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TOPICAL REVIEWS

Labeling and Functionalizing Amphipols for Biological Applications

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Abstract Amphipols (APols) are short amphipathic polymers developed as an alternative to detergents for handling membrane proteins (MPs) in aqueous solution. MPs are, as a rule, much more stable following trapping with APols than they are in detergent solutions. The best-characterized APol to date, called A8-35, is a mixture of short-chain sodium polyacrylates randomly derivatized with octylamine and isopropylamine. Its solution properties have been studied in detail, and it has been used extensively for biochemical and biophysical studies of MPs. One of the attractive characteristics of APols is that it is relatively easy to label them, isotopically or otherwise, without affecting their physical-chemical properties. Furthermore, several variously modified APols can be mixed, achieving multiple functionalization of MP/APol complexes in the easiest possible manner. Labeled or tagged APols are being used to study the solution properties of APols, their miscibility, their biodistribution upon injection into living organisms, their association with MPs and the composition, structure and dynamics of MP/APol complexes, examining the exchange of surfactants at the surface of MPs, labeling MPs to follow their distribution in fractionation experiments or to immobilize them, increasing the contrast between APols and solvent or MPs in biophysical experiments, improving NMR spectra, etc. Labeling or functionalization of APols can take various courses, each of which has its specific constraints and advantages regarding both synthesis and purification. The present review offers an overview of the various derivatives of A8-35 and its congeners that have been developed in our laboratory and discusses the pros and cons of various synthetic routes.

Keywords Membrane protein · A8-35 · Amphipathic polymers · Fluorophores · Immobilization · Isotopic labeling

Abbreviations

A8-35	Poly(sodium acrylate) based amphipol comprising 35 % of free carboxylate, 25 % of octyl chains, 40 % of isopropyl groups, and whose number-average molar mass is ~4.3 kDa
A8-75	Poly(sodium acrylate) based amphipol comprising 75 % of free carboxylate, 25 % of octylchains, and whose number-average molar mass is ~4 kDa
APol	Amphipol
AUC	Analytical ultracentrifugation
BAPol	Biotinylated A8-35
Đ	Molar mass dispersity
DAPol	A8-35 with deuterated octylamine and isopropylamine side chains
DCI	*N*,*N*′-dicyclohexylcarbodiimide
DCU	Dicyclohexylurea
DTT	Dithiothreitol
EDC	Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride
FAPol	Fluorescently labeled A8-35
$FAPol_{AF647}$	Alexa Fluor 647-labeled A8-35
$FAPol_{NBD}$	Nitrobenzoxadiazole-labeled A8-35
$FAPol_{rhod}$	Rhodamine-labeled A8-35
FRET	Förster resonance energy transfer
HAPol	Hydrogenated A8-35

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HMPA	Hydrophobically modified poly(acrylic acid)
HMPAS	HMPA synthesis
His_6PEG	*N*-(penta(histidyl)histidinamide)-8-amino-3,6-dioxa-octanamide
His-tag	Hexahistidine tag
HistAPol	Hexahistidine tag-carrying A8-35
HOBt	1-*N*-hydroxybenzotriazole
IMAC	Immobilized metal ion affinity chromatography
ImidAPol	Imidazole-carrying A8-35
INS	Inelastic neutron scattering
$\langle M_n \rangle$	Number-average molar mass
MP	Membrane protein
NBD	7-Nitrobenz-2-oxa-1,3-diazol-4-yl
NHS	*N*-hydroxysuccinimide
NOE	Nuclear Overhauser effect
NTA	Nitrilotriacetic acid
ODN	Oligodeoxynucleotide
OligAPol	ODN-carrying A8-35
PAA	Poly(acrylic acid)
perDAPol	Perdeuterated A8-35
SANS	Small angle neutron scattering
SAPol	Sulfonated amphipol derived from A8-75, comprising 40 % of taurine moieties
SPR	Surface plasmon resonance
TES	Triethylsilane
TFA	Trifluoroacetic acid
ThiAPol	Thiol-carrying APol
Tsv	Tosvinyl group
UAPol	Universal amphipol
UAPol-NH_2	Amine-carrying A8-35

Introduction

Over the past 18 years, specially designed amphipathic polymers called 'amphipols' (APols) have developed into versatile tools to handle membrane proteins (MPs) in aqueous solutions (Popot 2010; Popot et al. 2003, 2011; Sanders et al. 2004; Zoonens and Popot 2014). Trapping MPs with APols is particularly simple: the protein, usually in detergent solution, is supplemented with APol, and the detergent eliminated (Tribet et al. 1996; Zoonens et al. 2014). The resulting MP/APol complexes are highly soluble and, in most cases, the protein is considerably stabilized as compared to what can be achieved with detergents (see, e.g., refs. (Bazzacco et al. 2012; Champeil et al. 2000; Dahmane et al. 2009; Dahmane et al. 2013; Feinstein et al. 2014; Gohon et al. 2008; Tifrea et al. 2011; Tribet et al. 1996; Zoonens et al. 2014), and references therein). APols interact specifically with the hydrophobic transmembrane surface of MPs (Althoff et al. 2011; Catoire et al. 2009, 2010a; Etzkorn et al. 2014; Perlmutter et al. 2014; Planchard et al. 2014; Zoonens et al. 2005), leaving extramembrane surfaces freely accessible to ligands big and small (Bazzacco et al. 2012; Catoire et al. 2010a; Champeil et al. 2000; Charvolin et al. 2009; Dahmane et al. 2009; Martinez et al. 2002; Perlmutter et al. 2014; Tifrea et al. 2011). Due to the multipoint attachment of APols to the MP surface, as well as to their very low critical association concentration (Giusti et al. 2012), MP/APol complexes do not disassemble even at extreme dilution (Tribet et al. 2009; Zoonens et al. 2007), and APol-trapped MPs remain native and functional in surfactant-free buffers (Charvolin et al. 2009; Giusti et al. 2014a; Le Bon et al. 2014). Because MP-adsorbed APols do not desorb unless displaced by another surfactant (Tribet et al. 2009; Zoonens et al. 2007), trapping a MP with a functionalized APol will effectively functionalize the protein, without any covalent modification to it.

APols are relatively large molecules: the number-average molar mass of the most common APol, called A8-35 (see below), is ~4.3 kDa (Giusti et al. 2014b), that of more recently developed phosphocholine-based (Diab et al. 2007a, b), sulfonated (Dahmane et al. 2011; Picard et al. 2006) or glycosylated, non-ionic (Bazzacco et al. 2012; Sharma et al. 2012) APols similar or larger. APols assemble into small, globular, micelle-like particles, with, in the case of A8-35, a particle mass of ~40 kDa (Gohon et al. 2004, 2006), that is, on average, ~9 molecules. As previously observed for longer polymers (Morishima et al. 1995; Ringsdorf et al. 1991), a broad range of chemical modifications can therefore be brought to the basic structure of APols without compromising the solubility of the molecules, nor their ability to self-associate and to adsorb onto MP hydrophobic transmembrane surfaces, nor the solution properties of the resulting MP/APol complexes. Furthermore, because (1) each MP binds several APol molecules—bacteriorhodopsin, for instance, binds ~54 kDa of A8-35 (Gohon et al. 2008), i.e., ~12–13 molecules, and (2) APols mix freely both in particles and at the surface of MPs (Giusti et al. 2012; Zoonens et al. 2007), multiple functionalized complexes can be formed by the most simple device of trapping the protein with a mixture of APols [see, e.g., refs. (Della Pia et al. 2014a; Le Bon et al. 2014)].

As described in "Design, Synthesis and Characterization of APol A8-35" section, the best-characterized APol, A8-35 (Tribet et al. 1996), is obtained by derivatizing short-chain poly(acrylic acid) (PAA) with octylamine and isopropylamine (Fig. 1A). Most labeled or tagged APols reported to date are derivatives of either A8-35 or one of its analogs, A8-75 (Table 1), and it is to the synthesis and purification of those that this review will be devoted, but

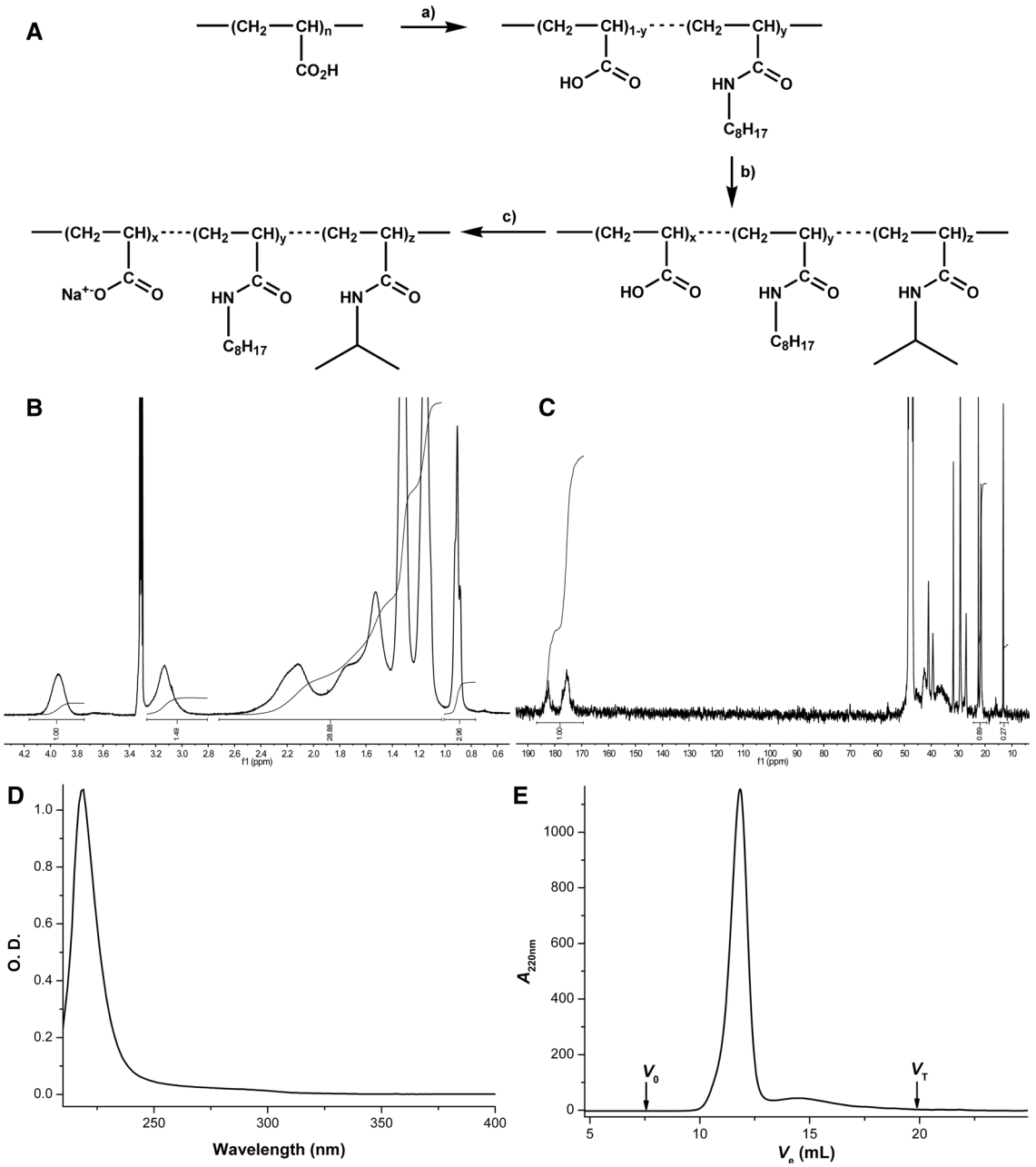

Fig. 1 **A** Synthesis of A8-35 by hydrophobic modification of a PAA precursor (HMPAS). (*a*) *n*-octylamine (0.25:1 molar ratio to PAA units), DCC/NMP, 60 °C, 1 h then RT 4 h; (*b*) isopropylamine (0.40:1 molar ratio to PAA units), DCC/HOBt/NMP, 50 °C, 1 h, then RT 4 h; (*c*) MeONa, followed by four cycles of precipitation in aqueous solution at pH < 2 and dissolution at pH > 8. **B** ^{1}H NMR spectrum of A8-35. **C** ^{13}C NMR spectrum of A8-35. **D** UV-visible light absorption spectrum of A8-35 recorded at 0.8 g L^{-1}. **E** SEC profile of A8-35. The chromatogram was obtained after injection of a 1 % A8-35 aqueous solution onto a Superose 12 10–300 GL column equilibrated with Tris buffer (20 mM Tris/HCl, 100 mM NaCl, pH 8.0), connected to a FPLC Äkta system. Detection at 220 nm. See refs. (Gohon et al. 2004, 2006) for further details

other functionalized APols are starting to appear, such as biotinylated derivatives of phosphocholine-based (Basit et al. 2012) or non-ionic (Ferrandez et al. 2014) APols. In the present review, we offer an overview of the strategies and techniques involved in the development of A8-35 and its derivatives. Some ensuing insights are provided as guidelines for further developments.

Design, Synthesis and Characterization of APol A8-35

A8-35 is obtained by grafting a certain percentage of akylamines onto the carboxylates of a PAA precursor, usually obtained from a commercial source (Tribet et al. 1996) (Fig. 1A). The length and ratio of the hydrophobic side chains are chosen so as to confer a sufficient hydrophobicity

Table 1 Tagged and labeled derivatives of A8-35 and A8-75 and their uses

Type of modification	Amphipol modified (short name of derivative)	Applications	References
Isotopic labeling			
^{14}C	A8-75	Following A8-75 distribution and exchange.	Tribet et al. (1997)
^{3}H	A8-35	Following A8-35 distribution; evaluating MP/A8-35 mass ratio in complexes.	Charvolin et al. (2014), Gohon et al. (2008)
^{2}H (on side chains)	A8-35 (DAPol)	Eliminating protons for NMR measurements. Contrast matching in SANS, AUC, INS.	Catoire et al. (2009, 2010a), Etzkorn et al. (2014), Gohon et al. (2004, 2006, 2008), Planchard et al. (2014), (Tehei et al. 2014, Zoonens et al. (2005)
^{2}H (perdeuteration)	A8-35 (perDAPol)	Eliminating protons for NMR measurements. Contrast matching in SANS, AUC, INS.	Giusti et al. (2014b)
Fluorescent labeling (λ_{exc}, λ_{em})			
Naphthalene (290 nm/310–370 nm)	A8-75	Studying A8-75 interactions with lipid vesicles.	Vial et al. (2005)
NBD (470 nm/530 nm)	A8-35 ($FAPol_{NBD}$)	Studying A8-35 distribution, binding and exchange. Determining the CAC of A8-35 by FRET with $FAPol_{rhod.}$	Charvolin et al. (2014), Giusti et al. (2012), Le Bon et al. 2014), Zoonens et al. (2007)
Fluorescein (495 nm/540 nm)	A8-35 ($FAPol_{fluo}$)	Mapping the transmembrane region of a MP by FRET with single-tryptophan mutants.	Opačić et al. (2014)
Rhodamine (555 nm/575 nm)	A8-35 ($FAPol_{rhod}$)	Examining the distribution of A8-35 upon delivery of a transmembrane peptide to cells in culture. Determining the CAC of A8-35 by FRET with $FAPol_{NBD}$. Following the biodistribution of APols in mice.	Fernandez et al. (2014), Giusti et al. (2012)
Atto 647 (651 nm/667 nm)	A8-35 ($FAPol_{atto647}$)	Following in vivo distribution and elimination of A8-35.	(unpublished results)
Alexa Fluor 647 (651 nm/668 nm)	A8-35 ($FAPol_{AF647}$)	Following in vivo distribution and elimination of A8-35. Tracer in APol-mediated immobilization of MPs.	Della Pia et al. (2014a, b, Fernandez et al. (2014), Le Bon et al. (2014)
Tags, adjuvants			
Biotin	A8-35 (BAPol)	Immobilizing MPs onto chips or beads for SPR or fluorescence measurements. Selecting soluble protein binders against immobilized MPs.	Charvolin et al. (2009), Della Pia et al. (2014), Fernandez et al. (2014a, b)
Polyhistidine	A8-35 (HistAPol)	Reversibly immobilizing MPs.	Giusti et al. (2014a)
Randomly distributed imidazole moieties	A8-35 (ImidAPol)	Reversibly immobilizing MPs.	Giusti et al. (2014a)
Oligodeoxynucleotide	A8-35 (OligAPol)	Vaccination, using the oligodeoxynucleotide as an adjuvant. Immobilizing MPs onto DNA chips.	Le Bon et al. (2014)

Adapted from ref. (Zoonens and Popot 2014)

to the polymer without compromising its solubility in aqueous media. In A8-35, *n*-octylamine and isopropylamine are grafted at molar ratios of ~25 and ~40 %, respectively, leaving free ~35 % of the original carboxylates. The latter, when all of them are ionized, i.e., at pH ≥ 7, make A8-35 highly water soluble (>200 g L^{-1}), whereas the octyl chains make it amphipathic. The isopropyl groups limit the charge density, which, if it is too high, seems to negatively affect MP stability (Bazzacco et al. 2012; Dahmane et al. 2011; Picard et al. 2006), without contributing much to the global hydrophobicity at low or ambient temperature (Takei et al. 1993).

The synthesis of A8-35 is carried out in two steps: octylamine is grafted onto PAA first, yielding an intermediate, called A8-75, which is itself an APol (Tribet et al. 1996; Tribet et al. 1997), isopropylamine second. Grafting is achieved in the presence of dicyclohexyl-carbodiimide (DCI) in *N*-methylpyrrolidone (NMP), as described in Ref. (Wang et al. 1988) (hydrophobically modified PAA synthesis, hereafter HMPAS). Under the conditions used, the grafts distribute randomly along the macromolecular chain (Magny et al. 1992). In order to reproducibly obtain batches of A8-35 with the nominal composition and, therefore, the physical-chemical properties of standard batches, careful attention must be paid to two critical points: (i) the initial alkylamine/PAA (R_1) and *n*-octylamine/isopropylamine (R_2) ratios must be strictly controlled; (ii) DCI must be used only in moderate excess (i.e., no more than 1.1 equivalent per alkylamine). When too large an excess of DCI is used, transposition of some of the activated intermediate *O*-acylurea to the very stable *N*-acylurea by-product takes place. This results in the artifactual grafting onto the PAA backbone of dicyclohexylurea (DCU), which increases the hydrophobicity of the polymer and perturbs its self-association behavior in water: instead of assembling into small, well-defined particles, DCU-grafted A8-35 forms collections of heterogeneous aggregates (Gohon et al. 2004, 2006), and so do MP/polymer complexes (Gohon et al. 2008).

Following synthesis, A8-35 is purified according to a procedure that itself requires some care. It takes advantage of the fact that the sodium salt of A8-35 is water soluble where as the acid form is not. Hence, insoluble hydrophobic by-products (DCU, mostly) can be removed by filtration or centrifugation of a basic solution, whereas when the pH is brought to 2, the precipitated acid can be separated from most of the water-soluble contaminants (mostly NMP and protonated unreacted alkylamines). In practice, because of the amphipathic nature of the polymer, which tends to trap both hydrophobic and hydrophilic contaminants, purification of A8-35 requires at least three cycles of dissolution-precipitation. pH changes must be instantaneous in order to avoid the formation of metastable aggregates: the size distribution of the particles formed by basic A8-35 must reach its thermodynamic equilibrium, and not become kinetically trapped (see below). Following the cycles of precipitation-dissolution, the last traces of hydrophilic co-solutes (NaCl, NMP) are removed by dialysis against a dilute solution of sodium hydroxide (pH = 8). Freeze-drying of the dialyzed solution, which yields the sodium salt of A8-35 as a white powder, is straightforward.

Prior to distribution, the chemical composition of each new batch of A8-35 is verified by NMR (Fig. 1B, C) and its behavior in aqueous solution is controlled by size exclusion chromatography (SEC) (Fig. 1E) (Gohon et al. 2004, 2006). A UV-visible light absorption spectrum of purified A8-35 (Zoonens et al. 2007) is shown in Fig. 1D.

The mass distribution of the molecules of A8-35 obtained by hydrophobization of a commercial PAA is relatively broad, with a molar mass dispersity $Đ \approx 2$ and a number-average molar mass $\langle M_n \rangle \approx 4.3$ kDa ($\sim$35 acrylate units), both of them deduced from those of the PAA (Giusti et al. 2014b). The extent of polydispersity of A8-35 molecules does not seem to influence the size nor the relative homogeneity of the particles formed in aqueous solution, batches of A8-35 with a much narrower mass distribution behaving, upon SEC, indistinguishably from classical batches (Tribet and F.G., unpublished data). As discussed elsewhere [see, e.g., ref. (Popot et al. 2011)], it is not certain that a narrower polydispersity would be an advantage when it comes to trapping MPs, because it reduces the variety of molecules that can come together to form an APol belt with a thermodynamically optimized geometry.

Functionalization Intermediates ('UAPols')

General Considerations

Labeling or functionalizing A8-35 can be achieved in several ways (Fig. 2). A labeled or functionalized amine may be grafted onto the polymer backbone during (Fig. 2A) or after (Fig. 2B, C) the synthesis of the APol. Grafting is performed after the synthesis if the grafted moiety is unstable under HMPAS conditions, but also when the amine is not soluble in NMP. In that case, labeling is carried out in another organic solvent, or in aqueous medium. In the latter case, grafting takes place at the surface of already formed A8-35 particles and not on isolated macromolecular chains as in NMP solution (Fig. 2C). Finally, labeling can be performed onto a pre-functionalized version of A8-35 bearing a reactive arm (Figs. 2B, 3).

Pre-functionalizing A8-35 presents a number of advantages: it provides a route to the synthesis of labeled or tagged versions of A8-35, but it also ensures that whole sets of derivatives will feature exactly the same R_1 and R_2 ratios (see "Design, Synthesis and Characterization of APol A8-35"), and therefore, in principle, the same solution behavior. For this reason, this kind of general precursor has been dubbed 'universal amphipol' (UAPol) (Zoonens et al. 2007).

Synthesis of UAPols: Successes and Failures

UAPol synthesis can in principle follow either of two distinct routes: (i) synthesis of a pre-functionalized PAA and subsequent hydrophobization; or (ii) functionalization during HMPAS. The synthesis of a pre-functionalized homopolymer is one of the most sophisticated and widespread strategies developed for polymer functionalization. It consists in the controlled radical polymerization of a

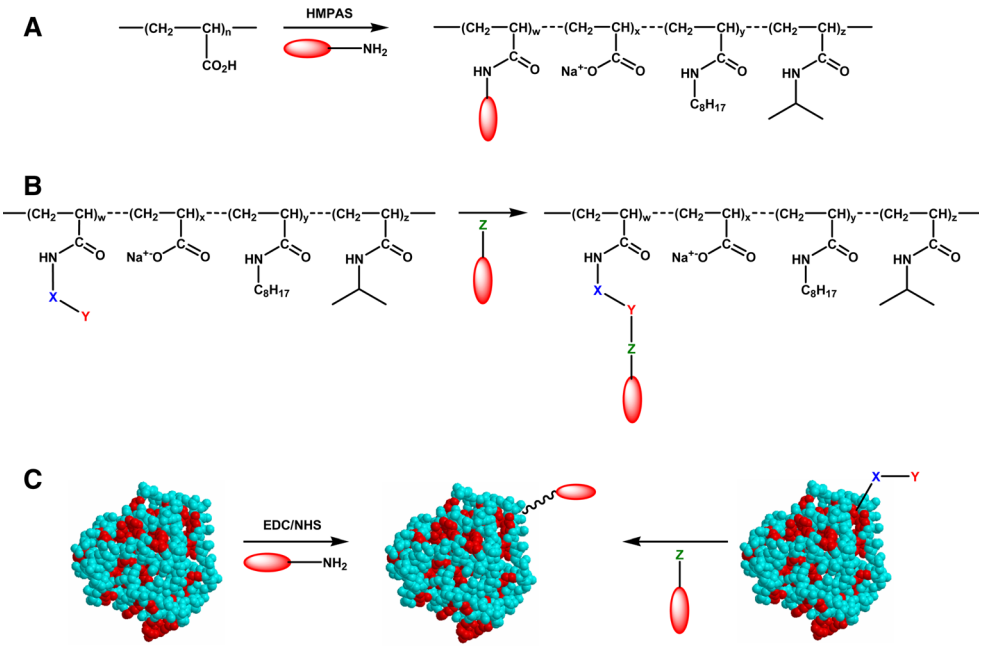

Fig. 2 Different approaches to labeling A8-35. **A** By reacting an amino derivative of the probe with the PAA carboxylic group via HMPAS. **B** By reacting a pre-functionalized APol (UAPol) in organic medium with an activated probe. **C** In aqueous buffer, by reacting an A8-35 or UAPol particle with a suitable probe derivative. An amino derivative of the probe can be directly reacted with activated carboxyl groups of the particle, formed after treatment with EDC in the presence of NHS. Labeling may also be achieved by reacting a UAPol particle with the suitable probe derivative (*inset*)

X	Y	Z	Co-reactant or catalysor
$-(CH_2)_n-$ or $-(CH_2CH_2O)_n-$	Amino group	NHS-ester	Weak basis
		Aldehyde Ketone Sugar	Reducer ($NaBH_3CN$, $NaBH(OAc)_3$)
	Aldehyde Ketone Sugar	Amino group	
	Sulfhydryl group	Maleimide Br, I	Weak basis
	Azido group	Alkyne	Cu I salts
	Alkyne	Azido group	

monomer performed in the presence of a functionalized transfer agent. This transfer agent, which allows the control of the length of the polymer, reacts in the first step of the polymerization and attaches the functional moiety at one or both extremities of the macromolecule (Grover et al. 2009; Javakhishvili and Hvilsted 2009; Roth et al. 2010). It is not straightforward, however, to use this approach to prepare functionalized APols with the same average length and dispersity as classical A8-35: first, the size distribution obtained in this way is narrower than that of the commercial PAA used for the synthesis of A8-35; second, the average length of this PAA would be difficult to match. This led to favoring a simpler route, according to which the reactive arm is incorporated into the polymer during HMPAS. To this end, a low molar ratio of an X-terminated short aminoalkane (~3 % of PAA units), where X is a reactive function, is added to octylamine at the first step of HMPAS (Fig. 2B). Under our usual conditions, *ca.* one linker is added per polymer chain. If there be a risk that X reacts with activated carboxylic groups, it is protected in order to avoid polymer cross-linking as a side reaction.

Among all the possibilities that could be envisioned for X, only those permitting the formation of a stable and non-hydrolysable bond were considered. Among them are as follows: an amine; a sulfhydryl; a carbonyl; a sugar; or an alkyne (or azide) (Fig. 2). In the following two subsections, we discuss the cases of amino- and thiol-carrying UAPols.

Amine-Carrying UAPol (UAPol-NH_2): A Highly Convenient Tool

UAPol-NH_2 was designed to form a stable covalent bond upon reacting with isocyanate, isothiocyanate or activated-ester derivatives of a probe. In principle, UAPol-NH_2 could also be used to attach a functional group by reductive amination (by reacting the amine with a carbonyl derivative), but this pathway was only briefly explored when trying to couple a carboxaldehyde-5′-terminated oligonucleotide (see section "Direct labeling of A8-35 particles: synthesis of an oligodeoxynucleotide-carrying APol"). It offers limited prospects, most of the commercially available functionalized probes that are designed to be

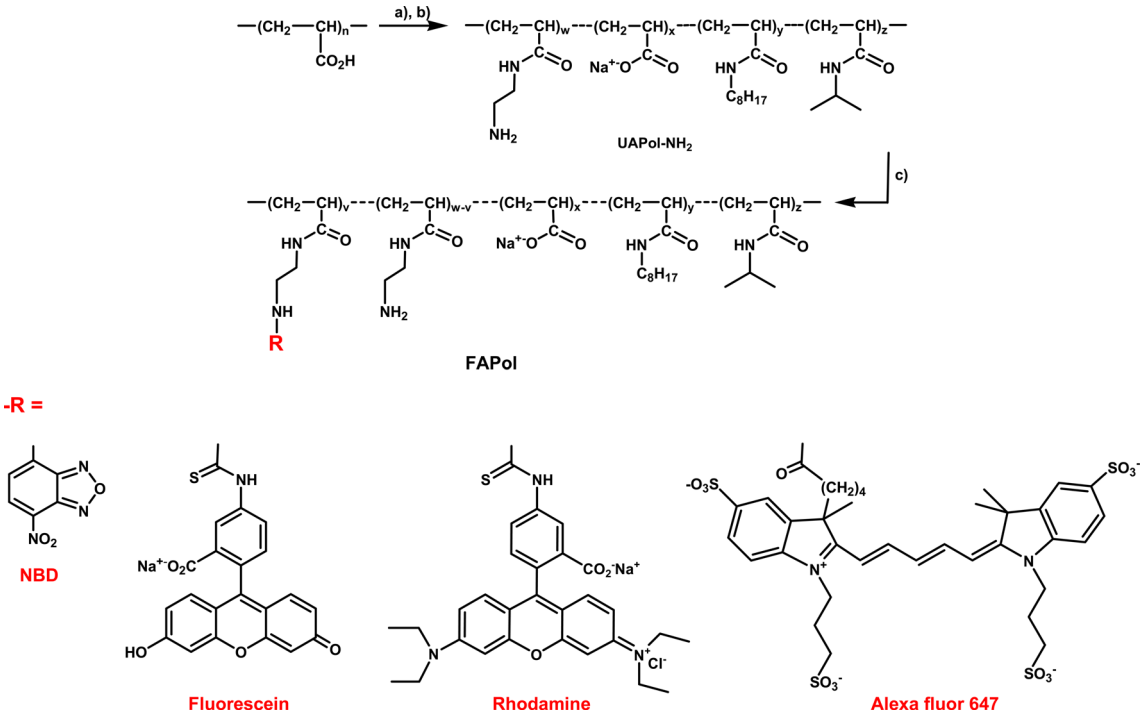

Fig. 3 Synthesis of UAPol-NH_2 and some fluorescent APols derived from it. (*a*) Z-$NHCH_2CH_2NH_3^+$,Cl^-, HMPAS; (*b*) HCO_2^-,NH_4^+/Pd/C, MeOH, 12 h; (*c*) either NBD-Cl/MeOH, TEA(cat.), Δ, 4 h, yielding $FAPol_{NBD}$ (Giusti et al. 2012; Zoonens et al. 2007) or FITC (4 eq.), DABCO (cat.)/DMF, 50 °C, 4 h, yielding $FAPol_{fluo}$ (Opačić et al. 2014) or RITC (4 eq.), DABCO (cat.)/DMF, 50 °C, 4 h, yielding $FAPol_{rhod}$ (Giusti et al. 2012) or NHS-Alexa Fluor 647, DMF, TEA(cat.), 40 °C, 12 h, yielding $FAPol_{AF647}$ (Fernandez et al. 2014; Le Bon et al. 2014)

bound using reductive amination carrying an amino group (amine or hydrazine), not a carbonyl one.

The amino function of UAPol-NH_2 was introduced by grafting a short home-made selectively mono-protected α,ω-diaminolinker, *N*-benzyloxycarbonyl-ethylenediamine (Z-ethylenediamine). The reactivity of the amino group was recovered after submitting the resulting UAPol-NH-Z to catalytic hydrogenolysis, yielding UAPol-NH_2 (Fig. 3) (Zoonens et al. 2007). Even though the amino linker was incorporated at the expected level, UAPol-NH_2 was found to react only weakly to moderately with isothiocyanate (Giusti et al. 2012; Opačić et al. 2014; Zoonens et al. 2007) and NHS-ester (Fernandez et al. 2014; Le Bon et al. 2014) derivatives of various fluorescent probes (Fig. 3). This point will be discussed in "Labeled and functionalized versions of A8-35". Nevertheless, given the high sensitivity of fluorescence-based approaches, the fluorescent APols (FAPols) thus obtained have been highly helpful in a broad variety of experimental circumstances (Table 1).

Thiol-Carrying A8-35: A Biochemist's Dream, A Chemist's Nightmare

It is not customary to draw at length on one's failures. However, given the great interest that a thiol-carrying APol (ThiAPol) would present and the considerable efforts we have invested trying to synthesize it, we deem it useful to say a word of the difficulties that were encountered in the process.

The concept of ThiAPol arises from the well-documented versatility offered by the chemistry of the couples thiol/maleimide (Gauvreau et al. 2004; Warnecke and Kratz 2003), thiol/alkyl halide (Frey et al. 1997) and thiol/activated thiol (Roth et al. 2010; Woghiren et al. 1993; Zugates et al. 2006). Indeed, thiols react quantitatively with maleimide or alkyl halides to form a stable thioether bond, and with activated thiols to form a covalent but labile disulfide bond. Thus, a ThiAPol could be expected to provide higher yields of coupling as compared to UAPol-NH_2. Moreover, the reversible character of the disulfide bond [free thiols are regenerated upon reduction (Kikuzawa et al. 2007)] would offer the opportunity to endow A8-35 with removable functions. Such derivatives could be used, for instance, to ensure the controlled release of drugs, probes or other conjugates following exposure of functionalized ThiAPols to a reducing cellular environment such as the cytosol (Sauer et al. 2010) or the lysosomal lumen (Stefano et al. 2009). ThiAPols could also serve to immobilize MP/APol complexes onto gold surfaces, as is classically done with soluble proteins (Yoshimoto et al. 2008), cells (Murphy et al. 2004; Roberts et al. 1998) or

integral MPs (Terrettaz et al. 2002). For all these reasons, developing a ThiAPol appeared as a promising endeavor.

It seemed, initially, that a ThiAPol could be obtained rather simply by incorporating an α,ω-aminoalkanethiol into A8-35 *via* HMPAS. The use of cysteine derivatives was avoided because of (i) their high tendency to spontaneous oxidation upon exposure to air [whether the cysteine is incorporated into a protein (Andreu et al. 1994; Gielens et al. 1997) or into a synthetic polymer (Bernkop-Schnurch et al. 1999; Kast and Bernkop-Schnurch 2001)] and (ii) the low stability of cysteamine and cysteine in basic media (Nicolet 1931). Instead, a longer thiolated spacer arm was designed. The length of the alkyl chain of the linker was fixed at six methylenes, so as to deal with non-volatile intermediates without excessively increasing the hydrophobicity.

It was expected that an *N*-(UAPol)-tethered aminohexanethiol would oxidize upon exposing the polymer to the very basic condition (pH > 10) used during purification. Conversion of free thiols into disulfides is not a major issue as long as those can be reduced again to thiols. However, disulfide bonds are known to be unstable in aqueous solution above pH 8, converting to aldehydes or sulfoxides, and, subsequently, to sulfones or sulfonates, depending on the pH of the solution (Danehy and Hunter 1967; Danehy and Kreuz 1961), making protection mandatory. Among the many protection methods available (Andreu et al. 1994; Greene and Wuts 2002), the use of a trityl group was particularly attractive, because this protecting group is known to be perfectly stable in basic media and it can be removed under non-hydrolytic acid conditions (Harding et al. 2002).

Two linkers were first synthesized (Fig. 4A), an *S*-tritylated aminoalkanethiol, 1-amino-6-tritylsulfanyl-hexane (**1**), and a 'mock' linker, 1-acetylmercapto-6-amino-hexane (**2**). Both the tritylated and the acetylated linkers were successfully bound onto the PAA backbone to yield, after further hydrophobization, the expected *S*-tritylated ThiAPol (ThiAPol-STr) and deacetylated ThiAPol (ThiAPol-S-R_2) (Fig. 4B).

The amount of free thiol (R_2 = -H) in ThiAPol-S-R_2, which was estimated by Ellman's method (Ellman 1959), was found to be only $\sim$2 % of that of the incorporated linker, indicating that a labile protecting group like acetyl is unable to prevent thiol oxidation under the rough conditions used for purifying ThiAPol. Treating the oxidized ThiAPol by various reducing agents (Hansen and Winther 2009; Humphrey and Potter 1965) did not regenerate the expected amount of free thiol, suggesting either the degradative oxidation of most of the disulfide bridges (Danehy and Hunter 1967; Danehy and Kreuz 1961) and/or a poor accessibility of the bridges to the reducing agent.

Surprisingly, however, similar observations were also done with the ThiAPol-SH yielded by deprotecting ThiAPol-STr in the presence of trifluoroacetic acid (TFA) and triethylsilane (TES). Subsequent treatment of ThiAPol-SH by dithiothreitol (DTT) did not regenerate any free thiols. Although, it seems unlikely that thiols oxidized in the presence of TFA and TES, it cannot be excluded that they reacted with carboxylic acid functions of the polymer to form thioester bonds, which would resist treatment with DTT.

Finally, an attempt was made to develop a ThiAPol that could be deprotected under non-hydrolytic basic conditions. A third linker (**3**) was synthesized, whose thiol was protected by a tosvinyl (Tsv) group (Arjona et al. 2003) (Fig. 4A). The resulting ThiAPol-S-Tsv was submitted to deprotection in a hot basic organic mixture (Fig. 4B), but this treatment yielded a disulfide-bridged ThiAPol (ThiAPol-S-S-R). Treatment by DTT yielded quantitatively the free thiol, but the latter quickly irreversibly oxidized once in contact with aqueous buffer. This brought an end to our attempts at producing ThiAPol.

To summarize, the thiol functions of ThiAPol spontaneous oxidize in basic organic media, forming disulfides, whereas they are converted into another, unidentified derivative [possibly a thioether; see Ref. (Blackburn and Lee 1956)], whatever the pH, when in contact with a solvent containing more than 5 % water.

Labeled and Functionalized Versions of A8-35

The labeled and functionalized APols that have been described to date are listed in Table 1, along with references to the studies in which they have been used. Three approaches have been used to obtain them: (1) introducing the label or tag at the time of hydrophobization (Fig. 2a) (section "Labeling A8-35 via HMPAS"), (2) grafting it onto UAPol-NH_2 in organic solution (Fig. 2b) (section "Labeling A8-35 by Reacting UAPol-NH_2 with an Activated Probe: Synthesis of Fluorescent APols") and (3) grafting it in aqueous solution onto already assembled particles of plain A8-35 (Fig. 2c) (section "Direct Labeling of A8-35 Particles: Synthesis of an Oligodeoxynucleotide-Carrying APol").

Labeling A8-35 via HMPAS

A number of derivatives of A8-35 could be obtained by incorporating the label or tag during PAA hydrophobization. They include several isotopically labeled versions of A8-35, a biotinylated derivative, and two derivatives carrying imidazole groups.

Isotopically Labeled APols

Deuterated A8-35 ('DAPol') (Fig. 5) was initially developed as a tool for studying the physical-chemical properties of A8-35 and MP/A8-35 complexes in aqueous solutions

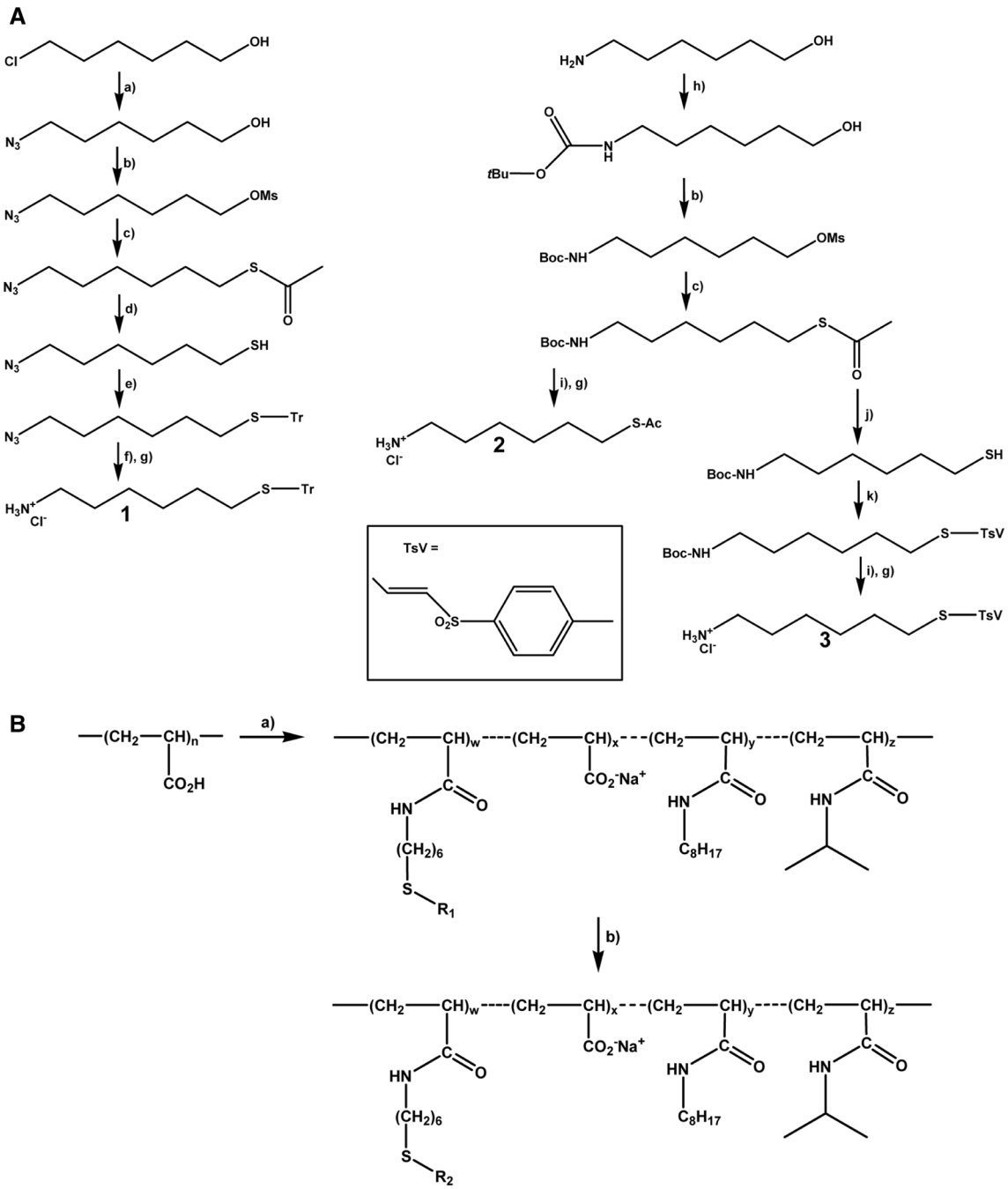

Fig. 4 **A** Synthesis pathway to the three thiolated spacer arms. (*a*) NaN_3/DMF, Δ, 12 h, 90 % [see refs. (Collman et al. 2004; Davis et al. 1999)], (*b*) CH_3SO_2Cl, TEA in 2:1 Et_2O:CH_2Cl_2, 100 % [see Ref. (Bette-Bobillo et al. 1985)], (*c*) $CH_3COS^-K^+$/EtOH, Δ, 4 h, 80 %, (*d*) MeOH/HCl 5 N, Δ, 5 h, 90 %, (*e*) Cl–$C(Ph)_3$/CH_3CN [see Ref. (Sharma et al. 2008)], (*f*) $LiAlH_4$/Et_2O, Δ, 2 h, (*g*) H_3O^+, 90 %, (*h*) $(Boc)_2O$, DMAP (cat.)/CH_2Cl_2, RT, 4 h, 80 %, (*i*) 50:50 TFA:CH_2Cl_2, (*j*) MeONa/MeOH, inert atmosphere, 3 h, then H_3O^+, 90 %, (*k*) HC ≡ C–$SO_2C_6H_4CH_3$, TEA/50:50 TFE:CH_3CN, 30 min, 90 % [see Ref. (Arjona et al. 2003)]. **B** Synthesis of *S*-protected ThiAPols and subsequent deprotection: (*a*) R_1-S-$(CH_2)_6$-NH_2, HMPAS. When R_1 was either a trityl (compound 1) or a tosvinyl group (compound 3), the protection was recovered after HMPAS, whereas it was not when R_1 was acetyl (compound 2). (*b*) R_1 = -Tr: TFA, TES/DMF, R_2 = 2:98 H:unidentified mixture; or R_1 = TsV-: 20:80 pyrrolidine:NMP 40 °C, 4 h, inert atmosphere. R_2 = -S-$(CH_2)_6$-A8-35

using small angle neutron scattering (SANS) and analytical ultracentrifugation (AUC) (Gohon et al. 2004, 2006, 2008). It was synthesized by grafting deuterated octylamine and isopropylamine onto a hydrogenated PAA backbone according the classical HMPAS procedure ("Design, Synthesis and Characterization of APol A8-35"). Its use

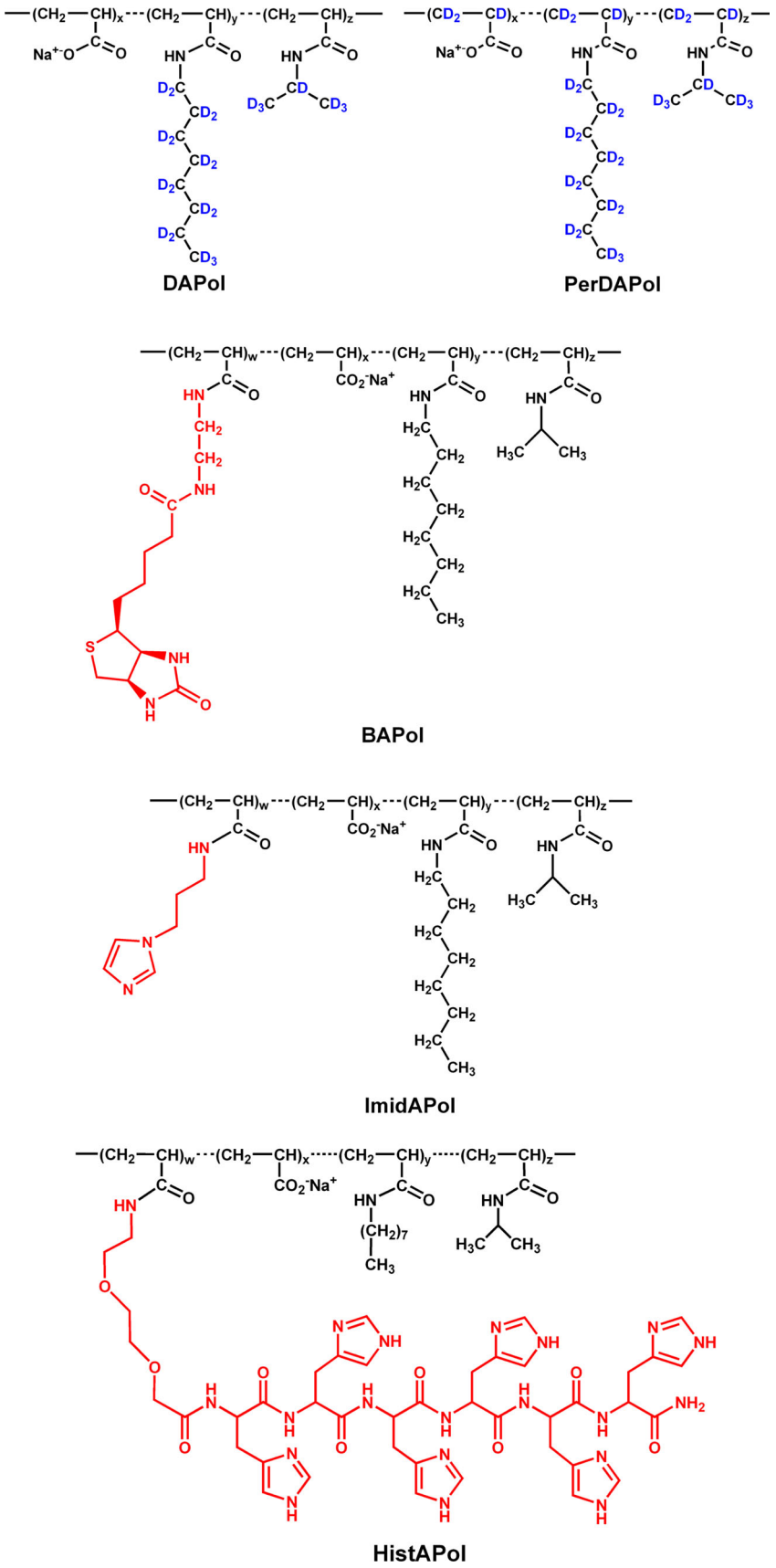

Fig. 5 Various labeled forms of A8-35 obtained by HMPAS. The aminoprobe moiety has been colored in red. For each derivative: $x \approx 0.35$, $y \approx 0.25$. DAPol (Gohon et al. 2004) and perDAPol (Giusti et al. 2014b): $z \approx 0.40$. BAPol (Charvolin et al. 2009): $w \approx 0.03$, $z \approx 0.37$. ImidAPol (Giusti et al. 2014a): $w \approx 0.06$, $z \approx 0.34$. HistAPol (Giusti et al. 2014a): $w \approx 0.015$, $z \approx 0.38$

was later extended to solution NMR studies of MPs and their ligands (Catoire et al. 2009, 2010a; Etzkorn et al. 2014; Planchard et al. 2014; Zoonens et al. 2005) and to inelastic neutron scattering (INS) investigations of the dynamics of A8-35 particles (Tehei et al. 2014) and MP/A8-35 complexes (unpublished data).

For NMR studies involving the examination of ^{1}H-^{1}H nuclear Overhauser effects (NOEs) and for INS studies, but also to improve the contrast in certain SANS and AUC studies, it is desirable to eliminate ^{1}H from the backbone as well. This led to the design of a perdeuterated version of A8-35 ('perDAPol'; Fig. 5). Its synthesis is more complex than that of DAPol, because it implies to first polymerize a perdeuterated PAA with a length and dispersity matching those of the commercial hydrogenated PAA used for the other syntheses, followed by grafting with perdeuterated side-chains (Giusti et al. 2014b).

From a practical point of view, the deuteration and perdeuteration of A8-35 are straightforward processes, which can be easily scaled up. A detailed comparison of the solution properties of A8-35 and DAPol has shown that, but for their different density and contrast matching point, the two polymers behave in exactly the same manner (Gohon et al. 2004, 2006).

In the early years of the APol project, radioactive forms of A8-75 (^{14}C-labeled) and A8-35 (^{3}H-labeled) were obtained by HMPAS, in order to study and quantify the binding of APols to MPs and their exchange for other surfactants (Table 1). Their production was discontinued with the advent of FAPols (section "Labeling A8-35 by Reacting UAPol-NH_2 with an Activated Probe: Synthesis of Fluorescent APols"), which can be put to most of the same uses (Zoonens et al. 2007) and are not subject to cumbersome safety regulations.

Biotinylated A8-35

Biotinylated A8-35 ('BAPol') was developed (Fig. 5) so as to mediate MP immobilization onto avidin-, neutravidin- or streptavidin-coated solid supports (Charvolin et al. 2009; Della Pia et al. 2014a, b; Ferrandez et al. 2014). Biotinylation was achieved either by reacting UAPol-NH_2 with an activated ester of biotin or by modifying PAA with an amino derivative of biotin during HMPAS. UAPol derivatization, however, was limited to a fraction of the amino arms, amounting to $\leq$7 biotins per particle, whereas BAPol obtained via HMPAS exhibited the levels of biotinylation that had been aimed for ($\sim$10 biotins per particle). These results are consistent with the observations mentioned in section "Amine-Carrying UAPol (UAPol-NH_2): A Highly Convenient Tool. They confirm that the modification of a PAA performed via HMPAS provides better yields than UAPol derivatization. Either approach could be easily extended to the labeling or tagging of sulfonated APols (SAPols; "Variants of A8-35 Obtained by Chemical Modification of A8-75 Particles" section).

The polyanionic character of A8-35, as well as its sensitivity to low pH (Gohon et al. 2004, 2006) and to the presence of multivalent cations (Diab et al. 2007a; Picard et al. 2006), can be problematical in some experimental situations. It is also responsible for high levels of non-specific binding when screening cationic ligands. These constraints have prompted the development of biotinylated versions of phosphocholine-based (Basit et al. 2012) and non-ionic (Ferrandez et al. 2014) APols.

A8-35 Carrying Either a Polyhistidine Tag or Distributed imidazole Groups

Polyhistidine tags are widely used to immobilize proteins onto metal-carrying beads or chips, be it for immobilized metal affinity chromatography (IMAC) or for ligand screening purposes, e.g., by surface plasmon resonance (SPR). Usually, the protein is genetically modified to this end. Developing APols that would be able to interact with such supports would have many applications, and, as in the case of BAPol, would present the advantage that a single APol could be used to immobilize any MP or mixture of MPs, without having to genetically engineer them. At variance with BAPol, the approach would provide reversibility. Two routes have been tested to date, grafting a polyhistidine (polyHis) tag onto A8-35 or endowing it with randomly distributed imidazole groups, the resulting polymers being dubbed 'HistAPol' and 'ImidAPol,' respectively (Fig. 5). Both of them were obtained via HMPAS, by grafting onto the PAA either *N*-(3-aminoprop-1-yl)imidazole (yielding ImidAPol) or *N*-(penta(histidyl)histidinamide)-8-amino-3,6-dioxa-octanamide (His_6-PEG) (yielding HistAPol).

His_6PEG is accessible only after multi-step peptidic coupling, which renders the synthesis of HistAPol particularly laborious. Furthermore, the cost and low-scale availability of His_6PEG severely limit the scale of HistAPol syntheses. ImidAPol, which features randomly distributed imidazole groups, was therefore developed in parallel as a cheap, somewhat simplistic but potentially useful alternative to HistAPol. ImidAPol indeed was obtained in large amounts following a simple synthesis route, the imidazole derivative being purchased at low cost from the industry. The batch of ImidAPol most extensively investigated carried $\sim$6 imidazole moieties per 100 PAA units, or $\sim$20 per 40 kDa particle. Its solution properties are interesting: in basic aqueous solution, its behavior resembles very much that of underivatized A8-35; in acidic solution, however, ImidAPol precipitates quantitatively only when the pH is close to 3, due to the additional

hydrophilicity provided, at acidic pH, by protonated imidazoles (Giusti et al. 2014a).

HistAPol was tagged to the level of ~4-5 polyHis per particle, or 25-30 imidazole groups, i.e., a total level similar to that of ImidAPol, but with quite a different distribution, since imidazoles come in groups of six. As for ImidAPol, the solution properties of HistAPol under acidic conditions differ from those of plain A8-35, a large fraction of the polymer remaining in solution at pH < 5 (Giusti et al. 2014a). At basic pH, HistAPol assembles into more heterogeneous and slightly larger particles than A8-35 or ImidAPol (*ibid.*).

From a practical point of view, care has to be paid to carefully select the pH at which to precipitate ImidAPol and HistAPol during their purification. Above ~9 imidazole moieties per 100 PAA units (~30 per particle), the solubility of ImidAPol at acidic pH increases to the point that purification by cycles of precipitation/resolubilization becomes impossible.

Both HistAPol and ImidAPol proved readily able to trap MPs and keep them water soluble, but they bound markedly differently onto nickel-nitrilotriacetic acid (Ni-NTA)-coated surfaces (Giusti et al. 2014a). In both cases, the complexes adsorbed and could be desorbed upon washing with either EDTA or imidazole. However, MP/HistAPol complexes bind with a much higher affinity and more permanently onto Ni-NTA-coated chips, making them highly suited to SPR applications, whereas MP/ImidAPol ones leach more rapidly and may be better adapted to such applications as IMAC (Giusti et al. 2014a).

Labeling A8-35 by Reacting UAPol-NH_2 with an Activated Probe: Synthesis of Fluorescent APols

When a high level of labeling is not required, as is the case, e.g., for fluorescent labeling, an efficient course is to derivatize a 'universal' intermediate such as UAPol-NH_2 (section "Amine-Carrying UAPol (UAPol-NH_2): A Highly Convenient Tool"): provided a suitably functionalized version of the group to be added is commercially available, which is frequently the case, a single step is required for the synthesis, purification remaining, usually, the most time-consuming task. This is the method of choice when the fluorophore is too fragile for HMPAS, or insoluble in NMP, or when no aminated version is available.

Fluorescently labeled forms of A8-35 (FAPols) (Fig. 3) were initially developed in order to characterize, by Förster resonance energy transfer (FRET), the associative properties of the polymer in aqueous solution, whether it interacts with itself to form a particle or it adsorbs onto the transmembrane surface of a MP to form a complex. The first FAPols to be synthesized were labeled with common, cheap probes convenient for FRET. 7-Nitrobenz-2-oxa-1,3-diazol-4-yl-labeled A8-35 ($FAPol_{NBD}$) was developed first and used for studying MP/APol interactions by measuring FRET between the tryptophan residues of the protein (donors) and NBD (acceptor) (Zoonens et al. 2007). Rhodamine-labeled FAPol ($FAPol_{rhod}$) was developed next as a FRET-exchanger with $FAPol_{NBD}$. The two FAPols were mixed in aqueous solution in a 1:1 ratio and the mixture diluted until the FRET signal disappeared, indicating that the particles had dissociated into single molecules. The critical aggregation concentration (CAC) of A8-35 thus determined is ~0.002 g L^{-1}, consistent with surface tension measurements (Giusti et al. 2012). Fluorescein-labeled A8-35 ($FAPol_{fluo}$) was developed for a dual purpose. First, it has been used as a quencher in experiments aiming at using APols for MP topological studies (Opačić et al. 2014). Second, it is a potential alternative FRET donor to rhodamine, whose quantum yield and stability are higher than those of NBD.

Attempts are under way to use APols to deliver either immunogens (Feinstein et al. 2014; Tifrea et al. 2011, 2014), in vaccine preparations, or therapeutic peptides (Fernandez et al. 2014; Popot et al. 2011). In order to examine how, following an injection, A8-35 distributes in the body, where it accumulates, and how it is eliminated, FAPols that absorb and emit in the red and far-red were developed, carrying as fluorophores either Atto-647 or Alexa Fluor 647. Their fate following injection to mice was followed by fluorescence imaging, as well as that of $FAPol_{rhod}$ (Fernandez et al. 2014). $FAPol_{AF647}$ and $FAPol_{NBD}$ have also been used to visualize, by fluorescence microscopy, the adsorption of APol particles and MP/APol complexes carrying either a biotin or an oligonucleotide tag onto appropriately functionalized gold nanoparticles (Della Pia et al. 2014a, b; Le Bon et al. 2014).

The synthesis of some FAPols, such as that of a naphthalene derivative of A8-75, can proceed by HMPAS (see section "Variants of A8-35 Obtained by Chemical Modification of A8-75 Particles"). However, this route is harsh, particularly due to the final treatment with sodium methanolate, and few fluorophores can stand it. Rather, an activated version of the fluorophore, carrying either an isothiocyanate or an *N*-hydroxysuccinimidyl ester (NHS-ester) is reacted with the free amine functions of UAPol-NH_2 (section "Amine-Carrying UAPol (UAPol-NH_2): A Highly Convenient Tool"). Whatever the dye used and the chemistry involved, the same two observations were repeatedly made: (i) the reaction is never complete, resulting in low to medium yields of labeling (typically 0.5-5 fluorophores per particle); (ii) traces of unreacted dye can be removed only, and not always quantitatively, after extensive purification by SEC and prolonged dialysis. The

limited yield of the labeling reaction is probably due to the low reactivity of the short amino linker randomly distributed along charged bulky macromolecular chains.

Only preparative SEC efficiently removes most of the free dye from FAPols. This step is fastidious and time consuming. Given that SEC is almost always necessary, synthesis by HMPAS could be considered if the fluorophore can stand it, and the precipitation/redissolution steps dispensed with. Derivatization of purified UAPol-NH_2, however, has the advantage that this intermediate can be synthesized once, and then used for multiple one-step FAPol syntheses.

Once purified, FAPols are very stable in aqueous solutions provided they are kept in the dark (Fernandez et al. 2014; Giusti et al. 2012).

Direct Labeling of A8-35 Particles: Synthesis of an Oligodeoxynucleotide-Carrying APol

Oligodeoxynucleotides (ODNs) are short strands of DNA with a phosphodiester or phosphorothioate backbone and a specific sequence of bases. The rationale behind tagging A8-35 particles with ODNs is twofold. First, vaccination studies show an increased protection from the pathogenic bacterium *Chlamydia trachomatis* when the major outer membrane protein of this organism, used as an immunogen, is trapped and stabilized in A8-35 rather than kept in detergent solution (Tifrea et al. 2011, 2014). Because adjuvants are known to be most effective when co-delivered along with the antigen (Krieg 2006), it seemed worthwhile to develop an APol coupled to an adjuvant, such as the CpG-1826 ODN (Krieg et al. 1995), so as to ensure co-delivery of the two molecules. Second, an ODN-tagged APol ('OligAPol') would provide a highly polyvalent mode of attachment of MPs onto solid supports (Le Bon et al. 2014), with the double advantage, over BAPol-mediated attachment, to be both reversible and open to multiplexing, with obvious applications in bionanotechnologies.

As compared to the syntheses discussed previously, coupling an ODN to an A8-35 particle presents special challenges: (i) the reaction involves two large particles ($\sim$6.6 and $\sim$40 kDa, respectively), both of them polyanions and, therefore, repulsing each other; (ii) the tag is larger than the molecule of polymer that carries it, whose average mass is $\sim$4.3 kDa (Giusti et al. 2014b). This may compromise the ability of the resulting OligAPol molecules to self-organize into globular particles and/or to trap and stabilize MPs.

The formation of bioconjugates with ODNs is well documented in the literature (Alemdaroglu and Herrmann 2007). Among the many synthesis routes that have been explored are the following: (i) formation of an amide bond between an ODN functionalized with a primary amine and appropriate functions carried by the polymer, such as a carboxylic acid, an anhydride or an NHS group (Ferraton et al. 1997; Jeong et al. 2005); (ii) creation of a disulfide bridge between thiols carried by the polymer and by a chemically modified ODN (Oishi et al. 2005); (iii) Michaël addition between a maleimide function carried by the polymer and a thiolated ODN; and (iv) reductive amination, with the formation of an alkylamine, between an aldehyde-carrying polymer and the amine function of a modified ODN (Delair et al. 1997; Ferraton et al. 1997).

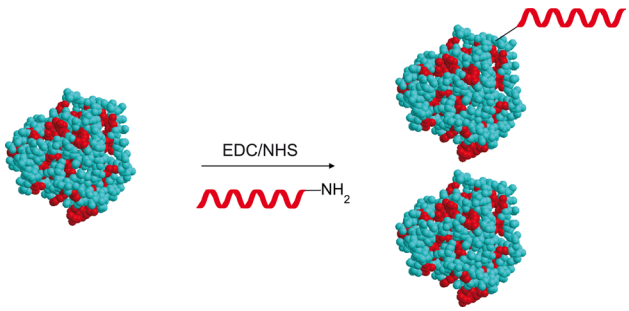

Fig. 6 Synthesis of OligAPol. Labeling A8-35 particles (*left*) with an amino-functionalized oligodeoxynucleotide (*red helix*). The grafting reaction was performed in aqueous medium (0.4 M NaCl) at room temperature: carboxylate moieties present on the polymer backbone were first activated in the presence of cross-linking agents (EDC/NHS) and then reacted with the amine function of the functionalized ODN to lead to an amide moiety. A two-step purification process led to an equal mixture of labeled and unlabeled particles (*right*). From ref. (Le Bon et al. 2014)

Because the synthesis of a ThiAPol turned out to be problematical (section "Thiol-Carrying A8-35: A Biochemist's Dream, A Chemist's Nightmare") and the grafting yield of UAPol-NH_2 is low (section "Labeling A8-35 by Reacting UAPol-NH_2 with an Activated Probe: Synthesis of Fluorescent APols"), OligAPol was obtained by forming, in aqueous solution, an amide bond between an aminated ODN and one of the activated carboxylate functions of A8-35, a chemical pathway that had been successfully used before for the synthesis of sulfonated APols (see section "Variants of A8-35 Obtained by Chemical Modification of A8-75 Particles"). Carboxylate functions of A8-35 were reacted with the amine-functionalized ODN in the presence of zero-length cross-linkers, ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), leading to the formation of an amide function (Fig. 6). Coupling being incomplete, a two-step purification was required, involving first partial separation of OligAPol from unbound ODN by selective precipitation at acidic pH, followed by preparative SEC to remove residual by-products and most of the remaining unreacted ODN. The initial objective of 1 ODN per particle could not be reached, the grafting ratio being limited to $\sim$0.5 ODN per A8-35 particle. This level

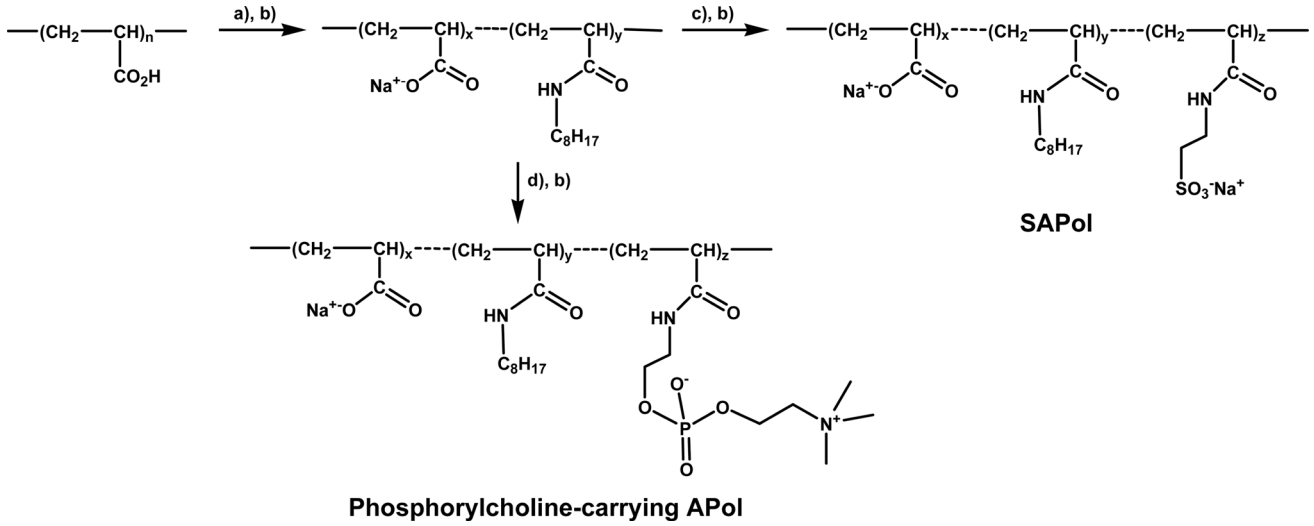

Fig. 7 Synthesis of SAPol (Dahmane et al. 2011; Picard et al. 2006) and of a phosphocholine-carrying APol (unpublished data) obtained by chemical modification of A8-75 particles in aqueous buffer. Conditions were: (*a*) first step of HMPAS; (*b*) NaOH and further purification; (*c*) taurine, EDC, water pH = 7, 4 h; or (*d*) NH_2CH_2-CH_2-OPO_3-$CH_2CH_2N(CH_3)_3$, EDC, water pH = 7, 4 h. For both polymers, the chemical composition was found to be $x \approx 0.35$, $y \approx 0.25$ and $z \approx 0.40$

of grafting was nevertheless sufficient to show that OligAPols assemble into well-behaved particles and to validate them as efficient tools to trap and handle MPs (Le Bon et al. 2014). Immobilization of MP/OligAPol complexes was demonstrated by hybridizing them with a complementary ODN bound to either magnetic beads or gold nanoparticles (Le Bon et al. 2014).

Variants of A8-35 Obtained by Chemical Modification of A8-75 Particles

A8-75 is an intermediate in the synthesis of A8-35, namely the product obtained after the first step of HMPAS (grafting of octylamine). It is easy to produce and can be purified using the same protocol as for A8-35. A8-75 molecules feature the same distribution of backbone lengths as A8-35, ~25 % of octylamide side chains and ~75 % of free carboxylates (Tribet et al. 1996). Using HMPAS, A8-75 was derived with traces of naphthalene to yield a fluorescent APol, which was used to study the interaction of APols with lipid vesicles (Vial et al. 2005). Derived with ~40 % isopropylamine, using HMPAS, A8-75 yields A8-35 (Fig. 1a). If isopropylamine is replaced with taurine, the end product is a sulfonated APol ('SAPol') (Fig. 7) (Dahmane et al. 2011; Picard et al. 2006). SAPol cannot be synthesized by HMPAS because of the poor solubility of taurine in NMP. Rather, taurine was grafted in aqueous buffer, in the presence of EDC, onto the surface of already formed A8-75 particles (Fig. 7). According to SEC, these particles are similar to those formed by A8-35. It can be assumed that taurine is grafted more or less randomly onto water-exposed carboxylates lying at their surface (Dahmane et al. 2011). Although the distribution of the octyl chains along the PAA backbone of SAPol is expected to be random (Magny et al. 1992), that of the taurine residues is likely biased, because carboxylate-rich regions of the chains must stand a higher probability of reacting than octylamide-rich ones.

Along with phosphocholine-based (Diab et al. 2007a, b) and non-ionic (Bazzacco et al. 2012; Sharma et al. 2012) APols, SAPol was developed as a pH-insensitive APol. It is also insensitive to the presence of multivalent cations (Picard et al. 2006). It can for instance be substituted to A8-35 for NMR measurements when working at an acidic pH is desirable (Dahmane et al. 2011) and has also been found to be useful for single-particle cryo-electron microscopy (Huynh et al. 2014). Its deuteration or perdeuteration, if needed, could be carried out following the same routes as for DAPol and perDAPol (section "Isotopically Labeled APols").

Another A8-75 derivative has been synthesized according to the same method, namely a phosphocholine-carrying variant, obtained by grafting A8-75 particles with ethanolamine-*O*-phosphocholine (unpublished results) (Fig. 7). The aim of this synthesis was essentially to explore the efficiency of the method. The reaction was found to be quantitative, the targeted amount of grafted amine (~25 % octyl chain, ~40 % taurine or phosphocholine derivative) having been reached in both cases.

Conclusion

Over the nearly twenty years that have elapsed since APols were designed and the first ones synthesized and validated, the chemistry of the field has branched into many directions: (i) developing and testing polymers with radically different structures [e.g., refs. (Klammt et al. 2011; Knowles et al. 2009; Long et al. 2013; Rajesh et al. 2011)]; (ii) developing variants of the original APols with improved properties, such as pH-insensitivity [e.g., refs. (Bazzacco et al. 2009, 2012; Dahmane et al. 2011; Diab et al. 2007a, b; Gorzelle et al. 2002; Nagy et al. 2001; Prata et al. 2001; Sharma et al. 2008, 2012)]; (iii) labeling or functionalizing the best-characterized APol to date, A8-35, to tailor it for various applications. This is what has been discussed here. It should be apparent, from this survey, that the chemistry of APols is extremely rich, polyvalent, and that its resources have only begun to be tapped.

There is an irony in the ease with which MPs can be labeled or functionalized, indirectly, by associating them with an appropriately modified APol. Membrane biochemists know that, as a rule, any experiment that is straightforward with soluble proteins becomes much more challenging with MPs. Here, on the contrary, a single functionalized APol can be used to functionalize any number of MPs, purified or not, without having to modify them either genetically or chemically, the only requirement being that the proteins expose a sufficiently extended hydrophobic surface for the polymer to adsorb, which any transmembrane protein does. Furthermore, because APols are freely miscible (Giusti et al. 2012; Zoonens et al. 2007), APol particles and MP/APol complexes can easily be multiply labeled, such as by using a mixture of a fluorescent and a tagged APol, the former becoming a convenient reporter of the behavior of the latter [see, e.g., Ref. (Le Bon et al. 2014)].

The applications of labeled or functionalized APols that have already been validated are listed in Table 1. They are discussed in refs. (Popot et al. 2011; Zoonens and Popot 2014). It is to be expected that their number will keep increasing in years to come, calling for new chemical developments.

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References

Alemdaroglu FE, Herrmann A (2007) DNA meets synthetic polymers—highly versatile hybrid materials. Org Biomol Chem 5:1311–1320

Althoff T, Mills DJ, Popot J-L, Kühlbrandt W (2011) Assembly of electron transport chain components in bovine mitochondrial supercomplex $I_1III_2IV_1$. EMBO J 30:4652–4664

Andreu D, Albericio F, Solé NA, Munson MC, Ferrer M, Barany G (1994) Formation of disulfide bonds in synthetic peptides and proteins. Methods Mol Biol, Springer, pp 91–169

Arjona O, Medel R, Rojas J, Costa AM, Vilarrasa J (2003) Chemoselective protection of thiols versus alcohols and phenols. The Tosvinyl group. Tetrahedron Lett 44:6369–6373

Basit H, Sharma S, Van der Heyden A, Gondran C, Breyton C, Dumy P, Winnik FM, Labbé P (2012) Amphipol mediated surface immobilization of FhuA: a platform for label-free detection of the bacteriophage protein pb5. Chem Commun 48:6037–6039

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

Bazzacco P, Billon-Denis E, Sharma KS, Catoire LJ, Mary S, Le Bon C, Point E, Banères J-L, Durand G, Zito F, Pucci B, Popot J-L (2012) Non-ionic homopolymeric amphipols: application to membrane protein folding, cell-free synthesis, and solution NMR. Biochemistry 51:1416–1430

Bernkop-Schnurch A, Schwarz V, Steininger S (1999) Polymers with thiol groups: a new generation of mucoadhesive polymers? Pharm Res 16:876–881

Bette-Bobillo P, Bienvenue A, Broquet C, Maurin L (1985) Synthesis and characterisation of a radioiodinated, photoreactive and physiologically active analog of platelet activating factor. Chem Phys Lipids 37:215–226

Blackburn S, Lee GR (1956) The reaction of wool keratin with alkali. Biochim Biophys Acta 19:505–512

Catoire LJ, Zoonens M, van Heijenoort C, Giusti F, Popot J-L, Guittet E (2009) Inter- and intramolecular contacts in a membrane protein/surfactant complex observed by heteronuclear dipole-to-dipole cross-relaxation. J Magn Res 197:91–95

Catoire LJ, Damian M, Giusti F, Martin A, van Heijenoort C, Popot J-L, Guittet E, Banères J-L (2010a) Structure of a GPCR ligand in its receptor-bound state: leukotriene B_4 adopts a highly constrained conformation when associated to human BLT2. J Am Chem Soc 132:9049–9057

Catoire LJ, Zoonens M, van Heijenoort C, Giusti F, Guittet E, Popot J-L (2010b) Solution NMR mapping of water-accessible residues in the transmembrane β-barrel of OmpX. Eur Biophys J 39:623–630

Champeil P, Menguy T, Tribet C, Popot J-L, le Maire M (2000) Interaction of amphipols with the sarcoplasmic reticulum Ca^{2+}-ATPase. J Biol Chem 275:18623–18637

Charvolin D, Perez J-B, Rouvière F, Giusti F, Bazzacco P, Abdine A, Rappaport F, Martinez KL, Popot J-L (2009) The use of amphipols as universal molecular adapters to immobilize membrane proteins onto solid supports. Proc Natl Acad Sci USA 106:405–410

Charvolin D, Picard M, Huang L-S, Berry EA, Popot J-L (2014) Solution behavior and crystallization of cytochrome bc_1 in the presence of amphipols (submitted for publication)

Collman JP, Devaraj NK, Chidsey CED (2004) "Clicking" functionality onto electrode surfaces. Langmuir 20:1051–1053

Dahmane T, Damian M, Mary S, Popot J-L, Banères J-L (2009) Amphipol-assisted in vitro folding of G protein-coupled receptors. Biochemistry 48:6516–6521

Dahmane T, Giusti F, Catoire LJ, Popot J-L (2011) Sulfonated amphipols: synthesis, properties and applications. Biopolymers 95:811–823

Dahmane T, Rappaport F, Popot J-L (2013) Amphipol-assisted folding of bacteriorhodopsin in the presence and absence of lipids. Functional consequences. Eur Biophys J 42:85–101

Danehy JP, Hunter WE (1967) The alkaline decomposition of organic disulfides. II. Alternative pathways as determined by structure. J Org Chem 32:2047–2053

Danehy JP, Kreuz JA (1961) The alkaline decomposition of organic disulfides. I. Some dithiodicarboxylic acids. J Am Chem Soc 83:1109–1113

Davis BG, Khumtaveeporn K, Bott RR, Jones JB (1999) Altering the specificity of subtilisin *Bacillus lentus* through the introduction of positive charge at single amino acid sites. Bioorg Med Chem 7:2303–2311

Delair T, Badey B, Charles MH, Laayoun A, Domard A, Pichot C, Mandrand B (1997) Conjugation of nucleic acid probes to 6-aminoglucose-based homo- and copolymers. 2. Application to diagnostics. Polym Adv Technol 8:545–555

Della Pia EA, Holm J, Lloret N, Le Bon C, Popot J-L, Zoonens M, Nygård J, Martinez KL (2014a) A step closer to membrane protein multiplexed nano-arrays using biotin-doped polypyrrole. ACS Nano 8:1844–1853

Della Pia EA, Westh Hansen R, Zoonens M, Martinez KL (2014b) Amphipols: a versatile toolbox suitable for applications of membrane proteins in synthetic biology (submitted for publication)

Diab C, Tribet C, Gohon Y, Popot J-L, Winnik FM (2007a) Complexation of integral membrane proteins by phosphorylcholine-based amphipols. Biochim Biophys Acta 1768:2737–2747

Diab C, Winnik FM, Tribet C (2007b) Enthalpy of interaction and binding isotherms of non-ionic surfactants onto micellar amphiphilic polymers (amphipols). Langmuir 23:3025–3035

Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82:70–77

Etzkorn M, Zoonens M, Catoire LJ, Popot J-L, Hiller S (2014) How amphipols embed membrane proteins: global solvent accessibility and interaction with a flexible protein terminus. J Membrane Biol (in press)

Feinstein HE, Tifrea D, Popot J-L, de la Maza LM, Cocco MJ (2014) Amphipols stabilize the *Chlamydia* major outer membrane protein vaccine formulation (submitted for publication)

Fernandez A, Le Bon C, Baumlin N, Giusti F, Crémel G, Popot J-L, Bagnard D (2014) In vivo characterization of the biodistribution profile of amphipols (submitted for publication)

Ferrandez Y, Dezi M, Bosco M, Urvoas A, Valério M, Le Bon C, Giusti F, Broutin I, Durand G, Polidori A, Popot J-L, Picard M, Minard P (2014) Amphipol-mediated screening of molecular orthoses specific for membrane protein targets (submitted for publication)

Ferraton N, Delair T, Laayoun A, Cros P, Mandrand B (1997) Covalent immobilization of nucleic acid probes onto reactive synthetic polymers. J Appl Polym Sci 66:233–242

Frey A, Neutra MR, Robey FA (1997) Peptomer aluminum oxide nanoparticle conjugates as systemic and mucosal vaccine candidates: synthesis and characterization of a conjugate derived from the C4 domain of HIV-1(MN) gp120. Bioconjugate Chem 8:424–433

Gauvreau V, Chevallier P, Vallières K, Petitclerc E, Gaudreault RC, Laroche G (2004) Engineering surfaces for bioconjugation: developing strategies and quantifying the extent of the reactions. Bioconjugate Chem 15:1146–1156

Gielens C, De Geest N, Xin X-Q, Devreese B, Van Beeumen J, Préaux JG (1997) Evidence for a cysteine-histidine thioether bridge in functional units of molluscan haemocyanins and location of the disulfide bridges in functional units *d* and *g* of the β_c-haemocyanin of *Helix pomatia*. Eur J Biochem 248:879–888

Giusti F, Popot J-L, Tribet C (2012) Well-defined critical association concentration and rapid adsorption at the air/water interface of a short amphiphilic polymer, amphipol A8-35: a study by Förster resonance energy transfer and dynamic surface tension measurements. Langmuir 28:10372–10380

Giusti F, Kessler P, Westh Hansen R, Della Pia EA, Le Bon C, Mourier G, Popot JL, Martinez KL, Zoonens M (2014a) Synthesis of polyhistidine- or imidazole-bearing amphipols and their use for immobilized metal affinity chromatography and surface plasmon resonance studies of membrane proteins (in preparation)

Giusti F, Rieger J, Catoire L, Qian S, Calabrese AN, Watkinson TG, Radford SE, Ashcroft AE, Popot J-L (2014b) Synthesis, characterization and applications of a perdeuterated amphipol. J Membrane Biol (in press)

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

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

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

Gorzelle BM, Hoffman AK, Keyes MH, Gray DN, Ray DG, Sanders CR II (2002) Amphipols can support the activity of a membrane enzyme. J Am Chem Soc 124:11594–11595

Greene TW, Wuts PGM (2002) Protection for the thiol group. Protective groups in organic synthesis. Wiley, Hoboken, pp 454–493

Grover GN, Alconcel SNS, Matsumoto NM, Maynard HD (2009) Trapping of thiol-terminated acrylate polymers with divinyl sulfone to generate well-defined semitelechelic Michael acceptor polymers. Macromolecules 42:7657–7663

Hansen RE, Winther JR (2009) An introduction to methods for analyzing thiols and disulfides: reactions, reagents, and practical considerations. Anal Biochem 394:147–158

Harding RL, Henshaw J, Tilling J, Bugg TDH (2002) Thioester analogues of peptidoglycan fragment MurNAc-L-Ala-gamma-D-Glu as substrates for peptidoglycan hydrolase MurNAc- L-Ala amidase. J Chem Soc Perkin Trans 1:1714–1722

Humphrey RE, Potter JL (1965) Reduction of disulfides with tributylphosphine. Anal Chem 37:164–165

Huynh KW, Cohen MR, Moiseenkova-Bell VY (2014) Application of amphipols for structure-functional analysis of TRP channels (submitted for publication)

Javakhishvili I, Hvilsted S (2009) Gold nanoparticles protected with thiol-derivatized amphiphilic poly(epsilon-caprolactone)-*β*-poly(acrylic acid). Biomacromolecules 10:74–81

Jeong JH, Kim SH, Kim SW, Park TG (2005) Polyelectrolyte complex micelles composed of c-raf antisense oligodeoxynucleotide-poly(ethylene glycol) conjugate and poly(ethylenimine): effect of systemic administration on tumor growth. Bioconjugate Chem 16:1034–1037

Kast CE, Bernkop-Schnurch A (2001) Thiolated polymers-thiomers: development and in vitro evaluation of chitosan-thioglycolic acid conjugates. Biomaterials 22:2345–2352

Kikuzawa A, Kida T, Akashi M (2007) Synthesis of stimuli-responsive cyclodextrin derivatives and their inclusion ability control by ring opening and closing reactions. Org Lett 9:3909–3912

Klammt C, Perrin M-H, Maslennikov I, Renault L, Krupa M, Kwiatkowski W, Stahlberg H, Vale W, Choe S (2011) Polymer-based cell-free expression of ligand-binding family B G-protein coupled receptors without detergents. Prot Sci 20:1030–1041

Knowles TJ, Finka R, Smith C, Lin Y-P, Dafforn T, Overduin M (2009) Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. J Am Chem Soc 131:7484–7485

Krieg AM (2006) Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov 5:471–484

Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, Koretzky GA, Klinman DM (1995) CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374:546–549

Le Bon C, Della Pia EA, Giusti F, Lloret N, Zoonens M, Martinez KL, Popot J-L (2014) Synthesis of an oligonucleotide-derivatized amphipol and its use to trap and immobilize membrane proteins. Nucleic Acids Res (accepted for publication)

Long AR, O'Brien CC, Malhotra K, Schwall CT, Albert AD, Watts A, Alder NN (2013) A detergent-free strategy for the reconstitution of active enzyme complexes from native biological membranes into nanoscale discs. BMC Biotechnol 13:41. doi:10.1186/1472-6750-13-41

Magny B, Lafuma F, Iliopoulos I (1992) Determination of microstructure of hydrophobically modified water-soluble polymers by ^{13}C NMR. Polymer 33:3151–3154

Martinez KL, Gohon Y, Corringer P-J, Tribet C, Mérola F, Changeux J-P, Popot J-L (2002) Allosteric transitions of *Torpedo* acetylcholine receptor in lipids, detergent and amphipols: molecular interactions *vs.* physical constraints. FEBS Lett 528:251–256

Morishima Y, Nomura S, Ikeda T, Seki M, Kamachi M (1995) Characterization of unimolecular micelles of random copolymers of sodium 2-(acrylamido)-2-methylpropanesulfonate and methacrylamides bearing bulky hydrophobic substituents. Macromolecules 28:2874–2881

Murphy WL, Mercurius KO, Koide S, Mrksich M (2004) Substrates for cell adhesion prepared via active site-directed immobilization of a protein domain. Langmuir 20:1026–1030

Nagy JK, Kuhn Hoffmann A, Keyes MH, Gray DN, Oxenoid K, Sanders CR (2001) Use of amphipathic polymers to deliver a membrane protein to lipid bilayers. FEBS Lett 501:115–120

Nicolet BH (1931) The mechanism of sulfur lability in cysteine and its derivatives. I. Some thio ethers readily split by alkali. J Am Chem Soc 53:3066–3072

Oishi M, Hayama T, Akiyama Y, Takae S, Harada A, Yarnasaki Y, Nagatsugi F, Sasaki S, Nagasaki Y, Kataoka K (2005) Supramolecular assemblies for the cytoplasmic delivery of antisense oligodeoxynucleotide: polylon complex (PIC) micelles based on poly(ethylene glycol)-SS-oligodeoxynucleotide conjugate. Biomacromolecules 6:2449–2454

Opačić M, Popot J-L, Durand G, Bosco M, Polidori A, Croce R (2014) Amphipols and photosynthetic light-harvesting pigment-protein complexes (submitted for publication)

Perlmutter JD, Drasler WJ, Xie W, Gao J, Popot J-L, Sachs JN (2011) All-atom and coarse-grained molecular dynamics simulations of a membrane protein stabilizing polymer. Langmuir 27:10523–10537

Perlmutter JD, Popot J-L, Sachs JN (2014) Molecular dynamics simulations of a membrane protein/amphipol complex (submitted for publication)

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

Planchard N, Point E, Dahmane T, Giusti F, Renault M, Le Bon C, Durand G, Milon A, Guittet E, Zoonens M, Popot J-L, Catoire LJ (2014) The use of amphipols for solution NMR studies of membrane proteins: advantages and limitations as compared to other solubilizing media (submitted for publication)

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

Popot J-L, Berry EA, Charvolin D, Creuzenet C, Ebel C, Engelman DM, Flötenmeyer M, Giusti F, Gohon Y, Hervé P, Hong Q, Lakey JH, Leonard K, Shuman HA, Timmins P, Warschawski DE, Zito F, Zoonens M, Pucci B, Tribet C (2003) Amphipols: polymeric surfactants for membrane biology research. Cell Mol Life Sci 60:1559–1574

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

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

Rajesh S, Knowles TJ, Overduin M (2011) Production of membrane proteins without cells or detergents. New Biotech 28:250–254

Ringsdorf H, Venzmer J, Winnik FM (1991) Fluorescence studies of hydrophobically modified poly(*N*-isopropylacrylamides). Macromolecules 24:1678–1686

Roberts C, Chen C, Mrksich M, Martichonok V, Ingber DE, Whitesides GM (1998) Using mixed self-assembled monolayers presenting RGD and (EG)(3)OH groups to characterize long-term attachment of bovine capillary endothelial cells to surfaces. J Am Chem Soc 120:6548–6555

Roth PJ, Jochum FD, Zentel R, Theato P (2010) Synthesis of heterotelechelic alpha, omega bio-functionalized polymers. Biomacromolecules 11:238–244

Sanders CR, Hoffmann AK, Gray DN, Keyes MH, Ellis CD (2004) French swimwear for membrane proteins. ChemBioChem 5:423–426

Sauer AM, Schlossbauer A, Ruthardt N, Cauda V, Bein T, Brauchle C (2010) Role of endosomal escape for disulfide-based drug delivery from colloidal mesoporous silica evaluated by live-cell imaging. Nano Lett 10:3684–3691

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

Sharma KS, Durand G, Gabel F, Bazzacco P, Le Bon C, Billon-Denis E, Catoire LJ, Popot J-L, Ebel C, Pucci B (2012) Non-ionic amphiphilic homopolymers: synthesis, solution properties, and biochemical validation. Langmuir 28:4625–4639

Stefano JE, Hou LH, Honey D, Kyazike J, Park A, Zhou Q, Pan CQ, Edmunds T (2009) In vitro and in vivo evaluation of a non-carbohydrate targeting platform for lysosomal proteins. J Controlled Release 135:113–118

Takei YG, Aoki T, Sanui K, Ogata N, Okano T, Sakurai Y (1993) Temperature-responsive bioconjugates. 1. Synthesis of temperature-responsive oligomers with react-ive end groups and their coupling to biomolecules. Bioconjugate Chem 4:42–46

Tehei M, Giusti F, Popot J-L, Zaccai G (2014) Thermal fluctuations in amphipol A8-35 measured by neutron scattering. (submitted for publication)

Terrettaz S, Ulrich WP, Vogel H, Hong Q, Dover LG, Lakey JH (2002) Stable self-assembly of a protein engineering scaffold on gold surfaces. Protein Sci 11:1917–1925

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

Tifrea D, Pal S, Cocco MJ, Popot J-L, de la Maza LM (2014) Increased immuno accessibility of MOMP epitopes in a vaccine formulated with amphipols may account for the very robust protection elicited against a vaginal challenge with *C. muridarum*. (submitted for publication)

Tribet C, Audebert R, Popot J-L (1996) Amphipols: polymers that keep membrane proteins soluble in aqueous solutions. Proc Natl Acad Sci USA 93:15047–15050

Tribet C, Audebert R, Popot J-L (1997) Stabilization of hydrophobic colloidal dispersions in water with amphiphilic polymers: application to integral membrane proteins. Langmuir 13:5570–5576

Tribet C, Diab C, Dahmane T, Zoonens M, Popot J-L, Winnik FM (2009) Thermodynamic characterization of the exchange of detergents and amphipols at the surfaces of integral membrane proteins. Langmuir 25:12623–12634

Vial F, Rabhi S, Tribet C (2005) Association of octyl-modified poly(acrylic acid) onto unilamellar vesicles of lipids and kinetics of vesicle disruption. Langmuir 21:853–862

Wang TK, Iliopoulos I, Audebert R (1988) Viscometric behavior of hydrophobically modified poly(sodium acrylate). Polymer Bull20:577–582

Warnecke A, Kratz F (2003) Maleimide-oligo(ethylene glycol) derivatives of camptothecin as albumin-binding prodrugs: synthesis and antitumor efficacy. Bioconjugate Chem 14:377–387

Woghiren C, Sharma B, Stein S (1993) Protected thiol polyethyleneglycol. A new activated polymer for reversible protein modification. Bioconjugate Chem 4:314–318

Yoshimoto K, Hirase T, Nemoto S, Hatta T, Nagasaki Y (2008) Facile construction of sulfanyl-terminated poly(ethylene glycol)-brushed layer on a gold surface for protein immobilization by the combined use of sulfanyl-ended telechelic and semitelechelic poly(ethylene glycol)s. Langmuir 24:9623–9629

Zoonens M, Popot J-L (2014) Amphipols for each season. (submitted for publication)

Zoonens M, Catoire LJ, Giusti F, Popot J-L (2005) NMR study of a membrane protein in detergent-free aqueous solution. Proc Natl Acad Sci USA 102:8893–8898

Zoonens M, Giusti F, Zito F, Popot J-L (2007) Dynamics of membrane protein/amphipol association studied by Förster resonance energy transfer. Implications for in vitro studies of amphipol-stabilized membrane proteins. Biochemistry 46:10392–10404

Zoonens M, Zito F, Martinez KL, Popot J-L (2014) Amphipols: a general introduction and some protocols, In: Membrane protein production for structural analysis (I Mus-Veteau, ed.), Springer (in press)

Zugates GT, Anderson DG, Little SR, Lawhorn IEB, Langer R (2006) Synthesis of poly(beta-amino ester)s with thiol-reactive side chains for DNA delivery. J Am Chem Soc 128:12726–12734