

# The use of amphipols as universal molecular adapters to immobilize membrane proteins onto solid supports

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**Because of the importance of their physiological functions, cell membranes represent critical targets in biological research. Membrane proteins, which make up  $\approx 1/3$  of the proteome, interact with a wide range of small ligands and macromolecular partners as well as with foreign molecules such as synthetic drugs, antibodies, toxins, or surface recognition proteins of pathogenic organisms. Whether it is for the sake of basic biomedical or pharmacological research, it is of great interest to develop tools facilitating the study of these interactions. Surface-based in vitro assays are appealing because they require minimum quantities of reagents, and they are suitable for multiplexing and high-throughput screening. We introduce here a general method for immobilizing functional, unmodified integral membrane proteins onto solid supports, thanks to amphipathic polymers called “amphipols.” The key point of this approach is that functionalized amphipols can be used as universal adapters to associate any membrane protein to virtually any kind of support while stabilizing its native state. The generality and versatility of this strategy is demonstrated by using 5 different target proteins, 2 types of supports (chips and beads), 2 types of ligands (antibodies and a snake toxin), and 2 detection methods (surface plasmon resonance and fluorescence microscopy).**

diagnostics | drug discovery | immobilization | chips bioreactors

The development of functional genomics and proteomics has increased the interest for identification and characterization of molecular interactions on minute amounts of proteins. Immobilization of target proteins onto a solid support allows one to combine low consumption of material with fast, parallel, and highly sensitive detection. Immobilizing membrane proteins (MPs) under a functional form is, however, complicated by their insolubility in water and their frequent instability in detergent solutions (1, 2). No method currently exists to immobilize unmodified, functional MPs without denaturation by using a simple and general protocol. Nonspecific adsorption onto solid surfaces, which is widely used for ELISA tests, immunoreplicas, and most of the protein arrays developed to date (see, e.g., refs. 3 and 4), does not require any protein modification, which facilitates massive parallel investigations, but it involves poorly characterized interactions between the target protein and the support, entailing an unknown degree of denaturation (see, e.g., ref. 5). Specific interactions mediated by an affinity tag, such as a polyhistidine tag or a biotin, covalently attached to the protein (see, e.g., refs. 6 and 7), improve the functionality and accessibility of immobilized proteins (8–10), but they require either a genetic or a chemical modification of each prospective target, and, in the case of single histidine tags, they are prone to leaching (11). Reintegrating MPs into tethered lipid vesicles or supported lipid bilayers (see, e.g., refs. 12 and 13) is a conceptually elegant alternative, but it requires a tedious optimization for each new target to be studied. Lipoprotein-stabilized bicelles have recently been used to immobilize a glycolipid (14), a procedure that

could, in principle, be extended to MPs. Up until now, however, none of the above approaches has been applied to arrays of purified MPs, which have exclusively resorted to nonspecific adsorption (see, e.g., refs. 15 and 16).

We show here that synthetic polymers called “amphipols” (APols) are tools of choice to circumvent such problems. APols (17, 18) are short soluble polymers carrying numerous hydrophobic side chains thanks to which they can associate with the transmembrane surface of MPs (19) by multiple attachment points. Thereby, they keep them water soluble in the absence of detergent and stabilize them biochemically (see ref. 20 and references therein). The most extensively used APol to date, A8-35, comprises a polyacrylate skeleton grafted with octyl and isopropyl groups (17, 21) (Fig. 1A). Its use has been validated on a large panel of MPs (18), including bacteriorhodopsin (BR) (20), the nicotinic acetylcholine receptor (nAChR) (22), the cytochrome *b<sub>6</sub>f* and *bc<sub>1</sub>* complexes (17, 18) and the transmembrane domain of *Escherichia coli* outer membrane protein A (tOmpA) (19, 23). Of particular relevance to the present work is the fact that, although it is noncovalent, its association to MPs is strictly irreversible as long as it is not displaced by another surfactant (23, 24).

In the present work, we have synthesized a functionalized version of A8-35 carrying a biotin moiety (BA8-35; Fig. 1A). Trapping a MP with BA8-35 results, in a single step, in the formation of a stable complex (Fig. 1B) in which the protein is made water soluble, stabilized, and functionalized for specific immobilization onto surfaces coated with streptavidin (SA) (Fig. 1C). The universality of the approach is demonstrated by using 5 MPs of different sizes, structures, functions, and origins (animal, vegetal, and bacterial membranes, inclusion bodies), which have been immobilized onto supports suitable for either label-free or fluorescence detection of ligand binding. The interaction of BA8-35-immobilized MPs with various types of ligands illustrates the potential of this versatile approach for immunodetection, drug screening, or the search for natural biological partners.

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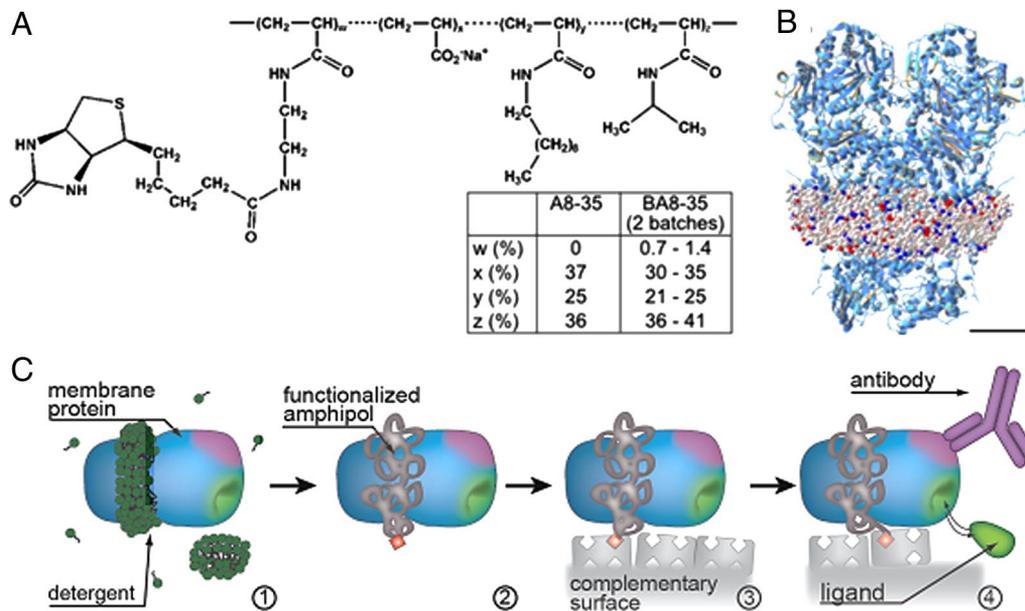
Conflict of interest statement: A patent application covering amphipol-mediated immobilization of membrane proteins and appropriately functionalized APols has been filed by the Centre National de la Recherche Scientifique and Université Paris-7.

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**Fig. 1.** Using a functionalized amphipol to immobilize membrane proteins onto solid supports. (A) Chemical structure of A8-35 and biotinylated A8-35 (BA8-35).  $w$ ,  $x$ ,  $y$ , and  $z$  are the percentages of each type of monomer: biotinylated, free, grafted with an octyl chain, and grafted with an isopropyl chain, respectively. Their distribution is random. On average, 1 molecule of A8-35 contains  $\approx 70$  acrylate units, carrying  $\approx 18$  octyl chains, for a molecular mass of 9–10 kDa. In the case of BA8-35, 2 batches were synthesized following different protocols (see *SI Text*). The structure shown is that of BA8-35-2. (B) Model of bovine cytochrome *bc*<sub>1</sub> (PDB ID code 1BGY) trapped in A8-35 [Reproduced with permission from ref. 18 (Copyright 2003, *Cellular and Molecular Life Sciences*)]. (Scale bar: 4 nm.) (C) Experimental principle of APol-mediated immobilization of MPs. A MP solubilized in detergent ① is transferred to a functionalized APol. The complex thus formed, ②, is applied to a support that exposes a functional group with which the APol can associate ③. Putative ligands of the protein are then flushed over the support ④ and their interaction with the immobilized protein detected by any convenient method.

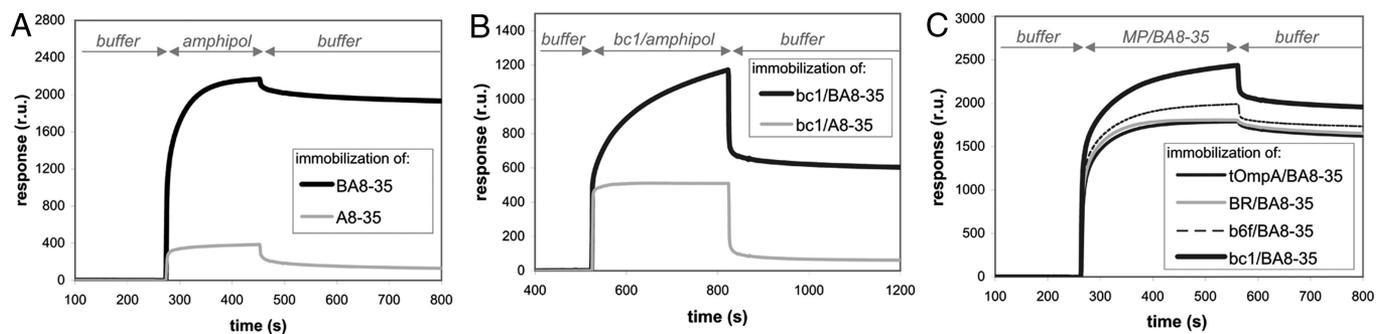
## Results

**Synthesis of Biotinylated Amphipols and Immobilization onto Streptavidin-Coated Surfaces.** Two slightly different forms of BA8-35 were obtained by different synthetic routes (see *Figs. S1 and S2*). Batches BA8-35-1 and -2 comprised an average of  $\approx 0.5$  and  $\approx 1$  biotin, respectively, per APol molecule (see *SI Text*).

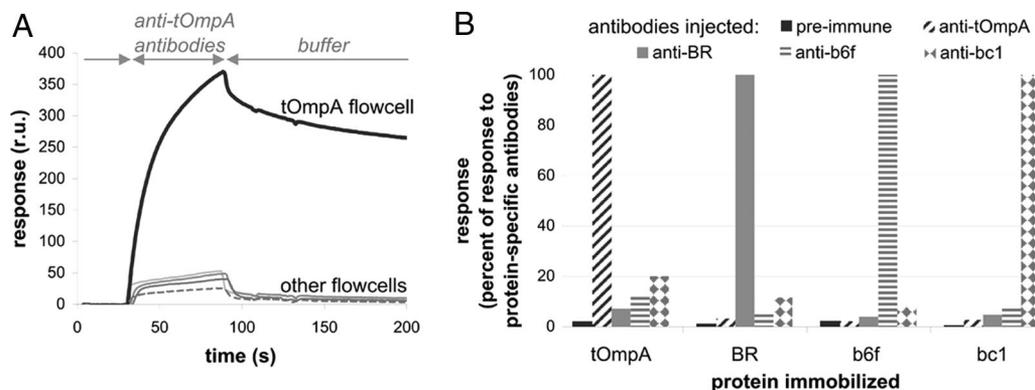
Upon injection of BA8-35 over an SA-coated sensor chip, a strong surface plasmon resonance (SPR) signal developed, most of which persisted after washing the chip with surfactant-free buffer (Fig. 2A). By contrast, a much smaller signal was observed upon injecting the same concentration of nonfunctionalized A8-35, most of which vanished upon washing with buffer. The SPR signal was  $\approx 15$  times higher with BA8-35-2 than with

A8-35, indicating that the biotin/SA interaction mediates most of the association with the chip and that nonspecific binding is low (Fig. 2A).

**Amphipol-Mediated Immobilization of Membrane Proteins.** MP immobilization and the binding of ligands to immobilized proteins were examined according to the experimental scheme shown in Fig. 1C. In a first series of experiments, we chose 4 MPs very different in terms of size, structure, function, and biological origin, namely tOmpA (19 kDa, a monomeric eubacterial  $\beta$ -barrel), BR (27 kDa, a monomeric archaebacterial protein folded into a 7- $\alpha$ -helix bundle), and 2 large eukaryotic complexes comprising numerous  $\alpha$ -helical subunits and cofactors, cytochrome *b<sub>6</sub>f* (228 kDa, from *Chlamydomonas reinhardtii*) and



**Fig. 2.** Immobilization of APols and of MP/APol complexes onto SA-coated chips followed by SPR. (A) Thirty microliters of a solution of BA8-35-2 (black) or of nonbiotinylated A8-35 (gray) were flushed over an SA sensor chip at a concentration of  $0.3 \text{ g} \cdot \text{l}^{-1}$  in NaPh buffer. SPR signals were recorded at  $25^\circ \text{C}$  in a Biacore 2000 instrument. (B) Fifty microliters of a solution of cytochrome *bc*<sub>1</sub> trapped either in BA8-35-1 (black) or in nonbiotinylated A8-35 (gray) were flushed over an SA sensor chip at a concentration of protein of  $2 \mu\text{M}$  in NaPh buffer. (C) Fifty microliters of solutions of tOmpA (thin black line), BR (thin gray line), cytochrome *b<sub>6</sub>f* (dashed thin black line) and cytochrome *bc*<sub>1</sub> (thick black line), each of them trapped in BA8-35-2, were flushed over various flow cells of 2 SA sensor chips at a concentration of  $0.3 \text{ g} \cdot \text{l}^{-1}$  BA8-35. The cytochrome *b<sub>6</sub>f* sensorgram, which was recorded in a different session, has been normalized by using as a reference the response to cytochrome *bc*<sub>1</sub>, which was measured each time. Experimental conditions differed in A, B, and C (in particular, 2 distinct BA8-35 batches were used, resulting in different binding capacities), so that sensorgrams cannot be directly compared.



**Fig. 3.** Specific recognition by antibodies of immobilized membrane protein/BA8-35 complexes. Ten microliters of polyclonal antibodies purified either from a preimmune serum or from postimmune sera raised against tOmpA, BR, cytochrome *b<sub>6f</sub>*, or cytochrome *bc<sub>1</sub>* were flushed over flow cells onto which either BA8-35-2 or each of the 4 MP/BA8-35-2 complexes had been immobilized. Purified antibodies were used at a 1/100 dilution in NaPh buffer. (A) An example of the SPR responses obtained, in this case by using anti-tOmpA antibodies. (B) Overview of the results. The intensity of the SPR response 10 s after the end of each injection is shown as the percentage of the specific response (that to antibodies raised against the protein immobilized on the flow cell under consideration).

cytochrome *bc<sub>1</sub>* (486 kDa, from beef heart mitochondria). Each protein was trapped with an excess of BA8-35 (17, 20, 23). The resulting preparations therefore comprised a mixture of MP/BA8-35 complexes and free BA8-35 particles. We have shown before that none of these proteins is denatured upon being trapped with APol A8-35 (17-20, 25). Upon flushing SA-coated chips with these preparations, an SPR signal developed, a large fraction of which resisted washing with buffer (Fig. 2B). As observed with pure APols, SPR signals were much higher than observed for the same MP trapped with underivatized A8-35, indicative of a biotin-mediated immobilization (Fig. 2B). For comparative purposes, injections of the 4 proteins were carried out at constant overall APol concentration. For the 2 small MPs (tOmpA and BR), where the overall protein/polymer mass ratio in the preparation is relatively low (0.2:1 and 0.25:1, respectively), the SPR signal was comparable (Fig. 2C) and close to that observed with pure BA8-35 (data not shown); for larger complexes (*b<sub>6f</sub>* and *bc<sub>1</sub>*, PM/BA8-35 = 0.33:1 and 0.66:1 wt/wt, respectively), it was significantly higher (Fig. 2C), strongly suggesting that the protein contributed to the signal.

**Label-Free Detection of the Binding of Antibodies to Immobilized Membrane Proteins.** The presence of the 4 proteins on the chips and their ability to recognize specific ligands were tested with protein-specific antibodies. Antisera were raised in rabbits against each of the 4 target proteins and the IgG fraction purified by affinity chromatography. After saturating nonspecific binding sites with preimmune antibodies, each flow cell was flushed with either preimmune or protein-specific antibodies at several dilutions. Strong, specific responses were obtained at 1/100 dilution (Fig. 3A). Responses to protein-specific antibodies were typically 10 times higher or more than those to nonspecific ones (Fig. 3B).

Comparative antibody binding experiments were carried out with proteins trapped with either BA8-35 or plain A8-35. Higher signals were observed when the protein had been trapped in BA8-35, with desorption kinetics compatible with the washing away of antibodies, whereas the signal observed in the absence of biotin was much weaker (data not shown). This confirms that most, if not all, of the specific response is due to the binding of antibodies to MPs immobilized via the biotin/SA interaction rather than via nonspecific interactions with the support.

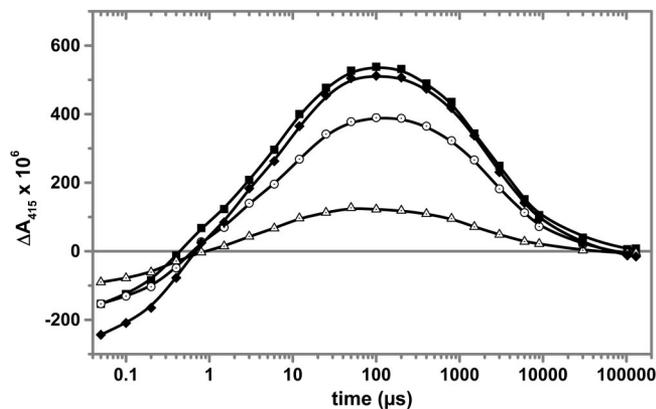
Taken together, these experiments establish (i) that each of the 4 MPs tested can be immobilized onto SA-coated surfaces via the biotinylated APol and (ii) that, once bound, each of them can be specifically recognized by antibodies directed against it.

Some interesting additional observations were made in the

course of these studies. In particular: (i) removing free BA8-35 from the trapped preparations, which suppresses the competition between MP/BA8-35 complexes and free BA8-35 particles for binding to SA, results, as expected, in increasing the protein fraction in the material adsorbed and, thereby, the signal-to-noise ratio observed upon challenging the chip with antibodies (Fig. S3); (ii) despite a lower signal-to-noise ratio, specific recognition of antibodies can also be observed with crude sera (Fig. S4); (iii) although A8-35 is insoluble in acidic solutions (21, 26), chips carrying BA8-35-immobilized PMs can be regenerated by washing away bound antibodies at pH 2.2 (data not shown).

**Functionality of Immobilized BR.** Recognition by polyclonal antibodies does not prove that MPs retain their functionality upon APol-mediated immobilization, because SPR signals could be due to the binding of conformation-insensitive antibodies to denatured proteins. The functionality of immobilized MPs was tested on BR and the nAChR. The photocycle of A8-35-trapped BR presents hybrid features between those of the membrane-bound and the detergent-solubilized protein (20). In the present work, we have used as a test of functionality the rise and fall of the M states, in which the retinal's Schiff base becomes deprotonated, driving the proton pump. BR trapped with BA8-35-2 was incubated with streptavidin-covered beads and photo-induced absorption changes followed at 415 nm, a wavelength close to the  $\lambda_{\text{max}}$  of the M states and where the other states transiently formed during the photocycle hardly contribute. Fig. 4 shows the transient absorption changes obtained with (i) the mixture of free and immobilized BR, (ii) immobilized BR separated by centrifuging the beads, and (iii) free BR present in the supernatant. In all 3 cases, an absorption increase develops in the 1- to 10- $\mu$ s time range, followed by a decrease in the 2- to 3-ms time range, as expected for the formation and decay of the M states. Although the present approach detects only functional BR and thus does not allow one to rule out fractional denaturation, it does unambiguously show that immobilized BR is still capable of cycling through the M states with normal kinetics. In addition, comparison of the amplitude of the photo-induced signals measured before and after separation of free from immobilized BR provides an estimate of the immobilization yield: 70–75% (Fig. 4).

**Binding of a Fluorescent Toxin to an Immobilized Pharmacological Receptor.** In a third series of experiments, the nAChR was chosen as a model to examine the potential of the immobilization strategy for drug screening. nAChR was solubilized with



**Fig. 4.** Kinetics of flash-induced absorption changes of free and immobilized bacteriorhodopsin at 415 nm. The photocycle was triggered by a saturating laser flash (532 nm, 5 ns full-width at half-maximum,  $5 \text{ mJ}\cdot\text{cm}^{-2}$ ). Solid squares, mixture of immobilized and nonimmobilized BR after incubating BR trapped with BA8-35-2 with streptavidin-covered beads for 1/2 h at 4 °C. Bead-associated BR (open circles) and nonimmobilized BR (open triangles) were then separated by centrifugation, and their kinetics measured separately. Solid diamonds show the sum of the signals obtained with the latter 2 samples.

CHAPS from postsynaptic membranes of *Torpedo marmorata* electric organs, purified, and trapped with a mixture of BA8-35 and A8-35 (see *SI Text*) as described in ref. 22 for nonfunctionalized A8-35. We have shown previously that A8-35-trapped nAChR, at variance with the CHAPS-solubilized receptor, binds a fluorescent analog of acetylcholine with kinetics comparable with that observed in native postsynaptic membranes (22). Free APols were removed by size exclusion chromatography, and the nAChR/APol complexes immobilized onto SA-coated beads. The binding kinetics of  $\alpha$ -bungarotoxin fluorescently labeled with Alexa Fluor 647 (BTx-647) was monitored by confocal fluorescence microscopy. In the absence of nAChR, or in the presence of an excess of nonfluorescent BTx, no fluorescence signal was detected at the bead surface (Fig. 5*A Lower*), whereas an intense signal appeared when BTx-647 was added to beads

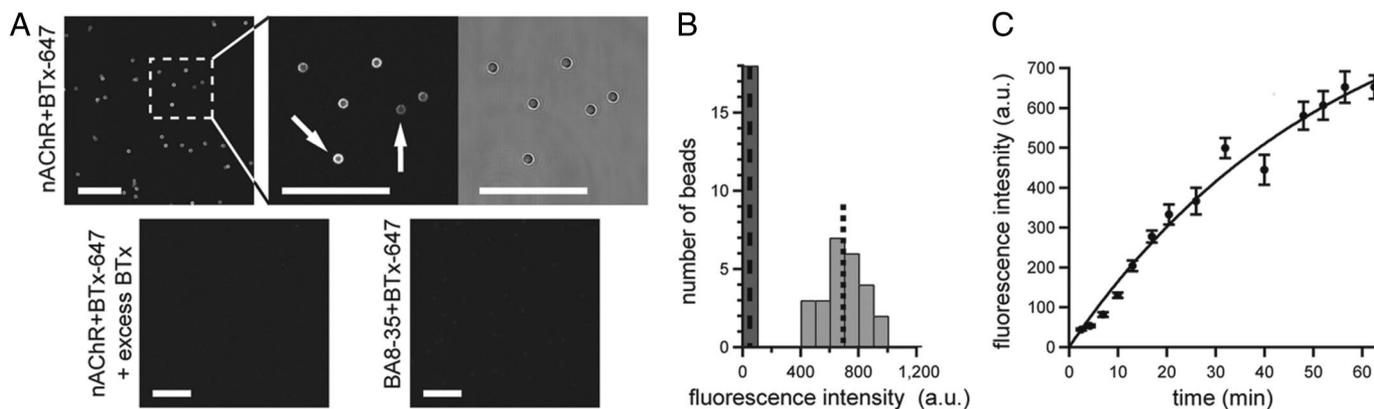
preincubated with BA8-35-trapped nAChR (Fig. 5*A Upper*), reflecting the interaction of the toxin with the agonist-binding sites of the receptor (27).

For a given concentration of BTx-647, each bead exhibited a homogeneous signal, indicating the absence of protein clusters at the surface and confirming the quality of the immobilization strategy. However, the analysis of populations of beads indicated that the intensity of fluorescence varied from bead to bead, most probably because of a variable degree of coating with SA (Fig. 5*B*). The initial binding kinetics of BTx-647 was recorded on-line and data collected at the single-bead level. Their analysis yielded an observed association constant  $k_{\text{obs}} = 0.018 \text{ min}^{-1}$  at 10 nM BTx-647, assuming a pseudofirst-order reaction (Fig. 5*C*), which corresponds to  $k_{\text{on}} = (3.0 \pm 0.7) \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$  (SD) for the bimolecular reaction. This rate is within the range of those reported in the literature for the binding of radiolabeled  $\alpha$ -bungarotoxin to purified *Torpedo* membranes or detergent-solubilized *Torpedo* nAChR ( $(0.8\text{--}33) \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$ ; see, e.g., refs. 28–30).

## Discussion

The present data establish an approach to immobilizing MPs for the purpose of ligand-binding studies. Instead of being kept soluble by a detergent and directly attached to the support, or reconstituted into a lipid bilayer that is itself somehow anchored, the target protein is immobilized via a functionalized APol. The latter fulfills simultaneously 3 functions: (i) it keeps the protein hydrosoluble; (ii) it stabilizes it biochemically; and (iii) it attaches it to the support in a specific and nondestructive manner.

Previous studies have firmly established the efficiency of APols at trapping and keeping any kind of MPs soluble (17, 18, 20, 31, 32). APol-mediated immobilization of MPs presents considerable advantages over existing approaches. It is universal, easy to implement, extremely mild, technologically very light (a single APol can be used for any number of proteins), remarkably versatile (a vast variety of tags or chemical functions can mediate noncovalent or covalent attachment to virtually any kind of support, opening the way to examining MP/ligand interactions by a very large panel of observation techniques), and it does not call for any genetic or chemical modification of the target protein:



**Fig. 5.** Immobilization of nAChR via BA8-35 onto SA-coated beads and application to monitoring the kinetics of ligand binding to the receptor by fluorescence microscopy. (A) SA-coated polystyrene beads ( $1.8\text{-}\mu\text{m}$  diameter) preincubated with nAChR/BA8-35-2/A8-35 complexes and washed with buffer were imaged after  $\approx 1\text{-h}$  incubation with 10 nM  $\alpha$ -bungarotoxin fluorescently labeled with Alexa Fluor 647 (BTx-647) (*Upper Left*). A homogeneous signal of fluorescence was observed at their surface (enlarged view, *Upper Center*; *Upper Left* shows the same field seen by light microscopy), whose intensity varied from bead to bead (arrows point to extreme cases). Under similar conditions, only very weak signals could be detected on nAChR-carrying beads preincubated with an excess of nonfluorescent  $\alpha$ -bungarotoxin (BTx) (*Lower Left*) or on beads carrying only BA8-35-2 (*Lower Right*). (Scale bars:  $25 \mu\text{m}$ .) (B) Distribution of the average fluorescence intensity per bead for nAChR- (light gray) or BA8-35-2-carrying beads (dark gray) after  $\approx 1\text{-h}$  incubation with 10 nM BTx-647 (data extracted from the experiments shown in *A Upper Left* and *Lower Right*). Dotted and dashed lines indicate the average value for each population (respectively  $695 \pm 29$  and  $49 \pm 2$  a.u., SEM). (C) Binding kinetics of BTx-647 to immobilized nAChR. The average intensity of the bead population (defined as shown in *B*) was determined at different times after addition of 10 nM BTx-647 and corrected for nonspecific binding (see *SI Text*) (error bars, SEM;  $n = 8\text{--}25$ ). The solid line is a fit with a first-order reaction model with  $k_{\text{obs}} = 0.018 \pm 0.004 \text{ min}^{-1}$  (SD). This value yields  $k_{\text{on}} = (3.0 \pm 0.7) \times 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$  (SD) for the bimolecular reaction.

Even a mixture of MPs, or a MP whose genetics is not under control and whose biochemistry is rudimentary, are eligible to the procedure, as long as they can be obtained in a detergent-soluble form. Because APols bind to membranes (31, 33), native membrane fragments or lipid vesicles could presumably be anchored to supports by the same strategy. Binding studies can be carried out in surfactant-free buffers, in the absence of any interference and methodological complications due to the presence of detergents. Unlike in protein-mediated immobilization methods, all extramembrane domains of the protein are expected to remain free from interactions with the support and to be comparably exposed to the solution, and, at variance with membrane-based schemes, all of them are simultaneously accessible from the same aqueous compartment. Finally, it is worth recalling that APols have proven to be an excellent medium for folding denatured MPs to their native state (ref. 25; T. Dahmane, M. Damian, S. Mary, J.-L.P., and J.-L. Banères, unpublished results), which ought to facilitate functional and structural studies of MPs overexpressed as inclusion bodies: folding can be achieved by using a functionalized APol, so that the folded protein can be immobilized thanks to the very polymer that has assisted its folding.

In most cases, trapping with APols is unlikely to interfere with ligand binding. It cannot be excluded, however, that, although APols appear to confine themselves to the transmembrane surface of the trapped protein (19), some of their tails or loops may explore its extramembrane surfaces and bind to hydrophobic ligand binding sites. This is not a purely theoretical possibility, because similar polymers have been shown to interact with serum albumin, a soluble protein, presumably via the hydrophobic groove that binds fatty acids (34). In the frontier region between the transmembrane and extramembrane surfaces of the protein, APols could sterically or otherwise hinder the binding of macromolecular ligands. Polyacrylate-based APols such as A8-35, being polyanionic, could also bind to strongly cationic pockets or patches (A8-35 does interact with lysozyme, a soluble, basic protein, ref. 31). The latter problem, should it arise, could be avoided by resorting to chemically different APols. Finally, APols could possibly affect ligand binding by damping large-scale conformational transitions of MP transmembrane domains (see ref. 32).

Available data encourage one not to exaggerate such concerns. No evidence for an interference with ATP binding has been found in the case of the calcium ATPase (31). Four G protein-coupled receptors, after being folded in A8-35, bind their specific ligands with normal affinities (T. Dahmane, M. Damian, S. Mary, J.-L.P., and J.-L. Banères, unpublished results). We show here that APol-trapped, immobilized nAChR binds a fluorescent derivative of  $\alpha$ -bungarotoxin with the expected kinetics. In solution, the binding to the same receptor of a fluorescent analog of acetylcholine, which is perturbed by detergents, is unaffected by APols (22). The absence of detergent in solution also appears to be an advantage in the case of the bacterial porin OmpF, which, after trapping by A8-35, binds the water-soluble R domain of colicin N with higher affinity than it does in detergent solution (Q. Hong and J. H. Lakey, unpublished observations quoted in ref. 18). A8-35-trapped OmpF also binds antibodies (Q. Hong and J. H. Lakey, in ref. 18). We show here that, after being trapped by BA8-35 and immobilized onto streptavidin-coated chips, all of the 4 proteins tested bound antibodies that had been raised against them. All things considered, this ensemble of data suggests that the possibility that APols interfere with ligand binding cannot be ignored, but that, in most cases, it is unlikely to be a serious hindrance.

The present work opens the way to an extremely wide range of applications. Those include the search for natural biological partners of MPs, drug discovery, diagnostics, the creation of biosensors, etc. APol-mediated immobilization of MPs, in addition, has applications beyond the study of protein/ligand interactions, such as the design of biomaterials or bioreactors.

## Methods

The *SI Text* includes a description of materials, BA8-35 synthesis, preparation and trapping of membrane proteins, preparation and purification of antibodies, detailed protocols of immobilization and ligand-binding experiments, and additional data about antibody-binding experiments.

**Immobilization of Proteins on SA Sensor Chips and Antibody Recognition.** SPR experiments were performed on a Biacore 2000 instrument, in HBS-N running buffer. MP immobilization and antibody recognition were conducted at 25 °C at a flow rate of 10  $\mu\text{L}\cdot\text{min}^{-1}$ .

**Immobilization of BR on Streptavidin-Coated Beads.** Immobilization was carried out by incubating for 1/2 h at 4 °C under agitation 1.5 mg of 1.8- $\mu\text{m}$ -diameter streptavidin-coated polystyrene beads in 12  $\mu\text{L}$  of NaPh buffer containing 6  $\mu\text{M}$  APol-trapped BR.

**Time-Resolved Absorption Spectroscopy.** Photocycle measurements were performed with a pump-probe spectrophotometer (35) in which the probe and pump flashes are respectively provided by an Optical Parametric Oscillator (Pantther; Continuum) and a frequency-doubled Nd:Yag laser (Brilliant; Quantel).

**Separation of nAChR/APol Complexes from Free APols and Immobilization on Streptavidin-Coated Beads.** nAChR was trapped with a 2:1 mixture of BA8-35-2 and A8-35. Free APol particles were separated from nAChR/APol complexes by size exclusion chromatography on a Superose 6HR column. SA-coated beads were incubated with nAChR/APol complexes for 1/2 h at room temperature under stirring before extensive washing with buffer.

**Monitoring Ligand Binding to Immobilized nAChR and Evaluation of Binding Kinetics.** Fluorescence images were recorded by using an SP5 confocal microscope (Leica Microsystems) with a 63.0  $\times$  1.20w HCX PL APO CS objective (Zeiss). Fluorescence was excited by a laser at 633 nm and detected in the 642- to 768-nm range. No background coming from the beads was detectable in our experimental conditions.

For kinetic experiments, beads were suspended in buffer and transferred to the holder. At  $t = 0$ , BTx-647 was added and images were recorded at different times over a period of  $\approx 1$  h. Each image was taken in a new area so as to limit photobleaching. The overall fluorescence intensity per bead was evaluated by using Igor Pro 5.03 (WaveMetrics) using filtered images to reduce the noise.

To estimate ligand binding kinetics, average intensities of bead populations were calculated for each image taken at increasing times after addition of BTx-647. Results were fitted by using a first-order reaction model,  $y(t) = y(0)(1 - e^{-k_{\text{obs}}t})$ , where  $y(t)$  is the fluorescence intensity,  $k_{\text{obs}}$  is the first-order reaction constant, and  $t$  is the time.

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