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An efficient mixed solid–liquid phase synthesis of a heterobifunctional amphiphilic PEG–NH_2 derivative and its conjugation to folic acid

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Abstract

A very efficient, versatile as well as simple to perform procedure was developed in order to prepare a heterobifunctional amphiphilic PEG–NH_2 derivative which can be used for conjugation to a targeting ligand (such as folic acid). This method proceeds by a mixed solid–liquid phase strategy using a TentaGel® PAP resin, a copolymer consisting of a polystyrene matrix on which a PEG (M_w 3400 Da) terminated by an amino function has been grafted. Solid phase chemistry was used for the conjugation of a highly hydrophobic moiety. After release from the resin, the amphiphilic PEG–OH conjugate was converted into its corresponding amphiphilic PEG–NH_2 derivative (four steps in 77% overall yields). This procedure allowed the preparation of ~330 mg batches. This derivative was then coupled to folic acid, a ligand that is used for the targeting of drug (gene) carrier and delivery systems to cells over-expressing the folate receptor. The low and high molecular weight of folic acid and its amphiphilic PEG–folate conjugate, respectively, allowed easy purification by dialysis and led to the targeted compound with high recovery.

Keywords: Heterobifunctional PEG; TentaGel® resin; Perfluoroalkylated amphiphile; DNA nanoparticles; Dimerizable cationic detergents; Non-viral gene delivery; Synthetic viruses; Folic acid

1. Introduction

Present attempts in drug delivery (including gene delivery) mediated with synthetic nanocarriers (liposomes, lipoplexes, polyplexes) are oriented toward the formulation of multifunctional systems.[1–5] Recent developments focused on the assembly of 'artificial' or 'synthetic' viruses in order to take advantages of viral vectors' properties such as their small size (<100 nm), capability to escape the immune system sentinels, cell tropism, cytoplasm delivery, and/or nuclear targeting. Such systems require the formulation of very small-sized as well as very homogeneous (quasi monodisperse) nanoparticles which can be further equipped with an arsenal boosting their biodisponibility as well as their specific cell targeting capabilities.[1–4,6]

Aiming at these goals, there is an increasing demand for amphiphilic polyethylene glycol (PEG)–ligand conjugates (see general structure shown in Fig. 1) dedicated to specific in vitro/in vivo studies and in vivo biomedical applications. Indeed, PEGs (which are chemically stable and non-toxic materials) of M_w in the 2000–5000 Da range are widely used for the formulation of 'stealth' nanoparticle-based drug carrier and delivery systems, owing to the steric and hydrophilic shields such long hydrophilic polymers provide for the nanoparticles avoiding their interactions with plasma proteins (opsonins), recognition and clearance by the reticuloendothelial system.[7–10] PEG-modified nanocarriers are efficient at maintaining circulation in blood and at facilitating tumur accumulation through the enhanced permeability and retention effect.[11] For preserving the targeting ability of surface PEG-functionalized nanoparticles, the ligand must further be placed at the distal extremity of the PEG allowing its recognition by and interactions with the receptors expressed at the cell surface.[12] The development of such surface PEG- and

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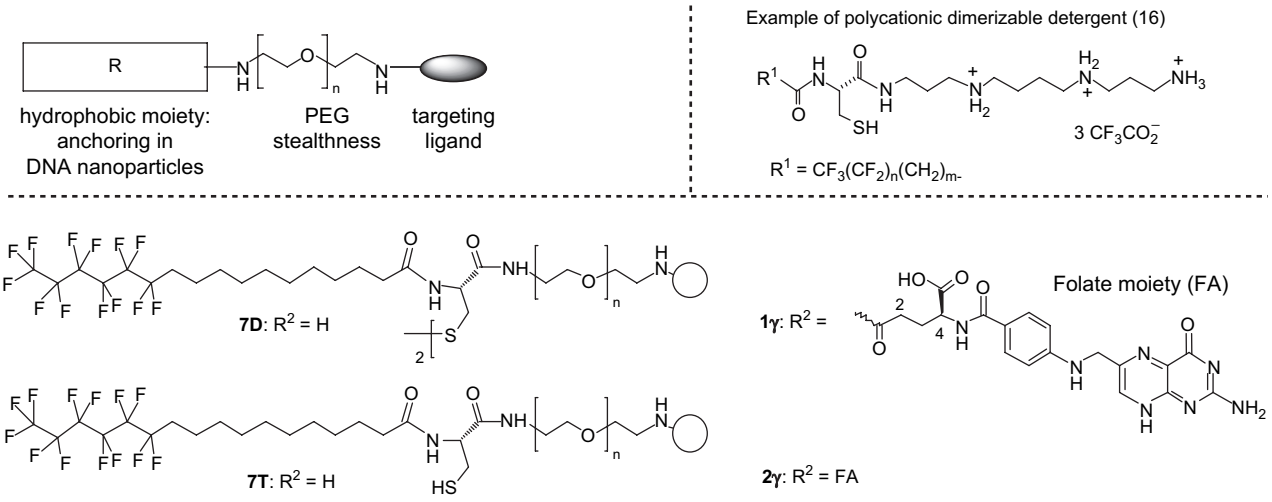


Figure 1. General structure of a heterobifunctional amphiphilic PEG–ligand conjugate (top left), the amphiphilic PEG–NH_2 compound **7D**, which is the key synthon for the access to our target amphiphilic PEG–folic acid conjugates **1** and **2** (bottom) for the formulation of targeted DNA nanoparticles with the polycationic perfluoroalkylated thiol-dimerizable detergents (top right).[16]

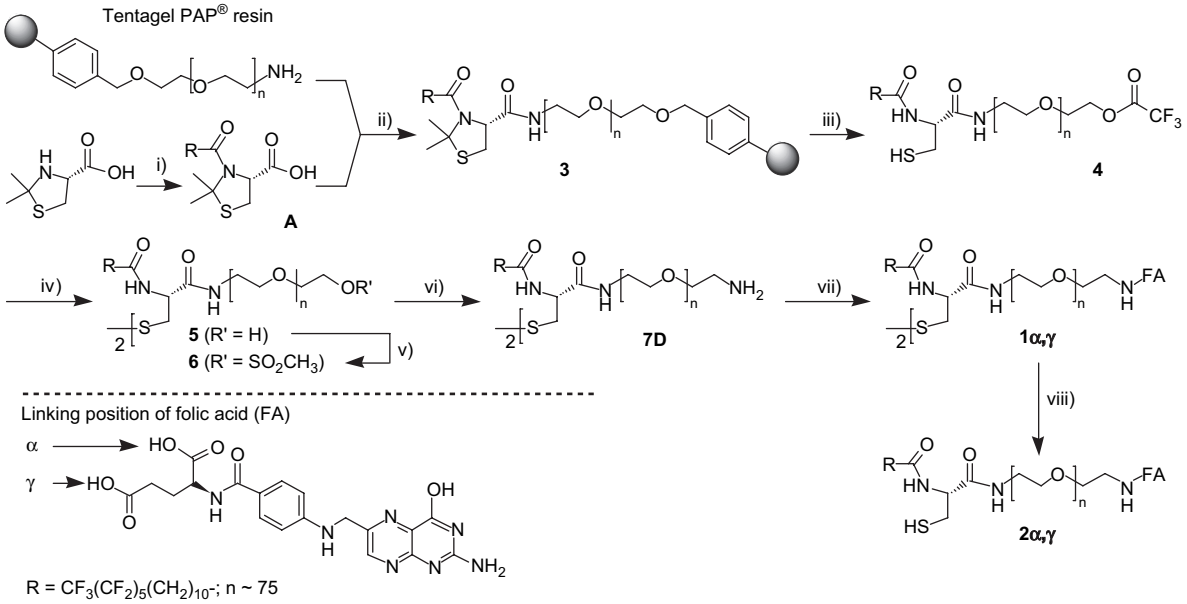
ligand-functionalized nanoparticles requires heterobifunctional PEG conjugates, i.e., PEGs bearing at each of its two extremities a different terminal group or function (such as a hydrophobic moiety for anchoring into a bilayer and a ligand for cell/tissue targeting). Straightforward access to such conjugates from commercially available PEGs and on a scale basis enabling in vivo testing remains however highly challenging. Indeed, preparations, purifications, and analyses of such high molecular weight conjugates may be cumbersome because of the polydisperse nature of the PEG and of its high hydrophilic character.[13–15] To circumvent these drawbacks, reactions that proceed quantitatively as well as regioselectively, and allow an easy purification and recovery of the formed products still need to be developed.

Our group has recently reported on the formulation of very small-sized (~40–50 nm) monodisperse, cationic DNA nanoparticles from the polycationic perfluoroalkylated, dimerizable detergents (see example in Fig. 1).[16] Indeed, these compounds condense DNA into stable nanoparticles after dimerization of the detergents into their disulfide lipids on the DNA matrix, each particle containing one copy of DNA (monomolecular DNA condensation).[17] For increasing the functionalities of these particles (stealthness, targeting), we designed the amphiphilic PEG–folic acid conjugates **1** and **2** shown in Figure 1. One extremity of each PEG unit in these conjugates is tethered to folic acid for targeting the folate receptor, which is over-expressed on various tumur cells.[18–20] We selected folic acid not only as targeting ligand but also as a model to validate our approach. The other PEG extremity of these conjugates is connected to a hydrophobic cystine (as in **1**) or cysteinamide segment (as in **2**), which contain a highly fluorinated tail, for increasing hydrophobic interactions, and for promoting their anchoring into the hydrophobic domains within the monomolecular DNA nanoparticles that are formed with the detergents (Fig. 1). Conjugate **2** with its cysteinyl thiol function was more particularly designed for its aggregation with the perfluoroalkylated, dimerizable detergents and its possibility to form a heterodisulfide with these detergents after subsequent oxidation onto the DNA matrix and, consequently, for promoting a more stable anchoring into or association with the monomolecular DNA nanoparticles that are formed with these detergents. Note that conjugates of type **1** (precursor of **2**) can also be used for the post-decoration of DNA nanoparticles that are formulated from (poly)cationic lipids/liposomes.

We report here on the synthesis of an amphiphilic PEG–NH_2 (e.g., compound **7D** in Fig. 1), which is the key synthon for the access to amphiphilic PEG–ligand conjugates such as **1** or **2**, and of its conjugation with folic acid using a straightforward mixed solid–liquid phase strategy shown in Scheme 1. It should be further emphasized that this strategy allows the preparation of conjugates on a scale (~300 mg) necessary for in vivo testing. To the best of our knowledge, the synthesis of heterobifunctional PEG–ligand conjugates that combine the advantages of a solid support with those of liquid-phase methodologies has never been reported. In this approach, the polymeric matrix acts as a protecting group for one PEG extremity allowing at the same time all benefits inherent in solid phase supported chemistry.

2. Results and discussion

The mixed solid–liquid phase synthesis of the amphiphilic PEG–folate cystine disulfide conjugate **1**α,γ, precursor of the target cystein thiol **2**α,γ, is presented on Scheme 1. It proceeded in six steps from TentaGel® PAP resin with a total yield of 35%. Large improvements of polymeric supports for SPPS and SPOS (Solid Phase for Peptide Synthesis and for Organic Synthesis, respectively) have led, among others, to graft all sort of PEGs on resins through one of its extremities and detachable linkers, allowing functionalization of the remaining terminal extremity.[21–32] The TentaGel® PAP resin which is a copolymer consisting of a polystyrene matrix on which a PEG (M_w 3000 Da) terminated by an amino function is grafted was selected among the various possible polymeric



Scheme 1. Synthesis of the amphiphilic PEG–folate conjugate **1**. (i) $CF_3(CF_2)_5(CH_2)_{10}C(O)Cl$ [prepared in situ from acid and $(COCl)_2$], Et_3N, CH_2Cl_2; (ii) **A**/HOBt/DIC/DMAP, DMF (twice); (iii) 95:5 TFA/thioanisole solution [95% yield for steps (ii)+(iii)]; (iv) O_2 bubbling, H_2O (100% yield); (v) methanesulfonyl chloride, Et_3N, CH_2Cl_2 (90% yield); (vi) aq NH_4OH, rt, 36 h (90% yield); (vii) folic acid (FA), DCC, pyridine, DMSO, rt, 12 h (63% yield); (viii) DTT (100% yield).

supports because the PEG is linked to the matrix by a cleavable benzyl ether linkage. Therefore, the PEG-containing conjugate can easily be cleaved and separated from the insoluble support.

We chose to couple first the hydrophobic moiety to the PEG-resin. This will result, after cleavage of the conjugate from the resin, in a synthon that can be used for coupling any type of ligands making this strategy very versatile.

The coupling reaction of the hydrophobic acid synthon **A** on the solid support was performed in DMF with the pre-swelled resin, and 1-hydroxybenzotriazole (HOBt), *N*,*N*-diisopropylcarbodiimide (DIC), and dimethylaminopyridine (DMAP) as condensing reagents. To fully complete the reaction, the process was repeated twice with an excess of **A**, which was besides totally recovered. The best results were further obtained by proceeding under anhydrous conditions. The completion of the reaction, i.e., the absence of unreacted amino groups, was confirmed by the sensitive Kaiser test, which proved negative.

The cleavage of the amphiphilic PEG conjugate from the support in **3** was performed with TFA containing 5 vol % of thioanisole as scavenger for 12 h at room temperature. However and unexpectedly,[33] these conditions led to conjugate **4** containing a trifluoroacetate ester (and not the hydroxy) and a thiol function, indicating that the thiazolidine ring protection did not resist these conditions probably due to water traces ascribable to the highly hydrophilic PEG substructure. The thiol function of the isolated material **4** was then best protected as its disulfide dimer, which was obtained through extensive oxygen bubbling in H_2O.[†] Under these conditions, hydrolysis of trifluoroacetate ester was also observed, giving quantitatively **5**. The chemical structure of the resulting disulfide **5** was unambiguously attested by ^1H, ^{19}F, ^{13}C NMR, and $^1H-^{13}C$ HSQC. The ^1H and ^{13}C NMR spectra of **5** exhibit a triplet of doublet at 5.19 ppm and a singlet at 46 ppm, respectively. These signals and chemical shifts are characteristic for the cystinyl CH. For comparison, the ^1H and ^{13}C resonances for the cysteinyl CH in the thiol **4** appear at 4.62 and 27 ppm, respectively. Hydrolysis of the ester **4** into the hydroxy derivative **5** is ascertained by (i) the disappearance of the characteristic $CF_3C(O)$ signal at −75.4 ppm in the ^{19}F NMR spectrum of **4**, and (ii) the concomitant deshielding of the ^1H and ^{13}C signal for the adjacent OCH_2 from 4.47 to 3.67 ppm and from 68.3/69.5 to 61.7 ppm, respectively. It should be noted that no traces of HO–PEG–NH_2 (or its NH_3^+ salt) resulting from an incomplete first-step coupling were detected by NMR.

The next two steps which consisted into the conversion of the hydroxy **5** into the amino derivative **7D** were performed almost quantitatively by the activation of the terminal hydroxyl group of **5** with a large excess of methanesulfonyl chloride followed by the nucleophilic substitution of the mesylate group in the isolated material **6** with aq NH_4OH. The structure of this key compound **7D** expecting to be the lead for any kind of ligand conjugation was demonstrated by ^1H, ^{19}F, ^{13}C, $^1H-^{13}C$ HSQC NMR experiments, and MALDI-TOF mass spectrometry. Typically, as expected, the ^1H (^{13}C) resonance of the adjacent methylene to the hydroxyl in the starting material **5** is shifted from 3.67 (61.7) to 4.35 ppm (69.0/69.3 ppm) for the mesylate **6** then to 2.82 ppm (41.8 ppm) for the amino derivative **7D** (^{13}C NMR spectra of **5** and **7D** for comparison are available as Supplementary data). The MALDI-TOF spectrum of **7D** (Fig. 2, left) exhibits a characteristic bell-shaped distribution of

[†] Alternatively, *S*-Boc protection of **4** was also performed. However, our attempts to convert the *S*-Boc derivative analogous to **6** into the amino derivative **7** were unsuccessful. Indeed, side reactions and extensive degradation were observed when the *S*-Boc derivative analogous to **6** was reacted with aq NH_4OH.

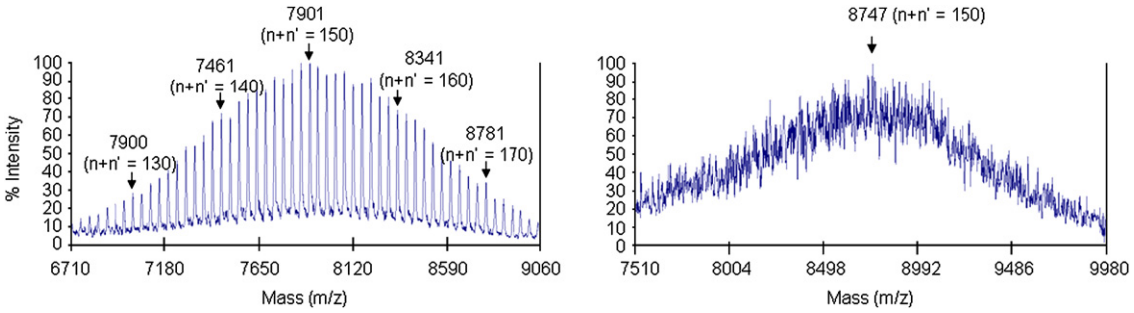


Figure 2. MALDI-TOF MS of (left) amphiphilic PEG–NH_2 **7D**, which shows the presence of compounds of m/z=[2×(648.19)+(n+n')×44.02+1] with n and n' in the range of 45–105, in agreement with the masses calculated for [2×($C_{22}H_{31}F_{13}N_3O_2S_1$)+($n$+$n'$)×($C_2H_4O$)]+$H^+$. The bell-shaped curve is centered at M+H^+ ~7901, which matches with the molecular weight of the compound for which n+n'=150 (calculated 7901); (right) amphiphilic PEG–folate conjugate **1**; this spectrum, though showing low S/N ratio due to the presence of DMSO into sample matrix, which contributed to poor quality of ionization, indicates the presence of several significant lines corresponding to the compounds of m/z=[2×(1071.32)+(n+n')×44.02+1 (or 23, 39)] with n and n' in the range of ~47–103, in agreement with the masses calculated for [2×($C_{41}H_{48}F_{13}N_{10}O_7S_2$)+($n$+$n'$)×($C_2H_4O$)]+$H^+$ (or Na^+, K^+). The curve is centered at (M+$H)^+$ ~8747, which matches with the molecular weight of the compound, which contains two PEG moieties for a total number of 150 ethylene oxide units, each PEG being connected to a folate residue of 423 Da (calculated 8747).

44 Da-spaced lines cenetred at m/z ~7901 Da, which matches with the molecular weight of compound **7D** with a total number of 150 ethylene oxide units (=44 Da) for the two PEG moieties (calculated mass for M+H: 7901 Da). This spectrum also indicates that the isolated PEG–NH_2 derivative **7D** consists of a polydispersed mixture of compounds deriving from a PEG ranging from ~45 to 105 ethylene oxide units.

Concerning the efficiency of this mixed solid–liquid phase synthetic approach, we were able to prepare batches of 330 mg of the amphiphilic PEG–NH_2 conjugate **7D** (0.17 mmol) starting from 1 g of TentaGel® PAP resin which are corresponding to 0.22 mmol of amino groups and a high-value hydrophobic acid synthon (e.g., the perfluoroalkylated thiazolidine carboxylic acid **A**). This represents 77% overall yields. The losses of materials are solely due to precipitation and manipulation processes, which did not allow, despite quantitative reactions, the total recovery at each step of the procedure.

The conjugation of **7D** to folic acid was realized in about 63% yield using conventional DCC coupling reagent, the resulting conjugate **1α,γ** being purified by dialysis. With respect to versatility of the process, the high molecular weight of **7D** allowed the use of large excesses of low molecular weight reagents (i.e., folic acid), which are easily separable and removable from **7D** by dialysis. Conjugation of the folate residue to the amino terminus of the PEG–NH_2 derivative **7D** was attested, among others, by NMR. In line with literature,[34] the folate adduct **1α,γ** was obtained as a mixture of isomers (8:2 ratio), the major isomer being **1γ** resulting from the coupling of the most active glutamic γ-acid function of folic acid, as disclosed by ^{1}H (^{13}C) 2D-NMR. Indeed, the ^{13}C signals at 31.95 and 52.60 ppm for the glutamic C-2 methylene and C-4 methyne of folic acid (see Fig. 3 for the atom numbering), respectively, are replaced by two characteristic sets of signals at 30.48 (i.e., CH_2-2 in α to amide) and 52.62 ppm (i.e., CH-4 in α to carboxyl), and 31.95 (i.e., CH_2-2 in α to carboxyl) and 52.33 ppm (i.e., CH-4 in α to amide) which are corresponding to isomer **1γ** and **1α**, respectively. Moreover, the ^{1}H NMR spectrum clearly exhibits for the CH-4 methyne proton a triplet of doublet at 4.36 ppm and a multiplet at 4.27 ppm, which integrate for 0.8 and 0.2H, respectively (the signal of the CH_2-2 methylene protons appears together with those of other methylenes). In addition, the ^{13}C signal of the distal $-CH_2NH_2$ of the starting PEG derivative **7D** at 41.78 ppm has been replaced by a signal at ~38.6 ppm for **1α,γ** characteristic of a CH_2NHCO. Furthermore, MALDI-TOF mass spectrometry confirmed this result. As shown in Figure 2 (right part), and although the isolated material **1α,γ** exhibits a rather poor resolute spectrum, this spectrum is centered at m/z ~8747 Da matching with the molecular weight of (M+$H)^+$ where M is corresponding to the compound which contains two PEG moieties for a total number of 150 ethylene oxide units, each PEG being connected to a folate residue of 423 Da (see caption of Fig. 2 for further details). The weak S/N ratio encountered on this particular spectrum is likely due to the presence of DMSO that was necessary to solubilize the target compound for analysis. The next step consisted into the reduction of the disulfide **1α,γ** into the thiol **2α,γ**: this was done by action of **1α,γ** with dithiothreitol (DTT) in NaOH

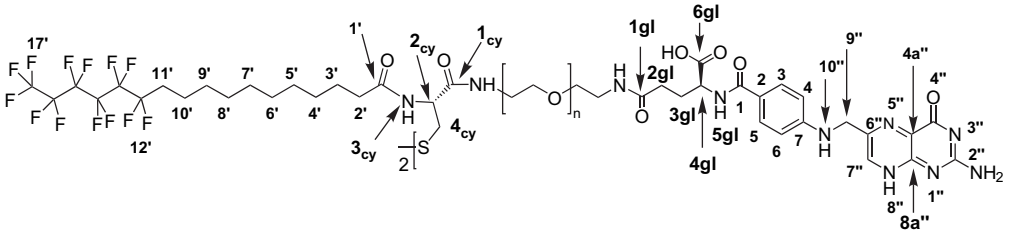


Figure 3. Folic acid–PEG conjugate **1γ** and numbering for NMR attribution.

0.05 N followed by precipitation upon acidification with TFA (data not shown). Unfortunately, full characterization of the thiol by NMR could not be realized owing to its relative sensitivity to reoxidation into the starting disulfide **1α,γ** and its poor solubility in other solvents than DMSO where reoxidation occurred very rapidly. Therefore, reduction of the disulfide into the thiol must be performed just before its further use.

3. Conclusion

A very efficient, versatile as well as simple to perform procedure was developed in order to prepare large amounts (~330 mg batches) of a heterobifunctional amphiphilic PEG–NH_2 derivative. Such derivatives can be used for the conjugation to a targeting ligand (that has an appropriate reactive function) and for the formulation of targeted drug (gene) carrier and delivery nanoparticles. This method proceeds by a mixed solid–liquid phase strategy using a commercially available TentaGel® PAP resin, a copolymer consisting of a polystyrene matrix on which a PEG terminated by an amino function has been grafted. Solid phase chemistry was used for the conjugation of a highly hydrophobic moiety. After release from the resin, the amphiphilic PEG–OH conjugate was then converted into its corresponding amphiphilic PEG–NH_2 conjugate (four steps with 77% overall yields). This derivative was then coupled to folic acid, a ligand that is used for the targeting of drug (gene) carrier and delivery systems to cells over-expressing the folate receptor. The low and high molecular weight of folic acid and its amphiphilic PEG–folate conjugate, respectively, allowed easy purification by dialysis and led to the target compound with high recovery and purity.

A detailed analysis of the ability of the amphiphilic PEG–folate conjugates **1** and **2** to modify the surface of negatively and positively charged monomolecular DNA nanoparticles formulated from the polycationic perfluoroalkylated, dimerizable detergents (such as the one shown in Fig. 1), the specific uptake of such functionalized DNA nanoparticles by folate over-expressing cells and cell transfection with these systems, which are currently under investigation, will be reported in due course.

4. Experimental

4.1. Generalities

Unless otherwise stated, all the reactions were performed in anhydrous solvents under dry and oxygen-free nitrogen. Anhydrous solvents were prepared by standard methods. Column chromatographies were performed on Merck silica gel 60 (mesh 70–230). Reactions were monitored by thin-layer chromatography (TLC) using silica plates (SDS $60F_{254}$), visualized by UV light (254 nm), and by spraying with ninhydrin reagent (Sigma) or with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma), or by charring with H_2SO_4. L-Cysteine hydrochloride, *N*,*N*-dicyclohexylcarbodiimide (DCC), 4-(dimethylamino)-pyridine (DMAP), folic acid, hydroxybenzotriazole (HOBt), thioanisole, triethylamine, trifluoroacetic acid (TFA) were purchased from Sigma, *N*,*N*-diisopropylcarbodiimide (DIC) from Alfa Aesar, methanesulfonyl chloride and oxalyl chloride from Fluka. TentaGel® PAP resin was bought from RAPP Polymer and dialysis membrane, Dialyse Float-A-Lyser (MWCO 1000 and 3500), was from VWR. These chemicals were used as received. 11-Perfluorohexyl-undecanoic acid was synthesized as described elsewhere.[35] 2,2-Dimethyl-thiazolidine-4(*R*)-carboxylic acid (hydrochloride salt) was prepared following a literature procedure.[33] ^1H, ^{13}C, and ^{19}F NMR spectra were recorded on a Bruker AC 200 (or AC 500) spectrometer at 200 (or 500), 50.3 (or 125.8), and 188.3 MHz, respectively. Chemical shifts (δ) were measured relative to $CDCl_3$ (7.26 ppm) or DMSO-d_6 (2.50 ppm) for ^1H, $CDCl_3$ (76.9 ppm) for ^{13}C, and $CFCl_3$ (0 ppm) for ^{19}F. In the ^{13}C NMR data, the carbon atoms attached to fluorine atoms were omitted, due to extensive couplings. The following abbreviations were used to describe the signal multiplicities: s (singlet), br s (broad singlet), d (doublet), t (triplet), br t (broad triplet), q (quadruplet), and m (multiplet). Chemical shifts were expressed in parts per million (ppm), coupling constants (*J*) were reported in hertz, and signals were listed as follows: shifts in parts per million (multiplicity, coupling, integration, and assignment). The atoms of the cysteine part are depicted as C-x_{cy} and H-y_{cy}, whereas those of the perfluoroalkylated part are depicted as C-x′, H-y′, and F-z′ according to their standard nomenclature numbering and those of the PEGylated part are depicted as CH_2N(or O); concerning the folic acid moiety, the atoms of the glutamic part are described as C-x_{gl} and H-y_{gl}, those of the benzoyl part as C-x and H-y, whereas those of the pteridine heterocycle following nomenclature numbering as C-x″ and H-y″(see Fig. 3 for numbering). COSY $^1H/^1H$, $^1H/^{13}C$ NMR correlation (on Bruker AC 500 spectrometer), and/or mass spectrometry data fully confirm the signal assignments and structure of the isolated materials. Electrospray ionization mass spectrometry (ESI-MS) in negative mode was performed on a Bruker Daltonics (Esquire 3000 plus) apparatus equipped with atmospheric pressure ionization source. MALDI-TOF spectra were recorded on a Perseptive Voyager DE-STR MALDI-TOF mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) equipped with a 337 nm pulsed nitrogen laser (20 Hz) and an Acqiris® 2 GHz digitizer board was used for all experiments. Mass spectra were obtained in linear positive ion mode with the following settings: accelerating voltage 22 kV, grid voltage 92% of accelerating voltage, extraction delay time of 300 ns. The laser intensity was set just above the ion generation threshold to obtain peaks with the highest possible signal-to-noise (S/N) ratio without significant peak broadening. The mass spectrometer was close externally calibrated using PEG 6000. 2,5-Dihydroxybenzoic acid was used as matrix at a concentration of 20 mg/mL in THF.

4.2. Synthesis of 3-(12,12,13,13,14,14,15,15,16,16,17,17,17-tridecafluoroheptadecanoyl)-2,2-dimethyl-4(R)-thiazolidine-4-carboxylic acid (*A*)

Oxalyl chloride (250 μL, 2.80 mmol) and DMF (10 μL) were successively added to 11-perfluorohexyl-undecanoic

acid (1.01 g, 2.0 mmol) dissolved in CH_2Cl_2 (30 mL).[36] After the sample was stirred overnight at room temperature, the solvent was removed under vacuum. The crude acid chloride dissolved in anhydrous CH_2Cl_2 (2.5 mL) was added dropwise to a CH_2Cl_2 solution (40 mL) of 2,2-dimethyl-thiazolidine-4(*R*)-carboxylic acid (600 mg, 2.80 mmol) and triethylamine (1.5 mL). The reaction mixture was stirred at room temperature for 3 h. The reaction mixture was washed with an aq 0.2 N $KHSO_4$ solution, then dried over Na_2SO_4, filtrated, and concentrated. The residue was purified by chromatography over silica gel (100:0–96:4 CH_2Cl_2/MeOH) to yield compound **A** as a white powder (1.13 g, 72%). R_f 0.41 (9:1 CH_2Cl_2/MeOH); ^{1}H NMR ($CDCl_3$): δ 8.65 (br s, 1H, COOH), 4.86 [br t, *J* 3.3 Hz, 1H, NHC*H*C(O)], 3.31 (br d, *J* 3.3 Hz, 2H, CH_2S), 2.23 (t, *J* 7.4 Hz, 2H, H-2′), 2.08 (m, 2H, H-11′), 1.99 and 1.90 (2s, 6H, CH_3), 1.50–1.68 (m, 4H, H-3′ and H-10′), 1.28 (br s, 12H, H-4′–9′); ^{19}F NMR ($CDCl_3$): δ −81.64 (3F, F-17′), −115.08 (2F, F-12′), −122.63 (2F, F-13′), −123.56 (2F, F-14′), −124.23 (2F, F-15′), −126.87 (2F, F-16′); ^{13}C NMR ($CDCl_3$): δ 174.60 (COOH), 171.88 (C-1′), 73.94 (*C*Me$_2$), 65.64 [NH*C*HC(O)], 36.93 (C-2′), 31.38 (CH_2S), 31.03 (t, J_{CF} 22.2 Hz, C-11′), 29.84 and 27.63 (CH_3), 29.55, 29.52, 29.48, 29.36, and 29.24 (C-4′–9′), 25.08 (C-3′), 20.24 (t, J_{CF} 3.5 Hz, C-10′). ESI-MS (negative mode): $(M-H)^-$=646.0 in agreement with the mass calculated for M=$C_{23}H_{30}O_3F_{13}S$ (647.17).

*4.3. Synthesis of α-[[[2-(12,12,13,13,14,14,15,15,16,16,17,17,17-tridecafluoro-heptadecanamido)-2-(2R)-(mercaptomethyl)-1-oxoethyl]amino]ethyl]-ω-[(trifluoroacetyl)oxy]poly(oxy-1,2-ethanediyl) (**4**)*

TentaGel® PAP resin (1 g, 0.22 mmol of amino group) in a rotary glass funnel was suspended in anhydrous DMF (10 mL) and swollen at room temperature for 12 h shaking at 120 rpm. The following reaction coupling was repeated twice under nitrogen: (i) compound **A** (341.5 mg, 0.53 mmol) and HOBt (74 mg, 0.53 mmol) in anhydrous DMF (4 mL) were added to the resin followed by DIC (85 μL, 0.53 mmol) and DMAP (16.8 mg, 0.26 mmol); (ii) the sample was stirred for 3 h then the solution was filtered and the grafted resin **3** washed successively with DMF, MeOH, and CH_2Cl_2. A 95:5 TFA/thioanisole solution (10 mL) was added to the dried resin and, after stirring for 12 h, the reaction mixture was filtered and the resin washed with CH_2Cl_2 and MeOH. The combined organic phase was concentrated under vacuum and remaining TFA removed by CH_2Cl_2–cyclohexane coevaporation. CH_2Cl_2 (5 mL) was added to the resulting colorless oil and Et_2O was added until precipitation. The solid was filtered out by centrifugation, dried under vacuum to afford the intermediate thiol **4** as a white solid (846.5 mg, 95%). ^{1}H NMR ($CDCl_3$): δ 7.00 (t, *J* 4.1 Hz, 1H, N*H*CH$_2$), 6.70 (d, *J* 7.2 Hz, 1H, NH-3$_{cy}$), 4.58 (q, *J* 6.8 Hz, 1H, H-2$_{cy}$), 4.47 (m, 2H, $CH_2CH_2OC(O)CF_3$), 3.78 (m, 4H, NHCH$_2$C*H*$_2$ and C*H*$_2$CH$_2$OC(O)CF$_3$), 3.60–3.70 [m, ∼340H (*n*∼85), O(C*H*$_2$C*H*$_2$O)$_n$], 3.48 (br t, *J* 4.6 Hz, 2H, NHC*H*$_2$), 2.96 (m, 1H, H$_A$-4$_{cy}$), 2.79 (m, 1H, H$_B$-4$_{cy}$), 2.45 (s, 1H, HS), 2.24 (t, *J* 7.8 Hz, H-2′), 1.93–2.07 (m, 2H, H-11′), 1.50–1.62 (m, 4H, H-3′ and H-10′), 1.24 (br s, 12H, H-4′–9′); ^{19}F NMR ($CDCl_3$): δ −75.36 [3F, $CF_3C(O)$], −81.20 (3F, F-17′), −114.81 (2F, F-12′), −122.39 (2F, F-13′), −123.35 (2F, F-14′), −124.04 (2F, F-15′), −126.60 (2F, F-16′); ^{13}C NMR ($CDCl_3$): δ 173.92 (C-1′), 169.92 (C-1$_{cy}$), 70.63 [O(*C*H$_2$*C*H$_2$O)$_n$], 70.35 (NHCH$_2$*C*H$_2$), 69.49, 68.25 (*C*H$_2$*C*H$_2$OC(O)CF$_3$), 54.45 (C-2$_{cy}$), 39.71 (NH*C*H$_2$), 36.53 (C-2′), 30.96 (t, *J* 22.3 Hz, C-11′), 29.41, 29.39, 29.32, 29.29, 29.27, and 29.17 (C4′–9′), 26.93 (C-4$_{cy}$), 25.70 (C-3′), 20.18 (t, *J* 3.6 Hz, C-10′).

*4.4. Synthesis of N,N′-[dithiobis[(1R)-1-[α-[2-(carboxyamino)ethyl]-ω-[hydroxy]-poly(oxy-1,2-ethanediyl)]-2,1-ethanediyl]]bis-(12,12,13,13,14,14,15,15,16,16,17,17,17-tridecafluoro)heptadecanamide (**5**)*

Thiol **4** (700 mg, 0.173 mmol) dissolved in water was submitted to oxygen bubbling for 4 h (^{1}H NMR monitoring) yielding, after evaporation, alcohol **5** as a white solid (683 mg, 100%). R_f 0.44 (95:5 CH_2Cl_2/MeOH); ^{1}H NMR ($CDCl_3$): δ 8.06 (t, *J* 5.4 Hz, 1H, N*H*CH$_2$), 6.51 (d, *J* 8.8 Hz, 1H, NH-3$_{cy}$), 5.19 (td, *J* 8.8, 4.8 Hz, 1H, H-2$_{cy}$), 3.73 (br t, *J* 4.8 Hz, 2H, NHCH$_2$C*H*$_2$), 3.67 (br t, *J* 4.5 Hz, 2H, C*H*$_2$OH), 3.50–3.70 [m, ∼340H (*n*∼85), O(C*H*$_2$C*H*$_2$O)$_n$ and C*H*$_2$CH$_2$OH], 3.45 (br t, *J* 4.8 Hz, 2H, NHC*H*$_2$), 2.93 (dd, J_{AB} 14.5 Hz, J_{Ax} 9.5 Hz, 1H, H$_A$-4$_{cy}$), 2.87 (dd, J_{AB} 14.5 Hz, J_{Ax} 4.5 Hz, 1H, H$_B$-4$_{cy}$), 2.20 (t, *J* 7.3 Hz, H-2′), 1.93–2.07 (m, 2H, H-11′), 1.50–1.62 (m, 4H, H-3′ and H-10′), 1.24 (br s, 12H, H-4′–9′); ^{19}F NMR ($CDCl_3$): δ −81.26 (3F, F-17′), −114.84 (2F, F-12′), −122.41 (2F, F-13′), −123.95 (2F, F-14′), −124.04 (2F, F-15′), −126.60 (2F, F-16′); ^{13}C NMR ($CDCl_3$): δ 173.36 (C-1′), 170.37 (C-1$_{cy}$), 72.62 (*C*H$_2$CH$_2$OH), 70.57 [O(*C*H$_2$*C*H$_2$O)$_n$], 70.27 (NHCH$_2$*C*H$_2$), 61.69 (*C*H$_2$OH), 53.03 (C-2$_{cy}$), 45.80 (C-4$_{cy}$), 39.39 (NH*C*H$_2$), 36.56 (C-2′), 30.87 (t, *J* 22.3 Hz, C-11′), 29.67, 29.43, 29.34, 29.23, 29.18, and 29.10 (C4′–9′), 25.57 (C-3′), 20.10 (C-10′).

*4.5. Synthesis of N,N′-[dithiobis[(1R)-1-[α-[2-(carboxyamino)ethyl]-ω-[(methylsulfonyl)oxy]-poly(oxy-1,2-ethanediyl)]-2,1-ethanediyl]]bis-(12,12,13,13,14,14,15,15,16,16,17,17,17-tridecafluoro)heptadecanamide (**6**)*

Methanesulfonyl chloride (80 μL, 1.07 mmol) was added dropwise to a solution of **5** (400 mg, 0.05 mmol) and triethylamine (150 μL) in CH_2Cl_2 (30 mL). The reaction mixture was stirred at room temperature for 12 h. The CH_2Cl_2 solution was washed with aq 50 mM $NaHCO_3$, dried over Na_2SO_4, concentrated up to 5 mL, and Et_2O was added until precipitation. The solid was filtered out by centrifugation, dried under vacuum to afford **6** as a white solid (366 mg, 90%). R_f 0.44 (95:5 CH_2Cl_2/MeOH); ^{1}H NMR ($CDCl_3$): δ 8.10 (t, *J* 5.5 Hz, 1H, N*H*CH$_2$), 6.49 (d, *J* 9.0 Hz, 1H, NH-3$_{cy}$), 5.22 (td, *J* 9.2, 4.7 Hz, 1H, H-2$_{cy}$), 4.35 (m, 2H, CH$_2$C*H*$_2$OMs), 3.73 (m, 4H, NHCH$_2$C*H*$_2$ and C*H*$_2$CH$_2$OMs), 3.50–3.65 [m, ∼360H (*n*∼90), O(C*H*$_2$C*H*$_2$O)$_n$], 3.45 (m, 2H, NHC*H*$_2$), 3.04 (s, 3H, CH_3), 2.93 (dd, J_{AB} 14.0 Hz, J_{Ax} 4.5 Hz, 1H, H$_A$-4$_{cy}$), 2.86 (dd, J_{AB} 14.0 Hz, J_{Ax} 9.5 Hz, 1H, H$_B$-4$_{cy}$), 2.20 (t, 2H, *J* 7.5 Hz,

H-2′), 1.93–2.06 (m, 2H, H-11′), 1.51–1.62 (m, 4H, H-3′ and H-10′), 1.24 (br s, 12H, H-4′–9′); ^{19}F NMR ($CDCl_3$): δ −81.22 (3F, F-17′), −114.88 (2F, F-12′), −122.44 (2F, F-13′), −123.38 (2F, F-14′), −124.06 (2F, F-15′), −126.62 (2F, F-16′); ^{13}C NMR ($CDCl_3$): δ 173.33 (C-1′), 170.35 (C-1_{cy}), 70.56 [O(CH_2CH_2O)$_n$], 70.25 (NHCH$_2$$CH_2$), 69.34, 69.02 ($CH_2CH_2$OMs), 53.09 (C-$2_{cy}$), 45.92 (C-$4_{cy}$), 39.35 (NH$CH_2$), 37.73 ($CH_3$), 36.57 (C-2′), 30.84 (t, J 22.2 Hz, C-11′), 29.44, 29.36, 29. 34, 29.24, 29.18, and 29.11 (C4′–9′), 25.57 (C-3′), 20.09 (t, J 3.7 Hz, C-10′).

4.6. Synthesis of N,N′-[dithiobis[(1R)-1-[α-[2-(carboxyamino)ethyl]-ω-[2-aminoethoxy]-poly(oxy-1,2-ethanediyl)]-2,1-ethanediyl]]bis-(12,12,13,13,14,14,15,15,16,16,17,17,17-tridecafluoro)heptadecanamide (**7D**)

Mesylate **6** (366 mg, 0.045 mmol) dissolved in aq NH_4OH (45 mL) was stirred at room temperature for 36 h. The aqueous phase saturated with NaCl was extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtrated, concentrated up to 5 mL, and Et_2O was added until precipitation. The solid was filtered out by centrifugation, dried under vacuum to afford **7** as a white solid (325 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 8.07 (t, J 5.4 Hz, 1H, NHCH$_2$), 6.49 (d, J 8.9 Hz, 1H, NH-3_{cy}), 5.21 (td, J 8.9, 4.8 Hz, 1H, H-2_{cy}), 3.72 (br t, J 4.8 Hz, 2H, NHCH$_2$CH$_2$), 3.5–3.65 [m, ∼320H (n∼80), O(CH_2CH_2O)$_n$], 3.48 (m, 4H, NHCH$_2$ and NH$_2$CH$_2$CH$_2$), 2.92 (dd, J_{AB} 14.5 Hz, J_{Ax} 4.5 Hz, 1H, H_A-4_{cy}), 2.86 (dd, J_{AB} 14.5 Hz, J_{Bx} 9.5 Hz, 2H, H_B-4_{cy}), 2.82 (t, J 5.2 Hz, 2H, CH_2NH_2), 2.18 (t, J 7.5 Hz, H-2′), 1.99–2.11 (m, 4H, NH$_2$ and H-11′), 1.51–1.62 (m, 4H, H-3′ and H-10′), 1.22 (br s, 12H, H-4′–9′); ^{19}F NMR ($CDCl_3$): δ −81.21 (3F, F-17′), −114.88 (2F, F-12′), −122.45 (2F, F-13′), −123.38 (2F, F-14′), −124.06 (2F, F-15′), −126.62 (2F, F-16′); ^{13}C NMR ($CDCl_3$): δ 173.71 (C-1′), 170.73 (C-1_{cy}), 73.27 (CH_2CH_2OH), 70.56 [O(CH_2CH_2O)$_n$], 70.28 (NHCH$_2$CH$_2$), 53.06 (C-2_{cy}), 45.92 (C-4_{cy}), 41.78 (NH$_2$CH$_2$), 39.37 (NHCH$_2$), 36.56 (C-2′), 31.04 (t, J 22.3 Hz, C-11′), 29.41, 29.33, 29.31, 29.21, 29.17, and 29.09 (C4′–9′), 25.56 (C3′), 20.11 (t, J 3.2 Hz, C-10′). MS (MALDI-TOF): presence of compounds of m/z= 2×(648.19)+(n+n')×44.02+1 with n and n' in the range of 45–105, in agreement with the masses calculated for M [=2×($C_{22}H_{31}F_{13}N_3O_2S_2$)+($n$+$n'$)×($C_2H_4O$)]+H$^+$. The bell-shape curve (see Fig. 3, left) is centered at [M+H]$^+$∼7901 Da, which is corresponding to the compound for which n+n'=150 (calculated 7901 Da).

4.7. Synthesis of N,N′-[dithiobis[(1R)-1-[α-[2-(carboxyamino)ethyl]-ω-[2-[[(2S)-2-[[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-amino]-2-carboxyethyl-1-oxoethyl]amino]ethoxy]-poly(oxy-1,2-ethanediyl)]-2,1-ethanediyl]]bis-(12,12,13,13,14,14,15,15,16,16,17,17,17-tridecafluoro)heptadecanamide (**1γ**) (major isomer) and N,N′-[dithiobis[(1R)-1-[α-[2-(carboxyamino)ethyl]-ω-[2-[[(4S)-4-[[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)-methyl]amino]benzoyl]amino]-4-carboxy-1-oxobutyl]-amino]ethoxy]-poly(oxy-1,2-ethanediyl)]-2,1-ethanediyl]]bis-(12,12,13,13,14,14,15,15,16,16,17,17,17-tridecafluoro)-heptadecanamide (**1α**) (minor isomer)

A solution of folic acid (45 mg, 0.1 mmol), DCC (41 mg, 0.2 mmol), and pyridine (1 mL) in anhydrous DMSO (5 mL) was stirred for 1.5 h at room temperature in the dark. After addition of compound **7** (80 mg, 0.01 mmol), the mixture was stirred for 12 h. The solvent was removed under vacuum, the solid redissolved in 3 mL of DMSO was filtered through 0.45 μm filter unit, dialyzed against DMSO and water (MWCO 3500), and lyophilized to afford compound **8α**,**γ** as a yellow powder (56 mg, 63%). ^{1}H NMR (DMSO-d_6): δ 8.64 (s, 1H, H-7″), 8.06 (d, J 8.3 Hz, 1H, NH-3_{cy}), 8.00 [t, J 5.3 Hz, 1H, NHC(O)-1_{cy}], 7.95 [d, J 7.9 Hz, C(O)NH-5_{gl}], 7.88 [t, J 5.8 Hz, C(O)NH-1_{gl}, isomer **1α**], 7.85 [t, J 5.6 Hz, C(O)NH-1_{gl}, isomer **1γ**], 7.65 (m, 2H, H-3, H-5), 6.93 (br m, 1H, NH-10″), 6.63 (m, 2H, H-4, H-6), 4.53 (m, 1H, H-2_{cy}), 4.48 (d, J 5.9 Hz, 2H, H-9″), 4.36 (td, J 8.5, 5.4 Hz, 0.8H, H-4_{gl}, isomer **1γ**), 4.27 (m, 0.2H, H-4_{gl}, isomer **1α**), 3.68–3.28 [m, ∼300H (n∼75), NHCH$_2$CH_2O(CH_2CH_2O)$_n$CH_2CH$_2$NH], 3.24–3.14 [m, 4H, NHCH_2CH$_2$O(CH$_2$CH$_2$O)$_n$CH$_2$CH_2NH], 3.03 (dd, J_{AB} 13.4 Hz, J_{Ax} 5.1 Hz, 1H, H_A-4_{cy}), 2.80 (dd, J_{AB} 13.4 Hz, J_{Bx} 9.2 Hz, 1H, H_B-4_{cy}), 2.38–1.82 (m, 8H, H-2′, H-11′, H-2_{gl}, H-3_{gl}), 1.66 (br s, 2H, NH-2″), 1.55–1.45 (m, 4H, H-3′, H-10′), 1.40–1.20 (m, 12H, H-4′–9′); ^{19}F NMR (DMSO-d_6): δ −80.83 (3F, F-17′), −113.93 (2F, F-12′), −122.31 (2F, F-13′), −123.55 (2F, F-14′), −124.06 (2F, F-15′), −126.33 (2F, F-16′); ^{13}C NMR (DMSO-d_6): δ 174.06 [C(O)OH, isomer **1γ**], 173.84 [C(O)OH, isomer **1α**], 172.30, 171.7, and 170.00 [C(O)NH-1′, C(O)NH-1_{cy}, C(O)NH-1_{gl}], 166.14 [C(O)NH-1], 153.77 (C-8a″), 150.73 (C-6″), 148.5 (br, C-7″, C-7), 128.99 (C-3, C-5), 121.40 (C-2), 120.64 (C-4a″), 111.13 (C-2, C-4), 70.10, 69.76, 69.59, 69.54, 69.05, 68.91, and 68.78 [NHCH$_2$CH_2O(CH_2CH_2O)$_n$CH_2CH$_2$NH], 52.62 (C-4_{gl}, isomer **1γ**), 52.33 (C-4_{gl}, isomer **1α**), 51.75 (C-2_{cy}), 45.91 (C-9″), 40.84 (C-4_{cy}), 38.69, and 38.52 [NHCH_2CH$_2$O-(CH$_2$CH$_2$O)$_n$CH$_2$CH_2NH], 35.18 (C-2′), 31.95 (C-2_{gl}, isomer **1α**), 30.48 (C-2_{gl}, isomer **1γ**), 29.72 (t, J 21.5 Hz, C-11′), 28.86, 28.78, 28.61, and 28.22 (C4′–9′), 27.06 (C-3_{gl}), 25.10 (C-3′), 19.69 (C-10′). MS (MALDI-TOF): presence of compounds of m/z=2×(1071.32)+(n+n')×44.02+1 (or +23 or +39) with n and n' in the range of 47–103, in agreement with the masses calculated for M [=2×($C_{41}H_{48}F_{13}N_{10}O_7S_2$)+($n$+$n'$)-×($C_2H_4O$)]+H (or Na or K)$^+$. The bell-shaped curve (see Fig. 3, right) is centered at m/z ∼8747 Da, which is matching the molecular weight of the compound for which n+n'=150 (calculated for (M+H)$^+$=8747 Da). For the MALDI-TOF analysis of **1**, the optimal conditions yielding the highest quality of MS data were obtained when **1** was used from a DMSO/H_2O (10:90 v/v) solution. Negative ion mode, other solvents (e.g., H_2O, 0.05 N NaOH, MeOH, MeCN—pure or in combination—with or without 0.1% TFA) and/or other matrices (e.g., cyano-4-hydroxycinnamic acid, 2-amino benzoic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, 9-amino-acridine) gave MS spectra of poorer quality. Conventional ESI and APCI MS were unsuccessful.

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Supplementary data

For comparison, ^{13}C NMR spectra of **5** and **7D** recorded at 125.8 MHz on a Bruker AC 500 spectrometer are available as supplementary materials. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2007.12.027.

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