Amphipols, Nanodiscs, and Fluorinated Surfactants: Three Nonconventional Approaches to Studying Membrane Proteins in Aqueous Solutions

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Key Words
nanolipoprotein particles, nanoscale apolipoprotein-bound bilayers, reconstituted high-density lipoprotein particles, hemifluorinated surfactants

Abstract
Membrane proteins (MPs) are usually handled in aqueous solutions as protein/detergent complexes. Detergents, however, tend to be inactivating. This situation has prompted the design of alternative surfactants that can be substituted for detergents once target proteins have been extracted from biological membranes and that keep them soluble in aqueous buffers while stabilizing them. The present review focuses on three such systems: Amphipols (APols) are amphipathic polymers that adsorb onto the hydrophobic transmembrane surface of MPs; nanodiscs (NDs) are small patches of lipid bilayer whose rim is stabilized by amphipathic proteins; fluorinated surfactants (FSs) resemble detergents but interfere less than detergents do with stabilizing protein/protein and protein/lipid interactions. The structure and properties of each of these three systems are described, as well as those of the complexes they form with MPs. Their respective usefulness, constraints, and prospects for functional and structural studies of MPs are discussed.
1. INTRODUCTION: STRANGER IN A STRANGE LAND

Thirty-five years have elapsed since the art of using detergents to handle membrane proteins (MPs) emerged from the “cooking recipe” age and entered that of physical chemistry (1, 2). Yet, most biochemists will confess to a feeling of nervousness when compelled to deal with membrane-associated proteins. MPs indeed have earned a well-deserved reputation for being hard to handle once extracted from their natural environment and made water soluble, and the search for the detergent and conditions that will confer upon them a modicum of stability is known to be time-consuming and, more often than not, frustrating.
How surfactants associate with a membrane protein. (a) Space-filling model of Escherichia coli’s trimeric outer membrane protein OmpF [Protein Data Bank (PDB) accession code 2OMF; see Reference 154]. Hydrophobic and aromatic amino acid side chains, shown in black, form an ∼2.5-nm wide belt, which, in situ, faces the hydrophobic interior of the membrane (approximated by horizontal lines). Reprinted from Reference 11. (b) The distribution of the detergent octyl-β-D-glucoside (purple cage) around OmpF (in skeleton representation), as determined by neutron crystallography, closely follows the belt of hydrophobic residues. Adapted from Reference 155.

A glimpse at Figure 1a makes it immediately obvious why integral MPs—the only ones we are concerned with in this review—cannot be handled in pure water: The part of their surface that, in situ, is in contact with lipid acyl chains and/or the transmembrane surface of other proteins is, as a rule, highly hydrophobic. A solution of MPs in an aqueous buffer does not stay monomeric because the hydrophobic effect, which tends to minimize the number of water molecules in contact with apolar surfaces (3), will drive MP transmembrane surfaces to interact with one another. This results in aggregation and, most often, in precipitation. Adding surfactants to the solution prevents this phenomenon. Surfactants are molecules that comprise at least one polar and at least one apolar moiety. In aqueous solutions, the polar groups are readily solvated, whereas the apolar ones are pushed toward the air-water interface from which they displace water molecules. This reduces the free-energy cost of creating this interface, which lowers the surface tension, hence the name surfactant.1 Surfactants also displace water from the hydrophobic surface of MPs onto which they adsorb, making it more polar. If their concentration is high enough, they may form a continuous belt that covers what, in situ, was the transmembrane surface (Figure 1b). The resulting complex can be readily soluble. For the protein to remain monomeric, surfactant/protein interactions must overcome protein/protein ones, a process that depends both on the properties of the surfactant and on its concentration.

Before being handled in aqueous solutions, MPs must be extracted from membranes. That is, the protein-lipid and protein-protein interactions that anchor them in the membrane must be replaced with protein-surfactant ones. This is usually achieved using a special class of surfactants that biologists call detergents (physicists hate the term, which they reserve for laundry). Detergents are surfactants whose solution properties allow them to disperse fats and other hydrophobic molecules. In contrast to the lipids that comprise biological membranes, which, as a rule, form bilayers, detergents self-organize in water in the form of small aggregates, comprising typically 40–100 molecules, called micelles. Their apolar

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1Most proteins are surfactants, but they are not included under this term in the present text.
Critical micellar concentration (cmc): the concentration of a surfactant above which its molecules start to organize into micelles.

Moieties are grouped in the core of the micelle, away from water, and the polar groups face the solution. The reason why biological lipids form extended planar structures and detergents form small closed ones is geometric: In projection on the plane of the membrane, the polar and apolar moieties of lipids occupy comparable areas—that is, on average, lipid molecules look more or less cylindrical—so that the juxtaposition of many molecules forms a flat monolayer, two of which appose to form the membrane. In detergents, the apolar part is less bulky than the polar one, which bends the interface, generating spheres, ellipsoids, or cylinders, called micelles (3, 4). The concentration above which micelles form—the critical micellar concentration (cmc)—depends on a balance between the hydrophobic effect and translational entropy, the first effect driving detergent molecules to assemble, whereas the second one favors their dispersion. Micelles are in rapid equilibrium with monomers (see, e.g., Reference 5). For straightforward thermodynamic (entropic) reasons, the formation of micelles essentially buffers the concentration of monomers at the cmc, so that every new addition of detergent to the solution results in the formation of more micelles. Above the cmc, the chemical potential of the detergent remains almost constant (3). Detergents dissolve hydrophobic or amphipathic molecules that partition into micelles.

Because they partition efficiently into membranes while having a molecular shape different from that of lipids, detergents stress the membrane/water interface, which they tend to bend. Ultimately, if the detergent is “strong” enough, the planar structure becomes energetically unfavorable, holes form, and membrane constituents become dispersed into mixed micelles comprising detergent, lipids, and MPs. For a detergent to be solubilizing, the membrane has to break up before micelles of pure detergent appear in the solution. If such is not the case, membranes into which some detergent has partitioned will coexist with micelles. This happens with some “weak” detergents and with non-detergent surfactants. For detergents to efficiently resolve a membrane into its constituents, protein/detergent and lipid/detergent interactions have to overcome the protein/protein, protein/lipid, and lipid/lipid interactions that keep the membrane together. Detergents used to solubilize biological membranes are, therefore, out of necessity dissociating.

It has long been observed that, once exposed to detergents, most MPs rapidly lose their functionality (for two examples among hundreds of studies, see, e.g., References 6 and 7). Why this is so is seldom studied in detail, is probably variable from one protein to the next, and is not the object of a consensus among membrane biochemists (for reviews, see, e.g., References 8–11). An extensive discussion of this matter is beyond the scope of the present review, and I present only my own views about it. Much of the data that my coworkers and I have collected in the course of more than 30 years of work with half-a-dozen MPs suggests that a major contribution to the destabilization of MPs by detergents is the dissociating properties of the latter, i.e., their ability to disrupt protein/protein and protein/lipid interactions, a property that is the very reason why they are used in the first place. Protein/protein and protein/lipid interactions, however, are essential to MP stability. Oligomeric MPs usually feature subunit/subunit interactions in the transmembrane region; the three-dimensional (3D) structure of monomeric MPs, as well as that of subunits, depends on protein/protein interactions between their transmembrane segments; and most MPs are extracted from membranes along with bound lipids, which stabilize them. Detergents compete with all of these interactions, and micelles act as a “hydrophobic sink” for molecules that, initially, were associated with the MP under study. Delipidation is one of the most, if not the most, common causes of MP inactivation. The destabilizing effect of diluting lipids, subunits, and/or hydrophobic or amphipathic cofactors among detergent micelles explains the fact that, for a given detergent, working close to the cmc, i.e., in the presence of few micelles, will often improve the stability of MPs.
The obvious underlying mechanism is that reducing the concentration of micelles limits the hydrophobic volume into which lipids, cofactors, and subunits can diffuse or, stated another way, increases their concentration in the mixed micelles. This favors their sticking together rather than coming apart. An equally frequent observation is that supplementing an MP in detergent solution with lipids very often will stabilize it. This can be due to the preservation of interactions with the lipids that help the protein to keep its 3D structure and/or to the lipids preventing access of the detergent to vulnerable spots at the transmembrane surface. Other causes of destabilization can also be considered. For example, it can be argued that the dynamics of transmembrane α-helices within helix bundles is restricted by the geometry of the bilayer more than it is within a detergent micelle or that the lateral pressure gradient within the bilayer is important for their stability (for a discussion of these effects, see Reference 12). Our own work has been based on the belief that finding ways to limit the disruption of protein/protein and protein/lipid interactions would be a decisive step toward improving MP stability.

2. KEEPING MEMBRANE PROTEINS WATER SOLUBLE IN THE ABSENCE OF DETERGENTS

Because dealing with solubilized MPs is necessary to understanding their structure and function and because most MPs become unstable once solubilized, many attempts have been made to develop countermeasures. One approach is to make the protein itself more stable, either by selecting it from appropriate organisms, e.g., hyperthermophiles, or by engineering it. The latter strategy has led to remarkable success (see, e.g., References 13–16), and it certainly holds great promise for the future.

An alternative or complementary approach is to make the environment less destabilizing. After being extracted from their original membrane environment, MPs traditionally are kept water soluble with a detergent. Investigators may resort to the same detergent that was used for extraction, which they generally use at a lower concentration, so as to limit MP destabilization. Alternatively, MPs, once solubilized, are often transferred to another less aggressive detergent, a detergent mixture, or a lipid/detergent mixture. There is indeed no fundamental necessity to use a strongly dissociating detergent once a protein has been extracted. Weak detergents, such as digitonin or surfactants of the Tween series, have long been used for this purpose, despite their chemical heterogeneity and less than satisfying micellar properties (very low cmc, large micelles). Once the protein has been extracted, however, what the biochemist needs is to keep it soluble and to prevent it from aggregating, conditions that can be provided by surfactants that are not necessarily able to solubilize membranes. This has led to the development of such molecules as tripod amphiphiles (17–19), surfactants with rigid hydrophobic tails containing cycles (20), peptigerns (21), lipopeptides (22, 23), peptigerns (24), fluorinated surfactants (FSs) (25, 26), or amphipathic polymers called amphipols (APols) (27), most of which are not good membrane solubilizers (for brief overviews, see References 11 and 28).

A major part of this review is devoted to the latter two approaches. Earlier reviews on APols and FSs are found in References 11 and 28–30.

Because replacing biological lipids with other surfactants is generally detrimental to MP stability, an obvious alternative is to reinsert them, after extraction, into a lipid environment. This may seem self-defeating as the lipids an MP is normally in contact with generally organize into bilayers, which can be dispersed as vesicle suspensions but do not form the small entities most suitable for biochemical and biophysical approaches. Bilayers can, however, be fragmented into small patches by mixing lipids with certain surfactants, e.g., bile salts or short-chain lipids such as dihexanoylphosphatidylcholine (DHPC), which tend to segregate from the surfactant-saturated lipid bilayers and form the rim of small lamellar discs called bicelles. MPs inserted into these discs find themselves

Fluorinated surfactant (FS): a surfactant whose hydrophobic chain is fluorinated

Amphipol (APol): an amphipathic polymer that can keep membrane proteins water soluble in detergent-free solutions as small individual entities by adsorbing onto their transmembrane surface

Bicelle: a patch of bilayer whose rim is stabilized by small surfactants such as DHPC
Nanodisc (ND): a small patch of bilayer stabilized by amphipathic rim proteins
Nonconventional surfactant (NCS): as used in this text, either a nanodisc, an amphipol, or a (hemi)fluorinated surfactant
Membrane scaffolding proteins (MSPs): nanodisc rim proteins derived from apoA-1

surrounded by a bilayer-like environment. Bicelles are a highly interesting medium that has been used, among other things, for MP NMR studies (31–36), as well as for MP crystallization (37, 38). For want of space, bicelles are not discussed here.

Over the past decade, a variant of bicelles has been actively investigated, the rim of which is formed by amphipathic proteins. These structures, which can integrate MPs up to a certain size determined by the structure of the rim proteins, are variously called nanodiscs, nanolipoprotein particles, nanoscale apolipoprotein-bound bilayers, or reconstituted high-density lipoprotein particles, hereafter lumped under the collective name of nanodiscs (NDs). NDs have been the object of two recent reviews (39, 40). This article includes a brief description of what NDs are and which MP studies have been done thanks to them and also compares their prospects with those of APols and FSs.

Throughout this text, APols, NDs, and FSs are collectively referred to as nonconventional surfactants (NCSs).

3. PRINCIPLE AND MOLECULAR ORGANIZATION OF NONCONVENTIONAL SURFACTANTS

NDs, FSs, and APols have quite different chemical compositions and molecular structures, which are reflected in the very distinct ways they assemble into particles when they are dispersed in aqueous solutions. This, in turn, has profound effects on the nature and properties of the complexes they form with MPs.

3.1. Nanodiscs

NDs were introduced by S.G. Sligar (University of Illinois) and his coworkers as the spin-off of a very large body of work on high-density lipoproteins (for a review, see Reference 39). Small NDs consist of a patch of, typically, 130–160 lipids, organized as a bilayer and surrounded by stabilizing proteins. The latter are often the so-called membrane scaffolding proteins (MSPs), which are derived from human high-density lipoprotein apoA-1 by modifications such as pruning away some undesired domains (41) and duplicating other domains so as to increase the protein’s length and, thereby, the perimeter of the ND (42). MSPs can also be endowed with a polyhistidine tag (42). Other proteins can be used as well (see References 43–45). The structure of NDs, whether empty or containing MPs, has been intensely studied by such approaches as small-angle X-ray scattering (SAXS), atomic force microscopy (AFM), size exclusion chromatography (SEC), native gel electrophoresis, electron microscopy (EM), solid-state NMR (ssNMR), Fourier-transform infrared spectroscopy (FTIR), and various other types of optical spectroscopy (reviewed in References 39, 40). The thickness of an empty ND is that of a bilayer (42, 46, 47). The size of the disc depends on the rim proteins, and for a given protein the number of encapsulated lipids depends on the surface area of the lipids (46). Overall diameters reported to date vary between ∼10 and ∼20 nm (reviewed in References 39, 40). How variable it is from disc to disc in a given population is under investigation (see Reference 47, and the references therein). The molecular mass of the smallest NDs is ∼150 kDa. ND lipids undergo phase transitions similar to those of extended bilayers, although, for entropic reasons, the transitions are broader, and the behavior of the first two rows of lipids in contact with the rim proteins is altered (48, 49).

Original versions of NDs contained two copies of MSP per disc, but these can also be fused one to another, resulting in objects containing a single protein per disc (42). Various models have been proposed for the arrangement of the MSPs in NDs, and extensive molecular dynamics (MD) simulations have been carried out. Most data, particularly ssNMR determination of the conformation of specific classes of MSP amino acid residues (50) and MD simulations (reviewed in
Figure 2
Molecular dynamics (MD) structures of nonconventional surfactants. (a, b) MD structure of a nanodisc. Model viewed (a) perpendicular to the bilayer and (b) in the plane of the bilayer, based on the molecular belt model of discoidal high-density lipoprotein (156). Two monomers of the membrane scaffold protein form an amphipathic helical belt around a segment of phospholipid bilayer. Model by S.C. Harvey from Reference 39. (c, d) MD structure of an amphipol (APol) particle. A snapshot from the end of a 50-ns MD simulation of a fully hydrated A8-35 particle. The particle ($R_g \approx 2.4$ nm) consists of four identical 10-kDa A8-35 molecules, whose side chains were distributed randomly along the backbone. In panel c, groups are colored as described in the key, with black lines indicating the polyacrylate backbone. Surrounding waters, hydrogens, and ions have been excluded for clarity. In panel d, only the octylamide side chains, backbone (black lines), and water within 0.5 nm of the particle center are shown in order to highlight the formation of submicellar domains and a hydrated particle core. The scale bar represents $\sim 5$ nm (J.N. Sachs, personal communication). Nanodiscs and APols are represented approximately to scale.

Reference 51), favor the “molecular belt” model in which the protein (or proteins) wraps around the rim of the disc (Figure 2a,b) (reviewed in References 39, 40, 51).

NDs have two unique properties compared to the two other systems discussed in this review. First, the MPs they harbor find themselves surrounded by a medium that is extremely similar to a normal bilayer. MP functions that depend, in one way or another, on interactions with lipids therefore stand a better chance to be faithfully reproduced than in APols or FSs. Second, the defined size of the rim proteins sets that of the disc and, therefore, the dimensions of the MP or MP complexes that can be encapsulated. In contrast to detergents, APols, or FSs, MSPs cannot accommodate MPs beyond a certain size, and the probability that discs can fuse, allowing MPs trapped in different discs to temporarily occupy the same disc, seems remote. NDs can be used, therefore, to investigate issues where the degree of association of MPs with one another is of primary importance (Section 7.3). The flip side, of course, is that, as of now, MPs with large transmembrane domains cannot be handled in NDs (Section 5.2).

3.2. Fluorinated Surfactants
The chemical structure of FSs resembles those of classical detergents, but their hydrophobic
tails contains fluorine atoms (Figure 3). The rationale behind the use of such compounds is based on the poor miscibility of alkanes and perfluorinated alkanes (52–55).

FSs indeed are not cytolytic (they are not detergents) because they do not partition well into lipid membranes (25, 55–61). Because they are not detergents, FSs originally attracted little attention from membrane biochemists (see, e.g., References 25, 62, 63), with the exception of perfluoroctanoate, which has been used for MP electrophoresis (64, 65). It could nevertheless be hoped that FSs might be less prone than detergents to destabilize MPs for the following two reasons: (a) Lipids, subunits, and hydrophobic cofactors should partition less favorably into FS micelles; and (b) because fluorinated alkyl chains are more bulky and more rigid than alkanes and have little affinity for hydrogenated transmembrane protein segments, they ought to be less efficient than detergents at disrupting protein/protein interactions. By the same token, however, FSs bearing perfluorinated chains could be expected to be ineffective in preventing MPs from aggregating, which early data seemed to bear out (25, 63). To try to improve interactions with the methyl group-covered transmembrane surfaces of MPs while preserving the overall lyophobic (“lipid-hating”) character of FS micelles, a hydrogenated tip was grafted onto the fluorinated tail, yielding “hemifluorinated surfactants” (HFSs) (Figure 3). Hereafter, FSs and HFSs are collectively referred to as (H)FSs.

The development of (H)FSs, which has gone through three main phases, has been carried out through a long-term collaboration between our laboratory and that of

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**Figure 3**

Chemical structure of some fluorinated surfactants. (a) F-TAC (C₈F₁₇-C₂H₄-S-poly-tris-(hydroxy-methyl)aminomethane) (see References 25, 56, and 57) and HF-TAC [C₂H₅-C₆F₁₂-C₂H₄-S-poly-tris-(hydroxymethyl)aminomethane] (see References 26 and 55). (b) (H)F-Mono-, Di- and TriGlu; F₆- and H₂F₆-, as used in the text and in Figure 7, refer to hydrophobic moieties, where R = F and R = C₂H₅, respectively. See References 71 and 72. (c) Phenyl-HF-NTANi. See Reference 138. (d) (H)F-TACs labeled with Oregon Green (OG₄₄₈). See Reference 61. (e) A fluorinated amidosulfo betaine, FASB-p,m. See Reference 74.
B. Pucci (University of Avignon). The first molecules to be tested had as a polar head group a short hydrophilic oligomer derived from tris-(hydroxymethyl)aminomethane (THAM) and a perfluorinated hydrophobic moiety (Figure 3a) (25, 56, 57). In a second stage, the tail was hemifluorinated (Figure 3a) (26, 57, 66). These molecules yielded very promising results, and most of the applications of (H)FSs that have been explored to date have been developed using them (Section 7). The oligomeric polar head of (H)F-TAC—where “(H)F” refers indifferently to the fluorinated or the hemifluorinated form—however, has been a concern from the start because it is chemically polydisperse. From the biochemist’s point of view, this means that (H)F-TAC batches consist of a mixture of molecules with slightly different properties and that this mixture will never be exactly the same from one batch to the next.

Replacing the poly-THAM oligomer with a monodisperse headgroup initially seemed a straightforward proposition, which was explored as a third stage of development. This step turned out to be highly frustrating. Indeed, the surfactants obtained upon grafting (hemi)fluorinated chains onto monodisperse polar heads that, when associated to alkyl chains, yield efficient detergents, such as an aminoxyde (67), a monodisperse polyethylene glycol group (see Reference 28), or saccharide groups derived from galactose (25, 68), lactose (69), or maltose (70) featured unsatisfactory properties. A recurrent problem was that most of the molecules thus obtained tended to form huge polydisperse micelles by themselves and highly polydisperse MP/(H)FS complexes (for a discussion, see Reference 71). This behavior suggested that the bulky hydrophobic moiety of (H)FSs requires a bulkier hydrophobic head than classical detergents do to create the overall molecular asymmetry that leads to the formation of small globular micelles (4). A systematic investigation was, therefore, undertaken in which polar heads carrying one, two, or three glucose moieties were grafted onto perfluorinated, hemifluorinated, or hydrogenated hydrophobic chains (72). This study led to the identification of two chemically defined (H)FSs, Fc-DiGlu and H2Fc-DiGlu (Figure 3b), which, as described below (Sections 5.3 and 6.2), form with MPs small, well-defined complexes in which MPs are stabilized as compared to detergent solutions (71).

A new kind of hemifluorinated surfactant has recently been introduced in which the tip of the hydrophobic chain is perfluorinated, but a more or less extended hydrocarbon region is inserted between it and an amidosulfobetaine polar head (Figure 3e) (73, 74). To avoid confusion, these compounds are designated here by the name used by their developers, FASBs (fluorinated amidosulfobetaaines). As (H)FSs, FASBs do not by themselves extract MPs (74).

3.3. Amphipathic Polymers (Amphipols)

Detergents and FSs are in a constant equilibrium between monomers, micelles, and the surfactant layer that covers the transmembrane region of the protein and makes it hydrophilic. MPs will aggregate if the surfactant concentration drops below its cmc, meaning that MP/detergent complexes must be handled in the presence of free micelles. The initial idea behind the concept of APols was to design molecules that would have such a high affinity for the surface of the protein that traces of free surfactant in the solution would suffice to keep the protein soluble. An MP transferred to such a medium would face no difficulty retaining its associated lipids, cofactors, and/or subunits and, therefore, should be strongly stabilized. This concept led my colleagues C. Tribet and R. Audebert (ESPCI, Paris) and myself to devise a family of short amphipathic polymers that carry a large number of hydrophobic chains and thus can associate with the transmembrane surface of MPs by multiple contact points. This was expected to result in a vanishing low rate of spontaneous desorption and a very high affinity for MP transmembrane surfaces. The new molecules were dubbed amphipols (APols) to distinguish them from other

Lyophobic: having little affinity for lipids; perfluoralkanes are both hydrophobic and lyophobic

Hemifluorinated surfactant (HFS): as used in this text, a surfactant with a fluorinated hydrophobic chain that ends with a hydrogenated tip

(Hemi)fluorinated surfactant, abbreviated (H)FS: is used whenever the matter under discussion applies indifferently to FSs and HFSs

THAM: tris-(hydroxymethyl)aminomethane

(HF)-TAC: an (H)FS with an oligomeric polar head derived from THAM

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amphipathic polymers, such as those used in the industry to stabilize dispersions of mineral particles or droplets or to control the rheology of solutions.

For an amphipathic polymer to form compact and stable complexes with an MP, the distribution of its hydrophobic chains must be dense, its solubility in water must be high, and it must be highly flexible. It is also desirable that the synthesis of tens of grams be reasonably simple and reproducible. A first series of APols was designed in 1994. One of its members, A8-35 (27), has since become by far the most extensively studied APol (75, 76). A8-35 (Figure 4a) is composed of a relatively short polyacrylate chain (~70 residues), in which some (~17) of the carboxylates have been grafted at random with octylamine and some (~28) with isopropylamine. The ~25 free acid groups are charged in aqueous solutions (75), which makes the polymer highly water soluble, whereas the octylamide moieties, which are spaced along the chain about every nanometer (statistically), render it highly amphipathic. The incorporation of isopropylamide groups is not essential: Indeed, the sister structure, A8-75, which does not contain any such groups, is just as good as A8-35 when it comes to keeping MPs water soluble (27, 77). However, A8-35 features a lower charge density along the chain than A8-75, which seems to have a favorable effect on the stability of MPs (J.-L. Popot and coworkers, unpublished observations). The average molecular weight of a molecule of A8-35 is 9–10 kDa. Batches of A8-35 are a complex mixture of molecules with a variable overall length and a variable distribution of lateral chains. Whether this heterogeneity represents a favorable or an unfavorable feature from the biochemist’s and biophysicist’s points of view remains an open question because it is not known to which extent the protein may select among the variety of chains offered to it. It is worth noting, however, that A8-35 batches with a much narrower length distribution did not appear to behave differently from more polydisperse batches (C. Tribet & F. Giusti, unpublished observations).

**Figure 4**

Chemical structures of amphipols (APols). (a) A8-35, a polyacrylate-based APol. See References 27 and 76. (b) C22-43, a phosphorylcholine-based APol. See References 82 and 85. (c) A sulfonated APol. See Reference 84. (d) A glucosylated, nonionic APol (NAPol). See References 87–89.
A8-35 is highly water soluble (>200 g.L\(^{-1}\)). In a process that is rather unusual for amphipathic polymers, it forms well-defined, small globular particles in aqueous solutions (76). Each A8-35 particle has a mass of \(\sim 40 \text{kDa}\). It comprises, therefore, slightly more than four average A8-35 molecules and a total of \(\sim 80\) octyl chains. The latter number is close to that of hydrophobic chains in a typical detergent micelle. Förster resonance energy transfer (FRET) studies of mixtures of A8-35 molecules labeled with pairs of complementary fluorophores have shown that their critical aggregation concentration (that above which individual molecules start self-assembling) is \(<2 \text{mg.L}^{-1}\) (F. Giusti, J.L. Popot & C. Tribet, unpublished observations), meaning that, under most usual conditions, nearly all of the free polymer is assembled into particles. An MD model of an A8-35 particle is shown in Figure 2c,d (J. N. Sachs, personal communication). Its calculated radius of gyration, \(R_g \approx 2.4 \text{nm}\), is identical to that measured experimentally (76). An unexpected feature of the MD model is the tendency of water molecules to occupy the center of the particle (Figure 2d). There is also a marked tendency for octyl chains to form submicellar clusters in which octyl chains belonging to distinct APol molecules clump together (Figure 2d).

Variations around the chemical structure of A8-35 have been experimented with. The original study included molecules that were longer than A8-35 and/or carried a higher charge density (27), some of which were used in subsequent works (77, 78, 79). Related structures have been proposed by others (80, 81). One of the constraints imposed by the chemical structure of A8-35 is that its solubility is provided by carboxylate groups. For this reason, it cannot be used below pH 7 (75, 76, 82, 83) nor in the presence of mM concentrations of Ca\(^{2+}\) (82, 84). These limitations have prompted the design of APols that are zwitterionic (Figure 4b) (81, 82, 85), sulfonated (Figure 4c) (84), or nonionic (Figure 4d)—the latter also the fruit of a long-term collaboration between our laboratory and that of B. Pucci (86–89). All of these structures have proven able to trap MPs and, generally, have been found to be pH and calcium insensitive. Charged, amphipathic derivatives of pullulan (90), by contrast, turned out to be very inefficient at keeping MPs soluble (91). Most of the background studies and developments to date have been carried out using A8-35, but as discussed below (Section 7), there are some applications for which A8-35 is not the most suitable APol or cannot be used at all, making it highly desirable to develop and validate alternative APols.

Because APols are relatively large molecules, grafting a small functional group onto them will generally not affect their solution properties. A8-35 has thus been derivatized with biotin (92) or with various fluorophores (93; F. Giusti, unpublished data), opening the way to many interesting experiments (Sections 5.1 and 7.9). The polymerization process used for the synthesis of nonionic APols (NAPols), known as telomerization (87, 88), makes them easily amenable to stoichiometric functionalization with a single group per chain. A8-35 has also been labeled isotopically with \(^{14}\text{C}\) (77), \(^{3}\text{H}\) (83), or \(^{2}\text{H}\) (75). Deuteration has been particularly useful for NMR (94, 95) and neutron scattering (75, 76, 83) studies.

### 4. TRANSFERRING MEMBRANE PROTEINS TO NONCONVENTIONAL SURFACTANTS

Although direct extraction of MPs by APols has been observed occasionally (reviewed in Reference 29), APols, NDs, and (H)FSs are not normally used to extract MPs. The usual procedure is to transfer the MP to the NCS following solubilization with a nondenaturing detergent. In the case of NDs and APols, scaffolding proteins and lipids or the polymer, respectively, are usually supplied to the protein in detergent solution. Upon detergent removal, the systems self-assemble (see, e.g., References 43, 44, 46, 83, 93, 94, 96). The ratio of NDs

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**NAPol**: nonionic amphipol
to MP can be adjusted so as to facilitate the trapping of oligomers or, on the contrary, to favor that of monomers (Section 7.3). With APols, an excess of polymer with respect to what the MP actually binds is necessary to obtain homogeneous MP/APol complexes, so that, after trapping, part—typically about half—of the APol is protein bound and part is present as free APol particles. Lipids, if retained by the protein or if present in the detergent solution, will be trapped along with the protein, forming ternary MP/lipid/APol complexes, which may be important for maintaining the stability and function of the protein (see, e.g., References 83 and 97–99 and Section 6.1). It is of great interest that complex mixtures of detergent-solubilized MPs, such as the whole supernatant obtained after solubilizing a biological membrane preparation, can be trapped, whether with NDs or with APols, in the form of discrete MPs or MP complexes, which can then be separated, e.g., by centrifugation in sucrose gradients, SEC, or isoelectrofocusing (IEF) (cf. Section 7.4) (89, 100).

Similar transfer procedures can be used for (H)FSs, but what has often been done in the course of developing these compounds has been to layer a ternary MP/detergent/(H)FS mixture on top of a sucrose gradient containing the (H)FS to be tested (25, 26, 71). Upon ultracentrifugation, MP/(H)FS complexes enter the gradient, leaving the detergent behind them (see below, Section 6.2). This procedure has the advantage of consuming relatively little (H)FS, as compared, for instance, to dialysis or molecular sieving, which is an important criterion when working with compounds whose synthesis is difficult and whose availability is generally limited.

As described below, MPs are not always transferred to NDs, APols, or (H)FSs fully folded from a solution in nondenaturing detergent: They can be directly folded in NCSs, either starting from the full-length, denatured protein in sodium dodecyl sulfate (SDS) or urea (Section 7.10) or in the course of in vitro cell-free protein synthesis (Section 7.11).

5. STRUCTURE OF MEMBRANE PROTEIN AND NONCONVENTIONAL SURFACTANT COMPLEXES

The composition, size, homogeneity, structure, and dynamics of MP/NCS complexes have been closely scrutinized because they largely determine the suitability of the complexes for various studies.

5.1. Membrane Protein/A8-35 Complexes

A8-35 has been shown to form a complex with, and maintain solubility of, a very broad range of MPs. Those include all of the 30-odd integral MPs that have been tested to date. Their molecular masses range from <5 kDa (a single transmembrane α-helix) to >1.1 MDa [mitochondrial complex I, which probably harbors ∼70 transmembrane α-helices (101)], and they represent any conceivable diversity of origins, functions, and structures (for a review, see Reference 29). A8-35 has recently been used to trap and purify mitochondrial supercomplex B (T. Althoff & W. Kühnbrandt, personal communication; see Section 7.7), which comprises ∼120 transmembrane helices and whose overall mass is ∼1.7 MDa (102). The interactions of APols with other types of objects are beyond the scope of the present review. However, one may mention that APols have been used to stabilize in solution a signal sequence peptide (103), human apolipoprotein B-100 (I. Waldner, A. Kriško, A. Johs, & R. Prassl, unpublished data), plant lipid storage proteins (Y. Gohon, personal communication), and semiconductor quantum dots (104, 105). Several studies have been carried out of their interactions with lipid vesicles or cells (78, 79, 104, 106). Under most circumstances (for some exceptions, see Reference 78), APols will not solubilize preformed biological or lipid membranes (see Reference 29). They can, therefore, be applied to lipid vesicles, black films, or living cells without lysing them, which opens up some extremely interesting applications (Section 7.8).
The composition, structure, dynamics, and solution properties of MP/A8-35 complexes have been studied by SEC, analytical ultracentrifugation (AUC), small-angle neutron scattering (SANS), SAXS, FRET, and solution NMR, yielding a rather detailed picture of what these complexes look like. In NMR studies using as models the transmembrane domains of outer MP A from *Escherichia coli* (tOmpA) or *Klebsiella pneumoniae* (KpOmpA) and E. coli’s outer MP X (OmpX), each of which consists of an eight-strand β-barrel, the only detectable contacts between A8-35 and the protein were observed at the barrel’s transmembrane surface (Section 7.5). AUC and SANS studies of bacteriorhodopsin (BR)/A8-35 complexes indicate that the polymer layer is compact and 1.5–2 nm thick (83), which is only slightly thicker than a detergent layer (107). Measuring precisely the amount of protein-bound APol is technically difficult, particularly for small proteins (83, 93). Estimates vary from ∼2 g per g protein for a small, mainly transmembrane protein like BR (Figure 5c) (83) to ∼0.13 g per g for a complex with large extramembrane regions such as cytochrome bc₁ (Figure 5a) (D. Charvolin, unpublished data).

For large MPs, this corresponds to significantly less (by 2–3 times) octyl chains than the number of detergent molecules bound by the same protein in detergent solutions (29). For small MPs, the difference is much less (83, 93). In most experiments, such as those by SAXS, SANS, AUC, or solution NMR, MP/A8-35 complexes behave like compact, globular particles (83, 94, 108). Upon SEC, tOmpA/A8-35 and BR/A8-35 complexes migrate as though they are somewhat bigger than they actually are (83, 94).

When properly prepared, MP/A8-35 complexes are essentially homogeneous (83, 93), although they do not appear as narrowly distributed in SEC as MP/detergent complexes (93). It is, however, difficult to totally avoid the presence of minor fractions of small oligomers, which can seriously complicate, in particular, radiation scattering experiments (83). Among the factors that can lead to polydispersity, if not aggregation, are (a) the use of too little APol at the trapping step (93), (b) working at pH ≤ 7 (82, 83, 94), (c) the presence of Ca²⁺ ions (82, 84, 108), (d) shifts from the nominal composition of the polymer that result in increasing its hydrophobicity (83, 84), and (e) separating the complexes from the free polymer that coexists with MP/APol complexes at the end of a trapping experiment (83, 93). The oligomerization that follows the removal of free APol is reversible. It has been interpreted as resulting from the poor dispersive power of APols: In the competition between protein/protein and protein/APol interactions, the presence of an excess of polymer favors the formation of MP monomers, whereas its removal shifts the equilibrium toward aggregation (93). Increasing the ionic strength of the solutions progressively turns the interactions between cytochrome bc₁/A8-35 particles from a repulsive mode to an attractive mode (29). In a solution that contains both protein-bound and free APols, free and bound polymers exchange at the surface of the protein at a rate that, at least for A8-35, strongly depends on the ionic strength of the solution (minutes in 100 mM NaCl, hours at low ionic strength) (93). The underlying mechanism has not been studied in detail, but it probably involves collisions between MP/APol complexes and free APol particles, followed by fusion, mixing, and fission. Such a mechanism is consistent with the fact that APol particles can deliver retinal, a highly hydrophobic cofactor, to refolded bacterio-opsin (99). By contrast, in the absence of exchange with another surfactant, APols do not desorb from MPs even under extreme dilution or extensive washing (29, 77, 93, 109). A thermodynamic analysis of MP/detergent versus MP/APol interactions has led to the conclusion that the stability of MP/APol complexes is essentially of entropic origin: When a solution of MP in detergent is diluted below the cmc of the detergent, the release of tens of detergent molecules that accompanies the association with one another of two MPs creates a strong entropic drive toward aggregation; when two APol-trapped MPs aggregate, the desorption of one or two APol molecules, which
Figure 5

Models of complexes between membrane proteins (MPs) and amphipols (APols) or nanodiscs (NDs).

(a–c) Models of MP/APol complexes. (a) Cytochrome bc$_1$/A8-35 complex (from Reference 29). (b) An “open” model of a complex between the transmembrane domain of E. coli’s OmpA (tOmpA) and A8-35 (from Reference 29). (c) Cross section through a bacteriorhodopsin (BR)/A8-35 complex. Lipids from the purple membrane, which were retained throughout the solubilization and trapping procedures, are shown in olive green (from Reference 83). The volume and distribution given to the APol belts in the three complexes are based on an ensemble of data including binding, SEC, AUC, SANS, and NMR measurements, obtained on each individual complex as well as on other MPs (see References 29 and 83 and Section 5.1) Models by D. Charvolin. (d, e) Models of rhodopsin (Rho) (d) and the serine chemotaxis receptor (Tsr) (e) embedded into NDs. From Reference 39. The five models are represented approximately to scale.
associate into particles, is entropically neutral (109).

Although few proteins have been tested yet, it seems that freezing MP/APol complexes is not detrimental, whereas lyophilizing them can be (83; Y. Gohon & L. Catoire, unpublished data).

In ternary mixtures containing an MP, a detergent, and an APol, the two surfactants mix at the transmembrane surface of the protein, forming ternary complexes (77, 93, 109). FRET and isothermal calorimetry studies show that mixing is almost ideal and that the exchange of one surfactant for the other is isenthalpic: Detergents and APols mix freely, and the composition of the mixed belt of surfactant around the MP reflects that of the mixed, protein-free APol-detergent particles (93, 109). The exchange of APol for detergent at the surface of an MP is extremely rapid (<1 s) (93). Because APols have no strong preference for the MP-bound belt versus free APol/detergent particles, they are easily and rapidly washed away by an excess of detergent (77, 93, 97, 109). Interestingly, MPs can be stabilized biochemically even by mixtures of detergent and APols (97) (see Section 6.1 below). Upon SEC, MP/APol/detergent particles appear more homogeneous in size than pure MP/APol complexes (93). These two observations may have interesting implications for the choice of crystallization conditions (Section 7.6).

5.2. Membrane Protein/Nanodisc Complexes

MPs that have been reconstituted into NDs include a number of P450 cytochromes as well as the NADPH-cytochrome P450 reductase (reviewed in References 39 and 40), BR (110, 111), the bacterial Tar chemoreceptor (112), the SecYEG translocon complex (113), and several G protein–coupled receptors (GPCRs), including the β2-adrenergic receptor (β2-AR) (114–116), rhodopsin (44, 117), and a μ-opioid receptor (118). The size of the largest transmembrane domain that can be accommodated into a ND depends on the rim protein employed.

In addition, recent single-particle data suggest that NDs can have a multimodal distribution of diameters and that MP encapsulation can shift this distribution toward larger diameters (45, 47). The largest system whose encapsulation has been reported to date is the BR trimer, which comprises 21 transmembrane helices (111). It is to be expected, however, that this constraint will be relaxed as protein engineering will produce MSPs and other proteins able to stabilize larger and larger discs (see, e.g., References 42, 43, and 45).

MP/ND complexes have been studied experimentally by SEC, SAXS, AFM, EM, circular dichroism (CD), fluorescence spectroscopy, and ssNMR (see, e.g., References 44, 47, 110–112, 115, 117, 118), as well as simulated by MD (119) (for reviews, see References 39 and 40).

Essential to the use of NDs for functional studies is the knowledge of the number of copies of MPs they have captured. Thus, trapping conditions have been designed so as to trap either monomers or trimers of BR (110, 111), monomers of the β2-AR (115) or the μ-opioid receptor (118), and monomers or dimers of rhodopsin (44, 117). The Tar chemoreceptor has been trapped either as dimers or trimers of dimers (112). Functional studies of biological systems where the number of copies of MP involved is a critical factor are one of the most exciting applications of NDs (see Section 7.3 below).

5.3. Membrane Protein/(Hemi)Fluorinated Surfactant Complexes

MP/(H)FS complexes have not yet been studied in great detail, particularly for those (H)FSs that appear to be most satisfying both from the point of view of the chemistry (defined chemical structure) (72) and biochemistry (small, well-defined MP/(H)FS complexes) (71). A striking observation is that MP/(H)FS complexes migrate much faster upon centrifugation in sucrose gradients than MP/detergent complexes.
Behavior of cytochrome \( b_6f \) in sucrose gradients containing either a detergent, dodecylmaltoside (DDM), or glucosylated fluorinated surfactants at various concentrations. The chemical formulas of \( F_6\)-Mono-, -Di- and -TriGlu are shown in Figure 3. In DDM, the complex remains primarily a superdimer (D) at a concentration just above the critical micellar concentration and starts to fragment into monomers (M) at higher concentrations (cf. Reference 7). In \( F_6\)-TriGlu and \( F_6\)-DiGlu, it forms well-defined bands of dimer whatever the surfactant concentration. In \( F_6\)-MonoGlu, the bands are fuzzy, owing to the polydispersity of the complexes. From Reference 71.

(\textbf{Figure 6}) (25, 26, 69, 71). A priori, this could be due to an aggregation of the protein and/or to the binding of very large amounts of surfactant. However, a detailed study of the complexes formed between cytochrome \( b_6f \) and a lactose-derived HFS, HF-Lac, has shown that neither is true: The \( b_6f \) is dimeric in HF-Lac solutions, as it is in detergent ones as long as it retains its native structure (7), and it binds about the same number of HF-Lac molecules (~260) as of dodecylmaltoside ones (69). Instead, it is the much higher density of the surfactant \( \varrho \approx 0.6 \text{ mL.g}^{-1} \) for HF-Lac, \( F_6\)- and \( H_2F_6\)-DiGlu (69, 71), owing to the presence of the fluorine atoms, which accounts for the increased sedimentation coefficient of the complexes.

In the course of comparing the behavior of (H)FSs carrying a variable number of glucose moieties, it was noted that at least two sugars are needed for (H)FSs to form small globular micelles and that only in these cases was it possible to obtain well-defined MP/(H)FS complexes. By contrast, molecules bearing a single glucose form long, cylindrical micelles and generate polydisperse MP/(H)FS complexes (71). This is illustrated in \textbf{Figure 6} in the case of cytochrome \( b_6f \).

6. FUNCTIONALITY AND STABILITY OF MEMBRANE PROTEINS IN NONCONVENTIONAL SURFACTANTS

The stability and functionality of an MP in aqueous solution is extremely variable, depending both on the protein and on the surfactant that keeps it soluble, as well as on the concentration of the latter. Existing data indicate that, used at comparable ratios of “micellar” (assembled) surfactant to MP, the three types of NCSs considered here tend to be less inactivating than detergents—that is, they keep MPs from irreversible denaturation for a longer time. Data about the functionality of MPs associated to NCSs, which is a distinct question, are still relatively scarce but, on the whole, very encouraging.

6.1. Membrane Protein/Amphipol Complexes

Complexation by APols, in most cases, biochemically stabilizes MPs as compared to detergent solutions (see, e.g., References 27, 29, 83, 84, 88, 89, 97, and 120 and the
This is illustrated in Figure 7 for the sarcoplasmic reticulum calcium ATPase (Figure 7a), BR (Figure 7b), and a GPCR, the BLT1 receptor of leukotriene LTB₄ (Figure 7c). As illustrated by the intermediate curve in Figure 7a, even adding APols to an MP in detergent solution, without removing the detergent or diluting it, may have a stabilizing effect. A detailed discussion of the mechanisms of MP stabilization by APols is beyond the scope of the present review. Existing data indicate that the following effects may contribute to stabilization: (a) Retention of MP-associated lipids, cofactors, and subunits (a consequence of reducing the hydrophobic sink, but also of the poorly dissociating character of APols); (b) less efficient competition with protein/protein interactions, and (c) damping the dynamics of conformational excursions of transmembrane α-helix bundles, which limits opportunities for unfolding and/or aggregation (for discussions, see References 29 and 84). Stability can be further improved by forming ternary MP/lipid/APol complexes (Figure 7c) (97, 120). Although data are still limited, a sprinkling of observations suggests that, as is the case for detergents, NAPols may be even less destabilizing than ionic ones are (86; Y. Pierre, unpublished observations).

The functionality of APol-trapped MPs is generally preserved (see, e.g., References 29, 81, 83, and 98). However, MPs whose functional cycle involves large rearrangements of the surface of their transmembrane region, as is the case for the sarcoplasmic calcium ATPase, may see their activity reversibly slowed or blocked, presumably because the adsorbed polymer damps such transconformations (for discussions, see References 29 and 84). For BR (83, 99) and, perhaps, the nicotinic acetylcholine receptor (nAChR) (98), indirect arguments suggest that transferring the protein from a detergent to an APol environment favors the rebinding of lipids at the surface of the protein, which probably contributes to restoring membrane-like functionality (Section 7.3). Ligand binding is, very generally, unaffected by APol trapping (see Sections 7.2, 7.9, and 7.10 below).

6.2. Membrane Protein/Hemifluorinated Surfactant Complexes

Following their transfer from detergent solutions to (H)FS ones, BR, cytochrome b₅ f, the human GPCR Smoothened and the mitochondrial ATP synthase exhibit improved biochemical stability (25, 26, 69–71, 121, 122). Such is also the case of dimers of BLT1 (J.-L. Banères, personal communication). The improvement is usually limited when the surfactant concentration is close to the cmc, but it becomes obvious at higher concentrations (Figures 6 and 7d), suggesting that it is mostly a consequence of the lyophobicity of (H)FS micelles, which makes them a poor sink for lipids. BR and b₅ f are markedly stabilized in mono- and diglucosylated (H)FSs, but destabilized in triglucosylated ones (71). Although other hypotheses could be considered (see Reference 71), my favorite interpretation of the latter phenomenon is that too large a repulsion between polar heads, which favors the formation of particles with a small radius of curvature, may tend to either fragment and/or unfold MPs. The same phenomenon may well contribute to explaining the well-known destabilizing character of charged detergents. Because (H)FSs with a single glucose moiety form cylindrical micelles (Section 3.2) and polydisperse MP/(H)FS complexes (Section 5.3), and those with three glucose moieties are destabilizing, F- and HF-DiGlu (Figure 3b) were identified as optimal among chemically well-defined (H)FSs (71).

The relative benefits of using either per- or hemifluorinated compounds should be further investigated. Among the factors to be taken into consideration are the following: (a) HFSs are much harder to synthesize than FSs; as a consequence, they are more costly and available in more limited quantities; this limitation is of particular importance if (H)FSs are to be used for MP purification, where large volumes of...
solution above the cmc of the surfactant are required. (b) Initial observations indicating that FSs are much less efficient at preventing MPs from aggregating (25) than HFSs are (26) have not been supported, or not strongly so, by more recent experiments, where the difference appears marginal (71), perhaps because MP preparations used in more recent studies contained less lipids. (c) A difference has been noted between the spectra of monomeric BR trapped in either HFSs or FSs: In HFSs, the spectrum of the protein resembles that of the native protein;
in FSs, it is shifted to the red (“blue BR”) (71). The spectral change results from the protonation, owing to a pK shift, of Asp85, a residue that interacts electrostatically with the protonated Schiff base that associates the retinal to the protein (C. Breyton, unpublished results). This indicates that, directly or indirectly (e.g., via the lipids), FSs can affect the 3D structure of the MPs with which they are complexed. The fact that BR oligomers are not affected by this effect (71) suggests that perhaps this will not be a general concern but may simply reflect events occurring at one specific site at the surface of one particular protein.

6.3. Membrane Protein/Nanodisc Complexes

Because MPs trapped within NDs are not exposed to detergents, and because they are surrounded by an environment very similar to the natural one, they can be expected to exhibit improved stability. Few studies of this sort seem to have been reported. Rhodopsin, however, was indeed found to be much more resistant to thermal denaturation in NDs than in detergent solutions (Figure 7e) (44).

Functional studies are one of the most interesting applications of NDs, and they have been pushed much farther than with any of the other NCSs considered here. They will be discussed below along with other applications (Section 7.3).

7. APPLICATIONS

Owing to space limitation, it is not possible to review here at length every application that has been explored using each of the three systems under discussion. My purpose is, rather, to illustrate the kinds of studies that may be facilitated by using one or the other NCSs instead of detergents and to provide directions for further reading. I will also try, even though I am fully conscious of the risks inherent in such an exercise, to present my feelings about the prospects and constraints of each of them.

A first benefit of transferring MPs to NCSs is the stabilization they afford, which may permit the study, using otherwise classical biochemical or biophysical approaches, of MPs that would be intractable in detergent solution. What one has to consider here is whether using NCSs rather than detergents comes at a price and, if so, what price? In addition, NCSs may permit studies for which detergents cannot be used (e.g., mediating MP immobilization, defining the maximal size of the objects trapped, or inserting MPs into membranes under equilibrium conditions) or are poorly efficient (e.g., MP folding).

7.1. Constraints for Optical Spectroscopy

A8-35 (93), NAPols (89), and (H)FSs (B. Pucci, unpublished data) do not absorb light significantly at wavelengths above ~245 nm. None

Figure 7

Stabilization of membrane proteins by nonconventional surfactants. (a) Stabilization of the sarcoplasmic reticulum calcium ATPase by A8-35. The destabilization of the ATPase was initiated by diluting solubilized sarcoplasmic reticulum (SR) into an ethyleneglycol-Ο, Ο′-bis(2-aminoethyl)-N, N, N′, N′-tetraacetic acid-containing solution, thereby leaving the ATPase in a Ca2+-deprived, solubilized state, an environment known to lead to very rapid, irreversible inactivation. (Lower curve) The dilution medium contained 5 g.L−1 (9.3 mM) C12E8; (middle curve) same medium but with the addition of 5 g.L−1 A8-35; (upper curve) same medium as the latter sample, but incubation took place after a 250× dilution with surfactant-free buffer. From Reference 97. (b) Stabilization of BR by A8-35 and by nonionic amphipols (NAPols), as compared to 18 mM octylthioglucoside (OTG). From Reference 89. (c) Stabilization of the LTβ4 BLT1 receptor by A8-35 and by A8-35/lipid mixtures. (top) Stability upon incubation at increasing temperature; (bottom) stability upon extended storage at 4°C. From Reference 120. Abbreviations: D+L, detergent + lipids (fos-choline-16/asolectin, 2:1 w/w); AP, A8-35; AP+L, A8-35 + asolecint (5:1 w/w). (d) Stabilization of cytochrome b5 by (hemifluorinated surfactants as compared to detergents [dodecylmaltoside (DDM) and H-DiGlu]; H-DiGlu has the same chemical structure as F- and HF-DiGlu, except for a fully hydrogenated alkyl chain). From Reference 71. (e) Stabilization of rhodopsin (Rho) upon integration into nanodiscs (NDs) (in this case, nanoscale apolipoprotein bound bilayers) containing either one or two copies of the protein per disc, versus in rod outer segments (ROS; rhodopsin’s native membrane environment), DDM (29 mM) or octylglucoside (OG; 51 mM). From Reference 44.
of them fluoresces upon excitation at 280 nm (93; B. Pucci, unpublished data). A8-35 does not interfere with CD studies of proteins down to at least 180 nm (99, 103, 123, 124; T. Dahmane & F. Wien, unpublished data). (H)F-TACs are not expected to interfere significantly at these wavelengths. Glucosylated NAPols and (H)FSs, however, may be expected to contribute to CD spectra below ~195 nm, owing to the presence of the sugar residues (125). FTIR studies of MP/A8-35 complexes in the amide region are intractable because of the amide bonds of the polymer (Y. Gohon & E. Goormaghtigh, unpublished observations). The same difficulty will arise with sulfonated APols, NAPols, phosphorylcholine-based APols, and current (H)FSs.

NDs pose a special problem because they include scaffold proteins. It should be possible to reduce the contributions of MSPs by eliminating the tryptophan residues they contain using site-directed mutagenesis. The contribution of MSPs to IR and CD spectra will remain, but it can conceivably be subtracted.

### 7.2. Solution Studies of Membrane Protein Mass, Shape, and Interactions

All three NCS systems have been studied by SEC, AUC, SAXS, and/or SANS (see, e.g., References 71, 83, and 126). A priori, each of these techniques can be used to characterize NCS-complexed MPs. APols and (H)FSs have the advantages of adding less bulk and complexity to the system and of being more easily contrast matched. However, obtaining perfectly monodisperse preparations of MP/APol complexes is challenging, which makes molecular weight and \( R_g \) determination of the trapped MP by radiation scattering quite delicate (see Reference 83). Factors that have to be kept in mind when monodispersity is essential have been listed in Section 5.1.

APols do not interfere with most of the MP/ligand interactions that have been studied to date (92, 97, 98, 120) (see also Sections 7.9 & 7.10 below). This does not mean that one should not be aware of the possibility of such interferences (for discussions, see References 29 and 92). Thus, preliminary experiments with rhodopsin/A8-35 complexes suggested that A8-35 hinders the binding of transducin and arrestin (see Reference 29). This point has been reinvestigated recently with the leukotriene receptor BLT1. It was observed that receptor-catalyzed G protein activation was significantly slower with A8-35-trapped BLT1 than in detergent/lipid mixed micelles. In contrast, when folded in NAPols, BLT1 catalyzed GDP\( \rightarrow \)GTP exchange on the \( G_{\alpha_i} \) subunit with kinetic features similar to those in lipid/detergent mixtures (J.-L. Banéres, personal communication).

Particularly illustrative of the perspectives opened by the ND system are studies exploiting NDs to trap monomers or defined oligomers of various MPs. Some of the applications of this remarkable feature are described in Section 7.3.

Few detailed ligand-binding studies have been carried out in (H)FSs yet. The human Sonic Hedgehog receptor Patched was shown by surface plasmon resonance (SPR) to be able to interact with Sonic Hedgehog after complexation by FSs (127). The sensitivity of the mitochondrial ATP synthase to the inhibitors dicyclohexyl carbodiimide and oligomycin was found to be higher and to remain more stable over time in (H)F-TACs than it is in detergent solutions (122).

### 7.3. Functional Studies

As already mentioned, NDs provide a unique approach to examining the role of oligomerization in the biological function of MPs because they make it more straightforward and safer than any other system to prepare and handle monomers and various types of oligomers while limiting the risk that transient oligomerization complicates the interpretation of the experiments. In the case of GPCRs, NDs have been used to study the activation of G proteins by preparations of monomeric \( \beta_2 \)-AR receptor, rhodopsin, and \( \mu \)-opioid receptor (44, 114, 115, 117, 118, 128). Similarly, trapping of single copies of translocin in NDs made it possible to
establish that SecYEG monomers are able to bring about the dissociation of dimeric SecA into monomers and (pre)-activate the SecA ATPase (113). Trapping the Tar chemoreceptor as either dimers or trimers of dimers led to the demonstration that the formation of the dimer suffices for transmembrane signal transduction (methylation and deamidation), but the super-trimer is required for downstream signaling (activation of histidine kinase) (112). A vast body of work has been carried out on P450 cytochromes and their reductase (reviewed in References 39 and 40). They concern, in particular, the origin of cooperativity in ligand binding curves and the role of lipids and the transmembrane anchor of the cytochromes in P450/reductase interactions. NDs have also proven useful in studying the activation of enzymes involved in blood clotting and the role played in it by specific lipids (129, 130). There seems to be little doubt that NDs will henceforth provide a privileged tool whenever such questions will have to be dissected.

To date, applications of APols to investigations of MP function have mostly focused on sorting out whether perturbations observed upon MP solubilization are the result of removing the membrane environment or of direct interference by the detergent. In the case of the nAChR, it was shown that allosteric equilibria, which are perturbed upon solubilization, are similar in the postsynaptic membrane and after trapping with A8-35, indicating that it is the presence of the detergent, and not the disappearance of physical constraints imposed by the membrane, that is responsible for the perturbation (98). It was hypothesized that detergents might act by displacing lipids from critical sites at the surface of the nAChR's transmembrane domain, where A8-35 would let them rebind. BR, a light-driven proton pump, undergoes severe perturbations of its photocycle upon solubilization by detergents. One such perturbation, the acceleration of the retinal's Schiff base deprotonation upon capture of a photon, largely persists after transfer to APols, suggesting that the conformational change that underlies this phenomenon results from the extraction of BR from the purple membrane rather than from detergent binding (83). The end of the cycle, when BR returns to its ground state, goes back to close to membrane-like features upon transfer from detergent solution to APols (83). Comparative studies of the photocycle of native BR, trapped in APols along with purple membrane lipids, and of BR refolded in APols in the presence or absence of lipids have led to the conclusion that it is probably the rebinding of lipids at critical sites at the surface of the protein upon transfer from detergent to APols that accounts for the recovery of normal kinetics in the last part of the photocycle (99).

7.4. Proteomics: Isoelectrofocusing, Two-Dimensional Gels, and Mass Spectrometry

Analysis of protein mixtures on two-dimensional (2D) gels usually starts with a separation by IEF followed by electrophoresis in polyacrylamide gels in the presence of sodium dodecylsulfate (SDS-PAGE) in a perpendicular direction. IEF of MPs using detergents is notoriously difficult (131). Most APols, which bear net charges, are not compatible with IEF, but NAPols are. The resolution of IEF observed with NAPols is similar to that in neutral detergents (89, 132). Whether the use of NAPols can improve on the yields obtained with detergents remains to be ascertained. However, they are certainly of potential interest as a tool to separate and analyze fragile MP complexes that do not resist purification in detergent solutions. (H)FSs could conceivably be used to the same end but are probably at more risk of raising aggregation problems. FASBs (see Section 3.2) have been used in 2D electrophoresis as a means to detach nonmembrane proteins from erythrocyte membranes prior to solubilizing MPs with a classical detergent (74). Separating MP/ND complexes by IEF may seem a priori an odd proposition because MSPs carry charges. However, provided a constant stoichiometry with the target MPs is maintained and the lipids are neutral, this should result in a simple
shift of the isoelectric point of the complex as compared to that of the MP alone, without necessarily compromising the resolution.

In keeping with their displacement by non-ionic detergents (77, 93, 109), APols are washed away by an excess of SDS. This is reflected in the fact that, upon SDS-PAGE, MPs migrate at the same position whether they were initially complexed by A8-35 or not (123) and by the absence of a FRET signal and lack of any CD change when fluorescent A8-35 is added to an MP in SDS solution as a prelude to renaturation (89). In the second dimension of 2D gels, MPs, therefore, separate as they do in the absence of APols, and they can be analyzed by mass spectrometry under the same conditions (132).

Recent data indicate that native MPs complexed by either A8-35 or NAPols can be analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, with the APols remaining undetected (95; C. Béchara, G. Bolbach, & S. Sagan, unpublished data).

7.5. Nuclear Magnetic Resonance Studies

APol-trapped MPs have been studied by solution NMR (94, 95, 108; M. Renault & A. Milon, personal communication). All published studies to date have been carried out using A8-35, and they all bear on β-barrel proteins. At this point, the objectives have been mainly to work out the technology and to obtain information about MP/APol complexes (size, folding state of the protein, protein/APol interactions, and protein dynamics). Not surprisingly, these studies have shown that the three proteins studied to date, E. coli’s tOmpA (94) and OmpX (95, 108) and K. pneumoniae’s KpOmpA (M. Renault & A. Milon, unpublished data), are correctly folded in A8-35 (Figure 8a). Early studies with E. coli’s tOmpA were carried out in the absence of (ethylenedinitrilo)tetraacetic acid (EDTA) (94). In the presence of EDTA, the resolution of TROSY-HSQC spectra improves, even though the complexes still tumble more slowly than in DHPC solutions: For OmpX at 30°C, τ_c = 31 ns versus 23 ns (108); for KpOmpA at 40°C, τ_c = 30 ns versus 26 ns (M. Renault & A. Milon, unpublished data). For OmpX, the resulting broadening of the peaks in the

![Figure 8](image_url)

**Figure 8**
Solution NMR spectra of APol- versus detergent-complexed OmpX.

(a) Two-dimensional [15N,1H]-TROSY spectra of [u-2H,13C,15N]OmpX complexed by APol A8-35 (left; pH 8.0) or by DHPC (right; pH 6.8) collected at 30°C at 700 MHz. (b) A comparison of peak width in the direct dimension. From Reference 108.
$^1$H dimension is $\sim 35\%$ as compared to their width in DHPC solutions (Figure 8b) (108). Solution NMR has been used to map the contacts between the protein and the lateral chains of the polymer, based on amide $^1$H peak broadening in hydrogenated versus deuteriated A8-35 (94), or on heteronuclear $^{13}$C-$^1$H (95), or $^{15}$N-edited $^1$H-$^1$H (M. Renault & A. Milon, unpublished data) nuclear Overhauser effects (NOEs). In all three cases, A8-35 was found to interact exclusively with the transmembrane surface of the protein. No contacts are observed between KpOmpA and the APol’s main chain (M. Renault & A. Milon, unpublished data). Whether contacts with the APol are homogeneously distributed over the protein’s transmembrane surface and whether the polymer’s octyl chains interact with it in the same way as detergent chains do are still relatively open questions (95; M. Renault & A. Milon, unpublished data). Studies of H/D exchange at amide bonds show that the dynamics and accessibility of OmpX’s (108) and KpOmpA’s (M. Renault & A. Milon, unpublished data) transmembrane $\beta$-strands are similar in DHPC and after trapping with A8-35. It is possible to determine, by transferred NOE studies, the structure of ligands bound to large APol-stabilized MPs (L. Catoire, unpublished observations).

In addition to the slower tumbling rate, an inconvenience that is likely to become less and less relevant as larger MPs are tackled and the technology improves, the major drawback of A8-35 in solution NMR studies is that it does not allow one to work at the slightly acidic pH at which the exchange of amide protons with the solution is slowest. This is of little import for transmembrane amide protons, which exchange slowly anyway, but it becomes critical for water-exposed protons in the extramembrane loops. This has prompted the development of alternative, pH-insensitive APols (Section 3.3). Recent data indicate that TROSY-HSQC spectra can be recorded of $\tau$OmpA trapped with sulfonated APols. At pH 6.8, it becomes possible to resolve loop amide protons that are not seen at pH 8.0. Encouraging data have been obtained recently with NAPols (L. Catoire, personal communication). It is to be expected that further progress in the chemistry of APols and their implementation will soon make it possible to establish by solution NMR the structure of MPs that are too unstable to be studied in detergent solutions.

Solution NMR studies in (H)FS solutions have not been attempted yet. A potential pitfall may be aggregation of the protein at the high concentrations required by NMR, but the attempt is certainly worthwhile. $^{19}$F NMR potentially offers some original angles, e.g., to follow the distribution of (H)FSs whether in vitro or in vivo.

Solution NMR of MP/ND complexes is handicapped by the large size of NDs and possible anisotropy problems. Theoretical calculations indicate that the tumbling rate of an empty ND should be comparable to that of an $\sim 200$-kDa protein (133). In practice, the resolution obtained for a 13-residue peptide adsorbed onto NDs, although not as good as in bicelles, was somewhat better than expected, probably owing to the peptide moving with respect to the surface of the ND. Signal attenuation by a spin label dissolved in the lipid phase yielded information about the secondary structure of the peptide and its arrangement with respect to the ND surface (133). Similar results were obtained in the study of a transmembrane fragment of human CD4, in which the signals observed were thought to originate from flexible, solvent-exposed regions (134). Much more complete data were obtained recently using perdeuterated VDAC-1 (135). The rotational correlation time, $\tau_c$, of VDAC-1 in NDs was estimated to be $\sim 93$ ns, only slightly longer than the value of $\sim 70$ ns observed in LDAO micelles. As larger and larger MPs are being studied, the extra bulk added by the MSPs and lipids ought to become less and less of a handicap. One may also note that, whereas the slow tumbling rate of MP/ND complexes complicates structural studies of the protein itself, it would not prevent, for instance, studying the

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structure of bound ligands by transferred NOE measurements.

ssNMR is well adapted to the study of large objects. It has been used, as mentioned above (Section 3.1), to study the structure of MSPs in NDs that had been precipitated using polyethylene glycol (50). Upon precipitation of NDs containing the human P450 cytochrome 3A4 (CYP3A4), the protein retained its ability to bind one of its ligands, bromocryptine. 13C chemical shifts were consistent with the known X-ray structure of CYP3A4, illustrating the feasibility of studying by ssNMR the structure of ND-embedded MPs (136).

ssNMR of MP/APol complexes has not been attempted yet. It should be feasible, given that the complexes can be precipitated or frozen and, at least in some cases, lyophilized without denaturing the protein (Section 5.1). However, a great advantage of ssNMR is that it gives access to the protein in its natural environment, which is maintained in lipid bilayers and, to a large extent, in NDs and in bicelles, but not in APols. APols could, however, be useful, e.g., to study the structure of bound ligands or of small isotopically labeled subunits integrated into large unlabeled complexes, which current NDs would not be able to trap.

7.6. Crystallization

A8-35, the best-characterized APol, is not a priori an excellent candidate for forming 3D crystals of MP/APol complexes because of the high density of charges it carries (no structure of MP has ever been solved using a charged detergent). Indeed, attempts at crystallizing cytochrome bc1/A8-35 complexes did not yield any crystals (D. Charvolin, unpublished data). Some poorly diffracting crystals were obtained in preliminary studies of ternary bc1/A8-35/detergent complexes (D. Charvolin, A.-N. Galatanu, & M. Picard, unpublished data). The color and space group of these crystals, as well as their fluorescence when they were prepared using a fluorescent APol, indicated that they contained the protein, the polymer, and, not shown directly but necessarily (see Section 5.1), the detergent. It has not yet been possible, unfortunately, to determine whether the poor quality of the diffraction patterns observed (~20-Å resolution) is inherent to this type of crystals or results from their handling, from the freezing step, or, simply, from too few crystals having been examined. Why crystals were obtained in ternary mixtures and not with pure APols can have several origins. One is that diluting A8-35 with detergent will diminish the charge density of the surfactant belt surrounding the protein as well as facilitate charge redistribution in the crystal. Another may be a more homogeneous size of the complexes, as suggested by observations on tOmpA/A8-35 versus tOmpA/A8-35/C8E4 complexes (93) (Section 5.1). Crystallization attempts with NAPols may be expected to have greater chances of success. For the time being, APols cannot be advocated as a favorable medium for MP crystallization. However, because even mixtures of APols and detergent can have a stabilizing effect on MPs (Section 6.1), supplementing an MP in detergent solution with APols, used as an additive, may perhaps be attempted when protein instability is suspected to be the reason for the failure to grow good-quality crystals in pure detergent solution.

Attempts to grow 3D crystals from solutions of MPs in (H)FSs should preferably be carried out using chemically defined molecules, which have become available only very recently (71, 72). One may speculate that, because their affinity for MP transmembrane surfaces is likely to be relatively low, (H)FSs may favor the growth of type I crystals, which are made of stacks of 2D crystals.

Detergents do not displace or dissolve monolayers of (H)FSs, which are stronger surfactants and do not mix well with them. Nickel-bearing (H)FSs have been synthesized (137, 138). They can be used, as nickel-bearing fluorinated lipids have been in the past (139), to adsorb detergent-solubilized polyhistidine-tagged MPs at the air-water interface. Thus, His-tagged tOmpA/C8E4 complexes can diffuse within the aqueous space separating the two F-TAC monolayers of a Newton black
film, where they form, depending on the conditions, one- or two-molecule thick layers, whose structure has been studied by X-ray reflectivity measurements (137).

HFS monolayers containing Phenyl-HF-NTANi (Figure 3c) may hold interesting promises for forming 2D MP crystals (138). To date, HFS monolayers have been shown to allow adsorption and orientation of the sulfonamide receptor, whose projection structure was then solved by single-particle EM imaging (C. Vénien-Bryan, unpublished data).

Solving MP structures by X-ray analysis of 3D crystals of MP/ND complexes would depend, in most cases, on the target MP locking itself in perfect register with the MSPs, which, although it could conceivably be engineered, may be a rare circumstance.

7.7. Single-Particle Electron Microscopy and Atomic Force Microscopy

As described in Section 3.1, empty NDs have been studied by EM and AFM. NDs can be used for single-particle analyses of embedded MPs, with the limitation that they will not be able to trap MPs with very large transmembrane domains. This is a handicap for EM single-particle studies, which work best on large objects. Upon exposure to a mica surface, NDs, whether or not they harbor an MP, will tend to lie flat on the surface, which greatly facilitates AFM studies of their structure and that of the MP. AFM, for instance, has been used to measure the extension of CYP2B4 and the cytochrome P450 reductase in a direction normal to the plane of the disc (Reference 41 and references therein). Single-particle EM imaging, coupled with the use of antibody Fab fragments, has shown that, in NDs containing two rhodopsin molecules, the two proteins adopt indifferently parallel or antiparallel orientations with respect to each other (44).

APols and (H)FSs have the potential to trap and stabilize very large MP complexes, which may facilitate their purification, the identification of their components, and the study of their structure by single particle EM and other approaches. This application, which seems both promising and relatively straightforward, has been underexploited. In an early study, scanning transmission EM, which is mostly useful to map the distribution of masses rather than for imaging, had been used to show that trapping with A8-35 captured the distribution of cytochrome b6f dimers and monomers that preexisted in the original detergent solution (140). To date, four single-particle EM studies have been published, two studies of the A8-75-trapped proton ATP synthase (141, 142), a negative-stain study of A8-35-trapped BR and of the curious filaments these complexes form upon elimination of free APol (83), and a low-resolution cryo-EM study of A8-35-trapped mitochondrial complex I (143). As already mentioned, A8–35 has been used to trap and purify under a functional form mitochondrial supercomplex B, which comprises complexes I, III, and IV in a 1:2:1 molar ratio. The complexes were imaged both after negative staining and by cryo-EM (Figure 9); image reconstruction is in progress (T. Althoff & W. Kühbrandt, personal communication).

No EM studies of MPs stabilized by (H)FSs have been reported yet.

7.8. Delivering Membrane Proteins to Preformed Lipid Bilayers

As mentioned above, APols and (H)FSs, under most conditions, will not dissolve lipid membranes, and they are not cytolytic (Sections 5.1 and 5.3). They can, therefore, be used to deliver MPs to lipid vesicles, planar bilayers, or cells without destroying them. This useful property has permitted integration of APol-trapped diacylglycerol kinase into lipid vesicles (80), of A8-35-trapped porins into lipid black films (123), of the mechanosensitive channel MscL, kept soluble by (H)F-TAC, into lipid vesicles (59), and of APol-trapped mimics of transmembrane peptides into living cells (G. Crémel, personal communication). In such studies, however, two caveats have to be kept in mind: (a) fragile MPs risk being denatured in the process (BR cannot
be directly transferred from (H)FSs to lipid vesicles without denaturation) (C. Breyton, unpublished observation); and (b) whereas (H)FSs can be washed away, as they will equilibrate between the solution and the membrane, APols will remain associated with the target membrane, which may have undesirable consequences (see Reference 123).

The unusual situation of being able to keep a hydrophobic protein soluble, thanks to a surfactant, without lysing the target membrane has been exploited in a series of studies of the thermodynamics of insertion of the T domain of diphteria toxin into membranes following a pH drop (Figure 10) (58, 61, 144). Protein/surfactant interactions were examined by FRET thanks to Oregon Green–labeled (H)F-TACs (Figure 3d) (61).

Transfer of an MP from an MP/ND complex to a preformed membrane, an intriguing possibility, has not been reported yet. The absence of transfer, however, would make it feasible to study the interaction of a membrane-embedded MP with an ND-trapped one, or of two ND-trapped MPs with one another, while the two proteins are kept in distinct bilayers. This would provide a novel way to study interactions between MPs that in vivo belong to distinct membranes, as occurs, for instance, in processes involving membrane adhesion or fusion.

7.9. Immobilizing Membrane Proteins onto Solid Supports

Surface-based in vitro assays of ligand binding to proteins present multiple advantages, especially their adaptability to the use of minimal amounts of proteins and reagents and to multiplexing and high-throughput screening. Immobilizing an MP without modifying it or subjecting it to nonspecific, potentially denaturing interactions is, however, a challenge. Because the association of APols with MPs resists extensive washing with surfactant-free buffer (see Section 5.1), trapping an MP with a functionalized APol results in the permanent association with the protein of any functional group carried by the APol (93). Tagged APols thus can be used to immobilize MPs onto solid supports (92). The ligand-binding properties of the immobilized proteins can then be studied in detergent-free solutions, e.g., by SPR or fluorescence.
Using (H)FSs to study the thermodynamics of protein insertion into membranes. The pore-forming T domain of diphteria toxin, which is water soluble at pH 7.0 (I), changes conformation as the consequence of a pH drop, which renders it hydrophobic (II). In the absence of surfactants, a competition engages between membrane insertion (III, pathway 1) and nonproductive aggregation (IIa). HF-TAC keeps the T domain soluble and monomeric without solubilizing the target membrane, at variance with what a conventional detergent would do. This makes it possible to study the thermodynamics of insertion under equilibrium conditions (see Section 7.8). From Reference 58.

Techniques, opening the way to applications in diagnostics, drug discovery, or the search for natural biological partners (Figure 11). The procedure is universal in the sense that any MP is a priori amenable to it. It is also mild, because the protein itself is not involved in any interaction with the support, and highly versatile.

NDs can adsorb onto solid supports, either spontaneously or via tagged lipids, which can be used to create patterned bilayer surfaces (145, 146). His-tagged scaffolding proteins were used to form NDs incorporating rhodopsin, which were immobilized onto a nickel-bearing support, and SPR and a modified version of MALDI-TOF mass spectrometry were used to demonstrate transducin binding upon exposure to light (147). Similarly, His-tagged NDs were used to capture glycolipid GM1, the membrane receptor for cholera toxin. After immobilization of the complexes onto Ni-NTA chips, the kinetics of the interaction of the soluble toxin with the immobilized lipid was studied by SPR (148). Recent studies have described the screening of ligands of immobilized ND-trapped MPs by SPR (149) or NMR (150).

7.10. Folding Membrane Proteins from a Denatured State

Folding MPs to their native state in vitro provides precious insights into the respective roles of the amino acid sequence, the insertion machinery, and the membrane environment in determining the 3D fold adopted by the polypeptide. From a practical point of view, overexpressing MPs as inclusion bodies and folding them in vitro may allow production of large amounts of naturally rare MPs, which is often difficult to achieve when trying to express them directly in a functional state. Mild surfactants, such as APols and (H)FSs, may a priori provide favorable media for MPs to fold because they are expected to interfere less than detergents...
Using functionalized amphipols to attach membrane proteins onto solid supports and detect ligand binding to them. (top) Principle. A membrane protein (MP) in detergent solution is trapped with a biotinylated amphipol (BAPol). It is then attached via the biotin groups to a streptavidin-coated chip. Ligands are flushed over it in plain, surfactant-free buffers, and their binding is detected by any of various methods.

(bottom) Validation. Channels of a streptavidin-coated chip were exposed either to pure BAPol or to complexes of BAPol with any of four different MPs: tOmpA, BR, and cytochromes b$_6$f and bc$_1$. The five channels were then flushed with buffer containing either preimmune antibodies or antibodies raised against each of the four MPs. Antibody binding, measured in response units (RUs) by surface plasmon resonance, is protein specific. Adapted from Reference 92.

The use of (H)FSs to fold MPs has not been extensively investigated yet. Preliminary tests show that BR can be refolded with more than 50% yield in the presence of either F- or HF-TAC (60). tOmpA could be refolded into (H)FSs as well, albeit to a lesser extent than when using conventional detergents; HF-TAC was more efficient than F-TAC (60). The usefulness of (H)FSs for MP folding clearly deserves further investigation.

Refolding MPs into NDs is a more complex proposition because it will generally call for simultaneous refolding of the target MP and the scaffolding proteins as well as their reassembly with the lipids. Nevertheless, it may be worth...
Figure 12
Amphipol-assisted folding of four G protein–coupled receptors (GPCRs) expressed in an inactive form in inclusion bodies. The BLT1 leukotriene receptor (left) and the 5-HT_{4(a)} serotonin receptor, the BLT2 leukotriene receptor, and the CB1 cannabinoid receptor (right), all of them class A GPCRs, were expressed in inclusion bodies and purified in an inactive form in sodium dodecyl sulfate (SDS) solution. They were folded by substituting SDS either with a lipid/detergent mixture (D+L), with pure A8-35 (A) at different protein/APol mass ratios (BLT1), with A8-35 (A) at a 1:5 mass ratio (BLT2, 5-HT_{4(a)} and CB1), or with A8-35 supplemented with asolectin in a 1:5:1 protein/APol/asolectin mass ratio (A+L). The extent of correct folding is expressed as the percentage of total receptor (on the basis of the protein concentration in the SDS solution) that is able to bind a specific ligand. Adapted from Reference 120.

examining whether NDs would not reform, for instance, from a mixture of their constituents in SDS solution, in which case MP folding experiments might be attempted.

7.11. Expressing Membrane Proteins in Cell-Free Systems
Cell-free expression of MPs in a lysate containing the transcription and translation machineries offers highly interesting opportunities, e.g., for expressing MPs that are toxic to cells as well as for specific labeling. Traditionally, MPs are expressed in vitro in the presence of a detergent, of lipid vesicles, or in the absence of any surfactant at all. In the latter case, MPs precipitate and are later solubilized using a detergent. Detergents have the drawback of being inactivating, and lipids have the disadvantage of offering limited yields. There is probably an advantage in the use of NCSs as less-aggressive environments, in which neosynthesized MPs should stand a better chance to correctly fold. All three systems can be used to this end. (H)F-TACs have been used to synthesize the mechanosensitive channel MscL (59). After synthesis, MscL was purified in solutions of the same surfactants and directly applied to liposomes, into which it spontaneously integrated. Its activity was then characterized by patch-clamp measurements (59). MscL synthesis has also been achieved using the new, chemically defined (H)FSs (71). NDs have been employed to synthesize under their functional form BR and the multidrug-resistance protein EmrE, as well as to express a large panel of other MPs (151–153). APols initially yielded disappointing results. Indeed, both A8-35 and sulfonated APols were found to inhibit the transcription-translation machinery (E. Billon-Denis & F. Zito, unpublished observations). More recently, however, it was observed that NAPols do not block protein synthesis and provide excellent yields of expression for several MPs (89; E. Billon-Denis, P. Bazzacco, & F. Zito, unpublished observations). BR expressed in the presence of NAPols is both functional and soluble.

These observations are potentially of great interest. Given that most MPs are more or less unstable in detergent solutions and that folding yields in detergent or detergent/lipid mixtures are generally low, it is to be expected that many, if not most, MPs, if exposed to detergents in...
### Table 1  Opportunities and constraints associated with the use of amphipols, nanodiscs, and fluorinated surfactants

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<th>Technology</th>
<th>Amphipols</th>
<th>Nanodiscs</th>
<th>Fluorinated surfactants</th>
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*a, ±, and – signs refer to how promising or problematical each application to MP studies looks: +, promising; ±, promising, but with limitations or difficulties; –, not promising if not plainly impossible.*

*bFor those applications that have actually been tested, cells have been colored as follows: green, tested with success; yellow, shown to work, but with some caveats or limitations; pink, problematical if not impossible. A white background indicates that the +/- signs represent what appears to be reasonable expectations, but that these assessments are not currently backed up by actual data.*

*cIn the case of nanodiscs, the table refers to the study of nanodisc-embedded MPs, not to that of rim proteins.*

*dSection of this article where this is discussed.*

Abbreviations: AFM, atomic force microscopy; AUC, analytical ultracentrifugation; EM, electron microscopy; SANS, small-angle neutron scattering; SAXS, small-angle X-ray scattering; SEC, size exclusion chromatography; UV, ultraviolet.

the course of their synthesis, will not efficiently achieve their native 3D fold. NCSs may therefore offer an extremely attractive alternative for cell-free MP synthesis.

### 8. CONCLUSION: OPPORTUNITIES VERSUS CONSTRAINTS

It is my hope that the present article, which, unfortunately, had to limit itself to three among many more innovative systems, will convince membrane protein biochemists and biophysicists that the time is over when MPs could be handled in vitro only in a detergent solution or in a membrane-bound form. New tools have been developed and validated—painstakingly! These tools can at least partially circumvent the instability and other problems encountered with detergents. They can also provide totally new experimental opportunities. In Table 1, I have summarized my personal view of the usefulness of the three NCS systems discussed here as well as the constraints and difficulties with which each is associated. Needless to say, these prospects will evolve as improved molecules are developed and applications are more thoroughly explored.
As should be evident from the present review, each type of NCS has its own privileged applications. Although some uses, such as MP folding or in vitro synthesis, can probably be advantageously developed in parallel with the three of them, specialization will occur. One may speculate that, ten years from now, membrane biochemists and biophysicists may be in the habit of resorting to one or the other NCS depending on which particular problem they have at hand.

### SUMMARY POINTS

1. Membrane proteins (MPs) can be handled in detergent-free aqueous solutions as individual, well-defined complexes after being transferred to nondetergent surfactants, including amphipols (APols), nanodiscs (NDs), or fluorinated surfactants (FSs).

2. Transfer is usually achieved by first solubilizing the target protein with a detergent and then replacing the detergent with any of these three media.

3. Transferring MPs to any of these three environments generally stabilizes them as compared to detergent solutions.

4. Some applications, such as cell-free synthesis of MPs, the study of fragile MP complexes, or single-particle imaging, have been shown or appear likely to be common to all three systems. Others are best performed using one or the other of them. The development of many applications, however, is still in its infancy.

5. NDs have the unique characteristic of featuring a well-defined area into which only MPs whose transmembrane domain does not exceed a defined size can be trapped in a membrane-like environment. This makes them exceptional tools to sort out functional issues related to MP oligomerization and interactions with lipids. Other applications whose development has started include electron microscopy, atomic force microscopy, solution and solid-state NMR, and MP immobilization.

6. Among applications for which APols appear particularly promising are MP folding, solution NMR, the study of large MP complexes, and immobilization of MPs onto solid supports.

7. Applications of FSs are at a less developed stage, but among those that may be particularly useful are the study of fragile complexes and the delivery of MPs to preexisting membranes.

8. All three systems have by now been studied extensively enough to be used for the exploration of biological systems whenever detergents are unsuitable.

### FUTURE ISSUES

1. One can expect that future developments will take, for each of the three systems that have been discussed here, two main paths: improving the tools and exploring applications.

2. Regarding the development of applications, several suggestions have been evoked in Section 7 of the review. Exploring higher and less-permanent levels of organization than that of the single macromolecule is one of the frontiers of today’s biology. Means to preserve fragile or transient interactions between MPs better than is possible using detergents, which nonconventional surfactants provide, are, therefore, particularly timely.
3. Regarding the development of the tools, the resources of genetic engineering will improve and diversify the proteins used to stabilize NDs, which will permit them to encapsulate larger MPs or to more precisely fix the size of the discs, to tailor their spectroscopic properties to the needs of biophysicists, or to functionalize them.

4. Functionalization is also a key to developing original applications of APols. The new APol generations, and in particular nonionic APols, will have to be as thoroughly validated as first-generation molecules have been, and optimized and functionalized in view of their specific uses.

5. Whether developing chemically well-defined APols—a difficult challenge to chemists—is worth the long effort required by such an attempt remains to date an open question. Developing and validating applications is probably more urgent.

6. Fluorinated surfactants have been brought to the stage where chemically defined molecules are, at long last, available. Much effort is still necessary, however, to more fully explore their specific applications.

7. Finally, one should not forget that the three systems analyzed here do not cover the whole range of approaches that have been explored to date and that novel ones will no doubt emerge.

**DISCLOSURE STATEMENT**

The author is coinventor on several granted or pending patents on amphipols and their uses.

**ACKNOWLEDGMENTS**

Particular thanks are due to T. Althoff, J.-L. Banères, S. Banerjee, P. Bazzacco, C. Béchara, E. Billon-Denis, G. Bolbach, J. Borch, C. Breyton, L. Catoire, P. Champeil, D. Charvolin, G. Crémel, T. Dahmane, F. Giusti, Y. Gohon, E. Goormaghtigh, J.-C. Guillemot, T. Hamann, A. Kriško, W. Kühnbrandt, A. Ladokhin, C. Le Bon, M. le Maire, K.L. Martinez, A. Milon, B.L. Møller, E. Pebay-Peyroula, M. Picard, Y. Pierre, R. Prassl, B. Pucci, M. Renault, C.M. Rienstra, J.N. Sachs, S. Sagan, T.P. Sakmar, C.R. Sanders, S.G. Sligar, R. Sunahara, C.G. Tate, C. Tribet, C. Vénien-Bryan, F. Wien, F. Winnik, F. Zito, and M. Zoonens for communication and permission to quote unpublished information and/or to reproduce published or unpublished figures, for help with the figures or the bibliography, and for comments on the manuscript. I am deeply indebted to J. Barra for her help with preparing the figures and with collecting the bibliography and to M.E. Dumont for his heroic attempts at improving the English wording. My own work has been mainly supported by the CNRS, the Human Frontier Science Program Organization (RG00223/2000-M), and the European Community (BIO4-CT98-0269 and STREP LSHG-CT-2005-513770 Innovative Tools for Membrane Protein Structural Proteomics).

This review is dedicated to the memory of my father, whose courage and thoroughness have been an inspiration for me.

**LITERATURE CITED**


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