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## Mismatch discrimination of lipidated DNA and LNA-probes (LiNAs) in hybridization-controlled liposome assembly†

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Assays for mismatch discrimination and detection of single nucleotide variations by hybridization-controlled assembly of liposomes, which do not require tedious surface chemistry, are versatile for both DNA and RNA targets. We report herein a comprehensive study on different DNA and LNA (locked nucleic acids) probe designs, including membrane-anchoring requirements, studies on different probes and target lengths (including overhangs), DNA and RNA targets (including sequences associated with pathogens) for lipidated nucleic acids (LiNAs). Advantages and limitations of the liposome assembly based assay in the context of mismatch discrimination and SNP detection are presented. The advantages of membrane-anchored LiNA-probes compared to chemically attached probes on solid nanoparticles (e.g. gold nanoparticles) are described. Key functionalities such as non-covalent attachment of LiNA probes without the need for long spacers and the inherent mobility of membrane-anchored probes in lipid-bilayer membranes will be described for several different probe designs.

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## Introduction

The demand for low cost methods for the detection and analysis of known disease related genetic markers has been increasing steadily as an ever growing number of disease associated genetic variations are reported. This requires additional detection methods, which do not rely on sequencing of DNA or RNA. Genetic variations such as single nucleotide substitutions, insertions and deletions are associated with a wide range of diseases, such as cancer,<sup>1–4</sup> venous thrombosis,<sup>5</sup> alpha-1-antitrypsin deficiency,<sup>6</sup> sickle cell anemia,<sup>7–9</sup> Parkinson's disease,<sup>10–12</sup> Bardet-Biedl syndrome,<sup>13</sup> diabetes,<sup>14</sup> arthritis,<sup>15,16</sup> and phenylketonuria.<sup>17</sup> Detection methods for genetic variations based on hybridization include e.g. dynamic allele specific hybridization (DASH),<sup>18,19</sup> TaqMan<sup>20–22</sup> and molecular beacons,<sup>23</sup> and also nanoparticle based methods have been reported. Among a broad range of nanoparticles, gold nanoparticles have proven to be very useful in assays for the detection of genetic variations. Typically, short oligonucleotides

(e.g. 15 nucleotides in the base pairing region) with different nucleic acid sequences covalently attached to different batches of gold nanoparticles have been used as assay for the detection of single nucleotide variations by aggregation of gold nanoparticles as the primary readout.<sup>24–26</sup> Despite the success of gold nanoparticle systems some challenges, intrinsic to solid nanoparticles, remain. Chemistry on solid nanoparticle surfaces, while well established for gold, is often tedious and generally not applicable for other inorganic materials. This is fundamentally different from lipid based soft nanoparticles, which possess lipid bilayer surfaces. Lipid bilayers allow strong non-covalent attachment of lipid-modified nucleic acid (LiNA) probes by membrane anchoring without chemical modifications on the surface and with full lateral probe mobility in the lipid membrane.<sup>27–29,43–45,56,57,64,67–69</sup>

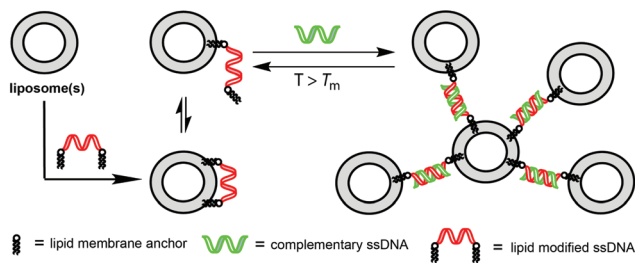
Herein, we report detection of single nucleotide variations by assembly of liposomes using membrane anchored DNA probes. The report is focused on DNA probe design and its impact on liposome assembly and subsequently on potential applications and limitations (e.g. DNA target size) for detection of single nucleotide variations. The general principle of the DNA-controlled assembly of liposomes has been reported<sup>27–29,56</sup> and is shown schematically in Fig. 1. Lipophilic membrane anchors attached at each end of a DNA probe strand adhere reversibly to the surface of liposomes when mixed with these. When a complementary oligonucleotide target strand is added, the resulting duplex is too rigid for both the membrane anchors to be anchored in the same liposome and one of the ends will be released during the

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**Fig. 1** Schematic representation of liposome assembly controlled by duplex formation. DNA strands (red and green ribbons) and liposomes (grey circles) are not drawn to scale.

hybridization process and anchor into another liposome, resulting in the rapid formation of liposome aggregates. The liposome assembly is a reversible process and the liposome aggregates will disassemble when heated to a temperature above the thermal denaturation temperature ( $T_m$ ) of the duplex linking the liposomes. Liposome aggregates scatter light considerably more than individual liposomes (as they are effectively larger particles) and the assembly/disassembly process can easily be monitored by *e.g.* dynamic light scattering (DLS), nanoparticle tracking analysis (NTA) or UV-spectroscopy at different wavelengths.

When the disassembly process is followed by UV-spectroscopy, the scattering of light by the liposome aggregates causes the apparent absorbance to be relatively large compared to the apparent absorbance of the individual liposomes after disassembly. For this reason the thermal denaturation curves ( $\Delta_{\text{abs}}$  versus  $T$ ) are inversed as compared to conventional  $T_m$ -experiments,<sup>27,28</sup> which monitor the change in absorbance of the nucleobases during duplex denaturation.<sup>30</sup> The change in signal intensity from the assembled to disassembled state is significantly increased compared to conventional  $T_m$ -experiments, which enables detection of significantly lower oligonucleotide concentrations.<sup>27,28</sup>

Additionally, the change in the apparent absorbance on disassembly of the liposome aggregates gives rise to very sharp thermal transitions (2–4 °C thermal window) as compared to conventional thermal denaturation experiments (10–20 °C thermal window).<sup>27,28</sup> The sharp thermal transitions are attributed to bundles of DNA-duplexes linking each pair of nano-

particles and the change in the local salt concentration upon melting of the DNA-strands as reported for DNA-functionalized gold-nanoparticles.<sup>31</sup> Compared to other systems consisting of oligonucleotides covalently linked to solid nanoparticles,<sup>24,25,32,33</sup> the DNA-controlled liposome aggregation does not require any chemical surface modification or subsequent purification. The surface loading can be controlled simply by changing the amount of oligonucleotide added and the oligonucleotides distributed freely over the liposome surface without steric crowding or hindrance between the individual oligonucleotides. Sharp melting transitions have been used to distinguish unmodified target strands with just one lesion (mismatch, insertion or deletion) from a matched strand at low nanomolar concentrations of DNA, even when the difference in melting temperature ( $\Delta T_m$ ) between the matched and mismatched duplex is small.<sup>27,28</sup> The ability of the liposome assembly based detection methods to distinguish between a matched target strand, in particular for weakly discriminating single mismatches, has to date not been studied.

## Results and discussion

In this study the DNA probe design has been varied to investigate the effect of different membrane anchor moieties, different hybridization schemes (*e.g.* DNA split probes), the application of non-natural building blocks (*e.g.* LNA – locked nucleic acid), different linker moieties and lengths on a number of DNA and RNA targets in the context of single nucleotide variation detection. The data presented here includes mismatches, insertions and deletions (for sequence details see Table 1 and ESI Tables S1–S25† including  $T_m$ -data for all systems). An aza crown ether with two palmitoyl or cholesterol substituents (**X** or **Y**, see Fig. 2) has been used as a membrane anchor which allows multiple incorporations anywhere in the sequence and has shown strong probe anchoring,<sup>34,35</sup> the concentration of oligonucleotides (both modified and unmodified strands) was 62 nM (*i.e.* a 16 times lower concentration than the 1.0  $\mu\text{M}$  used for regular  $T_m$ -measurements of oligonucleotides) unless noted otherwise, the liposomes had a diameter of 65 nm and the samples contained an amount of liposomes corresponding to 0.5 mM of 1-palmitoyl-2-oleoyl-*sn*-

**Table 1** DNA sequences of probes and targets containing mismatches, insertions and deletions. “\_” denotes a deleted nucleotide. X denotes the lipid anchor monomer

Probe	ON1 <sup>X</sup> 5'-TTT <sup>X</sup> TGTGGAAGAAGTTGGTG <sup>X</sup> TTT	DNA	ON2 <sup>X</sup> 5'-TTT <sup>X</sup> CACCAACTTCTTCCAC <sup>X</sup> TTT <sup>27</sup>	DNA
Match	3'-ACACCTTCTTCA <sup>A</sup> CCAC	1	3'-GTGGTTGAAGAA <sup>G</sup> GTGT	8
T-mismatch	3'-ACACCTTCTTCAT <sup>T</sup> CCAC	2	3'-GTGGTTGAAGAA <sup>A</sup> TGTGT	9
G/A-mismatch	3'-ACACCTTCTTCA <sup>G</sup> CCAC	3	3'-GTGGTTGAAGAA <sup>A</sup> GTGT	10
C-mismatch	3'-ACACCTTCTTCA <sup>C</sup> CCAC	4	3'-GTGGTTGAAGAA <sup>C</sup> GTGT	11
Terminal mismatch	3'- <sup>T</sup> CACCTTCTTCA <sup>A</sup> CCAC	5	3'-GTGGTTGAAGAA <sup>G</sup> GTG <sup>A</sup>	12
Deletion	3'-A <sup>_</sup> ACCTTCTTCA <sup>A</sup> CCAC	6	3'-G <sup>_</sup> GGTTGAAGAA <sup>G</sup> GTGT	13
Insertion	3'-ACAT <sup>C</sup> CCTTCTTCA <sup>A</sup> CCAC	7	3'-GTG <sup>A</sup> GTGAAGAA <sup>G</sup> GTGT	14



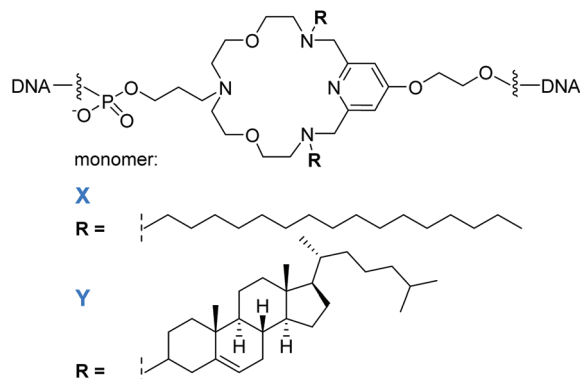


Fig. 2 Membrane anchor structure with palmityl (X) or cholesteryl (Y) moieties.

glycero-3-phosphocholine (POPC). Measurements were performed in 10 mM HEPES buffer, 110 mM Na<sup>+</sup> at pH 7.0 for all reported experiments. In Table 1 examples of the typical LiNA probes and the standard set of unmodified counter strands tested, as previously described for palmityl-anchored sequences (ON1<sup>X</sup> and ON2<sup>X</sup>),<sup>27</sup> are shown. The primary sequence design has been based on two complementary DNA sequences with an even distribution of the four bases to avoid sequence specific effects. A full list of all oligonucleotide sequences described here can be found in the ESI (Tables S24 and S25†). All LiNA probes used in this study have been modified with lipids at the terminal ends followed by three additional (non-hybridizing) thymidines to prevent (self)-aggregation of the otherwise surface active and amphiphilic DNA probes in aqueous solution.

### Palmityl-modified DNA probes

*T<sub>m</sub>*-Measurements without liposomes at a DNA concentration of 1.0 μM showed that the two palmityl-modified strands (ON1<sup>X</sup> and ON2<sup>X</sup>) were able to form duplexes with all tested target strands (Fig. S1†), and neither the modification nor the lesions affected the duplex forming ability of the oligonucleo-

tides (Fig. S1†). In measurements with liposomes, all duplexes, regardless of the sequence of the modified target strand and the lesion in the duplex, were able to induce liposome assembly (Fig. 3 and ESI Table S3†).

All internal single nucleotide mismatches from the matched duplex for both targets have been distinguished, even when the  $\Delta T_m$  was modest as for the G–T mismatch (3.5 °C for the G–T mismatch in Fig. 3a) which is known to form a wobble pair.<sup>36–40</sup> The relative stabilities of the studied mismatches are consistent with the literature.<sup>41</sup> A deletion or an insertion of a single nucleotide could easily be distinguished, even though the destabilization caused by these lesions was generally smaller than for the internal mismatches (Fig. 3). However, for neither of the target sequences could the terminal mismatch be distinguished from the matched duplex, but this was expected, as the *T<sub>m</sub>* of the duplex formed with this target strand is the same as the *T<sub>m</sub>* of the matched duplex (ESI Table S3†).

### Cholesteryl-modified DNA probes

The DNA probe design with cholesteryl (Y) moieties (Fig. 2) has been described by us,<sup>28,34</sup> but no *T<sub>m</sub>*-studies on mismatch discrimination with or without liposomes have been reported to date. The DNA probe with the same sequence as ON1<sup>X</sup> but containing Y at both ends (ON1<sup>Y</sup>) was found to induce liposome assembly on par with the equivalent palmityl-modified DNA probe (Fig. 4 and ESI Table S4†) and showed similar mismatch discrimination as seen for the palmityl-modified sequence (ON1<sup>X</sup>, Fig. 3a).

### DNA probes with increased membrane anchoring strength

We have previously shown that a single palmityl chain is not sufficient to anchor a DNA probe strongly enough to a POPC-membrane to function as a membrane anchor in the DNA-controlled assembly of liposomes.<sup>27,28</sup> As reported by Höök *et al.* for cholesteryl-modified oligonucleotides, two cholesteryl moieties are needed to anchor an oligonucleotide irreversibly (meaning practically no partitioning of the DNA probe between the lipid bilayer and the aqueous phase) to a POPC membrane,<sup>43</sup> as the anchoring of only one cholesteryl moiety

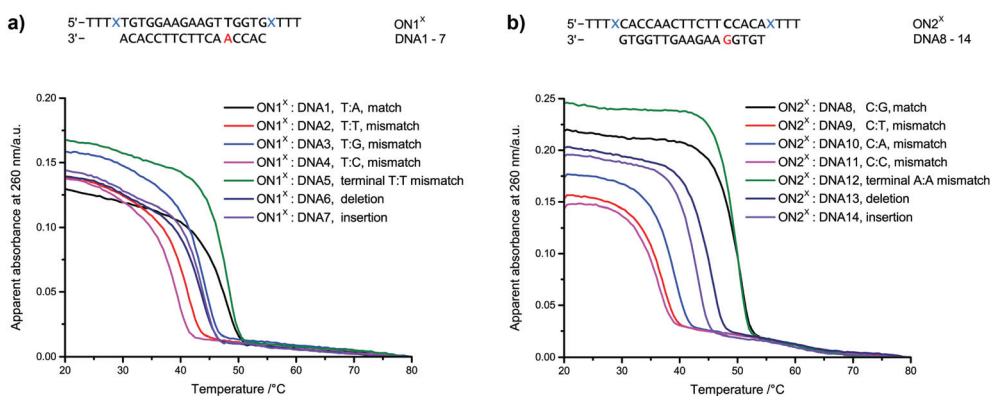


Fig. 3 Thermal dissociation curves for liposomes functionalized with LiNA in the presence of unmodified DNA target strands. The position of the internal mismatch is indicated in red and the membrane anchor in blue.

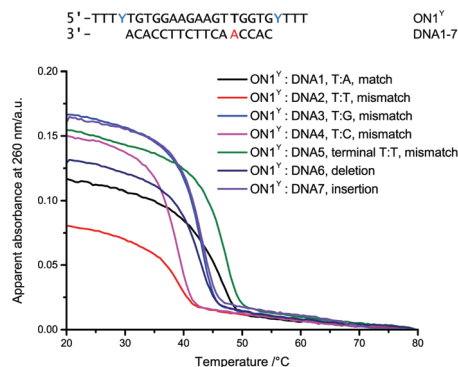


Fig. 4 Thermal dissociation curves for liposomes functionalized with cholesterol-modified LNA in the presence of unmodified DNA target strands (positions of lipid membrane anchors are indicated in blue and mismatches in red).

is too weak for permanent anchoring.<sup>44,45</sup> We therefore assumed that an even stronger anchored DNA probe with membrane anchors consisting of a total of four palmitoyl chains at one end of the oligonucleotide will irreversibly anchor the respective end into the lipid membrane (Fig. 5).

DNA-controlled assembly of liposomes was investigated for DNA probes with two X modifications in the 5'-end (**ON1**<sup>2X</sup> and **ON2**<sup>2X</sup>, Fig. 6a and b). In this design, the 5'-end of the probe is irreversibly bound to the liposomes, but the 3'-end is still reversibly bound and should be able to be released and promote liposome assembly. Despite the highly enforced anchoring and restricted motion out of the membrane (in the z-direction) of **ON1**<sup>2X</sup> and **ON2**<sup>2X</sup>, liposome assembly was indeed observed (Fig. 6a and b and ESI Table S7†) and clearly showed that it is sufficient if only one of the ends (here the 3'-end) of the modified oligonucleotide is able to leave the liposome surface. The insertion of two membrane anchors at the 5'-end does not affect the stability of the duplex as all DNA duplex melting transitions occurred at the same temperatures as observed for oligonucleotide probes with only one lipophilic modification at both ends (Tables S3 and S7 in the ESI†).

### RNA targets

RNA strands can be used as target strands as well and show similar mismatch discrimination as seen for the corres-

ponding DNA sequences (Fig. 6c and d and ESI Table S6†). As expected the matched duplex and the duplex with the terminal mismatch are difficult to distinguish for the same reason as mentioned for DNA targets.<sup>36–40,42</sup>

### LNA-modified DNA probes

Locked nucleic acids (LNA)<sup>46,47</sup> are an important class of non-natural nucleotides which are known to increase the  $T_m$  of a duplex when incorporated into oligonucleotides.<sup>46</sup> Incorporation of LNA changes the structure of the corresponding DNA duplex towards a more RNA type duplex, depending on the number of LNA modifications.<sup>48</sup> The mismatch discriminating power of duplexes consisting of one DNA/LNA and one DNA strand has previously been shown to be similar or better than for duplexes consisting of only DNA,<sup>49</sup> but the effect is dependent on a number of factors, including oligonucleotide sequence, strand length, mismatch type, and LNA position.<sup>50</sup> Moreover, for a system consisting of two oligonucleotides covalently attached to gold nanoparticles, an increased mismatch discrimination has been shown when DNA/LNA chimeras were used.<sup>51</sup> Based on the importance of LNA modifications in current oligonucleotide probes, a number of LNA thymidines were incorporated into two sequences (**ON1**<sup>LNA</sup> and **ON2**<sup>LNA</sup>) and the resulting oligonucleotides were tested in the presence of liposomes. All thermal transitions for liposome aggregate disassembly occurred at a higher temperature compared to **ON1**<sup>X</sup> and **ON2**<sup>X</sup> as expected for LNA-modified probe strands due to the increased thermal stability of the duplex (Fig. 6e and f and ESI Table S8†). The  $T_m$  values of all tested duplexes were increased by approximately the same degree (6–9 °C) resulting in a similar or moderately improved ability to detect SNPs compared to probe strands without LNA (Fig. 3). A series of  $T_m$ -experiments with different LNA probe concentrations was conducted to determine possible improvements in signal readout (larger difference in the apparent absorbance upon disassembly) and the effects of LNA modifications on the overall assembly/disassembly. LNA modified **ON1**<sup>LNA</sup> and **ON2**<sup>LNA</sup> were compared to the corresponding sequences without LNA (**ON1**<sup>X</sup> and **ON2**<sup>X</sup>) by recording  $T_m$ -curves with liposomes and probe concentrations of 12.5, 25 and 50 nM DNA (Fig. 7 and ESI Table S9†). Introduction of LNAs increased the  $T_m$  for both strands tested, but the intensity ( $\Delta_{abs}$ ) of the thermal transitions was not significantly altered. The results are in good agreement with our studies on mismatched duplexes, where duplexes with lower  $T_m$  compared to matched strands induced similar levels of liposome aggregation. Results for both parameters (LNA and single mismatches) indicate that differences in thermal duplex stability do not affect the amount of liposome assembly observed.

### Probe concentration dependence of hybridization-controlled assembly of liposomes

A lower concentration of oligonucleotides effectively gave a lower surface density of lipid-anchored oligonucleotides, since the amount of liposomes remained constant. Lower probe concentrations resulted in lower dissociation temperatures for the

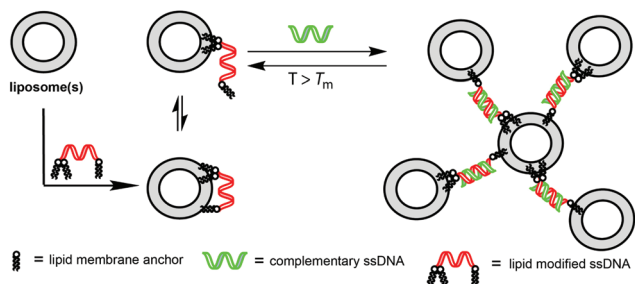
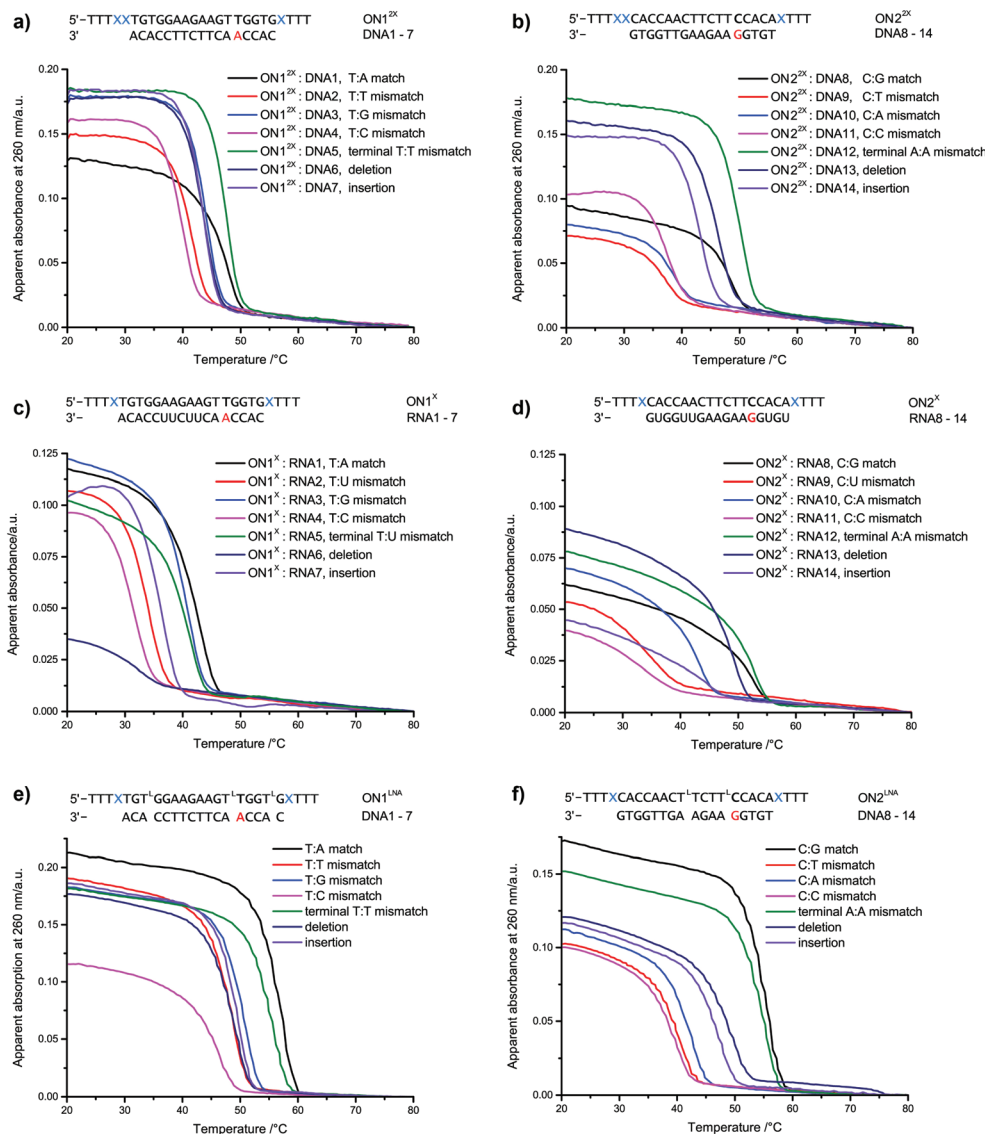


Fig. 5 Schematic illustration of liposome assembly with one end of the DNA probe strand permanently anchored.







**Fig. 6** (a and b) Thermal dissociation curves for liposomes functionalized with triple-modified LiNA strands in the presence of unmodified DNA target strands. (c and d) Thermal dissociation curves for liposomes functionalized with LiNA in the presence of unmodified RNA target strands. (e and f) Thermal dissociation curves for liposomes functionalized with LNA-modified LiNA strands in the presence of unmodified DNA target strands (lipid membrane anchors are indicated in blue and mismatches in red, LNA thymidines are denoted as T<sup>L</sup>).

assemblies and in broader transitions, *i.e.* the assembly is concentration dependent (Fig. 7). These results are consistent with data obtained for oligonucleotide-modified gold nanoparticles<sup>31</sup> as well as with the suggested mechanism for the aggregation of lipid vesicles reported by Beales *et al.*<sup>45</sup> To quantify the number of LiNAs necessary for interliposomal linkage, we compared our data on the concentration dependence of liposome assembly with the results from Beales *et al.*<sup>45</sup> The study reports visible liposome aggregate precipitation at an average of 155 cholesteryl-modified oligonucleotides per liposome.

The same observation, albeit with slower kinetics, was made for 39 oligonucleotides per liposome, whereas a smaller amount of aggregation was observed for 19 oligonucleotides

per liposome and no significant aggregation was observed for 2.5 oligonucleotides per liposome. For our assays, an average of only 4 oligonucleotides per liposome (corresponding to 62.5 nM DNA) was used, and the highest amount tested was 16 oligonucleotides per liposome (250 nM DNA), *i.e.* the lowest amount is well below the amounts used by others for effective aggregation and measured by a standard UV spectrophotometer system (Cary Varian 300 Bio). We assume that a larger percentage of oligonucleotides attaches to the first fraction of liposomes encountered upon mixing, and some liposomes have therefore considerably more oligonucleotides on their surface while other liposomes have fewer or none resulting in a non-homogeneous LiNA distribution.



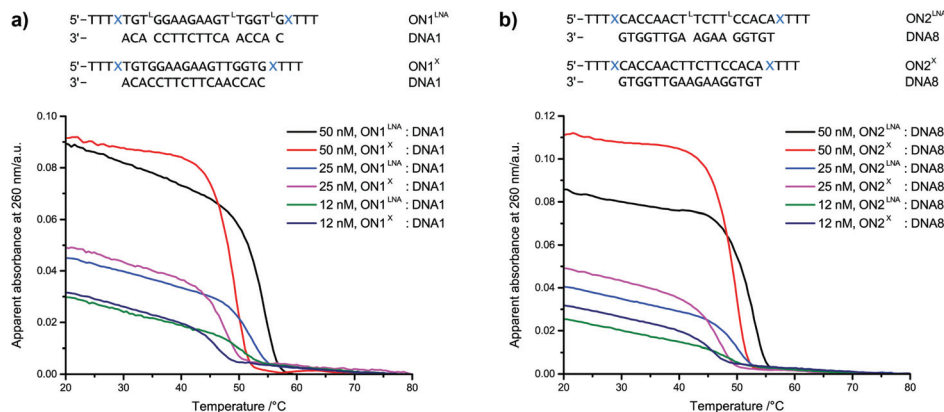


Fig. 7 Concentration dependence of thermal dissociation curves for liposomes functionalized with LNA-modified LiNA probe strands in the presence of unmodified DNA target strands (lipid membrane anchors are indicated in blue and mismatches in red, LNA thymidines are denoted as T<sup>L</sup>).

### DNA probe designs

A number of DNA probe designs for hybridization controlled assembly and mismatch discrimination studies in combination with solid nanoparticles are known.<sup>51–55</sup> In order to test the applicability of our methodology to soft nanoparticles (*e.g.* liposomes) and non-covalent anchoring of the LiNAs, different probe designs were synthesized and tested in  $T_m$ -studies.

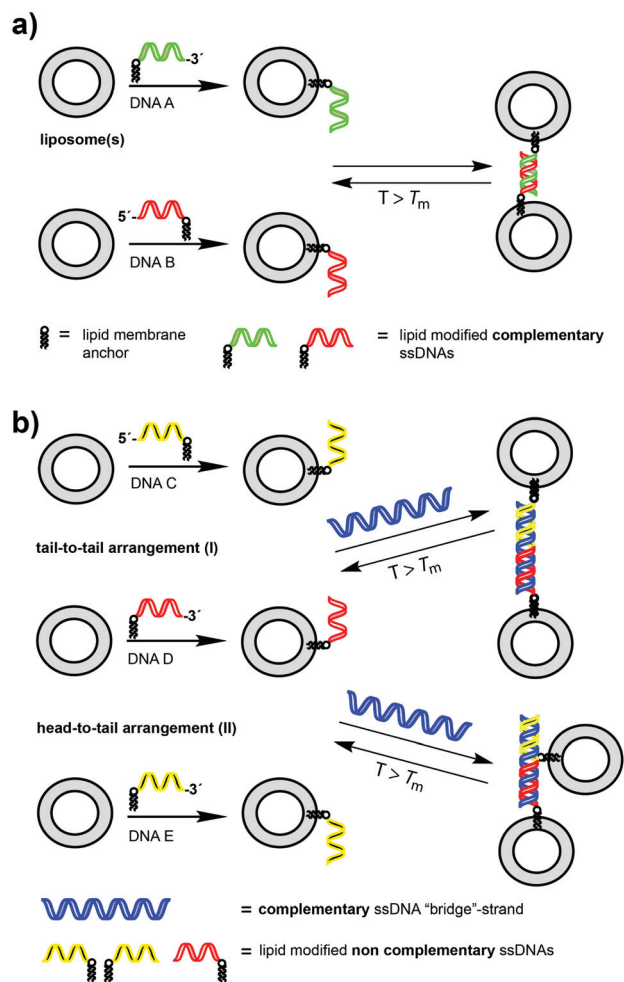
DNA probe designs with only one lipid-modified end (*i.e.* either the 3'- or the 5'-end) were able to initiate liposome aggregation upon hybridization (shown schematically in Fig. 8) as reported for *e.g.* oligonucleotides covalently attached to gold,<sup>51–53</sup> silver<sup>54</sup> and magnetic nanoparticles modified with complementary DNA and peptide nucleic acids (PNA) strands,<sup>55</sup> as well as for cholesterol-modified oligonucleotides attached to vesicles.<sup>45,56,65</sup> Even though all duplexes, whether modified in the 3'- or 5'-end, were able to hybridize (as seen from  $T_m$ -studies without liposomes at 1.0  $\mu$ M concentration, ESI Table S10†), oligonucleotides modified in the 5'-end were not able to induce liposome assembly for the systems investigated. The order of mixing components did not affect the outcome, and the same results were obtained regardless of whether the liposomes were mixed with both complementary strands before the  $T_m$ -experiment or the two strands were separately added to separate liposome batches and mixed afterwards. To verify that no assembly had occurred for the 5'-end modified oligonucleotides, the experiments were carried out with DNA probe concentrations of 62.5, 125 and 250 nM, but no transitions were observed even at the highest concentration, which is attributed to our probe design without spacers and relatively short interconnecting DNA. Liposome aggregation might be induced by longer DNA probe strands, which will allow the liposomes to be farther apart as well as have a stronger tether, by formation of more stable duplexes, but this has not been tested. Alternatively, a linker could be inserted between the membrane anchor and the DNA sequence, as the use of a linker (spacer) has been reported for the assembly of gold,<sup>52</sup> silver<sup>54</sup> and magnetic<sup>55</sup> nanoparticles (spacer effects are investigated and discussed later in this report). For LiNA

probes modified in the 3'-end very broad thermal transitions were seen (data not shown, for sequences see ESI Table S10†), indicating that liposome assembly occurred to some extent. The broad transitions are presumably due to a limited amount of assembly (effectively a “low concentration”). The same design was tested with LNA modified LiNA probes (ESI Table S11†) which, as expected, formed a duplex with a higher  $T_m$  in the absence of liposomes. However, only broad transitions were seen for LNA modified LiNA probes in measurements with liposomes, results corresponding to the DNA probes without LNA. The single-modified strands alone, duplexes consisting of single-modified strands and an unmodified strand as well as duplexes with the modifications juxtapositioned were also tested as references but as expected no transitions were seen (ESI Table S10†). The limited amount or absence of liposome assembly is in contrast to results reported for oligonucleotides modified with cholesterol, which have been reported to cause precipitation of vesicles due to aggregation.<sup>45,56</sup> However, these results were obtained at DNA concentrations around 40 times higher than the concentration used for the  $T_m$ -experiments in our study.<sup>45,56</sup>

### DNA split-probe designs

The bridging of two LiNA probes by a complementary target strand (DNA) represents another probe design for liposome assembly using LiNA probes (see Fig. 8b). Each LiNA probe has one membrane anchor and hybridizes to a target strand complementary to both probe strands, *i.e.* each modified oligonucleotide is complementary to half of the bridging target strand (Fig. 8b).<sup>28</sup> A similar design has been reported for the assembly of *e.g.* gold,<sup>25,26,51,58</sup> and silver nanoparticles<sup>59</sup> as well as quantum dots<sup>60</sup> with covalently attached oligonucleotides. For four different LiNAs, as described above (*i.e.* LiNAs with sequences such as ON1 and ON2 modified at either the 3'- or the 5'-end), there are only two possible combinations (the possibilities for complementary probe strands and the formation of hairpins were also tested and as expected no transitions were observed, ESI Table S13†). For both tail-to-tail (I)





**Fig. 8** Probe designs for liposome (grey circles) assembly using (a) complementary, single-modified DNA probes (red and green) or (b) non-complementary DNA probes (red and yellow) and a complementary bridging DNA strand (blue) in two different arrangements – tail-to-tail (I) or head-to-tail (II).

split-probe systems (I, Fig. 8b), the two modified strands have the same nucleic acid sequence (but modifications **X** were positioned at opposite ends of the strands).

Thermal transitions were observed for both systems (**ON3** + **ON4** + **DNA15** and **ON5** + **ON6** + **DNA16**, see Fig. 9), showing that liposome aggregation is induced by the split probes.

The ability of the three-strand systems (split-probe systems) to discriminate between matched and mismatched target strands was investigated using target strands with single nucleotide mismatches positioned as for the other mismatch studies described here. The triple-strand probe designs showed mismatch discrimination and all mismatched strands were distinguished from the matched complementary target strand (Fig. 9b and ESI Table S15†). It is of advantage that the two LiNA probe strands have the same sequence and thus the same  $T_m$ , as a mismatch for one of the strands always will cause the part of the duplex containing the mismatched

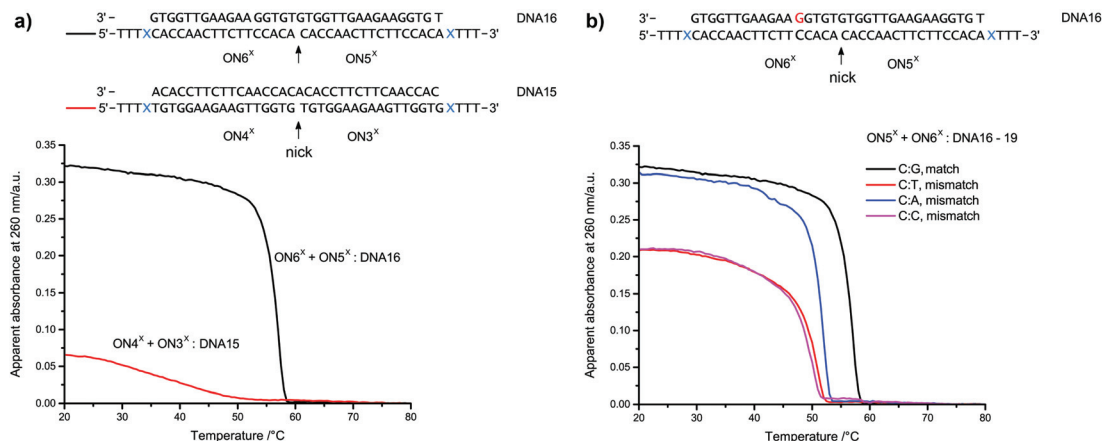
strand to have a lower  $T_m$  than the part with the matched probe, and thus the observed apparent  $T_m$  (dissociation temperature) is always a consequence of the presence of a mismatch in the target sequence. Finally a design with probe strands bearing modifications in the same end (e.g. the 5'-end) which could result in a "head-to-tail" arrangement of liposomes on the target strand (II, Fig. 8b) was investigated. However, no transitions were observed for this probe design (ESI Table S13,† entries 5 and 6), which is in contrast to observations for oligonucleotide-modified gold nanoparticles.<sup>31</sup>

### Influence of probe and target sequence length of liposome assembly on pathogen derived DNA sequences

Longer probe sequences and longer targets have been used to explore the limitations of liposome assembly in the context of longer DNA probes and target strands with overhangs of varying length (overhangs of 23 or 95 bp length). Initially a LiNA probe sequence (**ON11<sup>X</sup>**), associated with the anthrax lethal factor,<sup>61–63</sup> and its complementary sequence (**ON10<sup>X</sup>**), both containing 24 nucleotides in the base pairing region, were investigated. For both DNA probes, two targets of different length were tested; a target strand of equal length to the base pairing region of the DNA probe strands (24-mer) and two 119-mer targets with a region of 24 base pairs complementary to the probe sequences.

Both probe strands induced liposome assembly with the shorter target strands (Fig. S4 and ESI Table S20†), which shows the flexibility with respect to probe length. However, none of the probe strands was able to induce liposome aggregation with the 119-mer target strands at DNA concentrations of 62, 125 or 250 nM. This finding indicated that longer DNA probes are needed to accommodate the additional bulk of overhangs in the target sequences. We therefore synthesized two additional longer probe sequences related to the bacterium *Staphylococcus aureus*.<sup>66</sup> The probe strands contained 27 nucleotides in the base pairing region and were complementary to either a part of the *S. aureus* gene (**ON13<sup>X</sup>**) or a part of the *mecA* gene<sup>66</sup> causing methicillin-resistance in *S. aureus* (MRSA) (**ON12<sup>X</sup>**). The ability of these probe strands to initiate assembly with both 27-, 47- and 119-mer unmodified targets was investigated (overhangs of 20 or 92 bp length). Both probes gave sharp thermal transitions in the presence of liposomes and the 27-mer (equal length) target strands at a concentration of 62 nM (Fig. 10a, red curves and ESI Table S21†). Sharp thermal transitions were also observed for 47-mer target strands with overhangs (Fig. 10a, blue curves), showing equal efficiency as compared to targets of the same length. A thermal transition was only seen for one of the longer 119-mer targets at 62 nM DNA concentration (Fig. 10c, pink curve, target **DNA28**), albeit at a much lower intensity than transitions observed with shorter target strands (Fig. 10a). However, at 250 nM DNA, intense transitions were seen for both probes (Fig. 10b and Table S21†), showing that long target strands with more than 90 overhanging bases are able to trigger liposome assembly. However, DNA probe design is more demanding for longer sequences and targets. While the





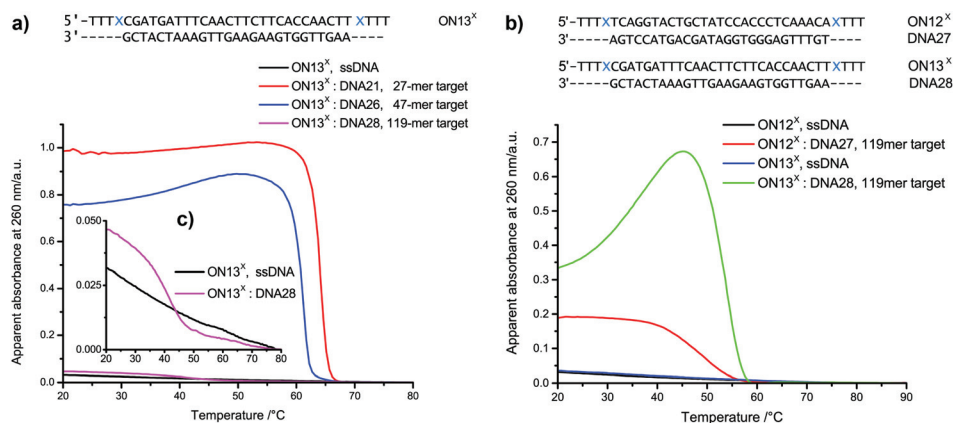
**Fig. 9** (a) Thermal dissociation curves for liposomes functionalized with two single-modified LiNA probes in the presence of one unmodified target strand (in a tail-to-tail arrangement I, see Fig. 8b) and (b) corresponding mismatch-discrimination data for the same DNA split-probe.

transition for the *S. aureus* probe (ON13<sup>x</sup>) occurred at a constant temperature, the observed transition for the MRSA-probe (ON12<sup>x</sup>) fluctuated within an interval of ~25 °C for multiple samples (with the same content, not shown) which limits the applicability of the assay for this particular DNA probe. The longer 27-mer probe strands thus allowed the use of target strands much longer than the complementary region of the probe.

The results for duplex structures as interliposomal linkages in comparison with the results for DNA and LNA triple helices, where overhangs in the target duplex were not tolerated,<sup>29</sup> show the importance of the overall linkage structure and charge density. The steric demands of overhangs are similar for dsDNA and triple helical DNA but overhangs are presumably easier to be accommodated between the liposomes and formed with less excessive local accumulation of negative charges and are conformationally more flexible than that seen for triple helices.<sup>29</sup>

Probe design is important and longer probe strands generally (27-mer *versus* 17-mer probes) allow detection of targets with longer overhangs. The longer probe ON12<sup>x</sup> was not only able to accommodate overhangs but also to distinguish between a complementary 47-mer target and target strands with single mismatches at target concentrations of 62 nM and 250 nM (Fig. 10 and ESI Table S23†).

Studies on the applicability of liposome assembly for the specific recognition of RNA targets with overhangs were less conclusive. The DNA probe (ON12<sup>x</sup>) was tested with the corresponding 27- and 47-mer RNA target strands (ESI Fig. S5 and Table S22†). The 27-mer target strand gave transitions within a broad interval (49–65 °C) as seen for the longest target strands but a transition was always seen, and the 47-mer target strand gave a transition at a lower temperature compared to the shorter target showing the destabilizing effect of overhangs for RNA targets. In conclusion, liposome assembly was observed for both RNA target strands also with overhangs in the target



**Fig. 10** (a) Thermal dissociation curves for liposomes functionalized with 27-mer LiNA probes associated with *Staphylococcus aureus* in the presence of unmodified DNA target strands (27-mer, 47-mer and 119-mer) at 62 nM DNA concentration. (b) Thermal dissociation curves in the presence of liposomes for 27-mer DNA probes associated with *Staphylococcus aureus* shown for both single strands alone and with 119-mer unmodified DNA-target strands at a concentration of 250 nM.





but with transition of lower intensity (apparent absorbance) and a larger reduction in thermal stability for the resulting interliposomal linkages. For all measurements the second cycle during thermal denaturation is shown (to show reproducibility and allow the soft nanoparticle with the surface attached LiNA system to equilibrate).

For many of the systems reported differences in apparent absorbance for closely related systems are observed (Fig. 3–6, 8 and 9), the observed differences are partially attributed to different kinetics of assembly. We assume that matched sequences and probe–target combinations with terminal mismatches hybridize faster and more efficiently compared to sequences with internal mismatches and are thermodynamically more stable. In addition to the kinetic and thermodynamic arguments also differences in effective oligonucleotide concentration may affect the apparent absorption. The problem is caused by the inherent uncertainty of OD measurements for oligonucleotides which is based on the absorption at 260 nm. However, the  $T_m$  is not affected strongly by differences in apparent absorbance and in all cases the expected destabilization by mismatches has been in full agreement with the literature and control experiments without liposomes.

## Conclusions

Hybridization controlled assembly of liposomes by LiNAs enables mismatch discrimination in thermal denaturation assays at low nanomolar concentrations in a setup which only requires a lipid label ( $\geq 10$  nM). The readout (apparent absorbance) for nanomolar target concentrations is an order of magnitude higher than for normal  $T_m$ -experiments at 1  $\mu$ M despite the 16 times lower DNA probe and target concentrations. The assay is, in addition to being compatible with a range of matrices (e.g. serum, urine), not sensitive to the presence of related sequences since most mismatches lower the corresponding  $T_m$  values considerably and can easily be distinguished or ultimately no assembly is observed for several mismatches present in the same target.<sup>28,29</sup> The simplicity of the method enables transfer of the method to polymer cartridges (lab-on-a chip) by immobilization of both the liposomes and membrane-anchored LiNAs by freeze drying. The wavelength independence of absorbance measurements allows both UV and VIS readout which would allow measurements of strongly absorbing samples with a reference cell on the same polymer cartridge. The method is not as sensitive as fluorescence based methods but has the advantage of being insensitive to the sample matrix (e.g. serum or salts). Several DNA probe designs have been successfully applied and all of them, except for the 5'- "head-to-tail" design, are capable of initiating liposome assembly at nanomolar target concentrations with similar readout intensity (apparent absorption). Most probe designs allow mismatch discrimination of weakly discriminating sequences enabled by remarkably sharp thermal transitions with the limitation, known for most  $T_m$  based assays,

that mismatches which do not affect the  $T_m$  are not discriminated. Both DNA and, with restrictions on length, also RNA targets are recognized and mismatches discriminated, both for targets with matching length and targets with up to 92 nucleobases in sequence overhangs. However, for the longest target sequences higher concentrations were necessary to achieve the same level of liposome assembly. The reported assay can easily be combined with important commercially available nucleic acid building blocks like LNA and no particular sequence context is required for efficient probe design. Both short linkers (spacers) in the probe strand and partially complementary targets with up to four non-complementary nucleobases are tolerated with a similar readout intensity. The versatile liposome assembly strategy can be monitored at different wavelengths and no tedious surface chemistry is needed for probe attachment to liposome nanoparticles. Multiple probes are easily attached and the probe ratio is conveniently controlled by the applied probe concentrations. Attachment of probes to the lipid bilayer nanoparticle surface is feasible without the use of long linkers, since LiNA probes redistribute after membrane-anchoring and show largely unrestricted hybridization due to the inherent lateral mobility of membrane-anchored probes in lipid-bilayer membranes. The method would be particularly useful as a low cost test in the context of environmental water samples with high concentrations of bacterial pathogens with known DNA sequences.

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