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Remote Control of Lipophilic Nucleic Acids Domain Partitioning by DNA Hybridization and Enzymatic Cleavage

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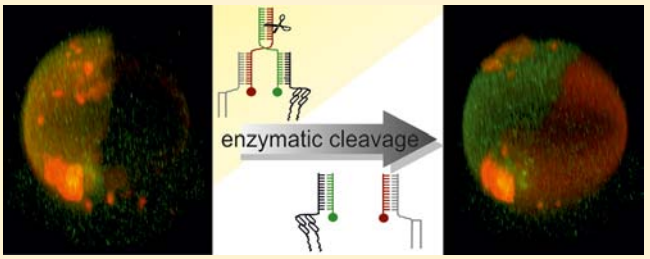
Supporting Information

ABSTRACT: Lateral partitioning of lipid-modified molecules between liquid-disordered (ld) and liquid-ordered (lo) domains depends on the type of lipid modification, presence of a spacer, membrane composition, and temperature. Here, we show that the lo domain partitioning of the palmitoylated peptide nucleic acid (PNA) can be influenced by formation of a four-component complex with the ld domain partitioning tocopherol-modified DNA: the PNA−DNA complex partitioned into the ld domains. Enzymatic cleavage of the DNA linker led to the disruption of the complex and restored the initial distribution of the lipophilic nucleic acids into the respective domains. This modular system offers strategies for dynamic functionalization of biomimetic surfaces, for example, in nanostructuring and regulation of enzyme catalysis, and it provides a tool to study the molecular basis of controlled reorganization of lipid-modified proteins in membranes, for example, during signal transduction.



INTRODUCTION

The segregation of integral and peripheral proteins together with specific lipids in small and dynamic regions is essential for cell signaling, endocytosis, and motility.[1−3] Small domains of 10−200 nm in diameter enriched in saturated lipids, sphingomyelin, and cholesterol are called rafts, whereas regions that are enriched in unsaturated lipids are referred to as the nonraft phase.[4] The lateral segregation of proteins is regulated by lipophilic modification of the proteins and by protein−protein, protein−lipid, and lipid−lipid interactions.[5,6] The most common lipophilic modifications of proteins are palmitoylation, myristoylation, prenylation (farnesylation, geranylgeranylation), and cholesterol attachment.[5,7−9] Studies on protein lipidation and lateral partitioning revealed that palmitoylation is a posttranslational and reversible modification, which controls the sequestration of peripheral and integral proteins into rafts. Proteins modified with farnesyl or geranylgeranyl moieties were found in less ordered nonraft regions.[10,11] Lipid-modified peptides, nucleic acids, and other lipophilic conjugates have been used to study the effect of lipidation on lateral partitioning of the lipid-modified molecule.[8,12−19] Model membrane systems such as domain-forming giant unilamellar vesicles (GUVs) and supported lipid bilayers as well as cell-derived giant plasma membrane vesicles provided valuable insight.[13,17,20−23] Studies on phase separation in membranes formed from mixtures of unsaturated, saturated lipids (sphingomyelin) and cholesterol showed that liquid-ordered (lo) domains coexist with liquid-disordered (ld) domains at physiological conditions. The lo domains are enriched in sphingomyelin and cholesterol.[24−27] Lipids in lo domains of model membranes have a higher order, due to the absence of proteins and due to a lower diversity of lipids, but a structure similar to that of lipids in raft regions of the plasma membrane.[22,28]

Specific and controllable organization of functional moieties for biotechnological applications, for example, for pathogens and nucleic acid detection, or for control of reaction cascades, might be achieved by incorporating protein or nucleic acid lipophilic conjugates into lipid membranes. In particular, nucleic acid-based conjugates offer unique opportunities. While chemical synthesis grants facile access to specific sequences and a variety of lipid anchors, Watson−Crick-based recognition can be used to control the proximity of the appended entities.[29]

Different lipophilic nucleic acid conjugates were found to incorporate into membranes; their specific recognition properties were used to attach vesicles to vesicles or to supported lipid

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bilayers and coated particles.[30−32] Lipophilic nucleic acids were shown to insert into plasma membrane of cells.[17,33] Nucleic acid conjugates were used to build defined three-dimensional cell clusters and layers via hybridization.[33,34] Lipophilic oligonucleotides were applied to mimic SNARE-induced fusion system,[35,36] and for DNA detection.[37]

Domain-specific partitioning of lipidated molecules would provide further options for nanotechnological applications, for example, soft membranes functionalization; however, most lipidated nucleic acid molecules partition into the ld domains or show no preference for a specific lipid domain. For example, tocopherol-modified DNA was found to partition into the ld domains,[16,38] whereas cholesterol-modified DNA showed, depending on lipid membrane composition, no or only a slight preference for the lo domains.[15,18] Only recently could we report the synthesis of a double palmitoylated peptide nucleic acid (PNA_C16) and found that its hybrid with DNA partitions almost exclusively into the lo domains in model and cell derived membranes.[17] Thus, a set of membrane anchors for either the lo or the ld domain was then available for membrane functionalization.

In the next step, we have developed and report here a system in which the domain partitioning of lipophilic constructs could be regulated first by nucleic acid hybridization and then through enzymatic cleavage. Using lateral organization of the membranes, domain-specific distribution of lipophilic nucleic acids and proteins will allow building of functionalized soft surfaces for remote control of interactions. For example, proteins can be enriched to form complexes or functional platforms in one lipid domain, and subsequently the complex stability can be manipulated by changing the temperature or by enzymatic cleavage.

RESULTS

Double Palmitoylated PNA Partitions into lo Domains (Independently of Hybridization with a Complementary DNA). Recently, we showed that a double palmitoylated peptide nucleic acid (*Pal*-Lys(*Pal*)-Gly-Glu-Glu-Gly-ttc ttc tcc tt-Glu-Glu-Gly-$CONH_2$, PNA_C16, Table 1) in complex with a complementary DNA partitions almost exclusively into lo domains.[17] To demonstrate that the PNA_C16 molecules by themselves also partition into the lo domains, we carried out ^2H solid-state NMR measurements on a POPC/PSM/cholesterol/PNA_C16 lipid mixture. To separately detect the NMR signals from each component of the mixture, molecules with perdeuterated palmitoyl chains were used. For measurements of the order parameters, in each experiment one of the compounds, either POPC, or PSM, or PNA_C16, was taken as ^2H labeled analogue so that ^2H NMR spectra could be detected for each component.[39,40] From the NMR spectra, chain order parameters were calculated; the order parameter profiles at 30 °C are shown in Figure 1. In agreement with literature,[39,41]

Table 1. Unlabeled and Labeled Oligonucleotide Sequences in Part or Fully Complementary to the Lipophilic Nucleic Acids PNA_C16 and DNA_tocopherol[a]

name	oligonucleotide sequence (5′→3′)
DNAc1_Rh	TG CCG GAA TTC GCG TTT TTT TTT AAG GAG AAG AAT − rhodamine
DNAc2_FITC	FITC-TTAT TTC TGA TGT CCA TTT TTT TTT C GCG AAT TCC GGC A
DNAc2	TTAT TTC TGA TGT CCA TTT TTT TTT C GCG AAT TCC GGC A
name	lipophilic nucleic acid sequence
DNA_tocopherol	TGG ACA TCA GAA ATA TTT LTT TTT LT
PNA_C16	Pal-Lys(Pal)-Gly-Glu-Glu-Gly-ttc ttc tcc tt-Glu-Glu-Gly-$CONH_2$

[a]The specific cleavage sequence characteristic for *Eco*R1-HF introduced into the DNAc1/DNAc2 complementary sequences is underlined. The oligonucleotides have sequences fully complementary to the corresponding sequences of PNA_C16 and DNA_tocopherol, respectively. Tocopherol-modified DNA (DNA_tocopherol) was a 24mer DNA sequence with two tocopherol-modified deoxyuridines each denoted as "L". The PNA_C16 sequence was the same as in Loew et al.[15] and is shown starting with the amino-terminal end with peptide nucleic acids shown in small letters.

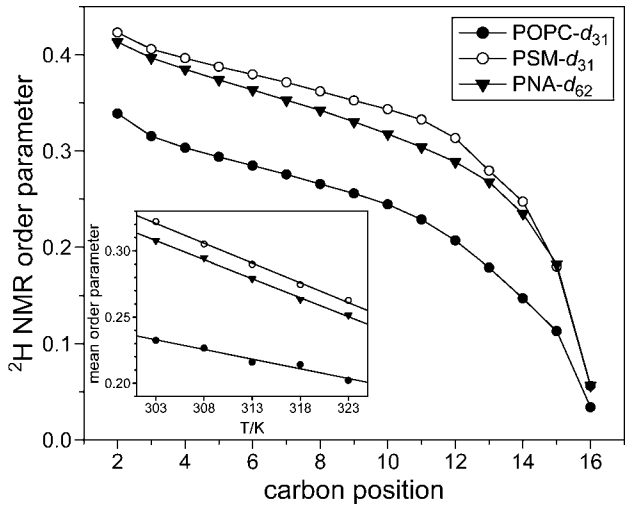

Figure 1. ^2H NMR chain order parameters of the individual molecules in the raft mixture. Similar order parameters are detected for the palmitoyl chains of PSM and the PNA_C16, confirming the colocalization in the lo domains. In contrast, the order parameters of the palmitoyl chains of POPC in the ld domains are much lower. The inset shows the temperature dependence of the average order parameters. Again, PSM and PNA_C16 order parameters show very similar temperature dependence as opposed to the temperature dependence of POPC order parameters.

high order parameters were measured for PSM that is known to be predominantly localized in the lo domains, while POPC, which is predominantly found in the ld domains, gave smaller order parameters (Figure 1). The order parameters detected for the PNA_C16 were similarly high as for the PSM, indicating a localization of PNA_C16 in the lo domains. The corresponding ^2H NMR spectra at different temperatures are shown in the Supporting Information, Figure S1.

Confocal fluorescence microscopy experiments showed that a PNA_C16 hybrid with a complementary rhodamine-labeled DNA strand, DNAc1_Rh (see Table 1 for the oligonucleotides sequences), partitions preferentially into the lo domains of the POPC/PSM/cholesterol GUVs at temperatures below 37 °C as shown in Figure S2, top (Supporting Information). Heating the samples to 40 °C led to mixing of lipids and disappearance of the microscopic lo domains and, therefore, to mostly liquid-disordered membranes and a homogeneous distribution of PNA_C16/DNAc1_Rh and C6-NBD-PC (Figure S2B). These results agree with that previously reported on the distribution of these nucleic acids.[17]

The conclusion that the palmitoylated PNA_C16 partitions into the lo domains was confirmed by analysis of the

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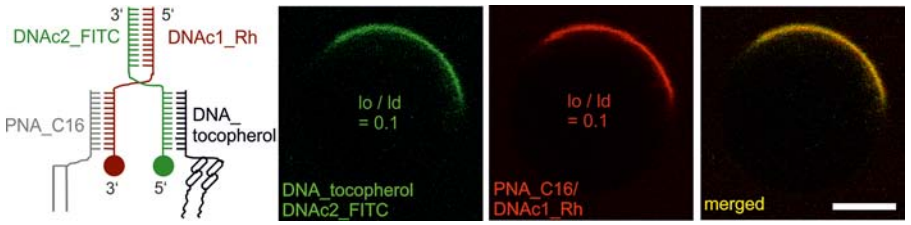

Figure 2. Co-localization of PNA_C16 and DNA_tocopherol in one domain upon formation of the four-component complexes. (Left) A sketch of the four-component complex: double palmitoylated PNA (PNA_C16) hybridized with a rhodamine-labeled (DNAc1_Rh) and tocopherol-modified DNA (DNA_tocopherol) hybridized with a FITC-labeled with a 3′-end overhang (DNAc2_FITC), which hybridizes with the 5′-end overhang of the DNAc1_Rh. Sequences are shown in Table 1. Rhodamine and FITC are illustrated as red and green circles, respectively. (Right) Incorporation of PNA_C16 and DNA_tocopherol each preincubated with the complementary oligonucleotides DNAc1_Rh and DNAc2_FITC at 5:4 molar ratio of lipophilic to fluorescent nucleic acids, respectively, into unlabeled 1:1:1 DOPC/SSM/cholesterol GUVs resulted in colocalization of FITC (green, left image) and rhodamine (red, middle image) in one lipid domain. The overlay (orange) of the two is shown on the right. Samples were in NEBuffer 4 with 50 mM NaCl at 37 °C. Ratios between the fluorescence intensities lo/ld are given (see Materials and Methods). Scale bar corresponds to 10 μm.

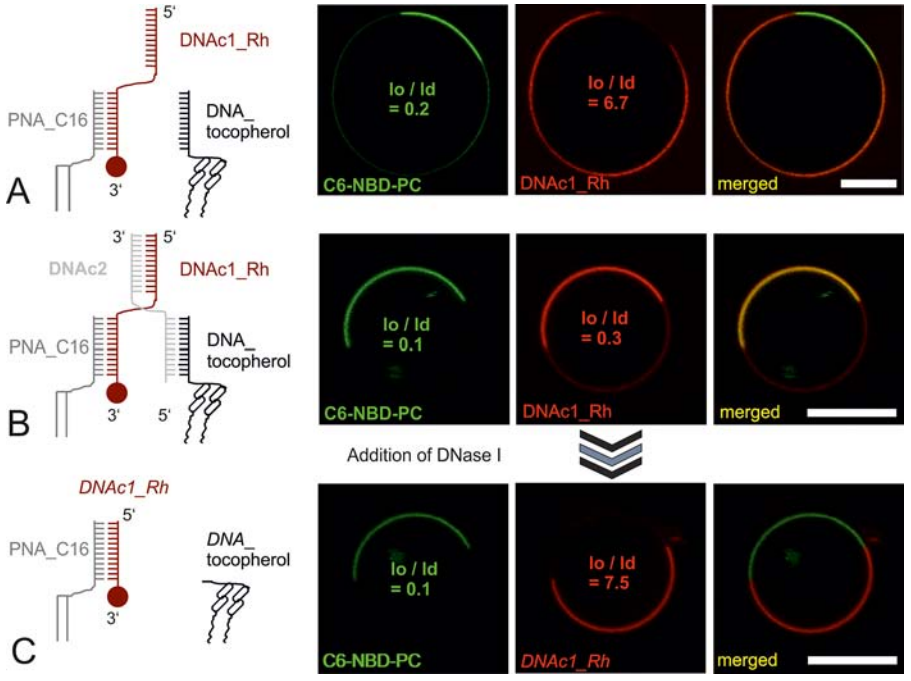

Figure 3. Controlling the distribution of lipophilic oligonucleotides in lipid domains. C6-NBD-PC (green) is used for the identification of ld domains in 1:1:1 DOPC/SSM/cholesterol GUVs.[43] (A) Equimolar amounts of PNA_C16, DNAc1_Rh (red), and DNA_tocopherol were incubated with GUVs in PBS. The red fluorescence signal observed originates from PNA_C16/DNAc1_Rh. (B) After subsequent incubation with the nonlabeled linker oligonucleotide DNAc2 for 30 min, a colocalization of the ld-marker C6-NBD-PC and rhodamine can be seen in the presence of DNaseBuffer. (C) Complete redistribution of red fluorescence into the domain not labeled by C6-NBD-PC after 10 min digestion with 50 U DNase I indicated a redistribution of the PNA_C16/DNA hybrids into the lo domain. Note that images in (B) and (C) reveal the same vesicle before and 10 min after addition of DNase I. Ratios between the fluorescence intensities lo/ld are given (see Materials and Methods). Scale bars correspond to 10 μm.

temperature dependence of the mean order parameter, shown in the inset in Figure 1. As expected, chain order parameters decrease with increasing temperature, but the slope of the decrease varies for PSM and POPC as previously reported.[39] The order parameters of the PNA_C16 palmitoyl chains follow the temperature dependence of the order parameters of PSM, which along with the majority of the cholesterol forms the lo domains, with slopes of -2.87×10^{-3} and -2.99×10^{-3} K^{-1}, respectively. In contrast, the slope of the temperature dependence of the order parameter of POPC in the ld domains is much shallower (-1.46×10^{-3} K^{-1}). These results confirm that PNA_C16 partitions into the lo domains even in the absence of a fluorescence labeled complementary DNA strand.

Repartitioning of the Double Palmitoylated PNA When Bound to Tocopherol-Modified DNA. To attest how the partitioning of the palmitoylated PNA can be influenced by interaction with a different conjugate featuring another lipid anchor and expressing, hence, different partitioning behavior, we designed a four-component system based on the two different lipophilic nucleic acids: PNA_C16 and DNA_tocopherol, the latter partitioning into the ld domains.[16,17,38] These were linked to each other via two partially complementary DNA oligonucleotides (DNA linker) to achieve the complex shown schematically in Figure 2.

The sequences of the nucleic acids used in this study are given in Table 1. The DNA oligonucleotides were designed to

allow (i) hybridization with lipophilic nucleic acids; (ii) maintaining stable incorporation of both palmitoyl and tocopherol chains into the membrane of the same vesicle before and after formation of the four-component complex; (iii) detection and confirmation of the membrane localization and hybridization by fluorescence microscopy even after enzymatic cleavage of the DNA linker; (iv) a flexible spacer between hybridizing sequences; (v) sterically unhindered enzymatic cleavage; and (vi) stability of the four-component complex up to 37 °C. Designing the complex-forming DNA strands, formation and stability of hybrids were estimated using NUPACK,[42] a software suite for the analysis and design of nucleic acid systems. The 5′-end overhang of DNAc1_Rh hybridizing with PNA_C16 was designed to recognize the 3′-end overhang of FITC-labeled DNAc2_FITC, which served to bind the DNA_tocopherol strand, building the hybrid of the DNA linker that holds the two lipophilic nucleic acids together. The chosen sequences were calculated to maintain integrity of the formed complexes in the used buffer (50−100 mM NaCl, 5 mM $Mg(OAc)_2$) within 20−37 °C. The 14-fold increase of Hoechst 33342 fluorescence that was observed when the complementary oligonucleotides were added proved duplex formation (Figure S3A). The colocalization of the green and red fluorescence signals at the membrane when only one of the lipophilic conjugates was incorporated proved formation of the three part complexes, for example, PNA_C16/DNAc1_Rh/DNAc2_FITC or DNA_tocopherol/DNAc2_FITC/DNAc1_Rh, at the vesicles surface (not shown). As expected from our previous work,[17] PNA_C16/DNAc1_Rh hybrids were localized in lo domains, when either DNA_tocopherol or linker DNAc2 were not added, and, hence, the four-component complex could not be formed (Figure 3A and Figure S4, Supporting Information). In the absence of lipophilic nucleic acids, no accumulation of fluorescence on the membrane surface upon addition of either DNAc1_Rh or DNAc2_FITC to GUVs was detected (Figure S5A,B, Supporting Information), meaning that no unspecific binding of the DNA oligonucleotides to lipid membranes was observed under our experimental conditions. Note, even in the presence of 10 mM $Mg(OAc)_2$ necessary for nuclease activity, no unspecific binding of the DNA oligonucleotides to GUVs was detected (Figure S5C,D, Supporting Information).

When the four nucleic acid conjugates, that is, the two lipophilic nucleic acids PNA_C16 and DNA_tocopherol as well as the long complementary strands DNAc1_Rh and DNAc2_FITC, were added to GUVs, a four-component complex was formed. The lateral distribution of the four-component complex in domain-forming 1:1:1 (molar ratios) DOPC/SSM/cholesterol GUVs was studied. As shown in Figure 2, formation of the complex led to the colocalization of the rhodamine and the fluorescein fluorescence signals, indicating that PNA_C16/DNAc1_Rh in hybrids with DNA_tocopherol/DNAc2_FITC partitioned into the same domains. To ensure that no three-component complexes with one lipophilic nucleic acid formed, and therefore only four-component complexes were observed with FITC and rhodamine fluorescence, lipophilic oligonucleotides were added in a slight excess over the complementary DNA strands at the molecular ratios of 5:4:4:5 for PNA_C16/DNAc1_Rh/DNAc2/DNA_tocopherol, respectively. We conclude that PNA_C16, which otherwise would be localized in the lo, and thus opposite to the ld domain with DNA_tocopherol, was included in the four-component complexes with DNA_tocopherol, and both lipophilic nucleic acids were located in the same domain.

To affirm the colocalization of the four-component complexes with the ld lipid domains, 1:1:1 (molar ratios) DOPC/SSM/cholesterol GUVs labeled with the ld marker C6-NBD-PC[43] and an unlabeled linker oligonucleotide, DNAc2, were used. Figure 3A shows the initial complementary distribution of PNA_C16/DNAc1_Rh hybrids (red) and C6-NBD-PC (green). In this experimental setup, DNA_tocopherol was already inserted into the membrane. Upon subsequent addition of DNAc2, hybridizing with DNA_tocopherol and DNAc1_Rh, and, therefore, linking the lipophilic nucleic acids to each other, the rhodamine signal colocalized with C6-NBD-PC (Figure 3B), proving that formation of the four-component complex led to a relocation of PNA_C16/DNA hybrids into the ld domains. The complexes were stable even at 37 °C for at least 3.5 h (Figure S6, Supporting Information).

Separation of PNA_C16 and DNA_tocopherol via Nuclease Cleavage of the Four-Component Complex Led to Redistribution of PNA_C16 Back into the lo Domains. A disruption of the four-component complexes, for example, by DNA cleavage, should restore the localization of PNA_C16 into the lo domains, as PNA and PNA/DNA duplexes are resistant against nucleases.[44,45] We used two different nuclease types to disrupt the four-component complexes. First, a nonspecific endonuclease DNase I, capable of cleaving both single- and double-stranded DNA, was used as a robust enzyme with no specific buffer requirements for activity except the presence of bivalent cations.

Note that DNAc1_Rh was labeled with rhodamine at the 3′-end, which hybridizes with PNA_C16 and, therefore, cannot be cleaved (Figure 2, Table 1). Second, we included a specific cleavage site for *Eco*R1-HF into the overhangs of DNAc1_Rh and DNAc2_FITC that together form the link between the two lipophilic constructs (Table 1, underlined).

The vesicles and four-component complexes were stable in the presence of buffers required for the activity of the enzymes. No change of the spatial distribution of either nucleic acids or fluorescent lipid marker was observed upon addition of the buffers (Figures 2 and S7, Supporting Information). Addition of heat-inactivated enzymes also caused no redistribution of either the conjugates or the ld-marker (Figure S7C,D, Supporting Information).

GUVs were preincubated with all four nucleic acids to achieve colocalization of rhodamine and C6-NBD-PC (Figure 3B). Addition of DNase I led to redistribution of red fluorescence observed on the same vesicle: rhodamine separated from C6-NBD-PC fluorescence on the membranes into the opposite domain (Figure 3C). To observe the DNase I caused redistribution directly, we tracked individual vesicles via confocal microscopy over 1 h. The same vesicles were imaged before and at constant intervals after addition of the enzyme: The longer the enzyme was present, the more complete was the change from a colocalized to a separated distribution. A moving front of red fluorescence was observed in the first 2−3 min (see Figure S8). Movie S1, Supporting Information, shows a three-dimensional reconstruction of a vesicle revealing that C6-NBD-PC distribution, and, therefore, the position of ld domain did not change upon addition of 10 U of DNase I, whereas PNA_C16/*DNAc1_Rh* hybrids quickly and permanently redistributed to the lo domain.

Hence, we could observe the DNase I induced disruption of the four-component complexes and the subsequent relocaliza-

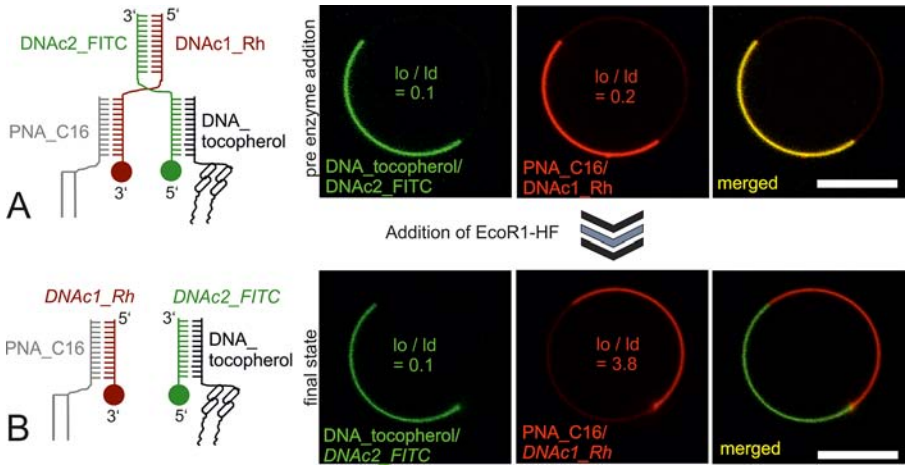

Figure 4. Redistribution of the lipophilic peptide nucleic acid upon cleavage of the four-component complexes by *Eco*R1-HF. Cartoons on the left illustrate the four-component complex and the DNA cleavage products with tags shown in cursive. Images A and B show the same 1:1:1 DOPC/SSM/cholesterol vesicle before and after addition of *Eco*R1-HF. (A) PNA_C16/DNAc1_Rh (red) and DNA_tocopherol/DNAc2_FITC (green) are colocalized as the four-component complexes in one domain as clearly seen in the merged image on the right. (B) Between 5.5 and 45 min after addition of 20 U of *Eco*R1-HF, fluorescence signals were not any longer colocalized but separated from each other, which was also indicative of a successful cleavage. For the corresponding sequence of images, see Figure S9 and movie S2, Supporting Information. Ratios between the fluorescence intensities lo/ld are given (see Materials and Methods). Scale bars correspond to 10 μm.

tion of the formed PNA_C16/DNA hybrids into lipid-ordered domains in real time. When DNAc2_FITC was used as the linker oligonucleotide, we observed a loss of FITC fluorescence from the membrane upon DNase I addition in parallel to the relocation of PNA_C16/DNA hybrids into the opposite domain (data not shown). The loss of FITC fluorescence from the membrane surface was expected as DNase I can cleave both single- and double-stranded DNA nonspecifically. Hence, this observation also proved that the nuclease was active under our experimental conditions.

We then studied redistribution induced by *Eco*R1-HF specific cleavage at the linker part of the DNAc1_Rh/DNAc2_FITC hybrid. A schematic representation of the predicted cleavage products is shown in Figure 4B: Both PNA_C16/*DNAc1_Rh* and DNA_tocopherol/*DNAc2*_FITC hybrids should be still detectable at the membrane upon the specific cleavage. PNA_C16/DNAc1_Rh and DNA_tocopherol/DNAc2_FITC hybrids were incubated with GUVs in PBS for 1 h; subsequently, 10% (v/v) of *Eco*R1-HF reaction buffer, NEBuffer 4, was added to achieve conditions necessary for the activity of the enzyme.

Prior to the addition of *Eco*R1-HF, colocalization of PNA_C16 and DNA-tocopherol in the four-component complexes was confirmed (Figure 4A). Next, 20 U of the enzyme was added, and the vesicles were scanned every 90 s, allowing observation of *Eco*R1-HF caused redistribution. Upon 5.5 min after addition of *Eco*R1-HF at 25 °C, FITC and rhodamine fluorescence signals were no longer colocalized, but distributed into opposite domains in the membrane (Figure 4B). Therefore, cleavage at the specific recognition sequence, disrupting the linker between the lipophilic nucleic acids, led to the restoration of the specific lateral partitioning of the separated hybrids into lo and ld domains, respectively. Again vesicles were imaged before and at constant intervals (90 s) after the addition of *Eco*R1-HF. A selection of images at different time points and the corresponding movie are shown in Figure S9 and movie S2, Supporting Information, respectively. We observed that distribution of the DNA_tocopherol/*DNAc2_FITC* hybrids was not influenced by (did not change in time upon) the addition of the enzyme, whereas PNA_C16/*DNAc1_Rh* gradually redistributed into the opposite domain. In the images recorded directly after addition of the enzymes, red fluorescence was not homogeneously spread through lo domain: A region of increased red fluorescence signal moving with time toward the center of the lo domain could be resolved (Figure S9B,C). The change of the distribution of PNA_C16/*DNAc1_Rh* and DNA_tocopherol/*DNAc2_FITC* hybrids from a colocalized to a complementary distribution was usually completed within 3−7 min after the enzyme addition.

■ DISCUSSION

In the present study, we have developed a modular system for remote control of lipid domain partitioning of lipophilic oligonucleotides using a double palmitoylated PNA (PNA_C16) and a tocopherol-modified DNA. While partitioning of the latter construct into the liquid-disordered (ld) domain has been well documented,[16,38] here in a first step, we verified partitioning of the palmitoylated PNA into the liquid-ordered (lo) domain. We have recently shown that hybrids of PNA_C16 with the complementary DNA oligonucleotides partition into the lo domains in domain-forming membranes.[17] However, this observation left open whether PNA_C16 would be localized to the lo domains in the absence of a complementary strand and/or fluorophore. Here, we show that unhybridized PNA_C16 also partitions into the lo domains. Using solid-state NMR measurements and confocal fluorescence microscopy, we observed that lateral partitioning of the double palmitoylated PNA construct with and without a fluorescently labeled complementary DNA strand partitioned into the lo domains, suggesting that the partitioning is driven by the intrinsic properties of the lipophilic PNA conjugate.

Many proteins modified with palmitoyl chains are found in more ordered membrane regions, rafts, enriched in cholesterol/sphingomyelin, whereas proteins modified with branched farnesyl or geranylgeranyl are always found in less ordered nonrafts regions enriched in unsaturated lipids that can be compared to the ld domains in GUVs.[5] Endothelial nitroxide synthase with N-terminal myristoylation and two palmitoyl

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chains was found in caveolae/raft regions of plasma membrane.[46] Hedgehog protein modified with cholesterol and adjacent palmitoyl was also found in rafts.[47] T cell-specific protein LAT (linker for activation of T cells), palmitoylated at two cysteines, is located to rafts; this partitioning is enhanced by oligomerization, and is abolished by inhibition of palmitoylation,[10] as was also previously found for Src-family tyrosine kinases Lck and Fyn located to rafts only if palmitoylated.[7] These examples demonstrate that in biological systems palmitoylation is an essential determinant for enrichment in rafts, although it may not always be sufficient to ensure a raft localization.[1] Nevertheless, our PNA_C16 construct and another palmitoylated synthetic molecule described recently[48] show that palmitoylation can be sufficient for partitioning of synthetic conjugates into lo domains.

Linking lo domain partitioning PNA_C16 with ld domain partitioning DNA-tocopherol through DNA linker into the four-component complex (Figure 2) leads to the partitioning of the whole complex into the ld domain. This demonstrates that lateral partitioning of nucleic acids with lipophilic anchors differing in their lipid domain preference can be changed by linking them into a complex. Obviously, in our system, the tocopherol moieties are the determinants of lipid domain localization of the complex. At present, the physicochemical background of the dominating character of tocopherol is not known. We may hypothesize that the perturbing effect of tocopherol on the lipid packing in the lo domains overrules the energetic favorable partition of palmitoyl chains being responsible for localization of the complex in the ld domain. Another nonexclusive reason could be that the complex formation adds an additional lo domain perturbing effect.

The redistribution upon complex formation reminds of the distribution of proteins and peptides modified by one palmitoyl and farnesyl chain; for example, small GTPase N-Ras was found sequestered in nonraft regions and at the border of the domains.[12,14,49] Another member of the Ras family, H-Ras with two palmitoyl and one farnesyl chains, was found to be sequestered in rafts in an inactive state and relocated to nonraft regions upon activation.[5,11,50] Hence, keeping in mind that nucleic acids can be linked to membrane embedding or transmembrane peptide sequences,[51−56] our modular system may serve as a model to understand dynamic relocation of components in biological membranes, in particular, proteins.

In a next step, we demonstrated remote control of the lateral partitioning of lipophilic nucleic acids with different anchors, for example, palmitoyl and tocopherol, first by linking them into a complex (located in ld) and then by an enzymatic cleavage of the linker leading to the redistribution. Specific and nonspecific nucleases, *Eco*R1 and DNase I, were used to cleave the duplexes formed by DNA oligonucleotides, while as expected PNA/DNA duplexes were resistant against nucleases. Direct observation of the redistribution upon nuclease cleavage was performed using confocal microscopy. Cleavage of the complex led to relocation of PNA_C16/DNA hybrids into the lo domains. We observed continuous changes in time of the fluorescently labeled lipophilic nucleic acid hybrids distribution upon the addition of the enzymes. During the first 2−3 min, we often observed a region with increased red fluorescence signal moving from the ld/lo domains border to the center of the lo domain over time. This front was formed probably due to the 5−10-fold slower diffusion of the PNA_C16 molecules in the lo, as compared to the diffusion in the ld domains.[57,58] Under the used conditions (enzyme amounts, activity, temperature), the lateral movement rather than the cleavage rate was probably the rate-limiting step.

Lipid modifications of many peripheral and transmembrane proteins are crucial for the proper functioning not only through the regulation of the affinity of the proteins to different membranes and, therefore, the correct subcellular localization, but also allowing specific and controlled dynamic lateral segregation necessary for activation or inactivation. Although the lateral distribution of many lipid modified proteins has been studied, the mechanisms of the controlled reorganization are still to be elucidated. Our modular system based on formation of a complex via hybridization of nucleic acids and subsequent separation via cleavage by nucleases reveals that reversible interaction of proteins with different lateral partitioning in the specific lipid surrounding can allow such dynamic control. Hence, model systems based on membrane attached lipophilic nucleic acid conjugates could be used to study membrane reorganization, for example, during a signaling platform formation at the plasma membrane, the dynamics of which in cells is otherwise difficult to study.

In summary, we have shown that the lateral partitioning of PNA_C16, natively enriching in the liquid ordered domain of GUVs, can be redistributed to the ld domain by complexation with a ld-localizing compound DNA_tocopherol. Releasing of PNA_C16 from the complex through enzymatic cleavage led to the reconstitution of the original lateral partitioning of PNA_C16. Our system thus allows addressing ordered and disordered domains of lipid membranes specifically, and, furthermore, a controllable mixing and separation of the membrane-anchored functional moieties in either domain at the same temperature within the coexistence of lo and ld domains in membranes.

■ MATERIALS AND METHODS

Lipids and fluorescent lipid analogues such as 1-palmitoyl-2-[6-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]hexanoyl]-*sn*-glycero-3-phosphatidylcholine (C6-NBD-PC), 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine (DOPC), palmitoyl-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC), palmitoyl(d_{31})-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC-d_{31}), *N*-palmitoyl-D-sphingomyelin (PSM), *N*-palmitoyl(d_{31})-D-sphingomyelin (PSM-d_{31}), *N*-stearoyl-D-sphingomyelin (SSM), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rh-DOPE), and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL) and stored in chloroform at −20 °C. Hoechst 33342 was bought from Invitrogen (Carlsbad, CA). Cholesterol, EDTA, and Triton X-100 were purchased from Sigma-Aldrich (Taufkirchen, Germany). PBS without Ca^{2+} and Mg^{2+}, pH 7.4, was bought from PAA (Pasching, Austria). HEPES was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). DNase I (50 U/μL) and 10x DNaseBuffer (with $MgCl_2$) were purchased from Fermentas (St. Leon-Rot, Germany); *Eco*R1-HF (20 U/μL) and NEBuffer 4 were purchased from New England Biolabs (Ipswich, MA).

All unmodified DNA sequences were purchased from BioTeZ (Berlin, Germany) in quantities of 1 μmol each, and aliquots dissolved in Milli-Q water were stored at −20 °C and kept at +4 °C during the experiments. The lipophilic nucleic acids were synthesized as described elsewhere.[17] Deuterated palmitic acid was used for the new synthesis of double palmitoylated PNA (PNA_C16). The assembly of PNA was performed as previously described.[59] In most experiments reported here, the deuterated PNA_C16 was used. Tocopherol-modified DNA (DNA_tocopherol) was a 24mer DNA sequence with two tocopherol-modified deoxyuridines denoted as "L" in Table 1.

For NMR spectroscopy, the phospholipids and cholesterol were codissolved in organic solvent at a molar ratio of POPC/PSM/cholesterol 1/1/0.6, dried under vacuum (10 mbar), and dissolved in

water. Large unilamellar vesicles were prepared by extrusion,[60] and the PNA_C16 was added at a 1:150 PNA_C16/lipids molar ratio. After incubation at 37 °C for 4 h, the sample was centrifuged at ∼90 000*g* for 14 h. The pellet was frozen in liquid nitrogen and lyophilized under a vacuum of approximately 0.1 mbar. Subsequently, the sample was hydrated to 50 wt % with deuterium-depleted H_2O, freeze−thawed, stirred, and gently centrifuged for equilibration. The samples were then transferred to 5 mm glass vials and sealed with Parafilm for NMR measurements. ^{2}H NMR spectra were acquired on a widebore Bruker Avance 750 NMR spectrometer operating at a resonance frequency of 115.1 MHz for ^{2}H. A single-channel solids probe equipped with a 5 mm solenoid coil was used. The ^{2}H NMR spectra were accumulated with a spectral width of ±250 kHz using quadrature phase detection, a phase-cycled quadrupolar echo sequence[61] with two ∼4 μs $\pi/2$ pulses separated by a 60 μs delay, and a relaxation delay of 0.5 s. Details of the order parameter determination have been described before.[62]

Domain-forming GUVs were formed in a sucrose buffer (280 mOsm/kg), slightly hypoosmotic to physiological phosphate buffered saline (PBS; 300 mOsm/kg), from 1:1:0.65 (molar ratios) POPC/PSM/cholesterol or 1:1:1 DOPC/SSM/cholesterol lipid mixtures at 100 μM lipid concentration.[24] Note that POPC/PSM/cholesterol GUVs were used in experiments to allow direct comparison with NMR results as only palmitoylated analogues are commercially available as deuterated compounds. C6-NBD-PC was added to the lipid mixture before or after the GUVs preparation to mark the ld lipid domains. For experiments involving DNase I, a stock solution in PBS containing GUVs at 33 μM, 100 nM DNA_tocopherol, 100 nM PNA_C16 preincubated with 60−80 nM DNAc1_Rh, 150 nM C6-NBD-PC, and 240 μM $MgCl_2$ was prepared and incubated at room temperature for 1 h; then 80 nM DNAc2 was added following a further 30 min incubation enabling four-component complex formation. One microliter of DNase I -diluted in 10 μL 1x DNaseBuffer in PBS—was added to the GUV-solution that was shortly preincubated with DNaseBuffer in PBS. For experiments involving *Eco*R1-HF, a stock solution in PBS containing GUVs at 33 μM, 100 nM DNA_tocopherol, and 100 nM PNA_C16 preincubated with 60−80 nM DNAc1_Rh was prepared at room temperature for 1 h; then 80 nM DNAc2_FITC was added for a further 30 min incubation, enabling four-component complex formation. *Eco*R1-HF, diluted in 10 μL of NEBuffer 4 and H_2O, was added to the GUV-containing stock solution shortly preincubated with 1x NEBuffer 4 (dilution in H_2O). Here, usage of water in place of PBS was required for maintaining a nearly equal osmolarity inside and outside of GUVs.

Oligonucleotides were usually added at equimolar amounts, or at a slight excess of lipophilic nucleic acids, to avoid formation of incomplete, for example, three-part complexes. The ratio of lipophilic nucleic acid:lipid was 1:300 unless otherwise indicated.

*Eco*R1-HF from an *E. coli* strain is a site-specific endonuclease with the recognition sequence shown underlined in Table 1. It was incorporated into the sequences of labeled and unlabeled DNAc1 and DNAc2. DNase I is an endonuclease that cleaves both single- and double-stranded DNA. Enzyme inactivation was performed using the protocol provided by the supplier. 10% (v/v) of 10x NEBuffer 4 stock was used to obtain optimal conditions (10 mM $Mg(OAc)_2$) for *Eco*R1-HF activity; correspondingly, 10% (v/v) of 10x DNaseBuffer was used for optimal conditions for DNase I activity. Controls were performed in the buffers with the same composition and with PBS containing 10 mM $Mg(OAc)_2$.

Microscopy was performed on an inverted confocal laser scanning microscope FluoView 1000 (Olympus, Hamburg, Germany) with a 60× (NA 1.2) water-immersion objective. NBD, FITC, and rhodamine were excited with a 488 nm argon laser and a 559 nm He−Ne laser, and detected sequentially at 500−545 and 570−630 nm, respectively. For experiments requiring 37 °C and/or for long-time observations, a custom built temperature chamber fitting μ-Slide VI 0.4 (ibidi GmbH, München, Germany) that allowed restricting overall GUVs mobility and protection against evaporation of buffers was used; otherwise, uncoated glass coverslips were employed. Experiments with enzymes were done at 25 or 37 °C. A controlled microscopy stage H117 (Prior Scientific, Cambridge, UK) was used to perform direct observation of multiple vesicles in time. After domain-separated vesicles were located, the coordinates were saved, and an automated scan of up to 20 vesicles within up to 2 min per cycle was performed. Enzyme (1 μL of 50 U/μL DNase I or 20 U/μL *Eco*R1-HF) was accurately added to the GUVs settled in μ-Slide VI 0.4 at the end of the initial image cycle (see pre-enzyme addition images), taking care not to disturb the vesicles. All times given in the text refer to the time since the addition of an enzyme. For all images and movies, a linear background subtraction was executed. Movies were assembled from time-resolved single slice or z-stack confocal acquisitions using FluoView 1000 software, assigning each frame a duration of 1/3 s.

To analyze and compare the fluorescence intensity of the different domains, the software Fluoview (Olympus, Hamburg, Germany) was used as follows: after subtracting linearly the image background, six regions of interests were equally spaced over each lipid domain with height and length being smaller than the average apparent membrane thickness, and the resulting average intensities were used to calculate the intensity ratios between lo and ld domains.

■ ASSOCIATED CONTENT

Supporting Information

Figures S1−S9 and movies S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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