Sensitive liposomes encoded with oligonucleotide amphiphiles: a biocompatible switch

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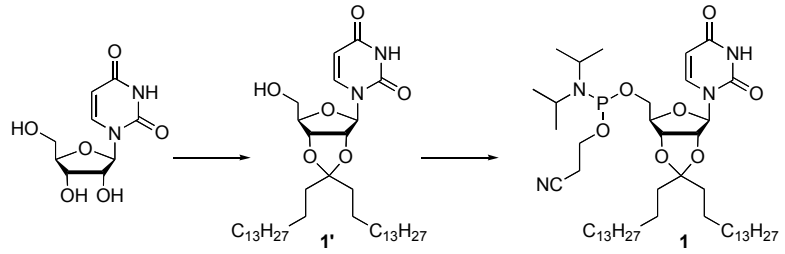
Preparation of the nucleoside amphiphile phosphoramidite 1:



2',3'-O-16-hentriacontanyliden-uridine **1'**. Palmitone (1g, 2.22 mmol) was added to a mixture of dry THF (20 mL), uridine (2.70 g, 11.11 mmol.), TsOH (0.42 g, 2.22 mmol) and triethylorthoformate (1.85 mL, 11.11 mmol.). The solution was refluxed for 1 day, quenched with triethylamine (0.6 mL) and poured into a solution of H_2O (50 mL), ice (50 g) and $NaHCO_3$ (4 g) then stirred for 15 min. The organic phase was dried over Na_2SO_4 and concentrated *in vacuo*. The remaining solid was triturated in MeOH to give compound **1'** (0.74 g, 49 %) as a white powder. ^{1}H NMR (300.13 MHz, $CDCl_3$) : 0.87 (t, *J* = 6.6Hz, 6 H, 2C*H*$_3$), 1.24 (m, 48H, 24C*H*$_2$), 1.41 (m, 4H, 2CH$_3$C*H*$_2$), 1.56 (m, 2H, CC*H*$_2$), 1.73 (m, 2H, CC*H*$_2$), 3.05 (m, 1H, O*H*), 3.80 (dd, *J* = 12.0 Hz, *J* = 3.6 Hz, 1H, *H*$_{5'}$), 3.91 (dd, *J* = 12.0 Hz, J = 2.7 Hz 1H, *H*$_{5'}$), 4.28 (m, 1H, *H*$_{4'}$), 4.95 (dd, *J* = 6.6Hz, *J* = 3.6Hz, 1H, *H*$_{3'}$), 5.04 (dd, *J* = 6.6 Hz, *J* = 2.7 Hz, 1H, *H*$_{2'}$), 5.55 (d, *J* = 2.7 Hz, 1H, *H*$_{1'}$), 5.72 (d, *J* = 8.1 Hz, 1H, C*H*), 7.33 (d, *J* = 8.1 Hz, 1H, C*H*), 9.43 (m, 1H, N*H*). ^{13}C NMR (75.47 MHz, $CDCl_3$) : 14,10 (*C*H$_3$), 22.70 (*C*H$_2$), 23.54 (*C*H$_2$), 24.15 (*C*H$_2$), 29.33-29.81 (*C*H$_2$), 31.89 (*C*H$_2$), 36.85 (*C*H$_2$COO), 36.98 (*C*H$_2$COO), 62.65 (*C*$_{5'}$), 80.29 (*C*$_{4'}$), 83.84 (*C*$_{3'}$), 87.29 (*C*$_{2'}$), 96.34 (*C*$_{1'}$), 102.56 (*C*H), 118.39 (O*C*O), 143.16 (*C*H), 150.32 (*C*O), 163.33 (*C*O). FAB MS ([M+H]$^+$ = 677).

Phosphoramidite **1**. Compound **1'** was dried over P_2O_5 overnight under reduced pressure before use. Diisopropylethylamine (0.052 mL, 0.299 mmol), **1'** (97mg, 0.149 mmol) and 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite were dissolved in 3 mL dichloromethane and the solution stirred at room temperature for 1h. Sodium bicarbonate 0.1M (3 mL) was poured into the flask and the aqueous phase extracted with dichloromethane. Flash chromatography (Hex/AcOEt/TEA 70/25/5) afforded 90 mg (69%) of the desired phosphoramidite as a white solid. ^{1}H NMR (300.13 MHz, $CDCl_3$) : 0.88 (t, *J* = 6.6 Hz, 6 H, 2C*H*$_3$), 1.19 (m, 12H, *iPr*), 1.27 (m, 50H, 25 C*H*$_2$), 1.43 (m, 2H, C*H*$_2$), 1.56 (m, 2H, C*H*$_2$), 1.74 (m, 2H, C*H*$_2$), 2.65 (m, 2H, *iPr*), 3.60 (m, 2H, C*H*$_2$CN), 3.85 (m, 4H, *OCH*$_2$, *H*$_{5'}$), 4.36 (m, 1H, *H*$_{4'}$), 4.83 (m, 2H, *H*$_{2',3'}$), 5.72 (d, *J* = 8.1 Hz, 1H, C*H*), 5.81 (s, 1H, *H*$_{1'}$, dia1), 5.90 (s, 0.5H, *H*$_{1'}$, dia2), 7.48 (d, *J* = 8.1 Hz, 0.5H, C*H*, dia1), 7.57 (d, *J* = 8.1 Hz, 0.5H, C*H*, dia2). ESI MS ([M-H]$^-$ = 876).

Oligonucleotide synthesis. ONA$_1$ and **ONA$_2$** were synthesized using phosphoramidite methodology on an automated Expedite 8909 DNA synthesizer at the mmol scale on 500 Å primer support (loading: 60-100 µmol/g, Link technologies, Synbase Control Pore Glass). Prior to use, the phosphoramidite **1** was co-evaporated several times with acetonitrile, and dissolved in dry CH_2Cl_2/CH_3CN 2/1 to a 0.06 M concentration. N-benzylthiotetrazole was used for activation of phosphoramidite prior to coupling. The phosphoramidite **1** was manually coupled last on the solid support by passing (via syringes) the activator and the phosphoramidite (0.25 mL) back and forth several times for 7 min. Deblocking and detachment from the solid support was achieved using 1 mL of a saturated aqueous NH_4OH/ethanol 3/1 (vol/vol) solution for 12 h at 55°C. The supernatant was collected and the CPG beads were washed 3 times with 0.25 mL of EtOH/CH_3CN/H_2O 3/1/1 (vol). The solutions were pooled and evaporated (speed vac). The crude oligonucleotide amphiphiles **ONA$_1$** and **ONA$_2$** were dissolved in 0.3 mL of water and purified on an analytical C_4-reverse phase HPLC using buffer A (0.1 M triethylammonium acetate, pH 6.5) with 20 % of buffer B (0.1 M triethylammonium acetate, pH 7.0, 80 % acetonitrile) over 2 min, followed by buffer B over 50 min (flow rate: 2 ml/min). The product oligoamphiphiles eluted after ca. 20 min. Product containing fractions were pooled and evaporated to dryness and dissolved in autoclaved milliQ water. Yields of final **ONA$_1$** (ε=141300) and **ONA$_2$**

(ε=132700) were good (falling in the 15-25% range). MALDI-TOF mass analyses: **ONA_1**: $[M-H]^-$ calculated MW: 4995.83 Da, found: 4997.84 Da (0.040% error); **ONA_2**: $[M-H]^-$ calculated MW: 5008.75 Da, found: 5011.10 Da (0.047% error).
9f and **14f** oligonucleotides were synthesized by the same protocol as **ONA_1** and **ONA_2** oligonucleotides using 5'-fluorescein phosphoramidite (6-FAM, Glen Research) at the final coupling step. These oligonucleotides were further purified by electrophoresis on denaturing 20% polyacrylamide, 7M urea gels. The band corresponding to the oligonucleotide of interest was crushed and the oligonucleotide eluted with 0.1M sodium acetate buffer overnight. Polyacrylamide residues were filtered off and the oligonucleotides precipitated with EtOH/3M AcONa. The precipitates were washed 3 times with 70% EtOH and finally dissolved in autoclaved milliQ water to the desired concentration. MALDI-TOF mass analyses: **9f**: $[M-H]^-$ calculated 3245.32 Da found: 3245.37 Da. **14f** $[M-H]^-$ calc: 4795.32 found: 4797.67 (0.049% error)

9 and **14** oligonucleotides were custom-made (Eurogentec) and further purified by electrophoresis on denaturing 20% polyacrylamide, 7M urea gels as described before.

Thermal melting curves. The purified oligonucleotides and the buffer 5X were preheated separately at 90°C for 4 min to remove air bubbles from the samples. The two strands (1 μM each, 0.08 mL) were then pooled with the buffer (20 mM hepes pH 7+ 10 mM NaCl or 15 mM cacodylate pH 6 + 10 mM NaCl or 40 mM phosphate pH 6.6, final concentrations). Melting experiments were performed with a Cary-1E UV/VIS spectrophotometer equipped with a temperature controller. Samples were pre-equilibrated at 5°C for 10 min, and then heated from 5 to 85°C at a rate of 0.4°/min. The absorbance at 260 nm was recorded every 30 s. The absorbance drift observed at high temperatures (see Figure Sup1) results from the partial evaporation of water during the course of the experiments. In conventional procedures, addition of mineral oil into the UV cells usually prevents water evaporation. Due to the presence of the hydrophobic residues in **ONA_1** and **ONA_2**, the experiments were carried out in the absence of mineral oil.

Liposome preparation. For preparation of large multilamellar vesicles (MLV), DOPC was dissolved in chloroform to a final concentration of 20 mg/mL. 50 μL of this solution was evaporated in vacuo forming a thin lipid layer on the flask wall. The lipid film was resuspended in 1 mL aqueous buffer (40 mM phosphate, pH 6.6). Subsequently, MLV (2 μm maximum diameter) were formed by subjecting this suspension to 10 freeze/thaw cycles (liquid nitrogen/50°C water bath) and extrusion through a polycarbonate filter (Nucleopore Track Etch membrane, 2 μm diameter) using an extruder (Avanti polar lipids). 0.1 mL of this suspension were diluted 10 times with the buffer, vortexed with **ONA_2** (10.6 μL of a 0.12 mM aqueous solution, 1 mol% total lipids) and incubated at 37°C for 30 min and used subsequently in SPR and epifluorescence microscopy experiments. Note that considering the way liposomes were prepared, **ONA_2** is inserted in the outer leaflets only.

Epifluorescence microscopy. The **9f** (or **14f**) fluorescent probe (0.6 μL of a 51 μM solution, 24 mol% compared to **ONA_2**) was added to 0.2 mL of the previous liposome suspension diluted twice with the buffer (40 mM phosphate, pH 6.6). Epifluorescence experiments were performed on an Olympus IX71 microscope with Hg lamp excitation. Emitted light was captured via a 100x, 1.4 NA oil-immersion objective and a Photometrics Cascade 512B camera (Roper Scientific). Fluorescein was excited at 485 +/- 10 nm and the emitted light collected at 525 +/- 20 nm. The solution temperature was controlled through the temperature of the metallic cover-slip holder with a TC-344B heater controller (Warner Instruments). The temperature could be more accurately monitored directly into the liposome suspension. Yet, evaporation of water in the course of the experiment takes place in that case as evidenced by an increase in T_m. As a result, our set up did not allow the precise measurement of the DNA duplexes T_m.

Surface plasmon resonance. SPR experiments were performed on a BiacoreTM 3000 apparatus (Biacore, GE Healthcare, Uppsala, Sweden). 70-80 resonance units (RU) of biotinylated **A_2** were immobilized onto a streptavidin-coated SA sensorchip (Biacore).[1] A L1 sensorchip was used to capture 7000-7500 RU of liposomes displaying **ONA_2**. Both sensorchips (Biacore) were prepared according to the manufacturer's instructions and a previously published study.[2] Briefly, SA sensorchips

[1] Wang R, Tombelli S, Minunni M, Spiriti MM, Mascini M. *Biosens Bioelectron.* **2004**, *20*, 967.
[2] C. L. Baird, E. S. Courtenay, D. G. Myszka, *Anal. Biochem.* **2002**, *310*, 93.

were conditioned by injecting three 1 min pulses of a mixture of 50 mM NaOH and 1 M NaCl. L1 sensorchips were prepared by injecting a 1 min pulse of 40 mM N-octyl ß-glucopyranoside (Sigma) in water followed by four 1 min pulses of a 50/50 (vol/vol) mixture of 100 mM HCl and isopropanol. One flow cell was left blank and the sensorgrams were double-referenced as described previously to remove instrument noises and the buffer contribution to the signal.[3] The experiments, performed at least in triplicate, were carried out at 25 °C in 10 mM sodium phosphate buffer, pH 7 at 21°C, containing 150 mM NaCl (running buffer). The oligonucleotides (**9**, **9f**, **14** and **14f**) were prepared in this buffer and injected at 20 µl/min across the sensor surface. The regeneration of the $\mathbf{A_2}$-coated surface (SA sensorchip) or the $\mathbf{ONA_2}$ one (L1 sensorchip) was achieved with two 1 min pulses of a freshly prepared 10 mM NaOH solution followed by a 1 min pulse of running buffer. These conditions perfectly regenerated the target without altering the amount immobilized, in particular when the $\mathbf{ONA_2}$ functionalized liposomes were captured.

Kinetic titration experiments that consist in running a binding experiment by injecting sequentially the ligand in order of increasing concentrations were performed to determine the rate constants and the binding equilibrium constant for complex formation, as described previously.[4] This method is advantageous for complexes that display very slow dissociation rates by minimizing the amount of regeneration steps that may alter the surface if harsh chemical conditions are required. In this work three concentrations of ligand were injected to determine the kinetic parameters. The association and dissociation rate constants, k_{on} and k_{off}, respectively, were determined from direct curve fitting of the sensorgrams using BiaEval 4.1 (Biacore), assuming a simple reversible mechanism according to Equations 1 and 2, for the association and the dissociation phases, respectively:

$$\frac{dRU}{dt} = k_{on}[Ligand]RU_{\max} - (k_{on}[Ligand] + k_{off})RU \quad (1)$$

$$\frac{dRU}{dt} = -k_{off}RU_{t0}.e^{-k_{-1}(t-t_0)} \quad (2)$$

where RU is the signal response; RU_{max}, the maximum response level; RU_{to}, the response at the beginning of the dissociation phase; and [Ligand], the molar concentration of the injected oligonucleotides. The binding equilibrium constant, K_d, was calculated as k_{off}/k_{on}.

[3] D. G. Myszka, *J. Mol. Recognit.* **1999**, *12*, 279.
[4] C. Di Primo, *J. Mol. Recognit.* **2008**, *21*, 37; R. Karlsson, P. S. Katsamba, H. Nordin, E. Pol, D. G. Myszka, *Anal. Biochem.* **2006**, *349*, 136

Figure Sup1. Thermal denaturation curves for a) the 4 different duplexes (200 pmoles) in 20 mM hepes buffer pH 7.0 containing 10 mM NaCl; b) **ONA$_2$-9f** duplex in 40 mM phosphate buffer pH 6.6 (a T_m of ca. 28°C can be read at the maximum of the derivative curve in blue).

a) b)

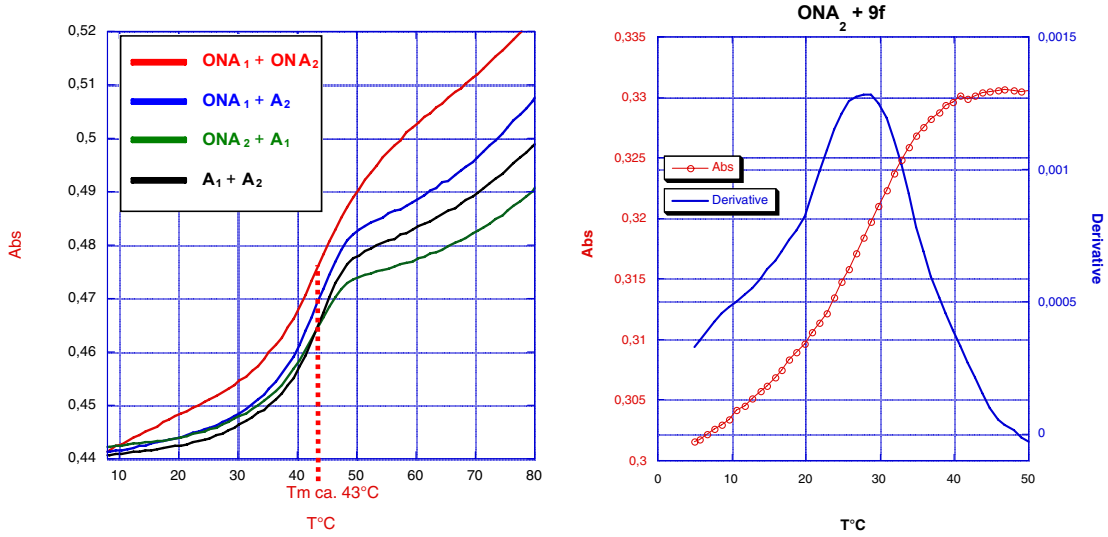


Figure Sup2. Kinetic analysis of DNA (**9** or **14**)-**A$_2$** or **ONA$_2$** complex formation. Single-stranded DNA prepared in the running buffer, were injected at 25 °C in order of increasing concentrations at 20 µl/min across the **A$_2$** (or **ONA$_2$** loaded liposome-coated) sensor surface. The sensorgrams were fitted as described above. The red curves represent the experimental data and the black one the fit of the sensorgrams to a kinetic titration data set of three analyte injections. a) Injection of oligonucleotide **9** (0.04 µM, 0.2 µM and 1 µM) to **A$_2$**-coated chip b) Injection of oligonucleotide **9** (0.04 µM, 0.2 µM and 1 µM) to **ONA$_2$**-coated chip. C) Injection of oligonucleotide **14** (5.5 nM, 33.3 nM and 200 nM) to **A$_2$**-coated chip. D) Injection of oligonucleotide **14** (5.5 nM, 33.3 nM and 200 nM) to **ONA$_2$**-coated chip.

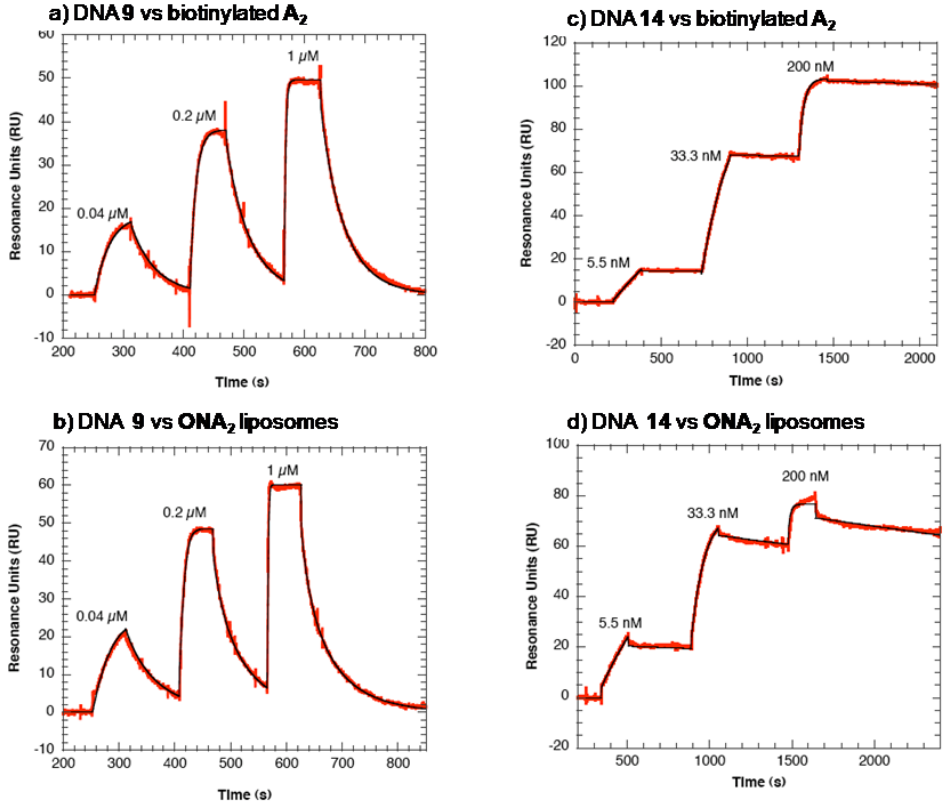


Figure Sup3. Control experiment. A 14 nt scrambled DNA sequence (5'-ACG ACG TGG CCA AC-3') prepared in the running buffer was injected for 1 min at 20 µl/min over an **A_2**-coated surface (grey) or an **ONA_2** loaded liposomes-coated surface (black) in order of increasing concentrations (40 nM, 200 nM and 1 µM) as indicated by the arrows.