Dynamics of Membrane Protein/Amphipol Association Studied by Förster Resonance Energy Transfer: Implications for in Vitro Studies of Amphipol-Stabilized Membrane Proteins[†]

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ABSTRACT: Amphipols (APols) are short amphipathic polymers that can substitute for detergents to keep membrane proteins (MPs) water-soluble while stabilizing them biochemically. We have examined the factors that determine the size and dispersity of MP/APol complexes and studied the dynamics of the association, taking as a model system the transmembrane domain of *Escherichia coli* outer membrane protein A (tOmpA) trapped by A8-35, a polyacrylate-based APol. Molecular sieving indicates that the solution properties of the APol largely determine those of tOmpA/APol complexes. Achieving monodispersity depends on using amphipols that themselves form monodisperse particles, on working in neutral or basic solutions, and on the presence of free APols. In order to investigate the role of the latter, a fluorescently labeled version of A8-35 has been synthesized. Förster resonance energy transfer measurements show that extensive dilution of tOmpA/A8-35 particles into an APol-free medium does not entail any detectable desorption of A8-35, even after extended periods of time (hours−days). The fluorescent APol, on the other hand, readily exchanges for other surfactants, be they detergent or unlabeled APol. These findings are discussed in the contexts of sample optimization for MP solution studies and of APol-mediated MP functionalization.

Membrane proteins (MPs)[1] perform numerous fundamental cell functions, including energy transduction, import and export of nutriments and drugs, assessment of and response to environmental conditions, cell-to-cell communication, etc. In vitro studies and, in particular, structural investigations often require monodisperse solutions of MPs in aqueous media. This raises difficult problems because the strongly hydrophobic transmembrane surface of MPs makes them insoluble in water. Detergents are generally used to provide an interface between this surface and aqueous solutions (4−6). Because detergents are dissociating surfactants, they tend, however, to destabilize MPs (see, for example, refs 7−9). This has prompted the development of alternative, less aggressive surfactants (reviewed in refs 10, 11), among which are a novel family of molecules dubbed "amphipols" (APols) (ref 1; reviewed in refs 12 and 13). APols are short amphiphilic polymers carrying multiple hydrophobic chains. They were thus designed so that they would bind to the transmembrane surface of MPs by multiple attachment points, which was expected to drastically decrease their dissociation rate and make their noncovalent association with the protein virtually irreversible. This should make it possible, in principle, to handle MP/APol complexes in the absence of free surfactant.

The detergency of APols is extremely weak and, in general, they do not efficiently extract MPs from cell membranes (1, 12). MPs solubilized with conventional detergents, however, can be transferred to APols, which keep them water-soluble, in their native state, and, as a rule, much more stable than they are in detergent solution (see, for example, refs 1, 12, and 14−17). MP/APol complexes can be fractionated by size-exclusion chromatography (SEC) or centrifugation in sucrose gradients, upon which they behave as soluble proteins, that is, they do not precipitate in surfactant-free media (1, 14, 17−20). APols thus have the potential to make amenable to in vitro studies MPs that are

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[1] Abbreviations: A8-35, a specific type of amphipol (1); APol, amphipol; AUC, analytical ultracentrifugation; BR, bacteriorhodopsin; C_8E_4, tetraethylene glycol monooctyl ether; $C_{12}E_8$, octaethylene glycol monododecyl ether; DAPol, A8-35 with perdeuteriated octyl and isopropyl chains (2, 3); DDM, dodecyl β-D-maltoside; DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; FAPol, fluorescent (NBD-labeled) amphipol A8-35; FRET, Förster resonance energy transfer; HHFW, half-height at full width; IMAC, immobilized metal affinity chromatography; MP, membrane protein; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; SANS and SAXS, small-angle neutron and X-ray scattering, respectively; SEC, size-exclusion chromatography; tOmpA, transmembrane domain of outer membrane protein A, including residues 1−171 and mutations F23L/Q34K/K107Y; tOmpA-His$_8$, tOmpA bearing an eight-histidine *N*-terminal tag; UAPol, nonfluorescent precursor of fluorescent amphipol.

too unstable to be studied in detergent solutions or whose function is perturbed by detergents. Another important asset of APols is the versatility of their chemistry, which makes it relatively straightforward to produce labeled or functionalized molecules (see, for example, refs *2*, *3*, *17*, *18*, *21*, and *22* and this work).

APols have multiple applications, ranging from solution studies of detergent-sensitive MPs (see, for example, refs *14*, *15*, and *19*) to electron microscopy (*23*, *24*), solution NMR (*3*, *25*), small-angle X-ray or neutron scattering (SAXS and SANS; refs *17* and *20*), MP renaturation (*26*), delivery of MPs to preformed lipid bilayers (*26*, *27*), or studies of ligand binding to immobilized MPs in surfactant-free solutions (*22*). Many biophysical applications, however, such as crystallography, solution NMR, SAXS, SANS, and, to some extent, electron microscopy, require particles to be monodisperse. In the case of solution NMR, it is, in addition, of great importance that they be as small as possible, since their rotational correlation time, τ_c, directly affects the resolution of the spectra. Identifying factors that control the dispersity and size of complexes between MPs and APols and understanding the dynamics of their interactions are the subjects of this work.

Previous studies of the solution behavior of MP/APol complexes have been carried out with bacteriorhodopsin (BR) as a model MP (*17*, *20*). These analyses in fact dealt with ternary complexes, because of the tendency of BR to retain lipids upon solubilization of the purple membrane with detergents. In addition, the partial quenching of BR tryptophans by its cofactor, retinal, increases the complexity of Förster resonance energy transfer (FRET) studies, which are the main tool used in this work. As an alternative, simpler system, we have chosen as a model MP the transmembrane domain of outer membrane protein A from *Escherichia coli* (tOmpA), which can be produced as inclusion bodies and refolded in detergent solution in the complete absence of lipids (*28*). tOmpA is a small (~19 kDa), rugged MP folded into an eight-strand β-barrel, whose structure has been studied by X-ray crystallography and NMR as complexes with detergents (*29–34*) or APols (*3*, *25*).

A8-35, the best characterized and most widely used APol to date, is composed of a polyacrylate backbone derivatized with octylamine and isopropylamine (*1*). In aqueous solutions, it assembles into well-defined micelle-like particles comprising, on average, ~4 molecules, with an aggregate molecular mass of ~40 kDa (*35*). NMR studies of tOmpA/A8-35 complexes have shown that the polymer associates exclusively with the transmembrane domain of the protein (*3*). Carrying out NMR studies required a careful optimization of the samples and, in particular, a search for conditions that would improve the monodispersity and diminish the size of the particles. As described here, a number of critical factors were identified, one of them the presence of free APols. This led us to reexamine our earlier conclusion that APols do not detectably desorb from MPs in the absence of a competing surfactant (*12*, *18*). To this end, we have synthesized a fluorescent APol (hereafter, FAPol) by grafting 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD) onto an unlabeled A8-35 analogue carrying a reactive arm (UAPol). FRET measurements of the rate of dissociation of FAPol from tOmpA, in the presence or absence of competing surfactants, offer new insights into the behavior of aqueous solutions of

MP/APol complexes, directly relevant to their use in biochemical or biophysical investigations.

EXPERIMENTAL PROCEDURES

Materials. Benzyl chloroformate, sodium azide (NaN$_3$), dicyclohexylcarbodiimide (DCC), ammonium formate, 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), octylamine, triethylamine (TEA) and isopropylamine were purchased from Aldrich; sodium methoxide (MeONa), poly(acrylic acid) (PAA) ($\langle M \rangle \approx 5.5$ kDa), palladium on activated charcoal (Pd/C), and Celite were from Acros; 2-bromoethylamine hydrobromide was from Merck; ethanol, methanol, dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), tetrahydrofuran (THF), chloroform (CHCl$_3$), ethyl acetate, and cyclohexane were from SDS; C$_8$E$_4$ was from Bachem; dihexanoylphosphatidylcholine (DHPC) was from Avanti Polar Lipids; and Bio-Beads SM2 adsorbent was from Bio-Rad. Except for THF, which was redistilled on sodium in the presence of benzophenone, all solvents were used as received. NMR spectra were recorded on a Bruker Advance 400 MHz instrument (Wissembourg, France), and UV−visible spectra were recorded on an Agilent 8453 UV−visible spectroscopy system (Massy, France). The melting point of the products (given uncorrected) was determined with an Electrothermal 9100 system. Chelating Sepharose fast-flow, XK 16/20 columns, 1-mL HiTrap chelating columns, 5-mL HiTrap desalting columns, HR-12 Superose columns, and the Äkta purifier system were from GE Healthcare Life Sciences (Orsay, France). Water ("MilliQ water") was purified on a MilliQ academic system equipped with a Q-Gard1 cartridge and two Organex cartridges from Millipore (Saint-Quentin-en-Yvelines, France).

Buffer. Tris-NaCl buffer contained 20 mM Tris-HCl, pH 8.0, and 100 mM NaCl.

Synthesis of NBD-Labeled Amphipol. FAPol was obtained by deriving a precursor (UAPol) endowed with a spacer arm carrying an amine function (Figure 2A). The synthesis of the monoprotected α,ω-alkanediamine arm is described in Supporting Information (Scheme S1). The synthesis and purification of UAPol starting from poly(acrylic acid) (PAA) were adapted from those of A8-35 (*1*, *2*, *35*). In a first step, octylamine and the spacer arm (compound **3** in Scheme S1) were grafted onto PAA dissolved in *N*-methylpyrrolidone (NMP) in the presence of dicyclohexylcarbodiimide (DCC) and a small amount of triethylamine (TEA). Isopropylamine was grafted in a second step. After several steps of purification (according to ref *2*), the aqueous solution was dialyzed and freeze-dried, yielding UAPol with the spacer arm protected by a benzyloxycarbonyl group (90% yield). For deprotection, the polymer (0.5 g) was dissolved in 20 mL of methanol and cooled to 0 °C. Palladium on activated charcoal (Pd/C, 0.1 g) and 0.5 g of ammonium formate were introduced, and the mixture was stirred overnight at room temperature. The methanolic solution was filtered on a pad of Celite, which was washed with 100 mL of methanol, and the filtrate was evaporated to dryness under reduced pressure, yielding the deprotected UAPol in 100% yield.

FAPol was obtained by dissolving 0.5 g of UAPol and 0.013 g of NBD-Cl in 20 mL of methanol and heating at 50 °C in the dark for 3 h. After cooling at room temperature, the solution was purified by several cycles of precipitation/

Table 1: Chemical Composition of Amphipol Batches Used in This Study

| | mol % of each type of unit | | | | | |
A8-35 batch[a] (laboratory code)	CO_2^-	octyl	isopropyl	impurity[b]	linker	$\langle M \rangle$ (kDa)
HAPol-1 (CT-961020H)	31	28	41	nd		9.9
HAPol-2 (PHRFG)	35	25	34	6		10.2
HAPol-3 (FGH10frac)[c]	33	24	32	11		10.8
DAPol (FGD2Rfrac)	57	20	21	2		9.4
UAPol (FGHU3frac), FAPol (FGHfluo2)	35	23.5	34	6	1.5[d]	10.1

[a] HAPol refers to three batches of unlabeled A8-35; DAPol refers to a batch whose octyl and isopropyl chains were deuterated (*2*, *3*). UAPol and FAPol are described in the text. Short batch names are used for convenience in the course of the article. Laboratory codes allow cross-reference between publications. [b] See Experimental Procedures. [c] HAPol-3 was further fractionated before use. The composition given is that of the unfractionated batch. [d] About a third of which is grafted with NBD in FAPol (see Experimental Procedures).

centrifugation/dissolution (*2*, *35*). In order to further remove all traces of free dye, FAPol was purified by SEC on an XK 26−100 Superose 12 column in a buffer composed of 20 mM Tris-HCl, pH 8.5, anf 100 mM NaCl. Finally, the solution was dialyzed against MilliQ water and freeze-dried, yielding 0.35 g of FAPol (70% yield).

The chemical composition was determined by microanalysis and ^1H and ^{13}C NMR spectroscopy in perdeuteriated methanol (*2*). NMR analysis was based on the measurement of isopropyl/octyl chain ratio and isopropyl/benzyl group ratio. The structure was close to that expected except for the presence of some unidentified impurities grafted onto the polymer, which gave rise to an additional peak at 3.6 ppm in the ^1H NMR spectrum (data not shown). This observation has already been reported for unlabeled A8-35 (*2*). The impurities, whose structure is quite similar to that of DCC, probably originate from a side reaction with the coupling reagent (*2*). In the batches of UAPol and FAPol used for the present studies, they were estimated to represent 5−6% of the carboxylic groups (Table 1), which is below the level at which their presence affects the physical−chemical properties of A8-35 (*35*). The optical density of FAPol solutions is 0.888 $L \cdot g^{-1} \cdot cm^{-1}$ at 476 nm in water (1.147 $L \cdot g^{-1} \cdot cm^{-1}$ at 467 nm in methanol) and 0.215 $L \cdot g^{-1} \cdot cm^{-1}$ at 340 nm in water (0.425 $L \cdot g^{-1} \cdot cm^{-1}$ at 334 nm in methanol). By reference to the molar extinction coefficients reported for NBD-alanine in water ($\epsilon_{476} = 24\ 000$ $M^{-1} \cdot cm^{-1}$) (*36*) and NBD-C_6 in methanol ($\epsilon_{476} = 22\ 000$ $M^{-1} \cdot cm^{-1}$) (*37*), this corresponds to 1 mol of NBD per 25 kDa of FAPol, that is, a grafting level of 0.54%, or, for an average molecular mass $\langle M \rangle \approx 10.0$ kDa, a molar extinction coefficient $\epsilon_{476} \approx 9000$ $M^{-1} \cdot cm^{-1}$ in water and 10 000 $M^{-1} \cdot cm^{-1}$ in methanol. This relatively low level of grafting ($\sim$0.54% of NBD vs $\sim$1.5% of linker) is certainly due to the poor reactivity of the short linker, randomly bound to a large polymer (*38*). From optical density measurements on concentrated solutions, the absorbance at 280 nm of FAPol (in water) can be estimated to be $\sim$0.12 $L \cdot g^{-1} \cdot cm^{-1}$ or $\epsilon_{280} \approx 1200$ $M^{-1} \cdot cm^{-1}$. The chemical compositions of all APol batches used in the present study are shown in Table 1.

Genetic Modification, Overexpression, Refolding, and Purification of tOmpA. Plasmid pET3b-OmpA171 (*28*), encompassing the coding region for tOmpA, was modified by polymerase chain reaction (PCR) so as to add an eight-histidine tag to the N-terminus. The following pair of primers was used: NHisCod, 5′-GCGCCATGGGCAGCAGCCAT-CATCATCATCATCACCACCACATGGCTCCGAA-AGATAACACCTGGTA-3′, and NHisRev, 5′-CGCCCATG-GTATATCTCCTTCTTAAAGTTAAACAAAATTATTTCTAGA-

GGGA-3′. The 5.1-kb amplified linear product was digested by *Nco*I and ligated onto itself to yield plasmid pET3b-OmpA171NHis₈. The sequence was checked and the plasmid was introduced by electroporation into *Escherichia coli* BL21 DE3 strain.

The protein was overexpressed as inclusion bodies. Those were purified and tOmpA was refolded as described in ref *28*. Protein purification in detergent micelles was then performed by immobilized metal affinity chromatography (IMAC) (*39*). The buffer contained 20 mM Tris-HCl, pH 8, and 0.6% (19.6 mM) C_8E_4. The absence of lipids in the final samples was checked by thin-layer chromatography.

Preparation of tOmpA/Amphipol Complexes. The trapping procedure has been described in ref *3*. Briefly, a sample of tOmpA in C_8E_4 is supplemented with APol in a 1:4 (w/w) protein/APol ratio. After 15 min of incubation, the concentration of the detergent is brought below the critical micelle concentration (cmc) by 2 h of incubation at room temperature with polystyrene beads (Bio-Beads SM2) in a 1:10 detergent/beads weight ratio. The beads are removed by a short centrifugation. Removal of free APol, when applicable, was achieved by injecting the sample onto a 1-mL HiTrap chelating column that had been loaded with nickel and washed with Tris-NaCl buffer. Complexes were eluted by adding 300 mM imidazole to the buffer. The fractions containing tOmpA/APol complexes were then desalted on a 5-mL HiTrap Desalting column equilibrated with 20 mM Tris-HCl, pH 8.

Size-Exclusion Chromatography. Samples (100 μL) were injected onto an HR-12 Superose column connected to an Äkta purifier system. Elution was carried out at room temperature and the protein was detected at 280 nm. The elution buffer was Tris-NaCl buffer, with or without 19.6 mM C_8E_4 or 28 mM DHPC.

Steady-State Fluorescence Measurements. Measurements were carried out on a PTI spectrofluorometer (Photon Technology International, London, Ontario, Canada). The excitation wavelengths of tOmpA and FAPol were respectively 280 and 476 nm. Emission fluorescence spectra were recorded between 290 and 550 nm (because of the Rayleigh scatter close to 280 nm and the scattered first harmonic of the exciting light at 560 nm). The excitation and emission slits were usually opened at 5 and 1 nm, respectively. Samples (2 mL) were placed in 10-mm-wide quartz cuvettes (Hellman) and stirred magnetically.

Since FAPol absorbs slightly at 280 nm (Figure 2C), the emission spectra were corrected for the contribution of direct excitation by subtracting the emission spectrum of a control sample containing the same total concentration of FAPol in

buffer, as determined from fluorescence emission spectra upon direct excitation of NBD at 476 nm (we assumed that the quantum yields of protein-bound and unbound FAPol are identical). The magnitude of this correction represents ∼30% of the emission signal at 533 nm.

The Förster distance between donor and acceptor molecules, R_0, at which energy transfer is 50% efficient, was estimated to be 22.5 Å, with the assumption of a spectral overlap of 4.9×10^{-15} $M^{-1} \cdot cm^3$, a quantum yield of 0.143, an orientation factor of $^2/_3$, and a refractive index of the solution of 1.33. This value is in agreement with estimates by M. Prieto and co-workers (ref *40* and personal communication). Radiative transfer from tryptophan to NBD was responsible for less than 1% of NBD fluorescence in all experiments and has not been corrected for.

For kinetic experiments, the ratio of emission intensities recorded at 533 and 325 nm is shown rather than absolute intensities, in order to correct for the photobleaching of tryptophans.

Stopped-Flow Measurements. Measurements were carried out at 20 °C in a SFM-3 stopped-flow instrument (Bio-Logic, Claix, France). The samples were excited at 280 nm and the fluorescence was detected at 330 nm. Light path was 0.15 cm. The dead time of the instrument is about 3 ms.

Evolution of the FRET signal following extensive dilution of tOmpA/FAPol complexes in surfactant-free buffer was recorded after tOmpA/FAPol (0.5 g·L^{-1} protein) was mixed with Tris-NaCl buffer. The dilution factors were 0, 2×, 5×, 10×, 20×, 50×, and 100×. As the total concentrations of donor and acceptor molecules varied, corrections were required for highly concentrated samples. For instance, the undiluted sample absorbs significantly at both the excitation and emission wavelengths, resulting in a lower signal than expected: if the signal from the 20-fold diluted sample, that is, 0.4 V, is taken as a reference, the undiluted sample should give a signal of 8 V in the absence of inner filter effects, while the signal actually recorded was 5.4 V. Measurements of sample absorbance indicated that the inner filter effect was 22% and 11% at excitation (280 nm) and emission (330 nm) wavelengths, respectively. When this correction is applied to the expected fluorescence intensity (8 V), the emission intensity is calculated to be 5.36 V, in accordance with the measurement.

The kinetics of exchange of protein-bound FAPol by $C_{12}E_8$ was followed at different detergent concentrations. For each condition, 180 μL of tOmpA/FAPol at 0.025 g·L^{-1} protein was mixed with 20 μL of Tris-NaCl buffer containing $C_{12}E_8$ such that final detergent concentrations in the detection cell were 0, 0.025, 0.05, 0.1, 0.2, 0.5, or 1.0 g·L^{-1}.

Steady-State Exchange Measurements. tOmpA/FAPol complexes (2 mL at 0.001 g·L^{-1} protein) were supplemented with 0.85−17 μL of 200 mM (102.2 g·L^{-1}) DDM stock solution. The resulting dilution factor (<1%) was neglected. After a few minutes of incubation at room temperature, fluorescence spectra were recorded upon excitation at 280 nm. Experimental data were fitted with the following assumptions: (i) the intensity of the FRET signal is directly proportional to the amount of FAPol bound to the protein; (ii) the mass of surfactant bound is the same ($p = 1.4$ g/g of protein) for FAPol, DDM, and mixtures thereof; (iii) all of the FAPol is distributed between the protein-bound surfactant layer and free mixed micelles; (iv) the mass fraction $X_{FAPol,free}$

in the mixed micelles is related to that in the protein-bound layer, $X_{FAPol,bound}$, by $X_{FAPol,bound} = PX_{FAPol,free}$, where P is a partition coefficient; and (v) the concentration of DDM monomers is equal to cmc$(1 - X_{FAPol,free})$. Assumptions i and ii clearly are rough approximations. Assumption iii is supported by the absence of desorption of FAPol upon extensive dilution of the complexes in the absence of detergent (present work, Figure 4). Assumption v is consistent with isothermal titration measurements of the enthalpy of mixing between APols and detergents (*41*). The choice of p is not critical to obtaining a good fit, while that of P is determinant. In Figure 6B, P has been taken to be 2.83. Significantly worse fits are obtained with $P = 2$ or $P = 4$. Fitting the equilibrium values for the FAPol/$C_{12}E_8$ exchange (Figure 6A) by use of the same equations required a value of $P \approx 1.15$, suggesting the existence of a significant effect of the detergent polar head upon the relative energies of mixing of the two surfactants in solution and at the surface of the protein. This simple analysis breaks down at low detergent concentrations, for which more complex equations would have to be used (ref *41* and Diab et al., manuscript in preparation).

RESULTS

Preparation and Solution Properties of tOmpA/A8-35 Complexes. The preparation of tOmpA/A8-35 complexes has been described in ref *3* and is summarized under Experimental Procedures. In brief, tOmpA in C_8E_4 solution is supplemented with A8-35 in a 4:1 APol/MP mass ratio and the concentration of detergent is lowered by adsorption onto polystyrene beads. Under our experimental conditions, the removal of C_8E_4 by the beads was only partial (see Supporting Information, Figure S1) but sufficient to reach a concentration (∼4.5 mM) at which, in the absence of APol, the protein is insoluble in water. Whenever required, removal of the detergent can be completed by several cycles of dilution followed by concentration on Centricon 10 membranes; it can be ascertained from the complete disappearance from ^{1}H NMR spectra of the peaks due to the protons of the poly(oxyethylene) polar head (*3*). A8-35, on the other hand, does not adsorb onto polystyrene beads (Figure S1) and does not cross Centricon 10 membranes under these conditions, so that all of the APol added initially, whether bound to the protein or not, was present in the final preparations. We first describe the behavior of samples containing free APols and then that of preparations from which they have been removed by IMAC.

Size and Composition of tOmpA/Detergent versus tOmpA/ APol Complexes. The size and dispersity of tOmpA/A8-35 complexes were analyzed by SEC on a Superose column equilibrated and eluted with surfactant-free buffer (Figure 1A). tOmpA/APol complexes migrated as a major, rather symmetrical peak, with an apparent Stokes radius (R_S) of 4.3−4.7 nm depending on the APol batch (Table 2). These values are slightly smaller than that observed for the slightly larger, lipid-associated bacteriorhodopsin ($R_S \approx 5.0$ nm) (*17*). A very small amount of aggregates eluted in the void volume. By comparison, the R_S of tOmpA/detergent particles was 3.3 nm in the presence of 19.6 mM C_8E_4 and 2.6 nm in the presence of 28 mM DHPC (Table 2). The ratio of the R_S for tOmpA/A8-35 versus tOmpA/DHPC complexes is surprisingly high, given the relative resolution of NMR spectra
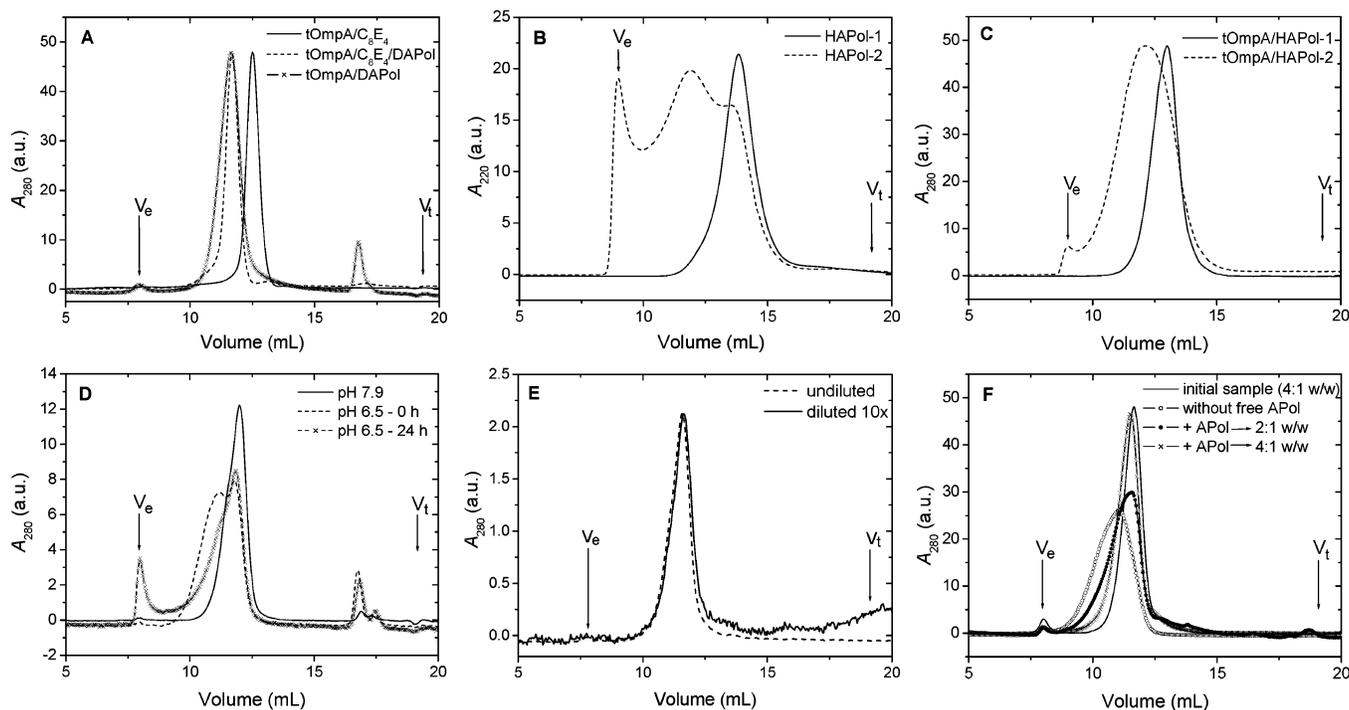
FIGURE 1: Size-exclusion chromatographic analysis of tOmpA/surfactant complexes. (A) SEC elution profiles at various steps during the trapping procedure. An aliquot of a sample of tOmpA (0.22 g·L^{-1}) in 6 g·L^{-1} (19.6 mM) C_8E_4 solution was injected onto an HR-12 Superose column equilibrated with Tris-NaCl buffer containing the same concentration of detergent. The tOmpA/C_8E_4 sample was then supplemented with the deuteriated form of A8-35 (DAPol) in a 4:1 APol/protein mass ratio and an aliquot of the ternary tOmpA/C_8E_4/DAPol complexes was injected on the column previously equilibrated with Tris-NaCl buffer containing a mixture of C_8E_4 and DAPol at 4 and 0.91 g·L^{-1}, respectively. A second aliquot of the tOmpA/C_8E_4/DAPol complexes was incubated for 2 h with polystyrene beads, and the resulting tOmpA/DAPol complexes were injected onto the same column, equilibrated with surfactant-free Tris-NaCl buffer (the peak at ~17 mL in the latter profile is due to a contaminant released by the Bio-Beads). Profiles have been normalized to the same maximum. (B, C) Influence of the dispersity of APol particles on that of tOmpA/APol complexes: (B) elution profiles of two APol batches (HAPol-1 and HAPol-2) used for tOmpA trapping and (C) size dispersity of the resulting tOmpA/A8-35 complexes in Tris-NaCl buffer. (D) Influence of pH on the size dispersity of MP/A8-35 complexes. An aliquot of tOmpA/HAPol-3 was injected onto an HR-12 Superose column equilibrated with Tris-NaCl buffer, pH 7.9. A second aliquot was diluted in Tris-NaCl buffer, pH 6.5, and injected onto a column equilibrated in the same buffer, either immediately or after 24 h of incubation at room temperature. (E) Absence of effect of dilution on the dispersity of tOmpA/APol complexes. tOmpA was trapped with FAPol at a 4:1 APol/protein mass ratio, and an aliquot of this sample (protein concentration 0.15 g·L^{-1}) was injected onto an HR-12 Superose column equilibrated with Tris-NaCl buffer, pH 7.9. The rest of the sample was diluted 10× in the same buffer and then injected onto the column. The profiles of absorbance at 280 nm have been normalized to the same maximum. (F) Effect of the presence of free APols on the dispersity of tOmpA/APol complexes. After tOmpA was trapped with DAPol at a 4:1 APol/protein mass ratio, an aliquot of this sample was injected onto an HR-12 Superose column equilibrated with Tris-NaCl buffer, pH 7.9. The rest of the sample was immobilized on a 1-mL nickel-chelating column and free APol particles washed away with the same buffer. The tOmpA/A8-35 complexes were eluted with 300 mM imidazole in the same buffer and desalted on a 5-mL desalting column. An aliquot of the fraction containing tOmpA/A8-35 was then injected onto the HR-12 Superose column. The half-height at full width (HHFW) of the peak of tOmpA/A8-35 complexes after IMAC (1.72 mL) is twice that of the initial sample (0.84 mL). Aliquots of the aggregated sample were then supplemented with A8-35 in a final overall APol/protein mass ratio of either 2:1 or 4:1. After one night of incubation, the aliquots were injected onto the HR-12 Superose column. The HHFW values decrease to 1.36 and 0.92 mL, respectively.

Table 2: Influence of Surfactant Composition on the Apparent Size and Dispersity of tOmpA/Surfactant Complexes

complexes	R_S^a (nm)	HHFW (mL)
tOmpA/C_8E_4	3.3	0.6
tOmpA/DHPC	2.6	0.5
tOmpA/C_8E_4/DAPol	4.7	0.6
tOmpA/DAPol	4.7	0.9
tOmpA/HAPol-1	4.6	b
tOmpA/HAPol-3	4.3	0.9
tOmpA/FAPol	4.7	1.0

^{a} Average Stokes radii of tOmpA complexed by either detergent (C_8E_4 or DHPC), APol, or a mixture thereof were determined by SEC by use of a calibration curve fitted with a 4-order polynomial function. ^{b} Sizing was performed on a different column; as a result, the HHFW is not comparable to those of the other experiments.

obtained with each type of preparation (3) (see Discussion). The half-height at full width (HHFW) of the peak was significantly larger for tOmpA/A8-35 complexes (0.9 mL)

than for tOmpA/detergent ones (0.6 and 0.5 mL), an indication that tOmpA/A8-35 particles are more polydisperse (Table 2).

Extrapolation to zero angle of Guinier plots of SANS data recorded at various percentages of D_2O yielded a contrast-match point for tOmpA/C_8E_4 complexes of 21% ± 1% D_2O (39). From this value, the mass ratio of detergent bound to tOmpA can be estimated to be 1.4 ± 0.2, corresponding to 86 ± 12 molecules of C_8E_4 bound per tOmpA monomer and an overall particle molecular mass of ~45 kDa (dry mass). This mass is compatible with that estimated by others by use of dynamic light scattering (70 kDa for the hydrated particle) (30).

Complexing tOmpA with a fluorescent APol (see below), removing free APols by IMAC, and measuring the ratio of absorbance at 280 and 476 nm yielded an estimate of ~1.3 g of bound APol per g of tOmpA (39), which is close to the

mass ratio obtained for C_8E_4. However, this measurement is only an estimate, since it implies separating free APols from the complexes. This, as described below, causes the latter to partially aggregate, which is likely to diminish the amount of APol they bind.

Factors Affecting the Size and Dispersity of tOmpA/A8-35 Complexes: (1) Presence or Absence of Detergent. Several earlier studies indicate that APols bind to MPs even in the presence of detergent above its cmc, leading to the formation of ternary complexes (see, for example, refs *14* and *18*). This was confirmed by SEC analysis of the complexes formed upon addition of A8-35 to tOmpA in C_8E_4 solution, prior to eliminating the detergent, the column being equilibrated with a mixture of detergent and APol (Figure 1A). Under these conditions, the apparent size of the particles was similar to that of pure tOmpA/A8-35 particles, but the HHFW was that observed in detergent alone (Table 2). Two phenomena therefore contribute to the behavior of tOmpA/A8-35 complexes upon SEC, an increase in apparent size that accompanies the binding of APols, and a heterogeneity that develops only after removal of the detergent. These observations were confirmed by AUC experiments showing that the presence of detergent improves the homogeneity of tOmpA/A8-35 particles (*39*).

The rate at which the concentration of detergent was lowered ($\sim 2\times$ under the cmc), whether by rapid dilution or by slow adsorption onto BioBeads, had no detectable effect on the behavior of the resulting particles upon SEC (*39*).

(2) Batch-to-Batch Variability of A8-35 Preparations. We have shown elsewhere that various factors, including relatively minor departures from the nominal chemical structure, can strongly affect the solution behavior of pure A8-35 and, in particular, the dispersity of the particles it forms in aqueous solutions (*2*, *35*) (Figure 1B). Controlling the latter parameter is of critical importance to limiting the polydispersity of MP/APol complexes, since APol polydispersity most often entails that of the complexes, as observed with tOmpA (Figure 1C) and with BR (*17*, *20*) and as surmised in the case of the sarcoplasmic Ca^{2+}-ATPase (*16*).

(3) pH. The solubility of A8-35 in water is ensured by carboxylate groups. When the pH is lowered, some of these groups protonate, solubility diminishes, and aggregative phenomena appear (*2*, *35*). With pure A8-35, this effect starts around neutrality and, by use of radiation scattering techniques, which are particularly sensitive to the presence of aggregates, is clearly seen already at pH 6.8 (*2*, *35*). Not surprisingly, similar effects are seen with tOmpA/A8-35 complexes, as illustrated in Figure 1D, where a preparation of complexes that was well-behaved at pH 7.9 was either injected onto a column equilibrated at pH 6.5 or incubated for 24 h at pH 6.5 before injection. Interestingly, examination of the profiles suggest that some of the complexes are more pH-sensitive than others. Indeed, a fraction of the initial complexes appears to immediately convert into larger particles, which, after 24 h, generate even larger aggregates, many of which elute in the void volume of the column. In the meantime, the size of that population of particles whose elution volume had remained essentially unchanged does not seem to evolve. This is reminiscent of observations done with the pure polymer: when a preparation yielding polydisperse particles is fractionated by SEC and analyzed again in the same manner, each fraction elutes at its initial position

(*2*, *14*, *35*). We have reasons to believe (see below) that this does not reflect a kinetically frozen composition and size of each APol particle but an equilibrium distribution, with some APol molecules tending to yield larger particles than others upon acidification. The same kind of phenomenon may well account for the behavior of tOmpA/A8-35 complexes: because of their length, the number or distribution of hydrophobic chains, or a combination thereof, some APol molecules may be more sensitive than others to a mild pH drop. Those MP/APol complexes that incorporate pH-sensitive molecules tend to aggregate, while the others are essentially unperturbed.

(4) Dilution. The SEC profile of tOmpA/APol complexes was unaffected by $10\times$ dilution into surfactant-free buffer (Figure 1E).

(5) Removal of Free Amphipols. APols were originally designed with the view that their multipoint attachment to the surface of MPs would ensure a quasi-irreversibility of the association, so that MP/APol complexes could be handled in the absence or near-absence of free polymers (*1*, *12*). This expectation appeared to be vindicated by the fact that MP/APol complexes would remain soluble and essentially monodisperse upon being centrifuged or chromatographed in surfactant-free solutions (*1*, *17−20*). It was also supported by the absence of detectable desorption when proteins trapped by a radioactive APol were centrifuged in an APol-free gradient (*18*), or when MP/A8-35 complexes were immobilized on a surface plasmon resonance chip and extensively washed with surfactant-free buffer (Q. Hong and J. H. Lakey, unpublished data quoted in ref *12*).

MP trapping with APols is always carried out in the presence of more polymer than the protein will ultimately bind (see, for example, ref *18*). In the case of tOmpA, where trapping was carried out at a 4:1 (w/w) APol/protein ratio, the amount of A8-35 retained by complexes washed with APol-free buffer is ~ 1.3 g per g of tOmpA (see above), suggesting that, following trapping, some two-thirds of the polymer initially added is still present as free particles (i.e., slightly more than one 40-kDa A8-35 particle per tOmpA/APol complex). The presence of free APols is not detrimental to most investigations, such as AUC, NMR, or spectroscopic ones, but it does interfere with others, such as SANS investigations of the protein-bound APol layer (*17*) or determination of the amount of bound APol. When the MP is large, many techniques, for example, SEC, can be used to separate MP/APol complexes from free APol particles. For small MPs like tOmpA, however, it is more difficult to efficiently separate the complex ($R_S = 4.3−4.7$ nm; this work) from free A8-35 particles ($R_S \approx 3.15$ nm; ref *35*). The tOmpA variant used for the present study therefore contained a polyhistidine tag, which made it possible to separate tOmpA/APol complexes from free APols by IMAC. Upon SEC analysis, the samples thus recovered, however, appeared markedly more polydisperse, even though no protein eluted in the void volume of the column (Figure 1F). This distribution was stable for several days at room temperature (not shown). As already reported for other proteins (*42*), Ni^{2+} ions leaching from the column can cause aggregation of histidine-tagged tOmpA/detergent complexes, a phenomenon that disappears upon addition of EDTA (*39*). EDTA however did not affect the aggregation observed after elimination of free APols by IMAC (*39*). Adding back free
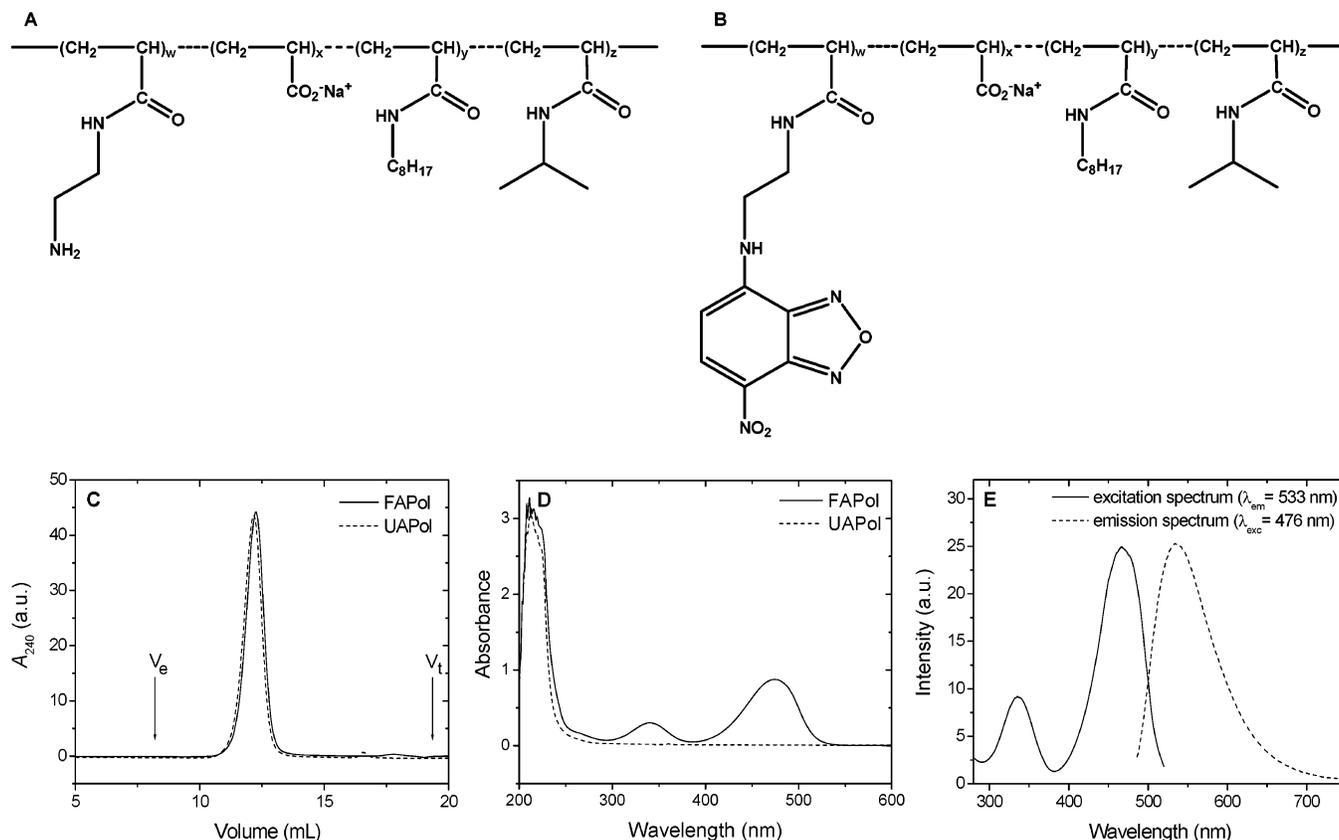
FIGURE 2: Structure and spectroscopic properties of UAPol and FAPol. (A, B) Chemical structures of UAPol and FAPol, respectively. The values of *w*, *x*, *y*, and *z* are given in Table 1. (C) SEC analysis of UAPol and FAPol on an HR-12 Superose column in Tris-NaCl buffer. (D) UV-visible spectra of UAPol and FAPol at 1 g·L^{-1} in water. (E) Excitation and emission spectra of FAPol at 0.08 g·L^{-1} in 20 mM Tris-HCl buffer, pH 8.0.

APol, on the other hand, did reverse aggregation (Figure 1F). At a final APol/protein overall mass ratio of 2:1, reversal was partial. It was essentially complete when the original 4:1 ratio was restored (Figure 1F) and did not change upon raising this ratio up to 20:1 (*39*).

The aggregation of tOmpA/APol complexes in the absence of free APol appears at odds with earlier experiments carried out on other MP/APol complexes (see, for example, refs *1* and *18*) but is in keeping with the behavior of BR/A8-35 ones (*17*). An obvious explanation for this phenomenon could be that the rate of desorption of APols from the transmembrane surface of MPs is *not* negligibly low: upon removal of free APols, some of the bound molecules would desorb, and this would induce the aggregation of partially depleted proteins. This hypothesis conflicts with earlier experiments that revealed no APol desorption when complexes between the MP OmpF and a radio-labeled, slightly different APol, [^{14}C]A8-75, were fractionated in surfactant-free sucrose gradients (*18*). However, the accuracy of these experiments was not such that a limited desorption—say, ≤10%—would have been detected with certainty. In order to explore the possible existence of an equilibrium between bound and free APol over a larger range of concentrations and time scales, we resorted to Förster resonance energy transfer (FRET) between tOmpA and an NBD-labeled, fluorescent variant of A8-35.

Synthesis of NBD-Labeled A8-35 (FAPol). Whatever care is brought to standardizing protocols, two batches of APols originating from different syntheses will necessarily feature somewhat different compositions. A labeled polymer used

as a tracer may therefore behave differently from the unlabeled molecules it is supposed to report upon if the two have been synthesized independently. In order to circumvent this problem, we synthesized a version of A8-35 carrying a reactive arm (UAPol) (Figure 2A and Scheme S1), to which any desirable label can be bound after the synthesis of the polymer itself has been completed. The subsequent binding of NBD (Scheme S2, Supporting Information) yielded a fluorescent polymer (hereafter FAPol) with precisely the same chain distribution and physical−chemical properties as the parent, unlabeled UAPol (Figure 2B). NBD was chosen as the fluorophore because its absorbance at 280 nm is low; it is weakly hydrophobic, which ought to make it a good reporter for the APol backbone, to which it is bound by a short spacer arm; and it is readily excited by FRET with tryptophans as donors (see below). The final ratio of NBD per mass of A8-35 was estimated from the UV−visible absorbance of the polymer in water, with the molar extinction coefficients of NBD-C$_6$ and NBD-alanine in water taken as references (see Experimental Procedures). The preparation of FAPol used for the present work contained ∼0.54% ± 0.05% NBD per acrylate unit, meaning that, on average, a little more than one out of three A8-35 molecules is NBD-labeled. Given the low hydrophobicity of NBD and the average length of UAPol and FAPol molecules (∼80 acrylate units, bearing ∼20 octyl chains), the presence of one label per molecule ought not to affect significantly the behavior of the labeled molecules as compared to that of the unlabeled ones. The two polymers, indeed, formed particles with the same R_S and size dispersity (Figure 2C). The UV−visible
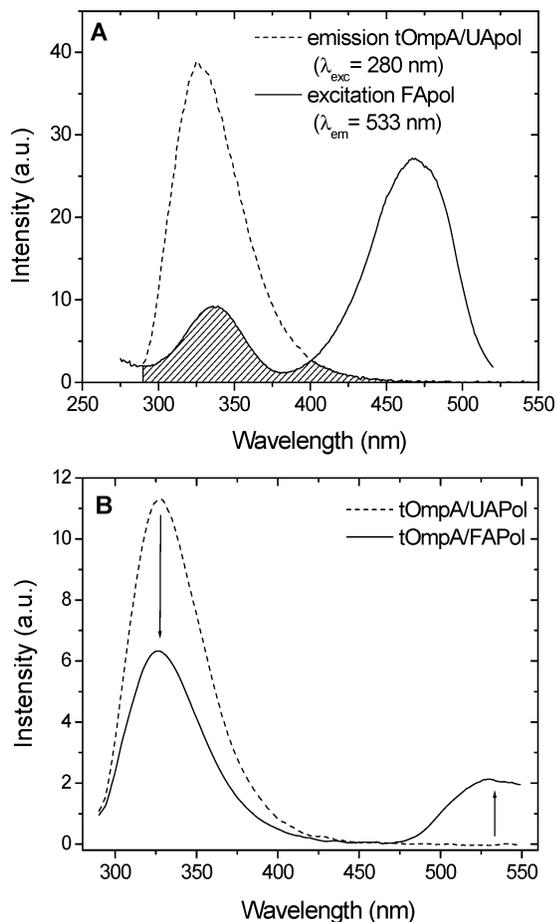
FIGURE 3: Förster resonance energy transfer between tOmpA and FAPol. (A) Emission spectrum of tOmpA/UAPol complexes and excitation spectrum of FAPol. tOmpA and APol were at 0.002 and 0.008 g·L^{-1}, respectively, in 20 mM Tris-HCl, pH 8.0. The spectral overlap that makes energy transfer possible is shown as a hatched area. (B) FRET between tOmpA and FAPol. tOmpA and APol were at 0.02 and 0.08 g·L^{-1}, respectively, in 20 mM Tris-HCl, pH 8.0. The fluorescence emission spectra of tOmpA/UAPol and tOmpA/FAPol have been corrected as described under Experimental Procedures and normalized according to protein concentration. Arrows indicate the decrease of emission at 325 nm and the increase at 533 nm due to FRET.

absorption spectra of UAPol and FAPol and the excitation and emission spectra of FAPol are shown in Figure 2D,E. As reported for NBD (*43*), the spectral properties of FAPol were sensitive to solvent polarity. In water, the maximum absorption and emission wavelengths were at 476 and 533 nm, respectively. At variance with UAPol, FAPol absorbs slightly at 280 nm (Figure 2D).

Examination by FRET of the Dynamics of tOmpA/A8-35 Association. The overlap between the emission spectrum of tOmpA and the excitation spectrum of FAPol allows for FRET from one to the other (Figure 3A). As compared to tOmpA/UAPol complexes, excitation of tOmpA/FAPol complexes at 280 nm indeed results in a reduced protein fluorescence emission at 325 nm and the appearance of a new signal at 533 nm, consistent with the occurrence of FRET (Figure 3B; the direct contribution of FAPol to the 533-nm emission signal has been subtracted as described under Experimental Procedures). The efficacy of transfer (*E*) from tryptophans to NBD was calculated to be 48% from Förster equations (*44*). The R_0 distance was estimated to be ~22.5 Å (see Experimental Procedures), in keeping with

estimates by others (M. Prieto, personal communication). Raising the concentration of NaCl from 0 to 200 mM slightly increased the FRET signal (the intensity at 330 nm dropped by ~7% and that at 533 nm rose by ~15%; Figure S2, Supporting Information).

Effect of Diluting tOmpA/A8-35 Solutions with Surfactant-Free Buffer. Following extensive dilution of tOmpA/FAPol complexes in surfactant-free buffer, the FRET signal was perfectly stable on a time scale ranging from milliseconds (Figure 4A) to a day (Figure 4C). After correction for inner filter effects (see Experimental Procedures), the relative intensity of FRET measured 5 s after dilution is seen to be independent of the final sample concentration in the range of 2−20× dilution (Figure 4B), consistent with the absence of desorption of FAPol from the protein. These four samples, however, appear slightly and comparably dequenched as compared to the undiluted one, a phenomenon that, if real, would have to take place during the dead time of the instrument (~3 ms), since it is not reflected in the kinetics of Figure 4A. Such a rapid desorption appears quite improbable. Indeed, the residence time in their micelles of single molecules of C_8E_4 or octyl glucoside, two detergents that carry a single octyl chain, is ~1 μs, a duration that, in keeping with the thermodynamics of micellization, increases by about 10× each time the chain is elongated by two methylenes (*45*, *46*). It should thus reach the dead time of the stopped-flow instrument for molecules comprising two octyl chains. Even with due allowance for the different environments of the chains and the polymeric nature of APols, it seems therefore highly unlikely that A8-35 molecules interacting hydrophobically with the transmembrane region of tOmpA by a large number of octyl chains could spontaneously desorb with millisecond kinetics. The argument, however, may not apply to molecules adsorbed as an outer layer by another mechanism. Perhaps more likely, the slight difference between the undiluted and 2−20× diluted samples could originate from small errors in correcting for the large inner filter effects that are present at high sample concentrations (see Experimental Procedures).

The two samples diluted 50× and 100× show an increase in relative fluorescence intensity at 330 nm, a phenomenon that can receive two alternative explanations. Either it is real, in which case desorption would have, once again, to occur within the dead time of the instrument, or it is an artifact due to difficult experimental conditions (unusually small volume ratios of sample to buffer injected into the mixing chamber, very small signal). The experiment shown in Figure 4C indicates that, upon manual 1000× dilution of another sample, no evolution of the FRET signal takes place over a period of a day.

In summary, FRET data indicate that extensive dilution does not entail any detectable desorption of the APol, unless the latter occurs, at very high dilution, in an improbably short time range. It is particularly significant that samples diluted either 2× or 5× exhibit the same relative fluorescence, even though their APol concentration is made to vary in a range in which lowering the APol/MP ratio is found to strongly affect the dispersity of tOmpA/A8-35 particles (Figure 1F).

Exchange of Protein-Bound FAPol for Other Surfactants. The above observations strongly suggest that the binding of A8-35 to the transmembrane region of tOmpA is, under our experimental conditions, irreversible, at least on the time
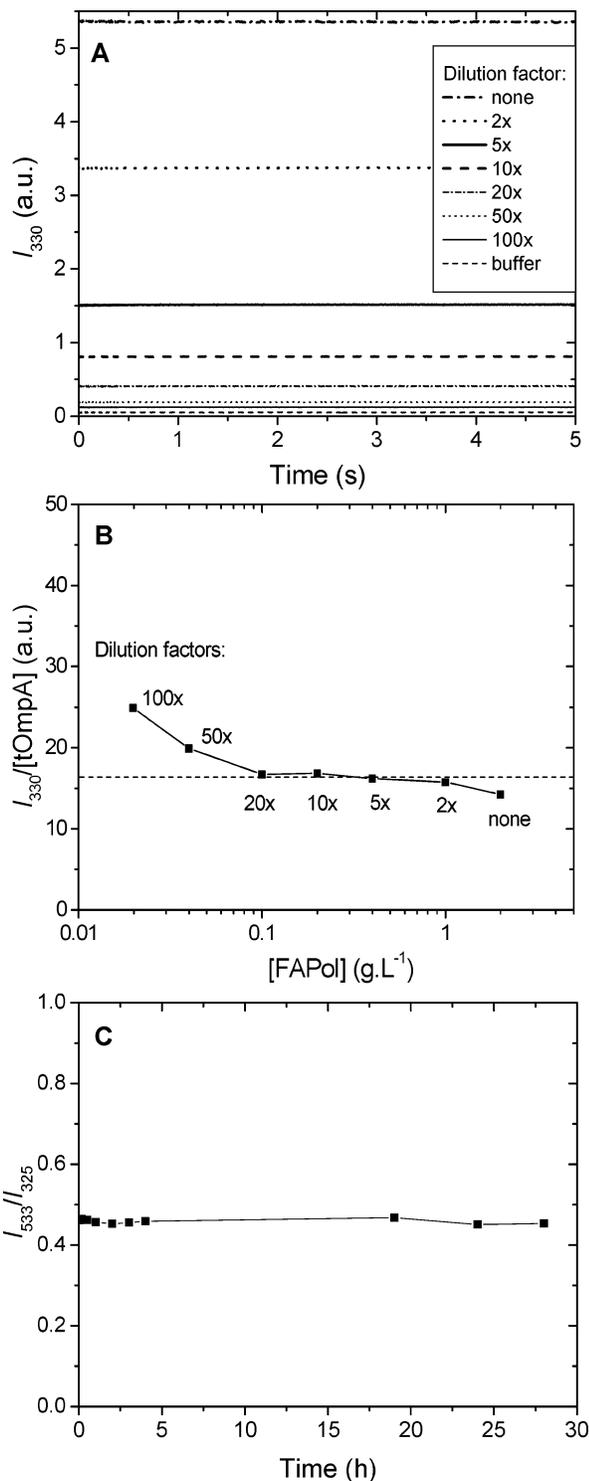
FIGURE 4: Stability of the FRET signal following dilution of tOmpA/FAPol complexes into surfactant-free buffer. (A) tOmpA/FAPol complexes (0.5 g·L^{-1} protein, 2 g·L^{-1} FAPol) in 20 mM Tris-HCl buffer, pH 8.0 were diluted with Tris-NaCl buffer in a stopped-flow instrument at the dilution factors indicated, and the protein fluorescence intensity at 330 nm was recorded as a function of time. (B) Fluorescence intensity of the same samples 5 s after dilution, normalized to the final tOmpA concentration and corrected for internal filter effects (see Experimental Procedures), as a function of the concentration of FAPol. (C) Evolution of the fluorescence of another sample of tOmpA/FAPol (2.5 g·L^{-1} protein, 10 g·L^{-1} FAPol) after 1000× dilution into 20 mM Tris-HCl, pH 8.0. Emission spectra were recorded as a function of the incubation time at room temperature. Data are plotted as the ratio of intensities at 325 and 533 nm so as to cancel out the effect of tryptophan photobleaching.
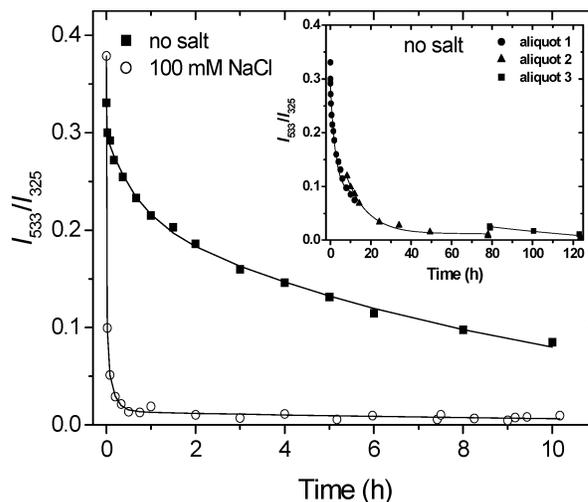

FIGURE 5: Kinetics of exchange of tOmpA-bound FAPol for UAPol: Evolution of the FRET signal after supplemention of tOmpA/FAPol samples with a 50× excess of UAPol. Emission spectra were recorded as a function of time and the ratio of intensities at 325 and 533 nm was plotted so as to cancel out the effects of tryptophan photobleaching. The kinetics of exchange was followed at room temperature either in the absence or in the presence of 100 mM NaCl in 20 mM Tris-HCl buffer, pH 8.0. tOmpA and FAPol concentrations were 0.001 and 0.004 g·L^{-1}, respectively. (Inset) The kinetics of exchange in the absence of NaCl was followed until the exchange of tOmpA-bound FAPol for UAPol was nearly complete (FRET signal down to <4% that in the absence of UAPol). To limit photobleaching effects, three aliquots of the same sample were used to cover different ranges of incubation times.

scale of a day, and even following extensive dilution in surfactant-free medium. This conclusion confirms and extends that from earlier experiments using other approaches (*12, 18*). On the other hand, it has been shown previously that [^{14}C]A8-75, a closely related APol, can be displaced from MPs upon migration of the complexes in sucrose gradients containing either unlabeled APol or detergent (*18*). Similarly, the functional effects of A8-35 on the sarcoplasmic Ca^{2+}-ATPase are reversed by adding back an excess of detergent, strongly suggesting displacement (*14*). FRET measurements provide an opportunity to re-examine these observations by an independent approach with higher sensitivity and time resolution.

Upon addition of a large excess of UAPol (50× the total mass of FAPol) to a solution of tOmpA/FAPol complexes, the FRET signal decreased with kinetics that depended on the ionic strength (Figure 5). Whatever the ionic strength, fitting the kinetics of FRET decrease required at least three exponentials. At low ionic strength, ~10% of the tOmpA-bound FAPol was displaced by UAPol with a $t_{1/2}$ of ~28 s, ~24% with a $t_{1/2}$ of ~28 min, and the remnant, ~66%, with a $t_{1/2}$ of ~7 h. In the presence of 100 mM NaCl in the buffer, the proportions were reversed: most (~79%) of the FAPol was displaced with a $t_{1/2}$ of ~21 s, ~17% with a $t_{1/2}$ of ~6 min, and ~4% with a $t_{1/2}$ of ~9 h. The basis for this effect most likely is an increased collision efficacy between tOmpA/FAPol complexes and free UAPol particles upon screening of repulsive electrostatic interactions. Under control conditions, namely, upon addition of UAPol-free buffer, the FRET signal was perfectly stable over time (Figure 4). We envision the exchange process as comprised of three steps: (i) a particle of UAPol collides with a protein/FAPol complex
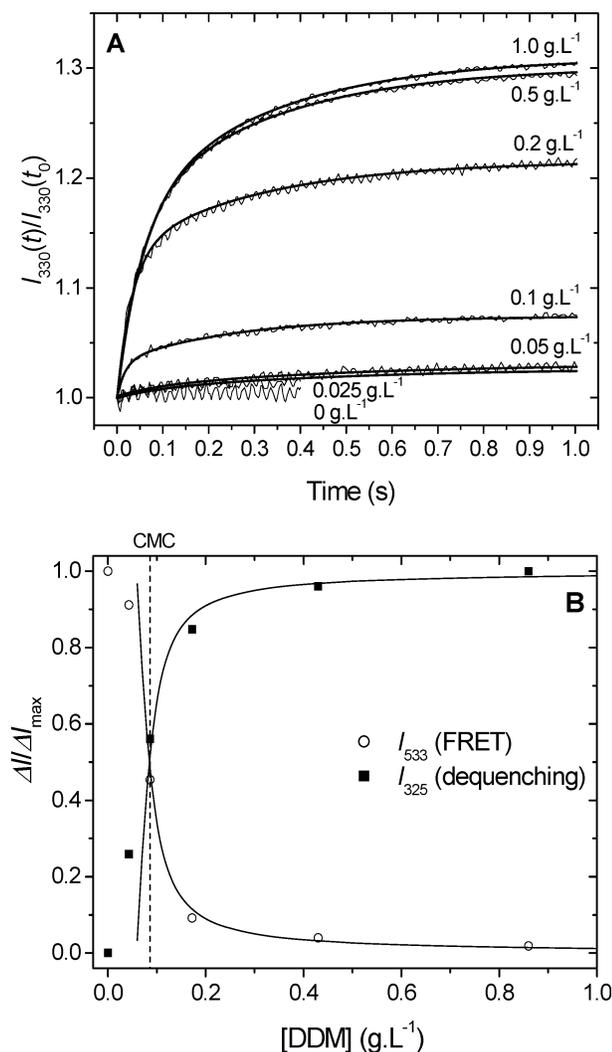
FIGURE 6: Displacement of tOmpA-bound FAPol by detergents. (A) Detergent ($C_{12}E_8$) was added to tOmpA/FAPol complexes in a stopped-flow instrument, and the kinetics of dequenching was followed as a function of time. The final concentrations of detergent extended from $2\times$ below the cmc to $20\times$ above it ($0.025-1$ g·L^{-1}). The final protein and APol concentrations were kept constant at 0.025 and 0.1 g·L^{-1}, respectively. Experimental data (thin lines) have been normalized to the intensity of tryptophan fluorescence at the first measurable point, taken as $I_{330}(t_0)$, and fitted with three exponentials (thick lines). (B) Steady-state fluorescence observed after addition of increasing concentrations of dodecyl β-D-maltoside (DDM). Aliquots of 200 mM (102 g·L^{-1}) DDM stock solution were added to tOmpA/FAPol samples (0.001 g·L^{-1} protein, 0.004 g·L^{-1} FAPol). Emission spectra were recorded after a few minutes of incubation, that is, at equilibrium. The rise of dequenching at 325 nm and the drop of FRET at 533 nm are plotted as percentages of the extent of quenching and FRET observed in pure FAPol, respectively. Curves are fits calculated as described under Experimental Procedures.

and fuses with it; (ii) in the transient complex thus formed, which contains an excess of APol, a "mixing step" takes place during which incoming UAPol molecules compete with FAPol ones for the surface of the protein; and (iii) an APol particle detaches from the ternary complex, possibly carrying off some FAPol. The kinetics of all these steps is likely to be quite complex. In particular, the probability that any given APol molecule will actually detach from the protein can be expected to depend on its individual chemical structure: as more FAPol molecules become washed away, the remaining ones will likely (for example, because of their

size and/or the number of octyl chains they carry) exchange more and more slowly, which probably accounts for the fact that the displacement of FAPol trails off (Figure 5). More detailed kinetic studies than those reported here—for example, studies of the exchange rate as a function of UAPol concentration—would be needed to better understand the underlying mechanism, but their analysis is unlikely to be straightforward.

Various concentrations of the detergent $C_{12}E_8$ were added to tOmpA/FAPol samples in a stopped-flow instrument and the protein's fluorescence was recorded as a function of time (Figure 6A). The kinetics of exchange was much faster than when the competing surfactant was UAPol (Figure 5), the equilibrium being reached within less than 1 s. The energetic barrier to exchange is obviously much lower in this case than it is when FAPol is exchanged for UAPol. More extensive kinetic studies would be required to establish whether, in that case, exchange results from the fusion of micelles or mixed micelles with the complexes, followed by fission, or, perhaps more likely, from the swelling of the surfactant layer surrounding the protein by an influx of detergent monomers, leading to fragmentation.

The competition for binding to tOmpA between FAPol and another detergent, dodecyl β-D-maltoside (DDM), was studied under equilibrium conditions (Figure 6B). At equilibrium (i.e., after a few minutes), the decrease of the FRET signal at 533 nm and the increase of the protein fluorescence at 325 nm evolved in parallel as the concentration of detergent was progressively increased. For a DDM concentration equal to its cmc, the FRET signal was halved. Although other factors can intervene, a simple explanation would be that half of the FAPol that made up the original protein-bound surfactant layer has transferred to APol/detergent mixed micelles. At 10-fold the cmc, that is, under conditions where the mass of DDM present in the sample is $\sim$200 times that of FAPol, the exchange appeared complete. At sufficiently high detergent concentration (at and above the cmc, that is, at overall detergent/APol mass ratios $\geq$20:1), the FRET data are well fitted by a simple model according to which FAPol freely equilibrates between the mixed surfactant layer associated with the protein and protein-free mixed micelles (solid lines in Figure 6B). Fitting the experimental data, however, requires the partition coefficient for FAPol between the two types of particles not to be 1, the concentration of APol being higher (in the case of DDM, by a factor of $\sim$2.8) in the protein-bound layer than in the protein-free particles. For $C_{12}E_8$, a partition coefficient of $\sim$1.1 can be estimated from the data of Figure 6A. When the detergent is not present in large excess over the APol, this simple analysis is no longer valid (see Experimental Procedures).

DISCUSSION

In the present work, we have examined some properties of MP/APol complexes that are relevant to their use in membrane protein biochemical and biophysical studies. In particular, we address the following questions: (i) What are the parameters that affect the size and dispersity of MP/APol complexes? (ii) To what extent is it possible to handle MP/APol complexes in the absence of free APols? (iii) To what extent can the binding of APols to the protein be considered

as irreversible in surfactant-free aqueous solutions? (iv) Can protein-bound APols be exchanged for other APols or washed away with detergents? (v) Can APol-trapped MPs interact with one another?

Factors Controlling Size and Dispersity of MP/APol Complexes. SEC experiments show that, following trapping of tOmpA with APol A8-35, well-defined particles are formed. Their apparent Stokes radius (4.3−4.7 nm) and size dispersity are larger than those of tOmpA/C_8E_4 (3.3 nm) or tOmpA/DHPC (2.6 nm) particles (*39*), suggesting that the layer of APol associated with the transmembrane surface of tOmpA is both thicker and less homogeneous, from one particle to the next, than that of detergent. To what extent the apparent ratio of hydrodynamic radii can be trusted is, however, somewhat problematical. Indeed, the rotational correlation times (τ_c) of tOmpA/A8-35 and tOmpA/DHPC particles, measured by NMR (*3*), differ by much less than should be expected: on the basis of SEC R_S estimates, the Stokes−Einstein relationship predicts that τ_c should increase by $1.81^3 = 5.9\times$, while the experimentally observed increase is only $1.6\times$ (*3*). Assuming homothetic particles, the τ_c ratio would indicate that tOmpA/A8-35 particles have a R_S of ∼3.0 nm, well below that yielded by SEC. The size derived from τ_c measurements is close to that (3.1−3.2 nm) expected from the assumptions of tOmpA/A8-35 mass ratio of 1:1.4, a spherical shape, and reasonable hydration values [for tOmpA, 0.4−1 g of water per g of protein; for A8-35, a hydrated volume per gram of dry APol identical to that of pure, spherical APol particles, that is, ∼3.3 nm^3/kDa; see ref *35*]. A similar apparent overestimation of R_S by SEC as compared to AUC, SANS, or electron microscopic data has been observed for BR/A8-35 complexes (*17*). It is difficult to argue that APol molecules adsorbed onto the column resin electrostatically or sterically repulse MP/APol complexes, resulting in an artifactual increase of the apparent size of the latter, since size estimates of free A8-35 particles obtained by SEC are consistent with those derived, in the absence of any solid phase, by both SANS and AUC (*35*). However, it cannot be excluded that MP-adsorbed APols exhibit a diffuse corona, which would increase the R_S of the complexes (cf. ref *47*). Partial decoupling between the movements of the whole particle and those of the MP within it may possibly account for the apparent inconsistency between R_S and τ_c measurements. This point will deserve further investigation.

SEC was used to compare the apparent size of MP/APol particles under various conditions. An interesting observation is that ternary tOmpA/APol/detergent complexes are more monodisperse than binary tOmpA/APol ones. Since the protective effect of APols against detergent-induced inactivation is observed even in the presence of detergent above its cmc (*14*), working with ternary complexes may be advantageous when studying detergent-sensitive MPs by techniques that require or benefit from monodispersity, such as crystallization, radiation scattering, or NMR.

Not unexpectedly, given that lowering the pH at or below neutrality induces the aggregation of A8-35 particles (*35*), initially well-dispersed tOmpA/A8-35 complexes prepared at pH 7.9 progressively aggregated when brought to pH 6.5. This effect, which can be a hindrance in some experimental situations, most obviously NMR (*3*), cannot be alleviated without resorting to APols with a different chemical structure, such as nonionic, zwitterionic, or sulfonated APols (see, for

example, refs *15*, *16*, *25*, *41*, *48*, and *49*). Another factor leading to heterogeneity of tOmpA/A8-35 complexes is, as already noted with BR (*17*, *20*), the use of APol batches that form polydisperse particles.

Dynamics of MP/APol Association. Less expectedly, polydispersity also developed upon removal of free APol from an initially monodisperse tOmpA/A8-35 preparation. The phenomenon is reversible and disappears when the overall APol/protein mass ratio is brought back to that used upon trapping. This led us to question our initial conclusion that, in the absence of a competing surfactant, the association of APols with MPs is irreversible, at least on the time scale of several hours (*12*, *18*). By use of a sensitive indicator, FRET between tOmpA and a fluorescent version of A8-35 (FAPol), and working on time scales ranging from 10 ms to more than a day, no evidence, however, was found for any significant desorption of MP-bound APol when the free APol concentration was lowered, even after the mixture of tOmpA/ FAPol complexes and free FAPol particles was diluted 1000× into surfactant-free buffer. In keeping with these observations, diluting the same mixture 10× did not cause any aggregation of the complexes. On the other hand, lowering the *relative* concentration of APol to MP by as little as a factor of 2 (e.g., lowering the overall APol/tOmpA mass ratio from 4:1 to 2:1) led to aggregation, as evidenced by SEC. FRET data indicate that desorption of a fraction of the protein-bound polymer upon lowering the concentration of free polymer cannot be the initial cause of aggregation. Rather, aggregation seems to reflect the poorly dissociating character of A8-35: in a MP/APol mixture, protein/APol interactions compete with protein/protein ones; upon removal of free APol, the equilibrium shifts toward protein aggregation. A likely possibility is that, in solution, tOmpA/A8-35 and pure A8-35 particles collide with each other, leading to transient fusion. Upon collision of two tOmpA/A8-35 complexes, the two proteins may associate, probably releasing some free APol. In the absence of free APol particles, this may lead to progressively building up aggregates. In their presence, on the other hand, the next collision may involve one of those and lead to fragmenting again the oligomers into monomeric MP/APol particles, with concomitant rebinding of APol. The aggregation state of MP/ APol complexes thus would be in a dynamic equilibrium and modulated by the overall MP/APol ratio −rather than the absolute free APol concentration−, reflecting the confinement of the protein to the APol "phase". In the case of tOmpA, the presence of ∼1 free APol particle per tOmpA/ A8-35 complex seems to be both necessary and sufficient, in the buffer and at the temperature used for the present experiments, to ensure monodispersity. It is to be anticipated that this ratio will depend on experimental conditions and may vary from one MP to the next. An interesting implication of this hypothesis is that biologically relevant MP/MP interactions, such as those leading to oligomerization, may possibly take place in the presence of APols.

The aggregation/dissociation mechanism hypothesized above suggests that free APol can exchange with protein-bound ones, despite the absence of spontaneous desorption. This is actually observed upon soaking FAPol-trapped tOmpA into an excess of unlabeled APol: progressive displacement of the protein-bound FAPol is evidenced by a decrease of the FRET signal. A likely mechanism is that

free APol particles transiently fuse with MP/APol complexes and separate again after the APols have mixed. The rate of FRET decrease indeed strongly depends on ionic strength, as expected from a mechanism involving collisions between highly charged particles. In keeping with this interpretation, displacement of FAPol by an excess of the neutral detergent $C_{12}E_8$ was much more rapid and reached completion within <1 s. These observations support and extend the results of earlier exchange experiments using less resolutive approaches (*14, 18*).

Implications. The present observations are of great practical interest on many counts. First, they indicate that, despite the irreversibility of the binding of A8-35 to the transmembrane surface of MPs, the presence of a minimal amount of free APols may be necessary to preserve the monodispersity of MP/APol complexes, an important parameter in many biophysical experiments. Second, they confirm that it is possible to exchange a protein-bound APol for another or to wash it away completely with detergent. Third, they establish that APol-trapped MPs can be extensively diluted into APol-free aqueous buffers without losing their protective APol coat. This is consistent with earlier surface plasmon resonance experiments by Q. Hong and J. H. Lakey (quoted in ref *12*), in which OmpF/A8-35 complexes adsorbed onto a solid support were exposed to a flux of surfactant-free buffer. It opens the way to using immobilized APol-trapped MPs for investigating MP interactions with biological macromolecules or drugs in surfactant-free buffers (Q. Hong and J. H. Lakey, unpublished data; ref *22*). Fourth, the present data strongly suggest that trapping MPs with APols does not prevent them from interacting with one another and that this interaction is reversible. APols have recently been shown to provide an efficient medium for MP renaturation (*26*). The present observations suggest that they may allow refolding proteins to oligomerize, an expectation supported by recent data (T. Dahmane, unpublished observations).

Finally, the present study exemplifies the vast perspectives opened by the versatility of APol chemistry. Because APols are relatively large molecules, discrete grafting with one or a small number of functionalized side chains will not necessarily affect their overall physical–chemical behavior, as shown here for UAPol and FAPol, while endowing them with specific properties. In this work, we have made use of this opportunity to synthesize a fluorescent APol. The same strategy can be used to create a whole range of polymers bearing chemical functions useful for specific biological, biochemical, or biophysical experiments. Upon being trapped with such APols, any MP will become noncovalently but permanently functionalized, without having to be genetically or chemically modified.

ACKNOWLEDGMENT

SUPPORTING INFORMATION AVAILABLE

Synthesis of the protected spacer arm (Scheme S1), synthesis routes to UAPol and FAPol (Scheme S2), relative rates and extents of C_8E_4 and APol adsorption onto polystyrene beads (Figure S1), comments about the extent of adsorption of polyhistidine-tagged tOmpA onto nickel-bearing and Sephadex columns, and description of ionic strength effects on the FRET process (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Dynamics of membrane protein/amphipol association studied by Förster resonance energy transfer.

Implications for *in vitro* studies of amphipol-stabilized membrane proteins

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Synthesis of the protected spacer arm (Scheme S1, Compound **3**)

Though the synthesis of the linker has already been reported (*50*), it has been performed here according to a different route, similar to that described in ref. *51*, so as to make sure that no traces of reactives contaminate the final product. A solution of 5 g 2-bromoethanamine hydrobromide in 50 mL chloroform was cooled to 0°C, supplemented simultaneously with 3.8 mL benzyl chloroformate and 8.2 mL triethylamine, and left to stand for 1 h. The mixture was then stirred during 12 h at room temperature and washed successively in a separatory funnel with 100 mL 1 N HCl, 100 mL distilled water (twice), 100 mL saturated $NaHCO_3$ solution and 100 mL distilled water (twice), before being dried over Na_2SO_4. After filtration and evaporation of the solvent, the residual oil was dissolved in 50 mL diethyloxyde/*n*-hexane 20/80 v/v mixture and kept at 0°C for 12 h. The crystals were filtered, washed with *n*-hexane, and dried under vacuum overnight. The yield of compound **1** is about 56% (3.5 g). Next, 2.7 g of compound **1** mixed with 0.755 g of sodium azide in 10 mL dimethylformamide were stirred and heated at 40°C during 4 h. After cooling, the solution was poured in 100 mL water and the mixture extracted with 100 mL diethyloxyde. The organic layer was washed with 50 mL water and dried over $MgSO_4$. After filtration, the solvent was removed by evaporation under reduced pressure and the residual oil purified on a short silica column (eluent: ethyl acetate/cyclohexane 30/70 v/v). Compound **2** was recovered with 100% yield (1.7 g) and dissol-

ved in 10 mL freshly distilled dry tetrahydrofurane. The solution was degassed and kept under nitrogen atmosphere at 10-15°C. Triphenylphosphine (2.16 g in 10 mL degassed dry THF) was added drop-wise and the mixture stirred at room temperature for ~24 h until total disappearance of compound **2** (as checked by TLC, eluent ethyl acetate/cyclohexane 30/70). 5.8 mL 2 N NaOH were then added and the mixture stirred for two more days at room temperature. The solvent was removed under reduced pressure. 30 mL 1 N chlorhydric acid and 30 mL ethyl acetate were added under vigorous stirring. The organic layer was removed by decantation and the aqueous layer extracted again with 30 mL ethyl acetate and evaporated to dryness under reduced pressure. Residual salts were dissolved in 30 mL methanol and the solvent removed under reduce pressure to dryness. This step was repeated twice, after which the salts were dissolved in 30 mL ethanol, and 4.2 g sodium methoxyde added. The mixture was stirred during 30 min and filtered under suction. 10 mL diethyloxyde were added to the filtrate and the mixture filtered again. The filtrate was acidified with 0.8 mL of concentrated HCl and evaporated under reduced pressure. The last traces of water were removed by two successive evaporations in methanol under reduced pressure. Ammonium salts were dissolved in a small amount of methanol and the methanolic solution added drop-wise to a large excess of diethyloxyde (100 mL). The precipitate was filtered and dried under vacuum overnight to yield compound **3** (1.59 g; 90% yield) as a white powder.

Synthesis of UAPol and FAPol

The synthesis routes to UAPol (Compound **5**) and FAPol (Compound **6**) are summarized in Scheme S2 and described in *Experimental Procedures*.

Detergent adsorption onto Bio-Beads

In Fig. S1 are compared the relative rates and extents of C_8E_4 and APol adsorption onto polystyrene beads.

Adsorption of polyhistidine-tagged tOmpA onto nickel-bearing and Sephadex columns

Possibly because the *N*-terminal his-tag is located very close to the TM β-barrel, which may give rise to steric hindrance, adsorption of the protein onto the immobilized nickel column was less efficient for tOmpA/A8-35 complexes (~65%) than for tOmpA/C_8E_4 ones (100%). Free APol was recovered in the flow-through. After elution with 300 mM imidazole, tOmpA/APol complexes were washed on a desalting Sephadex column. Overall, only 15-20% of tOmpA/APol complexes were recovered. One possible explanation for this low yield is that APols may mediate interaction of the complexes with the Sephadex gel, a step at which no salt was present in the buffer (see *Experimental Procedures*).

Ionic strength effects on the FRET process

In aqueous solution at neutral or basic pH, most of the carboxylate groups of A8-35 are charged (*2*). This confers a high density of negative charges to the APol layer surrounding an A8-35-trapped protein, suggesting that ionic strength may modulate the density of this layer. In Figure S2 are shown the variations of fluorescence intensity of tOmpA complexed by either detergent or FAPol, as well as that of the FRET signal, as a function of NaCl concentration.

There is no obvious salt effect on protein fluorescence in detergent solution. After complexation by FAPol, on the other hand, the protein fluorescence drops by ~7% at high salt concentration, while FRET rises by ~15% (data are corrected for the contribution of direct excitation of FAPol). The underlying molecular mechanism could be a slight compaction of the APol layer in the presence of salt. Because of the inverse sixth-power distance-dependence of the FRET signal, the intensity of FRET increase can be accounted for by a very small decrease (~1 Å) in donor/acceptor average distances. On the other hand, it cannot be excluded that decreasing electrostatic repulsion leads to an increase of FAPol binding (*cf.* ref. *18*). In this case, the excess bound FAPol would be ~15% if the average donor/acceptor distance does not change, more if it increases. The two mechanisms are not exclusive.

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Captions to supplementary schemes and figures

SCHEME S1: **Synthesis of the selectively monoprotected etylene diamine derivative (3) from 2-bromoethanamine hydrochloride (1).** Step **a**: benzyl chloroformate, TEA/chloroform, 0°C for 1 h, then 25°C for 12 h. Step **b**: NaN$_3$/DMF, 40°C, 3 h. Step **c**: P(Ph)$_3$/THF, neutral atmosphere, 24 h. Step **d**: NaOH 2 N, 48 h. Step **e**: HCl/H$_2$O.
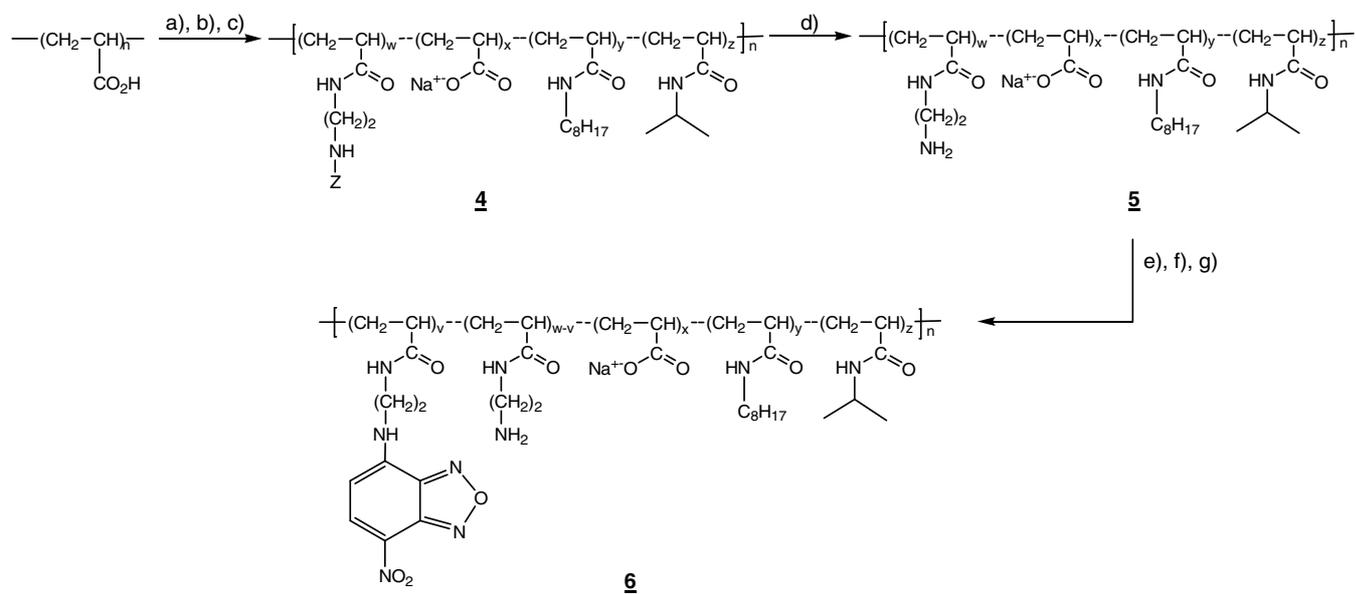
SCHEME S2: **Synthesis of UAPol (Compound 5) and FAPol (Compound 6) from the PAA precursor.**

FIGURE S1: **Specific removal of C$_8$E$_4$ by polystyrene beads.** The loss of surfactant by adsorption onto the beads was followed (in two parallel exeriments) by UV-visible measurements at 205 nm for the C$_8$E$_4$ sample and 475 nm for the FAPol one. The initial concentrations of detergent and FAPol were 6 g.L^{-1} and 1 g.L^{-1}, respectively. The surfactant:beads mass ratio was 1:10 in both cases. The buffer was 20 mM Tris/HCl, pH 8.
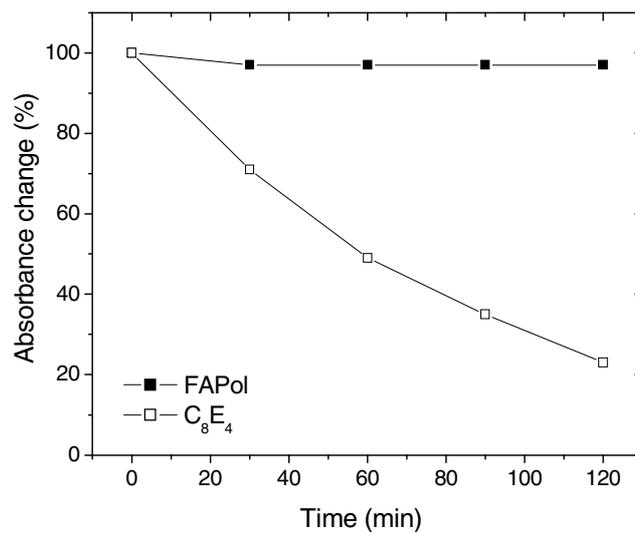
FIGURE S2: **Effect of salts on the FRET process.** Fluorescence intensity at 325 nm of tOmpA complexed by either detergent or FAPol, and, in the latter case, fluorescence intensity at 530 nm (FRET signal). FRET intensities have been corrected to take into account the higher quantum yield of NBD in the presence of salt (*39*). The protein concentration was the same in all samples (0.01 g.L^{-1}). The buffer contained 20 mM Tris-HCl, pH 8.0, and the concentrations of NaCl indicated.
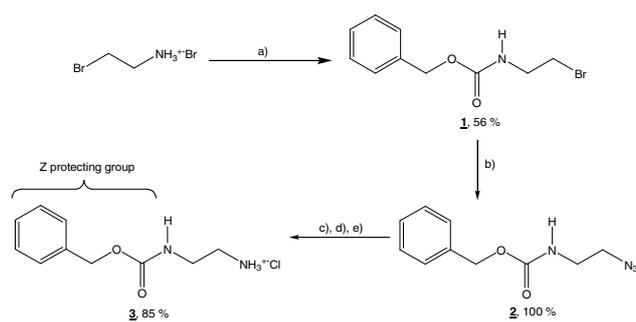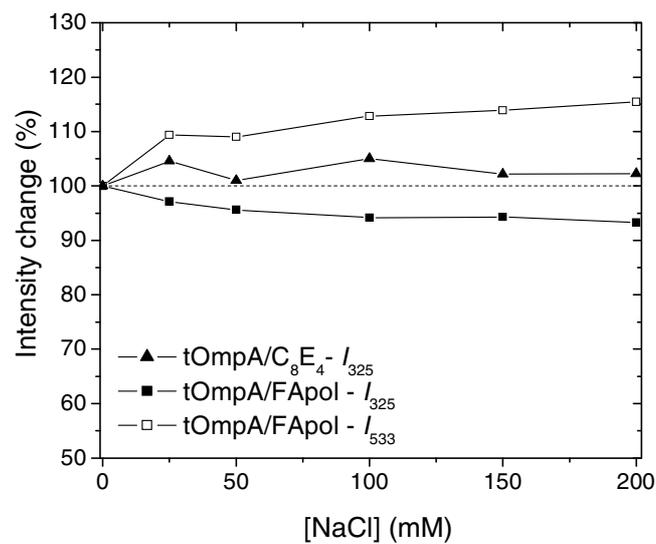
Scheme S1

Scheme S2

Figure S1

Figure S2