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Lipid Domain Specific Recruitment of Lipophilic Nucleic Acids: A Key for Switchable Functionalization of Membranes

Martin Loew,[†] Ralph Springer,[‡] Silvia Scolari,[†] Frank Altenbrunn,[‡] Oliver Seitz,[‡] Jürgen Liebscher,[‡] Daniel Huster,[§] Andreas Herrmann,[†] and Anna Arbuzova*[,†]

Institute of Biology/Biophysics, Humboldt-University Berlin, Invalidenstrasse 42, 10115 Berlin, Germany, Institute of Chemistry, Humboldt-University Berlin, Brook-Taylor-Strasse 2, 12489 Berlin, Germany, and Institute of Medical Physics and Biophysics, University of Leipzig, Härtelstrasse 16-18, 04107 Leipzig, Germany

Received June 29, 2010; E-mail: arbuzova@cms.hu-berlin.de

Abstract: Lipid domains in mammalian plasma membranes serve as platforms for specific recruitment or separation of proteins involved in various functions. Here, we have applied this natural strategy of lateral separation to functionalize lipid membranes at micrometer scale in a switchable and reversible manner. Membrane-anchored peptide nucleic acid and DNA, differing in their lipophilic moieties, partition into different lipid domains in model and biological membranes. Separation was visualized by hybridization with the respective complementary fluorescently labeled DNA strands. Upon heating, domains vanished, and both lipophilic nucleic acid structures intermixed with each other. Reformation of the lipid domains by cooling led again to separation of membrane-anchored nucleic acids. By linking appropriate structures/functions to complementary strands, this approach offers a reversible tool for triggering interactions among the structures and for the arrangement of reactions and signaling cascades on biomimetic surfaces.

Introduction

Lipophilic conjugates of oligonucleotides (DNA, RNA) and peptide nucleic acids (PNA) represent versatile tools for numerous applications in medicine and biotechnology. For example, they have been used for enhancing gene delivery and gene silencing by siRNA and antisense PNA.[1−7] By exploiting the self-assembly properties of complementary oligonucleotides, lipophilic nucleic acids allow controlled targeting and immobilization of specific molecules and liposomes to membranes and silica beads.[8−16] Cells can be guided to form three-dimensional microtissues either by using lipophilic oligonucleotides[17] or by oligonucleotides covalently attached to the surface of cells.[18]

As these examples demonstrate that lipophilic oligonucleotides and PNAs can efficiently target and functionalize membranes, applications could be strongly expanded if they could be recruited to specific lipid domains. Cell membranes, in particular mammalian plasma membranes, have been shown to form lipid domains, so-called rafts.[19] Rafts are small domains with a size of 10−100 nm[20] that are enriched in cholesterol (Chol), sphingomyelin (SM), and saturated phospholipids, while nonraft domains are formed essentially from unsaturated phospholipids. When composed from the appropriate lipid mixtures, model membrane systems as giant unilamellar vesicles (GUVs) form liquid-ordered (lo) and liquid-disordered (ld) domains in the micrometer range,[21] the lo domains mimicking the rafts.[22] Many membrane proteins partition preferentially either into rafts or into nonraft domains, depending on their (trans)membrane

[†] Institute of Biology/Biophysics, Humboldt-University Berlin.
[‡] Institute of Chemistry, Humboldt-University Berlin.
[§] University of Leipzig.

(1) Soutschek, J.; et al. *Nature* **2004**, *432*, 173–178.
(2) Lorenz, C.; Hadwiger, P.; John, M.; Vornlocher, H. P.; Unverzagt, C. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 4975–4977.
(3) Wolfrum, C.; Shi, S.; Jayaprakash, K. N.; Jayaraman, M.; Wang, G.; Pandey, R. K.; Rajeev, K. G.; Nakayama, T.; Charrise, K.; Ndungo, E. M.; Zimmermann, T.; Koteliansky, V.; Manoharan, M.; Stoffel, M. *Nat. Biotechnol.* **2007**, *25*, 1149–1157.
(4) Godeau, G.; Staedel, C.; Barthelemy, P. *J. Med. Chem.* **2008**, *51*, 4374–4376.
(5) Koppelhus, U.; Nielsen, P. E. *Adv. Drug Delivery Rev.* **2003**, *55*, 267–280.
(6) Koppelhus, U.; Shiraishi, T.; Zachar, V.; Pankratova, S.; Nielsen, P. E. *Bioconjugate Chem.* **2008**, *19*, 1526–1534.
(7) Hu, J.; Corey, D. R. *Biochemistry* **2007**, *46*, 7581–7589.
(8) Benkoski, J. J.; Höök, F. *J. Phys. Chem. B* **2005**, *109*, 9773–9779.
(9) Yoshina-Ishii, C.; Miller, G. P.; Kraft, M. L.; Kool, E. T.; Boxer, S. G. *J. Am. Chem. Soc.* **2005**, *127*, 1356–1357.
(10) Kurz, A.; Bunge, A.; Windeck, A. K.; Rost, M.; Flasche, W.; Arbuzova, A.; Strohbach, D.; Müller, S.; Liebscher, J.; Huster, D.; Herrmann, A. *Angew. Chem., Int. Ed.* **2006**, *45*, 4440–4444.
(11) Bunge, A.; Loew, M.; Pescador, P.; Arbuzova, A.; Brodersen, N.; Kang, J.; Dähne, L.; Liebscher, J.; Herrmann, A.; Stengel, G.; Huster, D. *J. Phys. Chem. B* **2009**, *113*, 16425–16434.
(12) Beales, P. A.; Vanderlick, T. K. *J. Phys. Chem. B* **2009**, *113*, 13678–13686.
(13) Loew, M.; Kang, J.; Dähne, L.; Hendus-Altenburger, R.; Kaczmarek, O.; Liebscher, J.; Huster, D.; Ludwig, K.; Böttcher, C.; Herrmann, A.; Arbuzova, A. *Small* **2009**, *5*, 320–323.
(14) Jakobsen, U.; Simonsen, A. C.; Vogel, S. *J. Am. Chem. Soc.* **2008**, *130*, 10462–10463.
(15) Stengel, G.; Simonsson, L.; Campbell, R. A.; Höök, F. *J. Phys. Chem. B* **2008**, *112*, 8264–8274.
(16) Chan, Y. H.; van Lengerich, B.; Boxer, S. G. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 979–984.
(17) Borisenko, G. G.; Zaitseva, M. A.; Chuvilin, A. N.; Pozmogova, G. E. *Nucleic Acids Res.* **2009**, *37*, e28.
(18) Gartner, Z. J.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 4606–4610.
(19) Simons, K.; Ikonen, E. *Nature* **1997**, *387*, 569–572.
(20) Lingwood, D.; Simons, K. *Science* **2010**, *327*, 46–50.

 10.1021/ja105714r

anchors.[23] By recruiting specific membrane proteins, domains are supposed to play an important role in many cellular functions such as signal transduction and endocytosis.[20,24,25]

Several efforts have recently been made to modulate lipid domain partitioning of lipophilic oligonucleotides using tocopherol and cholesterol-modified DNA.[10–12] Until now, however, a lipophilic oligonucleotide or PNA, which partitions exclusively into the lo phase, has not yet been described. To force partitioning into the lo phase, we took lessons from nature. Many membrane-associated proteins have distinct lipid modifications for their recruitment to specific membrane sites and, hence, for efficient regulation of their activity.[26] For example, palmitoylation plays a role in raft targeting.[27] As we are reporting, palmitoylation of DNA-recognizing PNA molecules paves the way to recruit nucleic acids exclusively to the lo phase. This allows combining the specific partitioning of lipophilic DNA/PNA molecules into defined membrane environments with the reversible temperature-dependent intermixing of laterally separated membrane domains. Two amphiphilic molecules capable of hybridizing with DNA were synthesized, one partitioning into the ld, the other into the lo phase of vesicles showing phase separation. Such two-sided vesicles are analogous to Janus particles[28] and Janus assemblies, for example, formed due to divalent cation-induced segregation of polyanionic amphiphiles,[29] and can be called Janus vesicles. Increasing the temperature leads to intermixing of lipid domains and, hence, of the initially separated lipophilic PNA and DNA constructs, while cooling restores separation. Janus vesicles could be used to trigger or abrogate reaction and signaling cascades at the membranes by mixing or separation, respectively, of lipophilic oligonucleotides/PNA carrying functional units on complementary strands.

Results and Discussion

Synthesis of Palmitoylated PNA. The palmitoylated PNA Pal-Lys(Pal)-Gly-Glu_2-Gly-ttcttctcctt-Glu_2-Gly-$CONH_2$ (PNA_C16), where Pal refers to the palmitoyl chain, was obtained by Fmoc-based solid-phase synthesis as described elsewhere[30,31] (Supporting Information, Synthesis and Figure S1). As the N-terminal amino acid we chose lysine, to which two palmitoyl groups were attached covalently via amide formation. We introduced four glutamic acid residues to improve the solubility of the lipidated PNA,[32] facilitating, thereby, labeling of preformed and cell (plasma) membranes.

PNA_C16 Incorporates into Phospholipid Membranes and Hybridizes with cDNA. GUVs were prepared from a mixture of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and PNA_C16 (molar ratio 300:1). After incubation with a stoichiometric amount of the complementary strand 5′-rhodamine-AAG GAG AAG AA-3′ (DNAc1) at room temperature for 10 min, GUV membranes showed a bright red fluorescence (Figure S2, Supporting Information). As no fluorescence on the vesicles was observed when a rhodamine-labeled noncomplementary oligonucleotide (5′-rhodamine-adenosine 25mer, Rh-A25) was added, we conclude that the lipophilic anchor of the PNA_C16 inserted into the lipid membrane, where the PNA sequence was accessible for hybridization with the DNAc1.

PNA_C16/DNAc1 Hybrids Insert into Cell Membranes. Insertion of PNA_C16/DNAc1 hybrids into membranes of Chinese hamster ovary (CHO-K1) cells was studied. PNA_C16 was mixed with a stoichiometric amount of DNAc1 in phosphate buffered saline (PBS), added to the cells, and incubated at 37 °C for 30 min. Confocal and differential interference contrast images of CHO-K1 cells after incubation with PNA_C16/DNAc1 hybrids are shown in Figure 1a and b, respectively. Rhodamine fluorescence is mainly localized to the plasma membrane, indicating that the PNA_C16/DNAc1 hybrids are inserted into the plasma membrane. As expected, some uptake of the membrane-incorporated PNA_C16/DNAc1 hybrids into endocytic vesicles was found after incubation at 37 °C (Figure 1a). In a control experiment, PNA_C16 was premixed with a noncomplementary rhodamine-labeled oligonucleotide (Rh-A25) and added to CHO-K1 cells. No fluorescence was observed either at the plasma membrane or inside the cells (Figure 1c and d). Therefore, we conclude that PNA_C16 (i) inserts into cell plasma membrane, PNA_C16 (ii) hybridizes specifically with the DNAc1, and (iii) the inserted PNA_C16/DNAc1 hybrids could be taken up by endocytosis. The inhomogeneous distribution of rhodamine fluorescence on the cell surface might be due to lateral segregation of PNA_C16/DNAc1 in rafts (see below) or accumulation in early endocytic vesicles.

Domain-Specific Partitioning of the PNA_C16/DNAc1 Hybrids in Model Membranes. Lipid vesicles composed of distinct lipid mixtures including Chol, saturated, and unsaturated lipids typically show well-defined domains at a specific temperature range.[33−35] For example, micrometer-scaled GUVs made from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), SM, and Chol show fluid−fluid phase separation into ld domains, consisting mainly of DOPC, and lo domains, enriched in Chol and SM.[36] GUVs consisting of a DOPC/SM/Chol mixture are often used as a model system for the plasma membrane, as the lo phase mimics lipid rafts due to the high Chol and SM content and the dense lipid packing.[22] Therefore, to address whether PNA_C16 has any preference for lipid domains, we studied the lateral distribution of PNA_C16 in GUVs prepared from a 1:1:1 (molar ratios) mixture of DOPC, *N*-stearoyl-D-*erythro*-sphingosylphosphorylcholine (SSM), and Chol. To distinguish between the lo and ld phases, a green fluorescent lipid analogue 1-palmitoyl-2-{6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (C6-NBD-PC) was used that

(21) Dietrich, C.; Bagatolli, L. A.; Volovyk, Z. N.; Thompson, N. L.; Levi, M.; Jacobson, K.; Gratton, E. *Biophys. J.* **2001**, *80*, 1417–1428.
(22) Brown, D. A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 10517–10518.
(23) Vogel, A.; Reuther, G.; Weise, K.; Triola, G.; Nikolaus, J.; Tan, K. T.; Nowak, C.; Herrmann, A.; Waldmann, H.; Winter, R.; Huster, D. *Angew. Chem., Int. Ed.* **2009**, *48*, 8784–8787.
(24) Hanzal-Bayer, M. F.; Hancock, J. F. *FEBS Lett.* **2007**, *581*, 2098–2104.
(25) Verma, S. P. *Curr. Drug Targets* **2009**, *10*, 51–59.
(26) Resh, M. D. *Sci. STKE* **2006**, *2006*, re14.
(27) Shogomori, H.; Hammond, A. T.; Ostermeyer-Fay, A. G.; Barr, D. J.; Feigenson, G. W.; London, E.; Brown, D. A. *J. Biol. Chem.* **2005**, *280*, 18931–18942.
(28) Wurm, F.; Kilbinger, A. F. *Angew. Chem., Int. Ed.* **2009**, *48*, 8412–8421.
(29) Christian, D. A.; Tian, A.; Ellenbroek, W. G.; Levental, I.; Rajagopal, K.; Janmey, P. A.; Liu, A. J.; Baumgart, T.; Discher, D. E. *Nat. Mater.* **2009**, *8*, 843–849.
(30) Köhler, O.; Jarikote, D. V.; Seitz, O. *ChemBioChem* **2005**, *6*, 69–77.
(31) Jarikote, D. V.; Köhler, O.; Socher, E.; Seitz, O. *Eur. J. Org. Chem.* **2005**, *2005*, 3187–3195.
(32) Marques, B. F.; Schneider, J. W. *Langmuir* **2005**, *21*, 2488–2494.
(33) Veatch, S. L.; Keller, S. L. *Biochim. Biophys. Acta* **2005**, *1746*, 172–185.
(34) Feigenson, G. W. *Biochim. Biophys. Acta* **2009**, *1788*, 47–52.
(35) Korlach, J.; Schwille, P.; Webb, W. W.; Feigenson, G. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 8461–8466.
(36) Veatch, S. L.; Keller, S. L. *Biophys. J.* **2003**, *85*, 3074–3083.

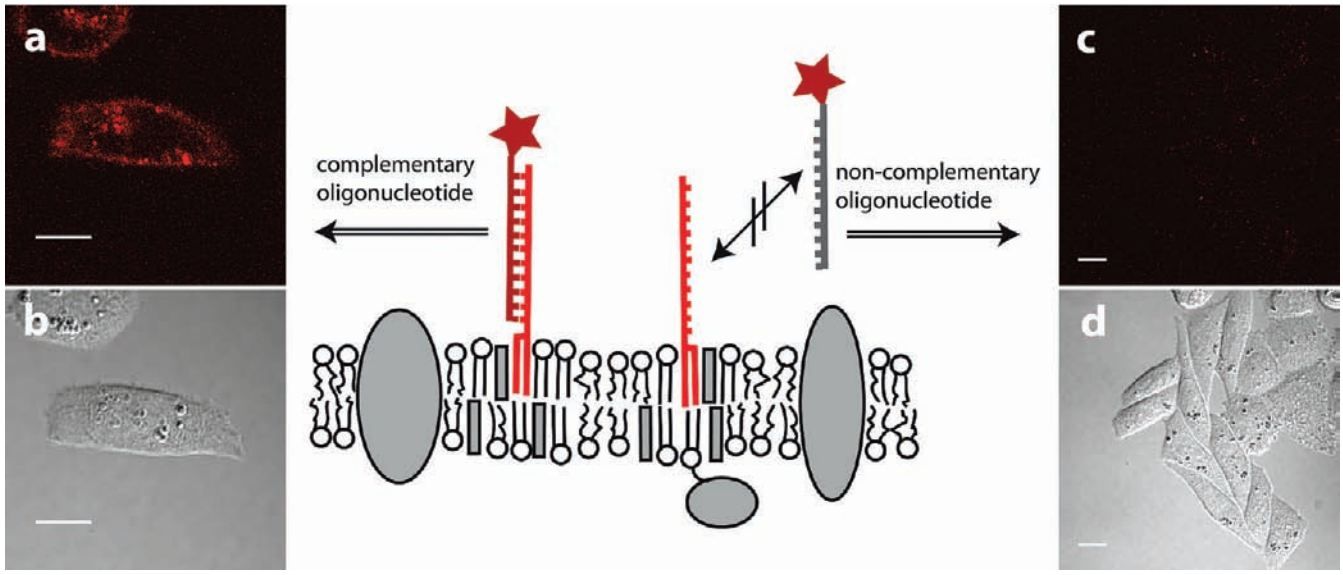

Figure 1. Palmitoyl-anchored PNA incorporates into the plasma membrane of CHO-K1 cells. A confocal fluorescence microscopy image (a) and a differential interference contrast image (b) show the localization of a complementary rhodamine-labeled oligonucleotide complementary to the PNA_C16. In contrast, a noncomplementary 5′-rhodamine-A25 oligonucleotide did not bind to PNA_C16, and no fluorescence could be detected at the cell membrane (c,d). Bars correspond to 10 μm. Cartoon (middle) summarizes the observations (gray rectangles: Chol).

preferentially segregates into the ld phase.[37] An equimolar mixture of PNA_C16 and DNAc1 was gently mixed with the vesicle suspension at a lipid to PNA_C16 molar ratio of 300:1 or 3000:1. The low molar ratio of 3000:1 was chosen (i) to minimize an influence of the lipophilic PNA on the membrane physical properties, and (ii) to exclude a reduction of NBD fluorescence due to a possible Förster resonance energy transfer (FRET) to rhodamine offering only one acceptor to 15 donor molecules. Figure 2a−c shows a typical domain-forming vesicle with inserted PNA_C16/DNAc1 hybrids at 3000:1 ratio. As judged from the green fluorescence, C6-NBD-PC (Figure 2a) almost exclusively partitions to the ld domain, whereas the red fluorescence from rhodamine on the hybrid PNA_C16/DNAc1 was only observed in the domain that could not be labeled with C6-NBD-PC (Figure 2b). This suggests that PNA_C16 is almost exclusively localized in the lo domain as was also seen in the overlay image in Figure 2c and illustrated in the sketch in Figure 2. An increase of the PNA_C16:lipid ratio to 1:300 did not change the partitioning behavior of the PNA_C16/DNAc1 hybrid (data not shown). To exclude the possibility of C6-NBD-PC influencing the PNA_C16/DNAc1 partitioning, we also prepared domain-forming vesicles lacking C6-NBD-PC. To illustrate the extent of segregation, Figure S3 (Supporting Information) shows a representative intensity plot of rhodamine fluorescence of PNA_C16/DNAc1 incorporated in a domain-forming GUV: almost all PNA_C16/DNAc1 hybrids were segregated into one domain, that is, into the lo phase when taking into account the results with C6-NBD-PC-labeled vesicles. Only low rhodamine fluorescence intensities were detected in the ld phase. Assuming that the fluorescence intensity is proportional to the concentration of PNA_C16/DNAc1 in the membrane, the ratio of the concentrations of PNA_C16/DNAc1 in the lo phase and PNA_C16/DNAc1 in the ld phase for a lipid:PNA_C16/DNAc1 molar ratio of 3000:1 was estimated to be 19:1.

Note that the interface energy contribution to the free energy of the vesicles is sometimes minimized by deformation of a domain-forming GUV to a nonspherical vesicle.[38] Therefore, the shape of the presented nonspherical GUVs (Figures 2a−c and 3d) is not caused by the incorporation of lipophilic PNA or DNA.

Janus Vesicles. Our finding that palmitoylated PNA partitions into lo domains in DOPC/SSM/Chol vesicles allowed us to produce Janus vesicles. Previous work showed that a tocopherol-modified oligonucleotide segregates entirely into the ld phase of domain-forming vesicles.[10,39] Therefore, we used a construct with the sequence 5′-TLT TTT TLT TTT ATT TCT GAT GTC CA-3′ (DNA_tocopherol), where L refers to a tocopherol-modified deoxyuridine (Figure S4, Supporting Information), and a fluorescein isothiocyanate (FITC)-labeled complementary oligonucleotide, 5′-FITC-TGG ACA TCA GAA ATA-3′ (DNAc2). We studied the lateral distribution of PNA_C16/DNAc1 and DNA_tocopherol/DNAc2 hybrids in domain-forming GUVs composed of DOPC/SSM/Chol raft mixture. The molar ratio of PNA_C16/DNAc1 to DNA_tocopherol/DNAc2 and to lipid was 1:1:300. Indeed, the DNA_tocopherol/DNAc2 (Figure 3a, FITC fluorescence) and the PNA_C16/DNAc1 (Figure 3b, rhodamine fluorescence) partitioned into well-separated domains, the ld and the lo domain, respectively (see also Figure 3c,d). Notably, domain partitioning was not affected by DNA sequence or position of the lipophilic anchors as partition behavior of DNA_tocopherol*/DNAc2* (DNA_tocopherol*, 5′-TGG ACA TCA GAA ATA TTT LTT TTT LT-3′; DNAc2*, 5′-TAT TTC TGA TGT CCA-FITC-3′) was identical to that of DNA_tocopherol/DNAc2 (see Figure 4a,e).

Domain-Specific Partitioning of the PNA_C16/DNAc1 Hybrids in Biological Membranes. We also used domain-forming giant plasma membrane vesicles (GPMVs) because their membranes

(37) Stöckl, M.; Plazzo, A. P.; Korte, T.; Herrmann, A. *J. Biol. Chem.* **2008**, *283*, 30828–30837.
(38) Semrau, S.; Schmidt, T. *Soft Matter* **2009**, *5*, 3174–3186.
(39) Bunge, A.; Kurz, A.; Windeck, A. K.; Korte, T.; Flasche, W.; Liebscher, J.; Herrmann, A.; Huster, D. *Langmuir* **2007**, *23*, 4455–4464.

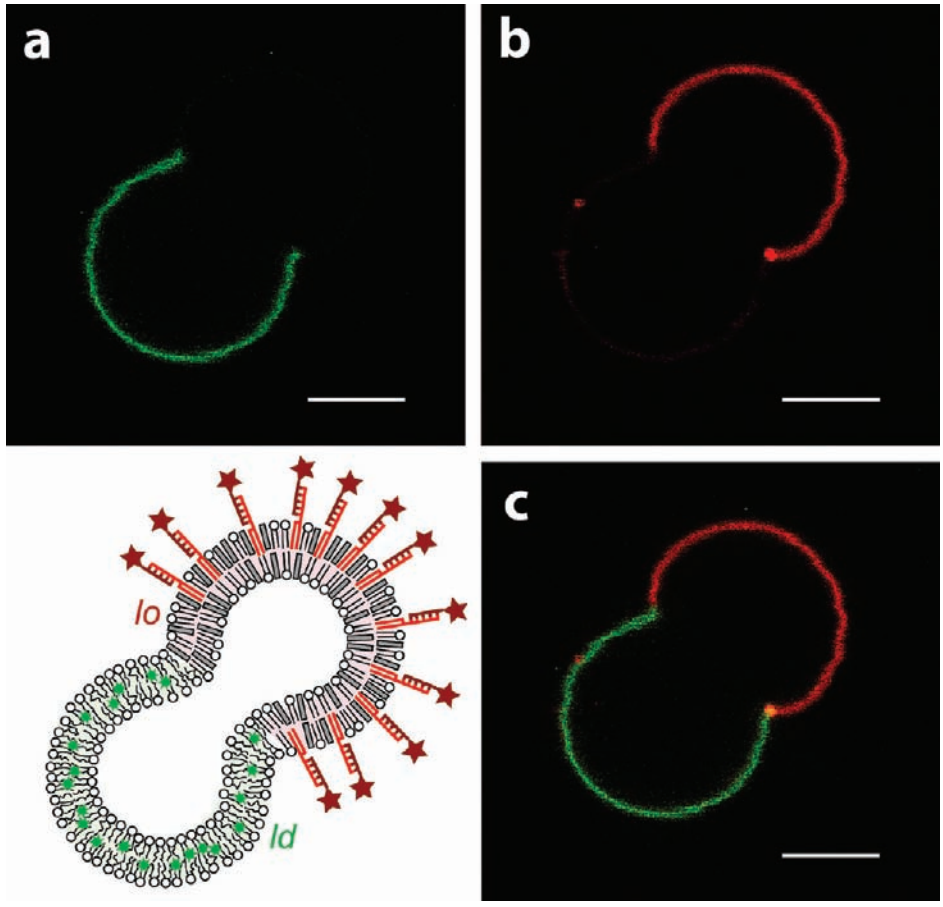

Figure 2. Lateral distribution of the palmitoyl-anchored PNA/DNA hybrid in the domain-forming GUVs made of 1:1:1 DOPC/SSM/Chol (molar ratio PNA_C16:lipid = 1:3000). The panels show confocal fluorescence microscopy images of (a) C6-NBD-PC fluorescence visualizing the ld phase, (b) the membrane-inserted PNA_C16/DNAc1 molecules, which are localized in the lo phase of the GUV, and (c) an overlay of the images shown in (a) and (b). Bars correspond to 5 μm. Cartoon summarizes the observations: C6-NBD-PC is localized to the ld domain (light green), while PNA_C16/DNAc1 (rose) is recruited to the cholesterol enriched lo domain (gray rectangles: Chol).

resemble cellular plasma membrane more than those of GUVs. First, GPMVs were derived from the plasma membrane of living CHO-K1 cells,[40,41] stably expressing the fluorescent raft marker GPI-mCFP. GPI anchored proteins are known to partition into cholesterol-enriched domains in the plasma membrane of cells.[42−45] At low temperatures, a significant fraction of GPMVs showed phase separation into micrometer-scaled lo and ld domains.[40,41] We observed that PNA_C16/DNAc1 colocalized with GPI-mCFP in the phase-separated GPMVs at 4 °C and therefore concluded that it partitioned into lo phase (Figure S5, Supporting Information). We then mixed PNA_C16, DNAc1, DNA_tocopherol, and DNAc2 with GPMVs prepared from cells that did not express GPI-mCFP. Two different populations of GPMVs were found: (i) in vesicles presenting no domain separation green fluorescence of DNA_tocopherol/DNAc2 and red fluorescence of PNA_C16/DNAc1 were spread over the whole membrane (not shown); and (ii) in vesicles showing domain formation DNA_tocopherol/DNAc2 (Figure 3e) and PNA_C16/DNAc1 (Figure 3f) partitioned into different domains. In conclusion, PNA_C16/DNAc1 partitioned into lo phase of domain-forming plasma membrane vesicles, as shown by colocalization with raft marker GPI-mCFP, whereas DNA_tocopherol segregated into ld phase.

Temperature-Controlled Mixing and Separation of the Functions on Janus Vesicles. Both domain formation and domain size are temperature dependent. Below the phase transition temperature, separation of lipid phases occurs, whereas above this temperature, the phases disappear building a uniform ld arrangement, and, hence, membrane compounds previously localized to different domains intermix. To take advantage of this mechanism, we incorporated PNA_C16/DNAc1 and DNA_tocopherol*/DNAc2* hybrids into the membranes of GUVs composed of a 1:1:1 (molar ratios) lipid mixture of POPC, SSM, and Chol. This lipid mixture was chosen because of its lower liquid−liquid phase transition temperature as compared to DOPC/SSM/Chol membranes[36] that is well below the melting temperature of the amphiphilic PNA/DNA and DNA/DNA hybrids. Movies showing temperature-controlled mixing and separation of PNA_C16/DNAc1 and DNA_tocopherol*/DNAc2* hybrids are provided in the Supporting Information (Movies S1 and S2). Figure 4 shows a sequence of fluorescence images of a vesicle that incorporated both DNA_tocopherol*/DNAc2*

(40) Baumgart, T.; Hammond, A. T.; Sengupta, P.; Hess, S. T.; Holowka, D. A.; Baird, B. A.; Webb, W. W. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 3165–3170.
(41) Sengupta, P.; Hammond, A.; Holowka, D.; Baird, B. *Biochim. Biophys. Acta* **2008**, *1778*, 20–32.
(42) Brown, D. A.; Rose, J. K. *Cell* **1992**, *68*, 533–544.
(43) Sharma, P.; Varma, R.; Sarasij, R. C.; Ira; Gousset, K.; Krishnamoorthy, G.; Rao, M.; Mayor, S. *Cell* **2004**, *116*, 577–589.
(44) Scolari, S.; Engel, S.; Krebs, N.; Plazzo, A. P.; De Almeida, R. F.; Prieto, M.; Veit, M.; Herrmann, A. *J. Biol. Chem.* **2009**, *284*, 15708–15716.
(45) Johnson, S. A.; Stinson, B. M.; Go, M. S.; Carmona, L. M.; Reminick, J. I.; Fang, X.; Baumgart, T. *Biochim. Biophys. Acta* **2010**, *1798*, 1427–1435.

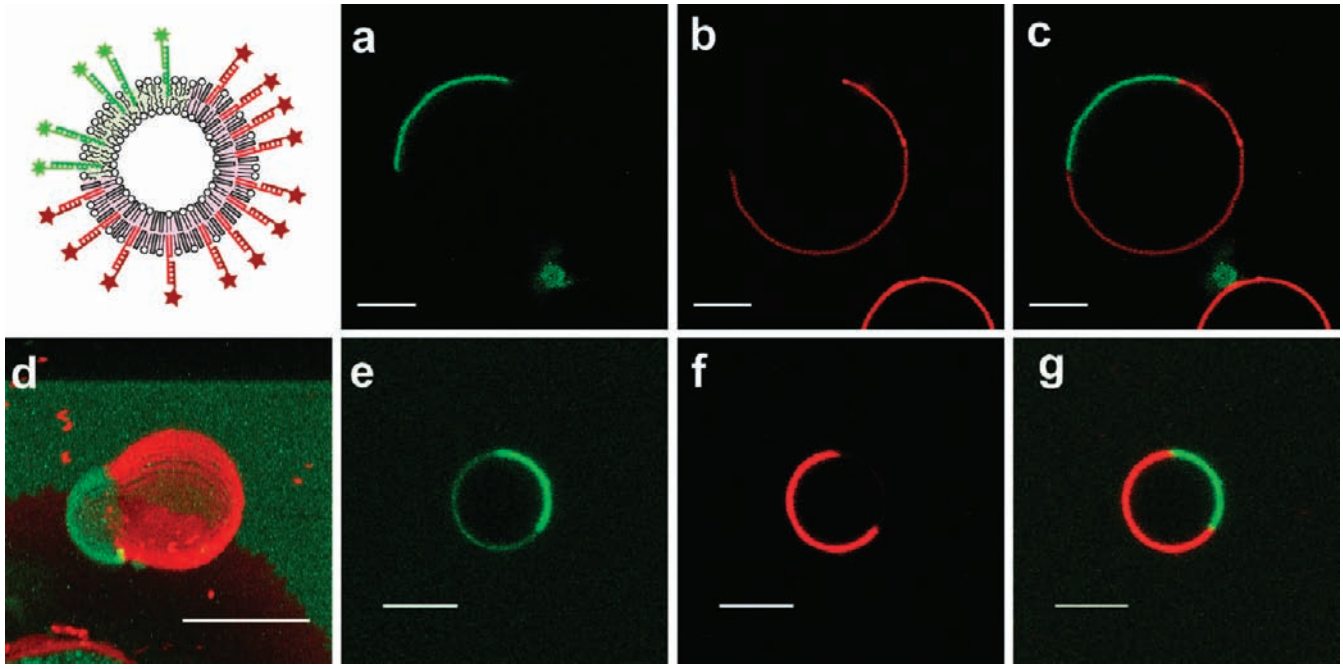

Figure 3. Lateral distribution of tocopherol-modified DNA (DNA_tocopherol/DNAc2; FITC-labeled) and palmitoyl-modified PNA (PNA_C16/DNAc1; rhodamine-labeled) in domain-forming GUVs made from 1:1:1 DOPC/SSM/Chol (a−d) and GPMVs prepared from CHO-K1 cells (e−g). (a) FITC channel − the membrane-inserted hybrids DNA_tocopherol/DNAc2 are localized to the ld domain. (b) Rhodamine channel − the membrane-inserted hybrids PNA_C16/DNAc1 are localized to the lo domain different from that in (a). (c) Overlay of the images shown in (a) and (b). (d) The three-dimensional overlay of the confocal images of another domain-forming GUV. (e−g) In domain-forming GPMVs, DNA_tocopherol/DNAc2 (e) partitioned into a domain other than PNA_C16/DNAc1 (f). (g) Overlay of the images shown in (e) and (f). Bars represent 5 μm (a−c) and 10 μm (d−g), respectively. Cartoon (left top) illustrates the results: PNA_C16/DNAc1 (red) is recruited to the lo domain (rose; gray rectangles: Chol), while DNA_tocopherol/DNAc2 is recruited to the ld domain (light green).

(a−d) and PNA_C16/DNAc1 (e−h) taken from Movie S1. At temperatures below phase transition, both lo and ld phases were present, and the constructs partitioned into different domains as described above (Figure 4a and e). Upon heating above the phase transition temperature, the two lipid domains merged causing colocalization of DNA_tocopherol*/DNAc2* (Figure 4b) and PNA_C16/DNAc1 (Figure 4f) hybrids in the entire vesicle. Subsequent cooling led to reformation of several small lo and ld domains and separation of lipophilic DNA and PNA molecules (Figure 4c,g). The small domains tend to fuse, minimizing the line tension until again only one lo and one ld domain remained containing PNA_C16/DNAc1 and DNA_tocopherol*/DNAc2*, respectively (Figure 4d,h). We observed that when the new domains were formed, DNA_tocopherol*/DNAc2* (c) was immediately excluded from the lo phase, whereas PNA_C16/DNAc1 (g) was still partially localized to both domains. Here, the redistribution process was remarkably slower as compared to DNA_tocopherol*/DNAc2*, nevertheless, leading again to almost complete segregation of PNA_C16/DNAc1 in lo domains. Taken together, Movies S1, S2, and Figure 4 show that functional moieties can be separated and mixed on demand using the phase-separated lipid membrane template.

Conclusions

The properties of membrane anchors are essential for the lateral distribution of lipophilic DNA and PNA constructs in lipid membranes. Here, we took advantage of those properties to organize lipophilic DNA and PNA constructs, which are accessible to complementary DNA strands, in lipid membrane domains in a controlled and reversible manner. Such a process has been unprecedented so far. We employed lipophilic PNA molecules featuring properties that are superior to those of DNA. Among other advantages, PNA is cleavable neither by nucleases nor by proteases, and PNA/DNA duplexes are more stable as indicated by their higher melting temperature in comparison to DNA/DNA duplexes.[5,46]

Various lipophilic anchors can be linked covalently to PNA, for example, diverse fatty acids,[6,47] cholesterol,[7] adamantyl residues,[48] and lipophilic cations.[49] So far, there are only a few reports on the distribution of lipophilic nucleic acids to lipid domains in model membrane systems. Tocopherol-based oligonucleotides were found to segregate to the ld phase of DOPC/SM/Chol GUVs.[10,39] Cholesterol-modified DNA was recently shown to partition equally into lo and ld phases, allowing equal functionalization of both domains of fluid−fluid phase-separated vesicles made of DOPC/SM/Chol.[11] In fluid−fluid phase-separated vesicles made of DOPC/dipalmitoyl PC/Chol, however, Beales and Vanderlick observed a preferential partitioning of cholesterol-modified DNA to the lo domain (ratio lo:ld $\approx$ 2:1).[12] Furthermore, the authors have shown that cholesterol-modified oligonucleotides were excluded from the solid phase in fluid−solid phase-separated GUVs and used these lipophilic oligonucleotides to functionalize the fluid phase the GUVs.[12] To the best of our knowledge, our study discloses the first example of a lipophilic nucleic acid segregating almost exclu-

(46) Janowski, B. A.; Kaihatsu, K.; Huffman, K. E.; Schwartz, J. C.; Ram, R.; Hardy, D.; Mendelson, C. R.; Corey, D. R. *Nat. Chem. Biol.* **2005**, *1*, 210–215.
(47) Savard, J. M.; Grosser, S. T.; Schneider, J. W. *Electrophoresis* **2008**, *29*, 2779–2789.
(48) Ljungstrom, T.; Knudsen, H.; Nielsen, P. E. *Bioconjugate Chem.* **1999**, *10*, 965–972.
(49) Filipovska, A.; Eccles, M. R.; Smith, R. A.; Murphy, M. P. *FEBS Lett.* **2004**, *556*, 180–186.

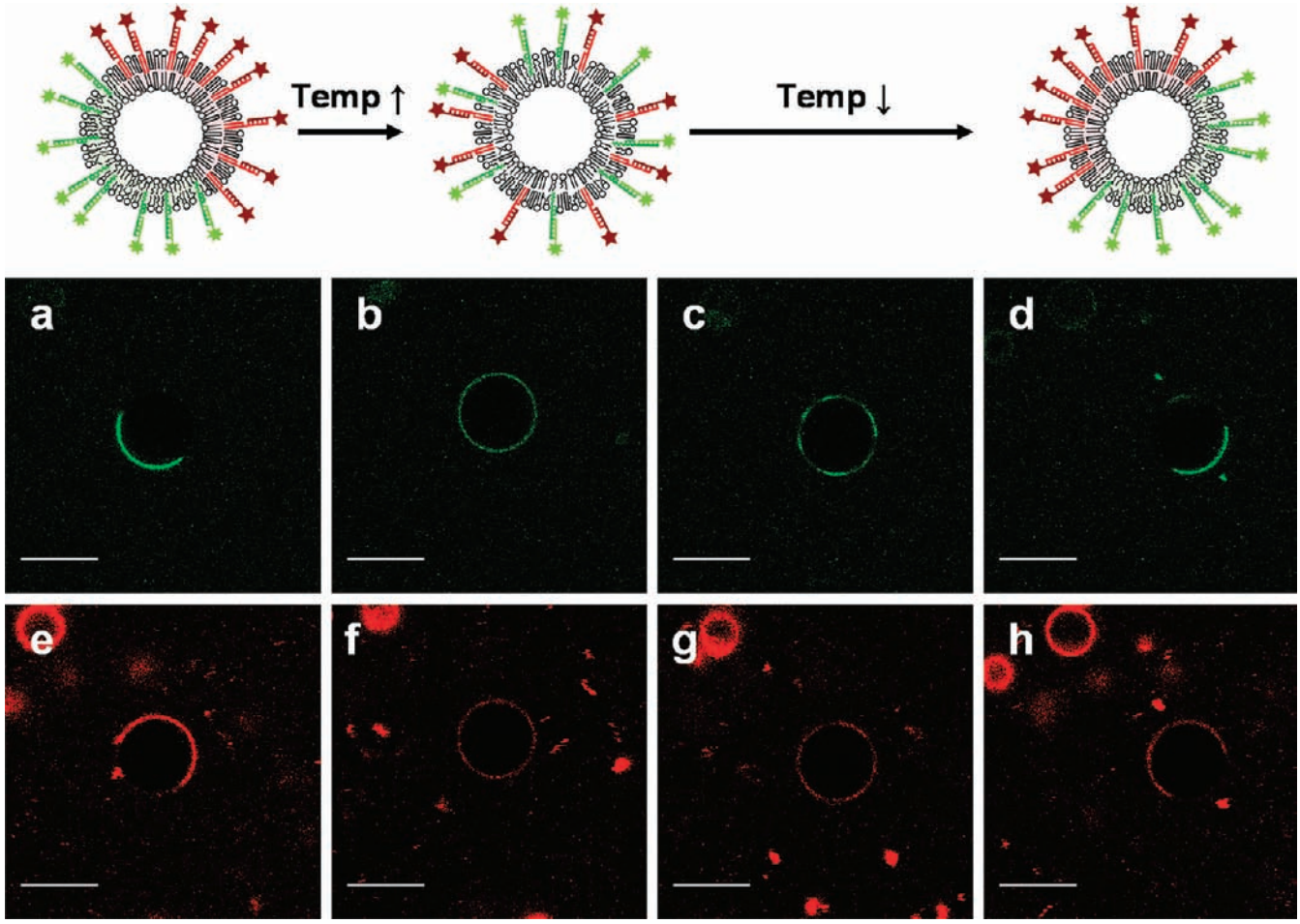

Figure 4. Mixing/demixing of DNA_tocopherol*/DNAc2* (a−d; FITC-labeled) and PNA_C16/DNAc1 (e−h; rhodamine-labeled) hybrids incorporated in POPC/SSM/Chol GUVs due to temperature increase and decrease. Below the phase transition temperature, DNA_tocopherol*/DNAc2* (a) and PNA_C16/DNAc1 (e) are located to different domains (∼5 °C). Above the phase transition temperature, domains disappeared, and both constructs mix (b,f) (∼55 °C). After cooling (∼46 °C), new smaller domains are formed (c,g), which then merge to larger domains separating again both types of constructs (d,h). Bars correspond to 10 μm. Scheme (top) illustrates the process: left, lipophilic DNA/DNA (green) and PNA/DNA (red) hybrids are separated to different domains (ld, light green; lo, rose) at low temperatures (corresponding to (a) and (e)); middle, upon heating, the whole membrane is liquid-disordered and both constructs intermix (as in (b) and (f)); right, subsequent cooling again leads to a two-sided vesicle with lipophilic DNA and PNA partitioning to opposing sites (as in (d) and (h)). Single images were taken from Movie S1 (Supporting Information). Fluorescence in the sequentially taken images was reducing due to the photobleaching. Vesicles in the upper left were not significantly affected by this process as they diffused into and out of the observed area. Note, the background fluorescence outside the vesicles in both channels is due to residual micelles of the hybrids, and submicroscopic lipid vesicles and aggregates, which also have incorporated PNA_C16/DNAc1 and DNA_tocopherol*/DNAc2*. For better visualization, a linear background correction was performed here, and the contrast of the images was enhanced; see Movie S1 (Supporting Information) for unprocessed images.

sively into the lo phase of fluid−fluid phase-separated vesicles and demonstrates the reversible, temperature-controlled mixing and separation of two different lipophilic nucleic acid units (DNA and PNA) into different domains of giant unilamellar and plasma membrane vesicles. This provides interesting prospects and applications as these lipophilic constructs can be targeted by complementary DNA strands that potentially can carry functional moieties: (1) The Janus vesicles can be used to enable controlled interaction between molecules attached to the complementary strands, for example, for signal transduction on the membrane surface, by switchable segregation and intermixing of the interacting molecules. Likewise, sequential reactions can be efficiently regulated by attaching to the DNA strands enzymes mediating such reactions. (2) We have shown that hybrids of the double palmitoylated PNA with the complementary DNA inserted spontaneously into the plasma membrane of CHO-K1 cells. Although further studies of the distribution in the cell plasma membrane are necessary, our results on giant plasma membrane vesicles imply that the double palmitoylated PNA will also partition into lipid rafts of the plasma membrane of living cells as multiple palmitoylation is a marker for raft partitioning.[27,50] By selecting appropriate lipophilic anchors, the uptake of those constructs and by that of the functional moieties linked via complementary DNA strands can be modulated. For example, it is known that lipid rafts are involved in clathrin-independent endocytosis.[24]

In summary, selecting specific lipid anchors for lipophilic DNA and PNA constructs offers a broad application for controlled, switchable functionalization of soft biocompatible fluid surfaces and biological membranes as well as for optimization of cellular uptake of substances.

Methods

Chemicals. 1-Palmitoyl-2-{6-[(7-nitro-2−1,3-benzoxadiazol-4-yl)amino]hexanoyl}-*sn*-glycero-3-phosphocholine (C6-NBD-PC),

(50) Zacharias, D. A.; Violin, J. D.; Newton, A. C.; Tsien, R. Y. *Science* **2002**, *296*, 913–916.

1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), *N*-stearoyl-D-*erythro*-sphingosylphosphorylcholine (SSM), and cholesterol were obtained from Avanti Polar Lipids, Inc. (Alabaster, U.S.). Neomycin was obtained from Sigma-Aldrich (Taufkirchen, Germany), Dulbecco's Modified Eagle Medium (DMEM) and penicillin/streptomycin (PS) were from PAN (Aidenbach, Germany), and fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA). DNA oligonucleotides were purchased from BioTeZ (Berlin, Germany), and tocopherol-modified deoxyuridine was synthesized as described elsewhere.[10,39] GUV buffer was 250 mM sucrose, 10 mM Hepes, pH 7.4. Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, pH 7.4, was used. Polymeric support Rink-Amid Tentagel Harz 80 NovaSyn TGR (capacity 0.12 mmol/g) was ordered from Novabiochem. All used chemicals were of highest purity grade.

Microscopy Images. All images were taken using an Olympus Fluoview 1000 with a 60× oil immersion objective. Laser light was directed to sample and separated from emitted light with a dichroic mirror (DM 405/488/559/635). FITC (NBD) was excited with a 488 nm argon laser, rhodamine with laser diode at 559 nm. Green fluorescence (FITC, NBD) was separated from red fluorescent light (rhodamine) with a dichroic long pass filter (SDM 560) reflecting light with a wavelength below 560 nm. FITC (NBD) fluorescence was recorded between 500 and 545 nm, and rhodamine fluorescence was recorded between 570 and 670 nm. To avoid crosstalk of FITC fluorescence in the rhodamine channel and vice versa, sequential scanning mode was used. Here, the sample is excited successively with the 488 and the 559 nm lasers, while the green and the red fluorescence is recorded only during excitation with the 488 and the 559 nm lasers, respectively. This setup also cuts off a possible FRET contribution of rhodamine fluorescence that might originate by excitation of NBD. Note, contrast of confocal fluorescence images shown in Figure 1a and c was equally enhanced.

Movies S1 and S2 were created by stacking several confocal images. Images were taken each 30 s. To minimize the effect of photobleaching on the quality of the images, the microscopic setup was changed as compared to the other microscopic images concerning laser power and amplification of the fluorescence signal. Heating and cooling was achieved using a water circulating bath and a heating block (self-construction) fitting ibidi μ-Slide VI (ibidi GmbH, München, Germany). Note, as we used these low volume channel chambers, the temperature could not be measured directly in the observation volume. Thus, the given temperature of the heating device is presumably a few degrees higher than the temperature of the sample.

For microscopy, 160 μL of PBS was mixed with oligonucleotides (10 μM in H_2O) and the lipophilic PNA (10 μM in MeOH), respectively. 40 μL of the GUV suspension was added, and the resulting suspension was mixed and incubated at room temperature for 1 h. GPMVs were incubated with oligonucleotides/lipophilic PNA at 4 °C for 30 min, while CHO-K1 cells were incubated at 37 °C for 1 h.

GUV Preparation. GUVs were produced using the electroformation method.[51] 100 nmol of a lipid or lipid mixture in $CHCl_3$ was spotted onto the bottom of a titan chamber. Lipid film was dried by heating the chamber to 50 °C for a few seconds and subsequent evaporation of solvent traces at about 10 mbar for at least 30 min. PNA_C16 was added as a powder to the thin lipid film of POPC at the lipid to PNA_C16 molar ratio of 300 to 1. Titan chamber was sealed and filled with 1 mL of GUV buffer. Immediately, an alternating voltage rising from 20 to 1100 mV with a frequency of 10 Hz was applied for 2.5 h. To detach GUVs from the chamber's surface, the frequency was reduced to 4 Hz, and a voltage of 1.3 V was applied for an additional 30 min. Pure POPC vesicles were produced at room temperature, whereas those composed of cholesterol, SSM, and DOPC or POPC (molar ratios 1:1:1) were produced on a heating plate at 60 °C adding buffer preheated to 70 °C.

Cell Culture and GPMVs Preparation. CHO-K1 cells were grown in DMEM without phenol red and supplied with 2 mM L-glutamine, 10% FBS, and 5% PS (complete medium) and incubated at 37 °C and 5% CO_2. Some CHO-K1 cells used were stably transfected with GPI-mCFP, a fusion protein of the monomeric cyan fluorescent protein (mCFP), and a glycosylphosphatidylinositol anchor (GPI). Note, the mCFP carries A206K mutation, which abolishes the natural tendency of fluorescent proteins to dimerize.[50] These cells were cultured in DMEM complete supplied with 250 μg/mL neomycin. Cells were forced to produce giant plasma membrane vesicles (GPMVs) or "blebs" upon treatment with buffer containing dithiothreitol (DTT) and formaldehyde as previously described.[40,41] Briefly, almost confluent cells (T25 flask) were washed twice with GPMV buffer, then 1.5 mL of GPMV buffer containing 2 mM DTT and 25 mM formaldehyde was added, and the flasks were incubated at 37 °C for 1 h, under gentle shaking (60−80 cycles per minute). GPMVs detached from cells were then collected from the bottom of the flask and transferred into a conical glass tube where they were allowed to sediment at 4 °C for about 30 min. Vesicles were imaged in ibidi-dishes. Images of the equatorial plane of the blebs were taken at 4 °C, and the temperature was controlled with a water circulating bath.

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Supporting Information Available: Schemes of palmitoylated PNA_C16 and tocopherol-modified uridine; additional confocal fluorescence and DIC images; FRET control measurement; synthesis of PNA_C16; movies of temperature-dependent mixing/demixing of PNA_C16/DNAc1 and DNA_tocopherol*/DNAc2*; movie legend. This material is available free of charge via the Internet at http://pubs.acs.org.

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(51) Angelova, M. I.; Soleau, S.; Meleard, P.; Faucon, J. H.; Bothorel, P. *Prog. Colloid Polym. Sci.* **1992**, *89*, 127–131.