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Biotinylated non-ionic amphipols for GPCR ligands screening

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ABSTRACT

We present herein the synthesis of biotin-functionalized polymers (BNAPols) that have been developed for the fixation of membrane proteins (MPs) onto surfaces. BNAPols were synthesized by free-radical polymerization of a tris(hydroxymethyl)acrylamidomethane (THAM)-derived amphiphilic monomer in the presence of a thiol-based transfer agent with an azido group. Then a Huisgen-cycloaddition reaction was performed with Biotin-$(PEG)_8$-alkyne that resulted in formation of the biotinylated polymers. The designed structure of BNAPols was confirmed by NMR spectroscopy, and a HABA/avidin assay was used for estimating the percentage of biotin grafted on the polymer end chain. The colloidal characterization of these biotin-functionalized polymers was done using both dynamic light scattering (DLS) and small angle X-ray scattering (SAXS) techniques. These BNAPols were used to stabilize a model G protein-coupled receptor (GPCR), the human Growth Hormone Secretagogue Receptor (GHSR), out of its membrane environment. Subsequent immobilization of the BNAPols:GHSR complex onto a streptavidin-coated surface allowed screening of ligands based both on their ability to bind the immobilized receptor and to trigger GHSR conformational changes using the fluorescence energy transfer (FRET)-based assay. This opens the way to the use of biotinylated NAPols to immobilize functional, unmodified, membrane proteins, providing original sensor devices for multiple applications including innovative ligand screening assays.

1. Introduction

Membrane proteins (MPs) play fundamental roles in biology because they control communication and material transfer within and between living cells and their environment. Traditionally, MPs are solubilized and studied in detergent micelles, although micellar assemblies are poor mimics of biological membranes. Detergents have major drawbacks for manipulating isolated membrane proteins that include denaturing properties and a negative impact on protein dynamics [1]. Alternative approaches have been developed to circumvent the destabilizing/denaturing effects of detergents. This includes facial amphiphiles [2], neopentyl-based detergents [3] as well as cyclic [4] and fluorinated detergents [5] to name but a few. Heterogeneous systems consisting in amphiphilic polymers have also been developed in parallel and have shown very promising properties. Among them one can cite amphipols (APols) [6] or styrene maleic acid (SMA) [7].

APols are amphipathic polymers that were designed and validated as mild alternatives to classical detergents. Accordingly, most integral membrane proteins are much more stable in APols than they are in detergent solutions. The first designed polyacrylate-based A8-35 [6] has been so far the most widely used amphipol [8]. However, acidic pH or the presence of multivalent cations can result in A8-35 aggregation. Besides, the ionic character of A8-35 can be a major drawback in the context of screening assays, as non-specific interactions between the polymer and charged compounds cannot be ruled out. These constraints prompted the development of chemically different APols (reviewed in reference [9], including zwitterionic APols [10], sulfonated APols (SAPols) [11], and nonionic amphipols (NAPols) [12,13]. The stabilizing efficiency of APols has been shown to inversely correlate with the charge density of the polymers, the NAPols being the most stabilizing derivatives [14]. NAPols therefore represent new, milder tools for the manipulation of membrane proteinszz.

MPs account for about one third of all proteins encoded in the human genome [15] and half of all drug targets [16]. Therefore, the screening of ligands for drug discovery has been of increasing interest over the recent years. In many cases, screening of specific ligands with isolated proteins involves their immobilization onto surfaces. For membrane proteins, functional immobilization can be carried out by covering bare and modified surfaces with native and reconstituted membrane fragments or by using proteins with engineered tags. This have been extensively reviewed in reference [17]. More recently, func-

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tionalized β-sheet peptides with stabilizing properties were successfully used for the immobilization of MPs onto solid supports [18]. Covalent immobilization of a MP on silica-based carriers was also reported [19].

In this context, amphipols offer an alternative and appear as highly versatile means for membrane protein immobilization [20–22]. Indeed, by grafting affinity tags on the polymer, MP/APol complexes have been successfully attached onto solid supports. The first example of such a functionalized amphipol was the biotinylated derivative of A8-35 called BAPol (Fig. 1), which was used to immobilize a model membrane protein for SPR and fluorescence microscopy studies [20]. Functionalization of A8-35 was further extended to poly-histidine and imidazole groups [22] as well as to oligodeoxynucleotide groups [23].

However, one of the drawbacks of tagged A8-35 is the random distribution of the tag along the polymer chain, as the synthesis protocol does not allow a control of the position of the tag on the polymer chain. To relieve this drawback, biotinylated phosphorylcholine amphipols B-PCAPols (Fig. 1) have been synthesized by RAFT polymerization in the presence of a transfer agent onto which the biotin group was further grafted [24]. This allowed attaching only one biotin group at the end of the polymer chain. B-PCAPols have been successfully used to study the interaction between *E. coli* outer membrane protein FhuA and a bacteriophage tail protein by SPR and Electrochemical Impedance Spectroscopy [24]. However, synthesis of these polymers is quite complex, and this limits large-scale preparation. In addition, ionic- and phosphocholine-based amphipols have a high electrostatic charge density that can induce non-specific binding or electrostatic repulsion [25]. This can be a limitation when using amphipol-trapped proteins for analyzing protein:ligand binding events where ionic interactions can have a prominent role. Development of non-ionic tagged amphipols (Fig. 1) is therefore to be considered as a very promising strategy for the design of an efficient, robust and versatile biosensor. We report herein the synthesis of the first series of biotinylated NAPols and the study of their colloidal properties by Dynamic Light Scattering (DLS) and Small Angle X-Ray Scattering (SAXS). These biotinylated NAPols were next investigated for their ability to allow immobilization onto a surface of a model membrane protein from the G protein-coupled receptor (GPCR) family. In particular, we investigated whether the polymer preserved the pharmacological properties of the receptor even after attachment to a functionalized surface and whether the immobilized receptor could then be used for subsequent screening of ligands.

2. Materials & methods

2.1. Synthesis

All starting materials were commercially available and were used without further purification. 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), triethylsilane and sodium chloride were purchased from Acros Organics. Triphenylmethyl chloride (TrCl) and trifluoroacetic acid were purchased from TCI. 3-mercaptopropionic acid, tris(hydroxymethyl)aminomethane, 2-bromoethylisocyanate, diisopropylethylamine (DIEA), triethylamine (Et_3N), sodium azide (NaN_3), sodium bicarbonate ($NaHCO_3$), sodium sulfate (Na_2SO_4) and copper iodide were purchased from Sigma-Aldrich. Biotin-$(PEG)_8$-alkyne was purchased from SiChem (Germany). Monomer DGC11 *i.e.* *N*-(1,1-(2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyloxymethyl)-1-(undecylcarbamoyloxymethyl)-methyl)acrylamide was synthesized according to earlier reported procedure [26]. All solvents were of reagent grade and used as received unless otherwise indicated. Tetrahydrofuran (THF) was dried over sodium and benzophenone, methanol (MeOH) over sodium, dichloromethane (CH_2Cl_2) over CaH_2 under argon atmosphere. Commercial anhydrous *N,N*-Dimethylformamide (DMF) was stored in the presence of activated molecular sieves 3 Å. Azobisisobutyronitrile (AIBN) was recrystallized twice from ethanol. The progress of the reactions was monitored by thin layer chromatography (silica gel coated with fluorescent indicator F254). The compounds were detected either by exposure to ultraviolet light (254 nm) or by spraying with sulfuric acid (5% ethanol) and/or ninhydrin (5% ethanol), followed by heating at ~150 °C. Flash chromatography purifications were carried out on silica gel (pore size 60 Å, 230–400 mesh particle size, 40–63 μm particle size). Size exclusion chromatography purifications were carried out on Sephadex LH-20 resin. ^1H and ^{13}C spectra were recorded on a Bruker AC 250 and AC 400 spectrometer at 250 and 400 MHz for ^1H and 62.86 and 100 MHz for ^{13}C respectively. Chemical shifts (δ values) were reported in ppm downfield from internal residual solvent as a heteronuclear reference. HR-MS (ESI+) was determined on a QStar Elite mass spectrometer. UV–Visible spectra were recorded on a Cary Win Varian Spectrophotometer with a double-compartment quartz cell of 10-mm length (Suprasil).

2.1.1. Synthesis of 3-(tritylthio)propionic acid (2)

To a solution of triphenylmethyl chloride (17.6 g, 63.1 mmol) in dichloromethane (150 mL) under argon atmosphere, 3-mercaptopropionic acid (6.0 mL, 69.4 mmol, 1.1 eq.) was added dropwise. The precipitate formed was collected by filtration and dried under vacuum to give compound **2** (20.0 g, 57.4 mmol, 90%) as a white powder. ^1H NMR (250 MHz, DMSO) δ 7.56–6.88

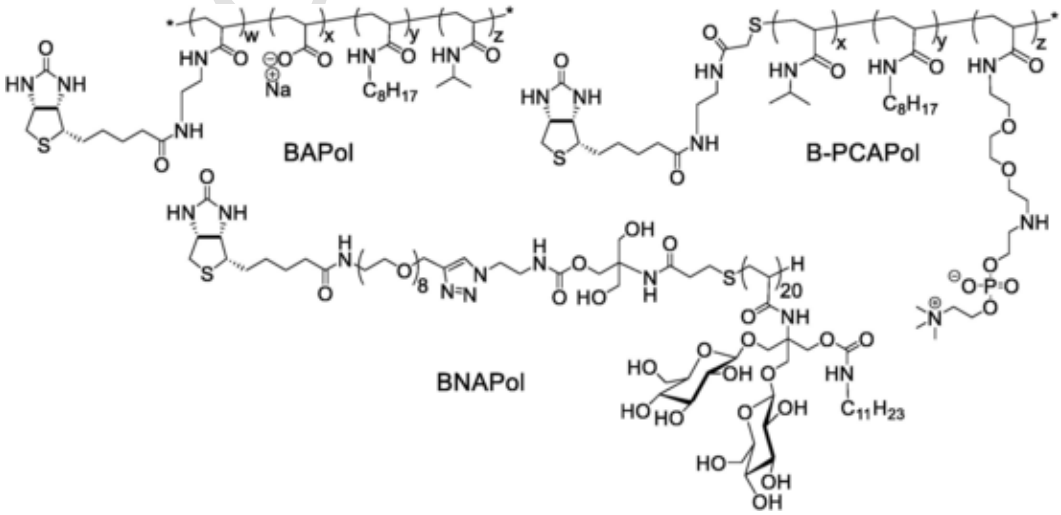

Fig. 1. Structure of BAPol (biotinylated amphipol with w = 0.7–1.4%, x = 30–35%, y = 21–25% and z = 36–41%), B-PCAPol (biotinylated phosphorylcholine amphipol with x = 29%, y = 29% and z = 42%) and BNAPol (biotinylated non-ionic amphipol).

(m, 15H), 2.29 (t, J = 6.6 Hz, 2H), 2.17 (t, J = 6.6 Hz, 2H). ^{13}C NMR (62.86 MHz, DMSO) δ 172.8, 144.4, 129.1, 128.1, 126.8, 66.2, 32.9, 26.7.

2.1.2. Synthesis of N-(1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl)-3-(tritylthio)propanamide (3)

To a solution of 3-tritylsulfanyl-propionic acid **2** (17.2 g, 49.2 mmol) in ethanol (80 mL) were added tris(hydroxymethyl)aminomethane (6.6 g, 54.1 mmol, 1.1 eq.) and EDDQ (14.6 g, 59.1 mmol, 1.2 eq.). After being stirred at 55 °C for 16 h, the reaction mixture was concentrated under vacuum. The crude mixture was recrystallized from ethanol to give compound **3** (19.1 g, 42.3 mmol, 86%) as a white solid. ^{1}H NMR (250 MHz, $CDCl_3$) δ 7.73–7.02 (m, 15H), 6.32 (s, 1H), 4.77 (s, 3H), 3.52 (s, 6H), 2.52 (t, J = 7.0 Hz, 2H), 1.94 (t, J = 7.0 Hz, 2H); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 173.1, 144.6, 129.7, 128.1, 126.9, 67.1, 62.0, 61.9, 35.9, 27.9.

2.1.3. Synthesis of 2-((((2-azidoethyl)carbamoyl)oxy)methyl)-2-(3-(tritylthio)propanamido)propane-1,3-diyl dibenzoate (4)

To a mixture of dimethylformamide (10 mL) and diisopropylethylamine (770 μL, 3.32 mmol, 1 eq.) compound **3** (2.0 g, 4.43 mmol, 1.33 eq.) was added portion wise, then 2-bromoethylisocyanate (300 μL, 3.31 mmol, 1 eq.) was added dropwise. After stirring for 4 h, sodium azide (1.07 g, 16.5 mmol, 5 eq.) was added to the reaction mixture and the stirring was continued for 16 h at 50 °C. The solvent was evaporated under vacuum and the resulting residue was solubilized in dichloromethane (100 mL). The organic layer was washed with a saturated solution of sodium bicarbonate (100 mL), brine (100 mL), then dried over sodium sulfate, filtered off and evaporated under vacuum to afford compound **4a**. Compound **4a** was directly used in the next step without purification. To a solution of compound **4a** in dichloromethane (25 mL), triethylamine (4.63 mL, 33.3 mmol, 10 eq.) and benzoyl chloride (2.6 mL, 22.2 mmol, 6.7 eq.) was added dropwise. After being stirred for 16 h, the reaction was diluted with dichloromethane (75 mL). The organic layer was washed with water (100 mL) and brine (100 mL), dried over sodium sulfate, filtered off and evaporated under vacuum. The crude mixture was purified by flash chromatography on silica gel, eluting with ethylacetate/cyclohexane (1:9 to 3:7, v/v) to afford compound **4** (1.36 g, 1.77 mmol, 53%) as a colorless oil. R_f 0.37 (ethylacetate/cyclohexane, 3:7, v/v); ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.16–6.96 (m, 25H), 6.29 (s, 1H), 5.12 (t, J = 5.9 Hz, 1H), 4.77 (s, 4H), 4.57 (s, 2H), 3.32 (t, J = 7 Hz, 2H), 3.25–3.06 (m, 2H), 2.49 (t, J = 7.0 Hz, 2H), 2.00 (t, J = 7.0 Hz, 2H); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 171.6, 166.2, 156.2, 144.6, 133.5, 130.2, 129.8, 129.6, 129.4, 128.6, 128.1, 126.8, 67.0, 64.1, 63.3, 59.0, 50.8, 40.5, 36.2, 27.6.

2.1.4. Synthesis of 2-((((2-azidoethyl)carbamoyl)oxy)methyl)-2-(3-mercaptopropanamido)propane-1,3-diyl dibenzoate (5)

To a solution of compound **4** (0.4 g, 518 μmol) in a 2:8 mixture of trifluoroacetic acid and dichloromethane (4 mL, v/v) triethylsilane (92 μL, 570 μmol, 1.1 eq.) was added dropwise under argon atmosphere at 0 °C. After being stirred at room temperature for 1 h, the solvents were evaporated under vacuum. The crude mixture was purified by flash chromatography on silica gel, eluting with ethylacetate/cyclohexane (2:8 to 3:7, v/v) to afford compound **5** (0.21 g, 396 μmol, 76%) as a colorless oil. R_f 0.57 (ethylacetate/cyclohexane, 4/6, v/v); ^{1}H NMR (250 MHz, $CDCl_3$) δ 8.13–7.30 (m, 10*H*), 6.78 (s, 1H), 5.47 (t, J = 5.7 Hz, 1H), 4.83 (s, 4H), 4.63 (s, 2H), 3.44–3.24 (m, 4H), 2.83–2.65 (m, 2H), 2.51 (t, J = 6.6 Hz, 2H), 1.59 (t, J = 8.3 Hz, 1H); ^{13}C NMR (62.86 MHz, $CDCl_3$) δ 171.4, 166.2, 156.5, 133.5, 129.8, 129.4, 128.6, 64.3, 63.3, 59.2, 50.9, 40.9, 40.6, 20.3; ESI-MS: Calcd for $C_{24}H_{28}N_5O_7S^+$ $[M+H]^+$: 530.2. Found: 530.3; ESI-HRMS: Calcd for $C_{24}H_{28}N_5O_7S^+$ $[M+H]^+$: 530.1709. Found: 530.1705.

2.1.5. Synthesis of BNAPol

The synthesis of biotinylated NAPoL involved three steps.

(i) NAPol-N_3

DGC11 (1.0 g, 0.97 mmol, 15.0 eq.), compound **5** (34.2 mg, 64.6 μmol, 1.0 eq.) and AIBN (5.3 mg, 32.3 μmol, 0.5 eq.) were dissolved in dry THF (1.0 mL) in a Schlenk tube. This solution was degassed by 5 freeze–pumpthaw cycles. Then the reaction mixture was refluxed under argon for 4 h. The reaction mixture was concentrated under vacuum and the crude was purified by gravity size-exclusion chromatography on Sephadex™ LH-20 resin eluting with a 1:1 (v/v) MeOH/CH_2Cl_2 mixture. The solvent was evaporated under vacuum to give the protected NAPol-N_3 as a white powder. Assuming that the conversion is complete (confirmed by ^{1}H NMR of the reaction crude), the polymerization yield was determined from the ratio of the amount determined by gravimetric analysis after the purification process (Sephadex) versus the theoretical expected amount of polymer. 0.85 g of NAPol-N_3 (Batch NV-08) was obtained, which correspond to ~ 85% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.1–7.4 (m, aromatic), 5.2–4.1 (m, glucose unit), 3.1 (m, -NH-CH_2), 2.1–1.9 (bs, -OCOCH_3), 1.7–1.2 (m, CH_2 of the alkyl chain), 0.8 (t, CH_3 of the alkyl chain).

(ii) BNAPol

To a solution of NAPol-N_3 (0.200 g, 11.2 μmol, 1 eq.) in dry THF (5 mL), diisopropylethylamine (2.63 μL, 15.2 μmol, 1.4 eq.), copper iodide (1.4 mg, 7.6 μmol, 0.7 eq.) and Biotin-$(PEG)_8$-alkyne (9.6 mg, 15.2 μmol, 1.4 eq.) was added. Then the reaction mixture was refluxed for 36 h under argon. The reaction mixture was concentrated under vacuum and the resulting crude polymer was purified by gravity size-exclusion chromatography on Sephadex™ LH-20 resin eluting with a 1:1 (v/v) MeOH/CH_2Cl_2 mixture. The solvent was evaporated under vacuum to give the protected BNAPol as a white powder (0.170 g, 80%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.1–7.4 (m, aromatic), 5.2–4.1 (m, glucose unit), 3.6 (m, PEG), 3.1 (m, -NH-CH_2), 2.1–1.9 (bs, -OCOCH_3), 1.7–1.2 (m, CH_2 of the alkyl chain), 0.8 (t, CH_3 of the alkyl chain).

(iii) Deprotection of BNAPol

To a solution of protected BNAPol (0.170 g) in dry methanol (20 mL) under argon atmosphere, a catalytic amount of sodium methoxide was added and the mixture was stirred overnight at room temperature. The reaction solution was neutralized by addition of a spatula of acidic resin (IRC 50) (pH ~ 8) and the solution was shaken for 15 min. The solution was filtered off, and then concentrated under vacuum. The crude polymer was dissolved in methanol (10 mL) and precipitated into cold ether (200 mL). The precipitate was filtered off and the solvent was evaporated under vacuum. Finally, the resulting BNAPol was solubilized in milliQ water at 10 g/L, filtered through a 0.45 μm PVDF syringe filter and then subjected for 24 h and under continuous stirring to dialysis against milliQ water (membrane MW Cut Off 6–8 kDa). The resulting dialyzed solution was freeze-dried to give the BNAPol (0.092 g, 80%, batch NV12-02) as a white powder. ^{1}H NMR (400 MHz, MeOD) δ 5.2–4.1 (m, glucose unit), 3.1 (m, -NH-CH_2), 1.7–1.2 (m, CH_2 of the alkyl chain), 0.8 (t, CH_3 of the alkyl chain).

2.2. Polymer analysis

2.2.1. ^{1}H NMr

The number-average molecular weight of polymers was obtained by ^{1}H NMR calibration, by comparing the integral area of protons from the phenyl groups at 7.4–8.1 ppm with those of the terminal methyl protons at δ 0.8 ppm of each amphiphilic monomer constituting the polymer backbone. NAPol-N_3 and BNAPols were analyzed at 5 g/L in $CDCl_3$. For BNAPols the appearance of peak at 3.6 ppm corresponds to PEGylated protons of biotin that was further estimated by HABA analysis.

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2.2.2. HABA test [27]

In a 1 mL cuvette, 0.9 mL of the red/orange solution of HABA/avidin reagent was analyzed. The absorbance was measured at $\lambda = 500$ nm *i.e.* A_{492}. Subsequently, 0.1 mL of biotinylated polymer solution was added into of the HABA/Avidin solution and then A_{500} was measured. The concentration of polymer was adjusted (from 1 to 5 g/L) so as to keep the decrease in the absorbance between 0.1 and 0.4. 2 min later A_{500} was measured again in order to make sure that the measurement was the same. Each measurement was repeated three times. The data were treated with the online HABA calculator from ThermoFischer Scientific and were expressed as a percentage of biotine per polymer chain.

2.3. Colloidal characterization.

2.3.1. Dynamic light scattering

The hydrodynamic particle size distribution and polydispersity of NAPols, BNAPols and deprotected monomer DGC11 were determined using a Zetasizer Nano-S model 1600 (Malvern Instruments Ltd., UK) equipped with a He-Ne laser ($\lambda = 633$ nm, 4.0 mW). The time-dependent correlation function of the scattered light intensity was measured at a scattering angle of 173° relative to the laser source. The stock solutions were prepared at 5.0 g/L in Milli-Q water and were stored overnight at room temperature. On the day of the experiment the solutions were centrifuged at room temperature (10000 rpm) for 30 min and then filtered through a 0.45 μm PVDF syringe filter, diluted to the final concentration, and the size of the particles was measured 1 h after filtration. Other conditions were as reported elsewhere [28].

2.3.2. Small-angle X-ray scattering

Self-assemblies were characterized on the bioSAXS beamline BM29 at the European Synchrotron Radiation Facility (Grenoble, France). The sample–detector distance was 2.85 m and the X-ray wavelength $\lambda = 0.1008$ nm leading to a q-range of 0.032–4.55 nm^{-1}. The beamline was equipped with a 2D-detector (Pilatus 1 M) and an automated sample changer. A stock solution was prepared at 10.0 g/L in Milli-Q water and a concentration series prepared by dilution down to 2.5 g/L. The sample storage and measurement temperatures were fixed at 20.0 °C. To prevent radiation damage during the scattering experiments, data were collected in 10 successive 2-s frames, and the solution was moved in the capillary during exposure. All data reduction and analysis were performed using a standard procedure via the ISPyB interface [29] and the program package PRIMUS [30].

2.4. Biochemical validation

2.4.1. GHSR preparation and assembly in BNAPols

The ghrelin receptor was produced and reconstituted in NAPols and in NAPols/BNAPols mixtures using the protocol previously described [14]. Briefly, the ghrelin receptor was expressed *in E. coli* inclusion bodies as a fusion protein with the α5 integrin fragment [31]. The receptor was then purified under denaturing conditions, *i.e.* in the presence of 0.8% SDS. NAPols or NAPols/BNAPols mixtures at different functionalized-to-non functionalized polymer weight ratios (see text) were added at a ratio of 10 g of total polymer per gram of SDS-unfolded protein. Asolectin (0.2:1 (w/w) lipid:amphipol ratio) and cholesteryl hemisuccinate (0.02%, w/v) were then added. After incubation at room temperature for 30 min, folding was initiated by precipitating dodecyl sulfate (SDS) with 200 mM KCl [32]. After 30 min, the precipitate was removed by two 10 min centrifugation runs at 16,100*g* and the supernatant was dialyzed against a 50 mM potassium phosphate, 150 mM KCl, pH 7.5 buffer. Active receptor fractions were finally purified using affinity chromatography with a GHSR ligand immobilized on a chromatography column [33]. To this end, the protein after assembly into amphipols was loaded on the column and the proteins bound to the matrix were subsequently recovered by washing the column with a 25 mM HEPES, 150 mM KCl, pH 7.5 buffer containing 0.5 mM of the neutral antagonist JMV2959. The latter was removed through extensive dialysis in a 25 mM HEPES, 150 mM KCl, 0.5 mM EDTA, pH 7.5 buffer. Monomeric GHSR was finally separated from protein aggregates through a size-exclusion chromatography step on a Superdex 200 increase 10/300 GL column (GE Healthcare) using a 25 mM Na-HEPES, 150 mM KCl, 0.5 mM EDTA, pH 7.4 buffer as the eluent. For ligand binding assays, the ghrelin receptor was labeled at its N-terminus with Lumi4-NHS (Cis-Bio), as previously described [33].

2.4.2. Receptor immobilization

The ghrelin receptor was immobilized onto 96-well streptavidin coated high-capacity plates (Pierce) following manufacturer instructions. Briefly, each well was washed with 200 μL of a 25 mM Tris-HCl, 150 mM NaCl, pH 7.5, 0.1% BSA buffer. The receptor was then added and incubated for two hours at room temperature and the plates washed. To optimize the coating efficiency, different dilutions of biotin-tagged receptor preparation were tested ranging from 1 to 0.001 g/L.

2.4.3. Ligand binding assays

Ligand binding was monitored through the homogenous time-resolved fluorescence (HTRF) signal between GHSR labeled with Lumi4-NHS at its N-terminus (see above) and a ghrelin peptide labeled with dy647 at its C-terminus. The immobilized receptor was incubated with the labeled ghrelin peptide (50 nM concentration range) and the compounds to be tested. Plates were then incubated at 4 °C for 3 h before signal detection. The signal was detected using a fluorescence microplate reader (PHERAstar plus, BMG Labtech) equipped with a HTRF optic module allowing a donor excitation at 337 nm and a signal collection at 620 nm.

3. Results and discussion

3.1. Synthesis of BNAPols

Functionalization of NAPols was achieved by post-functionalization of the polymer, which consists in the grafting of a tag of interest at the end of the polymer chain once the polymer is synthesized. To achieve such an end chain post-functionalization, a thiol-based transfer agent also called telogen, bearing an azido group, was first synthesized as described in Scheme 1. 3-Mercapto propionic acid was first protected by trityl chloride to afford compound **2**. Tris(hydroxymethyl)aminomethane was next condensated to compound **2** in the presence of EEDQ as a coupling agent to afford compound **3**. Bromoethylisocyanate was next put in reaction with compound **3** in the presence of diisopropylethylamine, then sodium azide was added to afford compound **4a**. Compound **4a** was directly used for protection of the two hydroxyl groups by benzoyl groups to lead after purification by flash chromatography to compound **4**. Finally, deprotection of the trityl group led to the transfer agent **5**.

BNAPols were therefore synthesized by free-radical telomerization of the amphiphilic monomer **DGC11** in the presence of the azido transfer agent **5** in refluxing THF and in the presence of the radical initiator AIBN (Scheme 2). Polymers with different degrees of polymerization (DP_n) were synthesized by varying the ratio of transfer agent (TA) and amphiphilic monomers (Table 1). The reaction was monitored by thin layer chromatography and carried on until full disappearance of the monomer spot. The number-average degree of polymerization (DP_n) was set by the initial ratio of **DGC11** to transfer agent TA (R_0), considering, in a first approximation, the transfer constant of **5** to **DGC11** (C_t) to be close to 1 [34]. The observed DP_n was rather close to the initial ratio (Table 1) suggesting that in THF C_t is close to 1, as usually observed during homotelomerization of THAM monomers [13,35,36].

The resulting azido functionalized NAPol was purified by size-exclusion chromatography on sephadex LH20 resin and the chemical structure was confirmed by ^{1}H NMR spectroscopy. In the second step NAPol-N_3 was reacted with the Biotin-$(PEG)_8$-alkyne in THF through a Huisgen-cycloaddition reaction in the presence of copper and the resulting biotinylated polymer was purified by size-exclusion chromatography. Then the final step consisted in the removal of the benzoyl and acetyl ester groups using the Zemplén method in the

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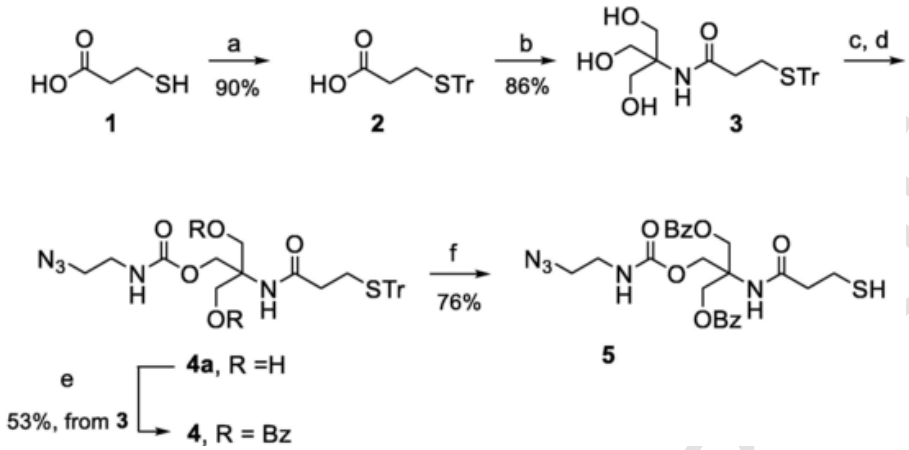
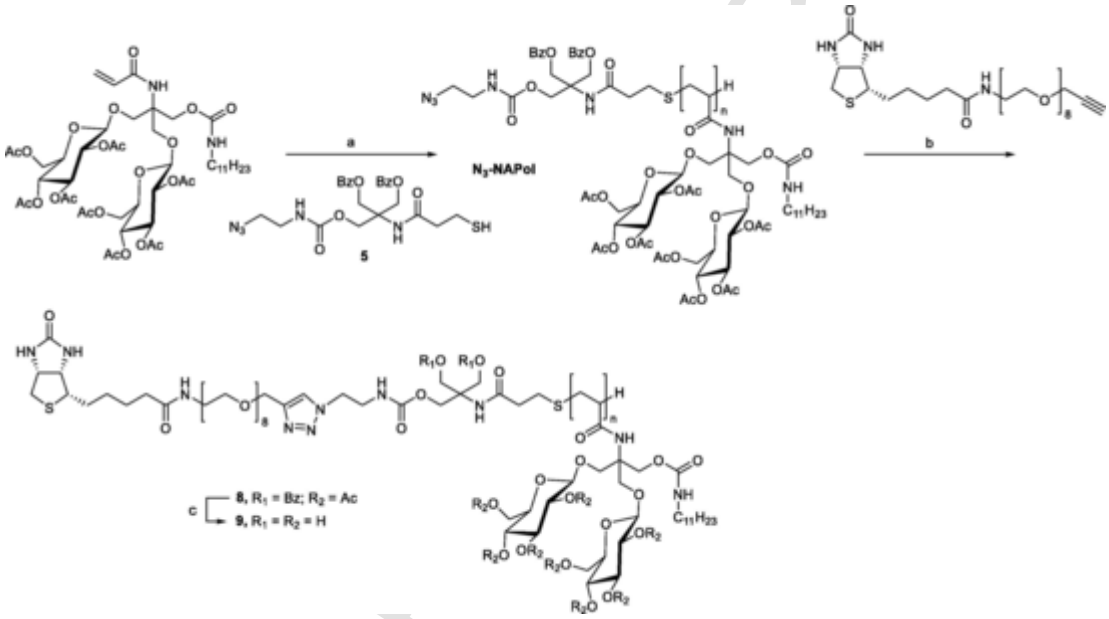


Scheme 1. a) TrCl, CH_2Cl_2, RT, 3.5 h; b) Tris(hydroxymethyl)aminomethane, EDDQ, EtOH, 55 °C, 16 h; c) $BrCH_2CH_2N = C = O$, DIEA, DMF; d) NaN_3, 50 °C; e) BzCl, Et_3N, CH_2Cl_2, RT, 14 h; f) Et_3SiH, CF_3COOH, CH_2Cl_2, 4 h, 0 °C.



Scheme 2. a) DGC11 (15 eq.), 5 (1 eq.), AIBN (0.5 eq.), THF, reflux, 16 h, 78%; b) Biotin-$(PEG)_8$-alkyne (2 eq.), NAPol-N_3 (1 eq.), CuI (1 eq.), DIEA (2 eq.), THF, 50 °C, 3 days; c) catalytic MeONa, MeOH, overnight, 90%.

presence of catalytic amount of sodium methoxide in methanol to afford water-soluble BNAPol. BNAPol was further purified by dialysis through cellulose-based membrane for 24 h to lead after freeze drying to a dry powder of pure BNAPol.

3.2. Polymer analysis

As shown in Fig. 2, the presence of the biotin group on the polymer chain was confirmed by ^{1}H NMR. The PEGylated chain of the Biotin-$(PEG)_8$-alkyne was easily monitored by ^{1}H NMR and was also observed in BNAPols at δ 3.54 ppm, indicating the grafting of biotin to the polymer. The presence of the benzoyl protons both in the protected N_3-NAPol and its biotinylated version allowed the determination of the degree of polymerization (DP_n) of the polymer chain (Table 1). The grafting of the biotin group onto the polymer chain was further estimated using spectrophotometric avidin - HABA test and was found to be between 14 and 40% (Table 1). The binding of HABA to avidin and the ability of biotin to displace HABA in stoichiometric proportions was monitored. A standard solution was titrated with BNAPol solutions and the decrease in absorption spectra of HABA/Avidin solutions witness the presence of biotin on the polymer (Fig. 3). Throughout the present article, BNAPols are denoted by a short name reflecting their chemical structure: the letters BNA stand for "biotinylated non-ionic amphiphilic polymer", followed by the average molecular weight expressed in kDa and the percentage of biotin group grafted onto the polymer chain. Batch numbers are given in Table 1.

3.3. Colloidal characterization

The colloidal properties of BNAPols were determined both by dynamic light scattering (DLS) and Small-angle X ray scattering (SAXS) methods in aqueous solution. For the sake of comparison, the deprotected monomer DGC11 was included in the study as well as two NAPols with no biotin group. Data are reported in Table 2.

3.3.1. Dynamic light scattering

DLS measurements were carried out at 5 g/L. The morphology of BNAPol aggregates in aqueous solution indicates the formation of small size aggregates having hydrodynamic diameter ranging from 6.4 to 8.0 nm (Fig. 4). A slight increase in the hydrodynamic diameter is observed when the DP_n increases while the percentage of biotin grafted seems to have no particular effect on the

Table 1
Represents the initial ratio of monomer (R_0), number-average degree of polymerization (DP_n), percentage of biotin grafting, number-average molecular weight (M_n) for azido-functionalized NAPols and their corresponding biotinylated version (BNAPol).

Polymers	R_0	DP_n	Biotin grafting (%)	Number-average Molecular Weight. (kDa)	Name	Batch
NAPol	20	20	N/A	14.1	NA14	MB105
	60	52	N/A	36.5	NA36	MB136
	20	24	N/A	17	NA17	MAS-C68762-EF
NAPol-N_3(OAc) [a]	20	20	N/A	21.1	N/A	MB134
BNAPol			17	14.9	BNA15_17	MB156
BNAPol			40	14.9	BNA15_40	MB160
NAPol-N_3(OAc) [a]	60	50	N/A	52.1	N/A	MB135
BNAPol			31	35.9	BN36_31	MB159
NAPol-N_3(OAc) [a]	15	17	N/A	17.9	N/A	NV08
BNAPol			40	12.6	BNA13_40	NV12-02
BNAPol			14	12.6	BNA13_14	NV12-03
NAPol-N_3(OAc) [a]	15	20	N/A	21.1	N/A	NV08-1
BNAPol			28	14.9	BNA15_28	NV12-04

[a] Protected NAPol from which biotin was grafted before removal of the acetyl groups.

self-aggregation. This is in full agreement with the previous report on the classical NAPols where hydrodynamic diameter of about 6 nm were observed.

3.3.2. Small-angle X-ray scattering

Self-assemblies were further characterized by SAXS. The radius of gyration (R_G) was determined from $I(q)$ at very small angles via the linear Guinier approximation $\mathrm{Ln}I(q) = \mathrm{Ln}I(0) - (qR_G)^2/3$, assuming that $qR_G < 1$. The pair distribution functions (PDF) and the maximum particle dimensions D_{max} were determined by inverse Fourier transformation of scattering intensity $I(q)$ using the program GNOM [37], to evaluate the geometry of the micelles. Finally, the SAXS data were fitted using SasView software (http://www.sasview.org/).

As already suggested by DLS, SAXS experiments showed that whatever the DP_n, NAPols and BNAPols adopt similar spherical shapes with radii of gyration of 2.70 nm and maximal dimensions of 6.2 to 6.5 nm. The shape of the PDF (Fig. 4C) suggests a core–shell sphere model, which fits well the experimental SAXS curves (Fig. 4B). The core radius fitted with SasView equal to 0.7 nm with the hydrophilic thickness of 2.42 nm are consistent with the value of D_{max} and hydrodynamic radius obtained from DLS. The presence of a biotin group on the polymer chain does not alter the shape and dimensions of the self-assemblies. While the polymers do not show any concentration effect on the shape of the spherical assemblies, the monomer micelles exhibit an elliptic core–shell shape that enlarges with concentration to an elongated core–shell cylinder (Table 2).

3.4. Biochemical validation.

3.4.1. GHSR assembly into BNAPols

To assess whether BNAPols could be efficiently used for stabilizing the native fold of membrane proteins (MP) and their subsequent immobilization on functionalized surfaces, we used here the Growth Hormone Secretagogue Receptor GHSR of ghrelin as a model. Besides being a typical MP, GHSR is a model for rhodopsin-like GPCRs, a class of most challenging proteins because

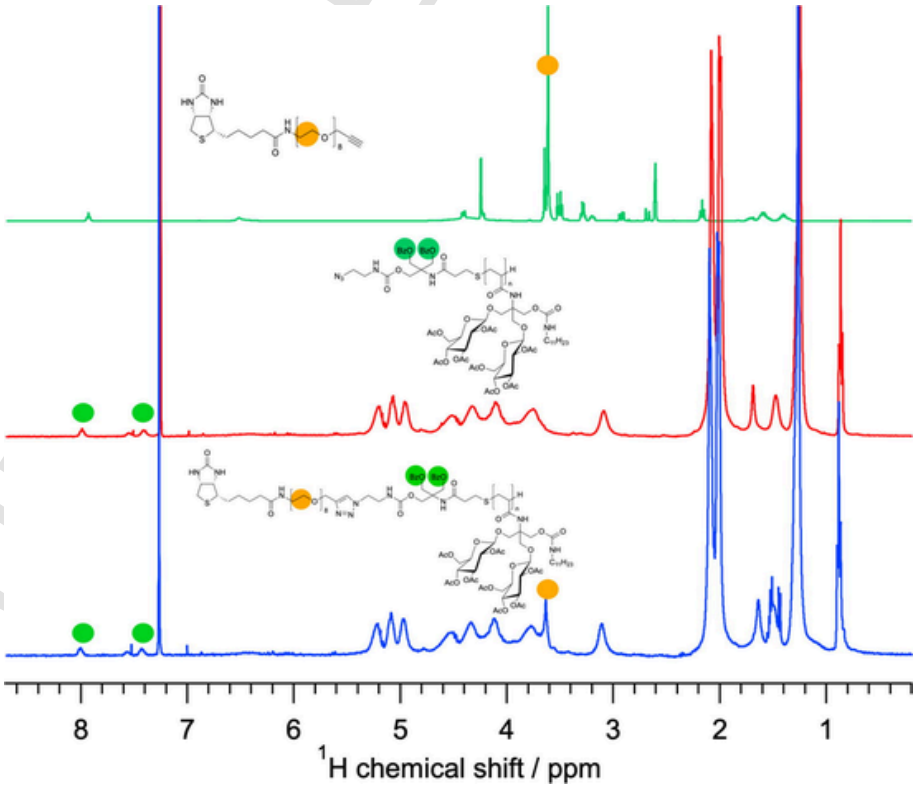


Fig. 2. Represents the NMR spectra of: (top) pure biotinylated polymer (5 g/L in), (middle) NAPol-N_3 (5 g/L in $CDCl_3$) and (bottom) Biotin-$(PEG)_8$-alkyne (5 g/L in DMSO-d_6). Yellow circles designate the PEG chain protons in biotinylated polymer (OAc) & Biotin-$(PEG)_8$-alkyne. Green circles designate aromatic protons of the benzoyl groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

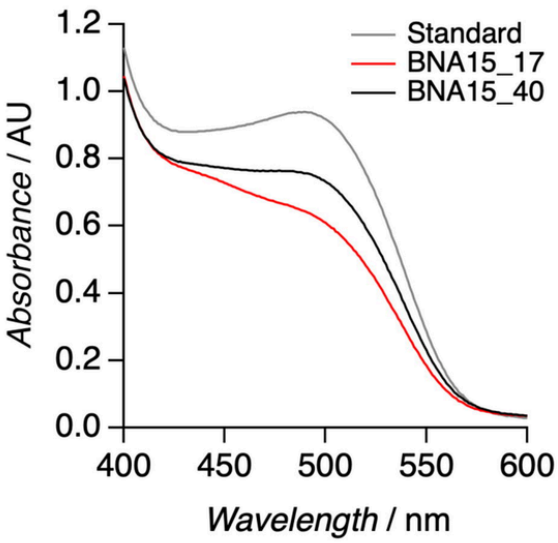


Fig. 3. Absorption spectra of avidin-HABA solution (standard) with addition of two different BNAPols. BNAPols solution were prepared at 5 mg/mL for BNA15_17 and at 1 mg/mL for BNA15_40.

Table 2
Structural parameters obtained from DLS (hydrodynamic radius) and from SAXS (Guinier and PDF) for monomer, NAPols and BNAPols at 5 g/L.

Name	Sample	Biotin grafting %	Molecular Weight. (kDa)	R_h [a] (nm)	R_G [b] (Guinier)	D_{max} [c] (PDF)
DGC11	MB103	N/A	0.69	3.2	8.40* 17.70 22.25**	28.4* 60 102**
NA14	MB105	N/A	14.2	3.2	2.72	6.52
NA36	MB136	N/A	36.5	3.7	2.66	6.48
BNA15_17	MB156	17	14.9	3.2	2.70	6.24
BNA13_40	NV12-02	40	12.6	3.6	–	–
BNA13_14	NV12-03	14	12.6	3.4		
BNA15_28	NV12-04	28	14.9	3.3	–	–
BNA15_40	MB160	40	14.9	3.2	–	–
BNA36_31	MB159	31	35.9	4.0	–	–

*2.5 g/L; ** 10 g/L.
[a] Hydrodynamic radius, values are the average of 5 experiments and are given ± 0.1 nm.
[b] Radius of gyration from SAXS, values are given ± 0.1 nm
[c] Maximum diameter from pair distribution fnctions (D_{max}), values are given ± 0.2 nm.

of their intrinsic instability out of the membrane. We have previously demonstrated that GHSR could be efficiently refolded in NAPols [14]. The same folding protocol, based on SDS precipitation in the presence of the polymer, was used here with BNAPols (see experimental section).

To first assess the impact of the biotin moiety on the folding efficiency, GHSR was folded in non ionic amphipols at varying NAPol-to-BNAPol ratios while keeping the protein-to-polymer ratio identical (1:10, w/w). As shown in Fig. 5, the yield of functional receptor after amphipol-mediated folding was very similar whatever the ratio of non biotinylated-to-biotinylated amphipol. This indicates that the occurrence of the biotin moiety on the polymer does not significantly affect the ability of NAPols to fold the ghrelin receptor to its native state. To reach a sufficient immobilization rate, a constant 1:1 NAPol-to-BNAPol ratio was used throughout this work. Such mixtures were successfully used with the functionalized A8-35 amphipol [20]. Such mixtures allowed an efficient immobilization of functional membrane proteins on surfaces. Another consideration for using mixtures of functionalized and non-functionalized amphipols is that multiplying the attachment points on the surface for a single protein-amphipol complex may be detrimental to its dynamics and therefore to the functional analysis (dynamics are essential in the binding process in the case of GPCRs).

3.4.2. GHSR immobilization

As GHSR could be refolded in BNAPols, we subsequently tested whether the receptor:polymer complex could be immobilized onto streptavidin plates. Immobilization was carried out under standard conditions (see experimental section). We estimated the amount of receptor immobilized under such conditions. To this end, the receptor was labeled at its N-terminus with Lumi4-Tb NHS and the emission intensity recovered in the unbound fraction after the immobilization step was measured. Only *ca.* 20% of the emission signal initially present was recovered after the immobilization process. This indicates that about 80% of the receptor could be immobilized on the streptavidin plates under these conditions.

We then analyzed whether the immobilization process affected the receptor ligand-binding properties. GHSR ligand-binding assays were performed using the fluorescence energy transfer (FRET)-based assay we previously developed [33]. This assay is based on measuring the FRET signal between the immobilized receptor labeled with lumi4-Tb at its N-terminus and ghrelin labeled with dy647 at its C-terminus in the presence of varying concentrations in a competing compound. Three pharmacologically-distinct compounds were tested here, *i.e.* the full agonist MK 0677, the antagonist JMV 3018 and the inverse agonist SPA. As shown in Fig. 6, the competition profile obtained for all three compounds were very closely related for the non-immobilized and immobilized receptor. This clearly suggests that immobilization onto the streptavidin surface through the polymer belt does not significantly imparts the pharmacological properties of the ghrelin receptor, as far as ligand binding is considered.

The values for solution and immobilized receptor were inferred from the titration plot in Fig. 6 and are summarized in Table 3. BNAPol-mediated immobilization of GHSR does not significantly affect its ligand binding properties compared to non-immobilized conditions. For the sake of comparison, we also included in Table 3 the values obtained for the recombinant GHSR in nanodiscs [38] and those obtained in HEK293T cells [39]. To be noted, the *K*i values obtained for MK0677 and JMV3018 are systematically higher for the isolated receptor than for GHSR expressed at the surface of HEK intact cells. This is consistent, as both compounds are agonists, full agonist for MK0677, and partial agonist for JMV3018 [40], and the isolated receptor is in a low affinity state, as it is uncoupled from its G protein partner. We previously demonstrated that high-affinity agonist binding can be restored upon adding isolated G proteins to the purified receptor [33].

As the ligand-binding properties were not significantly affected by the immobilization process, we then analyzed whether the ghrelin receptor assembled into BNAPols and immobilized onto a streptavidin surface could be used as a ligand screening device. To this end, we selected a set of known GHSR ligands. These include the natural full agonist ghrelin, a high-affinity synthetic agonist (JMV 2894), a low-affinity synthetic agonist (JMV 3091), a high-affinity synthetic antagonist (JMV 3002), a low-affinity synthetic antagonist (JMV 2959), the low-affinity inverse agonist SPA and JMV 1843, an agonist pseudopeptide which got the approval of the FDA for the diagnosis of GH defficiency in adults. All JMV compounds belong to the series of 1,2,4-triazole derivatives [41–44], with the exception of the pseudopeptide JMV 1843 compound [44]. In addition, two negative controls were included in the assay, *i.e.* unacylated ghrelin (UAG) that has been shown to be unable to bind GHSR because of the lack of the octanoyl moiety [45] and the unrelated adrenergic receptor antagonist atenolol. As shown in Fig. 7, a specific decrease in the ghrelin:GHSR FRET signal was observed for all ligands known to bind the receptor. Interestingly, the extent in the decrease in the FRET signal was directly related to the affinity of the compound considered. Overall, this indicates that the FRET signal obtained under such conditions indeed reports for the ability of the compound to compete with labeled ghrelin for binding GHSR.

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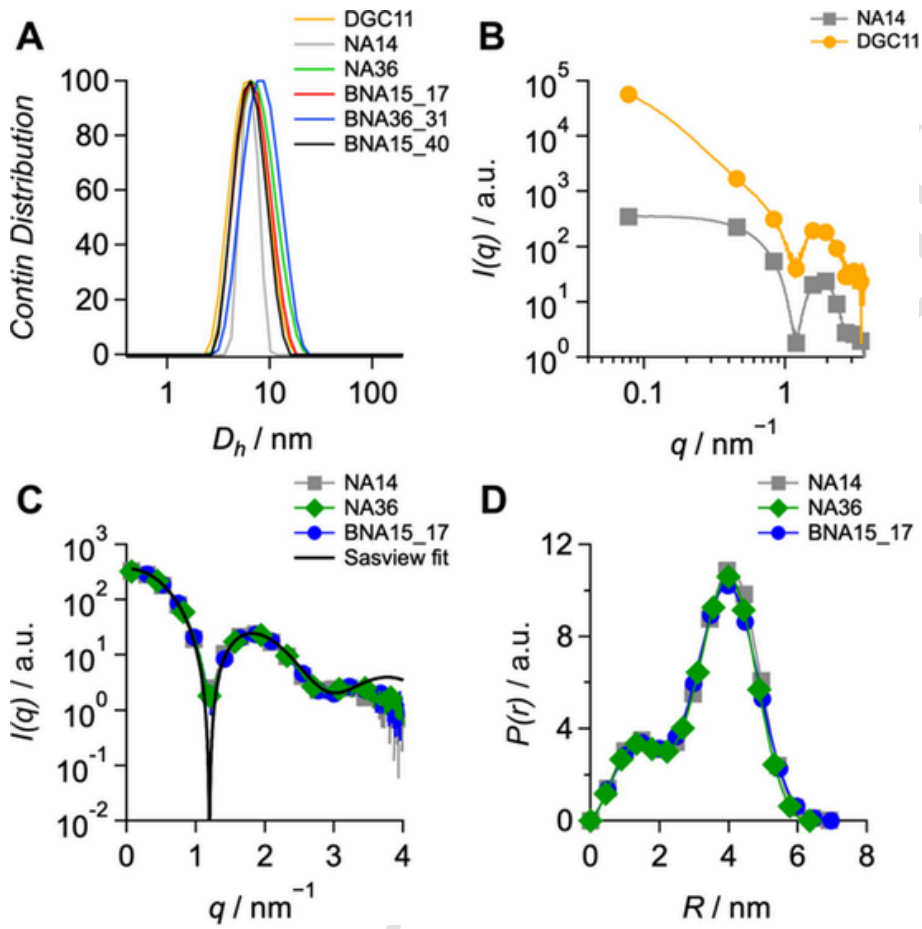

Fig. 4. (A) hydrodynamic volume distribution of DGC11, NAPols and BNAPols in aqueous phase at 5 g/L. (B) SAXS patterns of NAPol (DP_n 20) and DGC11 assemblies at 5 g/L (the curves for each series are shifted for more clarity). (C) SAXS patterns of NAPol (DP_n 20 ■ and 50♦) and BNAPol (DP_n 20 ●) at 5 g/L superimposed with a SASVIEW fit of a core–shell sphere model (black line). (D) Pair distribution function of NAPols (DP_n 20 ■ and 50♦) and BNAPol (DP_n 20 ●).

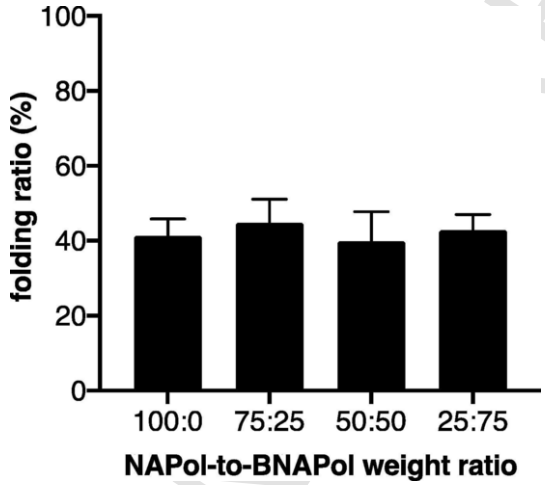

Fig. 5. Impact of NAPol-to-BNAPol ratio on the ability to fold GHSR. Polymers NA16 and BNA15_40 were used. Folding of GHSR from its SDS-state was carried out as described in the experimental section at varying NAPol-to-BNAPol weight ratios. Data are presented as the mean ± SEM of three experiments.

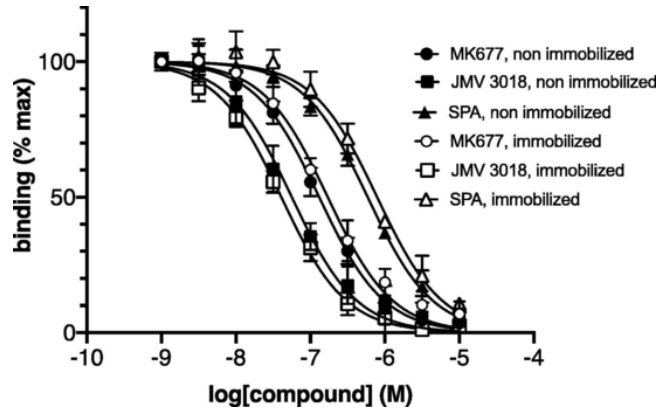

Fig. 6. Impact of immobilization on the ligand-binding properties of GHSR. FRET-monitored competition curves obtained with GHSR assembled into NAPols:BNAPols (1:1 wt ratio, polymers NA16 and BNA15_40 were used) in solution (closed symbols) or immobilized on a streptavidin plate (open symbols). Data are presented as the mean ± SEM of three experiments.

4. Conclusion

We have synthesized a series of biotin-functionalized non-ionic amphipols called BNAPols by free-radical telomerization of an amphiphilic monomer in the presence of a transfer agent that bears an azido group. By using a Huisgen-cycloaddition reaction the biotin group was grated on the polymer end chain. The BNAPols were found freely soluble in water and formed rather homogenous and globular micellar aggregates of about 6 nm in diameter.

The reconstitution of the ghrelin receptor GHSR in BNAPols was further demonstrated. Moreover, under our conditions, the receptor was efficiently bound to the surface while keeping its pharmacological properties essentially unaffected. This suggests that BNAPols could represent a valuable approach to immobilize functional GPCRs, and probably other MPs, onto surfaces for sub-

Table 3
Ligand-binding properties of GHSR in NAPols, in solution and immobilized compared to lipid nanodisc and HEK293T cells. In all cases, errors span the range of values observed in triplicate experiments.

Compound name	K_i (nM)			
	Solution	Immobilized	Lipid nanodiscs	HEK293T
MK 677	66.8 ± 4.7	73.3 ± 7.9	65.6 ± 1.5	8.4 ± 1.6
JMV3018	28.4 ± 6.3	20.5 ± 2.2	20.3 ± 1.4	3.4 ± 0.5
SPA	307 ± 27	317 ± 32	297 ± 5	226 ± 25

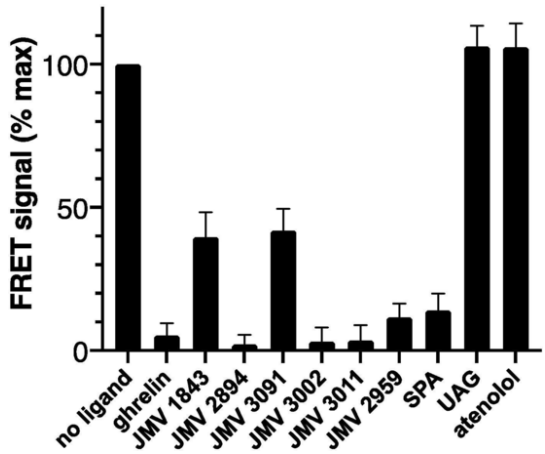

Fig. 7. Ligand-binding screening assay with the immobilized ghrelin receptor. FRET-monitored competition for binding GHSR assembled into NAPols:BNAPols (1:1 wt ratio, polymers NA16 and BNA15_40 were used) and immobilized on a streptavidin plate. Competing ligands were used at a 10 μM concentration while the fluorescent ghrelin tracer was used at 50 nM. All data are presented as the mean ± SEM of three experiments.

sequent screening assays. As such, this immobilization strategy of isolated MP could represent a valuable alternative to cellular systems in screening assays to identify a new generation of drugs from compound libraries. In particular, this assay could be well-adapted to fragment screening that is actually out of the scope of classical cell-based assays. Moreover, in contrast to other strategies already described, the method presented here is based on the use of a native, unmodified receptor, which can avoid any possible bias related to stabilizing mutations. Moreover, as the immobilization process involves the surfactant rather than the protein, such a process could be extended to untagged proteins as well.

CRediT authorship contribution statement

Michaël Bosco: Investigation, Formal analysis. **Marjorie Damian:** Investigation. **Vinay Chauhan:** Validation. **Mélanie Roche:** Investigation. **Pierre Guillet:** Investigation, Visualization. **Jean-Alain Fehrentz:** Resources. **Françoise Bonneté:** Investigation. **Ange Polidori:** Funding acquisition, Conceptualization. **Jean-Louis Banères:** Funding acquisition, Conceptualization. **Grégory Durand:** Supervision, Funding acquisition, Conceptualization.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2020.06.001.

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