Sensitive liposomes encoded with oligonucleotide amphiphiles: a biocompatible switch†

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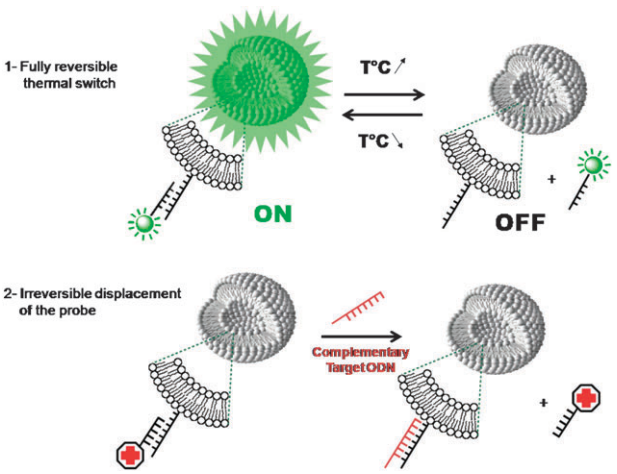
DNA-tagged liposomes made of DOPC specifically bind to a fluorescently labelled complementary ss-DNA with virtually no influence from the lipid bilayer despite the absence of a linker; depending on an external stimulus, either physical (temperature) or chemical (competitive complementary ON sequences), the liposomes switch between an *on* and *off* fluorescent state depending on the location of the probe either at the surface or in the bulk.

Life and oligonucleotides (DNA and RNA) are intimately intertwined. These somewhat chemically simple natural polymers indeed feature virtually infinite and programmable molecular recognition possibilities with exquisite specificity. Hence, many oligonucleotide-based supramolecular systems have been successfully used in biotechnology.[1] Most of these applications require the oligonucleotides (ON) to be bound to a support which is either solid (gold, glass or silicon *e.g.* in DNA chips) or fluid (lipid membranes). In the latter case, a lipophilic motif must somehow be tethered to the ON—in general covalently—to yield an oligonucleotide amphiphile (ONA) that is capable of binding to lipid bilayers. Supramolecular systems based on ONA have attracted considerable attention[2,3] owing to their biocompatibility, capability of molecular recognition[4] even at the single base level,[5] *e.g.* in sandwich hybridization assays,[6] cell biology,[2] and medicine.[7] These applications rely on the ability of the ONA to recognize and bind specific single-stranded ON targets at the lipid/water interface. Of particular interest is the design of encoded supramolecular systems that can undergo a transformation under the control of a physical or chemical stimulus. Despite the diversity of molecular switches reported so far,[8] it remains a challenge to develop a biocompatible model that can operate and be triggered under physiological conditions. Herein, we show that DNA-tagged liposomes (made of DOPC) specifically bind to a fluorescently labelled complementary DNA with no or little influence from the lipid bilayer. Depending on an external stimulus, either physical (temperature) or chemical (competitive complementary ON), the liposomes switch between an *on* and *off* fluorescent state depending on the location of the probe at the surface or in the bulk (Scheme 1).

The DOPC multilamellar liposomes were prepared using the freeze–thaw method (see ESI†). For this proof of principle investigation the sequence of the ON chain of the ONA was optimized (Hyther™, http://ozone3.chem.wayne.edu/) to (1) avoid self-complementary strands and (2) obtain a melting temperature (T_m) of 37 °C for a 14-mer DNA duplex in the presence of 100 mM monovalent cation. Membrane anchoring was brought about by the di-C_{15} ketal motif. It was expected to provide sufficient hydrophobic driving force for stable insertion of the ONA into bilayers[9] while avoiding too strong self-association.[10] Besides, given the zwitterionic phosphocholine polar head of DOPC at the liposome surface,[11,12] and for atom economy reasons, no spacer was inserted in between the ON headgroup and the hydrophobic tail. The two complementary **ONA$_1$** and **ONA$_2$** were then synthesized along with the control DNA **A$_1$** and **A$_2$** that lack the hydrophobic motif (Fig. 1). The synthesis of **ONA$_1$** and **ONA$_2$** is straightforward using classical phosphoramidite chemistry (see ESI†). Note that due to the chemical synthesis of DNA in the 3′–5′ direction, the phosphoramidite **1** used for the preparation of



Scheme 1 Illustrative sketch of (1) the thermo-controlled reversible switch and (2) the chemical irreversible switch. Below the T_m of the duplex, the probe is centered onto the liposome surface (*on* state). On increasing the temperature above T_m (or in the presence of a complementary ON), the fluorescent probe is expelled from the surface (*off* state).

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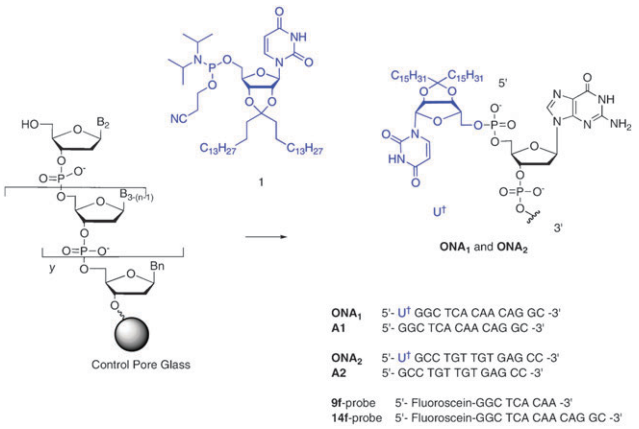

Fig. 1 Chemical structures of $\mathbf{ONA_1}$ and $\mathbf{ONA_2}$, control DNA $\mathbf{A_1}$ and $\mathbf{A_2}$, 9- and 14-mer fluorescein DNA probes (**9f** and **14f**, respectively).

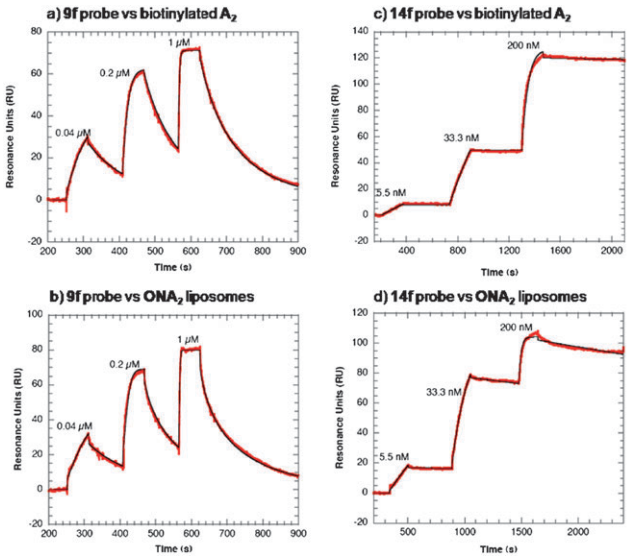

Fig. 2 Kinetic analysis of DNA duplex formation. Iterative injections of **9f** (0.04 μM, 0.2 μM and 1 μM) across (a) $\mathbf{A_2}$- and (b) $\mathbf{ONA_2}$-coated chip. Iterative injections of **14f** (5.5 nM, 33.3 nM and 200 nM) across (c) $\mathbf{A_2}$- and (d) $\mathbf{ONA_2}$-coated chip. The sensorgrams were fitted (black curves) as described in the ESI.†

ONA is attached in a head to head (5′–5′) fashion when incorporated last onto the solid support.

Duplex formation between a DNA and a complementary ONA present at the surface of a liposome was first visualized by Shohda.[12] DNA recognition principles on the surface of liposomes have also been utilized to induce liposome fusion.[13] Yet, the behaviour of flexible macromolecules attached to lipid bilayers[14] is greatly influenced by the surface (lipidic or other). The influence of the lipid membrane on the thermodynamics and kinetics of ON duplex formation is an important parameter for biotechnological applications. Bunge *et al.* have thoroughly investigated the thermodynamics,[15] but no kinetic data are available to our knowledge. Successful implementation of our ONA-based platform as molecular logic gates requires DNA pairing at the surface of the liposome to be at least as *fast* and *accurate* as in the bulk. Surface plasmon resonance (SPR) was used to compare hybridization kinetics of *unmodified* duplexes with related complexes formed between $\mathbf{ONA_2}$ embedded into the liposome surface and fully or partially complementary ON (modified or not with fluorescein, see Fig. 1). Although performed at a solid/liquid interface, the kinetic and thermodynamic parameters obtained with this surface-based technique usually match those in solution.[16] A streptavidin-coated surface was used to immobilize the unmodified target $\mathbf{A_2}$. A surface, referred to as L1, consisting of a carboxymethylated hydrogel with covalently attached lipophilic groups was used to capture the liposomes with embedded $\mathbf{ONA_2}$.[17] In addition, a stable baseline was observed after this capture indicating a stable insertion of $\mathbf{ONA_2}$ into the bilayer: no equilibrium exists with free or self-assembled aggregates of $\mathbf{ONA_2}$.[10] Sensorgrams—obtained by the kinetic titration method—for $\mathbf{ONA_2}$ and $\mathbf{A_2}$ binding to **9f** and **14f** are shown in Fig. 2 (see ESI Fig. Sup2 for binding to **9** and **14**, which lack the fluorescent probe†).

In both cases the sensorgrams were fitted assuming a one step reaction. The kinetic constants and the apparent equilibrium dissociation constants for each complex determined from direct curve fitting of the sensorgrams are listed in Table 1.

The fluorescent probe has no effect on the stable **14**–$\mathbf{A_2}$ duplex (K_d = 0.28 nM) while it slightly increases the stability of the **9**–$\mathbf{A_2}$ one by decreasing the off rate. This likely results from a favourable stacking of the probe on top of the shorter helix. Overall, the absolute values of the kinetic parameters obtained with 9 or 14 nt ligands and $\mathbf{ONA_2}$ loaded liposomes captured on the L1 sensorchip are similar compared to the unmodified $\mathbf{A_2}$-coated surface. A 14 nt scrambled sequence injected up to 1 μM did not interact either with $\mathbf{A_2}$ or $\mathbf{ONA_2}$ (see ESI, Fig. Sup3†). No non-specific binding of ON onto the liposome takes place and the association between $\mathbf{ONA_2}$ and its complementary ON is very likely mediated by specific Watson–Crick base pairing. The results clearly demonstrate that an ONA embedded into the liposome surface behaves as an unmodified DNA strand for recognizing complementary sequences. In particular, and despite the absence of spacer, the zwitterionic lipid membrane has virtually no effect on DNA duplex annealing and fusion even with **14** or **14f** complementary DNA whose 3′-ends lie in the Debye layer of the liposome. Furthermore, our results indicate that fusion of DNA duplexes onto DOPC liposomes is unaffected and can be used to develop our DNA-tagged liposomes platform for detection and delivery.

Our thermal switch concept—based on our DNA-tagged liposomes—relies on the thermo-controlled localization of fluorescence at the surface of liposomes. Below a threshold temperature, the fluorescence is concentrated at the surface of the liposomes and the system is *on*. Above that temperature, the fluorescence is expelled from the surface giving the *off* state. At the molecular level, the *reversible* switch is triggered by the annealing/melting of the **9f**–$\mathbf{ONA_2}$ DNA duplex (Scheme 1). Several limitations in our design are obvious: the melting temperature (T_m) of the duplex must be (1) above room temperature to observe the thermal switch and (2) low enough to preserve the integrity of the liposome suspension and thus ensure reversibility. Nevertheless, T_m are

Table 1 Kinetic parameters (k_{on} and k_{off}) and apparent dissociation constants (K_d) for complexes formed between DNA sequences and $\mathbf{A_2}$ or $\mathbf{ONA_2}$[a]

DNA sequence	$k_{on} \times 10^5$ M^{-1} s^{-1} Complexed with $\mathbf{A_2}$	$k_{off} \times 10^{-5}$ s^{-1}	K_d/nM	$k_{on} \times 10^5$ M^{-1} s^{-1} Complexed with $\mathbf{ONA_2}$	$k_{off} \times 10^{-5}$ s^{-1}	K_d/nM
5′-GGCTCACAA-3′ (**9**)	4.9 ± 0.8	2936 ± 298	59.6 ± 3.9	6.8 ± 2.2	3230 ± 886	48.9 ± 13.2
5′ F-GGCTCACAA-3′ (**9f**)	5.1 ± 0.7	1273 ± 76	25.4 ± 3.9	6.7 ± 2.6	1877 ± 367	31.2 ± 12.6
5′-GGCTCACAACAGGC-3′ (**14**)	1.8 ± 0.4	4.4 ± 2.6	0.25 ± 0.12	3.8 ± 0.2	16.3 ± 4.3	0.43 ± 0.10
5′ F-GGCTCACAACAGGC-3′ (**14f**)	1.7 ± 0.9	4.2 ± 2.9	0.28 ± 0.18	3 ± 1	14.6 ± 6.6	0.49 ± 0.10

[a] k_{on}, k_{off} and K_d are the means and standard deviations of at least three independent experiments. F = fluorescein.

experimentally finely tuned over a wide range of temperatures simply by optimizing the length of the duplex and/or the ionic strength of the medium. First attempts to UV-monitor the melting of the duplex at the surface of the liposomes proved impossible (results not shown), most probably because the colloid scatters light. In contrast, the T_m of the different duplexes were easily monitored in the absence of DOPC (see ESI Fig. Sup1†). We found a T_m of *ca.* 28 °C for the **9f**–$\mathbf{ONA_2}$ duplex in phosphate buffer (40 mM, pH 6.6) without added salts.[18] In practice, such a T_m for the **9f**–$\mathbf{ONA_2}$ duplex appeared ideal as it abides by the two rules mentioned above.

Liposomes loaded with $\mathbf{ONA_2}$ were then incubated with the **9f** probe at *ca.* 20 °C and the thermal switch investigated by epifluorescence microscopy. The switch proved fully operative and quite satisfyingly fully reversible (see movie 1 in ESI†). Hence, the fluorescence is localized at the liposome surface below the T_m of the **9f**–$\mathbf{ONA_2}$ duplex and the liposomes are *switched off* at higher temperatures. The reversibility of the switch was checked four times back and forth without any noticeable changes in the appearance of the suspension. Interestingly, and in line with SPR experiments, the T_m of the **9f**–$\mathbf{ONA_2}$ duplex seems to be virtually unaffected when present at the surface of the liposome. The whole process is thus being driven by specific Watson–Crick base pairing at the surface of the liposome. Hence, the fully complementary **14f** fluorescent probe (**14f**–$\mathbf{ONA_2}$ duplex T_m >50 °C) remains localized on the surface throughout the temperature interval tested (19–41 °C) (see movie 2 in ESI†).

Finally, liposomes are irreversibly switched off by addition of a 14-mer DNA complementary either to the fluorescent **14f** probe or $\mathbf{ONA_2}$ (see movie 3 in ESI†) while a scrambled 14-mer DNA complementary to $\mathbf{ONA_2}$ has no effect even after melting of the **14f**–$\mathbf{ONA_2}$ duplex (not shown).

In summary, this paper reports the synthesis and physicochemical properties of ONA inserted into DOPC liposomes. Interestingly, DNA recognition principles, thermodynamics and kinetics are unaffected at the liposome surface. Based on this simple sensitive platform, several applications have been demonstrated: (1) a thermo-responsive fully reversible molecular switch and (2) a potential delivery vehicle for biologically active molecules. In fact, provided a drug is tethered to the ON complementary to the ONA, the drug release could be triggered either by a competitive RNA or DNA (or proteins if the ONA features an aptameric ON) present in the targeted organs or by temperature. The latter approach could be advantageously applied in combination with ultrasound thermotherapy to release a molecule of interest in a regiocontrolled manner.[19] Interestingly, the temperature of release could be finely tuned simply by adjusting the sequence of the ON tag.

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