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Functionalized Amphipols: A Versatile Toolbox Suitable for Applications of Membrane Proteins in Synthetic Biology

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Abstract Amphipols are amphipathic polymers that stabilize membrane proteins isolated from their native membrane. They have been functionalized with various chemical groups in the past years for protein labeling and protein immobilization. This large toolbox of functionalized amphipols combined with their interesting physicochemical properties give opportunities to selectively add multiple functionalities to membrane proteins and to tune them according to the needs. This unique combination of properties makes them one of the most versatile strategies available today for exploiting membrane proteins onto surfaces for various applications in synthetic biology. This review summarizes the properties of functionalized amphipols suitable for synthetic biology approaches.

Keywords Biosensors · Self-assembly · Protein immobilization · Protein labeling · Multiple functionalization

Abbreviations

A8-35	Poly(sodium acrylate)-based amphipol comprising 35 % of free carboxylate, 25 % of octyl chains and 40 % of isopropyl groups
APol	Amphipol
BAPol	Biotinylated A8-35
BG	Benzyl guanine
BR	Bacteriorhodopsin
CMC	Critical micelle concentration
DAPol	Deuterated A8-35
E. coli	*Escherichia coli*
FAPol	Fluorescently labeled A8-35
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
HAPol	Hydrogenated A8-35
HistAPol	Histidine-tagged A8-35
ImidAPol	Imidazol-tagged A8-35
MPs	Membrane proteins
NAPol	Non-ionic glycosylated APol
NBD	7-Nitro-1,2,3-benzoxadiazole
ND	Nanodisc
NP	Nanoparticle
NTA	Nitriloacetic acid
OligAPol	Oligodeoxynucleotide tagged A8-35
PerDAPol	Perdeuterated A8-35
POR	Cytochrome P450 oxidoreductase
SAPol	Sulfonated APol
SPR	Surface plasmon resonance
tOmpA	Transmembrane domain of the *Escherichia coli* outer membrane protein A

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Membrane Proteins: Fascinating Key-Players in Synthetic Biology

Synthetic biology is an evolving interdisciplinary research area that combines biology, chemistry, physics and engineering to understand how biological systems are naturally structured and organized and to exploit this knowledge and

the intrinsic properties of biological systems to construct novel systems with beneficial functions. In addition to redesigning complex biological systems mimicking cells, such as artificial cells or genetically reprogrammed cells, a considerable part of novel initiatives in synthetic biology deals with the exploitation of the natural function of biological molecules for the design of novel biotechnological tools to be used as biosensing platforms and "smart devices" for various purposes, including personalized medicine, safety and molecular bioelectronics (Khalil and Collins 2010; Weber and Fussenegger 2012). Most of these applications require self-assembly of biomolecules onto various solid supports.

The intrinsic functions, sensitivity and selectivity of proteins in cells make them central components of many devices in synthetic biology and membrane proteins (MPs) are particularly relevant because of their functions and involvement in many cellular sensing processes. They are notably the targets for numerous compounds of various sizes (e.g., peptide hormones, neurotransmitters, small molecules), and their role in taste and smell perception is a good example of applications in synthetic biology. Novel sensing devices, called artificial noses, are currently under development in various formats for future use in safety, personalized medicine, food and perfume industries (Oh et al. 2011; Song et al. 2013). In addition to their sensitivity and selectivity, MPs exhibit also interesting functions to be exploited for new devices. They can act as channels with high selectivity—it is for example the case of aquaporins acting as selective channels for water molecules, which are presently exploited as the main component of new water filtration devices (Kumar et al. 2007; Qu et al. 2013). MPs involved in energy conversion or production in cells, for example via the exploitation of solar cells through photosynthesis processes, can be used to design sensing platforms converting a natural source of energy into another one representing a great potential in renewable resources (Jensen et al. 2011).

Most of the applications cited above require the use of functional MPs immobilized onto surfaces as functional unit/recognition motif of the device. The recognition motif can be a native MP extracted from natural sources, a recombinant MP, or a MP genetically modified to artificially optimize the properties of the protein (e.g., specificity and selectivity) and the devices (Goldsmith et al. 2011). However, several challenges have to be overcome for the generation of a successful device. Firstly, sufficient quantities of purified MPs are required for performing surface-based investigations. MPs can be overexpressed in heterologous organisms (e.g., prokaryotes, yeast, insect cells, plant cells and mammalian cells), by cell-free expression technology (Andrell and Tate 2013) or purified from natural sources. Secondly, MPs function and stability (i.e., the shelf-life of the proteins) is an essential criterion; the poor stability of MPs once isolated from their native membranes remaining the major issue in many cases. Solubilization of MPs in detergent causes often their denaturation or inactivation. Many options are available today to maintain isolated MPs stable, notably reconstitution into membrane mimetic environments such as nanodiscs (NDs) (Bayburt and Sligar 2010; Denisov et al. 2004), amphipathic peptides (Tao et al. 2013), bicelles (Ujwal and Bowie 2011), supported membrane bilayers or artificial vesicles (Fruh et al. 2011). Other environments have emerged with the development of novel surfactants less destabilizing toward MPs such as detergents with multiple, branched or cyclic hydrophobic chains (Hovers et al. 2011), fluorinated surfactants (Breyton et al. 2010) and small amphipathic polymers like amphipols (APols) (Popot et al. 2003, 2011). Thirdly, the structure, function and interaction of many MPs with other molecules must still be explored and the ability to specifically label MPs has been widely used to elucidate proteins physical properties. For example, spectroscopic techniques such as fluorescence and NMR that rely on the specific labeling of proteins with fluorophores or isotopes have allowed the investigation of proteins folding, conformation dynamics and assembly interaction. Further, immobilization of proteins onto surfaces for realizing new surface-based platforms in synthetic biology might alter their function and it is generally preferable to use site-specific immobilization via affinity tags (Fruh et al. 2011). A various panel of strategies for specific immobilization are available today (Jonkheijm et al. 2008), including biotin tag and Strep-tag® binding to surfaces modified with streptavidin or its derivatives, which allow stable immobilization but are poorly reversible. Desthiobiotin and peptide-like tags (e.g., polyhistidine tag or epitope peptide tags binding, respectively, to surfaces functionalized with Ni^{2+}:nitrilotriacetic acid (NTA) or antibodies) are also very useful strategies as they allow a reversible immobilization and facilitate the regeneration of the devices (Jonkheijm et al. 2008). All these strategies require the genetic modification of MPs prior to their expression in heterologous systems. Finally, the handling of the device has also to be as stable and direct as possible, for generic applications in medical and industrial sectors. In this respect, it is generally preferable to measure signals in simple aqueous buffer solutions. Solubilization of MPs in detergent micelles tends to complicate the handling of the device since it requires a constant concentration of detergent in the sink buffers during measurements. Moreover, detergent might interfere with signal detection due to variation of refractive indexes of the solutions.

The current review focuses on the perspectives offered by APols (Popot et al. 2011) in synthetic biology. They are

one of the most successful strategies to maintain MPs or MP complexes functional and stable in detergent-free solutions. They have been recently derived in several versions with numerous functions conferring them an extreme versatility for MPs immobilization and labeling, which makes them ideal molecules for numerous approaches in synthetic biology.

Amphipols Stabilize Functional Membrane Proteins in Solution

APols are short amphipathic polymers developed to keep MPs soluble as individual complexes in aqueous solution in the absence of detergent. The polymer consists of highly polar groups that separate hydrophobic chains along the polymer. By now, the best characterized type of APols is the polyacrylate-based APol named A8-35. A hydrophilic backbone with carboxylate groups keeps A8-35 soluble in aqueous solution with pH greater than 7 while hydrophobic chains drive the polymers to self-assemble into small well-defined particles ($\approx$40 kDa) (Gohon et al. 2004, 2006; Perlmutter et al. 2011). Compared with the critical micelle concentration (CMC) of detergents such as dodecyl maltoside (0.087 g/L) widely used in MPs purification, the critical self-association concentration of A8-35 is particularly low ($\approx$0.002 g/L) (Giusti et al. 2012).

MPs trapped in A8-35 form stable water-soluble complexes (Popot 2010; Popot et al. 2011). Because of the low concentration needed to drive the APols self-assembly and the high affinity of APols for the hydrophobic domain of MPs, MP/APol complexes can be handled in solution without further addition of APols, even at extreme dilutions (Zoonens et al. 2007). Furthermore, it has been shown that MPs trapped in APols and immobilized to a surface remain functional even though all free APol particles are removed from solution (Basit et al. 2012; Charvolin et al. 2009).

The general workflow for trapping MPs in APols is first to solubilize cell membranes with detergent. The target-solubilized MP is then purified and mixed with APols before the present detergent is removed by dilution, polystyrene beads or dialysis. After detergent removal, the amphipathic polymers surround the hydrophobic surface of the MPs and keep the protein water-soluble (Popot et al. 2011; Zoonens et al. 2014) (Fig. 1). The optimal ratio between APols and MPs depends both on the size of the MP hydrophobic domain and on the presence of lipids or other co-factors bound to the protein and therefore varies for each protein. The molecular size of MPs that can be trapped in APols is not limited. This is due to the high flexibility of the APol backbone chain, making APols suitable for stabilization of large membrane protein supercomplexes (Althoff et al. 2011).

In most cases, APols have been used to trap and stabilize MPs from native tissues (Althoff et al. 2011; Champeil et al. 2000; Gohon et al. 2008; Martinez et al. 2002; Tifrea et al. 2011) or expressed in heterologous systems (for review see Popot 2010). However, they have also been shown to be extremely useful for other strategies. APols can be used for folding denatured MPs after overexpression in large yields as inclusion bodies in *Escherichia coli* (*E. coli*) and for purification under denaturing conditions (Pocanschi et al. 2006). This method has been successfully used with a wide range of MPs of various structures, notably with several G protein-coupled receptors (GPCRs) (Baneres et al. 2011; Dahmane et al. 2009; Damian et al. 2012).

The most remarkable property of APols is their ability to preserve the structure and function of MPs and, in general, to improve the time stability of MPs compared with detergents (Champeil et al. 2000; Pocanschi et al. 2006; Popot 2010; Popot et al. 2011). The higher stability of the MPs is thought to depend on several factors. Of major importance is the non-destabilizing nature of APols that support binding of essential lipids or co-factors that are fundamental for MPs function and stability and which in detergent solution easily dissociate from the MP. In addition, non-ionic APols (NAPols), a special class of glucose-based APols featuring no charge compared with the parent compound A8-35, widen the scope of applications in isoelectrofocusing, study of MP-ligand interaction without unwanted electrostatic forces, NMR at pH below 7 and cell-free synthesis of MPs (Bazzacco et al. 2012).

After MPs are transferred to APols and are in a detergent-free environment, many biochemical and biophysical techniques can be used to explore the structural and functional properties of MPs in vitro. These include size-exclusion chromatography and small-angle neutron scattering (Gohon et al. 2008), fluorescence microscopy (Charvolin et al. 2009; Della Pia et al. 2014; Le Bon et al. 2014a), fluorescence spectroscopy (Martinez et al. 2003; Zoonens et al. 2007), analytical ultracentrifugation (Gohon et al. 2008), NMR (Catoire et al. 2010, 2009; Zoonens et al. 2005), electron microscopy (Althoff et al. 2011; Cvetkov et al. 2011), mass spectrometry (Bechara et al. 2012; Leney et al. 2012) and surface sensitive techniques (Basit et al. 2012; Charvolin et al. 2009).

MPs trapped in APols offer the unique feature of the hydrophobic belt that can be functionalized with a variety of small molecules. APols have been functionalized with many different chemical groups, such as (1) affinity tags with biotin (Charvolin et al. 2009), polyhistidine peptides, imidazole cycles (Giusti et al. 2014a), or oligodeoxynucleotide (Le Bon et al. 2014a), (2) fluorescent probes (Giusti et al. 2012; Zoonens et al. 2007) and (3) isotopic labels (Gohon et al. 2006; Ladaviere et al. 2001) opening

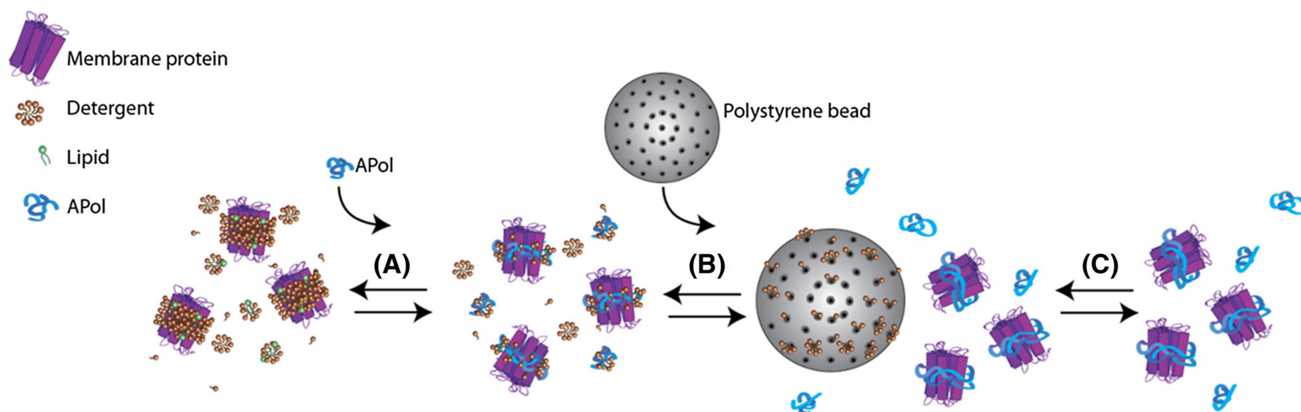

Fig. 1 Workflow for trapping MPs in APols. First MPs are extracted from the membrane by detergent and are optionally purified. (*A*) APols are added and mixed APol/detergent/MP complexes are formed. (*B*) Detergent is removed by adsorption onto polystyrene beads. (*C*) After detergent removal MP/APol complexes are soluble in detergent-free solutions

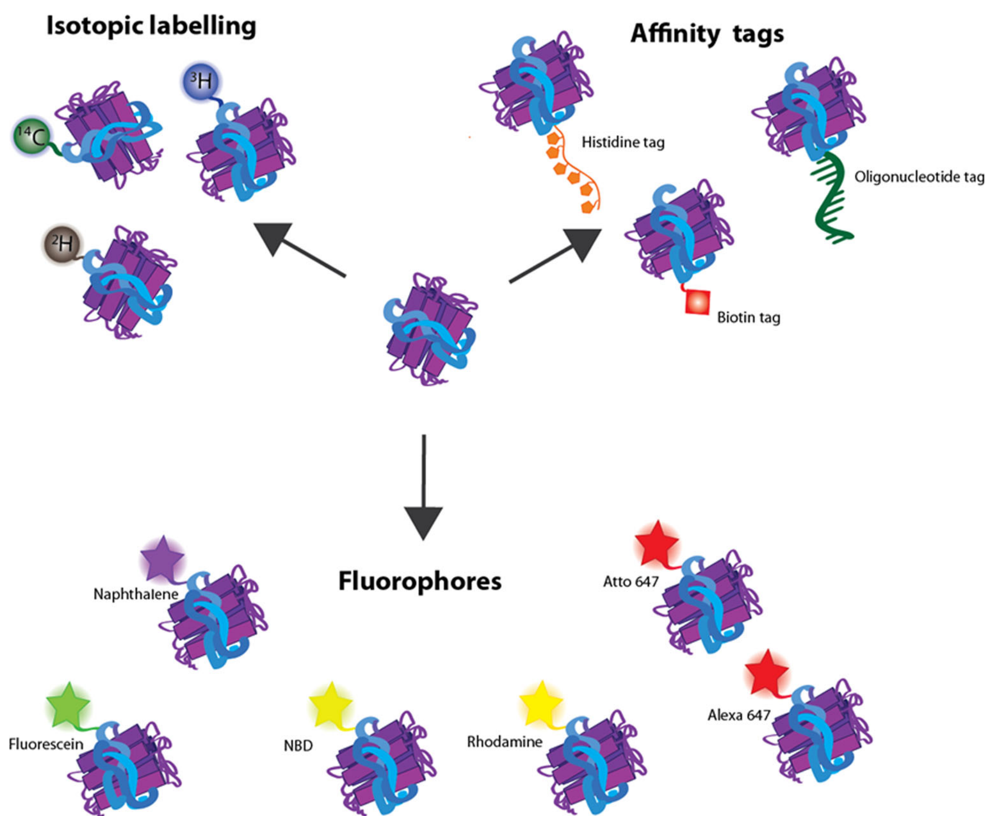

Fig. 2 Overview of the different functionalized APols

the way to many new experimental approaches in MPs characterization and applications in synthetic biology, notably for diagnostics and drug discovery (Popot 2010) (Fig. 2). Moreover, APols offer several advantages compared with other classes of surfactants and NDs (Bayburt and Sligar 2010; Nath et al. 2007): (1) Their versatile chemistry makes it relatively easy to synthetize a large variety of functionalized and labeled molecules (Le Bon et al. 2014b); (2) given their relatively large size, addition of small molecules tags does not affect their general physico-chemical properties; (3) as the association of APols with MPs is stable in the absence of competing surfactants or lipids (Popot et al. 2003; Tribet et al. 2009; Zoonens et al. 2007), the trapped MPs are permanently functionalized without need of chemical or genetic modification. The unique properties of APols to keep MPs in detergent-free solution and the possibility to design functionalities of the surrounding surfactant belt make them

ideal tools for stabilization of functional MPs necessary for applications in synthetic biology.

Functionalized APols: Fluorescent APols

Over the last two decades, fluorescence spectroscopy has become a powerful analytical and diagnostic tool both in fundamental biophysics, molecular and cellular biology studies and in the more applied research areas of biotechnology and medicine. It will also remain a method of choice for many devices developed in synthetic biology. The scientific interest in this method has grown thanks to the rapid development of a wide range of techniques and fluorescence detection instruments that can be used to characterize the most various and complex systems. Fluorescence spectroscopy is highly spatially and temporally sensitive, and great opportunities are offered by the wide range of fluorescence probes that can be synthesized. Currently, the most applied methods for specific labeling of purified proteins with fluorophores are based either (1) on fusion of a fluorescent protein (i.e., GFP) or (2) on post-translational modifications such as covalent labeling of organic fluorophores by amino and thiol chemistry, coordination to NTA fluorescent analogs via polyhistidine tag or use of fluorescent substrates recognized by enzyme tags (i.e., SNAP-tag) (Krueger and Imperiali 2013; Marks and Nolan 2006). All these strategies, besides the use of fluorescent NTA analogs, are irreversible labeling strategies, and each application requires a new modification of the protein. Although all these methods have been widely used, the main drawbacks are that the labeling processes require either the genetic engineering of the target protein DNA that could be not available or the chemical modification of the MP. The genetic incorporation of large proteins can also perturb the function of the target protein or protein–protein interactions.

An alternative and more versatile approach for labeling MPs with organic fluorophores is to exploit APols that can be functionalized with a fluorophore, thereby imparting fluorescence to the trapped protein. APols are not intrinsically fluorescent, and their chemistry offers the opportunity to finely tune the photophysical properties of the fluorescent probes; in principle, a plethora of small organic dyes could be grafted onto the APols. To date, APols have been modified with an array of bright multicolored and complementary organic fluorescent dyes of various brightness and photostability such as naphthalene (Vial et al. 2005), 7-nitro-1,2,3-benzoxadiazole (NBD) (Giusti et al. 2012; Zoonens et al. 2007), rhodamine B (Giusti et al. 2012), fluorescein (Opačić et al. 2014), Atto 647 (Le Bon et al. 2014b) and Alexa 647 (Le Bon et al. 2014a). The development of a series of fluorescent APols (FAPols) with excitation and emission wavelengths covering the full visible spectrum (Fig. 3) has allowed researchers to investigate the distribution of APols in lipid vesicles (Vial et al. 2005), in micelles (Giusti et al. 2012), in tridimensional crystals of MPs (Charvolin et al. 2014; Popot et al. 2011) and in living organisms (Ferrandez et al. 2014). FAPols have also been used to demonstrate the potential of APols as vectoring agents to cell and tissues (Popot et al. 2011). These results, together with the non-toxicity and non-cytolytic activity of APols (Popot et al. 2003), indicate that these surfactants could be successfully used as solubilizing, stabilizing and delivery agents. Finally, FAPols have shown their convenience for the visualization of immobilized MPs onto micro- and nano-surfaces (Della Pia et al. 2014; Le Bon et al. 2014a). All these applications illustrate the potentiality of FAPols in synthetic biology research. Novel FAPols can be further designed for additional applications. For example, the use of new dyes sensitive to their environment (i.e., pH, temperature) or reacting with ion species (i.e., calcium) would represent a novel generation of environmental sensitive FAPols suitable for novel structure/function studies of MPs covering protein activities, conformational changes, docking events, function, trafficking and distribution in living cells.

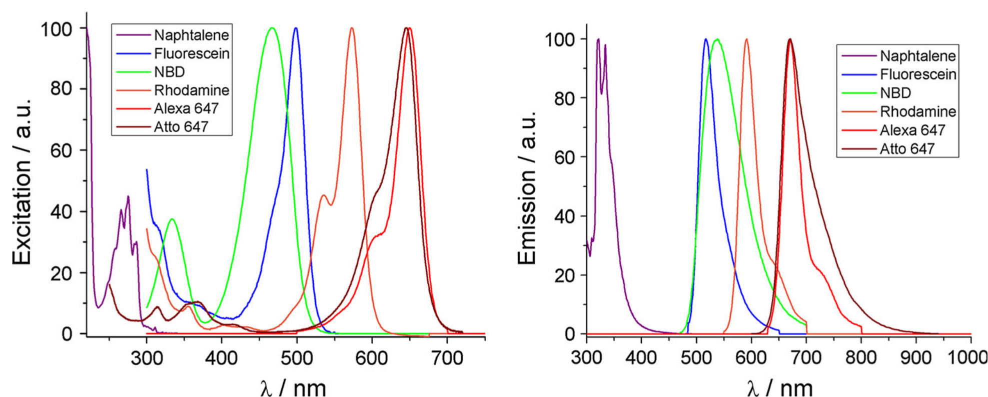

Fig. 3 Normalized excitation (*left*) and emission (*right*) spectra of the fluorescent dyes available as APols labels

Functionalized APols: Isotopically Labeled APols

Structural and functional characterization of MPs are fundamental for their applications in synthetic biology. Over the past 15 years, APols labeled with different isotopes have been synthesized including ^{14}C (Tribet et al. 1997), ^{3}H (Gohon et al. 2008) and ^{2}H (Catoire et al. 2010; Gohon et al. 2004, 2006, 2008; Zoonens et al. 2005). Their application to NMR (Catoire et al. 2010; Planchard et al. 2014; Zoonens et al. 2005) and neutron scattering experiments (Gohon et al. 2004, 2006, 2008) has provided interesting results regarding the organization, the size and the structure of MP-APols complexes. The first experiments performed with cytochrome b_6f trapped in ^{14}C-labeled APols indicated that surfactants used to stabilize MPs could be removed by an excess of APols and that the formed MP-APols complexes have a low mass polydispersity and appear to be irreversible in the absence of free APols or competing surfactants in solution (Tribet et al. 1997). Successively, a hydrogenated (HAPol) and a partially deuterated (DAPol) form of A8-35 were used to determine the size and composition of APols and MP-APols complexes in solution with analytical ultracentrifugation and small-angle neutron scattering (Gohon et al. 2004, 2006). The data indicated that, despite the variable length of the side chains and the number of hydrophobic groups carried by the single molecules, APols self-organize in small, compact micelle-like particles with a well-defined size. Further studies of BR trapped in HAPols and DAPols indicated that labeling the polymer with isotopes does not strongly affect its physical chemical properties (Gohon et al. 2008). Structural studies of tOmpA trapped in HAPols and DAPols by solution NMR led to the conclusion that the side chains of A8-35 associate exclusively with the hydrophobic transmembrane surface of the protein (Zoonens et al. 2005). More recently, the structure of the GPCR ligand LTB4 bound to its natural receptor BLT2 folded and stabilized in DAPols has been solved by NMR spectroscopy (Catoire et al. 2010). Apart from giving important information about the change in conformation of the ligand upon binding, the study validated a straightforward method for structural investigations of GPCRs by solution NMR suggesting the potential of APols as a valuable tool also in MPs structural biology. More recently, the synthesis of a perdeuterated APols (perDAPol) was performed in order to minimize overlapping of cross-correlation peaks of the hydrogenated backbone of DAPol with those of MPs and their ligands (Giusti et al. 2014b). The further development of NAPols and sulfonated APols (SAPols) provides new, attractive opportunities in this field, because these pH-insensitive APols eliminate the need to work at basic pH (7 or higher), as is the case for classical A8-35 APols, making protons exchange slower and high-resolution NMR spectra easier to obtain (Popot et al. 2011).

Functionalized APols: Tags for Immobilization

In the past few years, protein microarrays, protein sensor chips and microbeads have emerged as tools of choice for obtaining information about proteins functions and interactions (Weinrich et al. 2009). Protein arrays combined with sensitive optical detection systems such as surface plasmon resonance (SPR) and fluorescence microscopy offer multiplexing and high-throughput screening with a high potential in diagnostics. These techniques can also provide important information regarding protein affinities and binding kinetics of interaction using minimal amounts of reagents.

All these surface-based assays require the immobilization of proteins preserving their native structure and biological activity (Jonkheijm et al. 2008). Functional immobilization of MPs has been achieved by covering bare and modified surfaces with native (Gottschalk et al. 2000; Martinez et al. 2003; Perez et al. 2006; Yang and Lundahl 1994) and reconstituted membrane fragments (Bieri et al. 1999; Fruh et al. 2011). Although these methods allow the investigation of MPs in their native lipid environment, high levels of non-specific binding to surrounding biomolecules and the relatively unstable immobilization limit their applications (Fruh et al. 2011). Other approaches that have allowed a more stable and robust immobilization include the genetic or chemical fusion of small peptide or protein tags for reversible or irreversible immobilization (Friedrich et al. 2004; Harding et al. 2006; Schmid et al. 1998). However, these strategies present several technical hurdles due to the use of detergent, and APols can offer an alternative and highly versatile means for MPs immobilization. MPs trapped in functionalized APols are stabilized and permanently associated with any functional tag presented by the polymer. Therefore, fragile MPs or MP complexes functionalized in specifically tagged APols can be separated, purified, immobilized onto solid support in a gentle and stable manner and analyzed in detergent-free solutions (Popot 2010).

To date, several A8-35 APols modified with different affinity moieties have been synthesized and characterized. These include biotinylated APols (BAPols) (Basit et al. 2012; Charvolin et al. 2009; Ferrandez et al. 2014), polyhistidine- and imidazole-tagged APols (HistAPols and ImidAPol) (Della Pia et al. 2014; Giusti et al. 2014b) and oligodeoxynucleotide-derivatized APols (OligAPols) (Le Bon et al. 2014a). All these tags cover a large panel of interactions with different affinity, specificity and reversibility options (Fig. 4; Table 1).

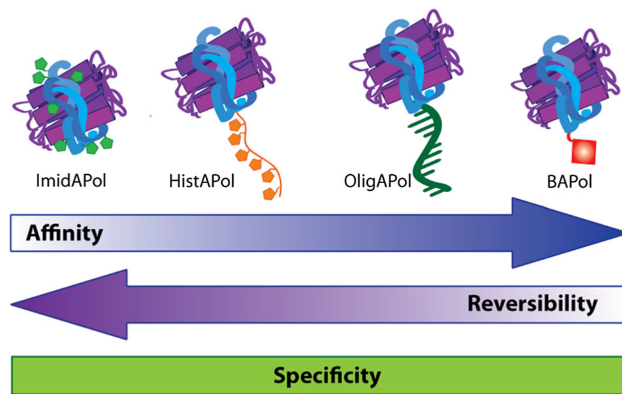

Fig. 4 Illustration presenting the physical properties of the immobilization tags of APols

Table 1 Properties of the immobilization tags presented by APols

Tag	K_D (M)	Specificity	Reversibility
Biotin	10^{-14}–10^{-16a}	High	Low
Oligonucleotide	10^{-9b}	High	High
Polyhistidine	10^{-7c}	Medium	High
Imidazole	$<10^{-7c}$	Medium	High

[a] (Jonkheijm et al. 2008), [b] (Laitinen et al. 2007), [c] (Niemeyer et al. 1998)

Upon APols-mediated immobilization, MPs can be investigated by a variety of label-free or fluorescence-based methods with a strong potential for protein arrays, diagnostics and drug screening as well as for structural and functional analysis of biomolecular interactions. In fact, even though the position of the functional tags in the APol belt surrounding the MP cannot be controlled and can change from protein to protein and over time, trapping MPs in APols leaves extra-membrane domains exposed to solution not hindering ligand binding. Furthermore, the immobilization procedure is very general; any kind of MP can in principle be trapped in functionalized APols.

Biotin has a high specificity and binding affinity for streptavidin ($K_D = 10^{-14}$–10^{-16} M) (Laitinen et al. 2007) and can be nearly irreversibly immobilized to streptavidin-modified surfaces. This strategy is often used for the development of biosensors as it guarantees a good stability of the proteins at the surface and thereby a stable signal. MPs of different biological origins, structures and sizes have been successfully reconstituted in BAPols and immobilized on streptavidin-modified beads or chips without interfering with their function and binding activity (Charvolin et al. 2009). The authors used SPR to investigate the kinetics of interaction between the soluble protein-specific antibodies and the immobilized MPs-BAPols complexes (Fig. 5). Furthermore, the binding of a fluorescently labeled toxin acting as antagonist for the nicotinic acetylcholine receptor trapped in BAPols and immobilized onto a solid support was confirmed by confocal fluorescence microscopy. Basit et al. 2012 showed that also the complex MP FhuA from the outer membrane of *E. coli* could be trapped in biotinylated phosphocholine-based APols and immobilized on streptavidin-modified surfaces. The MP retained its biological activity and its affinity for pb5 (a bacteriophage T5 protein) was confirmed by SPR and electrochemical impedance spectroscopy. BAPols have been recently shown to be compatible with nanoscale electrodes for functional MP arrays (Della Pia et al. 2014).

Many devices require the regeneration of the biosensing surfaces in addition to the stable immobilization of functional MP. For this purpose, polyhistidine tag is a very generic affinity tag used because it allows both a specific and a reversible immobilization (Liu et al. 2010). This feature is due to the low affinity of polyhistidine tag for the Ni^{2+}-NTA complex ($K_D \sim 10^{-7}$ M) and the easy dissociation by the addition of ethylenediaminetetraacetate (EDTA) or imidazole (Jonkheijm et al. 2008). Very recently, HistAPols have shown to be suitable for MP immobilization onto NTA surfaces loaded with Ni^{2+} (Fig. 5) (Giusti et al. 2014a). The experiments demonstrate that HistAPols allow for the stable binding of MPs to the chip surface; controlled amounts of MPs can be stably loaded onto the sensor surface, which is necessary to perform kinetic studies of bioaffinity interactions. Thanks to the presence of several polyhistidine tags on HistAPol particles, the immobilization stability of MP/HistAPol complexes is also improved, compared with a single polyhistidine tag fused to MPs. The NTA surface was reproducibly regenerated by simple incubation with EDTA that successfully removed all the immobilized MPs. Both the high stability and the reversibility of the immobilization make HistAPols an attractive alternative to BAPols for surface-based studies. Because the moieties of polyhistidine tag that interacts with nickel ions are only imidazole cycles of histidine residues, APols carrying imidazole groups randomly distributed along the APol backbone (ImidAPol) have also been synthetized (Giusti et al. 2014a). The advantage of ImidAPol compared with HistAPol is the easier chemical route for its synthesis leading to better yields. MPs trapped in ImidAPols were immobilized onto a metal-ion affinity chromatography (IMAC) column and recovered by incubation with an excess of imidazole in the buffer. MP/ImidApol complexes were also immobilized onto a SPR biosensing surface; however, their stability was lower than that provided by HistAPol binding.

In addition to the stability and reversibility of the immobilization, the specificity of the immobilization is usually an important criterion to take into account for the development of biosensing devices. Oligodeoxynucleotide

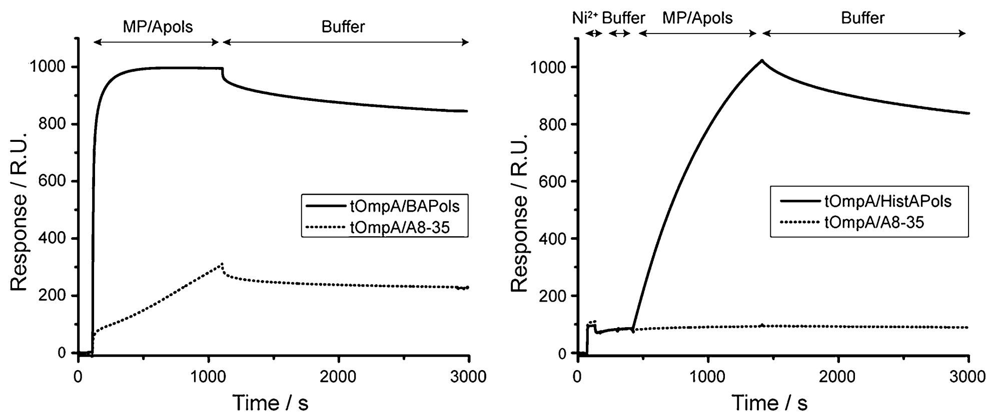

Fig. 5 SPR sensorgrams for different immobilization strategies. (*left*) Immobilization of MP-BAPols complexes onto streptavidin-modified surfaces (Charvolin et al. 2009). (*right*) Immobilization of MP-HistAPols complexes onto Ni–NTA-modified surfaces (Giusti et al. 2014a). *R.U.* response unit

tags present a considerable potential due to the high specificity and affinity for their complementary sequence ($K_D \sim 10^{-9}$ M—this value strongly depends on the oligodeoxynucleotide sequence—(Niemeyer et al. 1998)) and the easy reversibility of the association by chemicals or temperature. MPs have been recently trapped in a first generation of OligAPols (Le Bon et al. 2014a), and their immobilization on complementary oligodeoxynucleotide-modified surface has been shown to be specific, stable and reversible.

Several universal approaches to tether MPs to solid supports are thus available and offer new tools suited to creating new functional devices for synthetic biology applications. There is also a large potential for future developments of APols. In particular, APols have a high electrostatic charge density that, depending on the experimental technique, can be either an advantage or a constraint. Development of tagged NAPols would prevent either non-specific binding or electrostatic repulsion (Ferrandez et al. 2014; Popot et al. 2011).

Other Advantage of Functionalized APols: Multiple Functionalizations

The recent development of different tags for proteins isolation, immobilization and detection has provided us with a wide variety of tools for their studies. However, despite the distinct advantages of the single labels, the application of each tag is restricted by its range of capabilities. Therefore, studies based on multiple analytical methods often require labeling of the same protein with more than one tag and, as a result, many proteins are nowadays genetically engineered with multiple orthogonal fusions partners (Gautier et al. 2008). As an example, a recombinant SNAP-FLAG-His protein containing three tags can be (1) purified and immobilized on surfaces functionalized with Ni^{2+}-NTA motifs; (2) modified with a substrate for the SNAP-tag (benzyl guanine (BG)—biotin) and assembled on streptavidin-modified surfaces; (3) fluorescently labeled with a BG-600 fluorophore and imaged by fluorescence microscopy (Iversen et al. 2008; Liu et al. 2010). However, these methods rely on genetically encoded peptide and protein tags and cannot be applied to native proteins.

APols offer an interesting approach for generating multifunctional, multivalent MP complexes. In fact, as several APols molecules associate with a single MP (Popot et al. 2011), several functionalities can be added to a protein by simply trapping it in a mixture of custom-functionalized APols. The highly modular characteristics of the APols permit the facile and versatile construction of a multitude of constructs that could in principle match any experimental setup. Each APol module (either functionalized or non-functionalized) is synthesized and stored separately, can be paired with another one and combined on demand. The only limit of functionalities that can be added to a MP is essentially given by its size and the numbers of APols needed for its trapping.

In a first study, we have applied the modularity of APols to generate bi-functional MP-APols complexes by combining OligAPols with FAPol-Alexa 647 (Le Bon et al. 2014a). The latter allowed us to use epifluorescence microscopy to demonstrate highly specific and homogeneous binding of the trapped MPs to surfaces pre-functionalized with an oligodeoxynucleotide linker complementary to the sequence of the oligo-tag tethered to APols (Fig. 6a). The MP-OligAPols complexes could also be released from the gold nanoparticles (Au NPs) surface either chemically or by heat and the binding surface could be easily regenerated. These preliminary experiments suggest that OligAPols might have great potential for use in biosensing applications.

After establishing proof of principle with the OligAPol and FAPol modules, we next trapped two MPs in BAPol combined with two different FAPols labeled with either NBD or Alexa 647. The modularity of the APols showed a great potential in this application, allowing us to immobilize two different MPs onto streptavidin-modified surfaces while monitoring the specific interactions of the two MPs

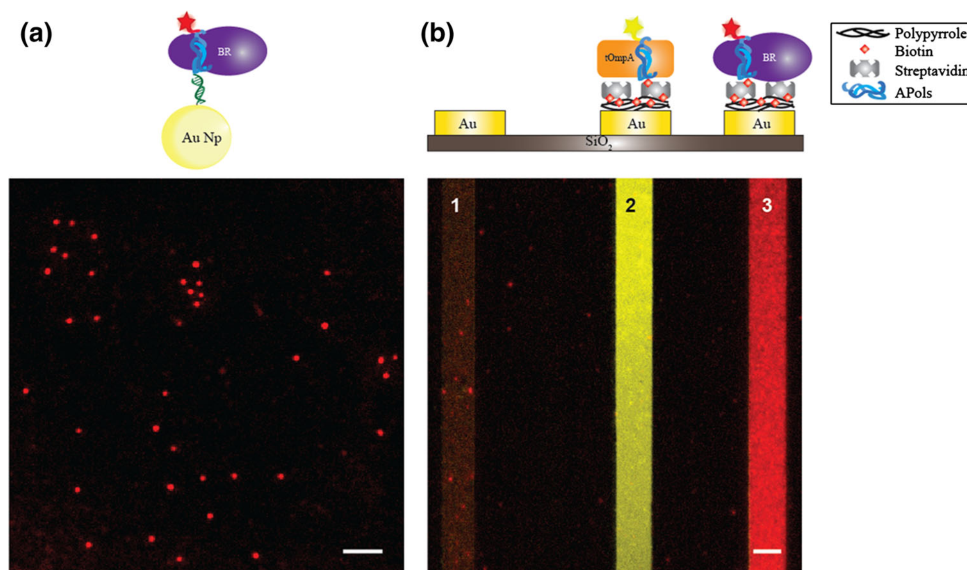

Fig. 6 Multiple functionalizations of MPs via APols. (**a**) Schematic illustration and fluorescent microscopy image of Au NPs functionalized with a thiolated oligodeoxynucleotide and BR trapped in 1:1 mixture of OligAPols and FAPols Alexa 647 (Le Bon et al. 2014a). The formation of the bi-labeled MP/OligAPol/FAPol-Alexa 647 complex was demonstrated by FPLC analysis combined with UV–Vis spectroscopy. (**b**) Cartoon and fluorescent microscopy image of a gold (Au) electrode (number 1) and two gold electrodes functionalized with a biotinylated polypyrrole film and streptavidin. By controlling the deposition of the polypyrrole film, tOmpA trapped in BAPols and FAPols NBD and BR trapped in BAPols and FAPols Alexa 647 were immobilized on electrode 2 and electrode 3, respectively. *Scale bar* is 5 µm. Adapted with permission from (Della Pia et al. 2014). Copyright 2014 American Chemical Society

with the solid supports at once (Della Pia et al. 2014). It also allowed us to demonstrate for the first time multiplexed functionalization of micro- and nano-meter electrodes with two different MPs (Fig. 6b) opening the way to attractive application in the bio-nanosensing field.

Other Advantage of APols: Reversibility of the Trapping

An extremely intriguing property of APols is the reversibility of their association with MPs. In fact, although APols bind strongly to the hydrophobic patches of MPs through several contact points and do not desorb even at extreme dilutions (Della Pia et al. 2014; Zoonens et al. 2007), they can be displaced by a competing surfactants such as free APols (Tribet et al. 1997; Zoonens et al. 2007) (Fig. 7), lipids (Damian et al. 2012; Nagy et al. 2001) or detergents (Tribet et al. 2009; Zoonens et al. 2007). The reversibility has innumerable applications in synthetic biology. In particular, MPs could be expressed, folded to their native state and stored in APols taking advantage of the polymers stabilizing and solubilizing properties, and then transferred to the desired environment simply by exchanging A8-35 with the surfactant of choice (Damian et al. 2012). As an example, this method could have interesting implications in structural biology studies where MPs stabilized by APols in aqueous solution can be transferred to tridimensional lipid phases suitable for the growth of 3D crystals of MPs for X-ray experiments (Polovinkin et al. 2014).

Proof of principle for the interchangeability of the APols has been first investigated by following sedimentation experiments of three MPs trapped in ^{14}C-labeled APols upon addition to the gradient of different amounts of unlabeled APols (Tribet et al. 1997). Indeed, the radioactive-labeled APols were not displaced in absence of free APols, while the presence of free APols led to the release of 40 % of the ^{14}C-labeled polymer bound to the MPs after 6 h of sedimentation. The MPs-bound APols could be completely removed by an excess of detergent added tenfolds above its CMC. Subsequent experiments investigated the kinetics of APols exchange by measuring variation in the FRET signal between the tryptophan residues of a MP and FAPols NBD in presence of different concentrations of unlabeled A8-35 APols (Zoonens et al. 2007). It was demonstrated that the ionic strength of the solution has an important effect not only on MP-APols association (Tribet et al. 1997), but also on their dissociation. The higher the salt concentration, the faster the APols could be displaced from the MPs; in the presence of 100 mM NaCl and an excess of free APols, the bound FAPol-NBD molecules were exchanged in only 10 min, whereas in the absence of NaCl, the exchange took more than 24 h. As at higher ionic strength, the electrostatic repulsion between negatively charged-free APols and MP/FAPol-NBD complexes is

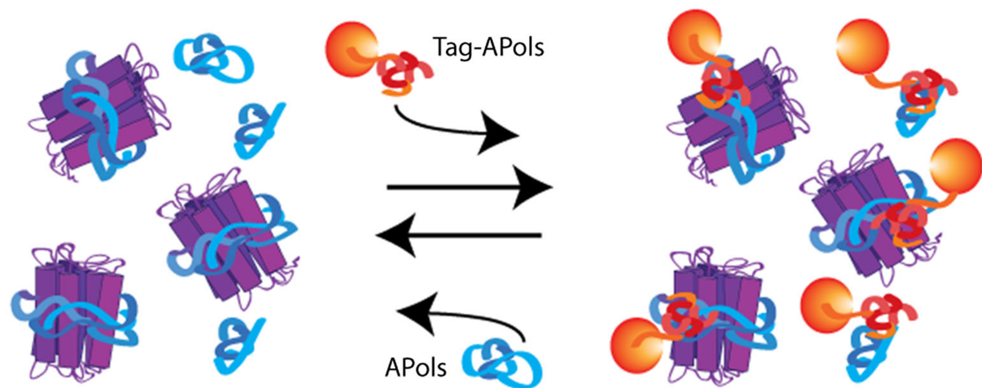

Fig. 7 Reversibility of MPs trapping in APols. APols surrounding MPs can be mixed or fully exchanged with different APols containing any kind of tags by simple incubation with an excess of the desired APols

hindered, the authors suggested that collisions between free and bound APols are the mechanism underlying the exchange of APols followed by their fusion, mixing and random detachment from the ternary MP/FAPol-NBD/APol complex. More recent experiments have also demonstrated that MP-bound APols can be partially displaced by small detergent molecules forming stable ternary MP/detergent/APol complexes (Tribet et al. 2009). These results open the way to novel and versatile functionalities that could be added to the MPs by simply using mixtures of APols and small grafted detergent molecules.

Conclusion

APols combine numerous advantages providing unique and versatile tools for applications of MPs. In addition to allow the handling of MPs in solution with the same facility than soluble proteins (without excess of APols and in detergent-free solution), they maintain the function of MPs and provide shelf-life time suitable for devices adapted to synthetic biology. The various functionalized APols now available for MPs immobilization (reversible or irreversible), labeling (fluorescent, isotopic-labeling) and the possibilities to combine several functionalities on the same protein and exchange them with limited efforts provide an unprecedented versatility of protein modification, which appears to be superior to any post-translational modification of soluble proteins. This versatile toolbox is generic as it is adapted to MPs of various sizes—even present as complexes—from various sources of proteins (tissues, heterologous systems and cell-free expression system). All these features make APols unique tools for the development of MPs–based devices in synthetic biology.

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