimize the formation of bridges between them.

The first APols were obtained by grafting poly(acrylic acid) (PAA) with octylamine (or other alkylamines), followed or not by blockade of a large fraction of the remaining carboxylates with isopropylamine, so as to diminish the charge density along the chain. Using NMR spectroscopy to document the vicinity between the various moieties, it has been shown that, under the conditions used,

the grafts distribute randomly along the macromolecular chain (Magny et al. 1992). This led to the structure of A8-35, the most intensively studied and broadly used APol (Fig. 1a), and several of its congeners, which differed from it by their length and/or their charge density (A8-75, A34-35 and A34-75; Table 1). All of them were validated as APols in the princeps publication (Tribet et al. 1996). Only A8-35, however, has been heavily used for MP studies. Its average length—which has been recently revised; see Giusti et al. 2014b—is $\sim$35 acrylate units, corresponding to an average mass of $\sim$4.3 kDa per molecule (Table 1). With a polydispersity index $Đ \approx 2$ (*ibid.*), the length distribution is quite broad: upon SEC analysis, the low-*R*s and high-*R*s half-height limits on each side of the maximum correspond to $\sim$15 and $\sim$200 units, or $\sim$1.2 and $\sim$18 kDa, respectively (Rieger and Giusti, unpublished data). A more densely charged homolog of A8-35, A8-75, with the same length distribution, has been used in several studies with lipid vesicles or cells (Table 1 and ref. Marie et al. 2014). Some of the PMAL compounds (Gorzelle et al. 2002; Nagy et al. 2001) resemble A8-35, whereas others diverge by carrying miscellaneous charges (Table 1). Several other APol-like polymers have been proposed, but the structure and properties of the complexes they form with MPs have not been studied in details and, in at least one case, they have been shown not to be *bonafide* APols, in the sense that they do not actually keep MPs soluble. The use of SMALPs (Knowles et al. 2009) (a combination of styrene-maleic acid copolymer and lipids) seems to develop particularly promisingly (Table 1).

Recent developments in APol chemistry fall in two categories. On the one hand, the basic structure of APols has been modulated. This has led, for instance, to the development of phosphorylcholine-based APols (hereafter, “PC-APols”) (Diab et al. 2007b) (Fig. 1b), non-ionic, glucosylated APols (“NAPols”) (Bazzacco et al. 2009, 2012; Sharma et al. 2008, 2012) (Fig. 1c), and sulfonated APols (“SAPols”) (Dahmane et al. 2011) (Fig. 1d). A second type of development is to label or functionalize a given APol so as to confer it additional, experimentally useful properties (Table 2). Thus, A8-35 and A8-75 have been isotopically labeled (Giusti et al. 2014b; Gohon et al. 2004, 2008; Tribet et al. 1997), or grafted with various fluorophores (Fernandez et al. 2014; Giusti et al. 2012; Opačić et al. 2014a; Vial et al. 2005; Zoonens et al. 2007) or with various tags and/or adjuvants, such as biotin, an oligodeoxynucleotide (ODN), polyhistidine, etc. (Charvolin et al. 2009; Giusti et al. 2014a; Le Bon et al. 2014a). PC-APols and NAPols have also been tagged with biotin (Basit et al. 2012; Ferrandez et al. 2014). Chemical approaches to labeling and functionalizing APols are reviewed elsewhere in this issue (Le Bon et al. 2014b).

Solution Properties of Amphipols (Table 3)

All APols in common use are highly soluble in water (>100 or >200 g L^{-1}) and all assemble into small particles resembling, by their dimensions and the number of sequestered hydrophobic chains, detergent micelles (Dahmane et al. 2011; Diab et al. 2007b; Gohon et al. 2006; Sharma et al. 2012). This behavior, which is uncommon among amphipathic polymers, is probably dictated by the choices made when defining their chemical structure (for a discussion, see Giusti et al. 2012).

A8-35 particles have been extensively studied by size-exclusion chromatography (SEC), dynamic light scattering (DLS), small angle neutron scattering (SANS) and analytical ultracentrifugation (AUC) (Gohon et al. 2004, 2006) (Table 3). They are globular, $\sim$6.3 nm in diameter, with an average molecular mass of $\sim$40 kDa, and comprise $\sim$80 octyl chains, i.e., $\sim$9 average A8-35 molecules. The critical aggregation concentration (CAC), at which individual A8-35 molecules assemble into particles, has been determined both by surface tension measurements and by observing the loss, upon dilution, of Förster resonance energy transfer (FRET) between two complementary fluorescent APols (Giusti et al. 2012). It is well-defined and very low: $\sim$0.002 g L^{-1}. This means that, under most circumstances, most of the APol in a solution is present as particles, which, for instance, will not cross dialysis membranes of standard cut-off. Judging from the translational diffusion coefficient measured by quasi-elastic neutron scattering (QENS), the size of the particles does not change up to at least 240 g L^{-1}—six orders of magnitude above the CAC—indicating that they occupy a very large region of the phase diagram (Tehei et al. 2014). The effect of varying the distribution of octyl chains has been examined with derivatives of poly-(methacrylic acid) (PMAA) (Table 1). It was observed that, whereas randomly grafted PMAA derivatives assemble into small, well-defined, A8-35-like particles, “blocky” polymers form much larger, probably cylindrical objects (Liu et al. 2007).

The assembly and structure of A8-35 particles have been examined by molecular dynamics (MD) (Perlmutter et al. 2011). Upon being released in aqueous solution, A8-35 molecules first collapse onto themselves, and then spontaneously assemble into globular particles in which, as expected, hydrophobic chains occupy the core and charged groups the surface. The viscosity of the polar surface is higher than that of detergent micelles, and even that of the polar head region of lipid bilayers. This conclusion has been recently substantiated by inelastic neutron scattering (INS) data, which indicates that, whereas the viscosity of the octyl chains of A8-35, at room temperature, is similar to that of lipid acyl chains in their fluid state, that of the

Table 1 Chemical structures of amphipols and amphipol-like compounds

Polymer name [source, if commercially available]	Chemical structure	Approximate average *MW* (number averaged)	Remarks	Selected references
Amphipol A8-35 (noted $5–25C_8–40C_3$ in some physical chemistry articles) [Anatrace]	Short polyacrylate (~35 units) grafted with octylamine (~25 %) and isopropylamine (~40 %). See Fig. 1a	~4.3 kDa[a]	As of today the most extensively studied and most widely used APol. Its main limitations originate from its being charged and sensitive to low pH and multivalent cations. Particles and solution properties thoroughly characterized by SANS, INS, AUC, DLS, SEC, FRET, surface tension measurements and MD	Giusti et al. (2012, 2014b), Gohon et al. (2004, 2006), Ladavière et al. (2001, 2002), Perlmutter et al. (2011), Tehei et al. (2014), Tribet et al. (1996), Tribet and Vial (2008), Vial et al. (2007, 2009)
Amphipol A8-75 (also noted $5\text{-}25C_8$)	Short polyacrylate (~35 units) grafted with octylamine (~25 %)	~4.1 kDa[a]	Properties appear similar to A8-35, but, due to its higher charge density, A8-75 is likely to be less stabilizing to MPs	Ladavière et al. (2001, 2002), Luccardini et al. (2006), Marie et al. (2014), Tribet et al. (1996, 1997), Tribet and Vial (2008), Vial et al. (2007, 2009), Vial et al. (2005)
Amphipol A34-35	Long polyacrylate (~140 units) grafted with octylamine (~25 %) and isopropylamine (~40 %)	~17 kDa[a]	Properties appear similar to those of A8-35. Not extensively used	Tribet et al. (1996)
Amphipol A34-75	Long polyacrylate (~140 units) grafted with octylamine (~25 %)	~16 kDa[a]	Properties appear similar to those of A8-75. Not extensively used	Tribet et al. (1996)
Tris-based non-ionic APols	Telomers derived from *Tris*(hydroxy-methyl)-acrylamido-methane (THAM). Their solubility is provided by multiple hydroxy groups	3-28 kDa	The first attempt at creating non-ionic APols. The molecules provided a proof of concept, but were not soluble enough for routine use	Prata et al. (2001)
Glucose-based non-ionic APols (NAPols)	Telomers derived from THAM (10–90 units). Their solubility is provided by multiple glucose moieties. The first versions were heteropolymers, obtained either by cotelomerization or by grafting. Current NAPols are homotelomers with typically ~30 units, each carrying two glucose moieties and one undecyl chain. See Fig. 1c	8–60 kDa (typically ~13 kDa)	Entirely non-ionic polymers. Insensitive to pH and multivalent cations. The only APols to date to have been validated for cell-free MP expression and isoelectrofocusing. Validated for NMR. Insensitive to pH and multivalent cations. Extension of their use depends on scaling-up the synthesis to industrial levels	Bazzacco et al. (2009, 2012), Sharma et al. (2008, 2012)
Phosphorylcholine-based APols (PC-APols C22-43 and C45-68)	Random copolymers of *n*-octylacrylamide, a phosphorylcholine-modified acrylamide carrying a secondary amine function, and, optionally, *N*-isopropylacrylamide. See Fig. 1b	22 or 45 kDa	Zwitterionic/cationic APols. Insensitive to pH and multivalent cations	Basit et al. (2012). Diab et al. (2007a, b), Tribet et al. (2009)
Sulfonated APols (SAPols)	Short polyacrylate (~35 units) grafted with octylamine (~25 %) and taurine (~40 %). See Fig. 1d	~5.6 kDa[a]	Polyanionic APols. Insensitive to pH and multivalent cations. Appear less stabilizing than A8-35. Validated for NMR and EM	Dahmane et al. (2011), Huynh et al. (2014), Picard et al. (2006)
PMAL series [Anatrace]	Polymers carrying ammoniumamide, carboxylate and dodecyl side chains in various proportions (~30 units)	~12 kDa	Polymers carrying mixed charges. Shown to stabilize DAGK and deliver it to lipid vesicles. PMAL-C12 forms small water-soluble complexes with the sarcoplasmic calcium ATPase while protecting it from denaturation. Also used to deliver quantum dots and magnetic nanoparticles	Gorzelle et al. (2002), Nagy et al. (2001), Picard et al. (2006), Qi and Gao (2008), Qi et al. (2012)

Table 1 continued

Polymer name [source, if commercially available]	Chemical structure	Approximate average *MW* (number averaged)	Remarks	Selected references
Poly(methacrylic acid) derivatives	Poly(methacrylic acid) grafted with octyl chains either randomly or in blocks	10–11 kDa (random form)	Used for interaction with lipid vesicles and solubilizing hydrophobic non-transmembrane proteins (oleosins)	Gohon et al. (2011), Liu et al. (2007)
Styrene-maleic acid/lipid particles (SMALPs; lipodisqs) [Malvern Cosmeceutics]	Particles comprised of a styrene/maleic acid copolymer and lipids	~9.5 kDa (size of the 3:1 SMA polymer)	The copolymer can extract directly MPs from membranes, without the use of detergents, forming MP/lipid/polymer ternary complexes. Has been used for EPR distance measurements in a MP	Banerjee et al. (2012), Jamshad et al. (2011), Knowles et al. (2009), Long et al. (2013), Orwick-Rydmark et al. (2012), Orwick et al. (2012), Rajesh et al. (2011), Sahu et al. (2013)
NVoy, NV10 [Expedeon]	Fructose-based polysaccharide carrying hydrophobic chains. The exact structure has not been released	~5 kDa	No report yet on the size and dispersity of MP/NVoy complexes. Has been used for cell-free synthesis	Guild et al. (2011), Klammt et al. (2011)
Amphibiopols	Hydrophobized derivatives of pullulan	~30 kDa	Do not qualify as APols, inasmuch as they cannot keep MPs soluble as small individual complexes. Have been used to stabilize suspensions of membrane fragments	Duval-Terrié et al. (2003), Picard et al. (2004)

The table lists a selection of polymers that have been used to handle membrane proteins (see "Chemical Structure of Amphipols" section). To prevent confusion in the literature, we have proposed to define amphipols as "amphipathic polymers that are able to keep individual MPs soluble (and native) under the form of small complexes" (Popot 2010; Popot et al. 2011). According to this definition, "amphibiopols" do not qualify as amphipols. SMALPs appear to incorporate MPs into small (10–12 nm diameter) disc-like, lipid-containing particles, not unlike nanodiscs. The status of NVoy is uncertain, as the composition and size of the complexes have not been described yet

[a] The average mass of A8-35 (and, by extension, those of A8-75, A34-35, A34-75 and SAPols) has been recently revised; see Giusti et al. (2014b)

Table 2 Modified amphipols and their uses

Type of modification	Amphipol modified (short name of derivative)	Applications	References
Isotopic labeling			
^{14}C	A8-75	Following A8-75 distribution and exchange	Tribet et al. (1997)
^{3}H	A8-35	Following A8-35 distribution, evaluating MP/A8-35 mass ratio in complexes	Gohon et al. (2008)
^{2}H (on side chains)	A8-35 (DAPol)	Contrast-matching in SANS, AUC, NMR. Distinguishing between backbone and side chains dynamics in INS measurements	Catoire et al. (2009, 2010a), Gohon et al. (2004, 2006, 2008), Planchard et al. (2014) Sverzhinsky et al. (2014), Tehei et al. (2014), Zoonens et al. (2005)
^{2}H (perdeuteration)	A8-35 (perDAPol)	NMR, SANS, AUC, INS	Giusti et al. (2014b)
Fluorescent labeling			
Naphtalene	A8-75	Studying A8-75 interactions with lipid vesicles	Vial et al. (2005)
NBD	A8-35 ($FAPol_{NBD}$)	Studying A8-35 distribution, binding and exchange. Determining the CAC of A8-35 by FRET with $FAPol_{rhod}$	Giusti et al. (2012), Zoonens et al. (2007)
Fluorescein	A8-35 ($FAPol_{fluo}$)	Mapping the transmembrane region of a MP by FRET with single-tryptophane mutants	Opačić et al. (2014a)
Alexa Fluor 488	A8-35 ($FAPol_{AF488}$)	Tracking multiplex MP immobilization	Unpublished data
Rhodamine	A8-35 ($FAPol_{rhod}$)	Examining the distribution of A8-35 upon delivery of a transmembrane peptide to cells in culture. Following in vivo distribution and elimination of A8-35. Determining the CAC of A8-35 by FRET with $FAPol_{NBD}$	Fernandez et al. (2014), Giusti et al. (2012)
Atto 647	A8-35 ($FAPol_{atto647}$)	Following in vivo distribution and elimination of A8-35	Unpublished data
Alexa Fluor 647	A8-35 ($FAPol_{AF647}$)	Following in vivo distribution and elimination of A8-35; visualizing immobilized MP/APol complexes	Della Pia et al. (2014a), Fernandez et al. (2014), Le Bon et al. (2014a)
Tags, adjuvants			
Biotin	A8-35 (BAPol)	Immobilizing MPs onto chips or beads for SPR or fluorescence measurements. Following in vivo distribution and elimination of A8-35. Selecting soluble protein binders against immobilized MPs. Locating APol belt in EM single-particle images of PM/APol complexes?	Charvolin et al. (2009), Della Pia et al. (2014a, b), Fernandez et al. (2014)
Biotin	PC-APol	Immobilizing a MP onto chips for SPR measurements	Basit et al. (2012)
Biotin	NAPol (BNAPol)	Selecting soluble protein binders against immobilized MPs	Ferrandez et al. (2014)
Polyhistidine	A8-35 (HistAPol)	Reversibly immobilizing MPs	Giusti et al. (2014a)
Distributed imidazole moieties	A8-35 (ImidAPol)	Reversibly immobilizing MPs	Unpublished data
Oligodeoxynucleotide	A8-35 (OligAPol)	Vaccination, using the oligodeoxynucleotide as an adjuvant. Immobilizing MPs onto DNA chips	Le Bon et al. (2014a)
Peptide EP67	A8-35 (PeptAPol)	Vaccination, using the peptide as an adjuvant	Unpublished data

Table 2 continued

Type of modification	Amphipol modified (short name of derivative)	Applications	References
Thiamorpholine	A8-35 (SulfidAPol)	Immobilizing MPs onto gold beads	Unpublished data

See "Chemical structure of amphipols" section. For a discussion of the chemistry of modifying APols, see Le Bon et al. (2014b)

backbone is higher (Tehei et al. 2014). MD and INS data are, therefore, consistent in pointing to the dynamics of the backbone as the probable cause of the damping of the conformational excursions of A8-35-trapped OmpX predicted by MD (Perlmutter et al. 2014). This rigidifying effect probably contributes to the stabilization observed for most APol-trapped MPs, and it has been proposed to underlie the inhibition by APols of the enzymatic cycle of the sarcoplasmic calcium ATPase pump (SERCA1a) (see below, "Membrane Protein Stabilization by Amphipols (Table 6, line A)" and "Ligand Binding and Functional Properties of Amphipol-Trapped Membrane Proteins (Table 6, line B)" sections).

APols (A8-35, PC-APols) mix freely with detergents, both as free particles and at the surface of MPs (Diab et al. 2007b; Tribet et al. 2009; Zoonens et al. 2007), which is of great practical importance (see below, "Trapping Membrane Proteins with Amphipols" and "X-ray Crystallography (Table 6, line L)" sections).

Because the solubility of A8-35 and MP/A8-35 complexes depends on the carboxylate groups being ionized, it decreases if the pH drops below ~7, or in the presence of multivalent cations, both of which lead to aggregation and/or precipitation (Diab et al. 2007a; Gohon et al. 2004, 2006, 2008; Picard et al. 2006). This property can be usefully exploited (see e.g., Ning et al. 2013, 2014), but it is a hindrance in some experimental circumstances (see e.g., "Solution NMR Studies of Amphipol-Trapped Membrane Proteins and their Ligands (Table 6, line H)"). This is one of the major reasons that have led to the development of PC-APols, SAPols, and NAPols, none of which present these drawbacks (Dahmane et al. 2011; Diab et al. 2007a; Picard et al. 2006; Sharma et al. 2012).

Trapping Membrane Proteins with Amphipols

APols adsorb onto and can permeabilize lipid vesicles, and, depending on their chemical structure, on that of the APols, and on experimental conditions (temperature, pH, and ionic strength), break them up into sheet fragments or even smaller objects, possibly ND- or bicelle-like structures and/or mixed micelles (Ladavière et al. 2001; Tribet and Vial 2008; Vial et al. 2005, 2007, 2009) (reviewed by Marie et al. 2014). The kinetics of these phenomena can be very slow (days). APols also adsorb at the surface of cells (Popot et al. 2011), but, under the conditions tested, they are not lytic (see "Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes" section). They do not solubilize biological membranes (Bazzacco 2009; Champeil et al. 2000) and, as a rule, do not extract MPs (a couple of possible exceptions has been noted, but not studied in detail; see ref. Popot et al. 2003). It may seem surprising that APols, on the one hand, can keep MPs

Table 3 Physical–chemical studies of amphipol particles

Information gathered	Amphipols used	Methods used	References
Particle mass, R_g and R_S, contrast-matching points, specific volumes	Plain and partially deuterated A8-35 (DAPol)	SANS, AUC, SEC, DLS, densimetry	Gohon et al. (2004, 2006)
Critical aggregation concentration	Plain A8-35, fluorescently labeled A8-35 ($FAPol_{rhod}$ and $FAPol_{NBD}$)	Surface tension measurements, FRET	Giusti et al. (2012)
Molecular mass and mass dispersity, particle mass, R_g and R_S, contrast-matching point, ^{1}N-^{1}H NOE signals	Plain A8-35, perdeuterated A8-35 (perDAPol)	SANS, mass spectrometry, SEC, NMR	Giusti et al. (2014b)
Particle organization and dynamics	Coarse-grain and all-atom models of A8-35	Molecular dynamics	Perlmutter et al. (2011), Tehei et al. (2014)
Dynamics of main chain and side chains	A8-35, DAPol	Inelastic neutron scattering	Tehei et al. (2014)
R_S, miscibility with detergent	A8-35, PC-APol	ITC, light scattering, capillary electrophoresis	Diab et al. (2007b)
Molecular mass, particle mass, R_g and R_S, contrast-matching point	Homopolymeric NAPol	DLS, SEC, surface tension measurements, densimetry, SANS	Sharma et al. (2012)
R_S	SAPols	SEC	Dahmane et al. (2011), Picard et al. (2006)
Sensitivity to pH and multivalent cations	A8-35, PC-APols, SAPols, NAPols	Centrifugation, SEC, light scattering, turbidimetry	Diab et al. (2007a), Gohon et al. (2004, 2006), Picard et al. (2006), Sharma et al. (2012)

See "Solution Properties of Amphipols (Table 3)" section

soluble, mix with detergents and lipids, adsorb onto cell plasma membranes and pure lipid bilayers (see below), and, under certain circumstances, break up lipid vesicles into very small objects, but, on the other hand, are inefficient at solubilizing biological membranes. This question has not been studied in great detail and can only be discussed with caution. We may note, however, that APols seem less efficient than detergents at prying apart MP/lipid interactions (see below, "Properties of Membrane Protein/Amphipol Complexes" section) and that, at the concentrations at which they are used by biochemists, they are self-assembled into particles, in which their hydrophobic chains are hidden. It is possible that thermodynamics favors an equilibrium in which membranes decorated with APols coexist with APol particles, without excluding that the latter can extract some lipids and the occasional protein. A second aspect that may be as important, if not more, is that of kinetics. It may be that, under appropriate conditions, APols are able to break up biological membranes into disk-like particles, for instance, much as SMALPs do (Orwick-Rydmark et al. 2012), but that these conditions were not met in the few experiments that have been carried out to-date, or that the kinetics was too slow. This question clearly deserves further exploration, if only because direct extraction with APols could possibly give access to MPs that do not stand even a brief exposure to detergents. It may be worth noting, in this context, that mixtures of detergent and APols (i) are less denaturing than pure detergent (Champeil et al. 2000), and (ii) can be used for controlled extraction of MPs from thylakoid membranes (Bazzacco 2009).

In general, MPs are, therefore, extracted from membranes using detergents and, most often, purified before replacing detergents by APols. In the case of particularly fragile, detergent-sensitive MPs or MP complexes, purification, however, can be carried out in the APol-trapped state (see e.g., Althoff et al. 2011). As of today, more than three dozens purified MPs have been trapped in APols (Table 4), as well as complex MP mixtures (Bazzacco, 2009; Ning et al. 2013, 2014). The ability of APols to keep MPs soluble in the absence of detergent depends neither on MP size (from a few kDa to several MDa), structure (α-helical bundle or β-barrel, monomeric or oligomeric) or distribution of hydrophilic and hydrophobic surfaces (mainly transmembrane or featuring very large extra membrane domains), but appears to be universal. It seems that even a single transmembrane α-helix or α-helix dimer presents enough hydrophobic surface for an APol belt to form around it and keep it soluble (Duarte et al. 2008; Gohon, 1996; Popot et al. 2003). At the other end of the spectrum, APols do arrange into a continuous belt around the very extended transmembrane surface of the 1.7-MDa respirasome (Althoff et al. 2011) (Table 4; Fig. 2b). This is probably due to the high flexibility and adaptability of

Table 4 Integral membrane proteins that have been shown to be kept soluble by amphipols

Protein(s) (abbreviation)	Organism	Membrane	Structure II	Number of chains	Overall mass	References
Bacteriorhodopsin (BR)	*Halobacterium salinarum*	Plasma	α	1 + retinal	27 kDa	Bazzacco et al. (2009, 2012), Bechara et al. (2012), Charvolin et al. (2009), Dahmane et al. (2013), Della Pia et al. (2014a), Diab et al. (2007a), Elter et al. (2014), Etzkorn et al. (2013, 2014), Ferrandez et al. (2014), Giusti et al. (2014a), Gohon et al. (2008), Knowles et al. (2009), Le Bon et al. (2014a), Orwick-Rydmark et al. (2012), Pocanschi et al. (2006), Polovinkin et al. (2014a, b), Prata et al. (2001), Sharma et al. (2008), Tribet et al. (1996)
Cytochrome b_6f	*Chlamydomonas reinhardtii*	Thylakoid	α	2 × 8 + cofactors	228 kDa	Bazzacco et al. (2012), Bechara et al. (2012), Charvolin et al. (2009), Diab et al. (2007a), Prata et al. (2001), Tribet et al. (1996, 1998)
Outer membrane protein F (OmpF)	*Escherichia coli*	Outer membrane	β	3	111 kDa	Arunmanee et al. (2014), Tribet et al. (1996)
Photosynthetic reaction center	*Rhodobacter sphaeroides*	Plasma	α	3	96 kDa	Tribet et al. (1996)
Glycophorin A transmembrane anchor (tGpA)	Human	Plasma	α	2	~8 kDa	Gohon (1996), Popot et al. (2003)
Sarcoplasmic calcium pump (SERCA1a)	*Oryctolagus cunniculus*	Sarcoplasmic reticulum	α	1	110 kDa	Champeil et al. (2000), Picard et al. (2006)
Diacylglycerol kinase (DAGK)	*E. coli*	Plasma	α	3	40 kDa	Gorzelle et al. (2002), Nagy et al. (2001)
Photosystem I	*Synechocystis PCC 6803*	Thylakoid	α	3 × 12 + >120 cofactors	>1 MDa	Kievit and Brudvig (2001)
Nicotinic acetylcholine receptor (nAChR)	*Torpedo marmorata*	Plasma	α	2 × 5	535 kDa	Charvolin et al. (2009), Martinez et al. (2002)
Rhodopsin	*Bos taurus*	Retina disks	α	1 + retinal	40 kDa	Popot et al. (2003), Vahedi-Faridi et al. (2013)
Cytochrome bc_1	*B. taurus*	Inner mitochondrial membrane	α	2 × 11 + cofactors	490 kDa	Bechara et al. (2012), Charvolin et al. (2009, 2014), Ferrandez et al. (2014), Popot et al. (2003)
Photosystem II reaction center	*Pisum sativum*	Thylakoid	α	5 + cofactors	107 kDa	Popot et al. (2003)
Photosystem II reaction center	*Thermosynechococcus elongatus*	Thylakoid	α	2 × 17 + cofactors	550 kDa	Nowaczyk et al. (2004)
Maltose transporter	*E. coli*	Plasma	α	4	150 kDa	Popot et al. (2003)
Outer membrane protein A (OmpA) or its transmembrane domain (tOmpA)	*E. coli; Klebsiella pneumoniae*	Outer membrane	β	1	35 kDa (OmpA) 19 kDa (tOmpA)	Bazzacco et al. (2009), Bechara et al. (2012), Charvolin et al. (2009), Dahmane et al. (2011), Della Pia et al. (2014a), Le Bon et al. (2014a), Planchard et al. (2014), Pocanschi et al. (2006, 2013), Renault, (2008), Sharma et al. (2008), Zoonens et al. (2005, 2007)

Table 4 continued

Protein(s) (abbreviation)	Organism	Membrane	Structure II	Number of chains	Overall mass	References
FomA	*Fusobacterium nucleatum*	Outer membrane	β	1	40 kDa	Pocanschi et al. (2006)
NADH dehydrogenase (Complex I)	*Neurospora crassa*	Plasma	α	~35, + cofactors	1.1 MDa	Flötenmeyer et al. (2007)
V-ATPase transmembrane peptides	*Saccharomyces cerevisiae*	Plasma	α	1	3-4 kDa	Duarte et al. (2008)
Outer membrane protein X (OmpX)	*E. coli*	Outer membrane	β	1	18.6 kDa	Bazzacco et al. (2012), Catoire et al. (2009, 2010b), Etzkorn et al. (2014), Perlmutter et al. (2014)
Leukotriene BLT1 receptor	Human	Plasma	α	1	38 kDa	Banères et al. (2011), Dahmane et al. (2009)
Leukotriene BLT2 receptor	*Mus musculus*	Plasma	α	1	41.5 kDa	Banères et al. (2011), Catoire et al. (2010a, 2011), Dahmane et al. (2009), Giusti et al. (2014b)
Serotonin 5-HT_{4a} receptor	*M. musculus*	Plasma	α	1	44 kDa	Banères et al. (2011), Dahmane et al. (2009)
Cannabinoid CB1 receptor	*M. musculus*	Plasma	α	1	53 kDa	Banères et al. (2011), Dahmane et al. (2009)
Respirasome	*B. taurus*	Mitochondrial inner membrane	α	Complex I + bc_1 dimer + cytochrome *c* oxidase	1.7 MDa	Althoff et al. (2011)
Transient receptor potential ankyrin 1 ion channel (TRPA1)	*M. musculus*	Plasma	α	4	512 kDa	Cvetkov et al. (2011)
Major outer membrane protein (MOMP)	*Chlamydia trachomatis*	Outer membrane	β	3	~120 kDa	Feinstein et al. (2014), Tifrea et al. (2014, 2011)
Ghrelin GHS-R1a receptor	Human	Plasma	α	1	41 kDa	Bazzacco et al. (2012), Damian et al. (2012)
Vasopressin type 2 receptor (V2R)	Human	Plasma	α	1	42 kDa	Rahmeh et al. (2012)
Outer membrane receptor (FhuA)	*E. coli*	Outer membrane	β	1	82 kDa	Basit et al. (2012)
Outer membrane protein T (OmpT)	*E. coli*	Outer membrane	β	1	35 kDa	Leney et al. (2012)
Lipid A palmytoyltransferase (PagP)	*E. coli*	Outer membrane	β	1	22 kDa	Knowles et al. (2009), Leney et al. (2012)
Aquaporin SoPIP2;1	Spinach	Plasma	α	4	30 kDa	Vahedi-Faridi et al. (2013)
Capsaicin receptor (TRPV1)	*Rattus norvegicus*	Plasma	α	4	380 kDa	Cao et al. (2013); Liao et al. (2013, 2014)
ABCA4 transporter	*B. taurus*	Retina disks	α	1	257 kDa	Tsybovsky et al. (2013)
Peripherin-ROM1 complex	*B. taurus*	Retina disks	α	4	153 kDa	Kevany et al. (2013)
KCNE1 (minK)	Human	Plasma	α	Uncertain	Uncertain	Sahu et al. (2013)

Table 4 continued

Protein(s) (abbreviation)	Organism	Membrane	Structure II	Number of chains	Overall mass	References
P450 cytochromes CYP79A1 and CYP71E1 and cytochrome P450 oxidoreductase	*Sorghum bicolor*	Endoplasmic reticulum	α	1	~60 kDa	Laursen et al. (2013)
Light-harvesting complex II (LHCII)	*Arabidopsis thaliana*	Thylakoids	α	3	~75 kDa	Opačić et al. (2014b)
ExbB – ExbD complex	*E. coli*	Inner membrane	α	6	~140 kDa	Sverzhinsky et al. (2014)
Mannitol permease (EII^{mtl})	*E. coli*	Inner membrane	α	2	136 kDa	Opačić et al. (2014a)

Proteins are listed in chronological order of first publication of their trapping by an APol

individual APol molecules and of the adsorbed layer they form, which make them apparently universal tools for keeping water-soluble any integral MP, as well as mineral particles such as quantum dots (Luccardini et al. 2006; Qi and Gao 2008). Oleosins (hydrophobic proteins from seeds) can be kept soluble both by classical APols and by blocky polymers, forming much bigger complexes in the latter case (Gohon et al. 2011). Several other studies, which will not be reviewed here, suggest that APols can also be of use for working with non-membrane proteins (Ma et al. 2012; Ning et al. 2014; Prassl and Laggner 2009; Udi et al. 2013; Wolff and Delepierre 1997).

Transferring MPs from a detergent to an APol environment is simple (Fig. 3a) (for detailed protocols, see Zoonens et al. 2014). In solution, APols freely mix with detergents both in micelles and at the hydrophobic surface of MPs (Tribet et al. 2009; Zoonens et al. 2007), forming mixed detergent/APol micelles and ternary MP/detergent/APol complexes. The amount of APols to be added to the sample containing pure MP/detergent complexes is proportional to the amount of protein present in solution. To ensure monodispersity of the complexes, more APol is added, at this stage, than the MP will actually bind (see below, "Composition and Organization" section). The optimal MP/APol mass ratio varies from one protein to another: it depends on the size of the transmembrane region, on the propensity of the protein to self-associate, and, as a result, on the ability of the APol to prevent it from oligomerizing. It is determined experimentally, by screening a series of mass ratios and examining, first, the ability of the APol to retain quantitatively the protein in solution following detergent removal, second, the dispersity of the complexes, as revealed for instance by SEC (see e.g., Charvolin et al. 2014; Le Bon et al. 2014a; Tribet et al. 1996; Zoonens et al. 2014). After a short incubation, the concentration of detergent is brought under its critical micellar concentration (cmc) either by dilution or, most commonly, by adsorption onto polystyrene beads. The use of beads, onto which APols do not adsorb significantly (Zoonens 2004; Zoonens et al. 2007), has the advantage of keeping constant the protein concentration. As APols replace detergent at the transmembrane surface of the protein, lipids that had been displaced by the detergent may rebind. This is strongly supported by functional studies of APol-trapped versus detergent-solubilized bacteriorhodopsin (BR) (Dahmane et al. 2013), and it appears probable in the case of the nicotinic acetylcholine receptor (nAChR) (Martinez et al. 2002).

Because of the particularly low CAC of APols and the high stability of the APol layer surrounding the transmembrane domain of MPs, MP/APol complexes can frequently be handled as though they were soluble proteins. They can be, for example, extensively diluted with APol-free buffer

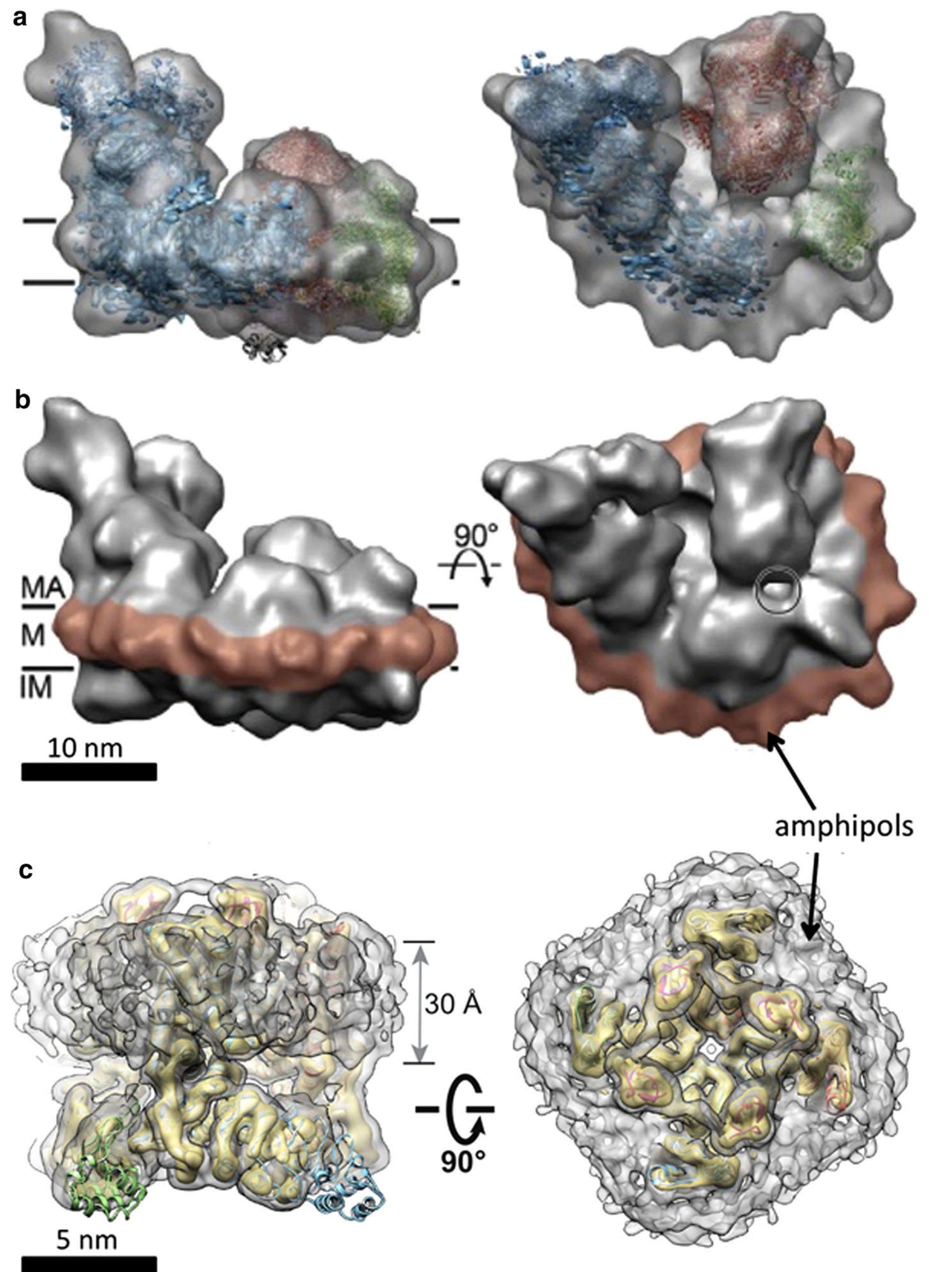

Fig. 2 Electron cryo-microscopy views of the A8-35 belt surrounding two large membrane proteins. **a**, **b** The mitochondrial respirasome ($M \approx 1.7$ MDa), comprising one copy of Complex I (*blue*), a dimer of cytochrome bc_1 (*red*) and one copy of cytochrome *c* oxidase (*green*), at 19-Å resolution. After the X-ray structures of the three complexes have been fitted in **a**, a band of unaccounted-for electron density, ~2-nm thick, is seen to follow the transmembrane surface of the supercomplex (in *brown* in **b**), corresponding to the amphipol belt. Reproduced, with permission, from Althoff et al. 2011. **c** The capsaicin (vanilloid) receptor, a cation channel (TRPV1; tetramer, $M \approx 380$ kDa), filtered at 6-Å resolution. After a 3D model of the structure has been fitted in, the APol belt appears as a low-density feature following the transmembrane surface (in *gray*). Reproduced, with permission, from Cao et al. 2013. The approximate position of the hydrophobic core of the membrane is indicated by two parallel lines. See text, "Composition and Organization" and "Electron Microscopy (Table 6, line I)"sections, and Huynh et al. 2014, Liao et al. 2014

(Tribet et al. 2009; Zoonens et al. 2007), washed with surfactant-free buffer after adsorption onto solid supports (Charvolin et al. 2009; Della Pia et al. 2014a, b; Giusti et al. 2014a; Le Bon et al. 2014a), or injected on a SEC column pre-equilibrated with surfactant-free buffer (Charvolin et al. 2014; Gohon et al. 2008; Zoonens et al. 2007). Nevertheless, the APol layer can be easily displaced and exchanged upon exposure to an excess of competing surfactants, be they detergents (Damian et al. 2012; Tribet et al. 1997, 2009; Zoonens et al. 2007), lipid vesicles (Nagy et al. 2001), black lipid membranes (BLM) (Pocanschi et al. 2006), lipidic three-dimensional (3D) phases (Polovinkin et al. 2014b), cell plasma membranes (Popot et al. 2011), or free APols (Tribet et al. 1997; Zoonens et al. 2007) (Fig. 3b). The rate of exchange between protein-bound APols and neutral detergents is extremely fast (<1 s) (Zoonens et al. 2007), and the mixing quasi-ideal (Tribet et al. 2009; Zoonens et al. 2007). Exchange between labeled and unlabeled forms of A8-35 occurs at a rate that strongly depends on the ionic strength, because of repulsive electrostatic interactions: depending on the presence or absence of 100 mM NaCl in a 20 mM Tris buffer, the exchange can be over in <10 min or require >24 h (Zoonens et al. 2007). The transfer of APol-trapped MPs to preformed membranes is discussed below in "Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes" section.

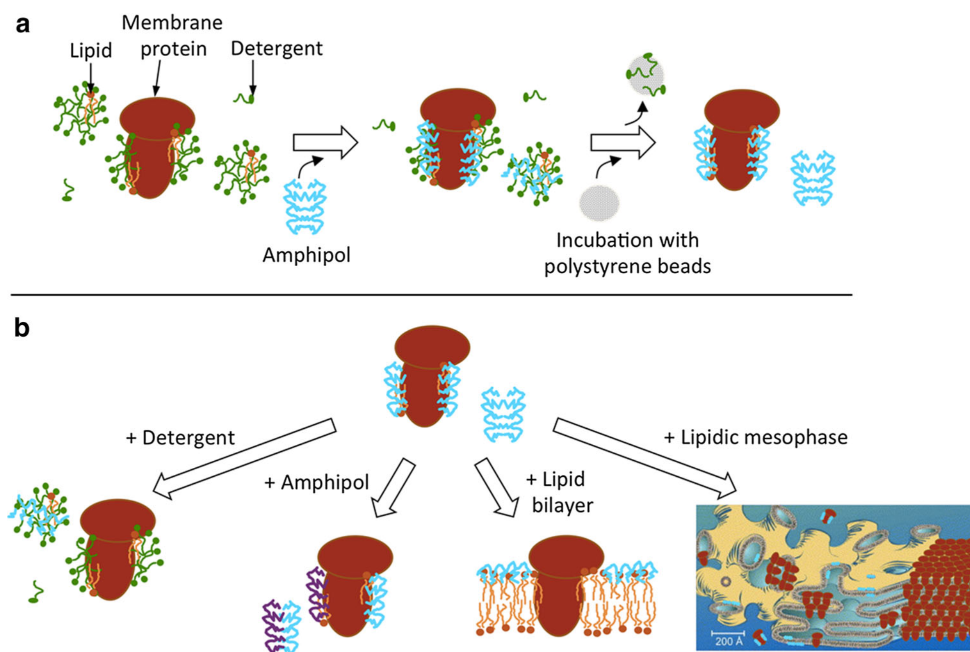

Fig. 3 Transferring a membrane protein (**a**) from a detergent solution to an amphipol and (**b**) from an amphipol to another surfactant. **a** Trapping a MP in APols. APol is added to the protein in detergent solution. After a short incubation, the detergent is removed, e.g., by adsorption onto polystyrene beads. **b** Displacement of MP-bound APol by other surfactants, be they a detergent, another APol, preformed membranes (vesicles, black films, biological membranes…), or a lipidic mesophase. The mesophase cartoon is adapted from Cherezov et al. 2006, with permission. See text, "Trapping Membrane Proteins with Amphipols", "X-ray Crystallography" and "Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes" sections, and Table 6, line N

After trapping in APols, all MPs that have been tested to date have been found to form small, compact, and stable water-soluble complexes (next section).

Properties of Membrane Protein/Amphipol Complexes (Table 5)

Composition and Organization

The most extensive data currently available concern MP/A8-35 complexes, with more limited information on MP/SAPol (Dahmane et al. 2011; Picard et al. 2006), MP/PMAL (Picard et al. 2006), MP/PC-APol (Diab et al. 2007a; Tribet et al. 2009), and MP/NAPol (Bazzacco et al. 2012; Sharma et al. 2012) ones. The major contributions to understanding the composition, organization, dynamics, and solution properties of MP/APol complexes are listed in Table 5. In the present section, we will concentrate on MP/A8-35 complexes, and only briefly mention differences between various types of complexes.

NMR (Catoire et al. 2009, 2010b; Etzkorn et al. 2014; Planchard et al. 2014; Zoonens et al. 2005) and electron microscopy (EM) (Althoff et al. 2011; Huynh et al. 2014; Kevany et al. 2013; Liao et al. 2013; Tsybovsky et al. 2013; Vahedi-Faridi et al. 2013) investigations show that A8-35 adsorbs specifically onto the transmembrane, hydrophobic surface of MPs, where it forms a relatively thin, 1.5–2 nm thick layer (Althoff et al. 2011; Gohon et al. 2008; Huynh et al. 2014; Liao et al. 2014). The same conclusions can be derived from recent MD simulations of OmpX/A8-35 complexes (Perlmutter et al. 2014). No experimental evidence has been found yet for interactions with, for instance, basic or hydrophobic patches at the water-exposed surfaces of MPs. In MD simulations, no contacts are observed between the hydrophobic moieties of the polymer and the extramembrane loops and turn of OmpX, but basic extramembrane residues are seen to interact transiently with the carboxylates of A8-35 (Perlmutter et al. 2014). This is reminiscent of the interactions observed between A8-35 and the basic soluble protein lysozyme (Champeil et al. 2000) and should be kept in mind when handling in polyanionic APols MPs that feature basic extramembrane domains.

If lipids are present, ternary MP/lipid/APol complexes will form (see e.g., Bechara et al. 2012; Gohon et al. 2008), and there is actually some evidence that transfer from detergent to APols facilitates the rebinding to the transmembrane surface of MPs of lipid molecules that had been displaced by the detergent (see below, "Ligand Binding and Functional Properties of Amphipol-Trapped Membrane Proteins" section).

The mass of APol-bound per MP has been determined in only a few cases (reviewed in Popot 2010; Popot et al.

Table 5 Composition, organization and solution properties of MP/APol complexes

Amphipols used	Methods used	Membrane proteins	Information gathered	References
Plain A8-35, DAPol, SAPol, NAPol	Solution NMR	tOmpA, OmpX, KpOmpA, BR, MOMP	τ_c, areas of contact between MP and APol, similarities and differences between MP/APol, MP/detergent and MP/ND complexes	Bazzacco et al. (2012), Catoire et al. (2014), Catoire et al. (2009, 2010b), Dahmane et al. (2011), Elter et al. (2014), Etzkorn et al. (2013, 2014), Feinstein et al. (2014), Planchard et al. (2014), Raschle et al. (2010), Renault (2008), Zoonens et al. (2005)
Plain A8-35, DAPol	SAXS, SANS	BR, cytochrome bc_1, ExbB-ExbD complexes	R_g, shape, composition, interactions between complexes	Charvolin et al. (2014), Gohon et al. (2008), Sverzhinsky et al. (2014)
A8-35	Negative-stain EM, cryo-EM, STEM	BR, b_6f, Complex I, respirasome, OmpF, ExbB-ExbD complexes, TRPA1, TRPV1, peripherin-ROM1 complex, ABCA4	Shape, arrangement of APol belt around MPs, modes of association of MPs upon APol depletion, mass	Althoff et al. (2011), Arunmanee et al. (2014), Cao et al. (2013), Cvetkov et al. (2011), Flötenmeyer et al. (2007), Gohon et al. (2008), Huynh et al. (2014), Kevany et al. (2013), Liao et al. (2013, 2014), Sverzhinsky et al. (2014), Tribet et al. (1998), Tsybovsky et al. (2013)
Coarse-grained model of A8-35	Molecular dynamics	OmpX	Arrangement of the complex, dynamics of APol-trapped *vs.* detergent-solubilized and bilayer-inserted OmpX	Perlmutter et al. (2014)
A8-35	Mass spectrometry, ion mobility spectrometry-mass spectrometry	OmpX, bc_1, b_6f, BR, tOmpA, OmpT, PagP, DAGK	MP mass and conformation, bound lipids	Bechara et al. (2012), Catoire et al. (2009), Hopper et al. (2013), Leney et al. (2012), Ning et al. (2013, 2014)
Plain A8-35, [^{14}C]A8-35, $FAPol_{NBD}$, PC-APols	FRET, ITC	tOmpA, BR, cytochrome b_6f	Thermodynamics of APol adsorption onto MPs, miscibility and exchange of surfactants at the surface of MPs	Tribet et al. (1997, 2009), Zoonens et al. (2007)
Plain A8-35, $FAPol_{NBD}$, BAPol, PC-APols	FRET, SPR, ITC, fluorescence microscopy	OmpF, tOmpA, BR, cytochrome bc_1, cytochrome b_6f, nAChR	Stability of MP/APol complexes upon extensive dilution and flushing	Charvolin et al. (2009), Popot et al. (2003), Tribet et al. (2009), Zoonens et al. (2007)
A8-35, NAPols	Mass spectrometry, biochemical analysis, functional studies	BR, cytochrome bc_1	Bound lipids	Bechara et al. (2012), Dahmane et al. (2013), Gohon et al. (2008)

See "Trapping Membrane Proteins with Amphipols" section

2003, 2011). For technical reasons, it can be difficult to measure it with great accuracy. The best determination to date, based on chemical analyses, AUC, and spectroscopic and SANS measurements, is for BR complexed by A8-35 in the presence of *Halobacterium* lipids. BR (~27 kDa, seven transmembrane α-helices) appears to be associated to the full complement of lipids (~9 kDa) extracted along with it from its native purple membrane and complexed by ~54 kDa of A8-35, yielding an overall particle mass of ~90 kDa (Gohon et al. 2008). The small (~19 kDa, eight β-strands) transmembrane β-barrel of *Escherichia coli* outer membrane protein A (tOmpA) binds a minimum of ~25 kDa of A8-35 (Zoonens et al. 2007). Due to experimental constraints, this is an estimate by default. MD calculations suggest that 33 kDa of A8-35 are insufficient to form a complete belt around OmpX, another small, 8-stranded β-barrel MP, whereas 44 kDa suffice (and may be an excess) (Perlmutter et al. 2014). The large

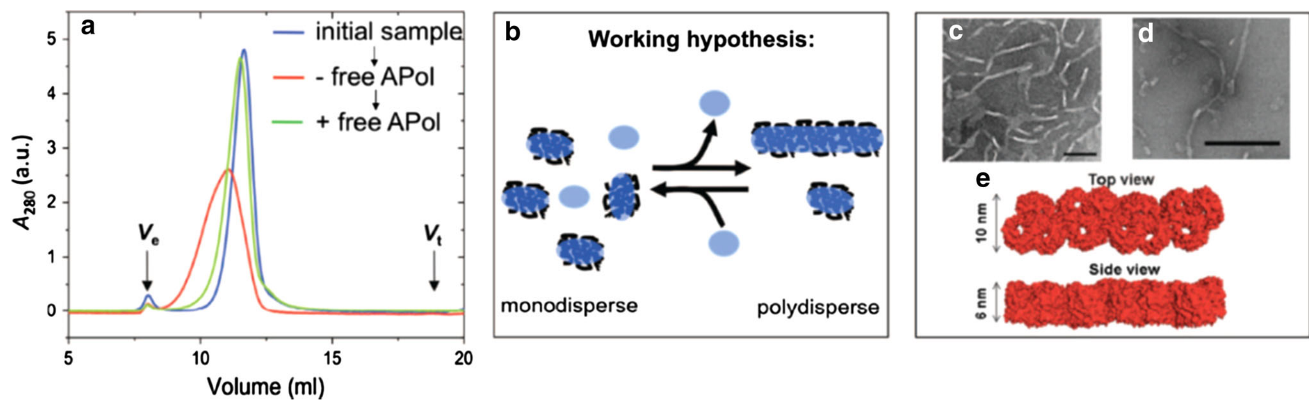

Fig. 4 Impact of free APol particles on the homogeneity of MP/APol complexes. **a** SEC profiles of tOmpA/A8-35 complexes after trapping with an excess of APol (*blue curve*), after separation of tOmpA/A8-35 complexes from free A8-35 particles by IMAC (*red curve*), and after adding back free A8-35 to the latter sample (*green curve*) (adapted with permission from Zoonens et al. 2007). **b** Working hypothesis: the equilibrium between protein/protein and protein/APol interactions is shifted one way or the other depending on the volume of the APol "phase". **c** EM image (in *negative stain*) of a BR/A8-35 sample depleted from free APol by repeated ultracentrifugations and kept at 4 °C for 2 years. Scale bar is 50 nm (reproduced with permission from Gohon et al. 2008). **d** EM image (in *negative stain*) of an OmpF/A8-35 sample 10 min after being separated from free APol by SEC. Scale bar is 100 nm. **e** Top and side views of a model for the structural organization of OmpF filaments (**d** and **e** reproduced with permission from Arunmanee et al. (2014)). See text, "Composition and Organization" section

mitochondrial cytochrome bc_1 complex (~490 kDa, 22 transmembrane helices) has been estimated to bind ~54 kDa (Charvolin et al. 2014; Popot et al. 2011)—surprisingly close to the amount bound by the 7-helix BR. In brief, it seems that the mass of A8-35 bound per MP increases only slowly with the perimeter of the transmembrane region, possibly indicating that, for very small proteins, it cannot decrease much below the ~40 kDa of the pure A8-35 particle.

The binding of NAPols has been estimated to be ~97 kDa per BR/lipid complex, whereas OmpX, ~18 kDa, binds ~74 kDa (Sharma et al. 2012).

A curious observation is that, in the cryo-EM single-particle image reconstruction of the very large mitochondrial supercomplex $I_1III_2IV_1$ (Althoff et al. 2011) (cf. "Electron Microscopy" section), the thickness of the A8-35 layer appears irregular, forming local bumps (Fig. 2a, b). The bumps are reproducible from one set of data to the other, suggesting that they are not noise, but not necessarily excluding that they be reconstruction artifacts. In the higher-resolution structure recently obtained of the tetrameric ion channel TRPV1, the A8-35 belt does not appear bumpy (Cao et al. 2013; Liao et al. 2013) (Fig. 2c) (reviewed in Huynh et al. 2014; Liao et al. 2014). It is not known whether the bumps, if real, betray the presence of underlying materials, such as clumps of lipids or unidentified subunits, or result from a mechanical response of the water/APol interface to the very large radius of curvature of the supercomplex, which stresses it well away from the spontaneous 3.15-nm radius of free A8-35 particles (Gohon et al. 2006). Perhaps related to this observation, the thickness of the A8-35 belt appears—reproducibly—irregular in MD models of OmpX/A8-35 complexes (Perlmutter et al. 2014). More data are clearly needed to clarify this issue.

Studies with tOmpA (Zoonens et al. 2007), BR (Gohon et al. 2008), the bc_1 complex (Charvolin et al. 2014), *E. coli*'s outer membrane protein F (OmpF) (Arunmanee et al. 2014), and the ExbB–ExbD complex (Sverzhinsky et al. 2014) have shown that the presence of some free APol particles is essential to keeping MP/APol complexes monodisperse, a 1:1 ratio between bound and free APol being typically sufficient (cf. Fig. 4a). The explanation, most likely, is that APols are not very good at preventing protein/protein interactions and need to be present in some excess to shift the equilibrium away from the formation of small oligomers (Fig. 4b). EM observations of APol-depleted preparations of BR/A8-35 complexes, kept for 2 years at 4 °C (Fig. 4c) (Gohon et al. 2008), or of OmpF/A8-35 ones kept for 10 min to a week (Fig. 4d) (Arunmanee et al. 2014), show linear filaments in which MPs seem to interact side-by-side via their transmembrane surfaces (Fig. 4e), in keeping with the idea that protein/protein contacts have replaced some of the protein/APol ones. In the presence of lipopolysaccharide, OmpF filaments tend to form small 2D crystals (Arunmanee et al. 2014). These observations have led to the suggestion that APol depletion could possibly be exploited to control the formation of MP assemblies, which could be of use for structural studies (Arunmanee et al. 2014).

MP-adsorbed layers of A8-35 exchange with free A8-35 in solution (Zoonens et al. 2007), most likely, given the

Table 6 A schematic overview of various validated or foreseeable applications of APols to MP studies and their current state of development

Type of application (section where discussed)	Rationale	Observations	Amphipols used	References
A. Stabilization ("Membrane Protein Stabilization by Amphipols" section)	A complex issue. Involves limitation of hydrophobic sink, preservation of MP/lipid interactions, and damping of transmembrane domain conformational excursions	Most MPs tested to date are more stable in APols than in detergent solutions. There seems to be a tendency for the less highly charged APols to be more stabilizing (see Bazzacco et al. 2012; Picard et al. 2006), but it may not be universal (Huynh et al. 2014)	A8-35, SAPols, NAPols, PMAL	Bazzacco et al. (2012), Champeil et al. (2000), Dahmane et al. (2011, 2013), Etzkorn et al. (2013), Feinstein et al. (2014), Gohon et al. (2008), Huynh et al. (2014), Picard et al. (2006), Pocanschi et al. (2013), Popot (2010), Popot et al. (2003, 2011), Tifrea et al. (2011, 2014), Tribet et al. (1996)
B. Ligand binding and functional studies ("Ligand Binding and Functional Properties of Amphipol-Trapped Membrane Proteins" section)	Avoid functional perturbations and/or destabilization by detergent	Ligand binding very generally unperturbed. Most MPs functional in APols, but the enzymatic cycle of the calcium ATPase is slowed down, possibly due to damping of large-scale transmembrane conformational changes	A8-35, NAPols, PMAL, SMALPs	Basit et al. (2012), Bazzacco et al. (2012), Champeil et al. (2000), Charvolin et al. (2009, 2014), Dahmane et al. (2009, 2013), Ferrandez et al. (2014), Gohon et al. (2008), Gorzelle et al. (2002), Knowles et al. (2009), Le Bon et al. (2014a), Martinez et al. (2002), Picard et al. (2006), Popot et al. (2003), Rahmeh et al. (2012)
C. Folding and refolding ("Amphipol-Assisted Folding and Refolding of Membrane Proteins" section)	The mildness of APols, along with other factors, seems to make them an excellent environment in which to fold or refold denatured MPs, such as those produced as inclusion bodies	To date applied to four outer MPs, BR, and six GPCRs (see Table 7)	A8-35, SAPols, NAPols	Banères et al. (2011), Bazzacco et al. (2012), Catoire et al. (2010a), Dahmane et al. (2009, 2011, 2013), Damian et al. (2012), Elter et al. (2014), Etzkorn et al. (2013), Gohon et al. (2011), Leney et al. (2012), Mary et al. (2014), Pocanschi et al. (2006, 2013), Popot and Kleinschmidt (2014)
D. Cell-free expression ("Amphipol-Assisted cell-Free Expression of Membrane Proteins" section)	Letting MPs synthesized in vitro fold in a mild environment	Validated to date for NAPols. A8-35 and SAPols block in vitro expression of MPs, presumably as a consequence of binding to hydrophobic segments as they exit the ribosome tunnel. NVoy also seems to be a favorable medium	NAPols, NVoy	Bazzacco et al. (2012), Guild et al. (2011), Klammt et al. (2011), Park et al. (2011), Shadiac et al. (2013)
E. Size exclusion, immobilized metal and affinity chromatographies, BN-PAGE	Purifying and studying MP/APol complexes	Most chromatographic methods can be resorted to. Reserve ion exchange chromatography to uncharged APols. Avoid fusing tags too close to the transmembrane domain to prevent steric/electrostatic interactions with the column that can reduce the efficiency of binding. SEC tends to overestimate the size of MP/A8-35 complexes	A8-35, SAPols, NAPols, PC-APols	Bazzacco et al. (2009, 2012), Champeil et al. (2000), Charvolin et al. (2014), Dahmane et al. (2011, 2013), Diab et al. (2007a), Etzkorn et al. (2013), Gohon et al. (2008, 2011), Le Bon et al. (2014a), Martinez et al. (2002), Picard et al. (2006), Prata et al. (2001), Sharma et al. (2012), Sverzhinsky et al. (2014), Tribet et al. (1996, 1997), Zoonens et al. (2007)

Table 6 continued

Type of application (section where discussed)	Rationale	Observations	Amphipols used	References
F. Ultracentrifugation	Purifying and studying MP/APol complexes	Sucrose gradient, equilibrium and sedimentation velocity methods can all be used. Separating the contributions of the protein and the APol can be facilitated by the use of deuterated or fluorescent APols	A8-35, DAPol, NAPols, PC-APols, SAPols	Althoff et al. (2011), Diab et al. (2007a), Gohon et al. (2008, 2011), Martinez et al. (2002), Prata et al. (2001), Sharma et al. (2012), Sverzhinsky et al. (2014), Tribet et al. (1996, 1997)
G. Light spectroscopy	Analyzing APol-trapped MPs	UV and visible absorption, fluorescence, CD and SR-CD spectroscopies can all be used. All current APols interfere with IR absorption spectroscopy in the amide band region, but resonance Raman spectroscopy is accessible	A8-35, NAPols, SAPols	See e.g., refs. Dahmane et al. (2013), Gohon et al. (2008), Pocanschi et al. (2006), Polovinkin et al. (2014a), Popot et al. (2011), Tifrea et al. (2011), Zoonens et al. (2007)
H. NMR ("Solution NMR Studies of Amphipol-Trapped Membrane Proteins and Their Ligands" section)	Usual conditions for solution NMR of MPs are aggressive (high detergent concentrations, high temperature). APols may permit to study MPs that do not stand them. A8-35 and SAPols are easier to deuteriate than most detergents	Solution NMR is a rapidly developing application. Stabilization by APols facilitates working for extended periods at relatively high temperature. Solid-state NMR remains to be validated	A8-35, DAPol, NAPols, SAPols	Bazzacco et al. (2012), Catoire et al. (2009, 2010a, b, 2011), Dahmane et al. (2011), Elter et al. (2014), Etzkorn et al. (2013, 2014), Feinstein et al. (2014), Planchard et al. (2014), Raschle et al. (2010), Renault (2008) Tifrea et al. (2014), Warschawski et al. (2011), Zoonens et al. (2005)
I. Electron microscopy ("Electron microscopy" section)	APols ought to be particularly useful to study MPs and MP supercomplexes that are either easily disrupted or prone to conformational changes	A particularly promising application. STEM has been validated. AFM and SMFS are certainly doable but remain to be validated	A8-35, SAPols	Althoff et al. (2011), Arunmanee et al. (2014), Cao et al. (2013), Cvetkov et al. (2011), Flötenmeyer et al. (2007), Gohon et al. (2008), Henderson (2013), Huynh et al. (2014), Kevany et al. (2013), Liao et al. (2013, 2014), Sverzhinsky et al. (2014), Tribet et al. (1998), Tsybovsky et al. (2013), Vahedi-Faridi et al. (2013)
J. Radiation scattering ("Radiation Scattering Studies" section)	Studying the mass, dimensions and organization of MP/APol complexes	DLS, SAXS and SANS have all been used. Avoiding the presence of small oligomers can be difficult. Separating the contributions of the protein and the APol to SANS signals is greatly helped by contrast-matching the APol using isotopically labeled MP or APol	Plain and deuterated A8-35	Charvolin et al. (2014), Gohon et al. (2008, 2011), Popot et al. (2003), Sharma et al. (2012), Sverzhinsky et al. (2014)

Table 6 continued

Type of application (section where discussed)	Rationale	Observations	Amphipols used	References
K. Mass spectrometry ("Mass Spectrometry" section)	Analysis of APol-trapped MPs and proteolytic peptides, identification of bound lipids	MALDI-TOF, ESI–MS and ESI-IMS-MS have all been validated. Subunits and lipids can be detected, and the folded and unfolded states of the proteins distinguished. A8-35 facilitates whole-proteome trypsinolysis and identification of tryptic peptides	A8-35, NAPols	Bechara et al. (2012), Catoire et al. (2009), Hopper et al. (2013), Leney et al. (2012) Ning et al. (2013, 2014)
L. X-ray crystallography ("Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes" section)	Stabilizing MPs under crystallization conditions	Chemical structure of A8-35 far from ideal (charges). NAPols untested yet. A8-35 has been used to transfer BR to a lipidic mesophase, where it forms highly ordered crystals	A8-35	Charvolin et al. (2014), Polovinkin et al. (2014b), Popot et al. (2011)
M. MP immobilization onto solid supports ("Amphipol-Mediated Immobilization of Membrane Proteins onto Solid Supports and Ligand-Binding Measurements" section)	Tagged APols will simultaneously make a MP water-soluble, stabilize it biochemically, and anchor it onto a solid support	Tags that have been validated to date include biotin, polyhistidine, an oligodeoxynucleotide, and distributed imidazole moieties. Cf. Table 2	A8-35, NAPols, PC-APols	Basit et al. (2012) Charvolin et al. (2009), Della Pia et al. (2014a, b), Ferrandez et al. (2014), Giusti et al. (2014a), Le Bon et al. (2014a)
N. Delivery of MPs to preexisting membranes ("Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes" section)	APols do no lyse target membranes (lipid vesicles or black films, cell plasma membrane) and can therefore be used to deliver to them hydrophobic cargoes	MPs have been delivered to lipid vesicles, black films, mesophases, and cell plasma membranes. Caveats: insertion process expected to be traumatic for fragile MPs; carrier APols will remain associated to the target membrane	A8-35, A8-75, PMAL	Kyrychenko et al. (2012), Nagy et al. (2001), Pocanschi et al. (2006), Polovinkin et al. (2014b), Popot et al. (2011)
O. Vaccination ("Vaccination" section)	Stabilizing biochemically and physically MPs used as immunogens. Co-delivering them along with APol-bound or co-trapped adjuvants	The A8-35-trapped major outer membrane protein (MOMP) from *Chlamydia trachomatis* offers a much better protection to vaccinated mice than its detergent-solubilized counterpart	A8-35	Tifrea et al. (2011, 2014)
P. Trapping labile supercomplexes, assembly intermediates, etc.	Stabilizing and studying MP assemblies that are too labile to resist exposure to detergents	Perhaps one of the most interesting applications of APols, but still underused	A8-35	Althoff et al. (2011)
Q. Isoelectrofocusing	Improving yields over that in detergent solutions?	Requires strictly neutral APols. Preliminary data indicate that the approach is feasible	NAPols	Bazzacco (2009)

The second column gives examples of the rationales for using APols for the application considered, the third one a very brief summary of current observations and, if applicable, caveats, the last one a selection of references

very low CAC and the near-absence of free individual APol molecules (Giusti et al. 2012), via a mechanism involving collisions between a MP/APol complex and a free APol particle, followed by fusion, mixing, and fission. As expected, the kinetics of exchange is highly dependent—from minutes to tens of hours—on the extent to which repulsive electrostatic interactions are screened (Zoonens et al. 2007). In contrast, A8-35 remains firmly

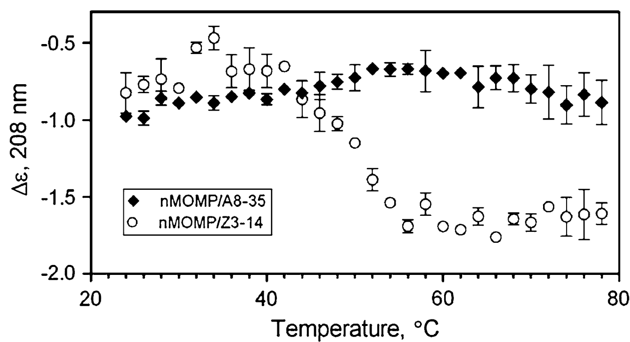

Fig. 5 Stabilization of the major outer membrane protein from *Chlamydia trachomatis* (MOMP) against thermal denaturation following transfer from Zwittergent 3–14 (*open circles*) to amphipol A8-35 (*solid circles*). Denaturation was followed by measuring the loss of ellipticity at 208 nm. Reproduced with permission from Tifrea et al. (2011). See also Feinstein et al. (2014), section "Membrane Protein Stabilization by Amphipols", and Table 6, line A

associated to MPs upon exposure to large volumes of surfactant-free buffer, as occurs upon extensive dilution (Tribet et al. 2009; Zoonens et al. 2007), or upon flushing of complexes attached to a surface plasmon resonance (SPR) chip via a histidine tag carried by the protein (Popot et al. 2003). Consistent with these observations, MPs bound to a solid support via a biotinylated APol neither desorb nor become inactivated upon extensive washing of the chips with surfactant-free buffer (Charvolin et al. 2009) (see "Amphipol-Mediated Immobilization of Membrane Proteins onto Solid Supports and Ligand-Binding Measurements" section).

As mentioned in the "Solution Properties of Amphipols" and "Trapping Membrane Proteins with Amphipols" sections, APols, whether present as free particles or as a MP-adsorbed layer, freely mix with detergents, in a nearly ideal manner (Tribet et al. 2009; Zoonens et al. 2007). This makes it very easy to exchange one type of surfactant for the other. The ease with which detergents can wash APols away from the surface of MPs may seem contradictory with the strong retention of APols by MPs upon extensive dilution or flushing with surfactant-free buffers. This apparent paradox is due to the fact that there is little or no free energy cost to displacing APols from a MP hydrophobic transmembrane surface to a mixed detergent/APol particle while replacing it with detergent, whereas it is extremely costly to bare the same surface from any surfactant (Giusti et al. 2012; Tribet et al. 2009).

Membrane Protein Stabilization by Amphipols (Table 6, line A)

Most MPs become more stable, generally much more so, when transferred from detergent to APols (reviewed in Popot 2010; Popot et al. 2011) (Fig. 5). The underlying mechanisms are several:

Fig. 6 Lemuel Gulliver's movements being restricted by the tiny strings of the Lilliputians (Swift 1726). See text, "Membrane Protein Stabilization by Amphipols" and "Ligand Binding and Functional Properties of Amphipol-Trapped Membrane Proteins" sections

(i) APols do not compete efficiently with the protein/protein and protein/lipid interactions that define the 3D structure of MPs and stabilize them (for the same reason, APols are not, or extremely weak, detergents). Indeed, lipids tend to rebind to MPs upon transfer from detergent solutions to APols (Dahmane et al. 2013; Martinez et al. 2002), which contributes to MP stabilization (Dahmane et al. 2013).

(ii) Whereas it is recommended to handle MP/APol complexes in the presence of some excess of APol to keep them from forming oligomers (see "Composition and Organization" section), this excess can be very small (for tOmpA, typically one free particle per complex; see Zoonens et al. 2007), which limits the volume of the hydrophobic sink into which lipids and subunits can disperse, a major cause of inactivation by detergent solutions (for a discussion, see Gohon and Popot, 2003; Popot 2010).

(iii) APols appear to damp large-scale conformational excursions by the transmembrane domains of MPs. This complex phenomenon, which has been discussed elsewhere under the nickname of

"Gulliver effect" (Picard et al. 2006; Popot et al. 2003, 2011) (Fig. 6) is thought to result from the activation free energy penalty for rearranging the backbone of the polymer to adapt to transconformations of the protein's transmembrane surface. It is probably related to the surface viscosity of A8-35 particles revealed by MD calculations (Perlmutter et al. 2011) and INS measurements (Tehei et al. 2014) and provides a tentative explanation for the correlation observed between stabilization of SERCA1a and inhibition of its enzymatic cycle (Picard et al. 2006) (see "Ligand Binding and Functional Properties of Amphipol-Trapped Membrane Proteins" section). Long highly hypothetical, the existence of this effect has recently received some strong support from three different types of observations. First, a detailed analysis of the mechanism of stabilization of OmpA by A8-35 against urea-induced denaturation shows that its origin is not thermodynamic, but kinetic: under the (rather extreme) conditions used, A8-35-trapped OmpA is thermodynamically *less* stable than in detergent solution, whereas the free energy barrier for moving from the folded to the unfolded state is strongly increased, resulting in a much slower denaturation rate (Pocanschi et al. 2013). Whether a similar mechanism accounts for the resistance of APol-trapped MPs to heat-induced denaturation in the absence of urea (see e.g., Dahmane et al. 2009, 2013; Feinstein et al. 2014; Tifrea et al. 2011) remains of course to be seen. The second line of support originates from a recent MD comparison of the dynamics of OmpX in complex with either A8-35, the detergent dihexanoylphosphatidylcholine (DHPC), or a lipid bilayer. It shows that the APol-trapped protein undergoes conformational excursions of restricted amplitude compared to the detergent-solubilized one, and even that spanning a bilayer (Perlmutter et al. 2014) (Fig. 7). Finally, inelastic neutron scattering (INS) measurements indicate that, whereas the fluidity of the inner core of A8-35 particles is similar to that of lipids in the fluid phase, the backbone is more viscous (Tehei et al. 2014). This observation seems consistent with the view that damping of MP dynamics, compared to that in lipids, originates from interactions with the backbone rather than with the octyl chains.

Current data suggests that, as is observed with detergents, APols whose charge density is lower are milder, so that A8-35 is more stabilizing than SAPols (Picard et al. 2006) and NAPols more than A8-35 (Bazzacco et al. 2012). How general this is remains to be seen, however. Indeed, the tetrameric ion channel TRAP1 is reported to be *more* stable in SAPols than in A8-35 (Huynh et al. 2014). It is to be expected that, depending on the mechanism of denaturation of individual MPs and on the APol they are transferred to, different stabilization mechanisms will come into play to different extents, and it may be more or less relevant to favor one type of APol over another.

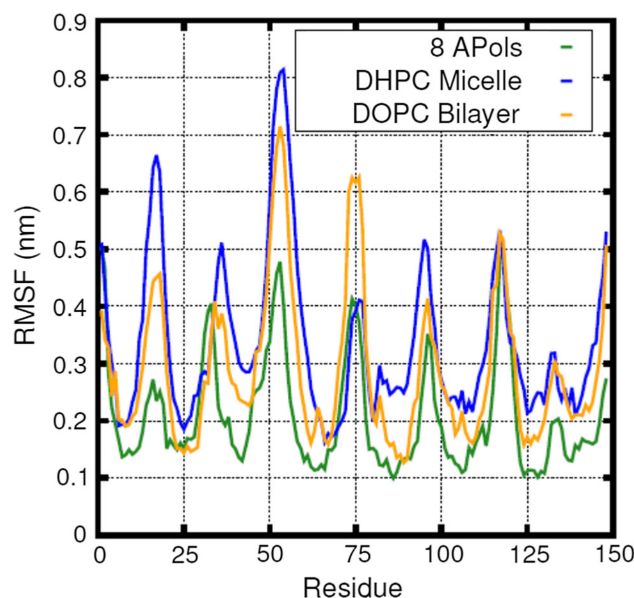

Fig. 7 Damping of the dynamics of the β-barrel MP OmpX by A8-35 as compared to a detergent (DHPC) or a lipid bilayer (dioleoylphosphatidylcholine) environment. The amplitude of root mean square backbone fluctuations is plotted against residue sequence number. Large excursions correspond to the loops and turns. Note that trapping with A8-35 damps the fluctuations of both transmembrane β-strands and extramembrane regions. Reproduced with permission from Perlmutter et al. (2014)

Ligand Binding and Functional Properties of Amphipol-Trapped Membrane Proteins (Table 6, line B)

As a rule, no interference is observed with the binding of small water-soluble ligands to APol-trapped MPs, such as that of calcium and ATP to SERCA1a (Champeil et al. 2000), of small acetylcholine analogs to the nAChR (Charvolin et al. 2009; Martinez et al. 2002), or of various ligands to six distinct G protein-coupled receptors (GPCRs) (Banères et al. 2011; Bazzacco et al. 2012; Catoire et al. 2010a, 2011; Dahmane et al. 2009; Rahmeh et al. 2012). Two GPCRs expressed in vitro in the presence of NVoy have been shown to bind their ligands (Klammt et al. 2011). It is to be noted that A8-35 does not interfere with the binding of leukotriene LTB_4 to the BLT1 or BLT2

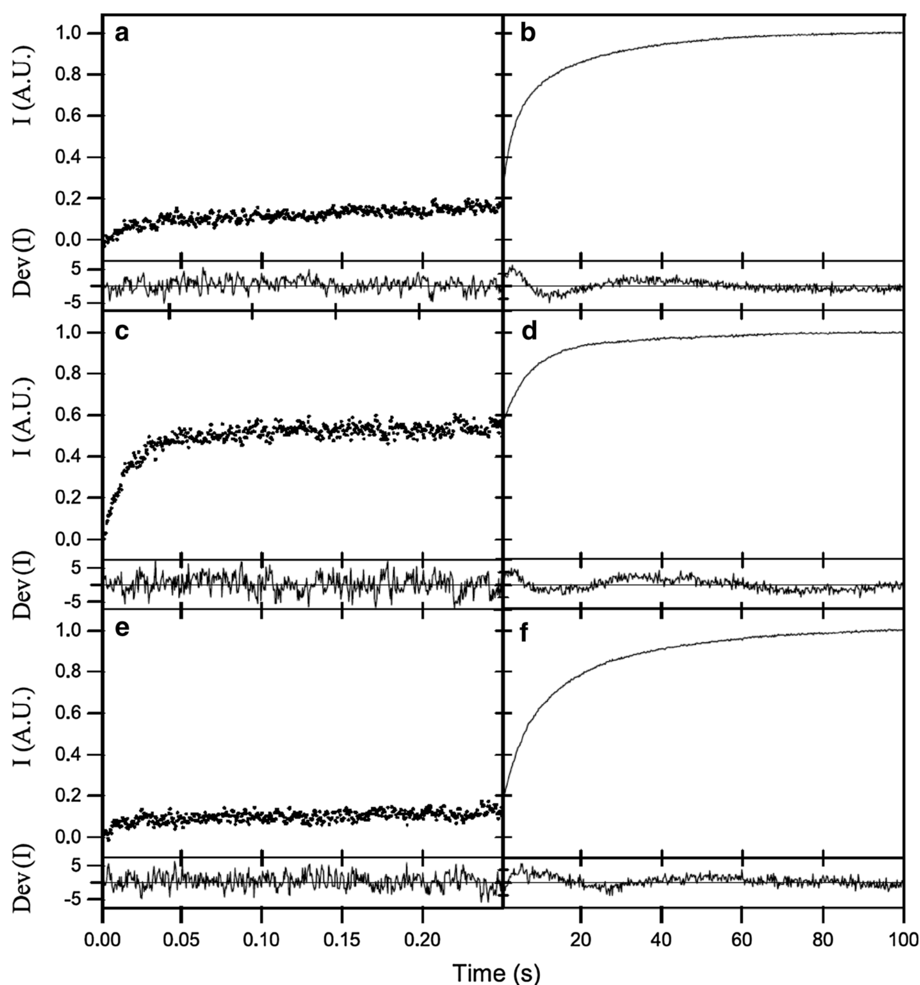

Fig. 8 Allosteric transitions of the nicotinic acetylcholine receptor in three different environments. Kinetics of binding of a fluorescent ligand to nAChR in (**a**, **b**) native membrane fragments from *Torpedo marmorata* electric organ; (**c**, **d**) after solubilization in detergent solution (CHAPS); (**e**, **f**) after addition of A8-35 and dilution below the cmc of CHAPS. In its native membrane environment, the nAChR pre-exists to the addition of ligands in an equilibrium between a low-affinity resting state and high-affinity, inactive state(s), in a proportion of about 9:1. Upon addition of a low concentration of fluorescent agonist, only the latter bind the ligand (panel **a**), relaxation of the resting state to high-affinity ones occurring more slowly (panel **b**). After solubilization, the ratio between high- and low-affinity states becomes about 1:1, explaining the higher level of fast binding seen in panel **c**. When most of CHAPS is replaced by A8-35 in the environment of the receptor, the allosteric equilibrium comes back to a situation similar to that in the membrane (**e**, **f**). Reproduced with permission from Martinez et al. 2002. See text, "Ligand binding and functional properties of amphipol-trapped membrane proteins" section

receptors (Catoire et al. 2010a; Dahmane et al. 2009), even though, given the hydrophobicity of LTB_4, its binding sites must be themselves quite hydrophobic and could in principle attract APol octyl chains. APols do not block either the binding of large water-soluble partners, such as that of α-bungarotoxin (8 kDa) to the nAChR (Charvolin et al. 2009), that of bacteriophage T5 protein pb5 (68 kDa) to FhuA (Basit et al. 2012), nor the recognition of several MP targets by synthetic proteins called αReps (15–20 kDa) (Ferrandez et al. 2014) or by antibodies (~150 kDa) (Charvolin et al. 2009; Le Bon et al. 2014a; Tifrea et al. 2011), nor the interaction of GPCRs with G proteins and arrestin (Bazzacco et al. 2012; Rahmeh et al. 2012). In the latter case, however, it has been observed that interactions are less efficient with A8-35-trapped than with NAPol-trapped GPCRs, presumably because of repulsive electrostatic interactions (J.-L. Banères, personal communication). Also, specific binding of cationic ligands can be difficult to measure in the presence of A8-35, because of a high background of non-specific binding (Ferrandez et al. 2014, and unpublished data by various groups). Experiments in which retinal was added to bacterio-opsin (BO) refolded in the presence of A8-35 indicate that this very hydrophobic ligand can be delivered a posteriori to the apoprotein (Dahmane et al. 2013). Retinal presumably moves from free APol particles, where it must partition, to the protein-

bound APol belt during collisions between the particles and BO/APol complexes, and then inserts itself into the α-helix bundle.

Following trapping with A8-35, the nAChR exhibits membrane-like allosteric transitions upon binding of an acetylcholine analog (Martinez et al. 2002) (Fig. 8), and BR undergoes its complete photocycle (Dahmane et al. 2013; Gohon et al. 2008). In both cases, the functional cycle in APols is closer to that observed in the membrane than is the case in detergent solution, which, at least in the case of BR, has been clearly traced to the rebinding of lipids (Dahmane et al. 2013). *Escherichia coli* diacylglycerol kinase (DAGK) retains full enzymatic activity upon transfer from decylmaltoside to APol PMAL-B-100 (Gorzelle et al. 2002). The bacterial outer membrane enzyme PagP retains phospholipase activity after being trapped in SMALPs (Knowles et al. 2009). OmpT and PagP are functional in A8-35 (Leney et al. 2012). The transmembrane domain of the bacterial EII^{mtl} mannitol permease performs the transphosphorylation from phosphoenolpyruvate to mannitol more rapidly after trapping in A8-35 than it does in detergent solution (Opačić et al. 2014a). Similarly, the basal ATPase activity of ABCA4, a photoreceptor-specific ABC transporter, is higher after trapping with A8-35 than in detergent solution (Tsybovsky et al. 2013). Cytochrome bc_1 transfers electrons from ubiquinol to oxidized cytochrome *c* at comparable rates whether solubilized in DDM or trapped by A8-35 (Charvolin et al. 2014).

Unexpectedly, the ATPase activity of the sarcoplasmic calcium pump SERCA1a was found to be reversibly inhibited by APols compared to what is observed in permeabilized membrane fragments or in detergent solutions (Champeil et al. 2000; Picard et al. 2006). The calcium pump is remarkable by the extensive rearrangement of the transmembrane helix bundle that takes place during the enzymatic cycle, which led to the suggestion that the inhibition by APols could be due to the free energy cost of rearranging the polymer around it during its transconformations (the "Gulliver effect" mentioned in "Membrane Protein Stabilization by Amphipols" section) (Picard et al. 2006; Popot et al. 2003, 2011). Furthermore, it has been observed that, when SERCA1a is exposed to mixtures of A8-35 and detergent, or trapped with SAPols, an intermediate level is observed both of the functional inhibition and of the protection against the denaturation induced by calcium removal (Champeil et al. 2000; Picard et al. 2006). Knowing that denaturation of SERCA1a starts with the opening of the transmembrane helix bundle, as inferred from its stabilization by calcium (Merino et al. 1994), this observation led to the hypothesis that damping of large-scale (nanometric) rearrangements by APols is the common cause underlying both phenomena (Picard et al. 2006; Popot et al. 2003, 2011). This proposal, as already mentioned ("Membrane Protein Stabilization by Amphipols" section), has received some support from MD (Perlmutter et al. 2011) and INS (Tehei et al. 2014) estimates of the viscosity of A8-35, and from an MD study of the dynamics of APol-trapped OmpX (Perlmutter et al. 2014), as well as from the thermodynamic analysis of unfolding experiments (Pocanschi et al. 2013).

Applications

The range of applications that can benefit from the use of APols is very broad:

(i) APols can be used to facilitate the production of properly folded MPs: they appear to be very efficient an environment for bringing MPs to their native state starting from a misfolded one ("Amphipol-Assisted Folding and Refolding of Membrane Proteins" section), and they can be used as a mild receiving medium during MP cell-free expression ("Amphipol-Assisted Cell-Free Expression of Membrane Proteins" section).

(ii) by stabilizing MPs compared to detergents, APols facilitate the purification of fragile MPs or MP complexes under a functional form (see e.g., "Electron Microscopy" section).

(iii) APols can make it easier to carry out studies under conditions that are not easily tolerated by detergent-solubilized MPs. Solution NMR, for instance, often requires long measurements at relatively high temperature and detergent concentration, two destabilizing factors ("Solution NMR Studies of Amphipol-Trapped Membrane Proteins and Their Ligands" section). Stabilization is also welcome when performing multiple cycles of ligand-screening measurements on immobilized MPs ("Amphipol-Mediated Immobilization of Membrane Proteins onto Solid Supports and Ligand-Binding Measurements" section).

(iv) Finally, the intrinsic properties of APols, or properties that can be conferred upon them by labeling or functionalization (cf. Table 2), open the way to a very wide range of original applications that are out of reach or impractical with detergent-solubilized preparations.

Table 6 compiles a list of publications involving those applications of APols that have been validated or that appear readily testable, with a brief indication of the advantages and constraints of resorting to APols. In the following sections, we provide a rapid update on a

Table 7 Integral membrane proteins that have been folded or refolded in amphipols

Protein	Origin	Structure	Function	Type of folding	Folding medium	Structural and functional evidence of folding, comments	References
Bacteriorhodopsin	*Halobacterium salinarum*	7-α-helix bundle	Light-driven proton pump	Refolding from SDS	A8-35 ± native lipids, ± retinal	UV–visible spectrum, CD, detailed study of photocycle, yielding functional evidence for lipid rebinding.	Dahmane et al. (2013), Etzkorn et al. (2013), Pocanschi et al. (2006)
BLT1 receptor	Human	7-α-helix bundle	GPCR	Folding from SDS	A8-35 ± lipids	Pharmacology	Dahmane et al. (2009)
BLT2 receptor	Human	7-α-helix bundle	GPCR	Folding from SDS	A8-35 ± lipids	Pharmacology. Structure of LTB_4 ligand bound to A8-35-folded and stabilized receptor solved by solution NMR	Catoire et al. (2010a), Dahmane et al. (2009)
5-$HT_{4(a)}$ serotonin receptor	Human	7-α-helix bundle	GPCR	Folding from SDS	A8-35 ± lipids	Ligand binding	Dahmane et al. (2009)
CB1 cannabinoid receptor	Human	7-α-helix bundle	GPCR	Folding from SDS	A8-35 ± lipids	Ligand binding	Dahmane et al. (2009)
GSH-R1a ghrelin receptor	Human	7-α-helix bundle	GPCR	Folding from SDS	A8-35 ± lipids, NAPols ± lipids	Ligand binding, activation of $G_{\alpha q}$, recruitment of arrestin-2	Banères et al. (2011), Bazzacco et al. (2012)
V2 vasopressin receptor	Human	7-α-helix bundle	GPCR	Folding from SDS	A8-35 ± lipids	Ligand binding	Banères et al. (2011)
OmpA (full length)	*Escherichia coli*	Monomeric 8-strand β-barrel + periplasmic domain	Structural + pore formation	Folding from urea	A8-35	SDS-PAGE, CD, channel formation upon transfer to BLM	Pocanschi et al. (2006, 2013)
OmpA (transmembrane domain)	*Escherichia coli*	Monomeric 8-strand β-barrel	Structural + pore formation	Folding from urea	SAPols	SDS-PAGE, NMR Trosy spectrum	Dahmane et al. (2011)
FomA	*Fusobacterium nucleatum*	Monomeric 14-strand β-barrel	Porin, adhesion	Folding from urea	A8-35	SDS-PAGE, CD, channel formation upon transfer to BLM	Pocanschi et al. (2006)
OmpT	*Escherichia coli*	Monomeric 10-strand β-barrel	Outer membrane protease	Folding from urea	A8-35	SDS-PAGE, CD, enzymatic assay, mass spectrometry	Leney et al. (2012)
PagP	*Escherichia coli*	Monomeric 8-strand β-barrel	Palmitoylation of Lipid A	Folding from urea	A8-35	SDS-PAGE, CD, enzymatic assay, mass spectrometry	Leney et al. (2012)

selection of applications, information and references about recent progress in the other fields being given in the Table. Five topical reviews in the present issue of *J. Membr. Biol.* provide more details and discussion about using APols for solution NMR studies of MPs (Planchard et al. 2014), in synthetic biology (Della Pia et al. 2014b), as a tool to manipulate biological membranes (Marie et al. 2014), or for the study of specific classes of MPs (Huynh et al. 2014; Mary et al. 2014). A sixth one covers the important subject of the chemistry of APol labeling and functionalization (Le Bon et al. 2014b).

Using Amphipols to Produce Membrane Proteins

Amphipol-Assisted Folding and Refolding of Membrane Proteins (Tables 6, line C, and 7)

Producing large amounts of properly folded MPs remains one of the most frustrating bottlenecks in membrane biology. MPs can be produced in vivo by homologous or heterologous overexpression, in which case they can be directed either to a membrane compartment or to inclusion bodies. The first approach tends to suffer from low expression levels, due to the restricted volume of membrane available for accumulating the protein, and to its toxicity. Alternatively, MPs can accumulate in large amounts in inclusion bodies without killing the host cell, but they do not fold properly and are recovered under a denatured form. Folding them to their native state is a highly challenging endeavor, protein-specific, very time-consuming to develop, and plagued with low folding yields (for recent general reviews about in vitro folding of MPs, see e.g., Buchanan et al. 2012; Harris and Booth 2012; Otzen and Andersen 2013; Popot 2014).

Probably because they compete less efficiently than detergents with the protein/protein and protein/lipid interactions that determine and stabilize the 3D structure of MPs, APols have turned out to be a remarkably efficient medium in which to fold or refold MPs that have been obtained in denatured state, typically as the result of solubilizing inclusion bodies in either sodium dodecylsulfate (SDS, for α-helical MPs) or urea (for β-barrel ones) (reviewed by Popot & Kleinschmidt 2014). At the date of this writing, seven α-helical MPs, including six GPCRs, have been folded in vitro using APols, and four β-barrel ones (see Table 7), with typical yields ranging between 60 and >90 %. For refolding from urea, the protocol generally involves diluting the urea-denatured protein into an APol solution, so as to lower the concentration of urea to non-denaturing levels (Dahmane et al. 2011; Leney et al. 2012; Pocanschi et al. 2006, 2013). For refolding from SDS, the most usual procedure is to precipitate the dodecylsulfate as its potassium salt in the presence of APols (Bazzacco et al. 2012; Catoire et al. 2010a; Dahmane et al. 2009, 2013; Pocanschi et al. 2006). Systematic studies using BR as a model have shown that other approaches can be made to work, such as dilution or dialysis, the precipitation method, however, providing the best yields (Dahmane et al. 2013; Elter et al. 2014).

In all cases that have been examined to date, lipids are not needed for the protein to fold, but their presence along with APols improves the folding yield, typically by ~10 % (see e.g., Dahmane et al. 2009, 2013). It has been suggested that lipids bind at the transmembrane MP surface as appropriate sites form in the course of folding, which stabilizes the newly acquired conformation and, thereby, steers folding in the direction of the correct native structure (Popot et al. 2011). Various facets of the use of APols to fold GPCRs are discussed in refs. Banères et al. 2011; Mary et al. 2014; Popot and Kleinschmidt 2014. Detailed protocols are provided in Zoonens et al. 2014. The remarkable rate of success and the high yields observed—thus far—upon folding MPs in APols have interesting general implications as regards the nature of the information that MPs require from their environment in order to reach a functional structure, which will be discussed elsewhere (Popot and Engelman 2014).

After folding in APols, MPs can be transferred to other environments, if need be, either by directly exposing the complexes to lipid vesicles, black films or mesophases ("Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes (Table 6, line N)" and "Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes" sections), or after displacing the APol with detergent (see "Composition and Organization" section). The latter route has been used to transfer to NDs a GPCR that had been folded in A8-35 (Damian et al. 2012).

Amphipol-Assisted Cell-Free Expression of Membrane Proteins (Table 6, line D)

Cell-free expression is an alternative approach to producing MPs under non-toxic conditions: the protein is expressed in vitro, in a cell lysate. It can be either left to precipitate, and then solubilized with a detergent, complexed by a detergent during synthesis, or integrated into lipid vesicles or NDs (see e.g., Etzkorn et al. 2013; Katzen et al. 2009; Klammt et al. 2006, 2011; Lyukmanova et al. 2012; Park et al. 2007; Shadiac et al. 2013; Shenkarev et al. 2013, and references therein). Because of their mild character and ability to facilitate folding, APols are an attractive medium into which to let newly expressed MPs fold. However, current data indicate that polyanionic APols—A8-35 and SAPols—block in vitro synthesis, possibly by interacting with basic proteins involved in the translation mechanisms (Park et al. 2011). Interestingly, blockade does

not affect a test soluble protein, GFP, suggesting that it follows binding of APols to the nascent MP. NAPols, on the contrary, have been shown to allow the synthesis of BR (Bazzacco et al. 2012). A detailed protocol is provided by Zoonens et al. 2014. Most of the protein is kept soluble, and a majority of it is properly folded, as shown by its ability to bind retinal and form the chromophore characteristic of the native holoprotein.

NVoy also appears to provide an interesting medium for MP cell-free expression (Guild et al. 2011; Klammt et al. 2011).

Structural and Analytical Studies of Amphipol-Trapped Membrane Proteins

APols were initially designed as tools that ought to facilitate in vitro studies of MPs by stabilizing them compared to detergent solutions (Popot et al. 2003; Tribet et al. 1996). This expectation has been largely validated. Some applications to structural biology are developing particularly rapidly, such as in NMR and electron microscopy. Others are only beginning to be exploited. We briefly discuss below some selected applications. A capsule summary and references are given in Table 6 for chromatographic methods (line E), ultracentrifugation (line F), light spectroscopy (line G), isoelectrofocusing (line N), and trapping of labile MPs and MP complexes (line P), which are not otherwise discussed here.

Solution NMR Studies of Amphipol-Trapped Membrane Proteins and Their Ligands (Table 6, line H)

Solution NMR is, along with EM, the structural biology application that has generated the most publications to date (Table 6). Early works aimed at investigating to which extent solution NMR of MP/APol complexes was practical. They were carried out with A8-35 and used small β-barrel MPs as models, namely tOmpA and OmpX from *E. coli* and tOmpA from *Klebsiella pneumoniae* (KpOmpA) (Catoire et al. 2009, 2010b; Planchard et al. 2014; Renault, 2008; Zoonens et al. 2005). They showed that, although MP/APol complexes are slightly bigger and, as a result, tumble slightly less rapidly than the best MP/detergent ones—typically formed with DHPC or dodecylphosphocholine (DPC)—leading to a somewhat degraded resolution, the latter is sufficient for structure determination. A fairly rapid tumbling (for OmpX, $\tau_c \approx 31$ ns; Catoire et al. 2010b) is consistent with A8-35 forming a thin layer at the surface of the protein, rather than a diffuse corona, in keeping with conclusions from AUC and SANS (Gohon et al. 2008) and from EM (Althoff et al. 2011; Huynh et al. 2014; Liao et al. 2014), as well as with MD calculations (Perlmutter et al. 2014).

A drawback of A8-35 for solution NMR studies is that it aggregates at the slightly acidic pH that is optimal for observing solvent-exposed amide protons ("Solution Properties of Amphipols" section). This has been one of the primary impetuses for developing pH-insensitive APols (Table 1). SAPols (Dahmane et al. 2011) and NAPols (Bazzacco et al. 2012) have both been validated for solution NMR, with resolutions comparable to those achieved with A8-35.

The respective advantages and drawbacks of APols, nanodiscs (NDs) and detergents for solution NMR have been investigated and discussed in several articles and reviews (Catoire et al. 2014; Etzkorn et al. 2013; Planchard et al. 2014; Raschle et al. 2010; Warschawski et al. 2011). In a nutshell, the principal advantage of APols seems to be the stabilization they provide and the simplicity of sample preparation. NDs also stabilize MPs, compared to detergents, and they have the added advantage of providing a bilayer-like environment, but this comes at the expense of much more demanding sample preparation protocols and some what less resolution (Etzkorn et al. 2013). Solution NMR spectra of BR either solubilized in DDM, trapped by A8-35, or inserted into NDs indicate that the transmembrane region of the protein is essentially the same in the three environments, but that there are some differences in the structure and/or dynamics of the extramembrane loops (Etzkorn et al. 2013). The NMR spectra of A8-35-trapped BR are of a sufficient quality to expect that, given proper labeling, it should be possible to collect high-resolution data on the structure and dynamics of APol-trapped GPCRs (Elter et al. 2014; Etzkorn et al. 2013). Preliminary data show that the extramembrane loops of the major outer membrane protein (MOMP) from *Chlamydia trachomatis* trapped in A8-35 are amenable to a solution NMR study (Feinstein et al. 2014). Tryptophan aromatic rings, which typically, in a membrane, interact with lipid headgroups, appear to be buried in MOMP/DPC complexes and accessible in MOMP/A8-35 ones, presumably because of weaker interactions with carboxylate polar moieties than with phosphocholine ones (Feinstein et al. 2014; Tifrea et al. 2014).

Early NMR experiments with tOmpA, OmpX and KpOmpA demonstrated that APols—in that case A8-35—interact specifically with the hydrophobic, transmembrane surface of MPs (Catoire et al. 2009; Planchard et al. 2014; Renault, 2008; Zoonens et al. 2005). In agreement with these observations, quenching with a water-soluble paramagnetic agent indicates that only the transmembrane region of OmpX is masked by A8-35 (Etzkorn et al. 2014). In the case of OmpX, these data are exactly supported by MD calculations (Perlmutter et al. 2014). Preferential interactions between specific groups of the APol and residues at the protein transmembrane surface have been

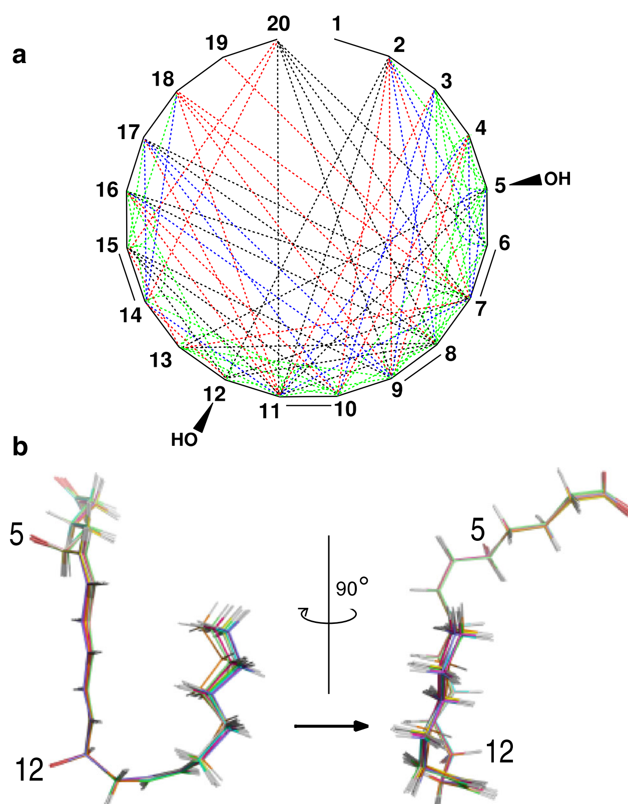

Fig. 9 Determination of the 3D structure of leukotriene LTB_4 bound to the BLT2 GPCR. The deuterated receptor was folded and stabilized in partially deuterated A8-35 (DAPol; see Table 2). Distances between the protons carried by each of the 20 carbon atoms of LTB_4 were deduced from the intensity of transferred NOE signals (color-coded in **a**) and used as constraints to deduce the 3D structure of the receptor-bound ligand (**b**). Reproduced with permission from Catoire et al. 2010a. See text, "Amphipol-Assisted Folding and Refolding of Membrane Proteins" and "Solution NMR Studies of Amphipol-Trapped Membrane Proteins and Their Ligands" sections, and Table 6, lines C and H

identified in the cases of KpOmpA and OmpX (Catoire et al. 2009; Planchard et al. 2014; Renault 2008), and the dynamics of various regions of OmpX investigated by H/D exchange measurements (Catoire et al. 2010b).

A8-35 has been used to fold and stabilize the BLT2 leukotriene B_4 (LTB_4) receptor, with the view of determining the 3D structure of the receptor-bound ligand (Catoire et al. 2010a). BLT2 was expressed in a perdeuterated form in inclusion bodies, solubilized in SDS, and folded using DAPol, a partially deuterated form of A8-35 obtained by grafting deuterated isopropylamine and octylamine chains onto hydrogenated polyacrylate (Gohon et al. 2004) (Table 2). The ligand itself was hydrogenated. Distances between the protons of the ligand while bound to the protein were deduced from transferred Nuclear Overhauser Effect (NOE) measurements. At variance with the free ligand, whose 3D structure is elongated and largely disordered, BLT2-bound LTB_4 adopts a constrained, sea-horse-like configuration (Catoire et al. 2010a) (Fig. 9). Model calculations suggest that this approach is applicable to many receptor/ligand complexes (Catoire et al. 2011). Because background signals from the hydrogenated backbone of DAPol prevent the observation of NOE signals originating from magnetization transfer between alkyl protons of the ligand, which entails the loss of structural constraints and would be crippling for the study of other ligands, a perdeuterated version of A8-35 has been developed (perDAPol), which involved synthesizing perdeuterated polyacrylic acid as a starting material (Table 2). This reduces the contribution of the polymer to ^1H-^1H NOE signals to ~6 % of that of unlabeled A8-35 (Giusti et al. 2014b), thus extending the approach to a wide range of ligands.

Electron Microscopy (Table 6, line I)

APols appear particularly suitable for single-particle EM studies. On the one hand, they stabilize fragile particles that do not stand well being exposed to detergents. On the other, they may facilitate controlling the spread of particles onto microscope grids (Flötenmeyer et al. 2007). Early EM work has been reviewed previously (Popot 2010; Popot et al. 2011). More recent work includes studies of TRP channels (Cao et al. 2013; Cvetkov et al. 2011; Huynh et al. 2014; Liao et al. 2013, 2014), of a mitochondrial supercomplex (Althoff et al. 2011), of two retina disk MPs (Kevany et al. 2013; Tsybovsky et al. 2013), of an aquaporin and a rhodopsin-transducin complex (Vahedi-Faridi et al. 2013), and of *E. coli*'s ExbB–ExbD complexes (Sverzhinsky et al. 2014) and OmpF (Arunmanee et al. 2014). As mentioned above, negative-stain EM images of BR/A8-35 (Gohon et al. 2008) and OmpF/A8-35 (Arunmanee et al. 2014) complexes depleted of free APol show linear or 2D assemblies of MPs (Fig. 4), leading to the tantalizing suggestion that this process could perhaps be somehow harnessed for image reconstruction (Arunmanee et al. 2014).

The cryo-EM study of supercomplex $I_1III_2IV_1$ (1.7 MDa) has revealed the relative arrangement of Complex I, the cytochrome bc_1 dimer and cytochrome c oxidase in the respirasome and mapped the distances between their electron-transfer sites (Fig. 2a). It has also directly visualized the distribution of A8-35 around the complex, confirming that it covers the transmembrane region with a relatively thin strip of polymer, and revealing an unexpected bumpiness (Althoff et al. 2011) (cf. "Composition and Organization" section) (Fig. 2b). Other views of MP-bound APol belts appear in Huynh et al. (2014), Kevany et al. (2013), Liao et al. (2014), Tsybovsky et al. (2013), Vahedi-Faridi et al. (2013). TRPA1 (transient receptor potential ankyrin 1) is a non-selective ion channel expressed in nociceptor sensory neurons. It transduces

chemical, inflammatory, and neuropathic pain signals. It has been studied in negative stain following trapping and stabilization by A8-35, revealing the arrangement of its subunits and leading to hypotheses about the conformational changes that lead to channel activation (Cvetkov et al. 2011). Subsequent work suggests that SAPols increase the stability of TRPA1 over that in A8-35 and may improve EM data (Huynh et al. 2014). A related channel, TRPV1, has been studied by EM after trapping by A8-35. Galleries of images of negatively-stained particles indicate that their overall shape is much more reproducible in A8-35 than it is in DDM, suggesting stabilization (Cao et al. 2013; Liao et al. 2013). Thanks to recent progress in collecting and treating cryo-EM images (see Henderson 2013; Liao et al. 2014), the structure could be solved to an exceptionally high resolution (3.4 Å), at which large amino acid side chains can be identified and an atomic structure built into the electron density map (Cao et al. 2013; Liao et al. 2013) (Fig. 2c). As mentioned above ("Composition and Organization" section), the APol belt around TRPV1 does not appear bumpy, an intriguing point that will deserve further investigation (see Huynh et al. 2014; Liao et al. 2014).

Altogether, APols seem to be turning into routine tools for single-particle EM studies, if only because of their usefulness for stabilizing target MPs. It is fair to say, however, that there does not seem to exist a clear consensus yet among specialists about what they contribute to improving imaging itself. It is worth noting that some of the tagged APols that have been developed for other purposes (see Table 2 and "Amphipol-Mediated Immobilization of Membrane Proteins onto Solid Supports and Ligand-Binding Measurements" section) could be advantageously exploited by microscopists either for locating MP transmembrane regions, by binding EM markers such as avidin or avidin-coated gold particles to the tags, or, perhaps, for organizing MPs onto nanoscale scaffolds (see "Amphipol-Mediated Immobilization of Membrane Proteins onto Solid Supports and Ligand-Binding Measurements" section).

Early work had shown the possibility to study the mass of APol-trapped complexes by scanning transmission EM (STEM) (Tribet et al. 1998). No other such study has been published since. Atomic force microscopy (AFM) and single-molecule force spectroscopy (SMFS) studies of APol-trapped MPs ought to be readily possible but have not been validated yet.

Radiation Scattering Studies (Table 6, line J)

Small angle scattering of X-rays and neutrons (respectively SAXS and SANS) has been used early on to characterize the size, composition, and arrangement of APol particles (Gohon et al. 2004, 2006) and MP/APol complexes and to study their interactions in aqueous solutions as a function of concentration and ionic strength (Charvolin et al. 2014; Gohon et al. 2008; Popot et al. 2003; Sharma et al. 2012). SANS analyses have been greatly helped by the availability of DAPol (Table 2), which permits to selectively cancel the contribution of the APol by adjusting to ~85 % the D_2O content of the buffers (Gohon et al. 2008). They ought to be further facilitated by the advent of perDAPol (Table 2), which will make it possible to contrast-match the APol belt at 100 % D_2O, thus further increasing the contrast of MPs with the solvent and decreasing the background noise from inelastic scattering by protons (Giusti et al. 2014b). SAXS studies have revealed, in particular, that in relatively concentrated (~20 g L^{-1}) solutions of cytochrome bc_1/A8-35 complexes, the particles repulse each other at moderate ionic strength (≤200 mM NaCl), whereas at high ionic strength (≥500 mM NaCl) the interactions become attractive (Charvolin et al. 2014; Popot et al. 2003), an important observation in the context of 3D crystallization (see "X-ray Crystallography (Table 6, line L" section). SANS studies also provided the first evidence about the limited thickness of the APol layer (Gohon et al. 2008).

Radiation scattering is a powerful approach, but its application to MP/APol complexes requires great care, because of the tendency of the complexes to form small oligomers unless a sufficient concentration of free APol particles shifts the equilibrium towards the monomeric state (Gohon et al. 2008; Zoonens et al. 2007) (see "Composition and Organization" section). Unimportant in most experiments, the presence of small oligomers becomes a redoubtable nuisance in radiation scattering ones. SAXS and SANS have recently been applied, in conjunction with EM and various biochemical techniques, to studying the overall shape and subunit organization of bacterial energy-transducing ExbB–ExbD complexes (Sverzhinsky et al. 2014). The study was rendered quite delicate by the tendency of the complexes to aggregate in the presence of too little free A8-35 and to come apart in the presence of an excess of it.

Mass Spectrometry (Table 6, line K)

MPs trapped in A8-35 (Bechara et al. 2012; Catoire et al. 2009; Hopper et al. 2013; Leney et al. 2012) or in NAPols (Bechara et al. 2012) are amenable to mass spectrometry (MS) using either matrix-assisted laser desorption ionization (MALDI) (Bechara et al. 2012; Catoire et al. 2009) or electron spray ionization (ESI) (Hopper et al. 2013; Leney et al. 2012) techniques. As a rule, most MPs and subunits can be detected, but there are, however, some exceptions (Bechara et al. 2012). A curious observation is that BR, which is readily detected when trapped in NAPols, escapes detection by MALDI-MS when it is trapped in A8-35. tOmpA is detected after trapping with either of the APols, but not if BR/

A8-35 and tOmpA/A8-35 complexes are mixed, suggesting some sort of segregation within the matrix during the dehydration of the samples (Bechara et al. 2012).

Under favorable circumstances, lipids bound to APol-trapped MPs can be identified by MS (Bechara et al. 2012). Given that APols seem to preserve MP/lipid interactions more faithfully than detergents ("Ligand Binding and Functional Properties of Amphipol-Trapped Membrane Proteins" section), this is a particularly interesting observation, because it could open the way to identifying lipids that interact with MPs in the membrane, but are displaced by detergents.

ESI–MS coupled with ion-mobility spectrometry (IMS) has been used to quantify the proportions of properly folded versus unfolded protein following A8-35-assisted folding of two β-barrel MPs, OmpT and PagP (Leney et al. 2012), as well as to compare the mass and dispersity of individual molecules of unlabeled and perdeuterated A8-35 (Giusti et al. 2014b). ESI–MS has been applied with limited success to investigating the oligomeric state of A8-35-trapped DAGK, part of the native trimer fragmenting into dimers and monomers (Hopper et al. 2013).

APol-trapped MPs can be subjected to proteolysis and the proteolytic fragments identified by MS (Bechara et al. 2012), which has been used to analyze complex mixtures of soluble and membrane proteins extracted in the presence of A8-35 (Ning et al. 2013, 2014).

X-ray Crystallography (Table 6, line L)

Crystallization of MP/APol complexes has proven a highly frustrating endeavor. The most extensive attempts have been carried out using cytochrome bc_1/A8-35 complexes as a model (Charvolin et al. 2014; Popot et al. 2011). Despite years of efforts, no crystals of pure bc_1/A8-35 complexes have ever been obtained. On the contrary, crystals of ternary bc_1/A8-35/detergent complexes readily formed, but circumstances—lack of funding, to speak plainly—did not permit to examine whether they can be made to diffract to high resolution (Charvolin et al. 2014; Popot et al. 2011).

Two factors can be supposed to come into play to explain why ternary complexes crystallize, whereas binary ones do not. First, mixing A8-35 with a neutral detergent (i) lowers the charge density at the surface of the surfactant belt, and (ii) permits the charges to reorganize as a function of the local electrostatic field, neutral polar heads being able to substitute for charged ones. These two processes ought to diminish the electrostatic repulsion between complexes. Second, it has been observed that ternary complexes of tOmpA/A8-35/detergent appear more homogeneous, when analyzed by SEC, than pure tOmpA/A8-35 ones (Zoonens et al. 2007) (Fig. 10). The reason for this behavior is not certain, but it seems probable that equilibration of the surfactant belt towards an energetically optimal volume be more efficient in the presence of small, rapidly diffusing detergent molecules. The latter can also permit the belt to adapt more easily to local constraints during crystal growth. Whatever the underlying mechanisms, homogeneity of the complexes and a better adaptability of the surfactant belt could obviously favor crystallization.

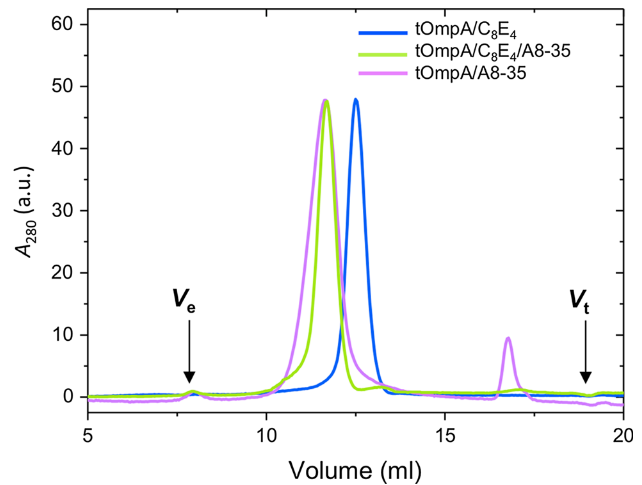

Fig. 10 Size distribution of a MP associated to A8-35, to a detergent (C_8E_4), or to an A8-35/detergent mixture. SEC profiles of tOmpA/surfactant complexes: in C_8E_4 (*blue curve*), after supplementing the sample with A8-35 (*green curve*), and after detergent removal (*mauve curve*). The full widths at half-height are 0.6, 0.6, and 0.9 mL, respectively (adapted from Zoonens et al. 2007, with permission). See text, "Trapping Membrane Proteins with Amphipols" and "Delivery of amphipol-trapped membrane proteins to preexisting membranes" sections

It is worth recalling that no crystals of MPs solubilized with a charged detergent have ever been reported (Privé 2007) (which may be related to crystallization itself, but also to the well-known destabilizing character of charged detergents). It is, in a sense, extremely encouraging that crystals could be obtained at all with highly charged MP/A8-35/detergent complexes. NAPols were not available at the time that these attempts were carried out. Trying to crystallize MP/NAPol and MP/NAPol/neutral detergent complexes is the obvious next step, but depends on scaling-up the synthesis of NAPols, which is not easy.

Whereas APols are not, at least at this point of their development, a good medium for MP crystallization, it has been noted above (i) that they are an excellent medium into which to fold and stabilize MPs (see "Amphipol-Assisted Folding and Refolding of Membrane Proteins" and "Membrane Protein Stabilization by Amphipols" sections) and (ii) that they can easily be displaced by other surfactants (see "Trapping Membrane Proteins with Amphipols" section). They can, therefore, conceivably be used as a shuttle to deliver MPs to a medium in which crystallization is readily possible. A proof of concept has been recently provided by transferring BR directly from A8–35 to a lipidic mesophase, where it

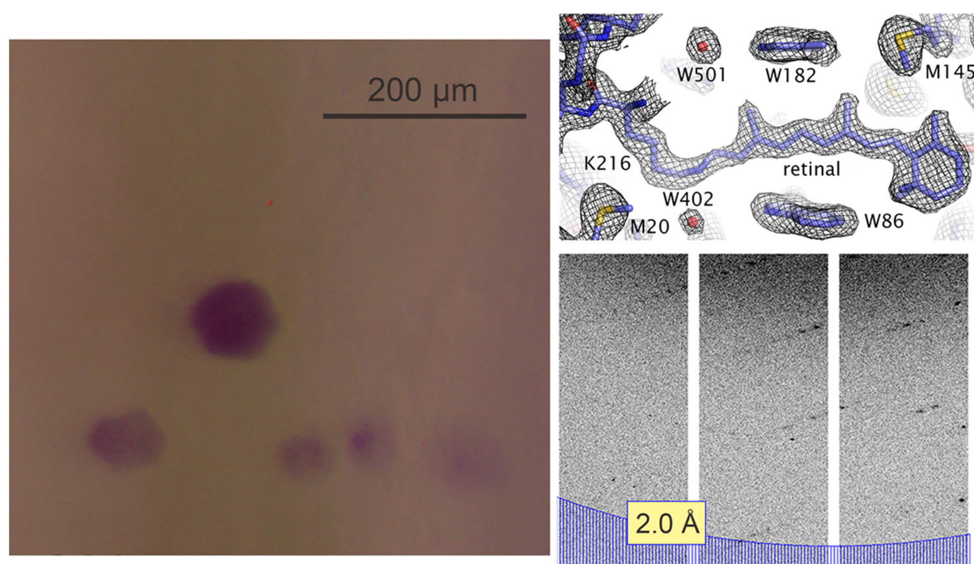

Fig. 11 Bacteriorhodopsin crystals grown in mesophase after transfer from BR/A8–35 complexes (*left*) and excerpts from the X-ray diffraction pattern (*lower right*) and from the retinal region of the electron density map, with an atomic model built in (*upper right*). See Polovinkin et al. (2014b), and text, "Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes" section

assembled into crystals diffracting to better than 2-Å resolution (Polovinkin et al. 2014b) (Fig. 11). The structure was solved to 2-Å resolution and found to be indistinguishable from that obtained after transfer from detergent solution, with evidence for the presence of bound lipids at the same position as in classic crystals. The packing of the crystals leaves no space for the APol. It seems highly likely that, upon mixing with the mesophase, the polymer and the protein, the latter possibly accompanied by bound purple membrane lipids, go their separate ways, and BR assembles into crystals without interference by the APol. Because APols appear to be a good environment into which to fold and stabilize MPs, including GPCRs (see "Amphipol-Assisted Folding and Refolding of Membrane Proteins" section), and lipid mesophases have proven a good medium in which to crystallize MPs in general and GPCRs in particular (Caffrey 2011; Cherezov 2011), the conjunction of the two technologies may open a very interesting route to obtain crystals of MPs that their fragility or other factors render difficult to crystallize from detergent solutions.

Pharmacological, Cell Biological and Biomedical Applications

Amphipol-mediated immobilization of membrane proteins onto solid supports and ligand-binding measurements (Table 6, line M)

Immobilization of MPs onto solid supports has numerous applications in both basic and applied research. Biochemical and biophysical studies of MPs exploit immobilization strategies for, on the one hand, separating the protein of interest from the other cell components, and, on the other, identifying biological partners or ligands that specifically interact with target MPs. In pharmaceutical research, the development of biosensors carrying proteins immobilized on the surface of a solid support enhances the sensitivity of the detection of molecular interactions and reduces the consumption of materials. It has, therefore, become one of the strategies for drug discovery (see e.g., Bano et al. 2009; Christman et al. 2006; Coyer et al. 2007; Wingren and Borrebaeck, 2007).

Immobilization of MPs under their native, functional form is of primary import for the identification of biologically relevant interactions. Because direct interactions of MPs with solid surfaces tend to be denaturing, immobilization is usually mediated by a tag fused at one extremity of the protein chain, implying its genetic modification. Functionalized APols bearing affinity tags provide a very promising alternative for attaching MPs onto solid supports (Charvolin et al. 2009) (reviewed by Della Pia et al. 2014b). APol-mediated immobilization indeed presents many practical advantages:

- (i) Anchoring via a functionalized APol suppresses the need for any genetic or chemical modification of the protein;
- (ii) The *N*- and *C*-termini of the protein remain unmodified and available for ligand binding or other functional roles;
- (iii) The protein is indirectly attached to the support, which limits the risks of denaturation or limited accessibility, particularly to large ligands such as antibodies;
- (iv) Because there is no fixed orientation of the tag relative to the protein, all regions of the latter's extramembrane surface are a priori equally accessible to analytes;

(v) The APol stabilizes the protein, extending the time period available for experiments compared to the use of detergent;
(vi) The stability of MP/APol complexes upon dilution in aqueous solutions makes it possible to work with surfactant-free buffers.

To date, four different types of tags have been grafted onto APols (Table 2) (reviewed by Della Pia et al. 2014b; Le Bon et al. 2014b). The adsorption/desorption properties of complexes between MPs and tagged APols have been studied by various biochemical and biophysical approaches, including SPR, fluorescence microscopy, etc.

APols bearing a biotin, whether derived from A8–35 (BAPol) (Charvolin et al. 2009; Della Pia et al. 2014a; Ferrandez et al. 2014), from PC-APols (Basit et al. 2012) or from NAPols (Ferrandez et al. 2014), achieve irreversible MP immobilization onto surfaces carrying avidin, streptavidin, or neutravidin. The very low dissociation constant of biotin/avidin complexes makes them tools of choice for the development of highly stable biosensors. In the princeps work, it was demonstrated that MPs immobilized onto chips or beads using BAPol are stable in surfactant-free buffers and can be recognized by specific ligands and by antibodies (Charvolin et al. 2009). This has recently been extended to micropatterning (Della Pia et al. 2014a). Biotinylated PC-APols have been used to study the interaction between *E. coli*'s outer membrane protein FhuA and a bacteriophage tail protein, pb5 (Basit et al. 2012). Biotinylated NAPols have made it possible, because of a lower background than is observed with BAPol, to select engineered soluble proteins that specifically recognize immobilized target MPs, with the view of using them as crystallization helpers and for other applications (Ferrandez et al. 2014).

Grafting an oligodeoxynucleotide (ODN) onto A8-35, yielding OligAPol, allows a stable immobilization of MP/OligAPol complexes onto surfaces carrying a complementary ODN (Le Bon et al. 2014a). By playing on the sequence and length of the ODN, and/or using diblock connectors, both the affinity and specificity of the attachment can be modulated essentially without limits, offering, among other applications, rich prospects for targeting and multiplexing, and the development of MP arrays. Dehybridization is feasible either chemically (with urea) or by heat, which permits the regeneration of the support (Le Bon et al. 2014a). Beyond anchoring MPs onto DNA chips or beads, OligAPols could conceivably be used to organize one or more MPs in space by attaching them at specific points of DNA filaments or of the sophisticated 2D or 3D lattices that can be built out of designed DNA fragments (see e.g., LaBean & Li, 2007). This could be of use, among many other applications, in structural biology—for example, for measuring residual dipolar coupling signals in NMR experiments (cf. Bellot et al. 2013), or for organizing MPs for EM studies—or for engineering purposes, such as building bioreactors (cf. Nowaczyk et al. 2004).

Recently developed functionalized versions of A8-35 carrying either hexahistidine tags (His-tags) or randomly distributed imidazole groups, respectively dubbed HistAPol and ImidAPol, further widen the spectrum of specific and reversible immobilization modes (Giusti et al. 2014a and unpublished data). Because MP/HistAPol complexes can carry several His-tags, their immobilization onto Ni:NTA-coated chips is almost as stable as that achieved with BAPol, with the advantage of reversibility. HistAPol requires a rather sophisticated synthesis, which would make it costly to produce in bulk. It can hardly be used for MP production. ImidAPol, on the other hand, is simple and cheap to make and can be used to immobilize sizeable amounts of MPs onto beads. The gentle mode of detachment with buffers containing either an excess of free imidazole or ethylenediaminetetraacetate (EDTA) allows recovery of HistAPol or ImidAPol immobilized MPs in their functional state.

NDs can also mediate the attachment of MPs onto solid supports, via a His-tag fused to the scaffold proteins (Goluch et al. 2008; Shaw et al. 2007). They present over APols the advantage of providing a bilayer environment. APols have for them more versatility in the modes of attachment, the simplicity of their implementation, and their ability to trap any MP without size limitation.

Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes (Table 6, line N)

Under most circumstances, most APols do not solubilize biological membranes, even though, in some cases, they can be made to disperse lipid bilayers, and they are known to be able to form pores (reviewed by Marie et al. 2014). Cells in culture resist moderate (0.05–0.1 g L^{-1}) concentrations of A8-35 (Popot et al. 2003, 2011), and mice survive without loss of weight being injected with amounts of A8-35 (10–50 μg) (Fernandez et al. 2014; Popot et al. 2003; Tifrea et al. 2011) sufficient to deliver either an anticancer peptide (Popot et al. 2011) or a MP used as a vaccine (Tifrea et al. 2011, 2014). Upon injection of MP/A8-35 complexes, antibodies are produced against the MP (Popot et al. 2003; Tifrea et al. 2011, 2014), but, according to ELISA tests and immunoreplicae, not against the APol (Popot et al. 2003 and unpublished data), potentially opening the way to medical applications. This sets the stage for a large range of applications based on the delivery to preexisting membranes and to cells, whether in vitro or in vivo, of MPs, transmembrane peptides, or other

hydrophobic cargoes, including quantum dots (Luccardini et al. 2006; Qi and Gao 2008).

In vitro, APols have been used to deliver DAGK (Nagy et al. 2001) and the pore-forming domain of diphteria toxin (Kyrychenko et al. 2012) to preformed lipid vesicles, as well as two outer membrane β-barrel proteins, OmpA from *E. coli* and FomA from *Fusobacterium nucleatum*, to lipid black films (Pocanschi et al. 2006). In all cases, the native state of at least some of the proteins inserted was demonstrated by functional tests. APols have also been used to deliver a synthetic peptide mimicking the single transmembrane helix of a growth factor receptor to cells in culture. In the hours and days that followed, fluorescence imaging showed that the peptide (and the APol) were endocytosed (Popot et al. 2011). The biodistribution and elimination of APols following intravenous (IV), intraperitoneal (IP), or subcutaneous (SC) administration to mice have been examined thanks to two fluorescent versions of A8-35 (FAPols) carrying either rhodamine or Alexa Fluor 647 (Table 2) (Fernandez et al. 2014). In brief, using the IV and IP routes results in a rapid distribution of FAPols throughout the organism, except in the brain and spleen (Fig. 12), followed by slow elimination (2–3 weeks), with a remarkable tendency for transient accumulation in fat pads. Following SC injection, FAPols remain mainly localized around the point of injection before being slowly eliminated. These observations provide interesting suggestions about the use of APols to deliver various types of hydrophobic cargoes to various organs over various time-scales.

A couple of caveats should be mentioned regarding the use of APols to deliver MPs to preformed membranes. First, the carrier APols will themselves become inserted into the target membrane (cf. Pocanschi et al. 2006; Popot et al. 2011), even though, very likely, they will dissociate from the protein and migrate independently from it (*cf.* the separation of BR from A8–35 upon transfer of the complexes to a lipid mesophase; "Delivery of Amphipol-Trapped Membrane Proteins to Preexisting Membranes" section). They may cause perturbations that have to be paid attention to (cf. Pocanschi et al. 2006). Second, not all MPs can be expected to survive such a drastic procedure. For the protein to adopt a transmembrane position, some of its hydrophilic regions have to somehow cross the bilayer, which can be a highly destabilizing process. It is reasonable to expect that the more robust (or the simpler) the protein is, the greater are the chances that it can be transferred without denaturation, or may be able to recover from it. Studying the transfer of a variety of MPs is clearly needed before a general view of the usefulness of this procedure can be formed.

Vaccination (Table 6, line O)

Because they are hard to produce and frequently instable, MPs are seldom used as immunogens in vaccinal preparations. Yet, they are among the first foreign molecules our organism encounters upon invasion by a virus, a bacterium, or a parasite. APols offer largely unexplored possibilities for improving vaccine preparations, because they can help preparing MPs in large amounts (see "Amphipol-Assisted Folding and Refolding of Membrane Proteins" and "Amphipol-Assisted Cell-Free Expression of Membrane Proteins" sections), stabilizing them (see "Membrane Protein Stabilization by Amphipols" section), and associating them with response-enhancing molecules, either by co-trapping or thanks to the use of appropriately functionalized APols.

The bacterium *C. trachomatis* is responsible for the most prevalent type of sexually transmitted bacterial disease over the planet, resulting in sterility, blindness, chronic pelvic pain, etc. No efficient and safe vaccine could be prepared from the whole organism, which has led to attempts at using as an immunogen its major outer MP (MOMP). In classical vaccines, MOMP is detergent-solubilized. Following transfer from Zwittergent 3–14 (Z3–14) to A8-35, MOMP is strongly stabilized (Feinstein et al.

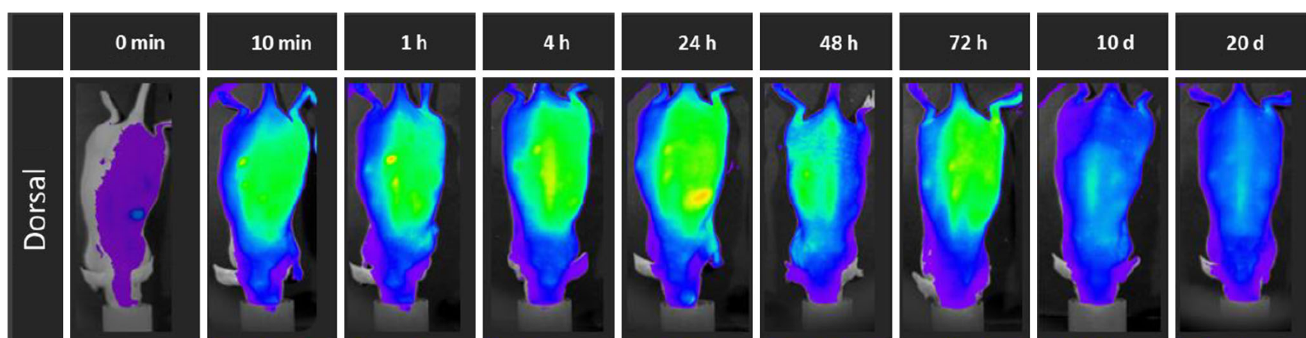

Fig. 12 Distribution and elimination of fluorescent A8-35 after intravenous injection to a mouse. Time-series images before (0 min) and after injection of 10 µg of Alexa Fluor 647-labeled A8-35 ($FAPol_{AF647}$; see Table 2). Dorsal view. Reproduced with permission from Fernandez et al. (2014)

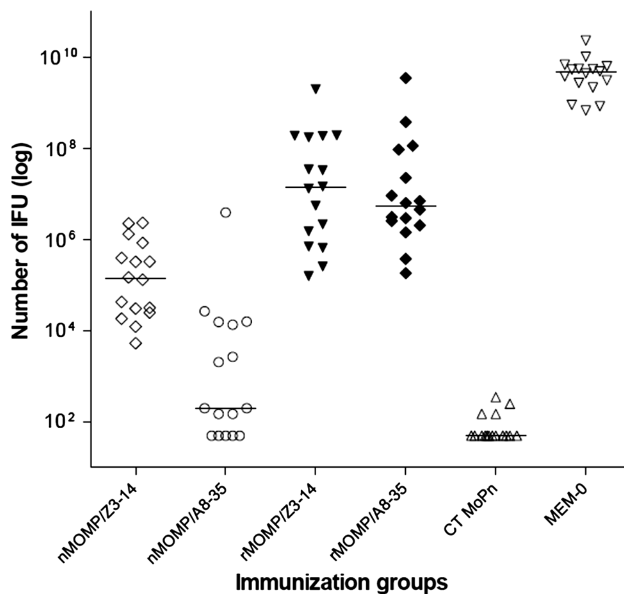

Fig. 13 Protection of mice against infection by *Chlamydia trachomatis* following vaccination with the native, trimeric major outer membrane protein (nMOMP) or recombinant, misfolded, monomeric MOMP (rMOMP) kept soluble by either Zwittergent 3-14 or amphipol A8-35. Controls include mock injection with buffer (Mem 0) and previous exposure to the live bacterium (CT MoPn). The parameter measured is the number of infective units in the lungs 10 days after intranasal challenge. Dots represent individual animals. Horizontal bars correspond to the medians for the different groups. Note the logarithmic scale. Reproduced with permission from Tifrea et al. (2011). See also Tifrea et al. (2014), and text, "Vaccination" section

2014; Tifrea et al. 2011) (Fig. 5). The efficiency of a vaccine incorporating A8-35-trapped MOMP is markedly improved compared with the formulation with Z3-14: indeed, when mice vaccinated with a MOMP/A8-35 preparation are subsequently infected with live *C. trachomatis*, they exhibit a level of protection close to that observed following pre-infection with the bacterium, three orders of magnitude, in terms of the number of infective units found in the lungs, over that observed with detergent-based vaccines (Tifrea et al. 2011, 2014) (Fig. 13). Whether this effect results from the biochemical stabilization of MOMP, from a more efficient presentation to the immune system or from a combination of factors is currently unknown. Attempts are on-going to examine the effect of delivering MOMP trapped in an adjuvant-carrying APol.

It is to be hoped that these promising results will incite other investigators to examine the efficiency of MP/APol-based vaccines against other infectious diseases.

Conclusion

It will be obvious, at the end of this survey, that, after slow beginnings, the use of APols is now becoming part of mainstream membrane biochemistry and biophysics. As illustrated by the two dozens of articles gathered in the present special issue of *J. Membr. Biol.*, it is branching into extremely varied fields of both basic and applied research, with contributions ranging from organic chemistry to immunology, from physical chemistry to biomedical applications. The implementation of these polymers, which has long depended on the expertise of a handful of laboratories, is now appropriated by totally unrelated groups—which is great. Indeed, enough experience has now accumulated about the properties of APols, those of MP/APol complexes, and the advantages and constraints of their applications for naive users to be able to form, a priori, a good view of what to expect, how to proceed, and what to pay attention to. Some fields of applications, like EM single-particle studies, solution NMR, or MP folding, have emerged from the exploratory stage and are on their way to become routine, whereas others, like mass spectrometry or MP immobilization, are developing rapidly. New exciting routes seem to open up, like in crystallography or vaccination.

The diversification of the chemical structures of APols continues, which has its advantages and its dangers. On the plus side, new molecules can permit to overcome physical–chemical limitations inherent to the chemical structure of A8-35, the workhorse of APol development, and to implement new applications. On the minus side, one should be conscious that mastering the synthesis of A8-35—which had its pitfalls—understanding its properties and those of MP/A8-35 complexes and exploring their applications has required a heavy, long-term investment in chemistry, physics, biochemistry and biophysics. Some of the conclusions from this work can likely be extended to other APols, others not. It will always remain desirable that biologists collaborate with chemists and physical chemists to provide a solid background to the use of new varieties of APols. Once one of them has been validated and well-characterized, however, it becomes relatively easy to develop modified versions of it featuring particular properties, like labels or tags. The range of modifications that can be implemented is enormous, as is the number of applications that they facilitate or just plainly render accessible. More worrying is the industrial development of APol production, which has been lagging behind. Research laboratories just cannot become production units, and convincing industrial chemists to bring out new, carefully validated molecules is an urgent necessity, which, given the relative narrowness of the market, will perhaps require some concerted initiative by membrane biology laboratories and funding organizations.

After 20 years of hard work, it is nevertheless satisfying to see APols take their modest but useful place in the increasingly rich box of tools available to membrane

biologists. We cannot wait to see what will have become of this technology 10 and 20 years from now!

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